BIOANALYTICAL SCREENING OF FISH MUSCLE TISSUE FOR DIOXINS AND DIOXIN-LIKE COMPOUNDS: RESULTS FROM APPLICATION OF THREE DIFFERENT EXTRACTION METHODS

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Introduction

A proficiency test on determination of PCDD/Fs and PCBs in fish (salmon filet) and fish oil was organized by the European Union Reference Laboratory (EU-RL) for Dioxins and PCBs in Feed and Food, Freiburg (Germany) in 2011. The PT was open for National Reference Laboratories (NRLs) and official laboratories of EU member states performing physico-chemical (GC/MS, GC/ECD) and bioanalytical screening methods. Bioanalytical results for salmon filet submitted by 11 laboratories, suggested a tendency towards underestimation of the consensus value being 9.76 pg WHO-PCDD/F-PCB-TEQ/g wet weight. The then applicable maximum tolerable level for the sum of PCDD/Fs and dl-PCBs in muscle meat of fish was 8 pg WHO-PCDD/F-PCB-TEQ/g wet weight[1].
Laboratory performance was assessed in terms of "bioassay-scores": The reported bioanalytical equivalents (BEQs), or "CALUX-TEQs" were compared with the WHO-TEQ consensus values based on results received from laboratories applying physico-chemical methods:

$$\textit{Bioassay-score} = (x - x_a) / \sigma_{bioassay}$$

x_a: assigned value (derived from physico-chemical methods); x: participant's result (BEQ, "CALUX-TEQ"); $\sigma_{bioassay}$: bioassay target deviation (20 %). Bioassay-scores serve as a tool for assessing method performance within the scope of external quality control measures. Bioanalytical results being an estimate of the TEQ level in the sample are semi-quanitative. Bioassay-scores are therefore based on a 20 % target standard deviation derived from the legal requirement[2] for repeatability of bioanalytical results not to exceed 20%, while 10% are used in calculation of z-scores for GC/HRMS methods providing quantitative results on the congener level.
Bioassay scores were below -2 for 6 laboratories, and for 3 laboratories even below -3 (figure 1). Probably based on underestimation of the consensus value, 6 out of these 9 laboratories decided that "the sample is compliant", therewith submitting false-compliant results, certainly not acceptable in feed and food control aiming at efficient and reliable consumer health protection. These false-compliant decisions might be attributable to various factors, including, but not limited to:

1. differences between TEF-values and REP-factors, the latter representing the relative response of e.g. H4IIe rat or H1L6 mouse hepatoma cells to various congeners relative to their response to 2,3,7,8-TCDD (REP-factor: 1). Main contributors to WHO-PCDD/F-PCB-TEQ were 2,3,4,7,8-PeCDF (TEF-value: 0.5; REP-factor depending on cell line and selected data set: 0.3 – 0.6) and PCB 126 (TEF-value: 0.1; REP-factor depending on cell line and selected data set: 0.04 – 0.07).
2. physico-chemical properties of the reference material used for recovery control and correction (congener pattern, matrix, level of contamination, fat content, etc.)
3. evaluation of results with, or possibly without performing recovery correction
4. efficiency of the method used for extraction of protein-bound lipids (solvents or solvent mixtures, mode of extraction: by shaking, ultra-turrax, ASE, etc.)

It is within the responsibility of each bioanalytical laboratory to duly take into account the first three of the above listed issues. In this study, the efficiency of the three extraction methods applied during the PT is therefore reviewed, being methods based on:

- EG Bligh and WJ Dyer (1959)[3]
- F Smedes (1999)[4]
- DR-CALUX, BioDetection Systems (BDS), Amsterdam (NL)[5]

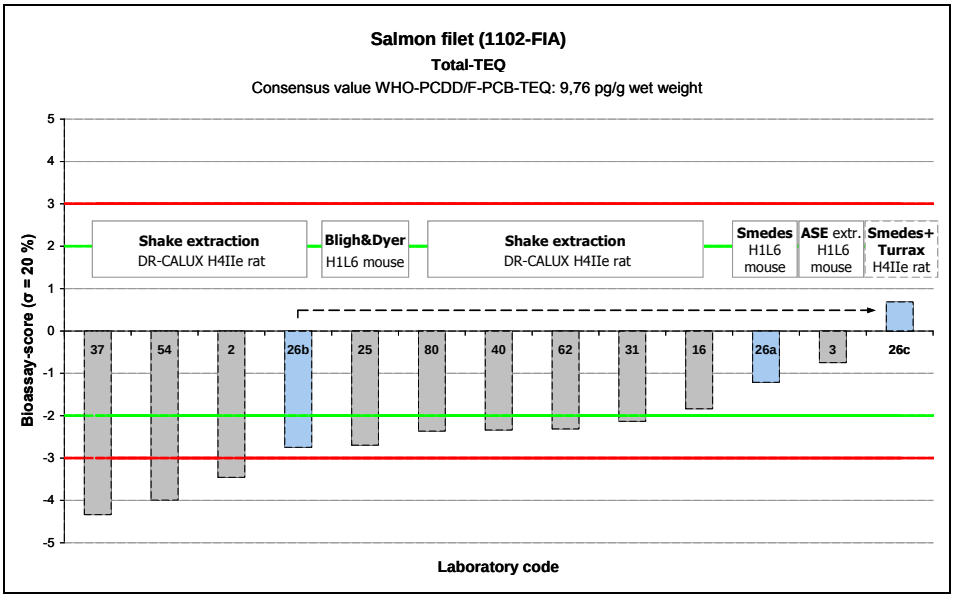

Figure 1. EU-RL proficiency test "Fish 2011": bioassay scores of participating laboratories using various extraction methods and CALUX cell lines. H4IIe rat hepatoma cells are from BDS (Amsterdam, NL), H1L6 mouse hepatoma cells are from XDS[6] (Durham, USA) (labs 3, 25) and from MS Denison, UC Davis[7] (USA) (lab 26). Lab 26 reported 3 results (26a-c) from 3 different extraction methods. Lipid content (assigned value): 6.2 %. Dotted line indicates change in bioassay-score when moving from shake extraction to a modified Smedes method dispersing the sample with an ultra-turrax (lab 26).

Materials and methods

Samples: 8 retail fish filet samples previously analyzed at EU-RL with GC/HRMS were screened in full duplicate analysis. GC/HRMS results and the %-contribution of PCB 126 to total-TEQ are given in table 1. Extracted lipid contents were determined after Twisselmann extraction of the freeze-dried sample material.

Table 1. Retail fish: extracted lipids, GC/HRMS results, contribution of PCB 126 to WHO-PCDD/F-PCB-TEQ

Retail fish (results based on TEF 1998)	Extracted lipids (%)	pg WHO-PCDD/F-PCB-TEQ/g wet weight	pg WHO-PCDD/F-TEQ/g wet weight	pg WHO-PCB-TEQ/g wet weight	Contribution of PCB 126 to total-TEQ (%)
1 Zander	0.4	3.79	0.25	3.54	49
2 Pike	0.4	2.07	0.27	1.79	56
3 Bream	1.4	2.06	0.50	1.56	50
4 Bream	2.3	4.40	0.77	3.63	54
5 Whitefish	2.7	1.81	0.39	1.43	55
6 Bream	2.0	5.72	1.17	4.55	50
7 Bream	2.9	4.75	1.30	3.45	43
8 Deep-fried fish	7.7	3.55	0.63	2.92	57

Bligh and Dyer extraction method, modified (included: vortex mixing): 5 mL chloroform and 10 mL methanol were added to 5 g homogenized sample in a 50 mL centrifuge tube (PP) and the mixture was vortexed for 15 s. 5 mL chloroform were added and the mixture was vortexed for 15 s, then 5 mL demin. water were added, followed by vortexing for 15 s. After 15 min, the lower phase was transferred with a pasteur pipette into a previously weighted 60 mL collection vial. Extraction with 5 mL chloroform was repeated once. After 15 min; the lower phase was transferred into the 60 mL collection vial. The combined extracts were evaporated to dryness at 37°C under a gentle stream of nitrogen. Subsequently, the weight of the extracted lipids was determined.

Smedes extraction method, modified (included: sample dispersion with ultra-turrax, extraction 2x repeated): In a 100 mL glass bottle, 16 mL 2-propanol and 20 mL cyclohexane were added to 5 g homogenized sample, followed by dispersion with an ultra-turrax high performance homogenizer (20000 rpm) for 15 s. 22 mL demin. water were added and the ultra-turrax again applied for 15 s. The rotor was rinsed with 10 mL 2-propanol into the glass bottle, 15 mL 2-propanol were added (for enhanced phase separation), the mixture gently agitated on a horizontal shaker for 30 min (110 mot*min-1). After 10 min standing (for enhanced phase separation) as much as possible of the upper phase was removed with a pasteur pipette and passed through a small glass column (lower end closed with a glass wool plug above which 1 g Na_2SO_4 were placed) into a 60 mL collection vial (weighted). 20 mL cyclohexane were added to the mixture, ultra-turraxed for 15 s, the rotor was rinsed with 5 mL 2-propanol into the glass bottle and the mixture gently agitated on a horizontal shaker for 10 min (110 mot*min-1). After 30 min standing, the upper phase was removed and passed through the Na_2SO_4 column into the 60 mL collection vial. Extraction with cyclohexane was repeated twice. The combined extracts were evaporated to almost dryness at 45°C under a gentle stream of nitrogen, the inner walls of the collection vial rinsed with 5 ml n-hexane, and evaporated to dryness. Finally, the weight of the extracted lipids was determined.

DR-CALUX extraction method[4]*:* 5 g of homogenized sample were mixed with water and 2-propanol. The lipid fraction was extracted 3 times with a mixture of n-hexane/diethyl ether 97/3 (v/v). For better phase separation, additional 2-propanol may be added at each step. The combined extracts were reduced to dryness and the weight of the extracted lipids was determined.

*Clean-up on acidic silica: E*xtracted lipids were re-dissolved in 2 - 3 mL n-hexane and the solution was transferred to a glass column containing (depending on the lipid amount) 10 - 15 g each of 33% (bottom) and 20% (middle) sulfuric acid activated silica, covered with 2 - 3 g of anhydrous sodium sulfate. Columns were eluted with 40 - 60ml of hexane/diethyl ether 97/3 (v/v), the eluate was evaporated to approx. 0,5 mL and transferred to a conical 1,2 mL vial. The reduced eluate was evaporated just to dryness (37°C, gentle stream of filtered N_2), the residue immediately re-dissolved in 25 µl DMSO.

Measurement of luciferase activity: Sample extracts were added to incubation medium and the mixtures subsequently added in triplicate to 96-well plates containing H4IIE rat hepatoma cells (BDS, Amsterdam, NL). Cells were exposed for 24 h, followed by lysis of the cells and measurement of luciferase activity in a Berthold Centro LB 960 microplate luminometer.

Evaluation of bioanalytical results: Sample response data were converted to assay concentrations via a dose-response curve fitted to the response data obtained from simultaneous exposure of the cells to a 2,3,7,8-TCDD standard dilution series. Weighted sum of squared residuals regression was applied. Sample-based results were calculated taking into account sample intake and final extract volume, and subsequently corrected for the procedure blank. Focus of the present evaluation was on apparent recoveries of the levels in the samples being the ratio of the estimated BEQ-level and the TEQ-level as determined by GC/HRMS. Therefore, sample results were not corrected for the apparent recovery of the positive control sample analyzed with each sample series.

Results and discussion

Bioanalytical results (not corrected for recovery) from application of various extraction methods to 8 fish samples analyzed in duplicate are displayed in figure 2, along with corresponding GC/HRMS results. Based on the TEF 1998 (TEF 2005) scheme, the modified Smedes method yields 63 % (83%) apparent recovery, the modified Bligh and Dyer method 34 % (45%), and the DR-CALUX method 41 % (54%) apparent recovery (mean values, see table 2). Each method meets the new requirements recently set by the EU[7] for an acceptable range of apparent recoveries when total-BEQ is determined, being 30 - 130%. However, the low apparent recoveries found in this study from the Bligh and Dyer and DR-CALUX method may explain underestimation of the assigned total-TEQ value for fish in the 2011 PT study, especially if recovery correction should not have been performed. We therefore recommend the combined use of a cyclohexane/2-propanol/water (20/16/22 v:v:v) solvent mixture and a method efficiently dispersing the fish muscle tissue (ultra turrax).

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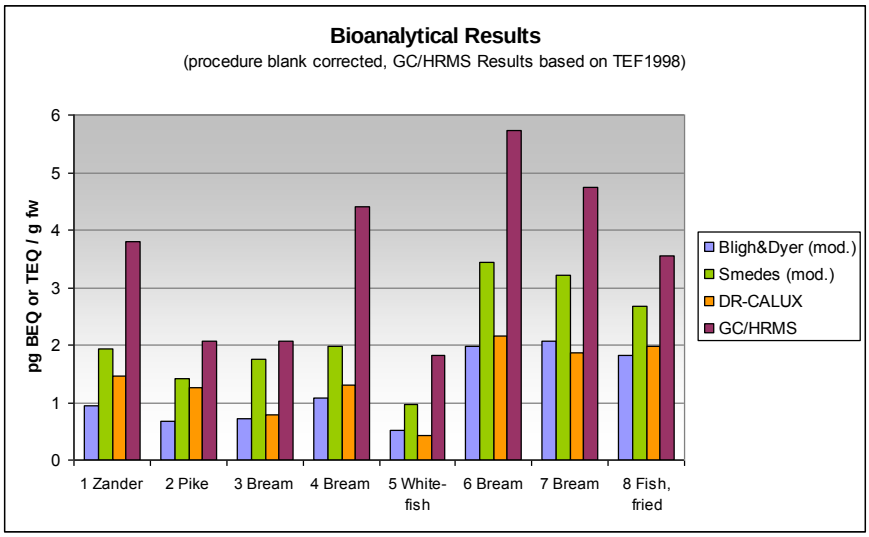

Figure 2. Application of various extraction methods to 8 fish samples: bioanalytical results (pg total-BEQ/g ww), not corrected for recovery, with corresponding GC/HRMS results (pg total-TEQ/g ww)

Table 2. Lipid contents and bioassay apparent recoveries (%) from application of various extraction methods

	Bligh & Dyer (modified)		Smedes (modified)		DR-CALUX	
Fish (recovery data based on TEF 1998)	Extracted lipids (%)	Apparent recovery (%)	Extracted lipids (%)	Apparent recovery (%)	Extracted lipids (%)	Apparent recovery (%)
1 Zander	0,3	24,7	0,6	51,0	0,5	38,6
2 Pike	0,5	32,2	0,7	69,0	0,7	61,2
3 Bream	0,9	34,7	1,6	84,9	0,8	38,0
4 Bream	0,9	24,4	1,8	44,8	0,9	29,4
5 Whitefish	0,9	28,3	2,7	53,1	1,1	23,6
6 Bream	1,5	34,6	2,6	60,2	1,2	37,8
7 Bream	1,6	43,6	3,0	67,8	1,4	39,3
8 Deep-fried fish	5,9	51,1	8,9	75,5	5,4	55,9
Median		33,4		64,0		38,3
Mean		34,2		63,3		40,5

References

1. Commission Regulation (EC) No 1881/2006 as regards maximum levels for dioxins, dioxin-like PCBs and non dioxin-like PCBs in foodstuffs, OJ L 320, 03.12.2011, p. 5
2. Commission Regulation (EU) No 252/2012 of 21 March 2012 laying down methods of sampling and analysis for the official control of levels of dioxins, dioxin-like PCBs and non dioxin-like PCBs in certain foodstuffs and repealing Regulation (EC) 1883/2006, OJ L 84, 23.03.2012, p. 1
3. Bligh EG and Dyer WJ, A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37 (1959) 911
4. Smedes F, Determination of total lipid using non-chlorinated solvents. *Analyst* 124 (1999) 1711–1718
5. DR-CALUX extraction and clean-up procedures (BioDetection Systems, Amsterdam, NL) are confidential and available only to licensed laboratories. However, principles of the applied method have been published and are as such part of public domain (see e.g: Gizzi G, Hoogenboom LAP, Von Holst C, Rose M, Anklam E, *Food Additives and Contaminants* 22 (2005) 472–481
6. Xenobiotic Detection Systems (XDS, Durham, NC, USA)
7. Prof. MS Denison, Department of Environmental Toxicology, University of California Davis, Davis (USA)