# Validation of extraction, clean-up and DR CALUX® bioanalysis. Part II: foodstuff

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## Introduction

**Food safety is a high priority issue for the food sector as it directly impacts the human health. Currently stringent EU limit values are in force for dioxins in foodstuffs[1] for 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 food allows the (pre)-selection of samples suspected of being contaminated above limit values with dioxins. To permit bioassays to be used for screening foodstuffs 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 foodstuffs 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 14 foodstuffs were evaluated, selected and validated. In this paper we present the results of this substantive multi-matrix foodstuff 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). Cells were exposed in triplicate to the purified extracts redisolved in DMSO for 24 hours in 96-well microtiterplates. Following incubation and addition of substrate, luminescence was measured using a luminometer equipped with 2 dispensers.

*Selection of extraction and clean-up method.* A total of 14 different food matrices were evaluated (see table 3). Samples were obtained either from local retailers or the food-industry. All matrices were extracted by sonification, shake-, soxhlet-, and 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.

*Validation*. The validation protocol followed was based on the validation protocol Netherlands Standardization Institute (NEN) 7777:2001, incorporating ISO 5725-2/4:1994.

In addition, the validation protocol complied with methods of analysis for the official control of dioxins and the determination of dioxins and/or dioxin-like PCBs in foodstuffs and specific requirements for cell-based bioassays as detailed in EU Commission Directive 2002/69/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). Samples analysed included the 0.5*maximum EU-limits and the 2* maximum EU-limits for the respective category. In addition, method robustness was evaluated by comparing alteration 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]. In summary, the percentage standard deviation between triplicate analyses on the same 96 well microtiterplate was well within 15%. Furthermore, 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 relative repeatability ($S_r$) and reproducibility ($S_R$) were 17.0 and 16.9% respectively (n = 20).

In figure 2, the results of comparison between various extraction (sonification vs shake solvent), clean-up (0.5 g column vs 1.75 g column) and analysis (0.4% DMS) vs 0.8% DMSO incubation) procedures for DR CALUX® analysis results are given. In all cases, the coefficient of determination was 0.95 or more, indicating the robustness of the methods under investigation.

The matrices were categorised on the basis of the most appropriate extraction and clean-up procedure and the maximum EU-limits for dioxins and related compounds in food products (table 1). Applying the given amounts of material for extraction, the LOQ for the studied matrices is at least 2.5 times below the maximum EU-limit values for dioxins and related compounds (see table 2). Lower LOQ can be obtained by increasing the amount of material processed.

| 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 | $x_1$ $x_2$ | | | | y | | | |
| 2 | y | | | | $x_1$ $x_2$ | | | |
| 3 | | $x_1$ $x_2$ | | | | y | | |
| 4 | | y | | | | $x_1$ $x_2$ | | |
| 5 | | | $x_1$ $x_2$ | | | | y | |
| 6 | | | y | | | | $x_1$ $x_2$ | |
| 7 | | | | $x_1$ $x_2$ | | | | y |
| 8 | | | | y | | | | $x_1$ $x_2$ |

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

$S_r$(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 reproducibility

Table 1 Categorisation of matrices and amount of material processed for validation

| Category | Type of matrix | Matrices tested | **Processed** (g) |
|---|---|---|---|
| 1 | Oils and fats of plant origin | Palm oil fatty acids / Soya fatty acids / Palm oil / Sunflower oil | 3.5 |
| 2 | Animal oils and fats | Animal fat / Poultr y fat / Pig fat | 1.75 (3.5 g for pig fat) |
| 3 | Milk and milk products | Milk / Cream / Butter | 2 – 50 (yield <1.75 g fat) |
| 4 | Egg | Egg | 17 (yield < 1.75 g fat) |
| 5 | Fish and fishproducts | Fish (various) | 9 |
| 6 | Fish oil | Fish oil | 1.75 |

For the validation of extraction, clean-up and DR CALUX® bioanalysis, the analysis results included the 0.5*maximum EU-limits and the 2* maximum EU-limits with the majority of results below the maximum EU-limit values for dioxins in foodstuff. This demonstrates clearly the performance of the DR CALUX® bioassay in the range of the level of interest[4]. Table 2 summarises the validation results. In all case, the repeatability is 26% or less and reproducibility is 32.4% 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 15.5% and average reproducibility was calculated to be 20.3%. Correctness of the applied methods for the extraction, clean-up and DR CALUX® bioassay for foodstuffs ranged from 70 to 120%.

Tabel 2 Matrix-specific Limit of Quantitation (LOQ), repeatability and reproducibility

| **Category** | **LOQ** (pg CALUX TEQ/g fat) | **Max. EU-limits** (pg WHO-TEQ/g fat) | **Repeatability** $S_r$ (%) | **Reproducibility** $S_R$ (%) |
|---|---|---|---|---|
| 1 | 0.29 | 0.75 | 18.1 | 32.4 |
| 2 | 0.57 (0.29 for pig fat) | 1-3 | 15.6 | 13.3 |
| 3 | 0.57 | 3 | 11.0 | 16.1 |
| 4 | 0.57 | 3 | 9.5 | 21.0 |
| 5 | 0.11[a] | 4[a] | 12.5 | 25.1 |
| 6 | 0.57 | 2 | 26.0 | 14.1 |

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

- Council Regulation (EC) No 2375/2001, amending Commission Regulation (EC) No 466/2001 setting maximum levels for certain contaminants in foodstuffs (6-12-2001)

[a] pg TEQ/g fresh weight

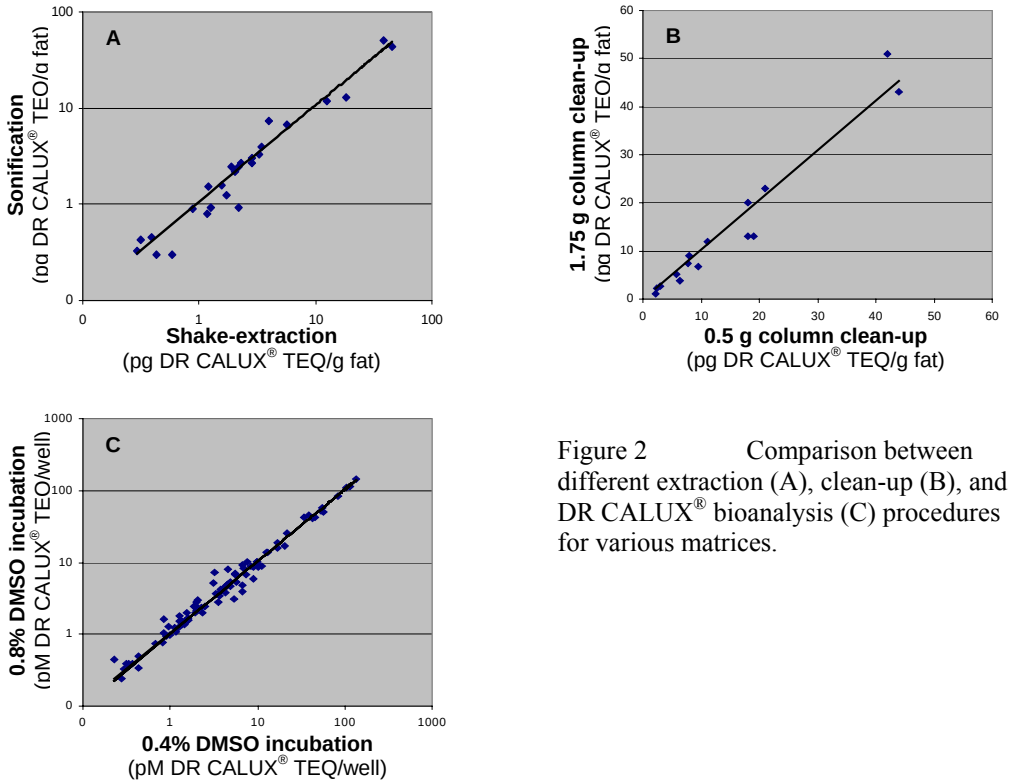


Figure 2 Comparison between different extraction (A), clean-up (B), and DR CALUX® bioanalysis (C) procedures for various matrices.

**Overall conclusions**

- Various food matrices required different extraction methods.
- The selected methods are 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 2.5 times below the maximum EU-limits set for the tested food 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 foostuffs dioxins compliance monitoring within the EU.

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