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CONTROL OF BOVINE MILK SAMPLES BY CALUX BIOASSAY AND GC-HRMS ANALYSIS

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Introduction

The CALUX bioassay has recently emerged as a rapid analysis method for the determination of dioxin-like toxicity in environmental, biological as well as food and feed samples.[1-4] Therefore matrix-specific optimisation and validation of the sample pre-treatment methods, both the extraction and purification techniques, is becoming increasingly important when the bioassay is used as a screening tool for different sample types.
In view of checking sample compliance with the regulatory limits for dioxin-like compounds by using the CALUX bioassay [3,5] and of validating the bioassay for TEQ determinations[6] the results obtained are often compared to TEQ values for PCDD/Fs and dioxin-like PCBs based on GC-HRMS analyses. Even if GC-HRMS results and CALUX TEQ results are difficult to compare for a number of reasons [7], comparisons are generally considered as a simple way to 'validate' the bioassay's accuracy. When analysing milk samples following different procedures to purify the extracted milk fat, it was observed that the correlation between CALUX and GC-HRMS results differed substantially depending on the cleanup steps.
The present paper focuses on the effects of the applied cleanup method on the CALUX TEQ results and discusses consequences and implications for using the CALUX assay as a method for compliance control.

Materials and Methods

- *Sample preparation for CALUX assay*

Milk fat was obtained by liquid-liquid extraction of milk (60 ml) using first acetone: hexane (3/1, v/v) and afterwards hexane. The pooled organic layers were washed with a 2 % aqueous Na_2SO_4 solution and dried over Na_2SO_4. After evaporation of the solvent under nitrogen the amount of extracted lipid was gravimetrically determined. In general 1.5 g of milk fat was further cleaned up by passing through an acid (33% H_2SO_4) silicagel column and a carbon (X-CARB) column. Either the carbon column was totally eluted (method A) or differentially eluted to yield a PCB fraction and a dioxin (DX) fraction (method B).
11 milk samples were analysed by using method A and 33 samples by method B.

- *CALUX assay*

Purified sample extracts in DMSO were suspended in cell culture medium prior to dosing monolayers of H1.L1.6 mouse hepatoma cells (from Xenobiotic detection Systems, Inc.) that were grown in 96-well culture plates. In addition to the samples, a 2,3,7,8-TCDD standard curve was generated on each plate. The plates were incubated for optimal induction of luciferase activity in a humidified CO_2 incubator at 37 °C. After incubation, the medium was removed and the cells were

examined microscopically for viability. The induced luciferase activity was quantified using the luciferase assay kit from Promega.

- *Sample preparation for chemical analysis and GC-HRMS determination*

After partly evaporating the milk and mixing with chemical drying agents, milk fat was extracted with 2/1 (v/v) hexane/acetone. For clean up ca. 7 g of extracted fat was spiked with 16 ^{13}C-labelled internal standards and subsequently purified by column chromatography on silica/H_2SO_4, HPLC using a carbon column, and column chromatography on basic alumina. The measurements were performed by GC-HRMS. A complete description of the analytical procedure and QA/QC measures applied is given elsewhere [8,9].

Results and discussion

- *Comparison of GC-HRMS data with CALUX data for samples analysed according to method A (PCBs+dioxins)*

GC-HRMS values for PCDD/Fs range from 0.73 to 5.80 pg WHO TEQ/g fat, the median value being 1.30 and corresponding CALUX data range from 0.71 to 5.08 pg TEQ/g fat with a median value of 1.52 (Figure 1).
The CALUX to GC-HRMS PCDD/F ratios vary from 0.65 to 1.61; the median value amounts to 1.18 indicating the obvious closeness of both results.
Similar CALUX results for these samples were obtained by VITO when a total TEQ was determined using the rat cell line H4IIE although a broader interquartile range is observed (CALUX* in Figure 1).

- *Comparison of GC-HRMS data with CALUX data for samples analysed according to method B (PCB and PCDD/F separation)*

When comparing the GC-HRMS values with CALUX values obtained for samples which were differentially eluted the pattern changes significantly.
GC-HRMS PCDD/F values range from 0.39 to 3.60 pg WHO TEQ/g fat, the median value being 1.00 and corresponding CALUX-DX data range from non detected (=0 in figure2) to 6.86 pg TEQ/g fat with a median value of 1.31 (Figure 2).
GC-HRMS PCB values (non-ortho+ mono-ortho PCBs) range from 1.00 to 8.60 pg WHO TEQ/g fat, with a median value of 1.40 while corresponding CALUX-PCB values range from non detected (=0 in figure2) to 2.99 pg TEQ/g fat with the median value being 0.54.
It must be noticed that for the CALUX PCB analyses the same amount of fat as for the DX TEQ determinations was used, which may have been insufficient. Nevertheless we chose to include the CALUX PCB results in the comparison with GC-HRMS data.
Total GC-HRMS TEQ values (PCDD/F+ PCBs) range from 1.47 to 12.20 pg WHO TEQ/ g fat, with a median value of 2.50. The sums of CALUX-DX and CALUX-PCB values range from 0.41 to 9.85 TEQ/ g fat, the median value being 2.18.
The CALUX DX to GC-HRMS PCDD/F ratios vary from 0 (for non detected values) to 5.43; the median value amounts to 1.58.
The CALUX PCB to GC-HRMS PCB ratios range from 0 to 2.30 with a median value of 0.26.
[CALUX-DX + CALUX-PCB] to GC-HRMS (PCDD/F + PCB) ratios range from 0.18 to 3.79 (median 0.89).

- *Compulsory control*

Currently, the EU regulation[10] is restricted to the 17 PCDD/F congeners, whereby the additivity principle is obeyed. In a near future the contribution of 12 mono-ortho and non-ortho PCBs will also be taken into consideration. Their contribution will be added to the one of the 17 PCDD/F

congeners by application of the additivity principle. On the long term, other contaminants, which have not yet been specified, will also be taken into account.
The presented results clearly indicate that CALUX-DX fractions hold other AhR agonists than the 17 PCDD/F congeners (notice the broad interquartile range for CALUX DX results (Figure 2)). This phenomenon has also been observed when analysing human plasma and marine samples.[11,12] The presence of these additional compounds is not negligible; it is moreover highly probable that their contribution exceeds the one of the 17PCDD/F congeners. The low response obtained for dioxin-like PCBs in the CALUX assay has been observed before[4] and is explained by the difference between CALUX REPs[13] and WHO TEFs for dioxin-like PCBs. Although the sum of the CALUX DX and CALUX PCB fractions seems in accordance with the total TEQ determined for 29 congeners by GC-HRMS, it is important to notice that for milk samples mainly the DX fraction contributes to the total CALUX TEQ.

Conclusion

Bovine milk samples were analysed by using the CALUX assay combined with a sample extract fractionation procedure to obtain a dioxin (DX) and a PCB fraction. It was observed that the bioassay DX TEQ tends to exceed the corresponding GC-HRMS PCDD/F TEQ. On the other hand, the bioassay PCB TEQ is inferior to the corresponding GC-HRMS PCB TEQ. These aspects have a pronounced effect on compliance control. Determination of bioassay based dioxin-like toxicity results in additional information, which is missed by the chemical methods, and might be more valuable in terms of risk assessment.

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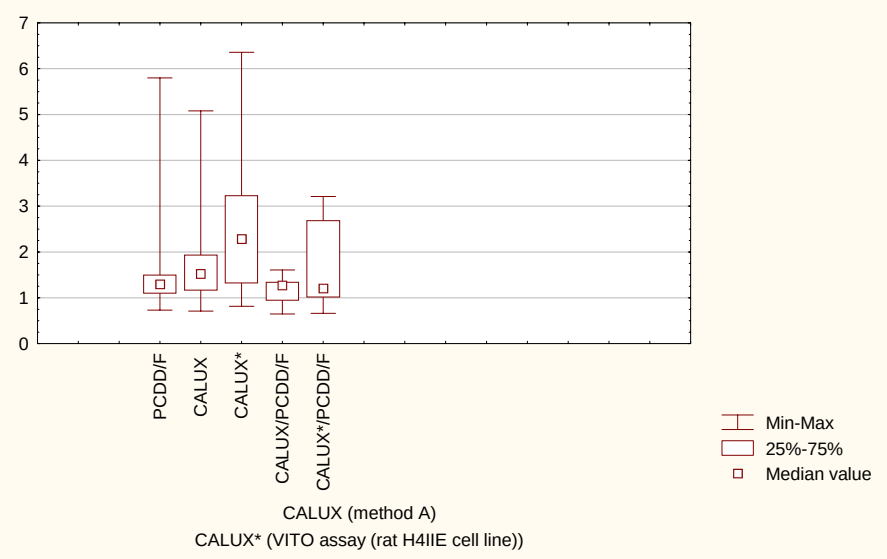

Figure 1: Box & Whisher plots for TEQ values (pg/g fat) of 11 milk samples analysed by CALUX according to method A and by GC-HRMS (PCDD/F).

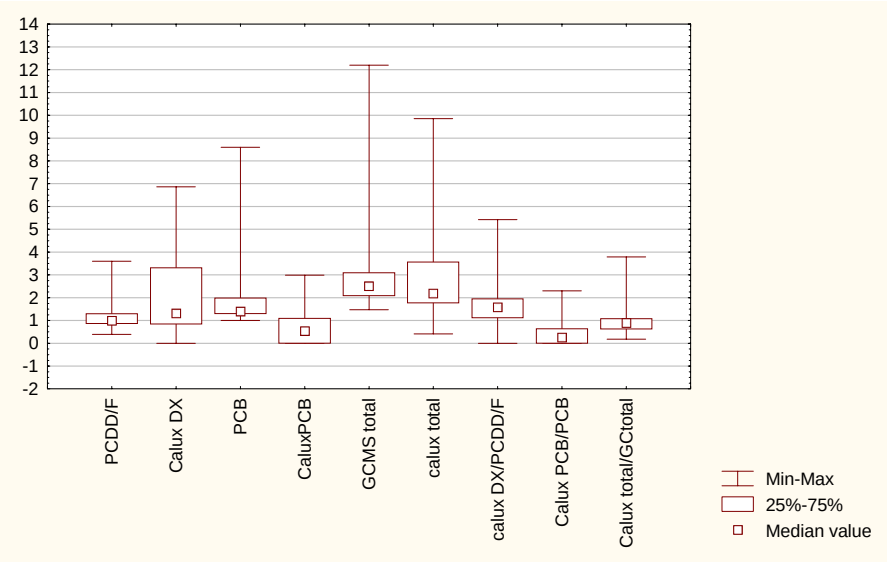

Figure 2: Box & Whisher plots for TEQ values (pg/g fat) of 33 milk samples analysed by CALUX according to method B and by GC-HRMS (PCDD/F and PCBs (=non-ortho + mono-ortho* PCBs)). (*mono-ortho PCB data for 23 samples).