Organohalogen Compounds, Volume 60, Pages 267-270 (2003)

DIFFERENCE: OPTIMISATION AND VALIDATION OF SCREENING METHODS FOR THE ANALYSIS OF DIOXINS AND DIOXIN-LIKE PCBs IN FOOD AND FEED AND THE PRODUCTION OF CERTIFIED REFERENCE MATERIALS

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Introduction

At 1 February 2002 a European research project called DIFFERENCE (Dioxins in Food and Feed – Reference Methods and New Certified Reference Materials) has started. It will continue for 3 years and aims at:

- the development, optimization and validation of alternative methods for dioxin analysis, including bio-analytical screening techniques (Ah-PCR and CALUX), GC-LRMS/MS and comprehensive multi-dimensional GC, in order to reduce the costs of routine dioxin analysis;
- the optimization and validation of new extraction and clean-up procedures including Accelerated Solvent Extraction (ASE), Microwave assisted Extraction (MAE) and Supercritical Fluid Extraction (SFE);
- the development of validation protocols and an instruction video;
- the feasibility testing of the production and certification of five high quality certified reference materials (CRMs) for dioxins, furans, indicator PCBs and dioxin-like PCBs in food and animal feed being i) fish tissue, ii) sterilised milk, iii) pork, iv) fish oil or fish meal, v) compound feed.

Methods and Materials

The project started with the optimization of the screening techniques for the analysis of dioxins and dioxin-like PCBs in food and feed. The following methods were optimized:
CALUX: evaluation of the role of interferences (both antagonists and agonists); fitting of the regression curve in the lower part of the calibration curve; sensitivity; role of DMSO in the extract and evaluation of the differences between the mouse and rat based cell lines.
Ah-PCR: evaluation of sensitivity, matrix interferences.
GCxGC-ECD: optimization of separation by various column combinations; evaluation of different modulator types, which are used for re-injection of the trapped analytes in the second column; sensitivity
GC-LRMS/MS: optimum ion trap MS/MS conditions like, sensitivity
ASE/MAE/SFE: evaluation of extraction efficiencies for high and low contaminated samples; evaluation of strategies; evaluation of possibilities of combined extraction and clean-up within the extraction cell. The focus will be on ASE as the most promising technique for within-cell extraction and clean-up of samples and ASE-CALUX.

The validation of CALUX, Ah-PCR, GCxGC-ECD, GC-LRMS/MS, ASE-GC-HRMS consists of three rounds, which are shown in Table 1. The first round primarily focussed on the goodness-of-

fit of the calibration curve and provided the first data concerning repeatability and reproducibility of the screening methods. The objective of the second round is to assess the detection capability and selectivity of the method. Furthermore, the accuracy of the results obtained with the methods applied can be investigated, since the exact amount added to the samples is known. Round three will provide more data on repeatability and reproducibility of the methods. The information obtained during the three rounds will be used to gauge the ruggedness of the analytical methods. During the whole validation process a quality control solution is used to assure the validity of the data. GC-HRMS serves as the reference technique throughout the validation.

Table 1: Validation protocol of bioanalytical and chemical analytical screening methods

Round 1	Round 2	Round 3
2,3,7,8-TCDD standard A to F (1x2-fold) Quality control sample 3 pg dioxin + 3 pg PCB TEQ/g Clean fish extract (2-fold) Fish oil (3x2-fold) Milk (3x2-fold)	Vegetable oils (4x1-fold) at the levels of 0 (blank), 0.2, 0.75, 1.5, 3.0 and 6.0 pg dioxin/dioxin like PCB TEQ. Vegetable oils (4x1-fold) at the levels 3.0 pg dioxin /dioxin like PCB TEQ, including potential interferences like PCNs, PCDEs and PCBs.	Quality control sample 3 pg dioxin + 3 pg PCB TEQ/g Cereal based feed (2-fold) Chicken (2-fold) Vegetable feed (2-fold) Egg (2-fold) Fish tissue (3x2-fold) Pork (3x2-fold)

Candidate CRMs

For the feasibility study to the preparation and certification of candidate CRMs, five materials have been selected and produced. The target levels of dioxins in the selected materials (see Table 2) are chosen to resemble the current EU limits[1,2]. Homogeneity and stability tests are currently being carried out in each material according to BCR and ISO guidelines[3,4]. No results on homogeneity and stability have been obtained so far. Finally, these materials will be used in an interlaboratory study to test the feasibility of certification. For that purpose ca. 20 expert laboratories will be invited to participate.

Table 2. Target levels of dioxins, dl-PCBs and indicator PCBs in the candidate CRMs

Material	Dioxins (pg TEQ/g fat)	dl-PCBs (pg TEQ/g fat)	Indicator-PCBs
Fish	4*	4*	background
Pork	1	0.5	200 µg/g fat
Milk	3	3	background
Fish oil	6	6	background
Feed	0.75*	0.75*	background

* pg TEQ/g ww

Results and Discussion

The different techniques have been optimized during the first half year of the project. For example for GCxGC-ECD the separation of (dioxin-like) PCBs and the 17 WHO dioxin and furan congeners has been optimized (in close cooperation with the EU research project DIAC- Dioxin Analysis using Comprehensive Gas Chromatography). For LR-MS/MS the isolation of the precursor ion and the fragmentation of the precursor ion were optimized.

After optimization of all techniques, round 1 of the validation scheme as mentioned in Table 1 has started. The resulting data were collected and statistically evaluated. Table 3 shows the results (z-scores) of the quality control oil analysed by different screening techniques and GC-HRMS.
The results of the quality control oil show generally good agreement among the different screening techniques and also compared with GC-HRMS. The z-scores for the total-TEQ determination range from –1.008 to 1.982, which is within the acceptance range of –2 to 2. The same accounts for the PCB-TEQ z-scores, but not for the dioxin-TEQ z-scores. GCxGC-ECD shows high z-scores for dioxins due to a combination of the use of upperbound and high detection limits for ECD. The ASE combined with CALUX and GC-HRMS shows also very good results. Obviously, the quality control oil was easy to 'extract', but based on results obtained during optimization (data not shown) it is expected that ASE will perform very well throughout the validation.
Lab A performed a separation of dioxins and dioxin-like PCBs prior to the CALUX determination, whereas lab E and H performed a total-TEQ determination.

Table 3. Z-scores of the quality control sample of round 1 for the different screening techniques compared with the GC-HRMS reference technique (bold).

Lab	**F**	**C**	E	A	G	I	B	H
Method	**GC-HRMS**	**GC-HRMS**	CALUX	CALUX	GC-LRMS	GCxGC-ECD	ASE+GC-HRMS	ASE+CALUX
Total TEQ	**-0.011**	**0.188**	-1.008	-0.416	0.182	1.982	-0.011	1.136
Dioxin-TEQ	**-0.112**	**-0.12**	n.a.	0.426	0.401	2.89	-0.031	n.a.
PCB-TEQ	**0.081**	**0.467**	n.a.	-1.18	-0.016	1.158	0.007	n.a.

Figure 1 shows the total-TEQ results of the fish oil sample with a bimodal distribution. Lab A, B, G, I and J show good agreement and are close to the GC-HRMS values (C, F and J). The laboratories D, E and H (CALUX or ASE-CALUX) show good agreement but are considerably lower compared with the GC-HRMS values.
For the CALUX bioassay it was e.g. shown that the use of a TCDD calibration curve for calculating the dioxin content in a sample, leads to a serious underestimation. The use of reference materials as required by EU-regulations is therefore a critical factor. Furthermore, the TEF values determined by the CALUX assay can deviate from the consensus values as used by the WHO[5].
Lab I (GCxGC-ECD) shows high levels due to reasons earlier explained.
The Ah-PCR assay was not yet available at the time of optimization of the methods and has therefore not yet been included in the first round of the interlaboratory study.

Conclusions

The first round of the validation of the different screening techniques shows promising results (accuracy, repeatability). Round 2 and 3 will focus on robustness, reproducibility and repeatability. It is expected that the laboratories will improve their methods between the different rounds, resulting in further improvement of validation results.
The candidate CRMs have successfully been prepared. Homogeneity, stability and levels of dioxins, dioxin-like PCBs and indicator PCBs will be evaluated in the near future. The project's web-site is www.dioxins.nl (DIFFERENCE).

Acknowledgements

The European Commission is gratefully thanked for supporting the DIFFERENCE project (contract G6RD-CT-2001-00623). The following partners and their co-workers and subcontractors are gratefully acknowledged for their hard work and stimulating contribution to the project J. de Boer

(RIVO), L. Goeyens and H. Beernaert (IPH), D. Fraise (CARSO), R. van Cleuvenbergen and G. Schoeters (VITO), E. Björklund (Lund University), J. Rivera (IIQAB-CSIC) R. Hoogenboom and W. Traag (RIKILT), C. von Holst (JRC-FPU), P. Haglund (Umeå University).

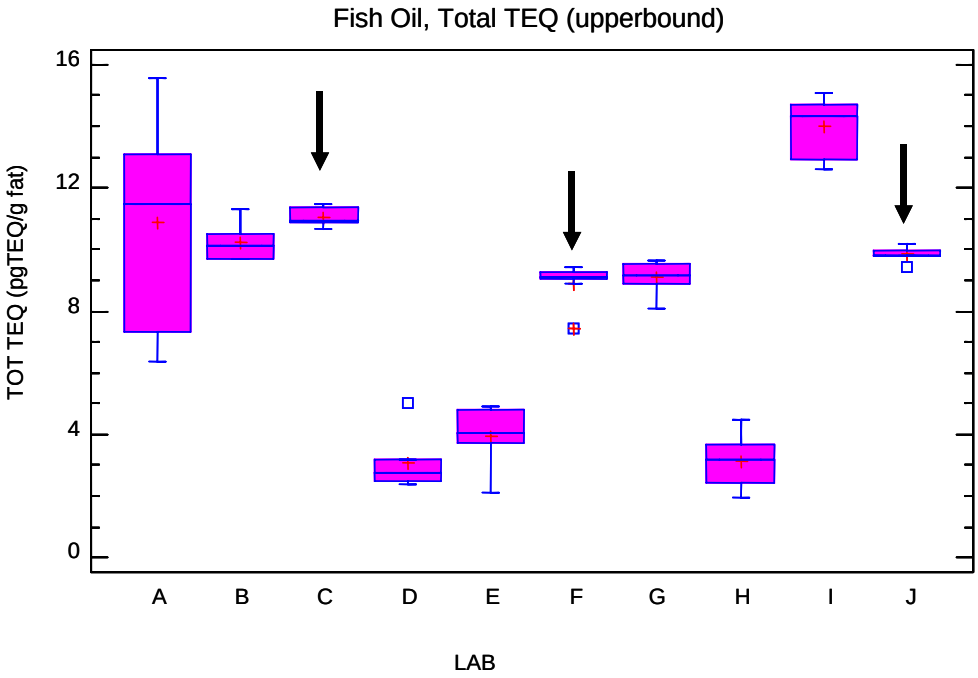

Figure 1. Results of the total-TEQ analysis in fish oil. The arrows indicate the GC-HRMS results (see Table 3 for corresponding techniques; additional labs: D=CALUX; J=GC-HRMS).

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