MEASURING PCDD/F IN EGGS USING THE PROCEPT RAPID DIOXIN ASSAY

Cariou R[1], Marchand P[1], McAlister DR[2], Fern MJ[2], Le Bizec B[1]

[1]LABERCA, Route de Gachet, BP50707, 44307 Nantes Cedex 3, France; [2]Eichrom Technologies, Inc., 8205 S. Cass Ave., Suite 106, Darien, IL 60561 USA

Abstract
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 the toxicity equivalent quotient (TEQ) of polychlorinated dibenzo-*p*-dioxins and furans (PCDD/F) in samples. Under appropriate conditions, when exposed to PCDD/F or similar compounds, the AhR forms an adduct including a DNA response element (DRE). A small DNA molecule mimicking this DRE can be tagged with a florescent probe and amplified using PCR to allow the measurement of very low levels of the DNA molecule and indirectly the amount of PCDD/F as a TEQ. In order to measure specifically a PCDD/F TEQ at very low levels, interfering compounds must be removed from the analyzed extract. This technique is well suited as a screening method but most of the work so far has been limited to measuring PCDD/F TEQ in soils. In this work, we focused on applying Procept Assay for measurement of PCDD/F in food samples, starting with different amounts of lipids extracted from naturally contaminated eggs. We then compared the Q-PCR to the GC-HRMS responses, and identified areas which need to be improved to achieve a well-suited screening method dedicated to food and feed samples.

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/F) in samples.[1] Under appropriate conditions, when exposed to PCDD/F or similar compounds, the AhR forms an adduct including a DNA response element (DRE). A small DNA molecule mimicking this DRE can be tagged with a florescent probe and amplified using PCR to allow the measurement of very low levels of the DNA molecule and indirectly the amount of PCDD/F as a TEQ. In order to measure specifically the PCDD/F TEQ at very low levels, interfering compounds including polychlorinated biphenyls (PCB), polycyclic aromatic hydrocarbons (PAH), brominated PCDD/F, non-2,3,7,8-PCDD/F or polychlorinated naphthalenes (PCN) have to be removed from the extract.[2-4] This technique has been shown to be an effective screening method for measuring PCDD/F TEQ in soil,[5] which fits the USA regulatory context. European Union regulation also focuses on food and feed samples including PCDD/F and PCB. Therefore, developing a screening method for PCDD/F and PCB in food and feed samples is important and will require lower detection levels than achieved in work with soils. Two areas have been identified for improving the detection limit of the Procept method: improving the PCR assay sensitivity and optimizing the sample clean-up. In this work, we focused on PCDD/F clean-up of egg fat samples.

Materials and Methods
Standard compounds were obtained from Cambridge Isotope Laboratories (Andover, USA) or Wellington Laboratories (Guelph, Canada). Solvents and sulfuric acid were Picograde® quality and provided by LGC Promochem (Wesel, Germany). Deionized water was obtained from a Milli-Q2 water purification system. Sodium sulfate and silver nitrate were from Merck (Darmstadt, Germany) and silica gel (G60) was provided by Fluka. PCR reagents were obtained from Stratagene, Inc.

The lipids were extracted from lyophilized eggs by Pressurized Liquid Extraction (PLE) (ASE300, Dionex, Sunnyvale, USA) with toluene/acetone 70:30 (v/v) mixture. Eight amounts of dried lipids, from 0.25 g to 2.5 g, including a triplicate at 2 g, were then purified on a multilayer silica column (including 22% H_2SO_4, 44% H_2SO_4 and 10% $AgNO_3$ silica layers) and a Florisil column (6 g phase containing 3 % of water and a 10% $AgNO_3$ silica layer at the bottom). The Procept Rapid Dioxin Assay was performed by Eichrom Technologies, Inc. Additional assays (blanks and biological samples) were performed using ^{13}C-labelled compounds as internal and external standards in order to be quantified by means of GC-HRMS.

Results and Discussion

The TEQ value obtained by GC-HRMS for the egg lipids sample was 4.58 $pg.g^{-1}$. Figure 1 reports the Q-PCR TEQ values obtained for each point *versus* the GC-HRMS value. The Q-PCR results (R^2 = 0.93) are well correlated to the sample amounts. However, the PCDD/F TEQ of samples are overestimated by the Procept assay by a factor of ~3.3. Moreover, the Q-PCR values are not corrected by a recovery yield. Indeed, ^{13}C-labelled congeners, that provide the same response in Q-PCR, can not be added as internal standards. This overestimation has already been observed in soil and sand samples[5] and work is on progress to understand the reasons. Some response elements can already be given here.

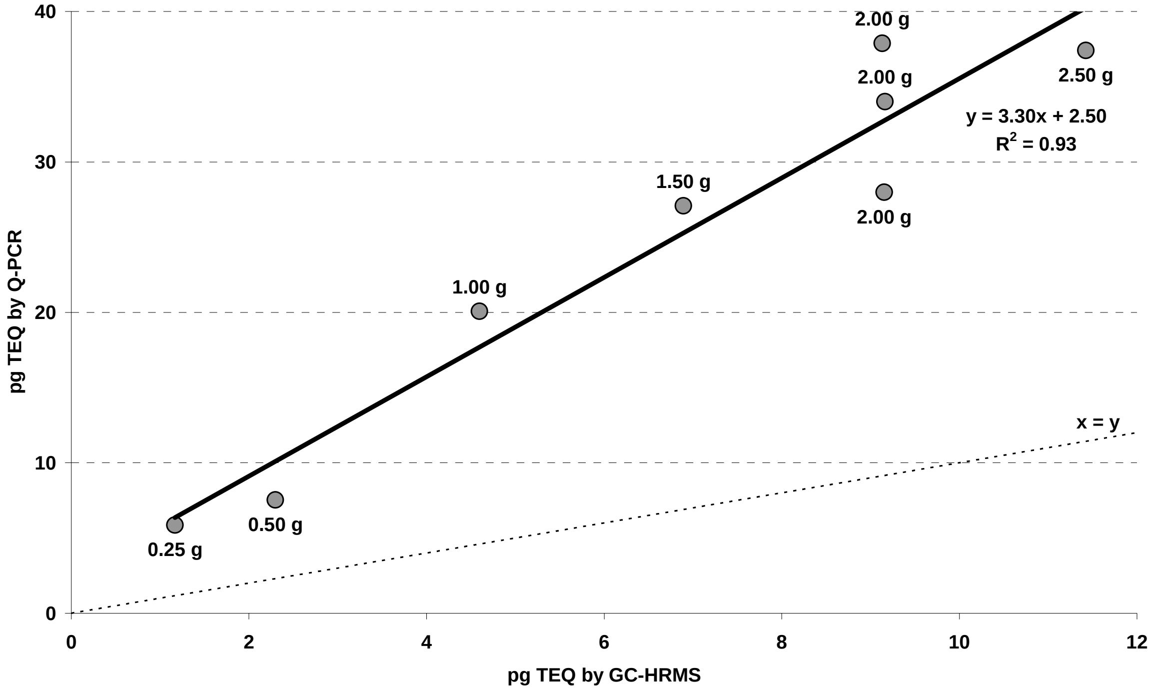

Figure 1. Comparison between PCDD/F TEQ values (in pg) obtained by means of Q-PCR and GC-HRMS for different amounts of lipids extracted from eggs.

Since the calibration solutions used in the two detection techniques are different, we controlled the PCDD/F TEQ values of the Q-PCR calibration curve solutions by GC-HRMS. The regression curve for the seven points from 5000 down to 78 $fg.\mu L^{-1}$ (successive factor 2 dilutions) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin correctly matches the expected parameters (y = 0.994 x - 23.925, R^2 = 0.998).

Standard mixtures were also compared in order to evaluate the possible occurrence of a synergistic phenomenon between PCDD/F on the Procept assay signal. Figure 2 reports the measured TEQ response of 5 known standards mixtures by Q-PCR *versus* the expected values calculated according to the WHO-1998 TEF or the Procept assay response factors. The concentration of each individual PCDD/F congener and the previously determined Procept response factors[6] are compiled in Table 1. The results exhibit a satisfactory relationship between the measurement and the expected value. These results tend to exclude the preponderance of a synergistic phenomenon. However, it could be interesting to prepare and measure the response of a standard mixture with the same congener profile as that of the biological sample.

Table 1. Toxicity equivalent factors (TEF) of PCDD/F congeners and composition ([12]C-native and [13]C-labelled compounds) of solutions compared.

	TEF			Concentration (pg/uL)									
	WHO-1998	WHO-2005	Q-PCR	Sol1		Sol2		Sol3		Sol4		Sol5	
				12C	13C	12C	13C	12C	13C	12C	13C	12C	13C
2,3,7,8-TCDD	1	1	1	-	2.5	0.05	2.5	2.0	2.5	5.0	2.5	10	-
1,2,3,7,8-PeCDD	1	1	0.55	-	2.5	0.05	2.5	2.0	2.5	5.0	2.5	10	-
1,2,3,4,7,8-HxCDD	0.1	0.1	0.35	-	2.5	0.05	2.5	2.0	2.5	5.0	2.5	10	-
1,2,3,6,7,8-HxCDD	0.1	0.1	0.1	-	2.5	0.05	2.5	2.0	2.5	5.0	2.5	10	-
1,2,3,7,8,9-HxCDD	0.1	0.1	0.49	-	2.5	0.05	2.5	2.0	2.5	5.0	2.5	10	-
1,2,3,4,6,7,8-HpCDD	0.01	0.01	0.013	-	2.5	0.05	2.5	2.0	2.5	5.0	2.5	10	-
OCDD	0.0001	0.0003	0.0000028	-	2.5	0.05	2.5	2.0	2.5	5.0	2.5	-	-
2,3,7,8-TCDF	0.1	0.1	0.06	-	2.5	0.05	2.5	2.0	2.5	5.0	2.5	10	-
1,2,3,7,8-PeCDF	0.05	0.03	0.14	-	2.5	0.05	2.5	2.0	2.5	5.0	2.5	10	-
2,3,4,7,8-PeCDF	0.5	0.3	0.32	-	2.5	0.05	2.5	2.0	2.5	5.0	2.5	10	-
1,2,3,4,7,8-HxCDF	0.1	0.1	0.39	-	2.5	0.05	2.5	2.0	2.5	5.0	2.5	10	-
1,2,3,6,7,8-HxCDF	0.1	0.1	0.17	-	2.5	0.05	2.5	2.0	2.5	5.0	2.5	10	-
1,2,3,7,8,9-HxCDF	0.1	0.1	0.28	-	2.5	0.05	2.5	2.0	2.5	5.0	2.5	10	-
2,3,4,6,7,8-HxCDF	0.1	0.1	0.1	-	2.5	0.05	2.5	2.0	2.5	5.0	2.5	10	-
1,2,3,4,6,7,8-HpCDF	0.01	0.01	0.053	-	2.5	0.05	2.5	2.0	2.5	5.0	2.5	10	-
1,2,3,4,7,8,9-HpCDF	0.01	0.01	0.016	-	2.5	0.05	2.5	2.0	2.5	5.0	2.5	10	-
OCDF	0.0001	0.0003	0.00046	-	2.5	0.05	2.5	2.0	2.5	5.0	2.5	-	-
1,2,3,4-TCDD	-	-	0.001	-	2.5	-	2.5	-	2.5	-	2.5	-	-

	Sol1	Sol2	Sol3	Sol4	Sol5
Expected WHO-1998 TEQ (pg/uL)	8.5	8.6	15.2	25.4	33.8
Expected WHO-2005 TEQ (pg/uL)	7.9	8.1	14.2	23.7	31.6
Expected Q-PCR TEQ (pg/uL)	10.4	10.6	18.6	31.1	41.4
Measured Q-PCR TEQ (pg/uL)	9.2	9.9	19.6	27.1	38.1

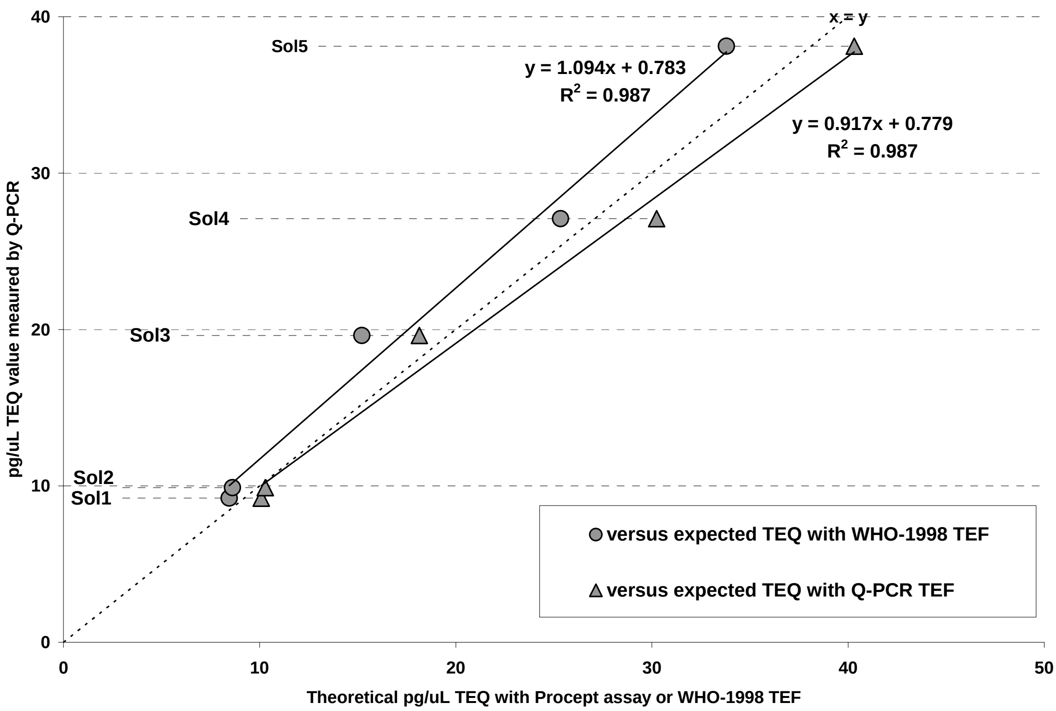

Figure 2. Measured TEQ response in Q-PCR of the 5 known standard mixtures *versus* the expected value.

A third possible bias source could come from a procedural contamination with cross-reacting compounds. However, this can only explain the *b* factor of the regression curve (2.50 pg TEQ) since such a contamination is not linked to the amount of sample. The quantification of PAH was carried out on extracts prepared at the same time (two blank assays, one herring liver oil sample and one egg lipids sample). A mean quantity of 4.3 pg (± 15%) of benzo(b)fluoranthene was found in final extracts. Yet, this compound has a Procept response factor[6] equal to 0.59, which means a TEQ contribution of 2.6 pg. Then, this compound could explain the entire constant bias observed in our assays on different amounts of egg lipids.

The major remaining question remains the origin of the proportional factor observed (~3.3). The expected Procept measurement can be calculated from the known congener profile of the sample, the recovery yield and the Procept response of each congener. This method leads to a 0.67 proportional factor that can be interpreted as a global TEQ recovery yield. Therefore, it seems that cross-reacting compounds present in the sample still remain in the final extract. Previous work suggests that PCB and PAH are not likely the source of the high bias, so other candidates have to be investigated. For example, bromo/chloro dioxins and furans, methylated analogues, non-2,3,7,8-PCDD/F, tetrachloroxanthene have already shown to cross-react,[4] and other compounds such as polychlorinated naphthalenes (PCN) could also be good candidates.

In the case of a constant proportional bias for every egg sample, the use of a recovery standard consisting of an egg sample of known PCDD/F composition could allow to apply a conversion factor. However, identifying the interfering compounds in order to improve the purification steps will be the purpose of our future work. The final goal will be to achieve a European regulatory screening method dedicated to the analysis of PCDD/F and PCB in food and feed samples.

References

1. Allen RL, Willey JJ. *Organohalogen Comp.* 2002;58:341.
2. Windal I, Dension MS, Birnbaum LS, Van Wouwe N, Baeyens W, Goeyens, L. *Environ. Sci. Technol*. 2005;39:7357.
3. Behnisch PA, Hosoe K, Sakai S. *Environ Int.* 2001;27:495-519.
4. McAlister DR, Fern ML, Allen RL, Sakata K. *Organohalogen Comp.* 2007, submitted.
5. McAlister DR, Fern ML, Allen RL. *Organohalogen Comp.* 2007, submitted.
6. McAlister DR, Fern ML, Allen RL, Sakata K, Le Bizec B, Marchand P. *Organohalogen Comp.* 2006;68:940.