Evaluation of the XDS-CALUX Assay for the analysis of Dioxins and Dioxin-like PCB's in Milk Powders and Butterfat according to EU Regulations

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Introduction

Food products sold in the European Union (EU) must comply with the following EU regulatory framework. Firstly, the EU regulation 2375/2001 sets maximum levels of polychlorodibenzodioxins (PCDD) and polychlorodibenzofurans (PCDF) in foodstuffs[1]. For milk and milk products the level is set at 3 pg PCDD/F-TEQ/g of fat based on the WHO TEF values. It should also be mentioned that this level does not apply to milk products that contain less than 1% fat content. Furthermore, method requirements for sampling and analysis of dioxins and dioxin-like PCB's in food and feed must comply with EU commission directives 2002/69/EC and 2002/70/EC respectively.[2,3] Provisions are provided for the monitoring foodstuffs along a strategy involving a screening method with confirmation carried out by a quantitative confirmatory method on samples whose results are less than 30-40% below or above the level of interest. Furthermore, minimum and strict requirements are established for screening and confirmatory methods respectively. For example, coefficient of variability CV must be < 15% for a confirmatory method and < 30% for a screening method. These directives also distinguish between between pure screening and quantitative screening methods of analysis. For both approaches, false negatives must be less than 1%. Traditionally, high resolution gas chromatography coupled to high resolution mass spectrometry (HRGC/HRMS) is the technique of choice for the determination of dioxins and dioxin-like PCB's. Since the discovery of the Aryl hydrocarbon receptor, bioassays based on this have made important advances making them an interesting technique as cost and time effective screening tools.[4] This purpose of this paper evaluates the performance of the XDS-CALUX Assay with respect to EU requirements for the analysis of milk powders and butterfat. HRGC/HRMS is used as the reference method for comparison.

Methods and Materials

Samples were chosen from representative raw materials of mainly eastern European countries. With reference to table 1, samples nos. 1,2, 16, 19 were butterfats; sample nos. 3 to 15 and 17, 18 were Full Cream Milk Powders and no. 20 was a Skim Milk Powder. In all 20 samples were sent to two accredited laboratories, XDS USA and Micropolluants Technologies France for analysis.

XDS-CALUX® Assay:

The XDS-CALUX (Chemical-Activated Luciferase Expression) assay is based on a genetically engineered cell line that contains the firefly luciferase gene under trans-activational control of the aryl hydrocarbon receptor. This cell line can be used for the detection and relative quantification of AhR agonists such as TCDD dioxin. Results from the CALUX assay provide a measure of TEQs in a sample. Sample processing separates polychlorinated biphenyls from chlorinated dioxins/dibenzofurans making it possible to determine what portion of the total TEQ in a sample is due to each of these classes of compounds. Samples stored at –20° C, were analyzed for PCDD/F and PCB TEQ activity. 10 grams of sample were transferred to hexane-rinsed glass vials with a PTFE-lined cap and were shaken and extracted three times with an acetone/hexane mixture. The three extracts were pooled and evaporated to dryness under nitrogen, and the remaining residue weighed to determine organic extractable lipid. The residues of extracts were re-suspended in hexane and processed for the bioassay using an XDS proprietary clean-up procedure. The sample extracts in DMSO were suspended in cell culture medium, just prior to dosing on monolayers of H1.6.1 mouse hepatoma cells that were grown in 96-well culture plates. Sample extracts and standards were applied to genetically engineered cells and induction of luciferase activity quantified. An estimate of dioxin-TEQ contamination of the sample was estimated from a least squares best fit using a four variable Hill Equation of

induction of luciferase activity from the standard curve of 2,3,7,8-tetrachlorodibenzo-p-dioxin. The response of the sample was analyzed and compared to associated method blanks. Quality control samples were included with each analysis.

HRGC/HRMS Analysis:

The complete description of the sample preparation and analytical procedure was published in a previous study.[5] Samples were spiked with 16 isotopically labeled dioxins and furans. Samples were extracted successively with methanol, diethyl ether and petroleum ether. The organic phase was collected. The combined organic extract was evaporated to dryness, and the fat content determined gravimetrically. The fat was then redissolved in n-hexane and cleaned-up according to the liquid chromatographic procedures described in the US EPA Method 1613. Prior to HRGC/HRMS analysis, purified extracts were reconstituted by adding 20µl of a standard solution containing ^{13}C 1,2,3,4 TCDD and ^{13}C 1,2,3,7,8,9 HxCDD (480 pg of each added to the extract) to monitor recoveries achieved during the HRGC/HRMS analysis. The levels of PCDD/Fs in the extracts were determined using HRGC/HRMS on a double focussing mass spectrometer of EB geometry Autoconcept, Mass Spectrometry International (MSI), Manchester, UK. The identification criteria specified in US EPA Method 1613 with respect to the GC column performance and mass spectrometer performance were fully satisfied by the data obtained in this study (separations, resolution and sensitivity capabilities). Laboratory blanks were analyzed with the samples, and showed no contamination (non-detectable PCDD/Fs).

Results and Discussion

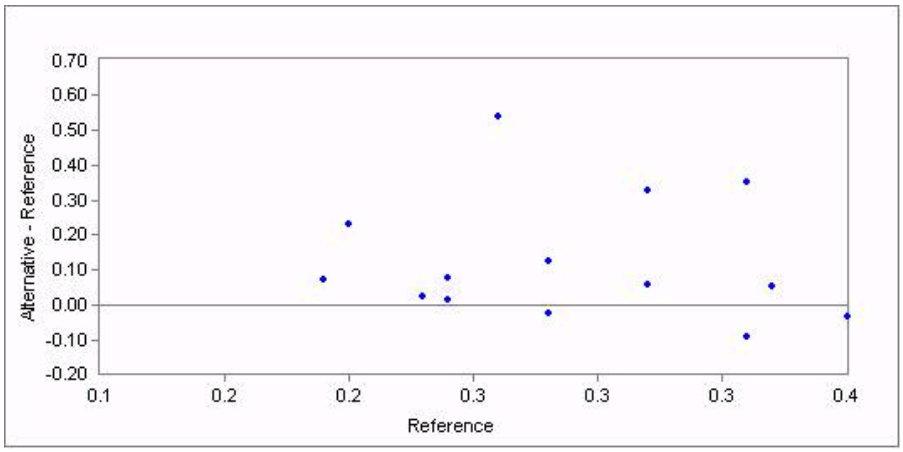
Table 1 summarizes the results obtained from the XDS-CALUX assay and the HRGC/HRMS analysis. The XDS analytical report express results as TEQ-ppt and are non-lipid adjusted. Thus, it is important to note that the values of fat content derived from the HRGC/HRMS sample preparation procedure were used for calculating final CALUX and HRGC/HRMS results.Values shown in table 1 are for total PCDD/F's and dioxin-like PCB's expressed as pg / gram fat using WHO TEF values. XDS values in italics indicate that raw data were at the limit of detection: 0.07 TEQ-ppt for dioxins and 0.08 TEQ-ppt for DL-PCB's.

Table 2 (Total TEQ WHO pg/g fat,)

Sample No.	Fat Content %	XDS-CALUX PCDD/F's	XDS-CALUX DL-PCB's	HRGC/HRMS PCDD/F's	HRGC/HRMS DL-PCB's
1	28	3.38	0.39	0.49	0.59
2	28	2.56	0.32	0.47	0.97
3	24	0.38	0.50	0.32	0.46
4	27	0.41	*0.30*	0.28	0.32
5	26	0.42	0.38	0.37	0.5
6	27	*0.26*	0.37	0.19	0.5
7	24	0.43	*0.34*	0.2	0.9
8	17	0.71	*0.47*	0.36	0.39
9	26	*0.27*	*0.31*	0.36	0.56
10	28	*0.25*	*0.29*	0.24	0.32
11	27	*0.26*	*0.30*	0.03	0.39
12	27	*0.26*	*0.29*	0.28	1.09
13	20	*0.37*	0.63	0.4	0.68
14	1.6	*4.40*	*5.03*	0.03	0.66
15	22	*0.32*	*0.36*	0.24	0.69
16	82	0.65	*0.09*	0.32	0.45
17	28	*0.25*	0.40	0.23	0.75
18	26	0.80	*0.30*	0.26	0.25
19	82	0.26	*0.09*	0.03	0.03
20	1.5	8.33	*5.56*	0.03	0.31

With respect to the 3 pg/g limit for milk products, the first observation is that the XDS-CALUX did not give false negatives. However, false positives were found for the two low fat samples nos.14, 20 and for the butterfat sample no.1. Also, a high value was found for a second butterfat sample no.2. In the case of the low fat samples, adjusting for fat content shifts the detection limit above the 3pg/g level. This is not the case for full fat samples where the LOD is roughly 10 times below the EU limit. The CV for repeatability on valid samples (ie. those that had enough raw data measurements) was <30%. The closeness between the average values of the XDS results were compared against the HRGC/HRMS reference values. Figure 1 presents the trueness of values after excluding two samples that required re-extraction, the false positives and the high value of sample 2. A bias is clearly evident for the assay where results are overestimated. This bias is believed to be due to interferences from other compounds in the sample matrice. To understand if the amount of bias is acceptable or not, the 3pg/g value was used to determine until when a CALUX value can be accepted. This was calculated at 2.77 pg / g fat. Above this value the XDS result could provide a positive result when the real value is below 3pg. Therefore, confirmatory testing by the HRGC/HRMS reference method is advisable when CALUX values are close to the 3pg limit and required when above. CALUX results below LOD compared very favourably with reference results. In this case all results were below the EU limit. Further work is proposed to be done on positive incurred samples. In conclusion, providing that the assay procedure is adapted for low fat samples it can be a viable screening method satisfying EU requirements in combination with HRGC/HRMS for confirmation of positives.

Figure 1 (Alternative = XDS-CALUX, Reference = HRGC/HRMS)



References

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