Development and validation of a ready to use cryo-EROD assay for the standardized screening of dioxins and dioxin-like compounds in foodstuffs

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Abstract

Recent European regulations have indicated the need for new bioanalytical screening methods capable of monitoring dioxin and dioxin-like compounds in foodstuffs and environmental samples, cost-effectively and with...
a quicker turnaround. Cryo-cells of the hepatic H4IIE line preserved in 96-well plates were exposed to sample extracts prepared from various foodstuffs and analysed for their content of dioxins and dioxin-like compounds by means of the 7-Ethoxycresolurfa-O-Deethylase (EROD)-assay in two laboratories. Assay data were compared between both laboratories and results from instrumental analysis used as a confirmatory method. Additionally, cut-off values for the different studied matrices were derived. The current European regulation regarding methods of analysis for the control of foodstuffs was applied with the aim of determining the feasibility of the cryo-methodology. Results obtained in both laboratories were in congruence with the required validation parameters of the Commission Regulation (EU) No 2017/644. Cut-off values should be established matrix-dependent to reduce the rate of false compliant results and to keep the rate of false non-compliant results under control. In summary, the ready-to-use cryo-assay method for the bioanalytical screening of foodstuffs in control laboratories without cell-culture facilities has successfully proven to be accurate, far quicker and more cost effective than current methods.

**Keywords:** Bio-screening methods; Cryo-EROD assay; Effect-based methods; Dioxins; Foodstuffs

### 1 Introduction

The planet's population is increasing and with it the need for sufficient staple foods. The ascent in global welfare standards has also created a major demand in protein sources and food diversity. Ever evolving methodologies for control of contaminants are needed to address the challenges that arise from growing consumption patterns for food and feed supplies in today's complex globalised world trade. Each country faces not only the challenge of assessing the quality of their consumers' food but also that of the raw materials for its food industry as well as feed used for farm animals and in aquacultures. In Europe particularly, the high rate of import/export volumes of foodstuffs within and outside the EU as well as food scandals related to recycled materials and open PCB applications (Weber et al., 2017), generate the need for new control methodologies that enable quick and cost-effective foodstuff control processes. Screening methods to optimise the detection of contaminated foodstuffs posing a potential health risk for the consumers are currently in development. Bioanalytical and effect-based screening methods are able to address among other compounds in food, the halogenated aromatic compounds (HAC) which are toxic, persistent, and can even be bio-accumulated through the food chain (Whitlock et al., 1996; Whyte et al., 2004; Stachel et al., 2007). These properties make HAC such as dioxins and dioxin-like compounds potential contaminants of natural food resources as well as industrialised products of foodstuffs (Baars et al., 2004; Bernard et al., 2002; Gizzi et al., 2005; Loutfy et al., 2006; Tard et al., 2007). These xenobiotics are able to activate the enzyme cytochrome P450 (CYP1A1) eliciting a response at the aryl hydrocarbon receptor (AhR) level and thus, giving a wholesome result of the investigated foodstuff mixture rather than its individual components. Based on this enzymatic activation, their detection and quantification is possible by means of 7-Ethoxycresolurfa-O-Deethylase (EROD) cell-based assay (Schwirzer et al., 1998; Hoffmaier, 1999; Behnisch et al., 2001; Petrusis et al., 2001; Eichbaum et al., 2014; Schwy et al., 2015). To allow the use of this assay in feed and food control laboratories not equipped with cell culture facilities, the development of a cryo-assay modified from the classical EROD assay is applied as a screening method for dioxin and dioxin-like compounds. Commission regulation (EU) No 252/2012 was the first legal document to set extensive validation criteria for screening dioxins and dioxin like compounds in food via cell based bioassays to achieve minimum standards throughout the laboratories. The most recent revision is Commission regulation (EU) No 2017/644. In the current study, the new cryo-assay was implemented and validated mainly based on the aforementioned European regulation. To achieve this, a set of 31 samples was chosen covering a varied range of contamination levels as well as different matrices of animal and plant origin. These samples were analysed in parallel in two independent laboratories using the cryo-plates. Additionally, EROD results from the same set of samples obtained with permanently cultured cells were compared with the results obtained by the ready-to-use cryo-plates. Instrumental analysis using high resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS) was used as confirmatory method. The cryo-assay data were analysed between laboratories and compared with the confirmatory method determining data compliance based on cut-off values. The current study addresses the issues of compliance, cost, and simplicity that are implicit in the current EU regulation for control of foodstuffs.

### 2 Materials and methods

#### 2.1 Samples

The 31 samples (Table 1) covered a broad range of matrices of animal and plant origin, namely: milk (breast milk), eggs (hen’s egg), mollusc (mussels), fish meat (salmon, bream, and carp), poultry test material, meat (bovine, sheep), liver (bovine, sheep), oil (seal, olive), and foodstuff additives (guar gum). As a quality assurance control, the sheep samples 18 and 19 were previously spiked with a mixture of dioxin congeners close to the maximum contamination allowance level for this type of food. The study samples were provided with the lipid content measurements (where applicable). If necessary, matrices were homogenized to assure a representative sample before sample treatment.

<p>| Table 1 | EROD assay results (pg BEQ/g sample) carried out with cryo-well plates (n = 3) and permanent cultured cells. HRGC/HRMS results of the sum of PCDD/F and dioxin-like PCB (pg WHO2005-TEQ/g sample) are also provided. n.d.: non-detectable. |</p>
<table>
<thead>
<tr>
<th>ID</th>
<th>Sample</th>
<th>Matrix</th>
<th>Type of sample</th>
<th>EROD assay with cryo-well plates BEQ (pg/g sample) Mean Laboratory 1 ± SD</th>
<th>Mean Laboratory 2 ± SD</th>
<th>Analytical results WHO_{2005} TEQ (pg/g sample)</th>
<th>EROD assay with cultured cells BEQ (pg/g sample)</th>
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<td>1</td>
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<td>3.86 ± 0.44</td>
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<td>6.57</td>
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<td>breast milk</td>
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<td>0.56</td>
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<tr>
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<td>15</td>
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<td>0.43</td>
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<td>1.62</td>
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<tr>
<td>22</td>
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<td>1.14 ± 0.26</td>
<td>1.04</td>
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<td>24</td>
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<td>n.d.</td>
<td>-</td>
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<td>10.8</td>
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<td>1965 ± 115</td>
<td>1249</td>
<td>1338</td>
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<tr>
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<td>poultry test material</td>
<td>0.33 ± 0.05</td>
<td>0.41 ± 0.12</td>
<td>0.21</td>
<td>n.d.</td>
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<tr>
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2.2 Bioanalytical screening

2.2.1 Extraction of liquid samples

Breast milk extracts were obtained by liquid-liquid extraction. After disruption of fat globule membranes with 8 ml of a saturated solution of potassium oxalate, the sample (100 ml) was vigorously shaken and extracted with a mixture of organic solutions, added successively as follows: 80 ml ethanol, 40 ml diethyl-ether, 60 ml n-pentane, and finally twice with 60 ml n-pentane. The final extract was then washed with 100 ml distilled water, dehydrated with sodium sulphate, and concentrated to approx. 2-3 ml for further clean-up.

2.2.2 Extraction of other samples

Homogeneous solid and colloidal samples (40 g) mixed with hydromatrix bulk material (Agilent Technologies, Oberhaching, Germany) were extracted by Accelerated Solvent Extraction (ASE 300) with two static cycles of 10 min at 120 °C and 120 bar. The solvent mixture was n-hexane/acetone 3:1 (v/v). Extracts were then dehydrated with sodium sulphate, and rotary evaporated to approx. 2-3 ml (450 mbar, 60 °C).

2.2.3 Clean-up without differentiation in lipid content

This clean-up was conducted according to Schirzewski et al. (1998) and consisted in a single chromatographic column containing the following components from bottom to top: 10 g activated silica-gel, 20 g sulphuric silica-gel (44% H2SO4 w/v), 40 g inactivated silica-gel (4% H2O, w/v) and 10 g anhydrous sodium sulphate (Na2SO4). Concentrated extracts were eluted with an organic mixture of n-hexane/dichloromethane 100:1 (v/v) and then rotary evaporated to 2-3 ml (550 mbar, 60 °C).

2.2.4 Differentiated clean-up based on sample lipid content

This clean-up was based on the official standard operation procedure of the European Union Reference Laboratory (EU-RL) for dioxins and PCBs in feed and food (CVUA, Freiburg, Germany). The amounts of materials within the clean-up column change dependently on the percentage range of fat content of the samples. A pre-oxidation step was carried out for samples with a fat amount ≥0.5% (g fat/g sample). The amount of sulphuric acid treated silica-gel in the pre-oxidation step and the oxidation clean-up step that followed were also dependently on the lipid content of the sample as well as on the type of sample.

2.2.5 Extracts for bio-screening

After clean-up procedures, extracts were transferred and concentrated in dimethyl-sulfoxide (DMSO) by nitrogen purge in vials. Sample extracts were then adjusted to a final organic solution of DMSO/isopropanol 4:1 (v/v). Extracts following a differentiated clean-up depending on their fat content, see clause 2.2.4., were concentrated to a final volume of 30 μl DMSO/isopropanol 4:1 (v/v) whereas extracts obtained from the other clean-up method were concentrated to a final volume of 500 μl DMSO/isopropanol 4:1 (v/v). Extracts concentrated to a final volume of 500 μl were divided into halves. One half each extracts were analyzed at each laboratory. 500 μl extracts allowed sequential dilution levels of the extracted sample in the bioassay medium (different final concentrations) and thus, enabled detection of potential cytotoxicity effects elicited in cells. The sequential dilution (D) levels were D = 2^n with 0 ≤ n ≤ 6 where D denotes the number of times the sample is being diluted and 1 is the undiluted extract sample. Procedure blanks, considering the clean-up, extraction, and concentration steps to obtain the extracts were also carried out.

2.2.6 Preparation of cryo-assay 96-well plates

H4IIEC3 rat hepatoma cells, used for preparation of cryo ready frozen assay plates (cryo-plates), were harvested from exponential growth phase. For detachment the cell layer was washed once with PBS and incubated with Accutase (A9604, Sigma-Aldrich) for 3 min at 37 °C. After detachment cells were centrifuged at 80 × g for 4 min and pellets were resuspended in NFM-G2 (serum-free freezing medium of acCELLerate). The cell suspension was counted in a semi-automated cell counter (CASY TT, Roche) and then, cell density was adjusted to 3 × 10^5 cells/ml. 96-well plates (0030601106, Eppendorf) were used for the preparation of the cryo-plates. For cell seeding a Multi-Channel Pipette (Xplorer® Plus 8 channels, Eppendorf) was used. Each well was filled with 10 μl of the cell suspension. After filling, each plate was packed under a slight vacuum in a foil bag and cooled down to storage at −80 °C under controlled conditions.

2.2.7 EROD-assay

The in vitro EROD assay (Donato et al., 1993) was carried out with cells of the H4IIEC3 rat hepatoma cell line in 96-well plates with the methods detailed in Schirzewski et al. (1998) and Schiwy et al. (2015). Two different approaches were carried out; 1) cultured hepatic cells plated in situ at a density of ~10,000 cells/well (classical EROD-assay) and 2) ready-to-use cryo-plates previously stored at ~−80 °C (cryo EROD-assay). Cryo-plates with a density ~30,000 cells/well were provided by acCELLerate GmbH (Hamburg, Germany). The basal culture media previously in use were kept in both laboratories but fetal bovine serum (FBS) was substituted with a complete chemically defined supplementation (Panexin, P04-95750, PAN Biotech, Germany) for assays carried out with cryo-plates. The culture media were Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma D5546, Germany) containing glucose (1 g/l), Na₂CO₃, and sodium pyruvate (1 mM) supplemented with L-Glutamine (2 mM), Panexin (10%),...
and penicillin/streptomycin (50 I.U./ml) or DMEM containing glucose (4.5 g/l) and Na$_2$CO$_3$ (3.7 g/l) (Biochrom F0475, Germany) supplemented with sodium pyruvate (1 mM), L-Glutamine (2 mM), Panexin (10%), and penicillin/streptomycin (50 I.U./ml). The 96-well plates plated in situ were incubated for 2 h (37 °C, 7% CO$_2$ (vol/vol), HR ≥ 95%) to assure stability and adherence of the cells to wells with 100 µl basal culture medium.

Cryo-cells are extremely temperature sensitive, thus gradients of temperature among the wells in cryo-plates during transport from and to storage must be avoided to prevent differences in well to well cell activity within a plate. Cryo-plates were taken from the −80 °C freezer and immediately placed on dry ice for transport. To thaw the cells the plates were transferred from dry ice directly in the incubator (37 °C, 7% CO$_2$ (vol/vol), HR ≥ 95%). After 20 min of re-activation, 90 µl media was added per well (giving a total of 100 µl including the cryo-well aliquot) under sterile conditions avoiding idle-times. Plates were agitated manually with caution for homogeneous distribution of cells in wells and culture medium (100 µl) containing 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) standards, blanks, or sample extracts was then directly added in classical 96-well plates and cryo-plates (supplementary information, Figs. 1S and 2S). The organic solvents in culture media were 0.5% of the final incubation mixture. After 72 h incubation medium was discarded, and hepatocytes were exposed to 8 µM 7-ethoxyresorufin and 10 µM dicumarol in 100 µl PBS per well for 30 min. Subsequently, 75 µl ethanol per well were added, gently mixed (10 min, Titrimax), and the generated resorufin was quantified by fluorescence (Excitation 535 nm/Emission 590 nm). Protein content was determined by the Pierce protein assay method (sample layout in supplementary information, Fig. 1S). For each extracted sample the classical assay was performed once and the assay with cryo 96-well plates in triplicate in two independent laboratories.

![Fig. 1](alt-text: EROD results (pg BEQ/g sample) for 3 samples carried out in cryo-plates with different storage times at −80 °C. Confirmatory results are also provided (pg WHO2005-TEQ/g sample).)
2.2.8 Calculation of BEQ equivalents

Each assay was carried out with a dose-response curve covering the activity cell range up to response saturation (2,3,7,8-TCDD standards: 0.0, 0.03, 0.06, 0.12, 0.2, 0.4, and 0.6 pg TCDD/well). This calibration curve was calculated with a standard concentration less than the minimal amount of concentrations recommended by the Commission Regulation (EU) No 2017/644 (from 8 to 12 calibration points). This decision was based on previous experience by achieving a good fitting of dose-response curves iterated by using the least-squared minimization method with seven concentration values. However, the incorporation of a low TCDD standard in the future (0.015 pg TCDD/well) to comply with the requirements of the Commission Regulation (EU) No 2017/644 and simultaneously to improve the sensitivity of the assay is not discarded. Each data point of the standard curve was calculated as the mean of the three response values with the least standard deviation among the four well determinations. The coefficient of variation of the triplicates of wells was ≤15%. Samples and procedure blanks were also calculated in this way. Fluorescence results were blank corrected. Then these values were adjusted dependent on the number of cells per well (protein amount). These so-called “specific fluorescence values” obtained from standards were then used to calculate a four-parameter dose-response curve by iterative least-squared minimization. The least-squared minimization method was applied giving equal weight to each data point. Response values of samples were then interpolated in the four-parameter curve to obtain the bioanalytical equivalents. Results are given as pg bioanalytical equivalents (BEQ) per gram sample (BEQ pg/g sample). When the regulation dictates an equivalence per gram fat (pg BEQ/g fat) to compare with the maximum levels and action thresholds of PCDD/F and PCB in different foodstuffs (EU No 1259/2011, EU No 277/2012, and No 1067/2013) gravimetric fat determination is performed. Details are given in the supplementary information.

2.3 Instrumental analysis

The accredited laboratory applies quality management system practices according to EN ISO/IEC 17025. HRGC/HRMS was used as the confirmatory method for PCDD/F and dioxin-like PCB content in samples. The compounds analysed according to Commission Regulation (EU) No 2017/644 were the seventeen 2,3,7,8-substituted congeners of PCDD/F, the non-dioxin-like PCB (28, 52, 101, 138, 153, 180) and the dioxin-like non-ortho PCB (77, 81, 126, 169) and the mono-ortho PCB (105, 114, 118, 123, 156, 157, 167, 189).

Internal reference materials were regularly run within the routine analysis as a quality control. From these control analyses, the coefficient of variation for all analytes was below or around 15%. Compounds were quantified by the isotope dilution method where only signals higher than a signal-to-noise ratio of 3:1 were considered valid for calculation. Limits of quantification ranged from 0.03 to 0.1 pg/g for PCDD/F and from 0.1 to 1.0 pg/g for PCB. Fulfillment of further quality criteria was based on EPA methods 1613 and 1668 for PCDD/F and PCB, respectively.

2.3.1 Sample processing

The extraction of samples for PCDD/F and PCB analysis was performed according to Çok et al. (2009). Briefly, previously spiked samples with 13C-labeled PCDD/F und PCB standard mixtures (Wellington Laboratories, Guelph, Canada) were
extracted using an ASE (ASE 300, Dionex, Sunnyvatem CA, USA). Breast milk samples were extracted by the separatory funnel method (liquid-liquid extraction) as described in 2.2.1. The clean-up was performed by use of an automated system (DETech, LTTeck GmbH, Dorfen, Germany) which was equipped with a column containing acidic and alkaline silica, a florasil, and two different carbon columns. The elution was performed according to the provided method using n-hexane, n-hexane/dichloromethane 1:1 (v/v), and toluene during the different steps. Two fractions were obtained: fraction 1 contained the indicator and mono-<i>ortho</i> PCB, fraction 2 the PCDD/F and non-<i>ortho</i> PCB. Fraction 1 was further cleaned by solid phase extraction using C18 modified silica (El-Kady et al., 2007). Procedural blanks were also carried out.

### 2.3.2 Instrumental measurements

After reducing the extract to 20 μl, samples were analysed by HRGC/HRMS. The GC columns used were Rtx-Dioxin2 and Rtx-CLPesticides2 for PCDD/F and PCB, respectively (Restek, Germany). The mass spectrometer (MAT95s, Thermo Electron GmbH, Germany) operated in selected ion monitoring (SIM) mode at a resolution power ≥ 9000. Detailed information about the instrumental analysis is provided in Wang et al. (2009). Percentage recoveries of 13C-Standards ranged from 62% to 101% for PCDD/F congeners and 59%-102% for PCB congeners. Results are given as the sum of PCDD/F and PCB equivalents in WHO<sub>2005</sub>-TEQ (pg/g sample).

### 3 Results and discussion

#### 3.1 Requirements regarding processing of the sample

Losses of compounds of interest during the sample processing (extraction, clean-up, and concentration steps) may cause a decrease in the assay response or even false non-compliant results (false negative). The EU regulation No 2017/644 indicates the need to include reference samples in each test series. Our set of samples is characterized by including various matrices of different origins, so we have no representative reference sample for the set under study. As we are not performing a test serial but the development and validation steps of this new cryo-assay, results of the extracted samples were used for checking performance of the cryo-assay comparing bioanalytical results without recovery correction by internal reference samples with their respective confirmatory results. Additionally, as a quality control, potential losses were determined quantifying the amounts of selected PCDD/F congeners in samples (extracted in triplicate) analysed by the cryo-assay and the confirmatory method. A procedure blank was spiked with a mixture of 17 PCDD/F native standards (EDF-5008, Cambridge Isotopes Laboratories, USA) achieving a representative congener pattern. The concentration of the standard mixture was selected to achieve a dilution in the bioassy medium close to the inflexion point of the 4-parameter curve (optimal range for quantification of samples). The recovery of samples was calculated as the ratio between the BEQ-values interpolated from the dose-response curve in cryo-plates and the PCDD/F WHO<sub>2005</sub>-TEQ-values obtained from the instrumental analysis. The bioassay's recoveries for the spiked samples were 92.0% ± 4.0% for extracts concentrated to 30 μl final volume and 104% ± 15% for extracted samples concentrated to 500 μl final volume. Differences in the amounts detected did not indicate a significant loss of compounds of interest during the processing of samples. The round robin test was carried out with samples treated according to the 500 μl clean-up procedure in order to generate enough sample extracts to perform dilution series.

#### 3.2 Requirements regarding the cryo-assay acceptance criteria

##### 3.2.1 Limit of detection and limit of quantification

The lowest TCDD-standard used in the dose-response curve had a final assay concentration of 0.03 pg/well, equivalent to 30 fg per well. Covering the upper femtogram range to potentially determine the most toxic compounds belonging to this concentration range is a requisite of the current EU regulation (Vo(EU) No 2017/644). As a quality control, even a low TCDD-standard of 0.015 pg/well was used in the assay (equivalent to a TCDD-stock solution of 30 pg/ml before dilution in culture media). From the 35 repetitions in cryo-assays, all of them detected a response signal at this concentration level and 32 were even able to quantify the response elicited by the 0.015 pg TCDD/well reference standard (20.4 ± 8.6 pg/ml, n = 32). However, the inclusion of this lowest standard is not enough to assure the data quality obtained from the dose-response curve close to the lowest asymptote. The highest errors are implicit per se in the asymptote zones of the 4-parameter curve where differences on dose are not always eliciting a significant differentiation in their response. Consequently, the standards were used for a good definition of the lower asymptote and the set of samples (Table 1) were used to define the limit of detection (LOD). Based on the obtained EROD results, all analysed samples with WHO<sub>2005</sub>-TEQ-values above 0.10 pg WHO<sub>2005</sub>-TEQ/g sample were detected or even quantified in the assay in both laboratories (Table 1). Based on the experimental data obtained from the varied foodstuff extracts we define a conservative LOD of 0.1 BEQ pg/g sample (40 g sample, 500 μl extract).

To determine the limit of quantification (LOQ) in the cryo-assay, a bovine liver sample (sample extracted in triplicate) was analysed in both laboratories. The matrix with the lowest maximum allowance level given per g whole sample in the current regulation (Amendment EU No 1067/2013) was chosen and within this matrix, a sample whose total WHO<sub>2005</sub>-TEQ-value (confirmed by HRGC/HRMS) was around 2/3 of this maximum value. This sample was detected and quantified in the low range of all dose-response curves. The mean value considering both laboratories was 0.26 ± 0.07 (BEQ pg/g sample), congruent with the analytical value of 0.30 (WHO<sub>2005</sub>-TEQ pg/g sample) given by the confirmatory method. Considering both laboratories independently, BEQ mean values were 0.31 ± 0.05 (BEQ pg/g sample) and 0.19 ± 0.01 (BEQ pg/g sample) with a repeatability of 16.6% and 2.5%, respectively and a reproducibility between them of 28%. Based on these experimental values, we set the LOQ as 0.30 BEQ pg/g sample (40 g sample, 500 μl final extract).
3.2.2 Working range and quantification of samples

The saturation of the assay response was achieved around 800 pg TCDD/ml (0.4 pg/well) to obtain well defined dose-response curves. EC$_{50}$ was chosen as the upper value of the working range to assure coefficient of variations lower than 15%. The calculation of BEQ was based on responses achieved in the middle region of the curve (highest slope) where quantifications are best, when possible. As a quality control, a standard of 240 pg TCDD/ml (0.12 pg/well) within this curve range was analysed as an additional sample, exhibiting a mean value of $220 \pm 58$ pg BEQ/ml (n = 35). Blanks (Table 1) and the positive controls (TCDD-standards 0.015 pg/well and 0.12 pg/well) carried out in all cryo-assays were in conformity with the obtained values. Mean EC$_{50}$ values (n = 35) were $0.11 \pm 0.03$ (pg BEQ/well) and $0.16 \pm 0.06$ (pg BEQ/well) at laboratories 1 and 2, respectively. Half of the samples studied in this round robin test had contamination levels ending up in the low concentration range of the dose-response curve providing the most challenging range for quantification of contaminants. The samples 18 and 19 (sheep liver) contaminated around 0.5 WHO$_{100}$TEQ pg/g sample were exhibiting responses in the lower range of the dose-response curves and quantified indicating contamination loadings close to the expected values given by the confirmatory method (Table 1). The response of samples contaminated near the concentrations of interest (maximum and action levels) was for all matrices within the working range of the standard curves.

3.2.3 Attachment of cryo-cells to wells and storage times

A critical step in the test is the microscopic control of the attachment of cells to the well-plate after thawing. The lack of cell homogeneity among wells generated by poor cell attachment may result in significant differences in the assay response being insufficiently minimized even after considering the explicit number of cells or protein per well in the cryo-assay. This generates variations in the dose-response curve leading to non-compliance of quality standards regarding accuracy and precision of results.

The homogeneity of the mono-layer in cryo-plates was thus, an issue of paramount importance and was controlled microscopically before cell incubation with samples. Two different charges of cryo-plates were controlled in both laboratories. The functionality of cryo-cells with time was determined by reiterative analyses of aliquots of extracts from three samples of different origin (breast milk 4, fish meat 15, and reference sheep liver 19) with cryo-well plates stored at different periods. Cryo-plates stored at $-80 ^{\circ}$C longer than 4 months exhibited a loss in functionality resulting in a lower sensitivity in the differentiation among samples (Fig. 1). In summary, cryo-well 96-plates exhibited the best functionality with storage times limited to a maximum of four months.

3.3 Interlaboratory cryo-assay results

Results obtained with cryo-plates were congruent between both laboratories (Fig. 2) resolutely proving the reproducibility of the assay beyond laboratory conditions and operators. Moreover, the linear correlation (R$^2$) between laboratories was R$^2$ = 0.89. Sample 15 (bream/carp meat) showed markedly lower EROD values than confirmatory results for all replicates in both laboratories independent of the time of analysis (Fig. 1). This can be attributed to antagonistic compounds accumulated in the fish matrix, in particular non-ortho PCB as also observed in sample 1 (salmon meat) and samples 2 and 12 (bream/carp meat). Previous studies have indicated that interactions between TCDD and non-ortho PCB generated non-additive antagonistic responses (Li et al., 1999; Binelli et al., 2006). Schlezing et al. (2006) determined not only the inhibition of EROD activity but also the release of reactive oxygen species from induced liver microsomes stimulated by non-ortho PCB. This release of reactive oxygen species was suggested as a participation of CYP1A in the oxidative stress associated with these AhR agonists. Data obtained in culture media using fetal bovine serum (EROD assay with permanent cultured cells, Table 1) exhibited less antagonistic effects in the fish matrices (samples 1, 2, 12, and 15) than data obtained using cryo-plates cultured in a serum-free medium. This can be partially attributed to natural components in the FBS composition that protect cells more effectively against oxidative stress and thus, hindering its functionality less than a serum free-medium culture would (Brunner et al., 2010).

Consequently, the assay could generate results biased towards lower cell-induced effects and thus, could generate false-compliant results. This methodology does not discriminate between agonists and antagonists exhibited in the sample, giving a total induced cell-effect response. Therefore, there is a need to determine potential interferences in the agonistic cell-effects induced in the cryo-assay. To determine antagonistic effects, the standard addition method was carried out in samples where the results obtained by the cryo-assay were not concordant with those obtained by the confirmatory method. Samples 1, 2, 12, and 15 were spiked with TCDD-standards, extracted and then analysed to determine a potential decrease in response generated by interference substances in this type of matrix. The concentration of TCDD-standard was chosen to obtain total responses below EC$_{50}$ and close to EC$_{50}$. Results elicited from spiked and unspiked sample extracts were summarized in Table 2.

<table>
<thead>
<tr>
<th>ID</th>
<th>Unspiked sample (BEQ pg/g sample)</th>
<th>TCDD-standard addition (0.06 pg TCDD/well)</th>
<th>Loss of response (%)</th>
<th>Mean (n = 3) Laboratory 2 (BEQ pg/g sample)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>4.25</td>
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<td>−72.6</td>
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</table>

Table 2: Extracted samples controlled routinely and applying standard addition method at laboratory 2. Mean values (n = 3) obtained during the round robin test were also included as comparison.
Loss of responses higher than 20% were considered to indicate a response decrease in the extracted samples. A decreased response of elicited dioxin-like activities in cryo-wells may be associated with the presence of AhR antagonists (such as observed here with samples of fish origin) but also by the effects of cytotoxicity. In effect, samples 1, 11, and 28 (salmon meat, guar gum, and spiked olive oil) elicited an exponential increase in response at higher dilutions. The other samples exhibited an increase in the assay response in the most concentrated samples of the two-fold dilution series. The contaminated samples 1, 11, and 28 could only be quantified after applying two-fold dilution steps. Microscopic observations of cells incubated with the sample extracts for 72 h indicated a tendency for the grouping of cells (clumping) in the wells. On the other hand, the reference standards elicited response values in agreement with the expected dioxin-like activities after 72 h incubation implying adequate cell culture conditions. Fig. 3 shows assay responses for sample 11 (guar gum) and sample 28 (olive oil) exhibited at different final assay dilutions in 96 well cryo-plates. The cytotoxicity of this guar gum sample could be related to the presence of the contaminant pentachlorophenol (PCP). A European study related to loadings of dioxins and PCP in guar gum indicated concentrations above the maximal allowance values for these compounds in guar gum batches coming from India (EU-RL, 2007). Sample 28 was spiked with TCDD-standard. This final TCDD concentration overloaded the dose-response system and thus, resulted in lower than expected dioxin-like activity in the cells. Two-fold dilutions are required in instances of assay cytotoxicity and when response values approach the upper asymptote (above the EC<sub>50</sub> value) of the TCDD-standard curve. We recommend at least two-fold dilution series with three different dilutions: 1:1 (undiluted), 1:2 and 1:4 in case of unknown matrices. If necessary, further dilutions can be performed based on the results of the first dilution series. The routine use of two-fold dilutions for a single sample as a part of the assay bio-screening requires additional work with all its associated costs. In future, elimination of the dilution series could be considered, provided we could apply a methodology that determines the possible compound-induced cell effects in the assay. Then it is possible to differentiate between cytotoxicity and antagonistic effects generated in hepatic cells exposed to extract samples. Consequently, the probability towards false negatives or sub evaluation of real risk samples is reduced. In the current study three samples (samples 2, 12, and 15) exhibited antagonistic effects, two samples (samples 11 and 28) were cytotoxic, and one sample (sample 1) induced both effects in the cryo-cells. This illustrates the need for further investigations in the development of techniques to determine induced cell-effects of tested samples in the cryo-assay. Some efforts have already been done in this direction where the discrimination of cytotoxicity and antagonistic effects was investigated (Schmitz et al., 2010; Marin-Kuan et al., 2017).

**Table 3**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>BEQ (pg/g)</th>
<th>BEQ (pg/g)</th>
</tr>
</thead>
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<tr>
<td>15</td>
<td>1.09</td>
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<table>
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<th>Concentration</th>
<th>BEQ (pg/g)</th>
<th>BEQ (pg/g)</th>
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<td>12</td>
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<td>15</td>
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![Fig. 3 Bioanalytical Equivalents (BEQ) (pg/g)](image)

Until now, public attention has been focused on foodstuffs like meat, avian products, or animal feedstuff which were historically more prone to being contaminated with dioxin-like compounds. However, some foods such as green tea extract, red wine, and peanuts contain natural inhibitors of CYP1A1 (Casper et al., 1999; Allen et al., 2001; Mikstacka et al., 2008). In these cases, we can even predict a compound-related inhibition in the cryo-assay. A further study with TCDD-standard addition would be advisable for this kind of foodstuffs with the purpose of determining the antagonistic effects of dietary compounds of natural occurrence not eliminated during the clean-up procedure in the cryo-assay. Table 3 summarizes parameters of importance for the test validation process, namely; cut-off values, false-compliant and false non-compliant samples, reproducibility (RSD<sub>r</sub>) between laboratories, and intra-laboratory repeatability (RSD<sub>i</sub>) of the samples at each laboratory. These parameters were in general, congruent with the expected values of RSD<sub>r</sub> < 20%, RSD<sub>i</sub> < 25%, and false-compliant rate <5%. Additionally, z-scores of single determinations based on the mean and standard deviation values were calculated with the experimental data of both laboratories (supplementary information, Table 1S). Cut-off values were established as 2/3 of the maximum levels given by the regulation (EU) No 2006/1881 and the amendment regulation (EC) No 1067/2013 and pursue the goal of detecting the potential contaminated samples but avoiding the generation of false-compliant results. These cut-off values were then
used to decide compliance of the analysed samples comparing bioanalytical results and their respective confirmatory results. According to the directive, maximum levels of some food matrices are defined depending on the lipid content of the sample. The accuracy in the determination of this parameter achieves importance when assay results obtained in a sample weight basis (pg BEQ/g sample) have to be transformed to a lipid basis (pg BEQ/g fat). Moreover, low lipid content in samples implies high conversion factors that achieve more numerical weight in the calculation of final results.

Table 3 Cut-off values, compliance, repeatability (RSD<sub>r</sub>), and reproducibility (RSD<sub>R</sub>) of samples at both laboratories. w.w.: wet weight.

<table>
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<tr>
<th>ID</th>
<th>Matrix</th>
<th>Lipid content</th>
<th>Maximal level (ML) TEQ&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Cut-off value TEQ</th>
<th>Compliance: BEQ- and TEQ- value of sample both ≤ or &gt; cut-off value</th>
<th>HRGC/HRMS WHO&lt;sub&gt;TEQ&lt;/sub&gt;</th>
<th>Repeatability RSD&lt;sub&gt;r&lt;/sub&gt; (%)</th>
<th>Repeatability RSD&lt;sub&gt;R&lt;/sub&gt; (%)</th>
<th>Reproducibility RSD&lt;sub&gt;R&lt;/sub&gt; (%)</th>
<th>Sample Type of sample</th>
<th>(%</th>
<th>pg/g wet weight or fat</th>
<th>2/3 of ML</th>
<th>Lab 1</th>
<th>Lab 2</th>
<th>pg/g sample</th>
<th>Lab 1</th>
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<td>21</td>
<td>sheep meat</td>
<td>33.7</td>
<td>4.0 fat</td>
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<td>22</td>
<td>sheep meat</td>
<td>37.7</td>
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<td>false non-compliant</td>
<td>1.04</td>
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From the 31 samples, three samples in laboratory 1 and one sample at laboratory 2 were false-compliant. Laboratory 2 had conformity with the current Regulation (EC No, 2017/644) requiring <5% of false-compliant results in bio-screening methods. Salmon meat (sample 1) was non-compliant in both laboratories and given the high repeatability in each laboratory (Table 1) this could be attributed to a significant amount of PCB in this sample acting as antagonists at the AhR level in the cryo-assay such as discussed above.

In the case of the other non-compliant samples at laboratory 1 (samples 13 and 14), their quantification was not always possible due to a lack of sensitivity in the low range of the dose-response curves. To counteract this in the future, we propose routinely incorporating the low standard of 0.015 pg/well to the dose-response curve of the cryo-assay. In this way, sensitivity in this range of the curve is improved and thus, assures quantification of potential samples eliciting a response in this curve range.

In the case of matrices of fish origin, an additional analysis of these samples applying a standard addition (spiked and unspiked extract) was necessary to determine antagonistic effects in the cryo-assay. Based on the current laboratory values, we recommend a more conservative cut-off value of the 2/5 value of the maximum allowance level for samples of fish origin. Applying this new experimental cut-off value for the fish matrix, all samples were compliant in laboratory 2 and laboratory 1 had a percentage of non-compliant-values close to 5%.

4 Conclusions and further research

This cryo-assay methodology successfully enables the control of food and feedstuffs that necessitate bio-screening processing thereby conclusively indicating the compliance or non-compliance of samples based on the current EU regulation regarding dioxins, dioxin-like compounds, and non-dioxin-like PCB in foodstuffs in a cost-effective and time-sensitive manner.

In order to accurately determine potential antagonistic or cytotoxic effects in new foodstuff matrices, the use of two-fold dilution series and standard addition procedures are paramount. Based on these experimental data, new cut-off values for new matrices can be defined.

Reductions in elicited dioxin-like activities can be attributed to two primary causes: antagonism and cytotoxicity effects of sample extracts. Both situations imply the risk of classifying a contaminated hazard sample as compliant according to the current allowance levels generating a false-compliant result. Therefore, the incorporation of a further technique able to differentiate between the antagonistic and cytotoxic induced cell-effects in the cryo-assay is a significant consideration. The issue may also be tackled by spiking the sample in question and checking for a possibly reduced response in comparison to the spiked amount.

The goal of an animal protein-free medium in the bio-screening of dioxin-like compounds in foodstuffs is the next step in achieving an ethical environment for assay testing. The standardisation of the culture media together with its further optimization will provide better cell functionality, and consequently a higher sensitivity to low concentrations of the target contaminants. This is an important issue because according to the policy of the European Commission to reduce the amounts of dioxins and dioxin-like compounds in the environment, in feed and in food, future regulations may tend to set lower maximum level values than the current ones.

Acknowledgements

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We are deeply thankful to the excellent laboratory teams at RTWH and HMGU. In particular, to Gabriele Engelhardt and Claudia Corsten for the assays carried out at RWTH (Aachen) and HMGU (Munich), Silke Bernholt for the processing of all samples as well as bioassay determinations at HMGU.

### Transparency document

Transparency document related to this article can be found online at [https://doi.org/10.1016/j.fct.2018.10.023](https://doi.org/10.1016/j.fct.2018.10.023).

### Appendix A. Supplementary data

Supplementary data to this article can be found online at [https://doi.org/10.1016/j.fct.2018.10.023](https://doi.org/10.1016/j.fct.2018.10.023).

### Uncited reference

*Cupp and Tracy, 1999.*

### References


Gizi G., Hoogenboom L.A., Von Holst C., Rose M. and Anklam E., Determination of dioxins (PCDDs/PCDFs) and PCBs in food and feed using the DR CALUX bioassay: results of an international validation study, *Food Addit. Contam.* **22** (5), 2005, 47-481.


**Transparency document**

**Appendix A. Supplementary data**

The following are the Supplementary data to this article:

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**Highlights**

- A novel method for bio-screening of dioxin and dioxin-like compounds was carried out in various foodstuffs.
- The cryo-assay validation was successfully performed based on the current European regulations.
- Toxicity and antagonistic effects that generate potential false-compliant samples were analysed.
- Cut-off concentrations should be established based on sample matrix and laboratory performance.
- Food control laboratories with basic cell culture facilities can easily use this new cryo-EROD assay.
- Further investigations toward greener bio-screening methodologies are ethically needed.

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Query: Highlights should consist of 3 to 5 bullet points. There are "6" bullet points provided. Please edit the highlights to meet the requirement.

Answer: I will take the last point out due it is based on further future activities. However, it is the only point of this list that mention the need of a more ethical behaviour in this topic. I hope writing this point again in a further related research soon!

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