Applicability of ELISA to screen for dioxin-like PCBs in retail fish

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Introduction

The group of dioxin-like PCBs, consisting of four non-*ortho* PCBs and eight mono-*ortho* PCBs, is classified among the dioxins. Humans are considerably exposed to dioxin through consumption of fish in Japan. The TEQ contribution of dioxin-like PCBs in fish is dominant, contributing about 70% of the total TEQ [1]. Therefore, it is important to reveal TEQ levels of dioxin-like PCBs as well as PCDD/Fs in retail fish. HRGC/HRMS is currently the most widely used method to determine levels of dioxin-like PCBs. This method is very reliable and highly sensitive, but it is also time-consuming and requires expensive equipment and highly trained analysts. One possible alternative method for screening dioxin-like PCBs is a bioassay, such as an ELISA. We recently developed an ELISA kit using a monoclonal antibody (MAb) specific to 2,3',4,4',5-pentachlorobiphenyl (PCB 118) [2]. PCB 118 is generally the most abundant isomer among dioxin-like PCBs in fish, although it has a relatively small contribution to the total TEQ derived from dioxin-like PCBs. Based on our HRGC/HRMS data, we found that the concentrations of PCB 118 correlated well to the TEQ levels of dioxin-like PCBs in retail fish ($r > 0.85$, $n = 181$). Here, we examined the application of the ELISA kit in determining TEQ levels of dioxin-like PCBs in retail fish.

Methods and Materials

Samples: Retail fish samples were purchased during 2002-2003 from supermarkets in Tokyo, Japan. The samples (muscular part) were homogenized using a food cutter and stored at -20°C until analysis.

Clean-up of fish for the ELISA: The homogenized fish samples (20 g) were added to aqueous KOH (2 mol/L) and then kept at room temperature for 16 hr to carry out alkali digestion. The alkaline hydrolysates were added to methanol (30 ml) and extracted three times by a mechanical stirrer (10 min) with *n*-hexane (40 ml), and extracts were washed twice with 2% aqueous NaCl (40 ml). The extracts were treated several times with concentrated sulfuric acid, and then passed through a multi-layer silica gel column (a 1/5 scale of a conventional volume). The elutes obtained

with *n*-hexane (50 ml) were then loaded onto an alumina column (2.5 g). After washing with *n*-hexane (10 ml), dioxin-like PCBs were eluted with 5% dichloromethane/*n*-hexane (45 ml). The elutes were dried out by nitrogen stream, and the residues were re-dissolved into DMSO (100 µl) and used for the ELISA.

Competitive ELISA: ELISA was performed as described by the manufacture, EnBioTec Laboratories (Tokyo, Japan). Briefly, samples or various concentrations of standard (3,3',4'-trichloro-4-methoxybiphenyl) mixed with PCB competitor-horseradish peroxidase conjugate were added to microtitre wells coated with MAb against PCB 118, and then incubated for 30 min at room temperature. After washing, enzyme substrate solution (3,3',5,5'-tetramethylbenzidine) was added to each well and incubated for 20 min. The enzyme reaction was stopped with 1 mol/L H_2SO_4, and the absorbance at 450 nm was measured. All experiments were conducted in duplicate. The standard curves were fitted by a four–parameter logistic model. The assay's quantification range for PCB 118 was 10 – 250 pg/ml (125 – 3,125 pg/assay). The quantitative limit corresponded to 50 pg/g for a 20 g fish sample.

HRGC/HRMS analysis: The extraction, cleanup and analysis of dioxins generally followed procedures described in a previous report [3]. Briefly, the samples (100 g) with $^{13}C_{12}$-labelled internal standards were digested with aqueous KOH. The alkaline hydrolysates were then extracted with *n*-hexane. After treatment with concentrated sulfuric acid, the extracts were purified on a silver nitrate/silica gel column followed by an alumina column. On the alumina column, the extracts were separated into mono-*ortho* PCBs fractions and non-*ortho* PCB and PCDD/Fs fractions. The latter fractions were further purified on an activated carbon column. Both the fractions were spiked with $^{13}C_{12}$-labelled recovery standards. The quantification of dioxin-like PCBs was conducted by HRGC/HRMS using a HP6890-plus gas chromatograph equipped with a HT-8 column and coupled to a JEOL JMS-700 mass spectrometer. The limits of quantification were approximately 0.05 pg/g for non-*ortho* PCBs and 0.5 pg/g for mono-*ortho* PCBs. The TEQ concentrations were calculated using the WHO-TEFs.

Results and Discussion

Performance of the ELISA for the determination of dioxin-like PCBs in fish

To examine the matrix effect for ELISA under the present clean-up procedure, a recovery test of dioxin-like PCBs in cleaned-up samples was carried out. Extracts of sea bass were spiked with known quantities of PCB 118 and assayed. Good recoveries (78.7 – 96.9%) over the assay's quantification range were obtained (Table 1). A dilution test was carried out to further examine the matrix effect for the ELISA. Two kinds of cleaned-up extracts from sea bass and mullet were serially 2-fold diluted with DMSO and assayed. The obtained levels with the dilutions were 74.1 – 107.9% of the expected levels based on their initial concentrations in the assay (data not shown). These results suggested that the matrix did not significantly interfere with the assay under the clean-up procedure.

The practical analysis of the ELISA combined with the clean-up procedure was examined in the following studies. A recovery test in which PCB 118 was added to fish samples also resulted in acceptable recoveries (60.2 – 82.3%), as shown in Table 2. This result indicated that no significant loss of the target isomer during the clean-up procedure occurred. Additionally, the repeatability of

the ELISA in combination with the clean-up procedure was tested by replicate analyses of the same fish sample on separate days. The assay gave acceptable results for dioxin-like PCBs: the coefficients of variation for the two kinds of fish were 5.7 – 29.0% (Table 3). It was concluded that the ELISA would perform well in the analysis of dioxin-like PCBs in retail fish.

Application of the ELISA in the analysis of dioxin-like PCBs in fish

The thirty-one retail fish samples were analyzed by both ELISA and HRGC/HRMS. A good correlation (r = 0.99) was observed between ELISA values and concentrations of PCB 118 in HRGC/HRMS analysis (Figure 1(a)). The slope of the linear regression equation was roughly 1, suggesting that the ELISA is reliable for determination of the target isomer in real samples. Furthermore, a good correlation (r = 0.91) between the ELISA and TEQ values of dioxin-like PCBs in HRGC/HRMS analysis was obtained (Figure 1(b)). This indicates that the ELISA would offer a practical method of predicting TEQ levels of dioxin-like PCBs in retail fish. According to the correlation study, ELISA could easily detect around 2 pg-TEQ/g on a fresh weight basis for dioxin-like PCBs in retail fish. The ELISA also allows for high throughput rates, low cost and small sample size compared to traditional HRGC/HRMS analysis. Thus, the ELISA would be a useful screening method for TEQ levels of dioxins-like PCBs in retail fish.

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Table 1. Recovery of PCB 118 from spiked cleaned up extract of fish [a)]

Sample	Spiked levels, ng/ml	Observed levels, ng/ml	Recovery, [b)] %
Sea bass	0	28.2	–
	15	41.8	90.7
	30	51.8	78.7
	45	71.8	96.9
	60	82.2	90.0

[a)] Cleaned up extract from sea bass was spiked with known quantities of PCB 118, and analyzed by the ELISA (n = 1).

[b)] Reveries were corrected by subtraction of the blank level (28.2 ng/ml).

Table 2. Recovery of PCB 118 from spiked fish samples [a)]

Samples	Spiked levels, pg/g	Observed levels, pg/g	Recovery, %
Tuna	0	ND	–
	150	115	76.7
	1,500	1,235	82.3
Yellowtail	0	194	–
	1,500	1,097	60.2 [b)]

[a)] Fish samples spiked with known quantities of PCB 118 were extracted, cleaned up and analyzed by the ELISA (n = 1).

[b)] Recovery was corrected by subtraction of the blank level (194 pg/g).

Table 3. Reproducibility of the ELISA in fish samples [a)]

Samples	ELISA, pg/g		CV, %
	Mean ± SD	Range	
Mullet	3,564 ± 204	3,330 – 3,700	5.7
Sea bass	417 ± 121	340 – 557	29.0

[a)] The two kinds of natively dioxin-contaminated fish samples were cleaned up and analyzed by the ELISA. Three examinations were carried out on different days.

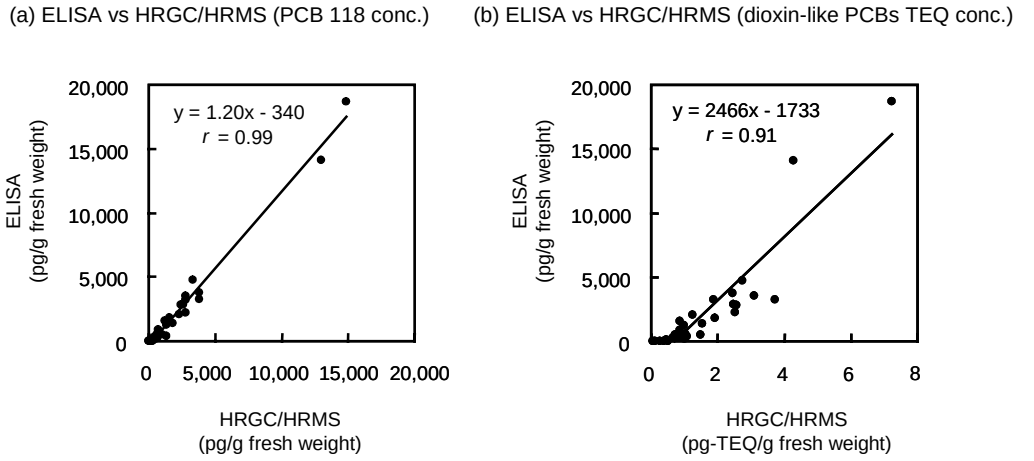

Figure 1. Comparison of the ELISA and HRGC/HRMS analyses of retail fish. Thirty one samples were analyzed by both assays.