Validation efforts for the analysis of animal fat using the CALUX method

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Introduction

As a measure to avoid food contamination incidents and to lower human exposure to dioxins via the food chain the European Commission has introduced maximal PCDD/F TEQ-levels for different food products and animal feed ingredients.[1,2] The lowest maximal levels are for animal fat and range between 1 and 3 pg WHO PCDD/F TEQ/g fat according to the origin of the fat. To check compliance to these limits appropriate validation of analytical procedures is required.
In addition, a thorough monitoring of the food chain is impossible without the use of rapid screening methods. The CALUX bioassay is one of such methods that can be applied as a screening tool for food and feed products.
The published data on TEQ levels in food obtained by using the CALUX method have increased during recent years. [3-6] The objective of this study is to investigate the feasibility of using the CALUX assay to screen animal fat samples at low TEQ contamination levels. The performance of the method at a level of 1 pg TEQ/g fat is demonstrated and the application of the procedure for pork, chicken and bovine fat is illustrated.

Methods and Materials

Fat samples: commercial bovine fat (for frying) was spiked with 2,3,7,8- TCDD (Wellington Laboratories); bovine and chicken fat were obtained from Belgian farms; pork fat came from a retail store. The non-commercial fats were melted and dried over Na_2SO_4 before clean-up.
Alternative clean-up method: 4 g of fat was suspended in hexane (30 ml) and acidic silicagel (33% H_2SO_4) (25 g) was added to it. The suspension was shaken and left for 30 minutes with occasional shaking. Then the supernatant hexane was transferred to a small acidic silica column (9 g) in series with an activated carbon column. A PCB and a dioxin fraction were separately eluted from this column.
For the *clean-up of 2 g of fat*, the fat was subjected to a column clean-up (acidic silicagel and carbon column) as previously described.[7,8]

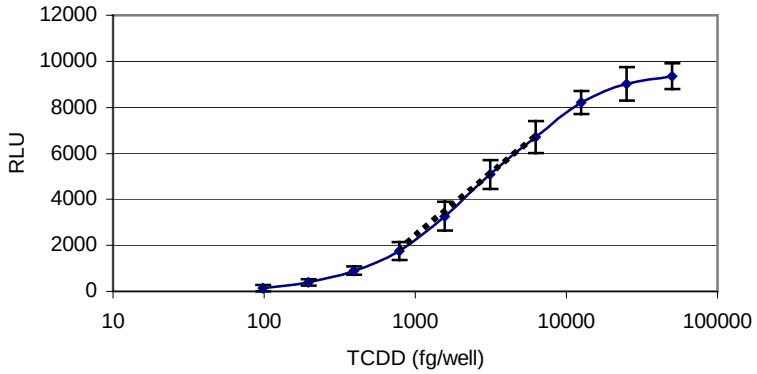
After clean-up of the fats (4 g or 2 g) only the dioxin fraction was analysed with the CALUX assay. All results were corrected for recovery (80 %). This recovery was determined using ^{14}C- labelled 2,3,7,8 TCDD that was spiked into the fat and counted by scintillation after the clean-up.
There was no correction for a blank. Instead , a QC criterium for the procedural blank was met: the procedural blank was spiked with the standard solution of 2,3,7,8 TCDD used for the quality control of the plates. The CALUX response (standard solution + procedural blank) can not exceed the range mean ±SD calculated for the standard solution.In this way both toxicity (lower response) and contamination (higher response) is detected but a value for the blank only can not be given. [9]
CALUX assay: The assay was performed using the recombinant mouse cell line H1L6.1 from Xenobiotic Detection Systems as described.[5] One well on a plate corresponds to 200 µl medium.

Results and Discussion

- *Quantification limit*

In Figure 1 a dose response curve for TCDD (0.098-50 pg/well or 1.5-776 pM) as well as the linear part (0.78-6.25 pg/well)are illustrated. It is stated in Commission directive2002/69/EC[10] that for cell-based bioassays, used for TEQ deteminations, the induction of the sample dilution used must be within the linear portion of the response curve. Therefore we consider the quantification limit for all samples as the lowest point of the linear part from the dose-response curve. The LOQ is in this manner fixed at 0.8 pg TCDD/well. Results below this value will not be quantified due to a large uncertainty on the calculated TEQ value**.**

Fig 1: calibration curve with TCDD concentrations in fg/well on a log scale; each point represents the mean ± SD of 17 curves. The linear part is indicated by the dotted line.



- *Validation study for 1 pg TEQ/g fat using alternative clean-up:*

The first goal of this study was to validate and optimise the performance of the CALUX method for a concentration level of 1 pg TEQ/g fat which is the maximal level for pig fat and the lowest of the maximal levels in animal fat.

To determine the performance of the method at levels around this TEQ value, a commercial bovine fat was used and a simplified validation protocol was followed. [8,11] The fat was spiked at 3 concentration levels (addition of 0.5 x; 1 x and 1.5 x the level of interest) and of each obtained concentration 2 replicate clean-ups per day were performed on 5 different days**.**
With the obtained data it is possible to calculate repeatability and within-laboratory reproducibility variances.[11,12]
Initially, we investigated the clean-up of 4 g of fat to attempt to lower the quantification limit and at the same time to have the possibility to obtain duplicate results (same extract measured on different plates). When the objective is to measure above 0.8 pg TEQ/well, this implies that using 1 g of fat per well results in a LOQ of 0.8 pg TEQ/g fat while using 2 g of fat per well should give a LOQ of 0.4 pg TEQ/g fat. Therefore, an alternative clean-up method was applied (see methods and materials). The major advantages of this method are the consumption of less solvent and the gain of time as compared to using large silica columns needed to purify that amount of fat. However, during the examination of this procedure it was observed that exposing the cells to 1/2 of the purified fat extract in 1 well (2 g fat/well), caused partial inhibition of the response of a control TCDD solution (at 1.5 pg/well or 0.75 pg/µl DMSO). Consequently, the quantity of purified extract that was measured on the plates was reduced to 1/4 per well. For this dilution the procedural blank extract did not significantly influence the response of the control solution. Finally for the 3 spiked concentration levels (fat + 0.5; +1 and + 1.5 pg TEQ/g) CALUX responses were measured for 1/4 of the purified extracts. Responses for the unspiked fat as well as most of the results for the fat to which 0.5 pg TEQ/g fat was added could not be quantified (TEQ< 0.8 pg/well). The results for the fat + 1 and + 1.5 pg TEQ/g fat are summarized in Table 1.

Table 1: results obtained by using alternative clean-up method to purify 4 g of fat (bovine fat for frying) and 1/4 extract per well measured in the CALUX assay

	Fat +1 pg TEQ/g (n=10)		**Fat +1.5 pg/g** (n=9)	
	Replicate 1	*Replicate 2*	*Replicate 1*	*Replicate 2*
Series1	1.70	1.33	1.83	1.77
Series2	1.13	1.25	1.61	1.86
Series3	1.04	1.35	1.95	.*
Series4	0.91	1.03	1.08	1.24
Series5	1.55	1.15	1.59	1.39
Mean pg TEQ/g	1.24		1.59	
SD	0.25		0.30	
RSD (%)	20		19	
Sr /mean (%)	17		8	
SRw/mean (%)	20		20	

*****Non-valid result, extremely low response of cells for unknown reason
Sr: standard deviation for repeatability; SRw: standard deviation for within-lab reproducibility

The results are for the 1/4 dilution of the purified extract per well. Series1-series 5 indicate clean-ups performed on different days. For each concentration level 2 replicates per day were performed. The associated coefficients of variation for repeatability (sr /mean) and for within-laboratory reproducibility (sRw/mean) are also shown in Table 1.

The following validation parameters can be determined using these results:
Precision *(*for fat spiked with 1 and with 1.5 pg TCDD/g): The relative standard deviations for repeatability are 17 and 8 %, and the intra-lab reproducibility CV's (sRw/mean) equal 20%. These values are in accordance with Commission Directive 2002/69 wherein a maximal CV of 30 % for screening methods for dioxin analyses is indicated.[10]
Accuracy/recovery*:(found value vs. added spike):* The mean CALUX values found for the fat enriched with 1 and with 1.5 pg TCDD/g are 1.24 pg TEQ/g fat and 1.59 pg TEQ/g fat respectively. This indicates a good agreement with the added concentration (mark that the non-spiked fat could not be quantified).
Although the LOQ was calculated as 0.8 pg TEQ/g fat (see above) the experimental results showed that 3 of the 10 results for fat + 1 pg TCDD/g fat were measured below the LOQ which indicates that the experimental LOQ approaches 1 pg TEQ/g fat.
The difference between the mean value for fat + 1.5 pg TCDD/g (1.59) and that for fat + 1 pg TCDD/g (1.24) is 0.35, being 70 % of the expected difference of 0.5 pg TCDD/g.

- *Applicability and adaptation of the method to determine maximal levels in animal fats*:

It could be concluded from these first experiments that exposing the cells to a quantity of purified extract that corresponds to 1 g of fat per well was needed to be able to determine levels of 1 and 1.5 pg TEQ/g fat. Since the maximal levels for chicken and bovine fat are 2 and 3 pg TEQ/g fat respectively, the purification of 2 g of fat (instead of 4 g) and the analysis of 1/2 of the purified extract per well (instead of 1/4) should be sufficient for the analyses of these types of fat.
Thus, for the animal fat samples 2 g of fat was purified using a column clean-up. Initial results for repeated analyses are shown in Table 2.

Table 2: CALUX results (pg TEQ/g) for animal fats (2 g purified and 1/2 extract per well)

	Pork +1pg TEQ/g fat	Chicken + 2 pg TEQ/g fat	Beef (*)	Beef + 3 pg TEQ/g fat (*)
	0.93	1.84	2.82	5.31
	0.81	2.10	2.46	4.77
	1.14	2.52	2.80	5.41
	1.54	2.18		
	1.16			
	1.57			
Mean	1.19	2.16	2.69	5.16
SD	0.31	0.28	0.20	0.35
RSD (CV) (%)	26	13	7	7

(*) results for bovine fat were obtained with 1/4 extract per well

For these initial studies the results for the pork, chicken and bovine fat were not determined by GC-HRMS. The CALUX responses for the non-spiked pork and chicken fat were too low and could not be quantified. Therefore, these fat samples were enriched with their respective maximal TEQ level by spiking with TCDD. The measured CALUX values approximate very well the added concentrations. The bovine fat resulted in a CALUX TEQ value close to the maximal EU limit of 3

pg WHO TEQ/g fat). When this bovine fat was spiked with 3 pg TEQ (TCDD)/g fat 82 % was recovered.
The results also indicate that the CV's are all below 25% apart from the pork fat at the lowest level. The RSD is inversely proportional to the concentration level but also for all concentrations (1-3 pg TEQ/g fat) below 30 % as required by Commission directive 2002/69/EC.
These initial studies indicate that the low maximal EU TEQ levels in animal fat can be detected by applying the CALUX assay. This enables the monitoring of animal fats in a fast and inexpensive way. For the application of the method on samples it will be of high importance to provide appropriate control samples and to maintain the quality control criteria set up for this bioassay.[9]
Further studies with real samples are needed to indicate the correlation between CALUX and HRGC-HRMS results at these low TEQ levels.

References

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