

BRAZILIAN PHARMACOPOEIA

6th EDITION



Brazilian Health Regulatory Agency – Anvisa

Brazilian Health Regulatory Agency

Brazilian Pharmacopoeia

6th edition

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Volume 2

MONOGRAPHS

1 PREFACE

Brazilian Pharmacopoeia (FB) is the national pharmaceutical compendium that establishes, via pharmacopoeial texts (chapters, methods and monographs), the minimum requirements for quality, authenticity and purity of pharmaceutical ingredients, drugs and other products subject to sanitary regulation. Failure to fully complying with the pharmacopoeial requirements may result in the product being classified as altered, adulterated or inappropriate for use, under the terms of Law 6.360/76, with those responsible incurring the sanctions and measures established in Law 6.437/77.

Therefore, FB is in charge of establishing the parameters of acceptability of the products made available to the Brazilian population, serving as an essential foundation for the achievement of health control and surveillance actions.

As technical-scientific development, by its very nature, is very dynamic and fast-moving, it is crucial that we have a pharmacopoeial compendium that fits simultaneously into pharmaceutical innovations. Since the 5th edition of FB, published on November 23, 2010, the Deliberative Council of the Brazilian Pharmacopoeia (CDFB) has been working day-to-day in the fulfillment of its mission to keep FB constantly updated.

The 1st errata of the 5th edition of the FB (RDC 18/2012) was published In 2012 with some specific corrections.

In 2016, RDC 59, on February 03, and RDC 101, on August 12th, were published, thus approving the First Supplement of the FB 5th edition. This supplement comprised 63 pharmacopoeial texts, 8 general texts/methods, 7 of which were unpublished, and 55 monographs, of which 38 were unpublished. This edition increased the number of FB monographs by about 10%, and covered monographs on synthetic drugs (16, 6 unpublished), biological drugs (15, being 12 unpublished), blood components/blood derivatives (7 unpublished) and pharmaceutical ingredients (17, 13 of which were unpublished). This publication highlights the incorporation of 6 general methods harmonized within the scope of the Mercosur Pharmacopoeia (Common Market Group).

In 2017, RDC 167 of 24 July was published, which approved the Second Supplement of the FB 5th edition. This supplement comprised 262 pharmacopoeial texts, 12 chapters and general methods, 8 of which were unpublished, and 247 monographs, of which 176 were unpublished. This edition raised the number of FB monographs by about 25%, and covered monographs on medical devices (03 unpublished), medical gases (02 unpublished), radiopharmaceuticals (03 unpublished), blood components (05 unpublished), synthetic drugs (11 new), biological drugs (18, 5 new), pharmaceutical ingredients (61, 58 new) and medicinal plants (147, 89 new). It is worth noting that the 58 monographs on medicinal plants from the 5th edition of the FB were duly revised, and there was also an increase of about 150% in the number of monographs in this class of products, fostering the development of products of plant origin and valuing the native flora. Still, in an unprecedented action, this supplement comprises the two prime monographs, and the respective chapter and general methods, of medical gases, a drug class not covered by FB yet.

In 2018 there was already content for a new supplementary update, with 15 new monographs of synthetic drugs (07), medical gases (02), active ingredient (01), medicinal plants (02), biological product (01) and radiopharmaceuticals (02). The desire to maintain a perennial annual update process was moderated by the urgent need to review and update the hundreds of pharmacopoeial texts in the 5th edition.

Accordingly, FB Deliberative Council decided to conduct a broad review process, which culminated in this FB 6th edition. This 6th edition comes with a new and modern visual identity, compatible with the relevance of this work, combined with a 100% digital publication.

Here, it is vital to praise the outstanding commitment of the Thematic Technical Committees (CTT) in carrying out such undertaking, which, despite the tight deadline, concluded the mission with honors.

Finally, we highlight Anvisa's support in promoting this work, in particular to the Pharmacopoeia Coordination (Cofar) team, whose dedication and extreme care were essential.

Varley Dias Sousa
President of CDFB

2 HISTORY

BRIEF HISTORY UPADTE OF THE BRAZILIAN PHARMACOPOEIA, 6th EDITION

Brazil, as a Portuguese domain, used as its official pharmaceutical code the *General Pharmacopoeia of the Kingdom and Domains*, published in 1794, and reprinted in 1824. In 1837 the French *Codex Medicamentarius* was also accepted in Brazil. On September 29, 1851, the Decree 828 established the French *Codex Medicamentarius* as the official compendium of Brazil, in addition to the recognition of the Portuguese Pharmacopoeia. On January 19, 1882, Decree 8387, and on December 31, 1923, Decree 16,300 reaffirmed the primacy and authority of the French Pharmacopoeia.

The 1st edition of the Brazilian Pharmacopoeia was approved on November 04, 1926, by Decree 17.509. This 1st edition was updated in 1943 – 1st supplement – (Ordinance nr 42, of March 2nd), in 1945 – 2nd supplement – (Ordinance nr 24, of April 14) and in 1950 – 3rd supplement – (Ordinance nr 39, of June 13).

The 2nd edition of the Brazilian Pharmacopoeia was approved in 1955 (Decree 37,843, of September 1st), and had updates and modifications in 1959 (Decree 45,502, of February 27th), with the separation of pharmaceutical formulations from the Pharmacopoeia at that time, thus emerging the National Form.

The 3rd edition of the Brazilian Pharmacopoeia was approved in 1976 (Decree 78,840, of 25 November).

The 4th edition of the Brazilian Pharmacopoeia (Part I) was approved in 1988 (Decree 96,607, of 30 August). Part II was published in fascicles in 1996 (first), 2000 (second), 2002 (third), 2003 (fourth), 2004 (fifth) and 2005 (sixth).

The 5th edition of the Brazilian Pharmacopoeia was approved in 2010 (Resolution of the Collegiate Board – RDC 49, of November 23). In 2012, the 1st erratum was approved (RDC 18, of March 23). The 5th edition was further complemented by the 1st Supplement in 2016 (RDC 59/2016 and RDC 101/2016) and by the 2nd Supplement in 2017 (RDC 167/2017).

Without further details, we have here a brief time recap of the evolution of this National Compendium.

Varley Dias Sousa
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4 MISCELLANEOUS

TITLE

The full title of this work is “Pharmacopoeia of the Federative Republic of Brazil, 6th edition”. It may be called “Brazilian Pharmacopoeia, 6th edition” or FB 6.

DEFINITIONS

Action, use and doses

The ones contained in the report for registration of the product at the sanitary agency, updated through a national and international bibliographic review, when applicable.

When indicated in the monographs, the doses represent the amount of medication usually prescribed, that has therapeutic efficacy for adult patients. The qualified prescriber, at his discretion and under his sole responsibility, considering pharmacokinetic and pharmacodynamic criteria, may vary the amounts and administration frequency of any medication. However, the prescription of doses much higher than the usual ones, established in the literature, leads the pharmacist to confirm, with the prescriber of the prescription, the established doses.

Acidity and alkalinity - quick tests

A solution is considered *neutral* when it does not change the color of the blue and red litmus papers, or when the universal indicator paper shows the colors of the neutral scale, or when 1 mL of the same solution is colored green with a drop of bromothymol blue TS (pH 7.0).

It is considered *acidic* when the blue litmus paper is stained red or 1 mL is stained yellow by a drop of phenol red TS (pH 1.0 to 6.6).

It is considered *weakly acidic* when the blue litmus paper is slightly stained red or 1 mL is stained orange by a drop of methyl red TS (pH 4.0 to 6.6).

It is considered *strongly acidic* when the Congo red paper is stained blue or 1 mL is stained red by the addition of a drop of methyl orange TS (pH 1.0 to 4.0).

It is considered *alkaline* when the red litmus paper is stained blue or 1 mL is stained blue by a drop of bromothymol blue TS (pH 7.6 to 13.0).

It is considered *weakly alkaline* when the red litmus paper is stained blue or 1 mL is stained pink by a drop of cresol red TS (pH 7.6 to 8.8).

It is considered *strongly alkaline* when stained blue by a drop of thymolphthalein TS (pH 9.3 to 10.5) or red by a drop of phenolphthalein TS (pH 10.0 to 13.0).

Adhesive

It is the system designed to produce a systemic effect by diffusing the active principle(s) at a constant rate/speed for an extended period of time.

Water for Injections

Water for injections is the liquid used in the preparation of drugs for parenteral administration, as a vehicle, or in the dissolution and dilution of substances or preparations.

Water for pharmaceutical purposes

Water for pharmaceutical purposes is the various types of water used in the synthesis of drugs, in the formulation and production of drugs, in testing laboratories, diagnostics and other applications related to the health area, including as a main component in the cleaning of utensils, equipment and systems.

Purified water

Purified water is drinking water that has undergone some type of treatment to remove possible contaminants and meet the purity requirements established in the monograph.

Ultrapure water

Ultrapure water is purified water that has undergone additional treatment to remove potential contaminants and meet the purity requirements established in the monograph.

Aromatic waters

They are saturated solutions of essential oils or other aromatic substances in water. They have a characteristic smell of the substances they are prepared with, also receiving their name.

Water bath and steam bath

It is a boiling water bath, unless the monograph specifies another temperature. The expressions *hot water* and *very hot water* indicate approximate temperatures between 60°C and 70°C and between 85°C and 95°C, respectively. Steam bath means exposure to flowing steam or another form of heat, corresponding in temperature to that of flowing steam.

Bioavailability

It indicates the speed and extent of absorption of an active ingredient in a dosage form, based on its concentration/time curve in the systemic circulation or its excretion in the urine.

Bioequivalence

It consists of proving pharmaceutical equivalence between products presented under the same pharmaceutical preparation, containing identical qualitative and quantitative composition of active principle(s), and which have comparable bioavailability, when studied under the same experimental design.

Tank truck

Vehicle containing a large-sized affixed container for transporting cryogenic liquids.

Capsule

It is the solid pharmaceutical preparation in which the active ingredient and excipients are contained in a hard or soft soluble envelope, of varying shapes and sizes, usually containing a single dose of the active ingredient. It is usually made of gelatin, but it can also be made of starch or other substances.

Hard capsule

It is the capsule that consists of two prefabricated cylindrical sections (body and cap) that fit together and whose ends are rounded. It is typically filled with active ingredients and excipients in solid form. It is usually made of gelatin, but it can also be made of other substances.

Prolonged release hard capsule

It is the capsule that consists of two prefabricated cylindrical sections (body and cap) that fit together and whose ends are rounded. It is typically filled with active ingredients and excipients in solid form. It is usually made of gelatin, but it can also be made of other substances. See general definition of prolonged release.

Delayed release hard capsule

It is the capsule that consists of two prefabricated cylindrical sections (body and cap) that fit together and whose ends are rounded. It is typically filled with active ingredients and excipients in solid form. It is usually made of gelatin, but it can also be made of other substances. See general definition of delayed release.

Soft capsule

It is a capsule made of a gelatin shell, of various shapes, which is more malleable than that of hard capsules. They are usually filled with liquid or semi-solid contents, but can also be filled with powders and other dry solids.

Prolonged release soft capsule

It is a capsule made of a gelatin shell, of various shapes, which is more malleable than that of hard capsules. They are usually filled with liquid or semi-solid contents, but can also be filled with powders and other dry solids. See general definition of prolonged release.

Delayed release soft capsule

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CAS

Chemical Abstract Service.

Medicinal tea

It consists exclusively of herbal drugs intended for oral aqueous preparations by means of decoction, infusion or maceration. The tea is prepared immediately before use.

Gas cylinder

It is the metallic container, perfectly closed, with resistant walls designed to contain gas under pressure, closed by an adjustable valve capable of maintaining the gas output at a determined flow rate.

Gas cylinder for medical use

Transportable and pressurized container with a capacity measured in water volume not exceeding 150 liters.

CNTP

Normal temperature and pressure.

Eye drops

It is a liquid pharmaceutical preparation intended for application over the ocular mucosa.

Total lyophilized human prothrombin complex

It is a fraction of plasmatic proteins that mandatorily contains Factors II, VII, IX and X of the human coagulation.

Tablet

It is a solid pharmaceutical preparation containing a single dose of one or more active principles, with or without excipients, obtained by compressing uniform volumes of particles. It can be of a wide variety of sizes, shapes, have surface markings, and be coated or uncoated.

Modified release tablet

It is the tablet that has a modified release dosage. It should be classified as modified release only when the classifications “delayed release” and “prolonged release” are not adequate.

Prolonged release tablet

It is the tablet whose excipients are specifically intended to modify the release of the active ingredient in digestive fluids. See definition of prolonged release.

Effervescent tablet

It is the tablet that contains, in addition to the active ingredients, acidic substances and carbonates or bicarbonates, which release carbon dioxide when the tablet is dissolved in water. It is intended to be dissolved or dispersed in water prior to administration.

Chewable tablet

It is the tablet formulated so that it can be chewed, producing a pleasant aftertaste in the oral cavity.

Orodispersible tablet

It is the tablet that quickly disintegrates or dissolves when placed on the tongue.

Mouthwash tablet

It is the tablet that must be dissolved in water to prepare the mouthwash, which is a liquid for mouth rinsing that acts on the gums and mucous membranes of the mouth and throat. It must not be swallowed.

Tablets for dissolution

It is intended to be dissolved in water prior to administration. The preparation produced can be slightly turbid due to the excipients used in the tablet manufacturing.

Tablets for suspension

It is the tablet that, when in contact with a liquid, quickly produces a homogeneous dispersion (suspension) and must be dispersed before administration.

Coated tablet

It is the tablet that has one or more thin coating layers, usually polymeric, designed to protect the drug from air or humidity; for drugs with unpleasant odor and taste; to improve the appearance of the tablets, or for some other property other than altering the speed or extent of the active ingredient release.

Prolonged release coated tablet

It is the tablet that has one or more thin coating layers, usually polymeric, designed to modify the speed or extent of release of the active ingredients. See general definition of prolonged release.

Delayed release coated tablet

It is the tablet that has one or more thin coating layers, usually polymeric, designed to modify the speed or extent of the release of the active ingredients, presenting a delayed release of the active ingredient. See definition of delayed release.

Uncoated tablet

It is the tablet in which excipients used are not specifically intended to modify the release of the active ingredient in digestive fluids.

Quality control

It is the set of measures designed to guarantee, at any time, the production of batches of drugs and other products that meet the standards of identity, activity, content, purity, efficacy and safety.

Dyes/Colorants

They are additional substances to drugs, diet products, cosmetics, perfumes, hygiene products and similars, household sanitizers and similars, to give them color and, in certain types of cosmetics, transfer it to the skin surface and skin appendages. For its use, observe the Federal legislation and the resolutions issued by Anvisa.

Surgical materials

Health product, such as equipment, apparatus, material, article or system for medical, dental or laboratory use or application, intended for the prevention, diagnosis, treatment, rehabilitation or contraception and which does not use pharmacological, immunological or metabolic means to carry out its main function in human beings being able, however, to be aided in its functions by such means.

Cosmetics

Products for external use; intended for the protection or beautification of different parts of the body, such as facial powders; body powders; beauty creams; hand cream and similars; face masks; beauty lotions; milky, creamy and astringent solutions; hand lotions; make-up foundation and cosmetic oils; blushes; lipsticks; lip balms; sunscreens; tanning lotions; mascaras; eye shadows; eyeliners; hair dyes; hair bleaching agents; hair curling and straightening preparations; hair fixatives; hairspray; shining hair gels and similars; hair lotions; depilation and epilation products; nail preparations and others.

Cream

It is the semi-solid pharmaceutical preparation that consists of an emulsion, formed by a lipophilic phase and a hydrophilic phase. It contains one or more active ingredients dissolved or dispersed in an appropriate base and is normally used for external application to the skin or mucous membranes.

Cryoprecipitates from fresh human plasma

They are constituted by cold-insoluble fractions containing mainly Factors I (140 to 250 mg) and VIII (70 to 120 IU) of human coagulation per unit of human blood collection. Other coagulation factors are also found in lower concentrations with cryoprecipitate such as Von Willebrand Factor (40 to 70%) and Factor XIII (20 to 30%).

Brazilian Common Denomination (DCB)

It is the name of the drug or pharmacologically active ingredient approved by the federal agency responsible for health regulation. It also includes the names of inactive ingredients, hyperimmune serums and vaccines, radiopharmaceuticals, medicinal plants, homeopathic and biological substances.

International Non-proprietary Names (INN)

It is the name of the drug or pharmacologically active ingredient, recommended by the World Health Organization.

Mass density and relative density

Mass density (ρ) of a substance is the ratio of its mass to its volume at 20°C. The usually adopted relative density (ρ) is defined as the ratio between the mass of a substance in the air at 20°C and the mass of an equal volume of water at the same temperature.

Disinfectants

Products designed to indiscriminately or selectively destroy microorganisms when applied to inanimate objects or environments.

Detergents

Products designed to dissolve fats; the hygiene of containers and canisters and domestic applications.

Blood Donors

Healthy and carefully selected individuals who, after medical examinations, laboratory blood tests and study of their medical history, are free of transmissible infectious agents can be accepted and used to collect their whole blood or their cellular or plasma fractions for prophylactic purposes, dressings or fractionation.

Sugar coated tablets

Tablets coated with layers consisting of mixtures of different substances, such as natural or synthetic resins, gums, gelatins, inactive and insoluble materials, sugars, plasticizers, polyols, waxes, authorized colorants and, sometimes, flavorings and active ingredients.

Drug of natural origin

They are obtained from living beings such as plants, bacteria, algae, fungi, lichens, animals, and minerals, which contain substances or classes of substances responsible for a therapeutic action and/or pharmaceutical purpose. The drug is specified by the part used and the scientific name (species, variety where applicable, and author(s)).

Herbal drugs

Herbal drugs are whole plants or their parts, usually dry, unprocessed, either whole or fragmented. Exudates such as gums, resins, mucilage, latex and waxes that have not been subject to specific treatment are also included.

Elixir

It is a pharmaceutical preparation for oral use, liquid, clear, hydroalcoholic, with a sweet and pleasant taste. Elixirs are prepared by simple dissolution and must be stored in amber colored flasks and kept in a cool place protected from light.

Packaging

It is the wrapping, container or any form of packaging, removable or not, intended to cover, package, fill, protect or maintain, specifically or not, medicines, drugs, pharmaceutical and related ingredients, cosmetics, sanitizers and other products.

Primary packaging

It is the one that maintains direct contact with its content. Examples of primary packaging: ampoule, tube, envelope, case, flacon, glass or plastic flask, ampoule flask, cartridge, can, pot, paper bag, among others. There must be no interaction whatsoever between the primary packaging material and its contents capable of altering the concentration, quality or purity of the packaged material.

Secondary packaging

It is the one that allows total protection of the packaging material under the usual conditions of transport, storage and distribution. Examples of secondary packaging: cardboard boxes, cardboard cartridges, wood or plastic material or cardboard case and others.

Patch

It is the semi-solid pharmaceutical preparation for external application. It consists of an adhesive base containing one or more active ingredients distributed in a uniform layer on an appropriate support made of synthetic or natural material. Intended to keep the active ingredient in contact with the skin acting as a protector or keratolytic agent.

Emulsion

It is the liquid pharmaceutical preparation of one or more active ingredients that consists of a two-phase system involving at least two immiscible liquids and in which a liquid is dispersed in the form of small drops (internal or dispersed phase) through another liquid (external or continuous phase). It is usually stabilized by means of one or more emulsifying agents.

Aerosol emulsion

It is a pressure-packed emulsion containing a propellant gas and therapeutically active ingredients that are released upon activation of an appropriate valve system.

Emulsion drops

It is the emulsion intended for administration in the form of drops.

Injectable emulsion

It is the sterile emulsion.

Emulsion for infusion

It is a sterile emulsion with water as the continuous phase normally, isotonic with blood and used primarily for large volume administration.

Spray emulsion

It is the emulsion administered in the form of a liquid finely divided by a jet of air or steam.

Biological assays

Procedures designed to assess the potency of active ingredients contained in raw materials and pharmacopoeial preparations, using biological reagents such as microorganisms, animals, fluids and isolated organs from animals.

Spirits

It is the alcoholic or hydroalcoholic liquid pharmaceutical preparation, containing aromatic or medicinal ingredients and classified into simple and compound. Spirits are obtained by dissolving aromatic substances in ethyl alcohol, usually in the proportion of 5% (w/v).

Sterility

Sterility is the absence of viable microorganisms.

Extracts

Semi-solid or solid liquid preparations, obtained from herbal drugs, using extraction methods and appropriate solvents. An extract is essentially defined by the quality of the herbal drug, the production process and its specifications. The material used in the preparation of extracts can undergo preliminary treatments, such as enzyme inactivation, grinding or degreasing. After extraction, unwanted materials can be eliminated.

Standardized extracts

Extracts adjusted to a defined content of one or more constituents responsible for the therapeutic activity. Content adjustment is achieved by adding inert excipients or blending other batches of extract.

Quantified extracts

Extracts adjusted to a content range of one or more active markers. Content range adjustment is achieved by mixing extract batches.

Other Extracts

Extracts not adjusted to a specific content of constituents. They are essentially defined by the parameters of their manufacturing process, such as the quality of the herbal drug, selection of the extraction liquid and extraction condition as well as their specifications. Markers do not necessarily have established therapeutic activity, being considered analytic markers. The content of the markers must not be inferior to the minimum value indicated in the monograph.

Fluid extract

It is the liquid preparation obtained by extraction with an appropriate liquid in which, in general, a part of the extract, by mass or volume, corresponds to a part, by mass, of the dry herbal drug used in its preparation. Preservatives can also be added. They must present specifications regarding the content of markers and dry residue. In the case of extracts classified as standardized, the proportion between the herbal drug and the extract can be modified due to the necessary adjustments to obtain the specified content of active constituents.

Soft extract

It is the semi-solid consistency preparation obtained by partial evaporation of the used extraction liquid, and can be used as solvents, solely, ethyl alcohol, water, or mixtures of ethyl alcohol and water in an adequate proportion. They have not less than 70% (w/w) of dry residue. Preservatives can be added if necessary.

Native extract (genuine)

Extracts prepared without the addition of excipients (simple or crude extracts). However, for soft extracts and liquid preparations, the native extract may have variable quantities of extraction liquid.

Dry extract

It is the solid preparation obtained by evaporating the solvent used in the extraction process. They can be added from suitable inert materials and have specifications as to the content of markers. In general, they have a desiccation loss of no more than 5% (w/w).

Manufacturing

All the operations necessary to obtain health care products.

Distillation range

Distillation range is the temperature range corrected for the pressure of 101.3 kPa (760 mm of Hg), within which the liquid, or specific fraction of the liquid, fully boils.

Melting range

Melting range of a substance is the temperature range between the start (at which the substance begins to liquefy) and the end of melting (which is evidenced by the disappearance of the solid phase).

Drug

See *Active pharmaceutical ingredient*.

Pharmacopoeial

The expression pharmacopoeial replaces the expressions official and officinal, used in previous editions, being equivalent to such expressions for all purposes.

Lyophilized human blood coagulation factor VII

It is the protein fraction of plasma that contains Factor VII (a single-chain glycoprotein derivative), which may also contain small quantities of its activated form (the two-chain derivative or Factor VIIa).

Lyophilized human blood coagulation factor VIII

It is a protein fraction of plasma that contains a glycoprotein called coagulation factor VIII and, depending on the purification method, variable quantities of von Willebrand factor. It is prepared from a mixture of plasma for fractionation obtained from healthy donors.

Lyophilized human fibrinogen

It is the soluble fraction of human plasma, obtained from *human Plasma for fractionation*, which, by adding thrombin, is transformed into fibrin. The preparation may contain additives (salts, buffers or stabilizers) and when reconstituted (addition of diluent) must contain not less than 10 g/L of fibrinogen.

FISPQ

Chemicals Information Sheet.

Pharmaceutical preparation

It is the final state of presentation of the active pharmaceutical ingredients after one or more pharmaceutical operations carried out with the addition or not of appropriate excipients in order to facilitate their use and obtain the desired therapeutic effect, with characteristics appropriate to a given route of administration.

Gas

Substance or mixture of substances whose vapor pressure is above 300 kPa absolute at 50 °C or remains in gaseous form at 20 °C at an absolute pressure of 101.3 kPa.

Compressed gas

Any gas or mixture of gases that exerts an absolute pressure greater than or equal to 280 kPa at 20 °C in the container where the gas is stored.

Excipient gas

Any component gas, which is not an active substance, intentionally added to the formulation of a gas mixture.

Liquefied gas

A gas at vapor pressure that remains partially liquefied at temperatures above –50°C.

Medicinal Gas

Gas, or mixture of gases, for treating or preventing disease in humans or administered for medical diagnosis purposes or to restore; correct; or modify physiological functions.

Cryogenic gas or liquid

Highly cooled gas in phase equilibrium (liquid and its vapor pressure) and boiling point less than or equal to –150 °C at an absolute pressure of 101.3 kPa.

Gel

It is the semi-solid dosage form of one or more active ingredients that contains a gelling agent to provide firmness to a colloidal solution or dispersion (a system in which particles of colloidal size – typically between 1 nm and 1 mm – are evenly distributed throughout the liquid) and may contain suspended particles.

Hydrophobic gel

It is the gel that usually consists of liquid paraffin with polyethylene or fatty oils with colloidal silica or aluminum or zinc soaps.

Lipophilic gel

It is the gel resulting from the preparation obtained by the incorporation of gelling agents — tragacanth, starch, cellulose derivatives, carboxyvinyl polymers and magnesium aluminum double-chain silicates in water, glycerol or propylene glycol.

Globule

It is the solid pharmaceutical preparation that is presented in the form of small spheres made of sucrose or a mixture of sucrose and lactose. They are impregnated with the desired potency and with alcohol above 70%

Chewing gum

It is a single-dose solid pharmaceutical preparation containing one or more active ingredients, which consists of insoluble, sweet and tasty plastic material. When chewed, it releases the active ingredient.

Granules

It is a solid pharmaceutical preparation containing a single dose of one or more active ingredients, with or without excipients. It consists of solid and dry agglomerates of uniform volumes of powder particles resistant to handling.

Effervescent granules

It is the granule that contains, in addition to the active ingredients, acidic substances and carbonates or bicarbonates, which release carbon dioxide when the granule is dissolved in water. It is intended to be dissolved or dispersed in water prior to administration.

Granule for dissolution

It is the granule intended to be dissolved in water prior to administration. The preparation produced can be slightly turbid due to the excipients used in the granule manufacture.

Granule for suspension

It is the granule that, in contact with a liquid, quickly produces a homogeneous dispersion (suspension). It is intended to be dispersed prior to administration.

Coated granule

It is the granule that has one or more thin coating layers, usually polymeric, designed to protect the drug from air or humidity; for drugs with unpleasant odor and taste; to improve the appearance of the granules, or for some other property other than altering the speed or extent of the active ingredient release.

Prolonged release coated granule

It is the granule that has one or more thin coating layers, usually polymeric, designed to modify the speed or extent of releasing the active ingredients. See definition of prolonged release.

Delayed release coated granule

It is the granule that has one or more thin coating layers, usually polymeric, designed to modify the speed or extent of releasing the active ingredients, presenting a delayed release of the active ingredient. See general definition of delayed release.

Anti-hepatitis A human immunoglobulin

It is a sterile, lyophilized or liquid preparation, containing immunoglobulins, mainly G.

Anti-hepatitis B human immunoglobulin

It is a sterile, lyophilized or liquid preparation, containing immunoglobulins, mainly G.

Anti-hepatitis B human immunoglobulin for intravenous administration

It is a sterile, lyophilized or liquid preparation, containing immunoglobulins, mainly G.

Anti-rabies human immunoglobulin

It is a sterile, lyophilized or liquid preparation, containing immunoglobulins, mainly G.

Anti-rubella human immunoglobulin

It is a sterile, lyophilized or liquid preparation, containing immunoglobulins, mainly G.

Anti-varicella human immunoglobulin

It is a sterile, lyophilized or liquid preparation, containing immunoglobulins, mainly G.

Anti-varicella human immunoglobulin for intravenous administration

It is a sterile, lyophilized or liquid preparation, containing immunoglobulins, mainly G.

Anti-D Antigen human immunoglobulin

It is a sterile, lyophilized or liquid preparation, containing immunoglobulins, mainly G.

Anti-measles human immunoglobulin

It is a sterile, lyophilized or liquid preparation, containing immunoglobulins, mainly G.

Anti-tetanus human immunoglobulin

It is a sterile, liquid or lyophilized preparation containing immunoglobulins, especially immunoglobulin G. It is obtained from plasma containing specific antibodies against the toxin of *Clostridium tetani*.

Normal Human Immunoglobulin

It is a sterile, lyophilized or liquid preparation, containing mainly IgG. Other proteins may also be present.

Normal Human Immunoglobulin for intravenous administration

It is a sterile, lyophilized or liquid preparation, containing immunoglobulins, mainly G (IgG). Other proteins may be present. It contains IgG antibodies from normal individuals.

Biological indicator

It is a preparation characterized by a specific microorganism that has defined and stable resistance to a certain sterilization process.

Refractive index

The refractive index (n) of a substance is the ratio between the speed of light in a vacuum and its speed inside the substance. For practical purposes refraction is measured with reference to air and substance and not with reference to vacuum and substance. The refractive index can be defined as the ratio between the sine of the angle of incidence and the sine of the angle of refraction, that is, $n = \sin i / \sin r$.

Injectable

It is a sterile preparation intended for parenteral administration. It is presented as a solution, suspension or emulsion.

Insecticides

Products for external use, intended for the prevention and control of insects, in homes, enclosures and places of public use and their surroundings.

Insulin

Insulin is a protein that affects glucose metabolism. It is obtained from the pancreas of healthy cattle and pigs, or both, used as food by humans.

Human insulin

Human insulin is a protein corresponding to an active principle made in the human pancreas that affects the metabolism of carbohydrates (particularly glucose), lipids and proteins.

Human isophane insulin suspension

Isophane human insulin suspension is a sterile suspension of human insulin crystals, zinc and protamine sulfate in buffered water for injection, combined in such a manner that the solid phase of the suspension is composed of human insulin crystals, protamine and zinc.

Human isophane insulin suspension and human insulin injection

Isophane human insulin suspension and human insulin injection is a sterile buffered suspension of human insulin, complexed with protamine sulfate, in human insulin solution.

Human Insulin Zinc Suspension

It is a sterile suspension of human insulin in buffered water for injection, modified by the addition of a suitable zinc salt so that the solid phase of the suspension is made up of a mixture of crystalline and amorphous insulin in a ratio of about seven parts of crystals and three parts of amorphous material.

Human Insulin Zinc Extended Suspension

It is a sterile suspension of human insulin in buffered water for injection, modified by the addition of a suitable zinc salt so that the solid phase of the suspension is predominantly crystalline.

Injectable Insulin

Injectable insulin is a sterile, isotonic insulin solution.

Insulin lispro

It is identical in structure to human insulin, except for the presence of lysine and proline at positions 28 and 29, respectively, of the B chain, while this sequence is inverted in human insulin. Insulin lispro is produced by microbial synthesis through a recombinant DNA process.

Active pharmaceutical ingredient.

It is an active chemical substance, medicine, drug or raw material that has pharmacological properties with a medicinal purpose used for diagnosis, relief or treatment, used to modify or explore physiological systems or pathological states for the benefit of the person to whom it is administered.

When intended for use in drugs, it must meet the requirements set in the individual monographs.

Insulators

Equipment that employs technology used for a dual purpose, to protect the product from contamination by the environment and by people during filling and closure and to protect people from toxic or harmful products that are produced.

Conventional release

It is the type of dosage form release that is not intentionally modified by a special formulation design and/or manufacturing method.

Parametric release

It is defined as the release of cargo or batches of products subject to terminal sterilization, through compliance with critical parameters of the sterilization process, without the need to perform a sterility test.

Prolonged release

It is the type of modified release of pharmaceutical preparations that allows at least a reduction in the dose frequency when compared to the drug presented in the conventional release form. It is obtained through a special formulation design and/or manufacturing method.

Delayed release

It is the type of modified release of pharmaceutical preparations that has a delayed release of the active ingredient. Delayed release is obtained through a special formulation design and/or manufacturing method. Gastro-resistant preparations are considered delayed release forms, as they are designed to resist gastric fluid and release the active ingredient into the intestinal fluid.

Extracting liquid

Solvents or mixture of solvents that are used in the extraction process.

Lotion

It is an aqueous or hydroalcoholic liquid preparation, with variable viscosity, for application to the skin, including the scalp. It can be a solution, emulsion or suspension containing one or more active principles or adjuvants.

Batch or item

It is the quantity of a drug, or other product, that is produced in a manufacturing cycle and whose essential characteristic is homogeneity.

Markers

Chemically defined constituents or groups of constituents, present in drugs, their preparations, phytotherapies or other drugs based on active ingredients of natural origin, which are used for quality control purposes, and may or may not have therapeutic activity.

Packaging material

By packaging material is understood the container; wrap; casing; or any other form of protection, removable or not, used for filling; protect; keep; cover; or wrap, specifically or not, raw materials; reagents and drugs.

Raw-materials

Active or inactive substances used in the manufacture of drugs and other products, both those that remain unchanged and those subject to modification.

Media fill

It is a test for simulating aseptic operations in which the product is replaced by a culture medium and serves to ensure that the processes used are capable of producing sterile products.

Drug

It is the pharmaceutical product, technically obtained or prepared, which contains one or more drugs and other substances, with a prophylactic, curative, palliative or diagnostic purpose.

Reference drug

It is the innovative product registered with the Brazilian federal agency, responsible for sanitary regulation and traded in the country, whose efficacy, safety and quality were scientifically proven by the competent federal agency upon registration.

Generic drug

It is the drug similar to a reference or innovative product, intended to be interchangeable with this product, usually produced after the expiration or waiver of patent protection or other exclusive rights, proven its efficacy, safety and quality, and designated by the Brazilian Common Nomenclature (DCB) or, in its absence, by the International Non-Proprietary Names (INN).

Interchangeable drug

It is the therapeutic equivalent of a reference drug, with essentially the same efficacy and safety effects proven.

Compounded drug

It is any drug whose prescription details the composition, pharmaceutical preparation and dosage. It is prepared in the pharmacy, by a qualified pharmaceutical professional or under his direct supervision.

Pressurized drug

It is a pressure-packed drug containing a propellant gas and therapeutically active ingredients that are released upon activation of an appropriate valve system.

Similar/Alternative drug

It is one that contains the same active principle(s), has the same concentration, pharmaceutical preparation, route of administration, dosage and therapeutic indication, and which is equivalent to the drug registered with the federal agency, responsible for health regulation, and may only differ in characteristics regarding the size and shape of the product, expiration date, packaging, labeling, excipients and vehicle, and must always be identified by trade name or brand.

Biological half-life

It is the time required for an organism to remove, by biological elimination, half the quantity of an administered substance.

Effective half-life

It is the time it takes for a radionuclide in an organism to halve its activity as a combined result of biological elimination and radioactive decay. The effective half-life is important for calculating the optimal dose of the radiopharmaceutical to be administered and for monitoring the quantity of radiation exposure.

Immunochemical methods

These methods are based on selective, reversible and non-covalent binding between antigens and antibodies.

Miscibility

The term miscible is used to describe a liquid or gas that produces a homogeneous mixture when mixed in any proportion with the indicated solvent in the same physical state.

Mixtures of surplus human plasma treated by viral inactivation

Frozen or lyophilized, sterile, pyrogen-free preparation obtained from surplus human plasma from donors having the same ABO and Rh(Du) blood group. The preparation is thawed or reconstituted prior to use, in order to obtain an injectable solution. The human plasma used should meet the requirements of the monograph *Human Plasma for Fractionation*.

Sterility assurance level

It is the degree of guarantee that the process in question sterilizes a group of items, being expressed as the probability of a non-sterile item in that population.

Chemical name

It is the name of the pharmacopoeial substance, according to the nomenclature recommended by the International Union of Pure and Applied Chemistry (IUPAC).

Batch number

Designation printed on the labeling of a drug and other products that allows identifying the batch or item to which they belong and, if necessary, locating and reviewing all manufacturing and inspection operations carried out during production.

Nutrients

They are constituent substances of food with nutritional value, including proteins, fats, carbohydrates, water, mineral elements and vitamins.

Fixed oil

They are non-volatile oils, liquid at room temperature. They are predominantly made up of triacylglycerols, esterified with different or identical fatty acids.

Volatile Oil

Oils obtained from plants, by physical processes, which evaporate at room temperature without leaving residue. They are made up of complex mixtures of low molecular weight substances which determine their odor and taste. They can be presented alone or mixed with each other, rectified, reduced or concentrated. They can also be called essential oils.

Oleoresin

They are semi-solid extracts consisting of a resin in solution in a volatile oil and/or fixed oil and are obtained by evaporation of the solvent(s) used for their production. This definition applies only to oleoresins produced by extraction.

Osmolality

It is a practical form that gives a total measure of the contribution of various solutes in a solution by the osmotic pressure of the solution. The osmolality unit is osmole per kilogram (osmole/kg), but the submultiple milliosmole per kilogram (mosmole/kg) is normally used.

Ovule

It is a solid pharmaceutical preparation, with a single dose, containing one or more active ingredients dispersed or dissolved in a suitable base which has several shapes, usually ovoid. They melt at body temperature.

Brazilian Pharmacopoeia Reference Standards

According to the WHO definition, pharmacopeial reference standards (PRef) are products of recognized uniformity, intended for use in tests where one or more of their properties will be compared with those of the substance under examination. They have a degree of purity suitable for their intended use.

The PRef is established and distributed by pharmacopeial authorities, whose value attributed to one or more of its properties is accepted without the need for comparison with another standard, intended for use in specific tests described in pharmacopoeial monographs. They include reference chemical substances, biological products, plant extracts and powders, radiopharmaceuticals, among others. The most commonly used related expression is: Pharmacopoeia Reference Chemical Substance.

Paste

It is an ointment containing a large quantity of dispersed solids (not less than 25%). It must meet the established specifications for ointments.

Losenge

It is a solid pharmaceutical preparation that contains one or more active ingredients, usually in a sweetened and flavored base. It is used for slow dissolution or disintegration in the mouth. It can be prepared by molding or by compression.

Hard losenge

Rigid losenge to be dissolved slowly.

Gummy losenge

Flexible and soft losenge of blends containing synthetic or natural polymers.

Perfume

It is the aromatic composition product obtained from natural or synthetic substances, which, in appropriate concentrations and vehicles, have as their main purpose scenting people or places, including extracts, scented waters, creamy perfumes, bath preparations and ambient scents, presented in liquid, gelled, creamy or solid form.

Atmospheric air gas separation plant (ASU – Air Separation Unit)

Industrial facility where atmospheric air is captured and, through processes of purification, cleaning, compression, cooling, liquefaction and distillation, it is fractionated in order to obtain and isolate oxygen, nitrogen and argon gases.

Fresh frozen plasma

It is the remaining liquid part of a whole blood unit obtained after centrifugation and separation of its cellular fractions, which must be completely frozen within four hours after collection of the whole blood that originated it, ensuring the maintenance of the integrity and concentrations of labile coagulation factors.

Human plasma for fractionation

It is the remaining liquid part of whole blood after separation of blood cell fractions using appropriate closed collection or centrifugation systems, which contains the labile coagulation factors. It contains an anticoagulant, conservative and preservative solution and is stored at a temperature of -30°C or lower. It is intended for the preparation of blood products in compliance with the Good Practices for Drugs Manufacturing.

Powder

It is the solid pharmaceutical preparation containing one or more dry active ingredients and with reduced particle size, with or without excipients

Aerosol powder

It is the pressure-packed powder containing a propellant gas and therapeutically active ingredients that are released upon activation of an appropriate valve system.

Effervescent powder

It is the powder that contains, in addition to the active ingredients, acidic substances and carbonates or bicarbonates, which release carbon dioxide when the powder is dissolved in water. It is intended to be dissolved or dispersed in water prior to administration.

Lyophilized powder for injectable solution

It is the sterile powder intended for the subsequent addition of liquid to form a solution. Prepared by lyophilization, a process that involves removing water from products by congealing at extremely low pressures.

Lyophilized powder for injectable suspension

It is the sterile powder intended for the subsequent addition of liquid to form a suspension. Prepared by lyophilization, a process that involves removing water from products by congealing at extremely low pressures.

Lyophilized powder for prolonged-release injectable suspension

It is the sterile powder intended for the subsequent addition of liquid to form a suspension. Prepared by lyophilization, a process that involves removing water from products by congealing at extremely low pressures. See general definition of prolonged release.

Mouthwash powder

It is the powder that must be dissolved in water to prepare the mouthwash, which is a liquid for mouth rinsing that acts on the gums and mucous membranes of the mouth and throat. It must not be swallowed.

Powder for solution

It is the powder intended to be reconstituted to form a solution.

Powder for injectable solution

It is the sterile powder intended for the subsequent addition of liquid to form a solution.

Powder for infusion solution

It is the sterile powder intended for reconstitution to form a solution for use by infusion. It is a sterile solution, normally isotonic with blood and used primarily for large volume administration.

Powder for suspension

It is the powder intended to be reconstituted to form a suspension.

Powder for injectable suspension

It is the sterile powder intended for the subsequent addition of liquid to form a suspension.

Powder for prolonged-release injectable suspension

It is the sterile powder intended for the subsequent addition of liquid to form a suspension. See definition of prolonged release.

Ointment

It is the semi-solid pharmaceutical preparation, for application to the skin or mucous membranes, which consists of a solution or dispersion of one or more active ingredients in low proportions in a suitable, usually non-aqueous base.

Expiry date

It is the time during which the product can be used, characterized as a useful life period and based on specific stability studies. The expiry date must be indicated on the primary and secondary packaging. When indicating month and year, the last day of that month is understood as the expiry date. The storage and transport conditions specified by the manufacturer must be maintained.

Topical semi-solid preparation

It is the preparation intended for application to the skin or certain mucous membranes for local action or percutaneous penetration of drugs, or for its emollient or protective action.

Herbal preparations

They are homogeneous preparations, obtained from herbal drugs subjected to specific treatments, such as extraction, distillation, expression, fractionation, purification, concentration or fermentation. Examples of herbal preparations: extracts, oils, juice capsules, processed exudates and herbal drugs that have been subject to size reduction for a specific application, for example, chopped herbal drugs for making medicinal teas or powdered for encapsulation.

Aseptic process

It is designed to prevent the contamination of sterile components by viable microorganisms or even in the intermediate phase of production.

Hygiene product

It is the product for external use; antiseptic or not; for cleaning or body disinfection, including soap, shampoo, toothpaste, mouthwash, antiperspirant, deodorant, shaving and after-shave products, astringent and others.

Dietary product

It is the product technically designed to meet the dietary needs of people with special physiological conditions.

Semi-finished product

It is any substance or mixture of substances still under the manufacturing process.

Purity

Degree to which a drug, raw material contains other foreign materials.

Off White

The term “almost white” is understood to be slightly grayish or yellowish, with a tendency to white. It is the translation of the English term “off white”.

Rodenticide

It is the preparation intended to combat rats, mice and other rodents, in homes, boats, enclosures and places of public use, containing active substances, isolated or in association, that do not pose a risk to life or health of humans and useful warm-blooded animals, when applied in accordance with the recommendations contained in its presentation.

Chemical identification reactions

They are reactions used to aid in the characterization of a substance. Although specific, they will only be sufficient to establish or confirm the substance's identity when considered in conjunction with other tests and specifications contained in the monograph. If the monograph does not specify otherwise, chemical reactions are carried out in test tubes with approximately 15 mm in internal diameter. Use 5 mL of the liquid or solution to be examined, adding three drops of reagent or of each reagent. The examination of the contents of the test tube must be carried out over the entire liquid layer, observing from top to bottom, in the direction of the longitudinal axis of the tubes, after five

minutes of standing. Usually, the order of preference of identification tests is presented in the monograph. When the order does not appear, all identification tests must be carried out.

Drug Extract Ratio (RDE)

It corresponds to the ratio between the amount of drug from natural origin used in the production of an extract and the final quantity of extract, expressed in weight (w/w) or volume (w/v).

Solvent Drug Ratio (SDR)

It corresponds to the ratio between the amount of herbal drug, expressed in mass, used in the preparation of an extract, and the amount of the first extraction solvent, expressed in mass (w/w) or volume (w/v).

Native Extract Drug Ratio (EDR_{native})

It corresponds to the ratio between the amount of herbal drug used in the preparation of an extract and the amount of native extract obtained. Thus, when there is no addition of adjuvants to a native extract, EDR and EDR_{native} must present the same values. On the other hand, the values observed for EDR and EDR_{native} should be different in operations where adjuvants are added to the native extract. Oleoresins are generally produced without the need for the addition of processing adjuvants, therefore EDR and EDR_{native} are generally identical. For soft and liquid extracts, for which the presence of excipients or processing adjuvants is required, EDR and EDR_{native} are identical (for example: generally 20 to 30% of water in soft extracts and ethyl alcohol in tinctures).

Reagents

They are substances used in tests, reactions, assays and pharmacopoeial assays, either as such or in solutions.

Tightly closed container

It is the one that protects its contents from loss and contamination by foreign solids, under the usual conditions of handling, storage, distribution and transport.

Hermetic container

It is impermeable to air, or any other gas, under the usual conditions of handling, storage, distribution and transport.

Opaque container

It is the one that prevents the visualization of the content, comprising all colors. It constitutes a light protection barrier.

Single dose container

It is the hermetic container that contains a certain quantity of the drug intended to be administered only once and that, once opened, cannot be closed with a guarantee of sterility.

Multi-dose container

It is the hermetic container that allows the removal of successive portions of its content, without modifying the concentration, purity and sterility of the remaining portion.

Perfectly closed container

It protects its contents from losses and contamination by foreign solids, liquids and foreign vapors, efflorescence, deliquescence or evaporation under the usual conditions of handling, storage, distribution and transport.

Translucent container

It is the one that allows the partial visualization of the content, comprising all colors except amber.

Transparent container

It is the one that allows the total visualization of the content, comprising all colors except amber.

Registration

It is the legal act that recognizes the adequacy of a product to health legislation, and its concession is granted by Anvisa. It is a control carried out before marketing, being used in the case of products that may present health risks. Products subject to sanitary regulation that are registered must meet the criteria established by laws and specific regulations in order to minimize any potential risks that may be related to the product.

Hydrolytic resistance or alkalinity

It is the test that quantifies the intensity of the chemical reaction between water and the alkali elements in the glass, especially sodium and potassium. This resistance determines the classification of the glass type.

Label

It is the printed or lithographed identification, as well as the words painted or engraved with fire, pressure or self-adhesive, applied directly on containers; casings; wraps; cartridges; or any other packaging protector, external or internal, which cannot be removed or altered during the use of the product and during its transportation or storage. Label manufacturing must comply with the regulations in force by the federal Health Regulation agency.

Cleanrooms

Room in which the concentration of airborne particles is controlled. It is built and used in such a manner as to minimize the introduction, generation and retention of particles within the room, in which other relevant parameters such as temperature, humidity and pressure are controlled as necessary.

Household sanitizer

It is the substance or preparation intended for sanitizing; home disinfection or disinfestation; of collective environments, private or public, in places of common use and in the treatment of water.

Human blood

It is a living tissue, circulating, connective, of a cellular, plasmatic and/or protein nature, contained within the cardiovascular system, performing multiple and complex functions that ensure the maintenance of life to the human body.

Human blood for transfusion

It is human whole blood *in vitro* from healthy donors collected in storing systems for the collection, storage and processing of human blood containing anticoagulant and preservative solution.

Oxygen Concentrator System (SCO)

System composed of equipment and tools that filters, retains moisture and concentrates oxygen from the atmospheric air through the process of molecular adsorption. This system is also known as an oxygen concentrator plant, *Pressure Swing Adsorption (PSA)*.

Closed system

System for administering parenteral solutions that, throughout the preparation and administration, does not allow the solution to come into contact with the environment.

Bottling systems for the collection, storage and processing of human blood or closed human blood collection systems

They are containers or plastic bags, containing or not an anticoagulant, conservative and preservative solution, intended for the collection, storage, fractionation and administration of human blood or derivatives. They are non-toxic, sterile, non-pyrogenic and disposable, and can be manufactured from one or several polymers, and depending on the case, from certain additives and are validated by their respective analytical methods.

Solution - pharmaceutical preparation

It is the liquid pharmaceutical preparation; clear and homogeneous, containing one or more active ingredients dissolved in a suitable solvent or mixture of miscible solvents.

Colorimetric solution

It is the solution used as a colorimetric standard for comparison purposes. It is called "CS".

Human albumin solution

Human albumin solution is a proteinaceous, sterile, pyrogen-free solution obtained from human plasma that meets the requirements of the *Human plasma for fractionation* monograph.

Molal solution

It is the solution that contains one mole of solute per kilogram of solvent.

Molar solution

It is the solution that contains one mole of the solute in 1000 mL of the solution. The multiples and submultiples of the molar solution are also designated by whole numbers or decimal fractions such as: 2 M; M; 0,5 M; 0,1 M; etc.

Volumetric solution

It is the reagent solution, of known concentration, intended for use in quantitative determinations. In FB 6 the concentrations of volumetric solutions are expressed in molarity. They are called “VS”.

Human blood-preserving anticoagulant solutions

They are solutions designed for the collection of human blood, aiming not only to make it incoagulable, but also to ensure the maintenance and morphofunctional and protein integrity of its cellular and plasmatic constituents.

Test solutions

They are indicator solutions in specific solvents and defined concentrations. They are called “TS”.

Reagent solutions

They are reagent solutions in specific solvents and defined concentrations. They are called “RS”.

Hyperimmune sera for human use

Hyperimmune sera are preparations containing purified immunoglobulins of animal origin that specifically neutralize bacterial toxins, bacteria, viruses or toxic components of the venom of one or more species of venomous animals.

Adjuvant substance

It is the specific purpose substance added to injectable preparations. This substance must be selected to increase the stability of the product; not cause interference with the therapeutic efficacy or with the active ingredient assay; or cause toxicity in the dose administered to the patient. The adjuvant substance can be solubilizing; antioxidant; chelating agent; buffer; antibacterial agent; antifungal agent; antifoaming agent and others, when specified in the individual monograph. The presence of the adjuvant substance must be clearly indicated on the labels of the primary and secondary packaging, in which the product is delivered for consumption. If there is no express contraindication, the air in the containers can be replaced by carbon dioxide or nitrogen. The addition of coloring substance is not allowed.

The maximum limits for some adjuvants are listed below, if the monograph does not specify otherwise:

- a) for agents containing mercury or cationic surfactant compounds — 0.01%;
- b) for chlorobutanol, cresol, and phenol-type agents — 0.5%;
- c) for sulfur dioxide, or equivalent quantity of potassium or sodium sulfite, bisulfite or metabisulfite — 0.2%.

Characterized chemical substance

CRS used in the absence of a Pharmacopeial CRS. This CRS must be characterized by means of adequate tests and the values obtained must be properly documented.

Brazilian Pharmacopoeia Chemical Reference Substance (CRS-BP)

It is established and made available by the Brazilian Pharmacopoeia Commission, following the WHO principles, and made official by Anvisa, with its use mandatory throughout the national territory. In the absence of a CRS-FB, the use of a CRS established by other recognized pharmacopoeias is allowed, in accordance with the legislation in force.

Standards for Atomic Absorption Spectrophotometry are identified by the name of the metal, followed by the acronym RSA (Reagent Solution for Atomic Absorption).

In house working standard

It is established by comparison with a Pharmacopoeial CRS, through pharmacopoeial tests, or duly validated, and registered by the laboratory that will use it. In this situation, analytical records must be kept and periodic controls carried out, using a Pharmacopoeial CRS.

Unsaponifiable substances

Unsaponifiable substances are those remaining from the saponification reaction, non-volatile at 100 – 105 °C and which were carried in the process of extracting the substance to be tested.

Suppository

It is a solid pharmaceutical preparation of various sizes and shapes adapted for introduction into the rectal, vaginal or urethral orifice of the human body, containing one or more active principles dissolved in a suitable base. They are usually absorbed, melt or dissolve at body temperature.

Suspension

It is the liquid pharmaceutical preparation that contains solid particles dispersed in a liquid vehicle, in which the particles are not soluble.

Aerosol suspension

It is a pressure-packed suspension containing a propellant gas and therapeutically active ingredients that are released upon activation of an appropriate valve system.

Prolonged release suspension

It is the liquid pharmaceutical preparation that contains solid particles dispersed in a liquid vehicle, in which the particles are not soluble. See definition of prolonged release.

Delayed release suspension

It is the liquid pharmaceutical preparation that contains solid particles dispersed in a liquid vehicle, in which the particles are not soluble. See definition of delayed release.

Suspension drops

It is the suspension intended for administration in the form of drops.

Injectable suspension

It is the sterile suspension.

Prolonged-release injectable suspension

It is the sterile suspension. See definition of prolonged release.

Spray Suspension

It is the suspension administered in the form of a liquid finely divided by a jet of air or steam.

Troche tablet

It is the solid pharmaceutical preparation prepared from a mass made with a hydroalcoholic solution, the active ingredient and lactose, or from the grinding process moistened with a hydroalcoholic solution. It is molded in tablet presses and is fragile and brittle.

Buffer

It is the preparation based on salts that are capable of supporting variations in the activity of hydrogen ions.

Fixed cryogenic tank or stationary tank

Container, thermally insulated, suitable for storing medicinal gases in the form of cryogenic liquid.

Mobile cryogenic tank or mobile storage tank

Mobile container, thermally insulated, suitable for storing medicinal gases in the form of cryogenic liquid.

Congealing point or temperature

The temperature or congealing point of a liquid or melted solid is the highest temperature at which it solidifies. For pure substances that melt without decomposition, the liquid congealing point is equal to its melting point.

Boiling point or temperature

The temperature or boiling point of a liquid is the corrected temperature at which the liquid boils under a vapor pressure of 101.3 kPa (760 mm of Hg).

Melting point or temperature

The temperature or melting point of a substance is the temperature at which it is completely melted.

Hydrostatic pressure test

Testing performed for safety reasons to ensure cylinders and tanks withstand the pressures for which they were designed.

Tincture

It is the alcoholic or hydroalcoholic preparation resulting from the extraction of herbal drugs or from the dilution of their extracts. They are obtained by liquid extraction using 1 part by mass of herbal drug and 10 parts of extraction solvent, or 1 part by mass of herbal drug and 5 parts of extraction solvent. The ratio can be in w/w or w/v. Alternatively, they can be obtained using either 1 part by mass of herbal drug and sufficient quantity of extraction solvent to produce 10 parts by mass or volume of tincture or 1 part by mass of herbal drug and sufficient quantity of extraction solvent to produce 5 parts, by mass or volume, of tincture. Other proportions of herbal drug and extraction solvent can be used. It is classified as simple or compound, as prepared with one or more herbal drugs.

Vaccines

Biological products that contain one or more antigenic substances that, when inoculated, are able to induce specific active immunity and protect against disease caused by the infectious agent that originated the antigen.

D-value (decimal reduction time)

It is the time, in minutes, required to reduce the microbial population by 90% or a logarithmic cycle.

F-Value

It is a measure of sterilizing effectiveness, that is, the number of minutes of thermal sterilization by steam at a given temperature supplied to a container or product unit, at a given Z value.

Z-value

It is the temperature rise, in degrees, necessary to reduce the *D-Value* by 90% or to produce a reduction of a logarithmic cycle in the thermal resistance curve.

Valve

Device capable of modifying the pressure or output (flow) of gases, or vacuum, either in the cylinder or in the centralized gas system.

Check, non-return or one-way valve

Valve that allows the passage of gas or provides vacuum in only one direction.

Routes of Administration

It is the place in the body through which the drug is administered.

Viscosity

It is the expression of the resistance of liquids to flowing, that is, to the displacement of part of their molecules on neighboring molecules. The viscosity of liquids comes from internal friction, that is, from the forces of cohesion between molecules relatively close together. As temperature rises, the average kinetic energy of the molecules increases, the amount of time the molecules spend together decreases (on average), the intermolecular forces become less effective and the viscosity is lower.

The dynamic unit, in the CGS System, of viscosity is the *poise*. The CGS System of Units is a system of physical measurement units, or dimensional system, of LMT typology (length, mass, time), whose base units are the centimeter for length, gram for mass, and second for time .

Syrup

It is an oral solution characterized by high viscosity, conferred by the presence of sucrose or other sugars or other thickening and sweetening agents in its composition. Syrups usually contain authorized flavoring and/or coloring agents. When not intended for immediate consumption, authorized antimicrobial preservatives must be added.

GENERAL INFORMATION

Water

The water mentioned in tests, reactions and tests is *purified water*. For injectable preparations, *water for injections*, described in an individual monograph, should be used. When the use of *carbon dioxide-free water* is prescribed, use purified water boiled for at least five minutes and protected from atmospheric air during cooling and storage.

Volumetric instruments

Volumetric instruments are used to measure volume in tests, assays and pharmacopoeial assays, and must be calibrated at a temperature of 25°C. If the volumetric instrument has not been calibrated at 25° C, the volume measurements must be carried out at the temperature indicated therein. In volume measurements, the lower level of the meniscus of the liquid contained in volumetric devices must touch the upper part of the reference line, with the line of sight in the same plane. In cases of strongly colored or opaque liquids, the upper edge of the meniscus is used as a reference, in the horizontal plane of vision. Volumetric devices for transferring liquids (pipettes or burettes), as they have been gauged with water, will only be able to supply exactly the indicated volume when the liquids to be measured have approximately the viscosity, surface tension and density of the water.

Conservation

Pharmacopoeial substances must be preserved under conditions such as to prevent their contamination or deterioration. The conservation conditions for pharmacopoeial substances are included in the respective monographs.

Protecting from light means that the substance must be kept in an opaque container or capable of preventing the action of light.

Protecting from dust means that the substance must be stored in a stoppered flask and wear protective hood.

In the monograph, the temperature conditions under which the substance must be preserved can be defined, using terms described below.

In the freezer – At a temperature between -20 °C and 0 °C.

In the refrigerator – At a temperature between 2 °C and 8 °C.

Cool place – Environment whose temperature remains between 8 °C and 15 °C.

Cold place – Environment whose temperature does not exceed 8 °C.

Room temperature – Temperature normally found in a working environment between 15°C and 30°C.

Hot place – Environment whose temperature remains between 30 °C and 40 °C.

Excessive heat – Indicates temperatures above 40 °C.

When it is necessary to keep a drug in a cool place, it can be kept in a refrigerator, if not indicated differently in the individual monograph.

When conservation conditions are not specified in the monograph, they include protection against humidity, congealing and excessive heat.

Substance description

Information relating to the description of a substance is generic and is intended for a preliminary assessment of its integrity. The description itself is not indicative of purity and should be combined with other pharmacopoeial tests to ensure that the substance complies with the monograph.

Desiccation to constant weight

This expression means that drying must continue until two consecutive weighing do not differ by more than 0.50mg per gram of the substance under examination, and the second weighing must be carried out after an additional hour of drying under the specified conditions.

Desiccator

A desiccator is understood to be a container that can be perfectly closed, of adequate shape and dimensions that allows maintaining an atmosphere of low humidity rate through desiccant agents introduced in it, such as: silica gel, calcium chloride, phosphorus pentoxide, sulfuric acid, among others.

Reduced pressure desiccator is what makes it possible to maintain an atmosphere of low humidity at a reduced pressure of a maximum of 6.7 kPa (approximately 50mm of mercury), or at the pressure indicated in the monograph.

Assay and determination of potency

When the result of a test or an assay is expressed in relation to the anhydrous or desiccated substance; in relation to the substance; or any other specific basis, the determination of the water content or loss on drying, or of another designated property, is carried out according to the method described in the monograph of the respective substance, or according to that described on the labeling.

Identification tests

Identification tests make it possible to verify, with an acceptable level of certainty, that the identity of the material under examination is in accordance with its packaging label. Although specific, they are not necessarily sufficient to establish absolute proof of identity. However, failure to comply with the requirements of an identification test can lead to errors in the material labeling. Other tests and specifications in the monograph contribute to confirming the identity of the article under examination.

Some identification tests must be considered conclusive, such as: infrared, spectrophotometry with specific absorption and high performance liquid chromatography coupled to spectrophotometry. These tests must be carried out in addition to the counter ion test, when applicable.

Structure of monographs

The raw material monographs are identified by their Common Brazilian Denominations (DCB), written in capital letters and centralized. Moreover, it is also comprised:

- whenever possible, the Latin name proposed by the INN – *International Non-proprietary Names* – international generic names of the World Health Organization;
- the substance chemical structure;
- molecular formula followed by molar mass;
- the Common Brazilian Denomination and related number;
- chemical name, according to the ACS – American Chemical Society;
- CAS – Chemical Abstracts Service registration;
- Monograph text

The monographs of pharmaceutical preparations are identified by the name of the corresponding raw material, followed by the name of the pharmaceutical preparation.

Expression of concentrations

Percent concentrations are expressed as follows.

Percent w/w (weight by weight) or % w/w – Expresses the number of g of a component in 100 g of mixture.

Percent w/v (volume weight) or % w/v – Expresses the number of g of a component in 100 mL of solution.

Percent v/v (volume in volume) or % v/v – Expresses the number of mL of a component in 100 mL of solution.

Percent v/w (volume by weight) or % v/w – Expresses the number of mL of a component in 100 g of mixture.

The expression percent, used without further category, means: mixture of solids and semi-solids, percent w/w; for solutions or suspensions of solids in liquids, percent w/v; for liquid solutions, percent v/v; for solutions of gases in liquids, percent w/v; to express essential oil content in herbal drugs, percent v/w.

Impurities

The tests described in the monographs limit the impurities to quantities that ensure the quality of the drug. The fact that the trials do not include an infrequent impurity does not mean that it can be tolerated.

Incineration to constant weight

This expression means that incineration must proceed at 800 ± 25 °C, or at another temperature indicated in the monograph, until two consecutive weighing do not differ by more than 0.5mg per gram of the substance under examination, and the second weighing must be carried out after fifteen minutes of additional incineration.

Interpreting numerical data accuracy and tolerance limits

The desired precision in tests, reactions and pharmacopoeial assays is indicated by the number of decimals presented in the text. For example, the numeric value 20 indicates values not lower than 19.5 and no higher than 20.5; the numeric value 2.0 indicates values not lower than 1.95 and not higher than 2.05; the numeric value 0.20 indicates values not lower than 0.195 and not higher than 0.205.

Tolerance limits, expressed numerically as a maximum and minimum value, indicate the purity of a pharmacopoeial substance. These values can be expressed as percentage or absolute numbers.

The variation range must be strictly observed, and values outside the maximum and minimum limits are not tolerated.

Pressure measurements

The expression pascal (Pa), used for pressure measurements such as arterial, atmospheric or internal to a device, refers to the use of manometers or barometers calibrated in relation to the pressure exerted by the force of one Newton uniformly distributed on a flat surface of 1 m² of area perpendicular to the direction of the force; one pascal is equivalent to 7.5×10^{-3} mm of mercury.

Nomenclature on the degree of molecules hydration

When the hydration degree of pharmaceutical ingredients is not mentioned in the nomenclature, it is an anhydrous substance.

Odor

The expressions: *odorless*; *practically odorless*; *slight characteristic odor*; or their variations, are used by examining the sample after being exposed to air for fifteen minutes, when dealing with packages of up to 25 g recently opened. In case of larger packages, transfer samples of approximately 25 g to a 100 mL capsule capacity.

The odor characterization is descriptive only and cannot be considered as a standard of purity, except in cases where a particular, disallowed odor is indicated in the individual monograph.

Preparation of solutions

All solutions used in tests, assays and reactions are prepared with purified water, unless otherwise indicated in the individual monograph.

The term *recently prepared*, referring to the preparation of solutions used in tests, assays and reactions, indicates that the solution must be prepared, maximum, 24 hours before the assay is carried out.

Reduced pressure

The term *reduced pressure* means pressure lower than or equal to 6.7 kPa (approximately 50 mm of mercury), if not otherwise indicated in the monograph. When *desiccation under reduced pressure on desiccant agent* is indicated in the monograph, the operation must be carried out under reduced pressure in a desiccator or other suitable equipment.

Manufacturing processes

In the manufacture of injectable products, tablets, capsules or other pharmacopoeial preparations, the use of adjuvant substances, described in the monographs and added for a specific purpose, is allowed. They must be innocuous and must not have an adverse influence on the therapeutic efficacy of the active substance contained in the preparation, nor interfere with tests and determinations. Whichever method is used, the final product must correspond to the specifications included in the Brazilian Pharmacopoeia, 6th edition.

Blank test

The terms: *carry out blank parallel*; *carry out a blank test*; or *carry out a blank assay* means repeating the determination under identical conditions and with identical quantities of reagents, omitting only the substance under examination.

Containers for injectables

Containers for injectable preparations must be made of materials that do not interact with the contents and have sufficient transparency to allow visual inspection. The lids, when used, cannot influence the drug composition or conservation, offering a perfect sealing, even after being perforated several times. Containers for injectable preparations are classified into:

- single dose containers;
- multi-dose containers;
- infusion container.

Single dose containers, ampoules and cartridges for dental use are flasks made of glass or suitable plastic material; closed by melting glass or with the use of fixed or movable plastic operculum. The content should only be used in a single dose and cannot be reused.

Multi-dose containers are strong-walled glass flasks which, after being filled with liquid preparations or with solids to be dissolved or suspended, are sealed with a lid of another material. The content of these flasks can be removed for single or multiple dose administration.

Infusion containers are flasks with a capacity of more than 50 mL, and may reach 1000 mL, sealed with a lid made of another material or not, made of glass or plastic. Medicines stored in these types of containers must be administered in one go, using sterile equipment, and cannot contain bactericidal or antifungal agents. The use of other types of adjuvants must be considered carefully.

Solubility

The indicated solubility should not be considered strictly as a physical constant, but as a complement to other tests, and may have a definitive value when the substance does not present the minimum solubility required, especially when the solvent is water.

The indications on the solubility to which reference is made are carried out at a temperature of 25 ± 5 °C. The term *parts* refers to the number of milliliters of solvent per gram of solid to be dissolved. The approximate solubilities established in the monographs are designated in descriptive terms, whose meanings are listed in **Table 1**:

Table 1 – Descriptive terms of solubility and their meanings.

<i>Descriptive term</i>	<i>Approximate volumes of solvent in milliliters per gram of substance</i>
Very soluble	Lower than 1 part
Freely soluble	From 1 to 10 parts
Soluble	From 10 to 30 parts

Sparingly soluble	From 30 to 100 parts
Slightly soluble	From 100 to 1000 parts
Very slightly soluble	From 1000 to 10000 parts
Practically insoluble or insoluble	Higher than 10000 parts

Temperature

All temperatures in FB 6 are expressed on the Celsius scale, and measurements are taken at 25°C, except for density measurement and if not indicated differently in the individual monograph.

Units of measurement

The units included in the International System of Units (IS) are adopted in this Pharmacopoeia, as listed in **Annex B**.

Aqueous vehicles

Water for injections is generally used as a vehicle for aqueous injectables. *Sodium chloride* solutions or *Ringer's solution* or other suitable solutions, prepared with *water for injections*, may be used in part or in whole rather than just *water for injections*, if monograph does not otherwise specify.

Non-aqueous vehicles

Non-aqueous vehicles used partially or fully to obtain injectable preparations can be miscible or immiscible with water. Among water miscible vehicles, the most used are polyalcohols and ethylene oxide polymers. Among those immiscible with water, the most used are fixed oils of vegetable origin and mono- and diglycerides of fatty acids.

Fixed oils are odorless or almost odorless and their odor and taste should not resemble rancidity. They must meet the requirements specified in the monographs and have the characteristics described below.

- cooling test — transfer a quantity of fixed oil, previously desiccated at 105 °C for two hours and cooled at room temperature in a desiccator containing silica gel, to a colorless cylindrical glass container, with an internal diameter of approximately 25 mm. Close the container and soak for four hours in water maintained at 10°C. The liquid must remain clear enough so that a 0.5mm thick black line can easily be seen when held vertically behind the cylinder and against a white background;
- Saponification value — between 185 and 200 (**5.5.29.8**);
- iodine value — between 79 and 128 (**5.5.29.10**);
- unsaponifiable substances — reflux 10 mL of the oil in water bath with 15 mL of sodium hydroxide (1:16) and 30 mL of ethyl alcohol, shaking occasionally until the mixture becomes clear. Transfer the mixture to a porcelain crucible, evaporate the ethyl alcohol in a water bath and mix the residue with 100 mL of water. Solution must result;
- free fatty acids — the free fatty acids in 10g of the oil should consume a maximum of 2 mL of 0.02 M sodium hydroxide.

Synthetic mono- or diglycerides of fatty acids must meet the following requirements:

- they are liquid and remain clear when cooled to 10°C;
- iodine value — not exceeding 140 (**5.5.29.10**).

Non-aqueous vehicles must be selected with special care, as they cannot be irritating, toxic or sensitizing and must not interfere with the therapeutic efficacy of the preparation.

5 GENERAL METHODS

5.1 METHODS APPLIED TO PHARMACEUTICAL PREPARATIONS

5.1.1 DETERMINATION OF WEIGHT

The test applies to solid dosage forms in unit dose (uncoated tablets, coated tablets, lozenges, hard and soft capsules and suppositories), solid dosage forms packaged in unit dose containers (sterile powders, lyophilized powders, powders for injectables and powders for reconstitution for oral use) and to solid and semi-solid pharmaceutical preparations packaged in multi-dose containers (granulates, powders, gels, creams, ointments and powders for reconstitution).

Weighing must be done on scales of adequate sensitivity.

PROCEDURE FOR UNIT DOSE PRODUCTS

For unit dose products, the test makes it possible to verify whether the units of the same batch have uniform weight. To carry out the test, it is necessary to determine, in advance, the average weight of the units in the batch.

Uncoated or film-coated tablets

Weigh 20 tablets individually and determine the average weight. A maximum of two units can be tolerated outside the limits specified in **Table 1**, in relation to the average weight, however, none may be above or below twice as much as the indicated percentages.

Sugar coated tablets (dragees)

Weigh 20 sugar coated tablets individually and determine the average weight. A maximum of five units can be tolerated outside the limits specified in **Table 1**, in relation to the average weight, however, none can be above or below twice as much as the indicated percentages.

Hard capsules

Weigh 20 units individually, remove the contents of each one, clean properly and weigh again. Determine the weight of the contents of each capsule by the difference in weight between the filled and empty capsules. With the values obtained, determine the average weight of the content. Not more than two units can be tolerated outside the limits specified in **Table 1**, in relation to the content average weight, however, none may be above or below twice as much as the indicated percentages.

Soft capsules

Proceed as described for *Hard capsules*. To determine the average weight of the contents, cut the pre-weighed capsules and wash them with ethyl ether or other suitable solvent. Place the casings exposed to air, at room temperature, until the solvent has completely evaporated. Weigh again.

Suppositories and ovules

Weigh 20 suppositories or ovules individually and determine the average weight. A maximum of two units can be tolerated outside the limits specified in **Table 1**, in relation to the average weight, however, none may be above or below twice as much as the indicated percentages.

Sterile powders, lyophilized powders and powders for injectables

Carry out the test with 20 units. Remove metal seals in case of ampoule flasks. Remove labels that could be damaged during testing. If necessary, dry the outer surface of the containers. Weigh the 20 units individually, with their respective lids. Remove the contents and wash the respective containers using water and then ethyl alcohol. Dry in an oven at 105 °C, for one hour, or at temperatures lower than that, depending on the nature of the material, until constant weight. Cool to room temperature, replace the lid and weigh again. The difference between the two weights represents the content weight. Determine the average weight of the content of the 20 units. Not more than two units can be tolerated outside the limits specified in **Table 1**, in relation to the content average weight, however, none may be above or below twice as much as the indicated percentages.

Powders or granules for reconstitution (oral use)

Proceed as described for *Sterile powders, lyophilized powders and powders for injectables*. Not more than two units can be tolerated outside the limits specified in **Table 1**, in relation to the content average weight, however, none may be above or below twice as much as the indicated percentages.

Table 1 – Evaluation criteria for determination of weight for pharmaceutical preparations in unit dose.

<i>Pharmaceutical preparations in unit dose</i>	<i>Average weight</i>	<i>Variation limits</i>
Uncoated or film-coated tablets, effervescent tablets, sublingual tablets, vaginal tablets and lozenges	80mg or less	± 10.0%
	more than 80mg and less than 250mg	± 7.5
	250mg or more	± 5.0
	25 mg or less	± 15.0%
Sugar coated tablets (dragees)	more than 25mg and up to 150mg	± 10.0%
	more than 150mg and less than 300mg	± 7.5
	300mg or more	± 5.0
Hard and soft capsules, vaginal capsules	less than 300 mg	± 10.0%
	300mg or more	± 7.5
Suppositories and ovules	Independent of average weight	± 5.0
Sterile powders, lyophilized powders and powders for injectables	more than 40mg*	± 10.0%
	less than 300 mg	± 10.0%
Powders for reconstitution (oral use)	300mg or more	± 7.5%

(*) If the average weight is 40mg or less, submit to the *Uniformity test of unit doses* (5.1.6).

PROCEDURE FOR MULTI-DOSE PRODUCTS

For products packed in containers for multiple doses, the test allows checking the homogeneity of the filling.

Powders for reconstitution (oral and parenteral use)

Weigh 10 units individually. Remove the contents and wash the respective containers using adequate solvent. Dry, cool to room temperature and weigh again. The difference between the two weights represents the content weight.

Determine the average weight of the content of the 10 units. Individual values do not differ by $\pm 10\%$ from the average weight.

Granules, powders, gels, creams and ointments

Note: to carry out the test, it is necessary to know the filling nominal quantity.

Weigh 10 units individually. Remove the contents and wash the respective containers using adequate solvent. Dry, cool to room temperature and weigh again. The difference between the two weights represents the content weight.

Determine the average weight of the content of the 10 units. The average weight of the contents is not inferior to the declared weight and the individual weight of none of the units tested is inferior to the percentage indicated in **Table 2**, in relation to the declared weight.

If this requirement is not met, determine the individual weight of the content of 20 additional units. The average weight of the 30 contents is not inferior to the declared weight and the individual weight of not more than one unit in 30 is inferior to the percentage indicated in **Table 2**, in relation to the declared weight.

Table 2 – Evaluation criteria for determination of weight for pharmaceutical preparations in multi-doses.

<i>Pharmaceutical forms in multi-doses</i>	<i>Declared weight</i>	<i>Minimum percentage in relation to the declared weight</i>
Granules, powders, gels, creams and ointments	up to 60 g	90.0
	above 60 g and up to 150 g	92.5
	above 150.0 g	95.0

5.1.2 DETERMINATION OF VOLUME

The determination of volume test is required for liquid products in multi-dose containers and liquid products in single-dose containers. The test applies to both liquid preparations and liquid preparations obtained from powders for reconstitution. The test is not required for liquid products in single-dose containers when, in the individual monograph, there is an requirement for *Uniformity of unit doses* (5.1.6).

PROCEDURE

Liquid products in multi-dose containers (except injectables)

Separate 10 units Remove metal seals, when applicable. Remove labels that could be damaged during testing. Weigh each container with their respective lids individually. Homogenize, remove and combine the contents and reserve for determination of mass density. Wash containers and lids with water and then with ethyl alcohol. Dry in an oven at 105 °C, for one hour, or at a temperature compatible with the container material, until constant weight. Cool to room temperature, replace the lid and further corresponding parts and weigh again.

The difference between the two weights represents the content weight. Determine the corresponding individual volumes (V), in mL, using the expression:

$$V = \frac{m}{\rho}$$

where

m = content weight, in g;

ρ = mass density of the product, in g/mL, determined at 20 °C, as described in *Determination of mass density and relative density* (5.2.5).

From the values obtained, calculate the average volume of the units tested. The average volume is not inferior to the declared volume and the individual volume of none of the units tested is inferior to 95.0% of the declared volume.

Liquid products in multi-dose containers obtained from powders for reconstitution (except injectables)

Separate 10 units Reconstitute each unit as indicated on the label. Proceed as described in *Liquid products in multi-dose containers (except injectables)*

From the values obtained, calculate the average volume of the units tested. The average volume is not lower than the declared volume and the individual volume of none of the units tested is lower than 95.0% or greater than 110.0% of the declared volume.

Liquid products in single-dose containers (except injectables)

Separate 10 units Pour, separately, the content of each unit into calibrated dry graduated cylinders with a capacity that does not exceed 2.5 times the volume to be measured, taking precautions to avoid the formation of bubbles. Allow the liquid to drain for five seconds, unless otherwise indicated in the individual monograph. Carry out the measurement.

From the values obtained, calculate the average volume of the units tested. The average volume is not lower than the declared volume and the individual volume of none of the units tested is lower than 95.0% or greater than 110.0% of the declared volume.

Injectable liquid products

The test applies to liquid injectable products packaged in containers such as ampoules, vials, plastic bags, plastic vials, carpule syringes or pre-filled syringes. The containers are filled with a small excess of volume, according to the product features, to allow the administration of the declared volume. The minimum volume excesses recommended in **Table 1** are generally sufficient to allow the removal and administration of the declared volume.

Table 1 – Recommended volume excess for injectable liquid products.

<i>Declared volume (mL)</i>	<i>Minimum volume excess recommended</i>	
	<i>movable (mL)</i>	<i>viscous (mL)</i>
0.5	0.10	0.12
1.0	0.10	0.15
2.0	0.15	0.25
3.0	0.20	0.35
4.0	0.25	0.45
5.0	0.30	0.50
10.0	0.50	0.70
20.0	0.60	0.90
30.0	0.80	1.20
50.0 or more	2%	3%

Suspensions and emulsions must be agitated before removing the content and before determination of density. Oily or highly viscous preparations can be heated, if necessary, as indicated on the label or to a maximum of 37°C, and shaken vigorously before removing the content. The contents are then cooled to between 20°C and 25°C before volume measurement.

For injectables in single-dose containers, test six units if the declared volume is equal to or greater than 10 mL, 10 units if the declared volume is greater than 3mL and less than 10mL, or 12 units if the declared volume is equal to or less than 3 mL. Remove the total contents of each unit with the aid of a syringe with a capacity that does not exceed three times the volume to be measured, fitted with a number 21 needle and not less than 2.5 cm long. Eliminate any bubbles that may exist in the needle and syringe and transfer the contents of the syringe, without emptying the needle, to a dry, calibrated measuring cylinder with a capacity not exceeding 2.5 times the volume to be measured. Alternatively, the contents of the syringe can be transferred to a dry tared graduated beaker, the volume being calculated by the weight of the liquid, in g, divided by its density. For containers with a declared volume of 2 mL or less, the contents of the containers can be collected to obtain the volume required for the assay, using separate dry syringes and needles for each container. The contents of containers with a declared volume of 10 mL or more can be determined by emptying the contents of each container directly into calibrated measuring cylinders or tared beakers.

The volume of each container examined is not inferior to the declared volume. In the case of containers with a declared volume of 2 mL or less, the volume of the collected contents is not inferior to the sum of the declared volumes of the containers used in the test.

For injectables in multi-dose containers labeled to contain a specific number of doses of a given volume, select a unit and proceed as described for injectables in single-dose containers, using the number of separate syringes and needles equivalent to the number of doses specified on the label . The volume dispensed by each syringe is not inferior to the declared volume per dose.

For injectables in cartridges or pre-filled syringes, test one unit if the declared volume is equal to or greater than 10 mL, three units if the declared volume is greater than 3 mL and less than 10 mL, or five units if the declared volume is equal or less than 3 mL. Adjust the necessary tools to the containers for their use (needle, plunger, syringe body), when applicable, and transfer the content of each container, without emptying the needle, to a tared dry graduated beaker, pushing the plunger slowly and regularly. Calculate the volume, in milliliters, by dividing the liquid weight, in g, by its density. The volume of each container is not inferior to the declared volume.

For large volume injectable preparations (parenteral infusions), select two units and transfer the content of each container to calibrated dry measuring cylinders with a capacity that does not exceed 2.5 times the volume to be measured. The volume of each container is not inferior to the declared volume.

5.1.3 DETERMINATION OF MECHANICAL STRENGTH IN TABLETS

Mechanical strength tests, such as hardness and friability, are considered official within the legal context of this Pharmacopoeia, constituting useful elements in evaluating the integral quality of tablets. These tests aim to demonstrate the tablets' resistance to breakage caused by falls or friction.

5.1.3.1 HARDNESS TEST

The hardness test makes it possible to determine the tablet strength to crushing or breaking under radial pressure. The hardness of a tablet is proportional to the compression force and inversely proportional to its porosity.

The test applies mainly to uncoated tablets. The test consists of submitting the tablet to the action of a device that measures the force, applied diametrically, necessary to crush it. Force is measured in newtons (N).

APPARATUS

Different types of apparatus can be used, which basically differ in the mechanism used to exert pressure. Force can be exerted manually or mechanically. As the pressure increases, a plunger, plate or piston applies a certain force on the tablet, supported by a fixed base. The apparatus is calibrated to a 1 N accuracy.

PROCEDURE

The test is carried out with 10 tablets, eliminating any surface residue before each determination. The tablets are individually tested, always following the same orientation (consider the shape, presence of groove and engraving). Express the result as the average of the values obtained in the determinations. The test result is informative.

5.1.3.2 FRIABILITY TEST

The friability test makes it possible to determine the tablets strength to abrasion, when subject to the mechanical action of a specific apparatus. The test applies solely to uncoated tablets.

The test consists of accurately weighing a determined number of tablets, submitting them to the action of the apparatus and removing after 100 rotations. After removing any powder residue from the tablets, they are weighed again. The difference between the initial and final weight represents the friability, measured from the percentage of powder lost.

APPARATUS

The apparatus (**Figure 1**) consists of a rotating cylinder, with (287.0 ± 4.0) mm in diameter and (38.0 ± 2.0) mm in depth, made of transparent synthetic polymer with polished internal faces, of low static activity, which rotates around its axis at a speed of (25 ± 1) revolutions per minute. One of the cylinder faces is removable. The tablets are collected at each turn of the cylinder by a curved projection with an inner radius of (80.5 ± 5.0) mm that extends from the center to the outer wall of the cylinder, and taken to a height of (156.0 ± 2.0) mm, from which they fall repeatedly.

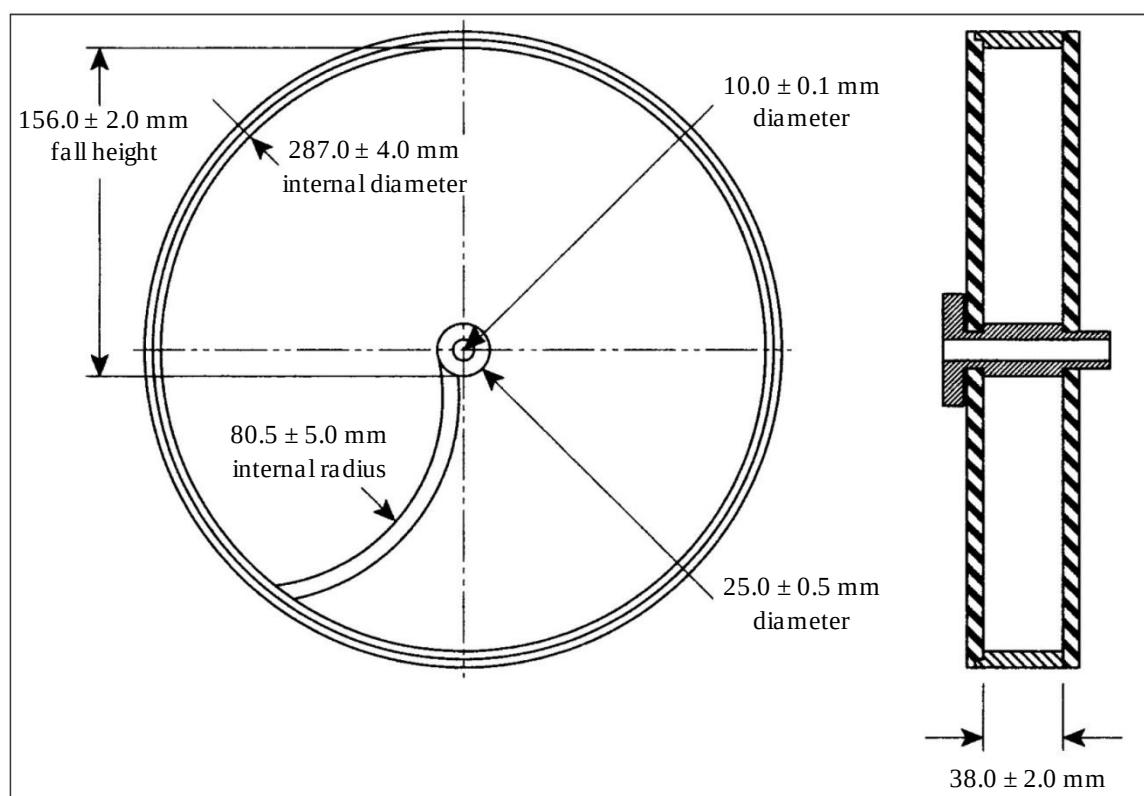


Figure 1 - Apparatus for friability test (friabilimeter).

PROCEDURE

For tablets with an average weight equal to or less than 0.65 g, use 20 tablets. For tablets with an average weight greater than 0.65 g, use 10 tablets. Accurately weigh the tablets and place them in the apparatus. Set speed to 25 revolutions per minute and test time to four minutes. When time elapsed, remove any residue of powder from the surface of the tablets and weigh again. No tablet may be broken, chipped, cracked or split at the end of the test. Tablets with a loss equal to or less than 1.5% of their weight or the percentage established in the monograph are considered acceptable. If the result is doubtful or if the loss is greater than the specified limit, repeat the test twice more, considering, in the evaluation, the average result of the three determinations.

5.1.4 DISINTEGRATION TESTS

5.1.4.1 DISINTEGRATION TEST FOR TABLETS AND CAPSULES

The disintegration test checks if tablets and capsules disintegrate within the specified time limit, when six batch units are subject to the action of specific apparatus under described experimental conditions.

The test applies to uncoated, film-coated or sugar-coated tablets (dragees), enteric-coated tablets, sublingual tablets, soluble tablets, dispersible tablets, hard capsules and soft capsules. It can be applied to chewable tablets; in this case, the conditions and evaluation criteria will appear in the individual monograph. The test does not apply to lozenges and tablets or controlled (prolonged) release capsules.

Disintegration is defined, for the purposes of this test, as the state in which no residue from the tested units (capsules or tablets) remains on the wire mesh of the disintegration apparatus, except for insoluble fragments of tablet coatings or capsule shells. Units that turn into a creamy mass during the test are also considered as disintegrated, as long as they do not present a palpable core.

APPARATUS

It consists of a system of baskets and tubes (**Figure 1**), a suitable container for the immersion liquid (a graduated beaker with a capacity of 1 liter), a thermostat to keep the liquid at $(37 \pm 1)^\circ\text{C}$ and a mechanism to move the basket and tubes in the immersion liquid, with constant frequency and specific path. The volume of the immersion liquid must be sufficient so that, when reaching the highest point of the route, the bottom of the basket is at least 25 mm below the surface of the liquid, and that at the lowest point it is at least 25 mm from the bottom of the beaker. Upward and downward movements should have the same speed and the change in direction of movement should be smooth.

The basket consists of six tubes of clear glass or acrylic, open on both sides. The dimensions of the tubes are: length (77.5 ± 2.5) mm, internal diameter between 20.7 mm and 23.0 mm, and wall thickness of approximately 2 mm.

The tubes are kept vertically, adapting at each end of the basket a disk of suitable transparent material, with a diameter between 88.0 mm and 92.0 mm and thickness between 5.0 mm and 8.5 mm, having six holes in which the tubes are introduced. The six holes are equidistant from the center of each disc, being equally spaced. On the external face of the lower disc is a stainless-steel wire mesh (wire diameter of (0.635 ± 0.030) mm, with an opening between 1.8 mm and 2.2 mm, attached to the disc by three screws.

For capsule disintegration testing, a stainless steel wire mesh, similar to the one fitted to the basket lower disc, or other suitable device can be fitted to the external face of the upper disc to prevent the capsules from escaping from the tubes during testing.

The parts that constitute the basket are assembled and held firmly together by a central metal axis, with a diameter of about 5 mm. The upper end of the central axis must have a device to attach the basket to the mechanism that produces the vertical movement of the system.

When indicated, a cylindrical disc of suitable transparent material must be added to each tube of the basket, with a relative density between 1.18 and 1.20, diameter of (20.70 ± 0.15) mm, and thickness of (9.50 ± 0.15) mm. Each disc has five holes, each of 2 mm in diameter, with one hole on the cylinder axis and the other four equidistant, arranged on a circle with a radius of 6 mm relative to the center of

the disc. The lateral surface of the disc has four equidistant dents, with a depth of (2.6 ± 0.1) mm, in a V shape, which, on the upper side of the disc, measure (9.4 ± 0.2) mm of width, and 1.6mm on the lower side. All disc surfaces are smooth. Basket design and assembly may vary as long as specifications for tubes and mesh opening are maintained.

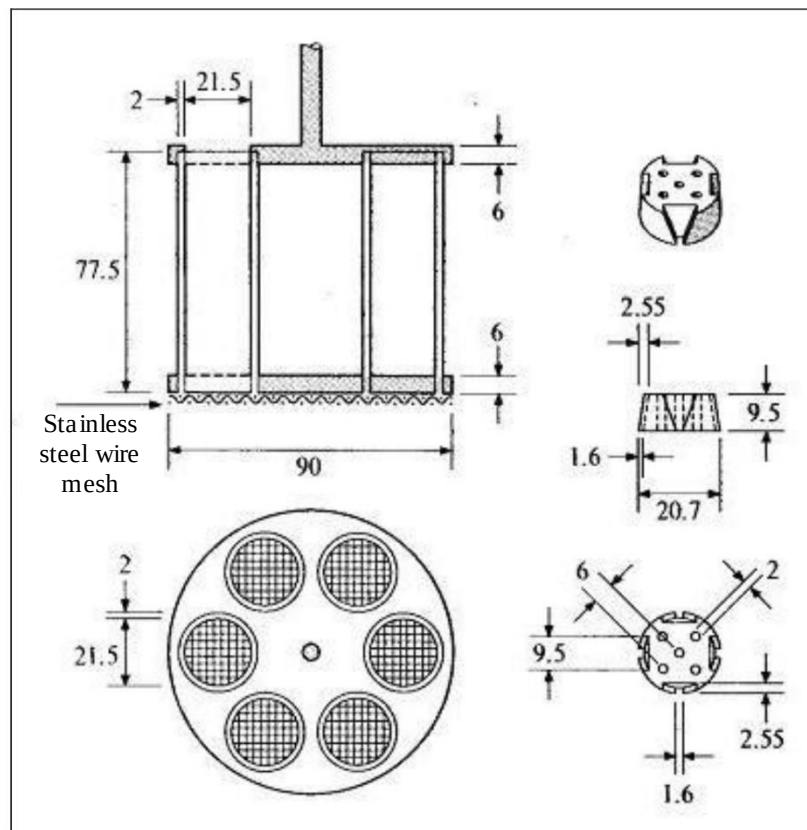


Figure 1 – Apparatus for tablet and capsule disintegration test (dimensions in mm).

PROCEDURE

Uncoated tablets

Use six tablets in the test. Place a tablet in each of the six tubes of the basket, add a disk to each tube and activate the apparatus, using water maintained at $(37 \pm 1)^\circ\text{C}$ as immersion liquid, unless another liquid is specified in the medication monograph. At the end of the specified time range, stop the movement of the basket and observe the material in each of the tubes. All tablets must be fully disintegrated. If tablets do not disintegrate due to sticking to the discs, repeat the test with six other tablets, omitting the discs. All tablets must be fully disintegrated upon test completion. The time limit established as a general criterion for disintegration of uncoated tablets is 30 minutes, unless otherwise indicated in the individual monograph.

Sugar coated (dragees) or film-coated tablets

Use six tablets in the test. Place one tablet in each one of the six tubes in the basket. Place a disk in each tube and activate the apparatus, using water maintained at $(37 \pm 1)^\circ\text{C}$ as immersion liquid. At the end of the specified time range, stop the movement of the basket and observe the material in each of the tubes. If the tablets are not fully disintegrated, test another six tablets using 0.1 M hydrochloric acid, kept at $(37 \pm 1)^\circ\text{C}$, as immersion liquid. At the end of the specified time range, stop the movement of the basket and observe the material in each of the tubes. All tablets must be fully disintegrated. If tablets do not disintegrate due to sticking to the discs, repeat the test with six other

tablets, omitting the discs. All tablets must be fully disintegrated upon test completion. The time limit established as a general criterion for disintegration of film-coated tablets is 30 minutes, and for sugar-coated tablets (dragees), 60 minutes, unless otherwise indicated in the individual monograph.

Enteric coated tablets or capsules (gastroresistant)

Use six units in the test. Place one unit in each one of the six tubes in the basket. Activate the apparatus, without adding the discs, using 0.1 M hydrochloric acid, kept at $(37 \pm 1)^\circ\text{C}$, as immersion liquid, for 60 minutes or for the time specified in the individual monograph. Cease basket movement and observe the tablets or capsules. No unit may show any sign of disintegration, cracking or softening that could allow its content to leak out. Use phosphate buffer solution pH 6.8, kept at $(37 \pm 1)^\circ\text{C}$, as immersion liquid. Place a disk in each tube and turn on the apparatus. After 45 minutes or the time specified in the monograph, cease basket movement and observe the material in each tube. All tablets or capsules must be completely disintegrated and only insoluble coating fragments may remain. If the tablets or capsules do not disintegrate due to sticking to the discs, repeat the test with six other units, omitting the discs. All tablets or capsules must be fully disintegrated at the end of the test. The test does not apply to uncoated capsules containing an enteric release preparation.

Sublingual tablets

Carry out the test as described for *Uncoated Tablets*, omitting the use of discs. All tablets must be fully disintegrated after five minutes.

Soluble tablets and dispersible tablets

Carry out the test as described for *Uncoated Tablets*, using water kept between 15°C and 25°C as immersion liquid. All tablets must be fully disintegrated after three minutes.

Gelatin capsules (hard)

Carry out the test as described for *Uncoated Tablets*, omitting the use of discs. Use a mesh with an opening of 1.8 mm to 2.2 mm, made of stainless-steel wire adapted to the external face of the upper disc, as described in the item *Apparatus*. Observe capsules after 45 minutes or as specified in the drug monograph. All capsules must be fully disintegrated, or only insoluble fragments of soft consistency remain on the mesh.

Soft capsules

Carry out the test as described for *Uncoated Tablets*, using the discs. Observe capsules after 30 minutes or as specified in the drug monograph. All capsules must be fully disintegrated, or only insoluble fragments of soft consistency remain on the mesh. If the capsules do not disintegrate due to sticking to the discs, repeat the test with six other units, omitting the discs. All capsules must be fully disintegrated at the end of the test.

5.1.4.2 DISINTEGRATION TEST OF SUPPOSITORIES, OVULES AND VAGINAL TABLETS

This test makes it possible to verify the greater or lesser capacity of these pharmaceutical preparations to soften or disaggregate in liquid medium, within the prescribed period.

Complete disintegration is considered when the suppository or ovule presents:

- full dissolution;
- complete separation of its components, accumulating melted fatty substances on the liquid surface, depositing insoluble powders at the bottom of the container and dissolving the soluble components of the sample, with the distribution of the components occurring in one or more of the manners described above;
- softening of the sample, which can be accompanied by a change in shape without complete separation of its components; the softening must be such that, when pressing the sample softened with a glass rod, it is not noticed the existence of a harder layer on its surface;
- rupture of the gelatinous capsule of ovules, allowing the release of its components;
- absence of residue on the perforated disc or, when present, that has the consistency of a soft mass that does not offer resistance to the pressure of a glass rod.

APPARATUS

The apparatus (**Figure 1**) consists of a transparent glass or plastic cylinder, with walls of appropriate thickness, inside which is attached, by three metal hooks, a metallic device consisting of two perforated stainless steel discs, each containing one 39 orifices of 4 mm in diameter each. The diameter of each disc is such that it can be inserted into the transparent cylinder, with the discs being spaced approximately 30 cm apart. The determination is carried out using three devices, each containing a single sample. Each apparatus is placed inside a beaker of not less than 4 liters of capacity, containing water at a temperature of 36°C to 37°C, unless otherwise indicated in the individual monograph. The beaker is equipped with a stirrer that operates at a slow speed and a device that allows the cylinder to be inverted without removing it from the water.

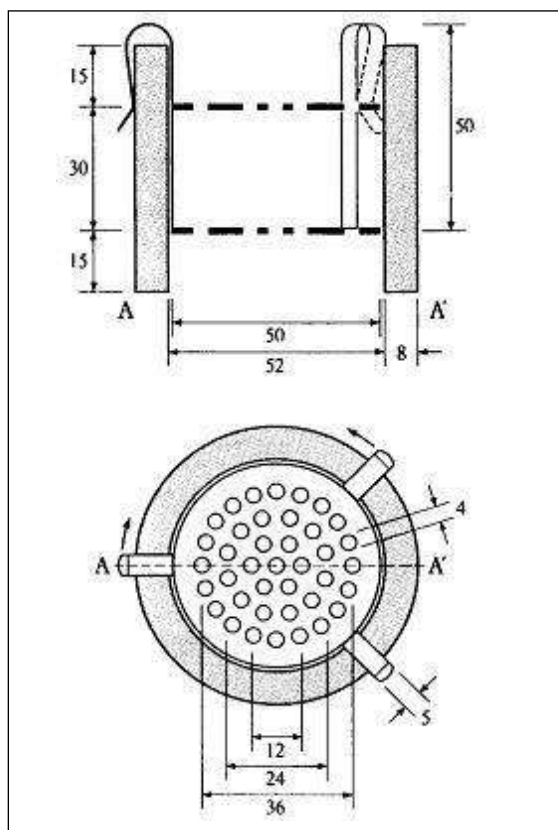


Figure 1 – Apparatus for disintegration test of suppositories, ovules and vaginal tablets (dimensions in mm).

PROCEDURE

Suppositories and ovules

Use three suppositories or ovules. Place each one on the device's lower disk and insert and fix the disk inside the cylinder. Invert the device every 10 minutes. Examine the samples after the time prescribed in the monograph has elapsed. The test is considered satisfactory if all samples are disintegrated. The time limit established as general criteria for disintegration is 30 minutes for suppositories, ovules and vaginal tablets with a hydrophobic base, and 60 minutes for suppositories with a hydrophilic base, unless otherwise indicated in the individual monograph.

Vaginal tablets

Use the device described in *Disintegration of suppositories and ovules*, assembled as shown in **Figure 2**. Introduce the cylinder into a beaker of a suitable diameter containing water between 36°C and 37°C, which should evenly cover the perforations of the disc. Use three devices, placing a vaginal tablet on the upper disc in each one. Cover the device with a glass plate to ensure adequate humidity. Examine each sample after the time prescribed in the monograph has elapsed. The test is considered satisfactory if all samples are disintegrated.

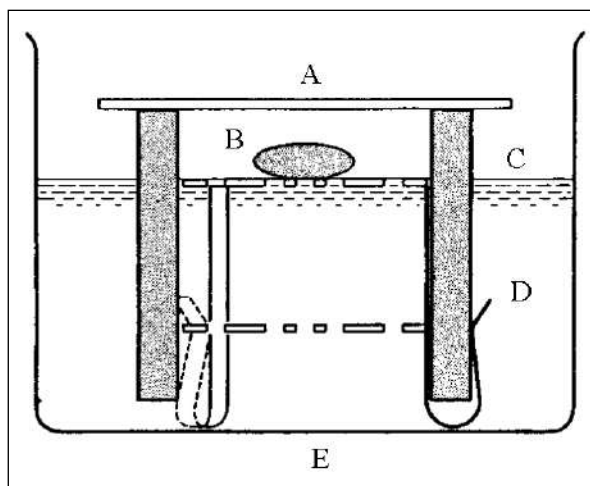


Figure 2 – Apparatus for disintegration test of suppositories, ovules and vaginal tablets.

A, glass plate; B, vaginal tablet; C, water surface; D, water; E, bottom of the container.

5.1.5 DISSOLUTION TEST

The dissolution test makes it possible to determine the quantity of active substance dissolved in the dissolution medium when the product is subject to the action of a specific apparatus, under described experimental conditions. The result is expressed as a percentage of the quantity stated on the label. This test is used to demonstrate whether the product meets the requirements in the drug monograph in tablets, capsules and other cases where testing is required.

APPARATUS FOR METHODS 1 AND 2

The dissolution apparatus consists of a three-component system, described below.

(1) Open containers in a cylindrical shape and hemispherical bottom (vats), made of boron silicate glass, plastic or other transparent and inert material, to which an inert material lid can be adapted, with suitable openings for the stirrer, sample collection and thermometer insert. The tanks can have the following dimensions and capacities: (185 ± 25) mm in height and (102 ± 4) mm in internal diameter for a nominal capacity of one liter; (290 ± 10) mm in height and (102 ± 4) mm in internal diameter for a nominal capacity of two liters; (290 ± 10) mm high and (150 ± 5) mm inside diameter for a nominal capacity of four liters.

(2) Stainless steel rods to provide agitation of the medium, which can be presented in two forms: baskets (*Method 1*) or paddles (*Method 2*) (**Figures 1 and 2**). The rod must be centered in such a manner that, when activated, its rotation axis does not distance more than 2 mm in relation to the vertical axis of the container containing the dissolution medium.

(3) A motor that makes it possible to adjust the rotation speed of the rod to that specified in the individual monograph, keeping it within the limits of $\pm 4\%$. The rotation must not pose undesirable effects on the hydrodynamics of the system.

The tanks are immersed in a thermostatic water bath, of transparent material and adequate size, in which the temperature is maintained at (37 ± 0.5) °C while carrying out the test. The device must be free from any source of vibration, including external ones, that could influence the hydrodynamics of the system. Preferably, the device should allow the visualization of samples and shakers during the test.

Method 1: Baskets

When specified in the monograph, a stainless-steel rod is used as a stirrer, at the end of which a basket of the same material is fitted (**Figure 1**). The standard mesh used in making the basket has a wire diameter of 0.25 mm and a square mesh opening of (0.40 ± 0.04) mm (40 mesh), unless otherwise specified in the individual monograph. The sample must be placed in the dry basket before starting the test. During its execution, a distance of (25 ± 2) mm must be maintained between the bottom of the basket and the inner bottom of the container containing the dissolution medium.

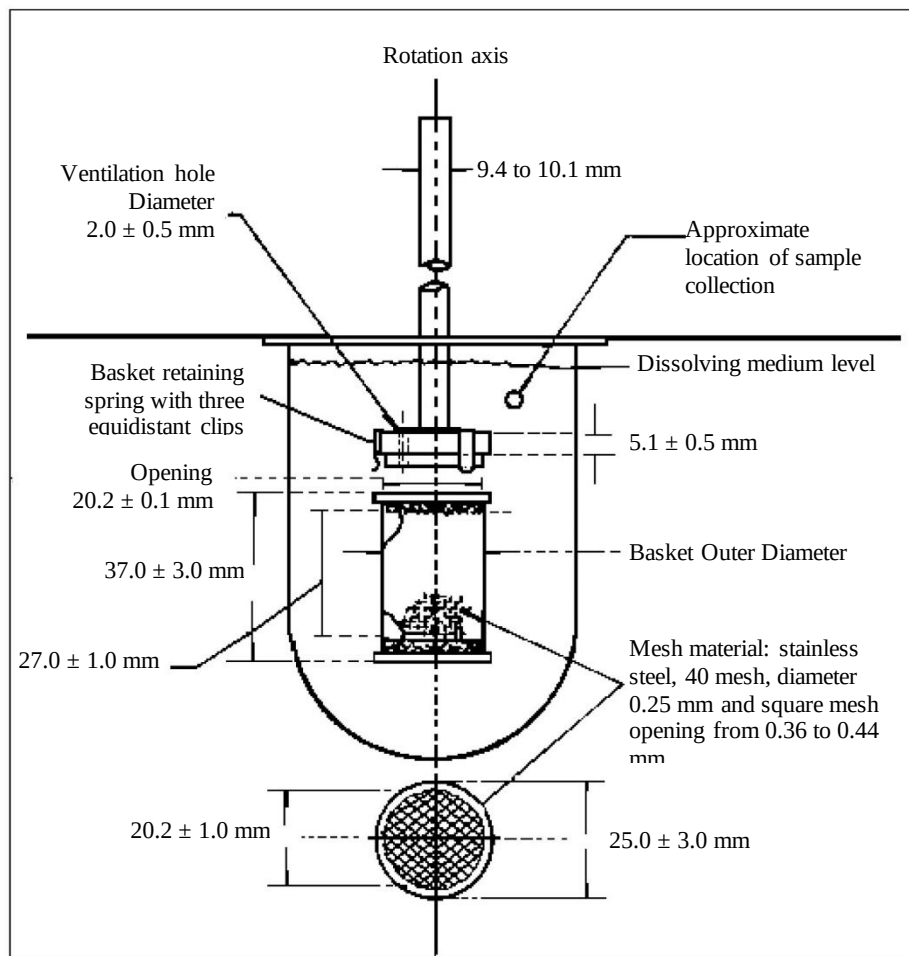


Figure 1 - Method 1 (Baskets). The basket and the tank are not in the same size proportion.

Method 2: Paddles

When specified in the monograph, a stainless-steel rod is used as a stirrer, coated or not with inert material, whose end has the shape of a paddle (**Figure 2**), capable of rotating smoothly and without axis deviation for the specified time and speed in the corresponding monograph. The sample should be added, whenever possible, before starting the test. During its execution, a distance of (25 ± 2) mm must be maintained between the bottom of the paddles and the inner bottom of the container containing the dissolution medium.

It is important that samples do not float in the dissolution medium. An appropriate device can be used, made of spiral steel wire in a few turns and with a sufficient diameter to trap the capsule or tablet without deforming them or reducing the area of contact with the medium.

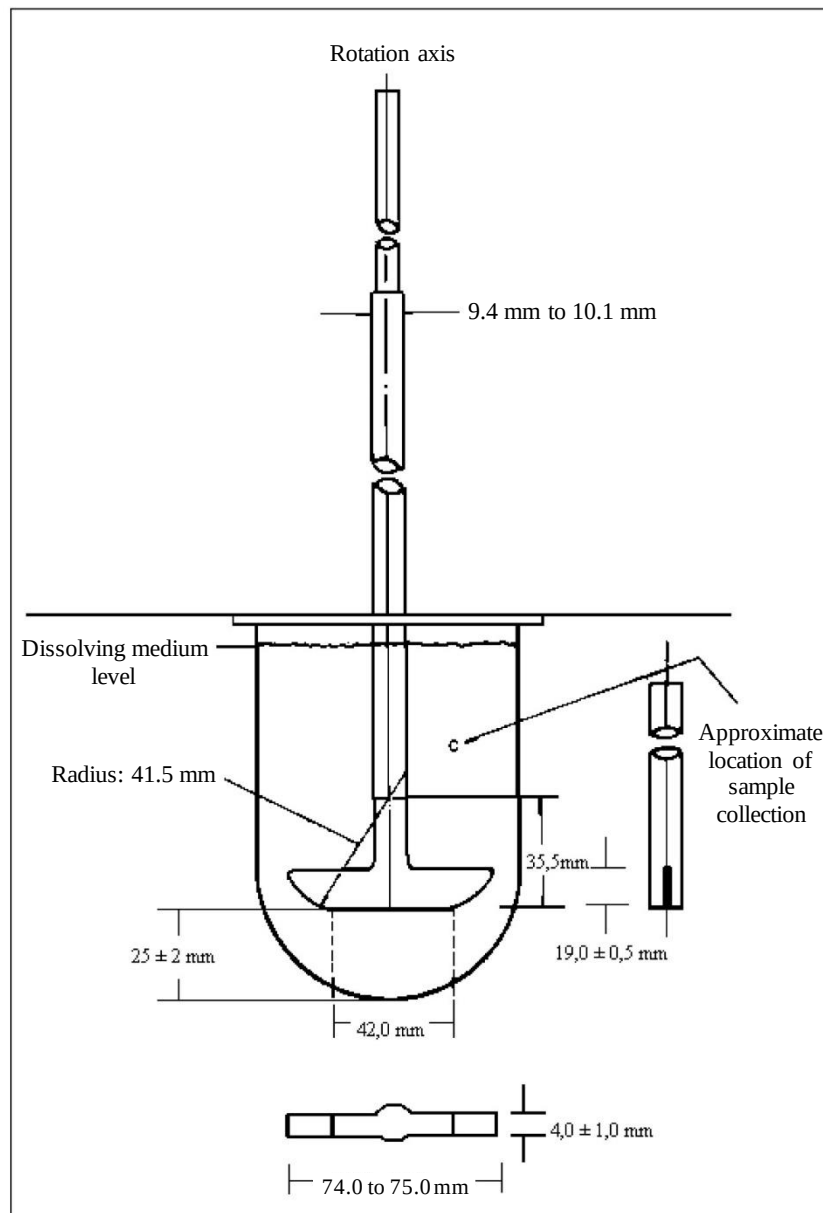


Figure 2 - Method 2 (Paddles). The paddle and the tank are not in the same size promotion.

APPARATUS FOR METHOD 3

Method 3: Alternating Cylinders

The dissolution apparatus for *Method 3* consists of a series of flat-bottomed cylindrical vials; a series of glass cylinders with a closing system made of inert material (stainless steel or other suitable material) and meshes made of non-adsorbent and non-reactive material, designed to be attached to the upper and lower parts of the cylinders. A motor and a cylinder fitting device must allow vertical alternating movement, ascending and descending, of the cylinders in the flasks and, also, providing horizontal displacement of the cylinder to another flask arranged in a different row.

The vials remain partially immersed in a water bath, of adequate dimensions, which allows thermostatzation at $(37 \pm 0.5) ^\circ\text{C}$ during the test period. The device must be free of any vibration, internal or external, that could influence the smooth upward and downward movement of the cylinders. The apparatus must have a device for adjusting the speed of alternating movement, as recommended in the individual monograph, with a maximum variation of $\pm 5\%$.

Preferably, the device should enable the visualization of the cylinders and samples under analysis inside. The flasks must have a suitable stopper which must remain fixed during the test. The assembly components have the dimensions shown in **Figure 3**, unless there is some differentiate specification in the monograph.

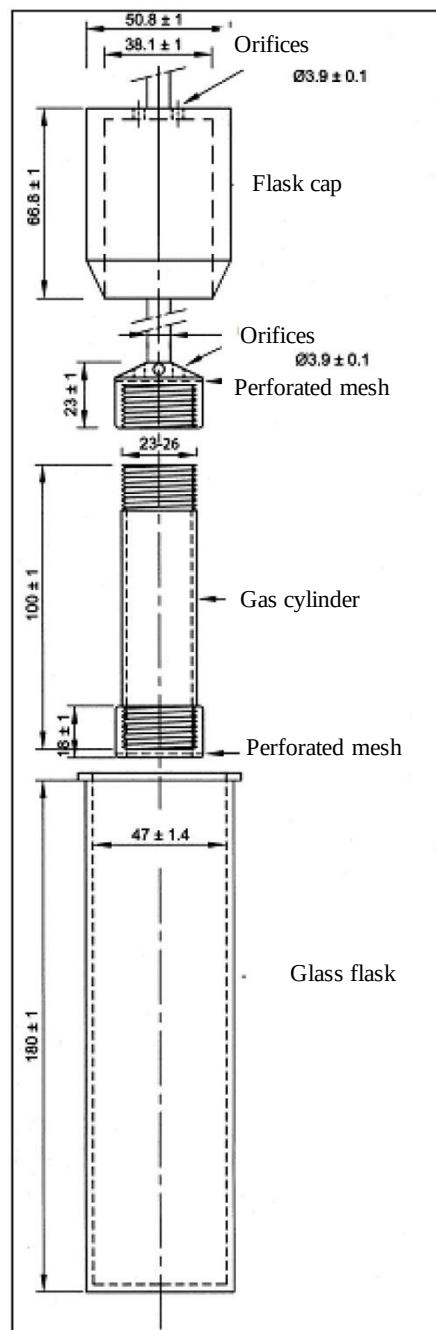


Figure 3 – Method 3 (Alternate Cylinders). Dimensions shown are in millimeters.

DISSOLUTION MEDIUM

The dissolution medium specified in the product monograph is used, previously degassed by a convenient procedure, when necessary, to avoid the formation of bubbles that could interfere with the dissolution speed of the pharmaceutical preparation. When the dissolution medium is a buffer solution, the pH should be adjusted to ± 0.05 units of the pH value specified in the product monograph.

DISSOLUTION TIME

When a single time is specified in the product monograph, it represents the maximum time within which the minimum quantity, in percentage, of active substance established must be dissolved. When more than one time is specified in the monograph, appropriately measured aliquots must be taken at the end of each time indicated.

GENERAL PROCEDURE FOR METHODS 1 AND 2

Assemble and check the apparatus according to the specifications previously mentioned in order to reduce, to a minimum, factors that significantly change the hydrodynamics of the system (axis deviation, vibration, etc.). Add the measured volume of the *Dissolution Medium* specified in the product monograph, suitably degassed, if necessary, to the dissolution apparatus container. Keep the temperature of the medium at $(37 \pm 0.5) ^\circ\text{C}$, removing the thermometer before starting to agitate. In *Method 1*, place the sample into the dry basket. In *Method 2*, place the sample into the dissolution container as described above. In both cases, upon observing the formation of bubbles on the surface of the samples, when in contact with the dissolution medium, check their influence on the result. Immediately start agitating, according to the pre-set speed. At the time range(s) specified in the product monograph, remove an aliquot for analysis of the intermediate region between the surface of the dissolution medium and the top of the basket or paddles, at least 1 cm from the inner wall of the container (**Figures 1 and 2**). While removing the aliquot, keep shaking. Immediately filter the samples, if not using filters coupled to the sampling system. The filters used must be inert, not adsorb a significant portion of the drug and have adequate porosity. As specified in the product monograph, the sample volume taken may or may not be replaced. If replacement is necessary, the same dissolution medium heated to $37 ^\circ\text{C}$ should be used. If the replacement of the dissolution medium is not carried out, correct the volume in the calculations. After filtration and dilution (when necessary) of the aliquot, the drug is quantified using the method indicated in the product monograph. Repeat the test with additional unit doses as necessary considering the *Acceptance Criteria*.

Dissolution of capsules: if the result is unsatisfactory, repeat the test as follows: when the dissolution medium is water or buffer with a pH lower than 6.8, use the same specified dissolution medium with addition of purified pepsin with activity of not more than 750 000 units/1000 mL. For dissolution media with a pH equal to or greater than 6.8, add pancreatin with an activity of not more than 1750 protease units/1000 mL.

PROCEDURE FOR DELAYED RELEASE PHARMACEUTICAL PREPARATIONS

Use *Method A* or *Method B* or the method indicated in the individual monograph.

Method A

Acid stage: use 750 mL of 0.1 M HCl as *Dissolution Medium* in the tanks when using Methods 1 and 2. Assemble the dissolution apparatus as described in *Apparatus for Methods 1 and 2* and add a test unit to each tank or basket, as appropriate. Carry out the test at the speed specified in the monograph for two hours. At the end of this period, remove an aliquot from the *Dissolution Medium* and immediately carry out *Buffer Stage* pH 6.8. Determine the amount of drug dissolved in the sampled aliquot, using appropriate analytical method.

Buffer stage pH 6.8: carry out the preparation of the buffer stage and pH adjustment in five minutes. With the dissolution apparatus operating at the speed specified for the product, add 250 mL of 0.20 M sodium phosphate tribasic solution, previously heated to $(37 \pm 0.5) ^\circ\text{C}$, to the *Acid Stage*

Dissolution Medium. If necessary, adjust pH to (6.8 ± 0.05) with 2 M HCl or 2 M NaOH. Continue operating the dissolution apparatus for 45 minutes or for the time specified in the monograph. At the end of this period, remove an aliquot from the *Buffer Stage Dissolution Medium pH 6.8* and determine the amount of drug dissolved, using appropriate analytical method.

Method B

Acid stage: use 1000 mL of 0.1 M HCl as *Dissolution Medium* in the tanks and assemble the dissolution device as described in *Apparatus for Methods 1 and 2*. Add a test unit to each tank or basket, as appropriate. Carry out the test at the speed specified in the monograph for two hours. Time elapsed, remove an aliquot of the *Dissolution Medium* and immediately carry out *Buffer Stage pH 6.8*. Determine the amount of drug dissolved in the sampled aliquot, using appropriate analytical method.

Buffer stage pH 6.8: use phosphate buffer pH 6.8, previously heated to $(37 \pm 0.5) ^\circ\text{C}$. Drain the *Acid Stage* dissolution medium from the tanks and add 1000 mL of pH 6.8 phosphate buffer dissolution medium. Alternatively, each tank with the *Acid Stage* medium can be removed from the dissolution apparatus and replaced by another tank with pH 6.8 *Stage buffer* medium, carefully transferring the test unit of the drug under test. Continue operating the dissolution apparatus for 45 minutes or for the time specified in the monograph. At the end of this period, remove an aliquot from the *Buffer Stage pH 6.8* dissolution medium and determine the amount of drug dissolved, using appropriate analytical method. The pH 6.8 buffer can be prepared by mixing three volumes of 0.1 M HCl and one volume of 0.20 M sodium phosphate tribasic solution, if necessary adjusting the pH to 6.8 ± 0.05 with 2 M HCl or 2 M NaOH.

PROCEDURE FOR METHOD 3

Immediate release dosage forms: using Method 3, add the volume of *Dissolution medium* specified in the product monograph to each flask of the device, place the flasks in the bath instrument to acclimatize to $(37 \pm 0.5) ^\circ\text{C}$ and remove the thermometers before starting the test. Place a sample dosage unit in each of the six alternating cylinders, avoiding the formation of air bubbles on the surface of the material, and immediately start operating the device as specified in the individual product monograph. During the upward and downward movement of the cylinders, the height range should be between 9.9 and 10.1 cm. At the time range(s) specified in the individual monograph, lift the cylinders and sample an aliquot of the *Dissolution Medium* from each vial, from the intermediate region between the liquid surface and the bottom of the vial. After filtering and diluting (when necessary) the aliquot, carry out a quantitative analysis of the dissolved drug according to what is recommended in the individual product monograph. If necessary, repeat the test with additional units of drug. Replace the volume of sampled medium with an equal volume of freshly prepared *Dissolution Medium* maintained at $(37 \pm 0.5) ^\circ\text{C}$ or, in situations where it is proven that it is not necessary to replace the medium, correct the volume change during the calculations. Keep the vials covered with their respective caps during the test and periodically check the temperature of the medium. For the medium and dissolution time, follow the general guidelines indicated in *Dissolution Medium* and *Dissolution Time*.

Prolonged-release dosage forms: using Method 3, perform the procedure as described in *Immediate-release dosage forms* and follow the general guidelines indicated in *Dissolution Medium* and *Dissolution Time*. Times are expressed in hours and normally at least 3 time ranges are indicated. *Delayed release dosage forms:* using Method 3, based on the procedure indicated in Method B for Delayed release dosage forms, using a row of vials for the Acid stage and the successive row of vials for the stage with buffer solution pH 6.8, adding the volume of medium specified in the monograph

(usually 300 mL). Collection times are those specified in the monograph or the general ones indicated in Method B for Delayed Release Dosage Forms.

ACCEPTANCE CRITERIA FOR IMMEDIATE RELEASE PHARMACEUTICAL PREPARATIONS

The product complies with the test if the results meet the requirements described in **Table 1**, unless otherwise specified in the individual monograph.

Table 1 – Acceptance criteria for the dissolution test of immediate release dosage forms.

<i>Stages</i>	<i>Number of samples tested</i>	<i>Acceptance criteria</i>
E_1	06	Each unit has a result greater than or equal to $Q + 5\%$.
E_2	06	Average of 12 units ($E_1 + E_2$) is equal to or greater than Q and no unit has a result lower than $Q - 15\%$.
E_3	12	Average of 24 units ($E_1 + E_2 + E_3$) is equal to or greater than Q , at most two units have results less than $Q - 15\%$ and no unit has results less than $Q - 25\%$.

The term Q corresponds to the quantity of dissolved drug, specified in the individual monograph, expressed as a percentage of the declared quantity. The 5%, 15% and 25% values also represent percentage of the declared quantity.

In special circumstances, the maximum dissolution percentage must be established experimentally. In these cases, ensure a value of Q_∞ (quantity dissolved in infinite time) checking that two consecutive dosages do not differ by more than 2% after 10 minutes.

Stage E₁

At *Stage E₁* six units are tested. If each unit individually presents a result equal to or greater than $Q + 5\%$, the product complies with what was specified, and *Stage E₂* is not necessary.

Stage E₂

If the criterion for *Stage E₁* is not met, repeat the test with another six units. If the average of the twelve units tested (*Stages E₁* and *E₂*) is greater than or equal to Q , and if none of the units tested presents a result lower than $Q - 15\%$, the product complies with what was specified, and it is not necessary to perform *Stage E₃*.

Stage E₃

If the criterion for *Stage E₂* is still not met, repeat the test with another 12 units. If the average of the 24 units tested (*Stages E₁*, *E₂* and *E₃*) is greater than or equal to Q , at most two units present results lower than $Q - 15\%$ and no unit presents results lower than $Q - 25\%$, the product complies with specification. If the criterion for *Stage E₃* is not yet met, the product is considered unsatisfactory.

ACCEPTANCE CRITERIA FOR PROLONGED RELEASE PHARMACEUTICAL PREPARATIONS

The product complies with the test if the results meet the requirements described in **Table 2**, unless otherwise specified in the individual monograph. The terms Q_1 and Q_2 correspond to the minimum

and maximum quantity of drug dissolved in each time range specified in the monograph, expressed as a percentage of the declared quantity. At the last time the specification can only be presented with a minimum Q value. The terms L_1 , L_2 and L_3 refer to the three possible stages of release evaluation (L).

Table 2 – Acceptance criteria for the dissolution test (release) carried out for extended-release dosage forms.

<i>Stages</i>	<i>Number of units tested</i>	<i>Acceptance criteria</i>
L_1	6	Each individual result fits in the established range (Q1 and Q2) for each given time and no individual result is inferior to the Q of the last time.
L_2	6	The average of 12 units ($L_1 + L_2$) fits in the established range (Q1 and Q2) for each given time and is not inferior to the Q of the last time. No individual unit presents a result that exceeds the limits of Q1 and Q2 by 10% of the declared quantity, for each given time, and no individual result provides a value lower than the Q of the last time that exceeds the declared quantity by 10%.
L_3	12	The average of 24 units ($L_1 + L_2 + L_3$) fits in the established range (Q1 and Q2) for each given time and is not inferior to the Q of the last time. A maximum of two units out of the 24 tested present results that exceed Q1 and Q2 limits by 10% of the declared quantity for each given time, and a maximum of two units out of the 24 tested present results with a value lower than the Q of the last time that exceeds in 10% the declared quantity in 10%. No individual unit presents a result that exceeds the limits of Q1 and Q2 by 20% of the declared quantity, for each given time, and no individual result provides a value lower than the Q of the last time that exceeds the declared quantity by 20%.

ACCEPTANCE CRITERIA FOR DELAYED RELEASE PHARMACEUTICAL PREPARATIONS

The product complies with the test if the results meet the requirements shown in **Table 3** in the *Acid Stage* (Methods A or B) and also the requirements indicated in **Table 4** in the *pH 6.8 buffer Stage* (Methods A or B), unless otherwise specified in the individual monograph. Use the Q value indicated in the product monograph and, when not specified, use 75% as the Q value in the *pH 6.8 Buffer stage*. The terms A_1 , A_2 and A_3 refer to the three possible evaluation stages in the *Acid Stage* (A) and the terms B_1 , B_2 and B_3 refer to the three possible evaluation stages in the *Buffer stage pH 6.8* (B).

Table 3 – Acceptance criteria for the *Acid stage* dissolution test (Methods A or B) carried out for delayed-release dosage forms.

<i>Stages</i>	<i>Number of units tested</i>	<i>Acceptance criteria</i>
A_1	06	No individual unit has a dissolved quantity greater than 10% of the declared.
A_2	06	The average of 12 units ($A_1 + A_2$) is not more than 10% of the declared quantity and no individual unit has a dissolved quantity greater than 25% of the declared quantity.
A_3	12	The average of 24 units ($A_1 + A_2 + A_3$) is, at most, 10% of the declared quantity and no individual unit has a dissolved quantity greater than 25% of the declared quantity.

Table 4 – Acceptance criteria for the *pH 6,8 Buffer stage* dissolution test (Methods A or B) carried out for delayed-release dosage forms.

<i>Stages</i>	<i>Number of units tested</i>	<i>Acceptance criteria</i>
B_1	06	Each unit has a result greater than or equal to $Q + 5\%$.
B_2	06	Average of 12 units ($B_1 + B_2$) is equal to or greater than Q and no unit has a result lower than $Q - 15\%$.
B_3	12	Average of 24 units ($B_1 + B_2 + B_3$) is equal to or greater than Q , at most two units have results less than $Q - 15\%$ and no unit has results less than $Q - 25\%$.

5.1.6 UNIFORMITY OF UNIT DOSES

To ensure the administration of correct doses, each unit of a drug batch must contain a quantity of the active ingredient close to the stated quantity. The unit dose uniformity test evaluates the quantity of active ingredient in individual units of the batch and verify that such quantity is uniform in the units tested. The specifications of this test apply to dosage forms with a single drug or with more than one active ingredient. Unless otherwise indicated in the individual monograph, the test applies individually to each active ingredient of the product.

The uniformity of unit doses of pharmaceutical preparations can be evaluated by two methods: *Weight variation* and *content uniformity*. The application of each method, considering the pharmaceutical preparation, dose and proportion of the drug, is shown in **Table 1**.

Table 1 – Application of the Content Uniformity (CU) or Weight Variation (WV) method according to the pharmaceutical preparation, dose and proportion of the drug.

Pharmaceutical preparation	Type	Subtype	Drug dose and proportion	
			≥ 25 mg and ≥ 25%	< 25 mg or < 25%
Uncoated	tablets		WV	CU
	coated	film	WV	CU
		others	CU	CU
Hard	capsules		WV	CU
	soft	suspensions, emulsions or gels	CU	CU
		solutions	WV	WV
	sole ingredient		WV	WV
Solids stored in single-dose containers	multiple ingredients	lyophilized solution in final container	WV	WV
		others	CU	CU
			WV	WV
Solutions stored in single-dose containers			WV	WV
Others			CU	CU

The *Content Uniformity* method for unit dose preparations is based on the assay for the individual content of the active ingredient from a number of unit doses to determine whether the individual content is within specified limits. The *Content Uniformity* method can be applied in all cases.

The *Weight Variation* method can be applied to the following dosage forms:

1. Solids packaged in single-dose containers and in soft capsules;
2. solids (including powders, granules and sterile solids) packaged in single-dose containers that do not contain other added substances, whether active or inactive;
3. solids (including sterile solids) packaged in single-dose containers, containing or not added active or inactive substances, which have been prepared from homogeneous lyophilized solutions in the final containers, and are labeled in such a manner as to indicate this mode of preparation;

4. hard capsules, uncoated or film-coated tablets, containing 25 mg or more of the active substance, comprising 25% or more by weight of the unit dose or, in the case of hard capsules, the content of the capsule. The uniformity of other active substances present in smaller proportions must be demonstrated by the *Content Uniformity* method.

The *Content Uniformity* method is required for all dosage forms that do not meet the specified conditions for applying the *Weight Variation* method.

CONTENT UNIFORMITY

To determine the uniformity of unit doses by the method of content uniformity, separate at least 30 units and proceed as described for the pharmaceutical preparations indicated. When the amount of active ingredient in a unit dose is different from that specified in the assay, make adjustments to the dilution of the solutions and/or to the volume of the aliquots to obtain the concentration of active ingredient in the final solution similar to that in the assay. In case of titration assay, use titrant with different concentration, if necessary, to consume an adequate volume of titrant. Consider any modification of dilutions to perform calculations.

When there is a special procedure for the content uniformity test in the individual monograph, make the necessary correction of the results obtained as described below.

1. Weigh amount of product units sufficient to carry out the assay and the special content uniformity test procedure presented in the individual monograph. Pulverize tablets to fine powder (or mix contents of capsules, solutions, suspensions, emulsions, gels or solids in single-dose containers) to obtain a homogeneous mixture. If obtaining a homogeneous mixture is not possible, use appropriate solvents or other procedures to obtain a solution containing the drug. Use appropriate aliquots of this solution for the specified tests.
2. Analyze, separately, portions of the sample, accurately measured, according to the procedure indicated for the assay (*D*) and the special procedure indicated for content uniformity (*E*), described in the individual monograph.
3. Calculate the amount of drug by average weight using the results obtained by the assay procedure (*D*) and the special procedure (*E*).
4. Calculate the correction factor (*F*) according to the formula:

$$F = D/S$$

where

D = amount of active ingredient by average weight of the pharmaceutical preparation obtained by the assay procedure;

E = amount of active ingredient by average weight of the pharmaceutical preparation obtained by the special procedure. If $(100|D - E|)/D$ is greater than 10, the use of *F* is not valid.

1. If *F* is between 0.970 and 1.030, there is no need for correction.
2. The correction will be applied when *F* value is between 0.900 and 0.970 and between 1.030 and 1.100 and must be carried out by calculating the amount of drug in each unit, multiplying the quantities obtained in the special procedure by the correction factor *F*.

Solid dosage forms

Analyze 10 units individually as indicated in the individual monograph for the assay, unless a special procedure for content uniformity is described in the monograph. Calculate the *Acceptance Value (AV)*.

Liquid dosage forms

Analyze 10 units individually as indicated in the individual monograph for the assay, unless a special procedure for content uniformity is described in the monograph. Carry out the test, individually, in a homogeneous quantity of material that is removed from each container under normal conditions of use. Express the result as quantity dispensed per unit. Calculate the *Acceptance Value (AV)*.

Acceptance Value for Content Uniformity

Calculate the *Acceptance Value (AV)* as per the formula:

$$AV = |M - \bar{X}| + ks$$

whose terms are defined in **Table 2**.

WEIGHT VARIATION

To determine the uniformity of unit doses by the method of weight variation, separate at least 30 units and proceed as described for the pharmaceutical preparations indicated. The amount of drug per unit is estimated from the result of the assay and the individual weights, assuming homogeneous distribution of the active ingredient. Estimated individual quantities (x_i) are calculated according to the formula:

$$x_i = p_i \times A/P$$

where

p_i = individual weights of units or contents of tested units;

A = amount of active ingredient, expressed as a percentage of the declared quantity, determined in the assay;

P = average weight of the units used in the assay.

Uncoated or film-coated tablets

Weigh, accurately and individually, 10 tablets. From the assay result and the individual weight of each tablet, estimate the quantity of active component in each unit and express the individual results as a percentage of the declared quantity. Calculate the *Acceptance Value (AV)*.

Hard capsules

Weigh, accurately and individually, 10 capsules, preserving the identity of each one. Carefully remove contents and weigh empty capsules. Calculate the content weight of each capsule and, from the assay result, estimate the amount of active ingredient in each capsule. The individual result is expressed as a percentage of the quantity stated. Calculate the *Acceptance Value (AV)*.

Soft capsules

Weigh, accurately and individually, 10 capsules, preserving the identity of each one. Cut the capsules with a slide and remove the contents, washing the shells with a suitable solvent. Place the shells at room temperature for 30 minutes for the complete evaporation of the solvent, taking precautions to avoid the addition or loss of humidity. Weigh the empty capsules and calculate the content weight of each capsule. Estimate the amount of active ingredient in each capsule from the assay result and from the content weight of each capsule. Calculate the *Acceptance Value (AV)*.

Solid dosage forms (except tablets and capsules)

Proceed as indicated in *Hard Capsules*. Calculate the *Acceptance Value (AV)*.

Liquid dosage forms

Weigh, accurately and individually, the quantity of liquid that is removed from each of 10 containers under normal use conditions. If necessary, calculate the equivalent volume of removed content after density determination. Estimate the amount of active ingredient in each container from the assay result and from the content weight removed from the individual containers. Calculate the *Acceptance Value (AV)*.

Acceptance Value for Weight Variation

Calculate the *Acceptance Value* as described in *Acceptance Value for Content Uniformity*, except that the individual active component quantities in the units are replaced by the estimated individual quantities.

CRITERIA

Apply the following criteria for both *Content Uniformity* and *Weight Variation*, unless otherwise indicated in the individual monograph.

Solid and liquid dosage forms

The product meets the unit dose uniformity test if the calculated *Acceptance Value* for the first 10 units tested is not greater than L_1 . If the *Acceptance Value* is greater than L_1 , test another 20 units and calculate the *Acceptance Value*. The product meets the unit dose uniformity test if the final *Acceptance Value* calculated for the 30 units tested is not greater than L_1 and the amount of active ingredient of any single unit is less than $(1 - L_2 \times 0.01)M$ or greater than $(1 + L_2 \times 0.01)M$. Unless otherwise indicated in the individual monograph, L_1 is 15.0 and L_2 is 25.0.

Table 2 – Terms and expressions for calculating the *Acceptance Value (AV)*.

<i>Variable</i>	<i>Definition</i>	<i>Conditions</i>	<i>Values</i>
X	Average of individual contents $(x_1, x_2, \dots, x_n)$, expressed as a percentage of the declared quantity.		
$x_1, x_2, \dots, x_n$	Individual contents of the tested units, expressed as a percentage of the declared quantity.		
n	Number of units tested		

Variable	Definition	Conditions	Values
k	Acceptability constant	If $n = 10$, then $k =$ If $n = 30$, then $k =$	2.4 2.0
s	Sample standard deviation		$\left[\frac{\sum_{i=1}^n (x_i - \bar{X})^2}{n - 1} \right]$
M to be used when $T \leq 101.5$ (case 1)	Reference value	If $98.5\% \leq \bar{X} \leq 101.5\%$, then If $\bar{X} < 98.5\%$, then If $\bar{X} > 101.5\%$, then	$M = \bar{X}$ ($AV = ks$) $M = 98.5\%$ ($AV = 98.5 - \bar{X} + ks$) $M = 101.5\%$ ($AV = \bar{X} - 101.5 + ks$)
M to be used when $T \leq 101.5$ (case 1)	Reference value	If $98.5\% \leq \bar{X} \leq T$, then If $\bar{X} < 98.5\%$, then If $\bar{X} > T$, then	$M = \bar{X}$ ($AV = ks$) $M = 98.5\%$ ($AV = 98.5 - \bar{X} + ks$) $M = T$ ($AV = \bar{X} - T + ks$)
Acceptance Value (AV).			General formula: $ M - \bar{X} + ks$ The calculations are specified above for the different cases.
L_1	Maximum value allowed for acceptance value		$L_1 = 15.0$ unless otherwise specified in the individual monograph
L_2	Maximum allowable deviation for each unit tested from the value of M used in the acceptance value calculations.	No individual result is less than $(1 - L_2 \times 0.01) M$ or greater than $(1 + L_2 \times 0.01) M$	$L_2 = 25.0$ unless otherwise specified in the individual monograph
T	Average of the limits specified in the individual monograph for the stated quantity or potency, expressed as a percentage.	T equals 100% unless another value has been approved for stability reasons; in these cases, T is greater than 100%.	

5.1.7 CONTAMINATION BY PARTICLES

5.1.7.1 SUBVISIBLE PARTICLES

Particle contamination of injectables is the presence of insoluble, foreign, and mobile materials other than air bubbles.

The specifications required for pharmaceutical preparations are described in the specific monographs.

Contamination by particles of preparations for parenteral use and of preparations for perfusion consists of non-soluble and mobile foreign particles, in addition to gas bubbles that are unintentionally found in these preparations. For the determination of particulate contamination, 2 methods are specified below: *method 1* (Light Obscuration Particle Count Test) and *method 2* (Optical Microscopy Particle Count Test). For the determination of non-visible particles in injectable preparations and in perfusion preparations, preferably use *method 1*. In certain preparations, however, it may be necessary to carry out particle counting tests by light blocking and, later, by optical microscopy in order to be able to conclude as to the conformity of the obtained results.

Screening for non-visible particles performed using one of these methods, or even both, is not possible for all injectable preparations. When *method 1* is not applicable, for example in the case of preparations that are not very clear or highly viscous, the test is carried out using *method 2* (in the case of emulsions, colloidal solutions and liposome preparations). Likewise, a particle counting test by optical microscopy may also be required in the case of products which form air or gas bubbles when passing through the detector. If the viscosity of the preparation is such that examination by either method is impossible, a quantitative dilution with an appropriate diluent may be carried out in order to reduce the viscosity to the degree considered sufficient to permit testing.

The results obtained when examining a unit or a group of units cannot be reliably extrapolated to other units that have not been analyzed. Consequently, it is advisable to establish statistically valid sampling plans to draw valid conclusions from the collected data for particulate material contamination in a large group of units.

The water used in the tests is free from particles. Particle-free water can be obtained by membrane filtration with a 0.22 µm porosity.

METHOD 1 - PARTICLE COUNT BY LIGHT BLOCKING

Apparatus

Use particle counter with operation based on the light blocking principle that allows the determination of the size of particles and their number according to their dimensions.

Calibration

Calibrate the apparatus with the aid of standard spherical particles with a size between 10 to 25 µm. These standard particles are dispersed in particle-free water. Avoid aggregation of particles during dispersion.

Precautions

Carry out the test under conditions of limited contamination, preferably in a laminar flow hood. Wash glassware and used filtration apparatus, with the exception of filtering membranes, with a warm detergent solution and rinse with water until all detergent is removed. Immediately before use, rinse the apparatus from top to bottom, internally and externally with particle-free water.

Take care not to introduce air bubbles into the sample to be analyzed, especially when sample aliquots are being transferred to the reading accessory.

To check the suitability of the ambient, the glassware and the water used, count the particles in five samples of 5 mL of particle-free water, according to the method described in this chapter. If the number of particles larger than 10 μm exceeds 25, for the total volume of 25 mL, the ambient is not suitable to carry out the test.

Procedure

Homogenize the sample through 25 consecutive slow, gentle inversions of the container. Remove bubbles by allowing the sample to stand for two minutes. Transfer four portions not smaller than 5 mL, and determine the number of particles with a size equal to or greater than 10 and 25 μm . Disregard the result obtained with the first aliquot, and calculate the average number of particles for the sample under examination.

Assessment

Use test A, test B or test C, as well as the number of samples, as indicated in the specific monograph of the pharmaceutical preparation.

Test A – Solutions for injectables in containers, with declared volume, greater than 100 mL. The sample complies with the test if the average number of particles with a size equal to or greater than 10 μm present in the units tested does not exceed 25 particles per mL and the number of particles with a size equal to or greater than 25 μm does not exceed three per mL.

Test B – Solutions for injectables in containers, with declared volume, equal to or less than 100 mL. The sample complies with the test if the average number of particles with a size equal to or greater than 10 μm present in the units tested does not exceed 6000 particles per container and the number of particles with a size equal to or greater than 25 μm does not exceed 600 particles per container.

Test C – Powders for injectables in containers, with declared volume, equal to or less than 100 mL. The sample reconstituted with water or appropriate particulate-free diluent complies with the test if the average number of particles, with a size equal to or greater than 10 μm present in the units tested, does not exceed 10.000 particles per container and the number of particles with the same size or greater than 25 μm does not exceed 1000 particles per container.

METHOD 2 - PARTICLE COUNT BY MICROSCOPY

Apparatus

Use an appropriate binocular microscope, a filtration device to trap particle contamination and a filter membrane. The microscope equipped with a calibrated ocular micrometer, with an objective micrometer, a cross-movement stage capable of maintaining and traversing the entire filtration surface of the filtering membrane, two appropriate illuminators that allow episcopic illumination and oblique illumination, adjusted for magnification of 100 ± 10 times.

The ocular micrometer is a circular reticle and comprises a large circle divided into quadrants, by crisscross lines, black and transparent reference circles with a diameter of 10 μ m and 25 μ m with a magnification of 100 and a linear graduated scale of 10 by 10 μ m (**Figure 1**).

The large circle is called the reticle field of view. Two illuminators are required, an episcopic illuminator for bright background, internal to the microscope, and an external adjustable auxiliary illuminator, adjustable to allow oblique illumination reflected at an angle of 10-20°. The filtering device for retaining the particular contamination comprises a filter holder made of glass or other suitable material, a vacuum source and a suitable filtering membrane. The filter membrane, of appropriate dimensions, is black or dark gray in color; is covered or not with a grid and the pore size is less than or equal to 1.0 μ m.

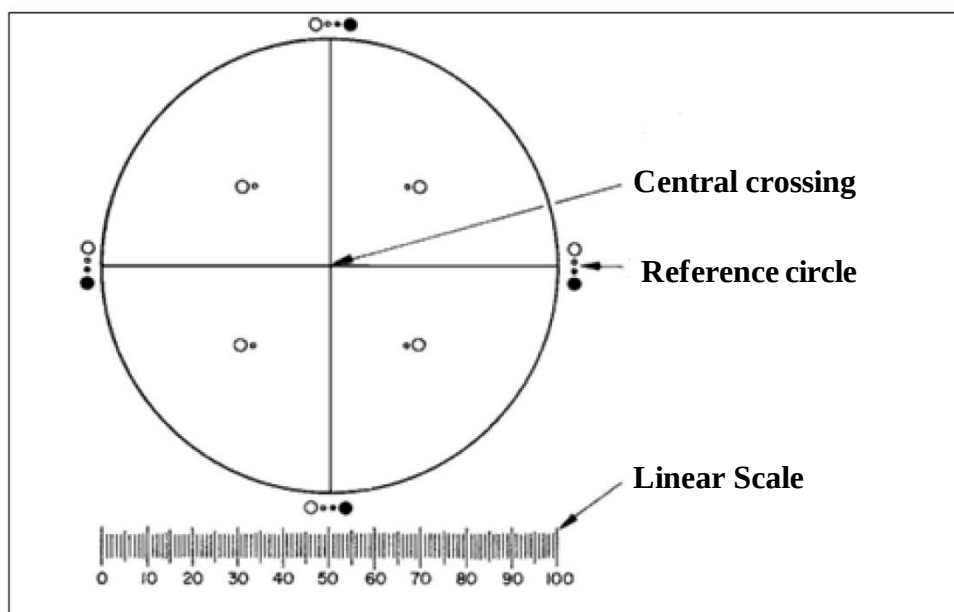


Figure 1 – Circular reticles.

Calibration

It is calibrated with an objective micrometer certified by an international or national standardization organization. A relative error of $\pm 2\%$ for the reticle linear scale is acceptable.

General precautions

Carry out the test under conditions of limited contamination, preferably in a laminar flow hood. Wash glassware and used filtration apparatus, with the exception of filtering membranes, with a warm detergent solution and rinse with water until all detergent is removed. Immediately before use, wash both sides of the filter membrane, thoroughly rinse the apparatus from top to bottom, internally and externally with particulate-free water.

To check the suitability of the ambient, the glassware and the water used, count the particles in 50 mL of particle-free water, according to the method described in this chapter. If the number of particles of 10 μ m or larger exceeds 20, or if more than 5 particles of 25 μ m or larger are present, the ambient is not suitable for testing.

Procedure

Homogenize the sample through 25 consecutive slow, gentle inversions of the container. If necessary, carefully remove the closing device. Wash the outer surfaces of the vial opening with a jet of particulate-free water and remove the closure avoiding any contamination of the contents.

For large volume parenteral preparations, carry out the test in separate units. In the case of large-volume or small-volume parenteral preparations equal to or greater than 25 mL, less than 10 packs may be sufficient for the test according to an appropriate sampling plan. For small volume parenteral preparations, less than 25 mL, bring together the content of 10 units or more in a clean container so as to obtain a minimum volume of 25 mL; in justified and authorized cases, the problem solution can be prepared by mixing the contents of an appropriate number of vials and making up 25 mL with particulate-free water or an appropriate particulate-free solvent, when particulate-free water is not appropriate. Small-volume parenteral preparations, whose volume is greater than or equal to 25 mL, can be examined individually.

In the case of powders for parenteral use, reconstitute the preparation with particulate-free water or a suitable solvent free from particle contamination, when particulate-free water is not suitable.

Moisten the inside of the filter holder fitted with the filter membrane with a few milliliters of particulate-free water. Pour the entire sample (mixture of test contents or the unit under test) onto the filter and apply vacuum. If necessary, add, little by little, portions of the solution until the total volume is filtered. After the last addition, start washing the inner walls of the filter holder using a jet of particulate-free water. Maintain the vacuum until the surface of the filter membrane is free of liquid.

Place the filter in a Petri dish and air dry, leaving the dish slightly open. When the filter is dry, place the Petri dish on the microscope stage, scan the entire filter membrane over the light reflected from the illuminator and count the number of particles with a size greater than or equal to 10 μm and the number of particles of size greater than or equal to 25 μm . It is also possible to carry out partial counting and determine by calculation the total number of particles retained in the filter. Calculate the average number of particles present in the sample. To determine the particle size with the aid of the circular crosshair, transform the image of each particle into a circle and then compare with the reference circles of the 10 μm and 25 μm crosshairs. Thus, the particles maintain their initial position within the reticle field of view and do not overlap the reference circles for comparison purposes. The inside diameter of the reticle transparent reference circles is used to determine the size of the white or transparent particles, whereas the dark particle size is determined with the outside diameter of the black and opaque reticle reference circles. When carrying out a particle counting test under a microscope, do not attempt to measure or enumerate amorphous, semi-liquid or morphologically indistinct matters that resemble a stain or discolored area of the filter membrane. These materials may have a low or no shine and have a gelatinous appearance or a filmy appearance. Assessment interpretation can be facilitated by carrying out a particle count test by retaining light on a sample of the solution.

Assessment

Use the criteria below, according to the volume of samples or as indicated in the specific monograph of the pharmaceutical preparation.

In preparations arranged in containers with a nominal content greater than 100 mL, the preparation satisfies the test if the average number of particles present in the units examined is, at most, 12 per milliliter for particles with a size greater than or equal to 10 μm and of , at most, two particles per milliliter for those with a size greater than or equal to 25 μm .

In preparations arranged in containers with a nominal content equal to or less than 100 mL, the preparation satisfies the test if the average number of particles present in the units examined is, at most, 3000 per container for particles of size greater than or equal to 10µm and not more than 300 per container for particles greater than or equal to 25µm.

5.1.7.2 VISIBLE PARTICLES

Contamination by particles of injectable preparations and of injectable preparations for perfusion consists of non-soluble and mobile foreign particles, in addition to gas bubbles that are unintentionally found in these preparations. The purpose of the test is to provide a simple method of visually evaluating the quality of solutions with respect to visible particles. Other validated methods can be used.

Apparatus

The apparatus (**Figure 1**) is composed of an observation post, comprising: a matte black panel, of appropriate dimensions, placed in a vertical position, a non-glare white panel of appropriate dimensions, placed in a vertical position next to the black panel, a ramp of adjustable lighting, with a shielded white light source and a suitable diffuser (a lighting system containing 2 fluorescent lamps of 13W, with a wavelength of 525 nm each, is appropriate).

The luminous intensity at the observation point is kept between 2000 lux and 3750 lux and a higher intensity is advisable for colored glass or plastic containers.

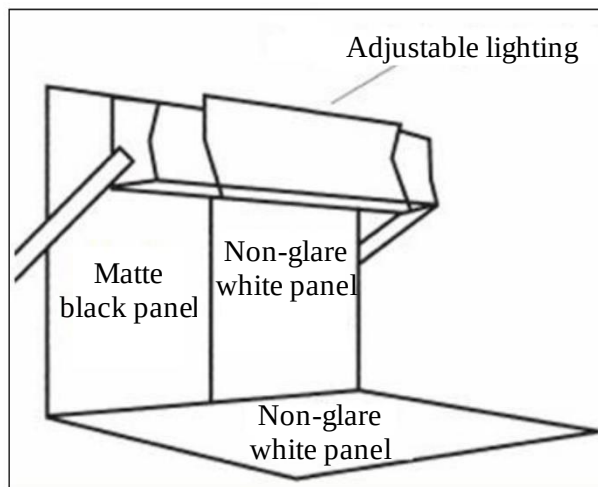


Figure 1 – Apparatus for visible particles.

Procedure

Remove the labels if necessary, wash and dry the outside of the container. Gently shake and invert each container carefully, avoiding the formation of air bubbles, and observe for approximately five seconds against the white panel. Repeat this procedure observing the container against the black panel. Note the presence of any particles.

5.1.8 DRIP TEST

The drip test is intended to determine the ratio between the number of drops per milliliter and the amount of drug per drop in liquid pharmaceutical preparations stored in containers with integrated measuring device. To carry out the test, it is necessary to know the declared number of drops per milliliter, or the declared amount of drug in mass per drop.

PROCEDURE

Determination of the number of drops per milliliter

Dripping must be carried out with the flask inverted in the vertical position or according to the drip angle declared by the manufacturer, allowing the flow by gravity, at a constant rate, without any type of additional pressure. Light pressure can be applied to polyethylene flasks.

Separate 30 units Carry out the test using 10 units, in an ambient with a controlled temperature of $(20 \pm 2) ^\circ\text{C}$. For each unit determine the mass relative to the number of drops corresponding to 1 mL, as declared by the manufacturer. If this ratio is not stated, use 20 drops for the test.

Calculate the number of drops per milliliter for each unit tested (N_t) according to the formula:

$$N_t = \frac{(N_1 \times \rho)}{m_i}$$

where

N_1 = number of drops used in the test, which can be the number of drops declared per milliliter (N_d) or 20 drops;

ρ = mass density of the product, in g/mL, determined at $20 ^\circ\text{C}$, as described in *Determination of mass density and relative density (5.2.5)*.

m_i = mass, in g, corresponding to the number of drops used in the test.

Determination of the amount of drug per drop

Calculate the amount of drug, in mg/drop, for each unit tested (q_t), as per the formula:

$$q_t = \frac{Q}{N_t}$$

where

Q = amount of drug in mg/mL determined in the assay;

N_t = number of drops per milliliter for each unit tested

Calculate the percentage in relation to the declared quantity, for each unit tested ($\%Q_t$ or $\%q_t$), using one of the formulas below:

$$\%Q_i = \frac{q_t}{(Q_d / N_d)} \times 100 \quad \text{or} \quad \%q_t = \frac{q_t}{q_d} \times 100$$

where

q_t = drug amount, in mg/drop, calculated for each unit tested;

Q_d = declared amount of drug, in mg/mL;

N_d = declared number of drops per milliliter;

q_d = declared amount of drug, in mg/drop;

Calculate the average of the individual percentage obtained ($\overline{\%Q}$) and the relative standard deviation (RSD) according to the formulas:

$$\overline{\%Q} = \frac{\sum \%Q_i}{n}$$

$$s = \sqrt{\frac{\sum (\%Q_i - \overline{\%Q})^2}{n - 1}}$$

$$DPR = \frac{100 \times s}{\overline{\%Q}}$$

where

$\%Q_i$ = percentage in relation to the declared quantity calculated for each unit tested;

s = standard deviation;

n = number of units tested;

CRITERIA

The product meets the test requirements if the individual percentage, for each of the 10 units tested, is between 85.0% and 115.0% of the declared quantity and the relative standard deviation (RSD) is not greater than 6.0 %.

If a unit is outside the range of 85.0% to 115.0% of the stated quantity, or if the RSD is greater than 6.0%, or if both conditions are met, test another 20 units.

The product meets the test if not more than one unit is outside the range of 85.0% to 115.0% of the declared quantity, no unit is outside the range of 75.0% to 125.0% and the RSD of the 30 units tested is not more than 7.8%.

5.2 PHYSICAL AND PHYSICAL-CHEMICAL METHODS

5.2.1 DETERMINATION OF MASS

In order to carry out the mass measurement, the scales must present capacity and sensitivity according to the required degree of precision and updated calibration certificate.

For activities that require exact weighing, in the determination of mass equal to or greater than 50mg, use an analytical balance of 100mg to 200mg capacity and 0.1mg sensitivity. For quantities lower than 50mg, use an analytical balance of 20g capacity and 0.01mg sensitivity.

APPARATUS

The analytical balances to be used in this test must be single-pan, preferably electronic.

Scales must have an adequate device that enables the verification of the applied load, provided they are periodically calibrated by means of measured reference masses.

Analytical balances must have the following features:

- protection cabinet or box, with appropriate openings to allow operations inside and exclude drafts;
- be installed on a base of compact and resistant material (marble, granite, metal or rubber, for example);
- level indicator (gravimetric or hydraulic) and device that allows its leveling; – be installed on a damping system (magnetic, pneumatic or hydraulic, for example) to promptly restore equilibrium;
- system that allows the reading of the mass (through dials and/or optical scale projection, etc.).

They must also support their full load without suffering undue tension that could compromise their sensitivity in serial weighing under these conditions.

The scale must not be overloaded.

Analytical balance location

The analytical balance must be placed leveled upon a firm and heavy table or shelf, protected by shock absorbers such as cork mats or rubber sheets, or on a concrete counter, supported by pillars that are fixed to the floor or connected to the elements of building construction to prevent vibrations. It must be in an isolated place, which offers safety and stability to the measuring, in an environment with a relatively dry atmosphere, protected from the attack of acid gases and vapors, away from heat sources (direct sunlight, ovens, heaters, muffle furnaces, etc.) and of drafts.

Conservation and cleaning

The pan and other parts of the scale, including its enclosure, must remain clean, free of dust and substances that accidentally fall onto the scale pan or the case floor. Such materials must be removed immediately.

The bodies to be weighed must not be placed directly on the pan. For this purpose, papers or containers suitable for the mass are used, such as beakers, watch glasses, crucibles, porcelain capsules and weighing bottle with or without a lid.

The moving parts of the scale and weights must not be touched by hand. For this purpose, appropriate tweezers are used, which must be kept in the weight box.

Desiccant agents such as silica gel or calcium chloride can be placed inside the enclosure to maintain a relatively dry atmosphere.

When the scale is not in use, its doors must remain closed and locked.

The sensitivity of analytical balances must be periodically inspected and checked by a qualified expert.

Use of Analytical balance

The material to be weighed must be in thermal equilibrium with the air inside the scale protective enclosure in order to avoid errors due to convection currents, as well as humidity condensation on cold bodies.

The scale must be leveled at the time of use. The balanced position with or without load should be checked several times at 10% of full load and with full load. The equilibrium difference found in two serial determinations made with equal weights must not exceed 0.1 mg for analytical balances (maximum 200 g) and 0.01 mg for analytical balances (maximum 20 g).

Both the weights and the material to be weighed must be placed in the center of the pan. During weighing operations, the enclosure doors must be closed.

5.2.2 DETERMINATION OF MELTING RANGE OR TEMPERATURE

The *Temperature* or *Melting point* of a substance is the temperature at which it is completely melted. It is an intrinsic property of substances, which is used, together with other tests, to confirm their identity, as well as an indicator of purity. In the case of substances that melt on decomposition, the temperature or melting point will be the temperature at which melting starts.

The *melting range* of a substance is defined as the range between the temperature at which the substance starts to liquefy or form drops on the walls of the capillary tube and the temperature at which the substance is completely melted.

A melting transition can be instantaneous for a highly pure material, but a gap is generally observed from the beginning to the end of the process. There are different factors that influence this transition and they should be standardized when describing the procedure. These factors include: sample quantity, particle size, heat diffusion performance, and speed of heating, among others.

For pharmacopoeial purposes, the melting point or melting range is reported as the temperature at which the first liquid phase is observed and the temperature at which there is no more apparent solid phase, except for those substances that melt with decomposition or specified otherwise in the individual monograph.

METHOD I

For samples that are easily reduced to powder.

Apparatus I

It consists of a glass container (C) for a transparent liquid bath, a mixing device (D), a thermometer (A) and a suitable heat source (see **Figure 1**). According to the required working temperature, the bath liquid can be one of the following or whatever is appropriate:

- Water for temperatures up to 60 °C;
- Glycerol for temperatures up to 150 °C;
- High boiling liquid paraffin for temperatures up to 250 °C;
- Sesame oil or a silicone oil at suitable grade for temperatures up to 300°C.

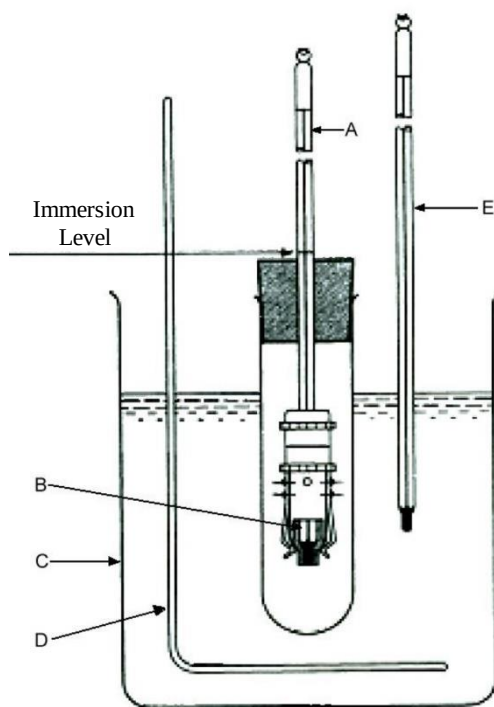


Figure 1 – Apparatus I for determining the melting point.

The bath liquid must be deep enough to allow the thermometer to immerse at the specified depth so that the bulb is approximately 2 cm from the bottom of the bath. Heat can be supplied by a flame or electrically. The capillary tube is approximately 10cm long and between 0.8mm and 1.2mm in internal diameter, with walls from 0.1mm to 0.3mm thick, and must be closed at one of its ends, unless otherwise specified in the individual monograph. A stirrer device that guarantees the homogeneity of the bath temperature must be used.

Procedure

If not otherwise specified in the individual monograph, proceed as follows:

Reduce the sample to a fine powder and dry it in a vacuum desiccator over a suitable desiccant for 24 hours.

Load the dry capillary tube with a sufficient quantity of powder to form a column of 3 mm to 4 mm in height, after having compressed with moderate strokes on a solid surface. Connect the capillary tube to the thermometer, both moistened with the bath liquid. Adjust height so that the sample contained in the capillary is close to the thermometer bulb (B).

Adapt an auxiliary thermometer (E) so that the center of the bulb is as close as possible to the stem of the main thermometer (A) at a point equidistant from the surface of the bath and from the division corresponding to the expected melting point.

Heat the bath to a temperature of 10°C below the expected melting point. Insert the thermometer with the capillary attached and continue heating in such a manner that the temperature rises at a rate of 1°C to 2°C per minute, depending on the stability of the substance.

Record the reading of the auxiliary thermometer at the end of the sample melting and, if necessary, apply the correction for the emerging column using the following formula:

$$t_c = k \times N(T - t)$$

where

t_c = correction that must be added to the observed melting temperature;

k = correction constant by the coefficient of liquid expansion of the thermometer. In the case of mercury, the value is 0.00016;

N = number of degrees of the main thermometer column between bath level and the observed melting temperature;

T = melting temperature;

t = temperature registered by the auxiliary thermometer.

Carry out the determination at least in triplicate. To do this, allow the bath to cool to 10°C below the melting point or to a lower temperature and repeat the procedure using new portions of the sample.

Apparatus II

It consists of a metal block that can be heated at a controlled speed, whose temperature can be monitored by a sensor or thermometer. The block allows the capillary tube containing the test substance to be inserted in it and to monitor the melting process through visual control or automatically.

Procedure

If not otherwise specified in the individual monograph, proceed as follows:

Reduce the sample to a fine powder and dry it in a vacuum desiccator over a suitable desiccant for 24 hours.

Load the dry capillary tube with a sufficient quantity of powder to form a column of 3 mm to 4 mm in height, after having compressed with moderate strokes on a solid surface.

Heat the bath to a temperature of 10°C below the expected melting point. Insert the capillary into the block and record the temperature at the beginning and end of melting. Continue heating in such a manner that the temperature rises at a rate of 1°C to 2°C per minute.

Carry out the determination at least in triplicate. To do this, allow the bath to cool to 10°C below the melting point or to a lower temperature and repeat the procedure using new portions of the sample.

METHOD II

For samples that are not easily reduced to powder.

Procedure

Carefully melt the sample at the lowest possible temperature and introduce the melted material into an capillary open at both ends to form a column about 10mm high. Cool the charged capillary to a temperature equal to or less than 10°C for approximately 24 hours. Join the capillary to the thermometer and adjust height so that the sample contained in the capillary is close to the thermometer bulb. Place in water bath and heat as indicated in *Method I, Apparatus I*, except that when it reaches a temperature of approximately 5°C below the expected melting point, the

temperature is increased at a rate of 0.5°C per minute. The temperature at which the sample begins to rise inside the capillary tube is recorded as the melting point. Carry out the determination at least in triplicate using different portions of the sample.

METHOD III

For petroleum jelly, fatty substances or others with a creamy consistency (semi-solid).

Procedure

Melt the sample under agitation until it reaches a temperature between 90°C and 92°C and allow the melted substance to cool to a temperature between 8 °C and 10°C above the expected melting point. Cool the thermometer bulb to 5°C, dry and, while it is still cold, submerge it in the melted sample approximately to the middle of the bulb. Remove immediately and keep in an upright position until the surface of the sample deposited on the bulb solidifies. Place in water bath at a temperature not exceeding 16°C for approximately five minutes.

Adapt the thermometer inside a test tube by means of a perforated stopper, so that its lower end is about 15 mm above the bottom of the tube. Suspend the test tube in water bath at a temperature of 16°C and raise the temperature of the bath to 30°C, at a rate of 2°C per minute, and then at a rate of 1°C per minute until the first drop detaches from the thermometer. The temperature at which this occurs is the melting point. For each determination use a fresh-fused portion of the sample. Carry out the determination in triplicate. If the maximum difference between the determinations is less than 1°C, determine the average of the obtained values. Otherwise, carry out two other determinations and calculate the average of the five.

5.2.3 DETERMINATION OF BOILING TEMPERATURE AND DISTILLATION RANGE

The temperature or boiling point of a liquid is the corrected temperature at which the liquid boils under a vapor pressure of 101.3 kPa (760 mm of Hg).

Distillation range is the temperature range corrected for the pressure of 101.3 kPa (760 mm of Hg), within which the liquid, or specific fraction of the liquid, fully distills.

APPARATUS

Use apparatus as suggested in **Figure 1** where A is a distillation flask with a capacity of 100 mL connected to condenser B. Adapter C is attached to the lower end of B. A 50 mL cylinder graduated in 0.2 mL is used as a collector. The thermometer must be fitted to the flask so that the temperature sensor is located in the center of the neck and about 5 mm below the level of the side tube. Heating (via gas, electric or bath) should be selected according to the nature of the substance.

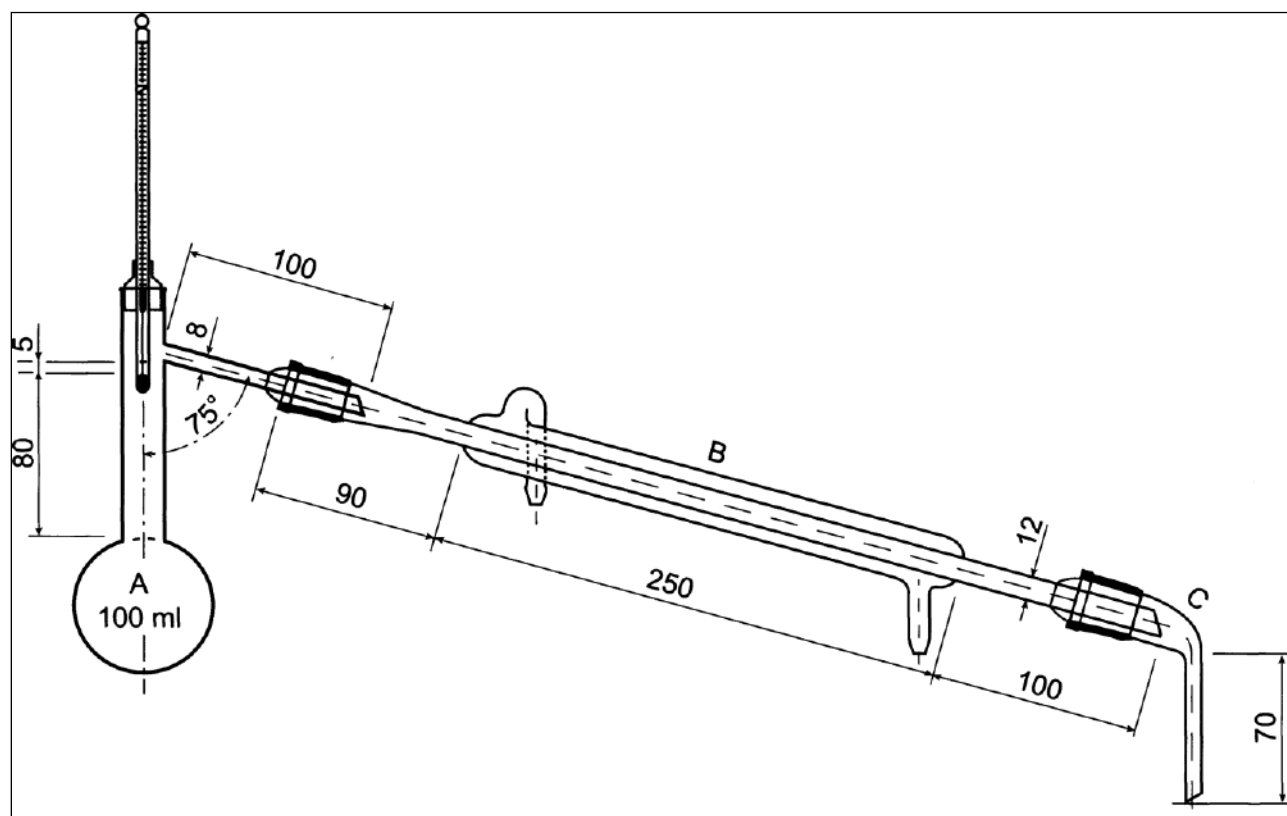


Figure 1 – Apparatus for determining the distillation range (dimensions in mm). A, distillation flask; B, condenser; C, adapter.

PROCEDURE

Add approximately 50 mL of the sample to the flask so that it does not leak into the side tube. Add glass beads or other suitable porous material. Adapt the thermometer to the flask and heat, slowly, protecting the system from drafts.

Record the temperature at which the first five drops of distillate are collected. Adjust heating to obtain distillate at a flow rate of 3 mL to 4 mL per minute. Record the temperature at which the last drop evaporates from the distillation flask or when the specified fraction is collected. Keep the distillate at

the same temperature at which the liquid was originally measured and record the volume of the distillate.

Compare the values obtained for the boiling point, distillation range and distillate volume with the respective specifications of the monographs.

Correct the readings for atmospheric pressure using the formula :

$$t_1 = t_2 + k (101.3 - b)$$

where

t_1 = temperature corrected;

t_2 = temperature observed at atmospheric pressure b ;

k = conversion factor (**Table 1**), unless this factor is not considered;

b = atmospheric pressure, expressed in kilopascals, during distillation.

Table 1 – Correction factors for different distillation temperatures.

<i>Distillation temperature</i>	<i>Correction factor k</i>
up to 100 g	0.30
above 100 g and up to 140 g	0.34
above 140 g and up to 190 g	0.38
above 190 g and up to 240 g	0.41
above 240 g	0.45

Note 1: When the liquid is pure, most of it boils at a constant temperature (in a range of 0.5°C). This temperature is the boiling point of the liquid.

Note 2: liquids that distill below 80°C must be cooled between 10°C and 15°C before measuring the volume and the cylinder that receives the distillate must be immersed in an ice bath.

Note 3: when the boiling point is above 150 °C, you can replace the water condenser with air condenser.

5.2.4 DETERMINATION OF CONGEALING TEMPERATURE

The *temperature* or *congealing point* of a liquid or melted solid is the highest temperature at which it solidifies.

For pure substances that melt without decomposition, the liquid congealing point is equal to its melting point.

APPARATUS

The device (**Figure 1**) consists of a test tube of approximately 25mm in internal diameter and 150mm in length suspended through a suitable stopper inside a second larger tube, 40mm in internal diameter and 160mm in length, forming an air jacket that prevents sudden changes in temperature. This system is fixed by a clamp in the center of the beaker with a capacity of 1000 mL containing water or a coolant solution.

The inner tube is sealed with a stopper in order to contain a stirring rod and a thermometer with 0.2°C divisions. The thermometer temperature sensor should be fixed approximately 15mm from the bottom of the tube. The stirrer is a glass rod fitted with a ring at its lower end (**Figure 1**).

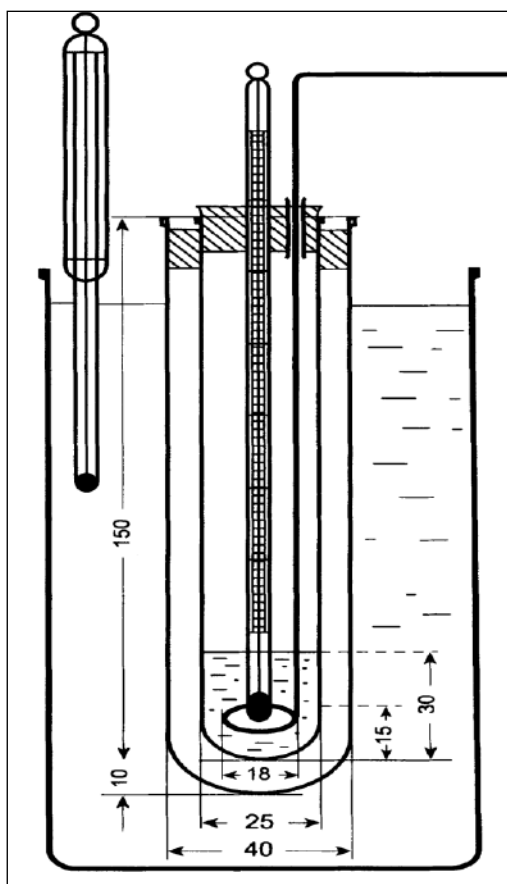


Figure 1 – Apparatus for determining congealing point.

PROCEDURE

Transfer the sample in sufficient quantity to reach 30 mm in the inner tube. Transfer the coolant mixture set at 5°C below the expected congealing point to the beaker. When the sample is cooled to

approximately 5°C above congealing point, move the stirrer vertically between the surface and the bottom for approximately 20 cycles per minute and record the thermometer temperature every 30 seconds. Stop shaking when the temperature remains constant or shows a slight increase. Record the temperature every 30 seconds for at least three minutes after the temperature starts to drop again.

Record the maximum on the temperature-time curve, which occurs after the temperature remains constant, or shows a slight increase, and before the temperature starts to decrease again. Congealing point is assigned to an average of not less than three consecutive maximum points that fall within a range of 0.4°C.

Note 1: *If the substance is solid at room temperature, melt the substance and heat to a maximum of 20°C above the expected congealing temperature before transferring to the inner tube.*

Note 2: *if the substance is liquid at room temperature, use bath at 15°C below the expected congealing temperature.*

5.2.5 DETERMINATION OF MASS DENSITY AND RELATIVE DENSITY

Mass density (ρ) of a substance is the ratio of its mass to its volume at 20°C. The mass density of the substance (ρ_t) at a given temperature (t) is calculated from its relative density (d^t) by the formula:

$$\rho^t = d_{(água)} \times d_t^t + 0,0012$$

expressed in g/mL or kg/L.

When the temperature is, for example, 20°C, the formula is expressed by:

$$\rho_{20} = 0,99820 \times d_{20}^{20} + 0,0012$$

Table 1 - Density of water from 0 to 40 °C.

Temp. (°C)	Density (g/mL)	Temp. (°C)	Density (g/mL)	Temp. (°C)	Density (g/mL)	Temp. (°C)	Density (g/mL)
0	0.99984	10	0.99970	20	0.99820	30	0.99565
1	0.99990	11	0.99961	21	0.99799	31	0.99534
2	0.99994	12	0.99950	22	0.99777	32	0.99503
3	0.99996	13	0.99938	23	0.99754	33	0.99470
4	0.99997	14	0.99924	24	0.99730	34	0.99437
5	0.99996	15	0.99910	25	0.99704	35	0.99403
6	0.99994	16	0.99894	26	0.99678	36	0.99368
7	0.99990	17	0.99877	27	0.99651	37	0.99333
8	0.99985	18	0.99860	28	0.99623	38	0.99297
9	0.99978	19	0.99841	29	0.99594	39	0.99259
10	0.99970	20	0.99820	30	0.99565	40	0.99222

The relative density of a substance is the ratio of its mass by the mass of an equal volume of water, both at 20°C (d_{20}^{20}) or by the mass of an equal volume of water at 4°C (d_4^{20}):

$$d_4^{20} = 0,998234 \times d_{20}^{20}$$

PROCEDURE

The relative density of the substance can be determined using a pycnometer, hydrostatic balance or densimeter. The use of these last two is conditioned to the type of apparatus available.

PICNOMETER METHOD

Use a clean and dry pycnometer, with a capacity of not less than 5 mL that has been previously calibrated. Calibration consists of determining the mass of the empty pycnometer and the mass of its contents with fresh-distilled and boiled water at 20°C.

Transfer the sample to the pycnometer. Adjust temperature to 20°C, remove substance excess, if necessary, and weigh. Obtain the sample weight from the mass difference of the full and empty pycnometer. Calculate the relative density (d_{20}^{20}) determining the ratio between the mass of the liquid sample and the mass of the water, both at 20°C. Use relative density to calculate mass density (ρ).

5.2.6 DETERMINATION OF REFRACTIVE INDEX

The refractive index (n) of a substance is the ratio between the speed of light in a vacuum and its speed inside the substance. When a ray of monochromatic light passes from a transparent medium to another with a different optical density, it is reflected or refracted, except when it is incident perpendicular to the interface. The ratio between the sine of the angle of incidence ($\sin i$) and the sine of the angle of refraction ($\sin r$) is constant. This ratio is equivalent to the refractive index (n).

$$n = \frac{\sin i}{\sin r}$$

For practical purposes, refraction is measured with reference to air and substance rather than with reference to vacuum and substance, as the differences between the values obtained with both measurements are not significant for pharmacopoeial purposes.

In isotropic substances, the refractive index is a constant feature at a given wavelength, temperature and pressure. For this reason, this index is useful not only to identify the substance, but also to detect the presence of impurities. It is used to characterize mainly fats, fatty oils, waxes, sugars and organic solvents, as well as to identify certain drugs. It is also used to determine the purity of volatile oils.

Generally, the refractive index is determined from the sodium light at a wavelength of 589.3 nm (lane D) and at $(20 \pm 0.5)^\circ\text{C}$. Hence, the value of the refractive index is expressed as n_D^{20} .

REFRACTOMETERS

Refractometers commonly used in pharmacopoeial analysis use white light, but are calibrated to provide the refractive index in terms of wavelength corresponding to that of sodium D-lane light.

The Abbé refractometer measures the range of refractive index values of pharmaceutical substances. Other refractometers of greater or equal precision can be used.

As the refractive index varies significantly with temperature, during the reading the sample must be adjusted and kept at 20°C .

The device is calibrated with a standard provided by the manufacturer. To control the temperature and clean the apparatus, the refractive index of distilled water must be determined, with values of 1.3330 at 20°C and of 1.3325 at 25°C .

5.2.7 DETERMINATION OF VISCOSITY

Viscosity is the expression of the resistance of liquids to flowing, that is, to the displacement of part of their molecules to neighboring molecules. The viscosity of liquids derives from internal friction, that is, from the cohesion forces between molecules relatively close together. As temperature rises, the average kinetic energy of the molecules increases, the amount of time the molecules spend together decreases (on average), the intermolecular forces become less effective and the viscosity is lower.

The dynamic unit of viscosity, in the CGS System, is the *poise*. The CGS System of Units is a system of physical measurement units, or dimensional system, of LMT typology (length, mass, time), whose base units are the centimeter for length, gram for mass, and second for time.

The analogous dynamic unit in the International System of Units (SI) is the pascal second. Poise is often used with the prefix *centi*; a centipoise (cP) is a millipascal second (mPa·s) in SI units.

CGS Sistema system – *poise (P)*

$$1 P = 1 g \cdot cm^{-1} \cdot s^{-1}$$

By definition, *poise* is the force, in dynes, necessary for the displacement of a flat liquid layer, with an area of 1 cm², on another identical layer, parallel and distanced from the first by 1 cm, at a speed of 1 cm/s. *Poise* is, however, too large for most applications, hence the *centipoise*, cP, corresponding to one hundredth of *poise*. Sometimes it is convenient to use kinematic viscosity, which is the ratio between dynamic viscosity and density. In this case, in the CGS system, the unit is the *stokes*. As an example of what happens with absolute viscosity (measured in *poise*), it is more convenient to express kinematic viscosity in *centistokes* (100 *centistokes* = 1 *stoke*) to characterize most liquids usual in Pharmacy and Chemistry.

International System of Units - *pascal second (Pa·s)*

$$1 Pa \cdot s = 1 kg \cdot m^{-1} \cdot s^{-1} = 10 P$$

Pascal second is equivalent to 10 *poise*, but usually millipascal second (mPa·s) is more commonly used. **Table 1** shows the viscosity of some liquids.

Table 1 - Viscosity of some liquids.

<i>Liquid</i>	<i>Viscosity (P)^a</i> <i>CGS Units</i>	<i>Viscosity</i> <i>N s m⁻² SI Units</i>	<i>Viscosity</i> <i>cP = mPa.s</i>
Water	0.0101 (298 K)	0.00101	0.890
Acetone	0.00316	0.000316	0.306
Ethanol	0.01200	0.001200	1.074
Glycerin	14.9	1.49	934

^a 1 poise (P) = 1 dyne. s. cm⁻² = 0.1 N s m⁻². cP = centi-poise = mPa.s = milli Pascal times sec.

The determination of viscosity – a test for which the specification of temperature is essential due to its decisive influence on the result (in general, viscosity is inversely proportional to temperature) – is performed based on different properties. The most frequent method is based on the flow time of liquids through capillaries (Ostwald, Ubbelohde, Baumé and Engler viscometers) due to the devices

simplicity and affordable price. Viscometers whose working principle is the determination of the free fall time of spheres through tubes containing the liquid under test (Höppler) or the rotation speed of metallic axes immersed in the liquid (Brookfield, among others) are also used.

Several methodologies that can be used:

- resistance of liquid to flow, time of flow of a liquid through a capillary (Oswald, Ubbelohde, Baumé and Engler viscometer);
- measure of the falling time of a sphere through tubes containing the liquid under test (Höppler);
- measurement of the resistance to rotational movement of metallic axis when immersed in liquid (Brookfield rheometer).

Although it is possible to determine the absolute viscosity, based on the exact dimensions of the viscometer used, it is more frequent to calibrate the device with a liquid of known viscosity, allowing, by comparison, the relative evaluation of the viscosity of the liquid under test. Thus, using Ostwald viscometer or similar, the flow times t_1 and t_2 of equal volumes of sample and reference liquids, of density d_1 and d_2 , respectively. If η_2 is the viscosity of the reference liquid, the absolute viscosity (cP) of the sample liquid can be calculated by the formula:

$$\frac{n_1}{n_2} = \frac{t_1 d_1}{t_2 d_2}$$

or yet

$$n_1 = n_2 \frac{t_1 d_1}{t_2 d_2}$$

The quotient $\eta_2/t_2.d_2$ has a constant value, k , for each reference liquid, in the same viscometer. Thus, once this value is known (usually found in the equipment manual), the formula is simplified:

$$n = k . t . d$$

The value of k can also be determined experimentally by measuring the flow time of a standard pure liquid and applying the formula:

$$k = \frac{n}{t . d}$$

Using water as the standard, usual for the determination of low viscosity liquids, the viscosity values recorded in **Table 2** are adopted, according to the test temperature:

Table 2 – Viscosity values, according to the test temperature.

<i>Temperature (°C)</i>	<i>η (cP)</i>
15	1.140
16	1.110
17	1.082
18	1.055
19	1.029
20	1.004
21	0.980
22	0.957
23	0.936

24	0.915
25	0.895

For highly viscous liquids (glycerin and oils in general), the relative viscosity can be determined by the method of velocity of falling balls through the liquid, using the Höppler viscometer. This method is also suitable for determining the absolute viscosity of liquids by applying the formula:

$$\eta = t(d_s - d_L)K$$

where

t = ball falling time (sec).

K = ball-specific cte (mPcm³), supplied by the manufacturer. d_s = ball density (g/cm³).

d_L = density of the liquid (g/cm³).

The density of the liquid (d_L), for a certain temperature, can be obtained from reference books (such as *handbooks*), or determined experimentally.

The relative viscosity in the Höppler method can be determined by applying the formula:

$$\frac{\eta_1}{\eta_2} = \frac{(d_s - d_1)t_1}{(d_s - d_2)t_2}$$

where η , d and t are, respectively, the coefficient of dynamic viscosity, the density and the flow time of equal volume of liquids 1 and 2.

OSTWALD VISCOMETER

The Ostwald viscometer is the simplest and most popular device available. It consists of a U-bent tube (**Figure 1**), with one of the branches fitted with an ampoule ending in a capillary. There are two reference lines, one immediately above the ampoule and the other above the capillary. The other branch is wide enough to be filled with the liquid under test to a height of about 5mm below the lower reference line. To enable the determination of viscosities over a wider range, collections of viscometers with different calibration levels are used. The device indicated for a certain evaluation is the one that allows the sample to flow in a period of maximum 60 seconds.

For the actual determination, transfer to the chosen viscometer, washed and dry, a sufficient quantity of liquid to reach a level of 5mm below the lower reference line. Set the device in a thermostat (20 °C). After waiting for the liquid inside the device to reach the controlled temperature, aspirate the liquid through the capillary tube/ampoule (by means of a rubber tube attached to the end) until the liquid level slightly exceeds the upper reference line. Then release the tube and, at the instant the meniscus reaches the upper reference line, start the precision timer, relocking it when the meniscus passes through the lower reference line. Record the time elapsed and repeat the test several times at ranges of a few minutes until serial times do not differ by more than 0.5 seconds. Determine the density of the liquid under test (5.2.5), correcting the value for the density relative to water, at 20°C, and calculate the viscosity of the liquid sample by the formula indicated, using the constant k provided or determined by a similar procedure.

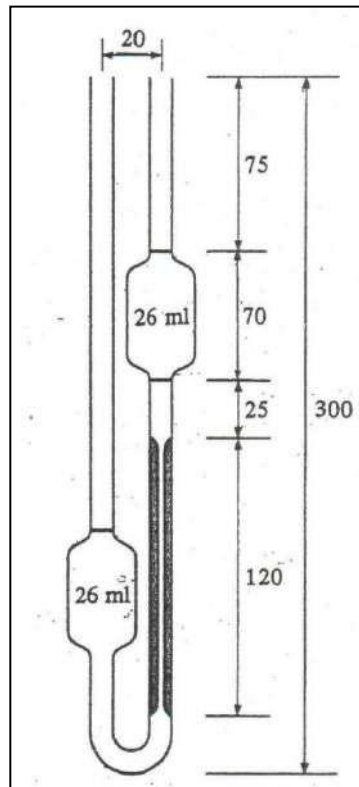


Figure 1 – Ostwald Viscometer (dimensions in mm).

HÖPPLER VISCOMETER

The Höppler measurement system measures the time a solid ball takes to travel a distance between two reference points within an inclined sample-filled tube. The results obtained are considered as dynamic viscosity in the measure standardized in the International System (mPa.s). It accurately determines the viscosity of Newtonian liquids and gases (with a special ball for gases). Among its applications we cite research, process control and quality control, mainly used for substances with low viscosity, between 0.6 and 100,000 mPa.s.

The Höppler Viscometer is composed of a glass tube with two marks (A and B) spaced from each other on the column by 10mm which define the measurement distance. A ball (in glass, nickel alloy and iron or steel) with a diameter compatible with the gauge of the glass tube is installed on top of its liquid content. The tube is surrounded by a glass cylinder filled with circulating water under controlled temperature. The entire set is arranged in a slightly inclined position (10% vertically), and can be rotated 180° around an axis perpendicular to both tubes, to allow the repetition of the determinations and the return of the ball to the initial position. The method consists of timing the falling time (fall) that a sphere (with variable density and diameter with the respective structural constitution) takes to traverse the space between those two marks (A and B) existing at the ends of the glass tube. The higher the viscosity, the longer the ball will take to travel through that space. The type of sphere to be used is chosen depending on the presumptive value of the viscosity of the liquid under observation. In the case of blood, glass beads are used. The viscosity results of Newtonian liquids are expressed in international standard absolute units (mPa.s)

For the actual determination, rinse the chosen viscometer, washed and dry, with the liquid used to determine the viscosity. Adjust the apparatus probe. Choose the appropriate ball for each liquid (water = glass ball). Completely fill the inner tube of the viscometer with liquid. Record the time for the ball to fall between marks A and B on the viscometer. Carry out two more determinations to get the best average.

BROOKFIELD VISCOMETER

The viscosity of a pharmaceutical preparation can be determined by a Brookfield viscometer, which measures viscosity by the force required to turn the spindle in the liquid being tested.

To use this device, proceed as follows:

- add the sample to be analyzed in the device collecting container up to the desired mark;
- set the device, choosing a spindle number and a rotation to be tested, according to the specific methodology;
- immerse the spindle in the sample to be analyzed;
- activate the device and, after stabilization of the value, which will appear on the device display, record the value which will be expressed in centipoise (cP);

If the value does not stabilize, test again using another spindle number or another rotation.

OUTFLOW VISCOMETER - FORD TYPE MODEL

Select the proper orifice. The guideline for orifice selection is to obtain a flow time of the liquid under test of around 60 seconds. There must be a flow time between 20 and 100 seconds for the sample at 25°C.

The sample must be perfectly mixed. At the time of testing, the viscometer and the material to be tested must be at $(25 \pm 0.1) ^\circ\text{C}$. Close the orifice with a flat glass slide and fill the sample cup to the highest level. Pour the sample slowly, avoiding the formation of bubbles. Level the sample in the cup using a flat glass plate. Remove the slide from the orifice. The sample will be retained in the cup. Remove the flat glass plate and activate the timer when the sample starts to flow through the orifice. When the first interruption in the flowing occurs, stop the timer and record the time elapsed in seconds. Carry out the test at least in triplicate. The viscosity will be the mean of the values obtained, expressed in mm^2/s or Centistokes, with a maximum standard deviation of 3% being allowed. The conversion from seconds to mm^2/s or Centistokes is given in accordance with the manual of the apparatus used.

5.2.8 DETERMINATION OF OPTICAL ROTATION

Optical rotation is the property that some liquid substances or solutes in solution have to rotate the plane of polarization of the polarized light that falls on them. This property is characteristic of many substances that have chiral centers, very often made up of carbon atoms with four different substituents (asymmetric center). The maximum number of possible optical isomers of a molecule is 2^n where n is the number of asymmetric centers. Substances that rotate the plane of polarization of light in a clockwise direction are called dextrorotatory or optical isomers (+); whereas those that rotate the plane of polarization of light in the opposite direction are called levorotary or optical isomers (-) (the symbols d- and l- which were previously used to indicate dextro- and levo- isomers, are no longer used, due to confusion with the symbols D- and L- which refer to configurations related to D-glyceraldehyde. The symbols R and S, as well as α and β , are also used to indicate the configuration, that is, the spatial ordering of the atoms or groups of atoms).

Chiral substances whose molecules are not superimposable but are mirror images are called enantiomers. They have the same physicochemical properties (density, refractive index, dipole-dipole moment, boiling and melting points), except that they rotate the plane-polarized light at the same quantity of degrees in opposite directions, and their reactions with other chiral substances have different characteristics.

Polarimetry is a convenient method to differentiate between optically active isomers by measuring the optical rotation of a substance; it is also an important criterion of identity and enantiomeric purity and can be used for quantitative purposes.

Optical rotation varies with temperature, the wavelength of incident light, the solvent used, the nature of the substance and its concentration. If a solution contains two optically active substances and they do not react with each other, the deviation angle will be the algebraic sum of the deviation angles of both.

POLARIMETER

Polarimeters are devices that detect optical rotation visually (by equaling the intensity of light over two fields) or through a photoelectric system, the latter being more accurate and precise than those for visual measurement.

The measurement of optical rotation must be performed using a polarimeter capable of measuring differences of not less than 0.05° , unless otherwise specified in the individual monograph. As a light source, sodium, mercury vapor, xenon or halogen-tungsten lamps are used, among others, equipped with a device that allows transmitting a monochromatic light beam. These last two lamps mentioned are usually less expensive, in addition to having greater durability and having a wider range of emission wavelengths compared to traditional light sources. The scale must be controlled using a polarization reference standard, which consists of certified quartz plates. Scale linearity should be checked periodically using a solution of standard dextrose and sucrose reference materials.

The use of lower wavelengths, such as the mercury lamp lines at 578 nm, 546 nm, 436 nm, 405 nm and 365 nm in a photoelectric polarimeter, can provide advantages in terms of sensitivity, with a consequent reduction test substance concentration. In general, the optical rotation observed at 436 nm is approximately double and that observed at 365 nm is approximately three times greater than that observed at 589 nm.

The reduction in the concentration of the test substance required for measurement can sometimes be achieved by converting it to another substance that has a significantly higher optical rotation. Optical rotation is also affected by the solvent used in the measurement and this must be specified in all cases.

PROCEDURE

The specific optical rotation is a reference value and is calculated from the observed optical rotation for a sample solution or liquid as specified in the monograph. Optical rotation measurements are performed at 589.3nm at 25°C, unless otherwise specified in the individual monograph. The experimental temperature must be maintained at ± 0.5 °C from the specified value.

When using a polarimeter with visual detection, the mean of at least five determinations must be used, corrected by the reading of the solvent blank, for solutions, and the air, for liquids. When using a photoelectric polarimeter, carry out a single measurement corrected by the solvent blank, for solutions, and by the air, for liquids. Use the same polarimeter tube in the same orientation for the sample and for the blank.

Optical rotation of solutions must be determined within 30 minutes of preparation. In the case of substances that can undergo racemization or mutarotation, special care must be taken in standardizing the time between which the solution is prepared and the polarimetric reading is carried out.

Unless otherwise indicated in the corresponding monograph, the specific rotation is calculated on dry substance when the monograph determines *Loss on Desiccation*, on anhydrous substance when specifying *Determination of water*, or free of solvents when specifying *Content of residual solvents*.

The accuracy and precision of optical rotation measurements can be enhanced if the following precautions are taken:

- 1) The formation of air bubbles must be avoided when filling the polarimeter tube, which is particularly necessary for micro and semi-micro tubes.
- 2) Samples of dissolved liquid or solid substances must be homogeneous and clear.
- 3) The optical elements must be perfectly aligned, as well as the light source in relation to the optical path.

CALCULATIONS

The specific optical rotation is calculated from the optical rotation observed in the sample solution, obtained as specified in the corresponding monograph.

Calculate the specific optical rotation using the following formulas:

For liquids: $[\alpha]^{25}_D = \alpha / l d^{25}$

For substances in solution: $[\alpha]^{25}_D = 100 \alpha / l d^{25}$

where

α = corrected observed rotation, in degrees, at 25°C;

l = length of the polarimeter tube in decimeters;

d^{25} = relative density of the liquid at 25°C;

c = concentration of substance in percent weight/volume;

$[\alpha]^{25D}$ = specific optical rotation determined at 25°C and 589.3nm (line D of the sodium light).

5.2.9 DETERMINATION OF WEIGHT LOSS

5.2.9.1 LOSS ON DESICCATION

This test is intended to determine the amount of volatile substance of any nature eliminated under the conditions specified in the individual monograph. For substances that have water as the only volatile constituent, it is appropriate to apply the procedure indicated in *Determination of Water* (5.2.20). The result is expressed as a percentage w/w, calculated as follows:

$$\frac{(P_u - P_s)}{P_m} \times 100$$

where

P_m = sample weight (g);

P_u = weight of the weighing bottle containing the sample before drying (g);

P_s = weight of the weighing bottle containing the sample after drying (g).

PROCEDURE

Gravimetric Analysis

If not otherwise specified in the individual monograph, proceed as follows:

If necessary, reduce the substance to a fine powder by grinding quickly. Weigh an approximate amount between 1 g to 2 g of the substance, in a weighing bottle previously desiccated for 30 minutes, under the same conditions as used in the sample test, and cooled to room temperature in a desiccator.

Distribute the sample as evenly as possible, gently shaking the weighing bottle so that a layer approximately 5mm thick is formed and a maximum of 10mm in the case of bulky materials. Place the uncapped weighing bottle containing the sample together with the cap in the drying chamber. Dry the sample under the conditions specified in the monograph (**Note:** *the temperature specified in the monograph must be considered to be within the range of +2 °C*). Open the drying chamber, cap the weighing bottle quickly, remove and allow to reach room temperature in a desiccator before weighing.

When the individual monograph specifies desiccation up to constant weight, drying must continue until two consecutive weighing determinations do not differ by more than 0.50 mg per gram of substance weighed, carrying out the second weighing after an additional hour of drying.

If the substance melts at a temperature lower than that specified for the determination of loss on drying, keep the weighing bottle with its contents for one to two hours at a temperature of 5°C to 10°C lower than the melting temperature and then dry at specified temperature.

For the analysis of capsules, use a portion of the mixed content of at least four units. In the case of tablets, use the powder of at least four units.

When indicated in the individual monograph:

- *drying under reduced pressure*, a desiccator, drying oven or other suitable apparatus must be used, under reduced pressure (vacuum);
- *dry, under reduced pressure, in a flask with a cap equipped with capillary perforation*, a flask or tube with a capillary cap of (225 ± 25) µm in diameter must be used and the heating chamber must

be maintained at a pressure of maximum 5mm of mercury. Heating period elapsed, allow dry air to enter the chamber, remove the flask and, with cap still in place, allow to cool to room temperature in a desiccator before weighing;

- *drying in a desiccator*, precautions must be taken to ensure that the desiccant remains active. Calcium chloride, silica gel and phosphorus pentoxide are among the most frequent desiccant agents.

Thermogravimetry

In the event that the individual monograph specifies that the loss on desiccation must be carried out by thermogravimetric analysis, proceed as described in *Thermal analysis (5.2.27)*.

Infrared or halogen lamp scale

In the event that the individual monograph specifies that the loss by desiccation must be carried out on an infrared scale or with a halogen lamp, proceed as follows:

- Remove moisture from the apparatus;
- Weigh quantity of the substance to be analyzed, distribute the material evenly in the sample collector and place inside the apparatus;
- Set the drying time and temperature as set in the individual monograph. Record the moisture value obtained.

5.2.9.2 LOSS ON IGNITION

This test is intended to determine the amount of substance of any nature volatilized and eliminated under the conditions specified in the monograph. The procedure is generally not destructive to the substance under test; however, the substance can be converted to another form, such as an anhydride or oxide. The result is expressed as a percentage w/w, calculated as follows:

$$\frac{P_u - P_s}{P_m} \times 100$$

where

P_m= sample weight (g);

P_u= weight of the crucible containing the sample before ignition (g);

P_s= weight of the crucible containing the sample after ignition (g).

PROCEDURE

Unless otherwise specified in the individual monograph, proceed as follows:

If necessary, reduce the substance to a fine powder by grinding quickly. Accurately weigh between 1 g and 2 g of the substance without further treatment into a crucible unless preliminary drying at a lower temperature or other special treatment is specified in the individual monograph. The crucible (for example: platinum, porcelain, silica, quartz) must be previously calcined at 500°C, cooled in a desiccator and tared. Distribute the sample as evenly as possible, gently shaking the crucible so that a layer approximately 5mm thick is formed and a maximum of 10mm in the case of bulky materials. Place the crucible in a muffle furnace, calibrated to control the temperature, and conduct the ignition at 500°C ± 25°C for a period of time between two and three hours. Open the apparatus, remove the crucible and allow to reach room temperature in a desiccator before weighing.

When the individual monograph specifies ignition up to constant weight, ignition must continue until two consecutive weighing determinations do not differ by more than 0.50 mg per gram of substance weighed, carrying out the second weighing after an additional hour of ignition.

Check the accuracy of the measurement and the circuit systems of the muffle furnace by controlling the temperature at different points in the muffle furnace. The temperature variation tolerated is ± 25 °C for each point evaluated.

5.2.10 DETERMINATION OF RESIDUE BY INCINERATION (SULPHATED ASH)

Residue on incineration (sulfated ash) is the non-volatile residue of a sample incinerated in the presence of sulfuric acid. This test is used to determine the content of inorganic impurities present in an organic substance. This method is also used for the determination of inorganic components in mixtures and impurities present in thermolabile inorganic substances.

PROCEDURE

Accurately weigh between 1 g and 2 g of the sample or the amount specified in the monograph, in an appropriate crucible (quartz, silica, platinum or porcelain, unless another material is specified in the individual monograph), previously submitted to incineration at temperature specified for the sample for 30 minutes, cooled in a desiccator and weighed. Moisten the sample with approximately 1 mL of sulfuric acid, heat gently to as low a temperature as possible until the sample is carbonized. Cool and moisten the residue with 1 mL of sulfuric acid, unless otherwise specified in the individual monograph. Heat gently until no white fumes are released and carbonize immediately. Incinerate at $(600 \pm 50)^\circ\text{C}$ for between two and three hours, unless another temperature and/or time is specified in the individual monograph. Cool in a desiccator, weigh and calculate the residue percentage. Unless otherwise specified in the individual monograph, if the residue obtained exceeds the specified limit, add 1 mL of sulfuric acid, heat and incinerate for an additional 30 minutes. Repeat this procedure until the difference between two consecutive weighing is, at most, 0.5 mg or until the residue meets the limit established in the individual monograph.

Calculate the percentage of the residue in relation to the substance under analysis using the following formula:

$$\% \text{ residue by incineration (sulfated ash)} = \frac{P_2 - P_1}{P_3} \times 100$$

P_1 = crucible weight after calcining and cooling (in g);

P_2 = crucible weight with the sample after calcining and cooling (in g);

P_3 = sample initial weight (in g);

100 = percentage factor.

Carry out this procedure in a fume hood that is well ventilated, but protected from drafts. A muffle furnace may be employed, if desired, and is recommended for final ignition at $(600 \pm 50)^\circ\text{C}$.

Check the accuracy of the measurement and the circuit systems of the muffle furnace by controlling the temperature at different points in the muffle furnace. The temperature variation tolerated is $\pm 25^\circ\text{C}$ for each point evaluated.

5.2.11 DETERMINATION OF POWDER GRANULOMETRY

The degree of division or granulometry of powders is expressed by referring to the nominal opening of the mesh of the sieve used. The sieves used are stainless steel or brass, and wire coating is not allowed.

In the description of the powders, the terms below are used.:

Coarse powder – one whose particles entirely pass through the sieve with a nominal mesh opening of 1.70 mm and, not more than 40% through the sieve with a nominal mesh opening of 355 µm.

Moderately coarse powder - one whose particles entirely pass through the sieve with a nominal mesh opening of 710 µm and, not more than 40% through the sieve with a nominal mesh opening of 250 µm.

Semi-fine powder - the one whose particles pass entirely through the sieve with a nominal mesh opening of 355 µm and not more than 40% by the sieve with a nominal mesh opening of 180 µm.

Fine powder - one whose particles pass in their entirety through the sieve with a nominal mesh opening of 180 µm.

Very Fine powder - one whose particles entirely pass through the sieve with a nominal mesh opening of 125 µm.

The determination of the granulometry of powders is made by the process described below, with the aid of sieves, whose features are standardized in the attached table.

PROCEDURE

Granulometry is determined with the aid of sieves operated by a mechanical device. This type of device reproduces the horizontal and vertical movements of manual operation through uniform mechanical action. To use this device, proceed as follows:

Separate at least four sieves that are described in **Table 1**, according to the characteristics of the sample. Assemble the kit with the larger opening sieve over the smaller opening. Place the set on the sieve receiver.

Weigh approximately 25 g of the sample (depending on the nature of the material, density of the powder or granule and the diameter of the sieves to be used). Transfer the sample to the upper sieve, distributing the powder evenly. Cap the set.

Activate the device for about 15 minutes with adequate vibration. After the end of this time, using a suitable brush, remove all the sample retained on the upper surface of each mesh onto waterproof paper, and weigh the powder. Also weigh the powder retained in the collector.

Calculate the percentage retained in each sieve, using the following calculation:

$$\% \text{ Retida pelo tamis} = \frac{P_1}{P_2} \cdot 100$$

where

P₁ = weight of the sample retained in each sieve (in g)

P₂ = sum of weights retained in each sieve and in the collector (in g);

Table 1 – Sieves mesh opening.

<i>Sieve number (ABNT/ASTM)</i>	<i>Sieve orifice</i>
2	9.5 mm
3.5	5.6 mm
4	4.75 mm
8	2.36 mm
10	2 mm
20	850 µm
30	600 µm
40	425 µm
50	300 µm
60	250 µm
70	212 µm
80	180 µm
100	150 µm
120	125 µm
200	75 µm
230	63 µm
270	53 µm
325	45 µm
400	38 µm
500	25 µm
635	20 µm

* The sieve number corresponds to the classification of the Brazilian Association of Technical Standards — ABNT (1984), ISO 3310-1:2000.

5.2.12 COLOR OF LIQUIDS

The evaluation of the color of liquids is performed by comparing the solution under analysis – prepared according to the instructions in the monograph – with the standard color solutions (SC).

The comparative process, unless otherwise specified, must be carried out in test tubes made of clear glass and flat bottom, with a diameter of 15mm to 25mm, of the type used in impurity limit testing. Tubes must be uniform.

For the evaluation, use volumes of 5 mL for both sample preparation and standard preparation, ensuring an approximate height of 2.5 cm for liquids in the tubes. View the tubes longitudinally against a white background, under diffused light. It is important to compare solutions under the same conditions, including temperature (25 °C).

The sample preparation is obtained so as to be similar in color to the specified reference preparation.

A solution is colorless when it has the appearance of water or of its constituent solvent or is less colored than the standard T color solution.

BASIC STANDARDS

Color reference solutions (SC) are obtained from three basic solutions to be prepared and stored in hermetic flasks. From them, prepare the specified solution or solutions for comparison, as recommended in Table 1, which contains volume indications for the preparation of 20 standard color solutions (CS), designated with the letters of the alphabet, from A to T. Transfer the indicated volumes (place the water last) and homogenize directly in the comparison tubes.

Cobalt II chloride base solution

Prepare a solution containing 25 mL of hydrochloric acid and 975 mL of water. Dissolve 65 g of cobalt(II) chloride ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) in approximately 900 mL of this solution, adjust volume to 1000 mL with the same solvent and homogenize. Transfer, using a pipette, 5 mL of this solution to a 250 mL iodine flask, add 5 mL of hydrogen peroxide RS and 15 mL of 5 M sodium hydroxide. Boil for ten minutes, cool and add 2 g of potassium iodide and 20 mL of diluted sulfuric acid (1 to 4). Titrate with 0.1 M sodium thiosulfate VS, adding 3 mL of starch TS as indicator. Correct the volume of titrant consumed by blank determination. Each mL of 0.1 M sodium thiosulfate VS is equivalent to 23.79 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$. Adjust the solution volume by adding enough hydrochloric acid solution and water to obtain a solution containing exactly 59.5 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ per mL of solution and homogenize.

Cupric sulfate base solution

Prepare a solution containing 25 mL of hydrochloric acid and 975 mL of water. Dissolve 65 g of cupric sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) in 900 mL of this solution, adjust volume to 1000 mL with the same solution and homogenize. Transfer, using a pipette, 10 mL of this solution to a 250 mL iodine flask, add 40 mL of water, 4 mL of glacial acetic acid, 3 mL of potassium iodide and 5 mL of hydrochloric acid. Titrate the released iodine with 0.1 M sodium thiosulfate VS, adding 3 mL of starch TS as indicator. Correct the volume of titrant consumed by blank determination. Each mL of 0.1 M sodium thiosulfate VS is equivalent to 24.97 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. Adjust the solution volume by adding

enough of a mixture of hydrochloric acid and water to obtain a solution containing exactly 62.4 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per mL of solution and homogenize.

Ferric chloride base solution

Prepare a solution containing 25 mL of hydrochloric acid and 975 mL of water. Dissolve about 55 g of ferric chloride($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) in approximately 900 mL of this solution, adjust volume to 1000 mL with the same solution and homogenize. Protect solution from light and filter if precipitation occurs. Transfer, using a pipette, 10 mL of this solution to a 250 mL iodine flask, add 15 mL of water, 3 g of potassium iodide and 5 mL of hydrochloric acid. Allow to stand for 15 minutes. Adjust the solution volume to 100 mL with water and titrate the released iodine with 0.1 M sodium thiosulfate VS, adding 3 mL of starch TS as indicator. Correct the volume of titrant consumed by blank determination. Each mL of 0.1 M sodium thiosulfate VS is equivalent to 27.03 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. Adjust the solution volume by adding enough hydrochloric acid solution and water to obtain a solution containing exactly 45.0 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ per mL of solution and homogenize.

Table 1 – Composition of standard color solutions (SC).

SC	Parts of			
	Cobalt II chloride base solution, in mL	Ferric chloride base solution, in mL	Cupric sulfate base solution, in mL	Water, to complete 5 mL
A	0.1	0.4	0.1	4.4
B	0.3	0.9	0.3	3.5
C	0.1	0.6	0.1	4.2
D	0.3	0.6	0.4	3.7
E	0.4	1.2	0.3	3.1
F	0.3	1.2	0.0	3.5
G	0.5	1.2	0.2	3.1
H	0.2	1.5	0.0	3.3
I	0.4	2.2	0.1	2.3
J	0.4	3.5	0.1	1.0
K	0.5	4.5	0.0	0.0
L	0.8	3.8	0.1	0.3
M	0.1	2.0	0.1	2.8
N	0.0	4.9	0.1	0.0
O	0.1	4.8	0.1	0.0
P	0.2	0.4	0.1	4.3
Q	0.2	0.3	0.1	4.4
R	0.3	0.4	0.2	4.1
S	0.2	0.1	0.0	4.7
T	0.5	0.5	0.4	3.6

5.2.13 ATOMIC SPECTROMETRY

5.2.13.1 ATOMIC ABSORPTION SPECTROMETRY

Atomic absorption spectrometry is used to determine various elements of the periodic table and basically consists of four methods: flame atomic absorption, hydride generation, cold vapor generation and graphite furnace. The methods that use flame and graphite furnace as atomizers allow the determination of about 70 elements, most of them metals. The hydride generation method allows the determination of arsenic, antimony, selenium, bismuth, tellurium, lead, indium, tin, germanium and thallium; whereas the generation of cold vapor is used basically for the determination of mercury.

For the determination of the analyte concentration by atomic absorption, the radiation from a source of specific wavelength according to the analyzed element falls on the atomic vapor containing free atoms of this element in the ground state. Radiation attenuation is proportional to the analyte concentration according to the Lambert-Beer law.

Instrumentation for atomic absorption basically consists of a radiation source, atomizer, monochromator, detector and data processing system. As light sources, hollow cathode lamps and electrodeless discharge lamps are used which emit intense radiation of the same wavelength as that absorbed by the element to be determined. The atomizer can consist of a flame or a graphite furnace. The monochromator is responsible for separating the desired wavelength. Radiation strikes the monochromator through a narrow slit; then, it is separated into different wavelengths in a diffraction grating and, later, directed to the detector. The detector is usually a photomultiplier that transforms light energy into electrical current, which is amplified and later interpreted by a reading system.

PROCEDURE

To operate atomic absorption spectrometers, it is recommended to follow the manufacturer's instructions. The determinations are made by comparison with reference solutions containing known concentrations of the analyte. Determinations can be carried out by the *Direct Calibration Method (Method I)* or by the *Standard Addition Method (Method II)*. *Method I* is recommended unless otherwise specified.

Direct Calibration Method (Method I): Prepare at least four reference solutions of the element to be determined using the concentration range recommended by the equipment manufacturer for the analyte. All reagents used in sample preparation must be equally included, at the same concentrations, in the preparation of reference solutions. After calibrating the apparatus with solvent, introduce each of the reference solutions into the atomizer three times and, after reading, record the result. Wash the sample introduction system with water after each operation. Plot the analytical curve for the mean of the absorbances of the three readings for each reference solution with its concentration. Prepare the sample as indicated in the monograph, adjusting its concentration so that it falls within the concentration range of the reference solutions for the analyte. Introduce the sample into the atomizer, record the reading and rinse the sample introduction system with water. Repeat this sequence twice. Determine the element concentration from the analytical curve using the average of the three readings.

Standard addition method (Method II): add to at least four volumetric flasks equal volumes of the solution of the substance to be determined prepared as indicated in the monograph.. To the flasks, except for one, add determined volumes of the specified reference solution in order to obtain a series of solutions containing increasing amounts of the analyte. Adjust volume of each flask with water and homogenize. After calibrating the spectrometer with water, record the readings for each solution

three times. Plot the analytical curve for the mean of the absorbances of the three readings for each solution versus the respective amount of analyte added to the solution. Record the amount of analyte in module in the sample by extrapolating the analytical curve on the abscissa axis.

5.2.13.1.1 FLAME ATOMIC ABSORPTION SPECTROMETRY

The system consists of a premix chamber, in which the fuel and oxidant are mixed, and of the burner which receives the fuel-oxidant mixture. The solution is introduced through a pneumatic nebulizer, in which a fine aerosol is generated and led to the flame. The amount of energy that can be supplied by the flame for the dissociation and atomization of the sample is proportional to the temperature. If a low temperature flame is used, the solution may not be converted to neutral atoms. On the other hand, if a flame with a very high temperature is used, a large amount of ions that do not absorb radiation from the source can be formed. By modifying the proportion of oxidant and fuel used for each type of flame, it is possible to significantly change its temperature. The most popularly used flames are those produced by air-acetylene (2100°C to 2400°C) and acetylene-nitrous oxide (2650°C to 2850°C). The air-acetylene mixture is used for elements with lower atomization temperatures such as Na, K, Mg, Cd, Zn, Cu, Mn, Co, etc. The flame generated by acetylene-nitrous oxide is applied to refractory elements such as Al, V, Ti, Si, U, among others.

INTERFERENCES

Physical Interferences: Using sample preparation with physical properties such as viscosity and surface tension different from the standard preparation may result in differences in relation to aspiration and nebulization, leading to incorrect readings. Whenever possible, preparations with the same physical properties and matrix constituents should be used.

Ionization interference: normally occurs for alkaline and alkaline earth elements that are easily ionizable. The greater the degree of ionization, the lower the absorbance. To minimize ionization interference, it is possible to use flames with lower temperatures or use "ionization suppressors", which are elements such as cesium that ionize more easily than analyte, thus increasing the number of atoms in the ground state.

Chemical interferences: the formation of thermally stable compounds in the flame, such as the oxides of some elements (Ca, Ti, Cr, V, Al, etc.), reduces the population of atoms in the ground state. This can be resolved by increasing the flame temperature, which results in the dissociation of these compounds. Another possibility is the use of a "suppressor agent" or "release agent" that has greater affinity for oxygen than the analyte, preventing the formation of oxides. The solution containing cesium chloride and lanthanum chloride, "Schinkel" solution, is the most commonly used.

Spectral interferences: occur through the absorption or scattering of selected radiation for the analyte. Spectral interferences caused by atoms are uncommon and can be resolved by changing the spectral line used. Interferences caused by molecular species are more severe, but are usually overcome by background correction.

5.2.13.1.2 ATOMIC ABSORPTION SPECTROMETRY WITH HYDRATE GENERATION

Atomic absorption spectrometry with hydride generation is a method used for the determination of volatile hydride-forming elements, most commonly for As, Se, Sb, Bi, Ge, Sn, Pb and Te. The process consists of three main steps: hydride generation, transport and atomization. The system can be built in batch or in flow. The generation of hydrides consists of the reaction of the analyte, usually in an

acidic medium, with a reductant (NaBH_4). The transport of the hydrides from the reaction flask to the quartz cell is done by an inert carrier gas, such as argon or nitrogen. For elements that absorb at a wavelength lower than 200 nm, before the hydride generation step, a purge must be carried out to remove atmospheric gases in order to prevent these gases from absorbing the radiation from the source. Atomization is done in an electrically heated quartz cell or with a burner typical of flame atomization systems; the internal temperature of the cell is from 850°C to 1000°C. The signal obtained is normally of the transient type; about 20 seconds are required for full signal integration for almost all elements.

INTERFERENCES

Influence of the oxidation state: analytes usually have more than one oxidation state. Arsenic and antimony, for example, have oxidation states III and V, whereas selenium and tellurium have oxidation states IV and VI, respectively. Higher oxidation states are generally inert for conversion to volatile hydrides; therefore, pre-reduction is necessary before determination in these cases.

Hydride-forming elements: Mutual interferences can occur between the hydride-forming elements, such as between arsenic and selenium. In these cases, the volatilization and atomization kinetics are decisive in the process.

Transition elements: some metallic ions such as Cu^{2+} and Ni^{2+} , if present in high concentrations, are reduced, forming precipitates that can adsorb volatile hydrides.

5.2.13.1.3 ATOMIC ABSORPTION SPECTROMETRY WITH COLD VAPOR GENERATION

Atomic absorption spectrometry with cold vapor generation is used for the determination of mercury. The apparatus and reagents are the same used in the hydride generation system, but the quartz cell does not need to be heated, as the mercury is reduced to metallic mercury, which is volatile at room temperature. However, water vapor can be transported by the carrier gas and interfere with the determination. To solve this problem, an infrared lamp is used to heat the quartz cell, preventing the condensation of water vapor. In this case, it is usually not necessary to carry out the purge, as the wavelength used for the determination of Hg is 253.7 nm, in which the absorption of radiation by gases from the atmosphere is rare.

5.2.13.1.4 GRAPHITE FURNACE ATOMIC ABSORPTION SPECTROMETRY

Graphite furnace atomic absorption spectrometry is a comprehensive method that has high sensitivity. The furnace consists of a graphite tube 3 to 5 cm long and 3 to 8 mm in diameter coated with pyrolytic graphite. The amount of sample injected into the oven ranges from 5 μL to 50 μL and is usually introduced by an automated system. The furnace is electrically heated by passing a longitudinal or transverse electric current. Streams of inert gases such as argon are maintained externally and internally to prevent furnace combustion. In addition, the internal flow expels atmospheric air from the furnace and also the vapors generated during the drying and pyrolysis steps. A graphite furnace lasts approximately 300 cycles, depending on the model.

Analysis with the graphite furnace can be divided into the following steps: sample drying, pyrolysis, atomization and cleaning. The transition from one step to another is marked by the increase in temperature, therefore, a special heating program must be planned. First, the sample is dried; in this step the residual solvents and acids are evaporated. After drying, the temperature is risen to remove

the matrix (pyrolysis step). Then, the increase in temperature leads to atomization of the analyte for further quantification. Finally, the furnace is cleaned at a high temperature (e.g. 2600 °C) for a few seconds. The temperature and duration of each heating step can be controlled; this is essential for developing analytical methods.

Atomization and pyrolysis curves are used to optimize temperatures for such processes. The pyrolysis curve allows determining the maximum temperature at which no loss of analyte occurs. The atomization curve determines the minimum atomization temperature of the analyte with adequate sensitivity. It is recommended that pyrolysis and atomization curves are made whenever an unknown sample is analyzed.

The atomization process in a graphite furnace is complex and depends on many factors, such as the material of the furnace and platform, the atmosphere inside the tube, the heating rate, the temperature and nature of the substances. For best results, it is recommended to use the L'Vov platform inside the tube as well as transversal heating. The signal obtained is of the transient type; a maximum of 12 seconds is required for signal integration.

INTERFERENCES

Spectral Interferences: Interferences caused by overlapping lines between atoms are uncommon. The attenuation of the species radiation beam generated during the atomization process, deriving from the matrix, is more frequent. To solve this problem, the matrix must be efficiently eliminated. The use of a matrix modifier and background correction is essential for the reliability of the results.

Formation of volatile substances: in samples with high levels of halogens (especially Cl) there is the possibility of formation of volatile substances from the analyte, which may be lost at low temperatures, causing error in the analysis. In this case, the use of a chemical modifier capable of forming thermally stable complexes with the analyte minimizes the formation of volatile substances. Furthermore, when the chemical modifier is combined with the L'vov platform, matrix interference effects are greatly reduced. It is important to point out that a given chemical modifier can be very effective for some elements, whilst ineffective for others.

5.2.13.2 ATOMIC EMISSION SPECTROMETRY

Atomic emission spectrometry is the method of determining the concentration of an element in a sample by measuring the intensity of one of the element emission lines. The determination is made at the wavelength corresponding to this emission line. Emission sources in atomic emission spectrometry must have energy to generate neutral atoms and to excite the elements of interest.

5.2.13.2.1 FLAME PHOTOMETRY

Flame photometry is a method with good sensitivity, being used mainly for the determination of alkali metals. The apparatus consists of a flame, normally produced by an air-liquid petroleum gas mixture, a monochromator and a detector. The solvent of choice for preparing the sample solution and reference solutions should preferably be aqueous. Organic solvents can be used as long as they do not interfere with flame stability.

INTERFERENCES

The interferences that occur in flame photometry are very similar to those observed in *Atomic Absorption Spectrometry* (5.2.13.1). However, spectral interferences can occur caused by the

emission of molecular rotation-vibration bands, such as OH (310-330 nm), NH (around 340 nm), N₂⁺ (around 390 nm), C₂ (around 450 nm), etc.

SOLVENTS

The solvent must be selected carefully. If there is a significant difference in surface tension or viscosity between the sample and the reference solution, variations in aspiration and nebulization rates will occur and, consequently, significant differences in the signals produced. Thus, the solvent used in the preparation of samples and references must be as similar as possible.

PROCEDURE

The apparatus must be operated in accordance with the manufacturer's instructions and at the specified wavelength. Adjust zero with the solvent. Then inject the most concentrated reference solution and adjust to the desired sensitivity. The determinations are made by comparison with reference solutions containing known concentrations of the analyte. Determinations can be carried out by the *Direct Calibration Method (Method I)* or by the *Standard Addition Method (Method II)*, as described in *Atomic Absorption Spectrometry (5.2.13.1)*.

5.2.13.2.2 OPTICAL EMISSION SPECTROMETRY WITH INDUCTIVELY COUPLED PLASMA

Inductively coupled plasma optical emission spectrometry is a very comprehensive method that has high sensitivity and multi-element characteristics. In general, in inductively coupled plasma spectrometry, the sample aerosol is introduced into a plasma source, where it is evaporated and dissociated into free atoms and ions, which are excited. The plasma consists of a partially ionized gas of high temperature (6000°C to 10000°C), electrically neutral and with good electrical conductivity. Due to the high temperature of the plasma, polychromatic radiation is generated as a result of the emission of various elements and ions present in the sample. Therefore, it is necessary to use a monochromator with high resolution capacity to separate the characteristic wavelengths of each element. Detection of radiation generated by specific wavelengths can be applied for qualitative analysis and the intensities of these wavelengths can be used for quantitative analysis.

APPARATUS

The apparatus used in inductively coupled plasma spectrometry basically consists of the signal generator and processor. The generator consists of a plasma source and a sample introduction system (propellant pump and nebulizer). The signal processor is comprised of optical and electronic systems and data acquisition unit.

Plasma sources: The most common is inductively coupled plasma. The plasma is generated in a torch that consists of three concentric tubes usually made of quartz. Gas flows, usually argon, are maintained in the three compartments formed by the concentric tubes. In the outer compartment, the gas is used to form the plasma. The intermediate compartment carries the auxiliary gas, which is responsible for keeping the plasma away from the internal compartment and preventing deposition of carbon and salts from the sample in that compartment. The internal argon flow carries the aerosol from the sample to the center of the plasma. When a certain power (between 700 W and 1500 W) is applied by the radio frequency generator to the induction coil, an alternating current is generated in the coil at a frequency of 27 MHz or 40 MHz. This oscillation in the coil results in an intense electromagnetic field at the tip of the torch. With argon flowing through the torch, a high-voltage electrical discharge is applied to the gas generating electrons and argon ions. Electrons are accelerated

by the magnetic field and collide with more argon atoms generating more ions and electrons. The ionization of argon continues in a chain reaction generating the plasma, which consists of argon atoms, electrons and argon ions.

Detection system for Optical Emission Spectrometry with Inductively Coupled Plasma: all elements present in the plasma emit radiation at the same time, therefore it is necessary to use a multi-element detection system. Spectrometers can be simultaneous or sequential. For inductively coupled plasma optical emission spectrometry, both sequential and simultaneous spectrometers are widely used. The most common configuration for sequential spectrometers is the *Czerny-Turner*. Simultaneous spectrometers, on the other hand, are basically found with *Echelle* and *Paschen-Runge* configurations.

INTERFERENCES

The overlapping of emission lines is one of the main interferences for inductively coupled plasma optical emission spectrometry. This type of interference can be eliminated using high resolution spectrometers and background correction procedures. Many spectral interferences are observed in the 200 nm to 400 nm range, in which more than 200.000 atomic emission lines and molecular bands are observed.

The physical interferences are similar to those in *Flame Atomic Absorption Spectrometry (5.2.13.1.1)*.

SOLVENTS

The ideal solvent for inductively coupled plasma optical emission spectrometry interferes as little as possible in the emission processes. The solvent must be carefully selected. If there is a significant difference in surface tension or viscosity between the sample and the reference solution, variations in aspiration and nebulization rates will occur and, consequently, significant differences in the signals produced. Thus, the solvents used in the preparation of samples and reference solutions should be as similar as possible.

PROCEDURE

The apparatus must be operated in accordance with the manufacturer's instructions and at the proper wavelength for each element. The determinations are carried out by comparison with reference solutions containing known concentrations of the analytes. Determinations can be carried out by the *Direct Calibration Method (Method I)* or by the *Standard Addition Method (Method II)*, as described in *Atomic Absorption Spectrometry (5.2.13.1)*.

5.2.13.3 MASS SPECTROMETRY WITH INDUCTIVELY COUPLED PLASMA

Inductively coupled plasma mass spectrometry is used for the determination of several elements with high sensitivity, in the ppt range (parts per trillion), and with multi-element capability.

APPARATUS

Similarly to inductively coupled plasma optical emission spectrometry (5.2.13.2.2), inductively coupled plasma mass spectrometry consists of two main units: the signal generator and the signal processor. The fundamental difference is that, in inductively coupled plasma mass spectrometry, the signal processor comprises an interface, a mass analyzer and a data acquisition unit. The interface is responsible for sampling and the efficient transport of ions from the plasma at atmospheric pressure

(760 Torr) to the mass separator (10^{-6} Torr) is done by reducing the pressure through vacuum application. The interface consists of two metallic cones with very small orifices (of 1 mm in diameter). After the ions are generated in the plasma, they pass through the first cone (sampling cone) and then through the second cone (*skimmer*). After the passage of ions through the *skimmer*, due to expansion, there is a need for them to be focused to ensure their arrival at the mass analyzer. The ions are focused by the action of an ionic lens or set of ionic lenses, which consists of a hollow metal cylinder (or series of cylinders or perforated plates) subjected to a potential difference (usually in the range of 2 to 15 V of continuous current). Most of the inductively coupled plasma mass spectrometers on the market today use the quadrupole as a mass separator. The quadrupole consists of four cylindrical or hyperbolic metal bars of the same length and diameter. By the combined application of direct current (dc) and alternating current (ac) to the electrodes (quadrupole), only ions with a given mass/charge ratio (m/z) are conducted through the quadrupole. The remaining ions collide with the electrodes or are removed from the interior of the quadrupole. Accordingly, the ions are sequentially separated by the quadrupole. Various types of detectors can be used to collect the ions at the quadrupole output and convert them into an electrical signal, but the most popular are discrete dynodes, *Faraday Cup* and *Chaneltron*.

INTERFERENCES

Similar to other spectrometric methods, inductively coupled plasma mass spectrometry has spectral and non-spectral interferences. Spectral interferences are species-dependent and can be divided into four main types: polyatomic, isobaric, double-charged ions and refractory oxide ions. This type of interference can be corrected by simulating the matrix composition, choosing another isotope (when possible) or using a reaction and/or collision cell. In some cases, spectral interference can be corrected using an appropriate computer program.

Non-spectral interferences can arise for several reasons: deposition on the interface cones, presence of another easily ionizable element, space charge effect, among others. However, most non-spectral interference can be corrected by using an internal standard. In this case, the internal standard must have a mass/charge ratio and ionization potential similar to the analyte. Scandium and Rhodium, for example, are widely used as an internal standard for elements with low and high mass/charge ratios, respectively.

SOLVENTS

The ideal solvent for inductively coupled plasma mass spectrometry interferes as little as possible in the emission processes. The solvent must be carefully selected. If there is a significant difference in surface tension or viscosity between the sample and the reference solution, variations in aspiration and nebulization rates will occur and, consequently, significant differences in the signals produced. Thus, the solvents used in the preparation of samples and reference solutions should be as similar as possible.

PROCEDURE

The apparatus must be operated in accordance with the manufacturer's instructions and at the proper isotope for each element. Adjust the zero with the solvent injected into the apparatus. The determinations are carried out by comparison with reference solutions containing known concentrations of the analytes. Determinations can be carried out by the *Direct Calibration Method (Method I)* or by the *Standard Addition Method (Method II)* or by the *Internal Standard Method (Method III)*.

Direct Calibration Method (Method I). Prepare at least four reference analyte solutions, covering the range of concentrations recommended by the equipment manufacturer for the elements under analysis. All reagents used in the sample solution must be equally included, at the same concentrations, in the preparation of the reference solutions. After calibrating the apparatus with solvent, inject each of the reference solutions three times and, after stabilization of the reading, record the result, washing the system with the solvent after each injection. Plot the analytical curve, plotting the average of the readings for each group of three, with their respective concentration. Prepare the solution of the substance to be determined as indicated in the monograph, adjusting its concentration so that it is within the range of concentrations of the reference solutions. Introduce the sample into the apparatus, record the reading and wash the system with solvent. Repeat this sequence twice and, by adopting the average of three measurements, determine the analyte concentration using the analytical curve.

Standard Addition Method (Method II). Add to each of four similar volumetric flasks minimum, equal volumes of prepared solution of the substance to be determined

as indicated in the monograph. Add to all, but one, flasks measured volumes of the specified reference solution in order to obtain a series of solutions containing increasing amounts of analytes. Conveniently dilute the volume of each flask with water. After calibrating the spectrometer with water, as indicated above, record the readings for each solution three times.

Internal Standard Method (Method III). Prepare at least four reference analyte solutions, covering the range of concentrations recommended by the equipment manufacturer for the analytes. All reagents used in the sample solution must be equally included, at the same concentrations, in the preparation of the reference solutions. The internal standard must be added to all solutions (solvent, reference solutions and samples), with a fixed concentration and in the same order of magnitude as the analytes. After calibrating the apparatus with solvent, inject each of the reference solutions three times and, after stabilization of the reading, record the result, washing the system with the solvent after each injection. Plot the analytical curve, plotting a graph of the ratio between the mean of the reading intensities of each group of three and the intensity of the internal standard, with the respective concentration. Prepare the solution of the substance to be determined as indicated in the monograph, adjusting its concentration so that it is within the range of concentrations of the reference solutions. Introduce the sample into the apparatus, record the reading and wash the system with solvent. Repeat this sequence twice and, by adopting the average of three measurements, determine the analyte concentration using the analytical curve.

5.2.14 SPECTROPHOTOMETRY IN ULTRAVIOLET, VISIBLE AND INFRARED

Spectrophotometric methods are based on the absorption of electromagnetic energy by molecules, which depends both on the concentration and on their chemical structures. According to the frequency range of the applied electromagnetic energy, absorption spectrophotometry can be divided into ultraviolet, visible and infrared, and can be used as a method for identifying and quantifying substances.

ELECTROMAGNETIC RADIATION

Electromagnetic radiation is a form of energy that propagates as waves and can generally be subdivided into regions of characteristic wavelength. Still, it can also be considered as a flux of particles called photons (or quanta). Each photon contains a certain energy whose magnitude is proportional to frequency and inversely proportional to wavelength. The wavelength (λ) is usually specified in nanometers, nm (10^{-9} m), and in some cases in micrometers, μm (10^{-6} m). In the case of infrared, electromagnetic radiation can also be described in terms of wave number and expressed in cm^{-1} . The electromagnetic energy wavelength ranges of interest for spectrophotometry are described in **Table 1**.

Table 1 – Wavelength ranges of interest for spectrophotometry.

<i>Region</i>	<i>Wavelength range</i>
Ultraviolet (UV)	100 – 380 nm
Visible (VIS)	380 – 780 nm
Near infrared (NIR)	780 – 2500 nm (13300 – 4000 cm^{-1})
Mid-infrared (MIR)	4 – 25 μm (2500 – 400 cm^{-1})
Far infrared	25 – 300 μm (400 – 33 cm^{-1})

ENERGY-MATTER INTERACTION

The total energy of the molecule involves the energy derived from vibration (vibrational energy, due to the relative motion of the constituent atoms or groups of atoms in the molecule); from rotation (rotational energy, due to the molecule rotation around an axis) and, usually, from electronic energy, generated by the configuration of electrons in the molecule.

Molecules when absorbing energy undergo a transition to a higher energy state or excited state. The transition to the excited state is not continuous in nature, generally taking place in stages called transitions. In the ultraviolet and visible region, transitions are electronic and occur in portions of the molecule called chromophores. These transitions comprise promotions of electrons from occupied molecular orbitals, usually σ and π bonding and non-bonding, to the next higher energy orbitals, non-bonding π^* and σ^* .

In the mid-infrared region (MIR), only vibrational energy transitions occur because the radiation in this region is insufficiently energetic to promote electronic transitions. Infrared radiation-induced vibrations comprise stretching and tensioning of inter-atomic bonds and modification of bond angles.

Near infrared (NIR) spectra are characterized by the absorption of radiation by overtones and combination of fundamental vibrational modes of bonds such as C-H, N-H, O-H and S-H. The bands of a NIR spectrum are generally weaker than the bands of the MIR spectrum. Chemical and physical information, with qualitative and quantitative characteristics, can be obtained from the NIR spectrum.

However, direct comparison between the spectrum of the sample and the reference chemical is not recommended.

NIR spectrophotometry is widely used for physical and chemical analysis, such as: quantification and identification of active principles and excipients, identification of crystalline and polymorphic forms, determination of particle size, disintegration pattern and process control.

SPECTRA ACQUISITION METHODS

Spectra can be obtained using different acquisition modes. In the case of UV/VIS spectrophotometry, the main mode is transmission. In NIR and MIR spectrophotometry the spectra can be acquired using the transmission and reflection mode. The latter is subdivided into diffuse reflection and attenuated total reflection. There is also the possibility of combining transmission and reflection modes, called transreflectance.

Transmission: is the measure of the decrease in radiation intensity at certain wavelengths when radiation passes through the sample. The sample is placed in the optical beam between the source and the detector. Transmission (T) can be calculated by the formula below:

$$T = \frac{I}{I_0}$$

I_0 = incident radiation intensity

I = transmitted radiation intensity

Transmission spectra can be converted to absorbance:

$$A = \log_{10} \left(\frac{I_0}{I} \right)$$

Diffuse reflection: is the measure ratio of the intensity of the light reflected by the sample and the light reflected by a reflective reference surface. Unabsorbed radiation is reflected towards the detector.

Attenuated total reflection: infrared radiation propagates inside an element with internal reflection (high refractive index) through reflections on the walls of this element. The sample is placed in contact with the wall of this reflection element where it interacts with infrared radiation (evanescent wave).

Transreflectance: This mode is the combination of transmission and reflection modes. In transreflectance measurement, a mirror or reflective surface is used to reflect the radiation transmitted through the sample, hitting it a second time to then bend the optical path. Unabsorbed radiation is reflected towards the detector.

INSTRUMENTATION USED IN ULTRAVIOLET (UV) AND VISIBLE (VIS)

Spectrophotometers used in the ultraviolet and visible regions are fundamentally equipped with a radiation source; wavelength selector; absorption cells (cuvettes) for inserting sample solutions into the monochromatic light beam; radiant energy detector and a signal reading and processing unit.

The lamps most used as a source of radiation in spectrophotometry in the ultraviolet and visible region are deuterium and tungsten, which provide radiation between 160 and 380 nm and 320 and 2500 nm, respectively. Instruments for the UV/VIS regions are usually equipped with one or more devices to restrict the radiation being measured within a narrow range that is absorbed or emitted by the analyte. Most devices use a monochromator or filter to isolate the desired wavelength band, so that only the band of interest is detected and measured. Monochromators generally have a diffraction grating, while filters can be interference or absorption filters. Photometers or colorimeters are simpler instruments that use a filter to select the wavelength and are generally used in the visible region. Spectrophotometers, in turn, use monochromators for wavelength selection and are used in the UV/VIS regions.

The compartments used to receive the sample are called cuvettes, which must have windows that are transparent in the spectral region of interest. For the UV region, quartz cuvettes are required, whereas for the VIS region, glass or acrylic cuvettes can be used.

The main types of detectors are phototubes, photodiode arrays and charge transfer devices. Phototubes are the simplest detectors and their answer is based on the photoelectric effect. The diode array detector allows all wavelengths to be monitored simultaneously. Charge transfer devices have been increasingly used in spectroscopic instruments.

Spectrophotometers can be found in single-beam, dual-beam, and multi-channel configurations. Dual-beam instruments have the advantage of compensating for any fluctuations in the radiant power of the source when compared to single-beam instruments. Multichannel instruments, on the other hand, are more recent, using diode array detectors and charge transfer devices, allowing the full spectrum of a sample to be obtained in less than one second. In these instruments, the dispersive system is a grating spectrograph placed after the sample cell.

Spectrophotometers may have graphic records that allow the acquisition of absorption spectra. This feature is important for the purpose of characterizing the substance from obtaining the wavelengths where the highest absorbances are obtained (λ_{maximum}). Currently, most spectrophotometers are connected to a microcomputer and an appropriate program, which allows obtaining the absorption spectra of substances in digital media.

INSTRUMENTATION USED IN MEDIUM INFRARED (MIR) AND NEAR INFRARED (NIR)

The spectrophotometers used to acquire mid and near infrared spectra consist of a light source, monochromator or interferometer and detector, and allow the acquisition of spectra in the region between 750 to 2500 nm ($13\,300$ to 4000 cm^{-1}).

Currently, mid-infrared spectrophotometers (4000 to 400 cm^{-1}) use the interferometer instead of the monochromator and the polychromatic radiation falls on the sample, with the spectra being obtained in the frequency domain with the aid of the Fourier transform.

Transmission cells, diffuse reflection and attenuated total reflection tools are the most common accessories for spectra acquisition.

Near infrared spectrophotometry (NIR) is a method that allows the acquisition of spectra in the region between 13300 and 4000 cm^{-1} (750 to 2500 nm). The spectrophotometers in the NIR region consist of an appropriate radiation source, monochromator or interferometer and detector. Conventional cuvettes, optical fibers, transmittance cells and accessories for diffuse reflectance are the most common accessories for spectra acquisition.

IDENTIFICATION BY SPECTROPHOTOMETRY

The identification of several pharmaceutical substances can be done using the ultraviolet, visible, mid-infrared and near-infrared regions.

In general, spectrophotometry in the UV/VIS regions requires solutions with a concentration in the order of 10 mg mL^{-1} of the substance, while for MIR and NIR, concentrations in the order of 100 mg mL^{-1} are required. Despite being more sensitive, the spectra obtained in the UV/VIS regions have less specificity when compared to the spectra in the MIR region. In the case of MIR, measurements performed using the reflection modes (diffuse and total attenuated) provide spectral information equivalent to that obtained by the transmission mode. When possible, a comparison of the obtained spectrum against the spectrum of the reference chemical should be made.

Ultraviolet (UV) and visible (VIS) diffuse and total attenuated

Several monographs include ultraviolet absorption spectra as proof of identification. In these cases, the scan length, solvent, solution concentration and cuvette thickness (optical path) will be specified. Some drugs require the use of reference standards. Standard and sample readings are taken simultaneously and under identical conditions for wavelength, cuvette size, etc.

For characterization using UV/VIS spectrophotometry, the drug is dissolved using appropriate solvent. Many solvents are suitable, including water, alcohols, ethers and dilute acid and alkaline solutions. It must be verified if the solvents do not absorb in the spectral region being used.

Mid-infrared (MIR)

The MIR spectrophotometry is an identification test par excellence, being able to differentiate substances with structural differences. Of the three infrared regions (near, medium and far), the region between 4000 and 400 cm^{-1} (mid infrared) is the most used for identification purposes.

The transmission spectra of solid samples are obtained by dispersing them in mineral oil or by preparing potassium and sodium halide lozenges. Sample dispersions are prepared by grinding about 5 mg of the substance in a drop of spectroscopic grade mineral oil. The paste obtained is spread between two windows of potassium bromide or sodium chloride. To prepare the lozenges, approximately 1 mg of the sample is ground with approximately 300 mg of spectroscopic grade potassium bromide.

For solid powder samples opaque to the transmission of infrared radiation, the spectrum can also be acquired by using an accessory for diffuse reflection. In this accessory, infrared radiation is directly incident on the powdered sample. Part of the radiation is absorbed and then diffusely reflected towards the detector. In this case, the sample in powder form is mixed with potassium bromide at a concentration of approximately 5% (w/w) and placed in the diffuse reflection accessory.

Finally, the spectrum of solid powder and creamy samples can be obtained using a total reflection attenuation accessory. The sample in powder form is placed under the high refractive index crystal where it comes into contact with infrared radiation, not requiring prior sample preparation.

QUANTITATIVE USE OF SPECTROPHOTOMETRY

UV/VIS spectrophotometry

Quantitative spectrophotometric analysis by absorption is based on the direct relation between the amount of light absorbed and the concentration of the substance, also known as Beer's law.

When concentration (c) is expressed in mole. L⁻¹ and the optical path (b) in centimeters, the formula is:

$$A = \varepsilon b c$$

where

A = absorbance, logarithm of the inverse transmittance ($A = -\log T$)

ε = molar absorptivity.

T = transmittance

Knowing that transmittance is the quotient between the intensity of the radiation transmitted by the solution (I) and the intensity of the incident radiation (I₀), you have:

$$\log_{10} (I_0/I) = A = \varepsilon b c$$

The intensity of ultraviolet light absorption by chromophore substances is generally expressed as molar absorptivity under maximum absorption conditions. If the molar mass of the substance is not known, it is possible to express the absorption intensity by the formula of specific absorptivity – A (1%, 1 cm):

$$A (1\%, 1 \text{ cm}) = A / b c$$

where A(1%, 1 cm) corresponds to the absorbance of the solution at 1% (w/v) of the substance when the optical path is 1 cm. To avoid possible deviations from Beer's law, one should try to work with diluted solutions (in the order of 0.01 M), avoiding associations between molecules, and with monochromatic radiation.

Near infrared spectrophotometry.

Quantification by means of NIR spectrophotometry can be performed using data obtained from a reference method or from a calibration set with samples of known composition. Spectra can be obtained using the transmission and reflection modes with the aid of suitable tools. Firstly, the spectral data are treated through mathematical transformations to reduce sources of unwanted variations before the calibration step. The calibration process consists of building a mathematical model that relates the spectrophotometer response to a sample property. There are a number of chemometric algorithms that can be used for calibration. These algorithms are usually available in software and available together with the spectrophotometer. The main calibration algorithms are: *multiple linear regression* (MLR), *partial least squares* (PLS) and *principal component regression* (PCR).

The validation of a method that employs NIR spectrophotometry is similar to that required for any analytical procedure and is generally based on chemometric methods. The main parameters to be evaluated are: specificity, linearity, working range, accuracy, precision and robustness.

The extent of specificity is dependent on the procedure used. The demonstration of the specificity of the NIR methods can be done in the following manners: (i) the wavelengths used in the calibration models must correspond to the bands of the analyte of interest; (ii) for calibration using PLS the coefficients must be plotted and the regions with the highest coefficient compared with the analyte spectrum; (iii) variations in the sample matrix should not significantly affect analyte quantification.

Validation of the NIR method linearity involves demonstrating its linear response for samples distributed over a defined calibration range. The correlation coefficient, r , is not an adequate tool for checking linearity, but it is the measure of data variation that is properly modeled by the formula. The best method to demonstrate the linearity of the NIR methods is through the statistical evaluation of the slope and intercept values obtained for the validation set.

The working range of the reference values for the analyte of the validation set defines the working range of the NIR method. Controls must be established to ensure that results out of the working range are not accepted. The validation of a NIR method should generate an anomalous value when a sample containing the analyte outside the working range is analyzed.

The accuracy of a NIR method is demonstrated by correlating NIR results with the reference method data. Furthermore, the accuracy can be verified from the proximity of the standard error of prediction (SEP) with the error of the reference method. Reference method error must be known based on historical values. Different statistical methods can be used to verify statistical differences between the results obtained by the NIR method and the reference method.

The precision of a NIR method expresses the agreement between a series of measurements obtained under predetermined conditions. There are two levels of precision that can be considered: repeatability and intermediate precision. The precision of a NIR method is typically expressed as a coefficient of variation.

The robustness of the NIR method can be verified through changes in method parameters, such as: environmental conditions, sample temperature, sample characteristics and instrumental changes.

5.2.15 FLUORESCENCE SPECTROPHOTOMETRY

Some substances can be analyzed with greater sensitivity and specificity using fluorimetric methods rather than other spectrophotometric methods. Fluorescence spectrophotometry, or spectrofluorimetry, comprises the measurement of the fluorescence emitted when these substances – called fluorescent – are exposed to ultraviolet, visible or electromagnetic nature radiation. Such radiations promote the excitation of the molecule electrons to higher energy levels. After a short stay in the excited state – about 10^{-8} to 10^{-4} seconds – the electrons return to the ground state through a non-radioactive process, called collision deactivation, combined with a radioactive process called luminescence (fluorescence or phosphorescence), contrary to most substances in which the return to a less energetic state does not involve light emission. In collision deactivation, energy is lost as heat in collisions between molecules. In the radiant process, the excess energy is re-emitted with maximum intensity at a longer wavelength (around 20 to 30 nm) than that of the absorbed excitatory radiation, due to the energy loss that occurs in the process. Being fluorescent in nature, the radiation emitted by the substance ceases when the energy source is removed and this characteristic distinguishes it from phosphorescence, which continues for some time after the end of excitation.

The intensity of light emitted by a fluorescent solution is, under certain conditions, proportional to the concentration of the solute and, therefore, used for analytical purposes. Fluorescence intensity measurement cannot be used directly for the determination of analyte concentration. Therefore, the determination is made by comparing the fluorescence intensity obtained for a sample solution with standard solutions, whose concentrations are known. The foundation of spectrofluorescence consists, therefore, in exciting the substance with radiation at the maximum absorption wavelength and comparatively measuring the intensity of the fluorescent light emitted against a standard.

DEFINITIONS

Fluorescence intensity: empirical expression of fluorescent activity, in arbitrary units proportional to the detector response.

Fluorescence excitation spectrum: graphical representation of the activation spectrum, showing the intensity of the radiation emitted by the activated substance (ordinate) and the wavelength of the incident excitatory radiation (abscissa).

Fluorescence emission spectrum: graphic representation of the spectral distribution of radiation emitted by an activated substance, showing the intensity of the emitted radiation as ordinate and the wavelength as abscissa.

EQUIPMENT

The determination of fluorescence intensity can be carried out in a simple filter fluorimeter (fluorimeter), in adapted absorption spectrophotometers or in a fluorescence spectrophotometer (spectrofluorimeter).

The filter fluorimeter comprises light source, primary filter, sample chamber, secondary filter and detection system. In this type of fluorimeters, the detector is arranged at 90° to the incident light. Such a right-angle arrangement allows incident light to pass through the sample solution without interfering with the fluorescent signal captured by the detector. This mechanism does not prevent part of the scattered light from reaching the detector due to the diffusing properties inherent to the solutions or due to the presence of suspended solid particles. This residual dispersion is controlled using filters.

The primary filter selects the appropriate wavelength radiation to excite the sample whereas the secondary filter selects the longer wavelength fluorescent radiation, blocking the access of the scattered radiation to the detector.

Most filter fluorimeter detectors are equipped with photomultiplier valves, although there are differences between types of apparatus regarding the spectral region of maximum sensitivity. Once the electric current generated in the photomultiplier is amplified, a corresponding reading is obtained in an analog or digital instrument.

Fluorescence spectrophotometers, in turn, differ from fluorimeters in that they do not have filters but rather prism monochromators or diffraction gratings, providing greater wavelength selectivity and flexibility.

Both fluorimeters and fluorescence spectrophotometers allow the use of different light sources. Mercury or tungsten lamps, although common, are advantageously replaced by the high-pressure xenon arc lamp, as it provides, unlike the others, a continuous spectrum from ultraviolet to infrared. In any case, radiation is very intense and should never be observed with the eyes unprotected, under the risk of permanent damage.

Monochromators, in turn, have slit width adjustment. Narrow slits provide greater resolution and less spectral noise while wide slits ensure greater light intensity to the detriment of these characteristics. The slit width to be adopted depends on the difference between the wavelengths of the incident and emitted light, as well as the level of sensitivity necessary for the analysis.

The sample chamber generally allows the use of round tubes and square cuvettes, similar to those employed in absorption spectrophotometry, except for the fact that the four vertical walls must be polished. Sample volumes of 2 to 3 mL are adequate, although some instruments may have small cuvettes, with a capacity of 0.1 to 0.3 mL, or even holders for capillaries that require even smaller volumes.

Apparatus calibration

Fluorimeters and spectrofluorimeters must be calibrated with stable, fluorophore substances to ensure reproducible results. Variations are generally due to changes in lamp intensity or sensitivity of the photomultiplier tube. The fluorophore can be the pure sample of the substance to be analyzed or any other fluorescent substance that is easily purified, whose wavelengths of absorption and fluorescence are similar to those of the substance being analyzed. For example, quinine in 0.05 *M* sulfuric acid is a suitable standard for blue fluorescence. On the other hand, fluorescein in 0.1 *M* sodium hydroxide is suitable for green fluorescence and rhodamine is the fluorophore of choice in red fluorescence. The fluorescence spectrophotometer wavelength scale also requires periodic calibration.

PREPARATION OF SOLUTIONS

The choice of solvent used to prepare fluorescent solutions requires precautions. The nature, purity and pH of the solvent are relevant parameters in the intensity and spectral distribution of fluorescence. As a result, it is recommended to comply with the volume specified in established methods. Many substances fluoresce in organic solvents but are practically non-fluorescent when dissolved in water. Thus, experimentation in various solvents is required to determine the fluorescent property of a substance.

For quantitative purposes, it is essential that the fluorescence intensity maintains a linear relation with the sample concentration within limits compatible with the method. If the solution is very

concentrated, a significant part of the incident light will be absorbed in the periphery of the cuvette and the amount of radiation reaching the central region will be lower. This means that the substance itself will act as an “internal filter”. However, this phenomenon is rare, considering that fluorescence spectrophotometry is a highly sensitive method, allowing the use of solutions with concentrations in the order of 10^{-5} to 10^{-7} M.

Due to the usually narrow concentration limits in which the fluorescence is proportional to the concentration of the substance, the rule is to obey the ratio $(c-d)/(a-b) = 0.40$ to 2.50 . In this case, a is the fluorescence intensity of the reference solution, b is the intensity of the corresponding blank, c is the intensity of the sample solution, and d is the intensity of the corresponding blank.

Fluorescence determinations are sensitive to the presence of solid particles in the solutions. Such impurities reduce the intensity of the incident beam, producing false high readings due to multiple reflections in the cuvette. It is therefore necessary to eliminate these solids by centrifugation or filtration prior to reading, bearing in mind, however, that some filter papers may contain fluorescent impurities.

The presence of dissolved oxygen in the solvent exerts a dampening effect on the fluorescence intensity and it should be eliminated using, for example, passage of a current of nitrogen, helium or any inert gas in the solution, prior to the reading.

Temperature control is also important. In some substances, the fluorescence emission can decrease by 1 to 2% with each temperature increase of 1°C . Accordingly, when maximum precision is required, the use of thermostatic cuvettes is recommended. However, for routine analysis, there is no need for this resource as long as the determinations are made quickly enough to avoid heating due to exposure of the solution to bright light.

Some fluorescent substances are sensitive to light and, when exposed to intense light radiation from the fluorescence spectrophotometer, they can decompose into more or less fluorescent products. This effect can be detected by observing the detector's response in relation to time and attenuated by reducing the incident light intensity using filters.

5.2.16 TURBIDIMETRY AND NEPHELOMETRY

Turbidimetry and nephelometry – variants of spectrophotometry – are intended for the quantitative evaluation of substances from the turbidity of their suspensions, proportional to their diffraction power on incident light (Tyndall effect).

In turbidimetry, also known as opacity, the intensity of light transmitted in the same direction as the incident light is measured. Although there are turbidimeters specifically designed to measure turbidity, conventional colorimeters and spectrophotometers are satisfactory for measuring transmitted light as long as they are adjusted to the appropriate wavelength.

Nephelometry (or diffusimetry), in turn, comprises the measurement of the intensity of light scattered (reflected) by suspended particles, at a right angle to the incident light beam. Once again, in addition to nephelometers, it is possible to use colorimeters and spectrophotometers in nephelometric measurements. Therefore, it is necessary to modify them so as to allow the capture perpendicular to the angle of the incident light, either by transferring the light source or by changing the position of the detector. Fluorimeters, similar to nephelometers, are used to measure scattered light (positioning the detector at an angle of 90° in relation to the incident light) and are therefore compatible with nephelometry.

Turbidity

Turbidance (S) in analogy to transmittance (T), defined in *UV, Visible and infrared absorption spectrophotometry* (5.2.14) is the official expression of light scattering produced by suspended particles, determinable by turbidimetry or nephelometry, according to the formula

$$s = \frac{P_0}{P} k \frac{bd^3}{d^4 + \lambda^4}$$

where

P_0 = incident radiation intensity;

P = transmitted radiation intensity;

b = sample thickness (cuvette);

d = particle average diameter;

λ = wavelength;

k = constant of proportionality, depending on the nature of the suspension and the method of measurement.

A suspension evaluated in a given instrument, under monochromatic light, presents turbidity that corresponds to the product of concentration C by a constant of proportionality k , which combines the other parameters of the formula above. Therefore, $S = kC$, expression of the Lambert-Beer law, allowing turbidimetric and nephelometric procedures to be analogous to those adopted in spectrophotometry. It is, however, relevant to note that proportionality is only true for very dilute suspensions, as secondary reflections cause excessive linearity deviation when the number of suspended particles exceeds a certain limit.

Another source of error in turbidimetric and nephelometric measurements is the decanting of suspended particles. Such occurrence can be minimized by increasing the viscosity, with the

incorporation of protective colloid – gelatin, gum arabic or starch – in the liquid medium of the suspension.

PROCEDURE

The basic procedure for the use of turbidimetric or nephelometric methods follows the principles of spectrophotometric methods, comprising the preparation of reference suspensions of known concentration. In practice, plotting against transmittance values rather than turbidity is allowed.

The steps of the procedure comprise, briefly: (1) adjust the instrument to the wavelength specified in the monograph (for colorimeters, in the absence of specification, use a filter that provides light in the blue range); (2) fill the cuvette with the most concentrated suspension and adjust the transmittance reading to 100% (transmittance offers more linearity than absorbance); (3) measure the transmittance of the other standard suspensions and plot the calibration curve (using the least squares method) and (4) measure the transmittance of the sample, determining its concentration using the calibration curve.

Visual comparison

Turbidity measurements can be performed by visual comparison, a method in which the suspension of the sample is compared with suspension or standard suspensions. To do so, use identical test tubes, flat-bottomed, with a capacity of 70 mL and approximately 23 mm in internal diameter. Tubes must be compared horizontally on a dark background, with lateral light incidence.

5.2.17 CHROMATOGRAPHY

5.2.17.1 THIN LAYER CHROMATOGRAPHY

It consists of the chromatographic system in which the separation of the components of a mixture occurs through differential migration over a stationary phase composed of a thin layer of adsorbent applied on a flat support, which can be made of different materials such as glass, aluminum or polyester. The mobile phase, in turn, consists of several mixtures of solvents and remains inside a container or tank made of transparent and inert material, usually glass, remaining sealed, where the chromatoplate is placed in a vertical position under a mobile phase saturated atmosphere.

APPARATUS AND PROCEDURES:

The apparatus used for thin layer chromatography consists of: plate, tank or elution chamber, stationary phase, mobile phase, TLC visualization reagent system. Plates are usually made of glass, aluminum or plastic material. Sizes vary as follows: 20 cm x 20 cm; 10 cm x 20 cm; 10 cm x 10 cm; 5 cm x 10 cm.

Stationary phases (adsorbents)

Silica – It is the most widely used adsorbent in TLC. It is an amorphous and porous adsorbent. It is also used in column chromatography; however, the silica used in TLC is thinner. Silica is prepared by spontaneous polymerization and dehydration of silicic acid. The substances are adsorbed by silica via hydrogen bonding and dipole-dipole interaction. A silica of satisfactory condition is one with 11 to 12% water by weight. A moisture level of 11 to 12% is achieved when the silica is in equilibrium with air, at a relative humidity of 50% and a temperature of 20°C.

Commercial silicas have variable pore sizes, between 40 and 150 Angstroms. Particle sizes range from 5 to 40 µm, averaging 10 to 15 µm, depending on the manufacturer.

By reducing the particle size, the silica performance is increased. Particle sizes from 5 to 6 µm are used to prepare HPTLC (High Performance Thin Layer Chromatography). Pore sizes affect selectivity and therefore can be used for migration rates and resolution of sample components.

The most common commercially available silica pore sizes are 40, 60, 80 and 100 Angstroms, with 60 Angstrom silica being the most versatile and widely used. Silica are used for the separation of lipophilic compounds, such as aldehydes, ketones, phenols, fatty acids, amino acids, alkaloids, terpenoids and steroids, using the adsorption mechanism.

Alumina – After silica, it is the most used adsorbent. The physical properties of alumina are similar to those of silica in terms of particle size, average pore diameter and surface. Acid (pH 4.0 –4.5), neutral (7.0 –8.0) and basic (9.0 –10.0) alumina are commercially available. Similarly to silica, alumina separates sample components by polarity, through hydrogen bonds, Lewis acid-base interactions or dipole-dipole interactions. The selectivity of alumina in adsorption TLC is similar to silica gel, with alumina being a better adsorbent than silica for the separation of lipophilic acid substances. Alumina with an acidic character strongly attracts basic substances, whereas alumina with a basic character attracts more strongly acidic substances. Alumina retains aromatic substances more strongly than silica gel. It has the inconvenience of promoting the catalysis of some reactions of labile substances. It is used in the separation of fat-soluble vitamins, alkaloids, certain antibiotics and polycyclic hydrocarbons.

Kieselguhr – It is the thermally treated Diatomaceous Earth, with a granulation of 5 to 40 µm. Its main constituent is SiO₂. A variety of other inorganic compounds are also present. Pore sizes vary greatly, its characteristics make it suitable for separating sugars, amino acids and other similar polar substances.

Cellulose – Cellulose is a polysaccharide that is highly polymerized by glucose monomers. The presence of large numbers of free hydroxyl groups allows hydrogen bonds with low molecular weight liquids such as water and alcohols. Cellulose is therefore suitable for separating hydrophilic substances such as carbohydrates and amino acids.

Polyamide – In contrast to cellulose, polyamide is a synthetic resin. Two types of polyamide are used: polyamide 6 and polyamide 11. Polyamide 6 comes from aminopolycaprolactam, whereas polyamide 11 is prepared from polyaminoundecanoic acid. Polyamides are used for the separation of polar compounds that are able to interact with the amide group by hydrogen bonds due to their molecular structure. Among them there are amino acids and derivatives, benzodiazepines, carboxylic acids, cyclodextrins, fatty acids, flavonoids, preservatives and pesticides.

Magnesium silicate – ideal for the separation of sugars, anthraquinones, flavones, glycosides, steroids, lipids, pesticide residues, vitamins, carbazoles, hydrocortisone acetate.

TLC visualization reagents and detection methods

After the development of chromatography and evaporation of the solvents, the stain development method is used. This, in turn, can be physical or chemical. As a physical method, ultraviolet light (lamps emitting radiation between 254 and 366 nm) is commonly used in the case of substances that become fluorescent when excited by UV or visible light. Chemical methods comprise the use of chromogenic reagents. There is an extensive list of TLC visualization reagents suitable for each group of substances.

Identification

The final position of each spot is designated by the R_f (retention factor). After developing the chromatoplate, the distance reached by each stain from the origin is measured. This distance is a fraction of the total distance traveled by the solvent in the stationary phase.

$R_f = (\text{distance reached by the stain from the origin}) / (\text{distance traveled by the solvent from the origin})$

5.2.17.2 CHROMATOGRAPHY ON PAPER

It uses differential migration on the surface of a special quality filter paper (stationary phase) to separate and identify the substances or components of the mixture. The mobile phase can be a pure solvent or a mixture of solvents.

In chromatographic paper, the adsorbent is a paper layer of suitable texture and thickness. Chromatographic separation occurs through the action of the liquid mobile phase similar to the adsorption process in column chromatography. Due to the intrinsic water content of the paper, or selective inhibition of the hydrophilic component of the liquid phase by the paper fibers, which can be considered as stationary phase, a partition mechanism can significantly contribute to the separation.

The chromatogram is developed by the slow passage of the mobile phase over the paper layer. Development can be upward, in the case of solvent carried upwards by capillary forces, or downward, in the case where the solvent flow is aided by the force of gravity.

The simplest form of paper chromatography is ascending chromatography, which uses a strip of paper of varying length and width, depending on the chromatographic chamber being used.

This method is very useful for separating very polar substances such as sugars and amino acids. It has the inconvenience of being able to apply a small amount of substance at a time. One should try to work in the closest possible conditions, of quality and quantity, between standard and sample, using the same paper, mobile phase, temperature, etc.

APPARATUS AND PROCEDURES

It consists of a glass chromatographic chamber or tank, provided with ground edges and lid and with adequate dimensions to contain the chromatographic paper, which can be adapted for ascending or descending chromatography. It is important that vapors from the mobile phase are not allowed to escape.

Use special filter paper for chromatography, cut along the fibers into strips of variable length and width of minimum 2.5 cm. There are several types of chromatography paper with different purposes for separating hydrophilic or hydrophobic, organic or inorganic, amphoteric or with many hydroxyl substances, among others.

For descending chromatography, use a tank with a lid provided with a central orifice, closed by a glass stopper or other inert material. At the upper part of the chamber, there is a suspended cuvette, which contains a device for holding the paper (usually a glass rod or stick). On each side of the cuvette there are glass guides, which support the paper so as not to touch the walls of the chromatographic chamber. The width of the chromatographic paper cannot be greater than that of the suspended cuvette and the height must be approximately equal to the height of the chromatographic chamber.

For ascending chromatography, there is a device in the upper part of the chamber that allows the chromatographic paper to be supported and that can be lowered without opening the chromatographic chamber. Handle the paper carefully and by the edges, and cut strips into sizes that can be placed in the chambers. It is important to cut the strips following the grain of the paper, as the cellulose is oriented to this direction, which will facilitate the passage of the mobile phase. The strip of paper must not touch the walls of the chamber.

When adding the paper to the chamber (paper addition should not be delayed so that there is no loss of saturation), make sure that the sample does not come into direct contact with the eluent, allowing it to ascend or descend on the surface of the paper, only by capillarity.

When the method used is ascending chromatography, plot a thin line with a pencil 3 cm from the lower edge of the paper; if the chromatography is descending, plot a line at a distance, such that it is a few centimeters below the rod that holds the paper in the eluent cuvette. The finish line of the mobile phase (or solvent front) should also be marked, usually at a distance of 10 cm from the starting point.

Apply the solutions in the form of circular spots (capillary tubes or micropipettes are used), containing 1 to 20 μg of the sample, with each spot having a width between 6 to 10 mm over the line plotted with a pencil. Depending on the width of the paper, only an aliquot of the standard or sample can be placed, centering this application on the starting line. In the case of placing more than one aliquot at

the starting point, leave 2 cm of distance from the lateral edges and a range between the application points of 3 cm. If each stain produced is larger than 6 to 10 mm, apply the sample in portions, allowing the solvent to evaporate before applying the next portion.

The level of the mobile phase must be below the starting point of the substance, and there must always be a good sealing in the chromatographic chamber so that the vapor in this phase is not lost. At the end of the procedure, wait for the paper to dry and submit it to some development process.

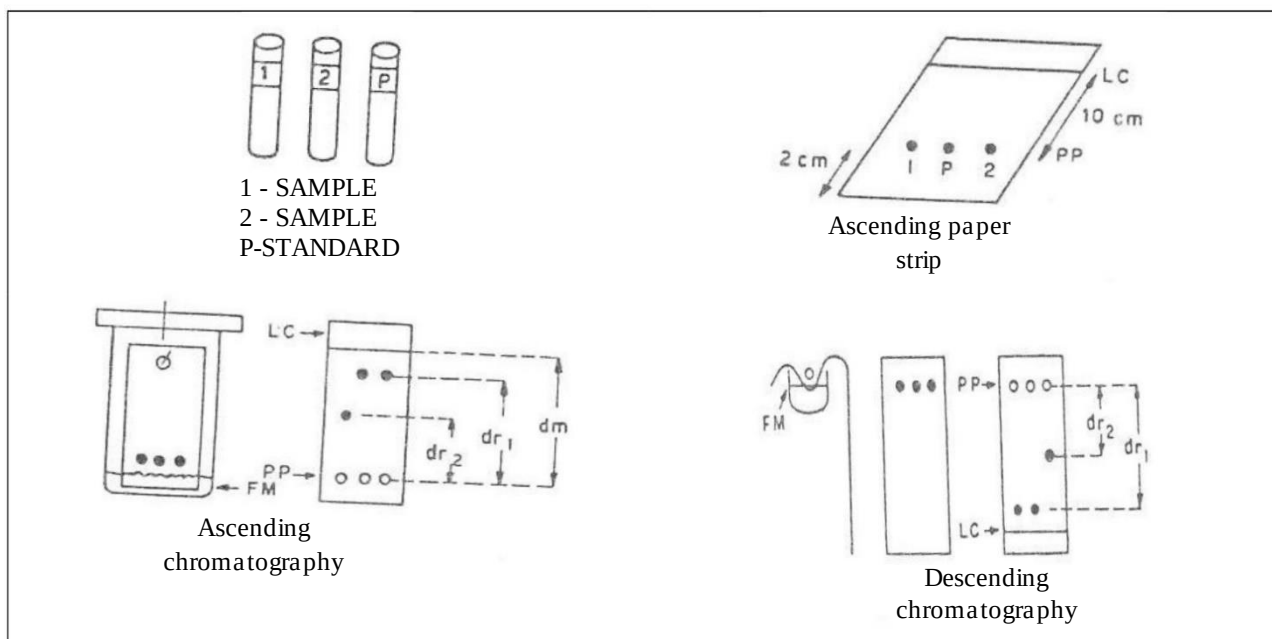


Figure 1 – Different types of paper chromatography according to development methods.

FM: Mobile phase; **PP** Starting point; **LC:** Finish line; **dr1 and dr2:** distances covered by substances; **dm:** mobile phase migration distance

DESCENDING CHROMATOGRAPHY

In descending chromatography, the mobile phase has a downward flow and relies on the action of gravity.

Introduce a layer of eluent specified in the monograph into the chamber, cap and allow to stand for 24 hours. Apply the sample to the paper, placing it properly on the guides so that the upper end remains inside the suspended cuvette and hold it with the glass rod. Close the chamber and allow to stand for an hour and a half. Then, through the hole in the lid, introduce the eluent into the cuvette. Develop the chromatogram up to the prescribed distance or time, protecting the paper from direct light incidence. Remove the paper, mark the path of the mobile phase, dry and visualize as prescribed in the monograph.

ASCENDING CHROMATOGRAPHY

The upward flow of the mobile phase over the chromatographic paper is allowed by capillary action.

Place the container with the eluent at the bottom of the chromatographic chamber, close the chamber and allow to stand for 24 hours. Apply the sample to the paper, introducing it into the chamber and allow to stand for an hour and a half. Without opening the chamber, lower the paper so that its lower

end is in contact with the eluent and develop the chromatogram up to the prescribed distance or time. Remove the paper, mark the path of the eluent, dry and visualize as prescribed in the monograph.

5.2.17.3 COLUMN CHROMATOGRAPHY

Preparative column chromatography is a separation method that plays an important role in the purification of substances of research value, in pilot plant operation and in the production of pharmaceuticals. It is a method that can be used, quickly and economically, to obtain substances with high purity. In practice, standardized adsorbents are used, as they provide a high degree of method reliability, direct analysis scale transfer and optimized processing. The types of column chromatography can be: by adsorption (liquid-solid), by partition (liquid-liquid) or by ion exchange.

EQUIPMENT

The apparatus used for procedures on chromatographic columns consists of a cylindrical chromatographic tube, in a vertical position, made of glass (or other inert and transparent material specified in the individual monograph), of variable length and diameter, in whose lower part there is narrowness (reduced passage) and a tap for regulating the flow of solvents used for elution. In some columns, the lower part has, at the base, a porous glass disk, whose purpose is to prevent the exit of the stationary phase (silica-gel). Columns have variable dimensions, however, in pharmaceutical analysis, the most commonly used ranges are 10mm to 30mm in diameter and 3mm to 6mm in the lower part, where the tap is attached. The tube length is usually from 150 mm to 400 mm. At the top of the column there may be a spherical expansion, intended to contain a greater volume of solvent, followed by a ground, cylindrical connection, which allows the fitting of a cylindrical plastic, glass, stainless steel, aluminum cap or other material specified in the individual monograph. The vein in the rod is substantially smaller than the diameter of the column and is at least 5 cm longer than the actual length of the column. The stopper has a smaller diameter by approximately 1 mm in relation to the inner diameter of the column.

PROCEDURE

Column adsorption Chromatography

Start the preparation of the column, if necessary, sealing the bottom, at the base of the tube, close to the tap, with a piece of cotton or glass wool in order to prevent the passage of adsorbent material and the entry of air (avoiding formation of bubbles). Then, uniformly fill the tube (according to the specified height) with this adsorbent material (such as activated alumina or silica gel, diatomaceous silica or calcined silica), previously suspended in the mobile phase (solvent system), removing the excess of eluent. After sedimentation of the adsorbent material, apply the mixture of substances previously dissolved in a small amount of solvent, at the top of the column, until it penetrates the adsorbent material. A certain amount of solvent can be added to the top to help adsorb the substances onto the adsorbent material, which is then allowed to settle by gravity or by applying positive air pressure, with the mixture being adsorbed in a narrow horizontal band at the top of the column. The movement rate of a given substance is determined or affected by several variables, including the low or high adsorptiveness of the adsorbent material, the particle size and surface area (contact surface), the nature and polarity of the solvent, the pressure applied and the temperature of the chromatographic system.

A flow chromatogram is widely used and is obtained by a process in which solvents travel through the column until the substance is separated into an effluent solution, known as an eluate. The eluate is controlled by collecting fractions as specified in the monograph and examining each fraction by an appropriate method. The substance can be determined in the eluate by several methods: titration,

colorimetry, spectrometry or be isolated (purified) upon evaporation of the solvent. The separation performance can be gauged by thin layer chromatography (TLC) of each fraction collected throughout the chromatographic run.

Partition Column Chromatography

In partition chromatography, the substances to be separated are partitioned into two immiscible liquids, one of which, the stationary phase, is adsorbed onto a solid support, thus presenting a very large surface area for the circulating solvent or mobile phase. The high number of serial liquid-liquid contacts allows an effective separation, which does not occur through the usual liquid-liquid extraction.

The solid support is generally polar, while the stationary adsorbent phase is more polar than the mobile phase. The most used solid support consists of chromatographic siliceous earth, whose particle size is satisfactory for proper eluent flow. In reverse phase partition chromatography, the stationary adsorbed phase is less polar than the mobile phase, and the solid adsorbent becomes nonpolar by treatment with a silanizing agent (e.g. dichlorodimethylsilane; paraffins) to produce a chromatographic silanized sand.

The sample to be chromatographed is usually inserted into a chromatographic system in two manners: (a) a sample solution in a small volume of mobile phase at the top of the column; or (b) a sample solution in a small volume of the stationary phase is mixed with the *solid support* and transferred to the column, forming a transverse layer over the adsorbent material.

Development and elution are achieved through the “run” of the circulating solvent. The solvent (mobile phase) is usually saturated with the solvent (stationary phase) before use. In the case of conventional liquid-liquid partition chromatography, the partition degree of a given compound between the two liquid phases is expressed by means of the partition or distribution coefficient.

In the case of substances that dissociate, the distribution can be controlled by modifying the pH, the dielectric constant, the ionic strength, among other properties of the two phases. Selective elution of the mixture components can be achieved by successfully changing the mobile phase to one that provides a more favorable partition coefficient, or by changing the pH of the stationary phase *in situ* with a stationary phase consisting of an acid solution. or an appropriate base in an organic solvent.

Unless otherwise specified in the individual monograph, assays and tests employing partition column chromatography are carried out in accordance with the conventional methods described below.

Solid support – Use purified silica sand. For reverse phase partition chromatography, use chromatographic silica sand.

Stationary phase – Use the solvent or solution specified in the individual monograph. If a mixture of liquids in the stationary phase is used, mix before introducing the solid support.

Mobile phase – Use the solvent or solution specified in the individual monograph. Equilibrate with water if the stationary phase is an aqueous solution; if the stationary phase is a polar organic solvent, equilibrate with this solvent.

Preparation of a chromatographic column – The chromatographic tube measures approximately 22 mm in internal diameter and 200 mm to 300 mm in length, without a porous glass disc, to which a distribution tube is attached, without a stopcock, measuring approximately 4 mm in inner diameter and approximately 50 mm in length. Insert a thin glass wool buffer into the base of the tube. Add the

specified amount of solid support in a beaker (cylinder) from 100 mL to 250 mL and mix until a homogeneous paste is produced. Transfer the mixture to the chromatographic tube, cap, pressing slightly, until a uniform mass is obtained. If the amount of solid support specified is more than 3 g, transfer the mixture to the column in portions of approximately 2 g, capping each portion. If the assay or test requires a multi-segment column, with a different stationary phase for each segment, cap after each segment is added, and add each next segment directly to the previous one. If an analyte solution is incorporated into the stationary phase, complete the quantitative transfer to the chromatographic tube by washing the beaker used to prepare the test mixture with a mixture of approximately 1 g of solid support and several drops of the solvent used for prepare the test solution. Insert a thin piece of glass wool on top of the complete filling column. The mobile phase flows through a properly filled column with moderate flow or, if reverse phase chromatography is used, slowly dropwise.

Transfer the mobile phase to the column space over the filled part, and allow to flow through the column under gravity. Wet the tip of the chromatographic column with about 1 mL of the mobile phase before each mobile phase composition change and after completing the elution. If the analyte is introduced into the column as a mobile phase solution, allow to elute completely through the filled column, then add the mobile phase in several smaller portions, allowing each to be completely removed before adding the stored mobile phase.

Ion exchange column chromatography

Use ion exchange resin as stationary phase. Ion exchange consists of reversible exchange of ions present in the solution with resin polymer ions (modified cellulose or silica gel support). The choice of resin, strong or weak, anionic or cationic, will depend largely on the pH at which the ionic exchange should take place and on the nature of the ions (anions or cations) to be exchanged. Strongly acidic and strongly basic resins are suitable for most analytical applications. In practice, a large excess (200% to 300%) of resin is used over the stoichiometrically calculated sample amount; resin capacity ranges from 2 mM/g to 5 mM/g (dry weight).

Resin treatment and column preparation – Suspend the ion exchange resin in water and allow to stand for 24 hours. Introduce into a suitable column and, in the case of anionic resin, convert into basic by passing through the column, sodium hydroxide RS solution, at a rate of 3 mL/minute, until the eluate provides a negative reaction for chloride ion. Then rinse with carbon dioxide-free water. In the case of cationic resin, the conversion to the acid form is done by passing hydrochloric acid RS through the column, followed by washing with carbon dioxide-free water until the eluate provides a neutral reaction.

An ion exchange column is developed similarly to that described for adsorption chromatography. After the operation, the resin is regenerated by washing with sodium hydroxide RS (anion-exchange columns) or hydrochloric acid RS (cation-exchange columns) and then with carbon dioxide-free water until a neutral reaction is produced.

5.2.17.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography (HPLC) is a separation method based on the distribution of the components of a mixture into two immiscible phases, the liquid mobile phase and the solid stationary phase, contained in a cylindrical column. Separations are achieved by partition, adsorption, ion exchange, size exclusion or stereochemical interactions, depending on the type of stationary phase used. HPLC has advantages over gas chromatography for the analysis of organic combinations. Samples of non-volatile and thermolabile substances are preferably analyzed by HPLC. Most pharmaceutical analyses are based on the partition separation method and must take place in a short

analysis time. Several chemical and physicochemical factors influence the chromatographic separation, which depend on the chemical nature of the substances to be separated, on the composition and flow of the mobile phase and on the composition and surface area of the stationary phase.

APPARATUS

The apparatus used consists of a reservoir containing the mobile phase, a pump for driving the mobile phase through the chromatographic system, an injector to introduce the sample into the system, a chromatographic column, a detector and a data capture device, such as software, integrator or recorder. In addition to receiving and sending information to the detector, software is used to control the entire chromatographic system, providing greater operability and analysis logistics.

Modern chromatographic systems consist of software-controlled mobile phase pressurizing pumps that can be programmed to vary the ratio of mobile phase components, as required for solvent gradient chromatography, or for isocratic mixing the mobile phase (mobile phases with fixed solvent ratio). Operating pressures up to 5800 psi (approximately 400 bar) and flow rates up to 10 mL per minute can be used. Higher pressures are conditioned to the evolution of the instrument.

After dissolving the sample in the mobile phase or in another suitable solvent, the solution is injected into the chromatographic system, manually, using an appropriate syringe, or through an injector or automatic sampler. This consists of a carousel or tray, capable of accommodating several vials containing the samples. Some autosamplers can be programmed to inject different volumes of sample, different amounts of injections, control the range between injections and other operational variables.

When working at high pressures, an injection valve is essential. This presents a calibrated system, with a defined volume, called an injection ring or sampling loop, which will be filled with the solution to be analyzed, which will later be transferred to the column.

For most pharmaceutical analyses, separation is achieved by partitioning the components, present in the solution to be analyzed, between the mobile and stationary phases. Systems consisting of polar stationary phases and nonpolar mobile phases are defined as *normal phase chromatography*, whereas the opposite, polar mobile phases and nonpolar stationary phases, are called *reverse phase chromatography*. The affinity of a substance for the stationary phase, and consequently the retention time on the column, is controlled by the polarity of the mobile phase.

Stationary phases used in *reverse phase chromatography* typically consist of an organic molecule chemically bonded to silica particles or other supports, such as porous graphite. The particle diameter is typically 3 µm to 10 µm. The smaller the diameter of the particles and the film covering the support, the faster and more efficient will be the transfer of substances between the stationary and mobile phases. The polarity of the column depends on the functional groups present, the most common being the non-polar *groups octyl, octadecyl, phenyl, cyanopropyl* and polar group, *nitrile*. The proportion of silanol groups not linked to the functional group significantly influences the performance of chromatographic separation and the shape of the eluted peak. Commercially, chromatographic columns with different qualities of stationary phases are available, including those with a small proportion of free silanol groups, called *endcapped*. Generally, reversed-phase silica columns have a shelf life in the pH range of 2 to 8, however, columns containing porous graphite or polymeric materials such as styrene-divinylbenzene are stable over a wider pH range. Less commonly, unalloyed liquids can be used as a coating of the silica support and therefore must be immiscible with the mobile phase. Columns commonly used for analytical separations have internal diameters from 1 mm to 5 mm. These can be heated, providing more efficient separations, but temperatures above 60°C are rarely used, due to the potential for degradation of the stationary phase or the volatility of the mobile phase. Unless specified in the analyte monograph, columns are used at room temperature.

The most frequently used detectors in high performance liquid chromatography are spectrophotometric (UV/Vis). Spectrophotometric detectors are used to detect substances with a **chromophore** group. Such detectors consist of a flow cell located after the chromatographic column. Ultraviolet radiation constantly passes through the flow cell and is received at the detector. With the system in place, substances elute from the column, pass through the flow cell and absorb radiation, resulting in measurable changes in the energy level. These detectors can present fixed, variable or multiple wavelength. Fixed wavelength detectors operate on a single value, typically 254 nm, emitted by a low-pressure mercury lamp. Those with variable wavelength contain a continuous source of emission, such as a high pressure deuterium or xenon lamp, and a monochromator or an interference filter, in order to generate monochromatic radiation of a wavelength selected by the operator, which can, further, be programmed to change the wavelength during the development of the analysis. Multiple wavelength detectors simultaneously measure absorbance at two or more wavelengths, called diode array detectors (DAD). In these, ultraviolet or visible radiation is transmitted through the flow cell, absorbed by the sample and then separated into their different wavelengths, which are individually detected by the photodiode detector, recording absorbance data across the entire spectrum range of the ultraviolet and visible and, additionally, the spectra of each peak recorded in the chromatogram.

Refractive index detectors measure the difference between the refractive index of the pure mobile phase and the mobile phase containing the substance to be analyzed. They are used to detect substances that do not absorb in the ultraviolet or visible light, however, they are less sensitive than spectrophotometric detectors. Refractive index detectors have the disadvantage of being sensitive to small changes in mobile phase solvent composition, flow rate and temperature.

Fluorimetric detectors are used to detect compounds with a **fluorophore** group or which can be converted to fluorescent derivatives, by chemical transformation or by adding fluorescent reagents to specific functional groups. If a chemical reaction is required, it can be carried out at the time of sample preparation or, alternatively, the reagent can be introduced into the mobile phase, with the reaction taking place before detection.

Potentiometric, voltammetric, or electrochemical detectors are useful for quantifying substances that can be oxidized or reduced in an electrode. These detectors are highly selective, sensitive and safe, but require mobile phases free of oxygen and reducible metal ions. A continuous flow pump must be used, ensuring that the pH, ionic strength, and temperature of the mobile phase remain constant. Electrochemical detectors with specific carbon electrodes can be used, advantageously, to quantify nanograms of easily oxidizable substances, such as phenols and catechols.

In mass spectrometry (MS) detection, the m/z ratio (mass/charge ratio) of the precursor ion of a substance is measured. The precursor ion is generated from the substance protonation (positive mode), from deprotonation (negative mode) or even from the formation of sodium, potassium, formate, etc. adduct ions. The combination of mass spectrometry with liquid chromatography provides good selectivity, since unresolved peaks can be isolated by monitoring a selected mass/charge (m/z) ratio value. These mass spectrometers can have only one mass analyzer, such as a single quadrupole, or sequential or *tandem* (MS/MS), such as a triple quadrupole, when two mass analyzers are combined. In this case, it is possible to fragment the precursor ion in a collision cell located before the second mass analyzer. Thus, the monitoring of the mass transitions (precursor ion $\rightarrow$ product ion), usually specific for each analyte, allows the analysis with high selectivity, since it is possible to obtain a chromatogram for each mass transition. The most commonly used ionization sources in the HPLC-MS coupling are the “*electrospray* ionization” (ESI) and the “atmospheric pressure chemical ionization” (APCI) type.

Conductivity detectors have application in ion exchange chromatography and measure the conductivity of the mobile phase continuously, which is modified in the presence of analytes in the flow cell.

Currently, modern data collection systems are available with the functions of receiving and storing the signals coming from the detector and, subsequently, providing the management of this information, generating chromatograms with the peak area and height data, sample identification, methods, among others. Information can also be collected in simple data recording systems, such as loggers, to ensure the integrity of the generated data.

PROCEDURE

Column length and internal diameter, stationary phase particle type and size, operating temperature, mobile phase composition and flow rate and detection type are described in the individual monographs.

The composition of the mobile phase has a significant influence on the chromatographic performance and on the separation of substances present in the solution to be analyzed. For accurate quantitative analysis, high purity reagents or chromatographic purity organic solvents should be used. Water, of adequate quality, must have low conductivity and low ultraviolet absorption. In partition chromatography, the partition coefficient and hence the separation can be modified by adding another solvent to the mobile phase. In ion-exchange chromatography, substance retention is affected by pH, ionic strength, and other changes in the composition of the mobile phase. The method of continuously modifying the composition of mobile phase solvents during the chromatographic run is called gradient elution, and is applied to separate complex mixtures of substances with different retention factors. However, detectors that are sensitive to changes in the composition of the mobile phase, such as refractometers, have limited use with the gradient elution method.

The detector must have a wide linear range and the substances to be analyzed must be separated from any interfering ones. The linear range for a substance is one in which the detector response is directly proportional to its concentration.

HPLC systems are calibrated by comparing the peak responses obtained with the respective concentrations of chemical reference standards (CRS). Reliable quantitative results are obtained through calibration with an external standard, when injectors or autosamplers are preferably used. This method involves direct comparison of the responses obtained with the separately analyzed peaks of standard and sample solutions. In cases where external standardization is used, calculations can be performed according to the formula:

$$Ca = Cp(Ra / Rp)$$

where

Ca = concentration of the sample solution.

Cp = concentration of the standard solution;

Ra = response (area or height) of the peak of the sample solution;

Rp = response (area or height) of the peak of the standard solution.

If the injection is performed by syringe, better quantitative results are obtained by means of internal standard calibration, by adding a known amount of a non-interfering reference chemical to the standard and sample solutions. The relation of responses obtained with the substance to be analyzed

and with the internal standard is used as a response to express the quantitative result. In cases where internal standardization is used, calculations can be performed according to the formula:

$$Ca = Cp \frac{(Ra / Rai)}{Rp / Rpi}$$

where

Rai = response (area or height) of the peak of the sample solution internal standard;

Rpi = response (area or height) of the peak of the internal standard of the standard solution;

Due to normal variations among devices, solvents, reagents and methods, a system suitability test is required to ensure that the described method is applied unrestrictedly. The main parameters of system suitability are described in *the Interpretation of chromatograms* and in *System suitability*.

INTERPRETATION OF CHROMATOGRAMS

In **Figure 1**, a typical chromatographic separation of two substances is represented, being t_1 and t_2 the respective retention times. The terms h , $h/2$ and $W_{h/2}$ correspond to the height, the half height and the width at half height, respectively, and W represents the peak width at the baseline, by the triangulation method. The dead time signal, t_0 refers to a substance not retained in the chromatographic column.

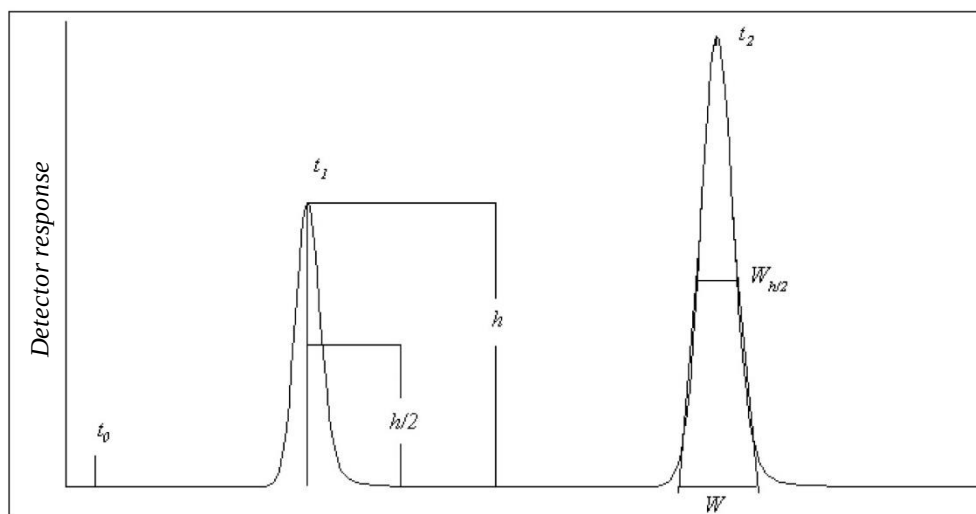


Figure 1 – Chromatographic separation of two substances.

Retention time (t), Retention factor (k) and Relative retention time

The retention time in chromatography is characteristic of the analyte, however it is not exclusive. The comparison between the retention times of the peak obtained with the sample and the peak obtained with the reference chemical can be used as an indication of the identity of the substance, but it is insufficient to guarantee the complete characterization of the sample. Absolute retention time may vary among devices and depending on the use of different solvents and reagents. In this sense, comparisons are made in terms of the retention factor, k , calculated according to the expression:

$$k = \frac{t - t_0}{t_0}$$

where

t = retention time of analyte;

t_0 = dead time.

The retention factor, k , is the ratio between the amount of substance with affinity for the stationary phase and the amount with affinity for the mobile phase. The greater the affinity of the substance for the stationary phase, the greater its retention.

The concept of relative retention time can also be applied. For that, a substance, of a mixture, must be defined as the main one. This will have a relative retention time of 1. All other substances will have their retention times related to the retention time of the main substance.

Number of theoretical plates (N)

The number of theoretical plates, N , is indicative of column performance. It can be expressed in numbers of theoretical plates per column or number of theoretical plates per meter. For peaks with a Gaussian shape, the number of theoretical plates per column is calculated using the expressions:

$$N = 16 \times \left(\frac{t}{W} \right)^2 \text{ ou } N = 5,54 \times \left(\frac{t}{W_{h/2}} \right)^2$$

The value of N depends on the substance to be analyzed and the analysis conditions such as mobile phase, temperature and stationary phase.

Peak/Valley Ratio (p/v)

The peak/valley ratio can be used as a system suitability criterion in a related substance assay, when separation between two peaks in the baseline is not sought. **Figure 2** represents an incomplete separation of two substances, where h_p is the height of the smallest peak above the extrapolated baseline, and h_v is the height at the lowest point of the curve, which separates the minor and major peaks above the extrapolated baseline.

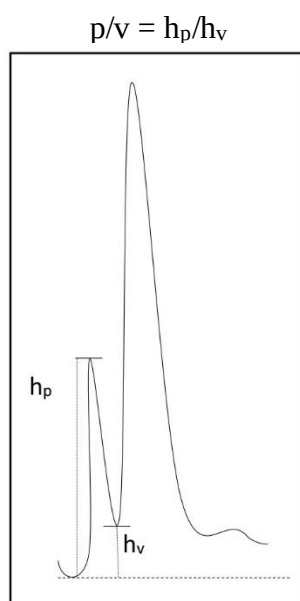


Figure 2 – Determination of peak/valley ratio.

Resolution (R)

The resolution, R , is the chromatographic parameter that indicates the degree of separation between two substances in a mixture, and is calculated using the expressions:

$$R = \frac{2(t_2 - t_1)}{W_1 + W_2} \text{ ou } R = 1,18 \frac{(t_2 - t_1)}{(W_{1,h/2} - W_{2,h/2})}$$

where

t_2 and t_1 = retention times of the two substances in the mixture;

W_1 and W_2 = respective peak widths in the baseline, by the triangulation method;

$W_{1,h/2}$ and $W_{2,h/2}$ = respective peak widths at half height.

The area or height of the peak is usually proportional to the amount of substance eluted. The area under the peak is generally used the most, however it may be less accurate if there are other interfering peaks. For manual measurements, the graph must be obtained at a speed greater than usual, minimizing errors in obtaining the width and width at half height of the peaks. For quantitative analysis, substances must be completely separated from any interfering substances.

Tailing factor (T)

The tailing factor, T , which indicates the symmetry of the peak, has a value equal to 1 when the peak is perfectly symmetric. This value increases as peak asymmetry becomes more pronounced. In some cases, values lower than 1 may be observed. As peak asymmetry increases, integration and accuracy become less reliable. The tailing factor is calculated using the expression:

$$T = \frac{W_{0,05}}{2f}$$

where

$W_{0,05}$ = peak width measured at 5% of the height;

f = width of the anterior portion of the peak at 5% of the height, according to **Figure 3**.

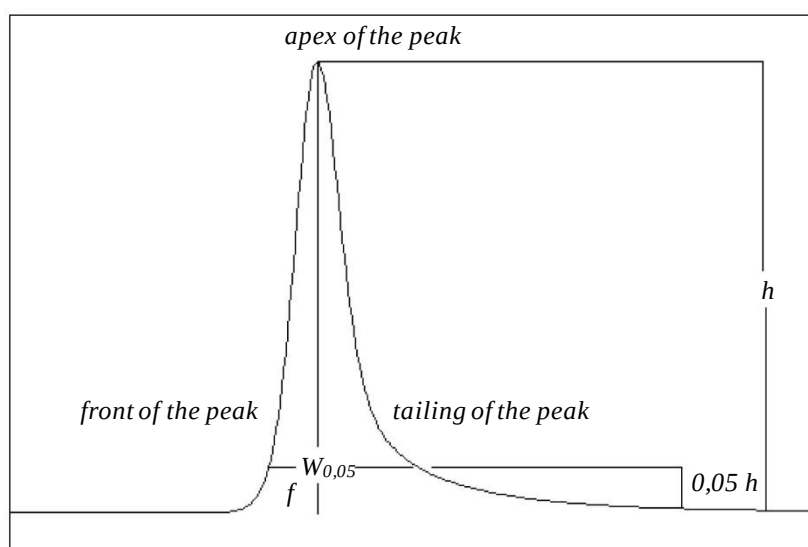


Figure 3 – Chromatogram representing peak asymmetry.

SYSTEM SUITABILITY

System suitability testing is an integral part of liquid chromatography methods. They are applied to verify if the resolution and reproducibility of the chromatographic system are adequate for the analyses to be carried out. The main parameters required to check the suitability of the system are described below.

The resolution, R , is a function of column performance, N , and is specified to ensure that substances eluting in close proximity to each other exhibit satisfactory separation without mutual interference.

Replicates of standard solution injections are statistically processed to verify that the requirements for analysis accuracy have been met. Unless specified in the individual monograph, data from five replicate injections is used to calculate the relative standard deviation (RSD) if the specification is equal to or less than 2.0%. If the specified relative standard deviation is greater than 2.0%, data from six replicates should be used.

The tailing factor, T , which indicates peak symmetry, is equal to 1 for perfectly symmetric peaks and greater than 1 for peaks that show asymmetry. In some cases, values lower than 1 may be observed.

These tests are performed after collecting the results of replicate injections of the standard solution or another solution specified in the individual monograph. The specification of these chromatographic parameters, in a monograph, does not prevent the modification of the analysis conditions. Adjustments in working conditions, in order to reach the parameters of suitability of the system, may be necessary. Unless specified in the individual monograph, system suitability parameters are determined from data obtained with the peak of the substance of interest. System accuracy, demonstrated through replicates of the standard solution, must be achieved prior to injections of sample solutions. The suitability of the system must be verified during the entire chromatographic analysis, by injection of standard solution at appropriate time ranges. When there is a significant change in apparatus or reagent, system suitability tests should be performed prior to sample injections. The analysis will not be valid unless the system suitability test requirements are met.

ADJUSTMENTS OF CHROMATOGRAPHIC CONDITIONS IN ISOCRATIC HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SYSTEMS

The analytical methods presented in this pharmacopoeia have been validated and, in most applications, are fully acceptable in terms of specificity, accuracy, precision, linearity, working range, robustness and, where appropriate, detection and quantification limits.

However, the methods must be checked in their state of validation, considering the formulations being analyzed, as there may be circumstances in which changes may be necessary, aiming to adapt them to specific needs. The acceptable limits for variations in some chromatographic parameters are presented in **Table 1**.

In no case are changes in the detector wavelength, changes in the qualitative composition of the mobile phase, increases in particle size or injection volume allowed.

Table 1 – Acceptable limits of variation for some chromatographic parameters.

<i>Parameter</i>	<i>Acceptable limits</i>
Length of column	± 70%
Column diameter	± 25%
Particle size	Can be reduced by up to 50%

Flow	± 50%
Temperature	± 10°C; without exceeding 60°C
pH	± 0.2 units
Concentration of salt in a buffer	± 10%
Mobile phase composition	±30% relative or ±2% absolute, minority component (whichever is greater). In no case can it exceed 10% absolute.
Injection volume	Reduction is acceptable, as long as the limit of quantification and accuracy of the chromatographic system are verified.

5.2.17.4.1 ION CHROMATOGRAPHY

Ion chromatography refers to the method of separation and determination of ions using high performance liquid chromatography (HPLC). This method is based on a process of separation of sample components into two phases: mobile phase and stationary phase. The separation process is the result of specific interactions between species present in the sample in both phases. The interaction mechanism with the stationary phase is the ion exchange, where the columns used are constituted by a charged functional group, usually SO_3^- , COO^- , NH_3^+ , NR_3^+ bonded to a polymeric matrix such as silica or copolymer of the polystyrene-divinylbenzene type. The mobile phase also contains ionic species, thus occurring a competition among the distribution of species present in the sample between the mobile phase and the stationary phase. For each ion, the exchange process is characterized by the distribution equilibrium between the mobile phase and the stationary phase.

The exchangers used can be classified into strong, medium and weak, depending on the functional group attached to the polymer matrix. Strong ion exchangers are those that completely ionize over a wide pH range, such as the sulfonic and quaternary ammonium group. The degree of dissociation of weak and medium ion exchangers is pH dependent and, therefore, the capacity of these exchangers varies as a function of pH. The carboxylic acid and polyamine group can be mentioned as an example.

This method allows electrical conductivity to be used for the detection and quantitative determination of ions in solution after separation. Generally, ion chromatography with an anion exchange column and conductivity detector can be used for the determination of F^- , Cl^- , Br^- , SO_4^{2-} , PO_4^{3-} , I^- ions, among others. Because electrical conductivity is a property common to all ionic species in solution, the conductivity detector has the ability to monitor all ionic species. The problem that occurs in the use of electrical conductivity to quantify the eluted ionic species can be caused by the high conductivity of the ions present in the mobile phase, mainly due to the sodium ion, making it impossible to quantify other ions. This problem is overcome with the use of an eluent suppressor, positioned after the separation column, where the eluent ions are converted into species that contribute to low or zero conductance. Carbonic acid, resulting from cation exchange, is poorly dissociated, having a low conductivity (baseline conductivity signal is less significant). Thus, the sensitivity for the determination of anions can be significantly increased, by a factor of 10 times or greater, when suppressors are used.

An ion chromatography apparatus basically consists of the same system used for HPLC. This system consists of a high propulsion pump, an injection valve with a suitable sampling loop, a separation column (for anion separation an anion exchange column must be used), a post-column, if necessary, for the conversion of eluent ions in species with lower conductivity and a conductivity detector.

PROCEDURE

To operate the ion chromatograph, it is recommended to follow the manufacturer's instructions. The determinations are carried out by comparison with reference solutions containing known concentrations of the analyte.

Mobile phase: prepare the mobile phase according to the specifications recommended by the manufacturer of the anion exchange column used. It is recommended to use a mobile phase composed of a mixture of carbonate and sodium bicarbonate ($\text{Na}_2\text{CO}_3/\text{NaHCO}_3$), in the concentration range of 1.0 to 4 mmole/L, depending on the column used. Use the mobile phase flow recommended by the equipment manufacturer and according to the ion exchange column used. During analyses using conductivity detection, regenerate the chemical suppression column as recommended by the manufacturer. It is recommended to use 0.005 mole/L H_2SO_4 and subsequent washing with purified water.

Calibration: prepare at least four reference solutions of the element to be determined, covering the concentration range recommended by the equipment manufacturer for the analyte under analysis, and separately inject each reference solution into the apparatus, using an appropriate sampling loop. The use of a 20 μL to 100 μL sampling loop is recommended. Record chromatograms and integrate signals in area or peak height. Then plot the calibration curve. Prepare the sample solution as indicated in the monograph, adjusting its concentration so that it is within the concentration range of the reference solutions. Inject the sample into the chromatograph, record the reading and repeat this sequence three times, taking the average of the three readings. Determine the element concentration from the calibration curve. If the simultaneous determination of several anions is carried out, reference solutions containing all analytes can be made.

5.2.17.5 GAS CHROMATOGRAPHY

Gas chromatography (GC) is a chromatographic separation method based on the difference in species distribution of a mixture into two immiscible phases, in which the mobile phase is a carrier gas that moves through the stationary phase contained in a column. GC is based on the adsorption, mass distribution or size exclusion mechanism. It is applicable to substances and their derivatives that volatilize at the temperatures used, and is used for identification, purity testing and quantitative determination.

When a vaporized constituent is driven by the carrier gas into the column, it is partitioned into the gaseous mobile phase and the stationary phase by a dynamic countercurrent distribution process, showing a greater or lesser retention due to sorption and desorption phenomena on the stationary phase.

EQUIPMENT

The apparatus consists of a carrier gas source and a flow controller, an injection chamber, a chromatographic column contained in an oven, a detector and a data acquisition system (or an integrator or recorder). Carrier gas passes through the column with controlled flow and pressure and goes directly to the detector.

Injector, column and detector are temperature controlled. Chromatography is carried out at constant temperature or using a suitable temperature program. The substances to be chromatographed, both in solution and gases, are injected, coming into contact with the carrier gas in the injection chamber. Depending on the equipment configuration, the mixture to be analyzed must be injected directly into

the column or must be vaporized in the injection chamber and mixed with the carrier gas before entering the column.

Once in the column, the constituents of the mixture are separated according to their different linear retention indices, which are dependent on the vapor pressure and the degree of interaction with the stationary phase. The retention index, which defines the resolution, retention time and column performance in relation to the components of the mixture, is also temperature-dependent. The use of temperature programs for the oven where the column is located has an advantage in the separation performance of compounds that behave differently in vapor pressure.

The compounds leave the column separately, passing through a detector, which provides a response related to the amount of each compound present. The type of detector to be used depends on the nature of the substances to be analyzed and is specified in each monograph. Detectors are heated to prevent condensation of eluted compounds. Detector output is given as a function of retention time, generating a chromatogram, which consists of a series of peaks on the time axis. Each peak represents a substance from the vaporized mixture, although some peaks may overlap. The elution time is characteristic of an individual substance and the instrument response, measured as peak area or peak height, is a function of the amount present.

Injectors

Direct injections of solutions is the usual mode of injection, unless otherwise indicated in the monograph. Injection can be carried out directly at the head of the column using a syringe or an injection valve, or in a vaporization chamber which can be equipped with a flow divider. The amount of sample that can be injected into a capillary column without saturating it is lower compared to the amount that can be injected into packed columns. Capillary columns, therefore, are often used with injectors capable of dividing the sample into two fractions (*split* mode), a smaller one, which enters the column, and a larger one, which is discarded. These injectors can be used without sample splitter (*splitless* mode) for component analysis in smaller quantities or in traces.

Vapor phase injections can be carried out by a static or dynamic confined space (*headspace*) injection system.

Static (*purge and trap*) confined space injection system (*headspace*) includes a concentration device, through which volatile substances from the solution are dragged to an adsorbent column, kept at low temperature, where they are adsorbed. The trapped substances are then desorbed in the mobile phase by rapid heating of the adsorbent column.

Dynamic confined space injection system (*headspace*) includes a thermostatically controlled sample heating chamber in which closed flasks (*vials*) containing solid or liquid samples are placed for a specified period of time so that volatile components of the samples reach equilibrium between the non-gas phase and the vapor phase. Once equilibrium is established, a predetermined amount of the vial confined space is injected into the chromatograph.

Stationary phases

Stationary phases are contained in columns, which can be:

- a fused silica capillary column whose wall is coated with the stationary phase;
- a column packed with inert particles impregnated with the stationary phase;
- a column packed with the solid stationary phase.

Capillary columns, usually made of fused silica, have an internal diameter ($\varnothing$) of 0.10 mm to 0.53 mm and a length of 5 m to 60 m. The liquid or stationary phase, which can be chemically bonded to the inner surface, is a film from 0.1 μm to 5.0 μm in thickness, although non-polar stationary phases can reach 5 μm in thickness.

The packed columns, made of glass or metal, have a length of 1 m to 3 m, with an internal diameter ($\varnothing$) of 2 mm to 4 mm. Stationary phases generally consist of porous polymers or solid supports impregnated with the liquid phase reaching approximately 5% (w/w). High-capacity columns, with the liquid phase reaching approximately 20% (w/w), are used for a wide range of substances and for the determination of substances with low molecular weight, such as water. The required capacity influences the choice of the solid support.

Supports for analysis of polar compounds in columns packed with a stationary phase of low polarity and low capacity must be inert to avoid excessive peak elongation. The reactivity of support materials can be reduced by silanization before filling with the liquid phase. Acid washed and calcined diatomaceous earth is generally used. The materials are available in a variety of particle sizes, with the most commonly used particles being 150 μm to 180 μm (80 mesh to 100 mesh) and 125 μm to 150 μm (100 mesh to 120 mesh).

Mobile phases

Carrier gas supply can be obtained from a high pressure cylinder or a high purity gas generator. In both cases, the gas passes through a pressure reducing valve and the flow is metered to then enter the injection chamber and column. Retention time and peak performance depend on carrier gas quality; retention time is directly proportional to column length and resolution is proportional to the square root of the column length. For packed columns, the average carrier gas flow is usually expressed in milliliters per minute at atmospheric pressure and room temperature. Average flow is measured at the detector outlet, either with a calibrated mechanical device or with a “bubbling” tube while the column is at operating temperature. The linear velocity of the carrier gas through the packed column is inversely proportional to the square root of the column inner diameter for a given flow volume. Flows of 60 mL/minute in a 2 mm ID column and 15 mL/minute in a 1 mm ID column provide identical linear velocities and therefore similar retention times. Unless specified in the monograph, the average flow rate for packed columns is approximately 30 to 60 mL/minute. For capillary columns, linear flow velocity is usually used instead of mean flow. This is determined from the column length and retention time of a diluted methane sample using a flame ionization detector. Operating at high temperatures, there is sufficient vapor pressure for a gradual loss of the liquid phase, a process called bleeding, to occur.

Helium or nitrogen are generally used as carrier gases for packed columns, whereas nitrogen, helium and hydrogen are used for capillary columns.

Detectors

Flame ionization detectors are the most used, but depending on the purpose of the analysis, other detectors can be used, including: thermal conductivity, electron capture, nitrogen-phosphorus, mass spectrometry, Fourier transform infrared spectrophotometry, among others. For quantitative analyses, detectors must have a wide linear dynamic range: the response must be directly proportional to the amount of substance present in the detector over a wide range of concentrations. Flame ionization detectors have a wide linear range and are sensitive to most compounds. The response of the detectors depends on the structure and concentration of the substance and the average flow of combustion, air and carrier gas. Unless otherwise specified in the monograph, flame ionization detectors operate with

either helium or nitrogen as the carrier gas for packed columns, and with helium or hydrogen for capillary columns.

Thermal conductivity detectors employ heated metal wire located in the carrier gas stream. When an analyte enters the detector with the carrier gas, the difference in the thermal conductivity of the carrier gas stream (sample gas and components), relative to a reference flow of the carrier gas without analyte, is measured. In general, thermal conductivity detectors respond uniformly to volatile substances regardless of their structure; however, they are considered less sensitive than the flame ionization detector.

Alkaline flame ionization detectors, also called NP or nitrogen-phosphorus detector, contain a thermionic source, with an alkali metal salt or glass element containing rubidium or another metal, which results in efficient ionization of organic nitrogen and phosphorus-containing substances. It is a selective detector that has a low response to hydrocarbons.

Electron capture detectors contain a radioactive source of ionizing radiation. They exhibit an extremely high response to halogenated compounds and a nitro group, but low response to hydrocarbons. Sensitivity increases with the number and atomic mass of halogen atoms.

Data processing devices

Data processing stations connected to the output of the detectors calculate the area and height of the peaks, and present the complete chromatograms containing the parameters from the run and the peak data. The chromatogram data can be stored and reprocessed by electronic integration or any other type of calculation as required. These data processing stations are also used to program chromatographic runs.

PROCEDURE

Packed columns and capillaries must be conditioned prior to use until the baseline is stable. This must be accomplished by operating at a temperature above that specified by the method or by repeated injections of the compound or mixture to be chromatographed. The column manufacturer usually provides instructions for the proper column conditioning procedure. In case of thermally stable methyl and phenyl substituted polysiloxanes, a special sequence increases performance and inactivity: keep the column at 250°C for one hour, with helium gas flow, to remove oxygen and solvent. For helium flow, heat to 340°C for four hours, then reduce heat to 250°C, and condition with helium flow until baseline stability.

After the conditioning procedure, equilibrate the column, injector and detector to the temperatures and gas flow specified in the monograph until a stable baseline is obtained. Prepare sample and reference solution(s) as described. Solutions must be free of solid particles.

Many drugs are polar reactive molecules. In that case, conversion to less polar and more volatile derivatives may be necessary by treating the reactive groups with appropriate reagents.

Assays require quantitative comparison of one chromatogram with another. The biggest source of error is the irreproducibility of the amount of sample injected, especially when manual injections are performed with the aid of a syringe. Variability effects can be minimized by adding an internal standard, a non-interfering substance added at the same concentration in the sample and standard solutions. The ratio of the analyte peak response to the internal standard peak response is compared between the sample and standard chromatograms. When the internal standard is chemically similar to the substance being analyzed, there is also a compensation for minor variations in column and

detector characteristics. In some cases, the internal standard can be used during sample preparation, prior to chromatographic analysis, to control other quantitative aspects of the assay. Automatic injectors increase the reproducibility of sample injections and reduce the need to use internal standards.

5.2.17.5.1 GAS CHROMATOGRAPHY IN CONFINED SPACE (*headspace*)

Confined space gas chromatography (*headspace*) is a particularly suitable method for the separation and determination of volatile compounds present in solid and liquid samples. This method is based on the analysis of a vapor phase in equilibrium with a solid or liquid phase.

APPARATUS

The apparatus consists of a gas chromatograph to which a device for introducing the sample is fitted, which can be connected to a programming module that automatically controls pressure and temperature. If necessary, a solvent removal device can be fitted. The sample to be analyzed is introduced into a flask equipped with a suitable shutter, which closes it, and with a valve system, which allows the entry of a carrier gas. The vial is placed in a thermostatic chamber at a certain temperature for the sample to be examined. The sample is left at this temperature long enough to allow for the balance between the solid phase and the gas phase. The carrier gas is introduced into the flask and, after a certain time, a valve is opened to allow the gas to expand into the chromatographic column, dragging out the volatile components.

Instead of using a specially adapted chromatograph for introducing samples, hermetic syringes and a conventional chromatograph can also be used. In this case, the equilibrium between the two phases is conducted in a separate chamber and the vapor phase is transferred to the column, taking the necessary precautions to avoid any modification of the equilibrium.

PROCEDURE

Adjust the apparatus working conditions in order to obtain a satisfactory response, using the reference solutions.

Direct calibration

Introduce, separately, in identical vials, the preparation to be examined and each of the reference solutions, according to the conditions described in the monograph and avoiding contact between the sample and the injection device. Hermetically close the flasks and place them in the thermostatic chamber at the temperature and pressure described in the monograph. After reaching equilibrium, proceed with the chromatographic analysis under the conditions described.

Standard addition

Add equal volumes of the solution to be examined to a series of identical vials. Add to all but one of the vials increasing amounts of a reference solution of known concentration of the substance to be examined. A series of preparations containing increasing amounts of a certain substance is then obtained. Hermetically close the vials and place them in the thermostatic chamber at the temperature and pressure described in the monograph. After reaching equilibrium, proceed with the chromatographic analysis under the conditions described.

Calculate the formula of the straight line by linear regression, using the method of least squares and, from it, obtain the concentration of the substance under examination in the sample preparation, indicated by the formula intercept.

5.2.18 POLAROGRAPHY

Polarography, an electrochemical analytical method, is based on measuring the electrical current resulting from the electrolysis of electroactive substances (reducible or oxidizable) under certain electrode potential and controlled conditions. In other words, the method involves recording the increase in the current in a polarizable electrode, during the electrolysis of a substance dissolved in the electrolytic medium, due to the increase in voltage applied to the system. The graph of this current evolution in relation to voltage – the polarogram – provides qualitative and quantitative information about electro-reducible or electro-oxidizable constituents of the sample.

Among the variants of the polarographic method, the simplest is the direct current method. It requires, like potentiometry, the use of two electrodes, the reference (usually saturated calomel electrode, SCE) and the indicator microelectrode (usually dropping mercury electrode, DME). In some cases, a third auxiliary electrode is used. The SCE – with a high surface area – provides constant potential during the test, while the DME – drops of mercury of reproducible dimensions flowing periodically from the end of a capillary connected to the metal reservoir – assumes the potential supplied to it by the external source. The polarographic apparatus comprises, in addition to the electrodes, the polarographic cell (electrolysis tank), variable power supply, equipped with a voltmeter and microammeter (galvanometer) and a graphic or digital recorder.

In simple terms, the method consists of dissolving the sample (the method is sensitive to concentrations of electroactive species in the range of 10^{-2} to 10^{-4} M) in a supporting electrolyte, responsible for maintaining a small residual current, but which is inert in the sample transformation potential range (electrochemical potential window). Initially, without applying voltage to the source (precision potentiostat), the voltage supplied to the microelectrode is null and there will be no indication of current in the microammeter. The increasing voltage rise will cause small potential to reach the electrodes. Under this voltage, which is still reduced, eventual impurities of the supporting electrolyte and small oxygen concentrations may suffer a reduction in the DME (cathode, in this case), reducing and causing the indication of a small current passage. The progressive increase in the applied voltage will accentuate the reduction process and the almost proportional increase in current. Finally, the necessary potential for the reduction of the analyte in the sample solution is reached, which is reflected in a sharp rise in the current read in the microammeter (galvanometer) and recorded in the polarogram. There is, however, a limit to the proportionality of the voltage-current rise. As the current rises (and the reduction proceeds), there is a progressive decrease in the concentration of the original electroactive species near the electrode surface. At a given moment - the speed of electrolysis being constant - such concentration reaches an insufficient level to allow additional current elevation and the latter becomes limited by the diffusion with which the electroactive species manages to diffuse into the interior of the electrolyte solution to the surface of the DME. The plateau observed in the polarogram appears (**Figure 1**), with the measured current – then called diffusion current – being a parameter proportional to the concentration of electroactive species in the sample (quantitative aspect of the polarography). Once a certain voltage level is exceeded, the current rises again. This increase is caused by the reaction of the supporting electrolyte. Its presence, in high concentrations, prevents the electroactive molecules in the sample from reaching the microelectrode by electrical migration and, therefore, ensures that the limit current is effectively regulated only by diffusion.

When using a dropping mercury microelectrode, the surface of the electrode is constantly renewed (a new drop is formed every three to five seconds), then there is a variation in the measured current within a given range; the current is lower when the drop forms, reaching its maximum at the moment of the drop. The phenomenon explains the “sawtooth” shape characteristic of the polarographic wave.

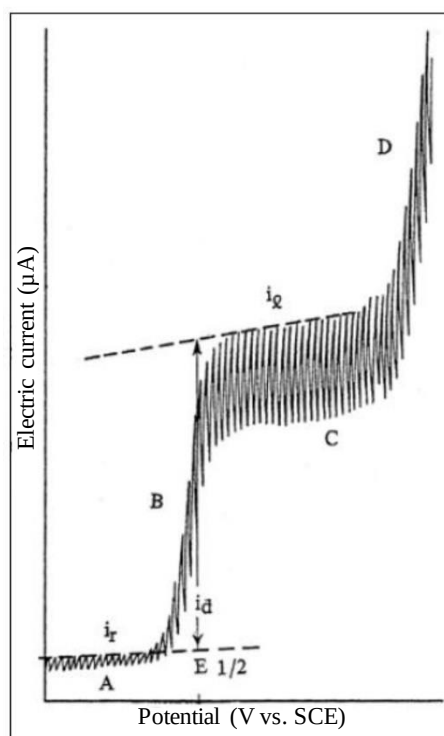


Figure 1- Electroreducible species polarogram.

POLAROGRAM

A typical polarogram (DME) is illustrated in **Figure 1**, characterized by 4 distinct phases. The A segment is due to the capacitive current, i_c , incorporated into the faradaic current, i_f , resulting from the oxidation or reduction of impurities in the supporting electrolyte, or in the sample and small concentrations of oxygen, when that is not completely removed from the solution. The set of these currents is called the residual current, i_r ($i_r = i_c + i_f$). In segment B of the polarogram, the faradaic current, i_f , occurs due to the conversion of the substance under test. Electrodecomposition leads to scarcity of this substance at the microelectrode, verifying the plateau (segment C) where the limit current, i_l , appears. This comprises the sum of the residual and diffusion currents ($i_l = i_r + i_d$), in which the diffusion current – proportional to the concentration of the electroactive species in the sample – has its value determined by:

$$i_d = i_l - i_r$$

Two other undesirable currents – migration and convection – can incorporate the limiting current. The first is suppressed by the use of an inert support electrolyte in the potential range used, in concentrations at least 100 times greater than those of the electroactive species. The convection current, in turn, is eliminated by not shaking the solution.

Finally, the D segment of the polarogram, in which there is a reversal of the voltage-current proportionality, corresponds to the reduction of other electroactive species, when present, or, more frequently, to the electrolysis of the support.

Ilkovic equation

Ilkovic equation establishes relations between variables included in the polarographic measurement and the DME diffusion current:

$$i_d = 708nD \frac{1}{2} C m \frac{2}{3} t \frac{1}{6}$$

where

i_d = diffusion current, in μA ;

708 = constant dependent on several parameters, including the unit adopted for the variables, dimension of the mercury drop and the instant measurement of i_d ;

n = number of electrons necessary for the reduction or oxidation of an electroactive substance molecule or ion;

D = diffusion coefficient, in cm^2/s ;

C = concentration of electroactive substance, in millimoles/L;

m = mass of mercury flow, in mg/s ;

t = drop lifetime, in s.

The constant 708 – encompassing the natural constant and the faraday value – is established for operation at 25 °C and is applicable to sampled direct current polarography, in which, instead of the continuous current record, only the current is read at the end of the life of the mercury drop, allowing to obtain a linear polarogram. However, when using instruments equipped with a “sawtooth” damper on the recorder, the average current of the pulses is considered. The diffusion current obtained according to the Ilkovic equation becomes the average for the entire life of the mercury drop. In this case the constant acquires the value 607.

The variables included in the Ilkovic equation must be controlled so that the diffusion current is effectively proportional to the concentration of electroactive species in the analyzed sample. Some ions and organic molecules in aqueous solution modify their diffusion coefficient at a rate of 1% to 2% for each degree Celsius increased, making it necessary for the polarographic cell to have its temperature controlled with a tolerance of ± 0.5 °C. The parameters m and t , related to the dimension and speed of renewal of the mercury drop, depend on the capillary geometry, the diffusion current being proportional to the square root of the height of the mercury column. Appropriate heights, measuring from the end of the capillary to the level of mercury in the reservoir – are between 40 cm and 80 cm. The internal diameter of the capillary in this case is 0.04 mm for lengths between 6 cm and 15 cm. The exact height of the capillary is adjusted to allow a drop to be formed every three to five seconds, with an open circuit and the capillary immersed in the electrolyte under test. Thus, if during a particular test, all parameters – with the exception of the concentration of the electroactive species – are kept constant, the Ilkovic equation can be written as

$$i_d = KC$$

where K represents the set of variables held constant.

This direct relation between diffusion current and concentration is usually adopted by previously determining the diffusion current of a reference standard solution of known concentration. Then, under identical conditions, the diffusion current of the sample is determined and, finally, its concentration:

$$\frac{(i_d)_P}{(i_d)_A} = \frac{C_P}{C_A}$$

where P and A correspond, respectively, to standard and sample.

Since most polarographs are equipped with automatic recorders, it is easier to graphically determine diffusion currents by measuring the height of the polarographic wave (see **Figure 1**). The values recorded, in cm, can be directly applied to the formula, without the need to convert them into units of electrical current:

$$\frac{A_P}{A_A} = \frac{C_P}{C_A}$$

where A_P and A_A correspond to the heights of the polarographic waves of the standard and the sample, respectively.

Half-wave potential

Measurement of the height of the polarographic wave for the purposes of quantitative analysis must be carried out by plotting straight lines close to the peaks of the oscillations of the residual current and the limit current and joining, by means of a third straight line parallel to the axis of the abscissa, the extensions of the first two. The vertical line is plotted passing through the inflection point of the polarographic wave, corresponding to half the distance between the residual current and the limit current ($I = I / 2i_a$). The projection of this line onto the ordinate axis provides the so-called half-wave potential, a parameter used to characterize electroactive substances (qualitative aspect of polarography). The half-wave potential, $E_{1/2}$, is given in volts *versus* SCE (reference electrode), unless otherwise specified, and its value as an identification parameter derives from its independence from concentration and DME characteristics. However, this parameter varies depending on the composition, pH and temperature of the electrolytic medium. It is noteworthy that for modern equipment the measurement of the height of the polarographic wave can be done automatically using specific programs for data acquisition and processing.

Oxygen removal

Oxygen is reduced in DME in two steps, first converting to hydrogen peroxide and then to water. The fact that such reactions occur at potentials more negative than zero volts, *versus* SCE, and may thus interfere with the polarographic wave of the sample, requires the elimination of the gas dissolved in the solution prior to determination. The best method is to bubble oxygen-free nitrogen through the solution for a period of 10 to 15 minutes immediately before the test, taking the precaution of previously saturating the nitrogen (to avoid changes in the electrolyte solution due to evaporation) by bubbling through small volume of electrolyte solution in a separate container.

It is important to keep the electrolytic tank still and vibration-free during the polarographic recording in order to avoid the formation of convection currents. As a result, it is necessary to remove the nitrogen tube from the solution during recording, and place the tube on the surface of the solution to fill the upper part of the polarographic cell with nitrogen ($N_{2(g)}$) thus preventing the entry of air in the polarographic cell. Alkaline solutions can be deoxygenated by the addition of sodium bisulfite, as long as it does not interact with constituents of the electrolyte solution.

Polarographic maxima

After the reduction of the electroactive species (cathodized DME), the polarographic wave often rises sharply, often before falling, equally sharply, to the value of the limit current. The phenomenon is called polarographic maxima and the corresponding current is called adsorption current (i_a). It has the inconvenience of making it difficult to measure the polarographic wave (diffusion current) and its causes – still poorly understood – comprise the adsorption of electrolyte to the surface of the mercury drop. The elimination of the polarographic maxima is, however, easily accomplished by

adding diminutive amounts of certain surfactants (maxima suppressors) to the electrolytic medium. For this purpose, the use of gelatin solution at 0.005% (w/v) and methyl red solution at 0.01% (w/v), among others, stand out.

Warning

Mercury vapors are toxic. When handling metal, work in a ventilated area and avoid spills which, if they occur, must be immediately and carefully collected.

PULSE POLAROGRAPHY

Pulse polarography is a variant of the method, superior, due to its precision and sensitivity, to direct current polarography in the assay and identification of a large number of substances at low concentrations, including trace level elements, metabolites and, of course, drugs. Its sensitivity, about 10 times higher than that of DC polarography, allows for determinations in the order of $10^{-6} M$.

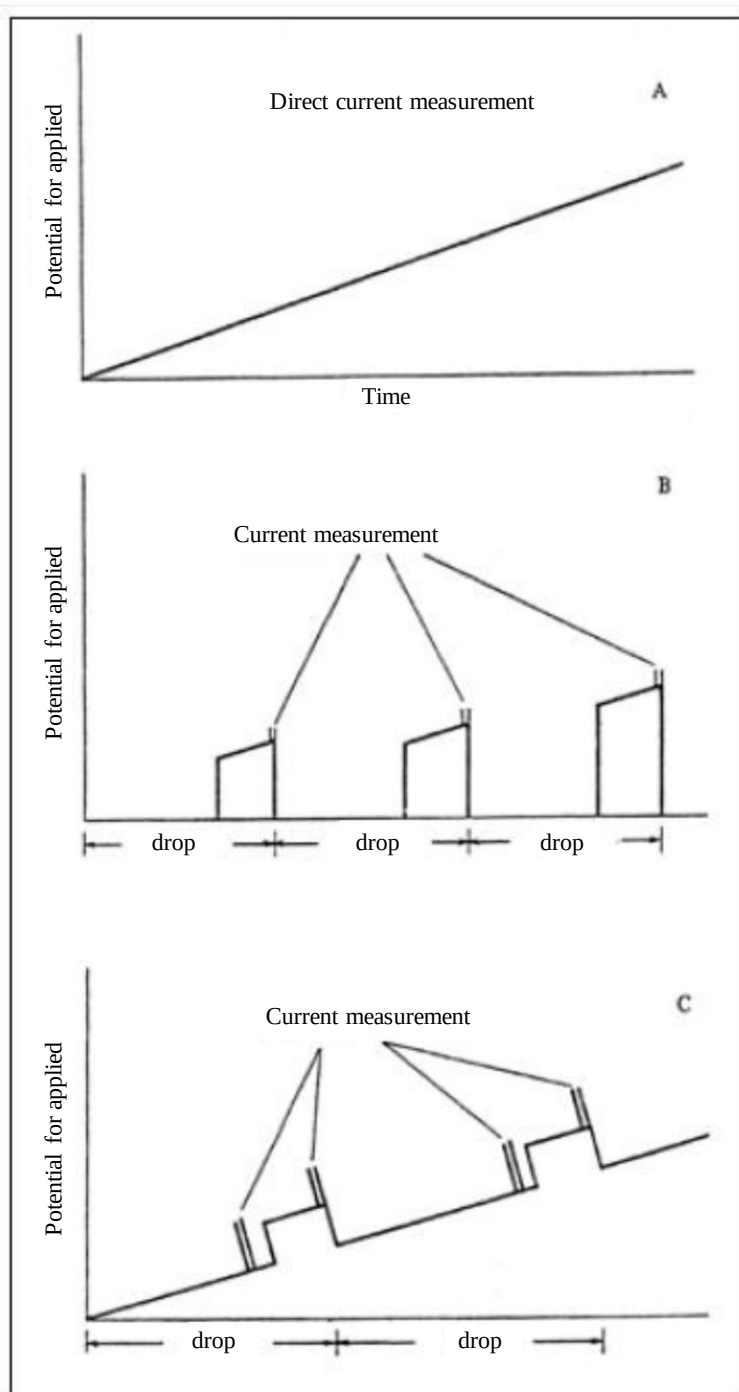


Figure 2 – Measure of current over time in direct current polarography (A); in pulse polarography (B); and in differential pulse polarography (C)

Instead of the linearly progressive application of potential and direct measure of the developed current, pulse polarography comprises the application of increasing potential pulses to the DME, coinciding with the final period of life of the mercury drops, each pulse presenting a potential slightly higher than the previous one. The current, in turn, is sampled at the final instant of the duration of the potential pulse, a period in which the capacitive current reaches practically zero and the residual current is composed almost exclusively of the diffusion current.

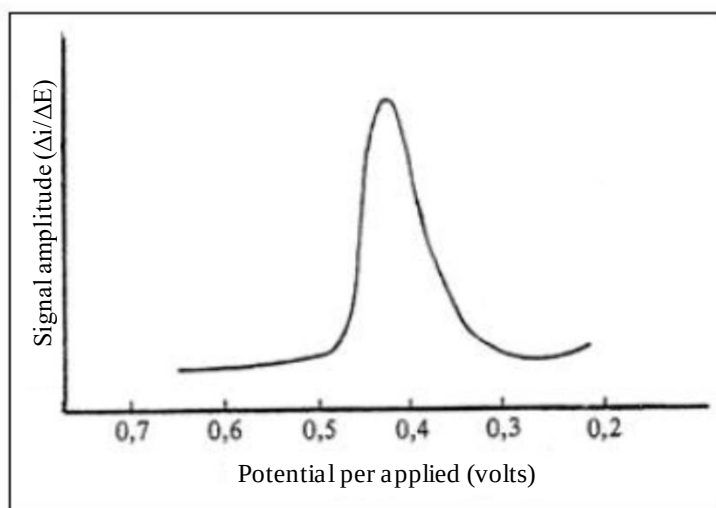


Figure 3 Polarogram obtained in differential pulse polarography.

On the other hand, the pulse method does not cause an accelerated reduction in the diffusion layer (concentration of electroactive species close to the electrode), allowing higher diffusion currents for equivalent concentrations. Hence the increased sensitivity inherent in the method. Another favorable aspect of pulse polarography is that it is easier to measure the limit current, free from oscillations, unlike what occurs in direct current polarography.

In differential pulse polarography, small-amplitude, constant pulses are superimposed on a linearly increasing voltage potential ramp. The current measurement is performed twice for each pulse – immediately before the application of the pulse and, again, at its final moment – recording only the difference between the two measured values (**Figure 2**). The graphic registration of this differential measurement system provides a curve similar to the polarographic wave derivative, showing a characteristic peak (**Figure 3**). The polarographic peak potential corresponds to $E_{1/2} - \Delta E/2$ where ΔE represents the height of the peak. Thanks to the nature of the polarogram, which has peaks rather than traditional polarographic waves, differential pulse polarography provides higher resolution, to the extent of allowing simultaneous determinations of electroactive species with close half-wave potentials in concentrations in the order of $10^{-7} M$.

5.2.19 DETERMINATION OF pH

POTENTIOMETRIC DETERMINATION OF pH

The pH value is defined as the measure of the hydrogen ion activity of a solution. The scale of the hydrogen ion concentration of the solution is conventionally used. Water is an extremely weak electrolyte, whose self-ionization produces hydronium ion (hydrated hydrogen) and hydroxide ion:



Hydronium ion concentrations in aqueous solutions can vary within wide limits, which experimentally range from 1 to 10^{-14} M, which is defined by the simplified relation:

$$\text{pH} = -\log [\text{H}_3\text{O}^+] = \log 1/[\text{H}_3\text{O}^+],$$

Thus, the pH scale is an inverted scale in relation to the hydronium ion concentrations, that is, the lower the hydronium ion concentration, the higher the pH value.

The potentiometric determination of pH is made by measuring the potential difference between two suitable electrodes immersed in the solution under examination. One of these electrodes is sensitive to hydrogen ions and the other is the constant potential reference electrode.

The formula that expresses the potentiometric measure of a cell is:

$$\text{pH} = \text{pH}_t = (\text{E} - \text{E}_t) / K,$$

where

E = potential measured when the cell contains the sample solution;

E_t = potential measured when the cell contains the buffer solution;

pH = pH value in the sample solution;

pH_t = pH value in the buffer solution;

K = change in potential per unit of change in pH – theoretically equivalent to $0.0591631 + 0.000198(t-25)$, where t corresponds to the temperature at which it operates.

The pH value is expressed by the formula in relation to the pH of the standard solution (pH_p) and determined in a pH meter using a glass electrode.

The devices commercially used for the determination of pH are potentiometric instruments, equipped with electronic current amplifiers with a glass-calomel cell, which are capable of reproducing values corresponding to 0.02 pH units. The pH scale is calibrated not only in millivolts, but also in corresponding pH units. Thus, there is no need to apply the formula above, which translates the electrometric pH measurement. As measurements of hydrogen ionic activity are sensitive to temperature variations, all pH meters are equipped with electronic temperature adjustment.

Buffer solutions for pH meter calibration

They are used for measuring the device, allowing linearity in the responses in relation to the observed potential changes. The most important are: 0.05 M potassium tetraoxalate, 0.05 M equimolar phosphate, 0.01 M sodium tetraborate, sodium carbonate and saturated calcium hydroxide at 25°C.

Buffer solutions are prepared as follows:

Potassium tetraoxalate, 0.05 M – Reduce potassium tetraoxalate to fine powder and desiccate in a silica desiccator. Dissolve exactly 12.71g of $\text{KH}_3(\text{C}_2\text{O}_4) \cdot 2\text{H}_2\text{O}$ in water. Adjust volume to 1000 mL with water and homogenize.

Potassium Biphthalate, 0.05M – Reduce potassium biphthalate to fine powder and dry at 110°C to constant weight. Dissolve exactly 10.21g of $\text{KHC}_8\text{H}_4\text{O}_4$, previously desiccated at 100°C for one hour, in water. Adjust volume to 1000 mL with water and homogenize.

Equimolar Phosphate, 0.05M – Reduce the Na_2HPO_4 and KH_2PO_4 to fine powder and dry at 110°C to constant weight. Dissolve 3.55g of Na_2HPO_4 and 3,40g of KH_2PO_4 in water. Adjust volume to 1000 mL with water and homogenize.

Sodium tetraborate, 0.01M – Desiccate the sodium tetraborate in a desiccator containing an aqueous solution of sodium bromide to constant weight. Dissolve 3.81g of $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, in water Adjust volume to 1000 mL with water and homogenize. Avoid carbon dioxide absorption.

Calcium hydroxide, saturated at 25°C – Reduce calcium hydroxide to fine powder and desiccate with silica in desiccator to constant weight. Transfer 5 g to volumetric flask and add water up to 1000 mL. Shake well and keep at a temperature of $(25 \pm 2)^\circ\text{C}$, for adequate saturation (approximately 0.02 M). Decant at 25°C before use. Protect to avoid carbon dioxide absorption.

Sodium carbonate – Desiccate the sodium carbonate in a desiccator with silica gel until constant weight. Weigh exactly 2.10 g. Dry the flask in an oven at 300°C to 500 °C until constant weight. Weigh 2.65g. Dissolve both samples in water. Transfer each solution to a 1000 mL volumetric flask, adjust volume with water and homogenize.

Such solutions must be freshly prepared with carbon dioxide-free water and used within three months, taking care to avoid the growth of fungi and bacteria. Preservatives are accepted as long as they do not interfere with the potentiometric PH measurement.

The water used to prepare the solutions must be freshly distilled, heated to boiling for at least 15 minutes, cooled and kept in a container impermeable to carbon dioxide. Prepare the six standard solutions individually and store them in suitable glass or polyethylene flasks. Observe the shelf life of the solutions, as the pH changes over time.

Table 1 – Relation between temperatures and pH values of buffer solutions for pH meter calibration.

<i>Temperature (°C)</i>	<i>Potassium tetraoxalate 0.05M</i>	<i>Potassium Biphthalate 0.05M</i>	<i>Equimolar phosphate</i>	<i>Sodium tetraborate 0,01M</i>	<i>Saturated calcium hydroxide at 25°C</i>	<i>Sodium carbonate</i>
10	1.67	4.00	6.92	9.33	13.00	10.18
15	1.67	4.00	6.90	9.27	12.81	10.12
20	1.68	4.00	6.88	9.22	12.63	10.07
25	1.68	4.01	6.86	9.18	12.45	10.02
30	1.68	4.01	6.85	9.14	12.30	9.97
35	1.69	4.02	6.84	9.10	12.14	9.93
40	1.70	4.03	6.84	9.07	11.99	-

PROCEDURE

pH meter gauge

Remove the beaker containing KCl solution in which the electrode is immersed when the meter is not in use;

Wash the electrode with jets of distilled water and dry with filter paper;

Immerse the electrode in a reference buffer solution, checking the temperature at which it will operate;

Adjust the pH value to the tabulated value using the calibration value;

Wash the electrode with several portions of the second reference buffer solution, immerse the electrode and check the pH value recorded. The pH value must not vary by more than 0.07 from the tabulated value for the second standard solution. There are devices that have flasks coupled with anionic detergents, used as washing solutions between each of the pH measurement operations. Water also lends itself to this function;

If the measurements are not accurate, check for possible damage to the electrodes and replace them.

Determination of pH in the sample solution

After the convenient measurement, wash the electrode with water (or proper solutions) and with several portions of the sample solution. For sample dilution, use carbon dioxide-free distilled water;

The first determination provides a variable value, requiring further readings. The values found later should not vary by more than 0.05 pH unit on three serial readings;

For determinations that require high precision, the temperatures of the buffer and sample solutions, of the electrodes and of the washing waters must not differ by more than 2°C from each other. Thus, to reduce the effects of thermal or electrical hysteresis on the electrodes, the solutions must be at the same temperature for minimum 30 minutes before starting the operation;

It is important that, after using the device, the electrode is kept in an appropriate solution, usually KCl.

Contamination of stock solutions must be avoided by adopting systematic procedures, such as the immediate closing of the vials containing the solutions, in order to prevent accidental introduction of pipettes or sticks, and the use of individual pipettes for each solution.

COLORIMETRIC DETERMINATION OF pH

It is based on the use of test solutions or indicator papers, which have the property of changing color as the pH changes. In this case, it is an approximate measure, indicating only a range of values, more or less wide, depending on the indicator used. The determination is carried out by adding drops of the indicator solution to the solution under examination or by moistening indicator papers with the solution under examination and observing the change in color. The colors produced by indicators in various pH ranges are listed in *Indicators and test solutions (7.1)*

ACIDITY AND ALKALINITY: QUICK TESTS

A solution is considered neutral when it does not change the color of the litmus blue and red papers, or when the universal indicator paper takes on the colors of the neutral scale, or when 1 mL of the same solution is colored green with a drop of bromothymol blue TS (pH 7.0).

It is considered *acidic* when the blue litmus paper is stained red or 1 mL is stained yellow by a drop of phenol red TS (pH 1.0 to 6.6).

It is considered *weakly acidic* when the blue litmus paper is slightly stained red or 1 mL is stained orange by a drop of methyl red TS (pH 4.0 to 6.6).

It is considered *strongly acidic* when the Congo red paper is stained blue or 1 mL is stained red by the addition of a drop of methyl orange TS (pH 1.0 to 4.0).

It is considered *alkaline* when the red litmus paper is stained blue or 1 mL is stained blue by a drop of bromothymol blue TS (pH 7.6 to 13.0).

It is considered *weakly alkaline* when the red litmus paper is stained blue or 1 mL is stained pink by a drop of cresol red TS (pH 7.6 to 8.8).

It is considered *strongly alkaline* when stained blue by a drop of thymolphthalein TS (pH 9.3 to 10.5) or red by a drop of phenolphthalein TS (pH 10.0 to 13.0).

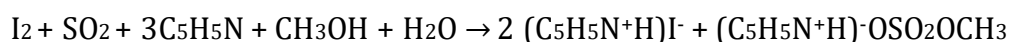
5.2.20 DETERMINATION OF WATER

Many substances are in the form of hydrate or contain adsorbed water, so it is important to determine them by specific methods.

Depending on the nature of the substance, the individual monograph will specify one of the methods described below.

5.2.20.1 VOLUMETRIC METHOD (KARL FISCHER METHOD)

The volumetric determination of water is based on the quantitative reaction of water with an anhydrous solution of sulfur dioxide and iodine in the presence of a buffering solution, which reacts with hydrogen ions, according to the following reaction:

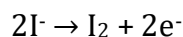


In the original volumetric solution, known as Karl Fischer Reagent, sulfur dioxide and iodine are usually dissolved in pyridine and methyl alcohol, and other solvents and/or bases may be used, thus requiring checking the stoichiometry and the absence of interferences. For this purpose, commercial reagents can be used, considering the manufacturer's recommendations.

There are two different methods based on the reaction with iodine: volumetric titration and coulometric titration.

In the first method, iodine is dissolved in the reagent and the water content is determined by measuring the amount of iodine consumed as a result of the reaction with water. The test sample can be titrated directly with the reagent or the analysis can be performed using an indirect titration procedure. The reaction stoichiometry is not accurate and the reproducibility of the determination depends on factors such as the relative concentrations of the reagent components, the nature of the inert solvent used to dissolve the test sample, and the method used in the determination. Therefore, it is necessary to standardize the method beforehand to achieve adequate accuracy. The accuracy of the method depends on the effectiveness of removing atmospheric moisture from the system.

In coulometric titration, iodine is produced by the electrolysis of a Karl Fischer reagent that contains the iodide ion. The water content in a sample can be determined by measuring the amount of electricity required to produce iodine during titration.



DIRECT VOLUMETRIC METHOD

Apparatus

Knowing that Karl Fischer reagent is highly hygroscopic, the apparatus must ensure an exclusion of atmospheric moisture. The endpoint determination must be adequate. In the direct titration of a colorless solution, the endpoint can be visually observed with a color change from deep yellow to amber. The inverse is observed when carrying out a backtitration (indirect) of a test sample. However, more commonly, the endpoint is determined by a voltametric method using an apparatus with a simple electrical circuit that generates an applied potential of approximately 200 mV between a pair of platinum electrodes submerged in the solution containing the sample that will be titrated. At the end

of the assay, a slight excess of the reagent increases the current flow between 50 μA and 150 μA for a period of 30 seconds to 30 minutes, depending on the solution being titrated. This period is shorter for substances that dissolve in the reagent. In some automatic volumetric titrators, the abrupt change in current or potential at the endpoint causes a valve to be closed by a solenoid that controls the burette that delivers the volumetric solution. Commercially available apparatus generally comprise a closed system, consisting of one or two automatic burettes and a hermetically closed titration vessel, equipped with the required electrodes and a magnetic stirrer. The air in the system is kept dry with a suitable desiccant, e.g. anhydrous calcium chloride or silica gel, and the titration flask can be purged by means of a stream of dry nitrogen or dry air.

Reagent

Karl Fisher reagent can be prepared by any of the methods listed below.

Note: Chloroform and methyl alcohol used for reagent preparation must have a water content inferior to 0.1 mg/mL. Methoxyethanol and diethylene glycol monomethyl ether must have a water content lower than 0.3 mg/mL.

Method a – Add 125 g of iodine to a solution containing 670 mL of methyl alcohol and 170 mL of pyridine, and cool. Place 100 mL of pyridine in a 250 mL graduated cylinder and, keeping the pyridine cold in an ice bath, introduce dry sulfur dioxide until reaching a volume of 200 mL. Slowly add this solution to the cooled iodine mixture, shaking until the iodine dissolves. Transfer the solution to the apparatus and allow the solution stand for 24 hours before standardizing. One mL of this fresh-prepared solution is equivalent to approximately 5 mg of water. Protect the solution from light while in use. To determine water in trace amounts (less than 1%), it is preferable to use a reagent with a water equivalence factor not greater than 2.0, which will generate the consumption of a more significant volume of the volumetric solution.

Method b – Dissolve 63 g of iodine in 100 mL of pyridine, with a water content of less than 1 mg/mL, cool the solution in an ice bath and pass dry sulfur dioxide through this solution until the weight gain reaches 32 g. Adjust to 500 mL with chloroform or methyl alcohol and allow to stand for minimum 24 hours before using.

Method c – Dissolve 102 g of imidazole, with a water content of less than 0.1%, in 350 mL of methoxyethanol or diethylene glycol monomethyl ether, cool the solution in an ice bath and pass dry sulfur dioxide through this solution until the increase in weight is 64 g, keeping the temperature between 25°C and 30°C. Dissolve 50 g of iodine in this solution and allow to stand for at least 24 hours before using.

Method d - Pass sulfur dioxide through 150 mL of methoxyethanol until the weight gain is 32 g. To this solution, previously cooled in an ice bath, add 250 mL of methoxyethanol or chloroform containing 81 g of 2-methylaminopyridine, with a water content lower than 1 mg per mL. Dissolve 36 g of iodine in this solution and allow to stand for at least 24 hours before using.

Karl Fischer reagent prepared by any of these methods should be standardized within one hour of use or daily if used continuously, as its activity for water determination varies with time. Store reagent refrigerated, protected from light and moisture.

A commercially available stabilized solution of Karl Fisher reagent can be used. Commercially available reagents which contain solvents or bases other than pyridine or alcohols rather than methyl alcohol can also be used. These can be individual solutions or reactants formed *in situ* by combining the components of the reactants present in two different solutions. The diluted reagent required in

some monographs should be diluted according to manufacturer's instructions. Methyl alcohol or other suitable solvent such as diethylene glycol monomethyl ether can be used as diluent,

Reagent Standardization

Place a sufficient amount of methyl alcohol or other suitable solvent in the titration flask to cover the electrodes and add sufficient amount of *Reagent* until the endpoint characteristic color is obtained, or $(100 \pm 50) \mu\text{A}$ of direct current with an applied potential of approximately 200 mV.

Purified water, sodium tartrate dihydrate, a pharmacopoeial reference standard, or a commercial standard with a certificate of analysis traceable to a pharmacopoeial standard may be used to standardize the *Reagent*. The reagent equivalence factor, the volume to be used in the assay, the size of the burette and the quantity of standard to be weighed are factors that must be considered when choosing the standard and the quantity to be used. For purified water or water standards, quickly add between 2mg and 250mg of water, accurately weighed, and titrate to endpoint. Calculate the water equivalence factor, F, in mg of water per mL of reagent, using the formula:

$$F = P/V$$

where

P = weight, in mg, of the water contained in the aliquot of the standard used;

V = volume, in mL, of the *Reagent* used in the assay.

For sodium tartrate dihydrate ($\text{C}_4\text{H}_4\text{Na}_2\text{O}_6 \cdot 2\text{H}_2\text{O}$), quickly add between 20 mg and 125 mg accurately weighed, and titrate to endpoint. The water equivalence factor, F, in mg of water per mL of reagent, is calculated by the formula:

$$F = (36.04/230.08) P/V$$

where

36.04 = twice the molar mass of water;

230.08 = molar mass of sodium tartrate dihydrate;

P = weight, in mg, of sodium tartrate dihydrate;

V = volume, in mL, of the *Reagent* used in the assay.

Note: the solubility of sodium tartrate dihydrate in methyl alcohol is such that the use of additional methyl alcohol may be required for further standard assays.

Sample preparation

If not otherwise specified in the individual monograph, use a weighed or accurately measured amount of the sample under analysis with an estimated water content between 2mg and 250mg. The amount of water depends on the water equivalence factor of the *Reagent* and the endpoint determination method. In most cases, the minimum sample quantity (P_m), in mg, can be estimated using the formula:

$$P_m = FCV/K_f$$

where

F = *Reagent* water equivalence factor, in mg/mL;

C = volume used, in percentage of burette capacity;

V = volume of the burette, in mL;

K_f = limit or expected content of water in the sample, in percentage.

C is generally between 30% and 100% for manual assay and between 10% and 100% for the instrumental method for determination of endpoint.

Note: *FCV product must be greater than or equal to 200 for the calculation, to ensure that the minimum amount titrated is greater than or equal to 2 mg.*

If the sample being analyzed is an aerosol with propellant, store in freezer for not less than two hours, open the container and analyze 10.0 mL of the sample thoroughly mixed. To titrate the sample, determine the endpoint at a temperature of 10°C or higher.

For the analysis of capsules, use a portion of the homogenized content of at least four units. If necessary, pulverize the contents into fine powder.

If the sample under analysis is for tablets, use the powder of at least four tablets pulverized to fine powder in an atmosphere with temperature and relative humidity values that do not affect the results.

In cases where the monograph specifies that the sample under analysis is hygroscopic, place a portion of the solid, accurately weighed, in a titration cup, proceeding with the determination of water immediately, to avoid absorption of atmospheric moisture.

If the sample consists of a defined amount of solid, such as a lyophilized product or powder in a flask, use a dry syringe to inject an appropriate volume of methyl alcohol or other appropriate solvent, accurately measured, into a tared container and shake until the sample dissolves. With the same syringe, remove the solution from the container, transfer to a titration flask prepared as described in *Procedure* and titrate immediately. Determine the consumption of reagent used in the titration of the solvent volume used to prepare the sample and subtract this value from that obtained in the titration of the sample under analysis. Dry the container and lid at 100°C for three hours, allow to cool in a desiccator and weigh. Determine the weight of the analyzed sample from the difference in weight in relation to the initial weight of the container.

When appropriate, water can be desorbed or released from the sample by heat in an external oven connected to the beaker, to which it is transferred with the aid of a dry inert gas such as pure nitrogen. Take care and correct any deviation due to carrier gas. Carefully select heating conditions to avoid water formation as a result of dehydration due to decomposition of sample components, which may invalidate the method.

Procedure

If not otherwise specified in the individual monograph, transfer a sufficient amount of methyl alcohol to the titration cup, ensuring that the volume is sufficient to cover the electrodes (approximately 30 to 40 mL), and titrate with the reagent up to the electrometric or visual endpoint to consume any moisture that may be present (do not consider the volume consumed in the calculation). Quickly add prepared sample as instructed in *Sample Preparation*, mix and titrate with *Reagent* up to electrometric or visual endpoint. Calculate the water content of the sample, in percentage, using the formula:

$$\% \text{ água} = \frac{(VF \times 100)}{m}$$

where

V = volume, in mL, of the *Reagent* used in the titration;

F = *Reagent* water equivalence factor

m = mass of the sample (mg).

BACKTITRATION METHOD (INDIRECT)

In this titration, an excess of *Reagent* is added to the sample, a sufficient time is allowed for the reaction to complete and the unused *Reagent* is titrated with a standard solution of water in a solvent such as methyl alcohol. The backtitration procedure is generally applied and avoids the problems that can arise in direct titration of substances in which the bonded water is slowly released.

Apparatus, reagent and sample preparation

Use the *Direct Volumetric Method*.

Preparation and standardization of the water solution

Prepare a water solution by diluting 2 mL of water with methyl alcohol or other suitable solvent to 1000 mL. Standardize this solution by titrating 25.0 mL with the *Reagent*, previously standardized as described in *Standardization of the reagent*. Calculate the water content (C_{water}), in mg per mL, of the Water Solution, using the formula:

$$C_{\text{água}} = VF/25$$

where

V = volume of the *Reagent* consumed, in mL;

F = *Reagent* water equivalence factor, in mg/mL;

Procedure

Transfer a quantity of methyl alcohol or other suitable solvent to the titration cup, ensuring that the volume is sufficient to cover the electrodes (approximately 30 mL to 40 mL) and titrate with the *Reagent* to the electrometric or visual endpoint. Quickly add the sample, homogenize and add an accurately weighed excess of *Reagent*. Wait a sufficient time for the reaction to complete and titrate the unused *Reagent* with the *Standard Water Solution* to the electrometric or visual endpoint. Calculate water content ($\%_{\text{water}}$) of the sample using the formula:

$$\% \text{ água} = \frac{F (X' - XR) 100}{m}$$

where

F = *Reagent* water equivalence factor, in mg/mL;

X' = volume, in mL, of the *Reagent* added after the introduction of the sample;

X = volume, in mL, of the standardized *Water Solution* necessary to neutralize the unused *Reagent*;

R = quotient, V/25 (mL of *Reagent* per mL of *Water Solution*), determined from the *Standardization of Water Solution* for volumetric backtitration (indirect);

m = mass of the sample (mg).

COULOMETRIC METHOD

For the coulometric determination of water, the Karl Fischer reaction is used. Iodine, however, is not added in the form of a volumetric solution, but is obtained by anodic oxidation in a solution containing iodide. The reaction cell usually consists of a large anode compartment and a small cathode

compartment, separated from each other by a diaphragm. Other suitable types of reaction cells (e.g. without diaphragm) can also be used. Each compartment has a platinum electrode that conducts current through the cell. Iodine, which is produced at the anode electrode, immediately reacts with the water in the compartment. When all the water is consumed, an excess of iodine is produced which is normally detected electrometrically, which indicates the endpoint. Moisture is removed from the system through pre-electrolysis. It is not necessary to change the solution in the cup after each determination. A requirement of this method is that each component of the sample is compatible with the other components and that no secondary reactions are produced. Samples are normally transferred to the cup as a solution by injection through a septum. Gases can be introduced into the cell using a suitable gas inlet tube. The accuracy of the method fundamentally depends on the degree of elimination of atmospheric moisture in the system; therefore, the introduction of solids into the cell may require precautions such as working in a dry inert gas atmosphere. System control can be performed by measuring the baseline derivative, which does not exclude the need for a blank correction when using sample introduction vehicle. This method is especially suitable for inert chemical substances such as hydrocarbons, alcohols and ethers. Compared to Karl Fischer volumetric assay, coulometric is a micromethod.

When appropriate, water can be desorbed or released from the sample by heat in an external oven connected to the beaker, to which it is transferred with the aid of a dry inert gas such as pure nitrogen. Take care and correct any deviation due to carrier gas. Carefully select heating conditions to avoid water formation as a result of dehydration due to decomposition of sample components, which may invalidate the method.

Apparatus

The use of any commercially available device that has an absolutely hermetic system, equipped with the necessary electrodes and a magnetic stirrer, is allowed. The equipment microprocessor controls the analytical procedure and displays the results.

Reagent

Electrolyte solutions can be prepared by any of the following procedures, and commercial reagents can also be used.

Note: Chloroform and methyl alcohol used for reagent preparation must have a water content inferior to 0.1 mg/mL. Methoxyethanol and diethylene glycol monomethyl ether must have a water content lower than 0.3 mg/mL.

Method a – ANOLYTE SOLUTION: Dissolve 102 g of imidazole in 900 mL of methyl alcohol, cool the solution in an ice bath and pass dry sulfur dioxide through the solution kept at a temperature below 30 °C, until weight is 64 g. Dissolve 12 g of iodine by agitating, add an appropriate amount of water to the solution until the liquid turns brown to yellow, and dilute to 1000 mL with methyl alcohol. **CATHOLYTE SOLUTION:** dissolve 24 g of diethanolamine hydrochloride in 100 mL of methyl alcohol.

Method b - ANOLYTE SOLUTION: Dissolve 40 g of 1,3-di(4-pyridyl)propane and 30 g of diethanolamine in approximately 200 mL of methyl alcohol and pass dry sulfur dioxide through the solution until the weight gain is 25 g. Add 50 mL of propylene carbonate and dissolve 6 g of iodine in the solution. Add methyl alcohol to bring the volume to 500 mL and add an appropriate amount of water until the liquid turns brown to yellow. **CATHOLYTE SOLUTION:** dissolve 30g of choline hydrochloride in methyl alcohol and dilute to 100 mL with the same solvent.

Method c - ANOLYTE SOLUTION: Dissolve 100 g of diethanolamine in 900 mL of methyl alcohol or in a mixture of methyl alcohol and chloroform (3:1) and pass sulfur dioxide through the solution until the weight increase of the solution is 64g. Dissolve 20 g of iodine in the solution and add an appropriate amount of water until the liquid turns brown to yellow. *CATHOLYTE SOLUTION:* Dissolve 25 g of lithium chloride in 1000 mL of a mixture of methyl alcohol and nitromethane (4:1).

Sample preparation

When the sample is a soluble solid, an appropriate amount, accurately weighed, can be dissolved in anhydrous methyl alcohol or other suitable solvents. When the sample is an insoluble solid, an appropriate amount can be extracted, accurately weighed, using a suitable anhydrous solvent and injected into the anolyte solution. Alternatively, an evaporation method can be used in which water is released and evaporates by heating the sample in a tube in a stream of dry inert gas. The gas soon passes into the cell.

When the sample is to be used directly without being dissolved in a suitable anhydrous solvent, an appropriate amount, accurately weighed, can be introduced directly into the anode compartment.

When the sample is a liquid miscible in anhydrous methyl alcohol or other suitable solvents, an appropriate amount, accurately weighed, may be added to the anhydrous methyl alcohol or other suitable solvents.

Procedure

Using a dry device, inject or add directly into the anolyte an accurately measured amount of the sample or sample preparation that contains between 0.5mg and 5mg of water, or the amount recommended by the instrument manufacturer, mix and carry out the coulometric titration up to the electrometric endpoint. Read the water content of the sample preparation directly from the instrument screen and calculate the percentage present in the substance. Carry out a blank determination, if necessary, and make the corresponding corrections.

5.2.20.2 AZEOTROPIC DISTILLATION METHOD - TOLUENE DISTILLATION

This method is based on distillation, with toluene steam, of the water contained in the sample of a product under the established conditions.

Apparatus

Use a 500 mL round bottom glass flask, A, connecting through a connection, B, to a reflux condenser, C, using ground glass joints (**Figure 1**).

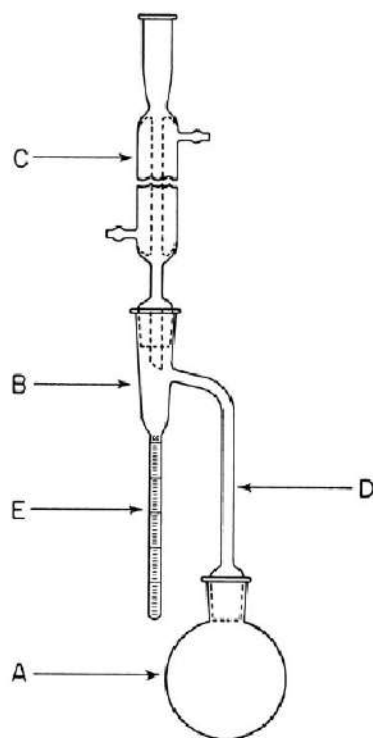


Figure 1— Apparatus for the determination of water by azeotropic distillation.

The critical dimensions of the apparatus parts are as follows: the connecting tube, D, has an inner diameter of 9 mm to 11 mm. The distillate collector has a length of 235 mm to 240 mm. The condenser should be of the straight tube type, with an approximate length of 400 mm and an internal diameter of not less than 8 mm. The receiving tube, E, has a capacity of 5 mL and its cylindrical part, with a length of 146 mm to 156 mm, is graduated in 0.1 mL subdivisions, so that the reading error is not greater than 0.05 mL for any volume indicated. The heat source is preferably a thermostatically controlled electric heater or an oil bath. The top of the flask and the connecting tube may be insulated.

Clean the receiver tube and condenser with a suitable cleaning solution, rinse thoroughly with water and dry. Prepare the toluene that will be used by shaking with a small amount of water, and distil until water excess is removed.

Procedure

Place in a dry flask an amount of the substance, accurately weighed, containing from 2 mL to 4 mL of water. If the substance is of the semi-solid type, weigh on an oval metal plate with a size that passes through the flask's neck. If there is a possibility of projections being produced when introducing the substance, add a quantity of porous material (for example: washed and dry sand, capillary tubes, porcelain). Place approximately 200 mL of toluene in the flask, connect the apparatus and fill the receiving tube, E, with toluene poured through the upper opening of the condenser. Heat the flask gently for 15 minutes and, as soon as the toluene has boiled, distill at a rate of approximately two drops per second until most of the water has been washed away, then increase the distillation rate to approximately four drops per second. When all the water appears to have been distilled, rinse the inside of the condenser tube with toluene. Continue the distillation for another five minutes; remove the heat source and allow the receiving tube to cool to room temperature and drag the water adhering to the walls. After completing the separation of water and toluene, read the volume of water and calculate the percentage present in the substance.

5.2.20.3 DETERMINATION OF WATER BY SEMI-MICRO METHOD

The determination of water by the semi-micro method is carried out in a titration device with a capacity of 60 mL, equipped with two platinum electrodes, an inlet tube for nitrogen, a stopper adapted to the end of a burette and an air intake tube protected by a drying agent. The sample intake is introduced through a side tube fitted with a ground stopper. During titration, agitation must be ensured by the support of a mechanical stirrer or by bubbling dry nitrogen.

Completion of the reaction is determined by the amperage. A suitable circuit, consisting of a potentiometer of approximately 2000 Ω , connected to a 1.5 V battery, allows applying a variable potential difference. This is adjusted so that it conducts a weak initial current through the platinum electrodes connected in series to a microammeter. The microammeter needle deviates after each addition of reagent, immediately returning to its initial position. The end of the reaction is indicated by a deviation that persists for minimum 30 seconds.

Use iodosulfuron RS after determining its equivalent in water. The solutions and reagents used must be stored in an anhydrous condition and protected from atmospheric moisture during the assay or upon any handling.

Iodosulfuron RS must be stored protected from light, preferably in a flask fitted with an automatic burette.

Commercially available iodosulfuron RS solutions have (or may have) a composition that differs from the iodosulfuron RS solution by replacing pyridine for several basic substances. The use of these reagent solutions must be preceded by an evaluation that allows, in each case, to verify the stoichiometry and the absence of incompatibility between the substance to be tested and the reagent. Unless otherwise indicated, *Method A* must be used.

Method A. Introduce about 20 mL of anhydrous methyl alcohol or the solvent prescribed in the monograph into the titration flask. Add iodosulfuron R solution to the reagent flask until the amperometric changeover. Quickly introduce the assay intake, shake for one minute and titrate with the iodosulfuron RS solution until new changeover.

Method B. Introduce in the titration flask about 10 mL of anhydrous methyl alcohol or of the solvent prescribed in the monograph. Add iodosulfuron RS until the amperometric changeover. Quickly introduce the substance sample intake and then a volume of iodosulfuron RS sufficient to obtain an excess of approximately 1 mL. In this case, as well, the volume prescribed in the monograph can be used. Allow to stand in a closed flask and protected from light for one minute or for the time prescribed in the monograph, shaking occasionally. Titrate the excess of iodosulfuron RS with anhydrous methyl alcohol or with another solvent prescribed in the monograph, adding a known quantity of water close to 2.5 g/L, until returning to the initial weak current.

5.2.20.4 GRAVIMETRIC METHOD

Procedure for chemical substances. Proceed as indicated in the individual monograph, preparing the sample as determined in *Loss on Desiccation* (5.2.9.1).

Procedure for herbal drugs. Proceed as indicated in *Methods of pharmacognosy* (5.4), as indicated in the individual monograph.

5.2.21 ANALYSIS OF PHASE SOLUBILITY

The solubility of a pure substance in a given solvent, at a constant temperature, is a characteristic parameter of the substance and can therefore serve for purposes of identification and assessment of its degree of purity. The analysis of phase solubility is based on this principle. The procedure consists of adding increasing portions of sample to constant volumes of solvent in which the analyzed substance shows only a slight solubility, aiming to obtain a saturated solution of this substance. Once the system equilibrium has been achieved - by prolonged agitation, under constant temperature - the total content of solute in the supernatant solution is determined (usually by gravimetric method) and the phase solubility diagram is plotted, plotting the solution composition in mg of solute per g of solvent (ordinate), by system composition in mg of sample added per g of solvent (abscissa). **Figure 1** illustrates such a diagram. Along segment AB, the entire solid dissolves and is found in solution (slope corresponds to unity). At point B the sample saturates the solution and subsequent additions do not increase its concentration. The slope of the line segment BC is therefore null and the intersection of the extension of this line with the Y axis gives the value of the solubility of the substance.

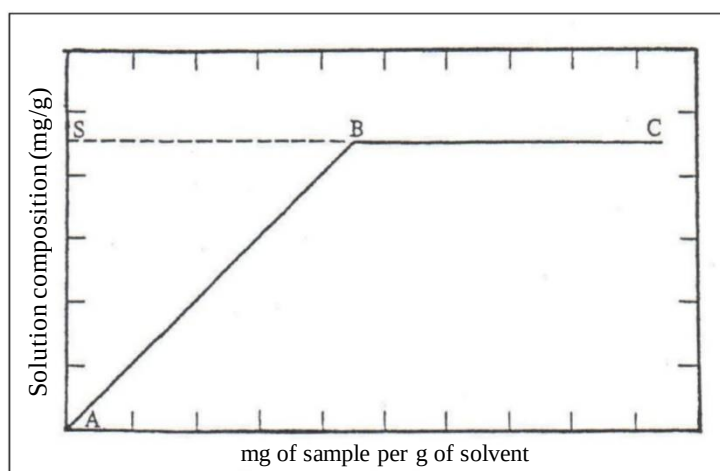


Figure 1 – Solubility diagram for phases of a sample consisting of a single substance.

If the sample consists of two substances (one of them impurity of the other, for example), the diagram takes the form shown in **Figure 2**. Segment AB has a unit slope; point B indicates saturation of the solution with respect to one of the sample components (generally the one that is present in greater proportion); the BC segment indicates the solubilization of the second component and the CD segment the saturation of the solution with the latter (zero slope).

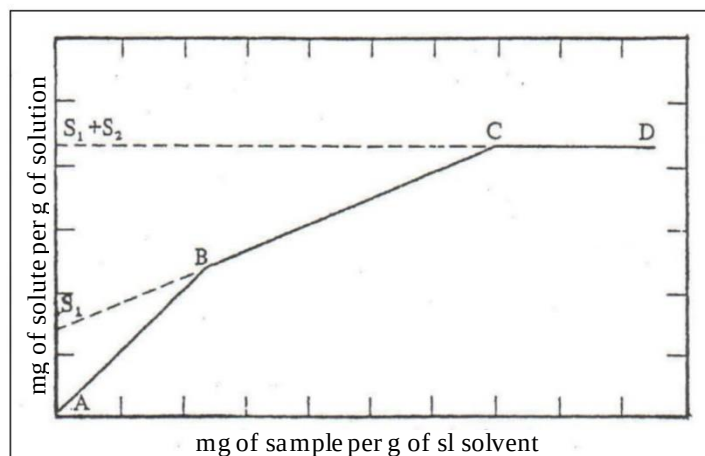


Figure 2 – Solubility diagram for phases of a sample consisting of two substances.

The slope value of the BC segment – phase in which only the second component is dissolved – corresponds to the proportion of this component in the sample. The subtraction of this unit value provides the content of the first component in the sample, allowing the use of the formula $(1-i) \cdot 100$ to obtain the content. The slope, i , is obtained by the formula $(Y_2 - Y_1) / (X_2 - X_1)$, where Y_1 , Y_2 and X_1 , X_2 correspond, respectively, to projections of points on the line segment BC on the ordinate (composition of the solution) and the abscissa (composition of the system). The extrapolation of the BC segment gives the solubility limit, S_1 , in mg of solute per g of solvent, of the first component, whereas the extension of the line from the segment CD to the Y axis leads to the sum of the solubilities of the two components, $S_1 + S_2$.

The occurrence of pronounced deviations at the points that form the straight segments of the diagram indicates a lack of equilibrium in the system, although these can also be attributed to the existence of a solid solution or to deviations from the theoretical behavior. If necessary, the slope i can be calculated by graphical approximation or using the least squares statistical method.

A peculiarity of the phase solubility analysis is that it is not a method applicable to mixtures whose components are present in the sample in proportion to their solubilities. In this particular case, both components promote saturation at the same point, providing, as a result, a phase diagram equivalent to that of pure substance.

CHOICE OF SOLVENT

The choice of solvent for phase solubility analysis is based on the solubility of the component present in greater proportion in the sample and on the assay method adopted to determine the concentration of the formed solution. As the gravimetric method is more common, the solvent should present sufficient volatility to allow evaporation in a vacuum, but insufficient to hinder transfer and weighing operations.

Solvents with a boiling point between 60 °C and 150 °C are recommended. In terms of solubility, it is convenient that the solvent has the capacity to dissolve the sample in a proportion not lower than 4 mg/g or greater than 50 mg/g. Optimum solubility is in the range of 10 to 20 mg/g. Additional recommendations include the solvent inertia towards the sample components (including the possibility of formation of solvates or salts) and the use of solvents of known purity and concentration (trace of impurities strongly affect the solubility), admitting, however, the use of mixtures.

APPARATUS

Comprises thermostated water bath, appropriate vials and ampoules and analytical balance, with accuracy of $\pm 10 \mu\text{g}$.

The water bath is provided with a thermostat with a temperature control tolerance of not more than 0.1°C , especially in the range of 25°C to 30°C , which is usual for testing. The bath is equipped with a horizontal rotating rod (25 rpm) provided with holding clamps for the ampoules. As an alternative, a vibrator (100 to 120 vibrations/second) can be used, also equipped with ampoule holding clamps.

The ampoule - with a capacity of 15 mL - next to the so-called solubility flask, also used in the tests, is illustrated in **Figure 3**. Containers of different specification are admissible as long as they are hermetic and suitable for the method described.

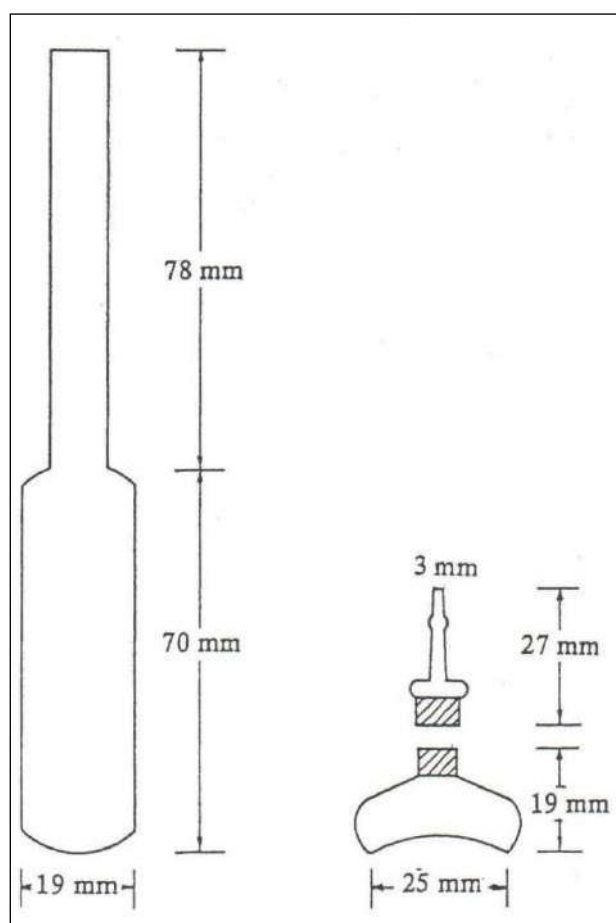


Figure 3 – Ampoule used in phased solubility analysis.

PROCEDURE

System composition

Accurately weigh a minimum of seven rigorously clean 15 mL ampoules. Transfer accurately weighed increasing amounts of sample into each ampoule so that the first contains only slightly less than the soluble one in 5 mL of solvent and the last contains a slight excess of sample. After transferring 5.0 mL of solvent to each ampoule, cool them in a mixture of dry ice and acetone and seal them with an air/gas blowtorch, taking care to keep glass fragments resulting from the process.

Allow the ampoules to reach room temperature and weigh them, together with their respective glass fragments. Calculate system composition, in mg/g, for each ampoule, using the formula: $1000(M_2 - M_1)/(M_3 - M_2)$, where M_2 corresponds to mass of the ampoule containing the sample; M_1 is the mass

of the empty ampoule and M_3 is the mass of the ampoule containing sample, solvent and any glass fragments.

Equilibrium

The period necessary to establish equilibrium in the systems contained in the ampoules varies according to nature of sample, method of agitation (rotation or vibration) and temperature. Experience indicates an average period of 1 to 7 days for shaking by vibration and 7 to 14 days for the rotational process. To confirm equilibrium promotion, heat the penultimate ampoule in the series to 40 °C to obtain supersaturation. Result is positive if the point corresponding to this ampoule is consistent with the others in the phase diagram. However, a different result does not necessarily mean that equilibrium has not been reached. There are substances that tend to remain in a supersaturated solution and, if this is the case, a series of analyses, varying the waiting period, is required to ensure the consistency of the points on the solubility curve.

Solution composition

Once equilibrium is reached, place the ampoules on an appropriate support so that they remain in a vertical position, with the necks above water level in thermostatic bath. Wait for solids to decant in the ampoules, open them and collect 2.0 mL of the supernatant from each ampoule using a pipette fitted with a cotton swab or other material capable of acting as a filter. Remove filtering material from the pipette and transfer the clear liquid to a solubility flask (**Figure 3**) tared and properly identified, weighing each flask after operation. Cool vials in dry ice and acetone bath, then evaporate the solvent under reduced pressure. Gradually increase evaporation temperature, taking care not to exceed the limit compatible with sample stability and dry residue to constant weight. Calculate solution composition in each vial, in mg/g, using the formula $1000 (P_3 - P_1)/(P_2 - P_3)$, where P_3 corresponds to the mass of the vial containing the evaporation residue; P_1 is the mass of the empty solubility vial (tare) and P_2 is the mass of the vial containing the solution.

Plot a phase diagram based on the values obtained and determine sample percentage purity as a function of the straight line slope.

APPLICATION OF PHASE SOLUBILITY ANALYSIS IN SUBSTANCE PURIFICATION

While solutions obtained in the analytical process described contain essentially all the impurities present in the sample in enhanced proportion compared to the original sample, leading – after solvent evaporation – to the qualitative determination of impurities, the phase is suitable, due to its high purity, for preparation of reference standards to other analytical tests.

Procedure

Weigh appropriate amount of sample and suspend it in suitable solvent so that – reached equilibrium – only dissolve 10% of the material. Close the flask and wait for equilibrium to be established at room temperature (usually 24 hours is sufficient). Then collect the clear supernatant solution and evaporate, at or near room temperature, to dryness. Since the solution contains impurities from the original sample, material in which the proportion of impurities is enhanced is obtained by this procedure, with the enrichment ratio being approximately equal to the ratio of sample mass by the mass of dissolved solids in the volume of solvent used. Purify undissolved residue by washing and drying (reference standard).

5.2.22 ELECTROPHORESIS

GENERAL PRINCIPLES

By the action of an electric field, charged particles dissolved or dispersed in an electrolyte solution migrate towards the opposite polarity electrode. In gel electrophoresis, the displacement of particles is slowed down by interactions with the matrix gel that constitutes the migration medium and behaves like a molecular sieve.

Opposing interactions of electrical force and molecular sieving result in the rate of migration differential according to particle size, shape, and charge. Due to their different physicochemical properties, the different molecules contained in a mixture will migrate at different speeds during electrophoresis, thus being separated into well-defined fractions. Electrophoretic separations can be conducted in systems without a support phase (e.g. free solution separation in capillary electrophoresis), and or in stabilized media such as thin-layer plates, films or gels.

MOVING-BOUNDARY, OR DIVISION, OR FREE FLOW, ELECTROPHORESIS

This method is mainly used in the determination of mobilities, with experimental characteristics being directly measurable and reproducible. It applies, above all, to substances of relatively high molar mass, not very diffusible. Divisions are initially demarcated by a physical process such as refractometry or conductimetry. After the application of a defined electric field, for a determined time, new divisions are obtained and their respective positions are observed. Operating conditions allow for the determination of divisions and constituents.

SUPPORT ELECTROPHORESIS, OR ZONE ELECTROPHORESIS

This method is only used for reduced samples. The nature of the support, such as paper, agarose gel, cellulose acetate, starch, methacrylamide or mixed gel, introduces a number of additional factors that modify mobility:

- a) due to the twisting of the support channeling, the distance apparently covered is shorter than the actual distance;
- b) certain supports are not electrically neutral and, as the medium constitutes a stationary phase, it can sometimes give rise to considerable electroosmotic current;
- c) heating due to the Joule effect can produce a certain evaporation of the liquid from the support, which leads, by capillarity, to a displacement of the solution from the extremities to the center; thus, the ionic strength tends to increase progressively.

The migration speed depends on four main factors: particle mobility, electroosmotic current, evaporation current and field intensity. For these reasons, it is necessary to proceed under well-defined experimental conditions and to use, if possible, reference standards.

Apparatus

An electrophoresis device consists of:

- a generator of direct, controllable voltage and preferably stabilized current;

– an electrophoresis tank. Usually rectangular, made of glass or rigid plastic material, with two separate compartments, anodic and cathodic, which contain the conductive buffer solution. An electrode, made of platinum or graphite, is immersed in each compartment; these are connected by a circuit duly isolated from the power supply of the corresponding terminal to form, respectively, the anode and cathode, connected by a circuit suitably isolated to the corresponding terminal of the generator. The liquid level in both compartments is equal to avoid siphoning effect. The electrophoresis tank must be equipped with a hermetic lid, allowing an atmosphere saturated with moisture to be kept inside and thus attenuating solvent evaporation during migration. A safety device is used that cuts the current when the lid is removed from the tank. If the measurement of electric current exceeds 10 W, it is preferable to cool the support;

– a support device:

Strip electrophoresis. In electrophoresis, the strips on the support are pre-impregnated with the same conductive solution and each end immersed in the electrode compartment. The strips are well extended, fixed on an appropriate support to prevent diffusion of the conductive solution, such as a horizontal frame, an inverted V support, or a uniform surface with contact points at suitable ranges.

Gel electrophoresis. In gel electrophoresis, the device consists of a glass plate, such as a simple microscope slide, on which an adherent gel layer of uniform thickness is deposited on the entire surface of the slide. The contact between the gel and the conductive solution varies depending on the type of device used. Any moisture condensation or drying of the solid layer is avoided;

– a measuring device or detection means.

Procedure. Place the electrolyte solution in the electrode compartments. Place the support, conveniently soaked with the electrolyte solution in the tank, according to the type of device used. Plot the starting line and apply the test sample. Allow the current to pass for the indicated time; then turn off the power, remove the tank support, dry and reveal.

ELECTROPHORESIS IN POLYACRYLAMIDE GEL IN CYLINDRICAL TUBE

In cylindrical tube polyacrylamide gel electrophoresis, the stationary phase consists of a gel prepared from acrylamide and N,N'-methylenebisacrylamide. The gels are prepared in tubes, usually 7.5 cm in length and 0.5 cm in internal diameter (cylindrical gel); a single sample is applied to each tube.

Apparatus. The apparatus consists of two containers intended to receive the buffer solutions and made of suitable material, such as polymethyl methacrylate. They are arranged vertically one above the other and are each equipped with a platinum electrode. These two electrodes are connected to a current source, enabling operating with constant intensity and voltage. For cylindrical gels, the device has, at the upper base of the reservoir, a number of elastomer joints located at equal distance from the electrode.

Procedure. In general, it is recommended to degas the solutions before polymerization and use the gel immediately after preparation. Prepare the gel as indicated in the monograph. Place the gel mixture in the appropriate glass tubes, closed at the lower end with a stopper, to an equal height in all of them, at a distance of about 1 cm from the upper edge of the tube. Avoid the introduction of air bubbles into the tubes. Cover the mixture with a layer of water to prevent contact with air and allow to stand. Gel formation generally requires about 30 minutes and is complete when a clear boundary between the gel and the aqueous layer appears. Eliminate the aqueous layer. Fill the lower reservoir with the prescribed buffer solution and remove the stoppers from the tubes. Fit the tubes into the

joints of the upper reservoir so that its lower part immerses in the buffer solution of the lower reservoir and adjusts so that the bottom of the tubes is immersed in the buffer solution of the lower reservoir. Gently fill the tubes with the solution from the lower reservoir. Prepare problem and standard solutions containing the prescribed colorant. Carefully fill the tubes with the indicated buffer solution. Apply the solutions, whose density has been increased, by adding sucrose, for example, to the gel surface, using a different tube for each solution. Place the same buffer solution in the upper reservoir. Connect the electrodes to the current source and proceed with electrophoresis, using current of constant intensity or voltage and at the temperature prescribed in the monograph. Stop the current when the colored indicator reaches the lower reservoir. Immediately remove the tubes and proceed with the gel extrusion. Locate the position of the bands on the electrophoretograms according to the indicated procedure.

SODIUM DODECYLSULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Application field. Polyacrylamide gel electrophoresis is used for the qualitative characterization of proteins contained in biological preparations, for purity controls and quantitative determinations.

Purpose. The analysis by gel electrophoresis is a process adapted to the identification and control of the homogeneity of proteins contained in pharmaceutical preparations. It is routinely used to assess the molar mass of protein subunits and determine the subunits that compose the purified proteins. There is a wide variety of ready-to-use gels and reagents on the market instead of those described below, provided that the results obtained are equivalent and that the validity conditions described in *Assay Validation* can be met.

Characteristics of polyacrylamide gels. The sieving properties of polyacrylamide gels are related to their particular structure, which is that of a three-dimensional network of fibers and pores resulting from the formation of cross-links between the bifunctional bisacrylamide and adjacent polyacrylamide chains. The polymerization is catalyzed by a free radical generator composed of ammonia persulfate (APS) and N,N,N',N'-tetramethylethylenediamine (TEMED). The smaller the actual pore size of a gel the higher its concentration of acrylamide. As the gel acrylamide concentration increases, the effective porosity decreases. The actual porosity of a gel is operationally defined by its molecular sieving properties, that is, the resistance it opposes to the migration of macromolecules. There are limits to the concentrations of acrylamide that can be used. At very high concentrations gels break down more easily and become difficult to manipulate. When the pore size of a gel decreases, the rate of migration of a protein in that gel decreases as well. By adjusting the porosity of a gel by changing the acrylamide concentration, it is possible to optimize the method resolution for a given protein product. Accordingly, the physical characteristics of a gel therefore depend on its acrylamide and bisacrylamide content. In addition to the gel composition, the state of the protein is another important factor for its electrophoretic mobility.

In the case of proteins, electrophoretic mobility depends on the pKa of the cleavable groups and the size of the molecule. It is also affected by the nature, concentration and pH of the buffer, the temperature, the intensity of the electric field and the nature of the support.

POLYACRYLAMIDE GEL ELECTROPHORESIS UNDER DENATURING CONDITIONS

The method described as an example is applicable to the analysis of polypeptides with a molar mass of between 14.000 and 100.000 daltons. It is possible to extend this range through different methods (for example, using gradient gels or special buffer systems), but these are not part of this text. Polyacrylamide gel electrophoresis under denaturing conditions using sodium dodecyl sulfate (SDS-PAGE) is the most widely used electrophoresis method to assess the pharmaceutical quality of protein products and is primarily the focus of this text. In general, analytical protein electrophoresis is

performed on a polyacrylamide gel under conditions that favor the dissociation of proteins into their polypeptide subunits and that limit the aggregation phenomenon. Sodium dodecyl sulfate (SDS), a strong anionic detergent, is often used to dissociate proteins prior to application to the gel, in combination with heat. Denatured polypeptides bind to SDS and acquire negative charges, being characterized by a constant charge/mass ratio, regardless of the type of protein considered. Since the amount of SDS bound is almost always proportional to the molar mass of the polypeptide and independent of its sequence, SDS-polypeptide complexes migrate in polyacrylamide gels with mobilities that are a function of the size of the polypeptide.

The electrophoretic mobility of the resulting detergent-polypeptide complexes always has the same functional relation with molar mass. The migration of the SDS complexes is, as expected, towards the anode at a higher velocity for the low molar mass complexes than for the high ones. It is, therefore, possible to determine the molar mass of a protein from its relative mobility, after comparison with standard solutions of known molar mass value with the observation of a single band constituting a purity criterion. However, eventual modifications in the constitution of the polypeptide, for example, an N- or an O-glycosylation, have a significant impact on the apparent molar mass of a protein since SDS does not bind to a carbohydrate medium in a similar manner it does to a polypeptide. In fact, SDS does not bind in the same manner to glycidic groupings or to peptide groupings, so that the constancy of the charge/mass ratio is no longer verified. The apparent molar mass of proteins that have undergone post-translational modifications does not really reflect the mass of the polypeptide chain.

Reducing conditions

The association of polypeptide subunits and the three-dimensional structure of proteins are often based on the existence of disulfide bridges. One of the objectives to be achieved in the SDS-PAGE analysis under reducing conditions is to break this structure by reducing the disulfide bridges. Complete denaturation and dissociation of proteins by treatment with 2-mercaptoethanol or with dithiothreitol (DTT) causes a splitting of the polypeptide chain, followed by a complexation with SDS. Under these conditions, the molar mass of the polypeptide subunits can be calculated by linear regression with the aid of appropriate molar mass standards.

Non-reducing conditions

For certain analyses, complete dissociation of the protein into peptide subunits is not desirable. In the absence of treatment by reducing agents such as 2-mercaptoethanol or DTT, the covalent disulfide bonds remain intact and the oligomeric conformation of the protein is preserved. SDS-oligomer complexes migrate more slowly than SDS-peptide subunits. Furthermore, unreduced proteins may not be fully saturated in SDS and therefore do not bind to detergent in a constant mass ratio. This circumstance makes the determination of the molar mass of these molecules by SDS-PAGE more difficult than the analysis of fully denatured polypeptides, as, for comparison to be possible, it is necessary that the standards and unknown proteins have similar configurations. However, obtaining a single stained band on the gel remains a criterion for purity.

CHARACTERISTICS OF GEL ELECTROPHORESIS IN DISCONTINUOUS BUFFER SYSTEM

The most widespread electrophoretic method for the characterization of complex protein mixtures is based on the use of a discontinuous buffer system that includes two continuous but distinct gels: a separation or resolving gel (lower) and a stacking gel (upper). These two gels have different porosity, pH and ionic strength. In addition, different mobile ions are used in gels and electrode buffers. The discontinuity of the buffer system leads to a large volume concentration of samples in the

concentrating gel and therefore an improvement in resolution. When the electric field is applied, a negative voltage gradient builds up through the sample solution and carries proteins from the concentration gel to the stacking gel. The glycinate ions contained in the electrode buffer follow the proteins in the stacking gel. A mobile division zone quickly forms, the front of which made of the highly mobile chloride ions and the back of the slower glycinate ions. A localized high voltage gradient establishes itself between the ionic fronts of the head and tail and causes the SDS-protein complexes to concentrate in a very narrow range that migrates between the chloride and glycinate fractions.

On a large scale, regardless of the volume of sample applied, the set of SDS-protein complexes undergoes a condensation effect and penetrates the separation gel in the form of a narrow, well-defined, high- protein density band. The large-pore stacking gel does not generally slow down the migration of proteins, but mainly plays the role of an anti-convective medium. At the interface of the stacking and separating gels, proteins are confronted with a sharp increase in the lag effect due to the small pore diameter of the separating gel. When they penetrate the separation gel, this delay proceeds due to the molecular slurry effect exerted by the matrix. The glycinate ions pass through the proteins whose migration then proceeds in a medium of uniform pH consisting of a buffered tromethamine solution (TRIS) and glycine. The molecular slurry effect leads to a separation of the SDS-polypeptide complexes based on their respective molar mass.

PREPARATION OF DISCONTINUOUS BUFFER VERTICAL SDS POLYACRYLAMIDE GELS

Mold assembly

With a mild detergent, clean the two glass plates (e.g. 10 cm x 8cm size), the polytetrafluoroethylene comb, the two spacers and the silicone rubber tube (e.g. 0.6 mm x 350 mm diameter), and rinse thoroughly with water. Dry all elements with a paper towel or fabric. Lubricate spacers and tube with non-silicone based lubricant. Place the spacers 2 mm from the edge along the two short sides and one of the long sides of the glass plate. The latter will correspond to the bottom of the gel. Start installing the tube on the glass plate using one of the spacers as a guide. When the end of the spacer is reached, carefully bend the tube so that it follows the long side of the glass plate. Hold the tube in place with one finger, bend it again to make it follow the second short side of the plate, using the spacer as a guide. Place the second plate in place, aligning it perfectly over the first, and hold the set by hand pressure. Place two tweezers on each of the short sides of the mold and then, with caution, four more tweezers on the long side that will form the base of the mold. Check that the tube follows the edge of the plates and has not shifted after placing the clamps. The mold is ready and the gel can be placed.

Preparation of gels

For the gels in discontinuous buffer system, it is recommended to place the separating gel first and allow to polymerize before placing the concentration gel, since the content of acrylamide-bisacrylamide in the two gels, the buffer and the pH are different.

Preparation of the separation gel. In an Erlenmeyer flask, prepare the appropriate volume of an acrylamide solution of the desired concentration, using the values indicated in **Table 1**. Mix the components in the order given. Before adding the ammonia persulfate solution and the tetramethylethylenediamine (TEMED) solution, filter, if necessary, by suction, using a cellulose acetate membrane (pore diameter 0.45,µm); keep under suction, shaking the filter unit until no more bubbles form in the solution. Add the appropriate amounts of APS and TEMED solution (**Table 1**), shake and immediately introduce into the space separating the two glass plates of the mold. Leave enough free height for the concentration gel (height of a comb tooth plus 1 cm). Using a tapered glass

pipette, carefully cover the solution with water-saturated isobutyl alcohol. Allow the gel to polymerize in an upright position at room temperature.

Preparation of the stacking gel. When polymerization is complete (about 30 minutes), exhaust the isobutyl alcohol and wash the gel surface several times with water to completely eliminate the isobutyl alcohol and, if necessary, the unpolymerized acrylamide. Place a minimum amount of liquid on the gel surface and, if necessary, absorb the residual water with the tip of a paper towel. In an Erlenmeyer flask, prepare the appropriate volume of an acrylamide solution of desired concentration, using the values indicated in **Table 2**. Mix the components in the order given.

Before adding the ammonia persulfate solution (APS) and the tetramethylethylenediamine (TEMED) solution, filter, if necessary, by suction, using a cellulose acetate membrane (pore diameter 0.45, μm); keep under suction, shaking the filter unit until no more bubbles form in the solution. Add the appropriate amounts of ammonia persulfate and TEMED solutions (**Table 2**), shake and immediately add onto the separating gel. Immediately place a clean polytetrafluoroethylene comb in the concentration gel solution, taking care to avoid the formation of air bubbles. Add the solution to the concentration gel to completely fill the comb interstices. Allow the gel to polymerize in an upright position at room temperature.

Table 1 – Preparation of the resolving gel.

Solution components	Component volume in mL per gel mold volume of:							
	5 mL	10 mL	15 mL	20 mL	25 mL	30 mL	40 mL	50 mL
<i>6% Acrylamide</i>								
Water	2.6	5.3	7.9	10.6	13.2	15.9	21.2	16.5
Acrylamide solution ⁽¹⁾	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
Tris 1,5 M pH 8.8 ⁽²⁾	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
(SDS) 100 g/L of Dodecyl Sulfate of Sodium ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
(APS) 100 g/L of Ammonia Persulfate ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
(TEMED) Tetramethylethylenediamine ⁽⁵⁾	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
<i>8% acrylamide</i>								
Water	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
Acrylamide solution ⁽¹⁾	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
Tris 1,5 M pH 8.8 ⁽²⁾	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
(SDS) 100 g/L of Dodecyl Sulfate of Sodium ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
(APS) 100 g/L of Ammonia Persulfate ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
(TEMED) Tetramethylethylenediamine ⁽⁵⁾	0.003	0.006	0.009	0.002	0.005	0.008	0.024	0.03
<i>10% Acrylamide</i>								
Water	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
Acrylamide solution ⁽¹⁾	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
Tris 1,5 M pH 8.8 ⁽²⁾	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
(SDS) 100 g/L of Dodecyl Sulfate of Sodium ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
(APS) 100 g/L of Ammonia Persulfate ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5

Solution components	Component volume in mL per gel mold volume of:							
	5 mL	10 mL	15 mL	20 mL	25 mL	30 mL	40 mL	50 mL
(TEMED) Tetramethylethylenediamine ⁽⁵⁾	0.002	0.004	0.006	0.008	0.01	0.002	0.016	0.02
12% Acrylamide								
Water	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
Acrylamide solution ⁽¹⁾	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
Tris 1,5 M pH 8.8 ⁽²⁾	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
(SDS) 100 g/L of Dodecyl Sulfate of Sodium ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
(APS) 100 g/L of Ammonia Persulfate ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
(TEMED) Tetramethylethylenediamine ⁽⁵⁾	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
14% Acrylamide								
Water	1.4	2.7	3.9	5.3	6.6	8.0	10.6	13.8
Acrylamide solution ⁽¹⁾	2.3	4.6	7.0	9.3	11.6	13.9	18.6	23.2
Tris 1,5 M pH 8.8 ⁽²⁾	1.2	2.5	3.6	5.0	6.3	7.5	10.0	12.5
(SDS) 100 g/L of Dodecyl Sulfate of Sodium ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
(APS) 100 g/L of Ammonia Persulfate ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
(TEMED) Tetramethylethylenediamine ⁽⁵⁾	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15% Acrylamide								
Water	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
Acrylamide solution ⁽¹⁾	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
Tris 1,5 M pH 8.8 ⁽²⁾	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
(SDS) 100 g/L of Dodecyl Sulfate of Sodium ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
(APS) 100 g/L of Ammonia Persulfate ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
(TEMED) Tetramethylethylenediamine ⁽⁵⁾	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

(1) Acrylamide solution: acrylamide/bisacrylamide (29:1) at 30% (w/v) RS

(2) 1.5 M Tris pH 8.8: 1.5 M trihydrochloride buffer pH 8.8.

(3) SDS 100 g/L: 10% sodium dodecyl sulfate solution (w/v).

(4) PSA 100 g/L: 10% ammonium persulfate solution (w/v). Ammonium persulfate provides the free radicals that induce the polymerization of acrylamide and bisacrylamide. The ammonia persulfate solution decomposes slowly and is renewed every week.

(5) TEMED: N,N,N',N'-tetramethylethylenediamine.

Table 2 – Preparation of the stacking gel.

Solution components	Component volume in mL per gel mold volume of:							
	1 mL	2 mL	3 mL	4 mL	5 mL	6 mL	8 mL	10 mL
Water	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
Acrylamide solution ⁽¹⁾	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
Tris M pH 6.8 ⁽²⁾	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
(SDS) 100 g/L of Dodecyl Sulfate of Sodium ⁽³⁾	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
(APS) 100 g/L of Ammonia Persulfate ⁽⁴⁾	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
(TEMED) Tetramethylethylenediamine ⁽⁵⁾	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

- (1) Acrylamide solution: acrylamide/bisacrylamide (29:1) at 30% (w/v) RS
- (2) 6.8 M Tris pH 8.8: 1.5 M trihydrochloride buffer pH 6.8.
- (3) SDS 100 g/L: 10% sodium dodecylsulfate solution (w/v).
- (4) APS 100 g/L: 10% ammonium persulfate solution (w/v). Ammonium persulfate provides the free radicals that induce the polymerization of acrylamide and bisacrylamide. The ammonium persulfate solution decomposes slowly and is renewed every week.
- (5) TEMED: N,N,N',N'-tetramethylethylenediamine.

When polymerization is complete (about 30 minutes), carefully remove the comb. Wash the wells immediately with water or SDS-PAGE electrophoresis buffer to remove any unpolymerized acrylamide. If necessary, straighten the teeth from the stacking gel with a blunt tip hypodermic needle attached to a syringe on one of the short sides of the plate, carefully remove the tube and place the clamps back. Proceed in the same manner on the other short side and then at the base of the mold. Introduce the gel into the electrophoresis device.

Insert the electrophoresis buffers into the upper and lower reservoirs. Eliminate any bubbles trapped in the base of the gel between the glass plates. It is recommended to use a bent hypodermic needle attached to a syringe for this purpose. Never establish electrical voltage on the gel without the samples, as this can destroy the discontinuity of the buffer system. Before depositing the sample, wash or fill the wells with caution with SDS-PAGE electrophoresis buffer. Prepare problem and standard solutions using the recommended sample buffer and handle them as specified in the analyte monograph. Apply the appropriate volume of the different solutions to the concentration gel wells. Carry out electrophoresis under the conditions recommended by the device manufacturer. Certain manufacturers of SDS-PAGE devices provide gels of various surfaces and thicknesses. To obtain an optimal separation, it may be necessary to vary the electrophoresis duration and electrical parameters as indicated by the manufacturer. Check that the staining front moves on the separating gel; if it reaches the base of the gel, stop the electrophoresis. Remove the mold from the appliance and separate the two glass plates. Remove the spacers, separate and discard the stacking gel and proceed immediately to staining.

DETECTION OF PROTEINS IN GELS

Coomassie blue staining is the most frequently used method for protein detection, with a detection level of 1µg to 10µg of protein per band. Silver nitrate staining is the most sensitive method for visualizing proteins in gels; it enables detection of bands with 10 ng to 100 ng of protein. All gel staining steps are carried out at room temperature, with moderate agitation and orbital movement in appropriate device. Gloves must be worn to avoid depositing fingerprints on the gel that would also be stained.

Coomassie blue staining. Soak the gel for at least one hour in a large excess of Coomassie blue RS. Discard the staining solution. Dip the gel in a large excess of bleach solution (consists of a mixture of one volume of glacial acetic acid, four volumes of methyl alcohol and five volumes of water). Renew the bleaching solution several times until the protein bands clearly appear on a light background. The stronger the gel discoloration, the less protein will be detected by this method. It is possible to accelerate the discoloration by incorporating a few grams of ion exchange resin or a sponge into the bleaching solution.

Note: The acid-alcoholic solutions used in this method do not fully bind the gel proteins. There may therefore be a loss of certain low molar mass proteins during the staining and bleaching operations of thin gels. Permanent fixation can be achieved by placing the gel for one hour in a mixture of one volume of trichloroacetic acid, four volumes of methyl alcohol and five volumes of water, before dipping into the Coomassie Blue RS Solution.

Staining with silver nitrate. Immerse the gel for one hour in a large volume of fixing solution (consists of adding 0.27 mL of formaldehyde in 250 mL of methyl alcohol and diluting to 500 mL with water). Discard and renew the fixation solution and allow to incubate for at least one hour, or overnight if more practical. Discard the fixation solution and place the gel in an excess volume of water for one hour, then soak for 15 minutes in 1% (v/v) glutaraldehyde solution. Wash the gel by placing it twice in an excess volume of water for 15 minutes, then immerse it for 15 minutes, protected from light, in fresh-prepared silver nitrate RS1. Wash the gel by placing it three times in an excess volume of water for 15 minutes and then immerse it for about one minute in the development solution (consisting of diluting 2.5 mL of 2% citric acid monohydrate (w/v) and 0.27 mL of formaldehyde in water and dilute to 500 mL with water) until obtaining satisfactory color.

Suspend the development by immersion for 15 minutes in a 10% (v/v) acetic acid solution. Wash with water.

Drying of stained SDS polyacrylamide gels

The treatment of gels is slightly different depending on the staining method used. In Coomassie staining, the discoloration step is followed by immersion of the gel in a 10% (w/v) glycerol solution for at least two hours (or overnight). In silver staining, the final wash is followed by immersion in a 2% (w/v) glycerol solution for five minutes. Soak two sheets of porous cellulose in water for five to ten minutes. Place one of the sheets in a drying frame. Gently lift the gel and place on the cellulose sheet. Eliminate any bubbles that may have been trapped and add a few milliliters of water along the edges of the gel. Cover with the second sheet and remove any trapped air bubbles. Finish the drying frame assembly. Place in the oven or allow to dry at room temperature.

DETERMINATION OF MOLECULAR MASS

The molecular mass of proteins is determined by comparing their mobility with the mobility of several known protein molecular mass markers. For the standardization of gels, there are mixtures of proteins of exactly known molecular masses that allow for obtaining a uniform coloration. Such blends are available for different molecular weight ranges. Concentrated stock solutions of proteins of known molecular weight are diluted in buffer for appropriate sampling and deposited on the same gel as the protein sample to be examined. Immediately after electrophoresis, determine the exact position of the marking dye (bromophenol blue) to identify the ion migration front. For this purpose, a small portion of the gel edge can be cut, or a needle wet in Indian ink can be dipped into the gel, at the level of the dye migration front. After staining the gel, determine the migration distance of each protein band (markers and unknown bands) from the upper edge of the separating gel and divide each of these migration distances by the distance traveled by the marking dye. The migration distances thus obtained are called relative protein mobilities (in reference to the staining front) and are conventionally represented by R_f . Plot a graph using the logarithms of the relative molecular mass (M_r) of the protein standards versus the corresponding R_f s. The graphics obtained are slightly sigmoid. The calculation of unknown molecular masses can be performed by linear regression, or by interpolation from the log variation curve (M_r) as a function of the R_f , as long as the values obtained for the unknown samples are located in the linear part of the graph.

Test validation

The test will only be valid if the proteins used as molecular mass markers are distributed over 80% of the length of the gel and if, within the desired separation range (for example, the range that covers the product and the dimer, or the product and the related impurities) there is for the protein bands concerned a linear relation between the log of molecular mass and the R_f value. Supplementary validation requirements regarding sample preparation may be specified in particular monographs.

QUANTITATIVE DETERMINATION OF IMPURITIES

When a particular content of impurities is specified in a monograph, it is convenient to prepare a standard solution corresponding to that content by diluting the problem solution. If, for example, this limit is 5%, the standard solution is a 1:20 dilution of the problem solution. The electrophoretogram obtained with the problem solution does not present any band due to impurities (besides the main band) that is more intense than the main band of the electrophoretogram obtained with the standard solution. As long as it operates under validated conditions, it is possible to quantify the impurities by normalization in relation to the main band, using an integrating densitometer. In this case, the linearity of the responses is verified.

5.2.22.1 CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE) is a physical analysis method based on the migration, inside a capillary, of charged solutes, dissolved in an electrolyte solution, under the influence of an electric current. Currently, CE comprises a family of electrokinetic separation methods that separate substances based, above all, on the difference in electrophoretic mobility, partition between phases, isoelectric point, molecular size, or even on the combination of one or more of these properties.

GENERAL PRINCIPLES

In CE, separation is governed by two factors. The first corresponds to the movement of solutes in the capillary due to the electric field (E), also called electrophoretic velocity. The second occurs as a function of the electrolyte flow due to the charged surface on the capillary wall, being called electroosmotic flow. The electrophoretic mobility of a solute (μ_{ep}) is related to specific characteristics such as molecular size, shape and electrical charge, as well as the inherent properties of the electrolyte in which migration occurs (electrolyte ionic strength, pH, viscosity and presence of additives). Under the influence of voltage, charged solutes migrate through the electrolyte with a given velocity, V_{ep} , given in cm/s, and calculated by the formula:

$$V_{ep} = \mu_{ep} \cdot E = \left(\frac{q}{6\pi\eta r} \right) \left(\frac{V}{L} \right)$$

where

μ_{ep} = electrophoretic mobility;

E = applied voltage;

q = effective charge of the solute;

η = electrolyte viscosity;

r = Stokes radius;

V = voltage applied to the system;

L = total capillary length.

When an electric field is applied across the capillary, an electrolyte flow is generated inside the capillary. The migration of different solutes along the capillary towards the detector, regardless of the presence of ionic charge, indicates that in addition to electrophoretic mobility, an additional force is involved. Without this additional force, positively charged compounds would migrate through the capillary while the anions would remain at a distance from the detector and the neutral solutes would simply not migrate. The additional force that directs all solutes through the capillary is called electroosmotic flow (EOF) and plays an important role in different types of CE.

EOF originates from the ionization of the silanol groups in the inner wall of the capillary, which are transformed into silanoate groups (Si-O⁻), at a pH above three. These negatively charged groups attract the electrolyte cations, forming an inner layer on the capillary wall. The double layer formed near the capillary surface is essentially static. The more diffuse layer, close to the double layer, is mobile and, under the action of an electrical voltage, migrates towards the cathode, carrying the hydration water together. Between the two layers there is a friction plane and the electrical imbalance generated corresponds to the potential difference that crosses the two layers, called zeta potential (ζ).

The speed of the electroosmotic flow is dependent on the electroosmotic mobility (μ_{eo}) which, in turn, is directly related to the charge density of the inner wall of the capillary and the characteristics of the electrolyte. The velocity of the electroosmotic flow (V_{eo}) can be calculated by the formula:

$$V_{eo} = \mu_{eo} \cdot E = \left(\frac{\varepsilon \cdot \zeta}{\eta} \right) \left(\frac{V}{L} \right)$$

where

ε = electrolyte dielectric constant;

ζ = capillary surface zeta potential;

η = electrolyte viscosity;

V = voltage applied to the system;

L = total capillary length.

The electrophoretic and electroosmotic mobilities of a solute can act in the same direction or in opposite directions, depending on the charge (positive or negative) of the solute and the speed of the solute (v), according to the formula below:

$$V = V_{ep} \pm V_{eo}$$

The sum or difference between the two speeds is used depending on whether the mobilities act in the same direction or in opposite directions. In capillary electrophoresis, in its most usual form, anions will migrate in the opposite direction to the electroosmotic flow and their velocities will be lower than the velocity of the electroosmotic flow. Cations will migrate in the same direction as the electroosmotic flow and their velocities will be higher than the velocity of the electroosmotic flow. In this condition, in which there is a fast electroosmotic flow velocity in relation to the electrophoretic velocity of solutes, cations and anions can be separated in the same electrophoretic run.

The time (t) required for the solute to migrate a distance (l) from the capillary injection terminal to the capillary detection window (effective capillary length) is defined by the formula:

$$t = \frac{l}{V_{ep} \pm V_{eo}} = \frac{l(L)}{V(U_{ep} \pm U_{eo})}$$

where

l = distance from the capillary injection terminal to the capillary detection window (effective capillary length);

V_{ep} = electrophoretic speed;

V_{eo} = speed of electroosmotic flow.

The reproducibility of solute migration speed is directly related to the maintenance of a constant value of the electroosmotic flux between different electrophoretic runs. For some specific applications, it

may be necessary to reduce or even suppress the electroosmotic flow through changes in the capillary wall or in the concentration, composition and/or pH of the electrolyte solution.

After introducing the sample into the capillary, each sample solute migrates along with the electrolyte as an independent band, according to its intrinsic mobility. Under ideal conditions, the only factor that can contribute to the widening of the band comes from the molecular diffusion of the solute along the capillary (longitudinal diffusion). In this case, the band performance is expressed as the number of theoretical plates (N) according to the formula:

$$N = \frac{(\mu_{ep} \pm \mu_{eo}) \cdot (Vl)}{2DL}$$

where

D = molecular diffusion coefficient of the solute in the electrolyte;

The remaining terms were covered earlier.

The separation between two bands can be achieved by modifying the electrophoretic mobility of the solutes, by the electroosmotic flow and by increasing the performance of the bands of each solute under analysis. The resolution can be calculated by the formula:

$$R_s = \frac{\sqrt{N}(\mu_{epb} - \mu_{epa})}{4(\mu_{ep} + \mu_{eo})}$$

where

μ_{epa} and μ_{epb} = electrophoretic mobilities of two solutes to be separated;

μ_{eo} = mobility of electroosmotic flow;

μ_{ep} = average electrophoretic mobility of solutes $\left(\frac{\mu_{epb} - \mu_{epa}}{2}\right)$

APPARATUS

Capillary electrophoresis apparatus is composed of:

- a high voltage source;
- two electrolyte reservoirs, kept at the same level, containing anodic and cathodic solutions;
- two electrodes (cathode and anode), immersed in the electrolyte reservoirs and connected to the high voltage source;
- a fused silica capillary provided with a detection window for alignment to certain types of detectors. The capillary ends are immersed in the reservoirs containing the electrolyte solutions. The capillary must be filled with the electrolyte solution prescribed in the monograph;
- system for injection of the solute(s) sample by hydrodynamic or electrokinetic action. The choice of the injection process and its automation are essential in quantitative analysis by capillary electrophoresis. The introduction of the sample through the electrokinetic mode must take into account the intrinsic electrophoretic mobility of each solute, allowing adequate discrimination of the different components of the sample;

- detector capable of monitoring the amount of solutes that pass through the capillary detection segment in a specific time range. The most common detectors are based on absorption spectrophotometry (UV and UV-VIS) or fluorimetry. Analyses can also be carried out using electrochemical detectors or by coupling with mass spectrometry;
- temperature control system capable of keeping it constant inside the capillary. Changes in temperature imply a lack of reproducibility in the separation of solutes;
- computerized system for recording and integrating electropherograms.

The monograph for each substance should detail the capillary type, electrolyte solutions, preconditioning method, sample conditions and electrophoretic migration.

The electrolyte solution must be filtered (0.45µm filter) to remove particles and de-aerated to avoid the formation of bubbles that could interfere with the detection system or break electrical contact in the capillary during electrophoretic migration. Electrophoretic methods must establish a detailed capillary washing procedure between each run to allow reproducible migration times of the solutes under analysis.

5.2.22.1.1 CAPILLARY ELECTROPHORESIS IN FREE SOLUTION

PRINCIPLE

In this method, solutes are separated in a capillary containing only electrolyte, without any anticonvective medium. The separation mechanism is based on the differences presented by the charge/mass ratio of the analyzed species that migrate as bands at different speeds. Solute are separated by the combination of intrinsic electrophoretic mobility and the magnitude of electroosmotic flow in the capillary. Internally coated capillaries, with reduced electroosmotic flow, can be used to increase the separation capacity of solutes that adsorb on the capillary surface.

The free solution method is suitable for the analysis of low molecular mass ($PM < 2000$) and high molecular mass ($2000 < PM < 100\,000$) solutes. Due to the high performance of the system, molecules with minimal differences in their mass/charge ratio can be discriminated. The method also allows for the separation of chiral solutes by adding chiral selectors to the separation electrolyte. Separation optimization requires the evaluation of different instrumental and electrolyte solution-related parameters.

INSTRUMENTAL PARAMETERS

Voltage – the separation time is proportional to the applied voltage. However, an increase in the voltage used can cause excessive heat production (*Joule effect*), causing an increase in temperature and viscosity gradients in the electrolyte inside the capillary, which are responsible for widening the band and reducing the resolution of the solutes under analysis;

Polarity – electrode polarity can be normal (inlet anode and outlet cathode). In this case the electroosmotic flow moves towards the cathode. If the electrode polarity is reversed, the direction of the electroosmotic flow is opposite to the exit and only charged solutes with electrophoretic mobility greater than that of the electroosmotic flow migrate towards the exit;

Temperature – the main effect of temperature is observed on the viscosity and electrical conductivity of the electrolyte. Changes in these two electrolyte properties determine differences in migration speed;

Capillary – the length and internal diameter influence analytical parameters such as total solute migration time, separation performance and loading capacity. Under constant voltage, the increase in the total and effective length of the capillary can reduce the electrical current, which, in turn, determines the increase in the migration time of the analytes. Capillaries with a smaller internal diameter have a better capacity to dissipate the heat generated by the electrical current (*Joule effect*), allowing for an increase in the applied voltage and a reduction in the analysis time. The detection limit of the method can also be influenced by the internal diameter, depending on the injected sample volume and the detection system used. The performance of separations can also be increased by reducing the internal diameter of the capillary.

Adsorption of sample components to the inner wall of the capillary can limit performance. For this reason, strategies to avoid these interactions should be considered when developing a capillary electrophoresis separation method. This is a critical factor, for example, in samples containing proteins. One of these strategies (using extreme pH(s) and adsorption of positively charged electrolytes) requires modifying the electrolyte composition to prevent adsorption of proteins. Alternatively, it is possible to cover the inner wall of the capillary with a polymer through covalent bonds, preventing the interaction of proteins with the surface of the negatively charged silica. For this proposal, capillaries with the inner wall previously covered with polymers of neutral-hydrophilic, cationic and anionic nature are commercially available.

PARAMETERS OF THE ELECTROLYTIC SOLUTION

Buffer nature and concentration – Electrolytes for capillary electrophoresis must have adequate buffering capacity in the chosen pH range and low mobility, in order to minimize the generation of electrical current. To decrease the distortion of the electrophoretic peak, matching the mobility of the electrolyte ion with the mobility of the solute is required. The choice of sample solvent is important to achieve solute uniformity, which allows for increased separation performance and improved detection. Furthermore, an increase in the concentration of the electrolyte at a specific pH determines a decrease in the electroosmotic flow and solute velocity.

Electrolyte pH – The pH of the electrolyte can affect separation by modifying the charge of solute or other additives, as well as altering the electroosmotic flow. The change in the pH value of the electrolyte above or below the isoelectric point of proteins and peptides influences the separation of these solutes, by changing the net charge from negative to positive. In general, an increase in the electrolyte pH causes an increase in the electroosmotic flow.

Organic solvents – Organic solvents, such as methyl alcohol, acetonitrile, among others, can be added to the aqueous electrolyte to increase the solubility of the solute and/or other additives present in the electrolyte, or even influence the degree of ionization of the solutes in the sample. The addition of these solvents to the electrolyte usually causes a reduction in the electroosmotic flow.

Additives for chiral separations – Enantiomeric separations should be carried out by adding chiral selectors to the running electrolyte. The most used chiral selectors are cyclodextrins. However, crown ethers, polysaccharides and proteins can also be used for this purpose. Enantiomeric discrimination is governed by different interactions between the chiral selector and each of the enantiomers of the solute under analysis. Accordingly, the correct choice of the selector directly influences the enantiomeric resolution obtained for chiral solutes. During the development of a method for enantiomeric separation, it is recommended to test cyclodextrins of different cavity sizes, (a, b, g),

cyclodextrins modified with neutral groups (methyl, ethyl, hydroxyalkyl, etc.), or with ionizable groups (aminomethyl, carboxymethyl, sulfobutylether, etc.). The resolution of chiral separations is also controlled by the concentration of the chiral selector, the composition and pH of the electrolyte, and the analysis temperature. Organic additives such as methyl alcohol and urea can be used to modify the obtained resolution.

5.2.22.1.2 MICELAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)

PRINCIPLE

In micellar electrokinetic chromatography, separation occurs in an electrolytic solution that contains a surfactant at a concentration above the critical micellar concentration (CMC). The solute molecules are distributed between the electrolyte and the pseudo-stationary phase composed of micelles, according to the solute partition coefficient. It is a method that can be used for the separation of neutral and/or ionized solutes, maintaining the performance, speed and instrumental suitability of capillary electrophoresis. The anionic surfactant sodium dodecyl sulfate (SDS) is one of the most used surfactants in MEKC, although others are also used, such as cationic surfactants (cetyltrimethylammonium salts).

At neutral or alkaline pH, a strong electro-osmotic flow is generated, moving the separating electrolyte ions towards the cathode. If SDS is used as a surfactant, the electrophoretic migration of the anionic micelle will be in the opposite direction, towards the anode. As a result, the total micellar migration speed is reduced compared to the flow of the electrolyte solution. In neutral solutes, since the analyte can be distributed between the micelle and the electrolyte, and there is no electrophoretic mobility, the rate of migration of the analyte will only depend on the partition coefficient between the micelle and the electrolyte. In the electropherogram, the peaks corresponding to each neutral solute are always located between the electroosmotic and micelle flow marker (the time between these two peaks is called separation window). For ionized solutes, the migration speed depends on the partition coefficient of the solute between micelle and electrolyte and on the electrophoretic mobility of the solute in the absence of the micelle.

In MEKC, the mechanism of neutral and weakly ionized solutes is essentially chromatographic. Therefore, solute migration and resolution can be represented in terms of the solute retention factor (k), also called the mass distribution ratio (D_m), which is the ratio between the number of moles of the solute within the micelle and in the mobile phase. For a neutral substance, k can be calculated using the following formula:

$$k = \frac{t_R - t_0}{t_0 \times \left(1 - \frac{t_R}{t_{mc}}\right)} = K \times \frac{V_S}{V_M}$$

where

t_R = solute migration time;

t_0 = migration time of an unretained solute (determined by injection of an electroosmotic flow marker that does not bind to the micelle, e.g. methyl alcohol);

t_{mc} = micelle migration time (determined by the injection of a micelle marker, such as Sudan III, which continuously migrates associated with the micelle throughout the electrophoretic migration);

K = solute partition coefficient;

V_S = micellar phase volume;

V_M = mobile phase volume;

Likewise, the resolution between 2 adjacent peaks (R_s) is given by:

$$R_s = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k_b}{k_b + 1} \times \frac{1 - \left(\frac{t_0}{t_{mc}}\right)}{1 + k_a \left(\frac{t_0}{t_{mc}}\right)}$$

where

N = Number of theoretical plates of each solute;

α = selectivity;

k_a and k_b = retention factors for both solutes respectively ($k_b > k_a$).

Similarly, but not identically, the formulas provide values of k and R_s for charged solutes.

OPTIMIZATION

The development of methods by MEKC involves instrumental and electrolyte solution parameters:

Instrumental parameters

Voltage – the separation time is inversely proportional to the applied voltage. However, an increase in voltage can cause excessive heat production, increasing the temperature and viscosity gradients of the electrolyte in the capillary cross-section. This effect can have a relevant impact on electrolytes with higher conductivity, such as those containing micellar systems. Systems that have lower heat dissipation capacity determine band broadening and lower resolution between peaks.

Temperature – Changes in capillary temperature affect the solute partition coefficient between the electrolyte and micelles, the critical micelle concentration, and the electrolyte viscosity. These parameters directly influence the solute migration time during electrophoretic separation. The use of an adequate refrigeration system increases the reproducibility of solute migration time.

Capillary – Capillary dimensions (length and internal diameter) contribute to analysis time and performance of separations. Under constant voltage, the increase in the total and effective length of the capillary can decrease the electrical current, which, in turn, determines the increase in the migration time of the analytes. The internal diameter of the capillary controls the dissipation of heat (in a given electrolyte and electrical current) and consequently the widening of the solute bands.

Electrolyte Solution Parameters

Nature of the surfactant and concentration – The nature of the surfactant, analogous to the stationary phase in chromatography, affects the resolution, as it modifies the selectivity of the separation. The log k of a neutral substance increases linearly with the concentration of the surfactant in the mobile phase. Since the resolution in MEKC reaches a maximum when k has a value close to

$$\sqrt{t_{mc}/t_0}$$

Modifications in the concentration of surfactant present in the mobile phase determine changes in the resolution of the bands.

Electrolyte pH – pH does not change the partition coefficient of non-ionized solutes, but may determine changes in electroosmotic flow in uncoated capillaries. A decrease in electrolyte pH

reduces the electroosmotic flow, providing an increase in the resolution of neutral solutes and in the analysis time.

Organic solvents – organic solvents (methyl alcohol, propanol, acetonitrile) can be added to the electrolyte solution to improve the separation of hydrophobic solutes. In general, the addition of these modifiers reduces migration time and separation selectivity. The percentage of added organic solvent must take into account the critical micellar concentration of the surfactant, considering that excessive values can affect or even inhibit the micelle formation process and, therefore, the absence of the partition phenomenon. The dissociation of micelles in the presence of high percentages of modifier does not necessarily mean better results in the separation. In certain situations, the hydrophobic interaction between the surfactant monomer and neutral solutes form solvophobic complexes that can be electrophoretically separated.

Modifiers for chiral separations – the separation of enantiomers in MEKC can be achieved through the inclusion of chiral selectors to the micellar system, covalently linked to the surfactant or added to the separation electrolyte. Micelles that have bonds with chiral discrimination properties include salts of *N*-dodecanoyl-*L*-amino acids, bile salts, among others. Chiral resolution can also be achieved through chiral selectors, such as cyclodextrins, added directly to electrolyte solutions containing non-chiral surfactants.

Other Additives – Selectivity can be modified through various strategies, by adding chemicals to the electrolyte. The addition of several types of cyclodextrins to the electrolyte can also be used to reduce the interaction of hydrophobic solutes with the micelle, thus increasing the selectivity for this type of solute.

The addition of substances capable of modifying the solute-micelle interactions by adsorption in the latter has been used to increase the selectivity of separations in MEKC. These additives can be a second surfactant (ionic or non-ionic) that originate a mixture of micelles or metallic cations that dissolve the micelle forming coordination complexes with the solutes.

QUANTIFICATION

The areas under the peaks must be divided by the corresponding migration time to provide the correct area in order to:

- compensate for the shift in migration time between runs, thus reducing response variation;
- compensate for the different responses of the sample components with different migration times.

When an internal standard is used, it must be verified that no solute peak to be analyzed overlaps with the peak of the internal standard.

CALCULATIONS

The content of the component (or components) under analysis must be calculated from the values obtained. When prescribed, the percentage content of one or more components of the sample to be analyzed is calculated by determining the corrected area(s) of the peak(s) as a percentage of the total corrected areas of all peaks, excluding those resulting from solvents or added reagents (standardization process). It is recommended to use an automatic integration system (integrator or data acquisition and processing system).

SYSTEM SUITABILITY

The suitability parameters of the system are used to verify the behavior of the method by capillary electrophoresis. The choice of these parameters depends on the type of Capillary Electrophoresis used. The factors are: retention factor (k) (only for micellar electrokinetic chromatography), apparent number of theoretical plates (N), symmetry factor (A_s) and resolution (R_s). The formulas that allow you to calculate the values of N and R_s through the electropherograms are given below.

Apparent number of theoretical plates

The apparent number of theoretical plates (N) can be calculated using the expression:

$$N = 5,54 \times \left(\frac{t_R}{w_h} \right)^2$$

where

t_R = migration time or distance from the baseline from the injection point to the perpendicular line of the maximum point of the peak corresponding to the component;

w_h = peak width at half height

Resolution

The resolution (R_s) between peaks of similar heights of 2 components can be calculated using the expression:

$$R_s = \frac{1,18 \times (t_{R2} - t_{R1})}{w_{h1} + w_{h2}}$$

$$t_{R2} > t_{R1}$$

where

t_{R1} and t_{R2} = migration times or baseline distances from the injection point to the perpendicular line of the maximum point of two adjacent peaks

w_{h1} and w_{h2} = peak width at half height

Where appropriate, resolution can be calculated by measuring the valley height (H_v) between 2 partially resolved peaks in a standard preparation and the height of the smaller peak (H_p), calculating the peak/valley ratio (p/v):

$$\frac{p}{v} = \frac{H_p}{H_v}$$

Symmetry factor

The symmetry factor (A_s) of a peak can be calculated using the expression:

$$A_s = \frac{w_{0,05}}{2d}$$

where

$w_{0,05}$ = peak width determined at 5% of height value;

d = distance between the perpendicular line of the maximum peak and the tangent of the peak at 5% of the height of the peak.

Tests for area repeatability (standard deviation of areas or the area/migration time ratio) and for migration time repeatability (standard deviation of migration time) are introduced as suitability parameters. The repeatability of the migration time provides a test for the adequacy of capillary washing procedures. An alternative practice to avoid lack of repeatability of the migration time is to use the migration time relative to an internal standard.

A test to check the signal-to-noise ratio of a standard preparation (or determination of the limit of quantification) can also be useful for the determination of related substances.

Signal: noise ratio

The limits of detection and quantification correspond to the signal:noise ratio of 3 and 10, respectively. The signal:noise ratio (S/N) is calculated using the expression:

$$\frac{S}{N} = \frac{2H}{h}$$

where

H = peak height corresponding to the specific component, in the electropherogram obtained with the reference solution, measured from the peak maximum to the extrapolated baseline of the observed signal over a distance equal to 20 times the width at half height of the peak;

h = baseline range in an electropherogram obtained after blank injection, observed at a distance equal to 20 times the width at half height of the peak in the electropherogram obtained with the reference solution, and if possible, located close to the retention time where this peak would be found.

5.2.23 ENANTIOMERIC ANALYSIS

CHIRAL DRUGS

Enantiomers generally exhibit different pharmacological and toxicological properties as the main molecular targets, such as proteins, nucleic acids and polysaccharides, are chiral. For example, the methyl ether enantiomers of levorphanol, dextromethorphan and levomethorphan are used differently in therapy. While dextromethorphan is indicated as an antitussive, levomethorphan is indicated as an analgesic.

Due to the recognition of the importance of the clinical use of enantiomerically pure drugs in the treatment of several diseases, pharmaceutical laboratories are constantly encouraged to make resolved drugs available in industrial quantities.

To ensure the safety and efficiency of drugs available and under development, it is necessary to resolve the enantiomers and examine each one for pharmacological and toxicological activities. After identifying the most active enantiomer (eutomer), the enantiomeric excess of the eutomer from synthesis to consumption must be evaluated to ensure the quality of the drug.

SEPARATION AND ENANTIOMERIC DETERMINATION OF DRUGS

Separation, or resolution, of enantiomers by high performance liquid chromatography (HPLC) began to be applied in the 1960s. In the seventies, with the advent of small particle columns for liquid chromatography, the development of chiral stationary phases for the resolution of racemic drugs started.

HPLC is considered one of the most efficient methods for the separation, detection and quantification of drugs. The use of adequate chiral stationary phase (CSP) becomes a powerful method for the separation of enantiomers.

The chromatographic resolution of enantiomers can be achieved by several methods, however, the use of some type of discriminator or chiral selector is always required. The indirect and direct methods are the two manners to separate the enantiomers using liquid chromatography.

In the indirect method, enantiomers are converted to diastereoisomers by reaction with a chiral substance. Diastereoisomers are substances that have different physicochemical properties and, therefore, can be separated using a non-chiral stationary phase.

The indirect method was widely used in the past. However, it has limitations such as the need to isolate the substance of interest and the related derivatization. These facts make it difficult to develop the automated process for large numbers of samples. Furthermore, the enantiomeric purity of the derivatizing agents is important to avoid false results. Another limitation is the different reaction rates and/or constants for the enantiomers since the reaction transition states are diastereoisomeric, which can result in a different proportion of the initial enantiomeric composition.

In the direct method, the mixture of enantiomers to be resolved is injected directly into the chromatograph. For the separation of the enantiomers, a CSP, a chiral solvent, or a mobile phase with a chiral additive can be used. Resolution occurs due to the formation of diastereoisomeric complexes between the enantiomeric mixture and the chiral selector used for resolution. The use of CSP is currently the most used method for resolution by HPLC.

In the following tables (**Tables 1, 2, 3, 4 and 5**) the main classes of stationary phases used for the resolution of racemic mixtures and some examples of chiral selectors in each class are presented. Consult the manufacturer for indication of the use of each selector.

Table 1 – Pirkle-type chiral stationary phases.

<i>Chiral discriminator*</i>
(R)-DNB-phenylglycine
(S)-DNB-phenylglycine
(R)-DNB-leucina
(S)-DNB-leucina
DNB-□-amino-2,2-dimethyl-4-pentynyl dimethyl phosphonate
DNB-tetrahydrophenanthrene
(Naphthyl) ethylamide

* Most Pirkle-type columns are available in both enantiomeric forms.

Table 2 – Protein-based chiral stationary phases.

<i>Chiral discriminator</i>
α1-acidic glycoprotein
Bovine serum albumin
Human serum albumin
Cellobiohydrolase I
Pepsin
Ovomucoid

Table 3 – Chiral stationary phases of cavity or inclusion type.

<i>Chiral discriminator</i>
α-Cyclodextrin
β-Cyclodextrin
γ-Cyclodextrin
O-(S)-2-Hydroxypropyl-β-cyclodextrin
O-(R/S)-2-Hydroxypropyl-β-cyclodextrin
O-(S)-Naphthylethylcarbamoyl-β-cyclodextrin

Table 4 – Carbohydrate-type chiral stationary phases.

<i>Chiral discriminator</i>
Tris (dimethylphenylcarbamoyl)cellulose
Tris (4-methylbenzoate)cellulose
Tris (phenylcarbamoyl)cellulose
Cellulose triacetate
Cellulose tribenzoate
Cellulose tribenzyl ether
Cellulose Tricinnamate

Table 5 – Macrocyclic antibiotic chiral stationary phases

Chiral discriminator

Vancomycin

Teicoplanin

Ristocetin

5.2.24 WATER CONDUCTIVITY

Water electrical conductivity is a measure of electrons flow, which is facilitated by the presence of ions. Water molecules dissociate into ions as a function of pH and temperature, resulting in a certain conductivity. Some gases, especially carbon dioxide, dissolve in water and interact to form ions that affect water conductivity and pH. These ions and their resulting conductivity can be considered to be intrinsic to water. Exposing the sample to the atmosphere can change the conductivity/resistivity due to the loss or gain of dissolved gases.

Chloride and ammonium ions are some of the main impurities found in water and also influence its conductivity. These external ions can significantly impact the chemical purity of water and compromise its use in pharmaceutical applications.

The combined conductivities of dry intrinsic ions and external ions vary according to pH and are the basis for the conductivity specifications described in **Table 3** and used when running step 3 of the test. Two preliminary steps are included in this test. If the test conditions and conductivity limits are met in any of these preliminary steps (Steps 1 and 2), water meets the requirements of this test and the application of Step 3 is not required. Only in case the sample does not meet the requirements of Step 3, water is judged not to comply with the requirements of the conductivity test.

OPERATIONAL INSTRUMENTATION AND PARAMETERS

The conductivity of water must be measured using calibrated instruments with a resolution of 0.1 $\mu\text{S}/\text{cm}$. The thermometer should have 0.1°C divisions and cover the range of 23°C to 27°C. Electrodes must be maintained as recommended by the device manufacturer.

The cell conductivity constant is a factor used as a multiplier for the conductivity meter scale values.

Cell constant: the value must be known in $\pm 2\%$. Generally conductivity cells show constants of 0.1 cm^{-1} , 1 cm^{-1} and 2 cm^{-1} . Most devices show the defined cell constant. It is necessary to measure this constant with the reference KCl solution described in **Table 1**. Verification is normally carried out using only a reference solution; in this case, use the lowest conductivity reference solution. However, it is recommended to periodically measure the conductivity of the other standards and observe the agreement between the conductivity meter reading and the nominal value of each reference solution.

Calibration: as per manufacturer's instructions. Since most multi-scale apparatus has a single calibration point, calibration is required whenever you use a different scale. The reading obtained must be within $\pm 0.1 \text{ mS}/\text{cm}$ of the nominal value of the reference solution.

To calibrate the conductivity meter, use the reference solutions described below.

Solution A (0.01 M): weigh exactly 0.7455 g of dry potassium chloride at 105 °C for two hours, transfer to a 1000 mL volumetric flask, adjust volume with water and homogenize.

Solution B (0.005 M): pipet 50 mL of *Solution A* into a 100 mL volumetric flask, adjust with water and homogenize.

Solution C (0.001 M): pipette 10 mL of *Solution A* into a 100 mL volumetric flask, adjust with water and homogenize.

Solution D (0.0005 M): pipet 5 mL of *Solution A* into a 100 mL volumetric flask, adjust with water and homogenize.

Solution E (0.0001 M): pipet 5 mL of *Solution A* into a 500 mL volumetric flask, adjust with water and homogenize.

Note 1: for the preparation of the solutions above, always use carbon dioxide-free water with a conductivity inferior to $0.10 \mu\text{S}\cdot\text{cm}^{-1}$.

Note 2: Do not use temperature compensation and keep reference solutions at 25°C during reading.

Table 1 – Conductivity of potassium chloride solutions (25°C).

<i>Solution</i>	<i>Concentration (mole/L)</i>	<i>Conductivity ($\mu\text{S}/\text{cm}$)</i>
A	0.01	1412
B	0.005	717.5
C	0.001	146.9
D	0.0005	73.9
E	0.0001	14.9

PROCEDURE

The procedure described below is established for measurements of purified water and water for injections. Alternatively, Step 1 can be performed (with appropriate modifications, in accordance with item 1 of Step 1) using "in-line" type instrumentation that has been properly calibrated, whose cell constants have been exactly determined, and whose temperature compensation functions have been disabled. The suitability of such "in-line" instruments for quality control testing is also dependent on their location in the water system. Obviously, the placement of the instrument needs to reflect the quality of the water that will be used.

Step 1

- 1 Rinse the cell with at least three portions of the sample.
- 2 The determination must be carried out in an appropriate container or as an "in-line" determination. The value obtained must be inferior to $1.3 \mu\text{S}/\text{cm}$, at a temperature of $(25.0 \pm 0.1)^\circ\text{C}$.
- 3 In **Table 2**, find the closest and lowest temperature value to the temperature at which the conductivity was measured. The conductivity value corresponding to this temperature is the limit. (Do not interpolate).
- 4 If the measured conductivity value is not greater than the corresponding value in **Table 2**, the water meets the requirements for conductivity. However, if the measured value is greater than that in the table, proceed with the determination according to *Step 2*.

Table 2 – Limit values for conductivity according to temperature (only for conductivity values without temperature compensation).

<i>Temperature ($^\circ\text{C}$)</i>	<i>Conductivity ($\mu\text{S}/\text{cm}$)</i>
0	0.6
5	0.8
10	0.9

15	1.0
20	1.1
25	1.3
30	1.4
35	1.5
40	1.7
45	1.8
50	1.9
55	2.1
60	2.2
65	2.4
70	2.5
75	2.7
80	2.7
85	2.7
90	2.7
95	2.9
100	3.1

Step 2

1 Transfer a sufficient amount of water (100 mL or more) to an appropriate container and shake the sample. Adjust to $(25 \pm 1) ^\circ\text{C}$ and shake the sample vigorously, periodically observing the conductivity meter reading. When the change in conductivity due to absorption of atmospheric carbon dioxide is lower than $0.1 \mu\text{S/cm}$ for five minutes, record the conductivity.

2 If the conductivity is not higher than $2.1 \mu\text{S/cm}$, the water meets the requirements for the conductivity test. If the conductivity is higher than $2.1 \mu\text{S/cm}$, proceed as per *Step 3*.

Step 3

Carry out this test maximum five minutes after *Step 2* with the same sample, keeping temperature at $(25 \pm 1) ^\circ\text{C}$. Add saturated potassium chloride solution (0.3 mL for 100 mL of sample) and determine the pH with an accuracy of 0.1 unit in accordance with *Determination of pH (5.2.19)*. Using **Table 3**, determine the limit value for conductivity according to pH.

Table 3 – Conductivity limit values according to pH (only for samples kept in balanced atmosphere and temperature).

<i>pH</i>	<i>Conductivity ($\mu\text{S/cm}$)</i>
5.0	4.7
5.1	4.1
5.2	3.6
5.3	3.3
5.4	3.0
5.5	2.8
5.6	2.6
5.7	2.5
5.8	2.4

<i>pH</i>	<i>Conductivity (μS/cm)</i>
5.9	2.4
6.0	2.4
6.1	2.4
6.2	2.5
6.3	2.4
6.4	2.3
6.5	2.2
6.6	2.1
6.7	2.6
6.8	3.1
6.9	3.8
7.0	4.6

After determining the pH and establishing the limit according to **Table 3**, the water meets the test if the conductivity measured in *Step 2* is not greater than this limit. If the conductivity is higher or the pH value is outside the range of 5 to 7, the water does not meet the test for conductivity.

ULTRAPURE WATER

For ultra-purified water, in general, conductivity meters or resistivity meters installed in water purification device have a temperature compensation circuit for 25.0,°C and provide direct reading. These devices must be periodically calibrated. The conductivity of ultrapurified water should be 0.055 μS/cm at 25.0°C (resistivity > 18.0 MOhm.cm) for a specific application.

Alternatively, if the apparatus does not provide a direct conductivity reading, proceed as follows:

- 1 Rinse the cell with at least three portions of the sample.
- 2 Simultaneously determine the temperature and conductivity of the water without automatic temperature compensation. The determination must be carried out in an appropriate container or as an “in-line” determination. The value obtained must be less than 0.055 μS/cm, at a temperature of (25.0 ± 0.1) °C.
- 3 In **Table 4**, find the closest and lowest temperature value to the temperature at which the conductivity was measured. The conductivity value corresponding to this temperature is the limit. (Do not interpolate).
- 4 If the measured conductivity value is not greater than the corresponding value in **Table 4**, the water meets the requirements for conductivity.

Table 4 – Limit values for conductivity according to temperature (only for conductivity values without temperature compensation).

<i>Temperature (°C)</i>	<i>Conductivity (μS/cm)</i>
0	0.012
5	0.017
10	0.023
15	0.031
20	0.042

25	0.055
30	0.071
35	0.090
40	0.113
45	0.140
50	0.171
55	0.207
60	0.247
65	0,294
70	0.345
75	0.403
80	0.467
85	0.537
90	0.614
95	0.696
100	0.785

5.2.25 CLARITY OF LIQUIDS

PROCEDURE

Use neutral, colorless and transparent glass tubes, with a flat bottom and 15 to 25 mm in internal diameter, unless otherwise indicated in the monograph. Introduce, in separate tubes, the liquid under examination and the *reference suspension* indicated in the monograph, preparing it during use, as specified in **Table 1**. The liquid under examination and the reference suspension must reach, in the tubes, a height of 40 mm. Five minutes after preparation of the *reference suspension*, compare the contents of the tubes, observing them vertically, under diffused visible light and against a black background. Light scattering must be such that the *reference suspension I* is easily distinguished from water and *reference suspension II*.

A liquid is considered clear when, when examined under the conditions described above, its transparency corresponds to that of the water or solvent used, or when its opalescence is not more pronounced than that of the *reference suspension I*.

Opalescence standard

Dissolve 1 g of hydrazine sulfate in water, adjust volume to 100 mL with the same solvent and homogenize. Allow to stand for four to six hours. Add 25 mL of this solution to a solution containing 2.5 g of methenamine in 25 mL of water. Mix well and allow to stand for 24 hours. This suspension is stable for two months if kept in a glass container, with a surface free of defects. The suspension must not adhere to the walls of the container and must be vigorously shaken in the original container before use. To prepare the *opalescence standard*, dilute 15 mL of the suspension to 1000 mL with water. The *opalescence standard* must be prepared at the time of use and can be kept for a maximum of 24 hours.

Table 1— Preparation of the reference suspensions.

<i>Reference suspension</i>	<i>I</i>	<i>II</i>	<i>III</i>	<i>IV</i>
Opalescence standard (mL)	5	10	30	50
Water (mL)	95	90	70	50

5.2.26 ALCOHOLOMETRY

Alcoholometry is the determination of the alcoholic grading or ethanol content of mixtures of water and ethyl alcohol.

The volumetric alcoholic grading of a mixture of water and alcohol is expressed by the number of volumes of ethyl alcohol at 20°C contained in 100 volumes of this mixture at the same temperature. It is expressed in % (v/v).

The weighed alcoholic grading is expressed by the ratio between the mass of ethyl alcohol contained in a mixture of water and ethyl alcohol and its total mass. It is expressed in % (w/w).

Ethyl alcohol contains minimum 95.1% (v/v), corresponding to 92.55% (w/w) and maximum 96.9% (v/v), corresponding to 95.16% (w/w) of C_2H_6O at 20°C. Ethyl alcohol contains minimum 99.5% (v/v), corresponding to 99.18% (w/w) of C_2H_6O at 20°C. These values can be seen in the alcoholometric table (Appendix D).

DETERMINATION OF ALCOHOLIC GRADING OR ALCOHOLOMETRIC CONTENT

The centesimal alcoholometer is intended to determine the alcoholic content of mixtures of water and alcohol, indicating only the concentration of alcohol in volume and expressed by its measurement unit, degree Gay-Lussac (°G.L.).

Alcoholmeter determinations are accurate only for the mixture of water and alcohol at 20°C on which the instrument was graduated. If the temperature during the test is lower or higher than 20°C, it is necessary to correct the alcohol temperature to 20°C.

The determination of the alcohol degree of water mixtures in volume is carried out by the alcoholometer.

For the determination of the alcohol degree of the mixtures of water and alcohol in mass, the relative density method can be used or the graduation in the alcoholometric table can be verified after the determination by the alcoholometer.

5.2.27 THERMAL ANALYSIS

Thermal analysis is a set of methods that enables to measure the physicochemical properties of a substance according to the temperature. The most commonly used methods are those that measure the changes in energy or mass of a substance.

THERMOGRAVIMETRY (TG)

Thermogravimetry is the thermal analysis method in which the mass variation of the sample is determined as a function of temperature, or heating time, using a temperature-controlled program.

Apparatus

It basically consists of a thermobalance that is an association between the electric oven and a high precision electronic balance, in which the substance is inserted into a sample holder under a specified atmosphere and a temperature controlled program. The device enables to simultaneously heat and measure the analyte mass. In certain cases, the device can be associated with a system that makes it possible to detect and analyze volatile products.

Thermobalance calibration and/or gauging. Transfer an adequate amount of CRS calcium oxalate monohydrate into the sample holder. The thermobalance will indicate its mass with great precision and accuracy. Use a heating rate of 10°C/minute and heat the sample to 900°C. At the end of the thermal process, record: i) the thermogravimetric curve (TG), marking the temperature on the abscissa axis (increasing values from left to right) and the percentage mass of the sample on the ordinate axis (increasing values from bottom to top); ii) the derived thermogravimetric curve (DTG), first derived from the TG curve, which enables to better define where the loss of mass began and ended. Determine, on the graph, the distance between the initial and final levels of the mass-temperature curve, the distance that represents the loss of sample mass in the given temperature range. The reported mass losses of CRS calcium oxalate monohydrate are calculated stoichiometrically from the three stages of mass losses due to serial releases of: a) H₂O; b) CO; c) CO₂. The verification of the temperature scale can be carried out using the melted metal hook method (In, Pb, Zn, Al, Ag and Au) according to the manufacturer's instructions.

Procedure

Use the same method as described for calibration and/or gauging by adding an adequate amount of sample. The TG and DTG curves, illustrated in **Figure 1**, indicate a stage of sample mass loss. On the DTG curve, the initial plateau is between points **ab**. The loss of mass starts at point **b** and ends at point **c**. The final plateau is between the **cd** points. Range **bc** corresponds to the reaction range. To calculate the sample mass loss on the TG curve, comparison with the DTG curve is used for greater precision in locating points **b** and **c**. Plot the extensions of the initial and final levels of the TG curve on the ordinate axis using points **b** and **c**. The measured distance corresponds to the loss of mass (Dm) of the sample. The projections of points **b** and **c** on the abscissa axis correspond, respectively, to the initial (Ti) and final (Tf) temperature of the mass loss. Record the result as a percentage of the w/w ratio.

Note 1: It is necessary to obtain a blank test curve (heating under the same experimental conditions using the empty sample holder) before testing the sample for baseline subtraction.

Note 2: in case of frequent use of the device, check and/or calibrate regularly. Otherwise, perform these operations before each determination.

Note 3: As the atmosphere can affect the results, the flow and gas composition are recorded for each test.

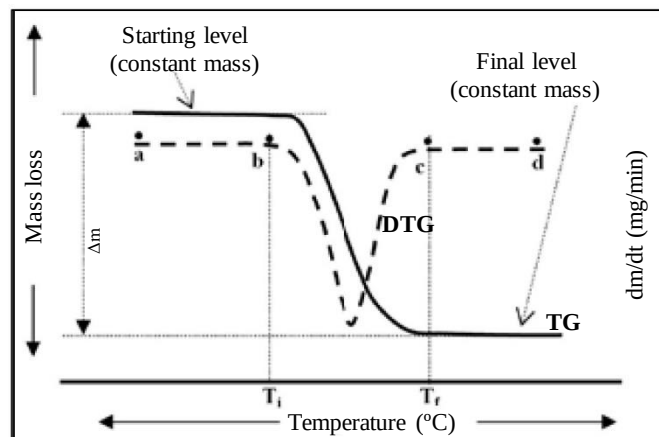


Figure 1 – Example of thermogravimetric curve and its measurements.

Uses

The determination of mass variation for a substance at certain temperature ranges can be used to assess thermal behavior; determination of moisture content and/or solvents; determination of boiling and sublimation temperature; determination of thermal decomposition temperature and determination of ash content.

DIFFERENTIAL SCANNING CALORIMETRY (DSC)

Differential scanning calorimetry is a technique that enables to evaluate the energetic, physical and/or chemical phenomena produced during the heating (or cooling) of a substance. This method enables measuring the differential heat flow between the sample and a thermally inert reference material as a function of temperature and/or heating time under a controlled temperature program. The sample and reference material are kept at approximately the same temperature during the experiment. The enthalpy variations can be determined; the changes in specific heat and temperature of endothermic and exothermic events. According to the measurement method used, there are two modes: DSC with power compensation and DSC with heat flow.

APPARATUS

The power-compensated DSC consists of a calorimetric cell that contains two ovens, one for the reference material and the other for the sample. The DSC with heat flow consists of a calorimetric cell containing a single oven that has a calorimetric sensor for reference and sample. The apparatus consists of a temperature-controlled programming device, one or more thermal detectors and a recording system that can be associated with a data processing system. Determinations are carried out under specified atmosphere.

Equipment calibration and/or gauging. Calibrate the instrument for the temperature and heat flow axis using high purity indium metal or any other suitable certified material in accordance with the manufacturer's instructions. To adjust the linearity, a combination of two metals such as indium and zinc is used to measure the temperature axis.

PROCEDURE

Transfer a precisely known quantity of the sample to a suitable sample holder. Fix the initial and final temperature of the test and the heating rate. Start heating. After the test, record the differential scanning calorimetry curve, writing on the abscissa axis the temperature or time (increasing values from left to right) and the heat flow on the ordinate axis, indicating the direction (endothermic or exothermic). In the DSC curve illustrated in **Figure 2**, the enthalpic variation between the points **a** and **d** is observed. The intersection point **b**, referring to the extension of the baseline with the tangent at the point of greatest slope (inflection point) of the curve, corresponds to the *onset* temperature (extrapolated start of the event, T_{onset}), used in melting events such as initial temperature of the change of state. The end of the thermal event is marked by point **c** (T_{peak}). However, for curve area calculation purposes, the point **d** (T_{final}) is considered. The enthalpy variation (ΔH) of the phenomenon is proportional to the area under the curve limited by the points **a**, **c**, and **d**, the proportionality factor being determined from the determination of the melting enthalpy of a known standard substance (indium, for example) under the same working conditions. Each thermoanalytical curve is recorded containing the following data: indication of the last calibration, sample size and identity, type of sample holder, reference material, atmosphere (flow rate and gas composition), heating rate and sensitivity of the calorimetric cell.

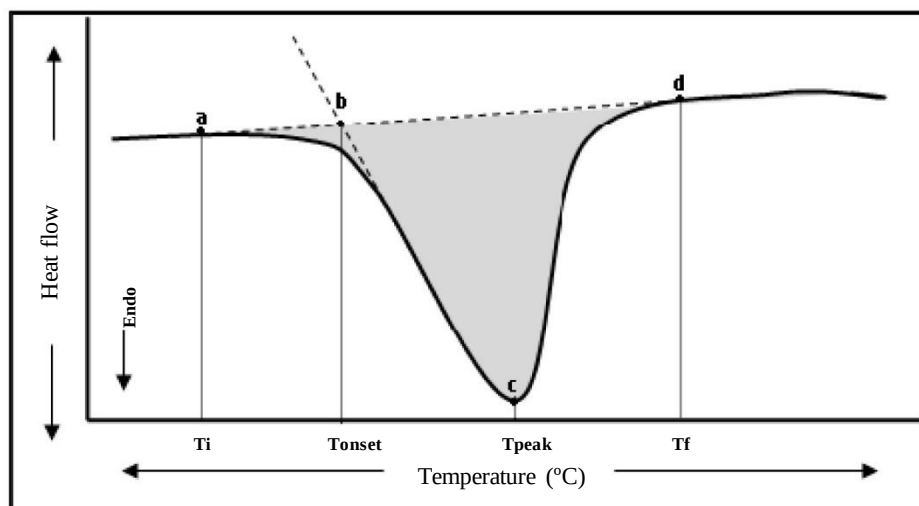


Figure 2 – Example of a typical DSC curve and its measurements.

Uses

The evaluation of the differential heat flow referring to variations in thermal capacity and enthalpy of the phase transitions of a substance as a function of temperature can be used to determine the melting point and range; determination of sublimation, evaporation and solidification temperature; determination of the glass transition temperature; polymorphism evaluation, phase diagram plotting, purity determination (except for amorphous substances, unstable polymorphs in the experimental temperature range, compounds that fuse with thermal decomposition and substances with purity lower than 95%).

Determination of purity

The method is based on the fact that the presence of small amounts of impurities in a given material lowers its melting point and widens its overall melting range. **Figure 3** illustrates this behavior for three hypothetical samples, one of which is standard and the other two contain small amounts of impurities.

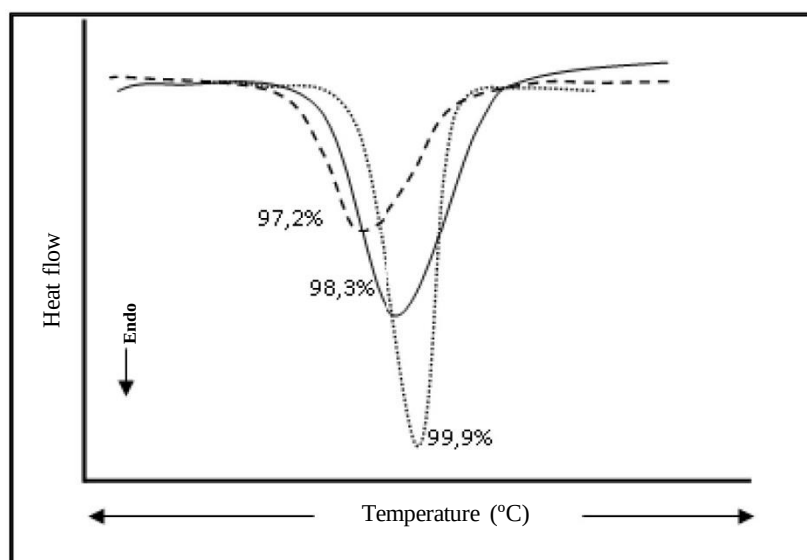


Figure 3 Example of DSC curves of a hypothetical sample with different purity levels.

Based on the van't Hoff equation (**Equation 1**), it is possible to determine the molar fraction of impurities X_2 (number of moles of impurities by the total number of moles of the sample), considering that there is no solid phase formation during the melting.

$$X_2 = \frac{(T_o - T_m)\Delta}{RT_o^2} \quad (\text{Equation 1})$$

where T_m represents the melting temperature of the sample; T_o is the melting point of pure substance in Kelvin; R is the gas constant ($8.3143 \text{ J.K}^{-1} \cdot \text{mole}^{-1}$); ΔH_f is the melting heat of the main component expressed in J.mole^{-1} .

When there is no solid phase formation, the concentration of impurity in the liquid phase, at a given temperature during melting, is inversely proportional to the melted fraction at that temperature, and the decrease in the melting point is directly proportional to the molar fraction of impurity. The graph of the sample temperature (T_s) versus the inverse of the molten fraction ($1/F$), at the temperature T_s , results in a straight line with a slope equal to the decrease in the melting point ($T_o - T_m$). The theoretical melting point of the pure substance can be obtained by extrapolation when $1/F = 0$.

$$T_s = T_o - \frac{RT_o^2 X_2 (1/F)}{\Delta H_f} \quad (\text{Equation 2})$$

By substituting the experimental values obtained for $T_o - T_m$, ΔH_f and T_o in **equation 1**, it is possible to calculate the molar fraction of the impurities in the sample.

5.2.28 DETERMINATION OF OSMOLALITY

Osmolality is a unit of concentration that refers to the number of osmotically active particles of solute present in a kilogram of solvent and provides a measure of the contribution of various solutes present in the solution to the osmotic pressure. An acceptable approximation of the osmolality in aqueous solution is given by: $\epsilon m = \nu m \Phi$, if the solute is not ionized, $\nu = 1$; however ν is the total number of ions always present or formed by the lysis solution of a solute molecule; m = solution molality, which is the number of moles of solute per kilogram of solvent; Φ = molar osmotic coefficient which is quantified from the interaction between oppositely charged ions in the solution. It is dependent on the value of m . If the complexity of the solution increases, Φ becomes difficult to measure. The osmolality unit is osmole per kilogram (osmole/kg), but the submultiple milliosmole per kilogram (mosmole/kg) is normally used.

As described otherwise, osmolality is determined by measuring the decrease in congealing point. There is a relation between osmolality and a decrease in the congealing point ΔT :

$$\epsilon m = \Delta T / 1.86 \times 1000 \text{ mosmole/kg}$$

APPARATUS

The apparatus – Osmometer – consists of: refrigerated container for the measurement; temperature measurement system equipped with a thermosensor, with a device for measuring different potentials that can be graduated to decrease the temperature or directly to osmolality; and a feature to homogenize the solution must be included.

PROCEDURE

Prepare the reference solution as described in **Table 1**. Adjust device to zero using water. Calibrate the apparatus using the reference solution: pipet 50 to 250 μL of the sample to be analyzed; transfer to the measuring cell and start cooling system. Typically, a homogenizing device is programmed to operate at a temperature below the expected cryoscopic temperature drop to prevent overcooling. A device indicates when equilibrium is reached. Before each measurement, rinse the measuring cell with the solution to be tested.

Table 1 – Information for preparing the reference solution for the Osmometer calibration.

<i>Mass in g of sodium chloride solution per kg of water</i>	<i>Actual osmolality (mosmole/kg)</i>	<i>Ideal osmolality (mosmole/kg)</i>	<i>Molal osmotic coefficient</i>	<i>Cryoscopic decrease ($^{\circ}\text{C}$)</i>
3.087	100	105.67	0.9463	0.186
6.260	200	214.20	0.9337	0.372
9.463	300	323.83	0.9264	0.558
12.684	400	434.07	0.9215	0.744
15.916	500	544.66	0.9180	0.930
19.147	600	655.24	0.9157	1.116
22.380	700	765.86	0.9140	1.302

Carry out the same operation with the test sample. Directly read osmolality or calculate by measuring congealing point decrease. The test is considered valid when the value found is between two values on the calibration scale.

5.2.29 PHYSICAL AND PHYSICO-CHEMICAL TESTS FOR FATS AND OILS

5.2.29.1 DETERMINATION OF RELATIVE DENSITY

Proceed as described in *Determination of Mass Density and Relative Density* (5.2.5).

5.2.29.2 DETERMINATION OF MELTING TEMPERATURE

Proceed as described in *Determination of temperature and melting range, Method III* (5.2.2).

5.2.29.3 DETERMINATION OF SOLIDIFICATION TEMPERATURE

SEPARATION OF FATTY ACIDS

Transfer 75 mL of 25% (w/v) potassium hydroxide solution in glycerol to a 1000 mL beaker and heat to 150°C. Add 50 mL of treated sample as indicated in the specific monograph and continue heating under agitation. The temperature must not exceed 150°C. Saponification is concluded when the mixture is homogeneous, without traces of particulate matter. Transfer the mixture to another 1000 mL beaker containing 500 mL of nearly boiling water. Slowly add 50 mL of 25% sulfuric acid solution (v/v) and heat, with shaking, until the defined separation of the clear phase (fatty acids). Transfer the fatty phase to a small beaker, wash it with boiling water to free it from sulfuric acid and keep it in a boiling water bath until the water decants, leaving the oil phase clear. Filter and collect the fatty acid mixture while still hot in a dry beaker and dry at 150°C for 20 minutes. Transfer the hot mixture to an appropriate flask and keep it in an ice bath until solidified.

To assess the purity degree of the fatty acids separated by the previous procedure, transfer, prior to congealing, 3 mL of the desiccated fatty acid solution to a test tube and add 15 mL of ethyl alcohol. Heat the solution to boil and add 15 mL of 6 M ammonium hydroxide. The resulting preparation must be clear.

PROCEDURE

Proceed as described in *Determination of congealing temperature* (5.2.4).

5.2.29.4 DETERMINATION OF REFRACTIVE INDEX

The refraction index n^t of a referred medium to air is equal to the ratio between the sine of the angle of incidence of a luminous ray in air and the sine of the angle of refraction of the ray refracted in the considered medium. Unless otherwise indicated, the refractive index is determined at $(20 \pm 0.5)^\circ\text{C}$ and at a wavelength of 589.3 nm, corresponding to that of sodium D-ray light. In this case, the symbol representing the refraction index is n^{20} .

Refractometers usually determine the limit angle. In some devices, the essential part is a prism of known refractive index, in contact with the liquid under test.

To calibrate the device, use the reference liquids mentioned in **Table 1**. The refractive index value of each reference liquid is indicated on its label.

Table 1 – Reference liquids in the determination of refractive index.

<i>Reference liquid</i>	$\Delta n/\Delta t$ (<i>temperature coefficient</i>)
Trimethylpentane	0.00049
Toluene	0.00056
Methylnaphthalene	0.0048

If white light is used to determine the refractive index, the refractometer has a compensation system. The device must provide accurate readings up to the third decimal place, at least, and have a device that allows it to operate at the prescribed temperature: the thermometer allows reading to the nearest 0.5 °C minimum.

5.2.29.5 DETERMINATION OF ROTATION POWER

Proceed as described in *Determination of Optical Rotation* (5.2.8).

5.2.29.6 DETERMINATION OF WATER

Proceed as described in *Volumetric Method (Karl Fischer Method)* (5.2.20.1).

5.2.29.7 ACID VALUE

The acid value, AV, expresses, in milligrams, the amount of potassium hydroxide required to neutralize the free fatty acids in 1 g of sample.

High acidity indices are suggestive of accentuated hydrolysis of the constituent esters of the fatty matter. Degradation causes include chemical treatments that are part of industrial extraction and purification processes, bacterial activity, catalytic action (heat, light), inadequate storage and the presence of impurities, such as moisture, among others.

PROCEDURE

Weigh approximately 10.0 g or exactly the prescribed amount of test substance and place in a 250 mL Erlenmeyer flask. Add 50 mL of a mixture of 96% (v/v) ethyl alcohol and ethyl ether (1:1). Unless otherwise indicated in the specific monograph, the solvent mixture must be previously neutralized with 0.1 M potassium hydroxide, or 0.1 M sodium hydroxide, in the presence of 0.5 mL of phenolphthalein TS. Heat the sample to 90°C if necessary to dissolve it. After complete solubilization titrate with 0.1 M potassium hydroxide until a pale pink color persists for minimum 15 seconds. Carry out the blank test and correct the volume of titrant consumed.

Calculate the I_A according to the formula:

$$I_A = \frac{5,610n}{m}$$

where

n = volume (in mL) of 0.1 M potassium hydroxide used in the titration;

m = sample mass in g.

5.2.29.8 DETERMINATION OF SAPONIFICATION VALUE

The saponification value S_v expresses, in milligrams, the amount of potassium hydroxide required to neutralize the free acids and hydrolyze the esters present in 1 g of the sample.

The I_s provides evidence of adulteration of the fatty matter with unsaponifiable substances (mineral oil, for example). Unless indicated in the specific monograph, use the amount of sample indicated in **Table 1**.

Table 1 – Sample quantity to determine saponification value.

<i>SV Expected value</i>	<i>Sample quantity (g)</i>
3 – 10	12 – 15
10 – 40	8 – 12
40 – 60	5 – 8
60 – 100	3 – 5
100 – 200	2.5 – 3
200 – 300	1 – 2
300 – 400	0.5 – 1

Weigh the indicated sample quantity (m), place in a 250 mL volumetric flask and add 25.0 mL of 0.5 *M* ethanolic potassium hydroxide VS and some boiling stones. Adapt the vertical reflux condenser. Heat in a water bath for 30 minutes, unless specifically indicated. Add 1 mL of phenolphthalein solution TS and immediately titrate excess potassium hydroxide with 0.5 *M* hydrochloric acid solution VS (n_1 , mL). Carry out blank assay under the same conditions and correct the titrant volume (n_2 , mL).

Calculate the saponification value (S_v), using the expression:

$$I_s = \frac{28,05(n_2 - n_1)}{m}$$

5.2.29.9 DETERMINATION OF ESTER VALUE

The ester value, E_v , expresses the amount of potassium hydroxide, in milligrams, necessary for the saponification of esters present in 1 g of sample. The (E_v) is calculated from the saponification value (S_v) and the acid value (A_v), according to the formula:

$$I_E = I_s - I_A$$

5.2.29.10 DETERMINATION OF IODINE VALUE

Iodine value (I_v) expresses, in g, the amount of iodine susceptible to complexation in 100 g of substance under the conditions described below. It is a quantitative measure of the degree of unsaturation of fatty acids, esterified and free, in the sample. The I_i , value found in the determination, is suggestive of the degree of purity of the tested material, as well as the presence of adulterants. If the monograph does not indicate the method to be used, carry out Method A.

METHOD A

Unless indicated in the specific monograph, use the amount of sample indicated in **Table 1**.

Table 1 – Sample quantity to determine iodine value.

<i>Expected value I_v</i>	<i>Sample quantity</i>
Inferior to 20	1.0
20 – 60	0.5 – 0.25
60 – 100	0.25 – 0.15
Greater than 100	0.15 – 0.10

In a 250 mL container equipped with a ground stopper, dry or washed with glacial acetic acid, introduce the sample (m , g) and dissolve it in 15 mL of chloroform, unless otherwise specified in the respective monograph. Add 25.0 mL of iodine bromide solution. Cap the container and keep it protected from light for 30 minutes, shaking frequently. After adding 10 mL of 10% potassium iodide solution (w/v) and 100 mL of water, titrate with 0.1 M sodium thiosulfate VS, shaking vigorously until the yellow color has almost faded. Add 5 mL of starch TS and proceed with the titration, adding 0.1 M sodium thiosulfate VS, dropwise, and shaking, until the color fades (n_1 , mL). Blank test must be carried out under the same conditions and without the sample (n_2 , mL).

Calculate the iodine content by the expression:

$$I_i = \frac{1,269(n_2 - n_1)}{m}$$

Unless indicated in the specific monograph, use the amount of sample indicated in **Table 2**.

Table 2 – Sample quantity to determine iodine value.

<i>Probable Iodine Value I_v</i>	<i>Mass (g) corresponding to a 150 percent excess of ICl</i>	<i>Mass (g) corresponding to a 100 percent excess of ICl</i>	<i>Iodine Chloride Solution (mL)</i>
<3	10	10	25
3	8.4613	10.5760	25
5	5.0770	6.3460	25
10	2.5384	3.1730	20
20	0.8461	1.5865	20
40	0.6346	0.7935	20
60	0.4321	0.5288	20
80	0.3173	0.3966	20
100	0.2538	0.3173	20
120	0.2115	0.2644	20
140	0.1813	0.2266	20
160	0.1587	0.1983	20
180	0.1410	0.1762	20
200	0.1269	0.1586	20

In a 250 mL container with a ground stopper, previously washed with glacial or dry acetic acid, introduce the amount of sample (m , g) and dissolve it in 15 mL of a mixture of equal volumes of cyclohexane and glacial acetic acid, unless stated otherwise. If necessary, pre-melt the sample (melting point higher than 50°C). Slowly add the volume of iodine chloride solution indicated in **Table 2**. Cover the container and shake, protected from light, for 30 minutes, unless otherwise indicated. Add 10 mL of 10% potassium iodide solution (w/v) and 100 mL of water. Titrate with 0.1 M sodium thiosulfate VS, shaking vigorously until the yellow color almost fades. Add 5 mL of starch TS and proceed with the titration, adding 0.1 M sodium thiosulfate VS, dropwise, and shaking, until

the color fades (n_1 , mL of 0.1 M sodium thiosulfate VS). Carry out a blank test under the same conditions (n_2 mL 0.1 M sodium thiosulfate VS). Calculate iodine value according to the following formula:

$$I_i = \frac{1,269(n_2 - n_1)}{m}$$

5.2.29.11 DETERMINATION OF PEROXIDE VALUE

The peroxide value V_p expresses, in milliequivalents of active oxygen, the amount of peroxide in 1000 g of substance.

If the monograph does not indicate the method to be used, carry out *Method A*. The replacement of *Method A* with *Method B* is always object of validation.

METHOD A

Weigh 5.00 g of the sample and transfer to a 250 mL Erlenmeyer with a ground stopper. Add 30 mL of a mixture of glacial acetic acid and chloroform (3:2). Shake until the sample dissolves and add 0.5 mL of saturated potassium iodide solution. Shake for exactly one minute and add 30 mL of water. Titrate with 0.01 M sodium thiosulfate VS, slowly, without ceasing vigorous agitation, until yellow color has almost faded. Add 5 mL of starch TS. Continue the titration, shaking vigorously until the color disappears (n_1 , mL of 0.01 M sodium thiosulfate VS). Carry out a blank test under the same conditions n_2 mL 0.01 M sodium thiosulfate VS). The blank assay consumes no more than 0.1 mL of 0.01 M sodium thiosulfate VS.

Calculate the peroxide value by the expression:

$$I_p = \frac{10(n_1 - n_2)}{m}$$

METHOD B

Note: operate protected from light.

In an Erlenmeyer flask, with a ground stopper, introduce 50 mL of a mixture of glacial acetic acid and trimethylpentane (3:2). Add the amount of sample to the Erlenmeyer flask as indicated in **Table 1**. Cork and shake until the sample dissolves. Add 0.5 mL of saturated potassium iodide solution, cork again and let the solution stand for (60 ± 1) seconds. During this standing period, shake at least three times and then add 30 mL of water. Titrate with 0.01 M sodium thiosulfate solution VS (v_1 , mL), added slowly, with constant and energetic agitation, until almost total disappearance of the yellow color characteristic of iodine presence. Add about 0.5 mL of starch TS and proceed with the titration, without stopping shaking, especially when it is close to the equivalence point, to ensure the release of iodine from the solvent. Add, drop by drop, the sodium thiosulfate solution until the blue color starts to fade.

If the peroxide value is equal to or greater than 70, and if there is a delay in the color change of the starch indicator for 15 to 30 seconds, shake vigorously until the yellow color disappears. This is due to the tendency of trimethylpentane to supernate in the aqueous phase and the time required to obtain an adequate mixture between the solvent and the aqueous titrant.

For peroxide numbers less than 150, 0.01 *M* sodium thiosulfate VS is used. A small amount (0.5 to 1.0% (w/w)) of an appropriate emulsifier may be added to the mixture to delay phase separation and shorten the time for iodine release (e.g. polysorbate 60). Perform a blank assay (v_0 , mL). If more than 0.1 mL of 0.01 *M* sodium thiosulfate VS is consumed, replace the reagents and repeat the titration. The peroxide value is calculated by the formula below.

$$I_p = \frac{1000(v_1 - v_0)c}{m}$$

where

c = concentration of the sodium thiosulfate solution in moles per liter.

Table 1 – Sample quantity to determine peroxide value.

<i>Value expected of I_p</i>	<i>Sample quantity (g)</i>
0 – 12	2.00 – 5.00
12 – 20	1.20 – 2.00
20 – 30	0.80 – 1.20
30 – 50	0.500 – 0.800
50 – 90	0.300 – 0.500

5.2.29.12 DETERMINATION OF HIDROXYL VALUE

The hydroxyl value (V_{OH}) expresses, in milligrams, the amount of potassium hydroxide necessary for the neutralization of acid that combines, by acylation, with 1 g of the sample.

METHOD A

Introduce the sample, accurately weighed, according to the quantity indicated in **Table 1**, in a 150 mL acetylation flask, unless another volume is specified in the specific monograph. Add the indicated volume of acetic anhydride solution (acetylation reagent) and fit the reflux condenser.

Table 1 – Sample quantity and volume of acetylation reagent.

<i>IOH expected</i>	<i>Sample quantity (g)</i>	<i>Acetylating reagent volume (mL)</i>
10 – 100	2.0	5.0
100 – 150	1.5	5.0
150 – 200	1.0	5.0
200 – 250	0.75	5.0
250 – 300	0.6 or 1.20	5.0 or 10
300 – 350	1.0	10.0
350 – 700	0.75	15.0
700 – 950	0.5	15.0

Heat in a water bath for one hour, taking care to maintain the level of the bath water approximately 2.5 cm above the level of the liquid contained in the flask. Remove the flask and allow to cool. Add 5 mL of water through the top end of the condenser. If the addition of water causes turbidity, add pyridine until the turbidity disappears and record the volume added. Shake, heat the flask again in a water bath for 10 minutes. Remove the flask and allow to cool. Wash the condenser and the sides of the flask with 5 mL of alcohol, previously neutralized in the presence of phenolphthalein TS. Titrate

with an alcoholic solution of 0.5 M potassium hydroxide VS (n_1 , mL), in the presence of 0.2 mL of phenolphthalein TS. Carry out a blank test under the same conditions (n_2 , mL).

Calculate the hydroxyl value using the expression:

$$I_{OH} = \frac{28,05(n_2 - n_1)}{m} + I_A$$

where

V_A = acid value.

METHOD B

In a dry erlenmeyer flask fitted with a ground stopper, introduce the sample solution (m , g). Add 2.0 mL of propionic anhydride, stopper the flask and shake gently, until the sample dissolves. After two hours of standing, unless otherwise indicated, remove the stopper from the Erlenmeyer flask and transfer its contents to another 500 mL with a wide mouth, containing 25.0 mL of 0.9% (w/v) aniline solution in cyclohexane and 30 mL of glacial acetic acid. Shake and, after standing for five minutes, add 0.05 mL of methylrosanilinium chloride TS. Titrate with 0.1 M perchloric acid VS until turning to emerald green (n_1 , mL). Carry out a blank test under the same conditions (n_2 , mL).

Calculate the hydroxyl value using the expression:

$$I_{OH} = \frac{5,610(n_1 - n_2)}{m} + I_A$$

where

A_v = acid value.

Due to the possibility of water presence, determine the moisture content (y , %) in the sample according to the specific method. The hydroxyl value is obtained by the formula:

$$V_{OH} = (\text{value obtained}) - 31.1y$$

5.2.29.13 DETERMINATION OF ACETYL VALUE

Acetyl value is the amount of alkali, in mg of potassium hydroxide, required to neutralize the acetic acid released by the hydrolysis of 1 g of acetylation substance. It is used to establish the degree of presence of free alcohols in fatty substances. It is calculated based on the difference between the saponification values of the acetylated substance by the method described below and of the non-acetylated substance.

PROCEDURE

Transfer 10 g of substance and 20 mL of acetic anhydride to a 200 mL Kjeldahl flask. Adapt the reflux condenser. Place the flask on an asbestos mesh in the center of which an orifice of about 4 cm in diameter has been cut and heat over a gas nozzle with a maximum height of 25 mm (preventing the flame from reaching the base of the flask). Keep at regular boiling for two hours, cool and transfer the contents of the flask to a 1000 mL beaker containing 600 mL of water. Add 0.2 g of pumice

powder and boil for 30 minutes. Cool and transfer the mixture to the separating funnel, discarding the lower aqueous layer. Wash the acetylated substance with three or more 50 mL portions of hot saturated sodium chloride solution, until the wash solution no longer provides an acidic reaction to the litmus paper. Add 20 mL of hot water to the funnel and shake, removing the water phase as much as possible. Transfer to porcelain capsule, add 1 g of powdered anhydrous sodium sulfate and filter through pleated filter paper. Determine the saponification value of the original, non-acetylation substance and of the acetylation substance by the procedure described and calculate the acetyl value using the formula:

$$I_{AC} = \frac{(b - a) \cdot 1335}{1335 - a}$$

where

a = original substance saponification value,

b = saponification value of acetylation substance.

5.2.29.14 DETERMINATION OF UNSAPONIFIABLE SUBSTANCES

Unsaponifiable substances are those remaining from the saponification reaction, non-volatile at 100 – 105 °C and which were carried in the process of extracting the substance to be tested.

If the specific monograph does not indicate the procedure, use *Method I*. Use glass material with a ground and degreased mouth.

METHOD I

Add from 2.0 to 2.5 g of the sample in a 250 mL flask. Add 25 mL of 0.5 *M* ethanolic potassium hydroxide. Attach a reflux condenser to the flask and boil in a water bath for one hour, under shaking. Transfer the contents of the flask to a separating funnel, using 50 mL of water and, while the liquid is still warm, extract, by vigorous shaking, with three 50 mL portions of peroxide-free ether. Wash the flask with the first ether aliquot. Mix the ethereal solutions in a separating funnel containing 20 mL of water. If the ethereal solutions contain suspended solids, filter into the separating funnel using a fat-free filter paper. Wash filter with peroxide-free ether. Shake carefully and discard the aqueous phase. Wash the organic fraction with two 20 mL portions of water and discard the aqueous phase. Then add three 20 mL portions of 0.5 *M* potassium hydroxide and shake vigorously for each addition. After each treatment, washing with 20 mL of water must be carried out. Finally, wash with serial amounts of 20 mL of water, until the aqueous phase does not show an alkaline reaction in the presence of phenolphthalein TS. Transfer the organic fraction to a previously tared flask, washing the separating funnel with peroxide-free ether. Remove the ether and add 3 mL of acetone to the flask. Eliminate the solvent completely at a constant temperature of maximum 80°C. Dissolve the contents of the flask in 10 mL of ethyl alcohol 96% (v/v) freshly boiled and previously neutralized. Titrate with 0.1 *M* ethanolic sodium hydroxide VS and phenolphthalein TS as indicator. If the volume of titrant solution spent does not exceed 0.1 mL, the amount of heavy residues should be taken as unsaponifiable matter. Calculate unsaponifiable matter as a percentage of the substance to be examined. If the volume of titrant solution spent exceeds 0.1 mL, the amount of heavy residues should not be taken as unsaponifiable matter and the test must be repeated.

METHOD II

In a 250 mL flask, coupled to a reflux condensation system, introduce the prescribed amount of sample (m , g). Add 50 mL of alcoholic 2 M potassium hydroxide solution and heat in a water bath for one hour while shaking. After cooling to a temperature below 25°C, transfer the contents of the flask to a separatory funnel. Add 100 mL of water. Add 100 mL of peroxide-free ether and shake carefully. Repeat the operation two more times with 100 mL of ethyl ether. Mix the ethereal solutions in a separating funnel containing 40 mL of water. Shake gently for a few minutes and let the phases separate. Discard the aqueous phase. Wash the ether phase twice with 40 mL of water. Then wash successively with 40 mL of 3% potassium hydroxide (w/v) and 40 mL of water. Repeat this operation three times. Repeatedly wash the ethereal phase with 40 mL of water, until the aqueous phase does not provide an alkaline reaction to the phenolphthalein TS. Transfer the ether phase to a previously tared flask, washing the separating funnel with peroxide-free ether. Evaporate to dryness. Add 6 mL of acetone to the residue. Carefully remove the solvent in a current of air. Dry at a temperature of 100 °C to 105°C, until mass is constant, allow to cool in a desiccator and weigh (a , g). The result is calculated as a percentage w/w.

$$\% \text{ de insaponificáveis} = \frac{100a}{m}$$

Dissolve the residue in 20 mL of alcohol, previously neutralized in the presence of phenolphthalein TS and titrate with an alcoholic solution of 0.1 M sodium hydroxide VS. If the volume of alcoholic solution of 0.1 M sodium hydroxide VS used in the titration is higher than 0.2 mL, it indicates that the separation of the two phases was incomplete and the residue obtained cannot be considered unsaponifiable. The test should be repeated.

5.2.29.15 IDENTIFYING FIXED OILS

5.2.29.15.1 IDENTIFYING VEGETABLE OILS BY THIN LAYER CHROMATOGRAPHY

Proceed as described in *Thin-layer chromatography* (5.2.17.1).

Stationary phase: octadecyl silanized silica gel (RP-18).

Sample solution. Unless indicated in a specific monograph, dissolve approximately 20 mg (one drop) of the sample, accurately weighed, in 3 mL of methylene chloride.

Standard solution. Dissolve about 20mg (one drop) of corn oil, accurately weighed, in 3mL of methylene chloride.

Procedure. Separately apply 1 µL of each solution to the plate. Develop twice to a distance of 0.5 cm with ether. Then develop twice up to a distance of 8 cm with a mixture of methylene chloride, glacial acetic acid and acetone (2:4:5). Allow the plate to air dry and nebulize with a 10% (w/v) phosphomolybdic acid solution in alcohol. Heat the plate to 120°C for about three minutes. Examine in daylight.

The chromatogram shows spots comparable to those reproduced in **Figure 1**.

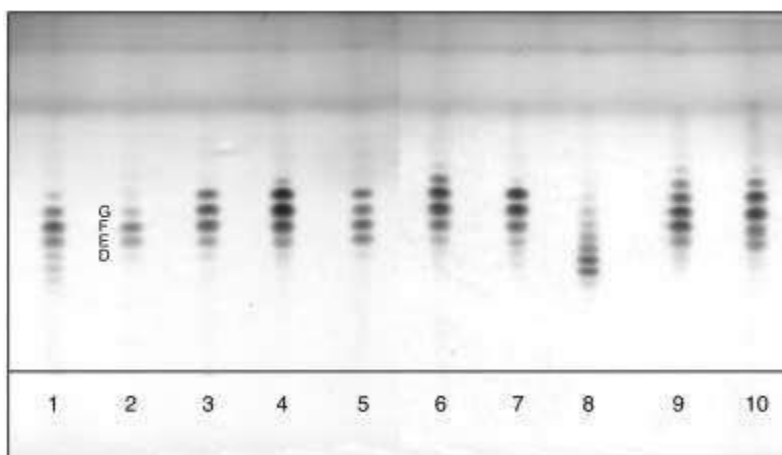


Figure 1 – Thin-layer chromatography to identify fixed oils.

1 Peanut Oil 2 Olive Oil 3 Sesame Oil 4 Corn Oil 5 Almond Oil 6 Soybean Oil 7 Sunflower Oil 8 Canola Oil 9 Canola Oil (Erucic Acid Free) 10 Wheat Germ Oil

(5.2.29.15.2) ALKALINE IMPURITIES

Add 10 mL of freshly distilled acetone, 0.3 mL of water, and 0.05 mL of 0.04% (w/v) bromophenol blue alcoholic solution to a test tube. Neutralize, if necessary, with 0.01 M hydrochloric acid or 0.01 M sodium hydroxide. Add 10 mL of the sample, shake and allow to stand. Changeover is indicated by the development of a yellow color in the top layer. Volume greater than 1.1 mL of 0.01 M hydrochloric acid is not required.

5.2.29.15.3 FOREIGN OILS IN VEGETABLE OILS BY THIN LAYER CHROMATOGRAPHY

Proceed as described in *Thin-layer chromatography* (5.2.17.1) using plate (kieselguhr G). Impregnate the plate, placing it in a closed chamber containing the necessary amount of ethyl ether and liquid paraffin mixture (90:10) so that the liquid surface reaches about 5 mm of the adsorbent layer. When the impregnation mixture has covered at least 12 cm of the layer, remove the plate from the chamber and allow the solvent to evaporate for five minutes. Develop in the same direction as the impregnation.

Preparation of fatty acid mixture. Heat, under reflux, for 45 minutes, 2 g of the sample with 30 mL of 0.5 M alcoholic solution of potassium hydroxide. Add 50 mL of water and allow to cool. Transfer to separation funnel. Shake three times with 50 mL of ethyl ether. Discard ethereal solutions. Acidify the aqueous phase with hydrochloric acid and shake three times with 50 mL of ethyl ether. Combine the ethereal solutions and wash three times with 10 mL of water. Discard wash water. Add anhydrous sodium sulfate to the ethereal fraction and filter. Evaporate at a temperature below 50°C. Use the residue to prepare the problem solution.

Fatty acids can also be obtained from the saponified solution resulting from the reaction to determine unsaponifiables.

Sample solution. Dissolve 40mg of the fatty acid mixture obtained from the sample in 4mL of chloroform.

Standard solution. Dissolve 40mg of the fatty acid mixture, obtained from a mixture of corn oil and canola oil (19:1), in 4mL of chloroform.

Procedure. Separately apply 3 μL of each solution to the plate. Develop the chromatogram with a mixture of glacial acetic acid and water (90:10) over a course of 8 cm. Dry the plate at 110°C for 10 minutes. Allow to cool. Place the plate, unless otherwise indicated, in a chromatographic chamber saturated with iodine vapors. To do this, place iodine in a crystallizer, low, at the bottom of the tank. After a while, brown or brownish-yellow spots appear. Remove the plate from the tank and wait a few minutes. When the brown background color of the layer disappears, pulverize with starch TS; blue stains then appear which, when dry, can turn brown and turn blue again after pulverizing with water. The chromatogram obtained with the *Sample Solution* shows spots corresponding to the spots of the chromatogram obtained with the *Standard Solution*: one with R_f close to 0.5 (oleic acid) and another with R_f close to 0.65 (linoleic acid). In certain oils, a spot with R_f close to 0.75 (linolenic acid) may appear. By comparison with the chromatogram obtained with the *Standard solution*, verify the absence of the spot with R_f 0.25 (erucic acid) in the chromatogram obtained with the *Sample solution*.

5.2.29.15.4 FOREIGN OILS IN FIXED OILS BY GAS CHROMATOGRAPHY

When there is no indication in the specific monograph, use *Method A*. The search for foreign oils is carried out on the methyl esters of the fatty acids in the oil under analysis. Proceed as described in *Gas chromatography* (5.2.17.5).

METHOD A

This method does not apply to oils containing fatty acid glycerides with epoxy, hydro epoxy, cyclopropyl or cyclopropenyl groups, or to those containing a large amount of fatty acids with a carbon chain lower than 8, or to those whose acid values are greater than 2.0.

Sample solution. If the monograph indicates, dry the sample before starting the assay. Weigh 1.0 g of the sample and transfer to a 25 mL Erlenmeyer with ground mouth. Couple a reflux condenser and a device to pass nitrogen stream inside the flask. Add 10 mL of anhydrous methyl alcohol and 0.2 mL of 6% (w/v) potassium hydroxide solution in methyl alcohol. Pass a stream of nitrogen with a flow of about 50 mL/minute until the air is eliminated. Shake and heat to boiling. When the preparation is clear (usually about 10 minutes later), heat it for another five minutes. Cool under running water and transfer to a separating funnel. Rinse flask with 5 mL of heptane, add to the contents of the separating funnel and shake. Add 10 mL of 20% sodium chloride solution (w/v) and shake vigorously. Allow the phases to separate and transfer the organic phase to a flask containing anhydrous sodium sulfate. Allow to stand and filter.

Standard solution (a). Prepare 0.50 g of a mixture of reference substances as prescribed in the specific monograph. If the monograph does not indicate the standard solution, use one of those described in **Table 1**. Dissolve in heptane, dilute to 50.0 mL with the same solvent and homogenize. Note: For capillary column chromatography and *Split* ratio, it is recommended that the long chain component of the mixture under analysis be added to the calibration mixture when quantitative analysis is performed by calibration curve.

Standard solution (b). Transfer 1.0 mL of the standard solution (a) to a 10 mL volumetric flask, adjust the volume with heptane and homogenize.

Standard solution (c). Prepare 0.50 g of a mixture of fatty acid methyl esters as indicated in the analyte monograph. Dissolve in heptane, dilute to 50 mL with the same solvent in volumetric flask. Commercial mixtures of fatty acid methyl esters can also be used.

Chromatographic conditions

Column:

- material: fused silica, glass or quartz;
- size: 10 to 30 m in length and 0.2 to 0.8 mm in internal diameter;
- stationary phase: poly (cyanopropyl) methylphenylmethylsiloxane or macrogol 20 000 (film thickness 0.1 to 0.5 μm) or other suitable stationary phase;

Carrier gas: helium or hydrogen for chromatography;

Carrier gas flow: 1.3 mL/minute (for 0.32 mm internal diameter columns);

Split ratio: 1:100 or smaller, according to the internal diameter of the column in use (1:50 when the diameter is 0.32 mm);

Detector: flame ionization; Temperature:

- column: 160 – 200 °C, according to stationary phase and length (200 °C for a 30 m long column, internally coated with macrogol 20.000). If necessary or indicated in the analyte monograph, raise the column temperature from 170 to 230°C with a heating ramp of 3°C per minute (column with macrogol 20,000).
- injector: 250°C;
- detector: 250°C;

Injection volume; 1 μL ;

Sensitivity: The height of the main peak in the chromatogram obtained with *Standard Solution* (a) is 50 to 70% of the recorder full scale.

Suitability of the system when mixtures of reference substances are used (Table 2).

Note. For capillary column chromatography and *split* ratio, it is recommended that the long chain component of the mixture under analysis be added to the calibration mixture when quantitative analysis is performed by calibration curve.

- resolution: at least 4 between the peaks of methyl caprylate and methyl caprate, calculated in the chromatogram obtained with the *Standard solution* (a);
- signal/noise ratio: at least 5 for the peak referring to methyl caprate, observed in the chromatogram obtained with the *Standard solution* (b);
- number of theoretical plates: minimum 15,000, calculated for the peak corresponding to methyl caproate.

Suitability of the system when mixtures of reference substances are used (Table 1 or Table 3).

Note. For capillary column chromatography and *split* ratio, it is recommended that the long chain component of the mixture under analysis be added to the calibration mixture when quantitative analysis is performed by calibration curve.

- resolution: at least 1.8 between the peaks of methyl oleate and methyl stearate, calculated in the chromatogram obtained with the *Standard solution* (a);

- signal/noise ratio: at least 5 for the peak referring to methyl myristate, observed in the chromatogram obtained with the *Standard solution (b)*;
- number of theoretical plates: minimum 30 000, calculated for the peak corresponding to methyl stearate.

Chromatogram evaluation. Avoid analysis conditions that allow the emergence of 'masked peaks' (presence of constituents with close retention times, such as linolenic and arachidonic acids, for example).

Qualitative analysis

Identify the peaks of the chromatogram obtained with the *Standard solution (c)* (under isothermal operating conditions or with linear temperature programming).

When isothermal operating conditions are used, the peaks can be identified by comparison with the chromatogram obtained with the *Standard Solution (a)* and information recorded in **Table 1**, **Table 2**, or **Table 3**:

a) measure reduced retention time (t'_R) of each peak obtained with the *Standard Solution (a)*. t'_R is the retention time measured in relation to the solvent peak and not in relation to the injection time. Plot the line through the formula:

$$(t'_R) \text{ Log} = f(\text{number of carbons in the equivalent chain})$$

b) the logarithms of the reduced retention times of unsaturated acids are points on the straight line, with non-integer values of carbon atoms called the 'equivalent chain length'. The equivalent chain length corresponds to the theoretical number of carbon atoms of saturated fatty acids that would have the same t'_R . For example, linoleic acid has t'_R s a theoretically saturated fatty acid with 18.8 carbon atoms. Identify the peaks of the chromatogram obtained with the test solution by calibration curve and by the reduced retention time. Chain lengths are recorded in **Table 4**.

Quantitative Analysis

Quantification is generally performed using the normalization method, in which the sum of the areas under the chromatogram peaks, with the exception of the solvent peak, is assumed to be equal to 100%. Preferably use an electronic integrator.

The percent content of each component is calculated by determining the area under the corresponding peak versus the sum of the areas under all peaks. Do not consider peaks whose area is inferior to 0.05 percent of the total area.

In certain cases, when the fatty acid chain is inferior or equal to twelve carbon atoms, correction factors can be indicated in the individual monographs to convert the area under the peaks to a percentage w/w.

Table 1 – Mixture of substances for calibration.

<i>Mixture of substances</i>	<i>Composition (% w/w)</i>
Methyl laurate	5
Methyl myristate	5
Methyl palmitate	10
Methyl stearate	20

Methyl arachidate	40
Methyl oleate	20

Table 2 – Mixture of substances for calibration.

<i>Mixture of substances</i>	<i>Composition (% w/w)</i>
Methyl caproate	10
Methyl caprylate	10
Methyl caprate	20
Methyl laurate	20
Methyl myristate	40

Table 3 – Mixture of substances for calibration.

<i>Mixture of substances</i>	<i>Composition (% w/w)</i>
Methyl myristate	5
Methyl palmitate	10
Methyl stearate	15
Methyl arachidate	20
Methyl oleate	20
Methyl Eicosanoate	10
Methyl behenate	10
Methyl lignocerate	10

Table 4 – Equivalent chain length (values calculated from calibration curve and analysis with macrogol 20 000 column).

<i>Fatty acid</i>	<i>Equivalent chain length</i>
Caproic acid	6.0
Caprylic acid	8.0
Capric acid	10.0
Lauric acid	12.0
Myristic acid	14.0
Palmitic acid	16.0
Palmitoleic acid	16.3
Margaric acid	17.0
Stearic acid	18.0
Oleic acid	18.3
Linoleic acid	18.8
Gamma-linolenic acid	19.0
Alpha-linolenic acid	19.2
Arachidic acid	20.0
Eicosanoic acid	20.2
Arachidonic acid	21.2
Behenic acid	22.0
Erucic acid	22.2
12-oxostearic acid	22.7
Ricinoleic acid	23.9
12-hydroxystearic acid	23.9
Methyl lignocerate	24.0

<i>Fatty acid</i>	<i>Equivalent chain length</i>
Nervonic acid	24.2

METHOD B

This method does not apply to oils containing fatty acid glycerides with epoxy, hydro epoxy, cyclopropyl or cyclopropenyl groups, or to those containing acid values greater than 2.0.

Sample solution. Add 0.100 g of the sample to a 10 mL centrifuge tube with a ground stopper. Dissolve with 1 mL of heptane and 1 mL of dimethylcarbonate. Shake vigorously, heating to a mild heat (50 –60 °C). Add 1 mL of 1.2% (w/v) sodium solution in anhydrous methyl alcohol to the still hot solution. Shake vigorously for about five minutes. Add 3 mL of distilled water and shake vigorously for about 30 seconds. Centrifuge for 15 minutes at 1500 *g*. Inject 1 µL of the organic phase.

Standard solutions and evaluation of chromatograms. In the absence of specific indication in the individual monograph, proceed as described in *Method A*.

Chromatographic conditions. Chromatographic separation can be carried out using:

- column of fused silica 30 m in length and 0.25 mm in internal diameter, covered with macrogol 20 000 (film thickness: 0.25 µm);
- carrier gas: helium for chromatography, with a flow of 0.9 mL/minute;
- flame ionization detector;
- *Split* ratio 1:100

Use the temperature schedule shown in **Table 1**.

Table 1 – Temperature programming for chromatographic separation.

	<i>Time (minutes)</i>	<i>Temperature (°C)</i>
Column	0 – 15	100
	15 – 36	100 → 225
	36 – 61	225
Injector		250
<u>Detector</u>		<u>250</u>

METHOD C

This method does not apply to oils containing fatty acid glycerides with epoxy, hydroperoxy, aldehyde, ketone, cyclopropyl and cyclopropenyl groups, as well as oils with conjugated polyunsaturated groups or with acetylenic groups due to the partial or total destruction of these groups.

Sample solution. In a 25 mL conical flask, dissolve 0.10 g of the sample in 2 mL of a 2% (w/v) sodium hydroxide solution in methyl alcohol. Fit the flask to the vertical reflux condenser and heat for 30 minutes. Through the condenser, add 2.0 mL of boron trifluoride-methanol solution and heat for 30 minutes. Through the condenser, add 4 mL of heptane and heat for five minutes. Cool the mixture and add 10.0 mL of saturated sodium chloride solution. Shake for 15 seconds and add enough saturated sodium chloride solution to bring the upper phase to the neck of the container vial. Remove a 2 mL aliquot from the upper phase. Wash three times with 2 mL of water and dry with anhydrous sodium sulfate.

Standard solutions, chromatographic conditions and chromatograms evaluation. In the absence of specific indication in the individual monograph, proceed as described in *Method A*.

5.2.29.16 DETERMINATION OF STEROLS IN FIXED OILS

SEPARATION OF THE STEROL FRACTION

Prepare the unsaponifiable fraction. Separate the sterol fraction from the fixed oil by thin-layer chromatography, using a silica-gel G plate (layer thickness between 0.3 mm and 0.5 mm).

Sample solution (a): In a 150 mL flask, add a volume of 0.2% (w/v) betulin solution in methylene chloride, which corresponds to approximately 10% of the sterol content of the sample used for the assay (for example, volume of 500 µL of betulin solution for virgin olive oil, and 1500 µL for other vegetable oils). If the monograph requires to calculate the percentage content of each sterol in the sterol fraction, the addition of betulin may be omitted. Evaporate to dryness in nitrogen stream. Add 5.00 g of the sample and 50 mL of 2M potassium hydroxide in ethyl alcohol. Couple the vertical reflux condenser. Heat in water bath for one hour, under agitation. Cool to a temperature below 25°C and transfer the contents of the flask to a separating funnel, using 100 mL of water. Carefully shake three times with 100 mL of peroxide-free ethyl ether. Mix the ethereal solutions in a separating funnel containing 40 mL of distilled water. Shake gently for a few minutes. Allow the phases to be separated by decantation and discard the aqueous phase. Wash the organic phase several times with 40 mL of water, until the aqueous phase does not show an alkaline reaction to phenolphthalein TS. Transfer the organic fraction to a previously tared flask, washing the separating funnel with ethyl ether. Evaporate the ether. Add 6 mL of acetone to the residue. Carefully remove the solvent with a stream of nitrogen. Dry in oven at 100 – 105 °C until constant mass. Dissolve the residue with minimal volume of methylene chloride.

Sample solution (b): Submit 5.00 g of canola oil to the same procedure described for *Sample Solution (a)* from “Add 50 mL of 2 M potassium hydroxide in ethyl alcohol...”.

Sample solution (c): Submit 5.00 g of sunflower oil to the same procedure described for *Sample Solution (a)* from “Add 50 mL of 2 M potassium hydroxide in ethyl alcohol...”.

Standard solution. Dissolve 25mg cholesterol and 10 mg betulin in 1mL methylene chloride. Use a different plate for each problem solution.

Separately apply to the plate 20 µL of the *Standard Solution* in a band of 20 mm by 3 mm and 0.4 mL of the problem solution (a), (b) or (c) in a band of 40 mm by 3 mm. Migrate over a distance of 18 cm with the mobile phase, consisting of a mixture of ether and *n*-hexane (35:65). Dry the plates in a stream of nitrogen. Develop with a 0.2% (w/v) dichlorofluorescein solution in ethyl alcohol. Examine in a UV lamp at 254 nm.

The chromatogram obtained with the *Standard Solution* shows bands corresponding, respectively, to cholesterol and betulin. The chromatograms obtained with the *Sample solutions* show *R_f* bands close to those corresponding to the sterols. From each of the chromatograms, scrape the region of the plate corresponding to the sterol bands, as well as an area located 2 – 3 mm above and below the visible areas corresponding to the standard solution. Place these regions in three different 50 mL Erlenmeyer flasks. Add 15 mL of hot methylene chloride to each and shake. Filter each solution separately on a porous glass filter (40) or on a suitable filter paper. Wash each filter three times with 15 mL of methylene chloride. Transfer the filtrate and washing liquids in a tared Erlenmeyer flask. Evaporate to dryness in nitrogen stream, then weigh.

STEROL ASSAY

Proceed as described in *Gas chromatography* (5.2.17.5). The assay must be carried out in a place with low moisture content and solutions prepared at the time of use.

Sample solution. To the sterols separated from the sample by thin layer chromatography, add 0.02 mL of fresh-prepared mixture of chlorotrimethylsilane, hexamethyldisilazane and anhydrous pyridine (1:3:9) per milligram of residue. Shake carefully until the sterols are fully dissolved. Allow to stand in a desiccator with phosphorus pentoxide for 30 minutes. Centrifuge if necessary and use the supernatant.

Standard solution (a). To nine parts of the sterols separated from the canola oil by thin layer chromatography, add one part of cholesterol. Add 0.02 mL of fresh-prepared mixture of chlorotrimethylsilane, hexamethyldisilazane and anhydrous pyridine (1:3:9) per milligram of residue. Shake carefully until the sterols are fully dissolved. Allow to stand in a desiccator with phosphorus pentoxide for 30 minutes. Centrifuge if necessary and use the supernatant.

Standard solution (b). To the sterols separated from the sunflower by thin layer chromatography, add 0.02 mL of freshly prepared mixture of chlorotrimethylsilane, hexamethyldisilazane and anhydrous pyridine (1:3:9) per milligram of residue. Shake carefully until the sterols are fully dissolved. Allow to stand in a desiccator with phosphorus pentoxide for 30 minutes. Centrifuge if necessary and use the supernatant.

Chromatographic conditions

- column of fused silica 20 to 30 m in length and 0.25-0.32 mm in internal diameter, covered with a film of poly[methyl(95) phenyl(5)] siloxane or poly[methyl(94) phenyl(5) vinyl(1)] siloxane (0.25 µm film thickness);
- carrier gas: hydrogen gas with a linear velocity from 30 to 50 cm/s or helium with a linear velocity from 20 to 35 cm/s;
- *Split* ratio (1/50 or 1/100);
- temperatures: column: 260 °C; injector 280 °C; detector: 290°C
- Injection volume: 1 µL.

Results. The chromatogram obtained with the *Standard solution (a)* presents four main peaks, corresponding, respectively, to cholesterol, brassicasterol, campesterol and β -sitosterol. The chromatogram obtained with the *Standard solution (b)* presents four main peaks, corresponding, respectively, to campesterol, stigmasterol, β -sitosterol and Δ^7 -stigmasterol. The relative retention times of the different sterols in relation to β -sitosterol are shown in **Table 1**.

The peak corresponding to the internal standard (betulin) is sharply separated from the peaks corresponding to the sterols to be quantified.

Table 1 – Relative retention times of sterols in relation to β -sitosterol obtained with two different columns.

<i>Sterols</i>	<i>Poly[methyl(95)phenyl(5) siloxane</i>	<i>Poly[methyl(94)phenyl(5)vinil(1) siloxane</i>
Cholesterol	0.63	0.67
Brassicasterol	0.71	0.73
24-Methylenecholesterol	0.80	0.82
Campesterol	0.81	0.83

Campestanol	0.82	0.85
Stigmasterol	0.87	0.88
Δ^7 -Campesterol	0.92	0.93
$\Delta^{5,23}$ -Stigmastadienol	0.95	0.95
Clerosterol	0.96	0.96
β -Sitosterol	1	1
Sitostanol	1.02	1.02
Δ^5 -Avenasterol	1.03	1.03
$\Delta^{5,24}$ -Stigmastadienol	1.08	1.08
Δ^7 -Stigmasterol	1.12	1.12
Δ^7 -Avenasterol	1.16	1.16
Betulin	1.4	1.6

Examine the *Sample solution* chromatogram. Identify the peaks and calculate the percentage content of each sterol in the sterol fraction using the formula:

$$\frac{A}{S} \times 100$$

where

A = area under the peak corresponding to the substance to be quantified;

S = sum of the areas under the peaks corresponding to the substances indicated in **Table 1**.

If there is a requirement in the monograph, calculate the content of each sterol in the sample, in milligrams per 100 grams, using the expression:

$$\frac{A \times m_s \times 100}{A_s \times m}$$

where

A = area under the peak corresponding to the substance to be quantified;

A_s = area under the peak corresponding to betulin;

m = mass of the sample taken for the test, in g;

m_s = mass, in milligrams, of added betulin

5.2.30 TOTAL ORGANIC CARBON

The determination of total organic carbon (TOC) is a sensitive and non-specific method of quantifying the covalently bound carbon atoms in organic molecules present in a sample. The analysis is used to identify water contamination by organic impurities and contribute to control purification and distribution processes. Low levels of TOC suggest the absence of potentially hazardous organic chemicals in the water used in drug manufacturing. The TOC content may be related to the occurrence of endotoxins, microbial growth and the development of biofilms on the pipe walls of pharmaceutical water distribution systems. The TOC content is independent of the oxidation state of the organic matter and is not interfered with by other atoms linked to the chemical structure, such as nitrogen and hydrogen. There are several suitable methods for analyzing TOC and determinations can take place on-line or in laboratory.

The methods generally rely on the complete oxidation of organic molecules to carbon dioxide, which is quantified as carbon. Normally, organic carbon is oxidized by combustion, applying heat, ultraviolet emission, or oxidizing agents such as sodium persulfate. Quantification of carbon dioxide is done by detecting the gas produced with infrared or by reading the solution conductivity.

The method covered in this chapter is only a suggestion and the user can adopt any one that is appropriate and accessible for his specific purposes, as long as the quantification limit is adequate for the expected reading range. The method uses a standard solution of easily oxidizable substance, such as sucrose, for example, in such a concentration that the obtained instrumental response corresponds to the established limit for the TOC. The method may also be carried out with an apparatus installed on-line, which has been properly calibrated and which satisfies the system compliance test.

Table 1 shows the average expected values for the main types of water purification.

Table 1 – Typical TOC values in water.

<i>Type of purification</i>	<i>Expected TOC range (mg/L)</i>
Potable water	0.5 to 7.0.
Distillation	Around 0.10
Deionization	0.05 to 0.50.
Reverse osmosis	0.04 to 0.10
Reverse osmosis + deionization	0.01 to 0.05
Combined technologies	0.003 to 0.005
Combined technologies + UV Oxidation	<0.002

APPARATUS

It consists of an injector, a device to decompose the sample, a system to separate the formed carbon dioxide, a detector and a recorder of the emitted electrical signal. The decomposition tube must be capable of generating minimum 0.450mg/L of organic carbon for a sample of 1.071mg/L of sucrose.

The detection limit of the apparatus, specified by the manufacturer, is equal to or less than 0.050 mg of carbon per liter (0.05 ppm). System compliance is periodically checked using a solution prepared with a substance that is difficult to oxidize, such as 1.4-benzoquinone. The location of the apparatus is chosen to ensure that the results obtained are representative of the water used. The reading must be taken immediately after collecting the water sample.

TOC WATER (TOCW)

Use high purity water that meets the following specifications:

- Conductivity: maximum $0.1 \mu\text{S}\cdot\text{cm}^{-1}$ at 25 °C;
- Total organic carbon: maximum 0.10mg/L.

Depending on the type of apparatus used, heavy metal and copper contents can be critical. Observe the manufacturer's instructions.

Use COT water as blank; in the preparation of standard solutions; system suitability solution and equipment cleaning. The preparation of the standard solution and the system compliance solution must be concurrent with the sample.

PREPARATION OF GLASS MATERIAL.

Carefully wash the glass material using a process that eliminates organic matter. Place the material immersed in a mixture of equal parts of a 30% diluted hydrogen peroxide solution and diluted nitric acid. Rinse with TOC water.

If a micro syringe is used to inject the sample, it should be washed with a mixture of 5% sodium hydroxide solution (w/v) and absolute ethyl alcohol (1:1), or in 25% hydrochloric acid. Rinse thoroughly with TOC water.

PREPARATION OF SOLUTIONS

Blank. Prepare blank solution, or any other solutions required to set the baseline, or proceed with calibration, as per the manufacturer's instructions. Use the appropriate blank to zero the device.

Standard solution. Dissolve sucrose mass, previously dried at a temperature of 105 °C for three hours, in TOC water, in order to obtain a solution containing 1.19 mg of sucrose per liter of solution (0.50 mg of carbon per liter), to check the instrument.

Use a potassium acid phthalate solution in COT water, previously dried at 105°C for four hours, at the concentration determined by the equipment manufacturer, for instrument calibration. Preserve the solution by acidifying with concentrated phosphoric acid or concentrated sulfuric acid at $\text{pH} < 2$. To determine organic and inorganic carbon, separately, also prepare a standard solution of sodium bicarbonate (dried in a desiccator for not less than 18 hours) and decayed sodium carbonate (dried at 500 – 600 °C for 30 minutes) at 1:1 carbon content ratio in TOC water.

The concentration of the standard solution was calculated for purified water, whose TOC limit is 500 ppb. For other types of water, adjust accordingly.

System compliance solution. Dissolve 1,4-benzoquinone in TOC water to obtain a solution of 0.75mg of 1,4-benzoquinone per liter (0.50mg of carbon per liter).

Sample. Collect the water sample in a clean, dry container with a lid, leaving a minimum of air. Take care not to have any kind of contamination. Do not use plastic material. Carry out the analysis as soon as possible to minimize the risk of deterioration or contamination of the sample.

SYSTEM COMPLIANCE

Proceed with the readings (L) of the *COT* water solutions (L_{toc}), *standard solution* (L_{Pa}), *system compliance solution* (L_{Cs}) and record. Calculate the efficiency of the system in percentage, using the expression:

$$\frac{L_{Cs} - L_{cot}}{L_{Pa} - L_{cot}} \times 100$$

The system will be in compliance if the obtained value is between 85% and 115% of the theoretical response.

PROCEDURE

Use the analytical method recommended by the manufacturer of the equipment used. Inject an adequate volume of the sample and read the total carbon.

Determine the sample reading (L_{Am}). The sample complies with the test if L_{Am} is not greater than $L_{Pa} - L_{Toc}$.

$$L_{Am} < L_{Pa} - L_{Toc}$$

For different calculations of the organic and inorganic carbon fractions, read the total organic carbon, change the apparatus configuration to the inorganic carbon reading and calculate the organic carbon by subtraction. Alternatively, one can measure organic carbon after removing the inorganic carbon and subtracting the total carbon. Normally, for high purity waters the inorganic carbon fraction is negligible.

5.2.31 X-RAY DIFFRACTION

X-ray diffraction by the powder method (DRXP) consists of measuring diffraction angles and intensities characteristic of a polycrystalline material, randomly oriented, irradiated by a monochromatic X-ray beam.

The DRXP method is non-destructive in nature (sample preparation is generally limited to milling to reduce the particle size to around 5 μm). Investigations using DRXP can also be carried out under *in situ* conditions on specimens exposed to non-environmental conditions, such as low or high temperature and humidity.

Each crystalline phase of a given substance produces a characteristic X-ray diffraction pattern, which is obtained from a crystalline powder, composed of crystallites or crystalline fragments of characteristic size and randomly oriented. Essentially three types of information can be obtained with a DRXP pattern: angular position of the diffraction lines (depending on the dimensions of the unit cell and its geometric crystallographic arrangement); intensity of diffraction lines (depending mainly on the type and arrangement of atoms, and the orientation of particles within the sample) and diffraction line profiles (depending on the instrumental resolution, crystallite size and micro strain of the sample).

DRXP tests that provide the angular positions and line intensities of the crystalline phases are used for phase identification, determination of grades, estimate of crystallinity degree, micro deformation and average crystallite size.

PRINCIPLE

Every crystalline material has an organization of atoms that defines a unit cell. The unit cell is defined by the dimensions a , b and c and the angles between them, α , β e γ (**Figure 1a**). Interplanar spacing for a set of parallel planes hkl is represented by d_{hkl} . Each set of crystal planes has a Bragg diffraction angle, θ_{hkl} , associated with it (for a specific wavelength λ).

X-ray diffraction results from the interaction between X-rays and the electron clouds of atoms. Depending on the atomic arrangement, scattered X-rays exhibit the phenomenon of constructive interference, when the path difference between two diffracted X-ray waves is equal to an integer number of wavelengths. This selective condition is described by the Bragg equation (Equation 1), also called Bragg's Law represented in **Figure 1b**.

$$2d_{hkl} \sin \theta_{hkl} = n\lambda \text{ (Equation 1)}$$

where

λ = X-ray wavelength characteristic of the tube anode;

θ_{hkl} = angle between the incident ray and the lattice plane family;

n = wavelength order, usually set to one;

d_{hkl} = distance among planes;

hkl = Miller indices.

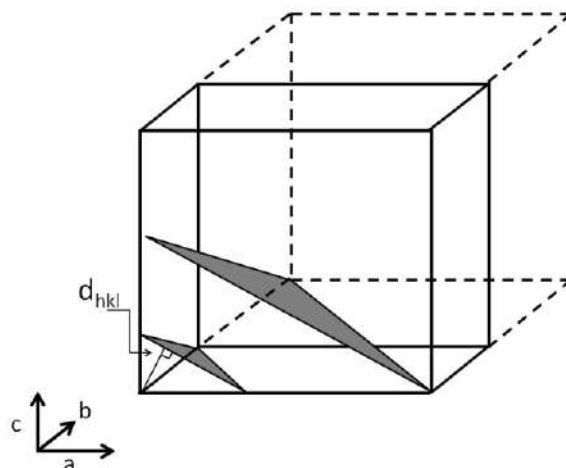


Figure 1a – Representation of the distances between crystalline planes (a, b and c) dimensions of the unit cell..

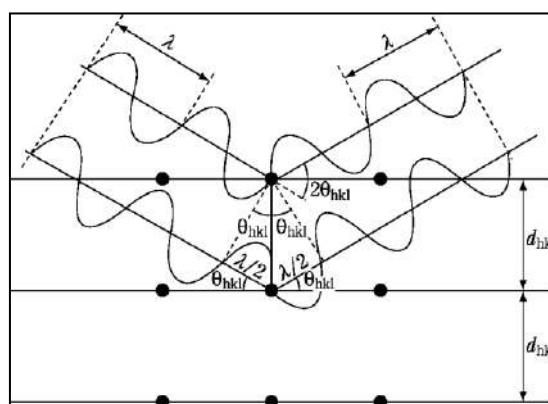


Figure 1b – X-ray diffraction by a crystal according to Bragg's law.

A powder sample is considered polycrystalline if, for any angle θ_{hkl} , there are always crystallites in one orientation, allowing diffraction according to Bragg's law. For a given wavelength of X-rays, the positions of the diffraction peaks (also referred to as 'lines', 'reflections' or 'Bragg reflections') are characteristic of the crystal structure (d spacings). The main characteristics of the diffraction line profiles are the 2θ position, height, area and shape of the peak (characterized, for example, by peak width or asymmetry, analytical function, empirical representation). **Figure 2** represents an example of the type of powder X-ray diffraction patterns obtained for five different solid phases of the same substance.

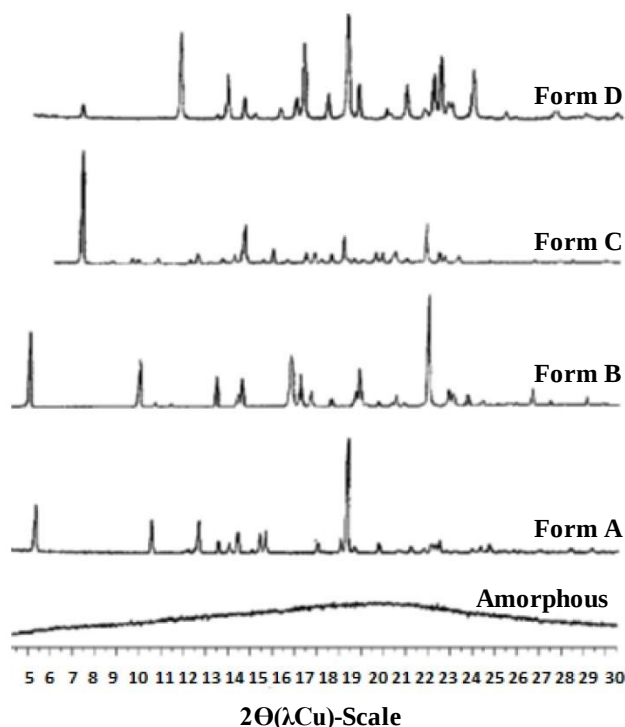


Figure 2 - Examples of X-ray powder diffraction patterns collected for five different solid phases of a substance.

In addition to the diffraction peaks, an X-ray diffraction experiment also generates a baseline, over which the peaks overlap. Factors contributing to the baseline are air diffuse scattering, apparatus and the presence of amorphous (**Figure 2**). The ratio of peak intensities to baseline can be increased by minimizing the baseline and choosing longer exposure times.

APPARATUS

X-ray diffraction experiments are usually performed using powder diffractometers. A powder diffractometer generally consists of five main parts: an X-ray source; incident beam optics (set of slits for collimation and beam focusing); sample port; diffracted beam optics (set of slits for collimation, beam focusing and convenient filter for radiation) and a detector. Data collection and processing systems are also required and comprise the X-ray diffraction measurement device. Currently, the Bragg-Brentano configuration is the most commonly used.

A given instrument can provide either a horizontal or vertical $\theta/2\theta$ geometry or a vertical geometry θ/θ . For both geometries, the X-ray beam is incident at an angle θ with the surface plane of the sample and the diffracted X-ray beam forms an angle 2θ with the direction of the incident X-ray beam (an angle θ with the sample surface plane). The basic diffraction geometry is shown in **Figure 3**. The divergent radiation beam from the X-ray tube (the so-called 'primary beam') passes through the parallel plate collimators, a divergence slit and illuminates the flat surface of the sample. All rays diffracted by the crystallites properly oriented in the sample at an angle of 2θ converge to a receiving slit. A second set of parallel plate collimators and a scattering slit can be placed behind or before the receiving slit. The axes of the focus of the X-ray source and the receiving slit are at equal distances from the axis of the goniometer. X-ray quanta are counted by a radiation detector, usually a scintillation counter, a gas-sealed proportional counter or a position-sensitive solid-state detector such as an image plate or a CCD detector. The receiving slit and detector are coupled and move tangentially to the focus circle. For $\theta/2\theta$ sweeps the goniometer rotates the sample about the same axis as the detector, but at half the rotation speed, in a $\theta/2\theta$ motion. Thus, the sample surface remains tangential

to the focus circle. The parallel plate collimator limits the axial divergence of the beam and therefore partially controls the shape of the diffracted line profile.

A diffractometer can also be used in transmission mode. The advantage with this technology is the effects reduction due to the preferential orientation. A capillary about 0.5 to 2 mm thick can also be used for small amounts of sample.

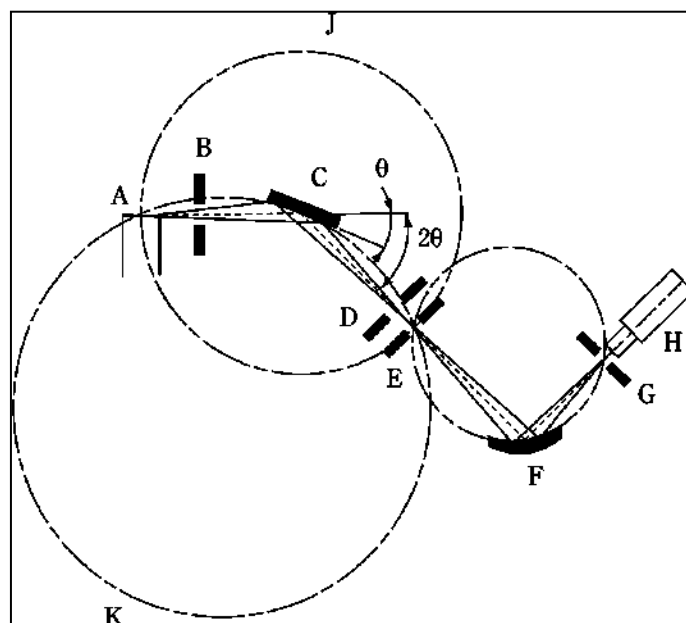


Figure 3 – Geometrical arrangement of the Bragg-Brentano para-focusing geometry.

A – X-ray tube; B – Divergence slit; C - Sample; D – Anti-scatter slit; E – Receiving slit; F – Monochromator; G – Detector receiving slit; H – Detector; J – Diffractometer circle; K – Focusing circle.

X-RAY RADIATION

In the laboratory, X-rays are obtained by bombarding a metal anode with electrons emitted by the thermoionic effect and accelerated in a strong electric field (using a high-voltage generator). Most of the kinetic energy of the electrons is converted into heat, which limits the power of the X-ray tubes and requires anode efficient cooling. A 20- to 30-fold increase in brightness can be obtained using rotating anodes and through X-ray optics. Alternatively, X-ray photons can be produced in a large-scale installation (synchrotron).

The spectrum emitted by an X-ray tube operating with sufficient voltage consists of a continuous background of polychromatic radiation and additional characteristic radiation that depends on the type of anode. Only this characteristic radiation is used in X-ray diffraction experiments. The main radiation sources used for X-ray diffraction are vacuum tubes, using copper, molybdenum, iron, cobalt or chromium as anodes; X-rays produced by copper (CuK_{α}), molybdenum or cobalt are most commonly used for organic substances (the use of cobalt anodes may be especially preferred to separate different X-ray lines). The choice of radiation to be used depends on the absorption characteristics of the sample and the potential fluorescence caused by atoms present in the sample. The wavelengths used in diffraction generally correspond to the radiation K_{α} from the anode. Consequently, it is advantageous to make the X-ray beam 'monochromatic', eliminating all other components of the emission spectrum. This can be achieved in part using K_{β} filters, i.e. metallic filters selected as having an absorption discontinuity between the wavelengths K_{α} and K_{β} emitted by the tube. The filter is usually inserted between the X-ray tube and the sample. Another method often used to obtain a

monochromatic X-ray beam is by means of a monochromator crystal. This crystal can be placed in the incident beam, obtaining a pure monochromatization $K_{\alpha 1}$, or after the sample, obtaining $K_{\alpha 1,2}$ at different angles, so that only one of them can be selected to focus on the detector.

RADIATION PROTECTION

Exposure of any part of the human body to X-rays can be harmful to health. Therefore, it is essential that proper precautions are taken to protect the operator and anyone else in the vicinity of X-ray equipment in use.

SAMPLE PREPARATION

The pulverized sample is often pressed into a sample holder made of aluminum, glass or polymer. As a rule, crystallites must be randomly oriented. The samples must be ground in an agate mortar to a fine powder.

In general, the morphology of many crystalline particles tends to generate a sample that has some degree of preferential orientation in the sample holder. This is particularly evident for needle or plate-shaped crystals when reducing the size of the crystals produces smaller needles or plates. The preferred orientation of the sample influences the intensities of various reflections. Thus, some are more intense whereas others less, compared to what would be expected in a sample with completely random crystallites. Various methods can be employed to minimize the randomness in crystallite orientation (and therefore to minimize the preferred orientation), but particle size reduction is often the best and simplest. In some cases, particle sizes of 10 μm will provide satisfactory results in phase identification. However, reducing this particle size may be convenient if no phase changes or amorphization of the material occur. Therefore, it is advisable to compare the diffraction pattern of the unground sample with that corresponding to a sample of smaller particle size (ground sample).

INSTRUMENTAL PERFORMANCE CONTROL

The goniometer and optical system corresponding to the incident and diffracted X-ray beams have many mechanical parts that need adjustment. The degree of alignment or misalignment directly influences the quality of the results of a DRXP investigation. Therefore, the different components of the diffractometer must be carefully adjusted (optical systems and mechanisms, etc.) to minimize systematic errors, optimizing the intensities received by the detector. The pursuit of maximum intensity and maximum resolution are always antagonistic when aligning a diffractometer. Accordingly, the best equilibrium must be sought while performing the alignment procedure. Each device has its own configuration and requires a specific alignment procedure.

The overall performance of the diffractometer should be periodically tested and monitored using properly certified reference materials. Depending on the type of analysis, other well-defined reference materials may also be employed, although the use of certified reference materials is preferred.

QUALITATIVE PHASE ANALYSIS

The identification, by XRPD, of the phases that constitute an unknown sample is based on a visual or computer-aided comparison with the peaks of a reference chemical substance, well characterized or calculated, from the crystal structure model or from certified databases. Ideally, these diffraction patterns are obtained on well-characterized single-phase specimens. This approach makes it possible, in most cases, to identify a crystalline substance through the spacings d and their relative intensities. The list of spacing d and normalized intensities I_{norm} , also called list (d, I_{norm}) extracted from the

pattern, is the crystallographic fingerprint of the material and can be compared with the lists (d , I_{norm}) of single-phase reference samples.

For most organic crystals, when $CuK\alpha$ radiation is used, it is useful to record the diffraction pattern in a 2θ range of 2° to not less than 50° . The variation of the 2θ diffraction angles between sample and reference must be lower than $0,2^\circ$ for the same crystal form, while the relative intensities between sample and reference can vary considerably due to preferential orientation effects. By their very nature, hydrates and solvates are recognized to have variations in unit cell dimensions, so changes can occur in the peak positions of the DRXP standards measured for these materials. In these cases, variation of 2θ positions higher than $0,2^\circ$ are expected.

It is sometimes difficult or even impossible to identify phases in the following cases: amorphous or non-crystallized substances; when the components to be identified are in low concentration; when the phase is not present in certified databases or does not have a determined crystal structure; or when the sample comprises many phases.

QUANTITATIVE PHASE ANALYSIS

If the sample under investigation is a mixture of two or more known phases, of which no more than one is amorphous, the percentage (by volume or mass) of each crystalline phase and the amorphous phase can, in many cases, be determined. Quantitative phase analysis can be based on integrated intensities, on the heights of several individual diffraction lines, or on the complete pattern. These integrated intensities; heights or data from complete standards are compared with corresponding values from reference materials. These reference materials must be single-phase or a known mixture of phases. Difficulties encountered during quantitative analysis are due to sample preparation (the accuracy and precision of the results require special homogeneity of all phases and an adequate particle size distribution in each phase) and matrix effects. If crystal structures of all components are known, the Rietveld method can be used to quantify them with acceptable precision. In favorable cases, amounts of crystalline phases as small as 10% in solid matrices can be determined.

5.2.32 NUCLEAR MAGNETIC RESONANCE

INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy is an analytical procedure that is based on the magnetic properties of certain atomic nuclei. It is similar to other spectroscopic methods in which energy is absorbed and emitted at a certain frequency, providing analytical information. NMR differs from other methods as it creates discrete levels of energy between the transitions of the nucleus of atoms in a molecule when subjected to a magnetic field.

Atomic nuclei, when magnetically charged, rotate about the nuclear axis, creating a magnetic dipole moment (μ) along this axis. Those that exhibit this behavior are called isotopes. The angular momentum of this nuclear spin is characterized by the nuclear spin quantum number (I). If the mass number is odd, the value of I is $\frac{1}{2}$ or an integer plus $\frac{1}{2}$; otherwise, it has a value of zero or an integer. When nuclei have a non-zero spin quantum number ($I \neq 0$) and are subject to a static and uniform external magnetic field of force (H_0), they align with the respective field with $(2I + 1)$ possible orientations. However, for nuclei with $I = \frac{1}{2}$, there will be two possible orientations, which correspond to two different energy states. Thus, a nuclear resonance is the transition between these spin states, caused by the absorption and emission of the corresponding amount of energy. In **Table 1** spin quantum numbers of some nuclei are presented.

Table 1– Quantum numbers and spin state of some nuclei.

Element	¹ H 1	¹ H 2	¹² C 6	¹³ C 6	¹⁴ N 7	¹⁶ O 8	¹⁷ O 8	¹⁹ F 9	³¹ P 15
Spin quantum numbers	$\frac{1}{2}$	1	0	$\frac{1}{2}$	1	0	$\frac{5}{2}$	$\frac{1}{2}$	$\frac{1}{2}$
Spin state	2	3	0	2	3	0	6	2	2

In a static magnetic field, the nuclear magnetic axis undergoes a precession movement (Lamor precession) around the axis of the external field. The precession angular velocity (ω_0) is related to the strength of the magnetic field through the formula $\omega_0 = \gamma H_0$; where γ is the gyromagnetic constant and is intrinsic to all nuclei of a given isotope. With the introduction of oscillating radiofrequency energy, the absorption of radiation by the paramagnetic core takes place according to the formula:

$$\Delta E = h\nu = \mu H_0 / I$$

where h is Planck's constant, and

$$\nu = \omega_0 / 2\pi = \gamma H_0 / 2\pi$$

Therefore, when frequency (ν_0) of external energy field ($E = h\nu$) is the same as the precession angular velocity, resonance occurs.

The difference in energy between the two levels corresponds to a specific electromagnetic radiation within the range of radio frequencies used. This is a function of γ , which is a property of the nucleus, and H_0 which represents the strength of the external field. The resonant frequency of a nucleus rises when the strength of the magnetic field increases.

The signal characteristics in the spectra that provide analytical information are: chemical shift, multiplicity, peak width, coupling constant and relative intensity.

NMR spectroscopy has a wide range of applications, such as structure elucidation; thermodynamic studies; kinetics, mechanics and in quantitative analysis.

APPARATUS

Currently, the most used spectrometer is the Fourier transform. The main components of an NMR spectrometer are: magnet to provide a constant magnetic field (B_0); temperature-controlled probe that receives the sample; conducts the radio frequency pulse and detects the radiation emitted by the sample; and electronic console (computer) for generating high energy radiofrequency pulses, collecting and digitizing the induced free decay signal (FID – free induction decay).

The devices use a single pulse of radio frequency energy to simultaneously excite all the nuclei. The excited nuclei return to the lowest energy level, generating an accumulation of FID signals at a given time. The decay time and frequency form a Fourier transform, generating a graph of amplitude versus frequency (spectrum). After the time in which the excited nuclei relax is possible, the pulse can be repeated and thus the response obtained accumulates in the computer memory, promoting greater resolution.

Figure 1 shows the instrumental parts constituting a high-resolution pulse spectrometer (NMR).

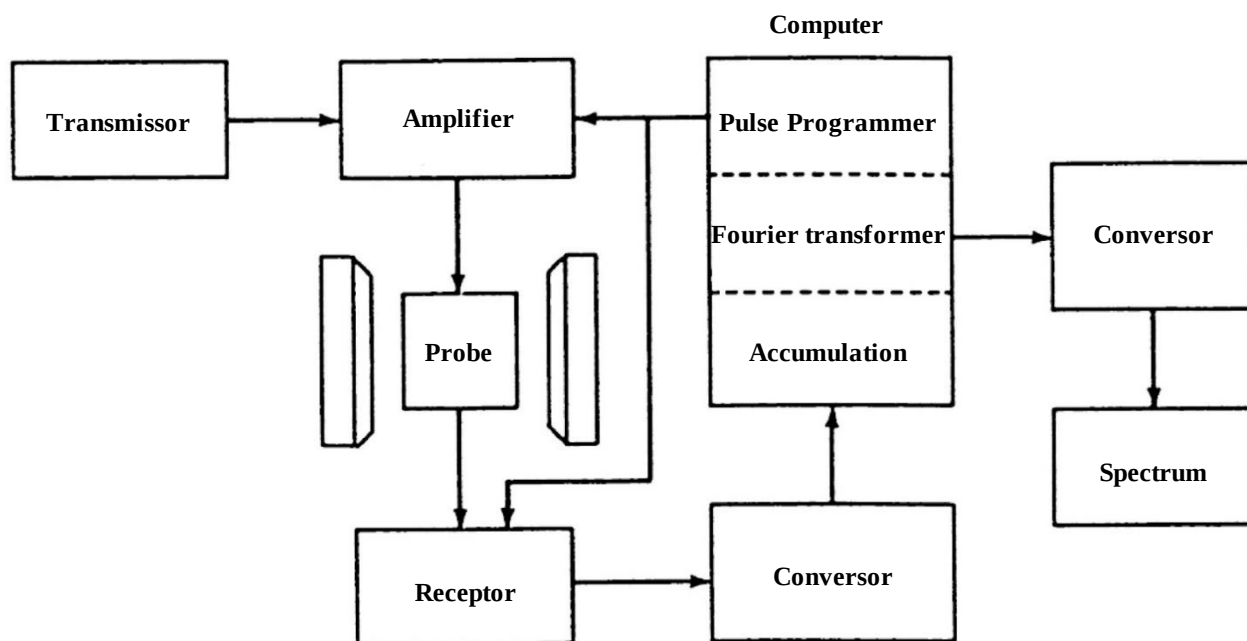


Figure 1 – Representative high resolution spectrometer (NMR) scheme.

The basic configuration of a NMR spectrometer contains a superconducting (cryogenic) solenoid as a magnetic field source, probe and electronic console. These devices can provide spectra of many chemical nuclei, making it possible to obtain spectra in a short time of analysis and with small amounts of sample.

PROTECTION IN FRONT OF MAGNETIC FIELDS AND RADIO WAVES

Exposure to magnetic fields and radio waves can be harmful to health. Thus, it is essential that proper precautions must be taken to protect the operator and anyone else in the vicinity of NMR equipment in use.

SPECTRUM

Peaks in an NMR spectrum are characterized by: frequency, multiplicity and relative intensity. The analytical utility of the NMR method lies in the fact that the same nuclei, when found in different molecular environments, show different resonance frequencies. The reason for this difference is that the electromagnetic field experienced by a particular nucleus is composed of an external field provided by the instrument and the field generated by the circulation of its electrons. The latter opposes the external field and the coupling phenomenon. Thus, it is possible to accurately measure the frequency difference between the resonant signals (peaks). The position of a signal in a NMR spectrum is described by its separation from another resonant signal taken as a reference. This separation is called chemical shift.

The chemical shift is directly proportional to the strength of the magnetic field (the frequency of the radio frequency emitter). However, the ratio of chemical shift (in frequency units) to instrument frequency is constant. This enables the definition of a dimensionless chemical shift parameter (δ) that is independent of the instrument frequency:

$$\delta = (\nu_s - \nu_r) / \nu_p + \delta_r$$

where

ν_s = frequency of the substance being analyzed;

ν_r = reference frequency;

ν_p = equipment frequency (in MHz);

δ_r = chemical shift from the reference.

The formula above is applicable to nearly all methods, with few exceptions. Tetramethylsilane (TMS) is the chemical shift reference most used to obtain hydrogen and carbon spectra since it is chemically inert, has a single signal at a higher field than most signals, and is volatile, enabling rapid sample recovery. Using the formula, it is possible to use the chemical shift of any species known as the chemical reference shift, for example, deuterated solvents that contain ^1H residue. Some care is necessary when TMS is not used as a solvent.

In NMR spectra, the strength of the magnetic field increases from left to right. Nuclei that resonate under high magnetic field strengths (to the right) are better protected (higher electronic density) than those that resonate under lower magnetic field strengths.

In **Figure 2**, the hydrogen NMR spectrum of benzoyl acetate is shown. This substance contains hydrogens in methyl, methylene and aromatic groups. These groups are located in distinct molecular environments, observed in the spectrum as three different hydrogen peaks referring to methyl (CH_3), methylene (CH_2), in addition to the peak corresponding to the resonance of aromatic hydrogens (H-Ar) (*a*, *b* and *c*, respectively).

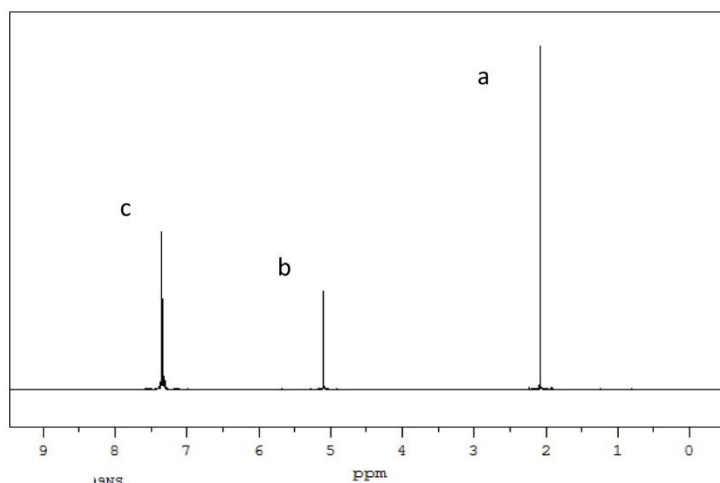


Figure 2– NMR spectrum of ^1H of benzoyl acetate.

Another information to be obtained in the NMR spectrum is the phenomenon of the *spin-spin* interaction. Accordingly, the coupling between the nuclei, called *spin-spin* coupling (J), corresponds to the separation (in Hertz) between the individual peaks of the signal (multiplet). When the interaction of the nuclei occurs in a reciprocal manner, the coupling constants observed in the multiplets are equal. In addition, J is independent of the magnetic field strength.

In a relatively simple first-order *spin* system, the number of individual peaks expected in a multiplet and the relative peak intensities are predictable. The number of peaks is determined by $2n + 1$ (only when J is equal), where n is the number of nuclei in adjacent groups that share the same signal. For hydrogen it is convenient $(n + 1)$ peaks. In general, relative intensity of each peak is the multiplet signal followed by the binomial expansion coefficient $(a + b)^n$. These coefficients can be found using Pascal's triangle, which produces the following relative areas for the related signals: doublet (1:1), triplet (1:2:1), quartet (1:3:3:1), quintet (1:4:6:4:1), sextet (1:5:10:10:5:1), and heptet (1:6:15:20:15:6:1). This ordered system, usually Dv referred to a first-order behavior, can be expected when the ratio and J is higher than 10; Dv is the chemical shift difference between the nuclei and the equivalent nuclei groups. Two examples of first-order coupled spectra are shown in **Figure 3**.

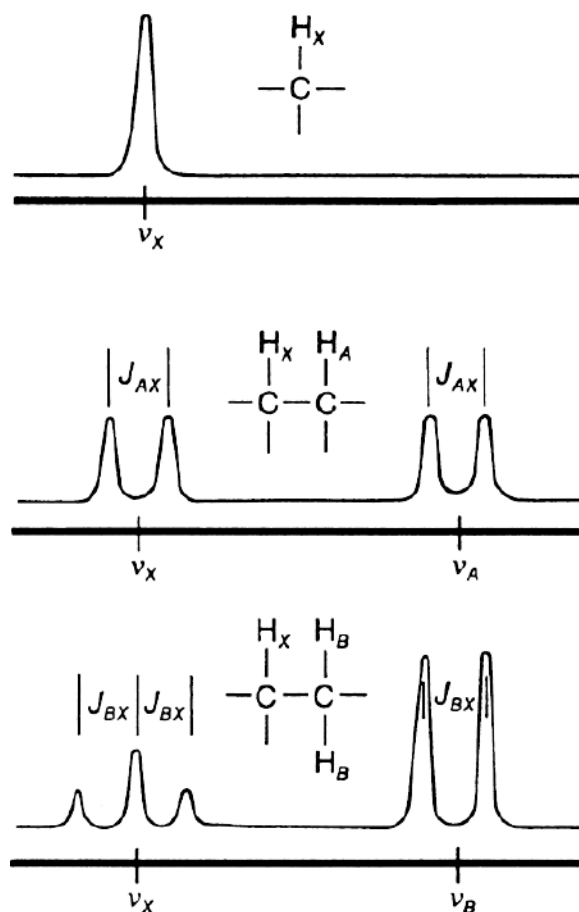


Figure 3 – First order coupling example.

In **Figure 4**, a spectrum with triplet and quartet signals is presented. We can note that the methylene hydrogens are split into a quartet (four peaks) and that the methyl group is split into a triplet (three peaks).

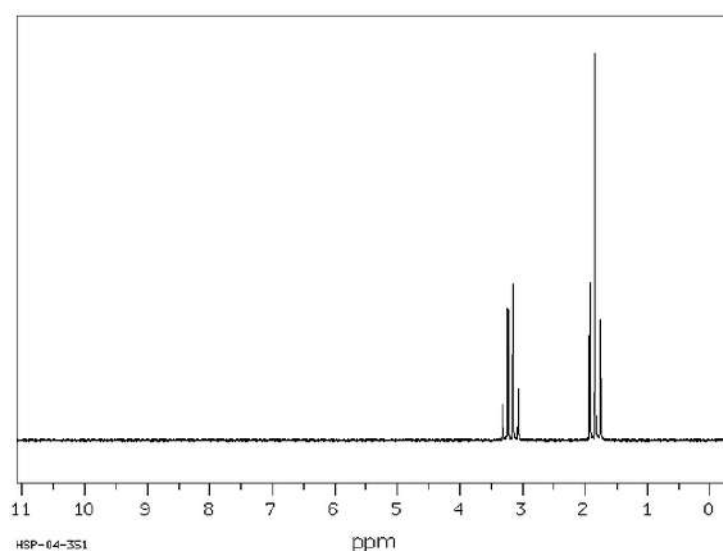


Figure 4 – NMR spectrum of ^1H of ethyl iodide.

Relative intensity is another feature of the experiment that has wide analytical applications. The area of a signal is directly proportional to the number of hydrogens present in a spectrum.

As a result, it is possible to determine the relative ratio of different types of hydrogens, or other nuclei, in a sample.

The NMR spectrum may contain signals unrelated to the sample due to the inhomogeneity of the magnetic field. These signals, called high rotation sidebands, indicate that it is necessary to adjust the compensation coils and are easily identifiable. The separation of the signals is equal to the frequency of the rotation speed of the sample tube.

METHOD

Sample preparation or incorrect instrumental settings and parameters can lead to decreased sensitivity (low resolution), spectral artifacts, and erroneous data. The user must be familiar with NMR theory, sample properties, and instrument operating principles. The instruction manuals provided by the manufacturers must be strictly followed and frequent checks on the equipment calibration and operation must be carried out.

The procedures described herein refer specifically to hydrogen (^1H NMR) and fluorine (^{19}F NMR) NMR, and can be applied, with some modifications, to other nuclei. The NMR spectrum is obtained from liquid or solid samples dissolved in an appropriate solvent.

Solvent selection – Appropriate solvents, in addition to having good dissolving properties, should not present resonance peaks that interfere with those originated by the sample under analysis. The most used solvents in hydrogen and carbon NMR are described in **Table 2**. Deuterated solvents also provide their signals to heteronuclear systems, enabling field fixation. The isotopic purity of the solvent should be as high as possible to avoid peaks of these impurities making it difficult to see any signal from the sample. Deuterium ($J = 1$) does not resonate under ^1H conditions, but can cause J coupling.

Table 2 – Chemical shift values of ^1H (ppm) for some solvents commonly used in NMR.

<i>Solvent</i>	<i>Deuterated form</i>	<i>Chemical Shift (Multiplicity)</i>
Acetone	Acetone- d_6	2.05 – 5
Acetonitrile	Acetonitrile- d_3	1.93 – 5
Benzene	Benzene- d_6	7.15 (wide)
Carbon tetrachloride	----	----
Chloroform	Chloroform- d	7.25 – 1
Dimethylsulfoxide	Dimethylsulfoxide- d_6	2.49 – 5
Water	Deuterium oxide	4.82 – 1
Methanol	Metanol- d_6	4.84 (1) hydroxyl 3.30 (5) methyl
Methylene chloride	Methylene chloride- d_2	5.32 – 3

In some solvents (D_2O and CD_3OD), rapid exchanges of deuterium with the hydrogen in the sample can occur, eliminating the signal of a series of groups: $-\text{COOH}$, $-\text{OH}$ and $-\text{NH}_2$. The hydrogens of alcohols and amines are not exchanged quickly, except in the presence of D_2O and some other solvents (CD_3OD), and can be restored with small concentrations of acid or base.

For ^{19}F NMR, most solvents used in ^1H NMR can be used, the most common being CHCl_3 , CCl_4 , H_2O , CS_2 , aqueous acids and bases, and dimethylacetamide. In general, any non-fluorinated solvent can be used whenever spectroscopic calibration is to be performed. Obviously, there is no interference

of protic functional groups with the solvent. However, protic and ^{19}F functional groups in the sample are *J*-coupled unless they are uncoupled.

Sample preparation – Solution is prepared in a suitable container and transferred to NMR analysis tube. Volume required depends on the analysis tube size and the instrument geometry. The solution level in the test tube should extend above the coils as it is inserted into the probe and rotated. Solute concentration depends on the experiment objective and type of instrument. Detecting small amounts of contaminants may require more concentrated solutions.

NMR sample tubes must strictly comply with the tolerance specifications for diameter, wall thickness and curvature. The most commonly used tubes have an external diameter of 5mm and measure between 15 and 20 cm. There are microtubes for analyzing small amounts of samples.

Procedure – The tube containing sample is introduced into a probe perpendicular to the magnetic field. The probe contains an electronic circuit that includes a radio frequency emitter, and accessories for rotating the tube containing the sample.

The instrument adjusts before each experiment. The rotation speed of the tube containing the sample is adjusted in such a manner that the rotating sidebands do not interfere with the peaks of interest and the vortex must not exceed the probe coils. To optimize the instrument performance, the magnetic compressor gradients can be adjusted in the NMR spectrometers so that the oscillation phenomenon does not occur.

The computer-aided operation of a NMR spectrometer enables to control the equipment; the programming of the experiment; data collection and processing. Programming the experiment involves recording the values of a large number of variables (peaks), including the width of the spectrum that will be examined (expansion); the duration of the excitation pulse; the data acquisition time and the waiting period between data acquisition of one sample and the next. The analysis time for an accumulated is in the order of seconds. The number of accumulations to be acquired depends on the concentration of the sample; the type of nucleus and the objective with the experiment. At the end of the experiment, the FID is digitally stored in memory, appearing on the video monitor. This FID can be processed mathematically to improve resolution or sensitivity and can be converted to frequency range spectra using Fourier transform. The integration of the peaks results in a graph with staggered lines. More accurate signals can be obtained when they are integrated separately.

Using NMR spectrometers, qualitative and quantitative data can be obtained. In quantitative experiments, special precautions must be taken so that the signal areas are proportional to the number of hydrogens. The waiting times between pulses must be sufficient to allow the total relaxation of all excited nuclei. This considerably increases the analysis time and some resolution is lost. Qualitative analysis is usually performed under non-quantitative conditions, with an experiment designed to obtain a rapid analysis with maximum resolution and sensitivity.

QUALITATIVE ANALYSIS

Comparison of a spectrum described in the literature or that of a reference substance with that of a sample under analysis can be used to confirm the identity of a compound and detect the presence of impurities. The NMR spectra of simple structures can be adequately described using numerical values for chemical shifts, coupling constants and the number of hydrogens corresponding to each signal (the instruments include programs that generate simulated spectra of substances with this data). Experimental details such as solvent, sample concentration and chemical shift reference can also be added to the spectra.

For unknown samples, NMR analysis, usually accompanied by other analytical information, is a powerful method for structural elucidation. Chemical shifts provide information about the chemical environment of the nuclei. There are many publications with correlation tables and rules for analyzing these chemical shifts. The multiplicity of signals provides important stereochemical information. The mutual partition of signals from functional groups indicates their proximities. The magnitude of the coupling constant J between residual hydrogens in substituted aromatic, olefinic or cycloalkyl structures is used to identify the relative position of the substituents.

There are several special methods (double resonance; chemical exchange; use of shift reagents; two-dimensional analysis; etc.) to simplify some of the more complex spectra; identify certain functional groups and determine coupling correlations.

In the double resonance method, in a simple hydrogen system often referred to as the AX system, each hydrogen appears as a doublet. If we introduce a fluent radiofrequency field at the frequency of X, while the normal radiofrequency field that maintains the frequency responsible for the resonance of A, the shift between A and X will be nullified (homonuclear shift). Accordingly, the A signal will not be coupled, and it now appears as a singlet. Routine ^{13}C spectra are obtained under proton decoupling conditions that nullify all ^{13}C - ^1H heteronuclear couplings. As a result of this decoupling, carbon signals appear as singlets unless other nuclei that are not uncoupled are present (e.g. ^{19}F , ^{31}P).

Functional groups containing interchangeable hydrogens bonded to heteroatoms such as $-\text{OH}$, $-\text{NH}_2$ or $-\text{COOH}$ can be identified by quickly exchanging the hydrogens with D_2O . To determine the presence and position of these groups it is necessary to test the substance in CDCl_3 or $\text{DMSO}-d_6$ and then add a few drops of D_2O to the sample tube, shake and analyze again. The resonance peaks of these groups collapse in this second analysis and are replaced by singlet HDO between 4.7 and 5.0 ppm.

This chemical exchange serves to exemplify the speed effect of the intermolecular and intramolecular processes on NMR spectra. If a hydrogen can experience different environments as a result of this process (tautomerism; rotation around an axis; exchange equilibria; ring inversion; etc.), the appearance of the spectrum will be a function of the process speed. Slow processes (time scale in NMR) provide more than one signal, whereas fast processes provide narrow signals, and other intermediate processes large signals.

The computing programs in NMR devices contain a sequence of varied pulses with the repetitive accumulation of transients described above. These experiments include two-dimensional homonuclear or heteronuclear analyses, which determine the correlation of couplings and can simplify the interpretation of more complex spectra.

QUANTITATIVE ANALYSIS

If the instrument is correctly calibrated for quantitative analysis, the areas under the peaks are proportional to the total number of hydrogens that generate them.

$$A_1/A_2 = N_1/N_2$$

If the peaks originate from functional groups of the same molecule, the formula can be simplified

$$A_1/A_2 = n_1/n_2$$

Where n_1 and n_2 are the number of hydrogens in the respective functional groups.

If signals originate from different molecular species,

$$A_1/A_2 = n_1m_1/n_2m_2 = (n_1W_1/M_1)/(n_2W_2/M_2)$$

Where m_1 and m_2 are the number of moles; W_1 and W_2 are the masses, and M_1 and M_2 are the molar masses of compounds 1 and 2 respectively.

The analysis of the formulas above makes it possible to verify that the quantitative analysis by NMR can be performed in an absolute or relative manner. In the absolute method, an internal standard must be added to the sample and the area under the resonance peak arising from the test substance must be compared with the area under the resonance peak of the internal standard. If such substances are exactly equivalent, the quantity of the substance can be calculated. A good internal standard must have the following properties: present a reference resonance peak, preferably singlet, in a field position distinct from all peaks in the sample; be soluble in the solvent used; no proton equivalent weight (when the weight divided between the number of hydrogens that generate a reference peak is low) and does not interact with the compound under analysis. The choice of internal standard will be dictated by the spectrum of the sample.

The relative method can be used to determine the molar fraction of an impurity in a sample (or components of a mixture) using the aforementioned formula.

Quantitative analyses and detection of impurity traces have improved with the development of modern instruments.

5.2.33 DETERMINATION OF RESIDUAL SOLVENTS

This chapter applies to active pharmaceutical ingredients, excipients and finished products. Every substance or product is subject to the determination of residual solvents that may be present, using an appropriate method.

Generally, tests for residual solvents are not mentioned in the individual monographs when the limits to be applied are in accordance with those indicated below, as the solvents employed may vary from one manufacturer to another.

The purpose of this chapter is to inform the acceptable amounts of residual solvents in pharmaceuticals for patient safety. The chapter recommends the use of less toxic solvents and describes levels considered toxicologically acceptable for some residual solvents.

For pharmacopoeial purposes, residual solvents in pharmaceuticals are defined as volatile organic chemicals that are used or produced during the manufacture of active pharmaceutical ingredients or excipients, or in the preparation of finished products. Residual solvents are not completely removed during manufacturing process.

This chapter does not deal with solvents that are used as excipients or with solvates. However, the solvent content in such products must be evaluated and justified.

Pharmaceutical products must not contain amounts of residual solvents higher than those allowing for safety data. Avoid the use of solvents that cause unacceptable toxicity (Class 1, **Table 3**) in the production of active pharmaceutical ingredients, excipients or finished products, unless their use can be strongly justified by a risk-benefit assessment. The use of solvents associated with less severe toxicity (Class 2, **Table 4**) should be limited to protect patients from potential adverse effects. In an ideal situation, the least toxic solvents (Class 3, **Table 5**) should be used. In Appendix 1, the complete list of solvents included in this chapter is presented. These tables and list are not mutually exclusive.

APPLICATION SCOPE

Active pharmaceutical ingredients, excipients and finished products must be analyzed to detect the presence of residual solvents when it is known that purification or production processes may result in the presence of such solvents.

It is necessary to carry out the tests for solvents that are used or produced in the purification or manufacture of active pharmaceutical ingredients, excipients or finished products, even when the test is not indicated in the individual monograph.

Although manufacturers may choose to test the finished product, a cumulative procedure can be used to calculate the levels of residual solvents present in the finished product from the levels in the ingredients used to produce the finished product. If the calculations result in a level equal to or lower than that given in this general chapter, carrying out the residual solvent test on the finished product is not required.

However, if the calculated level is above the recommended level, the finished product must be analyzed to determine if the formulation process has reduced the corresponding solvent level to the acceptable amount. A finished product must also be analyzed if any solvent is used during its manufacture.

GENERAL PRINCIPLES

Classification of residual solvents by risk assessment

The term permitted daily exposure (PDE) is defined as the maximum allowable intake of residual solvents from pharmaceuticals.

Residual solvents that are evaluated in this chapter are listed in Appendix 1 according to their chemical structure and common name. They were evaluated according to the risk they pose to human health and placed in one of the three classes below:

Residual solvent class	Assessment
Class 1	Solvents that should be avoided: • Substances known to be carcinogenic to humans. • Substances seriously suspected of being carcinogenic to humans. • Substances that pose environmental hazards
Class 2	Solvents that should be limited: – Non-genotoxic carcinogenic substances in animals, or possible causative agents of other irreversible toxicities, such as neurotoxicity or teratogenicity. – Solvents suspected of causing other significant but reversible toxic effects.
Class 3	Solvents with low toxic potential: – Low toxic potential solvents for humans; an exposure limit based on health risk is not required. These solvents have a PDE of 50 mg or higher per day.

Options to describe the limits of Class 2 residual solvents

There are two options to set the limits of Class 2 residual solvents.

Option 1 – Concentration limits in ppm shown in **Table 4** are used. These limits were calculated using the formula below, assuming a product weight of 10 g, administered daily.

$$\text{Concentration (ppm)} = (1000 \mu\text{g/mg} \times \text{PDE})/\text{dose}$$

In this case, PDE is expressed in mg per day and the dose is expressed in g per day.

These limits are considered acceptable for all active pharmaceutical ingredients, excipients and finished products. Therefore, this option can be applied if the daily dose is not known or has not been defined. If all active pharmaceutical ingredients and excipients in a formulation comply with the limits given in *Option 1*, these components can be used in any proportion. It is not necessary to perform additional calculations as long as the daily dose does not exceed 10 g. Products that are administered in doses higher than 10 g per day are listed in *Option 2*.

Option 2 - Each component of the finished product is not required to comply with the limits set in *Option 1*. PDE, expressed in mg per day, as indicated in **Table 4**, with the maximum known daily dose and the formula mentioned above, can be used to determine the concentration of residual solvent allowed in a finished product.

Such limits are considered acceptable if it can be proved that the residual solvent has been reduced to the minimum possible. Limits must be realistic regarding analytical accuracy, manufacturability, and

reasonable variation in the manufacturing process. The limits must also meet current manufacturing standards.

Option 2 can be applied by adding the amount of residual solvents present in each of the components of the finished product. The sum of the amount of solvent per day must be lower than indicated by the PDE.

Below is an example of the application of *Option 1* and *Option 2* for the concentration of acetonitrile in a finished product. The permitted daily exposure for acetonitrile is 4.1 mg per day; therefore, *Option 1* limit is 410 ppm. Maximum administered daily weight of a finished product is 5.0 g, which contains two excipients. The composition of the finished product and the calculated maximum amount of residual acetonitrile are shown in **Table 1**.

Table 1 – Example of application of Options 1 and 2 for acetonitrile concentration in a finished product.

<i>Component</i>	<i>Quantity in formulation (g)</i>	<i>Acetonitrile content (ppm)</i>	<i>Daily Exposure (mg)</i>
API	0.3	800	0.24
Excipient 1	0.9	400	0.36
Excipient 2	3.8	800	3.04
Finished product	5.0	728	3.64

Excipient 1 complies with *Option 1* limit, but API, Excipient 2 and finished product do not comply with *Option 1* limit. However, finished product complies with *Option 2* limit of not more than 4.1 mg acetonitrile per day and therefore complies with acceptance criteria in this chapter.

Below is another example using acetonitrile as the residual solvent. Maximum administered daily weight of a finished product is 5.0 g, which contains two excipients. Composition of finished product and calculated maximum amount of residual acetonitrile are shown in **Table 2**.

Table 2 –Example of application of Options 1 and 2 for acetonitrile concentration in a finished product.

<i>Component</i>	<i>Quantity in formulation (g)</i>	<i>Acetonitrile content (ppm)</i>	<i>Daily Exposure (mg)</i>
Drug	0.3	800	0.24
Excipient 1	0.9	2000	1.80
Excipient 2	3.8	800	3.04
Pharmaceutical product	5.0	1016	5.08

In this example, finished product does not meet *Option 1* or *Option 2* limit according to this sum. The manufacturer must analyze finished product to determine if the formulation process has reduced the acetonitrile concentration.

If, during formulation, acetonitrile concentration has not been reduced to allowable limits, the product does not meet the residual solvent limits as described in this chapter, and the pharmaceutical manufacturer must take other measures to reduce the amount of acetonitrile in the pharmaceutical product.

Analytical procedures

Typically, residual solvents are determined using chromatographic methods such as gas chromatography. The official methods for analyzing the content of residual solvents are described in the item *Identification, control and quantification of residual solvents* in this chapter.

If Class 3 solvents are present, a non-specific method such as loss on desiccation can be used.

Report residual solvent levels

Finished product manufacturers need certain information about the content of residual solvents in APIs or excipients to meet the criteria in this general chapter. The following statements are provided as acceptable examples of the information that a manufacturer of APIs or excipients could provide to a manufacturer of finished products. The manufacturer may choose one of the options below, as appropriate:

- It is likely that only Class 3 solvents are present. The loss on desiccation is less than 0.5%.
- It is likely that only X, Y, ... Class 2 solvents are present. All are below the *Option 1* limit (here the manufacturer would mention Class 2 solvents, represented by X, Y, ...)
- It is likely that only X, Y, ... Class 2 and Class 3 solvents are present. Class 2 residual solvents are below the *Option 1* limit and Class 3 residual solvents are below 0.5%.

The phrase "is likely to be present", as used in the examples above, refers to the solvent used or produced in the final manufacturing step and the solvents used or produced in the initial manufacturing steps that are not uniformly removed by a validated process.

If Class 1 solvents are likely to be present, these should be identified and quantified. If Class 2 or 3 solvents are present in an amount that exceeds the *Option 1* or 0.5% limits, respectively, these must be identified and quantified.

RESIDUAL SOLVENTS LIMITS

Class 1 (solvents that should be avoided)

Class 1 residual solvents (**Table 3**) should not be used in the manufacture of APIs, excipients or finished products due to unacceptable toxicity or their harmful environmental effects. However, if its use in the manufacture of a drug with a significant therapeutic advantage is unavoidable, its levels should be restricted, as shown in **Table 3**, unless otherwise indicated in the individual monograph. The 1,1,1-trichloroethane solvent was included in **Table 3** because it represents an environmental hazard. The stated limit of 1500 ppm is based on review of safety data.

When Class 1 residual solvents are used or produced in the manufacture or purification of APIs, excipients or finished products and they are not removed during the process, these must be identified and quantified. The procedures described in item *Identification, control and quantification of residual solvents* in this chapter must be applied whenever possible. If this is not possible, a validated procedure must be used.

Table 3 – Class 1 residual solvents.

<i>Solvent</i>	<i>Concentration limit (ppm)</i>	<i>Reason</i>
Benzene	2	Carcinogenic
Carbon tetrachloride	4	Toxic and poses risks to the environment
1,2-Dichloroethane	5	Toxic

Solvent	Concentration limit (ppm)	Reason
1,1-Dichloroethane	8	Toxic
1,1,1-Trichloroethane	1500	Poses risks to the environment

Class 2 (solvents that should be avoided)

Class 2 residual solvents (**Table 4**) should be limited in APIs, excipients and finished products due to their inherent toxicity. EDP is supplied to an approximate 0.1 mg per day and concentrations to an approximate 10 ppm. The values indicated do not reflect the necessary analytical precision of the determination process. Accuracy should be determined as part of procedure validation.

If Class 2 residual solvents are present in an amount that exceeds the *Option 1*, these must be identified and quantified. The procedures described in item *Identification, control and quantification of residual solvents* in this chapter must be applied whenever possible. If this is not possible, a validated procedure must be used.

Note: The following Class 2 residual solvents are not easily detected under the gas phase injection conditions described in the item *Identification, control and quantification of residual solvents* in this chapter: formamide, 2-ethoxyethanol, 2-methoxyethanol, ethylene glycol, N-methylpyrrolidone and sulfolane. Other appropriate validated procedures for quantification of these residual solvents must be employed.

Table 4 – Class 2 residual solvents.

Solvent	EDP (mg/day)	Concentration limit (ppm)
Acetonitrile	4.1	410
Cyclohexane	38.8	3880
Chlorobenzene	3.6	360
Chloroform	0.6	60
Methylene chloride	6.0	600
Cumene	0.7	70
1,2-Dichloroethane	18.7	1870
N,N-Dimethylacetamide	10.9	1090
N,N-Dimethylformamide	8.8	880
1,2-Dimethoxyethane	1.0	100
1,4-Dioxane	3.8	380
Ethylene glycol	6.2	620
2-Ethoxyethanol	1.6	160
Formamide	2.2	220
Hexane	2.9	290
Methanol	30.0	3000
Methylbutylketone	0.5	50
Methylcyclohexane	11.8	1180
Methylisobutylketone	45	4500
N-Methylpyrrolidone	5.3	530
2-Methoxyethanol	0.5	50
Nitromethane	0.5	50
Pyridine	2.0	200
Sulfolane	1.6	160

Solvent	EDP (mg/day)	Concentration limit (ppm)
Tetrahydrofuran	7.2	720
Tetralin	1.0	100
Toluene	8.9	890
Trichlorethylene	0.8	80
Xylene*	21.7	2170

*Generally 60% *m*-xylene, 14% *p*-xylene, 9% *o*-xylene with 17% ethylbenzene.

Class 3 (solvents with low toxic potential)

Class 3 residual solvents (**Table 5**) are considered to be less toxic and pose a lower risk to human health when compared to Class 1 and Class 2 residual solvents. Class 3 does not include solvents that pose a risk to human health at levels normally accepted in pharmaceutical products. However, there are no long-term carcinogenicity or toxicity studies for many of the Class 3 residual solvents. Available data indicate that they are less toxic in acute or short-term toxicity studies and negative in genotoxicity studies.

Residual solvent amounts of 50 mg per day or less (corresponding to 5000 ppm or 0.5% in *Option 1*) would be considered acceptable without justification. Higher amounts may also be acceptable, where authorized by the relevant regulatory authority, taking into account, among other things, process capability and good manufacturing practices.

If the limit of a Class 3 solvent in an individual monograph is greater than 50 mg per day, such residual solvent must be identified and quantified. The procedures described in item *Identification, control and quantification of residual solvents* in this chapter must be applied whenever possible with the required changes of the reference solutions. If this is not possible, another validated procedure must be used.

Table 5 – Class 3 residual solvents.

(Limited by good manufacturing practices or other quality-based requirements in API, excipients and finished products)

Butyl acetate	Ethanol
Ethyl acetate	Tert-butyl methyl ether
Isobutyl acetate	Ethyl ether
Isopropyl acetate	Ethyl formate
Methyl acetate	Heptane
Propyl acetate	3-Methyl-1-butanol
Acetone	Methyl ethyl ketone
Acetic Acid	2-Methyl-1-propanol
Formic acid	Pentane
Anisole	1-Pentanol
1-Butanol	1-Propanol
2-Butanol	2-Propanol
Dimethylsulfoxide	Triethylamine

Other residual solvents

The residual solvents listed in **Table 6** may also be of interest to manufacturers of APIs, excipients or finished products. However, adequate toxicological data to support PDE have not yet been found.

Table 6 –Other residual solvents.
(For which adequate toxicological data have not been found)

Trichloroacetic acid	Isopropyl ether
Trifluoroacetic acid	Petroleum ether
1.1-Diethoxypropane	Isoctane
1.1-Dimethoxymethane	Methyl isopropyl ketone
2.2-Dimethoxypropane	Methyltetrahydrofuran

IDENTIFICATION, CONTROL AND QUANTIFICATION OF RESIDUAL SOLVENTS

Whenever possible, the analyte needs to be dissolved to release the residual solvent. In some cases, it may be acceptable that some of the components of the formulation do not fully dissolve. In such cases, it may be necessary to first reduce the pharmaceutical product to a fine powder to release any residual solvent that may be present. This operation must be carried out as soon as possible to avoid the loss of volatile solvents during the procedure.

Note: these procedures must be carried out with water free of organic substances to avoid the presence of peaks that could significantly interfere with the chromatogram.

Class 1 and 2 residual solvents

The following procedures are useful to identify and quantify residual solvents when you do not have information about which ones might be present in the material, when information about them is not available. When information on the presence of specific residual solvents is available, it is only necessary to perform *Procedure C* to quantify the residual solvents present. **Figure 1** presents a flow diagram for the application of residual solvent limit tests.

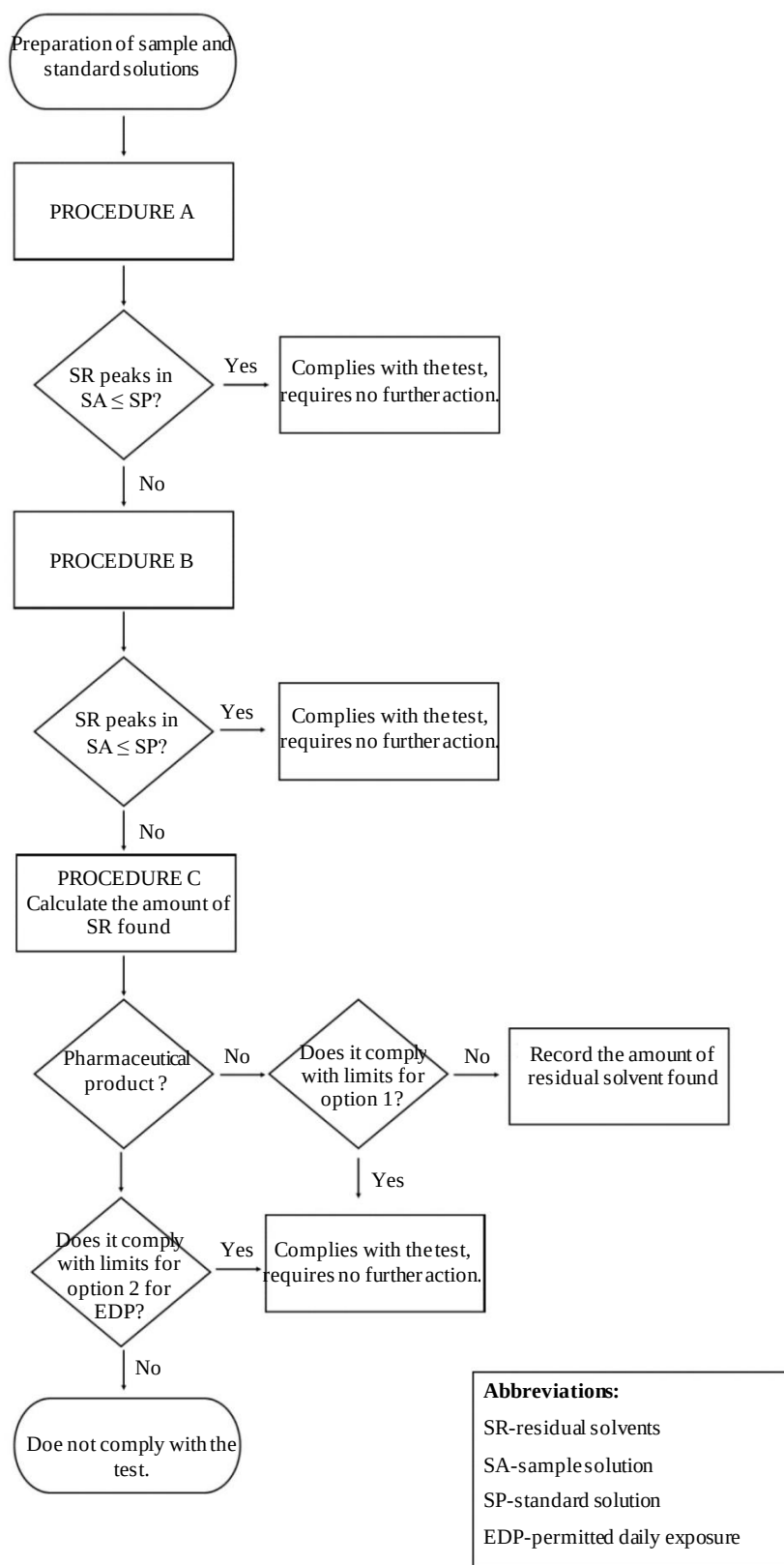


Figure 1 - Flowchart referring to the identification of residual solvents and the application of limit tests.

Water-soluble substances

Procedure A

Class 1 stock standard solution: [**Note:** when transferring solutions, place the pipette tip just below the liquid surface and mix]. Transfer 1.0 mL of the reference standard Class I residual solvent mixture to a 100 mL volumetric flask to which approximately 9 mL of dimethylsulfoxide has been previously added, adjust volume with water and homogenize. Transfer 1.0 mL of this solution to a 100 mL volumetric flask, to which about 50 mL of water were previously added, adjust volume with water and homogenize. Transfer 10.0 mL of this solution to a 100 mL volumetric flask, to which about 50 mL of water were previously added, complete the volume with water and homogenize.

Class 1 Standard Solution: transfer 1.0 mL of *Class 1 Stock Standard Solution* to a suitable gas phase sampling flask containing 5.0 mL of water (place the pipette tip just below the liquid surface for dispensing), cap and homogenize.

Class 2 stock standard solutions: transfer 1.0 mL of mixture A – *Class 2 Residual Solvents reference standard* to a 100 mL volumetric flask, adjust volume with water and homogenize. This is *Class 2 stock A Standard Solution*. Transfer 1.0 mL of mixture B – *Class 2 Residual Solvents reference standard* to a 100 mL volumetric flask, adjust volume with water and homogenize. This is *Class 2 stock B Standard Solution*.

Class 2 Standard Solution A: transfer 1.0 mL of *Class 2 Stock A Standard Solution* to a suitable gas phase sampling flask containing 5.0 mL of water), cap and homogenize.

Class 2 Standard Solution B: transfer 5.0 mL of *Class 2 Stock B Standard Solution* to a suitable gas phase sampling flask containing 1.0 mL of water, cap and homogenize.

Stock sample solution: transfer approximately 250 mg of the material under analysis, accurately weighed, into a 25 mL volumetric flask, dissolve and adjust volume with water and homogenize.

Sample Solution: Transfer 5.0 mL of *Stock Sample Solution* to a suitable gas phase sampling flask, add 1.0 mL of water, cap and homogenize.

Class 1 System Suitability Solution: Transfer 1.0 mL of *Class 1 Reference Stock Solution* to a suitable gas phase sampling flask, add 5.0 mL of *Stock Sample Solution*, cap and homogenize.

Chromatographic system: gas chromatograph equipped with flame ionization detector and 0.32mm × 30 m fused silica column, coated with a phase layer of 6% cyanopropyl phenyl – 94% 1.8 µm dimethylpolysiloxane, or column of 0.53 mm × 30 m macrocapillary layer, coated with a phase layer of 6% cyanopropyl phenyl – 94% dimethylpolysiloxane 3.0 µm. The carrier gas is nitrogen or helium at a linear velocity of approximately 35 cm/s and a partition ratio of 1:5 [**Note:** the partition ratio can be modified to optimize sensitivity]. Keep the column temperature at 40°C for 20 minutes; then raise the temperature to 240°C at a heating rate of 10°C per minute and hold at 240°C for 20 minutes. Maintain the injector and detector temperatures at 140°C and 250°C, respectively. Inject *Class 1 Standard Solution*, *Class 1 System Suitability Solution*, and *Class 2 Standard Solution A* into the chromatograph and record the chromatogram as indicated in *Procedure*. The signal/noise ratio of 1,1,1-trichloroethane in the *Class 1 Standard Solution* is minimum 5; the signal/ noise ratio of each peak in the *Class 1 System Suitability Solution* is minimum 3; and the resolution *R* between the peaks of acetonitrile and methylene chloride in *Class 2 Standard Solution A* is minimum 1.0.

Procedure: [**Note:** It is recommended to increase the transfer line temperature between runs to eliminate any potential condensation from the solvents.]. Inject, separately, into the chromatograph (following the operating parameters for the gas phase injector, as described in **Table 7**) equal volumes of the gas phase (1 mL) of *Class 1 Standard Solution*, *Class 2 Standard Solution A*, *Class 2 standard solution B* and *Sample Solution*, record the chromatograms and measure the main peak responses. If

the response of any peak other than the 1,1,1-trichloroethane peak in the *Sample Solution* is higher or equal to the corresponding peak obtained with the *Class 1 Standard Solution* or with any peak obtained with the *Class 2 Standard Solutions (A and B)*, or if the response of the 1,1,1-trichloroethane peak is higher or equal to 150 times the corresponding peak response of 1,1,1-trichloroethane obtained with the *Class 1 standard solution*, follow as *Procedure B* to verify peak identity; if no peak is detected, within the criteria specified above, the sample meets the requirements of this assay.

Table 7 – Operating parameters for the gas phase injector.

	<i>Operating parameters for the gas phase injector.</i>		
	<i>1</i>	<i>2</i>	<i>3</i>
Equilibrium temperature (*)	80	105	80
Equilibrium time (minutes)	60	45	45
Transfer line temperature (*) (if applicable)	85	110	105
Syringe temperature (*) (if applicable)	80-90	105-115	80-90
Pressurization time (s) (if applicable)	≥ 60%	≥ 60%	≥ 60%
Injection volume (mL)*	1	1	1
Carrier gas: nitrogen or helium at suitable pressure			

* Or follow the instrument manufacturer's recommendations, as long as the method criteria are met. It is allowed to inject a smaller amount than specified, as long as adequate sensitivity is sought.

Procedure B

Class 1 stock standard solution, Class 1 standard solution, Class 2 stock standard solution, Class 2 standard solution A, Class 2 standard solution B, Stock sample solution, Sample solution and Class 1 system suitability solution: prepare as indicated in *Procedure A*.

Chromatographic system: gas chromatograph equipped with a flame ionization detector and a 0.32 mm × 30 m fused silica column, coated with a 0.25 µm layer of polyethylene glycol (approximate molecular weight 15 000), or a column 0.53 mm × 30 m macrocapillary, coated with a 0.25 µm polyethylene glycol phase layer (approximate molecular weight 15 000). The carrier gas is nitrogen or helium at a linear velocity of approximately 35 cm/s and a partition ratio of 1:5 [**Note:** the partition ratio can be modified to optimize sensitivity]. Keep the column temperature at 50°C for 20 minutes; then raise the temperature to 165°C at a heating rate of 6°C per minute and hold at 165°C for 20 minutes. Maintain the injector and detector temperatures at 140°C and 250°C, respectively. Inject *Class 1 Standard Solution*, and *Class 1 System Suitability Solution* into the chromatograph and record the chromatogram as instructed in *Procedure*. The benzene signal/noise ratio, in *Class 1 Standard Solution*, is minimum 5; the signal/noise ratio of each peak in the *Class 1 System Suitability Solution* is minimum 3; and the resolution *R* between the acetonitrile and cis-dichloroethene peaks in *Class 2 Standard Solution A* is minimum 1.0.

Procedure: [**Note:** It is recommended to increase the transfer line temperature between runs to eliminate any potential condensation from the solvents.]. Inject, separately, into the chromatograph (following the operating parameters for the gas phase injector, as described in **Table 7**) equal volumes of the gas phase (1,0 mL) of *Class 1 Standard Solution*, *Class 2 Standard Solution A*, *Class 2 standard solution B* and *Sample Solution*, record the chromatograms and measure the main peak responses. If responses of the peaks obtained with the *Sample Solution*, identified in *Procedure A*, are equal or higher than the corresponding peaks obtained with the *Class 1 Standard Solution* or with either of the two *Class 2 Standard Solutions (A and B)*, proceed according to *Procedure C* to quantify analytes; if no peak is detected, within the criteria specified above, the sample meets the assay requirements

Procedure C

Class 1 stock standard solution, Class 1 standard solution, Class 2 stock A standard solution, Class 2 standard solution A, Stock sample solution, Sample solution and Class 1 system suitability solution: prepare as indicated in *Procedure A*.

Stock Standard Solution: [**Note:** Prepare, separately, a Stock Standard Solution for each identified and confirmed peak as per Procedures A and B. For Class 1 solvents other than 1,1,1-trichloroethane, prepare the first dilution as described for the first dilution of Class 1 Stock Standard Solution, Procedure A]. Transfer an accurately measured volume of each reference standard corresponding to each residual solvent peak identified and confirmed after performing Procedures A and B to a suitable flask and dilute quantitatively with water to obtain a solution with a final concentration of 1/20 of the value given in **Table 3** or **Table 4** (under *Concentration Limit*).

Standard Solution: Transfer 1.0 mL of the Stock Standard Solution to a suitable gas phase sampling flask, add 5.0 mL of water, cap and homogenize.

Sample Solution with a known amount added: [**Note:** separately prepare a Sample Solution with the addition of a known amount of the analyte from each identified and verified peak as per Procedures A and B]. Transfer 5.0 mL of Stock Sample Solution to an appropriate vial, add 1.0 mL of Stock Standard Solution, cap and homogenize.

Chromatographic System: [**Note:** If it is confirmed that the chromatographic analysis results for Procedure A are inferior to those observed for Procedure B, the Chromatographic System for Procedure B may be replaced]. Use a gas chromatograph equipped with a flame ionization detector and a 0.32 mm × 30 m fused silica column, covered with a 6% cyanopropyl phenyl-94% 1.8 µm dimethylpolysiloxane phase layer, or a macrocapillary column 0.53 mm × 30 m, overlaid with a phase layer of 6% cyanopropyl phenyl-94% dimethylpolysiloxane of 3.0 µm. The carrier gas is nitrogen or helium at a linear velocity of approximately 35 cm/s and a partition ratio of 1:5 [**Note:** the partition ratio can be modified to optimize sensitivity]. Keep the column temperature at 40°C for 20 minutes; then raise the temperature to 240°C at a heating rate of 10°C per minute and keep at 240°C for 20 minutes. Maintain the injector and detector temperatures at 140°C and 250°C, respectively. Inject Class 1 Standard Solution, Class 1 System Suitability Solution, and Class 2 Standard Solution A into the chromatograph and record the chromatogram as indicated in Procedure. The signal/noise ratio of 1,1,1-trichloroethane in the Class 1 Standard Solution is minimum 5; the signal/ noise ratio of each peak in the Class 1 System Suitability Solution is minimum 3; and the resolution *R* between the peaks of acetonitrile and methylene chloride, in Class 2 Standard Solution A, is minimum 1.0.

Procedure: [**Note:** It is recommended to increase the transfer line temperature between runs to eliminate any potential condensation from the solvents.]. Inject, separately, into chromatograph (following the operating parameters for the gas phase injector described in **Table 7**), equal volumes of the gas phase (1.0 mL) of each standard, Sample Solution and Sample Solution with a known amount added, record the chromatograms and measure the main peak responses. Calculate the amount, in ppm, of each residual solvent found in the material under analysis, using the formula:

$$5 (C / W)[rU / (rST - rU)]$$

where

C = concentration, in µg per mL, of the reference standard corresponding to the Stock standard solution;

W = weight, in g, of the material under analysis, weighed to prepare the *Stock Sample Solution*;
 rU and rST = peak responses of each residual solvent obtained from the *Sample Solution* and the *Sample Solution with a known added amount*, respectively.

Water-insoluble substances

Procedure A

Note: dimethylsulfoxide can be used as an alternative solvent to replace dimethylformamide.

Class 1 stock standard solution: transfer 1.0 mL of the reference standard Class I residual solvent mixture to a 100 mL volumetric flask to which approximately 80 mL of dimethylformamide has been previously added, adjust volume with the same solvent and homogenize. Transfer 1.0 mL of this solution to a 100 mL volumetric flask, to which approximately 80 mL of dimethylformamide has been previously added, complete the volume with the same solvent and homogenize (reserve a portion of this solution to prepare the *Class 1 System Suitability Solution*). Transfer 1.0 mL of this solution to a 10-mL volumetric flask, adjust volume with dimethylformamide and homogenize.

Class 1 Standard Solution: transfer 1.0 mL of *Class 1 Reference Stock Solution* to a suitable gas phase sampling vial, add 5.0 mL of water, cap and homogenize.

Class 2 stock standard solutions: transfer 1.0 mL of Mixture A – Class 2 Residual solvents reference standard to a 100 mL volumetric flask, to which approximately 80 mL of dimethylformamide has been previously added, adjust volume with the same solvent and homogenize. This is *Class 2 stock A Standard Solution*. Transfer 0,5 mL of Mixture B – Class 2 Residual Solvents reference standard to a 10 mL volumetric flask, adjust volume with dimethylformamide and homogenize. This is *Class 2 stock B Standard Solution*.

Class 2 Standard Solution A: Transfer 1.0 mL of *Class 2 Stock A Standard Solution* to a suitable gas phase sampling vial, add 5.0 mL of water, cap and homogenize.

Class 2 Standard Solution B: transfer 1.0 mL of *Class 2 Stock B Standard Solution* to a suitable gas phase sampling vial, add 5.0 mL of water, cap and homogenize.

Stock sample solution: transfer approximately 500 g of the material under analysis, accurately weighed, into a 10 mL volumetric flask, dissolve and adjust volume with dimethylformamide and homogenize.

Sample Solution: Transfer 1.0 mL of the *Stock Sample Solution* to a suitable gas phase sampling vial, add 5.0 mL of water, cap and homogenize.

Class 1 System Suitability Solution: homogenize 5 mL of the *Stock Sample Solution* with 0.5 mL of the reserved Intermediate dilution of the *Class 1 Stock Standard Solution*. Transfer 1.0 mL of this solution to a suitable gas phase sampling vial containing 5.0 mL of water, cap and homogenize.

Chromatographic system: gas chromatograph equipped with a flame ionization detector and a 0.53mm × 30m macrocapillary column, covered with a 6% cyanopropyl phenyl- 94% dimethylpolysiloxane phase layer of 3.0µm. The carrier gas is helium at a linear velocity of approximately 35 cm/s and a partition ratio of 1:3 [**Note:** the partition ratio can be modified to optimize sensitivity]. Keep the column temperature at 40°C for 20 minutes; then raise the temperature to 240°C at a heating rate of 10°C per minute and keep at 240°C for 20 minutes. Maintain the injector and detector temperatures at 140°C and 250°C, respectively. Inject *Class 1 Standard Solution*, *Class*

Class 1 System Suitability Solution, and *Class 2 Standard Solution A* into the chromatograph and record the chromatogram as indicated in *Procedure*. The signal/noise ratio of 1,1,1-trichloroethane in *Class 1 Standard Solution* is minimum 5; the signal/ noise ratio of each peak in *Class 1 System Suitability Solution* is minimum 3; and resolution *R* between acetonitrile and methylene chloride, in *Class 2 Standard Solution A*, is minimum 1.0.

Procedure: [Note: It is recommended to raise the transfer line temperature between runs to eliminate any potential condensation of solvents.] Inject separately into the chromatograph (use the operating parameters for the gas phase injector described in column 3 of **Table 7**, with a vial pressure of 10 psi), equal volumes of gas phase (1.0,mL) of *Class 1 Standard Solution*, *Class 2 Standard Solution A*, *Class 2 Standard Solution B*, and *Sample Solution*, record the chromatograms and measure the main peak responses. If the response of any peak other than the 1,1,1-trichloroethane peak in the *Sample Solution* is superior or equal to the corresponding peak obtained with the *Class 1 Standard Solution* or with any peak obtained with the *Class 2 Standard Solutions (A and B)*, or if the response of the 1,1,1-trichloroethane peak is higher than or equal to 150 times the corresponding peak response of 1,1,1-trichloroethane obtained with the *Class 1 standard solution*, follow as per *Procedure B* to verify peak identity; if no peak is detected, within the criteria specified above, the sample meets the requirements of this assay.

Procedure B

Class 1 Stock Standard Solution, *Class 1 Standard Solution*, *Class 1 System Suitability Solution*, *Class 2 Stock Standard Solutions*, *Class 2 Standard Solution A*, *Class 2 Standard Solution B*, *Stock Sample Solution* and *Sample Solution*: prepare as described in *Procedure A*.

Chromatographic system: proceed as described in *Procedure B* for *Water Soluble Materials* with a 1:3 partition ratio. **Note:** *The partition ratio can be modified to optimize sensitivity.*

Procedure: [Note: It is recommended to increase transfer line temperature between runs to eliminate any potential condensation from solvents]. Inject, separately, into chromatograph (use the operating parameters for the gas phase injector described in column 3 of **Table 7** with a vial pressure of 10 psi), equal volumes of the gas phase (approximately 1.0 mL) of *Class 1 standard solution*, *Class 2 Standard Solution A*, *Class 2 Standard Solution B*, and *Sample Solution*, record the chromatograms and measure the main peak responses. If responses of the peaks obtained with the *Sample Solution*, identified in *Procedure A*, are equal to or higher than the corresponding peaks obtained with the *Class 1 Standard Solution* or with either of the two *Class 2 Standard Solutions (A and B)*, proceed according to *Procedure C* to quantify analytes; if no peak is detected, within the criteria specified above, the sample meets the assay requirements.

Procedure C

Class 1 Stock Standard Solution, *Class 1 Standard Solution*, *Class 1 System Suitability Solution*, *Class 2 Stock A Standard Solution*, and *Class 2 Standard Solution A*: Proceed as described in *Procedure A*.

Stock Standard Solution: [Note: separately prepare a Stock Standard Solution for each identified and verified peak as per Procedures A and B. For Class 1 solvents other than 1,1,1-trichloroethane, prepare the first dilution as described for the first dilution of Class 1 Stock Standard Solution in Procedure A.] Transfer an accurately measured volume of each individual reference standard corresponding to each residual solvent peak identified and verified in *Procedures A and B* into a suitable container and dilute quantitatively with water to obtain a solution with a final concentration of 1/20 of the value specified in **Table 3** or **Table 4** (*Concentration Limit*).

Standard Solution: Transfer 1.0 mL of the *Stock Standard Solution* to a suitable gas phase sampling flask, containing 5.0 mL of water, cap and homogenize.

Stock sample solution: proceed as described in *Procedure A*.

Sample Solution: Transfer 1.0 mL of the *Stock Sample Solution* to a suitable gas phase sampling vial, containing 5.0 mL of water, cap and homogenize.

Sample Solution with a known amount added: [**Note:** separately prepare a *Sample Solution* with the addition of a known amount to each peak identified and verified as per *Procedures A and B*]. Transfer 1.0 mL of the *Stock Sample Solution* to a suitable gas phase sampling flask, add 1 mL of *Standard solution* and 4.0 mL of water, cap and homogenize.

Chromatographic system: proceed as described in *Procedure C* for *Water Soluble Materials*.

Procedure: [**Note:** It is recommended to increase transfer line temperature between runs to eliminate any potential condensation from solvents. Inject, separately, into chromatograph (use the operating parameters for the gas phase injector described in column 3 of **Table 7** with a vial pressure of 10 psi), equal volumes of the gas phase (approximately 1.0 mL) of *Standard Solution*, *Sample Solution* and *Sample Solution with a known amount added*, record the chromatograms and measure the main peak responses. Calculate the amount, in ppm, of each residual solvent found in the material under analysis, using the formula:

$$10 (C / W)[rU / (rST - rU)]$$

where

C = concentration, in µg per mL, of the reference standard corresponding to *Stock standard solution*;
W = weight, in g, of the material under analysis to prepare *Stock Sample Solution*;

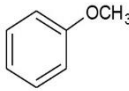
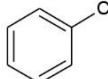
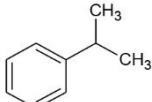
rU and *rST* = peak responses of each residual solvent obtained from *Sample Solution* and *Sample Solution with a known added amount*, respectively.

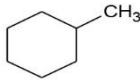
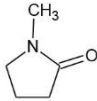
Class 3 residual solvents

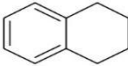
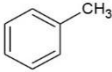
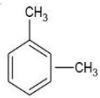
If Class 3 solvents are present, level of residual solvents can be determined according to the *Loss on Desiccation* method when the analyte monograph includes a loss on desiccation procedure that specifies an upper limit of maximum 0.5% (according to *Option 1* in this general chapter), or can be carried out by solvent specific determination. If analyte monograph does not include a loss on desiccation procedure or if Class 3 solvent limit in the individual monograph is higher than 50mg per day (which corresponds to 5000ppm or 0.5% - *Option 1*), the individual residual solvent of Class 3 or solvents present in the test substance must be identified and quantified, applying the procedures described above, with the necessary modifications in the standard solutions, whenever possible. If this is not the case, an appropriate validated procedure must be employed.

APPENDIX 1 – LIST OF RESIDUAL SOLVENTS INCLUDED IN THIS CHAPTER

<i>Dissolvent</i>	<i>Other terms</i>	<i>Structure</i>	<i>Class</i>
Butyl acetate	Acetic acid butyl ester	CH ₃ COO(CH ₂) ₃ CH ₃	Class 3
Ethyl acetate	Acetic acid ethyl ester	CH ₃ COOCH ₂ CH ₃	Class 3
Isobutyl acetate	Acetic acid isobutyl ester	CH ₃ COOCH ₂ CH(CH ₃) ₂	Class 3
Isopropyl acetate	Acetic acid isopropyl ester	CH ₃ COOCH(CH ₃) ₂	Class 3

<i>Dissolvent</i>	<i>Other terms</i>	<i>Structure</i>	<i>Class</i>
Methyl acetate	Acetic acid methyl ester	$\text{CH}_3\text{COOCH}_3$	Class 3
Propyl acetate	Acetic acid propyl ester	$\text{CH}_3\text{COOCH}_2\text{CH}_2\text{CH}_3$	Class 3
Acetone	2-Propanone Propan-2-one	CH_3COCH_3	Class 3
Acetonitrile		CH_3CN	Class 2
Acetic Acid	Ethanolic acid	CH_3COOH	Class 3
Formic acid		HCOOH	Class 3
Anisole	Methoxybenzene		Class 3
Benzene	Benzene		Class 1
1-Butanol	Butan-1-ol n-butyl alcohol	$\text{CH}_3(\text{CH}_2)_3\text{OH}$	Class 3
2-Butanol	Sec-butyl alcohol Butan-2-ol	$\text{CH}_3\text{CH}_2\text{CH}(\text{OH})\text{CH}_3$	Class 3
Cyclohexane	Hexamethylene		Class 2
Chlorobenzene			Class 2
Chloroform	Trichloromethane	CHCl_3	Class 2
Methylene chloride	Dichloromethane	CH_2Cl_2	Class 2
Cumene	Isopropylbenzene (1 -Methylethyl)benzene		Class 2
1,2-Dichloroethane	Sim-Dichloroethane Ethylene dichloride Ethylene chloride	$\text{CH}_2\text{ClCH}_2\text{Cl}$	Class 1
1,1-Dichloroethane	1,1-Dichloroethylene Vinylidene chloride	$\text{H}_2\text{C}=\text{CCl}_2$	Class 1
1,2-Dichloroethane	1,2-Dichloroethylene Acetylene dichloride	$\text{C}_1\text{HC}=\text{CHC}_1$	Class 2
N,N-Dimethylacetamide	DMA	$\text{CH}_3\text{CON}(\text{CH}_3)_2$	Class 2
N,N-Dimethylformamide	DMF	$\text{HCON}(\text{CH}_3)_2$	Class 2
Dimethylsulfoxide	Methylsulfinylmethane Methyl sulfoxide DMSO	$(\text{CH}_3)_2\text{SO}$	Class 3
1,2-Dimethoxyethane	Ethylene glycol dimethyl ether Monoglyme	$\text{H}_3\text{COCH}_2\text{CH}_2\text{OCH}_3$	Class 2
1,4-Dioxane	p-dioxane [1,4] Dioxane		Class 2
Ethanol	Ethyl alcohol	$\text{CH}_3\text{CH}_2\text{OH}$	Class 3

<i>Dissolvent</i>	<i>Other terms</i>	<i>Structure</i>	<i>Class</i>
Tert-butyl methylether	2-Methoxy-2-methylpropane	$(\text{CH}_3)_3\text{COCH}_3$	Class 3
Ethylether	Diethylether Ethoxyethane 1,1'-Oxybisethane	$\text{CH}_3\text{CH}_2\text{OCH}_2\text{CH}_3$	Class 3
Ethylene glycol	1,2-Dihydroxyethane 1,2-Ethanediol	$\text{HOCH}_2\text{CH}_2\text{OH}$	Class 2
2-Ethoxyethanol	Methanamide	$\text{CH}_3\text{CH}_2\text{OCH}_2\text{CH}_2\text{OH}$	Class 2
Formamide	Methanamide	HCONH_2	Class 2
Ethyl formate	formic acid ethyl ester	$\text{HCOOCH}_2\text{CH}_3$	Class 3
Heptane	n-Heptane	$\text{CH}_3(\text{CH}_2)_5\text{CH}_3$	Class 3
Hexane	n-Hexane	$\text{CH}_3(\text{CH}_2)_4\text{CH}_3$	Class 2
Methanol	methyl alcohol	CH_3OH	Class 2
3-Methyl-1-butanol	Isoamyl alcohol Isopentyl alcohol 3-Methylbutan-1-ol	$(\text{CH}_3)_2\text{CHCH}_2\text{CH}_2\text{OH}$	Class 3
Methylbutylketone	2-Hexanone Hexan-2-one	$\text{CH}_3(\text{CH}_2)_3\text{COCH}_3$	Class 2
Methylcyclohexane	Cyclohexylmethane		Class 2
Methyl ethyl ketone	2-Butanone MEK Butan-2-one	$\text{CH}_3\text{CH}_2\text{COCH}_3$	Class 3
Methyl isobutyl ketone	4-Methylpentan-2-one MIBK	$\text{CH}_3\text{COCH}_2\text{CH}(\text{CH}_3)_2$	Class 2
N-Methylpyrrolidone	4-Methyl-2-pentanone 1-Methylpyrrolidin-2-one 1-Methyl-2-pyrrolidone		Class 2
2-Methyl-1-propanol	Isobutyl alcohol 2-Methylpropan-1-ol	$(\text{CH}_3)_2\text{CHCH}_2\text{OH}$	Class 3
2-Methoxyethanol	Methyl cellosolve	$\text{CH}_3\text{OCH}_2\text{CH}_2\text{OH}$	Class 2
Nitromethane		CH_3NO_2	Class 2
Pentane	n-Pentane	$\text{CH}_3(\text{CH}_2)_3\text{CH}_3$	Class 3
1-Pentanol	Amyl alcohol Pentan-1-ol Pentyl alcohol	$\text{CH}_3(\text{CH}_2)_3\text{CH}_2\text{OH}$	Class 3
Pyridine			Class 2
1-Propanol	Propan-1-ol Propyl alcohol	$\text{CH}_3\text{CH}_2\text{CH}_2\text{OH}$	Class 3
2-Propanol	Propan-2-ol Isopropyl alcohol	$(\text{CH}_3)_2\text{CHOH}$	Class 3
Sulfolane	1,1-tetrahydrothiophene dioxide		Class 2

Dissolvent	Other terms	Structure	Class
Carbon tetrachloride	Tetrachloromethane	CCl_4	Class 1
Tetrahydrofuran	tetramethyleneoxide Oxacyclopentane		Class 2
Tetralin	1,2,3,4-Tetrahydronaphthalene		Class 2
Toluene	Methylbenzene		Class 2
1,1,1-Trichloroethane	Methylchloroform	CH_3CCl_3	Class 1
Trichlorethylene	1,1,2-Trichloroethane	$\text{HC}_1\text{C}=\text{CCl}_2$	Class 2
Triethylamine	N,N-diethylethanamine	$\text{N}(\text{CH}_2\text{CH}_3)_3$	Class 3
Xylene*	Dimethylbenzene Xylol		Class 2

*Generally 60% *m*-xylene, 14% *p*-xylene, 9% *o*-xylene with 17% ethylbenzene.

5.3 CHEMICAL METHODS

5.3.1 IDENTIFICATION REACTIONS

5.3.1.1 IONS, GROUPS AND FUNCTIONS

Classic methods for identification of functions or certain chemical groups present in drugs consist of reactions that result in precipitate formation, colored product, gas release, discoloration of the used reagent or any other easily noticeable phenomenon. Employ one of the methods described below for each ion, group, or function.

Acetate

- 1) Heat sample with an equal amount of oxalic acid; Acid vapors are released with a characteristic odor of acetic acid.
- 2) Heat sample with sulfuric acid RS and ethyl alcohol; ethyl acetate is released, with a characteristic odor.
- 3) Treat neutral sample solution with ferric chloride RS; a dark red coloration is produced which disappears when mineral acids are added.
- 4) Dissolve sample in water, add five drops of lanthanum nitrate RS, two drops of 0.1 M iodine and one drop of concentrated ammonia solution. Carefully heat to boiling. After a few minutes, a blue precipitate forms or an intense blue color appears.

Acetyl

Place sample in a test tube and add three drops of phosphoric acid RS. Close the tube with a lid crossed by another smaller test tube filled with water and on the outside of which a drop of lanthanum nitrate RS has been deposited. Heat in water bath for five minutes (certain acetylation substances hydrolyze with difficulty; in this case, the mixture must be heated slowly up to boiling over direct flame). Transfer the lanthanum nitrate RS drop to a porcelain dish and homogenize with a drop of iodine RS. Place a drop of 2 M ammonium hydroxide on the edge of the mixture. In the contact zone of the two liquids, a blue color slowly appears and persists for a short time.

Alkaloid

Dissolve a few milligrams of the sample in 5 mL of water, add hydrochloric acid RS until solution becomes acidic, add 1 mL of potassium iodobismuthate solution; an orange or red-orange precipitate is immediately formed.

Aluminum, ion

- 1) Transfer to sample, 6 M ammonium hydroxide; a white gelatinous precipitate is formed, insoluble in excess of the same reagent.
- 2) Transfer to sample sodium hydroxide M or sodium sulfide RS; a white gelatinous precipitate is formed, soluble in excess of the same reagent.

3) Transfer 5 M ammonium hydroxide to sample solution until turbidity is formed. Then add three to four drops of the freshly prepared solution of 0.05% quinalizarin in 1% sodium hydroxide (w/v). Heat to boiling, cool and acidify with excess 5 M acetic acid; a reddish-violet color is produced.

Primary aromatic amine

Acidify sample solution with 2 M hydrochloric acid and add four drops of sodium nitrite RS. After one to two minutes, add 1 mL of 2-naphthol RS; an intense orange or red color arises, generally forming a precipitate.

Ammonia and volatile aliphatic amine

Dissolve the sample in a test tube, add magnesium oxide and heat if necessary; alkaline vapors are gradually released, which darkens the silver-manganese paper placed in the upper part of the tube.

Ammonium, ion

Transfer excess M sodium hydroxide cold to sample; ammonia is released, with a characteristic odor, which changes litmus paper red color to blue. Decomposition is accelerated by heating.

Antimony(III), ion

1) Treat sample solution, strongly acidified with hydrochloric acid (maximum 2 M), with hydrogen sulfide RS; an orange precipitate of antimony sulfide forms, insoluble in 6 M ammonium hydroxide, but soluble in ammonium sulfide RS, 2 M sodium hydroxide and concentrated hydrochloric acid.

2) Dissolve sample in potassium sodium tartrate RS; after cooling, add, dropwise, sodium sulfide RS1; a red-orange precipitate is formed soluble in 2 M sodium hydroxide.

Arsenic

1) To a sample ammoniacal solution, transfer sodium sulfide RS and acidify with dilute hydrochloric acid; a yellow precipitate forms, insoluble in hydrochloric acid, but soluble in alkaline solutions.

2) Heat 5 mL of sample solution, strongly acidified with hydrochloric acid, in water bath with an equal volume of sodium hypophosphite RS; a brown to black precipitate is formed. In As(V), the reduction is slower; addition of potassium iodide RS will exert a catalytic effect.

Barbiturate without nitrogen substitute

To a sample methanolic solution, transfer a few drops of a solution containing 10% (w/v) cobalt(II) nitrate and 10% (w/v) calcium chloride, homogenize and add, while shaking, a few drops of 2M sodium hydroxide; a blue-violet precipitate is formed.

Barium ion

1) Treat sample solution with M sulfuric acid; a white precipitate is formed, insoluble in hydrochloric and nitric acids.

2) Place sample in the flame reducing zone; it takes on a yellowish-green color, which appears blue when viewed through green glass.

Benzoate

- 1) Treat neutral sample solution with ferric chloride RS; a dark yellow precipitate is formed, soluble in ethyl ether.
- 2) Acidify moderately concentrated sample solution with *M* sulfuric acid; a precipitate of benzoic acid is formed, freely soluble in ethyl ether.

Bicarbonate

- 1) Treat sample with mineral acid; effervescence is produced with the release of colorless gas which, when reacted with calcium hydroxide RS, immediately forms a white precipitate.
- 2) To a cold sample solution transfer phenolphthalein TS; the solution remains unchanged or is only slightly colored.

Bismuth, ion

Dissolve sample in a slight excess of nitric or hydrochloric acid and dilute with water; a white precipitate is formed which, treated with hydrogen sulfide, turns brown. The resulting compound is soluble in a hot mixture of equal parts of nitric acid and water, but insoluble in ammonium sulfide RS.

Bisulfite

Treat sample with 3 *M* hydrochloric acid; sulfur dioxide is released, recognized for its characteristic pungent odor and for darkening filter paper moistened with mercury(I) nitrate RS.

Borate

- 1) To a sample solution acidified with hydrochloric acid, transfer a few drops of 0.1% iodine solution (w/v) and 2% polyvinyl alcohol solution (w/v); an intense green color is produced. The reaction is altered by oxidizing or reducing agents.
- 2) Treat sample with sulfuric acid, add methyl alcohol and ignite the mixture; it burns with green edges flame.

Bromide

- 1) Transfer chlorine water RS to the sample solution acidified with sulfuric acid RS; bromine is released, which gives a brown color to the solution; by shaking with chloroform, solvent becomes red to reddish-brown in color whereas aqueous layer remains colorless.
- 2) Treat sample solution with nitric acid RS and silver nitrate RS; a slightly yellowish white caseous precipitate is formed, insoluble in nitric acid and slightly soluble in 6 *M* ammonium hydroxide.

Calcium, ion

- 1) Moisten sample with hydrochloric acid and take it to the flame reducing area; a transient red-orange color appears.

2) Dissolve the sample, add two drops of methyl red TS, neutralize with 6 M ammonium hydroxide, add 3 M hydrochloric acid, drop by drop, until solution becomes acidic and pour ammonium oxalate RS; a white precipitate of calcium oxalate is formed, insoluble in 6 M acetic acid, but soluble in hydrochloric acid RS.

Carbonate

- 1) Treat the sample with mineral acid; effervescence is produced with the release of colorless gas which, when reacted with calcium hydroxide RS, immediately forms a white precipitate.
- 2) To a cold solution of the soluble sample, transfer phenolphthalein TS; red color is formed.

Lead, ion

- 1) Treat sample solution with M sulfuric acid; a white precipitate forms, insoluble in 3 M hydrochloric acid or 2 M nitric acid, but soluble in heated M sodium hydroxide, 10% (w/v) ammonium acetate and in an excess of M sulfuric acid.
- 2) Treat sample solution, free of mineral acids, with potassium chromate RS; a yellow precipitate is formed, insoluble in 6 M acetic acid, but soluble in hot M sodium hydroxide and nitric acid.

Cyanide

Treat the sample solution with ferrous sulfate RS, sodium hydroxide RS and ferric chloride RS, heat to boiling and acidify with hydrochloric acid; a blue color or precipitate is formed. If the amount of cyanide present is small, a blue-greenish colloidal solution is formed.

Citrate

To 15 mL of pyridine, transfer a few milligrams of the sample dissolved or suspended in 1 mL of water, shake, transfer 5 mL of acetic anhydride to the mixture, shake again; a light red color appears.

Chlorate

- 1) Treat sample solution with silver nitrate RS in nitric acid RS medium; no precipitate is formed. Pour sulfurous acid or fresh sodium nitrite RS solution into this mixture; a white precipitate forms, insoluble in nitric acid RS, but soluble in 6 M ammonium hydroxide.
- 2) Expose sample to ignition; chloride is formed, identified by appropriate tests.
- 3) In a fume hood, treat the dry sample with sulfuric acid; crackles occur with greenish-yellow gas being released. For this test, use a small amount of chlorate, and extreme care must be taken when carrying it out, as the gas formed decomposes explosively above 45°C.

Chloride

- 1) With silver nitrate RS, chloride solutions produce a white lumpy precipitate, which is insoluble in nitric acid but soluble in a slight excess of 6 M ammonium hydroxide.
- 2) When analyzing amine hydrochlorides, including alkaloids, which do not respond to the above test, one drop of diluted nitric acid and 0.5 mL of silver nitrate RS in 2 mL of the solution under analysis containing approximately 2 mg of chloride ion. A white, lumpy precipitate should form.

Immediately centrifuge the mixture and decant the supernatant layer. Wash precipitate with three 1 mL portions of nitric acid solution (1 in 100) and discard supernatants. Transfer a few drops of ammonia RS to the precipitate. The precipitate will quickly dissolve.

3) When a monograph specifies that the solid substance responds to the tests for chlorides, homogenize the sample with the same quantity of manganese dioxide, moisten with sulfuric

acid and moderately heat the mixture. If chlorine is produced, it is recognized by the production of a blue color on paper moistened with iodized starch.

Copper (II), ion

1) Treat sample solution with potassium ferrocyanide RS; a reddish-brown precipitate is formed, insoluble in dilute acids but soluble in ammonium hydroxide.

2) Treat sample solution with hydrochloric acid and metallic iron filings; a red metallic copper film is deposited.

3) Treat sample solution with 6 M ammonium hydroxide excess; a bluish precipitate is formed first and then a strongly bluish solution.

Ester

Transfer to sample a 7% (w/v) hydroxylamine hydrochloride solution in methyl alcohol and 10% (w/v) potassium hydroxide solution in ethyl alcohol, heat to boiling, cool, acidify with hydrochloric acid RS and add ferric chloride TS solution; a bluish-red or red color is produced.

Iron

Treat sample with ammonium sulfide RS; a black precipitate is formed, which dissolves in 3 M hydrochloric acid, with the release of hydrogen sulphide gas, characterized by lead acetate paper.

Ferric, ion

1) Treat acidic sample solution with potassium ferrocyanide RS; a dark blue precipitate is formed, which does not dissolve by addition of hydrochloric acid RS, but is decomposed by 2 M sodium hydroxide.

2) Treat sample with ammonium thiocyanate RS; it produces an intense red color that does not disappear with the addition of dilute mineral acids, but can be extracted with ethyl ether, passing the red color to the ether layer.

Ferrous, Ion

1) Treat sample solution with potassium ferricyanide RS; a dark blue precipitate is formed, insoluble in 3 M hydrochloric acid, but decomposed by M sodium hydroxide.

2) Treat sample solution with M sodium hydroxide; a greenish-white precipitate is formed, which rapidly turns green and then, when shaken, brown.

Phosphate (or orthophosphate)

- 1) Treat sample neutral solution with silver nitrate RS; a yellow precipitate is formed, soluble in 2 M nitric acid or 6 M ammonium hydroxide.
- 2) Treat sample nitric solution with ammonium molybdate RS; a yellow precipitate is formed, soluble in 6 M ammonium hydroxide; the reaction is accelerated by heat.

Hypophosphite

- 1) Heat sample solution, acidified with sulfuric acid RS, with cupric sulfate RS; a red precipitate is formed.
- 2) Treat sample solution with mercuric chloride RS; a white precipitate is formed, which turns gray in the presence of hypophosphite excess.

Iodide

- 1) Treat sample solution with chlorine water RS, drop by drop; iodine is released, which changes the color of the solution from yellow to red; shaking this solution with chloroform makes it violet in color.
- 2) Treat acidified sample solution with nitric acid RS, with silver nitrate RS; a yellow caseous precipitate is formed, insoluble in nitric acid RS and 6 M ammonium hydroxide.

Lactate

Treat sample solution, acidified with sulfuric acid RS, with potassium permanganate RS and heat the mixture; acetaldehyde is released, identified by the characteristic odor.

Lithium, ion

- 1) Treat sample solution moderately concentrated and made alkaline by sodium hydroxide RS with sodium carbonate RS; a white precipitate is formed on heating, soluble in ammonium chloride RS.
- 2) Moisten sample with hydrochloric acid and heat in the flame reducing zone; this acquires an intense red color.

Magnesium, ion

- 1) Treat sample solution with sodium hydroxide RS; a white precipitate is formed, which dissolves with the addition of ammonium chloride RS.
- 2) Treat sample solution, in the presence of ammonium chloride RS, with ammonium carbonate RS; no precipitate is formed, but when dibasic sodium phosphate heptahydrate RS is added, a white crystalline precipitate is formed, insoluble in 6 M ammonium hydroxide.

Mercury

- 1) Treat sample solution with hydrogen sulfide RS; a black precipitate is formed, insoluble in ammonium sulfide RS and in boiling 2 M nitric acid.

2) Apply sample solution, without nitric acid excess, on a shiny copper slide; a deposit is formed which, when polished, becomes shiny and silvery.

Mercury (II), ion

- 1) Treat sample solution with *M* sodium hydroxide; a yellow precipitate is formed.
- 2) Treat sample neutral solution with potassium iodide RS; a scarlet precipitate is formed, very soluble in excess of reagent.

Mercury (I), ion

- 1) Treat sample with *M* sodium hydroxide; decomposition occurs, generating black color.
- 2) Treat sample solution with hydrochloric acid RS; a white precipitate is formed, which darkens when treated with 6 *M* ammonium hydroxide.
- 3) Treat sample solution with potassium iodide RS; a yellow precipitate is formed which, over time, may turn green.

Nitrate

- 1) In a fume hood, heat sample with sulfuric acid and metallic copper; brown red vapors are released.
- 2) Treat sample solution with an equal volume of sulfuric acid, cool the mixture and add 0.5 mL of 0.5 *M* ferrous sulfate solution; a brown to purple color is produced on the interface.

Nitrite

- 1) In a fume hood, treat sample with diluted mineral acids or 5 *M* acetic acid; brown vapors are released.
- 2) Treat iodized starch paper with sample solution; the indicator turns blue.
- 3) Transfer acidified potassium permanganate RS solution to sample; the color fades.

Oxalate

- 1) Treat neutral or alkaline solution of the sample with calcium chloride RS; a white precipitate is formed, insoluble in 6 *M* acetic acid but soluble in hydrochloric acid.
- 2) Treat hot acidified sample solution with potassium permanganate RS; the color fades.

Permanganate

- 1) Treat sample solution, acidified by sulfuric acid RS, with 3% hydrogen peroxide (w/v) RS; the color fades when cold.
- 2) Treat sample solution, acidified by sulfuric acid RS, with oxalic acid RS in a heated solution; the color fades.

Peroxide

Treat sample solution, slightly acidified by sulfuric acid RS, with potassium dichromate RS; an intense blue color appears. Shaking the mixture with an equal volume of ethyl ether and letting the liquids separate, the blue color passes into the ether layer.

Potassium, ion

- 1) Treat sample alkaline solution with 1% sodium tetraphenylborate (w/v); a white precipitate is formed.
- 2) Treat sample solution with acetic acid RS and 1 mL of sodium cobaltinitrite RS; a yellow or orange-yellow precipitate is formed immediately, in the absence of ammonium ions.
- 3) Place sample solution, acidified with hydrochloric acid RS, in the flame reducing zone; it acquires a violet color; the presence of a small amount of sodium masks the color.
- 4) Treat sample solution with perchloric acid RS; a white crystalline precipitate is formed.

Silver, ion

- 1) Treat sample solution with hydrochloric acid; a white caseous precipitate is formed, insoluble in nitric acid RS, but freely soluble in 6 M ammonium hydroxide.
- 2) Treat sample solution with 6 M ammonium hydroxide and a small amount of formaldehyde solution; by heating, a metallic silver mirror is deposited on the surface of the container.

Salicylate

- 1) Treat the diluted sample solution with ferric chloride RS; violet color is produced.
- 2) Treat moderately concentrated sample solution with mineral acid; a white crystalline precipitate of salicylic acid is formed, which melts at 156 to 160°C.

Sodium, ion

- 1) Place the sample solution, acidified, with hydrochloric acid RS, in the flame reducing zone; it acquires an intense yellow color.
- 2) Treat the sample solution with hydrochloric or nitric acid and then with uranyl acetate and zinc RS; a yellow-gold crystalline precipitate is formed after shaking for a few minutes.
- 3) Prepare a sample solution containing 0.1 g in 2 mL of water, add 2 mL of 15% potassium carbonate solution and heat to boiling. No precipitate is formed. Add 4 mL of potassium pyroantimoniate RS solution and heat to boiling point. Allow to cool in ice bath and, if necessary, rub the inside of the tube with a glass rod. A dense precipitate is formed.

Succinate

- 1) Treat neutral sample solution with ferric chloride RS; a light brown precipitate is formed.
- 2) Treat sample neutral solution with silver nitrate RS; a white precipitate is formed, freely soluble in 6 M ammonium hydroxide.

Sulfate

- 1) Treat sample solution with barium chloride RS; a white precipitate is formed, insoluble in hydrochloric acid RS and nitric acid RS.
- 2) Treat sample solution with lead acetate RS; a white precipitate is formed, soluble in ammonium acetate RS, but insoluble in hydrochloric or nitric acid RS.
- 3) Treat sample solution with hydrochloric acid RS; no precipitate is formed (*distinction from thiosulfate*).

Sulfite

- 1) Treat sample with 3 M hydrochloric acid; sulfur dioxide is released, recognized for its characteristic pungent odor and for darkening filter paper moistened with mercury(I) nitrate RS.
- 2) Acidify sample solution with hydrochloric acid RS, heat with a few drops of potassium permanganate RS and add drops of barium chloride RS; a white precipitate is formed.

Tartrate

- 1) Dissolve a few milligrams of the sample in water, acidified with acetic acid RS, add one drop of ferrous sulfate solution at 1% (w/v) and one drop of hydrogen peroxide at 3% (w/v); it produces a fleeting yellow color. Add 2 M sodium hydroxide drop by drop; an intense blue color is produced.
- 2) Acidify sample solution with M sulfuric acid, add a few drops of resorcinol 2% (w/v) and sulfuric acid, carefully, so as to form two layers; heating in water bath for a few minutes, a red ring appears on the interface.

Thiocyanate

Treat sample solution with ferric chloride RS; it produces a red color, which does not disappear with the addition of moderately concentrated mineral acids and can be extracted with ethyl ether, passing the red color to the ether layer.

Thiosulfate

- 1) Treat sample solution with hydrochloric acid; a white precipitate is formed, which soon turns yellow, and sulfur dioxide is released, recognized by the odor.
- 2) Treat sample acetic solution with ferric chloride RS; it produces a dark violet color that quickly disappears.

Xanthine

Treat sample with two drops of concentrated solution of concentrated hydrogen peroxide solution and five drops of 2 M hydrochloric acid, and heat to dryness in water bath; a yellowish-red residue is obtained which, treated with 2 M ammonium hydroxide, changes to red-violet.

Zinc, ion

- 1) Treat sample solution with potassium ferrocyanide RS; a white precipitate is formed, insoluble in 3 M hydrochloric acid
- 2) Treat neutral or alkaline solution of the sample with ammonium sulfide RS; a white precipitate is formed.
- 3) Treat sample solution with 2 M sodium hydroxide solution, dropwise; a white, flaky precipitate is formed, soluble in excess of sodium hydroxide RS.

5.3.1.2 IDENTIFICATION OF STEROIDS BY THIN LAYER CHROMATOGRAPHY

PROCEDURE

Prepare chromatoplate using Kieselguhr G as support. Introduce chromatoplate into the tank containing the impregnation solvent and allow to develop until the solvent reaches the chromatoplate top. Remove chromatoplate from the tank and allow solvent to evaporate. Prepare 0.25% (w/v) sample solution and 0.25% (w/v) standard solution using, as solvent, a mixture of nine volumes of chloroform and one volume of methyl alcohol. If not specified in the monograph, apply to chromatoplate 2 μ L of sample solution, 2 μ L of standard solution and 2 μ L of the 1:1 mixture of sample and standard solutions. Develop the chromatogram with the mobile phase specified in the monograph, letting it rise in the same direction as the impregnation solvent. Remove chromatoplate from the tank, allow mobile phase to evaporate, heat chromatoplate at 120°C for 15 minutes and nebulize with a 10% (v/v) sulfuric acid solution in 96% ethyl alcohol. Heat at 120°C for another 10 minutes, allow to cool and examine under normal visible and ultraviolet light (366nm). The main spot of the chromatogram obtained with the sample solution will correspond to the main spot obtained in the chromatogram of the standard solution. The main stain resulting from the application of the mixture of sample and standard solutions will appear as single and compact.

Impregnation solvents:

- I - Mixture of formamide and acetone (1:9).
- II - Mixture of 1,2-propanediol and acetone (1:9).
- III - Mixture of liquid paraffin and petroleum ether with a boiling range of 40°C to 60°C (1:9).

Mobile phases:

- A – Chloroform.
- B – Mixture of toluene and chloroform (3:1). C – Toluene
- D – Mixture of cyclohexane and toluene (4:1).
- E – Mixture of cyclohexane and petroleum ether with a boiling range of 40°C to 60°C (1:1).
- F – Mixture of glacial acetic acid and water (2:3).
- G – Mixture of hexane and dioxane (8:2).

5.3.1.3 THIN LAYER CHROMATOGRAPHY RESEARCH FOR FOREIGN STEROIDS

PROCEDURE I

Proceed as described in *Thin Layer Chromatography (5.2.17.1)*, using silica gel G as support. Prepare three solutions using, as a solvent, a mixture of chloroform and methyl alcohol (9:1) in the following concentrations: *Solution (1)*: 1.5% (w/v) sample solution; *Solution (2)*: Chemical Reference Substance (CRS) solution corresponding to 1.5% (w/v) and *Solution (3)*: Prednisolone CRS solution

and 0.03% (w/v) cortisone acetate CRS solution. Apply 1 µL of each newly prepared solution to the chromatoplate separately. Develop the chromatogram using, as a mobile phase, a mixture of methylene chloride, ethyl ether, methyl alcohol, water (77:15:8:1.2). Conduct chromatogram. Remove the plate and allow to air dry, heat at 105°C for 10 minutes and nebulize with alkaline tetrazolium blue RS solution. Examine under visible light. The main spot of the chromatogram obtained with *Solution (1)* corresponds, in position, color and intensity, to the main spot of the chromatogram obtained with *Solution (2)*. Any secondary spot obtained with *Solution (1)* is not more intense than the corresponding spot in the chromatogram obtained with *Solution (3)*.

PROCEDURE II

Chromatograph using silica gel G as support and, as mobile phase, a mixture of 1,2-dichloroethane, methyl alcohol and water (95:5:0.2). Apply on the chromatoplate, separately, 1 µL of each of the three solutions in a mixture of chloroform and methyl alcohol (9:1), as in *Procedure I*, except for *Solution 3*, in which CRS deoxycortone acetate is added.

5.3.1.4 STUDY OF SUBSTANCES RELATED TO SULFONAMIDES BY THIN LAYER CHROMATOGRAPHY

PROCEDURE I

Proceed as described in *Thin Layer Chromatography (5.2.17.1)*, using silica gel H as support. Prepare two solutions using a mixture of ethyl alcohol and 13.5 M ammonium hydroxide (9:1) as a solvent: *Solution (1)*: 0.005% (w/v) sample solution and *Solution (2)*: 0.005% (w/v) sulfanilamide CRS solution. Apply 10 µL of each prepared solution to chromatoplate separately. Develop chromatogram using a mixture of butyl alcohol and M ammonium hydroxide (15:3). Remove the plate and allow to air dry, heat at 105°C for 10 minutes and nebulize with a 0.1% (w/v) solution of 4-dimethylaminobenzaldehyde in ethyl alcohol, containing 1% hydrochloric acid (v/v). Examine under visible light. Any secondary stain obtained in the chromatogram with *Solution 1*, different from the main spot, is not more intense than that obtained in the chromatogram with *Solution 2*.

PROCEDURE II

Proceed as described in *Thin Layer Chromatography (5.2.17.1)*, using silica gel H as support. Apply on chromatoplate, separately, 10 µL of each of the following solutions prepared in a mixture of ethyl alcohol and 13.5 M ammonium hydroxide (9:1): *Solution (1)*: 0.25% (w/v) sample solution and *Solution (2)*: 0.00125% (w/v) sulfanilamide CRS solution. Develop the chromatogram using a mixture of chloroform, methyl alcohol and dimethylformamide (20:2:1) as the mobile phase. Remove plate and allow to air dry. Reveal as prescribed in *Procedure I*: any secondary spot obtained with *Solution 1*, other than the main spot, is not more intense than that obtained in the chromatogram with *Solution 2*.

5.3.1.5 IDENTIFICATION OF PHENOTHIAZINES BY THIN LAYER CHROMATOGRAPHY

Proceed as described in *Thin-layer chromatography (5.2.17.1)*. Use *Kieselguhr G* as support. Impregnate the dry chromatoplate, placing it in a chamber containing a mixture of 2-phenoxyethanol, macrogol 300 and acetone (10:5:85). Allow mobile phase to rise not less than 17 cm. Remove chromatoplate from the chamber and use immediately. Apply 2 µL of each of the following solutions to chromatoplate separately: *Solution (1)*: 0.2% sample solution in chloroform and *Solution (2)*:

corresponding reference chemical solution, operating under nitrogen atmosphere and reduced light. Develop the chromatogram using, as mobile phase, a mixture of diethylamine and petroleum ether with a boiling range 40–60 °C saturated with 2-phenoxyethanol (2:100). Remove plate from the chamber and allow to air dry. Examine under ultraviolet light with maximum intensity at 366 nm: fluorescence is observed, produced in a few minutes. Then nebulize chromatoplate with 10% (v/v) sulfuric acid solution in ethyl alcohol. The main stain in the chromatogram, obtained with *Solution 1*, corresponds in position, color and fluorescence intensity to that obtained in the chromatogram with *Solution 2* and has the same stability for a period of minimum 20 minutes after nebulization.

5.3.1.6 STUDY OF IMPURITIES RELATED TO PHENOTHIAZINES BY THIN LAYER CHROMATOGRAPHY

PROCEDURE

Proceed as described in *Thin Layer Chromatography (5.2.17.1)*, using silica-gel GF₂₅₄ as support, operating in a nitrogen atmosphere and protected from light. Prepare solutions, using a mixture of methyl alcohol and diethylamine (95:5) with solvent: *Solution (1)*: 2.0% sample solution (w/v) and *Solution (2)*: 0.01% sample solution (w/v). Apply separately to chromatoplate 10 µL of each fresh-prepared solution. Develop chromatogram using the mobile phase specified in the monograph up to 12 cm above application point. Remove plate and allow to air dry. Examine under ultraviolet light (254 nm). Discard any stain over the baseline. Any secondary stain obtained in the chromatogram with *Solution (1)*, except the main spot, is not more intense than the stain obtained with *Solution (2)*, unless monograph establishes differently.

Mobile phases:

- A – Mixture of cyclohexane, acetone and diethylamine (80:10:10).
- B – Mixture of hexane, acetone and diethylamine (85:10:5).
- C – Mixture of butyl alcohol and *M* ammonium hydroxide (15:3).

5.3.2 LIMIT TESTS FOR INORGANIC IMPURITIES

5.3.2.1 LIMIT TEST FOR CHLORIDE

Sample preparation: transfer the amount of sample specified in the monograph, or indicated in **Table 1**, or calculated, to a Nessler tube (50mL capacity and 22 mm internal diameter), adding a volume of 30 to 40 mL of distilled water. If sample solution is used, transfer volume of the solution specified in the monograph, or calculated, to the Nessler tube and adjust volume to 30 to 40 mL with distilled water. Neutralize, if necessary, with nitric acid RS. An amount of sample that allows the use of a volume higher than 0.2 mL of standard hydrochloric acid must be used.

Fixing the volume of standard solution at 1 mL, m (sample mass in g) can be calculated using the formula:

$$m = \frac{354,6}{l}$$

where l is chloride limit in ppm in the raw material.

Standard preparation: Transfer the volume of standard hydrochloric acid (0.01 M HCl VS), indicated in the monograph, in **Table 1**, or calculated, and a volume of 30 to 40 mL of distilled water, to a Nessler tube.

Procedure: Transfer 1 mL of nitric acid RS to the Nessler tubes containing standard preparation and sample preparation. If, after acidification, preparation is not perfectly clear, filter through a chloride-free filter paper, transfer filtrate and 1 mL of 0.1 M silver nitrate to Nessler tube. Adjust volume to 50 mL with distilled water and homogenize. Allow to stand for five minutes protected from light. Turbidity of sample preparation must not be greater than that of standard preparation.

Table 1 – Chloride impurity limits and corresponding quantities of raw material to carry out the test considering constant use of 1.0 mL of standard solution containing 3.546×10^{-4} g of chloride.

<i>Sample quantity (g)</i>	<i>Chloride limit (ppm)</i>	<i>Sample quantity (g)</i>	<i>Chloride limit (ppm)</i>
0.10	3546 (= 0.355%)	3.8	93
0.15	2364 (=0.236%)	4.0	88
0.20	1773 (=0.180%)	4.2	84
0.25	1418 (= 0.142%)	4.4	80
0.30	1182 (=0.120%)	4.6	77
0.35	1013 (=0.100%)	4.8	74
0.40	886	5.0	71
0.45	788	5.2	68
0.50	709	5.4	65
0.55	645	5.6	63
0.60	591	5.8	61
0.65	545	6.0	59
0.70	506	6.2	57
0.75	473	6.4	55
0.80	443	6.6	53

<i>Sample quantity (g)</i>	<i>Chloride limit (ppm)</i>	<i>Sample quantity (g)</i>	<i>Chloride limit (ppm)</i>
0.85	417	6.8	52
0.90	394	7.0	50
0.95	373	7.2	49
1.00	354	7.4	48
1.2	295	7.6	46
1.4	253	7.8	45
1.6	221	8.0	44
1.8	197	8.2	43
2.0	177	8.4	42
2.2	161	8.6	41
2.4	148	8.8	40
2.6	136	9.0	39
2.8	126	9.2	38
3.0	118	9.4	37
3.2	111	9.6	37
3.4	104	9.8	36
3.6	98	10.0	35

The amount of chloride (3.546×10^{-4} g) in standard preparation being fixed, if chloride limit in a given substance is, for example, 354 ppm, 1.0 g of the substance should be used to obtain up to the same standard turbidity; if limit is 71 ppm of chloride, 5.0 g of sample should be used, and so on.

Alternatively, proceed as described in *Ion Chromatography (5.2.17.4.1)*, using a chromatograph equipped with an anion exchange column and conductivity detector with chemical suppression.

5.3.2.2. LIMIT TEST FOR SULFATES

Sample preparation: transfer sample amount specified in the monograph, or indicated in **Table 2**, or calculated, to a Nessler tube (50mL capacity and 22 mm internal diameter), adding a volume of 30 to 40 mL of distilled water. If sample solution is used, transfer volume of the solution specified in the monograph, or calculated, to the Nessler tube and adjust volume to 30 to 40 mL with distilled water. If necessary, neutralize with hydrochloric acid RS. Eventually, acetic acid can be used. If preparation is not perfectly clear, filter through a sulfate-free filter paper and transfer filtrate to a Nessler tube. A sample amount allowing the use of a volume higher than 0.2 mL of standard sulfuric acid solution must be employed. Fixing standard solution volume at 2.5 mL, m (sample mass in g) can be calculated using the formula:

$$m = \frac{1200,8}{l}$$

where l is sulfate limit in ppm in the raw material.

Standard preparation: Transfer the volume of standard sulfuric acid (0,005 M H₂SO₄ VS), indicated in the monograph, in **Table 2**, or calculated, and a volume of 30 to 40 mL of distilled water, to a Nessler tube.

Procedure: Transfer 1 mL of 3 M hydrochloric acid and 3 mL of barium chloride RS to the Nessler tubes containing standard and sample preparation. Adjust volume to 50 mL with distilled water. Homogenize. Allow to stand for 10 minutes. Turbidity of sample preparation must not be greater than that of standard preparation.

Table 2 – Sulfate impurity limits and corresponding quantities of raw material to carry out the test considering the constant use of 2.5 mL of standard solution containing 1.2008×10^{-3} g of sulfate.

<i>Sample quantity (g)</i>	<i>Sulfate limit (ppm)</i>	<i>Sample quantity (g)</i>	<i>Sulfate limit (ppm)</i>
0.50	2401 (=0.240%)	4.6	261
0.55	2183 (=0.220%)	4.8	250
0.60	2001 (=0.200%)	5.0	240
0.65	1847 (=0.185%)	5.2	231
0.70	1715 (=0.171%)	5.4	222
0.75	1601 (=0.160%)	5.6	214
0.80	1501 (=0.150%)	5.8	207
0.85	1412 (=0.141%)	6.0	200
0.90	1334 (=0.133%)	6.2	194
0.95	1264 (=0.126%)	6.4	187
1.00	1200 (=0.120%)	6.6	182
1.2	1001 (=0.100%)	6.8	177
1.4	858	7.0	171
1.6	750	7.2	166
1.8	667	7.4	162
2.0	600	7.6	158
2.2	546	7.8	154
2.4	500	8.0	151
2.6	462	8.2	146
2.8	429	8.4	143
3.0	400	8.6	139
3.2	375	8.8	136
3.4	353	9.0	133
3.6	333	9.2	130
3.8	316	9.4	127
4.0	300	9.6	125
4.2	286	9.8	122
4.4	273	10.0	120

Sulfate amount being fixed (1.2008×10^{-3} g), if sulfate limit in a given substance is, for example, 500 ppm, 2.4 g of sample should be used to obtain up to the same standard turbidity; if limit is 151 ppm of sulfate, 8 g of sample should be used, and so on.

Alternatively, proceed as described in *Ion Chromatography (5.2.17.4.1)*, using a chromatograph equipped with an anion exchange column and conductivity detector with chemical suppression.

5.3.2.3 LIMIT TEST FOR HEAVY METALS

Determination of heavy metals can be carried out by two methods: limit test by formation of solid sulfide particles or determination by atomic spectrometry.

Limit test consists of the formation of solid particles of heavy metal sulfides, in suspension, and subsequent visual comparison of the color intensity in sample and standard preparations in a Nessler tube. The test is semi-quantitative and makes it possible to infer whether sample passes the test or not, representing the sum of the concentration of contaminating elements in the sample.

Atomic spectrometry method enables to quantify each contaminating element in the sample and differentiated limits are established for each element according to its toxicity, pharmaceutical preparation and route of administration. Elements such as As, Cd, Pb and Hg, due to their high toxicity, have lower limits than the others. Due to the greater bioavailability of elements eventually present in substances used in the manufacture of parenteral products, the specified limits are lower than those related to oral use.

LIMIT TEST METHOD

Special Reagents

Lead Nitrate Stock Solution: Accurately dissolve 159.8 mg of lead nitrate in 100mL of water and 1mL of nitric acid. Dilute with water to 1000 mL and homogenize. Prepare and store this solution in glass containers free from soluble lead salts.

Standard lead solution (10 ppm Pb): dilute 10 mL of lead nitrate stock solution to 100 mL with water. Each milliliter of this fresh-prepared solution contains the equivalent of 10 µg of lead (10 ppm Pb).

Acetate buffer pH 3.5: dissolve 25.0 g of ammonium acetate in 25 mL of water and 38 mL of 6 M hydrochloric acid. If necessary, adjust pH to 3.5 with 6 M ammonium hydroxide or 6 M hydrochloric acid. Dilute with water to 100 mL and homogenize.

Preparation of thioacetamide reagent: see thioacetamide RS. Heat in water bath for 20 seconds, cool and use immediately.

METHOD I

Sample preparation: transfer to a suitable tube sample solution prepared as specified in the monograph and dilute to 25 mL with water, or dissolve and dilute with water to 25 mL the sample amount, in g, specified in the monograph or calculated according to the formula:

$$2 / (1000l)$$

where

l = limit of heavy metals in sample in percentage (w/w).

Adjust pH between 3.0 and 4.0 with *M* acetic acid or 6 *M* ammonium hydroxide using narrow range indicator paper as external indicator. Dilute with water to 40 mL and homogenize.

Standard preparation: transfer 2 mL of standard lead solution (10 ppm Pb) to a suitable tube and dilute to 25 mL with water. Adjust pH between 3.0 and 4.0 with *M* acetic acid or 6 *M* ammonium hydroxide using narrow range indicator paper as external indicator. Dilute with water to 40 mL and homogenize.

Control preparation: transfer to a third tube volume of sample solution prepared as described in the monograph or in *sample preparation* and 2 mL of standard lead solution (10 ppm Pb). Adjust pH between 3.0 and 4.0 with *M* acetic acid or 6 *M* ammonium hydroxide using narrow range indicator paper as external indicator. Dilute with water to 40 mL and homogenize.

Procedure: for each preparation, transfer 2 mL of acetate buffer pH 3.5 and 1.2 mL of thioacetamide RS. Dilute with water to 50 mL, homogenize and allow to stand for two minutes. After two minutes, a shade ranging from yellow to black will be produced. Observe preparations from top to bottom, along the tube vertical axis, on a white background. The color produced in the sample preparation must not be more intense than in the standard preparation. Test is only valid if intensity of the color produced in the control preparation is equal to or greater than that of the standard preparation.

METHOD II

Sample preparation: transfer to a suitable tube the sample solution prepared as specified in the monograph and dilute to 25 mL with organic solvent (dioxane or acetone, containing not less than 15% v/v of water), or dissolve and dilute with the same solvent for 25 mL the sample amount, in g, specified in the monograph or calculated according to the formula:

$$2 / (1000l)$$

where

l = limit of heavy metals in sample in percentage (w/w).

Adjust pH between 3.0 and 4.0 with *M* acetic acid or 6 *M* ammonium hydroxide using narrow range indicator paper as external indicator. Dilute with water to 40 mL and homogenize.

Standard preparation: transfer 2 mL of standard lead solution (10 ppm Pb) to a suitable tube and dilute to 25 mL with the same solvent used to dissolve sample. Adjust pH between 3.0 and 4.0 with *M* acetic acid or 6 *M* ammonium hydroxide using narrow range indicator paper as external indicator. Dilute with the same solvent used to dissolve sample to approximately 40 mL and homogenize.

Control preparation: transfer to a third tube volume of sample solution prepared as described in the monograph or in *sample preparation* and 2 mL of standard lead solution (10 ppm Pb). Adjust pH between 3.0 and 4.0 with *M* acetic acid or 6 *M* ammonium hydroxide using narrow range indicator paper as external indicator. Dilute with the same solvent used to dissolve sample to approximately 40 mL and homogenize.

Procedure: for each preparation, transfer 2 mL of acetate buffer pH 3.5 and 1.2 mL of thioacetamide. Dilute with water to 50 mL, homogenize and allow to stand for two minutes. After two minutes, a color ranging from yellow to black will be produced. Observe preparations from top to bottom, along the tube vertical axis, on a white background. The color produced in the sample preparation must not be more intense than in the standard preparation. The test is only valid if intensity of the color produced in the control preparation is equal to or greater than that of the standard preparation.

METHOD III

Sample preparation: use sample amount, in g, specified in the monograph or calculated according to the formula:

$$2 / (1000l)$$

where

l = limit of heavy metals in sample in percentage (w/w).

Transfer sample to a suitable crucible, with enough sulfuric acid to moisten the substance, and carefully incinerate at low temperature. Transfer 2 mL of nitric acid and five drops of sulfuric acid to the carbonized mass. Heat, carefully, until no more white vapors are released. Incinerate in muffle furnace at 500–600 °C until carbon is completely combusted.

Cool at room temperature, add 4 mL of 6 M hydrochloric acid, cover, digest in water bath for 15 minutes, uncover and slowly evaporate in water bath until dry. Moisten residue with a drop of hydrochloric acid, 10 mL of hot water and digest in water bath for two minutes. Make litmus paper alkaline with 6 M ammonium hydroxide added dropwise. Dilute with water to 25 mL and adjust pH between 3.0 and 4.0 with M acetic acid, using narrow range indicator paper as external indicator. Filter if necessary, wash crucible and filter with 10 mL of water and combine filtrate and washing water in a suitable tube for color comparison. Dilute with water to 40 mL and homogenize.

Standard preparation: transfer 2 mL of standard lead solution (10 ppm Pb) to a suitable tube and dilute to 25 mL with the same solvent used to dissolve sample. Adjust pH between 3.0 and 4.0 with M acetic acid or 6 M ammonium hydroxide using narrow range indicator paper as external indicator. Dilute with the same solvent used to dissolve sample to approximately 40 mL and homogenize.

Control preparation: transfer to a third tube volume of sample solution prepared as described in the monograph or in sample preparation and 2 mL of standard lead solution (10 ppm Pb). Adjust pH between 3.0 and 4.0 with M acetic acid or 6 M ammonium hydroxide using narrow range indicator paper as external indicator. Dilute with the same solvent used to dissolve sample to approximately 40 mL and homogenize.

Procedure: for each preparation, transfer 2 mL of acetate buffer pH 3.5 and 1.2 mL of thioacetamide. Dilute with water to 50 mL, homogenize and allow to stand for two minutes. After two minutes, a color ranging from yellow to black will be produced. Observe preparations from top to bottom, along the tube vertical axis, on a white background. The color produced in the sample preparation must not be more intense than that in the standard preparation. The test is only valid if intensity of the color produced in the control preparation is equal to or greater than that of the standard preparation.

METHOD IV

Sample preparation: accurately weigh sample amount specified in the monograph or calculated according to the formula:

$$2 / (1000l)$$

where

l = limit of heavy metals in sample in percentage (w/w).

Transfer to a 100 mL borosilicate glass digestion tube with about 10 mL nitric acid. Proceed with the digestion on a heating plate or block digester at a temperature of 120°C, for three hours. It is recommended to heat the system slowly to avoid sample projection. If acid evaporates, add another 5 mL aliquot. If a clear preparation is not obtained, add, after cooling, 2 mL of 30% hydrogen peroxide (w/w) and heat at 140 °C for another hour. Cool and dilute cautiously with a small volume of water. Transfer, with washing, to a 50 mL Nessler tube, without exceeding 25 mL.

Standard preparation: transfer 2 mL of standard lead solution (10 ppm Pb) to a suitable tube and dilute to 25 mL with water. Adjust pH between 3.0 and 4.0 with *M* acetic acid or 6 *M* ammonium hydroxide using narrow range indicator paper as external indicator. Dilute with water to 40 mL and homogenize.

Control preparation: transfer to a third tube volume of sample solution prepared as described in the monograph or in sample preparation and add 2 mL of standard lead solution (10 ppm Pb). Adjust pH between 3.0 and 4.0 with *M* acetic acid or 6 *M* ammonium hydroxide using narrow range indicator paper as external indicator. Dilute with water to 40 mL and homogenize.

Procedure: for each preparation, transfer 2 mL of acetate buffer pH 3.5 and 1.2 mL of thioacetamide. Dilute with water to 50 mL, homogenize and allow to stand for two minutes. After two minutes, a color ranging from yellow to black will be produced. Observe preparations from top to bottom, along the tube vertical axis, on a white background. The color produced in the sample preparation must not be more intense than in the standard preparation. The test is only valid if intensity of the color produced in the control preparation is equal to or greater than that of the standard preparation.

METHOD V

Sample preparation: in cases where the previous sample preparation methods are not efficient, proceed as described in *Wet decomposition in closed system* or *Method of microwave-assisted combustion in pressurized system* described in *Atomic spectrometry method*.

Standard preparation: transfer 2 mL of standard lead solution (10 ppm Pb) to a suitable tube and dilute to 25 mL with water. Adjust pH between 3.0 and 4.0 with *M* acetic acid or 6 *M* ammonium hydroxide using narrow range indicator paper as external indicator. Dilute with water to 40 mL and homogenize.

Control preparation: transfer to a third tube volume of sample solution prepared as described in the monograph or in sample preparation and 2 mL of standard lead solution (10 ppm Pb). Adjust pH between 3.0 and 4.0 with *M* acetic acid or 6 *M* ammonium hydroxide using narrow range indicator paper as external indicator. Dilute with water to 40 mL and homogenize.

Procedure: for each preparation, transfer 2 mL of acetate buffer pH 3.5 and 1.2 mL of thioacetamide. Dilute with water to 50 mL, homogenize and allow to stand for two minutes. After two minutes, a color ranging from yellow to black will be produced. Observe preparations from top to bottom, along the tube vertical axis, on a white background. The color produced in the sample preparation must not be more intense than that in the standard preparation. The test is only valid if intensity of the color produced in the control preparation is equal to or greater than that of the standard preparation.

ATOMIC SPECTROMETRY METHOD

Use atomic spectrometry methods to determine As, Cd, Cr, Cu, Hg, Ir, Mn, Mo, Ni, Os, Pb, Pd, Pt, Rh, Ru and V, according to *Atomic spectrometry* (5.2.13). However, different sample preparation procedures can be applied, as shown in **Figure 1**.

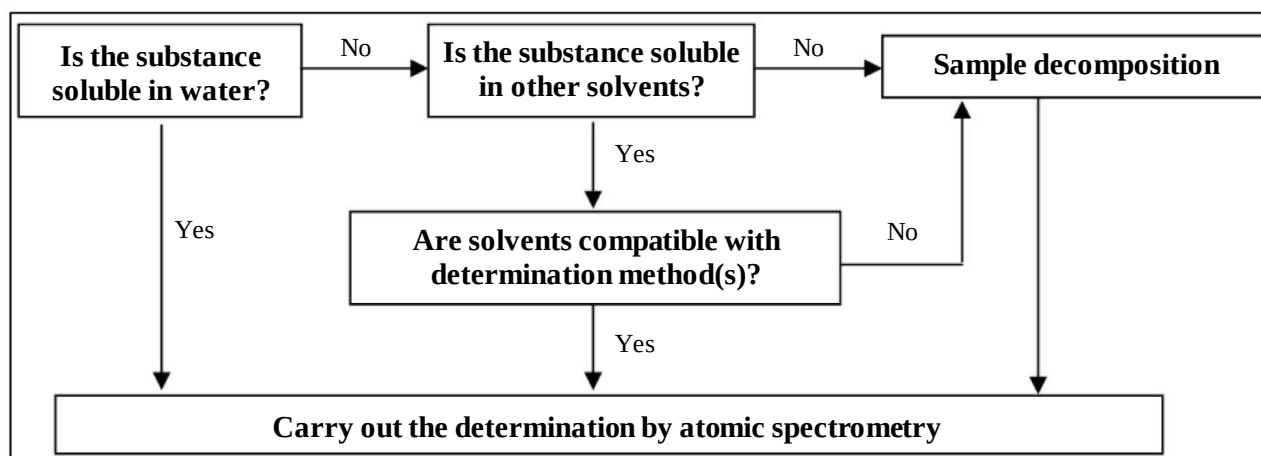


Figure 1 – Sample preparation procedures for atomic spectrometry method.

In water-soluble substances, there is no need to decompose the sample beforehand, it can be analyzed directly after dissolution. If it is not soluble in water and presents solubility in another solvent, the substance can be analyzed directly after dissolution if there is no incompatibility between solvent and the atomic spectrometry method used. When none of the above conditions are met, it is recommended to decompose the sample beforehand. In these cases, two procedures are recommended:

Wet decomposition in closed system

Accurately weigh the amount of sample between 0.1 and 0.5 g of sample and add nitric acid as recommended by the manufacturer and proceed with digestion in closed system with conventional heating or microwave at temperature of 180 °C or higher. In systems that use conventional heating and microwaves, when there is no specification in the monograph, it is recommended to digest for 240 minutes and 20 minutes, respectively.

Method of microwave-assisted combustion in pressurized system

Proceed as described in *Combustion methods* (5.3.3.3).

In principle, the two decomposition procedures described can be used interchangeably. However, the use of wet decomposition is recommended due to its higher simplicity and sample processing capacity. Other reagents such as hydrochloric acid, sulfuric acid, hydrogen peroxide and hydrofluoric acid (cannot be used in quartz flasks) can also be used in the digestion step, depending on the need. Decomposition by microwave-assisted combustion is recommended in cases where wet digestion is not efficient for organic samples.

The maximum allowed limits for each element are described in **Table 1**.

Table 1 – Permitted limits of metal and non-metal impurities.

<i>Element</i>	<i>Oral use Maximum limit ($\mu\text{g g}^{-1}$)</i>	<i>Parenteral use Maximum limit ($\mu\text{g g}^{-1}$)</i>
Arsenic (As)	1.5	0.15
Cadmium (Cd)	0.5	0.05
Lead (Pb)	1.0	0.1
Mercury (Hg)	1.5	0.15
Chromium (Cr)	25	2.5
Copper (Cu)	250	25

<i>Element</i>	<i>Oral use Maximum limit ($\mu\text{g g}^{-1}$)</i>	<i>Parenteral use Maximum limit ($\mu\text{g g}^{-1}$)</i>
Manganese (Mn)	250	25
Molybdenum (Mo)	25	2.5
Nickel (Ni)	25	2.5
Palladium (Pd)	10	1.0
Platinum (Pt)	10	1.0
Vanadium (V)	25	2.5
Iridium (Ir)	The concentration sum must not exceed 10	The concentration sum must not exceed 10
Osmium (Os)		
Rhodium (Rh)		
Ruthenium (Ru)		

5.3.2.4 LIMIT TEST FOR IRON

Standard iron solution (100 ppm Fe): weigh 0.8634 g of ferric ammonium sulfate dodecahydrated, transfer to a 1000 mL volumetric flask and dissolve in distilled water. Add 5 mL of sulfuric acid RS and adjust volume with distilled water.

Iron Standard Solution (10 ppm Fe): dilute 10 mL of *Iron Standard Solution* (100 ppm Fe) with distilled water and adjust to 100 mL.

Iron Standard Solution (2 ppm Fe): dilute 2 mL of *Iron Standard Solution* (100 ppm Fe) with distilled water and adjust to 100 mL.

METHOD I

Sample preparation: weigh the sample amount specified in the monograph, or in **Table 1**, or calculated, dissolve in a suitable solvent, transfer to a Nessler tube (capacity of 50 mL and 22 mm internal diameter). Add distilled water, or the solvent indicated in the monograph, in an amount sufficient for 40 mL. Add 2 mL of 20% citric acid (w/v).

Fixing the volume of *Iron standard solution (100 ppm Fe)* in 1 mL, the value of m (sample mass in g) can be calculated using the formula:

$$m = \frac{100}{l}$$

where l is the iron limit in ppm in the raw material.

Standard preparation: use 10 mL of *Iron Standard Solution* (10 ppm Fe) or 1 mL of *Iron Standard Solution* (100 ppm Fe), according to **Table 1**, or calculated volume, and add distilled water, or the solvent indicated in the monograph, in sufficient quantity for 40 mL. Add 2 mL of 20% citric acid (w/v).

Procedure: at the same time, add two drops of thioglycolic acid to the tubes containing the sample and standard preparations. Homogenize, make alkaline with ammonium hydroxide, adjust to 50 mL with distilled water and homogenize. Allow to stand for five minutes. The pink color produced in the sample preparation should not be more intense than in the standard preparation.

METHOD II

Sample preparation: weigh sample quantity specified in the monograph, or calculated, dissolve in suitable solvent, or use volume of sample solution as specified in the monograph. Transfer 2 mL of 2 M hydrochloric acid and 0.5 mL of bromine water RS. After five minutes, remove bromine excess by air current in a fume hood (CAUTION!) TOXIC REAGENT) and transfer to a Nessler tube (50 mL capacity and 22 mm internal diameter).

Standard preparation: submit the volume of *Iron Standard Solution (2 ppm or 10 ppm Fe)* indicated in the monograph, or the calculated volume, as described in the sample preparation.

Procedure: at the same time, add to the tubes containing the sample and standard preparations, 3 mL of potassium thiocyanate M, complete the volume to 50 mL, homogenize and allow to stand for five minutes. The color produced in the sample preparation must not be more intense than in the standard preparation.

METHOD III

Sample preparation: transfer to a Nessler tube (50 mL capacity and 22mm internal diameter) the amount of sample specified in the monograph, or calculated, or the volume of sample solution indicated in the monograph. Dilute to 40 mL with distilled water. Add 2 mL of M hydrochloric acid and homogenize.

Standard preparation: transfer the volume of *Iron Standard Solution (10 ppm Fe)* indicated in the monograph, or calculated volume, to a Nessler tube and proceed as described in the sample preparation.

Procedure: transfer 50mg of ammonium peroxydisulfate crystals to the tubes containing sample and standard preparations. Add 3 mL of ammonium thiocyanate RS, adjust to 50 mL with distilled water and homogenize. The color produced in the sample preparation must not be more intense than in the standard preparation.

Table 1 – Limits of iron impurity and corresponding amount of raw material to carry out the test considering the constant use of 1.0 mL of the iron standard 100 ppm solution, which contains 10^{-4} g of iron, in the standard preparation.

<i>Sample quantity (g)</i>	<i>Iron limit (ppm)</i>	<i>Sample quantity (g)</i>	<i>Iron limit (ppm)</i>
0.100	1000	0.4	250
0.105	950	0.5	200
0.111	900	0.667	150
0.116	850	1	100
0.125	800	1.111	90
0.133	750	1.250	80
0.143	700	1.429	70
0.154	650	1.667	60
0.167	600	2	50
0.182	550	2.5	40
0.200	500	3.333	30
0.222	450	5	20
0.250	400	10	10

<i>Sample quantity (g)</i>	<i>Iron limit (ppm)</i>	<i>Sample quantity (g)</i>	<i>Iron limit (ppm)</i>
0.285	350	20	5
0.333	300		

The amount of iron (10^{-4} g of Fe) in the *Standard Preparation* being fixed, if the limit of iron in a given substance is, for example, 1000 ppm, 0.1 g of sample should be used to obtain the same coloration of the standard preparation; if the limit is 200 ppm iron, 0.5 g of sample should be used, and so on.

METHOD IV

Alternatively, for iron determination, proceed with the preparation of the sample solution as described in *Limit test for heavy metals (5.3.2.3)* and carry out the determination by one of the Atomic spectrometry methods (5.2.13).

5.3.2.5 LIMIT TEST FOR ARSENIC

SPECTROPHOTOMETRIC METHOD

The method is based on the reaction between arsine (AsH_3) released and silver diethyldithiocarbamate that form a red complex; radiation absorption can be measured in a spectrophotometer or colorimeter. Two methods can be used, differing only in sample and standard preparation. *Method I* is generally used for inorganic substances, whereas *Method II* is used for organic substances.

The system used – **Figure 1** – comprises: (a) arsine generator; (b) and (d) together; (c) ground unit; (e) absorption tube. Another adapted system that has the essential characteristics of the presented one can, eventually, be used.

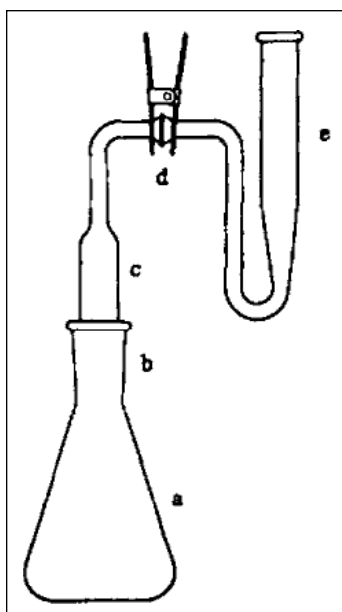


Figure 1 – Arsenic determination system using the spectrophotometric method.

Arsenic standard stock solution: dry arsenic trioxide for one hour at 105°C . Accurately weigh 132mg and dissolve in 5mL of sodium hydroxide solution (1:5) in a 1000mL volumetric flask. Neutralize

with *M* sulfuric acid and then add another 10 mL of *M* sulfuric acid. Adjust volume with freshly boiled and cooled water.

Arsenic standard solution: transfer 1 mL (or 0.5; or 0.25; or 0.1 mL) of the arsenic standard stock solution to a 100 mL (or 50, or 25, or 10 volumetric flask) mL, as required by the laboratory) – Preserve the environment. Add 1 mL of *M* sulfuric acid and complete the volume with freshly boiled water and, subsequently, cooled. Homogenize. Store the solution in a glass container and use within three days. Each mL of the solution obtained contains 1 µg of arsenic.

METHOD I

Sample preparation: transfer to the arsine generator flask the amount of substance indicated in the monograph, or the calculated amount. Fixing the volume of the standard arsenic solution at 3 mL, *m* (mass in g of the sample) can be calculated using the formula:

$$m = \frac{3}{l}$$

where *l* is the limit of arsenic in ppm in the raw material.

Dissolve with distilled water, adjusting the volume to 35 mL. Transfer 20 mL of sulfuric acid *M*, 2 mL of potassium iodide RS, 0.5 mL of strongly acidic stannous chloride RS and 1 mL of isopropyl alcohol. Homogenize. Allow to stand for 30 minutes at room temperature. In unit (c) of the device described, place two pieces of cotton soaked in a saturated solution of lead acetate, leaving a space of 2mm between them. Solution excess must be eliminated by squeezing the cotton pieces and drying them under reduced pressure at room temperature. The joints (b) and (d) must be lubricated with petroleum jelly and joined as in **Figure 1**.

Standard preparation: transfer 3 mL of *Arsenic Standard Solution* to the arsine generator vial. Dilute with distilled water to 35 mL. Proceed in the same manner as described for sample preparation.

Procedure: Transfer 3 mL of silver diethyldithiocarbamate RS to the absorption unit (e) of the generator flasks containing the sample and standard preparations. Transfer 3 g of granulated zinc to the mixture in each arsine generator vial. Immediately join units (c) and (e) to the generator flask. Place in water bath at $(25 \pm 3)^\circ\text{C}$ for 45 minutes. At 10-minute ranges, shake slowly. After this period, transfer the contents of the absorption unit to a 1 cm cell. Compare the red color produced in sample and standard preparations. The color produced in the sample preparation must not be more intense than in the standard preparation. If necessary, determine the absorption in a spectrophotometer or colorimeter at a wavelength between 535 and 540 nm using silver diethyldithiocarbamate RS as blank for zero adjustment.

METHOD II

This method additionally employs hydrogen peroxide in sample digestion. With certain substances it can cause a violent reaction. Therefore, it is important to proceed carefully at all stages. Caution must also be taken in the presence of halogenated compounds, especially when heating the sample with sulfuric acid and subsequently adding 26% hydrogen peroxide (v/v). Heating should be gentler preventing it from reaching the boiling temperature of the mixture and charring to prevent arsenic loss.

Sample preparation: transfer to the generator flask the quantity of sample specified in the monograph, or calculated. Fixing the volume of the standard arsenic solution at 3 mL, m (mass in g of the sample) can be calculated using the formula:

$$m = \frac{3}{l}$$

where l is the limit of arsenic in ppm in the raw material.

Add 5 mL of sulfuric acid and glass beads. If necessary, use a larger amount of acid to completely moisten the substance, taking care that the volume does not exceed 10 mL. Proceed with the digestion in fume hood, preferably using a hotplate with a temperature not exceeding 120°C for the time necessary to start the digestion. Once sample decomposition begins, add dropwise and carefully concentrated hydrogen peroxide. Wait for the reaction to slow down and then heat between addition of each drop. If there is foam excess, stop heating. As soon as the reaction intensity decreases, heat it carefully with flask shaking to promote homogeneous heating. It is necessary to maintain the oxidizing conditions throughout digestion. To do this, add small amounts of concentrated hydrogen peroxide whenever the mixture turns brown or darkens. Once the organic matter is destroyed, gradually increase the heating temperature allowing the sulfur trioxide vapors to be released and the solution to become colorless or slightly beige. Cool, carefully add 10 mL of distilled water, evaporate until the sulfur trioxide is again released and cool. If necessary, repeat the operation, removing traces of hydrogen peroxide. Cool and add 10 mL of distilled water. Wash the flask and dilute with distilled water, bringing the volume to 35 mL. Proceed as in the sample preparation of *Method I* starting with “Transfer 20 mL of sulfuric acid $M_{\dots}$ ”.

Standard preparation: transfer 3 mL of *Arsenic Standard Solution* and 2 mL of sulfuric acid to an arsine generator flask. Homogenize. Add the same volume of concentrated hydrogen peroxide used for sample preparation. Then, heat the solution obtained until vapors are formed. Cool and carefully add 10 mL of distilled water. Repeat the heating procedure with another 10 mL and, after that, cool again and dilute with distilled water to 35 mL. Proceed as for sample preparation.

Procedure: proceed as described in *Method I*.

Note: antimony interferes with the reaction, as it forms stibine (SbH_3) providing a falsely positive result in color development with silver diethyldithiocarbamate RS. In these cases, the preparations at wavelengths of 535 and 540 nm should be compared, in which the interference of the stibine is negligible.

ATOMIC ABSORPTION SPECTROMETRY METHOD WITH HYDRATE GENERATION

Proceed with sample preparation as described in item *Wet decomposition in a closed system – Limit test for heavy metals (5.3.2.3)* and determine by *Atomic absorption spectrometry with hydride generation (5.2.13.1.2)*. Proceed according to manufacturer specifications using a wavelength of 193.7 nm and monochromator resolution of (0.5 ± 0.1) nm.

OPTICAL EMISSION SPECTROMETRY METHOD WITH INDUCTIVELY COUPLED PLASMA

Proceed with sample preparation as described in item *Wet decomposition in a closed system – Limit test for Heavy Metals (5.3.2.3)* and determine by *Inductively coupled plasma optical emission spectrometry (5.2.13.2.2)*. Proceed according to manufacturer's specifications. It is recommended to use the wavelength of 188.979 to 189.042 nm.

5.3.2.6 LIMIT TEST FOR AMMONIA

Ammonia Standard solution (2.5 ppm NH₃): transfer 1 mL of the 0.00741% (w/v) ammonium chloride solution to a 10 mL volumetric flask. Adjust volume with distilled water.

Ammonia standard solution (1 ppm NH₃): dilute 40 mL of ammonia standard solution (2.5 ppm NH₃) to 100 mL with distilled water.

Sample preparation: dissolve the indicated amount of the test substance in 12 mL of distilled water, make alkaline, if necessary, with 2 M sodium hydroxide. Transfer to a 15 mL volumetric flask, add 0.3 mL of *alkaline potassium tetraiodomercurate (II) solution* and adjust volume with distilled water. Homogenize and allow to stand for five minutes. Transfer to a Nessler tube with a capacity of 50 mL and an internal diameter of 22 mm.

Standard preparation: transfer 10 mL of *ammonia standard solution (1 ppm NH₃)*, or the calculated volume, to a 15 mL volumetric flask, add 4.0 mL of distilled water, 0.3 mL of alkaline tetraiodomercurate solution (II) potassium and adjust volume with distilled water. Homogenize and allow to stand for five minutes. Transfer to a Nessler tube.

Procedure: compare the color produced in the preparations. The yellow color produced in the sample preparation must not be more intense than in the standard preparation.

5.3.2.7 LIMIT TEST FOR CALCIUM

Calcium standard solution alcoholic (100 ppm Ca): accurately weigh 2.5 g of calcium carbonate and transfer to a 1000 mL volumetric flask with 12 mL of acetic acid. Dissolve and complete the volume with distilled water. Immediately before use, transfer 10 mL of this solution to a 100 mL volumetric flask and complete the volume with ethyl alcohol.

Calcium Standard solution (10 ppm Ca): accurately weigh 0.624 g of calcium carbonate and transfer to a 250 mL volumetric flask with 3 mL of acetic acid. Dissolve and complete the volume with distilled water. Immediately before use, transfer 10 mL of this solution to a 1000 mL volumetric flask and complete the volume with water.

Sample preparation: transfer 1 mL of ammonium oxalate RS to a Nessler tube (50 mL capacity and 22 mm internal diameter) containing 0.2 mL of the calcium standard solution alcoholic (100 ppm Ca). Wait one minute, add a mixture of 1 mL of diluted acetic acid and 15 mL of the sample solution prepared as described in the monograph.

Standard preparation: transfer to a Nessler tube the same amounts of ammonium oxalate RS and the standard calcium solution alcoholic (100 ppm Ca) as per sample preparation. Wait one minute, add a mixture of 10 mL of the calcium standard solution (10 ppm Ca), 1 mL of diluted acetic acid and 5 mL of distilled water.

Procedure: homogenize the preparations in Nessler tubes. After 15 minutes, the turbidity of the sample preparation should not be more intense than that of the standard preparation.

Alternatively, proceed with the sample preparation as indicated in the monograph and carry out the determination of calcium by *Flame atomic absorption spectrometry (5.2.13.1.1)* using an acetylene type flame, wavelength of 422.7 nm and resolution of the monochromator of (0.7 ± 0.1) nm; or

Inductively Coupled Plasma Optical Emission Spectrometry (5.2.13.2.2) using the wavelength of 393.366 nm.

5.3.2.8 LIMIT TEST FOR MAGNESIUM

Sample preparation: transfer 0.1 g of sodium tetraborate to 10 mL of the sample solution prepared as specified in the monograph. Adjust pH between 8.8 and 9.2 with hydrochloric acid RS or sodium hydroxide RS.

Standard preparation: Transfer 0.1 g of sodium tetraborate to a mixture of 1 mL of magnesium standard solution (10 ppm Mg) and 9 mL of distilled water. Proceed as for sample preparation.

Procedure: Transfer the sample preparation to a separating funnel and extract twice, shaking for one minute each time, with 5 mL of a 0.1% (w/v) solution of hydroxyquinoline in chloroform. Discard the organic phases and transfer 0.4 mL of butylamine and 0.1 mL of triethanolamine to the aqueous phase. Adjust pH between 10.5 to 11.5 if necessary. Add 4 mL of 0.1% (w/v) hydroxyquinoline solution in chloroform and shake for one minute. Use the lower phase for comparison. Proceed in the same manner with the standard preparation. The color produced in the sample preparation must not be more intense than in the standard preparation.

Alternatively, proceed to the determination of magnesium by *Flame atomic absorption spectrometry (5.2.13.1.1)* using air-acetylene type flame, wavelength of 285.2 nm and monochromator resolution of (1.2 ± 0.1) nm; or *Inductively Coupled Plasma Optical Emission Spectrometry (5.2.13.2.2)* using a wavelength of 285.213 nm.

5.3.2.9 LIMIT TEST FOR MAGNESIUM AND ALKALINE EARTH METALS

To 200 mL of distilled water, transfer 0.1 g of hydroxylamine hydrochloride, 10 mL of ammonium chloride buffer pH 10, 1 mL of 0.1 M zinc sulfate solution and about 15 mg of eriochrome black T. Heat to approximately 40°C. Titrate with 0.01 M disodium EDTA VS until the violet color changes to blue. Transfer to this solution the recommended amount of the sample dissolved in 100 mL of distilled water or prepared as described in the monograph. If the color of the solution changes to violet, titrate with 0.01 M disodium EDTA VS until turning blue. The volume of 0.01 M disodium EDTA solution VS used in the second titration must not exceed the volume established in the monograph.

5.3.2.10 LIMIT TEST FOR ALUMINUM

Aluminum standard solution (200 ppm Al): treat a portion of metallic aluminum with 6 M hydrochloric acid at 80°C for a few minutes. Weigh 100 mg of the treated portion and dissolve in a mixture of 10 mL hydrochloric acid and 2 mL nitric acid at 80°C for approximately 30 minutes. Keep under heating until volume is reduced to about 4 mL. Cool to room temperature and add 4 mL of distilled water. Allow to evaporate until the volume is 2 mL. Cool and transfer the solution accurately, using distilled water, to a 100 mL volumetric flask. Adjust volume with distilled water and homogenize. Pipette 20 mL of this solution and transfer to another 100 mL volumetric flask. Adjust volume with distilled water and homogenize.

Aluminum Standard Solution (10 ppm Al): transfer, immediately before use, 5 mL of *Aluminum Standard Solution (200 ppm Al)* to a 100 mL volumetric flask; adjust volume with distilled water and homogenize.

Aluminum Standard Solution (2 ppm Al): transfer, immediately before use, 1 mL of *Aluminum Standard Solution (200 ppm Al)* to a 100 mL volumetric flask; adjust volume with distilled water and homogenize.

Nitric acid diluent solution: transfer 40 mL of nitric acid to a 1000 mL volumetric flask; adjust volume with distilled water and homogenize.

METHOD I

Sample preparation: use the specified amount of the sample, or calculated, prepared as specified in the monograph.

Standard Preparation: Use the specified or calculated volume of *Aluminum Standard Solution (10 ppm or 2 ppm)*.

Procedure: transfer the sample and standard preparations to separating funnels and extract with three portions (20, 20 and 10 mL) of the 0.5% (w/v) hydroxyquinoline solution in chloroform. Add the chloroform extracts and dilute to 50 mL with chloroform. Carry out a *blank preparation* using the same solvent. Measure the fluorescence intensity (**5.2.15**) of the *sample preparation* (I₁), the *standard preparation* (I₂) and the *blank preparation* (I₃) using an excitation wavelength of 392 nm and monochromator set at 518 nm. The fluorescence of the *sample preparation* (I₁), deducted from the *blank preparation* (I₃) must not be greater than that of the *standard preparation* (I₂), deducted from the *blank preparation* (I₃).

METHOD II

Sample preparation: transfer the quantity of the sample specified in the monograph, or calculated, and 50 mL of distilled water to a 100 mL plastic volumetric flask and submit to an ultrasonic bath for 30 minutes. Add 4 mL of nitric acid; adjust volume with distilled water and homogenize.

Standard preparations: prepare solutions containing 0.01, 0.02 and 0.04 ppm of aluminum, immediately before use, by diluting the *Aluminum Standard Solution (1 ppm Al)* with *Nitric Acid Diluent Solution* in a 100 mL volumetric flask.

Procedure: determine the absorbances of the standard preparations and the sample preparation by *Graphite furnace atomic absorption spectrometry (5.2.13.1.4)* equipped with a hollow aluminum cathode lamp. Adjust the wavelength to 309.3 nm using a monochromator resolution of (0.7 ± 0.1) nm. Use the *Nitric Acid Diluent Solution* as a blank and proceed with the calibration as described in (**5.2.13.1.4**) *Method I (Direct Calibration)*. Determine the Al concentration in the sample preparation in ppm. Calculate the amount of Al in the sample in ppm by multiplying the concentration of the sample preparation in ppm by 100/*P* where *P* is the mass in g of the substance used in the sample preparation.

METHOD III

Proceed as described in *Method II* and carry out the determination by *Optical Emission Spectrometry with Inductively Coupled Plasma (5.2.13.2.2)*. Proceed according to manufacturer's specifications. It is recommended to use the wavelength of 396.153 nm.

5.3.2.11 LIMIT TEST FOR PHOSPHATES

Phosphate standard solution (5 ppm): dissolve 0.716 g of monobasic potassium phosphate in distilled water and adjust to 1000 mL. Transfer 10 mL of this solution to a 1000-mL volumetric flask and adjust the volume with distilled water.

Sample preparation: transfer the specified, calculated amount of sample or the volume of sample solution prepared as described in the monograph to a 100 mL volumetric flask and adjust volume with the appropriate solvent. Transfer this solution, 4 mL of sulfomolybdic reagent, 0.1 mL of stannous chloride RS to a beaker and homogenize.

Standard preparation: Transfer 2 mL of *Phosphate Standard Solution (5 ppm)* to a 100 mL volumetric flask and adjust volume with the appropriate solvent. Continue as described in sample preparation.

Procedure: wait 10 minutes, transfer 20 mL of the contents of the sample and standard preparations to Nessler tubes (capacity of 50 mL and 22 mL of internal diameter) and compare the color of the preparations. The color produced in the sample preparation must not be more intense than in the standard preparation.

Alternatively, proceed as described in *Ion Chromatography (5.2.17.4.1)* using a chromatograph equipped with an ion exchange column for anion separation and conductivity detector with chemical suppression.

5.3.2.12 LIMIT TEST FOR LEAD

Lead Diluted Standard Solution (1 ppm Pb): dilute the accurately measured volume of *Lead Standard Solution (10 ppm Pb)* prepared as described in *Limit Test for Heavy Metals (5.3.2.3)* with nine volumes of nitric acid to 1 % (v/v).

Note: Store all reagent solutions in borosilicate glass containers. Rinse the entire glassware with a 20% (v/v) nitric acid solution and then with distilled water.

Sample preparation: in the absence of specification in the monograph, prepare the sample solution as follows. Proceed in a fume hood, as some substances can react violently when digested with hydrogen peroxide. Transfer 1.0 g of the sample to a suitable flask, add 5 mL of sulfuric acid, some glass beads and heat in a hotplate, in a hood, until the fumes evolve. Other suitable means of heating can be used. If necessary, add excess sulfuric acid to completely wet the sample not exceeding a total of 10 mL. Carefully add dropwise concentrated hydrogen peroxide, heating between additions, allowing the reaction to take place. Add the first drops slowly and very slowly, mixing carefully to prevent rapid reaction and stopping heating if excessive foaming occurs. Shake the solution in the vial to allow the sample adhering to the walls to react. Add hydrogen peroxide whenever the mixture turns brown or darkens. Continue digestion until sulfur trioxide vapors are released abundantly so that the reaction is complete and the solution becomes colorless. Cool, cautiously, with the addition of 10 mL of distilled water, evaporate again until the sulfur trioxide is fully released and cool. Repeat this procedure with 10 mL of distilled water to remove any traces of hydrogen peroxide. Carefully dilute with 10 mL of distilled water and cool.

Note: if, before heating, the sample reacts very quickly and starts to smoke with 5 mL of sulfuric acid, use 10 mL of cold 50% sulfuric acid (v/v) and add a few drops of peroxide hydrogen before heating.

Standard Preparation: Use the specified or calculated volume of *Lead diluted standard solution (1 ppm Pb)*. Subject to the same treatment as the sample preparation.

Procedure: Transfer the *Sample Preparation* and *Standard Preparation* to a separating funnel using 10 mL of distilled water. Add 6 mL of ammonium citrate RS and 2 mL of hydroxylamine hydrochloride RS1 (for the determination of lead in iron salts use 10 mL of ammonium citrate RS). Add two drops of 0.1% phenol red (w/v) in ethyl alcohol, make alkaline with ammonium hydroxide until red color and homogenize. Cool the solution, if necessary, and add 2 mL of potassium cyanide RS. Immediately extract with 5 mL portions of the dithizone extraction solution and collect each extract into another separating funnel until the dithizone solution maintains its green color. Shake the combined dithizone solutions for 30 seconds with 20 mL of 1% nitric acid (v/v) and discard the organic phase. Transfer 5 mL of dithizone standard solution and 4 mL of cyanide ammonia RS to the acid solution and shake for 30 seconds. The violet color produced in the organic phase of sample preparation must not be more intense than in the standard preparation.

Alternatively, prepare the sample as described in *Limit Test for Heavy Metals (5.3.2.3)* and determine lead by one of the *Atomic Spectrometry methods (5.2.13)*.

5.3.2.13 LIMIT TEST FOR N,N-DIMETHYLANILINE

METHOD I

Proceed as described in *Gas chromatography (5.2.17.5)*. Use a gas chromatograph equipped with a flame ionization detector; column of fused silica 25 m long and 0.32 mm internal diameter, covered with a 0.52 μm thick cross-linked polymethylphenylsiloxane film, maintained at a temperature of 150°C for five minutes, then increase the temperature in the ratio of 20°C per minute to 275°C and keep at this temperature for three minutes. The injector temperature should be 220 °C and the detector 300 °C. Use helium as carrier gas with a flow split ratio of 1:20, column head pressure of 50 kPa and flow divider of 20 mL per minute. The flow divider jacket consists of a column approximately 1 cm in length, filled with diatomaceous earth for gas chromatography impregnated with 10% polydimethylsiloxane.

Internal standard solution: dissolve 50mg of N,N-diethylaniline in 4 mL of 0.1 M hydrochloric acid and dilute to 50 mL with water. Dilute 1 mL of the solution to 100 mL with water.

Standard solution: dissolve 50.0mg of N,N-dimethylamine in 4 mL of 0.1 M hydrochloric acid and dilute to 50.0 mL with water. Dilute 1.0 mL of the solution to 100.0 mL with water. Dilute 1.0 mL of the solution to 30.0 mL with water. Add 1.0 mL of *Internal Standard Solution* and 1.0 mL of 10.5 M Sodium Hydroxide. Add 2.0 mL of trimethylpentane. Shake for two minutes and wait for phase separation. Use the clear supernatant solution.

Sample Solution: Transfer approximately 0.50 g of accurately weighed sample to a centrifuge tube and add 30 mL of water. Add 1.0 mL of *Internal Standard Solution* and adjust the solution temperature between 26 and 28 °C. Add 1.0 mL of 10.5 M sodium hydroxide and shake until complete dissolution. Add 2.0 mL of trimethylpentane. Shake for two minutes and wait for phase separation. Use the clear supernatant solution.

Procedure: Separately inject equal volumes (approximately 1 μL) of *Standard Solution* and *Sample Solution* into the chromatograph, record the chromatograms and main peak responses. Retention times should be approximately 3.6 minutes for N,N-dimethylaniline and 5.0 minutes for N,N-diethylaniline. The ratio between the peak responses of dimethylaniline and diethylaniline, obtained from the *Sample solution*, must not be greater than that obtained from the *Standard solution* (0.002%).

METHOD II

Proceed as described in *Gas chromatography* (5.2.17.5). Use gas chromatograph equipped with a flame ionization detector, a 2 m long and 2 mm internal diameter glass column, packed with a silanized diatom support for gas chromatography impregnated with 3% polymethylphenylsiloxane, kept at a temperature of 120°C and the temperature of the injector and detector at 150°C. Use nitrogen as carrier gas (30 mL/minute flow).

Internal standard solution: dissolve an accurately weighed amount of naphthalene in cyclohexane to obtain a solution of approximately 0.05mg/mL.

Standard solution: transfer approximately 50.0 mg of N,N-dimethylamine, accurately weighed, into a 50.0 mL volumetric flask, add 25.0 mL of *M* hydrochloric acid, shake until dissolved, dilute with water to volume and homogenize. Dilute 5.0 mL of the solution to 250.0 mL with water and homogenize. Transfer 1.0 mL of the resulting solution to a centrifuge tube, add 5.0 mL of *M* sodium hydroxide and 1.0 mL of the *Internal standard solution*, shake vigorously for one minute and centrifuge. Use the clear supernatant solution.

Sample Solution: Transfer approximately 1.0 g of accurately weighed sample to a centrifuge tube and add 5 mL of *M* sodium hydroxide until complete dissolution. Add 1.0 mL of the *Internal standard solution*, shake vigorously for one minute and centrifuge. Use the clear supernatant solution.

Procedure: Separately inject equal volumes (approximately 1 µL) of *Standard Solution* and *Sample Solution* into the chromatograph, record the chromatograms and main peak responses. The ratio between the peak responses of dimethylaniline and naphthalene, obtained from the *Sample solution*, must not be greater than that obtained from the *Standard solution* (0.002%).

5.3.3 CHEMICAL TESTS

5.3.3.1 DIAZOTIATION TITRATIONS

This type of titration is very useful in the analysis of drugs that contain a primary aromatic amino group, such as sulfonamides and local anesthetics derived from aminobenzoic acid. Titration is carried out with a volumetric solution of sodium nitrite, in an acidic medium, providing the diazonium salt of the primary aromatic amine.

Two methods of quantification are used.

In *Method I*, iodine starch solution or iodine starch paper is used as indicator. Excess nitrous acid converts iodide to iodine, which in contact with starch results in the characteristic blue color.

In *Method II*, the titration endpoint is determined potentiometrically. In this method, platinum-calomel or platinum-platinum electrodes with adequate potential difference and sensitivity are used. After use, the electrodes should be immersed for a few seconds in nitric acid RS to which 1mg/mL of ferric chloride was added and then wash with distilled water.

METHOD I

Procedure – Accurately weigh approximately 500mg of the sulfonamide or the amount specified in the monograph for other primary aromatic amines and transfer to a 250 mL Erlenmeyer flask. Add, while shaking, 100 mL of hydrochloric acid RS to dissolve the sample. Then add about 30 mL of water and cool in ice bath to approximately 15 °C. Titrate, under constant agitation, with a 0.1 M sodium nitrite solution VS previously standardized with CRS sulfanilamide. The titration endpoint is reached when a drop of the Erlenmeyer solution immediately turns blue with a iodine starch solution TS on a spot plate or on moist iodine starch TS paper. To check the titration completion, repeat the spot test two minutes after the last addition. This one should remain positive.

The weight, in mg, of the sample corresponding to each mL of 0.1 M sodium nitrite VS is described in the monograph of each drug.

METHOD II

Procedure – Accurately weigh approximately 500mg of the sulfonamide, or the equivalent in mass of the active ingredient for pharmaceutical specialties, or the amount specified in the monograph for other primary aromatic amines. In injectables or other liquid forms, an amount equivalent to 500 mg of active ingredient or the amount specified in the monograph must be used. Transfer to an Erlenmeyer flask and add 20 mL of hydrochloric acid RS and 50 mL of water. Shake until dissolved. Cool to approximately 15°C maintaining this temperature over the course of the titration. Add suitable catalyst when specified. Titrate, slowly and under constant agitation, with a 0.1 M sodium nitrite solution VS previously standardized with CRS sulfanilamide.

The weight, in mg, of the sample corresponding to each mL of 0.1 M sodium nitrite VS is described in the monograph of each drug.

Note: The tip of the burette must remain just above the surface of the solution to avoid oxidation of the sodium nitrite. Shake carefully, avoiding the formation of a vortex of air below the surface. When

the titration is approximately 1 mL from the calculated endpoint, add 0.1 mL volumes at ranges of not less than one minute.

5.3.3.2 DETERMINATION OF NITROGEN BY THE KJELDAHL METHOD

The Kjeldahl method described in the form of macro and semi-micro method is intended for the determination of nitrogen in relatively labile substances such as amides and amines. It comprises two phases: (1) catalytic digestion of the organic substance in sulfuric acid with the ensuing quantitative conversion of nitrogen into ammonium sulfate; (2) distillation of the digest made alkaline and volumetric titration of the ammonia released in the process.

5.3.3.2.1 MACRODETERMINATION (METHOD I)

Accurately transfer approximately 1 g of sample, 10 g of potassium sulfate, 0.5 g of cupric sulfate and 20 mL of sulfuric acid into a 500 mL Kjeldahl flask. Lean the flask about 45° and heat, slowly, keeping the temperature below the boiling point while foam is produced. Increase the temperature until the acid boils and continue heating for 30 minutes until the mixture is clear and becomes light green. Cool, add 150 mL of water, homogenize and cool again. Carefully add 100 mL of the 40% (w/v) sodium hydroxide solution, allowing the alkali to drain through the flask wall and form an independent phase under the acidic solution. Add small amount of granulated zinc; immediately, connect the flask to the isolation bulb previously fixed to the condenser, and immerse the collecting tube in 100 mL of 5% boric acid solution (w/v) in a 500 mL erlenmeyer flask. Homogenize the mixture in the flask by gently shaking and distill until approximately 80% of the volume contained in the flask is collected in the Erlenmeyer flask. Add about three drops of mixture of methyl red with methyl blue TS to the erlenmeyer flask and titrate with 0.25 M VS sulfuric acid. Carry out blank test and make the necessary corrections. Each mL of 0.25 M sulfuric acid VS is equivalent to 7.003 mg of nitrogen. For samples with low nitrogen content, use 0.05 M sulfuric acid VS. In this case, each mL is equivalent to 1.401 mg of nitrogen.

In the presence of nitrates or nitrites, accurately transfer the weighed amount of the sample containing about 150mg of nitrogen, 25 mL of sulfuric acid containing 1g of dissolved salicylic acid to a 500 mL Kjeldahl flask. Homogenize and wait for about 30 minutes shaking frequently. Add 5 g of sodium thiosulfate, homogenize and then add 0.5 g of cupric sulfate. Proceed as indicated in the previous procedure starting from “Lean the flask approximately 45°...”.

When the nitrogen content in the sample exceeds 10%, add, prior to digest, 0.5 to 1.0 g of benzoic acid to facilitate the decomposition of the substance.

5.3.3.2.2 Semi-microdetermination (METHOD II)

Accurately weigh the amount of substance corresponding to 2 – 3 mg of nitrogen and transfer to a Kjeldahl flask compatible with the device, together with 1 g of potassium sulfate and 0.1 g of cupric sulfate. If necessary, wash off solids adhering to the neck with a fine jet of water. Add 7 mL of sulfuric acid and then 1 mL of 30% hydrogen peroxide (v/v) so that the liquids flow down the wall of the flask. Heat the flask and keep digesting until the carbonization residues disappear and the light blue preparation is perfectly clear. Carefully add 70 mL of water and cool. Connect the flask to the distillation apparatus and, through the funnel, transfer 30 mL of 40% sodium hydroxide solution (w/v) allowing the alkali to flow through the flask wall and form an independent phase under the acidic solution. Rinse the funnel with water and immediately start the distillation. Collect the distillate in a 250 mL Erlenmeyer flask containing 15 mL of 5% (w/v) boric acid solution, amount of water sufficient to immerse the collection tube and three drops of a methyl red with methyl blue TS mixture.

Distill until the volume of distillate reaches 80 to 100 mL; remove the collecting flask, wash the walls with a small amount of water and titrate with 0.005 M sulfuric acid VS. Carry out blank test and make the necessary corrections. Each mL of 0.005 M sulfuric acid VS is equivalent to 0.1401 mg of nitrogen.

5.3.3.3 COMBUSTION METHOD

Combustion methods consist in the decomposition of organic substances in the presence of oxygen, through the oxidation of organic matter for the subsequent identification or assay step. These methods can be applied in two manners: atmospheric pressure flask combustion method and pressurized microwave-assisted combustion method.

ATMOSPHERIC PRESSURE FLASK COMBUSTION METHOD

Apparatus

It comprises a conical flask made of resistant refractory borosilicate glass, with an internal volume of 500 mL and a ground glass cap. For determination of fluoride, a quartz flask is used. The base of the ground cap that accompanies the flask has a glass extension on which a platinum wire is fixed with an end composed of a platinum support where the sample is introduced (**Figure 1**).

Solid samples

Weigh the amount specified in the monograph onto a piece of filter paper of appropriate shape and dimensions, fold and hold to the platinum mesh, leaving part of the edge free. Place the specified absorber solution inside the flask and bubble oxygen into this solution to saturate the interior of the flask. Ignite the end of the paper (see **Note 1**) and, without delay, place the cap on the flask, holding it firmly in place to prevent its displacement due to the pressure exerted by the combustion gases. Invert the flask to ensure liquid seal at the cap, taking care to prevent incompletely burnt material from falling into the liquid. Once combustion is complete, shake the flask until the gases formed in the process disappear. After 15 to 30 minutes, place a small amount of water on the rim of the flask and remove the cap, allowing this water to flow into the flask, washing the neck walls. Wash stopper, neck, wire and platinum mesh with water and transfer washing water to the absorbent solution. The solution obtained by this procedure is called sample solution. To prepare the blank, proceed likewise, omitting the sample (**Note 2**).

Liquid samples

Pack a small amount of absorbent cotton in a piece of filter paper and weigh, in this device, the specified amount of the sample, which is absorbed into the cotton. After fixing the cotton wrapped in the filter paper to the platinum mesh, proceed with combustion as described for solid samples.

Determination of chlorine and bromine

Burn the specified amount of the substance under examination, using as an absorbent solution 20 mL of water plus 1 mL of concentrated hydrogen peroxide and 3 mL of 0.1 M sodium hydroxide. After absorption, add two drops of bromophenol blue and sufficient amount of 0.1 M nitric acid to turn the indicator from blue to yellow, incorporating 0.5 mL of excess. If the test substance contains sulfur, add a few drops of 0.005 M barium nitrate. Add 100 mL of ethyl alcohol, using the addition to wash the inner walls of the flask, and then 15 drops of diphenylcarbazone TS. Titrate with 0.005 M mercury

(II) nitrate VS until permanent pink color. Each mL of 0.005 *M* mercury (II) nitrate VS is equivalent to 0.3550 mg of chlorine or to 0.79904 mg of bromine.

Alternatively, proceed as described in *Ion Chromatography (5.2.17.4.1)*, using a chromatograph equipped with an anion exchange column and conductivity detector with chemical suppression.

Determination of iodine

Burn the specified amount of the substance under examination as described, using as an absorbent liquid 10 mL of water plus 2 mL of *M* sodium hydroxide. After absorption, add 1 mL of 4 *M* hydrazine hydrate solution in water, cap the flask again and shake until the solution is pale. Then proceed as described in *Determination of Chlorine and Bromine* from “Once the absorption is completed...”. Each mL of 0.005 *M* mercury (II) nitrate VS is equivalent to 1.269 mg of iodine.

Alternatively, proceed as described in *Ion Chromatography (5.2.17.4.1)* using a chromatograph equipped with an ion exchange column for anion separation and conductivity detector with chemical suppression.

Determination of fluorine

Burn the specified amount of the substance under examination as described, using 15 mL of water as an absorbing solution. Once the operation is completed, wash the stopper, platinum wire, platinum mesh and flask sides (**Note 3**) with 40 mL of water. Add 0.6 mL of alizarin TS and then add dropwise 0.1 *M* sodium hydroxide until the color changes from pink to yellow. Add 5 mL of acetate buffer solution – hydrochloric acid pH 3.5 and titrate with 0.005 *M* thorium nitrate VS until yellow color changes to pinkish yellow. Each mL of 0.005 *M* thorium nitrate VS is equivalent to 0.380 mg of fluorine. If a difficulty arises when identifying the changeover, carry out a preliminary test with a standardized inorganic fluorine solution.

Alternatively, proceed as described in *Ion Chromatography (5.2.17.4.1)* using a chromatograph equipped with an ion exchange column for anion separation and conductivity detector with chemical suppression.

Determination of sulfur

Burn the specified amount of the substance under examination as described, using 12.5 mL of hydrogen peroxide RS as absorbing solution. Once the absorption is complete, add 40 mL of water, using it to wash the stopper, platinum wire and mesh and the flask sides. Boil the solution for 10 minutes, cool, add 2 mL of acetic acid RS and 20 mL of ethyl alcohol. Titrate with 0.01 *M* barium nitrate VS, using two drops of thorin TS and two drops of methylthioninium chloride TS as indicator until the yellow color changes to pink. Each mL of 0.01 *M* barium nitrate VS is equivalent to 0.3206 g of sulfur.

Alternatively, proceed as described in *Ion Chromatography (5.2.17.4.1)*, using a chromatograph equipped with an anion exchange column and conductivity detector with chemical suppression.

Notes:

1. It is recommended that the analyst wear safety glasses and adequate protection to prevent vial splinters from reaching her/him in the event of an accident. Currently, there are commercially systems that avoid manual ignition, using infrared radiation or electric current, reducing the risks to the operator.

2. Ensure that combustion vials are clean and free from traces of organic solvents.
3. Substances containing fluorine provide low contents if combustion is carried out in borosilicate glass vials. Satisfactory results are obtained in boron-free soda-glass flasks, but the ideal performance implies the use of quartz flasks.

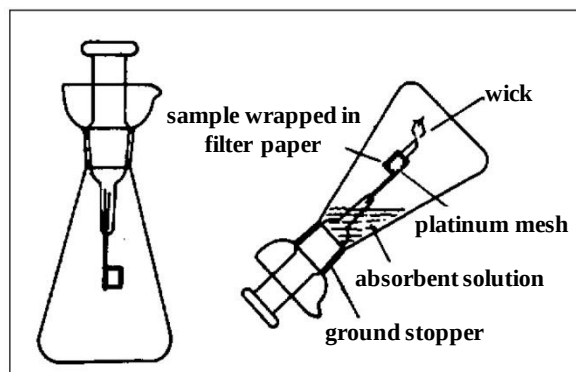


Figure 1 – Oxygen flask for the determination of sulfur and halogens.

MICROWAVE INITIATED COMBUSTION METHOD IN PRESSURIZED SYSTEM

Apparatus

It comprises a quartz flask with an internal volume of 80 mL and an operating pressure of 80 atm. The lid that comes with the flask is made of fluorinated polymer, which has an orifice to release gases in case of wet decomposition, which is used to pressurize the system with oxygen. A quartz sample holder is inserted inside the decomposition flask.

Solid samples

Weigh the specified amount of substance and press into tablet form (approximately 1.2 cm in diameter). Place the sample on the quartz support containing a piece of filter paper (approximately 10mg) moistened with 6M ammonium nitrate. Place the specified absorbent solution inside the flask and insert the holder containing the sample and paper into the flask. Close the system properly, as per manufacturer's specifications, and pressurize with 20 atm of oxygen. Insert the decomposition flasks into the microwave oven rotor and, without delay, place the rotor in the oven cavity, immediately starting irradiation. Once the ignition is started, using maximum power, the irradiation can be continued for another five minutes, so that the reflux of the absorbent solution takes place, allowing the complete absorption of the analytes in the solution. After cooling (20 minutes), the decomposition flask can be opened and the solution transferred to an appropriate container with the aid of water and measured to a known volume, for the subsequent identification or determination of the analytes of interest. The solution obtained by this procedure is called sample solution. To prepare the blank, proceed in the same manner, omitting the sample.

Determination of chlorine and bromine

Proceed as described in “*Determination of Chlorine and Bromine*” in the Atmospheric Pressure Flask Combustion Method.

Determination of iodine

Proceed as described in “*Determination of iodine*” in the Atmospheric Pressure Flask Combustion Method.

Determination of fluorine

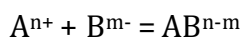
Proceed as described in “*Determination of fluorine*” in the Atmospheric Pressure Flask Combustion Method.

Determination of sulfur

Proceed as described in “*Determination of sulfur*” in the Atmospheric Pressure Flask Combustion Method.

5.3.3.4 COMPLEXOMETRIC TITRATIONS

Complexometry is the volumetric analytical method that comprises the titration of metallic ions (A) with a complexing agent (B). The reaction involved is of the following type:



Many complexants known as chelators are capable of forming cyclic structures through the simultaneous coordination of several groups with the metallic ion. Edetic acid (ethylenediaminetetraacetic acid, EDTA) is the typical example. This acid is the most used complexing agent. EDTA forms 1:1 complexes with many metals with an oxidation state greater than +1 and these complexes are highly soluble in water.

The stability of EDTA complexes is pH dependent for different metals. Therefore, ideal pH conditions must be established for the complexation analysis for each metal.

In complexometrics, changeover can be determined visually or instrumentally. Complexing indicators are used that exhibit profound color changes upon coordination with the metal. Typical examples are: xylenol orange, chalcone and eriochrome black

T. The complexometric indicator acts in a competitive manner with the titrant agent, so it must be effectively displaced close to the equivalence point.

PROCEDURES

Aluminum

Accurately weigh the amount of the substance indicated in the monograph, add 50 mL of water and acidify, if necessary, with a minimum amount of hydrochloric acid *M*, unless the monograph indicates another type of solvent. Add 25 mL of 0.1 *M* disodium edetate VS and 10 mL of the mixture, in equal volumes, of 2 *M* ammonium acetate with 2 *M* acetic acid. Heat the solution to boiling and keep for two minutes. Cool down. Add 50 mL of ethyl alcohol and 3 mL of fresh- prepared solution of 0.025% (w/v) dithizone in ethyl alcohol. Titrate excess disodium edetate with 0.1 *M* zinc sulfate VS until the color changes from blue-green to violet-pink. Each mL of 0.1 *M* disodium edetate VS is equivalent to 2.698 mg of aluminum.

Bismuth

Accurately weigh the amount of the substance indicated in the monograph and dissolve in a minimum amount of 2 *M* nitric acid. Add 50 mL of water and concentrated ammonia solution, drop by drop

while shaking, until the preparation becomes turbid. Add 0.5 mL of nitric acid and heat at 70 °C until the turbidity of the preparation disappears. Add a few drops of xylene orange TS. Titrate slowly with 0.05 M disodium edetate VS until color changes from pink-violet to yellow. Each mL of 0.05 M disodium edetate VS is equivalent to 10.45 mg of bismuth.

Calcium

Accurately weigh the amount of the substance indicated in the monograph, dissolve in a few milliliters of water and acidify, if required, with a minimum amount of 2 M hydrochloric acid. Dilute to 100 mL with water. Titrate with 0.05 M disodium edetate VS to approximately 2 mL before predicted equivalence point. Add 4 mL of 10 M sodium hydroxide and drops of chalcone TS. Continue titration until the color changes from pink to deep blue. Each mL of 0.05 M disodium edetate VS is equivalent to 2.004 mg of calcium.

Lead

Accurately weigh the amount of the substance indicated in the monograph and dissolve in 5 to 10 mL of water, or in a minimum amount of 5 M acetic acid. Dilute to 50 mL with water. Add drops of xylene orange TS and enough methenamine (about 5 g) for the solution to turn violet. Titrate with 0.05 M disodium edetate VS, or 0.1 M VS as indicated in the monograph until color changes from violet to yellow. Each mL of 0.05 M SV disodium edetate is equivalent to 10.36 mg of lead. Each mL of 0.1 M disodium edetate VS is equivalent to 20.72 mg of lead.

Magnesium

Accurately weigh the amount of the substance indicated in the monograph and dissolve in 5 to 10 mL of water, or in a minimum amount of 2 M hydrochloric acid. Dilute to 50 mL with water. Add 10 mL of ammonium chloride buffer pH 10.0, and a few drops of eriochrome black T TS. Titrate with 0.05 M disodium edetate VS, or 0.1 M VS as indicated in the monograph until color changes from violet to blue. Each mL of 0.05 M disodium edetate VS is equivalent to 1.215 mg of magnesium. Each mL of 0.1 M disodium edetate VS is equivalent to 2.431 mg of magnesium.

Zinc

Accurately weigh the amount of the substance indicated in the monograph and dissolve in 5 to 10 mL of water, or in a minimum amount of 5 M acetic acid. Dilute to 50 mL with water. Add drops of xylene orange TS and enough methenamine (about 5 g) for the solution to turn violet. Titrate with 0.05 M disodium edetate VS, or 0.1 M VS as indicated in the monograph until color changes from violet to yellow. Each mL of 0.05 M disodium edetate VS is equivalent to 3.268 mg of zinc. Each mL of 0.1 M disodium edetate VS is equivalent to 6.536 mg of zinc.

5.3.3.5 TITRATION IN NON-AQUEOUS MEDIUM

Drugs that are weak bases or acids cannot be quantified in an aqueous medium, but can be in a non-aqueous medium. Titration in a non-aqueous medium is based on the Brönsted-Lowry acid/basic concept, in which the acid is a substance that donates protons whereas the base is the one that receives protons. Potentially acidic substances are acidic only in the presence of a base to which they can donate protons and vice versa.

The solvent therefore plays a very important role in determining the acid/base character of a substance, as the strength of the acid or base depends on the solvent ability to receive or donate protons. Water should be the solvent of choice due to its easy availability. However, the strongest

acid that can exist in an aqueous medium is the hydronium ion (H_3O^+) whereas the strongest base is the hydroxide ion (OH^-), this is known as the *solvent leveling effect*. For assay of very weak acids or bases, the titrant must be a very strong base or acid, respectively, for the acid-base reaction to take place; however, due to water leveling effect, it is not possible to titrate such substances in an aqueous medium.

Using weakly protophilic solvents such as acetic acid, titration of very weak bases is possible as the acetonium ion ($\text{CH}_3\text{COOH}_2^+$) is a much stronger acid than the hydronium ion. Acids stronger than hydronium ion cannot be differentiated in aqueous media, but they can in acetic acid showing that the decreasing order of strength of acids is perchloric, hydrobromic, sulfuric, hydrochloric and nitric. Similarly, titration of weak acids is possible using basic solvents such as *n*-butylamine. Amidite ($\text{CH}_3\text{CH}_2\text{CH}_2\text{NH}^-$) is a much stronger base than hydroxide.

The solvents used for titration in a non-aqueous medium must meet certain requirements: (1) not react with the substance or the titrant; (2) dissolve the substance, allowing at least the preparation of a 0.01 *M* solution; (3) dissolve the titration product and, if precipitation is unavoidable, the precipitate must be compact and crystalline; (4) easily enable the visualization of the endpoint, whether it is measured with the use of indicators or potentiometers; (5) be inexpensive and easy to purify.

For the titration of substances of a basic character (amines, nitrogenous heterocyclics, quaternary ammonium compounds, alkaline salts of organic and inorganic acids and some amine salts) solvents of a relatively neutral or acidic nature are used, with glacial acetic acid being the most used. Acetic anhydride is reserved for very weak bases such as amides. The mixture of dioxane with acetic acid can occasionally be used to reduce the dielectric constant and consequently lower the ionization potential of acids favoring the neutralization reaction. As a titrant, a solution of perchloric acid in acetic acid is generally used. Other useful titrants are perchloric acid in dioxane; *p*-toluenesulfonic acid (tosic acid) and fluorosulfonic acid are commonly used with aprotic solvents such as chloroform.

For assay of salts of halogenated acids (hydrochloride, hydrobromide and hydroiodide), mercury acetate must be added; it does not dissolve in acetic acid solution. Halide ion is too weak a base to quantitatively react with perchloric acid in acetic acid. This ion can be quantitatively replaced by the acetate ion being removed as a mercuric complex that does not dissociate. Acetate, which is a relatively strong base in acetic acid, can be accurately titrated with perchloric acid.

For the titration of substances that behave like acids (acid halides, acid anhydrides, carboxylic acids, amino acids, enols, imides, phenols, pyrroles and sulphates) solvents of a basic or aprotic nature are used. For assay of substances with intermediate acidity, the use of dimethylformamide is common. Whereas for assay of weak acids, stronger bases such as morpholine, ethylenediamine and *n*-butylamine are used. Properly selected basic solvents can enable the selective determination of acid mixtures. Two classes of titrants can be used for the determination of acidic substances: the alkali metal alkoxides and the quaternary alkylammonium hydroxides. Sodium methoxide is the most used alkoxide in a mixture of methyl alcohol and toluene or methyl alcohol and benzene. Lithium methoxide in methyl alcohol and benzene is used for compounds that form a gelatinous precipitate in titrations with sodium methoxide. The most used among hydroxides is tetrabutylammonium. With quaternary ammonium hydroxides such as tetrabutylammonium hydroxides and trimethylhexadecylammonium hydroxides (in a mixture of benzene and methyl alcohol or isopropyl alcohol) there is the advantage that the salt of the titrated acid is, in general, soluble in the titration medium.

It is important to protect solvents for the titration of acidic substances from excessive exposure to the atmosphere due to CO_2 interference. Therefore, an inert atmosphere or special apparatus can be used

during the titration. To determine the absorption of CO₂ proceed to the titration of the blank which should not consume more than 0.01 mL of 0.1 M sodium methoxide VS per milliliter of solvent.

The assay endpoint can be determined potentiometrically or visually by color change. Generally the choice of method is based on the pK_a of the analytes in water. For bases with a pK_a of the order of 4, detection is, in general, through indicators; for those whose pK_a is between 1 and 4, detection is potentiometric. In this case, the glass/calomel electrode is useful. In acetic acid, such an electrode works as theoretically expected. For calomel electrode as reference, it is recommended to replace the aqueous potassium chloride salt bridge with 0.1 M lithium perchlorate in glacial acetic acid for acid solvent titration or with potassium chloride in methyl alcohol for basic solvent titration. The determination of the endpoint in the quantification of acids whose pK_a in water is around 7 can be done with the use of an indicator. For acids with pK_a between 7 and 11, potentiometric determination is recommended, although in certain cases indicators such as azoic violet or *o*-nitroaniline are used with less precision.

With the use of organic solvents, the high coefficient of cubic expansion of most of these in relation to that of water must be considered. This is due to the possibility of variation in the titrant content in a non-aqueous medium depending on the temperature. The titrant volume must be corrected, multiplying it by the correction factor below:

$$[1 + \text{coefficient of cubic solvent expansion } (t_0 - t)]$$

where

t_0 = titrant standardization temperature,

t = titrant utilization temperature.

The most used systems for titration in non-aqueous medium are listed in **Table 1**.

Table 1 - Systems for titration in non-aqueous medium.

<i>Type of Solvent</i>	<i>Solvent^a</i>	<i>Indicator</i>	<i>Electrodes</i>
<i>Acid</i> (for titration of bases or their salts)	Glacial acetic acid	Alphazurine 2 G	Mercury/ Mercury acetate
	Formic acid	Methylrosaniline chloride	Glass/calomel
	Propionic acid	<i>p</i> -Naphtholbenzein	Glass/Silver/Silver Chloride
	Acetic anhydride	Malachite green	
	Sulfonyl chloride	Quinaldine red	
<i>Relatively neutral</i> (for differential titration of bases)	Ethyl acetate	<i>p</i> -Naphtholbenzein	Calomel/silver/ Silver chloride
	Acetonitrile	Methyl red	Glass/calomel
	Alcohols	Methyl orange	
	Benzene		
	Chlorobenzene		
	Chloroform		
<i>Basic</i> (for titration of acids)	Dioxane	<i>p</i> -Hydroxyzobenzene <i>o</i> -Nitroaniline Thymolphthalein	Antimony/calomel Antimony/glass Platinum/calomel
	<i>n</i> -Butylamine		
	Dimethylformamide		
	Ethylenediamine		

	Morpholine	Azoic violet	Glass/calomel
<i>Relatively neutral</i> (for differential titration of acids)	Acetone	Bromothymol blue	Antimony/calomel
	Acetonitrile	Thymol blue	Glass/calomel
	<i>Tert</i> -butyl alcohol	<i>p</i> -Hydroxybenzene	Glass/platinum
	2-Butanone	Azoic violet	
	Isopropylacetone		

- a) relatively neutral solvents of low dielectric constant, such as benzene, chloroform, or dioxane, can be used in conjunction with any acidic or basic solvent to increase the sensitivity of titration changeover.
b) in the titrant.

Titration of basic substances

Accurately weigh the amount of the substance indicated in the monograph and dissolve it in a specified amount of solvent, or mixture of suitable solvents. In titration of salts of halogenated acids, 10 mL of mercury acetate RS should be added. Use the appropriate indicator or, in potentiometric determination, use a suitable electrode titrating with 0.1 M perchloric acid VS in acetic acid. To prepare the blank, proceed in the same manner, omitting the sample. If t_0 is different from t correct the volume by:

$$[1 + 0.0011(t_0 - t)]$$

where

t_0 = temperature at which the titrant was standardized;

t = temperature at which the titration was carried out.

Titration of acidic substances

METHOD I

Accurately weigh the amount of the substance indicated in the monograph and dissolve it in the solvent, or mixture of suitable solvents. Use the recommended indicator or, if applicable, use the appropriate electrode for potentiometric determination. Titrate with 0.1 M sodium methoxide VS previously standardized with benzoic acid. Avoid carbon dioxide absorption. Carry out titration on the blank preparation. Make the necessary corrections.

METHOD II

Accurately weigh the amount of the substance indicated in the monograph and dissolve it in the solvent, or mixture of suitable solvents. Titrate with 0.1 M tetrabutylammonium hydroxide VS using burette equipped with carbon dioxide absorber. Potentiometrically determine the endpoint. Carry out titration on the blank preparation. Make the necessary corrections.

5.3.3.6 DETERMINATION OF METHOXYL

The method is intended for the determination of methoxyl groups in organic substances by reaction with concentrated hydriodic acid. The methyl iodide formed is distilled off under a continuous stream of nitrogen or carbon dioxide, washed and absorbed in a bromine/acetic solution. Methyl iodide is converted to iodine and then titrated with sodium thiosulfate.

APPARATUS

The device (**Figure 1**) used in the determination of methoxyl consists of a round-bottomed flask with a capacity of 50 mL to which a capillary side-arm with 1 mm of internal diameter is sealed for the inflow of inert carrier gas - nitrogen or carbon dioxide. A vertical condenser 24 cm in height and 12 mm in external diameter is connected by ground joints to the flask, on the top of which a curved tube is affixed, whose capillary end with 3mm in diameter is immersed in a scrubbing vial. The outlet of the scrubber consists of a tube of about 10mm in diameter that ends in a removable tube of 6mm in diameter immersed in the absorbent liquid.

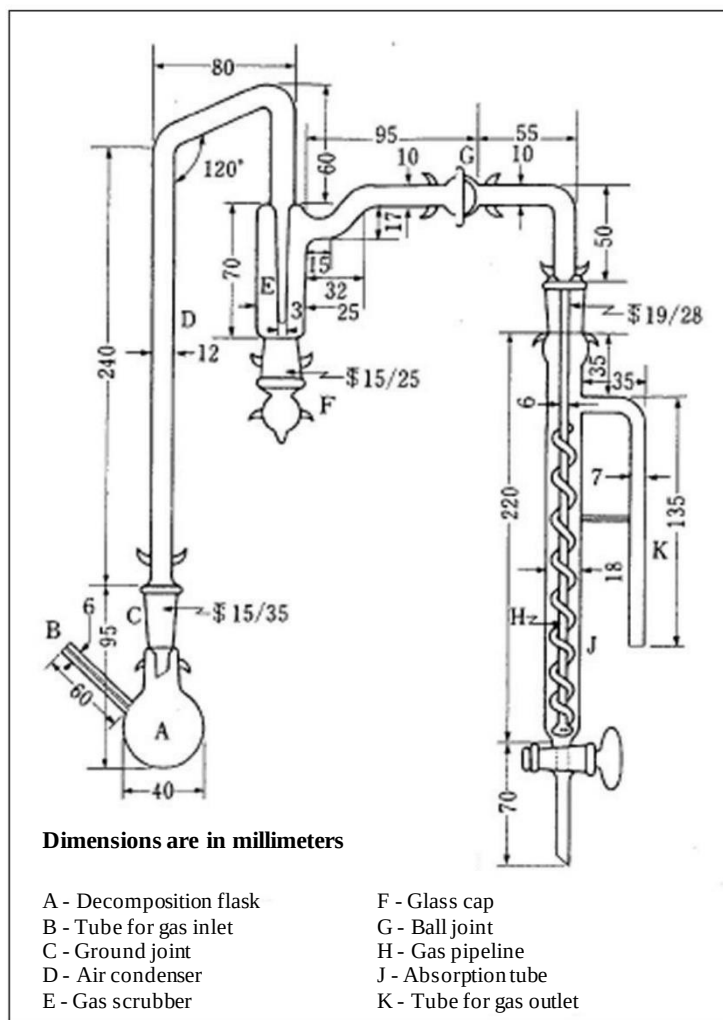


Figure 1 – Apparatus used in the determination of methoxyl.

PROCEDURE

Washer preparation: suspend 1 g of red phosphorus in 100 mL of water.

Absorbent liquid: weigh 15 g of potassium acetate and dissolve in 150 mL of a mixture of glacial acetic acid and acetic anhydride (9:1). Add 5 g of bromine to 145 mL of this solution. Prepare immediately before use.

Add enough washer preparation to cover half of the gas scrubber. Transfer 20 mL of the absorbent liquid to the absorption tube. Transfer to the flask the sample quantity corresponding to 6.5 mg of methoxyl or the quantity indicated in the monograph, together with glass beads and 6 mL of hydriodic acid. Moisten the ground joint with hydriodic acid and connect to the air condenser. Connect the two parts of the device by the ball joint using silicone grease for sealing. Adjust the gas inflow through tube B so as to form two bubbles per second in the gas scrubber E. Gradually heat the flask for 20 -

30 minutes to 150 °C and keep heating at this temperature for 60 minutes. After cooling the flask to room temperature under gas flow, pour the preparation contained in the absorption tube into an Erlenmeyer flask with a capacity of 500 mL with a ground cap containing 10 mL of the aqueous solution of sodium acetate trihydrate (1:5). Wash the tube walls with water, transfer the wash water to the Erlenmeyer flask and dilute to 200 mL with water. Add formic acid dropwise while shaking until the reddish color of bromine disappears, then 1 mL of formic acid. Add 3 g of potassium iodide and 15 mL of *M* sulfuric acid; cap, shake gently and allow to stand for five minutes.

Titrate the released iodine with 0.1 *M* sodium thiosulfate VS, using starch TS as indicator. Carry out titration on the blank preparation proceeding as described, omitting the sample and correcting if necessary. Each mL of 0.1 *M* Na₂S₂O₃ VS is equivalent to 0.5172 mg of methoxyl (CH₃O).

5.3.3.7 DETERMINATION OF SULFUR DIOXIDE

The method comprises the removal of SO₂ released by heating the substance in an acidified aqueous medium by a stream of carbon dioxide, followed by absorption of SO₂ in a hydrogen peroxide solution. The sulfuric acid formed in the process is titrated with standardized sodium hydroxide.

APPARATUS

The apparatus (**Figure 1**) used in the determination of sulfur dioxide consists of a three-neck round-bottom flask with a capacity of 1000 to 1500 mL. A device designed for the inflow of carbon dioxide is attached to one of the flask side outlets. A 100 mL capacity addition funnel and vertical reflux condenser, both fitted with ground joints, are coupled to another side outlet and to the central outlet, respectively. At the upper end of the condenser, the absorption tube D is connected.

PROCEDURE

Transfer approximately 300 mL of water to the flask, fix the flask to the device and promote a slow and uniform inflow of carbon dioxide for 15 minutes. Transfer 20 mL of 3% (w/v) hydrogen peroxide RS, previously neutralized with 0.1 *M* sodium hydroxide, to the absorption tube using bromophenol blue TS as indicator. Without interrupting the inflow of gas, momentarily remove the funnel and accurately transfer to the flask about 50 g of sample and 200 mL of water. Add, dropwise, 50 mL of 6 *M* hydrochloric acid through the funnel and reflux for 45 minutes. Exactly transfer, by washing with water, the liquid contained in the absorption tube to a 250 mL Erlenmeyer flask and titrate with 0.1 *M* sodium hydroxide VS using bromophenol blue TS as indicator. Carry out titration on the blank preparation proceeding as described, omitting the sample and correcting if necessary. Each mL of 0.1 *M* sodium hydroxide VS is equivalent to 3.203 mg of sulfur dioxide.

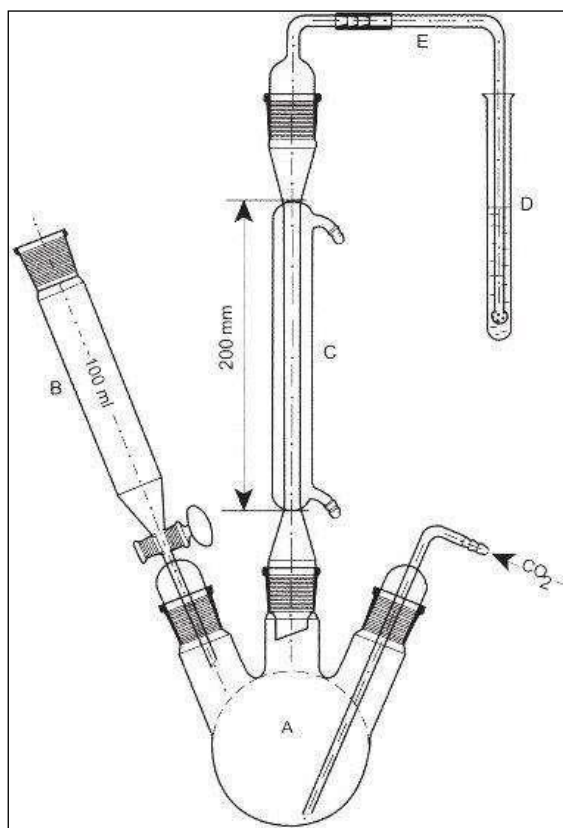


Figure 1 – Apparatus used in the determination of sulfur dioxide.

5.3.3.8 DETERMINATION OF ALCOHOL

5.3.3.8.1 DISTILLATION METHOD

This method is to be used for the determination of alcohol, in a solution containing alcohol, unless another method is specified in the monograph. It is suitable for analysis of most fluid extracts and tinctures.

PROCEDURE

Notes:

- 1 - A distilling flask with a capacity of two to four times the volume of the liquid to be heated must be used.
- 2 - During all manipulations, take care to minimize the loss of alcohol through evaporation.
- 3 - To avoid the occurrence of violent boiling, add fragments of insoluble and porous material, such as silicon carbonate or glass beads.
- 4 - Liquids that form too much foam during the distillation must be previously treated with phosphoric, sulfuric or tannic acid, up to a strong acid reaction or with a slight excess of calcium chloride solution, or with a small amount of paraffin or silicone oil, before starting the distillation.
- 5 - The speed of distillation must be such as to allow the production of clear distillates. Turbid distillates should be clarified by shaking with talcum powder or calcium carbonate, precipitated and filtered. Adjust the temperature of the filtrate and determine the alcohol content by density.

METHOD 1

Liquids with less than 30% alcohol – Transfer to a suitable distillation apparatus, accurately, a sample volume of minimum 35 mL of the liquid in which the alcohol content is being determined, record the temperature at which the volume was measured. Add an equal amount of water, distil and collect a volume of distillate that is about 2 mL smaller than the initial sample volume. Adjust the temperature of the distillate to that at which the sample was measured and add enough water to obtain the initial volume of the sample and homogenize. The distillate should be clear or, at most, slightly turbid. Determine the density of the liquid at 20°C. With the result, evaluate the percentage, in volume, of C₂H₅ OH contained in the examined liquid, using the Table of Alcohol.

METHOD 2

Liquids with more than 30% alcohol – Proceed as indicated in the previous method, with the following modification: dilute the sample with twice the volume of water and collect a volume of distillate approximately 2 mL less than twice the initial volume of the sample. Adjust the temperature of the distillate to that at which the sample was measured and complete with water at a volume equal to twice the initial volume of the sample. Homogenize and determine density at 20°C. The proportion of C₂H₅OH, by volume, in this distillate, evaluated by density, is equal to half that of the liquid examined.

Special treatments

Volatile Acids and Bases – Liquids containing volatile bases should be treated with dilute sulfuric acid RS until a slightly acidic reaction. If volatile acids are present, sodium hydroxide RS should be added to the preparation until slightly alkaline reaction.

Glycerol – Liquids containing glycerol must be added in such a volume of water that the residue, after distillation, contains minimum 50% of water.

Iodine – Solutions containing free iodine should be treated prior to distillation with pulverized zinc or bleached with a sufficient amount of 10% (w/v) sodium thiosulfate solution followed by the addition of a few drops of sodium hydroxide RS.

Other volatile substances – Elixirs, tinctures and similar preparations that contain significant proportions of volatile substances, in addition to alcohol and water, such as: volatile oils, chloroform, ether, camphor etc., must undergo, before distillation, one of the following treatments .

1) *Liquids with less than 50% alcohol* – Weigh accurately 35 mL of the sample, and homogenize with an equal volume of water, in a separating funnel, saturating this mixture with sodium chloride. Extract the volatile components by shaking with a 25 mL portion of hexane. Transfer the lower layer to a second separating funnel and repeat the extraction with two more portions of hexane. Collect the hexane portions and treat with 3 portions of 10 mL saturated sodium chloride solution. Collect the saline solutions and distil, collecting a volume of distillate corresponding to twice the initial volume of the sample.

2) *Liquids with more than 50% alcohol* – Measure a sample and dilute with water so that it contains approximately 25% alcohol and its final volume is approximately 35 mL. Then proceed as indicated for liquids with less than 50% alcohol, proceeding from “...saturating this mixture with sodium chloride”.

When preparing collodion for distillation, use water instead of the saturated sodium chloride solution indicated above. If treatment with hexane was not used in the sample and the distillate obtained is turbid (due to the presence of volatile oils present in small proportions), it can be clearer and suitable

for density determination, by shaking with about 1/5 of its volume of hexane or by filtration through a thin layer of talcum powder.

5.3.3.8.2 GAS CHROMATOGRAPHY METHOD

Proceed according to the *General Specifications for Gas Chromatography (5.2.17.5)*. Use efficient apparatus for the quantitative determination of alcohol.

Standard solution

For liquids containing more than 10% alcohol, prepare two standard solutions of alcohol in water so that the concentrations are respectively about 5% below (*Standard solution 1*) and about 5% above (*Standard solution 2*) of the expected alcohol concentration in the sample under analysis. Determine the density of each of the standard solutions at 20 °C (5.2.5) and obtain the exact concentration of C₂H₅ OH from the Table of Alcohol. For liquids containing less than 10% alcohol, accurately prepare two standard alcohol solutions so that the concentrations are respectively about 1% lower and about 1% higher than the expected concentration, diluting with water. Determine the densities of the solutions in the same procedure as above.

APPARATUS

Under typical conditions, the instrument contains a 2 m x 4 mm column loaded with 20% macrogol (polyethylene glycol) 400 on calcined chromatographic silica. The column is kept at a temperature of 100°C; the injector is equipped with a filter for solids and is kept at 160°C; as a conductor, inert gas such as helium is used, flowing at a flow rate of about 60 mL per minute.

PROCEDURE

Proceed with the sample and each of the standard solutions as follows: transfer 25 mL to a suitable ground-stopper container, add 1.0 mL of the internal standard (acetone, unless otherwise specified in the monograph) for each 6% alcohol estimated in the sample and homogenize. Add water only if necessary to make the solution. Inject the appropriate amount of the solution into the apparatus. Calculate the ratio between the area under the alcohol peak and the area under the peak of the internal standard in the chromatograms. Calculate the percentage of alcohol in the sample using the formula

$$\frac{P_1(Y - Z) + P_2(Z - X)}{(Y - Z)}$$

where

P₁ = percentage of alcohol in *standard solution 1*,

P₂ = percentage of alcohol in *standard solution 2*,

X = ratio between the area under the alcohol peak and the area under the peak of the internal standard of *Standard solution 1*,

X = ratio between the area under the alcohol peak and the area under the peak of the internal standard of *Standard solution 2*,

Z = ratio between the area under the alcohol peak and the area under the peak of the internal standard of *Sample solution*.

If the value obtained is outside the range of the values included by the standard solutions, repeat the procedure using those that provide a range that includes the sample value.

5.3.3.9 AMINO ACID ANALYSIS

Amino acid analysis is carried out through two steps: hydrolysis of peptide bonds and evaluation of each amino acid in the resulting hydrolyzate.

Method for hydrolysis of isolated proteins and peptides

Accurately weigh the amount of sample containing 4 to 10 mg of protein and transfer to a 20 x 150 mm test tube with screw cap and polytetrafluoroethylene disc previously washed with 0.2 M sodium hydroxide, rinsed and dried in oven. If the sample is solid, add 5 mL of hydrochloric acid and 5 mL of water. If the sample is liquid, add hydrochloric acid so that the final concentration of hydrochloric acid is 6 M. Remove oxygen from the tube by nitrogen flow for two to three minutes and then close the tube with a disc and screw cap. Place the tube in a vertical position in an oven regulated between 108 °C and 112 °C, keeping it for 22 hours. Time elapsed, remove the tube from the oven and, while still standing, cool it in running water or ice bath. Transfer the mixture to a 10-mL volumetric flask, quantitatively, adjust the volume with distilled water. If there is any residue or precipitate, remove it by centrifugation and filtration on a sintered glass plate or a 0.45 mm porosity filter membrane. Accurately measure 5.0 mL of the solution, transfer to a round-bottomed flask and remove the solvent under reduced pressure at a maximum of 50 °C. Transfer 10 mL of distilled water to the flask residue and re-evaporate. This operation must be repeated twice more, or until the residue does not present an odor of hydrochloric acid. Dissolve the dry film formed by the hydrolyzate in an appropriate volume of pH 2.2 citrate buffer (0.20 M in Na⁺). The resulting amino acid solution must then be kept in a glass flask, capped and refrigerated until the analysis is performed.

Method for hydrolysis of low-protein samples containing carbohydrates and/or lipids

Accurately weigh the amount of sample containing 10mg of protein and transfer to a 150 mL round-bottomed flask and ground mouth. Add 40 mL of 6 M hydrochloric acid and some glass beads to the medium. Connect a reflux condenser and start heating the flask using heating mantle. Keep the suspension under constant and gentle boiling for 24 hours. Cool to room temperature and quantitatively transfer the contents to a 50 mL volumetric flask, completing the volume with distilled water, proceeding from “...If there is any residue or precipitate...” of the procedure described in *Method for hydrolysis of isolated proteins and peptides*.

Amino acid mixtures in solution (serums) or in pharmaceutical preparations

Properly dilute the solution with pH 2.2 citrate buffer (0.20 M in Na⁺) and then analyze it. If it is in powder or tablet form, dissolve the sample in 0.1 M hydrochloric acid. Transfer the material to a volumetric flask and complete the volume with the same buffer as above. Filter the preparation and keep refrigerated (4°C) until analyzed.

Hydrolysis method with oxidation of cystine and methionine

Due to losses during the acid hydrolysis of proteins, sulfur amino acids are preferably analyzed by means of their respective oxidized derivatives. Oxidation is promoted by performic acid, which converts cystine and cysteine into cysteic acid and methionine into methionine/sulfone, both resistant to hydrolysis conditions.

Prepare performic acid by adding 1 mL of hydrogen peroxide 30 volumes to 9 mL of formic acid. Homogenize the solution, allowing to stand for one hour at room temperature. Cool the formed performic acid in ice bath.

Accurately weigh the sample quantity containing 10mg of protein and transfer to a 25 mL round bottom flask and add 2 mL of performic acid in ice bath. If the sample is soluble, keep the mixture in an ice bath for four hours and, if the sample is insoluble, for 16 hours. Add 0.5 mL of 40% hydrobromic acid to remove excess performic acid. Couple the flask to a rotary evaporator and remove the residual bromine by means of reduced pressure, passing the vapors through *M* sodium hydroxide solution. Proceed with the hydrolysis as described above.

Separation and quantitative analysis of isolated amino acids

The separation of amino acids into hydrolysates is usually performed by ion exchange chromatography using sulfonated polystyrene resins in amino acid analyzers. In these devices, after separation, the eluted amino acids from the chromatographic columns form blue/violet colored substances by reaction with ninhydrin and the quantitative determination is made spectrophotometrically. When using autoanalyzers of amino acids, the specifications of the respective manufacturers must be followed.

5.3.3.10 IODOMETRIC TEST OF ANTIBIOTICS

This antibiotic iodometric assay is intended for the assay of penicillin antibiotic drugs and their elaborated pharmaceuticals, for which iodometric titration is particularly suitable.

Standard solution: accurately weigh the quantity of the chemical reference substance (CRS), previously dried, specified in the individual monograph, and dissolve using the solvent described in **Table 1** or as described in the monograph. Quantitatively dilute with the same solvent to obtain a solution with known final concentration, specified in **Table 1** or as described in the monograph. Transfer 2.0 mL of this solution to a 250 mL erlenmeyer with lid.

Sample solution: if not specified in the individual monograph, accurately weigh the sample quantity and dissolve using the solvent described in **Table 1**. Quantitatively dilute with the same solvent to obtain a solution with known final concentration, specified in **Table 1**. Transfer 2.0 mL of this solution to a 250 mL erlenmeyer with lid.

Table 1 – Solvents and final concentrations.

Antibiotic	Solvent*	Final concentration
amoxicillin trihydrate	Water	2.00 mg/mL
ampicillin	Water	2.50 mg/mL
ampicillin sodium	<i>Solution 1</i>	2.50 mg/mL
ampicillin trihydrate	Water	2.50 mg/mL
Benzathine benzylpenicillin	<i>Solution 1</i>	4000 U/mL
Benzylpenicillin potassium	<i>Solution 1</i>	4000 U/mL
Procaine benzylpenicillin	<i>Solution 1</i>	4000 U/mL
benzylpenicillin sodium	<i>Solution 1</i>	4000 U/mL
cloxacillin sodium	Water	2.50 mg/mL
cyclacillin	Water	2.00 mg/mL
dicloxacillin sodium	<i>Solution 1</i>	2.50 mg/mL
Phenoxymethylpenicillin potassium	<i>Solution 1</i>	2.50 mg/mL
pheneticillin potassium	<i>Solution 1</i>	2.50 mg/mL
Methicillin sodium	<i>Solution 1</i>	2.50 mg/mL
Nafcillin sodium	<i>Solution 1</i>	2.50 mg/mL

Antibiotic	Solvent*	Final concentration
Oxacillin sodium	<i>Solution 1</i>	2.50 mg/mL

*If not specified, *Solution 1* is the one defined in the section *Solutions in Microbiological Assay of Antibiotics (5.5.3.3)*, except that sterilization is not required.

PROCEDURE

Inactivation and titration: to each erlenmeyer containing, respectively, 2.0 mL of the *Standard Solution* and the *Sample Solution*, add 2 mL of 1.0 M sodium hydroxide, homogenize with circular movements and allow to stand for 15 minutes. Add 2 mL of 1.2 M hydrochloric acid, 20.0 mL of 0.005 M iodine VS, cap immediately and allow to stand for 15 minutes. Titrate with 0.01 M sodium thiosulfate VS. Close to the titration endpoint, add three drops of starch TS and proceed with titration until the blue color disappears.

Blank assay: Transfer 20.0 mL of 0.005 M iodine VS to each erlenmeyer flask containing 2.0 mL of *Standard solution*. If the *Standard Solution* contains amoxicillin or ampicillin, immediately add 0.1 mL of 1.2 M hydrochloric acid. Titrate with 0.01 M sodium thiosulfate VS. Close to the titration endpoint, add three drops of starch TS and proceed with titration until the blue color disappears. Proceed similarly for an Erlenmeyer flask containing 2.0 mL of *Sample Preparation*.

Calculations: if not specified in the individual monograph, calculate potency, in µg or units per mg of the sample (active pharmaceutical ingredient or dosage form) using formula 1 and formula 2, or only formula 3, described below.

Calculate the equivalence factor (*F*), in micrograms or Unit, for each milliliter of 0.01 M sodium thiosulfate VS consumed by the standard preparation according to formula 1:

$$F = \frac{2(C_p \times P_p)}{V_{bp} - V_p} \quad (\text{Formula 1})$$

where

C_p = concentration, in mg/mL, of the reference chemical substance in the *Standard Solution*;

P_p = potency, in µg/mg or Units/mg, of the chemical reference substance;

V_{bp} = volume of titrant, in mL, consumed in *Standard Blank Assay*;

V_p = volume of titrant, in mL, consumed in *Standard inactivation and titration*.

Calculate the potency, in µg or Units per mg of the sample (active pharmaceutical ingredient or pharmaceutical preparation) according to formula 2:

$$\frac{F(V_{ba} \times V_a)}{2C_a} \quad (\text{Formula 2})$$

where

C_a = concentration, in mg/mL or Units/mL, of the drug in the *Sample Solution*;

V_{ba} = volume of titrant, in mL, consumed in *Sample Blank Assay*;

V_a = volume of titrant, in mL, consumed in *Sample inactivation and titration*.

Calculate the potency, in µg or Units per mg of the sample (active pharmaceutical ingredient or pharmaceutical form) according to Formula 3:

$$P = \frac{(V_{ba} - V_a) \times P_p \times C_p}{(V_{bp} - V_p) \times C_a} \quad (\text{Formula 3})$$

where

P_p = sample potency (µg/mg);

V_{ba} = volume of titrant, in mL, consumed in *Sample Blank Assay*;

V_a = volume of titrant, in mL, consumed in *Sample inactivation and titration*.

V_{bp} = volume of titrant, in mL, consumed in *Standard Blank Assay*;

V_p = volume of titrant, in mL, consumed in standard *inactivation and titration*.

P_p = potency, in µg/mg or Units/mg, of the chemical reference substance;

C_p = concentration, in mg/mL, of the chemical reference substance in the *Standard Solution*;

C_a = concentration, in mg/mL or Units/mL, of the drug in the *Sample Solution*;

5.4 METHODS OF PHARMACOGNOSIS

5.4.1 HERBAL DRUGS ANALYSIS METHODS

5.4.1.1 SAMPLING

Due to the characteristics of herbal drugs, in particular the lack of homogeneity, special procedures are required in relation to the tests to be carried out. The sampling procedures take into account three aspects: number of packages that contain the drug; degree of drug division and amount of drug available.

NUMBER OF PACKAGES

Examine the integrity of the packaging containers and the nature of the drug contained therein. If external examination of the packages and labels indicates that the batch is homogeneous, take individual samples from a number of randomly selected packages, as indicated in **Table 1**. If the batch is not homogeneous, it should be divided into sub batches as uniformly as possible. Carry out sampling with each fraction as a batch.

Table 1 – Number of packages to be sampled.	
<i>Total number of packages</i>	<i>Number of packages to be sampled</i>
1 to 3	All of them
4 to 10	3
11 to 20	5
21 to 50	6
51 to 80	8
81 to 100	10
Over 100	10%

DEGREE OF DIVISION AND AMOUNT OF DRUG

Carry out sampling of the top, middle and bottom of each package from top to bottom and bottom to top (vertical direction) and laterally (horizontal direction).

Fragments smaller than 1 cm: remove the sample with the aid of a sampler (tube fitted with a closing device at the base). Collect samples of at least 250 g for batches of up to 100 kg of drug. For batches larger than 100 kg, take 250 g samples for every 100 kg of drug and obtain a final 250 g sample by quartering.

Fragments larger than 1 cm: remove samples manually. Homogenize the samples taken from each open package, taking care not to increase the degree of fragmentation or significantly modify the moisture content during handling.

For drug quantities up to 100 kg, the sample must consist of at least 500 g. If there is more than 100 kg of drug, proceed with sampling followed by selection by quartering, generating a 500 g sample at the end of the process.

In both cases, drugs with dimensions less than or more than 1 cm, it is permitted to sample quantities lower than those specified above provided that the total amount of available drug is less than 10 kg. However, the final sample should not be inferior to 125 g.

In case of large bales or packages, samples must be collected from more than 10 cm from the edges, as the surface moisture content is different in relation to the inner layers.

QUARTERING

Combine and homogenize the samples taken from each open package, taking care not to increase the degree of fragmentation or significantly modify the moisture content during handling.

Evenly distribute the sample in the shape of a square, divide it into four equal parts and discard the portions into two opposite squares on one of the diagonals. Add the remaining two portions and repeat the process, until the indicated amount is obtained. If there is a marked difference in fragment dimensions, perform manual separation and record the approximate percentages of components with different degrees of division found in the sample.

5.4.1.2 SENSORY EXAMINATION AND MICROSCOPIC INSPECTION OF HERBAL DRUGS

The identity, purity and quality of a plant material must be established through detailed visual, macroscopic and microscopic examination. Whenever possible, the plant material should be compared with reference raw material, or derived from a perfectly identified sample according to pharmacopoeias. Samples that are not similar in color, consistency and odor should be discarded as they do not meet the minimum requirements specified in the monographs. Macroscopic identification of drugs, when whole, is based on the shape, size, color, surface, texture, fracture, and appearance of the fracture surface. As these identification characteristics are subjective, and similar adulterants exist, microscopic and physicochemical analyses of the sample are necessary. Microscopic inspection is essential when the material is scratched or powdered.

Size

Length, width and thickness measurements must coincide with those quoted in the monographs. Small fruits and seeds require a sample of ten units and further calculation of the mean and standard deviation.

Color

Examine the sample before any treatment, in daylight or under a lamp with a wavelength similar to that of daylight. The color of the sample must be compared to the reference material.

Surface, texture and fracture

Examine the untreated sample. When necessary, use a magnifying glass from five to ten times. When indicated in the monograph, moisten with water or a specified reagent to observe fracture surface characteristics. Touch the material to check whether it is soft or hard, bending and breaking the material to obtain information on the fragility and appearance of the fracture, whether fibrous, smooth, rough, grainy, among others.

Odor

Before checking the odor of the material, make sure there is no risk to health. Place a small sample in the palm of your hand or in a glass container and inhale slowly and repeatedly. If the odor is indistinct, press some of the material between your fingers and inhale again. When the monograph indicates a toxic risk, place some crushed material in hot water. Determine the intensity of the odor and classify it according to characteristics: none, weak, distinct or strong; and then in relation to the sensation caused by the odor: aromatic, fruity, musty or rancid. When possible, it is important to compare the odor with a defined substance, such as peppermint, which should have an odor similar to menthol and cloves, similar to eugenol.

Taste

Test the taste only when required in the monograph.

PREPARATION OF MATERIAL FOR MICROSCOPIC ANALYSIS

Hydration or softening of the material

Plant organs or their parts are normally dry, and to be sectioned and observed under an optical microscope, it is convenient to first soften them by treatment with hot water or a hydration solution. The time required for softening each plant organ or its parts varies according to its texture. In fresh organs, only those with firmer consistency need such treatment.

Hydration method for dry materials

Place the sample in a suitable container: a) on a heating plate, or metallic mesh with water, in an amount of 20 to 30 times the volume of the sample, gently heating until boiling, keeping for five minutes and if softening is not enough, add detergent and boil for another five minutes; or b) in a hydration solution, prepared with five parts of water, four parts of ethyl alcohol, one part of glycerol and five drops of commercial detergent for each 200 mL of solution, in an oven at 60 °C, for a variable period, according to the material texture. Flowers and leaves tend to hydrate in a few minutes, while hard materials such as husks and seeds require a variable amount of time in heated water as in a); according to the degree of lignification, or hours or days in the hydration solution b). In direct hydration in water a), carefully observe the time, as excessive softening may occur, preventing observation under an optical microscope. In both methods, periodically check the material consistency. For future analyses, determine the time each herbal drug needs to acquire the consistency that allows for sectioning.

Obtaining histological sections

Once hydrated and softened, proceed with the preparation of the sections of the vegetable organs or their parts. They must be performed transversally to the axis of the organ, or as established in the individual monographs. In these, longitudinal or tangential cuts (barks, roots, etc.) or paradermal sections are requested to observe the epidermis of foliaceous organs (leaves, sepals and petals). Freehand sections are made with the aid of cutting slides. Very small or very thin structures require the sample to be firmed or embedded in suitable material. Better quality sections can be obtained using microtomes. Select the thinnest slices to observe under the microscope at 10 times.

Coloring and preparation of slides

Submerge sections in 50% sodium hypochlorite solution to eliminate cell content. Allow to act until the sections are transparent (maximum 10 to 15 minutes). Wash the sections with distilled water to

eliminate sodium hypochlorite until neutral pH. Place sections in 0.05% Toluidine Blue solution for 10 seconds. Wash with distilled water, then with 0.5% acetic acid solution, and again with distilled water. Place between slide and cover slip with two to three drops of a mixture of glycerin-distilled water (1:1) and observe under an optical microscope at 10 and 40 times. The cellulosic walls will be dyed purple pink. The lignified walls and walls with tannins will be dyed a bright greenish-blue color and the color obtained may not remain stable.

TISSUE DISSOCIATION

This method is mainly used for the analysis of leaves, herbaceous stems and bark. The crystals remain intact. Starch grains lose their characteristic structure.

Place a portion of the plant material in a 30 mL beaker. Add 10 mL of 5% sodium hydroxide solution and boil for five minutes. Cool and transfer to a centrifuge tube. Centrifuge for two minutes, discard supernatant solution and wash with distilled water. Place a portion of the centrifuge on a slide with two or three drops of a mixture of glycerin-water (1:1). Insert the coverslip and press. Observe under an optical microscope, with 10x and 40x magnification.

Powder drug observation

Weigh 1 to 2mg of the drug and place a small portion, with a fine, soft brush, on a slide. Add two or three drops of 5% lactic acid solution (diaphanizing), and if necessary, before placing the coverslip, add one or two drops of water or a mixture of glycerol and ethyl alcohol (1:1), mixing well with the brush. Insert the coverslip. Observe under an optical microscope, with 10x and 40x magnification.

Determination of stomata value

The stomata value is used in the analysis of laminar structures, such as leaves, lamina leaves and bracts, counting the number of stomata in a given area of the epidermis. For this count, prepare portions of about 0.5 cm by 0.5 cm of the leaf slide, submerged in a mixture of 10 mL of chloral hydrate and water (5:2), in a beaker, boiling for 10 to 15 minutes, until the material is transparent. Carry out the operation in a fume hood. Place a piece of the prepared leaf on a slide, with the abaxial epidermis facing up. For very thick leaves, section each portion close to the abaxial epidermis, ensuring that this face is placed correctly on the slide, with the epidermal layer facing the coverslip. Add 2-3 drops of the mixture of chloral hydrate and water (5:2) and cover with a coverslip. Observe under an optical microscope, with 10x magnification. Count the epidermal cells and stomata that appear in the area. The value is calculated according to the formula $100S/(E+S)$, where S is the number of stomata in a determined area of the leaf surface whereas E is the number of epidermal cells, including trichomes existing in the same microscopic field observed. For each sample, carry out and calculate the average of minimum ten determinations.

Histochemical reactions

Reactions can be carried out with fresh or dry sectioned material, material cut in a microtome or with the powder of the herbal drug. The material is placed properly distributed on a slide, adding one or two drops of the reagent. Place coverslip and observe under an optical microscope, with 10x and 40x magnification.

Starch

Add one or two drops of iodine solution RS diluted (1:5) in water. Starch grains turn blue or blue-violet in color.

Calcium carbonate concretions (cystolites) and calcium oxalate crystals

Add one or two drops of 2 M hydrochloric acid or 6% (w/v) acetic acid. The presence of calcium carbonate is indicated by effervescence. Calcium oxalate crystals take longer to dissolve, do not release bubbles and are insoluble in 6% acetic acid.

Hydroxyanthraquinones

Add one drop of 5% potassium hydroxide (w/v). Cells containing 1,8-dihydroxyanthraquinones stain red.

Inulin

Add one drop of 20% 1-naphthol solution in methyl alcohol, followed by one drop of sulfuric acid. Inulin spherul crystals stain red or reddish-brown and dissolve.

Lignin

Add one drop of phloroglucin RS, quickly heat the slide and add one drop of 25% (w/v) hydrochloric acid. The lignin stains red.

Lipids (including cutin, waxes and suberin)

Add one or two drops of Sudan III RS or Sudan IV RS reagent, allowing to be in contact for 10 minutes, wash with 70% ethyl alcohol (v/v). Lipids, cutin and suberin stain from reddish-orange to red in a short time.

Pectins and mucilages

Submerge the dry sample in a thionine solution, allowing to stand for 15 minutes, washing in 20% ethyl alcohol (v/v). Mucilages appear as spherical globules with a reddish-violet color, while cellulose, pectin and lignified partitions are colored blue or blue-violet. Mucilage also appears as dilated and transparent spherical fragments on a black background, adding a drop of India ink to a dry sample.

Proteins

Carry out this procedure only with fresh material. Add 0.5% (w/v) ninhydrin in absolute ethyl alcohol, and keep at 37°C for 24 hours. Wash in absolute ethyl alcohol followed by distilled water, add bleached fuchsin RS and allow to be in contact for 10 to 30 minutes. Wash in water and add 2% sodium bisulfite (w/v), leave in contact for one to two minutes. Wash under running water for 10 to 20 minutes. The proteins stain purple red.

Saponins

Add a drop of sulfuric acid. A yellow color sequence occurs, followed by a red color, and finally a violet or blue-greenish color.

Tannins

Add 5% ferric chloride (w/v) and a small amount of sodium carbonate, leave in contact for two to three minutes, wash with distilled water. The tannins turn dark blue-greenish.

5.4.1.3 DETERMINATION OF FOREIGN MATTER

Foreign matter is any material that does not appear in the definition of the drug described in the corresponding monograph. Drugs must be free of fungi, insects and other contaminations of animal origin. Unless otherwise stated, the percentage of foreign elements must not exceed 2% (w/w).

Matter foreign to the drug is classified into three types: a) parts of the organism or organisms from which the drug is derived, except those included in the definition and description of the drug, above the tolerance limit specified in the monograph; b) any organisms, portions or products of organisms not specified in the definition and description of the drug, in its respective monograph; and c) mineral impurities or other dirt not inherent to the drug. During storage, products must be kept in clean areas to avoid contamination. Take special precautions to prevent the proliferation of fungi, as some of them can produce toxins.

Procedure

If not otherwise specified in the corresponding monograph, obtain by quartering the following sample quantities:

- roots, rhizomes, barks, whole plant and aerial parts: 500 g
- leaves, flowers, fruits and seeds: 250 g
- fragmented herbal drugs of 0.5 g or smaller: 50 g
- powders 25 g.

Spread the sample in a thin layer on a flat surface. Manually separate material foreign to the drug, initially with the naked eye and then with the aid of a magnifying glass (five to ten times). Weigh the separated material and determine the percentage of foreign matter based on the weight of the sample submitted for testing.

5.4.1.4 DETERMINATION OF WATER IN HERBAL DRUGS

For the determination of water in herbal drugs, three methods are used: gravimetric method (desiccation); azeotropic method (distillation with toluene) (5.2.20.2) and volumetric method (Karl Fischer) (5.2.20.1). The first, technically simpler and faster, is not applicable when the drug contains volatile substances. The azeotropic method is indicated for herbal drugs containing volatile oil. The other methods require special devices and comprise more complex techniques.

Sample preparation

Reduce the sample by cutting; granulation or fragmentation of the drugs not pulverized or crushed, to limit the dimension of their components to approximately 3 mm thick. Seeds and fruits, even smaller than 3 mm, must be broken. Avoid high speed mills to prepare the sample and take necessary precautions not to modify the moisture content of the sample.

Gravimetric method

Accurately weigh between 1 to 10 g, or specified in the monograph, of a previously prepared sample, transfer to an accurately weighed weighing bottle, previously dried under the same conditions to be adopted for the sample, for 30 minutes. Dry the sample between 100 °C and 105 °C for five hours, until constant weight, that is, until the difference between two serial weighings corresponds to a maximum of 0.25% of the sample. Calculate the percentage of water in relation to air-drying drug.

5.4.1.4 DETERMINATION OF ASH

Total ash includes physiological ash and non-physiological ash.

5.4.1.5.1 DETERMINATION OF TOTAL ASH

Procedure

Accurately weigh approximately 3 g of the pulverized sample, or the amount specified in the monograph, and transfer to a previously tared porcelain crucible. Distribute the sample evenly in the crucible and incinerate, gradually increasing the temperature to a maximum of $(600 \pm 50)^\circ\text{C}$, until all the carbon is eliminated. A temperature gradient (30 minutes at 200°C , 60 minutes at 400°C and 90 minutes at 600°C) can be used. At the end of the test, wait for cooling in a desiccator and weigh. In cases where the carbon cannot be completely eliminated, cool the crucible and moisten the residue with about 2 mL of water or saturated ammonium nitrate solution. Evaporate to dryness in water bath and then place on a hot plate and incinerate until the difference between two serial weighing is not more than 1.0 mg. Calculate the percentage of ash in relation to the dry drug.

5.4.1.5.2 DETERMINATION OF SULFATED ASHES

Procedure

Heat a porcelain crucible at red heat for 10 minutes, wait for it to cool in a desiccator and weigh. Accurately weigh about 1.0 g of the drug in the previously tared crucible and moisten the drug with concentrated sulfuric acid. Carbonize; and moisten again with concentrated sulfuric acid and incinerate with gradual heating up to 800°C . Wait for cooling, weigh again, and incinerate for another 15 minutes. Repeat this procedure until the difference between two serial weighing is, at most, 1.0 mg.

5.4.1.5.3 DETERMINATION OF ACID-INSOLUBLE ASH

Acid-insoluble ash constitutes the residue obtained from the boiling of total ash or ashes sulfated with dilute hydrochloric acid, after filtering, washing and incineration. The method is intended for the determination of silica and silicic drug constituents.

Procedure

Heat the ash obtained to boiling, as indicated in total ash, with 25 mL of 2 M hydrochloric acid for five minutes in a crucible covered by a watch glass. Wash the watch glass with 5 mL of hot water, adding the washing water to the crucible. Collect the acid-insoluble material on a filter paper with known ash content, rinsing with hot water until the filtrate is neutral. Transfer the filter paper containing the residue to the original crucible, dry on a plate and incinerate at approximately 500°C until the difference between two serial weighing is, at most, 1.0mg. Calculate the percentage of insoluble ash in relation to the dry drug.

5.4.1.6 DETERMINATION OF VOLATILE OILS

The content of volatile oils in herbal drugs is determined by the process of hydrodistillation or steam distillation, in devices described below.

Apparatus 1 (**Figure 1**), made of resistant glass, of appropriate quality, comprises:

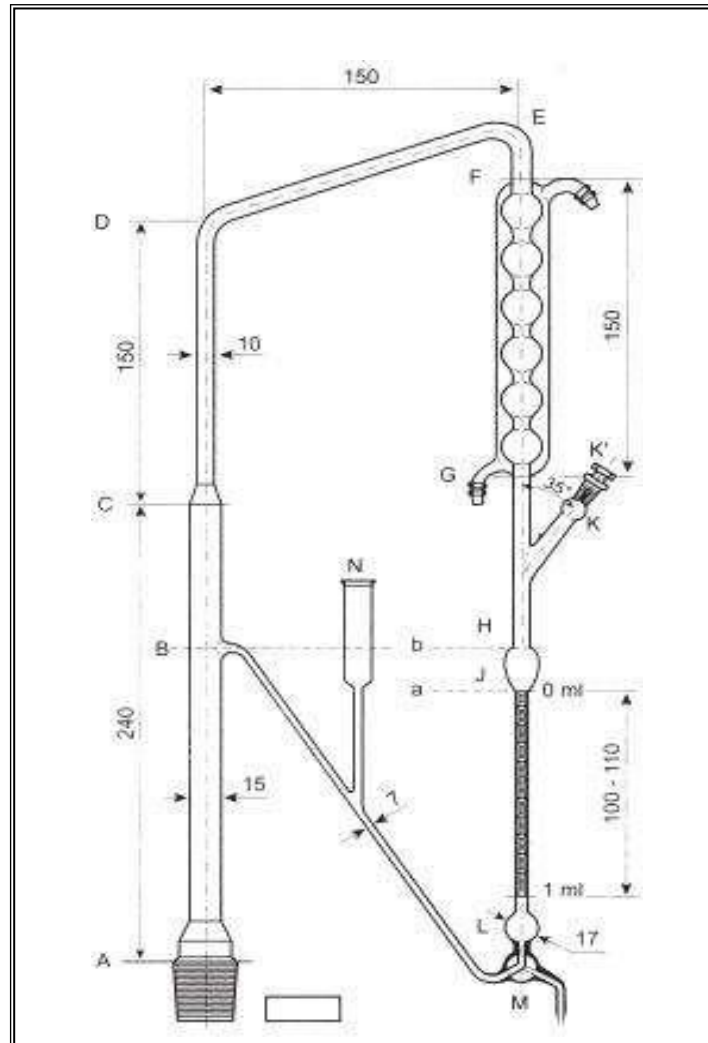


Figure 1 – Apparatus for determination of volatile oil content in herbal drugs by the hydrodistillation process

- 1) 500 mL to 1000 mL round bottom flask, short neck, fitted with a 24/40 female joint;
- 2) condenser, adaptable to the flask by means of a 24/40 male ground joint, built in a single piece of glass, comprising the parts described below, with the respective measurements:
 - 2.1) vertical tube (AC) 240mm in length and 13-15mm in internal diameter;
 - 2.2) bent tube, with segments (CD) and (DE) measuring 150 mm in length each and internal diameter of 10 mm;
 - 2.3) Ball condenser, Allihn type (FG), 150mm in length and 15mm in internal diameter on the bulbs and 8-10mm in the narrowing;
 - 2.4) stopper (14/20 ground joint) (K') containing an orifice of about 1 mm in diameter, which seals a side outlet (K) provided with a 14/20 female ground joint at the end;
 - 2.5) tube (GH) 30-40 mm in length and 7-8 mm in internal diameter, forming the parts (HK) angle (GHK) of 35°;
 - 2.6) pear-shaped widening (J) of 3 mL capacity;
 - 2.7) tube (JL) fitted with a graduated scale of 100-110 mm; 1 mL capacity and subdivided into 0.01 mL;
 - 2.8) bulb-shaped widening (L) of approximately 2 mL capacity;
 - 2.9) three-way valve;
 - 2.10) connecting tube (BM) 7-8mm in diameter, fitted with a safety tube. Insertion point (B) is 20mm above the highest part of the graduated scale;
- 3) appropriate heating device allowing precise regulation;

4) vertical support with a horizontal ring covered with insulating material.

Use a perfectly clean and dry apparatus. Assemble the system in a place protected from drafts. Check the graduated scale and, if necessary, establish a correction factor for each device. Proceed with the assay, as established in the individual monograph and according to the drug nature.

Procedure

Introduce the volume of liquid indicated in the corresponding monograph and porous porcelain fragments or glass beads into the flask. Adapt the condenser to the flask. Introduce the water through the tube (N) until reaching the level (B). Remove the ground stopper (K') and introduce the prescribed amount of xylene using a pipette, through the opening (K), supporting the pipette tip on the bottom of the lateral outlet (K). Place the K' cap ensuring that the K and K' orifices match each other. Heat the liquid inside the flask until boiling begins and distil at a rate of 2 to 3 mL per minute, or as indicated in the corresponding monograph.

To determine the speed of distillation, drain the water using a three-way valve, until the meniscus is in the inferior reference line *a* (**Figure 2**). Close the valve and time the necessary time until the liquid reaches the superior mark *b*. Open the valve and continue the extraction for 30 minutes, modifying the heat to regulate the distillation speed. Turn off the heating, allow to cool for at least 10 minutes and read the xylene volume in the graduated tube.

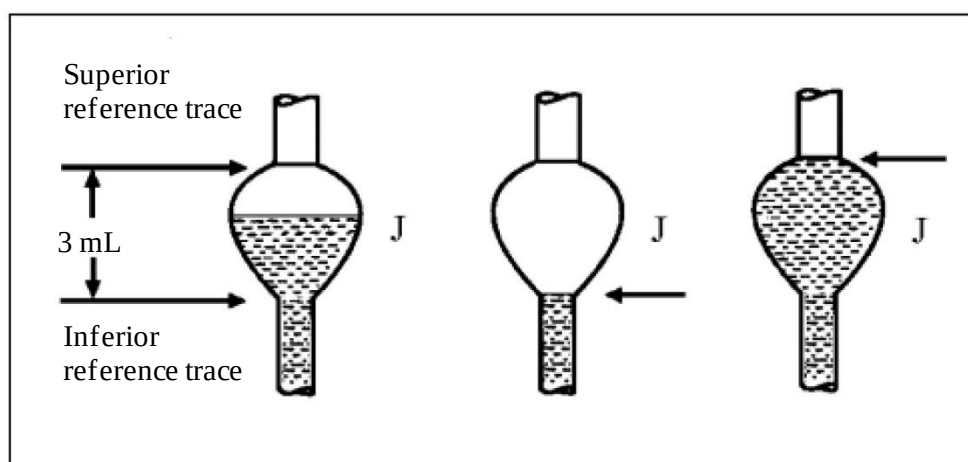


Figure 2 – Indication for determining the speed of distillation.

Introduce the amount of drug prescribed in the monograph into the flask and distil as described above, for the time and speed indicated in the monograph. After the operation, wait for 10 minutes to cool and read the volume of volatile oil collected in the graduated tube. Subtract the xylene volume determined previously from the reading. The difference represents the amount of volatile oil contained in the sample. Calculate the result in milliliters of volatile oil per 100 g of the drug.

When the determination of volatile oil is for analytical purposes, obtaining a mixture of volatile oil and xylene, free of water, is carried out as described below: remove the K' cap and transfer 1.1 mL of a solution of sodium fluorescein at 0.1% and 0.5 mL of water. Reduce the volume of the essential oil and xylene mixture inside tube L using the three-way valve. Allow to stand for five minutes and slowly remove the mixture up to the level of valve M. Open the valve anticlockwise so that water flows out of the BM connection tube. Thoroughly wash the tube with acetone, introduced through tube N. Turn the valve anticlockwise so that it is possible to collect the mixture of xylene and volatile oil in an appropriate container.

Apparatus 2 (**Figure 3**), made of resistant glass, of appropriate quality, comprises:

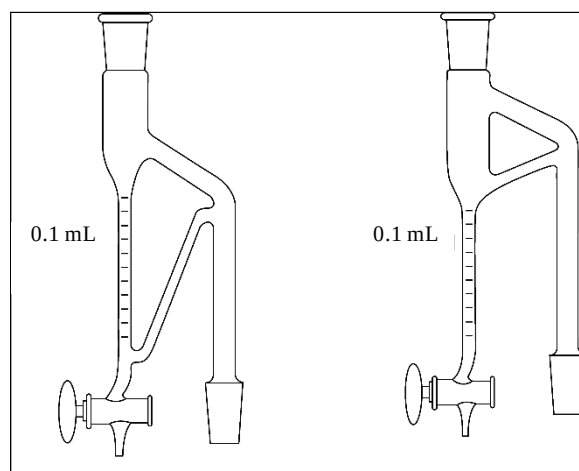


Figure 3 – Apparatus for determination of volatile oil content in herbal drugs by the hydrodistillation process, for oils denser and less dense than water.

Weigh enough of the drug to obtain 0.5 to 2 mL of volatile oil into an appropriate round-bottomed flask and add water to half of the container. Connect the condenser. Bring to a boil keeping a gentle boiling for two hours, or as specified in the monograph.

Allow to stand for 10 minutes at room temperature, open the apparatus valve and slowly drain the water until the surface of the oil layer reaches the zero line on the graduated tube. Allow to stand at room temperature for about an hour and then measure the volume (mL) of the oil obtained.

5.4.1.7 DETERMINATION OF FIXED OILS

The determination of fixed oils is based on their solvent extraction. After the solvent evaporation process, the residue obtained, which is determined by weighing, represents the amount of fixed oils present in the sample.

Procedure for samples with a high content of water-soluble substances (carbohydrates, urea, lactic acid, among others): pre-treatment of the sample may be necessary in order to avoid interference in the determination of fatty matters. Transfer the sample quantity to a funnel containing filter paper, wash with water and dry the residue in an oven at 105°C for two hours.

Use the Soxhlet device (**Figure 4**), previously cleaned and dry, assembled in a place free from air currents. The apparatus, made of resistant glass, of appropriate quality, comprises a round-bottomed flask (A), with 500 mL to 1000 mL capacity, connected to the Soxhlet extractor (B) and reflux condenser (C).

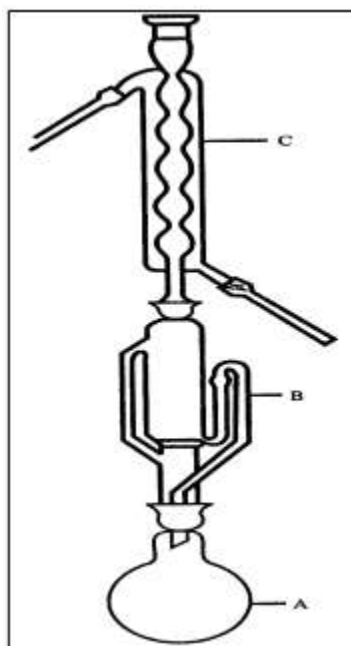


Figure 4 – Soxhlet device.

Procedure

Accurately weigh about 10 g of previously desiccated drug as described in *Determination of water in herbal drugs, Gravimetric method*, and transfer to a Soxhlet extractor device (B), covering it with degreased cotton. Weigh the clean and dry flask (A), containing fragments of porcelain or glass beads. Assemble the system over water bath, ensuring complete sealing in the ground flask joint, in a fume hood. Transfer petroleum ether to the extractor, in sufficient quantity to carry out three siphon separations and fit the reflux condenser (C). Proceed with the extraction, under sufficient heating to keep the solvent at moderate boiling, for four hours. Allow to cool, transfer the cartridge contents to a porcelain mortar and add an equivalent amount of washed and dry sand. Pulverize the drug and transfer, inside the cartridge, to the extractor. Restart extraction under the same conditions for another two hours. Disconnect the flask from the apparatus and evaporate the solvent, preferably under a stream of carbon dioxide. Transfer the flask to an oven at 105 °C, cool and weigh. Repeat the operation until weight is constant. Calculate the percentage of fixed oils in the drug based on the heavy drug mass and the obtained oil mass.

5.4.1.8 DETERMINATION OF FOAMING VALUE

Accurately weigh 1 g of the plant material reduced to fine powder and transfer to an Erlenmeyer flask containing 50 mL of boiling water. Boil for 30 minutes. Cool, filter into a 100 mL volumetric flask and complete the volume through the filter to 100 mL. Distribute the filtrate obtained in 10 test tubes with lids (16 mm in diameter by 16 cm in height), in successive series of 1, 2, 3, up to 10 mL. Complete the volume of liquid in each tube to 10 mL with water. Cap the tubes and shake with vertical movements for 15 seconds at two runs per second. Allow to stand for 15 minutes and measure the height of the foam. If the foam height of all tubes is less than 1 cm, the foaming value is lower than 100. If, in any of the tubes, the measured foam height is 1 cm, the dilution of the plant material in such tube (A) is the observed rate. If this tube is the first or second in the series, it is necessary to make an intermediate dilution, using the same method described above, to obtain a more accurate result. If the foam height is greater than 1 cm in all tubes, the foaming value is higher than 1000. In this case, the determination must be carried out with a new series of decoction dilutions to obtain an accurate result. The foaming value is calculated according to the $1000/A$ formula, where A is the

volume, in milliliters, of the decoction used to prepare the dilution in the tube where the foam was observed.

5.4.1.9 DETERMINATION OF EXTRACTABLE SUBSTANCES

This method determines the amount of active constituents extracted with solvents from a given amount of plant material, and is used in the absence of a suitable chemical or biological test.

METHOD A - COLD EXTRACTION

Accurately weigh approximately 4.0 g of the powdered and dry herbal drug and transfer to a 250-mL, polished-necked erlenmeyer flask, previously weighed. Macerate, with 100 mL of solvent specified in the individual monograph of the herbal drug, for 6 hours, shaking frequently, and allow to stand for 18 hours. Quickly filter and transfer 25 mL of the filtrate to a pre-weighed crystallizer and evaporate to dryness in water bath. Dry the flask in an oven at 105°C until constant weight. Calculate the percentage of extracted materials in mg/g of dry plant material.

METHOD B - HOT EXTRACTION

Accurately weigh approximately 4.0 g of the powdered and dry herbal drug and transfer to a 250-mL, polished-necked erlenmeyer flask, previously weighed. Add 100 mL of solvent specified in the test to the herbal drug and weigh to obtain the total weight, including the vial. Cap, shake and allow to stand for an hour. Attach a reflux condenser and heat gently for one hour, cool and weigh. Correct the original weight with the solvent used. Shake and filter quickly on a dry filter. Transfer 25 mL of the filtrate to a pre-weighed crystallizer and evaporate to dryness in water bath. Dry the flask in an oven at 105°C until constant weight. Calculate the percentage of extracted materials in mg/g of dry plant material.

METHOD C - SOXHLET EXTRACTION

Accurately weigh about 2 g of the herbal drug and transfer to a Soxhlet extraction cartridge, previously weighed and dried. Introduce 0.2 g of sodium hydroxide and sufficient absolute ethyl alcohol into the extractor flask. Extract for five hours, remove the cartridge with the residue and dry it in an oven at 105 °C until constant weight. Calculate the percentage of extracted materials in mg/g of dry plant material.

5.4.1.10 DETERMINATION OF BITTERNESS VALUE

The bitterness value of a substance, a liquid or an extract is the inverse of the limit dilution that still has a bitter taste. It is determined by comparing it with quinine hydrochloride whose bitterness value is set at 200 000.

Determination of correction factor

Each experimenter must rinse their mouth with fresh water before testing. To correct individual differences in the determination of bitterness among the experimenters it is necessary to determine the correction factor for each member. It is recommended that the group of experimenters consists of minimum six people.

Stock solution

Weigh 0.1 g of quinine hydrochloride, dissolve in drinking water and dilute to 100 mL with the same solvent. Transfer 1 mL to a 100 mL volumetric flask and adjust the volume with the same solvent

Reference solutions

Prepare a series of dilutions by adding 3.6 mL of the *Stock Solution* to the first tube and gradually increasing the volume by 0.2 mL in each subsequent tube up to a total of 5.8 mL. Complete the volume of each tube to 10.0 mL with drinking water.

Determine the highest dilution that still has a bitter taste. Place 10 mL of the weaker solution in the mouth and pass it back and forth under the tongue for 30 seconds. If no clear bitter taste is found, discard the solution and wait a minute. Rinse mouth with drinking water. After 10 minutes, test the subsequent solution in ascending order of concentration. Calculate the correction factor (*k*) for each experimenter using the following expression:

$$k = \frac{n}{5,00}$$

n = volume in milliliters of the highest dilution of the stock solution in which a clear bitter taste was found.

Note: experimenters unable to feel a distinct bitter taste in the reference solution prepared with 5.8 mL of the stock solution should be excluded from the group.

Sample preparation

If necessary, reduce the sample to powder (710 µm). Weigh 1.0 g of the sample and add 100 mL of boiling drinking water. Heat in water bath for 30 minutes, shaking continuously. Allow to cool and compensate the volume of evaporated water with drinking water. Shake vigorously and filter on filter paper, discarding the first 2 mL of the filtrate. The filtrate is called C-1 and has a dilution factor of 100.

For liquid samples, take 1 mL and dilute with an appropriate solvent to 100 mL, naming it C-1.

Determination of bitterness value

Sample solutions:

10.0 mL of C-1 is diluted with water to 100 mL: C-2	FD 1000
10.0 mL of C-2 is diluted with water to 100 mL: C-3	FD 10 000
20.0 mL of C-3 is diluted with water to 100 mL: C-3A	FD 50 000
10.0 mL of C-3 is diluted with water to 100 mL: C-4	FD 100 000

Starting with the C-4 dilution, each experimenter determines the dilution at which they experience the clear bitter taste. This solution is called D. The dilution factor of this solution D is equal to Y.

Starting with solution D, prepare the dilution sequence according to **Table 1**.

Table 1 – Sequence of dilution of solution D for determination of bitterness value.

Solution D (mL)	1.2	1.5	2.0	3.0	6.0	8.0
Drinking water (mL)	8.8	8.5	8.0	7.0	4.0	2.0

Determine the volume in milliliters of solution D which, when diluted to 10 mL, still has a clear bitter taste (X).

Calculate the Bitter Value for each experimenter according to the formula:

$$IA = \left(\frac{Y \times k}{X \times 0,1} \right)$$

The Bitter Value of the sample is the average value of experimenters.

5.4.1.11 DETERMINATION OF SWELLING INDEX

The swelling index is the measure of the volume occupied by the swelling of 1 g of the drug, by the addition of water or another swelling agent, under defined conditions.

Carry out the test simultaneously in triplicate. Accurately weigh approximately 1 g of the pulverized herbal drug and transfer to a 25-mL ground-mouth cylinder. The length of the graduated part should be approximately 125 mm and the inner diameter, close to 16 mm, with subdivisions of 0.2 mL marked from 0 to 25 mL, ascending. Add 25 mL of water, or other defined agent, and shake every 10 minutes for one hour. Let the mixture stand for three hours at room temperature. Measure the volume, in milliliters, occupied by plant material plus mucilage or any other adhered material subtracted from the initial drug volume. Calculate the average value obtained from the individual determinations carried out and relate it to 1 g of plant material.

5.4.2 METHODS OF PREPARATION AND ANALYSIS OF VEGETABLE EXTRACTS

5.4.2.1 METHODS OF PREPARATION OF VEGETABLE EXTRACTS

PRODUCTION

Extracts are produced by an appropriate process employing ethyl alcohol or other suitable extracting liquid. Different batches of a specific herbal drug can be combined prior to extraction. The drug can also be subject to preliminary treatments such as enzyme inactivation, grinding or degreasing. Additionally, unwanted substances (e.g. toxic or insoluble) can be removed after the extraction operation.

Herbal drugs, solvents and other materials used for the preparation of extracts must be of adequate quality and must comply with pharmacopoeial requirements and good manufacturing practices. Solvents recovered from the extract concentration or drying process can be reused as long as the recovery processes are controlled and monitored to ensure that the solvent meets specifications prior to reuse or mixing with other materials. The water used for the production of extracts must comply with the requirements of purified water monograph.

Where applicable, extractive solutions can be concentrated to the desired consistency using suitable methods, generally under reduced pressure and at a temperature at which the possibility of degradation of the constituents is reduced.

Volatile oils that have been separated during processing can be incorporated into the extracts at an appropriate stage of the production process.

Suitable excipients can be added at different stages of the production process to enhance technological properties (e.g. as part of the drying process, or to improve homogeneity or maintenance of extract qualities).

Extraction with a certain solvent leads to obtaining typical constituents from the drying of the extracting liquid. During the production of standardized and quantified extracts, purification procedures can be applied to increase the proportion of certain constituents. Such extracts are named purified.

IDENTIFICATION

Extracts are identified using suitable methods.

TESTS

In the extracts production process, tests to verify microbiological quality **(5.5.3.1)**, heavy metals **(5.3.2.3)**, mycotoxins **(5.4.4)**, and pesticide residues **(5.4.3)** may be necessary, as per specific regulation. Whenever a test for heavy metals is performed on the herbal drug, the same limits for heavy metals indicated in the herbal drugs monographs apply to the extracts, unless otherwise indicated in the individual extract monograph, or unless under justified and authorized reason.

ASSAYS

Extracts must be analyzed using recommended methods.

LABELING

The label must indicate:

- name of drug;
- the form of the extract (e.g., liquid, tincture, soft, dry, or oleoresin);
- whether the extract is standardized, quantified or others;
- for standardized extracts, the definition of the content of the constituents responsible for the therapeutic activity;
- for quantified extracts, the specified range of marker content;
- where applicable, whether the extract is purified;
- ratio between the amount of drug and the amount of liquid extract (extractive solution) (genuine RDE) expressed as mass/mass ratio for soft extracts, oleoresins and dry extracts, and both in mass/mass and mass/volume for preparations of liquid extracts;
- solvent or solvents used in the extraction;
- name and quantity of any excipients present in the extract, including stabilizers and preservatives;
- for quantified extracts and other extracts, the ratio between the amount of drug and the amount of liquid extract (extractive solution) (genuine RDE) expressed as mass/mass ratio for soft extracts, oleoresins and dry extracts, and both in mass/mass and mass/volume for liquid extract preparations;
- when applicable, the percentage of dry residue;
- storage conditions.

LIQUID EXTRACTIVE PREPARATIONS

Liquid extractive preparations consist of diversified products, obtained by liquid extraction, described by their extraction solvents, production methods and drug-solvent ratio or drug-extract ratio. They include products obtained using ethyl alcohol, water, glycerol, propylene glycol and fixed oils as extraction solvents. Fluid extracts and tinctures belong to this category.

5.4.2.1.1 FLUID EXTRACT

Fluid extracts are prepared using ethyl alcohol, hydroethyl mixtures in appropriate proportions, or water. When necessary, other substances may be added (e.g. glycerol or ammonia solution) to aid in the extraction or dissolution of a soft or dry extract of the herbal drug (which must be produced using the same extraction solvent that would be used to prepare the fluid extract by direct extraction) either in water or ethyl alcohol at the required concentration.

Fluid extracts can be adjusted, if necessary, to meet solvent content requirements. Fluid extracts can be filtered if necessary.

A light sediment can form when standing.

FLUID EXTRACT PREPARATION PROCESSES

Most are prepared by one of the four general processes described below and designated by the letters **A**, **B**, **C** and **D**.

The maceration time and percolation rate vary according to the drug, aiming to fully extract the drug-specific markers. Percolate flow rate is determined by the expressions percolate slowly (up to 1 mL

per minute); percolate quickly (3 to 5 mL per minute) and percolate at moderate speed (1 to 3 mL per minute), in reference to the extraction of 1000 g of drug.

An extract, which, over time, deposits some sediment, can be filtered or decanted, as long as the resulting liquid complies with pharmacopoeial specifications.

The following general production processes are:

Process A

This process is used in the preparation of fluid extracts by percolation, in which the extracting liquid is ethyl alcohol or a hydroethyl mixture.

Procedure: uniformly moisten 1000 g of the pulverized drug, with a sufficient quantity of the indicated extracting liquid, and allow to macerate in a suitable container until the herbal drug swells. Then transfer to a percolator. Strongly compress the drug and pour an additional and sufficient amount of extracting liquid over it until an excess of supernatant liquid remains. When the liquid starts to drip, close the lower outlet of the percolator, cap and allow to soak for the time prescribed in the monograph. Percolate at the specified speed, adding more extractant liquid until the drug is depleted. Separately collect the first 850 mL of percolate, if not otherwise specified in the monograph. Continue the percolation until the drug is depleted and concentrate this remaining percolate until a syrup consistency, with a maximum temperature of 60 °C. Add this concentrated extract to the previously separated percolate and, if necessary, add a sufficient amount of extracting liquid used to obtain 1000 mL of fluid extract.

This process can be replaced by **Process C**.

Process B

Use this process in the preparation of fluid extracts in whose extraction, in addition to ethyl alcohol or hydroethyl mixture, determined amounts of other components are used, such as acids, bases or polyols (glycerol, ethylene glycol etc.), used successively in two extracting liquids. *Extracting liquid I* contains a hydroethyl mixture and other components in the proportion required for the amount of drug used and *extracting liquid II*, a hydroethyl mixture, in the indicated proportion, used to complete the depletion of the drug.

Procedure: Uniformly moisten 1000 g of the pulverized drug with a sufficient amount of *Extracting Liquid I* (this operation requires 600 mL to 800 mL of Extracting Liquid). Allow the drug, moistened, to stand for about 15 minutes. Then transfer to a percolator, compress the drug strongly, and add the remainder of the *Extracting Liquid I*. When the liquid starts to drip, close the outlet of the percolator, cap and allow to soak for the time prescribed in the monograph. Percolate at the indicated flow rate and, when the level of the *extracting liquid I* reaches the drug surface, continue the percolation with the *extracting liquid II* until the drug is depleted. Separately collect the first 850 mL of percolate. Continue the percolation until the drug is depleted and concentrate the remaining percolate until a syrup consistency, with temperature not exceeding 60 °C. Add this concentrated extract to the previously separated percolate and add, if necessary, a sufficient quantity of *extracting liquid II*, to obtain 1000 mL of fluid extract or adjust the volume according to the assay.

The process can be replaced by **Process C**, with the proper adjustment in the extraction liquid.

Process C

This process is that of fractional percolation, especially in substitution for processes **A** or **B** and indicated for drugs that contain volatile or thermolabile constituents and/or when there is no adequate apparatus for concentration and distillation. When using **Process C** instead of **Process B**, use *extracting liquid I* throughout the percolation course.

Procedure: divide 1000 g of the drug previously pulverized into three portions of 500 g, 300 g and 200 g respectively. Evenly moisten the first portion with a sufficient amount of the extracting liquid. Transfer the moisten powder to a suitable percolator, the capacity of which should not greatly exceed the volume of drug in the percolator. Add extracting liquid until the drug is completely covered and macerate for the time prescribed in the monograph. Then proceed with the percolation, separately collecting the first 200 mL (F1) and then separately collect five serial fractions of 300 mL of percolate, numbering them in the order in which they are obtained (F2-F6).

Moisten the second portion of the drug with a sufficient amount of F2 percolate; percolate, proceeding as with the first portion of the drug, using the remaining portions of the percolate (F3-F6), obtained in the first operation, as the extracting liquid, and using them in the order in which they were collected. Collect and separate the first 300 mL of the new percolate (F7) and collect five more fractions of 200 mL each, numbering them in the order in which they are obtained (F8-F12).

Moisten the third portion of the drug with a sufficient amount of F8 percolate and proceed with the percolation as in the previous operation, using as extracting liquid the 200 mL fractions of percolate from the second portion (F9-F12), in the order in which they were collected. If there is no assay, collect and separate 500 mL of percolate (F13). Homogenize the three percolates (F1, F7 and F13) separated from the three portions of the drug, to obtain 1000 mL of fluid extract.

When necessary to carry out an assay for the fluid extract prepared by **Process C**, collect and separate only 420 mL of percolate from the third portion instead of the 500 mL previously determined (F13). Homogenize the three separate percolates (F1, F7 and F13) obtained from the three portions of the drug and carry out an assay for a fraction of the mixture. If the content is higher than recommended, adjust using the extracting liquid. If the content is lower than recommended, deplete the extracting liquid from the percolator and repeat the extraction starting with the most concentrated percolate (F1, F7 and F13) and then with the remaining percolates.

Process D

This process is used to prepare fluid extracts in which the extracting liquid is boiling water, adding ethyl alcohol to the concentrated percolate as a preservative.

Procedure: weigh 1000 g of the coarsely pulverized drug and add about 3000 mL of boiling water, homogenize thoroughly and allow to macerate in a suitable container for two hours. Transfer to a percolator and elute at the specified speed, adding, little by little, boiling water until complete depletion of the drug. Evaporate the percolates, in water bath or in a vacuum distiller, to the determined volume. Wait for cooling, transfer the ethyl alcohol and allow the mixture to stand in a closed container for 24 hours. Decant the clear liquid, filter the remaining, mixing them and wash the filter residue with a sufficient amount of extracting liquid to obtain 1000 mL.

TESTS

Determination of mass density and relative density (5.2.5). Where applicable, the fluid extract must comply with prescribed limits.

Determination of alcohol (5.3.3.8). For ethylic fluid extracts, the determination of the ethyl alcohol content must be carried out. Where applicable, the ethyl alcohol must comply with prescribed limits.
Methyl alcohol and isopropyl alcohol (5.4.2.2.1). Maximum 0.05% (v/v) for ethylic fluid extracts, unless otherwise prescribed or justified and authorized.

Dry residue (5.4.2.2.2). Where applicable, the fluid extract must comply with prescribed limits.

STORAGE

Protect from light LABELING

Labels must contain, in addition to the requirements listed above, the percentage of ethyl alcohol content (v/v), when applicable.

5.4.2.1.2 TINCTURES

Tinctures are usually prepared by maceration or percolation, using ethyl alcohol at an appropriate concentration to extract the herbal drug, or by dissolving a soft or dry extract of the herbal drug (which have been produced using the same extraction solvent that would be used to prepare the tincture by direct extraction) in ethyl alcohol in the required concentration.

Tinctures can be adjusted to meet solvent content requirements. Tinctures can be filtered if necessary and light sediment can form when standing.

5.4.2.1.2.1 Production of tinctures by maceration

Process in which, unless otherwise indicated, the herbal drug is comminuted to an appropriate granulometry, mixed with the defined extraction solvent and allowed to stand in a closed container for an appropriate period of time, shaking when necessary. The frame is separated from the extractive solution and, if necessary, pressed. In this case, the liquid is added to the extractive solution and the volume/mass of the final product is adjusted.

5.4.2.1.2.2 Production of tinctures by percolation

Process in which, unless otherwise indicated, the herbal drug is comminuted to an appropriate granulometry and mixed with a portion of the defined extraction solvent and left to swell for an appropriate period of time. The mixture is transferred to a percolator and the extraction solvent is added until the herbal drug is completely covered with a layer of extraction solvent. The percolate is allowed to flow slowly, collected at the base of the percolator, while more extraction solvent is slowly added to the top of the percolator, ensuring that the herbal drug is constantly covered with extraction solvent, until all the solvent has been added. Percolation continues until the percolation is recovered. If the frame is pressed, the two liquids are combined.

TESTS

Determination of mass density and relative density (5.2.5). Where applicable, the tincture must comply with prescribed limits.

Determination of alcohol (5.3.3.8). The content must comply with the limits defined in the individual monographs.

Methyl alcohol and isopropyl alcohol (5.4.2.2.1). Maximum 0.05% (v/v) unless otherwise defined or justified and authorized.

Dry residue (5.4.2.2.2). Where applicable, the tincture must comply with prescribed limits.

STORAGE

Protect from light

LABELING

Labels must contain, in addition to the requirements listed above, the percentage of ethyl alcohol content (v/v).

5.4.2.1.3 SOFT EXTRACTS

TESTS

Dry residue (5.4.2.2.2). Where applicable, the soft extract must comply with prescribed limits.
Solvents. Solvent residues must be controlled unless defined or justified and authorized.

STORAGE

In hermetic container, protected from light.

5.4.2.1.4 OLEORESIN

The requirements below apply to oleoresins produced by extraction and non-natural oleoresins.

TESTS

Determination of water (5.2.20) Where applicable, oleoresin must comply with prescribed limits.

Solvents. Solvent residues must be controlled unless defined or justified and authorized.

STORAGE

In hermetic container, protected from light.

5.4.2.1.5 DRY EXTRACT

TESTS

Loss by desiccation (5.2.9.1). Where applicable, dry extract must comply with prescribed limits.

Determination of water (5.2.20) When the desiccation loss test is not applicable, the dry extract must comply with the defined limits.

Solvents. Solvent residues must be controlled unless defined or justified and authorized.

STORAGE

In hermetic container, protected from light.

5.4.2.2 METHODS OF VEGETABLE EXTRACTS ANALYSIS

5.4.2.2.1 DETERMINATION OF METHYL ALCOHOL AND ISOPROPYL ALCOHOL IN FLUID EXTRACTS

Distill the extract as described in *Determination of ethyl alcohol (5.3.3.8.1)*. Examine the distillate by *Gas Chromatography (5.2.17.5)*, using chromatograph equipped with a flame ionization detector, a 2 m long, 2 mm internal diameter glass chromatographic column, packed with ethylvinylbenzene / divinylbenzene copolymer, particles of 125 μm to 150 μm , and nitrogen for chromatography as carrier gas, with a flow rate of 30 mL/minute.

Keep the column temperature at 130°C, the injector temperature at 200°C and the detector temperature at 220°C.

Internal standard solution: 2.5% (v/v) 1-propanol solution in water.

Sample solution: add 2 mL of the *internal standard solution* to a determined volume of distillate. Dilute to 50 mL with water or 90% ethyl alcohol (v/v), adjusting the ethyl alcohol content to 10% (v/v).

Standard solution: prepare 50 mL of solution containing 2 mL of *Internal standard solution*, 10% ethyl alcohol (v/v), 0.05% isopropyl alcohol (v/v) and 0.05% anhydrous methyl alcohol (v/v).

Procedure: Inject, separately, 1 μL of the *Standard Solution* and the *Sample Solution*, record the chromatograms and measure the peak areas. Calculate the contents of methyl alcohol and isopropyl alcohol in relation to the sample subject to distillation from the responses obtained with the *Standard solution* and the *Sample solution*.

5.4.2.2.2 DETERMINATION OF DRY RESIDUE OF EXTRACTS

In a crystallizer, about 50 mm in diameter and 30 mm in height, quickly and accurately weigh 2 g or measure 2.0 mL of extract. Evaporate to dryness in water bath and dry in an oven between 100 °C and 105 °C for three hours. Wait for cooling in a desiccator in the presence of concentrated phosphorus pentoxide or concentrated anhydrous silica gel and weigh. Express the result in percentage m/m or in g/L.

5.4.2.2.3 LOSS DUE TO DESSICATION OF EXTRACTS

In a crystallizer, of about 50 mm in diameter and 30 mm in height, weigh 0,5 g of dry and pulverized extract. Dry in an oven between 100°C and 105°C for three hours. Wait for cooling in a desiccator in the presence of concentrated diphosphorus pentoxide, or concentrated anhydrous silica gel and weigh. Express the result in % w/w.

5.4.3 DETERMINATION OF PESTICIDES RESIDUES

For the purposes of Pharmacopoeia, a pesticide is any substance or mixture of substances intended to prevent; eliminate or control any pest; unwanted species of plants or animals that cause harm or interfere in any other manner with the production, preparation, storage, transport or sale of herbal drugs. The definition also comprises substances used as growth regulators, hormones, defoliants and desiccants, as well as any other substance applied to crops before or after harvesting/cropping to prevent deterioration during storage and transport. Agrochemical residues must be controlled in herbal drugs and their derivatives.

Limits

Unless indicated in the specific monograph, the herbal drug to be examined must minimally comply with the limits indicated in **Table 1**. The limit to be applied to pesticides not established in **Table 1** and whose presence is suspected for any reason must comply with the limits referenced in Brazilian legislation. Limits that are not listed in **Table 1** or indicated in Brazilian legislation must be calculated according to the following formulas.

$$LAR_{DV} = (IDA \times M) / (DDD_{DV} \times 100)$$

where

LAR_{DV} = Acceptable Limit of Pesticide Residues in mg/kg of the herbal drug;

IDA = Acceptable Daily Intake of pesticides in mg/kg of body weight, according to current legislation;

M = body weight in kg (taking 60 kg as a convention);

DDD = DDD = Daily Drug Dose in kg;

A factor of 100 corresponds to the participation of drug intake in the daily diet (1%). For special cases, this factor can be changed according to the population dietary habits that are properly documented.

The maximum limits for pesticide residues in plant derivatives are calculated by the following formula:

$$\text{If } RDE \leq 10 \quad LAR_D = LAR_{DV} \times RDE$$

$$\text{If } RDE > 10 \quad LAR_D = (IDA \times M) / (DDD_{DX} \times 100)$$

where

LAR_{DV} = Acceptable Limit of Pesticide Residues in mg/kg of the herbal drug shown in **Table 1**, in the current legislation or calculated according to the formula above;

LAR_D = Acceptable pesticide residue limit in mg/kg of the vegetable derivative preparation.

RDE = Drug Extract Ratio.

The analyses may be totally or partially waived by the competent authority according to manners of obtaining the raw material that indicate the absence of risk in relation to the presence of pesticide residues.

Table 1 – Acceptable limit of pesticide residues in mg/kg of the herbal drug.

<i>Substance</i>	<i>Limit (mg/kg)</i>
Acephate	0.1
Alachlor	0.05
Aldrin and dieldrin (sum of)	0.05
Azinphos - ethyl	0.1
Azinphos - methyl	1
Bromophos - ethyl	0.05
Bromophos - methyl	0.05
Bromopropylate	3
Chlordan (sum of cis-, trans - and oxychlordan)	0.05
Chlorfenvinphos	0.5
Chlorpyrifos-ethyl	0.2
Chlorpyrifos-methyl	0.1
Chlorthal - dimethyl	0.01
Cyfluthrin (sum of)	0.1
λ -Cyalothrin	1
Cypermethrin and isomers (sum of)	1
DDT (sum of <i>o,p'</i> -DDE, <i>p,p'</i> -DDE, <i>o,p'</i> -DDT, <i>p,p'</i> -DDT, <i>o,p'</i> -TDE and <i>p,p'</i> -TDE)	1
Deltamethrin	0.5
Diazinone	0.5
Dichlofuanide	0.1
Dichlorvos	1
Dicofol	0.5
Dimethoate and omethoate (sum of)	0.1
Dithiocarbamates (expressed as CS ₂)	2
Endosulfan (sum of isomers and endosulfan sulfate)	3
Endrin	0.05
Ethion	2
Etrinfos	0.05
Phenclorphos/ Phenclorphos-oxone (sum of Phenclorphos and Phenclorphos-oxone)	0.1
Fenitrothion	0.5
Fenpropathrin	0.03
Fensulfothione (sum of fensulfothione, fensulfothione-oxone, fensulfothione-oxone and fensulfothione-sulfone)	0.05
Phenthione (sum of phenthione, phenthione-oxone, phenthione-oxone-sulfoxide, phenthione-oxone-sulfoxide, phenthion-sulfone and phenthione-sulfoxide)	0.05
Fenvalerate	1.5
Flucitrate	0.05
τ -Fluvalinate	0.05
Phonophos	0.05
Heptachlor (sum of heptachlor, cis – heptachlorepoxyde and trans – heptachlorepoxyde)	0.05
Hexachlorobenzene	0.1
Hexachlorocyclohexane (sum of α -, β -, δ - and ϵ isomers)	0.3
Lindan (γ – hexachlorocyclohexane)	0.6
Malathion and Malaoxon (sum of)	1

<i>Substance</i>	<i>Limit (mg/kg)</i>
Mecarban	0.05
Metacryphos	0.05
Methamidophos	0.05
Methadione	0.2
Methoxychlor	0.05
Mirex	0.01
Monocrotophos	0.1
Parathion - ethyl and Paraoxon - ethyl (sum of)	0.5
Parathion - methyl and Paraoxon - methyl (sum of)	0.2
Pendimethalin	0.1
Pentachloroanisole	0.01
Permethrin and isomers (sum of)	1
Phosalone	0.1
Phosmete	0.05
Piperonyl butoxide	3
Pyrimiphos – ethyl	0.05
Pyrimphos - methyl (sum of pyrimiphos - methyl and N - disethyl pyrimiphos - methyl)	4
Procymidone	0.1
Profenophos	0.1
Prothiophos	0.05
Pyrethrum (sum of cinerin I, cinerin II, jasmolin I, jasmolin II, pyrethrin I and pyrethrin II)	3
Quinalphos	0.05
Quintozene (sum of quintazene, pentachloroaniline and pentachlorophenyl methyl sulfide)	1
S-421	0.02
Tecnazene	0.05
Tetradiphone	0.3
Vinclozoline	0.4

Sampling

It must be performed in compliance with the criteria established in *Sampling (5.4.1.1)*.

Qualitative and quantitative analysis of pesticide residues

The analytical procedures used must be validated in compliance with the SANCO document in its most updated version "*Guidance Document on analytical quality control and validation procedures for pesticide residues in food and feed*" and meet at least the following criteria.

The chosen extraction method must be suitable for the mixture of pesticides to be investigated and not cause interference.

Possible matrix interferences, for example, interferences of sulfur compounds in brassicaceae and alliaceous, in the determination of dithiocarbamates such as CS₂ should be considered. Reference solutions and sample solutions must be within the linearity range of the detector.

Detection and quantification limits must be determined for each combination of pesticides and matrices to be analyzed.

Recovery should be between 70 and 110%.

The repeatability and reproducibility of the method must not be inferior to that indicated in **Table 2**.

Table 2 – Repeatability and reproducibility.

<i>Pesticide concentration (mg/kg)</i>	<i>Repeatability (±mg/kg)</i>	<i>Reproducibility (±mg/kg)</i>
0.01	0.005	0.01
0.1	0.025	0.05
1	0.125	0.25

5.4.4 DETERMINATION OF AFLATOXINS

Aflatoxins are very toxic and must be handled with extreme care. Aflatoxins are photosensitive, carry out the assays, protecting standard solutions and samples from the incidence of light.

METHOD I

Proceed as described in *Thin-layer chromatography (5.2.17.1)*. The determination of aflatoxin by the *Thin Layer Chromatography* method allows for detecting the possible presence of aflatoxins in Herbal drugs and derivatives, whenever no other specific method is recommended in the monograph. *Stationary phase*: silica gel (0.25 mm).

Mobile phase: chloroform and acetone (9:1).

TLC visualization reagent solution: examine under ultraviolet light at 360-365 nm; 30% (v/v) sulfuric acid solution.

Phosphate buffer solution pH 7.4: weigh 1.0 g of potassium chloride, 1.0 g of monobasic potassium phosphate, 5.8 g of dibasic sodium phosphate anhydrous and 40.0 g of sodium chloride and transfer to a 5 L volumetric flask. Add approximately 4.5 L of water and dissolve. Adjust the pH to 7.4 with hydrochloric acid or sodium hydroxide. Adjust volume with water, homogenize and check pH again.

Aflatoxin Stock Solution: Dissolve the contents of the aflatoxin standard in a mixture of benzene and acetonitrile (98:2). Dilute quantitatively and in steps with the same solvent until obtaining solutions with a concentration of 8 µg/mL to 10 µg/mL of each aflatoxin. Shake the solution vigorously for one minute. Determine the absorbance of each solution at 350 nm in an appropriate spectrophotometer, using a mixture of benzene and acetonitrile (98:02) as blank. Calculate the concentration of the respective aflatoxin, in mg/mL, using the following formula:

$$C = \frac{1000 \times A \times M}{\varepsilon}$$

M = molecular mass;

ε = molar absorptivity in the indicated solvent of the corresponding aflatoxin; and

A = solution absorbance

These values are shown in **Table 1**.

Table 1 – Correlation of molar absorptivity, molar mass and type of aflatoxin.

<i>Aflatoxin</i>	<i>M (molecular mass)</i>	<i>ε = (molar absorptivity)</i>
B1	312	19 800
B2	314	20 900
G1	328	17 100
G2	330	18 200

Standard solution: transfer aliquots of each of the previously prepared aflatoxin stock solutions to 3 mL vials with caps. Add sufficient amounts of benzene and acetonitrile (98:02) to obtain solutions with 1 µg/mL of B1, 0.5 µg/mL of B2, 1 µg/mL of G1 and 0.5 µg/mL of G2.

Sample preparation

Column: Immunoaffinity chromatographic column (IAC) with monoclonal antibodies specific for aflatoxins.

Extraction solvent: dissolve 5 g of sodium chloride in 200 mL of methyl alcohol and water (70:30)

Procedure: accurately weigh 25 g of sample, previously ground and sieved with sieve nr 20, transfer to a 500 mL Erlenmeyer flask. Add enough extraction solvent to soak the entire sample. Shake in a mechanical shaker for one hour or in a blender at high speed for five minutes. Filter and collect the filtrate in a 250 mL Erlenmeyer flask. Transfer accurately measured 80 mL of the extract to a 250 mL Erlenmeyer flask, add 160 mL of pH 7.4 phosphate buffer solution. Shake and filter through a membrane filter with a porosity between 0.8 –1.6 µm. Apply 120 mL of the filtrate (equivalent to 5 g of sample) on the immunoaffinity chromatographic column, maintaining a flow rate of one to two drops per second, taking care that the column does not dry out. Wash the column with 20 mL of buffer solution and dry by passing air through the column with the aid of a syringe. Discard the washing liquid. Slowly elute the aflatoxins adsorbed on the column, by gravity, with 2 mL of methyl alcohol. Collect the entire eluate in a 25 mL flask with a small container at the bottom, accurately pre-weighed. Dry using rotary evaporator at 60°C. Weigh to obtain the mass of the residue. Dissolve the residue in 100 µL of a mixture of benzene and acetonitrile (98:02).

Fortified solution with standard solution: homogenize 10 µL of *Sample solution* with 4 µL of *Standard solution*.

Aflatoxin analysis

Procedure: apply, separately, 10 µL of the *Sample Solution*, 2 µL, 4 µL and 6 µL of the *Standard Solution* and 10 µL of the *Solution fortified with standard solution*. Allow to dry and develop the chromatograms until the solvent front runs approximately 11 cm. Remove the plate and air dry, protected from light. Examine under ultraviolet light (360 nm). Aflatoxins B1 and B2 appear as blue stains and G1 and G2 as green stains. Rf values are approximately: 0.4 for G2, 0.5 for G1, 0.6 for B2 and 0.7 for B1, respectively. To confirm, pulverize the plate with the *TLC visualization reagent solution*. Allow to dry in the dark and observe under 360 nm ultraviolet light: the four aflatoxins are seen as yellow stains. Calculate the concentration of each aflatoxin, in µg/kg, in the portion of the sample taken, using the formula:

$$C = \frac{P \times C \times V}{S \times m}$$

P = volume, in µL, of the *Standard solution*;

C = aflatoxin concentration in the *Standard Solution* (G1 and B1 1 µg/mL and G2 and B2 0.5 µg/mL);

V = volume, in µL, of the final residue solution;

S = volume of *Sample solution*; and

m = weight of residue in g.

Acceptance criteria

Absence of stains in test solution applications in areas where stains of the *Standard Solution* are observed. If any stains are observed in the *Sample Solution*, check the correspondence with any fluorescent stains in the *Standard Solution* to identify this aflatoxin. The intensity of the aflatoxin stain, if present in the *Sample Solution*, when compared to the intensity of the corresponding aflatoxin stain in the standard solutions, will provide the approximate concentration of the aflatoxins in the *Sample Solution*. The maximum accepted limits are lower than 5 µg/kg for aflatoxin B1 and lower

than 20 µg/kg for the sum of aflatoxins B1, B2, G1 and G2, except when other values are recommended in specific monograph.

METHOD II

Determination of aflatoxins by *High Performance Liquid Chromatography (5.2.17.4)* with fluorescence detection, recommended for herbal drugs and derivatives of ginseng, ginger, cat's claw and senna fruit, with the exception that for other matrices the method should be tested, eventually adapted and validated.

Aflatoxin Primary Stock Solution: Dissolve aflatoxin B1 in a mixture of acetonitrile and toluene (2:98) to obtain a solution at 10 µg/mL. To determine the exact concentration of aflatoxin B1 in the stock solution, record the absorption curve between 330-370 nm in a quartz cell. Calculate the concentration of B1 aflatoxin, in µg/mL, using the following formula:

$$C = \frac{A \times M \times 100}{\varepsilon \times l}$$

where

A = absorbance determined at the maximum of the absorption curve;

M = molar mass of aflatoxin B1 (312 g/mol);

ε = molar absorptivity of aflatoxin B1 in the mixture of acetonitrile and toluene (1930 m²/mole); and

l = length of the optical path in the cell (1 cm).

Aflatoxin B1 secondary stock solution: prepare secondary solution containing 100 ng/mL of aflatoxin B1 by diluting the primary stock solution with a mixture of acetonitrile and toluene (2:98). Wrap the solution flask in aluminum foil and wait for the contents to reach room temperature. If the solution is to be stored for a long period (e.g. one month), weigh the flask and record the mass before each use of the solution.

Standard Aflatoxin Solutions: Transfer the volumes of secondary aflatoxin stock solution indicated in **Table 2** to volumetric flasks. Apply nitrogen gas to these solutions, at room temperature, until solvent evaporation. In each flask, add 75 mL of methyl alcohol, wait for complete solubilization and adjust to 250 mL with water and homogenize.

Table 2 – Aflatoxin B1 solutions for preparing analytical curve

<i>Standard solution</i>	<i>Secondary stock solution volume (µL)</i>	<i>Final concentration of AFB1 in standard solution (µL/mL)</i>
1	125	0.05
2	250	0.1
3	500	0.2
4	750	0.3
5	1000	0.4

Analytical curve: prepare the analytical curve using standard solutions of aflatoxin B1, from one to five, in a concentration range of 1 µg/kg to 8 µg/kg of aflatoxin B1 in the herbal drug. Check if the curve has adequate linearity. If the aflatoxin B1 content in the sample to be examined is outside the range of the analytical curve, the test solution must be diluted until the aflatoxin content is adequate for the established calibration curve.

Column: 250 mm in length and 4.6 mm in internal diameter, packed with silica chemically bonded to octadecylsilane group (5 µm)

Mobile phase A: mixture of acetonitrile, methyl alcohol and water (2:3:6) for post-column derivation with photochemical reactor or pyridinium bromide.

Mobile Phase B: add 0.12 g of potassium bromide and 350 mL of diluted nitric acid per liter of *Mobile Phase A* for post-column derivation with electrochemically derived bromide.

Mobile Phase Flow: 1.0 mL/minute.

Detection: fluorescence detector (excitation filter at 360nm and emission filter at 420nm). If using detector with adjustable wavelengths, use 365 nm for excitation and 435 nm for emission.

Post-column derivation with pyridinium bromide-perbromide (PBPB): use pulseless pump; T with dead volume equal to zero; Teflon reaction tube (PTFE) of 0.45 m length and internal diameter = 0.5 mm; *Mobile phase A; Post-derivation reagent:* dissolve 50 mg of PBPB in 1000 mL of water (protect from light and use within four days) and flow of derivation reagent: 0.4 mL/minute.

Post-column shunt with photochemical reactor (PHRED): use a low pressure mercury bulb reactor (minimum 8 W) at 254 nm; polished backplate; reaction coil with PTFE tube tightly braided around UV bulb, length 25 cm and diameter 0.25 mm, nominal dead volume 1.25 mL; two-minute exposure time; and *Mobile Phase A*.

Post-column derivation with electrochemically generated bromine (KOBRA): use KOBRA-cell: electrochemical cell that generates a reactive form of bromine for aflatoxin derivation, resulting in increased fluorescence; direct current source in series with KOBRA-cell, with constant current of about 100 µA; PTFE reaction tube of 0.12 cm length and 0.25 mm inside diameter; and *Mobile Phase B*.

Immunoaffinity Column (IAC): use an immunoaffinity column containing antibodies against aflatoxin B1, with a capacity of not less than 100 ng of aflatoxin B1 and with not less than 80% recovery for a solution of 5 ng of aflatoxin B1 in one mixture of methyl alcohol and water (12.5:87.5) is applied. Condition IAC at room temperature.

Procedure: weigh 5 g of dried and pulverized plant material, transfer and add 100 mL of water and methyl alcohol mixture (30:70) and extract by sonication for 30 minutes. Filter on pleated filter paper. Exactly transfer 10 mL of the clear filtrate to a 150 mL Erlenmeyer flask and add 70 mL of water. Pass 40 mL through the immunoaffinity column (IAC) at a flow rate of 3 mL/minute (do not exceed 5 mL/minute). Wash the column with two 10 mL volumes of water at a flow rate not to exceed 5 mL/minute. Dry the IAC using a light vacuum for 5 to 10 seconds or air using a syringe for 10 seconds. Apply 0.5 mL of methyl alcohol to the top of the column and allow to elute by gravity into a 5 mL volumetric flask. After one minute, apply another 0.5 mL of methyl alcohol. After one minute, apply a third portion of methyl alcohol. Collect most of the applied solvent by blasting compressed air across the top of the column or using light vacuum. Dilute with water to 5 mL and shake. If the solution is clear, it can be used directly. Otherwise, filter in a filter unit before analysis. Use a disposable filter unit (e.g. polytetrafluoroethylene filter with 0.45 µm pores) that does not cause aflatoxin loss by retention.

Injection volume: 500 µL.

Elution order: aflatoxin G2, G1, B2 and B1.

Calculation: Determine the formula of the analytical curve ($y = ax + b$) with the aflatoxin B1 concentration (ng/mL) on the x-axis and the sign (S) on the y-axis. The concentration of aflatoxin B1 in the test solution is equal to $C \frac{s-b}{a}$.

Calculate the content of aflatoxin B1 in the herbal drug, in ng/g, using the following expression:

$$C = \frac{V_1 \times V_2 \times C}{m \times V_i}$$

where

m = mass of herbal drug, in g;

V_1 = volume of solvent used in extraction, in mL;

V_i = aliquot used in the IAC, in mL;

V_2 = final volume of solution after IAC elution and dilution, in mL; and

C = concentration of aflatoxin B1 in the test solution in ng/mL.

The presence of aflatoxin B1 can be confirmed by recording the chromatogram without post-column derivation, which results in a large reduction (greater than 10-fold) in the response due to aflatoxin B1.

5.4.5 DETERMINATION OF HEAVY METALS IN HERBAL DRUGS

Proceed according to the *Limit Test for Heavy Metals* (5.3.2.3).

When not determined in the monograph, the maximum total value of heavy metals of 20 ppm must be observed, not exceeding the limits specified in **Table 1**.

Table 1 – Limit for heavy metals.

<i>Metals</i>	<i>Limit</i>
cadmium	1.0 ppm
<i>lead</i>	5.0 ppm
mercury	0.1 ppm
arsenic	5.0 ppm

5.5 BIOLOGICAL METHODS, BIOLOGICAL AND MICROBIOLOGICAL ASSAYS

5.5.1 BIOLOGICAL METHODS

5.5.1.1 DETERMINATION OF HEPARIN IN COAGULATION FACTORS

Heparin is determined in the form of a complex to antithrombin III (AT) via inhibition of the activity of Factor Xa of coagulation. An excess of AT is maintained in the reaction mixture to ensure a constant concentration of the heparin-AT complex. Factor Xa is neutralized by the heparin-AT complex and residual Factor Xa hydrolyzes a specific peptide chromogenic substrate releasing a chromophore. The amount of chromophore is inversely proportional to the activity of heparin.

Factor Xa chromogenic substrate: Factor Xa specific chromogenic substrate such as: N- α -benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitro-anilide hydrochloride. Reconstitute according to manufacturer's instructions.

Dilution buffer: 0.605% (w/v) tromethamine solution. If necessary, adjust to pH 8.4 with hydrochloric acid.

Sample solution: dilute the sample with the *Dilution Buffer* to obtain a solution that supposedly contains 0.1 IU of heparin per milliliter.

Standard solution: dilute the standard heparin solution with the *Dilution Buffer* to obtain a solution containing 0.1 IU of heparin per milliliter. The conditions described apply to microplates. If the test is carried out in tubes, adjust the volumes in order to maintain the proportions in the mixture. Shortly before testing, place all solutions at 37°C in water bath. Distribute 20 μ L of normal human plasma and 20 μ L of antithrombin III RS into a series of wells. Add a series of volumes (20 μ L, 60 μ L, 100 μ L and 140 μ L) of the *Sample Solution* or *Standard Solution* to the wells and complete the volume of each well with 200 μ L using the Dilution Buffer (0.02 – 0.08 IU of heparin per milliliter in the final reactive mixture).

EQUIVALENCE POINT METHOD

Transfer 40 μ L from each well to a second series of wells, add 20 μ L of the bovine Factor Xa solution and incubate at 37°C for 30 seconds. Add 40 μ L of *Chromogenic Substrate for Factor Xa solution* at 1 mmole/L and incubate at 37°C for three minutes. Stop the reaction by lowering the pH with an appropriate reagent such as a 20% (v/v) glacial acetic acid solution and measuring the absorbance at 405 nm (5.2.14). The reaction time is generally on the order of three minutes to 15 minutes, but certain variations are tolerated if they allow to improve the linearity of the dose-response curve.

KINETIC METHOD

Transfer 40 μ L from each well to a second series of wells, add 20 μ L of the bovine Factor Xa solution and incubate at 37°C for 30 seconds. Add 40 μ L of the *Chromogenic Substrate for Factor Xa solution* at 2 mmole/L, incubate at 37 °C and determine the substrate cleavage rate by continuously reading the absorbance variation at 405 nm (5.2.14), thus enabling calculate the initial rate of substrate cleavage. This velocity must be proportional to the residual concentration of Factor Xa. Check the validity of the test and calculate the activity of the sample through the statistical procedures applicable to biological tests (8).

5.5.1.2 DETERMINATION OF THE HUMAN VON WILLEBRAND FACTOR

The potency of the human Von Willebrand Factor is determined by comparing, under mandatory conditions given, its activity in collagen or as a ristocetin cofactor with the same activity, and calibrated using an international reference standard, in international units, when applicable. The International Unit is the activity of a declared amount of the international reference standard for Von Willebrand Factor existing in Factor VIII concentrate of human blood coagulation. The equivalence in international units from the international reference standard is indicated by the World Health Organization (WHO).

COLLAGEN BINDING ASSAY

Collagen binding is determined by enzyme immunoassay method in collagen-coated microplates. The method is based on the specific binding of Von Willebrand Factor to collagen fibers and the subsequent binding of an anti-Von Willebrand Factor polyclonal antibody conjugated to an enzyme. After the addition of a chromogenic substrate, a spectrophotometrically quantifiable product is formed. Under appropriate conditions, there is a linear relation between collagen, Von Willebrand Factor and the indicated absorbance.

MATERIALS

Collagen: use native equine or human collagen fibrils type I or III. To facilitate handling, collagen solutions can be used.

Collagen diluent: weigh 50 g of glucose and dissolve in water. Adjust the pH to between 2.7 and 2.9 with *M* hydrochloric acid and dilute to 1000 mL with water.

Chloride-phosphate buffer: Chloride-phosphate buffer: weigh 8g of sodium chloride, 1.05g of dibasic sodium phosphate dihydrate, 0.2g of monobasic sodium phosphate dihydrate and 0.2 g of potassium chloride and dissolve in water. If necessary, adjust pH to 7.2 with *M* sodium hydroxide or *M* hydrochloric acid. Dilute to 1000 mL with water.

Buffered Wash Solution: 0.1% (w/v) polysorbate 20 solution in *Phosphate Chloride Buffer*.

Neutralizing reagent: prepare *Chloride-phosphate buffer* containing 0.1% (w/v) polysorbate 20 and 1.0% (w/v) bovine albumin.

Dilution buffer: prepare *Chloride-phosphate buffer* containing 0.1% (w/v) polysorbate 20 and 5.0% (w/v) bovine albumin.

Conjugation: rabbit serum of human anti-Von Willebrand Factor conjugated to horseradish peroxidase, a histochemical marker, prepared as per the manufacturer's instructions.

Substrate solution: dissolve, immediately before use, one o-phenylenediamine hydrochloride tablet and one carbamide peroxide tablet in 20 mL of water, or use an adequate volume of hydrogen peroxide. Protect from light .

Microplates: they must have a flat bottom, polystyrene plates with optimized surface properties for immunoenzymatic assay and high binding capacity protein.

PROCEDURE

Sample solution: reconstitute the preparation to be analyzed as indicated on the label. Dilute with *Dilution Buffer* to prepare a solution containing about 1 IU/mL of Von Willebrand Factor. Prepare two independent runs with minimum three dilutions using the *Dilution Buffer*.

Standard solutions: reconstitute the standard solution as indicated by the manufacturer. Dilute with *Dilution Buffer* to prepare a solution containing about 1 IU/mL of Von Willebrand Factor. Prepare two independent runs with minimum three dilutions using the *Dilution Buffer*.

Dilute with *Collagen Diluent*, at room temperature, to obtain a solution containing 30 to 75 mg/mL of collagen. Gently homogenize to produce a uniform suspension of collagen fibers and then pipet 0.1 mL and transfer to each well of the microplate. Cover plate with plastic wrap and incubate at 37°C overnight. Empty collagen-coated plate wells by inverting and draining on a paper towel. Add 0.25 mL of *Buffered Wash Solution*. Empty plate wells by inversion and drain on a paper towel, repeating this operation three times. Add 0.25 mL of *Neutralizing Reagent* to each well, cover the plate with plastic film and incubate at 37°C for one hour. Plate wells should be emptied by inversion and draining on a paper towel. Add 0.25 mL of *Buffered Wash Solution*. Empty plate wells by inversion and drain on a paper towel. Repeat this operation three times.

Add 0.1 mL of each of the *Sample* or *Reference Solutions* to the wells. Add 0.1 mL of *Dilution Buffer* to a series of wells to obtain the negative control. Cover plate with plastic wrap and incubate at 37°C for two hours. Plate wells should be emptied by inversion and draining on a paper towel. Add 0.25 mL of *Buffered Wash Solution*. Empty plate wells by inversion and drain on a paper towel, repeating this operation three times.

Prepare an appropriate dilution of *Phosphate Chloride Conjugation Buffer* containing 0.5% (w/v) bovine albumin and add 0.1 mL to each well. Cover plate with plastic film and incubate at 37°C for two hours. Empty plate wells by inversion and drain on a paper towel. Add 0.25 mL of *Buffered Wash Solution*. Empty plate wells by inversion and drain on a paper towel. Repeat this operation three times.

Add 0.1 mL of *Substrate Solution* to each well and incubate at room temperature for 20 minutes in the dark. Add 0.1 mL of *M* hydrochloric acid to each well. Measure the absorbance at 492 nm (5.2.14). Use the absorbance values to estimate the potency of the preparation to be analyzed using the statistical procedures applicable to biological tests. The assay is valid if the absorbances measured for the negative controls are greater than 0.05.

5.5.1.3 DETERMINATION OF HUMAN BLOOD COAGULATION FACTOR II

The determination of Factor II of human coagulation is performed after specific activation in Factor IIa. Factor IIa is calculated by comparing its activity on a specific chromogenic peptide substrate with the same activity as the international standard or a standard preparation calibrated in International Units (IU). The International Unit of Factor II corresponds to the activity of a given quantity of the international standard, which is constituted by a lyophilized concentrate of Factor II of blood coagulation. The correspondence between the International Unit and the International Standard is indicated by the World Health Organization.

The chromogenic determination method includes two successive steps: activation of Factor II by the action of snake venom and the enzymatic cleavage of a chromogenic substrate by Factor IIa, which

releases a spectrophotometrically quantifiable chromophore. Under appropriate assay conditions, there is a linear relation between Factor IIa activity and chromogenic substrate cleavage.

REAGENTS

Factor II specific activator from viper venom (Ecarina): protein obtained from the venom of the *Echis carinatus* viper, specifically activates Factor II. Reconstitute the preparation following the manufacturer's instructions. Once reconstituted, store at 4°C and for not more than one month.

Chromogenic substrate for Factor IIa: Factor IIa specific chromogenic substrate such as: HD-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide hydrochloride, 4-toluenesulfonyl-glycyl-prolyl-L-arginine-4-nitroanilide, HD-cyclohexylglycyl - α -aminobutyryl-L-arginine-4-nitroanilide, D-cyclohexylglycyl-L-alanylarginine-4-nitroanilide-diacetate. Reconstitute following the manufacturer's instructions.

Dilution buffer: solution containing 0.606% (w/v) of tromethamine, 1.753% (w/v) sodium chloride, 0.279% (w/v) edetic acid and 0.1% bovine or human albumin (p/v). Adjust pH to 8.4 with hydrochloric acid.

PROCEDURE

Sample solution: dilute the sample in the *Dilution Buffer* to obtain a solution containing 0.015 IU of Factor II per milliliter. Prepare at least three more dilutions of this solution in *Dilution Buffer*.

Standard solution: dilute the sample in the *Dilution Buffer* to obtain a solution containing 0.015 IU of Factor II per milliliter. Prepare at least three more dilutions of this solution in the *Dilution Buffer*. Place the solutions contained in the microplates in water bath at 37°C, immediately before the assay. If the test is carried out in tubes, adjust the volumes to maintain the proportions in the mixture. Introduce 25 μ L of the different dilutions of *Sample Solution* and *Standard Solution* into a series of wells of the microplate kept at 37 °C. Transfer 125 μ L of *Dilution Buffer* and 25 μ L of *Factor II Specific Activator from viper venom* into each well and incubate for exactly two minutes. Transfer 25 μ L of *Chromogenic Substrate for Factor IIa* to each well.

Read the absorbance change rate at 405 nm (5.2.14 and continue for three minutes to obtain the average rate of absorbance change. If a continuous reading is not possible, determine the absorbance at 405 nm at appropriate standardized consecutive ranges, e.g. every 40 seconds. Plot the linear graph of the absorption values versus time and calculate the average rate of change in absorbance. From the individual values found for each standard and sample dilution, calculate the sample activity and check the validity of the assay using the usual statistical methods.

5.5.1.4 DETERMINATION OF HUMAN BLOOD COAGULATION FACTOR IX

Sample activity is determined by comparing the amount of sample required to reduce the clotting time of a test mixture that contains substances, in addition to Factor IX, necessary for blood clotting; with the amount of a standard solution, evaluated in international units, required to obtain the same effect.

The International Unit of Factor II corresponds to the activity of a given quantity of the international standard, constituted by a lyophilized concentrate of Factor IX of blood coagulation. The equivalence

in international units from the International Reference Standard is indicated by the World Health Organization.

Reconstitute the sample and standard solution respectively according to the label directions and use immediately. When applicable, determine the amount of heparin present and neutralize it by adding protamine sulfate (10 µg of protamine sulfate neutralizes 1.0 IU of heparin). Dilute the sample and standard solution with pH 7.3 imidazole buffer to obtain solutions with 0.5 to 2.0 IU per milliliter. With a mixture of 3.8% (w/v) sodium citrate and pH 7.3 (1:5) imidazole buffer, prepare a dilution series comprising 1/10, 1/20, 1/40 and 1/80. These dilutions must be accurately prepared and are used immediately.

For example, use incubation tubes kept in water bath at 37°C. Introduce 0.1 ml of plasma substrate and 0.1 ml of each of the standard and sample dilutions into each tube. Transfer to each tube 0.1 mL of an appropriate dilution of cephalin RS or platelet substitute and 0.1 mL of a suspension of 0.5 g of light kaolin in 100 mL of 0.9% sodium chloride (w/v) and allow to stand for about 10 minutes, tilting the tubes regularly. Add 0.1 mL of 0.74% (w/v) calcium chloride solution to each tube. With the aid of a chronometer, determine the clotting time, that is, the time range between the moment of addition of calcium chloride and the first indication of the formation of fibrin that is observed visually or with appropriate devices. Calculate the activity using the statistical procedure applicable to biological assays.

To ensure that there is no significant contamination of the plasma substrate by Factor IX, perform a blank assay using, in place of the sample, a corresponding volume of a mixture of 3.8% (w/v) sodium citrate and imidazole buffer of pH 7.3 (1:5). The test is only valid if the clotting time determined in the blank test is between 100 and 200 seconds.

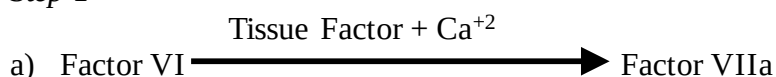
5.5.1.5 DETERMINATION OF HUMAN BLOOD COAGULATION FACTOR VII

The determination of Factor VII of coagulation is carried out by determining its biological activity as a cofactor in the activation of Factor X by Factor VIIa/Tissue Factor in the presence of calcium ions and phospholipids. The activity of a Factor VII preparation is calculated by comparing the respective amounts of that preparation and the international standard or a standard solution determined in international units that are necessary to obtain a rate of formation of Factor Xa in a reaction medium containing the different substances that intervene in the activation of the X Factor.

The International Unit of Factor VII corresponds to the activity of a given quantity of the international standard, which is currently constituted by a lyophilized plasma. The correspondence between the International Unit and the International Standard is indicated by the World Health Organization.

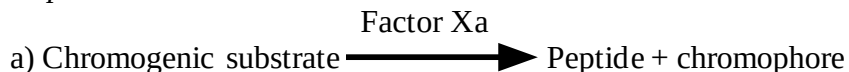
The chromogenic determination method comprises two successive steps: the activation of Factor X, under the action of Factor VIIa, in a reactive mixture containing Tissue Factor/phospholipid and calcium ion and the enzymatic lysis of a chromogenic substrate by Factor Xa that releases a chromophore quantifiable by spectrophotometry. Under proper assay conditions, there is a linear relation between the rate of formation of Factor Xa and the concentration of Factor VII. The following diagram summarizes the principle of determination:

Step 1





Step 2



Both steps use commercially available reagents from different suppliers. Although the composition of these reagents may vary slightly, their essential characteristics are described in the following specifications.

REAGENTS

The reactive mixture of coagulation factors contains, in particular, purified proteins of human or bovine origin, specifically Factor X, thromboplastin and Tissue Factor/phospholipid and a Factor VII activator. These proteins are partially purified and do not contain impurities capable of interfering with the activation of Factor VII or Factor X. Factor X is present in such quantity that its final concentration, outside the activation step, is 10 – 350 nmole/L, preferably 14 – 70 nmole/L. The thromboplastin used can be of natural origin (ox or rabbit brain) or synthetic. The thromboplastin used for the determination of Quick's time is diluted 5 to 50 times in a buffer solution so that the final concentration of Ca^{2+} is 15-25 nmole/L. The final stage of Factor Xa formation is carried out in a solution containing human albumin or bovine albumin at a concentration in which there is no loss in adsorption and, conveniently, buffered at a pH between 7.3 and 8.0. Factor VII is the only Factor that limits the formation of Factor Xa in the final incubation mixture and none of the reactive constituents in the mixture have the power to induce the formation of Factor Xa on their own.

The second step consists of quantifying the Factor Xa formed in the previous step, in a chromogenic substrate specific for Factor Xa. This substrate is usually a short peptide derived from 3 to 5 amino acids linked to a chromophore group. The sectioning of this group and the peptide substrate promotes a shift of the chromophoric activity to a wavelength that allows its quantification by spectrophotometry. The substrate is usually dissolved in water and used at a final concentration of 0.2 – 2 nmole/L. It may also comprise appropriate inhibitors preventing the further formation of Factor Xa (iodide addition).

PROCEDURE

Separately, reconstitute the contents of one ampoule of the standard solution and the sample by adding the required amount of water, and once reconstituted, use them within one hour at most. Add to the reconstituted preparations the amounts of pre-diluent necessary to obtain solutions of 0.5 – 2.0 IU of Factor VII per milliliter.

Prepare the following dilutions of standard solution and sample with an isotonic buffer solution without chelating agent, containing 1.0% (w/v) human or bovine albumin, and preferably buffered to pH 7.3 – 8.0. Make from each of the two preparations at least three separate, independent dilutions, preferably in duplicate. The concentrations of these Factor VII dilutions are adjusted so that the final concentration is less than 0.005 IU/mL.

Also prepare a control solution containing all the constituent kit of the reactive mixture except for Factor VII.

All dilutions are prepared in plastic tubes and used in no more than one hour.

Step 1. To each of the dilutions, obtained from the standard and sample solution, add an appropriate volume of pre-heated coagulation reagent (or a mixture of its separate constituents), homogenize and incubate at 37°C in plastic tubes or wells of a microplate. The concentration of the different constituents during the formation of Factor Xa is as specified in reagents. Allow the Factor X activation reaction to develop for an appropriate time; the end of the reaction takes place, preferably, before the Factor Xa concentration has reached its maximum level, so that the dose-response curve presents a satisfactory linearity. The reaction time is also chosen so that the linearity condition of the Factor Xa production curve as a function of time is satisfactory. It is usually on the order of two to five minutes, but certain variations are permitted to improve the linearity of the dose-response curve.

Step 2. Stop the activation reaction by adding a reactive mixture containing the chromogenic substrate. The substrate lysis rate, which is proportional to the Factor Xa concentration, is determined with the aid of a spectrophotometer by varying the absorbance at an appropriate wavelength. The absorbance can be determined continuously, which makes it possible to calculate the initial rate of substrate lysis, either by stopping the hydrolysis reaction after an appropriate time, lowering the pH with an appropriate reagent such as 50% acetic acid (w/v) or by a solution of *M* sodium citrate at pH 3.0. Adjust the hydrolysis time so that the linearity condition for chromophore formation as a function of time is satisfactory. It is usually on the order of 3 to 15 minutes, but certain variations are permitted to improve the linearity of the dose-response curve. Check the validity of the titration and calculate the activity of the sample preparation through the statistical procedures applicable to biological tests (8).

5.5.1.6 DETERMINATION OF HUMAN BLOOD COAGULATION FACTOR X

The determination of Factor X in human blood coagulation is carried out after specific activation in Factor Xa, which is calculated by comparing its activity in cleaving a specific peptide chromogenic substrate with the same activity as the International Standard or a standard solution measured in International Units.

The International Unit of Factor X corresponds to the activity of a given quantity of the international standard, which is constituted by a lyophilized concentrate of Factor X of blood coagulation. The correspondence between the International Unit and the International Standard is indicated by the World Health Organization.

The chromogenic determination method includes two steps: activation of Factor X by the action of snake venom and the enzymatic cleavage of a chromogenic substrate by Factor Xa, which releases a spectrophotometrically quantifiable chromophore. Under appropriate assay conditions, there is a linear relation between Factor Xa activity and the substrate cleavage.

REAGENTS

Specific X-Factor activator from the Russel's viper venom (RVV): a protein obtained from the venom of the Russel's viper (*Vipera russelli*) that specifically activates the X-Factor.

Reconstitute the preparation following the manufacturer's instructions. Once reconstituted, store at 4°C and use within one month.

Chromogenic substrate for Factor Xa: Factor Xa specific chromogenic substrate such as: N- α -benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide, Nbenzoyl-L-isoleucyl-L-glutamyl-hydrochloride glycyl-L-arginine-4-nitroanilide, methanesulfonyl-D-leucyl-glycyl-L-arginine-4-nitroanilide, methoxycarbonyl acetate-D-cyclohexylalanyl-glycyl-L-arginine-4-nitroanilide. Reconstitute following the manufacturer's instructions.

Dilution buffer: solution containing 0.37% (w/v) tromethamine, 1.8% (w/v) sodium chloride, 0.21% (w/v) imidazole, 0.002% hexadimethrine bromide (w/v) and bovine albumin, or 0.1% human albumin (w/v). If necessary, adjust to pH 8.4 with hydrochloric acid.

PROCEDURE

Sample solution: dilute the sample in the *Dilution Buffer* to obtain a solution containing 0.18 IU of Factor X per milliliter. Prepare at least three more dilutions of this solution in the *Dilution Buffer*.

Standard solution: dilute the standard preparation in the *Dilution Buffer* to obtain a solution containing 0.18 IU of Factor X per milliliter. Prepare at least three more dilutions of this solution in the *Dilution Buffer*. Shortly before testing, place all solutions at 37°C in water bath.

The conditions described apply to the microtitration plates. If the test is carried out in tubes, adjust the volumes to maintain the proportions in the mixtures.

Introduce 12.5 μ L of the different dilutions of *Sample Solution* and *Standard Solution* into a series of wells of the microplate kept at 37 °C. Add 25 μ L of RVV to each well. Incubate for exactly 90 seconds. Add to each well 150 μ L of *Chromogenic Substrate for Factor Xa*, diluted six times in *Dilution Buffer*. Read the absorbance change rate at 405 nm (**5.2.14**) and continue for three minutes to obtain the average rate of absorbance change. If a continuous reading is not possible, determine the absorbance at 405 nm at standardized consecutive ranges, e.g. every 40 seconds. Plot the linear graph of the absorbance values versus time and calculate the average rate of change in absorbance. From the individual values found for each standard and sample dilution, calculate the sample activity and check the validity of the assay using the usual statistical methods.

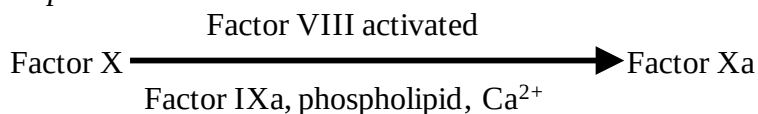
5.5.1.7 DETERMINATION OF FACTOR VIII OF LYOPHILIZED HUMAN BLOOD COAGULATION

It is carried out by determining the biological activity of Factor VIII as a cofactor in the activation of Factor X by Factor IX activated (IXa) in the presence of calcium ions and phospholipids. The activity of a Factor VIII preparation is calculated by comparing the respective amounts of that preparation and the International Standard; or a standard solution measured in international units that are necessary to obtain a certain formation rate of Factor Xa in a reaction medium containing the different substances that participate in the activation of Factor X.

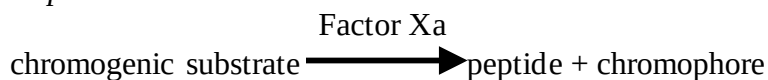
The International Unit of Factor VIII corresponds to the activity of a given quantity of the International Standard, which is constituted by a lyophilized concentrate of Factor VIII of human blood coagulation. The equivalence of the International Standard with International Units is established by the World Health Organization (WHO). Human blood coagulation Factor VIII concentrate is measured in international units against the International Standard. The colorimetric measurement method consists of two successive steps: the activation of Factor X under the action of Factor VIII in a reactive mixture of coagulation factors composed of purified substances and the enzymatic cleavage of a chromogenic substrate by Factor Xa that releases a quantifiable chromophore by spectrophotometry. Under proper dosing conditions, there is a linear relation between the

formation rate of Factor Xa and the concentration of Factor VIII. The following diagram summarizes the determination principle:

Step 1



Step 2



Both steps use commercially available reagents. Although the composition of these reagents may vary slightly, their essential characteristics are described in the following specifications. Deviations from such specifications may be permitted provided it is demonstrated, using the International Standard, that the results obtained do not differ significantly. Commercial packaging is used according to manufacturer's instructions; it is important to ensure that the packaging chosen is suitable.

The kits used must be properly validated, and in this case, the verification of Factor Xa generation time may be used to determine the time required to reach 50% of Factor Xa maximum formation.

REAGENTS

The reactive mixture of coagulation factors corresponds to purified proteins of human or bovine origin, specifically, Factor X, Factor IXa and a Factor VIII activator, usually thrombin. These proteins are partially purified, preferably minimum 50%, and do not contain impurities capable of interfering with the activation of Factor VIII or Factor X. Thrombin may be present in the form of its precursor, prothrombin, as long as its activation in reactive mixture is fast enough to allow complete and nearly instantaneous activation of Factor VIII in the assay. The reactive mixture must contain phospholipids that can be of natural origin (for example: brain, bovine spinal cord and soy extract) or obtained artificially, consisting of about 15 to 31% of phosphatidylserine. The final concentration of phospholipids during the Factor Xa formation step is approximately 10 to 35 $\mu\text{mole/L}$. The reactive mixture also contains calcium ions in such quantity that its final concentration is 5 to 15 mmole/L . The final stage of Factor Xa formation is carried out in a solution that must contain minimum 1 mg/mL of conveniently buffered human or bovine albumin (pH 7.3 to 8.0). The different constituents of the reactive medium are usually brought together in two separate preparations, which alone should not induce the formation of Factor Xa. After reconstitution, these two preparations can be combined provided that no amounts of Factor Xa are formed in the absence of Factor VIII. Factor VIII is the only factor that limits the formation of Factor Xa in the final incubation mixture. The second step consists of quantifying the Factor Xa formed in the previous step in a chromogenic substrate specific for Factor Xa. This substrate is usually a short peptide derived from 3 to 5 amino acids bonded to a chromophore group. The sectioning of this group and the peptide substrate promotes a shift of the chromophoric activity to a wavelength that allows its quantification by spectrophotometry. The substrate, usually dissolved in water and used in a final concentration of 0.2 to 2 mmole/L , must contain the appropriate inhibitors to prevent the additional formation of Factor Xa and suppress all thrombin activity, which enables to improve the assay selectivity in the presence of Factor Xa.

PROCEDURE

The entire content of an ampoule of the *Standard Solution* and the *Sample Solution* must be reconstituted by adding the amount of water and use immediately. Add amounts of pre-diluent necessary to obtain solutions between 0.5 to 2.0 IU/mL . The pre-diluent consists of plasma from a

donor with severe hemophilia A, or from an artificially prepared reagent, providing results equivalent to those obtained with hemophilic plasma and with the same standard and sample preparations. Pre-diluted solutions must present good stability beyond the time required for determination of at least 30 minutes at 20°C and be used within 15 minutes. Make the following dilutions of the standard and sample preparation using an isotonic buffer solution without a chelating agent, and containing 1.0% human or bovine albumin; the solution may contain, for example, tromethamine or imidazole and is preferably buffered (pH 7.3 to 8.0). Prepare at least three additional independent dilutions, preferably in duplicate. The solutions must be prepared in such a manner that the final Factor VIII concentration, apart from the Factor Xa formation stage, is less than 0.03 UI/mL and preferably than 0.01 UI/mL. Prepare a standard containing all the constituent kit of the reactive mixture except for Factor VIII. Prepare dilutions in plastic tubes and use immediately.

Step 1. To each of the dilutions, obtained from the standard and sample solution preparation, add an appropriate volume of pre-heated coagulation reagent (or a mixture of its separate constituents), homogenize and incubate at 37°C in plastic tubes or wells of a microplate. Allow the Factor X activation reaction to develop for an appropriate time; the end of the reaction takes place, preferably, before the Factor X concentration has reached its maximum level, so that the dose-response curve presents a satisfactory linearity. The reaction time is also chosen so that the linearity condition of the Factor Xa production curve as a function of time is satisfactory. It is usually on the order of two to five minutes, but certain variations are permitted to improve the linearity of the dose-response curve.

Step 2. Interrupt the activation reaction by adding a reactive mixture containing the chromogenic substrate. The substrate lysis rate, which is proportional to the Factor Xa concentration, is determined by a spectrophotometer by varying the absorbance at an appropriate wavelength. The absorbance can be determined continuously, which makes it possible to calculate the initial rate of substrate lysis, either by interrupting the hydrolysis reaction after an appropriate time, lowering the pH with an appropriate reagent such as acetic acid (50% v/v of C₂H₄O₂) or by a *M* citrate buffer at pH 3.0. Adjust the hydrolysis time so that the linearity condition for chromophore formation as a function of time is satisfactory. It is usually on the order of 3 to 15 minutes, but certain variations are permitted to improve the linearity of the dose-response curve. Check the validity of the test and calculate the activity of the sample through statistical procedures applicable to biological tests.

5.5.1.8 DETERMINATION OF ACTIVATED COAGULATION FACTORS

If the sample contains heparin, determine the existing amount and neutralize it with the addition of protamine sulfate (10 µg of protamine sulfate neutralizes 1 IU of heparin). With tris-sodium chloride buffer pH 7.5, prepare 1/10 and 1/100 dilutions. Place a series of polystyrene tubes in a 37°C water bath. Introduce 0.1 mL of platelet-poor plasma and 0.1 mL of an appropriate dilution of cephalin RS or a phospholipid preparation that acts as a platelet substitute into each tube. Allow to stand for 60 seconds and add 0.1 mL of one of the dilutions to each tube and 0.1 mL of the buffer solution to the blank test tube.

Immediately transfer 0.1 mL of a 0.37% (w/v) calcium chloride solution to each tube, previously heated to 37°C, and determine the time range between the addition of the calcium chloride solution. calcium and clot formation, this determination is carried out within 30 minutes following the first dilution. The assay is only valid if the blank clotting time is 200 to 350 seconds.

5.5.1.9 DETERMINATION OF ANTI-A AND ANTI-B HEMAGGLUTININS TITER (INDIRECT METHOD)

Prepare a serial duplicate dilution of the preparation to be examined in 0.9% (w/v) sodium chloride solution. For each dilution of a series, add a volume equal to 5.0% (v/v) of the group A1 red cell suspension. Red blood cells must be previously washed three times in a sodium chloride solution. For each dilution of the other series, add an equal volume of 5.0% (v/v) of the group B red cell suspension. The red cells must be previously washed three times in a 0.9% (w/v) sodium chloride solution. Incubate the dilution series at 37°C for 30 minutes and then wash three times with 0.9% sodium chloride (w/v). Leave the red cells in contact with the polyvalent human antiglobulin reagent for 30 minutes. Without centrifuging, examine each suspension for agglutination under a microscope.

5.5.1.10 NUCLEIC ACID AMPLIFICATION METHODS

INTRODUCTION

Nucleic acid amplification methods were established based on two different principles:

- a) amplification of a target nucleic acid sequence using polymerase chain reaction (PCR), ligase chain reaction (LCR), or isothermal amplification of a ribonucleic acid (RNA) sequence;
- b) amplification of a hybridization signal for deoxyribonucleic acid (DNA) using the branched DNA method (bDNA), for example. In this case, signal amplification takes place without subjecting the nucleic acid to repetitive amplification cycles.

Generally speaking, the PCR method is described as the reference method. Alternative methods can be used, provided they meet the quality requirements and are properly validated.

APPLICATION FIELD

Establish the requirements for sample preparation, amplification of DNA sequences, and specific detection of the PCR reaction product. PCR enables detection and amplification of defined DNA and RNA sequences (after their reverse transcription into complementary DNA – cDNA).

METHOD PRINCIPLE

PCR is the fundament of a method that enables the specific *in vitro* amplification of DNA or RNA segments. After denaturation of the double strand of DNA into single strands of DNA, two synthetic oligonucleotide primers, of opposite polarity, hybridize with their respective complementary sequences in the DNA to be amplified. In this case, the activity of the primers makes it possible to complete the single strand of DNA, giving rise to short, biquaternary sequences that surround the DNA fragment to be amplified; thus serving as a starting point for DNA synthesis. It should be noted that this process is carried out through the action of a thermostable DNA polymerase.

DNA amplification takes place in cycles consisting of:

- heat denaturation of nucleic acid (target sequence to be amplified) into two monoquaternary strands;
- specific hybridization of the primers with the sequence to be amplified, under suitable reaction conditions;
- elongation, through the action of DNA polymerase, of the primers linked to each of the two single strands, at a suitable temperature (favorable to the process of DNA synthesis).

Repeated cycles of heat denaturation, primer hybridization and DNA synthesis give rise to an exponential amplification of the DNA fragment then delimited by the primers. The specific product

of the PCR reaction, known as an amplicon, can be detected using a variety of methods of appropriate specificity and sensitivity. The Multiplex PCR assay uses several pairs of primers, intended for simultaneous amplification for different targets of a reaction.

TEST MATERIAL

Due to PCR great sensitivity, samples must be protected from the incidence of light and any external contamination. The sampling, conservation and transport of the material to be analyzed must be carried out under conditions capable of minimizing the risks of degradation of the sequence to be amplified. In tagged RNA sequences, special precautions must be taken since RNA is very sensitive to degradation by ribonucleases, as well as to some additives (anticoagulants and preservatives) that may interfere with the assays.

PROCEDURE

Contaminant Prevention

The risk of contamination requires the existence of restricted areas, depending on the nature of the materials and technology used. Points to consider include: personnel flow, work flow, material handling, ventilation systems, and decontamination procedures.

It is advisable to subdivide the system into areas, such as:

- primary preparation area (place where materials not contained in the matrix are exclusively manipulated, for example, primers and buffers);
- pre-PCR area (where reagents, samples and controls are handled);
- amplification area (where the amplified material is manipulated in a closed system);
- post-PCR detection area (only area where amplification products are manipulated in an open system).

Sample preparation

Sample preparation consists of extracting or releasing the target sequence to be amplified from the material to be examined. The method used for this purpose must be effective, reproducible and compatible with the amplification under the selected reaction conditions. A variety of physicochemical methods for extraction and/or enrichment can be used.

Potential additives in the material under analysis may interfere with the PCR method. The procedures described in the *Internal Control* item must be used to verify the absence of inhibition factors in the material to be examined.

As for the RNA models, precautions must be taken so that there is an absence of ribonuclease-like activity.

Amplification

Amplification of a target sequence by PCR method requires, minimum, a pair of primers, the four types of triphosphate deoxynucleotides (dNTPs), magnesium ions (MgCl₂), and a thermostable DNA polymerase for DNA synthesis.

Amplification of the target sequence by PCR is conducted under defined cyclic conditions: temperature profile for DNA double-stranded denaturation; annealing and extension of primers and incubation times at selected temperatures within a range.

The following parameters should be considered:

- the length and base composition of the primer and target sequence;
- the type of DNA polymerase, the composition of the buffer and the reaction volume used in the amplification;
- the type of thermal cycler used and the rate of thermal conductivity between the apparatus, reaction tube and reaction medium.

Amplification takes place in cycles consisting of:

- denaturation of the nucleic acid target sequence by heating the two single strands; reaction is heated between 92 and 96°C;
- specific annealing of the primers to the target sequence that will be synthesized, under suitable reaction conditions. The temperature is normally 55°C, depending on the homology of the primers to the target sequence to be amplified, the composition of the primers and the amount of cytosine and guanine bases;
- extension of the primers that are linked to the single strands, through the action of thermostable DNA polymerase, at a temperature suitable for DNA synthesis. Typically at 72°C;
- after the end of the cycle, it is cooled to 4°C and preserved.

Detection

The generated amplified sequence can be identified: by its size, sequence, chemical modification or a combination of these parameters. Detection and characterization by size can be performed by gel electrophoresis (using agarose gel plates, or polyacrylamide gel plates, or by capillary electrophoresis), or by column chromatography (for example, HPLC - *High performance liquid chromatography*). Detection and characterization by sequence composition can be carried out by specific hybridization with probes complementary to the target sequence or by fragmentation of the amplified material by means of a restriction enzyme at specific sites of the sequence to be amplified. Characterization through chemical modification can be performed by incorporating a fluorophore in the labeled sequences and subsequent excitation and detection of fluorescein. Labeled probes that enable subsequent radioisotopic or immunoenzymatic detection can also be used.

ANALYSIS AND INTERPRETATION OF RESULTS

An assay result is only valid if the positive control(s) is(are) unequivocally positive and the negative control(s) is(are) unequivocally negative. Due to the PCR method high sensitivity and the inherent risks of contamination, it is necessary to confirm positive results by performing the assay in duplicate or, when possible, with a new aliquot of the sample. The sample is considered positive if at least one of the repeated tests is positive.

QUALITY ASSURANCE

Validation of the PCR Assay System

The validation program must include the devices and the PCR method used. As references recommendations from the ICH (The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) should be used: Q2B, *Analytical Method Validation*, or equivalent alternative reference.

It is essential to carry out this validation using official biological reference standards, properly calibrated using International Standards for the target sequences used in the assay.

Validation must include the determination of the positive response threshold, that is, the minimum number of labeled sequences per volume unit that can be detected in not less than 95% of the assays. This value depends on several interrelated factors, such as: the volume of the sample submitted to extraction and the efficiency of the extraction method; the transcription of tagged RNA into complementary DNA; the amplification procedure and the detection system. To define the detection limit of the system used, it is necessary to consider the positive response threshold for each sequence to be amplified and the assay operating characteristics with the respective maximum and minimum limits of the positive response.

Reagents quality control

All crucial reagents used in the methodology put into practice must be subject to control before their routine use. Acceptance/rejection must be based on pre-defined quality criteria. Primers are one of the essential components of the PCR method, thus requiring particular attention to their design; purity and validation of their use in the assay. Each new batch of initiators should be checked for specificity; amplification efficiency and absence of inhibitory impurities. Primers can be modified (for example, by conjugation with a fluorophore, or an antigen) so as to allow the use of a specific method of detection of the target sequence to be amplified; provided that those modifications do not inhibit the precision and effectiveness of amplification of the target sequence.

ASSAY CONTROLS

External controls

To detect any contamination and ensure adequate sensitivity, the following external controls should be included in all PCR assays:

- a positive control with a defined number of copies of the target sequence, that number being specifically determined for each assay system and expressed as a multiple of the positive response threshold of the system in question;
- a negative control consisting of a matrix sample that was shown to be free of target sequences.

Internal control

The internal control is formed by defined nucleotide sequences containing the primer binding sites. Internal control must be amplified with defined effectiveness and products must be clearly discernible. This internal control must belong to the same type of nucleic acid (DNA/RNA) as the sample. The internal control is preferably added to the sample prior to nucleic acid isolation and therefore acts as a global control (extraction, reverse transcription, amplification and detection).

External quality assessment

For each laboratory and each operator, participation in external quality assessment programs is an important aspect of quality assurance in PCR.

5.5.1.10.1 RECOMMENDATIONS FOR THE VALIDATION OF NUCLEIC ACID AMPLIFICATION METHODS FOR THE DETECTION OF HEPATITIS C VIRUS (HCV) RNA IN PLASMA MIXTURES.

Most nucleic acid amplification methods are quantitative analytical assays designed to detect their presence. There are some quantitative tests traded or developed internally by the laboratories themselves. To detect HCV RNA contamination in plasma mixtures, qualitative assays are suitable,

and may even be considered as limit assays for the control of impurities. These recommendations describe the methods for validating nucleic acid amplification methods applicable only to qualitative assays designed to detect HCV RNA in plasma mixtures. Therefore, the two validation parameters considered to be the most important are specificity and detection limit. Robustness is also evaluated. However, this document can also be used as a basis for general validation of amplification methods.

This document defines the analytical method as the set of operations performed after nucleic acid extraction, followed by detection of amplified products. It should be noted that in cases of use of commercial kits, as part of the complete analytical procedure, the documented validation considerations already carried out by the manufacturer can replace the validation by the operator. However, performance of the traded kit related to its intended use has to be demonstrated by the user (e.g. detection limit, robustness and cross contamination).

SPECIFICITY

Specificity is the ability to unambiguously assess nucleic acid in the presence of components of unexpected presence. The specificity of analytical nucleic acid amplification procedures is dependent on the choice of primers, the choice of probe (for analysis of the final product) and the strictness of test conditions (for both the amplification and detection steps).

In the design of primers and probes, one of the aspects to be considered is their specificity in detecting HCV RNA; for this fact it is convenient to compare the target sequences with the sequences published in databases. For HCV, primers and probes are usually chosen from the areas of the 5' non-coding region (5'NCR) of the HCV genome, composed of 341 nucleotides, which are the most conserved among the different HCV isolates.

The amplified product must be unambiguously identified by the use of methods such as: amplification with stranded primers, restriction enzyme analysis, sequencing, or hybridization with a specific probe.

To validate the specificity of the analytical method, it is convenient to test minimum 100 plasma mixtures negative for HCV RNA, and all results obtained are negative. The World Health Organization (WHO) has suitable samples of non-reactive plasma.

The method capability to detect all HCV genotypes will depend on the choice of primers, probes and operating parameters. This capability should be demonstrated through the use of a collection of characterized reference preparations.

It has been suggested that the distribution pattern of HCV genotypes in Brazil is similar to that found in many European countries, with the prevalence of types 1 and 3. There is an epidemiological behavior typical of an exponential spread in recent years, probably as a result of blood transfusions. In this context, genotypes 1 and 3 must be detected at appropriate levels.

DETECTION LIMIT

The detection limit of an individual method is the smallest amount of nucleic acid that can be detected, but not necessarily quantified, with an exact value in the sample.

The amplification process used to detect HCV RNA in plasma mixtures generally provides qualitative results. The number of possible results is limited to two answers: positive or negative. Although determination of the detection limit is recommended, for practical reasons, the positive response threshold for nucleic acid amplification methods is determined. The positive response threshold is the minimum number of target sequences per unit volume that can be detected in 95% of assays. This

positive response threshold is influenced by the distribution of viral genomes in the individual samples tested and by factors such as enzyme efficacy, which can lead to differences of 95% in the positive response thresholds obtained in the individual analysis.

To determine the positive response threshold, it is essential to perform the method on different days with a series of dilutions of a working reagent or Hepatitis C virus (biological reference standard), calibrated against the International Standard for HCV 96/ 790 WHO to evaluate among the various assays. A minimum of three separate dilution series are tested with a sufficient number of replicates of each dilution to obtain a total number of 24 results per dilution, thus allowing for statistical analysis of the results.

For example, in a laboratory, three series of dilutions are tested with eight replicates for each dilution on different days; four dilution series with six replications for each dilution on different days, or six dilution series with four replications for each dilution on different days.

For the number of dilutions used to remain the same, it is essential to carry out a preliminary test (such as, for example, logarithmic dilutions in the sample of the plasma mixture to obtain a preliminary positive response threshold value, i.e., the highest dilution in which a positive sign occurs).

The distribution of dilutions can then be performed based on this pre-calculated preliminary value (using, for example, a 0.5 log dilution factor), or less than a mixture of negative plasma as the dilution matrix. The RNA content of HCV that can be detected is 95% in the assays and can be calculated using an appropriate statistical method. These results, also, serve to demonstrate the internal variation of the assay and the variation in several days of the analytical method.

ROBUSTNESS

The robustness of an analytical method is the measure of its ability to remain unchanged when subject to small but deliberate variations in operating parameters and provides an indication of the method feasibility under normal conditions of use. Robustness assessment is one of the aspects to be considered during the development phase. It enables establish the method feasibility under deliberate variations in operating parameters. In nucleic acid amplification methods, small variations in operating parameters may be of special importance. However, robustness can be demonstrated during method development, when small variations in the concentration of reagents are tested (for example: MgCl₂, primers or dNTPs). To demonstrate robustness during validation, at least twenty plasma mixtures (randomly chosen) negative for HCV RNA should be examined to which a final typical concentration corresponding to the positive response threshold is added, previously, determined. All results obtained are positive.

Problems with robustness can arise in methods that use, in their initial phase, ultracentrifugation prior to the extraction of viral RNA. Therefore, to test the robustness of these methods. At least twenty plasma mixtures containing varying concentrations of HCV RNA but free of HCV-specific antibodies are tested. All results obtained are positive.

It is convenient to demonstrate the absence of cross-contamination by accurately detecting a set of minimum twenty samples, alternating samples of negative plasma mixtures and negative plasma mixtures to which a high concentration of HCV has been added (at least 10² times 95 % of positive response threshold, or at least 10⁴ IU/mL).

QUALITY ASSURANCE

Biological assay methods, such as the nucleic acid amplification method, may present specific problems that interfere with the validation and interpretation of results.

Procedures must be described precisely in the form of standard operating procedures (SOPs), which must cover:

- sampling (type of containers, etc.);
- preparation of mini-mixtures (if applicable);
- storage conditions prior to analysis;
- exact description of the operating conditions (including precautions that must be taken to avoid cross-contamination or destruction of the viral RNA) as well as the standard reagents and preparations used;
- Detailed formulas for calculating results, including statistical evaluation. The use of appropriate control (e.g., appropriate dilution of hepatitis C virus, biological reference standard; or plasma to which a calibrated HCV sample has been added against the HCV International Standard 96/790 from WHO) it can be considered a satisfactory stable means of controlling the system and ensuring and maintaining the method feasibility in each use.

Technical qualification: for each critical element of the apparatus used, an appropriate installation and operational qualification program is created. After any modification of critical equipment (for example, thermal cyclers) it is essential to reconfirm the method acceptability, proceeding in parallel with the examination of eight samples of a plasma mixture to which was added a triple concentration of HCV RNA of that corresponding to the previously determined positive response threshold; all results obtained are positive.

Operator qualification: an appropriate qualification program is developed for the set of operators involved in the test. For this purpose, each operator should examine at least eight samples of a plasma mixture to which a triple HCV RNA concentration corresponding to the previously determined positive response threshold has been added. This test (eight samples) is repeated twice on different days for a total of twenty-four analysis carried out on three different days. All results obtained are positive.

5.5.1.11 DETERMINATION OF PRE-KALLIKREIN ACTIVATION

The prekallikrein activation transforms prekallikrein into kallikrein and can be titrated because it has the ability to cleave a chromophore from a synthetic peptide substrate, determining the reaction rate by spectrophotometry. The prekallikrein activation concentration is calculated by comparison with the standard preparation whose activity is expressed in international units. The International Unit corresponds to the activity of a certain quantity of International Standard consisting of lyophilized pre-kallikrein activator. The correspondence between the International Unit and the International Standard is indicated by the World Health Organization.

REAGENTS

Buffer A

Tromethamine	6.055 g
Sodium chloride	1.170 g
Hexadimethrin Bromide	50 mg
Sodium Azide	0.100 g

Dissolve the reagents in water, adjust pH to 8.0 with 2 M hydrochloric acid and adjust to 1000 mL with water.

Buffer B

Tromethamine	6.055 g
Sodium chloride	8.770 g

Dissolve the reagents in water, adjust pH to 8.0 with 2 M hydrochloric acid and adjust to 1000 mL with water.

PREPARATION OF PRE-KALLIKREIN SUBSTRATE

Blood or plasma used in the preparation of prekallikrein should be collected and handled only in plastic or silicone glass materials in order to avoid activation of prekallikrein resulting from clotting. Homogenize nine volumes of human blood with one volume of anticoagulant solution (ACD, CPD or a 38 g/L sodium citrate solution) added with 1 mg per milliliter of hexadimethrine bromide. Shake and centrifuge at 3600 g for five minutes. Separate plasma and centrifuge at 6000 g for 20 minutes to separate platelets. Separate the platelet-poor plasma and dialyze against 10 volumes of *Buffer A* for 20 hours. After dialysis, deposit the plasma on a chromatography column containing twice its volume of agarose-DEAE for ion exchange chromatography previously equilibrated with *Buffer A*. Carry out elution with *Buffer A* (flow rate 20 mL/cm²/hour). Collect the eluate in fractions and record the absorbance at 280 nm (5.2.14). Join the fractions containing the first protein peak so as to obtain a volume approximately 1.2 times that of platelet-poor plasma.

To verify that the substrate does not have active kallikrein, homogenize a volume with 20 volumes of chromogenic substrate solution that will be used in the assay, previously heated at 37°C, and keep the mixture at 37°C for two minutes. Substrate is suitable if absorbance does not increase by more than 0.001 per minute. Add 7 g per liter of sodium chloride to the substrate solution and filter through a membrane (0.45 µm). Freeze the filtrate after partitioning it into aliquots and store at -25°C; it is also possible to lyophilize the filtrate before storage. Carry out the operations between chromatography and congealing the aliquots on the same day.

TITRATION

Preferably, titration is carried out on an automated enzyme analyzer at 37°C. Adjust volumes, substrate concentration and incubation times so that the reaction rate is linear to not less than 35 IU per milliliter. If necessary, the standards, samples and prekallikrein substrate can be diluted with *Buffer B*.

Incubate standards or diluted samples with prekallikrein substrate for 10 minutes; the volume of standard or sample before dilution does not exceed 1/10 of the total volume of the mixture to be incubated to avoid errors resulting from differences in ionic strength or pH. Incubate the mixture or a portion of the mixture with an equal or higher volume of a solution of a known specific chromogenic substrate for kallikrein (e.g. N-benzoyl-L-prolyl-L-phenylalanyl-L-arginine acetate 4-nitroanilide or D-propyl-L-phenylalanyl-L-arginine 4-nitroanilide dihydrochloride) and dissolved in *Buffer B*. Record the change in absorbance per minute ($\Delta A/\text{minute}$) for 2 to 10 minutes at the appropriate wavelength for the substrate used. For each standard or sample mix, prepare a blank by replacing the prekallikrein substrate with *Buffer B*. Correct the change in absorbance per minute by subtracting the value obtained with the corresponding blank. Plot a calibration curve from the values of the change in absorbance per minute obtained with the standard and its respective concentrations and determine the prekallikrein activation content of the sample.

5.5.1.12 DETERMINATION OF HUMAN ANTITHROMBIN III

The antithrombin III content of the sample is determined by comparing its capacity to inactivate thrombin in the presence of an excess of heparin with the capacity of a standard preparation of human antithrombin III concentrate with concentration in international units. Varying amounts of sample are

added to a thrombin and residual thrombin activity is determined with an appropriate chromogenic substrate.

The International Unit corresponds to the activity of a specified amount of the International Standard for human antithrombin III concentrate. The equivalence of the International Unit with the International Standard is indicated by the World Health Organization.

PROCEDURE

For the sample and the standard, prepare with Tris-EDTA ASB buffer pH 8.4 containing 15 IU of heparin per milliliter, two independent series of three or four dilutions between 1/75 and 1/200 starting from 1 IU/mL. Heat at 37 °C for one to two minutes 200 µL of each dilution. Add to each dilution 200 µL of a bovine thrombin solution containing 2 IU/mL in Tris-EDTA ASB buffer pH 8.4. Homogenize and keep at 37°C for exactly one minute. Add 500µL of an appropriate chromogenic substrate (e.g. D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide; dissolve the substrate in water to obtain a solution containing 4 mmole/L and dilute with Tris-EDTA buffer ASB pH 8.4 without albumin to a concentration appropriate for the titration assay). Immediately determine absorbance at 405 nm (**5.2.14**) for at least 30 seconds. Calculate the change in absorbance ($\Delta A/\text{minute}$). An endpoint titration can also be used by interrupting the reaction with acetic acid and determining the absorbance at 405 nm. The change in absorbance ($\Delta A/\text{minute}$) is inversely proportional to the activity of human antithrombin III. Check the validity of the test and calculate the activity of the sample through the statistical procedures applicable to biological tests.

5.5.1.13 DETERMINATION OF ANTICOMPLEMENTARY ACTIVITY OF IMMUNOGLOBULIN

The determination of the anti-complementary activity (AAC) of the immunoglobulin is carried out by incubating a sample of immunoglobulin (10 mg) with a certain amount of guinea pig complement (20 CH₅₀). The titration of the remaining complement follows: the anti-complement activity is expressed by the proportion of complement consumed, taking the standard complement as 100%.

The hemolytic unit of complementary activity (CH₅₀) is the amount of complement that, under the stipulated reaction conditions, causes the lysis of 2.5×10^8 of a total number of 5×10^8 properly sensitized red cells

REAGENTS

Magnesium and calcium stock solution. Weigh 1.103 g of calcium chloride and 5.083 g of magnesium chloride, dissolve in water and adjust to 25 mL with the same solvent.

Barbital buffer stock solution. Weigh 207.5 g of sodium chloride and 25.48 g of sodium barbital, dissolve in 4000 mL of water and adjust pH to 7.3 with *M* hydrochloric acid. Add 12.5 mL of *Magnesium and calcium stock solution* and adjust to 5000 mL with water. Filter through membrane (0.22 µm) and store at 4 °C in a glass container.

Gelatin solution. Weigh 12.5 g of gelatin, dissolve in about 800 mL of water and heat to boiling in water bath. Cool to 20°C and adjust to 10 liters with water. Filter through membrane (0.22 µm) and store at 4 °C. Only use the solution if it is clear without fractioning.

Citrated solution. Weigh 8 g of sodium citrate, 4.2 g of sodium chloride and 20.5 g of glucose and dissolve in 750 mL of water. Adjust pH to 6.1 with 10% (w/v) citric acid solution and adjust to 1000 mL with water.

Barbital gelatin buffer. Add four volumes of *Gelatin Solution* to one volume of *Barbital Buffer Stock Solution* and homogenize. If necessary, adjust the pH to 7.3 with *M* hydrochloric acid or *M* sodium hydroxide and store at 4°C. Prepare a new solution daily.

Stabilized sheep blood. Collect one volume of sheep blood in one volume of *Citrated Solution* and homogenize. Store blood stabilized at 4°C for not less than seven days and not more than 28 days. Sheep blood or stabilized sheep erythrocytes are commercially available from a variety of suppliers.

Hemolysin. Sheep anti-erythrocyte serum, prepared in rabbits. Such sera are commercially available from a variety of suppliers.

Guinea pig complement. Homogenize the sera obtained from at least 10 guinea pigs. Separate serum from clotted blood by centrifugation at a temperature of about 4°C. Store the serum, in small portions, at a temperature below -70 °C.

PROCEDURE

Standardization of the 5.0% sheep red blood cell solution.

Separate sheep red blood cells by centrifugation of an appropriate volume of stabilized sheep blood; wash the cells at least three times with *Gelatin Barbital Buffer* and prepare a 5.0% (v/v) suspension in the same buffer. Determine cell concentration by the following method: add 0.2 mL of the suspension to 2.8 mL of water and centrifuge the lysate for five minutes at 1000 g. Cellular concentration is adequate if the supernatant absorbance (**5.2.14**), determined at 541 nm, is 0.62 ± 0.01 . Correct the cell concentration by adding *Gelatin-Barbital Buffer*, according to the formula:

$$V_f = \frac{V_i \times A}{0,62}$$

where

V_f = final volume;

V_i = initial volume;

A = absorbance determined at 541 nm for the original suspension.

Once the cell concentration is adjusted, the suspension contains about 1×10^9 cells per milliliter.

Hemolysin Titration

Prepare hemolysin dilutions according to **Table 1**.

Table 1 – Hemolysin dilutions.

<i>Dilution of hemolysin to be prepared</i>	<i>Barbital gelatin buffer</i>		<i>Hemolysin</i>
	<i>Volume (mL)</i>	<i>Dilution (1/...)</i>	<i>Volume (mL)</i>
7.5	0.65	not diluted	0.1
10	0.90	not diluted	0.1
75	1.80	7.5	0.2
100	1.80	10	0.2

Dilution of hemolysin to be prepared	Barbital gelatin buffer		Hemolysin
	Volume (mL)	Dilution (1/...)	Volume (mL)
150	1.00	75	1.0
200	1.00	100	1.0
300	1.00	150	1.0
400	1.00	200	1.0
600	1.00	300	1.0
800	1.00	400	1.0
1200	1.00	600	1.0
1600	1.00	800	1.0
2400	1.00	1200	1.0
3200(*)	1.00	1600	1.0
4800(*)	1.00	2400	1.0

(*) discard 1.0 mL of the mixture.

Transfer 1 mL of the 5.0% sheep red blood cell suspension to each tube of the hemolysin dilution series from the 1/75 dilution and homogenize. Incubate at 37°C for 30 minutes. Transfer 0.2 mL of each incubated hemolysin dilution mixture to new tubes and add 1.1 mL of *Barbital Gelatin Buffer* and 0.2 mL of a guinea pig complement dilution (e.g. 1/150). Carry out these manipulations in duplicate.

Prepare three control tubes of non-hemolyzed cells, transferring 1.4 mL of *Barbital Gelatin Buffer* and 0.1 mL of 5.0% sheep red blood cell suspension into each one.

Prepare three control tubes of fully hemolyzed cells by transferring 1.4 mL of water and 0.1 mL of the 5.0% sheep erythrocyte suspension to each tube.

Incubate all tubes at 37°C for 60 minutes and centrifuge at 1000 g for five minutes. Determine the absorbance (5.2.14) of the supernatants at 541 nm and calculate the percentage of hemolysis occurring in each tube using the formula:

$$\frac{A_a - A_1}{A_b - A_1} \times 100$$

where

A_a = absorbance of tubes containing the hemolysin dilution,

A_b = average absorbance of the three tubes with total hemolysis,

A₁ = average absorbance of the three control tubes without hemolysis.

Plot a graph containing the hemolysis percentages on the ordinate axis and the inverses of hemolysin dilutions on the abscissa axis. Determine the optimal hemolysin dilution from the graph by choosing a dilution such that an increase in the hemolysin amount does not produce a meaningful change in the hemolysis degree. This dilution is considered to contain 1 minimal hemolytic unit (1 MHU) in 1.0 mL. For the preparation of sensitized sheep red blood cells, the hemolysin dilution corresponding to the optimal hemolysis contains 2 MHU per milliliter.

Hemolysin titration is only valid if the degree of total hemolysis is between 50% and 70%. If the percentage of total hemolysis cannot be determined from the dilution used, repeat the titration using a more or less diluted complement solution.

Optimum preparation of sensitized sheep red blood cells (hemolytic system). Prepare an appropriate amount of diluted hemolysin containing 2 UHM per milliliter and an equal volume of 5.0% standardized sheep erythrocyte suspension. Add hemolysin dilution to the standardized cell suspension and homogenize. Incubate at 37°C for 15 minutes, store at 2°C to 8°C and use within six hours.

Complement titration

Prepare an appropriate dilution of complement (e.g. 1:250) using *Gelatin-Barbital Buffer* and carry out the titration, in duplicate, according to the information recorded in **Table 2**.

To each tube, add 0.2 mL of sensitized sheep erythrocytes, homogenize thoroughly and incubate all tubes at 37°C for 60 minutes. Cool tubes in ice water and centrifuge at 1000 g for five minutes. Determine the absorbance of the supernatants at 541 nm and calculate the percentage of hemolysis (Y), using the formula:

$$\frac{A_c - A_1}{A_b - A_1} \times 100$$

where

A_c = absorbance of tubes 1 to 12;

A_b = average absorbance of tubes with 100% hemolysis;

A_1 = average absorbance of the tubes with 0% of hemolysis,

Table 2 – Complement dilutions.

<i>Tube number</i>	<i>Complement volume diluted in milliliters (e.g. 1:250)</i>	<i>Gelatin-barbital buffer volume in milliliters</i>
1	0.1	1.2
2	0.2	1.1
3	0.3	1.0
4	0.4	0.9
5	0.5	0.8
6	0.6	0.7
7	0.7	0.6
8	0.8	0.5
9	0.9	0.4
10	1.0	0.3
11	1.1	0.2
12	1.2	0.1
3 tubes with 0% hemolysis	-	1.3
3 control tubes with 100% hemolysis	-	1.3 mL of water

Plot a graph by entering the values in abscissa, and the corresponding volume in milliliters of diluted complement in ordinates.

From the points, plot the ideal line and determine the ordinate of the hemolytic activity at 50% of the complement at the point where $Y/(1-Y) = 1.0$. Calculate the activity in terms of hemolytic units (CH₅₀/mL) according to the formula:

$$\frac{C_d}{C_a} \times 5$$

where

C_d = inverse value of the complement dilution,

C_a = volume in milliliters of diluted complement that produces 50% hemolysis,

5 = Scale factor to account for the number of erythrocytes.

The test is only valid if, between 15 and 85% of hemolysis, the curve obtained is a straight line whose slope is between 0.15 and 0.40, preferably between 0.18 and 0.30.

Determination of anti-complementary activity

Dilute the titrated guinea pig complement with *Gelatin-Barbital Buffer* to obtain 100 CH₅₀/mL. If necessary, adjust the sample to pH 7.0. For an immunoglobulin containing 50 mg/mL, prepare the incubation mixtures (**Table 3**).

Table 3 – Incubation mixtures.

<i>Preparation</i>	<i>Sample</i>	<i>Control complement</i>
Immunoglobulin (50 mg/mL)	0.2 mL	-
Barbital gelatin buffer	0.6 mL	0.8 mL
Complement	0.2 mL	0.2 mL

Carry out the determination on the sample and prepare the AAC negative and positive control from a human immunoglobulin international standard, according to the instructions provided on the label for the standard preparation. If the sample does not contain 50mg/mL of immunoglobulin, adjust the volumes of the preparation and the *Gelatin Barbital Buffer*; for example, pipette 0.33 mL of a preparation containing 30 mg/mL of immunoglobulin and add 0.47 mL of *Gelatin-Barbital Buffer* to obtain the same total volume of 0.8 mL. Close tubes and incubate at 37°C for 60 minutes. Add 0.2 mL of each incubation mixture to 9.8 mL of *Gelatin Barbital Buffer* to dilute the complement. In each tube, carry out complement titrations as described above to determine residual anti-complementary activity (**Table 2**). Calculate the anti-complementary activity of the sample, by reference to the complement control considered as 100%, according to the formula:

$$\frac{a - b}{a} \times 100$$

where

a = average complementary activity (CH₅₀/mL) of controls,

b = complementary activity (CH₅₀/mL) of the sample.

The test is only valid if:

- the anti-complementary activities found for the AAC Negative Control and AAC Positive Control fall within the limits indicated on the label accompanying the standard preparation;
- complementary activity of the complement control (a) is from 80 to 120 CH₅₀/mL.

5.5.1.14 DETERMINATION OF TOTAL PROTEIN

METHOD 1

Proteins in solution absorb ultraviolet light at a wavelength of 280 nm, due to the presence in their structure of aromatic amino acids (especially tyrosine and tryptophan), a property that can be used for protein assay. The use of a buffer as compensation liquid can remedy the interference produced in case the buffer used to dissolve the protein has high absorbance, which could compromise the results. At low concentrations, the protein adsorbed onto the curve can cause a significant decrease in the protein content of the solution. It is possible to prevent this phenomenon by preparing high-grade samples or using a non-ionic detergent during preparation.

Sample solution. Dissolve an appropriate amount of the sample in the chosen buffer to obtain a solution whose protein concentration is between 0.2mg/mL and 2mg/mL.

Standard solution. Prepare a solution of the appropriate reference substance corresponding to the protein to be dosed, in the same buffer as used for the sample solution, so as to obtain the same concentration.

Procedure. Keep the sample solution, standard solution and compensation liquid at the same temperature throughout the test. Determine the absorbance (5.2.14) of the sample solution and the standard solution at 280 nm in quartz cuvettes, using the specific buffer as the compensation liquid. For the accuracy of the results, the response is linear over the range of protein concentrations to be dosed.

Light diffusing. Light diffusing through the sample can affect the accuracy of protein assay. If the proteins in solution form particles whose size is of the same magnitude as the wavelength of the measuring beam (250 –300 nm), the diffusion of the light beam translates into an increase in the apparent absorbance of the sample. To calculate the contribution of this diffusion effect on the absorbance read at 280 nm, determine the absorbance of the sample solution at various wavelengths (320 nm, 325 nm, 330 nm, 335 nm, 340 nm, 345 nm and 350 nm). Plot a graph of the absorbance log read as a function of the log of the respective wavelength and determine, by linear regression analysis, the calibration curve that best fits the different points inserted on the graph.

Determine by extrapolation the absorbance log at 280 nm. The absorbance due to the diffusion effect is the antilog of this value. Correct the observed values by subtracting the total absorbance at 280 nm from the absorbance due to the diffusion effect to obtain the absorbance value from the protein in solution. It is possible to carry out a filtration using a 0.2 µ filter that does not absorb the proteins, or a clarification by centrifugation, to reduce the effects of light scattering in a solution.

Calculations Use corrected values for calculations. Calculate the protein content of the sample solution (C_u), using the expression:

$$C_u = C_s \left(\frac{A_u}{A_s} \right)$$

where

C_s = protein content of the standard solution;

A_u = corrected absorbance value of the sample solution;

A_s = corrected absorbance value of the standard solution;

METHOD 2

This method was conceived based on the property that proteins have to reduce phosphomolybdenum tungsten acids contained in the phosphomolybdenum and tungsten reagent; this reaction is chromogenic and translates into the existence of an absorption peak at 750 nm.

Phosphomolybdenum and tungsten reagent react primarily with protein tyrosine residues. Color production reaches a maximum after 20 – 30 minutes of incubation at room temperature; then progressive discoloration takes place. Since the method is sensitive to interfering substances, a treatment that produces precipitation of the proteins in the sample can be used. Most interfering substances reduce the intensity of the color obtained, but some detergents increase it slightly. A strong salt concentration can form a precipitate. Since the intensity of the color obtained may vary depending on the protein species considered, the protein to be dosed and the standard protein are the same. If necessary, separate interfering substances from sample protein, proceed as follows on interfering substances before preparing the sample solution. It is possible to minimize the effect of interfering substances by dilution, as long as the protein content to be determined remains high enough to allow for an accurate determination.

Use distilled water for the preparation of all buffers and reagents used in this method.

Sample solution. Dissolve an appropriate amount of the sample in the specified buffer to obtain a concentration within the range covered by the calibration curve. The pH of a solution prepared with an appropriate buffer is between 10.0 and 10.5.

Standard solutions. Dissolve the reference substance corresponding to the protein to be dosed in the specified buffer. Take samples of this solution and adjust with the same buffer to obtain at least five standard solutions of different concentrations between 5µg/mL and 100µg/mL and evenly distributed over the chosen range.

Blank solutions. Use the same buffer that was used to prepare the *Sample Solution* and *Standard Solutions*.

Copper Sulfate Reagent. Weigh 100 mg of copper sulphate and 0.2 g of sodium tartrate, dissolve in distilled water and adjust to 50 mL with the same diluent. Weigh 10 g of anhydrous sodium carbonate, dissolve in distilled water and adjust to 50 mL with the same diluent. Slowly pour the sodium carbonate solution into the copper sulphate solution, always mixing. This solution is used within 24 hours of its preparation.

Copper alkaline reagent. Prepare a mixture of one volume of *Copper Sulfate Reagent*, two volumes of 5.0% (w/v) sodium lauryl sulfate with one volume of 3.2% (w/v) sodium hydroxide. Store this mixture at room temperature. The mixture is used within two weeks upon preparation.

Diluted phosphomolybdenum and tungsten reagent. Homogenize 5 mL of phosphomolybdenum and tungsten reagent with 55 mL of distilled water. Store the reagent at room temperature in an amber glass flask.

Procedure. To 1 mL of each *Standard Solution*, *Sample Solution* and *Blank Solution* add 1 mL of *Alkaline Copper Reagent* and homogenize. Allow to stand for 10 minutes. Add 0.5 mL of *diluted Tungsten* and *Phosphomolybdenum Reagent*, homogenize and allow to stand at room temperature for 30 minutes.

Determine the absorbance **(5.2.14)** of the solutions at 750 nm, using the blank solution for zero adjustment.

Calculations The relation between absorbance and protein content is not linear; however, if the concentration range covered by the calibration curve is sufficiently narrow, the curve obtained will be substantially linear. Plot the absorbance of standard solutions against the protein content of these solutions and determine the calibration curve by linear regression analysis. From the calibration curve and the absorbance of the sample solution, determine the protein content of the sample solution.

Interfering substances. In this method, sodium deoxycholate and trichloroacetic acid are added to the sample to precipitate the proteins and separate them from interfering substances, before dosing. This method can also be used to concentrate proteins contained in a very dilute solution. To 1 mL of a sample solution add 0.1 mL of 0.15% sodium deoxycholate (w/v). Homogenize with a vortex shaker and allow to stand at room temperature for 10 minutes. Add 0.1 mL of 72% (w/v) trichloroacetic acid. Homogenize with a vortex mixer and centrifuge at 3000 g for 30 minutes. Discard the liquid supernatant and discard the residual liquid with a pipette. Dissolve the clot in 1 mL of *Alkaline Copper Reagent*.

METHOD 3

This method was based on the property that proteins have to shift from 470 nm to 595 nm the maximum absorption of acid blue 90 when they bind to the dye. The acid blue 90 dye has a marked affinity for arginine and lysine residues in the protein which can cause variations in the response to the assay of different proteins. The protein used as the reference substance must therefore be the same as the protein to be dosed. There are relatively few interfering substances, but it is preferable to avoid detergents and analytes in the sample to be dosed. Highly alkaline samples can interfere with the acid reagent.

Use distilled water for the preparation of all buffers and reagents to be used in this method.

Sample solution. Weigh an appropriate amount of sample and dissolve in the indicated buffer to obtain a concentration within the range covered by the calibration curve.

Standard solutions. Weigh the reference substance corresponding to the protein to be dosed and dissolve in the indicated buffer. Take samples from this solution and adjust volume with the same buffer to obtain at least five standard solutions with protein concentrations between 0.1 mg/mL and 1 mg/mL and uniformly distributed over the chosen range.

Blank solutions. Use the same buffer used to prepare the sample and standard solutions.

Acid Blue 90 reagent. Weigh 0.10 g of acid blue 90 and dissolve in 50 mL of ethyl alcohol. Add 100 mL of phosphoric acid, adjust to 1000 mL with distilled water and homogenize. Filter the solution and store it at room temperature in an amber glass flask. A slow precipitation of the dye occurs during storage. The precipitate is filtered before using the reagent.

Procedure. To 0.100 mL of each *Standard Solution*, *Sample Solution* and *Blank Solution* add 5 mL of *Acid Blue 90 reagent*. Homogenize the mixture by rotation, avoiding the formation of foam that can create reproducibility problems. Determine the absorbance **(5.2.14)** of the *Standard Solutions* and the *Sample Solution* at 595 nm using the *Blank Solution* for zero adjustment. The use of quartz (silica) cuvettes is avoided since the dye binds to this material.

Calculations The relation between absorbance and protein content is not linear. However, if the concentration range covered by the calibration curve is sufficiently narrow, the curve obtained will be substantially linear. Plot the absorbance of *Standard solutions* according to the protein content of these solutions and determine the calibration curve by linear regression analysis. From the calibration curve and the absorbance of the *Sample solution*, determine the protein content of the *Sample solution*.

METHOD 4

This method, also known as the bicinchoninic acid (BCA) method, was developed based on the property that proteins have to reduce cupric ion (Cu^{2+}) to cuprous ion (Cu^+). The bicinchoninic acid reagent serves to detect cuprous ions. There are few interfering substances. If there are interfering substances, it is possible to minimize their effects by dilution, as long as the protein content to be dosed remains high enough to allow for an accurate determination. The protein precipitation method described in Method 2 can be used to eliminate interfering substances. The intensity of the color obtained by the reaction with the reagent may vary from one type of protein to another and, therefore, the protein to be dosed and the reference protein are the same.

Use distilled water to prepare buffers and reagents to be used in this method.

Sample solution. Weigh an appropriate amount of the sample and dissolve in the indicated buffer so as to obtain a concentration within the concentration range of the *Standard Solutions*.

Standard solutions. Weigh the reference substance corresponding to the protein to be dosed and dissolve in the indicated buffer. Take samples from this solution and adjust to volume with the same buffer to obtain minimum five standard solutions with concentrations between 10 $\mu\text{g/mL}$ and 1200 $\mu\text{g/mL}$ and evenly distributed over the chosen range.

Blank solutions. Use the same buffer that was used to prepare the *Sample Solution* and *Standard Solutions*.

BCA Reagent. Weigh 10 g of disodium bicinchoninate, 20 g of sodium carbonate monohydrate, 1.6 g of sodium tartrate, 4 g of sodium hydroxide and 9.5 g of sodium bicarbonate and dissolve in distilled water. If necessary, adjust pH to 11.25 with sodium hydroxide or sodium bicarbonate solution. Adjust to 1000 mL with distilled water and homogenize.

Copper-BCA reagent. Homogenize 1 mL of 4% copper sulfate (w/v) with 50 mL of *BCA Reagent*.

Procedure. Homogenize 0.1 mL of each *Standard Solution*, *Sample Solution* and *Blank Solution* with 2 mL of *Copper-BCA Reagent*. Incubate solutions at 37°C for 30 minutes. Allow to cool to room temperature. Within exactly 60 minutes following the incubation period, determine the absorbance (5.2.14) at 562 nm of the *Standard Solutions* and the *Sample Solution* in quartz cuvettes using the *Blank Solution* for zero adjustment. When the temperature of the solutions returns to room temperature, the color intensity continues to progressively increase.

Calculations The relation between absorbance and protein content is not linear. However, if the concentration range covered by the calibration curve is sufficiently narrow, the curve obtained will be substantially linear. Plot the absorbance of the standard solutions against the protein content of these solutions and determine the calibration curve by linear regression analysis. From the calibration curve and the absorbance of the sample solution determine the protein content of the sample solution.

METHOD 5

This method is also known as the biuret method, based on the property that proteins have to interact with the cupric ion (Cu^{2+}), in an alkaline medium, providing a reaction product that presents absorbance at 545 nm. The use of this method makes it possible to obtain a minimum deviation between equivalent IgG and albumin samples. On the contrary, simultaneous addition of sodium hydroxide and biuret reagent (as a mixture), insufficient homogenization after addition of sodium hydroxide or a very long time lag between addition of sodium hydroxide and sodium hydroxide reagent. biuret leads to obtaining a higher response with IgG samples than with albumin samples. Treatment with trichloroacetic acid used to reduce interferences can also make it possible to quantify the protein when its concentration in the sample is inferior to 0.5 mg/mL.

Use distilled water to prepare buffers and reagents to be used in this method.

Sample solution. Weigh an appropriate amount of the sample and dissolve in 0.9% (w/v) sodium chloride solution so as to obtain a concentration within the concentration range of standard solutions.

Standard solutions. Weigh the reference substance corresponding to the protein to be measured and dissolve in 0.9% sodium chloride solution (w/v). Take samples of this solution and complete with 0.9% sodium chloride solution (w/v) to obtain at least three standard solutions with concentrations between 0.5 mg/mL and 10 mg/mL and, uniformly, distributed into the chosen range.

Blank solutions. Use 0.9% sodium chloride solution (w/v).

Biuret reagent. Weigh 3.46 g of copper sulfate and dissolve in 10 mL of hot distilled water. Allow to cool (solution A). Weigh 34.6 g of sodium citrate and 20 g of anhydrous sodium carbonate and dissolve in 80 mL of hot distilled water and allow to cool (solution B). Homogenize solutions A and B and adjust to 200 mL with distilled water. This reagent is used within 6 months upon preparation; not used if turbidity or precipitate is produced.

Procedure. To one volume of *Sample Solution* add an equal volume of 6% sodium hydroxide solution (w/v) and homogenize. Immediately add 0.4 volume (calculated in relation to the sample solution) of *Biuret reagent* and homogenize quickly. Keep the samples for at least 15 minutes at a temperature between 15 °C and 25 °C. Within 90 minutes of reagent addition, determine the absorbance (5.2.14), at a maximum of 545 nm, of the *Standard Solutions* and the *Sample Solution*, using the *Blank Solution* as compensation liquid. If turbidity or precipitates appear in the solutions, they are not used for calculating the protein content.

Calculations The relation between absorbance and protein content is substantially linear over the range of concentrations indicated for *Standard solutions*. Plot the absorbance of *Standard solutions* according to the protein content of these solutions and determine the calibration curve by linear regression analysis. Calculate the correlation coefficient for the calibration curve. The system satisfies if it obtains a straight line whose correlation coefficient is not less than 0.99. From the calibration curve and the absorbance of the *Sample solution*, determine the protein content of the *Sample solution*.

Interfering substances. It is possible to limit the effect of interfering substances by precipitating, as indicated below, the sample protein: add 0.1 volume of 50% (w/v) trichloroacetic acid solution to one volume of *Sample solution*, discard the supernatant and dissolve the precipitate in a small volume of 0.5 M sodium hydroxide. Use the solution obtained to prepare the sample solution.

METHOD 6

This fluorimetric method was designed based on a derivation of the protein by *o*-phthalaldehyde which reacts with the protein primary amines, that is, the *N*-terminal amino acid and the α -amine

function of the lysine residues. Assay sensitivity can be improved by pre-hydrolysis of the protein, prior to adding *o*-phthalaldehyde. Hydrolysis frees the α -amine function from the constituent amino acids of the protein and which enables it to react with the phthalaldehyde reagent. This method is applicable to small amounts of protein.

The primary amines contained in, for example, tromethamine buffers and amino acid buffers react with phthalaldehyde and are therefore to be avoided or eliminated. Ammonia in high

concentration also reacts with phthalaldehyde. The fluorescence resulting from the amine phthalaldehyde reaction may be unstable. Using automated processes to standardize the method can improve its accuracy and feasibility.

Use distilled water for the preparation of all buffers and reagents to be used in this method.

Sample solution. Weigh an appropriate amount of the sample and dissolve in 0.9% (w/v) sodium chloride solution to obtain a concentration within the concentration range of *Standard Solutions*. Adjust the pH of the solution to 8 –10.5 before adding the *Phthalaldehyde Reagent*.

Standard solutions. Weigh the reference substance corresponding to the protein to be measured and dissolve in 0.9% sodium chloride solution (w/v). Take samples of the solution and complete the volume with a 0.9% (w/v) sodium chloride solution to obtain at least five standard solutions with concentrations between 10 $\mu\text{g/mL}$ and 200 $\mu\text{g/mL}$ and uniformly distributed over the chosen range. Adjust pH of solutions to 8-10.5 before adding *Phthalaldehyde Reagent*.

Blank solutions. Use 0.9% sodium chloride solution (w/v).

Borate buffer. Weigh 61.83 g of boric acid, dissolve in distilled water and adjust pH to 10.4 with potassium hydroxide solution. Adjust to 1000 mL with distilled water and homogenize.

Phthalaldehyde stock solution. Weigh 1.20 g of phthalaldehyde and dissolve in 1.5 mL of methyl alcohol, add 100 mL of *Borate buffer* and homogenize. Add 0.6 mL of 30% (w/v) Macrogol 23 lauric ether solution and homogenize. Store the solution at room temperature and use within three weeks of preparation.

Phthalaldehyde Reagent. To 5 mL of *Phthalaldehyde Stock Solution* add 15 μL of 2-mercaptoethanol. Prepare the reagent at least 30 minutes before use and within 24 hours upon preparation.

Method. Homogenize 10 μL of the *Sample Solution* and each of the *Standard Solutions* with 0.1 mL of *Phthalaldehyde Reagent* and allow to stand at room temperature for 15 minutes. Add 3 mL of 0.5 M sodium hydroxide and homogenize. Determine the fluorescence intensity (**5.2.15**) of the *Standard Solution* and *Sample Solution* samples at the excitation wavelength of 340 nm and the emission wavelength of 440 nm to 455 nm. Determine the fluorescence intensity of a sample once as irradiation causes a decrease in fluorescence intensity.

Calculations The relation between fluorescence intensity and protein content is linear. Plot the fluorescence intensities obtained with the *Standard Solutions* according to the protein content of these solutions and determine the calibration curve by linear regression analysis. From the calibration curve and the fluorescence intensity of the *Sample Solution*, determine the protein content of the *Sample Solution*.

METHOD 7

This method was developed based on the quantification of proteins by nitrogen assay. The presence of other nitrogenous substances in the sample may affect the result of the protein assay. The techniques used to measure nitrogen lead to the destruction of the sample during the analysis, but are not limited to the determination of proteins in an aqueous medium.

Method A. Proceed as indicated for the measurement of nitrogen after mineralization by sulfuric acid (5.3.3.2) or use instruments available on the market adapted to the nitrogen assay by the Kjeldahl method.

Method B. There are devices on the market adapted for the nitrogen assay. Most of them use pyrolysis (combustion of the sample in the presence of oxygen at temperatures close to 1000°C), which causes the formation of nitrogen monoxide (NO) and other oxides to form NO_x from the nitrogen existing in the sample. Certain instruments convert these nitrogen oxides into nitrogen gas which is quantified by thermal conductimetry. Others mix nitrogen monoxide (NO) with ozone (O₃) to produce nitrogen dioxide in the excited state (NO₂) which emits light radiation when it decreases and is quantified by chemiluminescence. A reference product, relatively pure, and similar in composition to the protein to be dosed is used to optimize the injection and pyrolysis parameters and to assess the analysis reproducibility.

Calculations The protein content is calculated by dividing the nitrogen content of the sample by the (known) nitrogen content of the protein which can be determined either from the chemical structure of the protein or by comparison with an appropriate reference substance.

5.5.1.15 DETERMINATION OF ANTI-D HUMAN IMMUNOGLOBULIN

The activity of anti-D human immunoglobulin is evaluated by comparing the amount required to produce the agglutination of D-positive erythrocytes and that of a standard solution, measured in international units, necessary to produce the same effect. The International Unit corresponds to the activity of a certain quantity of the international reference preparation. Correspondence between international units and the international standard solution is indicated by the World Health Organization.

Use a mixture of D-positive erythrocytes, less than seven days old and stored under appropriate conditions, obtained from at least four donors from the OR₁R₁ group. To an appropriate volume of erythrocytes, previously washed three times with 0.9% (w/v) sodium chloride solution, add an equal volume of bromelain RS, allow to stand at 37°C for 10 minutes. Centrifuge, discard the supernatant liquid and wash the erythrocytes three times with 0.9% sodium chloride solution (w/v). Suspend 20 volumes of erythrocytes in a mixture of 15 volumes of inert serum, 20 volumes of 30% (w/v) bovine albumin solution, and 45 volumes of 0.9% (w/v) sodium chloride solution. Place the suspension in ice water under continuous shaking.

With a calibrated automatic dilution device prepare sample and standard solution dilutions in a 0.5% (w/v) bovine albumin and 0.9% (w/v) sodium chloride solution.

Use an appropriate device for continuous automatic analysis; keep the temperature in the kits at 15°C with the exception of the incubation spirals. Aspirate the erythrocyte suspension at a flow rate of 0.1 mL per minute and a 0.3% (w/v) methylcellulose 450 solution at a flow rate of 0.05 mL per minute into the device inlet kits.

Introduce the sample and standard solution dilutions at a rate of 0.1 mL per minute for two minutes and then the diluent at a rate of 0.1 mL per minute for four minutes before introducing the next

dilution. Introduce air at a rate of 0.6 mL per minute. Incubate at 37°C for 18 minutes and then disperse the spirals by introducing, at a rate of 1.6 mL per minute, a 0.9% (w/v) sodium chloride solution containing an appropriate wetting agent (e.g. polysorbate 20 at a final concentration of 0.2 g/L) to avoid changing the continuity of the bubbles. Allow the agglutinates to settle and decant twice, the first time at 0.4 mL per minute and the second time at 0.6 mL per minute. Lyse the residue of unagglutinated erythrocytes using a solution of 10 to 0.5% (w/v) of octoxynol, 0.02% (w/v) potassium ferricyanide, 0% sodium bicarbonate .1% (w/v) and potassium cyanide at 0.005% (w/v), with a flow rate of 2.5 mL per minute. A 10-minute delay coil is introduced to allow for the hemoglobin transformation.

Carry out continuous registration (5.2.14) of the hemolysate absorbance at a wavelength from 540 to 550 nm.

Determine antibody concentrations for which there is a linear relationship between concentration and change in absorbance (ΔA). Based on the results, plot a calibration curve and use the linear portion of the curve to determine sample activity. Calculate sample activity in international units per milliliter using the formula:

$$\frac{a \times d}{D}$$

where

a = activity of the reference preparation in international units per milliliter at a dilution of 1 in D ;

d = sample dilution factor that corresponds to a given value of ΔA ;

D = dilution factor of the standard solution corresponding to the same ΔA value.

5.5.1.16 DETERMINATION OF IMMUNOGLOBULIN Fc FUNCTION

REAGENTS

Stabilized human blood. Carry out a phlebotomy to collect group O human blood in an ACD-type conservative anticoagulant solution. Store stabilized human blood at 4°C for not more than three weeks.

Phosphate buffered saline solution pH 7.2. Weigh 1.022 g of anhydrous sodium phosphate dibasic, 0.336 g of monobasic sodium phosphate and 8.766 g of sodium chloride, dissolve in 800 mL of water and adjust to 1000 mL with the same diluent.

Magnesium and calcium stock solution. Weigh 1.103 g of calcium chloride and 5.083 g of magnesium chloride, dissolve in water and adjust to 25 mL with the same diluent.

Barbital buffer stock solution. Weigh 207.5 g of sodium chloride and 25.48 g of sodium barbital, dissolve in 4000 mL of water and adjust to pH 7.3 with M hydrochloric acid. Add 12.5 mL of the *Magnesium and calcium stock solution* and adjust to 5000 mL with water. Filter through membrane (0.22 μm) and store at 4 °C in a glass container.

Albumin-barbital buffer. Weigh 0.150 g of bovine albumin, dissolve in 20 mL of *Barbital Buffer Stock Solution* and adjust to 100 mL with water.

Tannic acid solution. Weigh 10mg of tannic acid and dissolve in 100 mL of pH 7.2 *Phosphate Buffered Saline Solution*. Prepare immediately before use.

Guinea pig complement. Homogenize the sera obtained from at least 10 guinea pigs. Separate serum from clotted blood by centrifugation at a temperature of approximately 4°C.

Store the serum, in small portions, at a temperature below -70 °C. Immediately before starting hemolysis by the action of complement, dilute to 125 –200 CH₅₀ per milliliter with *Barbital Albumin Buffer* and, during the assay, keep the diluted solution in ice bath.

Rubella antigen. Rubella antigen suitable for hemagglutination inhibition titrations. Titration > 256 HA units.

PROCEDURE

Tannic acid treatment of human erythrocytes. Separate erythrocytes by centrifugation of an appropriate volume of stabilized human blood. Wash erythrocytes at least three times with *Phosphate Buffered Saline Solution* at pH 7.2 and then suspend at 2% (v/v) in *Phosphate Buffered Saline Solution* at pH 7.2. Use 0.1 mL of *Tannic Acid Solution* and complete 7.5 mL with *Phosphate buffered saline solution* of pH 7.2 (final concentration 1.3mg/L); homogenize one volume of the fresh-prepared dilution with 1 volume of the red cell suspension and incubate at 37°C for 10 minutes. Collect the tannic acid-treated erythrocytes by centrifugation (400 –800 g, for 10 minutes), discard the supernatant and wash the erythrocytes once with *Phosphate buffered saline solution*, pH 7.2. Suspend 1.0% (v/v) tannic acid-treated erythrocytes in *phosphate buffered saline solution*, pH 7.2.

Addition of antigens to erythrocytes. In an appropriate volume (v_s) of tannic acid-treated erythrocytes, add 0.2 mL of rubella antigen per 1.0 mL of erythrocytes and incubate at 37°C for 30 minutes. Collect erythrocytes by centrifugation (400-800 g for 10 minutes) and discard the supernatant, leaving a volume of 200 µL. Add a volume of *Barbital Albumin Buffer* equal to the volume of the discarded supernatant, shake until erythrocytes are suspended, collect them as described above and repeat the wash. Complete the remaining volume obtained from 0.2 mL to three-quarters of v_s , thus obtaining the initial volume (v_i).

Homogenize 900 µL of *Barbital Albumin Buffer* with 100 µL of v_i , which is thus reduced to the residual volume, and determine the initial absorbance at 541 nm (A). Dilute v_r by a factor equal to A using *Barbital Albumin Buffer*. This gives the final adjusted volume $v_f = v_r \times A$ of sensitized human erythrocytes and a value for A of 1.0 ± 0.1 in the case of a 1/10 dilution. *Antibody binding to erythrocytes with tannic acid and covered with antigen.* Prepare, in duplicate, and successively, the following solutions using for each solution, separately, a semi-micro cuvette (for example, disposable plates) or a test tube for each solution:

(1) *Sample solutions.* If necessary, adjust the sample to pH 7 by adding, for example, M sodium hydroxide. Use sample volumes containing, respectively, 30 mg and 40mg of immunoglobulin and complete 900 µL with *Barbital Albumin Buffer*.

(2) *Standard solution.* Prepare the solution as described for *Sample Solution* from a human immunoglobulin reference standard.

(3) *Complement Evidence.* 900µL of *Barbital Albumin Buffer*. Add 100µL of sensitized human erythrocytes to each cuvette/test tube and homogenize carefully. Allow to stand for 15 minutes, add 1000 µL of *Barbital Albumin Buffer*, collect the erythrocytes by centrifugation (1000 g for 10 minutes) from the cuvette/test tube and remove 1900 µL of the supernatant. Replace this volume with 1900 µL of *Barbital Albumin Buffer* and repeat the washing procedure leaving a final volume of 200 µL. Samples can be stored in closed cuvettes/test tubes at 4°C for 24 hours.

Complement activation hemolysis. To determine hemolysis, add 600 µL of *Barbital Albumin Buffer* heated to 37 °C to the sample, carefully suspend the erythrocytes by pipetting them repeatedly (at least five times) and place the cuvette in the sample holder of a spectrophotometer with thermostat. After two minutes, add 200 µL of *Guinea Pig Complement* diluted to 125 – 200 CH₅₀/mL, carefully homogenize the mixture, pipetting the mixture twice and immediately after the second pipetting start recording the absorbance at 541 nm as a function of time, using *Barbital-albumin buffer* as compensation liquid. Stop recording if the absorbance versus time curve clearly exceeds the inflection point.

Assay. Determine the slope (S) of the hemolysis curve at the approximate point of inflection by segmenting the curve in the region of greatest slope by appropriate time ranges (for example, Δt = one minute) and calculating S, expressed in ΔA per minute between the points of adjacent intersection. The highest value of S corresponds to (S_{exp}). Also determine the absorbance at the beginning of the curve (A_s) by extrapolating the curve, which is almost always linear and parallel to the time axis in the first minutes of the registration. Correct S_{exp} according to the formula:

$$S' = \frac{S_{exp}}{A_s}$$

For each preparation, calculate the arithmetic mean of the S' values. Calculate the function index F_c (I_{Fc}) from the formula:

$$I_{Fc} = \frac{S' - S'_c}{S'_s - S'_c}$$

where

S' = arithmetic mean of the slope corrected for the sample;

S_s' = arithmetic mean of the slope corrected for the standard;

S_c' = arithmetic mean of the slope corrected for the complement evidence;

Calculate the index of the function F_c for the sample. The value is not inferior to that indicated by the standard manufacturer.

5.5.1.17 HUMAN LEUKOCYTE ANTIGENS (HLA)

The HLA complex comprises a group of genes located on the short arm of chromosome 6 and grouped into three sub-regions: HLA class I (A, B and C), HLA class II (DR, DQ and DP) and HLA class III, where only HLA class I and II genes determine tissue compatibility. This genetic complex comprises the Major Histocompatibility Complex (MHC) of human beings. In the HLA system, sets of codominant genes that express themselves as glycoprotein molecules on the surface of cells are observed. These molecules are known as "Human Leukocyte Antigens". Thus, it can be understood that we have HLA genes, which produce HLA antigens. These are classified into several Loci (A, B, C, DR, DQ, DP), each with several alleles.

Table 1 - HLA system.

HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQ
A1	B5	Cw1	DR1	DQ1
A2	B7	Cw2	DR103	DQ2
A203	B703	Cw3	DR2	DQ3

HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQ
A210	B8	Cw4	DR3	DQ4
A3	B12	Cw5	DR4	DQ5(1)
A9	B13	Cw6	DR5	DQ6(1)
A10	B14	Cw7	DR6	DQ7(3)
A11	B15	Cw8	DR7	DQ8(3)
A19	B16	Cw9(w3)	DR8	DQ9(3)
A23(9)	B17	Cw10(w3)	DR9	
A24(9)	B18	Cw12	DR10	
A2403	B21	Cw13	DR11(5)	
A25(10)	B22	Cw14	DR12(5)	
A26(10)	B27	Cw15	DR13(6)	
A28	B35	Cw16	DR14(6)	
A29(19)	B37	Cw17	DR1403	
A30(19)	B38(16)		DR1404	
A31(19)	B39(16)		DR15(2)	
A32(19)	B3901		DR16(2)	
A33(19)	B3902		DR17(3)	
A34(10)	B40		DR18(3)	
A36	B40(05)		DR51	
A43	B41		DR52	
A66(10)	B42		DR53	
A68(28)	B44(12)			
A69(28)	B45(12)			
A74(19)	B46			
	B47			
	B48			
	B49(21)			
	B50(21)			
	B51(5)			
	B5102			
	B5103			
	B52(5)			
	B53			
	B54(22)			
	B55(22)			
	B56(22)			
	B57(17)			
	B58(17)			
	B59			
	B60(40)			
	B61(40)			
	B62(15)			
	B63(15)			
	B64(14)			
	B65(14)			

HLA-A	HLA-B	HLA-C	HLA-DR	HLA-DQ
	B67			
	B70			
	B71(70)			
	B72(70)			
	B73			
	B75(15)			
	B76(15)			
	B77(15)			
	B7801			
	Bw4			
	Bw6			

SEROLOGICAL CLASSIFICATION OF THE HLA SYSTEM

The serological classification of the HLA system can be determined using the complement-dependent microlymphocytotoxicity assay, also known as the Terasaki and McClelland method modified by Bodmer, which is the most commonly used method. In this test, specific mono or polyclonal sera against HLA system antigens present in lymphocytes are used. Viable cells are extracted from peripheral blood, lymph nodes, spleen and others.

With this serological assay it is possible to measure cell mortality through complement activation (rabbit) in the presence of specific antigen-antibody combinations. The antigen-antibody complement reaction is measured by viewing the test under a microscope at 100 to 150 times magnification, with phase contrast illumination and a vital stain, using diluted eosin Y, propidium iodide or trypan blue in 1% aqueous solution (w/v). Dead cells (which have the antigen detected by the specific antiserum) will absorb the contrast and show an appropriate color change. Negative cells (those that do not have the antigen detected by the specific antiserum) remain viable and exclude the contrast.

In these tests, plates with a 72-well "V" bottom and with specific anti-HLA human serum of murine origin or ready-to-use HLA classification trays, also known as Terasaki plates, are used, provided they are regulated by the competent authority. In this type of ready-to-use plate, the operationally monospecific and polyspecific or multispecific antisera for HLA antigens are already fixed. Positive and negative controls must always be used.

Ready-to-use plates or trays are typically prepared with 1 µL of antisera in each well, 0.1% sodium azide as preservative and phenol red as pH indicator, and are coated with 4 µL to 5 µL of mineral oil to avoid dryness and prolong the time of use.

Sample preparation (lymphocyte suspension) using the density gradient separation method: collect 10 mL of whole blood in a vacuum test tube with heparinized solution, ACD or sodium citrate as anticoagulant. Gently homogenize by inversion a few times and centrifuge the blood for 10 minutes at 700 to 900 g to obtain a buffy coat.

Note: centrifugation can be replaced by the use of 2 mL of a 5% dextran solution, which must be added to the whole blood sample, using an anticoagulant. Allow the mixture to stand for 15 minutes at a temperature of 37 °C. The use of this solution allows the sedimentation of red blood cells.

Carefully remove the buffy coat, using a Pasteur pipette, the volume of which should be 2 mL, transfer to a clean 17 x 100 mm test tube containing 5 mL of phosphate-buffered saline solution (PBS) and

homogenize. Distribute 4 mL of gradient solution of polysucrose and meglumine sodium diatrizoate, which should be at a temperature between 20 °C to 22 °C, in a clean 17 x 100 mm test tube. Carefully add the buffy coat suspension onto the gradient solution of polysucrose and meglumine sodium diatrizoate, by flowing through the walls of the tube, which should be in an inclined position, without mixing the layers. Centrifuge for 20 minutes at 700 g. After centrifugation, mononuclear cells can be seen as a narrow band at the interface between the plasma/diluent and the gradient solution. Aspirate the entire mononuclear cell layer and transfer to a 17 x 100mm test tube. Dilute with 4 mL of PBS. Centrifuge for 10 minutes at 600 g. Remove supernatant, carefully resuspend cell aggregate, add 4 mL of PBS and centrifuge for 10 minutes at 600 g. Remove the supernatant and resuspend the cell aggregate in 1 ml of cell culture medium developed by Moore *et al* (RPMI-1640 medium with buffer for HEPES) with 20% of PBS. Examine the cell suspension on a cell counter. Assess purity and perform a cell count. Adjust cell concentration to $2-3 \times 10^6$ cells/mL.

Viability test: add one drop of 0.4% trypan blue dye in aqueous solution and one drop of cell suspension or 19 drops of 0.4% trypan blue dye in PBS and one drop of cell suspension in a clean test tube and then mix well. Incubate the mixture at room temperature for 15 minutes. Examine cell viability on a cell counter. Viable cells have intact cell membranes and appear smooth; are able to exclude trypan blue and consequently will not be stained. Non-viable cells do not have intact cell membranes and do not appear smooth; are unable to exclude trypan blue and consequently will be stained. If there are non-viable cells, the sample should not be used.

Separation of B and T cells

Use one of the methods described below.

Method I. Nylon Wool Column Separation

B and T lymphocytes are easily separated using the nylon wool column method. B cells and macrophages have the attribute of adhering to nylon wool, whereas T cells do not.

Method II. Separation through magnetic particles

Using magnetic particles, it is possible to separate T lymphocytes from B lymphocytes. The immunomagnetic spheres are made up of superparamagnetic particles with monoclonal antibodies anti-CD2 for T lymphocytes and anti-CD19 for B lymphocytes, coupled to their respective surfaces. The spheres can be collected using a magnetic field, which after removal does not leave any residual magnetism in the spheres.

PROCEDURE

Use one of the methods described below.

Method I. Microlymphocytotoxicity test – Contrast exclusion

Prepare a lymphocyte suspension with a minimum viability of 80%, without excessive contamination by non-lymphoid cells. Using ready-to-use HLA grading trays, thaw and allow trays to reach room temperature. Using blank plates, add 1 µL of the corresponding antiserum to each properly labeled well. Add 1 µL of lymphocyte suspension to be tested (approximately 3000 lymphocytes), using a 50 µL automatic pipettor, to the top of each test well, taking care not to touch the antisera. Homogenize and examine each well to ensure homogeneity of the lymphocyte suspension with the antiserum. Incubate trays at $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$ for 30 minutes. Add 5 µL of rabbit complement to the test wells using a 250 µL automatic pipettor, taking care not to touch the pipette tip in the antisera/lymphocyte mixture. Incubate trays at $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$ for 60 minutes. Add 2 µL of 5% eosin solution in pre-filtered

aqueous solution to each test well and incubate at $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$ for three to five minutes. Take care not to touch the pipette tip in the antiserum/lymphocyte mixture. Add 5 μL of 12-37% neutralized formalin with a pH of 7.0 ± 0.2 filtered to each test well. Take care not to touch the pipette tip in the antiserum/lymphocyte mixture. Read the test under a microscope at 100 to 150 times magnification under phase contrast illumination.

Method II. Microlymphocytotoxicity test – Fluorescence

Prepare a lymphocyte suspension with a minimum viability of 80%, without excessive contamination by non-lymphoid cells. Using ready-to-use HLA grading trays, thaw and allow trays to reach room temperature. Using blank plates, add 1 μL of the corresponding antiserum to each properly labeled well. Add 1 μL of lymphocyte suspension to be tested (approximately 3000 lymphocytes), using a 50 μL automatic pipettor, to the top of each test well, taking care not to touch the antisera.

Homogenize and examine each well to ensure homogeneity of the lymphocyte suspension with the antiserum. Incubate trays at $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$ for 30 minutes for HLA Class I (A, B and C) and 45 minutes for HLA Class II (DR and DQ). Add 5 μL of rabbit complement to the test wells using a 250 μL automatic pipettor, taking care not to touch the pipette tip in the antisera/lymphocyte mixture. Incubate trays at $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$ for 50 minutes for HLA Class I (A, B and C) and 60 minutes for HLA Class II (DR and DQ). Add 5 μL of 1% propidium iodide in aqueous solution to each of the test wells, taking care not to touch the pipette tip in the antisera/lymphocyte mixture. Cover the plates to avoid dehydration and allow to stand for 15 minutes for the lymphocytes to sediment. Read the test under a microscope at 100 to 150 times magnification under phase contrast illumination. Fluorescence-labeled viable cells are shown in green and non-viable cells are shown in red. The test can also be read using direct fluorescence microscopy, where viable cells will be fluorescent.

Results: non-viable cells (those that have the antigen) absorb propidium iodide, appear enlarged and darkened and demonstrate clear nuclear details. Viable cells (those that do not have the antigen) exclude propidium iodide and appear slightly brighter and smaller compared to non-viable cells. After correction for the percentage of non-viable cells in negative control cells, the test is classified according to **Table 2**.

Table 2 – Classification of cell viability assay.

<i>Non-viable cells (%)</i>	<i>Interpretation</i>
0-10	Negative
11-20	Equivocally negative
21-50	Slightly positive
51-80	Positive
81-100	Strongly positive
0	Illegible

5.5.2 BIOLOGICAL TESTS

5.5.2.1 PYROGENS

The pyrogen test is based on measuring the increase in body temperature in rabbits, after intravenous injection of the sterile solution under analysis. For products well tolerated by animals, use a dose that does not exceed 10 mL/kg, injected in a time not exceeding 10 minutes. For products that require preliminary preparation or special administration conditions, follow the recommendations established in the monograph.

General conditions

Use rabbits of same sex, healthy adults, preferably of the same breed, weighing not less than 1.5 kg. After selection, keep the animals in individual cages in a room with uniform temperature between 20 and 23 °C, free from disturbances that may stress them. The selected temperature can vary up to ± 3 °C.

Carry out conditioning to determine the temperature of the animals, minimum once, up to seven days before starting the test. The animals must be conditioned according to the same test procedure only without product inoculation. Animals that present a temperature rise equal to or greater than 0.5 °C, in relation to the initial temperature, should not be used in the test.

When carrying out the test, use only animals with a temperature equal to or lower than 39.8 °C and that do not present, from one to the other, a variation greater than 1.0 °C.

Temperature record

Use clinical thermometer calibrated with an accuracy of ± 0.1 °C or any other calibrated temperature recording device of equal sensitivity. Introduce the thermometer into the animal's rectum at a depth of approximately 6 centimeters. If a recording device is used, which must remain in the rectum during the test period, contain the rabbits so that they are in a natural standing posture. When using a clinical thermometer, allow the necessary time (previously determined) to elapse to reach the maximum temperature, before proceeding with the reading.

Material

Sterile and non-pyrogenic syringes, needles and glassware. Diluents and extracting or washing solutions must also be sterile and non-pyrogenic.

Procedure

Perform the test in an area specially designed for testing, under controlled environmental conditions, free from disturbances that may stress the rabbits. In the preceding two hours and during the test, withhold food supply. Access to water is allowed but may be restricted during testing.

Not more than 40 minutes before injecting the dose of the product to be tested, record the temperature of each animal through two readings taken with a 30 minute-range. The average of the two readings will be taken as the control temperature required to assess any individual temperature rise subsequent to sample injection.

Prepare the product to be tested as specified in the monograph and heat at $(37 \pm 2) ^\circ\text{C}$. For the pyrogen test of materials for hospital use, wash, with sterile saline solution, the surfaces of the material that come into contact with the product, injection site or the patient's internal tissue. Carry out the procedures ensuring that the solution is not contaminated.

Inject through the marginal ear vein of three rabbits not less than 0.5 mL or more than 10 mL of the solution per kg of body weight or the amount indicated in the monograph. The injection should not last longer than 10 minutes, unless a different time is specified in the monograph. Record each animal's temperature at 30-minute ranges for three hours after injection.

Interpretation

Do not consider the decreases in temperature shown by the animals during the test. The temperature rise is verified by the difference between the highest temperature presented by the rabbit during the test and its control temperature.

If none of the three rabbits present an individual increase in temperature equal to or greater than $0.5 ^\circ\text{C}$, in relation to their respective control temperatures, the product complies with the requirements of the pyrogen test.

If any rabbit shows an increase in temperature equal to or greater than 0.5°C , repeat the test using another five animals.

The product under examination meets the requirements for the absence of pyrogens if at most three of the eight rabbits show individual increases in temperature equal to or greater than $0.5 ^\circ\text{C}$, and if the sum of the individual increases of all rabbits does not exceed $3.3 ^\circ\text{C}$.

5.5.2.2 BACTERIAL ENDOTOXINS

The bacterial endotoxin test is used to detect or quantify gram negative bacterial endotoxins present in specimens for which the test is recommended. The aqueous extract of circulating amoebocytes from *Limulus polyphemus* or *Tachypleus tridentatus* prepared and characterized as LAL reagent is used.

There are two methods with different sensitivity for this test:

1. GEL-CLOT METHOD: based on clot or gel formation (semi-quantitative method)
2. Quantitative PHOTOMETRIC METHODS including:
 - *Turbidimetric method* (based on the development of turbidity after breaking down an endogenous substrate); and
 - *Chromogenic method* (based on color development after breakdown of a chromogenic synthetic peptide complex).

Any of these procedures can be performed, unless otherwise indicated in the monograph.

In the gel-clot method, the determination of the reaction endpoint is carried out from dilutions of the substance under test in direct comparison with parallel dilutions of the standard endotoxin. Levels of endotoxins are expressed in defined endotoxin units (EU). **Note:** 1 EU is equal to 1 IU (international unit).

LAL reagent (*Limulus Amebocyte Lysate sp.*) is prepared for turbidimetric or colorimetric readings and these procedures can be used if they meet the methods requirements. For its calibration, it is necessary to plot a standard curve, obtaining its linear regression, which determines, by interpolation, the endotoxin concentration of the substance under test.

The procedure includes incubation of standard endotoxin to obtain a calibration curve and control solutions with LAL reagent, for a predetermined time and spectrophotometric reading at the appropriate wavelength.

In the turbidimetric method procedure, the reading is taken immediately after the final incubation period whereas for the colorimetric procedure, the enzymatic reaction is interrupted at the end of the time predetermined by the reagent addition, before the readings. For kinetic turbidimetric and colorimetric procedures the absorbance values measured during the reaction period and velocity values are determined for those readings.

GLASSWARE AND DISPOSABLE MATERIALS

All glassware must be depyrogenized in an oven under a validated process. Use a minimum time and temperature of 250 °C for 30 minutes. If using plastic disposables, such as tips and pipettes, only use the certificates that indicate they are free of endotoxins so as not to interfere with the test.

PREPARATION OF REFERENCE STANDARD ENDOTOXIN AND ENDOTOXIN STANDARD

The reference endotoxin standard has a defined potency of 10 000 EU (endotoxin units) per flask. Reconstitute the vial with 5 mL of LAL reagent grade water (pyrogen-free) and vortex agitate intermittently for 30 minutes. Use this concentrated solution (stored in a refrigerator for not more than 14 days) to make serial dilutions. Shake vigorously before use for at least three minutes and proceed with serial dilutions, shaking at least 30 seconds before the next dilutions. After use, discard dilutions due to activity loss by adsorption. For the preparation of the endotoxin standard, follow the supplier's guidelines, certified in the endotoxin report.

Test Preparation

Use LAL reagent with confirmed stated sensitivity. The validity of test results for bacterial endotoxins requires demonstration that the samples, wash solutions or extracts under test do not inhibit or potentiate the reaction, nor do they interfere with the test. Validation is performed by means of the inhibition or potentiation test described for each of the methods indicated. Appropriate negative controls are included. The validation must be repeated if there is a change in the LAL reagent origin, the production method or the formulation of the substance under test.

Sample solution: prepare the sample solution by diluting in LAL reagent grade water. If necessary, adjust the pH of the sample solution so that the LAL reagent mixture with sample has a pH between 6 and 8. The pH can be adjusted using a suitable buffer recommended by the supplier. Acids and bases can be prepared with LAL reagent grade water and validated to be free of endotoxins and interfering factors.

DETERMINATION OF MAXIMUM VALID DILUTION (MVD)

The maximum valid dilution is the maximum allowable dilution of the sample under analysis where the endotoxin limit can be determined. It applies for injections or solutions for parenteral

administration in reconstituted or diluted form for administration, amount of drug by weight, if the volume of the dosage form is variable.

The formula for calculating the MVD is as follows:

$$MDV = \frac{\text{limite de endotoxina}}{\lambda}$$

λ = labeled sensitivity of the LAL reagent.

Note: formula used when the drug endotoxin limit specified in the monograph is in volume (EU/mL).

When the endotoxin limit of the drug specified in the monograph is in weight (EU/mg) or in active drug unit (EU/units), the MVD is calculated by the following formula:

$$MDV = \frac{\text{limite de endotoxina} \times \text{concentração da amostra na solução}}{\lambda}$$

where

λ = labeled sensitivity of the LAL reagent.

The MVD obtained is the limiting dilution factor for the test to be validated.

SETTING ENDOTOXIN LIMIT

The formula for setting endotoxin limit for parenteral drugs is:

$$LE = \frac{K}{M}$$

where

EL = endotoxin limit;

K = human limit dose of endotoxin per kilogram of body weight;

M = maximum dose of product per kg of weight in a period of one hour.

The endotoxin limit is specified in the parenteral drugs individual monographs in EU/mL, EU/mg or EU/unit of biological activity.

GEL-CLOT METHOD

The gel-clot method allows the detection and quantification of endotoxins based on the gelation reaction of the LAL reagent. Labeled LAL sensitivity is the concentration of endotoxin required to cause a gelation of the LAL reagent.

To ensure the accuracy and validity of the assay, tests are required to confirm labeled LAL sensitivity as well as tests to check interfering factors, as described in sample preparation for the test.

Test for confirmation of LAL sensitivity

Confirm the declared LAL sensitivity using not less than one vial of LAL reagent and prepare a series of endotoxin dilutions using the reference standard endotoxin (RSE) or the control standard endotoxin (CSE), with geometric ratio equal to 2 to obtain the concentrations of 0.25λ , 0.5λ , λ and 2λ s, where

λ is LAL declared sensitivity in EU/mL. Carry out the test with all four standard endotoxin concentrations in quadruplicate and include negative controls. The geometric mean of the endpoint concentration, calculated and interpreted below, must be greater than or equal to 0.5λ and lower than or equal to 2λ . Confirmation of LAL sensitivity must be performed for each new LAL batch.

Results and interpretation. The gelling endpoint is the last test in the descending series of standard endotoxin concentration that formed gel. Calculate the logarithmic geometric mean of the gelation endpoints and the mean antilog by the formula:

$$\text{geometric mean of the endpoint concentration} = \text{antilog} \left(\frac{E_e}{f} \right)$$

where

E_e = sum of endpoint concentrations log of the dilution series used;

f = number of replicates.

The LAL reagent sensitivity in EU/mL is calculated by the formula above and must be not less than 0.5λ and not more than 2λ .

Interference tests in the gel-clot method (Inhibition / Potentiation)

Carry out the test on sample aliquots in which there is no detectable endotoxin and on dilutions that do not exceed MVD (maximum valid dilution). Perform the test, as in the test procedure, on the sample without added endotoxin (solution A) and on the sample with added endotoxin (solution B), at concentrations of $\frac{1}{4} \lambda$, $\frac{1}{2} \lambda$, 1λ and 2λ , in quadruplicates, and also testing in parallel the same concentrations of endotoxin in water (solution C) and negative control in LAL reagent grade water (solution D) in duplicate.

Calculate the geometric mean endotoxin concentration of the sample gelling endpoint as described in the test procedure above (test for confirmation of LAL sensitivity).

The test is valid for the sample under analysis if the geometric mean of this concentration is greater than or equal to 0.5λ and less than or equal to 2λ . If the result obtained in the samples in which endotoxin was added is out of the specified limit, the endotoxin inhibition or potentiation test must be repeated after neutralization, inactivation or removal of interfering substances or after dilution of the sample by a factor that does not exceed the MVD. Repeat the test at a higher dilution not exceeding the MDV or use a higher LAL sensitivity so that interference can be eliminated in the analyzed sample. Interferences can be eliminated by suitable treatment such as filtration, neutralization, dialysis or heating.

GEL-CLOT - LIMIT TEST

This test is used when the monograph contains endotoxin limit requirements.

Procedure. Carry out the tests in duplicate with solutions A, B, C and D as follows. Prepare diluted sample solution without adding endotoxin (solution A); with addition of endotoxin (product positive control) at 2λ (solution B); LAL reagent grade water with endotoxin added at 2λ (solution C) and LAL reagent grade water without added endotoxin (solution D – negative control). Dilution of solution A and B must not exceed MVD.

Interpretation The test will only be valid if the replicas of positive controls from solutions B and C form a gel and the replicas of negative controls from solutions A and D do not form a gel. Contrary results will not be valid and must be repeated.

Gel-clot test assay

Homogenize one volume (e.g. 100 μ L) of LAL with an equal volume of the above solutions, sample, standards and negative control of the test in 10 x 75 mm test tubes, in duplicate. Incubate the tubes for one hour at $(37 \pm 1) ^\circ\text{C}$, avoiding vibrations. After this period, remove the tubes one by one, turning 180 degrees and checking the integrity of the gel; if the gel remains firm after inverting the tubes, consider the result as positive, and if there is no gel formation or it is not firm, consider it as negative.

The test will only be valid if the following conditions are met:

- If both replicas of the negative control (D) show negative reactions;
- If both replicas of the positive control of the product (B) show positive reactions;
- If the geometric mean of solution C is within the range of 0.5λ to 2λ .

To calculate the endotoxin concentration of solution A, calculate the endpoint concentration of each replicate in the dilution series by multiplying each endpoint dilution factor by the labeled sensitivity of the LAL reagent (λ). The endotoxin concentration in the test solution is the geometric mean of the concentration of the replicas limit.

If the test is carried out on the diluted sample, determine the endotoxin concentration in the original solution by multiplying the result by the sample dilution factor. If none of the test sample dilutions are positive, express the endotoxin concentration result as lower than the LAL sensitivity (λ) or lower than the LAL sensitivity multiplied by the lowest sample dilution factor. If all sample dilutions show positive reactions, the endotoxin concentration is expressed as equal to or greater than λ multiplied by the highest sample dilution factor.

The sample meets the test requirements if the endotoxin concentration is lower than the individual limit specified in the monograph.

PHOTOMETRIC METHODS

Quantitative photometric methods include:

- A. Turbidimetric kinetic method: (based on the development of turbidity after breaking down an endogenous substrate); and
- B. Chromogenic kinetic method: (based on color development after breakdown of a chromogenic synthetic peptide complex).
- C. Chromogenic limit method (*endpoint*).
- D. Turbidimetric limit method (*endpoint*).

TURBIDIMETRIC METHOD

This method is based on measuring turbidity increase and, depending on the principle employed, it can be classified into two types:

- A. Turbidimetric limit: based on the relation between the endotoxin concentration and the reaction turbidity (absorbance or transmission).
- B. Kinetic turbidimetric: method based on the reaction time (*onset time*) necessary for the reaction mixture to reach a predetermined absorbance or on the turbidity development rate.

The test is performed at a recommended incubation temperature of $(37 \pm 1) ^\circ\text{C}$.

CHROMOGENIC METHOD

This method is based on the measurement of a chromophore released by a chromogenic peptide by the reaction of the endotoxin with the lysate and, depending on the principle employed, it can be classified into two types:

- A. Chromogenic limit test: based on the relationship between the endotoxin concentration and the amount of chromophore released at the end of an incubation period.
- B. Kinetic chromogenic test: method based on the reaction time (*onset time*) necessary for the reaction mixture to reach a predetermined absorbance or on the coloring development rate.

The test is performed at a recommended incubation temperature of $(37 \pm 1) ^\circ\text{C}$.

Blank preparation:

To ensure the accuracy and validity of turbidimetric and chromogenic tests, preparatory tests are performed to ensure that the criteria for the standard curve are satisfactory and that the sample under test does not interfere with the test. Method validation is required when any change in experimental conditions is made and may interfere with the test.

Standard curve criteria

Prepare a standard curve using three endotoxin concentrations, using a prepared endotoxin standard solution, and perform the test at least in triplicate of each concentration, as recommended by the LAL supplier (volume ratio, incubation time, temperature and pH, etc.)

If a range greater than 2 logs is desired, a standard concentration should be added to increase the range of the standard curve. The absolute value of linear correlation R must be greater than or equal to 0.980 for the endotoxin concentration range indicated by the LAL supplier.

TEST FOR INTERFERENCE FACTORS FOR PHOTOMETRIC METHODS

Prepare diluted sample solutions without exceeding the MDV (maximum valid dilution) without endotoxin (solution A) and with added endotoxin (solution B) at a concentration equal to or close to the midpoint of the standard curve. Prepare a series of positive control with endotoxin solutions (solution C) with three different concentrations and also the negative control with pyrogenic water (solution D) and carry out the tests by adding LAL reagent, at least, in duplicate (follow the guidelines of the reagent used with respect to sample and reagent volume, incubation time, etc.), the lowest point of the curve is considered λ .

Calculate the mean recovery of endotoxin added to the sample by subtracting the endotoxin concentration mean in the test solution (solution A), if any, from the mean of the solution whose endotoxin was added (solution B).

The test solution is considered to be interference-free if the level of endotoxin concentration added to the test solution (solution B) is in the range of 50% to 200% recovery, after subtraction of any endotoxin detected in the solution without endotoxin addition.

When endotoxin recovery is within the specification range, interference factors should be removed as described in the gel-clot method section.

Procedure

Follow the procedures described above in the items: *Test preparation and Testing for interference factors*.

Calculations for Photometric Methods

Calculate the endotoxin concentration for each replicate of solution A using the standard curve generated by the positive control series solution C.

The test is only valid if the three following requirements are met:

- The result obtained from solution D (negative control) does not exceed the limit of the blank value required in the description of the lysate used;
- The result obtained with the positive control series, solution C, is in accordance with the validation requirements defined in the standard curve criteria;
- The endotoxin recovery, calculated from the endotoxin found in solution B after subtracting the endotoxin concentration found in solution A, is within the range of 50 to 200%.

Interpretation of results in photometric tests

The sample solution to be examined will comply with the test if the endotoxin concentration mean found in the replicates (solution A), after correction for dilution and concentration, is lower than the endotoxin limit of the product tested.

REAGENTS

Amoebocyte lysate

The amoebocyte lysate is a lyophilizate obtained from the amoebocyte lysate of a horseshoe-shaped crustacean (*Limulus polyphemus* or *Tachypleus tridentatus*). This reagent refers only to the product manufactured in accordance with the regulations of competent authority. The lysate also reacts with some B-Glucans in addition to endotoxins. Lysate preparations that do not react with B-Glucans are also available; they are prepared either by removing or inhibiting factor G, which reacts with glucans. These preparations can be used for endotoxin testing in the presence of glucans.

Reagent Reconstitution

Dissolve the amoebocyte lysate (LAL) in reagent grade water for BET (bacterial endotoxin test) or buffer, without agitation, and store in refrigerator or freezer according to supplier's recommendation.

Bacterial endotoxin test water

Test water is water for injections or water produced by other procedures that demonstrate no reaction with the lysate used at the reagent detection limit.

5.5.2.3 TOXICITY

The toxicity test detects unexpected and unacceptable biological reactivity of drugs and medications. This *in vivo* test is recommended for evaluating the safety of biological products and biotechnology derivatives.

GENERAL TEST

Choice of animals.

Use healthy mice, of either sex, of known strain, not previously used in biological tests. Keep them under uniform diet, unrestricted water and at constant room temperature of $(21 \pm 3) ^\circ\text{C}$. On the test day, select mice weighing between 17 g and 22 g.

Sample preparation

The sample must be prepared as specified in the respective monograph and administered immediately.

Procedure

Use sterile syringes, needles and glassware. Administer, in five mice, the volume of the sample preparation indicated in the monograph, by one of the routes described below.

Intravenous – Inject the dose into the tail vein, maintaining a constant rate of 0.1 mL per second or as indicated in the monograph.

Intraperitoneal – Inject the dose into the peritoneal cavity.

Subcutaneous – Inject the dose in the cervical or abdominal region.

Oral – Administer the dose through a tube or other suitable device.

Interpretation

Keep the animals under observation for 48 hours upon administration or for the time indicated in the monograph. The sample meets the test if all animals survive and not more than one shows abnormal symptoms within the established time range. If one or two animals die, or more than one show abnormal symptoms or unexpected toxicity, repeat the test using another five or fifteen mice, weighing between 19 g and 21 g. The sample meets the test requirements if the number of dead mice does not exceed 10% of the total animals tested, including the original test, and no animals in the second group show symptoms indicative of abnormal toxicity.

TEST FOR BIOLOGICAL PRODUCTS, SERA AND VACCINES

Choice of animals.

Use not less than five mice weighing between 17 g and 22 g and minimum two healthy guinea pigs weighing between 250 g and 350 g.

Procedure

Weigh the animals and record in a proper form before injecting the sample. Unless otherwise specified in the monograph, intraperitoneally inject each animal with the equivalent of one human dose of the preparation, not exceeding 1.0 mL for mice and 5.0 mL for guinea pigs. The human dosage is defined on the label of the preparation under test or on the accompanying package insert.

Interpretation

For a period of at least seven days, observe the animals for signs of illness, weight loss, abnormalities or death. If, during the observation period, all animals survive, do not show responses that are not specific or expected for the product, and do not undergo weight loss, the preparation complies with the test. Otherwise, the test must be repeated for species for which the requirements have not been met. The preparation meets the test if all animals in the second group meet the criteria specified for the initial test.

If, after the second test, the preparation does not meet the requirements, but no deaths are observed in a percentage equal to or greater than 50% of the total number of animals tested, a second retest can be performed in the species in which requirements were not complied with. Use twice as many animals as the initial test. If the animals meet the specified criteria for the initial test, the preparation meets the test.

5.5.2.4 VASOPRESSOR SUBSTANCES

Reference standard preparation

As a standard preparation, use epinephrine bitartrate. This preparation must be kept in opaque, hermetic vials and dried on silica gel for 18 hours before use.

Reference standard solution

Dissolve 91 mg of epinephrine bitartrate (equivalent to 50 mg of epinephrine base $C_9H_{13}NO_3$) in fresh 0.4% (w/v) sodium bisulfite solution. Dilute with water to 50 mL and homogenize. The final solution will contain 1.0 mg of epinephrine (free base) per milliliter. Store, under refrigeration, in an amber hermetic vial. Use for a maximum of six months. Discard the solution when it shows any sign of deterioration, such as a change in color.

Standard dilution

Dilute the epinephrine standard reference solution in saline solution so that the administration of a dose between 0.1 mL and 0.5 mL produces an increase of 20mm to 70mm of mercury in blood pressure.

Method

Select rats, weighing between 275 g and 325 g, and anesthetize with an anesthetic that allows the maintenance of constant blood pressure (free of effect on blood pressure). Immobilize the animal and keep it warm to prevent loss of body heat. Surgically carry out tracheal intubation, if necessary, and expose the femoral or jugular vein, preparing it for intravenous injections. Administer 200 units of heparin per 100 g of body weight. Surgically expose the carotid and cannular artery, connecting it to the manometer set for continuous recording of blood pressure.

Inject, intravenously, 0.1% (w/v) atropine sulfate solution at the rate of 1 mL per kilogram of body weight. Consider the muscarinic receptor sufficiently blocked only if subsequent injections of the recent 0.001% (w/v) acetylcholine chloride solution at a dose of 1 mL per kilogram of weight do not produce a transient drop in blood pressure. If this mechanism is not sufficiently paralyzed, inject a 0.5 mL dose of atropine sulfate solution until complete paralysis.

Procedure

Select dose of standard dilution that produces an increase between 2.7 kPa and 9.3 kPa (20mm to 70mm mercury) in blood pressure. Inject the dosage at constant ranges of minimum five minutes to allow the blood pressure to return to baseline. After each injection, immediately administer 0.2 mL of saline solution to wash the cannula. Ensure response reproducibility by repeating the dose two or more times. Administer a new dose of the standard dilution so as to obtain hypertensive responses approximately 20% greater than the mean of the lower dose responses. Consider the animal apt for the test if (1) the responses to the first selected dose are reproducible between 2.7 kPa and 9.3 kPa (20mm to 70mm of mercury) and (2) significantly lower in relation to the response of higher dose.

Keeping the established time range constant, inject a series of five doses in which the selected dosage of the standard dilution and the dosage of the same volume of the substance under test, diluted conveniently, are alternated. After each of the five injections, measure the change in blood pressure.

Calculate the difference between each sample response and the response mean of the immediately prior and subsequent standard dilution doses. The sample meets the test requirements if the average of these differences means that the responses obtained with the sample solution are not higher than those with the standard dilution. Results must match the pressor activity limit specified for that test in the corresponding monograph.

5.5.2.5 HISTAMINE

Euthanize a guinea pig weighing between 250 g and 350 g, fasting for approximately 24 hours. Resect approximately 10 cm of the ileum distal portion. Wash internally with nutrient solution. Select a portion about two or three centimeters long and tie two thin threads at the ends. Make a small incision in the central portion of the tissue. Transfer to an isolated organ bath, with a capacity of 10 mL to 20 mL, at a controlled temperature between 34 °C and 36 °C under an air stream or a mixture of 95% oxygen and 5.0% CO₂. Attach one of the threads to the bottom of the tank and tie the other to the lever designed to record muscle contractions in the kymograph or other suitable recording system. Adjust the lever for recording ileum contractions with an amplification degree of the order of 20 times. Wash the preparation with the solution and allow to stand for 10 minutes.

Add known volumes – 0.2 mL to 0.5 mL of histamine reference standard solution (1 g/mL) – to obtain an optimal response (higher dose). Wash the ileum three times with nutrient solution. Add successive additions at regular ranges of approximately two minutes. Add new doses of histamine reference standard solution – obtained by diluting the original solution to keep the dose volumes always the same – establishing the dose responsible for the response whose intensity is half the highest dose (lower dose).

Proceed with the test by adding sequences of three doses: lower reference standard dose, solution dose of the substance under test and higher reference standard dose. Adjust the sample dilution so that, in the event of ileum contraction, it is lower than that produced by the higher reference standard dose. Establish the contraction reproducibility by serial repetitions of dose sequences.

Calculate the activity of the substance under test in terms of its equivalent in micrograms per milliliter of histamine (free base), based on the dilutions made. The value found must not exceed the limit established in the monograph.

If no contraction occurs in the above-mentioned test due to the effect of the sample tested, prepare a new sample solution, adding the amount of histamine corresponding to the maximum limit specified in the monograph and observe if the contraction produced is proportional to the amount of histamine added. Consider the test valid if this response is proportional and the reproducibility of the

contractions induced by the dose sequence is confirmed: lower reference standard dose, test substance solution dose and higher reference standard dose. Otherwise, test for vasodepressor substances.

Nutrient solution (prepare at the time of use)

Solution A	50 mL
Atropine sulfate	0.5 mg
Sodium bicarbonate	1.0 g
Anhydrous dextrose (for parenteral use)	0.5 g
Sufficient water for injections for	1000 mL

Solution A	
Sodium chloride	160.0 g
Potassium chloride	4.0 g
Anhydrous calcium chloride	2.0 g
Anhydrous magnesium chloride	1.0 g
Sodium Phosphate Dibasic	0.05 g
Sufficient water for injections for	1000 mL

5.5.2.6 VASODEPRESSOR SUBSTANCES

Preparation of the reference standard. use histamine dihydrochloride, storing in an opaque and hermetic flask, dried on silica gel for two hours, before use.

Reference standard solution. Dissolve, in sterile water for injection, a sufficient and accurately weighed amount of histamine dihydrochloride to obtain a solution containing the equivalent of 1 mg/mL of histamine (free base). Store under refrigeration in an amber glass container with a ground lid, protected from light, for one month. On the test day, prepare a reference standard solution containing the equivalent of 1 µg/mL of histamine (free base), in saline solution.

Sample solution. Proceed as described in the specific monograph.

Method

Carry out the test using a cat weighing not less than 2 kg (weigh adult and healthy cat) (if a female cat, not pregnant) and anesthetize with chloralose or barbiturate injection that allows for the maintenance of uniform blood pressure. Immobilize the animal and protect it to prevent loss of body heat, perform rectal temperature monitoring to maintain physiological limits.

Dissect the femoral or jugular vein, preparing it by inserting a cannula filled with heparin (1000 units/mL of saline solution) for administration of reference standard and sample solutions.

Surgically expose the carotid artery, dissecting it completely from surrounding structures, including the vagus nerve. Insert a cannula connecting it directly to a mercury manometer or other suitable device for continuous recording of blood pressure.

Assess the cat's sensitivity to histamine, injecting at uniform ranges of not less than five minutes, doses corresponding to 0.05 µg (dose A); 0.10 µg (dose B) and 0.15 µg (dose C) of histamine (free base) per kilogram of body weight. After each administration, immediately wash the cannula by injecting approximately 0.5 mL of saline solution to remove residual activity. Repeat administration of dosage B three times to observe response uniformity to the same dose. The animal is considered

apt for the test if the responses to the three dosage levels are clearly differentiated and the responses to the B dose sequence are approximately similar, corresponding to blood pressure drops of not less than 2.7 kPa (20 mm of mercury).

Inject two series of four doses, each series consisting of two injections of the dose specified in the sample monograph, alternated with dose B, always at a uniform range of minimum five minutes.

Measure the change in blood pressure after each injection. Upon analyzing the results, the sample is considered to meet the test requirements if the mean of its depressor responses is lower than that of dose B.

Finish the test by administering standard dose C to check that the response remains higher than dose B: if this does not occur, the test is not valid.

The animal can be used as long as it remains stable and responds adequately to administration of the reference standard solution.

5.5.3 MICROBIOLOGICAL TESTS

5.5.3.1 MICROBIOLOGICAL TESTS FOR STERILE PRODUCTS

Microbial contamination of a product can lead to changes in its physical and chemical properties besides posing a risk of infection and toxi-infection for the user. Thus, pharmaceutical products for oral and topical use (capsules, tablets, suspensions, creams, patches, etc.) that are not required to be sterile must be subject to microbial contamination control.

The quality assurance and manufacturing control provided in best practices must ensure that the product meets the specified requirements, that is, it meets, in addition to other parameters, the acceptable limits for microorganisms.

To carry out the test, microbial limits, the most likely type of contamination in different categories of products and the route of administration must be considered.

Testing nature and frequency varies depending on the raw material and the finished product. Certain categories must be routinely tested for total microbial contamination, such as: those of vegetable, mineral and/or animal origin, as well as those with a high water content (aqueous oral solutions, creams, etc.). For other categories such as synthetic raw materials, tablets, powders, capsules, non-aqueous liquid products, ointments and suppositories, the test frequency can be established based on historical data from both environmental and equipment microbiological monitoring tests. Other criteria to be considered would be microbial load of raw material, manufacturing process, product formulation and the results of water activity determination, when applicable. Low water activity results (less than or equal to 0.75 measured at 25°C), as well as low or high pH, absence of nutrients and addition of preservatives contribute to prevent microbial contamination. These parameters must be considered when establishing a minimum frequency for carrying out microbiological tests.

5.5.3.1.1 GENERAL CONDITIONS

For microbiological tests on non-sterile products, aseptic methods must be used for sampling and testing. The test should preferably be performed in a laminar flow hood and, when possible, use membrane filtration method.

If the sample has antimicrobial activity, it must be conveniently removed or neutralized.

The efficacy and absence of toxicity of the inactivating agent for the considered microorganisms must be demonstrated. If using surface-active substances in sample preparation, the absence of toxicity for microorganisms and compatibility with the inactivating agent must also be demonstrated, as described in *Mesophilic microorganisms total count* (5.5.3.1.2).

SOLUTIONS AND CULTURE MEDIA

The solutions and culture media described are considered satisfactory to perform the prescribed microbial contamination limit tests. However, other media can be used that have similar nutrient and selective properties for the microbial species studied.

<i>pH 7.0 Sodium chloride-peptone buffer solution</i>	
Monobasic potassium phosphate	3.6 g
Disodium phosphate dihydrate	7.2 g

Sodium chloride	4.3 g
Peptone (meat or casein)	1.0 g
Purified water	1000 mL
Sterilize in autoclave using validated cycle.	

pH 7.2 Phosphate Buffer Solution – Stock Solution

Monobasic potassium phosphate	34.0 g
4% Sodium hydroxide	Add approximately 175 mL
Purified water	1000 mL

Dissolve monobasic potassium phosphate in 500 mL of water, adjust pH to 7.2 ± 0.2 with 4% sodium hydroxide. Adjust the volume with water, sterilize and store under refrigeration. When using, dilute the stock solution with water in a proportion of 1 to 800 (v/v) and sterilize.

Washing fluid

Peptic digest meat peptone	1.0 g
Polysorbate 80	1.0 g (if necessary)
Water	1000 mL

Weigh the reagents and dissolve in distilled water under constant agitation. Heat if necessary. Adjust pH so that it is 7.1 ± 0.2 . Sterilize in autoclave using validated cycle.

Universal diluent

Monobasic potassium phosphate	3.6 g
Disodium phosphate dihydrate	7.2 g
Sodium chloride	4.3 g
Meat or Casein Peptone	1.0 g
egg yolk lecithin	3.0 g
L-histidine	1.0 g
Polysorbate 80	30.0 g
Purified water	1000 mL

Weigh the reagents and dissolve in distilled water under constant agitation. Heat if necessary. Adjust pH so that it is 6.8 ± 0.2 . Sterilize in autoclave using validated cycle.

DEY-ENGLEY neutralizing broth

Enzymatic hydrolyzed casein	5.0 g
Bromocresol purple	20.0 mg
Yeast extract	2.50 g
Sodium thiosulfate	6.00 g
Sodium thioglycolate	1.0 g
Sodium bisulfite	2.50 g
Polysorbate 80	5.00 g
Dextrose	10.0 g
Lecithin	7.0 g
Water	1000 mL

Weigh the reagents and dissolve in distilled water under constant agitation. Heat if necessary. Adjust pH so that it is 7.6 ± 0.2 . Sterilize in autoclave using validated cycle.

Soybean-casein digest broth

Peptone from pancreatic casein	17.0 g
Soybean flour obtained by papain digestion	3.0 g
Sodium chloride	5.0 g
Dibasic potassium phosphate	2.5 g
Glucose monohydrate	2.5 g
Purified water	1000 mL

pH 7.3 ± 0.2. Sterilize in autoclave using validated cycle.

Soybean-casein digest agar

Peptone from pancreatic casein	15.0 g
Soybean flour obtained by papain digestion	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Purified water	1000 mL

pH 7.3 ± 0.2. Sterilize in autoclave using validated cycle.

Neutral violet red glucose agar

Yeast extract	3.0 g
Peptone from pancreatic casein	7.0 g
Bile salts	1.5 g
Sodium chloride	5.0 g
Glucose monohydrate	10.0 g
Agar	15.0 g
Neutral red	30.0 mg
Crystal violet	2.0 mg
Purified water	1000 mL

pH 7.4 ± 0.2. Heat to boiling point and do not sterilize in autoclave.

Mossel Enterobacteria enrichment broth

Pancreatic gelatin hydrolysate	10.0 g
Glucose monohydrate	5.0 g
dehydrated bovine bile	20.0 g
Monobasic potassium phosphate	2.0 g
Disodium phosphate dihydrate	8.0 g
Brilliant green	15.0 mg
Purified water	1000 mL

pH 7.2 ± 0.2. Incubate at 100°C for 30 minutes. Cool immediately.

MacConkey Broth

Pancreatic gelatin hydrolysate	20.0 g
Lactose monohydrate	10.0 g
dehydrated bovine bile	5.0 g
Bromocresol purple	10.0 mg
Purified water	1000 mL

pH 7.3 ± 0.2. Sterilize in autoclave using validated cycle.

MacConkey Agar

Pancreatic gelatin hydrolysate	17.0 g
Peptone (meat or casein)	3.0 g
Lactose monohydrate	10.0 g
Sodium chloride	5.0 g
dehydrated bovine bile	1.5 g
Neutral red	30.0 mg
Crystal violet	1.0 mg
Agar	13.5 g
Purified water	1000 mL

pH 7.1 ± 0.2 . Boil for one minute under constant agitation. Sterilize in autoclave using validated cycle.

Xylose lysine deoxycholate agar

Xylose	3.5 g
L-Lysine	5.0 g
Lactose monohydrate	7.5 g
Sucrose	7.5 g
Sodium chloride	5.0 g
Yeast extract	3.0 g
Phenol red	80.0 mg
Agar	13.5 g
Sodium deoxycholate	2.5 g
Ferric ammonium citrate	0.8 g
Sodium thiosulfate	6.8 g
Purified water	1000 mL

Adjust so that after heating pH is 7.4 ± 0.2 . Heat to boiling point and do not sterilize in autoclave.

Salmonella Rappaport Vassiliadis enrichment broth

Soybean peptone	4.5 g
Magnesium chloride hexahydrate	29.0 g
Sodium chloride	8.0 g
Dibasic potassium phosphate	0.4 g
Monobasic potassium phosphate	0.6 g
Malachite green	36.0 mg
Purified water	1000 mL

pH 5.2 ± 0.2 . Sterilize in autoclave at a temperature not exceeding 115°C.

Cetrimide agar

Pancreatic gelatin hydrolysate	20.0 g
Magnesium chloride	1.4 g
Dipotassium sulfate	10.0 g
Cetrimide	0.3 g
Agar	13.6 g
Purified water	1000 mL

Glycerol	10.0 mL

Boil for one minute under constant agitation. Adjust pH to 7.2 ± 0.2 . Sterilize in autoclave using validated cycle.

Mannitol salt agar

Pancreatic casein hydrolysate	5.0 g
Animal tissue peptone	5.0 g
Meat extract	1.0 g
D-mannitol	10.0 g
Sodium chloride	75.0 g
Agar	15.0 g
Phenol red	25.0 mg
Purified water	1000 mL

Boil for one minute under constant agitation. Adjust pH to 7.4 ± 0.2 . Sterilize in autoclave using validated cycle.

Potato dextrose agar

Potato infusion	200.0 g
Dextrose	20.0 g
Agar	15.0 g
Purified water	1000 mL

Disperse 39 g in 1000 mL of water. pH 5.6 ± 0.2 . Sterilize in autoclave using validated cycle. If pH 3.5 is desired, add approximately 14 mL of sterile 10% (w/v) tartaric acid solution to medium cooled at 45°C to 50°C.

Sabouraud dextrose agar 4%

Dextrose	40.0 g
Peptones	10.0 g
Agar	15.0 g
Purified water	1000 mL

pH 5.6 ± 0.2 . Sterilize in autoclave using validated cycle.

Sabouraud dextrose broth

Dextrose	20.0 g
Peptones	10.0 g
Purified water	1000 mL

pH 5.6 ± 0.2 . Sterilize in autoclave using validated cycle.

Nickerson Candida selective agar

Yeast extract	1.0 g
Soy flour peptone	2.0 g
Glycine	10.0 g
Glucose	10.0 g
Bismuth-sulfite indicator	2.0 g
Agar	15.0 g
Purified water	1000 mL

Dissolve 40 g in 1000 mL of water. pH 6.5 ± 0.2 . Sterilize under flowing steam.

Enriched Clostridium medium

Meat extract	10.0 g
Peptone	10.0 g
Yeast extract	3.0 g
Soluble starch	1.0 g
Glucose monohydrate	5.0 g
Cysteine Hydrochloride	0.5 g
Sodium chloride	5.0 g
Sodium acetate	3.0 g
Agar	0.5 g
Purified water	1000 mL

Allow the agar to swell and dissolve by heating to boiling under constant agitation. Adjust pH to 6.8 ± 0.2 . Sterilize in autoclave using validated cycle.

Columbia Agar

Pancreatic casein hydrolysate	10.0 g
Peptic digestion meat peptone	5.0 g
Heart pancreatic digest	3.0 g
Yeast extract	5.0 g
Maize starch	1.0 g
Sodium chloride	5.0 g
Agar, according to gelling power	10.0 g – 15.0 g
Purified water	1000 mL

Allow the agar to swell and dissolve by heating to boiling under constant agitation. Adjust pH to 7.3 ± 0.2 if necessary. Sterilize in autoclave using validated cycle. Cool to 45°C to 50°C and add, if necessary, gentamicin sulfate corresponding to 20mg of gentamicin base, pour into Petri dishes.

5.5.3.1.2 MESOPHILIC MICROORGANISMS TOTAL COUNT

This test enables determining the total number of mesophilic bacteria and fungi in non-sterile products and raw materials and is applied to determine if the product meets the pharmacopoeial microbiological requirements, according to **Table 1** – Microbial limits for non-sterile products (**5.5.3.1.5**). When used for this purpose, the indications given must be followed, including the number of samples taken and results interpretation. The test is not applied to products that contain viable microorganisms as an active ingredient.

This test consists of counting the population of microorganisms that show visible growth, within five days, on *Soybean-casein digest agar* at $(32.5 \pm 2.5)^{\circ}\text{C}$ and within seven days, on *Sabouraud-dextrose agar* (22.5 ± 2.5) $^{\circ}\text{C}$.

Alternative microbiological methods, including automated ones, can be used as long as their equivalence with the pharmacopoeial method has been properly validated.

PREPARATION OF SAMPLES

Water-soluble products: transfer 10 g or 10 mL of the sample mixture to 90 mL of *Sodium Chloride-Peptone Buffer Solution pH 7.0*, *Phosphate Buffer Solution pH 7.2*, *Soybean-casein digest broth* or

other suitable diluent. If necessary, adjust the pH to 6.0 ± 8.0 with 0.1 M HCl or 0.1 M NaOH solution. Prepare serial decimal dilutions with the same diluent.

Water-insoluble non-lipid products: prepare a suspension of 10 g or 10 mL of the sample mixture in *Sodium Chloride-Peptide pH 7.0 Buffer Solution*, *Soybean-casein digest broth* or other suitable diluent. In general, the ratio of diluent to sample is 10:1, but product characteristics may request a change in this ratio. A surfactant such as polysorbate 80, at a concentration of 1 g/L, can be added to facilitate diffusion. If necessary, adjust pH to 6.0 to 8.0. Prepare serial decimal dilutions with the same diluent.

Lipidic-based products:

Membrane filtration method: dissolve 1 g or 1 mL of the sample mixture in 100 mL of membrane filtration sterilized isopropyl myristate (its aqueous extract must have a pH of not less than 6.5) and heated at 40 °C to 45°C. Sterile polysorbate 80 or another non-inhibitory surfactant agent can be used.

Plate counting method: Transfer 10 g or 10 mL of the sample mixture to a flask containing not more than of 5 g of sterile polysorbate 20 or 80 or other non-inhibitory surfactant. Heat, if necessary, at a temperature between 40°C and 45°C.

Carefully homogenize keeping the temperature from 40°C to 45°C. Add suitable diluent from those presented in *General Conditions (5.5.3.1.1)* – Solutions and culture media, previously heated, in the amount necessary to obtain a 1:10 dilution of the initial product.

Homogenize carefully, maintaining a maximum temperature of 40°C to 45°C for the time necessary to form an emulsion; in any case, not more than 30 minutes. Adjust pH between 6.5 to 7.5 if necessary. Prepare serial decimal dilutions with the same diluent added to polysorbate 20 or 80.

Insoluble creams and ointments in isopropyl myristate

Transfer 10 g of the sample mixture to make a 1:10 dilution in *Soybean-casein digest broth* containing 0.1 sodium tetradecyl sulfate, heated at 40°C to 45°C. Homogenize until homogeneous mixture.

Homogenize carefully, maintaining temperature for the time necessary to form an emulsion; in any case, not more than 30 minutes. If necessary, adjust pH to 6.5 to 7.5. Prepare serial decimal dilutions with the same diluent added to 0.1% sodium tetradecyl sulfate.

Aerosols: cool not less than 10 containers of the product in a mixture of alcohol and dry ice for one hour. Open the containers and place them at room temperature for the propellant to be eliminated. Remove 10 g or 10 mL from the containers and transfer the product to filtration apparatus or to a flask containing a pH 7.2 phosphate buffer solution or other suitable diluent to obtain a 1:10 dilution. If necessary, adjust pH to 6.0 to 8.0. Prepare serial decimal dilutions with the same diluent.

Empty capsules: Transfer 10 g of empty capsules to 90 mL of *pH 7.2 Phosphate Buffer Solution* heated at 40°C to 45°C and homogenize for not more than 30 minutes. Adjust volume to 100 mL with water (5:10 dilution). If necessary, adjust pH to 6.0 to 8.0. Prepare serial decimal dilutions with the same diluent.

Gelatin: transfer 10 g of the sample mixture to a vial containing sterile water heated at 40°C to 45°C and allow to stand for one hour (1:10 dilution). Then transfer the flask to water bath at 45°C, shaking

vigorously at frequent ranges. If necessary, adjust pH to 6.0 to 8.0. Prepare serial decimal dilutions in sterile water.

Transdermal device: with sterile tongs, remove the protective film from 10 transdermal devices and place them, adhesive side up, on sterile plates and cover the adhesive side with sterile gauze. Transfer the 10 devices to a minimum of 500 mL of *pH 7.0 Sodium Chloride-Peptone Buffer Solution* containing an appropriate inactivating agent such as polysorbate 80 or soy lecithin. Homogenize vigorously for not more than 30 minutes.

Correlates:

Cotton and gauze: transfer three 3.3 g portions from the most internal parts of the samples to *pH 7.0 Sodium chloride-peptone buffer solution* containing an appropriate inactivating agent. Prepare serial decimal dilutions with the same diluent.

Other correlates: transfer 10 units whose shape and size allow for fragmentation or total immersion in not more than 1000 mL of *pH 7.0 sodium chloride-peptone buffer solution* or other suitable diluent. Allow to be in contact between 10 and 30 minutes. Prepare serial decimal dilutions with the same diluent. For those that cannot be fragmented or immersed, aseptically introduce 100 mL of *pH 7.0 Sodium Chloride-Peptone Buffer Solution* in the container. Homogenize. Use 0.45 µm membrane filtration method.

The preparation method depends on the physical characteristics of the product to be tested. If none of the procedures described are satisfactory, develop a suitable procedure. Some products may require more heat in sample preparation, but sample preparation should not exceed 48°C.

PRODUCT ANALYSIS

Sample quantity

Unless otherwise indicated, use a sample mixture containing 10 g or 10 mL of the product to be examined. Use 10 units for aerosol - liquid or solid form and for transdermal devices.

The quantity to be tested may be reduced in the case of active substances that are formulated under the following conditions: the quantity per unit dose (example: tablet, capsule) is less than or equal to 1 mg. In this case, the quantity of sample to be tested must not be less than the quantity present in 10 unit doses.

For products where the batch size is extremely small (i.e., less than 1000 mL or 1000g), the quantity to be tested must be 1.0% of the batch or less when justified or authorized.

For products where the total number of units in the lot is less than 200, use two units or one unit if the batch is less than or equal to 100 units.

When sampling products in process, collect three samples from the beginning, four from the middle and three from the end of the process. Carry out the test on the mixture of these samples.

PROCEDURES

The determination can be carried out by the *Membrane Filtration*, *Plate Count* or *Multiple Tube Method (NMP)*. The latter is reserved for bacterial determinations that cannot be carried out by one of the other methods and when the product is expected to have a low bacterial density.

The choice of method is determined by factors such as the product nature and the expected number of microorganisms, with chosen method being properly validated.

MEMBRANE FILTRATION

Use filtration apparatus that allows for the transfer of the membrane to the culture media. Cellulose nitrate membranes, for example, can be used for aqueous, oily or weak alcoholic solutions, and cellulose acetate membranes, for strong alcoholic membranes. Prepare the sample using the most suitable method previously determined.

Transfer 10 mL, or the amount of dilution representing 1 g or 1 mL of the sample to be tested, to two membranes and filter immediately. If necessary, dilute the sample to obtain a colony count between 10 and 100 CFU. Wash membranes at least three times with approximately 100 mL of the appropriate wash fluid. Transfer one of the membranes to the surface of a plate containing *Soybean-casein digest agar*, incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for three to five days to determine the number of total aerobic microorganisms. Transfer the other membrane to the surface of a plate containing *Sabouraud-dextrose Agar* and incubate at $(22.5 \pm 2.5)^\circ\text{C}$ for five to seven days for the determination of molds and yeasts.

Calculate the number of UFC per gram or milliliter of product.

When analyzing transdermal devices and medical products, separately filter 10% of the preparation volume, according to the product suitability procedure, and proceed with washing and incubation as described above.

PLATE COUNT

Depth method - Add 1 mL of the sample prepared as described in *Sample preparation*, to a Petri dish and pour, separately, 15 mL to 20 mL of *Soybean-casein digest agar* and *Sabouraud-dextrose agar* kept at 45°C to 50°C . Use two plates for each medium and dilution. Incubate plates containing *Soybean-casein digest agar* at $(32.5 \pm 2.5)^\circ\text{C}$ for three to five days and plates containing *Sabouraud dextrose Agar* at $(22.5 \pm 2.5)^\circ\text{C}$ for five to seven days for determination of the number of total aerobic microorganisms and molds and yeasts, respectively. Only plates with a number of colonies lower than 250 (bacteria) and 50 (molds and yeasts) per plate should be considered for results recording. Use the arithmetic mean of the plates of each medium and calculate the number of CFU per gram or mL of the product.

Surface method - Add 15 mL to 20 mL of *Soybean-casein digest agar* and *Sabouraud-dextrose agar* separately to Petri dishes and allow to solidify. Dry the plates. Add to the culture medium surface 0.1 mL of the sample prepared as described in *Preparation of Samples*. Incubate plates containing *Soybean-casein digest agar* at $(32.5 \pm 2.5)^\circ\text{C}$ for 3 to 5 days and plates containing *Sabouraud dextrose agar* at $(22.5 \pm 2.5)^\circ\text{C}$ for 5 to 7 days for determination of the number of total aerobic microorganisms and molds and yeasts, respectively. Use the arithmetic mean of the plates of each medium and calculate the number of CFU per gram or mL of the product.

Calculation example:

<i>Dilution</i>	<i>Colonies per plates</i>	<i>CFU/g or mL</i>
1:100	293	2.93×10^4
1:100	100	1.00×10^4
1:1000	41	4.10×10^4

1:1000

12

 1.20×10^4

$$Média = \frac{(2,93 + 1,00 + 4,10 + 1,20)}{4} \times 10^4 = 2,30 \times 10^4$$

If the number of colonies on the plates of all dilutions is less than 20, record the count corresponding to the lowest dilution and express it as CFU/g or mL. If plates from all dilutions show no colonies, record the count as less than once the corresponding lowest dilution. For example, if no growth is detected at the 1:100 dilution, express the count as less than 100 CFU/g or mL.

MOST PROBABLE NUMBER

Prepare the sample according to product suitability procedures. Prepare 1:10; 1:100; 1:1000 dilutions. Transfer 1 mL of each dilution to three tubes with 9 mL of *Soybean-casein digest broth*. Incubate all tubes at $(32.5 \pm 2.5)^\circ\text{C}$ for three to five days. Record the number of positive tubes and the number of negative tubes.

If the sample nature makes reading difficult, a suspension for example, perform a subculture for the same broth or for *Soybean-casein digest agar* for two days at the same temperature.

Determine the most probable number of viable microorganism per gram or milliliter of the product according to information on **Table 1**.

Table 1 – Value of Most Probable Number of Microorganisms – MPN.

Number of positive tubes			MPN per g or mL of product	Confidence limit of 95%
Number of g or mL of product per tube				
10 ⁻¹ (0.1)	10 ⁻² (0.01)	10 ⁻³ (0.001)		
0	0	0	<3	0.0 – 9.4
0	0	1	3	0.1 – 9.5
0	1	0	3	0.1 – 10
0	1	1	6.1	1.2 – 17
0	2	0	6.2	1.2 – 17
0	3	0	9.4	3.5 – 35
1	0	0	3.6	0.2 – 17
1	0	1	7.2	1.2 – 17
1	0	2	11	0.4 – 35
1	1	0	7.4	1.3 – 20
1	1	1	11	0.4 – 35
1	2	0	11	0.4 – 35
1	2	1	15	0.5 – 38
1	3	0	16	0.5 – 38
2	0	0	9.2	1.5 – 35
2	0	1	14	0.4 – 35
2	0	2	20	0.5 – 38
2	1	0	15	0.4 – 38
2	1	1	20	0.5 – 38
2	1	2	27	0.9 – 94
2	2	0	21	0.5 – 40

Number of positive tubes			MPN per g or mL of product	Confidence limit of 95%
Number of g or mL of product per tube				
10 ⁻¹ (0.1)	10 ⁻² (0.01)	10 ⁻³ (0.001)		
2	2	1	28	09 – 94
2	2	2	35	09 – 94
2	3	0	29	09 – 94
2	3	1	36	09 – 94
3	0	0	23	05 – 94
3	0	1	38	09 – 104
3	0	2	64	16 – 181
3	1	0	43	09 – 181
3	1	1	75	17 – 199
3	1	2	120	30 – 360
3	1	3	160	30 – 380
3	2	0	93	18 – 360
3	2	1	150	30 – 380
3	2	2	210	30 – 400
3	2	3	290	90 – 990
3	3	0	240	40 – 990
3	3	1	460	90 – 1980
3	3	2	1100	200 – 4000
3	3	3	>1100	

5.5.3.1.3 STUDY OF PATHOGENIC MICROORGANISMS

This method enables verifying the presence or absence of specific microorganisms in selective media. Experimental procedures must include pre-enrichment steps to ensure the recovery of microorganisms, if present in the product. The result must meet the pharmacopoeial microbiological requirements, according to **Table 1** - Microbial limits for non-sterile products (5.5.3.1.5).

Alternative microbiological methods, including automated ones, can be used as long as their equivalence with the pharmacopoeial method has been properly validated.

PROCEDURE

Bile-tolerant gram-negative bacteria

Sample preparation and pre-incubation: prepare the sample using a 1:10 dilution of minimum 1 g or 1 mL of the product to be tested, as described in *Mesophilic microorganisms total count* (5.5.3.1.2), using *Soybean-casein digest broth (Dilution A)* as diluent. Homogenize and incubate at $(22.5 \pm 2.5)^\circ\text{C}$ for two hours and not more than five hours (time required to reactivate the bacteria, but not enough to stimulate the microorganism to multiply).

Absence test: homogenize *Dilution A* and transfer a volume corresponding to 1 g or 1 mL of the product to the *Mossel Enterobacteria enrichment broth* (*Aeromonas* and *Pseudomonas* can also grow on this medium, as well as other types of bacteria). Incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for 24 to 48 hours. Prepare subculture on plates containing *Glucose Neutral Red Violet Agar*. Incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for 18 to 24 hours. The product meets the test if there is no colony growth.

Quantitative test (selection and subculture): dilute the appropriate amount of *Dilution A* into the *Mossel Enterobacteria enrichment broth* to obtain 0.1 dilutions; 0.01 and 0.001 g (or 0.1; 0.01 and 0.001 mL) of the product to be tested. Incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for 24 to 48 hours. For each positive tube, subculture on *Glucose Neutral Red Violet Agar*. Incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for 18 to 24 hours.

Interpretation: Growth of well-developed colonies of Gram-negative bacteria, usually red or reddish, indicates contamination (positive result). Record the positive and negative results. Determine the most probable number of bacteria per gram or milliliter of product according to **Table 1**.

Table 1 – Interpretation of quantitative test results for bile tolerant gram-negative bacteria.

Results for product quantity of			Probable number of bacteria per gram or milliliter of product
0.1 g or 0.1 mL	0.01 g or 0.01 mL	0.001 g or 0.001 mL	
+	+	+	Over 10^3
+	+	-	Less than 10^3 and more than 10^2
+	-	-	Less than 10^2 and more than 10
-	-	-	less than 10 g

Escherichia coli

Sample preparation and pre-incubation: prepare the sample using a 1:10 dilution of not less than 1 g of the product to be tested, as described in *Mesophilic microorganisms total count* (5.5.3.1.2).

Use 10 mL of the dilution for 90 mL of enrichment broth (*Soybean-casein digest broth*) or an amount corresponding to 1 g or 1 mL. Homogenize and incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for 18 to 24 hours.

Selection and subculture: homogenize and transfer 1 mL of the enriched sample to 100 mL of *MacConkey Broth*. Incubate at $(43 \pm 1)^\circ\text{C}$ for 24 to 48 hours. Carry out subculture on *MacConkey Agar* plate and incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for 18 to 72 hours.

Interpretation: The growth of red colonies, usually non-mucous, with characteristic micromorphology of Gram-negative bacillus, indicates probable presence of *E. coli* that must be confirmed by microbial identification tests. The product meets the test if no growth of such colonies is observed or if the microbial tests are negative.

Salmonella

Sample preparation and pre-incubation: prepare the sample using a 1:10 dilution of not less than 10 g or 10 mL of the product to be tested, as described in *Mesophilic microorganisms total count* (5.5.3.1.2). Homogenize and incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for 18 to 24 hours.

Selection and subculture: homogenize and transfer 0.1 mL of the content to 10 mL of *Salmonella Rappaport Vassiliadis enrichment broth*. Incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for 18 to 24 hours. Carry out subculture on a plate containing *Xylose Lysine Deoxycholate Agar* and incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for 18 to 48 hours.

Interpretation: The growth of well-developed red colonies with or without a black center indicates probable presence of *Salmonella* which must be confirmed by microbial identification tests. The product meets the test if no growth of such colonies is observed or if the microbial tests are negative.

Pseudomonas aeruginosa

Sample preparation and pre-incubation: Prepare the sample using a 1:10 dilution of not less than 1 g of the product to be examined, as described in *Mesophilic microorganisms total count (5.5.3.1.2)*. Use 10 mL of the dilution for 90 mL of *Soybean-casein digest broth* or an amount corresponding to 1 g or 1 mL. Homogenize and incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for 18 to 24 hours.

When testing the transdermal device, filter 50 mL of *Soybean-casein digest broth* through sterile membrane and transfer the membrane to 100 mL *Soybean-casein digest broth*. Incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for 18 to 24 hours.

Selection and subculture: homogenize and transfer a loop to a plate containing *Cetrimide Agar*. Incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for 18 to 72 hours. Colony growth indicates probable presence of *Pseudomonas aeruginosa* which must be confirmed by microbial identification tests. The product meets the test if no growth of such colonies is observed or if the identification tests are negative.

Staphylococcus aureus

Sample preparation and pre-incubation: Prepare the sample using a 1:10 dilution of not less than 1 g of the product to be examined, as described in *Mesophilic microorganisms total count (5.5.3.1.2)*. Use 10 mL of the dilution for 90 mL of enrichment broth (*Soybean-casein digest broth*) or an amount corresponding to 1 g or 1 mL. Homogenize and incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for 18 to 24 hours.

When testing the transdermal device, filter 50 mL of enrichment broth through sterile membrane and transfer the membrane to 100 mL of *Soybean-casein digest broth*. Incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for 18 to 24 hours.

Selection and subculture: homogenize and transfer a loop to a plate containing *Mannitol Salt Agar*. Incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for 18 to 72 hours.

Interpretation: the growth of yellow or white colonies surrounded by a yellow zone indicates probable presence of *S. aureus* which must be confirmed by microbial identification tests.

The product meets the test if no growth of such colonies is observed or if the identification tests are negative.

Clostridium

Sample preparation and pre-incubation: Prepare the sample as described in *Mesophilic microorganisms total count (5.5.3.1.2)*. Use two equal fractions corresponding to at least 1 g or mL of the product to be examined. Heat one portion at 80°C for 10 minutes and cool immediately. Inoculate 10 mL of each mixed fraction mixed in two flasks containing 100 mL of *Enriched Clostridium medium*. Incubate in anaerobiosis at $(32.5 \pm 2.5)^\circ\text{C}$ for 48 hours.

Selection and subculture: transfer a loop of each flask to the plate containing *Agar Columbia*. Incubate in anaerobiosis at $(32.5 \pm 2.5)^\circ\text{C}$ for 48 hours.

Interpretation: The growth of catalase negative colonies, with Gram-positive bacillus micromorphology (with or without endospores) indicates probable presence of *Clostridium*. The product meets the test if no anaerobic microorganism growth is observed or if the catalase test is negative.

Candida albicans

Sample preparation and pre-incubation: prepare the sample using a 1:10 dilution of not less than 1 g or mL of the product to be tested, as described in *Mesophilic microorganisms total count (5.5.3.1.2)*. Use 10 mL of the dilution to 90 mL of *Sabouraud-dextrose Broth*. Incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for three to five days.

Selection and subculture: transfer a loop to a plate containing *Sabouraud-dextrose Agar* or *Candida Selective Agar according to Nickerson*. Incubate at $(32.5 \pm 2.5)^\circ\text{C}$ for 24 to 48 hours.

Interpretation: Growth of white colonies on *Sabouraud-dextrose Agar* or brown/black colonies on *Candida selective Agar according to Nickerson* indicates probable presence of *C. albicans* which should be confirmed by microbial identification tests. The product meets the test if there is no colony growth.

5.5.3.1.4 SUITABILITY OF PHARMACOPEIC METHODS

For the suitability of pharmacopoeial methods to non-sterile products, the elimination of any antimicrobial property must be demonstrated before checking the existence of microbial contamination in the products.

The suitability test protocol should adapt the microbial limit test, sample preparation, type of culture medium and buffer solutions, number and type of membrane wash solution as well as incubation conditions. This protocol requires the use of microorganisms for microbial recovery testing.

During the adaptation, demonstrate that the choice of method for qualitative and/or quantitative estimate of viable microorganisms is sensitive, accurate and reliable and that it is capable of eliminating any interference or inhibition during the recovery of viable microorganisms.

Revalidate the suitability method if the test conditions are modified and/or changes occur in the product that could affect it.

For indication purposes, the microorganisms available on the ATCC were listed. The same microorganisms can also be obtained from other sources: INCQS, CIP, NBRC, NCIMB, NCPF, NCTC, NCYC, IMI and IP. The correspondence between the microorganisms and the addresses of the entities that provide them is indicated in *Microorganisms used in tests and assays (5.5.3.5)*.

MESOPHILIC MICROORGANISMS TOTAL COUNT***Maintenance and preparation of test microorganisms***

The lyophilized cultures must be rehydrated according to instructions from suppliers and maintained by transfers to fresh culture media or by freezing or cooling process for period of storage that maintains the culture original characteristics.

Use standardized suspensions of microorganisms as established below. Use maintenance method so that the inoculum does not exceed five passages of the original culture. Perform subcultures of each microorganism (bacterium and fungus) separately as described in **Table 1**.

Table 1 – Preparation and use of microorganisms.

Microorganism	Culture media for maintenance	Culture media for enrichment		Culture media for suitability of count method in product presence	
		Total count of aerobic bacteria	Total count of mold and yeast	Total count of aerobic bacteria	Total count of mold and yeast
<i>Staphylococcus Aureus</i> (ATCC 6538)	Soybean-casein digest agar or Soybean-casein digest broth (32.5 ± 2.5) °C, 18-24 hours	Soybean-casein digest agar and Soybean-casein digest broth ≤ 100 CFU (32.5 ± 2.5) °C, ≤ 3 days	-	Soybean-casein digest agar/MPN Soybean-casein digest broth ≤ 100 CFU (32.5 ± 2.5) °C, ≤ 3 days	-
<i>Pseudomonas aeruginosa</i> (ATCC 9027)	Soybean-casein digest agar or Soybean-casein digest broth (32.5 ± 2.5) °C, 18-24 hours	Soybean-casein digest agar and Soybean-casein digest broth ≤ 100 CFU (32.5 ± 2.5) °C, ≤ 3 days	-	Soybean-casein digest agar/MPN Soybean-casein digest broth ≤ 100 CFU (32.5 ± 2.5) °C, ≤ 3 days	-
<i>Bacillus subtilis</i> (ATCC 6633)	Soybean-casein digest agar or Soybean-casein digest broth (32.5 ± 2.5) °C, 18-24 hours	Soybean-casein digest agar and Soybean-casein digest broth ≤ 100 CFU (32.5 ± 2.5) °C, ≤ 3 days	-	Soybean-casein digest agar/MPN Soybean-casein digest broth ≤ 100 CFU (32.5 ± 2.5) °C, ≤ 3 days	-
<i>Candida albicans</i> (ATCC 10231)	Sabouraud dextrose Agar or Sabouraud Broth (22.5 ± 2.5) °C, 2-3 days	Soybean-casein digest agar ≤ 100 CFU (32.5 ± 2.5) °C, ≤ 5 days	Sabouraud dextrose agar ≤ 100 CFU (22.5 ± 2.5) °C, ≤ 5 days	Casein-soy agar ≤ 100 CFU (32.5 ± 2.5) °C, ≤ 5 days MPN: not applicable	Sabouraud dextrose agar ≤ 100 CFU (22.5 ± 2.5) °C, ≤ 5 days
<i>Aspergillus brasiliensis</i> (ATCC 16404)	Sabouraud dextrose agar or Potato dextrose agar (22.5 ± 2.5) °C 5-7 days or until evident sporulation	Soybean-casein digest agar ≤ 100 CFU (32.5 ± 2.5) °C, ≤ 5 days	Sabouraud dextrose agar ≤ 100 CFU (22.5 ± 2.5) °C, ≤ 5 days	Soybean casein digest agar ≤ 100 CFU (32.5 ± 2.5) °C, ≤ 5 days MPN: not applicable	Sabouraud dextrose agar ≤ 100 CFU (22.5 ± 2.5) °C, ≤ 5 days

Use a sodium chloride-peptone buffer solution pH – 7.0 or phosphate buffer solution pH 7.2 to prepare the suspensions. When preparing the solution of *A. brasiliensis* spores, add to the buffer solution 0.05% of polysorbate 80. Use the suspensions within two hours or within 24 hours if maintained at temperature of 2-8 °C. Longer times may be used, provided that validated.

Nutritional capacity of culture media

For the media indicated on **Table 3**, inoculate a small amount of microorganism, below 100 CFU. Use a plate or tube for each microorganism.

Test each lot of culture medium for its nutritional capacity as described below: *Liquid culture medium*: inoculate less than 100 CFU of the test microorganism in the culture medium indicated. Incubate at adequate temperature and observe the visible growth, comparing with a (blank) control of the same culture medium.

Solid culture medium: inoculate each plate that has the culture medium indicated with less than 100 CFU of the test microorganism. Incubate at adequate temperature and compare the growth achieved, which must not be below 50% in relation to the standardized inoculum.

Negative control: to check the sterility of culture media, put them in incubation for no less than 72 hours in adequate temperature. There must not be growth of microorganisms.

Inoculation of test microorganisms in the sample

Add to the sample diluted and to the control (diluent without sample), as described on *Preparation of sample*, in *Total count of the amount of mesophilic microorganisms (5.5.3.1.2)*, a sufficient amount of microorganism to obtain a concentration of no more than 100 CFU/mL. The inoculum suspension volume must not exceed 1.0% of the volume of the product diluted.

The capacity of the culture medium to detect microorganisms at the presence and absence of the sample must be demonstrated.

To demonstrate the microorganism recovery in the product, use the lowest dilution factor possible. If the recovery is not adequate, an alternative method, such as neutralization, dilution or filtration, must be executed.

A) Neutralization/removal of antimicrobial activity

The number of microorganisms recovered in the diluted sample is comparable with the number of microorganisms in control.

If the growth is inhibited (reduction lower than 50%), modifications must be made to the counting procedure to ensure the validity of results. The modifications include:

- Increase the volume of the diluent or culture medium, keeping the product amount constant.
- Incorporate a specific neutralizing agent or an universal neutralizing agent.
- Associate both procedures above.
- Conduct membrane filtration.

If the modifications in the neutralization method are ineffective, it is possible to explain that the failure is due to the product's antimicrobial activity, which does not allow the development of the control microorganism tested.

B) Neutralizing agents

Neutralizing agents for inhibition of the antimicrobial activity must be added to the diluent selected or to the culture medium, preferably before the sterilization (**Table 2**). Demonstrate its efficacy and

absence of toxicity to the test microorganism by using diluent with neutralizer and product and conducting a blank with diluent and neutralizer, respectively.

Table 2 – Preservative and neutralizing agents.

Preservatives	Neutralizing agent/neutralization method
Alcohol	Dilution
Aldehydes	Dilution, Thiosulfate, Glycine
Bisbiguanides	Lecithin
Mercury chloride and other mercurial components	Thioglycolate*; Sodium thiosulfate
Chlorhexamide	Polysorbates and Lecithin
Quaternary ammonium compounds	Lecithin, Polysorbate 80
Phenolic compounds	Dilution and Polysorbate 80
EDTA	Mg ⁺⁺ and Ca ⁺⁺ ions
Glutaraldehyde	Glycine and Sodium bisulfite
Halogens	Thiosulfate
Sodium hypochlorite	Sodium thiosulfate
Organic acids and their esters	Dilution and Polysorbate 80
Parabens	Polysorbate 80 and Lecithin
Sorbates	Dilution
Beta-lactam antibiotic	Beta-lactamase
Chloramphenicol	Chloramphenicol acetyltransferase
Sulfonamide	P-aminobenzoic acid
Trimethoprim	Thymidine

* Thioglycolate may be toxic to certain microorganisms, especially spores and staphylococci; thiosulfate may be toxic to staphylococci. Use Dey-Engley Neutralizing Broth or Universal Neutralizer.

If the neutralization is not adequate, it is possible to admit that the failure in recovering the inoculated microorganism is attributed to the product's antimicrobial activity. This information is used to indicate that the product is not susceptible to contamination by the microorganisms tested, but it may not inhibit others not included in the list, which are not representative and may be used as a replacement to the ones established.

Recovery of microorganisms in the product

Conduct the tests separately for each test microorganism listed on **Table 3**. Use the sample as prepared on *Inoculation of test microorganisms in the sample*.

A) Membrane filtration

Use a filtrating membrane with 0.45 µm of pore diameter and proven retention effectiveness. Cellulose nitrate membranes, for example, can be used for aqueous, oily or weakly alcoholic solutions, and cellulose acetate membranes, for strongly alcoholic membranes. Use one membrane for each test microorganism.

From the sample prepared, as described in *Inoculation of test microorganisms in the sample*, transfer 10 mL to a membrane filtration equipment and filter immediately. Wash the membrane with an appropriate volume of washing liquid.

For determination of the aerobic microorganism count and for the count of molds and yeasts, transfer the membranes to *Soybean-casein digest agar* and *Sabouraud dextrose agar*, respectively. Incubate in the conditions described on **Table 3** and count the colonies.

B) Plate count

Depth method: use plates with 9 cm of diameter. Add 1 mL of the sample prepared as described on *Inoculation of test microorganisms in the sample* and add 15 mL to 20 mL of *Soybean-casein digest agar* or *Sabouraud dextrose agar* kept at 45 °C to 50 °C. For each microorganism tested, use two plates for each medium and each dilution. Incubate in the conditions described on **Table 1**. Use the arithmetic mean of the plates with each culture medium and calculate the number of CFU.

Surface method: for each 9-cm Petri dish, add 15 mL to 20 mL of *Soybean casein agar* or *Sabouraud dextrose agar* and let it solidify. Dry the plates. Add to the culture medium surface 0.1 mL of the sample prepared as described in inoculation of the microorganism in the sample. For each microorganism tested, use two dishes. Count and calculate the CFU number.

C) Most Probable Number

From the sample prepared as described in *Inoculation of test microorganisms in the sample* (1:10), prepare dilutions 1:100 and 1:1000. Transfer 1 mL of each dilution to three tubes with 9 mL of Soybean Casein Broth each. If necessary, add inactivating agent.

Incubate all tubes at (32.5 ± 2.5) °C for no more than five days. Write down the number of positive tubes. If the sample nature makes reading difficult, execute a subculture to other tubes with the same culture medium or for *Soybean-casein digest agar* for two days at the same temperature. Determine the most probable number of microorganism per gram or milliliter of the product according to information on **Table 3**.

Results and interpretation

When the membrane filtration method and the plate count method are employed, the number of colonies obtained must be no less than 50% (factor 2) of the initial inoculum for each microorganism in the absence of product, and the number of colonies obtained in the diluent must be no less than 50% (factor 2) of the standard inoculum. When the MPN method is employed, the value calculated is in the confidence interval of 95% of the results obtained.

STUDY OF PATHOGENIC MICROORGANISMS

General conditions

Neutralize the sample conveniently if it has antimicrobial activity. If a surfactant agent is used for the sample preparation, demonstrate absence of toxicity for the microorganisms and its compatibility with the inactivating agent, as described in *Neutralization/removal of antimicrobial activity* from item *Total count of the amount of mesophilic microorganisms* from this general method.

Microorganisms isolated from the environment or other species can be included in challenge tests, especially if they represent contaminants that may be introduced during the product manufacturing or during the product use.

Maintenance and preparation of test microorganisms

The lyophilized cultures must be rehydrated according to instructions from suppliers and maintained by transfers to fresh media or by freezing or cooling process for duly qualified storage periods.

Use standardized suspensions of the test strains as established below. Employ maintenance technique so that the inoculum does not exceed five passages of the original cultivation. Cultivate each microorganism (bacterium and fungus) separately.

Use the *Sodium chloride-peptone buffer solution pH 7.0* or the *Phosphate buffer solution pH 7.2* to prepare the suspensions of microorganisms. When preparing the solution of *A. brasiliensis* spores, add to the buffer solution 0.05% of polysorbate 80. Use the suspensions within two hours or within 24 hours if maintained at temperature of 2 °C to 8 °C.

Microorganisms

A) Aerobic microorganisms:

Staphylococcus aureus – ATCC 6538 P

Pseudomonas aeruginosa – ATCC 9027

Escherichia coli – ATCC 8739

Salmonella enterica ssp serotype *typhimurium* – ATCC 14028

Candida albicans – ATCC 10231

Perform subcultures separately in tubes with culture medium *Soybean-casein broth* or *Soybean-casein digest agar* at (32.5 ± 2.5) °C for 18 to 24 hours. Cultivate *Candida albicans* in *Sabouraud dextrose agar* at (22.5 ± 2.5) °C for two to three days. Use *Sodium chloride-peptone buffer solution pH 7.0* or *Phosphate buffer solution pH 7.2* to prepare the suspensions. Use them within two hours or within 24 hours if stored at 2 °C to 8 °C.

B) Anaerobic microorganism:

Clostridium sporogenes – ATCC 11437

Cultivate the strain *Clostridium sporogenes* under anaerobic conditions in *Reinforced Clostridial medium* at (32.5 ± 2.5) °C for 24 to 48 hours. As an alternative method, perform dilutions of a suspension of vegetative cells from *Clostridium sporogenes*. This suspensions of spores can be used as inoculum if maintained at 2 °C to 8 °C for an adequate period..

Nutritional and selective capacity of culture media

For the culture media indicated on **Table 3**, inoculate a small amount of test microorganism, (no more than 100 CFU). Use a Petri dish or a tube for each microorganism. Each lot of culture medium sterilized must be tested for its capacity to promote growth of microorganisms, as described below.

Liquid culture medium: inoculate less than 100 CFU of the test microorganism in the culture medium indicated. Incubate at adequate temperature and observe the visible growth, comparing with a (blank) control of the same culture medium.

Solid culture medium: inoculate each plate that has the culture medium indicated with less than 100 CFU of the test microorganism. Incubate at temperature. The growth obtained must have the standard characteristics of the microorganisms in the medium used.

Negative control

To check the assay conditions, conduct a test on the sterility of culture media. There must not be growth of microorganisms.

Table 3 – Promotion of growth, inhibitory properties and indicative of culture medium.

<i>Culture medium</i>	<i>Property</i>	<i>Test microorganism</i>
Bile-tolerant gram-negative bacterium		
Mossel Enterobacteria enrichment broth	Promotion of growth	<i>Escherichia coli</i> <i>Pseudomonas aeruginosa</i>
	Inhibitory	<i>Staphylococcus aureus</i>
Neutral violet red glucose agar	Presumptive growth	<i>E. coli</i> <i>P. aeruginosa</i>
<i>Escherichia coli</i>		
MacConkey Broth	Inhibitory Promotion of growth	<i>E. coli</i> <i>S. aureus</i>
MacConkey Agar	Presumptive growth	<i>E. coli</i>
<i>Salmonella</i>		
Rappaport Vassiliadis Salmonella enrichment broth	Promotion of growth	<i>Salmonella enterica</i> ssp serotype typhimurium or <i>S. enterica</i> ssp serotype abony <i>S. aureus</i>
	Inhibitory	
Xylose lysine deoxycholate agar	Presumptive growth	<i>S. enterica</i> ssp serotype typhimurium or <i>S. enterica</i> ssp serotype abony
<i>Pseudomonas aeruginosa</i>		
Cetrimide agar	Inhibitory Presumptive growth	<i>P. aeruginosa</i> <i>E. coli</i>
<i>Staphylococcus aureus</i>		
Mannitol salt agar	Inhibitory Presumptive growth	<i>S. aureus</i> <i>E. coli</i>
<i>Clostridium</i>		
Reinforced Clostridial medium	Promotion of growth	<i>Clostridium sporogenes</i>
Columbia Agar	Promotion of growth	<i>C. sporogenes</i>
<i>Candida albicans</i>		
Sabouraud dextrose broth	Promotion of growth	<i>Candida albicans</i>
Sabouraud dextrose agar	Presumptive growth	<i>C. albicans</i>
Nickerson Agar	Presumptive growth	<i>C. albicans</i>

Recovery of microorganisms in the product

For each product to be analyzed, conduct the test as described on *Procedure*, in *Study of pathogenic microorganisms* (5.5.3.1.3).

When homogenizing, add each strain described in the growth promotion. Inoculate the microorganisms individually in inoculums with no more than 100 CFU. The test must be conducted in the smallest period of time.

The microorganisms must be detected by the reactions indicated in the corresponding paragraph, described on *Procedure*, in *Study of pathogenic microorganisms* (5.5.3.1.3).

If the product has antimicrobial activity and it is necessary to modify the methodology, proceed as stated in *Neutralization/removal of antimicrobial activity* from this chapter, using *Soybean casein broth* as diluent.

5.5.3.1.5 MICROBIAL LIMITS

The microbial contamination of a non-sterile product (specialty and pharmaceutical ingredient) may lead not only to its deterioration, with the associated physical and chemical changes, but also to the risk of infection and poisoning to the user. Consequently, oral and topic pharmaceutical products (capsules, tablets, suspensions, creams, etc.), which are not sterile, must be submitted to microbial contamination controls.

Quality assurance and production controls must be such that the microorganisms capable of proliferating and contaminating the product are within the limits specified. The microbial limits must be adequate to several product categories that reflect the most likely type of contamination introduced during the manufacturing, as well as the route of administration, the end consumer (neonates, children, seniors, debilitated people), use of immunosuppressive agents, corticosteroids and other factors. When assessing the results from microbiological tests, the number and types of microorganisms present must be considered in the context of use of the product proposed.

The microbiological test of non-sterile products and pharmaceutical ingredients is conducted according to the methodology described in *Microbiological assays for non-sterile products (5.5.3.1)* and the acceptance limits are described on **Table 1**.

Table 1 – Microbial limits for non-sterile products.

<i>Route of administration</i>	<i>Total aerobic bacteria count CFU/g or mL^a</i>	<i>Total fungi count CFU/g or mL^a</i>	<i>Study of pathogens^{b, c}</i>
1 Finished products of synthetic or biological nature^d			
Aqueous for oral use	10 ²	10 ¹	Absence of <i>Escherichia coli</i> in 1 g or mL.
Not aqueous for oral use	10 ³	10 ²	Absence of <i>Escherichia coli</i> in 1 g or mL.
For rectal use	10 ³	10 ²	-
For topical use (oral, nasal, gingival, cutaneous, auricular mucosa)	10 ²	10 ¹	Absence of <i>Staphylococcus aureus</i> and <i>Pseudomonas aeruginosa</i> in 1 g or mL.
For inhalation	10 ²	10 ¹	Absence of <i>Staphylococcus aureus</i> , <i>Pseudomonas aeruginosa</i> and bile-tolerant Gram-negative bacterium in 1 g or mL.
For vaginal use	10 ²	10 ¹	Absence of <i>Staphylococcus aureus</i> , <i>Pseudomonas aeruginosa</i> and <i>Candida albicans</i> in 1 g or mL.
Transdermal device (limit per unit)	10 ²	10 ¹	Absence of <i>Staphylococcus aureus</i> and <i>Pseudomonas aeruginosa</i> /device.
2 Products of vegetable origin^d			
2.1 Finished product			
For oral use, with active ingredient that was submitted to pre-treatment that reduces the microbial burden.	10 ⁴	10 ²	Absence of <i>Escherichia coli</i> and <i>Staphylococcus aureus</i> in 1 g or mL. Absence of <i>Salmonella</i> in 10 g or 10 mL. Maximum limit of 10 ² bile-tolerant Gram-negative bacterium ^e in 1 g or mL.
For oral use that will be submitted to hot extraction process (infusions or decoctions, for example)	10 ⁷	10 ⁴	Absence of <i>Escherichia coli</i> in 1 g or mL. Absence of <i>Salmonella</i> in 10 g or mL. Maximum limit of 10 ⁴ bile-tolerant Gram-negative bacterium ^e in 1 g or mL.
For oral use, with active ingredient that was not submitted to pre-treatment that reduces the microbial burden.	10 ⁵	10 ³	Absence of <i>Escherichia coli</i> in 1 g or mL. Absence of <i>Salmonella</i> in 10 g or mL. Maximum limit of 10 ³ bile-tolerant Gram-negative bacterium ^e in 1 g or mL.
For topical use (oral, nasal, gingival, cutaneous, auricular mucosa)	10 ²	10 ¹	Absence of <i>Staphylococcus aureus</i> , <i>Pseudomonas aeruginosa</i> and <i>Clostridium</i> in 1 g or mL. Maximum limit of 10 ² bile-tolerant Gram-negative bacterium ^e in 1 g or mL.
For vaginal use	10 ²	10 ¹	Absence of <i>Staphylococcus aureus</i> , <i>Pseudomonas aeruginosa</i> and <i>Candida albicans</i> in 1 g or mL.
For inhalation use	10 ²	10 ¹	Absence of <i>Staphylococcus aureus</i> , <i>Pseudomonas aeruginosa</i> and bile-tolerant Gram-negative bacterium ^e in 1 g or mL.
2.2 Vegetable pharmaceutical ingredients			
Herbal drug (scratched or ground) that will be submitted to pre-treatment that reduces the microbial burden.	10 ⁷	10 ⁴	Absence of <i>Escherichia coli</i> in 1 g or mL. Absence of <i>Salmonella</i> in 10 g or mL. Maximum limit of 10 ³ bile-tolerant Gram-negative bacterium ^e in 1 g.
Herbal drug (scratched or ground) that will not be submitted to pre-treatment that reduces the microbial burden.	10 ⁵	10 ³	Absence of <i>Escherichia coli</i> in 1 g. Absence of <i>Salmonella</i> in 10 g or mL. Maximum limit of 10 ³ bile-tolerant Gram-negative bacterium ^e in 1 g or mL.
Dry extract, fluid extract and tincture	10 ⁴	10 ²	Absence of <i>Escherichia coli</i> in 1 g or mL. Absence of <i>Salmonella</i> in 10 g or mL.

Route of administration	Total aerobic bacteria count CFU/g or mL ^a	Total fungi count CFU/g or mL ^a	Study of pathogens ^{b, c}
3 Products of animal or mineral origin^d			
For oral use	10 ⁴	10 ²	Absence of <i>Escherichia coli</i> and <i>Staphylococcus aureus</i> in 1 g or mL. Absence of <i>Salmonella</i> in 10 g or 10 mL. Maximum limit of 10 ² bile-tolerant Gram-negative bacterium ^e in 1 g or mL.
For topical use	10 ²	10 ¹	Absence of <i>Staphylococcus aureus</i> and <i>Pseudomonas aeruginosa</i> in 1 g or mL.
4 Substances for pharmaceutical use			
Raw material, galenic base	10 ³	10 ²	Absence of <i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i> and <i>Staphylococcus aureus</i> in 1 g or mL.

- (a) A result that is twice the specification value is acceptable due to the higher variability of execution of tests for determination of bioburden. However, when these values are found frequently, it is necessary to review the production process. Thus:
- 10¹ CFU: maximum acceptable value = 20
 - 10² CFU: maximum acceptable value = 200
 - 10³ CFU: maximum acceptable value = 2000 and so forth.
- (b) Results from bacteria and fungi counts within the acceptable limits do not exclude the need for studying pathogens.
- (c) In addition to the microorganisms listed on the Table, the significance of other microorganisms recovered must be assessed taking into account:
- the product use: consider the route of administration to be used;
 - the product nature: is the product susceptible to microbial growth?
 - the user: consider the risk to neonates, babies and debilitated people.
- (d) For products that fit more than one situation, the more restrictive limits will prevail.
- (e) Other enterobacteria.
- (f) For raw materials, the frequency of execution of tests for counting and identifying pathogens must be determined considering their susceptibility to microbial contamination. Results from low water activity (equal to or below 0.75 measured at 25 °C), as well as high or low pH, reduce this susceptibility.

Based on historical data from microbiological monitoring tests, low microbial burden of the raw material, aqueous composition, manufacturing process, formulation, the frequency of the test for determining the microbial limit can be changed for the pharmaceutical dosage forms if they present water activity (Aa) below 0.75 measured at 25 °C. For the correlates, consider as microbial limit the ones expressed according to the route of application.

5.5.3.2 MICROBIOLOGICAL ASSAYS FOR STERILE PRODUCTS

5.5.3.2.1 STERILITY TEST

The sterility tests apply to pharmaceutical ingredients, medicines and health care products that, according to the Pharmacopoeia, must be sterile, being adequate to reveal the presence of bacteria and fungi. However, a satisfactory result indicates that a contaminant microorganism was not found only on the sample examined.

Extending this result to what remains of the lot requires the security that all units from the same lot have been prepared in order to ensure a high probability that the entire lot would be approved on the test. Obviously, this depends on the precautions taken during the operational manufacturing processes, according to Good Manufacturing Practices.

PRECAUTIONS DURING THE TEST

For conducting the sterility test, it is important that the people are adequately trained and qualified.

The tests must be conducted under aseptic conditions using, for example, a Class II type A biosafety cabinet, which must be installed in a clean area, with classification compatible with the environmental control required for conducting the sterility test. For sterility tests on oncogenic drugs, mutagenic drugs, antibiotics, hormones, steroids and others, the tests must be conducted in the class II type B2 biosafety cabinet, which has an exhaust system external to the laboratory environment.

Tests must not be conducted under direct exposure to ultraviolet light or in areas under treatment with aerosols. The conditions must be adequate in order to avoid accidental sample contamination during the test, and also not affect the detection of possible contaminants. Environmental controls of the work areas must be executed regularly (air and surface control, particle counts, determination of speed and direction of air flow, among others).

CULTURE MEDIA

The culture media used for sterility tests are *Fluid thioglycolate medium* and *Soybean casein broth*. The first is used primarily for culture of anaerobic bacteria, although it may also detect the growth of aerobic bacteria. The second is adequate for culture of yeasts, fungi and aerobic bacteria. The media used must meet the requirements from *Tests for promotion of growth of culture media*. Prepare the culture media as described below. Dehydrated formulations can also be used, and it is necessary to demonstrate that, after reconstitution according to the manufacturer's indications, the requirements from *Tests for promotion of growth of culture media* are met. The culture media must be sterilized by a validated process.

Fluid thioglycolate medium

L-cystine	0.5 g
Sodium chloride	2.5 g
Dextrose	5.5 g
Granulated agar (humidity not superior to 15%)	0.75 g
Yeast extract (soluble in water)	5.0 g
Casein obtained by pancreatic digestion	15.0 g
Sodium thioglycolate (or thioglycolic acid)	0.5 g (0.3 mL)
0.1% (w/v) resazurin sodium freshly prepared	1.0 mL
Purified water	1000 mL
pH of medium after sterilization	7.1 ± 0.2

Mix L-cystine, sodium chloride, dextrose, yeast extract and casein from pancreatic digestion with 1000 mL of purified water and heat until complete dissolution. Dissolve the sodium thioglycolate or thioglycolic acid in this solution and adjust the pH with sodium hydroxide *M* so that, after the sterilization, the solution pH is 7.1 ± 0.2. If there is need for filtration, heat the solution again, without letting it reach ebullition, and filter, still warm, in a paper filter. Add the resazurin sodium solution, homogenize and distribute in adequate vials. The medium must present a pink color on its surface that does not exceed one third of the height of its liquid mass. In case of obtaining a medium with pink color in more than one third of its liquid mass, restore the medium by a single heating in water bath or in flowing vapor.

Sterilize employing a validated process. If it is not used immediately, store at temperature between 2 °C and 25 °C according to guidance from the manufacturer. Do not use the medium for a storage period superior to the one it was validated for. The *Fluid thioglycolate medium* must be incubated at (32.5 ± 2.5) °C under aerobic conditions.

Alternative fluid thioglycolate medium

Proceed as described for *Fluid thioglycolate medium* without adding agar and resazurin sodium. The pH after sterilization is 7.1 ± 0.2 . The *Alternative fluid thioglycolate medium* must be incubated at $(32.5 \pm 2.5)^\circ\text{C}$ under anaerobic conditions.

Soybean casein broth

Casein from pancreatic digestion	17.0 g
Soybean flour from papain digestion	3.0 g
Sodium chloride	5.0 g
Dibasic potassium phosphate	2.5 g
Dextrose	2.5 g
Purified water	1000 mL
pH of medium after sterilization	7.3 ± 0.2

Dissolve all components in purified water, heating slowly. Cool down at room temperature and adjust the pH with sodium hydroxide *M* so that, after sterilization, the solution pH is 7.3 ± 0.2 . If necessary, filter for medium clarification. Distribute in adequate vials and sterilize employing a validated process. If it is not used immediately, store at temperature between $(22.5 \pm 2.5)^\circ\text{C}$ or according to guidance from the manufacturer. Do not use the medium for a storage period superior to the one it was validated for. The *Soybean casein broth* must be incubated at $(22.5 \pm 2.5)^\circ\text{C}$ under aerobic conditions.

Media for penicillins and cephalosporins

In cases when the culture media are used for the sterility test of penicillins and cephalosporins through the direct inoculation method, the preparation of *Fluid thioglycolate medium* and of *Soybean casein broth* must be modified as described below. Transfer, aseptically, to sterilized vials with each medium a sufficient amount of β -lactamase to inactivate the antibiotic present in the sample. A representative number of vials having medium with β -lactamase without sample must be incubated during the test period (negative control). Positive controls must also be included to check if all the penicillin or cephalosporin was inactivated. Proceed to the validation test for bacteriostasis and fungistasis, using *Staphylococcus aureus* (ATCC 6538/INCQS 00039) as test microorganism. The observation of typical microbial growth is a confirmation that the concentration of β -lactamase used is adequate.

STANDARDIZATION OF INOCULUM

Usually, it is necessary to make adjustments to obtain a specific density of viable microbial cells (no more than 100 CFU) in the culture medium. To establish a volume that has the recommended density of cells, serial dilutions must be made from a stock suspension, proceeding to count in plates to determine the microbial density obtained with each dilution.

If the procedure is properly standardized, it is possible to reproduce the results with the same microbial strain.

It is recommendable to use subcultures of microorganism up to no more than five transfers, from the original culture.

Note: the culture media used in the standardization of inoculum are the ones described in the chapter *Total count of the amount of mesophilic microorganisms (5.5.3.1.2)* for each microorganism.

Procedure

Using a cultivation loop, transfer the growth of the specific microorganism to a test tube with agar slant indicated for its growth. Seed the culture over the surface of agar slant, in order to obtain an uniform growth film. Incubate in optimal conditions for growth of the test microorganism.

As a suggestion of dilutions for the inoculum, after the period of incubation wash the microorganism growth with 1 mL of 0.9% (w/v) sterile sodium chloride solution or 0.1% (w/v) or sterile peptone water and transfer to a flask with 99 mL of 0.9% (w/v) sterile sodium chloride solution or 0.1% (w/v) sterile peptone water – (stock suspension). Homogenize the suspension manually or in a vortex-type tube shaker.

Prepare serial dilutions (1:100, 1:10000 and 1:1000000) from the stock suspension using 0.9% (w/v) sterile sodium chloride solution or 0.1% (w/v) or sterile peptone water as diluent. Incorporate 1 mL of each dilution in a solid medium that is adequate for the microorganism, previously molten and cooled down at approximately 45 °C. Homogenize and incubate.

Proceed to counting the number of colonies that have developed in the solid medium and select, from the results, the dilution to be used to obtain no more than of 100 CFU per culture medium vial.

Repeat the procedure for each microorganism used.

For preparing the fungal suspension, the 0.9% (w/v) sterile sodium chloride solution can be replaced with sterile purified water.

CULTURE MEDIA SUITABILITY TESTS

The culture media used must comply with the tests described below, conducted before or in parallel with the *Sample sterility test*.

Sterility

To confirm the sterility, a sample that is representative of each lot of the medium must be incubated in the conditions specified for 14 days. For a lot of up to 100 units, it is suggested that 3% to 5% of the samples are submitted to the assay. For a bigger batch, 10 plates or tubes taken randomly must be tested. There must not be microbial growth.

The existence of microbial growth renders the lot of medium useless for the sterility test.

Promotion of growth

Each lot of culture medium sterilized must be tested for its capacity to promote growth of microorganisms. Inoculate, separately, in duplicate, tubes of each medium with a volume of inoculum containing no more than 100 CFU of each microbial strain listed on **Table 1** and incubate according to the conditions specified for each medium. The test for promotion of growth is considered valid if there is evidence of microbial growth, viewed by turbidity and/or by microscopic methods, after three days of incubation of media inoculated with bacteria and after five days of incubation of media inoculated with fungi.

Table 1 – Microorganisms indicated for use in tests for promotion of growth and validation.

<i>Medium</i>	<i>Microorganism</i>	<i>Strain</i>
Fluid thioglycolate medium	<i>Staphylococcus aureus</i>	ATCC 6538, NCTC 10788, NCIMB 9518, CIP 4.83, NBRC 13276, INCQS 00039

Alternative thioglycolate	<i>Pseudomonas aeruginosa</i>	ATCC 9027, NCIMB 8626, CIP 82.118, NBRC 13275, INCQS 00230
	<i>Clostridium sporogenes</i>	ATCC 19404, NCTC 532, CIP 79.3, INCQS 00352 or ATCC 11437, NCIMB 14239, CIP 100651, NBRC 14293
	<i>Clostridium sporogenes</i>	ATCC 19404, NCTC 532, CIP 79.3, INCQS 00352 or ATCC 11437, NCIMB 14239, CIP 100651, NBRC 14293
Soybean casein broth	<i>Bacillus subtilis</i>	ATCC 6633, NCIMB 8054, CIP 52.62, NBRC 3134, INCQS 00001
	<i>Candida albicans</i>	ATCC 10231, NCPF 3179, IP 48.72, NBRC 1594, INCQS 40006
	<i>Aspergillus brasiliensis</i>	ATCC 16404, IMI 149007, IP 1431.83, NBRC 9455, INCQS 40036

Bacteroides vulgatus (ATCC 8482, NCTC 11154, INCQS 00059) can be used alternatively to *Clostridium sporogenes*, when it is not necessary to use a sporulated microorganism.

An alternative to *Staphylococcus aureus* is the *Bacillus subtilis* (INCQS 00001, ATCC 6633, CIP 52.62, NBRC 3134, NCIMB 8054, NCTC 10400).

An alternative microorganism to *Pseudomonas aeruginosa* is the *Kocuria rhizophila* (INCQS 00010, ATCC 9341, CIP 53.65, NCTC 8340).

STORAGE OF MEDIA

If the media prepared are stored in non-hermetically closed vials, they can be used for one month, provided that they are tested for promotion of growth within 15 days from the time of use and that they meet the requisite for the color indicator.

If the media are stored in hermetically closed vials, they can be used for one year, provided that they are tested for promotion of growth within three months from the time of use and that they meet the requisite for the color indicator.

DILUTION AND WASHING FLUIDS

Fluid I

Peptone from meat	1.0 g
Purified water	1000 mL
pH after sterilization	7.1 ± 0.2

Dissolve the peptone from meat in purified water, filter or centrifuge for medium clarification, if necessary, and adjust the pH to 7.1 ± 0.2. Distribute in adequate vials and sterilize employing a validated process.

Preparation for penicillins or cephalosporins. For conducting the assay on sterility of penicillins or cephalosporins through the membrane filtration method, add, aseptically, to the sterilized Fluid I a sufficient amount of β -lactamase to inactivate any residual antibiotic activity in the membrane after filtering the sample.

Fluid II

For every liter of *Fluid I*, add 1 mL of polysorbate 80 before the sterilization. Adjust the pH to 7.1 ± 0.2 . Distribute in adequate vials and sterilize employing a validated process. Use this fluid for products that have lecithin or oil and for healthcare products.

Fluid III

Peptone from meat	5.0 g
Meat extract	3.0 g
Polysorbate 80	10.0 g
Purified water q.s.p.	1000 mL
pH after sterilization	6.9 ± 0.2

Homogenize all components and heat, slowly, until dissolution. Filter, if necessary, and adjust the pH to obtain, after the sterilization, the value of 6.9 ± 0.2 . Distribute in adequate vials and sterilize employing a validated process.

VALIDATION OF ASSAY FOR BACTERIOSTASIS AND FUNGISTASIS

Before establishing a procedure for the test on sterility of pharmaceutical ingredients, medicines or healthcare products, it is necessary to ensure that any bacteriostatic or fungistatic activity inherent to the product has no adverse influence over the test reliability, demonstrating that the procedure employed is adequate for the product being examined.

The test of validation for bacteriostasis and fungistasis must be conducted when the sterility test is executed for the first time for a product and whenever there are modifications in the product formulation and/or in the experimental conditions of the test. The validation must be done before the test on sterility of the product being examined.

Procedure

To conduct the validation test, proceed as described in *Procedures for the sterility test*, employing exactly the same methods, except for the following modifications.

Note: for both methods described below, use the microorganisms previously specified (**Table 1**). Conduct tests on Promotion of growth as positive control. Incubate all vials with the media for no more than five days.

Membrane filtration method. After transferring content from the vial(s) to be tested (as specified on **Table 3**) for the filtration device, add no more than 100 CFU of the test microorganism to the last aliquot of the sterile filter used for membrane washing.

Direct inoculation method. After transferring the content from the vial(s) to be tested (as specified on **Table 3**) to vials with the culture media, add no more than 100 CFU of the test microorganisms to the media.

Interpretation

If the growth of microorganisms obtained after incubation is visibly comparable with the one obtained on positive control (vial without addition of sample), the sample does not present antimicrobial activity under the test conditions or such activity was satisfactorily eliminated. The sterility test can then be conducted without the need for modifications.

If the growth of microorganisms is not obtained in the presence of sample, or if it is not visibly comparable with the one obtained on positive controls, the sample presents antimicrobial activity that was not satisfactorily eliminated under the test conditions. In this case, modifications must be made in the test conditions to eliminate the antimicrobial activity, such as dilution, use of neutralizing substances, increase in the number of washes on the membrane filtration method, or a combination of them. The validation test must be repeated to check if the antimicrobial activity was eliminated by the modification proposed.

PROCEDURES FOR THE STERILITY TEST

The sterility test can be conducted using the membrane filtration or direct inoculation methods according to the nature of the product, except when one of the methods is specified in the individual monograph. In both cases, appropriate negative controls must be included.

Before proceeding to the test, make the asepsis of the external surface of vials and ampoules, dipping them into an adequate antiseptic solution, or employing other procedures for external disinfection of vessels, such as, for example, hydrogen peroxide vapors. In case of articles which packages do not resist this treatment, make the asepsis of samples with a fabric that does not release particles, soaked in antiseptic solution.

Sampling

If not specified in the individual monograph, test the number of units of the sample as listed on **Table 2**. If the sample units present content in sufficient amount (**Table 3**), the content from each unit can be divided into two equal portions for each type of culture medium used. If the sample units do not present content in sufficient amount for each medium, separate double the number of units specified on **Table 2** for conducting the test.

Table 2 – Minimum number of units to be tested due to the lot size.

<i>Number of units from the lot</i>	<i>Minimum number of units to be tested^{a,b}</i>
Parenteral preparations	
Up to 100	10% or 4 units (whichever is highest)
Above 100 to 500	10 units
Above 500	2% or 20 units (whichever is lowest)
Parenteral with large volume	2% or 10 units (whichever is lowest)
Solid antibiotics	
Vials with capacity <5 g	20 units
Vials with capacity ≥ 5 g	6 units
Ophthalmic and other non-injectable preparations	
Up to 200	5% or 2 units (whichever is highest)
Above 200	10 units
Product presented in single-dose package	apply the same recommended for parenteral preparations
Healthcare products	
Up to 100	10% or 4 units (whichever is highest)
Above 100 to 500	10 units
Above 500	2% or 20 units (whichever is lowest)
Bulk solid products	
Up to 4	each unit
Above 4 to 50	20% or 4 units (whichever is highest)
Above 50	2% or 10 units (whichever is highest)

<i>Number of units from the lot</i>	<i>Minimum number of units to be tested^{a,b}</i>
Surgical medical devices	
Catgut and other sutures	2% or 5 packages (whichever is highest) up to a maximum of 20 packages
Up to 100	10% or 4 units (whichever is highest)
Above 100 to 500	10 units
Above 500	2% or 20 units (whichever is highest)

^a sampling specified considering that the content of a vessel is sufficient to inoculate both culture media.
^b for raw materials, the satisfactory sampling can be based on the square root of the total number of vessels in the lot.

Table 3 – Minimum amounts to be used for each culture medium.

<i>Amount per vessel</i>	<i>Minimum volume to be inoculated in each medium (mL)</i>
Liquids (non-antibiotic)	
less than 1 mL	the entire content
from 1 to 40 mL	half the content, but no less than 1 mL
above 40 mL up to 100 mL	20 mL
above 100 mL	10% of the product content, but no less than 20 mL
Antibiotics (liquids)	1 mL
Other preparations soluble in water or in solvent like isopropyl myristate	total content, but no less than 0.2 g
Insoluble creams and pomades to be suspended and emulsified, total content, but no less than 0.2 g	total content, but no less than 0.2 g
Solids	
less than 0.05 g	the entire content
above 0.05 g up to 0.3 g	half the content, but no less than 0.05 g
above 0.3 g up to 5 g	0.15 g
above 5 g	0.5 g
Healthcare products	
surgical sutures	three parts of the thread (30 cm of length each)
surgical medical tape/gauze/cotton in multiple package	0.1 g per package
sutures and other materials in individual packages	the entire material
other medical correlates	the entire material cut in pieces, or disassembled

MEMBRANE FILTRATION METHOD

Use filtering membranes with nominal porosity of no more than 0.45 µm which efficiency in retaining microorganisms has been established. Cellulose nitrate filters, for example, are used for aqueous, oily and weakly alcoholic solutions, and cellulose acetate membranes, for strongly alcoholic membranes, for example. Specially adapted filters can be requested for certain products, such as antibiotics.

For extremely aggressive oncologic products – replace the cellulose ester membrane with polyvinylidene difluoride (PVDF) or polytetrafluoroethylene (PTFE).

The procedures described below apply to membranes with diameter of approximately 50 mm. If filters with different diameters are used, the volumes of dilutions and washes must be adjusted according to the diameter of the membrane used. The filtration device and the membrane are sterilized by an adequate process. The device has such a setup that the solution to be examined can be introduced and filtered under aseptic conditions. The filtration device must also allow the aseptic removal of the membrane for its transfer to the culture medium, or be adequate for proceeding with the incubation after adding the culture medium to the own device. The type of fluid used in membrane washing depends on the product nature, being specified in the individual monograph, whenever it is the case.

Negative controls or blanks must be included for the fluids and solvents used, for which no microbial growth must be observed. It is also necessary to verify if the fluids used do not present antimicrobial activity in the test conditions.

Liquids miscible in aqueous vehicles: transfer a small amount of sterile diluent, such as *Fluid I*, to the membrane and filter. The diluent must have neutralizing or inactivating substances, such as in the case of antibiotics. Transfer to the membrane the contents from the vessels to be tested or the appropriate dilution (previously defined in the *Validation test for bacteriostasis and fungistasis*) in amounts not inferior to the ones recommended on **Tables 2** and **3** and filter immediately. If the product presents antimicrobial activity, wash the membrane no less than three times, filtering, each time, the volume of sterile diluent established on *Validation test for bacteriostasis and fungistasis*. The amount of washing fluid used must not be superior to five portions of 200 mL, even if during the validation test it has been demonstrated that such washing cycle does not completely eliminate the antimicrobial activity. Transfer the entire membrane **or cut, aseptically, in two equal parts, to the media selected, being one half to each**. Use the same volumes of medium used in the validation test. Incubate the media for no less than 14 days.

Oils and oily solutions: use, for each culture medium, the amount of sample specified on **Tables 2** and **3**. Oils and oily solutions with low viscosity can be filtered without dilution through the dry membrane. Viscous oils must be diluted in adequate sterile solvent, such as, for example, isopropyl myristate, provided that it is demonstrated they don't have antimicrobial activity in the test conditions. Let the oil penetrate in the membrane, filter using vacuum gradually. Wash the membrane with no less than three portions of *Fluid III*. Carry on as described in *Liquids miscible in aqueous vehicles*.

Pomades and creams: use, for each culture medium, the amount of sample specified on **Tables 2** and **3**. Pomades with oily base and emulsion of the water in oil type can be diluted to 1.0% in adequate solvent (isopropyl myristate or another) as describe din the previous item, heating, if necessary, at 40 °C (in exceptional cases, heat up to no more than 44 °C). Filter, as quickly as possible, and carry on as described in *Oils and oily solutions*. In case of using isopropyl myristate as diluent, provided that demonstrated it has no antimicrobial activity in the test conditions, it must be sterilized before use, by membrane filtration, and its aqueous extract must present pH not below 6.5.

Soluble solids (non-antibiotics): use, for each culture medium, the amount of sample specified on **Tables 2** and **3**. Dissolve the product in adequate fluid, such as *Fluid I*, and carry on as described in *Liquids miscible in aqueous vehicles*.

*Solids for injectable preparations (non-antibiotics):*reconstitute the product as described on the label and proceed as described for *Liquids miscible in aqueous vehicles* or *Oils and oily solutions*, depending on the case. If necessary, excess diluent can be used to help in the product reconstitution and filtration.

Solid antibiotics for injectable preparations: for packages with less than 5 g, take aseptically, from each one of the 20 vials recommended, approximately 0.3 g of sample, dissolve in 200 mL of *Fluid I* and homogenize. Alternatively, reconstitute the product as described on the label, transfer the equivalent, in liquid, to 0.3 g of sample and dilute to 200 mL with *Fluid I*. For packages with 5 g or more, transfer aseptically, from every six vessels, 1 g of sample to an adequate vial, dissolve in 200 mL of *Fluid I* and homogenize.

Alternatively, reconstitute the six vials of product as recommended by the manufacturer, transfer an amount of liquid equivalent to 1 g of sample to an adequate vial, dilute to 200 mL with *Fluid I* and homogenize. Carry on as described in *Liquids miscible in aqueous vehicles*.

Sterile aerosols: for pressurized liquid products, freeze the content in a mixture of ethyl alcohol and dry ice at no less than -20 °C, for approximately one hour. If possible, before opening the package, let the propellant escape and transfer the content aseptically to an adequate sterile vial. Add 100 mL of *Fluid II* and softly homogenize. Carry on as described in *Liquids miscible in aqueous vehicles* or *Oils and oily solutions*, according to the case.

Syringes already filled, with or without a coupled needle: expel the content from each syringe over the membrane(s) or in separate vials and then filter. Carry on as described in *Liquids miscible in aqueous vehicles*.

Sterile devices: Pass aseptically one volume of *Fluid II* not inferior to 10% of the volume of each unit from the total of devices to be tested as established on **Tables 2 and 3**. Collect the fluid in an adequate sterile vessel and proceed as indicated for liquids miscible in aqueous vehicles or aqueous solutions of oils and oily solutions, according to the case. In the case of sterile empty syringes, extract the sterile diluent from the vessel with the sterile needle, if coupled, or through a sterile needle coupled to proceed with the assay, and expel the content in a sterile vessel. Proceed as indicated previously.

METHOD FOR DIRECT INOCULATION IN CULTURE MEDIUM

Transfer, directly and aseptically, to the culture media the amount of product specified on **Tables 2 and 3**, in such a way that the product volume is not higher than 10% of the culture medium volume, unless specified differently in the individual monograph or in this section. If the sample presents antimicrobial activity, conduct the test after neutralizing the activity with an adequate neutralizing substance or by dilution in sufficient amount of culture medium. When it is necessary to use large volumes of the product, it is possible to work with concentrated culture medium, prepared taking into account the dilution subsequent to the product addition. If the vessels can handle it, the concentrated medium can be added directly to the sample.

Non-oily liquids: transfer the indicated volume of each sample according to **Table 3** to tubes containing fluid thioglycolate medium and soybean casein broth, using a sterile pipette or sterile syringe and needle. Homogenize the liquid with the medium, without aerating excessively. Incubate in the conditions specified for each medium for 14 days.

Oily liquids: use a culture medium with appropriate emulsifying agent in a concentration shown to be adequate in the validation, for example, 1.0 (w/v) polysorbate 80.

Pomades and creams: prepare a sample dilution at 10% using an adequate emulsifying agent added to a sterile diluent such as *Fluid I*. Transfer the diluted sample to culture media without emulsifier. Incubate the inoculated media for no less than 14 days. Observe the media throughout the incubation period. Homogenize, softly, the culture medium vials with oil, every day, throughout the incubation period. The vials with *Liquid thioglycolate medium* or another similar medium must be agitated in order to not affect the anaerobiosis conditions.

Solids: transfer the amount of sample specified on **Tables 2 and 3** or prepare a solution or suspension of the product adding a volume not superior to 20 mL of sterile diluent to the vessel. Transfer the material obtained to 200 mL of *Fluid thioglycolate medium*. Likewise, transfer the same amount of material to 200 mL of *Soybean casein broth* and homogenize. Carry on as described for *Non-oily liquids*.

Catgut and other surgical sutures: For each medium, use the amount of sample specified on **Tables 2 and 3**. Open the package aseptically and take three portions of threat for each culture medium. Such

portions must be taken from the beginning, the middle and the end and have 30 cm of length. Cover each part of the thread with sufficient volume of the media (20 mL to 150 mL).

Purified cotton, gauze, bandage and related material: take from each package of cotton, gauze in roll or in bandage to be analyzed, with sterile instruments, two portions of 0.1 g to 0.5 g from the innermost parts of the sample. For materials in individual package, such as gauze pad, take two individual portions of 0.25 g to 0.5 g, or two total units, in case of small units (e.g.: bandages smaller than 25 mm to 75 mm). Transfer one portion to a tube with 40 mL of *Fluid thioglycolate medium* and the other to tubes with 40 mL of *Soybean casein broth*. Carry on as described for *Liquids*.

Parenteral devices: For devices in shapes and dimensions that allow their immersion in volume of medium that does not exceed 1000 mL, make their immersion using the amounts specified on **Tables 2 and 3** and proceed as described in *Liquids*. For very large devices, make the immersion of parts that come into contact with the patient in a sufficient volume of medium for immersion of all parts. For catheters which internal and external lumens must be sterile, pass the medium inside the lumen or fill the lumen with the medium and promote the immersion of the entire device.

OBSERVATIONS AND INTERPRETATION OF RESULTS

During the period of incubation and until its end, examine the media for macroscopic evidence of microbial growth. If the sample being examined causes turbidity of culture media, preventing the observation of microbial growth, transfer adequate portions from each vial (no less than 1 mL) to new vials of the same media 14 days after the beginning of incubation. Incubate the original vials and the new vials by an additional period of no less than four days. If, at the end of the incubation period, there is no evidence of microbial growth, the sample being examined complies with the requirement of sterility. If there is evidence of microorganism growth, the sample does not comply with the requirement of sterility, unless there is evident fault during the test execution, such as, for example, contamination not related to the product being analyzed.

The sterility test may be considered invalid if one or more of the following conditions are met.

a) the data on microbiological monitoring of the area where the test was conducted demonstrate fault; b) a review of the analytical procedures employed during the test reveals fault; c) microbial growth is observed in negative controls; d) after identification of microorganism(s) isolated from the test, the growth of such species can be attributed, undoubtedly, to faults related to the material used and/or techniques employed in the execution of the sterility test.

If considered invalid, the sterility test must be repeated with the same number of units from the initial test. If, after the test repetition, no microbial growth is observed, the sample complies with the requirement of sterility. If microbial growth is observed after the test repetition, the sample being examined does not comply with the requirement of sterility.

Conventional microbiological/biochemical techniques are usually satisfactory for identification of microorganisms recovered in a sterility test. In the case of considering only that, after determining the identity of microorganisms isolated in the test, the growth of such species can be undoubtedly attributed to faults concerning the material and/or technique used in the procedure from the sterility assay, it may be necessary to employ more sensitive techniques to demonstrate that the microorganism isolated in the product is identical to the one isolated in materials or in the environment. While the routine microbiological/biochemical identification techniques may demonstrate that two isolates are not identical, these methods may not be sufficiently sensitive or reliable to provide unequivocal evidence that two isolates come from a single source. Molecular

methods can be employed to determine if two microorganisms belong to the same clone and have an origin in common.

APPLICATION OF STERILITY TEST TO PARENTERAL, OPHTHALMIC AND OTHER NON-INJECTABLE PREPARATIONS WITH REQUIREMENT FOR STERILITY.

When employing the membrane filtration technique, use, whenever possible, the entire content of the vessel, but no less than the amount indicated on **Tables 2** and **3**, diluting, when necessary, to approximately 100 mL with an adequate sterile solution, such as *Fluid I*.

When employing the direct inoculation technique, use the amounts indicated on **Tables 2** and **3**, unless authorized and justified otherwise. The tests for bacteria and fungi are conducted with the same unit of sample being examined. When the volume or amount in a single vessel is insufficient for conducting the test, the contents from two or more vessels are used to inoculate the different media.

APPLICATION OF STERILITY TEST TO RADIOACTIVE PHARMACEUTICAL PRODUCTS

Due to the fast radioactive decay, it is not feasible to delay the release of some pharmaceutical products because of the sterility test.

In such cases, the results from sterility tests provide only confirmatory retrospective evidence for guarantee of sterility and, therefore, depend on the initial methods established in the manufacturing and in the validation/certification procedures.

5.5.3.3 MICROBIOLOGICAL ASSAY OF ANTIBIOTICS

The potency or activity of a product with antibiotic is determined by comparing the dose that inhibits the growth of a susceptible microorganism in relation to the dose of a standard substance or biological preparation of reference of the antibiotic that produces similar inhibition.

INTERNATIONAL UNIT AND STANDARD PREPARATION

International Unit is the specific activity included in an amount (mass) of International Biological /Standard or International Biological Reference Preparation. The equivalent amount of units for international use is established, whenever necessary, by the World Health Organization.

International Chemical Reference Substances do not present defined biological activity units. When biological assays are necessary, the potency of these products is in terms of mass equivalent to the one from the pure substance.

The number of units, or the equivalent mass of the pure substance, in micrograms, contained in 1 mg of antibiotic substance is indicated in the monograph from each of the products registered in the Pharmacopoeia.

For microbiological assays registered in the Pharmacopoeia, Standard Preparations (Primary Standards) are the International Standards and Reference Preparations established by the World Health Organization and by the European Pharmacopoeia or the Brazilian Standards and Reference Preparations. Other adequate preparations, of current international use, in which the potency has been determined in relation to the standard preparations from the World Health Organization, have identical legal value.

It is recommended that (secondary) work standards are prepared and used; however, it is mandatory that the potency has been determined by an adequate number of comparative assays in relation to a primary or pharmacopoeial standard, validated by appropriate statistical analysis, and that the data and results are archived and made available to the relevant inspection for a period that is identical to the one of the validity of tested products.

For assay on lots of antibiotic substances or which there are national Standard Preparations, referenced by international organizations, it is mandatory to use these preparations.

SOLUTIONS

Solution 1 (potassium phosphate buffer 1.0%, sterile, pH 6.0) – Dissolve 2.0 g of dibasic potassium phosphate and 8 g of monobasic potassium phosphate in 800 mL of purified water. If necessary, adjust the pH to 5.9 – 6.1 with 6 M phosphoric acid or 10 M potassium hydroxide, complete the volume to 1000 mL with water and homogenize. Sterilize the solution for 20 minutes in autoclave at 121 °C.

Solution 2 (potassium phosphate buffer 0.1 M, sterile, pH 8.0) – Dissolve 16.73 g of dibasic potassium phosphate and 0.523 g of monobasic potassium phosphate in 800 mL of purified water. If necessary, adjust the pH to 7.9 – 8.1 with 6 M phosphoric acid or 10 M potassium hydroxide, complete the volume to 1000 mL with water and homogenize. Sterilize the solution for 20 minutes in autoclave at 121 °C.

Solution 3 (potassium phosphate buffer 0.1 M, sterile, pH 4.5) – Dissolve 13.6 g of monobasic potassium phosphate in 800 mL of purified water. If necessary, adjust the pH to 4.4 – 4.5 with 6 M phosphoric acid or 10 M potassium hydroxide, complete the volume to 1000 mL with water and homogenize. Sterilize the solution for 20 minutes in autoclave at 121 °C.

Solution 4 (potassium phosphate buffer 10%, sterile, pH 6.0) – Dissolve 20.0 g of dibasic potassium phosphate and 80.0 g of monobasic potassium phosphate in 800 mL of purified water. If necessary, adjust the pH to 5.9 – 6.1 with 6 M phosphoric acid or 10 M potassium hydroxide, complete the volume to 1000 mL with water and homogenize. Sterilize the solution for 20 minutes in autoclave at 121 °C.

Solution 5 (potassium phosphate buffer 0.2 M, sterile, pH 10.5) – Dissolve 35.0 g of dibasic potassium phosphate and add 2.0 g of potassium hydroxide 10 M in 800 mL of purified water. If necessary, adjust the pH to 10.4 – 10.6 with 6 M phosphoric acid or 10 M potassium hydroxide, complete the volume to 1000 mL with water and homogenize. Sterilize the solution for 20 minutes in autoclave at 121 °C.

Solution 6 (0.1 M methanolic hydrochloric acid) – Dilute 10.0 mL of hydrochloric acid 1.0 M in sufficient methyl alcohol to make 1000 mL.

Solution 7 (isopropyl alcohol solution 80%) – Dilute 800 mL of isopropyl alcohol in sufficient purified water to make 1000 mL.

Solution 8 (potassium phosphate buffer 0.1 M, sterile, pH 7.0) – Dissolve 13.6 g of dibasic potassium phosphate and 4.0 g of monobasic potassium monobasic in 800 mL of purified water. If necessary, adjust the pH to 6.8 – 7.2 with 6 M phosphoric acid or 10 M potassium hydroxide, complete the volume to 1000 mL with water and homogenize. Sterilize the solution for 20 minutes in autoclave at 121 °C.

CULTURE MEDIA

Dehydrated culture media, commercially available, which have the same composition as the medium produced with the ingredients individually indicated for obtaining them when reconstituted with purified water, according to specifications from the manufacturer, can be used.

Culture medium n. 1 – Dissolve 6.0 g of dry peptone, 4.0 g of casein from pancreatic digestion, 3.0 g of yeast extract, 1.0 g of dextrose and 15.0 g of agar in sufficient purified water to make 1000 mL. The pH after sterilization must be 6.6.

Culture medium n. 2 – Dissolve 6.0 g of dry peptone, 3.0 g of yeast extract, 1.5 g of meat extract and 15.0 g of agar in sufficient purified water to make 1000 mL. The pH after sterilization must be 6.6.

Culture medium n. 3 – Dissolve 5.0 g of dry peptone, 1.5 g of yeast extract, 1.5 g of meat extract, 2.5 g of sodium chloride, 1.0 g of dextrose, 3.68 g of dibasic potassium phosphate and 1.32 g of monobasic potassium phosphate, in sufficient purified water to make 1000 mL. The pH after sterilization must be 7.0.

Culture medium n. 4 – Dissolve 6.0 g of dry peptone, 3.0 g of yeast extract, 1.5 g of meat extract, 1.0 g of D-glucose and 15.0 g of agar in sufficient purified water to make 1000 mL. The pH after sterilization must be 6.6.

Culture medium n. 5 – Use culture medium n. 2, but the pH after sterilization must be 7.8.

Culture medium n. 6 – Dissolve 40.0 g of dextrose and 10.0 g of dry peptone in sufficient purified water to make 1000 mL. The pH after sterilization must be 5.6.

Culture medium n. 7 – Use culture medium n. 1, sterilized and cooled down at 50 °C. Prepare aqueous solution with 10 mg of neomycin per mL and sterilize by membrane filtration on membrane with porosity of 0.22 µm. Add, aseptically, a sterile solution of neomycin sulfate, to obtain final concentration with potency of 100 µg of neomycin per mL of medium.

Culture medium n. 8 – Use culture medium n. 2, but the pH after sterilization must be adjusted to 5.8 to 6.0.

Culture medium n. 9 – Dissolve 17.0 g of casein from pancreatic digestion, 3.0 g of soybean from papain digestion, 5.0 g of sodium chloride, 2.5 g of dibasic potassium phosphate, 2.5 g of dextrose and 20.0 g of agar in sufficient purified water to make 1000 mL. The pH after sterilization must be 7.3.

Culture medium n. 10 – Use culture medium n. 9, but adding, instead of 20.0 g, 12.0 g of agar and 10.0 mL of polysorbate 80 (the latter added after heating the medium to dissolve agar, immediately diluting with water to make 1000 mL). The pH after sterilization must be 7.3.

Culture medium n. 11 – Use culture medium n. 1, but the pH after sterilization must be adjusted to 8.0.

Culture medium n. 12 – Prepare like culture medium n. 1, but adding 300 mg of manganese sulfate monohydrate (MnSO₄.H₂O) for every 1000 mL of medium.

Culture medium n. 13 – Dissolve 10.0 g of dry peptone and 20.0 g of dextrose in sufficient purified water to make 1000 mL. The pH after sterilization must be 5.6.

Culture medium n. 14 – Dissolve 10.0 g of glycerol, 10.6 g of dry peptone, 10.6 g of meat extract and 3.0 g of sodium chloride in sufficient purified water to make 1000 mL. The pH after sterilization must be 7.0.

Culture medium n. 15 – Prepare like culture medium n. 14, but adding 17.0 mg of agar for every 1000 mL of medium.

Culture medium n. 16 – Dissolve 15.0 g of casein from pancreatic digestion, 5.0 g of soybean from papain digestion, 5 g of sodium chloride and 15.0 g of agar in sufficient purified water to make 1000 mL. The pH after sterilization must be 7.3.

Culture medium n. 17 – Dissolve 17.0 g of casein from pancreatic digestion, 3.0 g of soybean peptone, 2.5 g of dextrose, 5.0 g of sodium chloride and 2.5 g of dibasic potassium phosphate in sufficient purified water to make 1000 mL. The pH after sterilization must be 7.3.

Culture medium n. 18 – Use culture medium n. 11, but, after heating the solution to dissolve the ingredients, add 20.0 mL of polysorbate 80. The pH after sterilization must be 8.0.

Culture medium n. 19 – Dissolve 9.4 g of dry peptone, 4.7 g of yeast extract, 2.4 g of meat extract, 15.0 g of sodium chloride, 10.0 g of dextrose and 23.5 g of agar in sufficient purified water to make 1000 mL. The pH after sterilization must be 6.1.

Culture medium n. 20 – Dissolve 40.0 g of dextrose, 10.0 g of dry peptone, 15.0 g of agar and 0.05 g of chloramphenicol (in potency) in sufficient purified water to make 1000 mL. The pH after sterilization must be 5.6.

Culture medium n. 21 – Use culture medium n. 20, sterilized and cooled down at 50 °C. Add, aseptically, 2.0 mL of sterile cycloheximide solution for every 100 mL of melted agar. Prepare a solution with 10.0 mg of cycloheximide per mL, in purified water, and sterilize by membrane filtration in membrane with porosity of 0.22 µm.

Culture medium n. 22 – Dissolve 15.0 g of dry peptone, 5.0 g of soybean flour from papain digestion, 4.0 g of sodium chloride, 0.2 g of sodium sulfite, 0.7 g of L-cystine, 5.5 g of dextrose and 15.0 g of agar in sufficient purified water to make 1000 mL. The pH after sterilization must be 7.0.

PREPARATION OF INOCULUM

Recommended microorganisms

- *Staphylococcus aureus* (ATCC 6538p)
- *Micrococcus yunnanensis* (ATCC 7468)
- *Kocuria rhizophila* (ATCC 9341)
- *Staphylococcus epidermidis* (ATCC 12228)
- *Saccharomyces cerevisiae* (ATCC 9763)
- *Bordetella bronchiseptica* (ATCC 4617)
- *Bacillus cereus* var. *mycoides* (ATCC 11778)
- *Bacillus subtilis* (ATCC 6633)
- *Klebsiella pneumoniae* (ATCC 10031)
- *Escherichia coli* (ATCC 10536)

- *Enterococcus hirae* (ATCC 10541)
- *Micrococcus luteus* (ATCC 10240)
- *Microsporium gypseum* (ATCC 14683)
- *Saccharomyces kudriavzevii* (ATCC 2601)
- *Micrococcus luteus* (ATCC 14452)
- *Pseudomonas aeruginosa* (ATCC 25619)
- *Mycobacterium smegmatis* (ATCC 607)

For indication purposes, the microorganisms available on the ATCC were listed. The same microorganisms can also be obtained from other sources: INCQS, CIP, NBRC, NCIMB, NCPF, NCTC, NCYC, IMI and IP. The matching between the microorganisms and the addresses from entities that provide them is indicated in *Microorganisms used in tests and assays* (5.5.3.5).

Procedure 1 – *Staphylococcus aureus*, *Micrococcus luteus*, *Kocuria rhizophila*, *Staphylococcus epidermidis*, *Bordetella bronchiseptica*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa*.

Preparation of suspension: transfer the microorganism from a stock culture to tubes with 10 mL of slant culture medium n. 1. Incubate the tube at 32 °C to 35 °C, for 24 hours. After the incubation, wash the microorganism growth with 50 mL of the sterile physiological solution.

Standardization of the suspension: dilute the suspension prepared, with sterile physiological solution, to obtain the transmittance of 25% at the wavelength of 580 nm, using an adequate spectrophotometer and test tubes with 13 mm of diameter as absorption vat. Determine the amount of suspension to be added to each 100 mL of agar or nutrient broth to produce clear and defined inhibition zones or a satisfactory dose/response relationship in the turbidimetric method. The suspensions of microorganisms submitted to procedure 1 can be stocked at temperature of 4 °C, respectively, for the following periods: one week, two weeks, two weeks, two weeks, two weeks, six months, one week, two weeks, and two weeks.

Micrococcus luteus ATCC 14452. Execute as indicated on Procedure 1. However, employ on the tube with slant medium and on the Roux bottle the culture medium n. 7, incubating the vial for 48 hours. The suspension can be stocked for two weeks, at temperature not superior to 4 °C.

Procedure 2 – *Bacillus subtilis*.

Execute as indicated on Procedure 1. However, in the preparation of the suspension, use culture medium n. 12, which incubation time is of five days. In the standardization of suspension, proceed to thermal shock and standardize the suspension as follows: centrifuge and decant the supernatant liquid. Resuspend the sediment with 50 mL to 70 mL of sterile physiological solution and heat the suspension for 30 minutes at 70 °C. Execute tests in plates, to ensure the viability of spores, and determine the amount that must be added to every 100 mL of medium, to obtain adequate inhibition zones. The suspension can be stocked for six months, at temperature not superior to 4 °C.

Procedure 3 – *Bacillus cereus*.

Execute as indicated on Procedure 1. However, incubate the tube with the microorganism for one week. In the standardization of suspension, proceed to thermal shock and standardize the suspension as follows: heat the suspension for 30 minutes, at 80 °C. Wash the suspension of spores three times with 20 mL to 25 mL of sterile water. Resuspend the microorganisms in 50 mL to 70 mL of sterile water and promote a new thermal shock for 30 minutes at 70 °C. Execute tests in plates, to ensure the viability of spores, and determine the amount that must be added to every 100 mL of agar, to obtain

adequate inhibition zones. The suspension can be stocked for six months, at temperature not superior to 4 °C.

Procedure 4 – *Microsporum gypseum*.

Incubate the microorganism for six to eight weeks, at 25 °C, in three-liter Erlenmeyer flasks with 200 mL of culture medium n. 6. Check the growth by sporulation. When the sporulation is 80% or more, collect the conidia from the mycelium layer with a sterile spatula or another adequate instrument. The conidia will be on the upper part of the floating layer. Keep the conidia in 50 mL of physiological solution. Determine, experimentally, the amount of conidia for the assay. The suspension can be stocked for two months, at temperature not superior to 4 °C.

Procedure 5 – *Enterococcus hirae*.

Transfer the microorganism from a stock culture to medium n. 33 and incubate, for 16 to 18 hours, at 37 °C. Determine, experimentally, the amount of microorganisms for the assay. Keep this culture under refrigeration for no more than 24 hours.

Procedure 6 – *Saccharomyces cerevisiae*. (ATCC 9763).

Keep the microorganism in tubes with 10 mL of slant culture medium n. 19. Incubate the tubes at 32 °C to 35 °C, for 24 hours. Inoculate 100 mL of nutrient broth — culture medium n. 13 — and incubate, for 16 to 18 hours, at 37 °C. Standardize the suspension as described in the Procedure 1. The suspension can be stocked for four weeks, at temperature not superior to 4 °C.

Procedure 7 – *Saccharomyces cerevisiae* (ATCC 9763) and *Saccharomyces kudriavzevii* (ATCC 2601).

Follow what is indicated on Procedure 1. However, incubate the slant tube with the culture medium n. 19, at 30 °C, the latter for a period of 48 hours. The suspension can be stocked for four weeks, at temperature not superior to 4 °C.

Procedure 8 – *Mycobacterium smegmatis*.

Keep the microorganisms in tubes with slant medium with 10 mL of culture medium n. 16 and re-plate every week. Incubate the tube at 37 °C, for 48 hours. Using 3 mL of sterile physiological solution, transfer the cultures that grew on agar slant to a 500 mL Erlenmeyer flask, with 100 mL of culture medium n. 14 and 50 g of glass beads. Homogenize the culture by rotation speed of 130 cycles per minute, in a radius of 3.5 cm and at temperature of 27 °C, for a period of five days. Determine the amount of suspension to be added to every 100 mL of agar through assay on plates. The suspension can be stocked for two weeks at temperature not superior to 4 °C.

* the microorganisms can be used in conditions that ensure no more than five passages of the culture of origin.

DESICCATION OF ANTIBIOTIC SUBSTANCES

Employ for desiccation of standards the procedures indicated below, and recommended according to the information described on **Table 2** in **5.5.3.3.1** and **5.5.3.3.2**.

Method 1: transfer a sufficient amount of standard for a tared weighing bottle with a ground cap. Weigh the bottle and put it in an oven under reduced pressure, tilting the cap over the bottle mouth to

ensure it remains open during the desiccation. Desiccate at 60 °C, under pressure of 0.67 kPa or less, for three hours. With the process concluded, introduce dry air into the oven, submitting it to a desiccating agent such as sulfuric acid or silica gel. Put the cap back on and put the weighing bottle in a desiccator with a desiccating agent such as phosphorus pentoxide or silica gel. Let it cool down at room temperature and weigh, calculating the percentage of loss of mass from the standard.

Method 2: proceed according to Method 1. However, use a tared weighing bottle with a cap with capillary tube with 0.20 mm to 0.25 mm of internal diameter, and desiccate without removing the cap.

Method 3: proceed according to Method 1. However, desiccate the sample at 110 °C, under pressure of 0.67 kPa or less, for three hours.

Method 4: proceed according to Method 1. However, desiccate the sample at 40 °C, under pressure of 0.67 kPa or less, for two hours.

Method 5: proceed according to Method 1. However, desiccate the sample at 100 °C, under pressure of 0.67 kPa or less, for four hours.

Method 6: proceed according to Method 1. However, desiccate the sample at 40 °C, under pressure of 0.67 kPa or less, for three hours.

Method 7: proceed according to Method 1. However, desiccate the sample at 25 °C, under pressure of 0.67 kPa or less, for three hours.

Method 8: the antibiotic substance is not submitted to desiccation.

PROCEDURE

Every material must be adequate to the intended use and be thoroughly cleaned, after each use, to remove any vestige of antibiotic. The material must remain covered while it is not being used. All glassware used in contact with the microorganism must be sterilized in oven, at temperature between 200 °C and 220 °C for two hours. When diluting the standard solution and sample, use volumetric flasks, pipettes or carefully calibrated equipment items.

5.5.3.3.1 AGAR-DIFFUSION MICROBIOLOGICAL ASSAY

PROCEDURE

For each antibiotic listed on **Table 1**, check the culture medium (according to the list of culture media), the amount of medium to be used in the base layer and in the inoculated layer, and the assay microorganism. The volume of inoculum to be added to every 100 mL of culture medium must be determined experimentally.

However, as an initial reference, the amount of inoculum to be added per 100 mL of medium is suggested.

Prepare the base layer through addition of the appropriate number of molten agar on the Petri dishes, which must be especially selected, have flat bottom, have dimensions of 20 x 100 mm and cap in appropriate material. Distribute the agar evenly on the plates, which must be placed on a leveled surface so that the middle layer has even depth. Put the cap from each plate beside it; if a non-porous cap is used, leave it slightly opened to avoid buildup of condensed humidity from the hot agar layer.

After the agar hardening, cover the plates. To prepare the inoculated layer – surface, add the volume of inoculum determined for the appropriate amount of culture medium that has been melted and cooled down between 46 °C and 48 °C. Shake the flask, by rotation, to obtain an homogeneous suspension, and add the indicated amount of medium inoculated on each Petri dish, with the non-inoculated base layer. Spread the layer evenly, cover the plates and allow it to harden over a flat surface.

After the medium hardening, put six stainless steel cylinders, with external diameter of (8 ± 0.1) mm, internal diameter of (6 ± 0.1) mm and length of (10 ± 0.1) mm, over the inoculated agar surface, so that they form a 60° angle with each other and a radius of 2.8 cm. Cylinders made of glass, porcelain or aluminum and sterilized in the conditions already described can also be used. Instead of cylinders, wells with 5 mm to 8 mm of diameter can be perforated, in the middle, with sterile perforator. Paper discs, made with paper of appropriate quality or stainless-steel molds, can also be used. When paper discs are used, they must be sterile. It is recommendable for obtaining satisfactory results that the halos formed by the diffusion of drug in the medium have no less than 14 mm of diameter and a maximum diameter so that there is no overlap between them.

Preparation of the standard working solution, sample and standard curve

The preparation of samples of antibiotics is indicated on the respective monograph. The concentrations of antibiotic used on the assay must be in geometric progression; for example, by the preparation of series of dilution in the 2:1 ratio, or another determined experimentally, provided that the linear relation between the logarithm of the antibiotic concentration and the diameter of the zone of inhibition is proven;

The **Table 2** indicates, for each antibiotic, the preparation of the standard working solution and the standard curve, including:

- conditions of desiccation, as described on item *Desiccation of antibiotic substances (5.5.3.3)*;
- initial solvent for dissolution of antibiotic, if necessary, and up to what concentration it is used;
- solution for dilution until the working concentration, as described in *Solutions*;
- concentration of the working solution, expressed in weight or International Units per mL of solution;
- validity period of the standard working solution under refrigeration;
- solution used for dilution of the working solution, due to the preparation of the standard curve, according to *Solutions*;
- concentration ranges suggested, in weight or International Units per mL, within which the adequate concentrations for the standard curve can be found.

Procedure for parallel lines design (3 x 3 or 2 x 2): use, on the assay, no less than six Petri dishes. Arrange the standard and sample solutions, on each plate, with three concentrations for 3 x 3 assay (low, medium and high) or two concentrations for 2 x 2 assay (low and high). The solutions must be distributed in such a way that the solutions from the standard preparation and sample are alternated in the inoculated layer (high and low concentration) to avoid overlapping of zones of inhibition.

Procedure for 5 x 1 design: for the standard curve, use a total of 12 plates, three for each of the standard solutions (P₁, P₂, P₄, P₅), except for the medium concentration of the curve (P₃), which is included in all plates. In each set of three plates, use three cylinders for the medium concentration (P₃) and alternate three cylinders for low concentration (P₁) and so forth with the other solutions from the standard. This way, 36 zones of inhibition are obtained for the concentration (P₃) and nine zones of inhibition are obtained for each of the other four concentrations of the curve. For each sample, use

three plates, where three cylinders will be placed for the medium concentration of standard (P₃) and three with the sample solution prepared on the same concentration of the standard (A₃).

Apply 0.2 mL of the solutions in the cylinders or stainless-steel molds through pipette or another calibrated instrument, if not specified in the individual monograph. When the well system is used, the volume of liquid applied must be sufficient to fill them completely.

After executing the adequate procedures for the design selected, incubate the plates at the indicated temperature, which variation shall not exceed ± 0.5 °C, for a period of 16 to 18 hours. Then, measure the diameter of the zones of inhibition, using an adequate device for measuring, such as the caliper, or an optical projector with precision of 0.1 mm or less.

For some microorganisms, the procedure can be improved if the plates prepared remain at room temperature for a period of 30 minutes to two hours before the incubation, a period when there is diffusion of antibiotic to the medium

Table 1 – Agar-diffusion microbiological assay.

<i>Antibiotic</i>	<i>Microorganism</i>	<i>Culture medium</i>		<i>Volume (mL) of medium applied on the layers</i>		<i>Volume of inoculum mL/100 mL</i>	<i>Temperature of incubation (°C)</i>
		<i>Base</i>	<i>Surface</i>	<i>Base</i>	<i>Surface</i>		
Amoxicillin	<i>Kocuria rhizophila</i> (ATCC 9341)	11	11	21	4	0.5	32 to 35
Ampicillin	<i>Kocuria rhizophila</i> (ATCC 9341)	11	11	21	4	0.5	32 to 35
Amphotycin	<i>Micrococcus luteus</i> resistant to neomycin (ATCC 14452)	2	1	21	4	0.5	36 to 38
Amphotericin B	<i>Saccharomyces cerevisiae</i> (ATCC 9763)	–	19	–	8	1.0	29 to 31
Bacitracin	<i>Micrococcus yunnanensis</i> (ATCC 7468)	2	1	21	4	0.3	32 to 35
Bacitracin	<i>Micrococcus luteus</i> (ATCC 10240)	2	1	21	4	0.3	32 to 35
Benzylpenicillin	<i>Staphylococcus aureus</i> (ATCC 6538p)	2	1	21	4	1.0	32 to 35
Bleomycin	<i>Mycobacterium smegmatis</i> (ATCC 607)	15	15	10	6	1.0	32 to 35
Kanamycin	<i>Bacillus subtilis</i> (ATCC 6633)	5	5	21	4	(1)	36 to 38
Carbenicillin	<i>Pseudomonas aeruginosa</i> (ATCC 25619)	9	10	21	4	(1)	36 to 38
Cefacetrile	<i>Staphylococcus aureus</i> (ATCC 6538p)	2	1	21	4	0.5	32 to 35
Cefadroxil	<i>Staphylococcus aureus</i> (ATCC 6538p)	2	1	21	4	0.05	36 to 38
Cefalexin	<i>Staphylococcus aureus</i> (ATCC 6538p)	2	1	21	4	0.05	32 to 35
Cephaloglycin	<i>Staphylococcus aureus</i> (ATCC 6538p)	2	1	21	4	0.2	32 to 35
Cephaloridine	<i>Staphylococcus aureus</i> (ATCC 6538p)	2	1	21	4	0.1	32 to 35
Cephalothin	<i>Staphylococcus aureus</i> (ATCC 6538p)	2	1	21	4	0.1	32 to 35
Cefapirin	<i>Staphylococcus aureus</i> (ATCC 6538p)	2	1	21	4	0.08	32 to 35
Cefazolin	<i>Staphylococcus aureus</i> (ATCC 6538p)	2	1	21	4	0.05	32 to 35
Cefoxitin	<i>Staphylococcus aureus</i> (ATCC 6538p)	2	1	21	5	0.1	32 to 35
Cefradine	<i>Staphylococcus aureus</i> (ATCC 6538p)	2	1	21	4	0.05	32 to 35
Cyclacillin	<i>Kocuria rhizophila</i> (ATCC 9341)	11	11	21	4	0.5	36 to 38
Cycloserine	<i>Staphylococcus aureus</i> (ATCC 6538p)	2	1	10	4	0.04	29 to 31
Clindamycin	<i>Kocuria rhizophila</i> (ATCC 9341)	11	11	21	4	1.5	36 to 38
Chloramphenicol	<i>Kocuria rhizophila</i> (ATCC 9341)	1	1	21	4	2.0	32 to 35

Table 1 (conclusion)

Antibiotic	Microorganism	Culture medium		Volume (mL of medium applied on the layers)		Volume of Inoculum mL/100 mL	Temperature of incubation (°C)
		Base	Surface	Base	Surface		
Cloxacillin	<i>Staphylococcus aureus</i> (ATCC 6538p)	2	1	21	4	0.1	32 to 35
Colistin	<i>Bordetella bronchiseptica</i> (ATCC 4617)	9	10	21	4	0.1	36 to 38
Dactinomycin	<i>Bacillus subtilis</i> (ATCC 6633)	5	5	10	4	(1)	36 to 38
Dicloxacillin	<i>Staphylococcus aureus</i> (ATCC 6538p)	2	1	21	4	0.1	32 to 35
Dihydrostreptomycin	<i>Bacillus subtilis</i> (ATCC 6633)	5	5	21	4	(1)	36 to 38
Erythromycin	<i>Kocuria rhizophila</i> (ATCC 9341)	11	11	21	4	1.5	32 to 35
Streptomycin	<i>Bacillus subtilis</i> (ATCC 6633)	5	5	21	4	(1)	36 to 38
Phenethicillin	<i>Kocuria rhizophila</i> (ATCC 9341)	11	11	21	4	0.5	32 to 35
Phenoxymethylpenicillin	<i>Staphylococcus aureus</i> (ATCC 6538p)	2	1	21	4	1.0	32 to 35
Gentamicin	<i>Staphylococcus epidermidis</i> (ATCC 12228)	11	11	21	4	0.03	36 to 38
Griseofulvin	<i>Microsporum gypseum</i> (ATCC 14683)	20	21	6	4	(1)	29 to 31 for 48 hours
Mitomycin	<i>Bacillus subtilis</i> (ATCC 6633)	8	8	10	4	0.5	36 to 38
Neomycin	<i>Staphylococcus aureus</i> (ATCC 6538p)	11	11	21	4	1.0	32 to 35
Neomycin	<i>Staphylococcus epidermidis</i> (ATCC 12228)	11	11	21	4	1.0	36 to 38
Nystatin	<i>Saccharomyces kudriavzevii</i> (ATCC 2601)	–	19	–	8	1.0	29 to 31
Novobiocin	<i>Staphylococcus epidermidis</i> (ATCC 12228)	2	1	21	4	4.0	34 to 36
Ofloxacin	<i>Kocuria rhizophila</i> (ATCC 9341)	1	11	20	5	0.5	30 to 35
Oxacillin	<i>Staphylococcus aureus</i> (ATCC 6538p)	2	1	21	4	0.3	32 to 35
Paromomycin	<i>Staphylococcus epidermidis</i> (ATCC 12228)	11	11	21	4	2.0	36 to 38
Polymyxin B	<i>Bordetella bronchiseptica</i> (ATCC 4617)	9	10	21	4	0.1	36 to 38
Rifampicin	<i>Bacillus subtilis</i> (ATCC 6633)	2	2	21	4	0.1	29 to 31
Rifampicin	<i>Staphylococcus aureus</i> (ATCC 6538p)	2	2	21	4	0.1	36 to 38
Sisomicin	<i>Staphylococcus epidermidis</i> (ATCC 12228)	11	11	21	4	0.03	36 to 38
Tetracycline	<i>Staphylococcus epidermidis</i> (ATCC 12228)	1	1	20	5	2.0	33 to 37
Vancomycin	<i>Bacillus subtilis</i> (ATCC 6633)	8	8	10	4	(1)	36 to 38

(1) Determine the amount of inoculum, on the assay, through diffusion on plates.

Table 2 – Preparation of the standard working solution and standard curve.

<i>Antibiotic</i>	<i>a. Condition of desiccation (5.5.3.3)</i>	<i>b. Initial solvent</i>	<i>c. Solution for dilution (5.5.3.3)</i>	<i>d. Concentration of working solution (/mL)</i>	<i>e. Validity period of the solution under refrigeration</i>	<i>f. Solution for dilution (5.5.3.3)</i>	<i>g. Concentration range (/mL)</i>
Amoxicillin	8	-	Sterile water	1 mg	7 days	2	0.05 to 0.2 µg ⁷
Ampicillin	8	-	Sterile water	0.1 mg	7 days	2	0.05 to 0.2 µg ⁷
Amphoterycin ⁸	1	-	2	0.1 mg	14 days	2	5 to 20 µg
Amphotericin B	1	-	Dimethylsulfoxide	1 mg ¹	Use on the same day	5	0.5 to 2 µg ⁷
Bacitracin	1	-	HCl 0.01 M	100 IU	Use on the same day	1	1 to 4 IU
Benzylpenicillin	8	-	1	1000 IU	4 days	1	0.2 to 2 IU
Bleomycin	7	-	8	2 IU	14 days	8	0.01 to 0.2 IU
Kanamycin B	8	-	2	1 mg	30 days	2	0.5 to 2 µg
Carbenicillin	8	-	1	1 mg	14 days	1	10 to 40 µg
Cefacetile	8	-	1	1 mg	7 days	1	5 to 20 µg
Cefadroxil	8	-	1	1 mg	Use on the same day	1	10 to 40 µg
Cefalexin	8	-	1	1 mg	7 days	1	10 to 40 µg
Cephaloridine	1	-	1	1 mg	5 days	1	0.5 to 2 µg
Cephaloglycin	8	-	Sterile water	100 µg	7 days	3	5 to 20 µg
Cephalothin	1	-	1	1 mg	5 days	1	0.5 to 2 µg
Cefapirin	8	-	1	1 mg	3 days	1	0.5 to 2 µg
Cefazolin	8	10,000 µg per mL in the solution 4	1	1 mg	5 days	1	0.5 to 2 µg
Cefoxitin	8	-	1	1 mg	Use on the same day	1	10 to 40 µg
Cyclacillin	8	-	Sterile water	1 mg	1 day	2	0.5 to 2 µg ⁷
Cefradine	8	-	1	1 mg	5 days	1	5 to 20 µg
Cycloserine	1	-	Sterile water	1 mg	30 days	1	20 to 80 µg
Clindamycin	8	-	Sterile water	1 mg	30 days	2	0.5 to 2 µg

Table 2 (conclusion)

<i>Antibiotic</i>	<i>a. Condition of desiccation (5.5.3.3)</i>	<i>b. Initial solvent</i>	<i>c. Solution for dilution (5.5.3.3)</i>	<i>d. Concentration of working solution (/mL)</i>	<i>e. Validity period of the solution under refrigeration</i>	<i>f. Solution for dilution (5.5.3.3)</i>	<i>g. Concentration range (/mL)</i>
Chloramphenicol	8	10,000 µg per mL in <i>ethyl alcohol</i>	1	1 mg	30 days	1	20 to 80 µg
Cloxacillin	8	—	1	1 mg	7 days	1	2 to 8 µg
Colistin	1	10,000 µg per mL in <i>ethyl alcohol</i>	4	1 mg	14 days	4	0.5 to 2 µg
Dactinomycin	1	10,000 µg per mL in <i>methyl alcohol</i>	2	1 mg	90 days	2	0.5 to 2 µg
Dicloxacillin	8	—	1	1 mg	7 days	1	2.5 to 10 µg
Dihydrostreptomycin	5	—	2	1 mg	30 days	2	0.5 to 2 µg
Erythromycin ⁵	1	10,000 µg per mL in <i>methyl alcohol</i>	2	1 mg	14 days	2	0.5 to 2 µg
Streptomycin	1	—	2	1 mg	30 days	2	0.5 to 2 µg
Phenethicillin	8	—	Sterile water	1000 IU	7 days	2	0.05 to 0.2 IU
Phenoxyethylpenicillin	8	—	1	100 IU	4 days	1	0.2 to 2 IU
Gentamicin	3	—	2	1 mg	30 days	2	0.5 to 2 µg
Griseofulvin	8	—	Dimethylformamide	1 mg ⁴	90 days	2	2 to 10 µg
Mitomycin	8	—	1	1 mg	14 days	1	0.5 to 2 µg
Neomycin	1	—	2	1 mg	14 days	2	5 to 20 µg (<i>S. aureus</i>)
Neomycin	1	—	2	1 mg	14 days	2	0.5 to 2 µg (<i>S. epidermidis</i>)
Nystatin	4	—	Dimethylformamide	1000 IU ²	Use on the same day	4	10 to 40 IU ⁷
Novobiocin	5	10,000 µg per mL in <i>ethyl alcohol</i>	2	1 mg	5 days	4	0.2 to 1 µg
Oxacillin	8	—	1	1 mg	3 days	1	2 to 10 µg

<i>Antibiotic</i>	<i>a. Condition of desiccation (5.5.3.3)</i>	<i>b. Initial solvent</i>	<i>c. Solution for dilution (5.5.3.3)</i>	<i>d. Concentration of working solution (/mL)</i>	<i>e. Validity period of the solution under refrigeration</i>	<i>f. Solution for dilution (5.5.3.3)</i>	<i>g. Concentration range (/mL)</i>
Paromomycin	1	—	2	1 mg	21 days	2	0.5 to 2 µg
Polymyxin B	1	<i>Sterile water</i> ³	4	10,000 IU	14 days	4	200 to 800 IU
Rifampicin	8	—	Methyl alcohol	1 mg	1 day	1	2 to 10 µg
Sisomicin ⁶	8	—	2	1 mg	14 days	2	0.05 to 0.2 µg
Vancomycin	1	—	Sterile water	1 mg	7 days	2	5 to 20 µg

1 Dilute aliquots of the working solution with dimethylsulfoxide, to obtain a concentration between 10 and 40 mg per mL according to the standard curve points.

2 Dilute aliquots of the working solution with dimethylformamide, to obtain concentrations between 10 and 40 units per mL according to the standard curve points.

3 Add 2 mL of sterile water for every 5 mg of standard.

4 Dilute aliquots of the working solution with dimethylformamide, to obtain concentrations between 40 and 200 mg per mL according to the standard curve points.

5 When using erythromycin under the form of estolate, hydrolyze the working solution in water bath at 60 °C, for two hours.

6 Sisomicin is hygroscopic, use precautions during the weighing. The working standard must remain at 20 °C, in nitrogen atmosphere.

7 Prepare concomitantly the standard and sample solutions. The sample dilutions must have the same amount of dimethylformamide than the standard dilutions.

8 The standard working solution must remain for one night at room temperature for complete dissolution.

5.5.3.3.2 TURBIDIMETRIC MICROBIOLOGICAL ASSAY

PROCEDURE

Preparation of the working solution, sample and standard curve

The preparation of samples of antibiotics is indicated on the respective monograph.

On the **Table 2**, presented below, there is an indication, for each antibiotic, of the preparation of the standard working solution and the standard curve, including:

- conditions of desiccation, as described on item *Desiccation of antibiotic substances* (5.5.3.3);
- initial solvent for dissolution of antibiotic, if necessary, and up to what concentration it is used;
- solution for dilution of antibiotic until the working concentration, according to *Solutions*;
- concentration of working solution, expressed in weight or International Units per mL of solution;
- validity period of the standard working solution under refrigeration;
- solution used for dilution of the working solution, for the preparation of the standard curve, according to *Solutions*;
- concentration range, in weight or International Units per mL, within which the adequate concentrations for the standard curve can be found.

Use for each antibiotic the microorganism and nutritive broth listed on **Table 1**. Determine experimentally the volume of inoculum to be added to 100 mL of broth from the amount suggested as initial reference. The inoculated medium must be prepared and used immediately.

Procedure for parallel lines design (3 x 3 or 2 x 2): distribute, in identical tubes, an equal volume of each of the standard and sample solutions. Add to each tube an equal volume of inoculated nutrient broth, for example, 1 mL of solution with antibiotic and 9 mL of medium (0.1 mL of solution for gramicidin and tyrothricin). No less than eighteen tubes are used for parallel lines 3 x 3 assay and twelve tubes for parallel lines 2 x 2 assay. The number of replicas per concentration in each assay must be sufficient to ensure the statistical precision specified on the monograph, but no less than three tubes must be executed for each concentration of standard and sample. It may be necessary to conduct the assay with a higher number of doses of standard and sample, or repeat it and match the results to obtain the precision required. The doses used must be in geometric progression.

Procedure for 5 x 1 curve design: for the 5 x 1 design, prepare dilutions that represent five concentrations of the standard (P1, P2, P3, P4 and P5) and one concentration of sample (A3). The sample solution must correspond to the same dilution of standard that corresponds to the average concentration of the curve (P3). Use no less than three tubes for every concentration of the standard and sample. This way, no less than 18 tubes are necessary on the assay.

After executing the adequate procedures for the design selected, inoculate the culture medium recommended with a known amount of suspension of the antibiotic-sensitive microorganism, so that, after incubation of approximately four hours, the bacterial turbidity in the medium is easy to measure and maintains correlation between the dose and the response of the substance being analyzed.

The **Table 1** describes the antibiotics to be analyzed by the turbidimetric method with description of the microorganism, culture medium, volume of standardized inoculum suggested as initial reference, and temperature of incubation for each case.

Incubate in water bath for three to four hours, taking the precaution of ensuring adequate and constant temperature for all tubes. The adequate time must be checked by observation of growth in the tube

with the average concentration (P3) used on the assay. After the incubation period, interrupt the multiplication of microorganisms by adding 0.5 mL of formaldehyde solution 12%, to each tube.

Determine the absorbance for each tube in spectrophotometer, on the wavelength of 530 nm. Standardize the device in absorbance through the blank with the same amount of nutrient broth and formaldehyde, 12%.

In routine assays, when the system linearity is proven by an adequate number of experiments employing the three-point assay (3 x 3), the two-point assay (2 x 2) can be used. The 5 x 1 design, adopted by other pharmacopoeias of current international use, will also be accepted. However, in case of controversy or litigation, the three-point assay must be applied.

Calculation of potency

From the results, calculate the potency of the sample and its confidence limits, through standard statistical method described on *Statistical procedures applicable to biological assays – indirect quantitative assays*.

Confidence interval (CI)

The precision of an assay is verified by the confidence interval, which ensures that the true potency is within the limits specified. In the absence of CI from the product monograph, it is recommendable to have upper and lower confidence limits of 5.0% or less, in relation to the potency calculated, with limit values of up to 10% being accepted.

Table 1 – Turbidimetric microbiological assay.

<i>Antibiotic</i>	<i>Microorganism</i>	<i>Nutrient broth</i>	<i>Volume of inoculum mL/100 mL</i>	<i>Temperature of incubation (°C)</i>
Amikacin	<i>Staphylococcus aureus</i> (ATCC 6538p)	3	0.1	37
Kanamycin	<i>Staphylococcus aureus</i> (ATCC 6538p)	3	0.2	37
Candididin	<i>Saccharomyces cerevisiae</i> (ATCC 9763)	13	0.2	28
Capreomycin	<i>Klebsiella pneumoniae</i> (ATCC 10031)	3	0.05	37
Cycloserine	<i>Staphylococcus aureus</i> (ATCC 6538p)	3	0.4	37
Chloramphenicol	<i>Escherichia coli</i> (ATCC 10536)	3	0.7	37
Chlortetracycline	<i>Staphylococcus aureus</i> (ATCC 6538p)	3	0.1	36
Demeclocycline	<i>Staphylococcus aureus</i> (ATCC 6538p)	3	0.1	37
Dihydrostreptomycin	<i>Klebsiella pneumoniae</i> (ATCC 10031)	3	0.1	37
Doxycycline	<i>Staphylococcus aureus</i> (ATCC 6538p)	3	0.1	37
Spectinomycin	<i>Escherichia coli</i> (ATCC 10536)	3	0.1	37

Antibiotic	Microorganism	Nutrient broth	Volume of inoculum mL/100 mL	Temperature of incubation (°C)
Streptomycin	<i>Klebsiella pneumoniae</i> (ATCC 10031)	3	0.1	37
Gramicidin	<i>Enterococcus hirae</i> (ATCC 10541)	3	1.0	37
Lincomycin	<i>Staphylococcus aureus</i> (ATCC 6538p)	3	0.1	37
Minocycline	<i>Staphylococcus aureus</i> (ATCC 6538p)	3	0.2	37
Oxytetracycline	<i>Staphylococcus aureus</i> (ATCC 6538p)	3	0.1	37
Rolitetracline	<i>Staphylococcus aureus</i> (ATCC 6538p)	3	0.1	37
Tetracycline	<i>Staphylococcus aureus</i> (ATCC 6538p)	3	0.1	37
Tyrothricin	<i>Enterococcus hirae</i> (ATCC 10541)	3	1.0	37
Tobramycin	<i>Staphylococcus aureus</i> (ATCC 6538p)	3	0.15	37

Table 2 – Preparation of the standard solution and standard curve – Turbidimetric method.

<i>Antibiotic</i>	<i>a. Condition of desiccation (5.5.3.3)</i>	<i>b. Initial solvent</i>	<i>c. Solution for dilution (5.5.3.3)</i>	<i>d. Concentration of working solution (/mL)</i>	<i>e. Validity period of the solution under refrigeration</i>	<i>f. Solution for dilution (5.5.3.3)</i>	<i>g. Concentration range (/mL)</i>
Amikacin	8	-	Sterile water	1 mg	14 days	Sterile water	6 to 14 µg
Kanamycin	8	-	Sterile water	1 mg	30 days	Sterile water	6 to 14 µg
Candimycin ¹	6	-	Dimethyl sulfoxide	1 mg	Use on the same day	Sterile water	0.02 to 0.14 µg ³
Capreomycin	5	-	Sterile water	1 mg	7 days	Sterile water	60 to 180 µg
Cycloserine	1	-	Sterile water	1 mg	30 days	Sterile water	20 to 80 µg
Chloramphenicol	8	10,000 µg per mL in ethyl alcohol	1	1 mg	30 days	1	1 to 4 µg
Chlortetracycline	8	-	HCl 0.01 M	1 mg	4 days	Sterile water	0.03 to 0.09 µg
Demeclocycline	1	-	HCl 0.1 M	1 mg	4 days	Sterile water	0.06 to 0.14 µg
Dihydrostreptomycin	5	-	Sterile water	1 mg	30 days	Sterile water	20 to 60 µg
Doxycycline	8	-	HCl 0.1 M	1 mg	5 days	Sterile water	0.06 to 0.14 µg
Spectinomycin	8	-	Sterile water	1 mg	30 days	Sterile water	20 to 60 µg
Streptomycin	1	-	Sterile water	1 mg	30 days	Sterile water	20 to 60 µg
Gramicidin	1	-	Ethyl alcohol 95%	1 mg	30 days	Ethyl alcohol 95%	0.02 to 0.08 µg
Lincomycin	8	-	Sterile water	1 mg	30 days	Sterile water	0.3 to 0.8 µg
Minocycline	8	-	HCl 0.1 M	1 mg	2 days	Sterile water	0.06 to 0.12 µg
Oxytetracycline	8	-	HCl 0.1 M	1 mg	4 days	Sterile water	0.16 to 0.32 µg
Rolitetraacycline	1	-	Sterile water	1 mg	1 day	Sterile water	0.16 to 0.32 µg
Tetracycline	8	-	HCl 0.1 M	1 mg	1 day	Sterile water	0.16 to 0.32 µg
Tyrothricin ²	1	-	Ethyl alcohol 95%	1 mg	30 days	Ethyl alcohol 95%	0.02 to 0.08 µg
Tobramycin	8	-	Sterile water	1 mg	14 days	Sterile water	1 to 4 µg

¹ In the candimycin assay, use sterile equipment in all steps.² For the tyrothricin assay, use the standard working solution and the dose-response curve of gramicidin.³ Prepare, simultaneously, the standard and sample solutions.

5.5.3.4 ANTIMICROBIAL EFFECTIVENESS TEST

The antimicrobial effectiveness test ensures the effectiveness of antimicrobial preservatives added to pharmaceutical products. Antimicrobial preservatives are substances added to non-sterile pharmaceutical dosage forms with the purpose of protecting them from any microbial growth. For sterile pharmaceutical dosage forms, stored in multiple dose packages, antimicrobial preservatives are added to inhibit the growth of contaminant microorganisms during the repeated use of individual doses.

The amount of preservative used in a formulation must be the minimum necessary for protecting the product without harming the patient or the consumer.

The antimicrobial effectiveness, whether inherent to the product or due to the addition of preservatives, needs to be demonstrated for topical multiple-dose products, oral products, ophthalmic products, otologic products, nasal products, fluids for dialysis, irrigation, etc.

The test and the criteria established apply to the product how it is found in the market.

MICROORGANISMS USED

- *Candida albicans* ATCC 10231
- *Aspergillus brasiliensis* ATCC 16404
- *Escherichia coli* ATCC 8739
- *Pseudomonas aeruginosa* ATCC 9027
- *Staphylococcus aureus* ATCC 6538

The microorganisms used in the test must have no more than five passages counted from the original ATCC culture. A passage is defined as the transfer of a established culture to a sterile culture medium.

In case of cultures maintained by freezing techniques, each cycle of freezing, thawing and reactivation is considered a passage. Lyophilized cultures received from ATCC must be reconstituted according to instructions provided with the material.

Recover the material in a liquid or solid culture medium. The conditions for culture preparation are registered on **Table 1**.

Table 1 – Conditions for reconstitution of strains.

<i>Microorganism</i>	<i>Culture medium</i>	<i>Temperature of incubation</i>	<i>Inoculum incubation time</i>	<i>Incubation time for microbial recovery</i>
<i>Escherichia coli</i> ATCC 8739	Soybean-Casein Digest Broth/Soybean-Casein Digest Agar	(32.5 ± 2.5) °C	18 – 24 hours	3 to 5 days
<i>Pseudomonas aeruginosa</i> ATCC 9027	Soybean-Casein Digest Broth/Soybean-Casein Digest Agar	(32.5 ± 2.5) °C	18 – 24 hours	3 to 5 days
<i>Staphylococcus aureus</i> ATCC 6538	Soybean-Casein Digest Broth/Soybean-Casein Digest Agar	(32.5 ± 2.5) °C	18 – 24 hours	3 to 5 days
<i>Candida albicans</i> ATCC 10231	Sabouraud Dextrose Broth/Sabouraud Dextrose Agar	(22.5 ± 2.5) °C	44 – 52 hours	3 to 5 days

<i>Microorganism</i>	<i>Culture medium</i>	<i>Temperature of incubation</i>	<i>Inoculum incubation time</i>	<i>Incubation time for microbial recovery</i>
<i>Aspergillus brasiliensis</i> ATCC 16404	Sabouraud Dextrose Broth/Sabouraud Dextrose Agar	(22.5 ± 2.5) °C	6 – 10 days	3 to 7 days

If the microorganism is recovered in liquid culture medium, after incubation, centrifuge and discard the supernatant. Suspend the sediment with a dilution 1/20 of the sterile maintenance culture medium and add an equal volume of sterile glycerol solution 20% v/v in water.

If the microorganism is recovered in a solid culture medium, transfer the surface growth to the sterile liquid maintenance culture medium, added with sterile 10% glycerol. The test and the criteria established apply to the product how it is found in the market.

In both cases, dispense small aliquots of the suspension in sterile cryogenic tubes, appropriate for freezing microorganisms.

Store the cryogenic tubes in liquid nitrogen or ultra-freezer (no more than -50 °C). This stock culture can be used to inoculate a series of working culture.

CULTURE MEDIA USED

All culture media used in the test must be tested for their capacity of growth.

PREPARATION OF INOCULUM

From the stock culture, inoculate the surface of the solid culture medium specified on **Table 1**.

To collect the growth of bacteria and yeasts, use sterile saline solution. Collect the suspension obtained in an appropriate sterile tube or vial and add a sufficient amount of sterile saline solution to obtain a concentration of 1×10^8 CFU/mL.

To collect the growth of *A. niger*, use sterile saline solution with 0.05% of polysorbate 80. Collect the suspension obtained in an appropriate sterile tube or vial and add a sufficient amount of sterile saline solution to obtain a concentration of 1×10^8 CFU/mL.

Alternatively, the stock culture can be inoculated in liquid medium (**Table 1**), incubated and then centrifuged. Discard the supernatant and suspend the sediment with sufficient amount of sterile saline solution to obtain a concentration of 1×10^8 CFU/mL.

Refrigerate the suspensions if you do not use them in a period of two hours.

Determine the number of CFU/mL of each suspension by turbidimetry or counting on plate, checking the conditions of time and temperature of incubation and the incubation time for microbial recovery described on **Table 1**, with the purpose of confirming the count in initial CFU. These values will be used to calibrate the size of inoculum to be used in the contaminations of the product being tested.

The suspension of bacteria and yeasts must be used in 24 hours. The suspension of molds can be used in up to seven days if maintained under refrigeration.

PROCEDURE

When the type of package allows the introduction of suspension of microorganisms and when its content is sufficient to execute all steps, conduct the test in five original packages of the product to be tested. Otherwise, transfer the content from one or more original packages to a vial with cap, previously sterilized and of adequate size to contain the necessary amount of sample for executing all steps of the test.

Inoculate each original package or vial with sterile cap with each one of the microorganisms required.

The concentration of the inoculum used must be sufficient to obtain a final concentration on the product between 1×10^5 and 1×10^6 CFU/g or mL – applicable to the categories 1, 2 and 3 (see **Table 2** – column “Type of Product”).

For category 4, the concentration of inoculum must be sufficient to obtain a final concentration on the product between 1×10^3 and 1×10^4 CFU/g or mL.

The volume of inoculum to be introduced must be between 0.5% and 1.0% in relation to the total volume (liquid sample) or weight (solid or semisolid sample) of the product.

Incubate the inoculated samples in oven with temperature between $(22.5 \pm 2.5)^\circ\text{C}$.

Sample each package or vial with inoculated sample in intervals of 7, 14 and 28 days.

Determine by the plating method the number of Colony-Forming Unit (CFU) of each sample, in the initial time and in each time interval specified.

A neutralizing agent specific for the preservative(s) present in the product formulation, determined in the validation study, must be incorporated to the counting plates or in the dilution of the sample prepared for plating.

Calculate the concentration of each microorganism (CFU/mL) present in the sample, compare with the counting at the initial time, and express the change in terms of logarithmic reductions.

PRODUCT CATEGORY AND CRITERIA FOR ANTIMICROBIAL EFFECTIVENESS

For the purpose with the test, the products have been separated in four categories according to **Table 2**, which lists the criteria established for antimicrobial effectiveness.

Table 2 – Product categories and criteria for antimicrobial effectiveness.

<i>Type of product</i>	<i>Microorganism</i>	<i>7th day</i>	<i>14th day</i>	<i>28th day</i>
Category 1 - Injectables, other parenteral products, including emulsions, otologic products, sterile nasal products, ophthalmic products, made of aqueous base or vehicle	Bacteria	There must be a reduction of, at least, 1 log of the number of CFU initially inoculated	There must be a reduction of 3 logs of the number of CFU initially inoculated	The count must not increase in relation to the 14 th day
	Molds and yeasts	There must not be an increase in the number of CFU initially inoculated	There must not be an increase in the number of CFU initially inoculated	There must not be an increase in the number of CFU initially inoculated

<i>Type of product</i>	<i>Microorganism</i>	<i>7th day</i>	<i>14th day</i>	<i>28th day</i>
Category 2 – Products of topical use, made of aqueous base or vehicle, non-sterile nasal products and emulsions, including the ones applied to mucous membranes	Bacteria	----	There must be a reduction of 2 logs of the number of CFU initially inoculated	There must not be an increase of count in relation to the 14 th day
	Molds and yeasts	----	There must not be an increase in the number of CFU initially inoculated	There must not be an increase in the number of CFU initially inoculated
Category 3 – Oral products, made of aqueous base or vehicle, except for anti-acids	Bacteria	----	There must be a reduction of 1 log of the number of CFU initially inoculated	The count must not increase in relation to the 14 th day
	Molds and yeasts	----	There must not be an increase in the number of CFU initially inoculated	There must not be an increase in the number of CFU initially inoculated
Category 4 – Anti-acids made of aqueous base	Bacteria	----	There must not be an increase in the number of CFU initially inoculated	There must not be an increase in the number of CFU initially inoculated
	Molds and yeasts	----	There must not be an increase in the number of CFU initially inoculated	There must not be an increase in the number of CFU initially inoculated

Note: the “non-increase” in the number of CFU inoculated is defined as no more than 0.5 log₁₀ of units bigger than the value previously obtained.

5.5.3.5 MICROORGANISMS USED IN TESTS AND ASSAYS

The microorganisms listed on **Table 1** are indicated for assays and tests outlined in the pharmacopoeia.

Main suppliers of microorganism cultures:

ATCC American Type Culture Collection
<http://www.atcc.org>

CIP Collection de l’Institut Pasteur
<http://www.pasteur.fr/ip/index.jsp>

IMI United Kingdom National Culture Collection (UKNCC)
<http://www.cabi.org> Email: cultures@cabi.org

- INCQS** Instituto Nacional de Controle de Qualidade em Saúde
Department of Microbiology – Laboratory of References Material
Av. Brasil, 4365, Manguinhos, Rio de Janeiro,
RJ, Brazil, ZIP Code: 21.040-900
<http://www.incqs.fiocruz.br>
Email: coleção@incqs.fiocruz.br
- NCIMB** National Collection of Industrial Bacteria
<http://www.ncimb.com>
Email: enquiries@ncimb.com
- NBRC** NITE Biological Resource Center
<http://www.nbrc.nite.go.jp>
Email: collection@nbrc.nite.go.jp
Email: collection@nbrc.nite.go.jp
- NCPF** National Collection of Pathogenic Fungi
<http://www.hpacultures.or.uk>
Email: hpacultures@hpa.org.uk
- NCTC** National Collection of Type Cultures
<http://www.hpacultures.or.uk>
Email: hpacultures@hpa.org.uk
- NCYC** National Collection of Yeast Cultures
<http://www.ncyc.co.uk>
Email: ncyc@ncyc.co.uk

Table 1 – Microorganisms used in tests and assays.

Microorganism	ATCC	CIP	INCQS	NBRC	NCIMB	NCTC	NCPF	NCYC	IMI	IP
Molds and yeasts										
<i>Aspergillus brasiliensis</i>	16404	-	-	9455	-	-	2275	-	149007	1431.83
<i>Candida albicans</i>	10231	-	40006	1594	-	-	3179	1363	-	48.72
<i>Microsporum gypseum</i>	14683	-	40005	-	-	-	-	-	-	-
<i>Saccharomyces kudriavzevii</i>	2601	-	40001	-	-	-	-	-	-	-
<i>Saccharomyces cerevisiae</i>	9763	1432.83	40002	-	-	10716	-	87	-	-
Bacteria										
<i>Bacillus atrophaens</i>	9372	-	-	-	-	-	-	-	-	-
<i>Bacillus cereus</i> var. <i>mycoides</i>	11778	64.52	003	-	-	10230	-	-	-	-
<i>Bacillus pumilis</i>	27142	77.25	-	-	10692	10327	-	-	-	-
<i>Bacillus subtilis</i>	6633	52.62	001	3134	8054	10400	-	-	-	-
<i>Bacteroides vulgatus</i>	8482	103717	059	-	-	11154	-	-	-	-
<i>Bordetella bronchiseptica</i>	4617	53.157	023	-	-	8347	-	-	-	-
<i>Clostridium sporogenes</i>	19404	79.3	-	-	532	532	-	-	-	-
<i>Clostridium sporogenes</i>	11437	-	060	-	-	-	-	-	-	-
<i>Enterococcus hirae</i>	10541	-	019	-	-	-	-	-	-	-
<i>Escherichia coli</i>	8739	53.126	-	3972	8545	12923	-	-	-	-
<i>Escherichia coli</i>	10536	54.127	031	-	8879	10418	-	-	-	-
<i>Geobacillus stearothermophilus</i>	7953	-	-	-	-	-	-	-	-	-
<i>Klebsiella pneumoniae</i>	10031	53.153	030	-	9111	7427	-	-	-	-
<i>Kocuria rhizophila</i>	9341	53.65	010	-	-	8340	-	-	-	-
<i>Micrococcus yunnanensis</i>	7468	-	009	-	-	-	-	-	-	-
<i>Micrococcus luteus</i>	10240	53.160	011	-	8166	7743	-	-	-	-
<i>Micrococcus luteus</i> resistant to neomycin	14452	-	012	-	10418	-	-	-	-	-
<i>Mycobacterium smegmatis</i>	607	-	021	-	-	-	-	-	-	-
<i>Pseudomonas aeruginosa</i>	9027	82.118	-	13275	8626	-	-	-	-	-
<i>Pseudomonas aeruginosa</i>	25619	-	026	-	-	-	-	-	-	-
<i>Salmonella enterica</i> enteric subspecies	-	80.39	-	100797	-	6017	-	-	-	-
<i>Staphylococcus aureus</i>	6538p	53.156	013	-	8625	7447	-	-	-	-
<i>Staphylococcus aureus</i>	6538	4.83	039	13276	9518	10788	-	-	-	-
<i>Staphylococcus epidermidis</i>	12228	68.21	016	-	8853	-	-	-	-	-

5.5.3.6 MICROBIOLOGICAL ASSAYS ON WATER FOR PHARMACEUTICAL USE

SAMPLING

Sampling is an important step in the water quality assessment, since the sample taken for analysis must precisely reflect the performance of the production and distribution system and the quality of the water used. An inadequate sampling may lead to an incorrect assessment, generating unnecessary interventions in the purification system, or compromising the accuracy of the water quality status through an imprecise result.

Considering the peculiarities of each system, the water samples must be taken from the place of their generation to the points of use, since the results obtained in the generation may not reflect the quality of water in the points of use.

The collection of samples in the points of use must be conducted employing practices identical to the ones used routinely in the use of water at that point, mimicking the system operation (valve purge, use of hoses, sanitization of the point, etc.).

The initial sampling plan is usually developed for a program of validation of the system of production of water for pharmaceutical use, in order to characterize its capacity of purification, distribution and supply of water. The sampling plan has short duration (for example, two to four weeks) and determines a high frequency of collection of samples, to generate a significant volume of data that offers an initial assessment of the system performance, to guide the decisions about the use of the water produced.

The initial sampling plan is reassessed when the system is placed in operation, usually to look for a reduction in the amount of data being generated without compromising the capacity to identify abnormal operations/events, especially during the initial phase of the water system life cycle. In the absence of such deviations of quality during the initial sampling period, the frequency of sampling can be reduced to ensure that, in a slightly longer period (for example, for no less than another two to four weeks), there are no adverse quality trends. During this period of time for validation of the second sampling plan, the use of water in routine may be considered a risk. After concluding the assessment, if successful, the monitoring may eventually be reduced again for the sampling plan to be adopted as routine.

Periodically, it is necessary to review the system operation and monitoring to assess the seasonal variability of the water source, the effectiveness of sanitization, and the routine maintenance events. This review must be conducted during the entire life cycle of the water production system, usually every year, to make evident the trend of quality deviations from long-term data.

The sampling plan adopted in the routine must also be reassessed periodically, based on data available, to reassess the frequency and adequate sampling sites. This step offers an opportunity to improve the data assessment and reduce the work loads based on what the data indicate about the process and quality control. The sampling plan adopted as routine must have a rational base for the frequency and sampling sites established, in order to justify how the resulting data will be used to characterize the general system operation and for clearing water for use.

The samples must be collected in sterile borosilicate glass vessels or in sterile plastic bags adequate for microbiological use. The volume of sample must be sufficient to conduct all analysis necessary.

The amount of sample added to the vessels must allow homogenization before conducting the assays, being suggested a space of no less than 2.5 cm above the water surface (headspace).

Disinfectant agents, such as chlorine or other halogenated compounds, when present in water samples, must be neutralized before conducting tests, to ensure adequate recovery of microorganisms that are possibly present. A neutralizing agent commonly used is the sodium thiosulfate solution (0.1 mL of a solution 3% neutralizes above 5 mg/L of residual chlorine in a 120 mL sample).

STORAGE CONDITIONS

The tests must be conducted in the sample up to two hours after the collection and, if it is not possible to conduct the test within this interval, the sample must be kept at refrigeration temperature in the range of 2 °C to 8 °C for no more than 12 hours, to maintain the microbiological characteristics until the analysis. In situations when not even this is possible (such as when laboratories hired out of the site are used), the assay of these refrigerated samples must be conducted within 24 hours after the collection.

CULTURE MEDIA

Soybean-casein digest agar

Peptone from pancreatic casein	15.0 g
Soybean flour obtained by papain digestion	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Purified water q.s.p.	1000 mL

pH 7.3 ± 0.2. Sterilize in autoclave using validated cycle.

Endo C agar

Peptic hydrolysate from animal tissue	10.0 g
Lactose	10.0 g
Dibasic potassium phosphate	3.5 g
Sodium sulfite	2.5 g
Basic fuchsin	0.5 g
Agar	15.0 g
Purified water q.s.p.	1000 mL

pH 7.4 ± 0.2. Sterilize in autoclave using validated cycle.

Eosin methylene blue agar

Pancreatic gelatin hydrolysate	10.0 g
Lactose	5.0 g
Sucrose	5.0 g
Dibasic potassium phosphate	2.0 g
Agar	13.5 g
Eosin Y	0.4 g
Methylene blue	0.065 g
Purified water q.s.p.	1000 mL

pH 7.2 ± 0.2. Sterilize in autoclave using validated cycle.

M-Pa-C Agar

L-lysine	5.0 g
Sodium chloride	5.0 g

Yeast extract	2.0 g
Xylose	1.25 g
Sucrose	1.25 g
Lactose	1.25 g
Phenol red	0.08 g
Ammonium iron citrate III	0.8 g
Magnesium sulfate	1.5 g
Agar	12.0 g
Sodium thiosulfate	5.0 g
Kanamycin	8.0 mg
Nalidixic acid	37.0 mg
Sterile purified water q.s.p.	1000 mL

pH 7.1 ± 0.2 . Do not autoclave. Boil the medium for one minute. Determine the validity period of the medium prepared.

MacConkey Agar

Pancreatic gelatin hydrolysate	17.0 g
Pancreatic casein hydrolysate	1.5 g
Peptic hydrolysate from animal tissue	1.5 g
Lactose	10.0 g
Bile salts	1.5 g
Sodium chloride	5.0 g
Neutral red	0.03 g
Crystal violet	0.001 g
Agar	13.5 g
Purified water q.s.p.	1000 mL

pH 7.1 ± 0.2 . Sterilize in autoclave using validated cycle.

m-HPC Agar

Peptone	20.0 g
Gelatin	25.0 g
Glycerol	10.0 mL
Agar	15.0 g
Purified water q.s.p.	1000 mL

Homogenize the reagents, except glycerol. Adjust the pH to 7.1 ± 0.2 , heat to dissolve and transfer the glycerol. Sterilize in autoclave using validated cycle.

Plate count agar (PCA)

Peptone from casein	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
Agar	15.0 g
Purified water q.s.p.	1000 mL

pH 7.0 ± 0.1 . Sterilize in autoclave using validated cycle.

R2A agar

Peptone (casein or animal tissue)	0.5 g
Casamino Acid	0.5 g
Yeast Extract	0.5 g

Sodium Pyruvate	0.3 g
Glucose	0.5 g
Magnesium Sulfate, heptahydrate	0.05 g
Soluble starch	0.5 g
Dipotassium Hydrogen Phosphate	0.3 g
Agar	15.0 g
Purified water q.s.p.	1000 mL

pH 7.2 ± 0.1. Sterilize in autoclave using validated cycle.

EC or EC-MUG broth

Tryptose or trypticase	20.0 g
Lactose	5.0 g
Mixture of bile salts	1.5 g
Dipotassium hydrogen phosphate	4.0 g
Potassium dihydrogen phosphate	1.5 g
Sodium chloride	5.0 g
4-methyl-umbelliferyl-β-D-glucuronide (MUG)	0.05 g
Purified water q.s.p.	1000 mL

pH 6.9 ± 0.2. Before the sterilization, dispense in tubes that do not presence in high UV light wavelengths (366 nm). The Durham tube is not necessary. Sterilize in autoclave using validated cycle.

Brilliant green bile lactose broth

Peptone	10.0 g
Lactose	10.0 g
Oxgall	20.0 g
Brilliant green	0.0133 g
Purified water q.s.p.	1000 mL

pH 7.2 ± 0.2. Before the sterilization, place an inverted Durham tube in each test tube to detect the production of gas. Sterilize in autoclave using validated cycle.

Lauryl tryptose broth

Tryptose	20.0 g
Lactose	5.0 g
K ₂ HPO ₄	2.75 g
KH ₂ PO ₄	2.75 g
NaCl	5.0 g
Sodium lauryl sulfate	0.1 g
Purified water q.s.p.	1000 mL

pH 6.8 ± 0.2. Before the sterilization, place an inverted Durham tube in each test tube to detect the production of gas. Sterilize in autoclave using validated cycle.

5.5.3.6.1 COUNT OF TOTAL NUMBER OF HETEROTROPHIC BACTERIA

The methods presented are optional and may or may not be ideal for recovery of microorganisms, including the undesirable ones. The selection must be made through experiments, determining which are the adequate methods for monitoring its process, and also for the recovery of specific microorganisms that can be found in water purification systems and may be undesirable for the products to be handled.

There are two categories of culture media for count of the total number of heterotrophic bacteria: the ones with high concentration of nutrients, such as the plate count agar (PCA), soybean-casein digest agar, and m-HPC agar, being adequate for general isolation and count of heterotrophic or copiotrophic bacteria, and the ones with low concentration of nutrients, such as R2A agar, which is indicated for recovery of oligotrophic bacteria.

The temperature and the incubation time are critical aspects for microbiological tests of water, due to the types of microorganisms found in the water systems. Incubations at low temperatures (for example, 20 °C to 25 °C or 25 °C to 30 °C) for longer periods, no less than four days, may lead to higher recoveries of microorganisms than classic temperatures. Media with low amount of nutrients require longer incubation periods (no less than five days), because such media promote a slower growth. Even the ones with high concentration of nutrients may sometimes result in high microbial recovery for long periods of incubation and lower temperatures.

The decision about the type of culture medium and the incubation temperature to test a water purification system must be based on comparative cultivation studies using a native microbiome from the water purification system being analyzed.

PROCEDURE

Plate depth method: add 1 mL of the sample to a Petri dish and pour 15 mL to 20 mL of culture medium maintained at 45 °C to 50 °C, according to **Table 1**. Conduct the test at least on duplicate.

Membrane filtration method: use filtration equipment that allows transferring the membrane to culture media. Use a sterile membrane with 47 mm of diameter and 0.45 µm of porosity, washing the membrane, after the sample filtration, with three portions of 20 mL to 30 mL of sterile purified water. The volume to be filtered may vary according to the sample, obeying a maximum volume that provides 20 CFU to 200 CFU per membrane.

Table 1 – Conditions for count of total number of heterotrophic bacteria.

<i>Method</i>	<i>Types of water</i>		
	<i>Potable water</i>	<i>Purified water</i>	<i>Water for injection and ultrapure water</i>
Type of method	Plate depth or Membrane filtration	Plate depth or Membrane filtration	Membrane filtration
Suggested size of sample ^a	1.0 mL/100 mL ^b	1.0 mL/100 mL ^b	200.0 mL
Medium ^c	R2A agar, PCA	R2A agar, PCA	R2A agar, PCA
Incubation period	R2A agar: 4-7 days (or longer) PCA: 48-72 hours (or longer)	R2A agar: 4-7 days (or longer) PCA: 48-72 hours (or longer)	R2A agar: 4-7 days (or longer) PCA: 48-72 hours (or longer)
Temperature of incubation	R2A agar: 20-25 °C or 30-35 °C PCA: 30-35 °C	R2A agar: 20-25 °C or 30-35 °C PCA: 30-35 °C	R2A agar: 20-25 °C or 30-35 °C PCA: 30-35 °C

^a The sample size must be adequate for the expected microbial count of water to obtain statistically valid colony counts.

^b 1.0 mL for plate depth and 100.0 mL for membrane filtration.

^c To optimize the recovery, an alternative medium may be more adequate (m-HPC, Soybean-casein digest agar).

5.5.3.6.2 RESEARCH ON TOTAL AND FECAL COLIFORMS

The coliform group consists of several bacteria belonging to the Enterobacteriaceae family. The historical definition of this group was based on the method used for detection: fermentation of lactose in detriment of the systematic bacteriology principles. Consequently, when the fermentation technique is used, this group is defined as facultative anaerobic, Gram-negative rods, that do not form spores, and fermenters of lactose with formation of gas and acid when incubated for 48 hours at 35 °C. The standard test for the coliform group may be conducted by the following methods: fermentation in multiple tubes, membrane filtration, or chromogenic.

For all types of water for pharmaceutical use, regardless of the method used, total and fecal coliforms must be absent.

PROCEDURE

Method of fermentation in multiple tubes

Presumptive phase – Use Lauryl tryptose broth. If the medium is refrigerated after the sterilization, leave it at room temperature before use. Discard the tubes that present growth or bubbles. Prepare the Lauryl tryptose broth at such a concentration that, by adding 100 mL, 20 mL or 10 mL of the sample to the medium, the concentration of ingredients from the formula will not be reduced, as described on **Table 2**. Make sure that the inverted Durham tubes are free of bubbles.

Table 2 – Preparation of the Lauryl tryptose broth.

Sample (mL)	Amount of medium/tube (mL)	Volume of medium + sample (mL)	Lauryl tryptose broth required (g/L)
1	10 or more	11 or more	35.6
10	10	20	71.2
10	20	30	53.4
20	10	30	106.8
100	50	150	106.8
100	35	135	137.1
100	20	120	213.6

Shake the sample vigorously. Use five portions of 20 mL, 10 portions of 10 mL or a single sample of 100 mL. Mix the portions of sample in the medium slightly stirring. Incubate at (35 ± 0.5) °C. After (24 ± 2) hours, shake each tube slightly and observe for presence of growth or formation of gas; if no gas is evident, re-incubate the tubes and reexamine at the end of (48 ± 3) hours. Register the presence or absence of growth and gas. Turbidity or production of gas in the tubes within (48 ± 3) hours comprises a positive presumptive reaction. In this case, carry on with the confirmatory phase.

Confirmatory phase – Use Brilliant green bile lactose broth. Make sure that the inverted Durham tubes are free of bubbles. Slightly shake the positive Lauryl tryptose broth fermentation tubes and inoculate one or more loops of culture in Brilliant green bile lactose broth. Incubate the Brilliant green bile lactose broth tubes at (35 ± 0.5) °C. The growth and formation of any amount of gas within (48 ± 3) hours comprises a positive confirmatory phase, indicating the presence of total coliforms.

To estimate the density of coliforms, calculate the Most Probable Number (MPN) from the number of positive tubes of Brilliant green bile lactose broth (**Tables 3 and 4**).

Table 3 – MPN index and confidence limits of 95% for all combinations of positive and negative results when five portions of 20 mL of sample are used.

Number of tubes with positive result (20 mL of sample each)	MPN index/100 mL	Confidence limit of 95% (exact)	
		Low	High
0	< 1.1	-	3.5
1	1.1	0.051	5.4
2	2.6	0.40	8.4
3	4.6	1.0	13
4	8.0	2.1	23
5	8.0	3.4	-

Table 4 – MPN index and confidence limits of 95% for all combinations of positive and negative results when 10 portions of 10 mL of sample are used.

Number of tubes with positive result (10 mL of sample each)	MPN index/100 mL	Confidence limit of 95% (exact)	
		Low	High
0	< 1.1	-	3.4
1	1.1	0.051	5.9
2	2.2	0.37	8.2
3	3.6	0.91	9.7
4	5.1	1.6	13
5	6.9	2.5	15
6	9.2	3.3	19
7	12	4.8	24
8	16	5.8	34
9	23	8.1	53
10	>23	13	-

Complete phase: – Slightly shake the positive Brilliant green bile lactose broth fermentation tubes and inoculate one or more loops of culture in EC or EC-MUG broth. Alternatively, the inoculation in EC or EC-MUG broth may be done from the culture in Lauryl tryptose broth simultaneously to the inoculation in Brilliant green bile lactose broth in the confirmatory phase. Incubate the tubes of EC or EC-MUG at temperature of $(44 \pm 0.2)^\circ\text{C}$ for (24 ± 2) hours.

The observation of growth and the production of gas in the EC or EC-MUG tubes indicate the presence of fecal coliforms or *E. coli*, respectively. In parallel, positive cultures from Brilliant green bile lactose broth with negative results on EC or EC-MUG broths indicate the presence of non-fecal coliforms.

Confirmatory tests using selective and differential media and biochemical tests for identification of species may be conducted when necessary.

Membrane filtration method: use filtration equipment that allows transferring the membrane to culture media. Use a sterile membrane with 47 mm of diameter and 0.45 μm of porosity, washing the membrane, after the sample filtration, with three portions of 20 mL to 30 mL of sterile purified water. The volume to be filtered may vary according to the sample, obeying a maximum volume that provides 20 CFU to 200 CFU per membrane. For research of coliforms, the membrane must be incubated in specific medium (for example, MacConkey, endo C, eosin methylene blue, etc.), at the temperatures established for studies on total and fecal coliforms for 24 hours.

Chromogenic method: culture media that have in their formulation specific enzyme substrates allow significant improvements in the recovery of microorganisms and in their identification.

In case of research of total coliforms and *E. coli*, there are alternative methods correlated to the traditional membrane filtration and multiple tube methods. There are tests based on using a specific substrate, which allow the simultaneous research on fecal coliforms and *E. coli* in a shorter period of time. For example, tests based on the activity of β -galactosidase over the ONPG (O-nitrophenyl- β -D-galactopyranoside) substrate and of β -D-glucuronidase over the MUG (4-methylumbelliferyl- β -D-glucuronide) substrate. The test is based on the addition of 100 mL of sample to the substrates and incubation at 35 °C to 37 °C for 24 hours. The activity of total coliforms over the ONPG substrate produces a yellow coloration, indicating their presence. The presence of *E. coli* can be confirmed by fluorescence under UV light, due to their activity over the MUG substrate.

5.5.3.6.3 RESEARCH ON *Pseudomonas aeruginosa*

The research on *Pseudomonas aeruginosa* in water for pharmaceutical use can be conducted by the membrane filtration method. For all types of water for pharmaceutical use, *P. aeruginosa* must be absent.

PROCEDURE

Membrane filtration method: filter 200 mL of sample through sterile filtration membrane. Put each membrane on the M-Pa-C agar plate so that there is no room between the membrane and the agar surface. Invert the plates and incubate at (41.5 ± 0.5) °C for 72 hours. Typically, the *P. aeruginosa* colonies have 0.8 mm to 2.2 mm of diameter and are apparently flat, with clear edge and brownish to dark green core. Count typical colonies, preferably from the filter with 20 to 80 colonies. Confirm the presence of *P. aeruginosa* through adequate biochemical tests. Other methods and culture media can be used, provided that duly validated.

5.6 IMMUNOCHEMICAL METHODS

The immunochemical methods are based on selective, reversible and non-covalent binding between antigens and antibodies. These methods are used to detect or dose antigens and antibodies. The detection or assay of the antigen-antibody complex can be made by several techniques. The requisites from this method apply to the immunochemical methods used, in the case of labeled or unlabeled reagents.

The results from immunochemical methods depend on the conditions of the experience, the nature and the quality of the reagents used. It is essential to measure the components of an immunological assay and use international preparations of reference for immunoassay whenever available. The reagents necessary to many of the immunochemical methods are available in the market in the form of kits that include reagents (especially the antigen or antibody) and the materials targeted at *in vitro* assessment of a certain substance, as well as the necessary instructions for their correct use. The kits must be used according to instructions from the manufacturer, and it is important to make sure they are adequate to the sample analysis, especially concerning selectiveness and sensitivity. The requisites related to kits for immunoassay are provided by the World Health Organization.

METHODS USING LABELED ANTIGENS OR ANTIBODIES

The techniques that use labeled substances must use appropriate labels, such as enzymes and radioisotopes. When the label is a radioisotope, we call the technique *radioimmunoassay*. All techniques conducted with radioactive substances must be in compliance with the national and international legislation for protection against the risk from radiations.

METHODS USING UNLABELED ANTIGENS OR ANTIBODIES

Immunoprecipitation methods. The immunoprecipitation methods include flocculation and precipitation reactions. When a solution from an antigen is mixed with the corresponding antibodies, in adequate conditions, the reagents form flocculant or precipitating aggregates. The ratio between the amounts of reagents corresponding to the shortest flocculation time, or to the most accentuated precipitation, is called optimum ratio. It is usually obtained in presence of equivalent amounts of antigen and antibody. The immunoprecipitation can be assessed visually or through measuring the light dispersion.

Turbidimetric immunochemical methods. An increase in the method sensitivity can be obtained by using particles coated of antibodies or antigens (for example, latex). In the flocculation methods, usually successive dilutions of one of the reagents are used, while in the immunodiffusion (ID) method the dilution is obtained by diffusion in a gel. Concentration gradients from one or two reagents are obtained, in order to create on the gel zones where the proportions of reagents favor the precipitation. While the flocculation methods are conducted on test tubes, the immunodiffusion methods can be conducted using different supports, such as beakers, plates, slides, tubs or chambers. It is called *single immunoprecipitation* when the antigen reacts only with its corresponding antibody; it is called *complex* when several reagents that are serologically related are used; and *multiple* when several reagents not serologically related are used. In the single diffusion method, a concentration gradient is established only for one of the reagents diffused from an external source inside the gel that has the reagent corresponding to a relatively low concentration.

Single radial immunodiffusion (SRID). It is a single quantitative immunodiffusion technique. When the equilibration between internal and external reagents is established, the area of the circular

precipitation zone, originated from the external reagent, is directly proportional to the concentration of antigen applied and inversely proportional to the concentration of antibodies in gel.

Double diffusion methods. The concentration gradients are established for two reagents. Both the antigen and the antibody diffuse from separate places in a gel that is initially neutral under the immunological standpoint. The double immunodiffusion methods are used to compare, qualitatively, several antigens in relation to an appropriate antibody, or vice-versa. The comparison is based on the presence or absence of interaction between the precipitation standards. It is possible to distinguish reactions of identity, non-identity or partial identity between antigens and antibodies.

Immunoelectrophoresis methods. Immunoelectrophoresis (IE) is a qualitative technique of two associated methods: gel electrophoresis, followed by immunodiffusion.

Crossed immunoelectrophoresis. The crossed immunoelectrophoresis is a modification of the immunoelectrophoresis (IE), adapted to the qualitative and quantitative analysis. Initially, a classic electrophoresis is conducted. A strip of gel that contains the fractions to be analyzed, separated by electrophoresis, is then cut out and transferred to another plate. This new plate is subjected to a second electrophoresis perpendicularly to the previous strip, with the use of a gel that has a relatively low content of antibodies corresponding to the antigen. For a given concentration of antibodies and gel thickness, the ratio between the area of each precipitation peak and the amount of corresponding antigen is linear.

Electroimmunology method or rocket immunoelectrophoresis. The electroimmunoassay, many times referred to as rocket immunoelectrophoresis, is a quick method to dose antigens which charge is different from the antibody and vice-versa. The electrophoresis of the antigen to be dosed is conducted in a gel that must have a concentration relatively lower than the one from the corresponding antibody. The substance to be analyzed and the dilutions of the antigen used for calibration must be placed on different wells of the gel. During the electrophoresis, fusiform precipitation zones are formed and migrate from the wells. When the antigen is no longer in excess, the precipitation line becomes stationary. For a given concentration of antibodies, the ratio between the distance traveled by the precipitation line and the amount of antigen applied is linear.

Contra-immunoelectrophoresis. It is a quick quantitative method that allows establishing gradients of concentration of external antigens and antibodies, in an electric field depending on their different charges. The standard and sample dilutions must be organized on a row of wells in the gel. A known amount of corresponding reagent is placed on an opposite row of wells. The titer of the substance to be dosed may be considered the highest dilution where a precipitation line is observed. There are variants of crossed immunoelectrophoresis and immunoelectrodosage. Other techniques associate the separation of antigen with the molecular size and serological properties. The visualization and characterization of immunoprecipitation lines can be conducted by selective, or non-selective, coloring, by fluorescence, enzyme labeling, isotope labeling, or other appropriate techniques. Selective coloring is normally used for characterizing non-protein substances on the precipitates.

In translucent gels, such as agar or agarose, the precipitation line becomes clearly visible in the gel, provided that the concentration of each of the reagents is adequate.

VALIDATION OF METHOD

A quantitative immunochemical method will only be valid if:

a) the antigen or antibody does not discriminate, significantly, the substance being analyzed from the standard. In case of a labeled reagent, the corresponding reagent must not distinguish, significantly, the labeled substance from the non-labeled substance;

- b) the method is not influenced by the assay matrix, that is, all components of the sample being analyzed, or its excipients, that may vary from one sample to the other. They may include high concentrations of other proteins, salts, preservatives in high concentrations, or have a proteolytic contamination activity;
- c) the limit of quantification is lower than the criteria for acceptance indicated in the individual monograph;
- d) the accuracy of assay is such that the variation of results corresponds to the requirements established in the individual monograph;
- e) absence of systematic errors when conducting the assay.

Parameters for validation of method

For these criteria to be checked, the validation includes the following elements:

- a) the assay must be conducted at least in triplicate;
- b) the assay must include no less than three different dilutions of standard and three different dilutions of sample with supposed activity similar to the one from the standard preparation;
- c) the samples must be distributed randomly;
- d) if the sample is present in serum, or if it is mixed with other constituents, the standard must be prepared in the same way;
- e) the assay must include a measure of non-specific bond of the labeled reagent;
- f) for radioimmunoassays with shift: the maximum bond (zero shift) must be determined and the dilutions must cover the full range of responses to the closest values from the non-specific bond to the maximum bond, preferably both for the sample and for the standard.

STATISTICAL CALCULATION

For analysis of results, the response curves from sample and standard can be analyzed by the statistical procedures applicable to biological assays. The significant non-parallelism indicates that the antigen or antibody distinguishes the sample from standard and implies in invalidation of result. On immunoassays with shift, the values from non-specific bond and maximum shift at a high concentration of sample or standard must not be significantly different. The differences may reflect effects due to the matrix, whether by inhibition of bond or degradation of label.

5.7 PHYSICAL METHODS APPLIED TO SURGICAL AND HOSPITAL MATERIALS

5.7.1 TENSILE STRENGTH

The determination of tensile strength of surgical sutures must be made in an environment with constant humidity and temperature. The relative air humidity must be between 60% and 80% and the temperature, between 20 °C and 25 °C.

EQUIPMENT

When determining the tensile strength of surgical sutures, the equipment must have an electric motor that applies to the suture being analyzed a constant load rate per time unit.

INCLINED PLANE EQUIPMENT

Specifications: the clamps must be of roll type with flat surfaces for fastening the sutures. The roll diameter must be of 1.8 cm to 1.9 cm and the flat surfaces must have no less than 2.5 cm of length. The distance between clamps must be 1.25 cm. The load carriage friction must allow the recording pen to slide up to 2.5% of the registration capacity when there is no sample. The plan inclination speed must be adjusted so that 20 seconds are necessary from the beginning of the test for the maximum slope of 30 degrees to be achieved.

PROCEDURE

Determine the tensile strength of surgical sutures with the same preliminary precautions required for the diameter determination test. Adjust the weight of the carriage so that, at the moment the rupture occurs, the position of the recording pen is between 20% and 80% of the registration capacity.

Direct traction: insert the suture in the equipment by fastening one of the ends and passing the free end through the other clamp. Apply on it a tension equivalent to 25% of the minimum strength required for the suture being tested and tighten the clamp. Adjust the recording pen on the point zero of the chart and turn the equipment on; record the reading and assess the strength. Discard the determination when the suture ruptures, near the clamps.

Traction on knot: determine the tensile strength over surgical knot by making on the suture being tested a surgeon's knot (**Figure 1**) over a segment of a flexible rubber tube with 5 cm of length, 6.5 mm of internal diameter, and 8.1 mm of external diameter. Place the suture on the equipment so that the knot is positioned equidistantly from the clamps. Adjust the recording pen on the point zero of the chart and turn the equipment on; record the reading and assess the strength. Discard the determination when the suture ruptures, near the clamps.

Execution of surgeon's knot

To make a surgeon's knot, proceed as follows:

- hold the ends of the surgical suture, one in each hand;
- place the tip on the left hand over the tip on the right hand, forming a circle;
- introduce the overlapped end in the loop;
- repeat the operation;
- fasten on the flexible rubber tube;

- f) put the right side end over the left side end, forming a second loop;
- g) close the knot.

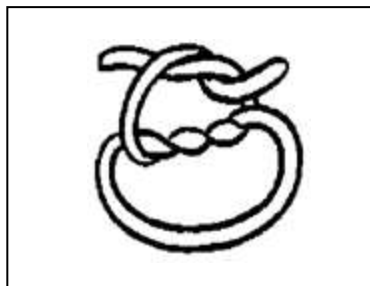


Figure 1 – Surgeon's knot.

Results: The results must comply with what is described on the respective monographs.

5.7.2 DIAMETER OF SUTURES

The determination of diameter of surgical sutures must be made in an environment with constant humidity and temperature. The relative air humidity must be between 60% and 80% and the temperature, between 20 °C and 25 °C. The weights for pre-tension to determine the diameter of multifilament threads are registered on **Table 1**.

APPARATUS

The gauge used to determine the diameter of sutures is of “dead weight” type, mechanical or electronic, and is equipped with a direct, digital or printed reading output dial. The scale resolution is of no less than 0.002 mm and the presser foot must have approximately (12.70 ± 0.02) mm of diameter. The presser foot and the moving parts connected to it must have total load of (210 ± 3) g to the sample. For sutures of surgical number 9-0 and smaller, remove the additional weight from the presser so that the total weight on the sample does not exceed 60 g. The presser foot and the equipment base must present parallelism and flatness of 0.005 mm.

Table 1 – Weights for pre-tension to determine the diameter of multifilament threads.

<i>Number according to metric system</i>	<i>Surgical number</i>	<i>Absorbable sutures</i>	<i>Non-absorbable sutures</i>
0.01	12-0	-	-
0.1	11-0	-	-
0.2	10-0	12.5	12
0.3	9-0	25	27
0.4	8-0	35	38
0.5	7-0	70	69
0.7	6-0	125	125
1.0	5-0	340	250
1.5	4-0	475	375
2	3-0	885	600
3	2-0	1340	900
3.5	0	1950	1350
4	1	2540	1700
5	2	3175	2200
6	3 and 4	3645	3050
7	5	-	3850
8	6	-	4550
9	7	-	5650

PROCEDURE

The diameter of surgical sutures of natural origin, packaged without preservative liquid, is determined after they remain for no less than four hours in atmosphere with temperature and humidity previously specified. The sutures packaged with preservative liquid are submitted to test immediately after they are removed from the liquid, without prior drying.

Multifilament sutures

To determine the diameter of multifilament surgical sutures, the measurements must be made keeping them tensioned with the help of a roller system fastened to a table, according to **Figure 1**, and proceeding as detailed below:

- fasten one of the suture ends through a fastening clamp;
- on the other free end, put a weight with a mass according to **Table 1**. Note: It is necessary to be careful to not twist the suture;
- position the suture on the gauge so that it passes through the center of the circular base and, with the help of a lever, lower the moving rod foot slowly until the entire load is applied;
- measure the suture diameter on three points, approximately at $1/4$, $1/2$ and $3/4$ of its total length;
- in case of braided sutures with diameters larger than the surgical number 3-0, make two perpendicular measurements in each point.

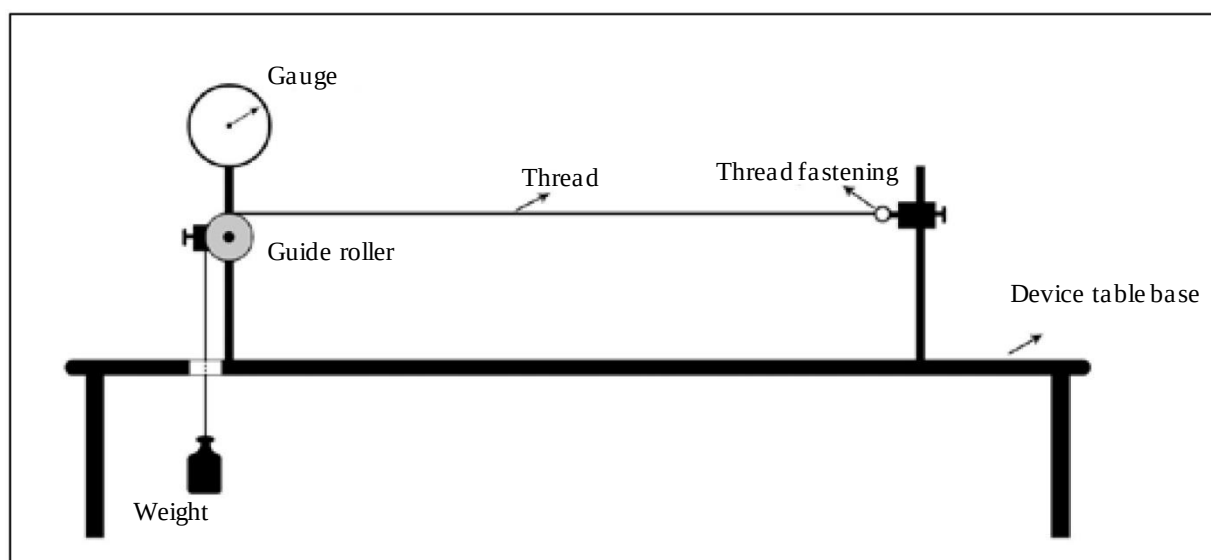


Figure 1 – Model of table suggested for measuring the diameter of multifilament sutures.

Monofilament sutures

To determine the diameter of monofilament sutures, it is necessary to proceed as follows:

- make the measurements on sutures in the dry form or with fluid, immediately after they are removed from the package, without prior drying;
- position the suture on the gauge, between the fixed base and the moving lock base;
- lower the lever slowly so that the entire load is on the suture;
- measure the suture diameter on three points, approximately at $1/4$, $1/2$ and $3/4$ of its total length.

Result: The average of the measurements made in the sutures must be between the limits established on the respective monographs. Individual values must be comprised between the averages of limits for surgical numbers, immediately inferior and posterior to the one analyzed.

5.7.3 RESISTANCE TO NEEDLE THREADING

The purpose of this assay is to assess the fastening of threads for sutures in atraumatic needles.

APPARATUS

Use an universal traction machine equipped with electric motor that applies a constant load rate per time unit. The load cell used must be compatible with the necessary traction force for checking.

PROCEDURE

Fasten the needle to one of the equipment clamps so that the threaded part is free and aligned with the direction where the force will be applied by the moving clamp. Measure the strength required to unthread the suture from the needle.

Results: they must be assessed considering **Table 1**.

Note: the assessment of resistance to threading must consider simultaneously the individual limits for threads and the limits for the average of five threads from the lot analyzed. If one of the individual limit results, and no more than one, does not meet the minimum limits for individual values, repeat the assay with another ten threads. The assay requisite will be met if none of the 10 samples is below the limits described.

Table 1 – Limits of resistance to needle threading in relation to the surgical number.

Surgical number	Number according to metric system			Minimum resistance limits			
	Absorbable		Non-absorbable	Average		Individual	
	Natural	Synthetic		kgf	N	kgf	N
11-0	-	0.1	0.1	0.007	0.07	0.005	0.05
10-0	-	0.2	0.2	0.014	0.14	0.010	0.10
9-0	0.4	0.3	0.3	0.021	0.21	0.015	0.15
8-0	0.5	0.4	0.4	0.05	0.49	0.025	0.25
7-0	0.7	0.5	0.5	0.08	0.78	0.045	0.44
6-0	1	0.7	0.7	0.17	1.67	0.08	0.78
5-0	1.5	1.0	1.0	0.23	2.26	0.11	1.08
4-0	2	1.5	1.5	0.45	4.41	0.23	2.26
3-0	3	2	2	0.68	6.67	0.34	3.33
2-0	3.5	3	3	1.10	10.79	0.45	4.41
0	4	3.5	3.5	1.50	14.71	0.45	4.41
1	5	4.0	4.0	1.80	17.65	0.60	5.88
2	6	5	5	1.80	17.65	0.70	6.86
3	7	6	6	2.00	19.61	0.90	8.83
4	8	6	6	2.00	19.61	0.90	8.83
≥ 5	-	≥ 7	≥ 7	2.00	21.57	1.10	10.79

5.7.4 DETERMINATION OF ABSORPTION

To conduct tests on *Determination of absorption*, remove the cotton from its original package and store it previously, for no less than four hours, in a place with relative air humidity of $(65 \pm 2)\%$ and temperature of $(21 \pm 1.1)^\circ\text{C}$.

PROCEDURE

Use a basket that weighs no more than 3 g, made of copper wire with approximately 0.4 mm of diameter, in the shape of a cylinder with approximately 5 cm of diameter and 8 cm of depth, with spaces of approximately 2 cm between wires. Transfer portions of hydrophile cotton with, exactly, approximately (1 ± 0.05) g, from five different parts of the package, through pulls, not cuts of the sample. Place the matched portions in the basket and weigh. Hold the basket by its side approximately 12 mm from the water surface at $(25 \pm 1)^\circ\text{C}$ and let it drop in the water. Determine, preferably by using a timer, the time in seconds required for complete submersion.

Remove the basket from the wire, let it drain for 10 seconds in the same horizontal position, then place it immediately in a vessel tared and covered, and weigh. Calculate the mass of water absorbed from the mass of the test basket and the mass of hydrophile cotton.

5.7.5 DETERMINATION OF THE FIBER LENGTH

To conduct tests on *Determination of fiber length*, remove the cotton from its original package and store it previously, for no less than four hours, in a place with relative air humidity of $(65 \pm 2)\%$ and temperature of $(21 \pm 1.1)^\circ\text{C}$.

This procedure applies to the Suter-Webb cotton fiber duplex sorter device. With changes in the procedure, it may be applied to two Baer sorters arranged sequentially, or to a Johannsen or another similar device.

APPARATUS

The sorter consists of two banks with combs rigidly assembled side by side over a common base. Each bank of combs consists of no less than 12 individual combs spaced 3.2 mm apart, one behind the other, and fitted so that, as they are approached during the fractioning process and no longer necessary, they can be released to fall below the work plan. Each comb has a single series of teeth precisely aligned and very sharp, with 12 mm of length, comprised of needles with 0.38 mm of diameter. The teeth are spaced 62 mm to 25 mm apart in an extension of approximately 50 mm.

The accessories consists of fiber sorter forceps, fiber depressing grid, flat fiber depressor plate, and velvet-covered plates. The sorter forceps consists of two brass pieces, with approximately 75 mm of length, hinged on one side and slightly curved on the other, thus presenting a beak shape to catch the fibers that are out and near the comb surfaces. Usually, one of the catcher ends has padding in leather or another fibrous material. The catcher end has approximately 19 mm of width.

The fiber depressing grid consists of series of metal rods spaced 3.2 mm apart, so that the fibers can be placed between the combs to press the fibers down between the teeth. The flat fiber depressing plate consists of a polished metal plate, with approximately 25 mm by 50 mm, with a round knob or handle on the upper surface through which the plate can be smoothed over the fibers as they are placed on the surface of the velvet-covered plates. The velvet-covered plates, on which the fibers can be placed in order, are aluminum plates with approximately 100 mm by 225 mm and 2.4 mm of thickness, covered on both sides by high-quality velvet, preferably black.

SELECTION OF COTTON

After unrolling the cotton, prepare a representative sample by taking, from a package with 225 g to 450 g, 32 samples (each one with approximately 75 mg) well distributed along the lap, being 16 taken from one longitudinal half and the rest, from the other half.

Avoid the lap ends and carefully make sure that the portions are taken considering the lap thickness. To avoid the selection of only long fibers or short fibers, remove all fibers from each sample and do not let them pass through your fingers.

Weigh eight samples from packages with no more than 112.5 g and weigh 16 samples from packages weighing 112.5 g to 225 g, all of them well distributed.

Mix the samples in pairs, randomly, and match each pair by pulling and rolling softly on the fingers. Then, divide each matched pair lengthwise in two approximately equal parts and use one part in the further mixture (the other part can be discarded or reserved for any other tests or controls).

Repeat the process described on the previous paragraph with the successive halves of the series bifurcated until it results in only one sample. Arrange the final sample fibers softly in parallel position, pulling and rolling them on the fingers. Retain all fibers, including, as much as possible, the ones tangled and the masses of braided fibers, discarding only unripe seed fragments with fibers and non-fibrous foreign material, such as petioles, leaves and fragments of integuments.

From the final sample described on the previous paragraph, separate lengthwise a sample with (75 ± 2) mg, weighed accurately. Retain the residue for any necessary test.

PROCEDURE

Using the fiber depressing grid, carefully insert the weighed sample in a comb bank from the cotton sorter, so that it extends through the combs in approximately straight angles.

With the sorter forceps, hold, by the free ends, a small portion of fibers that extends through the teeth from the comb closest to the operator; softly take it from the combs and transfer it to the tips of teeth from the second banks, laying the fibers parallel with each other, linearly and in approximately straight angles in relation to the teeth sides, releasing as close as possible to the front comb side.

Using the depressing grid, carefully press down the fibers transferred on the comb teeth. Continue the operation until all fibers are transferred to the second comb bank. During this fiber transfer, let the combs from the first bank fall successively when and while all protruding fibers are removed.

Turn the equipment 180° and transfer the cotton fibers back to the first comb bank as described previously.

The ends of the fibers must be carefully smoothed during both transfers, being arranged as close as possible to the front surface of the proximal comb. Such smoothing may involve removing isolate fibers from both sides, frontal and distal, of the comb banks and depositing them again in the main bundle of the combs.

Turn the equipment 180° again. Let successive combs fall, if necessary, to expose the ends of the longer fibers. It may be necessary to deposit again some loose fibers. Using the forceps, remove the few more protruding fibers. Thus, continue removing successively the remaining protruding fibers back to the front side of the proximal comb. Let this comb fall and repeat the series of operations in the same way until all fibers have been removed. To not disturb the sample seriously and, therefore, vitiate the fractioning in groups, pull several times (eight to ten) between each pair of combs.

Place the pulls over the velvet-covered plates parallel with each other, as straight as possible, with the ends as clearly defined as possible, and with the distal parts arranged in a straight line, pressing them down gently with the flat fiber depressor plate before releasing the pull from the forceps. Use no less than 50 and no more than 100 pulls to fraction the sample.

Group all fibers that have length of 12.5 mm or more and weigh the group until tenths of milligram. Likewise, group all fibers that have length of 6.25 mm or less and weigh the same manner. Finally, group the remaining fibers, of intermediate lengths, and weigh. The sum of the three weights must not be more than 3 mg different from the initial sample weight. Divide the mass of each one of the two first groups by the sample mass to obtain the percentage in weight of fiber on the two length ranges.

5.8 GENERAL METHODS APPLIED TO MEDICINAL GASES

5.8.1 DETERMINATION OF GASES

5.8.1.1 DETERMINATION OF GASES USING DETECTOR TUBES

Detector tubes are sealed cylindric tubes comprised of an inert clear material, built in order to enable the passage of gas. They have reagents adsorbed in inert substrates appropriate to view the substance to be detected and may have preliminary layers and/or adsorbent filters to eliminate the impurities that interfere with the substance to be detected. Such layers have a single reagent for detecting a certain impurity or several reagents to detect several substances (single-layer or multi-layer tube).

The assay is conducted passing the necessary volume of gas to be analyzed through the indicator tube. The extension of the colored layer or the intensity of color change in a graded scale allows assessing the presence of impurities.

The user must ensure the adequacy of detector tubes for the intended use and they must be used according to the procedure below or to instructions from the manufacturer.

The gas supply must be connected to adequate pressure regulator and needle valve. Connect a flexible tube to a T for adjusting the flow of gas to be analyzed and for the tube purge, in order to obtain adequate flow (**Figure 1**). Couple the indicator tube to the dosing pump and connect the other end to the T. Operate the pump so that an adequate volume of the gas to be analyzed passes through the tube. Read the value corresponding to the extension of the colored layer or intensity of color in the graded scale. If the result is negative, the indicator tube may be checked through a calibration gas with the adequate impurity.

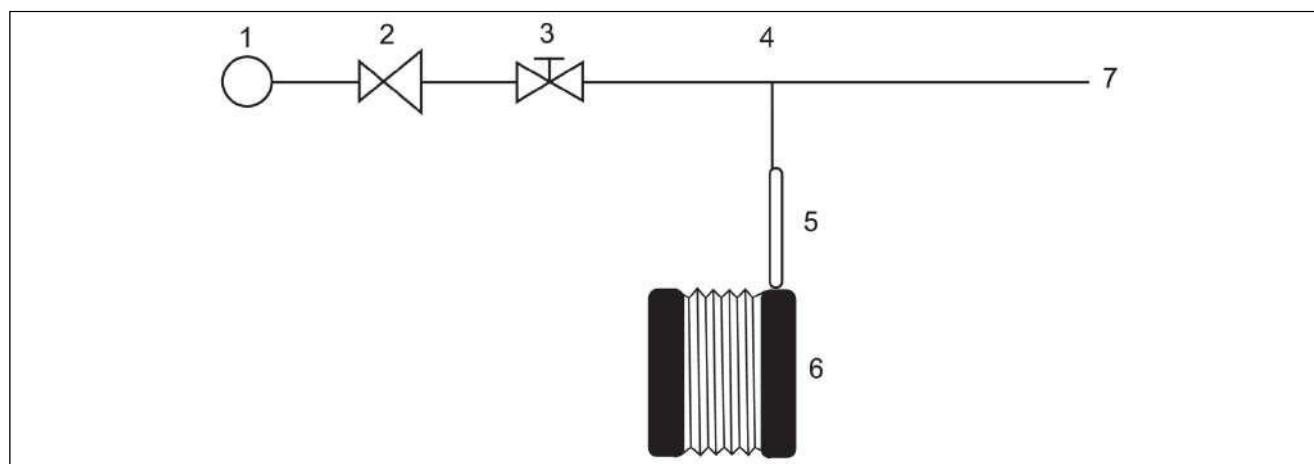


Figure 1 – Assembly for gas detector tubes.

1 Gas supply; 2 Pressure regulator valve; 3 Needle valve; 4 T-shaped part; 5 Indicator tube; 6 Dosing pump; and 7 Outlet to atmosphere.

Carbon monoxide: the carbon monoxide detector tube must indicate the minimum concentration of 5 micromol/mol (ppm), with the relative standard deviation of results of no more than 15.0%.

Carbon dioxide: the carbon dioxide detector tube must indicate the minimum concentration of 100 micromol/mol (ppm), with the relative standard deviation of results of no more than 15.0%.

Sulfur dioxide: the sulfur dioxide detector tube must indicate the minimum concentration of 0.5 micromol/mol (ppm), with the relative standard deviation of results of no more than 15.0%.

Nitrogen monoxide and nitrogen dioxide: the nitrogen monoxide and nitrogen dioxide detector tube must indicate the minimum concentration of 0.5 micromol/mol (ppm), with the relative standard deviation of results of no more than 15.0%.

5.8.1.2 DETERMINATION OF GASES BY NON-DISPERSIVE INFRARED SPECTROPHOTOMETRY

Gases absorb light in one or more specific wavelengths and this property is broadly used to determine their concentrations. The analytical technique that uses the infrared analyzer may be used to determine impurities, such as carbon monoxide, carbon dioxide, among others.

The analyzer is comprised of two identical generators of infrared beams: one beam crosses a cell with the sample and the other crosses a cell with the reference gas. The infrared radiation emitted by a source is converted to intermittent light through a rotary switch (chopper) and enters the detector chamber after going through one of the measurement cells. Part of the radiation is absorbed by the impurity that may exist in the sample cell, producing a difference in the intensity on the two beams that enter the detector chambers. This difference produces an electric current that is proportional to the amount of impurity that exists in the sample.

Whenever the equipment is turned on, it must be adjusted as described in the specific monograph for each gas.

5.8.1.3 DETERMINATION OF GASES BY PARAMAGNETIC ANALYSIS

The paramagnetic signal identifies the oxygen in the sample. The principle of the method is based on the high paramagnetic sensitivity of the oxygen molecule, which has a strong interaction in the magnetic field, which signal is measured electronically, amplified and converted to a value transmitted in the oxygen concentration reader.

The equipment must be calibrated periodically. The equipment reading range is from 0 to 100.0% v/v, with resolution of 0.1%, linearity of $\pm 0.1\%$ and precision of 0.1%.

For calibrating the equipment, adjust the zero passing standard certified nitrogen gas with minimum purity of 99.99% v/v through the device until a constant reading is achieved; configure to the scale of 100% passing the calibration gas (standard certified oxygen with minimum purity of 99.99% v/v) with the same flow used when conducting the calibration from zero until constant reading is achieved.

Procedure: pass the gas to be analyzed through the analyzer with the same flow used at calibration until constant reading is achieved. Register the sample gas concentration read on the instrument. The result must be in percentage of volume of oxygen.

5.8.1.4 DETERMINATION OF GASES BY ULTRAVIOLET SPECTROPHOTOMETRY

The analytical technique that uses the ultraviolet fluorescence analyzer can be used to determine impurities, such as sulfur dioxide, nitrogen dioxide, total of sulfur compounds, among others.

The device is comprised of an ultraviolet radiation generator system, with specific wavelength to determine each gas impurity, formed by an ultraviolet lamp, a collimator and a selective filter, a reaction chamber, where the gas being assayed circulates, and a system for detection of the radiation emitted in the specific wavelength, comprised of a selective filter, a photomultiplier tube and an amplifier. The light beam is periodically interrupted by a rotary switch (chopper) spinning at high speed.

Whenever the equipment is turned on, it must be calibrated as described in the specific monograph for each gas.

5.8.1.5 DETERMINATION OF GASES BY CHEMICAL LUMINESCENCE

The analyzer operates through the principle that nitric oxide (NO) and ozone (O₃) react producing a characteristic luminescence, with intensity linearly proportional to the concentration of NO. The chemical luminescence reaction is described below:



For the chemiluminescence reaction to happen, the nitrogenated species must be in the form of NO. The sample stream is divided into two streams, one going through a converter of NO_x into NO and the other not passing through the converter. The stream that passes through the converter will have a higher NO concentration than the real one, and the stream that does not go through the converter will have the actual NO concentration. The difference between both will reveal the concentration of NO₂.

Principle of operation: the sample enters the equipment, crosses the capillary and goes to a valve that divides the sample flow into two streams, one for the reaction chamber and the other for the converter of NO₂ into NO. A flow meter indicates the flow of sample that goes to the chamber. Dry air enters on the other hole, passes through a flow controller, and goes to the ozone generator that produces the necessary ozone for the chemiluminescence reaction. Right at the outlet of the reaction chamber, the PMT (photomultiplier tube) detects the luminescence generated. The concentrations of NO and NO_x are determined and the difference between these concentrations is the concentration of NO₂.

5.8.2 DETERMINATION OF WATER VAPOR IN MEDICINAL GASES

5.8.2.1 DETERMINATION OF WATER VAPOR USING ELECTROLYTIC HYGROMETER

This equipment has a detection cell that consists of a thin phosphorus pentoxide film between two spiraled platinum wires that work as electrodes. Water vapor on the gas to be examined is absorbed by the diphosphorus pentoxide, which is turned into phosphoric acid, an electric conductor. A direct voltage applied to the electrodes produces water electrolysis and regeneration of diphosphorus pentoxide. The value obtained from the electric current, which is proportional to the concentration of water vapor in the gas examined, is determined. The system is self-calibrated provided that the Faraday's law is complied with.

Procedure: collect a sample of the gas to be examined and wait for its stabilization at room temperature. Purge the cell continuously until a constant value is obtained. Measure the concentration of water vapor in the gas examined, making sure that the temperature is constant in the system for introduction of gas in the device.

5.8.2.2 DETERMINATION OF WATER VAPOR USING DETECTOR TUBES

Proceed as described in *Determination of gases using detector tubes (5.8.1.1)*. The water vapor detector tube must indicate the minimum concentration of 67 micromol/mol (ppm), with the relative standard deviation of results of no more than 20%.

5.8.3 DETERMINATION OF OIL IN MEDICINAL GASES

Proceed as described in *Determination of gases using detector tubes* (5.8.1.1). The water vapor detector tube must indicate the minimum concentration of 0,1 micromol/mol (ppm), with the relative standard deviation of results of no more than 30%.

6 CONTAINERS FOR MEDICINES AND CORRELATES

6.1 GLASS CONTAINERS

CLASSIFICATION

Type I glass. Neutral glass of borosilicate, non-alkaline type, with high thermal, mechanic and hydrolytic resistance, with alkalinity of up to 1.0 mL of H_2SO_4 0.01 M (assay in crushed glass vial). Targeted at containing medicines; for intravascular application and parenteral use.

Type II glass. Alkali glass of the sodic / calcic type, with high hydrolytic resistance, resulting from appropriate treatment of the internal surface of type III glass, so that its alkalinity is no more than 0.7 mL of H_2SO_4 0.01 M for vials with up to 100 mL and 0.2 mL of H_2SO_4 0.01 M for capacity over 100 mL (assay in whole glass vial). Targeted at containing solutions of parenteral use; neutral and acid solutions that don't have their pH changed.

Type III glass. Alkali glass of sodic / calcic type, with medium hydrolytic resistance, but with good mechanical resistance, without any surface treatment, with maximum alkalinity of 8.5 mL of 0.01 M H_2SO_4 (assay in crushed glass vial). Targeted at containing solutions of oral and topical use; it can be used for parenteral solutions, when approved by stability assays.

NP (non-parenteral) type glass. Alkali glass of the sodic / calcic type, with low hydrolytic resistance and high alkalinity, of no more than 15 mL of H_2SO_4 0,01 M (assay in crushed glass vial). Indicated for containing non-parenteral products, that is, of oral and topical use.

6.1.1 HYDROLYTIC RESISTANCE OR ALKALINITY

Test that quantifies the intensity of the chemical reaction between water and the alkali elements in the glass, especially sodium and potassium. This resistance determines the classification of the glass type.

EQUIPMENT, MATERIALS AND REAGENTS

- Autoclave with temperature control of $(121 \pm 1.0)^\circ\text{C}$, equipped with thermometer, manometer, safety valve and shelf for supporting no less than 12 vials.
- Ball mill with hardened steel stricture and polished steel spheres or mortar in tempered steel with the specifications on **Figure 1**.

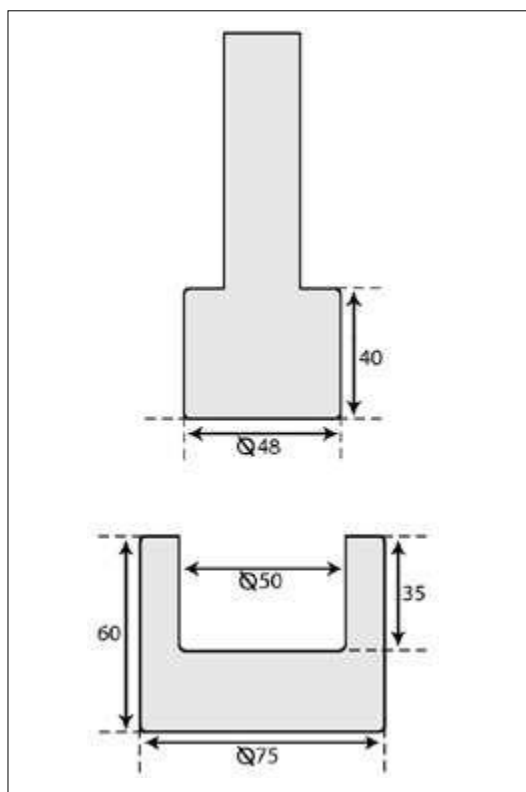


Figure 1 – Mortar and pestle for glass powdering.

- Oven for drying with temperature of 140 °C;
- Precision balance with two decimal places;
- Kit of sieves in stainless steel, n. 20, n. 40 and n. 50, with diameter of 20.3 cm (8”), including pan and lid;
- Magnet;
- Beaker or aluminum foil;
- 250 mL Erlenmeyer flask;
- Desiccator;
- Burette and micro-burette for titration;
- 100 mL graded cylinder;
- Bi-distilled or deionized water, with maximum conductivity of 0.15 $\mu\text{S}/\text{cm}$ (or 6.67 $\text{M}\Omega/\text{cm}$) at 25 °C;
- Methyl red solution (24 mg in 100 mL of water);
- Acetone PA;
- H_2SO_4 solution 0.01 M;
- HCl solution 0.01 M.

PROCEDURE FOR ASSAY IN CRUSHED GLASS VIAL

Wash no less than six vials, selected randomly, with bi-distilled or deionized water, and dry them in a clean and dry air current.

If necessary, cut the vials and transfer and crush 30 g to 40 g of glass using the ball mill or mortar. Pass the crushed glass through a sieve n. 20 and transfer the portion retained in the sieve to the ball mill or mortar again. Repeat the operations of grinding and passing the fragments through the sieve until no less than 2/3 of the material has gone through sieve n. 20. Combine all portions of crushed glass that went through sieve n. 20 and pass through a sieve n. 40. Grind the portion retained on the sieve and repeat the operation.

Combine the portions of crushed glass that went through sieve n. 40 and transfer to the assembled set of sieves n. 40 and n. 50. Shake horizontally for five minutes. Collect 12.0 g of crushed glass that went through the n. 40 sieve, but did not go through the n. 50 sieve, and store it in a desiccator until being used in the test.

Spread the sample of crushed glass over a piece of satin paper and pass the magnet, to remove possible iron fragments that may have been introduced during the crushing procedure.

Transfer the sample to a 250 mL Erlenmeyer flask and wash the glass particles with six portions of 30 mL of acetone PA, agitating for approximately 30 seconds in each procedure, and carefully decant the acetone. After washing, the sample must be free from glass powder blocks and the grain surface must be practically free from adherence of fine particles. Dry the material for 20 minutes at 140 °C.

The sample must be tested up to 48 hours after drying and, in this case, it must be maintained in a desiccator.

Weigh 10.0 g of crushed glass, transfer to a 250 mL Erlenmeyer flask, prepared in advance with bi-distilled or deionized water in bath at 90 °C for no less than 24 hours or at 121 °C for one hour, and add 50 mL of bi-distilled or deionized water.

As *blank*, use a 250 mL Erlenmeyer flask, prepared in advance in bi-distilled or deionized water in bath at 90 °C for no less than 24 hours or at 121 °C for one hour, and add 50 mL of bi-distilled or deionized water.

Close the Erlenmeyer flasks with an inverted beaker or aluminum foil, washed in advance with bi-distilled or deionized water.

Put them in autoclave and submit them to the following treatment:

- increase the autoclave temperature after closing the exhaust valve, between 19 to 23 minutes, until achieving (121 ± 1) °C;
- maintain at temperature of (121 ± 1) °C for 30 minutes;
- discharge the pressure in a period of 38 to 46 minutes, until achieving atmospheric pressure.

Take the vials and cool them immediately in running water. After cooling, decant the water from the Erlenmeyer flask and wash the crushed glass with four portions of 15 mL of bi-distilled or deionized water. Add five drops of methyl red solution and titrate, immediately, with sulfuric acid 0.01 M. If the expected volume of solution to be used in titration is inferior to 10 mL, use a micro-burette. Register the volume of sulfuric acid used in titration and correct the value in relation to the volume of *blank*.

Limits:

The maximum alkalinity value for the **type I glass** vial is of 1.0 mL of H₂SO₄ 0.01 M for 10 g of crushed glass.

The maximum alkalinity value for the **type III glass** vial is of 8.5 mL of 0.01 M H₂SO₄ for 10 g of crushed glass.

The maximum alkalinity value for the **NP type glass** vial is of 15 mL of H₂SO₄ 0.01 M for 10 g of crushed glass.

PROCEDURE FOR ASSAY IN WHOLE GLASS VIAL

Wash vials, selected randomly, with bi-distilled or deionized water, and dry them in a clean and dry air current. Add a volume of bi-distilled or deionized water corresponding to 90% of the total capacity of the vial, determined as described in *Total volumetric capacity* (6.1.3).

Close the vials with aluminum foil washed in advance with bi-distilled or deionized water and put them in autoclave. Submit them to the following treatment:

- heat the autoclave at 100 °C, with the exhaust valve opened, for 10 minutes;
- increase the autoclave temperature after closing the exhaust valve, by 1 °C/minute, until reaching (121 ± 1) °C;
- maintain the temperature of (121 ± 1) °C for 60 minutes;
- lower the temperature by 0.5 °C/minute, until reaching 100 °C, discharging the pressure until achieving atmospheric pressure;
- open the autoclave only after reaching the temperature of 95 °C;
- transfer the vials to a water bath at 80 °C. Add cold water, taking care to avoid contamination of the extraction solution, and the cooling time must not exceed 30 minutes.

After cooling, combine the extraction solution from each of the vials. Measure the volume as registered on **Table 1** and transfer to a 250 mL Erlenmeyer flask.

As *blank*, use a 250 mL Erlenmeyer flask and add the same volume of bi-distilled or deionized water.

Table 1 – Volume of extraction solution according to the total volumetric capacity of the container.

<i>Volumetric capacity of the flask (mL)</i>	<i>Volume of extraction solution (mL)</i>
≤ 3	25.0
From 3 to 30	50.0
From 30 to 100	100.0
≥ 100	100.0

Transfer five drops of methyl red solution for each 25 mL of extraction solution and titrate, immediately, with hydrochloric acid 0.01 M, using a micro-burette. Register the volume of hydrochloric acid 0.01 M used in titration and correct the value in relation to the volume of *blank*.

Limits:

The maximum alkalinity value must not exceed the values indicated on **Table 2**.

Table 2 – Maximum alkalinity according to the glass type and the volumetric capacity of the vial.

<i>Volumetric capacity of the flask (mL)</i>	<i>Maximum volume of HCl 0.01 M (mL) for 100 mL of extraction solution</i>	
	<i>Types I and II</i>	<i>Type III</i>
≤ 1	2.0	20.0
From 1 to 2	1.8	17.6
From 2 to 5	1.3	13.2
From 5 to 10	1.0	10.2
From 10 to 20	0.80	8.1
From 20 to 50	0.60	6.1
From 50 to 100	0.50	4.8
From 100 to 200	0.40	3.8
From 200 to 500	0.30	2.9
> 500	0.20	2.2

PROCEDURE FOR ASSAY OF WATER ATTACK AT 121 °C – TO QUALIFY TYPE II GLASS.

Rinse three or more vials, selected randomly, with bi-distilled or deionized water twice and dry them in a clean and dry air current. Add a volume of bi-distilled or deionized water corresponding to 90% of the total capacity of the vial, determined as described in *Total volumetric capacity* (6.1.3). Close the flasks with an inverted beaker or aluminum foil, washed in advance with bi-distilled or deionized water.

Put them in autoclave and submit them to the following treatment:

- increase the autoclave temperature after closing the exhaust valve, between 19 to 23 minutes, until achieving $(121 \pm 1) ^\circ\text{C}$;
- maintain at temperature of $(121 \pm 1) ^\circ\text{C}$ for 60 minutes;
- discharge the pressure in a period of 38 to 46 minutes, until achieving atmospheric pressure.

Combine the volume of extraction solution from several vials in a graded cylinder and transfer 100.0 mL to a 250 mL Erlenmeyer flask. Add five drops of methyl red solution and immediately titrate with sulfuric acid 0.01 M. Complete the titration within 60 minutes after opening the autoclave. Register the volume of sulfuric acid used in titration and correct the value in relation to the volume of *blank* (100 mL of bi-distilled or deionized water at the same temperature and with the same amount of indicator).

Limits:

The maximum alkalinity value for the **type II glass** vial is of 0.7 mL of H_2SO_4 0.01 M for vials with up to 100 mL of volumetric capacity.

The maximum alkalinity value for the **type II glass** vial is of 0.2 mL of H_2SO_4 0.01 M for vials with more than 100 mL of volumetric capacity.

6.1.2 ARSENIC

EQUIPMENT, MATERIALS AND REAGENTS

- Autoclave with temperature control of $(121 \pm 1.0) ^\circ\text{C}$, equipped with thermometer, manometer, safety valve and shelf for supporting no less than 12 vials;
- oven for drying with temperature of $140 ^\circ\text{C}$;
- beaker or aluminum foil;
- 250 mL Erlenmeyer flask;
- 100 mL graded cylinder;
- bi-distilled or deionized water, with maximum conductivity of $0.15 \mu\text{S}/\text{cm}$ (or $6.67 \text{ M}\Omega/\text{cm}$) at $25 ^\circ\text{C}$.

PROCEDURE

Wash vials, selected randomly, with bi-distilled or deionized water and dry them in a clean and dry air current. Add a volume of bi-distilled or deionized water corresponding to 90% of the total capacity of the vial, determined as described in *Total volumetric capacity* (6.1.3).

Close the vials with aluminum foil washed in advance with bi-distilled or deionized water and put them in autoclave. Submit them to the following treatment:

- heat the autoclave at $100 ^\circ\text{C}$, with the exhaust valve opened, for 10 minutes;

- increase the autoclave temperature after closing the exhaust valve, by 1 °C/minute, until reaching (121 ± 1) °C;
- maintain at temperature of (121 ± 1) °C for 60 minutes;
- lower the temperature by 0.5 °C/minute, until reaching 100 °C, discharging the pressure until achieving atmospheric pressure;
- open the autoclave only after reaching the temperature of 95 °C;
- transfer the vials to a water bath at 80 °C. Add cold water, carefully, to avoid contamination of the extraction solution, and the cooling time must not exceed 30 minutes.

After cooling, combine the extraction solution from each of the flasks to obtain 35 mL and transfer to a 250 mL Erlenmeyer flask.

Proceed as described for *Limit test for arsenic (5.3.2.5)*. No more than 1 µg/g.

6.1.3 TOTAL VOLUMETRIC CAPACITY

Assay to determine the volume of liquid product the vial can contain, when full, until the upper termination surface.

EQUIPMENT, MATERIALS AND REAGENTS

- Balance with minimum resolution of 0.1 g;
- thermometer from 0 °C to 100 °C, with resolution of 0.5 °C;
- bi-distilled water.

PROCEDURE

Select six units randomly. Tare the balance with the dry and empty vial. Fill the vial with bi-distilled water until the termination sealing surface (vial closing region, also named neck, finish or finishing), keeping the external surface completely dry; for ampoules, the filling must be conducted until the height of point A (**Figure 1**).

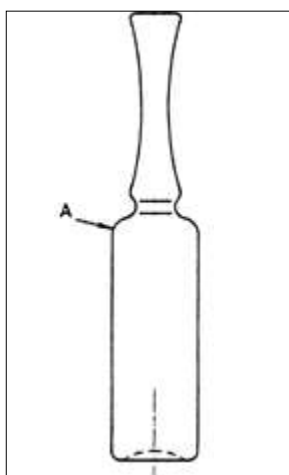


Figure 1 – Filling of volume of ampoules (up to point A).

Determine the water temperature during the execution of the assay and make sure that the water temperature does not vary more than 1 °C. Weigh the full vial and determine the mass of water contained in it.

Calculate the volume of the vial dividing the mass of water by its density, at the assay temperature, using the data listed on **Table 1** for distilled water.

Table 1 – Density of distilled water due to temperature.

<i>Temperature (°C)</i>	<i>Water density (g/mL)</i>	<i>Temperature (°C)</i>	<i>Water density (g/mL)</i>
10	0.99970	23	0.99754
11	0.99961	24	0.99730
12	0.99950	25	0.99704
13	0.99938	26	0.99678
14	0.99924	27	0.99651
15	0.99910	28	0.99623
16	0.99894	29	0.99594
17	0.99877	30	0.99565
18	0.99860	31	0.99534
19	0.99841	32	0.99503
20	0.99820	33	0.99470
21	0.99799	34	0.99437
22	0.99777	35	0.99403

RESULTS

The results expressed in mL, with one decimal place, must comply with the specifications indicated.

6.2 PLASTIC CONTAINERS

The intended goal with this section is to establish rules for plastic materials and components used to contain medicines and correlates. The rules and tests for functional properties of containers and their components are provided in *Plastic containers – Performance tests* (6.2.3).

Plastic items are identified and characterized by infrared spectroscopy and differential scanning calorimetry. This section describes the procedures from tests and rules for identification and characterization of different types of plastic. The level of verification is based on direct or indirect contact with the medicine, and the risk is based on the route of administration.

Plastics may contain residues from the polymerization process, plasticizers, stabilizers, antioxidants, pigments and lubricants. Factors such as the composition of plastic, processing and procedures for cleaning, surface treatment, means of contact, colorants, adhesives, absorption and permeability of preservatives, as well as storage conditions, may also affect the suitability of a plastic for a specific use. Tests on extractable substances are planned to characterize the components extracted and identify possible migrants. The degree or extension of tests to extract substances from a component depends on the purpose of use and the level of risk of impacting negatively the product efficacy. This chapter describes the tests on specific extractable substances for resins of polyethylene, polypropylene, poly(ethylene terephthalate) and poly(ethylene terephthalate glycol). All other plastics must be tested as described on *Physicochemical tests* from *Test methods* (6.2.1.3). The *Buffer capacity* test must be tested for containers targeted at containing a liquid product.

Plastic components used for high risk products, such as the ones targeted at inhalation, parenteral and ophthalmic preparations, are tested using the *Biological Assays* from *Test methods* (6.2.1.3).

Plastic containers targeted at containing parenteral products must meet the requirements from *Biological assays* and from *Physicochemical tests*. Rules are also provided for polyethylene containers used to contain dry oral pharmaceutical dosage forms, not targeted at constitution in solution.

6.2.1 PLASTIC CONTAINERS AND CORRELATES

6.2.1.1 POLYETHYLENE CONTAINERS

High-density polyethylene and low-density polyethylene are long chain polymers, synthesized under controlled heat and pressure conditions, with the help of catalysts and from no less than 85.0% of ethylene and a total of 95.0% of olefins. Both high-density and low-density polyethylene have a specific infrared absorption spectrum and have characteristic thermal properties. High-density polyethylene has density between 0.941 and 0.965 g/cm³. Low-density polyethylene has density between 0.850 and 0.940 g/cm³. Other properties that may affect the suitability of polyethylene include elasticity modulus, flow index, resistance to breaking under environmental tension, and level of crystallinity after molding.

The rules and assays described in this section characterize containers and components, made from low- or high-density polyethylene from homopolymeric or copolymeric resins.

All polyethylene components are subject to infrared spectroscopy and differential scanning calorimetry tests. When stability studies are conducted to determine the validity date of a special pharmaceutical preparation in a suitable polyethylene container, any other polyethylene container that

complies with these requisites may be equally used to contain the pharmaceutical preparation in question, provided that the adequate stability programs are extended to include the alternative container to ensure that the identity, strength, quality and purity of the pharmaceutical preparation are maintained throughout the validity period.

ASSAYS

High-density polyethylene

Infrared spectroscopy. Use the attenuated total reflection accessory, as described on the item *Mid-infrared* (5.2.14). The corrected sample spectrum must present bands with higher absorption only on the same wavelengths as the reference standard spectrum.

Differential scanning calorimetry. Proceed as described in *Thermal analysis* from *Test methods* (6.2.1.3). The sample thermogram must be similar with the one from the reference standard, determined in a similar manner, and the endothermal (thawing) temperature on the sample thermogram must not differ by more than 6.0 °C from reference standards.

Heavy metals and non-volatile residue. Prepare extracts from the sample as described on *Physicochemical tests*, in *Test methods* (6.2.1.3), with area sample equivalent to 60 cm², without considering the thickness, for every 20.0 mL of *Extraction medium*.

Heavy Metals. The containers must meet the requirements for *Heavy metals* on *Physicochemical tests*, in *Test methods* (6.2.1.3).

Non-volatile residue. Proceed as described in *Non-volatile residue* on *Physicochemical tests* in *Test methods* (6.2.1.3), and the *Blank* must be the same solvent used in each of the test conditions. The difference between the amounts obtained of *Sample and blank preparation* must not exceed 12.0 mg when water maintained at 70 °C is used as *Extraction medium*; not exceed 75.0 mg when alcohol maintained at 70 °C is used as *Extraction medium*; and not exceed 100.0 mg when hexane maintained at 50 °C is used as *Extraction medium*.

Substances used in contact with oral liquids. Proceed as described in the *Buffer capacity* from *Physicochemical tests*, *Test methods* (6.2.1.3).

Low-density polyethylene

Infrared spectroscopy. Use the attenuated total reflection accessory, as described on the item *Mid-infrared* (5.2.14). The corrected sample spectrum must present bands with higher absorption only on the same wavelengths as the reference standard spectrum.

Differential scanning calorimetry. Proceed as described on *Thermal analysis*, in *Test methods* (6.2.1.3). The sample thermogram must be similar with the one from the reference standard, determined in a similar manner, and the endothermal (thawing) temperature on the sample thermogram must not differ by more than 8.0 °C from reference standards.

Heavy metals and non-volatile residue. Prepare extracts from the sample as described on *Sample preparation* in *Physicochemical tests* from *Test methods*, with area sample equivalent to 60 cm², without considering thickness, for every 20.0 mL of *Extraction medium*.

Heavy metals. The containers must meet the requirements for *Heavy metals* from *Physicochemical tests*, in *Test methods* (6.2.1.3).

Non-volatile residue. Proceed as described in *Non-volatile residue* on *Physicochemical tests* in *Test methods* (6.2.1.3), and the *Blank* must be the same solvent used in each of the test conditions. The difference between the amounts obtained of *Sample and blank preparation* must not exceed 12.0 mg when water maintained at 70 °C is used as *Extraction medium*; not exceed 75.0 mg when alcohol maintained at 70 °C is used as *Extraction medium*; and not exceed 350.0 mg when hexane maintained at 50 °C is used as *Extraction medium*.

Substances used in contact with oral liquids. Proceed as described in the *Buffer capacity* from *Physicochemical tests*, in *Test methods* (6.2.1.3).

6.2.1.2 POLYPROPYLENE CONTAINERS

Polypropylene polymers are long chain polymers, synthesized with the help of catalysts under controlled heat and pressure conditions. Factors such as the composition of plastic, processing and procedures for cleaning, means of contact, colorants, adhesives, adsorption, permeability of preservatives, and storage conditions, may also affect the suitability of a plastic for a specific use. The suitability of a characteristic polypropylene must be established through adequate tests.

Polypropylene has a distinctive infrared spectrum and characteristic thermal properties. It has density of 0.880 g/cm³ to 0.913 g/cm³. The permeability properties of molded polypropylene containers may be altered when re-powdered polymer is incorporated, depending on its ratio in the final product. Other properties that may affect the suitability of polypropylene used in containers for packaging medicines include permeability to oxygen and humidity, elasticity modulus, flow index, resistance to breaking under environmental tension, and level of crystallinity after molding.

The rules and assays provided characterize containers in polypropylene, made from homopolymers or copolymers, which are adequate for containing dry solid and liquid pharmaceutical dosage forms. Considering that adequate stability studies have been conducted to determine the validity date of a specific pharmaceutical preparation in a suitable polypropylene container, any other polypropylene container that complies with these requisites may be equally used to contain the same pharmaceutical preparation, provided that the adequate stability programs are extended to include the alternative container, to ensure that the identity, strength, quality and purity of the pharmaceutical preparation are maintained throughout the validity period.

ASSAYS

Infrared spectroscopy. Use the attenuated total reflection accessory, as described on the item *Infrared absorption spectrophotometry* (5.2.14). The corrected spectrum of the sample must present bands with higher absorption only in the same wavelengths of the spectrum of the respective reference standard (polypropylene homopolymer or copolymer) determined in a similar manner.

Differential scanning calorimetry. Proceed as described in *Thermal analysis* from *Test methods* (6.2.1.3). The endothermal (thawing) temperature on the thermogram must not differ by more than 6.0 °C from the reference standards for homopolymers. The endothermal temperature obtained from the polypropylene copolymer sample thermogram must not differ by more than 12.0 °C from the standards of this substance.

Heavy metals and non-volatile residue. Prepare extracts from samples as described on *Sample preparation*, from *Physicochemical tests*, in *Test methods* (6.2.1.3), with a portion of 60 cm², without considering the thickness, for every 20.0 mL of *Extraction medium*.

Heavy metals. The containers must meet the requirements for *Heavy metals* from *Physicochemical tests*, in *Test methods* (6.2.1.3).

Non-volatile residue. Proceed as described in *Non-volatile residue* on *Physicochemical tests* in *Test methods* (6.2.1.3), and the *Blank* must be the same solvent used in each of the test conditions. The difference between the amounts obtained of *Sample* and *blank preparation* must not exceed 10.0 mg when water maintained at 70 °C is used as *Extraction medium*; not exceed 60.0 mg when alcohol maintained at 70 °C is used as *Extraction medium*; and not exceed 225.0 mg when hexane maintained at 50 °C is used as *Extraction medium*. The containers must meet the requirements for *Non-volatile residue* for all extraction media.

Note: hexane and alcohol are flammable. When evaporating these solvents, use an air current with water bath; when drying the residue, use explosion-proof oven.

Substances used in contact with oral liquids. Proceed as described in the *Buffer capacity* from *Physicochemical tests*, in *Test methods* (6.2.1.3).

6.2.1.3 POLY(ETHYLENE TEREPHTHALATE) AND POLY(ETHYLENE TEREPHTHALATE GLYCOL) CONTAINERS

Poly(ethylene terephthalate) (PET) resins are crystalline long chain polymers prepared by condensing ethylene glycol with dimethyl terephthalate or terephthalic acid. PET copolymer resins are prepared in a similar manner, except that they may also have a small amount of isophthalic acid (lower than 3% of mol of resin) or 1,4-cyclohexanedimethanol (lower than 5% of mol of resin). Polymerization is conducted under controlled heat and vacuum conditions; with the help of catalysts and stabilizers.

PET copolymer resins have physical and spectral properties similar to PET and, for practical purposes, are handled as PET. Assays and specifications provided in this section to characterize PET resins and containers also apply to copolymer residues and to containers made from them.

Usually, PET and its copolymer resins present a high level of order in their molecular structure. As a result, they present a characteristic thermal behavior dependent on the composition, including a glass transition temperature of approximately 76 °C and a melting temperature of approximately 250 °C. These resins have a particular infrared absorption spectrum that allows their differentiation from other plastic materials, such as polycarbonate; polystyrene; polyethylene; poly(ethylene terephthalate glycol) (PETG) resins. PET and its copolymer resins have density between 1.3 and 1.4 g/cm³ and minimum intrinsic viscosity of 0.7 dL/g, which corresponds to an average molecular mass of approximately 23,000 Da.

PETG resins are polymers with high molecular mass prepared by condensing ethylene glycol with dimethyl terephthalate, or terephthalic acid, and with 15 to 34% of molar 1,4-hexanedimethanol. PETG resins are clear, amorphous polymers with glass transition temperature of approximately 81 °C and without a crystalline melting point, as determined by the differential scanning calorimetry. PETG resins have a particular infrared absorption spectrum that allows distinguishing it among other plastic materials, including PET. PETG resins have density of approximately 1.27 g/cm³ and minimum intrinsic viscosity of 0.65 dL/g, which corresponds to an average molecular mass of approximately 16,000 Da.

PET and PETG resins do not have any plasticizer, processing support or antioxidants. When colorants are used in manufacturing PET and PETG containers, they must not migrate to the liquid.

The rules and assays provided in this section characterize polyethylene terephthalate (PET) and polyethylene terephthalate glycol (PETG) containers that are used to contain liquid oral pharmaceutical dosage forms. Considering that adequate stability studies have been conducted to determine the validity date of a particular liquid pharmaceutical preparation in a container that complies with the requisites for PET or PETG containers, any other container from these substances that complies with these requisites may be equally used to contain the same pharmaceutical preparation, provided that the adequate stability programs are extended to include the alternative container, to ensure that the identity, strength, quality and purity of the pharmaceutical preparation are maintained throughout the validity period. The suitability of a specific PET or PETG container to be used for dispensing a specific liquid oral pharmaceutical preparation must be established through adequate tests.

ASSAYS

Infrared spectroscopy. Use attenuated total reflection accessory, proceed as described in *Ultraviolet, visible and infrared spectrometry (5.2.14)*. The corrected sample spectrum presents bands with higher absorption only on the same wavelengths as the spectrum from reference standards, determined in a similar manner.

Differential scanning calorimetry. Proceed as described on the item *Thermal analysis* in *Test methods*. For polyethylene terephthalate, the sample thermogram must be similar to the one from the reference standard, determined in a similar manner; the sample melting point (T_m) must not differ from the reference standards by more than 9 °C and from the glass transition temperature by more than 4 °C. For polyethylene terephthalate glycol, the sample thermogram must be similar to the one from the reference standard, determined in a similar manner; the glass transition temperature (T_g) from the sample must not differ by more than 6 °C from the reference standards.

Extraction of colorants. Select three containers for the assay. Cut a relatively flat part from the side wall of a container and trim it to the necessary dimension to adjust the sample to the spectrophotometer support. Perform a scan (5.2.14) to obtain the visible spectrum of 350-700 nm from the side wall. With approximation of 2 nm, determine the maximum absorbance wavelength. Fill the two remaining containers with 50% ethyl alcohol for PET containers and 25% ethyl alcohol for PETG containers. Prepare the containers with impermeable seals, such as an aluminum foil, and close with the lids. Fill with the corresponding solvent from a glass container of the same capacity as the containers being tested, prepare it with a impermeable seal, such as an aluminum foil, and close with a lid. Incubate the containers being tested and the glass container at 49 °C for 10 days. Remove the containers and wait until they reach room temperature. Concomitantly, determine the absorbances (5.2.14) of the solutions being tested in cells with 5 cm on the maximum absorbance wavelength, using the corresponding solvent from the glass container as blank. For both solutions being tested, the absorbance values obtained must be lower than 0.01.

Heavy metals; Total terephthaloyl and ethylene glycol.

Extraction media.

Purified water

Ethyl alcohol 50%. Dilute 125 mL of ethyl alcohol in water for 238 mL of solution and homogenize.

Ethyl alcohol 25%. Dilute 125 mL of *Ethyl alcohol 50%* in water for 250 mL of solution and homogenize.

n-Heptane.

General procedure. Use an extraction medium of *Ethyl alcohol* 50% for PET containers and *Ethyl alcohol* 25% for PETG. For each extraction medium, fill a sufficient number of test containers with 90% of their nominal capacity to obtain no less than 30 mL. Fill a corresponding number of glass containers with *Purified water*, the same amount of containers with *Ethyl alcohol* 50%, or *Ethyl alcohol* 25% and the same number of glass containers with *n-Heptane* to be used as blank of extraction media. Put impermeable seals, such as aluminum foil, on the containers and cover them. Incubate the test containers and the glass containers at 49 °C for 10 days. Remove the test containers with the samples and the blanks from the extraction medium and store them at room temperature. Do not transfer samples of extraction medium to alternative storage containers.

Heavy metals. Pipette 20 mL of *Purified water* extracted from the test containers, filtered as necessary, put in one or two 50 mL tubes for comparing the color, and store the remaining *Purified water* to use in the *Ethylene glycol* test. Adjust the pH of the extract between 3.0 and 4.0 with acetic acid *M* or ammonium hydroxide 6 *M* using a narrow range pH indicator paper. Dilute with water to approximately 35 mL and homogenize. Pipette 2 mL of the *Standard lead solution* (10 ppm Pb) (5.3.2.3), prepared on the day of use; transfer to a second color comparison tube and add 20 mL of *Purified water*. Adjust the pH between 3.0 and 4.0 with acetic acid *M* or ammonium hydroxide 6 *M* using a narrow range pH indicator paper. Dilute with water to approximately 35 mL and homogenize. Add to each tube 1.2 mL of thioacetamide RS and 2 mL of *Buffer acetate pH 3.5* (5.3.2.3), dilute with water to 50 mL of solution and homogenize. Any color produced within 10 minutes in the tube with *Purified water* extracted from test containers must not be more intense than the one from the tube with the *Standard lead solution* (10 ppm Pb), both viewed on a white surface (limit 1 ppm).

Total terephthaloyl. Determine the absorbance of extract of *Ethyl alcohol* 50% or *Ethyl alcohol* 25% in a cell with 1 cm, on the maximum absorbance wavelength at approximately 244 nm (5.2.14), using as blank the one corresponding to the extraction medium. The extract absorbance must not exceed 0.150, which corresponds to no more than 1 ppm of the total terephthaloyl from the medium. Determine the absorbance of extract of *n-Heptane* in a cell with 1 cm, on the maximum absorbance wavelength at approximately 240 nm (5.2.14), using as blank the *n-Heptane* extraction medium. The extract absorbance must not exceed 0.150, which corresponds to no more than 1 ppm of terephthaloyl from the medium.

Ethylene glycol.

Periodic acid solution. Dissolve 125 mg of periodic acid in 10 mL of water.

Diluted sulfuric acid. For 50 mL of water, add slowly and in constant agitation 50 mL of sulfuric acid and wait until it reaches room temperature.

Sodium bisulfite solution. Dissolve 0.1 g of sodium bisulfite in 10 mL of water. Use this solution in up to seven days.

Disodium chromotropate solution. Dissolve 100 mg of disodium chromotropate in 100 mL of sulfuric acid. Protect the solution from light and use it in up to seven days.

Standard solution. Dissolve an amount, accurately weighed, of ethylene glycol in water and dilute, quantitatively, step by step if necessary, to obtain a solution with concentration of approximately 1.0 µg/mL.

Sample solution. Use the extract in *Purified water*.

Procedure. Transfer 1 mL of the *Standard solution* to a 10 mL volumetric flask. Transfer 1 mL of the *Sample solution* to a second 10 mL volumetric flask. Transfer 1 mL of the extraction medium in *Purified water* to a third 10 mL volumetric flask. For each of the three flasks, transfer 100 µL of the *Periodic acid solution*, shake to homogenize and allow to stand for 60 minutes. Transfer to each flask 1 mL of the *Sodium bisulfite solution* and homogenize. Transfer 100 µL of the *Disodium chromotropate solution* to each flask and homogenize. All solutions must be analyzed up to one hour

after the addition of *Disodium chromotropate solution*. Add, carefully, 6 mL of sulfuric acid to each flask, homogenize and wait for the solutions to reach room temperature.

Note: the dilution of sulfuric acid produces considerable heat and may cause ebullition of solution. Make this addition carefully. Sulfur dioxide gas will be released. Using an exhaust chamber is recommended.

Dilute each solution with sulfuric acid diluted until completing the volume and homogenize. Concomitantly, determine the absorbances (5.2.14) of the solutions from the *Standard solution* and the *Sample solution* in cells with 1 cm, in the maximum absorbance wavelength at approximately 575 nm, using as blank the solution taken from the extraction medium in *Purified water*. The absorbance of the solution obtained from the *Sample solution* is not superior to the one of the solution obtained from the *Standard solution*, corresponding to no more than 1 ppm of ethylene glycol.

TEST METHODS

Multiple internal reflection

Equipment. Use an infrared spectrophotometer capable of correcting to the white spectrum and equipped with an attenuated total reflection and an internal reflection KRS-5 plate. The KRS-5 crystal with 2 mm of thickness, and incidence angle of 45°, provides a sufficient number of reflections.

Sample preparation. Cut two flat portions representing the average thickness of the container wall, and trim them as necessary, to obtain adequate segments to assemble in the multiple internal reflection accessory; To avoid scratching the surface, clean the samples with dry paper or, if necessary, with a soft cloth moistened with methyl alcohol and wait until drying. Firmly couple the samples on both sides of the KRS-5 internal reflection plate, ensuring the adequate surface of contact. Before placing the samples on the plate, compress them obtaining thin even films to be exposed at temperatures of approximately 177 °C, under high pressure (15,000 psi or more).

Procedure. Put the coupled parts of the sample in the multiple internal reflection accessory and place the set in the infrared spectrophotometer light beam. Adjust the position of the sample and the equipment mirrors to allow maximum transmission of light by the non-attenuated reference beam. Complete the adjustments of the accessory and attenuate the reference beam, to allow total deflection scale during the sample scanning. Determine the infrared spectrum from 3500 cm⁻¹ to 600 cm⁻¹ for polyethylene and polypropylene and from 4000 cm⁻¹ to 400 cm⁻¹ for PET and PETG.

Thermal analysis

Procedure. Cut a section with an approximate weight of 12 mg and place it in the compartment for the sample. The close contact between the compartment and the thermoelement is essential for the reproducibility of results. Determine the thermogram under nitrogen, using the heating and cooling conditions as specified for the type of resin and use an equipment capable of making the determinations.

For polyethylene. Determine the thermogram under nitrogen at temperatures between 40 °C and 200 °C, at a heating rate between 2 °C and 10 °C per minute, followed by cooling to 40 °C, at a rate between 2 °C and 10 °C per minute.

For polypropylene. Determine the thermogram under nitrogen at temperatures that range between room temperature and 30 °C above the melting point. Maintain the temperature for 10 minutes, then cool down to 50 °C below the maximum crystallization temperature at a rate of 10 °C to 20 °C per minute.

For poly(ethylene terephthalate). Heat the sample from room temperature to 280 °C at a heating rate of approximately 20 °C per minute. Maintain the sample at 280 °C for one minute. Cool the sample quickly down to room temperature and reheat it to 280 °C at a heating rate of approximately 5 °C per minute.

For poly(ethylene terephthalate) glycol. Heat the sample from room temperature to 120 °C at a heating rate of approximately 20 °C per minute. Maintain the sample at 120 °C for one minute. Cool the sample quickly down to room temperature and reheat it to 120 °C at a heating rate of approximately 10 °C per minute.

Biological assays

The biological assays *in vitro* are conducted according to the procedures established in *Biological reactivity tests in vitro* (6.2.5). The components that comply with requisites from tests *in vitro* do not need to be submitted to additional tests. No designation of class of plastic is assigned to these materials. The materials that do not comply with requisites from tests *in vitro* are not adequate for use as medicine containers.

If the class designation is necessary for plastics and other polymers that comply with the requisites included in *Biological reactivity tests in vitro* (6.2.5), conduct the adequate test *in vivo* specified for *Classification of plastics in Biological reactivity tests in vivo* (6.2.6).

Physicochemical tests

The following tests, targeted at determining the physical and chemical properties of plastics and their extracts, are based on the extraction of plastic material, being essential that the designated amount of plastic is used. Additionally, the surface area specified must be available for extraction at the temperature determined.

Test parameters:

Extraction medium. Unless directed otherwise in a specific test to be followed, use *Purified water* as extraction medium, maintaining the temperature at 70 °C during the extraction for the *Sample preparation*.

Blank. Use *Purified water* where the blank is specified on the following tests.

Equipment. Use water bath and *Extraction containers*, as described in *Biological reactivity tests in vivo* (6.2.6). Proceed as described on *Equipment preparation in Biological reactivity tests in vivo* (6.2.6). The containers and equipment do not need to be sterile.

Sample preparation. From a homogeneous sample of plastic, use one aliquot for every 20 mL of extraction medium, equivalent to 120 cm² of the total surface area (combining both sides), and subdivided into strips of, approximately, 3 mm of width and close to 5 cm of length. Transfer the subdivided sample to a 250 mL type I glass graded cylinder, with cap, and add approximately 150 mL of *Purified water*. Shake for approximately 30 seconds, empty it, discard the liquid and repeat a second wash.

Extraction for sample preparation. Transfer the ready *Sample preparation* to an adequate extraction vial and add the requested amount of extraction medium. Extract for 24 hours by heating in water bath at the temperature specified for the extraction medium. Cool to temperatures not below

20 °C. Pipette 20 mL of the extract prepared to an adequate container. Use this part on the test for *Buffer capacity*. Immediately decant the residual extract in an adequate clean container and close it.

Non-volatile residue. Transfer, in adequate aliquots, 50 mL of *Extract from sample preparation* to an adequate tared crucible (preferably a fused silica crucible that has been cleaned with acid) and evaporate the volatile part in a vapor bath. Evaporate, in a similar manner, 50 mL of *Blank* in another crucible. If an oily residue is expected, examine the crucible repeatedly during the evaporation and drying process and reduce the amount of heat, if oil tends to slide down the crucible wall. Dry at 105 °C for one hour. The difference between the amounts obtained of *Extract for sample preparation* and *Blank* must not be superior to 15 mg.

Residue by incineration (5.2.10). It is not necessary to perform this test when the result from the *Non-volatile residue* test does not exceed 5 mg. Proceed with obtaining residues, from the *Extract for sample preparation* and *Blank* described in the test for *Non-volatile residue* above, using, if necessary, more sulfuric acid for the same amount in each crucible. The difference between the amounts obtained of ignition residue from the *Extract for sample preparation* and *Blank* must not be superior to 5 mg.

Heavy metals. Pipette 20 mL of the *Extract from sample preparation*, filtered, if necessary, to one of the two 50 mL tubes for color comparison. Adjust the pH between 3.0 and 4.0 with acetic acid *M* or ammonium hydroxide 6 *M* using a narrow range pH indicator paper. Dilute with water to approximately 35 mL and homogenize.

Pipette 2 mL of *Standard lead solution (10 ppm Pb) (5.3.2.3)*, transfer to the second tube for color comparison and add 20 mL of *Blank*. Adjust the pH between 3.0 and 4.0 with acetic acid *M* or ammonium hydroxide 6 *M* using a narrow range pH indicator paper. Dilute with water to approximately 35 mL and homogenize. Add to each tube 1.2 mL of thioacetamide RS and 2 mL of *Buffer acetate pH 3.5 (5.3.2.3)*, dilute with water to 50 mL of solution and homogenize. Any color produced within 10 minutes in the preparation that has the *Extract from sample preparation* extracted from test containers must not be more intense than in the *Standard preparation*, both viewed on a white surface (1 ppm in the extract).

Buffer capacity. Titrate, potentiometrically, the 20 mL aliquots, collected in advance, from the *Extract from Sample preparation* to a pH 7.0, using 0.010 *M* hydrochloric acid or 0.010 *M* sodium hydroxide, as necessary. Treat, in a similar manner, a 20 mL aliquot of *Blank*. If the same titrant is necessary for both titrated, the difference between the two volumes must not be superior to 10 mL; and if acid is necessary either for the *Extract from sample preparation* or for the *Blank*, and the alkali for the other, the total of the two volumes requested must not be superior to 10 mL.

6.2.2 ELASTOMER CAPS

Elastomer caps are made in materials obtained from polymerization, polyaddition or polycondensation of organic substances. The polymers obtained are usually vulcanized. The cap formulations have natural or synthetic elastomers, and inorganic and organic additives, to help or control the vulcanization, offer physical and chemical properties, coloring, or stabilize the cap formulation.

For caps formulated with natural or synthetic elastomer substances, used for long-term storage. It is not applicable to caps made of silicone elastomer, but it applies to silicone-treated caps, such as dimethicone, and caps coated with other lubricant materials, such as materials chemically bonded or mechanically bonded to the cap.

The following comments refer only to laminated caps or caps coated with materials targeted at providing or working as a barrier to the elastomer base, such as poly(tetrafluorethylene) (PTFE) or varnished coatings. It is not permitted to use a material with the purpose of transforming a cap that is not within the specific requirements for compliance. However, all physicochemical tests apply to the base formula of such caps, as well as to the laminated or coated caps. The functionality tests must be conducted using laminated or coated elastomer caps. The biological assays apply to coated or laminated materials, as well as to the base formula. The biological assays may be conducted in laminated or coated caps or materials and in non-laminated and non-coated caps, and the results must be reported separately. The base formula, used in physicochemical or biological assays, must comply with the specifications from a cap with coating barrier that must be similar to the cap coating in configuration and size.

The tests from this section are limited to elastomer caps from Types I and II, with the Type I caps being used for aqueous preparations and the Type II caps usually being targeted at non-aqueous preparations. If a cap does not comply with all requirements from the Type I test, but complies with requirements for the Type II test, the cap receives the final classification of Type II.

This section proposes an initial triage to identify elastomer caps that can be adequate for use with injectable preparations, based on their biological compatibilities, the physicochemical properties of their aqueous extracts, and their functionalities. All elastomer caps adequate for use in injectable preparations comply both with the Type I and Type II test limits. However, this specification does not have the purpose of being a single criterion of assessment for selection of such caps.

Among the requisites for assessing caps that are beyond the scope of this section, there is the establishment of identification tests and specifications for the cap, the cap checking, physicochemical compatibility of the product, identification and determination of safety of filterable caps found in the product package, checking of the product package functionality under real storage conditions, and conditions for use.

The cap user must obtain from the supplier a guarantee that the cap composition does not vary and that it is the same one used in the compatibility test. When the supplier informs the end user about changes in the composition, the compatibility test must be repeated, totally or partially, depending on the nature of changes.

CHARACTERISTICS

The elastomer caps are translucent or opaque, and do not have characteristic coloring, depending on the additives used. They are homogeneous and practically free from luminous and accidental materials, such as fibers, foreign particles, and rubber residues.

IDENTIFICATION

The caps are made from a broad variety of elastomeric materials and optional polymeric coatings. Therefore, this section does not specify identification tests involving all possible presentations of caps. The cap supplier and the finished product manufacturer are responsible for checking the cap formulation and any coated or laminated materials used, according to the adequate identification tests. Examples of some analytical tests that can be used include specific density, ash analysis, determination of sulfur content, chromatography in thin layer of extract, ultraviolet absorption spectrophotometry of the extract, or absorption spectrophotometry.

TEST PROCEDURES

The elastomer caps must be in compliance with the biological, physicochemical and functional requirements. Since the elastomer caps are processed by the supplier before the distribution to the end user, the supplier must demonstrate the compliance of the caps exposed with the processing or sterilization steps. Likewise, if the elastomer caps received by the end user are processed, or sterilized, subsequently, the end user is responsible for proving the continued compliance of the subsequent caps to the processing or sterilization conditions. This is important if the caps are exposed to processes or conditions that may have significant impact on the biological, physicochemical or functional characteristics of the cap, such as gamma radiation.

For caps that are usually lubricated with silicone before use, it is permitted to conduct the physicochemical test in non-lubricated caps to avoid potential interference from method and/or difficulties in interpreting the test results. For caps provided with other non-occlusive lubricants, all tests must be conducted using the coated cap.

For coated caps, or laminated with coatings targeted at granting a barrier function, such as PTFE, or varnished coatings, physicochemical tests will be applied to the elastomer with non-coated base, as well as to coated caps. The non-coated cap submitted to physicochemical tests must be similar to the coated cap in size and configuration. The end users of coated caps are also responsible for proving the compliance of these caps with the physicochemical specifications, processed or treated in a way that simulates the conditions normally employed by the end user before use.

In all cases, it is adequate to document the conditions for cap processing, pre-treatment, sterilization or lubrication when the results are reported.

The **Table 1** summarizes the requirements from cap tests and responsibilities from the supplier and the end user.

Table 1 – Requirements from cap tests and responsibilities from the supplier.

<i>Types of caps (as supplied or used)</i>	<i>Physicochemical tests</i>	<i>Functionality tests</i>	<i>Biological assays</i>
Caps with or without silicone coating	The tests must be conducted The use of silicone is optional Responsibility: supplier and end user	The tests must be conducted The use of silicone is optional Responsibility: supplier and end user	The tests must be conducted The use of silicone is optional Responsibility: supplier and end user
Caps with lubricant coatings (non-occlusive materials, not silicone)	The tests must be conducted in coated caps Responsibility: supplier and end user	The tests must be conducted in coated caps Responsibility: supplier and end user	The tests must be conducted in coated caps Responsibility: supplier and end user
Caps with occlusive coatings	The tests must be conducted in coated caps Responsibility: supplier and end user AND: The tests must be conducted in non- coated caps (base formula) Responsibility: supplier	The tests must be conducted in coated caps Responsibility: supplier and end user	The tests must be conducted in coated caps OR: The tests must be conducted in non- coated caps (base formula) and laminated/coated material (report the results separately) Responsibility: supplier and end user.

Biological assays

Two test stages are indicated. The first stage is conducting the test *in vitro*. The materials that do not comply with requirements from the test *in vitro* are submitted to the second stage of tests *in vivo*, as described in *Biological reactivity tests in vivo* (6.2.6). Materials that meet the requirements for tests *in vitro* do not need to be submitted to test *in vivo*. Type I and Type II caps must be in compliance with the biological reactivity tests *in vitro* and *in vivo*.

Physicochemical tests / Development of preparation S.

Put entire caps, not cut out, corresponding to a surface area of (100 ± 10) cm² in an adequate glass container. Cover the caps with 200 mL of purified water or water for injection. If it is not possible to obtain a cap with the surface area prescribed using caps not cut, select a number of caps that will get close to 100 cm², and adjust the volume of water used to the equivalent of 2 mL for every 1 cm² of actual surface area of the cap used. Boil for 5 minutes and rinse five times with purified water or cold water for injection.

Put the washed caps in a Type I glass vial with broad neck, add the same amount of purified water or water for injection initially added to the caps and weigh. Cover the vial mouth with a Type I glass beaker. Sterilize in an autoclave, so that the temperature of (121 ± 2) °C is reached within 20 to 30 minutes, and maintain this temperature for 30 minutes. Let it cool until reaching room temperature for a period of approximately 30 minutes. Add purified water or water for injection to return to the original mass. Shake, immediately decant and collect the liquid. This liquid must be stirred before being used in each of the tests.

Blank preparation: The blank preparation must be conducted in a similar manner, using 200 mL of purified water, or water for injection, omitting the caps.

Appearance of preparation (turbidity and coloring)

Determination of turbidity. The determination of turbidity can be made through visual comparison (Procedure A) or instrumentally, using an adequate turbidimeter (Procedure B). The instrumental assessment of turbidity provides a test that does not depend on the visual acuity of the analysts.

Hydrazine sulfate solution. 1.0 g of hydrazine sulfate in water and dilute with water to 100.0 mL. Allow to stand for four to six hours.

Hexamethylenetetramine solution. Dissolve 2.5 g of hexamethylenetetramine in 25.0 mL of water in a 100 mL glass vial, with stopper.

Opalescence stock suspension. Transfer 25.0 mL of the hydrazine sulfate solution to the hexamethylenetetramine solution in the vial, mix and allow to stand for 24 hours. This suspension is stable for two months, if stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be mixed before use.

Opalescence standard preparation. Prepare a suspension by diluting 15.0 mL of opalescence stock suspension with water to 1000.0 mL. The opalescence standard preparation is stable for, approximately, 24 hours after the preparation.

Reference suspensions. Prepare according to **Table 2**. Mix and shake before use. Stable suspensions of formazin that may be used to prepare stable standards are commercially available and can be used after comparison with standards prepared as described.

Table 2 – Preparation of the reference suspensions.

	<i>Reference Suspension A</i>	<i>Reference Suspension B</i>	<i>Reference Suspension C</i>	<i>Reference Suspension D</i>
Opalescence standard	5.0 mL	10.0 mL	30.0 mL	50.0 mL
Water	95.0 mL	90.0 mL	70.0 mL	50.0 mL
Nephelometric turbidity unit	3 NTU	6 NTU	18 NTU	30 NTU

Procedure A. Visual comparison – Use identical test tubes, in colorless glass; clear and neutral; with a flat base and an internal diameter of 15 mm to 25 mm. Fill a tube with 40 mm of length with the *Preparation S*, a tube of the same length with water, and four other tubes of the same length with the Reference Suspensions A, B, C and D. Compare the preparations in diffuse daylight five minutes after the preparation of the *Reference suspensions*, viewing, vertically, against a black background. The light conditions must be such that the Reference suspension A can be readily distinguished from water and that the Reference suspension B can be readily distinguished from the Reference suspension A. **Limit.** The *Preparation S* must not be more opalescent than Reference suspension B for Type I caps, and no more opalescent than the Reference suspension C for Type II caps. The *Preparation S* is considered clear if the clarity is the same as the water when examined as described above, or if its opalescence is not more pronounced than the one from Reference suspension A (refer to **Table 3**).

Procedure B. Instrumental comparison: measure the turbidity of the *Reference suspensions* on an adequate calibrated turbidimeter. The blank must be tested and the results, corrected to the blank. The Reference suspensions A, B, C and D represent 3, 6, 18 and 30 Nephelometric Turbidity Units (NTU), respectively. Measure the turbidity of *Preparation S* using the calibrated turbidimeter. **Limit.** The turbidity of *Preparation S* must not be higher than the one for the Reference suspension B (6 NTU) for Type I caps, and it is not higher than the one from the Reference suspension C (18 NTU) for Type II caps (**Table 3**).

Table 3 – Method for comparison of turbidity developed in the preparations.

<i>Opalescence requirements</i>	<i>Procedure A (visual)</i>	<i>Procedure B (instrumental)</i>
Type I caps	No more opalescent than Suspension B	No more than 6 NTU
Type II caps	No more opalescent than Suspension C	No more than 18 NTU

Determination of color

Standard color. Prepare a dilution of 3.0 mL of Matching Fluid O with 97.0 mL of diluted hydrochloric acid.

Procedure. Use identical tubes, in neutral, colorless, clear glass, with flat bottom and internal diameter of 15 to 25 mm. Put in a tube the *Preparation S*, forming a liquid column of 40 mm of length and, in a second tube, the Color standard forming the same liquid column. Compare the liquids in diffuse daylight, viewing, vertically, against a white backdrop.

Limit. The *Preparation S* must not be more intensely colored than the Color Standard.

Acidity or alkalinity

Bromothymol blue solution. Dissolve 50 mg of bromothymol blue in a mixture with 4 mL of 0.02 M sodium hydroxide and 20 mL of alcohol. Dilute with water to 100 mL.

Procedure. Add 0.1 of the bromothymol blue solution to 20 mL of *Preparation S*. If the preparation turns yellow, titrate with 0.01 M sodium hydroxide until the blue endpoint is reached. If the preparation turns blue, titrate with hydrochloric acid 0.01 M until the yellow endpoint is reached. If the preparation turns green, it is neutral and the titration is not necessary.

Correction of blank. Test 20 mL of blank in a similar manner. Correct the results obtained for *Preparation S* through subtraction or addition of the volume of titrant required for the blank, as appropriate.

Limit. No more than 0.3 mL of 0.01 M sodium hydroxide produces a blue color, or no more than 0.8 mL of 0.01 M hydrochloric acid produces a yellow color, or the titration is not necessary.

Absorbance

Procedure. Conduct this test in the time period of five hours after developing the *Preparation S*. Filter the *Preparation S* through a filter with pore of 0.45 μm , discarding the first mL of the filtrate. Measure the absorbance of the filtrate in wavelengths between 220 and 360 nm on a well with 1 cm using the blank in a matching cell in a reference beam. If the filtrate dilution is necessary before measuring the absorbance, correct the results from the test for dilution.

Limit. The absorbances in all these wavelengths must not exceed 0.2 for Type I caps or 4.0 for Type II caps.

Reducing substances

Procedure. Conduct this test in the time period of four hours after developing the *Preparation S*. Transfer to 20.0 mL of *Preparation S* 1 mL of diluted sulfuric acid and 20.0 mL of potassium permanganate 0.002 M. Boil for three minutes. Cool down, add 1 g of potassium iodide and titrate, immediately, with sodium thiosulfate 0.01 M, using 25.0 mL of starch solution TS as indicator. Titrate using 20.0 mL of blank and notice the difference in the volume of sodium thiosulfate 0.01 M necessary.

Limit. The difference between the titration volumes must not be higher than 3.0 mL for Type I caps and must not be higher than 7.0 mL for Type II caps.

Heavy metals

Procedure. Proceed as guided for Method 1 in Heavy Metals. Use 10.0 mL of *Preparation S* in the problem preparation.

Limit. 2 ppm of heavy metals such as lead.

Extractable Zinc.

Sample solution. Prepare a *Sample solution* through dilution of 10.0 mL of *Preparation S* to 100 mL with hydrochloric acid 0.1 M. Prepare the test blank in a similar manner, using the blank for *Preparation S*.

Zinc standard solution. Prepare a solution (10 ppm of Zn) dissolving zinc sulfate in hydrochloric acid 0.1 M.

Reference solutions. Prepare no less than three *Reference solutions* through dilution of the *Standard zinc solution* with hydrochloric acid 0.1 M. The concentrations of zinc in these *Reference solutions* are the extension of the limit expected from the *Sample solution*.

Procedure. Use an atomic absorption spectrometer, adequate and equipped with an adequate source of electromagnetic radiation and air acetylene flame. An alternative procedure, such as an analysis by mass spectrometry or optical emission spectrometry with inductively coupled plasma, appropriately validated, may be used.

Assess each one of the *Reference solutions* in wavelength for Zinc selected in 213.9 nm, no less than three times. Register the stable readings. Rinse the equipment with the blank solution every time, to ensure that the reading returns to the initial blank value. Prepare a calibration curve from the average of readings obtained for each *Reference solution*. Register the absorbance of the *Sample solution*. Determine the concentration of zinc in ppm of the *Sample solution* using the calibration curve.

Limit. The *Preparation S* contains no more than 5 ppm of extractable zinc.

Ammonium

Alkaline potassium tetraiodomercurate (II) solution. Prepare a solution of 100 mL with 11 g of potassium iodide and 15 g of mercury iodide in water. Immediately before use, mix a volume of this solution with equal volume of a solution at 250 g/L of sodium hydroxide.

Sample solution. Dilute 5 mL of *Preparation S* in 14 mL of water. Make it alkaline, if necessary, by adding sodium hydroxide 1 M, and dilute in water to 15 mL. Add 0.3 mL of the alkaline potassium tetraiodomercurate (II) solution and close the container.

Ammonium standard solution. Prepare an ammonium chloride solution in water (1 ppm of NH_4). Mix 10 mL of the solution of 1 ppm of ammonium chloride with 5 mL of water and 0.3 mL of alkaline potassium tetraiodomercurate (II) solution. Close the container.

Limit. After five minutes, any yellow color in the *Sample solution* must not be darker than in the *Standard ammonium solution* (no more than 2 ppm of NH_4 on *Preparation S*).

Volatile sulfides

Procedure. Put the caps, cut if necessary, with a total surface area of $(20 \pm 2) \text{ cm}^2$ in a 100 mL vial, and add 50 mL of a citric acid solution at 20 g/L. In the same manner and at the same time, prepare a control solution in a separate 100 mL vial through dissolution of 0.154 mg of sodium sulfide in 50 mL of a citric acid solution at 20 g/L. Put a piece of lead acetate paper on the mouth of each vial, and hold the paper in position, placing an inverted weighing bottle over it. Heat the vials in autoclave at $(121 \pm 2) ^\circ\text{C}$ for 30 minutes.

Limit. Any black coloring in the paper produced by *Preparation S* is not more intense than the one produced by the control solution.

FUNCTIONAL TESTS

The samples treated as described for obtaining the *Preparation S* and air dried must be used for the tests on functionality, penetrability, fragmentation and auto-sealing capacity. The functionality tests are conducted on caps targeted at being penetrated by a hypodermic needle. The auto-sealing capacity

test is necessary only for caps targeted at multiple-dose containers. The needle specified for each test is a lubricated hypodermic needle with long bevel (bevel angle $12 \pm 2^\circ$)¹.

Penetrability

Procedure. Fill 10 vials adequate to the nominal volume with water, adjust the caps to be examined, and close the vials with the respective caps. Using a new hypodermic needle, as already described, perforate the cap with the needle perpendicular to the surface.

Limit. The force for perforating each cap must not be greater than 10 N (1 kgf), determined with precision of ± 0.25 N (25 gf).

Fragmentation

Caps for liquid preparations. Fill 12 clean vials with water until 4 mL less than the nominal capacity. Adjust the caps to be examined, close with a lid and allow to stand for 16 hours.

Caps for dry preparations. Adjust the caps to be examined in 12 clean vials and close each one with a lid.

Procedure. Using a hypodermic needle as previously described, adjusted to a clean needle, inject inside each vial 1 mL of water, while removing 1 mL of air. Repeat this procedure four times for each cap, perforate each time at a different place. Use a new needle for each cap, checking if its tip is not blunt during the test. Filter the total volume of the liquid in all vials through a simple filter with nominal pore size not larger than 0.5 μm . Count the rubber fragments on the filter surface that are visible to the naked eye.

Limit. There are no more than five visible fragments. This limit is based on the assumption that fragments with diameter superior to 50 μm are visible to the naked eye. In case of doubt or controversy, the particles are examined microscopically to check their natures and sizes.

Auto-sealing capacity

Procedure. Fill 10 adequate vials with water up to the nominal volume. Adjust the caps to be examined and cover. Using a new hypodermic needle like before for each cap, perforate each cap 10 times, every time at a different place. Immerse the 10 vials in a methylene blue solution 0.1% (1 g/L) and reduce the external pressure by 27 kPa for 10 minutes. Restore atmospheric pressure and let the vials immersed for 30 minutes. Rinse the external part of the vials.

Limit. None of the vials must have any trace of blue solution.

6.2.3 PLASTIC CONTAINERS – PERFORMANCE TESTS

This section proposes standards for the functional properties of plastic containers and their components used to package medicines. The following tests are established to determine the permeability to humidity and transmission of light from the plastic containers applicable to each type of packaging. A container targeted at providing protection from light, or presented as a container resistant to light, must meet the requirement from the *Light transmission test (6.2.3.4)*, where the protection or resistance is due to specific properties of the material the container is made of, including any coating applied to it. A clear and colorless, or translucent, container, manufactured as *resistant*

¹ Refers to ISO 7864, sterile hypodermic needles for single use with external diameter of 0.8 mm (21 gauge).

to light by inclusion of an opaque compound, is free from the requirements of item *Light transmission tests* (6.2.3.4). The way it is used in this chapter, the term “container” refers to the full system encompassing the container itself, the container when used, the closure in the case of multiple-unit containers, and caps and blister in the cases of single-dose containers.

6.2.3.1 MULTIPLE-UNIT CONTAINERS FOR CAPSULES AND TABLETS

Desiccant. Pour an amount of anhydrous calcium chloride (1) with 4 mesh to 8 mesh in a shallow container, being careful to exclude any fine powder, dry at 110 °C for one hour, and cool down in a desiccator.

Procedure. Select 12 containers of uniform sizes and type, clean the closing surfaces with a fiber-free cloth, close and open each container 30 times. Cover tightly and uniformly every time the container is closed. Close the containers with a threaded cap with a torque movement that is within the range specified on **Table 1**. Transfer desiccants to 10 containers, designated as *test containers*, fill each one to 13 mm of the closure if the volume is 20 mL or higher, or fill each one to two thirds of the capacity if the container volume is below 20 mL. If the internal part of the container has more than 63 mm of depth, an inert funnel or spacer must be placed on the bottom to minimize the total weight of the container and the desiccant; the layer of desiccant in such container must not be inferior to 5 cm in depth. Close each one immediately after adding the desiccant, applying the torque designated on **Table 1** in the case of containers with screw cap. For each of the two remaining containers, designated as *controls*, transfer a sufficient number of glass beads to achieve an approximately equal weight to the one of *test containers* and close applying the torque designated on **Table 1** in the case of containers with screw cap. Register the weight of the containers, individually, thus prepared to the approximation of 0.1 mg if the volume of the container is below 20 mL, or to the approximation in closer mg if the container volume is 20 to 200 mL, or to approximation in centigrams (10 mg) if the volume is 200 mL or higher. Store at relative humidity of $(75 \pm 3)\%$ and at temperature of $(23 \pm 2)^\circ\text{C}$. A saturated system of 35 g of sodium chloride for every 100 mL of water placed on the bottom of the desiccator maintains the specified humidity, or other methods can be used to maintain such conditions. After (336 ± 1) hours (14 days), register the weight of containers individually, in the same manner. Completely fill five empty containers of the same size and type as the *test containers* with water or a non-compressible, free-flow solid, such as properly accommodated glass beads, to the level indicated by the closure surface. Transfer the content from each container to a graded cylinder and determine the average volume of the container in mL. Calculate the humidity permeability rate, in mg per day, per L, through the formula:

$$(1000/14V)[(T_F - T_I) - (C_F - C_I)]$$

where

V is the volume in mL of the container,

$(T_F - T_I)$ is the difference in mg between the final and initial weights of each *test container*;

$(C_F - C_I)$ is the difference in mg between the final and initial average weights from the two controls.

For containers used in medicines dispensed with prescription, the containers thus tested are of the sealed type, if no more than one of the 10 *test containers* exceeds 100 mg per day per L in humidity permeability, and none exceeds 200 mg per day per L. For containers used for medicines dispensed with prescription, the containers are tightly closed if no more than one of the 10 *test containers* exceeds 2000 mg per day per L in humidity permeability and none exceeds 3000 mg per day per L.

Table 1 – Torque applicable to the container with screw cap.

<i>Closure diameter^a</i> <i>(mm)</i>	<i>Tightening range suggested with torque</i> <i>applied manually^b</i> <i>(inches / pounds)</i>
8	5
10	6
13	8
15	5-9
18	7-10
20	8-12
22	9-14
24	10-18
28	12-21
30	13-23
33	15-25
38	17-26
43	17-27
48	19-30
53	21-36
58	23-40
63	25-43
66	26-45
70	28-50
83	32-65
86	40-65
89	40-70
100	45-70
110	45-70
120	55-95
132	60-95

a the torque designated for the next biggest closure diameter must be applied to *test containers* that have an intermediate closure diameter in relation to the diameters listed.

b use an adequate equipment for measuring the torque.

6.2.3.2 MULTIPLE-UNIT CONTAINERS FOR CAPSULES AND TABLETS (without closure)

Polyethylene container. Close the containers, with impenetrable seals obtained by hot sealing with a polyethylene-laminated aluminum foil or another adequate sealing. Test the containers as described above. The high-density polyethylene containers tested comply with the requisites if the permeability to humidity exceeds 10 mg per day per L, at maximum, in 1 of the 10 *test containers* and does not exceed 25 mg per day per L in none of them. The low-density polyethylene containers thus tested comply with the requisites if the permeability to humidity exceeds 20 mg per day per L, at maximum, in 1 of the 10 *test containers* and does not exceed 30 mg per day per L in none of them.

Polypropylene containers. Close the containers, with impenetrable seals obtained by hot sealing with a polyethylene-laminated aluminum foil or another adequate closure. Test the containers as described above. The containers comply with the requisites if the permeability to humidity exceeds

15 mg per day per L, at maximum, in 1 of the 10 *test containers* and does not exceed 25 mg per day per L in none of them.

6.2.3.3 SINGLE-UNIT AND UNIT-DOSE CONTAINERS FOR CAPSULES AND TABLETS

To allow a substantiated assessment in relation to the suitability of the packaging for a specific type of product, the following procedures and classification schemes are presented to assess the characteristics of permeability to humidity from single-unit and unit-dose containers. Since the equipment and operator performances can affect the penetration of humidity in a closed or formed container, the humidity penetration characteristics of the packaging system used must be determined.

Desiccant. Dry the appropriate desiccant pellets at 110 °C for one hour before use. Use pellets with approximate weight of 400 mg each one and with diameter of approximately 8 mm. If necessary, due to the limited dimension of the unit-dose container, pellets weighing less than 400 mg each and with diameter smaller than 8 mm can be used.

PROCEDURE

Method I. Seal no less than 10 unit-dose containers with one pellet each, and seal another 10 empty unit-dose containers for *control*, using glove fingers or a padded tweezer to handle the sealed containers. Number the containers and register the weights, individually, with the closest approximation in mg. Weigh the *controls* as one unit and divide the total weight by the number of controls to obtain the average. Store all containers at relative humidity of $(75 \pm 3)\%$ and at temperature of (23 ± 2) °C. A saturated system of 35 g of sodium chloride for every 100 mL of water placed on the bottom of a desiccator maintains the specified humidity, or other methods can be used to maintain such conditions. After an interval of 24 hours, and in each of their multiples, remove the containers from the chamber and let them equilibrate for 15 to 60 minutes in the weighing area. Register again the weight of the containers individually and the matched controls in the same manner. If no indicator pellet turns pink during the procedures, or if the pellet weight increase exceeds 10%, finish the test and consider only the first determinations as valid. Return the containers to the humidity chamber. Calculate the humidity penetration rate in mg per day from each container using the formula:

$$(1/N)[(W_F - W_I) - (C_F - C_I)]$$

where

N is the number of days expired in the test period (starting after the initial 24 hours of the equilibration period);

$(W_F - W_I)$ is the difference in mg between the final and initial weights of each test container;

$(C_F - C_I)$ is the difference in mg between the final and initial average weights of the controls, with the data calculated in relation to two significant figures. When the penetration measured is lower than 5 mg per day, and when it is observed that the controls reach equilibration in a period of seven days, the individual penetration can be determined more precisely, using the *test container from the 7th day* and the *control container* as W_I and C_I , respectively, in the calculations. In this case, an adequate test interval for Class A must not be inferior to 28 days from the equilibration period of the 7th day (a total of 35 days).

Method II. Use this procedure for packages, such as punch-out cards, that incorporate a number of blisters or unit-dose containers sealed separately. Seal a sufficient number of packages, no less than 4, and a total of no less than 10 unit-dose containers or blisters filled with one pellet in each unit to

be tested. Seal a corresponding number of empty packages, each one containing the same number of unit-dose containers or blisters like the ones used in test packages, as *controls*. Store all containers at relative humidity of $(75 \pm 3)\%$ and at temperature of $(23 \pm 2)^\circ\text{C}$. A saturated system of 35 g of sodium chloride for every 100 mL of water placed on the bottom of the desiccator maintains the humidity required, or other methods can be used to maintain such conditions. After 24 hours and at each subsequent 24 hours, remove the packages from the chamber and let them equilibrate at room temperature for approximately 45 minutes. Register the weights of individual packages and return them to the chamber. Weigh the *control* packages as one unit and divide as one unit and divide the total weight by the number of control packages to obtain the average weight of empty packages. If any indicator pellet turns pink during the procedure, or if the average pellet weigh exceeds 10% in any of the packages, finish the test and consider only the first determinations as valid. Calculate the average humidity penetration rate, in mg per day, for each unit-dose container or blister, in each package according to the formula:

$$(1/NX)[(W_F - W_I) - (C_F - C_I)]$$

where

N is the number of days elapsed in the test period (starting after the initial 24 hours of the equilibration period);

X is the number of units sealed separately per package;

$(W_F - W_I)$ is the difference in mg between the final and initial weights of each test package;

$(C_F - C_I)$ is the difference in mg between the final and initial average weights of control packages, and these rates are calculated up to two significant figures.

Limits. Individual unit-dose containers, as tested on *Method I*, are classified as *Class A* if no more than 1 of the 10 containers tested exceeds 0.5 mg per day in humidity penetration rate and none exceeds 1 mg per day; they are classified as *Class B* if no more than 1 of the 10 containers tested exceeds 5 mg per day and none exceeds 10 mg per day; they are classified as *Class C* if no more than 1 of the 10 containers tested exceeds 20 mg per day and none exceeds 40 mg per day; and they are classified as *Class D* if the containers tested do not meet any of these requisites for humidity penetration rate.

The packages, as they are tested on *Method II*, are classified as *Class A* if no package tested exceeds 0.5 mg per day of average humidity penetration rate per blister; they are classified as *Class B* if no package tested exceeds 5 mg per day of average humidity penetration rate per blister; they are classified as *Class C* if no package tested exceeds 20 mg per day of average humidity penetration rate per blister; and they are classified as *Class D* if no package tested meets the aforementioned requisites for average humidity penetration rate per blister.

With the use of *desiccant* described on *Method I* and *Method II*, after every 24 hours, the *test containers and controls* are weighed; the adequate test intervals for final weighing, W_F and C_F , must be: 24 hours for *Class D*; 48 hours for *Class C*; 7 days for *Class B*; and no less than 28 days for *Class A*.

6.2.3.4 MULTIPLE-DOSE AND UNIT-DOSE CONTAINERS FOR LIQUIDS

The standards and tests presented in this section are used to measured functional and performance characteristics of plastic containers used to package aqueous products through measuring the loss of weight of liquid water as a percentage of its content. This test can also be used to demonstrate a functional and performance comparison. Throughout the procedure, determine the weights from individual container closure systems (container, internal sealing if used, and closure), both as tare

weights and fill weights, at an approximation of 0.1 mg if the maximum capacity is lower than 200 mL; an approximation in mg if the maximum capacity is between 200 and 1000 mL; or an approximation in centigrams (10 mg) if the maximum capacity is 1000 mL or higher.

Procedures for tests in commercialized closed containers (cap liner if applicable, internal sealing and cap). Select 10 containers of uniform type and size and clean the sealing surfaces with a fiber-free cloth. Assemble each container with the cap liner, if applicable, and closure system. Number each closure system and register the tared weight.

Remove the closures and, with the help of a pipette, fill the containers to maximum capacity with water. Assemble the container with the seals and apply the closures. If threaded caps are used, apply the torque specified on **Table 1** in *Multiple-unit containers for capsules and tablets (6.2.3.1)* and store the closed containers at temperature of $(25 \pm 2)^\circ\text{C}$ and relative humidity of $(50 \pm 2)\%$. After (168 ± 1) hours (seven days), register the weight of containers individually. Return the containers to the storage place for another (168 ± 1) hours. After the second period of (168 ± 1) hours elapses, remove the containers, register the weights of each container system, individually, and calculate the water vapor penetration rate, in percentage of water weight loss, for each container through the formula:

$$(W_7 - W_{14}) 365 \times 100 / (W_7 - W_T) 7 = \text{Percentage per year}$$

where

W_7 is the weight in mg of the container at seven days;

W_{14} is the weight in mg of the container at 14 days;

W_T is the tare weight in g;

7 is the test time in days, after seven days of the equilibration period. The containers thus tested comply with the requisites and are considered tightly sealed containers if the percentage of water weight loss exceeds 2.5% per year, at maximum, in 1 of the 10 containers tested and does not exceed 5.0% per year in none of them.

The unit-dose containers for liquids comply with the requisites for a tightly sealed container if the average weight in water weight loss is inferior or equal to 2.5% (w/w) per year and 5% at the end of two years.

Procedure for tests on multiple-dose containers in conditions of use. Select 10 containers of uniform type and sizes. If internal sealing is used, open the containers carefully and remove the internal seals from each one. Assemble each container with the cap liner, if applicable, and its closure system. Number each container closure system and register the tare weight. Open and close the containers 30 times, being careful to not lose liquid during this procedure. Close the containers with screw cap within the torque range presented on **Table 1** in *Multiple-unit containers for capsules and tablets (6.2.3.1)* and store the sealed containers at temperature of $(25 \pm 2)^\circ\text{C}$ and relative humidity of $(50 \pm 2)\%$. After (168 ± 1) hours (seven days), register the weight of containers individually. Return to the storage place for another (168 ± 1) hours. After the second period of (168 ± 1) hours, remove the containers, register the weights of each container system, individually, and calculate the water vapor penetration rate, in percentage of water weight loss, for each container through the formula:

$$(W_7 - W_{14}) 365 \times 100 / (W_7 - W_T) 7 = \text{Percentage per year}$$

where

W_7 is the weight in mg of the container at seven days;

W_{14} is the weight in mg of the container at 14 days;

W_T is the tare weight in g;

The containers thus tested comply with the requisites and are considered tightly sealed containers if the percentage of water weight loss exceeds 2.5% per year, at maximum, in 1 of the 10 containers tested and does not exceed 5.0% in none of them.

6.2.3.5 LIGHT TRANSMISSION TEST

Equipment. Use a spectrophotometer with adequate sensitivity and precision, adapted to measure the amount of light transmitted by plastic materials, used as pharmaceutical containers. Additionally, the spectrophotometer must measure and register the diffuse transmitted light, as well as parallel rays.

Procedure. Select sections to represent the average thickness of the container wall. Cut circular sections from two or more areas of the container and trim if necessary to provide segments of convenient sizes to be inserted in the spectrophotometer. Cut, wash and dry each sample, being careful to avoid scratches in the surface. If the sample is too small to cover the opening on the sample support, cover the uncovered portion of the opening with an opaque paper or adhesive tape, making the sample length larger than the opening in the spectrophotometer. Immediately before assembling the sample support, clean the sample with a cloth suitable for cleaning lenses. Assemble the sample with the help of viscous wax, or through other convenient media, being careful to not leave fingerprints or other marks on the surfaces the light must go through. Put the section in the spectrophotometer with its cylindrical axis parallel to the opening plan and approximately centralized in relation to the opening. When placed adequately, the light beam is normal at the section surface and the losses by reflection are minimal. Measure, continuously, the section transmittance with reference to air in the wavelength of interest, with a registration equipment or in intervals of approximately 20 nm with a manual equipment, in the wave amplitude between 290 and 450 nm.

Limit. The light transmission observed must not exceed the limits included on **Table 1** for containers targeted at parenteral use.

Table 1 – Limits for plastics classes I-VI.

<i>Nominal size (mL)</i>	<i>Maximum percentage of light transmission in any wavelength between 290 and 450 nm</i>	
	<i>Heat-sealed containers</i>	<i>Hermetically sealed containers</i>
1	50	25
2	45	20
5	40	15
10	35	13
20	30	12
50	15	10

Any container, with a size intermediate to the ones listed on **Table 1**, presents transmission not greater than the next biggest size listed on the table. For containers larger than 50 mL, the limits for 50 mL apply. The light transmission observed for plastic containers for products targeted at oral or topical administration must not exceed 10% in any wavelength on the range between 290 nm and 450 nm.

6.2.4 BIOCOMPATIBILITY

In this section there are guidelines about procedures for assessment of biocompatibility of plastic containers for medicines, elastomer caps and correlates. Biocompatibility refers to the trend of these products to remain biologically inert when in contact with the body. In combination with chemical

assays, the biological processes can be used to detect and identify the inherent or acquired toxicity of correlates, before or during their manufacturing and processing.

The procedures used to assess the biocompatibility of a correlate or its constituents were classified in a panel of biological effects or toxicity procedures, such as cytotoxicity, sensitivity, irritation or intracutaneous reactivity, acute systemic toxicity, sub-chronic toxicity (sub-acute toxicity), genotoxicity, implantation, hemocompatibility, chronic toxicity (extends by 10% the life expectancy of the test animal, or to more than 90 days), carcinogenicity, reproductivity or development toxicity, and biodegradation.

Pyrogenicity, in a special toxicity area, is assessed by the *Bacterial endotoxins* (5.5.2.2) test and the *Pyrogens* (5.5.2.1) test. Currently, there are no chapters detailing about sensitivity, sub-chronic toxicity, genotoxicity, chronic toxicity, carcinogenicity, hemotoxicity, reproductive toxicity, or requisites for biodegradation test.

6.2.4.1 PLASTIC CONTAINERS AND ELASTOMER CAPS

Plastic containers can be made of polymers that, by extraction, do not present toxicity or do not change the stability of the packaged product. The requisites for the biocompatibility test of containers for medicines are related to plastic containers. Plastic, or other polymeric portions of these products, are tested according to the procedures established in *Biological reactivity tests in vitro* (6.2.5), and the ones that do not comply with requirements from these tests are not adequate for a medicine container. The materials that comply with *in vitro* requisites are qualified as biocompatible materials, without the need for other tests, and can be used to manufacture a medicine container. If a class designation (classes I-VI) is requested for plastics or other polymers, the adequate test procedures are conducted as presented on *Biological reactivity tests in vivo* (6.2.6) and Class designation.

The biocompatibility of an elastomeric material is assessed in two phases, as described on Procedures from *Biological assay in Elastomer caps* (6.2.2).

Contrary to plastics or other polymers, an elastomeric material that does not meet the requirements from the first phase of the test *in vitro* can be considered a biocompatible material if it is approved on the second phase – *in vivo*, which consists on the systemic injection test and the intracutaneous test in *Biological reactivity tests in vitro* (6.2.5). No distinction of class or type is made between the elastomeric materials that meet the requisites from the first phase of test and the ones that meet the second phase, qualifying as biocompatible materials. Elastomeric materials are not classified on classes I-VI.

6.2.4.2 CORRELATES

The biocompatibility of plastic, other polymers and elastomeric parts of these products is tested according to the procedures described in *Biological reactivity tests in vitro* (6.2.5). If a class designation is also necessary for a plastic or another polymer, the adequate test procedures described in *Biological reactivity tests in vivo* (6.2.6) are conducted.

6.2.4.3 TESTS IN VITRO, TESTS IN VIVO AND CLASS DESIGNATION FOR PLASTICS AND OTHER POLYMERS

The requisites for tests *in vitro* and *in vivo* are developed to determine the biological reactivity of mammal cell cultures and the biological response from animals to elastomeric materials, plastics and other polymers, when in direct or indirect contact with the patient. The biological reactivity of these

materials may depend both on their surface characteristics and their extractable chemical components. The test procedures can be conducted with the material, or an extract of the material being tested, except if indicated otherwise.

Preparation of extracts

Usually, the assessment of compatibility of an entire correlate is not realistic, and using representative portions, or extracts from selected materials, may be a practical alternative for conducting the assays. When portions or extracts are used, it is important to consider that the raw material may suffer chemical changes during the manufacturing, processing and sterilization of a correlate. Assays *in vitro* of a raw material may serve as an important triage process, but the final assessment of biocompatibility of the correlate must be conducted with parts of the product, finished and sterilized.

The extractions can be made in several temperatures (121, 70, 50 or 37 °C), in several time intervals (1, 24 or 72 hours) and in different extraction media. The selection of extraction medium for tests *in vitro* includes sodium chloride solution injectable 0.9%, or culture medium for tissues with or without serum. When medium with serum is used, the extraction temperature cannot exceed 37 °C. When selecting the extraction conditions, select the temperature, solvent and time variables the best conditions of use of the product. The performance from several tests in different conditions can be used to simulate variations of the conditions “in use”. A biocompatibility assessment is conducted with the product, finished and sterilized, although a careful selection of the extraction conditions allows to simulate the raw material production and test conditions.

Test in vitro

When tests *in vitro* are conducted, the sample is biocompatible if the cell cultures do not present reactivity greater than mild (grade 2), as described on the *Biological reactivity tests in vitro* (6.2.5).

Test in vivo and class designation

According to the definition of injection and implantation described in *Biological reactivity tests in vivo* (6.2.6), plastics and other polymers are classified in classes I to VI. To obtain designation of plastics, or other polymers, extracts from the test substance are produced according to the procedures described in different media. To assess the biocompatibility, the extracts are inoculated, systemically and intracutaneously, in mice and rabbits. According to the requisites for injection, a plastic or other polymer can be initially classified as I, II, III, or V. If, in addition to the injection test, the implantation test is conducted with the same material, the plastic or polymer can be classified as class IV or VI.

6.2.4.4 BIOCOMPATIBILITY OF CORRELATES

In addition to assessing the correlates for sterility, tests *in vitro* and *in vivo*, the correlates are assessed for sensitivity, sub-chronic toxicity, genotoxicity, hemocompatibility, chronic toxicity, carcinogenicity, reproductive or development toxicity, and biodegradation.

In international guidelines, there is an indication that the extension of tests executed for a correlate depends on the following factors: product similarity and exclusivity in relation to products previously commercialized, as considered on the *Decision flowchart*; extension and duration of contact between the product and the patient, as described in *Categorization of correlates*, and the composition of the product material, as considered in the sections *Decision flowchart*, *Tests in vivo* and *Class designation*.

DECISION FLOWCHART

The guidance for comparing a correlate with products commercialized previously is provided by the Biocompatibility decision flowchart (**Figure 1**).

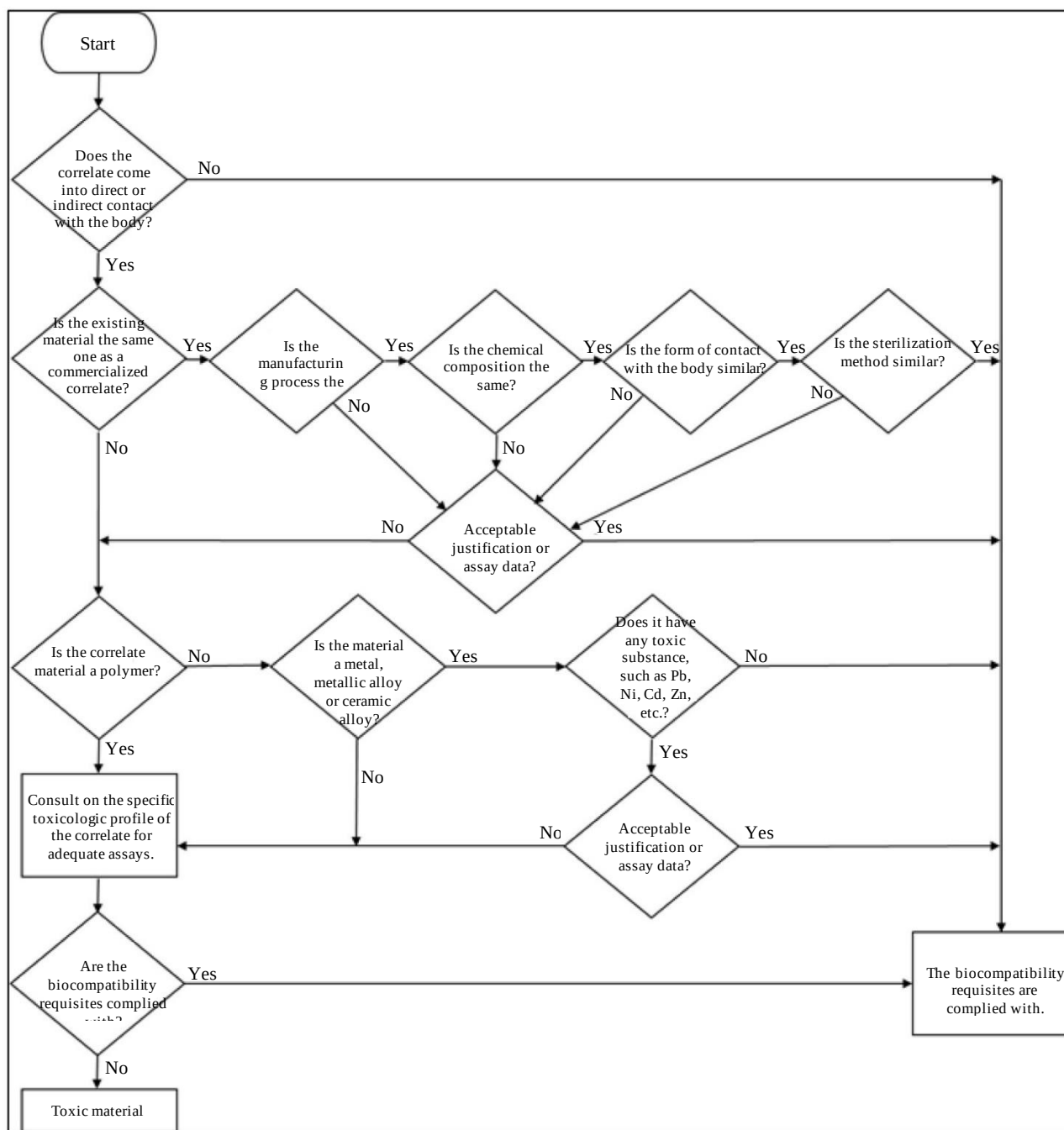


Figure 1 – Biocompatibility flowchart adapted from the FDA Blue Book Memorandum # G95-1.

The goal with the flowchart is to determine whether the data available on previously commercialized correlates are sufficient to ensure the safety of the correlate in question. As indicated on the flowchart, the material composition and manufacturing techniques of a product are compared with correlates already commercialized that come into direct contact with the body. Additionally, there is a requirement on the flowchart for an assessment of the toxicity of an exclusive material that has not been previously used in correlate products. The answers to the questions made on the flowchart lead to the conclusion that the data available are sufficient, or that additional tests are necessary to ensure the product safety. The guidance about the identification of appropriate procedures for additional tests is provided in the Test selection matrix.

CATEGORIZATION OF CORRELATES

To facilitate the identification of adequate test procedures, the correlates are divided and subdivided, as registered on **Table 1**, according to the nature and extension of their contact with the body. The main categories of correlates are surface, extracorporeal communication and implantable. Then, these classifications are subcategorized with examples of correlates belonging to each of the subcategories (**Table 1**).

Table 1 – Classification and examples of correlates.

<i>Category of correlate</i>	<i>Subcategory of correlate</i>	<i>Nature or extension of contact</i>	<i>Examples</i>
Surface	Skin	Correlates that only come into contact with the intact surface of the skin.	Electrodes, external prostheses, fixation tapes, compression bandages, and monitors of different types.
	Mucosa	Correlates that communicate with intact mucous membranes.	Contact lenses, urinary catheters, intravaginal and intra-intestinal devices (stomach tubes, sigmoidoscopes, colonoscopes, gastroscopes), endotracheal tubes, bronchoscopes, dental prostheses, orthodontic and intrauterine devices. Dressings, healing devices, and occlusive bandages for ulcer, burn and granulated tissue.
	Compromised or Breached Surfaces	Correlates that come into contact with compromises or breached body surfaces.	
	Blood Vessel, Indirect	Correlates that come into contact with blood vessel in one point and serve as channel for entrance to the vascular system.	Solution administration set, blood transfer and administration, extensors.
Extracorporeal communication	Communication with Tissue, Bone or Dentin	Correlates and materials that communicate with tissue, bone or Dentin/pulp system.	Laparoscopes, arthroscopes, draining systems, dental cement, dental filling material, and skin staples.
	Blood circulation	Correlates that come into contact with blood circulation.	Intravascular catheters, temporary pacemaker electrodes, oxygenators, extracorporeal oxygenator tube and accessories, dialyzers, dialysis tube and accessories, hemoadsorbents and immunoadsorbents.
Implantable items	Tissue or Bone	Correlates that come into contact especially with bone, tissue or tissue fluid.	Examples of cast with orthopedic pins, plates, replacement joints, bone prostheses, cements and intraosseous devices. Examples of the latter are pacemakers, medicine administration devices, neuromuscular sensors and stimulators, replacement tendons, breast implants, artificial larynx, sub-periosteal implants, and ligation clips.

Category of correlate	Subcategory of correlate	Nature or extension of contact	Examples
	Blood	Correlates in contact especially with blood.	Pacemaker electrodes, artificial arteriovenous fistula, heart valves, valve graft, catheters for internal medicine administration, and ventricular assistance devices.

TEST SELECTION MATRIX

In the matrix there is guidance for identification of adequate procedure for biological assays for the three categories of correlates: Tests for surface devices (**Table 1** in *Guide for selection of plastic and other polymers (6.2.4.5)*), Tests for extracorporeal communication devices (**Table 2** in *Guide for selection of plastic and other polymers (6.2.4.5)*), and Tests for implantable devices (**Table 3** in *Guide for selection of plastic and other polymers (6.2.4.5)*). Each category of correlates is subcategorized and subdivided according to the duration of contact between the device and the body. The duration of contact is defined as limited (less than 24 hours); prolonged (24 hours to 30 days), or permanent (more than 30 days). The biological effects included in the matrix are: cytotoxicity, sensitivity, intracutaneous irritation or reactivity, systemic toxicity, sub-chronic toxicity, genotoxicity, implantation, hemocompatibility, chronic toxicity, carcinogenicity, reproductive or development toxicity, and biodegradation. In the matrix, for each subcategory there is a board associated to the test requisites and, usually, the number of tests increases as the duration of the contact between the device and the body is extended and according to the proximity of contact between the device and the circulatory system. In the subcategories, the option of conducting additional tests must be considered on a case-by-case basis. Specific situations, such as the use of permanent implantable devices or with extracorporeal communication in pregnant women, must be considered by the manufacturer, who will decide about including the reproduction or development test. Guidance about identifying eventual additional procedures for test is provided in the matrix of each subcategory of correlates.

6.2.4.5 GUIDE FOR SELECTING PLASTIC AND OTHER POLYMERS

Class designation for correlate

In **Figure 1** there is guidance for selecting the appropriate class designation of the plastic or other polymer for a correlate and each subcategory of Surface devices, and on **Figure 2**, for Communication devices. The class designations can be found on *Biological reactivity tests in vivo (6.2.6)*.

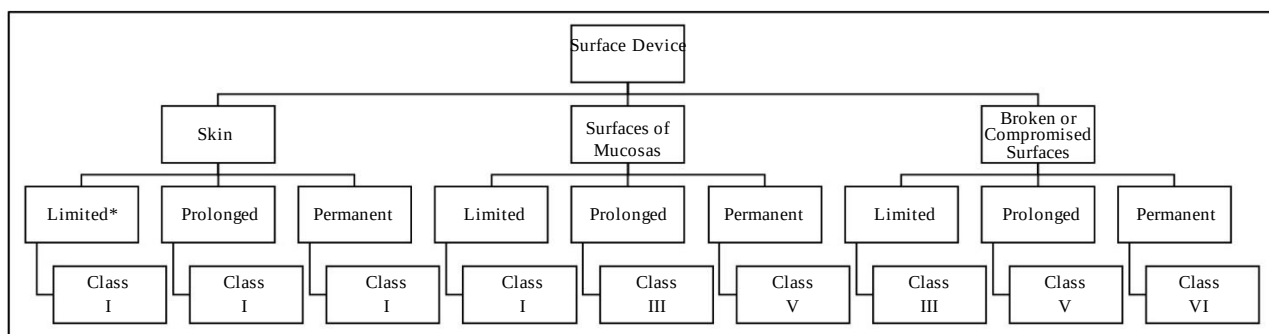


Figure 1 – Class Requisites of plastics and other polymers for surface devices.

* Categorization based on the duration of contact. Limited: less than 24 hours; prolonged: 24 hours to 30 days; permanent: more than 30 days.

† Designation of Class of Plastics.

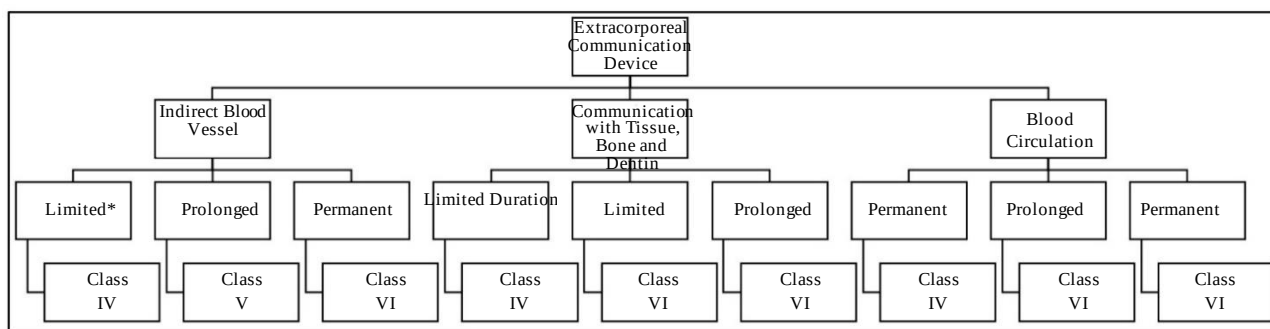


Figure 2 – Class Requisites of plastics and other polymers for extracorporeal communication devices.

* Categorization based on the duration of contact. Limited: less than 24 hours; prolonged: 24 hours to 30 days; permanent: more than 30 days.

† Designation of Class of Plastics.

The class number indicated increases according the duration of contact between the device and the body (risk). In the category of Implantable devices, the exclusive use of class VI is mandatory. The designation of plastic classes is based on the test selection matrixes illustrated on **Tables 1, 2 and 3**.

The assignment of class of a plastic or another polymer to a subcategory is not targeted at restricting the use of superior categories of plastics or other polymers. Although the designation assigned defines the lower numerical class of plastic or other polymer that can be used in the corresponding correlate, the use of a numerically higher plastic class is optional. When a correlate belongs to more than one category, the plastic or other polymers must meet the requirements from the higher numerical class.

Table 1 – Test selection matrix for surface and devices.*

<i>Categories of correlates</i>	<i>Biological effect^b</i>												
<i>Contact with the body</i>	<i>Duration of contact^a</i>	<i>Cytotoxicity</i>	<i>Sensitivity</i>	<i>Intracutaneous irritation or reactivity</i>	<i>Systemic toxicity (acute)</i>	<i>Sub-chronic (sub-acute) toxicity</i>	<i>Genotoxicity</i>	<i>Implantation</i>	<i>Hemocompatibility</i>	<i>Chronic toxicity</i>	<i>Carcinogenicity</i>	<i>Reproductive or development toxicity</i>	<i>Biodegradation</i>
Surface devices	Skin	A	X	X	X	-	-	-	-	-	-	-	-
		B	X	X	X	-	-	-	-	-	-	-	-
		C	X	X	X	-	-	-	-	-	-	-	-
	Mucosa	A	X	X	X	-	-	-	-	-	-	-	-
		B	X	X	X	O	O	-	O	-	-	-	-
		C	X	X	X	O	X	X	O	-	O	-	-
	Compromised or breached surfaces	A	X	X	X	O	-	-	-	-	-	-	-
		B	X	X	X	O	O	-	O	-	-	-	-
		C	X	X	X	O	X	X	O	-	O	-	-

a Key A: limited (less than 24 hours); B: prolonged (24 hours to 30 days); C: permanent (more than 30 days).

b Key X: ISO assessment tests for consideration; O: additional tests that may be applied.

* Adapted from FDA's *Blue Book Memorandum #G95-1* (**Table 1**. Initial Assessment tests for Consideration and **Table 2**. Complementary Assessment Tests for Consideration).

Table 2 – Test Selection Matrix for Extracorporeal Communication Devices.*

<i>Categories of correlates</i>		<i>Biological effect^b</i>												
<i>Contact with the body</i>		<i>Duration of contact^a</i>	<i>Cytotoxicity</i>	<i>Sensitivity</i>	<i>Intracutaneous irritation or reactivity</i>	<i>Systemic toxicity (acute)</i>	<i>Sub-chronic (sub-acute) toxicity</i>	<i>Genotoxicity</i>	<i>Implantation</i>	<i>Hemocompatibility</i>	<i>Chronic toxicity</i>	<i>Carcinogenicity</i>	<i>Reproductive or development toxicity</i>	<i>Biodegradation</i>
Extracorporeal communication device	Blood vessel, indirect	A	X	X	X	X	-	-	-	X	-	-	-	-
		B	X	X	X	X	O	-	-	X	-	-	-	-
		C	X	X	O	X	X	X	O	X	X	X	-	-
	Communication with tissue, bone or dentin	A	X	X	X	O	-	-	-	-	-	-	-	-
		B	X	X	O	O	O	X	X	-	-	-	-	-
		C	X	X	O	O	O	X	X	-	X	X	-	-
	Blood circulation	A	X	X	X	X	-	O	-	X	-	-	-	-
		B	X	X	X	X	O	X	O	X	-	-	-	-
		C	X	X	X	X	X	X	O	X	X	X	-	-

a Key A: limited (less than 24 hours); B: prolonged (24 hours to 30 days); C: permanent (more than 30 days).

b Key X: ISO assessment tests for consideration; O: additional tests that may be applied.

* Adapted from FDA's *Blue Book Memorandum #G95-1* (**Table 1**. Initial Assessment tests for Consideration and **Table 2**. Complementary Assessment Tests for Consideration).

Table 3 – Test Selection Matrix for Implantable Devices.*

<i>Categories of correlates</i>	<i>Biological effect^b</i>												
<i>Contact with the body</i>	<i>Duration of contact^a</i>	<i>Cytotoxicity</i>	<i>Sensitivity</i>	<i>Intracutaneous irritation or reactivity</i>	<i>Systemic toxicity (acute)</i>	<i>Sub-chronic (sub-acute) toxicity</i>	<i>Genotoxicity</i>	<i>Implantation</i>	<i>Hemocompatibility</i>	<i>Chronic toxicity</i>	<i>Carcinogenicity</i>	<i>Reproductive or development toxicity</i>	<i>Biodegradation</i>
Implantable devices	Tissue or bone	A	X	X	X	O	-	-	-	-	-	-	-
		B	X	X	O	O	X	X	-	-	-	-	-
		C	X	X	O	O	X	X	-	X	X	-	-
	Blood	A	X	X	X	X	-	-	X	X	-	-	-
		B	X	X	X	X	O	X	X	X	-	-	-
		C	X	X	X	X	X	X	X	X	X	-	-

a Key A: limited (less than 24 hours); B: prolonged (24 hours to 30 days); C: permanent (more than 30 days).

b Key X: ISO assessment tests for consideration; O: additional tests that may be applied.

* Adapted from FDA's *Blue Book Memorandum #G95-1* (**Table 1**, Initial Assessment tests for Consideration and **Table 2**, Complementary Assessment Tests for Consideration).

1 Document from ISO 10993-1:1997 titled *Biological Evaluation of Medical Devices – Part 1: Evaluation and Testing*.

* Adapted from FDA's *Blue Book Memorandum #G95-1* ("Use of International Standard ISO-10993." Biological Evaluation of Medical Devices - Part 1: Evaluation and Testing.")

6.2.5 BIOLOGICAL REACTIVITY TESTS IN VITRO

The following tests are developed to determine the biological reactivity of mammal cell cultures, after the contact with elastomeric plastics and other polymeric materials, which come into direct or indirect contact with the patient, or after contact with specific extracts elaborated from the materials being tested. It is essential that the tests are conducted on the surface area specified. When the sample surface cannot be determined, use 0.1 g of elastomer or 0.2 g of plastic, or another material, for every mL of extraction fluid.

Three assays are described: *Agar diffusion test*, *Direct contact test* and *Elution test*. The decision on what type or the number of assays to be conducted to assess the potential of biological response from a specific sample or an extract depends on the material, the end product, and its intentions of use. Other factors that may also affect the suitability of the sample for a specific use are: polymer composition; procedures for processing and cleaning; means of contact; colorants; adhesives; absorption, adsorption and permeability of preservatives; and storage conditions. Such factors must be assessed by specific appropriate additional assays, before determining that a product produced through a specific material is adequate for its intention of use.

Preparation of cell culture. In a minimum essential medium supplemented with seeding density serum of approximately 105 cells per mL, prepare multiple cultures of fibroblastic cells L-929 (ATCC cell line CCL 1, NCTC clone 929). Incubate the cultures at $(37 \pm 1)^\circ\text{C}$ in a humidified incubator, with atmosphere of $(5 \pm 1)\%$ carbon dioxide, for no less than 24 hours until obtaining the single layer, with confluence superior to 80%. Examine the cultures prepared with a microscope to ensure an uniform level of nearly confluent monolayers.

Extraction solvents. Injectable sodium chloride solution (refer to the corresponding monograph). Alternative, free media or supplemented with serum for mammal cell culture can be used. The serum supplementation is used when the extraction is performed at 37°C , for 24 hours.

Equipment

Autoclave. Use an autoclave capable of maintaining the temperature of $(121 \pm 2)^\circ\text{C}$ and capable of cooling the assay containers down around 20°C .

Oven. Preferably use a model with mechanic convection, capable of maintaining the operating temperatures in the range of 50°C to $70^\circ\text{C} \pm 2^\circ\text{C}$.

Incubator. Use an incubator capable of maintaining the temperature of $(37 \pm 1)^\circ\text{C}$ and a humid atmosphere with $(5 \pm 1)\%$ carbon dioxide in the air.

Extraction containers. Use only Type I glass containers, such as culture test tube with screw cap, or equivalent. The screw cap must have appropriate elastomeric coating. The exposed surface of this coating must be completely protected with an inert solid disc of 50-75 μm of thickness.

Preparation of equipment. Completely clean all glassware with chromic acid cleaning solution and, if necessary, with warm nitric acid, followed by prolonged rinsing with sterile water for injection. Sterilize and dry the containers and equipment used for extraction, transfer or administration of the assay material, through adequate process. If ethylene oxide is used as a sterilizing agent, wait no less than 48 hours until complete degassing.

Procedure

Preparation of sample for extract. Prepare as described in the Procedure from *Biological reactivity tests in vivo* (6.2.6).

Preparation of extracts. Prepare as described in the Procedure from *Biological reactivity tests in vivo* (6.2.6), using an injectable sodium chloride solution (0.9% NaCl) or serum-free medium for mammal cell culture as described in *Extraction solvents*. If the extraction is made at 37 °C for 24 hours in incubator, use cell culture media supplemented with serum. In no case must the extraction conditions cause physical changes, such as fusion or melting of portions of material, except a slight adherence.

AGAR DIFFUSION TEST

This test was developed for elastomeric materials of different models. The agar layer works as a support to protect the cells from mechanical damages, allowing the diffusion of leachable chemical products from polymeric samples. The extracts from materials to be tested are applied to a piece of filter paper.

Sample preparation. Use extracts prepared as described or portions of samples with flat surfaces not inferior to 100 mm².

Preparation of positive control. Proceed as described in *Preparation of sample*.

Preparation of negative control. Proceed as described in *Preparation of sample*.

Procedure. Use 7 mL of the cell suspension prepared as described in the *Preparation of cell culture* and prepare the layers on plates with 60 mm of diameter. After the incubation is done, aspirate the culture medium from the layers and replace it with medium supplemented with serum with amounts of up to 2% of agar. The quality of agar must be adequate to sustain the cell growth. The agar layer must be sufficiently thin to allow the diffusion of leachable chemical products. Put the flat surfaces of the sample, negative control and positive control, or their extracts, in contact with the solidified surface of agar, in duplicate. Do not use more than three samples in each plate prepared. Incubate all cultures at (37 ± 1) °C for no less than 24 hours, in an adequate incubator. Examine, visually or with a microscope, each culture around the sample; negative control and positive control, using adequate coloring agent, if necessary.

Interpretation of results. The biological reactivity, that is, the cell malformation and degeneration, is described and classified on a scale from 0 to 4 (**Table 1**). Measure the responses of cell cultures from the sample, negative control and positive control. The cell culture assay system is adequate if the responses observed are classified as 0 (no reactivity) for negative control and no less than 3 (moderate) for positive control. The sample complies with the test requisites if the response is not superior to the classification 2 (mildly reactive). Repeat the procedure if the system suitability is not confirmed.

Table 1 – Classification of reactivity for Agar diffusion test and Direct contact test.

<i>Classification</i>	<i>Reactivity</i>	<i>Description of reactivity zone</i>
0	None	No zone detectable around or under the sample.
1	Slight	Some malformed or degenerated cells under the sample.
2	Mild	Zone limited to the area under the sample.
3	Moderate	Zone extends from 0.5 to 1.0 cm beyond the sample.
4	Strong	Zone extends more than 1.0 cm beyond the sample.

DIRECT CONTACT TEST

This test is defined for materials in different formats. The procedure allows simultaneous extractions and test on leachable chemical products from the sample in a medium supplemented with serum. The procedure is not appropriate for materials with very high or very low density, because it may cause mechanical damages to the cells.

Sample preparation. Use a portion of sample with flat surface not inferior to 100 mm².

Preparation of positive control. Proceed as described in *Preparation of sample*.

Preparation of negative control. Proceed as described in *Preparation of sample*.

Procedure. Use 2 mL of the cell suspension prepared as described in *Preparation of cell culture* and prepare the layers on plates with 35 mm of diameter. After the incubation, aspirate the culture medium and replace it with 0.8 mL of fresh culture medium. Put a single sample, negative control and positive control in each of the culture medium duplicates. Incubate all cultures at $(37 \pm 1)^\circ\text{C}$ for no less than 24 hours in an adequate incubator. Examine, visually or with a microscope, each culture around the sample, negative control and positive control, using adequate coloring agent, if necessary.

Interpretation of results. Proceed according to the interpretation of results from the *Agar diffusion test*. The sample complies with the test requisites if the sample response is not superior to the classification 2 (mildly reactive). Repeat the procedure if the system suitability is not confirmed.

ELUTION TEST

This assay is defined for assessment of extracts from polymeric materials. The procedure allows extracting samples by varied time intervals and in physiological and non-physiological temperatures. It is appropriate for high-density materials and dose-response assessments.

Sample preparation. Prepare as described in *Preparation of extracts*, using injectable sodium chloride solution (0.9% NaCl) or serum-free medium for mammal cell culture according to *Extraction solvents*. If the sample size cannot be readily measured, a mass with no less than 0.1 g of elastomeric material or 0.2 g of plastic or polymeric material per mL of extraction medium can be used. Alternatively, to simulate conditions that are closer to the physiological ones, use for extraction a mammal cell culture medium, supplemented with serum. Prepare the extracts through heating at $(37 \pm 1)^\circ\text{C}$ for 24 hours, in an appropriate incubator. Higher temperatures may cause denaturation of serum proteins.

Preparation of positive control. Proceed as described in *Preparation of sample*.

Preparation of negative control. Proceed as described in *Preparation of sample*.

Procedure. Use 2 mL of the cell suspension prepared as described in the *Preparation of cell culture* and prepare the monolayers on plates with 35 mm of diameter. After the incubation, aspirate the medium from the layers and replace it with extract from the sample, negative control and positive control. Extracts from media, supplemented or not with serum, are tested in duplicate, without dilution (100%). The extract of injectable sodium chloride solution is diluted with cells from the culture medium supplemented with serum and tested, in duplicate, at a concentration of 25%. Incubate all cultures at $(37 \pm 1)^\circ\text{C}$ for 48 hours, in an adequate incubator. Examine with a microscope each culture after 48 hours, using adequate coloration, if necessary.

Interpretation of results. Proceed according to the interpretation of results from the *Agar diffusion test*, but using **Table 2**. The sample complies with the test requisites if the sample response is not superior to the classification 2 (mildly reactive). Repeat the procedure if the system suitability is not confirmed. For dose-response assessments, repeat the procedure using quantitative dilutions of the sample extract.

Table 2 – Classification of reactivity for elution test.

Classification	Reactivity	Conditions of cultures
0	None	Discrete intracytoplasmic granules; no cell lysis.
1	Slight	Up to 20% of the cells are round, vaguely united, with no intracytoplasmic granules; lysed cells are occasionally present.
2	Mild	Up to 50% of the cells are round and have no intracytoplasmic granules; no extensive cell lysis and empty areas between cells.
3	Moderate	Up to 70% of the layers have rounded or lysed cells.
4	Strong	Nearly integral destruction of the cell layers.

6.2.6 BIOLOGICAL REACTIVITY TESTS *IN VIVO*

The following tests are developed to determine the biological response from animals to elastomeric materials, plastics and other polymeric materials, which come into direct or indirect contact with the patient, or the response to the inoculation of specific extracts elaborated from the materials being tested. It is essential to make a specific surface area for extraction available. When the sample surface area cannot be determined, use 0.1 g of elastomer or 0.2 g of plastic, or another material, for every mL of extraction fluid.

Three assays are described to classify plastics and other polymers, which are applicable to materials and correlates, based on *in vivo* biological reactivity assays. The *Systemic injection test* and the *Intracutaneous test* are used for elastomeric materials, especially for materials where the adequate *Biological reactivity test in vitro* (6.2.5) indicated significant biological reactivity. The *Implant test* is used to check the suitability of plastics and other polymers, used in manufacturing containers and accessories; in parenteral preparations, in correlates, implants and other systems.

The following definitions are applied in this chapter: sample is the material being tested, or the extract prepared from a certain material. The blank consists of the same amount of the medium that is used for the sample extraction, being treated the same way than the medium that has the sample analyzed. The negative control is a sample that does not present any reaction in the assay conditions.

Classification of plastics. Six classes of plastic are defined (**Table 1**), based on the responses for a series of assays *in vivo* where the extracts, materials and routes of administration are specified. These tests are directly related to the end use of plastic articles. In preparations where plastics are susceptible to coming into contact with the vehicles, the selection of extraction solution is representative. The classification registered on **Table 1** summarizes the tests to be conducted in containers for injection and in medical devices, if there is need for classification.

Table 1 – Classification of plastics and tests to be conducted.

Classes of plastics ^a						Tests to be conducted			
I	II	III	IV	V	VI	Test material	Animal	Dose	Procedures ^b
x	x	x	x	x	x		Mouse	50 mL/kg	A (IV)

Classes of plastics ^a						Tests to be conducted			
I	II	III	IV	V	VI	Test material	Animal	Dose	Procedures ^b
x	x	x	x	x	x	Extract of sample in injectable sodium chloride solution	Rabbit	0.2 mL/ animal in each of the 10 sites	B
	x	x	x	x	x	Extract of sample of alcohol solution 1:20 in injectable sodium chloride solution	Mouse	50 mL/kg	A (IV)
	x	x	x	x	x		Rabbit	0.2 mL/ animal in each of the sites	B
		x		x	x	Extract of sample in polyethylene glycol 400	Mouse	10 g/kg	A (IP)
				x	x		Rabbit	0.2 mL/ animal in each of the 10 sites	B
		x	x	x	x	Extract of sample in vegetable oil	Mouse	50 mL/kg	A (IP)
			x	x	x		Rabbit	0.2 mL/ animal in each of the 10 sites	B
			x		x	Sample implant strips	Rabbit	4 strips/animal	C

^a Tests required for each class indicated with an “x” on the appropriate column.

^b Key: A (IP) *Systemic Injection Test* (intraperitoneal); A (IV) *Systemic Injection Test* (intravenous); B *Intracutaneous Test* (intracutaneous);

^c *Implantation Test* (intramuscular implantation).

With exception of the *Implantation test*, the procedures are based on the use of extracts that, due to the thermal resistance of the material, are prepared in one of the three standard temperatures: 50, 70 and 121 °C. For this reason, the class designation of a plastic must be followed by an indication of the extraction temperature (for example: IV-121 °C is the designation of class IV, from a plastic extracted at 121 °C; I-50 °C is the designation of class I, from a plastic extracted at 50 °C). Plastics can be classified on classes I to VI, based on the response criteria registered on **Table 1**.

This classification does not apply to plastics that are used as containers for topical or oral products, or that may be used as an integral part of a medicine formulation. The information registered on **Table 1** does not apply to natural elastomers, which are only tested through injectable sodium chloride solution and vegetable oils.

The *Systemic injection test* and the *Intracutaneous test* are developed to determine, respectively, the systemic and local biological responses; in animals exposed to plastics and other polymers, by inoculation of single dose of specific extracts of the sample. The *Implantation test* is developed to assess the reaction of live tissue to plastic and other polymers, through implanting the own sample in animal tissue. The adequate preparation and placement of the samples in asepsis conditions are important for conducting the *Implantation test*.

These tests are developed for application in materials in the conditions they are used. If the material must be exposed to any cleaning or sterilization process before its end use, the tests must be conducted in a *sample* submitted to such processes.

Extraction media

Injectable sodium chloride solution. Refer to the corresponding monograph.

Alcohol solution 1:20 in injectable sodium chloride solution.

Macrogol. Refer to the corresponding monograph.

Vegetable oil. Use sesame oil, cottonseed oil, or other appropriate vegetable oils (refer to the monograph). If possible, obtain freshly refined oils. Use three animals duly prepared and inoculated intracutaneously in each animal one dose of 0.2 mL of oil, in each of the 10 sites, and observe the animals for 24, 48 and 72 hours after the inoculation. Classify the observations from each site, according to the numeric scale indicated on **Table 2**. At any point of observation, the average response on the three rabbits (30 inoculation sites) must not be superior to 0.5 for erythema, must be inferior to 1.0 for edema, and in no site there can be a tissue reaction larger than 10 mm of total diameter. The oil residue in the inoculation site must not be interpreted as edema. When softly pressed, the tissue edema turns whitish.

Water for injection. Refer to the corresponding monograph.

Table 2 – Assessment of skin reactions.

<i>Erythema and formation of ulcers</i>	<i>Score</i>
No erythema	0
Mild erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet red) to mild formation of ulcer (deep wounds)	4

<i>Formation of edema</i>	<i>Score</i>
No edema	0
Very mild edema (barely perceptible)	1
Mild edema (edges with well-defined area by precise magnification)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and beyond the exposure area)	4

* Excludes non-inflammatory (mechanical) edema from the blank or extraction fluid.

Equipment

Autoclave. Use an autoclave capable of maintaining the temperature of $(121 \pm 2) ^\circ\text{C}$ and capable of cooling the assay containers down around $20 ^\circ\text{C}$.

Oven. Preferably use a model with mechanic convection, capable of maintaining the operating temperatures in the range of $50 ^\circ\text{C}$ to $70 ^\circ\text{C} \pm 2 ^\circ\text{C}$.

Extraction containers. Use only Type I glass containers, such as culture test tube with screw cap, or equivalent. The screw cap must have appropriate elastomeric coating. The exposed surface of this coating must be completely protected with an inert solid disc of 50-75 μm of thickness.

Preparation of equipment. Completely clean all glassware with chromic acid cleaning solution and, if necessary, with warm nitric acid, followed by prolonged rinsing with water. Before using in the subdivision of sample, clean cutting equipment items with an adequate method, such as successive cleanups with acetone and methylene chloride. Clean all other equipment items through complete washing with adequate detergent and prolonged rinsing with water. Sterilize and dry the containers and equipment used for extraction, transfer or administration of the assay material, through adequate process. If ethylene oxide is used as a sterilizing agent, allow an adequate time until complete degassing.

Procedure.

Sample preparation. The *Systemic injection test* and the *Intracutaneous test* can be conducted with the same extract or with different extracts. Select and subdivide the sample in parts with the size indicated on **Table 3**. Remove the particulate material from each subdivided sample, or negative control, putting the sample in a 100 mL type I glass graded cylinder, clean and with lid, and add approximately 70 mL of water for injection. Shake for about 30 seconds and drain the water, repeat this step and dry the parts prepared for extraction with oil in an oven up to 50 °C. Do not clean the sample with dry or damp cloth or wash and rinse with organic solvent, surfactant, etc.

Table 3 – Surface area of the sample to be used.

<i>Form of material</i>	<i>Thickness</i>	<i>Amount of sample for every 20 mL of extraction medium</i>	<i>Subdivided in 1</i>
Film or slide	< 0.5 mm	Equivalent to 120 cm ² of the total surface area (both sides combined)	Strips with approximately 5 × 0.3 cm
	0.5 to 1 mm	Equivalent to 60 cm ² of the total surface area (both sides combined)	
Tube	< 0.5 mm (wall)	Length (in cm) = 120 cm ² / (sum of circumferences of internal and external diameter)	Parts with approximately 5 × 0.3 cm
	0.5 to 1 mm (wall)	Length (in cm) = 60 cm ² / (sum of circumferences of internal and external diameter)	
Strips, tube and molded items	> 1 mm	Equivalent to 60 cm ² of the total surface area (all exposed surfaces combined)	Pieces with up to 5 × 0.3 cm
Elastomers	> 1 mm	Equivalent to 25 cm ² of the total surface area (all exposed surfaces combined)	With no subdivision ²

Preparation of extracts. Put a sample, duly prepared, to be tested in an extraction container and transfer 20 mL of the adequate medium. Repeat these instructions for each extraction medium necessary for the test. Also prepare a blank of 20 mL of each medium for parallel injections and comparisons. Extract by heating, in an autoclave at 121 °C, for 60 minutes, and in case of an oven at 70 °C, for 24 hours, or at 50 °C for 72 hours. Allow sufficient time for the liquid in the container to achieve the extraction temperature. In no moment must the extraction conditions cause physical changes, such as fusion or melting of sample parts, to not result in a reduction of the surface available. A slight adherence of the parts can be tolerated. Always transfer, individually, the clean parts to the extraction medium. If the culture tubes are used for extracting vegetable oil with autoclave, properly seal the thread caps with pressure-sensitive adhesive tape. Cool down to room temperature, but not inferior to 20 °C, shake, vigorously, for several minutes, and immediately decant each extract aseptically, in a sterile and dry container. Store the extracts at a temperature between 20 °C and 30 °C and do not use for tests after 24 hours.

SYSTEMIC INJECTION TEST

This test is developed to assess the systemic responses to extracts from materials tested through inoculation in mice.

Test animal. Use healthy albino mice, not used before, weighing between 17 and 23 g. For each test group, use only mice from the same origin. Water and foods with known composition, commonly used in laboratory animals, are freely permitted.

Procedure. Before taking the inoculation dose, shake vigorously each extract to ensure the even distribution of the matter extracted. Visible particles must not be administered intravenously. In a test group, inoculate in each of the five mice the sample or blank, as described on **Table 4**, diluting each g of the extract of sample prepared with polyethylene glycol 400 and the corresponding blank, with 4.1 volumes of injectable sodium chloride solution, to obtain a solution with concentration of approximately 200 mg of Macrogol per mL.

Table 4 – Procedure for inoculation – Systemic Injection Test.

<i>Extract or blank</i>	<i>Dose per kg</i>	<i>Route of administration*</i>	<i>Speed of inoculation, μL per second</i>
Alcohol solution 1:20 in injectable sodium chloride solution	50 mL	IV	100
Polyethylene glycol 400	10 g	IP	-
Medicine vehicle (when applicable)	50 mL	IV	100
	50 mL	IP	-
Vegetable oil	50 mL	IP	-

* IV = intravenous (aqueous sample and blank); IP = intraperitoneal (oily sample and blank).

Observe the animals on the following times: immediately after inoculation, after four hours, and, at minimum, after 24, 48 and 72 hours. If during the observation period none of the animals treated with the extract of sample presents biological reactivity significantly higher than the ones treated with blank, the sample complies with the requisites from this test. If two or more mice die or present abnormal behavior, such as convulsions or prostration, or if there is loss of body weight superior to 2 g in three or more mice, the sample does not comply with the requisites from the test. If an animal treated with the sample displays only slight signs of biological reactivity, and if only one animal presents severe symptoms of biological reactivity or dies, repeat the test using groups of 10 mice. In the repetition test, during the observation period, none of 10 animals treated with the sample must present any significant biological reactivity more than the ones treated with blank.

INTRACUTANEOUS TEST

This test was developed to assess local responses to the extracts from materials tested, after intracutaneous inoculation in rabbits.

Test animal. Select healthy albino rabbits, which hair can be tied close to the skin, which must be thin and free from irritation or trauma. When handling animals during the observation periods, avoid touching the inoculation sites, except for differentiating an edema and a residue of oil. Rabbits previously used in independent tests, such as the pyrogen test (5.5.2.1), and which have rested during the period expected can be used for this test, provided they have a clear, blemish-free skin.

Procedure. Before taking the inoculation dose, shake vigorously each extract, to ensure the even distribution of the matter extracted. On the test day, carefully tie the hair on the animal's back, on both sides of the spine, over a sufficiently large test area. Avoid irritation and trauma. Remove loose hair by vacuum. If necessary, before the inoculation, slightly clean the skin with diluted alcohol and let it dry. More than one extract from a certain material can be used per rabbit, if it is determined that the results will not be affected. For every sample, use two animals and inoculate intracutaneously,

using one side of the animal for the sample and the other for blank, as described on **Table 5**. Dilute every g of the extract of sample prepared with polyethylene glycol 400 and the corresponding blank with 7.4 volumes of injectable sodium chloride solution to obtain a solution with concentration of approximately 120 mg of polyethylene glycol per mL.

Table 5 – Intracutaneous test.

<i>Extract or blank</i>	<i>Number of sites (per animal)</i>	<i>Dose, µL per site</i>
Sample	5	200
Blank	5	200

Examine the inoculation sites to make evident any tissue reaction, such as erythema, edema and necrosis. If necessary, slightly clean the skin with diluted alcohol to facilitate the reading of inoculation sites. Observe all animals 24, 48 and 72 hours after the inoculation. Classify the observations in a numeric scale for the extract sample and for the blank, using **Table 2**. If necessary, tie the hair again during the observation period. The average score of erythema and edema for the sample and blank sites is determined for each rabbit and each score interval after 24, 48 and 72 hours of inoculation. After the score related to 72 hours, all scores from erythema, plus the ones from edema, are totaled, separately, for each sample and blank. Divide each total by 12 (2 animals × 3 score periods × 2 score categories) to determine the total average for each sample versus each corresponding blank. The test requisites are complied with if the difference between the average score of the sample and the blank is inferior or equal to 1.0. If in any observation period the average for the sample reaction is questionable for being higher than the average for the blank reaction, repeat the test using three additional rabbits. The test requisites are complied with if the difference between the average score of the sample and the blank is equal or inferior to 1.0.

IMPLANT TEST

The implant test is developed to assess plastic materials and other polymers when they come into direct contact with live tissue. The adequate preparation of the implant strips and their implantation must be conducted under asepsis conditions. Prepare for implanting 8 strips of sample and 4 strips of standard. Each strip must measure no less than 10 × 1 mm. The strip edges must be as soft as possible, to avoid additional mechanical traumas in the implantation. The strips of minimum size specified are implanted through a hypodermic needle (15 to 19 gauge) with intravenous tip and sterile trocar. Use one or another pre-sterilized needle where the sterile plastic strips are inserted aseptically, or insert each clean strip in a needle which cannula and central orifice are protected with an adequate cap, and then submitted to the appropriate sterilization procedure.

Test animal. Select healthy adult rabbits with minimum weight of 2.5 kg, with sufficiently large paravertebral muscles to allow the implantation of test strips. Do not use any muscle tissue in addition to the one located in the paravertebral area. The animals must be anesthetized with an anesthetic agent that is commonly used for a sufficient level of depth to prevent muscle movements, such as spasms.

Procedure. Conduct the test in a clean area. On the test day or up to 20 hours previously, tie the animals' hair on both sides of the spine. Remove loose hair with vacuum. Before the inoculation, slightly clean the skin with diluted alcohol and dry it. Implant four strips of sample in paravertebral muscles, spaced approximately 2.5 cm apart, in one side of the spine of each of the two rabbits, 2.5 cm to 5.0 cm away from the medial line and parallel to the spine. Likewise, implant two strips of standard on the opposite muscle of each animal. Insert a sterile catheter in the needle to hold the implant strip on the tissue with the needle removal. After implanting one strip, if there is excessive bleeding, put another piece in duplicate on another site. Keep the animals for a minimum period of 120 hours and euthanize them at the end of the observation period with an overdose of anesthetic agent or other

adequate agents. Allow sufficient time to elapse to cut the tissue without bleeding. Examine macroscopically the tissue area around the central part of every implant strip. Use magnifying glasses and an additional source of light. Observe if there are hemorrhages, necroses, discolorations and infections in the site of implantation of sample and control and register the observations. If there is encapsulation, measure and register the capsule width, rounding to the closest 0.1 mm, from the periphery of the space occupied by the control implant or sample to the periphery of the capsule. Score the encapsulation, according to **Table 6**. Calculate the differences between the average score for the sample and control sites. The test requisites are complied with if the difference is not superior to 1, or if the difference for more than one of the four implant sites does not exceed 1 in any of the animals.

Table 6 – Assessment of encapsulation in the Implant test.

<i>Width of capsule</i>	<i>Score</i>
None	0
up to 0.5 mm	1
0.6-1.0 mm	2
1.1-2.0 mm	3
Superior to 2.0 mm	4

7 REAGENTS

7.1 INDICATORS AND TEST SOLUTIONS

Indicators are colorants used to indicate the endpoint of a volumetric analysis or to assess the pH of non-colored solutions. The indicators more frequently used are listed on **Table 1**, in increasing order from the lower limit of their pH transition range. Then, the indicators and test solutions (TS) used in different pharmacopoeia procedures are described.

Table 1 – Most frequently used indicators.

<i>Indicator</i>	<i>Transition range</i>	<i>Change of color</i>
Malachite green	0.0 to 2.0	Yellow to green
Cresol red	0.2 to 1.8	Red to yellow
M-cresol purple	0.5 to 2.5	Red to yellow
Tropaeolin OO	1.0 to 2.8	Red to yellow
Thymol blue	1.2 to 2.8	Red to yellow
Naphthol yellow	2.0 to 3.2	Colorless to yellow
Dimethyl yellow	2.8 to 4.6	Red to yellow
Bromophenol blue	2.8 to 4.6	Yellow to violet-blue
Methyl orange	2.9 to 4.0	Red to yellow
Congo Red	3.0 to 5.0	Blue to red
Bromocresol green	3.6 to 5.2	Yellow to blue
Methyl red	4.2 to 6.3	Red to yellow
Resazurin	5.0 to 7.0	Pink to violet
Litmus	5.0 to 8.0	Red to blue
Bromocresol purple	5.2 to 6.8	Yellow to violet-blue
Bromothymol blue	6.0 to 7.6	Yellow to blue
Phenol red	6.8 to 8.4	Yellow to red
Cresol red	7.2 to 8.8	Yellow to red
M-cresol purple	7.5 to 9.2	Yellow to violet
Thymol blue	8.0 to 9.6	Yellow to blue
Phenolphthalein	8.3 to 10.0	Colorless to intense violet
Nile Blue A	9.0 to 13.0	Blue to red
Thymolphthalein	9.3 to 10.5	Colorless to blue
Alizarin yellow GG	10.0 to 12.0	Pale yellow to brown
Tropaeolin O	11.0 to 12.7	Yellow to orange
Titan yellow	12.0 to 13.0	Yellow to red

Methyl orange (CI 13025)

CAS – [547-58-0].

Molecular formula and molar mass – C₁₄H₁₄N₃NaO₃S – 327.34.

Description – Orange-yellow crystalline powder.

Solubility – Slightly soluble in water and practically insoluble in ethyl alcohol.

Methyl orange TS

Preparation – Dissolve 0.1 g in 100 mL of 20% (v/v) ethyl alcohol.

pH range – 2.9 – 4.0.

Change of color – Provides red color in moderately acidic medium and yellow color in weakly acidic and alkaline medium.

Sensitivity test – The mixture of 0.1 mL of test solution with 100 mL of carbon dioxide-free water presents a yellow color. No more than 0.1 mL of hydrochloric acid 0.1 M is necessary to determine the change of color to red.

Methyl orange, solution

Preparation – Dissolve 20 mg of methyl orange and 0.1 g of bromocresol green in 1 mL of 0.2 M sodium hydroxide and complete the volume to 100 mL using water.

pH range – 3.0 – 4.0.

Change of color – Provides orange color in moderately acidic solutions and olive green color in weakly acidic and alkaline solutions.

Xylenol orange

CAS – [3618-43-7].

Molecular formula and molar mass – $C_{31}H_{28}N_2Na_4O_{13}S$ – 760.59.

Description – Red-brown crystalline powder.

Solubility – Soluble in water and ethyl alcohol.

Xylenol orange TS

Preparation – Dissolve 0.1 g of xylenol orange in 100 mL of ethyl alcohol.

Change of color – Presents pale yellow color in acidic medium. Reacting with certain metals (such as lead and zinc), forms a complex of intense red color. In presence of excess disodium edetate, acquires a yellow color.

Alizarin

CAS – [130-22-3].

Molecular formula and molar mass – $C_{14}H_7NaO_7S \cdot H_2O$ – 360.27.

Description – Orange-yellow powder.

Solubility – Freely soluble in water and in ethyl alcohol.

Alizarin TS

Preparation – Dissolve 0.1 g of alizarin in 100 mL of water.

Alizarin yellow GG (CI 14025)

CAS – [584-42-9].

Molecular formula and molar mass – $C_{13}H_8N_3NaO_5$ – 309.21.

Description – Yellow powder.

Solubility – Slightly soluble in cold water and soluble in hot water.

Alizarin yellow GG TS

Preparation – Dissolve 0.1 g of alizarin yellow GG in 100 mL of water.

pH range – 10.0 – 12.0.

Change of color – Provides pale yellow color in weakly alkaline solutions and brown color in strongly alkaline solutions.

Dimethyl yellow (CI 11020)

CAS – [60-11-7].

Molecular formula and molar mass – $C_{14}H_{15}N_3$ – 225.30.

Description – Yellow crystals.

Solubility – Insoluble in water, soluble in ethyl alcohol, benzene, chloroform, ethyl ether, and diluted mineral acids.

Dimethyl yellow TS

Preparation – Dissolve 0.2 g of dimethyl yellow in 100 mL of 90% (v/v) ethyl alcohol.

pH range – 2.8 – 4.6.

Change of color – Provides red color in moderately acidic solutions and yellow color in weakly acidic and alkaline solutions.

Homogeneity test – Prepare 0.01% (w/v) dimethyl yellow solution in methylene chloride and apply 0.01 mL of this solution on silica-gel G TLC plate. Use methylene chloride as eluent. The chromatogram must present a single stain.

Sensitivity test – Prepare a solution with 2 g of ammonium chloride in 25 mL of carbon dioxide-free water. This solution, added with 0.1 mL of dimethyl yellow TS, must present a yellow color. The color turns red by adding no more than 0.1 mL of hydrochloric acid 0.1 M.

Metanil yellow (CI 13065)

CAS – [587-98-4].

Molecular formula and molar mass – $C_{18}H_{14}N_3NaO_3S$ – 375.38.

Description – Brownish-yellow powder.

Solubility – Soluble in water and in ethyl alcohol.

Metanil yellow TS

Preparation – Dissolve 0.1 g of metanil yellow in 100 mL of methyl alcohol.

Change of color – In titrations developed in non-aqueous medium, it changes the color from yellow (basic medium) to crimson (acidic medium).

Sensitivity test – Dissolve 0.1 mL of metanil yellow TS in 50 mL of glacial acetic acid. This solution must present a pink-red color. Add 0,05 mL of perchloric acid 0.1 M. The color must change to violet.

Naphthol yellow (CI 10315)

CAS – [887-79-6].

Molecular formula and molar mass – $C_{10}H_5N_2NaO_5$ – 256.15.

Description – Orange-yellow powder or crystals.

Solubility – Freely soluble in water and slightly soluble in ethyl alcohol.

pH range – 2.0 – 3.2.

Change of color – Provides colorless solution in strongly acidic medium and yellow color in less acidic solutions.

Titan yellow (CI 19540)

CAS – [1829-00-1].

Molecular formula and molar mass – $C_{28}H_{19}N_5Na_2O_6S_4$ – 695.71.

Description – Yellow-brown powder.

Solubility – Freely soluble in water and in ethyl alcohol.

Titan yellow TS

Preparation – Dissolve 0.05 g of titan yellow in water and complete the volume to 100 mL using the same solvent.

pH range – 12.0 – 13.0.

Change of color – In acidic and moderately alkaline solutions, it provides yellow color. In strongly alkaline solutions, it presents red color.

Sensitivity test – Prepare a mixture with 10 mL of water, 0.2 mL of standard magnesium solution (10 ppm Mg) and 10 mL of sodium hydroxide M. Add 0.1 mL of titan yellow TS. Prepare the blank test

in a similar manner, but omitting the magnesium standard. Compare the two solutions. An intense rose color develops in comparison with the blank test.

Titan yellow, paper

Preparation – Impregnate a common filter paper with titan yellow solution TS. Air-dry it at room temperature.

Starch (Soluble starch)

CAS – [9005-84-9].

Molecular formula – $(C_6H_{10}O_5)_x$.

Description – White or nearly white powder.

Starch TS

Specification – 2% (w/v) soluble starch solution in hot water. The solution may present slight opalescence.

Sensitivity test – Mix 1 mL of starch TS, 20 mL of water, approximately 50 mg of potassium iodide and 0.05 mL of iodine 0.01 M. A blue color develops.

Iodized starch TS

Preparation – Weigh 0.5 g of starch, add 50 mL to 60 mL of water and dissolve by heating. Dissolve 0.5 g of potassium iodide in the solution and complete the volume to 100 mL with water. Protect from light. Use within 24 hours after the preparation.

Iodide-free starch TS

Preparation – Crush 1 g of soluble starch with 5 mL of water and add, with constant agitation, water in sufficient ebullition to complete 100 mL.

Stability – Prepare immediately before use.

Iodized starch, paper

Preparation – Impregnate filter paper with freshly prepared starch TS, added with 0.5 g of potassium iodide.

Bromophenol blue

CAS – [115-39-9].

Molecular formula and molar mass – $C_{19}H_{10}Br_4O_5S$ – 669.96.

Description – Light orange-yellow powder.

Solubility – Very slightly soluble in water, slightly soluble in ethyl alcohol, and freely soluble in alkaline hydroxide solutions.

Bromophenol blue TS

Preparation – Dissolve, heating slowly, 0.2 g of bromophenol blue in 3 mL of 0.1 M sodium hydroxide and 10 mL of ethyl alcohol. Let it cool down and complete the volume to 100 mL with ethyl alcohol.

pH range – 2.8 – 4.6.

Change of color – Provides yellow color in moderately acidic solutions and violet-blue color in weakly acidic and alkaline solutions.

Bromothymol blue

CAS – [76-59-5].

Molecular formula and molar mass – $C_{27}H_{28}Br_2O_5S$ – 624.38.

Description – Brown or light red powder.

Solubility – Practically insoluble in water, soluble in ethyl alcohol and diluted alkali hydroxide solutions.

Bromothymol blue TS

Preparation – Heat 1 g of bromothymol blue with 3.2 mL of 0.05 M sodium hydroxide and 5 mL of ethyl alcohol. After dissolution, complete the volume to 250 mL with ethyl alcohol.

pH range – 6.0 – 7.0.

Change of color – Provides yellow color in weakly acidic solutions and blue color in weakly alkaline solutions. In neutral medium, provides green color.

Sensitivity test – The mixture of 0.3 mL of bromothymol blue TS with 100 mL of carbon dioxide-free water presents a yellow color. The color changes to blue with the addition of no more than 0.1 mL of sodium hydroxide solution 0.02 M.

Hydroxynaphthol blue

CAS – [63451-35-4].

Molecular formula and molar mass – $C_{20}H_{11}N_2Na_3O_{11}S_3$ – 620.46.

Hydroxynaphthol blue TS

Preparation – Dissolve 0.1 g in ethyl alcohol and complete the volume to 100 mL with the same solvent.

Change of color – On the pH range between 12.0 and 13.0, its solution has a red-pink color in presence of calcium ions. With excess disodium edetate, it presents intense blue color.

Oracet blue B

CAS – [12769-16-3].

Molecular formula and molar mass – $C_{21}H_{16}N_2O_2$ – 328.37.

Specification – It is a mixture of 1-methylamino-4-anilino-anthraquinone with 1-amino-4-anilino-anthraquinone.

Oracet blue B TS

Preparation – Dissolve 0.5 g of oracet blue B in glacial acetic acid and complete the volume to 100 mL using the same solvent.

Change of color – When used in titrations in non-aqueous medium, it changes from blue color (basic medium) to purple (neutral medium) and to pink (acidic medium).

Thymol blue

CAS – [76-61-9].

Molecular formula and molar mass – $C_{27}H_{30}O_5S$ – 466.60.

Description – Brownish-green or greenish-blue crystalline powder.

Solubility – Slightly soluble in water, soluble in ethyl alcohol and in diluted alkali hydroxide solutions.

Thymol blue TS

Preparation – Heat 0,1 g of thymol blue with 4.3 mL of 0.05% (w/v) sodium hydroxide and 5 mL of 90% (v/v) ethyl alcohol. After dissolution, complete the volume to 250 mL with 20% (v/v) ethyl alcohol.

pH range – 1.2 – 2.8 and 8.0 – 9.6.

Change of color – Presents red color in strongly acidic solutions (pH range: 1.2 – 2.8), yellow color in weakly acidic and alkaline solutions, and blue color in more alkaline solutions (pH range: 8.0 – 9.6).

Sensitivity test – The mixture of 0.1 mL of thymol blue TS, 100 mL of carbon dioxide-free water and 0.2 mL of 0.02 M sodium hydroxide presents a blue color. The color changes to yellow by adding no more than 0.1 mL of hydrochloric acid 0.2 M.

Trypan blue

CAS – [72-57-1]

Formula and molecular mass – $C_{34}H_{24}N_6Na_4O_{14}S_4$ – 960.81

Nile blue A (CI 51180)

CAS – [3625-57-8].

Molecular formula and molar mass – $C_{40}H_{40}N_6O_6S$ – 732.86

Description – Green crystalline powder with bronze luster.

Solubility – Moderately soluble in ethyl alcohol, in glacial acetic acid and in pyridine.

Nile blue A TS

Preparation – Dissolve 1 g in glacial acetic acid and complete the volume to 100 mL using the same solvent.

pH range – 9.0 – 13.0.

Change of color – Provides blue color to strongly alkaline solutions and red color to weakly alkaline solutions.

Sensitivity test – The mixture of 0.25 mL of Nile blue A TS in 50 mL of glacial acetic acid presents blue color. The color turns greenish-blue by adding no more than 0.1 mL of perchloric acid 0.1 M to glacial acetic acid.

Identification test – The solution at 0.0005% (w/v) in 50% (v/v) ethyl alcohol presents maximum absorption (5.2.14) in 640 nm.

Calcon

CAS – [2538-85-4].

Molecular formula and molar mass – $C_{20}H_{13}N_2NaO_5S$ – 416.38.

Description – Dark black powder with violet nuances.

Solubility – Very soluble in water and freely soluble in ethyl alcohol and acetone.

Calcon TS

Preparation – Dissolve 0.1 g of calcon in 100 mL of methyl alcohol.

Change of color – Provides purple-red color with calcium ions in alkaline medium. In presence of excess disodium edetate, the solution acquires a blue color.

Calcon, compound mixture

Preparation – Mix one part of calcon with 99 parts of sodium sulfate.

Sensitivity test – Dissolve 0.2 g of the compound calcon mixture in 5 mL of water. Mix 1 mL of colorant solution, 50 mL of water, 10 mL of sodium hydroxide M and 1 mL of 1% (w/v) magnesium sulfate. The solution formed is blue, turning violet with the addition of 0.1 mL of 0.15% (w/v) calcium chloride. The addition of 0.1 mL of disodium edetate 0.01 M provides an intense blue color.

Methylrosanilinium chloride (CI 42555)

CAS – [548-62-9].

Synonym – Crystal violet.

Molecular formula and molar mass – $C_{25}H_{30}ClN_3$ – 407.99.

Description – Dark green powder or crystals.

Solubility – Soluble in water and in ethyl alcohol.

Methylrosanilinium chloride TS

Preparation – Dissolve 0.5 g of methylrosanilinium chloride in 100 mL of glacial acetic acid. *Change of color* – In titrations in non-aqueous medium, the color changes from violet (less acidic medium) to greenish-blue to yellow-blue (more acidic medium).

Sensitivity test – The mixture of 0.1 mL of methylrosanilinium chloride TS with 50 mL of glacial acetic acid shows a blueish-purple color. The addition of 0.1 mL perchloric acid 0.1 M in acetic acid changes the color to green.

Ferric chloride

CAS – [10025-77-1].

Synonym – Iron chloride.

Molecular formula and molar mass – $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ – 270.30.

Description – Orange-yellow crystallized, deliquescent mass.

Solubility – Very soluble in water and soluble in ethyl alcohol and ethyl ether. Salt and its solutions, exposed to light, suffer partial reduction.

Ferric chloride TS (approximately 0.4 M)

Specification – Dissolve 10.5 g of ferric chloride in water and complete the volume to 100 mL using the same solvent.

Conservation – In tightly closed containers.

Storage – Protect from light.

BRP colorant

Preparation – Dissolve 0.1 g of bromothymol blue, 0.02 g of methyl red, and 0.2 g of phenolphthalein in ethyl alcohol. Complete the volume to 100 mL with the same solvent. Filter.

Diphenylcarbazide

CAS – [140-22-7].

Molecular formula and molar mass – $\text{C}_{13}\text{H}_{14}\text{N}_4\text{O}$ – 242.28.

Description – White or nearly white crystalline powder, gradually turns pink with exposure to air.

Solubility – Very slightly soluble in water, soluble in acetone, in ethyl alcohol and in glacial acetic acid.

Diphenylcarbazide TS

Preparation – Dissolve 1 g of diphenylcarbazide in 100 mL of hot ethyl alcohol. Store sheltered from light.

Diphenylcarbazone

CAS – [538-62-5].

Molecular formula and molar mass – $\text{C}_{13}\text{H}_{12}\text{N}_4\text{O}$ – 240.27.

Description – Crystals or yellow-orange crystalline powder.

Solubility – Practically insoluble in water and freely soluble in ethyl alcohol.

Diphenylcarbazone TS

Preparation – Dissolve 0.1 g in 100 mL of ethyl alcohol. Store sheltered from light.

Eosin Y (CI 45380)

CAS – [17372-87-1].

Molecular formula and molar mass – $\text{C}_{20}\text{H}_6\text{Br}_4\text{Na}_2\text{O}_5$ – 691.86.

Description – Brown powder.

Solubility – Soluble in water.

Eosin Y TS

Preparation – Dissolve 1 g of eosin Y in 100 mL of water.

Change of color – The addition of 20 mL of 40% (w/v) sodium hydroxide over 10 mL of eosin Y TS forms a red precipitate.

Tetrabromophenolphthalein, ethyl ester

CAS – [1176-74-5].

Synonyms – Bromophenolphthalein magenta E., Ethyl ester from 2-[(3,5-dibromo-4-hydroxyphenyl)(3,5-dibromo-4-oxo-2,5-cyclohexadien-1-ylidene)methyl]-benzoic acid

Molecular formula and molar mass – $C_{22}H_{14}Br_4O_4$ – 661.97.

Tetrabromophenolphthalein, ethyl ester TS

Preparation – Dissolve 0.1 of tetrabromophenolphthalein ethyl ester in 90 mL of glacial acetic acid and complete the volume to 100 mL with the same solvent. Prepare immediately before use.

Phenolphthalein

CAS – [77-09-8].

Molecular formula and molar mass – $C_{20}H_{14}O_4$ – 318.33.

Description – Crystalline or amorphous powder, white or slightly yellow. Odorless.

Solubility – Insoluble in water and soluble in ethyl alcohol.

Phenolphthalein TS

Preparation – Dissolve 0.1 g in 100 mL of 80% (v/v) ethyl alcohol.

pH range – 8.3 – 10.0.

Change of color – Provides colorless solutions in acidic and weakly alkaline medium. Presents intense violet color in stronger alkaline solutions.

Sensitivity test – The mixture of 0.1 mL of phenolphthalein TS with 1000 mL of carbon dioxide-free water is colorless. No more than 0.2 mL of 0.02 M sodium hydroxide is necessary for the pink color to appear.

Phenolphthalein, paper

Preparation – Immerse common filter paper strips in phenolphthalein TS for a few minutes and air-dry at room temperature.

Ferroin

CAS – [14634-91-4].

Molecular formula and molar mass – $C_{36}H_{24}FeN_6O_4S$ – 692.53

Ferroin TS

Preparation – Dissolve 0.7 g of ferrous sulfate heptahydrate and 1.49 g of 1,10-phenanthroline in 70 mL of water and complete the volume to 100 mL with the same solvent.

Sensitivity test – Add to 50 mL of M sulfuric acid 0.15 mL of osmium tetroxide RS and 0.1 mL of ferroin TS. After adding 0.1 mL of ceric ammonium sulfate 0.1 M VS, the color changes from orange-red to pale green.

Conservation – In tightly closed containers.

Magneson

CAS – [74-39-5].

Molecular formula and molar mass – $C_{12}H_9N_3O_4$ – 259.22

Description – Red-brown powder.

Magneson TS

Preparation – Dissolve 0,2 g of magneson in 100 mL of toluene.

Change of color – In titrations in non-aqueous medium, it changes from orange (acidic medium) to blue (basic medium), going through the pink color.

Magneson, reagent

Preparation – Dissolve 0.1 g of magneson in 100 mL of 1% (w/v) sodium hydroxide.

1-Naphtholbenzein

CAS – [6948-88-5].

Synonym – Phenylbis(4-hydroxynaphthyl)methanol.

Molecular formula and molar mass – $C_{27}H_{20}O_3$ – 392.45.

Description – Red-brown powder.

Solubility – Insoluble in water, soluble in benzene, in ethyl ether and in glacial acetic acid.

1-Naphtholbenzein TS

Preparation – Dissolve 0.2 g of 1-naphtholbenzein in 100 mL of glacial acetic acid.

Change of color – When used in titrations in non-aqueous medium, it changes from blue or blueish-green color (basic medium) to orange (neutral medium) and to dark green (acidic medium).

Sensitivity test – Add 0.25 mL of solution of 1-naphtholbenzein TS to 50 mL of glacial acetic acid. No more than 0.05 mL of perchloric acid 0.1 M in glacial acetic acid is necessary to change the color from brown-yellow to green.

1-Naphtholphthalein

CAS – [596-01-0].

Molecular formula and molar mass – $C_{28}H_{18}O_4$ – 418.45.

Description – Colorless powder when pure, usually is greyish-red.

Solubility – Practically insoluble in water and soluble in ethyl alcohol.

1-Naphtholphthalein TS

Preparation – Dissolve 0.5 g in 100 mL of ethyl alcohol.

Change of color – Provides colorless or pale red solution in acidic and neutral media and blue color in moderately alkaline solutions.

Eriochrome black T (CI 14645)

CAS – [1787-61-7].

Molecular formula and molar mass – $C_{20}H_{12}N_3NaO_7S$ – 461.38.

Description – Dark brown powder.

Solubility – Soluble in water and in ethyl alcohol.

Eriochrome black T TS

Preparation – Dissolve 0.5 g of eriochrome black T and 4.5 g of hydroxylamine hydrochloride in methyl alcohol and complete the volume to 100 mL using the same solvent. Prepare immediately before use.

Change of color – In a medium comprised of hydrochloric acid it produces a brown-violet precipitate; in medium comprised of sulfuric acid, it forms a dark blue precipitate that, when diluted, turns brown. In aqueous sodium hydroxide solution, it presents a violet color.

Ammonium oxalate

CAS – [6009-70-7].

Molecular formula and molar mass – $C_2H_8N_2O_4 \cdot H_2O$ – 142.11.

Description – Colorless clear crystals or white crystalline powder. Odorless.

Solubility – Soluble in water.

Ammonium oxalate TS

Specification – Contains 4% (w/v) of ammonium oxalate in water.

Bromocresol purple

CAS – [115-40-2].

Molecular formula and molar mass – $\text{C}_{21}\text{H}_{16}\text{Br}_2\text{O}_5\text{S}$ – 540.22.

Description – Pink crystalline powder.

Solubility – Practically insoluble in water, soluble in ethyl alcohol and diluted alkali hydroxide solutions.

Bromocresol purple TS

Preparation – Heat 0.1 g of bromocresol purple with 5 mL of 90% (v/v) ethyl alcohol until dissolution. Add 3.7 mL of 0.05 M sodium hydroxide and complete the volume to 250 mL with 20% (v/v) ethyl alcohol.

pH range – 5.2 – 6.8.

Change of color – Provides yellow color in weakly acidic solutions and violet-blue color in alkaline, neutral and acidic solutions that are very close to neutrality.

Sensitivity test – Mix 0.2 mL of bromocresol purple TS with 100 mL of carbon dioxide-free water. Add 0.05 mL of 0.02 M sodium hydroxide. This solution has a violet-blue color. To change the color to yellow, no more than 0.2 mL of hydrochloric acid 0.02 M is necessary.

Bromocresol purple, reagent

Solution A – Dissolve 38 g of monobasic sodium phosphate and 2 g of sodium phosphate dibasic in water and complete the volume to 1000 mL with the same solvent. Adjust the pH to 5.3.

Solution B – Dissolve 0.4 g of bromocresol purple in 30 mL of water, add 6.3 mL of 0.1 M sodium hydroxide and complete the volume to 500 mL with water.

Preparation – Mix equal volumes of *Solution A*, *Solution B* and chloroform.

Shake for five minutes, let it decant and discard the chloroform layer.

M-cresol purple

CAS – [2303-01-7].

Molecular formula and molar mass – $\text{C}_{21}\text{H}_{16}\text{O}_5\text{S}$ – 380.41.

Description – Olive green crystalline powder.

Solubility – Slightly soluble in water, soluble in ethyl alcohol, glacial acetic acid and methyl alcohol.

M-cresol purple TS

Preparation – Dissolve 0.1 g of m-cresol purple in 100 mL of 0.001 M sodium hydroxide.

pH range – 0.5 – 2.5 and 7.5 – 9.2.

Change of color – Presents red color in strongly acidic solutions (pH range between 0.5 and 2.5); yellow color in less acidic and neutral solutions; and violet color in moderately alkaline solutions (pH range between 7.5 and 9.2).

Resazurin

CAS – [550-82-3].

Molecular formula and molar mass – $\text{C}_{12}\text{H}_7\text{NO}_4$ – 229.19.

Description – Small dark red crystals with greenish luster.

Solubility – Insoluble in water and ethyl ether, slightly soluble in ethyl alcohol and soluble in diluted alkali hydroxide solutions.

Resazurin TS

Preparation – Dissolve 0.1 g of resazurin in 100 mL of 0.02 M sodium hydroxide.

Prepare immediately before use.

pH range – 5.0 – 7.0.

Change of color – Provides pink color in weakly acidic solutions and violet color in weakly alkaline solutions.

Resorcinol

CAS – [108-46-3].

Synonym – Resorcine.

Molecular formula and molar mass – $C_6H_6O_2$ – 110.11.

Description – Colorless or pale yellow crystalline powder or crystals; when exposed to light and air, acquires a pink color.

Solubility – Soluble in water and ethyl alcohol.

Resorcinol TS

Preparation – Dissolve 0.2 g of resorcinol in 100 mL of benzene. Let it decant.

Thymolphthalein

CAS – [125-20-2].

Molecular formula and molar mass – $C_{28}H_{30}O_4$ – 430.54.

Description – White or light yellow powder.

Solubility – Practically insoluble in water, soluble in ethyl alcohol and in alkali hydroxide solutions.

Thymolphthalein TS

Preparation – Dissolve 0.1 g of thymolphthalein in 100 mL of ethyl alcohol.

pH range – 9.3 – 10.5.

Change of color – It is colorless in acidic and weakly alkaline medium.

It provides a blue color in more intense alkaline solutions.

Sensitivity test – The mixture of 0.05 mL of thymolphthalein TS with 100 mL of carbon dioxide-free water is colorless. No more than 0.05 mL of 0.1 M sodium hydroxide is necessary to change the color to blue.

Ammonium thiocyanate

CAS – [1762-95-4].

Molecular formula and molar mass – NH_4SCN – 76.12.

Description – Colorless and deliquescent crystals.

Solubility – Very soluble in water and soluble in ethyl alcohol.

Ammonium thiocyanate TS

Preparation – Dissolve 7.6 g of ammonium thiocyanate in 100 mL of water.

Litmus

CAS – [1393-92-6].

Specification – It is comprised of indigo blue pigment prepared from several species of *Rocella*, *Lecanosa* or other lichens. The pigment has a characteristic odor.

Litmus TS

Preparation – Boil under reflux, for one hour, 25 g of litmus, finely powdered, with 100 mL of 90% (v/v) ethyl alcohol. Discard the ethyl alcohol and repeat the operation twice, using in each extraction 75 mL of 90% (v/v) ethyl alcohol.

Add 250 mL of water to the litmus extracted. Filter.

pH range – 5.0 – 8.0.

Change of color – Provides red color in acidic medium and blue in alkaline medium.

Blue litmus, paper

Preparation – Boil 10 parts of litmus, finely powdered, with 100 parts of ethyl alcohol, under reflux, for one hour. Decant and discard the ethyl alcohol. Add a mixture of 45 parts of ethyl alcohol with 15 parts of water to the residue. Let it macerate for two days. Decant the supernatant and impregnate common filter paper strips with the extract. Dry at room temperature.

Sensitivity test – Immerse a strip of blue litmus paper strip, measuring 10 mm x 60 mm, in 100 mL of mixture with 10 mL of hydrochloric acid 0.02 M and 90 mL of water. Shake. The paper acquires a red color after 45 seconds.

Red litmus, paper

Preparation – Add hydrochloric acid 2 M to the extract obtained in the process of preparing blue paper, drop by drop, until the solution presents a red color. Impregnate filter paper strips with this solution and let them dry at room temperature.

Sensitivity test – Immerse red litmus paper strip in 100 mL of 0.002 M sodium hydroxide. Shake. The paper acquires a blue color after 45 seconds.

Tropaeolin O (CI 14270)

CAS – [547-57-9].

Molecular formula and molar mass – $C_{12}H_9N_2NaO_5S$ – 316.27.

Description – Brown powder.

Solubility – Soluble in water and ethyl alcohol.

Tropaeolin O TS

Preparation – Dissolve 25 mg of tropaeolin in 50 mL of methyl alcohol and complete the volume to 100 mL with water.

pH range – 11.0 – 12.7.

Change of color – Provides yellow color solutions in moderately alkaline medium and orange color in strongly alkaline solutions.

Homogeneity test – Apply 10 μ L of tropaeolin O TS in a cellulose G TLC plate. Develop the chromatogram with the mixture of *n*-propyl alcohol, ethyl acetate and water (5:1:4).

The chromatogram must show a single stain with R_f of, approximately, 0.9.

Tropaeolin OO (CI 13080)

CAS – [554-73-4].

Molecular formula and molar mass – $C_{18}H_{14}N_3NaO_3S$ – 375.38.

Description – Yellow or orange-yellow powder.

Solubility – Soluble in water.

pH range – 1.0 – 2.8.

Change of color – Provides red color in strongly acidic solutions and yellow color in less acidic solutions.

Bromocresol green

CAS – [76-60-8].

Molecular formula and molar mass – $C_{21}H_{14}Br_4O_5S$ – 698.02.

Description – Brownish-white powder.

Solubility – Slightly soluble in water, soluble in ethyl alcohol and diluted alkali hydroxide solutions.

Bromocresol green TS

Preparation – Heat 0.1 g of bromocresol green with 2.9 mL of 0.05 M sodium hydroxide and 5 mL of 90% (v/v) ethyl alcohol. After dissolution, complete the volume to 250 mL with 20% (v/v) ethyl alcohol.

pH range – 3.6 – 5.2.

Change of color – Provides yellow color in moderately acidic solutions and blue color in weakly acidic and alkaline solutions.

Sensitivity test – The mixture of 0.2 mL of bromocresol green TS with 100 mL of carbon dioxide-free water presents a blue color. No more than 0.2 mL of hydrochloric acid 0.02 M is necessary to change the color to yellow.

Malachite green, oxalate

CAS – [2437-29-8].

Molecular formula and molar mass – $C_{48}H_{52}N_4O_4 \cdot 2HC_2O_4$ – 927.02.

Description – Green crystalline solid.

Solubility – Very soluble in water.

Malachite green TS

Preparation – Dissolve 1 g of malachite green oxalate in 100 mL of glacial acetic acid.

pH range – 0.0-2.0.

Change of color – Provides yellow color in acidic solutions and green color in less acidic and alkaline solutions.

Methyl green (CI 42590)

CAS – [14855-76-6].

Molecular formula and molar mass – $C_{27}H_{35}BrClN_3$ – 516.95.

Description – Green powder. Usually, it is presented in the form of salt with $ZnCl_2$.

Solubility – Soluble in water.

Methyl green TS

Preparation – Dissolve 0.1 g of methyl green in 100 mL of water.

Change of color – In sulfuric acid solution, it presents a yellow color. By dilution, it returns to the green color.

Cresol red

CAS – [1733-12-6].

Molecular formula and molar mass – $C_{21}H_{18}O_5S$ – 382.44.

Description – Red-brown crystalline powder.

Solubility – Slightly soluble in water, soluble in ethyl alcohol and diluted alkali hydroxide solutions.

Cresol red TS

Preparation – Heat 50 g of cresol red with 2.65 mL of 0.05 M sodium hydroxide and 5 mL of ethyl alcohol 90%. After dissolution, complete the volume to 250 mL with 20% ethyl alcohol. *pH range* – 0.2 – 1.8 and 7.2 – 8.8.

Change of color – Provides red color in strongly acidic solutions (pH range between 0.2 and 1.8), yellow color in less acidic and neutral solutions, and presents red color in moderately alkaline solutions (pH range between 7.2 and 8.8).

Sensitivity test – The mixture of 0.1 mL of cresol red TS with 1000 mL of carbon dioxide-free water, added with 0.15 mL of 0.02 M sodium hydroxide, presents a purple red color. The color changes to yellow by adding no more than 0.15 mL of hydrochloric acid 0.02 M.

Congo Red (CI 22120)

CAS – [573-58-0].

Molecular formula and molar mass – $C_{32}H_{22}N_6Na_2O_6S_2$ – 696.66.

Description – Brownish-red powder.

Solubility – Soluble in water.

Congo Red TS

Preparation – Dissolve 0,25 mg of Congo red in 50 mL of 90% (v/v) ethyl alcohol and complete the volume to 250 mL with water.

pH range – 3.0 – 5.0.

Change of color – Presents blue color in moderately acidic solutions and red color in weakly acidic and alkaline solutions.

Sensitivity test – The mixture of 0.2 mL of Congo red TS, 100 mL of carbon dioxide-free water and 0.3 mL of hydrochloric acid 0.1 M has a blue color. No more than = 0.3 mL of 0.1 M sodium hydroxide is necessary to change the color to pink.

Congo Red, paper

Preparation – Immerse common filter paper strips in Congo red TS and let them dry at room temperature.

Phenol red

CAS – [143-74-8].

Molecular formula and molar mass – $C_{19}H_{14}O_5S$ – 354.38.

Description – Light red or dark red crystalline powder.

Solubility – Very slightly soluble in water and slightly soluble in ethyl alcohol.

Phenol red TS

Preparation – Heat 0.1 g of phenol red with 1.42 mL of 0.2 M sodium hydroxide and 5 mL of 90% (v/v) ethyl alcohol. After dissolution, complete the volume to 250 mL with 20% (v/v) ethyl alcohol.

pH range – 6.8-8.4.

Change of pH – Provides yellow color in neutral medium and red color in weakly alkaline solution.

Sensitivity test – The mixture of 0.1 mL of phenol red TS with 100 mL of carbon dioxide-free water presents a yellow color. No more than 0.1 mL of 0.02 M sodium hydroxide is necessary to change the color to red-violet.

Methyl red (CI 13020)

CAS – [493-52-7].

Molecular formula and molar mass – $C_{15}H_{15}N_3O_2$ – 269.30.

Description – Violet crystals or dark red powder.

Solubility – Practically insoluble in water and soluble in ethyl alcohol.

Methyl red TS

Preparation – Heat 0.1 g of methyl red with 1.85 mL of 0.2 M sodium hydroxide and 5 mL of 90% (v/v) ethyl alcohol. After dissolution, complete the volume to 250 mL with 50% (v/v) ethyl alcohol.

pH range – 3.0 – 4.4.

Change of color – Provides red color in weakly acidic solutions and yellow color in very weakly acidic and alkaline solutions.

Sensitivity test – The mixture of 0.1 mL of methyl red TS, 100 mL of carbon dioxide-free water and 0.05 mL of hydrochloric acid 0.02 M presents a red color. No more than 0.1 mL of 0.02 M sodium hydroxide is necessary to change the color to yellow.

Quinaldine red

CAS – [117-92-0].

Molecular formula and molar mass – $C_{21}H_{23}IN_2$ – 430.33.

Description – Dark blue powder.

| *Solubility* – Moderately soluble in water and freely soluble in ethyl alcohol.

Quinaldine red TS

Preparation – Dissolve 0.1 g of quinaldine red in 100 mL of methyl alcohol.

Change of color – There is a change of color from crimson to nearly colorless.

Used in titrations of bases with perchloric acid.

7.2 REAGENTS AND REAGENT SOLUTIONS

Reagents are substances used as such or as constituents of solutions, when conducting pharmacopoeia assays.

Acetal

CAS – [105-57-7].

Molecular formula and molar mass – $\text{C}_6\text{H}_{14}\text{O}_2$ – 118.18.

Description – Colorless, clear and volatile liquid.

Physical characteristics – Density (20 °C): approximately 0.824.

Refractive index (20 °C): approximately 1.382. Boiling temperature: approximately 103 °C.

Miscibility – Miscible with water and ethyl alcohol.

Acetaldehyde

CAS – [75-07-0].

Synonym – Ethanal.

Molecular formula and molar mass – $\text{C}_2\text{H}_4\text{O}$ – 44.05.

Description – Colorless and clear liquid.

Physical characteristics – Density (20 °C): approximately 0.788.

Refractive index (20°C): approximately 1.332. Boiling temperature: approximately 21 °C.

Miscibility – Miscible with water and ethyl alcohol.

Safety – Flammable.

Acetanilide

CAS – [103-84-4].

Synonym – *N*-Phenylacetamide.

Molecular formula and molar mass – $\text{C}_8\text{H}_9\text{NO}$ – 135.17.

Description – White crystalline, odorless powder.

Physical characteristic – Melting range: 114 °C to 116 °C.

Solubility – Slightly soluble in water, freely soluble in chloroform and ethyl alcohol, soluble in water in ebullition, ethyl ether and glycerin.

Conservation – In closed containers.

Ammonium acetate

CAS – [631-61-8].

Molecular formula and molar mass – $\text{C}_2\text{H}_7\text{NO}_2$ – 77.08.

Specification – Contains no less than 98.0% (w/w).

Description – Colorless crystals, very deliquescent, with faint acetic odor.

Solubility – Very soluble in water and in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from humidity.

Ammonium acetate RS

Specification – Contains 15% (w/v) of ammonium acetate in water.

Conservation – In tightly closed containers.

Stability – Prepare immediately before use.

Bornyl acetate

CAS – [5655-61-8].

Molecular formula and molar mass – $\text{C}_{12}\text{H}_{20}\text{O}_2$ – 196.29.

Description – Colorless crystals or colorless liquid.

Physical characteristic – Melting temperature: approximately 28 °C.

Solubility – Very slightly soluble in water and soluble in ethyl alcohol.

Butyl acetate

CAS – [123-86-4].

Molecular formula and molar mass – C₆H₁₂O₂ – 116.16.

Description – Colorless, flammable liquid, with sweet fruit odor.

Physical characteristics – Density (20 °C): approximately 0.88.

Refractive index (20 °C): approximately 1.395. Boiling range: 125 °C to 126 °C.

Solubility – Slightly soluble in water; miscible with ethyl alcohol and ethyl ether.

Conservation – In closed containers.

Cellulose acetate

CAS – [9004-35-7].

Specification – Partially acetylated cellulose, with varied degrees of acetylation.

Description – Amorphous white solid.

Category – Adsorbent in thin layer chromatography.

Lead acetate, trihydrate

CAS – [6080-56-4].

Synonym – Lead (II) acetate trihydrate.

Molecular formula and molar mass – C₄H₆PbO₄·3H₂O – 379.33.

Specification – Contains no less than 99.0% (w/w).

Description – Colorless clear crystals or crystalline white powder, with faint acetic odor. Efflorescent.

Physical characteristics – Melting temperature: 75 °C (quick heating); completely decomposes at 200 °C.

Solubility – Freely soluble in water and soluble in ethyl alcohol.

Conservation – In hermetic containers.

Safety – Toxic. Pollutant.

Lead acetate, paper

Preparation – Impregnate an adequate paper (usually sized 6 mm x 80 mm) with lead acetate solution RS. Dry the reagent paper at 100 °C, avoiding contact with metal.

Conservation – In tightly closed containers.

Storage – Protect from light and humidity.

Lead acetate RS (approximately 0.25 M)

Specification – Contains 9.5 g of lead acetate in 100 mL of carbon dioxide-free water.

Conservation – In tightly closed containers.

Safety – Toxic. Pollutant.

Lead acetate, saturated solution

Specification – Contains, approximately, 35 g of lead acetate in 50 mL of carbon dioxide-free water.

Conservation – In tightly closed containers.

Safety – Toxic. Pollutant.

Chlorhexidine acetate

CAS – [56-95-1].

Molecular formula and molar mass – C₂₆H₃₈Cl₂N₁₀O₄ – 625.56.

Description – White to pale beige crystals or powder; odorless.

Physical characteristic – Melting range: 154 °C to 155 °C.

Conservation – In tightly closed containers.

Storage – Protect from light.

Safety – Irritant.

Therapeutic class – Antimicrobial.

0.1% (w/v) chlorhexidine acetate

Specification – Contains 0.1 g of chlorhexidine acetate in 100 mL of water.

Conservation – In tightly closed containers.

Safety – Toxic.

Therapeutic class – Antimicrobial.

Copper acetate

CAS – [142-71-2].

Molecular formula and molar mass – $C_4H_6CuO_4 \cdot H_2O$ – 199.65.

Description – Bluish-green powder or crystals.

Solubility – Freely soluble in water in ebullition, soluble in water and ethyl alcohol, slightly soluble in glycerol.

Cortisone acetate

CAS – [50-04-4].

Molecular formula and molar mass – $C_{23}H_{30}O_6$ – 402.49.

Specification – Contains no less than 96.0% (w/w) in relation to the desiccated substance.

Description – Colorless faintly yellowed crystals or white or nearly white crystalline powder. Odorless; initially insipid, then bitter.

Physical characteristics – Melting temperature: approximately 240 °C.

Specific optical rotation: +209 to +219 (determine in solution 1.0% [w/v] in dioxane).

Conservation – In tightly closed containers.

Storage – Protect from light.

Therapeutic class – Corticosteroid.

Cortisone acetate, injectable

Description – Consists of a suspension in adequate aqueous medium, with pH between 5.0 and 7.0.

Specification – Contains no less than 90.0% (w/w).

Conservation – In single-dose ampoules.

Desoxycortone acetate

CAS – [56-47-3].

Synonym – Desoxycorticosterone acetate.

Molecular formula and molar mass – $C_{23}H_{32}O_4$ – 372.51.

Specification – Contains no less than 96.0% (w/w), calculated over the desiccated substance.

Description – Colorless crystals or white crystalline powder. Odorless.

Physical characteristics – Melting range: 157 °C to 161 °C.

Specific optical rotation: +171 to +179 (determine in solution 1.0% [w/v] in dioxane).

Conservation – In tightly closed containers.

Storage – Protect from light.

Therapeutic class – Corticosteroid.

Ethyl acetate

CAS – [141-78-6].

Molecular formula and molar mass – $C_4H_8O_2$ – 88.11.

Specification – Contains no less than 99.9% (w/v).

Description – Clear, colorless, volatile liquid, with characteristic odor.

Physical characteristics – Density: approximately 0.90.

Boiling temperature: approximately 77 °C. Refractive index (20 °C): 1.371 to 1.373.

Conservation – In tightly closed containers.

Storage – Protect from heat.

Safety – Flammable.

Phenylmercury acetate

CAS – [62-38-4].

Molecular formula and molar mass – $C_8H_8HgO_2$ – 336.74.

Specification – Contains no less than 98.0% (w/w).

Description – Small crystals or white or bright beige crystalline powder.

Physical characteristic – Melting range: 149 °C to 153 °C.

Conservation – In tightly closed containers.

Storage – Protect from light.

Safety – Toxic. Pollutant.

Indophenol acetate RS

Synonym – 2,6-dichlorophenol-indophenol sodium in acetate buffer.

Preparation – Dilute 12 mL of the standard solution of 2,6-dichlorophenol-indophenol sodium in 100 mL of water. Add to this solution 100 mL of acetate buffer pH 7.0.

Conservation – In tightly closed containers. *Stability* – Use in within two weeks. *Storage* – Keep under refrigeration.

Magnesium acetate

CAS – [16674-78-5].

Molecular formula and molar mass – $C_4H_6MgO_4 \cdot 4H_2O$ – 214.45.

Description – Colorless and deliquescent crystals.

Solubility – Freely soluble in water and in ethyl alcohol.

Conservation – In tightly closed containers.

Menthyl acetate

CAS – [2623-23-6].

Molecular formula and molar mass – $C_{12}H_{22}O_2$ – 198.31.

Description – Colorless liquid.

Physical characteristics – Density (20 °C): approximately 0.92. Refractive index (20 °C): approximately 1.447. Boiling temperature: approximately 228 °C.

Solubility – Slightly soluble in water; miscible with ethyl alcohol.

Mercury acetate

CAS – [1600-27-7].

Synonym – Mercuric acetate.

Molecular formula and molar mass – $C_4H_6HgO_4$ – 318.68.

Description – Crystals or white or nearly white crystalline powder, with faint acetic odor. *Physical characteristic* – Melting range: 178 °C to 180 °C (under heating results in decomposition).

Conservation – In tightly closed containers

Storage – Protect from light.

Safety – Toxic.

Mercury acetate RS

Preparation – Dissolve 6 g of mercury acetate in glacial acetic acid and complete the volume to 100 mL with the same solvent.

Conservation – In closed containers.

Storage – Protect from direct sunlight.

Methyl acetate

CAS – [79-20-9].

Molecular formula and molar mass – $C_3H_6O_2$ – 74.08.

Description – Colorless and clear liquid.

Physical characteristics – Density (20 °C): approximately 0.933. Refractive index (20 °C): approximately 1.361. Boiling range: 56 °C to 58 °C.

Solubility – Soluble in water; miscible with ethyl alcohol.

Potassium acetate

CAS – [127-08-2].

Molecular formula and molar mass – $C_2H_3KO_2$ – 98.14.

Specification – Contains no less than 99.0% (w/w) in relation to the desiccated substance.

Description – Colorless crystals or white crystalline powder, odorless or with faint acetic odor. Deliquescent.

Physical characteristic – Melting temperature: 292 °C.

Conservation – In tightly closed containers.

Potassium acetate RS

Specification – Contains 10 g of potassium acetate in 100 mL of water.

Conservation – In tightly closed containers.

Prednisolone acetate

CAS – [52-21-1].

Molecular formula and molar mass – $C_{23}H_{30}O_6$ – 402.49.

Specification – Contains no less than 96.0% (w/w) calculated in relation to the desiccated substance.

Description – White or nearly white crystalline powder. Odorless. Bitter.

Physical characteristics – Melting temperature: approximately 247 °C.

Specific optical rotation: +112 to +119 (determine in solution 1.0% [w/v] in dioxane).

Conservation – In tightly closed containers.

Therapeutic class – Corticosteroid.

Sodium acetate

CAS – [6131-90-4].

Molecular formula and molar mass – $C_2H_3NaO_2 \cdot 3H_2O$ – 136.08 (if anhydrous – 82.03).

Specification – Contains no less than 99.0% (w/w).

Description – Colorless crystals or white crystalline powder, odorless or with faint acetic odor. Efflorescent.

Conservation – In tightly closed containers.

Sodium acetate RS (approximately 0.02 M)

Specification – Contains 0.272% (w/v) of sodium acetate trihydrate in water.

Conservation – In tightly closed containers.

Uranyl acetate

CAS – [6159-44-0].

Molecular formula and molar mass – $C_4H_6O_6U \cdot 2H_2O$ – 424.15.

Description – Yellow crystalline powder, with faint acetic odor.

Conservation – In tightly closed containers.

Safety – Radioactive substance.

Uranyl and zinc acetate RS

Preparation – Dissolve 10 g of uranyl acetate in 50 mL of hot water and 5 mL of 30% (w/v) acetic acid. Dissolve 30 g of zinc acetate in 30 mL of hot water and 3 mL of 30% (w/v) acetic acid. Mix the previous preparations. Allow to cool. Filter.

Conservation – In tightly closed containers.

Storage – Protect from light.

Safety – Radioactive substance.

Zinc acetate

CAS – [5970-45-6].

Molecular formula and molar mass – $C_4H_6O_4Zn \cdot 2H_2O$ – 219.50.

Specification – Contains no less than 98.0% (w/w).

Description – Colorless or white crystals, or crystalline scales or granules, with faint acetic odor and astringent metallic flavor. Efflorescent.

Physical characteristic – Melting temperature: 237 °C.

Solubility – Freely soluble in water and soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Safety – Irritant.

Acetylacetone

CAS – [123-54-6].

Molecular formula and molar mass – $C_5H_8O_2$ – 100.12.

Description – Clear, colorless or yellow liquid, with aromatic odor.

Physical characteristics – Boiling temperature: approximately 139 °C.

Density: approximately 0.97. Refractive index (20 °C): 1.4505 to 1.4525.

Miscibility – Miscible with acetone and ethyl alcohol.

Conservation – In tightly closed containers.

Safety – Irritant. Flammable.

Acetone

CAS – [67-64-1].

Molecular formula and molar mass – C_3H_6O – 58.08

Specification – Contains no less than 98.0% (w/v).

Description – Clear, colorless, volatile liquid, with characteristic odor.

Physical characteristics – Density: 0.790 to 0.793. Refractive index (20 °C): 1.358 to 1.360.

Boiling temperature: approximately 56 °C.

Conservation – In hermetic containers.

Safety – Flammable. Irritant and toxic.

Dehydrated acetone

Specification – Acetone, dehydrated in anhydrous sodium sulfate.

Conservation – Prepare immediately before use.

Buffered acetone RS

Preparation – Dissolve 8.15 g of sodium acetate trihydrate and 42 g of sodium chloride in water, add 68 mL of hydrochloric acid 0.1 M and 150 mL of acetone.

Complete the volume to 500 mL with water.

Acetonitrile

CAS – [75-05-8].

Molecular formula and molar mass – $\text{C}_2\text{H}_3\text{N}$ – 41.05.

Description – Clear and colorless liquid. Odor similar to ether.

Physical characteristics – Density (20 °C): approximately 0.78. Refractive Index (20 °C): approximately 1.344.

Miscibility – Miscible with water, acetone and methyl alcohol.

Conservation – In hermetic containers.

Safety – Toxic. Flammable.

Acetic acid M

Specification – Contains 6% (w/v) of glacial acetic acid in water.

Conservation – In hermetic containers.

Additional information – Confirm the titer before using.

6 M acetic acid

Specification – Contains 34.8% (w/v) of glacial acetic acid in water.

Conservation – In tightly closed containers.

Storage – Protect from heat.

Safety – Corrosive. Flammable.

Diluted acetic acid

Specification – Contains 12% (w/v) of glacial acetic acid in water.

Conservation – In hermetic containers.

Acetic acid RS

Specification – Contains 30% (w/v) of glacial acetic acid in water.

Corresponds to acetic acid 5 M.

Description – Clear, colorless liquid, with irritant odor.

Conservation – In hermetic containers.

Glacial acetic acid

CAS – [64-19-7].

Molecular formula and molar mass – $\text{C}_2\text{H}_4\text{O}_2$ – 60.05.

Specification – Contains no less than 98.0% (w/w).

Description – Clear, colorless, volatile liquid, with characteristic irritant odor.

Crystallizable at low temperatures.

Physical characteristics – Density (20 °C): approximately 1.05.

Boiling temperature: approximately 118 °C.

Freezing temperature: approximately 14 °C.

Conservation – In hermetic containers.

Safety – Corrosive. Flammable. Protect eyes, skin and mucous membranes.

7-aminodesacetoxycephalosporanic acid

CAS – [22252-43-3].

Synonym – 7-ADCA.

Molecular formula and molar mass – $\text{C}_8\text{H}_{10}\text{N}_2\text{O}_3\text{S}$ – 214.24

Ascorbic acid

CAS – [50-81-7].

Molecular formula and molar mass – $\text{C}_6\text{H}_8\text{O}_6$ – 176.12.

Specification – Contains no less than 99.0% (w/w).

Description – Colorless crystals or white crystalline powder. Odorless.

Physical characteristics – Solution at 5% (w/v) presents pH from 2.2 to 2.5.

Melting temperature: approximately 190 °C, with decomposition.

Specific optical rotation: between +20.5 and +21.5°, determine in 1% (w/v) aqueous solution.

Conservation – In tightly closed, non-metallic containers.

Storage – Protect from light.

Barbituric acid

CAS – [67-52-7].

Synonym – 1H,3H,5H-Pyrimidine-2,4,6-trione.

Molecular formula and molar mass – C₄H₄N₂O₃– 128.09.

Description – White or nearly white powder.

Solubility – Moderately soluble in water, freely soluble in water in ebullition and in diluted acids.

Physical characteristic – Melting temperature: approximately 253 °C.

Benzoic acid

CAS – [65-85-0].

Molecular formula and molar mass – C₇H₆O₂– 122.12.

Specification – Contains no less than 99.0% (w/w).

Description – Colorless crystals or white crystalline powder, with characteristic odor.

Physical characteristic – Melting temperature: approximately 122 °C.

Solubility – Moderately soluble in water, soluble in water in ebullition and freely soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Boric acid

CAS – [10043-35-3].

Molecular formula and molar mass – H₃BO₃– 61.83.

Specification – Contains no less than 99.5% (w/w).

Description – Colorless bright crystals or white fine crystalline powder, unctuous to touch, with weakly acid and bitter flavor.

Solubility – Soluble in water and in ethyl alcohol, freely soluble in water in ebullition.

Conservation – In tightly closed containers.

Boric acid, saturated solution

Preparation – Dissolve 5 g in 100 mL of water.

Conservation – In tightly closed containers.

Hydrobromic acid

CAS – [10035-10-6].

Molecular formula and molar mass – HBr – 80.91.

Specification – Contains 48.0% (w/v).

Description – Colorless or faint yellow liquid, with strong and irritant odor.

Turns dark slowly by exposure to air and light.

Conservation – In tightly closed containers.

Storage – Protect from air and light.

Safety – Irritant. Corrosive.

Caffeic acid

CAS – [331-39-5].

Molecular formula and molar mass – C₉H₈O₄– 180.16.

Description – White or nearly white crystals.

Physical characteristic – Melting temperature: approximately 225 °C, with decomposition.

Solubility – Freely soluble in hot water and ethyl alcohol, moderately soluble in cold water.

Calconcarboxylic acid

CAS – [3737-95-9].

Molecular formula and molar mass – $C_{21}H_{14}N_2O_7S$ – 438.41.

Description – Black-brown powder.

Solubility – Slightly soluble in water, very slightly soluble in acetone and in ethyl alcohol, moderately soluble in diluted sodium hydroxide solutions.

Conservation – In tightly closed containers.

Cyclobutane-1,1-dicarboxylic acid

CAS – [5445-51-2].

Molecular formula and molar mass – $C_6H_{10}O_4$ – 146.14.

Description – White crystals.

Physical characteristic – Melting temperature: approximately 160 °C.

Conservation – In closed containers.

1,2-cyclohexylene-dinitrilo-tetracetic acid

CAS – [125572-95-4].

Synonyms – 1,2-cyclohexylene-diamino-tetracetic acid, CDTA.

Molecular formula and molar mass – $C_{14}H_{22}N_2O_8.H_2O$ – 364.35. *Description* – White powder.

Conservation – Tightly closed containers, protected from heat.

Safety – Irritant.

Cinnamic acid

CAS – [140-10-3].

Molecular formula and molar mass – C_9H_8O – 132.16.

Description – Colorless crystals.

Physical characteristic – Melting temperature: 133 °C.

Solubility – Very slightly soluble in water and freely soluble in ethyl alcohol.

Monohydrate citric acid

CAS – [5949-29-1].

Molecular formula and molar mass – $C_6H_8O_7.H_2O$ – 210.14.

Description – Colorless crystals or granules, or white crystalline or nearly white powder. Efflorescent.

Solubility – Very soluble in water and freely soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Hydrochloric acid

CAS – [7647-01-0].

Synonyms – Hydrogen chloride and concentrated hydrochloric acid.

Molecular formula and molar mass – HCl – 36.46.

Specification – Contains no less than 35.0% (w/w) comprised of gaseous HCl solution in water.

Description – Clear, colorless liquid, fuming, with irritant odor.

Physical characteristics – Density: approximately 1.18.

Conservation – In hermetic containers, of material inert to the reagent.

Storage – Protect from heat (maintain at temperatures below 20 °C).

Safety – Corrosive. Avoid external contact, eye and skin, inhalation and ingestion.

Brominated hydrochloric acid RS

Preparation – Add 1 mL of bromine RS to 100 mL of hydrochloric acid.

Conservation – In tightly closed containers.

Diluted hydrochloric acid

Specification – Use hydrochloric acid RS.

Hydrochloric acid M

Specification – Contains 10.3% (w/v) of hydrochloric acid in water.

Conservation – In tightly closed containers.

Stability – Protect from heat.

Safety – Corrosive.

Additional information – Confirm the titer before using.

Hydrochloric acid RS

Specification – Contains 27.4% (w/v) of concentrated hydrochloric acid in water.

Physical characteristics – Density: approximately 1.05.

Conservation – In tightly closed containers.

Stability – Protect from heat.

Safety – Corrosive.

0.01 M methanolic hydrochloric acid

Preparation – Transfer 0.85 mL of hydrochloric acid to a 1000 mL volumetric flask and complete the volume with methyl alcohol.

Tin hydrochloric acid RS

Preparation – Mix 1 mL of tin (II) chloride RS1 with 100 mL of hydrochloric acid.

Chlorogenic acid

CAS – [327-97-9].

Molecular formula and molar mass – $C_{16}H_{18}O_9$ – 354.31.

Description – White or nearly white crystalline powder or needles.

Physical characteristic – Melting temperature: approximately 208 °C.

Solubility – Freely soluble in water in ebullition, in acetone and in ethyl alcohol.

Chloroplatinic acid

CAS – [18497-13-7].

Synonyms – Platinic chloride, platinum chloride, chloroplatinic (IV) acid.

Molecular formula and molar mass – $H_2PtCl_6 \cdot 6H_2O$ – 517.90

Specification – Contains no less than 37.0% (w/w) platinum.

Description – Brownish-yellow crystalline mass, very deliquescent.

Physical characteristics – Density: 2.431. Melting temperature: 60 °C.

Solubility – Freely soluble in water and soluble in ethyl alcohol.

Conservation – In closed containers.

Storage – Protect from light.

Safety – Toxic.

Chromic acid

Use chromium trioxide (CrO_3).

3,5-dinitrobenzoic acid

CAS – [99-34-3].

Molecular formula and molar mass – $C_7H_4N_2O_6$ – 212.12

Description – Practically colorless crystals.

Physical characteristic – Melting temperature: approximately 206 °C.

Edetic acid

CAS – [60-00-4].

Synonyms – Ethylenediaminetetraacetic acid, EDTA.

Molecular formula and molar mass – $C_{10}H_{16}N_2O_8$ – 292.24

Specification – Contains no less than 98.0% (w/w).

Description – Colorless crystals.

Physical characteristic – Decomposes around 220 °C, can decarboxylate at 150 °C.

Conservation – In tightly closed containers.

Phenoldisulfonic acid RS

CAS – [96-77-5].

Molecular formula and molar mass – $C_6H_6O_7S_2$ – 254.24.

Description – Clear to light brown liquid.

Preparation – Dissolve 2.5 g of phenol in 15 mL of sulfuric acid.

Add 7.5 mL of fuming sulfuric acid. Heat at 100 °C for two hours.

Transfer the fluid product to an adequate container. To use, liquefy in water bath.

Conservation – Glass container with ground cap.

Safety – Irritant. Corrosive.

Phenoxyacetic acid

CAS – [122-59-8].

Molecular formula and molar mass – $C_8H_8O_3$ – 152.15.

Description – Nearly white crystals.

Physical characteristic – Melting temperature: approximately 98 °C.

Solubility – Moderately soluble in water and freely soluble in ethyl alcohol and glacial acetic acid.

Hydrofluoric acid

CAS – [7664-39-3].

Molecular formula and molar mass – HF – 20.01.

Specification – Contains no less than 40% (w/w) of HF.

Description – Colorless and clear liquid.

Conservation – In tightly closed polyethylene containers.

Formic acid

CAS – [64-18-6].

Synonym – Methanoic acid.

Molecular formula and molar mass – CH_2O_2 – 46.03.

Specification – The anhydrous form contains no less than 98.0% (w/w).

Description – Colorless, very caustic liquid, with pungent odor.

Physical characteristics – Boiling temperature: 100.5 °C.

Density: approximately 1.22. Refractive index (20 °C): 1.3714 Solidifies at 70 °C.

Conservation – In tightly closed containers.

Safety – Caustic.

Phosphomolybdic acid

CAS – [51429-74-4].

Synonym – Molybdatophosphoric acid.

Molecular formula – Approximately $12MoO_3.H_3PO_4.xH_2O$.

Description – Faint yellow crystals.

Conservation – In tightly closed containers.

Phosphomolybdic acid RS

Preparation – Dissolve 4 g of phosphomolybdic acid in 40 mL water while heating. Add 60 mL of sulfuric acid after cooling.

Phosphoric acid

CAS – [7664-38-2].

Synonym – Orthophosphoric acid.

Molecular formula and molar mass – H_3PO_4 – 97.99.

Specification – Contains no less than 85.0% (w/w).

Description – Clear, colorless, odorless liquid. Hygroscopic. Syrupy consistence.

Physical characteristic – Density (20 °C): approximately 1.7.

Conservation – In hermetic containers.

Safety – Corrosive. Avoid contact with skin and mucous membranes.

Phosphoric acid RS

Preparation – Mix an amount corresponding to 15 g of concentrated phosphoric acid with water until getting to 100 mL.

Physical characteristic – Density: approximately 1.15.

Phosphotungstic acid RS

Preparation – Heat under reflux for three hours the mixture of 10 g of sodium tungstate with 8 mL of phosphoric acid and 75 mL of water. Let it cool down and dilute to 100 mL with water.

Phthalic acid

CAS – [88-99-3].

Molecular formula and molar mass – $\text{C}_8\text{H}_6\text{O}_4$ – 166.13.

Description – White or nearly white crystalline powder.

Solubility – Soluble in hot water and in ethyl alcohol.

Gallic acid

CAS – [5995-86-8].

Molecular formula and molar mass – $\text{C}_7\text{H}_6\text{O}_5 \cdot \text{H}_2\text{O}$ – 188.14.

Description – Long needles or colorless or light yellow crystalline powder.

Physical characteristics – Loses crystallization water at temperature of 120 °C and fuses at approximately 206 °C, with decomposition.

Solubility – Soluble in water, freely soluble in hot water, in ethyl alcohol and in glycerol.

p-hydroxybenzoic acid

CAS – [99-96-7].

Molecular formula and molar mass – $\text{C}_7\text{H}_6\text{O}_3$ – 138.12.

Description – Colorless crystals.

Physical characteristic – Melting range: 213 °C to 214 °C.

Conservation – In tightly closed containers.

Hypophosphorous acid

CAS – [6303-21-5].

Synonym – Diluted hypophosphorous acid.

Molecular formula and molar mass – H_3PO_2 – 66.00.

Specification – Contains no less than 48% (w/v) of H_3PO_2 .

Description – Colorless or slightly yellow liquid.

Miscibility – Miscible with water and ethyl alcohol.

Hydriodic acid

CAS – [10034-85-2].

Molecular formula and molar mass – HI – 127.91.

Description – Aqueous solution of hydriodic acid. When freshly prepared, it is colorless, but with exposure to air and light it presents a yellow to brown color.

Conservation – In tightly closed containers.

Storage – Protect from light and from contact with air. Maintain at a temperature below 30 °C.

Lactic acid

CAS – [50-21-5].

Synonym – 2-hydroxypropionic acid.

Molecular formula and molar mass – C₃H₆O₃ – 90.08.

Specification – Mixture of 2-hydroxypropionic acid and its condensation products.

The equilibration between lactic acid and polylactic acid depends on concentration and temperature.

Lactic acid is usually a racemate ((*RS*)-lactic acid).

Description – Colorless or slightly yellow viscous liquid.

Miscibility – Miscible with water and ethyl alcohol.

Conservation – In closed containers.

Metaphosphoric acid

CAS – [10343-62-1].

Molecular formula and molar mass – (HPO₃)_n, monomer – 79.98.

Specification – Contains a certain ratio of sodium metaphosphate.

Description – Colorless solid or vitreous mass. Hygroscopic.

In aqueous solution, slowly turns into phosphoric acid (H₃PO₄).

Physical characteristic – Volatilizes under intense heating.

Conservation – In hermetic containers.

Metaphosphoric acetic acid RS

Specification – Contains 3 g of metaphosphoric acid and 8 mL of glacial acetic acid in water, completing the volume to 100 mL.

Conservation – In tightly closed containers.

Stability – Limited to two days.

Storage – Keep under refrigeration.

Methanesulfonic acid

CAS – [75-75-2].

Molecular formula and molar mass – CH₄O₃S – 96.11.

Description – Clear and colorless liquid (solidifies at 20 °C).

Physical characteristics – Density (20 °C): approximately 1.48.

Refractive index: approximately 1.430. Melting temperature: 20 °C.

Solubility – Miscible with water; slightly soluble in toluene and practically insoluble in hexane.

Conservation – In tightly closed containers.

Safety – Irritant.

Methoxyphenylacetic acid

CAS – [7021-09-2].

Molecular formula and molar mass – C₉H₁₀O₃ – 166.18

Synonym – (*RS*)-2-Methoxy-2-phenylacetic acid.

Description – White and crystalline powder, or white or nearly white crystals.

Physical characteristic – Melting temperature: approximately 70 °C.

Solubility – Slightly soluble in water, very soluble in ethyl alcohol.

Nitric acid

CAS – [7697-37-2].

Molecular formula and molar mass – HNO_3 – 63.01.

Specification – Contains no less than 63.0% (w/w).

Description – Clear, practically colorless solution, with characteristic odor.

Physical characteristic – Density (20 °C): 1.384 to 1.416.

Conservation – In hermetic containers, protected from light. *Safety* – Corrosive.

Fuming nitric acid

Specification – Contains no less than 95.0% (w/w).

Description – Clear, slightly yellow liquid, fuming on air.

Nitric acid RS

Specification – Contains approximately 12.5% (w/v) of nitric acid.

Physical characteristic – Density: approximately 1.5.

4-nitrobenzoic acid

CAS – [62-23-7].

Molecular formula and molar mass – $\text{C}_7\text{H}_5\text{NO}_4$ – 167.12.

Description – Yellow crystals.

Physical characteristic – Melting temperature: approximately 240 °C.

Oxalic acid

CAS – [6153-56-6].

Synonym – Ethanedioic acid.

Molecular formula and molar mass – $\text{C}_2\text{H}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$ – 126.06.

Specification – Contains no less than 99.0% (w/w).

Description – Colorless crystals or white crystalline powder.

Physical characteristic – Melting temperature: approximately 101 °C.

Safety – Poison!

Oxalic acid RS

Specification – 6.3% (w/v) solution of oxalic acid in water.

p-toluene sulfonic acid

CAS – [6192-52-5].

Molecular formula and molar mass – $\text{C}_7\text{H}_8\text{O}_3\text{S} \cdot \text{H}_2\text{O}$ – 190.22.

Specification – Contains no less than 87.0% (w/w) of p-toluene sulfonic acid.

Description – Crystalline powder or white crystals.

Perchloric acid

CAS – [7601-90-3].

Molecular formula and molar mass – HClO_4 – 100.46.

Specification – Contains no less than 70.0% (w/w) and no more than 72.0% of perchloric acid.

Description – Clear, colorless, volatile liquid, with pungent odor. Hygroscopic.

Physical characteristic – Density: approximately 1.7.

Conservation – Decomposes spontaneously, may explode especially in contact with oxidizable substances.

Safety – Irritant. Corrosive.

Perchloric acid M

Specification – Contains 8.5 mL of perchloric acid in water, completing the volume to 100 mL.

Stability – Prepare immediately before use.

Perchloric acid RS

Use perchloric acid M.

Performic acid

CAS – [107-32-4].

Synonym – Peroxyformic acid.

Molecular formula and molar mass – CH_2O_3 – 62.02.

Preparation – Mix 1 mL of hydrogen peroxide at 30.0% (v/v), or 9.0% (w/w), with 90 mL of formic acid.

Conservation – Prepare immediately before use.

Storage – Protect from heat.

Safety – Irritant. It may explode in contact with metals, their oxides, reducing substances, or in distillation.

Periodic acid

CAS – [10450-60-9].

Molecular formula and molar mass – H_5IO_6 – 227.93.

Description – White to colorless crystals.

Physical characteristics – Melting temperature: 122 °C.

Decomposes between 130 °C and 140 °C, forming I_2O_5 , H_2O and O_2 .

Solubility – Freely soluble in water and soluble in ethyl alcohol.

Picric acid

CAS – [88-89-1].

Synonym – 2,4,6-Trinitrophenol.

Molecular formula and molar mass – $\text{C}_6\text{H}_3\text{N}_3\text{O}_7$ – 229.10.

Specification – Yellow crystals or plates wetted with water.

Conservation – In tightly closed containers, mixed with an equal mass of water.

Storage – At room temperature.

Safety – Explodes when heated quickly or submitted to shock.

For safe transportation, 10% to 20% of water are usually added.

Picric acid RS

Preparation – Add 0.25 mL of sodium hydroxide 10 M to 100 mL of saturated picric acid solution in water.

Picric acid RS1

Preparation – Dissolve the equivalent to 1 g of picric acid in 100 mL of hot water.

Cool down and filter, if necessary.

Rosmarinic acid

CAS – [20283-92-5].

Molecular formula and molar mass – $\text{C}_{18}\text{H}_{16}\text{O}_8$ – 360.32.

Description – Orange-red powder.

Physical characteristic – Melting range: 170 °C to 174 °C.

Salicylic acid

CAS – [69-72-7].

Synonym – 2-hydroxybenzoic acid.

Molecular formula and molar mass – $C_7H_6O_3$ – 138.12.

Specification – Contains no less than 99.0% (w/w) in relation to the desiccated substance.

Description – White crystalline powder or colorless crystalline needles. Odorless.

Sweet acid and irritant flavor.

Physical characteristic – Melting range: 156-160 °C.

Solubility – Slightly soluble in water, freely soluble in ethyl alcohol, moderately soluble in methylene chloride.

Conservation – In tightly closed containers.

Selenious acid

CAS – [7783-00-8].

Molecular formula and molar mass – H_2SeO_3 – 128.97.

Specification – Contains no less than 93.0% (w/w) of selenious acid.

Description – White or colorless crystals. Efflorescent to dry air and hygroscopic to humid air.

Solubility – Soluble in water and in ethyl alcohol.

Conservation – In tightly closed containers.

Sulfamic acid

CAS – [5329-14-6].

Synonym – Amidosulfonic acid.

Molecular formula and molar mass – H_3NO_3S – 97.09.

Specification – White crystals or crystalline powder.

Physical characteristic – Melting temperature: approximately 205 °C, with decomposition.

Solubility – Freely soluble in water, moderately soluble in acetone, in ethyl alcohol and in methyl alcohol.

Conservation – In tightly closed amber glass containers.

Safety – Moderately irritating to skin and mucous membranes.

Sulfanilic acid

CAS – [6101-32-2].

Synonym – 4-aminobenzenesulfonic acid.

Molecular formula and molar mass – $C_6H_7NO_3S \cdot H_2O$ – 191.20; anhydrous – 173.19.

Specification – Contains no less than 99.0% (w/w).

Description – Colorless crystals or white powder.

Physical characteristic – The monohydrate acid decomposes without melting at approximately 288 °C.

Solubility – Moderately soluble in water, practically insoluble in ethyl alcohol.

Diazotized sulfanilic acid RS

Preparation – Dissolve, carefully, 0.2 g of sulfanilic acid in 20 mL of hydrochloric acid *M*, cool down in an ice bath and add, drop by drop, with constant stirring, 2.2 mL of 4% (w/v) sodium nitrite solution. Leave in an ice bath for 10 minutes and add 1 mL of 5% (w/v) sulfamic acid solution.

Sulfanilic acid RS

Preparation – Dissolve 0.5 g of sulfanilic acid finely powdered in water. Add 6 mL of 6 *M* hydrochloric acid. Complete the volume to 100 mL with water.

Sulfuric acid

CAS – [7664-93-9].

Molecular formula and molar mass – H_2SO_4 – 98.07.

Specification – Contains no less than 95.0% (w/w).

Description – Colorless, caustic liquid, of oily consistency, very hygroscopic.

Physical characteristic – Density: 1.834 to 1.839.

Conservation – In tightly closed containers.

Safety – Irritant. Corrosive.

Diluted sulfuric acid RS

Use sulfuric acid RS.

Nitrogen-free sulfuric acid

Specification – Conduct this test: add to 5 mL of water, carefully, 45 mL of sulfuric acid, wait until cooling down to 40 °C and add 8 mg of diphenylbenzidine. The resulting solution presents slightly rose or pale blue color.

0.1 M methanolic sulfuric acid

Preparation – Dilute 5.4 mL of sulfuric acid with 20 mL of methyl alcohol.

Complete the volume to 1000 mL with the same solvent.

Additional information – Prepare 24 hours before use.

Sulfuric acid/Methyl alcohol RS

Preparation – Add, slowly, 10 mL of sulfuric acid to 90 mL of methyl alcohol.

Additional information – Keep the system cooled.

Methanolic sulfuric acid RS

Preparation – Carefully add, to 30 mL of methyl alcohol cooled in ice bath, sulfuric acid in small amounts, under agitation. Cool down to room temperature and complete the volume to 100 mL with sulfuric acid. Homogenize.

Sulfuric acid, ethanolic solution

Preparation – Add, carefully and constantly cooling, 20 mL of sulfuric acid to 60 mL of ethyl alcohol. Continue cooling down and dilute to 100 mL with ethyl alcohol. Prepare immediately before use.

Sulfuric acid RS

Specification – Contains 10% (w/v) of sulfuric acid in water.

Preparation – Carefully add 57 mL of sulfuric acid to 100 mL of water, cool down and complete the volume to 1000 mL with water.

Conservation – In tightly closed containers.

Sulfurous acid

CAS – [7782-99-2].

Molecular formula and molar mass – H_2SO_3 – 82.07.

Specification – Contains 5.0 to 6.0% (w/w) of pure sulfur dioxide.

Prepare according to the consumption.

Description – Acid, clear, colorless liquid, with suffocating odor of sulfur dioxide.

On air, oxidizes gradually to sulfuric acid.

Conservation – In almost full containers, tightly closed, in a cool place.

Tartaric acid

CAS – [87-69-4].

Synonym – L-(+)-tartaric acid.

Molecular formula and molar mass – $\text{C}_4\text{H}_6\text{O}_6$ – 150.09.

Description – White crystalline crystals or powders.

Physical characteristics – Melting range: 168 °C to 170 °C. Density (20 °C): 1.756.

Solubility – Very soluble in water and freely soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Thioglycolic acid

CAS – [68-11-1].

Synonym – Mercaptoacetic acid.

Molecular formula and molar mass – $C_2H_4O_2S$ – 92.11.

Specification – Contains no less than 79.0% (w/w).

Description – Colorless or nearly colorless liquid, with strong unpleasant odor.

Physical characteristic – Density: approximately 1.33.

Miscibility – Miscible with water and ethyl alcohol.

Conservation – Protect from air.

Safety – May cause severe burns to the skin.

Additional information – Its decomposition releases hydrogen sulfide.

p-toluenesulfonic acid

CAS – [6192-52-5].

Molecular formula and molar mass – $C_7H_8O_3S \cdot H_2O$ – 190.21.

Specification – Contains no less than 87.0% of p-toluenesulfonic acid ($C_7H_8O_3S$).

Description – White or nearly white crystalline powder or crystals.

Solubility – Freely soluble in water and soluble in ethyl alcohol.

Trichloroacetic acid

CAS – [76-03-9].

Molecular formula and molar mass – $C_2HCl_3O_2$ – 163.39.

Specification – Contains no less than 98.0% (w/w).

Description – Colorless crystals or crystalline mass, deliquescent, with characteristic faintly pungent, irritant odor.

Physical characteristic – Melting range: 55 °C to 61 °C.

Conservation – In hermetic containers.

Storage – Protect from heat and humidity.

Safety – Very corrosive acid.

Trichloroacetic-chloramine-T acid RS

Solution A – 3% (w/v) chloramine-T.

Solution B – 25% (v/v) trichloroacetic acid in absolute ethyl alcohol.

Preparation – Mix 10 mL of *Solution A* with 40 mL of *Solution B*.

Trifluoroacetic acid

CAS – [76-05-1].

Synonym – TFA.

Molecular formula and molar mass – $C_2HF_3O_2$ – 114.02.

Description – Clear, volatile liquid, with characteristic irritant odor.

Physical characteristics – Boiling temperature: 72.4 °C. Density: 1.535.

Miscibility – Miscible with acetone, benzene, ethyl alcohol, ethyl ether, hexane and carbon tetrachloride.

Conservation – In tightly closed containers.

Safety – Corrosive. Flammable. Protect eyes, skin and mucous membranes.

Acrylamide

CAS – [79-06-1].

Synonym – 2-Propenamide.

Molecular formula and molar mass – C_3H_5NO – 71.08.

Specification – Appropriate quality for electrophoresis.

Description – White or nearly white crystalline powder, or colorless or white scales.

Physical characteristic – Melting temperature: approximately 84 °C.

Solubility – Very soluble in water and methyl alcohol, freely soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Safety – Highly toxic and irritant. Causes paralysis of the central nervous system. It may be absorbed by intact skin.

30% (w/v) acrylamide/bisacrylamide (29:1) RS

Preparation – Prepare a solution with 290 g of acrylamide and 10 g of methylenebisacrylamide in 1000 mL of hot water. Filter.

Conservation – In tightly closed containers.

Agar

CAS – [9002-18-0].

Synonyms – Agar-agar, gum agar.

Specification – Polysaccharide extracted from *Gelidium cartilagineum* (L) Gaillon (Gelidiaceae), *Gracilaria confervoides* (L) Greville (Sphaerococcaceae) and similar red algae (Rhodophyceae).

Description – Fine, colorless or slightly yellowed dry, hydrophilic powder.

Conservation – In hermetic containers.

Agarose, gel

CAS – [9012-36-6].

Specification – Linear, neutral polysaccharide, component of agar.

Description – White or nearly white powder.

Solubility – Practically insoluble in cold water and very slightly soluble in hot water.

Use – Electrophoresis.

DEAE agarose for ion exchange chromatography

Specification – Reticulated agarose with diethylaminoethyl groups. Presents as beads.

Bromine water RS

Preparation – Mix 3 mL of bromine with 100 mL of water until saturation. Shake before use. After decanting, use the clear supernatant solution.

Conservation – In hermetic containers.

Storage – Preserve with excess bromine and protected from light.

Safety – Toxic.

Chlorine water RS

Specification – Saturated chlorine solution in water.

Conservation – In tightly closed containers.

Storage – Protect from light and from air. Keep in a cold, dark place.

Carbon dioxide-free water

Specification – Water maintained in ebullition for five minutes or more and protected from atmosphere, during cooling down and conservation.

Conservation – Protect from air (from absorption of CO_2).

Ammonia-free water

Preparation – Transfer 0.1 mL of 96% (w/w) sulfuric acid to 100 mL of water and distill using an equipment with ammonia-free walls.

Nitrate-free water

Preparation – Transfer 5 mg of potassium permanganate and 5 mg of barium hydroxide to 100 mL of water and distill using an equipment with nitrate-free walls.

Particle-free water

Specification – Water obtained by membrane filtration with 0.22 µm of pore size.

Bovine albumin

CAS – [9048-46-8].

Synonym – Bovine serum albumin.

Description – White or light yellowed-brown powder.

Specification – Contains no less than 96% of proteins.

Water (5.2.20.3) – Determine in 0.8 g of the sample. No more than 30%.

Storage – At temperatures between 2 °C and 8 °C.

Human albumin

Synonym – Human serum albumin.

Specification – Contains no less than 96% of albumin.

Human Albumin, reagent solution

Preparation – Dilute the human albumin solution with 15% to 25% (w/v) in 0.9% (w/v) sodium chloride solution until a concentration of 0.1% (w/v) in proteins. Adjust the pH to 3.5-4.5 with glacial acetic acid.

Butyl alcohol

CAS – [71-36-3].

Synonyms – 1-butanol, *n*-butanol, *n*-butyl alcohol.

Molecular formula and molar mass – C₄H₁₀O – 74.12.

Description – Clear, colorless, retractive liquid, with characteristic odor.

Physical characteristics – Boiling range: 117 °C to 118 °C. Density (20 °C): 0.810. Refractive index (20 °C): 1.3993

Conservation – In tightly closed containers.

Safety – Irritant. Flammable.

Ethyl alcohol

CAS – [64-17-5].

Synonym – Ethanol.

Molecular formula and molar mass – C₂H₆O – 46.07.

Specification – Contains no less than 96.0% (v/v).

Description – Clear, colorless, volatile liquid, with characteristic odor.

Physical characteristics – Boiling temperature: approximately 78 °C. Density: 0.803 to 0.808.

Miscibility – Miscible with water and with methylene chloride.

Conservation – In tightly closed containers.

Storage – Protect from heat.

Safety – Toxic. Flammable.

Absolute ethyl alcohol

CAS – [64-17-5].

Synonym – Anhydrous alcohol.

Molecular formula and molar mass – $\text{C}_2\text{H}_6\text{O}$ – 46.07.

Specification – Contains no less than 99.5% (v/v).

Description – Clear, colorless, volatile liquid, with characteristic odor. Hygroscopic.

Physical characteristics – Boiling temperature: 78-79 °C.

Density: 0.791 to 0.794. Refractive index: (20 °C): 1.361.

Conservation – In hermetic containers.

Storage – Protect from heat and humidity. *Safety* – Toxic. Flammable.

Glycerinated ethyl alcohol

Preparation – Mix 20 mL of glycerin and 80 mL of 70% (v/v) ethyl alcohol.

Isoamyl alcohol

CAS – [123-51-3].

Synonym – 3-Methyl-1-butanol.

Molecular formula and molar mass – $\text{C}_5\text{H}_{12}\text{O}$ – 88.15.

Description – Colorless liquid.

Physical characteristic – Boiling temperature: approximately 130 °C.

Solubility – Slightly soluble in water; miscible with ethyl alcohol.

Isobutyl alcohol

CAS – [78-83-1].

Synonyms – 2-Methylpropanol, 2-methyl-1-propanol, isobutanol.

Molecular formula and molar mass – $\text{C}_4\text{H}_{10}\text{O}$ – 74.12.

Description – Colorless and clear liquid.

Physical characteristics – Density (20 °C): approximately 0.80. Refractive index (15 °C): 1.397 to 1.399. Boiling temperature: approximately 107 °C.

Conservation – In tightly closed containers.

Safety – Flammable.

Isopropyl alcohol

CAS – [67-63-0].

Synonyms – Isopropanol, 2-propanol.

Molecular formula and molar mass – $\text{C}_3\text{H}_8\text{O}$ – 60.10.

Specification – Contains no less than 99.0%.

Description – Colorless liquid, with characteristic odor.

Physical characteristics – Boiling temperature: approximately 82 °C.

Density: approximately 0.785. Refractive index (20 °C): 1.376 to 1.378.

Miscibility – Miscible with water and ethyl alcohol.

Conservation – In tightly closed containers.

Safety – Flammable.

Methyl alcohol

CAS – [67-56-1].

Synonym – Methanol.

Molecular formula and molar mass – CH_4O – 32.04.

Specification – Contains no less than 99.5% (w/v).

Description – Clear, colorless, flammable liquid, with characteristic odor.

Physical characteristics – Boiling temperature: 64 °C to 65 °C.

Density: 0.791 to 0.793. Refractive index (20 °C): 1.328 to 1.330.

Conservation – In hermetic containers.

Safety – Toxic. Flammable.

n-amyl alcohol

CAS – [71-41-0].

Synonyms – 1-Pentanol, pentyl alcohol.

Molecular formula and molar mass – $C_5H_{12}O$ – 88.15.

Description – Colorless liquid.

Physical characteristics – Refractive index (20 °C): approximately 1.41.

Boiling temperature: approximately 137 °C. Melting temperature: approximately -79 °C.

Solubility – Moderately soluble in water; miscible with ethyl alcohol.

Conservation – In tightly closed containers.

Safety – Irritant.

n-propyl alcohol

CAS – [71-23-8].

Synonyms – 1-Propanol, propanol.

Molecular formula and molar mass – C_3H_8O – 60.10.

Description – Clear, colorless liquid, with faint alcoholic odor.

Physical characteristics – Boiling temperature: approximately 97 °C

Density (20 °C): 0.802 to 0.806.

Miscibility – Miscible with water and ethyl alcohol.

Conservation – In tightly closed containers.

Safety – Flammable.

Polyvinyl alcohol

CAS – [9002-89-5].

Molecular formula – $(C_2H_4O)_n$.

Description – White powder.

Solubility – Soluble in water and insoluble in organic solvents.

Tert-amyl alcohol

CAS – [75-85-4].

Synonym – 2-Methyl-2-butanol.

Molecular formula and molar mass – $C_5H_{12}O$ – 88.15.

Description – Clear and colorless liquid. Volatile.

Physical characteristics – Density (20 °C): approximately 0.81.

Melting temperature: approximately -8 °C. Boiling temperature: 102 °C.

Miscibility – Easily miscible with water. Miscible with ethyl alcohol and in glycerol.

Conservation – In tightly closed containers.

Storage – Protect from light.

Safety – Flammable.

Tert-butyl alcohol

CAS – [75-65-0].

Synonym – 2-Methyl-2-propanol.

Molecular formula and molar mass – $C_4H_{10}O$ – 74.12.

Description – Colorless and clear liquid, or crystalline mass with camphorated odor.

Physical characteristics – Density (25 °C): 0.778 to 0.782.

Melting temperature: 25.7 °C. Boiling temperature: 82.5 °C to 83.5 °C.

Solubility – Soluble in water; miscible with ethyl alcohol and with ethyl ether.

Aluminum, metallic

CAS – [7429-90-5].

Element and atomic mass – Al – 26.98.

Description – White or nearly white to blueish, malleable, flexible metal.
Available on bar, powder, strips or threads.

Aluminon

CAS – [569-58-4].

Molecular formula and molar mass – $C_{22}H_{23}N_3O_9$ – 473.44.

Description – Red-brown crystals.

Solubility – Freely soluble in water.

Amaranth

Use amaranth red.

Iodized starch RS

Use iodized starch TS.

Iodized starch RS1

Preparation – Dissolve 0.75 g of potassium iodide in 100 mL of water. Heat until ebullition and add, stirring constantly, a solution with 0.5 g of soluble starch in 35 mL of water. Leave in ebullition for two minutes and cool down.

Iodide-free starch RS

Use iodide-free starch TS.

Soluble starch

Synonyms – Amylodextrin, amylogen. *Description* – Fine, odorless, insipid white powder.

Conservation – In tightly closed containers.

Storage – Protect from humidity.

Starch RS

Use starch TS.

Starches

Description – Extracted from mature caryopsis of *Zea mays* L., *Triticum aestivum* L. or *Oryza sativa* L. (fam. *Graminae*). Fine, odorless, insipid white powder that produces slight crepitation when compressed.

Conservation – In tightly closed containers.

Storage – Protect from humidity.

Additional information – The labeling must indicate the botanical origin.

4-Aminoantipyrine

CAS – [83-07-8].

Synonym – Aminopyrazolone.

Molecular formula and molar mass – $C_{11}H_{13}N_3O$ – 203.24

Description – Crystals or light yellow crystalline powder.

Physical characteristic – Melting temperature: approximately 109 °C.

Conservation – In tightly closed containers.

Aminobutanol

CAS – [96-20-8].

Synonym – 2-Amino-1-butanol.

Molecular formula and molar mass – $C_4H_{11}NO$ – 89.14.

Description – Oily liquid.

Physical characteristic – Boiling temperature: approximately 180 °C.

Solubility – Miscible with water; soluble in alcohols.

2-Aminoheptane

CAS – [123-82-0].

Synonyms – 2-Heptanamine; 2-heptylamine; 1-methylhexanamine.

Molecular formula and molar mass – C₇H₁₇N – 115.22.

Description – Volatile liquid.

Physical characteristic – Boiling temperature: approximately 143 °C.

Miscibility – Slightly miscible with water, easily miscible with chloroform, ethyl alcohol and ethyl ether.

4-Aminophenol

CAS – [123-30-8].

Molecular formula and molar mass – C₆H₇NO – 109.13.

Description – White crystalline powder or slightly colored due to exposure to air and light.

Physical characteristic – Melting temperature: approximately 186 °C, with decomposition.

Solubility – Moderately soluble in water and soluble in ethyl alcohol.

Conservation – In closed containers.

Storage – Protect from light.

2-Aminopyridine

CAS – [504-29-0].

Synonyms – α -Aminopyridine, 2-pyridinamine.

Description – Large crystals or leaflets.

Physical characteristic – Melting temperature: approximately 58 °C.

Ammonia RS

Description – Contains 37.5 mL of concentrated ammonia solution in 100 mL of aqueous solution.

Specification – Contains no less than 10% (w/v) of ammonium hydroxide (approximately 6 M).

Ammonia 6 M

Use ammonia RS

Ammonia 10 M

Preparation – Dilute 56 mL of ammonia to 100 mL with water.

Ammonia, concentrated solution

Synonym – Ammonium hydroxide.

Molecular formula and molar mass – NH₃ – 17.03.

Specification – Contains no less than 28.0% (w/w) and no more than 30.0% (w/w).

Description – Clear, colorless liquid, with characteristic and asphyxiating odor.

Conservation – In hermetic containers, not completely full.

Storage – Protect from air and light.

Safety – Caustic.

Anethol

CAS – [4180-23-8].

Synonym – *trans*-Anethol.

Description – White or nearly white crystalline mass at temperature between 20 °C and 21 °C, liquid at temperature above 23 °C.

Physical characteristics – Refractive index (25 °C): approximately 1.56. Boiling temperature: approximately 230 °C.

Solubility – Practically insoluble in water, freely soluble in ethyl alcohol and soluble in ethyl acetate and petroleum ether.

Acetic anhydride

CAS – [108-24-7].

Molecular formula and molar mass – C₄H₆O₃ – 102.09.

Specification – Contains no less than 97.0% (w/w).

Description – Mobile, colorless liquid, intense and irritant acetic odor.

Physical characteristics – Density: approximately 1.075. Boiling range: 136 °C to 142 °C.

Conservation – In hermetic containers.

Safety – Easily combustible. Strong irritant.

Pyridine-acetic anhydride RS

Synonym – Pyridine-acetic anhydride mix RS.

Description – Carefully mix, under refrigeration, 25 g (or 23 mL) of acetic anhydride in 50 mL of anhydrous pyridine.

Conservation – In hermetic containers.

Storage – Protect from air and light.

Stability – Prepare immediately before use. *Safety* – Toxic.

Phthalic anhydride

CAS – [85-44-9].

Molecular formula and molar mass – C₈H₄O₃ – 148.12.

Description – White or nearly white flakes.

Physical characteristic – Melting range: 130 °C to 132 °C.

Solubility – Slightly soluble in water and soluble in ethyl alcohol.

Conservation – In closed containers.

Propionic anhydride

CAS – [123-62-6].

Molecular formula and molar mass – C₆H₁₀O₃ – 130.14.

Description – Colorless liquid with pungent odor.

Physical characteristics – Density: 1.01. Boiling temperature: approximately 167 °C.

Solubility – Soluble in ethyl alcohol.

Aniline

CAS – [62-53-3].

Molecular formula and molar mass – C₆H₇N – 93.13.

Description – Colorless or slightly yellow liquid.

Physical characteristics – Density (20 °C): 1.02. Boiling temperature: 183 °C to 186 °C.

Conservation – Protected from light.

Anisaldehyde

CAS – [123-11-5].

Synonyms – Anisic aldehyde and *p*-methoxybenzaldehyde.

Molecular formula and molar mass – C₈H₈O₂ – 136.15.

Description – Oily, colorless and yellow liquid, with aromatic odor.

Physical characteristics – Density: approximately 1.12.

Boiling temperature: approximately 248 °C.

Solubility – Slightly soluble in water; miscible with ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from light.

Anisaldehyde, solution

Preparation – Mix, on the following order: Mix 0.5 mL of anisaldehyde, 10 mL of glacial acetic acid, 85 mL of methyl alcohol and 5 mL of sulfuric acid.

Anisaldehyde RS

Preparation – Add to 10 mL of anisaldehyde 90 mL of ethyl alcohol, mix, add 10 mL of sulfuric acid and homogenize.

Anisaldehyde RS1

Preparation – Mix 25 mL of glacial acetic acid with 25 mL of ethyl alcohol, add 0.5 mL of anisaldehyde and 1 mL of sulfuric acid.

Antithrombin III

CAS – [90170-80-2].

Specification – Antithrombin III is purified from human plasma by chromatography in gum agar-heparin and must have specific activity of no less than 6 IU/mg.

Antithrombin III RS

Preparation – Reconstitute the antithrombin III according to specifications from the manufacturer and dilute with sodium trichloride buffer of pH 7.5, to obtain a solution at 1 IU/mL.

Aprotinin

CAS – [9087-70-1].

Description – Nearly white powder.

Solubility – Soluble in water and in isotonic solutions, practically insoluble in organic solvents.

Asparagine

CAS – [5794-13-8].

Molecular formula and molar mass – $C_4H_8N_2O_3 \cdot H_2O$ – 150.13.

Description – Colorless, odorless crystals.

Physical characteristics – Isomer L: Melting temperature: 234-235 °C.

Isomer D: Melting temperature: 215 °C.

Solubility – Slightly soluble in water, practically insoluble in ethyl alcohol and in methylene chloride.

Sodium Azide

CAS – [26628-22-8].

Molecular formula and molar mass – NaN_3 – 65.01.

Description – White or nearly white crystalline powder or crystals.

Solubility – Freely soluble in water and slightly soluble in ethyl alcohol.

Acid blue 83

CAS – [6104-59-2].

Synonym – Brilliant blue.

Molecular formula and molar mass – $C_{45}H_{44}N_3NaO_7S_2$ – 825.97.

Description – Brown powder.

Solubility – Insoluble in cold water, slightly soluble in water in ebullition and in ethyl alcohol, soluble in sulfuric acid and in glacial acetic acid, soluble in diluted alkali metal hydroxide solutions.

Acid blue 90

CAS – [6104-58-1].

Molecular formula and molar mass – $C_{47}H_{48}N_3NaO_7S_2$ – 854.04.*Description* – Dark brown powder, with violet reflexes and particles with metallic reflexes.*Solubility* – Soluble in water and in ethyl alcohol.**Astra blue**

CAS – [82864-57-1].

Molecular formula and molar mass – $C_{47}H_{52}CuN_{14}O_6S_3$ – 1068.75.**Coomassie Blue RS***Preparation* – Prepare a 0.125% (w/v) acetic blue 83 solution in a mixture of glacial acetic acid, methyl alcohol and water (1:4:5) and filter.**Disulfin blue (CI 42045)**

CAS – [129-17-9].

Synonym – Acid blue I.*Molecular formula and molar mass* – $C_{27}H_{31}N_2NaO_6S_2$ – 566.66.*Description* – Violet powder. In diluted solutions, presents blue color.

After addition of concentrated hydrochloric acid, there is change of color to yellow.

Solubility – Soluble in water.**Tetrazolium blue**

CAS – [1871-22-3].

Molecular formula and molar mass – $C_{40}H_{32}N_8O_2Cl_2$ – 727.65.*Description* – Yellow crystals.*Physical characteristic* – Melting temperature: approximately 245 °C, with decomposition.*Solubility* – Slightly soluble in water, freely soluble in chloroform, ethyl alcohol and methyl alcohol, insoluble in acetone and ethyl ether.**Canada Balsam**

CAS – [8007-47-4].

Description – Yellow or greenish oily liquid, extracted from *Abies balsames* L., Pinaceae.

With pleasant odor of pine. If exposed to air, solidifies gradually into a non-crystalline mass.

Physical characteristics – Density: 0.987 to 0.994. Refractive index: 1.53.*Miscibility* – Miscible with water, benzene, chloroform and xylene.*Additional information* – Used to mount slides for microscope.**Barbaloin**

CAS – [1415-73-2].

Synonym – Aloin.*Description* – Yellow needles or yellow to dark yellow crystalline powder.

Turns dark with exposure to air and light.

Solubility – Moderately soluble in water and in ethyl alcohol, soluble in acetone, in ammonia and in alkali hydroxide solutions.**Barbital**

CAS – [57-44-3].

Molecular formula and molar mass – $C_8H_{12}N_2O_3$ – 184.20.*Specification* – Contains no less than 99.0% (w/w) calculated in relation to the desiccated substance.*Description* – Colorless crystals or white crystalline powder, odorless, with faintly bitter flavor.*Physical characteristic* – Melting temperature: approximately 190 °C.

Solubility – Slightly soluble in water, soluble in water in ebullition and in ethyl alcohol.

Sodium barbital

CAS – [144-02-5].

Molecular formula and molar mass – $C_8H_{11}N_2NaO_3$ –206.18

Specification – Contains no less than 99.0% (w/w) in relation to the desiccated substance.

Description – Colorless crystals or white crystallized powder, odorless, with bitter flavor and weakly caustic.

Solubility – Freely soluble in water and slightly soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Barium RSA – 1 mg/mL

Specification – Contains 1.775 g of barium chloride in 1000 mL of aqueous solution.

Conservation – In tightly closed, inert containers (like polyethylene).

Benzene

CAS – [71-43-2].

Synonym – Benzol.

Molecular formula and molar mass – C_6H_6 –78.11.

Description – Clear, colorless, refractive, volatile liquid, with characteristic odor.

Physical characteristics – Boiling range: 79 °C to 81 °C. Density: 0.878 to 0.880.

Refractive index: 1.5016.

Solubility – Practically insoluble in water; miscible with ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from heat.

Safety – Highly flammable. Carcinogenic.

Additional information – Use toluene whenever possible.

Benzenesulfonamide

CAS – [98-10-2].

Molecular formula and molar mass – $C_6H_5SO_2NH_2$ –157.19.

Description – White or pale beige crystals.

Physical characteristic – Melting range: 150 °C to 153 °C.

Benzil

CAS – [134-81-6].

Synonym – Diphenylethanedione.

Molecular formula and molar mass – $C_{14}H_{10}O_2$ –210.23.

Description – Yellow crystalline powder.

Physical characteristic – Melting temperature: approximately 95 °C.

Solubility – Practically insoluble in water and soluble in ethyl alcohol, ethyl acetate and toluene.

Benzyl benzoate

CAS – [120-51-4].

Description – Oily, clear and colorless liquid. By cooling, forms colorless crystals. *Physical characteristics* – Freezing temperature: approximately 17 °C.

Boiling temperature: approximately 324 °C.

Solubility – Practically insoluble in water and glycerol; miscible with ethyl alcohol, ethyl ether and chloroform.

Cholesteryl benzoate

CAS – [604-32-0].

Molecular formula and molar mass – $C_{34}H_{50}O_2$ – 490.77.

Description – White solid.

Solubility – Insoluble in water.

Methyl benzoate

CAS – [93-58-3].

Molecular formula and molar mass – $C_8H_8O_2$ – 136.15.

Description – Colorless liquid.

Physical characteristics – Density (20 °C): 1.088.

Boiling temperature: approximately 200 °C.

Benzophenone

CAS – [119-61-9].

Molecular formula and molar mass – $C_{13}H_{10}O$ – 182.22.

Description – White crystalline powder.

Characteristics – Melting temperature: approximately 48 °C.

Solubility – Practically insoluble in water, freely soluble in ethyl alcohol.

Benzoin

CAS – [119-53-9].

Synonym – 2-Hydroxy-1,2-diphenylethanone.

Molecular formula and molar mass – $C_{14}H_{12}O_2$ – 212.25.

Description – Slightly yellow crystals.

Solubility – Very slightly soluble in water, freely soluble in acetone, soluble in heated ethyl alcohol.

Sodium bicarbonate

CAS – [144-55-8].

Synonym – Sodium acid carbonate, sodium hydrogen-carbonate.

Molecular formula and molar mass – $NaHCO_3$ – 84.01.

Specification – Contains no less than 99.0% (w/w) and no more than 101.0% (w/w) in relation to the desiccated substance.

Description – White crystalline, odorless powder, of salty flavor and weakly alkaline. By heating, turns into sodium carbonate.

Solubility – Soluble in water, practically insoluble in ethyl alcohol.

Disodium bichinchoninate

CAS – [979-88-4].

Molecular formula and molar mass – $C_{20}H_{10}N_2Na_2O_4$ – 388.29.

Potassium biphthalate

CAS – [877-24-7].

Synonyms – Acid potassium phthalate, potassium hydrogen phthalate, potassium diphtalate.

Molecular formula and molar mass – $C_8H_5KO_4$ – 204.22.

Specification – Contains no less than 99.9% (w/w) and no more than 100.3% (w/w) in relation to the substance desiccated at 120 °C for two hours.

Description – Colorless crystals or white crystalline powder.

Solubility – Soluble in water and moderately soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Potassium biphthalate 0.05 M

Preparation – Dissolve 10.21 g in water and complete the volume to 1000 mL with the same solvent.

Conservation – In tightly closed containers.

Potassium bisulfate

CAS – [7646-93-7].

Synonyms – Potassium hydrogen sulfate; acid potassium sulfate.*Molecular formula and molar mass* – KHSO_4 – 136.16.*Specification* – Contains no less than 98.0% (w/w) in relation to the desiccated substance.*Description* – Colorless crystals or white mass. Hygroscopic.*Physical characteristics* – Aqueous solution with strongly acidic character. Melting temperature: 197 °C.*Solubility* – Freely soluble in water, resulting in a very acidic solution.*Conservation* – In tightly closed containers.**Sodium bisulfate**

CAS – [7681-38-1].

Synonyms – Acid sodium sulfate, sodium hydrogen sulfate, sodium pyrosulfate.*Molecular formula and molar mass* – NaHSO_4 – 120.06.*Physical characteristic* – Melting temperature: approximately 315 °C.*Solubility* – Freely soluble in water, very soluble in water in ebullition. Decomposes in ethyl alcohol, forming sodium sulfate and free sulfuric acid.**Sodium bisulfite**

CAS – [7631-90-5].

Synonyms – Sodium hydrogen sulfite, acid sodium sulfite.*Molecular formula and molar mass* – NaHSO_3 – 104.06.*Description* – White or nearly white crystalline powder. The exposure to air may cause loss of sulfur dioxide and the substance is gradually oxidized to sulfate.*Solubility* – Freely soluble in water and moderately soluble in ethyl alcohol.**Sodium bitartrate**

CAS – [6131-98-2].

Synonym – Acid sodium tartrate.*Molecular formula and molar mass* – $\text{C}_4\text{H}_5\text{NaO}_6 \cdot \text{H}_2\text{O}$ – 190.08.*Description* – White crystals or crystalline powder.*Solubility* – Soluble in water.**Sodium bitartrate RS***Preparation* – Dissolve 1 g of sodium bitartrate in water and complete the volume to 10 mL.

Prepare immediately before use.

Biuret

CAS – [108-19-0].

Molecular formula and molar mass – $\text{C}_2\text{H}_5\text{N}_3\text{O}_2$ – 103.08.*Description* – White or nearly white crystals. Hygroscopic.*Physical characteristic* – Melting range: 188 °C to 190 °C, with decomposition.*Solubility* – Soluble in water, moderately soluble in ethyl alcohol, very slightly soluble in ethyl ether.*Conservation* – In closed container.**Biuret, reagent***Preparation* – Dissolve 1.5 g of pentahydrate copper sulfate and 6 g of sodium tartrate and potassium in 500 mL of water. Add 300 mL of carbonate-free sodium hydroxide solution to 10% (w/v), complete the volume to 1000 mL with the same solution and mix.

Boldine

CAS – [476-70-0].

Molecular formula and molar mass – $C_{19}H_{21}NO_4$ – 327.38.

Description – White or nearly white crystalline powder.

Physical characteristic – Melting temperature: approximately 163 °C.

Solubility – Very slightly soluble in water, soluble in ethyl alcohol and in diluted acid solutions.

Conservation – In closed containers.

Borneol

CAS – [507-70-0].

Molecular formula and molar mass – $C_{10}H_{18}O$ – 154.25. *Description* – Colorless crystals, sublimate quickly. *Physical characteristic* – Melting temperature: approximately 208 °C.

Solubility – Practically insoluble in water, freely soluble in ethyl alcohol and in petroleum ether.

Potassium bromate

CAS – [7758-01-2].

Molecular formula and molar mass – $KBrO_3$ – 167.00.

Description – White or nearly white crystals or granular powder.

Solubility – Soluble in water and slightly soluble in ethyl alcohol.

Bromelain

CAS – [37189-34-7].

Specification – Concentrate of proteolytic enzymes derived from *Ananas comosus* Merr.

Description – Yellow powder.

Bromelain RS

Preparation – Dissolve 1 g of bromelain in 100 mL of a mixture comprised of phosphate buffer pH 5.5 and 0.9% (w/v) sodium chloride solution (1:9).

Dimidium bromide

CAS – [518-67-2].

Molecular formula and molar mass – $C_{20}H_{18}BrN_3$ – 380.29.

Description – Dark red crystal.

Solubility – Slightly soluble in water at 20 °C, moderately soluble in water at 60 °C and in ethyl alcohol.

Dimidium bromide - sulfan blue RS

Preparation – Dissolve, separately, 0.5 g of dimidium bromide and 0.25 g of sulfan blue in 30 mL of a hot mixture of ethyl alcohol and water (1:9) (v/v) and shake. Mix the two solutions and complete the volume to 250 mL with the same mixture of solvents. Mix 20 mL of this solution with 20 mL of a sulfuric acid solution 14 % (v/v), previously diluted with approximately 250 mL of water, and complete the volume to 500 mL with water.

Conservation – In closed containers.

Storage – Protect from exposure to light.

Hexadimethrin bromide

CAS – [28728-55-4].

Molecular formula – $(C_{13}H_{30}Br_2N_2)_n$.

Description – White or nearly white powder. Hygroscopic. Amorphous polymer.

Solubility – Soluble in water.

Conservation – In closed containers.

Iodine bromide

CAS – [7789-33-5].

Molecular formula and molar mass – IBr – 206.80.

Description – Dark brown or dark blue crystals.

Physical characteristics – Boiling temperature: approximately 116 °C.

Melting temperature: approximately 40 °C.

Solubility – Freely soluble in water, in ethyl alcohol and in glacial acetic acid.

Conservation – In closed containers.

Storage – Protect from light.

Iodine bromide RS

Preparation – Dissolve 13.2 g of iodine in glacial acetic acid and complete the volume to 1000 mL with the same solvent. Determine the content of iodine in 20 mL of this solution, by titrating with sodium thiosulfate 0.1 M VS. Add to the remaining iodine solution (980 mL) an amount of bromine equivalent to the iodine determined.

Conservation – In tightly closed glass containers.

Storage – Protect from light.

Potassium bromide

CAS – [7758-02-3].

Molecular formula and molar mass – KBr – 119.00.

Specification – Contains no less than 98.0% (w/w) in relation to the desiccated substance.

Description – Colorless crystals or white crystalline powder, with markedly salty flavor.

Solubility – Freely soluble in water and in glycerol, slightly soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Tetrabutylammonium bromide

CAS – [1643-19-2].

Molecular formula and molar mass – C₁₆H₃₆BrN – 322.38.

Description – White crystalline powder.

Physical characteristic – Melting range: between 103 °C and 105 °C.

Tetraheptylammonium bromide

CAS – [4368-51-8].

Molecular formula and molar mass – C₂₈H₆₀BrN – 490.70.

Description – White, scaly powder.

Physical characteristic – Melting range: between 89 °C and 91 °C.

Mercuric bromide

CAS – [7789-47-1].

Synonym – Mercury(II) bromide.

Molecular formula and molar mass – Br₂Hg – 360.40.

Specification – White crystals or crystalline powder, sensitive to light.

Physical characteristics – Melting temperature: approximately 237 °C.

Solubility – Slightly soluble in water and soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from light.

Safety – Poison!

Bromine

CAS – [7726-95-6].

Molecular formula and molar mass – Br₂ – 159.81.

Description – Brown-red liquid, irritant, suffocating and fuming.

Physical characteristic – Density (20 °C): approximately 3.1.

Solubility – Slightly soluble in water and soluble in ethyl alcohol.

Conservation – In hermetic containers or ampoules.

Safety – Toxic.

Bromine 0.2 M in glacial acetic acid

Preparation – Add 15 g of potassium bromide and 5.5 mL of bromine to glacial acetic acid and complete the volume to 1000 mL. Shake and allow to stand for 24 hours. Titrate before use.

Conservation – In hermetic containers.

Storage – Protect from heat.

Safety – Toxic.

Bromine RS

Preparation – Dissolve 30 g of bromine and 30 g of potassium bromide in water and complete the volume to 100 mL with the same solvent.

Sodium butanesulfonate

CAS – [2386-54-1].

Molecular formula and molar mass – C₄H₉NaO₃S – 160.16.

Description – White or nearly white crystalline powder.

Physical characteristic – Melting temperature: higher than 300 °C.

Solubility – Soluble in water.

Butyl hydroxyanisole

CAS – [25013-16-5].

Synonym – BHA.

Molecular formula and molar mass – C₁₁H₁₆O₂ – 180.245.

Specification – Mixture of two isomers: 2-*tert*-butyl-4- hydroxyanisole and 3-*tert*-butyl-4- hydroxyanisole

Description – Solid, of waxy aspect.

Physical characteristic – Melting range: from 48 °C to 55 °C.

Solubility – Practically insoluble in water and soluble in petroleum ether.

Conservation – In closed containers.

Butylamine

CAS – [109-73-9].

Synonym – *n*-Butylamine.

Molecular formula and molar mass – C₄H₁₁N – 73.14.

Description – Colorless liquid, with ammoniacal odor.

Physical characteristic – Boiling temperature: approximately 78 °C.

Miscibility – Miscible with water and ethyl alcohol.

Additional information – Distill and use within 30 days.

Butylparaben

CAS – [94-26-8].

Molecular formula and molar mass – C₁₁H₁₄O₃ – 194.23.

Description – White crystalline powder.

Physical characteristic – Melting range: from 68 °C to 69 °C.

Solubility – Very slightly soluble in water and freely soluble in acetone, ethyl ether and chloroform.

Conservation – In closed containers.

Calciferol

CAS – [50-14-6].

Synonym – Ergocalciferol, vitamin D₂.

Molecular formula and molar mass – C₂₈H₄₄O – 396.66.

Specification – 1 g corresponds, in anti-rickets activity, to 40,000,000 IU.

Description – Colorless crystals or white crystalline powder.

Solubility – Practically insoluble in water, freely soluble in ethyl alcohol, soluble in greasy oils.

Conservation – In hermetic containers, under inert gas.

Storage – Protect from heat and light.

Calcium RSA – 400 µg/mL

Specification – Contains 1.001 g of calcium carbonate in 25 mL of hydrochloric acid *M*. Boil. Complete the volume to 1000 mL with water.

Conservation – In tightly closed, inert containers (like polyethylene).

Camphene

CAS – [79-92-5].

Molecular formula and molar mass – C₁₀H₁₆ – 136.24.

Camphor

CAS – [76-22-2].

Molecular formula and molar mass – C₁₀H₁₆O – 152.24.

Light kaolin

CAS – [1332-58-7].

Specification – Natural, hydrated, purified aluminum silicate. It contains an appropriate dispersing agent.

Description – White power, little dense, free from gritty particles, unctuous to tact.

Solubility – Practically insoluble in water and in inorganic acids.

Coarse particles – Add 5 g of the sample to a cylinder with stopper (with 160 mm of length and 35 mm of internal diameter) and add 60 mL of 1% (w/v) sodium pyrophosphate solution. Shake vigorously and allow to stand for five minutes. Using a pipette, take 50 mL of the supernatant liquid, from a position approximately 5 cm below the preparation surface. Add to the remaining liquid 50 mL of water, shake, allow to stand for five minutes and take 50 mL of the liquid the same way as previously described. Repeat the process until taking a total of 400 mL. Transfer the suspension to a porcelain capsule, evaporate until dry in water bath, and desiccate at temperature between 100 °C and 105 °C until constant weight. The residue mass is not superior to 25 mg (0.5%).

Fine particles – Disperse 5 g of the sample in 250 mL of water, shake vigorously for 2 minutes, and transfer immediately to a glass cylinder (with 50 mm of internal diameter). Using a pipette, transfer 20 mL of liquid to a watch glass. Evaporate until dry in water bath, and desiccate at temperature between 100 °C and 105 °C until constant weight (*m*₁). Allow the remaining suspension to stand at 20 °C for four hours. Take 20 mL of the liquid, from a position approximately 5 cm below the preparation surface, avoiding to disperse the sediment. Transfer to a watch glass, evaporate until dry in water bath, and desiccate at temperature between 100 °C and 105 °C until constant weight (*m*₂). The value of *m*₂ is not inferior to 70% of the value of *m*₁.

Ammonium carbonate

CAS – [506-87-6].

Molecular formula and molar mass – (NH₄)₂CO₃ – 96.09.

Specification – Mixture in variable proportions of ammonium bicarbonate (NH₄HCO₃ – 79.06) and ammonium carbamate (H₂NCOONH₄ – 78.07).

It contains no less than 30.0% of NH_3 (MM – 17.03) (w/w).

Description – White crystalline, translucent masses, with strong ammoniacal flavor.

Solubility – Soluble in water. Decomposes in water in ebullition.

Conservation – In tightly closed containers.

Storage – Protect from light and from heat.

Ammonium carbonate RS

Specification – Contains 15.8 g of ammonium carbonate in 100 mL of aqueous solution.

Conservation – In tightly closed containers.

Storage – Protect from light and from heat.

Calcium carbonate

CAS – [471-34-1].

Molecular formula and molar mass – CaCO_3 – 100.09.

Specification – Contains no less than 98.5% (w/w) in relation to the desiccated substance.

Description – Odorless, insipid white powder.

Solubility – Practically insoluble in water.

Conservation – In tightly closed containers.

Strontium carbonate

CAS – [1633-05-2].

Molecular formula and molar mass – SrCO_3 – 147.63.

Description – Odorless, insipid white powder.

Conservation – In tightly closed containers.

Lithium carbonate

CAS – [554-13-2].

Molecular formula and molar mass – Li_2CO_3 – 73.89.

Specification – Contains no less than 98.5% in relation to the desiccated substance.

Description – White, light, odorless powder.

Solubility – Sparingly soluble in water and very slightly soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Potassium carbonate

CAS – [584-08-7].

Molecular formula and molar mass – K_2CO_3 – 138.20.

Description – Granular powder or white or nearly white granules. Hygroscopic.

Physical characteristic – Melting temperature: 891 °C.

Solubility – Moderately soluble in water and practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

Potassium carbonate sesquihydrate

CAS – [6381-79-9].

Molecular formula and molar mass – $\text{K}_2\text{CO}_3 \cdot 1\frac{1}{2}\text{H}_2\text{O}$ – 165.23.

Description – Small granular crystals.

Conservation – In tightly closed containers.

Safety – Irritant. Caustic.

Sodium carbonate

CAS – [497-19-8].

Formula and molecular mass – Na_2CO_3 – 105.99.

Specification – Contains no less than 99.0% (w/w) in relation to the desiccated substance.

Description – White, hygroscopic powder.

Solubility – Freely soluble in water.

Conservation – In hermetic containers.

Storage – Protect from humidity.

Sodium carbonate decahydrate

CAS – [6132-02-1].

Molecular formula and molar mass – $\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$ – 286.14.

Specification – Contains no less than 36.7% (w/w).

Description – Clear, colorless, efflorescent crystals or crystalline white powder; odorless, of alkaline and salty flavor.

Solubility – Freely soluble in water and practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

Sodium carbonate monohydrate

CAS – [5968-11-6].

Molecular formula and molar mass – $\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$ – 124.00.

Specification – Contains no less than 83.0% (w/w).

Description – Colorless crystals or crystalline white powder, odorless, with alkaline and salty flavor.

Solubility – Freely soluble in water and practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

Additional information – When sodium carbonate is prescribed for mixture in powder, use $\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$.

Sodium carbonate RS

Specification – Contains 10.6 g of sodium carbonate in 100 mL of aqueous solution.

Conservation – In tightly closed containers.

Carvone

CAS – [2244-16-8].

Molecular formula and molar mass – $\text{C}_{10}\text{H}_{14}\text{O}$ – 150.22.

Description – Colorless liquid.

Physical characteristics – Density (20 °C): approximately 0.965. Refractive index (20 °C): approximately 1.500. Boiling temperature: approximately 230 °C. Rotation power (20 °C): approximately +61°.

Solubility – Practically insoluble in water; miscible with ethyl alcohol.

Catechin

CAS – [154-23-4].

Molecular formula and molar mass – $\text{C}_{15}\text{H}_{14}\text{O}_6 \cdot x\text{H}_2\text{O}$ – 290.27 (for the anhydrous substance)

Physical characteristics – Melting range: 93 °C to 96 °C, or 175 °C to 177 °C when in anhydrous form.

Cephalin

Specification – Consists of esters of glycerophosphoric acid with long-chain fatty acids, with the phosphate group being esterified with ethanolamine.

Description – Amorphous yellow substance, of characteristic odor and flavor.

Category – Local hemostatic and laboratory reagent in hepatic function tests.

Cephalin RS

Preparation – Add 20 mL of acetone to an amount of 0.5 to 1 g of ox brain powder, allow to stand for two hours. Centrifuge for two minutes and decant the supernatant liquid. Dry the residue under reduced pressure. Add 20 mL of chloroform to the dry material. Allow to stand for two hours, with occasional agitation. After eliminating the solid material, by filtration or centrifugation, evaporate the chloroform under reduced pressure. Put the residue in suspension in 5 to 10 mL of 0.9% (w/v) sodium chloride solution. The solvents used to prepare this reagent have an appropriate antioxidant, such as 0.002% (w/v) butyl hydroxyanisole.

Conservation – Use within three months, after freezing or lyophilization.

Chromatographic cellulose

CAS – [9004-34-6].

Synonym – Cellulose for chromatography.

Description – Fine, homogeneous white powder. The average particle size is no less than 30 µm.

Category – Support to chromatography.

Lead RSA – 100 µg/mL

Specification – Contains 0.16 g of lead(II) nitrate in 5 mL of nitric acid.

Complete the volume to 1000 mL with water.

Conservation – In tightly closed, inert containers (like polyethylene).

Potassium cyanide

CAS – [151-50-8].

Molecular formula and molar mass – KCN – 65.12.

Specification – Contains no less than 96.0% (w/w) in relation to the desiccated substance.

Description – Crystalline powder, masses or white granules; deliquescent.

Physical characteristic – Melting temperature: 634 °C.

Conservation – In hermetic containers.

Storage – Protect from light.

Stability – Decomposes gradually by exposure to air, carbon dioxide and humidity.

Safety – Poison!

Potassium cyanide RS

Preparation – Dissolve 50 g of potassium cyanide in distilled water and complete the volume to 100 mL. Remove lead from this solution by extraction with successive portions of the dithizone extractor solution. Extract the remaining dithizone in the cyanide solution stirring with chloroform. Dilute the cyanide solution with enough distilled water so that every 100 mL has 10 g of potassium cyanide

Conservation – In hermetic containers.

Safety – Poison!

Ammonia cyanide RS

Preparation – Dissolve 2 g of potassium cyanide in 15 mL of ammonium hydroxide and dilute to 100 mL with distilled water.

Ethyl cyanoacetate

CAS – [105-56-6].

Molecular formula and molar mass – C₅H₇NO₂ – 113.12.

Description – Colorless or pale yellow liquid.

Physical characteristics – Density (25 °C): 1.056.

Boiling range: 205 °C to 209 °C, with decomposition.

Solubility – Slightly soluble in water; miscible with ethyl alcohol and ethyl ether.

Conservation – In tightly closed containers.

Cyclohexane

CAS – [110-82-7].

Molecular formula and molar mass – C_6H_{12} – 84.16.*Description* – Clear, colorless, volatile liquid, with characteristic odor (similar to gasoline). *Physical characteristics* – Boiling temperature: approximately 80 °C

Density: approximately 0.78. Refractive index (20 °C): 1.426 to 1.427.

Solubility – Practically insoluble in water; miscible with organic solvents.*Conservation* – In tightly closed containers.*Safety* – Flammable.**Benzyl cinnamate**

CAS – [103-41-3].

Molecular formula and molar mass – $C_{16}H_{14}O_2$ – 238.29.*Description* – Colorless or yellowed crystals.*Physical characteristic* – Melting temperature: approximately 39 °C.**Methyl cinnamate**

CAS – [103-26-4].

Molecular formula and molar mass – $C_{10}H_{10}O_2$ – 162.19.*Description* – Colorless crystals.*Physical characteristics* – Melting range: 34 °C to 36 °C.

Boiling temperature: approximately 260 °C. Refractive index (20 °C): approximately 1.56.

Solubility – Practically insoluble in water and soluble in ethyl alcohol.**Cinchonine**

CAS – [118-10-5].

Molecular formula and molar mass – $C_{19}H_{22}N_2O$ – 294.40.*Description* – White or nearly white crystalline powder.*Physical characteristics* – Specific rotation power (20 °C): +225° to +230°, determined on a 5% (w/v) solution in ethyl alcohol. Melting temperature: approximately 263 °C.*Conservation* – In closed containers.*Storage* – Protect from exposure to light.**1,8-Cineole**

CAS – [470-82-6].

Synonym – Eucalyptol.*Molecular formula and molar mass* – $C_{10}H_{18}O$ – 154.25.*Description* – Colorless liquid.*Physical characteristics* – Density (20 °C): 0.922 to 0.927. Refractive index (20 °C): 1.456 to 1.459.*Solubility* – Practically insoluble in water; miscible with ethyl alcohol.**Citral**

CAS – [5392-40-5].

Molecular formula and molar mass – $C_{10}H_{16}O$ – 152.24.*Description* – Light yellow liquid.*Solubility* – Practically insoluble in water; miscible with ethyl alcohol and glycerol.**Ammonium citrate RS***Preparation* – Dissolve 40 g of citric acid in 90 mL of distilled water. Add two or three drops of 0.1% (w/v) phenol red in ethyl alcohol. Carefully add ammonium hydroxide until the solution acquires a

red color. Remove any lead present by extraction with portions of 20 mL of dithizone extractor solution until the orange-green color in the dithizone solution is maintained.

Alkaline cupric citrate RS

Preparation – Under heating, dissolve 173 g of sodium citrate and 177 g of sodium carbonate monohydrate in 700 mL of water. Filter if necessary to obtain a clear solution. On a separate vial, dissolve 17.3 g of cupric sulfate pentahydrate in 100 mL of water. Add (slowly and under constant agitation) to this solution the first solution prepared. Complete the volume to 1000 mL with water.

Cupric citrate RS

Preparation – Dissolve 25 g of cupric sulfate pentahydrate, 50 g of citric acid monohydrate, and 144 g of sodium carbonate in water and dilute to 1000 mL with the same solvent.

Sodium citrate

CAS – [6132-04-3].

Synonym – Trisodium citrate.

Molecular formula and molar mass – $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$ – 294.10.

Specification – Contains no less than 99.0% (w/w) in relation to the desiccated substance.

Description – Crystals or crystalline white powder, odorless, with salty flavor, refreshing. Deliquescent.

Solubility – Freely soluble in water and practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

Citronellal

CAS – [106-23-0].

Molecular formula and molar mass – $\text{C}_{10}\text{H}_{18}\text{O}$ – 154.25.

Description – Colorless or light yellow liquid.

Physical characteristics – Density (20 °C): 0.848 to 0.856. Refractive index (20 °C): approximately 1.446.

Solubility – Very slightly soluble in water and soluble in ethyl alcohol.

Citronellol

CAS – [106-22-9].

Molecular formula and molar mass – $\text{C}_{10}\text{H}_{20}\text{O}$ – 156.27.

Description – Colorless and clear liquid.

Physical characteristics – Density (20 °C): 0.857.

Refractive index (20 °C): 1.456. Boiling range: 220 °C to 222 °C.

Solubility – Practically insoluble in water; miscible with ethyl alcohol.

Chloramine T

CAS – [7080-50-4].

Synonyms – *N*-chloro-*p*-toluene sulfonamide sodium salt trihydrate.

Molecular formula and molar mass – $\text{C}_7\text{H}_7\text{ClNNaO}_2\text{S} \cdot 3\text{H}_2\text{O}$ – 281.69.

Description – Efflorescent white or slightly yellow crystals or crystalline powder.

Physical characteristic – Melting range: 167 °C to 170 °C.

Solubility – Freely soluble in water, soluble in ethyl alcohol with decomposition, insoluble in benzene, chloroform and ethyl ether.

Conservation – In perfectly closed containers, protected from light, in a refrigerator.

Potassium chlorate

CAS – [3811-04-9].

Molecular formula and molar mass – KClO_3 – 122.55.

Description – White or nearly white crystals, granules or powder.

Physical characteristic – Melting temperature: 368 °C.

Solubility – Soluble in water.

Conservation – In tightly closed containers.

Safety – Avoid contact with organic materials or other oxidizable substances.

Cobalt chloride

CAS – [7791-13-1].

Synonym – Cobalt(II) chloride.

Molecular formula and molar mass – $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ – 237.93.

Specification – Contains no less than 99.0% (w/w).

Description – Crystalline powder or red-violet crystals.

Solubility – Very soluble in water and soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Cobalt chloride RS

Specification – Contains 6.5 g of cobalt chloride, added with 70 mL of hydrochloric acid RS and sufficient water to complete 100 mL.

Conservation – In tightly closed containers.

Acetyl chloride

CAS – [75-36-5].

Molecular formula and molar mass – $\text{C}_2\text{H}_3\text{ClO}$ – 78.50.

Description – Clear and colorless liquid. Flammable. Decomposes in contact with water and with ethyl alcohol.

Physical characteristics – Density (20 °C): approximately 1.10. Boiling temperature: 52 °C.

Miscibility – Miscible with ethylene chloride, ethyl ether and glacial acetic acid.

Conservation – In tightly closed containers.

Safety – Irritant to the eyes.

Acetyl chloride 2% in antimony chloride RS

Solution A – 110 g of antimony chloride in 400 mL of 1,2-dichloromethane. Add 2 g of anhydrous aluminum oxide, mix and filter in sintered glass filter to a 500 mL volumetric flask. Dilute to 500 mL with 1,2-dichloromethane and mix. The absorbance of the resulting solution measured at 500 nm in a 2-cm cell is not more than 0.07 using 1,2-dichloromethane as blank.

Solution B – in a hood, mix 100 mL of distilled, colorless, acetyl chloride and 400 mL of 1,2-dichloromethane and store in refrigeration.

Preparation – Mix 90 mL of *Solution A* with 10 mL of *Solution B*. Store in amber, polished-necked vials and use within seven days. Discard if color appears.

Aluminum chloride hexahydrate

CAS – [7784-13-6].

Molecular formula and molar mass – $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ – 241.43.

Description – White or slightly yellow, deliquescent powder or colorless crystals.

Solubility – Very soluble in water, freely soluble in ethyl alcohol, soluble in glycerol.

Conservation – In hermetic containers.

Aluminum chloride RS

Preparation – Dissolve two parts of aluminum chloride hexahydrate in water sufficient for three parts. Treat the solution with activated charcoal, filter and, if necessary, adjust the pH to 1.5 with 1% (w/v) sodium hydroxide.

Ammonium chloride

CAS – [12125-02-9].

Molecular formula and molar mass – NH_4Cl – 53.49.*Specification* – Contains no less than 99.5% (w/w) in relation to the desiccated substance.*Description* – Colorless crystals or crystalline white powder, odorless, with salty flavor. Hygroscopic.*Physical characteristic* – Sublimates without melting at 338 °C.*Solubility* – Freely soluble in water.*Conservation* – In hermetic containers. *Storage* – Protect from humidity.**Ammonium chloride RS***Specification* – Contains 10.7 g in 100 mL of aqueous solution (approximately 2 M).*Conservation* – In tightly closed containers.**Ammonium chloride - ammonium hydroxide RS***Preparation* – Mix equal volumes of ammonium hydroxide and water and saturate with ammonium chloride.**Barium chloride**

CAS – [10326-27-9].

Molecular formula and molar mass – $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ – 244.27.*Specification* – Contains no less than 99.0% (w/w).*Description* – Colorless crystals or white crystalline powder.*Solubility* – Freely soluble in water and slightly soluble in ethyl alcohol.*Conservation* – In tightly closed containers.*Safety* – Toxic.**Barium chloride RS***Specification* – Contains 10 g in 100 mL of aqueous solution.*Conservation* – In tightly closed containers.**Benzalkonium chloride**

CAS – [8001-54-5].

Molecular formula and molar mass – $[\text{C}_6\text{H}_5\text{CH}_2\text{N}(\text{CH}_3)_2\text{R}]^+ \text{Cl}^-$ – 360.00 (average)*Chemical composition* – Mixture of alkyldimethylbenzylammonium chlorides, where R represents alkyl, from $n\text{-C}_8\text{H}_{17}$ and superior homologs: $n\text{-C}_{12}\text{H}_{25}$, $n\text{-C}_{14}\text{H}_{29}$, $n\text{-C}_{16}\text{H}_{33}$, in higher proportion.*Specification* – Contains no less than 95.0% in relation to the desiccated substance. Content of alkyl homologs present, in relation to the total calculated over dried basis: $n\text{-C}_{12}\text{H}_{25}$: no less than 40.0% (w/w); $n\text{-C}_{14}\text{H}_{29}$: no less than 10.0% (w/w); sum of the two homologs: no less than 70.0% (w/w).*Description* – Amorphous powder or gelatinous white or yellow-white mass, of aromatic odor and bitter flavor.*Solubility* – Very soluble in water and in ethyl alcohol. In aqueous solution, it forms foam under agitation.*Conservation* – In tightly closed containers.*Storage* – Protect from light and from air.*Category* – Disinfectant. Detergent. Preservative.**Benzethonium chloride**

CAS – [121-54-0].

Molecular formula and molar mass – $\text{C}_{27}\text{H}_{42}\text{ClNO}_2 \cdot \text{H}_2\text{O}$ – 466.10.*Description* – Colorless crystals or fine, white or nearly white powder.*Physical characteristic* – Melting temperature: approximately 163 °C.

Solubility – Soluble in water and ethyl alcohol.

Conservation – In closed containers.

Storage – Protect from exposure to light.

Benzyl chloride

CAS – [100-44-7].

Synonym – Chloromethyl benzene.

Molecular formula and molar mass – C_7H_7Cl – 126.58.

Description – Colorless liquid.

Physical characteristics – Density (20 °C): 1.100.

Boiling temperature: 179 °C. Melting range: -48 °C to -43 °C.

Solubility – Insoluble in water; miscible with ethyl alcohol, chloroform and ethyl ether.

Conservation – In hermetic containers.

Storage – Protect from heat.

Calcium chloride

CAS – [10043-52-4].

Molecular formula and molar mass – $CaCl_2$ – 110.98.

Specification – Contains no less than 98.0% (w/w) in relation to the desiccated substance.

Description – Dry, white granules. Deliquescent.

Solubility – Very soluble in water, freely soluble in ethyl alcohol and in methyl alcohol.

Conservation – In hermetic containers.

Storage – Protect from humidity.

Category – Desiccant.

Calcium chloride dihydrate

CAS – [10035-04-8].

Molecular formula and molar mass – $CaCl_2 \cdot 2H_2O$ – 147.01.

Specification – Contains no less than 96.0% (w/w).

Description – White crystalline powder or granules, odorless, of salty flavor and strongly bitter. Hygroscopic.

Solubility – Freely soluble in water and soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from humidity.

Calcium chloride RS

Specification – Contains 7.35 g of calcium chloride in 100 mL of aqueous solution (approximately 0.5 M).

Conservation – In tightly closed containers.

Cesium chloride

CAS – [7647-17-8].

Molecular formula and molar mass – $CsCl$ – 168.36.

Description – White or nearly white powder.

Solubility – Very soluble in water, freely soluble in methyl alcohol and practically insoluble in acetone.

Tin(II) chloride RS

Preparation – Heat 20 g of tin with 85 mL of hydrochloric acid until no more hydrogen is released.

Magnesium chloride

CAS – [7791-18-6].

Molecular formula and molar mass – $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ – 203.30.

Specification – Contains no less than 98.0% (w/w).

Description – Colorless crystals, with bitter flavor. Hygroscopic.

Solubility – Very soluble in water and freely soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from humidity.

Mercury(II) chloride

CAS – [7487-94-7].

Synonym – Mercuric chloride.

Molecular formula and molar mass – HgCl_2 – 271.50.

Specification – Contains no less than 99.0% (w/w) in relation to the desiccated substance.

Description – Colorless crystals or white or nearly white crystalline powder, or crystallized mass; odorless.

Physical characteristic – Melting temperature: 277 °C (volatilizes at temperature of approximately 300 °C).

Solubility – Soluble in water and in glycerol, freely soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from light.

Safety – Irritant. Caustic. Toxic. Pollutant.

Additional information – Antidote: dimercaprol.

Methylene chloride

CAS – [75-09-2].

Synonym – Dichloromethane.

Molecular formula and molar mass – CH_2Cl_2 – 84.93.

Description – Clear, colorless, volatile liquid, with odor similar to chloroform.

Physical characteristics – Boiling temperature: approximately 40 °C.

Density: approximately 1.32. Refractive index (20 °C): 1.424.

Solubility – Moderately soluble in water; miscible with ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from light.

Safety – Irritant. Toxic.

Methylene chloride saturated with ammonia

Preparation – Mix 100 mL of methylene chloride with 30 mL of concentrated ammonia solution in separation funnel. Let the phases separate and use the bottom layer.

Methylthionine chloride

CAS – [7220-79-3].

Synonyms – Methylthionine chloride trihydrate, methylene blue.

Molecular formula and molar mass – $\text{C}_{16}\text{H}_{18}\text{ClN}_3\text{S} \cdot 3\text{H}_2\text{O}$ – 373.90.

Description – Dark green or bronze crystalline powder. It may be found in different hydrate forms.

Solubility – Freely soluble in water and in ethyl alcohol.

Methylthionine chloride RS

Synonym – Methylene blue RS.

Preparation – Dissolve 23 mg of methylthionine chloride in sufficient amount of water to prepare 100 mL.

Methylthionine chloride RS1

Synonym – Methylene blue RS1.

Preparation – Dissolve 125 mg of methylthionine chloride in 100 mL of ethyl alcohol and dilute in ethyl alcohol to prepare 250 mL.

Nickel(II) chloride

CAS – [7791-20-0]

Molecular formula and molar mass – $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ – 237.68.

Description – Green crystalline powder. Hygroscopic.

Nitrobenzoyl chloride

CAS – [122-04-3].

Molecular formula and molar mass – $\text{C}_7\text{H}_4\text{ClNO}_3$ – 185.57.

Description – Yellow crystals, with pungent odor.

Physical characteristic – Melting temperature: approximately 73 °C.

Gold chloride

CAS – [16961-25-4].

Molecular formula and molar mass – $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ – 393.82.

Description – Red-yellow to golden yellow monoclinic crystals. Very hygroscopic and deliquescent.

Conservation – In tightly closed containers.

Storage – Protect from light.

Gold chloride RS

Preparation – Dissolve 1 g of gold chloride in 35 mL of water.

Conservation – In tightly closed containers.

Storage – Protect from light.

Palladium chloride

CAS – [7647-10-1].

Molecular formula and molar mass – PdCl_2 – 177.32.

Specification – Contains no less than 59.0% (w/w) of palladium.

Description – Red-brown crystalline powder.

Physical characteristic – At high temperatures, decomposes to palladium and chlorine.

Conservation – In tightly closed containers.

Safety – Toxic.

Potassium chloride

CAS – [7447-40-7].

Molecular formula and molar mass – KCl – 74.55.

Specification – Contains no less than 99.0% (w/w) in relation to the desiccated substance.

Description – Colorless crystals or white crystalline powder, with saline, faintly bitter flavor.

Conservation – In tightly closed containers.

Potassium chloride, saturated solution

Specification – Contains 17 g in 50 mL of aqueous solution.

Conservation – In tightly closed containers.

Sodium chloride

CAS – [7647-14-5].

Molecular formula and molar mass – NaCl – 58.44.

Specification – Contains no less than 99.0% (w/w) in relation to the desiccated substance.

Description – Colorless crystals or crystalline white powder, odorless, with saline flavor.

Solubility – Freely soluble in water and practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

Additional information – Additive-free salt.

Sodium chloride 0.9% (w/v)

Synonyms – Sodium chloride approximately 0.15 M, isotonic sodium chloride solution, physiological solution, saline solution.

Description – Contains 9 g of sodium chloride in 1000 mL of aqueous solution.

Conservation – In closed containers.

Tin chloride

CAS – [10025-69-1].

Molecular formula and molar mass – $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ – 225.64.

Specification – Contains no less than 97.0% (w/w).

Description – Colorless or almost colorless crystals.

Solubility – Very soluble in water, freely soluble in ethyl alcohol, in glacial acetic acid, and in diluted and concentrated hydrochloric acid.

Conservation – In tightly closed containers.

Storage – Protect from air and heat.

Tin chloride RS

Specification – Contains 10% (w/v) of tin chloride in hydrochloric acid.

Conservation – Prepare immediately before use.

Storage – Protect from light.

Tin chloride RS1

Synonym – Tin(II) chloride RS.

Preparation – Heat 20 g of tin with 85 mL of hydrochloric acid until no more hydrogen is released.

Ferric chloride

CAS – [10025-77-1].

Synonym – Iron chloride hexahydrate.

Molecular formula and molar mass – $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ – 270.30.

Specification – Contains 99.0% (w/w) in relation to the desiccated substance.

Description – Orange-yellow or brown crystallized mass. Deliquescent.

Physical characteristic – Melting temperature: approximately 37 °C.

Conservation – In tightly closed containers.

Storage – Protect from light.

Ferric chloride RS (approximately 0.4 M)

Use ferric chloride TS.

Acid ferric chloride RS

Preparation – Dissolve 15 mg of ferric chloride hexahydrate in 20 mL of mixture of glacial acetic acid and sulfuric acid (1:1).

Methanolic ferric chloride

Preparation – Dissolve 1 g of ferric chloride in 100 mL of methyl alcohol.

Mercuric chloride RS (approximately 0.2 M)

Specification – Contains 5.4 g of mercury(II) chloride in 100 mL of aqueous solution.

Conservation – In tightly closed containers.

Safety – Toxic. Pollutant.

Platinic chloride RS

Synonym – Platinum chloride RS.

Preparation – Dissolve 2.6 g of chloroplatinic acid in water and complete the volume to 20 mL.

Conservation – In tightly closed containers.

Storage – Protect from light.

Benzoyl hydrochloride

CAS – [98-88-4].

Molecular formula and molar mass – C_7H_5ClO – 140.57.

Description – Colorless liquid. Decomposes in water and in ethyl alcohol.

Physical characteristics – Density (20 °C): approximately 1.21. Boiling temperature: approximately 197 °C.

(2-chloroethyl)diethylamine hydrochloride

CAS – [869-24-9].

Molecular formula and molar mass – $C_6H_{14}ClN.HCl$ – 172.10.

Description – White crystalline powder.

Solubility – Very soluble in water and in methyl alcohol, freely soluble in methylene chloride, practically insoluble in *n*-hexane.

Physical characteristic – Melting temperature: approximately 211 °C.

Dimethyl-*p*-phenylenediamine hydrochloride

CAS – [536-46-9].

Synonym – *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride.

Molecular formula and molar mass – $C_8H_{12}N_2.2HCl$ – 209.12.

Description – White or nearly white crystalline powder. Hygroscopic.

Solubility – Freely soluble in water and soluble in ethyl alcohol.

Conservation – In tightly closed containers.

O-phenylenediamine hydrochloride

CAS – [615-28-1].

Synonym – 1,2-benzenediamine dihydrochloride.

Molecular formula and molar mass – $C_6H_8N_2.2HCl$ – 181.06.

Description – White or slightly pink powder.

P-phenylenediamine hydrochloride

CAS – [624-18-0].

Synonym – 1,4-benzenediamine dihydrochloride.

Molecular formula and molar mass – $C_6H_8N_2.2HCl$ – 181.14.

Description – White crystalline powder, becomes reddish by exposure to air.

Solubility – Freely soluble in water, slightly soluble in ethyl alcohol and in ethyl ether.

Phenylhydrazine hydrochloride

CAS – [59-88-1].

Molecular formula and molar mass – $C_6H_8N_2.HCl$ – 144.60.

Description – White or nearly white crystalline powder, becomes brown by exposure to air.

Physical characteristic – Melting temperature: approximately 245 °C, with decomposition.

Solubility – Soluble in water and in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protected from light.

Phenylhydrazine hydrochloride RS

Preparation – Dissolve 0.9 g of phenylhydrazine hydrochloride in 50 mL of water. Decolor with activated charcoal and filter. Collect the filtrate in a 250 mL volumetric flask, add 30 mL of hydrochloric acid and complete the volume with water.

Hydrastine hydrochloride

CAS – [5936-28-7].

Molecular formula and molar mass – $C_{21}H_{22}ClNO_6$ – 419.86.

Description – White or nearly white powder. Hygroscopic.

Physical characteristic – Rotation power (17 °C): approximately +127°. Melting temperature: approximately 116 °C.

Solubility – Very soluble in water and in ethyl alcohol.

Hydroxylamine hydrochloride

CAS – [5470-11-1].

Molecular formula and molar mass – NH_4ClO – 69.49.

Specification – Contains no less than 96.0% (w/w).

Description – Colorless crystals or white crystalline powder.

Physical characteristic – Melting temperature: approximately 151 °C.

Solubility – Very soluble in water and soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from humidity.

Hydroxylamine hydrochloride RS

Preparation – Dissolve 5 g in 5 mL of hot water. Complete the volume to 100 mL with ethyl alcohol.

Conservation – In tightly closed containers.

Safety – Flammable.

Hydroxylamine hydrochloride RS1

Preparation – Dissolve 20 g of hydroxylamine hydrochloride in distilled water to obtain, approximately, 65 mL. Transfer to separation funnel. Add five drops of thymol blue TS and add ammonium hydroxide until the solution acquires a yellow color. Add 10 mL of 4% (w/v) aqueous sodium diethyldithiocarbamate solution, shake and allow to stand for five minutes. Extract this solution with successive portions of 10 mL to 15 mL of chloroform until a portion of 5 mL of the chloroform extract does not acquire a yellow color when stirred with 12.5% (w/v) copper sulfate. Add hydrochloric acid 3 M until obtaining a pink color (if necessary, add one or two drops of thymol blue TS) and dilute to 100 mL with distilled water.

Chlorine RS

Specification – Saturated chlorine solution in water.

Conservation – In completely full and tightly closed containers.

Storage – In a cool place, protected from light and air.

Stability – Prepare the solution immediately before use.

Additional information – The solution tends to deteriorate even if protected from light and air.

p-Chloroacetanilide

CAS – [539-03-7].

Molecular formula and molar mass – C_8H_8ClNO – 169.61.

Description – Crystalline powder.

Physical characteristic – Melting temperature: approximately 178 °C.

Solubility – Practically insoluble in water and soluble in ethyl alcohol.

Chlorobenzene

CAS – [108-90-7].

Molecular formula and molar mass – $\text{C}_6\text{H}_5\text{Cl}$ – 112.56.

Description – Colorless, refringent liquid, with characteristic odor.

Physical characteristics – Boiling temperature: approximately 132 °C.

Density: approximately 1.11. Refractive index (20 °C): 1.5251.

Conservation – In tightly closed containers.

Safety – Toxic. Flammable.

1-Chloro-2,4-dinitrobenzene

CAS – [97-00-7].

Molecular formula and molar mass – $\text{C}_6\text{H}_3\text{ClN}_2\text{O}_4$ – 202.55.

Description – Pale yellow crystals or crystalline powder.

Physical characteristic – Melting temperature: approximately 51 °C.

Chloroform

Synonym – Trichloromethane.

Molecular formula and molar mass – CHCl_3 – 119.37.

Specification – Contains no less than 99.9% (w/w).

Description – Mobile, colorless liquid, with sweet odor.

Physical characteristics – Density: approximately 1.48.

Boiling temperature: approximately 62 °C.

Conservation – In tightly closed containers.

Safety – Toxic.

Alcohol-free chloroform

Preparation – Prepare immediately before use. Shake, carefully, 20 mL of chloroform with 20 mL of water for three minutes. Remove the organic phase carefully and wash with two portions of 20 mL of water. Filter chloroform in dry paper. Add 5 g of anhydrous sodium sulfate, shake for five minute and allow to stand for two hours. Decant or filter.

Chlorothiazide

CAS – [58-94-6].

Molecular formula and molar mass – $\text{C}_7\text{H}_6\text{ClN}_3\text{O}_4\text{S}_2$ – 295.73.

Description – White or nearly white, odorless crystalline powder.

Physical characteristic – Melting temperature: approximately 340 °C, with decomposition.

Solubility – Very slightly soluble in water, moderately soluble in acetone, slightly soluble in ethyl alcohol. Soluble in diluted alkali hydroxide solutions.

Sodium cobaltnitrite

CAS – [13600-98-1].

Molecular formula and molar mass – $\text{Na}_3\text{CoN}_6\text{O}_{12}$ – 403.93.

Description – Orange-yellow crystalline powder.

Solubility – Freely soluble in water and slightly soluble in ethyl alcohol.

Conservation – In tightly closed containers

Sodium cobaltnitrite RS

Specification – Contains 10 g of sodium cobaltnitrite in 100 mL of water.

Conservation – Prepare immediately before use.

Copper

CAS – [7440-50-8].

Element and atomic mass – Cu – 63.546.*Description* – Slide, thread, powder or fragment, of reddish color and metallic luster.*Conservation* – In non-metallic containers.**Copper RSA – 1 mg/mL***Specification* – Contains 1 g of copper dissolved in the lowest volume possible of 50% (v/v) nitric acid. Complete the volume to 1000 mL with 1% (v/v) nitric acid.*Conservation* – In tightly closed, inert containers (like polyethylene).**o-Cresol**

CAS – [95-48-7].

Synonym – 2-Methylphenol.*Molecular formula and molar mass* – C₇H₈O – 108.14.*Description* – Colorless to brown-yellow liquid or solid, which acquires color by light and in the presence of oxygen, of phenolic odor. Deliquescent.*Physical characteristics* – Melting temperature: approximately 30 °C.

Boiling temperature: approximately 191 °C.

Density: approximately 1.03. Refractive index (20 °C): 1.540 – 1.550.

Solubility – Moderately soluble in water and soluble in alkali hydroxide solutions; miscible with ethyl alcohol.*Conservation* – In hermetic containers.*Storage* – Protect from light, humidity and oxygen. *Safety* – Irritant. Caustic. Toxic.*Category* – Disinfectant.**Potassium chromate**

CAS – [7789-00-6].

Molecular formula and molar mass – K₂CrO₄ – 194.19.*Specification* – Contains no less than 99.0% (w/w) in relation to the desiccated substance.*Description* – Crystals or yellow crystalline powder.*Solubility* – Freely soluble in water.*Conservation* – In tightly closed containers.*Safety* – Oxidizing agent. Pollutant.**Potassium chromate RS***Specification* – Contains 10% (w/v) of potassium chromate in water.*Conservation* – In tightly closed containers.*Safety* – Oxidizing agent. Pollutant.**Disodium chromotropate**

CAS – [5808-22-0].

Synonym – Chromotropic acid dihydrate disodium salt.*Molecular formula and molar mass* – C₁₀H₆Na₂O₈S₂·2H₂O – 400.28.*Description* – Yellow-white powder.*Solubility* – Soluble in water and practically insoluble in ethyl alcohol.**Sodium deoxycholate**

CAS – [302-95-4].

Molecular formula and molar mass – C₂₄H₃₉NaO₄ – 414.56.*Description* – White or nearly white crystalline powder.

Dextran

CAS – [9004-54-0]

Description – Powder.

Solubility – Soluble in water.

Dextran 5%

Preparation – Weigh 5 g of dextran and transfer to a 100 mL volumetric flask. Add distilled water in sufficient amount for 100 mL. If possible, maintain the sterile solution using a sterilizing filtration system with 0.22 µm pore filters.

Dextrose

Use glucose.

0.1% (w/v) dextrose

Use 0.1% (w/v) glucose in pyridine.

Chlorhexidine diacetate

Use chlorhexidine acetate.

1,8-Diaminonaphthalene

CAS – [479-27-6].

Synonym – 1,8-Naphthalenediamine.

Molecular formula and molar mass – C₁₀H₁₀N₂– 158.20.

Description – Sublimable crystals.

Physical characteristic – Melting range: 63 °C to 67 °C.

Meglumine sodium diatrizoate

CAS – [8064-12-8]

Molecular formula – C₁₁H₉I₃N₂O₄.C₁₁H₉I₃N₂O₄.C₇H₁₇NO₅.Na

Diaveridine

CAS – [5355-16-8].

Synonym – 5-[(3,4-Dimethoxyphenyl)methyl]-2,4- pyrimidinediamine.

Molecular formula and molar mass – C₁₃H₁₆N₄O₂– 260.30.

Physical characteristic – Melting temperature: approximately 233 °C.

2,6-Dibromoquinone-4-chlorimide

CAS – [537-45-1].

Molecular formula and molar mass – C₆H₂Br₂ClNO – 299.35.

Description – Yellow crystalline powder.

Physical characteristic – Melting range: between 82 °C and 84 °C.

Solubility – Insoluble in water and soluble in ethyl alcohol and in diluted alkali hydroxide solutions.

Conservation – In closed containers.

Dibutylamine

CAS – [111-92-2].

Molecular formula and molar mass – C₈H₁₉N – 129.25.

Description – Colorless liquid.

Physical characteristics – Boiling temperature: approximately 159 °C.

Refractive index (20 °C): approximately 1.417.

Solubility – Soluble in water and in ethyl alcohol.

Conservation – In tightly closed containers.

Ethylene dichloride

CAS – [107-06-2].

Synonym – 1,2-Dichloroethane.

Molecular formula and molar mass – $C_2H_4Cl_2$ – 98.96.

Description – Colorless and clear liquid, with odor similar to chloroform.

Physical characteristics – Boiling temperature: approximately 83 °C.

Density (20 °C): approximately 1.25. Refractive index (20 °C): 1.444.

Solubility – slightly soluble in water and freely soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Safety – Irritant. Toxic. Flammable.

N-(1-naphthyl)ethylenediamine dihydrochloride

CAS – [1465-25-4].

Synonyms – N-1-naphthalenyl-1,2-ethanediamine dihydrochloride.

Molecular formula and molar mass – $C_{12}H_{14}N_2 \cdot 2HCl$ – 259.18.

Description – White or yellow-white powder.

Physical characteristic – Melting range: 188 °C to 190 °C.

Solubility – Soluble in water and slightly soluble in ethyl alcohol.

N-(1-naphthyl)ethylenediamine dihydrochloride RS

Synonym – Bratton-Marshall reagent.

Preparation – Dissolve 0.1 g of N-(1-naphthyl)ethylenediamine dihydrochloride in 100 mL of water.

Conservation – In tightly closed containers.

2,6-Dichloroquinone-4-chlorimide

CAS – [101-38-2].

Synonyms – Gibbs reagent, 2,6-dichloro-4-(chloroimino)-2,5-cyclohexadien-1-one.

Molecular formula and molar mass – $C_6H_2Cl_3NO$ – 210.45.

Description – Orange or yellow crystalline powder.

Physical characteristic – Melting temperature: approximately 66 °C.

Solubility – Practically insoluble in water, soluble in ethyl alcohol and in diluted alkaline solutions.

1-(2,6-Dichlorophenyl)-1,3-dihydro-2H-indol-2-one (Impurity A of diclofenac)

CAS – [15362-40-0].

Synonym – 1-(2,6-dichlorophenyl)indolin-2-one.

Molecular formula and molar mass – $C_{14}H_9Cl_2NO$ – 278.14.

Description – White crystalline powder.

Conservation – In tightly closed containers.

Storage – Protect from exposure to light.

2,6-Dichloroindophenol sodium

CAS – [620-45-1].

Synonym – 2,6-dichlorophenol-indophenol sodium.

Molecular formula and molar mass – $C_{12}H_6Cl_2NNaO_2$ – 290.08.

Description – Dark green powder. The aqueous solution presents a dark blue color and turns pink when acidified.

Solubility – Freely soluble in water and in ethyl alcohol.

Conservation – In tightly closed containers.

Potassium dichromate

CAS – [7778-50-9].

Molecular formula and molar mass – $\text{K}_2\text{Cr}_2\text{O}_7$ – 294.18.

Specification – Contains no less than 99.8% (w/w) in relation to the desiccated substance.

Description – Orange-red crystals. Odorless.

Solubility – Soluble in water and practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

Safety – Caustic. Oxidizing agent. Pollutant.

Potassium dichromate RS

Specification – Contains 5% (w/v) of potassium dichromate in water.

Conservation – In tightly closed containers.

Safety – Caustic. Oxidizing agent. Pollutant.

Diethylamine

CAS – [109-89-7].

Molecular formula and molar mass – $\text{C}_4\text{H}_{11}\text{N}$ – 73.14.

Description – Clear, colorless, volatile liquid, with ammoniacal odor, strongly alkaline.

Physical characteristics – Boiling range: 55 °C to 58 °C. Refractive index (20 °C): 1.386.

Density (20 °C): approximately 0.707.

Miscibility – Miscible with water and ethyl alcohol.

Conservation – In tightly closed containers.

Safety – Irritant. Flammable.

Diethylaminoethyl-dextran

CAS – [9015-73-0].

Molecular formula and molar mass – $\text{C}_{12}\text{H}_{28}\text{N}_2\text{O}$ – 216.37.

Description – Powder.

Solubility – Soluble in water.

Silver diethyldithiocarbamate

CAS – [1470-61-7].

Molecular formula and molar mass – $\text{C}_5\text{H}_{10}\text{AgNS}_2$ – 256.13.

Description – Light yellow to greyish-yellow powder.

Solubility – Practically insoluble in water and soluble in pyridine.

Conservation – In tightly closed containers.

Silver diethyldithiocarbamate RS

Specification – Contains 0.5% (w/v) of silver diethyldithiocarbamate in pyridine.

Stability – Prepare immediately before use.

Safety – Toxic.

Sodium diethyldithiocarbamate

CAS – [20624-25-3].

Molecular formula and molar mass – $\text{C}_5\text{H}_{10}\text{NNaS}_2 \cdot 3\text{H}_2\text{O}$ – 225.30.

Description – White, nearly white or colorless crystals.

Solubility – Freely soluble in water and soluble in ethyl alcohol.

N,N-diethylethylenediamine

CAS – [100-36-7].

Synonym – N,N-Diethyl-1,2-diaminoethane.

Molecular formula and molar mass – $C_6H_{16}N_2$ – 116.21.

Description – Liquid with slightly oily appearance, colorless or slightly yellow, with strong ammoniacal odor, irritant to the eyes, skin and mucous membranes.

Physical characteristics – Density (20 °C): 0.827. Boiling range: 145 °C to 147 °C.

Water (5.2.20.1) – Determine in 0.5 g. No more than 1.0%.

Diethyl phthalate

CAS – [84-66-2].

Molecular formula and molar mass – $C_{12}H_{14}O_4$ – 222.24.

Description – Colorless and practically odorless oily liquid.

Specification – Contains no less than 99.0% (w/w).

Physical characteristics – Density: 1.118. Boiling temperature: 295 °C.

Miscibility – Miscible with water, ethyl alcohol, ethyl ether and other organic solvents. *Conservation* – In tightly closed containers.

Safety – Irritant.

Diphenylamine

CAS – [122-39-4].

Molecular formula and molar mass – $C_{12}H_{11}N$ – 169.23.

Description – White or nearly white crystals.

Physical characteristics – Melting temperature: approximately 55 °C. Boiling temperature: 302 °C. Loses color in presence of light.

Solubility – Slightly soluble in water and soluble in ethyl alcohol. Forms salt in solution with strong acids.

Conservation – In tightly closed containers.

Storage – Protect from light.

Diphenylamine RS

Preparation – Dissolve 1 g of diphenylamine in 100 mL of sulfuric acid.

Conservation – In tightly closed containers.

Storage – Protect from exposure to light.

Diphenylbenzidine

CAS – [531-91-9].

Synonym – *N,N'*-Diphenylbenzidine.

Molecular formula and molar mass – $C_{24}H_{20}N_2$ – 336.44.

Description – White or slightly gray crystalline powder.

Physical characteristic – Melting temperature: approximately 248 °C.

Solubility – Practically insoluble in water, slightly soluble in ethyl alcohol.

Conservation – In closed containers.

Storage – Protect from exposure to light.

Aminoethanol diphenylborate

CAS – [524-95-8].

Molecular formula and molar mass – $C_{14}H_{16}BNO$ – 225.10.

Description – White or yellow crystalline powder.

Physical characteristic – Melting temperature: approximately 193 °C.

Solubility – Practically insoluble in water and soluble in ethyl alcohol.

Aminoethanol diphenylborate RS

Preparation – Dissolve 1 g of aminoethanol diphenylborate in methyl alcohol and complete the volume to 100 mL with the same solvent.

Diphenylcarbazine

CAS – [140-22-7].

Molecular formula and molar mass – $C_{13}H_{14}N_4O$ – 242.28.*Description* – White crystalline powder; becomes pink by exposure to air.*Physical characteristic* – Melting range: 168 °C to 171 °C.*Solubility* – Very slightly soluble in water, soluble in acetone, in ethyl alcohol, and in glacial acetic acid.*Conservation* – In hermetic containers.*Storage* – Protect from light and from air.**Diphenylcarbazine RS***Specification* – Contains 1% (w/v) of diphenylcarbazine in ethyl alcohol.*Conservation* – In tightly closed containers.*Storage* – Protect from light.*Safety* – Flammable.**Diphenylcarbazone**

CAS – [538-62-5].

Molecular formula and molar mass – $C_{13}H_{12}N_4O$ – 240.27.*Description* – Crystals with red-orange color.*Physical characteristic* – Melting temperature: approximately 157 °C, with decomposition.*Solubility* – Practically insoluble in water and freely soluble in ethyl alcohol.*Conservation* – In tightly closed containers.**Diphenylcarbazone-bromophenol blue RS***Preparation* – In a 25 mL volumetric flask, dissolve 12 mg of diphenylcarbazone and 12.5 mg of bromophenol blue in 15 mL of ethyl alcohol. Complete the volume with ethyl alcohol.*Conservation* – Keep the solution in an amber glass container at temperature between 4 °C and 8 °C.**Mercuric diphenylcarbazone RS***Solution A* – Dissolve 0.1 g of diphenylcarbazone in ethyl alcohol and complete the volume to 50 mL with the same solvent.*Solution B* – Dissolve 1 g of mercury(II) chloride in ethyl alcohol and complete the volume to 50 mL with the same solvent.*Preparation* – Mix equal volumes of *Solutions A* and *B* at the moment of using.***N,N'*-Diisopropylethylenediamine**

CAS – [4013-94-9]

Molecular formula and molar mass – $C_8H_{20}N_2$ – 144.26.*Description* – Colorless or yellow liquid. Corrosive, flammable and hygroscopic.*Physical characteristics* – Density (20 °C): approximately 0.798. Refractive index (20 °C): approximately 1.429. Boiling temperature: approximately 170 °C.**Dimethylacetamide**

CAS – [127-19-5].

Molecular formula and molar mass – C_4H_9NO – 87.12.*Description* – Colorless and clear liquid.*Physical characteristics* – Boiling temperature: approximately 165 °C.

Refractive index (20 °C): approximately 1.437. Density (20 °C): approximately 0.94.

Miscibility – Miscible with water and with most organic solvents.

Conservation – In closed containers.

***p*-Dimethylaminobenzaldehyde**

CAS – [100-10-7].

Synonyms – 4-Dimethylaminobenzaldehyde and Ehrlich's Reagent.

Molecular formula and molar mass – C₉H₁₁NO – 149.19.

Description – White to slightly yellow crystalline powder.

Physical characteristic – Melting temperature: approximately 74 °C.

Solubility – Soluble in ethyl alcohol and in diluted acid solutions.

Conservation – In tightly closed containers.

Storage – Protect from light.

***p*-Dimethylaminobenzaldehyde RS**

Preparation – Dissolve, without heating, 0.2 g of *p*-dimethylaminobenzaldehyde in a mixture of 4.5 mL of water and 5.5 mL of hydrochloric acid. Prepare immediately before use.

***p*-Dimethylaminobenzaldehyde RS1**

Preparation – Dissolve 0.2 g of *p*-dimethylaminobenzaldehyde in 20 mL of ethyl alcohol and add 0.5 mL of hydrochloric acid. Shake the solution with activated charcoal and filter. The solution color is less intense than of a freshly prepared iodine solution at 0.0001 *M*. Use immediately after preparation.

***p*-Dimethylaminobenzaldehyde RS2**

Synonym – Wasicky Reagent.

Preparation – Dissolve 0.5 g of *p*-dimethylaminobenzaldehyde in 8.5 mL of sulfuric acid and carefully add 8.5 mL of water.

4-(Dimethylamino)cinnamaldehyde

CAS – [6203-18-5].

Molecular formula and molar mass – C₁₁H₁₃NO – 175.23.

Description – Orange or orange-brown crystals or powder.

Physical characteristic – Melting temperature: approximately 138 °C.

Solubility – Soluble in ethyl alcohol, acetone and benzene.

2,6-Dimethylaniline

CAS – [87-62-7].

Synonym – 2,6-Xylidine.

Molecular formula and molar mass – C₈H₁₁N – 121.18.

Description – Colorless liquid.

Physical characteristic – Density (20 °C): approximately 0.98.

***N,N*-Dimethylaniline**

CAS – [121-69-7].

Synonym – *N,N*-Dimethylbenzenamine.

Molecular formula and molar mass – C₈H₁₁N – 121.18.

Description – Clear, practically colorless oily liquid, turns dark during the storage.

Physical characteristic – Distillation range: 192 °C to 194 °C.

Solubility – Practically insoluble in water, freely soluble in ethyl alcohol and ethyl ether.

1,1-Dimethylethylamine

CAS – [75-64-9].

Synonym – *tert*-Butylamine.

Molecular formula and molar mass – $C_4H_{11}N$ – 73.14.

Description – Colorless liquid.

Physical characteristics – Density (20 °C): approximately 0.694.

Refractive index (20 °C): approximately 1.378. Boiling temperature: approximately 46 °C.

2,5-Dimethylphenol

CAS – [95-87-4].

Synonym – *p*-Xylenol.

Molecular formula and molar mass – $C_8H_{10}O$ – 122.17.

Description – White or nearly white crystals.

Physical characteristic – Melting temperature: approximately 74.5 °C.

Dimethylformamide

CAS – [68-12-2].

Molecular formula and molar mass – C_3H_7NO – 73.10.

Description – Clear, colorless liquid, with odor similar to amines.

Physical characteristics – Boiling temperature: approximately 153 °C.

Density: approximately 0.95. Refractive index (20 °C): 1.424. 1.428.

Miscibility – Miscible with water and ethyl alcohol.

Conservation – In tightly closed containers.

Safety – Irritant. Toxic.

Dimethylsulfoxide

CAS – [67-68-5].

Synonym – DMSO.

Molecular formula and molar mass – C_2H_6OS – 78.13.

Description – Colorless and odorless liquid. Hygroscopic.

Physical characteristics – Boiling temperature: approximately 189 °C.

Density: approximately 1.10. Refractive index (20 °C): 1.424. 1.479.

Miscibility – Miscible with water and ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from humidity and exposure to light.

Safety – Irritant.

1,3-Dinitrobenzene

CAS – [99-65-0].

Molecular formula and molar mass – $C_6H_4N_2O_4$ – 168.11.

Description – Yellow crystals.

Physical characteristic – Melting temperature: approximately 89 °C.

Solubility – Practically insoluble in water and slightly soluble in ethyl alcohol.

Conservation – In tightly closed containers.

1,3-Dinitrobenzene RS

Specification – Contains 1% (w/v) of 1,3-dinitrobenzene in ethyl alcohol.

Conservation – Tightly closed container.

Dioxane

CAS – [123-91-1].

Synonyms – 1,4-Dioxane, ethylene dioxide, dioxane.

Molecular formula and molar mass – $C_4H_8O_2$ – 88.11.

Description – Clear, colorless liquid, with odor similar to ethyl ether.

Physical characteristics – Boiling temperature: approximately 101 °C.

Density: approximately 1.03. Refractive index (20 °C): 1.421 to 1.424.

Miscibility – Miscible with water and with most organic solvents.

Conservation – In tightly closed containers.

Safety – Irritant. Toxic. Flammable.

Sulfur dioxide

CAS – [7446-09-5].

Synonym – Sulfur anhydride.

Molecular formula and molar mass – SO₂ – 64.06.

Specification – Contains no less than 97.0% (v/v).

Description – Colorless gas, with characteristic, suffocating odor.

Conservation – In pressurized cylinders.

Safety – Irritant. Toxic.

Manganese dioxide

CAS – [1313-13-9].

Molecular formula and molar mass – MnO₂ – 86.94.

Description – Black or dark brown fine powder.

Conservation – In tightly closed containers.

Storage – Protect from heat.

Safety – Energetic oxidizing agent.

Dipropylene glycol

CAS – [25365-71-8].

Synonym – 1,1'-Oxide-2-propanol.

Molecular formula and molar mass – C₆H₁₄O₃ – 134.18.

Description – Colorless, practically odorless liquid.

Physical characteristics – Density: approximately 1.02. Boiling temperature: approximately 230 °C.

Conservation – In tightly closed containers.

Storage – In properly ventilated places.

Carbon disulfide

CAS – [75-15-0].

Molecular formula and molar mass – CS₂ – 76.14.

Description – Colorless or yellow liquid.

Physical characteristics – Density (20 °C): approximately 1.26. Boiling range: 46 °C to 47 °C.

Solubility – Practically insoluble in water and miscible with ethyl alcohol.

Conservation – In tightly closed containers.

Safety – Poisonous! Flammable.

Dithiol

CAS – [496-74-2].

Synonyms – 1,2-Dimercapto-4-methylbenzene; toluene-3,4-dithiol.

Molecular formula and molar mass – C₇H₈S₂ – 156.27.

Description – White or nearly white crystals.

Physical characteristic – Melting temperature: 31 °C.

Solubility – Soluble in methyl alcohol and in alkali hydroxide solutions.

Dithiol RS

Specification – Contains 0.5 g of dithiol in 100 mL of ethyl alcohol.

Stability – Prepare immediately before use.

Safety – Flammable.

Dithiothreitol

CAS – [3483-12-3].

Molecular formula and molar mass – $C_4H_{10}O_2S_2$ – 154.24.

Description – White crystals.

Solubility – Freely soluble in water, in acetone and in ethyl alcohol.

Conservation – In tightly closed containers.

Dithizone

CAS – [60-10-6].

Synonym – Diphenylthiocarbazone.

Molecular formula and molar mass – $C_{13}H_{12}N_4S$ – 256.33.

Specification – Contains no less than 98.0% (w/w).

Description – Dark brown crystalline powder.

Physical characteristic – Melting temperature: 168 °C, with decomposition.

Solubility – Practically insoluble in water and soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from exposure to light.

Dithizone RS

Specification – Contains 0.05% (w/v) of dithizone in carbon tetrachloride.

Conservation – In hermetic containers.

Storage – Protect from heat.

Safety – Poison!

Dithizone, concentrated solution

Preparation – Dissolve 35 mg of dithizone in 80 mL of chloroform. Transfer to a 500 mL volumetric flask and complete the volume with chloroform.

Conservation – In closed amber containers.

Storage – Protect from exposure to light and keep at temperature of 4 °C to 8 °C.

Stability – This solution is stable for five months.

Dithizone, diluted solution

Preparation – Dilute the concentrated dithizone solution in chloroform (1:7).

Dithizone, extractor solution

Preparation – Dissolve 30 mg of dithizone in 1000 mL of chloroform and add 5 mL of ethyl alcohol. Before use, shake an adequate volume of extractor dithizone solution with half its volume of nitric acid 1% (v/v) discarding the acid phase.

Storage – In refrigerator.

Disodium edetate

CAS – [6381-92-6].

Synonyms – EDTA disodium; Disodium salt, dihydrate, of (ethylenedinitrilo)acetic acid

Molecular formula and molar mass – $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$ – 372.24.

Specification – Contains no less than 97.0% (w/w) in relation to the desiccated substance.

Description – White crystalline powder, of faint saline flavor.

Solubility – Soluble in water and practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

Category – Chelating agent.

Disodium edetate, solution 0.05 M

Specification – Contains 1.861 g, added with 10 mL of sodium hydroxide *M*, and dilute in water to 100 mL.

Conservation – In tightly closed containers.

Emodin

CAS – [518-82-1]

Molecular formula and molar mass – $C_{15}H_{10}O_5$ – 270.25

Description – Orange-red needles.

Solubility – Practically insoluble in water, soluble in ethyl alcohol and in alkali hydroxide solutions.

Sulfur

CAS – [7704-34-9]

Element and atomic mass – S – 32.1

Description – Fine grayish yellow or greenish yellow powder.

Aescin

CAS – [11072-93-8]

Specification – Mixture of saponins obtained from *Aesculus hippocastanum* L. seeds.

Description – Amorphous, fine, nearly white, red or yellow powder.

Metallic tin

CAS – [7440-31-5]

Element and atomic mass – Sn – 118.71

Specification – Purity of no less than 99.5%.

Description – Gray granules.

Physical characteristic – Melting temperature: approximately 231.9 °C.

Conservation – In tightly closed containers.

Storage – Protect from exposure to light and from heat.

Safety – Irritant.

Methyl stearate

CAS – [112-61-8]

Molecular formula and molar mass – $C_{19}H_{38}O_2$ – 298.50

Description – White crystals or white or pale yellow crystalline mass.

Physical characteristic – Melting temperature: approximately 38 °C.

Solubility – Soluble in ethyl alcohol and petroleum ether.

Conservation – In tightly closed containers.

Tetrabromophenolphthalein, ethyl ester

CAS – [1176-74-5]

Synonym – Bromophenolphthalein magenta E.

Molecular formula and molar mass – $C_{22}H_{14}Br_4O_4$ – 661.96

Erythromycin estolate

CAS – [3521-62-8]

Molecular formula and molar mass – $C_{52}H_{97}NO_{18}S$ – 1056.43.

Physical characteristic – Melting range: 135 °C to 138 °C.

Solubility – Practically insoluble in water, freely soluble in ethyl alcohol, soluble in acetone. It is practically insoluble in diluted hydrochloric acid.

Conservation – In hermetic containers.

Storage – Protect from light and heat.

Therapeutic class – Antibacterial.

Strontium RSA – 1 mg/mL

Specification – Contains 1.685 g of strontium carbonate in 10 mL of 50% (v/v) hydrochloric acid. Complete the volume to 1000 mL with water.

Conservation – In tightly closed, inert containers (like polyethylene).

Petroleum ether

CAS – [8032-32-4].

Synonym – Benzine.

Description – Clear, colorless, volatile liquid, with characteristic odor. Non-fluorescent.

Physical characteristics – Boiling range: 40 °C to 60 °C. Density: 0.630 to 0.656.

Solubility – Practically insoluble in water; miscible with ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from heat.

Safety – Flammable.

Ethyl ether

CAS – [60-29-7].

Molecular formula and molar mass – C₄H₁₀O – 74.12

Specification – Contains no less than 96.0% (v/v).

Description – Clear, colorless, very volatile liquid, with characteristic pungent odor.

Hygroscopic. *Physical characteristics* – Boiling temperature: approximately 35 °C. Density: approximately 0.715. Refractive index (20 °C): 1.355.

Miscibility – Miscible with water and ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from light and from heat (do not exceed the temperature of 15 °C).

Category – Anesthetic.

Safety – Flammable. Risk of explosion.

Isopropyl ether

CAS – [108-20-3].

Synonym – Diisopropyl ether.

Molecular formula and molar mass – C₆H₁₄O – 102.18.

Description – Colorless and clear liquid.

Physical characteristics – Boiling range: 67 °C to 69 °C. Density (20 °C): 0.723 to 0.728.

Solubility – Very slightly soluble in water; miscible with ethyl alcohol.

Conservation – In closed containers.

Storage – Protect from light.

Safety – Flammable.

Tert-butyl methyl ether

CAS – [1634-04-4].

Synonym – MTBE.

Molecular formula and molar mass – (CH₃)₃COCH₃ – 88.15.

Description: Colorless volatile liquid.

Miscibility: Miscible with water.

Safety – Flammable.

Ethylene glycol

CAS – [107-21-1].

Synonym – 1,2-Ethanediol.

Molecular formula and molar mass – C₂H₆O₂ – 62.07.

Description – Colorless, viscous liquid.

Physical characteristics – Boiling temperature: approximately 196 °C. Density (20 °C): 1.113 to 1.115.

Miscibility – Miscible with water and ethyl alcohol.

Ethylparaben

CAS – [120-47-8].

Molecular formula and molar mass – C₉H₁₀O₃ – 166.18.

Description – Small and colorless crystals or white powder.

Physical characteristics – Melting temperature: 116 °C. Boiling range: 297 °C to 298 °C, with decomposition.

Solubility – Slightly soluble in water, freely soluble in acetone, ethyl alcohol and ethyl ether.

Conservation – In closed containers.

Category – Preservative.

Eugenol

CAS – [97-53-0].

Molecular formula and molar mass – C₁₀H₁₂O₂ – 164.20.

Description – Colorless or slightly yellow oily liquid. Gets dark and becomes more viscous with the exposure to light and contact with air.

Physical characteristics – Density (20 °C): approximately 1.07. Boiling temperature: approximately 250 °C.

Solubility – Practically insoluble in water; miscible with ethyl alcohol, fatty oils and essential oils.

Fast green (CI 42053)

CAS – [2353-45-9].

Molecular formula and molar mass – C₃₇H₃₄N₂Na₂O₁₀S₃ – 808.86.

Description – Dark green powder or granule, with metallic luster.

Solubility – Soluble in water and moderately soluble in ethyl alcohol.

Conservation – In closed containers.

Blood coagulation factor Xa, bovine

Specification – Enzyme that enables the conversion of prothrombin into thrombin. The semi-purified substance is obtained from liquid bovine plasma and is prepared by activation of the Factor X zymogen through an appropriate agent, such as Russel viper venom.

Storage – The lyophilized preparation must be stored at a temperature of -20 °C. The frozen preparation must be stored at a temperature lower than -20 °C.

Bovine factor Xa, solution

Preparation – Reconstitute, according to instructions from the manufacturer, and dilute with tromethamine - sodium chloride buffer of pH 7.4.

Absorbance (5.2.14) – Any modification to the absorbance of the solution at 405 nm, using the tromethamine - sodium chloride buffer with pH 7.4 as blank, is not superior to 0.15-0.20 per minute.

1,10-Phenanthroline

CAS – [5144-89-8].

Synonym – Orthophenanthroline.

Molecular formula and molar mass – C₁₂H₈N₂.H₂O – 198.23.

Description – White crystalline powder.

Physical characteristic – Melting range: 100 °C to 104 °C.

Solubility – Slightly soluble in water, soluble in acetone and in ethyl alcohol.

Category – Indicator for redox systems; reagent for colorimetry.

DL-Phenylalanine

CAS – [150-30-1].

Molecular formula and molar mass – $C_9H_{11}NO_2$ – 165.19.

Specification – Contains no less than 99.0%.

Description – Monoclinic crystals.

Physical characteristic – Sublimates in vacuum.

Phenol

CAS – [108-95-2].

Molecular formula and molar mass – C_6H_6O – 94.11.

Specification – Contains no less than 98.0% (w/w).

Description – Crystalline mass or colorless, faintly pink or yellow crystals, with characteristic odor. Deliquescent.

Physical characteristics – Melting temperature: approximately 43 °C. Boiling temperature: approximately 180 °C.

Solubility – Soluble in water, very soluble in ethyl alcohol, in glycerol and in methylene chloride.

Conservation – In hermetic containers.

Storage – Protect from light and from heat.

Labeling – It must indicate the name and amount of stabilizer.

Category – Disinfectant.

Safety – Caustic. Toxic.

Phenolphthalein

CAS – [77-09-8].

Molecular formula and molar mass – $C_{20}H_{14}O_4$ – 318.33.

Specification – Contains no less than 97.0% (w/w) in relation to the desiccated substance.

Description – Crystalline or amorphous powder, white or slightly yellow. Odorless.

Physical characteristic – Melting temperature: approximately 258 °C.

Solubility – Practically insoluble in water and soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Category – Acid-base indicator.

Phenolphthalein 0.1% (w/v)

Specification – Contains 0.1% (v/v) of phenolphthalein in ethyl alcohol 80% (v/v).

Conservation – In tightly closed containers.

Safety – Flammable.

Additional information – For preparation of indicator paper.

2-Phenoxyethanol

CAS – [122-99-6].

Molecular formula and molar mass – $C_8H_{10}O_2$ – 138.17.

Description – Colorless, slightly viscous liquid, with faint aromatic odor and ardent flavor.

Physical characteristics – Density (20 °C): approximately 1.11. Boiling temperature: approximately 245 °C. Refractive index (20 °C): 1.534.

Solubility – Slightly soluble in water, freely soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Category – Preservative.

Potassium ferricyanide

CAS – [13746-66-2].

Molecular formula and molar mass – $K_3Fe(CN)_6$ – 329.25.

Specification – Contains no less than 99.9% (w/w) in relation to the desiccated substance.

Description – Red crystals.

Solubility – Freely soluble in water. *Conservation* – In tightly closed containers.

Storage – Protect from light.

Potassium ferricyanide RS

Specification – Contains 5% (w/v) of potassium ferricyanide in water.

Conservation – Prepare immediately before use.

Storage – Protect from light.

Ammoniacal potassium ferricyanide

Preparation – Dissolve 2 g of potassium ferricyanide in 75 mL of water. Add 25 mL of ammonium hydroxide and homogenize.

Potassium ferricyanide

CAS – [14459-95-1].

Molecular formula and molar mass – $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$ – 422.39.

Specification – Contains no less than 99.0% (w/w) in relation to the desiccated substance.

Description – Clear crystals or yellow crystalline powder. Efflorescent. Becomes anhydrous at 100 °C.

Solubility – Freely soluble in water and practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

Potassium ferricyanide RS

Specification – Contains 5.3% (w/v) of potassium ferricyanide in water (approximately 0.125 M).

Conservation – Prepare immediately before use.

Fibrinogen

CAS – [9001-32-5].

Refer to the *Lyophilized human fibrinogen* monograph.

Phloroglucin RS

Preparation – Dissolve 1 g of phloroglucinol in ethyl alcohol and dilute to 100 mL with the same solvent.

Conservation – In tightly closed containers.

Storage – Protect from light.

Phloroglucinol

CAS – [6099-90-7].

Molecular formula and molar mass – $\text{C}_6\text{H}_6\text{O}_3 \cdot 2\text{H}_2\text{O}$ – 162.14.

Description – White or light yellow crystals or crystalline powder.

Solubility – Slightly soluble in water and soluble in ethyl alcohol and ethyl ether.

Simulated gastric fluid

Preparation – Dissolve 2 g of sodium chloride and 3.2 g of purified pepsin in 7 mL of hydrochloric acid and complete the volume to 1000 mL with water. It presents pH of approximately 1.2.

Simulated gastric fluid (without enzyme)

Preparation – Dissolve 2 g of sodium chloride in 100 mL of water. Add 7 mL of hydrochloric acid and dilute to 1000 mL with water. Adjust the pH to 1.2 ± 0.1 with hydrochloric acid or sodium hydroxide 10 M.

Simulated intestinal fluid with pancreatin, pH 6.8

Preparation – dissolve 0.68 g of monobasic potassium phosphate in 25 mL of water, mix and add 7.7 mL of 0.2 M sodium hydroxide and 50 mL of water. Add 1 g of pancreatin, mix and adjust the pH to 6.8 ± 0.1 with 0.2 M sodium hydroxide or with 0.2 M hydrochloric acid.

Simulated intestinal fluid (without enzyme) pH 6.8

Preparation – Mix 250 mL of 0.2 M monobasic potassium phosphate solution and 112 mL of 0.2 M sodium hydroxide solution and dilute to 1000 mL with water. Adjust pH to 6.8 ± 0.1 with 0.2 M sodium hydroxide.

Simulated intestinal fluid without pancreatin, pH 7.5

Preparation – Dissolve 6.8 g of phosphate potassium monobasic in 900 mL of water, add 77 mL of 0.2 M sodium hydroxide and adjust the pH to 7.5 ± 0.1 with 0.2 M sodium hydroxide. Adjust volume to 1000 mL with water and homogenize.

Ammonium fluoride

CAS – [12125-01-8].

Molecular formula and molar mass – NH_4F – 37.04.

Description – Colorless crystals.

Physical characteristic – Melting temperature: approximately 100 °C.

Conservation – Protect from light, heat and humidity.

Safety – Irritant.

Calcium fluoride

CAS – [7789-75-5].

Molecular formula and molar mass – CaF_2 – 78.07.

Description – White crystals or powder.

Conservation – In tightly closed containers.

Sodium fluoride

CAS – [7681-49-4].

Molecular formula and molar mass – NaF – 41.99.

Description – Colorless crystals or white or nearly white powder.

Physical characteristics – Density: 2.78. Melting temperature: 993 °C.

Solubility – Soluble in water and practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

Safety – Poisonous!

Sodium fluoride RS

Preparation – Dry approximately 0.5 g of sodium fluoride at temperature of 200 °C for four hours. Weigh, accurately, about 0.222 g of dry material, dissolve in water and complete the volume to 100 mL with the same solvent. Pipette 10 mL of this solution, transfer to a 1000 mL volumetric flask and complete the volume with water. Each mL of this solution is equivalent to 10 µg of fluorine.

Conservation – In tightly closed containers.

Formaldehyde, solution

Synonyms – Formol, formalin.

Molecular formula and molar mass – CH_2O – 30.03.

Specification – Contains no less than 34.0% (w/v) and no more than 37.0% (w/v).

Description – Colorless, clear liquid. Produces irritant vapors.

Physical characteristics – Density: approximately 1.08. Refractive index (20 °C): 1.374.

Conservation – In tightly closed containers.

Storage – Protect from light, from air and from temperatures below 9 °C.

Stability – It may contain methyl alcohol as stabilizer.

Safety – Irritant. Toxic.

Category – Disinfectant.

Formamide

CAS – [75-12-7].

Molecular formula and molar mass – CH_3NO – 45.04.

Description – Clear, colorless, viscous liquid, with faint ammoniacal odor.

Physical characteristics – Boiling temperature: approximately 210 °C. Density (20 °C): approximately 1.13. Refractive index (20 °C): 1.447.

Conservation – In hermetic containers.

Storage – Protect from humidity.

Safety – Irritant.

Ammonium formate

CAS – [540-69-2].

Molecular formula and molar mass – CH_5NO_2 – 63.06.

Description – Deliquescent granules and crystals.

Physical characteristic – Melting range: between 119 °C and 121 °C.

Solubility – Very soluble in water and soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Alkaline phosphatase, solution

Solution A – Dissolve 3.1 g of boric acid in 500 mL of water. Add 21 mL of sodium hydroxide *M* and 10 mL of magnesium chloride 0.1 *M*. Dilute and complete the volume with water to 1000 mL.

Preparation – Dissolve 95 mg of alkaline phosphatase enzyme in *Solution A*. Dilute to 50 mL with the same solvent.

Ammonium phosphate dibasic

CAS – [7783-28-0].

Molecular formula and molar mass – $(\text{NH}_4)_2\text{HPO}_4$ – 132.06.

Description – White or nearly white granules or crystals. Hygroscopic.

Physical characteristic – Presents pH of approximately 8.0 in aqueous solution 20% (w/v).

Solubility – Very soluble in water and practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

Ammonium phosphate monobasic

CAS – [7722-76-1].

Synonym – Ammonium dihydrogen phosphate.

Molecular formula and molar mass – $(\text{NH}_4)\text{H}_2\text{PO}_4$ – 115.03.

Description – White crystals or crystalline powder.

Physical characteristic – The pH of the solution at 0.2 *M* is approximately 4.0.

Solubility – Freely soluble in water, slightly soluble in ethyl alcohol, insoluble in acetone.

Codeine phosphate

CAS – [41444-62-6].

Synonym – Codeine phosphate hemihydrate.

Molecular formula and molar mass – $\text{C}_{18}\text{H}_{21}\text{NO}_3 \cdot \text{H}_3\text{PO}_4 \cdot 1/2\text{H}_2\text{O}$ – 406.37.

Description – White or nearly white crystalline powder, or small colorless crystals.

Solubility – Freely soluble in water. Slightly or very slightly soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Potassium phosphate

CAS – [7778-53-2].

Synonym – Potassium phosphate tribasic.

Molecular formula and molar mass – K_3PO_4 – 212.27.

Description – White crystals or crystalline, deliquescent powder.

Solubility – Soluble in water and insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

Monobasic potassium phosphate

CAS – [7778-77-0].

Synonyms – Potassium biphosphate, potassium dihydrogen phosphate, acid potassium phosphate, monopotassium phosphate, Sorensen's potassium phosphate.

Molecular formula and molar mass – KH_2PO_4 – 136.08.

Specification – Contains no less than 98.0% in relation to the desiccated substance.

Description – Colorless crystals or white crystalline powder.

Conservation – In tightly closed containers.

Dibasic potassium phosphate

CAS – [7758-11-4].

Synonym – Potassium monoacid phosphate.

Molecular formula and molar mass – K_2HPO_4 – 174.17.

Description – Colorless crystals or white or nearly white powder. Very hygroscopic.

Solubility – Very soluble in water, very slightly soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Sodium phosphate dibasic

CAS – [7558-79-4].

Molecular formula and molar mass – Na_2HPO_4 – 141.96.

Description – White hygroscopic powder.

Conservation – In tightly closed containers.

Storage – Protect from humidity.

Sodium phosphate dibasic dihydrate

CAS – [10028-24-7].

Molecular formula and molar mass – $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ – 177.99.

Specification – Contains no less than 99.5% (w/w) in relation to the desiccated substance.

Description – Colorless crystals.

Solubility – Soluble in water and practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from heat and humidity.

Sodium phosphate dibasic dodecahydrate

CAS – [10039-32-4].

Molecular formula and molar mass – $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ – 358.14.

Specification – Contains no less than 98.5% (w/w) in relation to the desiccated substance.

Description – Colorless, clear, odorless crystals or granules, with saline flavor, weakly alkaline. Efflorescent.

Conservation – In tightly closed containers.

Storage – Protect from heat.

Sodium phosphate dibasic dodecahydrate RS

Specification – Contains 9 g in 100 mL of aqueous solution.

Conservation – In tightly closed containers.

Sodium phosphate dibasic heptahydrate

CAS – [7782-85-6].

Molecular formula and molar mass – $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ – 268.06.

Description – Colorless or white granular powder or crystal. It is stable on air. The aqueous solution is alkaline.

Conservation – In tightly closed containers.

Sodium phosphate dibasic heptahydrate RS

Specification – Contains 12 g of sodium phosphate dibasic heptahydrate in 100 mL of water.

Conservation – In tightly closed containers.

Monobasic sodium phosphate

CAS – [7558-80-7].

Synonym – Sodium dihydrogen orthophosphate.

Molecular formula and molar mass – NaH_2PO_4 – 119.98.

Description – White or nearly white powder. Hygroscopic.

Conservation – In hermetic containers.

Sodium phosphate monobasic monohydrate

CAS – [10049-21-5].

Molecular formula and molar mass – $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ – 137.99.

Description – White or nearly white, slightly deliquescent crystals or granules.

Solubility – Freely soluble in water and practically insoluble in ethyl alcohol. The aqueous solution is acid.

Conservation – In tightly closed containers.

Sodium phosphate monobasic dihydrate

CAS – [13472-35-0].

Formula and molecule mass – $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ – 156.01.

Description – Colorless crystals or white or nearly white powder.

Physical characteristic – Melting temperature: 60 °C.

Solubility – Very soluble in water and very slightly soluble in ethyl alcohol.

Conservation – In closed containers.

Sodium phosphate tribasic dodecahydrate

CAS – [10101-89-0].

Synonyms – Sodium phosphate tribasic, trisodium phosphate. *Molecular formula and molar mass* – $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ – 380.12. *Description* – Colorless or white crystals. Efflorescent.

Physical characteristic – Melting temperature: 75 °C, by quick heating.

Solubility – Freely soluble in water.

Conservation – In tightly closed containers. *Storage* – Protect from heat.

Tetrabutylammonium phosphate

CAS – [5574-97-0].

Molecular formula and molar mass – $\text{C}_{16}\text{H}_{38}\text{NO}_4\text{P}$ – 339.46.

Description – White or nearly white powder. Hygroscopic.

Solubility – Soluble in water.

Conservation – In closed containers.

Tributyl phosphate

CAS – [126-73-8].

Molecular formula and molar mass – $C_{12}H_{27}O_4P$ – 266.32.

Description – Colorless or slightly yellow, odorless liquid.

Solubility – Slightly soluble in water.

Equimolar phosphate 0.05 M

Specification – Contains 3.53 g of sodium phosphate dibasic and 3.39 g of monobasic potassium phosphate in 1000 mL of aqueous solution.

Conservation – In closed containers.

Bromocresol purple - phosphate RS

Solution A – Dissolve 38 g of monobasic sodium phosphate and 2 g of sodium phosphate dibasic in water and dilute to 1000 mL with the same solvent. Adjust the pH, if necessary, to 5.3 ± 0.1 using sodium hydroxide 5 M or phosphoric acid.

Solution B – Dissolve 400 g of bromocresol purple in 30 mL of water, add 6.3 mL of 0.1 M sodium hydroxide and dilute with water to 500 mL.

Preparation – On the day of use, mix the *Solutions A* and *B* and chloroform (1:1:1) in a separation funnel. Shake and discard the organic phase. Repeat the extraction with equal portions of chloroform until the organic layer is colorless. Use the aqueous phase.

Red phosphorus

CAS – [7723-14-0].

Description – Dark red powder.

Solubility – Insoluble in water and in diluted acids.

Safety – Flammable.

Fructose

CAS – [57-48-7].

Synonyms – β -D-Fructose, levulose.

Molecular formula and molar mass – $C_6H_{12}O_6$ – 180.16.

Specification – Contains no less than 98.0% (w/w) in relation to the desiccated substance.

Description – White, odorless crystalline powder, with strong sweet flavor.

Physical characteristic – Melting temperature with decomposition: approximately 103 °C.

Solubility – Very soluble in water and soluble in ethyl alcohol.

Conservation – In tightly closed containers.

0.1% (w/v) fructose

Specification – Contains 0.1 g of fructose in 100 mL of solution prepared with pyridine.

Conservation – In tightly closed containers.

Safety – Toxic.

Phthalaldehyde

CAS – [643-79-8].

Molecular formula and molar mass – $C_8H_6O_2$ – 134.13.

Description – Yellow crystalline powder.

Physical characteristic – Melting temperature: approximately 55 °C.

Conservation – In closed containers.

Storage – Protect from exposure to light and from contact with air.

Dibutyl phthalate

CAS – [84-74-2].

Synonyms – Phthalic acid dibutyl ester, di-*n*-butyl phthalate and phthalate dibutyl.

Molecular formula and molar mass – C₁₆H₂₂O₄– 278.35.

Description – Colorless or slightly colored clear, oily liquid.

Physical characteristics – Boiling temperature: 340 °C. Density: 1.043 to 1.048.

Solubility – Very slightly soluble in water, very soluble in acetone, ethyl alcohol and ethyl ether.

Phthalazine

CAS – [253-52-1].

Molecular formula and molar mass – C₈H₆N₂– 130.15

Description – Pale yellow crystals.

Physical characteristic – Melting range: between 90 °C and 91 °C.

Solubility – Freely soluble in water and soluble in absolute ethyl alcohol, in ethyl acetate and in methyl alcohol.

Basic fuchsine (CI 42510)

CAS – [632-99-5].

Synonyms – Magenta I, rosaniline hydrochloride.

Molecular formula and molar mass – C₂₀H₂₀ClN₃– 337.85.

Description – Lustrous metallic green crystals.

Physical characteristic – Decomposes at temperatures above 200 °C.

Solubility – Soluble in water and in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from light.

Category / Therapeutic class – Colorant. Antifungal.

Bleached fuchsine RS

Synonym – Schiff's Reagent.

Preparation – Dissolve 1 g of basic fuchsine in 600 mL of water, add 100 mL of 10% (w/v) sodium sulfite. Cool down externally with ice, under agitation. Slowly add 10 mL of hydrochloric acid, dilute with water to 1000 mL and filter. If the solution gets dark, shake with 0.2 to 0.3 g of activated charcoal until bleaching and filter immediately. If the pink color still remains, add 2 to 3 mL of hydrochloric acid and shake.

Conservation – Allow to stand for one hour before use, keep protected from light.

Galactose

CAS – [59-23-4].

Molecular formula and molar mass – C₆H₁₂O₆– 180.16.

Description – White crystalline powder.

Physical characteristic – Melting temperature: 167 °C.

Solubility – Freely soluble in water.

Conservation – In tightly closed containers.

0.1% (w/v) galactose in pyridine

Specification – Contains 0.1 g of galactose in 100 mL of solution prepared with pyridine.

Conservation – In tightly closed containers.

Storage – Protect from heat.

Safety – Toxic.

Gelatin

CAS – [9000-70-8].

Specification – It is the mixture of water-soluble proteins obtained by extracting material with collagen.

Description – Clear, brilliant, colorless or slightly yellow powder, granules, scales or sheets. Hygroscopic, with characteristic odor and mild flavor.

Conservation – In tightly closed containers.

Storage – Protect from heat and humidity.

Glycerol gelatin

Preparation – Dissolve 1 g of gelatin in 100 mL of water heated at temperature not superior to 30 °C. Add 1 mL of 2% (w/v) sodium salicylate and 15 mL of glycerol; shake and filter the heated mixture in glass wool.

Gelatin RS

Preparation – Dissolve 2.5 g of gelatin in 100 mL of hot water. Use after cooling down to room temperature.

Glycerol

CAS – [56-81-5].

Synonym – Glycerin.

Molecular formula and molar mass – $C_3H_8O_3$ –92.09.

Specification – Contains no less than 97.0% (w/w).

Description – Clear, colorless, odorless, hygroscopic, viscous liquid, with sweet flavor.

Physical characteristics – Density: 1.255 to 1.263. Refractive index (20 °C): 1.470 to 1.474.

Solubility – Miscible with water and with ethyl alcohol; slightly soluble in acetone and practically insoluble in fatty oils and essential oils.

Conservation – In hermetic containers.

Storage – Protect from oxidizing agent.

Glycine

CAS – [56-40-6].

Molecular formula and molar mass – $C_2H_5NO_2$ –75.07.

Description – White and odorless crystalline powder.

Physical characteristic – Melting range: 232 °C to 236 °C, with decomposition.

Solubility – Freely soluble in water, slightly soluble in ethyl alcohol and very slightly soluble in ethyl ether.

Glucose

CAS – [50-99-7].

Synonym – Dextrose.

Molecular formula and molar mass – $C_6H_{12}O_6$ –180.16.

Description – White, odorless crystalline powder, with sweet flavor.

Physical characteristic – Specific rotation power (20 °C): + 52.5° to + 53.0° (dissolve 10 g of glucose in 100 mL of water and add 0.2 mL of ammonia).

Solubility – Freely soluble in water and moderately soluble in ethyl alcohol.

Conservation – In tightly closed containers.

0.1% (w/v) glucose in pyridine

Specification – Contains 0.1 g of glucose in 100 mL of solution prepared with pyridine.

Conservation – In tightly closed containers.

Storage – Protect from heat.

Safety – Toxic.

Glyoxal-hydroxyanil

CAS – [1149-16-2].

Synonym – Glyoxal di(2-hydroxyanil).

Molecular formula and molar mass – $C_{14}H_{12}N_2O_2$ – 240.26.

Description – White or nearly white crystals.

Physical characteristic – Melting temperature: approximately 200 °C. *Solubility* – Soluble in hot ethyl alcohol.

Glutaraldehyde

CAS – [111-30-8].

Molecular mass formula – $C_5H_8O_2$ – 100.12.

Description – Oily liquid.

Physical characteristics – Refractive index (25 °C): approximately 1.434. Boiling temperature: approximately 188 °C.

Miscibility – Miscible with water.

Guaiacol

CAS – [95-05-1].

Synonym – 2-methoxyphenol, methylcatechol.

Molecular formula and molar mass – $C_7H_8O_2$ – 124.14.

Description – White or slightly yellow crystals, or colorless or slightly yellow liquid. Hygroscopic.

Physical characteristics – Melting temperature: approximately 28 °C. Boiling temperature: approximately 205 °C.

Solubility – Slightly soluble in water, very soluble in methylene chloride and freely soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from light.

Guanine

CAS – [73-40-5].

Molecular formula and molar mass – $C_5H_5N_5O$ – 151.13.

Description – White or nearly white amorphous powder.

Solubility – Practically insoluble in water, slightly soluble in ethyl alcohol. Dissolves in diluted alkali hydroxide solutions.

Heparin sodium

CAS – [9041-08-1].

Description – Consists of mixing active ingredients, with the property of extending the blood coagulation time. Usually obtained from the intestinal mucosa, lungs or another adequate tissue from domestic mammals used for human consumption.

Solubility – Freely soluble in water.

Conservation – In hermetic containers.

Labeling – The label must include the organ and species of origin. The potency must be indicated in IU.

Therapeutic class – Anticoagulant.

Heptane

Specification – Usually contains a mixture of hydrocarbons – fraction of petroleum – with predominance of *n*-heptane.

Description – Clear, colorless, volatile, highly flammable liquid, with characteristic odor. *Physical characteristics* – Boiling range: 95 °C to 99 °C. Density: approximately 0.69. *Solubility* – Practically

insoluble in water and miscible with absolute ethyl alcohol. Miscible with ethyl ether, chloroform, benzene and with the majority of volatile and non-volatile oils.

Conservation – In hermetic containers.

Storage – Protect from heat. Keep away from flame/spark.

Safety – Irritant to the respiratory tract. Flammable.

***n*-Heptane**

CAS – [142-82-5].

Molecular formula and molar mass – C_7H_{16} – 100.21.

Specification – Main component of heptane.

Description – Clear and flammable liquid.

Physical characteristics – Boiling temperature: 98.4 °C. Density: 0.684. Refractive index (20 °C): 1.3855.

Solubility – Practically insoluble in water; miscible with absolute ethyl alcohol.

Sodium heptanesulfonate

CAS – [22767-50-6].

Molecular formula and molar mass – $C_7H_{15}NaO_3S$ – 202.25.

Description – White or nearly white crystalline mass.

Solubility – Freely soluble in water and soluble in methyl alcohol.

Conservation – In hermetic containers.

Hexane

Specification – Usually contains a mixture of C_6H_{14} isomers, predominantly *n*-hexane and methylcyclopentane (C_6H_{12}).

Description – Clear, colorless, volatile, highly flammable liquid, with characteristic odor.

Physical characteristics – Boiling range: 67 °C to 70 °C. Density: 0.66.

Conservation – In hermetic containers.

Storage – Protect from heat. Keep away from flame/spark.

Safety – Irritant to the respiratory tract. Flammable.

***n*-Hexane**

CAS – [110-54-3].

Molecular formula and molar mass – C_6H_{14} – 86.18.

Specification – Main component of petroleum ether and hexane.

Description – Clear, volatile liquid, with odor similar to petroleum.

Physical characteristics – Boiling temperature: 69 °C. Density: 0.66. Refractive index (20 °C): 1.375.

Solubility – Practically insoluble in water; miscible with absolute ethyl alcohol.

Conservation – In hermetic containers.

Storage – Protect from heat. Keep away from flame/spark.

Safety – Flammable.

1- Sodium hexanesulfonate

CAS – [2832-45-3].

Molecular formula and molar mass – $C_6H_{13}NaO_3S$ – 188.22.

Description – White or nearly white powder.

Hexylamine

CAS – [111-26-2].

Synonym – Hexanamine.

Formula and molar mass – $C_6H_{15}N$ – 101.19.

Description – Colorless liquid.

Physical characteristics – Density (20 °C): approximately 0.766. Refractive index (20 °C): approximately 1.418. Boiling temperature: 127 °C to 131 °C.

Solubility – Slightly soluble in water and soluble in ethyl alcohol.

Chloral hydrate

CAS – [302-17-0].

Synonym – Hydrated chloral.

Molecular formula and molar mass – $\text{C}_2\text{H}_3\text{Cl}_3\text{O}_2$ – 165.40.

Specification – Contains no less than 98.5% (w/w).

Description – Clear, colorless crystals, with characteristic pungent odor and slightly bitter, caustic taste. Deliquescent.

Physical characteristic – Melting temperature: 57 °C.

Solubility – Very soluble in water and freely soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from light and from heat. *Safety* – Irritant to the skin.

Therapeutic class – Sedative, hypnotic.

Hydrazine, hydrate

CAS – [7803-57-8].

Molecular formula and molar mass – $\text{N}_2\text{H}_4 \cdot \text{H}_2\text{O}$ – 50.06.

Description – Colorless and clear liquid.

Miscibility – Miscible with water.

Conservation – In tightly closed containers.

Ammonium hydroxide

Use ammonia, concentrated solution.

Barium hydroxide

CAS – [12230-71-6].

Molecular formula and molar mass – $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ – 315.46.

Description – Colorless crystals.

Physical characteristic – Melting temperature: 78 °C.

Solubility – Soluble in water.

Conservation – In tightly closed containers.

Calcium hydroxide

CAS – [1305-62-0].

Molecular formula and molar mass – $\text{Ca}(\text{OH})_2$ – 74.09.

Specification – Contains no less than 93.0% (w/w).

Description – White, soft, odorless granules or powder.

Solubility – Practically insoluble in water.

Conservation – In tightly closed containers.

Storage – Protect from carbon dioxide.

Calcium hydroxide, saturated solution

Use calcium hydroxide RS.

Calcium hydroxide RS

Specification – Contains 0.15 g of calcium hydroxide in 100 mL of preparation with carbon dioxide-free water (saturated solution).

Conservation – In tightly closed containers.

Stability – Prepare immediately before use.

Storage – Protect from carbon dioxide. *Category* – Astringent.

Lithium hydroxide

CAS – [1310-66-3].

Molecular formula and molar mass – $\text{LiOH} \cdot \text{H}_2\text{O}$ – 41.96.

Description – White or nearly white granular powder.

Solubility – Soluble in water, forming a strongly alkaline solution. Moderately soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Safety – Corrosive.

Potassium hydroxide

CAS – [1310-58-3].

Molecular formula and molar mass – KOH – 56.11.

Specification – Contains no less than 85.0% (w/w), calculated as KOH , and no more than 3.5% of K_2CO_3 .

Description – White, hard, dry mass, with crystalline, odorless and very hygroscopic structure. Absorbs carbon dioxide. Liquefies on air. Presented in form of pellets, cylinders or scales.

Solubility – Very soluble in water and freely soluble in ethyl alcohol.

Conservation – In hermetic, inert containers.

Storage – Protect from humidity and from carbon dioxide. *Safety* – Very caustic.

Ethanollic potassium hydroxide RS (approximately 0.5 M)

Preparation – Dissolve 34.04 g of potassium hydroxide in 20 mL of water; complete the volume to 1000 mL with (aldehyde-free) ethyl alcohol. Allow to stand to decant for 24 hours in hermetic containers. Use the clear supernatant.

Conservation – In hermetic containers.

Storage – Protect from light.

2 M ethanollic potassium hydroxide

Preparation – Dissolve 6.6 g of potassium hydroxide in 5 mL of water, cool down and complete the volume to 50 mL with ethyl alcohol. Allow to stand to decant for 24 hours and use the clear supernatant.

Sodium hydroxide

CAS – [1310-73-2].

Synonym – Caustic soda.

Molecular formula and molar mass – NaOH – 40.00.

Specification – Contains no less than 95.0% (w/w) total alkali, calculated as NaOH , and no more than 3.0% (w/w) Na_2CO_3 .

Description – White hard mass, with crystalline structure, in the form of flakes, pellets and sticks. Deliquescent and absorbs carbon dioxide.

Solubility – Very soluble in water and freely soluble in ethyl alcohol.

Conservation – In hermetic containers.

Storage – Protect from humidity and from carbon dioxide.

Safety – Caustic, corrosive.

Sodium hydroxide RS

Specification – Contains 8% (w/v) of sodium hydroxide in water.

Conservation – Refer to sodium hydroxide *M*.

Sodium hydroxide M

Specification – Contains 40 g of sodium hydroxide in 1000 mL of preparation with carbon dioxide-free water.

Conservation – In alkali-resistant glass or polyethylene containers.

Storage – Protect from humidity and from carbon dioxide.

Sodium hydroxide, concentrated solution RS (approximately 10 M)

Specification – Contains 20 g of sodium hydroxide in 50 mL of preparation with water.

Conservation – In tightly closed containers.

Storage – Protect from carbon dioxide.

Safety – Caustic.

Tetrabutylammonium hydroxide

CAS – [2052-49-5].

Molecular formula and molar mass – $(C_4H_9)_4NOH$ – 259.48.

Description – White or nearly white crystals.

Solubility – Soluble in water.

Tetramethylammonium hydroxide

CAS – [75-59-2].

Molecular formula and molar mass – $C_4H_{13}NO$ – 91.15.

Description – It is a stronger base than ammonia and quickly absorbs carbon dioxide from air. A preparation in aqueous medium at 25% (w/v) is clear and colorless.

Physical characteristic – Melting temperature: 63 °C.

Conservation – In tightly closed containers.

Tetramethylammonium hydroxide RS

Specification – Contains no less than 10% (w/w) tetramethylammonium hydroxide.

Description – Colorless or light yellow clear liquid.

Miscibility – Miscible with water and ethyl alcohol.

Preparation – To 1.000 g, add 50 mL of water and titrate with sulfuric acid 0.05 M VS, using 0.1 mL of methyl red TS as indicator. Each mL of sulfuric acid 0.05 M VS is equivalent to 9.12 mg of $C_4H_{13}NO$.

D- α -4-hydroxyphenylglycine

CAS – [22818-40-2].

Molecular formula and molar mass – $C_8H_9NO_3$ – 167.16.

Description – Brilliant leaflets.

Physical characteristic – Decomposition range: between 220 °C and 247 °C.

Solubility – Moderately soluble in water, in ethyl alcohol, ethyl ether and acetone. Soluble in alkaline and acid minerals.

Hydroxylamine in ethyl alcohol

Preparation – Dissolve 3.5 g of hydroxylamine hydrochloride in 95 mL of 60% (v/v) ethyl alcohol, add 0.5 mL of a methyl orange solution at 2 g/L in 60% (v/v) ethyl alcohol and sufficient amount of potassium hydroxide 0.5 M in ethyl alcohol 60% for producing a yellow color. Dilute to 100 mL with 60% (v/v) ethyl alcohol.

Hydroxymethylfurfural

CAS – [67-47-0].

Synonym – 5-Hydroxymethylfurfural.

Molecular formula and molar mass – $C_6H_6O_3$ – 126.11.

Description – Needle-shaped crystals.

Solubility – Freely soluble in water, acetone and alcohol, soluble in ethyl ether.

Physical characteristic – Melting temperature: approximately 32 °C

Hydroxyquinoline

CAS – [148-24-3].

Synonym – 8-hydroxyquinoline.

Molecular formula and molar mass – C₉H₇NO – 145.16.

Description – White or slightly yellow crystalline powder.

Physical characteristic – Melting temperature: approximately 75 °C.

Solubility – Slightly soluble in water, freely soluble in acetone, in ethyl alcohol and in diluted mineral acid solutions.

Butylated hydroxytoluene

CAS – [128-37-0].

Synonym – BHT.

Molecular formula and molar mass – C₁₅H₂₄O – 220.36.

Specification – Contains no less than 99.0% (w/w).

Description – White or yellow-white crystalline powder.

Physical characteristics – Freezing temperature: no less than 69.2 °C. Boiling temperature: 265 °C.

Density: 1.048.

Solubility – Practically insoluble in water, very soluble in acetone, freely soluble in ethyl alcohol and in vegetable oils.

Safety – May cause contact dermatitis.

Hyperoside

CAS – [482-36-0].

Molecular formula and molar mass – C₂₁H₂₀O₁₂ – 464.38.

Description – Pale yellow needles.

Physical characteristic – Melting temperature: approximately 240 °C, with decomposition.

Solubility – Soluble in methyl alcohol.

Sodium hypochlorite

CAS – [7681-52-9].

Molecular formula and molar mass – NaClO – 74.44.

Description – White crystals. It is usually obtained in the pentahydrate form, and its anhydrous form is explosive.

Physical characteristic – Melting temperature: 18 °C (pentahydrate form).

Solubility – Very soluble in water.

Conservation – In tightly closed containers. *Safety* – Irritant.

Sodium hypochlorite RS

Refer to the *Diluted sodium hypochlorite solution* monograph.

Sodium hypophosphite

CAS – [10039-56-2].

Molecular formula and molar mass – NaH₂PO₂·H₂O – 105.99.

Specification – Contains no less than 99.0% (w/w) in relation to the desiccated substance.

Description – White granulated or crystalline powder or colorless, odorless crystals, with saline flavor. Hygroscopic.

Solubility – Freely soluble in water and soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from heat.

Sodium hypophosphite RS

Specification – Contains 5 g of sodium hypophosphite in 10 mL of water, added to 50 mL with hydrochloric acid. Separate eventual crystals formed. The solution must be clear and colorless.

Imidazole

CAS – [288-32-4].

Synonym – Glyoxaline.

Molecular formula and molar mass – $C_3H_4N_2$ – 68.08.

Description – White crystalline powder.

Physical characteristic – Melting range: 90 °C to 91 °C.

Solubility – Soluble in water and in ethyl alcohol.

Iminodibenzyl

CAS – [494-19-9].

Molecular formula and molar mass – $C_{14}H_{13}N$ – 195.27.

Description – Pale yellow crystalline powder.

Physical characteristic – Melting temperature: approximately 106 °C.

Solubility – Practically insoluble in water and freely soluble in acetone.

Conservation – In tightly closed containers.

Inosine

CAS – [58-63-9].

Molecular formula and molar mass – $C_{10}H_{12}N_4O_5$ – 268.23.

Physical characteristic – Melting temperature: 222 °C to 226 °C.

Potassium iodate

CAS – [7758-05-6].

Molecular formula and molar mass – KIO_3 – 214.00.

Description – White, odorless crystals or crystalline powder.

Physical characteristic – Melting temperature: approximately 560 °C, with partial decomposition.

Solubility – Soluble in water, insoluble in ethyl alcohol.

Category – Oxidizing agent.

Mercury(II) iodide

CAS – [7774-29-0].

Synonyms – Mercury diiodide, red mercury iodide.

Molecular formula and molar mass – HgI_2 – 454.39.

Description – Scarlet red, crystalline, dense, odorless, and nearly insipid powder.

Physical characteristic – Melting temperature: 259 °C.

Solubility – Slightly soluble in water, moderately soluble in acetone and in ethyl alcohol, soluble in excess potassium iodide solution.

Conservation – In tightly closed containers.

Storage – Protect from light.

Safety – Poison!

Potassium iodide

CAS – [7681-11-0].

Molecular formula and molar mass – KI – 166.00.

Specification – Contains no less than 99.0% (w/w) in relation to the desiccated substance.

Description – Colorless crystals or white crystalline powder, odorless, with salty and bitter flavor. Faintly deliquescent.

Physical characteristic – Melting temperature: 680 °C.

Solubility – Very soluble in water, freely soluble in glycerol, soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from light and humidity.

Approximately M potassium iodide

Use potassium iodide RS.

Potassium iodide RS

Specification – Contains 16.5% (w/v) of potassium iodide in water.

Conservation – In opaque, tightly closed containers.

Storage – Protect from light.

Alkaline mercuric potassium iodide RS

Synonyms – Nessler's reagent, alkaline potassium tetraiodomercurate(II) solution, mercury potassium chloride iodide.

Preparation – Dissolve 5 g of potassium iodide in 5 mL of water and gradually add 10 mL of the 25% (w/v) mercury(II) chloride solution, controlling the addition so that the precipitate formed in the beginning is not completely dissolved. Allow to cool. Then, add 50% (w/v) potassium hydroxide solution, dilute with water until completing the volume to 100 mL and add 0.5 mL of 25% (w/v) mercury(II) chloride solution. Let it decant and use the supernatant.

Alkaline mercuric potassium iodide RS1

Synonym – Alkaline potassium tetraiodomercurate RS.

Preparation – Dissolve in water 11 g of potassium iodide and 15 g of mercury(II) iodide and complete the volume to 100 mL with the same solvent. Immediately before use, mix the previous solution with equal volume of 25% (w/v) sodium hydroxide.

Potassium mercury iodide RS

Synonym – Mayer's reagent.

Solution A – Dissolve 13.5 g of mercury(II) chloride in 600 mL of water.

Solution B – Dissolve 50 g of potassium iodide in 100 mL of water.

Preparation – Mix the *Solutions A* and *B* and complete the volume to 1000 mL with water.

Propidium iodide

CAS – [25535-16-4]

Formula and molecular mass – $C_{27}H_{34}I_2N_4$ – 668.39

Safety – Carcinogenic

Preparation – Weigh 0.1 mg of reagent and add 10 mL of bi-distilled. Mix well.

Sodium iodide

CAS – [7681-82-5].

Molecular formula and molar mass – NaI – 149.89.

Specification – Contains no less than 99.0% (w/w) in relation to the desiccated substance.

Description – White crystalline powder or colorless, hygroscopic, odorless crystals.

Solubility – Very soluble in water and freely soluble in ethyl alcohol.

Conservation – In hermetic containers.

Sodium iodide in acetic acid

Specification – Contains 20% (w/v) of sodium iodide in glacial acetic acid.

Conservation – In tightly closed containers.

Storage – Protect from light.

Tetrabutylammonium iodide

CAS – [311-28-4].

Synonym – Tetra-*n*-butylammonium iodide.

Molecular formula and molar mass – C₁₆H₃₆IN – 369.37.

Description – White or faintly colored crystalline powder or crystals.

Solubility – Slightly soluble in water and soluble in ethyl alcohol.

Indigo carmine

CAS – [860-22-0].

Molecular formula and molar mass – C₁₆H₈N₂Na₂O₈S₂ – 466,36.

Description – Blue granules with copper luster, or blue or violet-blue powder.

Solubility – Moderately soluble in water, practically soluble in ethyl alcohol. Precipitates in aqueous sodium chloride solutions.

Indigo carmine RS

Preparation – In a mixture of 10 mL hydrochloric acid and 990 mL of 20% (w/v) sulfuric acid, add 0.2 g of indigo carmine.

Iodine

CAS – [7553-56-2].

Molecular formula and molar mass – I₂ – 253.80.

Description – Blue-black or violet-gray scales, plates or small crystals; metallic luster, of irritant odor.

Physical characteristics – Slowly sublimates at room temperature; when heated, releases violet vapors. Melting temperature: 113.6 °C

Solubility – Very slightly soluble in water, soluble in ethyl alcohol and slightly soluble in glycerol.

Conservation – In hermetic glass containers.

Safety – Corrosive vapors.

Iodine RS

Synonyms – Aqueous iodine solution – iodized, lugol reactive.

Specification – Contains 1 g of iodine and 2 g of potassium iodide in 100 mL of aqueous preparation.

Preparation – Dissolve 1 g of iodine in 100 mL of water, add 2 g of potassium iodide, shake, allow to stand for a few hours, and filter through glass wool.

Conservation – In tightly closed amber glass containers.

Storage – Protect from light.

Iodine 0.05 M

Preparation – Dissolve 20 g of potassium iodide in the minimum amount of water, add 13 g of iodine, then add water to complete the volume to 1000 mL.

0.5% (w/v) iodine in chloroform

Specification – Contains 0.5% (w/v) of iodine in chloroform.

Conservation – In tightly closed containers.

Storage – Protect from light.

Safety – Toxic.

1% (w/v) iodine in ethyl alcohol

Synonyms – Iodine alcoholic solution, iodine ethanolic solution.

Specification – Contains 1% (w/v) of iodine in ethyl alcohol.

Conservation – In tightly closed glass containers.

Storage – Protect from light.

Safety – Flammable.

Potassium iodobismuthate

Use aqueous-acetic potassium iodobismuthate.

Aqueous-acetic potassium iodobismuthate

Preparation – Mix 58 mL of water, 1.21 g of bismuth subnitrate, 14 mL of glacial acetic acid and 28 mL of 40% (w/v) potassium iodide solution.

Diluted potassium iodobismuthate RS

Preparation – Dissolve 100 g of tartaric acid in 500 mL of water. Separately, dissolve 100 g of tartaric acid in 400 mL of water and add 8.5 g of bismuth subnitrate. Shake for one hour, add 200 mL of 40% (w/v) potassium iodide solution and shake well. Allow to stand for 24 hours and filter. Mix the first solution with 50 mL of the second solution.

Potassium iodide and bismuth subnitrate RS

Synonym – Dragendorff's reagent.

Preparation – Mix equal volumes of 40% (w/v) aqueous potassium iodide solution prepared by dissolving 0.85 g of bismuth subnitrate in a mixture of 10 mL of glacial acetic acid and 40 mL of water. Dilute 1 volume of this mixture with 2 volumes of glacial acetic acid and 10 volumes of water immediately before use.

Storage – Protect from light.

Potassium iodobismuthate RS

Preparation – Dissolve 16.6 g of tartaric acid in 67 mL of water and add 1.41 g of bismuth subnitrate. Shake for one hour, add 33 mL of 40% (w/v) aqueous potassium iodide solution. Shake for one more hour. Allow to stand for 24 hours. Filter.

Conservation – In tightly closed containers.

Storage – Protect from light.

Potassium iodobismuthate RS1

Preparation – Dissolve 10 g of tartaric acid in 40 mL of water and add 0.85 g of bismuth subnitrate. Shake for one hour. Add 20 mL of 40% (w/v) aqueous potassium iodide solution and homogenize. Allow to stand for 24 hours and filter.

Potassium iodobismuthate RS2

Preparation – Suspend 1.7 g of bismuth subnitrate and 20 g of tartaric acid in 40 mL of water. Add to the suspension 40 mL of 40% (w/v) aqueous potassium iodide solution. Shake for one hour and filter. Protect the solution from exposure to light. Immediately before use, mix 5 mL of the previous solution with 15 mL of water.

Conservation – In tightly closed containers.

Storage – Protect from exposure to light.

Iodosulfuron RS

Preparation – Use a round flask with 3 L to 4 L, with three tubes, one stirrer, one thermometer and one drying tube. The flask must be dry and closed during the preparation. Mix 700 mL of pyridine with 700 mL of methoxyethanol; add, stirring, 220 g of iodine, finely powdered and dried in advance, under phosphorus pentoxide. The agitation must be maintained until complete dissolution (approximately 30 minutes). Cool down at -10 °C and, stirring, quickly introduce 190 g of liquid sulfur dioxide. The temperature must not exceed 30 °C. Cool down.

Assay – Determine the titer in the moment of use, always working protected from light. Introduce in an Erlenmeyer flask approximately 20 mL of methyl alcohol and proceed to the *Water determination* by the semi-micro method (5.2.20.3), with the sample, until the titration endpoint. Introduce in the Erlenmeyer flask an accurately weighed amount of water and perform a new titration. Calculate the equivalent in water of the sample, in mg/mL. Each mL of iodosulfuron RS is equivalent to no less than 3.5 mg of water.

Conservation – In a dry container.

Irganox 1010

CAS – [6683-19-8].

Molecular formula and molar mass – $C_{73}H_{108}O_{12}$ – 1177.66.

Description – White to slightly yellow powder. Odorless, insipid.

Physical characteristics – Melting range: 110 °C to 125 °C. Crystallizes in two forms: alpha form, melting range 120 °C to 125 °C; and beta form, melting range 110 °C to 115 °C. The melting range varies according to the proportion of crystalline forms in the mixture; this proportion does not influence the product efficiency.

Category – Stabilizer for organic substances, such as polyethylene and polypropylene, protecting them from thermo-oxidative degradation.

Irganox 1076

CAS – [2082-79-3].

Molecular formula and molar mass – $C_{35}H_{62}O_3$ – 530.88.

Description – White to slightly yellow powder. Odorless, stable to light.

Physical characteristic – Melting range: 49 °C to 54 °C

Category – Antioxidant for organic substrates, such as polyethylene and polypropylene, protecting them from thermo-oxidative degradation.

Irganox PS 800

CAS – [123-28-4].

Molecular formula and molar mass – $C_{30}H_{58}O_4S$ – 514.85.

Description – White crystals.

Physical characteristic – Melting range: 38 °C to 40 °C

Category – Stabilizer of polyolefins, especially high-density polyethylene and polypropylene.

Isooctane

CAS – [540-84-1].

Synonym – 2,2,4-Trimethylpentane.

Molecular formula and molar mass – C_8H_{18} – 114.23.

Description – Colorless liquid.

Physical characteristics – Density (20 °C): 0.691 to 0.696. Refractive index (20 °C): 1.391 to 1.393.

Solubility – Practically insoluble in water and soluble in ethyl alcohol.

Conservation – In closed containers.

Safety – Flammable.

Fluorescein isothiocyanate

CAS – [27072-45-3].

Molecular formula and molar mass – $C_{21}H_{11}NO_5S$ – 389.38. *Specification* – Mixture of isomers: 5-isothiocyanate and 6-isothiocyanate. *Description* – Orange solid, decomposes with heating.

Lactose

CAS – [5989-81-1].

Synonym – Lactose monohydrate.

Molecular formula and molar mass – $C_{12}H_{22}O_{11}.H_2O$ – 360.31.

Description – White crystalline powder or granules. Odorless, with faint sweet flavor.

Physical characteristics – Specific optical rotation (20 °C): +52.2° to + 52.8° (determine in anhydrous lactose solution at 0.1 g/mL). Melting temperature: 202 °C

Conservation – In tightly closed containers.

Additional information – Adsorbs foreign odors.

0.1% (w/v) lactose in pyridine

Specification – Contains 0.1% (w/v) in pyridine.

Conservation – In tightly closed containers.

Safety – Toxic

Methyl laurate

CAS – [111-82-0].

Molecular formula and molar mass – $C_{13}H_{26}O_2$ – 214.35.

Specification – Contains no less than 98.0% (w/v).

Description – Colorless or yellow liquid.

Physical characteristics – Density (20 °C): approximately 0.870. Refractive index (20 °C): approximately 1.431. Melting temperature: approximately 5 °C.

Conservation – In tightly closed containers.

Sodium lauryl sulfate

CAS – [151-21-3].

Synonyms – Sodium dodecyl sulfate, dodecyl sulfate sodium.

Molecular formula and molar mass – $C_{12}H_{25}NaO_4S$ – 288.38.

Specification – Mixture of no less than 85.0% (w/w) sodium alkyl sulfates, comprised mainly of sodium lauryl sulfate $[CH_3(CH_2)_{10}.H_2SO_4.Na]$. The total content of sodium chloride and sodium sulfate is no more than 8.0% (w/w).

Description – White or light yellow powder, scales or crystals, with faint characteristic odor.

Solubility – Freely soluble in water and partially soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Sodium lauryl sulfate RS

Description – Contains 1 g of sodium lauryl sulfate in 100 mL of water.

Conservation – In tightly closed containers.

Lecithin

Specification – Mixture of diglycerides, especially of stearic, palmitic and oleic acids, bonded to the phosphoric acid from choline. Variable structure and composition according to the source.

Description – Fatty yellow-brown mass, with faint characteristic odor.

Conservation – In tightly closed containers.

Labeling – Specify origin.

Nickel-aluminum alloy

Description – Gray fine powder.

Solubility – Practically insoluble in water, soluble in mineral acids with formation of salt.

Linalool

CAS – [78-70-6].

Molecular formula and molar mass – $C_{10}H_{18}O$ – 154.25.

Description – Liquid. Mixture of two stereoisomers (licareol and coriandrol).

Physical characteristics – Density (20 °C): approximately 0.860. Boiling temperature: approximately 200 °C. Refractive index (20 °C): approximately 1.462.

Solubility – Practically insoluble in water.

Lithium

CAS – [7439-93-2].

Element and atomic mass – Li – 6.94.

Solubility – Reacts violently with water. Soluble in methyl alcohol, forming lithium methoxide. Practically insoluble in petroleum ether.

Lithium RSA – 2 mg/mL

Specification – Contains 1.064 g of lithium carbonate in 5 mL of hydrochloric acid. Complete the volume to 100 mL with water.

Conservation – In tightly closed, inert containers (like polyethylene).

Macrogol 300

CAS – [25322-68-3].

Synonyms – PEG 300, polyethylene glycol 300.

Molecular formula and molar mass – $\text{H}(\text{OCH}_2\text{CH}_2)_n\text{OH}$ – Molar mass not inferior to 95.0% of the nominal value labeled. Presents the average number of 6 or 7 oxyethylene groups ($n = 6$ or 7).

Specification – Mixture of products from the polycondensation of ethylene oxide and water.

Description – Colorless or nearly colorless, clear, viscous liquid, with faint and characteristic odor. Hygroscopic.

Physical characteristics – Density: approximately 1.125. Refractive index (20 °C): approximately 1.465. Viscosity: approximately 80 cP.

Conservation – In hermetic containers.

Labeling – It must state the average molar mass. *Storage* – Protect from humidity.

Macrogol 1000

CAS – [25322-68-3].

Synonyms – PEG 1000, polyethylene glycol 1000.

Molecular formula and molar mass – $\text{H}(\text{OCH}_2\text{CH}_2)_n\text{OH}$ – Molar mass not inferior to 95.0% of the nominal value labeled.

Description – White or nearly white solid with waxy appearance. Hygroscopic.

Physical characteristics – Density: approximately 1.080. Freezing range: between 35 °C and 40 °C.

Solubility – Very soluble in water, freely soluble in ethyl alcohol and in methylene chloride. Practically insoluble in fatty oils and in mineral oils.

Conservation – In hermetic containers.

Labeling – It must state the average molar mass. *Storage* – Protect from humidity.

Magnesium RSA – 1 mg/mL

Specification – Contains 9 g of magnesium chloride in 500 mL of aqueous solution.

Standardization – Add to 25 mL of this solution 25 mL of water, 10 mL of ammonium chloride buffer pH 10.7 and 0.1 g of eriochrome black T indicator. Titrate with 0.05 M disodium edetate VS. Each mL of 0.05 M disodium edetate VS is equivalent to 0.001215 g of Mg. For use, dilute at concentration of 1 mg/mL of magnesium.

Conservation – In tightly closed, inert containers (like polyethylene).

Magneson

CAS – [74-39-5].

Molecular formula and molar mass – $\text{C}_{12}\text{H}_9\text{N}_3\text{O}_4$ – 259.22.

Description – Red-brown powder.

Category – Indicator for magnesium and molybdenum.

Melamine

CAS – [108-78-1].

Molecular formula and molar mass – $\text{C}_3\text{H}_6\text{N}_6$ – 126.12.

Description – White or nearly white amorphous powder.

Solubility – Very slightly soluble in water and in ethyl alcohol.

2- Mercaptoethanol

CAS – [60-24-2].

Molecular formula and molar mass – $\text{C}_2\text{H}_6\text{OS}$ – 78.14.

Description – Clear and colorless liquid.

Physical characteristics – Density (20 °C): approximately 1.116. Boiling temperature: approximately 157 °C.

Miscibility – Miscible with water.

Mercaptopurine

CAS – [6112-76-1].

Molecular formula and molar mass – $\text{C}_5\text{H}_4\text{N}_4\text{S} \cdot \text{H}_2\text{O}$ – 170.20.

Description – Yellow crystalline powder.

Solubility – Practically insoluble in water and slightly soluble in ethyl alcohol. Soluble in alkali hydroxide solutions.

Storage – Protect from light.

Mercury

CAS – [7439-97-6].

Element and atomic mass – Hg – 200.59.

Specification – Liquid, mobile, dense, silvery metal, of mirrored surface.

Physical characteristics – Density (°C): approximately 13.5. Boiling temperature: approximately 357 °C.

Conservation – In tightly closed containers.

Safety – Poison! Volatile at room temperature.

Mercury RSA – 1 mg/mL

Specification – Contains 1.080 g of mercuric oxide dissolved in the lowest volume possible of 2 M hydrochloric acid. Complete the volume to 1000 mL with water.

Conservation – In tightly closed, inert containers (like polyethylene).

Sodium metabisulfite

CAS – [7681-57-4].

Synonyms – Sodium disulfite, sodium pyrosulfite.

Molecular formula and molar mass – $\text{Na}_2\text{S}_2\text{O}_5$ – 190.10.

Specification – Contains no less than 95% (w/w). Contains an amount of sodium metabisulfite equivalent to no less than 65.0% and no more than 67.4% of sulfur dioxide (SO_2).

Description – Colorless crystals or white or cream-white crystalline powder, with sulfurous odor and acid and saline flavor.

Solubility – Freely soluble in water and slightly soluble in ethyl alcohol.

Conservation – In tightly closed, full containers.

Storage – Protect from excessive heat, air and humidity.

Stability – Slowly oxidizes at sulfate, by exposure to air and to humidity, with disintegration of crystals.

Methenamine

CAS – [100-97-0].

Synonym – Hexamethylenetetramine.

Molecular formula and molar mass – $C_6H_{12}N_4$ – 140.19.

Specification – Contains no less than 99.0% (w/w), after desiccation on atmosphere of phosphorus pentoxide for four hours.

Description – Colorless crystalline powder.

Physical characteristics – Sublimates without melting and with partial decomposition at approximately 263 °C. The pH of the 0.2 M solution is 8.4.

Solubility – Very soluble in water. Conservation – In tightly closed containers. Therapeutic class – Urinary antiseptic.

Methylcellulose 450

CAS – [9004-67-5].

Specification – Partially O-methylated cellulose with viscosity of 450 mPa/second. Description – White, yellow-white or grey-white granule or powder. Hygroscopic. Solubility – Practically insoluble in hot water, in acetone, absolute ethyl alcohol and toluene.

4,4-Methylenebis-N,N-dimethylaniline

CAS – [101-61-1].

Synonym – Tetramethyldiaminediphenylmethane.

Molecular formula and molar mass – $C_{17}H_{22}N_2$ – 254.38.

Description – White or bluish-white crystals or leaflets.

Physical characteristic – Melting range: 90 °C to 91 °C.

Solubility – Practically insoluble in water, slightly soluble in ethyl alcohol and soluble in mineral acids.

Conservation – In closed containers.

Methylenebisacrylamide

CAS – [110-26-9].

Synonyms – N,N'-methylenebisacrylamide, methylenebispropenamide.

Molecular formula and molar mass – $C_7H_{10}N_2O_2$ – 154.17.

Description – White or nearly white fine powder.

Physical characteristic – Melting temperature: above 300 °C, with decomposition.

Methyl-ethyl-ketone

CAS – [78-93-3].

Synonyms – Ethylmethylketone, 2-butanone.

Molecular formula and molar mass – C_4H_8O – 72.11.

Description – Clear and colorless liquid. Characteristic odor of acetone.

Physical characteristics – Density (20 °C): approximately 0.81. Boiling temperature: 79.6 °C.

Conservation – In hermetic containers.

Safety – Toxic. Flammable.

Methylisobutylketone

CAS – [108-10-1].

Synonym – 4-Methyl-2-pentanone, isopropylacetone.

Molecular formula and molar mass – $C_6H_{12}O$ – 100.16.

Description – Colorless liquid, with ketonic and camphor odor.

Physical characteristics – Boiling temperature: approximately 115 °C

Methylparaben

CAS – [99-76-3].

Synonym – 4-hydroxybenzoic acid methyl ester.*Molecular formula and molar mass* – $C_8H_8O_3$ – 152.15.*Description* – White crystals, slightly soluble in water, freely soluble in acetone, in ethyl alcohol and in ethyl ether.*Solubility* – Very slightly soluble in water and freely soluble in ethyl alcohol and in methyl alcohol.*Category* – Preservative.**4-Methyl-2-pentanol**

CAS – [108-11-2].

Molecular formula and molar mass – $C_6H_{14}O$ – 102.18.*Description* – Colorless, clear and volatile liquid.*Physical characteristics* – Density (20 °C): approximately 0.802. Refractive index (20 °C): approximately 1.411. Boiling temperature: approximately 132 °C.**3-Methyl-2-pentanone**

CAS – [565-61-7].

Molecular formula and molar mass – $C_6H_{12}O$ – 100.16.*Description* – Colorless and flammable liquid.*Physical characteristics* – Boiling temperature: approximately 118 °C. Density (20 °C): approximately 0.815. Refractive index (20 °C): approximately 1.400.*Conservation* – In closed containers.**Methoxyazobenzene**

CAS – [2396-60-3].

Molecular formula and molar mass – $C_{13}H_{12}N_2O$ – 212.25.*Description* – Orange blades.*Solubility* – Practically insoluble in water, soluble in ethyl alcohol, in petroleum ether and in other organic solvents.*Thin layer chromatography (5.2.17.1)* – Apply, on a silica gel G plate, a solution with 5 mg of methoxyazobenzene in benzene and develop a chromatogram with the same solvent. A single stain appears, with R_f of approximately 0.6.**Methoxyazobenzene RS***Specification* – Solution at 0.2% (w/v) in a mixture of one volume of benzene with four volumes of petroleum ether.**Potassium methoxide**

CAS – [865-33-8].

Molecular formula and molar mass – CH_3OK – 70.13.*Stability* – Extemporaneous preparation.**Sodium methoxide**

CAS – [124-41-4].

Molecular formula and molar mass – CH_3ONa – 54.02.*Description* – White fine powder. Reacts violently with water with formation of heat. Sensitive to air. May be presented in the form of: $CH_3ONa \cdot 2CH_3OH$, white powder. In solution, it can be prepared *in situ*.*Solubility* – Soluble in ethyl alcohol and in methyl alcohol.*Conservation* – In hermetic containers.*Storage* – Protect from humidity.

Methoxyethanol

CAS – [109-86-4].

Synonyms – 2-Methoxyethanol, ethylene glycol monomethyl ether.

Molecular formula and molar mass – $C_3H_8O_2$ – 76.10.

Description – Colorless and clear liquid.

Physical characteristics – Density (20 °C): approximately 0.9663. Refractive index (20 °C): approximately 1.4028. Boiling temperature: approximately 125 °C.

Miscibility – Miscible with water, acetone and ethyl alcohol.

Conservation – In tightly closed containers.

Safety – Poisonous! Use in places with proper ventilation.

Methyl myristate

CAS – [124-10-7].

Molecular formula and molar mass – $C_{15}H_{30}O_2$ – 242.40.

Specification – Contains no less than 98.0% (w/v).

Description – Colorless or faintly yellow liquid.

Physical characteristics – Density (20 °C): approximately 0.868. Refractive index (20 °C): approximately 1.437. Melting temperature: approximately 20 °C.

Miscibility – Miscible with ethyl alcohol and petroleum ether.

Conservation – In tightly closed containers.

Mixture of eriochrome black T

Preparation – Mix 0.2 parts of eriochrome black T with 100 parts of sodium chloride.

Conservation – In tightly closed containers.

Category – Indicator for calcium and magnesium.

Reducer mixture

Preparation – Powder the substances, added on the following order, to obtain a homogeneous mixture: 20 mg of potassium bromide, 0.5 g of hydrazine sulfate and 5 g of sodium chloride.

Sulfochromic mixture

Preparation – Dissolve 50 g of potassium dichromate in approximately 50 mL of water and add 1000 mL of sulfuric acid.

Conservation – In tightly closed containers.

Ammonium molybdate

CAS – [12054-85-2].

Molecular formula and molar mass – $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ – 1235.92.

Specification – Contains no less than 99.0% (w/w).

Description – Colorless to slightly yellow or blue-green, brilliant crystals.

Solubility – Soluble in water and practically insoluble in ethyl alcohol.

Physical characteristics – Loses water and ammonia by heating.

Conservation – In tightly closed containers.

Ammonium molybdate RS

Specification – Contains 10 g of ammonium molybdate in 100 mL of aqueous solution.

Conservation – In tightly closed containers.

Ammonium molybdate RS1

Preparation – Dissolve 6.5 g of finely ground molybdic acid in a mixture of 14 mL of water with 14.5 mL of ammonium hydroxide. Cool down the solution and add it, slowly and stirring, to a cooled

mixture of 32 mL of nitric acid with 40 mL of water. Allow to stand for 48 hours and filter in a crucible with sintered bottom of fine porosity. This solution deteriorates under storage and is inadequate for use if, after adding 2 mL of sodium phosphate dibasic dodecahydrate RS in 5 mL of solution, an abundant yellow precipitate does not form immediately or after slight heating. If there is formation of precipitate during the storage, employ only the clear supernatant solution.

Storage – Protect from light.

Ammonium molybdate, acid solution

Preparation – Dilute 25 mL of 7% (w/v) ammonium molybdate to 200 mL with water. Slowly add 25 mL of 3.75 M sulfuric acid and homogenize.

1% (w/v) ammonium molybdate in M sulfuric acid

Preparation – Weigh 1 g of ammonium molybdate and dissolve with 50 mL of M sulfuric acid solution. Complete the volume to 100 mL with the same solvent.

Sodium molybdate

CAS – [10102-40-6].

Molecular formula and molar mass – $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ – 241.95.

Description – Colorless crystals or white or nearly white crystalline powder.

Solubility – Freely soluble in water.

Molybdovanadate RS

Synonyms – Molybdovanadate reagent, vanadate-molybdate reagent.

Preparation – Using finely powdered substances, prepare a suspension of 4 g ammonium molybdate and 0.1 g of ammonium vanadate in 70 mL of water. Add 20 mL of nitric acid. Complete the volume to 100 mL with water.

Conservation – In tightly closed containers.

Storage – Protect from light.

Morpholine

CAS – [110-91-8].

Synonyms – Tetrahydro-2H-1,4-oxazine, diethylene oximide.

Molecular formula and molar mass – $\text{C}_4\text{H}_9\text{NO}$ – 87.12.

Description – Colorless liquid. Hygroscopic.

Physical characteristic – Boiling temperature: approximately 128 °C.

Miscibility – Miscible with water and ethyl alcohol.

Conservation – In hermetic containers.

Morin

CAS – [6472-38-4].

Molecular formula and molar mass – $\text{C}_{15}\text{H}_{10}\text{O}_7 \cdot 2\text{H}_2\text{O}$ – 338.27.

Naphthalene

CAS – [91-20-3].

Molecular formula and molar mass – C_{10}H_8 – 128.17.

Description – White or nearly white crystals.

Physical characteristics – Melting temperature: approximately 80 °C. Boiling range: between 217 °C and 219 °C.

Solubility – Practically insoluble in water, soluble in ethyl alcohol and freely soluble in benzene and chloroform.

Conservation – In tightly closed containers.

1,3-Naphthalenediol

CAS – [132-86-5].

Molecular formula and molar mass – $C_{10}H_8O_2$ – 160.17.*Description* – Crystalline, usually brown-violet powder.*Physical characteristic* – Melting temperature: approximately 125 °C.*Solubility* – Freely soluble in water and in ethyl alcohol.**2,7-Naphthalenediol**

CAS – [582-17-2].

Molecular formula and molar mass – $C_{10}H_8O_2$ – 160.17.*Description* – Yellow to nearly white crystalline solid or powder.*Physical characteristics* – Melting range: 187 °C to 191 °C.*Solubility* – Soluble in water and in ethyl alcohol.**Naphthalenediol, reagent***Preparation* – Dissolve 20 mg of 1,3-naphthalenediol in 10 mL of ethyl alcohol with 0.2 mL of sulfuric acid.**1-Naphthylamine**

CAS – [134-32-7].

Synonym – α -Naphthylamine.*Molecular formula and molar mass* – $C_{10}H_9N$ – 143.19.*Description* – Colorless crystals or white crystalline powder. Becomes red by exposure to air and light. Unpleasant odor.*Physical characteristic* – Melting range: 49 °C to 51 °C.*Solubility* – Slightly soluble in water and freely soluble in ethyl alcohol.*Conservation* – In tightly closed containers.*Storage* – Protect from light and from air.*Safety* – Harmful vapor and powder.**1-Naphthol**

CAS – [90-15-3].

Synonyms – Alpha-naphthol, α -naphthol.*Molecular formula and molar mass* – $C_{10}H_8O$ – 144.17.*Description* – Colorless, white or nearly white crystals, or white or nearly white crystalline powder. Turns dark with exposure to light.*Physical characteristic* – Melting temperature: approximately 95 °C.*Solubility* – Slightly soluble in water and freely soluble in ethyl alcohol.*Conservation* – In closed containers.*Storage* – Protect from light.**1-Naphthol RS***Specification* – Contains 20% (w/v) of 1-naphthol in ethyl alcohol.*Conservation* – In tightly closed containers.*Stability* – Prepare immediately before use. *Storage* – Protect from light.**1-Naphthol**

CAS – [135-19-3].

Synonyms – Beta-naphthol, b-naphthol.*Molecular formula and molar mass* – $C_{10}H_8O$ – 144.17*Description* – White to slightly pink crystalline powder, of faint phenolic odor.*Physical characteristic* – Melting temperature: approximately 122 °C

Solubility – Very slightly soluble in water and very soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from light.

2-Naphthol RS

Synonyms – Beta-naphthol RS, b-naphthol RS.

Specification – Contains 1 g of 2-naphthol in 100 mL of 1% (w/v) sodium hydroxide.

Conservation – In tightly closed containers.

Stability – Prepare immediately before use.

Storage – Protect from light.

2-Naphthol RS1

Synonyms – Beta-naphthol RS1, b-naphthol RS1.

Preparation – Dissolve 5 g of freshly recrystallized 2-naphthol in 40 mL of sodium hydroxide 2 M and complete the volume to 100 mL with water.

Conservation – In tightly closed containers.

Stability – Prepare immediately before use.

Storage – Protect from light.

Naringin

CAS – [10236-47-2].

Molecular formula and molar mass – $C_{27}H_{32}O_{14}$ – 580.54.

Description – White or nearly white crystalline powder.

Physical characteristic – Melting temperature: approximately 171 °C.

Solubility – Slightly soluble in water, soluble in methyl alcohol and in dimethylformamide.

Amido black 10B

CAS – [1064-48-8].

Molecular formula and molar mass – $C_{22}H_{14}N_6Na_2O_9S_2$ – 616.50.

Description – Dark brown to black powder.

Solubility – Moderately soluble in water, soluble in ethyl alcohol.

Amido black 10B RS

Specification – 0.5% (w/v) amido black 10B solution in a mixture of acetic acid with methyl alcohol (10:90).

Ninhydrin

CAS – [485-47-2].

Synonym – Indantrione hydrate.

Molecular formula and molar mass – $C_9H_4O_3 \cdot H_2O$ – 178.14

Specification – Contains no less than 96.0% (w/w).

Description – White to faintly pale yellow crystalline powder.

Solubility – Soluble in water and in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from light.

Ninhydrin acetic ethanol RS

Preparation – Dissolve 1 g of ninhydrin in 50 mL of ethyl alcohol and add 10 mL of glacial acetic acid.

Ninhydrin RS

Synonym – Indantrione hydrate RS.

Specification – Contains 0.2% (w/v) in a mixture of butyl alcohol and 2 M acetic acid (95:5).

Conservation – In tightly closed containers.

Storage – Protect from light.

Safety – Flammable.

Ceric ammonium nitrate

CAS – [16774-21-3].

Molecular formula and molar mass – $(\text{NH}_4)_2[\text{Ce}(\text{NO}_3)_6]$ – 548.22.

Description – Orange-yellow crystalline powder or clear orange crystals.

Solubility – Soluble in water.

Aluminum nitrate, nonahydrate

CAS – [7784-27-2].

Molecular formula and molar mass – $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ – 375.14

Description – Deliquescent crystals.

Solubility – Very soluble in water and ethyl alcohol, very slightly soluble in acetone.

Conservation – In hermetically closed containers

Ammonium nitrate

CAS – [6484-52-2].

Molecular formula and molar mass – NH_4NO_3 – 80.04.

Description – Colorless, deliquescent crystals or white powder, with salty flavor.

Physical characteristics – Melting temperature: approximately 155 °C, decomposes around 210 °C in water and nitrogen oxides.

Solubility – Very soluble in water, freely soluble in methyl alcohol and soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Ammonium nitrate RS

Specification – Contains 5 g of ammonium nitrate in 100 mL of aqueous solution.

Ammonium nitrate, saturated solution

Specification – Contains 20.1 g of ammonium nitrate in 10 mL of water.

Conservation – In tightly closed containers.

Barium nitrate

CAS – [10022-31-8].

Molecular formula and molar mass – BaN_2O_6 – 261.34.

Description – Crystals or crystalline powder.

Physical characteristic – Melting temperature: approximately 590 °C.

Solubility – Freely soluble in water, very slightly soluble in ethyl alcohol and in acetone.

Conservation – In tightly closed containers. *Safety* – Poison!

Cadmium nitrate

CAS – [10022-68-1].

Molecular formula and molar mass – $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ – 308.48.

Description – Colorless crystals. Hygroscopic.

Solubility – Very soluble in water and soluble in acetone and in ethyl alcohol.

Lead nitrate

CAS – [10099-74-8].

Synonym – Lead(II) nitrate.

Molecular formula and molar mass – $\text{Pb}(\text{NO}_3)_2$ – 331.21.

Specification – Contains no less than 99.0% (w/w).

Description – Colorless, translucent crystals or white crystalline powder.

Solubility – Freely soluble in water.

Conservation – In tightly closed containers.

Safety – Poison!

Cobalt(II) nitrate

CAS – [10026-22-9].

Synonym – Cobaltous nitrate.

Molecular formula and molar mass – $\text{CoN}_2\text{O}_6 \cdot 6\text{H}_2\text{O}$ – 291.03.

Specification – Contains no less than 99.0% (w/w).

Description – Small, red, hygroscopic crystals.

Physical characteristic – Melting temperature: approximately 55 °C.

Solubility – Soluble in water.

Conservation – In tightly closed containers.

Storage – Protect from heat.

Cobalt(II) nitrate RS

Description – Contains 1.0% (w/v) cobalt(II) nitrate in methyl alcohol.

Conservation – In tightly closed containers.

Safety – Flammable. Toxic.

Lanthanum nitrate

CAS – [10277-43-7].

Molecular formula and molar mass – $\text{LaN}_3\text{O}_9 \cdot 6\text{H}_2\text{O}$ – 433.01.

Description – Colorless, deliquescent crystals.

Solubility – Freely soluble in water.

Conservation – In tightly closed containers.

Lanthanum nitrate RS

Specification – Contains 5% (w/v) of lanthanum nitrate in water.

Conservation – In tightly closed containers.

Magnesium nitrate

CAS – [13446-18-9].

Molecular formula and molar mass – $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ – 256.40.

Description – Colorless and deliquescent crystals.

Solubility – Very soluble in water and freely soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Mercury(I) nitrate

CAS – [14836-60-3].

Synonym – Mercurous nitrate.

Molecular formula and molar mass – $\text{Hg}_2\text{N}_2\text{O}_6 \cdot 2\text{H}_2\text{O}$ – 561.22.

Description – Colorless crystals, usually with faint odor of nitric acid.

Physical characteristic – Melting temperature: approximately 70 °C, with decomposition.

Conservation – In tightly closed containers.

Storage – Protect from light.

Safety – Poison!

Mercury(I) nitrate RS

Synonym – Mercurous nitrate RS.

Specification – Contains 15 g of mercury(I) nitrate in a mixture with 90 mL of water and 10 mL of 10% (v/v) nitric acid.

Conservation – In closed amber glass containers.

Stability – Add a small pellet of metallic mercury.

Storage – Protect from light.

Mercury(II) nitrate

CAS – [7783-34-8].

Synonym – Mercuric nitrate.

Molecular formula and molar mass – $\text{HgN}_2\text{O}_6 \cdot \text{H}_2\text{O}$ – 342.61.

Description – Colorless or faintly colored crystals. Hygroscopic.

Solubility – Soluble in water in presence of a small amount of nitric acid.

Conservation – In hermetic containers.

Storage – Protect from light and humidity. *Safety* – Poison!

Potassium nitrate

CAS – [7757-79-1].

Molecular formula and molar mass – KNO_3 – 101.10.

Specification – Contains no less than 99.5% (w/w).

Description – Colorless and clear crystals or white, crystalline or granular powder.

Solubility – Very soluble in water.

Conservation – In tightly closed containers.

Silver nitrate

CAS – [7761-88-8].

Molecular formula and molar mass – AgNO_3 – 169.87.

Specification – Contains no less than 99.0% (w/w).

Description – Colorless clear crystals, or white crystalline powder. Odorless.

Physical characteristic – Melting temperature: 212 °C.

Solubility – Very soluble in water and soluble in ethyl alcohol.

Conservation – In closed non-metallic containers.

Storage – Protect from light.

Safety – Caustic. Poison!

0.1 M silver nitrate

Specification – Contains 17 g of silver nitrate in 1000 mL of aqueous solution.

Conservation – In tightly closed containers.

Storage – Protect from light.

Silver nitrate RS

Specification – Contains 4.25% (w/v) of silver nitrate in water.

Conservation – In tightly closed containers.

Storage – Protect from light.

Silver nitrate RS1

Synonym – Silver nitrate reagent.

Preparation – Mix 3 mL of concentrated ammonia solution and 40 mL of M sodium hydroxide, add, drop by drop and stirring, 8 mL of 20% (w/v) silver nitrate solution. Dilute to 200 mL with water.

Sodium nitrate

CAS – [7631-99-4].

Molecular formula and molar mass – NaNO_3 – 84.99.

Description – Colorless and clear crystals, or white or nearly white granule or powder. Deliquescent.
Physical characteristic – Melting temperature: 308 °C.
Solubility – Freely soluble in water and slightly soluble in ethyl alcohol.
Conservation – In tightly closed containers.

Sodium nitrate RS

Specification – Contains 10 g of sodium nitrate in 100 mL of aqueous solution.
Stability – Prepare immediately before use.

Thorium nitrate

CAS – [13470-07-0].
Molecular formula and molar mass – $\text{ThN}_4\text{O}_{12} \cdot 4\text{H}_2\text{O}$ – 552.12.
Description – White crystals or crystalline, slightly deliquescent powder.
Solubility – Very soluble in water and ethyl alcohol.
Conservation – In tightly closed containers.
Storage – Protect from humidity.

Zirconyl nitrate

CAS – [14985-18-3].
Synonym – Zirconium nitrate.
Molecular formula – $\text{ZrO}(\text{NO}_3)_2 \cdot x\text{H}_2\text{O}$.
Description – White or nearly white powder or crystals.
Conservation – In tightly closed containers.

Zirconyl nitrate RS

Preparation – Dissolve 0.1 g of zirconyl nitrate in a mixture of 60 mL of hydrochloric acid with 40 mL of water.
Conservation – In tightly closed containers.

Phenylmercuric nitrate

CAS – [55-68-5].
Synonyms – Basic phenylmercury nitrate and phenylmercury nitrate.
Molecular formula and molar mass – $\text{C}_6\text{H}_5\text{HgNO}_3$ – 339.70.
Specification – Consists of a mixture of nitrate and phenylmercury ion hydroxide ($\text{C}_6\text{H}_5\text{Hg}^+$). Contains no less than 87.9% (w/w) phenylmercuric ion and no less than 62.75% (w/w) mercury (Hg).
Description – White crystalline powder or white lustrous scales. Odorless.
Physical characteristic – Melting range: between 175 °C and 190 °C, with decomposition.
Solubility – Very soluble in water and in ethyl alcohol, slightly soluble in hot water. Soluble in glycerol and fatty oils.
Conservation – In hermetic containers.
Storage – Protect from light.

Nitrazepam

CAS – [146-22-5].
Molecular formula and molar mass – $\text{C}_{15}\text{H}_{11}\text{N}_3\text{O}_3$ – 281.27.
Description – Yellow crystalline powder.
Physical characteristic – Melting range: 226 °C to 230 °C.
Solubility – Practically insoluble in water and slightly soluble in ethyl alcohol.
Conservation – In closed containers.
Storage – Protect from exposure to light.

Sodium nitrite

CAS – [7632-00-0].

Molecular formula and molar mass – NaNO_2 – 69.00.

Specification – Contains no less than 97.0% (w/w).

Description – White or slightly yellow granulated powder or colorless crystals. Hygroscopic.

Physical characteristics – Melting temperature: 271 °C. Decomposes above 320 °C.

Solubility – Freely soluble in water.

Conservation – In tightly closed containers.

Stability – Oxidizes on air very slowly, forming nitrate.

Sodium nitrite RS

Specification – Contains 10 g of sodium nitrite in 100 mL of aqueous solution.

Conservation – Prepare immediately before use.

***p*-Nitroaniline**

CAS – [100-01-6].

Molecular formula and molar mass – $\text{C}_6\text{H}_6\text{N}_2\text{O}_2$ – 138.13.

Description – Light crystalline powder.

Physical characteristic – Melting range: from 146 °C to 148 °C.

Solubility – Insoluble in water and soluble in ethyl alcohol and ethyl ether. It forms a salt soluble in aqueous solution with strong mineral acid.

Conservation – In tightly closed containers.

***p*-Nitroaniline and sodium nitrite RS**

Solution A – Dissolve 0.3 g of *p*-nitroaniline in 100 mL of 10 M hydrochloric acid. *Solution B* – Dissolve 2.5 g of sodium nitrite in 50 mL of water.

Preparation – Mix 90 mL of *Solution A* with 10 mL of *Solution B* at the moment of use.

2-Nitrobenzaldehyde

CAS – [552-89-6].

Molecular formula and molar mass – $\text{C}_7\text{H}_5\text{NO}_3$ – 151.12.

Description – Yellow crystals, with odor similar to almond oil.

Physical characteristic – Melting temperature: approximately 42 °C.

Solubility – Slightly soluble in water and freely soluble in ethyl alcohol.

Nitrobenzene

CAS – [98-95-3].

Synonym – Nitrobenzol.

Molecular formula and molar mass – $\text{C}_6\text{H}_5\text{NO}_2$ – 123.11.

Description – Colorless to pale yellow liquid, with odor similar to almond oil.

Physical characteristics – Boiling temperature: approximately 211 °C. Density: approximately 1.20.

Solubility – Practically insoluble in water; miscible with ethyl alcohol.

Conservation – In tightly closed containers.

Safety – Poison!

Nitromethane

CAS – [75-52-5].

Molecular formula and molar mass – CH_3NO_2 – 61.04.

Description – Colorless oily liquid, with characteristic odor.

Physical characteristic – Boiling temperature: approximately 102 °C.

Solubility – Slightly soluble in water; miscible with ethyl alcohol.

Sodium nitroprusside

CAS – [13755-38-9].

Synonyms – Disodium pentacyanonitrosylferrate(III) dihydrate, nitroprusside sodium, sodium nitroferrocyanide.*Molecular formula and molar mass* – $\text{Na}_2[\text{Fe}(\text{CN})_5(\text{NO})] \cdot 2\text{H}_2\text{O}$ – 297.95.*Description* – Dark red, clear powder or crystals.*Solubility* – Freely soluble in water and slightly soluble in ethyl alcohol.**Sodium nitroprusside and piperazine RS***Specification* – Contains 0.1 g of sodium nitroprusside and 0.25 g of piperazine in 5 mL of water.*Conservation* – In tightly closed containers.**Sodium 1-octanesulfonate**

CAS – [5324-84-5].

Molecular formula and molar mass – $\text{C}_8\text{H}_{17}\text{NaO}_3\text{S}$ – 216.27.*Specification* – Contains no less than 98.0% of $\text{C}_8\text{H}_{17}\text{NaO}_3\text{S}$.*Description* – White or nearly white flakes or crystalline powders.**Sodium octyl sulfate**

CAS – [142-31-4].

Molecular formula and molar mass – $\text{C}_8\text{H}_{17}\text{NaO}_4\text{S}$ – 232.27.*Description* – White or nearly white flakes or crystalline powder.*Solubility* – Freely soluble in water and soluble in methyl alcohol.**Octoxynol 10**

CAS – [9002-93-1].

Molecular formula and molar mass – $(\text{C}_2\text{H}_4\text{O})_{10}\text{C}_{14}\text{H}_{22}\text{O}$ – 646.86.*Description* – Light yellow clear, viscous liquid.*Solubility* – Miscible with water, acetone and ethyl alcohol; soluble in toluene.*Conservation* – In tightly closed container.**Olive oil**

CAS – [8001-25-0].

Specification – Fixed oil obtained from the ripe fruit of *Olea europaea* L. – Oleaceae.*Description* – Pale yellow or green-yellow oil.*Physical characteristic* – Density: 0.910 to 0.915.*Solubility* – Practically insoluble in ethyl alcohol; miscible with chloroform, ethyl ether and petroleum ether.**Ammonium oxalate**

CAS – [6009-70-7].

Molecular formula and molar mass – $\text{C}_2\text{H}_8\text{N}_2\text{O}_4 \cdot \text{H}_2\text{O}$ – 142.11.*Specification* – Contains no less than 99.0% (w/w).*Description* – Colorless clear crystals or white crystalline powder. Odorless.*Physical characteristic* – Melting temperature: 212 °C.*Solubility* – Soluble in water. *Conservation* – In tightly closed containers.*Safety* – Caustic. Corrosive. Poison!**Ammonium oxalate RS**

Use ammonium oxalate TS.

Potassium oxalate

CAS – [6487-48-5].

Molecular formula and molar mass – $\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$ – 184.23; if anhydrous – 166.21.

Description – Colorless, odorless crystals, efflorescent to hot and dry air.

Physical characteristic – Loses water at approximately 160 °C

Conservation – In hermetic containers.

Storage – Protect from humidity.

Safety – Poison!

Sodium oxalate

CAS – [62-76-0].

Molecular formula and molar mass – $\text{Na}_2\text{C}_2\text{O}_4$ – 134.00.

Description – White or nearly white crystalline powder.

Solubility – Soluble in water and practically insoluble in ethyl alcohol.

Malachite green oxalate

CAS – [633-03-4].

Synonym – Brilliant green.

Molecular formula and molar mass – $\text{C}_{27}\text{H}_{34}\text{N}_2\text{O}_4\text{S}$ – 428.64.

Description – Golden yellow brilliant crystals.

Conservation – In tightly closed containers.

Aluminum oxide

CAS – [1344-28-1].

Synonym – Alumina.

Molecular formula and molar mass – Al_2O_3 – 101.96.

Description – White fine granulated powder.

Physical characteristic – The pH (5.2.19) of the suspension at 10.0% (w/v) is between 9.0 and 10.0.

Conservation – In hermetic containers.

Holmium oxide

CAS – [12055-62-8].

Molecular formula and molar mass – Ho_2O_3 – 377.86.

Specification – Contains no less than 99.9% (w/w).

Description – Yellow powder.

Solubility – Practically insoluble in water.

Conservation – In tightly closed containers.

Magnesium oxide

CAS – [1309-48-4].

Synonym – Light or heavy magnesium oxide.

Molecular formula and molar mass – MgO – 40.30.

Specification – Contains no less than 95.0% (w/w).

Description – White, odorless, fine amorphous powder, with faint alkaline flavor.

Conservation – In tightly closed containers.

Storage – Protect from contact with air and with humidity.

Silver oxide

CAS – [20667-12-3].

Molecular formula and molar mass – Ag_2O – 231.74.

Description – Dark gray powder.

Solubility – Practically insoluble in water and in ethyl alcohol, freely soluble in diluted nitric acid and in ammonium hydroxide.

Conservation – In closed containers.

Storage – Protect from light.

Mercuric oxide

CAS – [21908-53-2].

Synonyms – Yellow mercury oxide, mercury(II) oxide.

Molecular formula and molar mass – HgO – 216.59.

Specification – Contains no less than 99.5% (w/w).

Description – Dense, odorless orange-yellow powder.

Solubility – Practically insoluble in water and in ethyl alcohol.

Storage – Protect from light.

Safety – Poison!

Palladium RSA – 1 mg/mL

Specification – Contains 1.67 g of palladium chloride in 200 mL of 50% (v/v) hydrochloric acid. Heat until complete dissolution. Cool down and complete the volume to 1000 mL with water.

Conservation – In tightly closed, inert containers (like polyethylene).

Methyl palmitate

CAS – [112-39-0].

Molecular formula and molar mass – $\text{C}_{17}\text{H}_{34}\text{O}_2$ – 270.46.

Description – White or yellow crystalline mass.

Physical characteristics – Density (30 °C): approximately 0.86. Melting temperature: approximately 30 °C.

Solubility – Soluble in ethyl alcohol and in petroleum ether.

Conservation – In tightly closed containers.

Silver manganese paper

Preparation – Add to a mixture of equal volumes of 0.1 M silver nitrate and 1.5% (w/v) manganese sulfate, drop by drop, 0.1 M sodium hydroxide until a persistent precipitate is formed. Filter. Immerse filter paper strips in the solution for 15 minutes. Dry at room temperature, protected from light and from acid or alkaline vapors. Silver manganese paper must be colorless.

Sensitivity assay – In a cylinder of approximately 40 mL, introduce 1 mL of 1% (w/v) ammonium chloride. Add 9 mL of water and 1 g of magnesium oxide. Close the container immediately with a polyethylene cap, under which silver manganese paper is placed. Shake the solution, being careful to not let magnesium particles come into contact with the paper. Keep the cylinder at 50 °C to 60 °C for one hour. A gray color appears on the reagent paper.

Liquid paraffin

Specification – Purified mixture of liquid saturated hydrocarbons obtained from petroleum.

Description – Colorless and clear oily liquid.

Physical characteristics – Density: 0.827 to 0.890. Viscosity: 110 mPa to 230 mPa.

Solubility – Practically insoluble in water and slightly soluble in ethyl alcohol; miscible with hydrocarbons.

Conservation – In tightly closed containers.

Storage – Protect from light.

Sodium 1-pentanesulfonate monohydrate

CAS – [207605-40-1].

Molecular formula and molar mass – $\text{C}_5\text{H}_{11}\text{NaO}_3\text{S} \cdot \text{H}_2\text{O}$ – 192.21.

Description – White or nearly white crystalline solid.

Solubility – Soluble in water.

Conservation – In tightly closed containers.

Phosphorus pentoxide

CAS – [1314-56-3].

Synonym – Phosphoric anhydride.

Molecular formula and molar mass – P_2O_5 – 141.94.

Description – White, amorphous, very deliquescent powder.

Physical characteristics – Melting temperature: 340 °C. Sublimation temperature: 360 °C.

Conservation – In hermetic containers.

Storage – Protect from humidity.

Safety – Irritant. Corrosive to skin, mucous membrane and eyes.

Vanadium pentoxide

CAS – [1314-62-1].

Molecular formula and molar mass – V_2O_5 – 181.88.

Specification – Contains no less than 99.5% (w/w).

Description – Yellow to orange-yellow fine powder.

Physical characteristic – Melting temperature: 690 °C.

Solubility – Slightly soluble in water and soluble in strong mineral acids and alkali hydroxide solutions with formation of salts.

Conservation – In tightly closed containers.

Purified pepsin

Specification – Derived from porcine gastric mucosa, with activity of 800 to 2500 units/mg of protein.

Description – White or yellow, amorphous or crystalline powder. Hygroscopic.

Solubility – Soluble in water, practically insoluble in ethyl alcohol. The solution in water may turn slightly opalescent with a small amount of acid.

Conservation – In closed container.

Storage – Protected from light and at temperature between 2 °C and 8 °C.

Labeling – It must express the pepsin activity.

Peptone

Specification – Mixture of products of polypeptide nature coming from animal proteins (meat, casein). The origin determines the physical characteristics, composition and production process.

Description – Light yellow to brown powder. Characteristic odor and flavors. Minimum content in nitrogen: 12.0% (w/w) of casein and 14.2% (w/w) of meat.

Conservation – In tightly closed containers.

Storage – Protect from humidity.

Labeling – It must express the origin and content in nitrogen.

Sodium perchlorate

CAS – [7791-07-3].

Synonym – Perchloric acid monohydrate sodium salt.

Molecular formula and molar mass – $\text{NaClO}_4 \cdot \text{H}_2\text{O}$ – 140.45.

Specification – Contains no less than 99.0% (w/w).

Description – Colorless, deliquescent crystals.

Solubility – Very soluble in water, soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Potassium periodate

CAS – [7790-21-8].

Synonym – Potassium metaperiodate.

Molecular formula and molar mass – KIO_4 –229.99.

Description – White crystalline powder or colorless crystals.

Physical characteristic – Melting temperature: 582 °C.

Safety – Highly irritant to skin, eyes and mucous membranes.

Ferric potassium periodate RS

Preparation – Dissolve 1 g of potassium periodate in 5 mL of freshly prepared 12% (w/v) potassium hydroxide solution. Add 20 mL of water and 1.5 mL of ferric chloride RS. Complete the volume to 50 mL with freshly prepared 12% (w/v) potassium hydroxide solution.

Sodium periodate

CAS – [7790-28-5].

Synonym – Sodium metaperiodate.

Molecular formula and molar mass – NaIO_4 –213.89.

Specification – Contains no less than 99.0% (w/w) of sodium periodate.

Description – White tetragonal crystals.

Physical characteristic – Melting temperature: approximately 300 °C, with decomposition.

Solubility – Soluble in water, acetic acid, nitric acid and sulfuric acid.

Conservation – In tightly closed containers.

Storage – In ventilated places.

Safety – Strong oxidizing agent.

Potassium permanganate

CAS – [7722-64-7].

Molecular formula and molar mass – KMnO_4 –158.03.

Specification – Contains no less than 99.0% (w/w) in relation to the desiccated substance.

Description – Dark violet crystals, with metallic luster, odorless, of sweet astringent flavor.

Solubility – Soluble in cold water and freely soluble in water in ebullition.

Conservation – In tightly closed containers.

Storage – Protect from light.

Safety – The substance and its solutions present risk of explosion when in contact with oxidizable materials.

Category – Energetic oxidizing agent.

Potassium permanganate RS (approximately 0.2 M)

Specification – Contains 3% (w/v) of potassium permanganate in water.

Stability – Prepare immediately before use.

Conservation – In tightly closed containers.

Storage – Protect from light.

Safety – Irritant. Caustic.

Carbamide peroxide

CAS – [124-43-6].

Synonym – Urea hydrogen peroxide.

Molecular formula and molar mass – $\text{CH}_6\text{N}_2\text{O}_3$ –94.07.

Description – White crystals or crystalline powder. Decomposes at contact with air in urea, oxygen and water.

Solubility – Soluble in water.

Conservation – In tightly closed containers. *Category* – Oxidizing agent.

Concentrated hydrogen peroxide

CAS – [7722-84-1].

Synonym – Perhydrol.

Molecular formula and molar mass – H_2O_2 – 34.01.

Specification – Contains no less than 29.0% (w/w) of hydrogen peroxide. Corresponds to, approximately, 100 parts in volume. May contain stabilizer.

Description – Clear, irritant liquid, with faint odor.

Physical characteristic – Density: 1.11.

Conservation – In partially full containers that have a relief closing system.

Storage – Protect from light and from heat.

Safety – Strong oxidizing agent.

Hydrogen peroxide, 30 volumes, RS

Molecular formula and molar mass – H_2O_2 – 34.01.

Specification – Contains no less than 9.7% (w/v) and no more than 10.7% (w/v) hydrogen peroxide, corresponding to approximately 30 parts in volume. May contain stabilizer.

Description – Dilute the concentrated hydrogen peroxide.

Conservation – In closed containers.

Stability – Avoid long storage periods.

Storage – Protect from light and from heat.

3% (w/v) hydrogen peroxide

Molecular formula and molar mass – H_2O_2 – 34.01.

Specification – Contains no less than 2.5% (w/v) and no more than 3.5% (w/v) hydrogen peroxide, corresponding to approximately 10 parts in volume. May contain stabilizer.

Description – Clear, colorless liquid.

Conservation – In closed containers. Avoid long storage periods.

Storage – Protect from light and from heat.

Methanolic hydrogen peroxide

Preparation – On the day of use, dilute 2 mL of concentrated hydrogen peroxide to 100 mL with methyl alcohol and store in a refrigerator. Immediately before use, dilute 2 mL of this solution to 100 mL with methyl alcohol.

Sodium peroxide

CAS – [1313-60-6].

Molecular formula and molar mass – Na_2O_2 – 77.98.

Description – Yellow-white granular powder.

Solubility – Freely soluble in water, forming sodium hydroxide and hydrogen peroxide, which decomposes into oxygen gas and water.

Conservation – In tightly closed containers, protected from organic and oxidizable substances.

Ammonium persulfate

CAS – [7727-54-0].

Synonym – Ammonium peroxydisulfate.

Molecular formula and molar mass – $\text{H}_8\text{N}_2\text{O}_8\text{S}_2$ – 228.19.

Specification – Contains no less than 95.0% (w/w).

Description – White crystals or granulated powder. Odorless. Stable for months when pure and dry; decomposes in presence of humidity.

Solubility – Freely soluble in water.

Conservation – In hermetic containers.

Storage – Protect from humidity, heat and organic material.

Additional information – Strongly oxidizing agent.

Potassium persulfate

CAS – [7727-21-1].

Molecular formula and molar mass – $\text{K}_2\text{S}_2\text{O}_8$ – 270.32.*Description* – Colorless crystals or white or nearly white crystalline powder.*Solubility* – Moderately soluble in water, practically insoluble in ethyl alcohol. In aqueous solution, decomposes at room temperature, and the increase in temperature leads to higher decomposition speed.*Conservation* – In tightly closed containers.*Storage* – In a ventilated place.**Sodium persulfate**

CAS – [7775-27-1].

Molecular formula and molar mass – $\text{Na}_2\text{O}_8\text{S}_2$ – 238.09.*Description* – White crystalline powder. Slowly decomposes with humidity and by heat.*Conservation* – In hermetic containers.*Storage* – Protect from humidity and heat. *Safety* – Irritant.**Alkaline sodium picrate RS***Preparation* – Mix 20 mL of 1% (w/v) picric acid with 10 mL of 5% (w/v) sodium hydroxide and complete the volume to 100 mL with water.*Stability* – Use within two days.**Piperazine**

CAS – [110-85-0].

Molecular formula and molar mass – $\text{C}_4\text{H}_{10}\text{N}_2$ – 86.14.*Description* – White or nearly white lumps or flakes. Ammoniacal odor.*Solubility* – Soluble in water and in ethyl alcohol, insoluble in ethyl ether.**Pyridine**

CAS – [110-86-1].

Molecular formula and molar mass – $\text{C}_5\text{H}_5\text{N}$ – 79.10.*Description* – Colorless liquid, with characteristic and unpleasant odor.*Physical characteristics* – Boiling range: 115 °C to 116 °C.

Density (25 °C): approximately 0.980. Refractive index (20 °C): 1.5092.

Miscibility – Miscible with water and ethyl alcohol.*Conservation* – In tightly closed containers.*Storage* – Protect from humidity.*Safety* – Flammable. Toxic.**Pyridine anhydrous***Specification* – Contains no more than 0.01% (w/w) water.*Preparation* – Dry the pyridine with anhydrous sodium carbonate. Filter and distill.*Conservation* – In tightly closed containers.*Storage* – Protect from humidity.*Safety* – Flammable. Toxic.**Potassium pyroantimonate RS***Preparation* – Dissolve 2 g of potassium pyroantimonate in 85 mL of hot water. Cool it down quickly and add 50 mL of the potassium hydroxide solution 5% (w/v) potassium hydroxide solution and 1 mL of the 8.5% (w/v) sodium hydroxide solution. Allow to stand for 24 hours, filter and dilute with water to 150 mL.

Sodium pyrophosphate

CAS – [13472-36-1].

Molecular formula and molar mass – $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ – 446.05.*Description* – Colorless, slightly efflorescent crystals.*Physical characteristic* – Melting temperature: 79.5 °C.*Solubility* – Freely soluble in water.*Conservation* – In tightly closed containers.**Pyrogallol**

CAS – [87-66-1].

Molecular formula and molar mass – $\text{C}_6\text{H}_6\text{O}_3$ – 126.11.*Description* – White or nearly white crystals. Becomes brown by exposure to air and light.*Physical characteristic* – Melting temperature: approximately 131 °C.*Solubility* – Very soluble in water and in ethyl alcohol. Aqueous solutions become brown by exposure to air.*Conservation* – In closed containers.*Storage* – Protect from light.**Polyacrylamide**

CAS – [9003-05-8].

Synonym – Acrylamide polymer.*Molecular formula and molar mass* – $(\text{C}_3\text{H}_5\text{NO})_n$; monomer – 71.08.*Specification* – Polymer in several forms, soluble and insoluble in water, obtained by heating with several polymerization catalysts.*Conservation* – In tightly closed containers.*Safety* – Highly toxic and irritant. Causes paralysis of the central nervous system. It may be absorbed by intact skin.**Polysucrose**

CAS – [26873-85-8]

Molecular formula – $(\text{C}_{12}\text{H}_{22}\text{O}_{11} \cdot \text{C}_3\text{H}_5\text{ClO})_n$ *Description* – White or nearly white powder.**Polysucrose and meglumine sodium diatrizoate***Preparation* – Dilute 36 mL of polysucrose (9% aqueous solution) in 100 mL of distilled water, add 10 mL of meglumine sodium diatrizoate (50% aqueous solution). Check the density (1.077 g/cm³) and sterilize by moist heat at 121 °C for 15 minutes.**Polysorbate 20**Refer to the *Polysorbate 20* monograph.**Polysorbate 80***Specification* – Mixture of sorbitol oleates and their copolymerized anhydrides with, approximately, 20 M ethylene oxide for every mol of sorbitol and anhydride.*Description* – Yellow or dark yellow clear liquid. Oily. Faint characteristic odor.*Physical characteristics* – Density: approximately 1.08. Viscosity: approximately 400 cP.*Conservation* – In tightly closed containers.*Category* – Surfactant.**Potassium RSA – 600 µg/mL***Specification* – Contains 1.144 g of potassium chloride in 1000 mL of aqueous solution.

Conservation – In tightly closed, inert containers (like polyethylene).

Prednisolone

CAS – [50-24-8].

Molecular formula and molar mass – $C_{21}H_{28}O_5$ – 360.45.

Specification – Contains no less than 97.0% (w/w) in relation to the desiccated substance.

Description – White or nearly white crystalline powder. Hygroscopic. Presented in anhydrous form or containing one or half a molecule of hydration water.

Physical characteristic – Melting temperature: 240 °C to 241 °C, with decomposition.

Solubility – Very slightly soluble in water, soluble in ethyl alcohol and in methyl alcohol, moderately soluble in acetone, and slightly soluble in methylene chloride.

Conservation – In tightly closed containers.

Therapeutic class – Corticoid.

Prednisone

CAS – [53-03-2].

Molecular formula and molar mass – $C_{21}H_{26}O_5$ – 358.43.

Specification – Contains no less than 97.0% (w/w) in relation to the desiccated substance.

Description – White or nearly white crystalline powder.

Physical characteristic – Melting temperature: approximately 233 °C, with decomposition.

Solubility – Practically insoluble in water and slightly soluble in ethyl alcohol and in methylene chloride.

Conservation – In tightly closed containers.

Therapeutic class – Corticoid.

Brilliant black BN

CAS – [2519-30-4].

Molecular formula and molar mass – $C_{28}H_{17}N_5Na_4O_{14}S_4$ – 867.69.

Description – Fine crystals, violet blue or grey-black powder. Redox indicator. Oxidized form: violet blue. Reduced form: brown-yellow.

Physical characteristic – Specific absorptivity A (1%, 1 cm) is higher than 0.390 at 570 nm.

Conservation – In tightly closed containers.

Propylene glycol

CAS – [57-55-6].

Synonym – 1,2-Propanediol.

Molecular formula and molar mass – $C_3H_8O_2$ – 76.10.

Description – Colorless, viscous, hygroscopic liquid.

Physical characteristics – Density (25 °C): 1.035 to 1.037. Boiling range: 187 °C to 189 °C.

Miscibility – Miscible with water and ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from humidity.

Propylparaben

CAS – [94-13-3].

Synonym – 4-hydroxybenzoic acid propyl ester.

Molecular formula and molar mass – $C_{10}H_{12}O_3$ – 180.20.

Description – White crystals.

Solubility – Very slightly soluble in water, freely soluble in ethyl alcohol and in ethyl ether.

Category – Preservative.

Phthalein purple

CAS – [2411-89-4].

Synonym – Metal phthalein.

Molecular formula and molar mass – $C_{32}H_{32}N_2O_{12}$ – 636.61.

Description – Light yellow to brown powder. It may be found in the form of sodium salt: light yellow to pink powder.

Solubility – Practically insoluble in water and soluble in ethyl alcohol. In the form of sodium salt, it is soluble in water and practically insoluble in ethyl alcohol.

Sensitivity assay – Dissolve 10 mg of phthalein purple in 1 mL of concentrated ammonia solution and dilute to 100 mL with water. Add to 5 mL of the solution 95 mL of water, 4 mL of concentrated ammonia solution, 50 mL of ethyl alcohol and 0.1 mL 0.1 M barium chloride VS. The solution presents a violet blue color. Add 0.15 mL of 0.1 M disodium edetate VS. The solution must become colorless.

Quinalizarin (CI 58500)

CAS – [81-61-8].

Synonym – Mordant violet 26.

Molecular formula and molar mass – $C_{14}H_8O_6$ – 272.21.

Description – Dark red powder.

Conservation – In tightly closed containers.

Quinidine

CAS – [56-54-2].

Molecular formula and molar mass – $C_{20}H_{24}N_2O_2$ – 324.42.

Description – White or nearly white crystals.

Physical characteristics – Specific rotation power (20 °C): approximately +260°, determined on a 1% (w/v) quinidine solution in ethyl alcohol. Melting temperature: approximately 172 °C.

Solubility – Very slightly soluble in water, moderately soluble in ethyl alcohol and slightly soluble in methyl alcohol.

Conservation – In closed containers.

Storage – Protect from exposure to light

Quinhydrone

CAS – [106-34-3].

Molecular formula and molar mass – $C_{12}H_{10}O_4$ – 218.21.

Description – Lustrous crystals or dark green crystalline powder.

Physical characteristic – Melting temperature: 170 °C, may sublime and decompose partially.

Solubility – Moderately soluble in cold water, soluble in hot water, ammonia and ethyl ether.

Conservation – In closed containers.

Quinine

CAS – [130-95-0].

Molecular formula and molar mass – $C_{20}H_{24}N_2O_2$ – 324.42.

Description – White or nearly white micro-crystalline powder.

Physical characteristics – Specific rotation power (20 °C): approximately -167°, determined on a 1% (w/v) quinine solution in ethyl alcohol. Melting temperature: approximately 175 °C. Solubility – Very slightly soluble in water, slightly soluble in water in ebullition and very soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from light.

Rhaponticin

CAS – [155-58-8].

Molecular formula and molar mass – $C_{21}H_{24}O_9$ – 420.41.

Description – Yellow-gray crystalline powder.

Solubility – Soluble in ethyl alcohol and in methyl alcohol.

Aluminon reagent

Solution A – Dissolve 250 g of ammonium acetate in 500 mL of purified water. Add 40 mL of glacial acetic acid; 0.5 g of aluminon dissolved in 50 mL of purified water; 1 g of benzoic acid dissolved in 150 mL of isopropyl alcohol; and 225 mL of isopropyl alcohol. Complete the volume to 1000 mL with purified water.

Solution B – Dissolve 5 g of gelatin in 125 mL of hot purified water and mix with 250 mL of cold purified water. Filter and complete the volume to 500 mL with purified water.

Preparation – Mix by stirring the *Solutions A* and *B*. The mixture must be completely clear when cold. Store in a polyethylene container, protected from light.

Coloring reagent

Preparation – Mix 50 mL of glacial acetic acid and 50 mL of sulfuric acid. Allow to stand for two hours before use. Store in a refrigerator for no more than 24 hours.

Modified Ehrlich's reagent

Preparation – Dissolve 0.1 g of *p*-dimethylaminobenzaldehyde in 1 mL of hydrochloric acid and dilute with ethyl alcohol to 100 mL.

Folin-Denis reagent

Preparation – Add to 75 mL of water 10 g of sodium tungstate, 2 g of phosphomolybdic acid and 5 mL of phosphoric acid. Keep the mixture in reflux for two hours, cool down and complete the volume to 100 mL with water. The solution presents a green color.

Hantzsch reagent

Preparation – Dissolve 150 g of ammonium acetate in 500 mL of distilled water with 3 mL of acetic acid and 2 mL of acetylacetone. Complete the volume to 1000 mL.

Conservation – In a closed amber glass container.

Jones reagent

Preparation – Add to 40 mL of water 5.3 g of chromium trioxide and 24 mL of a mixture of water and sulfuric acid (1:1).

Marquis reagent

Preparation – Mix 4 mL of formaldehyde solution with 100 mL of sulfuric acid.

Xanthydrol reagent

Preparation – Dissolve 0.125 g of xanthydrol in 100 mL of glacial acetic acid. Add 1 mL of hydrochloric acid before use.

Phosphomolybdotungstic reagent

Preparation – Dissolve 100 g of sodium tungstate and 25 g of sodium molybdate in 700 mL of water. Add 100 mL of hydrochloric acid and 50 mL of phosphoric acid. Heat the mixture under reflux in glass apparatuses, for 10 hours. Add 150 g of lithium sulfate, 50 mL of water and a few drops of bromine. Boil to remove the excess bromine (for approximately 15 minutes), let it cool down and dilute to 1000 mL with water. Filter. The reagent presents a yellow color. If the solution presents a green color, it must not be used, and must be regenerated with the addition of a few drops of bromine to the reagent in ebullition. Then, boil the reagent to eliminate the excess bromine.

Storage – Keep at temperature between 2 °C and 8 °C.

Iodoplatinate reagent

Preparation – Mix equal volumes of 0.3% (w/v) chloroplatinic acid and 6% (w/v) potassium iodide.

Methoxyphenylacetic reagent

Preparation – Dissolve 2.7 g of methoxyphenylacetic acid in 6 mL of tetramethylammonium hydroxide solution and add 20 mL of absolute ethyl alcohol.

Storage – In a polyethylene container.

Sulfomolybdic reagent

Preparation – Dissolve, by heating, 2.5 g of ammonium molybdate in 20 mL of water. Dilute 28 mL of sulfuric acid in 50 mL of water and cool down. Mix the two solutions and complete the volume to 100 mL with water.

Ammonium reineckate

CAS – [13573-16-5].

Synonym – Ammonium tetrathiocyanatodiamminechromate.

Molecular formula and molar mass – $C_4H_{10}CrN_7S_4 \cdot H_2O$ – 354.45.

Description – Dark red crystals or crystalline red powder.

Solubility – Moderately soluble in chilled water, soluble in hot water and ethyl alcohol. Slowly decomposes in solution.

Ammonium reineckate RS

Preparation – Constantly shake approximately 0.5 g of ammonium reineckate in 20 mL of water for one hour and filter.

Stability – Use within two days.

Resazurin

CAS – [550-82-3].

Synonym – Diazo-resorcinol.

Molecular formula and molar mass – $C_{12}H_7NO_4$ – 229.19.

Description – Crystals or dark red crystalline powder.

Conservation – In tightly closed containers.

Resorcinol

CAS – [108-46-3].

Synonym – Resorcine.

Molecular formula and molar mass – $C_6H_6O_2$ – 110.11.

Specification – Contains no less than 99.0% (w/w).

Description – Crystals, or colorless or pale yellow crystalline powder. When exposed to light and air, acquires a pink color.

Physical characteristic – Melting range: 109 °C to 111 °C.

Solubility – Soluble in water and ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from light and from air.

Ristocetin

CAS – [1404-55-3].

Synonym – Ristocetin A.

Molecular formula and molar mass – $C_{94}H_{108}N_8O_{44}$ – 2053.91.

Description – White solid. Also found as ristocetin sulfate.

Rhodamine B

CAS – [81-88-9].

Synonyms – Tetraethylrhodamine, Basic violet 10.*Molecular formula and molar mass* – $C_{28}H_{31}ClN_2O_3$ – 479.02.*Description* – Green crystals or red powder.*Solubility* – Very soluble in water and in ethyl alcohol.*Conservation* – In tightly closed containers.*Storage* – Protect from exposure to light and from heat.*Safety* – Irritant.**Rutin**

CAS – [153-18-4].

Molecular formula and molar mass – $C_{27}H_{30}O_{16}$ – 610.52.*Description* – Pale yellow needle-shaped crystals. Turns dark at presence of light.*Physical characteristic* – Melting temperature: approximately 210 °C, with decomposition.*Solubility* – Very slightly soluble in water and soluble in pyridine.*Conservation* – In closed containers.*Storage* – Protect from exposure to light.**Sucrose**

CAS – [57-50-1].

Molecular formula and molar mass – $C_{12}H_{22}O_{11}$ – 342.30.*Specification* – It is obtained from *Saccharum officinarum* Linné (Gramineae family), *Beta vulgaris* Linné (Chenopodiaceae family) and other sources.*Description* – White or colorless crystals; crystalline powder or crystalline mass or white blocks. Odorless. Sweet flavor. Stable on air. Finely divided, it is hygroscopic and absorbs up to 1% of humidity. Contains no additives.*Physical characteristic* – Decomposition: between 160 °C and 186 °C.*Solubility* – Very soluble in water, slightly soluble in ethyl alcohol and practically insoluble in absolute ethyl alcohol.*Conservation* – In tightly closed containers.**0.1% (w/v) sucrose in pyridine***Specification* – Contains 0.1 g of sucrose in 100 mL of solution with pyridine.*Conservation* – In tightly closed containers.*Safety* – Toxic.**Safranin O**

CAS – [477-73-6].

Description – Dark red powder. It consists of a mixture of 3,7-diamino-2,8-dimethyl-5-phenylphenazinium ($C_{20}H_{19}ClN_4$ – 350.85) chloride and 3,7-diamino-2,8-dimethyl-5,6-tolylphenazinium ($C_{21}H_{21}ClN_4$ – 364.88) chloride. Redox indicator. Oxidized form: acid medium, violet blue color; alkaline medium, brown color. Reduced form: colorless both in acidic and in alkaline medium.*Physical characteristic* – In the ultraviolet absorption spectrum (5.2.14), there is a maximum between 530 nm and 533 nm.*Conservation* – In tightly closed containers.**Sodium salicylate**

CAS – [54-21-7].

Molecular formula and molar mass – $C_7H_5NaO_3$ – 160.10.*Description* – Small colorless crystals; or white crystalline powder; or bright flakes.

Physical characteristic – Melting temperature: 440 °C.

Solubility – Freely soluble in water and moderately soluble in ethyl alcohol.

Conservation – In closed containers.

Storage – Protect from light.

Santonin

CAS – [481-06-1].

Molecular formula and molar mass – C₁₅H₁₈O₃– 246.31.

Description – Colorless crystals. If exposed to light, may acquire a yellow color.

Physical characteristic – Melting range: 174 °C to 176 °C.

Solubility – Very slightly soluble in water, freely soluble in hot ethyl alcohol and moderately soluble in ethyl alcohol.

Saponins

CAS – [8047-15-2].

Description – Light yellow powder.

Solubility – Soluble in water and, under agitation, forms foam.

Conservation – In closed containers.

Silica, desiccated

CAS – [7631-86-9].

Molecular formula and molar mass – SiO₂– 60.08.

Specification – Colloidal, polymerized, previously dehydrated silicic acid; contains cobalt chloride as indicator.

Description – Vitreous, amorphous granules, of variable granulometry, with granules impregnated with adsorption capacity indicator of blue to pink color.

Conservation – In hermetic containers.

Storage – Protect from humidity.

Category – Desiccant.

Silica-gel “G”

CAS – [112926-00-8].

Synonym – Silica gel “G”.

Specification – Contains, approximately, 13.0% (w/w) calcium sulfate hemihydrate.

Description – White fine powder with variable granulometry between 10 µm and 40 µm, homogeneous.

Physical characteristic – The pH (5.2.19) of the 10% (w/v) suspension in carbon dioxide-free water, obtained by stirring during 15 minutes is of, approximately, 7.0.

Conservation – In tightly closed containers.

Category – Support to chromatography.

Silica-gel “GF₂₅₄”

Synonym – Silica gel “GF₂₅₄”.

Specification – Contains, approximately, 13.0% (w/w) of calcium sulfate hemihydrate and approximately 1.5% (w/w) of maximum fluorescence intensity indicator at 254 nm.

Description – White fine powder with variable granulometry between 10 µm and 40 µm, homogeneous.

Physical characteristic – As described for silica-gel “G”.

Conservation – In tightly closed containers.

Category – Support to chromatography.

Silica-gel “H”

Synonym – Silica gel “H”.

Description – White fine powder, with variable granulometry between 10 µm and 40 µm, homogeneous.

Physical characteristic – As described for silica-gel “G”.

Conservation – In tightly closed containers.

Category – Support to chromatography.

Silica-gel “HF₂₅₄”

Synonym – Silica gel “HF₂₅₄”.

Specification – Contains, approximately, 1.5% (w/v) of maximum fluorescence intensity indicator at 254 nm.

Description – White fine powder with variable granulometry between 10 µm and 40 µm, homogeneous.

Physical characteristic – As described for silica-gel “G”.

Conservation – In tightly closed containers.

Category – Support to chromatography.

Kieselguhr silica

Description – White or light yellow powder.

Solubility – Practically insoluble in water, diluted acid solutions and organic solvents.

Kieselguhr silica “G”

Specification – Kieselghur silica treated with hydrochloric acid and calcinated, where approximately 15% (w/w) calcium sulfate hemihydrate is added.

Description – Grey-white fine powder. With average particle size from 10 µm to 40 µm.

Sodium RSA – 200 µg/mL

Specification – Contains 0.5084 g of sodium chloride in 1000 mL of aqueous solution.

Conservation – In tightly closed, inert containers (like polyethylene).

Tin chloride and ninhydrin solution

Preparation – Dissolve 0.2 g of ninhydrin in 4 mL of hot water. Add 5 mL of 0.16% (w/v) tin chloride and allow to stand for 30 minutes. Filter and store in refrigerator. At the moment of use, dilute 2.5 mL with 5 mL of water and 45 mL of isopropyl alcohol.

Jeffrey’s solution

Preparation – Mix equal parts of 10% (w/v) nitric acid and 10% (w/v) chromic acid.

Conservation – In tightly closed containers.

Karl-Fischer solution

Synonym – Sulfur iodine reagent.

Specification – Comprised of two solutions.

Solution 1: add to a water-free mixture of 70 mL of methyl alcohol with 35 mL of pyridine, under refrigeration and with absence of humidity, dry sulfur dioxide until obtaining a weight increase of 9 g. Homogenize.

Solution 2: contains 12.6 g of iodine in 100 mL of solution with methyl alcohol.

Conservation – In hermetic containers.

Stability – Decomposes continuously.

Storage – Protect from humidity and from light. Keep under refrigeration.

Safety – Toxic. Flammable.

Additional information – For determination of water content.

Chromic acid cleaning solution

Preparation – Add to 100 mL of sulfuric acid, gradually and with constant stirring, 3 g of potassium dichromate. Shake until salt dissolution, let it cool down to 40 °C and store in a glass container.

Calcium standard solution, ethanolic (100 ppm Ca)

Preparation – Dissolve 2.5 g of calcium carbonate, desiccated in advance, in 12 mL of 5 M acetic acid and dilute with water to 1000 mL. Dilute 1 volume of this solution in 10 volumes of ethyl alcohol, immediately before use.

Acetaldehyde standard solution (100 ppm C₂H₄O)

Preparation – Dissolve 1 g of acetaldehyde in isopropyl alcohol and complete the volume to 100 mL with the same solvent.

Dilute 1 volume of this solution in 100 volumes of isopropyl alcohol, immediately before use.

Stability – Extemporaneous preparation.

Ammonium standard solution (1 ppm NH₄)

Preparation – Dissolve 0.4444 g of ammonium nitrate in 1000 mL of distilled water, corresponding to 100 mg/mL of ammonium.

Dilute 1 volume of this solution in 100 volumes of distilled water, immediately before use.

Barium standard solution (10 ppm Ba)

Specification – Contains 1.779 g of BaCl₂·2H₂O in 1000 mL of aqueous solution. Dilute 1 volume of this solution in 100 volumes of water, immediately before use.

Conservation – In tightly closed and inert containers (like polyethylene).

Barium standard solution (0.1% Ba)

Preparation: Dissolve 0.178 g of barium chloride dihydrate in water and complete the volume to 100 mL with the same solvent.

Cadmium standard solution (0.1% Cd)

Preparation – Dissolve an amount of cadmium nitrate with 0.1 g of cadmium in minimum amount of mixture of water with hydrochloric acid (1:1) and dilute to 100 mL with 1% (v/v) hydrochloric acid.

Cadmium standard solution (5 ppm Cd)

Specification – Contains 0.229 g of cadmium sulfate in 100 mL of aqueous solution; corresponds to 1000 µg/mL of cadmium.

Dilute 1 volume of this solution in 200 volumes of water, immediately before use.

Conservation – In tightly closed and inert containers (like polyethylene).

Calcium standard solution (10 ppm Ca)

Preparation – Dissolve 0.624 g of calcium carbonate, desiccated in advance, in distilled water with 3 mL of 5 M acetic acid. Dilute to 250 mL with water. Dilute 1 volume of this solution in 100 volumes of distilled water, immediately before use.

Lead standard solution (0.1% Pb)

Preparation – Dissolve 0.4 g of lead(II) nitrate in water and dilute to 250 mL with the same solvent.

Copper standard solution (10 ppm Cu)

Preparation – Dissolve 392.9 mg of cupric sulfate pentahydrate in 100 mL of water. Dilute 1 mL of this solution with water to 100 mL immediately before use.

Chloride standard solution (8 ppm Cl)

Specification – Contains 1.318 g of sodium chloride in 1000 mL of aqueous solution. Dilute 1 volume of this solution in 100 volumes of water, immediately before use.

Conservation – In tightly closed containers.

Chloride standard solution (5 ppm Cl)

Specification – Contains 0.824 g of sodium chloride in 1000 mL of aqueous solution. Dilute 1 volume of this solution in 100 volumes of water, immediately before use.

Conservation – In tightly closed containers.

Dithizone standard solution

Preparation – Dissolve 10 mg of dithizone in chloroform and complete the volume to 1000 mL with chloroform.

Conservation – Store in a lead-free container, with glass lid and adequately packaged to protect from light.

Storage – In refrigerator.

Tin standard solution (5 ppm Sn)

Specification – Contains 1.225 g of tin acetate hemihydrate in 25 mL of hydrochloric acid in water at 1000 mL. Dilute 1 volume of this solution in 100 volumes of 2.5% (w/v) hydrochloric acid, immediately before use.

Conservation – In tightly closed containers.

Magnesium standard solution (10 ppm Mg)

Preparation – Dissolve 1.010 g of magnesium sulfate heptahydrate in water and complete the volume to 100 mL with the same solvent. Dilute 10 mL of the solution to 1000 mL with water.

Nitrate standard solution (100 ppm NO₃)

Preparation – Dissolve 163.1 g of potassium nitrate in 100 mL of water. Dilute 10 mL of this solution with water to 100 mL immediately before use.

Nitrate standard solution (2 ppm NO₃)

Preparation – Dissolve 1.2903 g of ammonium nitrate in 1000 mL of water. Corresponds to 1000 mg/mL of nitrate. Dilute 1 volume of this solution in 500 volumes of water, immediately before use.

Silver standard solution (5 ppm Ag)

Preparation – Dissolve 79 g of silver nitrate in 100 mL of water. Dilute 1 mL of this solution with water to 100 mL immediately before use.

Selenium standard solution (100 ppm Se)

Preparation – Dissolve 0.1 g of selenium in nitric acid, evaporate until dry, dissolve the residue in 2 mL of water and evaporate until dry. Repeat the procedure three times. Dissolve the residue with 2 M hydrochloric acid and complete the volume to 1000 mL with the same solvent.

Sodium standard solution (200 ppm Na)

Preparation – Dissolve 0.509 g of sodium chloride in 100 mL of water. Dilute 1 volume of this solution in 10 volumes of water, immediately before use.

Sulfate standard solution (10 ppm SO₄)

Preparation – Dissolve 0.182 g of potassium sulfate in 100 mL of water. Dilute 1 mL of this solution in 100 mL of water immediately before use.

Zinc standard solution (100 ppm Zn)

Preparation – Dissolve 0.440 g of zinc sulfate in water with 1 mL of 5 M acetic acid and dilute to 100 mL with water. Immediately before use, dilute 1 volume to 10 volumes with water.

Zinc standard solution (10 ppm Zn)

Preparation – Dilute 1 volume of the zinc standard solution (100 ppm Zn) to 10 volumes with water immediately before use.

Brown standard solution

Preparation – Make a solution comprised of 30 mL of *Ferric chloride base solution (5.2.12)*, 30 mL of *Cobalt(II) chloride base solution (5.2.12)*, 24 mL of *Cupric sulfate base solution (5.2.12)* and 16 mL of 1% (w/v) hydrochloric acid.

Reducer solution

Preparation – Dissolve 5 g of sodium tetrahydroborate in 500 mL of 1% (w/v) sodium hydroxide.

Bismuth subnitrate

CAS – [1304-85-4].

Synonym – Bismuth oxynitrate.

Molecular formula and molar mass – $\text{Bi}_5\text{O}(\text{OH})_9(\text{NO}_3)_4$ – 1461.98.

Specification – It is a basic salt that contains, at minimum, the equivalent to 79.0% of bismuth trioxide (Bi_2O_3) (w/w).

Description – White, dense, hygroscopic, odorless and insipid powder. Presents alkaline reaction with litmus paper.

Solubility – Practically insoluble in water.

Conservation – In tightly closed containers.

Storage – Protect from light.

Therapeutic class – Antacid.

Platelet substitute

Preparation – Add to an amount between 0.5 g and 1 g of phospholipids 20 mL of acetone and frequently shake the mixture for two hours. Centrifuge for two minutes and eliminate the supernatant liquid. Dry the residue with the help of a water suction pump, add 20 mL of chloroform and shake for two hours. Filter under reduced pressure and suspend the residue obtained in 5 mL to 10 mL of 0.9% (w/v) sodium chloride solution.

Determination of Factor IX activity – Prepare a dilution in 0.9% (w/v) sodium chloride solution, in such a way that the difference between the coagulation times from successive dilutions of the reference preparation is approximately 10 seconds.

Conservation – Diluted suspensions can be used for six weeks after the preparation, if preserved at -30 °C.

Plasma substrate

Preparation – Separate the plasma from human or bovine blood collected at 1/9 of its volume from 3.8% (w/v) sodium citrate solution, or at 2/7 of its volume from a solution with 2% (w/v) sodium acid citrate and 2.5% (w/v) glucose. On the first case, the substrate is prepared on the day blood is collected; on the latter case, plasma substrate can be prepared within two days after the collection.

Conservation – In plastic tubes, in small amounts, at a temperature of no more than -20 °C.

Plasma substrate 1

Preparation – Use hydrophobic equipment made of appropriate plastic material or silicone glass for collecting and handling blood. From an adequate number (no less than five) of sheep, alive or during the slaughter, collect an appropriate volume of blood from each animal (a volume of 285 mL of blood collected over 15 mL of anticoagulant solution is considered appropriate). The collection is made through a needle adapted to a cannula with sufficient length to reach the bottom of the collecting container. Reject the first milliliters and collect only blood that flows freely. Mix the blood with a sufficient amount of anticoagulant solution with 8.7 g of sodium citrate and 4 mg of aprotinin in 100 mL of water, to obtain a final ratio of 19 volumes of blood to 1 volume of anticoagulant solution. During and immediately after the collection, make a rotation movement with the container to mix without forming foam. As soon as the collection is finished, close the flask and let it cool down to 10 °C – 15 °C. After cooling, gather the content from all flasks, except from the ones that present evident signs of hemolysis or coagulation, and keep the blood collected at 10 °C to 15 °C. As soon as possible and within four hours after the collection, centrifuge the blood collected at 1000 g – 2000 g at 10 °C to 15 °C, for 30 minutes. Separate the supernatant liquid and centrifuge it at 5000 g, for 30 minutes. If necessary, perform a faster centrifugation, at 20,000 g, for example, for 30 minutes, to clarify the plasma (do not use filtration processes). Separate the supernatant liquids and immediately mix, carefully, and distribute the plasma substrate to small containers, which must be closed at the end of the procedure, in sufficient amounts that allow complete heparin titration (for example, 10 mL to 30 mL). Immediately freeze, quickly, at a temperature below -70 °C (for example, immersing the containers in liquid nitrogen) and keep at a temperature below -30 °C. The plasma prepared in these conditions can be used as plasma substrate in heparin titration if a coagulation time that is appropriate to the detection method used is obtained in the titration conditions and if reproducible dose-response / log curves with major slope are obtained. At the moment of use, thaw a certain amount of plasma substrate in water bath at 37 °C, slowly mixing until complete liquefaction. Once liquefied, the plasma must be maintained at 10 °C to 20 °C and used immediately. The thawed plasma substrate can be slightly centrifuged, if necessary (do not use filtration processes).

Plasma substrate 2

Preparation – Prepare from human blood that has Factor IX content below 1% of the normal content. Collect blood at 1/9 of its volume from a 3.8% (w/v) sodium citrate solution. *Conservation* – In plastic tubes, in small amounts, at a temperature of no more than -30 °C.

Factor V deficient plasma substrate

Specification – Use preferably a congenitally deficient plasma or plasma prepared as described next: separate plasma from human blood that has been collected at 1:10 of its volume from a 1.34% (w/v) sodium oxalate solution. Incubate at 37 °C for 24 to 36 hours. Plasma presents a coagulation time between 70 and 100 seconds. If the coagulation time is inferior to 70 seconds, incubate the plasma again for 12 to 24 hours.

Conservation: in small amounts, at a temperature of no more than -20 °C.

Sudan III

CAS – [85-86-9].

Molecular formula and molar mass – $C_{22}H_{16}N_4O$ – 352.40.

Description – Brown-red powder.

Conservation – In tightly closed containers.

Sudan III RS

Preparation – Dissolve 0.5 g of Sudan III in 100 mL of 80% (v/v) ethyl alcohol, heated at 60 °C, cool down and filter.

Sudan IV

CAS – [85-83-6].

Molecular formula and molar mass – $C_{24}H_{20}N_4O$ – 380.45.

Description – Brown or red-brown powder.

Physical characteristics – Melting range: 181 °C to 188 °C. Decomposes completely at 260 °C.

Solubility – Practically insoluble in water, soluble in paraffin and phenol, slightly soluble in acetone and in ethyl alcohol.

Conservation – In tightly closed containers.

Sudan IV RS

Preparation – Dissolve 2 g of Sudan IV in 100 mL of 92% (v/v) ethyl alcohol, heated at 60 °C, cool down and add 5 mL of glycerin.

Ammonium sulfamate

CAS – [7773-06-0].

Molecular formula and molar mass – $NH_4SO_3NH_2$ – 114.13.

Description – White crystalline powder or colorless crystals.

Physical characteristic – Melting temperature: approximately 131 °C.

Solubility – Very soluble in water and slightly soluble in ethyl alcohol.

Conservation – In perfectly closed containers.

Sulfanilamide

CAS – [63-74-1].

Synonym – 4-Aminobenzenesulfonamide.

Molecular formula and molar mass – $C_6H_8N_2O_2S$ – 172.20.

Description – Crystals or fine white or yellow-white powder.

Physical characteristic – Melting temperature: approximately 165 °C.

Solubility – Soluble in glycerol and practically insoluble in chloroform and in ethyl ether.

Conservation – In tightly closed containers.

Therapeutic class – Antibacterial.

Ceric sulfate

CAS – [13590-82-4].

Synonym – Cerium disulfate.

Molecular formula and molar mass – $Ce(SO_4)_2$ – 332.23.

Description – Orange-yellow crystal or powder.

Physical characteristic – Melting temperature: approximately 350 °C.

Conservation – Protect from light, heat and humidity.

Safety – Toxic and oxidizing agent.

Ceric ammonium sulfate

CAS – [10378-47-9].

Molecular formula and molar mass – $(NH_4)_4Ce(SO_4)_4 \cdot 2H_2O$ – 632.53.

Description – Orange-yellow crystals.

Physical characteristic – Melting temperature: approximately 130 °C.

Solubility – Soluble in water.

Conservation – In tightly closed containers.

Category – Standard for de-redox titration.

Cupric sulfate, pentahydrate

CAS – [7758-99-8].

Synonym – Copper sulfate pentahydrate.

Molecular formula and molar mass – $CuSO_4 \cdot 5H_2O$ – 249.68.

Specification – Contains no less than 98.5% (w/w) in relation to the substance desiccated at 250 °C.

Description – Blue crystals, powder or granules. Slowly effloresces in contact with air.

Physical characteristic – Heated at 250 °C until constant weight, loses 33.0% to 36.5% of its weight.

Solubility – Very soluble in water and slightly soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from air.

Safety – Irritant.

Cupric sulfate RS

Specification – Contains 12.5 g of cupric sulfate pentahydrate in 100 mL of aqueous solution.

Conservation – In tightly closed containers.

Cupric ammonium sulfate RS

Synonym – Copper ammonium sulfate RS and Schweizer's reagent.

Preparation – Dissolve 10 g of cupric sulfate in 100 mL of water, add a sufficient amount of sodium hydroxide solution (1:5) to precipitate copper hydroxide. Filter and collect the precipitate. Wash with cold water. Dissolve the precipitate, which must be kept moist during the process, in the smallest amount of ammonia RS necessary to form the solution.

Potassium aluminum sulfate dodecahydrate

CAS – [7784-24-9].

Synonym – Potassium alum.

Molecular formula and molar mass – $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ – 474.38.

Description – Colorless, clear granular powder or mass.

Solubility – Very soluble in water in ebullition, soluble in glycerin, practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers

Ammonium sulfate

CAS – [7783-20-2].

Molecular formula and molar mass – $(\text{NH}_4)_2\text{SO}_4$ – 132.13.

Specification – Contains no less than 99.0% (w/w).

Description – Colorless, odorless crystals.

Physical characteristic – Decomposes at temperatures above 280 °C.

Solubility – Very soluble in water, practically insoluble in acetone and in ethyl alcohol.

Conservation – In tightly closed containers.

Barium sulfate

CAS – [7727-43-7].

Molecular formula and molar mass – BaSO_4 – 233.39.

Specification – Contains no less than 97.5% (w/w).

Description – White, fine, dense powder. Odorless and insipid.

Solubility – Practically insoluble in water, in organic solvents and in acids and alkali hydroxide solutions.

Conservation – In tightly closed containers.

Therapeutic class – Radiology contrast for gastrointestinal tract.

Cadmium sulfate

CAS – [7790-84-3].

Molecular formula and molar mass – $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ – 769.52.

Specification – Contains no less than 99.0% (w/w).

Description – Colorless, odorless crystalline powder.

Conservation – In tightly closed containers.

Calcium sulfate hemihydrate

CAS – [10034-76-1].

Molecular formula and molar mass – $\text{CaSO}_4 \cdot 1/2\text{H}_2\text{O}$ – 145.14.

Specification – Contains no less than 98.0% (w/w) in relation to the desiccated substance. Contains approximately 7.0% of water.

Description – White fine powder.

Solubility – Very slightly soluble in water, practically insoluble in ethyl alcohol. When mixed with half its mass in water, it is quickly solidified into a porous hard mass.

Conservation – In tightly closed containers.

Calcium sulfate RS

Preparation – Shake 5 g of calcium sulfate hemihydrate with 100 mL of water, for one hour. Filter before use.

Conservation – In tightly closed containers.

N,N-dimethyl-p-phenylenediamine sulfate

CAS – [536-47-0].

Synonym – N,N-dimethyl-1,4-benzenediamine sulfate.

Molecular formula and molar mass – $\text{C}_8\text{H}_{12}\text{N}_2 \cdot \text{H}_2\text{SO}_4$ – 234.28.

Physical characteristic – Melting range: 200 °C to 205 °C, with decomposition.

Storage – Protect from light.

Safety – Toxic.

Dimethyl sulfate

CAS – [77-78-1].

Synonyms – Dimethyl sulfate, DMS.

Molecular formula and molar mass – $(\text{CH}_3)_2\text{SO}_4$ – 126.13.

Description – Colorless liquid.

Physical characteristics – Boiling temperature: approximately 188 °C, with decomposition.

Refractive index (20 °C): 1.3874.

Miscibility – Miscible with water (with hydrolysis) and with ethyl ether and acetone.

Conservation – In closed containers.

Safety – Corrosive. Poisonous!

Hydrazine sulfate

CAS – [10034-93-2].

Molecular formula and molar mass – $\text{H}_6\text{N}_2\text{O}_4\text{S}$ – 130.12.

Description – Colorless crystals.

Solubility – Moderately soluble in cold water, soluble in hot water (50 °C) and freely soluble in water in ebullition. Practically insoluble in ethyl alcohol.

Lithium sulfate

CAS – [10102-25-7].

Molecular formula and molar mass – $\text{Li}_2\text{SO}_4 \cdot \text{H}_2\text{O}$ – 127.95.

Description – Colorless crystals.

Solubility – Freely soluble in water and practically insoluble in ethyl alcohol.

Magnesium sulfate, heptahydrate

CAS – [10034-99-8].

Molecular formula and molar mass – $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ – 246.46.

Description – White crystalline powder or colorless brilliant crystals, of saline flavor, soluble in water, very soluble in water in ebullition, practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

Manganese sulfate

CAS – [10101-68-5].

Molecular formula and molar mass – $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ – 223.05.

Specification – Contains no less than 98.0% (w/w) of MnSO_4 , in relation to the substance desiccated at temperature between 450 °C and 500 °C

Description – Pink crystals or crystalline powder. Odorless. Effloresces slowly.

Physical characteristic – Loses water at approximately 450 °C.

Solubility – Freely soluble in water, very soluble in water in ebullition and practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

Additional information – The commercial product is usually a mixture of manganese sulfate tetrahydrate and pentahydrate.

4-methylaminophenol sulfate

CAS – [55-55-0].

Molecular formula and molar mass – $\text{C}_{14}\text{H}_{20}\text{N}_2\text{O}_6\text{S}$ – 344.38.

Description – Colorless crystals.

Physical characteristic – Melting temperature: approximately 260 °C, with decomposition.

Solubility – Very soluble in water and slightly soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from light.

4-methylaminophenol sulfate RS

Preparation – Dissolve 0.35 g of 4-methylaminophenol sulfate in 50 mL of water. Add 20 g of sodium bisulfite and homogenize. Dilute to 100 mL with water.

Potassium sulfate

CAS – [7778-80-5].

Molecular formula and molar mass – K_2SO_4 – 174.25.

Specification – Contains no less than 99.0% (w/w) in relation to the desiccated substance.

Description – Colorless crystals or white crystalline powder, with bitter flavor.

Physical characteristics – Aqueous solution with neutral character. Melting temperature: 1067 °C.

Conservation – In closed containers.

Protamine sulfate

CAS – [9009-65-8].

Specification – Consists of mixture of simple proteins, obtained from sperm and testicles of adequate species of fish. It has the property of neutralizing heparin.

Description – White or faintly colored, fine, crystalline or amorphous powder.

Conservation – In tightly closed containers, under refrigeration.

Storage – Protect from heat.

Sodium sulfate

CAS – [7757-82-6].

Molecular formula and molar mass – Na_2SO_4 – 142.04.

Specification – Prepared from $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ by heating at, approximately, 100 °C. Contains no less than 99.0% (w/w) in relation to the desiccated substance.

Description – White, odorless, fine powder, of salty and faintly bitter flavor. Hygroscopic.

Physical characteristic – Melting temperature: approximately 800 °C.

Solubility – Freely soluble in water.

Conservation – In tightly closed containers.

Storage – Protect from humidity.

Sodium sulfate decahydrate

CAS – [7727-73-3].

Synonym – Glauber's salt.

Molecular formula and molar mass – $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ – 322.19.

Specification – Contains no less than 99.0% (w/w) of Na_2SO_4 , in relation to the desiccated substance.

Description – Clear, colorless crystals or white crystalline powder; efflorescent, odorless, of faintly bitter flavor.

Physical characteristic – Melting temperature: 32.5 °C. Dissolves in its own crystallization water, at temperature of approximately 33 °C.

Solubility – Freely soluble in water, practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers

Storage – Protect from heat.

Tetrabutylammonium sulfate

CAS – [32503-27-8].

Synonyms – *N,N,N*-tributyl-1-butanaminium sulfate, tetrabutylammonium hydrogen sulfate

Molecular formula and molar mass – $\text{C}_{16}\text{H}_{36}\text{N} \cdot \text{HSO}_4$ – 339.54.

Description – White crystalline powder.

Physical characteristic – Melting range: 169 °C to 173 °C.

Solubility – Freely soluble in water and in methyl alcohol.

Zinc sulfate, heptahydrate

CAS – [7446-20-0].

Molecular formula and molar mass – $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ – 287.54.

Specification – Contains no less than 99.0% (w/w) of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, or no less than 55.6% (w/w) of ZnSO_4 .

Description – White crystalline powder or colorless clear crystals. Odorless, with astringent flavor. Efflorescent.

Physical characteristic – At temperature of 280 °C, becomes anhydrous.

Solubility – Very soluble in water and practically insoluble in ethyl alcohol.

Conservation – In tightly closed non-metallic containers.

Storage – Protect from humidity.

0.1 M zinc sulfate

Description – Contains 28.75 g of zinc sulfate heptahydrate in 1000 mL of aqueous solution.

Conservation – In tightly closed non-metallic containers.

Ferric sulfate

CAS – [10028-22-5].

Synonym – Ferric persulfate.

Molecular formula and molar mass – $\text{Fe}_2(\text{SO}_4)_3 \cdot x\text{H}_2\text{O}$.

Specification – The commercial product usually contains approximately 20% (w/w) of water.

Description – White to yellow powder, very hygroscopic; decomposes in presence of air.

Solubility – Slightly soluble in water and in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from light and from air.

Ferric ammonium sulfate

CAS – [7783-83-7].

Molecular formula and molar mass – $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ – 482.18.

Description – Colorless to pale violet clear crystals. Odorless. Efflorescent.

Physical characteristic – Melting temperature: approximately 37 °C.

Solubility – Very soluble in water and practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

Acid ferric ammonium sulfate RS

Preparation – Dissolve 20 g of ferric ammonium sulfate in 70 mL of water, add 10 mL of 0.05 M sulfuric acid and complete the volume to 100 mL with water.

Ferric ammonium sulfate RS

Specification – Contains 10% (w/v) of ferric ammonium sulfate in water.

Conservation – In tightly closed containers.

Ferric ammonium sulfate RS1

Preparation – Dissolve 30 g of ferric ammonium sulfate in 40 mL of nitric acid and complete the volume to 100 mL with water.

Conservation – In tightly closed containers.

Storage – Protect from light.

Ferric ammonium sulfate RS2

Preparation – Dissolve 0.2 g of ferric ammonium sulfate in 50 mL of water, add 5 mL of nitric acid and complete the volume to 100 mL with water.

Ferric sulfate - potassium ferricyanide RS

Preparation – Mix equal volumes of the 0.5% (w/v) ferric sulfate solution in sulfuric acid 0.5 M and of the 0.2% (w/v) potassium ferricyanide solution.

Stability – Prepare immediately before use.

Acidified ferrous sulfate RS

Preparation – Dissolve 0.45 g of ferrous sulfate heptahydrate in 50 mL of 0.1 M hydrochloric acid and complete the volume to 100 mL with carbon dioxide-free water.

Ferrous ammonium sulfate

CAS – [7783-85-9].

Molecular formula and molar mass – $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ – 392.14.

Description – Crystalline powder or pale blue-green crystals. Oxidizes slowly to air, becoming efflorescent.

Physical characteristic – Melting temperature: approximately 100 °C, with decomposition.

Solubility – Soluble in water, practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from light and from air.

Ferrous sulfate, heptahydrate

CAS – [7782-63-0].

Synonym – Iron sulfate, heptahydrate.

Molecular formula and molar mass – $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ – 278.01.

Specification – Contains no less than 98.0% (w/w) of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$.

Description – Green-blue crystals, or granules, or green crystalline powder. Odorless. Efflorescent. Oxidizes by humidity and luminosity to basic iron(III) sulfate in brown color.

Physical characteristic – From temperature of 65 °C, becomes monohydrate.

Solubility – Freely soluble in water, very soluble in water in ebullition and practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

Storage – Protect from air and from humidity.

Additional – Do not use when it has brown color.

Ferrous sulfate RS

Specification – Contains 8% (w/v) of ferrous sulfate heptahydrate in cold, recently boiled water. Prepare immediately before use.

Conservation – In tightly closed containers.

Storage – Protect from light, from air and from heat.

Ammonium sulfide RS

Preparation – Saturate 60 mL of ammonia RS with hydrogen sulfide and add 40 mL of ammonia RS. Prepare immediately before use.

Conservation – In a small, full and tightly closed container.

Storage – Protect from light and from heat.

Stability – In case of abundant sulfur precipitation, discard the solution.

Hydrogen sulfide

CAS – [7783-06-4].

Synonym – Hydrosulfuric acid.

Molecular formula and molar mass – H_2S – 34.08.

Specification – Produced by treatment of ferrous sulfide (or other sulfides) with diluted sulfuric acid or hydrochloric acid.

Description – Colorless gas with characteristic odor and sweet flavor; denser than air.

Physical characteristics – Vapor density: 1.19. Ignition temperature: 260 °C.

Safety – Flammable. Toxic. Poison!

Hydrogen sulfide RS

Specification – The aqueous solution saturated at 20 °C contains approximately 0.4 to 0.5% (w/v). Prepared by the passage of hydrogen sulfide in cold water.

Physical characteristic – the pH (**5.2.19**) of the freshly prepared aqueous solution is 4.5.

Stability – Prepare immediately before use.

Safety – Flammable. Toxic. Poison!

Sodium sulfide

CAS – [1313-84-4].

Molecular formula and molar mass – $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ – 240,18.

Description – Colorless deliquescent crystals, which turn yellow by exposure to air or by action from light. Odor similar to hydrogen sulfide.

Physical characteristic – Melting temperature: approximately 50 °C.

Conservation – Tightly closed container, in a cold place.

Storage – Protect from air, light and heat.

Sodium sulfide RS

Specification – Contains 10% (w/v) of sodium sulfide in water.

Stability – Prepare immediately before use.

Sodium sulfide RS1

Preparation – Dissolve, by heating, 12 g of sodium sulfide in 45 mL of a mixture of water with 85% (v/v) glycerol (10:29). Cool down and dilute to a 100 mL volumetric flask with the same solvent. The solution must be colorless. Prepare immediately before use.

Sodium sulfite

CAS – [7757-83-7].

Molecular formula and molar mass – Na_2SO_3 – 126.04.

Description – White or nearly white odorless powder.

Solubility – Freely soluble in water and very slightly soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Tannin

CAS – [1401-55-4].

Synonym – Tannic acid.

Specification – Obtained from the bark of different plants, consisting especially of a mixture of polyphenolic substances.

Description – Yellow to brown powder. Faintly characteristic odor and astringent flavor. *Solubility* – Very soluble in water, freely soluble in ethyl alcohol and soluble in acetone. *Conservation* – In tightly closed containers.

Storage – Protect from light.

Labelling – The labeling must indicate the botanical source.

Adrenaline acid tartrate

CAS – [51-42-3].

Synonym – Epinephrine bitartrate.

Molecular formula and molar mass – $\text{C}_{13}\text{H}_{19}\text{NO}_9$ – 333.29.

Specification – Contains no less than 97.0% (w/w) in relation to the desiccated substance.

Description – Crystals, or white or light gray crystalline powder. Odorless.

Physical characteristic – Melting temperature: approximately 150 °C, with decomposition.

Solubility – Freely soluble in water and slightly soluble in ethyl alcohol.

Conservation – In hermetic containers.

Storage – Protect from air and light.

Stability – Turns dark slowly by exposure to air and light.

Therapeutic class – Adrenergic.

Alkaline cupric tartrate RS

Synonym – Fehling's solution.

Solution A – Dissolve 34.6 mg of cupric sulfate pentahydrate in 500 mL of water.

Solution B – Dissolve 173 g of potassium sodium tartrate and 50 g of sodium hydroxide in 400 mL of water and heat until ebullition. Cool down and complete the volume to 500 mL with carbon dioxide-free water.

Preparation – Mix equal volumes of *Solutions A* and *B* immediately before use.

Antimony potassium tartrate

CAS – [28300-74-5].

Synonym – Antimony potassium salt.

Molecular formula and molar mass – $\text{C}_8\text{H}_4\text{K}_2\text{O}_{12}\text{Sb}_2 \cdot 3\text{H}_2\text{O}$ – 667.85.

Description – Colorless crystals or white powder.

Physical characteristic – Melting range: 332 °C to 335 °C.

Conservation – In tightly closed containers.

Safety – Toxic.

Sodium tartrate

CAS – [6106-24-7].

Molecular formula and molar mass – $\text{C}_4\text{H}_4\text{O}_6\text{Na}_2 \cdot 2\text{H}_2\text{O}$ – 230.08.*Specification* – Contains 84.34% of $\text{C}_4\text{H}_4\text{O}_6\text{Na}_2$ and 15.66% of water. Heated at 150 °C, loses a minimum of 15.6% and a maximum of 15.7% of its weight.*Description* – White or nearly white crystals.*Solubility* – Freely soluble in water and practically insoluble in ethyl alcohol.*Conservation* – In tightly closed containers.**Potassium sodium tartrate**

CAS – [6381-59-5].

Synonyms – Rochelle salt or Seignette salt, tartaric acid potassium sodium salt.*Molecular formula and molar mass* – $\text{C}_4\text{H}_4\text{KNaO}_6 \cdot 4\text{H}_2\text{O}$ – 282.22; anhydrous – 210.16.*Specification* – Contains no less than 99.0% (w/w) of $\text{C}_4\text{H}_4\text{KNaO}_6$, in relation to the desiccated substance.*Description* – Colorless crystals or crystalline white powder, odorless, with salty flavor. Efflorescent on hot air.*Solubility* – Very soluble in water and practically insoluble in ethyl alcohol.*Conservation* – In hermetic containers.*Storage* – Protect from heat.**Potassium sodium tartrate RS***Specification* – Contains 20% (w/v) of potassium sodium tartrate.*Conservation* – In tightly closed containers.**Ferrous tartrate RS***Preparation* – Dissolve 1 g of ferrous sulfate heptahydrate, 2 g of potassium sodium tartrate and 0.1 g of sodium bisulfite in water. Complete the volume to 100 mL with water. Prepare immediately before use.**Sodium tetraborate**

CAS – [1303-96-4].

Synonyms – Sodium borate, di-sodium tetraborate, borax.*Molecular formula and molar mass* – $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ – 381.37.*Specification* – Contains no less than 99.0% (w/w).*Description* – Colorless crystals or white crystalline powder, odorless, with caustic flavor. Efflorescent.*Solubility* – Soluble in water, very soluble in water in ebullition and freely soluble in glycerol.*Conservation* – In tightly closed containers; effloresces on dry air.*Storage* – Protect from air.**Carbon tetrachloride**

CAS – [56-23-5].

Molecular formula and molar mass – CCl_4 – 153.82.*Specification* – Contains no less than 99.0% (w/w).*Description* – Clear, colorless, dense liquid, with characteristic odor.*Physical characteristics* – Boiling range: 76 °C to 77 °C. Density: 1.588 to 1.590. Refractive index (20 °C): 1.4607.*Solubility* – Practically insoluble in water; miscible in ethyl alcohol.*Conservation* – In hermetic containers.*Storage* – Protect from light and from heat.*Safety* – Poison (in liquid and gaseous forms)!*Additional information* – It is not flammable, but releases phosgene (toxic) in presence of flame.

Tetradecane

CAS – [629-59-4].

Molecular formula and molar mass – $C_{14}H_{30}$ – 198.39.

Specification – Contains no less than 99.5% (w/w).

Description – Clear and colorless liquid.

Physical characteristics – Density (20 °C): approximately 0.76. Refractive index (20 °C): approximately 1.429. Melting temperature: approximately -5 °C. Boiling temperature: approximately 252 °C.

Conservation – In closed containers.

Sodium tetraphenylborate

CAS – [143-66-8].

Molecular formula and molar mass – $NaB(C_6H_5)_4$ – 342.22.

Description – White or nearly white powder or crystals.

Solubility – Freely soluble in water and in acetone.

Conservation – In tightly closed containers.

Sodium borohydride

CAS – [16940-66-2].

Molecular formula and molar mass – $NaBH_4$ – 37.83.

Description – Colorless and hygroscopic crystals.

Solubility – Freely soluble in water, soluble in absolute ethyl alcohol.

Storage – In tightly closed containers.

3,3'-Diaminobenzidine tetrahydrochloride

CAS – [7411-49-6].

Molecular formula and molar mass – $C_{12}H_{18}Cl_4N_4$ – 360.12.

Description – White or yellow, occasionally purple crystals.

Physical characteristic – Melting temperature: approximately 280 °C, with decomposition.

Solubility – Soluble in water.

Conservation – In tightly closed containers, under refrigeration.

Safety – Irritant.

3,3'-Diaminobenzidine tetrahydrochloride RS

Specification – Contains 1 g of 3,3'-diaminobenzidine tetrahydrochloride in 200 mL of water.

Conservation – In tightly closed containers, under refrigeration.

Safety – Irritant.

Tetrahydrofuran

CAS – [109-99-9].

Molecular formula and molar mass – C_4H_8O – 72.11.

Specification – The product is added with stabilizers (*p*-cresol, hydroquinone) in the ratio of 0.05% to 0.1% (w/v), to avoid excessive formation of peroxides.

Description – Colorless liquid. Intense odor similar to ethyl ether.

Physical characteristics – Boiling temperature: 65 °C to 66 °C. Density (20 °C): approximately 0.889. Refractive index (20 °C): 1.4070.

Miscibility – Miscible with water and ethyl alcohol.

Conservation – In small, tightly closed and full containers.

Storage – Protect from contact with light.

Safety – Irritant to skin, eyes and mucous membranes.

1,1,3,3-Tetramethylbutylamine

CAS – [107-45-9].

Molecular formula and molar mass – $C_8H_{19}N$ – 129.25.

Description – Colorless and clear liquid.

Physical characteristics – Density (20 °C): approximately 0.805. Refractive index (20 °C): approximately 1.424. Boiling temperature: approximately 140 °C.

Tetramethylethylenediamine

CAS – [110-18-9].

Synonyms – *N,N,N',N'*-Tetramethylethylenediamine, TEMED.

Molecular formula and molar mass – $C_6H_{16}N_2$ – 116.21.

Specification – Appropriate quality for electrophoresis.

Description – Colorless liquid.

Physical characteristics – Density (20 °C): approximately 1.418. Boiling temperature: approximately 121 °C.

Miscibility – Miscible with water, with ethyl alcohol and with ethyl ether.

Potassium tetraoxalate

CAS – [6100-20-5].

Molecular formula and molar mass – $C_4H_3KO_8 \cdot 2H_2O$ – 254.19.

Description – White crystalline powder or colorless or white crystals.

Solubility – Moderately soluble in water and soluble in water in ebullition, slightly soluble in ethyl alcohol.

Conservation – In tightly closed containers.

Osmium tetroxide

CAS – [20816-12-0].

Molecular formula and molar mass – OsO_4 – 254.20.

Description – Yellow crystalline mass, or light yellow needles, hygroscopic, sensitive to light.

Solubility – Soluble in water, ethyl alcohol and ethyl ether.

Conservation – In hermetic containers.

Safety – Poisonous vapors!

Osmium tetroxide RS

Specification – Contains 0.25% (w/v) of osmium tetroxide in 0.05 M sulfuric acid.

Conservation – In tightly closed containers.

Thiomersal

CAS – [54-64-8].

Molecular formula and molar mass – $C_9H_9HgNaO_2S$ – 404.81.

Description – Light yellow crystalline powder.

Solubility – Very soluble in water and freely soluble in ethyl alcohol.

Thymidine

CAS – [50-89-5].

Synonym – 1-(2-Deoxy- β -D-ribofuranosyl)-5-methyluracil.

Molecular formula and molar mass – $C_{10}H_{14}N_2O_5$ – 242.23.

Description – Needle-shaped crystals or white powder.

Solubility – Soluble in water, in hot ethyl alcohol and in glacial acetic acid.

Thymine

CAS – [65-71-4].

Synonym – 5-Methyl-2,4-(1*H*,3*H*)-pyrimidinedione.Molecular formula and molar mass – C₅H₆N₂O₂ – 126.12.

Description – Small needle-shaped crystals or plates.

Solubility – Slightly soluble in cold water, soluble in hot water. Dissolves in diluted alkali hydroxide solutions.

Thymol

CAS – [89-83-8].

Synonym – 5-Methyl-2-(1-methylethyl)phenol.

Molecular formula and molar mass – C₁₀H₁₄O – 150.22.

Description – Colorless crystals, with aromatic odor.

Physical characteristic – Melting temperature: approximately 50 °C.

Solubility – Very slightly soluble in water, very soluble in ethyl alcohol, freely soluble in essential oils and in fatty oils, moderately soluble in glycerol. Dissolves in alkali hydroxide solutions.

Thioacetamide

CAS – [62-55-5].

Molecular formula and molar mass – C₂H₅NS – 75.13.

Description – White or nearly white crystalline powder or crystals. Faint odor of mercaptan.

Physical characteristic – Melting temperature: 113 °C to 114 °C.

Solubility – Freely soluble in water and in ethyl alcohol.

Conservation – In tightly closed containers.

Thioacetamide RSPreparation – Mix 0.2 mL of the 4% (w/v) thioacetamide solution with 1 mL of the following mixture: 1.5 mL of *M* sodium hydroxide, 0.5 mL of water and 2 mL of 85% (w/v) glycerol. Heat in a water bath for 20 seconds.

Stability – Prepare immediately before use.

Ammonium thiocyanate

CAS – [1762-95-4].

Synonym – Ammonium rhodanide.

Molecular formula and molar mass – NH₄SCN – 76.12.

Description – Colorless and deliquescent crystals.

Physical characteristic – Melting temperature: approximately 149 °C.

Solubility – Very soluble in water and soluble in ethyl alcohol.

Conservation – In hermetic containers.

Storage – Protect from humidity.

Ammonium thiocyanate RS

Specification – Contains 8% (w/v) of ammonium thiocyanate in water.

Conservation – In tightly closed containers.

Mercury thiocyanate

CAS – [592-85-8].

Molecular formula and molar mass – Hg(SCN)₂ – 316.76.

Description – White or nearly white crystalline powder.

Solubility – Very soluble in water, slightly soluble in ethyl alcohol, soluble in sodium chloride solutions.

Conservation – In tightly closed containers.

Storage – Protect from light.

Mercury thiocyanate RS

Preparation – Dissolve 0.3 g of mercury thiocyanate in ethyl alcohol. Complete the volume to 100 mL with the same solvent.

Conservation – In tightly closed containers.

Stability – Limited to one week.

Potassium thiocyanate

CAS – [333-20-0].

Synonym – Potassium rhodanide.

Molecular formula and molar mass – KSCN – 97.18.

Specification – Contains no less than 99.0% (w/w).

Physical characteristic – Melting temperature: approximately 173 °C.

Solubility – Very soluble in water and in ethyl alcohol.

Conservation – In tightly closed containers.

Safety – May cause skin rashes.

Sodium thioglycolate

CAS – [367-51-1].

Molecular formula and molar mass – C₂H₃NaO₂S – 114.09.

Specification – Contains no less than 95.0% (w/w).

Description – White, hygroscopic crystalline powder, with characteristic faint odor. Oxidizes in contact with air.

Solubility – Freely soluble in water and in methyl alcohol, slightly soluble in ethyl alcohol.

Conservation – In hermetic containers.

Storage – Protect from light and from air.

Thionine (CI 52000)

CAS – [135-59-1].

Molecular formula and molar mass – C₁₂H₁₀ClN₃S – 263.75.

Description – Dark green needles, with luster.

Solubility – Freely soluble in hot water.

Thionine RS

Preparation – Add 1 g of thionine to 2.5 g of phenol and complete the volume to 100 mL with water.

Conservation – In closed containers.

Thionine RS1

Preparation: prepare a 0.2% thionine acetate solution in 25% (v/v) ethyl alcohol by immersing the dry sample in the solution. After 15 minutes, wash the excess reagent with 25% (v/v) ethyl alcohol.

Sodium thiosulfate

CAS – [10102-17-7].

Synonym – Sodium hyposulfite R.

Molecular formula and molar mass – Na₂S₂O₃·5H₂O – 248.17.

Specification – Contains no less than 99.0% (w/w) in relation to the desiccated substance.

Description – Colorless crystals, or white crystalline powder, easily efflorescent, with faintly bitter flavor.

Physical characteristics – Melting temperature: approximately 48 °C. Dissolves in its own crystallization water, at temperature of approximately 49 °C.

Solubility – Very soluble in water, practically insoluble in ethyl alcohol.

Conservation – In tightly closed containers.

0.1 M sodium thiosulfate

Preparation – Dissolve 2.5 g of sodium thiosulfate and 0.02 g of sodium carbonate in carbon dioxide-free water and complete the volume to 100 mL with the same solvent.

Conservation – In tightly closed containers.

Thiourea

CAS – [62-56-6].

Molecular formula and molar mass – $\text{CH}_4\text{N}_2\text{S}$ – 76.12.

Description – White or nearly white crystalline powder or crystals.

Physical characteristic – Melting range: from 176 °C to 178 °C.

Solubility – Soluble in water and in ethyl alcohol.

Conservation – In closed containers.

Tyrosine

CAS – [60-18-4].

Molecular formula and molar mass – $\text{C}_9\text{H}_{11}\text{NO}_3$ – 181.19.

Description – Colorless, white or nearly white crystals, or white or nearly white crystalline powder.

Solubility – Slightly soluble in water, practically insoluble in acetone and in ethyl alcohol, soluble in diluted hydrochloric acid and alkali hydroxide solutions.

p-Tolualdehyde

CAS – [104-87-0].

Molecular formula and molar mass – $\text{C}_8\text{H}_8\text{O}$ – 120.15.

Description – Colorless or yellow clear liquid.

Physical characteristic – Refractive index (20 °C): between 1.544 and 1.546.

Toluene

CAS – [108-88-3].

Synonym – Methylbenzene, toluol.

Molecular formula and molar mass – C_7H_8 – 92.14.

Description – Colorless liquid with characteristic odor.

Physical characteristics – Boiling temperature: 110 °C to 111 °C. Density: approximately 0.87. Refractive index (20 °C): 1.4967.

Solubility – Very slightly soluble in water; miscible with ethyl alcohol.

Safety – Toxic. Flammable.

p-Toluidine

CAS – [106-49-0].

Synonym – 4-Methylaniline.

Molecular formula and molar mass – $\text{C}_7\text{H}_9\text{N}$ – 107.16.

Description – White or slightly yellow flakes or crystals.

Physical characteristics – Melting temperature: approximately 44 °C. Density (20 °C): 1.046.

Solubility – Freely soluble in ethyl alcohol, methyl alcohol, acetone and in diluted acids, and slightly soluble in water.

Conservation – In tightly closed containers.

Thorin

CAS – [3688-92-4].

Synonym – Thoron.

Molecular formula and molar mass – $\text{C}_{16}\text{H}_{11}\text{AsN}_2\text{Na}_2\text{O}_{10}\text{S}_2$ – 576.30.

Description – Red powder.

Solubility – Soluble in water.

Thorin RS

Preparation – Dissolve 0.2% (w/v) thorin in water.

Conservation – In closed container.

Storage – Protect from light.

Stability – Use within one week after the preparation.

Tricine

CAS – [5704-04-1].

Molecular formula and molar mass – $C_6H_{13}NO_5$ – 179.17.

Specification – Appropriate quality for electrophoresis.

Physical characteristic – Melting temperature: approximately 183 °C.

1,1,1-Trichloroethane

CAS – [71-55-6].

Molecular formula and molar mass – $C_2H_3Cl_3$ – 133.40.

Description – Non-flammable liquid.

Physical characteristics – Density (20 °C): approximately 1.34. Boiling temperature: approximately 74 °C.

Solubility – Practically insoluble in water, soluble in acetone and in methyl alcohol.

Trichlorethylene

CAS – [79-01-6].

Synonym – Trichloroethene.

Molecular formula and molar mass – C_2HCl_3 – 131.39.

Specification – Contains no less than 99.5% (w/w).

Description – Colorless liquid, characteristic odor.

Physical characteristics – Density (20 °C): approximately 1.46. Refractive index (20 °C): approximately 1.477. Boiling temperature: approximately 87 °C.

Solubility – Practically insoluble in water, soluble in acetone and in methyl alcohol.

Conservation – In tightly closed containers.

Safety – Toxic.

Triethanolamine

CAS – [102-71-6].

Synonym – 2,2',2''-nitrilotriethanol.

Molecular formula and molar mass – $C_6H_{15}NO_3$ – 149.19.

Description – Colorless, viscous, very hygroscopic liquids, turns brown by exposure to air.

Physical characteristic – Density: approximately 1.13.

Miscibility – Miscible with water, acetone, ethyl alcohol and methyl alcohol.

Conservation – In tightly closed containers protected from light.

Triethylamine

CAS – [121-44-8].

Molecular formula and molar mass – $C_6H_{15}N$ – 101.19.

Description – Colorless liquid, with strongly ammoniacal odor.

Physical characteristics – Density: approximately 0.727. Boiling range: 89 °C to 90 °C.

Conservation – In tightly closed containers.

Safety – Irritant. Flammable.

Triphenylmethanol

CAS – [76-84-6].

Molecular formula and molar mass – $C_{19}H_{16}O$ – 260.34.

Description – Colorless crystals or white or nearly white powder.

Solubility – Practically insoluble in water and freely soluble in ethyl alcohol.

Conservation – In tightly closed containers

Boron trifluoride

CAS – [7637-07-2].

Molecular formula and molar mass – BF_3 – 67.81.

Description – Colorless gas, with pungent, suffocating odor.

Boron trifluoride, methanolic solution

Specification – Commercial solution with approximately 14% (w/v) boron trifluoride in methyl alcohol.

Trinitrophenol RS

Use picric acid RS1.

Arsenic trioxide

CAS – [1327-53-3].

Synonym – Arsenous acid.

Molecular formula and molar mass – As_2O_3 – 197.84.

Description – White or clear crystalline powder or amorphous mass.

Solubility – Slightly soluble in water and soluble in water in ebullition.

Conservation – In tightly closed containers.

Safety – Poison!

Chromium trioxide.

CAS – [1333-82-0].

Synonym – Chromic anhydride.

Molecular formula and molar mass – CrO_3 – 99.99.

Description – Red-brown scales or granulated powder or crystals, deliquescent.

Physical characteristic – Melting temperature: approximately 197 °C.

Solubility – Very soluble in water.

Conservation – In hermetic glass containers.

Storage – Avoid proximity to flammable items.

Safety – Energetic oxidizing agent. Irritant.

Bovine thrombin

CAS – [9002-04-4].

Specification – Biological preparation obtained from bovine plasma, with an enzyme that converts fibrinogen into fibrin.

Description – Yellow-white powder.

Conservation – In closed containers.

Storage – At temperatures below 0 °C.

Human thrombin

CAS – [9002-04-4].

Specification – Biological preparation obtained from human plasma, by appropriate fractioning techniques.

Description – Cream-colored amorphous powder.

Conservation – In tightly closed containers, under refrigeration, specifying the preparation date and potency.

Storage – Protect from light, humidity and from oxygen.

Category – Enzyme. Local hemostatic.

Thromboplastin

CAS – [9035-58-9].

Synonym – Factor III (blood coagulation).

Specification – Biological preparation of animal origin, obtained by extraction of certain organs: brain, lung.

Description – Yellow powder or suspension, with characteristic odor.

Physical characteristic – In the presence of appropriate calcium ion concentrations, it presents thrombokinase activity in blood coagulation.

Conservation – In hermetic containers.

Labeling – Specify in the composition: ions and antimicrobial agents, their concentrations, as well as origin, date of preparation and activity.

Storage – Protect from heat and humidity. Keep under refrigeration.

Category – Preparation with enzymatic activity. Local hemostatic.

Thromboplastin, reagent

Preparation – Shake 1.5 g of ox brain powder dried with acetone, with 60 mL of water at 50 °C, for 10 to 15 minutes. Centrifuge at 1500 rpm for two minutes and decant the supernatant liquid.

Conservation – The extract, stored at temperature below 0 °C, maintains the activity for several days. Cresol may be added, in the amount of 3 g/L, as antimicrobial agent.

Tromethamine

CAS – [77-86-1].

Molecular formula and molar mass – $C_4H_{11}NO_3$ – 121.14.

Synonym – Trometamol, tris(hydroxymethyl)aminomethane.

Specification – Contains no less than 99.0% in relation to the desiccated substance.

Description – White or nearly white crystalline powder or crystals.

Physical characteristics – Melting range: 168 °C to 172 °C. The pH (5.2.19) of the 0.1 M tromethamine solution is 10.4.

Solubility – Freely soluble in water, moderately soluble in ethyl alcohol and very slightly soluble in ethyl acetate.

Conservation – In tightly closed containers.

Sodium tungstate

CAS – [10213-10-2].

Molecular formula and molar mass – $Na_2WO_4 \cdot 2H_2O$ – 329.85.

Description – Colorless crystals or white or nearly white crystalline powder.

Solubility – Freely soluble in water, forming a clear solution, and practically insoluble in ethyl alcohol.

Urea

CAS – [57-13-6].

Synonym – Carbamide.

Molecular formula and molar mass – $(NH_2)_2CO$ – 60.06.

Description – White crystals or powder, strong odor.

Physical characteristic – Melting temperature: approximately 132.7 °C.

Solubility – Very soluble in water, soluble in ethyl alcohol and practically insoluble in methylene chloride.

Conservation – In tightly closed containers in ventilated places.

Safety – May cause damage if aspired or inhaled.

Ammonium vanadate

CAS – [7803-55-6].

Molecular formula and molar mass – NH_4VO_3 – 116.98.

Description – White or light yellow crystalline powder.

Solubility – Slightly soluble in water.

Vanillin

CAS – [121-33-5]

Molecular formula and molar mass – $\text{C}_8\text{H}_8\text{O}_3$ – 152.15.

Description – Needle-shaped crystals, or white or yellow crystalline powder.

Physical characteristic – Melting range: between 81 °C and 84 °C.

Solubility – Slightly soluble in water, freely soluble in ethyl alcohol and methyl alcohol.

Conservation – In tightly closed containers.

Vanillin RS

Preparation – Dissolve 1 g of vanillin in ethyl alcohol and complete the volume to 100 mL with the same solvent. Carefully add 2 mL of sulfuric acid and homogenize. Use the solution within 48 hours.

Sulfuric vanillin RS

Preparation – Dissolve 1 g of vanillin in 100 mL of methyl alcohol. Add 4 mL of hydrochloric acid and 5 mL of sulfuric acid.

Warfarin sodium

CAS – [129-06-6].

Molecular formula and molar mass – $\text{C}_{19}\text{H}_{15}\text{NaO}_4$ – 330.31.

Specification – Contains no less than 97.0% (w/w) in relation to the desiccated substance.

Description – Crystalline or amorphous powder, with faintly bitter flavor.

Solubility – Very soluble in water and in ethyl alcohol, soluble in acetone, very soluble in methylene chloride.

Conservation – In tightly closed containers.

Storage – Protect from light.

Therapeutic class – Anticoagulant.

Bromocresol green RS

Solution A – Dissolve 0.2 g of bromocresol green in 30 mL of water and 6.5 mL of 0.1 M sodium hydroxide.

Solution B – Dissolve 38 g of monobasic sodium phosphate and 2 g of sodium phosphate dibasic in water and complete the volume to 1000 mL with the same solvent.

Preparation – Dilute the *Solution A* to 500 mL using *Solution B* as diluent and homogenize. If necessary, adjust the pH to 4.6 ± 0.1 with 0.1 M hydrochloric acid.

Amaranth red (CI 16185)

CAS – [915-67-3].

Molecular formula and molar mass – $\text{C}_{20}\text{H}_{11}\text{N}_2\text{Na}_3\text{O}_{10}\text{S}_3$ – 604.46.

Solubility – Fine powder, freely soluble in water, practically insoluble in ethyl alcohol, acetone, ethyl ether and chloroform.

Conservation – In tightly closed containers.

Phenol red RS

Solution A – Dissolve 33 mg of phenol red in 1.5 mL 2 M sodium hydroxide and dilute to 100 mL with water.

Solution B – Dissolve 25 g of ammonium sulfate in 235 mL of water. Add 105 mL of 2 M sodium hydroxide and 135 mL of 2 M acetic acid.

Preparation – Add 25 mL of *Solution A* to *Solution B*. If necessary, adjust pH to 4.7.

Conservation – In small containers resistant to alkalis.

Vitexin

CAS – [3681-93-4].

Molecular formula and molar mass – $C_{21}H_{20}O_{10}$ – 432.38.

Description – Yellow powder.

Conservation – In tightly closed containers.

Storage – Protect from exposure to light.

Xanthidrol

CAS – [90-46-0].

Molecular formula and molar mass – $C_{13}H_{10}O_2$ – 198.22.

Specification – Contains no less than 90.0% (w/w) of xanthidrol.

Description – White or light yellow powder.

Solubility – Very soluble in water, soluble in ethyl alcohol and glacial acetic acid.

Storage – Protect from light.

Xylene

CAS – [1330-20-7].

Synonym – Xylol.

Molecular formula and molar mass – C_8H_{10} – 106.17.

Specification – Mixture of isomers: *o*-xylene, *p*-xylene and *m*-xylene, with predominance of *m*-xylene.

Description – Clear and colorless liquid.

Physical characteristics – Density (20 °C): approximately 0.867. Refractive index (20 °C): approximately 1.497. Boiling temperature: approximately 138 °C.

Conservation – In hermetic containers.

Safety – Toxic. Flammable.

Zinc, activated

Preparation – Cover an amount of granulated zinc with chloroplatinic acid solution at 50 mg/mL. Allow to stand for 10 minutes. After washing, drain and dry immediately.

Conservation – In tightly closed containers.

Zinc, granulated

CAS – [7440-66-6].

Element and atomic mass – Zn – 65.38.

Description – Blue-white lustrous metal. Stable on dry air. Converts into basic carbonate when exposed to humidity.

Physical characteristics – Becomes malleable between 100 °C and 150 °C. Burns in presence of air, presenting a blue-green flame.

Conservation – In tightly closed containers.

Storage – Protect from humidity.

Safety – Toxic.

Zinc RSA – 5 mg/mL

Specification – Contains 2.5 g of granulated zinc in 20 mL of 5 M hydrochloric acid. Complete the volume to 500 mL with water.

Conservation – In tightly closed, inert containers (like polyethylene).

7.3 VOLUMETRIC SOLUTIONS

Volumetric solutions (VS) are accompanied with a method of standardization, although there may be other methods that lead to the same degree of accuracy.

The values obtained in standardization are valid for all pharmacopoeia uses.

The reagents employed must have a chemically pure degree and, when necessary, be submitted to desiccation. Volumetric solutions are standardized and used at temperatures of approximately 25 °C. When there are significant variations in temperature, the volumetric solution must have its titration confirmed at the same temperature or be measured with a correction factor.

M hydrochloric acid VS

Specification – Contains 85 mL of hydrochloric acid in 1000 mL of aqueous solution.

Standardization – Weigh, accurately, about 1.5 g of anhydrous sodium carbonate. Add 100 mL of water and two drops of methyl red TS. Add acid slowly, from the burette, until a faint pink color. Heat the solution until boiling, cool down and continue the titration. Repeat this sequence of operations until heating no longer affects the pink color. Calculate the molarity. Each 52.99 mg of sodium carbonate is equivalent to 1 mL of M hydrochloric acid.

Conservation – Hermetic containers.

Storage – Protect from heat.

0.05 M oxalic acid VS

Specification – Contains 6.45 g of oxalic acid in 1000 mL of aqueous solution.

Standardization – Transfer 15 mL of the sample to a 250 mL Erlenmeyer flask. Add 100 mL of water and 7 mL of sulfuric acid. Heat at approximately 70 °C and titrate with freshly standardized 0.02 M potassium permanganate VS, slowly adding the titrant, constantly stirring, until a pale pink color appears and persists for 15 seconds. The temperature at the end of titration must not be inferior to 60 °C.

Conservation – Tightly closed glass containers.

Storage – Protect from light.

0.1 M perchloric acid VS

Specification – Contains 10 g of perchloric acid in 1000 mL of solution with acetic acid.

Standardization – Dissolve, by stirring, 8.5 mL of perchloric acid in 200 mL to 300 mL of glacial acetic acid. Add 20 mL of acetic anhydride, dilute the mixture to 1000 mL with glacial acetic acid and allow to stand for 24 hours. Determine the content of water, which must be between 0.02% and 0.5%. Weigh, accurately, about 700 mg of potassium biphthalate previously powdered and desiccated at 120 °C for two hours and dissolve it in 50 mL of glacial acetic acid in an Erlenmeyer flask with 250 mL of capacity. Add two drops of methylrosanilinium chloride TS and titrate with the perchloric acid solution until the violet color turns to emerald green. Each 20.422 mg of potassium biphthalate is equivalent to 1 mL of 0.1 M perchloric acid.

M sulfuric acid VS

Specification – Contains 98.07 g of sulfuric acid in 1000 mL of aqueous solution.

Preparation – Add 800 mL of water to a 1000 mL volumetric flask and carefully add, on the center of the liquid layer, 54 mL of sulfuric acid. Homogenize, cool down to room temperature, complete the volume with water and homogenize.

Standardization – Weigh, accurately, about 3 g of anhydrous sodium carbonate. Add 100 mL of water and two drops of methyl red TS. Add acid slowly, from the burette, until getting a faint pink color.

Heat the solution until boiling, cool down and continue the titration. Repeat this sequence of operations until heating no longer affects the pink color. Calculate the molarity. Each 105.98 mg of anhydrous sodium carbonate is equivalent to 1 mL of *M* sulfuric acid.

0.1 M potassium bromate VS

Specification – Contains 16.704 g of potassium bromate in 1000 mL of aqueous solution.

Standardization – Measure, accurately, about 40 mL of the 1.67% (w/v) potassium bromate solution. Add 3 g of potassium iodide and 3 mL of hydrochloric acid RS. Wait five minutes and titrate the released iodine with 0.1 *M* sodium thiosulfate VS, using 3 mL of starch TS as indicator. Prepare a blank. Correct and calculate the molarity. Each mL of potassium bromate is equivalent to 6 mL of 0.1 *M* sodium thiosulfate VS.

Conservation – In tightly closed containers.

Storage – Protect from light.

0.05 M bromine VS

Preparation – Dissolve 3 g of potassium bromate and 15 g of potassium bromide in water and complete the volume to 1000 mL with the same solvent. Homogenize.

Standardization – Transfer 25 mL of the bromine solution to a 500 mL Erlenmeyer flask with stopper and add 120 mL of water. Add 5 mL of hydrochloric acid, put the stopper and softly shake. Add 5 mL of potassium iodide RS, put the stopper again, shake and allow to stand for five minutes protected from light. Titrate the released iodine with 0.1 *M* sodium thiosulfate VS, adding 3 mL of starch TS near the endpoint. Calculate the molarity. Each mL of 0.05 *M* bromine VS is equivalent to 1 mL of 0.1 *M* sodium thiosulfate VS.

Conservation – Tightly closed amber glass containers.

Storage – Protect from light.

0.1 M barium chloride VS

Preparation – Dissolve 24.4 g of barium chloride in water and complete the volume to 1000 mL with the same solvent. Homogenize.

Standardization – Add to 10 mL of the barium chloride solution 60 mL of water, 3 mL of concentrated ammonia solution and 1 mg of phthalein purple. Titrate with 0.1 *M* disodium edetate VS. When the solution is discolored, add 50 mL of ethyl alcohol and continue the titration until the violet blue color disappears.

0.004 M benzethonium chloride VS

Preparation – After desiccating in oven at temperature between 100 °C and 105 °C, until constant mass, dissolve 1.792 g of benzethonium chloride in water and complete the volume to 1000 mL with the same solvent.

Standardization – Dissolve 0.350 g of benzethonium chloride, after dried in oven at temperature between 100 °C and 105 °C until constant mass, in 30 mL of acetic acid and add 6 mL of mercury acetate solution RS. Titrate with 0.1 *M* perchloric acid VS in presence of 0.05 mL of methylrosanilinium chloride TS. Conduct a blank test. Each mL of 0.1 *M* perchloric acid VS is equivalent to 44.81 mg of benzethonium chloride.

Dichlorophenol-indophenol VS

Preparation – Dissolve 50 mg of 2,6-dichlorophenol-indophenol sodium in 50 mL of water with 42 mg of sodium bicarbonate. Shake vigorously. After dissolution, complete the volume to 200 mL with water and homogenize. Filter.

Standardization – Weigh, accurately, about 50 mg of ascorbic acid, dissolve with metaphosphoric acetic acid RS and complete the volume to 50 mL with the same solvent. Transfer to a 50 mL flask, immediately, 2 mL of the ascorbic acid solution and add 5 mL of metaphosphoric acetic acid RS. Titrate quickly with the dichlorophenol-indophenol solution until the pink color persists for, at least,

five seconds. Perform a blank titration, titrating 7 mL of metaphosphoric acetic acid RS, added with an amount of water equal to the amount of dichlorophenol-indophenol solution used in the ascorbic acid titration. Express the concentration of the volumetric solution in terms of equivalent in mg of ascorbic acid.

Conservation – Tightly closed amber glass containers.

Stability – Use within three days and standardize immediately before use.

0.05 M disodium edetate VS

Specification – Contains 18.6 g of disodium edetate dihydrate in 1000 mL of aqueous solution.

Standardization – Weigh, accurately, about 200 g of calcium carbonate. Transfer to a 400 mL beaker and add 10 mL of water. Shake and cover the beaker with watch glass. Add 2 mL of diluted hydrochloric acid and shake until dissolving the calcium carbonate. Wash the beaker walls and the watch glass with water until approximately 100 mL. Continue stirring, magnetically. Add 30 mL of the disodium edetate solution from the 50 mL burette. Add 15 mL of sodium hydroxide RS and 300 mg of hydroxynaphthol blue indicator. Continue titrating the disodium edetate solution until a blue color. Calculate the molarity.

Conservation – In tightly closed containers.

0.1 M disodium edetate VS

Preparation – Dissolve 37.5 g of disodium edetate in 500 mL of water, add 100 mL of *M* sodium hydroxide and complete the volume to 1000 mL with water.

Standardization – Dissolve 0.12 g of zinc in powder (with purity grade 99.9%) in 10 mL of *M* hydrochloric acid. Add 0.1 mL of bromine water RS. Eliminate excess bromine by boiling the solution. Add 8.5% (w/v) sodium hydroxide solution until weakly acid or neutral reaction, and proceed as described in *Complexometric titrations (5.3.3.4)* for *Zinc*. Each mL of 0.1 *M* disodium edetate VS is equivalent to 6.536 g of zinc.

Conservation – In tightly closed containers.

***M* potassium hydroxide VS**

Preparation – Dissolve 60 g of potassium hydroxide in water and complete the volume to 1000 mL with the same solvent. Add saturated barium hydroxide solution, freshly prepared, until precipitate is no longer formed. Shake and allow to stand for approximately 12 hours. Decant the clear liquid, or filter, and transfer to inert material containers (like polyethylene). *Standardization* – Use the same procedure adopted for *M* sodium hydroxide VS. *Conservation* – In tightly closed, inert containers (like polyethylene).

Safety – Caustic.

0.5 M ethanolic potassium hydroxide VS

Synonym – 0.5 *M* alcoholic potassium hydroxide VS.

Preparation – Dissolve 3 g of potassium hydroxide in 5 mL of water and complete the volume to 100 mL with ethyl alcohol. Allow the solution to stand for approximately 24 hours. Decant the clear liquid and transfer to inert material containers protected from light.

Standardization – Titrate 20 mL of the potassium hydroxide solution with 0.5 *M* hydrochloric acid VS using 0.5 mL of phenolphthalein TS as indicator. Each mL of 0.5 *M* hydrochloric acid VS is equivalent to 28.060 mg of potassium hydroxide.

***M* sodium hydroxide VS**

Preparation – Prepare a 50% (w/v) sodium hydroxide solution with carbon dioxide-free water. Cool to room temperature and allow to settle. Take 82 mL of supernatant and dilute with water, completing the volume to 1000 mL with the same solvent.

Standardization – Weigh, accurately, about 5 g of desiccated potassium biphthalate and dissolve in 75 mL of carbon dioxide-free water. Add two drops of phenolphthalein TS and titrate with the sodium hydroxide solution until forming a permanent pink color. Each mL of *M* sodium hydroxide VS is equivalent to 204.220 mg of potassium biphthalate.

Conservation – Tightly closed, inert containers (like polyethylene). Stoppers with tube containing the mix of sodium hydroxide and calcium oxide.

Storage – Protect from exposure to carbon dioxide.

Safety – Caustic.

Additional information – Check the titer frequently.

0.1 *M* ethanolic sodium hydroxide VS

Preparation – Prepare a 50% (w/v) sodium hydroxide solution in carbon dioxide-free water. Cool down to room temperature and allow to settle. Transfer 2 mL of the supernatant to a 250 mL volumetric flask and complete the volume with ethyl alcohol.

Standardization – Weigh, accurately, about 0.2 g of benzoic acid and dissolve in a mixture of 10 mL ethyl alcohol with 2 mL of water. Add two drops of phenolphthalein TS and titrate with the ethanolic sodium hydroxide solution until forming a permanent pink color. Each mL of 0.1 *M* sodium hydroxide VS is equivalent to 12.212 mg of benzoic acid.

Conservation – In tightly closed, inert containers (like polyethylene).

Storage – Protect from exposure to carbon dioxide.

Safety – Caustic.

0.1 *M* tetrabutylammonium hydroxide VS

Preparation – Dissolve 40 g of tetrabutylammonium iodide in 900 mL of methyl alcohol, in an Erlenmeyer flask with ground stopper. Put in an ice bath, add 20 g of powdered silver oxide, put the stopper on the flask and shake vigorously for 60 minutes. Take a few mL and centrifuge. Check for the presence of iodide in the supernatant liquid. If the test results positive, add another 2 g of silver oxide and allow to stand for 30 minutes, stirring occasionally. Filter in a porous plate filter, wash the Erlenmeyer flask and the funnel with three portions of 50 mL of toluene and join the washing toluene to the filtrate. Complete the volume to 1000 mL with the mixture of three volumes of toluene and one volume of methyl alcohol. Flush the solution, for 10 minutes, with carbon dioxide-free nitrogen. Store in a container protected from carbon dioxide and humidity. Use within 60 days. Determine the molarity on the day of use, dissolving approximately 400 mg of benzoic acid accurately weighed, in 80 mL of dimethylformamide. Add to this solution three drops of 1% (w/v) thymol blue solution in dimethylformamide and titrate with tetrabutylammonium hydroxide solution until obtaining a blue color. Protect the solution from contact with air during the titration. Use a burette with a carbon dioxide absorption tube. Conduct a blank test. Each mL of tetrabutylammonium hydroxide is equivalent to 12.212 mg of benzoic acid.

Indigo carmine VS

Preparation – Crush 4 g of indigo carmine with successive portions of water until dissolution, without exceeding 900 mL. Transfer to a 1000 mL volumetric flask, add 2 mL of sulfuric acid and complete the volume with water. Homogenize.

Standardization – Add to 10 mL of standard nitrate solution (100 ppm NO₃) 10 mL of water, 0.05 mL of indigo carmine VS and, carefully, 30 mL of sulfuric acid. Titrate immediately with indigo carmine VS until turning into a stable blue color. The total volume, in mL, of indigo carmine VS required is equivalent to 1 mg of NO₃.

0.02 *M* potassium iodate VS

Specification – Contains 4.28 g of potassium iodate in 1000 mL of aqueous solution.

Standardization – Dilute 50 mL of the potassium iodate solution to 100 mL with water. Add to 25 mL

of this solution 2 g of potassium iodide and 10 mL of *M* sulfuric acid. Titrate with 0.1 *M* sodium thiosulfate VS using starch TS, added near the endpoint, as indicator. Each mL of 0.1 *M* sodium thiosulfate VS is equivalent to 3.566 mg of KIO_3 .

0.1 *M* potassium iodate VS

Preparation – Weigh, accurately, about 21.4 g of potassium iodate desiccated in advance at 110 °C, until constant weight, dissolve in water and complete the volume to 1000 mL with the same solvent.. The standardization is not necessary, because this reagent is primary standard.

0.005 *M* iodine VS

Preparation – Dissolve approximately 1.3 g of iodine in 10 mL of potassium iodide at 36% (w/v). Add three drops of hydrochloric acid and complete the volume to 1000 mL with water.

Standardization – Add to 25 mL of iodine solution 1 mL of *M* hydrochloric acid and titrate with 0.01 *M* sodium thiosulfate VS until pale yellow color. Add three drops of starch TS and continue with the titration until the blue color disappears. Calculate the molarity.

Conservation – Tightly closed glass containers.

Storage – Protect from light.

0.05 *M* iodine VS

Preparation – Dissolve 13 g of iodine in 100 mL of 20% (w/v) potassium iodide solution. Add three drops of hydrochloric acid and dilute to 1000 mL with water.

Standardization – Dissolve, accurately, about 0.15 g of arsenic trioxide in 20 mL of *M* sodium hydroxide. Heat if necessary. Add 40 mL of water, two drops of methyl orange and hydrochloric acid until the color turns pink. Add 50 mL of 4% (w/v) sodium carbonate, 3 mL of starch TS and titrate with 0.05 *M* iodine VS until permanent blue color. Each mL of 0.05 *M* iodine VS is equivalent to 4.946 mg of arsenic trioxide.

Conservation – In tightly closed glass container protected from light.

0.1 *M* iodine VS

Preparation – Dissolve approximately 13 g of iodine in 100 mL of potassium iodide at 36% (w/v). Add three drops of hydrochloric acid and complete the volume to 1000 mL with water.

Standardization – Weigh, accurately, about 150 mg of arsenic trioxide. Dissolve in 20 mL of *M*, sodium hydroxide heating if necessary. Add 40 mL of water, two drops of methyl orange S1 and hydrochloric acid diluted until the color turns pink. Add 50 mL of 4% (w/v) sodium carbonate and 3 mL of starch TS. Titrate with the iodine solution, from the burette, until permanent blue color. Calculate the molarity. Each 4.946 mg of arsenic trioxide is equivalent to 1 mL of 0.1 *M* iodine. *Conservation* – Tightly closed glass containers.

Storage – Protect from light.

0.1 *M* lithium methoxide VS

Preparation – Dissolve carefully, in a 1000 mL volumetric flask, 0.694 g of lithium in 150 mL of methyl alcohol and complete the volume with toluene.

Standardization – Always standardize before use. Add to 10 mL of dimethylformamide 0.05 mL of 0.3% (w/v) thymol blue in methyl alcohol and titrate with 0.1 *M* lithium methoxide VS until the color turns blue. Immediately, add 0.2 g of benzoic acid, shake and titrate with 0.1 *M* lithium methoxide until the color turns blue. Avoid atmospheric carbon dioxide absorption. The volume of titrant spent on the second titration represents the amount of lithium methoxide required. Each mL of 0.1 *M* lithium methoxide VS is equivalent to 12.212 mg of benzoic acid.

0.1 *M* sodium methoxide VS

Specification – Contains 5.402 g in 1000 mL of solution with toluene.

Preparation – Cool in ice bath 150 mL of methyl alcohol, in a 1000 mL volumetric flask. Add, in small portions, approximately 2.5 g of recently fragmented metallic sodium. After the metal dissolution, add toluene until 1000 mL and homogenize. Keep this solution in a container protected from carbon dioxide.

Standardization – Weigh, accurately, about 400 mg of benzoic acid, dissolve in 80 mL of dimethylformamide, add three drops of 1% (w/v) thymol blue solution in dimethylformamide and titrate with the sodium methoxide solution until a blue color appears. Each 12.212 mg of benzoic acid is equivalent to 1 mL of 0.1 M sodium methoxide.

0.01 M ceric ammonium nitrate VS

Preparation – Add to 100 mL of 0.1 M ceric ammonium nitrate VS, carefully, by cooling, 30 mL of sulfuric acid and dilute to 1000 mL with water.

0.1 M ceric ammonium nitrate VS

Preparation – Shake the solution with 56 mL of sulfuric acid and 54.82 g of ceric ammonium nitrate for two minutes, and carefully add five successive portions of 100 mL of water, stirring after each addition. Dilute the clear solution to 1000 mL with water. Standardize 10 days after the preparation.

Standardization – Add to 25 mL of ceric ammonium nitrate solution 2 g of potassium iodide and 150 mL of water. Titrate immediately with 0.1 M sodium thiosulfate SV, using starch TS as indicator. Each mL of 0.1 M sodium thiosulfate VS is equivalent to 54.822 mg of ceric ammonium nitrate.

Storage – Protect from light.

0.01 M barium nitrate VS

Specification – Contains 2.614 g of barium nitrate in 1000 mL of water.

Standardization – Add 10 mL of 0.01 M sulfuric acid solution to a vial and dilute with water. Add two drops of 0.2% (w/v) thorin and two drops of 0.02% (w/v) methylthionine chloride and titrate slowly with barium nitrate solution until the color changes from yellow to pink. Calculate the molarity.

0.1 M lead nitrate VS

Preparation – Transfer, accurately, about 8.28 g of lead nitrate to a 250 mL volumetric flask, dilute in water and complete the volume with the same solvent. Homogenize.

Standardization – Transfer 5 mL of 0.1 M lead nitrate to a 125 mL Erlenmeyer flask, add 50 mL of water and, under magnetic stirring, add five drops of 0.1% (w/v) xylene orange and 5 g of methenamine, until the color turns violet. Titrate with 0.05 M disodium edetate VS until the yellow color. Each mL of 0.05 M disodium edetate VS is equivalent to 16.560 g of lead nitrate.

0.1 M mercury(II) nitrate VS

Synonym – 0.1 M mercuric nitrate VS.

Preparation – Dissolve, accurately, about 35 g of mercury(II) nitrate in 5 mL of nitric acid and 500 mL of water. Complete the volume to 1000 mL with water.

Standardization – Add to 20 mL of the mercury nitrate solution 2 mL of nitric acid RS and 2 mL of ferric ammonium sulfate RS. Cool to a temperature below 20 °C and titrate with 0.1 M ammonium thiocyanate VS until a permanent brown color appears. Calculate the molarity.

Conservation – In tightly closed containers.

0.1 M silver nitrate VS

Preparation – Dissolve, accurately, about 17.5 g of silver nitrate in water and complete the volume to 1000 mL with the same solvent.

Standardization – Weigh, accurately, about 100 mg of desiccated sodium chloride, transfer to a 150 mL beaker and dissolve in 5 mL of water. Add 5 mL of acetic acid RS, 50 mL of methyl alcohol and three drops of eosin Y TS. Shake, preferably with a magnetic stirrer, and titrate with the silver

nitrate solution. Calculate the molarity. Each mL of 0.1 M silver nitrate VS is equivalent to 5.844 mg of sodium chloride.

Conservation – In tightly closed containers.

Storage – Protect from light.

0.005 M thorium nitrate VS

Specification – Contains 2.401 g of thorium nitrate in 1000 mL of aqueous solution.

Standardization – Transfer, accurately, about 0.05 g of sodium fluoride, desiccated in advance, to a 250 mL volumetric flask and complete the volume with water. Add to 20 mL of this solution 0.6 mL of alizarin TS and titrate with 0.1 M sodium hydroxide VS until the color changes from pink to yellow. Add 5 mL of acetate buffer pH 3.0 and titrate with 0.005 M thorium nitrate solution until the color changes from yellow to yellow-pink. Each 0.8398 mg of sodium fluoride is equivalent to 1 mL of 0.005 M thorium nitrate VS.

0.1 M sodium nitrite VS

Specification – Contains 6.9 g of sodium nitrite in 1000 mL of aqueous solution.

Standardization – Weigh, accurately, about 500 mg of sulfanilamide desiccated in advance for three hours at 105 °C. Transfer to a beaker. Add 20 mL of hydrochloric acid and 50 mL of water. Shake until dissolving and cool down to 15 °C. Keeping the temperature around 15 °C, titrate slowly with sodium nitrite solution, using iodized starch TS as external indicator, until turning. Each 17.220 mg of sulfanilamide is equivalent to 1 mL of 0.1 M sodium nitrite.

0.02 M potassium permanganate VS

Specification – Contains 3.161 g of potassium permanganate in 1000 mL of aqueous solution.

Preparation – Dissolve approximately 3.2 g of potassium permanganate in 1000 mL of water and heat until boiling for 15 minutes. Allow to stand in an amber vial with glass stopper, protected from light, for two days and filter in a sintered glass funnel.

Standardization – Dissolve, accurately, about 0.2 g of sodium oxalate, desiccated in advance at 110 °C until constant weight, in 250 mL of water. Add 7 mL of sulfuric acid, heat at approximately 70 °C, and slowly titrate with the potassium permanganate solution, with constant stirring, until a pale pink color that persists for 15 seconds. The temperature at the end of titration must not be inferior to 60 °C. Each mL of 0.02 M potassium permanganate VS is equivalent to 6.700 mg of sodium oxalate.

Conservation – Tightly closed amber glass containers, with glass stopper.

Storage – Protect from light.

Additional information – Check the titer frequently.

0.05 M ceric sulfate VS

Specification – Contains 16.61 g of ceric sulfate or 20.2 g of ceric sulfate tetrahydrate $[\text{Ce}(\text{SO}_4)_2 \cdot 4\text{H}_2\text{O}]$ in 1000 mL of water.

Standardization – Weigh, accurately, about 0.2 g of sodium oxalate, desiccated in advance, and dissolve in 75 mL of water. Add, stirring, 2 mL of sulfuric acid mixed in advance with 5 mL of water. Homogenize. Add 10 mL of hydrochloric acid and heat for another 75 minutes. Titrate with 0.05 M ceric sulfate until permanent light yellow color. Each 6.700 mg of sodium oxalate is equivalent to 1 mL of 0.05 M ceric sulfate VS.

0.1 M ceric ammonium sulfate VS

Preparation – Dissolve 65 g of ceric ammonium sulfate in a mixture of 30 mL of sulfuric acid with 500 mL of water. Cool and complete the volume with water to 1000 mL.

Standardization – Dissolve 80 g of arsenic trioxide in 15 mL of 0.2 M sodium hydroxide, heating if necessary. Add 50 mL of M sulfuric acid, 0.15 mL of 0.25% (w/v) osmium tetroxide and 0.1 mL of ferroin TS. Titrate with 0.1 M ceric ammonium sulfate until the color changes. Each mL of 0.1 M ceric ammonium sulfate is equivalent to 4.496 mg of arsenic trioxide.

0.1 M zinc sulfate VS

Specification – Contains 16.144 g of zinc sulfate heptahydrate in 1000 mL of aqueous solution.

Preparation – Dissolve 28.8 g of zinc sulfate in water and complete the volume to 1000 mL with the same solvent. Pipette 20 mL of the 0.05 M disodium edetate solution and transfer to a 250 mL Erlenmeyer flask. Add, in this order, 20 mL of acetic acid-ammonium acetate buffer solution, 100 mL of ethyl alcohol and 2 mL of dithizone RS. Titrate with the zinc sulfate solution until a light pink color appears. Calculate the molarity.

0.02 M sodium tetraphenylborate VS

Preparation – Dissolve 6.845 g of sodium tetraphenylborate in 1000 mL of aqueous solution.

Standardization – Pipette two portions of 75 mL of the sodium tetraphenylborate solution and transfer to two beakers. Add to each one of them 1 mL of acetic acid RS, 25 mL of water and, slowly and stirring, 25 mL of 5% (w/v) potassium biphthalate. Allow to stand for two hours. Filter one of the mixtures in a sintered glass filtering crucible (porosity 100 µm to 160 µm) and wash the precipitate with cold water. Transfer the precipitate with 50 mL of water and shake intermittently for 30 minutes. Filter and use the filtrate as saturated potassium tetraphenylborate solution in the following standardization procedure. Filter the mixture in a sintered glass filtering crucible, tared, and wash with three portions of 5 mL of the saturated potassium tetraphenylborate solution. Dry the precipitate at 105 °C for one hour. Each g of potassium tetraphenylborate is equivalent to 955.1 mg of sodium tetraphenylborate. From the weight of the sodium tetraphenylborate obtained, calculate the molarity of the solution.

Conservation – In tightly closed containers.

Stability – Use fresh solutions.

0.1 M ammonium thiocyanate VS

Preparation – Dissolve approximately 8 g of ammonium thiocyanate in water and complete the volume to 1000 mL with the same solvent.

Standardization – Mix, accurately, about 30 mL of 0.1 M silver nitrate VS with 50 mL of water, 2 mL of nitric acid RS and 2 mL of ferric ammonium sulfate RS. Titrate with the ammonium thiocyanate solution until a red-brown color appears. Each mL of 0.1 M silver nitrate is equivalent to 7.612 mg of ammonium thiocyanate.

Conservation – In tightly closed containers.

0.01 M sodium thiosulfate VS

Preparation – Dissolve, accurately, about 2.5 g of sodium thiosulfate pentahydrate and 20 mg of sodium carbonate in water, recently boiled and cooled. Transfer the solution quantitatively to a 100 mL volumetric flask, complete the volume with the same water and homogenize.

Standardization – Weigh, accurately, about 21 g of potassium dichromate, powdered and desiccated, and dissolve in 10 mL of water. Transfer to a 50 mL volumetric flask, add 0.3 g of potassium iodide, 0.2 g of sodium bicarbonate and 0.5 mL of hydrochloric acid. Put a stopper, shake and allow to stand, for 10 minutes, in the dark. Titrate the iodine released with the sodium thiosulfate solution until a yellow-green color. Add 3 mL of starch TS and continue with the titration until the blue color disappears. Calculate the molarity of the solution.

0.1 M sodium thiosulfate VS

Preparation – Dissolve approximately 25 g of sodium thiosulfate pentahydrate and 200 mg of sodium carbonate in water, recently boiled and cooled. Complete the volume to 1000 mL with the same solvent.

Standardization – Weigh, accurately, about 210 g of potassium dichromate, powdered and desiccated, and dissolve in 100 mL of water. Transfer to a 500 mL volumetric flask and add 3 g of potassium iodide, 2 g of sodium bicarbonate and 5 mL of hydrochloric acid PA. Shake and allow to

stand for 10 minutes in the dark. Titrate the iodine released with the sodium thiosulfate solution until having a yellow-green color. Add 3 mL of starch TS and continue with the titration until the blue color disappears. Calculate the molarity. Each mL of 0.1 M sodium thiosulfate VS is equivalent to 4.903 mg of potassium dichromate.

Conservation – In tightly closed containers.

Additional information – Check the titer frequently.

7.4 BUFFERS

Certain pharmacopoeia assays require the adjustment or maintenance of pH. For such, they use solutions named buffers, capable of withstanding variations in the activity of hydrogen ions. The components used for preparing buffer solutions are described on the item Reagents. Components of crystalline nature must be desiccated in advance in an oven between 110 °C and 120 °C, for one hour. For the preparation of buffer solutions, use carbon dioxide-free water. They must be stored in hermetic and appropriate containers. Consider the stability in the preparation of amounts for consumption. The solutions are listed in increasing order of pH values. Other buffers with particular characteristics are described on the texts from the respective assays.

Acetate buffer pH 3.0

Preparation – Dissolve 12 g of sodium acetate in water, add 6 mL of glacial acetic acid, complete the volume to 100 mL with water and homogenize. Adjust pH to 3.0, if necessary.

Acetate buffer pH 3.5

Preparation – Dissolve 25 g of ammonium acetate in 35 mL of water, add 38 mL of 7 M hydrochloric acid, adjust the pH to 3.5 with hydrochloric acid RS or 6 M ammonium hydroxide, complete the volume to 100 mL with water and homogenize.

Acetate buffer pH 4.0

Preparation – Transfer 900 mL of water to a 1000 mL volumetric flask, add 2.86 mL of glacial acetic acid and 1 mL of 50% (w/v) sodium hydroxide, complete the volume with water and homogenize. If necessary, adjust the pH to 4.0 with glacial acetic acid or 50% (w/v) sodium hydroxide.

Acetate buffer pH 4.4

Preparation – Dissolve 136 g of sodium acetate and 77 g of ammonium acetate in water and complete the volume to 1000 mL with the same solvent. Add 250 mL of glacial acetic acid and homogenize.

0.05 M acetate buffer pH 4.5

Preparation – Transfer 2.99 g of sodium acetate trihydrate and 1.66 mL of glacial acetic acid to a 1000 mL volumetric flask. Dissolve in water and complete the volume with the same solvent. Homogenize. Adjust pH to 4.5, if necessary.

0.05 M acetate buffer pH 5.3

Preparation – Transfer 5.61 g of sodium acetate trihydrate and 4.4 mL of 2 M acetic acid to a 1000 mL volumetric flask. Dissolve in water and complete the volume with the same solvent. Homogenize. Adjust the pH to 5.3, if necessary, with 0.1 M acetic acid or 0.1 M sodium hydroxide.

Sodium acetate buffer pH 4.5

Preparation – Dilute 2.8 mL of glacial acetic acid in sufficient water to prepare 1000 mL. Adjust the pH to 4.5 ± 0.05 with 50% (w/v) sodium hydroxide.

0.1 M sodium acetate buffer pH 5.0

Preparation – Transfer 13.61 g of sodium acetate trihydrate to a 1000 mL volumetric flask, dissolve in sufficient amount of water and complete the volume with the same solvent. Homogenize. Adjust pH to 5.0 with 0.1 M acetic acid.

Hydrochloric acid buffer pH 2.0

Preparation – Mix 50 mL of 0.2 M potassium chloride aqueous solution with 13 mL of 0.2 M hydrochloric acid aqueous solution. Complete the volume to 200 mL with water and adjust pH to 2.0, if necessary.

Biphthalate buffer pH 4.4

Preparation – Dissolve 2.042 g of potassium biphthalate in 50 mL of water, add 7.5 mL of 0.2 M sodium hydroxide and dilute to 200 mL of water. Adjust pH to 4.4, if necessary.

Phosphate-citrate buffer pH 5.0

Solution A – Dissolve 0.8 g of sodium phosphate dibasic heptahydrate in 500 mL of water.

Solution B – Dissolve 3.5 g of monohydrate citric acid in 500 mL of water.

Preparation – Mix, by stirring, the *Solutions A* and *B* until obtaining pH of 5.0. Distribute in containers with 50 mL each. Autoclave at 121 °C, pressure of 1 atm, for 20 minutes. Store at 4 °C.

Phosphate buffer pH 2.2

Preparation – Dissolve 1.38 g of monobasic sodium phosphate in 800 mL of water. Adjust pH with phosphoric acid and dilute to 1000 mL with water. Homogenize.

Phosphate buffer pH 5.5

Solution A – Dissolve 13.61 g of monobasic sodium phosphate in water and complete the volume to 1000 mL with the same solvent.

Solution B – Dissolve 35.81 g of sodium phosphate dibasic dodecahydrate in water and complete the volume to 1000 mL with the same solvent.

Preparation – Mix 96.4 mL of *Solution A* with 3.6 mL of *Solution B*. Adjust pH to 2.2, if necessary.

Phosphate buffer pH 5.8

Preparation – In a 200 mL volumetric flask, add 3.6 mL of 0.2 M sodium hydroxide to 50 mL of 0.2 M monobasic potassium phosphate and complete the volume with water. Homogenize. Adjust pH to 5.8, if necessary.

Phosphate buffer pH 6.0

Preparation – Mix 50 mL of 0.2 M monobasic potassium phosphate and 5.7 mL of 0.2 M sodium hydroxide. Complete the volume to 200 mL with water. Homogenize. Adjust pH to 6.0, if necessary.

Acetate buffer pH 6.0

Preparation – Dissolve 100 g of ammonium acetate in 300 mL of water, add 4.1 mL of glacial acetic acid, if necessary adjust pH to 6.0, using 10 M ammonium hydroxide or 5 M glacial acetic acid and complete the volume to 500 mL with water. Homogenize.

Citro-phosphate buffer pH 6.0

Synonym – Sodium phosphate buffer pH 6.0.

Preparation – Mix 36.8 mL of 2.1% (w/v) citric acid with 63.2 mL of 7.15% (w/v) sodium phosphate dibasic. Adjust pH to 6.0, if necessary.

Phosphate buffer pH 6.5

Preparation – Dissolve 2.75 g of sodium phosphate dibasic dihydrate and 4.5 g of sodium chloride in 500 mL of water. Homogenize. Adjust pH to 6.5 with phosphate buffer pH 8.5.

Phosphate buffer pH 6.8

Preparation – Mix 50 mL of 0.2 M monobasic potassium phosphate solution and 22.4 mL of 0.2 M sodium hydroxide. Complete the volume to 200 mL with water. Homogenize. Adjust pH to 6.8, if necessary.

Sodium lauryl sulfate-phosphate buffer pH 6.8.

Preparation – Mix 19 parts of 0.1 M hydrochloric acid with 17 parts of sodium lauryl sulfate-phosphate buffer pH 11.0. If necessary, adjust the pH to 6.8 with 20% (v/v) phosphoric acid or 40% (w/v) sodium hydroxide.

M tris-hydrochloride buffer pH 6.8

Preparation – Dissolve 60.6 g of tromethamine in 400 mL of water. Adjust pH to 6.8 with hydrochloric acid and complete the volume to 500 mL with water. Homogenize. Adjust pH to 6.8, if necessary.

0.025 M phosphate buffer pH 6.86

Preparation – Dissolve 3.53 g of sodium phosphate dibasic and 3.39 g of monobasic potassium phosphate in water and complete the volume to 1000 mL with the same solvent. Homogenize. Adjust pH to 6.86, if necessary.

Acetate buffer pH 7.0

Preparation – Dissolve 2.73 g of sodium acetate in, approximately, 70 mL of water. Adjust pH to 7.0 with 0.5 M acetic acid. Complete the volume to 100 mL with water. Homogenize. Adjust pH to 7.0, if necessary.

Conservation – In tightly closed containers.

Citro-phosphate buffer pH 7.0

Synonym – Sodium phosphate buffer pH 7.0.

Preparation – Mix 82.4 mL of 7.15% (w/v) sodium phosphate dibasic dodecahydrate with 17.6 mL of 2.1% (w/v) citric acid. Adjust pH to 7.0, if necessary.

Phosphate buffer pH 7.0

Solution A – M sodium hydroxide.

Solution B – Dissolve 13.6 g of monobasic potassium phosphate in water and complete the volume to 100 mL with the same solvent.

Preparation – Mix 29.5 mL of *Solution A* with 50 mL of *Solution B*. Adjust pH to 7.0 ± 0.1 using *Solutions A* and *B* and complete the volume to 100 mL with water. Homogenize.

M/15 phosphate buffer pH 7.0

Preparation – Dissolve 0.908 g of monobasic sodium phosphate in water, dilute to 100 mL with the same solvent and homogenize. Separately, dissolve 2.38 g of sodium phosphate dibasic in water, dilute to 100 mL with the same solvent and homogenize. Mix 38.9 mL of the monobasic potassium phosphate solution with 61.1 mL sodium phosphate dibasic solution. Adjust pH to 7.0, if necessary.

Phosphate buffer pH 7.1

Preparation – Transfer 1 g of monobasic potassium phosphate and 1.8 g of sodium phosphate dibasic to a 1000 mL volumetric flask, add 900 mL in water and adjust pH to 7.1 ± 0.1 with phosphoric acid or 10 M sodium hydroxide. Complete the volume with the same solvent and homogenize.

Phosphate-albumin buffer pH 7.2

Preparation – Dissolve 4.26 g of sodium phosphate dibasic, 7.6 g of sodium chloride and 10 g of bovine albumin in water. Complete the volume to 1000 mL and homogenize. Adjust pH to 7.2 with 2 M sodium hydroxide or with phosphoric acid.

Phosphate buffer pH 7.2

Preparation – Mix 250 mL of 0.2 M monobasic potassium phosphate and 175 mL of 0.2 M sodium hydroxide. Complete the volume to 1000 mL. Homogenize. Adjust pH to 7.1, if necessary.

Phosphate buffer pH 7.3

Preparation – Dissolve 20.8 g of sodium phosphate dibasic heptahydrate and 3.08 g of sodium phosphate monobasic monohydrate in 900 mL of water, adjust pH to 7.3 ± 0.1 with phosphoric acid RS or sodium hydroxide RS and dilute to 1000 mL with the same solvent. Homogenize.

Barbital buffer pH 7.4

Preparation – Mix 50 mL of a solution with 1.94% (w/v) sodium acetate and 2.95% (w/v) of sodium barbital in water with 50.5 mL of 0.1 M hydrochloric acid. Add 20 mL of 8.5% (w/v) sodium chloride solution and complete the volume to 250 mL with water. Homogenize.

Potassium phosphate buffer pH 7.4 with 2% (v/v) polysorbate 80

Preparation – Dissolve 27.22 g of monobasic sodium phosphate in 1000 mL of water. Transfer 250 mL from the previous solution to a 1000 mL volumetric flask, add 195.5 mL of 0.2 M sodium hydroxide and 450 mL of water. Adjust pH to 7.4 with phosphoric acid or sodium hydroxide and complete the volume with water. Polysorbate 80 must be added later, due to the difficulty of dissolving it. Homogenize.

Imidazole buffer pH 7.4

Preparation – Dissolve 3.4 g of imidazole and 5.84 mL of sodium chloride in water. Add 18.6 mL of M hydrochloric acid and complete the volume to 1000 mL with water. If necessary, adjust pH to 7.4 ± 0.1 .

Tromethamine-sodium chloride buffer pH 7.4

Preparation – Dissolve 6.08 g of tromethamine and 8.77 mL of sodium chloride in 500 mL of distilled water. Add 10 g of bovine albumin. Adjust pH to 7.4 with hydrochloric acid and complete the volume to 1000 mL with water. Homogenize.

Tris-sodium chloride buffer pH 7.5

Preparation – Dissolve 7.27 g of tromethamine and 5.27 g of sodium chloride in 950 mL of water. Adjust pH to 7.5 with 2 M hydrochloric acid and complete with water at 1000 mL. Homogenize.

Borate buffer pH 8.0

Preparation – Mix 0.619 g of boric acid and 0.746 g of potassium chloride in 50 mL of water. Add 3.97 mL of 0.2 M sodium hydroxide and dilute to 200 mL with water. Homogenize. Adjust pH to 8.0, if necessary.

Barbital buffer pH 8.4

Preparation – Dissolve 8.25 g of sodium barbital in water and complete the volume to 1000 mL with the same solvent. Homogenize. Adjust pH to 8.4, if necessary.

Tris-EDTA ASB buffer pH 8.4

Preparation – Dissolve 6.1 of tromethamine, 2.8 g of sodium iodide, 10.2 g of sodium chloride and 10 g of bovine albumin in water, adjust pH with M hydrochloric acid and complete to 1000 mL with water. Homogenize.

Tromethamine-EDTA buffer pH 8.4

Preparation – Dissolve 5.12 g of sodium chloride, 3.03 g of tromethamine and 1.40 g of sodium iodide in 250 mL of water. Adjust pH to 8.4 with hydrochloric acid and complete the volume to 500 mL with water. Homogenize.

Ammonium acetate buffer pH 8.5

Preparation – Dissolve 50 g of ammonium acetate in 1000 mL of 20% (v/v) ethyl alcohol. Adjust pH to 8.5 with 6 M ammonium hydroxide.

Phosphate buffer pH 8.5

Preparation – Dissolve 3.5 g of dibasic potassium phosphate and 4.5 g of sodium chloride in 500 mL of water. Adjust pH to 8.5 with a mixture of water and phosphoric acid (1:1).

Barbital buffer pH 8.6

Preparation – Mix 129 mL of 0.1 M hydrochloric acid with sufficient volume of 0.1 M sodium barbital to complete 1000 mL. Adjust pH to 8.6, if necessary.

Phosphate buffer pH 8.6

Preparation – Mix 2.3 volumes of 0.2 M sodium hydroxide with 2.5 volumes of 0.2 M monobasic potassium phosphate and 2 volumes of methyl alcohol. Cool and mix with water to obtain 10 volumes of solution. Adjust pH, if necessary, to 8.60 ± 0.05 with sodium hydroxide.

1.5 M tris-hydrochloride buffer pH 8.8

Preparation – Dissolve 90.8 g of tromethamine in 400 mL of water. Adjust pH to 8.8 with hydrochloric acid and dilute to 500 mL with water.

Borate buffer pH 9.0

Preparation – Dissolve 12.37 g of boric acid and 14.91 g of potassium chloride in water and complete the volume to 1000 mL with the same solvent. Transfer 50 mL from the solution obtained to a 200 mL volumetric flask, add 20 mL of 0.2 M sodium hydroxide and 120 mL of water. Homogenize. Adjust pH to 9.0 with sodium hydroxide RS or hydrochloric acid RS, complete the volume with water and homogenize.

0.05 M tris buffer pH 9.0

Preparation – Transfer 6.05 g of tromethamine to a 1000 mL volumetric flask. Dissolve in water and complete the volume with the same solvent. Homogenize. Adjust pH to 9.0 ± 0.05 using phosphoric acid. Dissolve 10 g of sodium lauryl sulfate in approximately 600 mL of buffer. Mix the solution obtained with the remaining buffer.

Borate buffer pH 9.6

Preparation – Transfer 3.093 g of boric acid and 3.728 g of potassium chloride to a 1000 mL volumetric flask, add 250 mL of water and shake until dissolution. Add 182 mL of 0.2 M sodium hydroxide and complete the volume with water. Homogenize. Adjust pH to 9.6, if necessary.

Carbonate-sodium bicarbonate buffer pH 9.6

Preparation – Dissolve 0,75 g of sodium carbonate and 1.5 g of sodium bicarbonate in 500 mL of water. Distribute in containers with 50 mL each. Autoclave at 121 °C, pressure of 1 atm, for 20 minutes. Store at 4 °C.

Ammonium chloride buffer pH 10.0

Preparation – Dissolve 5.4 g of ammonium chloride in 70 mL of 5 M ammonium hydroxide and complete the volume to 100 mL with water.

Ammonium chloride buffer pH 10.7

Preparation – Dissolve 67.5 g of ammonium chloride in water, add 570 mL of concentrated ammonia solution and dilute to 1000 mL with water. Homogenize. Adjust pH to 10.7, if necessary.

Sodium lauryl sulfate-phosphate buffer pH 11.0

Preparation – Dissolve in water 16.35 g of monobasic sodium phosphate, 7.05 g of sodium hydroxide and 3 g of sodium lauryl sulfate and dilute with water to 1000 mL. Homogenize. Adjust pH to 11.0, if necessary.

Acetic acid-ammonium acetate buffer

Preparation – Dissolve 77.1 g of ammonium acetate in water, add 57 mL of glacial acetic acid and complete the volume to 1000 mL with water. Homogenize. Adjust pH between 4.5 and 4.6 if necessary.

Concentrated buffer for DSS-EGPA samples.

Preparation – Dissolve 1.89 g of tromethamine, 5 g of sodium lauryl sulfate and 50 mg de of bromophenol blue in water. Add 25 mL of glycerin and complete the volume to 100 mL with water. Homogenize. Adjust pH to 6.8 with hydrochloric acid and complete the volume to 125 mL with water.

Concentrated buffer for DSS-EGPA samples in reducer conditions

Preparation – Dissolve 3.78 g of tromethamine, 10 g of sodium lauryl sulfate, 100 mg de of bromophenol blue and 50 mL of glycerin in 200 mL water. Add 25 mL of 2-mercaptoethanol. Homogenize. Adjust pH to 6.8 with hydrochloric acid and complete the volume to 250 mL with water. As an alternative, dithiothreitol can be used instead of 2-mercaptoethanol as reducer agent. In this case, proceed as described: dissolve 3.78 g of tromethamine, 10 g of sodium lauryl sulfate, 100 mg de of bromophenol blue and 50 mL of glycerin in 200 mL water. Homogenize. Adjust pH to 6.8 with hydrochloric acid and complete the volume to 250 mL with water. Immediately before use, add dithiothreitol, to obtain a final concentration of 0.1 M.

DSS-EGPA electrophoresis buffer

Preparation – Dissolve 151.4 g of tromethamine, 721 g of glycine and 50 g of sodium lauryl sulfite in water and complete the volume to 5000 mL with the same solvent. Homogenize. Dilute this solution 10 times with water, immediately before use. The pH of the diluted solution must be between 8.1 and 8.8.

Phosphate-buffered saline (PBS)

Preparation – Dissolve, by stirring, 24 g of sodium chloride, 0.6 g of potassium chloride, 4.3 g of sodium phosphate dibasic dodecahydrate and 0.6 g of monobasic potassium phosphate in 4 L of water. Autoclave at 121 °C, pressure of 1 atm, for 20 minutes. Store at 4 °C.

Cupric sulfate buffer

Solution A – Dissolve 15.22 g of sodium phosphate dibasic in sufficient amount of water. Add 9.75 g of monohydrate citric acid and dilute to 1000 mL with water. Homogenize. Adjust pH to 5.2 with the help of sodium hydroxide or citric acid.

Solution B – Dissolve 0.313 g of cupric sulfate pentahydrate in water and dilute to 100 mL with the same solvent. Homogenize.

Preparation – At the moment of use, mix 15 mL of *Solution B* with 985 mL of *Solution A*.

8 GENERAL INFORMATION

8.1 STERILE PRODUCTS

STERILIZATION AND STERILITY ASSURANCE

Sterility is the absence of viable microorganisms. Since the sterility of any item isolated from a population submitted to the sterilization process cannot be assured or demonstrated to having been obtained, the sterility of a lot is defined in probabilistic terms through an adequately validated production process.

The invalidation of microorganisms by physical or chemical means follow an exponential law; therefore, there is a statistic probability that microorganisms may survive the sterilization process. For a determined process, the survival probability is determined by the number, type and resistance of microorganisms present and by the environment during the sterilization. The sterility assurance level from a sterilization process translates the safety with which the process in question sterilizes a set of items, being expressed as the probability of a non-sterile item in that population. The sterility assurance level of 10^{-6} , for example, indicates the probability of no more than one viable microorganism in 1×10^6 sterilized items from the final product. The sterility assurance level from a process for a certain product is established through appropriate validation studies and it is usually accepted that injectable products or critical sterile devices submitted to terminal sterilization achieve a microbial survivor probability of 10^{-6} . With heat-stable products, the frequent approach is to exceed the critical time necessary to achieve the microbial survivor probability of 10^{-6} (overkill). However, for thermosensitive products, the overkill approach cannot be employed and the development of the sterilization cycle depends on knowledge of the product's microbial burden.

The D value, decimal reduction time, is the time in minutes necessary for reducing the microbial population by 90%, or 1 log cycle, at a specific condition, that is, to a surviving fraction of 1/10. Therefore, where the D value of a biological indicator preparation from, for example, *Geobacillus stearothermophilus* spores is 1.5 minutes under the total process parameters, that is, at 121 °C, if treated for 12 minutes under the same conditions, it is possible to state that the lethality input is 8 D. Applying this input to product sterilization depends on the initial microbial burden. Assuming that the product's microbial burden presents resistance to the sterilization process equal to the biological indicator resistance and that the product's initial burden is 10^2 microorganisms, the lethality input of 2 D would reduce the microbial burden to 1 (theoretically 10^0) and, consequently, additional 6 D would result in a microbial survivor probability calculated as 10^{-6} . Under the same conditions, a lethality input of 12 D can be employed as a typical approach to obtain overkill. Usually, the microbial burden survival probability in the material, which sterilization validation process is being executed, is not the same as the biological indicator. For valid use, therefore, it is essential that the biological indicator resistance is higher than the bioburden of the material to be sterilized, being necessary to assume the worst case scenario during the validation. The D value of the biological indicator to be used must be determined or verified for each validation program, and also in case such program is changed.

The determination of survivor curves, or fractional cycle approach, can be used to determine the D value of the biological indicator selected for the specific sterilization process. This approach can also be used to assess the product bioburden resistance. Fractional cycles are used to assess the reduction in microbial count or achieve negative fraction. These numbers can be used both to determine the process lethality under production conditions and to establish appropriate sterilization cycles. An adequate biological indicator, such as the *Geobacillus stearothermophilus* preparation, must also be used during routine sterilization. Any microbial burden method used for sterility assurance requires adequate surveillance of the item's microbial resistance to detect any changes.

METHODS OF STERILIZATION

With a method of sterilization, the purpose is to remove, or destroy, all forms of life, animal or vegetable, macroscopic or microscopic, saprophytic or not, from the product considered, without assuring the inactivation of cell toxins and enzymes. The procedure selected to achieve the sterility assurance level depends on knowledge of the nature of the material to be sterilized; the sterilization process to be employed; and changes that may occur in the material, due to sterilization. The knowledge on the type, amount and source of contaminants in the products, before sterilization, and the employment of methods to minimize such contamination and prevent it after processing contribute to ensure a successful sterilization.

This chapter reports concepts and principles involved in quality assurance and control for products that must meet the sterility requirement and includes descriptions of the sterilization methods and instructions for an aseptic process.

PHYSICAL METHODS

STERILIZATION BY HEAT

Heat is the simplest, most economic and safer sterilizing agent available. However, the sensitivity of different microorganisms to action from heat is highly varied, with the spored forms being the most resistant. The efficiency in the inactivation of microorganisms depends on temperature, exposure time and presence of water, because lower exposure times and temperatures are required in presence of water. Sterilization by moist heat causes coagulation of cell proteins from microorganisms, while sterilization by dry heat occurs due to oxidizing processes, which need high temperature and long exposure time.

Moist heat

The sterilization process employing saturated vapor under pressure is executed in a chamber called autoclave. The basic operation principle is replacing air in the chamber with saturated vapor. To displace air more efficiently in the chamber and inside the products, the sterilization cycle may include air and vapor evacuation steps. For this sterilization method, the reference condition for sterilization of aqueous preparations is of heating at no less than 121 °C for at least 15 minutes. Different time and temperature combinations may be used, provided that validated and that they demonstrate the effectiveness of the process selected, offering adequate and reproducible lethality level when operated, as routine, within the tolerances established. Procedures and precautions are applied to achieve a sterility safety level of 10^{-6} or better. Time and temperature combinations must be established based on factors such as nature of the material and its thermolability, penetrability of vapor in the product to be sterilized, and other parameters defined in the validation process. When a sterilization temperature different from 121 °C is used, the concept of F_0 must be used. F_0 in a particular temperature different from 121 °C is the time in minutes necessary to provide lethality equivalent to the one provided at 121 °C for a certain time. F_0 is a measure of sterilizing effectiveness, that is, the number of minutes of thermal sterilization by steam at a given temperature supplied to a container or product unit, at a given Z value.

To assure the sterilization process efficiency, the burden must be distributed in the chamber to allow contact of vapor with regions of most difficult access. For materials sterilized by moist heat, it is acceptable that a microbial survivor probability of 10^{-6} is achieved. For heat-stable products, the necessary time to achieve the previous condition may be exceeded, resulting in overkill, which does

not apply to products that may undergo change due to excessive exposure to heat. In this situation, the development of the sterilization cycle depends, especially, on knowing the microbial burden in the product, which must be determined in a substantial amount of product batches, before sterilization. The D value of the adequate biological indicator used, such as *Geobacillus stearothermophilus*, must be assessed in the validation program or if there is any change to this program.

Dry heat

Thermal sterilization by dry heat is carried out in an oven with homogeneous heat distribution, which can be achieved by forced air circulation. Items such as glassware, metals, powders, petrolatum, fats, waxes, oily solutions and suspensions and special fabrics can be sterilized. This process is applied especially to materials sensitive to sterilization by moist heat. For this sterilization method, the reference condition is a minimum temperature of 160 °C for, at least, two hours. Different time and temperature combinations may be used, provided that validated and that they demonstrate the effectiveness of the process selected, offering adequate and reproducible lethality level when operated as routine within the tolerances established.

A sterility assurance level of 10^{-12} is considered acceptable for heat-stable products. An example of biological indicator to validate and monitor the sterilization by dry heat is the *Bacillus atrophaeus* spore preparation.

The process employing dry heat can also be used for sterilization and depyrogenation as integral part of the aseptic filling process, which requires very high temperatures due to the lower time of exposure to heat. In continuous processes, it is usually necessary to have a cooling stage previous to the packaging process. Due to the lower material dwell time, the validation program must include parameters such as uniformity of temperature and dwell time.

Dry heat at temperatures higher than 220 °C can be used to sterilize and depyrogenate glassware. In this case, a challenge with bacterial endotoxin must be part of the validation program, demonstrating a reduction of no less than three log cycles of heat-resistant endotoxin, that is, test materials inoculated with at least 1000 units of bacterial endotoxin. The test, with *Limulus* lysate, can be used to demonstrate that the endotoxin was inactivated to no more than 1/1000 of the original amount, and the remaining endotoxin is measured to ensure the reduction of 3 log cycles.

STERILIZATION BY IONIZING RADIATION

Ionizing radiations are high energy emissions, in the form of electromagnetic waves or particles that, when colliding with atoms from the irradiated material, change their electric charge by electron displacement, turning the irradiated atoms into positive or negative ions. When these radiations cross the cells, they create free hydrogen, hydroxyl radicals and some peroxides, which in turn may cause different intracellular injuries. The main sources of radiation are: alpha; beta; gamma rays and X rays. The two types of ionizing radiation employed are radioisotope decay (gamma radiation) and radiation by electron beam. The products are exposed to ionizing radiation in the form of gamma radiation from an adequate source of radioisotopes (cobalt 60, for example) or a beam of electrons energized through an electron accelerator.

In addition to the possibility of processing at low temperatures, which allows the sterilization of heat-sensitive products, the sterilization by ionizing radiation has advantages such as low chemical reactivity and the fact that there are few parameters to be controlled, being mandatory to control the radiation dose absorbed. The dose of radiation established for sterilization must assure the materials to be sterilized are not compromised. For gamma radiation, the process validation includes establishing the compatibility of the material, establishing the product loading model and mapping

the dose in the sterilization container, identifying zones for maximum and minimum radiation doses, defining the exposure time, and proving the application of the sterilization dose required. For irradiation by electron beams, voltage, current, conveyor speed and the electron beam scanning dimension must also be controlled. For this sterilization process, the absorbed reference dose is 25 kGy, but in some situations there is the need for selecting a higher or lower dose. The dose selected must offer an adequate and reproducible level of lethality when the process is operated as routine within the tolerances established. Procedures and precautions must be applied to achieve a sterility safety level of 10^{-6} or better.

To validate the effectiveness of this sterilization, especially when lower doses are used, it is necessary to determine the resistance to radiation of the product's microbial burden. Specific product loading patterns and the distribution of minimum and maximum doses absorbed must be established. The doses absorbed are usually measured through specific dosimeters, such as standardized plastic support that shows the color intensity proportional to the amount of radiation absorbed. The fractional cycle approach provides the data used to determine the D_{10} value of the biological indicator, an information applied to extrapolate the amount of radiation absorbed, to establish an adequate microbial survivor probability. Currently, the dose is based on the resistance to radiation of the natural heterogeneous microbial burden in the product to be sterilized. The validation procedures can be used the exposure of the inoculated product, using resistant organisms, such as *Bacillus pumilus*, or exposure of samples of the finished product from the production line to the sublethal process dose.

The procedure for selecting the radiation dose, based on the assessment of the resistance of microorganisms that are constituents of the microbial burden of the product to be sterilized, allows a more representative determination of its resistance when working with different susceptibilities to radiation. This procedure requires enumerating the microbial population in a representative sampling of different product batches. With knowledge on the microbial burden, the radiation dose is established based on a table available on literature. Another method that allows establishing the radiation dose is based on employing radiation dose increases until obtaining, at maximum, a positive sample in 100 units irradiated. This information provides the base for extrapolating this dose and obtaining the radiation dose. Periodic assessments must be made to ensure that the values established remain effective (reference: ISO 11137-1: 2006 – *Sterilization of health care products – Radiation – Part 1: Requirements for development, validation and routine control of a sterilization process for medical devices*).

The effectiveness of the sterilization cycle must be assessed, periodically, by determining the product's microbial burden, or by employing the biological indicator and using calibrated dosimeters.

STERILIZATION BY FILTRATION

The filtration is employed for sterilization of heat-sensitive solutions through physical removal of contaminant microorganisms. The filtering material cannot shed fibers or extractable materials that are undesirable for the filtered solution, which restricts the nature of the filtering element to glass, metal, synthesized polymers and polymeric membranes.

A filter assembly consists of a porous matrix inserted in an impermeable housing. The effectiveness of a filtering medium or substrate depends on the material pore size, the adsorption of microorganisms on or in the filter matrix, and the sieving or exclusion mechanism. The effect of exclusion by size is a function of the pore opening (diameter), and the adsorption depends on the composition, thickness of the filtering element and fluid being filtered.

The pore size of filtering membranes is estimated by a nominal value that reflects the filter membrane capacity of retaining microorganisms represented by specific strains. The filtration for sterilization purposes is usually conducted with graduation membranes of nominal pore size of 0.2 μm or smaller. Such sterilizing filtration membranes, classified as 0.22 μm or 0.2 μm , depending on the manufacturer, are capable of retaining 100% of a culture with 10^7 microorganisms of *Brevundimonas diminuta* (ATCC 19146), per cm^2 of filtering membrane surface, under minimum pressure of 30 psi (2.0 bar).

The user is responsible for selecting the filter due to the nature of the material to be filtered, which meets the needs from the sterilization process, and must also determine whether the parameters employed in the production will influence the effectiveness of microbial retention. Since the filtration process effectiveness is also influenced by the bioburden of the solution to be filtered, it is important to determine the microbial quality of the solutions before the filtration, as well as establish parameters such as pressure, flow rate and characteristics of the filtering unit.

The log reduction value can also be used to assess the retention capacity of the filtering membrane. For example, a 0.2 μm filter, which can retain 10^7 microorganisms from a specific strain, will have a minimum log reduction value of 7, under the conditions stated.

Commercially available filtering membranes include cellulose acetate, cellulose nitrate, fluorocarbonate, acrylic polymers, polyester, polycarbonate, polyvinyl chloride, vinyl, nylon, polytetrafluoroethylene, and also metallic membranes. Membrane filters, for being polymeric films, offer many advantages and some disadvantages when compared to depth filters such as porcelain or sintered material. Since a considerable part of the membrane surface is a void or open space, the correct filter assembly and sterilization offer the advantage of a high flow rate. A disadvantage is that, since the membrane is fragile, it is necessary to ensure there is no rupture during the assembly, sterilization or use.

The filtration system must be tested before and after the filtration process to ensure its integrity is maintained during the filtration process. Typical use tests include bubble point test, diffusive airflow test, pressure hold test, and progressive flow test. The bubble point test consists of a non-destructive test, and derives its name from viewing bubbles after applying a certain pressure on the filter. For example, after filtration of approximately two liters of sterile distilled water, constant nitrogen pressure is applied for five minutes for 0.2 μm cellulose ester membranes. For each type of filter there is a limit pressure value to be supported, without presenting the formation of bubbles, indicating the resistance of the filtering material. The tests must be correlated to the retention of microorganisms. Additional tests conducted by the filter manufacturer, such as the microbial challenge test, are not usually repeated by the user.

CHEMICAL METHOD

STERILIZATION BY ETHYLENE OXIDE GAS

Gas sterilization can be the method selected for materials that do not resist high temperatures, such as in the processing by dry heat or moist heat. The active agent generally employed in gas sterilization is ethylene oxide. Among the disadvantages of this sterilizing agent, there are its mutagenic properties, the possibility of toxic residues in the materials treated and its highly flammable nature, except when in certain mixtures with inert gases. The sterilization process is usually conducted in a pressurized chamber with design similar to autoclave, but with specific characteristics, such as system for degassing after sterilization and minimization of exposure from operators to gas.

The qualification program for the process of sterilization with ethylene oxide is broader than for other sterilization processes, since humidity, vacuum/positive pressure and concentration of ethylene oxide, as well as temperature, must be controlled. It is important to determine and demonstrate that all critical process parameters are adequate inside the sterilization chamber throughout the cycle. Since the sterilization parameters applied to the products to be processed are critical, it is recommendable to precondition the load to minimize the time of exposure to the temperature required.

The validation program is usually conducted by employing the inoculated product, or simulate inoculated product, with appropriate preparations, such as *Bacillus atrophaeus* spores. Biological indicators are usually employed to establish the final microbial survivor probability, using the fractional cycle concept, to design a cycle of sterilization with ethylene oxide, and they must be used in product, or simulated product, loads with full chamber.

The biological indicator must be employed in routine cycle monitoring, and also in the planning of the cycle of sterilization by ethylene oxide. Another important aspect of the sterilization process planning is the definition of the type of packaging of the material to be processed and its distribution in the sterilization chamber, due to the limited capacity of diffusion of ethylene oxide in the innermost areas of the product.

RESIDUES FROM STERILIZATION BY ETHYLENE OXIDE GAS

The control of residues in products sterilized by ethylene gas is necessary to ensure minimum risk for the patient when using the product. Residual levels of ethylene oxide (EO), ethylene chlorohydrin (ECH) and ethylene glycol (EG) must be followed to minimize the exposure by professionals and patients.

The maximum residues admissible for EO and ECH are presented. Local effects, such as irritation, are considered as incorporated to the tolerable contact limit (TCL). No limit is defined for EG, because the risk assessment available indicates that the admissible levels calculated are higher than the residual levels that can be found in the products.

The protection against systemic effects was considered on the limits described. The residual level of EO is considered at the moment of product release.

To establish the maximum daily doses of EO and ECH, the products must be classified according to the duration of contact with the patient:

Limited exposure

Products with one-time, multiple or repeated use or cumulative contact of up to 24 hours;

Prolonged exposure

Products with one-time, multiple or repeated use or cumulative contact of long duration – from 24 hours to 30 days;

Permanent contact

Products with one-time, multiple or repeated use or cumulative contact of long duration – above 30 days.

“Multiple use” means the repeated use of the same type of product, such as, for example, dialysis cartridges.

If a product is classified in more than one duration category, the stricter considerations from test and/or assessment must be enforced. For multiple exposures, the classification must take into account the potential cumulative effect, considering the time period when these exposures occur.

The limits for permanent contact and prolonged exposure products are expressed as maximum average daily doses. If there are data available, one must consider the proportional reduction of limits if multiple products are used at once, or the proportional increase of limits when the product is used only in part of the exposure period.

For permanent contact products, the average daily dose of EO is no more than 0.1 mg. The maximum dose of EO is 4 mg on the first 24 hours; 60 mg on the first 30 days; and 2.5 g through the lifetime. The average daily dose of ECH is no more than 0.4 mg. The maximum dose of ECH is 9 mg on the first 24 hours; 60 mg on the first 30 days; and 10 g through the lifetime.

For prolonged exposure products, the average daily dose of EO is no more than 2 mg. The maximum dose of EO is 4 mg on the first 24 hours and 60 mg on the first 30 days. The average daily dose of ECH is no more than 2 mg/day. The maximum dose of ECH is 9 mg on the first 24 hours and 60 mg on the first 30 days.

For limited exposure products, the average daily dose of EO is no more than 4 mg, and the average daily dose of ECH is no more than 9 mg.

The tolerable contact limit (TCL) is established to prevent localized irritation due to the release of EO or ECH by the product, being expressed in micrograms per square centimeter ($\mu\text{g}/\text{cm}^2$) for EO and in milligrams per square centimeter (mg/cm^2) for ECH, where the square centimeter unit represents the surface area of the product interface with the patient.

For implants and contact products, the TCL of EO is no more than $10 \mu\text{g}/\text{cm}^2$ or negligible irritation. The TCL of ECH is no more than $5 \text{ mg}/\text{cm}^2$ or negligible irritation.

The norm ISO 10993-7 describes the acceptable limits for special situations, such as intraocular lenses; blood cell separators, used in blood collection; blood oxygenators and separators; products used in cardiopulmonary bypass procedures; blood purification devices, extracorporeal devices and gauze bandages.

The procedure for determining the compliance with limits consists of extracting the residue from samples, determining the product contact surface, analyzing and interpreting data.

A validated extraction and measurement method must be used to determine the residual levels of EO and ECH in the product. Many analytical methods have been investigated and the broad variety of materials and formats of sterile products may eventually present issues in determining residues. Therefore, any analytical method must be validated before use.

When residues are within the requirements for the products tested by exhaustive extraction, there is no need to test the product by extraction of simulated use, provided that the results are below the limits specified. When exhaustive extraction is used, attention must be given to the limits for the first 24 hours and for the first 30 days. Small products must be extracted in an adequate container. When a product is too large to be entirely extracted, it may be necessary to extract many representative portions of the product components to ensure the reliability of derived data.

Many factors influence the initial levels of residues and their dissipation rate, and precautions must be taken, such as the moment of taking the sample from the sterilized load; transportation to the laboratory; effects of laboratory conditions on the aeration rate, as well as the safety of the analyst and the operator. The samples must remain with the product load until the day of analysis, or until the test samples are taken and frozen immediately. The samples must be sealed, shipped in dry ice and stored by freezing until their analysis. The test samples can also be taken directly from the product load in the desired aeration range and immediately placed in a confined space (*headspace*) vial, sealed and sent to the laboratory for analysis. Alternatively, the samples can be extracted and the extraction fluid, sent for analysis. If the extraction fluid is water, the shipment must be made so that the liquid is maintained at low temperatures ($<10\text{ }^{\circ}\text{C}$) until it arrives to the laboratory. The test must be conducted to measure the hydrolysis of EO into EG.

The samples must be placed in a fume hood, removed from the package, prepared according to the instructions of use from the product, and the extractions must be started soon after removal from the package or conclusion of the pre-use preparations.

A sample blank must be tested to check for the presence of interferences. The blank must be prepared by using a non-sterilized sample submitted to the same procedure applied to test samples. The chromatographic conditions must be modified to separate the interference peak from the analyte peak, or an alternative analytical procedure may be used when the retention times are conflicting or overlapped.

The nature and size of the product sample will be considered to establish the ideal volume of fluid for extraction, and the sample/extraction fluid ratios must not compromise the detection sensitivity. To maximize the analytical sensitivity, a minimum amount of extraction fluid must be used, and products with highly absorbing materials may require increased sample/extraction fluid ratios.

Two methods for basic extraction are used for determining EO residues: simulated use extraction and exhaustive extraction. The method selected must be adequate to the product use and examples can be seen on the norm ISO 10993-7.

Temperatures and extraction times must be determined based on the nature of patient exposure and duration of contact with the product. The norm ISO 10993-12 is a reference for extraction temperatures.

For certain products, the simulated use extraction may result in relatively large elution volumes, and may considerably increase the limit of detection for the residual material, compromising the determination of compliance. In very large products, the extraction of representative portions can be used to ensure data reliability.

Simulated use aqueous extraction is the method of reference, because it is the only method that produces results directly comparable with the limits specified.

Water, normally used for recovery of EO and ECH residues in simulated use extractions, is employed for elution of EO residues from the sample, not to dissolve the sample. In the case of packaging use simulation, the product must be filled to eliminate air bubbles, at $37\text{ }^{\circ}\text{C}$, for products in total or partial contact with the body during the use (body temperature) and at $25\text{ }^{\circ}\text{C}$ for products with no immediate contact with the body during their use (room temperature). If the test is not executed immediately, the extract must be taken from the sample and sealed in a poly-tetrafluoroethylene coated vial. The standard solution or extract vial headspace must be inferior to 10% of the total volume. The extract can be stored in the refrigerator for several days. It is necessary to use caution when employing water,

since EO can be converted to EG and/or ECH during the extraction period, as well as during the extract storage.

The amounts of EO or ECH extracted through normal product use simulation are not necessarily equal to the total residual content of the product.

Exhaustive extraction methods are targeted at recovering the entire residual content of product, being an acceptable alternative in certain situations.

A variety of extraction fluids can be used for exhaustive recovery of residual EO, and its selection depends on the composition of the product material and its components.

The norm ISO 10993-7 is a detailed guide on assessment of residues from sterilization by EO and indicates how to convert the concentration of residue observed in extracts for the amount administered to a patient, in milligrams.

If sufficient experimental data on the kinetic of residue diffusion are available, it may be possible to group products for tests based on similarity of materials, manufacturing processes and use.

Dissipation curves may be used to estimate the post-sterilization time required for the products or families of similar products, to reach the limits of residues, especially for EO. The products must be released according to the post-sterilization times and conditions, pre-determined and defined by experimental dissipation curves, so that the target levels of EO residues for the product are assured. Concerns with product aeration must be considered by gathering sterilization load data collected from the quarantine storage or aeration in different times of the year, if the aeration temperatures are different.

The product re-sterilization and the presence of other products sterilized with EO in adjacent areas must also be considered when obtaining experimental data to generate such dissipation curves.

When dissipation curve data are not available, the product can be released if the data are obtained from tests conducted according to the procedures and the limits described for EO and, if applicable, for ECH are met.

The product is compliant when it complies with the limits described for EO and, if applicable, for ECH.

The products manufactured and sterilized in controlled conditions may be cleared considering data from at least three sterilization batches at different times.

VALIDATION OF THE STERILIZATION PROCESS

The validation must demonstrate in a documented manner that the sterilization process established will consistently provide products that achieve the sterility assurance level required. The products sterilized according to the validated process must meet the pre-determined specifications and the quality characteristics related to functionality and safety.

Once the process is validated, it must be revalidated periodically, and after changes in product, equipment and process that may compromise the sterility assurance level specified.

The main validation elements are: installation qualification; operation qualification; and performance qualification.

INSTALLATION QUALIFICATION

The execution of the plan for installation qualification must offer documented evidence that the equipment and all auxiliary items were provided, installed and operate according to specifications. It must be demonstrated that the sterilization equipment, its components, auxiliary items and supplies, such as vapor, water and air, have been correctly designed, installed and calibrated.

In order to meet the parameters and limits recommended for sterilization, it is necessary to use appropriate instrumentation to monitor and control critical parameters such as temperature, time, humidity, concentration of sterilizing gas, or radiation absorbed. These instruments must be assessed in the installation qualification.

The installation qualification comprises the following elements: equipment, installation and function.

Concerning equipment and installation, the specifications of the sterilizer; auxiliary items and services; operating procedures; the location of the facility and the documentation must be defined in advance and verified in the installation qualification, ensuring its compliance. To ensure the function, it is necessary to verify that the equipment and operating safety systems work according to their specifications; that the operation cycles are as defined; and that there is no evidence of leakage of utilities or in the sterilizer, when applicable.

In the documented procedures for installation qualification, it must be specified how each qualification element is planned, executed and reviewed. The documentation that provides support to the installation qualification includes description of physical and operational characteristics of the equipment; its components and services. Designs and process and instrumentation diagrams must be verified against the setup proposed and updated, when necessary. Safety systems applicable must be assessed to ensure the performance, quality and safety of equipment and operators.

The installation qualification is necessary for new equipment or when the existing sterilizer is replaced or relocated. The qualification must be redone at defined time intervals, and at least partially when there are changes that may change the sterilization process effectiveness, such as replacement or overhaul of equipment, or parts, modifications in process supplies, and change to radioactive source.

OPERATIONAL QUALIFICATION

In the operational qualification, it is necessary to demonstrate that the equipment installed is capable of conducting the sterilization process specified within the intervals defined. The interval of parameters and the limits of operation must be established in the process definition. Before the operational qualification, the calibration status for the entire instrumentation used to monitor, control, indicate and register must be confirmed.

For autoclaves and other sterilizers that employ thermal process, studies on heat distribution in different positions must be conducted considering the chamber size and the load. It is necessary to confirm that the chamber (empty and full) operates within the critical parameters in all its main locations. The number and position of thermocouples are determined by the load type and configuration; equipment size; type of instrument; and cycle employed. An acceptable temperature range in the empty chamber is ± 1 °C when the chamber temperature is 121 °C. For ethylene oxide sterilizers, relative humidity, concentration of gas and temperature must be monitored by sensors

distributed in adequate positions. Applicable safety systems must be tested. Control software must be validated and challenged in fault conditions. The penetration and distribution of ionizing radiation on the load must be performed and monitored by dosimeters. The operation for qualification of sterilizing filters is conducted through filter integrity test, differential pressure measurements, and flow rate. Since fluids sterilized by filtering membranes can be exposed to the environment during the following processing, the environmental control and the qualification and/or validation of the aseptic handling area must be an integral part of the process of sterilization by filtration.

PERFORMANCE QUALIFICATION

In the performance qualification it is necessary to demonstrate that the sterilization process is capable of repeatedly reaching the sterility assurance level pre-determined for defined product loads; that the apparatus works consistently according to pre-determined criteria; and that the product meets the specified safety, quality and performance requirements.

The performance qualification comprises physical and microbiological assessments that demonstrate the effectiveness and reproducibility of the sterilization process, maintaining the product characteristics specified.

In physical studies, it is necessary to consider: criteria such as test load representative of the process; package identical to the product; pre-conditioning; temperature profile and temperature on the point of reference; response from chemical indicators; package integrity; documentation; among others. The load for sterilization must be established and documented, taking into account parameters such as configuration, distribution, orientation, density, dimension, composition of material, use and type of pallets. The product or material with similar characteristics to the product (simulated product) used for qualification must have a package identical to the product and represent, as a minimum, the worst case of production routine load, that is, the most difficult configuration to sterilize. Criteria for reutilization of load must be defined, and it must be equilibrated with the environmental conditions or aerated before reuse. With the data generated, it is necessary to demonstrate the compliance with physical and chemical parameters applicable. The relation between the conditions of monitoring positions during the qualification and the routine must be established.

In the physical performance qualification, it is necessary to demonstrate the process reproducibility with a minimum of three consecutive cycles to verify if all acceptance criteria are met.

In microbiological qualification, it is necessary to follow specific requirements for each sterilizing agent. Different methods can be used in the validation of the sterilization process and include three categories: process based on inactivation of the natural microbial burden (bioburden); combined process based on the inactivation of reference microorganism and knowledge of the microbial burden (bioburden and biological indicator); and process based on the inactivation of reference microorganism (overkill). It is indicated that the microbial challenge must be executed in the minimum process parameters and must achieve the sterility assurance levels for all load combinations, and may use the worst case of product representing the families. For each type of load to be sterilized, the process reproducibility must be demonstrated by employing at least three consecutive cycles. The biological indicators used must be positioned in and/on the product at a defined location.

The performance qualification must be repeated when significant changes are proposed, such as changes in product design and package, load configuration or density, and sterilization equipment or process. The effects of these changes in the stages of validation of the sterilization process must be assessed.

REVIEW AND APPROVAL OF VALIDATION

The documented review of the validation data generated in the installation, operation and performance qualifications must be conducted to confirm the acceptability of the sterilization process and define the process specification, including parameters and tolerance.

The final stage of the validation program requires the documentation of support data developed in the execution of this program.

BIOLOGICAL INDICATORS

The biological indicator is defined as a preparation characterized by a specific microorganism that provides defined and stable resistance to a certain sterilization process. Spore-forming bacteria are microorganisms recognized as appropriate for use as biological indicators, since, except for ionizing radiation, these microorganisms are significantly more resistant to sterilization processes than microorganisms from the natural microbial burden of the product.

A biological indicator can be used in the performance qualification for the sterilization equipment and in the development and establishment of the sterilization process for a specific product. Biological indicators are used in processes for obtaining the sterile product in its final container and in sterilization of equipment, materials and components of the package, employed in the aseptic process. Biological indicators can also be used to monitor sterilization cycles in periodic revalidations and to assess the capacity of the process used in decontamination of isolators or clean rooms.

TYPES OF BIOLOGICAL INDICATORS

There are at least three types of biological indicators, and each type incorporates a microbial species with known resistance to the sterilization process.

One type of biological indicator includes *spores that are added to a support or carrier* (disc or strip in filter paper, glass, plastic or another material) packaged in order to maintain the integrity and viability of the material inoculated. Carriers and primary packaging must not have any type of chemical, physical or microbiological contamination that may compromise the performance and stability of the biological indicator and cannot undergo change due to the sterilization process submitted. Carriers and primary packaging must withstand transport and handling until the moment of use and must avoid loss of the original inoculum during the transportation, handling and storage until the expiration of the validity period.

Another type of biological indicator consists of a *spore suspension inoculated in representative units of the product* to be sterilized. When it is not possible to employ the actual product, it is possible to inoculate a simulated product, which differs from the actual product in some characteristics, but behaves in a similar manner when submitted to test or sterilization conditions. A spore suspension with known D value must be used for inoculation of the actual or simulated product, ensuring that the simulated product does not compromise the resistance of the biological indicator when used. The physical design of the (actual or simulated) product to be inoculated may affect the resistance of the microbial suspension inoculated. In case of liquid products, it is recommendable to determine the D value and the Z value of the biological indicator in the liquid product specified. The population, D value, Z value where applicable and microorganism destruction time must be determined. Z value is the temperature rise, in degrees, necessary to reduce the D value by 90% or to produce a reduction of a log cycle in the thermal resistance curve.

The third type is the *self-contained indicator*, presented in such a way that the primary packaging targeted at incubation after sterilization has the growth medium required for the microorganism recovery. In this case, the system comprised of the biological indicator and the microorganism growth medium must be resistant to the sterilization process and must allow the penetration of sterilizing agent. The D value, microorganism destruction time and survivor time must be determined for the system, not only for the paper strip or disk that contains the microorganisms. After sterilization, the contact of strips or disks with microorganisms and the culture medium is permitted.

The self-contained biological indicator may consist of a spore suspension in a culture medium with pH indicator that allows view the presence or absence of growth after the incubation. The self-contained system resistance depends on the penetration of the sterilizing agent in the package, which must be controlled by the manufacturer through the design and composition of the material that comprises the package, ampoule or container. The self-contained indicator in the form of ampoule may be incubated directly after exposure to the sterilization process, in the conditions specified. The absence or presence of microbial growth is determined visually, from the change of color of an indicator incorporated to the medium, by the turbidity resulting from the development of the microorganism, or also by microscopic examination of the inoculated medium. The self-contained biological indicator must withstand the transport and handling during the use without any ruptures or loss of the original inoculum. During or after the sterilization process, the material of which the self-contained system is comprised must not retain or release any substance that may inhibit the growth of surviving microorganisms. The growth promoting capacity of the culture medium after exposure to the sterilization process must be proven.

PREPARATION OF BIOLOGICAL INDICATOR

All operations involved in the preparation of biological indicators must be monitored through a documented quality system that allows traceability of all materials and components incorporated to the microbial suspension, the inoculated carrier or the biological indicator. The preparation of stock spore suspensions of the microorganisms selected as biological indicators requires the development of appropriate procedures, including cultivation, harvesting, purification and maintenance. The stock suspensions must predominantly have dormant (non-germinative) spores maintained in non-nutritive liquid. The final product provided by manufacturers (microbial suspension, inoculated carrier or biological indicator) must not contain a microorganism different from the test microorganism in sufficient number that may affect the product. To minimize the presence of microorganisms different from the test microorganism, the system must be validated, monitored and registered.

SELECTION OF BIOLOGICAL INDICATOR

The selection of the biological indicator requires knowledge of its resistance to the specific sterilization process to ensure that the biological indicator system provides a higher challenge than the product microbial burden system.

The efficient use of biological indicator for development of the cycle, process and validation, or for monitoring the routine sterilization process, requires knowledge on the material to be sterilized, including its components and packaging material. Only biological indicators recognized and specified on monographs must be used in the development or validation of a sterilization process, to ensure that the biological indicator selected offers a greater challenge to the sterilization process than the microbial burden in the product.

In case of using biological indicators with different characteristics from the ones commercially available, it is possible to cultivate microorganisms described in scientific literature to prepare biological indicators. The user must be capable of determining the D and Z values for in-house

indicators. When a non-commercial biological indicator is used, the population, purity and validity must be confirmed to ensure the legitimacy of the tests to be performed using this indicator.

When the sterilization process definition is based on the product microbial burden, it must be quantified and the resistances of the biological indicator and the microbial burden must be compared. The sterilization process must result in a minimum sterility assurance level of 10^{-6} .

The overkill method may be employed in the development of the sterilization process and, in this case, specific considerations related to the supposed resistance used in establishing the process lethality requirements must be made. In general, overkill processes are developed with the assumption that the microbial burden is equal to 10^6 highly resistant microorganisms. A 12 D process is defined as the process that provides sufficient lethality for reduction of 12 log cycles, equivalent to 12 times the D value for microorganisms with resistance above the average resistance from microorganisms present in the product microbial burden. By assuming a microbial burden of 10^6 , an overkill process will result in a probability of non-sterility inferior to 10^{-6} . The use of the overkill process and its validation may minimize or avoid the need for quantification and identification of the product microbial burden.

For the moist heat process, spores from appropriate strains of *Geobacillus stearothermophilus* are commercially available as biological indicators. Other spore-forming microorganisms resistant to moist heat, such as *Clostridium sporogenes*, *Bacillus atrophaeus* and *Bacillus coagulans*, can also be used in the development and validation of a process of sterilization by moist heat.

For validation of the process of sterilization via dry heat, *Bacillus atrophaeus* spores can be employed. During the validation, studies can be conducted for assessment of depyrogenation instead of microbial inactivation, since the bacterial endotoxin inactivation rate is much slower than the inactivation of *Bacillus atrophaeus* spores. In practice, a reduction of at least three log cycles of the endotoxin level results in a non-sterility probability lower than 10^{-6} .

To monitor sterilization processes employing ionizing radiation, *Bacillus pumilus* spores have been used, despite this not being an usual practice. The method for establishing the radiation dose, more employed, does not use biological indicators. Some microorganisms in the microbial burden of the material to be sterilized may present higher resistance to the process of sterilization by radiation in comparison with *Bacillus pumilus* spores.

For the process of ethylene oxide sterilization, spores from subspecies of *Bacillus atrophaeus* var. *niger* are used, when 100% ethylene oxygen or different mixtures of gases are used.

Table 1 – Examples of characteristics of commercially available biological indicator systems.

Sterilization process	D value (minutes)	D value range for selection of biological indicator (minutes)	Limits for adequate resistance (dependent on D value in minutes)	
			Survival time	Kill time
Dry heat ^a (160 °C)	1.9	Min. 1.0	Min. 4.0	10.0
		Max. 3.0	Max. 14.0	32.0
Ethylene oxide ^b (600 mg/L, 54 °C, 60% RH)	3.5	Min. 2.5	Min. 10.0	25.0
		Max. 5.8	Max. 27.0	68.0
Moist heat ^c (121 °C)	1.9	Min. 1.5	Min. 4.5	13.5
		Max. 3.0	Max. 14.0	32.0

^a for 1.0×10^6 to 5.0×10^6 spores / carrier

^b for 1.0×10^6 to 5.0×10^7 spores / carrier

^c for 1.0×10^5 to 5.0×10^6 spores / carrier

PERFORMANCE EVALUATION

The manufacturer's responsibilities are: determining and providing the performance characteristics of the lot of biological indicator through certificate of analysis that confirms the validity of the performance declared on the product package; definition of the sterilization process for which the biological indicator is recommended; characterization of each type of biological indicator, using standardized conditions and adequate equipment; D value and the method through which this value was defined; microbial count; stability of resistance until the validity indicated on the label; storage conditions, including temperature and relative humidity; guidance about the culture medium to be employed; and the conditions for recovery of microorganisms after exposure to the sterilization process and disposal.

COMMERCIAL PRODUCTS

When a biological indicator is commercially acquired, its suitability for use in a sterilization process must have been established in studies, unless there are data available to confirm the employment of the indicator in a specific process. The user must establish, in their institution, the acceptance criteria for the lots of biological indicator. When a biological indicator is acquired, it must be accompanied with a certificate issued for each lot. If the biological indicator is employed in a different manner than the one indicated by the manufacturer, the user must proceed to the registration of conditions of use, verifications and performance of the biological indicator.

After receiving a lot of biological indicator, the user must quantify the burden of spores per unit and proceed to the verification of morphology and purity of microorganisms, confirming, as a minimum, the genus of the microorganism. Information related to D value, to storage conditions, to validity period and to stability of the biological indicator must be followed and registered. The user may consider the need to audit the D value before accepting the lot of biological indicator. For long-term storage, it is important to verify the D value and the stability of count. In case of spore suspension storage for a period superior to 12 months, under documented conditions, the confirmation of count and proof of resistance of the spores must be conducted, unless the performance from a previous culture has been validated after a long storage period. The results from resistance assays and spore count must be within the acceptance range established during the approval of the spore suspension lot.

NON-COMMERCIAL PRODUCTS

The user may decide to cultivate microorganisms for development of biological indicators to be employed in the development or validation of a sterilization process. If the user becomes a producer, the biological indicator performance requirements must be met. If a biological indicator system is employed for the development of a new sterilization process or validation of an existing process, the same performance criteria for commercial products must be met.

PREPARATION OF THE SPORE SUSPENSION

The spore suspension identification records must be maintained by commercial or non-commercial producers and must include information on the source of the initial microorganism culture; identification; traceability of the spore mother culture; frequency of subculture; culture medium used

for sporulation; changes occurred in the preparation of medium; observations about contamination of suspension; data before and after the thermal shock; records of use of spore suspension and resistance to sterilization (particularly D values and Z values, where applicable).

USE OF BIOLOGICAL INDICATOR FOR VALIDATION

Regardless of the sterilization process to be employed, the initial production of microorganisms, their resistance to the sterilizing process, and the product inoculation place may influence the biological indicator inactivation rate. During the validation process, the biological indicator must be inoculated in several product locations, ensuring the challenge both of the package and of the product stored in it, to ensure a sterility assurance level of 10^{-6} for the product and for the package.

It may be necessary to determine, through laboratory studies, if the product components are more difficult to sterilize than, for example, a solution or medicine included in it. The product performance qualification phase must identify the most important parameters from the process for inactivation of microorganisms in the locations most difficult to sterilize. The biological indicator survival is consequence of the resistance and size of the microbial population. Therefore, not always a biological indicator with population of 10^6 is necessary for confirming a sterility assurance level of 10^{-6} . The appropriate use of biological indicators is to confirm that the parameters established in the sterilization process ensure the sterility assurance level desired. In the sterilization by moist heat, the employment of biological indicator confirms the lethality determined by physical parameters. Biological indicators with relevant D value and populations substantially lower than 10^6 are adequate to validate many sterilization and decontamination processes. It is important that the users are qualified to justify, scientifically, the selection of a biological indicator.

ASEPTIC PROCESS

Although the terminal sterilization of a packaged product is the procedure that ensures minimum risks of microbial contamination in the production of a lot, there are classes of products that cannot be sterilized on their final packaging and that must be prepared employing an aseptic process. This process is designed to prevent the contamination of sterile components by viable microorganisms or even in the intermediate phase of production, when a component must be provided free from microorganisms. A product defined as processed aseptically consists of components that were sterilized by one of the sterilization processes, such as, for example, filtration, if it is a liquid. In the case of packaging material made of glass, dry heat can be employed; when it is a polymeric packaging material, such as lids, autoclave or ethylene oxide can be used.

In the aseptic process, the environment where the ingredients are handled and the aseptic filling step are considered critical points. The requirement for a properly validated project that maintains the necessary conditions for the aseptic process encompass an environment free from viable microorganisms, where the air quality is ensured by suitable equipment, by personnel trained and equipped according to the environment requirements and by operation to be performed. The desired environment may be obtained through the air filtration technology that provides supply of air with the microbial quality required. The plant planning must provide an airflow cascade system with higher positive pressure, of the most critical (aseptic) areas for those of intermediate requirement (preparation areas), and, finally, the ones with lower requirement of control; and must also allow frequent air exchange, in addition to the employment of unidirectional airflow in the immediate surroundings of the product or components exposed and the control of temperature and humidity (when applicable). The installations must include primary (near the product) and secondary (where the aseptic process is conducted) isolation systems through physical barriers. Surfaces such as walls and ceiling must be smooth, allowing frequent sanitization. Changing rooms must have adequate

space for the personnel and storage of sterile clothing. The personnel training concerning apparel must include the correct use of clothing, such as overalls, gloves and other items that provide body surface coverage. The entire sanitization process must be documented. The certification and validation of the process and aseptic installations are conducted through confirmation of efficiency of the filtration systems; by the environment microbiological monitoring procedures; and by simulation of the product aseptic filling, employing a sterile culture medium. The monitoring of the aseptic installation must include the periodical examination of environmental filter, the routine monitoring of particulate and viable material, and simulated filling with sterile culture medium.

CLEAN ROOMS AND ASSOCIATED CONTROLLED ROOMS

This section includes some aspects related to the aseptic processing of products with the establishment, maintenance and control of the microbiological quality of clean rooms and zones. It includes the classification of these controlled environments based on particle count limits; on the microbiological assessment program for controlled environments; in personnel training; in critical factors on the design and implementation of a microbiological assessment program; in the development of a sampling plan; in the establishment of microbiological levels for alert and action; in methods and equipment used for microbiological sampling; in culture media and diluents used; in the identification of microbiological isolates; and in the operational assessment through culture medium packaging (media fill).

Media fill is a test for simulating aseptic operations in which the product is replaced by a culture medium and serves to ensure that the processes used are capable of leading to sterile products.

There are alternative methods to assess and control the microbiological status of clean rooms and zones, with a variety of apparatuses and methods for microbiological sampling. The inadequate application of sampling and microbiological analysis may cause significant variability and potential for inadvertent contamination. A considerable number of sterile products is manufactured by aseptic processing, which depends on the exclusion of microorganisms from the processing line and, therefore, on the prevention of entry of microorganisms in open containers during the packaging, and the packaging and microbial burden of the product and the manufacturing environment are important factors related to the sterility assurance level of these products.

CLASSIFICATION OF CLEAN ROOMS AND ASSOCIATED CONTROLLED ROOMS

Clean room is the room where the concentration of particles in suspension on air is controlled; it is built and used in such a manner as to minimize the introduction, generation and retention of particles within the room, in which other relevant parameters such as temperature, humidity and pressure are controlled as necessary.

The classification of air cleanliness in clean rooms and zones, through analysis of concentration of particles suspended on air, is ruled by the norm ABNT NBR ISO 14644-1 – Clean rooms and associated controlled environments – Part 1: classification of air cleanliness. This document applies to particles suspended on air inside a controlled environment, but does not intend to characterize the viable or non-viable nature of particles.

The enforcement of this norm has been used by manufacturers of clean rooms and zones to guide the construction, preparation and maintenance of these facilities. However, it does not provide a relation between the number of non-viable particles and the concentration of viable microorganisms.

The pharmaceutical industry is concerned with the count of viable particles and, in the case of injectable products, there is the additional concern with the count of total particles. The justification that the smallest the number of particles present in a clean room, the less probable the presence of microorganisms carried by air, is acceptable and directive in the design, construction and operation of clean rooms and zones.

The **Table 1** describes the air cleanliness classes according to the norm ABNT NBR ISO 14644-1, which is based on limits of particles with sizes from 0.1 to 5 µm. The **Table 2** presents a list of different systems for classification of clean rooms.

It is acceptable that, if a smaller number of particles is present in the clean room or controlled environment, the microbial count under operational conditions will be lower, provided there are no changes in the airflow, temperature and humidity. Clean rooms are maintained under an operational control status based on dynamic (operational) data.

Table 1 – Classes of air cleanliness for particles in suspension, selected for clean rooms and zones.

Classification number (N)	Maximum limits of concentration (particles/m ³ of air) for particles equal to or larger than the sizes considered					
	0.1 µm	0.2 µm	0.3 µm	0.5 µm	1 µm	5 µm
ISO Class 1	10	2				
ISO Class 2	100	24	10	4		
ISO Class 3	1 000	237	102	35	8	
ISO Class 4	10 000	2 370	1 020	352	83	
ISO Class 5	100 000	23 700	10 200	3 520	832	29
ISO Class 6	1 000 000	237 000	102 000	35 200	8 320	293
ISO Class 7				352 000	83 200	2 930
ISO Class 8				3 520 000	832 000	29 300
ISO Class 9				35 200 000	8 320 000	293 000

Table 2 – Comparison between different air cleanliness classification systems.

WHO and EEC (GMP)	United States (usual)	ISO
Class A	Class 100	ISO 5
Class B	Class 100	ISO 5
Class C	Class 10.000	ISO 7
Class D	Class 100,000	ISO 8

MICROBIOLOGICAL EVALUATION PROGRAM FOR CLEAN ROOMS AND ASSOCIATED CONTROLLED ENVIRONMENTS

The monitoring of total particles in suspension on the air in clean rooms and zones does not provide information about the microbiological content of the environment. The basic limitation of particle counters is that they usually measure particles with 0.5 µm or larger, and microorganisms carried on air are not cells that flow freely, are not alone, and are frequently associated with particles from 10 to 20 µm. Particle counts, as well as microbial counts in clean rooms and zones, vary with the activities conducted during the sampling and their location. The environment monitoring for non-viable particles and microorganisms is important because both are necessary to meet the requirements related to particulate material and sterility established for the products.

Microbiological monitoring programs for clean rooms and zones must assess the effectiveness of cleaning and disinfecting practices that may have impact on the microbial burden of the environment.

Usually, the microbiological monitoring does not identify and quantify all microbial contaminants in the environments; however, the routine monitoring must provide sufficient information to ensure that the environment is operating within the adequate control status.

The environmental microbiological monitoring and the data analysis conducted by qualified personnel allow the control status to be maintained in clean rooms and zones. The environment must be sampled during regular operations to allow significant data to be collected, and the microbial sampling must occur when the materials are in the area, the processing activities are ongoing, and all employees are working in the location.

The microbiological monitoring of clean rooms and zones must include the quantification of the microbial content in the environment air; the compressed air that enters the critical area; surfaces; devices; containers; floors; walls; and personnel clothing. The goal intended with the program is to obtain representative estimates of the microbial burden of the environment, and once compiled and analyzed any trends must be assessed by trained personnel. It is important to review environmental results based on the frequency specified, as well as review results for prolonged periods to determine whether there are trends. Trends can be viewed through statistic control boards, which include alert levels and action. The microbiological control of controlled environments can also be evaluated based on trend data. Periodic reports or summaries must be issued to alert the person responsible for the area.

When the microbiological level specified for a controlled environment is exceeded, there must be documentation review and investigation. The investigation must include the review on the area maintenance documentation; the disinfection documentation; inherent physical or operational parameters, such as changes in environment temperature and relative humidity; and the training phase of the personnel involved. After the investigation, the actions adopted may include reinforced personnel training to emphasize the microbiological control of the environment; additional sampling in higher frequency; additional disinfection; additional product testing; the identification of microbial contaminant and its possible source; and reevaluation and revalidation of current standardized operating procedures, if necessary. Based on the review of the investigation and on test results, the meaning of the exceeded microbiological level and the acceptability of operations or products processed under such condition can be defined. Every investigation and justification of actions must be documented and become part of the quality management system.

Clean room and zone are defined by certification according to the applicable rule, and the parameters assessed include integrity of filters, pressure and speed differentials, patterns and changes of air. An example of method for conducting the particle challenge test for the system consists of increasing the concentration of particles in the environment through smoke around the critical work areas and view the air displacements. The presence of vortices and turbulent zones can be viewed and the airflow pattern can be fine-tuned to eliminate or minimize undesirable effects. This evaluation is made under simulated production conditions, but with equipment and employees in the location.

The appropriate test and optimization of the physical characteristics of the clean room or controlled environment are essential before concluding the microbiological monitoring program validation. The assurance that the environment is operating properly and according to its specifications will give more assurance that the microbial burden of the environment will be appropriate for aseptic processing. These tests must be repeated during the routine certification of the clean room or zone and whenever changes considered significant are made in the operation, such as changes in the flow of people, processing, operation, material flow, air manipulation system or equipment layout.

ALTERNATIVE TECHNOLOGIES FOR ASEPTIC PROCESS

Due to the strong correlation between human involvement and intervention and the potential risk for product contamination in aseptic packaging, production systems where people are removed from critical zones have been implemented, employing, therefore, advanced aseptic processing strategies, with reduced requirements of environmental monitoring of viable and non-viable particles.

Here are some definitions of systems used to reduce the aseptic process contamination rate.

Barriers: device that restricts contact between operator and aseptic field. Barriers cannot be sterilized and do not always have transfer systems that allow the passage of materials in and out of the system without exposure to the surrounding environment. There are different types of barriers, from plastic curtains in critical zones to hard barriers in devices, which may incorporate elements such as glove support and transfer port.

Blow/Fill/Seal: this system combines the container assembly with the product packaging and sealing in a single equipment. From the microbiological standpoint, the sequence of forming the container, filling the sterile product and forming and applying the seal is obtained aseptically, in a seamless operation with minimum exposure to the environment. These systems have existed for many years and the contamination rates are lower than 0.1%.

Isolators: technology used for a dual purpose, to protect the product from contamination by the environment and by people during filling and closure and to protect people from toxic or harmful products during the production. This technology is based on the principle of placing materials sterilized previously, such as containers, products and lids, in a sterile environment, and they remain sterile throughout the operation, since people or non-sterile components are not inside the isolator. The isolator barrier is an absolute barrier that does not allow exchanges between protected and non-protected environments. Isolators may be physically sealed against the entry of external contaminants or may be effectively sealed by the continuous application of overpressure. The material is handled by employees wearing globes or half-size or full-size suits. The air coming into the isolator goes through a HEPA or ULPA filter and the air exhaust usually goes through a HEPA filter. Vapors from hydrogen peroxide or peracetic acid are usually employed for sterilization of surfaces or internal environment. The sterilization inside the isolators and the entire content is usually validated for a sterility assurance level 10^{-6} .

The introduction of equipment, components and materials can be done in several ways, such as the use of double-door autoclave, continuous introduction of components through a conveyor belt that goes through a sterilization tunnel, or using a docking system. It is necessary to monitor the integrity, calibration and maintenance of the isolator.

The requirements for controlled environments adjacent to these new technologies employed on aseptic processing depend on the type of technology used.

Blow/Fill/Seal equipment items that limit the contact between operator and product can be installed in a controlled environment, especially if an intervention by the operator is possible during the production.

Barrier systems require some form of controlled environment. Due to different types and applications, the requirements for the adjacent environment may vary. The design and operation strategies for the environment where these systems circulate must be developed by producers using a logical and

rational criterion and the system capacity to provide sterile products must be validated according to pre-established criteria.

In isolators, air enters through integral filters of HEPA quality or better, and their interior is typically sterilized with a sterility assurance level of 10^{-6} . Therefore, isolators that have sterile air do not exchange air with the surrounding environment and are free from human operators. However, when the isolator is in a controlled environment, the potential for contaminated product is reduced in case of leak in gloves or suits.

The extension and scope of environmental microbiological monitoring depend on the system used. Producers must balance the frequency of environmental sampling that requires human intervention with the benefits accumulated by the monitoring results. Since barriers are designed to reduce human intervention, remote sampling systems must be used to replace intervention by people. In general, once the validation has established the barrier efficacy, the sampling frequency to monitor the microbiological status of the aseptic processing area can be reduced when compared to the frequency of a classic aseptic process system.

Isolator systems require lower frequency of microbiological monitoring. The continuous monitoring of total particles may offer assurance that the air filtration system inside the isolator is operating adequately. Traditional methods for quantitative microbiological sampling of air may not be sufficient to test the environment inside the isolator. Experiences with isolators indicated that, under normal operation conditions, leak or pinhole in gloves represent the highest potential for microbiological contamination, which requires frequent glove integrity tests and monitoring of their surfaces. The infrequent monitoring of surfaces inside the isolator must be assessed and may be beneficial.

TRAINING OF PERSONNEL

Products processed aseptically require close attention to details, rigorous discipline and the strict supervision from people, to maintain the appropriate level of environmental quality to ensure the product sterility.

Training all employees who work in clean rooms and zones is critical. This training is also important for people responsible for the microbiological monitoring program, since the work area may be inadvertently contaminated during the microbiological sampling, due to the use of inadequate techniques. In highly automated operations, the monitoring can be performed by people who have more direct contact with critical zones inside the processing area. The employee monitoring must be conducted before and after work in the processing area.

The facility management must ensure that all personnel involved in operations in clean rooms and zones knows the relevant microbiological principles, including basic principles on aseptic processing and the relation between manufacturing procedures and manipulation with potential sources of product contamination. They must also have knowledge on basic principles of microbiology; microbial physiology; cleaning, disinfection and sterilization; selection and preparation of culture media, according to the involvement of employees in the process. The personnel involved in microbial identification requires specialized training on applicable laboratory methods. Additional training on managing the environmental data collected must be provided. Knowledge and understanding of standard operating procedures applicable are critical, especially the ones related to corrective measures that are taken when the environmental conditions require. Understanding the policies for compliance with regulatory requirements and the responsibility from each individual, related to Good Manufacturing Practices, must be an integral part of the training program, in addition to training on how to conduct investigations and analyze data.

The control of microbial contamination associated to people is one of the most important elements from the environmental control program. The contamination may occur from the dissemination of microorganisms by individuals, particularly those with active infections and, therefore, only healthy individuals must be authorized to access controlled environments. Good personal hygiene and careful attention to details of the aseptic gowning procedures are important items. Appropriately gowned employees must be careful to maintain the integrity of their gloves and suits throughout the period they are in controlled environments.

Since the environmental monitoring program is not capable of detecting all aseptic processing events that could compromise the microbiological quality of the environment, periodical studies on culture medium packaging or process simulation are necessary to ensure that the appropriate operating controls and training are effectively maintained.

DESIGN AND IMPLEMENTATION OF THE ENVIRONMENTAL MICROBIOLOGICAL CONTROL PROGRAM

The manufacturer is responsible for developing, starting, implementing and documenting and environmental microbial monitoring program that is capable of detecting an adverse event in the microbiological conditions in time to allow significant and effective corrective actions. It is imperative that the program is tailor-made for the specific conditions and installations.

A culture medium of general microbiological growth, such as soybean-casein medium, should be adequate in most cases. This medium can be supplemented with additives to overcome or minimize effects from disinfecting agents or antibiotics, if used or processed in these environments. The detection and quantification of yeasts and molds must be considered. Media such as Sabouraud and modified Sabouraud are usually accepted. Other media validated to promote fungal growth can be used, such as Soybean-casein digest agar. In general, the analysis of mandatory anaerobic microorganisms is not performed as routine, unless conditions or investigations require so. The capacity of the culture media selected to detect and quantify anaerobic or microaerophilic microorganisms must be assessed.

The sterilization processes used to prepare the culture media for the environmental program must be validated and examined for sterility and promotion of growth. The media must be capable of maintaining growth when inoculating with less than 100 CFU. The selection of incubation time and temperature is made once the appropriate media have been selected. Typically, incubation temperatures within the ranges of $(22.5 \pm 2.5) ^\circ\text{C}$ and $(32.5 \pm 2.5) ^\circ\text{C}$ have been used with incubation times of 72 hours and 48 hours, respectively.

The environmental control program must include identification and evaluation of locations for sampling and validating the environment microbiological sampling methods.

SAMPLING PLAN AND SITES

During the initial phase of activities, as well as in the preparation of a clean room, or another controlled environment, specific sites for sampling air or surfaces must be determined. It is necessary to consider the proximity of the product, whether air and surfaces in the room are in contact with it or with internal surfaces from the container closure systems.

The sampling frequency will depend on the criticality of the sites specified and the treatment subsequent to the aseptic process.

As manual interventions during the operation and the potential for personal contact with the product increase, the importance of the environmental monitoring program also increases. It is most critical for products processed aseptically than for those submitted to terminal sterilization. When the terminal sterilization cycle is not based on the overkill concept, the microbial burden program before the sterilization is critical. The sampling plans must be dynamic with monitoring frequencies and sites adjusted based on the trend performance. It is appropriate to increase or reduce the sampling based on this performance.

MICROBIOLOGICAL ALERT AND ACTION LIMITS IN CLEAN ROOMS AND ZONES

The principles and concepts of statistic process controls are useful to establish alert and action levels, as well as mechanisms for controlling trends.

The alert level in environmental microbiological monitoring reveals a contamination level significantly superior to regular operation conditions. Exceeding the alert level must not necessarily require corrective action, but it must generate a documented monitoring investigation, which may include modifications in the sampling plan.

The indication of action in environmental microbiological monitoring, when exceeded, requires immediate monitoring and, if necessary, corrective action.

Alert levels are usually based on historical information obtained from process routine operations in a specific controlled environment.

In a new facility, these levels are usually based on the previous experience from similar facilities and processes and in data obtained throughout several weeks. These levels are usually reexamined for suitability to a frequency established. Environmental quality deterioration trends require attention to determine the cause and implement a corrective action plan, to bring the conditions back to expected levels. An investigation must be implemented and the assessment of potential impact on the product must be made.

METHODS AND EQUIPMENT USED FOR ENVIRONMENTAL MONITORING

Viable microorganisms from air may influence the microbiological quality of the products manufactured in clean rooms and zones. The quantification of these microorganisms may be influenced by instruments and procedures used on the assays. Employing alternative equipment or methods must be preceded by verification on the equivalence of results. There are different ways of monitoring different types of equipment available to quantify viable microorganisms, including deposit, impaction and centrifugal samplers. The user is responsible for the selection and suitability of the method to be used.

The method using deposit plates is still the one most broadly disseminated thanks to its simplicity and low cost and provides qualitative information about the environment of exposure for prolonged times, but the exposure of open Petri dishes containing agar medium is not for quantitative assessment of the microbial contamination levels of critical environments.

One of the main limitations of mechanical air samplers is the size of the air sample being tested, because the level of microorganisms in the air of a controlled environment is usually reduced and a large volume of air must be tested for the result to be accurate and precise, which many times is not practical. To demonstrate that the microbial counts in the environment are not increasing after sampling, it can be extended to determine if the sampling time is a limiting factor to obtain a representative sample. There are equipment items capable of sampling high air volume rates, but it is

necessary to consider the airflow rupture in critical areas or the creation of turbulence that may increase the probability of contamination.

Centrifugal samplers demonstrate selectiveness for larger particles and, therefore, using this equipment may result in higher counts of particles in air. When using these samplers, it is necessary to consider their effect in the airflow linearity in the controlled zone where it is positioned for sampling. The use of remote probes requires determining whether the extra tube used has no adverse effect in the count of viable particles, because such effect must be eliminated, or a correction factor must be used for the results obtained.

METHODS AND EQUIPMENT USED FOR MONITORING VIABLE PARTICLES ON SURFACES

Sampling surfaces from equipment, areas and employees is a component from the microbiological control program for controlled environments. To minimize the disruption of critical operations, the sampling is usually performed at the end of the operations. The sampling can be performed using contact plates or swab.

The monitoring is usually performed in areas that come into contact with the product and in adjacent areas. Plates of contact with nutrient agar are used to sample flat surfaces and incubated in adequate temperature for quantification of viable particles. Specific agar may be used to quantify fungi, spores, etc. The swab is used in irregular surfaces, especially on equipment. The swab is placed in an adequate diluent and the microbial count is estimated by plating an appropriate aliquot in specific nutrient agar. The area to be sampled using swab is defined using a sterile template of appropriate size, usually between 24 cm² and 30 cm². The result is given per contact plate or per swab.

CULTURE MEDIA AND DILUENTS FOR SAMPLING AND QUANTIFICATION OF VIABLE PARTICLES

The culture media and diluents used for sampling and quantification of microorganisms in clean rooms and zones depend on the procedures and equipment used. Soybean-casein digest agar is the solid medium usually employed, but there are different media and diluents available for different purposes. Alternative media must be validated to the purpose used. When disinfectants or antibiotics are used in the controlled area, it is necessary to consider employing media with appropriate inactivating agents.

IDENTIFICATION OF MICROBIAL ISOLATES

The environmental control program includes an appropriate level of identification of the flora obtained in sampling. Knowledge of the regular flora of clean rooms and zones is important to define the area monitoring, the effectiveness of cleaning and disinfection procedures, and the microbial disinfection methods. The information obtained using the identification program may be useful in the investigation of contamination sources, especially when the action limits are exceeded. The identification of isolated microorganisms from critical areas is important.

OPERATIONAL EVALUATION OF THE MICROBIOLOGICAL STATUS OF ASEPTICALLY FILLED PRODUCTS

Clean rooms and zones are monitored by an appropriate environmental monitoring program. To ensure minimum microbial burden, additional information in the evaluation of the environment biological status can be obtained through culture medium aseptic fill test (media fill). The media fill test is employed to assess the aseptic processing using sterile culture medium instead of the product.

Satisfactory media fill results demonstrate the line suitability for manufacturing the product. However, other factors are important, such as construction of areas, environmental monitoring and training of personnel.

When an aseptic process is developed and installed, it is necessary to qualify the microbiological status of the process, executing no less than three consecutive media fills. Issues in the media fill program development to be considered include procedures for filling the medium; selection of medium; packaging volume; time and temperature of incubation; inspection of packaged units; interpretation of results and possible corrective actions required.

Once the media fill is conducted to simulate the aseptic processing of a product, it is important that it is conducted in normal production conditions. This includes maximum number of people and use of all steps and materials used in the normal production process. While conducting the media fill, known pre-documented interventions must be planned during the normal production runs, such as change of filling nozzles, fastening components, etc. Alternatively, to add a safety margin, a combination of possible conditions can be used and examples include frequent downtimes, unexpected repairs, change of filters, etc.

The qualification of an aseptic process must be performed for all products and for each line. Provided that the container geometry (such as size and opening) and the line speed are variable factors, the appropriate combination of these factors, preferably on the extremes, must be used in the qualification. A reasonable analysis of the products used must be documented.

It is recommended that the media fill is conducted to cover all production shifts for line/product/combination of containers for initial qualification and periodic revalidations. The media fill program must simulate production practices in prolonged times and may be executed at the end of the production shift.

Rich culture media, such as soybean-casein broth, may be used. After aseptic processing of the culture medium, they must be incubated at $(22.5 \pm 2.5)^\circ\text{C}$ or $(32.5 \pm 2.5)^\circ\text{C}$, for no less than 14 days. If two temperatures are used for incubation of culture medium samples, they must be incubated at least seven days in each one of them. After incubation, the samples must be inspected for growth. Isolates must be identified for genus and, when possible, for species, to allow the investigation of sources of contamination.

Critical points when conducting the media fill are number of containers to qualify the aseptic process; number of units filled for the media fill; interpretation of results; and implementation of corrective actions. Usually, three media fill runs are employed for initial qualification, or in the beginning of an area to demonstrate consistency in the aseptic packaging line. The minimum number to demonstrate the contamination rate of no more than 0.1%, acceptance criterion for a media fill run, is of no less than 3000 units. Pilot plans that prepare small batches can use a smaller number of units.

Since employees are a critical source of contamination in clean rooms, visual documentation may be useful to check the correlation of production activities with contamination events.

PROCEDURES FOR RELEASE

A quality assurance program that describes in details the steps and documentation required for releasing the cargo or batch must be established. The release of sterilized products will depend on the type of release, which may be conventional or parametric.

PARAMETRIC RELEASE OF PRODUCTS WITH TERMINAL STERILIZATION

Parametric release is defined as the release of cargo or batches of products subject to terminal sterilization, through compliance with critical parameters of the sterilization process, without the need to perform a sterility test. The parametric release is a possibility when the sterilization process is very well known, the important process control points are defined, predictable and measurable, and the sterilization cycle lethality has been validated with adequate biological indicator or, in the case of sterilization by ionizing radiation, the execution of appropriate microbiological and dosimetry tests. The use of parametric release for sterilization processes requires prior approval from the regulatory agency, which must assess the scientific justification for the sterilization process employed and the documented validation data.

It is important to consider the sterility test limitations in the assessment of products submitted to terminal sterilization, which has compromised sensitivity and is statistically limited due to the low probability of presence of contaminated units. Therefore, once the sterilization process is completely validated and operating consistently, the physical sterilization data, combined with other methods, such as biological indicators, thermochemical indicators and physicochemical indicators, may provide more accurate information than the sterility test for releasing products submitted to terminal sterilization.

Four sterilization processes can be qualified for parametric release: moist heat, dry heat, ethylene oxide and ionizing radiation. Products submitted to terminal sterilization represent the lowest risk category among sterile pharmaceutical products. Contrary to sterile products obtained by aseptic production in controlled environments, products submitted to terminal sterilization present a measurable sterility assurance level.

Sterile products obtained by terminal sterilization must meet a sterility assurance level of 10^{-6} , that is, no more than one contaminated unit in one million units produced. The appropriate application of methods used for development of terminal process requires vast scientific knowledge of the sterilization method selected, in three categories, for use with a specific product:

- a) process based on microbial burden (bioburden);
- b) combined process: biological indicator and bioburden;
- c) overkill process.

The process based on bioburden requires vast knowledge of the product microbial burden. It must be observed that different procedures for establishing the dose in the process of sterilization by radiation use the knowledge on the product microbial burden and its resistance to radiation. This method also requires a sterility assurance level of at least 10^{-6} . The method based on determination of bioburden needs the development of critical process control points concerning the product microbial burden. Risk analysis procedures, such as Hazard Analysis and Critical Control Point (HACCP), are useful to establish manufacturing control conditions and appropriate parameters of control in process.

For products that allow microbial burden survival, more controlled production environments and more precise process controls are necessary. This process is more indicated for clean products, with reduced level of microbial burden and low frequency of spore-forming microorganisms. This process may also be useful for products that may suffer changes when submitted to more drastic sterilization processes.

The combined process that uses biological indicator and bioburden is usually employed for products that may lose attributes when using overkill process and when a sterilization process that demonstrates the inactivation of high numbers of microorganisms from biological indicators, recognized as more resistant to the sterilization process, is desired. This process requires knowledge of the product microbial burden and data related to its resistance to the sterilization process. The relative resistance

of the biological indicator selected must be established by inoculation of microbial spores in the product. Usually, biological indicators with 10^6 spores and D value higher than one minute are employed. Fractional cycles are used to determine the relative resistance (D value) between the product inoculated with microorganisms from the biological indicator and those frequently found on the microbial burden. This process is usually employed for development of cycles for sterilization of parenteral products using terminal sterilization and sterilization of correlates by ethylene oxide.

The overkill process is used when the product to be sterilized is not harmfully influenced by the sterilizing agent or sterilization process conditions. When employing this process, it is important to know the product microbial burden and the prevalence of spore-forming microorganisms. In this case, data on microbial burden do not need to be thorough like for the other two processes (bioburden and biological indicator/bioburden). Usually, biological indicators are resistant to the process. Overkill is demonstrated by the log reduction of spores from the biological indicator, calibrated in a process that allows obtaining minimum F_0 of 12 minutes.

VALIDATION OF THE STERILIZATION PROCESS

The parametric release requires the sterilization process selected to be developed and constantly validated, for inactivation of the microbial burden and for achieving a sterility assurance level of 10^{-6} . The validation of most sterilization processes includes the validation of physical parameters and the microbiological effectiveness through the use of biological indicators to demonstrate a reasonable correlation between the lethality obtained through physical measurements (F_0) and the biological lethality determined with the use of biological indicators.

Once the efficacy of the terminal sterilization process defined due to the bioburden is associated to the number and resistance of microorganisms in the product, one of the components of parametric release is the active microbiological control program to monitor the count and resistance of the product microbial burden. The control of microbial burden and its enumeration is not a crucial factor when the overkill method is employed, because, in general, the overkill method does not require extensive assessment of the microbial burden throughout the process and requires less control in production environment process.

8.2 STATISTIC PROCEDURES

APPLICABLE TO BIOLOGICAL ASSAYS

GLOSSARY OF SYMBOLS

<i>Symbol</i>	<i>Definition</i>
$a_1 \dots z_1$	doses of assayed preparations (samples) A...Z.
a	statistic significance of a result or estimated measure of the degree where this result is "true"
b_0	intersection of responses (y) over log doses (x) in the regression line.
b, b_1	estimated slope of the response regression line (y) in relation to the dose logarithm (x).
bl	number of blocks (animals) in a crossover trial.
c'	constant used in the assessment of confidence limits (Table 15).
d	number of levels of doses for each preparation in a balanced assay.
f	number of differences in paired responses between standard and samples, on assays conducted by 5 x 1 design.
gl	degrees of freedom.
h	number of preparations in an assay, including the standard preparation.
h'	number of samples tested.
k	number of different treatments in an assay $k = dh$.
k'	number of potency logs in assays conducted by the 5 x 1 design, for the same sample.
n	number of replicas for each treatment.
n'	number of individual potency estimates.
n''	degrees of freedom used to estimate the s^2_M variance on the 5 x 1 assay
p	probability
$p_1 p_2 p_3$	lowest, average and highest doses of the standard preparation P; in assays with only two levels of doses, p_2 represents the highest dose.
r	Pearson correlation coefficient
s^2	estimate of variance provided by the average square of error in the variance analysis. Also used as an index letter; for example, s^2 represents the M log potency variance.
s	estimate of standard variation, that is, the square root of s^2 .
t	Student statistic (Table 3).
t'	Dunnett statistic (Table 12).
v	variance for heterogeneity between assays.
w	weighting coefficient.
x	log dose – also used with index to indicate a particular preparation.
$\bar{x}$	average of log doses.
y	individual response or transformed individual response.
y'	calculated response to replace a missing value.
$\bar{y}_P \dots \bar{y}_Z$	average of responses for standard and sample preparations.
A...Z	samples tested.
$A_1 A_2 A_3$	sum of responses for the lowest, average and highest doses of sample A. For an assay with two levels of doses, A_2 represents the response to the highest dose. Similarly for other samples tested.

Symbol	Definition
$B_1 \dots B_{2n}$	sum of responses for each subject (1 to 2n) in double crossover trial.
B'	incomplete total of responses in row or block that has a missing value.
C	statistics used in the calculation of confidence limits (Formula 14).
$C_1 \dots C_n$	sum of responses in each column (1 to n) in Latin square design.
C'	incomplete sum of responses in a Latin square design column with a missing value.
CV	coefficient of variation.
X^2	statistics constant from Table 18 .
X^2_M	statistics constant to test the homogeneity of individual log potency estimates.
E	sum of squares for regression (Table 10).
F	ratio of two estimates of independent variances (Tables 4 and 5).
F_I, F_{II}	sum of responses on phase I or phase II in a crossover trial.
$F_1 \dots F_n$	sum of responses in each one of the row 1 to n in Latin square design or in each block from a randomized block design.
G_1, G_2, G_3	statistics used in the outlier values test.
G'	incomplete total of responses in an assay with exclusion of missing value.
I	interval between adjacent log doses, in the parallel straight assay.
K	correction term used in the analysis of variance $K = (\sum y)^2/N$.
L	Confidence interval in logarithms.
L_C	confidence interval in logarithms for semi-weighted average.
$L_p \dots L_z$	linear contrasts for standard and sample preparations.
M	estimative of log potency or log potency ratio used with an index letter in a multiplex assay, to denote a particular preparation ($M = \log R$).
M_i, M_s	confidence limits of the log potency estimate.
M	average of several independent estimates from M .
M'	estimate of log potency from sample A or log potency ratio before correcting by the supposed potency ($M' = \log R'$).
M'_s, M'_i	upper and lower limits of the log potency estimate, before correcting with the supposed estimate.
N	total number of responses from assay.
N_P, N_A	total number of responses for preparations P and A .
P	standard preparation.
P	sum of responses for standard preparation.
P_1, P_2, P_3	sum of responses for lowest, average and highest doses of standard preparation P . For assays on only two levels of dosage, P_2 represents the responses for the highest dose.
Q	sum of squares for linearity in the same direction (Table 10).
QM	sum of squares due to a source of variation divided by the respective degree of freedom.
$Q_p \dots Q_z$	quadratic contrast for standard and sample preparations (Table 9).
R	estimated potency of the sample.
R_i, R_s	upper and lower confidence limits of the estimated potency.
R'	estimated ratio of potencies before the correction by the assumed potency.
$R+$	specific constant to test atypical values (Table 2).
S_A	assumed potency for sample A , when the doses are prepared.
S_Q	sum of squares due to a source of variation.
T'	incomplete total of responses to a treatment excluding the missing value.
$V = 1/W$	variance of the individual log potency.

Symbol	Definition
X	differences in paired responses between sample and standard, divided by the regression coefficient (b1), in the 5 x 1 design.
W	statistical weighting used in the combination of several independent estimates of log potency.
W'	semi-weighting of each log potency in a series of assays.
χ^2	chi-square statistics (Table 18).

Note: Tables 1 to 20 are available on section **8.9 STATISTICAL TABLES**. Tables 21 to 47 are available on section **8.10 EXAMPLES OF STATISTICAL TESTS**.

FUNDAMENTS

BIOLOGICAL ASSAYS

Procedures designed to assess the potency of active ingredients contained in raw materials and pharmacopoeial preparations, using biological reagents such as microorganisms, animals, fluids and isolated organs from animals. The characteristic of biological reagents is their variability. While physicochemical reagents may be defined and standardized to provide identical results in all laboratories, it is impossible to completely define the biological reagents, despite efforts from international entities in this sense. This variability inherent to biological reagents makes it mandatory to: 1) employ adequate reference standards to obtain relative potencies, and 2) employ statistical methods for experimental designs and analysis of results.

EXPERIMENTAL DESIGNS

The design of an assay includes: a) selection of the set of standard doses (*P*) and samples from the unknown (*A*) that will be tested; b) specification of experimental units (animals, microorganisms, antisera, blood, etc.); c) rules by which the doses will be distributed to the experimental units; d) specifications on measures or other registrations that must be carried out in each experimental unit. The best experimental design is the one that produces the information desired with the most efficiency. Due to practical difficulties, it may be impossible to achieve this goal. Therefore, for each assay different experimental designs can be used, according to the availability of personnel, reagents and time. All designs that provide valid assays with adequate precision as result are scientifically acceptable. Additionally, they must include a system that ensures random distribution of experimental units for the different doses used.

CHANCE AND BIAS

The distribution must be made randomly using a device employed in games of chance or a random number table. It is worth highlighting that this procedure does not eliminate all biases. For example, due to chance, animals with more weight may be targeted to a certain dose, and this difference in weights will bias the results. Therefore, a balance must be created, that is, animals must be classified by weight range and those with the same weight must be distributed randomly for all doses and preparations (standard and sample).

STATISTICAL ANALYSIS

It is the mathematical procedure applied to experimental results with the goal of estimating the sample potency and assessing the validity and precision of the assay. The analysis methods are related to the experimental designs used.

RESULTS

Express the results from the biological evaluation as an estimate of the assumed potency for a sample (R), which will be the expression of the true relative potency of the sample in relation to the standard (ρ). The latter is impossible to be calculated accurately due to the variability of biological reagents. Such estimate of the assumed potency (R) must be followed by the lower and upper confidence limits (R_i , R_s), or interval that encompasses the true relative potency of the sample (ρ). The monographs establish specifications for the acceptable amplitude of these intervals in relation to the estimated potency. These specifications take into account the difficulty of the methods and the practical need to estimate the true potency with certain precision. To achieve the confidence limits specified, it sometimes is necessary to conduct more than one assay. To obtain an estimate of potency with reduced confidence interval, the results from these independent assays must be combined statistically.

The probability, which measures the degree of confidence that the potency is out of the upper and lower confidence limits, is given by the statistical significance (α) of a result or estimated measure of the degree in which such result is “true”. The level of significance most used in biological assays is 5% ($\alpha = 0.05$) or 1% ($\alpha = 0.01$). In cases not explicitly specified, it will be understood that the level of significance used in the calculation of limits is $\alpha = 0.05$.

The calculation procedures are planned for assay in single sample. If several samples are tested simultaneously, employ the modifications described in this volume.

ATYPICAL VALUES

All responses obtained without strictly complying with the protocol pre-established must be eliminated. When apparently atypical values are observed after the responses are registered, the decision to maintain or eliminate them must be based on statistical criteria, like the ones described below:

Criterion based on variation inside a single group of supposedly equivalent responses

In average, for relatively few identical responses inside the group, valid observations will be disregarded in 2% or 4% of the tests. Starting with the supposedly atypical value, indicate responses in order of magnitude from y_1 to y_n , where n represents the number of observations in the group or replicas of the same treatment. Calculate:

$$\begin{aligned} G_1 &= (y_2 - y_1)/(y_n - y_1), \text{ when } n = 3 \text{ to } 7 \\ G_2 &= (y_3 - y_1)/(y_{n-1} - y_1), \text{ when } n = 8 \text{ to } 13 \text{ or} \\ G_3 &= (y_3 - y_1)/(y_{n-2} - y_1), \text{ when } n = 14 \text{ to } 24 \end{aligned}$$

If G_1 , G_2 or G_3 exceeds the critical value registered on **Table 1** for the corresponding value of n , there is a statistical base for elimination of the suspicious value.

Criterion that includes the amplitude of a series $K = 2$ or more groups of equal size

The groups may receive different treatments, but all n responses inside each group result from the same treatment. In this test, the variation of values for each treatment, obtained by the difference between the highest and the lowest values, is studied. The value obtained with greater difference must be divided by the sum of all differences and must not exceed the listed value of (R+) on **Table 2** for k = number of doses and n = number of replicas. If the value calculated exceeds the value listed, the

suspicious column must be investigating to detect the discrepant value. If k is lower than or equal to 10, use the values presented on **Table 2**; if it is higher, multiply R_+ by $(k + 2)$ and interpolate, if necessary, between the values presented on **Table 2a**.

If R_+ exceeds the listed or interpolated value, the group with highest interval is suspicious ($\alpha = 0.05$) and the observation of its data will allow identifying the value that is then considered atypical. The procedure can be repeated with the other intervals if there an atypical value is suspected in a second group.

DIRECT ASSAYS

The doses from each preparation (sample and standard) necessary to produce pre-determined responses in each experimental unit from two equivalent groups of animals or other biological reagents are measured directly. A typical example is the biological assays of a fingerprint. Prepare the standard and sample solutions so that they have approximately the same potency, taking into account the declared activity of the sample or the activity estimated in previous tests (S_A). Transform each result (effective dose) into logarithms (x) and calculate the average values of the logarithms from effective doses for the standard ($\bar{x}_P$) and the sample ($\bar{x}_A$). Calculate the relative potency of the sample (R'), before adjusting by the assumed potency, such as the M' antilog, where:

$$M' = \bar{X}_P - \bar{X}_A \quad (1)$$

Calculate the variance of M' as the sum of variances from the two averages, from the equation

$$S_{M'}^2 = S_x^2 \left(\frac{1}{N_P} + \frac{1}{N_A} \right) \quad (2)$$

where

$$S_x^2 = \frac{[\sum_P x_P^2 - (\sum_P x_P)^2 / N_P] + [\sum_A x_A^2 - (\sum_A x_A)^2 / N_A]}{N_P + N_A - 2} \quad (3)$$

N_P and N_A are numbers of animals treated as standard and sample; $\sum_P$ e $\sum_A$ representam sum of results calculated for the two preparations. Calculate the confidence limits as:

$$\frac{R'_s}{R'_i} = \text{antilog} (M' \pm t_{SM'}) \quad (4)$$

Obtain the appropriate value of t on **Table 3**, according to the degrees of freedom (gl) provided by the equation denominator (3).

Calculate the relative potency of the sample and the confidence limits, taking into account the assumed potency of the sample (S_A) used to prepare the dilutions:

$$R = \text{antilog} M \quad (5)$$

where

$$M = M' + \log S_A \quad (6)$$

with confidence limits

$$\frac{R'_s}{R'_i} = \text{antilog} (M' \pm t_{SM'}) \quad (7)$$

In this assay, S_M equals $S_{M'}$.

For the assay to be valid, the variance of x_P must be the same as x_A , only differing by sampling errors. To test, calculate the variances and divided the highest by the lowest. This way, a relation of variances (F) is obtained.

Calculate the variance of x_P as follows:

$$S_{x_P}^2 = \frac{\sum_P x_P^2 - (\sum_P x_P)^2 / N_P}{N_P - 1} \quad (8)$$

Calculate in an analog manner $S_{x_P}^2$ (8a)

The distribution of ratio of variances (F) is available on **Tables 4** and **5**, but for this test the values on **Table 4** correspond to the significance levels $\alpha = 0.05$ and the ones on **Table 5** to $\alpha = 0.01$. The F value of the assay must not exceed the value on the table, corresponding to the degrees of freedom of the numerator and denominator with which F was obtained. The degrees of freedom are the ones of the denominators from variances of equations (8) and (8a).

QUANTITATIVE INDIRECT ASSAYS

NATURE AND VALIDITY

In general, it is not possible to directly measure the effective dose. For this reason, the potency is determined indirectly, comparing the responses produced in quantitative scale, such as weight, for example, by known doses of standard with the ones produced by one or more doses of sample.

In a restricted interval of doses, the responses or their convenient transformation (logarithm, probit, etc.) present a linear relation with the logarithm from corresponding doses. Use two or more levels of doses of standard or, preferably, of standard and sample to determine the position and straight line slope. Proceed the same way in each assay, because, depending on the sensitivity of the biological reagents used, both the position and the straight line slope may vary.

Each treatment consists of a fixed dose of standard (p_1, p_2, p_3 , etc.) or of sample (a_1, a_2, a_3 , etc.) and is administered to a certain number (n) of experimental units (animals, organs, cultures, tubes, etc.). Register n responses, that is, one for each experimental unit. For the methods presented in this chapter to be valid, the following conditions must be met:

- 1) the experimental units corresponding to each treatment must be selected randomly;
- 2) for each treatment, the responses or their transformations used in the calculation (y) constitute a sample of normal distribution;
- 3) the standard deviation of the response or its transformation is independent from the response level, that is, it is equal for all treatments, only differing by sampling errors;
- 4) the response, or its transformation used in calculations (y), has linear relation with the logarithm from dose (x) in the interval of doses used;
- 5) the straight line corresponding to one or more samples must be parallel to the one from the standard.

From preliminary studies on the assay method, it is possible to suppose that the conditions 2 and 3 are met. With the results from each assay, it is possible to test the conditions 4 and 5. The condition 4 (linearity) can only be verified in assays that apply at least three dilutions from each preparation. When an assay is conducted with only two dilutions, it is presumed that the system linearity was established in advance. The condition 5 (parallelism) must be tested in each assay. Less than two dilutions from each preparation must never be used in it.

If any of the conditions from 1 to 5 is not met, the calculation methods described in this chapter cannot be applied and it is necessary to conduct studies to establish the conditions recommended.

It is convenient that the sample is tested with doses which responses are approximately equal to the ones obtained with the corresponding doses of the standard. This increases the precision of the result. Name the assumed potency for the sample S_A .

EXPRESSION OF POTENCY AND RESTRICTIONS

When the corresponding validity tests are conducted and the results are satisfactory, it is possible to express the relative potency of each sample in relation to the standard with a potency ratio or convert into appropriate units for each sample, for example, international units, national units, weight units, etc. The confidence limits can also be calculated from the set of data obtained in the assay.

To simplify the calculations from the statistical analysis presented in this chapter, it is necessary to impose the following limits to the assay design:

- a) test each preparation, standard and sample with the same number of dilutions. Formulas are presented for pharmacopoeial assays, using two and three levels of doses for each preparation, as well as the 5 x 1 design;
- b) keep constant in each assay the ratio of consecutive doses for all treatments and
- c) obtain the same number of responses for each treatment. If any response is missing, it may be estimated by methods suitable to each design presented in this chapter; if there is loss of a treatment, follow the specification on the section *Partially balanced assays*.

TYPES OF DESIGN

Random

When experimental units are, in their totality, reasonably homogeneous and there is no indication that the variability of response may be smaller in certain subgroups, proceed to the distribution of experimental units for different treatments randomly.

If there is the possibility that some subgroups, such as layers, positions on shelves or experiment days, are more homogeneous than the totality of units, the accuracy of the assay may be increased by introducing one or more restrictions to the experimental design.

Randomized blocks

It allows to segregate a source of variation, such as the sensitivity of different animal litters or the variation between Petri dishes in the microbiological assay by diffusion. This planning makes each treatment be applied once in each block (litter, plate, etc.) and can only be executed when the block is sufficiently large to accommodate all treatments.

Crossover

Use this planning when the experiment can be adjusted in blocks. However, it is only possible to apply two treatments per block. For example, a block can be an animal that can be tested in two different occasions. The goal is to increase the precision, eliminating the influence from the variation of animals, at the same time the effects from any difference between the overall response levels, in the two steps of the assay, are balanced. Denominate as *double crossover* the assay with two doses of standard and sample and as *triple crossover* the one with three doses of each preparation. Proceed to the assay in two phases according to the period of time defined in the method. Distribute the animals in four or six groups and make a treatment in each group on the first phase. On the second phase, the animals that received a preparation will receive another; the animals that received lower doses will receive higher doses in this step. Follow the scheme on **Table 6**.

Latin square

Adequate when the response can be affected by two sources of variation, and each one may have different k levels. For example, the experiment is conducted in k different day and by k testers, or an antibiotic assay by diffusion on plate is conducted, where the treatments can be applied on a $k \times k$ scheme, where each treatment occurs only once in each row and each column. Use only when the number of columns, rows and treatments are equal.

The responses are registered in the form of a square named Latin. There are many possibilities of Latin squares found in specialized literature. From one square other squares can be made, alternating randomly rows and/or columns. On **Table 7** there is an example of Latin square with two doses of standard and of sample.

For any design, the distribution of experimental units in the blocks must be done randomly by procedure, with the units being maintained as seamlessly as possible before and during the experiment.

ANALYSIS OF VARIANCE

When conducting this analysis, the goal is to study the validity of the assay and calculate the residual error. Except for the calculation of residual error, the analysis of data from an assay is identical for random, randomized blocks and Latin square designs. The formulas for analysis of each type of assay are described below. Please refer to the glossary of symbols. The formulas are appropriated for the case where a single sample (A) is compared against the reference standard (P), and also for the case of multiple assays where $h-1$ samples are included ($A...Z$). The formulas for crossover assays do not fit the general scheme and will be presented separately.

If necessary, transform the responses (y) to meet the validity conditions described. Sum all y values for each treatment and for each preparation, as seen on **Tables 8** and **9**. From these data, obtain the linear contrasts related to the dose-response line slopes.

When three doses of each preparation are tested, quadratic contrasts that represent the line curvatures are also obtained. See the formulas on **Tables 8** and **9**.

The total variation of responses resulting from different treatments can be as displayed on **Table 10**. The sums of squares are obtained from values on **Tables 8** or **9**. K represents the square of the sum of all responses obtained on the assay divided by their total number:

$$K = \{(\sum y)^2/N\}$$

Calculate the residual error from the assay by subtracting the controlled variations in the design from the total variation in responses (**Table 11**). In this table, Σy^2 represents the sum of squares from all responses registered in the assay. It is worth highlighting that the sum of squares, reduced, corresponding to the item Treatments is equal to the sum of reduced square sums (**Table 10**) and that, for Latin square, the number of responses replicated (n) is equal to the number of rows, columns or treatments (k).

TESTS OF VALIDITY

To test the significance of the sources of variation listed on **Table 10**, each reduced sum of square obtained on the table must be divided by the corresponding degree of freedom to obtain the average square. The average square of residual error (s^2) is a similar quotient, obtained from the appropriated row on **Table 11**.

To obtain the ratio known as F, divide the average square from each source of variation to be tested by the variance (s^2). Calculate the significance of each source and compare with the values listed (**Tables 4 and 5**) to the significance level of 5% ($\alpha = 0.05$) and 1% ($\alpha = 0.01$). The F values are obtained in the column corresponding to the number of degrees of freedom associated to the average square of the source tested (gl_1) and on the table row corresponding to the number of degrees of freedom associated with s^2 (gl_2). If the F value calculated is higher than the value listed, the source of variation tested is considered “significant” for the level of probability used.

Consider the assays “statistically valid” if the tests present the following results:

Parallel line design

- 1) **Significant regression**, that is, F calculated is higher than the one listed at the significance level of 1% ($\alpha = 0.01$). It indicates that the dose-response row is satisfactory;
- 2) **Non-significant quadratic terms**, that is, the F values calculated must be smaller than the ones listed at the significance level of 5% ($\alpha = 0.05$). It is equivalent to meeting the linearity condition of the relation between the transformation of the response used and the log dose;
- 3) **Non-significant parallelism**, that is, the F calculated must be lower than the value listed at the significance level of 5% ($\alpha = 0.05$) indicating that the straight lines from the sample and standard are parallels. If several samples are being tested simultaneously, and a significant deviation of parallelism is obtained, this may occur due to the use of a preparation that provided a dose-response row with a different slope in relation to the other samples. In this case, use the value of t' for each A...Z preparation, using the equation

$$t' = \frac{L_P - L_A}{2s\sqrt{n}} \quad (9)$$

Each t' calculated must be compared with the value from **Table 12**, where $gl_1 = h - 1$ and gl_2 equals the number of degrees of freedom associated with s^2 . If a “significant” value of t for a sample is found, all data related to this preparation must be eliminated from the assay and the analysis must be repeated from the beginning.

In assays with very large residual error, a “significant” F ratio for the term Preparations may indicate that the assumed potency that was the base for preparation of dilutions was not correct. This is not a condition for invalidity. When this conclusion is reached, the potency estimated on the assay can be used as assumed potency in future assays.

On parallelism and quadratic assays, very low F values, lower than 1, may occur randomly. If this occurs repeatedly, it may be an indication that the assumed conditions were not met, which must be investigated more deeply.

5 x 1 design

The validity is established when:

- 1) **Significant regression**, that is, F calculated must be higher than the value listed for the significance level $\alpha = 0.01$. It indicates that the dose-response row is satisfactory;
- 2) **Non-significant deviation from linearity**. The relation between both variables (log dose and zone of inhibition) must be linear. The F value calculated must be smaller than the one listed for significance level $\alpha = 0.05$. Another calculation to be made is the Pearson correlation coefficient (r), which must be higher than 0.98;
- 3) **Coefficient of variation (CV)** lower than 5% is appropriate. The variability of response in the calibration curve must be constant.

In the case of *crossover assays*, with a special calculation scheme, the formulas to be used are available on **Tables 13** and **14**.

There are three terms of interactions due to replicas within each group: Phases X Preparations, Phases X Regression, and Phases X Parallelism.

Like in designs discussed previously, each reduced sum of squares must be divided by the corresponding number of degrees of freedom to obtain the average squares.

In the case of double crossover design, two average squares are obtained corresponding to errors I and II, named s^2_{I} and s^2_{II} . Divide the average square of each source of variation by the appropriate s^2 to obtain the F ratio.

For the sources Parallelism, Phases X Preparations, Phases X Regression, s^2_{I} is used. For the other sources, s^2_{II} is used.

Calculate the significance of the source using the **Tables 4** and **5**. If F calculated is higher than the value listed, for the degrees of freedom of the source tested (gl_1) and the corresponding s^2 (gl_2), the source of variation is considered “significant” for the significance level used ($\alpha = 0.05$ or $\alpha = 0.01$).

For the assay to be valid, the *regression* must be significant and the *parallelism and three interactions* must not be significant. In the crossover assay, the parallelism test is not very sensitive, because it depends on the variation between blocks (animals).

With the statistical validity of the assays done with any design being established, calculate the potency and the confidence limits by the methods described below.

ESTIMATED POTENCY AND CONFIDENCE LIMITS

Calculations for parallel line design (3 x 3 or 2 x 2)

Calculate first the average response for each preparation ($\bar{y}_P, \bar{y}_A, \dots, \bar{y}_Z$)

$$\bar{y}_P = \frac{P}{N_P} \quad (10)$$

and, in an analog manner, for other preparations.

Naming I the interval in log of the concentrations, for each preparation, the common slope (b) is obtained on assays with two doses, from the equation

$$b = \frac{L_P + L_A + \dots L_Z}{Inh} \quad (11)$$

For assays with three doses of each preparation, the denominator Inh must be replaced with $2 Inh$.

The log potency ratio from sample A (M'_A), before correcting by the value of S_A , is

$$M'_A = \frac{\bar{y}_A - \bar{y}_P}{b} \quad (12)$$

The potency calculated is the estimate of the true potency from each sample. The confidence limits (with 5% of probability to exclude the true potency or $\alpha = 0.05$) can be calculated as the antilog of the formula

$$\frac{M'_{As}}{M'_{Ai}} = M'_A \pm \frac{ts\sqrt{C}}{b} \sqrt{\frac{1}{N_P} + \frac{1}{N_A} + \frac{(\bar{y}_P - \bar{y}_A)^2}{E - s^2t^2}} \quad (13)$$

where

$$C = \frac{E}{(E - s^2t^2)} \quad (14)$$

Obtain E from **Table 10**. s^2 is the residual error from **Table 11** divided by its degrees of freedom and t is available on **Table 3** according to the degrees of freedom from s^2 .

For balanced assays of two to three doses per preparation, the formula for the limits from equation 13 can be simplified:

$$\frac{M'_{As}}{M'_{Ai}} = M'_A \pm \sqrt{(C - 1)(CM'^2_A + c'I^2)} \quad (15)$$

where c' is the coefficient obtained on **Table 15** and C is the measure of significance of regression. In an assay with a well defined slope, the C value will be very close to the unit.

Calculation for 5 x 1 design

Procedure for building the dose-response curve In the turbidimetric method, measure the turbidity on tubes with liquid medium.

In the agar diffusion method, measure the zones of inhibition for each concentration of standard (P_1 , P_2 , P_3 , P_4 , and P_5) in the four sets of plates. The average of 36 readings of intermediate concentration of standard (P_3) is used to correct the averages from each one of the other concentrations of standard P_1 , P_2 , P_4 , P_5 .

The correction is made as follows: measure the 36 readings of P_3 in all plates and calculate the average. Measure the nine readings of P_3 in the set of plates (3) for the other concentrations (P_1 , P_2 , P_4 and P_5) and calculate the average. Calculate the difference between the total average and the average on the three plates of each concentration, which must be added to the measures of the other concentrations.

Example:

average value of P_3 on the 36 readings: 18.2 mm

average value of P_3 on plates with P_1 : 18.0 mm

value of P_1 on the first reading of the nine plates: 17.3 mm

value corrected on the first point P_1 : $(18.2 - 18.0) + 17.3 = 17.5$ mm

value of P_1 on the second reading of the nine plates: 16.9 mm

value corrected on the second point P_1 : $(18.2 - 18.0) + 16.9 = 17.1$ mm

Build the table with the responses corrected for the respective concentrations (P_1 to P_5) according to **Table 19** and conduct the analysis of variance. With the validity of results confirmed, calculate the differences in paired responses between sample and standard on the central point of the curve by the equation

$$X = \frac{(y_A - y_P)}{b^1} \quad (16)$$

where y_A is one of the responses from the sample among the f repetitions, y_P is the paired response from the standard, and b^1 is the regression coefficient given by **Table 20**.

The log potency ratio is

$$M'_A = \sum X/f \quad (17)$$

where f is the number of differences in the paired responses between the sample and the respective standard.

When a number of assays from the same sample is obtained through the same curve, calculate the coefficient of variation (CV) for the results from samples.

$$CV = \frac{\text{desvio padrão (s)}}{\text{média } (\bar{y}) \text{ de cada amostra}} \times 100 \quad (18)$$

$$\text{where } s = \sqrt{\frac{\sum y^2 - (\sum y)^2 / N}{N - 1}} \quad \text{and } y \text{ is the response from 1 to } N \text{ for the same sample.} \quad (18a)$$

The variance is calculated over the f values of X for the total of samples tested as

$$S_M^2 = \frac{\sum X^2 - \sum (Tx^2/f)}{n''} \quad (19)$$

where $Tx = \sum X$ for a single sample and $n'' = \sum f - h'e h'$ is the number of samples tested.

The logarithm of the confidence interval from each sample is

$$L = \frac{2S_M^t}{\sqrt{k'}} \quad (20)$$

where s is the standard deviation for the total of differences X , t is available on **Table 3** with the degrees of freedom from S_M^2 and k' is the number of differences paired by sample tested.

The confidence limits (with 5% of probability to exclude the true potency) can be calculated with the antilog of the formula

$$\frac{M'_{A_s}}{M'_{A_i}} = M'_A \pm 1/2L \quad (21)$$

Obtain the potency ratio (R_A) and the confidence limits (R_s , R_i) taking the antilogs of the values obtained from formulas 12 and 15 (parallel line design 3 x 3 or 2 x 2) and 17 and 21 (5 x 1 design), after adding $\log S_A$ to both:

$$M'_A = M'_A + \log S_A \quad (22)$$

$$R_A = \text{antilog } M_A \quad (23)$$

$$M'_{A_s} = M'_{A_s} + \log S_A \quad (24)$$

$$M'_{A_i} = M'_{A_i} + \log S_A \quad (25)$$

$$R_{A_s} = \text{antilog } M_{A_s} \quad R_{A_i} = \text{antilog } M_{A_i}$$

Missing values

In balanced assays, the same number of observations is required for each concentration. If any response is missing due to a cause not related to the treatments applied, such as death of an animal or rupture of a test tube, the statistical analysis becomes much more complex. The equilibration may be reestablished in two manners:

1) reduce the number of observations in larger groups until the number of responses is the same for each treatment. If the design is completely random, the average from each larger group can be subtracted, as many times as necessary, or one or more responses from each larger group can be eliminated, by selecting them randomly. For randomized blocks assay, maintain only the full blocks;

2) alternatively, a group, casually smaller, may be recomposed to the original size, when the number of missing responses is not higher than one in any treatment or 5% in the total from the assay. In this case, calculate the missing value replacement. *A degree of freedom is lost in the variance from error s^2 for each value replaced:*

a) if the design is completely random, replace the missing value with the average of the remaining responses from the incomplete group;

b) if the design is of randomized blocks, replace the missing value by applying the formula

$$y' = \frac{nB' + kT' - G'}{(n-1)(k-2)} \quad (26)$$

where B' is the incomplete total of responses in the block that has the missing value, T' is the incomplete total of responses in the treatment that has the missing value, G' is the total sum of responses obtained on the assay. As defined previously, n is the number of blocks and k is the number of treatments or doses;

c) if the assay is based on Latin square design, the missing value (y') is obtained by the equation

$$y' = \frac{k(B' + C' + T') - 2G'}{(k-1)(k-2)} \quad (27)$$

where B' and C' are the sums of responses on rows and columns, respectively, that have the missing value. In this case, $k = n$.

If more than one value is missing, replace, temporarily, with the average from the respective treatment, all empty places, except one. Substitute this place with the value y' , calculated by equation 27. Substitute one by one the values that had been placed, temporarily, with the average until obtaining a stable set of values for all missing responses.

If the number of values replaced is small in relation to the total number of observations in the assay (lower than 5%), the resulting approximation of replacements described and the reduction in the degrees of freedom, equivalent to the number of values replaced, is usually satisfactory. However, the analysis must be interpreted carefully, especially if there is predominance of missing values in a particular treatment or block. The same is valid for the case of missing values in crossover plannings.

Partially balanced assays

If the assumed potency of samples (used to calculate the assay doses) is very different from the actual potency, it is possible that the larger dose provides maximum response or that the smaller dose provides very low or null response. These responses will be out of the linear zone from the log dose-response curve and the validity tests will indicate the curvature and/or "significant" deviation from parallelism.

In this case, the responses to the larger or smaller dose from the sample can be disregarded, calculating a relative potency value from remaining data. This potency can be taken as the supposed potency to select doses of sample for another assay, with the goal of obtaining responses similar to standard and, thus, increase the precision of the result. The equation employed to calculate the potency is:

$$M'_A = \frac{\bar{y}_A - \bar{y}_P}{b} \pm \frac{1}{2} \quad (28)$$

This formula is similar to formula 12, but half of the log dose interval is subtracted when the responses from the lower dose are omitted and the same interval is added when the larger dose is disregarded.

The average responses $\bar{y}_A$ and $\bar{y}_P$ are obtained from the same formulas in totally balanced assays (formula 10), but a modification must be introduced in the calculation of slope (b) according to the assay design.

For *multiple assays*, which would necessarily have *two doses* of each preparation, the linear contrasts ($L_P \dots L_Z$) must be formed excluding L_A (since the responses for 1 or 2 were eliminated, it is not possible to form a contrast L_A). Calculate the slope from the average of values from L divided by $\ln$:

$$b = \frac{L_P + \dots + L_Z}{\ln(h-1)} \quad (29)$$

For simple assays with one sample:

$$b = \frac{L_P}{\ln n} \quad (30)$$

For *multiple assays with three doses* from each preparation, obtain L_A and the other contrasts from **Table 9**. The equation for the slope is:

$$b = \frac{2(L_P + \dots + L_Z) + L_A}{\ln(4h-3)} \quad (31)$$

If there is a *single sample*, the equation is reduced to:

$$b = \frac{2L_P + L_A}{5\ln n} \quad (32)$$

MOVING AVERAGES

In the particular case of *heparin bioassay*, the interval between the dose that allows coagulation and the one that inhibits it is so small that the dose-response curve cannot be determined explicitly. To interpolate the log dose corresponding to 50% of coagulation, both for the standard and for the sample, moving averages are used.

Calculation of potency

Transform in a logarithm the volumes of standard preparation used in five or six tubes that comprise the series, so that two or three tubes present coagulation degrees equal to or lower than 0.5 and two or three tubes have degrees equal to or higher than 0.5.

Build a table correlating the tubes numerated, consecutively, with the degree of coagulation observed.

Denominate x the logarithms of the volumes used and y the corresponding degrees of coagulation. Calculate the paired averages x_i and y_i from tubes 1, 2 and 3; from tubes 2, 3 and 4; and from tubes 3, 4 and 5, and when the series is comprised of six tubes, from tubes 4, 5 and 6, respectively. If for one of these pairs of averages the average degree of coagulation y_i is exactly 0.50, the corresponding x_i is the median of the log volume of standard preparation x_p . If this does not occur, interpolate x_p from the paired values of y_i , x_i and y_{i+1} , x_{i+1} that occur, immediately below and above the degree 0.50, as:

$$x_p = x_i + (y_i - 0.5)(x_{i+1} - x_i)/(y_i - y_{i+1}) \quad (33)$$

From paired data obtained on the sample tubes, calculate in the same manner the median of the log volume x_A . The log potency of the sample is:

$$M_A = x_P + x_A + \log S_A \quad (34)$$

where S_A is the assumed potency of the sample made in the preparation of the corresponding solution from the sample tubes.

Repeat the assay, independently, and calculate the average from two or more M values to obtain $\bar{M}$. If the second determination of M is different from the first one more than 0.05, continue conducting assays until the logarithm of the confidence interval, calculated according to the end of section *Combination of potency estimates*, does not exceed 0.20.

The potency of heparin sodium is:

$$R = \text{antilog } \bar{M}$$

INDIRECT “ALL OR NOTHING” ASSAYS

In some assays, it is not possible or convenient to measure the effect in each experimental unit (for example, animal) in a quantitative scale. In this case, all-or-nothing effects can be measured, such as death or occurrence of a pre-established symptom. The proportion of experimental units that present the symptom comprises the result. These assays are called quantal. This chapter will present an approximate calculation. If there are computing facilities, it is possible to resort to the exact theoretical calculation. It is necessary to register, for each dose, the percentage of animals with positive effect. For example: percentage of mice in convulsion. Transform the percentages in probits, using **Table 16**. Each probit will be considered the value of the transformed response (y). The following method is used when there are no responses equivalent to zero or 100 percentages. In this case, employ full statistical methods of maximum probability (logit or probit). For each y value, it is necessary to obtain a weighting coefficient (w) value on **Table 17**.

The formulas of sums of squares for the validity test are the same used in quantitative indirect assays (**Table 10**), taking $n = 1$, except for the term error (s^2), which has degrees of freedom equal to infinite, and is calculated as:

$$S^2 = \frac{k}{n \sum w} \quad (35)$$

where k = number of treatments, n = number of animals used in each treatment.

Calculate the potency and the confidence limits using the formulas 12 and 25. This approximate method is useful when the assay is designed so that the responses in percentages corresponding to the lower and higher doses are evenly spaced around 50%. If one of the doses tested provides zero or 100% responses, they can be disregarded. In this case, obtain the estimated potency by the methods described on section *Partially balanced assays*.

COMBINATION OF ESTIMATES OF POTENCY

When n' independent assays are conducted for each sample, the results can be combined to obtain an estimated potency with reduced confidence interval that meets the limits established in each monograph. There are several methods to combine repeated assays.

Adopt simplifications, taking into account two aspects:

- correct estimates of log potency (M') with the assumed potency (S_A) before making the combinations ($M = M' + \log S_A$);
- the estimates must be independent, that is, obtained in separate assays.

WEIGHTED AVERAGE POTENCY AND CONFIDENCE LIMITS

Assume that results from n assays were analyzed to provide n' M values with confidence limits (in logarithms) associated to each M value, obtained according to equations 13 to 15 and 22 to 25. For each assay, obtain the log confidence interval (L), subtracting the lower from the upper limit. Also calculate a weight (W) for each M value from equation 36, where t is the same value employed in the calculation of the confidence interval:

$$W = \frac{4t^2}{L^2} \quad (36)$$

For each assay, calculate the product WM and divide its sum by the sum of all weights, to obtain the logarithm of the weighted average potency ($\bar{M}$), according to equation 37:

$$\bar{M} = \sum_{n'} WM / \sum_{n'} W \quad (37)$$

$$\bar{M} = \sum_{n'} WM / \sum_{n'} W$$

The standard error of the average potency ($s_{\bar{M}}$) is the square root of the converse from total weight:

$$S_{\bar{M}} = \sqrt{1 / \sum_{n'} W} \quad (38)$$

$$S_{\bar{M}} = \sqrt{1 / \sum_{n'} W} \quad (38)$$

Calculate the approximate confidence limits ($\alpha = 0.05$), from the antilog of values obtained through formula 39:

$$\bar{M} \pm ts_{\bar{M}} \quad (39)$$

The t value on **Table 3** is obtained, with degrees of freedom equivalent to the sum of the degrees of freedom from the variance of error from individual assays.

This approximate method of combination gives satisfactory results when:

- C is lower than 1.1 for each one of the n' assays;
- the individual estimates of potency comprise a homogeneous set according to the homogeneity test conducted, applying the statistics χ^2 . It is calculated by squaring the difference between each M value in relation to the weighted average ($\bar{M}$), multiplying this square by the corresponding weight (W) and adding the values for all assays:

$$X_M^2 = \sum_{n'} W(M - \bar{M})^2 \quad (40)$$

$$X_M^2 = \sum_{n'} W(M - \bar{M})^2$$

If the X_M^2 value calculated is lower than the corresponding one on **Table 18** for $(n' - 1)$ degrees of freedom, it is considered that there are no elements to suspect on the heterogeneity of potencies. In this case, the average potency and the calculated limits are correct.

If the X_M^2 value is higher than the one on **Table 18**, it is considered that the potencies are heterogeneous, that is, that the dispersion of individual M values is higher than expected, according to the respective confidence limits. In this case, do not apply the formulas 37 and 39, investigate the origin of this heterogeneity and, if considered adequate, calculate $\bar{M}$ using semi-weights W' :

$$W' = 1/(V + v) \quad (41)$$

where

$$V = 1/W = \frac{L^2}{4t^2} \quad (42)$$

and v is the variance of heterogeneity between assays and calculated by the equation:

$$v = \frac{\sum M^2 - (\sum M)^2/n'}{n' - 1} - \frac{\sum V}{n'} \quad (43)$$

When V varies in such a way that the v calculated is a negative number, it is possible to calculate an approximate v , omitting the term after the minus sign on equation 43.

To calculate the semi-weighted average ($\bar{M}$), substitute on equation 37 the W and $\sum W$ values with the respective W' and $\sum W'$ values:

$$\bar{M} = \sum_{n'} (W'M) / \sum_{n'} W' \quad (44)$$

$$M = \sum_{n'} (W'M) / \sum_{n'} W'$$

It is possible to consider this value ($\bar{M}$) near the center of a confidence interval of approximate size L' , which is the square root of:

$$L'^2_c = 4t^2 / \sum W' \quad (45)$$

where t , from **Table 3**, has degrees of freedom equal to the sum of degrees of freedom from the variance of error from n' individual assays.

In the *special case of heparin assay*, all logarithms of potency (M) have the same weight and the confidence interval from log estimated potency $\bar{M}$ is determined as follows:

Calculation of error variance with $n' - 1$ degrees of freedom:

$$S^2 = \left\{ \sum M^2 - (\sum M)^2 / n' \right\} / n' - 1 \quad (46)$$

Determine the confidence limit as logarithms (L):

$$L = 2st/\sqrt{n'} \quad (47)$$

where

$s = \sqrt{s^2}$, t (**Table 3**) with $n' - 1$ degrees of freedom, n = number of individual estimates of potency.

Calculate the confidence limits:

$$M_s = \bar{M} + 1/2 L \quad (48)$$

$$M_i = \bar{M} - 1/2 L \quad (49)$$

$$R_s = \text{antilog } M_s \quad (50)$$

$$R_i = \text{antilog } M_i \quad (51)$$

STATISTICAL TABLES

Table 1 – Table G for atypical values.

<i>n</i>	3	4	5	6	7						
G1	0.976	0.846	0.729	0.644	0.586						
n	8	9	10	11	12	13					
G2	0.780	0.725	0.678	0.678	0.605	0.578					
n	14	15	16	16	18	19	20	21	22	23	24
G3	0.602	0.579	0.559	0.559	0.527	0.514	0.502	0.491	0.481	0.472	0.464

Table 2 – Test for groups with atypical values.

<i>k</i>	<i>Critical R+ value for interval of n observations each, at the significance level $\alpha = 0.05$</i>								
	2	3	4	5	6	7	8	9	10
2	0.962	0.862	0.803	0.764	0.736	0.717	0.702	0.691	0.682
3	0.813	0.667	0.601	0.563	0.539	0.521	0.507	0.498	0.489
4	0.681	0.538	0.479	0.446	0.425	0.410	0.398	0.389	0.382
5	0.581	0.451	0.398	0.369	0.351	0.338	0.328	0.320	0.314
6	0.508	0.389	0.342	0.316	0.300	0.288	0.280	0.273	0.267
7	0.451	0.342	0.300	0.278	0.263	0.253	0.245	0.239	0.234
8	0.407	0.305	0.267	0.248	0.234	0.225	0.218	0.213	0.208
9	0.369	0.276	0.241	0.224	0.211	0.203	0.197	0.192	0.188
10	0.339	0.253	0.220	0.204	0.193	0.185	0.179	0.174	0.172

Table 2a – Test for groups with atypical values.

k	Critical R+ value (k + 2) for interval of n observations each, at the significance level $\alpha = 0.05$								
	2	3	4	5	6	7	8	9	10
10	4.06	3.04	2.65	2.44	2.30	2.21	2.14	2.09	2.05
12	4.06	3.03	2.63	2.42	2.29	2.20	2.13	2.07	2.04
15	4.06	3.02	2.62	2.41	2.28	2.18	2.12	2.06	2.02
20	4.13	3.03	2.62	2.41	2.28	2.18	2.11	2.05	2.01
50	4.26	3.11	2.67	2.44	2.29	2.19	2.11	2.06	2.01

Table 3 – Student's t-distribution.

$gl \backslash \alpha$	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	.05	.02	.01	.001
1	0.158	0.325	0.510	0.727	1.000	1.376	1.963	3.078	6.314	12.706	31.821	63.657	636.619
2	0.142	0.289	0.445	0.617	0.816	1.061	1.386	1.886	2.920	4.303	6.965	9.925	31.598
3	0.137	0.277	0.424	0.584	0.765	0.978	1.250	1.638	2.353	3.182	4.541	5.541	12.924
4	0.134	0.271	0.414	0.569	0.741	0.941	1.190	1.533	2.132	2.776	3.747	4.604	8.610
5	0.132	0.267	0.408	0.559	0.727	0.920	1.156	1.476	2.015	2.571	3.365	4.032	6.869
6	0.131	0.265	0.404	0.553	0.718	0.906	1.134	1.440	1.943	2.447	3.143	3.707	5.959
7	0.130	0.263	0.402	0.549	0.711	0.896	1.119	1.415	1.895	2.365	2.365	3.499	5.408
8	0.130	0.262	0.399	0.546	0.706	0.889	1.108	1.397	1.860	2.896	2.896	3.355	5.041
9	0.129	0.261	0.398	0.543	0.703	0.883	1.100	1.383	1.833	2.821	2.821	3.250	4.781
10	0.129	0.260	0.397	0.542	0.700	0.879	1.093	1.372	1.812	2.764	2.764	3.169	4.587
11	0.129	0.260	0.396	0.540	0.697	0.876	1.088	1.363	1.796	2.718	2.718	3.106	4.437
12	0.128	0.259	0.395	0.539	0.695	0.873	1.083	1.356	1.782	2.681	2.681	3.055	4.318
13	0.128	0.259	0.394	0.538	0.694	0.870	1.079	1.350	1.771	2.650	2.650	3.012	4.221
14	0.128	0.258	0.393	0.537	0.692	0.868	1.076	1.345	1.761	2.624	2.624	2.977	4.140
15	0.128	0.258	0.393	0.536	0.691	0.866	1.074	1.341	1.753	2.602	2.602	2.947	4.073
16	0.128	0.258	0.392	0.535	0.690	0.865	1.071	1.337	1.746	2.583	2.583	2.921	4.015
17	0.128	0.257	0.392	0.534	0.689	0.863	1.069	1.333	1.740	2.567	2.567	2.898	3.965
18	0.127	0.257	0.392	0.534	0.688	0.862	1.067	1.330	1.734	2.552	2.552	2.878	3.922
19	0.127	0.257	0.391	0.533	0.688	0.861	1.066	1.328	1.729	2.539	2.539	2.861	3.883
20	0.127	0.257	0.391	0.533	0.687	0.860	1.064	1.325	1.725	2.528	2.528	2.845	3.850
21	0.127	0.257	0.391	0.532	0.686	0.859	1.063	1.323	1.721	2.518	2.518	2.831	3.819
22	0.127	0.256	0.390	0.532	0.686	0.858	1.061	1.321	1.717	2.508	2.508	2.819	3.792
23	0.127	0.256	0.390	0.532	0.685	0.858	1.060	1.319	1.714	2.500	2.500	2.807	3.767
24	0.127	0.256	0.390	0.531	0.685	0.857	1.059	1.318	1.711	2.492	2.492	2.797	3.745
25	0.127	0.256	0.390	0.531	0.684	0.856	1.058	1.316	1.708	2.485	2.485	2.787	3.726
26	0.127	0.256	0.390	0.531	0.684	0.856	1.058	1.315	1.706	2.479	2.479	2.779	3.707
27	0.127	0.256	0.389	0.531	0.684	0.855	1.057	1.314	1.703	2.473	2.473	2.771	2.690
28	0.127	0.256	0.389	0.530	0.683	0.855	1.056	1.311	1.699	2.462	2.462	2.756	3.674
29	0.127	0.256	0.389	0.530	0.683	0.854	1.055	1.310	1.697	2.457	2.457	2.750	3.659
30	0.127	0.255	0.389	0.530	0.683	0.854	1.055	1.310	1.697	2.457	2.457	2.750	3.646
40	0.126	0.254	0.388	0.529	0.681	0.851	1.050	1.303	1.684	2.423	2.423	2.704	3.551
60	0.126	0.254	0.387	0.527	0.679	0.848	1.046	1.296	1.671	2.390	2.390	2.660	3.460
120	0.126	0.253	0.386	0.526	0.677	0.845	1.041	1.289	1.658	2.358	2.358	2.617	3.373
∞	0.126	0.256	0.385	0.524	0.674	0.842	1.036	1.282	1.645	2.326	2.326	2.576	3.291

gl = degrees of freedom; α = significance level

Table 4 – Fisher F distribution for $\alpha = 0.05$.

<i>gl1 Denominator</i>	<i>gl2 Numerator</i>									
	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>	<i>6</i>	<i>8</i>	<i>12</i>	<i>24</i>	∞
<i>1</i>	161.40	199.50	215.70	224.60	230.20	234.00	238.90	243.90	249.00	254.30
<i>2</i>	18.51	19.00	19.16	19.25	19.30	19.33	19.37	19.41	19.45	19.50
<i>3</i>	10.13	9.55	9.28	9.12	9.01	8.94	8.84	8.74	8.64	8.53
<i>4</i>	7.71	6.94	6.59	6.39	6.26	6.16	6.04	5.91	5.77	5.63
<i>5</i>	6.61	5.79	5.41	5.19	5.05	4.95	4.82	4.68	4.53	4.36
<i>6</i>	5.99	5.14	4.76	4.53	4.39	4.28	4.15	4.00	3.84	3.67
<i>7</i>	5.59	4.74	4.35	4.12	3.97	3.87	3.73	3.57	3.41	3.23
<i>8</i>	5.32	4.46	4.07	3.84	3.69	3.58	3.44	3.28	3.12	2.93
<i>9</i>	5.12	4.26	3.86	3.63	3.48	3.37	3.23	3.07	2.90	2.71
<i>10</i>	4.96	4.10	3.71	3.48	3.33	3.22	3.07	2.91	2.74	2.54
<i>11</i>	4.84	3.98	3.59	3.36	3.20	3.09	2.95	2.79	2.61	2.40
<i>12</i>	4.75	3.88	3.49	3.26	3.11	3.00	2.85	2.69	2.50	2.30
<i>13</i>	4.67	3.80	3.41	3.18	3.02	2.92	2.77	2.60	2.42	2.21
<i>14</i>	4.60	3.74	3.34	3.11	2.96	2.85	2.70	2.53	2.35	2.13
<i>15</i>	4.54	3.68	3.29	3.06	2.90	2.79	2.64	2.48	2.29	2.07
<i>16</i>	4.49	3.63	3.24	3.01	2.85	2.74	2.59	2.42	2.24	2.01
<i>17</i>	4.45	3.59	3.20	2.96	2.81	2.70	2.55	2.38	2.19	1.96
<i>18</i>	4.41	3.55	3.16	2.93	2.77	2.66	2.51	2.34	2.15	1.92
<i>19</i>	4.38	3.52	3.13	2.90	2.74	2.63	2.48	2.31	2.11	1.88
<i>20</i>	4.35	3.49	3.10	2.87	2.71	2.60	2.45	2.28	2.08	1.84
<i>21</i>	4.32	3.47	3.07	2.84	2.68	2.57	2.42	2.25	2.05	1.81
<i>22</i>	4.30	3.44	3.05	2.82	2.66	2.55	2.40	2.23	2.03	1.78
<i>23</i>	4.28	3.42	3.03	2.80	2.64	2.53	2.38	2.20	2.00	1.76
<i>24</i>	4.26	3.40	3.01	2.78	2.62	2.51	2.36	2.18	1.98	1.73
<i>25</i>	4.24	3.38	2.99	2.76	2.60	2.49	2.34	2.16	1.96	1.71
<i>26</i>	4.22	3.37	2.98	2.74	2.59	2.47	2.32	2.15	1.95	1.69
<i>27</i>	4.21	3.35	2.96	2.73	2.57	2.46	2.30	2.13	1.93	1.67
<i>28</i>	4.20	3.34	2.95	2.71	2.56	2.44	2.29	2.12	1.91	1.65
<i>29</i>	4.18	3.33	2.93	2.70	2.54	2.43	2.28	2.10	1.90	1.64
<i>30</i>	4.17	3.32	2.92	2.69	2.53	2.42	2.27	2.09	1.89	1.62
<i>40</i>	4.08	3.23	2.84	2.61	2.45	2.34	2.18	2.00	1.79	1.51
<i>60</i>	4.00	3.15	2.76	2.52	2.37	2.25	2.10	1.92	1.70	1.39
<i>120</i>	3.92	3.07	2.68	2.45	2.29	2.17	2.02	1.83	1.61	1.25
∞	3.84	2.99	2.60	2.37	2.21	2.10	1.94	1.75	1.52	1.00

Table 5 – Fisher F distribution for $\alpha = 0.01$.

<i>gl1 Denominator</i>	<i>gl2 Numerator</i>									
	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>	<i>6</i>	<i>8</i>	<i>12</i>	<i>24</i>	∞
<i>1</i>	4052	4999	5403	5625	5764	5859	5982	6106	6234	6366
<i>2</i>	95.50	99.00	99.17	99.25	99.30	99.33	99.37	99.42	99.46	99.50
<i>3</i>	34.12	30.82	29.46	28.71	28.24	27.91	27.49	27.05	26.60	26.12
<i>4</i>	21.20	18.00	16.69	15.98	15.52	15.21	14.80	14.37	13.93	13.46
<i>5</i>	16.26	13.27	12.06	11.39	10.97	10.67	10.29	9.89	9.47	9.02
<i>6</i>	13.74	10.92	9.78	9.15	8.75	8.47	8.10	7.72	7.31	6.88
<i>7</i>	12.25	9.55	8.45	7.85	7.46	7.19	6.84	6.47	6.07	5.65
<i>8</i>	11.26	8.65	7.59	7.01	6.63	6.37	6.03	5.67	5.28	4.86
<i>9</i>	10.56	8.02	6.99	6.42	6.06	5.80	5.47	5.11	4.73	4.31
<i>10</i>	10.04	7.56	6.55	5.99	5.64	5.39	5.06	4.71	4.33	3.91
<i>11</i>	9.65	7.20	6.22	5.67	5.32	5.07	4.74	4.40	4.02	3.60
<i>12</i>	9.33	6.93	5.95	5.41	5.06	4.82	4.50	4.16	3.78	3.36
<i>13</i>	9.07	6.70	5.74	5.20	4.86	4.62	4.30	3.96	3.59	3.16
<i>14</i>	8.86	6.51	5.56	5.03	4.69	4.46	4.14	3.80	3.43	3.00
<i>15</i>	8.68	6.36	5.42	4.89	4.56	4.32	4.00	3.67	3.29	2.87
<i>16</i>	8.53	6.23	5.29	4.77	4.44	4.20	3.89	3.55	3.18	2.75
<i>17</i>	8.40	6.11	5.18	4.67	4.34	4.10	3.79	3.45	3.08	2.65
<i>18</i>	8.28	6.01	5.09	4.58	4.25	4.01	3.71	3.37	3.00	2.58
<i>19</i>	8.18	5.93	5.01	4.50	4.17	3.94	3.63	3.30	2.92	2.49
<i>20</i>	8.10	5.85	4.94	4.43	4.10	3.87	3.56	3.23	2.86	2.42
<i>21</i>	8.02	5.78	4.87	4.37	4.04	3.81	3.51	3.17	2.80	2.36
<i>22</i>	7.94	5.72	4.82	4.31	3.99	3.76	3.45	3.12	2.75	2.31
<i>23</i>	7.88	5.66	4.76	4.26	3.94	3.71	3.41	3.07	2.70	2.26
<i>24</i>	7.82	5.61	4.72	4.22	3.90	3.67	3.36	3.03	2.66	2.21
<i>25</i>	7.77	5.57	4.68	4.18	3.86	3.63	3.32	2.99	2.62	2.17
<i>26</i>	7.72	5.53	4.64	4.14	3.82	3.59	3.29	2.96	2.58	2.13
<i>27</i>	7.68	5.49	4.60	4.11	3.78	3.56	3.26	2.93	2.56	2.10
<i>28</i>	7.64	5.45	4.57	4.07	3.75	3.53	3.23	2.90	2.52	2.06
<i>29</i>	7.60	5.42	4.54	4.04	3.73	3.50	3.20	2.87	2.49	2.03
<i>30</i>	7.56	5.39	4.51	4.02	3.70	3.47	3.17	2.84	2.47	2.01
<i>40</i>	7.31	5.18	4.31	3.83	3.51	3.29	2.99	2.66	2.29	1.80
<i>60</i>	7.08	4.98	4.13	3.65	3.34	3.12	2.82	2.50	2.12	1.60
<i>120</i>	6.85	4.79	3.95	3.48	3.17	2.96	2.66	2.34	1.95	1.38
∞	6.64	4.60	3.78	3.32	3.02	2.80	2.51	2.18	1.79	1.00

Table 6 – Order of doses in crossover assays.

Group	Double Crossover		Triple Crossover	
	Phase I	Phase II	Phase I	Phase II
1	ρ_1	a_2	ρ_1	a_3
2	ρ_2	a_1	ρ_2	a_2
3	a_1	ρ_1	ρ_3	a_1
4	a_2	ρ_1	a_1	ρ_3
5	-	-	a_2	ρ_2
6	-	-	a_3	ρ_1

Table 7 – Example of order of doses on Latin square.

a_2	ρ_1	a_1	ρ_2
ρ_2	a_1	ρ_1	a_2
a_1	a_2	ρ_2	ρ_1
ρ_1	ρ_2	a_2	a_1

Table 8 – Formulas for assays with two doses of each preparation.

	Standard (P)	1st Sample (A)	Sample h -1 (Z)
Lower dose (sum of responses)	P_1	A_1	Z_1
Higher dose (sum of responses)	P_2	A_2	Z_2
By preparation (sum of responses)	$P_1 + P_2 = P$	$A_1 + A_2 = A$	$Z_1 + Z_2 = Z$
Linear contrast	$P_2 - P_1 = L_P$	$A_2 - A_1 = L_A$	$Z_2 - Z_1 = L_Z$

Table 9 – Formulas for assay with three doses of each preparation.

	Standard (P)	1st Sample (A)	Sample h -1 (Z)
Lower dose (sum of responses)	P_1	A_1	Z_1
Medium dose (sum of responses)	P_2	A_2	Z_2
Higher dose (sum of responses)	P_3	A_3	Z_3
By preparation (sum of responses)	$P_1 + P_2 + P_3 = P$	$A_1 + A_2 + A_3 = A$	$Z_1 + Z_2 + Z_3 = Z$
Linear contrast	$P_3 - P_1 = L_P$	$A_3 - A_1 = L_A$	$Z_3 - Z_1 = L_Z$
Quadratic contrast	$P_1 - 2P_2 + P_3 = Q_P$	$A_1 - 2A_2 + A_3 = Q_A$	$Z_1 - 2Z_2 + Z_3 = Q_Z$

Table 10 – Validity tests (variance analysis).

Source of variation	Degrees of freedom (gl)	Reduced sum of squares	
		Two-dose assay	Three-dose assay
Preparations	h-1	$\frac{P^2 + A^2 + \dots Z^2}{2n} - K$	$\frac{P^2 + A^2 + \dots Z^2}{3n} - K$
Regression	1	$\frac{(L_P + L_A + \dots L_Z)^2}{2nh} = E$	$\frac{(L_P + L_A + \dots L_Z)^2}{2nh} = E$
Parallelism	h-1	$\frac{L^2_P + L^2_A + \dots L^2_Z}{2n}$	$\frac{L^2_P + L^2_A + \dots L^2_Z}{2n} - E$
Quadratic	1	--	$\frac{(Q_P + Q_A + \dots Q_Z)^2}{6nh} = Q$
Difference of Quadratics	h-1	--	$\frac{(Q^2_P + Q^2_A + \dots Q^2_Z)^2}{6n} - Q$

Table 11 – Estimate of residual error.

Source of variation	Degrees of freedom (gl)	Reduced sum of squares		
		Random design	Randomized blocks	Latin square
Treatments	k - 1	$\frac{P^2_1 + P^2_2 + \dots Z^2_d}{n} - K$	$\frac{P^2_1 + P^2_2 + \dots Z^2_d}{n} - K$	$\frac{P^2_1 + P^2_2 + \dots Z^2_d}{n} - K$
Blocks (rows)	n - 1	--	$\frac{F^2_1 + F^2_2 + \dots F^2_n}{k} - K$	$\frac{F^2_1 + F^2_2 + \dots F^2_n}{k} - K$
Blocks (columns)	n - 1	--	--	$\frac{C^2_1 + C^2_2 + \dots C^2_n}{k} - K$
Residual error	By difference	*	*	*
TOTAL	N - 1	$\sum y^2 - K$	$\sum y^2 - K$	$\sum y^2 - K$

* Obtained by subtracting, from the total reduced sum of squares, all other reduced sums of squares calculated for the corresponding design.

Table 12 – t' table for two-tailed comparison between (h-1) samples and one standard for a coefficient of

gl_2	$gl_1 = (h-1) = \text{number of samples (excluding standard)}$								
	1	2	3	4	5	6	7	8	9
5	2.57	3.03	3.29	3.48	3.62	3.73	3.82	3.90	3.97
6	2.45	2.86	3.10	3.26	3.39	3.49	3.57	3.64	3.71
7	2.36	2.75	2.97	3.12	3.24	3.33	3.41	3.47	3.53
8	2.31	2.67	2.88	3.02	3.13	3.22	3.29	3.35	3.41
9	2.26	2.61	2.81	2.95	3.05	3.14	3.20	3.26	3.32
10	2.23	2.57	2.76	2.89	2.99	3.07	3.14	3.19	3.24
11	2.20	2.53	2.72	2.84	2.94	3.02	3.08	3.14	3.19
12	2.18	2.50	2.68	2.81	2.90	2.98	3.04	3.09	3.14
13	2.16	2.48	2.65	2.78	2.87	2.94	3.00	3.06	3.10
14	2.14	2.46	2.63	2.75	2.84	2.91	2.97	3.02	3.07
15	2.13	2.44	2.61	2.73	2.82	2.89	2.95	3.00	3.04
16	2.12	2.42	2.59	2.71	2.80	2.87	2.92	2.97	3.02
17	2.11	2.41	2.58	2.69	2.78	2.85	2.90	2.95	3.00
18	2.10	2.40	2.56	2.68	2.76	2.83	2.89	2.94	2.98
19	2.09	2.39	2.55	2.66	2.75	2.81	2.87	2.92	2.96
20	2.09	2.38	2.54	2.65	2.73	2.80	2.86	2.90	2.95
24	2.06	2.35	2.51	2.61	2.70	2.76	2.81	2.86	2.90
30	2.04	2.32	2.47	2.58	2.66	2.72	2.77	2.82	2.86
40	2.02	2.29	2.44	2.54	2.62	2.68	2.73	2.77	2.81
60	2.00	2.27	2.41	2.51	2.58	2.64	2.69	2.73	2.77
120	1.98	2.24	2.38	2.47	2.55	2.60	2.65	2.69	2.73
∞	1.96	2.21	2.35	2.44	2.51	2.57	2.61	2.65	2.69

Table 13 – Totals and contrasts in assay with double crossover design.

	<i>Standard (P)</i>	<i>Sample (A)</i>	<i>Total</i>
PHASE I			
Lower dose (sum of responses)	P _{1I}	A _{1I}	-
Higher dose (sum of responses)	P _{2I}	A ₂	-
Total	P _I	A _I	P _I + A _I = F _I
PHASE II			
Lower dose (sum of responses)	P _{1II}	A _{1II}	-
Higher dose (sum of responses)	P _{2II}	A _{2II}	-
Total	P _{II}	A _{II}	P _{II} + A _{II} = F _{II}
By preparation (sum of responses)	P	A	$\sum y$
Linear contrast			
PHASE I	P _{2I} - P _{1I} = L _{PI}	A _{2I} - A _{1I} = L _{AI}	L _{PI} + L _{AI} = L _I
PHASE II	P _{2II} - P _{1II} = L _{PII}	A _{2II} - A _{1II} = L _{AII}	L _{PII} + L _{AII} = L _{II}
TOTAL	P ₂ + P ₁ = L _P	A ₂ + A ₁ = L _A	L _P + L _A = $\sum L$

Table 14 – Tests of validity in double crossover assay.

<i>Source of variation</i>	<i>Degrees of freedom (gl)</i>	<i>Reduced sum of squares</i>
Parallelism	1	$\frac{L^2_P + L^2_A}{2n} - E$
Phases X Preparations	1	$\frac{P^2_I + P^2_{II} + A^2_I + A^2_{II}}{n} - K - (\text{Phases} - \text{Preparations})$
Phases X Regression	1	$\frac{L^2_I + L^2_{II}}{2n} - E$
Error I	Difference	Blocks – Parallelism – (Phases X Preparations) – (Phases X Regression)
Blocks (animals)	bl-1	$\frac{B^2_I + B^2_2 + \dots + B^2_{2n}}{2} - K$
Preparations	1	$\frac{P^2 + A^2}{2n} - K$
Regression	1	$\frac{(L_P + L_A)^2}{N} = E$
Phases	1	$\frac{F^2_I + F^2_{II}}{2n} - K$
Phases X Parallelism	1	$\frac{L^2_{PI} + L^2_{PII} + L^2_{AI} + L^2_{AII}}{n} - E - \text{Parallelism} - (\text{Phases X Regression})$
Error II	Difference	Total – Blocks – Preparations – Regression – Phases – (Phases X Parallelism)
TOTAL	N – 1	$\sum y^2 - K$

$$K = (\sum y)^2 / N$$

N = total number of responses

n = total number of replicas per dose including the two phases; bl = number of blocks (animals)

B = sum of the two responses for each block (animal)

Table 15 – Constant used in the formula for the confidence limits

<i>Dose of each preparation (d)</i>	<i>Number of samples tested (h – 1)</i>	<i>c'</i>
2	1	1
	2	3/2
	3	2
	4	5/2
	5	3
3	1	8/3
	2	4
	3	16/3
	4	20/3
	5	8

Table 16 – Probits corresponding to percentage.

%	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
%	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
97	6.88	6.90	6.91	6.93	6.94	6.96	6.98	7.00	7.01	7.03
98	7.05	7.07	7.10	7.12	7.14	7.17	7.20	7.23	7.26	7.29
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

Table 17 – Coefficients of weight for probits (w).

<i>Probits</i>	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
1	0.001	0.001	0.001	0.002	0.002	0.003	0.005	0.006	0.008	0.011
2	0.015	0.019	0.025	0.031	0.040	0.050	0.062	0.076	0.092	0.110
3	0.131	0.154	0.180	0.208	0.238	0.269	0.302	0.336	0.370	0.405
4	0.439	0.471	0.503	0.532	0.558	0.581	0.601	0.616	0.627	0.634
5	0.637	0.634	0.627	0.616	0.601	0.581	0.558	0.532	0.503	0.471
6	0.439	0.405	0.370	0.336	0.302	0.269	0.238	0.208	0.180	0.154
7	0.131	0.110	0.092	0.076	0.062	0.050	0.040	0.031	0.025	0.019
8	0.015	0.011	0.008	0.006	0.005	0.003	0.002	0.002	0.001	0.001

Table 18 – Values of χ^2 ($\alpha = 0.05$).

<i>gl</i>	χ^2	<i>gl</i>	χ^2
1	3.84	9	16.92
2	5.99	10	18.31
3	7.81	11	19.67
4	9.49	12	21.03
5	11.07	13	22.36
6	12.59	14	23.69
7	14.07	15	25.00
8	15.51	20	31.41
		25	37.65

Table 19 – Matrix of responses in the antibiotic assay by 5 x 1 design, after correction.

	<i>Standard</i>					<i>Sample</i>		
	P₁	P₂	P₃	P₄	P₅	A	B	C
Responses	y ₁₁	y ₂₁	y ₃₁	y ₄₁	y ₅₁	A ₃₁	B ₃₁	...
	y ₁₂	y ₂₂	y ₃₂	y ₄₂	y ₅₂	A ₃₂	B ₃₂	...
	y ₁₃	y ₂₃	y ₃₃	y ₄₃	y ₅₃	A ₃₃	B ₃₃	...
	y ₁₄	y ₂₄	y ₃₄	y ₄₄	y ₅₄	A ₃₄	B ₃₄	...
	y ₁₅	y ₂₅	y ₃₅	y ₄₅	y ₅₅	A ₃₅	B ₃₅	...
	y ₁₆	y ₂₆	y ₃₆	y ₄₆	y ₅₆	A ₃₆	B ₃₆	...
	y ₁₇	y ₂₇	y ₃₇	y ₄₇	y ₅₇	A ₃₇	B ₃₇	...
	y ₁₈	y ₂₈	y ₃₈	y ₄₈	y ₅₈	A ₃₈	B ₃₈	...
	y ₁₉	y ₂₉	y ₃₉	y ₄₉	y ₅₉	A ₃₉	B ₃₉	...
Total	y _{1.}	y _{2.}	y _{3.}	y _{4.}	y _{5.}	A _{3.}	B _{3.}	

ynz: n is the concentration (P1 to P5) and z is the response (1 to 9)

Anz: n is the median concentration (P3) and z is the response (1 to 9)

Table 20 – Table of Analysis of variance for the simple linear regression model – 5 x 1 design.

$b_1 = \frac{\sum(x - \bar{x})(y - \bar{y})}{\sum(x - \bar{x})^2}$	$Y = b_0 + b_1x$	$r = \frac{\sum(x - \bar{x})(y - \bar{y})}{(N - 1)\sqrt{\frac{\sum(x - \bar{x})^2}{N - 1}}\sqrt{\frac{\sum(y - \bar{y})^2}{N - 1}}}$
	$b_0 = \bar{y} - b_1\bar{x}$	

<i>Source of variation</i>	<i>gl</i>	<i>Sum of squares</i>	<i>Average square</i>	<i>F calculated</i>
Regression	1	$SQ_{reg} = b_1\sum xy + b_0\sum y - (\sum y)^2/N$	$QM_{reg} = SQ_{reg}$	QM_{reg}/QM_{res}
Residual error	$N - 2$	$SQ_{res} = \sum y^2 - b_1\sum xy - b_0\sum y$		--
Linear deviation	3	$SQ_{dev} = SS_{res} - SQ_{ep}$		QM_{dev}/QM_{ep}
Pure error (ep)	$N - 5$	$SQ_{ep} = \sum y^2 - (\sum yi)^2/k'$		--
Total	$N - 1$	$SQ_{reg} + SQ_{res}$	--	

$$(\sum yi)^2 = (y_{11} + y_{12} + y_{13} + \dots + y_{19})^2 + \dots + (y_{51} + y_{52} + y_{53} + \dots + y_{59})^2$$

$$\sqrt{\frac{(x - \bar{x})^2}{N - 1}} = S_{xx} = \text{standard deviation from variable (x)}$$

$$\sqrt{\frac{(y - \bar{y})^2}{N - 1}} = S_{yy} = \text{standard deviation from variable response (y)}$$

EXAMPLES OF STATISTICAL CALCULATIONS APPLIED ON BIOLOGICAL ASSAYS

DIRECT ASSAY

Example 1: direct assay with a sample.

Direct fingerprint assay by the guinea pig cardiac arrest method. The standard solution was used in the concentration of 0.0658 IU/mL. An equivalent dilution of sample was prepared from the assumed potency of $S_A = 1.3$ IU/100 mg.

The guinea pigs were perfused randomly with standard or sample solution. The precise volume necessary to produce cardiac arrest in each animal was registered.

The responses are listed on **Table 19**.

Calculation of the estimated potency and confidence limits.

From equation 1:

$$M' = 1.3974 - 1.4089 = -0.0115$$

From equation 6:

$$M = -0.0115 + \log 1.3 = 0.1024$$

From equation 5:

$$R = \text{antilog } 0.1024 = 1.2660$$

From equation 3:

$$\begin{aligned} S^2_x &= \frac{1}{22} \left[\left(1,4404^2 + \dots + 1,3304^2 - \frac{19,5641^2}{14} \right) + \left(1,5317^2 + \dots + 1,4072^2 - \frac{14,0890^2}{10} \right) \right] \\ &= \frac{1}{22} [27,3829 - 27,3396 + 19,8879 - 19,8500] \\ &= 0,003691 \end{aligned}$$

From equation 2:

$$S^2_M = 0,003691 \left(\frac{1}{14} + \frac{1}{10} \right) = 0,000632$$

$$S_M = \sqrt{0,000632} = 0,0251$$

For $\alpha = 0.05$ with 22 gl, $t = 2.07$ (**Table 3**)

From equation 7:

$$\frac{R_s}{R_i} = \text{antilog } [0,1024 \pm (2,07 \times 0,0251)]$$

$$R_s = \text{antilog} [0,1024 \pm (2,07 \times 0,0251)] = 1,43$$

$$R_i = \text{antilog} [0,1024 \pm (2,07 \times 0,0251)] = 1,12$$

The average estimate of the potency from the fingerprint sample is 1.27 IU/100 mg.

The confidence limits ($p=0.05$) for the true potency are 1.12 IU/100 mg and 1.43 IU/100 mg.

Table 21 – Example 1: Effective doses to produce cardiac arrest.

<i>Standard P</i>		<i>Sample A</i>	
<i>Lethal dose mL/kg</i>	<i>Log lethal dose X_p</i>	<i>Lethal dose mL/kg</i>	<i>Log lethal dose X_A</i>
27.57	1.4404	34.02	1.5317
25.97	1.4145	21.90	1.3404
27.74	1.4431	28.33	1.4523
30.94	1.4905	24.87	1.3957
28.31	1.4519	27.56	1.4403
27.29	1.4360	24.73	1.3932
22.13	1.3450	21.67	1.3359
23.63	1.3735	21.30	1.3284
21.39	1.3302	29.10	1.4639
22.13	1.3450	25.54	1.4072
20.97	1.3216		
29.23	1.4658		
23.78	1.3762		
21.40	1.3304		
$\sum x$	19.5641		14.0890
$\bar{X}$	1.3974		1.4089
$\sum x^2$	27.3829		19.8879
S^2	0.003331		0.004211
N	14		10

QUANTITATIVE INDIRECT ASSAYS

Example 2: assay with three doses, design completely random.

Human chorionic gonadotropin assay by the method of increased weight of seminal vesicles

The doses used from standard were: $p_1 = 1.0$ IU/mL, $p_2 = 2.0$ IU/mL and p_3 IU/mL. Equivalent doses of the sample were prepared from the assumed potency $S_A = 3000$ IU/mg. The rats were injected subcutaneously with 0.20 mL of the respective solution, for three consecutive days, in a total of 0.6 mL/rat.

The weights of the seminal vesicles are listed on **Table 22**.

Table 22 – Example 2: weights of seminal vesicles (mg).

P_1	P_2	P_3	a_1	a_2	a_3
8.5	12.5	14.8	10.5	16.8	16.7
10.4	13.1	14.1	10.5	14.3	16.9

P_1	P_2	P_3	a_1	a_2	a_3
11.4	8.3	14.9	9.1	14.9	18.8
11.6	13.1	13.8	9.9	12.3	16.7
10.2	9.0	14.6	10.5	15.4	12.7
9.1	14.4	15.2	8.4	14.9	16.2
9.5	11.7	12.3	10.1	12.8	17.3
7.7	11.72*	15.5	10.1	10.0	12.8

* missing value replaced by the average from the treatment.

Table 23 – Example 2: totals and contrasts.

	<i>Standard P</i>	<i>Sample A</i>
Lower dose	P1 = 78.40	A1 = 79.10
Medium dose	P2 = 93.82	A2 = 111.10
Higher dose	P3 = 115.20	A3 = 128.10
Preparation	P = 287.42	A = 318.60
Linear contrast	LP = 36.80	LA = 49.00
Quadratic contrast	QP = 5.96	QA = 15.60

Results obtained with formulas from **Table 9**.

Table 24 – Example 2: analysis of variance.

<i>Source of variation</i>	<i>gl</i>	<i>Sum of squares</i>	<i>Average square</i>	<i>F</i>	<i>A</i>
Preparations	1	20.26	20.26		
Regression	1	230.05	230.05	79.05	<0.01
Parallelism	1	4.65	4.65	1.60	>0.05
Quadratic	1	0.97	0.97	0.33	>0.05
Difference of squares	1	4.84	4.84	1.66	>0.05
Treatments	5	260.11	52.15		
Error	41*	119.18	s ² = 2.91		
TOTAL	47	379.95			

* Removed one degree of freedom because one missing value was substituted.
The sums of squares were obtained by employing the formulas from **Tables 9, 10 and 11**.

$$N = 48$$

$$n = 8$$

$$K = (\sum y)^2 / N = 7\,651.25$$

$$\sum y^2 = 8\,031.21$$

$$\text{Preparações} = \frac{287,42^2 + 318,6^2}{24} - 7651,25 = 20,26$$

$$\text{Regressão} = \frac{(36,8 + 49,0)^2}{32} = 230,05 = E$$

$$\text{Paralelismo} = \frac{36,8^2 + 49,0^2}{16} - 230,05 = 4,65$$

$$\text{Quadrático} = \frac{[5,96 + (-15,6)]^2}{96} = 0,97 = Q$$

$$\text{Diferença de quadráticos} = \frac{5,96^2 + (-15,6)^2}{48} - 0,97 = 4,84$$

$$\text{Tratamentos} = \frac{78,40^2 + 93,83^2 + \dots + 128,10^2}{8} - 7651,25 = 160,77$$

$$\text{Total} = 8\,031,21 - 7\,651,25 = 379,95$$

$$\text{Error} = 379,95 - 260,77 = 119,18$$

Validity of assay:

The assay meets the validity conditions:

- significant regression, F calculated 79.05 is higher than the critical value from **Table 5** for $\alpha = 0.05$, gl1 = 1 and gl2 = 41;
- non-significant deviation from parallelism, F calculated 1.60 is lower than the critical value from Table 4 for $\alpha = 0.05$, gl1 = 1 and gl2 = 41 and
- non-significant deviation from linearity, F = 0.33 and 1.66.

Calculation of the estimated potency and confidence limits: Use formulas 10 to 15.

$$I = \log 2.0 = 0.3010$$

$$t = 2.02 \text{ with } 41 \text{ gl from Table 3}$$

$$b = \frac{36,8 + 49,0}{2 \times 0,301 \times 8 \times 2} = 8,90$$

$$y_A = \frac{318,60}{24} = 13,27$$

$$y_P = \frac{287,42}{24} = 11,97$$

$$\bullet = \frac{13,27 - 11,97}{8,90} = 0,1460$$

$$SA = 3\,000 \quad \log SA = 3.4771$$

$$M = 0.1460 + 3.4771 = 3.6231$$

$$R = \text{anti log } 3.6231 = 4\,198.56 \text{ IU/mg}$$

$$C = \frac{230,05}{230,05 - 2,91(2,02)^2} = 1,05$$

$$C' = 8/3, \text{ from Table 15}$$

$$\frac{M'_s}{M'_i} = 1,05 \times 0,146 \pm \sqrt{(1,05 - 1)[1,05(0,146)^2 + 8/3(0,3010)^2]}$$

$$M'_s = 0,2679$$

$$M'_i = 0,0381$$

Logarithm of confidence limits from potency

$$M'_s = 0,2679 + 3,4771 = 3,7449$$

$$M'_i = 0,0381 + 3,4771 = 3,5151$$

Confidence limits from potency

$$R_s = \text{anti log } 3.7445 = 5552.64 \text{ IU/mg} = \text{anti log } 3.5151 = 3274.16 \text{ IU/mg}$$

Example 3: assay with three doses, randomized blocks design.

Antibiotic assay using Petri dishes

The doses used from standard were: $p_1 = 0.25 \text{ IU/mL}$, $p_2 = 0.50 \text{ IU/mL}$ and $p_3 = 1.00 \text{ IU/mL}$.

Equivalent doses of the sample were prepared based on the assumed potency $S_A = 1\,650 \text{ IU/mg}$. The diameters of the zones of inhibition are available on **Table 25**.

Table 25 – Example 3: diameter of zones of inhibition.

Plates (Blocks)	Standard P			Sample A			Total Block
	p_1	p_2	p_3	a_1	a_2	a_3	
1	17.0	20.4	24.0	17.4	20.7	24.4	123.9
2	14.9	19.7	22.7	14.9	19.3	22.2	113.7
3	15.0	18.6	22.0	15.0	18.0	22.3	110.9
4	14.6	18.3	22.4	14.8	19.0	22.2	111.3
5	14.7	18.0	22.3	14.4	17.8	22.6	109.8
6	14.4	19.1	23.3	14.5	19.3	23.0	113.6
7	14.9	19.0	22.5	15.0	19.4	22.4	113.2

Table 26 – Example 3: totals and contrasts.

	Standard P	Sample A
Lower dose	$P_1 = 105.5$	$A_1 = 106.0$
Medium dose	$P_2 = 133.1$	$A_2 = 133.5$
Higher dose	$P_3 = 159.2$	$A_3 = 159.1$
Preparation	$P = 397.8$	$A = 398.6$
Linear contrast	$L_P = 53.7$	$L_A = 53.1$
Quadratic contrast	$Q_P = -1.5$	$Q_A = -1.9$

Results obtained with formulas from **Table 9**

Table 27 – Example 3: analysis of variance.

<i>Source of variation</i>	<i>gl</i>	<i>Sum of squares</i>	<i>Average square</i>	<i>F</i>	<i>A</i>
Preparations	1	0.0150	0.0150	0.09	>0.05
Regression	1	407.3657	407.3657	2396	<0.01
Parallelism	1	0.0129	0.0129	0.080	>0.05
Quadratic	1	0.1376	0.1376	0.81	>0.05
Difference of squares	1	0.0019	0.0019	0.01	>0.05
Treatments	5	407.53	3020.25		
Plates	6	22.18	3.70	21.8	<0.01
Error	30	4.99	$s^2 = 0.17$		

The sums of squares were obtained by employing the formulas from **Tables 9, 10 and 11**.

$$N = 42$$

$$n = 7$$

$$K = (\Sigma y)^2 / N = 15101.26$$

$$\Sigma y^2 = 15\,535.96$$

$$\text{Preparações} = \frac{397,8^2 + 398,6^2}{21} - 15101,2610 = 0,0152$$

$$\text{Regressão} = \frac{(53,7 + 53,1)^2}{28} = 407,3657 = E$$

$$\text{Paralelismo} = \frac{53,7^2 + 53,1^2}{14} - 407,3657 = 0,0129$$

$$\text{Quadrático} = \frac{[-1,5 + (-1,9)]^2}{84} = 0,1376 = Q$$

$$\text{Diferença de quadráticos} = \frac{-1,5^2 + (-1,9)^2}{42} - 0,1376 = 0,0019$$

$$\text{Tratamentos} = \frac{105,5^2 + 133,1^2 + \dots + 159,1^2}{7} - 15101,261 = 407,53$$

$$\text{Blocos (Placas)} = \frac{123,9^2 + 113,7^2 + \dots + 113,2^2}{6} - 15101,261 = 22,18$$

$$\text{Total} = 15\,535.96 - 15\,101.261 = 434.7$$

$$\text{Error} = 434.7 - 22.18 - 407.53 = 4.99$$

Validity of assay

The assay meets the validity conditions:

- a) significant regression, F calculated 2390 is higher than the critical value from **Table 5** for $\alpha = 0.01$, $gl_1 = 1$ and $gl_2 = 30$;

- b) non-significant deviation from parallelism, F calculated 0.08 is lower than the critical value from **Table 4** for $\alpha = 0.05$, $g/1 = 1$ and $g/2 = 30$, and
- c) non-significant deviation from linearity, F calculated = 0.81 and 0.01.

Calculation of the estimated potency and confidence limits

Use formulas 10 to 15.

$$I = \log 1,00 - \log 0,50 = 0,301 \quad t =$$

$t = 2.04$ with 30 *gl* from **Table 3**.

$$b = \frac{53,7 + 53,1}{28 \times 0,301} = 12,67$$

$$\bar{y}_A = \frac{398,6}{21} = 18,98$$

$$\bar{y}_P = \frac{397,8}{21} = 18,94$$

$$M' = \frac{18,92 - 18,94}{12,672} = 0,003157$$

$$SA = 1650 \text{ IU/mg}$$

$$M = M' + \log 1650 = 0,003157 + 3,217480 = 3,2206$$

$$R = \text{anti log } 3,2206 = 1662$$

$$C = \frac{407,3657}{[407,3657 - 0,17(2,04)^2]} = 1,0017$$

$$C' = 8/3, \text{ from Table 15}$$

$$M'_s = 0,0235$$

$$M'_i = -0,0171$$

Logarithm of confidence limits from potency

$$M_s = 0,0235 + 3,2175 = 3,2410$$

$$M_i = -0,0171 + 3,2175 = 3,2004$$

Confidence limits from potency

$$R_s = \text{anti log } 3,2410 = 1742 \text{ IU/mg}$$

$$R_i = \text{anti log } 3,2004 = 1586 \text{ IU/mg}$$

Example 4: assay with two doses, Latin square design.

Assay of oxytocin – method of contraction of female rat's isolated uterus: The administered doses of standard were: $p_1 = 0.2$ mL and $p_2 = 0.25$ mL of solution with 0.02 IU/mL. Equivalent doses of the sample were prepared based on the assumed potency of 10 IU/mL diluted 1:500.

Table 28 – Example 4: order of addition of doses.

Rows	Columns			
	1	2	3	4
1	p_1	p_2	a_1	a_2
2	p_2	p_1	a_2	a_1
3	a_1	a_2	p_1	p_2
4	a_2	a_1	p_2	p_1

Table 29 – Example 4: records of contractions in mm.

Rows	Columns				Total Rows
	1	2	3	4	
1	38	43	35	40	F1 = 156
2	38	30	44	38	F2 = 150
3	39	45	37	40	F3 = 161
4	45	38	45	37	F4 = 165
Total Column	C1 = 160	C2 = 156	C3 = 161	C4 = 155	
Total of doses	P1 = 142	P2 = 166	A1 = 150	A2 = 174	

Table 30 – Example 4: totals and contrasts.

	Standard P	Sample A
Lower dose	$P_1 = 142$	$A_1 = 150$
Higher dose	$P_2 = 166$	$A_2 = 174$
Preparation	$P = 308$	$A = 324$
Linear contrast	$L_P = 24$	$L_A = 24$

Table 31 – Example 4: analysis of variance

Source of variation	gl	Sum of squares	Average square	F	A
Preparation	1	16.0	16.0	1.65	> 0.05
Regression	1	144.0	144.0	14.89	< 0.01
Parallelism	1	0.0	0.0	0.00	> 0.05
Treatment	3	160.0			
Rows	3	31.5	10.5	1.08	> 0.05
Columns	3	6.5	2.2	0.23	> 0.05
Error	6	58.0	$s^2 = 9.67$		
Total	15	256.0			

The sums of squares were obtained by employing the formulas from **Tables 8, 10** and **11**.

$$N = 16$$

$$n = 4$$

$$K = (\Sigma y)^2 / N = 632^2 / 16 = 24\,964$$

$$\text{Preparações} = \frac{308^2 + 324^2}{8} - 24\,964,0 = 16,0$$

$$\text{Regressão} = \frac{(24 + 24)^2}{2 \times 4 \times 2} = 144,0 = E$$

$$\text{Paralelismo} = \frac{24^2 + 24^2}{2 \times 4} - 144,0 = 0$$

$$\text{Tratamentos} = \frac{142^2 + 166^2 + 150^2 + 174^2}{4} - 24\,964 = 160$$

$$\text{Filas} = \frac{152^2 + 150^2 + 161^2 + 155^2}{4} - 24\,964 = 31,5$$

$$\text{Colunas} = \frac{160^2 + 156^2 + 161^2 + 155^2}{4} - 24\,964 = 6,5$$

$$\text{Total} = 25\,220 - 24\,964 = 256,0$$

$$\text{Error} = 256,0 - 160,0 - 31,5 - 6,5 = 58,0$$

The analysis did not present significant differences ($p > 0.05$) between rows and between columns.

Validity of assay

The assay meets the validity conditions:

- a) significant regression, F calculated 14.9 is higher than the critical value from **Table 5** for $\alpha = 0.01$, $gl_1 = 1$ and $gl_2 = 6$;

Calculation of the estimated potency and confidence limits: Use formulas 10 to 15.

$$I = \log 0.25 - \log 0.20 = 0.0969$$

$t = 2.45$ with 6 *gl* from **Table 3**

$$b = \frac{24 + 24}{0,0969 \times 4 \times 2} = 61,91$$

$$\bar{y}_A = \frac{324}{8} = 61,91$$

$$\bar{y}_P = \frac{308}{8} = 38,5$$

$$M' = \frac{40,5 - 38,5}{61,91} = 0,0323$$

$$S_A = 10 \quad \log S_A = 1$$

$$M = 0.0323 + 1 = 1.0323$$

$$R = \text{anti log } 1.0323 = 10.8 \text{ IU/mL} = \text{Estimated potency}$$

$$C = \frac{144,0}{144,0 - 9,67 \times 2,45^2} = 1,67$$

$$c' = 1, \text{ from Table 15}$$

$$\frac{M'_s}{M'_i} = 1,67 \times 0,0323 \pm \sqrt{(1,67 - 1,0)[1,67(0,0323)^2 + 1(0,09691)^2]}$$

$$M'_s = 0,1402$$

$$M'_i = 0,0324$$

Logarithm of confidence limits from potency

$$M_s = 0.1402 + 1 = 1.1402$$

$$M_i = 0.0324 + 1 = 0.9676$$

Confidence limits from potency

$$R_s = \text{anti log } 1.1402 = 13.81 \text{ IU/mL}$$

$$R_i = \text{anti log } 0.9676 = 9.78 \text{ IU/mL}$$

Example 5: double crossover assay.

Assay of insulin in mice. The doses used from standard were $p_1 = 60 \text{ mIU/mL}$ and $p_2 = 120 \text{ mIU/mL}$. Equivalent doses of the sample were prepared, $a_1 = 60 \text{ mIU/mL}$ and $a_2 = 120 \text{ mIU/mL}$ from the assumed potency $S_A = 27.4 \text{ IU/mL}$. The mice were injected with 0.1 mL of the respective solution for each 10 g of average weight, according to **Table 6**.

The responses are listed on **Table 30**.

Table 32 – Example 5: concentration of blood glucose (mg/100 mL), forty minutes after the injection.

Group 1			Group 2			Group 3			Group 4		
p_1	a_2	total	p_1	a_2	total	p_1	a_2	total	p_1	a_2	Total
37.1	16.6	53.7	32.4	32.4	80.8	36.8	17.0	53.8	30.9	52.1	83.0
35.2	40.1	75.3	35.2	35.2	103.0	53.2	24.9	78.1	27.8	59.4	87.2
43.1	33.9	77.0	35.3	35.3	108.4	71.2	58.2	129.4	35.4	39.1	74.5
41.3.	16.2	57.5	32.9	32.9	78.1	37.1	24.8	61.9	49.8	79.0	128.8
54.2	33.2	87.4	31.9	31.9	65.0	45.9	22.7	68.6	28.2	37.3	65.5
41.4	13.1	54.4	51.2	51.2	113.6	82.2	42.7	124.9	49.9	51.1	101.0
48.6	32.7	81.3	38.2	38.2	114.4	64.8	33.9	98.7	28.3	59.5	87.8

Group 1			Group 2			Group 3			Group 4		
p_1	a_2	total	p_1	a_2	total	p_1	a_2	total	p_1	a_2	Total
57.8	50.4	108.2	39.7	39.7	89.8	49.1	37.6	86.7	39.6	55.8	95.4
71.1	47.3	118.4	37.0	37.0	110.8	44.1	10.4	54.5	32.2	40.6	72.8
60.8	26.1	86.9	38.9	38.9	103.5	64.7	34.7	99.4	55.1	68.2	123.3
78.2	50.9	129.1	42.6	42.6	97.2	88.0	61.6	149.6	40.6	61.4	102.0
76.1	54.4	130.5	30.4	30.4	80.0	90.1	60.3	150.4	43.5	52.8	96.3

Table 33 – Example 5: totals and contrasts.

	Standard P	Sample A	Total
PHASE I			
Lower dose	P11 = 644.9	A11 = 727.2	
Higher dose	P21 = 445.7	A21 = 461.3	
TOTAL	P1 = 1090.6	A1 = 1188.5	F1 = 2279.1
PHASE II			
Lower dose	P1II = 656.3	A1II = 704.9	
Higher dose	P2II = 428.8	A2II = 414.9	
TOTAL	P11 = 1085.1	A11 = 1119.8	F11 = 2204.9
Linear Contrast Preparation	P = 2175.7	A = 2308.3	$\sum y = 4484.0$
PHASE I	LP1 = -199.2	LA1 = -256.9	L1 = -465.1
PHASE II	LP11 = -227.5	LA11 = -290.0	L11 = -517.5
TOTAL	LP = -426.7	LA = -555.9	$\sum L = -982.6$

Results obtained with formulas from **Table 13**

Table 34 – Example 5: analysis of variance.

Source of variation	gl	Sum of squares	Average square	F	A
Parallelism	1	173.88	173.88	0.53	> 0.05
Phases × Preparations	1	41.61	41.61	0.13	> 0.05
Phases × Regressions	1	28.60	28.60	0.09	> 0.05
Error I	44	14 545.64	330.58		
Blocks	47	14 789.73	314.67		
Preparations	1	183.15	183.15	3.01	> 0.05
Regression	1	10 057.32	10 057.32	165.52	< 0.01
Phases	1	57.35	57.35	0.94	> 0.05
Phases × Parallelism	1	0.19	0.19	0.00	> 0.05
Error II	44	2 673.39	60.76		
TOTAL	95	27 761.13			

The sums of squares were obtained by employing the formulas from **Tables 13** and **14**.

$$N = 96$$

$$n = 24$$

$$bl = 48$$

$$K = (\sum y)^2 / N = 4\,484.0^2 / 96 = 209\,440.17$$

$$\sum y^2 = 237\,201.30$$

$$\text{Total} = 237\,201.30 - 209\,440.17 = 27\,761$$

$$\text{Blocks} = \frac{448459,8}{2} - 209440,17 = 14789,73$$

$$\text{Preparation} = \frac{2175,7^2 + 2308,3^2}{48} - 209440,17 = 183,15$$

$$\text{Phase} = \frac{2279,1^2 + 2204,9^2}{48} - 209440,17 = 57,35$$

$$\text{Regression} = \frac{[(-426,7) + (-555,9)]^2}{96} = 10057,32 = E$$

$$\text{Parallelism} = \frac{(-426,7)^2 + (-555,9)^2}{48} = 10057,32 = 173,88$$

$$\text{Phase} \times \text{Regression} = \frac{(-465,1)^2 + (-517,5)^2}{48} - 10057,32 = 28,60$$

$$\text{Phase} \times \text{Parallelism} = \frac{1090,6^2 + 1085,1^2 + 1188,5^2 + 1119,8^2}{42} - 209440,17 - 57,35 - 183,15 = 41,61$$

$$\text{Error I} = 14\,789.73 - 173.88 - 41.61 - 28.60 = 14\,545.64$$

$$\text{Erro II} = 27\,761.13 - 14\,789.73 - 183.15 - 10\,057.32 - 57.35 - 0.19 = 2\,673.39$$

Validity of assay:

The assay meets the validity conditions:

- significant regression, F calculated 165.52 is higher than the critical value from **Table 5**, for $\alpha = 0.01$, $gl1 = 1$ and $gl2 = 44$;
- non-significant parallelism, F calculated 0.53 is lower than the critical value from **Table 4**, for $\alpha = 0.05$, $gl1 = 1$ and $gl2 = 44$.
- None of the three interactions was significant – the F values calculated: 0.13, 0.09 and 0.00 were lower than the critical value from **Table 4** for $\alpha = 0.05$, $gl1 = 1$ and $gl2 = 44$.

Calculation of the estimated potency and confidence limits:

Use formulas 10 to 15.

$$I = \log 120 - \log 60 = 2.0792 - 1.77820.301$$

t = 2.01 with 44 gl from **Table 3**

$$b = \frac{(-426,7) + (-555,9)}{24 \times 2 \times 0,301} = -68,01$$

$$\bar{y}_P = \frac{2175,7}{2 \times 24} = 45,33$$

$$\bar{y}_A = \frac{2308,3}{2308,3} = 48,09$$

$$M' = \frac{48,09 - 45,33}{-68,01} = -0,0406$$

$$S_A = 27.4 \quad \log S_A = 1.4377$$

$$M = -0.0406 + 1.4377 = 1.3971$$

Estimated potency: $R = \text{antilog } 1.3971 = 24.95 \text{ IU/mL}$

$$C = \frac{10057,32}{[10057,32 - 60,76(2,01)^2]} = 1,025$$

$c' = 1$, from **Table 15**

$$\frac{M'_s}{M'_i} = 1,025(-0,0406) \pm \sqrt{(1,025 - 1)[1,025(0,0406)^2 + 1(0,301)^2]}$$

$$M'_s = 0,0064$$

$$M'_i = -0,0064$$

Logarithm of confidence limits:

$$M_s = 0.0064 + 1.4377 = 1.4441$$

$$M_i = -0.0896 + 1.4377 = 1.3481$$

Confidence limits from potency:

$$R_s = \text{anti log } 1.4441 = 27.80 \text{ IU/mL}$$

$$R_i = \text{anti log } 1.3481 = 22.29 \text{ IU/mL}$$

Example 6: moving averages.

Assay of heparin by the method of inhibition of coagulation of citrated sheep plasma: The doses used of standard, in mL, were: $p_1 = 0.78$; $p_2 = 0.76$; $p_3 = 0.74$; $p_4 = 0.72$; $p_5 = 0.70$; and $p_6 = 0.68$. Equivalent doses (a) of the sample were prepared from the assumed potency $S_A = 140.6 \text{ IU/mg}$. The assay was developed as described in the heparin assessment method in this volume. Three assays were conducted. As an example of calculation of M , only the assay number 1 will be developed.

The degrees of coagulation are available on **Table 35**.

Table 35 – Example 6: degrees of coagulation = y.

Tube	Standard P		Sample A	
	p (mL)	y	A (mL)	Y
1	0.78	0.00	0.78	0.00
2	0.76	0.00	0.76	0.25
3	0.74	0.50	0.74	0.75
4	0.72	0.75	0.72	1.00
5	0.70	1.00	0.70	1.00
6	0.69	1.00	0.68	1.00

Calculation of the estimated potency and confidence limits: Use formulas 27, 28 and 40 to 45.

$$x_{iP} = 0.8691$$

$$x_{iP} = 0.8572$$

$$x_{(i+1)} = 0.8691$$

$$y_{(i+1)} = 0.750$$

$$x_P = 0.8691 + (0.4171 - 0.5) \frac{0.8572 - 0.8691}{0.417 - 0.750} = 0.8661$$

$$x_{iA} = 0.8807$$

$$y_{iA} = 0.333$$

$$x_{(i+1)}^A = 0.8691$$

$$y_{(i+1)}^A = 0.667$$

$$x_A = 0.8807 + (0.333 - 0.5) \frac{0.8691 - 0.8807}{0.333 - 0.667} = 0.8749$$

$$SA = 140.6 \text{ IU/mg}$$

$$M_1 = 0.8661 - 0.8749 + \log 140.6 = 2.1392$$

Assuming that the other two assays conducted with the same sample provided the estimates:

$$M_2 = 2.1995 \text{ and } M_3 = 2.1805, \text{ calculate } \bar{M}$$

$$\bar{M} = (2.1392 + 2.1995 + 2.1805)/3 = 2.1731$$

$$R = \text{anti log } \bar{M} = 149.0 \text{ IU/mg} = (2.1392 + 2.1995 + 2.1805)/3 = 2.1731 = \text{anti log } M = 149.0 \text{ IU/mg}$$

Calculate the variance of error:

$$s^2 = \{14.1686 - 42.4999/3\}/2$$

$$s^2 = 0.001$$

$$s = \sqrt{0.001} = 0.0316$$

$$n' = 3$$

$$t = 4.3 \text{ (Table 3 } gl=2)$$

Calculate the confidence interval:

$$L = \frac{2 \times 0.0316 \times 4.3}{1.7321} = 0.1569$$

$$L/2 = 0.0784$$

$$M_s = 2.1731 + 0.0784 = 2.2515$$

$$M_i = 2.1731 - 0.0784 = 2.0947$$

$$R_s = 178.4$$

$$R_i = 124.4$$

Table 36 – Example 6: paired averages.

Tube	Standard P			Sample A		
	Log dose (mL × 10) x_P	Averages log dose x_{iP}	Averages degree of coagulation y_{iP}	log dose (mL × 10) x_A	Averages log dose x_{iA}	Averages degree of coagulation y_{iA}
1	0.8921	-	-	0.8921	-	-
2	0.8808	0.8807	0.167	0.8808	0.8807	0.333
3	0.8692	0.8491	0.417	0.8692	0.8691	0.667
4	0.8572	0.8572	0.750	0.8573	0.8572	0.917

Tube	Standard P			Sample A		
	Log dose (mL × 10) x_P	Averages log dose x_{iP}	Averages degree of coagulation y_{iP}	log dose (mL × 10) x_A	Averages log dose x_{iA}	Averages degree of coagulation y_{iA}
5	0.8450	0.8450	0.917	0.8451	0.8450	1.000
6	-	-	-	0.8325	-	-

Example 7: microbiological assay with five doses of standard and one dose of sample (5 × 1).

Assay of antibiotic using Petri dishes – Microbiological dosage of Benzathine benzylpenicillin powder for injectable. The doses of standard used were: 0.15 IU/mL; 0.30 IU/mL; 0.60 IU/mL; 1.20 IU/mL; 2.40 IU/mL. Equivalent doses of the sample were prepared based on the assumed potency. S_A of 600,000 IU/vial.

Table 37 – Example 7: readings of zones of inhibition.

	P_1	P_3	P_2	P_3	P_4	P_3	P_5	P_3
Diameter of zones of inhibition	13.87	19.88	16.24	19.54	23.47	19.21	27.41	19.32
	12.95	20.60	16.35	19.85	23.04	18.97	27.62	19.61
	13.08	20.43	16.88	19.86	23.19	19.39	26.67	19.72
	12.86	19.85	15.34	18.49	23.04	19.68	27.50	19.65
	13.24	20.07	15.98	19.06	22.65	19.14	27.41	19.27
	13.08	20.06	15.50	19.20	23.01	19.65	26.53	20.04
	12.88	19.75	16.26	19.96	23.99	19.81	27.30	19.25
	13.39	20.30	16.70	19.70	23.85	19.72	27.49	19.53
	13.31	20.30	16.70	19.95	23.82	19.55	27.27	19.90
Average	13.184	20.138	16.217	19.512	23.340	19.458	27.244	19.588

Average of P_3 (36 readings): 19.674

Table 38 – Example 7: readings of zones of inhibition after correction.

	$P1$	$P2$	$P3$	$P4$	$P5$
	$x_1 = -0.82391$	$x_2 = -0.52288$	$x_3 = -0.22185$	$x_4 = 0.079181$	$x_5 = 0.380211$
Diameter of zones of inhibition	13.406	16.402	19.674	23.686	27.496
	12.486	16.512	19.674	23.256	27.706
	12.616	17.042	19.674	23.406	26.756
	12.396	15.502	19.674	23.256	27.586
	12.776	16.142	19.674	22.866	27.496
	12.616	15.662	19.674	23.226	26.616
	12.416	16.422	19.674	24.206	27.386
	12.926	16.862	19.674	24.066	27.576
	12.846	16.862	19.674	24.036	27.356
$(\sum y_i)^2$	13106.59	21729.12	31352.37	44945.7	60503.21

x = concentration of antibiotic in logarithm

Totals:

$N = 45$

$$\begin{aligned} \sum x &= -9,983205 \\ \sum y &= 896,936 \\ \sum y^2 &= 19.076,73 \\ \sum (\sum yi^2)/9 &= 19.070,78 \\ \sum xy &= -100,374 \\ \sum (x - \bar{x})(y - \bar{y}) &= 98,61069 \\ \sum (x - \bar{x})^2 &= 8,155715 \\ \sum (y - \bar{y})^2 &= 1.199,081 \\ \sqrt{\frac{\sum (x - \bar{x})^2}{N - 1}} &= 0,4305 \\ \sqrt{\frac{\sum (y - \bar{y})^2}{N - 1}} &= 5,2203 \\ b_0 &= 22.61426 \\ b_1 &= 12.09086 \end{aligned}$$

Table 39 – Example 7: analysis of variance.

$$b_1 = 12.09 \quad b_0 = 22.61 \quad r = 0.997$$

Source of variation	gl	Sum of squares	Average square	F calc.
Regression	1	1,192.3	1192.3	5691.5
Residual error	43	6.80	0.2	---
Deviation of linearity	3	0.85	0.3	3
Pure error	40	5.95	0.1	---
Total	44	1,199.1	---	

Average of P_3 (36 readings): 19.674

Table 40 – Example 7: reading of samples.

A_1	P_3	A_2	P_3	A_3	P_3
19.66	19.78	19.57	18.73	18.69	18.57
19.49	19.45	18.91	19.12	19.04	18.89
19.94	19.50	19.02	19.70	19.28	19.12
19.38	19.68	19.41	19.55	19.38	19.14
19.88	19.90	19.32	19.38	19.22	19.40
19.88	19.91	19.55	19.74	19.22	19.10
19.74	19.45	19.41	19.33	20.03	19.45
19.15	19.04	19.47	19.68	19.33	19.45
19.45	19.32	19.48	19.46	19.45	19.45

A_1	P_3	A_2	P_3	A_3	P_3
$\Sigma y_i^2 =$ 31,176.96	$\Sigma y_i^2 = 30,986.56$	$\Sigma y_i^2 = 30,324.74$	$\Sigma y_i^2 = 30,516.6$	$\Sigma y_i^2 =$ 30,150.85	$\Sigma y_i^2 =$ 29,780.40

Table 41 – Example 7: differences in paired responses or $X = (y_A - y_P)/b_1$ (formula 16)

X_1	X_2	X_3	X_4
- 0.0099256	0.0694789	0.0099256	- 0.0198511
0.0033085	- 0.0173697	0.0124069	- 0.0256410
0.0363937	- 0.0562448	0.0132341	0.03300852
- 0.0248139	0.0115798	0.0198511	- 0.0281224
- 0.0016543	- 0.0049628	- 0.0148883	0.0066170
- 0.0024814	- 0.0157155	0.0099256	0.0016543
0.0239868	0.0066170	0.0479735	- 0.0281224
0.0090984	- 0.0173697	- 0.0099256	0.0645161
0.0107527	0.0016543	0.0000000	- 0.0264682
$T_x = 0.044665$	$T_x = - 0.04549$	$T_x = 0.088503$	$T_x = - 0.02233$
$T_x^2/9 = 0.000222$	$T_x^2/9 = 0.00023$	$T_x^2/9 = 0.00087$	$T_x^2/9 = 0.0000554$

$$\Sigma X^2 = 0.024058$$

$$\Sigma (T_x^2/9) = 0.001377$$

$$t = 2.042$$

$$k' = 9$$

$$f = 9$$

$$n = 32$$

$$S_M^2 = \frac{0.024058 - 0.001377}{32} = 0.00071 \text{ (formula 19)}$$

$$s = 0.02662$$

$$\frac{1}{2} L = 0.01812 \text{ (formula 20)}$$

Table 42 – Example 7: log potency ratio and confidence limits for samples A1, A2, A3 and A4.

	$A1$	$A2$	$A3$	$A4$
Logarithm of potency ratio (log)	0.004963	- 0.00505	0.009833	- 0.00248
M'_{AS} and M'_{AI}	0.02308	- 0.1316	0.01307	0.01564
	0.01564	0.02795	0.01564	- 0.02060

Calculation of estimated potency and confidence limits for sample A1: Using formulas 17 and 20 to 25.

$$M' (A_1) = \Sigma X_1/9 = 0.004963$$

$$M = M' + \log 600,000 = 5.78311$$

$$R = \text{anti log } 5.78311 = 606,895.97 \text{ IU/vial}$$

$$M'_{as} (A_1) + \frac{1}{2} L = 0.004963 + 0.01812 = 0.02308$$

$$M'_{Ai} (A_1) - \frac{1}{2} L = 0.004963 - 0.01812 = -0.01316$$

Logarithm of confidence limits from potency:

$$M_{AS} (A_1) = 0.02308 + \log 600,000 = 5.80123$$

$$M_{Ai} (A_1) = -0.01316 + \log 600,000 = 5.76499$$

Confidence limits from potency:

$R_s = \text{antilog } 5.80123 = 632,746.9 \text{ IU/vial}$

$R_i = \text{antilog } 5.76499 = 582,091.5 \text{ IU/vial}$

Table 43 – Example 7: coefficient of Variation (formula 18).

	A1	A2	A3	A4
Standard deviation (s)	0.269	0.232	0.356	0.229
Average	19.62	19.35	19.24	19.26
Coefficient of variation (CV)	1.37	1.20	1.85	1.19

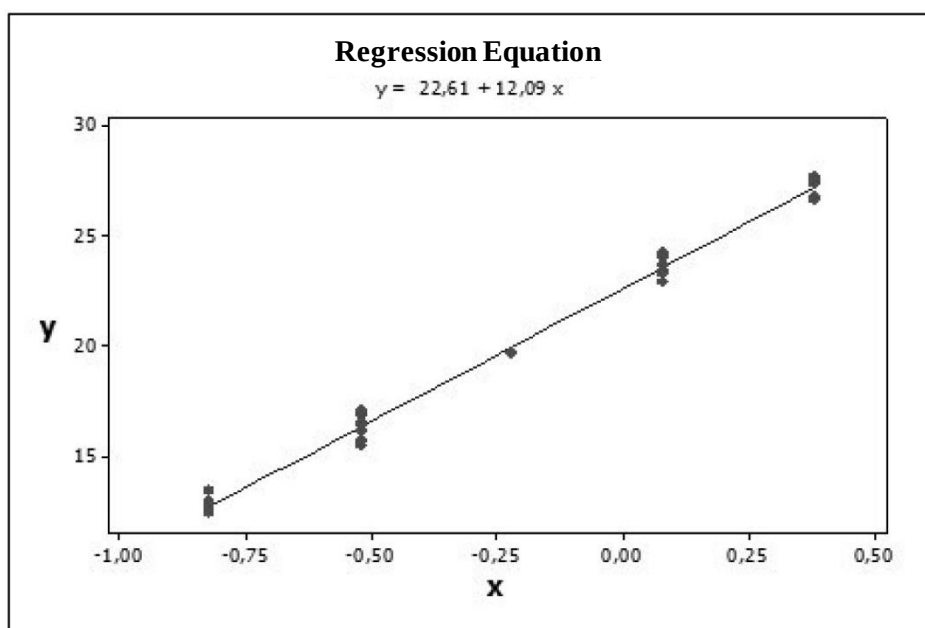


Figure 1 – Example 7: Regression curve chart

INDIRECT “ALL OR NOTHING” ASSAY

Example 8: Dichotomous assay of two doses, simplified probits method.

Assay of insulin by the method of convulsion in mice: The doses used from standard were $p_1 = 18$ mIU/mouse and $p_2 = 30$ mIU/mouse. Equivalent doses of the sample ($a_1 = 18$ mIU/mouse and $a_2 =$ mIU/mouse) were prepared based on the assumed potency $S_A = 40 \text{ IU/mL}$.

The mice, divided randomly into four groups, were submitted to subcutaneous injection with 0.25 mL/mouse of the respective solution.

Table 44 – Example 8: Responses (% of mice in convulsion).

	Standard P		Sample A	
	p_1	p_2	a_1	a_2
Number of mice injected (n)	30	28	28	24
Number of mice in convulsion	9	17	11	18
Percentage of responses (%)	30.0	60.7	39.3	75.0

$$K = \frac{(P + A)^2}{k} = \frac{20,15^2}{4} = 101,5056$$

$$\text{Preparations} = \frac{9,75^2 + 10,4^2}{2} - 101,5056 = 0,1056$$

$$\text{Regression} = \frac{(0,79 + 0,94)^2}{4} = 0,7482 = E$$

$$\text{Paralelism} = \frac{0,79^2 + 0,94^2}{2} - 0,748 = 0,0056$$

$$s^2 \frac{k}{\sum wn} = \frac{4}{30(0,576) + 28(0,619) + 28(0,619) + 24(0,540)} = 0,0616$$

Validity of assay. The assay meets the validity conditions:

- significant regression, F calculated 12.15 is higher than the critical value from Table 5, for $p = 0.01$, $gl_1 = 1$ and $gl_2 = \text{infinite}$; and
- non-significant deviation from parallelism, F calculated 0.09 is lower than the critical value from Table 4, for $p = 0.05$; $gl_1 = 1$ and $gl_2 = \text{infinite}$.

Table 45 – Example 8: Transformation in probits, totals and contrasts.

	<i>Standard P</i>		<i>Sample A</i>	
	<i>p</i> ₁	<i>p</i> ₂	<i>a</i> ₁	<i>a</i> ₂
Probit (Table 16)	P1 = 4.48	P2 = 5.27	A1 = 4.73	A2 5.67
Weight w (Table 17)	0.576	0.619	0.540	0.540
Preparation	P = 9.75		A = 10.4	Σy = 20.15
Linear contrast	LP = 0.79		LA = 0.94	ΣL = 1.73

Results obtained with formulas from **Table 8**.

Table 46 – Example 8: Analysis of variance.

<i>Source of variation</i>	<i>gl</i>	<i>Sum of squares</i>	<i>Average square</i>	<i>F</i>	<i>p</i>
Preparation	1	0.1056	0.1056	1.71	> 0.05
Regression	1	0.7482	0.7482	12.15	< 0.01
Parallelism	1	0.0056	0.0056	0.09	> 0.05
Error	Infinite		$s^2 = 0.0616$		

The sums of squares were obtained by employing the formulas from **Table 10**, taking $n = 1$.

Calculation of the estimated potency and confidence limits: Use formulas 10 to 15.

$$I = \log 30 - \log 18 = 1.4771 - 1.2553 = 0.2219$$

$t = 1.96$ with $gl = \text{infinite}$ and $p = 0.05$ (from **Table 3**)

$$b = \frac{0,79 + 0,94}{2(0,2219)} = 3,9318$$

$$\bar{y}_p = \frac{9,75}{2} = 4,87$$

$$\bar{y}_A = \frac{10,4}{2} = 5,20$$

$$M' = \frac{5,20 - 4,87}{3,9318} = 0,0839$$

$$S_A = 40.0 \quad \log S_A = 1.6021$$

$$M = 0.0839 + 1.6021 = 1.6860$$

$$R_A = \text{anti log } 1.6860 = 48.53 \text{ IU/ mL} = \text{Estimated potency}$$

$$C = \frac{0,7482}{[0,7482 - 0,0616(1,96)]^2} = 1,4625$$

$$c' = 1 \text{ (from Table 15)}$$

$$\frac{M'_s}{M'_i} = 1,4625 \times 0,0839 \pm \sqrt{0,4625[1,425(0,0839)^2 + (0,2219)^2]}$$

$$\frac{M'_s}{M'_i} = 0,1227 \pm 0,1658$$

$$M'_s = 0,2885$$

$$M'_i = -0,0431$$

Logarithm of confidence limits:

$$M_s = 0.2885 + 1.6021 = 1.8906$$

$$M_i = 0.0431 + 1.6021 = 1.5590$$

Confidence limits from potency:

$$R_s = 77.73 \text{ IU/mL}$$

$$R_i = 36.22 \text{ IU/mL}$$

Using the complete method of analysis of probits, an estimated potency of 48.48 was obtained with limits of 35.9 and 75.92 IU/mL.

COMBINATION OF ESTIMATES OF POTENCY

Example 9: Combination of estimates of potency.

Combination of assays of corticotropin by the method of depletion of adrenal ascorbic acid in hypophysectomized female rats: three independent assays on the same sample were conducted according to the procedure described on Combination of Estimates of Potency (8.2.8). The results from the assays are available on Table 47.

Table 47 – Example 9: Data for combination of potencies.

	Assay 1	Assay 2	Assay 3
<i>M</i>	1.24797	1.25164	1.42193
<i>L</i>	0.29097	0.90082	0.11555
<i>t</i> ²	4.1209	4.1209	4.2025
<i>gl</i>	34	33	27

Calculation of weighted average potency:

$$\bar{M} = \frac{\sum MW}{\sum W} = \frac{2058,6174}{1474,0148} = 1.3966$$

$$R = \text{anti log } 1.3966 = 24.9$$

Test of homogeneity of log estimates of potency:

$$X^2M = \sum W(M - \bar{M})^2 = 5.5$$

$$X^2_{gl} = 2p = 0.05 \text{ (Table 18)} = 5.9$$

Since X^2 calculated is lower than the critical value, there are no elements to suspect of heterogeneity.

Calculation of confidence limits:

$$\bullet = \sqrt{1/\sum W} = \sqrt{1/1474,0198} = 0,0260$$

$$\frac{M'_s}{M'_i} = \bar{M} \pm 1,98 \times 0,0260$$

$$M_s = 1.4226$$

$$M_i = 1.3700$$

$$R_s = 26.5$$

$$R_i = 23.5$$

8.3 RADIOPHARMACEUTICALS

GLOSSARY

Specific activity (or specific radioactivity)

Radioactivity of the radionuclide related to the unit mass of the element or compound. It is commonly referred to the activity of 1 g of the substance specified on the monograph:

$$S = \frac{N \times 0,693}{W \text{ ou } M \times T_{1/2}} \text{ desintegrations/s/g}$$

where

S = specific radioactivity;

N = Avogadro's number;

W = atomic mass;

M = molecular mass.

Carrier

Stable isotope of the radionuclide in question, added to the radioactive preparation in the chemical form identical to the one where the radionuclide is present.

Non-radioactive components for labeling

Preparation or set of reagents that must be reconstituted or combined with a radionuclide for the synthesis of the final radiopharmaceutical, before administration to the patient. They may come in the form of lyophilized reagents or other substances and are most commonly known as labeling kits.

Radioactive concentration

The radioactive concentration of the solution is the radioactivity of the radionuclide contained in the unit volume and usually referred to as activity per milliliter. As it occurs with all specifications involving radionuclides, it is necessary to state the date and, in the case of radionuclides with short half-life, the time when the radioactive concentration was determined.

Radioactive decay

The nuclei from radioactive elements (radionuclides) suffer loss of particles and/or energy according to their own characteristics. These characteristics include the speed of decay and the type of emission. The emission of particles by the nuclei determines the modification of their mass number. When the particle emitted has positive or negative charge, the nucleus suffers a change in the atomic number and, consequently, in the number of electrons in the electron cloud of the atom corresponding to it, determining a change in the chemical properties of the atom. The radioactivity decays in exponential ratio, which is characteristic for each radionuclide. The activity at any time can be calculated by the expression:

$$A = A_0 e^{-\lambda t}$$

where

A = activity on t time;

A₀ = initial time;

λ = decay constant – also named disintegration constant or transformation constant, that is, the fraction of radioactive atoms that suffer transformation in the time unit, provided this time is short when compared to the physical half-life;

t = time elapsed;

e = base from Napierian logarithms.

Disintegration

Transformation in which the nucleus emits one or more particles.

Generator

System that incorporates a parent radionuclide that, by decay, produces a daughter radionuclide that can be removed by elution or by any other method to be used as an integral part of a radiopharmaceutical.

Isotopes

Nuclides from a same chemical element which nuclei have the same atomic number and different atomic mass.

Starting material

All constituents that are used in the preparation of radiopharmaceuticals.

Effective half-life

The time it takes for a radionuclide in an organism to halve its activity as a combined result of biological elimination and radioactive decay. The effective half-life is important for calculating the optimal dose of the radiopharmaceutical to be administered and for monitoring the quantity of radiation exposure. It can be calculated from the formula:

$$T_{1/2e} = \frac{T_{1/2p} \times T_{1/2b}}{T_{1/2p} + T_{1/2b}}$$

where

T_{1/2e} = time of effective half-life of the radiopharmaceutical;

T_{1/2p} = time of physical half-life of the radionuclide;

T_{1/2b} = time of effective half-life of the radiopharmaceutical;

Physical half-life

Time necessary for half a population of atoms from a radionuclide to decay to another nuclear form. The half-life is related to the decay constant (λ) by the equation:

$$T_{1/2} = \frac{0,693}{\lambda}$$

Neutrino

Particle difficult to detect, with negligible mass, but with energy emitted simultaneously to the emission of beta particle. The sum of energies from the beta particle and the neutrino corresponds to a value quantified for each beta disintegration process.

Nuclides

Species of atoms characterized by the constitution of their nuclei, particularly by their number of protons and electrons, and also by their nuclear energy state.

Precursors or raw material for synthesis

Usually, these precursors are not produced in large scale. Some precursors are synthesized by the radiopharmaceuticals production laboratory, others are supplied by specialized producer laboratories. Tests for identity, chemical purity and assay must be conducted through validated procedures. When batches of precursors are accepting using the certificates of analysis, adequate evidence must be established to demonstrate the reliability of the supplier and at least one identity test must be conducted. It is recommended to test precursor materials before their use in the radiopharmaceutical production routine, to ensure that, under specified production conditions, the precursor allows the preparation of the radiopharmaceutical in the amount and quality specified.

Expiry date

Limit date specified by the manufacturer for the use of a radiopharmaceutical, before and after the reconstitution and/or radioactive labeling of the product, taking into account chemical, radiochemical and radionuclidic degradation products, maintaining the established conditions of storage and transportation.

Chemical purity

Percentage ratio of the molecule mass from the compound of interest in its chemical state indicated, in relation to the total mass of the preparation. The relevant chemical impurities and corresponding limits are listed in the individual monographs.

Radionuclidic purity

It is the ratio, expressed in percentage, of the radioactivity of the radionuclide in relation to the total radioactivity of the radiopharmaceutical. The relevant radionuclidic impurities are listed, with their limits, in the individual monographs.

Radiochemical purity

Percentage ratio of radioactivity of the radionuclide of interest in its chemical state indicated, in relation to the total radioactivity of the radiopharmaceutical preparation. The relevant radiochemical impurities are listed, with their limits, in the individual monographs.

Specific radioactivity

Expresses the radioactivity of a radionuclide per unit of mass of the element or chemical product of interest.

Total radioactivity

Expresses the radioactivity of the nuclide expressed by unit (vial, capsule, ampoule, generator, etc.).

Radioisotopes

Radioactive isotopes or radionuclides. They are unstable isotopes that suffer radioactive decay and turn into a new element. These are atoms that disintegrate by emission of corpuscular (particle) or electromagnetic radiation. Every radioisotope is characterized by its half-life time ($T_{1/2}$), expressed in time units (seconds, minutes, hours, days and years) and by the nature and energy of its radiation. The energy can be expressed in electron volts (eV), kilo-electron volts (keV) or mega-electron volts (MeV).

INTRODUCTION

Radiopharmaceuticals are pharmaceutical preparations with diagnostic or therapeutic purpose that, when ready to use, contain one or more radionuclides. Radiopharmaceuticals also include the non-radioactive components for labeling and radionuclides, including the components extracted from generators of radionuclides.

The production of radiopharmaceuticals must meet the requirements from Good Manufacturing Practices (GMP) for Radiopharmaceuticals, and also comply with pharmacopoeial specifications. Radiopharmaceuticals have their production, supply, storage, use and disposal regulated by the current national legislation.

The radiopharmaceutical has the radionuclide in one of the following forms: as an atomic or molecular element, an ion, or included or bonded to organic molecules, by chelation process or by covalent bond.

The forms of obtaining radionuclides used in the production of radiopharmaceuticals are by bombarding neutrons in nuclear reactors, bombarding with charged particles in particle accelerators, nuclear fission of heavy nuclides after bombarding with neutrons or with particles, and radionuclide generator systems that involve the physical or chemical separation of a daughter radionuclide with a shorter half-life than the parent radionuclide.

STORAGE

The radiopharmaceuticals must be maintained in sealed containers and in a sufficiently protected place to avoid irradiation from personnel by primary or secondary emissions, according to national and international regulations on handling of radioactive substances.

STABILITY

The preparations of radiopharmaceuticals tend to be less stable than their corresponding inactive materials, their decomposition occurs by radiolysis and, therefore, they must be used within a short period. The effects of primary radiation include the disintegration of the radioactive atom and the decomposition of molecules when the fraction of energy from particle emitted or from gamma ray is absorbed by these molecules.

The stability of radiopharmaceuticals depends on many factors, including the energy and nature of the radiation, the specific activity and the storage time. The effects of primary radiation may induce

secondary effects due to the formation of excited species, which may degrade other molecules, such as the ones from solvents or preservatives, for example.

The susceptibility to oxidation and reduction of a small amount of chemical species present must also be considered. The initial exclusion of all traces of oxidation and reduction agents is occasionally not enough, because such agents may be continuously produced due to effects of radiation. During storage, containers and solution may turn dark due to the radioactivity emitted. Such factor does not necessarily indicate deterioration of the preparation.

PRESERVATIVES

Injectable radiopharmaceutical preparations are usually stored in multi-dose containers. Antimicrobial preservatives may suffer decomposition due to the influence of radiation, which restricts their use for some injectable radiopharmaceuticals. Therefore, the requirement that injectable preparations have an adequate antimicrobial preservative, in adequate concentration, does not necessarily apply to radiopharmaceutical preparations.

Injectable radiopharmaceutical preparations with a shelf life period larger than one day and that do not have an antimicrobial preservative must be provided in single-dose vials. However, if the preparation is provided in a multi-dose container, it must be used within 24 hours after the first dose is taken, aseptically.

Injectable radiopharmaceutical preparations in which the shelf life period is larger than one day and that have antimicrobial preservative can be provided in multi-dose containers. After the first dose is taken, aseptically, the container must be stored at temperature from 2 °C to 8 °C and the contents, used within seven days.

DILUTION

If dilution is necessary, it is preferable to use vehicles with the same composition as the ones present in the preparation. In case of injectable radiopharmaceuticals, sterile solutions and materials, free from particles and traces of organic matter, must be used.

The amount of radioactive material present in the preparation is frequently very small to be measured by the chemical or physical methods available.

Considering the formula:

$$S_{max} = \frac{1,16 \times 10^{20} Bq g^{-1}}{W \times T_{1/2}}$$

where

S_{max} = maximum specific activity,

W = atomic mass,

$T_{1/2}$ = half-life time in hours.

It is verified that, for example, for the sodium pertechnetate (^{99m}Tc) solution with radioactive concentration of 37 MBq (1 mCi) per mL, the concentration of pertechnetate can be as low as 3×10^{-10} g/mL. The behavior of such small masses in very diluted solutions may require the addition of an inert carrier to limit the adsorption to the surface of the container, as well as facilitate the chemical reactions for preparation of radiopharmaceuticals.

BIOLOGICAL CONTROL

Sterility (5.5.3.2.1)

Injectable radiopharmaceuticals must be prepared according to Good Manufacturing Practices, in order to ensure sterility, meeting the criteria from the *Sterility test*. Due to the radioactive characteristics of preparations, it is not technically feasible to wait the incubation time of the assay for using the product. Thus, the results from sterility tests provide only confirmatory retrospective evidence for guarantee of sterility and, therefore, its quality is related to controls performed during the manufacturing and in the validation and certification procedures. In the case of radiopharmaceuticals prepared in small batches and for which the execution of sterility test presents a high level of radiological risk, the amount of sample required in the sterility test must be considered. If the radiopharmaceutical preparation is sterilized by filtration or processed aseptically, the process validation is necessary.

Bacterial endotoxins (5.5.2.2)

When specified, an individual monograph for a radiopharmaceutical preparation requires compliance with the test for bacterial endotoxins. When conducting the test, the necessary precautions must be taken to limit the irradiation from personnel who conducts the test.

The limit of bacterial endotoxins is indicated in the monographs on radiopharmaceuticals. The test validation is necessary to exclude any interference due to the nature of the radiopharmaceutical. Radioactivity levels must be standardized, since some types of radiation and radionuclides, especially high levels of activity, may interfere with the test. The pH of some radiopharmaceutical preparations must be adjusted to pH 6.5 – 7.5 to promote optimal results.

When the nature of the radiopharmaceutical preparation results in an interference by inhibition or potentiation and it is not possible to eliminate the interfering factor, the compliance with the test for bacterial endotoxins must be specified. In some cases, it is difficult to conclude the test before clearing the batch for use, when the half-life of the radionuclide in the preparation is short. The test is then comprised of a production quality control.

RADIOACTIVITY

Property that certain nuclides have to emit radiation by spontaneous transformations of their nuclides. In general, the term “radioactivity” is used to describe the phenomenon of radioactive decay and to express the physical amount (activity) of this phenomenon. The activity of a preparation is the number of nuclear transformations per time unit that occur in the preparation. These transformations can involve the emission of charged particles, capture of electrons or isomeric transition. The charged particles emitted by the nucleus can be alpha particles (helium nuclei, with mass number 4) or beta particles (negative or positive charge electrons, respectively -1β – negatron or $+1\beta$ – positron). The emission of beta particles is accompanied by the emission of neutrino.

The emission of charged particles can be accompanied of gamma rays, which are also emitted in the isomeric transition process. This emission of gamma rays can be partially replaced with the ejection of electrons, known as internal conversion electrons. This phenomenon, just like the electron capture process, causes secondary emission of X-rays, due to the reorganization of electrons in the atom. This secondary emission also causes the ejection of low-energy electrons known as Auger electrons. X-rays, eventually accompanied of gamma rays, are emitted in the electron capture process. $+1\beta$ particles are annihilated in contact with another electron ($-1e$) present in the matter, and this process

is followed by the emission of two gamma photons, each one with energy of 511 keV, usually emitted 180° from each other and which is named annihilation radiation.

The penetrating power of each radiation varies considerably according to its nature and energy. Alpha particles are completely absorbed by thicknesses of solids or liquids that vary from a few to dozens of micrometers; beta particles are completely absorbed in the thickness of some millimeters to several centimeters. Gamma rays are not completely absorbed, but only attenuated, and a ten-fold reduction may require, for example, a few centimeters of lead. The denser the absorbent, the lower is the reach of alpha and beta particles and the higher the attenuation of gamma rays is.

Radioactivity measurement

The absolute measurement of a sample radioactivity can be made if the decay scheme of the nuclide is known, but, in practice, many corrections are required to obtain accurate results. For this reason, it is common to take measurements using a primary standard source.

Primary standards may not exist for radionuclides with short half-life, such as, for example, positron emitters. The measurement instruments are calibrated using appropriate standards for particle emitter radionuclides.

The Geiger-Müller counter can be used to measure beta and beta-gamma emitters. Scintillation counters, semiconductors or ionization chambers can be used to measure gamma rays. Low-energy beta emitters need a liquid scintillation counter.

In this case, the sample is dissolved in the solution of one or more (generally two) fluorescent organic substances (primary and secondary scintillators), which convert part of the disintegration energy into light photons, which are detected and converted into electric impulses in the photomultiplier. When the liquid scintillation counter is used, comparative measurements must be corrected due to the effects from light interference. Direct measurements must be taken in conditions that ensure that the geometric conditions are constant (identical volumes of containers and solutions).

Whatever the equipment used, it is essential to work in extremely well defined geometric conditions, so that the radioactive source is always on the same position in the device and, consequently, its distance from the measurement device is constant and remains the same, while the sample is substituted with the standard.

All reactivity measurements must be corrected by subtraction of the background radiation activity, due to the radioactivity of the medium and the spurious signals generated in the device itself. In certain devices, where the count is performed in high levels of activity, the correction may be necessary because of losses by coincidence, due to the resolution time of the detector and the electronic equipment associated.

For the counting system with fixed dead time (τ), after each count the correction is given by the equation:

$$N = \frac{N_0}{1 - N_0\tau}$$

where

N = actual counting rate per second;

N₀ = counting rate measured per second;

τ = dead time in seconds.

In certain devices, the correction is made automatically. Corrections of coincidence loss must be made before the corrections for background radiation. In the determinations of radioactivity, there are statistical variations because they are related to the probability of nuclear disintegration. A sufficient number of counts must be made to compensate for variations in the number of disintegrations per time unit. At least 10,000 counts are necessary to obtain a standard deviation of no more than 1%.

The activity decays in exponential ratio, which is characteristic to each radionuclide. Its determination is only true in the reference time specified. The activity in other times can be calculated from exponential equation or by decay table, or also be obtained graphically from the curve established for each radionuclide. All determinations of activity must be accompanied by statement of date and, if necessary, the time when the measurements were made. The measure of activity from sample in solution is calculated in relation to its original volume and expressed by unit of volume – radioactive concentration.

Radioactivity units

In the International System (SI), the radioactivity is expressed in becquerel (Bq), which means one transformation per second. The historical unit of activity is curie (Ci), equivalent to 3.7×10^{10} Bq.

The conversion factors between becquerel and curie and their submultiples are indicated on **Table 1**.

Table 1 – Radioactivity units used in radiopharmacy and the conversions between SI units and historical units.

<i>Number of atoms transformed per second</i>	<i>SI unit: becquerel (Bq)</i>	<i>Historical unit: curie (Ci)</i>
1	1 Bq	27 picocurie (pCi)
1000	1 kilobecquerel (Kbq)	27 nanocurie (nCi)
1×10^6	1 megabecquerel (MBq)	27 microcurie (μ Ci)
1×10^9	1 gigabecquerel (GBq)	27 millicurie (mCi)
37	37 Bq	1 (nCi)
37,000	37 KBq	1 (μ Ci)
3.7×10^7	37 MBq	1 (mCi)
3.7×10^{10}	37 GBq	1 Ci

Identification of radionuclides

The radionuclide is usually identified by the physical half-life, by the nature and energy of its radiation (or radiations), or by both.

Determination of half-life

The half-life is measured with the help of detection devices such as ionization chamber, Geiger-Müller counter, scintillation counter, or semiconductor detector. The amount of activity, considering the experimental conditions, must be sufficiently high to allow detection during several presumable half-lives, but not too high, to avoid the phenomenon of coincidence loss due to, for example, the equipment dead time.

The radioactive source is prepared in order to avoid losses during its handling. Liquid samples must be contained in sealed vials or tubes. Solid products must be protected by a layer of cellulose acetate

adhesive sheet, or another material which mass per unit of area is negligible to avoid the attenuation of significant amount of radiation in study. The same source is measured in identical geometric conditions and at intervals that usually correspond to half of the half-life and by the time corresponding to approximately three half-lives. The correct equipment operation is verified through the use of a permanent source and the count variations are corrected, if necessary, as described in

To make the radioactivity measurement, a curve is traced by launching the time on the abscissa axis and the logarithm of the number of counts per time unit, or the electric current, according to the type of equipment used, in the ordinate axis. The half-life calculated from this curve must meet the specification described in the respective monograph.

Determination of nature and energy of radiation

The nature and energy of the radiation emitted can be determined by several procedures that include outlining the attenuation curve and using spectrometry. The attenuation curve is generally used for determining the energy from beta radiation, and spectrometry is mainly used for determining the energy from gamma radiation.

The attenuation curve is developed for pure beta emitters or for beta-gamma emitters when there is no availability of gamma ray spectrometer. This method for determination of maximum energy from beta radiation provides only approximate values.

The source, assembled conveniently to provide constant geometric conditions, is placed in front of the thin window of the Geiger-Müller counter and protected as described on *Determination of half-life*.

The source count is then measured. No less than six aluminum absorbers, with increasing mass per area unit, are placed between the source and the counter, until the counter rate is not affected by the addition of more absorbers. The absorbers are inserted in such a way that the geometric conditions are maintained constant.

A curve is built by putting in abscissas the mass per unit of area from the absorber expressed in mg cm^{-2} and, in ordinates, the logarithm of the number of counts per time unit for each of the absorbers used. An identical curve is developed using the standard. The mass attenuation coefficient is calculated in relation to the median, practically straight, part of the curves.

The mass attenuation coefficient, expressed in $\text{cm}^2 \text{mg}^{-1}$, depends on the energy from beta emission and on the chemical and physical properties of the absorber. This allows the identification of beta emission and the coefficient is calculated, from curves built as described previously, by the expression:

$$\mu_m = \frac{2,303}{m_2 - m_1} (\log A_1 - \log A_2)$$

where

m_1 = mass per area unit from the lighter absorber;

m_2 = mass per area unit of the heavier absorber (measure m_1 and m_2 within the straight part of the curve);

A_1 = count rate for mass per area unit m_1 ;

A_2 = count rate for mass per area unit m_2 .

The attenuation coefficient thus calculated must not differ by more than 10% from the coefficient obtained in identical conditions with the standard from the same radionuclide.

Gamma spectrometry is used to identify radionuclides by the energy and intensity of X or gamma rays. It is based on the property that certain substances (scintillators) have of emitting light when they interact with electromagnetic radiation. The number of photons produced is proportional to the energy absorbed by the scintillator. The light is turned into electric impulses of amplitude approximately proportional to the energy dissipated by gamma photons.

With the analysis of output pulses by percentage, the spectrum of energy from the source is obtained, with the help of pulse analyzer. In the gamma ray scintillation spectra, there is one or more characteristic peaks corresponding to the energies from gamma radiation in the source. These peaks are accompanied by others, more or less large, due to secondary effects from the radiation in the scintillator or the material around it. The shape of the spectrum varies according to the equipment used, and it is necessary to calibrate it with the help of standard of the radionuclide in question.

The gamma ray spectrum from the radionuclide that emits the rays is from the own radionuclide, and is characterized by the number of gamma rays with individualized energy produced by transformation. This property can be used to identify which radionuclides are present in the source and the amounts of each of them. It also allows to assess the degree of impurities present, by detection of peaks foreign to the ones expected.

The preferred detector for the gamma ray spectrometry is a germanium semiconductor detector activated with lithium. Sodium iodide scintillation detectors activated with thallium, although they present lower resolution, can also be used. The output from each of these detectors occurs in the form of electric pulses, which amplitude is proportional to the energy from gamma rays detected. After the amplification, these pulses are analyzed in multichannel analyzer, which provides the gamma ray spectrum from the source. The relation between gamma energy and the channel number can be easily established using gamma ray sources with known energy. The detection system must be calibrated, because the detector efficiency is a function of the gamma radiation energy, the source shape, and the distance from source to detector. The detection efficiency can be measured with the help of the calibrated source of the radionuclide in question or, for a more generic work, an efficiency curve versus gamma radiation can be built from a series of calibrated sources of several radionuclides.

Using a low-resolution detector may bring some difficulty to identify impurities, because the peaks on the spectrum may not be well resolved. In this case, it is recommendable to determine the half-life by repeated measurements of sample.

If radioactive impurity with different half-life is present in a source, it is easily detectable by the identification of characteristic peaks, which amplitudes decrease in different rates from the ones of the radionuclide expected. The determination of half-life from interfering peaks by repeated sample measurements will help identify the impurity. It is possible to establish the radioactivity decay rate using gamma spectrometry, provided that the peaks decrease in amplitude due to the half-life.

Information about the physical characteristics of radionuclides of relevance in the production of radiopharmaceuticals are provided on **Table 2**.

RADIONUCLIDIC PURITY

To establish the radionuclidic purity of the preparation, the radioactivity and the identity of each radionuclide present must be known. The most commonly used method to examine the radionuclidic purity is the gamma spectrometry. It is not an entirely precise method because alpha-emitter and beta-

emitter impurities are usually not detectable, and when sodium iodide detectors are used the peaks due to impurities are frequently hidden by the spectrum of the main radionuclide.

This monograph establishes the overall requirements for radionuclidic purity (for example, the spectrum of gamma rays must not be significantly different from the one of the standard source) and may establish limits for specific radionuclidic impurities (for example, molybdenum-99 in technetium-99). These requirements are necessary, although they are not sufficient by themselves to ensure that the radionuclidic purity of the preparation is adequate for human use. The manufacturer must analyze its products, especially preparations of radionuclides with short half-life, for the presence of impurities with long half-life, after a convenient period of decay. This way, information can be obtained about the suitability of manufacturing processes and the control procedures.

Due to differences in the half-lives from different radionuclides present in the pharmaceutical preparation, the radionuclidic purity changes with time. The specification of radionuclidic purity must be ensured throughout the validity period. Sometimes, it is difficult to conduct this test before clearing a batch produced for use, when the half-life of the radionuclide in the preparation is short. In this case, the test is comprised of a production quality control.

RADIOCHEMICAL PURITY

The determination of radiochemical purity requires the separation of different chemical substances that have the radionuclide and the estimated percentage of radioactivity associated to the chemical substance stated.

Analytical methods of separation can be used in the determination of radiochemical purity, such as chromatographic methods (paper, thin layer, molecular exclusion, gas chromatography or high-performance liquid chromatography), electrophoresis and extraction by solvents.

In chromatography, the volume of sample to be used depends on the technique employed. It is preferable to not dilute the preparation in analysis, but it is important to use such an amount of radioactivity that coincidence losses of count do not occur while measuring the radioactivity.

Considering the very small masses of the radioactive material applied to chromatograms, the use of carriers is sometimes necessary and they can be added when prescribed by the monograph.

After developing the paper or thin layer chromatography, the support is dried and the positions of the radioactive areas are detected or by autoradiography or by measuring the radioactivity along the chromatogram, with the help of counters duly collimated, or by cutting the strips and counting each portion.

The positions of stains or areas allow chemical identification by comparison with solutions of the same chemical (non-radioactive) substances, viewed by color reaction or examination under ultraviolet light. Viewing by the direct color reaction from the radioactive sample is not always possible or desirable, since the revelation may cause diffusion of the radioactive substance beyond the stains or areas identified.

Radioactivity measurements can be taken by integration, using an automatic equipment or digital counter. The proportions of areas below peaks provide the relations of radioactive concentrations from chemical substances. When the strips are cut into portions, the ratios of amounts of radioactivity quantities measured provide the proportions of concentrations of radioactive chemical species.

Since the radiochemical purity can change over time, especially due to decomposition by radiation, the test result must indicate that the product presents values specified throughout the validity period of the radiopharmaceutical.

SPECIFIC ACTIVITY

The specific activity is calculated by relating the radioactive concentration (radioactivity per volume unit) with the concentration of the chemical substance being analyzed, after verification that the radioactivity is only due to the radionuclide (radionuclidic purity) and the chemical species (radiochemical purity) in question.

The specific activity changes over time, and must be expressed having as reference the date and, if necessary, the time. The specification must be ensured throughout the validity period of the radiopharmaceutical.

Table 2 – Information about the physical characteristics of radionuclides of relevance in the production of radiopharmaceuticals.

<i>Radionuclide</i>	<i>Physical half-life</i>	<i>Type of decay</i>	<i>Energy (MeV)</i>	<i>Probability of transition (%)</i>	<i>Energy from photon (MeV)</i>	<i>Photons emitted (%)</i>	<i>Internally Converted Transitions (%)</i>
Cesium-137	30.1 y	β^+	0.512 1.174	94.6 5.4	Via 2.6 min ^{137m}Ba 0.662 0.032-0.038	85.1 8 (Ba K X-ray)	9.5
Carbon-11	1223.1 s	β^+	0.960	99.76	0.511	Coming from annihilation	
Carbon-14	5730 y	β^-	0.158	100	-	-	
Chromium-51	27.7 d	c.e.		100	0.320 0.005-0.006	9.83 ~22 (V K X-ray)	
Cobalt-57	270 d	c.e.		100	0.114 0.122 0.136 0.570 0.692 others	9.4 85.2 11.1 0.02 0.16 low intensity	
Cobalt-58	70.8 d	β^+ c.e.	0.475	15.0 85	0.006-0.007 0.511 0.811 0.864 1.675	~55% (Fe K X-ray) p^+ 99.4 0.7 0.5	
Cobalt-60	5.27 y	β^-	0.318 1491	99.9 0.1	0.006-0.007 1.173 1.333 others	~26 (Fe K X-ray) 99.86 99.98 <0.01	0.02 0.01
Dysprosium-165	2.32 h	β^+, γ	0.205 0.290 1.190 1.285	0.1 1.6 14.6 83.4	0.046 0.047 0.053 0.094 0.279 0.361 0.545 0.008	2.5 4.6 1.8 3.5 0.5 0.8 0.16 0.15	
Erbium-169	9.4 d	β^+, γ	0.341	45			

Table 2 (continued)

<i>Radionuclide</i>	<i>Physical half-life</i>	<i>Type of decay</i>	<i>Energy (MeV)</i>	<i>Probability of transition (%)</i>	<i>Energy from photon (MeV)</i>	<i>Photons emitted (%)</i>	<i>Internally Converted Transitions (%)</i>
Fluorine-18	111 min	β^+ , K	0.350 0.649	55 97	0.511	Coming from annihilation	
Gallium-67	78.3 h	c.e.		100	0.091 0.185 0.209 0.300 0.394 0.494 0.704 0.795 0.888 0.008-0.010 via 9.2 μs $^{67\text{m}}\text{Zn}$ 0.093 0.008-0.010	3.6 23.5 2.6 16.7 4.4 0.1 0.02 0.06 0.17 43 (Zn X-ray) 37.6 13 (Zn X-ray)	0.3 0.4 0.02 0.06 0.01
Holmium-166	27.3 h	β^+ , γ	0.191 0.394 1.773 1854	0.2 1 48 51	0.007 0.048 0.049 0.055 0.080 1.379 1.581 1.662 0.172 0.247	7.6 2.8 5.0 2.0 6.2 0.9 0.18 0.12 89.6 94	32.4
Indium-111	2.81 d	c.e.		100	0.392	64.9	10.4 6
Indium-113 m	99.5 min	t.i.		100	0.024-0.028	24 (in K X-ray)	35.1
Iodine-123	13.2 h	c.e.		100	0.159 0.347 0.440	83.0 0.10 0.35	16.3

Table 2 (continued)

<i>Radionuclide</i>	<i>Physical half-life</i>	<i>Type of decay</i>	<i>Energy (MeV)</i>	<i>Probability of transition (%)</i>	<i>Energy from photon (MeV)</i>	<i>Photons emitted (%)</i>	<i>Internally Converted Transitions (%)</i>	
Iodine-124	4.1 d	β^+ , γ , K	1.53		0.506	0.26		
					0.529	1.05		
					0.539	0.27		
					0.027-0.032	~86 (Te K X-ray)		
					0.511 and	Coming from annihilation		
					2.13			
					0.605	66		
					0.644	12		
					0.730	14		
					1.320	1.0		
Iodine-125	60.0 d	c.e.		100	1.510	4.2		
					1.695	14		
					2.09	2.0		
					2.26	1.5		
					0.035	7		
Iodine-131	8.06 d	β^-	0.247	1.8	0.027-0.032	138 (Te K X-ray)	93	
					0.080	2.4		
					0.304	5.9		
					0.334	81.9		
					0.606	7.2		
(Xenon-131m)		t.i.	0.806	0.7	0.637	1.8	98	
					0.723			
					1.3% of ^{131}I decay via 12d $^{131\text{m}}\text{Xe}$			
					100			
					(percentage related to the disintegration of $^{131\text{m}}\text{Xe}$)			
Iron-59	44.6 d	β^-	0.084	0.1	0.164	2		
					0.143	0.8		
					0.132	2.8		
					0.274	0.3		
					0.467	0.02		
			1.566	0.3	1.099	55.8		
					1.292	43.8		

Table 2 (continued)

<i>Radionuclide</i>	<i>Physical half-life</i>	<i>Type of decay</i>	<i>Energy (MeV)</i>	<i>Probability of transition (%)</i>	<i>Energy from photon (MeV)</i>	<i>Photons emitted (%)</i>	<i>Internally Converted Transitions (%)</i>
Krypton-81m	13 s	γ	-	-	1.482	0.06	
Lutetium-177	6.71 d	β^- , γ	0.175	12.3	0.193	82	
			0.384	9.0	0.071	0.16	
			0.497	78.7	0.113	6.3	
					0.208	11.0	
					0.250	0.2	
Molybdenum-99	66.2 h	β^-	0.454	18.3	0.041	1.2	4.8
			0.866	1.4	0.141	5.4	0.7
			1.232	80	0.181	6.6	1.0
			others	0.3	0.366	1.4	
					0.412	0.02	
					0.529	0.05	
					0.621	0.02	
					0.740	13.6	
					0.778	4.7	
					0.823	0.13	
					0.961	0.1	
					via 6,02h ^{99m}Tc in balance	~0	93.9
					0.002	83.9	10
					0.141	0.03	0.8
					0.143		
Nitrogen-13	10 min	β^+	1.19		0.511	Coming from annihilation	
Oxygen-15	2.04 s	β^+	1.723		0.511	Coming from annihilation	
Phosphorus-32	14.3 d	β^+	1.709	100			
Rhenium-186	88.9 h	β^- , γ , K	0.939	22	0.123	0.7	
			1.076	71	0.137	9.5	
					0.631	1.9	
					0.768	0.8	

Table 2 (continued)

<i>Radionuclide</i>	<i>Physical half-life</i>	<i>Type of decay</i>	<i>Energy (MeV)</i>	<i>Probability of transition (%)</i>	<i>Energy from photon (MeV)</i>	<i>Photons emitted (%)</i>	<i>Internally Converted Transitions (%)</i>
Rhenium-188	18 h	β^- , γ	1.964	25.3	0.155	14.9	
			2.119	71.4	0.477	1.0	
					0.633	1.2	
					0.635	0.14	
					0.673	0.11	
					0.829	0.41	
					0.931	0.56	
					1.13	0.7	
					1.306	0.01	
Rubidium-81	4.7 h	β^+ , γ , K	0.33	0.253			
			0.58	0.450			
Samarium-153	47 h	β^- , γ	1.05	1.10			
			0.26	0.1	0.0058	11.8	
			0.632	34.1	0.0409	17.2	
			0.702	44.1	0.0415	31.2	
			0.81	21	0.0470	12.2	
					0.0696	5.1	
					0.103	28.3	
					0.422	0.2	
Selenium-75	118.5 d	c.e.		100	0.066	1.1	
					0.097	2.9	
					0.121	15.7	
					0.136	54	
					0.199	1.5	
					0.265	56.9	
					0.280	18.5	
					0.401	11.7	
					others	<0.05 each ~50 (as K X-ray)	
					0.010-0.012		
					via 16.4 ms ^{75m}As		
					0.024	0.03	5.5

Table 2 (continued)

<i>Radionuclide</i>	<i>Physical half-life</i>	<i>Type of decay</i>	<i>Energy (MeV)</i>	<i>Probability of transition (%)</i>	<i>Energy from photon (MeV)</i>	<i>Photons emitted (%)</i>	<i>Internally Converted Transitions (%)</i>
Strontium-89	50.5 d	β ⁻ , (γ)	0.582	0.01	0.280	5.4	0.1
					0.304	1.2	
					0.010-0.012	~2.6	
					(as K X-ray)	0.01	
Technetium-99m	6.02 h	t.i.	1.491	99.98	0.909	0.01	99.1
				100	0.002	~0	
					0.141	88.5	
					0.143	0.03	
			daughter ⁹⁹ Tc				

Table 2 (conclusion)

Radionuclide	Physical half-life	Type of decay	Energy (MeV)	Probability of transition (%)	Energy from photon (MeV)	Photons emitted (%)	Internally Converted Transitions (%)
Thallium-201	73.5 h	c.e.		100	0.031 0.032 0.135 0.166 0.167 0.255 0.024-0.028	0.29 0.25 2.9 0.13 8.81 21 73 (in K X-ray)	10.1 9.6 8.9 0.2 16 0.1
Aluminum-113	115 d	c.e.		100			
		daughter ^{131m}In					
Tritium (^3H)	12.35 to 1.393	β^-	0.0186	100			
Tungsten-188	69.5 d	β^-	0.3	0.227 0.291			
		daughters $^{188m+188}\text{Re}$					
Xenon-131m	11.9 d	t.i.		100	0.164 0.029-0.035	2 ~52 (Xe K X-ray)	98
Xenon-133	5.25 d	β^-	0.266 0.346	0.9 99.1	0.080 0.081 0.160 0.030-0.036	0.4 36.6 0.05 ~46 (Cs K X-ray)	0.5 63.3
Xenon-133m	2.26 d	t.i.		100	0.233 0.029-0.035	8 ~59 (Xe K X-ray)	92
		daughter ^{133}Xe					
Ytterbium-169	32.0 d	c.e.		100	0.021 0.063 0.094 0.110 0.117 0.118 0.131 0.177 0.198 0.240 0.261 0.308	0.21 45.16 0.78 3.82 0.04 1.90 11.42 17.31 26.16 0.12 1.74 11.04	12.3 50.4 12.3 56.2 3, 2 13.5 17.7 25.7 0.7

Yttrium-90	64.5 h	β^-	2.281	100	-	-

8.4 PHARMACEUTICAL EQUIVALENCE AND BIOEQUIVALENCE OF MEDICINES

INTRODUCTION

This chapter addresses scientific and technical aspects related to assays of Pharmaceutical Equivalence, Dissolution, Bioavailability and Bioequivalence applicable to medicines, with emphasis to immediate-release solid pharmaceutical dosage forms (IRSPDF) of oral use and suspensions, in the context of exchangeability between medicines. Biological medicines (vaccines, sera, blood derivatives, etc.), biotechnological medicines, radiopharmaceuticals and phytotherapy medicines require other considerations and, therefore, are not addressed.

The Brazilian Health Regulatory Agency (Anvisa) is responsible for the minutes of registration and post-registration of medicines. The technical and scientific aspects presented in this chapter are in line with the criteria adopted internationally and with the technical regulation in force in Brazil about the related topics.

Generic medicines were implemented in Brazil in 1999, and in 2003 a specific technical regulation was published for registration of similar/alternative medicines, as well as another regulation for adjustment of registration of similar medicines that were already being sold in Brazil. Similar/alternative medicines that were available in the market had been registered in compliance with norms that allowed their sanitary registration through the concept of similarity to a previously registered medicine, without the need to present, for the registration, results from *in vitro* or *in vivo* tests related to proof of efficacy and safety. The new regulations for similar medicines published in 2003 were targeted at establishing isonomy of criteria for registration and renovation of registration of non-innovative medicines (generic and similar), based on the precepts of quality assurance, efficacy and safety.

Pharmaceutical Equivalence and Bioequivalence are criteria applicable to generic and similar medicines. Bioequivalence may also be necessary for innovative medicines when changes or adjustments are made to the formulation during the clinical development for extrapolation of efficacy and safety data. There are also cases where Bioequivalence may replace phase II and III clinical trials for registration of medicines with new concentrations, under new pharmaceutical dosage forms and new associations.

For registration of a generic or similar medicine, the pharmaceutical industry must request to the Brazilian Health medicine Agency (Anvisa) the indication of the reference medicine for conducting the necessary assays for developing the formulation, the pharmaceutical preparation and the manufacturing process, establishing the conditions for stability tests and specifications of the medicine, to prove its Pharmaceutical Equivalence (*in vitro*) and Bioequivalence (*in vivo*) with the reference medicine, a mandatory condition to prove the Therapeutic Equivalence between the candidate to generic or similar and the reference medicine (usually, the innovative medicine which bioavailability is known and the clinical efficacy and safety were proven for the sanitary registration).

The Therapeutic Equivalence between the generic medicine and the reference medicine, or between the similar medicine and the reference medicine, allows the exchangeability in the moment the pharmacist sells the medicine. When the medicines are considered Therapeutic Equivalents, it is

assumed that both will present the same efficacy and safety when being administered to the body, and also have the same potential to cause adverse effects.

PHARMACEUTICAL EQUIVALENCE

The Pharmaceutical Equivalence corresponds to the evidence that two medicines are equivalent in relation to results from tests *in vitro*. By definition, Pharmaceutical Equivalents are medicines that have the same drug, that is, the same salt or ester from the same therapeutically active molecule, the same pharmaceutical preparation and route of administration, and are identical in relation to potency or concentration.

They must be formulated to meet the same updated specifications from the Brazilian Pharmacopoeia and, in their absence, specifications from other codes authorized by the current legislation, or also other applicable quality standards, related to identity, content, purity, potency, uniformity of content, disintegration and dissolution time, as the case may be. However, they may differ in characteristics such as aspect, excipients, mechanism of release, package, validity period and, within certain limits, labeling.

Pharmaceutical Equivalence studies are targeted at assessing the quality of medicines through comparative analysis between the test medicine and the reference medicine, and must necessarily be conducted by laboratories authorized by Anvisa. Additionally, the studies must be conducted in samples within their validity period, using Chemical Reference Substances from the Brazilian Pharmacopoeia, made official through Resolution of the Collegiate Board of Anvisa or originated from other pharmacopoeias. If such substances do not exist, the use of Characterized Chemical Substances is admitted according to the current legislation.

The analytical methods employed for quality assessment of medicines have considerable importance in the Pharmaceutical Equivalence study. The analytical methods described in the individual monograph on the medicine present in the Brazilian Pharmacopoeia must be used, and if such monograph does not exist in this compendium, the use of methods included in other pharmacopoeias authorized by the current legislation is permitted. When there are no monographs for the product in official pharmacopoeias, the study must be conducted using validated analytical methods, complemented with assays described in general methods from the Brazilian Pharmacopoeia. The compendial analytical method or the ones transferred by the study sponsor must have their suitability demonstrated through partial validation employing, as a minimum, the parameters of selectiveness, linearity, precision and accuracy.

The Pharmaceutical Equivalence tests must be conducted simultaneously in the medicine candidate to generic or similar and in the reference medicine. It is worth highlighting that the medicine being tested must not be developed and formulated to be superior to the reference medicine, but to present the same characteristics related to drug release and to quality already established for the reference medicine. The demonstration of Pharmaceutical Equivalence between the two medicines is an indication that the candidate to generic, or similar, may present the same efficacy and safety as the reference medicine.

BIOAVAILABILITY, ABSOLUTE BIOAVAILABILITY, RELATIVE BIOAVAILABILITY, AND BIOEQUIVALENCE.

Bioavailability (BA) is defined as the speed and extension of absorption of a drug, from a pharmaceutical preparation that becomes available to make the pharmacological effect intended.

Depending on the goal and design employed in the study, the Absolute Bioavailability (ABA) of a medicine or the Relative Bioavailability (RBA) between medicines is determined.

The ABA applies to innovative medicines that are developed as pharmaceutical dosage forms for administration via extravascular routes. In general, it corresponds to a crossover assay, conducted in healthy volunteers, comprised of two periods separated by a time interval named washout.

In the first period, the volunteers are distributed randomly in two groups (A and B). Volunteers from group A receive the medicine being tested by extravascular route, while the same dose of the medicine is administered, if possible, to volunteers from group B by intravascular route. The collections of biological fluid are made according to procedures established in advance and, after the washout interval, the second period starts, repeating the procedures with the same volunteers, but inverting the groups. The drug concentrations in the samples are quantified employing a validated bioanalytical method, which allows building the curves of concentrations versus time to make the calculations of pharmacokinetic parameters in relation to the bioavailability.

When determined for a pharmaceutical preparation administered orally, for example, the ABA corresponds to the systemic fraction calculated in relation to the dose administered by intravascular route, which bioavailability is, by definition, equal to 100%. If it is possible to administer the same dose of medicine by oral and intravascular routes and the ABA calculated equals 80%, this means that the oral dose application is not complete, because there was a loss of 20% that may be related to characteristics of the drug, the individual or the formulation.

The bioavailability is calculated using the following pharmacokinetic parameters:

- area under the curve of drug concentration in the biological fluid versus time (ASC_{0-t}), which expresses the amount of drug absorbed, that is, the extension of absorption;
- maximum concentration achieved after the dose administration (C_{max}), which is related to the speed of the absorption process and occurs on the time named T_{max} (**Figure 1**).

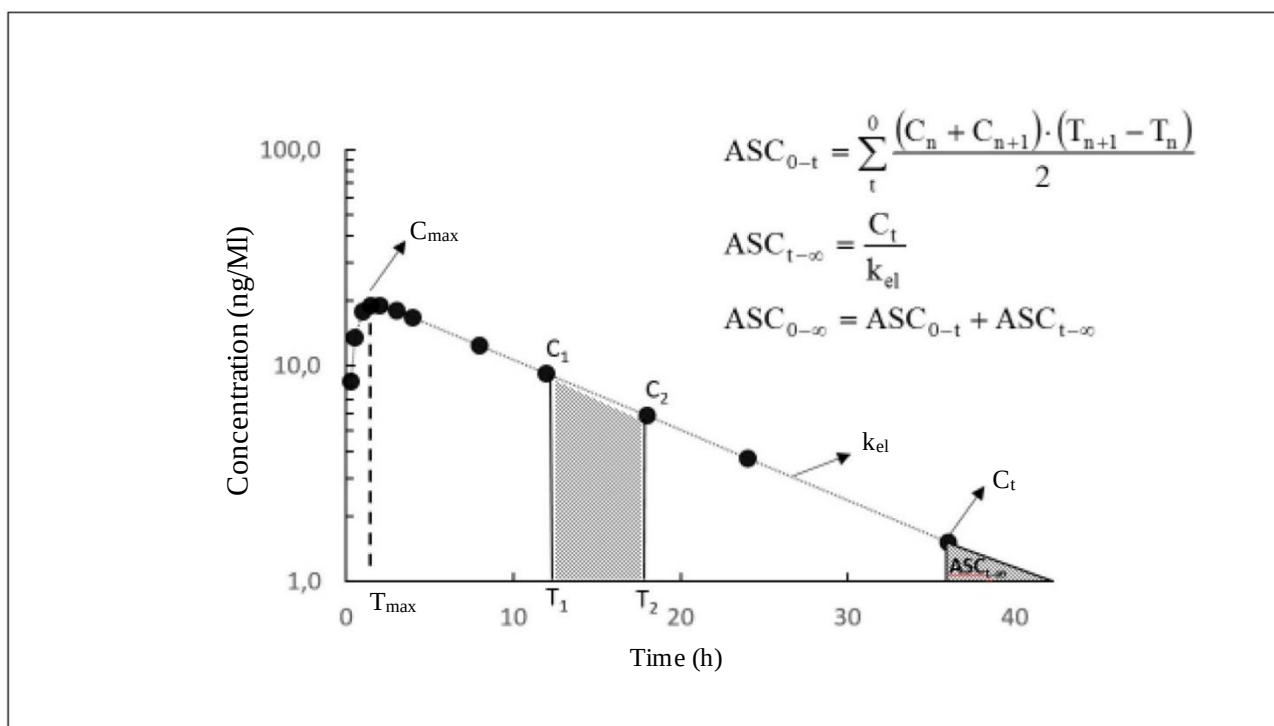


Figure 1 – Representation of curve of drug concentration on plasma with time after administering one dose of medicine by extravascular route. C_t Drug concentration in the final collection point; k_{el} . Elimination speed constant, $ASC_{t-\infty}$ Area under the curve estimated from time t to infinite.

In the case of a generic medicine or a similar medicine, the development of the formulation must be targeted at obtaining a pharmaceutical equivalent that, *in vivo*, does not present significant differences in relation to the bioavailability of the reference medicine, which is assessed by employing a RBA study adequately planned and an acceptance criterion applicable.

The Bioequivalence (BE) corresponds to a particular case of RBA and involves acceptance criterion and statistical analysis that allow to conclude on the comparison of bioavailability between two medicines with risk established in advance. Two medicines are considered bioequivalent and, therefore, exchangeable when the Confidence Intervals (CI) of 90% calculated for the ratios of geometric averages $ASC_{0-t}(T) / ASC_{0-t}(R)$ and $C_{max}(T) / C_{max}(R)$ are between 80% and 125%, considering T the medicine being tested and R the reference medicine, a criterion adopted internationally for acceptance of bioequivalence.

FACTORS RELATED TO BIOAVAILABILITY AND DISSOLUTION OF MEDICINES

In general, the main factors that may change the bioavailability of medicines are related to the individual (age, gender, body weight, associated pathophysiological factors) and to characteristics of the medicine (drug, formulation and manufacturing process). In case of factors related to the individual, their influence must be as minimized as possible, which occurs when the bioavailability assay planning is properly executed, through defined inclusion and exclusion criteria, selection of a representative group of volunteers in relation to the population for the study, and the employment of an adequate experimental design.

Among the factors related to the medicine, there are: chemical nature of the drug; solubility; particle size; polymorphism; type and amount of excipients; mixing and drying time; granulation and compression technique; instability of drug. In this sense, it is considered mandatory to conduct studies on pre-formulation and increase of scale to obtain a stable formulation, to be administered through a pharmaceutical preparation and a route that are adequate to the therapeutic goal. Thus, the professional involved in the pharmacotechnical development must have vast knowledge on the physicochemical, pharmacokinetic and pharmacodynamic characteristics of the drug, also selecting the most adequate pharmacotechnical adjuvants (excipients), as well as appropriate unit operations for manufacturing.

Among the pharmaceutical dosage forms most commonly used in therapeutics, the solid forms of oral use are those most subject to variability of bioavailability results due to characteristics of drug, formulation, processes employed in manufacturing, and route of administration. In these cases, after the administration, the drug dissolution process is essential for it to be in solution and be absorbed, and may be a limiting factor for absorption. Likewise, suspensions of oral or intramuscular use may generate challenges, since there is the drug dissolution process that suffers the influence of the factors mentioned.

DISSOLUTION PROFILE

The dissolution profile can be defined as an *in vitro* assay that allows building the curve of percentage of drug dissolved in relation to time, being proposed from conditions established in the dissolution test described on the medicine monograph included in the Brazilian Pharmacopoeia or, in its absence, in other compendia authorized by the current legislation. If the dissolution method does not exist in the pharmacopoeia, the company requesting the registration must develop an analytical method adequate to the product.

The assessment of the dissolution profile is applicable in cases of development of formulations, exemption of bioequivalence study for other dosages (possible when the bioequivalence study is conducted with another dosage and the dissolution profiles for the dosages considered are similar to the bio-batch profile), exemption of bioequivalence study by application of the Biopharmaceutical Classification System (classification of drugs according to solubility and permeability characteristics), and changes post-registration.

In case of medicines that will be submitted to the bioequivalence study, the assessment of comparative dissolution profile in relation to the reference medicine allows knowing the behavior of formulations. Similar dissolution profiles are an indicator that the test medicine may be bioequivalent to the reference medicine.

8.5 WATER FOR PHARMACEUTICAL PURPOSES

INTRODUCTION

This chapter considers as water for pharmaceutical use the various types of water used in the synthesis of drugs, in the formulation and production of drugs, in testing laboratories, diagnostics and other applications related to the health area, including as a main component in the cleaning of utensils, equipment and systems.

Water has a peculiar chemical structure, with one dipole moment and great facility to form hydrogen bonds. These properties make water an excellent medium to dissolve, absorb, adsorb or suspend different compounds, and also to carry contaminants and undesirable substances, which may change the purity and efficacy of a pharmaceutical product.

Due to its characteristics, the processes of purification, storage and distribution must ensure that the pharmacopoeia specifications are met, maintained and controlled adequately.

The water quality requisites will depend on the purpose and use, and the selection of the purification system is targeted at achieving the purity grade established. The user is responsible for selecting the type of water adequate to their goals, as well as for the necessary controls and verifications, at intervals that ensure the quality desired is maintained. They must ensure that the system presents adequate performance and capacity to provide water with the quality level established to meet the parameters specified in individual monographs.

This chapter does not exhaust the theme and does not intend to replace the legislation, guidelines or existing official monographs on water for pharmaceutical purposes. The purpose is to present subsidies that provide users with a better understanding on fundamental points related to the quality of water in the moment of obtaining it and during its distribution and use.

Controlling water contamination is crucial, since water has a high capacity to aggregate compounds and also be contaminated again after purification. Water contaminants are represented by two major groups: chemical and microbiological.

Chemical contaminants

Organic and inorganic contaminants have different origins from the feed source; extraction of materials with which water comes into contact; absorption of gases from the atmosphere; pollutant residues; or residues from products used in equipment cleaning and sanitization, among many others. This includes bacterial endotoxins, resulting from Gram-negative water microorganisms, critical contaminants that must be removed adequately.

These contaminants can be assessed, especially, by the total organic carbon – TOC (5.2.30) and conductivity (5.2.24) assays. The conductivity, measured in microsiemens/cm, is recommended to assess water with a large amount of ions and its reciprocal, resistivity, is measured in megohm.cm when there is low concentration of dissolved ions.

Most organic compounds can be removed by reverse osmosis. However, those with low molecular mass demand additional techniques, such as ion exchange resin, electrodeionization, activated charcoal, or oxidation by ultraviolet or ozone, to be removed.

The limits established for parameters of organic and inorganic chemical contaminants are targeted at protecting the health and preventing critical chemical compounds from interfering with the water system pre-treatment phase, considering that they may be difficult to remove on a later step.

Microbiological contaminants

They are represented mainly by bacteria and present a major challenge to water quality. They come from the own microbiota of the water source and from some purification devices. They may also appear due to inadequate cleaning and sanitization procedures, that allow the formation of biofilms and, consequently, install a continued cycle of growth from organic compounds that, ultimately, are nutrients for the microorganisms.

Bacteria may affect the water quality by deactivating reagents or changing substrates by enzyme action, increasing the content in TOC, changing the base line (background noise) in spectral analysis, and produce pyrogens, such as endotoxins.

The bacterial count is reported in colony-forming units per milliliter (CFU/mL) and, in general, increases with water storage time. The most frequent contaminants are Gram-negative rods, especially from the genera *Alcaligenes*, *Pseudomonas*, *Escherichia*, *Flavobacterium*, *Klebsiella*, *Enterobacter*, *Aeromonas* and *Acinetobacter*.

The microbiological standard is specified, in parallel to chemical contaminants, and consists of the absence of total and thermotolerant coliforms (pathogenic microorganisms of fecal origin), as well as enteroviruses, cysts and oocysts from protozoans, such as *Giardia* sp and *Cryptosporidium* sp in a 100 mL sample.

To comply with these limits, treatment stations use disinfection processes with chemical substances that have chlorine or other oxidants, employed for decades and considered relatively safe for humans. However, these oxidants may react with the organic material of natural origin and generate secondary products from disinfection, such as trihalomethanes, chloramines, or also leave residues from the own disinfectants. Such undesirable products require special attention from legislators and users.

Chloramines, in particular, may irreversibly damage a dechlorination equipment that is part of a purification system, and also present risk of formation and release of ammonia.

In addition to these two essential groups of contaminants, there are the particulates, comprised of silica, residues from pipes or colloids and that, in addition to being a risk to the quality of purified water, may cause clogging and severely harm the purification process, for reducing its performance, or even cause irreversible damages to equipment. They can be detected by filtration combined with gravimetry or microscopy. In general, it is not necessary to identify the type of particle, only remove it.

This chapter addresses some concerns about the main purification systems usually employed in the production of water for pharmaceutical use, their main applications, monitoring and maintenance. It also encompasses the purity parameters established for the types of water that are not addressed in the current legislation.

TYPES OF WATER

Basically, there are three types of water for pharmaceutical use: purified water (PW), water for injection (WFI), and ultrapure water (UPW), which monographs are included in this Pharmacopoeia. Official international compendia specify, in addition to them, other types of water, such as: contained in vials, sterile or bacteriostatic, for irrigation or inhalation. However, all of them have purity characteristics similar to the fundamental types already mentioned.

In addition to them, there is potable water, which is widely used and has direct application in pharmaceutical facilities, especially in general cleaning procedures. Thus, the four types of water below are considered, in relation to their main characteristics and suggestions of application. The specific monographs, when available, detail the purity parameters established for each type.

Potable water

As a fundamental guideline, the starting point for any process of purification of water for pharmaceutical purposes is potable water. It is obtained by treatment of water taken from springs, through adequate processes to meet specifications from the Brazilian legislation related to physical, chemical, microbiological and radioactive parameters, for a certain standard of potability and, therefore, has no specific monograph in this compendium.

Potable water is usually employed in the initial steps of cleaning procedures and as a source for obtaining water of the highest purity grade. It may also be used in the thermal climate control of some apparatuses and in the synthesis of intermediate ingredients.

The strict control and the maintenance of compliance with water potability parameters are fundamental, critical and of responsibility from the user of the purification system that will be fed. The control must be periodical to ensure that the purification system used is adequate for the conditions of the feed source and that there was no change in the quality of the water provided. However, most applications require additional potable water treatments, whether through distillation, deionization, ion exchange, reverse osmosis, isolated or coupled, or another adequate process to produce purified water, free from interference of contaminants that may affect the quality of the medicines produced.

Purified water (PW)

Purified water is produced from potable water and must meet the specifications established on the respective monograph. It does not have any other substance added. It is obtained by a combination of purification systems in a logical sequence, such as: multiple distillation; ion exchange; reverse osmosis; electrodeionization; ultrafiltration; or another process capable of meeting, with the desired efficiency, the limits specified for different contaminants.

It is employed as excipient in the production of non-parenteral pharmaceutical dosage forms and in compounding formulations, provided there is no recommendation of higher purity in its use or that it does not need to be pyrogen-free. It can also be used in washing material, preparation of reagent solutions, culture media, buffers, different dilutions, general microbiology, clinical analyses, Elisa or radioimmunoassay techniques, different applications in most laboratories, especially in less demanding qualitative or quantitative analysis (determinations in percentage). It is used in assays and determinations that indicate the use of water, unless specified otherwise concerning the level of purity required, such as, for example, some instrumental analytical methods and analysis that require

apyrogenic water or higher chemical purity. It may be employed in high-performance liquid chromatography, when confirmed that its use does not affect the accuracy or precision of results.

Depending on the application, it may be sterilized, without necessarily achieving the limit of bacterial endotoxins established for *Water for injection*.

It needs monitoring on the count of total viable aerobic organisms, in production and storage, since it has no added growth inhibitor. As a minimum, it is characterized by a conductivity of no more than 1.3 $\mu\text{S}/\text{cm}$ at 25.0 $^{\circ}\text{C}$ (resistivity $> 1.0 \text{ MW}\cdot\text{cm}$) and $\text{TOC} \leq 0.50 \text{ mg/L}$ and total bacterial count $\leq 100 \text{ CFU/mL}$, unless specified otherwise. The entire system for obtaining, storing and distributing must be duly validated and monitored for conductivity and microbial count parameters.

Even if a maximum microbial count of 100 CFU/mL is specified in the monograph, each facility must establish its alert or action limit, if the specific characteristics of use are more restrictive.

Ultrapure water (UPW)

Ultrapure water has low ion concentration, low microbial burden, and low TOC level. This type of water is required in more demanding applications, especially in test laboratories, for dilution of the reference substances, in quality control, and in the final cleaning of equipment and utensils used in processes that come into direct contact with the sample that requires water with this level of purity. It is ideal for analysis methods that require minimum interference and maximum precision and accuracy. The use of ultrapure water in quantitative analysis of low analyte contents is essential for obtaining precise analytical results. Other examples of application of ultrapure water are: analysis of residues, such as traces of mineral elements, endotoxins, preparations of calibrators, controls, chemical reference substance, atomic absorption spectrometry in general, ICP/IOS, ICP/MS, mass spectrometry, enzyme procedures, gas chromatography, high-performance liquid chromatography (determination of residues in ppm or ppb), methods in molecular biology and with cell culture, etc. It must be used at the moment it is produced, or in the same day of collection.

The laboratory must use the same type of water required for final reading of analysis in the preparation of samples, when obtaining the standard curve, controls, preparation of solutions, blanks, final material washing, and in all glassware that will come into direct contact with the sample, whenever appropriate.

Ultrapure water is characterized by a maximum conductivity of not more than 0.055 $\mu\text{S}/\text{cm}$ at 25.0 $^{\circ}\text{C}$ (resistivity $> 18.0 \text{ MW}\cdot\text{cm}$), $\text{TOC} \leq 0.50 \text{ mg/L}$, endotoxins $< 0.25 \text{ EU/mL}$ (when high biological quality is required) and total bacterial count $\leq 10 \text{ CFU}/100 \text{ mL}$.

Water for injection (WFI)

Water for injection is used as excipient in the preparation of parenteral pharmaceutical products of small and large volume, in the manufacturing of active ingredients of parenteral use, sterile products, other products that require control of endotoxins and are not submitted to a later step of removal. It is also used in cleaning and preparation of processes, equipment and components that come into contact with drugs and sterile medicines during their production.

The purification process of first choice is distillation, in an equipment with internal walls made of appropriate metal, such as AISI 316L stainless steel, neutral glass or quartz. Alternatively, WFI can be obtained by a process equivalent to distillation or higher for removal of chemical contaminants and microorganisms, provided that validated and monitored for the parameters established. The feed

water must be, as a minimum, potable and, in general, will need to be pre-treated to feed the equipment. The process is thus specified due to the robustness such equipment presents concerning operation and performance.

Water for injection must comply with the physicochemical tests established for purified water, as well as with tests on total bacterial count ≤ 10 CFU/100 mL and bacterial endotoxins, which value must be lower than 0.25 EU/mL.

Some quality parameters and suggestions for applications are registered on **Table 1**, for each type of water for pharmaceutical use.

Table 1 – Types of water for pharmaceutical use and quality parameters.

<i>Type of water</i>	<i>Characteristics</i>	<i>Critical parameters suggested</i>	<i>Examples of application</i>
Potable water	Obtained from springs or from the public distribution network.	Has specific legislation.	General cleaning and treatment system feed source.
Purified water	Variable levels of organic and bacterial contamination. Requires care to avoid chemical and microbiological contamination. May be obtained by reverse osmosis or by a combination of techniques of purification from potable water.	Maximum conductivity of 1.3 $\mu\text{S}/\text{cm}$ at 25.0 °C (resistivity > 1.0 $\text{M}\Omega\text{-cm}$); TOC $\leq$ 0.50 mg/L; Count of total number of heterotrophic bacteria: maximum 100 CFU/mL; Absence of <i>Pseudomonas sp</i> and coliforms.	Production of medicines and cosmetics in general, pharmaceutical dosage forms, washing material, preparation of reagent solutions, culture media, buffers, different dilutions, general microbiology, clinical analysis, Elisa techniques, radioimmunoassay, different applications in most laboratories, especially in less demanding qualitative or quantitative analysis (in %). In HPLC (in %).
Water for injection	Purified water treated by distillation or similar process.	Meets the chemical requirements from purified water and requires control of endotoxins. Count of total number of heterotrophic bacteria: maximum 10 CFU/100 mL. Endotoxins < 0.25 EU/mL. Absence of <i>Pseudomonas sp</i> and coliforms.	As vehicle or solvent for injectables, manufacturing of active ingredients of parenteral use, final washing of equipment, tubes and containers used in parenteral preparations. Use as diluent of parenteral preparations.
Ultrapure water	For analysis that require minimum interference and maximum precision and accuracy. Low ion concentration, low microbial burden, and low total organic carbon level. Purified water treated by complementary process.	Maximum conductivity of 0.1 $\mu\text{S}/\text{cm}$ at 25.0 °C (resistivity > 18.0 $\text{M}\Omega\text{-cm}$); TOC $\leq$ 0.50 mg/L; Count of total number of heterotrophic bacteria: maximum 10 CFU/100 mL. Endotoxins: < 0.25 EU/mL (when high biological quality is required) Absence of <i>Pseudomonas sp</i> and coliforms.	Dosage of mineral or organic residues, endotoxins, preparations of calibrators, controls, CRS, atomic absorption spectrometry, ICP/IOS, ICP/MS, mass spectrometry, enzyme procedures, gas chromatography, HPLC (ppm or ppb), molecular biology and cell culture, etc. Eventually in pharmaceutical preparations that require high-purity water.

TOC = Total organic carbon;

CFU = Colony-forming units; viable microbiological population.

WATER PURIFICATION SYSTEMS AND PURIFICATION TECHNOLOGIES

Designs, installations and operations of systems for production of purified water (PW), ultrapure water (UPW) and water for injection (WFI) have similar components, controls and procedures. The difference is in the presence of the parameter bacterial endotoxins on water for injection and in their methods of preparation, specifically in the final stage. These similarities of quality parameters allow to establish a common base for the design of systems targeted at obtaining PW, UPW or WFI, being differential critical points the degree of control from the system and the final stages of purification necessary for removing bacteria, bacterial endotoxins and reducing conductivity.

The processes for obtaining employ sequential unit operations – the purification stages – that are targeted at removing certain contaminants and protecting the subsequent purification stages. Notice that the final unit operation for obtaining water for injection is limited to distillation or another equivalent process or higher, in the removal of chemical contaminants, as well as microorganisms and their components. The distillation technique is renowned for its long history of reliability and can be validated for production of water for injection. However, other technologies or combination of technologies may be equally effective and validated for this purpose. Ultrafiltration placed in a sequence after other chemical contaminant purification technologies may be adequate for the production of water for injection, if it demonstrates the same effectiveness and reliability of distillation in the validation.

For producing water for injection, there are new and promising applications that can be validated thanks to the development of new materials for technologies such as reverse osmosis and ultrafiltration, that allow operating and sanitizing at a higher temperature, allowing a more effective microbial reduction.

The project for installing a water purification system must take into account the quality of the supply water and the desired water at the end, the necessary flow, the distance between production system and points of use, the layout of tubes and connections, the material employed, technical assistance and maintenance facilities, and the adequate instruments for monitoring.

The purification technologies are targeted at removing contaminants in different stages of the purification sequence. The main technologies presented below are in a logical sequential order, but the selection of which ones will be used and the order they are applied will depend on the quality of the potable water of admission and the type of water one aims at obtaining.

Pre-filtration

Also known as depth filtration or initial filtration, it is targeted at removing particulate contaminants in the size range from 5 to 10 μm , essentially to protect subsequent technologies, using sand filters or combination of filters.

Adsorption by vegetable activated charcoal

This technology employs the adsorption capacity of vegetable activated charcoal in contact with organic compounds or contaminants, such as chloramines. Additionally, it removes oxidizing agents by chemical reduction, especially free chlorine, which affects other membrane-based technologies, such as reverse osmosis or ultrafiltration.

The removal of sanitizing agents favors bacterial growth and formation of biofilm, which implies the need for sanitization of the activated charcoal, with direct vapor or hot water, for example, and for control of particles and microbial count of its effluent.

Treatment with chemical additives

The use of chemical additives refers to the ones targeted at adjusting the pH or removing carbonates and ammonia, for protection of other technologies, such as reverse osmosis.

As chemical additives, ozone, commonly used in the control of microorganisms, and metabisulfite, applied as reducer agent for free chlorine, may be used as substitutes to vegetable activated charcoal.

Chemical additives are necessarily removed in some later stage of purification and cannot leave residue in the final water.

Treatment with softeners

In cases where the feed water is “hard”, it is necessary to use softeners. This technology employs regenerable ion exchange resins, which capture calcium and magnesium ions and release sodium ions in water. Softening is used in the protection of technologies sensitive to incrustation, such as reverse osmosis.

It is necessary to control the microbial count, with frequent regeneration, recirculation or other forms of reduction of microbial count, to avoid the formation of biofilm.

Deionization and continuous electrodeionization

Deionization and continuous electrodeionization are effective technologies for removal of dissolved inorganic salts. Deionization systems, also known as conventional deionization, produce purified water for routine use, through specific ion exchange resins for cations or for anions. They are organic polymers, generally sulfonated, in the form of small particles. Cationic resins capture ions releasing the ion H^+ in water, and anionic resins release OH^- . They are regenerable with acids and bases, respectively. This process alone does produce high purity water, due to leak of small fragments of resin, facility of microbial growth, and low removal of organics.

Continuous electrodeionization systems combine cationic and anionic resins with semipermeable membranes and the application of an electric field, promoting the continuous removal of ions, that is, without the need to stop for regeneration. In both cases, it is necessary to have control over the generation of particles resulting from successive regenerations, as well as microorganisms. This can be done by controlling regenerations, in the case of deionization, using water recirculation and applying UV radiation for control of microorganisms in the outlet, which effectiveness must be proven.

Reverse osmosis

Reverse osmosis is a purification technology based on semipermeable membranes and with special properties for removal of ions, microorganisms and bacterial endotoxins. It removes 90% to 99% of most contaminants. However, different factors, such as pH, differential pressure along the membrane, temperature, type of polymer of the membrane, and the construction of the reverse osmosis cartridge may significantly affect this separation.

Reverse osmosis membranes must be duly control for the formation of incrustations coming from calcium salts, magnesium salts and other salts, and of biofilm, a critical source of microbial contamination and endotoxins. For this reason, it is mandatory to install a pre-treatment system before reverse osmosis that removes particles and oxidizing agents and, in parallel, the system sanitization

must be conducted periodically. This practice helps increase the work life of membranes and reduces the frequency of their regeneration.

There are double pass reverse osmosis systems, where water purified by the first step feeds the second step, increasing and complementing the purification.

Ultrafiltration

Ultrafiltration is frequently used in systems of water for pharmaceutical use for removal of endotoxins. The ultrafiltration is conducted using a special membrane with the property of retaining molecules according to their molecular mass and stereochemistry. The range used for particle separation is named Molecular Weight Cutoff, characterized by the size of molecular mass. Filters in the range of 10.000 Da, which retain molecules with molecular mass of 10,000 Da or higher are used in the removal of endotoxins.

This technology can be used in a final or intermediate step of the purification system, provided that validated, and, like reverse osmosis, requires pre-treatment, adequate control of the operating conditions, and adequate cleaning and sanitization procedures, to maintain the water quality as established.

Filtration with electrostatic charge

This type of filtration employs positive charges on the surface of membranes and is targeted at reducing the levels of endotoxins that have negative electric nature. It presents marginal capacity for removal of microorganisms, but its greater efficiency is due to the removal of endotoxins. It has an important limitation: when the charges are completely neutralized, by saturation due to the capture of endotoxins, the removal is paralyzed. For this reason, filters with electrostatic charge are extremely difficult to validate, due to this unpredictability concerning the moment they effectively no longer retain such contaminants.

Microfiltration – removal of microorganisms

This technology uses microporous membranes, with pore size specification of 0.2 or 0.22 µm. They must be validated for retention, through a bacteriological test, which determines the value of the log reduction of microorganisms on the membranes. The model used employs a suspension of *Brevundimonas diminuta* at 10⁷ CFU/cm² of filtering area and tests the sterility of the filtrate. Even if the membrane is specified as 0.2 or 0.22 µm of pore diameter, it will not necessarily be sterilizing if it does not produce a sterile filtrate through this test, that is, a log reduction value equal to 7. If the log reduction obtained is not around seven, the membrane can be used to reduce the microbial count, but must not be used to sterilize.

Microfiltration is applied to the filtration of gases or ventilation of storage tanks, to avoid the contamination of water stored in them. In such cases, hydrophobic membranes are used, for the filter to operate without condensed water buildup, from air humidity.

Ultraviolet radiation (UV)

UV radiation is used in water purification systems in two wavelengths: 185 nm and 254 nm, which promote two effects:

- 185 nm and 254 nm – Oxidation of organic compounds and consequent reduction of their concentration, to achieve the limits from PW, UPW and WFI;

- 254 nm – Germicide action at different points of the purification sequence, to reduce microbial count.

For the oxidation of organics, the water must be on the final stage of purification, and this removal will be more effective the lower the contaminant load is. It is necessary to monitor the intensity of the lamp and consider the depth/thickness of the bed and the water flow at the radiation site.

Distillation

In industrial facilities, there may be simple, multiple effect and vapor compression distiller, which are generally used for large volume production systems. Feed water for such equipment requires controls different from the ones used in reverse osmosis. In this case, the concentration of silicates is critical, like in any vapor generation system. Another important aspect is the possibility of carrying volatile compounds in the condensate. This is specially important when it comes to organic impurities, such as trihalomethanes and gases dissolved in water, such as carbon dioxide and ammonia. Thus, the control of inlet potable water is essential, as mentioned about feed water for purification systems.

DISTRIBUTION, SANITIZATION, STORAGE AND VALIDATION

Distribution

The distribution system design must take into account the constant recirculation of purified water and water for injection and the maintenance of temperature of the water stored in the tank. If necessary, it must have a heat exchanger to provide colder water to the points of use.

Tubes, valves, instruments and other devices must have sanitary construction and finishing, so that they do not contribute for microbial contamination and are sanitized.

Microbiological retention filters must not be used on the outlet or in the return from distribution systems, because they are deposits of retained microorganisms and, therefore, a critical source for the formation of endotoxins. The points of use must be designed in order to avoid dead volumes and allow water to completely recirculate in them when they are closed.

Sanitization

There are different methods for sanitization of production, storage and distribution systems. The system construction material must be resistant to the agents employed and the temperature used in the process is critical. It is common to use temperatures of 80 °C or 65 °C, with continuous water circulation. However, to prevent the formation of biofilms, a combination of heat and chemical agents is usually employed in sanitization. The sanitization procedure must be duly validated.

Oxidizing agents, such as halogenated compounds, hydrogen peroxide, ozone or a combination of them, are usually employed as chemical agents. The frequency of sanitization is determined by the history of results from monitoring and trend curves, so that the system works without exceeding the alert limit.

Storage

The storage conditions must be adequate to the water quality. Ultrapure water must not be stored for a period larger than 24 hours. The fundamental guideline for storage of purified water, ultrapure water or water for injection is to weight that, the higher the water purification grade, the faster it tends to recontaminate.

Thus, water must be maintained in constant recirculation, through its distribution system, whenever applicable. The first portions of water produced by a purification system that has been inactive for more than four hours must be discarded proportionally to the dead volume of the container. Such variables must be validated for the specific conditions of each system, and the parameters to be considered in the validation must also be established.

The reservoir used for its maintenance must be appropriate to the purposes it is targeted at, being comprised of inert, clean material and not be a source of contamination to the content. The construction material must present appropriate characteristics and rugosity to prevent the adherence of residues, formation of biofilm and corrosion by sanitizing agents. Electropolished 316L stainless steel, with rugosity below 0.5 microRA, is the most frequent choice to meet these requirements. The reservoir must be protected from improper sources of light and heat, and the geometry must allow its total drainage from the bottom, without dead volumes.

Adequate procedures must be adopted to avoid contamination by particulates, organics or microorganisms. They must have an “exhaust”/ventilation filter to prevent contamination of the tank volume by admission of contaminated air/humidity and avoid recontamination by this route.

In particular, but not exclusively, reservoirs of water for injection must be sleeved to maintain the circulating water at temperature higher than 80 °C, which significantly restricts the bacterial growth.

Validation

The fundamental purpose of validation is to ensure the reliability of a water purification system, involving acquisition, storage, distribution and quality at the point of use. Validation includes the project qualification (PQ); facility qualification (FQ); operation qualification (OQ); and performance qualification (PQ).

The validation plan for a water system involves the following phases:

- a) knowing the feed source quality standard;
- b) establishing the purified water quality standard;
- c) defining the purification technologies and their sequence, from inlet water quality;
- d) selecting the construction materials for the production, storage, distribution and monitoring systems of the points of use;
- e) developing protocols for qualification of design, installation, operation and performance;
- f) establishing critical parameters, alert and action levels, and periodicity of sanitization and monitoring;
- g) establishing a maintenance plan for validation, which will include mechanisms for control of changes to the water systems and will provide subsidies for a preventive maintenance program.

The qualification protocols must be approved before their execution.

WATER QUALITY MONITORING

The process employed in the production of water for pharmaceutical use must be validated and, systematically, the parameters established in the legislation and in the specific monographs for each type of water must be verified.

Water quality monitoring must encompass all critical and representative points of the system, according to the planning established, in a consistent and continuous manner. Water quality monitoring must be conducted through physicochemical and microbiological analysis.

Thus, it is necessary to establish operating and sanitization procedures, an encompassing monitoring program, with preventive maintenance, and a change control system, which determines judiciously whether the system will need to be revalidated after any modification. Seasonal issues that may affect the quality of the water from the supply source must be considered in the development of the plan. The frequency of sample collection is defined in the system validation, as well as the necessary assays to ensure the maintenance of water quality required. Any change to the original plan must be reassessed.

Equipment and apparatuses used in verifications must be capable of providing the reading in the range required for the purity established. The equipment used must be duly calibrated. The verifications made must be registered in an adequate form, which includes, as a minimum, the parameter(s) measured, measurement date, value obtained, acceptance range, and person responsible for the reading. The personnel who executes this task must know the sampling plan and the methods used, as well as the alert and action limits established. If the user outsources this control, they must ensure that the outsourced party meets the requirements and procedures defined.

The data obtained are compared with typical specifications and with alert and action limits. They are established by the user, based on validation data, history of the purification and distribution system, and quality requirements for a certain application.

The user must define the alert and action limits, to avoid obtaining a product with quality specification inferior to the one required for a given application. The alert limit indicates that a deviation in quality may happen and not necessarily requires a corrective measure. It may be established based on a statistical analysis of the history of trends, using two standard deviations, for example, or approximately 70% of the action limit, or 50% of the count of number of viable units, whichever is lowest. The action limit indicates that the quality deviation exceeded the tolerable parameters and requires activity interruption for correction. Non-compliant results require extraordinary intervention at the site, in addition to regular operations, to restore the system so that the quality standard expected is maintained.

PHYSICOCHEMICAL MONITORING

The physicochemical monitoring follows, especially, the conductivity and total organic carbon, which can also be monitored in line. These assays cover a large number of inorganic contaminants. If the sample is not analyzed after the collection, it must be maintained and stored in conditions that guarantee its integrity and preservation for an adequate period. Depending on the application required, the critical parameters to be monitored may vary.

MICROBIOLOGICAL MONITORING

The importance of controlling water for pharmaceutical use is in ensuring its quality in order to comply with the parameters determined in each monograph and avoid contamination carrying to the products. Thus, it requires strict microbiological quality control, since, due to its intrinsic characteristics and the processes involved in its production, it is highly susceptible to microbial contamination.

The target of microbiological control are pathogenic bacteria, with the need for identifying or selecting certain species of microorganisms that may be harmful to processes and products, such as, for example, *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Escherichia coli* and *Salmonella* sp. Tests intended for microbiological monitoring of water for pharmaceutical use are described on *Microbiological assays on water for pharmaceutical use (5.5.3.6)*.

8.6 CHEMICAL REFERENCE SUBSTANCES

According to the WHO definition, pharmacopeial reference standards (PRef) are products of recognized uniformity, intended for use in tests where one or more of their properties will be compared with those of the substance under examination. They have a degree of purity suitable for their intended use.

The PRef is established and distributed by pharmacopeial authorities, whose value attributed to one or more of its properties is accepted without the need for comparison with another standard, intended for use in specific tests described in pharmacopoeial monographs. They include chemical reference substances, biological products, plant extracts and powders, radiopharmaceuticals, among others. The most commonly used related expression is: Pharmacopoeial Chemical Reference Substance.

GENERAL ASPECTS OF CHEMICAL REFERENCE SUBSTANCES FROM THE BRAZILIAN PHARMACOPOEIA (CRS-BP)

The Chemical Reference Substances from the Brazilian Pharmacopoeia (CRS-BP) are pharmacopoeial reference standards, whose production is under coordination by the Thematic Technical Committee on Chemical Reference Substance (CTT CRS) from the Brazilian Pharmacopoeia, in line with guidelines from the Brazilian Pharmacopoeia Commission.

The CRS-BF are established and monitored according to principles from the WHO, with collaboration from public and private laboratories, through interlaboratory studies that use an analytical protocol developed and validated in advance, originating a product with high quality, whose value attributed to one or more of its physical and/or chemical properties does not need comparison with another CRS.

Analytical methods frequently use sophisticated equipment to facilitate the precision and agility of the procedure used, based on relative measures, that need reference standards for obtaining results.

The CRS-BF are developed to help in the conduction of assays described in monographs from the BF. Their purity grade may vary according to the assay it is targeted at. The stated value is specific to the assay described on the BF.

The CRS-BF must be stored and handled adequately in order to obtain reliable results when used. They must be stored in the original vials, closed and at temperature and humidity conditions according to specifications included in the label and/or certificate of analysis.

The amounts provided in each vial of CRS-BF are adequate for a certain number of analysis, in order to avoid issues with excessive exposure of material. However, the amounts and their value are targeted at stimulating the direct use of CRS-BF, without the need to establish derivative standards.

When drying the material before use is indicated, this procedure will never be conducted in its original package, but by transferring part of the material to another container. After use, the desiccated material must not be returned to the original vial, avoiding possible contaminations.

The validity of a certain batch must be monitored by the user through the Brazilian Pharmacopoeia website, which will inform the batch in force, the removal of batches in use and the availability of new batches. This website also offers information for acquisition of pharmacopoeial reference standards.

8.7 COLORING AGENTS

Coloring agent is any organic or inorganic compound, natural or synthetic, which, regardless of having pharmacological activity or not, is added to medicines, foods, cosmetics or correlates with the single purpose of coloring them or changing their original color.

Two types of coloring agents are used:

- dyes/colorants;
- pigments.

The basic difference between pigments and dyes is in the particle size and solubility in the medium it is inserted. Pigments, in general, have larger particle size and are insoluble in water, while dyes are water-soluble molecules. It is possible to state that dyes are employed in solutions, and pigments, in suspensions. Additionally, pigments have higher chemical and thermal stability than dyes.

The solubility of the dye can be determined by the presence of certain chemical groups in the structure of the compound, which may cause differentiations between pigments and dyes.

The dyes used are mostly of synthetic origin and can be, in general, classified in one of the seven chemical groups described below:

- Indigoid Group;
- Xanthine Group;
- Azo Group;
- Nitro Group;
- Triphenylmethane Group;
- Quinolone Group;
- Anthraquinone Group.

Dyes can also be divided into azoic dyes (which have $-N=N-$ groups) and non-azoic dyes (belonging to a wide range of chemical classes). Most dyes of more frequent use are of the non-azoic type, with erythrosine, indigo / carmine and quinoline yellow being the three most widely known.

Concerning pigments, two types are used: iron oxide (black, red and yellow), and titanium dioxide, which is white and also used in tablet coating, to prevent the photodegradation of components from the formulation that are sensitive to light, or also to obtain opaque capsule shells.

Dyes can be classified, according to the Food and Drug Administration (FDA), into:

- dyes designated as Food, Drug and Cosmetics (FD&C) can be employed in foods, medicines and cosmetic products;
- dyes designated as Drugs and Cosmetics (D&C) are authorized for use in medicines and cosmetics;
- D&C dyes of external use have their employment restricted to medicines and cosmetics applied externally;

Coloring agents included on the table below (**Table 1**) or a mixture of these agents can be added to medicines targeted at application via oral, rectal, vaginal or cutaneous route in cases and in amounts compatible with good pharmaceutical manufacturing practices.

The coloring agents employed must meet the requirements described in the respective monographs.

This table also identifies dyes for use in correlates such as: contact lenses, general surgery sutures, sutures for ophthalmic surgery, intraocular lenses (for haptic coloring), bone cement, and gelatinous contact lenses (to identify left or right size).

Table 1 – List of dyes permitted.

<i>Color</i>	<i>Dye</i>	<i>CAS</i>	<i>Synonym</i>	<i>Name in English</i>	<i>Description (IUPAC)</i>	<i>Color Index</i>	<i>Reference 21 CFR</i>	<i>Reference European Union</i>	<i>Uses, restrictions and requirements</i>
Yellow	Sunset yellow	2783-94-0	yellow 6 INS 110	FD&C yellow #6	disodium (5E)-6-oxo-5-[(4-sulfonatophenyl)hydrazinylidene]naphthalene-2-sulfonate	15985	741.706	E110	Used on foods (including nutritional supplements), cosmetics and medicines in general. Use not permitted in the eye area, in medical devices, and in injectable pharmaceutical dosage forms.
Yellow	sunset yellow, aluminum lake	15790-07-5	yellow 6 aluminum lake INS 110	FD&C yellow #6 aluminum lake	aluminum 6-oxido-5-(4-sulfonatophenyl)diazenylnaphthalene-2-sulfonate	15985:1	741.706	E110	Used on foods, cosmetics and medicines in general. Use not permitted in the eye area, in medical devices, and in injectable pharmaceutical dosage forms.
Yellow	quinoline yellow	8004-92-0	yellow 10	D&C yellow #10; quinolone yellow	2-(2-quinolyl)-1,3-indandione disulfonic acid disodium salt	47005	74.1710 74.3710	E104	Used on cosmetics and medicines in general. Use not permitted in the eye area, in medical devices, and in injectable pharmaceutical dosage forms. It can be used for coloring contact lenses in amounts that do not exceed the reasonably necessary minimum to achieve the coloring effect intended.
Yellow	quinoline yellow, aluminum lake	68814-04-0	yellow 10 aluminum lake	D&C yellow #10 aluminum lake; quinoline yellow aluminum lake	aluminum; 2-(2-quinolyl)-1,3-indandione disulfonic acid disodium salt	47005:1	74.1710 74.3710	E104	Used on cosmetics and medicines in general. Use not permitted in the eye area, in medical devices, and in injectable pharmaceutical dosage forms. Used in contact lenses.
Yellow	Fluorescein yellow	2321-07-5	fluorescein; yellow 7	D&C yellow #7; fluorescein	3',6'-dihydroxyspiro [2-benzofuran-3,9'-xanthene]-1-one	45350:1	741707	N/C	Used on cosmetics and medicines of topical application. Use not permitted in the eye area, in medical devices, and in injectable pharmaceutical dosage forms.

<i>Color</i>	<i>Dye</i>	<i>CAS</i>	<i>Synonym</i>	<i>Name in English</i>	<i>Description (IUPAC)</i>	<i>Color Index</i>	<i>Reference 21 CFR</i>	<i>Reference European Union</i>	<i>Uses, restrictions and requirements</i>
Yellow	curcumin	458-37-7	Curcumin yellow; turmeric	Turmeric oleoresin	(1E,6E)-1,7-bis (4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3,5-dione	75300	73615	E100	Used on foods.
Yellow	yellow iron oxide	51274-00-1	yellow iron oxide	yellow iron oxide	iron oxides obtained by synthesis, including they hydrated forms or combinations of more than one of these oxides	77492	731.200	E172	Used in medicines administered orally (not exceeding the daily dose of 5 mg of Fe) and topical use. Not permitted in the eye area, in medical devices, and in injectable pharmaceutical dosage forms.
Yellow	riboflavin	83-88-5	vitamin B2 lactoflavin	riboflavin	7,8-dimethyl-10-[(2S,3S,4R)-2,3,4, 5- tetrahydroxypentyl] benzo[G]pteridine-2,4-dione	N/C	73.450	E101	Used on foods.
Yellow	tartrazine	1934-21-0	tartrazine yellow; yellow 5 INS 102	FD&C yellow #5	trisodium (4E)-5-oxo-1-(4- sulfonatophenyl)-4-[(4-sulfonatophenyl) hydrazinylidene] pyrazole-3-carboxylate	1910	741.705	E102	Used on foods, cosmetics and medicines of internal and external use. Not permitted in medical devices and in injectable pharmaceutical dosage forms. Products for human use by oral, nasal, rectal or vaginal route, or for use in the eye area, must specifically state the dye on the label.
Yellow	tartrazine, aluminum lake	12225-21-7	tartrazine yellow aluminum lake; yellow 5 aluminum lake INS 103	FD&C yellow #5 aluminum lake	aluminum; 4-[[[3-carboxy-5-oxo-1-(4- sulfophenyl)-4H-pyrazol-4-YL]diazenyl] benzenesulfonate; 4-[[[3-carboxy-5-oxo-1-(4-sulfophenyl)-4H-pyrazol-4-YL]diazenyl] benzenesulfonate	19140:1	741.705	E102	Used on foods, cosmetics and medicines of internal and external use. Not permitted in medical devices and in injectable pharmaceutical dosage forms. Products for human use by oral, nasal, rectal or vaginal route, or for use in the eye area, must specifically state the dye on the label.

<i>Color</i>	<i>Dye</i>	<i>CAS</i>	<i>Synonym</i>	<i>Name in English</i>	<i>Description (IUPAC)</i>	<i>Color Index</i>	<i>Reference 21 CFR</i>	<i>Reference European Union</i>	<i>Uses, restrictions and requirements</i>
Blue	Brilliant blue	3844-45-9	blue n. 1 INS 133	FD&C blue #1	disodium 2-[[4-[ethyl- [(3-sulfonatophenyl)methyl]amino] phenyl]-4-[ethyl-[(3-sulfonatophenyl)methyl]azanumylidene] cyclohexa-2,5-dien-1-ylidene]methyl] benzenesulfonate	42090	741.101	E133	Used on foods (including nutritional supplements), cosmetics and medicines in general, including the eye area. Use not permitted in medical devices and in injectable pharmaceutical dosage forms.
Blue	brilliant blue, aluminum lake	68921-42-6	blue n. 1 aluminum lake INS 133	FD&C blue #1 aluminum lake	3-[[ethyl-4-[[4-[ethyl-[(3-sulfophenyl)methyl]amino]phenyl]-(2-sulfophenyl)methylidene] cyclohexa-2,5-dien-1-ylidene]azaniumyl] methyl] benzenesulfonate	42090:2	741.101	E133	Used on foods (including nutritional supplements), cosmetics and medicines in general, including the eye area. Use not permitted in medical devices and in injectable pharmaceutical dosage forms.
Blue	indigotin blue	860-22-0	blue n. 2; indigotin; indigo carmine; INS 132	FD&C blue #2	disodium (2E)-3-oxo-2-(3-oxo-5-sulfonato-1H-indol-2-ylidene)-1H-indole-5-sulfonate	73015/ 75781	74.1102 74.3102	E132	Used on foods, cosmetics and medicines administered by oral route. It may be used for coloring nylon and surgical sutures in general surgery, bone cements, subject to the following restrictions: the amount of color additive must not exceed 1% in weight of the suture thread; not be absorbed by surrounding tissues.
Blue	indigotin blue, aluminum lake	16521-38-3	blue n. 2 aluminum lake INS 132	FD&C blue #2 aluminum lake	aluminum (2E)-3-oxo-2-(3-oxo-5-sulfo-1H-indol-2-ylidene)-1H-indole-5-sulfonic acid	73015	741.102	E132	Used on foods, cosmetics and medicines administered by oral route. It may be used for coloring bone cement, provided it does not exceed 0.1% in weight of bone cement.

<i>Color</i>	<i>Dye</i>	<i>CAS</i>	<i>Synonym</i>	<i>Name in English</i>	<i>Description (IUPAC)</i>	<i>Color Index</i>	<i>Reference 21 CFR</i>	<i>Reference European Union</i>	<i>Uses, restrictions and requirements</i>
Blue	patent blue, calcium salt	3536-49-0	patent V blue; acid blue 3;	acid blue 3	ethanaminium, N-[4-[[4-(diethylamino)phenyl](5-hydroxy-2,4-disulphophenyl)methylene]-2,5-cyclohexadien-1-ylidene]-N-ethyl-, inner salt, calcium salt (2:1)	42051		E131	Used on foods and in radiographic study of structures from the lymphatic system (lymphangiography study). It may cause skin sensitivity, rash, pruritus, nausea, decrease blood pressure, cause tremors and respiratory issues. Not permitted for children. Prohibited in the USA, Norway and Australia.
Blue	patent blue, sodium salt	129-17-9	carmin blue; patent blue VS	acid blue 1	ethanaminium, N-[4-[[4-(diethylamino)phenyl](2,4-disulphophenyl)methylene]-2,5-cyclohexadien-1-ylidene]-N-ethyl-, inner salt, sodium salt (1:1)	42045		E131	Permitted exclusively in products that do not come into contact with mucous membranes in regular or predictable conditions of use. Not permitted for children. Prohibited in the USA, Norway and Australia.
Blue	methylthionine chloride	61-73-4	methylene blue	methylthioninium chloride; basic blue 9	3,7-bis(dimethylamino)phenothiazin-5-ium chloride	52015			Used in medicines, including bacteriological dye, indicated in contrast.
White	calcium carbonate	471-34-1	calcium carbonate	calcium carbonate	calcium carbonate	77220	731.070	N/C	Used on medicines Use not permitted in the eye area, in medical devices, and in injectable pharmaceutical dosage forms.
White	titanium dioxide	13463-67-7	titanium dioxide	titanium dioxide	dioxotitanium	77891	731.575	E171	Used on medicines for external (including the eye area) and internal use (excluding the eye area). Use not permitted in medical devices and in injectable pharmaceutical dosage forms.

<i>Color</i>	<i>Dye</i>	<i>CAS</i>	<i>Synonym</i>	<i>Name in English</i>	<i>Description (IUPAC)</i>	<i>Color Index</i>	<i>Reference 21 CFR</i>	<i>Reference European Union</i>	<i>Uses, restrictions and requirements</i>
Orange	annatto	1393-63-1	orange n. 4	annatto	(2E,4E,6E,8E,10E,12E,14E,16Z,18E)- 4,8,13,17-tetramethyl-icos-2,4,6,8,10,12,14,16,18-nonaenedioic acid	75120	731.030	E160B	Used on medicines for external (including the eye area) and internal use (excluding the eye area). Use not permitted in medical devices and in injectable pharmaceutical dosage forms.
Orange	Beta carotene	7235-40-7	orange food 5	beta-carotene	1,3,3-trimethyl-2-[(1E,3E,5E,7E,9E,11E,13E,15E,17E)- 3,7,12,16-tetramethyl-18-(2,6,6-trimethylcyclohexen-1-yl)octadeca-1,3,5,7,9,11,13,15,17-nonaenyl] cyclohexene	40800	731.095	E160E	Used on medicines for external (including the eye area) and internal use (excluding the eye area). Use not permitted in medical devices and in injectable pharmaceutical dosage forms.
Orange	beta-apo-8'-carotenal	1107-26-2	INS 160E	all-trans-beta-apo-8'-carotenal	(2E,4E,6E,8E,10E,12E,14E,16E)- 2,6,11,15-tetramethyl-17-(2,6,6-trimethylcyclohexen-1-yl) heptadeca-2,4,6,8,10,12,14,16-octaenal	40820	N/C	E160E	Used on medicines for external (including the eye area) and internal use (excluding the eye area). Use not permitted in medical devices and in injectable pharmaceutical dosage forms.
Orange	solar orange	633-96-5	Persian orange	D&C orange # 5	sodium 4-[(2E)-2-(2-oxonaphthalen-1-ylidene)hydrazinyl] benzenesulfonate	15510	741.255	N/C	Used in cosmetics (in lipsticks no more than 5% per weight of final product) and medicines of external and topical use (not exceeding the daily dose of 5 mg), dentifrices and mouthwashes. Not permitted in medical devices and in injectable pharmaceutical dosage forms.
Brown	caramel	8028-89-5	natural brown 10	caramel	NA	-	-	E150A	Used in medicines of topical use and oral administration. Not permitted for children. Use not permitted in the eye area, in medical devices, and in injectable pharmaceutical dosage forms.

<i>Color</i>	<i>Dye</i>	<i>CAS</i>	<i>Synonym</i>	<i>Name in English</i>	<i>Description (IUPAC)</i>	<i>Color Index</i>	<i>Reference 21 CFR</i>	<i>Reference European Union</i>	<i>Uses, restrictions and requirements</i>
Black	black iron oxide	12227-89-3	black iron oxide	black iron oxide	iron oxides obtained by synthesis, including they hydrated forms or combinations of more than one of these oxides	77499	731.200	E172	Used in medicines of topical use and administered orally (not exceeding the daily dose of 5 mg of Fe). Use not permitted in the eye area, in medical devices, and in injectable pharmaceutical dosage forms.
Green	chlorophyll	1406-65-1	chlorophyll INS 140I	chlorophyll	mixture of chlorophylls A and B	75810	N/C	E 140(1)	Used on medicines and cosmetics.
Green	chlorophyllin	15611-43-5	INS 140II	chlorophyllins	magnesium; 3-[18-(dioxidomethylidene)-8-ethenyl-13-ethyl-3,7,12,17-tetramethyl-20-(2-oxido-2-oxoethyl)-2,3-dihydroporphyrin-23-id-2-yl]propanoate; hydron	75810	N/C	E140(II)	Used on medicines and cosmetics.
Green	Brilliant green	4403-90-1	Alizarin green	D&C green # 5	disodium 5-methyl-2-[[4-(4-methyl-2-sulfonatoanilino)-9,10-dioxoanthracen-1-yl]amino] benzenesulfonate	61570	741.205	N/C	Used on cosmetics and medicines (including the eye area). It may be used for coloring nylon 66 and 6 from surgical sutures in general surgery, subject to the following restrictions: the amount of color additive must not exceed 0.6% in weight of the suture thread; not be absorbed by surrounding tissues. Use not permitted in injectable pharmaceutical dosage forms.
Green	fast green	2353-45-9	food green 3	FD&C green # 3	ethyl-[4-[[4-[ethyl-[(3-sulfophenyl)methyl]amino]phenyl]- (4-hydroxy-2-sulfophenyl)methylidene]-1-cyclohexa-2,5-dienylidene]-(3-sulfophenyl)methyl] azanium	42053	741.203	N/C	Used on foods and medicines of external use (including the eye area). Use not permitted in surgical sutures and in injectable pharmaceutical dosage forms.

<i>Color</i>	<i>Dye</i>	<i>CAS</i>	<i>Synonym</i>	<i>Name in English</i>	<i>Description (IUPAC)</i>	<i>Color Index</i>	<i>Reference 21 CFR</i>	<i>Reference European Union</i>	<i>Uses, restrictions and requirements</i>
Green	Soluble green	128-80-3	Anthraquinone green	D&C green # 6	1,4-bis(4-methylanilino)anthracene-9,10-dione	61565	74.1206 74.3206	N/C	Used on cosmetics and medicines of external use (including the eye area). Used in simple, synthetic and absorbable surgical sutures. Use not permitted in injectable pharmaceutical dosage forms. Used in contact lenses; polyethylene terephthalate surgical sutures, including sutures for ophthalmic use; polyglycolic acid surgical sutures with diameters lower than the diameter USP 8-0, including sutures for ophthalmic use; poly sutures (co-trimethylene glycolic acid carbonate) in general sutures, and intraocular lenses for support haptics.
Green	Solvent green	6358-69-6	Pyranine green	D&C green # 8	Trisodium 8-hydroxypyrene-1,3,6-trisulfonate	59040	741.208	N/C	Used on cosmetics and medicines of external use. No more than 0.01% of the weight of the final product. Use not permitted in the eye area, in medical devices, and injectable pharmaceutical dosage forms.
Red	amaranth	915-67-3	Bordeaux S INS 123	amaranth; D&C red 2; acid red 27, trisodium salt	trisodium (4Z)-3-oxo-4-[(4-sulfonatophthalen-1-yl)hydrazinylidene]naphthalene-2,7-disulfonate	16185	N/C	E123	It may cause asthma, eczema, hyperactivity, allergic reactions and/or intolerance, rashes, especially in asthmatic individuals or with intolerance to aspirin. Not permitted for children. Prohibited in Norway, United States, Russia and Austria. Very restricted use (foods) in France and Italy.

<i>Color</i>	<i>Dye</i>	<i>CAS</i>	<i>Synonym</i>	<i>Name in English</i>	<i>Description (IUPAC)</i>	<i>Color Index</i>	<i>Reference 21 CFR</i>	<i>Reference European Union</i>	<i>Uses, restrictions and requirements</i>
Red	amaranth, aluminum lake	12227-62-2	Bordeaux S aluminum lake	amaranth aluminum lake; pigment red 193; acid red 27 aluminum lake; FD and C red no. 2 aluminum lake	aluminum; trisodium (4Z)-3-oxo-4-[(4-sulfonatophthalen-1-yl)hydrazinylidene]naphthalene-2,7-disulfonate	16185:1	N/C	E123	It may cause asthma, eczema, hyperactivity, allergic reactions and/or intolerance, rashes, especially in asthmatic individuals or with intolerance to aspirin. Not permitted for children. Prohibited in Norway, United States, Russia and Austria. Very restricted use (foods) in France and Italy. Used on foods.
Red	azorubine	3567-69-9	carmoisine	carmoisine	disodium (3Z)-4-oxo-3-[(4-sulfonatophthalen-1-yl)hydrazinylidene]naphthalene-1-sulfonate	14720	N/C	E122	It may cause eczema, allergic reactions and/or intolerance, rashes, especially in individuals with intolerance to aspirin. Not permitted for children. Prohibited in Japan, Norway, Sweden and the United States. Used on foods.
Red	azorubine, aluminum lake	84041-67-8	carmoisine, aluminum lake	carmoisine aluminum lake	disodium salt of 2-(4'-sulfo-1'-naphthyl-azo)-1-naphthol-4-sulfonic acid	14720	N/C	E122	It may cause eczema, allergic reactions and/or intolerance, rashes, especially in individuals with intolerance to aspirin. Not permitted for children. Prohibited in Japan, Norway, Sweden and the United States.
Red	erythrosine	16423-68-0	erythrosine; red n. 3; sodium erythrosine. INS 127	FD&C red #3 erythrosine	disodium 2-(2,4,5,7-tetraiodo-3-oxido-6-oxoxanthen-9-yl) benzoate	45430	741.303	E127	Used on foods (including nutritional supplements), cosmetics and medicines of oral administration. Use not permitted in the eye area, in medical devices, and in injectable pharmaceutical dosage forms.

<i>Color</i>	<i>Dye</i>	<i>CAS</i>	<i>Synonym</i>	<i>Name in English</i>	<i>Description (IUPAC)</i>	<i>Color Index</i>	<i>Reference 21 CFR</i>	<i>Reference European Union</i>	<i>Uses, restrictions and requirements</i>
Red	erythrosine, aluminum lake	12227-78-0	erythrosine, aluminum lake, red n. 3 aluminum lake; sodium erythrosine aluminum lake INS 127	FD&C red #3 aluminum lake	dialuminum; 1',3',6',8'-tetraiodo-3-oxospyro[2-benzofuran-1,9'-xanthene]-2',7'-diolate; 1',3',6',8'-tetraiodo-3-oxospyro[2-benzofuran-1,9'-xanthene]-2',7'-diolate	45430	741.303	E127	Used on foods (including nutritional supplements), cosmetics and medicines of oral administration. Use not permitted in the eye area, in medical devices, and in injectable pharmaceutical dosage forms.
Red	red iron oxide	1309-37-1	red iron oxide	red iron oxide	iron oxides obtained by synthesis, including they hydrated forms or combinations of more than one of these oxides	77491	731.200	E172	Used in medicines administered orally or of topical use (not exceeding the daily dose of 5 mg of Fe). Not permitted in the eye area, in medical devices, and in injectable pharmaceutical dosage forms.
Red	red pigment 63	74336-37-1	red 34	D&C red # 34	calcium (4Z)-3-oxo-4-[(1-sulfonatophthalen-2-yl)hydrazinylidene]naphthalene-2-carboxylate	15880	74.1334 74.2334	N/C	Used on cosmetics and medicines of external use. Not permitted in the eye area, in medical devices, and in injectable pharmaceutical dosage forms.
Red	ponceau red SX	4548-53-2	red 4	FD&C red # 4	disodium (3E)-3-[(2,4-dimethyl-5-sulfonatophenyl)hydrazinylidene]-4-oxonaphthalene-1-sulfonate	14700	74.1304 74.2304	N/C	Used on foods, cosmetics and medicines of external use. Not permitted in the eye area, in medical devices, and in injectable pharmaceutical dosage forms.
Red	red 27	13473-26-2	red phloxine O	D&C red #27	disodium 2',4',5',7'-tetrabromo-4,5,6,7-tetrachloro-3-oxospiro [2-benzofuran-1,9'-xanthene]-3',6'-diolate	45410:2	74.1327 74.2327	N/C	Used on cosmetics and medicines in general. Not permitted in the eye area, in medical devices and in pharmaceutical dosage forms.
Red	red 30	2379-74-0	red 30	D&C red #30; indanthren brilliant pink R	(2Z)-6-chloro-2-(6-chloro-4-methyl-3-oxo-1-benzothiophen-2-ylidene)-4-methyl-1-benzothiophen-3-one	73360	74.1330 74.2330	N/C	Used on cosmetics and medicines in general. Not permitted in the eye area, in medical devices and in pharmaceutical dosage forms.
Red	red 33	3567-66-6	Hispanic red	D&C red	disodium (3E)-	17200	74.1333	N/C	Used in cosmetics (in lipsticks no more than

Color	Dye	CAS	Synonym	Name in English	Description (IUPAC)	Color Index	Reference 21 CFR	Reference European Union	Uses, restrictions and requirements
				#33	5-amino-4-oxo-3-(phenylhydrazinylidene)naphthalene-2,7-disulfonate		74.2333		3% per weight of final product) and medicines of external and topical use (not exceeding the daily dose of 0.75 mg), dentifrices and mouthwashes. Not permitted in medical devices and in injectable pharmaceutical dosage forms.
Red	red 40	25956-17-6	FD&C red n. 40; Allura red AC INS 129	FD&C red #40	disodium (5E)-5-[(2-methoxy-5-methyl-4-sulfonatophenyl)hydrazinylidene]-6-oxonaphthalene-2-sulfonate	16035	74.340 74.1340 74.2340	E129	Used on foods (including nutritional supplements), cosmetics and medicines in general. Not permitted in medical devices and in injectable pharmaceutical dosage forms.
Red	red 40, aluminum lake	68583-95-9	red 40 aluminum lake; Allura red AC aluminum lake	FD&C red #40 aluminum lake	aluminum lake or calcium and aluminum lake, in substrate of disodium salt of 6-hydroxy-5-(2-methoxy-5-methyl-4-sulfophenyl)azo-2-naphthalenesulfonic acid	16035:1	74.340 74.1340 74.2340	E129	Used on foods (including nutritional supplements), cosmetics and medicines in general. Not permitted in medical devices and in injectable pharmaceutical dosage forms.
Red	red 7, calcium lake	09/04/5281	red 7 calcium lake; lithol rubine red calcium lake	D&C red #7 calcium lake; D&C red #7	calcium (4Z)-4-[(4-methyl-2-sulfonatophenyl)hydrazinylidene]-3-oxonaphthalene-2-carboxylate	15850:1	74.1307 74.2307	N/C	Used on cosmetics and medicines in general. In medicines, the combination of D&C RED # 6 and D&C RED # 7 must not be larger than 5 mg/daily dose of medicine. Not permitted in the eye area, in medical devices, and in injectable pharmaceutical dosage forms.
Red	Beet red, betanin	7659-95-2	beet red; betanin INS 162	beet powder beetroot red	(2S)-4-[2-[(2S)-2-carboxy-6-hydroxy-5-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-2,3-dihydroindol-1-yl]ethenyl]-2,3-dihydropyridine-2,6-dicarboxylic acid	N/C	N/C	E162	Used on foods. Not permitted for children.
Red	cochineal red	1260-17-9	cochineal carmine; carmine; natural red 4	Carmine cochineal	3,5,6,8-tetrahydroxy-1-methyl-9,10-dioxo-7-[3,4,5-trihydroxy-6-(hydroxymethyl)-oxan-2-yl]anthracene-2-carboxylic	75470	731.100	E120	Used on medicines in general.

<i>Color</i>	<i>Dye</i>	<i>CAS</i>	<i>Synonym</i>	<i>Name in English</i>	<i>Description (IUPAC)</i>	<i>Color Index</i>	<i>Reference 21 CFR</i>	<i>Reference European Union</i>	<i>Uses, restrictions and requirements</i>
			INS 120		acid)				
Red	Eosin red	62342-51-2	red 21	D&C red # 21	2',4',5',7'-tetrabromo-3',6'-dihydroxyspiro [2-benzofuran-3,9'-xanthene]-1-one	45380:2	74.1321 74.2321	N/C	Used on cosmetics and medicines in general. Not permitted in the eye area, in medical devices and in injectable pharmaceutical dosage forms.
Red	pure eosin red	95917-83-2	red 22	D&C red # 22	disodium 2-(2,4,5,7-tetrabromo-3-oxido-6-oxoxanthene-9-yl)benzoate	45380	74.1322 74.2322	N/C	Used on cosmetics and medicines in general. Not permitted in the eye area, in medical devices, and in injectable pharmaceutical dosage forms.
Red	Permanent red	70632-40-5	red 36	D&C red # 36	(1Z)-1-[(2-chloro-4-nitrophenyl)hydrazinylidene]naphthalen-2-one	12085	74.1336 74.2336	N/C	Used in cosmetics and medicines of internal and external use, except mouthwashes and dentifrices. Provided that the amounts do not exceed 1.7 mg/daily dose of the drug for continuous use in less than 1 year. For medicines of continuous use for more than 1 year, the amounts must not exceed 1.0 mg/daily dose of the medicine prescribed. Not permitted in the eye area, in medical devices, and in injectable pharmaceutical dosage forms.
Red	ponceau red 4R	2611-82-7	ponceau 4R; red 2 INS 124	ponceau 4R	trisodium (8Z)-7-oxo-8-[(4-sulfonatophthalen-1-yl)hydrazinylidene]naphthalene-1,3-disulfonate	16255	N/C	E124	Used on medicines and foods. It may cause eczema, allergic reactions and/or intolerance, rashes, especially in individuals with intolerance to aspirin. Not permitted for children. Prohibited in Norway and in the United States.

<i>Color</i>	<i>Dye</i>	<i>CAS</i>	<i>Synonym</i>	<i>Name in English</i>	<i>Description (IUPAC)</i>	<i>Color Index</i>	<i>Reference 21 CFR</i>	<i>Reference European Union</i>	<i>Uses, restrictions and requirements</i>
Red	ponceau red 4R, aluminum lake	15876-47-8	ponceau 4R aluminum lake; red 2 aluminum lake	ponceau 4R aluminum lake	aluminum lake or calcium and aluminum lake, in substrate of trisodium salt of 1-(4'-sulfo-1'-naphthyl-azo)-2-naphthol-6,8-disulfonic acid	16255	N/C	E124	Used on medicines and foods. It may cause eczema, allergic reactions and/or intolerance, rashes, especially in individuals with intolerance to aspirin. Not permitted for children. Prohibited in Norway and in the United States.
Red	Ruby red	5858-81-1	red 6	D&C red # 6	disodium (4E)-4-[(4-methyl-2-sulfonatophenyl)hydrazinylidene]-3-oxonaphthalene-2-carboxylate	15850	74.1306 74.2306	N/C	Used on cosmetics and medicines in general. In medicines, the combination of D&C RED # 6 and D&C RED # 7 must not be larger than 5 mg / daily dose of medicine. Not permitted in the eye area, in surgical sutures and in injectable pharmaceutical dosage forms.
Red	Scarlet red	85-86-9	red 17	D&C red # 17	(1Z)-1-[(4-phenyldiazenylphenyl)hydrazinylidene] naphthalen-2-one	26100	74.1317 74.2317 74.3317 74.3230	N/C	Used on cosmetics, medicines of external use and contact lenses. Not permitted for injectable pharmaceutical dosage forms. Used in contact lenses.

<i>Color</i>	<i>Dye</i>	<i>CAS</i>	<i>Synonym</i>	<i>Name in English</i>	<i>Description (IUPAC)</i>	<i>Color Index</i>	<i>Reference 21 CFR</i>	<i>Reference European Union</i>	<i>Uses, restrictions and requirements</i>
Violet	Alizarin violet	81-48-1	violet 2	D&C violet # 2	1-hydroxy-4-(4-methylanilino)anthracene-9,10-dione	60725	74.1602 74.2602 74.3602	N/C	Used on cosmetics, medicines of external use. Used in absorbable synthetic sutures for use in general and ophthalmic surgery; Haptic media from polymethyl metacrylate intraocular lenses. Not permitted in the eye area and in injectable pharmaceutical dosage forms. Used for synthetic surgical sutures of copolymers with 90% glycolide and 10% L-lactide in general and ophthalmic surgeries; synthetic polydioxanone surgical suture in general and ophthalmic surgeries; synthetic absorbable sutures of polyglecaprone 25 (ε-caprolactone/glycolide polymers) in general surgeries; poly(ε-caprolactone) absorbable surgical sutures in general surgeries; tripolymer absorbable sutures in glycolide carbonate / dioxanone / trimethyl-ethylene in general surgeries; surgical glycolide homopolymer sutures in general surgeries; intraocular lenses for support haptics; and absorbable meniscus implants made of poly(L-lactic) acid.

8.8 MEDICAL GASES

INTRODUCTION

The intended goal with this chapter is to present basic and necessary information for interpretation of monographs on the main gases and mixtures used in Health Services, gases that comprise the mixtures used in clinical tests, and gases used in the preservation of biological material.

This chapter encompasses gases classified by ANVISA as medicines and other gases used in Health Services and in clinical laboratories.

Gases used in Health Services may be stored compressed under pressure; liquefied under high pressure (saturated vapor pressure); liquefied at low pressure (cryogenic gases); or produced in the site of consumption.

Medicinal gases are comprised of active substances or a mixture of active substances and gas excipients.

The mixtures may consist of two or more substances or an active substance diluted in an excipient gas. The percentage formula (v/v) is obtained from standard temperature and pressure (STP) conditions. The density or compressibility factor for each gas under standard conditions (21 °C, 1 atm) are obtained from scientific data.

The monographs described the methods for analysis and the reference standards for quality assurance of the product manufactured, and include details on physical state, molecular mass, CAS (Chemical Abstracts Service) registration number, DCB (Brazilian Common Denomination) of minimum purity, impurities and physical and organoleptic characteristics for each gas, storage and labeling.

Note – Medical compressed air or oxygen 93% v/v coming from compression systems or oxygen concentrator systems, when produced *in situ* in Health Service centers, must follow the analysis methods specified in their respective monographs.

USES

Medicinal gases described in this chapter represent the main gases available in the market.

Medical oxygen (O₂)

In medical practice, the use of oxygen is aimed at keeping the patient respiration in condition of saturation in arterial blood equal or superior to 90% v/v. In situations when oxygen is administered diluted in another gas, its minimum concentration in the mixture must be 21% v/v. Medical oxygen is usually especially in intensive care and in anesthetic procedures, as ventilatory support for patients with respiratory failure of different clinical origins. In acute respiratory emergency situations, it allows the cardiopulmonary reanimation of patients. In residential use, it is employed in oxygen therapy, in case of pulmonary diseases. Oxygen is also used in hyperbaric therapy and as vehicle in the administration of medicines by nebulization or inhalation.

Medical nitrous oxide (N₂O)

Nitrous oxide is used in medicine and in odontology as an analgesic gas and, when associated to other anesthetic agents in anesthesia procedures, as an anesthetic of regular potency. It must not be administered at high concentrations (above 79% v/v) to patients, due to the risk of hypoxia. Nitrous oxide, in mixtures with different concentrations, is always diluted with medical oxygen. The main applications of medical nitrous oxide are in inhalation anesthesia procedures, always along with other volatile anesthetics, in odontology procedures as a mild analgesic, and as an assistant in the control of stress level in patients.

Medical carbon dioxide (CO₂)

Carbon dioxide is used in the medical area especially in endoscopic surgeries, where it works as an insufflator, such as in exploratory laparoscopy, for example. In situations where the gas is mixed with oxygen and nitrogen, an atmosphere close to physiological is produced, applied in the creation of anaerobic and aerobic atmospheres, for microorganism cultures. Mixtures of carbon dioxide with nitrogen, or with nitrogen and oxygen, or pure carbon dioxide gas are used for calibration of blood analysis and pulmonary diffusion devices.

Medical air (synthetic and compressed)

Medical air (synthetic and compressed) is used as a source of fresh air in inhalation anesthetic procedures and as ventilatory support to patients with respiratory failure of different origins and clinical situations, associated or not to oxygen. It may be used for nebulization of patients, as a vehicle for medicines in bronchial asthmatic episodes. In hyperbaric therapy, it is used as large chamber filling gas, to create environments where patients are submitted to above-atmospheric pressures. It is also used as gas for moving different types of pneumatic devices in Health Services.

Medical nitrogen (N₂)

Medical nitrogen has application in the medical practice in gaseous state and in cryogenic liquid state. In gaseous state, it is used as impulsion agent for pneumatic devices in orthopedic, neurological and other surgeries. As a cryogenic liquid, it is used in processes for freezing blood derivatives, cells and embryos, bone marrow and in the preservation of organs. It can also be used in surgical techniques named cryosurgeries, for example, in surgical treatment of infectious dermatoses.

Mixture of compressed gas v/v with 50% N₂O + 50% O₂

With this mixture, the main goal is to reduce the stress level of the patient and, consequently, increase the tolerance to pain. The mixture of compressed gas v/v 50% N₂O + 50% O₂ is used in analgesia procedures, such as: orthopedic trauma; burns; esthetic and pediatric treatments. This mixture must not be used in equipment and mixers for analgesia procedures, such as pure medical nitrous oxide or pure medical oxygen.

MANUFACTURING METHODS

Medical nitrogen (N₂)

The fractional distillation process is started with the capture of atmospheric air in large volumes, passing through two filters in air gas plants, named ASU – Air Separation Unit. The first filter, named pre-filter, retains larger particles, while the second filter, of the pocket type, retains smaller particles.

After being filtered, the atmospheric air is suctioned by the main compressor. This air, after compression, is sent to the purification system, where contaminants, such as CO₂, H₂O, THC (Total Hydrocarbon Content), are eliminated. After purification, the compressed air goes through the heat exchanger, which is comprised of two stages, where it is cooled to low temperatures and sent to the high-pressure distillation column. In the distillation column, the air is cooled at low temperatures, allowing the separation of nitrogen in form of gas that goes up through separation trays and, when reaching the top of the column, one part is recycled and the other enters the condenser. In it, on its turn, one part returns to the reflux column, the other part is sent to the fixed liquid nitrogen cryogenic tank.

Medical oxygen (O₂)

For obtaining oxygen, a remaining part of the nitrogen distillation is used. Oxygen is transferred from the high pressure column to the low pressure column, causing a new liquefaction. The liquid oxygen obtained in the lower extremity of the low-pressure column, is sent to the fixed liquid oxygen cryogenic tank. Basically, there are no residues in the separation process, even if one of the gases is released to the atmosphere and will automatically reintegrate the air composition. *Note:* If the atmospheric air distillation process continues, the next product to be obtained is argon.

Medical compressed air

Through liquefaction of atmospheric air. After being filtered, the atmospheric air is suctioned by the main air compressor. The compressed air is sent to the purification system, where contaminants, such as CO₂, H₂O, THC (Total Hydrocarbon Content), for example, are eliminated. After purification, compressed air goes through the main heat exchanger, where it is cooled down to low temperatures. Atmospheric air in cryogenic form is sent to suction by compressor. This air obtained after the compressor outlet is used for packaging in medical compressed air cylinders.

Through compressors. Atmospheric air can be compressed through pumps and compressors. The compressed atmospheric air is obtained from compressors where the atmospheric air is suctioned and compressed. The suction points must be out of contaminated places, such as combustion sources, vehicle exhaust pipes, hospital garbage, or environments with outlet discharge of air conditioning ducts and other discharge points. It is necessary to invest in compressors, filters, dryers and other equipment that will provide adequate quality to use.

Medical synthetic air

The process of manufacturing synthetic air for medicinal use is comprised of a binary mixture of medical-grade oxygen and nitrogen. Oxygen is introduced in the cylinders and, then, nitrogen is added. The introduction of nitrogen and then oxygen does not change or modify the final composition, and its final concentration must be in compliance with the monograph on this compendium.

Medical nitrous oxide (N₂O)

The manufacturing of medical nitrous oxide, also known as dinitrogen oxide, dinitrogen monoxide or as laughing gas, occurs from the thermal decomposition of ammonium nitrate. Ammonium nitrate is transported in two ways: in solid scales packaged in natural fiber bags, or transported already in liquid form at temperature of 80 °C, in specific trucks. Solid scales or the product in liquid form are transferred to reactors where the thermal decomposition

is held, occurring around 250 °C and, consequently, nitrous oxide and water are separated from the other components, according to Equation 1.



Nitrous oxide is purified with potassium permanganate, sulfuric acid and sodium hydroxide. Purified nitrous oxide is stored in fixed tanks from where they are transferred to tanker trucks, or then used for packaging in cylinders, both targeted at final application.

Medical carbon dioxide (CO₂)

The process for obtaining medical carbon dioxide, also known as carbon anhydride or carbonic gas, can be executed from fermentation in sugar-alcohol industries, or from the combustion of materials, or even as a byproduct of different chemical processes. According to the process for obtaining medical carbon dioxide, there are different types of impurities, for example high content of hydrocarbons in a combustion process and high content of nitrogen oxides in a fermentation process. The purification of carbon dioxide for medicinal use is carried out during the acquisition process.

Mixture of compressed gas v/v of 50% N₂O + 50% O₂

It is a compressed, homogenized gas mixture, packaged under high pressure. The process for manufacturing the binary mixture for medicinal use is carried out by adding medical oxygen to medical nitrous oxide. Medical nitrous oxide is initially introduced into the cylinder, followed by the addition of medical oxygen. Introducing medical oxygen and then medical nitrous oxide does not change or modify the final composition, but the final pressure of the mixture will be reduced due to the critical pressure of nitrous oxide.

Oxygen 93% v/v (O₂)

Oxygen 93% v/v for hospital use can be obtained by an oxygen concentrator system (OCS). In this system, the oxygen concentrator is installed in the Health Service facilities and allows oxygen concentration by submitting atmospheric air to a molecular sieve under low pressure. The process retains nitrogen from air, which will be discarded, and allows oxygen, at the nominal concentration of 93% v/v, to cross the adsorbent bed (molecular sieve) as a product. For use in Health Services, it is mandatory to monitor the concentration of oxygen (through oxygen analyzers) during the procedures.

HANDLING AND SAFETY

For medicinal gases, there are several recommendations for appropriate handling, in order to ensure safety in product use. There are specific recommendations that vary according to the type of packaging and type of medicinal gas.

Cylinders for medicinal gases, for being a narrow and heavy cylindrical container, must always be used in vertical position, duly fastened or over supports on their base. An exception is permitted to cylinders targeted for transportation, which have lower dimensions and weights.

Medicinal gases packaged in cylinders are usually maintained under high pressure, requiring additional care when opening the valve or connecting accessories. Medicinal gas may also be in liquid form, usually in vacuum insulated cryogenic tanks. In this case, such gases may be at a cryogenic

temperature below $-185\text{ }^{\circ}\text{C}$. Gases packaged in this liquid form may burn skin and other body organs when in direct contact.

Depending on the type of gas, they may be highly combustible, asphyxiating or toxic. It is recommended that the environment for using medicinal gases has good natural ventilation or a good ambient air renovation system.

All safety recommendations, concerning the adequate handling of medicinal gases, must be verified in the Chemical Material Safety Data Sheet (MSDS), in safety labels and in the hazard symbols made available by the manufacturers in compliance with requisites from Associação Brasileira de Normas Técnicas – ABNT NBR 14725 – Chemical Products – information about health, safety and environment.

REFERENCE MATERIALS USED IN THE ANALYSIS OF MEDICINAL GASES

Monographs on medicinal gases from the Brazilian Pharmacopoeia require the standards used for analytical determinations to be certified reference materials (CRM), with stated metrological traceability. These CRM are produced by metrology institutes from several countries or organizations, Brazilian or from other countries, acknowledged as certified reference material producers. In the absence of such CRM, other reference materials (RM) produced according to norms and guides internationally known, such as ISO 17034 – General requirements for the competence of reference material producers, can be used.

STORAGE FORMS

Medicinal gases are, for the most part, stored in cylinders under pressure or in cryogenic tanks.

Packaging or filling

Operation that allows storing medicinal gases in cylinders and medical cryogenic liquids in mobile cryogenic tanks.

Cylinders for gases

They are usually made of metallic material, such as, for example, carbon steel or aluminum, without seams or splices, and equipped with valves on top. Such valves may have connections with differentiated threads for each type gas, to prevent the undue application of gas. The threads in these connections have different diameters and steps, are internal or external, to the left or to the right to minimize the possibility of connecting gases incompatible with the application.

Cylinders for gases have different sizes and support different levels of pressure, which determine the capacity of storing gas in the compressed form. Usually, pure gas is stored in cylinders in a range between 125 and 200 bar; however, this may vary in narrower or wider ranges for some specific applications. In cylinders for gases, there may be medicinal gases in compressed form, under high pressure, or in liquefied form, where the product is packaged as liquid and consumed in gaseous form.

The construction of cylinders follows the international norms DOT 3AA, DOT 3A and the Brazilian norms ABNT EB 1199, ABNT EB 926 and others, offering safety to people who handle them according to the correct safety procedures.

Knowledge about the information stamped on the cylinder dome is also important, as registered on **Figure 1**.

Handling cylinders with missing or incomplete information is extremely dangerous. In these circumstances, the most indicated is to refer to the Chemical MSDS or to companies specialized in the gas area to check the specific conditions for using the container.

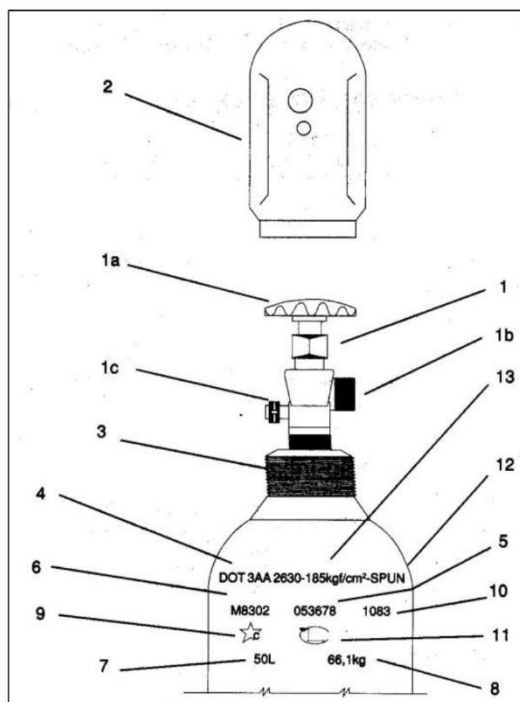


Figure 1 – Information included on cylinder and valve.

(1) Valve; (1a) Valve handwheel; (1b) CGA connection; (1c) Safety device; (2) Cylinder cap; (3) Neck; (4) Cylinder manufacturing specification; (5) Serial number; (6) Manufacturer's DOT registration number; (7) Hydraulic capacity; (8) Cylinder tare; (9) Manufacturer's symbol; (10) Date of latest hydrostatic pressure test; (11) Certifier entity code; (12) Manufacturing process; (13) Maximum service pressure

The valve is the least robust part of the set. For this reason, when the cylinder is not in use, it is recommended to keep it with the cap or another protection device for protecting the valve in case the cylinder falls or there are other unexpected events. On **Figure 2** there is an example of a cylinder valve used for medicinal gases.

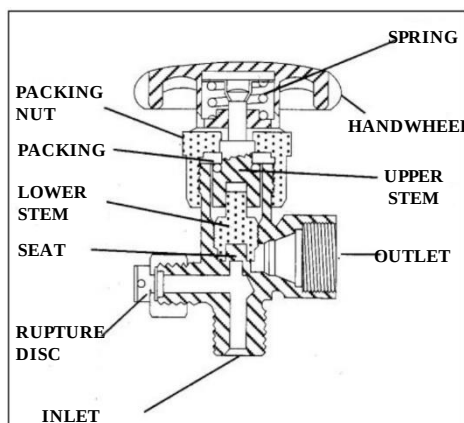


Figure 2 – Cylinder valve.*Cryogenic tanks*

Cryogenic tanks store medicinal gases in liquid form at extremely low temperatures. These tanks are comprised of two chambers, one inside the other, usually in carbon steel or stainless steel. These chambers are insulated from each other by vacuum and specific coatings, to preserve the product under cryogenic temperature. The goal of storing medicinal gas in liquid form is to increase its autonomy, since the use for human treatments is always under gaseous form.

In some situations, medicinal gas is used in liquid form, such as, for example, for dripping nitrogen for dermatological treatments or for storing liquid nitrogen for maintaining long-preservation biological samples.

Cryogenic tanks can be of the fixed or stationary type. Fixed cryogenic tanks are installed in health service centers and replenished with medicinal gases by tanker trucks. The replenishment of mobile cryogenic tanks, with medicinal gases in liquid form, is not made in the Health Service center, because they are replaced after consumption.

Safety device

Medicinal gases stored in the forms described earlier must be in equipment with safety devices that allow relieving the gas pressure, if there is an increase above expected, thus avoiding serious accidents. The relief or rupture pressure of these devices is calculated due to characteristics of the gas and cylinder in question. Basically, there are four types of safety devices:

Fusible plug. Contains an alloy with low melting point that, in case of cylinder heating, allows the passage of gas.

Rupture disc. It is a metallic disc calculated to break at a certain pressure and allow total depletion of the cylinder or tank.

Rupture disc + fusible plug. Combination of the two previous devices;

Spring cage. Allows pressure relief and closes automatically when the cylinder or tank pressure decreases.

FORMS OF TRANSPORTATION

Medicinal gases, packaged in cylinders or mobile cryogenic tanks, are transported in trucks that ensure good ventilation, to prevent gas confinements due to safety issues, in vertical position and properly fastened, since the gas containers, in case of fall, may cause severe accidents.

Liquefied medicinal gases are transported in tanker trucks, which are comprised of two chambers, one inside the other, insulated by vacuum and other coatings that ensure the maintenance of temperature at cryogenic levels. With medicinal gases transported in this manner, the fixed cryogenic tanks installed in health service centers are supplied.

The transportation of medicinal gases must comply with rules on transportation of hazardous chemical products, according to resolutions from ANTT – National Road Transportation Agency,

since medicinal gases are also classified as hazardous chemical products and the delivery vehicles must be appropriate and have the due safety identifications. Therefore, medicinal gases can only be transported in duly suitable vehicles.

Some precautions that must be taken in the transportation of gas cylinders are listed below:

- when handling cylinders, hands and gloves must be maintained free from oil or grease;
- protect cylinders against shock. Do not let them fall and suffer impacts;
- always transport cylinders with the valve protection device;
- never use them as rolls or supports, even empty;
- do not lift or transport cylinders with steel cables adapted to the valve and/or cap;
- check, beforehand, if all valves are completely closed and if there are no leaks;
- disconnect from the cylinders all devices (regulators, hoses) before their transportation;
- all cylinders must be adequately fastened to the vehicles, always in vertical position.

8.9 DETERMINATION OF SOLUBILITY APPLIED TO BIOWAIVER ACCORDING TO THE BIOPHARMACEUTICAL CLASSIFICATION SYSTEM

This chapter addresses topics related to determining the equilibrium solubility of active pharmaceutical ingredient (API) by the method of orbital shaking in flasks (shake flask method). The API solubility is one of the parameters used to determine its class according to the Biopharmaceutical Classification System (BCS). With the information, the goal is to contribute for conducting assays necessary for submitting the registration of a medicine considering the biowaiver, that is, the possibility of waiving a pharmaceutical company from conducting an *in vivo* study on bioequivalence or relative bioavailability. Information about the API solubility in dissolution media in the physiological pH range contribute to the execution of assessment of risks inherent to the decision to biowaiver using the BCS as criterion.

It is also worth highlighting that the technical and scientific aspects presented in this chapter are in line with the ones adopted internationally, as well as with the technical regulation in force in Brazil concerning the themes addressed. In this context, it is important to stress that the actions for registration and post-registration of medicines are the responsibility of the relevant sanitary authority.

BIOWAIVER

Biowaiver consists on waiving or replacing studies on relative bioavailability/bioequivalence with tests *in vitro* related to solubility and permeability of the API and to its dissolution from the immediate-release solid pharmaceutical form of oral use, in conditions established in advance according to the BCS.

BIOPHARMACEUTICAL CLASSIFICATION SYSTEM

The BCS was proposed by Amidon et al in 1995 and consists of four classes according to characteristics of solubility and permeability of the API (**Box 1**).

Box 1 – Biopharmaceutical classification system according to Amidon et al, 1995.

Class	Solubility	Permeability
I	High	High
II	Low	High
III	High	Low
IV	Low	Low

An API is considered highly soluble when its higher dose administered orally as an immediate-release pharmaceutical dosage form (maximum dose per administration described in the package insert) is

completely dissolved in up to 250 mL of each of the buffer solutions used within the physiological pH range (1.2 to 6.8), at $(37 \pm 1)^\circ\text{C}$. Active pharmaceutical ingredients of high permeability are those which extension of absorption in humans is equal or superior to 85%.

EQUILIBRIUM SOLUBILITY

The equilibrium solubility of an API is a type of dynamic balance that occurs when the API in solid state is in equilibrium with its solution. It may be obtained by determining the concentration of API in a saturated solution, after a certain time and by stirring.

DETERMINATION OF THE EQUILIBRIUM SOLUBILITY

In the context of biowaiver, according to the BCS, the method of orbital agitation in vial (shake flask method) is established for determining the API solubility.

Shake flask method

In the method of orbital agitation in vial, better known as “shake flask”, the equilibrium solubility is assessed through addition of excess of API to have its solubility determined in buffered aqueous solutions with pH between 1.2 and 6.8. The API must be added to the aqueous solution and submitted to agitation at controlled speed and temperatures. After achieving the equilibrium state of the saturated solution, the equilibrium solubility can be determined. To confirm the equilibrium was achieved between phases, the solubility must be constant in determinations made at consecutive times. During the experiments, do not take aliquots of buffer solution that, when added, exceed 10% of the total volume of the solution, because there may be dilution after the volume taken is replenished. This procedure is necessary for maintaining the system hydrodynamics and the amount of API above the saturation point.

For solubility studies, some rules must be followed: high purity of solvents; temperature maintained at 37°C throughout the experiment; the saturation must be achieved; and the method for quantification of analyte in the saturated solution must be validated. Immediately after collecting the aliquot of medium containing the analyte, the filtration is necessary, making sure that the API is not adsorbed by the filtration system components.

For active pharmaceutical ingredients known to have high solubility (classes I and III from BCS), it may be necessary to have a large amount of API to observe the formation of undissolved solid. In these cases, to avoid using a large amount of API, it is acceptable to demonstrate that the maximum dose of API per administration described in the package insert dissolves in up to 250 mL of the three buffer solutions in the physiological pH range established.

Experimental part

Equipment: shaker with orbital movement, by bearings and eccentric shaft, with temperature control adjusted to 37.0°C and variation of no more than 1.0°C .

Buffer solutions: for determining the equilibrium solubility, it is necessary to use: 0.1 M HCl solution or simulated enzyme-free gastric fluid pH 1.2; 0.05 M acetate buffer solution pH 4.5 and phosphate buffer solution pH 6.8 or simulated enzyme-free intestinal fluid pH 6.8 described on *Reagents (14)*. If the buffer solutions described are not adequate due to

physical or chemical nature, other buffer solutions may be used maintaining the pH values specified, provided that technically justified.

PROCEDURE

Definition of amount of API and time for the experiment (preliminary assay)

From the API solubility data available on literature, weigh an excess of no less than 10% in mass and transfer to an Erlenmeyer flask containing the adequate volume of buffer solutions pH 1.2; 4.5 and 6.8. Check for the presence of undissolved solid.

In case of absence of solubility data in literature, weigh a sufficient amount to check for the presence of undissolved solid. Homogenize and measure the pH value and, if there is a change in relation to the pH value of the buffer solution without the API, use a solution which buffer capacity maintains the pH value specified for the medium. Cover the Erlenmeyer flask and adapt it to the orbital shaker.

Adjust the temperature to $(37.0 \pm 1.0) ^\circ\text{C}$ and the agitation speed to between 50 and 150 rpm. The agitation speed must be established according to the volume of medium, in order to favor the balance between phases, but avoiding the formation of vortex. When taking aliquots and replenishing the solution, prevent the sum of volumes replenished from exceeding 10% from the total medium. After this procedure, filter the aliquot and quantify the API.

Determine the amount of API dissolved with time and plot a chart, which will present an ascending line followed by a plateau, which will indicate the equilibrium between the API dissolved and the saturated solution. The determination of solubility must be made in time and with the amount of API used to reach the plateau defined in this assay. Measure the pH value of buffer solutions after defining the time to achieve equilibrium.

Composition of system and determination of solubility

Weigh, accurately, at minimum in triplicate, for each pH condition to be assessed, an amount of API no less than 10% above the mass determined during the preliminary assay and transfer to an Erlenmeyer flask with an adequate volume of buffer solutions at pH 1.2; 4.5 and 6.8. Homogenize and measure the pH value. Take an aliquot and quantify the API. Adapt the Erlenmeyer flasks to the orbital shaker, adjust the temperature to $(37.0 \pm 1.0) ^\circ\text{C}$ and the agitation speed. Collect an aliquot of the supernatant solution at the previously determined time, in which the balance was established. Filter and quantify the API through a method previously validated and indicative of stability. Record the pH of the solution at the end of the experiment. There must not be a variation superior to 0.1 unit between the pH measured in the beginning and at the end of the test. The medium pH must not be adjusted during the procedure, since it may displace the dissociation equilibrium, changing the solubility.

Calculations

Determine the solubility in mg/mL. Calculate the relative standard deviation (RSD) between the solubility results obtained. The RSD results must be of no more than 5% between replicates of each assay condition.

For classification of API concerning solubility, according to the BCS, it is necessary to calculate the ratio between the maximum dose API by single administration described on the package insert

(em mg) and its solubility (in mg/mL). With results below or equal to 250 mL, the API is classified as having high solubility. Results higher than 250 mL indicate that the API has low solubility.

VALIDATION OF ANALYTICAL METHOD

The analytical method must be validated for quantification of API in the three mediums used. For such, it is necessary to observe the suitability of results from the following validation parameters: linearity, precision, accuracy, limit of quantification and selectivity. It is also necessary to confirm that the method proposed is capable of quantifying the API regardless of possible degradation products originated during the experiments.

8.10 ALTERNATIVE MICROBIOLOGICAL ASSAYS

The use of microbiological assays is very important in the quality control for products and pharmaceutical ingredients, since microbial contamination may cause changes to their physical and chemical properties and affect the product quality and the consumer safety.

Despite being efficient, simple and having low cost, traditional microbiological assays present some limitations, such as delayed result, low selectivity of culture medium, and variability of biological response from microorganisms.

In the attempt to overcome the deficiencies presented in traditional tests, alternative microbiological assays have been developed. These tests may provide an improvement in the quality of tests, higher sensitivity and faster results, allowing corrective actions to be taken early.

There are major advantages with the application of these tests, but it is necessary to have adequate understanding of their potential for application and a prior and judicious validation duly documented.

In alternative tests, there are specific principles and knowledge requiring adequate qualification from the professionals who will apply them. When selecting the test, it is necessary to consider its applicability and the compatibility with the product to be analyzed.

This chapter has the purpose of presenting the main alternative microbiological assays, their applications and general criteria for validation. The intention is not to recommend the assay to be used nor to present an exhaustive list, but to help in the selection and validation process.

TYPES OF TESTS

The alternative microbiological tests may be grouped into quantitative, qualitative and identification. Quantitative tests are those used when there is the goal of determining the amount of microorganisms in a sample. With qualitative tests, the presence or absence of microorganisms is presented. On the other hand, identification tests are used with the goal of characterizing the microorganism present.

MAIN METHODS

Methods based on growth

The alternative microbiological methods based on growth technologies are different from conventional methods since they use biochemical or physiological parameters or artifacts from them for detection of microbial growth. One of the advantages of these methods in comparison with traditional methods is the ability to process simultaneously a large number of samples and obtain results in a lower time. Examples of alternative microbiological method based on growth technologies:

Electrochemical methods Microorganisms that multiply in a specific culture medium produce highly charged ionic metabolites, from weakly charged organic nutrients, leading to modification of electrical properties of these media. Such changes in impedance (measured by conductance or capacitance) are monitored with electrodes in contact with the culture medium. The measurable endpoint is the necessary time to detect a change in the initial impedance, being inversely proportional to the size of the inoculum. For molds and yeasts, which produce only small changes in electric impedance, making an indirect conductance measurement is common, using a potassium hydroxide reservoir. The direct capacitance measurement can also be made. The automated detection with generation of electronic data and mapping of impedance variation reflects the microorganism growth curve, allowing to reduce the test duration to 48 hours.

Bioluminescence. Adenosine triphosphate (ATP) is present in all living cells and its detection is an indicator of the presence of viable microorganisms. The test consists of extracting ATP from microbial cells, followed by the quantitative or qualitative test using the enzyme system luciferin/luciferase, and measuring the light generated by a luminometer, or a camera with coupled charging device. The relative light (measured in relative light units – RLU) is directly proportional to the amount of ATP present in the atmosphere and depends on factors such as sensitivity of reagents and number of microorganisms present. In the case of a reduced number of microorganisms in the sample, that is, less than 10^2 – 10^3 , there may be the need for a system pre-incubation step. It may also be necessary to enhance the sample preparation steps to reduce the presence of non-microbial ATP, employing, for example, a sample pre-treatment with enzymes apyrase or somase. The ideal extraction of ATP must be fast and active, in order to avoid its degradation, thus ensuring efficient parameters of sensitivity and reproducibility. Since the reaction that generates bioluminescence is of enzymatic nature, it is subjected to interferences from products that may inhibit or reduce the enzymatic activity, and must be investigated during the process validation.

Detection of production or consumption of gas. The multiplication and metabolism of microorganisms in specific culture media produce metabolites or eliminate specific nutrients. In this sense, changes in gas compositions, such as production of CO₂ or consumption of O₂, may be monitored and detected through colorimetric detection, fluorimetric detection, pressure transducers or other types of detection. These systems are referred to as non-invasive microbial detection systems and can accommodate a large number of samples.

Use of chromogenic substrates. Chromogenic substrates are frequently used to detect the presence of specific enzymes in the identification of microorganisms, employing manual or automated methods. Liquid or solid culture media with chromogenic substrates are used to reveal specific enzyme activities for detection and differentiation of microorganisms. In these particular media, substrates defined are introduced in the formulation and hydrolyzed by the specific cell enzyme of a determined bacterium or fungus during growth. These substrates are selected according to the enzyme activity and are related to the presence of colored indicators. These products allow better differentiation of colonies in mixed cultures, being easy to use and interpret. Additionally, the response time is lower, because the growth and identification of the microorganism are simultaneous. However, the validation of culture media must be made carefully to guarantee a combination of selectivity, specificity and robustness. The signal quality is based not only on the judicious selection of enzymes to be assessed, because they may be present in different genera, but also in the physicochemical characteristics of the medium, such as pH.

Methods based on direct viability measurement

Methods based on direct viability measurement are fast and independent from microbial proliferation. They are based on the use of colorants for biochemical components of microbial cells or fluorescence obtained by enzyme cleavage of fluorogenic substrates in microorganisms with functional cell walls.

Solid phase cytometry. In solid phase cytometry, a fluorophore viability indicator is used, retained in the cytoplasm of microorganisms with intact membrane. The conjugated non-fluorescent substrate requires intracellular enzyme activity to be cleaved and release the fluorescent portion. An automatic laser reader allows reading fluorescent cells and appropriate software programs allow the differentiation between viable cells and fluorescent particles. The differentiation between viable and non-viable cells is based on the presence or absence of esterase activity and in the intact cell membrane. This technique uses membrane filtration to separate possible microbial contaminants from filtrable samples and subsequent labeling of cells retained with the viability substrate. This is hydrolyzed by non-specific esterase enzymes in the cytoplasm of metabolically active microorganisms, releasing free fluorophore. Before labeling, a counterstain can be added to minimize the background fluorescence, and a pre-incubation step may be necessary for activation of spores or recovery of stressed or fastidious microorganisms. The filtering membrane is scanned by a laser system from the cytometer and the fluorescent light is detected by photomultiplier cells.

Flow cytometry. The flow cytometry allows to detect microorganisms suspended in a liquid medium. The labeling and detection mechanisms are similar to the solid phase cytometry, but the sample does not need to be filtered. The microorganisms labeled by the non-fluorescent viability indicator can be detected in suspension, when passing through a flow cytometer. The sample labeled is injected in a quartz flow cell and passes through an excitatory laser beam for detection of microorganisms. The flow cytometry also provides quick results, but is less sensitive than the solid phase cytometry. To improve the performance, a prior step of incubation in culture medium can be conducted, which would make the method based on growth.

Direct epifluorescence. In this method, as well as in solid phase cytometry, the samples are filtered and stained with a fluorescent viability indicator, such as the acridine orange stain or 4',6-diamidino-2-phenylindole (DAPI). The microorganisms are detected by epifluorescence microscopy. Epifluorescence allows the fast detection of microorganisms and its sensitivity depends on the filtered volume and number of fields examined. Systems with image analysis increase the method usefulness. The direct epifluorescent filter technique is applicable to liquid products and fluids with low viscosity, and can also be applicable to particulate products diluted in advance. A modification of this method employs sampling using adhesive sheet to collect surface cells, staining on the sheet and subsequent direct observation in epifluorescence microscope.

Methods based on the analysis of cell components

They encompass methods on which the expression of some cell components provides the indirect measure of microbial presence. There is a high degree of specificity and they allow quick results. A high number of cells may be necessary.

Phenotypical

Immunologic. With immunologic methods, microorganisms are detected and quantified through antigen-antibody interaction. They are useful for the identification of specific microorganisms or unique cell determinants. The antigen-antibody interaction may be related to phenomena

of agglutination or endpoint visualization by colorimetry or fluorometry. There is great sensitivity and specificity.

The ELISA (Enzyme-Linked Immunosorbent Assay) test is a labeling technique to indicate the presence or absence of an antibody or antigen. In this technique, there is at least one antibody with specificity for an antigen in particular. The sample with the antigen is immobilized in a solid support (usually a polystyrene microtitration plate). After the antigen immobilization, the antibody connected to an enzyme is added and forms a complex with the antigen. Then, an enzyme substrate is added, producing a visible signal, usually by change of color, that indicates the presence of antigen in the sample.

The immunologic methods depend on the unique expression of specific identifiers. Therefore, they do not indicate necessarily the presence of microorganisms. Immunoassays are simple, cheap and can be used for qualitative and quantitative analysis.

Fatty acid profile. The cellular fatty acid profile is unique and stable, with a high level of homogeneity and reproducibility within a taxonomic group. The differentiation and identification of a wide variety of microorganisms can be made through the amount and types of fatty acids extracted from the microbial sample. Fatty acids from the ramified chain are common in many Gram-positive bacterial, while Gram-negative bacteria are predominantly comprised of linear chain fatty acids. The isolate is cultivated in a standard culture medium, and the use of cultures with 22 to 26 hours is important, to ensure that the cells are in the exponential growth phase. Fatty acids are extracted by saponification process, followed by methylation to convert them in the respective methyl ester (FAME – Fatty Acid Methyl Ester), which is extracted from the aqueous phase by the use of organic solvent, and the resulting extract is analyzed by high-resolution gas chromatography. The profile of fatty acid methyl esters extracted from a sample is compared with known isolates in a database. This technique requires a high level of standardization, including culture media, incubation temperature and operation conditions.

Fourier-transform infrared spectroscopy. Fourier-transform infrared spectroscopy applied to microbiology had a remarkable impulse in the past few years, considering the quickness and simplicity of application of the technique. The infrared region can be comprised of three zones (NIR, MIR and FIR) according to the wavelength. With the technique, the interaction between infrared radiation and matter is studied.

When matter absorbs radiation in the infrared region, there are changes in the vibrational level of chemical bonds. The wavelengths in which these transitions occur are characteristic to each functional group. Thus, it is possible to elaborate tables that allow the identification of molecules in samples which composition is unknown, with the construction of specific databases that allow the identification of microorganisms. The microorganism researched must be isolated in a cultivation medium, collected, analyzed in Fourier-transform infrared spectrometer and the spectrum obtained must be compared with a database for identification.

Mass spectrometry. The mass spectrometry allows the determination and identification of chemical structures through mass assessment and ionic charge. It is a technique that can be used in the analysis of biomolecules and large organic molecules, such as DNA, proteins, peptides, sugars and polymers.

The MALDI-TOF (matrix-assisted laser desorption/ionization – time of flight) is a matrix-assisted laser desorption and ionization technique that irradiates crystallized biological samples associated to chemical compounds. The ionized and accelerated molecules are launched in a tube under vacuum

and without electric field, to measure their time of flight, proportional to the molecule's molar mass, to the detector. The MALDI-TOF technique is used for fast identification of microorganisms that are treated as complex chemical entities. The microbial culture is placed on a plate well and covered with solvent matrix. The plate is exposed to laser, causing desorption of ionized cell components, which travel the tube toward the mass detector. The detection time differs for each molecule and the full analysis of each microbial cell provides a mass spectrum from the macromolecules. The detector signal is captured as a single fingerprint for each microorganism. The spectrum obtained is compared in a database.

SELDI-TOF is a variation of MALDI-TOF where the culture sample is placed directly on a chemically modified surface. In the mass spectrometry technique the microbial isolates require culture before the analysis.

Biochemical assays based on physiological reactions. The microorganisms may be characterized by biochemical reactions with certain chemical substances or by the use of specific sources of carbon. The identification of the microorganism can be made by comparing the profile of biochemical reactions with a database. The analysis can be conducted manually or employing specific equipment.

Preliminary differentiation tests, such as Gram staining, allow deciding for the adequate use of the series of biochemical and/or enzymatic reactions. Usually, microbial suspensions are tested using biochemical test kits. These assays require pure colonies with up to 3 days of cultivation. The system is easy to operate, but the result interpretation may be subjective. The result can be quick, depending on the system used and on the microorganism investigated.

Genotypic

Amplification of nucleic acids. The techniques that employ the amplification of nucleic acids may allow the identification of microorganisms from the exponential increase of a specific fragment of nucleic acid. Several methods can be employed in the analysis of fragments: size, specific sequence, reamplification with a second pair of primer, or specific detection by hybridization with fluorescent probe. The method selection must consider the purpose of the analysis. The use of DNA as marker may detect non-viable microorganisms that also have DNA, while mRNA is quickly degraded in non-viable microorganisms, being considered a good marker for viability.

Fingerprints. This technique characterizes and identifies microorganisms in subspecies level, using fragments of restriction of nucleic acids from bacterial and fungal genomes. From a pure and lysed culture, the DNA is extracted and fragmented by restriction enzymes. DNA fragments are separated by size through electrophoresis, viewed and compared with standards obtained from known species. Ribotyping is a typical example of this technique. There are also methods based on PCR fingerprint with primers that bond to different sites of the microbial genome, creating amplified fragments with a characteristic size distribution. For employing this technique, it is necessary to use pure colonies.

VALIDATION OF ALTERNATIVE MICROBIOLOGICAL METHODS

The main goal intended with this item is to provide general guidelines for validation of alternative microbiological methods, to demonstrate the non-inferiority between this and the traditional method.

Before the formal validation, a critical assessment of the alternative method one wants to validate must be made. Some aspects must be verified, such as the compatibility of the alternative method

with the product and its suitability to routine. Additionally, it is necessary to assess the production process to identify possible sources of microbial contamination and their microbiological profile. This assessment must take into account, for example, microorganisms isolated from raw materials, control in process or in the process development, environmental monitoring, as well as microorganisms of slow growth and contaminant microorganisms common to the product, reported in literature. This assessment will be important to determine the most adequate samples and identify the types and number of microorganisms associated to the process, which must be addressed in the validation of the new method. Regardless of the new method to be validated, the equipment, when used, including computer hardware and software, must be qualified/validated according to Good Practices. Some alternative methods depend on the use of database. The extension of coverage by this database must be described in the goal intended with the validation. According to the nature of the microbiological assay, the validation parameters follow **Table 1**.

Table 1 – Parameters for validation by microbiological test type.

Parameters	Quantitative tests	Qualitative tests	Identification tests
Specificity	Yes	Yes	Yes
Limit of detection	Yes	Yes	No
Accuracy	Yes	No	Yes
Precision	Yes	No	Yes
Limit of quantification	Yes	No	No
Linearity	Yes	No	No
Interval	Yes	No	No
Robustness	Yes	Yes	Yes

Considering the intrinsic characteristics of microorganisms, the number of colony-forming units (CFU) follows the Poisson distribution and, in this case, the Poisson statistical techniques are adequate for quantitative tests. If the normal distribution is used, the prior transformation of raw data is frequently necessary through adoption of a logarithm conversion or square root of the count +1. The latter requires special attention when there is zero result. After the transformation, it is necessary to apply a data normality assessment test.

The spectrum of microorganisms selected for validation of the alternative method and for demonstration of non-inferiority in relation to the traditional method must be carefully justified. The microorganisms described in general methods, specifically on items *Microbiological assays for non-sterile products (5.5.3.1)*, **Table 1**, or *Microbiological assays for sterile products (5.5.3.2)*, **Table 1**, are a reference to be followed. Additionally, it is recommended to use microbial isolates that are recurrent to the sample tested.

In the validation of the non-inferiority method, the non-inferiority in relation to the traditional method must be proven. With this attribute, the purpose is to demonstrate that the alternative method is not less sensitive and is as effective as the traditional method, considering a tolerance margin established in advance, named non-inferiority margin.

The non-inferiority of an alternative method (A) in relation to the traditional method (T) is established from the construction of bilateral, CI 95%, or unilateral, IC 97.5%, confidence intervals, where the upper limit for the difference $T - A$ is smaller than the margin (M).

The null (H_0) and alternative (H_1) hypotheses, related to non-inferiority studies, are, respectively:

H_0 : $T - A \geq M$ (the alternative method is inferior to the traditional method).

H_1 : $T - A < M$ (the alternative method is not inferior to the traditional method).

Rejecting the null hypothesis above represents that the alternative method (A) is not inferior to the traditional method (T).

In the demonstration of non-inferiority, the same types of products and the same microbial suspensions used in the assessment of alternative method must be submitted to the traditional method. However, it is necessary to pay attention to tests which sample contamination profile is zero. In these cases, the demonstration of non-inferiority with the use of contaminated samples is more appropriate. For qualitative tests, the demonstration of non-inferiority must be made for the parameter Limit of Detection. For quantitative tests, the demonstration must be made for Limit of Detection, Limit of Quantification, Accuracy and Precision. In the case of identification tests, the demonstration of non-inferiority must be made for the parameters of Accuracy and Precision.

PARAMETERS

Specificity

The specificity, in the context of non-selective microbiological methods, represents the method's capacity to promote a positive response for the different microorganisms one expects to be present in a sample. When it comes to selective microbiological methods, the demonstration of specificity also involves obtaining negative results for the microorganisms that are not of interest for the method. In both cases, when it comes to methods which interpretation of result through direct reading of microbial growth is not obtained, it is also necessary to demonstrate that components from the matrix, contaminants or foreign materials are not capable of promoting false positive or false negative results. For this parameter, it is important to test the highest possible number of microorganisms.

Limit of detection

The limit of detection is the lowest number of microorganisms in a sample that can be detected under the experimental conditions established. It refers to the number of microorganisms present in the original sample before any dilution or incubation.

The alternative method and the traditional method must be assessed using an inoculum with a low concentration of microorganisms. The concentration of this inoculum must be adjusted along the study so that at least 50% of the results obtained by the traditional method are positive. A minimum number of five replicates must be used for each concentration selected.

The non-inferiority with the traditional method must be demonstrated through statistical comparison of the number of positive and negative results obtained between the two methods. The Chi-squared test can be used for this purpose. The limit of detection achieved must be compatible with the purpose proposed for the method, such as, for example, there must be significant confidence for a method proposed for sterility analysis to be capable of detecting 1 CFU in the sample to be tested.

Limit of quantification

The limit of quantification is the lowest number of microorganisms that can be determined with precision and accuracy under the experimental conditions established. This parameter must be determined with, at least, five concentrations within the work range for each microorganism and in

five replicates for each concentration. Alternatively, results from linearity and accuracy can be used and, in this case, the limit of quantification corresponds to the lowest concentration found in the interval.

Accuracy

Accuracy represents the proximity of results obtained experimentally in relation to the results expected for microbial dilution used or the ones obtained by the traditional method. Generally, it is expressed as the percentage of recovery of microorganisms. The accuracy can be demonstrated by employing microbial suspension with concentration in the upper limit of the work interval defined for the method. From this suspension, serial dilutions must be prepared covering the entire work range, which must be overlapped with the one from the traditional method. The original suspension and its dilutions must comprise the different microbial concentrations. This parameter must be determined with, at least, five concentrations within the work range for each microorganism and in five replicates for each concentration. The percentage of recovery of the alternative method must be located on $(100 \pm 30)\%$.

To demonstrate the non-inferiority, in the two methods, it is not necessary to obtain statistically equal degrees of recovery. The verification that the results obtained with the alternative method meet the acceptance criterion is sufficient most of the time. However, the results obtained with the alternative method can be compared with results with the traditional method to determine which one is more accurate. For this, the data normality must be verified through a statistical test for this purpose. Once the data normality is found, the Snedecor F-test can be executed to verify the equality of variances. If they are equal, the comparison of recovery averages can be made by Student's t-test or by Analysis of Variance (ANOVA).

Precision

Precision is the degree of concordance between individual test results when the procedure is applied repeatedly in homogeneous suspensions of microorganisms including the work range. It is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of the results obtained. This parameter can be demonstrated by employing microbial suspension with a defined concentration and, from it, serial dilutions must be prepared.

The precision must be determined with, at least, two concentrations, one located in the limit of quantification and the other, in the limit of specification of the product for each microorganism and in 10 replicates for each concentration. For assessment of intermediate precision, the experiment must be repeated in a different work day.

For identification tests, the precision must be determined with 10 replicates in different work days.

In general, values lower than 30% for the coefficient of variation demonstrate an acceptable precision for the methods. However, the non-inferiority between the alternative method and the traditional method must be demonstrated through an appropriate statistical test.

Linearity

Linearity is the method's capacity to produce results that are proportional to the concentration of microorganisms present in the sample within a certain interval.

This parameter must be determined with, at least, five microbial concentrations for each microorganism and in five replicates for each concentration.

It is necessary to be aware of limitations from existing methods, both for the alternative method and for the traditional method, so that the concentrations used do not produce overlapped results. Therefore, adjustments to the number of concentrations can be made if the work limit is restricted.

The linearity assessment can be conducted by calculating the square of the correlation coefficient, r^2 , from a linear regression analysis of the data generated. Despite the fact that the correlation coefficient does not provide an estimate of linearity, it is commonly applied to give an idea of relation. The alternative method must not have a r^2 value lower than 0.95.

Interval

The interval is the range between the lowest and the highest concentration of microorganisms that have been determined with precision, accuracy and linearity following instructions in the method. The interval is determined from precision, accuracy and linearity studies.

Robustness

The robustness is the degree of reproducibility of results obtained in the test by analysis of the same sample with variations of the normal test conditions, such as instruments, reagent lots and laboratories. It enables establish the method feasibility under deliberate variations in operating parameters.

MAIN APPLICATIONS

The selection of the alternative microbiological method requires knowledge about its scientific base and must consider the purpose of the test and its compatibility with the product to be analyzed. Guidelines/suggestions for applications of the methods available are listed on **Table 2**.

Table 2 – Main applications of alternative microbiological methods.

<i>Methods</i>	<i>Technology</i>	<i>Applications</i>
Growth	Electrochemistry	Microbiological assay of antibiotics; antimicrobial efficacy test.
	Detection of consumption or production of gas	Sterility test.
	Bioluminescence	Microbial count in raw materials, water and products; environment monitoring; sterility test; antimicrobial efficacy test.
	Use of chromogenic substrates	Microbial identification
Viability	Solid phase cytometry	Microbial count in raw materials, water and products; environment monitoring; sterility test.
	Flow cytometry	Microbial count in raw materials, water and products.

<i>Methods</i>	<i>Technology</i>	<i>Applications</i>
Phenotypical	Direct epifluorescence	Microbial count in raw materials, water and products; environment monitoring.
	Immunologic	Analysis of bacterial endotoxins; microbial identification.
	Fatty acid profile	Microbial identification.
	Fourier-transform infrared spectroscopy (FTIR)	Microbial identification
	Mass spectrometry	Microbial identification
Genotypic	Biochemical assays based on physiological reactions	Microbial identification
	Amplification of nucleic acids	Microbial identification.
	Fingerprints	Microbial identification.

ANNEX A – PERIODIC TABLE OF CHEMICAL ELEMENTS – NAMES, SYMBOLS AND ATOMIC MASSES

Table A.1 is recommended by the International Union of Pure and Applied Chemistry – IUPAC, 2016. The atomic masses are based on the mass of $^{12}\text{C} = 12$.

Table A.1 – Chemical elements – names, symbols and atomic masses.

1																	18	
1 H Hydrogen 1.008																	2 He Helium 4.003	
3 Li Lithium 6.941	4 Be Beryllium 9.012											13 B Boron 10.811	14 C Carbon 12.011	15 N Nitrogen 14.007	16 O Oxygen 15.999	17 F Fluorine 18.998	18 Ne Neon 20.180	
11 Na Sodium 22.990	12 Mg Magnesium 24.305											13 Al Aluminum 26.982	14 Si Silicon 28.086	15 P Phosphorus 30.974	16 S Sulfur 32.066	17 Cl Chlorine 35.453	18 Ar Argon 39.948	
19 K Potassium 39.098	20 Ca Calcium 40.078	21 Sc Scandium 44.956	22 Ti Titanium 47.88	23 V Vanadium 50.942	24 Cr Chromium 51.996	25 Mn Manganese 54.938	26 Fe Iron 55.933	27 Co Cobalt 58.933	28 Ni Nickel 58.693	29 Cu Copper 63.546	30 Zn Zinc 65.39	31 Ga Gallium 69.723	32 Ge Germanium 72.61	33 As Arsenic 74.922	34 Se Selenium 78.09	35 Br Bromine 79.904	36 Kr Krypton 84.80	
37 Rb Rubidium 84.468	38 Sr Strontium 87.62	39 Y Yttrium 88.906	40 Zr Zirconium 91.224	41 Nb Niobium 92.906	42 Mo Molybdenum 95.94	43 Tc Technetium [98]	44 Ru Ruthenium 101.07	45 Rh Rhodium 102.906	46 Pd Palladium 106.42	47 Ag Silver 107.868	48 Cd Cadmium 112.411	49 In Indium 114.818	50 Sn Tin 118.71	51 Sb Antimony 121.760	52 Te Tellurium 127.6	53 I Iodine 126.904	54 Xe Xenon 131.29	
55 Cs Cesium 132.905	56 Ba Barium 137.327	57-71		72 Hf Hafnium 178.49	73 Ta Tantalum 180.948	74 W Tungsten 183.85	75 Re Rhenium 186.207	76 Os Osmium 190.23	77 Ir Iridium 192.22	78 Pt Platinum 195.08	79 Au Gold 196.967	80 Hg Mercury 200.59	81 Tl Thallium 204.383	82 Pb Lead 207.2	83 Bi Bismuth 208.980	84 Po Polonium [209]	85 At Astatine [210]	86 Rn Radon [222]
87 Fr Francium [223]	88 Ra Radium [226]	89-103		104 Rf Rutherfordium [261]	105 Db Dubnium [268]	106 Sg Seaborgium [269]	107 Bh Bohrium [270]	108 Hs Hassium [277]	109 Mt Meitnerium [278]	110 Ds Darmstadtium [281]	111 Rg Roentgenium [281]	112 Cn Copernicium [285]	113 Nh Nihonium [286]	114 Fl Flerovium [289]	115 Mc Moscovium [288]	116 Lv Livermorium [293]	117 Ts Tennessine [294]	118 Og Oganesson [294]
57 La Lanthanum 138.906	58 Ce Cerium 140.115	59 Pr Praseodymium 140.908	60 Nd Neodymium 144.24	61 Pm Promethium [144.913]	62 Sm Samarium 150.36	63 Eu Europium 151.966	64 Gd Gadolinium 157.25	65 Tb Terbium 158.925	66 Dy Dysprosium 162.50	67 Ho Holmium 164.930	68 Er Erbium 167.26	69 Tm Thulium 168.934	70 Yb Ytterbium 173.04	71 Lu Lutetium 174.967				
89 Ac Actinium [227]	90 Th Thorium 232.038	91 Pa Protactinium 231.036	92 U Uranium 238.029	93 Np Neptunium [237]	94 Pu Plutonium [244]	95 Am Americium [243]	96 Cm Curium [247]	97 Bk Berkelium [247]	98 Cf Californium [251]	99 Es Einsteinium [252]	100 Fm Fermium [257]	101 Md Mendelevium [258]	102 No Nobelium [259]	103 Lr Lawrencium [262]				

Table A.2 – Chemical elements ordered by atomic number.

<i>Atomic number (Z)</i>	<i>Name</i>	<i>Symbol</i>	<i>Atomic mass (A)</i>	<i>Density at 20 °C</i>	<i>Melting point (°C)</i>	<i>Boiling point (°C)</i>	<i>Year of discovery</i>	<i>Discoverer(s)</i>
1	Hydrogen	H	1.00794 g/mol	0.084 g/L	-259.1 °C	-252.9 °C	1766	Cavendish
2	Helium	He	4.002602 g/mol	0.17 g/L	-272.2 °C	-268.9 °C	1895	Ramsay and Cleve
3	Lithium	Li	6.941 g/mol	0.53 g/cm ³	180.5 °C	1317 °C	1817	Arfvedson
4	Beryllium	Be	9.012182 g/mol	1.85 g/cm ³	1278 °C	2970 °C	1797	Vauquelin
5	Boron	B	10.811 g/mol	2.46 g/cm ³	2300 °C	2550 °C	1808	Davy and Gay-Lussac
6	Carbon	C	12.011 g/mol	3.51 g/cm ³	3550 °C	4827 °C	Pre-history	Unknown
7	Nitrogen / (Azote)	N	14.00674 g/mol	1.17 g/L	209.9 °C	195.8 °C		Rutherford
8	Oxygen	O	15.9994 g/mol	1.33 g/L	218.4 °C	182.9 °C	1774	Priestley and Scheele
9	Fluorine	F	18.9984032 g/mol	1.58 g/L	219.6 °C	188.1 °C	1886	Moissan
10	Neon	Ne	20.1797 g/mol	0.84 g/L	248.7 °C	246.1 °C	1898	Ramsay and Travers
11	Sodium	Na	22.989768 g/mol	0.97 g/cm ³	97.8 °C	892 °C	1807	Davy
12	Magnesium	Mg	24.305 g/mol	1.74 g/cm ³	648.8 °C	1107 °C	1755	Black
13	Aluminum	Al	26.981539 g/mol	2.70 g/cm ³	660.5 °C	2467 °C	1825	Oersted
14	Silicon	Si	28.0855 g/mol	2.33 g/cm ³	1410 °C	2355 °C	1824	Berzelius
15	Phosphorus	P	30.973762 g/mol	1.82 g/cm ³	44 (P4) °C	280 (P4) °C	1669	Brandt
16	Sulfur	S	32.066 g/mol	2.06 g/cm ³	113 °C	444.7 °C	Pre-history	Unknown
17	Chlorine	Cl	35.4527 g/mol	2.95 g/L	34.6 °C	101 °C		Scheele
18	Argon	Ar	39.948 g/mol	1.66 g/L	189.4 °C	185.9 °C	1894	Ramsay and Rayleigh
19	Potassium	K	39.0983 g/mol	0.86 g/cm ³	63.7 °C	774 °C	1807	Davy
20	Calcium	Ca	40.078 g/mol	1.54 g/cm ³	839 °C	1487 °C	1808	Davy
21	Scandium	Sc	44.95591 g/mol	2.99 g/cm ³	1539 °C	2832 °C	1879	Nilson
22	Titanium	Ti	47.88 g/mol	4.51 g/cm ³	1660 °C	3260 °C	1791	Gregor and Klapproth
23	Vanadium	V	50.9415 g/mol	6.09 g/cm ³	1890 °C	3380 °C	1801	Del Rio
24	Chromium	Cr	51.9961 g/mol	7.14 g/cm ³	1857 °C	2482 °C	1797	Vauquelin
25	Manganese	Mn	54.93805 g/mol	7.44 g/cm ³	1244 °C	2097 °C	1774	Gahn
26	Iron	Fe	55.847 g/mol	7.87 g/cm ³	1535 °C	2750 °C	Pre-history	Unknown
27	Cobalt	Co	58.9332 g/mol	8.89 g/cm ³	1495 °C	2870 °C		Brandt
28	Nickel	Ni	58.69 g/mol	8.91 g/cm ³	1453 °C	2732 °C	1751	Cronstedt
29	Copper	Cu	63.546 g/mol	8.92 g/cm ³	1083.5 °C	2595 °C	Pre-history	Unknown
30	Zinc	Zn	65.39 g/mol	7.14 g/cm ³	419.6 °C	907 °C		Unknown
31	Gallium	Ga	69.723 g/mol	5.91 g/cm ³	29.8 °C	2403 °C	1875	Lecoq de Boisbaudran
32	Germanium	Ge	72.61 g/mol	5.32 g/cm ³	937.4 °C	2830 °C	1886	Winkler

Table A.2 (continued)

<i>Atomic number (Z)</i>	<i>Name</i>	<i>Symbol</i>	<i>Atomic mass (A)</i>	<i>Density at 20 °C</i>	<i>Melting point (°C)</i>	<i>Boiling point (°C)</i>	<i>Year of discovery</i>	<i>Discoverer(s)</i>
33	Arsenic	As	74.92159 g/mol	5.72 g/cm ³	613 °C	613 °C	1250	Albertus Magnus
34	Selenium	Se	78.96 g/mol	4.82 g/cm ³	217 °C	685 °C	1817	Berzelius
35	Bromine	Br	79.904 g/mol	3.14 g/cm ³	-7,3 °C	58.8 °C	1826	Balard
36	Krypton	Kr	83.8 g/mol	3.48 g/L	156.6 °C	152.3 °C	1898	Ramsay and Travers
37	Rubidium	Rb	85.4678 g/mol	1.53 g/cm ³	39 °C	688 °C	1861	Bunsen and Kirchhoff
38	Strontium	Sr	87.62 g/mol	2.63 g/cm ³	769 °C	1384 °C	1790	Crawford
39	Yttrium	Y	88.90585 g/mol	4.47 g/cm ³	1523 °C	3337 °C	1794	Gadolin
40	Zirconium	Zr	91.224 g/mol	6.51 g/cm ³	1852 °C	4377 °C	1789	Klaproth
41	Niobium	Nb	92.90638 g/mol	8.58 g/cm ³	2468 °C	4927 °C	1801	Hatchet
42	Molybdenum	Mo	95.94 g/mol	10.28 g/cm ³	2617 °C	5560 °C	1778	Scheele
43	Technetium	Tc	98.9063 g/mol	11.49 g/cm ³	2172 °C	5030 °C	1937	Perrier and Segrè
44	Ruthenium	Ru	101.07 g/mol	12.45 g/cm ³	2310 °C	3900 °C	1844	Claus
45	Rhodium	Rh	102.9055 g/mol	12.41 g/cm ³	1966 °C	3727 °C	1803	Wollaston
46	Palladium	Pd	106.42 g/mol	12.02 g/cm ³	1552 °C	3140 °C	1803	Wollaston
47	Silver	Ag	107.8682 g/mol	10.49 g/cm ³	961.9 °C	2212 °C	Pre-history	Unknown
48	Cadmium	Cd	112.411 g/mol	8.64 g/cm ³	321 °C	765 °C	1817	Stromeyer and Hermann
49	Indium	In	114.82 g/mol	7.31 g/cm ³	156.2 °C	2080 °C	1863	Reich and Richter
50	Tin	Sn	118.71 g/mol	7.29 g/cm ³	232 °C	2270 °C	Pre-history	Unknown
51	Antimony	Sb	121.75 g/mol	6.69 g/cm ³	630.7 °C	1750 °C	Pre-history	Unknown
52	Tellurium	Te	127.6 g/mol	6.25 g/cm ³	449.6 °C	990 °C	1782	Von Reichenstein
53	Iodine	I	128.90447 g/mol	4.94 g/cm ³	113.5 °C	184.4 °C	1811	Courtois
54	Xenon	Xe	131.29 g/mol	4.49 g/L	111.9 °C	107 °C	1898	Ramsay and Travers
55	Cesium	Cs	132.90543 g/mol	1.90 g/cm ³	28.4 °C	690 °C	1860	Kirchhoff and Bunsen
56	Barium	Ba	137.327 g/mol	3.65 g/cm ³	725 °C	1640 °C	1808	Davy
57	Lanthanum	La	138.9055 g/mol	6.16 g/cm ³	920 °C	3454 °C	1839	Mosander
58	Cerium	Ce	140.115 g/mol	6.77 g/cm ³	798 °C	3257 °C	1803	Von Hisinger and Berzelius
59	Praseodymium	Pr	140.90765 g/mol	6.48 g/cm ³	931 °C	3212 °C	1895	Von Welsbach
60	Neodymium	Nd	144.24 g/mol	7.00 g/cm ³	1010 °C	3127 °C	1895	Von Welsbach
61	Promethium	Pm	146.9151 g/mol	7.22 g/cm ³	1080 °C	2730 °C	1945	Marinsky and Glendenin
62	Samarium	Sm	150.36 g/mol	7.54 g/cm ³	1072 °C	1778 °C	1879	Lecoq de Boisbaudran
63	Europium	Eu	151.965 g/mol	5.25 g/cm ³	822 °C	1597 °C	1901	Demarçay
64	Gadolinium	Gd	157.25 g/mol	7.89 g/cm ³	1311 °C	3233 °C	1880	De Marignac

Table A.2 (continued)

<i>Atomic number (Z)</i>	<i>Name</i>	<i>Symbol</i>	<i>Atomic mass (A)</i>	<i>Density at 20 °C</i>	<i>Melting point (°C)</i>	<i>Boiling point (°C)</i>	<i>Year of discovery</i>	<i>Discoverer(s)</i>
65	Terbium	Tb	158.92534 g/mol	8.25 g/cm ³	1360 °C	3041 °C	1843	Mosander
66	Dysprosium	Dy	162.5 g/mol	8.56 g/cm ³	1409 °C	2335 °C	1886	Lecoq de Boisbaudran
67	Holmium	Ho	164.93032 g/mol	8.78 g/cm ³	1470 °C	2720 °C	1878	Soret
68	Erbium	Er	167.26 g/mol	9.05 g/cm ³	1522 °C	2510 °C	1842	Mosander
69	Thulium	Tm	168.93421 g/mol	9.32 g/cm ³	1545 °C	1727 °C	1879	Cleve
70	Ytterbium	Yb	173.04 g/mol	6.97 g/cm ³	824 °C	1193 °C	1878	De Marignac
71	Lutetium	Lu	174.967 g/mol	9.84 g/cm ³	1656 °C	3315 °C	1907	Urbain
72	Hafnium	Hf	178.49 g/mol	13.31 g/cm ³	2150 °C	5400 °C	1923	Coster and von Hevesy
73	Tantalum	Ta	180.9479 g/mol	16.68 g/cm ³	2996 °C	5425 °C	1802	Ekeberg
74	Tungsten	W	183.85 g/mol	19.26 g/cm ³	3407 °C	5927 °C	1783	Gebrüder de Elhu
75	Rhenium	Re	186.207 g/mol	21.03 g/cm ³	3180 °C	5627 °C	1925	Noddack, Tacke and Berg
76	Osmium	Os	190.2 g/mol	22.61 g/cm ³	3045 °C	5027 °C	1803	Tenant
77	Iridium	Ir	192.22 g/mol	22.65 g/cm ³	2410 °C	4130 °C	1803	Tenant et al
78	Platinum	Pt	195.08 g/mol	21.45 g/cm ³	1772 °C	3827 °C	1557	Scaliger
79	Gold	Au	196.96654 g/mol	19.32 g/cm ³	1064.4 °C	2940 °C	Pre-history	Unknown
80	Mercury	Hg	200.59 g/mol	13.55 g/cm ³	-38.9 °C	356.6 °C	Pre-history	Unknown
81	Thallium	Tl	204.3833 g/mol	11.85 g/cm ³	303.6 °C	1457 °C	1861	Crookes
82	Lead	Pb	207.2 g/mol	11.34 g/cm ³	327.5 °C	1740 °C	Pre-history	Unknown
83	Bismuth	Bi	208.98037 g/mol	9.80 g/cm ³	271.4 °C	1560 °C	1540	Agricola
84	Polonium	Po	208.9824 g/mol	9.20 g/cm ³	254 °C	962 °C	1898	Marie and Pierre Curie
85	Astatine	At	209.9871 g/mol		302 °C	337 °C	1940	Corson and MacKenzie
86	Radon	Rn	222.0176 g/mol	9.23 g/L	71 °C	61.8 °C	1900	Dorn
87	Francium	Fr	223.0197 g/mol		27 °C	677 °C	1939	Perey
88	Radium	Ra	226.0254 g/mol	5.50 g/cm ³	700 °C	1140 °C	1898	Marie and Pierre Curie
89	Actinium	Ac	227.0278 g/mol	10.07 g/cm ³	1047 °C	3197 °C	1899	Debieme
90	Thorium	Th	232.0381 g/mol	11.72 g/cm ³	1750 °C	4787 °C	1829	Berzelius
91	Protactinium	Pa	231.0359 g/mol	15.37 g/cm ³	1554 °C	4030 °C	1917	Soddy, Cranston and Hahn
92	Uranium	U	238.0289 g/mol	18.97 g/cm ³	1132.4 °C	3818 °C	1789	Klaproth
93	Neptunium	Np	237.0482 g/mol	20.48 g/cm ³	640 °C	3902 °C	1940	McMillan and Abelson
94	Plutonium	Pu	244.0642 g/mol	19.74 g/cm ³	641 °C	3327 °C	1940	Seaborg
95	Americium	Am	243.0614 g/mol	13.67 g/cm ³	994 °C	2607 °C	1944	Seaborg
96	Curium	Cm	247.0703 g/mol	13.51 g/cm ³	1340 °C	3110 °C	1944	Seaborg

Table A.2 (conclusion)

<i>Atomic number (Z)</i>	<i>Name</i>	<i>Symbol</i>	<i>Atomic mass (A)</i>	<i>Density at 20 °C</i>	<i>Melting point (°C)</i>	<i>Boiling point (°C)</i>	<i>Year of discovery</i>	<i>Discoverer(s)</i>
97	Berkelium	Bk	247.0703 g/mol	13.25 g/cm ³	986 °C		1949	Seaborg
98	Californium	Cf	251.0796 g/mol	15.1 g/cm ³	900 °C		1950	Seaborg
99	Einsteinium	Es	252.0829 g/mol		860 °C		1952	Seaborg
100	Fermium	Fm	257.0951 g/mol		1527 °C		1952	Seaborg
101	Mendelevium	Md	258.0986 g/mol				1955	Seaborg
102	Nobelium	No	259.1009 g/mol				1958	Seaborg
103	Lawrencium	Lr	260.1053 g/mol				1961	Ghiorso
104	Rutherfordium	Rf	261.1087 g/mol				1964/69	Flerov and Ghiorso
105	Dubnium	Db	262.1138 g/mol				1967/70	Flerov and Ghiorso
106	Seaborgium	Sg	263.1182 g/mol				1974	Oganessian
107	Bohrium	Bh	262.1229 g/mol				1976	Oganessian
108	Hassium	Hs	265 g/mol				1984	Institute for Heavy Ion Research
109	Meitnerium	Mt	266 g/mol				1982	Institute for Heavy Ion Research
110	Darmstadtium	Ds	269 g/mol				1994	Institute for Heavy Ion Research
111	Roentgenium	Rg	272 g/mol				1994	Institute for Heavy Ion Research
112	Unumbium	Uub	277 g/mol				1996	Institute for Heavy Ion Research
113	Ununtrium	Uut						
114	Ununquadium	Uuq						
115	Ununpentium	Uup						
116	Ununhexium	Uuh						
117	Ununseptium	Uus						
118	Ununoctium	Uuo						

ANNEX B – INTERNATIONAL SYSTEM (SI) UNITS

The international system has seven base units, used as a reference in all measurements and listed on **Table B.1**

Table B.1 – The seven base units from the SI.

<i>Quantity</i>	<i>Unit</i>	<i>Symbol</i>	<i>Definition of unit</i>
Length <i>l, h, r, x</i>	Meter	m	The meter is the distance traveled by light in the vacuum during a time interval of $1/299792458$ of the second. Thus, the speed of light in vacuum, c_0 , is precisely equal to 299792458 m/s.
Mass <i>M</i>	kilogram	kg	It is equal the mass of the international prototype of kilogram, $m(K)$, which is exactly equal to 1 kg.
Time <i>T</i>	second	s	The second is the duration of $9,192,631,770$ periods of radiation, corresponding to the transition between the two hyperfine levels of the ground state of the cesium ¹³³ atom and refers to the cesium atom at rest, at a temperature of 0 K.
Electric current <i>I, i</i>	ampere	A	The ampere is the intensity of a constant electric current that, maintained in two parallel, straight conductors, of infinite length, negligible circular cross-section and positioned 1 meter apart, in vacuum, would produce between those conductors a force equal to 2×10^{-7} newton per meter of length.
Thermodynamic Temperature <i>T</i>	kelvin	K	Kelvin, a thermodynamic temperature unit, is the fraction $1/273.16$ of the thermodynamic temperature of the triple point of water. <i>Thus, the temperature of the triple point of water, T_{ptw}, is exactly equal to 273.16 K.</i>
Amount of substance <i>N</i>	mol	mol	1. The mole is the amount of matter from a system that has as many elementary entities as there are atoms in 0.012 kilogram of carbon 12. 2. When mole is used, the elementary entities must be specified; they can be atoms, molecules, ions, electrons, as well as other particles or specified groupings of these particles. <i>Thus, the molar mass of carbon 12, $M(^{12}\text{C})$, is exactly equal to 12 g/mol. It refers to free atoms of carbon 12, at rest and at their fundamental state.</i>
Luminous intensity <i>I_v</i>	candela	cd	Candela is the luminous intensity in a given direction of a source that emits monochromatic radiation with frequency 540×10^{12} hertz and which radiant intensity in this direction is $1/683$ watt per steradian. <i>Thus, the spectral luminous efficacy, K, of the monochromatic radiation of frequency 540×10^{12} Hz is exactly equal to 683 lm/W.</i>

All other quantities are described as derived quantities and measured as derived units. **Table B.2** lists some of these derived quantities.

Table B.2 – Some derived quantities.

<i>Derived quantities</i>	<i>Representation</i>	<i>Derived unit</i>	<i>Symbol</i>
area	A	square meter	m ²
volume	v	cubic meter	m ³
speed	v	meter per second	m/s
acceleration	a	meter per second squared	m/s ²
wave number	σ, ν	reciprocal meter	m ⁻¹
specific mass	ρ	kilogram per cubic meter	kg/m ³
surface density	ρA	kilogram per square meter	kg/m ²
specific volume	v	cubic meter per kilogram	m ³ /kg
current density	j	ampere per square meter	A/m ²
magnetic field	H	ampere per meter	A/m
concentration	c	mole per cubic meter	mol/m ³
mass density	ρ, γ	kilogram per cubic meter	kg/m ³
luminance	Lv	candela per square meter	cd/c
refractive index	n	um	1
relative permeability	μ_r	um	1

Notice that the refractive index and the relative permeability are examples of dimensionless quantities, for which the SI unit is the number 1 (one), although this unit is not written.

Some derived units receive a special name, which is simply a compact form of expression of combination of base units that are frequently used. For example, joule, with the symbol J, is by definition equal to m² kg s⁻². There currently are 22 special names for units approved for use on the SI, and they are listed on **Table B.3**.

Table B.3 – Derived units with special name on SI.

<i>Derived quantities</i>	<i>Name of derived unit</i>	<i>Unit symbol</i>	<i>Expression in terms of other units</i>
plane angle	radian	rad	m/m = 1
solid angle	steradian	sr	m ² /m ² = 1
frequency	Hertz	Hz	s ⁻¹
force	newton	N	m kg s ⁻²
pressure, stress	pascal	Pa	N/m ² = m ⁻¹ kg s ⁻²
energy, work, quantity of heat	Joule	J	N m = m ² kg s ⁻²
potency, radiant flux	Watt	W	J/s = m ² kg s ⁻³
electric charge, quantity of electricity	coulomb	C	s A
electric potential difference	Volt	V	W/A = m ² kg s ⁻³ A ⁻¹
capacitance	Farad	F	C/V = m ⁻² kg ⁻¹ s ⁴ A ²
electric resistance	Ohm	Ω	V/A = m ² kg s ⁻³ A ⁻²
electric conductance	siemens	S	A/V = m ⁻² kg ⁻¹ s ³ A ²
magnetic induction flux	weber	Wb	V s = m ² kg s ⁻² A ⁻¹
magnetic induction	Tesla	T	Wb/m ² = kg s ⁻² A ⁻¹

<i>Derived quantities</i>	<i>Name of derived unit</i>	<i>Unit symbol</i>	<i>Expression in terms of other units</i>
inductance	Henry	H	$\text{Wb/A} = \text{m}^2 \text{ kg s}^{-2} \text{ A}^{-2}$
Celsius temperature	degree Celsius	°C	K
luminous flux	lumen	lm	$\text{cd sr} = \text{cd}$
illuminance	Lux	lx	$\text{lm/m}^2 = \text{m}^{-2} \text{ cd}$
activity of a radionuclide	becquerel	Bq	s^{-1}
absorbed dose, specific energy (imparted), kerma	Gray	Gy	$\text{J/kg} = \text{m}^2 \text{ s}^{-2}$
dose equivalent, ambient dose equivalent	sievert	Sv	$\text{J/kg} = \text{m}^2 \text{ s}^{-2}$
catalytic activity	Katal	kat	$\text{s}^{-1} \text{ mol}$

Although hertz and becquerel are equal to the inverse second, hertz is used only for cyclic phenomena, and becquerel is used for stochastic processes in radioactive decay.

The Celsius temperature unit is degree Celsius, °C, which is equal in magnitude to kelvin, K, the thermodynamic temperature unit. The Celsius temperature quantity t is related to the thermodynamic temperature T by the equation $t/^{\circ}\text{C} = T/\text{K} - 273.15$.

Sievert is also used for the quantities: directional dose equivalent and individual dose equivalent.

The final four special names of units on **Table B.3** were adopted specifically to protect measurements related to human health.

For each quantity, there is only one SI unit (although it may be frequently expressed in different manners, by the use of special names). However, the same SI unit can be used to express values from several different quantities (for example, the SI unit for the J/K ratio can be used to express both the thermal capacity and the entropy values). Therefore, it is important to not use the unit alone to specify the quantity. This applies both to scientific texts and to measurement instruments (that is, the output reading of an instrument must indicate the quantity measured and the unit).

Dimensionless quantities, also known as dimension one quantities, are usually defined as the ratio between two quantities of the same nature (for example, the refractive index is the ratio between two speeds, and relative permeability is the ratio between the permeability of a dielectric medium and the permeability of vacuum). Therefore, the unit of a dimensionless quantity is the ratio between two identical units of the SI, and therefore it is always equal to (1). However, when expressing the values of dimensionless quantities, the unit 1 (one) is not written.

MULTIPLES AND SUBMULTIPLES OF THE SI UNITS

A set of prefixes has been adopted for use with the SI units, in order to express the values of quantities that are much higher or much lower than the SI unit used without a prefix. The SI prefixes are listed on **Table B.4**. They can be used with any base unit and with derived units with special names.

Table B.4 – Multiples and submultiples from the SI – Prefixes and symbols.

<i>Quantity</i>	<i>Unit</i>	<i>Symbol</i>	<i>Relation with SI</i>
Time	Minute	min	1 min = 60 s
	Hour	h	1 h = 3600 s
	Day	d	1 d = 86400 s

Volume	Liter	L	1 L = 1 dm ³
Mass	Ton	t	1 t = 1000 kg
Energy	Electron volt	eV	1 eV ~ 1.602 x 10 ⁻¹⁹ J
Pressure	Bar	bar	1 bar = 100 kPa = 750.064 mm Hg = 0.987 atm
	millimeter of mercury	mm Hg	1 mm Hg = 133.322 Pa = 10 ⁻³ bar = 10 ⁻³ atm 760 mm Hg = 1 atm = 1.013 bar = 101.324 kPa
Length	Angstrom	Å	1 Å = 10 ⁻¹⁰ m
	nautical mile	M	1 M = 1852 m
Force	Dyne	dyn	1 dyn = 10 ⁻⁵ N
Energy	Erg	erg	1 erg = 10 ⁻⁷ J

When prefixes are used, the prefix name and the unit name are combined to form a single word and, similarly, the prefix symbol and the unit symbol are written without spaces, to form a single symbol that can be raised to any power. For example, it is possible to write: kilometer, km; microvolt, μV ; femtosecond, fs; $50 \text{ V/cm} = 50 \text{ V}(\text{10}^{-2} \text{ m})^{-1} = 5000 \text{ V/m}$.

When the base units and the derived units are used without any prefix, the resulting set of units is considered coherent. The use of a set of coherent units has technical advantages. However, the use of prefixes is convenient because it avoids the need to employ 10^n factors to express values of vary large or very small quantities. For example, the length of a chemical bond is more conveniently expressed in nanometers, nm, than in meters, m, and the distance between London and Paris is more conveniently expressed in kilometers, km, than in meters, m.

Kilogram, kg, is an exception because, although it is a base unit, its name already includes a prefix, for historical reasons. Multiples and submultiples of kilogram are written by combining prefix with gram: it is written milligram, mg, not microkilogram, μkg .

UNITS OUT OF THE SI

SI is the only system of units acknowledged universally, so it has a distinct advantage when an international dialogue is established. Other units, that is, the non-SI units, are usually defined in terms of SI units. Using SI also simplifies teaching science. For all these reasons, employing SI units is recommended in all fields of science and technology.

Although some non-SI units are still widely used, others, such as minute, time and day, as time units, will always be used, because they are deeply ingrained in our culture. Others are used for historical reasons, to meet the needs from groups with special interests, or because there isn't a convenient SI alternative.

Scientists must have the freedom to use non-SI units if they consider them more adequate to their purpose. However, when non-SI units are used, the factor of conversion to SI must always be included. Some non-SI units are listed on **Table B.5**, with their factor of conversion to SI.

Table B.5 – Some non-SI units.

<i>Quantity</i>	<i>Unit</i>	<i>Symbol</i>	<i>Relation with SI</i>
Time	Minute	min	1 min = 60 s
	Hour	h	1 h = 3600 s
	Day	d	1 d = 86400 s
Volume	Liter	L	1 L = 1 dm ³

Mass	Ton	t	1 t = 1000 kg
Energy	Electron volt	eV	1 eV $\sim$ 1.602 x 10 ⁻¹⁹ J
Pressure	Bar	bar	1 bar = 100 kPa = 750.064 mm Hg = 0.987 atm
	millimeter of mercury	mm Hg	1 mm Hg = 133.322 Pa = 10 ⁻³ bar = 10 ⁻³ atm
			760 mm Hg = 1 atm = 1.013 bar = 101.324 kPa
Length	Angstrom ¹	Å	1 Å = 10 ⁻¹⁰ m
	nautical mile	M	1 M = 1852 m
Force	Dyne	dyn	1 dyn = 10 ⁻⁵ N
Energy	Erg	erg	1 erg = 10 ⁻⁷ J

¹The *Houaiss Dictionary of Portuguese Language* admits this word spelled without the symbol over the letter “a”.

The unit symbols start with an upper-case letter when the unit is named after a person (for example, ampere, A; kelvin, K; hertz, Hz; coulomb, C). In the other cases, they always start with a lower-case letter (for example, meter, m; second, s; mole, mol). The symbol for liter is an exception: the upper-case letter is used to avoid confusion between the lower-case letter l and the number 1 (one). The symbol for nautical mile is presented here as M; however, there is no general agreement around any symbol for nautical mile.

Table B.6 lists other examples of units outside the SI and still being used, but which must be avoided. When mentioned in a document, it is worth indicating their equivalence to a SI unit.

Table B.6 – Other examples of units outside the SI.

Name	Symbol	Value in SI unit	Description
curie	Ci	1 Ci = 3.7 x 10 ¹⁰ Bq	Expresses the radionuclide activity.
roentgen	R	1 R = 2.58 x 10 ⁻⁴ C/kg	Expresses the exposure to X or γ radiations.
rad	Rad or rd	1 rad = 1 cGy = 10 ⁻² Gy	Expresses the absorbed dose of ionizing radiations.
rem	rem	1 rem = 1 cSv = 10 ⁻² Sv	Expresses the dose equivalent in radiation protection.
torr	Torr	1 torr = (101 325/760) Pa	Expresses pressure. Currently not used.
normal atmosphere	atm	1 atm = 760 mm Hg = 1.013 bar = 101.324 kPa	Expresses standard atmospheric pressure. Currently not used.
calorie	cal	1 cal = 4.18 J	Expresses the amount of heat (energy) necessary to increase from 14.5 °C to 15.5 °C the temperature of 1 gram of water. Currently, it is established that 1 Cal = 1000 cal = 1 kcal.

The unit symbols are printed in roman (upright) font, regardless of the font used in the rest of the text. They are mathematical entities, not abbreviations. They are never followed by a dot (except at the end of a sentence) or an “s” to form the plural. It is mandatory to use the correct form for the unit symbols, as illustrated by examples presented in the full publication by the SI. Sometimes, the unit symbols may have more than one letter. They are written in lower-case letters, except the first letter when they are named after a person, which is then written in upper-case. However, when a unit’s name is written in full, it must start with a lower-case letter (except in the beginning of a sentence), to differentiate the unit’s name from the person’s name.

When writing the value of a quantity, as the product of a numeric value and a unit, both number and unit must be treated by the ordinary algebra rules. For example, the equation $T = 293 \text{ K}$ can be also written as $T/\text{K} = 293$. This procedure is described as the use of calculation of quantities, or algebra of quantities. Sometimes, this notation is useful to identify the header of table columns, or the denomination of chart axes, so that entries on the table or the identification of points over the axes are simple numbers.

In the formation of products or quotient of units, the normal algebra rules apply. In the formation of products of units, it is necessary to leave a space between the units (alternatively, it is possible to raise a multiplication dot on half the row's height).

In the formation of numbers, the decimal separator can be a dot or a comma, according to the appropriate circumstances. For documents in English, the dot is usual, but in Brazil and form many Continental Europe languages and in other countries, the comma is more commonly used.

When the number has many digits, it is usual to group them in blocks of three, before and after the dot, to facilitate reading. This is not essential, but done frequently, and in general it is very useful. When this is done, the three-digit groups must be separated by only one narrow space; a dot or a comma must not be used between them. The uncertainty of the numeric value of a quantity may be conveniently expressed, making evident the uncertainty of the last significant digits, between parentheses, after the number. Example: 123 456.789 0

For additional information, please refer to the website of BIPM <http://www.bipm.org> or the full Publication of the SI, 8th edition, available on the website <http://www.bipm.org/en/si>.

ANNEX C – SOLVENTS FOR CHROMATOGRAPHY

Solvents for chromatography and their characteristics are listed on **Table C.1**.

Table C.1 – Solvents for chromatography and their properties.

<i>Solvent</i>	<i>Polarity index</i>	<i>Formula</i>	<i>Molecular mass (g/mol)</i>	<i>Refractive index n_D^{20}</i>	<i>Boiling point (°C)</i>	<i>Vapor pressure (mbar 20 °C)</i>	<i>Dielectric constant</i>	<i>Dipole moment</i>
<i>n</i> -heptane	-	C ₇ H ₁₆	100.21	1.388	98.4	48		
<i>n</i> -hexane	0.0	C ₆ H ₁₄	86.18	1.375	68.9	160	1.9	0
Cyclohexane	0.0	C ₆ H ₁₂	84.16	1.427	80.7	104	2.0	0
Isooctane	0.4	C ₈ H ₁₈	114.23	1.392	99.2		1.9	
Carbon tetrachloride	1.7	CCl ₄	153.82	1.460	76.5	120	2.2	0
Toluene	2.3	C ₆ H ₅ CH ₃	92.14	1.496	110.6	29	2.4	0.36
Chloroform	4.4	CHCl ₃	119.38	1.446	61.7	210	4.8	1.01
Dichloroethane	3.7	ClCH ₂ CH ₂ Cl	98.96	1.445	83.5	87	10.6	
Methylene chloride	3.4	CH ₂ Cl ₂	84.93	1.424	40.0	453	9.1	1.60
1-Butanol	3.9	CH ₃ (CH ₂) ₃ OH	74.12	1.399	117.2		17.8	1.66
Acetonitrile	6.2	CH ₃ CN	41.05	1.344	81.6		37.5	3.92
2-Propanol	4.3	CH ₃ CH(OH)CH ₃	60.11	1.378	82.4	43	18.3	1.66
Ethyl acetate	4.3	CH ₃ COOC ₂ H ₅	88.12	1.372	77.1	97	6.0	1.78
Acetone	5.4	CH ₃ COCH ₃	58.08	1.359	56.2	233	20.7	2.88
Ethanol	5.2	C ₂ H ₅ OH	46.07	1.361	78.5	59	24.3	1.70
1,4-Dioxane	4.8	C ₄ H ₈ O ₂	88.11	1.422	101.0	41	2.2	
Tetrahydrofuran	4.2	C ₄ H ₈ O	72.11	1.405	67.0	200	7.4	1.63
Methanol	6.6	CH ₃ OH	32.04	1.329	65.0	128	32.6	1.70
Water	9.0	H ₂ O	18.01	1.333	100.0	23	80.2	1.85

ANNEX D – ALCOHOLOMETRY

Table D.1 – Alcoholometric table (20 °C).

% v/v	% w/w	ρ_{20} (kg/m ³)	d (g/cm ³)
0.0	0.0	998.20	0.999997
0.1	0.08	998.05	0.999846
0.2	0.16	997.90	0.999696
0.3	0.24	997.75	0.999546
0.4	0.32	997.59	0.999386
0.5	0.40	997.44	0.999235
0.6	0.47	997.29	0.999085
0.7	0.55	997.14	0.998935
0.8	0.63	996.99	0.998785
0.9	0.71	996.85	0.998644
1.0	0.79	996.70	0.998494
1.1	0.87	996.55	0.998344
1.2	0.95	996.40	0.998194
1.3	1.03	996.25	0.998043
1.4	1.11	996.11	0.997903
1.5	1.19	995.96	0.997753
1.6	1.27	995.81	0.997602
1.7	1.35	995.67	0.997462
1.8	1.43	995.52	0.997312
1.9	1.51	995.38	0.997172
2.0	1.59	995.23	0.997021
2.1	1.67	995.09	0.996881
2.2	1.75	994.94	0.996731
2.3	1.82	994.80	0.996591
2.4	1.90	994.66	0.996450
2.5	1.98	994.51	0.996300
2.6	2.06	994.37	0.996160
2.7	2.14	994.23	0.996020
2.8	2.22	994.09	0.995879
2.9	2.30	993.95	0.995739
3.0	2.38	993.81	0.995599
3.1	2.46	993.66	0.995449
3.2	2.54	993.52	0.995308
3.3	2.62	993.38	0.995168
3.4	2.70	993.24	0.995028
3.5	2.78	993.11	0.994898
3.6	2.86	992.97	0.994757
3.7	2.94	992.83	0.994617
3.8	3.02	992.69	0.994477
3.9	3.10	992.55	0.994337

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
4.0	3.18	992.41	0.994196
4.1	3.26	992.28	0.994066
4.2	3.34	992.14	0.993926
4.3	3.42	992.00	0.993786
4.4	3.50	991.87	0.993655
4.5	3.58	991.73	0.993515
4.6	3.66	991.59	0.993375
4.7	3.74	991.46	0.993245
4.8	3.82	991.32	0.993104
4.9	3.90	991.19	0.992974
5.0	3.98	991.06	0.992844
5.1	4.06	990.92	0.992704
5.2	4.14	990.79	0.992573
5.3	4.22	990.65	0.992433
5.4	4.30	990.52	0.992303
5.5	4.38	990.39	0.992173
5.6	4.46	990.26	0.992042
5.7	4.54	990.12	0.991902
5.8	4.62	989.99	0.991772
5.9	4.70	989.86	0.991642
6.0	4.78	989.73	0.991512
6.1	4.87	989.60	0.991381
6.2	4.95	989.47	0.991251
6.3	5.03	989.34	0.991121
6.4	5.11	989.21	0.990991
6.5	5.19	989.08	0.990860
6.6	5.27	988.95	0.990730
6.7	5.35	988.82	0.990600
6.8	5.43	988.69	0.990470
6.9	5.51	988.56	0.990339
7.0	5.59	988.43	0.990209
7.1	5.67	988.30	0.990079
7.2	5.75	988.18	0.989959
7.3	5.83	988.05	0.989828
7.4	5.91	987.92	0.989698
7.5	5.99	987.79	0.989568
7.6	6.07	987.67	0.989448
7.7	6.15	987.54	0.989318
7.8	6.23	987.42	0.989197
7.9	6.32	987.29	0.989067
8.0	6.40	987.16	0.988937
8.1	6.48	987.04	0.988817
8.2	6.56	986.91	0.988686
8.3	6.64	986.79	0.988566

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
8.4	6.72	986.66	0.988436
8.5	6.80	986.54	0.988316
8.6	6.88	986.42	0.988196
8.7	6.96	986.29	0.988065
8.8	7.04	986.17	0.987945
8.9	7.12	986.05	0.987825
9.0	7.20	985.92	0.987695
9.1	7.29	985.80	0.987574
9.2	7.37	985.68	0.987454
9.3	7.45	985.56	0.987334
9.4	7.53	985.44	0.987214
9.5	7.61	985.31	0.987084
9.6	7.69	985.19	0.986963
9.7	7.77	985.07	0.986843
9.8	7.85	984.95	0.986723
9.9	7.93	984.83	0.986603
10.0	8.01	984.71	0.986482
10.1	8.10	984.59	0.986362
10.2	8.18	984.47	0.986242
10.3	8.26	984.35	0.986122
10.4	8.34	984.23	0.986002
10.5	8.42	984.11	0.985881
10.6	8.50	983.99	0.985761
10.7	8.58	983.88	0.985651
10.8	8.66	983.76	0.985531
10.9	8.75	983.64	0.985411
11.0	8.83	983.52	0.985290
11.1	8.91	983.40	0.985170
11.2	8.99	983.29	0.985060
11.3	9.07	983.17	0.984940
11.4	9.15	983.05	0.984819
11.5	9.23	982.94	0.984709
11.6	9.32	982.82	0.984589
11.7	9.40	982.70	0.984469
11.8	9.48	982.59	0.984359
11.9	9.56	982.47	0.984238
12.0	9.64	982.35	0.984118
12.1	9.72	982.24	0.984008
12.2	9.80	982.12	0.983888
12.3	9.89	982.01	0.983778
12.4	9.97	981.89	0.983657
12.5	10.05	981.78	0.983547
12.6	10.13	981.67	0.983437
12.7	10.21	981.55	0.983317
12.8	10.29	981.44	0.983207

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
12.9	10.37	981.32	0.983086
13.0	10.46	981.21	0.982976
13.1	10.54	981.10	0.982866
13.2	10.62	980.98	0.982746
13.3	10.70	980.87	0.982636
13.4	10.78	980.76	0.982525
13.5	10.87	980.64	0.982405
13.6	10.95	980.53	0.982295
13.7	11.03	980.42	0.982185
13.8	11.11	980.31	0.982075
13.9	11.19	980.19	0.981954
14.0	11.27	980.08	0.981844
14.1	11.36	979.97	0.981734
14.2	11.44	979.86	0.981624
14.3	11.52	979.75	0.981514
14.4	11.60	979.64	0.981403
14.5	11.68	979.52	0.981283
14.6	11.77	979.41	0.981173
14.7	11.85	979.30	0.981063
14.8	11.93	979.19	0.980953
14.9	12.01	979.08	0.980842
15.0	12.09	978.97	0.980732
15.1	12.17	978.86	0.980622
15.2	12.26	978.75	0.980512
15.3	12.34	978.64	0.980402
15.4	12.42	978.53	0.980291
15.5	12.50	978.42	0.980181
15.6	12.59	978.31	0.980071
15.7	12.67	978.20	0.979961
15.8	12.75	978.09	0.979851
15.9	12.83	977.98	0.979740
16.0	12.91	977.87	0.979630
16.1	13.00	977.76	0.979520
16.2	13.08	977.65	0.979410
16.3	13.16	977.55	0.979310
16.4	13.24	977.44	0.979199
16.5	13.32	977.33	0.979089
16.6	13.41	977.22	0.978979
16.7	13.49	977.11	0.978869
16.8	13.57	977.00	0.978759
16.9	13.65	976.89	0.978648
17.0	13.74	976.79	0.978548
17.1	13.82	976.68	0.978438
17.2	13.90	976.57	0.978328

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
17.3	13.98	976.46	0.978218
17.4	14.07	976.35	0.978107
17.5	14.15	976.25	0.978007
17.6	14.23	976.14	0.977897
17.7	14.31	976.03	0.977787
17.8	14.40	975.92	0.977677
17.9	14.48	975.81	0.977566
18.0	14.56	975.71	0.977466
18.1	14.64	975.60	0.977356
18.2	14.73	975.49	0.977246
18.3	14.81	975.38	0.977136
18.4	14.89	975.28	0.977036
18.5	14.97	975.17	0.976925
18.6	15.06	975.06	0.976815
18.7	15.14	974.95	0.976705
18.8	15.22	974.85	0.976605
18.9	15.30	974.74	0.976495
19.0	15.39	974.63	0.976384
19.1	15.47	974.52	0.976274
19.2	15.55	974.42	0.976174
19.3	15.63	974.31	0.976064
19.4	15.72	974.20	0.975954
19.5	15.80	974.09	0.975843
19.6	15.88	973.99	0.975743
19.7	15.97	973.88	0.975633
19.8	16.05	973.77	0.975523
19.9	16.13	973.66	0.975413
20.0	16.21	973.56	0.975312
20.1	16.30	973.45	0.975202
20.2	16.38	973.34	0.975092
20.3	16.46	973.24	0.974992
20.4	16.55	973.13	0.974882
20.5	16.63	973.02	0.974771
20.6	16.71	972.91	0.974661
20.7	16.79	972.80	0.974551
20.8	16.88	972.70	0.974451
20.9	16.96	972.59	0.974341
21.0	17.04	972.48	0.974230
21.1	17.13	972.37	0.974120
21.2	17.21	972.26	0.974010
21.3	17.29	972.16	0.973910
21.4	17.38	972.05	0.973800
21.5	17.46	971.94	0.973689
21.6	17.54	971.83	0.973579
21.7	17.63	971.73	0.973479

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
21.8	17.71	971.62	0.973369
21.9	17.79	971.51	0.973259
22.0	17.87	971.40	0.973149
22.1	17.96	971.29	0.973038
22.2	18.04	971.18	0.972928
22.3	18.12	971.08	0.972828
22.4	18.21	970.97	0.972718
22.5	18.29	970.86	0.972608
22.6	18.37	970.75	0.972497
22.7	18.46	970.64	0.972387
22.8	18.54	970.53	0.972277
22.9	18.62	970.42	0.972167
23.0	18.71	970.31	0.972057
23.1	18.79	970.20	0.971946
23.2	18.87	970.09	0.971836
23.3	18.96	969.98	0.971726
23.4	19.04	969.87	0.971616
23.5	19.13	969.76	0.971506
23.6	19.21	969.65	0.971395
23.7	19.29	969.54	0.971285
23.8	19.38	969.43	0.971175
23.9	19.46	969.32	0.971065
24.0	19.54	969.21	0.970955
24.1	19.63	969.10	0.970844
24.2	19.71	968.99	0.970734
24.3	19.79	968.88	0.970624
24.4	19.88	968.77	0.970514
24.5	19.96	968.66	0.970404
24.6	20.05	968.55	0.970293
24.7	20.13	968.43	0.970173
24.8	20.21	968.32	0.970063
24.9	20.30	968.21	0.969953
25.0	20.38	968.10	0.969843
25.1	20.47	967.99	0.969732
25.2	20.55	967.87	0.969612
25.3	20.63	967.76	0.969502
25.4	20.72	967.65	0.969392
25.5	20.80	967.53	0.969272
25.6	20.89	967.42	0.969161
25.7	20.97	967.31	0.969051
25.8	21.05	967.19	0.968931
25.9	21.14	967.08	0.968821
26.0	21.22	966.97	0.968711
26.1	21.31	966.85	0.968590

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
26.2	21.39	966.74	0.968480
26.3	21.47	966.62	0.968360
26.4	21.56	966.51	0.968250
26.5	21.64	966.39	0.968130
26.6	21.73	966.28	0.968019
26.7	21.81	966.16	0.967899
26.8	21.90	966.05	0.967789
26.9	21.98	965.93	0.967669
27.0	22.06	965.81	0.967548
27.1	22.15	965.70	0.967438
27.2	22.23	965.58	0.967318
27.3	22.32	965.46	0.967198
27.4	22.40	965.35	0.967088
27.5	22.49	965.23	0.966967
27.6	22.57	965.11	0.966847
27.7	22.65	964.99	0.966727
27.8	22.74	964.88	0.966617
27.9	22.82	964.76	0.966497
28.0	22.91	964.64	0.966376
28.1	22.99	964.52	0.966256
28.2	23.08	964.40	0.966136
28.3	23.16	964.28	0.966016
28.4	23.25	964.16	0.965895
28.5	23.33	964.04	0.965775
28.6	23.42	963.92	0.965655
28.7	23.50	963.80	0.965535
28.8	23.59	963.68	0.965415
28.9	23.67	963.56	0.965294
29.0	23.76	963.44	0.965174
29.1	23.84	963.32	0.965054
29.2	23.93	963.20	0.964934
29.3	24.01	963.07	0.964804
29.4	24.10	962.95	0.964683
29.5	24.18	962.83	0.964563
29.6	24.27	962.71	0.964443
29.7	24.35	962.58	0.964313
29.8	24.44	962.46	0.964192
29.9	24.52	962.33	0.964062
30.0	24.61	962.21	0.963942
30.1	24.69	962.09	0.963822
30.2	24.78	961.96	0.963692
30.3	24.86	961.84	0.963571
30.4	24.95	961.71	0.963441
30.5	25.03	961.59	0.963321
30.6	25.12	961.46	0.963191

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
30.7	25.20	961.33	0.963060
30.8	25.29	961.21	0.962940
30.9	25.38	961.08	0.962810
31.0	25.46	960.95	0.962680
31.1	25.55	960.82	0.962549
31.2	25.63	960.70	0.962429
31.3	25.72	960.57	0.962299
31.4	25.80	960.44	0.962169
31.5	25.89	960.31	0.962039
31.6	25.97	960.18	0.961908
31.7	26.06	960.05	0.961778
31.8	26.15	959.92	0.961648
31.9	26.23	959.79	0.961518
32.0	26.32	959.66	0.961387
32.1	26.40	959.53	0.961257
32.2	26.49	959.40	0.961127
32.3	26.57	959.27	0.960997
32.4	26.66	959.14	0.960866
32.5	26.75	959.01	0.960736
32.6	26.83	958.87	0.960596
32.7	26.92	958.74	0.960466
32.8	27.00	958.61	0.960335
32.9	27.09	958.47	0.960195
33.0	27.18	958.34	0.960065
33.1	27.26	958.20	0.959925
33.2	27.35	958.07	0.959795
33.3	27.44	957.94	0.959664
33.4	27.52	957.80	0.959524
33.5	27.61	957.66	0.959384
33.6	27.69	957.53	0.959254
33.7	27.78	957.39	0.959113
33.8	27.87	957.26	0.958983
33.9	27.95	957.12	0.958843
34.0	28.04	956.98	0.958703
34.1	28.13	956.84	0.958562
34.2	28.21	956.70	0.958422
34.3	28.30	956.57	0.958292
34.4	28.39	956.43	0.958152
34.5	28.47	956.29	0.958011
34.6	28.56	956.15	0.957871
34.7	28.65	956.01	0.957731
34.8	28.73	955.87	0.957591
34.9	28.82	955.73	0.957450
35.0	28.91	955.59	0.957310

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
35.1	28.99	955.45	0.957170
35.2	29.08	955.30	0.957020
35.3	29.17	955.16	0.956879
35.4	29.26	955.02	0.956739
35.5	29.34	954.88	0.956599
35.6	29.43	954.73	0.956449
35.7	29.52	954.59	0.956308
35.8	29.60	954.44	0.956158
35.9	29.69	954.30	0.956018
36.0	29.78	954.15	0.955867
36.1	29.87	954.01	0.955727
36.2	29.95	953.86	0.955577
36.3	30.04	953.72	0.955437
36.4	30.13	953.57	0.955286
36.5	30.22	953.42	0.955136
36.6	30.30	953.28	0.954996
36.7	30.39	953.13	0.954846
36.8	30.48	952.98	0.954695
36.9	30.56	952.83	0.954545
37.0	30.65	952.69	0.954405
37.1	30.74	952.54	0.954255
37.2	30.83	952.39	0.954104
37.3	30.92	952.24	0.953954
37.4	31.00	952.09	0.953804
37.5	31.09	951.94	0.953653
37.6	31.18	951.79	0.953503
37.7	31.27	951.63	0.953343
37.8	31.35	951.48	0.953193
37.9	31.44	951.33	0.953042
38.0	31.53	951.18	0.952892
38.1	31.62	951.02	0.952732
38.2	31.71	950.87	0.952582
38.3	31.79	950.72	0.952431
38.4	31.88	950.56	0.952271
38.5	31.97	950.41	0.952121
38.6	32.06	950.25	0.951960
38.7	32.15	950.10	0.951810
38.8	32.24	949.94	0.951650
38.9	32.32	949.79	0.951500
39.0	32.41	949.63	0.951339
39.1	32.50	949.47	0.951179
39.2	32.59	949.32	0.951029
39.3	32.68	949.16	0.950868
39.4	32.77	949.00	0.950708
39.5	32.86	948.84	0.950548

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
39.6	32.94	948.68	0.950388
39.7	33.03	948.52	0.950227
39.8	33.12	948.37	0.950077
39.9	33.21	948.21	0.949917
40.0	33.30	948.05	0.949756
40.1	33.39	947.88	0.949586
40.2	33.48	947.72	0.949426
40.3	33.57	947.56	0.949266
40.4	33.66	947.40	0.949105
40.5	33.74	947.24	0.948945
40.6	33.83	947.08	0.948785
40.7	33.92	946.91	0.948614
40.8	34.01	946.75	0.948454
40.9	34.10	946.58	0.948284
41.0	34.19	946.42	0.948124
41.1	34.28	946.26	0.947963
41.2	34.37	946.09	0.947793
41.3.	34.46	945.93	0.947633
41.4	34.55	945.76	0.947462
41.5	34.64	945.59	0.947292
41.6	34.73	945.43	0.947132
41.7	34.82	945.26	0.946961
41.8	34.91	945.09	0.946791
41.9	35.00	944.93	0.946631
42.0	35.09	944.76	0.946461
42.1	35.18	944.59	0.946290
42.2	35.27	944.42	0.946120
42.3	35.36	944.25	0.945950
42.4	35.45	944.08	0.945779
42.5	35.54	943.91	0.945609
42.6	35.63	943.74	0.945439
42.7	35.72	943.57	0.945268
42.8	35.81	943.40	0.945098
42.9	35.90	943.23	0.944928
43.0	35.99	943.06	0.944758
43.1	36.08	942.88	0.944577
43.2	36.17	942.71	0.944407
43.3	36.26	942.54	0.944237
43.4	36.35	942.37	0.944066
43.5	36.44	942.19	0.943886
43.6	36.53	942.02	0.943716
43.7	36.62	941.84	0.943535
43.8	36.71	941.67	0.943365
43.9	36.80	941.49	0.943185

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
44.0	36.89	941.32	0.943014
44.1	36.98	941.14	0.942834
44.2	37.07	940.97	0.942664
44.3	37.16	940.79	0.942483
44.4	37.25	940.61	0.942303
44.5	37.35	940.43	0.942123
44.6	37.44	940.26	0.941952
44.7	37.53	940.08	0.941772
44.8	37.62	939.90	0.941592
44.9	37.71	939.72	0.941411
45.0	37.80	939.54	0.941231
45.1	37.89	939.36	0.941051
45.2	37.98	939.18	0.940871
45.3	38.08	939.00	0.940690
45.4	38.17	938.82	0.940510
45.5	38.26	938.64	0.940330
45.6	38.35	938.46	0.940149
45.7	38.44	938.28	0.939969
45.8	38.53	938.10	0.939789
45.9	38.62	937.91	0.939598
46.0	38.72	937.73	0.939418
46.1	38.81	937.55	0.939238
46.2	38.90	937.36	0.939047
46.3	38.99	937.18	0.938867
46.4	39.08	937.00	0.938687
46.5	39.18	936.81	0.938496
46.6	39.27	936.63	0.938316
46.7	39.36	936.44	0.938126
46.8	39.45	936.26	0.937945
46.9	39.54	936.07	0.937755
47.0	39.64	935.88	0.937565
47.1	39.73	935.70	0.937384
47.2	39.82	935.51	0.937194
47.3	39.91	935.32	0.937004
47.4	40.00	935.14	0.936823
47.5	40.10	934.95	0.936633
47.6	40.19	934.76	0.936443
47.7	40.28	934.57	0.936252
47.8	40.37	934.38	0.936062
47.9	40.47	934.19	0.935872
48.0	40.56	934.00	0.935681
48.1	40.65	933.81	0.935491
48.2	40.75	933.62	0.935301
48.3	40.84	933.43	0.935110
48.4	40.93	933.24	0.934920

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
48.5	41.02	933.05	0.934729
48.6	41.12	932.86	0.934539
48.7	41.21	932.67	0.934349
48.8	41.30	932.47	0.934148
48.9	41.40	932.28	0.933958
49.0	41.49	932.09	0.933768
49.1	41.58	931.90	0.933577
49.2	41.68	931.70	0.933377
49.3	41.77	931.51	0.933187
49.4	41.86	931.32	0.932996
49.5	41.96	931.13	0.932806
49.6	42.05	930.92	0.932596
49.7	42.14	930.73	0.932405
49.8	42.24	930.53	0.932205
49.9	42.33	930.34	0.932015
50.0	42.43	930.14	0.931814
50.1	42.52	929.95	0.931624
50.2	42.61	929.75	0.931424
50.3	42.71	929.55	0.931223
50.4	42.80	929.35	0.931023
50.5	42.90	929.16	0.930832
50.6	42.99	928.96	0.930632
50.7	43.08	928.76	0.930432
50.8	43.18	928.56	0.930231
50.9	43.27	928.36	0.930031
51.0	43.37	928.16	0.929831
51.1	43.46	927.96	0.929630
51.2	43.56	927.77	0.929440
51.3	43.65	927.57	0.929240
51.4	43.74	927.36	0.929029
51.5	43.84	927.16	0.928829
51.6	43.93	926.96	0.928629
51.7	44.03	926.76	0.928428
51.8	44.12	926.56	0.928228
51.9	44.22	926.36	0.928027
52.0	44.31	926.16	0.927827
52.1	44.41	925.95	0.927617
52.2	44.50	925.75	0.927416
52.3	44.60	925.55	0.927216
52.4	44.69	925.35	0.927016
52.5	44.79	925.14	0.926805
52.6	44.88	924.94	0.926605
52.7	44.98	924.73	0.926395
52.8	45.07	924.53	0.926194
52.9	45.17	924.32	0.925984

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
53.0	45.26	924.12	0.925783
53.1	45.36	923.91	0.925573
53.2	45.46	923.71	0.925373
53.3	45.55	923.50	0.925162
53.4	45.65	923.30	0.924962
53.5	45.74	923.09	0.924752
53.6	45.84	922.88	0.924541
53.7	45.93	922.68	0.924341
53.8	46.03	922.47	0.924130
53.9	46.13	922.26	0.923920
54.0	46.22	922.06	0.923720
54.1	46.32	921.85	0.923509
54.2	46.41	921.64	0.923299
54.3	46.51	921.43	0.923089
54.4	46.61	921.22	0.922878
54.5	46.70	921.01	0.922668
54.6	46.80	920.80	0.922457
54.7	46.90	920.59	0.922247
54.8	46.99	920.38	0.922037
54.9	47.09	920.17	0.921826
55.0	47.18	919.96	0.921616
55.1	47.28	919.75	0.921406
55.2	47.38	919.54	0.921195
55.3	47.47	919.33	0.920985
55.4	47.57	919.12	0.920774
55.5	47.67	918.91	0.920564
55.6	47.77	918.69	0.920344
55.7	47.86	918.48	0.920133
55.8	47.96	918.27	0.919923
55.9	48.06	918.06	0.919713
56.0	48.15	917.84	0.919492
56.1	48.25	917.63	0.919282
56.2	48.35	917.42	0.919071
56.3	48.45	917.22	0.918871
56.4	48.54	916.99	0.918641
56.5	48.64	916.77	0.918420
56.6	48.74	916.56	0.918210
56.7	48.84	916.35	0.917999
56.8	48.94	916.13	0.917779
56.9	49.03	915.91	0.917559
57.0	49.13	915.70	0.917348
57.1	49.23	915.48	0.917128
57.2	49.32	915.27	0.916917
57.3	49.42	915.05	0.916697

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
57.4	49.52	914.83	0.916477
57.5	49.62	914.62	0.916266
57.6	49.72	914.40	0.916046
57.7	49.81	914.18	0.915826
57.8	49.91	913.97	0.915615
57.9	50.01	913.75	0.915395
58.0	50.11	913.53	0.915174
58.1	50.21	913.31	0.914954
58.2	50.31	913.09	0.914734
58.3	50.40	912.87	0.914513
58.4	50.50	912.65	0.914293
58.5	50.60	912.43	0.914072
58.6	50.70	912.22	0.913862
58.7	50.80	912.00	0.913642
58.8	50.90	911.78	0.913421
58.9	51.00	911.55	0.913191
59.0	51.10	911.33	0.912970
59.1	51.19	911.11	0.912750
59.2	51.29	910.89	0.912530
59.3	51.39	910.67	0.912309
59.4	51.49	910.45	0.912089
59.5	51.59	910.23	0.911868
59.6	51.69	910.01	0.911648
59.7	51.79	909.78	0.911418
59.8	51.89	909.56	0.911197
59.9	51.99	909.34	0.910977
60.0	52.09	909.11	0.910746
60.1	52.19	908.89	0.910526
60.2	52.29	908.67	0.910306
60.3	52.39	908.44	0.910075
60.4	52.49	908.22	0.909855
60.5	52.59	908.00	0.909634
60.6	52.69	907.77	0.909404
60.7	52.79	907.55	0.909184
60.8	52.89	907.32	0.908953
60.9	52.99	907.10	0.908733
61.0	53.09	906.87	0.908502
61.1	53.19	906.64	0.908272
61.2	53.29	906.42	0.908052
61.3	53.39	906.19	0.907821
61.4	53.49	905.97	0.907601
61.5	53.59	905.74	0.907370
61.6	53.69	905.51	0.907140
61.7	53.79	905.29	0.906920
61.8	53.89	905.06	0.906689

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
61.9	53.99	904.83	0.906459
62.0	54.09	904.60	0.906228
62.1	54.19	904.37	0.905998
62.2	54.30	904.15	0.905777
62.3	54.40	903.92	0.905547
62.4	54.50	903.69	0.905317
62.5	54.60	903.46	0.905086
62.6	54.70	903.23	0.904856
62.7	54.80	903.00	0.904625
62.8	54.90	902.77	0.904395
62.9	55.00	902.54	0.904165
63.0	55.11	902.31	0.903934
63.1	55.21	902.08	0.903704
63.2	55.31	901.85	0.903473
63.3	55.41	901.62	0.903243
63.4	55.51	901.39	0.903013
63.5	55.61	901.15	0.902772
63.6	55.72	900.92	0.902542
63.7	55.82	900.69	0.902311
63.8	55.92	900.46	0.902081
63.9	56.02	900.23	0.901850
64.0	56.12	899.99	0.901610
64.1	56.23	899.76	0.901380
64.2	56.33	899.53	0.901149
64.3	56.43	899.29	0.900909
64.4	56.53	899.06	0.900678
64.5	56.64	898.83	0.900448
64.6	56.74	898.59	0.900207
64.7	56.84	898.36	0.899977
64.8	56.94	898.12	0.899737
64.9	57.05	897.89	0.899506
65.0	57.15	897.65	0.899266
65.1	57.25	897.42	0.899035
65.2	57.36	897.18	0.898795
65.3	57.46	896.94	0.898554
65.4	57.56	896.71	0.898324
65.5	57.67	896.47	0.898084
65.6	57.77	896.23	0.897843
65.7	57.87	896.00	0.897613
65.8	57.98	895.76	0.897372
65.9	58.08	895.52	0.897132
66.0	58.18	895.28	0.896892
66.1	58.29	895.05	0.896661
66.2	58.39	894.81	0.896421

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
66.3	58.49	894.57	0.896180
66.4	58.60	894.33	0.895940
66.5	58.70	894.09	0.895699
66.6	58.81	893.85	0.895459
66.7	58.91	893.61	0.895218
66.8	59.01	893.37	0.894978
66.9	59.12	893.13	0.894738
67.0	59.22	892.89	0.894497
67.1	59.33	892.65	0.894257
67.2	59.43	892.41	0.894016
67.3	59.54	892.17	0.893776
67.4	59.64	891.93	0.893535
67.5	59.74	891.69	0.893295
67.6	59.85	891.45	0.893055
67.7	59.95	891.20	0.892804
67.8	60.06	890.96	0.892564
67.9	60.16	890.72	0.892323
68.0	60.27	890.48	0.892083
68.1	60.37	890.23	0.891832
68.2	60.48	889.99	0.891592
68.3	60.58	889.75	0.891352
68.4	60.69	889.50	0.891101
68.5	60.80	889.26	0.890861
68.6	60.90	889.01	0.890610
68.7	61.01	888.77	0.890370
68.8	61.11	888.52	0.890119
68.9	61.22	888.28	0.889879
69.0	61.32	888.03	0.889628
69.1	61.43	887.79	0.889388
69.2	61.54	887.54	0.889138
69.3	61.64	887.29	0.888887
69.4	61.75	887.05	0.888647
69.5	61.85	886.80	0.888396
69.6	61.96	886.55	0.888146
69.7	62.07	886.31	0.887905
69.8	62.17	886.06	0.887655
69.9	62.28	885.81	0.887404
70.0	62.39	885.56	0.887154
70.1	62.49	885.31	0.886904
70.2	62.60	885.06	0.886653
70.3	62.71	884.82	0.886413
70.4	62.81	884.57	0.886162
70.5	62.92	884.32	0.885912
70.6	63.03	884.07	0.885661
70.7	63.13	883.82	0.885411

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
70.8	63.24	883.57	0.885160
70.9	63.35	883.32	0.884910
71.0	63.46	883.06	0.884650
71.1	63.56	882.81	0.884399
71.2	63.67	882.56	0.884149
71.3	63.78	882.31	0.883898
71.4	63.89	882.06	0.883648
71.5	63.99	881.81	0.883397
71.6	64.10	881.55	0.883137
71.7	64.21	881.30	0.882886
71.8	64.32	881.05	0.882636
71.9	64.43	880.79	0.882375
72.0	64.53	880.54	0.882125
72.1	64.64	880.29	0.881875
72.2	64.75	880.03	0.881614
72.3	64.86	879.78	0.881364
72.4	64.97	879.52	0.881103
72.5	65.08	879.27	0.880853
72.6	65.19	879.01	0.880592
72.7	65.29	878.75	0.880332
72.8	65.40	878.50	0.880081
72.9	65.51	878.24	0.879821
73.0	65.62	877.99	0.879570
73.1	65.73	877.73	0.879310
73.2	65.84	877.47	0.879049
73.3	65.95	877.21	0.878789
73.4	66.06	876.96	0.878539
73.5	66.17	876.70	0.878278
73.6	66.28	876.44	0.878018
73.7	66.39	876.18	0.877757
73.8	66.50	875.92	0.877497
73.9	66.61	875.66	0.877236
74.0	66.72	875.40	0.876976
74.1	66.83	875.14	0.876715
74.2	66.94	874.88	0.876455
74.3	67.05	874.62	0.876194
74.4	67.16	874.36	0.875934
74.5	67.27	874.10	0.875673
74.6	67.38	873.84	0.875413
74.7	67.49	873.58	0.875152
74.8	67.60	873.32	0.874892
74.9	67.71	873.06	0.874632
75.0	67.82	872.79	0.874361
75.1	67.93	872.53	0.874101

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
75.2	68.04	872.27	0.873840
75.3	68.15	872.00	0.873570
75.4	68.26	871.74	0.873309
75.5	68.38	871.48	0.873049
75.6	68.49	871.21	0.872778
75.7	68.60	870.95	0.872518
75.8	68.71	870.68	0.872247
75.9	68.82	870.42	0.871987
76.0	68.93	870.15	0.871716
76.1	69.04	869.89	0.871456
76.2	69.16	869.62	0.871185
76.3	69.27	869.35	0.870915
76.4	69.38	869.09	0.870654
76.5	69.49	868.82	0.870384
76.6	69.61	868.55	0.870113
76.7	69.72	868.28	0.869843
76.8	69.83	868.02	0.869582
76.9	69.94	867.75	0.869312
77.0	70.06	867.48	0.869041
77.1	70.17	867.21	0.868771
77.2	70.28	866.94	0.868500
77.3	70.39	866.67	0.868230
77.4	70.51	866.40	0.867960
77.5	70.62	866.13	0.867689
77.6	70.73	865.86	0.867419
77.7	70.85	865.59	0.867148
77.8	70.96	865.32	0.866878
77.9	71.07	865.05	0.866607
78.0	71.19	864.78	0.866337
78.1	71.30	864.50	0.866056
78.2	71.41	864.23	0.865786
78.3	71.53	863.96	0.865515
78.4	71.64	863.69	0.865245
78.5	71.76	863.41	0.864964
78.6	71.87	863.14	0.864694
78.7	71.98	862.86	0.864413
78.8	72.10	862.59	0.864143
78.9	72.21	862.31	0.863862
79.0	72.33	862.04	0.863592
79.1	72.44	861.76	0.863311
79.2	72.56	861.49	0.863041
79.3	72.67	861.21	0.862760
79.4	72.79	860.94	0.862490
79.5	72.90	860.66	0.862209
79.6	73.02	860.38	0.861929

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
79.7	73.13	860.10	0.861648
79.8	73.25	859.83	0.861378
79.9	73.36	859.55	0.861097
80.0	73.48	859.27	0.860817
80.1	73.60	858.99	0.860536
80.2	73.71	858.71	0.860256
80.3	73.83	858.43	0.859975
80.4	73.94	858.15	0.859695
80.5	74.06	857.87	0.859414
80.6	74.18	857.59	0.859134
80.7	74.29	857.31	0.858853
80.8	74.41	857.03	0.858573
80.9	74.53	856.75	0.858292
81.0	74.64	856.46	0.858002
81.1	74.76	856.18	0.857721
81.2	74.88	855.90	0.857441
81.3	74.99	855.62	0.857160
81.4	75.11	855.33	0.856870
81.5	75.23	855.05	0.856589
81.6	75.34	854.76	0.856299
81.7	75.46	854.48	0.856018
81.8	75.58	854.19	0.855728
81.9	75.70	853.91	0.855447
82.0	75.82	853.62	0.855157
82.1	75.93	853.34	0.854876
82.2	76.05	853.05	0.854585
82.3	76.17	852.76	0.854295
82.4	76.29	852.48	0.854014
82.5	76.41	852.19	0.853724
82.6	76.52	851.90	0.853433
82.7	76.64	851.61	0.853143
82.8	76.76	851.32	0.852852
82.9	76.88	851.03	0.852562
83.0	77.00	850.74	0.852271
83.1	77.12	850.45	0.851981
83.2	77.24	850.16	0.851690
83.3	77.36	849.87	0.851400
83.4	77.48	849.58	0.851109
83.5	77.60	849.29	0.850819
83.6	77.72	848.99	0.850518
83.7	77.84	848.70	0.850228
83.8	77.96	848.41	0.849937
83.9	78.08	848.11	0.849637
84.0	78.20	847.82	0.849346

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
84.1	78.32	847.53	0.849056
84.2	78.44	847.23	0.848755
84.3	78.56	846.93	0.848454
84.4	78.68	846.64	0.848164
84.5	78.80	846.34	0.847863
84.6	78.92	846.05	0.847573
84.7	79.04	845.75	0.847272
84.8	79.16	845.45	0.846972
84.9	79.28	845.15	0.846671
85.0	79.40	844.85	0.846371
85.1	79.53	844.55	0.846070
85.2	79.65	844.25	0.845770
85.3	79.77	843.95	0.845469
85.4	79.89	843.65	0.845169
85.5	80.01	843.35	0.844868
85.6	80.14	843.05	0.844567
85.7	80.26	842.75	0.844267
85.8	80.38	842.44	0.843956
85.9	80.50	842.14	0.843656
86.0	80.63	841.84	0.843355
86.1	80.75	841.53	0.843045
86.2.	80.87	841.23	0.842744
86.3	81.00	840.92	0.842434
86.4	81.12	840.62	0.842133
86.5	81.24	840.31	0.841823
86.6	81.37	840.00	0.841512
86.7	81.49	839.70	0.841211
86.8	81.61	839.39	0.840901
86.9	81.74	839.08	0.840590
87.0	81.86	838.77	0.840280
87.1	81.99	838.46	0.839969
87.2	82.11	838.15	0.839659
87.3	82.24	837.84	0.839348
87.4	82.36	837.52	0.839028
87.5	82.49	837.21	0.838717
87.6	82.61	836.90	0.838406
87.7	82.74	836.59	0.838096
87.8	82.86	836.27	0.837775
87.9	82.99	835.96	0.837465
88.0	83.11	835.64	0.837144
88.1	83.24	835.32	0.836824
88.2	83.37	835.01	0.836513
88.3	83.49	834.69	0.836192
88.4	83.62	834.37	0.835872
88.5	83.74	834.05	0.835551

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
88.6	83.87	833.73	0.835231
88.7	84.00	833.41	0.834910
88.8	84.13	833.09	0.834590
88.9	84.25	832.77	0.834269
89.0	84.38	832.45	0.833948
89.1	84.51	832.12	0.833618
89.2	84.64	831.80	0.833297
89.3	84.76	831.48	0.832977
89.4	84.89	831.15	0.832646
89.5	85.02	830.82	0.832315
89.6	85.15	830.50	0.831995
89.7	85.28	830.17	0.831664
89.8	85.41	829.84	0.831334
89.9	85.54	829.51	0.831003
90.0	85.66	829.18	0.830673
90.1	85.79	828.85	0.830342
90.2	85.92	828.52	0.830011
90.3	86.05	828.19	0.829681
90.4	86.18	827.85	0.829340
90.5	86.31	827.52	0.829010
90.6	86.44	827.18	0.828669
90.7	86.57	826.85	0.828338
90.8	86.71	826.51	0.827998
90.9	86.84	826.17	0.827657
91.0	86.97	825.83	0.827316
91.1	87.10	825.49	0.826976
91.2	87.23	825.15	0.826635
91.3	87.36	824.81	0.826295
91.4	87.49	824.47	0.825954
91.5	87.63	824.13	0.825613
91.6	87.76	823.78	0.825263
91.7	87.90	823.44	0.824922
91.8	88.02	823.09	0.824572
91.9	88.16	822.74	0.824221
92.0	88.29	822.39	0.823870
92.1	88.42	822.04	0.823520
92.2	88.56	821.69	0.823169
92.3	88.69	821.34	0.822818
92.4	88.83	820.99	0.822468
92.5	88.96	820.63	0.822107
92.6	89.10	820.28	0.821757
92.7	89.23	819.92	0.821396
92.8	89.37	819.57	0.821045
92.9	89.50	819.21	0.820685

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
93.0	89.64	818.85	0.820324
93.1	89.77	818.49	0.819963
93.2	89.91	818.12	0.819593
93.3	90.05	817.76	0.819232
93.4	90.18	817.40	0.818871
93.5	90.32	817.03	0.818501
93.6	90.46	816.66	0.818130
93.7	90.59	816.30	0.817769
93.8	90.73	815.93	0.817399
93.9	90.87	815.55	0.817018
94.0	91.01	815.18	0.816647
94.1	91.15	814.81	0.816277
94.2	91.29	814.43	0.815896
94.3	91.43	814.06	0.815525
94.4	91.56	813.68	0.815145
94.5	91.70	813.30	0.814764
94.6	91.84	812.92	0.814383
94.7	91.98	812.54	0.814003
94.8	92.13	812.15	0.813612
94.9	92.27	811.77	0.813231
95.0	92.41	811.38	0.812840
95.1	92.55	810.99	0.812450
95.2	92.69	810.60	0.812059
95.3	92.83	810.21	0.811668
95.4	92.98	809.82	0.811278
95.5	93.12	809.42	0.810877
95.6	93.26	809.02	0.810476
95.7	93.41	808.63	0.810086
95.8	93.55	808.23	0.809685
95.9	93.69	807.82	0.809274
96.0	93.84	807.42	0.808873
96.1	93.98	807.01	0.808463
96.2	94.13	806.61	0.808062
96.3	94.27	806.20	0.807651
96.4	94.42	805.78	0.807230
96.5	94.57	805.37	0.806820
96.6	94.71	804.96	0.806409
96.7	94.86	804.54	0.805988
96.8	95.01	804.12	0.805567
96.9	95.16	803.70	0.805147
97.0	95.31	803.27	0.804716
97.1	95.45	802.85	0.804295
97.2	95.60	802.42	0.803864
97.3	95.75	801.99	0.803434
97.4	95.90	801.55	0.802993

% v/v	% w/w	$\rho_{20} \text{ (kg/m}^3\text{)}$	$d \text{ (g/cm}^3\text{)}$
97.5	96.05	801.12	0.802562
97.6	96.21	800.68	0.802121
97.7	96.36	800.24	0.801680
97.8	96.51	799.80	0.801240
97.9	96.66	799.35	0.800789
98.0	96.81	798.90	0.800338
98.1	96.97	798.45	0.799887
98.2	97.12	798.00	0.799436
98.3	97.28	797.54	0.798976
98.4	97.43	797.08	0.798515
98.5	97.59	796.62	0.798054
98.6	97.74	796.15	0.797583
98.7	97.90	795.68	0.797112
98.8	98.06	795.21	0.796641
98.9	98.22	794.73	0.796161
99.0	98.38	794.25	0.795680
99.1	98.53	793.77	0.795199
99.2	98.69	793.28	0.794708
99.3	98.86	792.79	0.794217
99.4	99.02	792.30	0.793726
99.5	99.18	791.80	0.793225
99.6	99.34	791.29	0.792714
99.7	99.50	790.79	0.792213
99.8	99.67	790.28	0.791703
99.9	99.83	789.76	0.791182
100.0	100.00	789.24	0.790661