

Evaluation of the DR-CALUX screening of food and feed, according to regulation levels including DL-PCB

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Introduction

Maximum levels of dioxins in food and feed are set in the European legislation^{1,2} for 17 congeners of PCDD/Fs and levels for 12 congeners of DL-PCBs will be added soon³. The reference gas chromatography-high resolution mass spectrometry (GC-HRMS) method allows the individual identification and quantification of these 29 congeners at the ultra-trace level. Congener-specific data can be used as such for source tracking or be converted in toxic equivalents quantities (TEQs) based on the use of toxic equivalent factors⁶ (TEFs). Because the monitoring of dioxins in the food chain is very expensive using HRMS, sample screening methods are needed. The CALUX cell based assay has widely been proposed to screen dioxins in food and feed samples^{4,5}. Compared to GC-HRMS, the CALUX AhR-activation mediated response directly yield to a TEQ estimation based on a correlation with 2,3,7,8-TCDD induction of the assay.

Comparison between GC-HRMS and CALUX results often shows discrepancies, due to differences between the WHO-TEF⁶ values and the CALUX REP⁷ (relative equivalent potency) assigned to each congener of dioxins, furans and DL-PCBs for a specific assay. Such differences make difficult the decision of compliance or suspicion of non compliance of samples submitted to biological screening.

We present a retrospective evaluation of the dioxin response (DR)-CALUX screening by comparing DR-CALUX results to GC-HRMS results for 51 samples analyzed in parallel.

Material

Samples: DIFF-01(fish), DIFF-02 (pork), DIFF-03 (milk), DIFF-04 (fish oil), DIFF-05 (feed), as well as matrix-specific reference samples (for milk, pork and fish) are samples issued from the DIFFERENCE European project. Other samples are the reference material BCR-533 (PCDD/F-spiked low level milk powder), internal quality control (QC) or unknown samples issued from the routine activity of the laboratory of mass spectrometry. QC samples and method blanks (BCs) were regularly run for QA/QC purposes.

Extraction: For HRMS analyses of PCDD/Fs and dioxin-like PCBs, samples were extracted using pressurized liquid extraction (PLE) (Dionex ASE 200) and cleaned-up using the automated Power-PrepTM system (Fluid Management Systems Inc.)⁸. For DR-CALUX analyses, samples were liquid-liquid (LLE) extracted and cleaned-up according to the method proposed by BioDetection System (BDS)⁹.

Analysis : HRMS. PCDD/Fs and non-*ortho*-PCBs were measured on an AutospecUltima (Micromass) coupled to an Agilent 6890 Series GC. The column was a 50 m VF-5MS (0.20 mm ID x 0.33 µm df) (Varian). Mono-*ortho*-PCBs and indicator PCBs were measured on an MAT95XL (ThermoFinniganMAT) coupled to an Agilent 6890 Series GC. The column was a 25 m HT-8 (0.22 mm ID x 0.25 µm df) (SGE)⁸. **DR-CALUX.** It originated from BioDetection System (BDS, NL). Analyses were performed by exposing the cells (triplicates, 96-well plates) during 24h to sample extracts and to 2,3,7,8-TCDD standard solutions in DMSO diluted in culture medium. See previous report for details⁷.

Results

Correction of CALUX results with a reference sample

During the DIFFERENCE project, we evaluated the use of matrix-specific reference samples for raw DR-CALUX data correction. Each reference sample (milk, pork and fish) followed the entire matrix-specific sample preparation procedure. The ratio of the total TEQ (sum of PCDD/Fs and dioxin-like PCBs) concentration measured by GC-HRMS over the DR-CALUX result for the reference samples were used as correction factors applied to raw DR-CALUX data obtained for DIFF-01(fish), DIFF-02 (pork) and DIFF-03 (milk) samples. Both pork and fish reference samples had a congener profile that corresponded to a classical background contamination (similar to the pattern of the unknown samples) and good correlations between DR-CALUX-reference sample corrected values and GC-HRMS data were observed (Figure 1). The situation was not as good for milk because the pattern observed in the reference sample (PeCB-118 was unusually high) was different from a classical background congener distribution for milk and this influenced the raw data correction, as if a non-matrix specific reference sample had been used. The direct consequence led to an unexpected low recovery for the milk sample, inducing an over-estimation of the reference sample corrected CALUX data. This correction was compared to a correction using an internal QC of spiked beef fat (1.7 pg PCDD/F WHO-TEQ/ g fat and 1.4 DL-PCB WHO-TEQ/g fat). For pork and fish samples, Figure 1 shows that both the reference sample and the internal QC correction are efficient to improve the correlation with the HRMS results. In the case of milk, because of the reasons described above, only the correction with the internal QC improves this correlation.

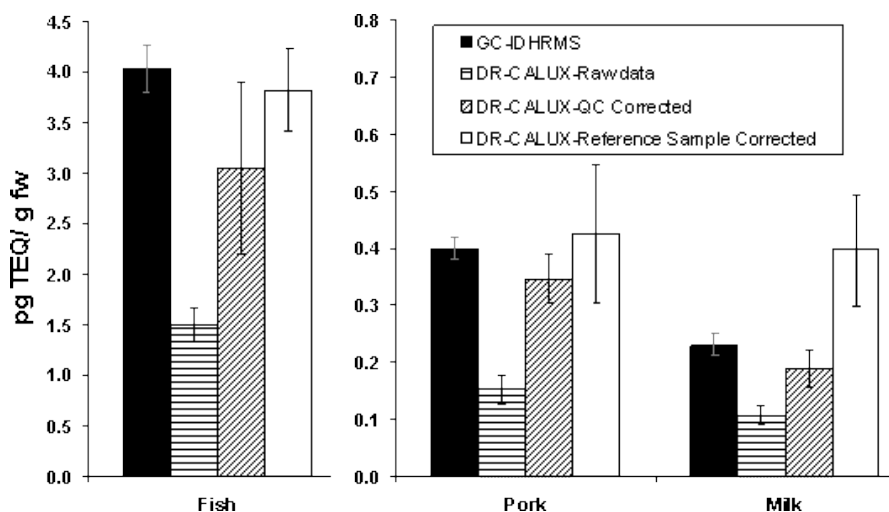


Figure 1 Raw and corrected responses of the DR-CALUX assay versus GC-HRMS for the investigated samples (DIFF-01(fish), DIFF-02 (pork), DIFF-03 (milk)).

DR-CALUX analysis of 51 food or feed samples

DR-CALUX and GC-HRMS results issued from the analysis of 51 food and feed samples are displayed in Table I, and illustrated in Figure 2 in more details for milk samples.

Apart from feed unknown samples, reference samples (REF sample), BCR-533 and internal quality controls (IQC), DR-CALUX results have been corrected using either a matrix specific reference sample or an internal QC sample (raw milk for unknown milk samples or spiked beef fat for the other samples). For GC-HRMS, the compliance is reported when “the analytical result exceeds the maximum level (of PCDD/F) beyond reasonable doubt, taking into account the measurement uncertainty”¹⁰. In practice, the measured value minus the estimated uncertainty (including additional parameters than just the RSDs issued from multiple measurements) must be above the regulation level for the sample to be declared non-compliant¹¹. For the DR-CALUX screening approach, high rates of false compliance (false negative) are avoided by deciding of “non compliance” (sample “to be confirmed”) when the measured value plus the estimated uncertainty (for samples in figure 2, the CV of at least a duplicate determination) is above the

PCDD/F action level (Figure 2).

Regulatory maximum^{1,2} and action¹² levels for PCDD/Fs as well as for the sum of PCDD/Fs and dioxin-like (DL)-PCBs (Total) are indicated in Table II. The value for 'Total action level' is set at 60% of the corresponding maximum level.

| Product | pg WHO-TEQ / g fat or product * | | | |
|--------------------------|---------------------------------|-------|--------------|-------|
| | MAXIMUM LEVEL | | ACTION LEVEL | |
| | PCDD/F | TOTAL | PCDD/F | TOTAL |
| PORK | 1 | 1,5 | 0.6 | 1 |
| MILK | 3 | 6 | 2 | 4 |
| FISH* | 4 | 8 | 3 | 5 |
| FISH OIL (food) | 2 | 8 | 1.5 | 5 |
| FEED* | 0,75 | 1,25 | 0.5 | 0.8 |
| ANIMAL FAT (feed) | 2 | 3 | 1.2 | 2 |
| FEEDING STUFF for FISH * | 2,25 | 7 | 1.5 | 4.7 |

Table II : PCDD/F^{1,2} and preliminary total (PCDD/F + DL-PCB)³ maximum levels. PCDD/F¹² and proposed total action levels (60% of the total maximum level) for feed and food samples.

Comparisons to these regulatory levels are made in Table I. The evaluation of the DR-CALUX screening efficiency was set as "true":

- 1) when samples declared "compliant" (indicated as "-") after screening were confirmed compliant by GC-HRMS, or,
- 2) when samples declared "to be confirmed" (suspicious) (indicated as "+") after screening were confirmed non-compliant, or,
- 3) when samples declared "to be confirmed" (suspicious) after screening were found to be compliant but > total (PCDD/F + PCB) maximum level by GC-HRMS, or,
- 4) when samples declared "to be confirmed" (suspicious) after screening were found to be compliant but > PCDD/F or total action level by GC-HRMS.

From Table I, the DR-CALUX screening efficiency was as following :

% of false compliant samples : 2%

% of false non compliant samples : 20%

% of true decision (compliant or non compliant) : 78%

Conclusions

Our evaluation of the use of the DR-CALUX for screening of food and feed samples show a very low rate of false negative (2% of false compliant, i.e. one sample out of 51 exceeded the total action level), as well as an acceptable rate of false non compliant of 20 %. However, results from GC-HRMS show that those 20% of false non compliant samples contain significant amounts of PCDD/F or DL-PCBs, and thus deserve consideration as indicator of background contamination of food and feed samples as recommended by the EC^{13,14}.

The DR-CALUX can be considered as a valuable low cost screening tool for the implementation of the PCDD/F level monitoring in food and feed. The upcoming extension of the European regulations to DL-PCBs will additionally reinforce this position.

Acknowledgements

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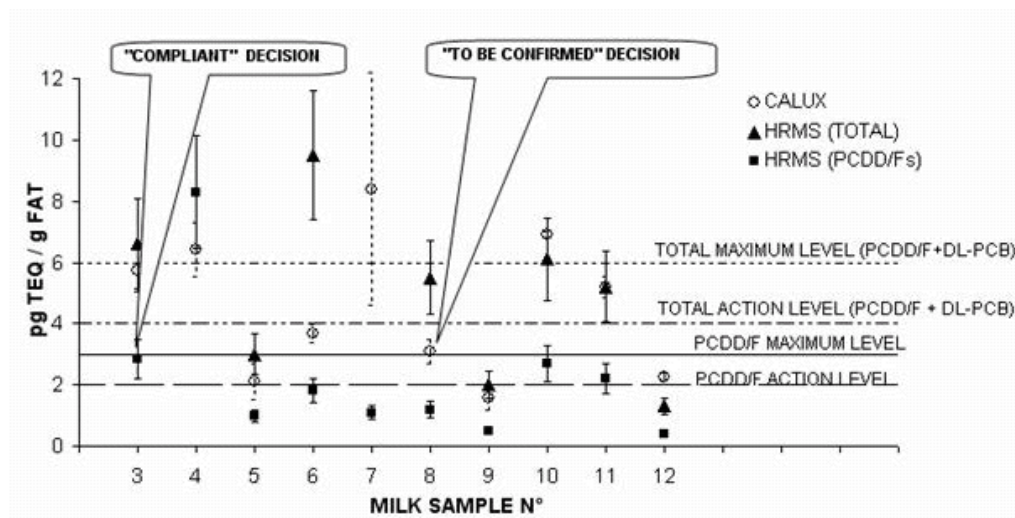


Figure 2 : Comparison of DR-CALUX and GC-HRMS analysis of milk samples. DR-CALUX : mean \pm standard deviation (at least duplicate determination). GC-HRMS : the uncertainty has been estimated to 22%.

References

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[12] COMMISSION RECOMMENDATION 2002/201/EC of 4 March 2002 on the reduction of the presence of dioxins, furans and PCBs in feedingstuffs and foodstuffs.

[13], [14] COMMISSION RECOMMENDATION 2004/704/EC (2004/705/EC) of 11 October 2004 on the monitoring of background levels of dioxins and dioxin-like PCBs in feedingstuffs (foodstuffs).

[15], [16] COMMISSION DIRECTIVE 2002/69/EC (2002/70/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 (feeding stuffs).

| Product | Sample # | TOTAL DR-CALUX-TEQ | DR-CALUX SCREENING Conclusion | PCDD/F WHO-TEQ (HRMS) | HRMS Conclusion (PCDD/F WHO-TEQ) | PCDD/F + PCB WHO-TEQ (HRMS) | HRMS Conclusion (PCDD/F + PCB WHO-TEQ) | DR-CALUX SCREENING EVALUATION | |
|-----------------------|-----------------|--------------------|-------------------------------|-----------------------|----------------------------------|-----------------------------|--|-------------------------------|---------------------|
| | | pg/g fat | | pg/g fat | | pg/g fat | | | |
| PORK | DIFF-02 | 1 2.08 | + | 1.54 | + | 2.00 | + | TRUE | |
| | REF SAMPLE | 2 0.52 | + | 0.76 | -* | 1.14 | - | TRUE | |
| MILK | DIFF-03 | 3 5.74 | + | 2.84 | + | 6.61 | -** | TRUE | |
| | BCR-633 | 4 6.41 | + | 8.30 | + | | | TRUE | |
| | IQC | 5 2.10 | + | 0.98 | - | 3.00 | - | FALSE NON COMPLIANT | |
| | UNKNOWN | 6 3.68 | + | 1.80 | - | 9.50 | + | TRUE | |
| | UNKNOWN | 7 8.40 | + | 1.10 | - | 19.00 | + | TRUE | |
| | UNKNOWN | 8 3.09 | + | 1.20 | - | 5.50 | -** | TRUE | |
| | UNKNOWN | 9 1.57 | - | 0.50 | - | 2.00 | - | TRUE | |
| | UNKNOWN | 10 6.92 | + | 2.70 | -* | 6.10 | -** | TRUE | |
| | UNKNOWN | 11 5.18 | + | 2.20 | -* | 5.20 | -** | TRUE | |
| | UNKNOWN | 12 2.27 | + | 0.40 | - | 1.30 | - | FALSE NON COMPLIANT | |
| | FISH OIL (Food) | DIFF-04 | 13 10.95 | + | 5.46 | + | 11.00 | + | TRUE |
| | | UNKNOWN | 14 47.22 | + | 6.94 | + | 43.32 | + | TRUE |
| UNKNOWN | | 15 43.91 | + | 6.91 | + | 42.27 | + | TRUE | |
| UNKNOWN | | 16 49.45 | + | 6.72 | + | 42.50 | + | TRUE | |
| UNKNOWN | | 17 33.69 | + | 6.85 | + | 42.22 | + | TRUE | |
| UNKNOWN | | 18 53.73 | + | 6.97 | + | 43.06 | + | TRUE | |
| UNKNOWN | | 19 32.76 | + | 6.93 | + | 42.18 | + | TRUE | |
| UNKNOWN | | 20 34.56 | + | 6.99 | + | 37.70 | + | TRUE | |
| UNKNOWN | | 21 34.47 | + | 13.85 | + | 82.04 | + | TRUE | |
| UNKNOWN | | 22 < LOQ | - | 0.02 | - | 2.53 | - | TRUE | |
| UNKNOWN | | 23 3.37 | + | 0.09 | - | 12.80 | + | TRUE | |
| UNKNOWN | | 24 1.61 | + | 0.03 | - | 4.62 | - | FALSE NON COMPLIANT | |
| UNKNOWN | | 25 1.70 | + | 0.03 | - | 4.00 | - | FALSE NON COMPLIANT | |
| FISH | | DIFF-01 | 26 3.82 | + | 1.89 | - | 4.03 | - | FALSE NON COMPLIANT |
| | | REF SAMPLE | 27 0.63 | - | 0.81 | - | 1.59 | - | TRUE |
| | UNKNOWN | 28 1.03 | - | 1.80 | - | 6.70 | -** | FALSE COMPLIANT | |
| | UNKNOWN | 29 0.77 | - | 0.80 | - | 2.60 | - | TRUE | |
| | UNKNOWN | 30 1.05 | - | 1.20 | - | 4.70 | - | TRUE | |
| | UNKNOWN | 31 1.62 | - | 0.80 | - | 2.80 | - | TRUE | |
| | UNKNOWN | 32 2.09 | - | 1.40 | - | 3.80 | - | TRUE | |
| | UNKNOWN | 33 0.18 | - | < LOQ | - | 0.01 | - | TRUE | |
| | UNKNOWN | 34 0.55 | - | 0.04 | - | 0.14 | - | TRUE | |
| | FEED | DIFF-05 | 35 0.84 | + | 0.46 | - | 1.23 | -** | TRUE |
| | | UNKNOWN | 36 0.44 | + | 0.64 | -* | 0.64 | - | TRUE |
| UNKNOWN | | 37 0.83 | + | 0.68 | -* | 0.68 | - | TRUE | |
| UNKNOWN | | 38 0.51 | + | 0.63 | -* | 0.64 | - | TRUE | |
| UNKNOWN | | 39 < LOQ | - | < LOQ | - | < LOQ | - | TRUE | |
| UNKNOWN | | 40 0.61 | + | 0.16 | - | 0.35 | - | FALSE NON COMPLIANT | |
| UNKNOWN | | 41 1.09 | + | 0.88 | -* | 0.88 | - | TRUE | |
| UNKNOWN | | 42 7.13 | + | 5.56 | + | 5.56 | + | TRUE | |
| UNKNOWN | | 43 0.26 | - | < LOQ | - | < LOQ | - | TRUE | |
| UNKNOWN | | 44 0.38 | - | 0.03 | - | 0.06 | - | TRUE | |
| UNKNOWN | | 45 < LOQ | - | < LOQ | - | < LOQ | - | TRUE | |
| UNKNOWN | | 46 4.74 | + | 6.35 | + | 6.36 | + | TRUE | |
| FEEDINGSTUFF FOR FISH | UNKNOWN | 47 8.09 | + | 2.90 | + | 8.30 | + | TRUE | |
| | UNKNOWN | 48 1.37 | + | 0.61 | - | 2.11 | - | FALSE NON COMPLIANT | |
| | UNKNOWN | 49 1.55 | + | 0.84 | - | 2.26 | - | FALSE NON COMPLIANT | |
| ANIMAL FAT | UNKNOWN | 50 2.31 | + | 0.38 | - | 0.74 | - | FALSE NON COMPLIANT | |
| | UNKNOWN | 51 2.28 | + | 0.26 | - | 0.64 | - | FALSE NON COMPLIANT | |

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| | | | | | | | | |
|---------|-----|------|---|------|---|------|---|---------------------|
| UNKNOWN | 0.1 | 0.30 | + | 0.20 | - | 0.31 | - | FALSE NON-COMPLIANT |
|---------|-----|------|---|------|---|------|---|---------------------|

Table I : DR-CALUX and GC-HRMS results of the analysis of 51 food or feed samples. For compliance decision (see text), the GC-HRMS uncertainty has been estimated to 22% and the DR-CALUX uncertainty was estimated to 30%.

DR-CALUX : - : compliant (< PCDD/F action level) ; + : suspicious (> PCDD/F action level).

HRMS : - : compliant (< PCDD/F or PCDD/F + PCB maximum level); + : non compliant (> PCDD/F or PCDD/F + PCB maximum level); -* : compliant but > PCDD/F action level; -** : compliant but > total (PCDD/F + PCB) action level.