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EVALUATION OF THE DR-CALUX BIOASSAY FOR THE DETERMINATION OF DIOXINS IN FOOD AND FEED ACCORDING TO THE REQUIREMENTS OF THE COMMISSION DIRECTIVES 2002/69 AND 2002/70 RESPECTIVELY.

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Introduction

Although European Commission directives 2002/69 and 2002/70 allow the use of screening methods for the monitoring of dioxins in food or feed samples, dioxin analyses are directly performed using gas chromatography coupled to high resolution mass spectrometry (GC-HRMS) in most laboratories. Screening based on GC-low resolution MS (GC-LRMS such as GC-ion trap MS/MS) is not yet applicable, due to a lack of sensitivity for food and feed samples.
Cell-based assays seem to be very interesting methods for the screening : it is possible to analyse a large number of samples and these methods are cheaper than MS-based methods.

Although cell-based assays could be sensitive enough considering the presently tolerable limits in food (Regulation N° 2375/2001 of the European Council) and feed (directive 2001/102 of the European Council), their lack of specificity make their use rather difficult in control laboratories. Indeed, until now, tolerable levels for dioxins are expressed in TEQ calculated from the concentrations of 17 PCDD/F congeners and do not take into account dioxin-like PCBs. Cell-based assays are sensitive to all dioxin-like compounds able to activate the Ah receptor. Therefore, samples, considered as positive after a cell-based screening assay, can become negative after the GC-HRMS confirmatory step if the first positive result is mostly due to PCBs dioxin-like, for example. In other words, the percentage of false positive samples can be very high. False negative samples may also occur, due to possible toxic or antagonistic effects of some compounds in the sample.Therefore, the use of cell-based assays as screening methods in control laboratories has to be evaluated.

We present here and compare results obtained from the analysis of three kinds of samples (cod liver, feed and spiked beef fat) by both GC-HRMS and cell-based assay methods. The parameters of the cell-based assay are evaluated according to the criteria of the Commission directives 2002/69 and 2002/70.

Materials and methods

The cell-based assay used here was the DR-CALUX developed by Bio Detection System[1].
Extraction of samples: fat from cod liver has been extracted with hexane using an ASE (Accelerated Solvent Extraction) and feed samples have been extracted by a shaken solvent extraction method. The clean-up was performed on an acidic silica column and dioxins were eluted with hexane. After hexane evaporation, residues were dissolved in DMSO.
DR-CALUX determination was performed by exposing the cells (in 96 wells plates) to sample extracts or to standard TCDD solutions in DMSO during 24h before cell lysis, substrate addition

and luminescence determination. All determinations were made in triplicate. A standard calibration curve was established on each 96 wells plate.
GC-HRMS analyses have been performed as already described[2].

Results and discussion

Both 2002/69 and 2002/70 directives require the application of the same specific criteria for cell-based assays:

- every test run requires a series of reference concentrations of TCDD (full dose-response curve with a R^2>0.95).
- a TCDD reference concentration (about 3 x LOQ) should be recorded on a quality control sheet over the time period of the assays.
- Quality control (QC) charts should be recorded for reference material.
- The induction of the sample dilution must be within the linear portion of the response curve.
- The percent standard deviation should not be above 15% in a triplicate determination and not above 30% between three independent experiments.
- The limit of detection (LOD) may be set as 3x the standard deviation of the solvent blank. The limit of quantification (LOQ) may be set as 5x to 6x the standard deviation of the solvent blank.

An example of **calibration curve** obtained with the DR-CALUX method is illustrated in figure 1.

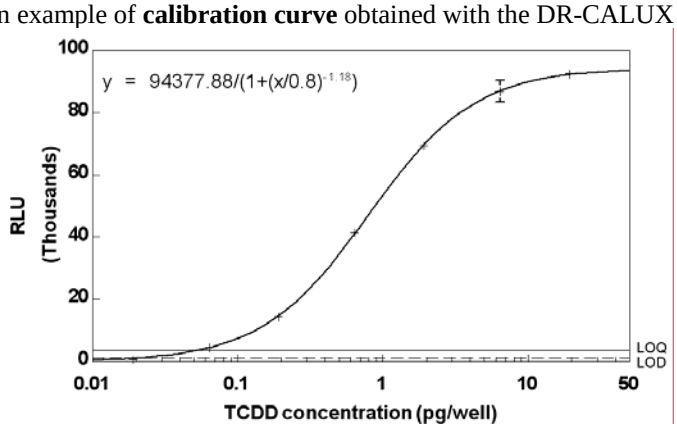

Figure 1: representative calibration curve obtained with the DR-CALUX assay. The dotted line corresponds to the LOD and the full line corresponds to the LOQ.

Mean values of various parameters (R^2, EC50, TCDD reference concentration, …) calculated from 22 curves are reported in table I. LOD and LOQ were calculated as indicated above: 3x and 6x the standard deviation of the solvent blank, respectively.
These results show that all the requirements of the 2002/69 and 2002/70 directives indicated above are met.

For fat samples, from the LOD and LOQ shown in table I, expressed in pg TCDD/well, we can calculate the LOD and LOQ expressed in pg TEQ/g fat: LOD = 0.63 pg TEQ/g fat and LOQ =1.56 pg TEQ/g fat.

Table I: Mean values of various parameters obtained from 22 standard calibration curves. Ranges are indicated between brackets.

R^2	EC50 (pg TCDD/ well)	Standard TCDD (0.18 pg/well)	Standard deviation (%) (calculated on triplicates)*	LOD (pg TCCD/ well)	LOQ (pg TCDD/ well)
0.99 (0.98-0.99)	0.63 (0.38-1.08)	0.19 (0.17-0.22)	5.6 (0.5-11.9)	0.02 (0.01-0.03)	0.05 (0.02-0.08)

** The mean standard deviation indicated is the mean of the 22 standard deviations obtained for the triplicate determination of the 0.18 pg/well standard TCDD amount.*

Tables II shows results of DR-CALUX determinations compared to those of GC-HRMS analyses of two **beef fat samples** spiked with PCDD/F and PCB-dioxin like congeners.

Table II: DR-CALUX and HR-MS analysis of spiked beef fat samples.

	DR-CALUX	HRGC-HRMS		
	pg TEQ/g fat	pg PCDD/F TEQ / g fat	pg Dioxin-like PCBs TEQ/g fat	Total pg TEQ/g fat
Sample 1	5.59 ± 28 % (n= 8)	4.81 ± 10.7 % (n=81)	6.84 ± 11.5 % (n=81)	11.65 ± 8 % (n=81)
Sample 2	10.56 ± 28 % (n=10)	10.89 ± 5.6 % (n=28)	0.92 ± 10.5 % (n=28)	11.81 ± 5.2 % (n=28)

Using DR-CALUX, 8 and 10 independent experiments were performed respectively for the analysis of sample 1 and sample 2 (table II). In both cases the reproducibility was 28%, meeting the requirements of European directives 200/69 and 2002/70.

In table II, if we compare DR-CALUX results with GC-HRMS results, we observe that the dioxin concentration measured with DR-CALUX is, in both cases (sample 1 and sample 2), very close to the concentration expressed in PCDD/F TEQ/g fat measured by GC-HRMS. So, although both samples display almost the same total TEQ concentration (sum of PCDD/F and dioxin-like PCBs TEQ) when measured by GC-HRMS, different results were obtained with DR-CALUX, the results being the same with both methods only for the sample displaying a very high percentage of PCDD/F.

For the analysis of **cod liver** with DR-CALUX, we can calculate the following LOD and LOQ: LOD = 0.78 pg TEQ/g fresh weight and LOQ = 1.95 pg TEQ/g fresh weight.

Table III compares DR-CALUX results for one cod liver sample analyzed during an inter-calibration study[3] with the GC-HRMS measured concentration reported in that study.

Table III: DR-CALUX and HR-MS analysis of a cod liver sample.

	DR-CALUX	HRGC-HRMS[3]		
	pg TEQ/g fresh weight	Total PCDD/F TEQ (pg/g fresh weight)	Dioxin-like PCBs TEQ (pg/ g fresh weight)	Total TEQ (pg/g fresh weight)
Cod liver sample	24.3 ± 11% (n=3)	4.92	22.26	27.18

Compared to the HR-MS measured TEQ concentration reported in the intercalibration exercise, our DR-CALUX measured result is close to the total TEQ value, even if the percentage of dioxin–like PCBs congeners is higher than that of the PCDD/F congeners.

The mean DR-CALUX concentration reported in the intercalibration study was 17 pg TEQ/g fresh weight (n = 5, including a very low result of 1.8 pg TEQ/g fresh weight).

For **feed sample** analysis, calculated LOD and LOQ were the following: LOD = 0.07 ng TEQ/Kg and LOQ = 0.17 ng TEQ/Kg.

A reference feed material[4], for which a consensus mean value on TEQ was established by an intercalibration exercise, was also analysed and compared by both techniques (table IV).

Table IV: DR-CALUX and HR-MS analysis of a reference feed material.

DR-CALUX			
	pg TEQ/g fat		RSD_r (%) (Repeatability)
Experiment 1	2.73		30 % (n = 4)
Experiment 2	1.44		24 % (n=4)
Experiment 3	1.67		5 % (n = 3)
Experiment 4	1.99		15 % (n=2)
Mean	**1.96**		
RSD R (%) Reproducibility	29 %		
GC-HRMS analysis			
	Total PCDD/F TEQ	PCBs Dioxin-like TEQ	**Total TEQ**
Feed sample	1.95 ng / Kg	0.07 ng / Kg	**2.02 ng / Kg**

It is clear from table IV that the RSD for repeatability ranges between 5 and 30 %, the maximum tolerated RSDr, while the RSD for reproducibility, for 4 independent experiments, is 29 %.

Conclusions

The evaluation of 22 calibration curves (table I) indicates that the specific requirements for cell-based bioassays of both 2002/69 and 2002/70 European directives are met.

With the experimental conditions used here, the LOQ for dioxin determination in fat is about 1.5 pg TEQ/g fat. Although this LOQ is low enough to analyse the samples described here (spiked beef fat and cod liver), this is too high if we consider the maximum permitted levels set in the Regulation n° 2375 (2001), which are 1 pg TEQ/g pork fat, 2 pg TEQ/g poultry fat and 3 pg/g beef fat. The LOQ could be lowered by increasing the amount of fat (1g instead of 0.5 g) and/or the proportion of DMO extract in the medium (1% instead of 0.4 %).
LOQ for feed samples (0.17 ng TEQ/Kg) is low enough compared to the maximum permitted level of 0.75 ng TEQ/Kg set in feed (directive 2001/102 of the European Council).
These preliminary results of comparing HR-MS results with cell-based assays results have to be confirmed on a large number of different kinds of samples.

References

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