MEASURING PCDD/Fs IN FISH USING THE PROCEPT® DNA-BINDING BIOASSAY WITH Q-PCR MEASUREMENT IN THE LOWER PPT RANGE

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Introduction

The Procept® Rapid Dioxin Assay (Eichrom Technologies, Inc.) is an Aryl hydrocarbon-Receptor (AhR) based bioassay which utilizes Quantitative Polymerase Chain Reaction (Q-PCR) to determine levels of polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs) in samples[1]. When a mammalian living cell is exposed to PCDD/Fs, the AhR forms an adduct including the AhR Nuclear Translocator protein (ARNT) and a Dioxin Response Element (DRE) located in the nuclear genome, in a proportion depending on the affinity between the ligand and the AhR. The Procept® bioassay allows quantifying a DNA sequence mimicking the DRE which has interacted with the ligand/AhR/ANRT complex. An appropriate calibration curve then permits to express the response as a Bioanalytical Equivalency Quotient (BEQ or bio-TEQ) for screening purposes. In many cases, this BEQ correlates well with TEQ values measured by gas chromatography-high resolution mass spectrometry.

Based on this bioassay, the Method 4430 has been recently approved by the US-EPA[2-3] for the screening of PCDD/Fs in soil and sediment. Another challenge is the screening of PCDD/Fs in foodstuff, which requires significantly lower detection levels than those required in soils. The removal of fat and of agonistic compounds, including PCBs and PAHs[4-6], is mandatory but the key point to be successful is the minimizing of the procedural blank assay. In the present work, we focused on fish samples since there is an identified need for the screening of PCDD/Fs in this matrix in the European Union and other regions.

Materials and Methods

Solvents and sulfuric acid were of high purity grades from several suppliers. Sodium sulphate was provided by Merck (Darmstadt, Germany), silica gel (G60) by Fluka (Buchs, Switzerland) and silver nitrate silica by Sigma (St. Louis, MO, USA). PCR reagents were obtained from Applied Biosystem and Procept® Kits from Eichrom Technologies (Darien, IL, USA).

Three naturally contaminated fish samples - organs (F.Org) and eggs (F.Egg) from *Chelon macrolepis* species, muscle (F.Mus) from *Oreochromis sp.* - and one naturally contaminated bovine butter sample were obtained from polluted sites in Asian and European countries in 2007.

Lipids have been extracted from 9 g of lyophilised samples by 3 successive liquid-liquid partitioning using a mixture of water, propan-2-ol and *n*-hexane containing 3% diethyl ether (1:1:2, v/v/v) for fish samples or by heating and centrifuging for butter sample. Extracted lipids dissolved in *n*-hexane have been purified on a carbon column (7 mm i.d., 200 mg, 1200 $m^2.g^{-1}$) with dichloromethane as washing solvent. PCBs have been removed in the forward direction with 30 mL toluene and PCDD/Fs have been finally back-flushed using 130 mL toluene. Then, an activated silica column permitted to remove residual fat and PAHs from PCDD/Fs fraction (7 mm i.d., 350 mg SiO_2/H_2SO_4 44%, 150 mg $SiO_2/AgNO_3$ 10%, anhydrous sodium sulphate layers at the top and the bottom). Extracts have been loaded in 1 mL *n*-hexane and eluted using 12 mL *n*-hexane. Final extracts have been suspended o 24 µL of *n*-heptane before performing the Procept® kit assay and Q-PCR measurement on 2 x 6 µL aliquots. The concentrations determined in all the assay blanks appeared lower than the lowest calibration point (468 fgBEQ per well) and all measurements performed on the analysed samples were in the linear ranges of the calibration curves.

Table 1: Concentrations **(fg/g f.w.)** of PCDD/Fs determined using the reference method (isotopic dilution and GC-HRMS measurement), and calculated TEQs using WHO 2005 consensual TEFs or expected BEQs using the Procept® Relative Effect Potencies (REP), for each analysed fish sample.

	Concentration			WHO	Procept®	WHO 2005 TEQ			Expected BEQ		
	F.Org	F.Egg	F.Mus	2005 TEF	REP	F.Org	F.Egg	F.Mus	F.Org	F.Egg	F.Mus
2,3,7,8-TCDD	ND(<40)	190	98	1	1	-	190	98	-	190	98
1,2,3,7,8-PeCDD	249	427	135	1	0.55	249	427	135	137	235	74
1,2,3,4,7,8-HxCDD	177	122	54	0.1	0.35	18	12	5	62	43	19
1,2,3,6,7,8-HxCDD	379	234	185	0.1	0.1	38	23	18	38	23	18
1,2,3,7,8,9-HxCDD	223	96	41	0.1	0.49	22	10	4	109	47	20
1,2,3,4,6,7,8- HpCDD	10037	719	2368	0.01	0.013	100	7	24	130	9	31
OCDD	148246	4912	33509	0.0003	0.0000028	44	1	10	0	0	0
2,3,7,8-TCDF	366	789	1122	0.1	0.06	37	79	112	22	47	67
1,2,3,7,8-PeCDF	136	155	92	0.03	0.14	4	5	3	19	22	13
2,3,4,7,8-PeCDF	440	777	207	0.3	0.32	132	233	62	141	249	66
1,2,3,4,7,8-HxCDF	281	206	114	0.1	0.39	28	21	11	110	80	45
1,2,3,6,7,8-HxCDF	364	438	898	0.1	0.17	36	44	90	62	75	153
1,2,3,7,8,9-HxCDF	128	ND(<80)	ND(<30)	0.1	0.28	13	-	-	36	-	-
2,3,4,6,7,8-HxCDF	515	230	203	0.1	0.1	52	23	20	52	23	20
1,2,3,4,6,7,8 -HpCDF	7945	3821	23495	0.01	0.053	79	38	235	421	203	1245
1,2,3,4,7,8,9 -HpCDF	653	ND(<90)	132	0.01	0.016	7	-	1	10	-	2
OCDF	31877	1111	22387	0.0003	0.00046	10	0	7	15	1	10
Total WHO 2005 TEQ or expected BEQ						**869**	**1114**	**837**	**1364**	**1246**	**1882**

Additional confirmatory analyses (blanks, biological samples and extracts) were performed using isotope dilution method and GC-HRMS measurement by accredited laboratories

Results and Discussion

Detection limit. The levels measured in procedural blank (including extraction, purification, kit assay and Q-PCR measurement) corresponded to 1.12 pqBEQ per well (±21%), with one third due to extraction step alone. This leads to a detection limit lower than 0.4 $pgBEQ.g^{-1}$ f.w. for a 20 g sample size (mean plus 3 times SD), which is already satisfying regarding a target level set at 4 $pgTEQ.g^{-1}$ f.w. (European Regulation). Moreover, an other procedural blank excluding the extraction step tends to corroborate the fact that a lower solvent volume (200 µL instead of 2 mL) used to transfer the final extract to the vial can lead to less than 0.2 pqBEQ response per well, which is very close to an assay blank. These results have to be confirmed but the silver nitrate silica already appears to be an important tool for minimizing the detection limit.

At least four reasons may contribute to an observed difference between a confirmatory result and a screening result: 1- the difference between WHO 2005 consensual TEFs[7] and real REPs[8], 2- the recovery yield if the screening value is not corrected by a reference sample, 3- the influence of interfering compounds (such as PCBs, PAHs, etc…) and 4- the method reproducibility, which is higher in the screening case.

Reason 1- (REPs ≠ TEFs). The concentrations measured in the 3 tested fish samples using the reference method (GC-HRMS) are shown in Table 1. Strong differences

can be observed between these samples in terms of pattern (*i.e.* contamination profile). The resulting calculated WHO 2005 TEQs are 0.869, 1.114 and 0.837 pgTEQ/g^{-1} f.w., respectively for samples F.Org, F.Egg and F.Mus. Using the Procept® REPs, the expected screening values are 1.57, 1.12 and 2.25 times higher. Only one congener, 1,2,3,4,6,7,8-HpCDF, is responsible for most of this bias, due to its relatively high concentration and a sensitive difference between its TEF and REP. Indeed, without considering this congener, the ratios respectively fall to 1.19, 0.97 and 1.06, which would indicate a very good concordance between the two methods. As a comparison, these ratios were found between 0.74 and 1.33 for 135 fish muscle samples from the 2004 French monitoring program. In these data, the 1,2,3,4,6,7,8-HpCDF contribution was generally not higher than 1%. Then, the pattern can lead to factor 2-3 variability, as illustrated by Figure 1. As a conclusion, the nature and associated pattern of the reference sample chosen for REPs deviation and recovery yield correction appears to be highly important regarding the accuracy.

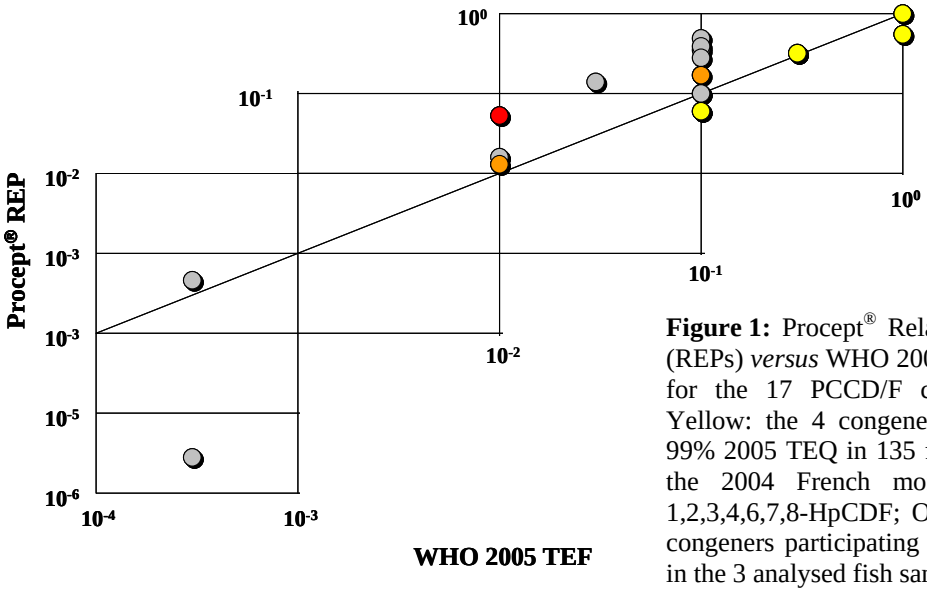

Figure 1: Procept® Relative Response Potencies (REPs) *versus* WHO 2005 consensual TEF values for the 17 PCCD/F congeners (see Table 1). Yellow: the 4 congeners participating to 70 to 99% 2005 TEQ in 135 fish muscle samples from the 2004 French monitoring program; Red: 1,2,3,4,6,7,8-HpCDF; Orange+red+yellow: the 7 congeners participating to 73 to 91% 2005 TEQ in the 3 analysed fish samples;

Table 2: Global BEQ recovery yield information on the screening sample preparation. Experiment realized on naturally contaminated bovine butter. Lipids sample size: 1.25 g for confirmatory method or 5 g for screening sample preparation.

GC-HRMS measurement (isotopic dilution)	**Procept® REPs**	**WHO 2005 TEFs**
Sample (pgBEQ.g^{-1} or pgTEQ.g^{-1} l.w.)	3.16	2.35
Final extract from screening sample preparation	1.44	1.08
Ratio (recovery yield)	46%	46%
Q-PCR measurement	**Blank corrected value**	**Procedural blank ratio**
Screening sample preparation (pgBEQ.g^{-1} l.w.)	1.27 ± 1%	10%
Ratio with GC-HRMS final extract measurement	88%	

Reason 2- (Recovery yield). An experiment (Table 2) performed on naturally contaminated bovine butter sample reported a ratio of 46% between isotope dilution method quantifications on the final extract (sample preparation described above) and on the butter sample (confirmatory method). This calculation can be considered as a global BEQ recovery yield. The results presented in Table 3 show that most of the bias observed between the Obtained Procept® response and the reference TEQ corrected by REPs can be explained by an assumed recovery yield of 46% with a low variability.

Table 3: Results on the 3 fish samples, with bias reasons impact estimations.

	F.Org	F.Egg	F.Mus
Sample size (g f.w.)	27.80	24.60	35.13
Extracted fat (g)	0.96 (3.5%)	1.32 (5.4%)	0.24 (0.7%)
WHO 2005 TEQ (pgTEQ.g^{-1} f.w.)	0.869	1.114	0.837
…corrected by reason 1- (Procept® REPs)	1.364	1.246	1.882
…corrected by reasons 1- and 2- (46% recovery yield)	0.623	0.573	0.860
Obtained Procept® response (pgBEQ.g^{-1} f.w.)	0.482 ± 8%	0.445± 28%	0.806± 10%
Procedural blank ratio	26%	30%	14%
Obtained response *versus* reasons 1- and 2- corrected TEQ	77%	78%	94%
Obtained response *versus* reference WHO 2005 TEQ	55%	40%	96%

Reasons 3- and 4- (Interfering compounds and reproducibility). The occurrence of remaining interfering compounds seems to be relatively limited. Various sources of variability from the confirmatory method, the recovery yields, the procedural blanks and the kit assay can easily explain the limited deviation between Procept® measurements and expected values corrected by REPs and recovery yield approximation.

Finally, 3 fish samples contaminated at the low ppt level have been analysed using the Procept® assay, with satisfying procedural blank levels. When compared to confirmatory method measurement, most of the deviation can be explained by the difference between WHO 2005 TEFs and Procept® REPs, and recovery yields. Using an appropriated reference sample allowing getting free from these two biases, the Procept® technology should show high accuracy and performances for the screening purpose in fish matrix. Consequently, the next step will be to conduct a whole validation procedure for the fish matrix but some minor improvements have to be previously obtained. The relatively poor recovery yield obtained on the carbon column has to be improved, changing some elution conditions. Moreover, minimizing the solvent volume used after the silver nitrate silica layer (or using some purified solvent) should be helpful to obtain highly satisfying procedural blanks. Still lowering a little LOD/LOQ is mandatory regarding the European Union regulation. At last, the PCB fraction will have to be considered to fully match the European Regulation.

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