The international validation of Chemical and Biological Screening Methods for dioxins and dl-PCB's: The DIFFERENCE Project round 3

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Introduction

The European research project DIFFERENCE focuses on the development, optimisation and validation of screening methods for dioxin analysis, including bio-analytical and chemical screening techniques (CALUX, GC-LRMS/MS, GCxGC-ECD) and on the optimisation and validation of new extraction and clean-up procedures[1]. The performance of these techniques is assessed in an international validation study and the results are compared with the reference technique GC-HRMS.

The purpose of the validation study is to ensure that the bio-analytical and chemical analytical screening methods for dioxins and dioxin-like PCBs (dl-PCBs) respond to the EU criteria. Screening methods are used to distinguish between compliant and non-compliant samples. The requirements for analytical methods for the official control of dioxins and dioxin-like PCBs in food and feeding stuffs are laid down in the EU commission directives 2002/69/EC and 2002/70/EC[2,3]. The analytical procedures must have a high sensitivity, a low limit of detection and a high accuracy.

This international validation protocol, which is based on the International Harmonized Protocol for Proficiency Testing[4], will provide information about the accuracy (trueness and precision), ruggedness, detection capability and selectivity of the bio and chemical analytical screening methods in three rounds. The first round focussed on the goodness-of-fit of the calibration curve and on the accuracy of the methods. In round 2 the detection capability and selectivity are assessed. The results of the first 2 rounds of the project have been reported by Van Loco *et*

al.[5] In round 3, the accuracy and robustness of the methods are evaluated on several samples of different origin. This paper summarizes some of the results of round 3 of the validation study.

Methods and Material

Materials

The following materials have been prepared by the Netherlands Institute for Fisheries Research: vegetable oil spiked at a concentration of about 3pg dioxin + 3pg PCB TEQ/g (QC-oil), pork tissue and chicken compound feed. With the exception of the QC-oil, all the materials are natural contaminated samples with a concentration nearby the level of interest. The results of the stability and homogeneity studies on the materials did not show any significant findings.

Methods

The following methods[6-8] are evaluated in the validation study: Chemical Activated Luciferase Gene Expression (CALUX), multi-dimensional GC with Electron Capture Detection (GCxGC-ECD), GC- Low Resolution Mass Spectrometry (GC-LRMS/MS), and GC- High Resolution MS (GC-HRMS). Accelerated Solvent Extraction (ASE)[9] is evaluated as a combined extraction and clean-up technique. Details on the methods used are provided at the web-site of the DIFFERENCE-project[10].

Statistical evaluation of the results

Proficiency scoring

The z-scores are evaluated according to the international harmonized protocol for proficiency testing of chemical analytical laboratories[4]: $z = (x-X) / \sigma_p$. The target value for the standard deviation (σ_p) can be determined via the (modified) Horwitz function[11], but preference is given to the use of the acceptance criteria in the European directives 2002/69/EC and 2002/70/EC[2,3]. The σ_p is therefore derived from: $\sigma_p = 0.3X$. The assigned value (X) is calculated using the added concentration (QC-oil) or using the median of the results obtained from the 3 laboratories using the GC-HRMS.

The sum of the squared z-scores (SSZ) is calculated to give a composite score of the individual results for each laboratory: $SSZ = \Sigma z^2$. The SSZ is evaluated by comparing it with critical χ^2 values with n degrees of freedom (where n is the number of scores) and a probability of 0.95 and 0.997 which corresponds with z-scores of 2 and 3.

Method validation parameters
The repeatability (r), the within-lab reproducibility (W) and the reproducibility (R) are calculated using a two factor nested ANOVA as explained in the ISO 5725-3[12].

Results and Discussion

<u>QC-oil</u>
The QC-oil was analysed during all the 3 rounds of the project: vegetable oil was spiked with a mixture of dioxins and dl-PCB at a concentration of 5.52 pg total TEQ / g oil. The mean found concentration, the bias, the within-lab reproducibility coefficient of variation (CV_W) and the reproducibility coefficient of variation (CV_R) are summarized in Table 1. The CV_W for the biological and chemical screening methods are all, except labs E and H, lower than 30%. The European directives [2,3] require that the variation of screening methods is below 30%. A very small variation (CV_W < 3%) for the GC-LRMS/MS screening method is noticed. Furthermore, the results of the CALUX laboratories underestimate the total TEQ concentration in the sample. However, the CALUX results are not corrected for recovery. Two CALUX laboratories (A and E) have also reported results with applying recovery correction. This is indicated by "*". The SSZ-scores for the recovery corrected CALUX results are satisfactory.

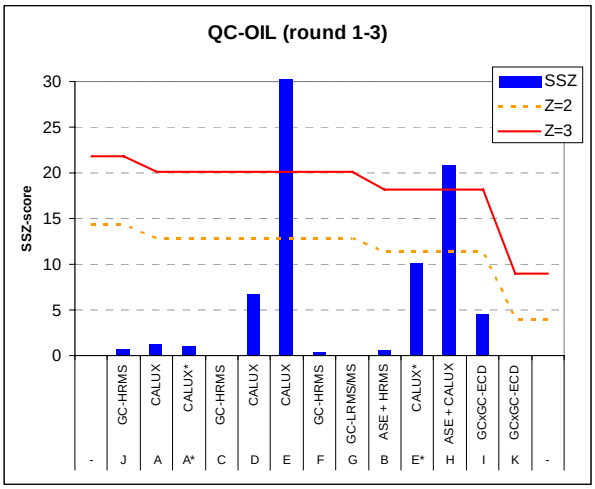

Figure 1: SSZ-scores of the total TEQ QC-oil results. The interpretation of the SSZ-scores is performed by the full and the dotted line, which represents the acceptance criteria with

Table 1: QC-oil validation data (the oil was spiked at a concentration of 5.52 pg total TEQ / g oil).

Lab	Method	n	Mean concentration (pg total TEQ/g oil)	Bias	CV_W	CV_R
A	CALUX	6	5.05	-8.5%	15.1%	
D	CALUX	6	3.97	-28.2%	22.8%	50.5%
E	CALUX	6	1.60	-71.0%	89.0%	
A*	CALUX*	6	5.68	2.8%	15.1%	23.5%
E*	CALUX*	5	7.64	38.4%	18.2%	
I	GCxGC-ECD	5	6.69	21.2%	27.9%	28.3%
K	GCxGC-ECD	1	6.10	10.5%	-	
C	GC-HRMS	6	5.73	3.7%	15.8%	
F	GC-HRMS	6	5.82	5.3%	6.0%	8.2%
J	GC-HRMS	7	5.24	-5.0%	11.3%	
G	GC-LRMS/MS	6	5.68	2.8%	2.2%	-
H	ASE+CALUX	5	3.06	-5.0%	127.1%	-
B	ASE+GC-HRMS	5	6.00	2.8%	3.6%	-

Table 2: Chicken compound feed validation data

Lab	Method	n	Mean concentration (ng total TEQ/kg)	Bias	CV_r	CV_W	CV_R
A	CALUX	6	0.730	-55.6%	12.0%	13.0%	
D	CALUX	6	0.262	-84.1%	9.6%	9.6%	50.3%
E	CALUX	6	0.740	-55.0%	17.4%	22.9%	
A*	CALUX*	6	1.043	-36.6%	12.0%	12.8%	42.9%
E*	CALUX*	6	1.895	15.2%	13.7%	13.7%	
C	GC-HRMS	6	1.642	-0.2%	1.1%	1.1%	
F	GC-HRMS	6	1.716	4.3%	3.4%	3.6%	14.5%
J	GC-HRMS	6	1.302	-20.9%	1.0%	4.2%	
K	GCxGC-ECD	1	1.415	-14.0%	-	-	-
G	GC-LRMS/MS	6	1.741	5.8%	4.9%	5.3%	-
H	ASE+CALUX	6	0.475	-70.7%	6.9%	23.4%	-
B	ASE+GC-HRMS	6	1.733	5.4%	2.8%	19.3%	-

For each reported result (total TEQ) z-scores are calculated. The squared z-scores are presented in Figure 1. Only the results of lab E and H are unsatisfactory. However, it cannot be concluded that the ASE+CALUX (extraction and clean-up followed by analysis with CALUX) is unsuitable, since the CALUX part was performed by the same lab E and the results were not corrected for recovery. The

results reported with GCxGC-ECD (1 outlier removed), with GC-LRMS/MS and with ASE+GC-HRMS (extraction and clean-up followed by GC-HRMS) are satisfactory.

Chicken compound feed

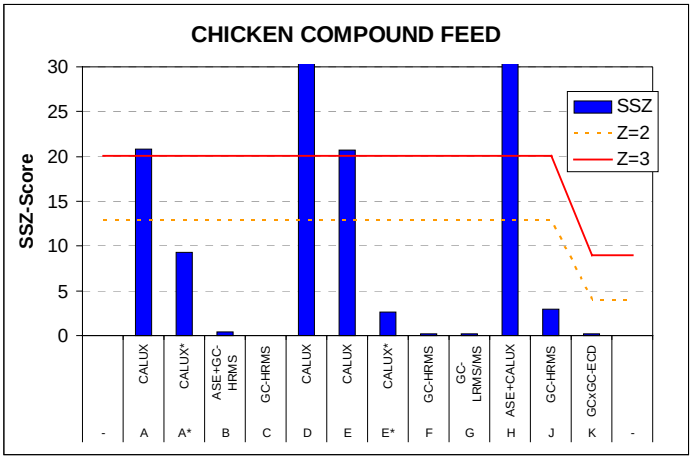

Figure 2: SSZ-scores for the total TEQ Chicken Compound Feed results

The feed samples were analysed in duplicate on 3 different analytical runs with different equipment and operators whenever feasible. The results are summarized in Table 2. The CV_W is below the required 30% for all the labs (no precision data are available for GCxGC-ECD). The not corrected CALUX results underestimate the total TEQ concentration of the feed sample. The results of the CALUX labs (= lab A, D, E and H) are more than 50% lower than the median of the GC-HRMS results. The SSZ-scores of the CALUX labs are unsatisfactory (Fig 2). Applying recovery correction improves the total TEQ results of these labs, since the SSZ-scores for CALUX* are satisfactory. The results of the GC-LRMS/MS and GCxGC-ECD are all satisfactory, however only 1 result for GCxGC-ECD was reported. The results of the ASE + GC-HRMS show a significant larger variation than the GC-HRMS results. Their SSZ-score is still satisfactory.

Pork Tissue

The pork samples were analysed in duplicate on 3 different analytical runs with different equipment and operators whenever feasible. The results are summarized in Table 3. The CV_W is below the required 30% for all the labs, except E, E* and H. No precision data are available for GCxGC-ECD. A relative large variation (CV_W = 28%) is noticed for the ASE + GC-HRMS method, although the SSZ-score is still satisfactory. This larger variation is explained by carry over from the feed samples during analysis. Also for LAB J (GC-HRMS) a larger repeatability

and within-lab reproducibility CV, in comparison with the other GC-HRMS labs, is observed.

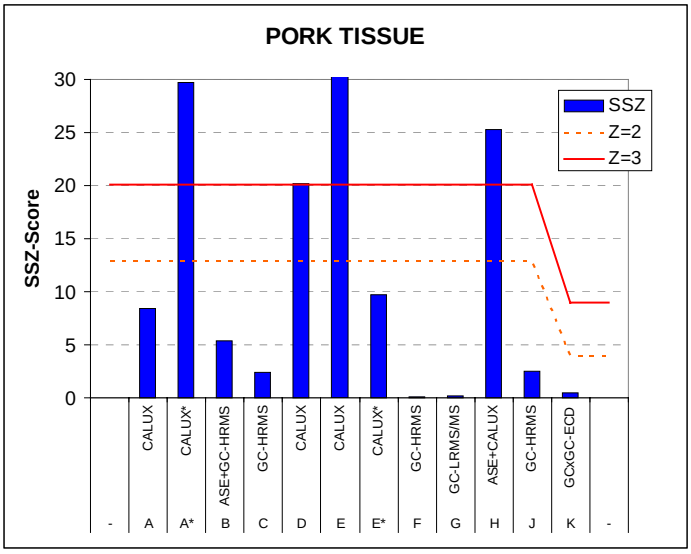

Figure 3: SSZ-scores for the total TEQ pork tissue

Once more it is shown that uncorrected CALUX results underestimate the total TEQ concentration in the sample. Indeed, the SSZ-scores for the labs D, E and H are unsatisfactory (Fig 3). However, the lab A results are inconsistent with the other CALUX results. The corrected results (A*) are unsatisfactory. The total TEQ concentration is over-estimated by 56%. No explanation was found for this inconsistency. The SSZ-scores of the GC-LRMS/MS, GCxGC-ECD (only 1 reported result) are satisfactory.

Table 3: Pork tissue validation data.

Lab	Method	n	Mean concentration (pg total TEQ/g fat)	Bias	CV_r	CV_W	CV_R
A	CALUX	6	1.622	21.9%	16.5%	26.8%	122.8%
D	CALUX	6	0.603	-54.6%	18.0%	18.0%	
E	CALUX	6	0.347	-73.9%	14.2%	58.3%	
A*	CALUX*	6	2.079	56.3%	16.5%	26.8%	96.8%
E*	CALUX*	6	0.885	-33.5%	28.6%	30.7%	
C	GC-HRMS	6	1.572	18.2%	4.2%	5.4%	29.0%
F	GC-HRMS	6	1.312	-1.4%	4.5%	4.5%	
J	GC-HRMS	6	1.185	-10.9%	16.4%	20.4%	
K	GCxGC-ECD	1	1.600	20.3%	-	-	-
G	GC-LRMS/MS	6	1.288	-3.2%	4.9%	4.9%	-
H	ASE+CALUX	6	0.545	-59.0%	34.3%	49.3%	-
B	ASE+GC-HRMS	6	1.053	-20.8%	19.8%	28.3%	-

Conclusion

The CALUX results, when not corrected for recovery, mostly underestimate the total TEQ concentration in the samples. The CALUX* results after recovery correction are better correlated with the GC-HRMS results. Most of the within-lab reproducibility CV's for the CALUX labs are below the required 30%.

The results of the GC-LRMS/MS and the GCxGC-ECD screening techniques are satisfactory. The variation of the GC-LRMS/MS method is, for all the samples, smaller than 6%. The within-lab CV of the GCxGC-ECD method for vegetable oil is below the required 30%. No precision data for GCxGC-ECD are available for chicken feed and pork tissue.
The within-lab reproducibility CV's of the ASE+GC-HRMS method (extraction and clean-up followed by GC-HRMS analysis) for pork tissue and chicken feed samples are significantly higher than the CV's of the GC-HRMS methods with a classical extraction and clean-up procedure, but are still below the 30%.

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), D. Fraise (CARSO), R. van Cleuvenbergen and G. Schoeters (VITO), E. Björklund (Lund University), J. Rivera (IIQAB-CSIC), J. Santos (University of Barcelona), R. Hoogenboom and W. Traag (RIKILT), P. Haglund (Umeå University).

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