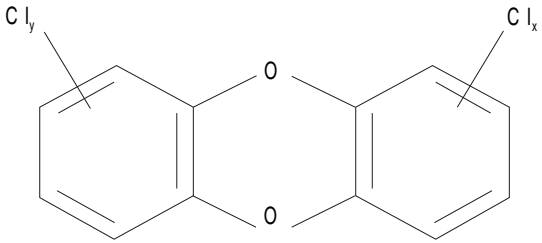
BioDetection Systems

Principles of the CALUX® method

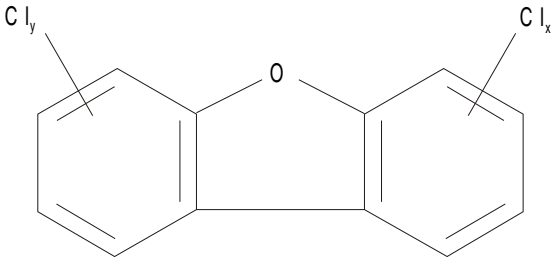
Dr. Peter A. Behnisch

Commercial Director BDS

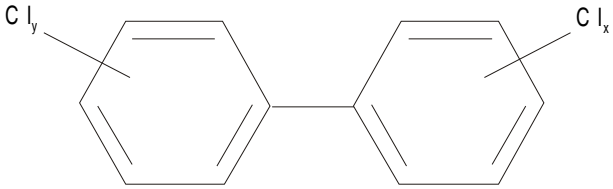
What are dioxins?



Dioxins (75)



Dibenzofurans (135)



PCBs (209)

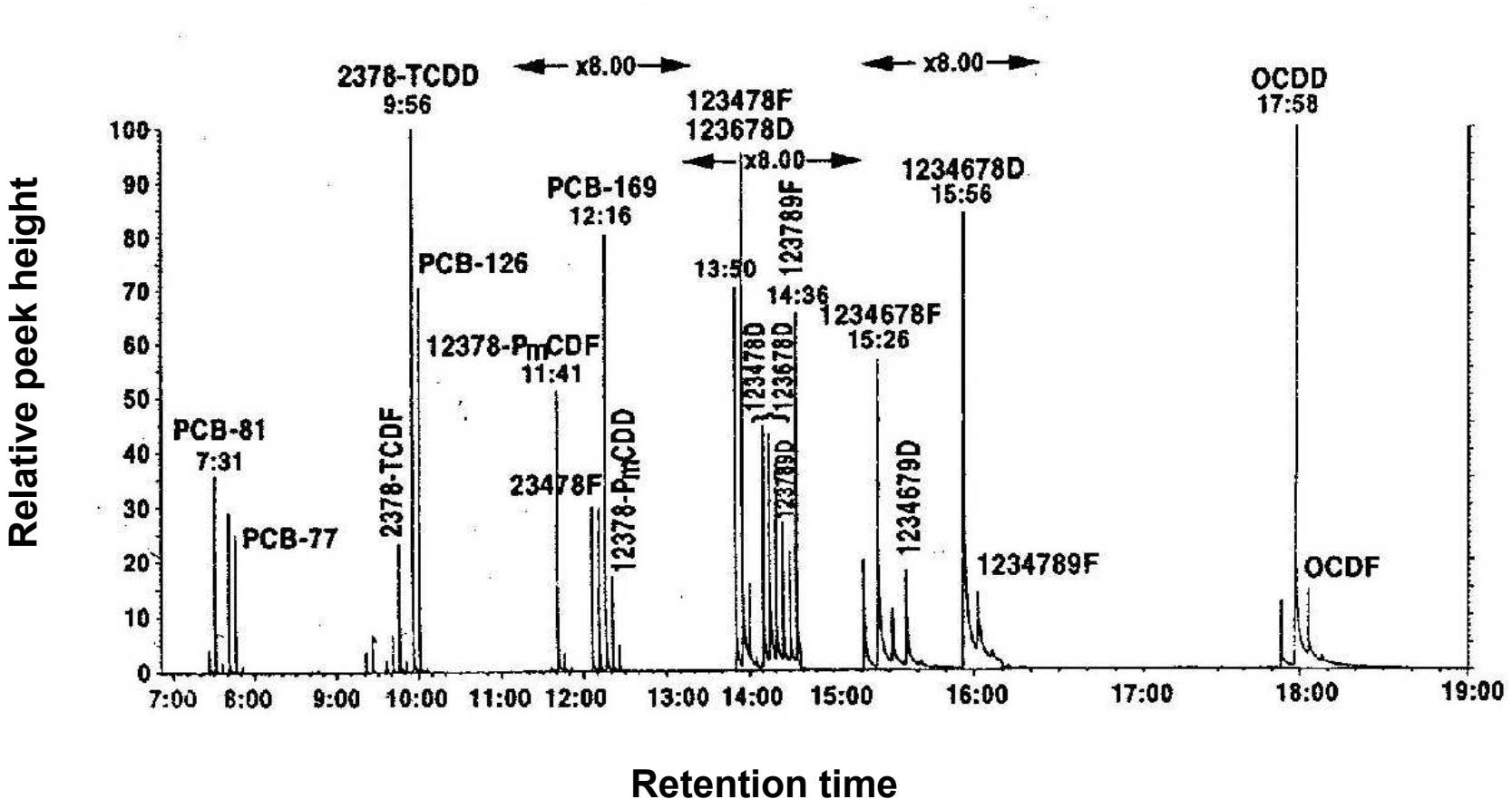
WHO-TEF values (1998 and 2006) vs CALUX-REP

PCDDs / PCDFs Structure	*WHO-TEF 1998	**WHO-TEF 2006	***CALUX REP
Dioxins			
2,3,7,8-TCDD	1	1	1
1,2,3,7,8-PeCDD	1	1	0.54
1,2,3,4,7,8-HxCDD	0.1	0.1	0.3
1,2,3,6,7,8-HxCDD	0.1	0.1	0.14
1,2,3,7,8,9-HxCDD	0.1	0.1	0.066
1,2,3,4,6,7,8-HpCDD	0.01	0.01	0.05
OCDD	0.0001	0.0003	0.0001
Furans			
2,3,7,8-TCDF	0.1	0.1	0.32
1,2,3,7,8-PeCDF	0.05	0.03	0.21
2,3,4,7,8-PeCDF	0.5	0.3	0.5
1,2,3,4,7,8-HxCDF	0.1	0.1	0.13
1,2,3,6,7,8-HxCDF	0.1	0.1	0.039
1,2,3,7,8,9-HxCDF	0.1	0.1	0.11
2,3,4,6,7,8-HxCDF	0.1	0.1	0.18
1,2,3,4,6,7,8-HpCDF	0.01	0.01	0.032
1,2,3,6,7,8,9-HpCDF	0.01	0.01	0.041
OCDF	0.0001	0.0003	0.0001

PCBs Structure		*WHO-TEF 1998	**WHO-TEF 2006	***CALUX REP
Non ortho				
3,3,4,4,-tetraCB	PCB-77	0.0001	0.0001	0.0013
3,4,4,5-tetraCB	PCB-81	0.0001	0.0003	0.0001
3,3,4,4,5-pentaCB	PCB-126	0.1	0.1	0.067
3,3,4,4,5,5-hexaCB	PCB-169	0.01	0.03	0.0034
Mono ortho				
2,3,3,4,4-pentaCB	PCB-105	0.0001	0.00003	0.000012
2,3,4,4,5-pentaCB	PCB-114	0.0005	0.00003	0.000048
2,3,4,4,5-pentaCB	PCB-118	0.0001	0.00003	0.0000073
2,3,4,4,5-pentaCB	PCB-123	0.0001	0.00003	0.000024
2,3,3,4,4,5-hexaCB	PCB-156	0.0005	0.00003	0.00021
2,3,3,4,4,5-hexaCB	PCB-157	0.0005	0.00003	0.00008
2,3,4,4,5,5-hexaCB	PCB-167	0.00001	0.00003	0.00001
2,3,3,4,4,5,5-heptaCB	PCB-189	0.0001	0.00003	0.0001

*vd Berg et al., 1998; **vd Berg et al., 2006; ***Hosoe et al., 2002

GC-HRMS analysis of dioxins



Toxic Equivalent (TEQ) calculation

GCMS method

Compound 1:	concentration 1	x TEF1 =	TEQ1
Compound 2:	concentration 2	x TEF2 =	TEQ2
Compound 3:	concentration 3	x TEF3 =	TEQ3
Compound n:	concentration n	x TEFn =	TEQn +
Total dioxin toxicity of mixture:			SumTEQ

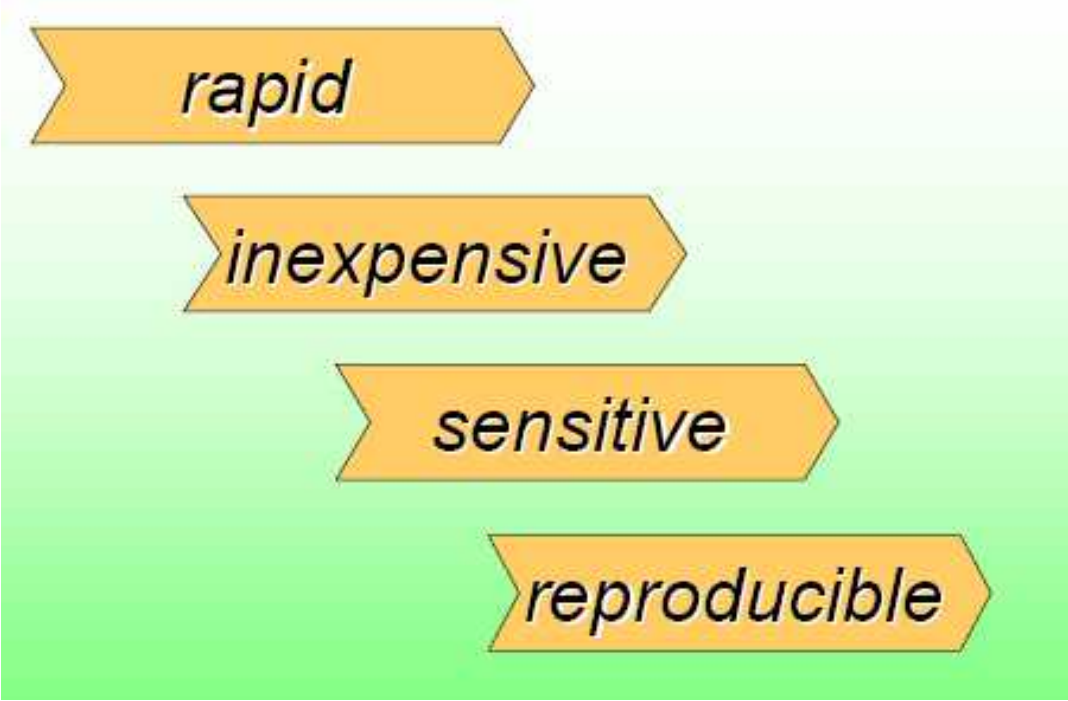
CALUX® method

Direct measurement of TEQ value of sample ≈ SumTEQ

Fair Cost Calculation for DR CALUX and HRGC/HRMS for Total-TEQ

	DR CALUX	HRMS+FMS+ASE	DR CALUX	HRMS+FMS+ASE
Costs in Euro/sample	500/yr	500/yr	First 500	First 500
1) Equipment	45K/3.5K= 13	450K/3.5K=130	45K/500= 90	450K/500=900
2) Material Costs	15	20/80/30=130	15	20/80/30/10=140
3) Training	2	7	7k/500=14	25k/500=50
4) Working hours	CT: 15	CT/Eng: 30	15	30
5) License	20	0	20	0
6) Per sample Fee	25	0	25	0
7) 13C12 Stds	0	3	0	10
8) ISO 17025	20K/4yr=5	40k/4yrs=12	20	40
9) ca 5% 2nd analysis	0	25 x 200 = 10	0	10
10) Repair Machine	0	10	0	0
11) Running Cost Analyser	0	10	0	10
12) Cell Culture: incl in 2)	1	0	1	0
Costs/Sample	96	342	200	1190
Final Result: Report	PCDD/F/PCB-TEQ	PCDD/F/PCB-TEQ	PCDD/F/PCB-TEQ	PCDD/F/PCB-TEQ
Approved by	EC/1883/2006	EC/1883/2006	EC/1883/2006	EC/1883/2006

Advantages DR CALUX



EU- Strategy Dioxins and dl-PCBs in Feed and Food

6.8.2002 EN Official Journal of the European Communities L 209/5

COMMISSION DIRECTIVE 2002/69/EC

of 26 July 2002

laying down the sampling methods and the methods of analysis for the official control of dioxins and the determination of dioxin-like PCBs in foodstuffs

(Text with EEA relevance)

(7) A screening method of analysis with proven, widely acceptable validation and high throughput could be used to select the samples with significant levels of dioxins. The levels of dioxins in these samples need to be determined by a confirmatory method of analysis. It is therefore appropriate to establish strict requirements for the confirmatory methods of analysis and minimum requirements for the screening method.

EC Project DIFFERENCE

- **Food/Feed dioxin/PCB Testing according to EC/1883/2006 guideline**
- **9 EU partners from 5 European Countries**
- **2002 to 2005 with a budget of ca. 500.000 Euro**
- **Rapid (min. 24 hrs) and cost efficient (60% reduction) dioxin/dl-PCB analysis by DR CALUX**
- **See at www.dioxins.nl**

Conclusion:

International intercalibration test shows benefits (speed, cost, reliable, easy to learn) of using CALUX screening technology for PCDD/PCDF/co-PCBs analysis of a wide range of food/feed

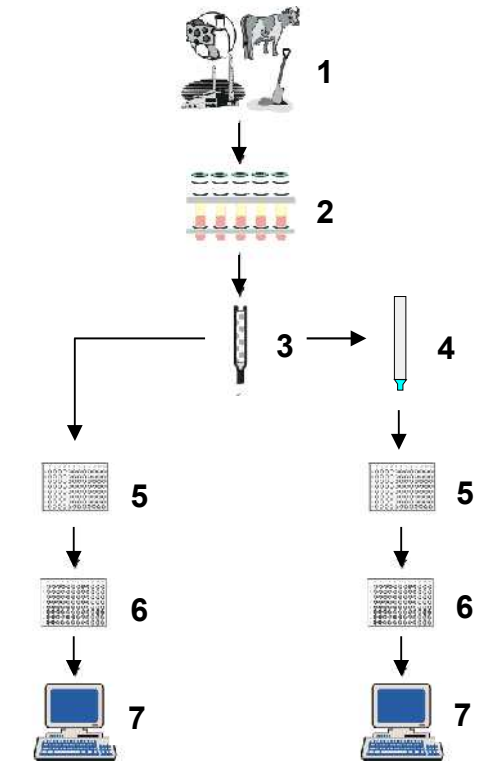
EU- Strategy Dioxins and dl-PCBs in Feed and Food

(5) A screening method of analysis with proven, widely acceptable validation and high throughput should be used to select the samples with significant levels of dioxins and dioxin-like PCBs. The levels of dioxins and dioxin-like PCBs in these samples need to be determined by a confirmatory method of analysis. It is therefore appropriate to establish strict requirements for the confirmatory methods of analysis and minimum requirements for the screening method.

Monitoring for the presence of dioxins in foodstuffs may be performed by a strategy involving a screening method in order to select those samples with levels of dioxins and dioxin-like PCBs that are less than 25 % below or exceed the maximum level. The concentration of dioxins and sum of dioxins and dioxin-like PCBs in those samples with significant levels needs to be determined/confirmed by a confirmatory method.

DR CALUX® bioassay: analysis scheme

Methodology



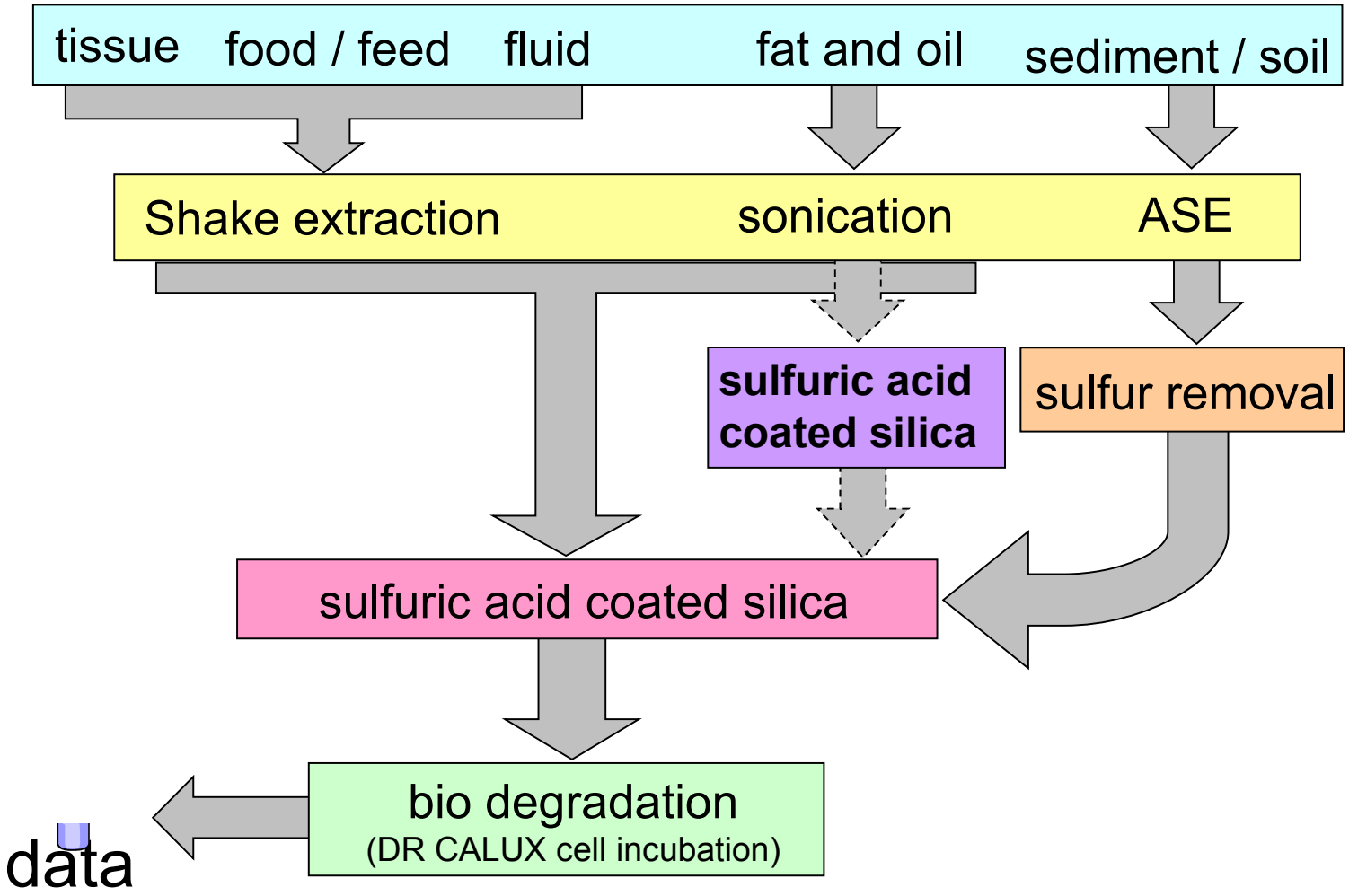
1. Sampling
2. Standard fat extraction
3. Acid silica clean-up
4. Separation of dioxins and PCBs on carbon (75/25 %hexane/toluene)
5. Exposure in 96-well plates
6. Quantification light emission
7. Data handling

Dioxin/furan TEQs = Total TEQs – "PCB" TEQs

Extraction equipment

CALUX Exchange 2006

Extraction and clean-up Overview



Extraction methods for Food and Feed

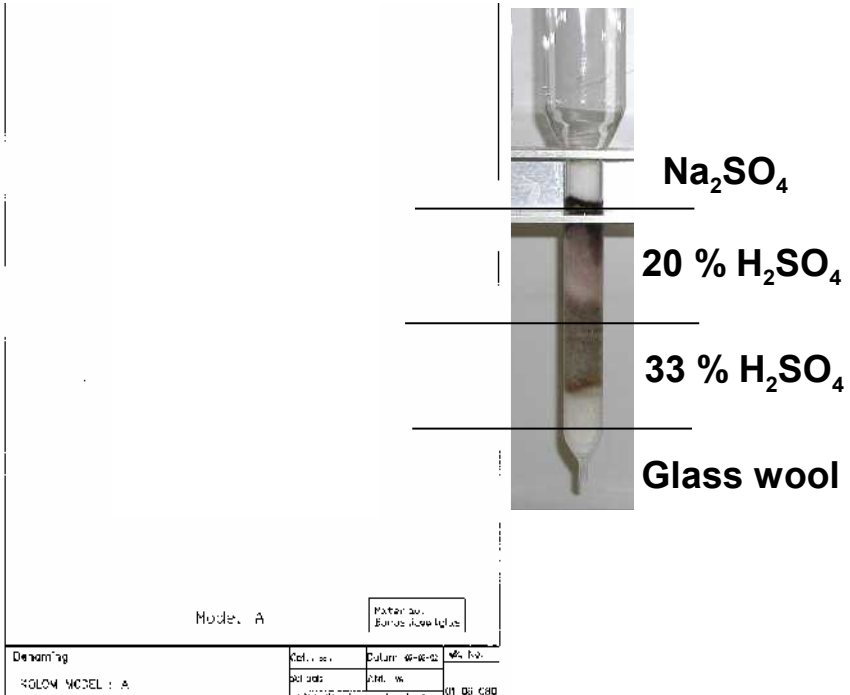
Matrix	ASE	Soxhlet	Shake extraction with HCl	Shake extraction without HCL	sonification
Palm oil fatty acids				X	X
Soya fatty acids				X	X
Palm oil				X	X
Sunflower oil				X	X
Technical fat (animal)				X	X
Poultry fat				X	X
Pig fat				X	X
Citruspulp				X	
Maize gluten feed				X	
Soya shred				X	
Cocos shred			X		
Koekjesmeel			X		
Pig feed			X		
Poultry feed			X		
Palm kernel cake		X			
Sunflower shred		X			
Milk				X	
Butter				X	
Egg				X	
Fish feed				X	
Fish				X	
Fish meal				X	
Fish oil				X	X
Meat				X	
Meat products				X	

Clean-up techniques – acid silica column (1)

Oxidation/breakdown of fat by sulfuric acid and chromatographic fractionation

- rapid (15 samples per hour)
- low solvent volume (70 – 130 ml)
- high recovery (multi layer)

0.5 g fat capacity



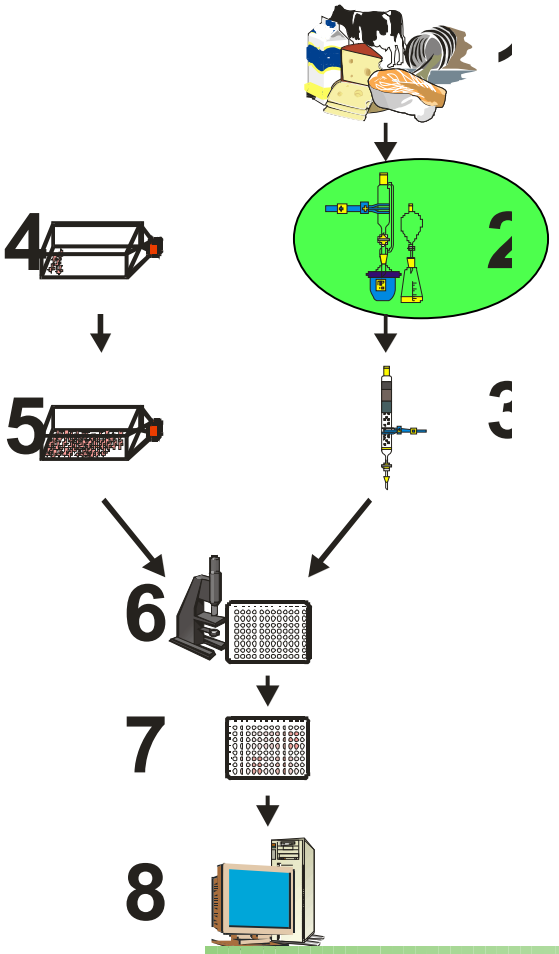
Clean-up techniques – carbon column (2)
Separation coplanar PCBs dioxins/furans

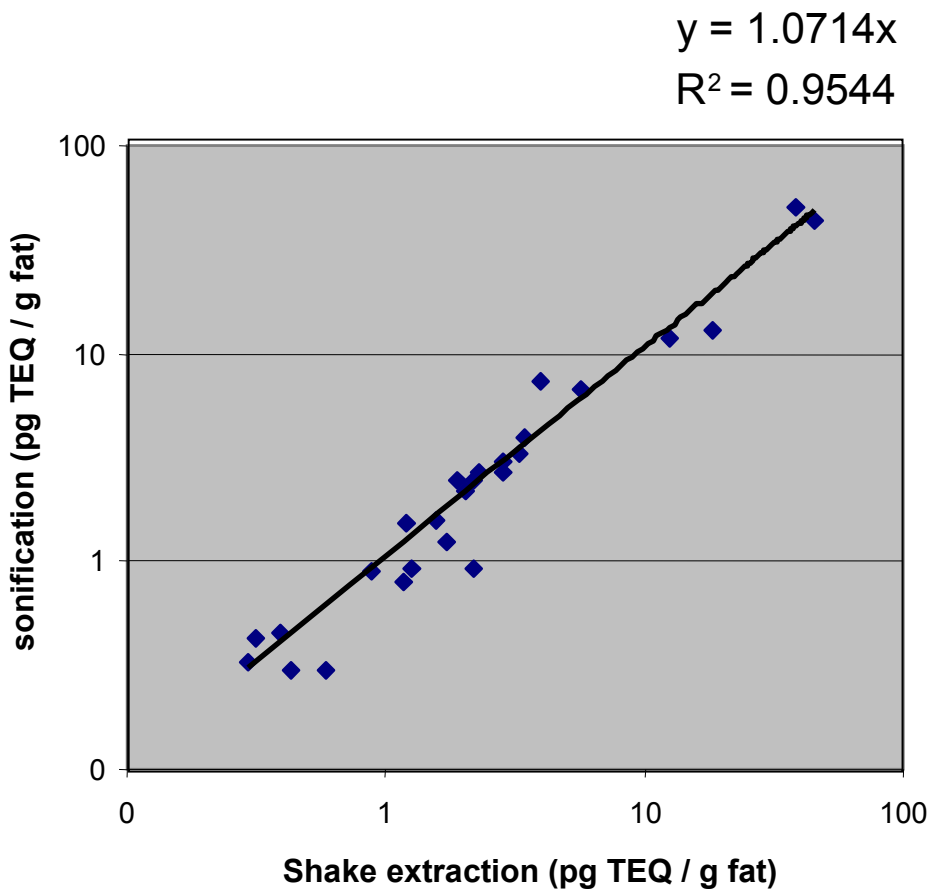
Protocol for the Envi-Carb column from Supelco see at:

Concejero et al. J. of Chromatography A, 917 (2001), 227

Validation of extraction/clean-up and DR CALUX®

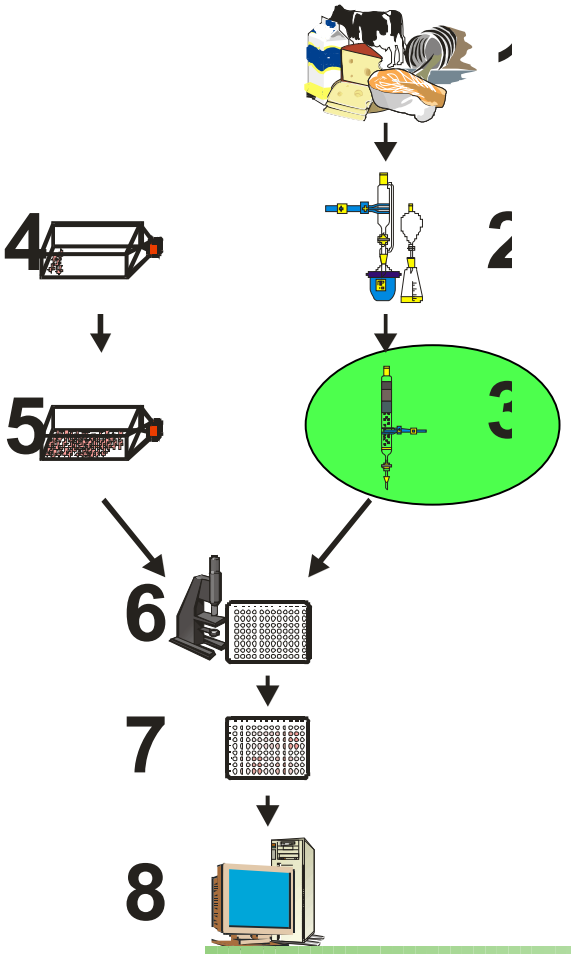
Robustness - sonification vs shake extraction

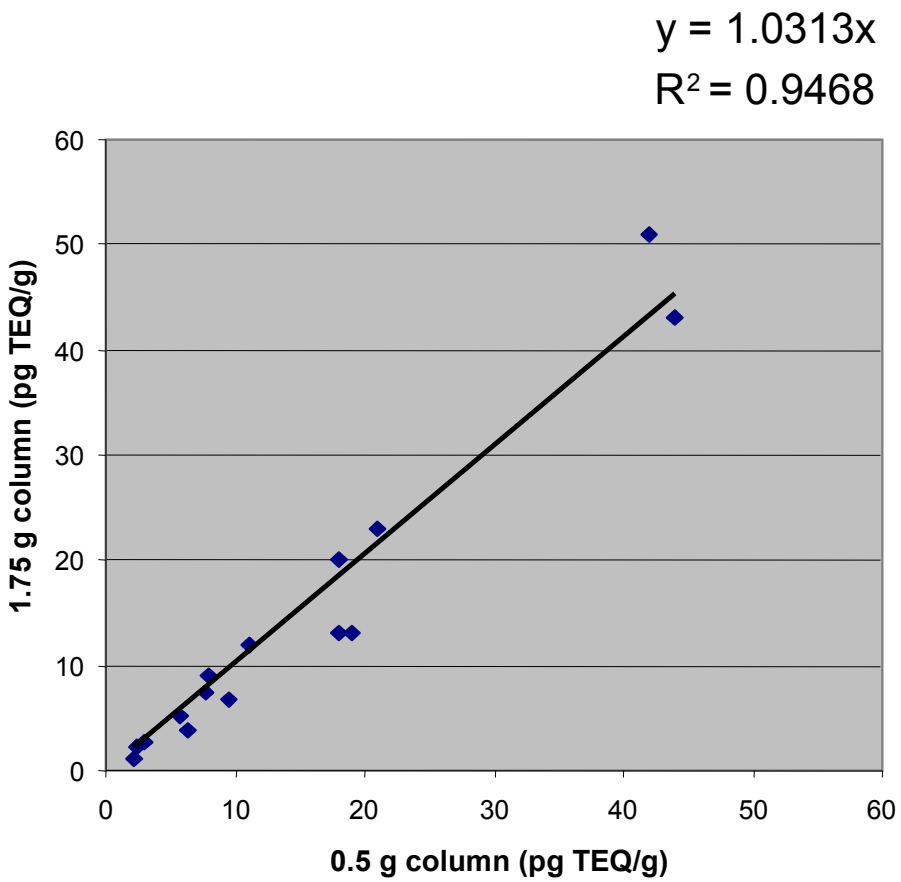




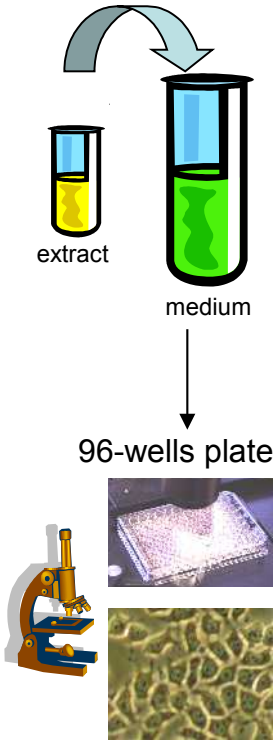
Validation of extraction/clean-up and DR CALUX®

Robustness – small vs large column clean-up

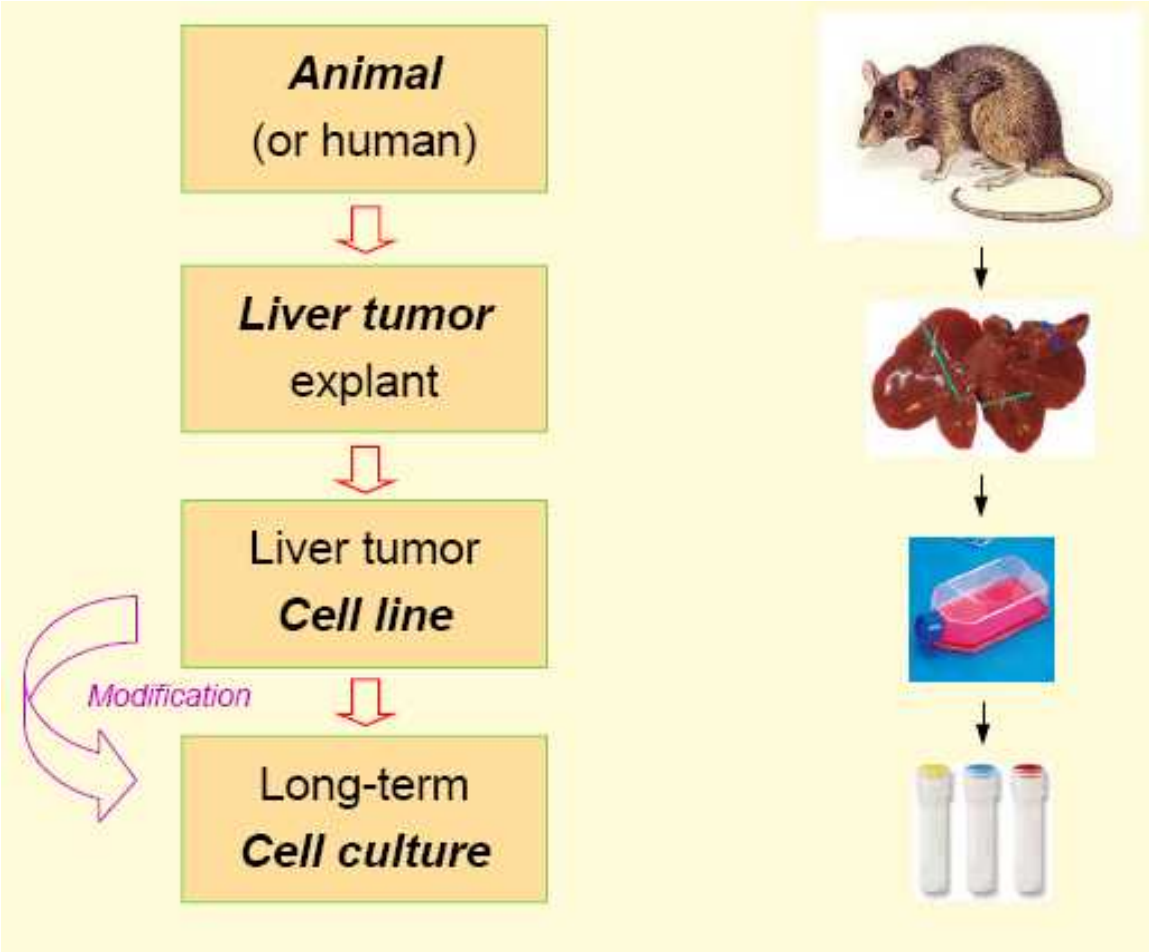




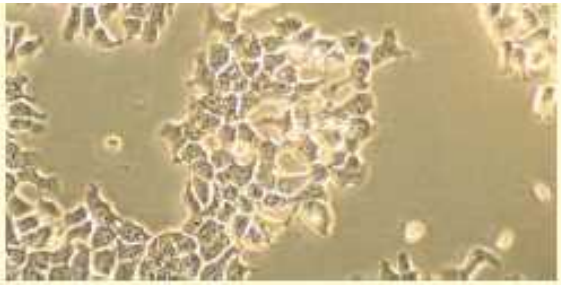
Cell culture (1)



Rat H4IIE Cell line



DR CALUX: Monolayer Cell Lines



- Hepatocytes: High detoxication capacity
- 4000 – 5000 Ah-Receptors per cell (R.Pollenz)
- Expression of the respective enzymes:
  Cytochrome P450 (1A1, 1A2, 1B1 ...) …
- Hepatocytes grow fast and in monolayers on the specially modified surface of sterile plastic cell culture flasks

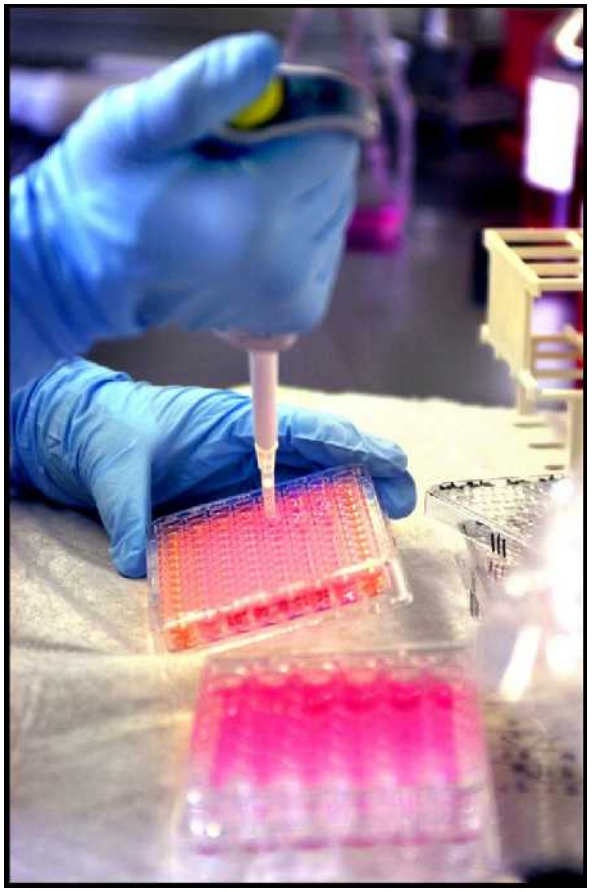
Main steps in Cell Culturing

1. *Thawing* of frozen cells (-196 °C)
2. *Growing* the cells in sterile culture flasks
3. *Sub-culturing* the cells into new sterile culture flasks
4. Checking cells for *contamination*
5. *Seeding* the cells in microtiter plates
6. *Exposing* the cells with a sample extract
7. Checking cells for *cytotoxicity*, *Lysing the cells*
8. *Measuring* gene expression (luciferase / light)

Culture vessels with medium for animal cell culture

Typical Cell Culture Room

Cell culture (3)



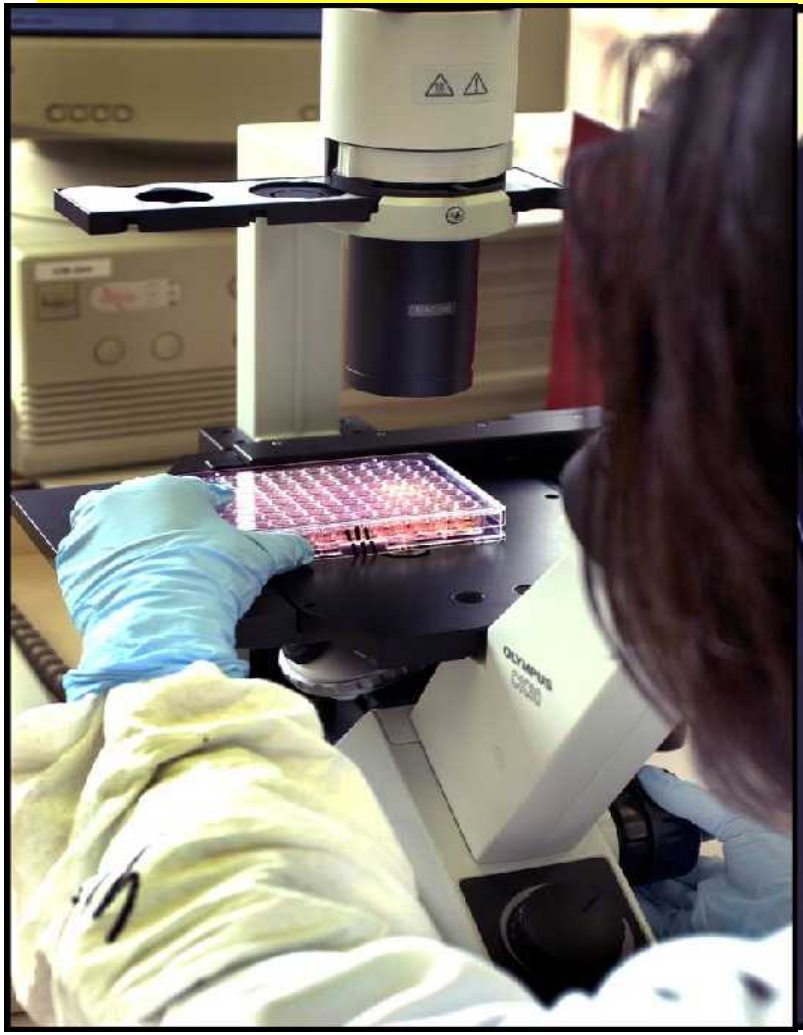
Preparing Microtiter Plates

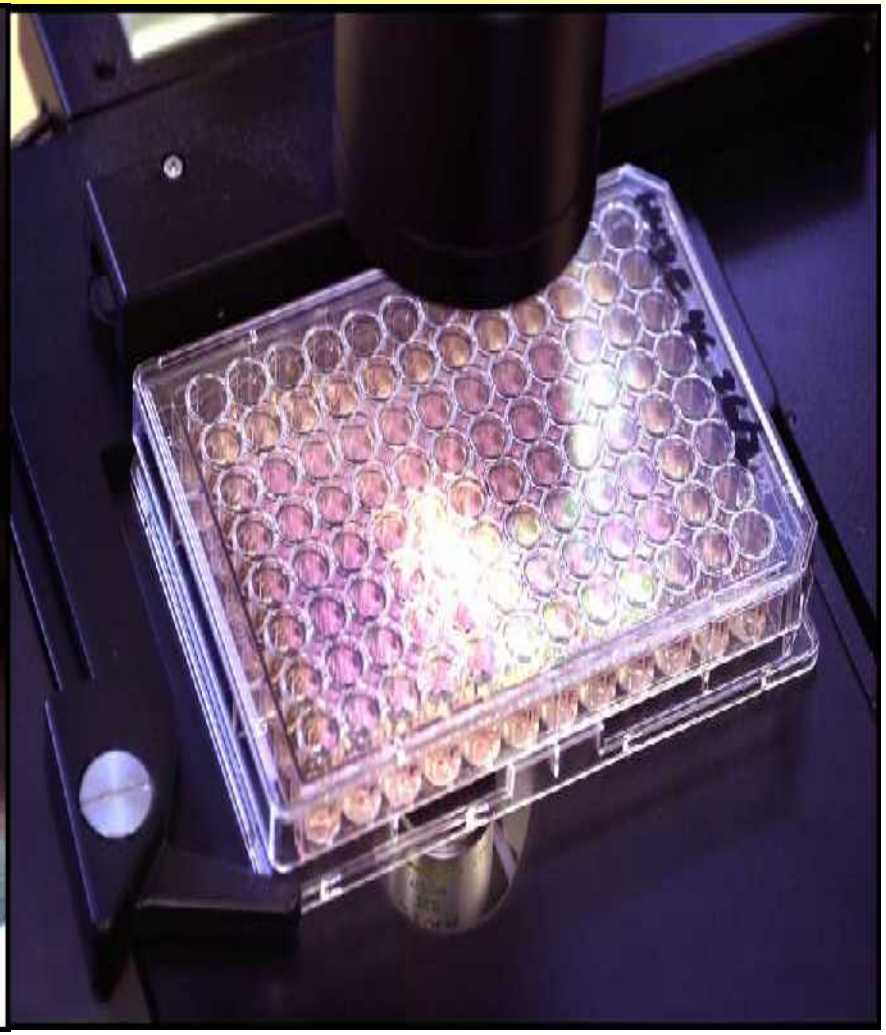
1. Seeding cells
2. Incubation 24 h

3. Preparing Premix for standards and sample extracts

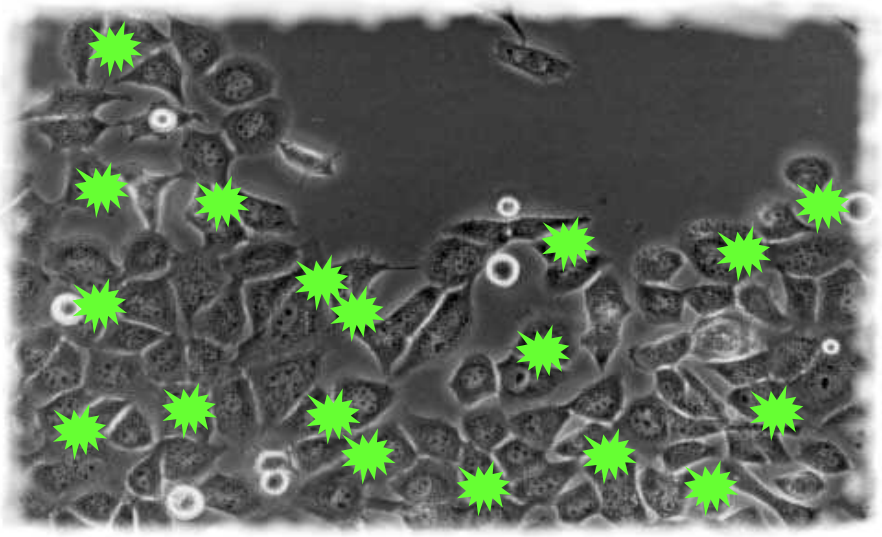
4. Add sample and standard premix to seeded cells
5. Incubation 24 h

Microscope





Easy detection of light emission (6)

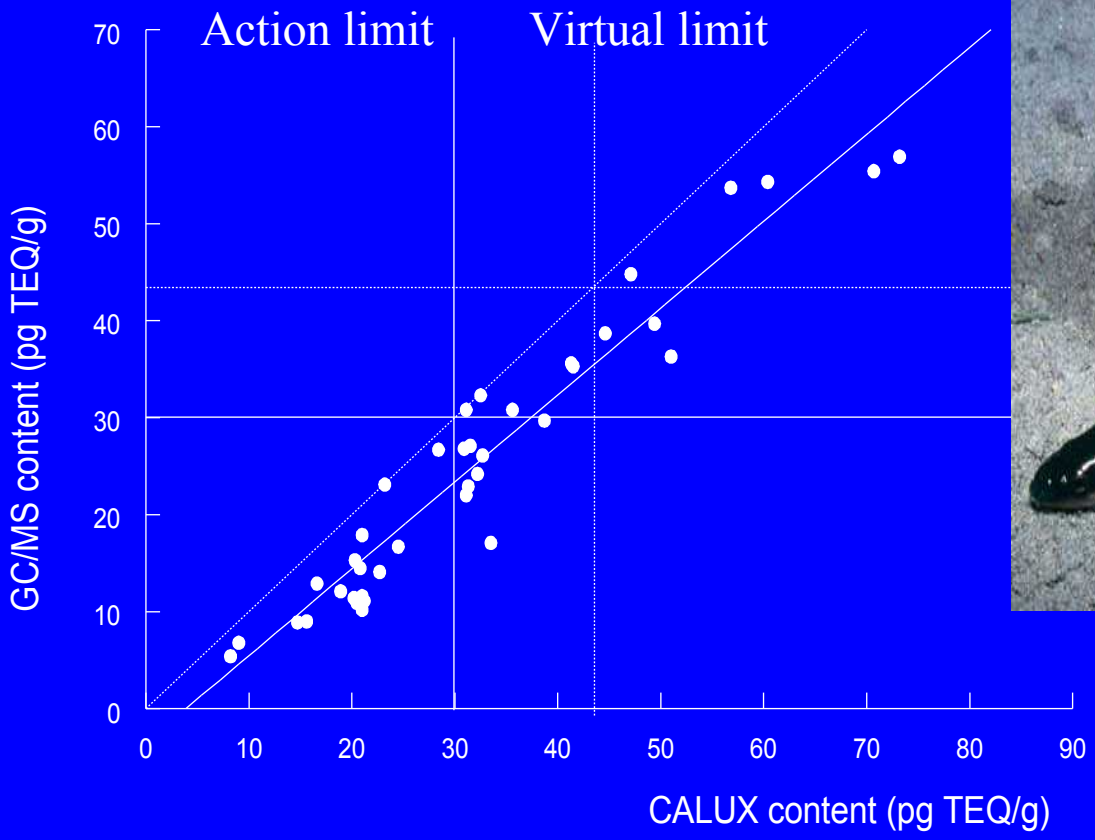


DR CALUX® cells emit light whenever dioxin-like substances are present

Luminometer Centro XS3

Berthold Detection Systems

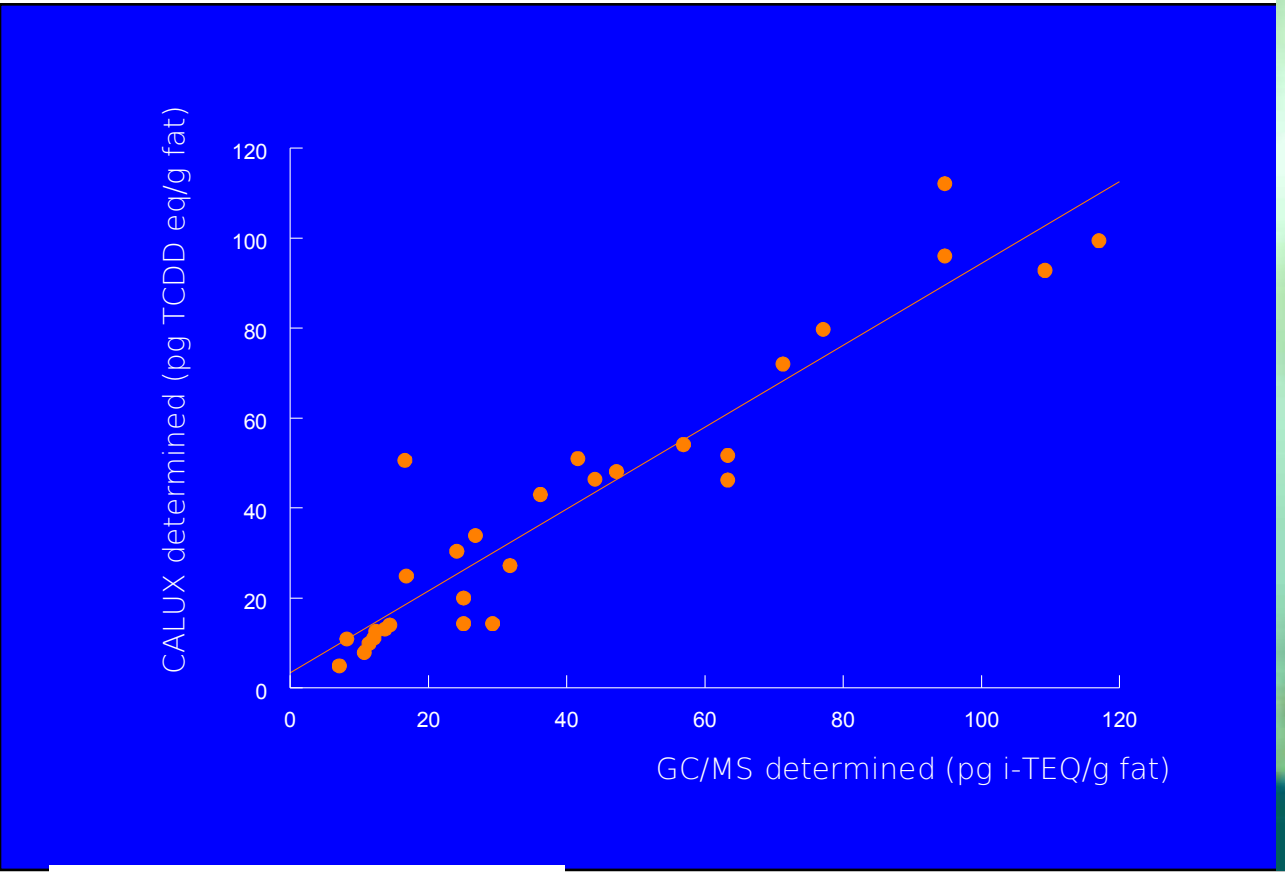
Comparison GC/MS vs CALUX of individual eel samples



Good relation total TEQ and CALUX-response

RIKILT INSTITUTE OF FOOD SAFETY WAGENINGEN UR

GC/MS vs DR CALUX®: fish oil



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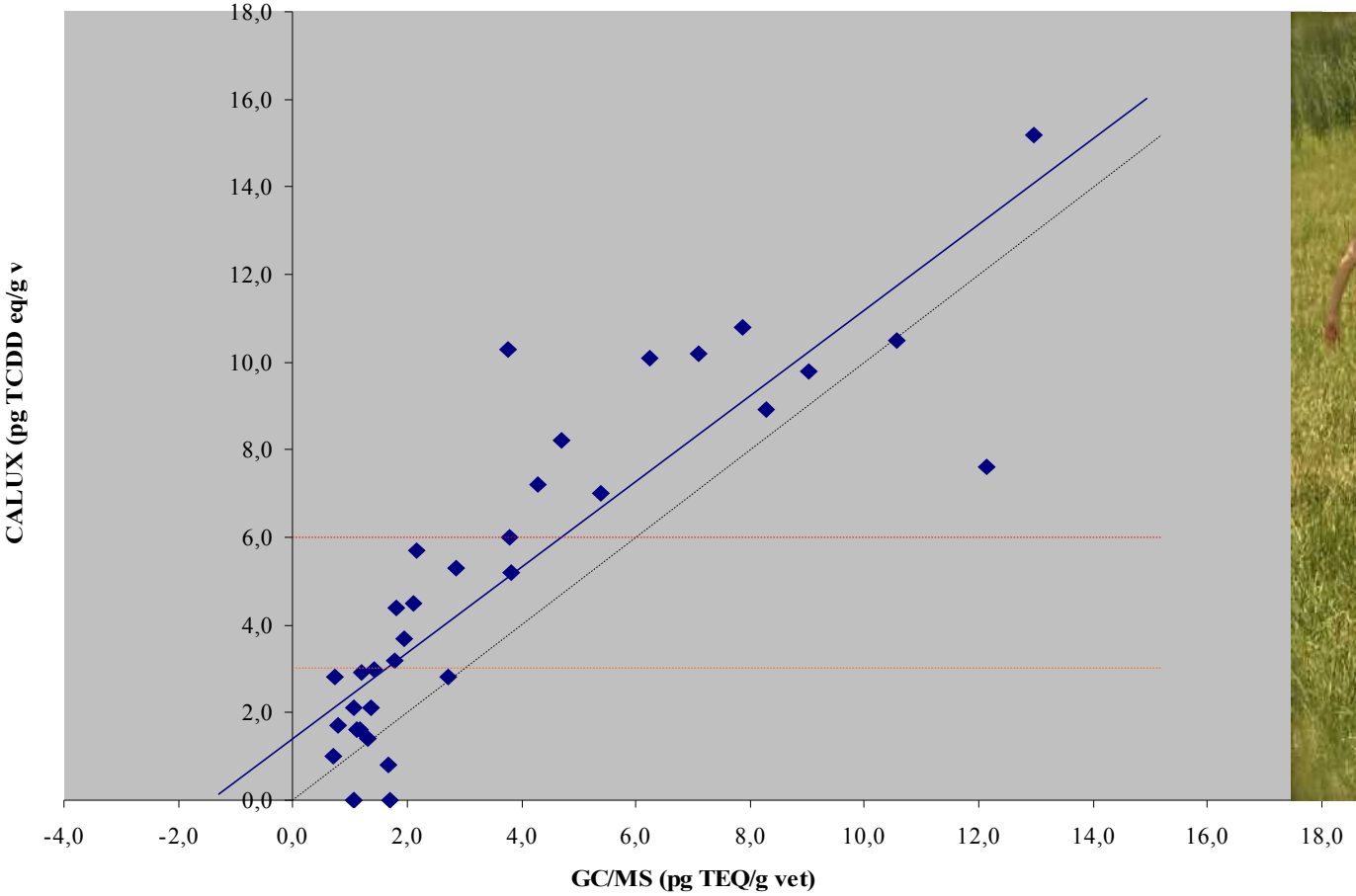
WAGENINGEN UR

Dioxins and dl-PCBs in free-range eggs (2005)



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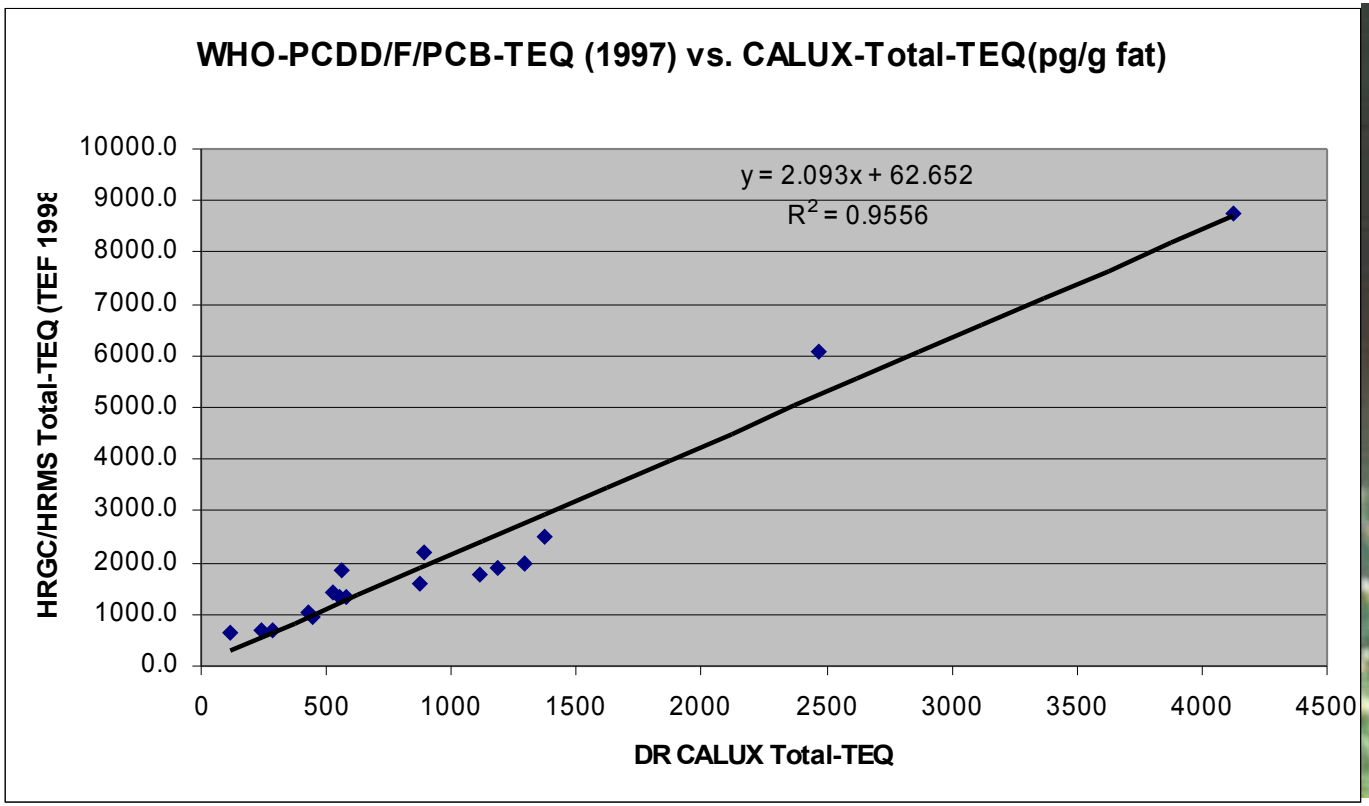
Endangered animal species – Peregrine falcon eggs from Germany



Good Correlation R^2 between DR CALUX Total-TEQ and chemical WHO-TEQ : 0.96

Training in the Sciencepark at BDS

Training at BDS

- Located in *Science Park, Amsterdam* (NL)
- Training of CRL Bioassay-staff (2 weeks)
- BDS staff competent, attentive and friendly
- Very detailed dokumentation available
- Focus on:
  Cell culturing, Extraction and Clean-up procedures, Measurement
- Workspace during training a bit scarce
- Trainees should definitely have experience in cell culturing

Training program

Presentations

- Introduction training
- BDS safety-rules
- Extraction and clean up
- TEF/REP/TEQ concept
- Analysis strategy and EU-directives
- QA/QC and troubleshooting
- Validation

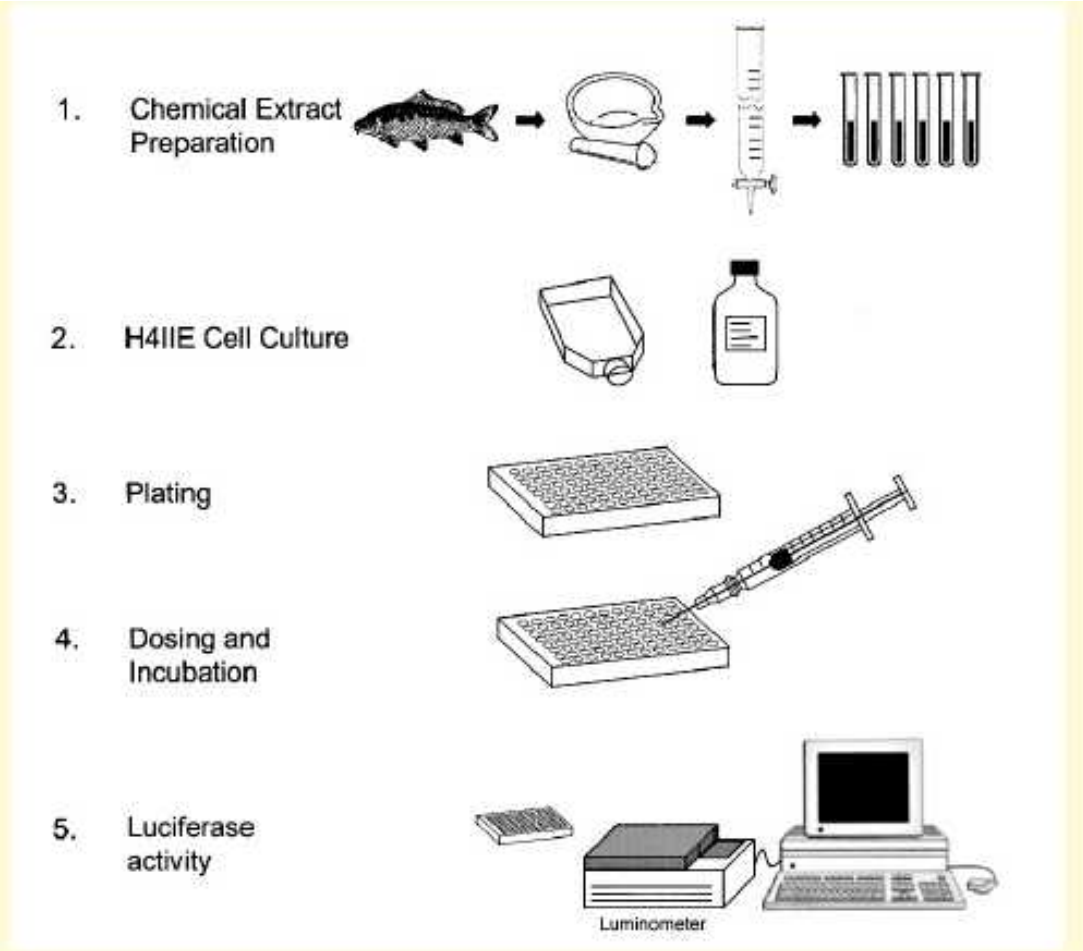
Desk-work

- DR CALUX® analysis plan
- Workplan
- Calculations DR CALUX® results

Lab-work

- Extraction
- Clean up
- Cell maintenance /cultivation
- Exposure of cells
- Detection of luminescence

Training Overview



Lab equipment

Cross-Validation

May – June 2007

- Phase 1

  Evaluation of Raw Data, measuring Standard Curves, Linearity Test of Luminometer Response

- Phase 2

  Analysis of DMSO- and sample Extracts

- Phase 3

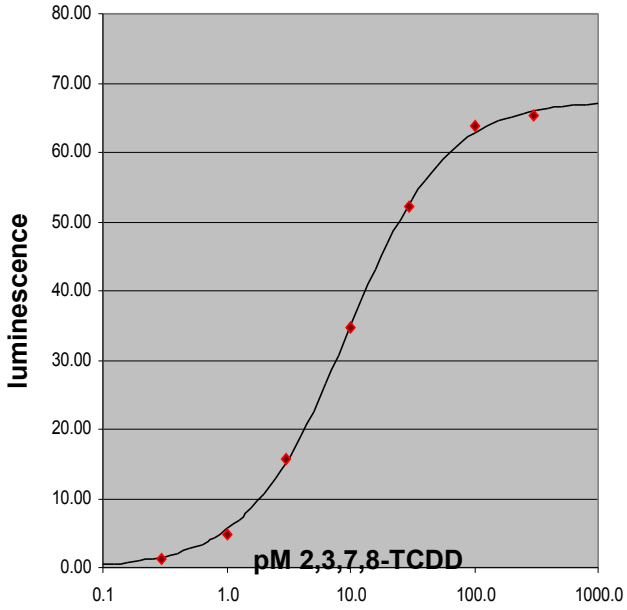
  Analysis of Samples (Milk, Sediment, Fish, Water)

CRL Freiburg now licenced by BDS

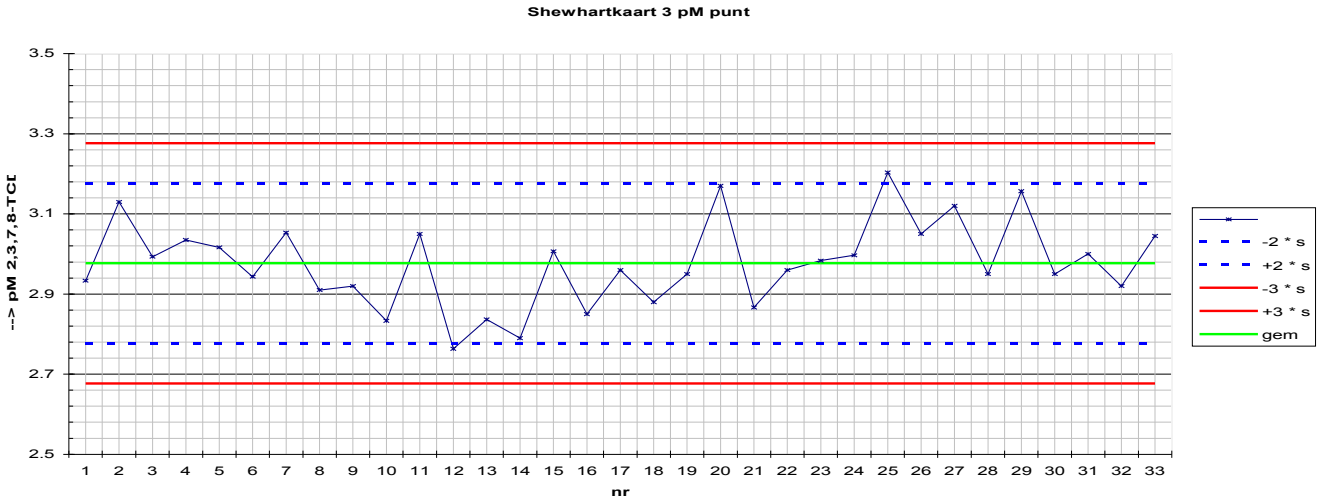
- **When performing a bioassay, every test run requires a series of reference concentrations of TCDD or a dioxin/furan/dl-PCB mixture (full dose-response curve with a $R^2 > 0.95$). However for screening purposes an expanded low level curve for analysing low level samples may be used**

TCDD-0	TCDD-0.3	TCDD-1.0	TCDD-3.0	TCDD-10	TCDD-30	TCDD-100	TCDD-300	DMSO	5157
TCDD-0	TCDD-0.3	TCDD-1.0	TCDD-3.0	TCDD-10	TCDD-30	TCDD-100	TCDD-300	DMSO	5157
TCDD-0	TCDD-0.3	TCDD-1.0	TCDD-3.0	TCDD-10	TCDD-30	TCDD-100	TCDD-300	DMSO	5157
100xx	100xx	100xx	100xy	100xy	100xy	BW1	BW2	IRM1	IRM2
100xx	100xx	100xx	100xy	100xy	100xy	BW1	BW2	IRM1	IRM2
100xx	100xx	100xx	100xy	100xy	100xy	BW1	BW2	IRM1	IRM2

5.123	5.994	9.908	20.613	39.727	57.749	67.479	69.566	4.603	9.216
4.668	6.25	9.467	21.192	40.627	55.697	68.427	70.366	4.737	8.985
5.568	6.824	10.229	20.922	39.386	57.989	70.621	71.425	5.008	9.68
57.662	25.233	13.727	39.678	10.102	7.164	6.325	5.685	14.41	13.912
55.122	25.129	13.698	39.721	10.095	7.231	6.185	5.458	14.356	13.9225
56.335	24.956	13.721	39.796	10.147	7.211	6.421	5.777	14.421	13.856



- **A TCDD reference concentration (about 3 times the limit of quantification) on a quality control sheet shall be used for the outcome of the bioassay over a constant time period. An alternative may be the relative response of a reference sample in comparison to the TCDD calibration line since the response of the cells may depend on many factors**
- **A quality control (QC) chart for each type of reference material shall be recorded and checked to make sure the outcome is in accordance with the stated guidelines**



Food/feed analysis by DR CALUX®

DR CALUX® recovery

matrix	final TEQ	avg TEQ	% std	% recovery
animal fat	2.0	2.5	9.2	124
egg	5.0	3.6	3.5	72
milk products	3.0	3.0	8.1	98
vegetable fat	1.5	1.1	7.6	73
feed components	1.5	1.4	12.6	89
feed	3.8	3.0	21.9	79

Overview of DR CALUX reference materials

BDDs reference materials fish oil and feed material:

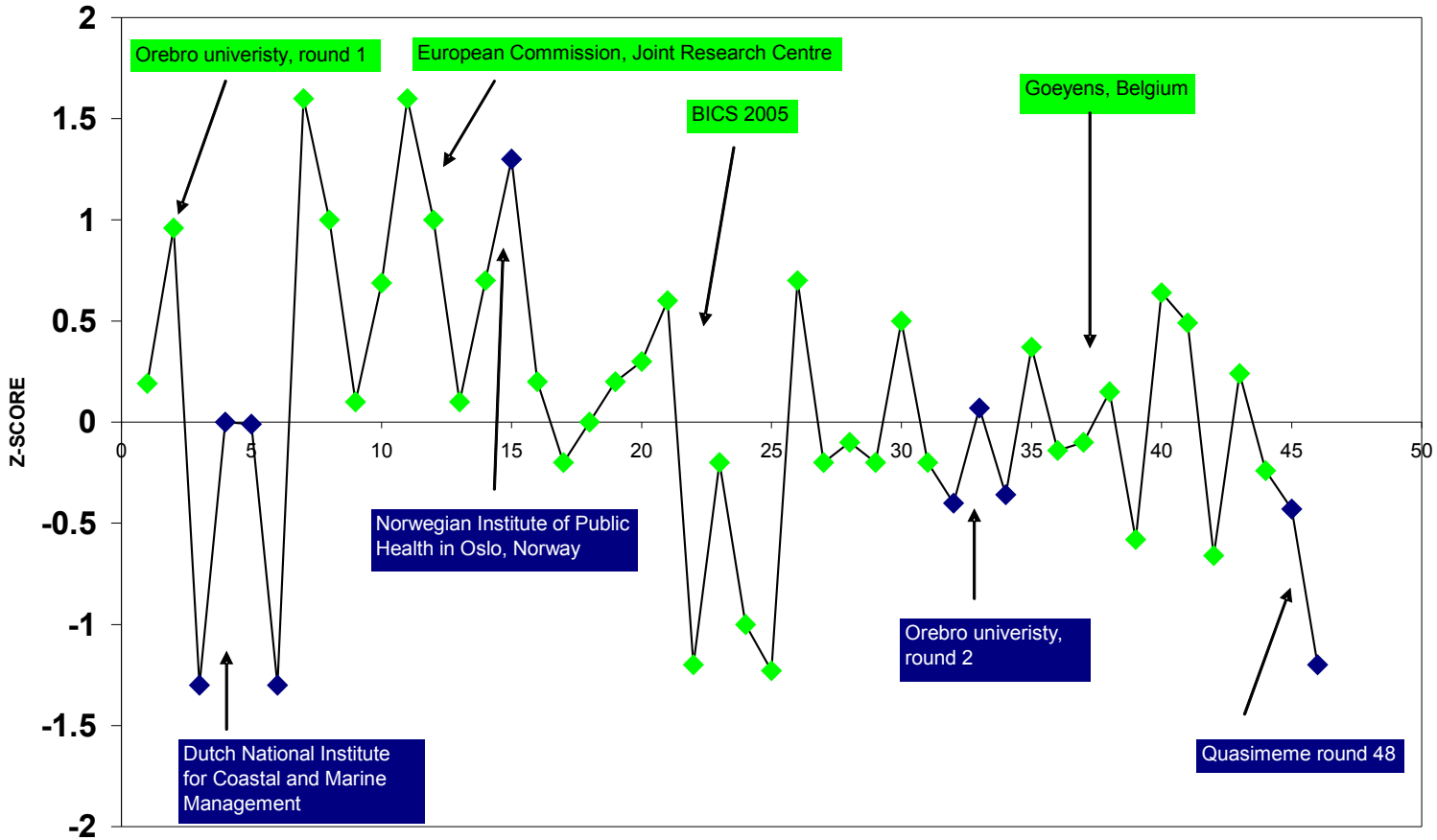
- **half of the EU limit value for dioxins,**
- **at the EU level for dioxins and**
- **at the EU limit for Total TEQ (Dioxins and dl- PCBs)**

Performance of DR CALUX® for various food and feeding stuffs

Foodstuffs	Weight (g)	EU-Limit TEQ pg/g lipid	LOQ	Repeatability (%)	Reproducibility (%)
Oils/fats plant origin	7.0	0.75	0.14	18	32
Animal oils/fats	5.0	1.0	0.20	16	13
Milk/Milk products	2-50	3	0.60	11	16
Egg	17	3	0.60	10	21
Fish/Fishproducts	9	4	0.11	13	25
Fish oil	3.5	2	0.28	26	14
Feedingstuffs					
Feed plant origin*	9	0.75	0.11	11-19	13-26
Feedingstuff for fish	9	2.25	0.11	14	27
Fish/Fish products	9	1.25	0.11	13	25
Fish oil	1	6	1.0	26	14

ng TEQ/kg

Proficiency test performance BDS



Sample report of DR CALUX® results

Table 2. Results of the DR CALUX® bioassay.

Client code	BDS-code	DR CALUX® TEQ			ISO17025 (RvA-L401)
		PCDD/F specific	dl-PCB specific		
C	12	1.5	4.7	(pg TEQ/g fresh weight)	yes
F	15	0.85	1.5	(pg TEQ/g fat)	yes

Note 1: All DR CALUX® analysis results comply with EU requirements as indicated in COMMISSION DIRECTIVE 2002/70/EC (laying down the sampling methods and the methods of analysis for the official control of dioxins and the determination of dioxin-like PCBs in feedstuffs) and in COMMISSION REGULATION (EC) No 1883/2006 (laying down the sampling methods and the methods of analysis for the official control of dioxins and the determination of dioxin-like PCBs in foodstuffs). Intralaboratory repeatability and reproducibility are less than 15 and 30% respectively.

Note 2: Data are quantifiable between the limit of quantification (LOQ) and the EC_{50}. Only results within this range are included in the final analysis results. For results below the limit of quantification, an estimate is given (between parentheses).

Note 3: PCDD/F-PCB-TEQ are analysed as DR CALUX® TEQs and benchmarked against a 2,3,7,8-TCDD calibration curve

Council Requirements 1883/2006 and 152/2009

- Determine TEQ selectively as sum of TCDD/F + dl-PCB
- False-negative rate < 1 % *(matrix-dependent calibration/validation?)*
- Repeatability: cv < 30 % *(with well-defined control samples)*
- Response of sample must be compared with response of a *(well defined)* reference sample at the level of interest
- Extra reference samples should be included at 0,5 x and 2,0 x the level of interest *(limited space on the microtiter plates)*
- Maximum blank levels should be defined *(matrix-dependent)*

Council Requirements 1883/2006 and 152/2009

- Full dose-response curve ($r^2 > 95\%$) required
- TCDD reference concentration (3 x LOQ) to be monitored
- QC-charts for reference materials
- Induction must be within the linear part of the curve;
  3 or more dilutions are recommended to be tested
- Extract triplicates cv < 15 %
- LOD = 3 x SD of solvent blank or „background response“
- LOQ = 5 - 6 x SD of solvent blank or „background response“

ISO 17025 Accreditation

Appendix to ISO/IEC 17025 accreditation certificate
number: **L 401**

of **BioDetection Systems BV (BDS)**

Amsterdam

RAAD VOOR ACCREDITATIE

Valid from: **09-10-2006** till **09-10-2010**

Replaces appendix dated: **n.a.**

Nr.	Material or Product	Type of activity	Internal reference number
1	Feedingstuff, fats, oils, meat products, dairy products and fish products	Determination of activity of dioxins and dioxin-like compounds; DR CALUX® bioassay	Pe-bds-051 Conform EC Commission Directive 2002/69/EC, Conform EC Commission Directive 2002/70/EC
2		Determination of activity of dioxin-like PCB's; DR CALUX® bioassay and C-SPLIT	Pe-bds-051 Conform EC Commission Directive 2002/69/EC, Conform EC Commission Directive 2002/70/EC
3	Soil, fly-ash, blood and blood products	Determination of activity of dioxins and dioxin-like compounds; DR CALUX® bioassay	Pe-bds-051 In-house method
4	Sludge	Determination of activity of dioxins and dioxin-like compounds; DR CALUX® bioassay	Pe-bds-051 Conform RIKZ protocol Specie*07
5		Determination of activity of estrogen and estrogen-like compounds; ER CALUX® bioassay	Pe-bds-052 Conform RIKZ protocol Specie*08
6	Surface water, influent and effluent extracts	Determination of activity of estrogen and estrogen-like compounds; ER CALUX® bioassay	Pe-bds-052 In-house method