BIOASSAY DIRECTED DETECTION OF BROMINATED DIOXINS IN THE FEED ADDITIVE CHOLIN CHLORIDE

Wim Traag[1], Alexander Kotz[2], Guido van der Weg[1], Rainer Malisch[2], and Ron Hoogenboom[1]

[1] RIKILT Institute of Foodsafety, Wageningen UR, P.O. Box 230, 6708PD Wageningen, The Netherlands
[2] EU Community Reference Laboratory (CRL) for Dioxins and PCBs in Feed and Food (at Chemisches und Veterinäruntersuchungsamt, CVUA), Bissierstr. 5, 79114 Freiburg, Germany

Abstract
During the past two years, several samples of the feed additive cholin chloride tested positive in the DR CALUX® assay. However, GC/MS analysis could not confirm the presence of dioxins or dioxin-like PCBs. Examination by GC-TOFMS revealed the presence of various brominated flame retardants (BFRs), such as PBDEs and 2,4,6-tribromophenol, but also the new BFR Octabromo-1,3,3-trimethylphenyl-1-indan (OBIND). These compounds could not explain the positive CALUX-result but indicated the presence of brominated dioxins. This was confirmed by GC/MS analysis, showing in particular 2,3,7,8-TBDF. This study shows that the combined use of a bioassay and chemical analytical methods may result in the identification of novel risks introduced into the food chain.

Introduction
The contamination of our food chain with dioxins and PCBs remains a concern, as shown by the recent incidents in Ireland and Chile. In both cases feed prepared from contaminated ingredients was shown to be the cause of the incident. At the same time it is clear from these and other recent incidents that sources of the contaminants may be variable. A targeted approach on suspected samples seems not to be a real option since other sources may be overlooked. Only intensive screening of samples may offer a reasonable guarantee for the early detection of contaminated ingredients or feeds. Bioassays, like the CALUX assay offer the possibility to set up large monitoring programs, since many samples can be screened in relatively short time and with limited resources. GC/MS confirmation is then required for samples showing an elevated response in the screening assay.

An advantage of the use of bioassays in combination with chemical confirmation methods is the possible detection of novel contaminants. This requires the follow-up of false-positive test results by further investigations, a strategy referred to as bioassay directed identification. Previously we described the use of such an approach for the identification of the furanocoumarin bergapten as the most important natural Ah-receptor agonist in marmalade (1). The present paper reports the identification of a number of brominated flame retardants and in addition brominated dioxins in cholin chloride, a widely used feed additive.

Materials and methods

CALUX-analysis
CALUX-analysis was performed as described previously. Chicken feed samples containing various levels were used as reference standards. These standards were used to estimate the actual levels in the cholin chloride, thereby expressing the result in BEQs since the response could be caused by compounds not obeying the TEQ-principle.

HRGC/HRMS analysis of dioxins and dl-PCBs
HRGC/HRMS analysis for dioxins and dl-PCBs was performed after extraction of cholin chloride with accelerated solvent extraction (ASE) and clean-up over a Powerprep system.

GCxGC-TOFMS analysis
Two suspected samples were further investigated by GC-TOF analysis, using a broad screening approach initially. Samples of choline chloride were extracted with toluene/acetone (90:10 v/v) using ASE. The obtained extract was evaporated to 0.5 ml and 2 µl was without any clean-up injected on the GCxGC–TOFMS.

Subsequent analysis focused on quantification of the compounds detected by comparison with relevant standards of 2,4,6-Tribroomfenol, 2,4,5- Tribroomfenol, several PBDEs and Octabromotrimethylphenyllindane .

Analysis of brominated dioxins
Analysis of PBDD/Fs and PXDD/Fs (tetra- and pentasubstituted congeners) was performed using the following extraction and clean-up steps: Twisselmann hot extraction with ethanol/toluene (70/30, v/v) and afterwards with toluene, multi-layer silica-gel column with neutral silica, acidified and basic silica, florisil column, deactivated with 3 % of water and Carbopack B. The instrumental method is described elsewhere (2).

Results and Discussion
Based on information on false-positive results in the CALUX-assay in other laboratories, the sampling of this material by the Dutch authorities (VWA) was intensified. This rapidly resulted in a number of samples that showed a high response in the bioassay which was not supported by the finding of elevated levels of chlorinated dioxins and dioxin-like PCBs. Figure 1 shows the response of a sample compared to the feed reference samples, clearly demonstrating an elevated response. This confirmed the findings obtained by the other laboratories. In 2008 26 samples were analyzed by CALUX of which 10 showed a response indicating a level higher than 1 ng BEQ/kg. The highest observed level was 11 ng BEQ/kg.

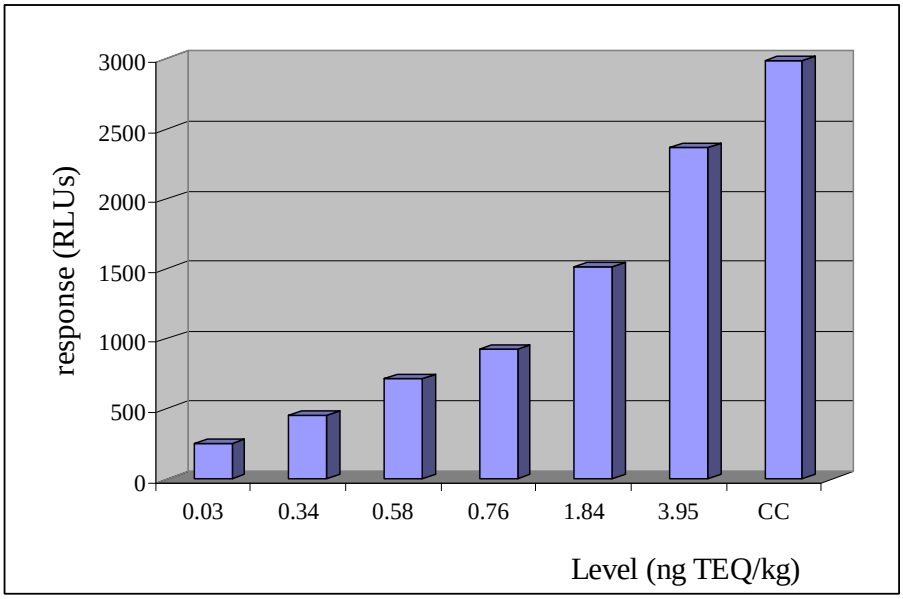

Figure 1. Response obtained in the DR CALUX® assay with a sample of cholin chloride (CC) in comparison to that of the reference feed samples, spiked with dioxins and dl-PCBs

Two samples (no 208908 and 210099) were selected with indicative BEQ-levels of respectively 1.2 and 4.9 ng BEQ/kg. Samples were investigated by GCxGC-TOFMS, resulting in the identification of a number of polybrominated diphenylethers (Table 1). In addition 2,4,6-tribromophenol was detected at levels of 1.1 and 3.6 ng/gram (Table 1). Both samples also showed an unknown peak with a molecular mass of 868. The fragmentation indicated the presence of eight bromines. Searching on the internet indicated that this compound could be a new brominated flame retardant, name FR-1808 or Octabromo-1,3,3-trimethylphenyl-1-indan (OBIND) (Figure 2). A standard was ordered and the identity of the compound was confirmed. Estimated levels were 140 and 700 ng/kg respectively (Table 1). OBIND was tested in the CALUX assay but showed no response.

Table 1. Results of the GC-TOF analysis of two samples of cholin chloride

RIKILT nr.	Material	Brominated flame retardants	Level (ng/kg)
208908	Cholin Chloride 60%	2,4,6-Tribroomfenol	1100
		Octabromo-1,3,3-trimethylphenyl-1-indan	140
210099	Cholin Chloride	2,4,6-Tribroomfenol	3600
		Octabromo-1,3,3-trimethylphenyl-1-indan	700
		PBDE 99	150
		PBDE 49	131
		PBDE 66	123
		PBDE 153	78
		PBDE 47	78
		PBDE 77	26
		PBDE 119	25
		PBDE 100	17

Table 2. Levels of brominated dioxins in two batches of cholin chloride

RIKILT nr.	Material	Brominated dioxins	Level (pg/kg)
208908	Cholin Chloride 60%	2,3,7,8-TBDD	<1.5
		2,3,7,8-TBDF	349
		1,2,3,7,8-PeBDF	69
		2,3,4,7,8-PeBDF	19
		1,2,3,4,7,8-HxBDF	34
210099	Cholin Chloride	2,3,7,8-TBDD	189
		2,3,7,8-TBDF	2263
		1,2,3,7,8-PeBDF	99
		2,3,4,7,8-PeBDF	139
		1,2,3,4,7,8-HxBDF	236

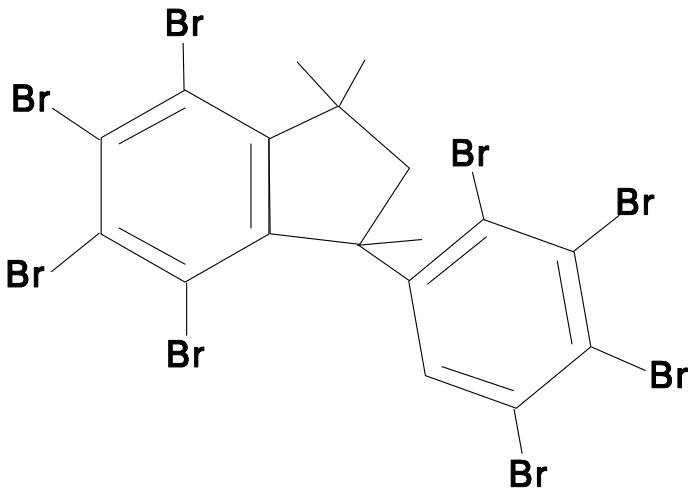

Figure 2: Structure of FR 1808 or Octabromo-1,3,3-trimethylphenyl-1-indan (OBIND)

Levels of the BFRs were in line with the differences in response of the two samples in the CALUX-assay, but these compounds could not explain the elevated response. Therefore, subsequent research focused on the possible presence of brominated dioxins in the two samples. This was performed at the CRL for dioxins in Freiburg. Table 2 shows the levels of the tetra- and pentabrominated dioxins that could be detected, with 2,3,7,8-TBDF being the most important congener. These data confirm that brominated dioxins could at least partly be responsible for the observed response in the bioassay. When applying the same TEFs as for the chlorinated dioxins, levels amounted to respectively 51 and 513 pg TEQ/kg. This is lower than the estimated levels from the bioassay response. However, the response of the different congeners may be different than the TEF values and furthermore, not all congeners were analysed.

Conclusion

This case shows that the follow-up of false-positive samples in the CALUX bioassay may actually result in the detection of novel emerging risks, in this case the presence of brominated flame retardants and brominated dioxins in a widely used feed additive. This included a novel BFR, OBIND, that was not detected in feed or food before. Although the levels observed in the current case may not directly present a risk for the animals and the consumers, it is evident that during the production or handling of this material, it becomes contaminated with industrial chemicals whose introduction in the food chain seems highly undesirable. Follow-up actions focus on tracing the origin of this material.

References

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2. Kotz A., Malisch R., Kypke K., Oehme M. *Organohalogen Compounds* 2005;67,1540.