EFFICIENT SCREENING OF DIOXINS IN FOOD AND FEED USING SHAPE-SELECTIVE PRESSURIZED LIQUID EXTRACTION AND CELL BASED BIOASSAY ANALYSIS

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Introduction

Incidents recent years have made the need for risk reduction strategies of persistent organic pollutants clear. During the Belgian dioxin crisis in 1999, for instance, contaminated feed caused increased levels of polychlorinated dibenzo-p-dioxins and dibenzofurans (dioxins) and polychlorinated biphenyls (PCBs) in pork and chicken meat, sometimes exceeding the tolerable level, set by the European Commission, as much as 250 times[1]. Implementing risk assessment and reduction strategies may involve random monitoring of dioxins and PCBs in food and feed. Screening methods with high sample throughput and low rate of false negatives would enhance the procedure considerably.

Cell based bioassays with enhanced green fluorescent protein (EGFP) detection[2] are potential screening methods for determination of aryl hydrocarbon receptor (AhR) ligands, such as dioxins and similar compounds, in environmental samples. With this technique, it is possible to detect dioxins at levels normally found in food and feed, i.e. pg toxic equivalents (TEQ)/g. Since the signal from the bioassay might be caused by compounds other than dioxins binding to the AhR, determination of the dioxin TEQ generally involve extraction with organic solvents or solvent mixtures, e.g. using a Soxhlet apparatus, followed by clean-up with sulphuric acid or sulphuric acid impregnated silica gel and carbon fractionation in order to exclude possible interferences from the extracts.

Until now, sample preparation has been time consuming and labour intensive, but alternatives to traditional methods have recently been developed, with the benefits of shorter analysis times and reduced organic solvent consumption[3,4,5]. Pressurized liquid extraction (PLE) may, for instance, be used with a fat retainer in the PLE cell to selectively extract PCBs from food, feed, and biota matrices[6,7,8,9]. In order to further streamline the sample preparation, new assemblies have been

developed to fit into a commercially available PLE-equipment[9] (Figure 1). The assemblies are packed with an activated carbon/celite mixture and the sample. In the subsequent extraction, the pollutants are fractionated into three fractions according to their planarity (shape-selective extraction). In the first fraction (I) bulk lipids and PCBs are eluted, in the second fraction (II) the majority of planar (non-*ortho*) PCBs, and in the third fraction (III), which is back-flushed, the dioxins are recovered. In this way, a pure dioxin fraction may be isolated and analysed separately with the cell based bioassay described above.

This study was conducted to meet the imperative demands for dioxin monitoring. The aim was to develop a comprehensive method for efficient screening of dioxin TEQ in food and feed based on enhanced PLE, for clean-up and fractionation, and a cell based bioassay for detection.

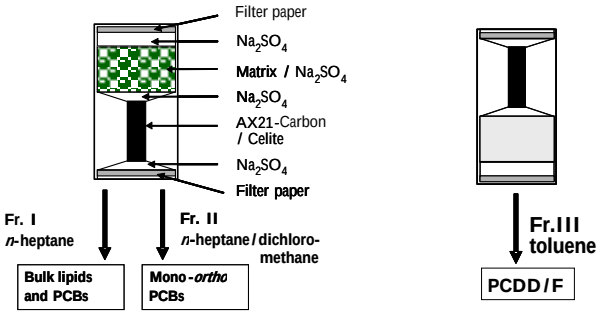

Figure 1. Combined extraction, clean-up and fractionation of PCBs and dioxins using PLE.

Materials and Methods

Two types of food and feed were analyzed, namely herring from the North Sea, distributed within the EU DIFFERENCE project[10], and naturally contaminated fish meal, delivered by the State Official Laboratory (ROLT, Tervuren, Belgium). Solvents used were of pesticide grade and purchased from Fluka/Riedel-de Haën (Buchs, Switzerland), with the exception of dimethyl sulfoxide (DMSO), reagent grade, which was purchased from Acros Organics (Geel, Belgium). Silica (Kieselgel 60) was from Merck (Darmstadt, Germany). The AX-21 carbon originated from Anderson Development Co. (Adrian, MI, USA), but is currently not commercially available. Tissue culture media and additives were obtained from Invitrogen Corporation (Carlsbad, CA, USA).

Sample preparation was performed by combined extraction, clean-up, and fractionation using an ASE 300™ (Dionex, Sunnyvale, CA). The extraction cells were packed as seen in Figure 1. Triplicates of fish oil and fish meal were processed on two consecutive days. Three grams of sample were placed in the extraction cell and extracted as previously described[9], using *n*-heptane (1 cycle), then dichloromethane/*n*-heptane (1:1, 2 cycles) and finally toluene (2 cycles in back-flush). In fraction 1 and 2 the fat content was determined by gravimetric analysis. Fat residues in the toluene fractions were removed by a miniaturized multi-layer column packed with KOH-silica, silica, and 40% sulphuric acid-silica. Thereafter, the solvent was changed to DMSO and a dilution series of each extract was prepared.

Analysis was carried out using a cell based bioassay (gift from Prof. Michael Denison, University of California, Davis). The cells (H1G1.1c3) in this bioassay have been genetically modified to

produce EGFP upon activation of the AhR by ligands such as dioxins[2]. The bioassay was performed according to the protocol of Prof. Denison[11]. Briefly, about 70 000 cells were plated into each well of a black 96-well clear-bottomed microplate and allowed to attach for 24 h prior to chemical exposure. The cells were treated with dilution series of the PLE extracts and for each microplate, a standard curve was created by using a dilution series of 2,3,7,8-TCDD. All dilutions were tested in triplicates and at two separate occasions for the two sets of samples prepared by PLE. The final concentration of DMSO in each well was 1%. The microplates were read to determine the EGFP levels in the intact cells after 24 h incubation at 33 ºC. The microplate fluorometer (Fluostar Galaxy, BMG Labtechnologies) was set at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The TEQ of a sample was obtained using a dilution producing an EGFP induction in the EC_{10} to EC_{50} range of the TCDD calibration curve.

Results and Discussion

Examples of fluorometer readings are given in Figure 2 and the results for all experiments are compiled in Figure 3. In Figure 4, the average TEQ values of the fish oil obtained in this study are compared to the chemically activated luciferase expression (CALUX) assay and high resolution mass spectrometry (HRMS) values obtained in an interlaboratory study performed within the EU DIFFERENCE project[10]. The average TEQ values of the fish meal are compared to HRMS values obtained after traditional extraction and clean-up performed at Environmental Chemistry, Umeå University.

All values are corrected for the amount of TEQs found in the blank. The AhR-EGFP blank values were significant, 1.9 pg TEQ/g (set 1) and 3.2 pg TEQ/g (set 2), whereas the HRMS blank values were negligible. In the CALUX assay, recovery correction was performed using a reference sample.

The developed method allows for gravimetrical lipid weight determination. This was demonstrated for both fish oil and fish meal. The lipid recoveries for fish oil were 102 and 104% in set 1 and 2, respectively (n = 3), while for the fish meal corresponding values were 101 and 103% (n = 3). All RSD values were less than 3%.

When comparing the results obtained in this study with those acquired elsewhere (Figure 4), it can be concluded that the AhR-EGFP method performs similar to HRMS and CALUX for fish oil and to HRMS for fish meal. However, it is important to eliminate the blank problem and to test the method using a larger set of samples. It may even be possible to enhance the performance by further optimization of the extraction technique or by application of recovery correction in the cell based bioassay.

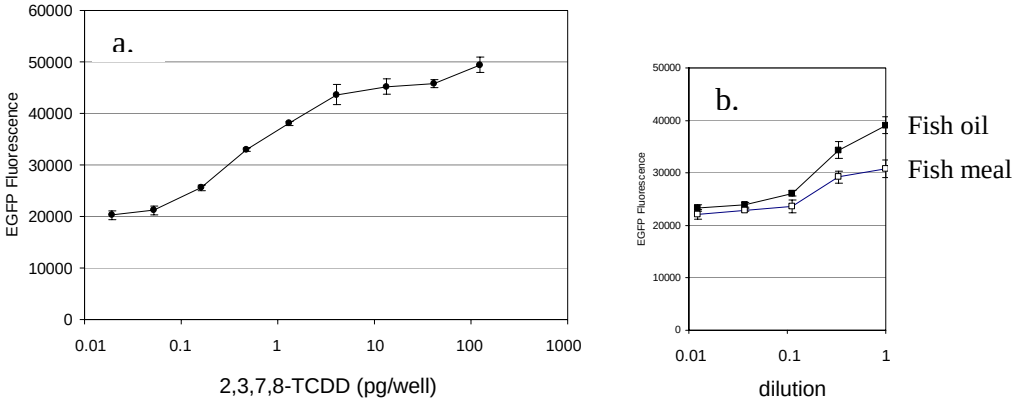

Figure 2. Cell based bioassay results for (a) TCDD calibration solutions (corresponding to 0.3, 0.8, 2.5, 7.3, 20, 63, 210, 640, and 1900 pM per well) and (b) dilutions of fish oil extracts (solid squares), and fish meal extracts (empty squares). DMSO treated cells gave a fluorescence of 20 000. Error bars correspond to one standard deviation (n = 3).

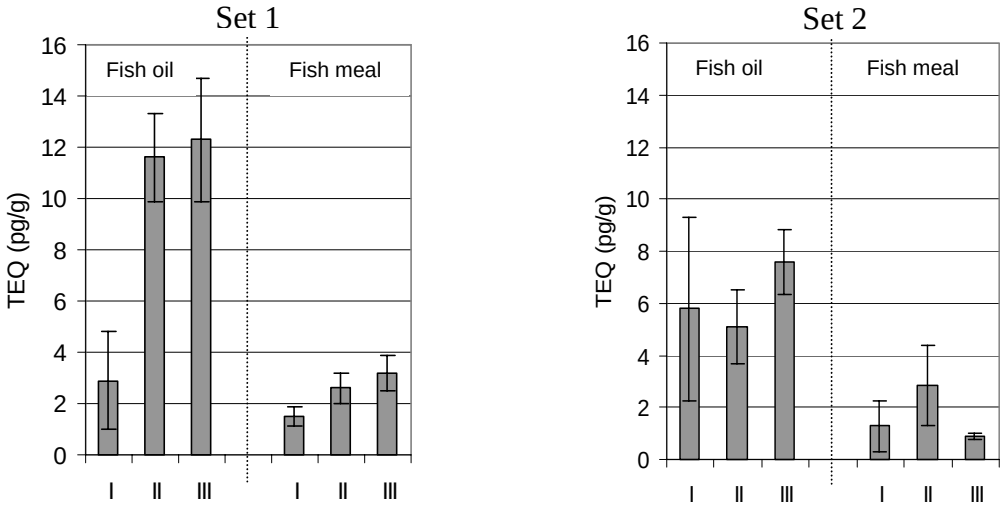

Figure 3. Dioxin content (pg TEQ/g sample) of six sub-samples of fish oil and fish meal as determined with PLE followed by triplicate analysis of each extract on a AhR-GFP cell based bioassay. Error bars correspond to one standard deviation.

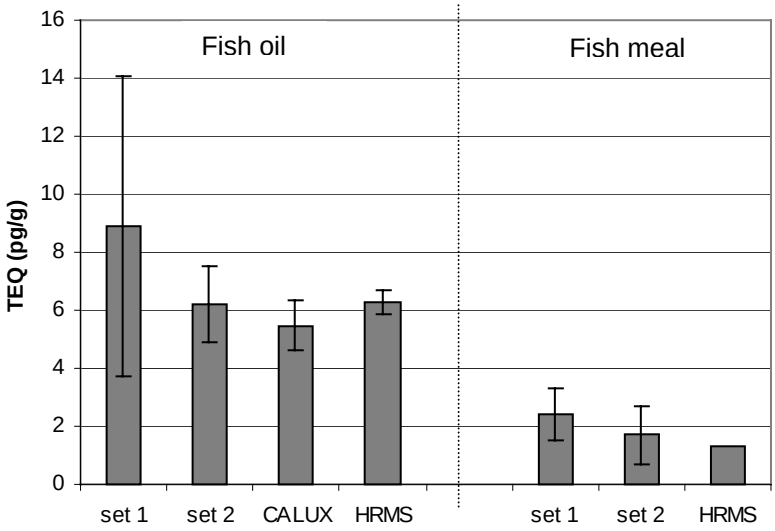

Figure 4. Dioxin content (pg TEQ/g sample) in fish oil and fish meal determined with different methods. Set 1 and 2 refer to analyses based on extractions with PLE (n = 3). The CALUX and HRMS values (lower bound TEQs) for the fish oil are produced within the EU DIFFERENCE project based on traditional extraction procedures (n = 6). For the fish meal, the HRMS values (lower bound TEQs) are based on traditional extraction procedures (n = 1) performed at Environmental Chemistry, Umeå University. Error bars correspond to one standard deviation.

Conclusions

In all, the method under study is promising and the results suggest that the quality criteria on screening methods for control of dioxins in foodstuffs laid down in the EU Commission Directive 2002/69/EC can be met by using shape-selective PLE and a cell based bioassay with EGFP detection. The extraction method is cheaper and less time consuming, and the solvent consumption is decreased, as compared to traditional methods. Additionally, sample throughput is increased by using the cell based bioassay, providing batch analysis. All together, the efficiency of the analysis procedure is immensely increased and high efficiency screening, at a low cost, of large sets of food and feed samples becomes both possible and feasible. It will even be achievable to meet future demands on reporting TEQ values for dioxin-like PCBs, since these elute in a separate fraction. If this holds true, it will lead to considerable improvements in the field of dioxin analysis of food and feed.

Acknowledgements

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