FACTORS AFFECTING THE TEST RESPONSE IN THE DR CALUX® BIOASSAY

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Introduction

Thus far the CALUX assay is the only bioanalytical test used for official control and monitoring of food and feed samples. Since negative samples need no further examination by a confirmatory method, it is essential that the application of this test does not lead to false-compliant results. For this purpose a number of performance criteria have been set which are described in EU-regulations EC 152/2009 (food) and EC 1883/2006 (feed). Any analysis performed to demonstrate that samples are compliant with the EU-limits should fulfill these criteria. These criteria were originally drafted by a group of experts and presented at Dioxin 2001. Recently the criteria for bioanalytical screening methods were reevaluated and adapted by an expert group within the EURL/NRL network. A draft proposal was presented at Dioxin 2010, but some slight changes were made afterwards. It is foreseen that these revised criteria will become effective in 2011.

A critical issue are the requirements for assuring that application of the test does not result in false-negative (false-compliant) results. It was proposed to increase the acceptable false-negative rate from 1 to 5% primarily to facilitate the validation of the test. A false-negative rate of 1% requires the testing of many positive samples to show that the test will not overlook more than 1 in 100 positive samples. This can be performed by repeated analysis of spiked samples during validation and appropriate statistical analysis of the results, but again a percentage of less than 1% is hard to prove. This is also reflected by the criteria set for screening methods for other components like veterinary drugs and hormones where also 5% is applied. In addition, a rate of 1% would also affect the setting of the so-called decision limit, being the cut-off value where it is decided whether samples are negative or suspected, meaning that they should be send to a confirmatory analysis. This calculation is based on levels around the limit and the variation in the test result. Application of a 1% criterium would result in decision limits that would end up in the background levels and as such render the application of any screening tool useless, since it would create too many suspected samples.

At the same time it is clear that application of a screening assay should not result in overlooking of any sample that clearly exceeds the limit. It is therefore of interest to know which factors may influence the test result. Sample extracts might e.g. contain compounds that could decrease the response of the cells. This can partly be overcome by looking at the appearance of the cells after incubation with the extracts, in order to exclude potential cytotoxic effects. However, even if this is not the case, a potential effect of compounds on the cell response can never be excluded entirely. For that reason the analysis of a certain fraction of the samples with a negative test result by GC/HRMS is thought to be essential for quality control. Even better is the analysis and test evaluation of many positive samples during an incident (1), but such samples are not always available. Also proficiency tests for screening methods should focus on testing of a set of positive and negative samples rather than one sample. In order to further exclude false-compliant results due to the presence of compounds suppressing the response, it was included in the revised criteria that at least part of the extracts should also be tested with a spike of TCDD. If the response deviates too much from that of a blank extract with TCDD, the test result cannot be evaluated.

In practice the congener pattern may also affect the response of samples in the test, due to differences between the official TEF values and the relative responses (REPs) of the different congeners in the bioanalytical tests on the so-called apparent recovery. This apparent recovery is determined from ratio between the estimated level (in BEQs) and the level determined by GC/HRMS (TEQs), and as such covers not only the real recovery of the analytes during extraction and clean-up, but also other factors influencing the response. Previous studies showed that there is a linear correlation but that the actual REPs are different from the TEFs. At the same time there is some variation in the REPs established by different laboratories. For this reason we reinvestigated the REPs of the 29 compounds of interest and studied the impact on a number of different congener patterns.

At the same time it is clear that the test may not only detect these 29 compounds but also other compounds that activate the Ah-receptor pathway and actually end up in the final extracts of test samples. It is important to investigate which compounds may actually cause a response in the test under routine conditions. It has e.g. been

shown that brominated dioxins will cause such a response and the question is whether this should be regarded as a false-positive result, despite the fact that they are not (yet) included in the limits. Regarding an overestimation of levels in corn and eggs during the dioxin incident in 2010, and the detection of several non-2,3,7,8-substituted PeCDFs in these samples, we studied the response of these congeners in the CALUX-assay.

Materials and methods

Materials
Standards of the seventeen 2,3,7,8-chlorinated PCDD/Fs and 12 dioxin-like PCBs were purchased from CIL. They were dissolved and diluted in DMSO and subsequently analysed by GC/HRMS in order to determine the levels and the presence of potential impurities. Standards of 16 different pentachloro-CDFs were purchased from Wellington, dissolved in DMSO and diluted.

DR CALUX-assay
The DR CALUX assay was performed as described previously (1,2). The test is based on the use of a modified rat H4IIE cell-line with the 1.1-GudLuc transcript and is commercialized by BDS in Amsterdam.

GC/HRMS
GC/HRMS was performed as described previously (3).

Results and discussion

REP values
The different PCDFs, PCDDs and dl-PCBs were tested in the DR CALUX® assay. The concentrations were corrected for the levels determined by GC/HRMS in the DMSO stock solutions. The levels were not in all cases identical to the levels intended. The data were subsequently fitted with the Hill-equation, following subtraction of the blank DMSO response. This resulted in a maximal response value and an EC50 value, being the concentration showing a half-maximal response. The ratio between the EC50 values of a certain PCDD/F or dl-PCB and TCDD was calculated to determine the REP. These are presented in Table 1 and compared with the data we obtained in 1998 and those obtained by other groups (4,5,6). In our previous study we only investigated the REPs of the compounds that contribute most to the TEQ-level in the normal background levels in Dutch milk. In general there was a good agreement between the REPs obtained now and back in 1998. However, the REPs of PeCDD and PCB 126 seemed to be even lower. It is also clear that the mono-ortho PCBs show in general a very poor response which is acknowledged by the new TEFs 2005. These values first of all stress the need to obtain consensus on these REP-values both in rat and in mouse cells used for CALUX-assays but also in other bioanalytical assays. In addition these differences between TEFs and REPs support the requirement to correct for these values when estimating the level in samples and deciding on compliance of samples. Only using a TCDD-curve seems to result in a serious underestimation of the levels, at least for the H4IIE-cells.

Impact of REP-values on apparent recoveries for different profiles
In order to estimate the impact of the difference between REP and TEF values for different congener profiles we applied these values on the TEQ-levels for PeCDD/Fs in a number of incidents with different congener patterns. This included the PCB-contaminated feed from Belgium, choline chloride mixed with sawdust contaminated with pentachlorophenol, carbosan copper, the dried bakery waste from the 2003 incident, the organic Ukrainian corn from 2010 and the fatty acids from the 2011 incident in Germany. When compared with the TEFs of 1998, the REP-based results were respectively 62, 107, 66, 66, 61%, 75 and 61% of the value, meaning an underestimation up to 39%. Only in the case of the choline chloride, there was an overestimation of the level due to the relatively high REPs for the higher chlorinated HpCDD, OCDD and OCDF. Since PCB 126 is by far the most important dl-PCB in most samples, this would result in a 50% underestimation of the level. For wild eel from Dutch rivers the difference is much higher due to the relative contribution of the mono-ortho PCBs. This will however change with the new TEFs 2005.

Table 1. Comparison of TEF values and REP values obtained in the DR CALUX®-bioassay with H4IIE pGudLuc 1.1 cells, as reported by four different groups. A check on the correct concentration of the stock solutions in DMSO by GC/HRMS was only reported by Bovee *et al.* (1998) and in this study.

Congener	WHO-TEF 1998	REP DR CALUX® Bovee *et al.* 1998	Laier *et al*. 2003	Benisch *et al*. 2004	Scippo *et al* 2004.	This study
2,3,7,8-TCDF	0.1			0.3	0.4	0.07
1,2,3,7,8-PeCDF	0.05			0.2	0.1	0.13
2,3,4,7,8-PeCDF	0.5	0.3	0.6	0.5	0.4	0.29
1,2,3,4,7,8-HxCDF	0.1			0.1	0.1	0.07
1,2,3,6,7,8-HxCDF	0.1	0.07		0.04	0.09	0.05
2,3,4,6,7,8-HxCDF	0.1			0.2	0.1	0.05
1,2,3,7,8,9-HxCDF	0.1			0.1	0.1	0.09
1,2,3,4,6,7,8-HpCDF	0.01			0.03	0.01	0.02
1,2,3,4,7,8,9-HpCDF	0.01			0.04	0.05	0.03
OCDF	1.10^{-4}			0.007	0.004	0.01
2,3,7,8-TCDD	1	1	1	1	1	1
1,2,3,7,8-PeCDD	1	0.5	1	0.5	0.5	0.27
1,2,3,4,7,8-HxCDD	0.1			0.3	0.1	0.08
1,2,3,6,7,8-HxCDD	0.1		0.3	0.1	0.06	0.05
1,2,3,7,8,9-HxCDD	0.1			0.07	0.06	0.05
1,2,3,4,6,7,8-HpCDD	0.01			0.05	0.03	0.01
OCDD	1.10^{-4}			0.0005	0.0008	0.002
PCB 81	1.10^{-4}			0.004	0.002	0.01
PCB 77	1.10^{-4}			0.001	0.0004	0.001
PCB 126	0.1	0.07	0.2	0.07	0.04	0.05
PCB 169	0.01	0.002		0.003	0.0008	0.002
PCB 123	1.10^{-4}			2.10^{-5}	nr	3.10^{-6}
PCB 118	1.10^{-4}	5.10^{-6}	3.10^{-6}	ndrc	nr	1.10^{-6}
PCB 114	5.10^{-4}			5.10^{-5}	2.10^{-5}	1.10^{-4}
PCB 105	1.10^{-4}	2.10^{-6}		1.10^{-5}	nr	4.10^{-6}
PCB 167	1.10^{-5}			1.10^{-5}	nr	1.10^{-6}
PCB 156	5.10^{-4}	4.10^{-5}	2.10^{-4}	2.10^{-4}	2.10^{-5}	6.10^{-5}
PCB 157	5.10^{-4}			1.10^{-4}	nr	5.10^{-5}
PCB 189	1.10^{-4}			1.10^{-5}	nr	2.10^{-9}

nr: no response obtained
ndrc: no dose-response curve

Overall this demonstrates that reference materials used to estimate the apparent recovery or estimate the BEQ-level should not contain only TCDD but rather a mix of the different congeners with a similar 30-40% underestimation of the level as in the case of most samples tested in practice.

Impact of non 2,3,7,8-chlorinated congeners

In 2010 there was an incident with contaminated eggs in the Netherlands, with levels up to 2-3x the dioxin limit. Samples of corn were also tested by DR CALUX assay and in general showed a strong overestimation of the level. Similar was observed for eggs from hens fed with this corn (Figure 1). Although there was an excellent correlation between results from CALUX and GC/HRMS, levels were overestimated by a factor of 2.7. The overestimation up to 1.5-fold could be explained by the difference in the congener patterns between the reference samples and the samples from the incident and the impact of the REP/TEF differences. However,

analysis by GC/HRMS revealed that in addition to the TCDF and the two PeCDFs a number of other peaks showed up in the chromatograms indicating the presence of other non-2,3,7,8-substituted PeCDFs, not only in the corn but also in the eggs. A number of these PeCDFs were purchased and tested in the bioassay, showing that at a concentration of 100 nM most of these compounds showed a maximum response after a 24 h incubation of the cells. This level is about 100x the level of TCDD showing a maximum response and further studies are performed to determine the REP-values of these compounds. In addition GC/HRMS analysis will be performed to determine which PeCDFs are present in the corn and eggs. Also in other incidents it has been shown that especially the original products may contain many other PeCDD/Fs which may as such contribute to the response. Once being absorbed by animals this may change due to specific metabolism, although this does not seem to be the case in laying hens. These data clearly show that an overestimation of levels in products, especially during incidents, may actually be explained by other dioxins. This may require an adaptation of the test strategy during an incident. At the same time this overestimation decreases the chance on false-compliant results during routine monitoring.

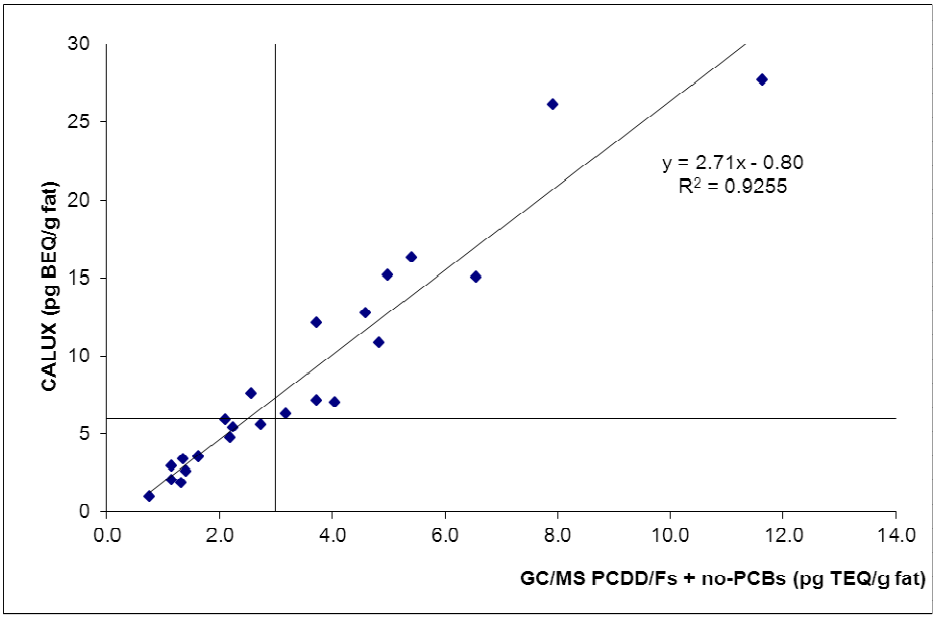

Figure 1. Comparison the GC/HRMS determined levels of PCDD/Fs and no-PCBs in eggs and the levels estimated by DR CALUX. Levels were estimated by comparison of the response with that of a set of butter fat samples spiked with PCDD/Fs and dl-PCBs.

An essential point in this regard is the use of so-called incurred materials as reference samples. Since these may also contain non-2,3,7,8-substituted congeners or other compounds that cause a response in the test, their use for recovery correction could lead to an underestimation of the level in the test-sample. Therefore, reference samples should be blank samples with a low response in the test and spiked with a set of standards.

References

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