Validation of extraction, clean-up and DR CALUX® bioanalysis. Part I: feedingstuff

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Introduction

Feed safety is a high priority issue for the feed sector as it directly impacts at the beginning of the food-chain. Currently stringent EU limit values are in force for dioxins in feedingstuffs[1] for animal and public health protection. Biodetection Systems BV's (BDS) DR CALUX® bioassay is a cost-effective and rapid method to measure low levels of dioxins and dioxin-like compounds in various matrices[2,3]. The use of bioassays for monitoring dioxins in feed allows the (pre)-selection of samples suspected of being contaminated above limit values with dioxins. To permit bioassays to be used for screening feedingstuffs for the presence of dioxins and related compounds, the EU has laid down general requirements for the determination of dioxins and dioxin-like PCBs in feedingstuffs and specific requirements for cell-based bioassays[4]. To ensure reliability the DR CALUX® bioassay, validated methods for extraction and DR CALUX® analysis are necessary. Within the framework of the development of DR CALUX® analysis methods, extraction and clean-up methods for 13 feedingstuffs were evaluated, selected and validated. In this paper we present the results of this substantive multi-matrix feedingstuffs validation study.

Methods and materials

DR CALUX® bioanalysis The procedure for the DR CALUX® by BDS bioassay is described in details previously[5]. Briefly, the bioassay is performed using a rat hepatoma H4IIE cell line stably transfected with an AhR-controlled luciferase reporter gene construct. Cells were cultured in α-MEM culture medium supplemented with 10% ($^{v}/_{v}$) FCS under standard conditions (37°C, 5% CO_2, 100% humidity). Following fat extraction and clean-up on a acidic silica column, samples are redissolved in DMSO. Cells were exposed in triplicate to cleaned extracts for 24 hours in 96-well microtiterplates. After incubation, the cells were lysed. A luciferine containing solution was added and the luciferase activity was measured using a luminometer equipped with 2 dispensers. Each 96-well microtiterplate contained a complete 2,3,7,8-TCDD calibration range (0 – 300 pM 2,3,7,8-TCDD per well). Total DR-CALUX® TEQ content in the samples analysed was determined by interpolation in the fitted 2,3,7,8-TCDD calibration curve.

Selection of extraction and clean-up method. A total of 13 different feed matrices were evaluated (see table 3). Samples were obtained either from local retailers or the feed-industry. All matrices were extracted by sonification, shake-, soxhlet-, and/or ASE-extraction. Spiked samples (mixture of dioxins, furans and non-ortho-PCBs) were extracted, cleaned and analysed using the DR CALUX® bioassay to determine the recovery. For each matrix tested, the most suitable extraction method was chosen for further validation.

Sample	Week 1		Week 2		Week 3		Week 4	
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
1	$\mathbf{x_1\ x_2}$				**y**			
2	**y**				$\mathbf{x_1\ x_2}$			
3		$\mathbf{x_1\ x_2}$				**y**		
4		**y**				$\mathbf{x_1\ x_2}$		
5			$\mathbf{x_1\ x_2}$				**y**	
6			**y**				$\mathbf{x_1\ x_2}$	
7				$\mathbf{x_1\ x_2}$				**y**
8				**y**				$\mathbf{x_1\ x_2}$

$$S_{r(rel)} = 100 * \left(\frac{\sqrt{\frac{\sum_{i=1}^{n} (x_{i1} - x_{i2})^2}{2n}}}{\left(\frac{\sum_{i=1}^{n} x_{i1} + \sum_{i=1}^{n} x_{i2}}{2n} \right)} \right) \qquad S_{R(rel)} = 100 * \left(\frac{\sqrt{\frac{\sum_{i=1}^{n} (x_{i1} - y_i)^2}{2n}}}{\left(\frac{\sum_{i=1}^{n} x_{i1} + \sum_{i=1}^{n} y_i}{2n} \right)} \right)$$

$S_r(rel)$ = repeatability (relative)
$S_R(rel)$ = reproducibility (relative)
n = number of samples tested
x_1 = first determination
x_2 = second determination
y = third determination

Figure 1 Validation scheme used to determine the repeatability and reproducibilty

Validation. The validation protocol followed was based on Netherlands Standardization Institute (NEN) 7777:2001, incorporating ISO 5725-2/4:1994. In addition, the validation protocol complied with the general requirements for the determination of dioxins and dioxin-like PCBs in feedingstuffs and specific requirements for cell-based bioassays as detailed in EU Commission Directive 2002/70/EC. Matrices were categorised according to extraction method and maximum EU levels. For each category, a total of 8 samples were selected, extracted, purified and analysed 3 times by DR CALUX® as indicated in the validation scheme (Figure 1). For a limit number of samples per category, HRGCMS analysis results were available. Method robustness was evaluated by comparing alterations in extraction, clean-up and bioanalysis procedures.

Results and Discussion

The validation of the DR CALUX® bioassay itself has been described in detail elswhere[1]. Table 1 summarises the major findings of the DR CALUX® bioassay validation study. The limit of detection (LOD) was calculated as 3 times the standard deviation of the DMSO blank (0 pM 2,3,7,8-TCDD) whereas the limit of quantitation (LOQ) was calculated as 10 times the standard deviation of the DMSO blank. For 10 determinations of the LOD and LOQ, the LOD varied between 0.04 and 0.25 pM 2,3,7,8-TCDD per well whereas the LOQ varied between 0.12 and 0.88 pM 2,3,7,8-TCDD per well. Finally, an overall LOD and LOQ was calculated as the average of 10 observations plus 3 times the standard deviation resulting in a LOD and LOQ of 0.3 and 1 pM 2,3,7,8-TCDD per well respectively. The percentage standard deviation between triplicate analyses

on the same 96 well microtiterplate was well within 15% in accordance with performance criteria set by BDS for the DR CALUX® bioassay.

Since the DR CALUX® is used as a rapid and reliable screening tool (either quantitative or qualitative), the choice of extraction and clean-up method for each matrix was based on the recovery, turnaround for extraction and simplicity of the method. The robustness of the chosen methods for extraction, clean-up and analysis were tested by comparing the effect of alterations in standard procedures

Tabel 1 LOD, LOQ, repeatability and reproducibility of the DR CALUX® by BDS bioassay.

	Range (pM DR CALUX® TEQ/well)	**99% confidence**		
LOD	0.04 – 0.25 (n = 10)	0.3	**Repeatability S_r (%)**	17.0% (n = 20)
LOQ	0.12 – 0.88 (n = 10)	1.0	**Reproducibility S_R (%)**	16.9% (n = 20)

Tabel 2 Comparison between various extraction, clean-up and exposure procedures to determine the robustness of the applied methods

Comparison of various procedures	**n**	**slope (a)**	**R^2**
sonification vs shake-extraction	26	1.1	0.95
1.75 g vs 0.5 g fat clean-up capacity	15	1.0	0.95
0.8% vs 0.4% DMSO incubation	90	1.0	0.99

Table 3 Categorisation of matrices and amount of material processed for validation

Category	**Type of matrix**	**Matrices tested**	**Processed** (g)
1	Feed materials of plant origin (extraction method A)	Citrus pulp / Maize gluten feed / Soya shred	9
2	Feed materials of plant origin (extraction method B)	Coconut meal / Wheat meal / Pig feed / Chicken feed	9
3	Feed materials of plant origin (extraction method C)	Palm kernel cake / Sunflower shred	9
4	Feedingstuffs for fish	Fish feed / Fish meal	9
5	Fish and fish products	Fish (various)	9
6	Fish oil	Fish oil	1

on the final DR CALUX® result. In table 2 correlations between the original procedure and the altered procedure are given. The coefficient of determination between the various methods was in all cases greater than 0.95 indicating the robustness of the methods under investigation.

To further categorise the matrices, the maximum EU-limits for dioxins and related compounds were taken into account. Table 3 shows the 6 categories established for the tested feed matrices. In addition, the amount of material processed for validation is given. Applying the amount of material for extraction, the LOQ for all of the matrices is at least 6 times lower than the maximum EU-limit values (see table 4). Lower LOQs can readily be obtained by increasing the amount of material processed.

The majority of the DR CALUX® analysis results for all samples in all 6 categories were below the maximum EU-limit levels (data not shown). Furthermore, analysis results included

Table 4 Matrix-specific Limit of Quantitation (LOQ), repeatability and reproducibility

Category	**LOQ** (ng CALUX® TEQ /kg product)	**Max. EU-limits** (ng WHO-TEQ /kg product)	**Repeatability** S_r (%)	**Reproducibility** S_R (%)
1	0.11	0.75	19.2	15.6
2	0.11	075	11.2	12.7
3	0.11	0.75	12.1	26.4
4	0.11	2.25	13.9	26.6
5	0.11	1.25	12.5	25.1
6	1.0	6	26.0	14.1

Maximum EU-limit values for dioxins and dioxin-like compounds in feed based on:

- Commission Directive 2003/57/EC, amending Commission Directive 2002/32/EC of the European Parliament and of the Council on undesirable substances in animal feed (19-6-2003)

samples at 0.5 times and 2 times maximum EU-limit values, demonstrating the performance of the DR CALUX® bioassay in the range of the level of interest[4]. In table 4 the repeatability and reproducibility of the validated methods for the various categories are given. In all case, the repeatability is 26% or less and reproducibility is 27% or less. In some cases observed repeatability or reproducibility is biased by a single analysis result that is different compared to the two other analysis results. Average repeatability for the 6 categories was calculated to be 15.9% and average reproducibility was calculated to be 20.1%. Correctness of the applied methods for the extraction, clean-up and DR CALUX® bioassay for feedingstuffs ranged from 79 to 93%.

Overall conclusions

The following conclusions can be made:

- It appeared that several different feed matrices required different extraction methods. Accordingly, feed materials were categorized in groups sharing identical extraction methods.
- The selected methods are shown to be robust and practical. Straightforward and practical methods for extraction were selected to avoid additional variation due to complex handling.
- For all matrices tested, the LOQ observed was at least 6 times below the maximum EU-limits set for the tested matrices.
- The repeatability and reproducibility were all within the required range of precision.

- The performance criteria for bioassays as described by Behnish et al[6] and incorporated in Commission Directive 2002/70/EC[4] are met for the methods used, showing that the DR CALUX bioassay can be used for feedstuffs dioxins compliance monitoring within the EU.

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References

[1] Commission Directive 2003/57/EC, amending Commission Directive 2002/32/EC of the European Parliament and of the Council on undesirable substances in animal feed (19-6-2003).
[2] Bovee, T.F.H., Hoogenboom, L.A.P., Traag, W.A., Zuidema, T., Hortsman, J.H.J., Aarts, J.M.M.J.G., Murk, T.J., Brouwer, A., Denison, M.S., Kuiper, H.A. (1996). Organohalogen compounds 27, 303.
[3] Hoogenboom, R., Portier, L., Onstenk, C., Polman, T., Hamers, A. and Traag, W. (2000) Organohalogen Compounds. 45, 180.
[4] Commission Directive 2002/70/EC of 26 July 2002. Establishing requirements for the determination of levels of dioxins and dioxin-like PCBs in feedingstuffs
[5] Besselink, H.T., Schipper, C., Klamer, H., Leonards, P., Verhaar, H., Felzel, E., Murk, A.J., Thain, J., Hosoe, K., Schoeters,G., Legler, J. and Brouwer, B. (2004) Environm. Toxicol. Chem. In press.
[6] Behnisch, P.A., Allen, R., Anderson, J., Brouwer, A., Brown, D.J., Campbell, T.C., Goeyens, L., Harrison, R.O., Hoogenboom, R., Van Overmeire, I., Traag, W. and Malisch, R. (2001) Organohalogen Compounds. 50, 59.