MODIFICATION OF THE MICRO EROD-BIOASSAY AND VALIDATION FOR ROUTINE ANALYSIS DEMONSTRATED FOR BEEF AND MILK

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Introduction

Biological test systems for routine application should be simple and robust to use. Aim of the study was to reduce handling time and material costs for the Micro EROD-Bioassay for dioxin screening in food samples. The measurement procedure used so far contained a laborious transfer step which needed accurate timing and thus limited the number of plates per batch[1,2]. Concepts of in vitro Micro EROD-Bioassays with lysed[3,4] and live cells[5,6] were compared. Some steps which were critical in terms of time and skill were eliminated. Protein contents was measured to detect cytotoxicity but was not used for normalisation as described before[5]. The resulting procedure was validated according to the criteria for bioassays defined by the European Commission[7].

Materials and methods

Following samples were tested: Beef (50), bovine milk (22) and human breast milk (1). In addition one specimen each of beef, reindeer, milk and milk powder were from proficiency tests, provided by NIH (Oslo, Norway) and EU-RL for Dioxins and PCBs (Freiburg, Germany) respectively. All other samples were measured in routine HRGC/HRMS analysis at the LAVES Food and Veterinary Institute Oldenburg, Germany. For re-evaluation of the bioassay some samples were extracted and measured twice: 19 beef, the reindeer and all 25 milk samples.
2,3,7,8-TCDD for standard curves was from LGC-Standards. Acetone and n-hexane for dioxin analysis were from Biosolve. Na_2SO_4 and silica (mesh 63 to 200 µm) were purchased from Merck. H_2SO_4 (95%) from VWR was used. Substrates for bio analysis (resorufin ethyl ether and 3,3′-methylenebis(4-hydroxycoumarin)), BCA test kit and DMSO (cell culture tested) were from Sigma-Aldrich. All glass material, silica and Na_2SO_4 were heated for eight hours at 300 °C. Rat hepatoma cells (H4IIEC/T3) were courtesy of Prof. Schramm (Helmholtz Institute Neuherberg, Germany)[1]. They were cultured in DMEM without phenol red (Biochrom), with 3.5 mM L-glutamine, 22 mM HEPES buffer, 10 % FCS (Invitrogen) at 37 °C, 95 % humidity and 7 % CO_2.

Chemical Methods

Preparation of milk products:
40 g raw milk was centrifuged at 4 °C and 3000 rpm for 30 min. The upper cream layer was further processed.
5 g of milk powder and 8 ml acetone/water 4/1 (v/v) were agitated for 5 min. Best results were achieved with an orbital shaker (IKA VIBRAX) at 2000 rpm. The mixture was allowed to settle and coagulate for another 5 min.

Lipid extraction and clean up:
Cream of milk, coagulated milk powder and 10 g of homogenized muscle tissue respectively were used. Lipids were extracted by addition of approximately 40 g Na_2SO_4 per sample and thorough mixing. The resulting powder was allowed to rest for 20 to 90 minutes. Subsequently the mixture was agitated again and filled in glass columns with glass wool. The samples were eluted using 200 ml n-hexane/acetone 2/1 (v/v)[8].
1g fat per sample was cleaned up as described before[5]. In brief: Silica columns (top to bottom: 3 g Na_2SO_4; 5 g 22 w% sulphuric acid on silica; 5 g 33 w% sulphuric acid on silica, glass wool) were eluted with 3x 2 ml + 34 ml n-hexane. Dichloromethane was used for the transfer of the reduced extracts into glass vials with 10 µl DMSO. Sample stems were prepared by adding 10 µl DMSO/isopropanol (3+2) and 1980µl culture medium aseptically to each reduced extract. Then they were shaken at ambient temperature at 600 rpm for at least 10 min.

Bioanalytical Methods

The Micro EROD-Bioassay was performed in 96-well tissue culture plates (TPP) [1,5]. 10000 cells in 50 µl DMEM were seeded per well. After two to four hours at 33 to 37°C, 50µl/well of controls and sample stems were added. The final solvent concentration was 0.5 % DMSO/isopropanol 4/1 (v/v). Each plate contained a TCDD standard curve. All concentrations were measured threefold. To test the signal response another well per sample was spiked with 0.048 pg TCDD. After 72 hours at culture conditions media were removed. Substrate composition and stopping procedure differed between the established (DMEM/transfer) and the new method (PBS/dispense).

DMEM/transfer[1,2,5,6] :100µl DMEM (with phenol red, Biochrom) with 8 µM 7-ethoxyresorufin (ETX) and 10 µM Dicumarol were dispensed per well. Each plate was incubated for exactly 30 minutes at 33 to 37 °C. Then 80µl medium per well were transferred to a second plate which already contained 178µl ethanol per well.

PBS/dispense: 100µl phosphate buffered saline[10] (PBS, Biochrom) with 8 µM 7-ethoxyresorufin (ETX) and 10 µM dicumarol were dispensed per well. The plates were incubated for 30 minutes at 33 to 37 °C. To stop the reaction 75µl of cold methanol were dispensed in all wells.

Plates were shaken for 2 min at 300 rpm. EROD fluorescence was determined at 544 nm for excitation and 590 nm for emission[2,3,4]. For detection of toxicity 100µl BCA-mixture per well were added to the cell culture plates. DMEM/transfer plates were rinsed twice with 100µl PBS/well in advance to BCA addition. Each plate contained 8 concentrations of bovine serum albumin including a blank. Absorbance at 550 nm was measured 90 min later.

Sample fluorescence was compared to the respective 2,3,7,8-TCDD standard curve (optimised by 4-parameter fit) and expressed in bio equivalents (BEQ)[7,9]. All data had to fulfil the following premises:
- $CV_{Response}$ of concentration triplicates <15%[7, 9]
- Standard curve with ≥ 5 valid concentrations and EC80 must be reached by one of the two high standards[9]
- Addition of TCDD-solution to sample wells must result in at least 75% of the calculated sum[7]

Final sample results were calculated by blank and recovery correction according to the applicable regulation[7].

Results and discussion

The results of the handling pretest are shown in figure 1. Higher fluorescence for the PBS/dispense method is probably due to less absorbance in PBS compared to DMEM with phenol red. The curves inductions were calculated as the fluorescence ratio of the highest standard to the negative control. The differences of slope, induction and CV of replicates were not significant between procedures. EC50 of the PBS/dispense method was significantly higher but still within the limit set for quality control (0.12 ±0.06). The difference of the relative response of PCB-126 dilution series tested on the plates was not significant (data not shown). For longer stability of the fluorescence plates can be covered, e.g. with fluorescence permeable polyolefin film, see figure 2.

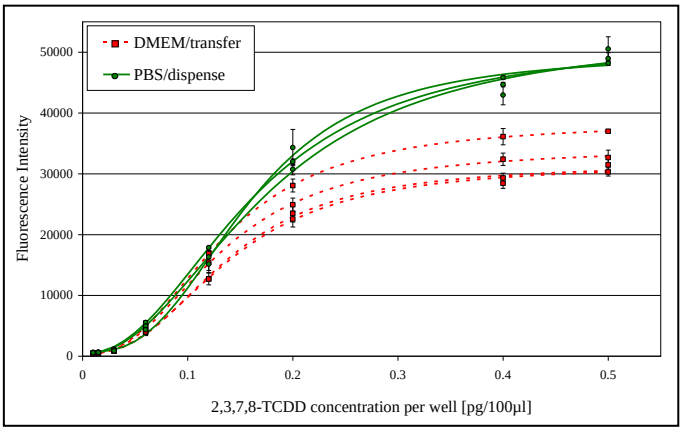
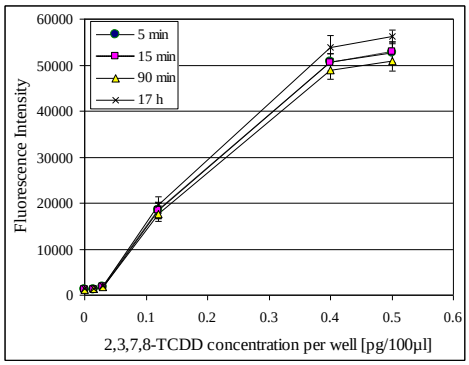


Figure 1: Test of substrate handling, each data point represents the mean of three wells. Error bars are SD of triplicates. Green slopes and circles are data of plates with ETX in PBS and methanol added to the wells (PBS/dispense). Red squares and lines represent the former routine (DMEM/transfer) ETX in DMEM and transfer to a second plate. Results of relevant curve parameters (mean ± SD) were:

	DMEM/transfer	PBS/dispense
Slope	2.5 ±0.1	2.4 ±0.4
EC50	0.137 ±0.005	0.166 ±0.009
CV	5.7 ±0.6	6.0 ±1.4
Induction	75 ±17	85 ±5

Figure 2: Evaluation of the time dependence of the fluorescence signal using a plate with ETX in PBS and methanol added to the wells (PBS/dispense). Each data point represents the mean of three wells. Error bars are standard deviations (SD) of triplicates. The fluorescence signal was stable between 5 minutes and 17 hours after addition of methanol. The CVs between data points of one 2,3,7,8-TCDD-concentration were between 4.2 % and 9.2 %.
Addition of BCA-solution for protein tests resulted in increasingly darker mixtures with time. Thus fluorescence was reduced. With a higher gain of the photometer (FLUOstar OPTIMA) it was still measurable after several days (data not shown).



Further experiments using PBS/dispense showed that cooling and pre-flush of pipette tips with cold methanol further reduced variations between triplicates of concentrations. After these basic tests the PBS/dispense method was used for matrix validation according to the current European regulations[7]. During initial validation beef and milk fat samples were measured 6fold to determine the repeatability (RSDr). Results are shown in tables 1 and 2.

Table 1: Initial validation of beef (representing ruminant muscle) and milk fat (representing diary products). Each concentration was extracted and measured via EROD-Bioassay 6fold. WHO-TEQs of 2005 were used[7]. Mean values of HRGC/HRMS (GC/MS), bioassay results and coefficients of variation (CV) are shown.

Beef			Milk fat	
GC/MS pg TEQ/g fat (Σ PCDD/F +dl PCB)	EROD pg BEQ/g fat ± CV %		GC/MS pg TEQ/g fat (Σ PCDD/F +dl PCB)	EROD pg BEQ/g fat ± CV %
-	-		0.53	0.84 ±16.5 %
2.88	3.01 ±8.60 %		1,53	1.49 ±18.4 %
4.84	5.15 ±18.0 %		3.03	3.08 ±13.2 %
6.84	6.85 ±8.40 %		5.53	4.80 ±17.2 %
8.88	9.78 ±15.1 %		10.53	10.2 ±12.2 %

The following step was the re-evaluation of the matrix cut offs with samples of different origin. Figures 3 and 4 show the data for beef and milk. Results from proficiency tests and routine analysis were included to further enlarge the data pool.

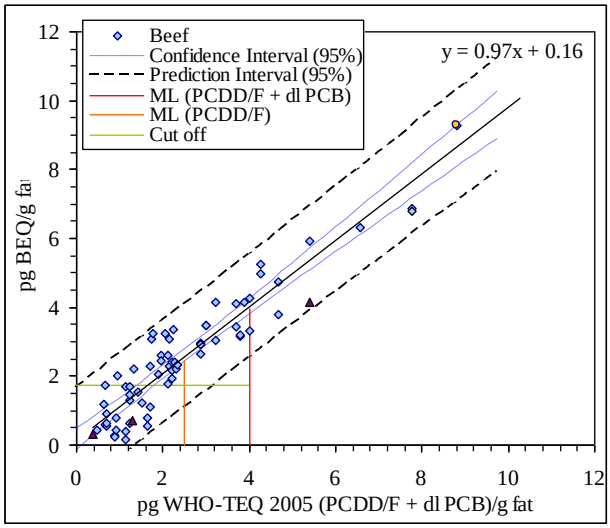


Figure 3: Comparison of sample results determined via Micro EROD-Bioassay and HRGC/HRMS for matrix re-evaluation of ruminant muscle. 19 beef samples (◇) and one reindeer (○) were extracted and measured in duplicate. Further 31 data points are results from routine analysis.
In three beef samples from routine the cells signal response was reduced. The results shown (▲) are from second cleanups and bioassays. All extracts showed a regular signal response during repetition**.**

For screening purpose the maximum level (ML) for PCDD/Fs (—) was chosen. The resulting cut off for EROD-Bioassay values suspected to be non conform used in routine analysis was 1.75 pg BEQ/g fat (—). For this cut off the false negative rate was 0 %.

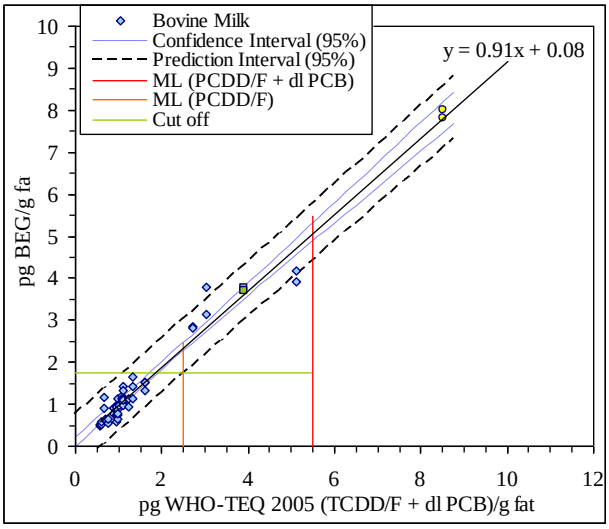


Figure 4: Comparison of sample results determined via Micro EROD-Bioassay and HRGC/HRMS for matrix re-evaluation in milk. Samples were extracted and measured twice: 23 bovine milks (◇), one sample of human breast milk (○) and one specimen of milk powder (□). The cells signal response was regular for all extracts.

For screening purpose the maximum level (ML) for PCDD/Fs (—) was chosen. The resulting cut off for EROD-Bioassay values suspected to be non conform was 1.88 pg BEQ/g fat (—). For this cut off the false negative rate was 0 %.

Table 2: Summary of relevant criteria for dioxin screening according to the current European regulation[7] compared to the data from EROD-bioassay validation for ruminant muscle and milk products

	Criterion[7]	Ruminant validation	Beef from routine included	Milk validation
n	≥ 20	2x 20	Additional 31	2x 25
RSDr*	< 20 %	16.9 %	-	17.8 %
RSDR	<25 %	18.9 %	23.6 %	16.4 %
False negative	< 5 %	0	0	0

***** These data result from the initial validation using 6fold analysis of samples contaminated at different levels

To summarize the study the current European regulation and the results of the validation were compared[7]. Table 2 shows the most relevant numerical parameters. During validation the apparent recovery of reference samples was >30 % for all batches. Procedure blanks allowed discriminating between the blank and samples above cut off value with a factor of at least 3. During routine dioxin screening of beef the cut off was applied and no false negative result was found via HRGC/HRMS confirmation. Signal response was tested on every sample. 3 samples showed a reduced signal response and were forwarded to HRGC/HRMS confirmation. Traces of acid silica or similar artefacts could be responsible for the signal suppression. Clean up and bioassay were repeated for these three samples. Here the signal response was regular. In this respect all routine samples are tested for signal suppression. The current European regulation demands tests for at least 20 % of the samples[7].

Compared to the previous method[5] the handling time from stopping the EROD reaction to BCA addition is reduced by 70 %. The skill and time dependant transfer[6] of ETX-medium is no longer necessary. The amount of stopping solvent was reduced from 178 µl ethanol/well[5,9] to 75 µl methanol/well. When using buffer[4,10] instead of culture medium[2,5,6] material costs were reduced. The absence of glucose allowed the application of BCA within the same plate. Working with live cells[1,2] does not require rinsing and freezing of cell culture plates and no additional NADPH[4,10] for the EROD reaction is necessary. Some authors prefer the utilisation of acetonitrile with fluorescamin[3, 10]. Thus protein is measured via fluorescence as well. For an analysis which requires a comparison of resorufin/mg protein this might be useful. For dioxin screening standards or reference samples are used to determine sample contamination. Here the application of the less expansive and less hazardous methanol combined with BCA for exclusion of toxicity is sufficient. In case the measurement needs to be postponed the fluorescence can be preserved using polyolefin film to prevent solvent evaporation.

The introduced procedure (PBS/dispense) is able to meet the criteria of the current European regulation[7]. For milk data around the cut off and up to the maximum levels were rare. If the amount of data increases it can be necessary to re-calculate the cut off. Since using the presented procedure and applying the current European regulations the stopping procedure of the Micro EROD-Bioassay is no longer limiting the sample throughput[7].

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