Determination of polychlorinated dibenzodioxins and polychlorinated dibenzofurans (PCDDs/PCDFs) in food and feed using a bioassay: Result of a validation study

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Introduction

It is estimated that more than 90% of dioxins consumed by humans come from foods derived from animals. The European Commission through a Council Regulation (No 2375/2001) and a Directive (2001/102/EC), both revised by the Commission Recommendation (2002/201/EC), has set maximum levels for dioxins in food and feedstuffs. To implement the regulation, dioxin-monitoring programs of food and feedstuffs will be undertaken by the Member States requiring the analysis of large amounts of samples. Food and feed companies will have to control their products before putting them into the market. The monitoring for the presence of dioxins in food and feeds needs fast and cheap screening methods in order to select samples with potentially high levels of dioxins to be then analysed by a confirmatory method like HRGC/HRMS. Bioassays like the DR CALUX®-assay have claimed to provide a suitable alternative for the screening of large number of samples, reducing costs and the required time of analysis[i]. These methods have to comply with the specific characteristics considered into two Commission Directives (2002/69/EC; 2002/70/EC), establishing the requirements for the determination of dioxin and dioxin-like PCBs for the official control of food and feedstuffs. The European Commission's Joint Research Centre is pursuing validation of alternative techniques in food and feed materials. In order to evaluate the applicability of the DR CALUX® technique as screening method in compliance with the Commission Directives, a validation study was organised in collaboration with CSL and RIKILT.

The aim of validating an analytical method is first to determine its performance characteristics (e.g. variability, bias, rate of false positive and false negative

results), and secondly to evaluate if the method is fit for the purpose. Two approaches are commonly used: an in-house validation is preferentially performed first in order to establish whether the method is mature enough for being tested in a collaborative trial. A number of reports have described the in-house validation of the DR CALUX®-assay, thus allowing proceeding to the second step, an interlaboratory trial. In this step a set of chicken feeds and fish oil samples at four different levels of contamination were analysed by both experienced and newly trained laboratories.

Methods and Materials

Cells and chemicals

Cells were kindly supplied by BDS in Amsterdam, The Netherlands, which also participated in a training course at the JRC in Ispra, Italy. The DR CALUX® technique uses a genetically modified cell-line (rat-hepatoma H4IIE GudLuc 1.1) that expresses the firefly luciferase upon exposure to dioxins or dioxin-like compounds. The amount of luciferase is related to the amount of dioxin-like compounds in the exposure mixture and can be easily quantified with a luminometer.

Prior to analysis, samples require a simple clean-up procedure based on fat extraction (feed), clean-up over acid-silica columns and removal of the solvents. With some exceptions, sample intake was 9 gram for feed and 1 gram for fish oil. Blank reagents were included in each series. The final extract was dissolved in 25 to 40 µl DMSO that was then added to the culture medium at a final DMSO concentration of 0.4 or 0.8%. Cells were exposed for 24 h and subsequently lysed, allowing quantification of the luciferase. Each 96-multiwell test plate contained a TCDD calibration curve, prepared from stock solutions in DMSO that in most cases were supplied by BDS (prepared from a CIL-standard in DMSO).

Validation study

The DR CALUX® technique was evaluated in a collaborative trial, based on international recognised guidelines [ii,iii]. Fourteen laboratories participated in the validation study which was carried out in three steps with increasing complexity of the test material, from simple TCDD standard solutions at eight different concentrations, a clean feed extract both with and without spiked dioxins (internal control), and eventually the two sets of nine coded samples. The two matrices chosen were fish oil and a compound feedstuff, each at four different concentrations and in duplicate. The proposed concentration levels were set in line

with recently established limits for dioxins respectively in food and feed (Council Directive 2375/2001 and 2001/1002). In addition, one sample from each matrix was provided for allowing a recovery correction. The feed was a chicken feed prepared with soy oil spiked with dioxins, dioxin-like PCBs and indicator PCBs at three different levels. Relative contribution of dioxins and dioxin-like non-orho and mono-ortho PCBs to the total TEQ was 52, 30 and 18%. The fish oil was an incurred sample, used in a FAPAS ring trial and shown to contain 9, 24 and 8 pg TEQ/g of dioxins, non-ortho and mono-ortho PCBs. The sample was diluted with linseed oil that contained no detectable levels of dioxin-like compounds and showed no elevated response in the DR CALUX®-assay.

With the statistical evaluation the submitted results were used to estimate the average and the standard deviations under repeatability and reproducibility conditions for the determination of the method performance characteristics. Prior to this, the results were screened for the presence of extremes mainly due to either high background levels or a total lack of a dose-related response. Statistical assessment has been carried out applying the analysis of variance approach as recommended in the ISO guideline[ii] applying robust statistics.

Results and Discussion

The 14 laboratories participating in the study were six laboratories that purchased/obtained the test from BDS previously and eight governmental institutes volunteering to participate after a request from JRC. The latter partners were trained during a ten-days course at the JRC. They subsequently set up the test within their institute, trained with standards, milk fat samples and spiked feed extracts, and subsequently received the coded samples, all within a period of 1 year.

In principle, samples were tested according to the protocols supplied by BDS, with minor modifications at some laboratories that were more experienced with the assay itself or the analysis of dioxins in general. A TCDD calibration curve was used to translate the response obtained with the sample extracts into a dioxin level in feed or fish oil. All partners were able to produce the required calibration curves with comparable characteristics, as shown by the EC50 value of 11 ± 3 pM (mean ± SD, n=14). Most partners tested the extracted fish oil and feed samples only at one dilution, i.e. without further dilutions of the final extracts prepared in DMSO or medium. This may result in an underestimation of the result, although only occasionally the response obtained with the extracts of the higher contaminated samples showed a response higher than 50% of the maximum response obtained in the TCDD calibration curve. None of the sample extracts showed a response below

5% of the maximum response. Respectively four and three laboratories were excluded prior to statistical analysis of the data for feed and fish oil, due to high background levels and/or the total lack of a dose-related response.

Tables 1 and 2 show the results obtained with feed and fish oil by the different partners. Data were not corrected for blank chemicals. Some laboratories (e.g. numbers 7 and 11) suffered from a high background, which is unlikely to be caused by dioxins or dioxin-like PCBs in the chemicals. In the case of number 7, the response of the blank chemicals accounted for 72 and 56% of the levels obtained respectively in the clean feed and fish oil (or better: linseed oil) samples. In the case of partner number 11 the contribution was even less, being 42 and 45%. This may either be explained by other Ah-receptor agonists in the feed or oil, or by the fact that the behaviour of contaminants on e.g. the acid silica columns may be different in the presence of sample matrix. This may be overcome by using cleaner chemicals and by using blank samples rather than blank chemicals in the test series for correction.

Individual results were used to determine the average, repeatability and reproducibility, using robust statistics. Overall the laboratories were able to differentiate the levels in the different feed and fish oil samples. This is demonstrated in Figure 1, showing a comparison between the GC/MS level in feed and the mean of the duplicate analysis with DR CALUX®, corrected for the blank samples, as obtained by the ten laboratories. Results of RIKILT (no. 0) are included and were at the higher range of the levels. The five best correlations showed a correlation coefficient higher than 0.97. Three of these laboratories were experienced with DR CALUX® analysis and two new ones were very experienced with GC/MS analysis of dioxins. In the case of the fish oil, the 5 best laboratories showed a correlation coefficient higher than 0.99, again with the best results for the laboratories most experienced with the test or dioxin analysis in general. This shows that the clean-up procedure, in the absence of suitable recovery standards, requires proper training and experience.

Figure 1 also shows that after correction for the blank feed, the levels determined in feed by DR CALUX® were only 30-50% of the levels determined by GC/MS. Part of this apparent recovery loss is due to the difference in TEF values and the relative response in the CALUX-assay (REP). Based on the levels of the individual congeners and the REP factors reported previously, it can be calculated that the maximum recovery could be around 55% for both the feed and fish oil. This is partly due to the insensitivity of the cells for the mono-ortho PCBs and the difference for the relatively important PCB 126 with an REP around 0.07 as compared to the TEF of 0.1. In the case of the fish oil, recoveries appeared to be

much higher, but, being an incurred sample, it cannot be excluded that this oil contains other Ah-receptor agonists.

The overall figures obtained for repeatability and reproducibility (Table 1, 2) are relatively high, especially when calculating safe action levels based on the obtained repeatability. Such an action level is used to decide whether a sample is subjected to GC/MS analysis or whether it can be considered as negative. As shown by the data, correction for background seems a requirement, but only partly resulted in a clear improvement of repeatability and reproducibility. Similar is true for recovery correction. When using the means of the duplicate analysis for each laboratory, the reproducibility improved, showing values of 43, 23 and 37% for the 3 feed samples, and 44, 28 and 30% for the 3 fish oil samples. According to the EU guideline, the use of duplicates is a requirement, when applying the quantitative approach. In practice the variation should be lower within part of the laboratories but the current set-up did not allow the evaluation.

Based on the experience with the calibration curves, the DR CALUX® bioassay on itself is a suitable test, which can easily be introduced in a laboratory with tissue culture experience. The weak spot of this test is the requirement of a clean-up without internal standards that could be used for correction for recovery losses. This requires proper standardization of the initial extraction, the clean-up on acid silica and the evaporation of the solvents. Furthermore, the data clearly show that the EU-requirement to include a set of control samples for correction for background, recovery and differences between the TEF values and the response factors in the test, is essential for the test.

Table 1. Total levels of dioxins and dioxin-like PCBs in feed, as determined by 10 laboratories with the DR CALUX® bioassay.

Feed	GC/MS	Laboratory										Statistical parameters					
	ng TEQ/kg	1	2	3	4	5	6	7	8	9	10	Av	Unc.	sr	RSD_r	sR	RSD_R
1	0.04	0.39	0.17	0.30	0.30	0.23	0.01	1.18	0.16	0.08	0.21	0.25	0.14-0.36	0.09	37	0.18	71
		0.33	0.19	0.50	0.34	0.12	0.05	1.00	0.19	0.05	0.44						
2	0.76	1.00	0.66	0.73	0.92	0.80	0.36	1.96	0.21	0.31	0.94	0.69	0.46-0.93	0.25	37	0.37	53
		0.80	0.32	1.22	0.53	0.63	0.29	1.41	0.40	0.43	0.68						
3	1.85	1.00	1.01	1.13	1.30	0.90	0.84	2.61	0.49	0.83	1.03	1.03	0.87-1.18	0.14	14	0.25	24
		0.88	0.91	1.14	0.73	1.48	0.87	2.18	0.53	0.68	1.01						
4	3.95	2.14	2.17	1.40	0.45	1.80	2.20	3.61	1.25	1.33	0.37	1.63	1.15-2.10	0.85	52	0.75	46
		1.91	1.49	1.81	1.17	1.18	0.68	2.88	0.72	1.64	2.07						
Rec	1.85	1.23	1.08	1.21	1.23	0.73	0.74	1.86	0.55	0.68	0.36						

Table 2. Total levels of dioxins and dioxin-like PCBs in feed, as determined by 11 laboratories with the DR CALUX® bioassay.

Fish oil	GC/MS												Statistical parameters					
	ng TEQ/kg	1	2	3	5	6	7	8	9	10	11	12	Av	Unc.	sr	RSD$_r$	sR	RSD$_R$
1	0.0	2.9	2.7	3.0	2.5	1.0	8.5	1.4	1.3	1.5	5.2	2.8	2.2	1.5-3.0	0.8	35	1.3	57
		2.7	2.3	1.6	2.7	0.8	6.0	0.8	1.1	1.9	2.6	1.4						
2	4.3	4.3	2.4	5.3	3.9	1.9	10.5	2.3	2.9	5.4	6.2	4.1	4.5	3.4-5.6	0.8	19	1.8	40
		4.8	6.4	5.8	3.5	2.8	10.6	3.9	2.7	7.0	5.3	3.1						
3	8.6	7.8	8.2	7.1	9.5	4.6	16.2	5.8	4.8	8.2	6.6	4.8	7.2	5.5-9.0	1.2	16	3.0	41
		6.0	4.1	8.1	10.2	4.1	17.2	5.2	5.2	13.6	8.5	4.0						
4	17.1	14.1	13.2	6.6	13.9	6.7	22.2	9.5	8.2	21.2	10.8	6.8	11.4	8.7-14.1	2.9	25	4.5	40
		12.4	10.3	13.2	13.3	7.2	27.6	5.8	10.0	14.4	12.2	9.7						
Rec	8.6	1.9	3.5	8.6	12.5	4.3	12.7	5.3	1.1	9.0	4.5	6.8						

Av: average; Unc.: uncertainty interval with α=0.05, lower and upper confidence limits; sr: within laboratory standard deviation (repeatability); RSD$_r$: relative within laboratory standard deviation (repeatability); sR: between laboratory standard deviation (reproducibility); RSD$_R$: relative between laboratory standard deviation

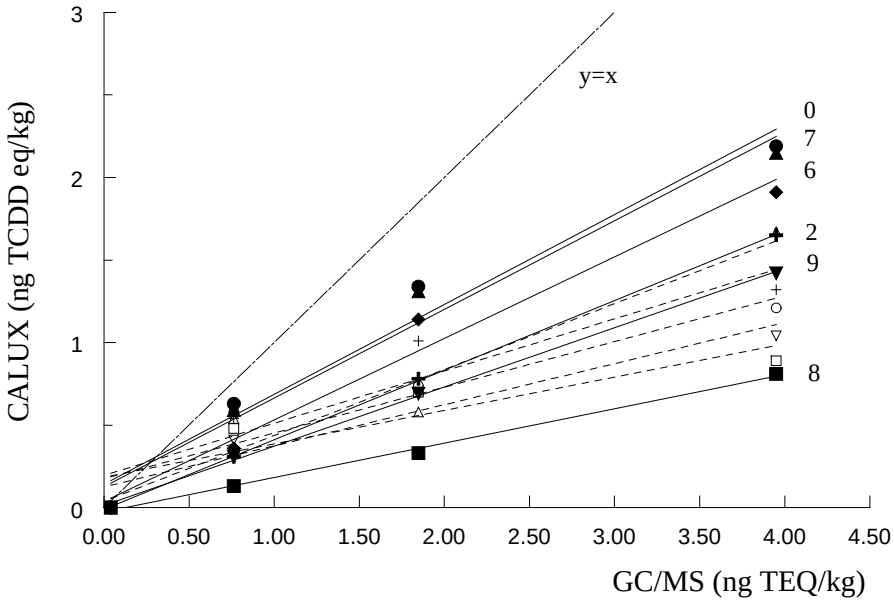

Figure 1. Comparison of GC/MS and DR CALUX® determined levels in the chicken feeds, as determined by the different laboratories (corrected for the blank feed). Linear regression curves are shown and those with a correlation coefficient higher than 0.97 are indicated with solid lines and marked with the laboratory. Data from RIKILT are included (no. 0).

References

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