STRATEGY FOR DR-CALUX DIOXIN SCREENING IN FEED UNDER EC REGULATION

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Introduction

Since 2001, the European Commission set a legislation defining maximum levels of polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) in feed (and food) for 17 congeners[1]. Maximum level values for 12 additional congeners of dioxin-like polychlorinated biphenyls (DL-PCBs) have recently been added to the list and will enter in force soon (4th November 2006)[2].

The reference gas chromatography-isotope dilution high resolution mass spectrometry (GC-IDHRMS) methodology routinely 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 and identification or can be converted in toxic equivalent quantities (TEQs) based on the use of toxic equivalent factors (TEFs) to assess the overall toxicity[3]. Because the high quality GC-IDHRMS monitoring of dioxins in the food chain is time and resource consuming, alternative sample screening methods are needed. The DR-CALUX (Dioxin Response-Chemically Activated LUciferase gene eXpression) cell-based assay has widely been proposed to screen dioxin and dioxin-like compounds in food and feed samples. Compared to congener-specific GC-IDHRMS data, the CALUX AhR (Aryl hydrocarbon receptor)-activation mediated response directly yield to a TEQ estimation based on a correlation with 2,3,7,8-TeCDD induction of the assay.

A comparison between GC-IDHRMS and DR-CALUX results often shows discrepancies, partly due to differences between the WHO-TEF values and the CALUX REP (relative equivalent potency) values[4,5] measured for each of the PCDD, PCDF and DL-PCB congeners. In addition, although analyte recovery rates are taken into account for calculations in GC-IDHRMS, no correction for analytes loss based on internal standards is possible in a cell based assay.

Such differences make difficult the strict decision of compliance or suspicion of non compliance for samples submitted to biological screening. Another difficulty of the screening stage is to determine a limit of decision, allowing "to select those samples with levels of dioxins and DL-PCBs that are less than 30-40% below or exceed the level of interest"[6] but yielding to a rate of false negative decision lower than 1%[6]. Furthermore, the rate of false positive decision should be very low to ensure profitable use of the screening procedure (all samples suspected to be positive at the screening stage have to be tested by a confirmatory method, i.e., the GC-IDHRMS).

We show and evaluate here a DR-CALUX screening strategy for dioxin monitoring in feed samples, allowing correction of DR-CALUX raw data and a rate of false negative les than 1%. The level of contamination with PCDD/F and DL-PCB congeners of a large number of feed samples analyzed routinely by GC-IDHRMS is also presented.

Materials and methods

Samples are unknown samples issued from the routine monitoring activity (with GC-IDHRMS) of the laboratory of mass spectrometry. QC samples and method blanks (BCs) were regularly run for QA/QC purposes.

Extraction: For GC-IDHRMS analyses of PCDD/Fs and dioxin-like PCBs, samples were extracted and cleaned-up as already described[7]. For DR-CALUX analyses, samples were liquid-liquid (LLE) extracted and cleaned-up according to the method proposed by the manufacturer.

Analysis : GC-IDHRMS analysis of PCDD/Fs and DL-PCBs were performed as already described[7]. **DR-CALUX** originates from BioDetection System (BDS, NL). Briefly, samples extracts were cleaned-up manually using liquid chromatography on acidic silica columns. Final extracts were concentrated in DMSO prior to analysis. All details are available elsewhere[4].

Results are expressed as pg total TEQ/g, because no separation between PCDD/Fs and DL-PCBs is performed.

Results

Dioxin monitoring of feed samples using GC-IDHRMS

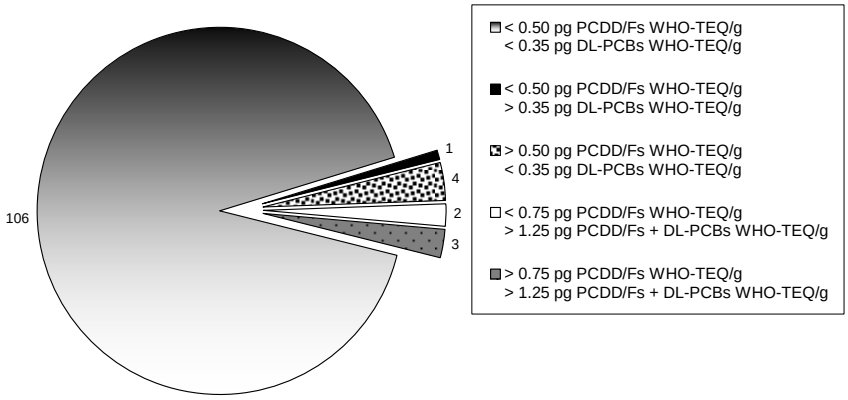

***Figure 1** : Distribution of results obtained from the monitoring of 116 feed samples (GC-IDHRMS method).*

Figure 1 shows results of dioxin (17 PCDD/Fs and 12 DL-PCBs) monitoring of 116 feed samples classified as "Feed materials of plant origin with the exception of vegetable oils and their by products"[1,2]. For these samples, PCDD/Fs and total (PCDD/Fs + DL-PCBs) WHO-TEQ maximal levels are respectively 0.75 and 1.25 pg/g, while separate action levels exist for PCDD/Fs and DL-PCBs[2], which are respectively 0.5 and 0.35 pg WHO-TEQ/g.

113 samples (97%) were below the maximum level for dioxins and furans, from which only two displayed levels above the maximum level for the sum of dioxins, furans and DL-PCBs.

From these 113 samples, 4 are above the PCDD/Fs action level and one is below the PCDD/Fs action level and slightly above the DL-PCBs action level. Only 3 samples (3%) were above both dioxin/furans and total maximum limits, but with a DL-PCBs contamination close to or below their action level.

DR-CALUX screening strategy

Correction of raw data

Usually, when comparing total TEQ levels (dioxins, furans and DL-PCBs) obtained for the same samples, CALUX measurements are lower than GC-IDHRMS. The main reason of this is that REP for DL-PCBs are lower than WHO-TEF[4,5]. Other causes such as loss of analytes during extraction and purification and antagonistic effects of some PCBs congeners can be mentioned.

As shown in a previous project (DIFFERENCE)[8], the use of reference samples to correct DR-CALUX results show a good improvement in the comparison of GC-IDHRMS and DR-CALUX measurement but, unfortunately, these kind of samples are not commercially available.

Compounds	WHO-TEF	DR-CALUX REP[4]	pg/g	pg WHO-TEQ / g	pg DR-CALUX-TEQ / g
2,3,7,8-TCDD	1	1	0.011	0.0110	0.0110
1,2,3,7,8-PeCDD	1	0.5	0.04	0.0400	0.0200
1,2,3,4,7,8-HxCDD	0.1	0.1	0.16	0.0160	0.0160
1,2,3,6,7,8-HxCDD	0.1	0.06	1.985	0.1985	0.1191
1,2,3,7,8,9-HxCDD	0.1	0.05	0.779	0.0779	0.0390
1,2,3,4,6,7,8-HpCDD	0.01	0.03	146.286	1.4629	4.3886
1,2,3,4,6,7,8,9-OCDD	0.0001	0.0005	742.237	0.0742	0.3711
2,3,7,8-TCDF	0.1	0.4	0.092	0.0092	0.0368
1,2,3,7,8-PeCDF	0.05	0.1	0.029	0.0015	0.0029
2,3,4,7,8-PeCDF	0.5	0.4	0.051	0.0255	0.0204
1,2,3,4,7,8-HxCDF	0.1	0.07	0.073	0.0073	0.0051
1,2,3,6,7,8-HxCDF	0.1	0.08	0.044	0.0044	0.0035
2,3,4,6,7,8-HxCDF	0.1	0.1	0.049	0.0049	0.0049
1,2,3,7,8,9-HxCDF	0.1	0.1		0.0000	0.0000
1,2,3,4,6,7,8-HpCDF	0.01	0.01	1.582	0.0158	0.0158
1,2,3,4,7,8,9-HpCDF	0.01	0.04	0.195	0.0020	0.0078
1,2,3,4,6,7,8,9-OCDF	0.0001	0.004	13.129	0.0013	0.0525
TOTAL PCDD/Fs			**907**	**1.95**	**5.11**
PCB 77	0.0001	0.0004	7.33	0.0007	0.0029
PCB 126	0.1	0.04	0.45	0.0446	0.0178
PCB 169	0.01	0.0008	0.08	0.0008	0.0001
PCB 81	0.0001	0.002	0.43	0.0000	0.0009
PCB 105	0.0001	0	28.64	0.0029	0.0000
PCB 114	0.0005	0.00002	2.36	0.0012	0.0000
PCB 118	0.0001	0	93.68	0.0094	0.0000
PCB 123	0.0001	0	2.72	0.0003	0.0000
PCB 156	0.0005	0.00002	12.51	0.0063	0.0003
PCB 157	0.0005	0	2.16	0.0011	0.0000
PCB 167	0.00001	0	6.76	0.0001	0.0000
PCB 189	0.0001	0	1.42	0.0001	0.0000
TOTAL dioxin-like PCBs			**159**	**0.1**	**0.02**
TOTAL			**1065**	**2.0**	**5.14**

***Table I** : GC-IDHRMS results of the "home made" feed QC sample.*

We propose here to correct DR-CALUX data using a "home made" quality control (QC) sample. That QC sample, which is a real contaminated feed material, previously used in an interlaboratory study[10], is incorporated in the series of unknown samples and analyzed accordingly. The GC-IDHRMS analysis of this samples showed a very high contamination with the 1,2,3,4,6,7,8-HpCDD congener, which displays a DR-CALUX REP 3 fold higher than its WHO-TEF (0.03 and 0.01 respectively) (table 1). The DR-CALUX measurement of this sample shows a level of 3.7 pg total TEQ/g, which is higher than the GC-IDHRMS measurement (2.0 pg total TEQ/g). This difference comes from the high contribution of the 1,2,3,4,6,7,8-HpCDD congener.

When using DR-CALUX REP instead of WHO-TEF to calculate the TEQ content, we find a calculated concentration of 5.1 pg calculated "DR-CALUX TEQ"/g (last column of Table 1).
This concentration is our reference concentration for the DR-CALUX measurement. The average DR-CALUX response found during the validation of the method was 72% of that DR-CALUX reference concentration (3.7 pg TEQ/g measured with DR-CALUX versus 5.1 pg "REP-calculated" TEQ/g). The resulting average multiplicative correction factor is 1.4. Because this QC contains very low levels of DL-PCBs, it allows correction of unknowns for recovery (analyte loss during extraction and purification steps) assuming that it is roughly the same for all congeners. Practically, the DR-CALUX result found for the unknown is multiplied by the correction factor, calculated from the result obtained for the QC sample analyzed in the same series than the unknown.

Limit of decision at the DR-CALUX screening stage

To calculate a CALUX decision limit allowing meeting the criteria of less than 1% of false negative set for screening techniques[7], we used a statistical approach. To determine the rate of false negative samples (true positive samples declared negative at the screening stage), we have first to define the limit at which a sample is declared positive. The non-compliance of a sample (true positive sample) is only declared at the confirmatory step (GC-IDHRMS) if its concentration is above the maximal limit taking into account the measurement uncertainty[10] (what we call here the GC-IDHRMS decision limit). Until now, regulatory limits exist for the TEQ concentration of PCDD/F congeners only (no DL-PCBs included). By November 4th 2006, regulatory limits for total (PCDD/Fs and DL-PCBs) TEQ concentrations will enter into force, but PCDD/Fs TEQ maximum limits will still be applicable until 31 December 2008[2].

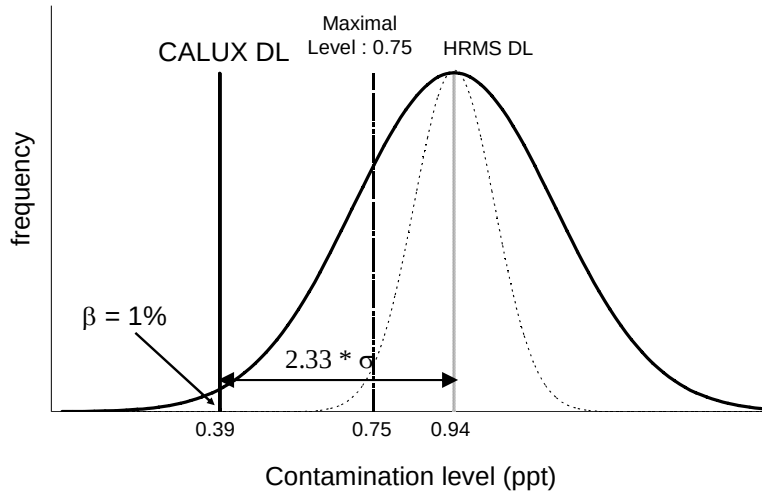

Figure 2 *: Distribution of the contamination measurement of a population of positives samples containing a PCDD/F WHO-TEQ corresponding to the GC-IDHRMS decision limit (i.e. the PCDD/F WHO-TEQ maximal level + the measurement uncertainity). DL : Decision limit. σ : standard deviation of the mean of the CALUX measurements calculated with a CV of 25%. β : beta error, percentage of positive samples below the CALUX decision limit (rate of false negative samples).*

For that reason, and to reduce the false negative rate as much as possible, we choose to calculate a CALUX decision limit that takes into account the lowest maximum limit (e.g. which is the PCDD/Fs TEQ maximum limit). In Figure 2, the dotted distribution corresponds to the expected GC-IDHRMS results for a population of positive samples, contaminated with a PCDD/F WHO-TEQ corresponding to the maximum legal limit plus the expanded uncertainty (we consider that an average expanded uncertainty of 20% is associated to a GC-IDHRMS measurement). The plain line distribution represents the expected DR-CALUX measurements for the same samples. In order to obtain less than 1% of false negative decision (which corresponds to the beta error) at the screening stage, the DR-CALUX decision limit is calculated as the inferior limit of the 99% unilateral confidence interval of a population of results characterized by a mean being equal to the GC-IDHRMS decision limit and a coefficient of variation (CV) of 25%, which is an average reproducibility CV of the DR-CALUX analysis (Figure 2). This CALUX decision limit has been calculated assuming that the recovery of the DR-CALUX method is 100 % (after correction).

Evaluation of the DR-CALUX screening

Table 3 shows results obtained with both DR-CALUX and GC-IDHRMS methods when analyzing real feed samples. From the 26 samples analyzed, only 3 are above the GC-IDHRMS decision limit for the PCDD/Fs TEQ content. As mentioned in the European Legislation, the screening has to detect samples 30% to 40% below the maximal level, so we evaluated the CALUX screening decision by comparing the number of samples above the DR-CALUX decision limit to the number of samples above 60% of the regulatory limit. Ten samples are above 60% of both the PCDD/Fs and the total (PCDD/Fs + DL-PCBs) WHO-TEQ maximum limits, all detected with the DR-CALUX screening, and 2 samples are above the total TEQ maximal limit only, also detected with the DR-CALUX screening. From the 15 remaining negative samples, four are detected as suspicious after the DR-CALUX screening and are thus false positive.

	pg DR-CALUX-TEQ/g product	DR-CALUX SCREENING Conclusion	pg PCDD/F WHO-TEQ/g product (HRMS)	pg PCDD/F + PCB WHO-TEQ/g product (HRMS)	PCDD/F WHO-TEQ (HRMS) > maximal level - 40% ?	PCDD/F + PCB WHO-TEQ (HRMS) > maximal level - 40% ?	DR-CALUX SCREENING EVALUATION
1	0.19	-	0.11	0.23	-	-	TRUE
2	0.19	-	0.11	0.23	-	-	TRUE
3	0.19	-	0.11	0.22	-	-	TRUE
4	0.19	-	0.11	0.22	-	-	TRUE
5	0.19	-	0.11	0.22	-	-	TRUE
6	0.19	-	0.11	0.22	-	-	TRUE
7	0.19	-	0.11	0.28	-	-	TRUE
8	0.19	-	0.11	0.22	-	-	TRUE
9	0.19	-	0.11	0.23	-	-	TRUE
10	0.50	+	0.11	0.23	-	-	FALSE
11	0.19	-	0.11	0.23	-	-	TRUE
12	0.75	+	0.12	0.25	-	-	FALSE
13	0.19	-	0.13	0.23	-	-	TRUE
14	0.46	+	0.20	0.36	-	-	FALSE
15	1.42	+	0.21	0.40	-	-	FALSE
16	0.51	+	0.37	0.91	-	+	TRUE
17	0.94	+	0.44	0.86	-	+	TRUE
18	1.67	+	0.46	1.23	+	+	TRUE
19	0.44	+	0.64	0.64	+	-	TRUE
20	2.24	+	0.67	0.79	+	+	TRUE
21	1.73	+	0.70	1.42	+	+	TRUE
22	0.65	+	0.71	0.84	+	+	TRUE
23	1.05	+	0.72	0.84	+	+	TRUE
24	2.45	+	0.95	1.07	+	+	TRUE
25	13.34	+	5.64	5.76	+	+	TRUE
26	5.10	+	6.42	6.55	+	+	TRUE

Table 3 *: DR-CALUX (pg DR-CALUX TEQ/g product, QC corrected) and GC-IDHRMS (HRMS) results of the analysis of 26 feed samples. The DR-CALUX screening conclusion is + if the DR-CALUX measured level is above the DR-CALUX decision limit.*

Conclusions

We have developed a strategy to screen feed samples with the DR-CALUX cell-based assay, with a rate of less than 1% of false negative decisions and a "home made" QC sample to correct for recovery. Even if this QC sample does not allow a correction for the difference between REP and TEF, it seems to work well to detect those samples containing a high level of DL-PCBs (samples 16 to 26 of Table 3).

We can conclude that the strategy shown here is good to screen samples in a situation of routine monitoring where a high rate of samples (more than 90%) are negative (compliant with both PCDD/Fs and total (PCDD/Fs + DL-PCBs) WHO-TEQ maximum levels) and a very low rate are non compliant to the total WHO-TEQ maximum level while compliant to the PCDD/Fs one. The congener profile found in the compliant samples corresponds to the background contamination.

On the contrary, in a crisis situation, the congener pattern found in samples depends of the origin of the contamination (such as for example in the Belgian dioxin crisis in feed at the begin of 2006, where feed was contaminated with specific congeners). It would be then interesting to prepare a dedicated reference sample displaying the same congener profile as the one expected in contaminated samples to be used for DR-CALUX correction.

References

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