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READY TO USE EROD ASSAY BY CRYO-PRESERVED CELLS AND QUANTIFICATION BY STANDARD ADDITION METHOD

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Introduction

Bioanalytical screening methods for persistent organic pollutants are applied for biomonitoring of food, feed and environmental samples. Especially the cell-based bioluminescent 7-ethoxyresorufin-O-deethylase (EROD) assay is one of the most widely non-commercial used bioassays for the analysis of dioxins and dioxin-like compounds (1).

Besides chemical analysis of dioxins and dioxin-like compounds, effect related analysis of food and feed stuff has been approved and suggested by new regulations in the European Union (2). Nevertheless many control laboratories are not equipped with cell culture facilities for routine effect related analysis of samples. To offer a simplified effect related analysis of food and feed stuff a ready-to-use EROD assay based cryo-preserved cells is in development. The assay will enable effect related analysis from thawed cells without the need for pre-culturing cells. Hence no routine cell culture laboratory will be mandatory to perform bioanalytical analysis based on the EROD assay due to ready to use frozen cells.

Commonly with the EROD assay, dioxins and PCBs are quantified by a standard curve based on 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Here, the unknown sample concentration is interpolated from the known concentration of the standard curve. For reliable quantification the same measuring properties for the standard and the unknown sample have to be assumed. However in biomonitoring it is common sense that there are matrix effects present from the sample material, which can significantly influence sample quantification. The standard addition method offers the possibility to incorporate these matrix effects into sample quantification. Herein we evaluate alternative strategies in quantitative biomonitoring based on the standard addition method.

Material and Methods

For analysis 2 to 40 gram homogenized sample was mixed with hydromatrix and extracted by an Accelerated Solvent Extractor (Dionex ASE 300) with hexane:acetone (75:25, v/v) at 120 °C and 12 MPa with two static cycles of extraction (10 min each). Water was removed from the organic phase with anhydrous sodium sulfate following reduction to 2-3 mL by a vacuum rotary evaporator. Sample clean-up was performed on a chromatography column packed with silica gel (activated silica-gel, sulphuric silica-gel, inactivated silica-gel, anhydrous sodium sulfate) pre conditioned with hexane. The sample extract was eluted with dichloromethane:hexane (1:100, v/v) and concentrated to 2-3 mL by a vacuum rotary evaporator. After evaporation with 200 µL DMSO under a gentle stream of nitrogen the sample was dissolved in DMSO:hexane (4:1, v/v) with a final volume of 500 µL.

Bioanalytical equivalents were measured by the EROD assay in 96-well plate format using the rat hepatoma cell line H4IIE according to (3) with modifications(4-6). Cells were seeded at a density of 1x10^4 cells/well in Dulbecco's MEM (w 3,7 g/l NaHCO3, w 4.5 g/l D-Glucose, w/o L-Glutamin, w/o phenol red) supplemented with 10 % Fetal Bovine Serum, 3.5 mM L-Glutamin and 25 mM HEPES.
The cells were exposed to decreasing concentrations (dilution series: 1/1 to 1/32) of samples together with a control sample (TCDD: 0, 0.03, 0.06, 0.12, 0.2, 0.4, 0.6, 1.2 pg/well) added in 50 µL to the wells in quadruplicates. For standard addition experiments the sample was added to the TCDD dilution series. The EROD assay was performed after 72 h of exposure in incubation conditions of 37 °C and 7.5% CO2. Fluorimetrical measurement was performed by a Tecan Fluorostar with excitation: 590 and emission: 535 nm.

Protein measurement was performed with the BCA Protein Assay Kit (Novagen) according to the manufacturer directly after the EROD assay including a BSA standard protein curve in rising concentrations of 0, 3.91, 7.81, 15.6, 62.5, 125, 250, 500 µg/mL on the same plate. Absorbance was recorded at 540 nm after 90 min incubation.

Sample quantification was interpolated from to the TCDD standard curve by a four-parameter log-logistic curve fit approach by the statistical software "R" and the package "drc" (7). The EROD values were normalized by the protein values and the protein standard curve. Additionally one outlier was removed from the quadruplicate measurement.

Results and discussion

Evaluation of three different cryo media for cells frozen either in 96 well plates or cryo vials revealed varied efficacies in the EROD assay. The EROD assay was performed directly after thawing of cells and compared to the EROD assay performed with cells from permanent culture; results are summarized in fig 1. For cells frozen in 96 well plates maximum relative fluorescence units (RFU) reached only 19.5 % (NFM), 38.4 % (cryoSO) and 36.0 % (standard) compared to permanently cultured cells. For cells frozen in cryo vials maximum RFUs were up to 77.7 % of permanently cultured cells, additionally ED50 values were increased for cryo cells (ED50: 0.11) compared to permanently cultured cells (ED50: 0.21). Therefore, appropriately applied freezing and thawing condition enables the use of cryo preserved cells for the EROD assay without the need of permanently culturing cells.

Quantification of dioxins and dioxin-like compounds by standard addition measurements was based on linear extrapolation of RFU differences between the standard curve and the standard addition curve (fig. 2). In total ten (soil and food) samples were analyzed and the measurements also compared to chemical analysis by HRGC/HRMS (fig. 3). Only for one soil sample the standard addition method compared to external calibration revealed a lower BEQ. In comparison to TEQs, the standard addition method marginally underestimated the BEQs for three samples, whereas the external calibration underestimated the BEQs in eight from ten samples. Therefore using a standard addition method might offer the possibility not to underestimate the amount of dioxins and dioxin-like compounds found in samples which are commonly under food and environmental safety surveillance.

Acknowledgments

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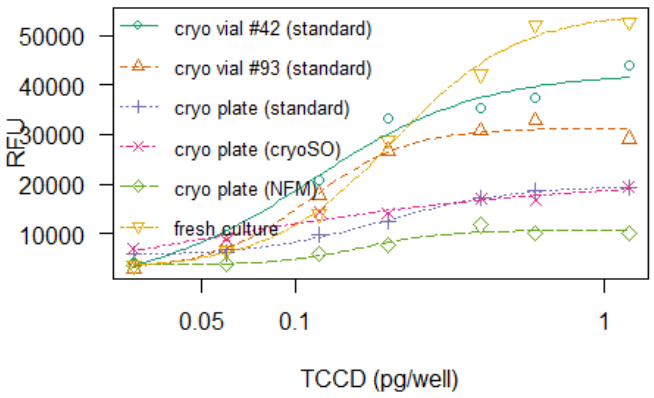
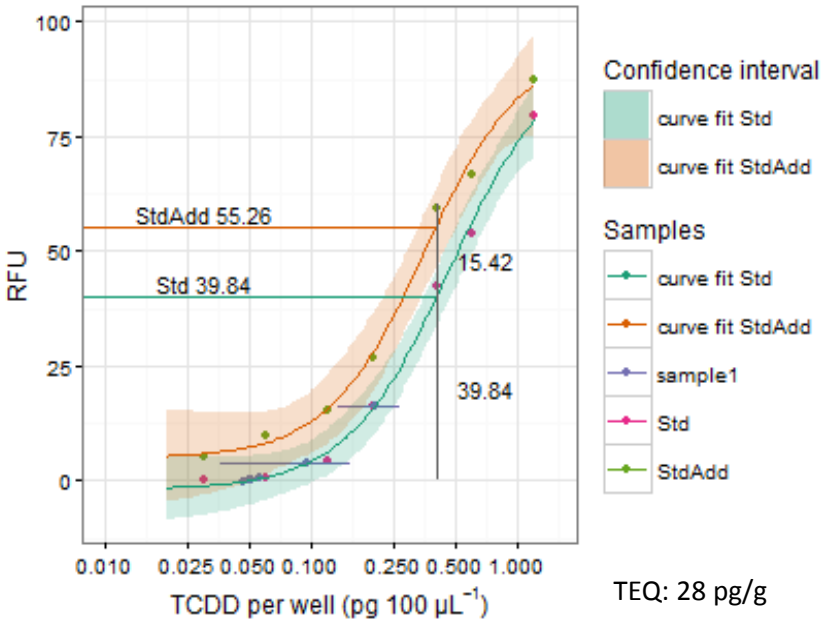


Figure 1: Comparison of cryo preserved cells with permanently cultured cells in EROD assay. Rat hepatoma cells (H4IIE) were cryo preserved with different cryo protectants (standard, cryoSO and NFM). After thawing of cells in 96-well plates and from cryo vials, direct EROD assay using TCDD was performed in comparison to permanently cultured cells.



TEQ: 28 pg/g

Sample concentration by external calibration

dilution	RFU sample	RFU Estimate	BEQ [pg/g]	BEQ z-score	BEQ (z: -1/1)
32	-0.11	0.047	93.9	1.8	22.6
16	0.1	0.051	50.2	0.5	22.6
8	0.06	0.050	24.8	-0.3	22.6
4	0.46	0.056	13.8	-0.6	22.6
2	3.88	0.096	11.9	-0.7	22.6
1	16.2	0.201	12.5	-0.7	22.6

Sample concentration by standard addition

TCDD [pg]	RFU Std	RFU StdAdd	RFU diff	BEQ [pg/g]	z-score	BEQ (z: -1/1)
0	-1.9	5.0	6.9	0.0	#N/A	39.0
0.03	-1.1	5.9	7.0	48.7	-0.1	39.0
0.06	0.8	8.3	7.5	144.5	2.2	39.0
0.12	6.4	15.8	9.4	43.3	-0.3	39.0
0.2	16.1	28.4	12.3	37.9	-0.4	39.0
0.4	39.8	55.3	15.4	38.5	-0.4	39.0
0.6	56.6	70.5	13.9	36.7	-0.4	39.0
1.2	78.7	86.4	7.7	29.2	-0.6	39.0

Figure 2: Measurement of soil sample by standard addition and external calibration method. For standard addition method, the sample concentration was extrapolated from the difference between standard and standard addition curve at respective TCDD standard points. Sample concertation was calculated within z-score range of -1 to 1. Sample: BFHNR 30504.

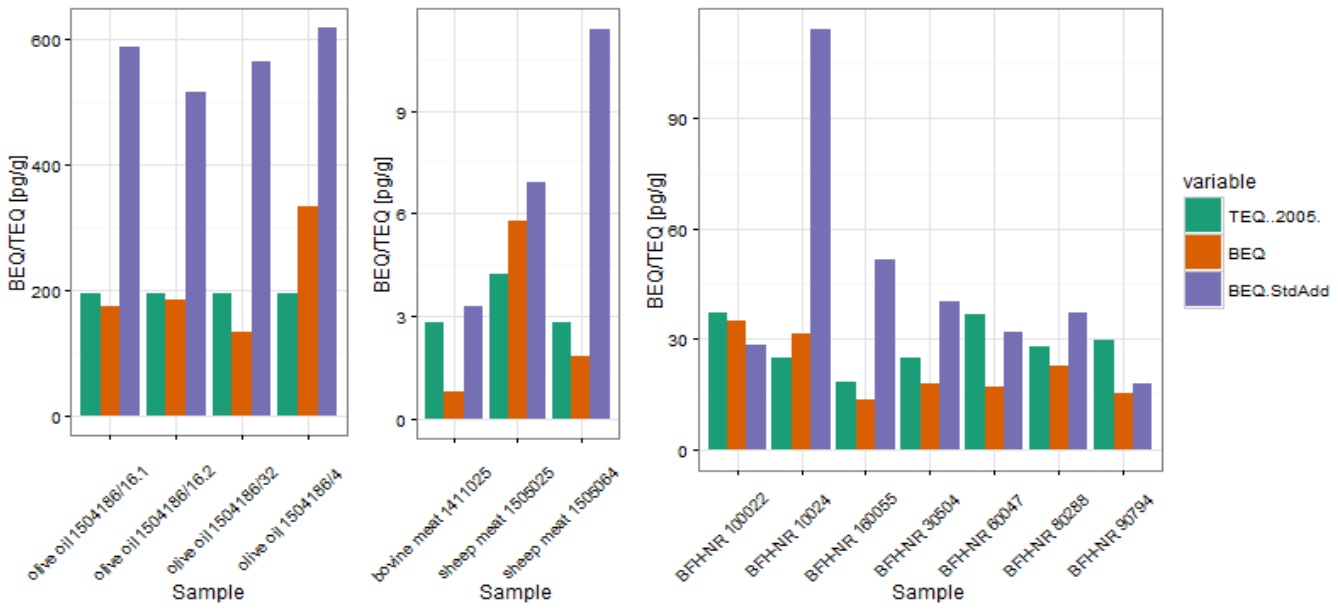


Figure 3: Comparison of measurements by standard addition and external calibration method for food and soil samples.