Analytical methods and performance criteria

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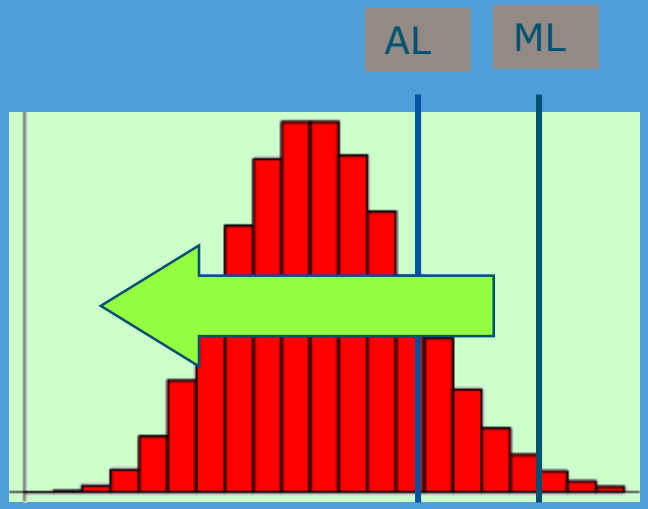
EU Limits for food and feed

- Exposure population around TWI
- Food levels should be further reduced
  - Limits should not result in high non-compliance rates: "strict but feasible"
- Eventual goal is reduction of exposure below TWI

Establishment of EU-limits (since 2001)

to gradually reduce the levels and exposure

- Inventory of existing levels
  - First maximum level (ML) dioxins only
  - In 2006: dioxin-like PCBs: sum TEQ, ML dioxins kept
  - Limit around 90-95th percentile; so 5-10% above limit
- Also action levels (2/3 of ML)
  - For dioxins and for dl-PCBs



“Strict but feasible”

- Kind of ALARA but “reversed”
  - ALARA: as low as reasonable achievable
  - M(R)Ls may be lower than required for protection
  - Eg based on GAP in case of pesticides
- Some confusion: witch hunt on dioxins and PCBs

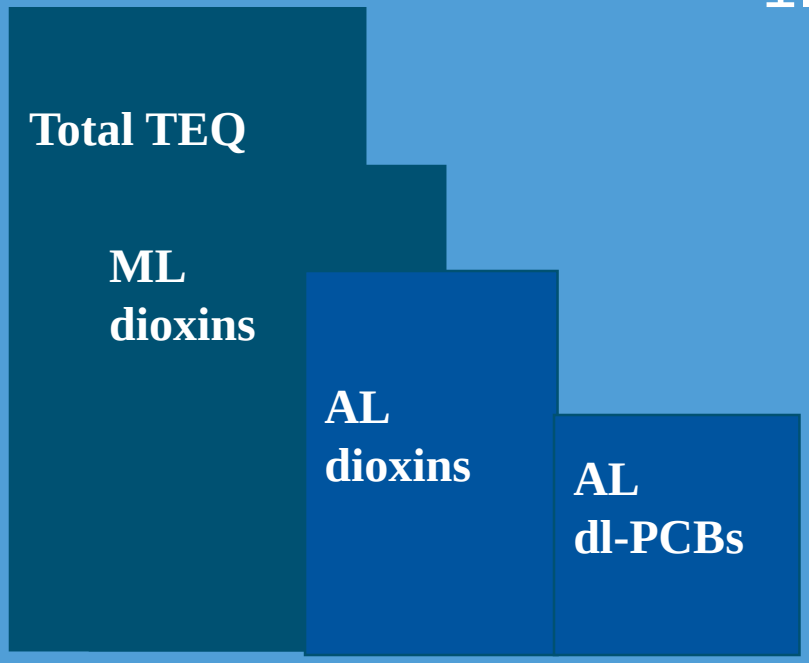
MLs protective?

- Not necessarily
- Example:
  - ML fish dioxins and dl-PCBs: 6.5 pg TEQ/g fish
  - Recommended intake: 300 g/week (2 portions)
  - Intake: 1950 pg TEQ, about 30 pg TEQ/kg bw/week
  - TWI: 14 pg TEQ/kg bw/week
- In practice lower but fish is important source
- Similar applies for other food products
- So, products just below the MLs not necessarily safe

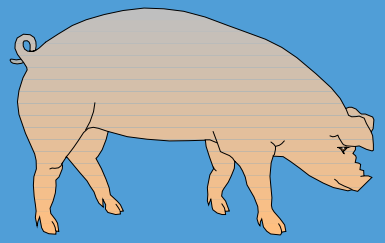
Food limits (since July 2002)

- Many different limits
  - Limits for pork (1), poultry (2), beef, milk and eggs (3), expressed in pg TEQ/g fat
  - Limit for fish: 4 pg TEQ/g fish
- First only dioxins; since 4-11-2006 also planar PCBs
- New limits in 2012: change to "new" TEFs 2005
  - Food: Regulation (EU) No 1259/2011 amending Regulation (EC) No 1881/2006
  - Feed: Regulation (EU) No 277/2012 amending Annexes I and II to Directive 2002/32/EC

Action and maximum levels



1.25 pg TEQ/g



1.0 pg TEQ/g

0.75 pg TEQ/g

0.5 pg TEQ/g

(EC) 1881/2006 and 2011/516/EU

Maximum and action levels

- If higher than maximum levels
  - Not allowed to sell the products
  - Recall
  - Not allowed to dilute
- If higher than action levels
  - Further action required to find the source of the contamination
  - Follow-up still limited

Screening and/or confirmation

- Both screening and confirmatory methods can be applied
- Screening methods are:
  - high throughput, often more rapid and require cheaper equipment: overall cheaper
  - Can be used to separate negative samples from suspected samples
  - Cannot be used for final confirmation of the positive result in official control
- Proper discrimination between negative and suspected samples essential

Analytical methods

Confirmatory methods

- Required for proving the identity of compounds
- Required for establishing level
- Requires use of MS-technologies
- Therefore relatively expensive, low throughput
- Confirms known compounds

Screening methods (examples)

- Bioanalytical methods
  - Immunoassays
  - Receptor assays
  - Bioassays
- Chemical analytical methods
  - Non-MS based techniques (UV, fluorescence)
  - MS-based LC- or GC, including
  - Multi-methods (e.g. pesticides, mycotoxins, veterinary drugs, dioxins and PCBs)
  - Untargeted screening GC- or LC-MS

Bioassays at RIKILT

- Bacterial assays for antibiotics
- DR CALUX-assay for dioxins and dioxin-like PCBs
- Yeast and cell assays for hormonal compounds
    - Estrogens, androgens, corticosteroids, etc.
- N2a-assay to replace mouse bioassay for marine biotoxins
    - MBA still widely applied
- PDE-5 inhibitor test for viagra-like compounds (supplements)
- Beta-receptor assay for beta-agonists (supplements)

Quality control on application of methods

Quality of results?

- Proper validation and accreditation
  - ISO 17025
  - Method is “fit for purpose”
- Demonstration of correct performance
  - Analysis of internal control samples
  - Participation in proficiency tests

Role of reference laboratories

- Various classes of residues, bacteria and contaminants
- For each class EURL (European Reference Laboratory) appointed
    - Existing national institutes or JRCs (Joined Research Centre)
    - Based on tenders and application
- Per class in each country at least one NRL (National Reference Laboratory)
- In addition OLs (Official Laboratories)

Some examples

- EURL dioxins and PCBs: CVUA Freiburg
- EURL mycotoxins: JRC Geel
- EURL heavy metals: JRC Geel
- EURL marine biotoxins: ASEAN Vigo
- EURL pesticides: 4 different EURLs
- EURL hormones: RIKILT Wageningen

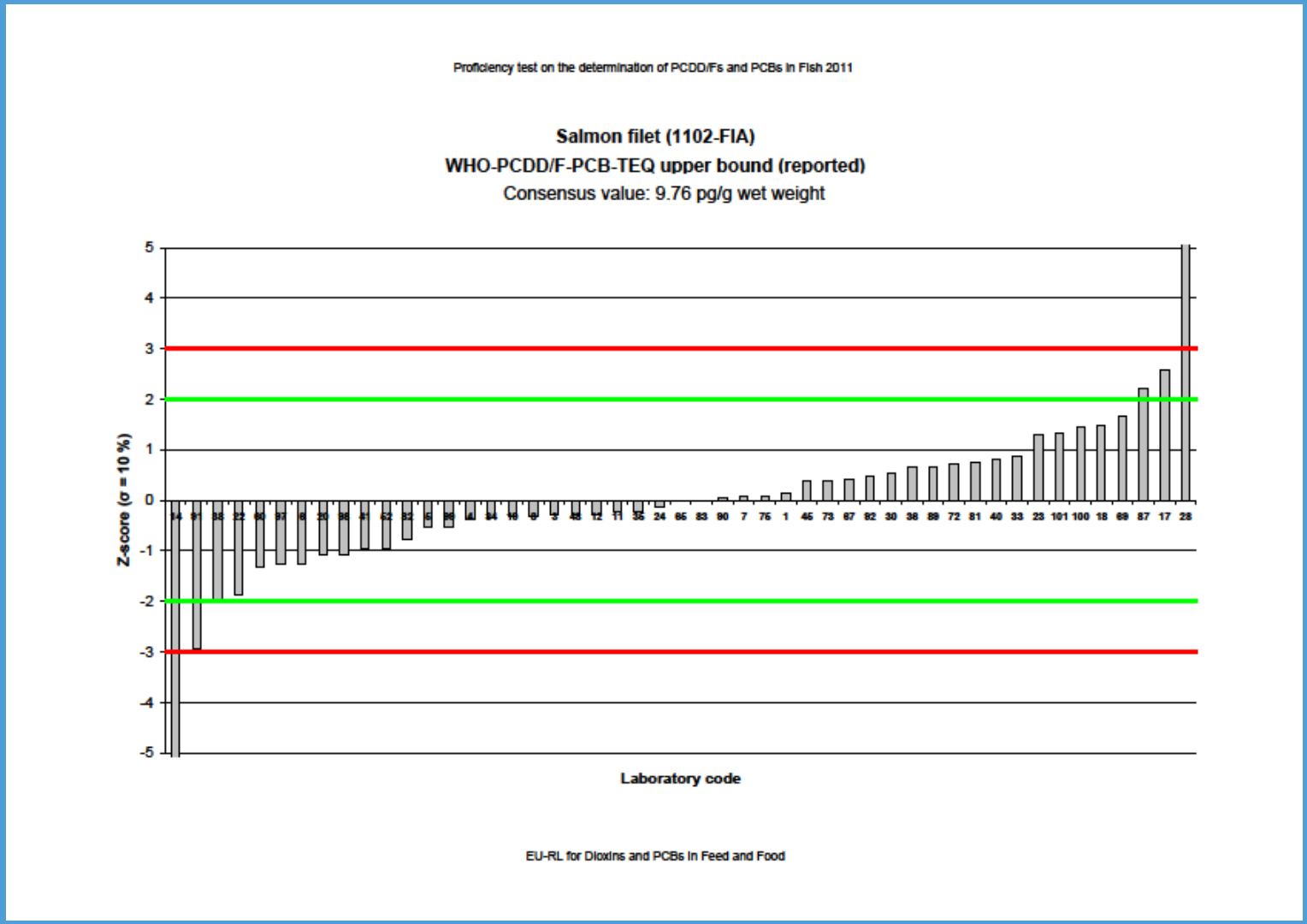
Task EURL

- Described in EU Regulation 882/2004
- Tasks include
  - Organization workshops for NRLs (at least once a year)
  - Discussion on new developments in legislation
  - Discussion of methods
  - Support of EU authorities (DG SANTE)
  - Organization of PT-tests for NRLs (OLs)
  - Support of NRLs to improve methods
  - Support of NRLs in case of conflicts

Task NRLs

- Participation in EURL workshops
- Participation in PT-tests
- Support national authorities
- Support Official Laboratories
    - Exchange of samples
    - Advice on improving methods
    - Confirmation of conflicting results

PT-test EURL: dioxins/PCBs in salmon



LOQs (limit of quantification)

- Should be low enough to check for compliance
  - Often reporting limit close to maximum level (50%)
- But would be better if detect background levels detected
  - Reduce upperbound levels
  - Exposure assessment (e.g. by EFSA)
  - Trend analysis
  - In case of dioxins/dl-PCBs LOQ <1/5 of ML

Performance criteria or prescribed methods?

- Criteria give more flexibility for changes
- For dioxins and dl-PCBs
  - Commission Directives 2002/69/EC (food) and 2002/70/EC (feed): also application of bioassays
  - Upgraded to Commission Regulations EC (No) 1883/2006 (food) and 152/2009 (feed)
  - Replaced by Commission Regulations EC (No) 252/2012 (food) and 278/2012 (feed)
- Changes based on the work of expert groups
  - Nowadays EURL/NRL network

Performance criteria for whom?

- Apply for official control
- And in the field of fats for feed also for private laboratories (EC 225/2012)
    - Will be extended to other matrices

Methods for dioxins and PCBs

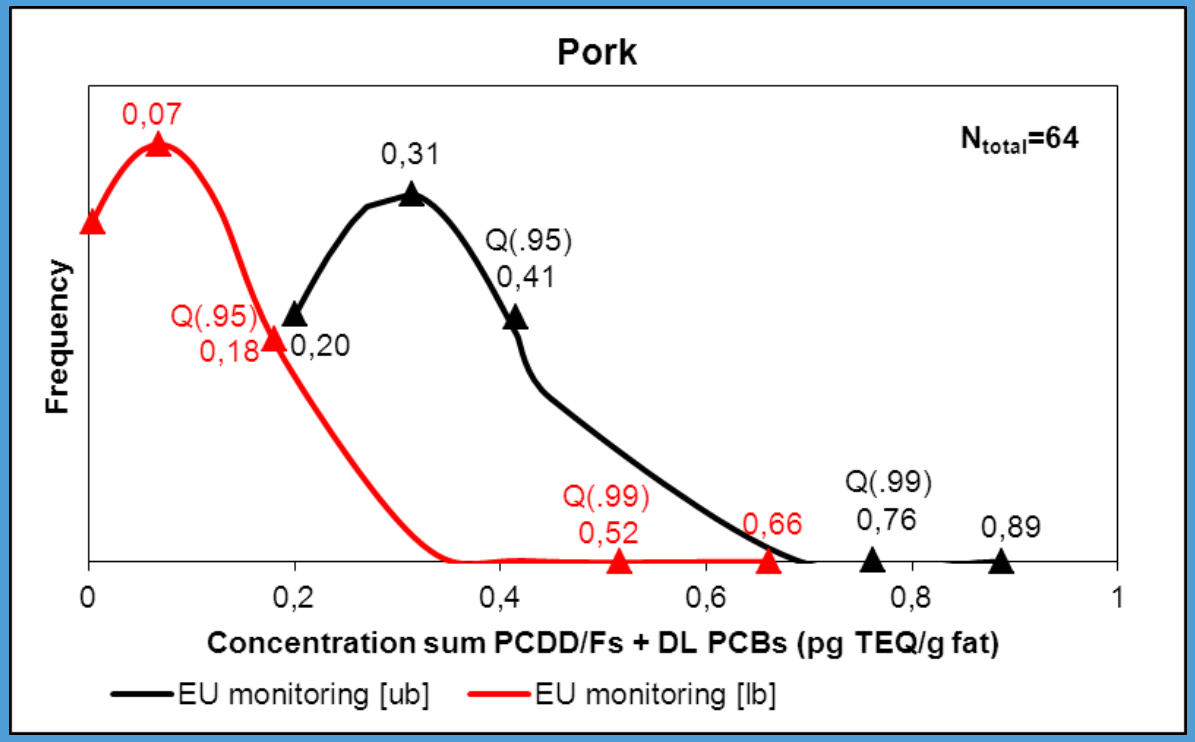
GC/HRMS or GC/MS/MS: reference method

- GC/HRMS: confirmation
  - detection at pg/g levels
  - removal of fat
  - removal of pesticides
  - removal non-dl PCBs
  - detection with GC/HRMS or GC/MS/MS
- Different columns needed
  - Automated clean-up
- Use of 13C-standards
- Expensive method

Relevant issues confirmatory methods

- Application of lower- and upperbound principle
  - Upperbound: levels of non-detected congeners are assumed to be equal to zero/LOQ
  - Lowerbound: non-detects set to zero
  - Upperbound level used for checking compliance
  - And for exposure assessment

Effect ub vs lb for samples with low levels

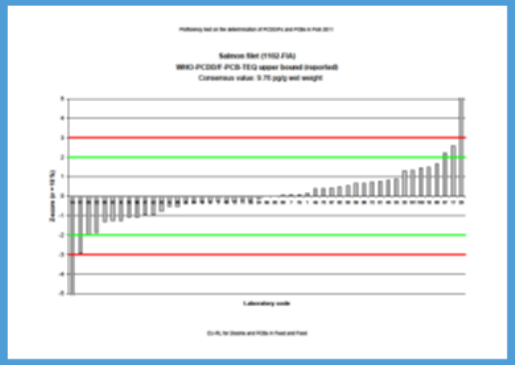


- Upperbound levels are a clear overestimation of the level
- So GC/HRMS levels look OK but are not

Relevant issues confirmatory methods

- Measurement uncertainty
  - The measurement uncertainty of the method should be established and levels corrected for this
  - Based on reproducibility of the analysis
  - So e.g. 1.15 may be reduced to 1.0 before checking compliance (15% MU)
- Also take into account result PT test (bias)
  - E.g. due to less good standards

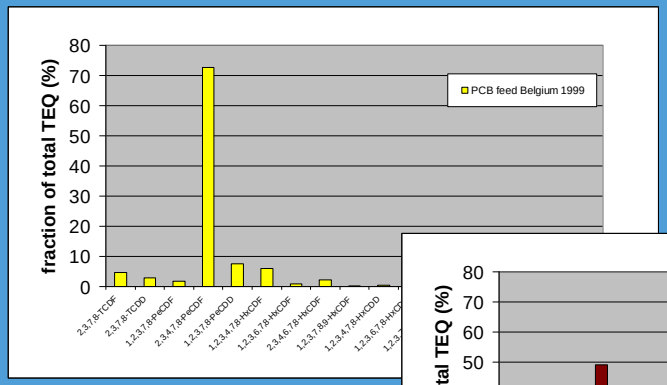
PT-test EURL: dioxins/PCBs in salmon

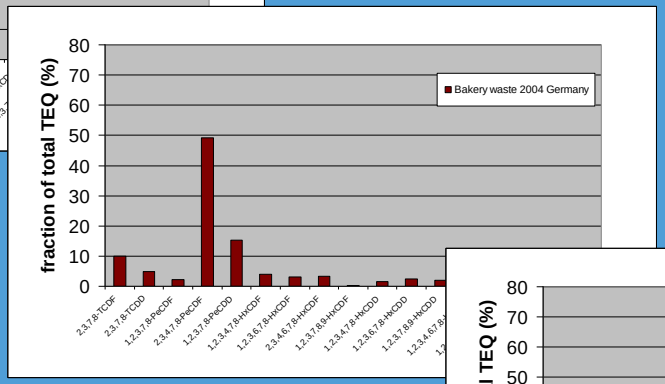


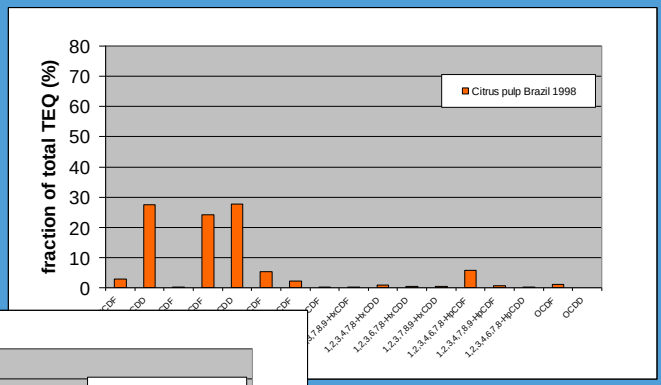
Non-compliance of samples

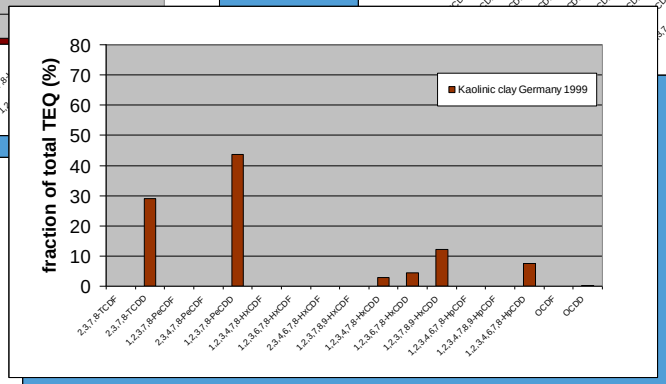
- Can only be based on confirmatory analysis
  - After application of measurement uncertainty
- And when reproduced in second independent analysis
  - Also to exclude mix-up
- Applies also to action levels/thresholds

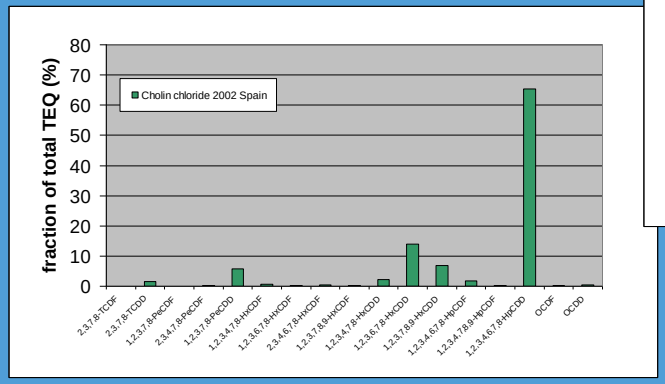
GC/HRMS: allows use of patterns











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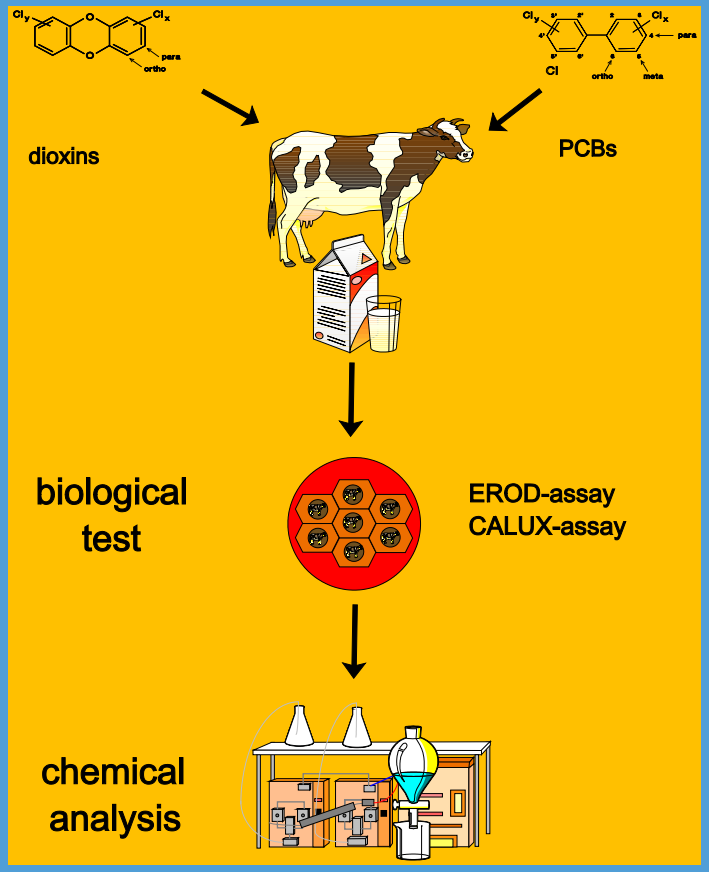
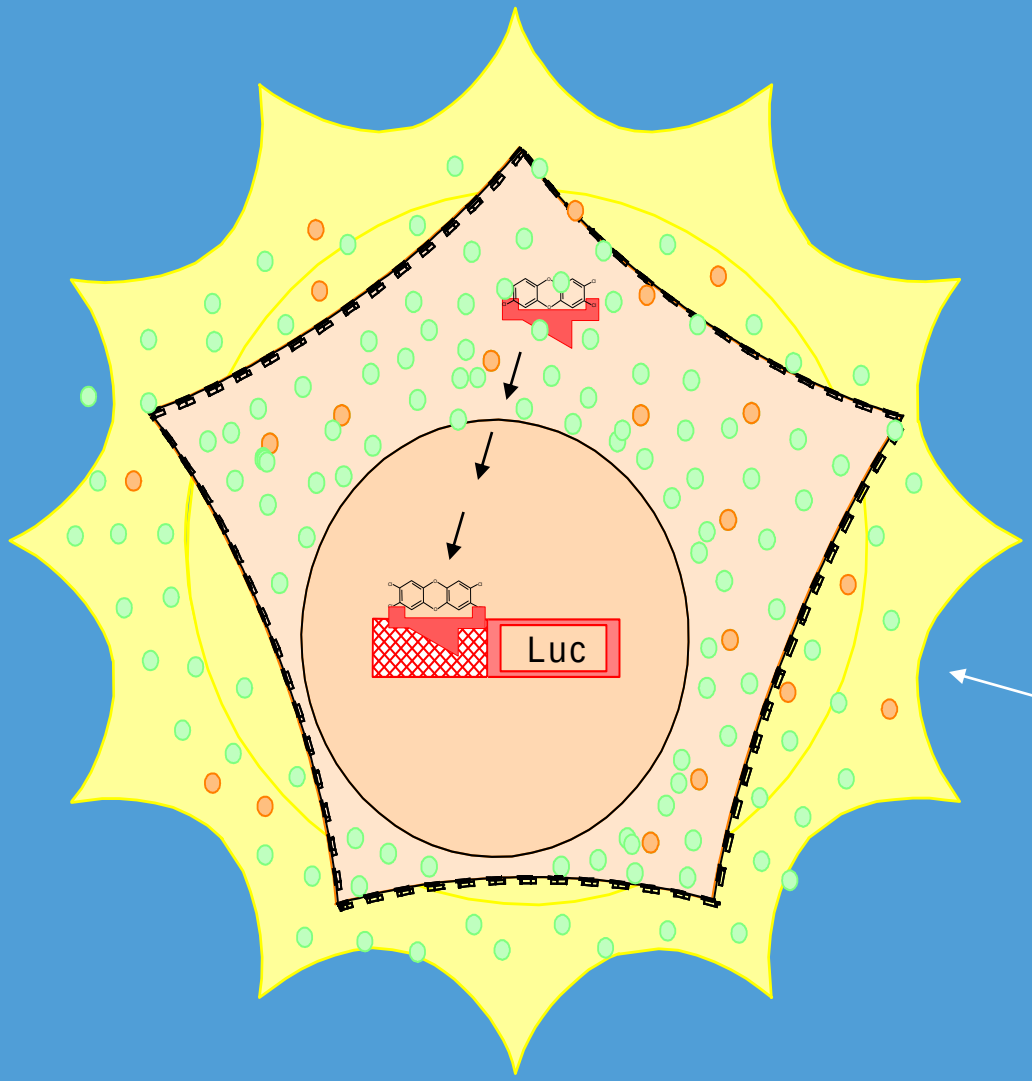
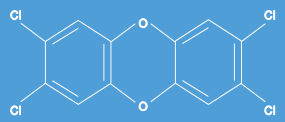
CALUX bioassay

- (DR) CALUX: screening
  - removal negative samples
  - confirmation suspects
- At RIKILT used since 1998
  - Almost every week



CALUX screening assay





Luciferin
+
ATP

Aarts et al. 1993

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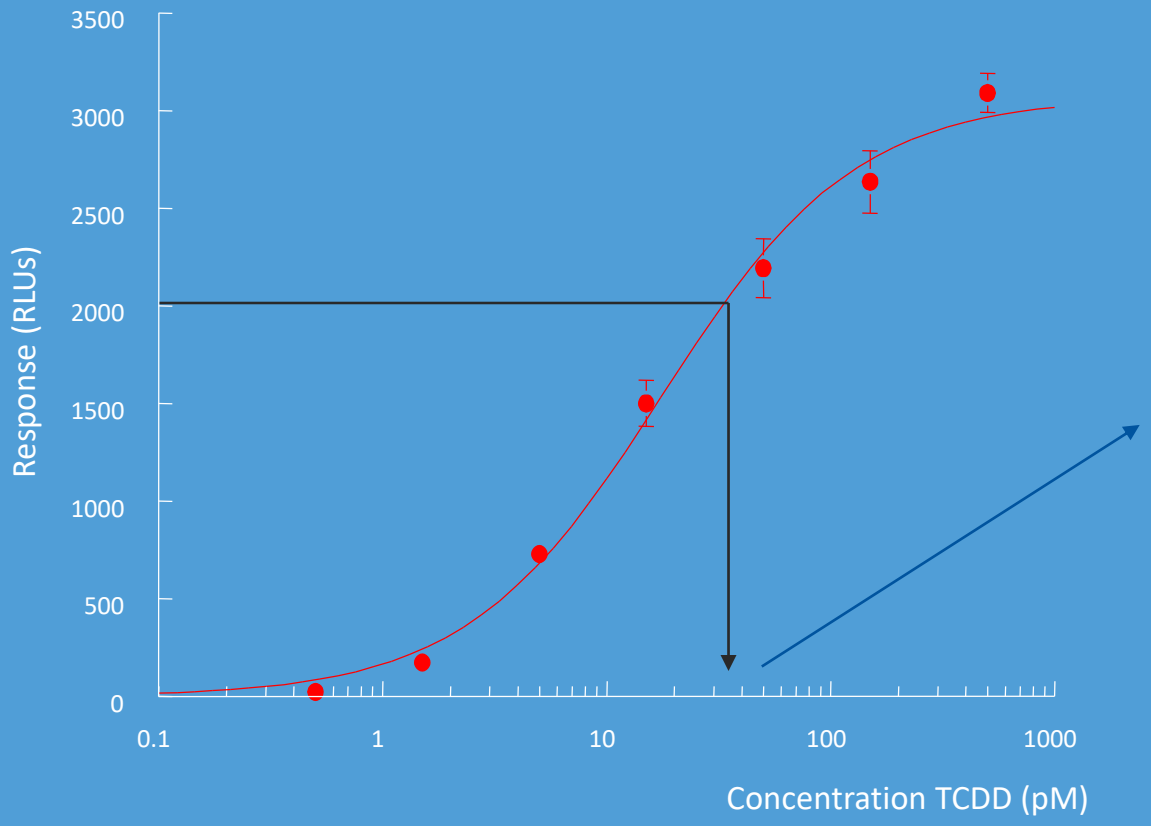
Estimation of level in sample



EU Directive performance criteria

- Development
  - Initially from expert group (2001)
  - Revised by WG EURL/NRLs
- CALUX is a screening method (yes/no answer)
  - Estimation may be given; support confirmation analysis
- Should be in BEQs and not TEQs
  - Relative response congeners in test not identical to TEFs
  - Also other compounds (w/o TEF) may show response
  - **Screening result should be recognizable**

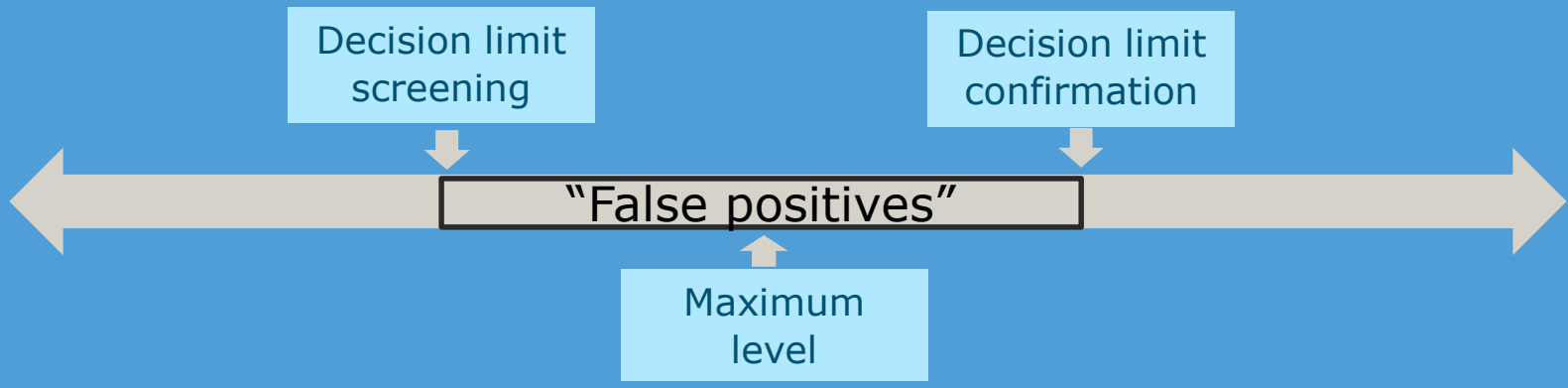
False-compliant rate

What fraction of positives can be missed?

- Initially set at 1%
  - Difficult to prove compliance
  - Hundreds of positive samples need to be analyzed
  - Not clear if towards ML or AL
- In other areas 5% is used (EC 2002/657)
- Therefore proposal to set it at 5%
- Refers to maximum limit, not action limit
  - Performance towards action limit should be evaluated

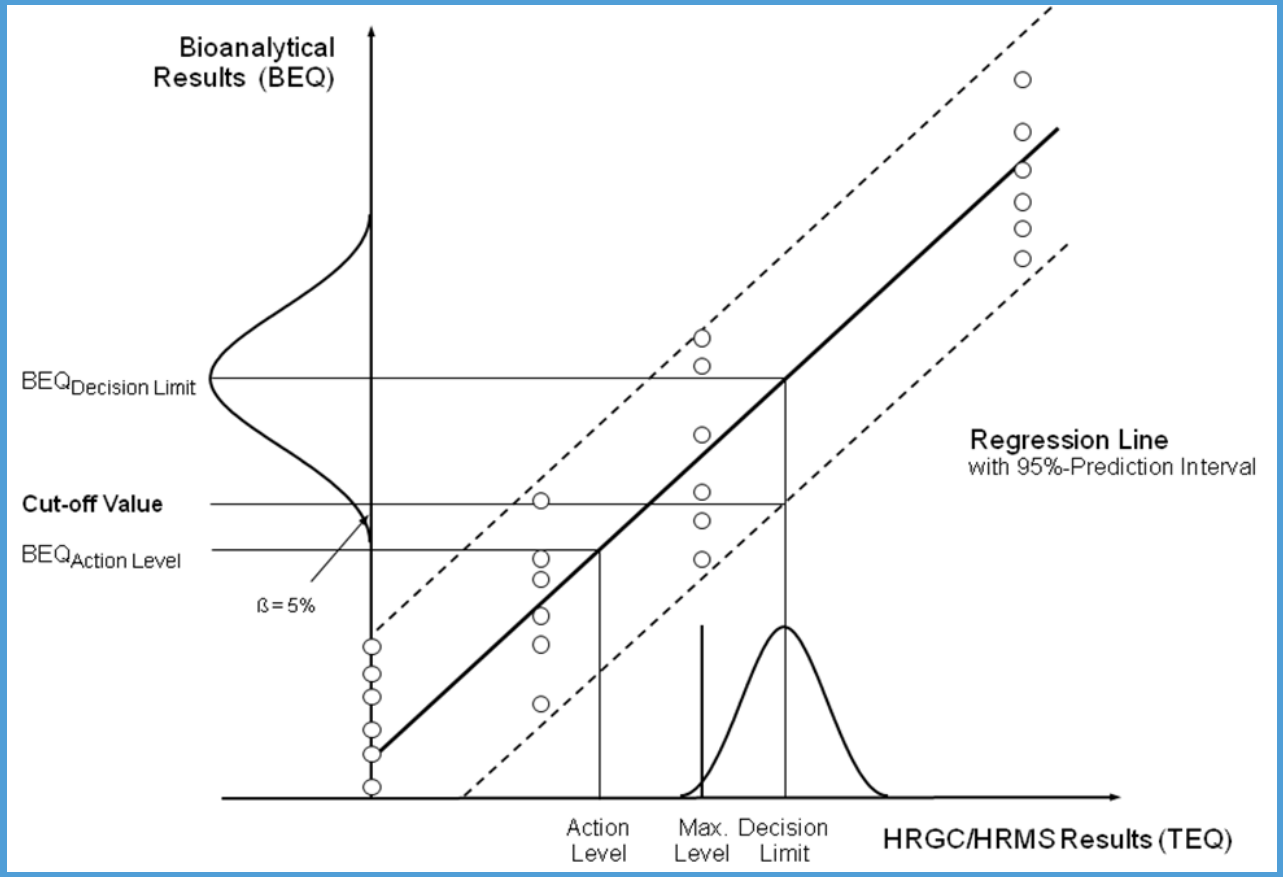
Screening versus confirmation

- Screening should not miss positive samples
  - Chance less than 5%
- Confirmation should not falsely decide on positive result
  - Chance less than 5%
  - Application of measurement uncertainty



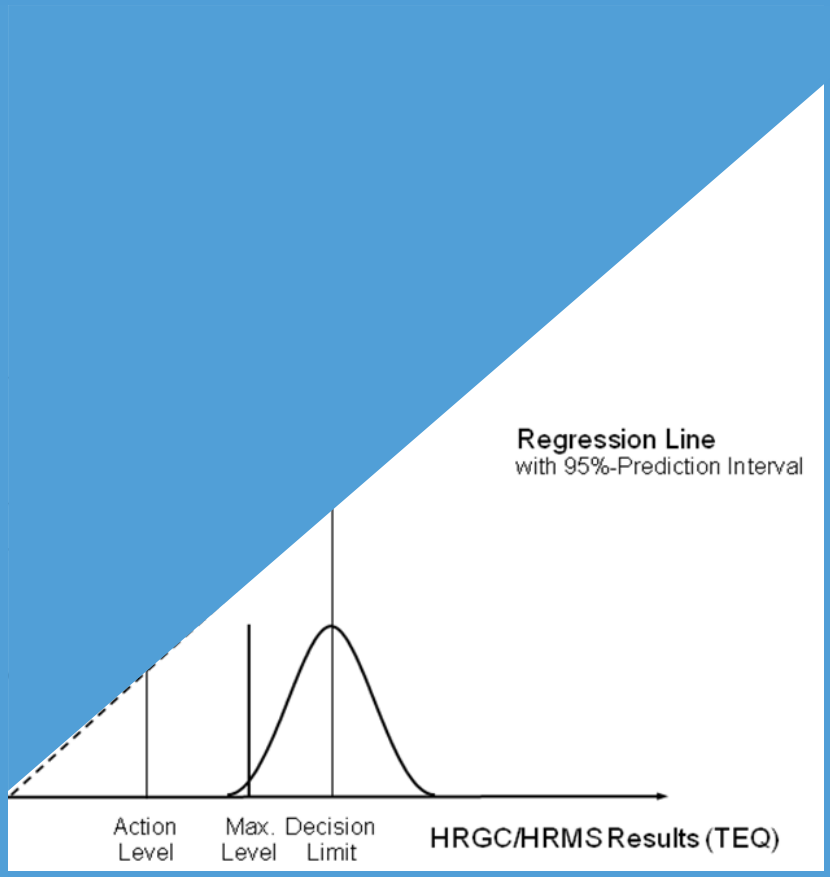
Setting of cut-off levels for screening

establish relation between screening and confirmatory method



(No) 252/2012 (food) and 278/2012 (feed)

Setting of cut-off levels for screening



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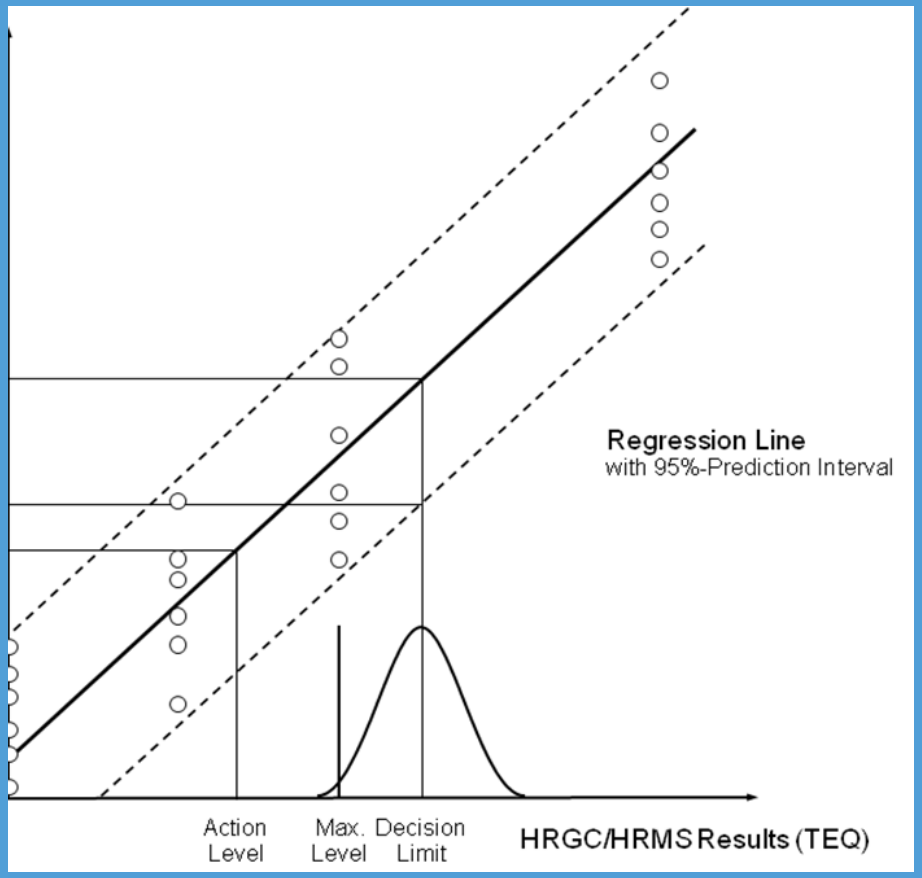
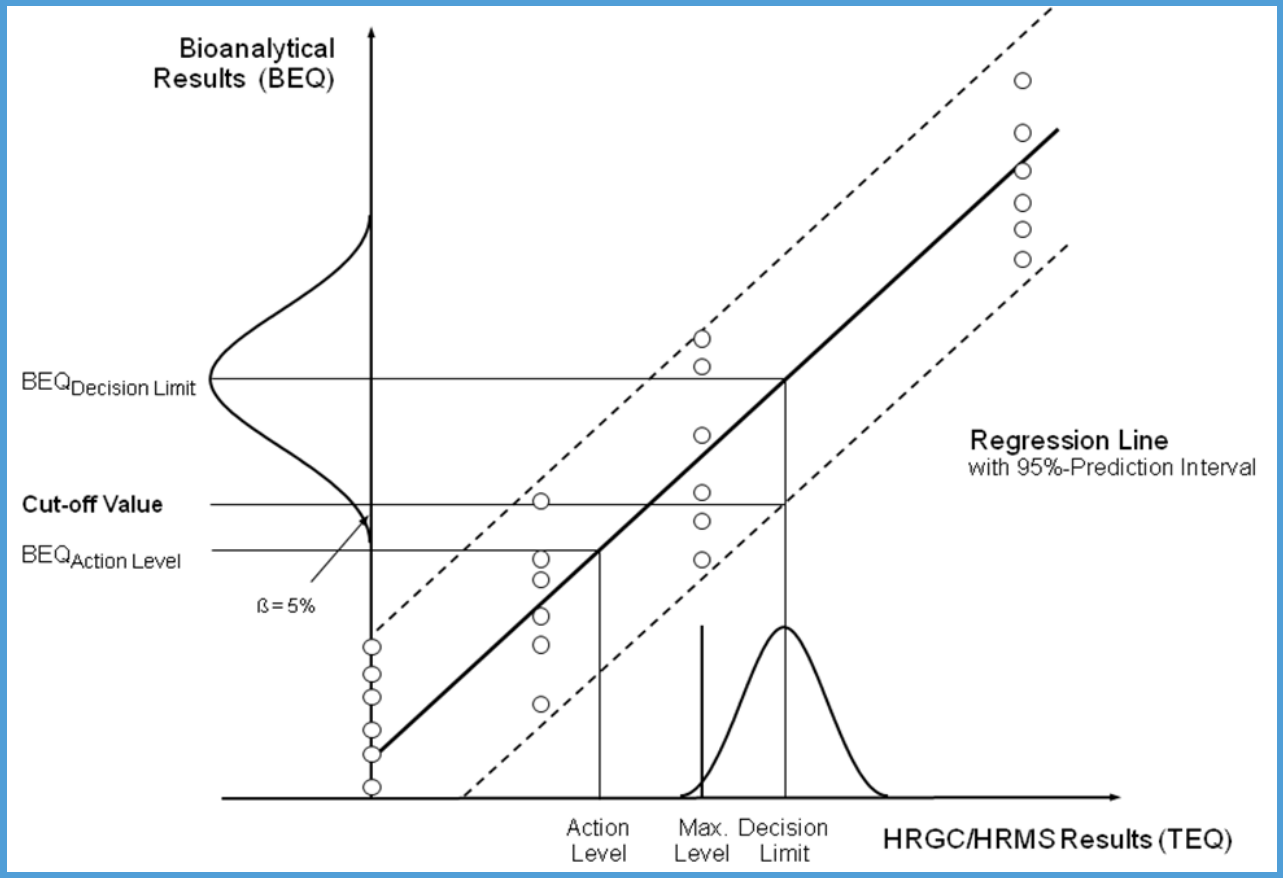
Setting of cut-off levels for screening

Analyze 4 samples
in 6-fold with CALUX
Levels around ML



Setting of cut-off levels for screening



Proposal: use 2/3 ML (≈AL) as cut-off for screening

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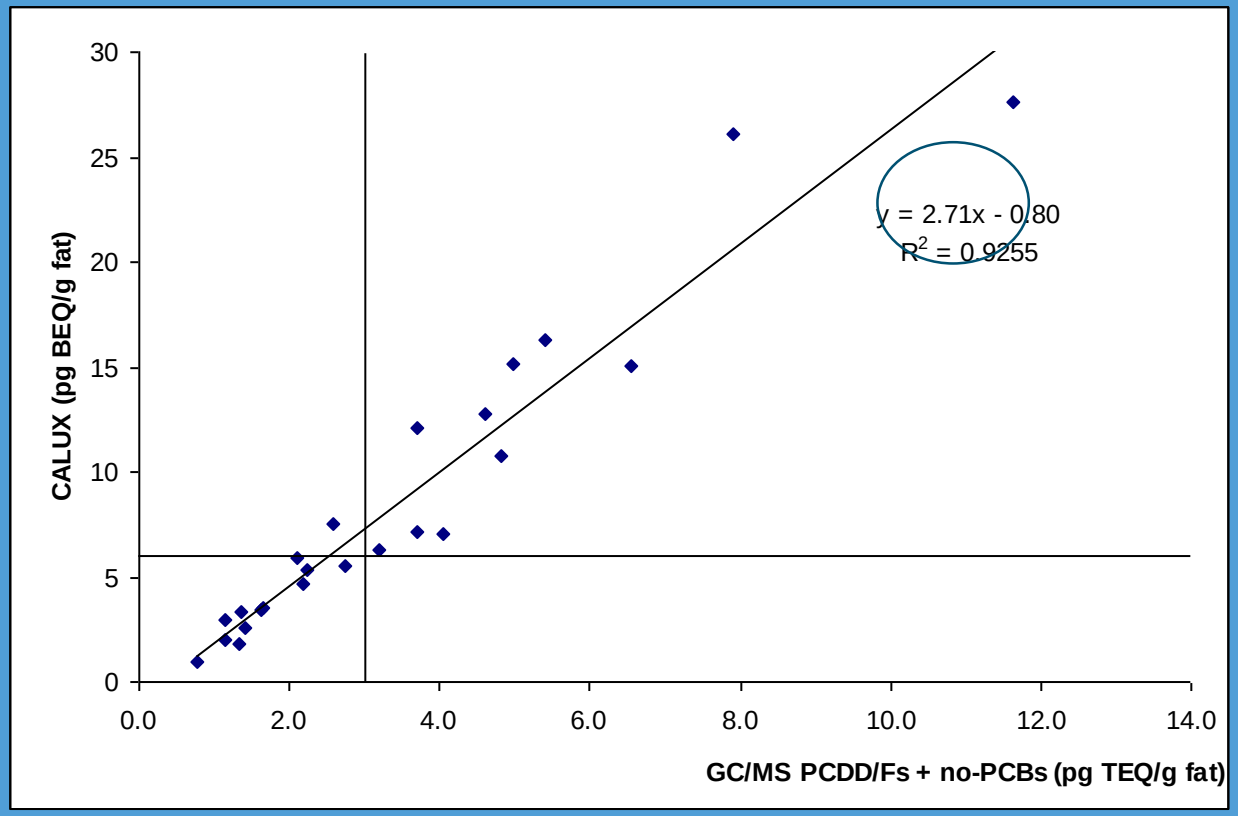
Estimation of levels

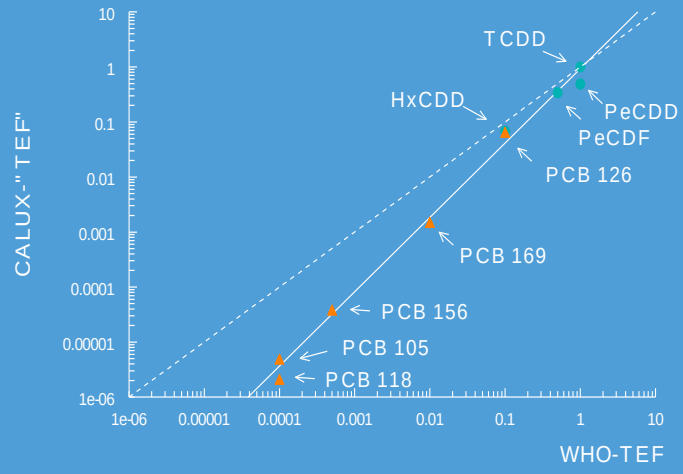
Eggs during incident with corn (2010)



Overestimation of levels in eggs, also in the corn

Apparent recovery

- Estimated by comparison of screening and confirmatory result
- When solely based on TCDD curve, includes:
  - Recovery during extraction and clean-up
  - Differences CALUX-REPs and WHO-TEFs
  - Possible other effects (e.g. DMSO)



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Effect of REPs on apparent recovery (rat cells)

	Belgium	cholin chloride	kaolinic clay	carbosan-Cu	bakery waste
2,3,7,8-TCDF	25.8		0.0	11.0	0.5
2,3,7,8-TCDD	23.0	2.2	86.0	19.0	0.4
1,2,3,7,8-PeCDF	35.6	0.1	0.0	71.4	0.4
2,3,4,7,8-PeCDF	329.4	0.2	0.0	308.3	2.2
1,2,3,7,8-PeCDD	15.9	2.4	35.1	59.1	0.4
1,2,3,4,7,8-HxCDF	31.7	0.7	0.0	196.6	0.2
1,2,3,6,7,8-HxCDF	3.5	0.2	0.0	89.6	0.1
2,3,4,6,7,8-HxCDF	8.4	0.3	0.0	123.8	0.1
1,2,3,7,8,9-HxCDF	2.0	0.2	0.0	117.0	0.1
1,2,3,4,7,8-HxCDD	3.3	2.7	6.6	50.6	0.1
1,2,3,6,7,8-HxCDD	0.0	10.9	6.5	35.8	0.1
1,2,3,7,8,9-HxCDD	0.4	4.8	16.4	28.4	0.1
1,2,3,4,6,7,8-HpCDF	2.6	4.4	0.0	59.8	0.1
1,2,3,4,7,8,9-HpCDF	0.0	1.3	0.0	31.1	0.0
1,2,3,4,6,7,8-HpCDD	0.1	111.1	24.9	86.7	0.1
OCDF	0.2	11.0	0.0	33.6	0.0
OCDD	0.0	13.4	21.8	18.4	0.1
	482.1	165.9	197.2	1340.1	4.9
	781.7	154.7	297.3	2025.6	8.1
	62	107	66	66	61

BEQs
TEQs
%

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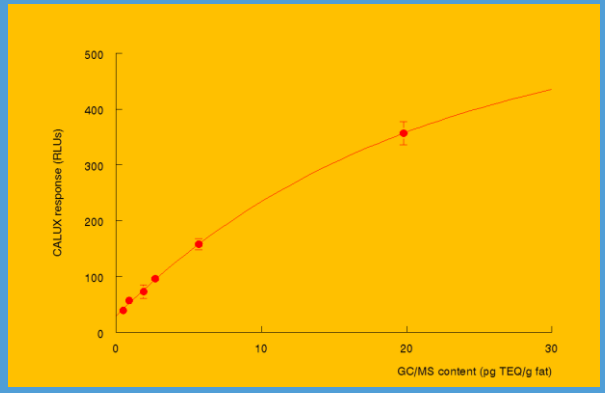
Use of reference samples

- Required to correct for background and apparent recovery
- Levels should be in the range of interest (AL/ML)
- Should not lead to underestimation of the levels
  - Would cause false-negatives
  - Mixture better than TCDD only
- References might be used to make calibration curve
  - Automatic correction for background and recovery



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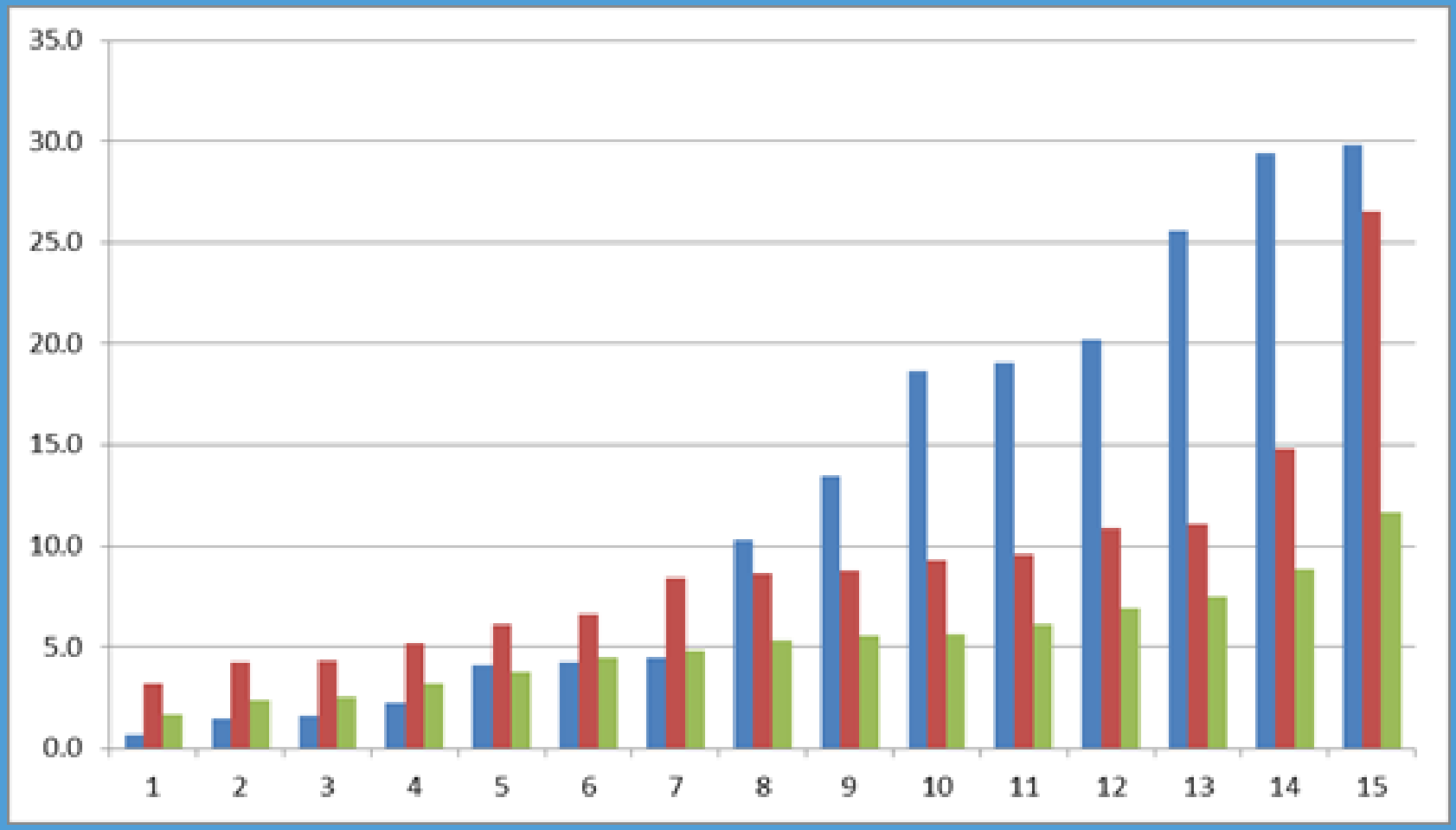
Choice for screening vs confirmation

- Both have advantages/disadvantages
- Choice depends on purpose analysis and number of samples
- Screening allows higher throughput (incidents)
    - Especially if most samples negative
    - GC/MS ub level often not be a real level
- Confirmation gives a figure
    - But often below LOQ: <LOQ or upperbound level
    - No clear advantage confirmation method

CALUX-analyses individual eggs



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Practical performance

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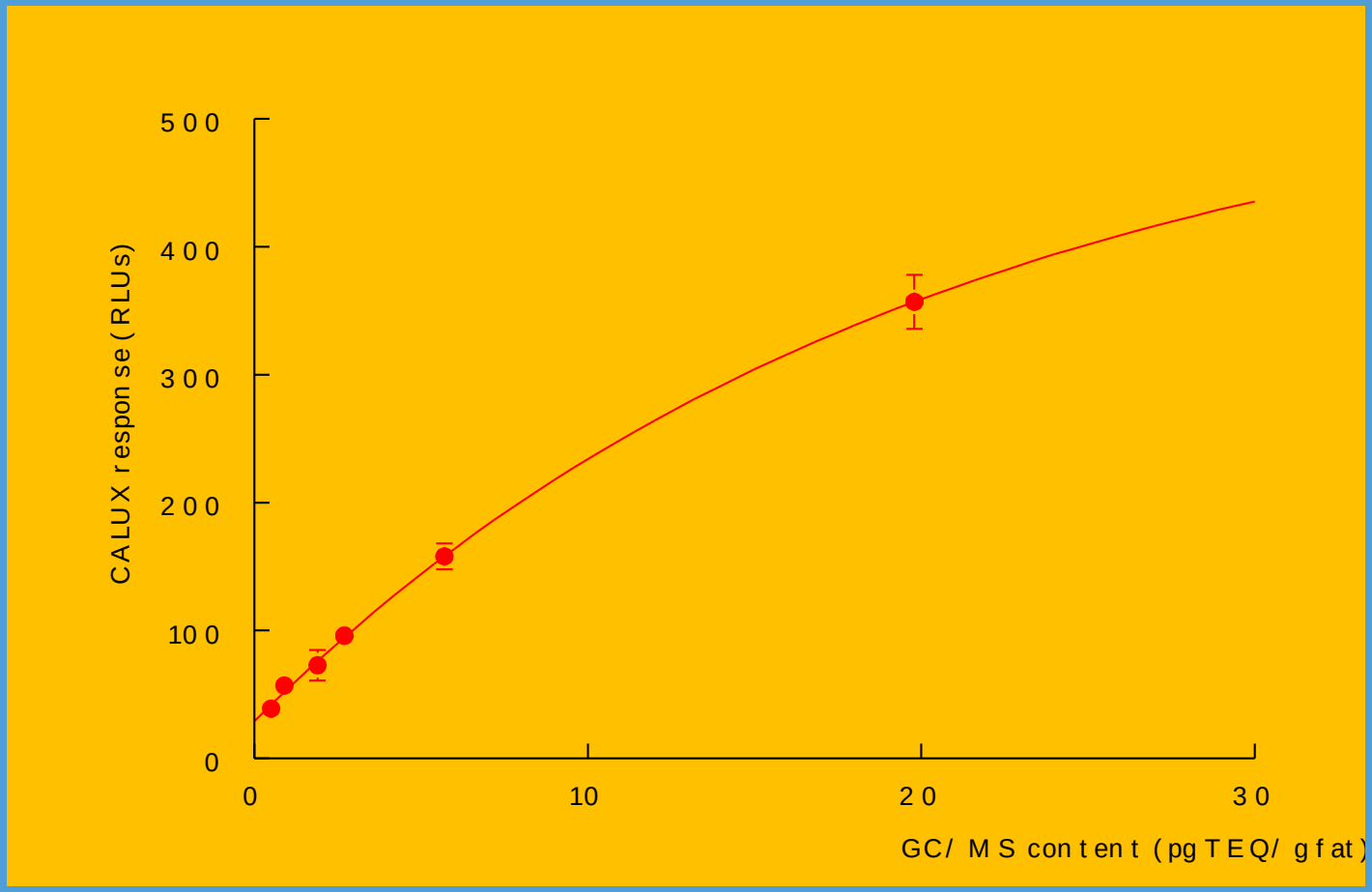
Application at RIKILT

- Certain fraction of false-positives not a big issue
  - Keep GC/HRMS running (emergency task)
- Screening approach
  - Response > reference sample: suspected
  - Based on AL dl-PCBs for most matrices
- Also possible to construct calibration curve with reference samples
  - Less dependent on variation in response reference sample
  - Cut-off closer to real AL

Calibration curve of reference samples (butter fat)



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January 2006: Dioxins in recycled fat from gelatin production

Sample number	Level pg TEQ/g	Response (RLUs) mean	SD	Decision
Blank fat		67	13	
Ref 1	0.4	75	2	
Ref 2	1.0	102	6	
Ref 3	1.5	168	13	
Ref 4	3.1	246	37	
200163193	?			

Hoogenboom et al. 2007

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Ref 1	0.4	75	2	
Ref 2	1.0	102	6	
Ref 3	1.5	168	13	
Ref 4	3.1	246	37	
200163193	?	941	77	**suspected**

Hoogenboom et al. 2007

Performance at RIKILT

	ALs[1]	REF	DR CALUX	
Matrix	pg TEQ/ g fat	pg TEQ/ g fat	tested	suspected > REF
Pork	0.6/0.5	0.5	94	0
Poultry	1.5/1.5	0.9	54	0
Bovine	1.5/1.0	0.9	87	10
Sheep	1.5/1.0	0.9	79	36
Deer[4]	1.5/1.5	0.9	6	6
Eggs	2.0/2.0	1.9	106	17
Milk	2.0/2.0	1.9	78	3
Total			504	72

1. ALs for dioxins/dl-PCBs,

Performance at RIKILT

	ALs[1]	REF	DR CALUX		HRGC/HRMS (AL/ML+mu)				
Matrix	pg TEQ/ g fat	pg TEQ/ g fat	tested	suspected > REF		Samples >AL[2,5]	Samples >ML[3,5]		
Pork	0.6/0.5	0.5	94	0		0 (0/0)			
Poultry	1.5/1.5	0.9	54	0		0 (0/0)			
Bovine	1.5/1.0	0.9	87	10		5 (0/5)			
Sheep	1.5/1.0	0.9	79	36		11 (6/10)	1 (0/1)		
Deer[4]	1.5/1.5	0.9	6	6		5 (3/5)	4 (3/4)		
Eggs	2.0/2.0	1.9	106	17		0 (0/0)			
Milk	2.0/2.0	1.9	78	3		0 (0/0)			
Total			504	72		21 (9/20)	5 (3/5)		

1. ALs for dioxins/dl-PCBs,
2. Samples exceeding one or both ALs (samples exceeding ALs for dioxins/dl-PCBs),
3. Samples exceeding one or both MLs (samples exceeding MLs for dioxins/sum),
4. No official limit for deer; for comparison the limits for game were used,
5. evaluation against AL and ML included 20% measurement uncertainty

Performance at RIKILT

	ALs[1]	REF	DR CALUX		HRGC/HRMS (AL/ML+mu)				
Matrix	pg TEQ/ g fat	pg TEQ/ g fat	tested	suspected > REF	Samples >REF	Samples >AL[2,5]	Samples >ML[3,5]		
Pork	0.6/0.5	0.5	94	0	0	0 (0/0)			
Poultry	1.5/1.5	0.9	54	0	0	0 (0/0)			
Bovine	1.5/1.0	0.9	87	10	8	5 (0/5)			
Sheep	1.5/1.0	0.9	79	36	35	11 (6/10)	1 (0/1)		
Deer[4]	1.5/1.5	0.9	6	6	6	5 (3/5)	4 (3/4)		
Eggs	2.0/2.0	1.9	106	17	4	0 (0/0)			
Milk	2.0/2.0	1.9	78	3	2	0 (0/0)			
Total			504	72	55	21 (9/20)	5 (3/5)		

1. ALs for dioxins/dl-PCBs,
2. Samples exceeding one or both ALs (samples exceeding ALs for dioxins/dl-PCBs),
3. Samples exceeding one or both MLs (samples exceeding MLs for dioxins/sum),
4. No official limit for deer; for comparison the limits for game were used,
5. evaluation against AL and ML included 20% measurement uncertainty

Performance at RIKILT

	ALs[1]	REF	DR CALUX		HRGC/HRMS (AL/ML+mu)				
Matrix	pg TEQ/ g fat	pg TEQ/ g fat	tested	suspected > REF	Samples >REF	Samples >AL[2,5]	Samples >ML[3,5]	Neg's tested	>AL
Pork	0.6/0.5	0.5	94	0	0	0 (0/0)		16	0
Poultry	1.5/1.5	0.9	54	0	0	0 (0/0)		15	0
Bovine	1.5/1.0	0.9	87	10	8	5 (0/5)		17	0
Sheep	1.5/1.0	0.9	79	36	35	11 (6/10)	1 (0/1)	7	0
Deer[4]	1.5/1.5	0.9	6	6	6	5 (3/5)	4 (3/4)	0	na
Eggs	2.0/2.0	1.9	106	17	4	0 (0/0)		22	0
Milk	2.0/2.0	1.9	78	3	2	0 (0/0)		21	0
Total			504	72	55	21 (9/20)	5 (3/5)	98	0

1. ALs for dioxins/dl-PCBs,
2. Samples exceeding one or both ALs (samples exceeding ALs for dioxins/dl-PCBs),
3. Samples exceeding one or both MLs (samples exceeding MLs for dioxins/sum),
4. No official limit for deer; for comparison the limits for game were used,
5. evaluation against AL and ML included 20% measurement uncertainty

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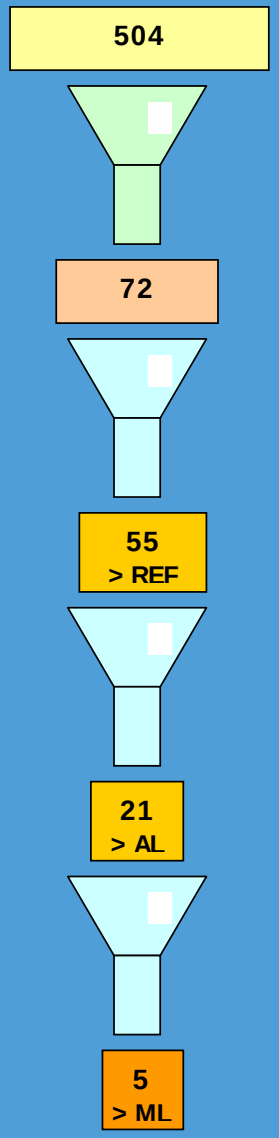
Or ....



Samples tested

Samples suspected

Level > DL (REF)

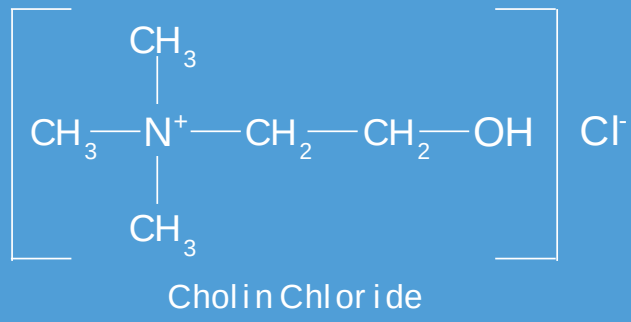
Level > AL

Level > ML

Conclusions

- CALUX assay is a valuable tool for screening, especially for routine monitoring where most samples will be negative
- Suspected samples should be analyzed by GC/HRMS
  - Confirmation of dioxins/dl-PCBs
  - Determination of TEQ-level
  - Determination of congener pattern: source identification
- Use of a bioassay in combination with a confirmation method is the best strategy for detecting novel risks

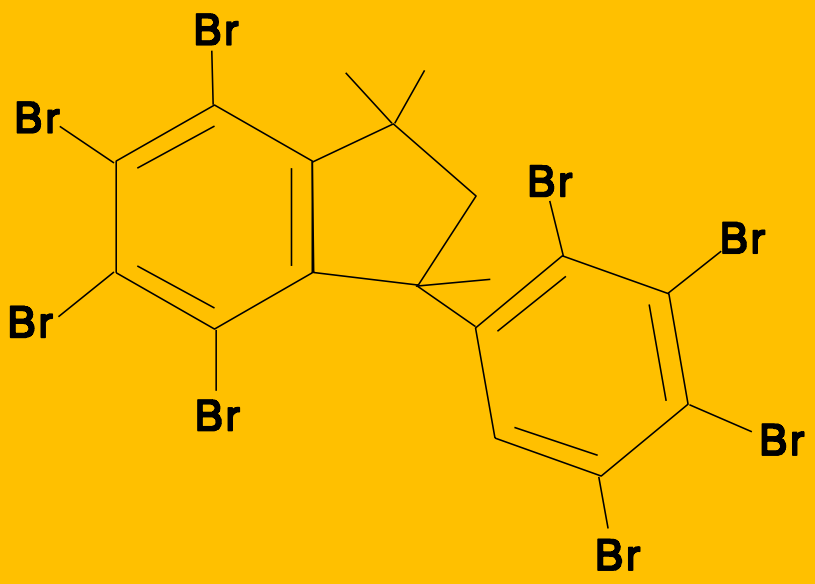
Case: cholin Chloride



Cholin Chloride

- Feed additive (up to 1 g/kg)
- Positive test response in DR CALUX (different samples)
- Indicative level around 5 ng BEQ/kg
- GC/HRMS: dioxins and dioxin-like PCBs below LOQ
- Various flame retardants present, including tribromophenols
- But also brominated dioxins, considered equally toxic as chlorinated dioxins (but no limits or TEFs (yet))

FR-1808 (OBIND): new flame retardent



octabromo-1,3,3-trimethyl-1-phenylindan
FR-1808 (Mw 867.52)

How do flame retardants get into a feed additive?

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Questions?

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