Comparison of the results obtained by CALUX bioassay and GC-HRMS for different matrices.

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Introduction

The reference method used to analyse polychlorodibenzo-p-dioxins (PCDDs), polychlorodibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (dl-PCBs) is gas chromatography with high resolution mass spectrometry (GC-HRMS).
It is interesting to check the suitability of screening methods that are faster and less expensive.
Different matrices (milk, fish oil, chicken compound feed, pork tissue, chicken tissue, sepiolitic clay, whole egg and herring tissue) were analysed in the frame of the European project DIFFERENCE[1]. One of the aims of this project is to optimise screening methods. The CALUX bio-assay was one of the screening techniques used.
This paper presents the extraction and purification methods used for the analyses. The CALUX results for dioxins and for dl-PCBs were compared to the corresponding GC-HRMS results.

Methods and Materials

Sample preparation
The extraction of the milk samples was done by liquid-liquid extraction. Fifty mL of milk was extracted with 180 mL acetone and 60 mL hexane. An aqueous solution of sodium sulfate (100 mL, 2%) was added to wash the organic layer. The upper phase was dried through a sodium sulfate column. After evaporation of the solvent, the amount of fat was determined gravimetrically.
The extractions of the fat from pork tissue, chicken tissue, whole egg were performed with hexane using the Dionex ASE 200 extractor (Accelerated Solvent Extraction). The samples were lyophilised during 24 to 48 hours. The freeze-dried samples were mixed with sodium sulfate and inserted in the 33 mL cells of the Dionex ASE 200 extractor. Two static cycles of 5 minutes were performed under 125°C and 1500 PSI. After evaporation of the solvent, the amount of fat was determined gravimetrically.

The chicken compound feed and sepiolitic clay were extracted by shaking during 2 hours with toluene/methanol 20/4.5 (v/v) and after filtration over a glass wool filter the latter was rinsed with hexane.
No extraction was needed for fish oil.
For the purification, aliquots of maximum 1.5 g fat were dissolved in hexane and passed through an acid silica column and an activated Carbon Column. After elution, the acid silica column was discarded. The Carbon Column was washed with hexane/acetone 9/1 (v/v), the dioxin-like PCB fraction was eluted with hexane/toluene/ethyl acetate 8/1/1 (v/v) and afterwards the dioxin fraction was eluted with toluene. Toluene was evaporated and the samples were diluted in hexane.

CALUX bio-assay
CALUX analyses were performed using the mouse hepatoma H1L6.1 cell line developed by Xenobiotic Detection System (Durham, US).[2,3]
Hexane extracts were added to DMSO. The hexane was evaporated and cell culture medium was added.
The cells were exposed to the purified extracts in 96-well plates during 20 to 24 hours in an incubator at 37°C and 5% CO_2. On each plate, 10 standard solutions of 2,3,7,8-TCDD (50000 fg/well, 25000 fg/well, 12500 fg/well, 6250 fg/well, 3125 fg/well, 1563 fg/well, 781 fg/well, 391 fg/well, 195 fg/well, 98 fg/well) were added for the calibration curve. After incubation, the cells were lysed and the amount of luciferase produced by the cells was determined by addition of the substrate luciferine. The light emission was measured with a luminometer. This value was reported on the TCDD calibration curve and translated in bio-assay TEQ value.

GC-HRMS
The GC-HRMS results used in this paper were performed by VITO. A description of the procedure for fatty matrices is given elsewhere[4]. Feed and related samples were soxhlet extracted with hexane/acetone 2/1 (v/v) during 8 h, after addition of ^{13}C-labelled internal standards.

Results and Discussion

All results were obtained by independent extraction and purification steps.
The results obtained for the dioxin fraction are shown in table 1.

Table 1: Results obtained for the dioxin fraction (pg TEQ/g fat, excepted for chicken compound feed and sepiolitic clay: pg TEQ/ g product) by CALUX and GC-HRMS

Sample	CALUX mean (pg TEQ/g fat or product*)	Relative standard deviation (%) and number of analyses	GC-HRMS mean (pg TEQ/g fat or product*)	Relative standard deviation (%) and number of analyses
Milk	7,88	24% (n=6)	3,97	2% (n=6)
Fish oil	10,65	28% (n=6)	5,49	5% (n=6)
Chicken compound feed	0,82*	10% (n=6)	0,81*	1% (n=6)
Pork tissue	1,65	26% (n=6)	0,98	7% (n=6)
Chicken tissue	2,47	12% (n=2)	2,55	8% (n=2)
Sepiolithic Clay	0,57*	21% (n=2)	0,34*	13% (n=2)
Egg	3,75	8% (n=2)	3,25	2% (n=2)
Herring tissue	2,51	27% (n=2)	0,90	3% (n=2)

As can be seen in Table 1, the values for the dioxin fraction obtained with CALUX are higher than or nearly the same (for chicken compound feed, chicken tissue and sepiolitic clay) as the values obtained by GC-HRMS. This can be due to other compounds that bind to the Ah receptor. Indeed, the CALUX results represent the TEQ value of the dioxin fraction in which other compounds than the 17 PCDD/F can be present and interact with the Ah receptor. On the other hand, the GC-HRMS results represent the TEQ value for the 17 PCDD/F congeners.
The standard deviation is higher for CALUX (maximum value 28%) than for GC-HRMS but this value is not above the 30% as required by the European commission directives 2002/69 and 2002/70. It must be noted that for the four last matrices in the table, there are only 2 values.

The results obtained for the dioxin-like PCBs fraction are shown in table 2.

Table 2: Results obtained for the PCB fraction (pg PCB TEQ/g fat or product*) by CALUX and GC-HRMS

Sample	CALUX mean (pg PCB TEQ/g fat or product*)	Relative Standard deviation (%) and number of analyses	GC-HRMS mean (pg PCB TEQ/g fat or product*)	Relative Standard deviation (%) and number of analyses
Milk	1,74	38% (n=6)	10,09	2% (n=6)
Fish oil	1,86	67% (n=6)	5,54	13% (n=6)
Chicken compound feed	0,22*	27% (n=6)	0,84*	2% (n=6)
Pork tissue	0,43	65% (n=6)	0,60	4% (n=6)
Chicken tissue	0,46	62% (n=2)	3,00	21% (n=2)
Sepiolithic Clay	0,07*	85% (n=2)	0,03*	0% (n=2)
Egg	0,37	5% (n=2)	3,45	2% (n=2)
Herring tissue	0,17	51% (n=2)	1,12	1% (n=2)

Table 2 shows that all the values (pg PCB TEQ/g fat) obtained with CALUX are much lower than the corresponding values obtained by GC-HRMS. The lower PCB TEQ values measured by CALUX can be explained by lower REP values[3], antagonistic effects[5] and losses during extraction and purification.

The relative standard deviation is much higher for CALUX than for GC-HRMS. The relative standard deviations are higher than 30% excepted for chicken compound feed and egg.

These high relative standard deviations can be due to the low values measured (in the lower part of the calibration curve, under the LOQ for some samples).

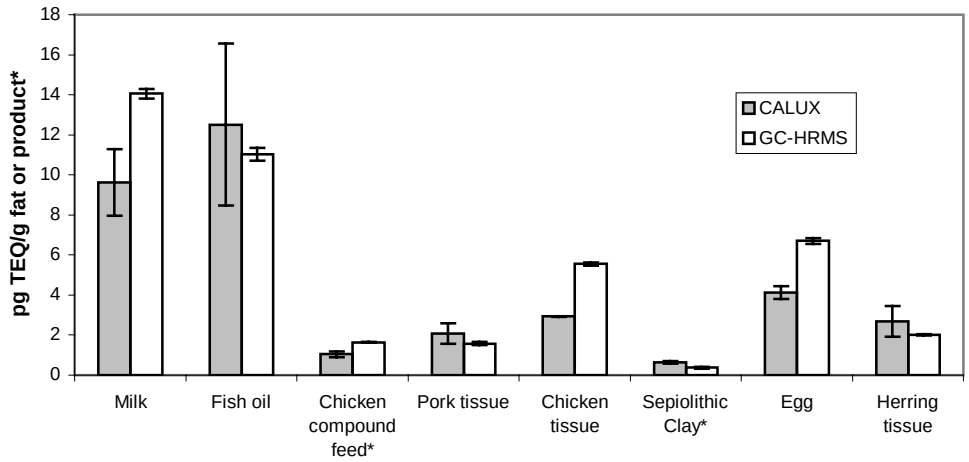

Figure 1: Comparison of the CALUX (sum of the results for the dioxin and PCB fractions) and GC-HRMS total TEQ results (pg total TEQ/g fat or product) for the different matrices.

The sum of the CALUX results for the dioxin fraction and those for the dl-PCB fraction is represented in Figure 1. This sum is compared to the sum of the PCDD/F TEQ and PCB TEQ obtained by GC-HRMS. On this figure, we can see that the results obtained by the two techniques are closer. The reason therefore is that the overestimation by CALUX for the dioxin fraction is compensated by the underestimation for the dl-PCB fraction.

For the dioxin fraction, the risk on false negative results is low due to the overestimation by CALUX. On the other hand, for the PCB fraction, the risk is very high because the CALUX results are largely under the GC-HRMS results. Calculating the sum of the two fractions, we can not avoid false negative results because the underestimation for the PCB fraction by CALUX is greater than the overestimation for the dioxin fraction for some matrices.
To avoid this kind of problem, the results should be corrected by a conversion factor as suggested by Besselink et al.[6] or with reference sample. It is suggested that reference samples should be similar in matrix and contaminants' profile. Obviously, it will be very difficult to find a "perfect" reference sample. The extraction and purification should also be improved to reduce losses.
The CALUX results presented here were only corrected for extraction and purification recovery using ^{14}C 2,3,7,8-TCDD.

Acknowledgements

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References

1 http://www.dioxins.nl/difference/
2 Tsutsumi T., Amakura, Y., Nakamura M., Brown D., Clark G., Sasaki K., Toyoda M., Maitani T. (2003) Analyst 128, 486.
3 Brown D., Chu M., Van Overmeire I., Chu A., Clark G. (2001) Organohalogen Compounds, 53, 211.
4 Schoeters G., Goyvaerts M.-P., Ooms D., Van Cleuvenbergen R. (2004) Chemosphere 54, 1289.
5 Windal I., Schroijen C., Van Wouwe N., Carbonnelle S., Van Overmeire I., Brown D., Clark G., Baeyens W., Goeyens L. (2003) Organohalogen Compounds, 60, 239.
6 Besselink H., Arjen J., Pijnappels M., Swinkels A., Fjellanger K., Brouwer B. (2003) Organohalogen Compounds, 60, 203.