

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

Walkiria [Levy](#)^{a, *}

walkiria.levy@helmholtz-muenchen.de

Karl-Werner [Schramm](#)^{a, d}

Florian [Mertes](#)^a

Bernhard [Henkelmann](#)^a

Martina [Maywald](#)^b

Peter [Uciechowski](#)^b

Alexander [Loa](#)^c

Johannes [Haedrich](#)^e

Ines [Thiem](#)^f

Henner [Hollert](#)^g

Roland [Goerlich](#)^{b, h}

Thorsten [Bernsmann](#)ⁱ

Lothar [Rink](#)^d

^aHelmholtz Zentrum München - German Research Center for Environmental Health (GmbH), Molecular EXposomics (MEX), Ingolstädter Landstrasse 1, 85764, Neuherberg, Germany

^bInstitute of Immunology, Medical Faculty, RWTH Aachen University, Pauwelsstrasse 30, 52074, Aachen, Germany

^cAcCELLerate GmbH, Osterfeldstraße 12-14, 22529, Hamburg, Germany

^dTUM, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Department für Biowissenschaftliche Grundlagen, Weihenstephaner Steig 23, 85350, Freising, Germany

^eChemisches und Veterinäruntersuchungsamt Freiburg, Bissierstrasse 5, 79114, Freiburg, Germany

^fLower Saxony State Institute for Consumer Protection and Food Safety (LAVES), Food and Veterinary Institute Braunschweig/Hannover Dresdenstrasse 2, 38124, Braunschweig, Germany

^gInstitut für Umweltforschung, RWTH Aachen University, Worringer Weg 1, 52074, Aachen, Germany

^hInstitut für wirkungsbezogene Analytik e.v., Kerpen, Germany

ⁱChemisches und Veterinäruntersuchungsamt Münsterland-Emscher-Lippe, Postfach 1980, 48007, Münster, Germany

*Corresponding author.

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-Ethoxyresorufin-*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-Ethoxyresorufin-*O*-Deethylase (EROD) cell-based assay (Schwirzer et al., 1998; Hoffmaier, 1999; Behnisch et al., 2001; Petrulis et al., 2001; Eichbaum et al., 2014; Schiwiy 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.

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.

alt-text: Table 1

ID	Matrix	EROD assay with cryo-well plates BEQ (pg/g sample)		Analytical results	EROD assay with cultured cells
Sample	Type of sample	Mean Laboratory 1 \pm SD	Mean Laboratory 2 \pm SD	WHO ₂₀₀₅ -TEQ (pg/g sample)	BEQ (pg/g sample)
1	salmon meat	3.86 \pm 0.44	3.30 \pm 0.24	8.01	6.57
2	bream/carp meat	0.84 \pm 0.28	1.53 \pm 0.59	3.60	3.89
3	hen eggs	0.70 \pm 0.23	1.11 \pm 0.30	0.68	2.67
4	breast milk	0.90 \pm 0.23	1.17 \pm 0.34	0.97	2.01
5	breast milk	0.05 \pm 0.03	0.20 \pm 0.06	0.13	n.d.
6	bovine liver	0.45 \pm 0.04	0.24 \pm 0.19	0.56	<0.20
7	bovine liver	0.19 \pm 0.01	0.31 \pm 0.05	0.30	0.40
8	bovine liver	0.16 \pm 0.03	0.34 \pm 0.07	0.27	<0.20
9	blank of ID 1, 2 and 10 to 15	n.d.	n.d.	-	n.d.
10	guar gum (powder)	0.09 \pm 0.05	0.10*	0.08	n.d.
11	guar gum (powder)	239 \pm 64	263 \pm 46	71	122
12	bream/carp meat	2.24 \pm 0.94	1.73 \pm 0.86	4.32	2.43
13	bovine meat	0.03*	0.06 \pm 0.05	0.08	1.60
14	bovine meat	0.05*	0.13 \pm 0.04	0.06	1.05
15	bream/carp meat	1.37 \pm 0.25	0.86 \pm 0.40	3.40	2.78
16	hen eggs	0.33 \pm 0.07	0.54 \pm 0.18	0.89	2.30
17	hen eggs	0.46 \pm 0.02	0.42 \pm 0.19	0.86	2.35
18	sheep liver	0.22 \pm 0.03	0.29 \pm 0.13	0.43	0.75
19	sheep liver	0.34 \pm 0.07	0.46 \pm 0.25	0.56	1.17
20	blank of ID 6,7,8, 21 and 22	n.d.	n.d.	-	n.d.
21	sheep meat	0.90 \pm 0.09	1.53 \pm 0.54	1.42	1.62
22	sheep meat	0.73 \pm 0.15	1.14 \pm 0.26	1.04	0.71
23	blank of sample ID 24	n.d.	n.d.	-	n.d.
24	mussel	0.28 \pm 0.11	0.85 \pm 0.16	0.66	1.63
25	blank of sample ID 4 and 5	n.d.	n.d.	-	n.d.
26	blank of sample ID 27 and 28	n.d.	n.d.	-	n.d.
27	seal oil	2.90 \pm 2.05	2.29*	2.30	10.8
28	olive oil spiked with TCDD	1949 \pm 110	1965 \pm 115	1249	1338
29	blank of sample ID 30	n.d.	n.d.	-	n.d.
30	poultry test material	0.33 \pm 0.05	0.41 \pm 0.12	0.21	n.d.
31	blank of sample ID 3 and 16 to 19	n.d.	n.d.	-	n.d.

Notes: * sample detected only once in 3 repetitions.

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 [Schwirzer 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 depend~~ent~~ on the ~~percentage ranges of fat contained fat content of in~~ the samples. A pre-oxidation step was carried out for samples with a fat amount $\geq 0.5\%$ (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 ~~was were~~ also depend~~anting~~ 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 μ l DMSO/isopropanol 4:1 (v/v) whereas extracts obtained from the other clean-up method were concentrated to a final volume of 500 μ l DMSO/isopropanol 4:1 (v/v). Extracts concentrated to a final volume of 500 μ l were divided into halves. ~~One and half each was~~ ~~extracts were~~ analysed at ~~each the~~ laboratory ~~ries~~. 500 μ l 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 $0 \leq n \leq 6$ where D denotes the number of times the sample is being diluted and $D = 1$ 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

H4IIEC/T3 rat hepatoma cells, used for preparation of ~~f-assay~~ 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[®] (A6964, Sigma-Aldrich) for 3 min at 37 °C. After detachment cells were centrifuged at $80 \times g$ for 4 min and pellets were resuspended in NFM-G2 (serum-free freezing medium of acCELLerate). The cell suspension was counted in a semi-automatic cell counter (CASY TT, Roche) and then, cell density was adjusted to 3×10^6 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 μ l 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 H4IIEC/T3 rat hepatoma cell line in 96-well plates with the methods detailed in [Schwirzer 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 $\sim 10,000$ 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 $\sim 30,000$ 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 Na₂CO₃ (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% CO₂ (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% CO₂ (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.

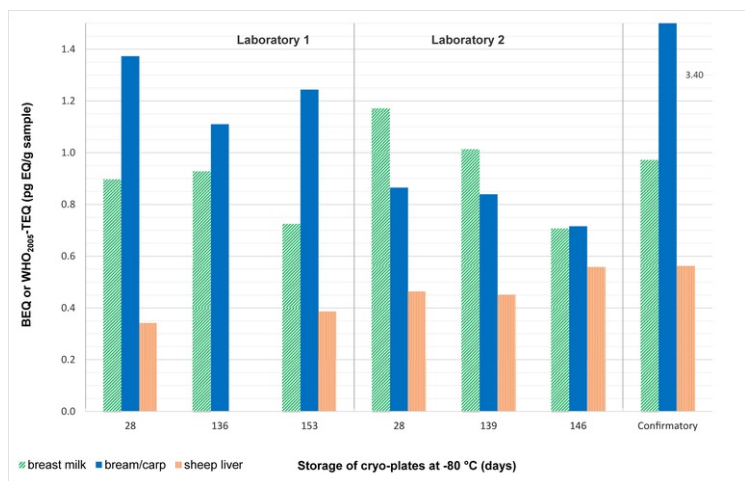


Fig. 1 EROD results (pg BEQ/g sample) for 3 samples carried out in cryo-plates with different storage times at -80 °C. Confirmatory results are also provided (pg WHO₂₀₀₅-TEQ/g sample).

alt-text: Fig. 1

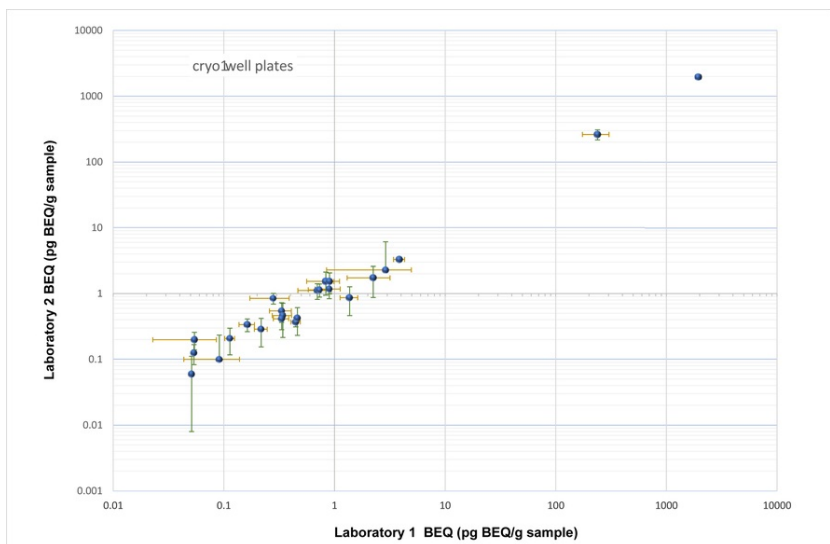


Fig. 2 Bioanalytical Equivalents (BEQ) (pg/g) in 31 samples $n = 3$ analysed independently in both laboratories. Data are mean values \pm SD ($n = 3$).

alt-text: Fig. 2

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 response](#) 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 $\leq 15\%$. 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 pg/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 ^{13}C -labeled PCDD/F and PCB standard mixtures (Wellington Laboratories, Guelph, Canada) were

extracted using an ASE (ASE 300, Dionex, Sunnyvale 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 (DEXTech, LCTech GmbH, Dorfen, Germany) which was equipped with a column containing acidic and alkaline silica, a florisil, 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-*ortho* PCB, fraction 2 the PCDD/F and non-*ortho* 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 ^{13}C -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 WHO₂₀₀₅-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 bioassay 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 WHO₂₀₀₅-TEQ-values obtained from the instrumental analysis. The bioassay's recoveries for the spiked samples were $92.0\% \pm 4.0\%$ for extracts concentrated to 30 µl final volume and $104\% \pm 15\%$ 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 quality 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 WHO₂₀₀₅-TEQ-values above 0.10 pg WHO₂₀₀₅-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 WHO₂₀₀₅-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 (WHO₂₀₀₅-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. EC₇₀ 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 ± 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 EC₅₀ values (n = 35) were 0.11 ± 0.03 (pg BEQ/well) and 0.16 ± 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 WHO₂₀₀₅-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 °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 (R²) between laboratories was R² = 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). Schlezinger 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 EC₇₀ and close to EC₅₀. Results elicited from spiked and unspiked sample extracts were summarized in Table 2.

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.

alt-text: Table 2

ID Sample	Unspiked sample BEQ (pg/g sample)	TCDD-standard addition Loss of response (%)		Mean (n = 3) Laboratory 2 BEQ (pg/g sample)
		0.06 pg TCDD/well	0.12 pg TCDD/well	
1	4.25	-34.0	-19.5	3.30
2	1.16	-23.0	-71.4	1.53

2	1.10	-23.9	-7.14	1.55
12	2.23	-8.9	-21.5	1.73
15	1.09	-12.4	-52.2	0.86

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 EC₇₀ 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).

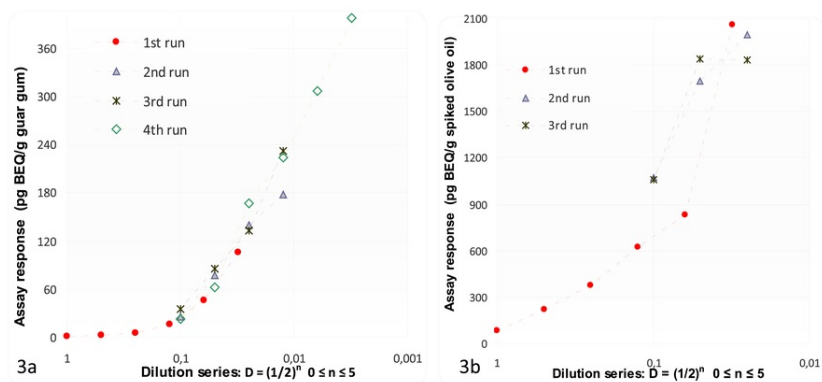


Fig. 3 Bioanalytical Equivalents (BEQ) (pg/g) in guar gum (3a) and in olive oil (3b) spiked with TCDD obtained at different extract concentrations of the samples in the culture media.

alt-text: Fig. 3

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_R) between laboratories, and intra-laboratory repeatability (RSD_I) of the samples at each laboratory. These parameters were in general, congruent with the expected values of RSD_I < 20%, RSD_R < 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_r), and reproducibility (RSD_R) of samples at both laboratories. w.w.: wet weight.

alt-text: Table 3

ID	Matrix	Lipid content	Maximal level (ML) TEQ ²	Cut-off value TEQ	Compliance: BEQ- and TEQ- value of sample both ≤ or > cut-off value		HRGC/HRMS WHO ₂₀₀₅ -TEQ	Repeatability RSD _r (%)	Repeatability RSD _r (%)	Reproducibility RSD _R (%)
Sample	Type of sample	(%)	pg/g wet weight or fat	2/3 of ML	Lab 1	Lab 2	pg/g sample	Lab 1	Lab 2	Between both laboratories
1	salmon meat	6.20	6.5 w.w.	4.3	false-compliant	false-compliant	8.01	11	7	11
2	bream/carp meat	2.90	6.5 w.w.	4.3	-	-	3.60	33	38	42
3	hen eggs	8.30	5.0 fat	3.3	-	-	0.68	33	27	32
4	breast milk	4.51	5.5 fat	3.7	-	-	0.97	26	29	19
5	breast milk	3.55	5.5 fat	3.7	-	-	0.13	58	30	81
6	bovine liver	unknown	0.5 w.w.	0.33	-	-	0.56	10	16	13
7	bovine liver	unknown	0.5 w.w.	0.33	-	-	0.30	3	17	41
8	bovine liver	unknown	0.5 w.w.	0.33	-	-	0.27	16	22	49
9	blank of 1, 2 and 10 to 15	0			-	-	-	-	-	-
10	guar gum (powder)	0	1.5 w.w.	1.0	-	-	0.08	52	0	7
11	guar gum (powder)	0	1.5 w.w.	1.0	-	-	71	27	17	7
12	bream/carp meat	2.00	6.5 w.w.	4.3	-	false non-compliant	4.32	42	50	18
13	bovine meat	0.90 ¹	0.08 w.w.	0.05	false-compliant	-	0.08	***	87	-
14	bovine meat	2.20	4.0 fat	2.7	false-compliant	-	0.06	***	34	57
15	bream/carp meat	2.30	6.5 w.w.	4.3	-	false non-compliant	3.40	18	46	32
16	hen eggs	8.20	5.0 fat	3.3	-	-	0.89	22	33	34
17	hen eggs	8.40	5.0 fat	3.3	-	-	0.86	4	45	7
18	sheep liver	3.70	2.0 w.w.	1.3	false non-compliant	false non-compliant	0.43	13	46	19
19	sheep liver	5.90	2.0 w.w.	1.3	false non-compliant	false non-compliant	0.56	20	54	21
20	blank of 6,7,8, 21 and 22	0			-	-	-	-	-	-
21	sheep meat	33.7	4.0 fat	2.67	-	-	1.42	10	35	36
22	sheep meat	37.7	4.0 fat	2.67	-	false non-compliant	1.04	21	23	31
23	blank of 24	0			-	-	-	-	-	-

24	mussel	unknown	6.5 w.w.	4.3	-	-	0.66	39	18	71
25	blank of 4 and 5	0			-	-	-	-	-	-
26	blank of 27 and 28	0			-	-	-	-	-	-
27	seal oil	100	2.5 fat	1.7	-	-	2.30	71	173	12
28	spiked olive oil	100	1.25 fat	0.8	-	-	1249	6	6	1
29	blank of sample 30	0			-	-	-	-	-	-
30	poultry test material	8.80	3.0 fat	2.0	-	-	0.21	16	32	15
31	blank of 3 and 16 to 19	0			-	-	-			

Notes: ¹ Samples with lipid content <2% were calculated with cut-off values in a wet weight basis assuming 2% fat content (amendment EU No 1259/2011). ²: Maximal allowance levels are based on Regulation (EC) No 1259/2011 amending Regulation 1881/2006 and on amendment EC No 1067/2013 for liver samples. ***: Only one determination of three replicates.

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

This research project was supported by the Federal Office of Agriculture and Food (BLE), Germany (Project funding code 314-06.01-2813HS021).

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 Bernhöft 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>.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fct.2018.10.023>.

Uncited reference

[Cupp and Tracy, 1998](#).

References

- Allen W.S., Mueller L., Williams N.S., Quattrochi C.L. and Raucy J., The use of a high-volume screening procedure to assess the effects of dietary flavonoids on human CYP1A1 expression, *Drug Metab. Dispos.* **29** (8), 2001, 1074–1079.
- Baars A.J., Bakker M.I., Baumann R.A., Boon P.E., Freijer J.I., Hoogenboom L.A.P., Hoogerbrugge R., van Klaveren J.D., Liem A.K.D., Traag W.A. and de Vries J., Dioxins, dioxin-like PCBs and non-dioxin-like PCBs in foodstuffs: occurrence and dietary intake in the Netherlands, *Toxicol. Lett.* **151** (1), 2004, 51–61.
- Behnisch P.A., Hosoe K. and Sakai S., Bioanalytical screening methods for dioxins and dioxin-like compounds a review of bioassay/biomarker technology, *Environ. Int.* **27** (5), 2001, 413–439.
- Bernard A., Broeckaert F., De Poorter G., De Cock A., Hermans C., Saegerman C. and Houins G., The belgian PCB/dioxin incident: analysis of the food chain contamination and health risk evaluation, *Environ. Res.* **88** (1), 2002, 1–18.
- Binelli A., Ricciardi F., Riva C. and Provini A., New evidences for old biomarkers: effects of several xenobiotics on EROD and AChE activities in Zebra mussel (*Dreissena polymorpha*), *Chemosphere* **62** (4), 2006, 510–519.
- Brunner D., Frank J., Appl H., Schöffl H., Pfaller W. and Gstraunthaler G., Serum-free cell culture: the serum-free media interactive online database, *ALTEX* **27**, 2010, 53–62, <https://doi.org/10.14573/altex.2010.1.53>
<https://www.fcs-free.org/>.
- Casper F.R., Quesne M., Rogers M.I., Shirota T., Jolivet A., Milgrom E. and Savouret J.-F., Resveratrol has antagonist activity on the aryl hydrocarbon receptor: implications for prevention of dioxin toxicity, *Mol. Pharmacol.* **56** (4), 1999, 784–790.
- Çok I., Donmez M.K., Uner M., Demirkaya E., Henkelmann B., Shen H., Kotalik J. and Schramm K.-W., Polychlorinated dibenzo-p-dioxins, dibenzofurans and polychlorinated biphenyls levels in human breast milk from different regions of Turkey, *Chemosphere* **76** (11), 2009, 1563–1571.
- Commission Regulation (EC), Setting maximum levels for certain contaminants in foodstuffs, *Orkesterjournalen L* **364**, 1881/2006, 5–24, of 19 December 2006.
- Commission Regulation (EU), Amending Annexes I and II to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels and action thresholds for dioxins and polychlorinated biphenyls, *Orkesterjournalen L* **91**, 277/2012, 1–7, of 28 March 2012.
- Commission Regulation (EU), Amending Regulation (EC) No 1881/2006 as regards maximum levels of the contaminants dioxins, dioxin-like PCBs and non-dioxin-like PCBs in liver of terrestrial animals, *Orkesterjournalen L* **289**, 2013, 56–57, of 30 October 2013.
- Commission Regulation (EU), Laying down methods of sampling and analysis for the control of levels of dioxins, dioxin-like PCBs and non-dioxin-like PCBs in certain foodstuffs and repealing Regulation (EU) No 589/2014 *Orkesterjournalen L* **92**, 2017/644, 9–34, of 5 April 2017.

- Cupp M.J. and Tracy T.S., Cytochrome P450: new nomenclature and clinical implications, *Am. Fam. Physician* **57** (1), 1998, 107-116.
- Donato M.T., Gómez-Lechón M.J. and Castell J.V., A microassay for measuring cytochrome P450IA1 and P450IIB1 activities in intact human and rat hepatocytes cultured on 96-well plates, *Anal. Biochem.* **213** (1), 1993, 29-33.
- Eichbaum K., Brinkmann M., Buchinger S., Reifferscheid G., Hecker M., Giesy J.P., Engwall M., van Bavel B. and Hollert H., In vitro bioassays for detecting dioxin-like activity-application potentials and limits of detection, a review, *STOTEN* **487** (1), 2014, 37-48.
- El-Kady A., Abdel-Wahhab M., Henkelmann B., Belal M., Morsi K., Galal S. and Schramm K.-W., Polychlorinated biphenyl, polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofuran residues in sediments and fish of the river Nile in the Cairo region, *Chemosphere* **68** (9), 2007, 1660-1668.
- European Union Reference Laboratory (EU-RL) for Dioxins and PCBs in Feed and Food, Freiburg, Germany. http://www.cvua-freiburg.de/pdf/dioxine/guar_gum_correlation_270807.pdf.
- Gizzi G., Hoogenboom L.A., Von Holst C., Rose M. and Ankam 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.
- Hofmaier A.M., Evaluierung eines Testsystems auf zellulärer Basis zur Detektion von Dioxinen und verwandten Verbindungen in Matrices der metallrecyclenden Industrie sowie die Bestimmung der gentoxischen Wirkung dieser Proben, PhD Thesis 1999, Technical University Munich.
- Li W., Wu W.Z., Schramm K.-W., Xu Y. and Kettrup A., Toxicity of mixtures of polychlorinated dibenzo-p-dioxins, dibenzofurans, and biphenyls determined by dose-response curve analysis, *Bull. Environ. Contam. Toxicol.* **62** (5), 1999, 539-546 <https://doi.org/10.1007/s001289900909>.
- Loutfy N., Fuerhacker M., Tundo P., Raccanelli S., El Dien A.G. and Tawfic A.M., Dietary intake of dioxins and dioxin-like PCBs, due to the consumption of dairy products, fish/seafood and meat from Ismailia city, Egypt, *STOTEN* **370** (1), 2006, 1-8.
- Marin-Kuan M., Fussell K.C., Riederer N., Latado H., Serrant P., Mollergues J., Coulet M. and Schilter B., Differentiating true androgen receptor inhibition from cytotoxicity mediated reduction of reporter-gene transactivation in-vitro, *Toxicol. Vitro* **45** (3), 2017, 359-365.
- Mikstacka R., Baer-Dubowska W., Wieczorek M. and Sobiak S., Thiomethylstilbenes as inhibitors of CYP1A1, CYP1A2 and CYP1B1 activities, *Mol. Nutr. Food Res.* **52** (1), 2008, 77-83, <https://doi.org/10.1002/mnfr.200700202>.
- Petrulis J.R., Chen G., Benn S., LaMarre J. and Bunce N.J., Application of the ethoxyresorufin-O-deethylase (EROD) assay to mixtures of halogenated aromatic compounds, *Environ. Toxicol.* **16** (2), 2001, 177-184.
- Schiwy A., Brinkmann M., Thiem I., Guder G., Winkens K., Eichbaum K., Nüßer L., Thalmann B., Buchinger B., Reifferscheid G., Seiler T.B., Thoms B. and Hollert H., Determination of the CYP1A-inducing potential of single substances, mixtures and extracts of samples in the micro-EROD assay with H4IIE cells, *Nat. Protoc.* **10** (11), 2015, 1728-1741.
- Schlezing J.J., Struntz W.D.J., Goldstone J.V. and Stegeman J.J., Uncoupling of cytochrome P450 1A and stimulation of reactive oxygen species production by co-planar polychlorinated biphenyl congeners, *Aquat. Toxicol.* **77**, 2006, 422-432.
- Schmitz M., Seiler A., Watt M.-A., Alexandridis R., Walch H., Boess F. and Roth A.B., Improved cellular analysis for the early detection of compound-induced hepatic cytotoxicity to reduce animal testing, *Roche Applied Science Cancer Research Application Note* **5**, 2010.
- Schwirzer S.M.G., Hofmaier A.M., Kettrup A., Nerdinger P.E., Schramm K.-W., Thoma H., Wegenke M. and Wiebel F.J., Establishment of a simple cleanup procedure and bioassay for determining 2,3,7,8-Tetrachlorodibenzo-p-dioxin toxicity equivalents of environmental samples, *Ecotoxicol. Environ. Saf.* **41** (1), 1998, 77-82.
- Stachel B., Christoph E.H., Gotz R., Herrmann T., Kruger F., Kuhn T., Lay J., Löffler J., Papke O., Reincke H., Schroter-Kermani C., Schwartz R., Steeg E., Stehr D., Uhlig S. and Umlauf G., Dioxins and dioxin-like PCBs in different fish from the river Elbe and its tributaries, Germany, *J. Hazard Mater.* **148** (1-2), 2007, 19-29.
- Tard A., Gallotti S., Leblanc J.-C. and Volatier J.L., Dioxins, furans and dioxin-like PCBs: occurrence in food and dietary intake in France, *Food Addit. Contam.* **24** (9), 2007, 1007-1017.

Wang J., Bi Y., Pfister G., Henkelmann B., Zhu K. and Schramm K.-W., Determination of PAH, PCB, and OCP in water from the Three Gorges Reservoir accumulated by semipermeable membrane devices (SPMD), *Chemosphere* **75** (8), 2009, 1119-1127.

Weber R., Herold C., Hollert H., Kamphues J., Ungemach L., Blepp M. and Ballschmiter K., Life cycle of PCBs and contamination of the environment and food producing animals, *Environ. Sci. Pollut. Res.* **25** (17), 2017, 16325-16343.

Whitlock J.P., Jr., Okino S.T., Dong L., Ko H.P., Clarke-Katzenberg R., Ma Q. and Li H., Induction of cytochrome P4501A1: a model for analyzing mammalian gene transcription, *FASEB J* **10** (8), 1996, 809-818.

Whyte J., Schmitt C. and Tillitt D., The H4IIE cell bioassay as an indicator of dioxin-like chemicals in wildlife and the environment, *Crit. Rev. Toxicol.* **34** (1), 2004, 1-83.

Transparency document

[Multimedia Component 1](#)

coi_disclosure

alt-text: coi_disclosure

Appendix A. Supplementary data

The following are the Supplementary data to this article:

[Multimedia Component 2](#)

Supplementary Information

alt-text: Supplementary Information

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.~~

Queries and Answers

Query: Please confirm that the provided email “walkiria.levy@helmholtz-muenchen.de” is the correct address for official communication, else provide an alternate e-mail address to replace the existing one, because private e-mail addresses should not be used in articles as the address for communication.

Answer: The email: walkiria.levy@helmholtz-muenchen.de is the correct one.

Query: Please check whether the designated corresponding author is correct, and amend if necessary.

Answer: The corresponding author is correct.

Query: Please confirm that the provided emails “walkiria.levy@helmholtz-muenchen.de” are the correct address for official communication, else provide an alternate e-mail address to replace the existing one, because private e-mail addresses should not be used in articles as the address for communication.

Answer: The provided email is the correct email for official communication, however you should also please add the email schramm@helmholtz-muenchen.de

Query: Have we correctly interpreted the following funding source(s) and country names you cited in your article: Federal Office of Agriculture and Food, Germany?

Answer: Federal Office of Agriculture and Food (BLE), Germany (Project funding code 314-06.01-2813HS021)

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!

Query: Uncited references: This section comprises references that occur in the reference list but not in the body of the text. Please cite each reference in the text or, alternatively, delete it. Any reference not dealt with will be retained in this section.

Answer: I deleted it.

Query: Please confirm that given names and surnames have been identified correctly and are presented in the desired order and please carefully verify the spelling of all authors' names.

Answer: Yes

Query: Your article is registered as a regular item and is being processed for inclusion in a regular issue of the journal. If this is NOT correct and your article belongs to a Special Issue/Collection please contact s.bhawarlal@elsevier.com immediately prior to returning your corrections.

Answer: Yes