



Evaluation of the use of CALUX results for dioxins and dioxin-like PCBs analysis for quantitative human exposure assessments

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ABSTRACT

The objective of this study was to evaluate if the analysis results of dioxins and dioxin-like PCBs in food matrices obtained using the screening CALUX (Chemically Activated Luciferase gene eXpression) method, could be used for quantitative risk assessment.

Contamination data of dioxins and dioxin-like PCBs in food matrices (dairy product, eggs, fish, animal fat and vegetable oil) obtained by the CALUX screening method and by the reference method (GC-HRMS) have been compared. Significant differences between the two methods were observed. Median concentrations of dioxins with the CALUX methods for the food matrices are about twice the median concentration of dioxins with the GC-HRMS. These discrepancies indicate that the data obtained using the CALUX method within the framework of the Belgian official control program cannot be used in risk assessment, specifically for an accurate estimation of the dietary intake of dioxins and dioxin-like PCBs. This can be explained, on one hand, by the promiscuous nature of the Aryl hydrocarbon Receptor (AhR) (whose ligand activation is responsible of the response measured in the CALUX assay) as it effectively binds to a large number of diversely shaped compounds not belonging to the 17 dioxins/furans and the 12 dioxin-like PCBs congeners. On the other hand, the assay is implemented as a screening tool for compliance monitoring where emphasis on method performance is required around the maximum levels, leading to a lack of accuracy for lower or higher concentrations.

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1. Introduction

Halogenated aromatic hydrocarbons, such as polychlorinated-dibenzo-p-dioxins (PCDD), polychlorinated-dibenzofurans (PCDF), polychlorinated-biphenyls (PCB) and other related compounds represent a group of ubiquitous environmental contaminants.

Many of them are toxic and persistent and their lipophilic properties facilitate bioaccumulation in the food chain (Eppe et al., 2006; Scippo et al., 2008).

Depending on the number of chlorine atoms and on their position on the aromatic rings, 209 PCBs and 210 PCDD/Fs congeners are distinguished. The toxicological properties of these congeners are directly related to their chemical structure, namely the number and position of chlorine atoms. The 17 PCDD/Fs congeners with chlorine atoms substituted in position 2,3,7 and 8 present such a sterical configuration that they bind with high affinity to the aryl hydrocarbon receptor (AhR) with which a stable complex is formed (Denison et al., 2004). The 12 non-ortho and

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mono-*ortho* substituted PCB congeners show toxicological properties that are similar to dioxins (Safe, 1992). They are therefore often termed 'dioxin-like PCB (DL PCB)'. These 17 PCDD/Fs and 12 DL PCBs congeners have an assigned toxic equivalent factor or TEF value (Van den Berg et al., 1998; Van den Berg et al., 2006) and contribute to the toxic equivalency (TEQ) value of the samples (Epe et al., 2006). As the PCDD/Fs and DL PCBs molecules are very stable in the cell and not metabolized, AhR is abnormally maintained activated with adverse effects on physiologic functions (Scippo et al., 2008).

Different methods are used to determine the levels of PCDD/Fs and DL PCBs (Baeyens, Verstraete, & Goeyens, 2004; Scippo et al., 2008) in environmental as well as in food and feed matrices. A distinction is made between the so-called 'reference method' and the alternative or screening methods. Gas chromatography (GC) in combination with high resolution mass spectrometry (HRMS) is used as the reference method ('the golden standard') for the identification and quantification of PCDD/Fs and DL PCBs (Baeyens et al., 2004; Behnisch, Hosoe, & Sakai, 2001; Firestone, 1991; Liem, 1999). For screening, bioassays can be used to determine a biological response towards PCDD/Fs and DL PCBs such as enzyme activity, the expression of reporter genes, the binding between ligand and receptor or antigen and antibody. Bioassays such as CALUX (Chemical-Activated Luciferase gene expression), Ah immunoassays, ethoxyresorufin-O-deethylase (EROD) bioassays and enzyme immunoassays have been developed for rapid screening of various matrices (food, sediments, soil, fly ash) (JECFA, 2002). So far, only the cell based assay CALUX, a widely used screening method, has been used successfully for feed and food.

The CALUX assay is a reporter-gene-cell based assay using genetically modified murine hepatoma cells which respond to chemicals able to activate the AhR by producing firefly luciferase (Denison et al., 2004; Han, Nagy, & Denison, 2004; Van Overmeire et al., 2001). The AhR is a ligand-dependent transcription factor that not only binds and is activated by dioxins and related chemicals but is also responsible for mediating the toxicity of these chemicals. A common response resulting from activation of the AhR signalling pathway in cells is the induction of gene expression, and this forms the basis of this bioassay system, where the gene coding for the firefly luciferase is used as a reporter gene (Windal, Denison et al., 2005). The AhR mechanism and the CALUX bioassay system have been described in detail by others authors (Denison et al., 2004; Han et al., 2004; Van Overmeire et al., 2001). Recently Denison et al. (2007) and Hoogenboom et al. (2010) suggested that responses obtained with a bioassay should be expressed as bio-analytical equivalents (BEQ), to make the difference with the TEQ measured with GC-HRMS.

In Belgium the Federal Agency for the Safety of the Food Chain (FASFC) uses the CALUX method as a screening tool for compliance monitoring. Responses exceeding the action levels of PCDD/Fs and DL PCBs for feed and food require confirmation with GC-HRMS (European Commission, 2002a, 2002b, 2006b, 2006c, 2009).

The objective of this study was to evaluate if analysis results of PCDD/Fs and DL PCBs in food matrices, obtained within the framework of the official control program carried out in Belgium by the FASFC and using the CALUX screening method, could be used for quantitative risk assessment.

2. Materials and methods

2.1. Sample collection

Food items were sampled within the framework of the control program of the Belgian FASFC during the period 2005–2007. The goal of this program is to test the conformity with regulations and

to monitor food safety (Maudoux et al., 2006). The food matrices discussed here are dairy products (cow milk, goat milk, mare milk, butter, cheese, and yogurt), eggs, fish, animal fat (from beef, veal, pork, poultry, sheep, horse) and vegetable oil. The number of food samples analyzed was 271 for PCDD/Fs and 211 for DL PCBs and the sum of PCDD/Fs and DL PCBs.

2.2. Bioassay analysis

The CALUX analyses were performed by the laboratory of the FASFC. According to the ministerial decree of 19 April 2007 to appoint national reference laboratories in Belgium (M.B. 24.IV.2007), this laboratory is the national reference laboratory for the analysis of PCDD/Fs and DL PCBs in food and feed by the CALUX method.

The CALUX analyses were carried out as described by Vanderperren et al. (2004). Briefly, fat of milk and eggs were extracted using an acetone/hexane mixture (6/1 v/v and 1/4 v/v respectively). The fish lipids were extracted with a chloroform/methanol mixture according to Bligh and Dyer (1959) and animal fat was melted and dried with Na₂SO₄. Vegetable oil and the obtained extracts were defatted on 33% H₂SO₄ silica columns and further purified using activated carbon columns, which were differentially eluted in order to obtain 2 separate fractions, one containing dioxin-like PCBs (DL PCBs), the other containing PCDD and PCDF.

Mouse hepatoma H1L6.1 cells, obtained from Xenobiotic Detection Systems (Durham, US), were exposed 20–24 h to both purified extract fractions together with a PCB 126 and/or a 2,3,7,8-TCDD solution to establish appropriate calibration curves. All results were corrected for blank and apparent recovery (from matrix specific quality control samples), and are expressed in BEQ (bioanalytical equivalents).

The sampling and the analysis of PCDD/Fs and DL PCBs in food was performed in accordance with the European legislation, i.e. Directive 2002/69/EC (European Commission, 2002c) for samples taken before the first of March 2007 and Regulation (EC) No 1883/2006 (European Commission, 2006b) for samples taken after the first of March 2007.

Samples with CALUX responses exceeding the corresponding action level set by Commission Recommendation 2006/88/EC (European Commission, 2006c) (i.e. above 70% of the maximum level) were declared suspected to be non compliant and analyzed by GC-HRMS for confirmation, and the rate of false-non compliant results was calculated. Samples with CALUX responses below these action levels were declared compliant. In addition, 2–10% of samples considered safe (compliant) were also analyzed by GC-HRMS, as required by the European legislation (European Commission, 2002c and 2006b), allowing to get some information on possible false compliant results (Vanderperren et al., 2010).

2.3. GC-HRMS determination

The analysis of the 29 congeners (7 PCDDs, 10 PCDFs and 12 DL PCBs) was performed by the CART (University of Liege, Belgium) as well as by SGS (Antwerpen, Belgium). According to the ministerial decree of 19 April 2007 to appoint national reference laboratories in Belgium (M.B. 24.IV.2007), CART is the national reference laboratory for the analysis of PCDD/Fs and DL PCBs by GC-HRMS in food and feed. Sample extraction and clean-up, as well as GC-HRMS analysis has been described in detail by Focant, Epe, Pirard, and De Pauw (2001), Focant, Pirard and De Pauw (2004) and Pirard, Focant, and De Pauw (2002). Briefly, solid and semi-solid samples were extracted by pressurized liquid extraction (PLE). All milk samples were Soxhlet extracted. Sample clean-up was carried out

using multi-layer (acidic, basic and neutral) silica columns, basic alumina columns, and carbon-Celite columns. The mono-*ortho* PCB fraction was collected when flushing the carbon column in a forward direction with a hexane/dichloromethane mixture (1:1, v/v). The PCDD/Fs and non-*ortho* PCB fractions were collected when back-flushing the carbon column with toluene.

Analytical methods used by the two labs were accredited following ISO 17025.

The obtained concentrations are then multiplied by their specific toxic equivalent factor (TEF) to take into account the different toxicity of the 29 congeners (Van den Berg et al., 1998; Van den Berg, Peterson & Schrenk, 2000). The concentrations expressed in TEQ are then summed, assuming additivity of the responses. The TEQ value of individual congeners was calculated using the TEFs reported by Van den Berg et al. (1998) (Table 1).

The sum of the analysis results obtained for the PCDD/Fs fraction and for the DL PCBs fraction is named the sum of PCDD/Fs and DL PCBs.

2.4. Statistics

For the statistical analysis, left censored data were replaced by the value of the limit of quantification (upper bound principle) since in accordance with Regulation (EC) No 1881/2006 (European Commission, 2006a) results of official controls have to be expressed this way. However, uncertainty was not taken into account in the treatment of the results.

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS®, version 16.0; SPSS Inc., Chicago, United States). The Wilcoxon matched-pairs signed-ranks test was used to compare the results obtained with the analytical methods. Moreover, the agreement between the CALUX and the GC-HRMS results was assessed by adopting the approach described by Bland and Altman (1986, 1999). For each sample, the difference *d* between the two methods was calculated. The mean difference *d* and the standard deviation (SD) were determined for each food matrix. The

Table 1

WHO Toxic Equivalency Factors (TEFs) (Van den Berg et al., 1998) and CALUX relative potencies (REP) (Brown et al., 2001) used to express the toxic potency of PCDD, PCDF and DL PCB congeners.

Congener	WHO-TEF value	REP value
2,3,7,8 - TCDD	1	1
1,2,3,7,8 - PeCDD	1	0.73
1,2,3,4,7,8 - HxCDD	0.1	0.075
1,2,3,6,7,8 - HxCDD	0.1	0.098
1,2,3,7,8,9 - HxCDD	0.1	0.061
1,2,3,4,6,7,8 - HpCDD	0.01	0.031
OCDD	0.0001	0.0003
2,3,7,8 - TCDF	0.1	0.067
1,2,3,7,8 - PeCDF	0.05	0.14
2,3,4,7,8 - PeCDF	0.5	0.58
1,2,3,4,7,8 - HxCDF	0.1	0.13
1,2,3,6,7,8 - HxCDF	0.1	0.14
1,2,3,7,8,9 - HxCDF	0.1	0.31
2,3,4,6,7,8 - HxCDF	0.1	0.11
1,2,3,4,6,7,8 - HpCDF	0.01	0.024
1,2,3,4,7,8,9 - HpCDF	0.01	0.04
OCDF	0.0001	0.0016
PCB-77	0.0001	0.0014
PCB-81	0.0001	0.0045
PCB-126	0.1	0.04
PCB-169	0.01	0.0011
PCB-105	0.0001	0.000001
PCB-114	0.0005	0.00014
PCB-118	0.0001	0.000001
PCB-123	0.0001	0.0000003
PCB-156	0.0005	0.00014
PCB-157	0.0005	0.000003
PCB-167	0.00001	0.0000003
PCB-189	0.0001	0.0000002

95% confidence interval for *d* was determined from the appropriate t-distribution.

3. Results

Table 2 presents the descriptive statistics of the PCDD/Fs and DL PCBs concentrations measured with CALUX and GC-HRMS in dairy product, egg, fish, animal fat and vegetable oil samples. Mean and median levels of PCDD/Fs obtained with the CALUX method are about 2–3 times higher than those measured with the GC-HRMS method (Table 2). Median levels of DL PCBs obtained with the CALUX method are systematically higher than those measured with the GC-HRMS methods whereas the mean level of DL PCBs obtained with GC-HRMS method is higher for dairy products. Mean levels of the sum of PCDD/Fs and DL PCBs are higher with the CALUX method than with the GC-HRMS method, except for dairy products. Median levels of the sum of PCDD/Fs and DL PCBs are 1–5 times higher with the CALUX method than with the GC-HRMS method.

The statistical comparison of the analytical results with the Wilcoxon matched-pairs signed-rank test showed that the difference between the two techniques was significant for PCDD/Fs ($p < 0.0005$) in all the food matrices and for DL PCBs ($p < 0.01$) in eggs, animal fat and vegetable oil. The difference was not significant for DL PCBs in dairy products ($p = 0.83$) and in fish ($p = 0.11$). The statistical comparison for the sum of PCDD/Fs and DL PCBs showed the same trend. The difference was significant ($p < 0.0001$) in eggs, animal fat and vegetable oil and not significant in dairy products ($p = 0.25$) and in fish ($p = 0.06$).

The dispersion of the results between the two methods is shown in Fig. 1 for PCDD/Fs, DL PCBs and the sum of PCDD/Fs and DL PCBs in dairy products, eggs, fish, animal fat and vegetable oil. These graphs illustrate that the CALUX results generally exceed the

Table 2

Descriptive statistics of the results obtained with GC-HRMS (in pg WHO-TEQ/g fat, except for fish: pg WHO-TEQ/g fresh weight) and with CALUX (in pg BEQ/g fat, except for fish: pg BEQ/g fresh weight) for the analysis of PCDD/Fs, DL PCBs and the sum of PCDD/Fs and DL PCBs in dairy product, egg, fish, animal fat and vegetable oil samples.

	PCDD/Fs		DL PCBs		Sum of PCDD/Fs and DL PCBs	
	GC-HRMS	CALUX	GC-HRMS	CALUX	GC-HRMS	CALUX
Dairy products	N = 53		N = 45		N = 45	
Mean	1.89	3.41	30.24	28.08	32.18	31.36
Median	1.16	2.79	1.85	1.98	3.77	4.84
Minimum	0.01	0.08	0.01	0.50	0.02	1.58
Maximum	15.25	31.12	1195.90	1109.23	1200.32	1140.35
Eggs	N = 25		N = 22		N = 22	
Mean	1.15	2.15	0.62	1.58	1.77	3.82
Median	0.79	2.10	0.43	1.50	1.21	4.15
Minimum	0.24	0.36	0.09	0.57	0.37	0.93
Maximum	2.88	3.90	2.32	3.54	4.48	5.63
Fish	N = 28		N = 21		N = 21	
Mean	0.68	1.89	2.19	2.60	2.93	4.62
Median	0.55	1.00	0.39	1.89	0.84	3.00
Minimum	0.03	0.24	0.04	0.28	0.06	0.65
Maximum	5.95	13.54	15.70	10.36	21.65	23.90
Animal Fat	N = 127		N = 96		N = 96	
Mean	1.05	2.34	1.72	1.71	2.77	3.98
Median	0.67	2.04	0.68	1.24	1.43	3.43
Minimum	0.24	0.17	0.15	0.17	0.41	0.40
Maximum	9.37	14.03	28.69	9.79	38.05	23.82
Vegetable oil	N = 38		N = 27		N = 27	
Mean	0.29	0.86	0.20	1.16	0.51	2.10
Median	0.24	0.87	0.17	1.00	0.39	2.00
Minimum	0.12	0.25	0.12	0.50	0.24	0.75
Maximum	0.77	2.12	0.75	2.81	1.14	3.73

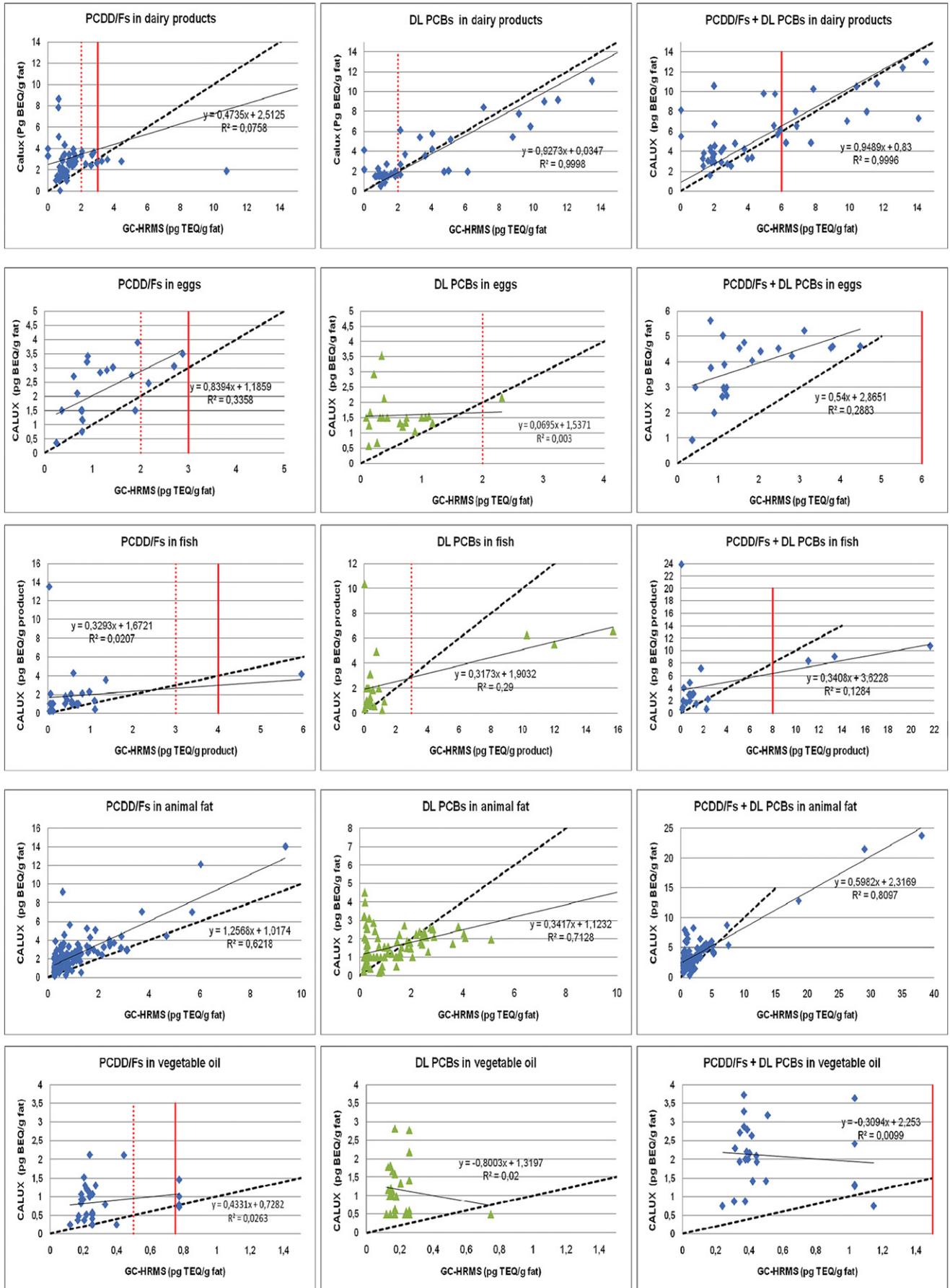


Fig. 1. Comparison of the CALUX and GC-HRMS results for the analysis of PCDD/Fs, DL PCBs and the sum of PCDD/Fs and DL PCBs in dairy products, eggs, fish, animal fat and vegetable oil. (—) regression line, (---) represents the bisecting line, vertical line represents the maximal levels established by Regulation (EC) No 1881/2006. Dotted vertical line represents the action level established by Recommendation 2006/88/EC.

GC-HRMS results. Most results plot above the 45° line and this is more pronounced for the lower concentrations. The proportion of samples with positive ranks (i.e. sample with CALUX results higher than the GC-HRMS results) is on average 84%. The slope of the trend line different from 1 indicates a proportional systematic error. Following Hádrič et al. (2010), Y intercepts above zero indicate that other AhR active compounds may be present in the PCDD/Fs or DL PCBs fraction.

The ratio of experimental CALUX over GC-HRMS results is presented in Fig. 2 as a function of the concentration of PCDD/Fs, DL PCBs and the sum of PCDD/Fs and DL PCBs measured by GC-HRMS for the food matrices. This underlines the systematic difference between CALUX and GC-HRMS results. From these graphs it can be deduced that the CALUX results are much higher than the GC-HRMS results at lower concentrations than at higher concentrations. For concentrations above the maximum level, the ratio between experimental CALUX concentrations and GC-HRMS concentrations tends towards one, while for lower concentrations, the ratio is higher.

The agreement between the CALUX and GC-HRMS results has been analyzed following the approach of Bland and Altman (1986; 1999) (Table 3).

For PCDD/Fs, the mean difference between the results obtained by the two methods ranged from 0.56 pg TEQ/g fat (95% Confidence Interval (95% CI): 0.41, 0.72) for vegetable oil to 1.52 pg TEQ/g fat (95% CI: 0.34, 2.70) for dairy products.

For DL PCBs, the mean difference between the results obtained by the two methods was the lowest for animal fat (−0.01 pg TEQ/g fat (95% CI: −0.56, 0.55)) and the highest for dairy product (−2.17 pg TEQ/g fat (95% CI: −6.17, 1.78)). A concentration up to 1200 pg TEQ/g fat has been found in a sample of milk in 2007 (Sci Com, 2007). This concentration was out of the working range of the calibration curve for the CALUX method. If this sample is excluded, the difference between the two methods for dairy products decreases to −0.24 pg

TEQ/g fat (95% CI: −1.00, 0.52). Negative values indicate that some GC-HRMS results are higher than the CALUX result.

The difference for the sum of PCDD/Fs and DL PCBs is the addition of the differences for PCDD/Fs and DL PCBs.

4. Discussion

The CALUX assay is a relatively inexpensive and reliable screening tool allowing a high number of samples to be analyzed within a short period of time (Vanderperren et al., 2010). Validation of the CALUX method on marine biological matrices and human plasma carried out by Windal, Van Wouwe et al. (2005) and Van Wouwe, Windal, Vanderperren, Eppe, Xhrouet, Massart et al., 2004 showed good correlations between the PCDD/Fs fraction measured by CALUX and GC-HRMS. Results of the CALUX method show a good correlation with GC-HRMS for food matrices analyzed even though it generally leads to an overestimation, especially at low levels of contaminants. The CALUX method, as practiced in Belgium for the official control program of the FASFC shows satisfactory analytical performance. Moreover, Vanderperren et al. (2010) have shown a 0% rate of false compliant samples in meat from ruminants, poultry, pigs, in milk, egg, fish and feed samples for PCDD/Fs and the sum of PCDD/Fs and DL PCBs.

Samples suspected to be non compliant (ie above the action level) after the CALUX screening are analyzed by the GC-HRMS confirmatory method, which allows the identification and quantification of 17 PCDD/Fs and 12 DL PCBs congeners. On the contrary, the screening method CALUX is not able to identify and quantify these congeners. It is a semi-quantitative method which detects all Ah receptor agonists still present in the sample extract (most of the natural agonists such as flavonoids are eliminated during the extraction/purification step).

Non-linear regression models are used to calibrate the CALUX response versus TCDD or PCB 126 standards and to convert the

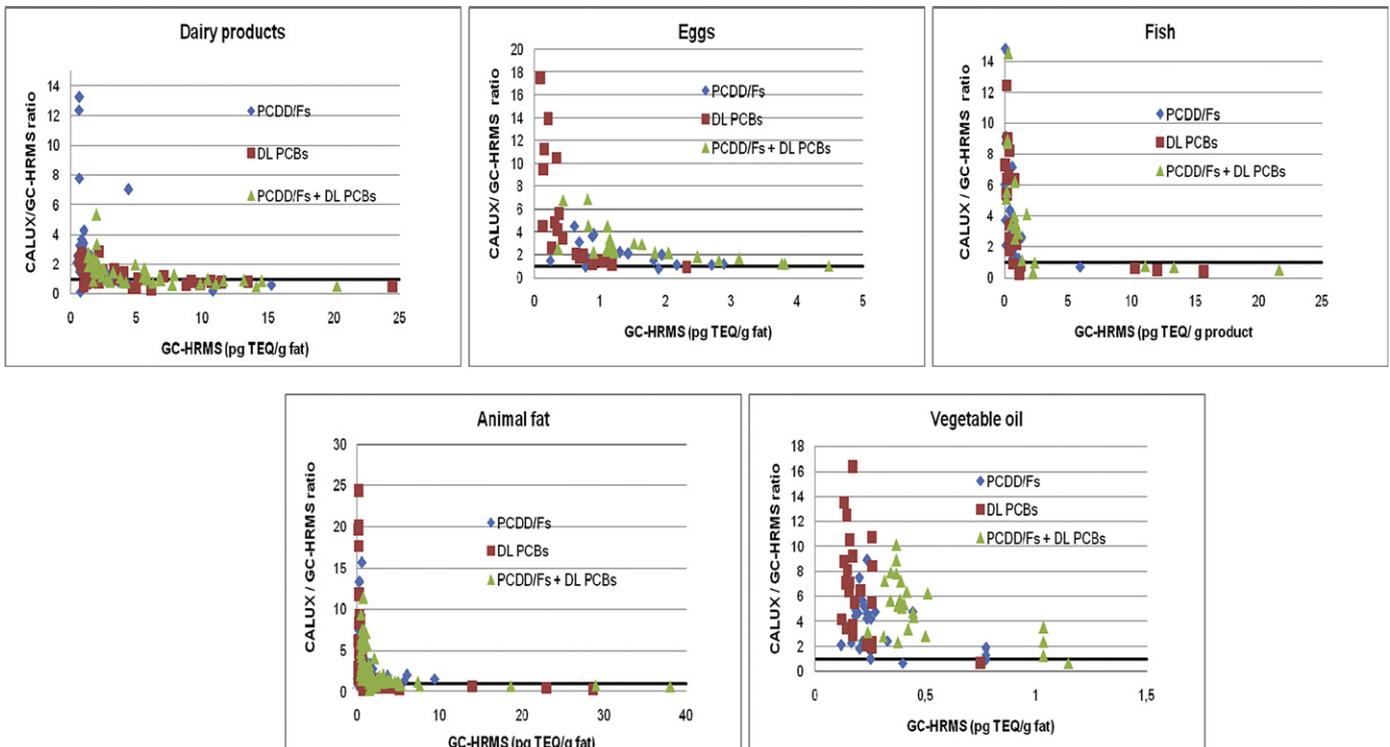


Fig. 2. CALUX/GC-HRMS ratio as a function of the concentration in PCDD/Fs and in DL PCBs as well as of the sum of PCDD/Fs and DL PCBs measured by GC-HRMS in dairy products (one outlier excluded), eggs, fish, animal fat and vegetable oil.

Table 3

Mean difference d and 95% Confidence Interval (95% CI) between the GC-HRMS results and the CALUX results for PCDD/Fs, DL PCBs and the sum of PCDD/Fs and DL PCBs in dairy products, eggs, fish, animal fat and vegetable oil (pg TEQ/g fat, excepted for fish: ln pg TEQ/g product).

	PCDD/Fs		DL PCBs		Sum of PCDD/Fs and DL PCBs				
	Mean difference d	95% CI	Mean difference d	95% CI	Mean difference d	95% CI			
Dairy products	1.52	0.34	2.70	−2.17	−6.11	1.78	−0.81	−3.72	2.10
Eggs	1.00	0.66	1.34	0.96	0.61	1.32	2.05	1.56	2.55
Fish	1.22	0.21	2.22	0.41	−1.31	2.13	1.69	−1.09	4.47
Animal fat	1.29	1.06	1.51	−0.01	−0.56	0.55	1.20	0.69	1.72
Vegetable oil	0.56	0.41	0.72	0.95	0.68	1.23	1.59	1.23	1.95

sample response into Bioanalytical Equivalents (BEQs). Several problems may arise in terms of statistical inference, specifically and most important is the uncertainty of the predicted BEQ. [Elskens et al. \(2011\)](#) have developed a linear calibration function based on Box-Cox transformations to overcome the issue of uncertainty.

In this study, the classical sigmoid-like calibration curves model was used. As the CALUX screening was used for compliance monitoring at the screening stage, the method was developed for optimal performance around the action level and the maximum level. The method may be adapted for the analysis of lower concentrations but with loss in accuracy for values around the maximal level.

The objective of this study was to evaluate if the analysis results of PCDD/Fs and DL PCBs in food matrices obtained within the framework of the official FASFC control program using the screening method CALUX, could be used for quantitative risk assessment.

The risk assessment of a chemical contaminant is subdivided in four steps: hazard identification, hazard characterisation, exposure assessment and risk characterisation ([WHO/IPCS, 2009](#)). For exposure assessment, the availability of reliable analytical techniques is critical to detect and quantify chemicals in environmental and food samples (occurrence data) with a high level of accuracy ([Dorne, Bordajandi, Amzal, Ferrari, & Verger, 2009](#)). The comparison of the PCDD/Fs and DL PCBs results obtained by both the CALUX screening and the GC-HRMS confirmatory methods have shown significant differences for the food matrices studied. Median concentrations of PCDD/Fs measured with the CALUX method in food matrices are about twice the median concentrations of PCDD/Fs resulting from the GC-HRMS analysis. Calculation of the dietary exposure of PCDD/Fs and DL PCBs with the CALUX results would be different (higher) from the dietary exposure determined with the GC-HRMS results. This evidently leads to wrong risk characterisation.

The graphs ([Figs. 1 and 2](#)) show that the ratio between CALUX and GC-HRMS results is much higher at low contamination levels. These high CALUX figures may be due to

- (i) The emphasis on method performance around the maximum levels, which implies that accuracy at very low levels is impaired.
- (ii) The presence of agonist compounds co-eluted during sample preparation. The effect of interfering compounds increases when PCDD/Fs and DL PCBs are present in low concentrations.

As mentioned by other authors ([Goeyens et al. \(2010\)](#); [Scippo et al. \(2008\)](#); [Vanderperren et al. \(2010\)](#); [Van Wouwe, Windal, Vanderperren, Eppe, Xhrouet, Massart et al. \(2004\)](#) and [Windal, Van Wouwe et al. \(2005\)](#)), factors affecting the discrepancy between the GC-HRMS method and the CALUX may be the quantification limits, the difference between relative potency (REP) and TEF values, and the potential of the bioassay to measure all compounds with AhR affinity.

For samples with low concentrations of PCDD/Fs and DL PCBs, most of the congeners will be lower than the quantification limit (LOQ) of the GC-HRMS method. For these non-detected congeners, a concentration equal to zero (lower bound approach), half of the LOQ (middle bound approach) or the value of the LOQ (upper bound approach) can be reported. In the European legislation, it is recommended to report the upper bound approach. If the lower bound approach is used, when concentrations of the non-detected congeners are set to zero, they do not contribute to the TEQ value. For low levels, below the LOQ, but above zero, this results in an underestimation of the TEQ value. Since the CALUX bioassay gives an overall response for all congeners, the differences between CALUX and GC-HRMS results may increase as the number of not detected congeners raise, when applying the lower bound approach ([Van Wouwe, Windal, Vanderperren, Eppe, Xhrouet, Massart et al., 2004](#)). On the opposite, the use of the upper bound values will probably reduce the discrepancy between CALUX and GC-HRMS results.

[Van Wouwe, Windal, Vanderperren, Eppe, Xhrouet, Massart et al. \(2004\)](#) have shown for dioxins in human blood plasma samples that the mean difference between upper bound and lower bound WHO-TEQ values decreases when the concentration range increases. However, [Hui et al. \(2007\)](#) have observed that the analytical difference between the upper bound and the lower bound results in milk samples was negligible (<0.1%).

For the food matrices, analyzed here, the difference between the upper bound and the lower bound results tends to decrease when the concentration range increase. In the study presented here, GC-HRMS results are presented as upper bound values.

In order to compare GC-HRMS results in a better way with the CALUX results, the relative potency (REP) values can be used instead of TEF values. REP values refer to potencies relative to 2,3,7,8-TCDD obtained in one single *in vitro* or *in vivo* study. The REP values are calculated directly by dividing the 50% effective concentration (EC50) of the dose–response curve of the 2,3,7,8-TCDD by the EC50 of the test compound curve ([Brown, Chu, Van Overmeire, Chu, & Clark, 2001](#); [Hosoe, Behnish, Takigami, & Sakai, 2002](#)). REP values are specific to the cell line and differ slightly from WHO-TEF ([Scippo et al., 2008](#)). Results named “expected CALUX” are obtained when multiplying concentrations of each congener measured by GC-HRMS with the corresponding REP ($= \sum \text{conc}_i \times \text{REP}_i$; [Table 1](#)). [Van Wouwe, Windal, Vanderperren, Eppe, Xhrouet, Massart et al. \(2004\)](#) have shown that experimental CALUX values for PCDD/Fs are closer to expected CALUX than GC-HRMS concentrations in human blood plasma. [Carbonnelle et al. \(2004\)](#) observed that mean or median levels in PCDD/Fs are slightly higher than those obtained with WHO-TEF. This may partially be explained by some REP values exceeding the WHO-TEF value. For DL PCBs, [Carbonnelle et al. \(2004\)](#) observed that expected CALUX results as well as experimental CALUX results are significantly lower than the GC-HRMS results, which indicate an underestimation of the CALUX responses with respect to the chemo-analysis results.

The potential of the CALUX assay to detect all compounds with AhR affinity, still present in the sample extract, in addition to the observed non-additive effects of some molecules, could explain the biggest part of the discrepancy between the two methods (Goeyens et al., 2010; Scippo et al., 2008; Vanderperren et al., 2010; Van Wouwe, Windal, Vanderperren, Eppe, Xhrouet, Massart et al., 2004; Windal, Van Wouwe et al., 2005). Environmental contaminants like brominated dioxins, mixed chlorinated/brominated dioxins, polychloroterphenyls (PCT), polychloronaphtalenes (PCN), polycyclic aromatic hydrocarbon (PAH) possibly end up in the final extract and have a significant contribution to the CALUX response.

The effects of these compounds appear to be relatively more important in samples with low concentrations of PCDD/Fs and DL PCBs. As a consequence, the uncertainty on the CALUX result regarding the presence of PCDD/Fs and DL PCBs will be higher for lower concentration.

The proportion of samples with negative ranks (i.e. sample with GC-HRMS results higher than the CALUX results) was higher for DL PCBs in dairy product (44%) and animal fat (36%). This higher proportion of GC-HRMS results above the CALUX results may be probably due to the antagonistic effect of some PCBs as well as other AhR ligands that may be present in the PCBs fraction (Safe, 1997; Van Wouwe, Windal, Vanderperren, Eppe, Xhrouet, De Pauw et al., 2004; Windal et al., 2003; Windal, Van Wouwe et al., 2005).

The screening of choline chloride, a widely used feed ingredient, resulted in the discovery of a contamination with brominated flame retardants and bromodioxins (PBDD/PBDFs) (Traag, Kotz, Van der Weg, Malisch, & Hoogenboom, 2009). Initially, the positive CALUX test result could not be confirmed by HRGC/HRMS but follow-up on this “false-positive” result led to the discovery of the contamination. Although not included in the legislation, brominated dioxins are well-known to be equitoxic to their chlorinated counterparts and it is expected that TEF will be assigned as soon as experimental potency results are obtained. Examination of the sample by gas-chromatography time-of-flight mass spectrometry (GC-TOFMS) revealed also the presence of a new brominated flame retardants Octabromo-1,3,3-trimethylphenyl-1-indan (OBIND) (Traag et al., 2009). This example demonstrates that routine screening may yield false-non compliant results but that a follow-up on the compounds responsible for this result may reveal novel risks (Hoogenboom et al., 2010). It is actually, the difference in the principles behind the two types of methods, either based on effects or on the structure of the compound, that makes that their combined use is very suitable for the detection of novel risks (Goeyens et al., 2010).

Not only contaminants such as PCDD/Fs and PCBs and PAHs bind to the Ah receptor but a lot of other compounds of natural origin such as flavones and carbinols or therapeutic agents such as omeprazole may bind and activate this receptor (Vanderperren et al., 2010).

Extracts of potatoes (French fries), cruciferous, bread, hamburgers and citrus fruit juice show an increased response in the CALUX assay (De Waard et al., 2008). The group of furocoumarins or flavonoids presents in various vegetables and citrus fruits is an example of such compounds (Hoogenboom et al., 2008; Van Der Heiden et al., 2009). These compounds are able to activate the Ah receptor but it is more than likely that they are destroyed during the clean-up step on acidic silica of the CALUX analysis. Furthermore, PAHs, even if they partially resist to the acidic treatment, are quickly metabolized in the hepatoma cells, and could contribute only negligibly to the CALUX response. Vegetable food was not analyzed within the framework of this study.

5. Conclusion

For the analyzed food matrices, the screening CALUX assay generally overestimates the PCDD/Fs and DL PCBs content

compared to GC-HRMS results, especially for low PCDD/Fs and DL PCBs levels. The implementation of the CALUX method as a screening tool for compliance monitoring using the above mentioned methods has proven to be successful. This study shows, however, that results obtained with the CALUX method are not appropriate for use in quantitative risk assessment in order to assess the specific dietary exposure to PCDD/Fs and DL PCBs. An estimation of the intake of PCDD/Fs and DL PCBs may not be conducted with data obtained with screening methods such as the CALUX method because these methods don't respond exclusively to the 17 PCDD/Fs congeners and 12 DL PCBs congeners.

Nevertheless it is recommended that an integrated approach to identify and characterize hazards, to assess human exposure and to evaluate risks should preferably rely on analytical tools that quantify both the chemical contaminants' concentrations as well as their resulting biological (toxicological) effects (Dorne et al., 2009). The growing demand for the combined use of chemo- and bio-analytical approaches in order to link chemical contamination to measurable toxic and biological effects (Blasco & Picó, 2009; Streck, 2009) is inspired by the current concern about the extremely complex and highly diverse contaminant cocktails.

When important differences between the CALUX and the GC-HRMS results are observed, it is recommended to carry out further chemical analyses in order to identify the nature of any co-eluting compounds with a dioxin-like activity (Sci Com, 2008). In addition, it is advisable, when performing official control programs, to include, a subsequent and limited random sampling that will be systematically analyzed both by the screening (e.g. CALUX) and reference (i.e. GC-HRMS) methods, enabling a more accurate quantitative assessment of the dietary exposure to PCDD/Fs and DL PCBs along the food chain.

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