STEP BY STEP ANALYSIS OF RECOVERY RATES OF DIOXIN LIKE COMPOUNDS IN BEEF USING THE MICRO-EROD-BIOASSAY

Thiem I, Böhmler G

Lower Saxony State Office for Consumer Protection and Food Safety, Food Institute Braunschweig (LAVES LI-BS); Dresdenstraße 2+6, 38124 Braunschweig, Germany

Introduction

During an interlaboratory comparison on Dioxins in food in 2009[1] eight labs showed z-scores below -4 and thus considerably underestimated the dioxin like contents of a beef sample. As none of these teams reported congener results for PCDD/Fs or PCBs we assumed that some of theses labs used bio analytical tools. For this study with the Micro-EROD-Bioassay we worked our way from single standards to complex mixtures and added a standard mixture to beef with low contamination. Finally we measured the beef sample tested in the comparison.

Materials and methods

Material

Chemical standards (2,3,7,8-TCDD; PCB-126; PCB-153; mixture of eight monoortho PCBs 10µg/ml each) were custom solutions in DMSO from LGC-Standards. Solvents for pesticide analyses, Na_2SO_4 and H_2SO_4 were purchased from VWR International, Silica 60 from Applichem. Substrates for bioanalysis (Resorufin ethyl ether and 3,3′-Methylenebis(4-hydroxycoumarin)), BCA test kit and DMSO (cell culture tested) were from Sigma-Aldrich. Some materials were heated for eight hours (glass, silica 300°C; Na_2SO_4 600°C). Reuber rat hepatoma cells (H4IIE) were courtesy of Prof. Schramm (Helmholtz Institute Neuherberg) and cultured in DMEM with Phenolred (Biochrom), 10% FCS (Invitrogen) at 37°C with 95% humidity. Beef samples originated from routine analysis of LAVES and the interlaboratory comparison of the Norwegian Institute of Public Health (FHI).

Bioanalytical Methods

The Micro-EROD-Bioassay with H4IIE cells was used to determine the dioxin like response[2,3]. 10.000 cells/well in 50µl DMEM were seeded on 96-well-plates. Two hours after seeding 50µl of sample or standard in DMEM were added[4]. 20µl of sample extract in DMSO/Isoproanol (4/1 v/v) were dissolved in 1980µl DMEM (10% FCS) and shaken at 600rpm for at least 10 minutes in glass vials. 20µl standard (TCDD, PCBs) were added to 1980µl DMEM, shaken for 5 seconds and immediately loaded onto the plate. The final concentration of solvent (DMSO/Isopropanol 4/1 v/v) was always 0.5%. Standard and sample concentrations were tested fourfold for one data point. Each plate contained a full TCDD standard curve (0.5; 0.4; 0.2; 0.12; 0.06; 0.03; 0.015pg/well). After 72h incubation at 37°C, 7% CO_2 and 95% humidity the medium was removed. 100µl substrate[2,4] per well were incubated (30min) and 80µl than transferred to 178µl Ethanol. Fluorescence (Ex: 530nm, Em: 590nm) was measured. Sample results were compared to the TCDD standard curve and expressed in bioequivalents (BEQ)[4]. The BCA protein test was only performed to guarantee no negative result due to cytotoxicity.

Chemical Methods

Samples with a complete extraction were split to two sub samples. One sub sample was spiked with 20pg 2,3,7,8-TCDD at the first handling step. Fat was extracted on a glass column two hours after mixture with Na_2SO_4 using 300ml dichloromethane/cyclohexane 1/1 v/v. The cleanup[5] was modified as follows: Silica columns (top to bottom: 3g Na_2SO_4; 5g 22w% sulphuric acid on silica; 5g 33w% sulphuric acid on silica) were eluted with 2x5ml +28ml n-hexane. Extracts were reduced and transferred to glass vials with dichloromethane. The final sample extracts were reduced with 10µl DMSO as keeper.

Test protocol

Step 1: Each solution was measured on its own: PCB-126 (8; 4; 2; 1; 0.5; 0.25pg/well), monoortho PCB mix (32, 16, 8, 4, 3.2, 2.8, 2.5, 2, 1, 0.8, 0.4, 0.2pg/well) and PCB-153 (4, 2, 1, 0.5, 0.25pg/well) respectively.

Step 2: The concentration of exactly one partner changed. Dosing was done in two rounds. The solutions for fix concentrations were mixed and 25µl added to the cells. Immediately afterwards the standard for the variable concentration was given to DMEM and usually diluted 5 times to half of the preceding concentration. 25µl of

each dilution was then dosed to the cells. A compressed summary of the tested concentrations can be seen in Tab. 1 and 2. Almost all possible combinations were tested in the EROD-Bioassay.

Tab. 1: Concentrations (C) of mixtures from two standard solutions (one fix – per row, one variable - columns)

	C_{fix} [pg/well]	TCDD [pg/well]	PCB-126 [pg/well]	Monoortho PCBs [ng/well]	PCB-153 [ng/well]
TCDD	**0.25**	/	/	16 to 2, 1.6, 1.3	2 to 0.063
	0.1	/	/	/	2 to 0.063
PCB-126	**4 & 2**	0.25, 0.2, 0.1, 0.06, 0.03, 0.015	/	/	2 to 0.063
Monoortho PCB-Mix	**16000**	0.25, 0.2, 0.1, 0.06, 0.03, 0.015	/	/	2 to 0.063
	2000 & 200	0.25, 0.2, 0.1, 0.06, 0.03, 0.015	/	/	/
PCB-153	**2000**	0.25, 0.2, 0.1, 0.06, 0.03, 0.015	4 to 0.125	16 to 0.5	/

Tab. 2: Concentrations (C) of mixtures from three standard solutions (two fix – per row, one variable - columns)

C_{fix}	TCDD [pg/well]	PCB-126 [pg/well]	Monoortho PCBs [ng/well]	PCB-153 [ng/well]
TCDD 0.03pg/well PCB-153 1000pg/well	/	4 to 0.125	16 to 0.5	/
PCB-126 2pg/well PCB-153 1000pg/well	0.25, 0.2, 0.1, 0.06, 0.03, 0.015	/	16 to 0.5	/
Monoortho PCBs 2400pg/well PCB-153 1000pg/well	0.25, 0.2, 0.1, 0.06, 0.03, 0.015	4 to 0.125	/	/
TCDD 0.03pg/well Monoortho PCBs 2400pg/well	/	/	/	2 to 0.063
TCDD 0.03pg/well PCB-126 2pg/well	/	/	/	2 to 0.063

When all four solutions were tested together the fix concentrations represented the value of 4g fat of the FHI-beef and were: 0.083pg/well (TCDD), 1.3pg/well (PCB-126), 2.666pg/well (monoortho PCBs) and 666pg/well (PCB-153) respectively. The variable partner had concentration ranges of 0.25 to 0.015pg/well (TCDD), 4 to 0.125pg/well (PCB-126), 16000 to 500pg/well (monoortho PCBs) and 2000 to 2pg/well (PCB-153).

Step 3: These tests simulated the FHI-beef with 0.85pg TCDD, 14pg PCB-126, 24pg monoortho PCBs and 0.1µg PCB-153. They escalated from the addition of the test solution in DMEM to the assay, inclusion of the mix into an extraction process without and with blank beef. Finally the FHI-beef itself was extracted and tested.

Results and discussion:

Step 1: TCDD had a mean EC50 of 0.11 ±0.01pg/well and a mean slope of 2.92 ±0.44 (n=51). The mean TCDD/PCB-126 ratio of EC50s was 0.083 ±0.01 pg/well (n=9). The monoortho PCBs provided no upper asymptote (see Fig. 1). For PCB-153 no CYP1A1 related signal could be seen.

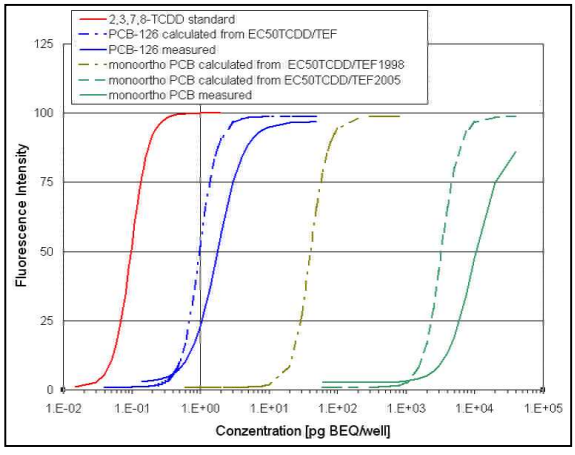
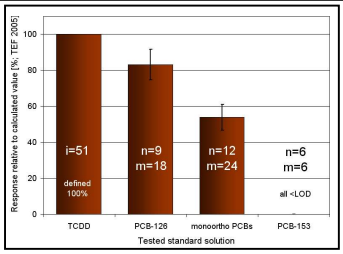
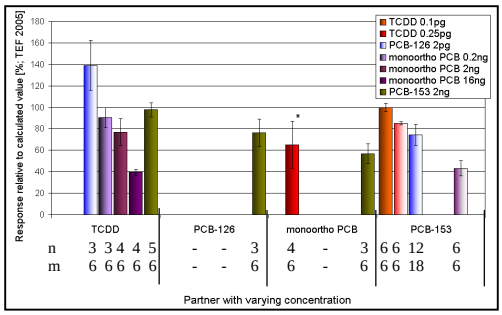


Fig. 1: Comparison of predicted and measured response using 2,3,7,8-TCDD as reference, assuming similar slope and asymptotes for PCB-126 and monoortho PCBs. PCB-126 (n=9) showed a smaller slope and upper asymptote value than TCDD. Using the EC50 values PCB-126 approached 82.7 ±9.75% of its WHO-TEF but monoortho PCBs reached only 59.1% of their WHO-TEF (2005)[6].

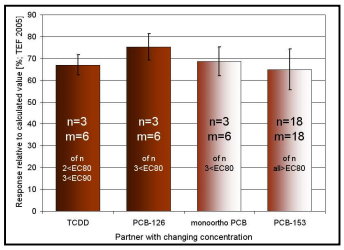
At *Step 2* with one or more standard solutions kept at constant concentrations substance dependent differences were observed (s. Fig. 2). All results were compared to the expected value from addition of the sum of WHO-TEFs from 2005[6]. Considering only the stronger Ah-receptor agonists (TCDD and PCB-126) the measured BEQ represented at least 69.5% (PCB-126) and 94.4% (TCDD) respectively of their expected value. Mixtures containing PCB-126 and TCDD together achieved at least 82.8% of the expected TCDD+PCB-126 TEQ. For some mixtures (TCDD + monoortho PCBs with or without PCB-153) higher ratios were obtained with less monoortho PCBs and more TCDD respectively. Thus monoortho PCBs are underestimated down to below 40% of their expected TEQ, even considering the WHO-TEF of 2005.



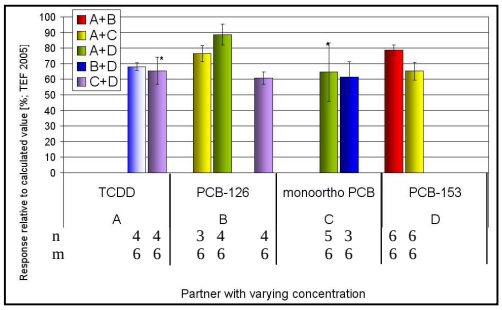
2.1: Tests of different concentrations for single standards and monoortho PCB mix. For TCDD i represents the number of full curves (*Step 1*).



2.2: Tests of two standard solutions each. One concentration is constant. The partners concentration varies. For monoortho PCBs and PCB-126 not all possible combinations were tested. PCB-153 does not change the result for TCDD or PCB-126 as shown at Fig. 2.1.
* 0.25pg/well TCDD plus monoortho PCBs has a clear trend to higher ratios of expected values with less monoortho PCBs. Monoortho PCBs are underestimated below 40% of their expected value.



2.4: Test of all four standard solutions. Three concentrations per test are kept constant the fourth partner is varying.



2.3: Test of three standard solutions each. Two concentrations are kept constant and the third mix partner has varying concentrations. All mixtures approach a mean of 60% of their expected values (WHO-TEFs 2005). The mixture "A+D plus B" (TCDD, PCB-153, PCB-126) shows almost 100% of its expected value.
* Trend to higher ratios of the expected values with more TCDD or less monoortho PCBs.

Fig. 2: Recovery rates from simple to complex of *Step 1*and *Step 2*. Ratio [%] of measured BEQ relative to the expected value using WHO-TEFs of 2005. In the mixtures only the standard given in the X-axis had a variable concentration. Half transparent columns ( ) indicate that all results of the corresponding mixture were above working range (here EC80). Error bars show the standard deviation, n represents the number of data between LOD and EC80 (if n was at least 3, else data <EC90 were included) used for the calculation, the number of tested concentrations is m.

Step 3: When applying increasingly more complex handling a good signal (>LOD) was achieved for all tests. The results of the original sub sample decreased (see Fig. 3). Through correcting these data by the apparent recovery of the TCDD spike the results were almost identical (2.58 ±0.08pg BEQ).

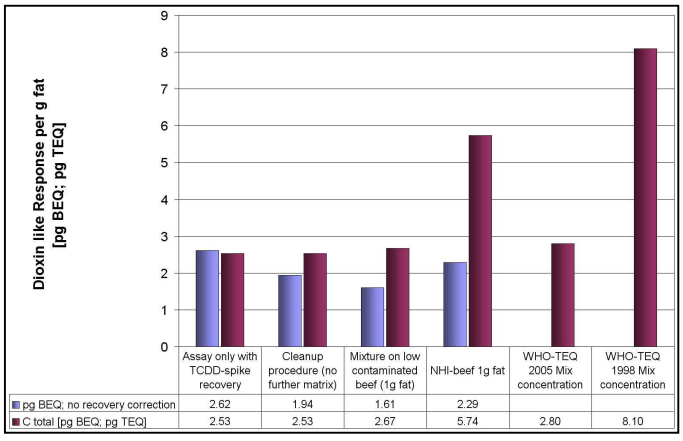


Fig. 3: Cascade of mixture testing (*Step 3*). The results of the sub sample without spike decreased from direct addition of the mixture to the cells over cleanup procedure and the final experiment with beef. Finally the FHI-beef result and its WHO-TEQs are shown.

When the beef of the interlaboratory comparison (FHI-beef) was extracted we found 2.29pg BEQ/g fat in the original sub sample (LOD=0.53pg/g fat). Due to an apparent spike recovery of 39.9% a total of 5.74pg BEQ/g fat resulted. The GC/MS consensus values for total PCDD/Fs + dl PCBs contents were 8.1pg TEQ/g fat (WHO-TEF 1998) and 2.8pg/g fat (WHO 2005)[1]. The total BEQ values of the handling tests and the result of the original sub sample were between 82 and 95% of the beefs consensus value considering WHO-TEQ 2005.
After correction of the 20pg-TCDD-spike recovery our samples value reached 205% of the consensus result for WHO-TEF of 2005 and 70.9% of the WHO-TEQ 1998. This can be due to losses of spike solution during the cleanup process or by not observe interactions between other sample contents.

Not all tested congeners interacted in the EROD-Bioassay according to their TEF value. Monoortho PCBs were used in a mixture of equal concentration, as the WHO-TEF 2005 gives the same value for all eight congeners. But the monoortho PCB mix showed only about half of the expected value considering the WHO-TEFs of 2005 and even less than 1% regarding the WHO-TEFs of 1998. PCB-126 achieved over 80% of its expected result.
The measured data were within the range given by other authors[7,8]. When combining several solutions of TCDD, PCB-126 and monoortho-PCBs, the expected value changes according to the ratio of the congeners. The main impact on underestimation of the total result was due to monoortho PCBs.

Antagonistic effects of PCB-153 were described at very high levels[7]. For better comparison of the data the ratio of expected and measured results were reevaluated using not the WHO-2005 TEF but the relative potency (REP) seen in the study. A REP of 0.08 for PCB-126 was used and 0.000015 for monoortho PCBs.
For mixtures of PCB-153 with PCB-126 93.4 ±10.0% (n=15) of the expected value were measured. Monoortho PCBs (REP 0.000015) combined with PCB-153 reached 95.4 ±15.9% (n=9). Across 63 data points containing PCB-153 mixed with two or three other standard solutions the ratio of expected to measured result was similar (91.2 ±10.1%). The differences between these results are not significant (α=0.05). So we conclude that using up to 2ng/well PCB-153 shows no clear evidence of Ah-receptor antagonism and thus it does not seem responsible for the underestimation in the FHI-beef.

Acknowledgements:
We would like to thank Prof. Schramm for providing us with his cells and profound knowledge.

References:
1. Norwegian Institute of Public Health (2009); url: http://www.fhi.no/dokumenter/31af7a765d.pdf
2. Schwirzer S (1998); Dissertation, Technische Universität München, Germany
3. Schwirzer S M G, Hofmaier A M, Kettrup A, Nerdinger P E, Schramm K-W, Thoma H, Wegenke M, Wiebel FJ (1998); *Ecotox. & Environ. Safety* (41): 77-82.
4. Thiem I, Böhmler G, Borowski U (2006); *J. Verbr. Lebensm. 1*: 310–316
5. Gizzi G, Hoogenboom LAP, von Holst C, Rose M, Anklam E (2005); *Food Additives and Contaminants 22(5)*: 472-481
6. Van den Berg M, Birnbaum LS, Denison M, De Vito M, Farland W, Feeley M, Fiedler H, Hakansson H, Haneberg A, Haws L, Rose M, Safe S, Schrenk D, Tohyama C, Tritscher A, Tuomisto J, Tysklind M, Walker N, Peterson RE *Toxicol. Sci.* 2006; 93: 223
7. Hofmaier A (1999); Dissertation, Technische Universität München, Germany
8. Behnisch P, Hosoe K, Sakai S-I (2001); *Environment International (27)*: 495-519