Screening for dioxins in retail fish using a combination of a PCB ELISA and an aryl hydrocarbon receptor immunoassay (Ah-immunoassay)

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Introduction

Our study of the overall human diet in Japan showed that fish and shellfish are the main sources of PCDD/Fs and dioxin-like PCBs (dioxins)[1]. To assess the risk posed by retail fish, it is therefore important to develop screening methods for dioxins. A reporter-gene assay, such as the CALUX assay, could be a useful methodology for this application, but has the drawback of involving cell culture, which requires skilled personnel and elaborate equipment, and their introduction is also likely to require that the assays used are licensed. An ELISA-based screening tool, in the form of a commercially available kit, would be a simpler and very attractive alternative. In this study, we evaluated the effectiveness of two commercially available kits, a PCB ELISA (PCB-EIA) kit and an Ah-immunoassay (Ah-I) kit, for screening for dioxins in fish. We tested the PCB-EIA, a competitive immunoassay specific for PCB 118, as a screening method for mono-*ortho* PCBs and the Ah-I, an ELISA-based aryl hydrocarbon receptor (AhR) binding assay, as a screening method for non-*ortho* PCBs and PCDD/Fs.

Materials and Methods

Sample preparation for PCB-EIA and Ah-I: The procedure for preparing the fish samples is shown schematically in Figure 1. Samples of 20 g of retail fish were homogenized and incubated in aqueous KOH for 16 h at room temperature. The alkaline hydrolysates were extracted three times by shaking with *n*-hexane. These extracts were treated several times with concentrated sulfuric acid, and loaded onto a multi-layer silica gel column. The eluate obtained with *n*-hexane was loaded onto an alumina column. After washing with *n*-hexane, the first fraction, containing mono-*ortho* PCBs, was eluted with 2% dichloromethane/*n*-hexane, and the second fraction, containing non-*ortho* PCBs and PCDD/Fs, was eluted with 60% dichloromethane/*n*-hexane. The first fraction was dried by evaporation; the residue was re-dissolved in 100 µl DMSO and used in the PCB-EIA.The second fraction was further purified with a sulfuric acid-silica gel column. The eluate obtained with *n*-hexane was dried by evaporation, and the residue was re-dissolved in 20 µl DMSO and used in the Ah-I.

PCB-EIA: This kit was used according to the manufacturer's instructions (EnBioTec Laboratories, Japan)[2]. Briefly, PCBs in samples competed with a competitor-horseradish peroxidase (HRP) conjugate for binding to an anti-PCB 118 monoclonal antibody, coated onto microtiter plate wells. The bound competitor-HRP was detected with the enzyme substrate, 3,3',5,5'-tetramethylbenzidine. The assay used a standard curve with varying concentrations of 3,3',4'-trichloro-4-methoxybiphenyl, which is a surrogate standard for PCB 118, and had a detection limit for PCB 118 of 10 ng/ml (125 pg/well), corresponding to 50 pg/g in the test samples.

Ah-I: This kit was used according to the manufacturer's instructions (KUBOTA Co., Japan and Paracelsian Inc., USA)[3]. Briefly, samples were mixed with a reagent containing dioxin receptor element (DRE) DNA oligomers, AhR nuclear translocator protein (ARNT) and cytosol components containing Ah receptors. The mixtures were added to microtiter wells coated with DRE binding protein. The presence of dioxins promotes the formation of the AhR-ARNT complexes, which then bind DRE and so bind to the wells. Binding was detected with an anti-ARNT antibody and a second antibody conjugated to alkaline phosphatase. The assay used a standard curve with varying concentrations of 2,3,7,8-TCDD for which the detection limit was 5.0 pg/ml (1.0 pg/well). Measurements for samples containing

dioxin-like compounds were converted into Ah-I-based, 2,3,7,8-TCDD equivalents (DEQs), and were corrected by subtraction of the blank concentration for the sample preparation procedure. The minimum concentration measurable in samples was 1.0 pg/g.

HRGC/HRMS: Dioxins were extracted, prepared and analyzed as described previously[4].

Results and Discussion

Two-fold serial dilutions of the prepared extracts of the fish samples were tested in the PCB-EIA and Ah-I, to check for any interference by contaminants from the natural environment. Two or three different fish extracts were diluted with DMSO and assayed. In the PCB-EIA, the concentrations measured were within 83.5 – 107.9% of those expected from the starting concentrations (Figure 2a), suggesting that the matrix did not significantly interfere with this assay, using samples prepared in this way. In contrast, in the Ah-I, dioxin concentrations in some samples, in particular sea bass and yellowtail, appeared to increase with dilution (Figure 2b). This suggested interference, in some cases, with the Ah-I by the sample matrix or AhR antagonists in the samples. Therefore, serial dilutions of the prepared samples were measured in the Ah-I, and the maximum concentration obtained was taken as the concentration of non-*ortho* PCBs and PCDD/Fs fraction.

Dioxin concentrations were measured in twenty samples of retail fish by PCB-EIA and Ah-I, and compared to TEQ concentrations obtained by HRGC/HRMS. Both the concentrations of mono-*ortho* PCBs fraction obtained in the EIA (Figure 3a) and the concentrations of non-*ortho* PCBs and PCDD/Fs fraction obtained in Ah-I (Figure 3b) showed good correlations with TEQ concentrations measured by HRGC/HRMS ($r > 0.98$ and $r = 0.97$, respectively). These results showed that using a combination of the PCB-EIA and the Ah-I would offer a practical method for estimating the TEQ levels of dioxins in retail fish.

Further, the concentrations by the PCB-EIA and the concentrations of PCB 118 by HRGC/HRMS showed a good correlation (Figure 4), with the slope of the linear regression equation being roughly 1. This suggested that a positive reading in the EIA was mainly attributable to PCB 118 in the samples. Results obtained for concentrations in the Ah-I were compared to the expected results, calculated by multiplying the concentrations of 4 non-*ortho* PCBs and 17 PCDD/Fs determined by HRGC/HRMS and their relative potency values in the Ah-I[5]. As shown in Figure 5, a good correlation was observed between obtained and expected values, with the slope of the linear regression equation being roughly 1. This showed that a positive reading in the Ah-I was largely attributable to the target compounds in the samples, and that the difference in the toxic equivalent measurements in the Ah-I and HRGC/HRMS was mainly due to the differences between the relative potency values in the Ah-I and the WHO-TEFs in HRGC/HRMS.

Overall our results indicate that using the PCB-EIA and Ah-I in combination is a useful approach to measuring TEQs of dioxins in retail fish.

Acknowledgements

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References

1. Tsutsumi, T., Yanagi, T., Nakamura, M., Kono, Y., Uchibe, H., Iida, T., Hori, T., Nakagawa, R., Tobiishi, K., Matsuda, R., Sasaki, K. and Toyoda, M. (2001) Chemosphere 45: 1129-1137.

2. EnBio Tec Laboratories Co., Ltd.,EnBio Coplanar PCB EIA system Instruction booklet, Chiyoda-Parion Bldg., 6th Floor, Kandasuda-cho 2-3-16, Chiyoda-ku, Tokyo 101-0041, Japan.

3. KUBOTA Corporation, Ah-immunoassay Instruction booklet, Nihonbashi-muromachi 3-1-3, Chuo-ku, Tokyo 103-8310, Japan.

4. Tsutsumi,T., Amakura,Y., Sasaki, K., Toyoda, M. and Maitani, T. (2003) Anal. Bioanal. Chem. 375: 792-798.

5. Kobayashi,Y., Lundquist,A., Uechi, T., Ashieda, K., Sasaki, K., Hughes, B. and Kaise, T. (2002) Organohalogen Compounds58: 337-340.

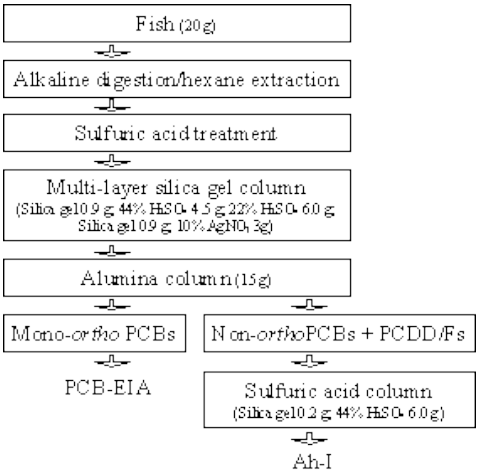

Figure 1. Sample preparation for retail fish

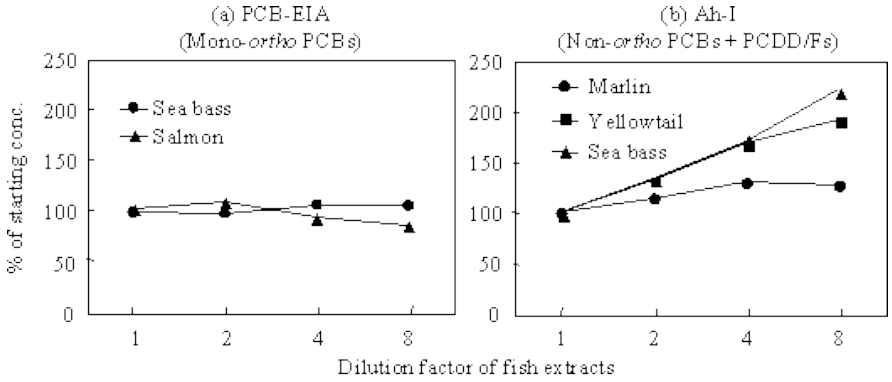

Figure 2. Effect of dilution factor on the determination of dioxins in fish

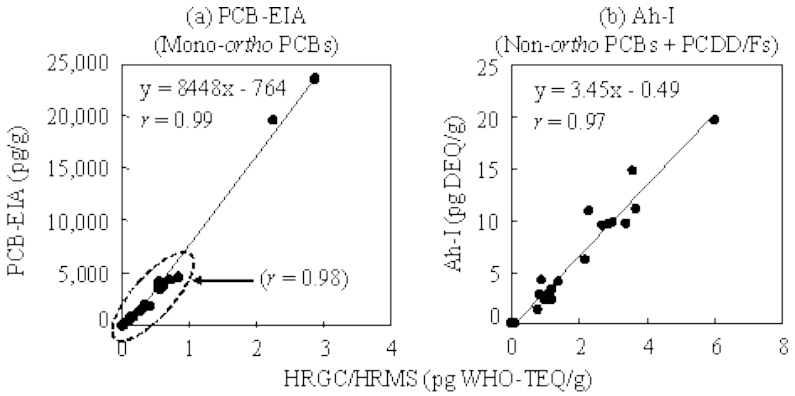

Figure 3. Comparison of EIA and Ah-I with HRGC/HRMS measurements of 20 fish samples

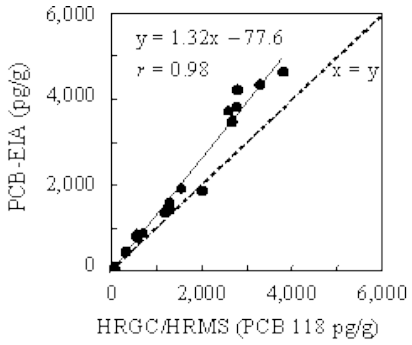

Figure 4. Comparison of concentrations measured in EIA with PCB 118 concentrations measured by HRGC/HRMS in fish samples
Two highly contaminated samples (> 5,000 pg/g of PCB 118) were excluded from the regression calculation.

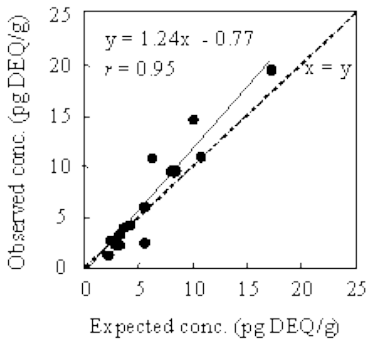

Figure 5. Comparison of observed and expected concentrations in fish, in the Ah-I
Two samples with undetectable levels were excluded from the regression calculation.