We did not minimize the gel and elution volumes used in the sample preparation procedure for the assays to speed the process. At present, it takes about three days to produce a final extract for the assays. However, it is possible to determine dioxin concentrations in a final extract using the assays in one day. Total time for determination of dioxins in a fish sample is similar for other bioassays for dioxins, like the CALUX assay, in retail fish, as we have reported previously (*10*).

In conclusion, the present study evaluated a novel combination of the PCB-EIA and Ah-I for the determination of dioxin concentrations in retail fish. Our method has increased throughput and reduced costs compared with conventional HRGC-HRMS analysis and is a useful dioxin TEQ screening method prior to HRGC-HRMS analysis.

ABBREVIATIONS USED

Ah-I, Ah immunoassay; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; CALUX, chemical-activated luciferase gene expression; DEQs, dioxin equivalents; DMSO, dimethyl sulfoxide; DRE, dioxin-responsive element; ELISA, enzyme-linked immunosorbent assay; HpCDDs, heptachlorodibenzo-*p*-dioxins; HpCDFs, heptachlorodibenzofurans; HRGC, high-resolution gas chromatography; HRMS, high-resolution mass spectrometry; HxCDDs, hexachlorodibenzo-*p*-dioxins; HxCDFs, hexachlorodibenzofurans; LOQ, limits of quantification; mAb, monoclonal antibody; OCDD, octachlorodibenzo-*p*-dioxins; OCDF, octachlorodibenzofuran; PCB-EIA, coplanar PCB EIA system; PCBs, polychlorinated biphenyls; PCDDs, polychlorinated dibenzo-*p*-dioxins; PCDFs, polychlorinated dibenzofurans; PeCDDs, pentachlorodibenzo-*p*-dioxins; PeCDFs, pentachlorodibenzofurans; SDs, standard deviations; TCDDs, tetrachlorodibenzo-*p*-dioxins; TCDFs, tetrachlorodibenzofurans; TEF, toxic equivalency factor; TEQ, toxic equivalent; WHO, World Health Organization.

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Biosensor immunoassay for the screening of dioxin-like polychlorinated biphenyls in retail fish

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ABSTRACT

Dioxin-like polychlorinated biphenyls (DL-PCBs) often make up the majority of the toxic equivalent (TEQ) contribution of dioxins found in fish samples. For the purpose of making risk assessments, it is therefore important to develop screening methods for determining TEQ concentrations of DL-PCBs in retail fish. We have developed a rapid biosensor immunoassay (BIA) for DL-PCBs that uses a surface plasmon resonance sensor (Biacore 3000). The BIA is highly specific for 2,3′,4,4′,5-pentachlorobiphenyl (PCB 118) that is generally the most abundant DL-PCB isomer found in fish. The fish extracts were first cleaned up on a multilayer silica gel column followed by an alumina column, then subjected to the assay. The quantitative limit of the assay was 1 ng PCB 118 per gram of tested sample. Dilution and recovery tests using purified fish extracts suggested that the matrix effect was minimized in the assay by diluting the analyzed samples. The assay results for retail fish samples ($n = 7$) agreed well with those obtained by an enzyme-linked immunoassay (ELISA) using the same monoclonal antibody: ELISA has been already validated for determining DL-PCBs in fish samples, so BIA performs well in this analysis. Finally, BIA results for the TEQ concentrations of DL-PCBs in retail fish samples ($n = 10$) correlated well with those obtained by high-resolution gas chromatography coupled to high-resolution mass spectrometry ($r = 0.89$). Our method is therefore useful for screening retail fish to determine the TEQ concentrations of DL-PCBs.

1. Introduction

Dioxin-like polychlorinated biphenyls (DL-PCBs) together with polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are collectively known as dioxins. Four non-*ortho* PCBs and eight mono-*ortho* PCBs are currently classified as DL-PCBs, and these have been assigned toxicity equivalent factors (TEFs) relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) [1,2]. People living in Japan are subject to exposure to high levels of dioxins through consumption of fish [3–5]. The DL-PCBs often make up the majority of the toxic equivalent (TEQ) contribution of dioxins in fish samples [6–9]. Therefore, it is important to determine the TEQ concentrations of DL-PCBs, in addition to those of PCDD/Fs, in retail fish.

High-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) is widely accepted as the most reliable method for determining the TEQ levels of dioxins. Although this technique is reliable and sensitive, it is time-consuming, requires expensive equipment, and must be performed by highly trained staff. Reporter-gene assays, such as the chemical-activated luciferase gene expression (CALUX) assay, are currently considered to be the best method for screening TEQ concentrations of dioxins in food [for review, see 10]. However, its drawbacks include the need for cell culture, which requires skilled personnel and elaborate

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equipment, and the probable need for a license to conduct the assay.

An enzyme-linked immunosorbent assay (ELISA) could be a simpler alternative that does not require cell culture. 2,3′,4,4′,5-Pentachlorobiphenyl (PCB 118) is generally the most abundant DL-PCB isomer found in fish [7,11,12]. On the basis of HRGC/HRMS data obtained in our national survey of dioxins in Japan [13], we found that the concentrations of PCB 118 correlates well with the TEQ concentrations of DL-PCBs in retail fish, although PCB 118 makes a relatively small contribution to the total TEQ of DL-PCBs as a result of its low TEF. Therefore, we recently developed an ELISA that uses a monoclonal antibody (Mab) that is highly specific for PCB 118 to screen retail fish for the TEQs of DL-PCBs [13]. The ELISA is simple, quick, and suitable for screening DL-PCBs in retail fish samples.

Various immunosensor techniques are growing in popularity for residue analysis because of their speed. Although electrochemical immmunosensors, like screen-printed electrodes, are widely used and cost-effective and allowing on site analysis, surface plasmon resonance (SPR) immunosensors have proved to be the most widely reported for immunosensor applications [for review, see 14]. Additionally, SPR techniques can eliminate the need for enzyme-labeled reagents and are mostly highly automated, they are quite simple methods. In particular, SPR-based biosensor immunoassays (BIAs) using Biacore instruments have been proposed, mainly for the determination of residues of veterinary drugs in foodstuffs [for reviews, see 15 and 16]. BIA has also been applied in the detection of dioxins and PCBs [17]; however, there are no previously published reports on their performance in determining these compounds in food samples. We developed an SPR-based BIA by using a Mab that is specific to PCB 118, and we assessed the performance of the BIA in screening TEQ concentrations of DL-PCBs in retail fish.

2. Experimental

2.1. Materials

Dioxin-analysis-grade acetone, dichloromethane, and *n*-hexane were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). A multilayer silica gel column filled from bottom to top with 0.9 g silica gel, 3 g of 2% (w/w) potassium hydroxide-impregnated silica gel, 0.9 g silica gel, 4.5 g of 44% (w/w) sulfuric acid-impregnated silica gel, 6 g of 22% (w/w) sulfuric acid-impregnated silica gel, 0.9 g silica gel, 3 g of 10% (w/w) silver nitrate-impregnated silica gel, and 6 g of anhydrous sodium sulfate was purchased from GL Science Inc. (Tokyo, Japan). Alumina B-Super I was obtained from ICN Pharmaceuticals Inc. (Costa Mesa, CA, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma–Aldrich (Milwaukee, USA). An activated carbon/dispersed silica gel reversible column was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). DL-PCBs were obtained from AccuStandard Inc. (New Haven, CT, USA). PCB analogue–bovine serum albumin (BSA) conjugate, a Mab against PCB 118, and the ELISA kit using the Mab were purchased from EnBioTec Laboratories (Tokyo, Japan).

Sensor chips (CM5), HBS-EP buffer [0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant polysorbate 20; pH 7.4], and an amine coupling kit were supplied by Biacore AB (Uppsala, Sweden).

Fish samples were purchased during 2002 and 2004 from supermarkets in Tokyo, Japan. The samples (muscle tissue) were homogenized by using a food processor and stored at −20 °C until analysis.

2.2. Equipment

The Biacore 3000 was supplied by Biacore AB (Uppsala, Sweden). HRGC/HRMS was performed by using an HP-6890 plus gas chromatograph coupled to a JEOL JMS-700 MStation mass spectrometer supplied by JEOL Ltd. (Tokyo, Japan).

2.3. Purification of fish tissue for the BIA

The homogenized fish sample (20 g) was added to aqueous 2 M KOH (100 mL) and subjected to alkali digestion at room temperature for 16–20 h. The alkaline hydrolysate was added to methanol (150 mL) and extracted three times by mechanically shaking (10 min) with *n*-hexane (100 mL). The extract was washed twice with 2% (w/v) aqueous NaCl (150 mL), treated several times with concentrated sulfuric acid, and then passed through the multilayer silica gel column (see above). The eluate obtained with *n*-hexane (200 mL) was then loaded onto an alumina column (15 g) and washed with *n*-hexane (150 mL). The mono-*ortho*-PCBs containing PCB 118 was eluted with 2% (v/v) dichloromethane/*n*-hexane (150 mL). The fraction was dried by using a nitrogen stream, and the residue was dissolved in DMSO (200 μL). Alternatively, the eluate from the alumina column was loaded onto an active carbon-dispersed silica gel reversible column and washed with *n*-hexane (50 mL). The mono-*ortho*-PCBs containing PCB 118 were then eluted with 25% (v/v) dichloromethane/*n*-hexane (50 mL). The fraction was dried using a nitrogen stream and the residue was dissolved into DMSO (200 μL). The solution was centrifuged at 10,000 rpm for 5 min and the supernatant was subjected to BIA.

2.4. BIA

2.4.1. Preparation of the biosensor chip

A PCB analogue–BSA conjugate was immobilized (yielding approximately 1500 relative response (RU)) on the surface of a CM5 sensor chip by using an amine coupling kit, following the manufacturer's instructions. Briefly, the chip surface was activated by injecting a 1:1 (v/v) mixture of 0.4 M 1-ethyl-3-(3-demethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.1 M *N*-hydroxysuccinimide (NHS) at a flow rate of 10 μL min^{-1} and with a contact time of 7 min. The reactants were removed and the PCB analogue–BSA conjugate (50 μg mL^{-1} in 10 mM acetate buffer, pH 4.0) was injected over the surface during 10 min at a flow rate of 10 μL min^{-1}. To deactivate the remaining active sites, 1 M ethanolamine was injected during 7 min at a flow rate 10 μL min^{-1}. The sensor chip surface was washed repeatedly with 50 mM NaOH for 5 s at a flow rate of 60 μL min^{-1}.

2.4.2. BIA for DL-PCBs in fish

Mixing of all the reagents, injection, and washing were performed automatically by the Biacore 3000. The running buffer was a 9:1 (v/v) mixture of HBS-EP buffer and DMSO at a flow rate of 20 μL min^{-1}. An aliquot of Mab against PCB 118 (5.5 μg mL^{-1} in HBS-EP buffer) was mixed with the samples or with various concentrations of PCB 118 (0–600 ng mL^{-1} in DMSO) in a ratio of 9:1 (v/v). The mixture was injected for 2 min over the immobilized surface and the reference (non-immobilized) surface. After the injection, the needle was washed with DMSO to eliminate the risk of carry-over of PCB 118 from a highly contaminated sample. The surface was regenerated with 50 mM NaOH for 5 s at a flow rate of 60 μL min^{-1}. The total run time between the samples was about 12 min. RUs were recorded on each sensorgram 15 s after the injection. All RU values were corrected by subtraction of the RUs from the reference surfaces. Duplicate injections for standard solutions were performed and the mean responses were used to construct a calibration curve. The calibration curves were fitted by using a four-parameter logistic model.

2.5. ELISA for DL-PCBs in fish

The purification procedure was performed in a similar way to that for the BIA. The ELISA kit was used according to the manufacturer's instructions (EnBioTec Laboratories, Tokyo, Japan) [18]. Briefly, samples or various concentrations of 3,3',4-trichloro-4'-methoxybiphenyl, a surrogate standard for PCB 118, mixed with competitor–horseradish peroxidase conjugate were added to microtitre wells coated with Mab against PCB 118, incubated for 30 min at room temperature, then washed with the solution provided. An enzyme substrate solution containing 3,3',5,5'-tetramethylbenzidine was added to each well and incubated for 20 min. The enzyme reaction was stopped with 0.5 M H_2SO_4 and the absorbance at 450 nm was measured. All experiments were conducted in duplicate. The calibration curves were fitted by using a four-parameter logistic model.

2.6. Measurement of DL-PCBs in fish by HRGC/HRMS

The extraction, purification, and analysis of dioxins were performed following general procedures described in a previous report [19]. Briefly, the homogenized sample with $^{13}C_{12}$-labelled internal standards was digested with aqueous KOH. The alkaline hydrolyzate was then extracted with *n*-hexane. The extracts were treated with concentrated sulfuric acid then purified on a silver nitrate/silica gel column. This was followed by further purification on an alumina column. The alumina column separated the extract into mono-*ortho* and non-*ortho* PCB fractions. The latter fraction was purified further on an activated-carbon column. Both the fractions were spiked with $^{13}C_{12}$-labelled recovery standards. Four non-*ortho* PCBs and eight mono-*ortho* PCBs were quantified by the HRGC/HRMS. The measurement of non-*ortho* and mono-*ortho* PCBs was performed in an HT-8 fused silica capillary column (SGE, TX, USA). The TEQ was calculated by using the World Health Organization (WHO) TEFs (TEF_{1998}) [1]. The TEQ was also calculated by using the new TEFs (TEF_{2005}) revised recently [2].

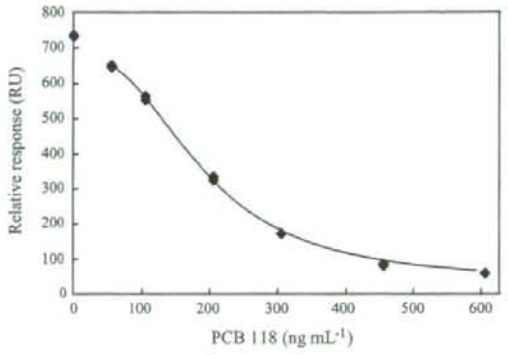

Fig. 1 – Typical calibration curve of the BIA. Each standard solution was assayed in duplicate. The calibration curve was fitted by using a four-parameter logistic model based on the data ranging from 50 to 600 ng PCB 118 mL^{-1}.

The limits of quantitation (LOQ) were around 0.1 pg g^{-1} for non-*ortho* PCBs and 1.0 pg g^{-1} for mono-*ortho* PCBs. Calculation of the total TEQ in a sample was carried out by assuming that all isomer concentrations below the LOQs were equal to zero.

3. Results and discussion

3.1. BIA calibration curve

Fig. 1 shows a typical calibration curve for BIA. To determine the accuracy and precision of the quantification, six concentrations of PCB 118 were assayed repeatedly on different days. As shown in Table 1, at PCB 118 concentrations of 100–600 ng mL^{-1}, the residual errors were small (−5.6 to +5.1%). Coefficients of variations (CVs) in the same concentration range were also extremely low (<2.8%). From these results, the BIA's quantitative range was selected to be 100–600 ng mL^{-1} determined as tolerable concentrations defined as being less than 10% residual errors and CVs. The LOQ of 100 ng of PCB 118 per milliliter corresponds to 1 ng of PCB 118 per gram of sample, when a 20-g fish sample is tested.

Table 1 – Accuracy and precision of the BIA calibration curve[a]

PCB 118 conc. (ng mL^{-1})	PCB 118 found (n = 6)		
	Mean ± S.D. (ng mL^{-1})	CV%	Bias%
50	48.5 ± 14.7	30.4	−3.0
100	101.3 ± 2.6	2.6	1.3
200	198.1 ± 2.7	1.4	−1.0
300	315.3 ± 8.8	2.8	5.1
450	463.6 ± 7.8	1.7	3.0
600	566.7 ± 10.3	1.8	−5.6

[a] The accuracy and precision were determined by analyzing each concentration of PCB 118 on 6 different days.

Table 2 – Cross-reactivity of the BIA against DL-PCBs

DL-PCBs	Cross-reactivity (%)	
	BIA[a]	ELISA[b]
Non-ortho PCBs		
PCB 77	40.3	17.8
PCB 81	2.7	<3.0
PCB 126	1.5	<3.0
PCB 169	<1.0	<0.1
Mono-ortho PCBs		
PCB 105	10.3	2.5
PCB 114	9.8	3.4
PCB 118	100	100
PCB 123	<1.0	<0.1
PCB 156	33.2	7.2
PCB 157	<1.0	<0.1
PCB 167	<1.0	<0.1
PCB 189	<1.0	<0.1

[a] The percentage of cross-reactivity relative to PCB 118 was calculated as follows: (Conc. of PCB 118 giving a certain RU)/(Conc. of DL-PCBs isomers giving the same RU) × 100.
[b] Data quoted from the EnBio Coplanar PCB EIA system instruction booklet [18].

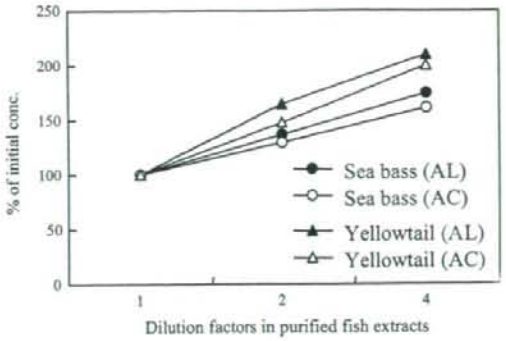

Fig. 2 – Effect of the dilution factor on concentrations measured in retail fish. Purified extracts from two varieties of fish contaminated in the natural environment were diluted with DMSO and assayed in duplicate by the BIA. The extracts were purified by an alumina column (AL) or an activated-carbon column (AC) after a multilayer silica gel column.

3.2. Cross-reactivity of the BIA

The cross-reactivity of the BIA to other DL-PCBs was examined. Serial dilutions of DL-PCBs were assayed and the concentrations of each compound required for around 70% of the maximal RU were compared with that of PCB 118 (Table 2). The BIA was specific to PCB 118; however, the assay cross-reacted to some extent with PCB 77, PCB 105, PCB 114, and PCB 156 (9.8–40.3% of the value for PCB 118). However these isomers are usually present in much lower concentrations than PCB 118 in fish samples. The cross-reactivity of the ELISA using the same Mab [18] is also shown in Table 2. The cross-reactivity of the BIA with some of the isomers (PCB 77, PCB 105, PCB 114 and PCB 156) appeared to be a little higher than the ELISA. This might be responsible for the difference in estimations for the cross-reactivities of both methods. The cross reactivity of the ELISA was determined by the concentrations of each compound required for 50% of the maximal binding [18]. Overall, the cross-reactivity of the BIA was similar to that of the ELISA.

3.3. Effect of fish matrix on the BIA

The effect of the fish tissue matrix on the BIA was measured by the dilution test. Purified extracts of fish samples contaminated in the natural environment were subjected to two-fold serial dilutions with DMSO before being assayed (Fig. 2). The extracts were purified by two different procedures (alumina or activated-carbon column) after the multilayer silica gel column. The concentrations in the assay appeared to increase with dilution. This shows that the dilution process can eliminate the matrix effect in the assay. No significant differences in the concentrations were found between the two purification procedures.

A recovery test using purified fish extracts was also carried out to examine in more detail the effect of the matrix on the BIA. Serial dilutions of purified extracts spiked with PCB 118 were assayed. The recovery was 51.8–77.2% and 33.4–85.4% for extracts purified by using the alumina and activated-carbon column, respectively (Table 3). The recovery tended to be greater when diluted samples were tested. For this reason,

Table 3 – Recovery of PCB 118 from spiked purified fish extracts[a]

Samples	Spiked levels (ng mL^{-1})	Dilution factors	Observed levels (ng mL^{-1})		Recovery (%)[b]	
			AL	AC	AL	AC
Procedural blank	100	1	–	–	99.6	93.9
	200	1	–	–	95.4	93.7
Yellowtail	200	1	323	229	51.8	33.4
	200	2	290	211	66.6	46.1
	200	4	250	207	77.2	63.5
Salmon	200	1	320	342	66.3	73.3
	200	2	274	292	77.2	85.4

[a] Serial dilutions of purified extracts from fish samples were spiked with known quantities of PCB 118, and analyzed by the BIA ($n=2$). The extracts were purified on an alumina column (AL) or an activated carbon column (AC) after a multilayer silica gel column.
[b] Recoveries were corrected by subtraction of the native levels of contamination for each sample.

Table 4 – Reproducibility of the BIA combined with the purification procedure[a]

Samples	n	BIA (ng g^{-1})		CV (%)
		Mean ± S.D.	Range	
Sea bass	4	10.7 ± 0.6	10.1–11.4	5.9
Salmon	4	2.5 ± 0.1	2.3–2.6	4.4

[a] The two varieties of fish contaminated in the natural environment were extracted, cleaned up and assayed by the BIA in four separate runs on different days.

serial dilutions of fish extracts were measured in the assay, and the maximum concentration was used to minimize the matrix effect in the assay. Finally, the use of the alumina column was selected as the purification procedure after the multilayer silica gel column, although no significant differences were found between the recovery rates for the two purification procedures.

3.4. Reproducibility of the BIA

The reproducibility of the BIA in combination with the sample-preparation procedure was tested by replicate analyses of the same homogenized fish samples. The fish were extracted, cleaned, and assayed in four separate analyses on different days. The assay showed excellent results for the measured concentrations: the CVs for the two varieties of fish were 4.4–5.9% (Table 4).

3.5. Comparison of the BIA with ELISA

The performance of the BIA was investigated by using seven retail fish samples that were analyzed by both the BIA and the ELISA using the same Mab. A good correlation ($r = 0.99$) was observed between the results of the BIA and those of the ELISA (Fig. 3). The slope of the linear regression equation was

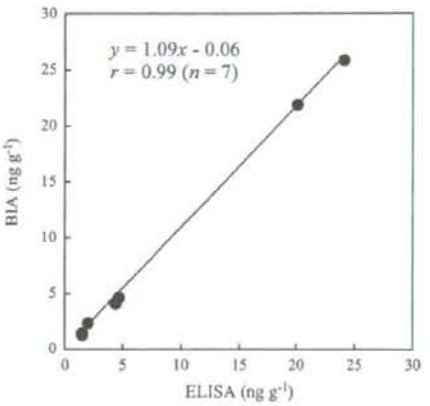

Fig. 3 – Comparison of data from the BIA and the ELISA. Seven fish samples (one marlin, two salmon, two sea bass, and two yellowtail) were analyzed by the BIA and the ELISA using the same Mab.

nearly 1 and y-intercept was near to zero, suggesting that the BIA gave almost the same values as the ELISA. The ELISA has been already validated for the analysis of DL-PCBs in retail fish samples, and the fish matrix did not greatly interfere with the ELISA [13]. Therefore, we concluded that the fish matrix caused no significant interference to the BIA results.

3.6. Comparison of the BIA with HRGC/HRMS

The values obtained in the BIA for ten retail fish samples tested were also compared with the results obtained by the HRGC/HRMS analysis. A good correlation ($r = 0.99$) was observed between the BIA values and the concentrations of PCB 118 obtained by HRGC/HRMS analysis [Fig. 4(a)]. At low

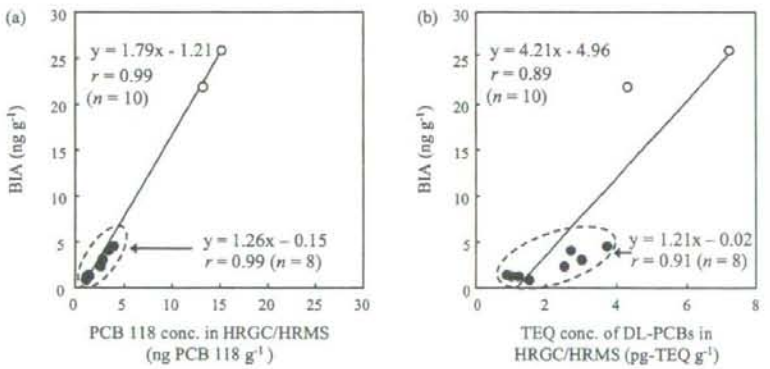

Fig. 4 – Comparison of data from the BIA and the HRGC/HRMS analysis in retail fish. Ten samples (two mackerel, one marlin, two salmon, two sea bass, and three yellowtail) were analyzed by the BIA and HRGC/HRMS. (a) BIA values versus PCB concentrations. The sea bass samples containing significantly high levels of DL-PCBs are indicated by the open circles. The regression equation for the samples, excluding the sea bass (indicated by the dashed circle), is also shown in (a). (b) BIA values versus TEQ concentrations of DL-PCBs calculated by using TEF_{1998} values. The sea bass samples are shown by the open circles. The regression equation for the samples excluding the sea bass (shown by the dashed circle) is also shown in (b).

PCB concentrations (<5.0 ng g^{-1}), a good correlation (r = 0.99) was also obtained between the two methods. However, the slopes of the linear regression equation tended to be a little higher than 1, and this became more conspicuous when two highly contaminated samples of sea bass were included. This suggests that BIA cross-reacted with some compounds, although the positive BIA results for the retail fish samples were caused mainly by their reactivity with PCB 118. The mono-*ortho* PCBs fractions tested by the BIA usually contain PCB 105, PCB 114, and PCB 156, which can cross-react with the BIA (Table 2). However, levels of these compounds were much lower than those of PCB 118 in the samples tested by HRGC/HRMS analysis (data not shown). Therefore, the reactivity of the BIA to these compounds must be negligible. The Mab is also known to have slight cross-reactivity with other PCBs [PCB 31, PCB 66, and PCB 70; 12.9–17.8% of PCB 118] in the ELISA [18]. The presence of large amounts of these isomers could have a slight effect on the results of the BIA, although it is not clear whether these isomers were present in the samples tested in the present study.

The values obtained in the BIA for the ten retail fish samples tested were plotted against the TEQ concentrations of DL-PCBs obtained by the HRGC/HRMS analysis [Fig. 4(b)]. A good correlation (r = 0.89) was obtained between the BIA and TEQ values calculated by using TEF_{1998}. This indicates that the BIA offers a practical approach for screening TEQ levels of DL-PCBs in retail fish. However, two highly contaminated samples of sea bass, shown as open circles in Fig. 4(b), appeared to distort the correlation. These samples gave much higher values in the BIA against their corresponding TEQ concentrations. However, these samples would result in false-positive results in the BIA; they would not, therefore, cause a serious problem in a screening method. The concentrations of DL-PCBs in retail fish on the Japanese market were in the range of 0.022–4.8 pg-TEQ g^{-1} (n = 91, median: 0.57, mean: 1.0) based on the HRGC/HRMS data produced by our national survey of dioxins in Japan [20]. The BIA will easily detect highly contaminated fish samples from the comparative data shown in Fig. 4(b). These in shellfish were in the range of 0.00012–2.6 pg-TEQ g^{-1} (n = 44, median: 0.070, mean: 0.24). These in meat and dairy products were in the range of 0–1.2 pg-TEQ g^{-1} (n = 173, median: 0.021, mean: 0.047). Therefore, due to the low concentrations of DL-PCBs in these samples, it is currently very difficult to apply the BIA to detect DL-PCBs in them.

TEF values have been revised recently [2]. WHO advises that the TEF_{2005} are used as they replace the previous TEF_{1998}. We therefore compared the BIA results with the TEQ concentrations calculated by using the TEF_{2005} values (Fig. 5). To eliminate any distortion of the results, the two highly contaminated samples of sea bass were excluded. A good correlation (r = 0.87) was observed between the BIA and TEQ values. The slope of the linear regression equation was slightly higher than the slope when the previous TEF_{1998} were used for the same samples, as shown by the dashed circle in Fig. 4(b). This is mainly because the TEF_{2005} of PCB 118 (0.00003) is a little lower than the TEF_{1998} value (0.0001).

There are currently no internationally recognized maximum limits for DL-PCBs in foods. However, the European Union established action levels for DL-PCBs in foodstuffs including fish [21]. This directive has increased the demand for screening methods to detect these compounds. The BIA is very rapid (about 12 min per cycle) and can be automated. The BIA will enable us to screen retail fish for TEQ concentrations and will be a useful option for screening DL-PCBs in retail fish. In future assessments of the BIA technique, it will be necessary to analyze a greater number of retail samples in comparative studies.

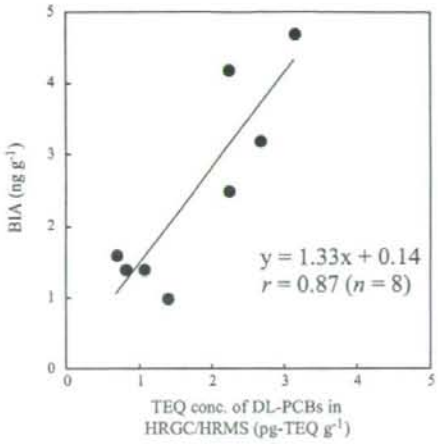

Fig. 5 – Comparison of BIA results and TEQ concentrations calculated by using the TEF_{2005} values. TEQ concentrations of DL-PCBs were calculated by using new TEFs (TEF_{2005}) and compared with the BIA results.

4. Conclusions

The BIA performed well in the analysis of PCB 118 in retail fish. When a 20-g fish samples was tested, the quantitative limit for PCB 118 was 1 ng per gram of sample. The assay was very quick and automated. A comparative study with conventional HRGC/HRMS showed that the BIA is suitable for the screening of DL-PCBs in retail fish. Thus, the BIA will be useful for the preliminary screening of large numbers of fish samples before HRGC/HRMS analysis.

Acknowledgement

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DIETARY EXPOSURE TO DIOXINS IN JAPAN: NATIONWIDE TOTAL DIET STUDY 2002-2006

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Introduction

Food is generally recognized as the main route of the human intake of polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofuranes (PCDFs) and dioxin-like polychlorinated biphenyls (dl-PCBs), which are known collectively as dioxins. It is therefore important to estimate the dietary intake of dioxins for risk assessments. A total diet study (TDS), also known as a market basket study, is a useful method for estimating the average dietary intake of contaminants. Therefore, we have been performing a nationwide TDS of dioxins in Japan annually since 1998. Here, we report the TDS result for fiscal year 2006 and also discuss the time trend of the dietary intake of dioxins from TDS results obtained over the last 5 years (fiscal years 2002-2006).

Materials and Methods

Sample preparations: TDS samples were prepared in 7 regions (Hokkaido, Tohoku, Kanto, Chubu, Kansai, Chugoku-Shikoku and Kyushu) across Japan. This involved 9 locations in fiscal year 2006 (12 locations in fiscal year 2002, 11 locations in fiscal year 2003 and 9 locations in fiscal years 2004 and 2005). More than one hundred food items were collected from supermarkets in each location. The TDS samples in these locations were designed based on official food classification and consumption data obtained by the National Health and Nutrition Survey in Japan. These food samples were cooked or prepared for consumption in typical ways. These samples were then blended to form 14 food group composites. Table 1 shows the classification of the 14 food groups and their daily consumption in Kanto, one of the 7 regions. The consumption pattern does not differ significantly from the consumption patterns in the other regions. We prepared three samples (shown as A, B and C in Table 1) in food groups 10–12. As far as possible, we chose different food items, such as different fish species and different edible parts of meat. This was because food items classified into these groups have a relatively wide range of dioxin concentrations[1]. Therefore, the choice of food items has a great influence on the estimated dioxin intake. With the remaining food groups (1–9, 13, and 14), the 7 regions were classified into five blocks, and composite food groups in each block were prepared by mixing regional samples according to regional consumption.

Dioxin analyses: The dioxins were extracted, purified and analysed according to the guideline for the analysis of dioxins in foods in Japan. In brief, all the samples were spiked with $^{13}C_{12}$-labelled internal quantification standards. Food groups 1, 2 and 5–8 (50 g) were twice extracted by shaking them with 50% acetone/*n*-hexane. The extracts were treated with concentrated sulphuric acid, and then purified on a silver nitrate/silica gel column, followed by an alumina column. The mono-*ortho* PCBs fraction and non-*ortho* PCB and PCDD/Fs fraction were separated on the alumina column. The former fraction was further purified by DMSO partition followed by a silica gel column. The latter fraction was further purified on an activated carbon-dispersed column. Both fractions were spiked with $^{13}C_{12}$-labelled recovery standards, and subjected to HRGC/HRMS equipped with a solvent-cut system with a large volume injector. Food groups 3, 4 and 9–13 (10–50 g) were digested with aqueous potassium hydroxide at room temperature. The alkaline hydrolysates were extracted with *n*-hexane. The extracts were treated with concentrated sulphuric acid, and then purified on a nitrate/silica gel column, followed by an activated carbon-dispersed column. The mono-*ortho* PCBs fraction and the non-*ortho* PCBs and PCDD/Fs fraction were separated on the activated carbon-dispersed column. The former fraction was further purified by DMSO partition followed by a silica gel column. The latter fraction was further purified on an alumina column. Both the fractions were spiked with $^{13}C_{12}$-labelled recovery standards, and subjected to HRGC/HRMS. Food group 14 (5 L) was filtered through a C18 disk, and then the disk was subjected to Soxhlet extraction with acetone followed by toluene. The extract was treated with concentrated sulphuric acid, and then purified on a nitrate/silica gel column, followed by an activated carbon-dispersed column. The fraction was spiked with $^{13}C_{12}$-labelled recovery standards, and subjected to HRGC/HRMS. The PCDD/Fs and non-*ortho* PCBs were

determined using an SP-2331 and DB-17 column. The mono-*ortho* PCBs were determined using an HT8-PCB column. The limits of detection (LODs) for PCDD/Fs were as follows: 0.01–0.05 pg/g in food groups 1–3 and 5–13; 0.05–0.2 pg/g in food group 4; 0.1–0.5 pg/L in food group 14. The limits of detection for dl-PCBs were as follows: 0.1–1 pg/g in food groups 1–3 and 5–13; 0.05–0.2 pg/g in food group 4; 1–10 pg/L in food group 14. The TEQ concentrations were calculated using WHO-TEFs (1998). The total TEQ in a sample was calculated assuming that all isomer concentrations lower than the LODs were equal to zero (ND=0) or half of the LODs (ND=1/2LOD).

Results and Discussion

Table 2 summarizes the daily dietary intakes of dioxins in the 7 regions in fiscal year 2006. The national mean intake calculated at ND=0 was 52.2 pg-TEQ/day, corresponding to 1.04 pg-TEQ/kg/day for an adult weighing 50 kg. The intake was about one fourth of the tolerable daily intake (TDI) of 4 pg-TEQ/kg/day set by the Japanese government in 1999[2]. The intakes in the 7 regions ranged from 0.38 to 1.94 pg-TEQ/kg/day. The maximum intake was still about half of the TDI. As the result of analysing three samples in each of the food groups 10–12, we found that there were 1.5– to 4.5–fold differences between the minimum and maximum intakes of dioxins in each region (shown as columns #1 and #3 in Table 2). This is mainly because the three samples of food group 10 (fish and shellfish) had a relatively wide range of dioxin concentrations owing to the different varieties of fish species used to prepare them. The dietary intakes calculated at ND=1/2LOD are also given as a reference. The national mean intake was 111.9 pg-TEQ/day (2.24 pg-TEQ/kg/day), which is about twice the mean intake calculate at ND=0. Recently, Sasamoto *et al.* carried out TDS in metropolitan Tokyo, Japan[3], and they reported that the daily dioxin intake was 1.55 pg-TEQ/kg/day at ND=0 (2.20 pg-TEQ/kg/day at ND=1/2LOD) in 2004. Their result is within the range of dietary intakes of dioxins obtained from the 7 regions in our study.

Table 3 shows the mean intakes of dioxins from each food group in fiscal year 2006. The dioxin intakes were highest from fish and shellfish (group 10) followed by meat and eggs (group 11), and milk and dairy products (group 12) at ND=0, and were highest from fish and shellfish (group 10) followed by beverages (group 9), and rice and rice products (group 1) at ND=1/2LOD. The TEQ contributions of the fish and shellfish group were noticeable in the total TEQs (about 90% at ND=0 and 43% at ND=1/2LOD). A much greater difference of intake was observed between the estimates obtained at ND=0 and ND=1/2LOD for beverages, and rice and rice products, because these food groups contain high percentages of non-detected data as well as having high daily consumptions.

Figure 1 shows the dioxin intakes obtained from our TDS results between fiscal years 2002 and 2006. The national mean intakes were within a range of 1.0 to 1.5 pg-TEQ/kg/day at ND=0, which is well below the Japanese TDI. Additionally, the maximum dioxin intakes observed during the same period were below the TDI, although some maximum intakes were near the TDI. The latest mean intake was the lowest value in the last 5 years. The mean intakes appeared to be decreasing slowly, although the each TDS sample had a wide range of dioxin intakes.

TEF values have been revised recently. WHO advises that the TEF_{2005} be used to replace the previous TEF_{1998}. The recalculated average intake using TEF_{2005} in fiscal year 2006 showed a decrease of about 15% in the intake calculated with TEF_{1998} (Figure 2). This is mainly because the TEF_{2005} of PCB 118 (0.00003) is slightly lower than that of TEF_{1998} (0.0001).

Thus, the estimated dioxin intakes in the last 5 years were below the Japanese TDI. However, in some cases, the maximum intakes were near the TDI, and so continuous monitoring of the dietary intake of dioxins is recommended.

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Table 1: Composition of 14 food groups in Kanto region (FY 2006)

No.	Food group[a]		No. of food stuffs	Daily consumption (g/day)
1	Rice and rice products		3	325.3
2	Cereals, seeds and potatoes		21	181.3
3	Sugars and confectioneries		9	35.7
4	Fats and oils		6	12.1
5	Pulses		7	55.3
6	Fruits		11	130
7	Green vegetables		8	106
8	Other vegetables, mushrooms and seaweed		21	206.7
9	Beverages		8	583.2
10	Fish and shellfish	A	26	85.8
		B	17	
		C	17	
11	Meat and eggs	A	12	116.1
		B	11	
		C	11	
12	Milk and dairy products	A	6	152.5
		B	6	
		C	6	
13	Other foods (seasoning)		16	94.2
14	Drinking water		1	600

[a] Three samples, shown as A, B and C, in food groups 10-12 were prepared using different food items as far as possible classified into each food group.

Table 2: Daily dietary intake of dioxins in Japan (FY 2006)

Region		Dioxin intake (pg-TEQ/day)[a]							
		ND=0				ND=1/2LOD			
		#1	#2	#3	Mean	#1	#2	#3	Mean
Hokkaido		18.9	22.3	85.4	42.2	78.2	81.9	140.7	100.3
Tohoku		26.3	53.1	92.5	57.3	83.6	108.0	145.4	112.3
Kanto	I	29.8	46.8	73.4	50.0	87.5	103.1	129.2	106.6
	II	39.4	50.1	69.0	52.8	97.6	107.9	126.0	110.5
Chubu	I	33.6	43.6	50.2	42.5	92.1	101.9	108.1	100.7
	II	23.2	35.1	62.1	40.1	84.6	95.9	122.0	100.8
Kansai		49.0	74.8	88.2	70.7	116.6	140.9	153.2	136.9
Chugoku-Shikoku		46.7	53.9	97.2	65.9	108.8	116.0	159.2	128.0
Kyushu		30.7	32.5	82.6	48.6	93.6	95.8	142.9	110.8
National mean	pg-TEQ/day	52.2 (18.9 - 97.2)				111.9 (78.2 - 159.2)			
(min - max)	pg-TEQ/kg/day[b]	1.04 (0.38 - 1.94)				2.24 (1.56 - 3.18)			

[a] Dioxin intakes in each region are shown in three different ways: #1, #2 and #3 were calculated by using minimum, median and maximum intakes, respectively, of food groups 10-12 in each region.

[b] Assuming an adult weight of 50 kg.

Table 3: Daily dietary intakes of dioxins from each food group (FY 2006)

No.	Food group	Dioxin intake (pg-TEQ/day)			
		ND=0		ND=LOD/2	
		Mean	Ratio (%)	Mean	Ratio (%)
1	Rice and rice products	< 0.1	< 0.1	13.0	11.6
2	Cereals, seeds and potatoes	0.3	0.5	7.1	6.3
3	Sugars and confectioneries	0.1	0.2	1.3	1.2
4	Fats and oils	0.1	0.1	1.5	1.3
5	Pulses	0.0	< 0.1	1.7	1.5
6	Fruits	< 0.1	< 0.1	3.4	3.1
7	Green vegetables	0.1	0.1	2.6	2.3
8	Other vegetables, mushrooms and seaweed	0.1	0.1	5.6	5.0
9	Beverages	< 0.1	< 0.1	14.4	12.8
10	Fish and shellfish	47.0	90.0	47.9	42.8
11	Meat and eggs	3.5	6.7	5.7	5.1
12	Milk and dairy products	1.1	2.0	4.9	4.3
13	Other foods (seasoning)	0.1	0.1	2.6	2.3
14	Drinking water	< 0.1	< 0.1	0.2	0.1
	Total	52.2	100.0	111.9	100.0

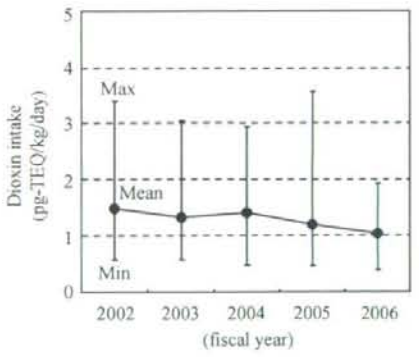

Figure 1: Time trend for dietary intake of dioxins (ND=0)

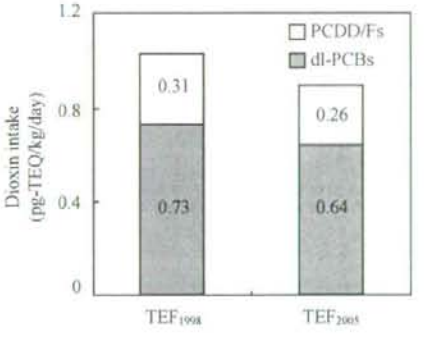

Figure 2: Comparison of dioxin intakes calculated using TEF_{1998} and TEF_{2005} (FY 2006)

HEXABROMOCYCLODODECANES IN MARINE PRODUCTS COLLECTED FROM FOUR REGIONS OF JAPAN

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Introduction

The Japan domestic consumption of hexabromocyclododecane (HBCDs) has recently increased as it is used to replacce or supplement other brominated flame retardants (BFRs). The demand in 2004 was reported to be 35,000 tons for tetrabromobisphenol A (TBBPA), 2,000 tons for decabrominated diphenyl ether (OCDE) and 2,600 tons for HBCDs. HBCDs are extraordinarily residual and accumulative in aqueous mammals, however, there is still little information about the extent of human exposure. At Dioxin 2007, we reported that the Japanese populace would be exposed to HBCDs mostly via fish among the market basket food group samples investigated [1]. Therefore, it is critical to clarify the status of seafood pollution by HBCDs. We determined the levels of α-, β-, and γ-HBCDs in fish (natural feeding habitat) collected from four regions of Japan and compared them with the previously reported levels of TBBPA and PBDEs[2], from which the harmful brominated dioxins could be generated during the formulation process of BFRs and when recycled plastic waste containing BFRs is burned.

Materials and Methods

Fish samples: Forty-three fish samples belonging to 33 kinds having natural feeding habitats were collected from four regions of Japan (Kyushu, Seto Inland Sea, Nagoya and Tohoku) between 2004 and 2005. Each of homogenized fish sample was prepared as to weigh 200-300 g with edible parts of single or plural fish caught at the same time and location. Samples were stored below -20 ° C until analysis.

Sample preparation: Five grams of a homogenized sample with an addition of $^{13}C_{12}$ -α-,β- and γ-HBCD were extracted twice with 20mL of dichloromethane (DCM) using a POLYTRON®. The extracts were dried over anhydrous sodium sulfate and concentrated. The residue was dissolved in 10% DCM/n-hexane and was treated twice with 5mL of sulfuric acid. After centrifuging at 2000 rpm, the upper hexane layer was collected and evaporated. The residue was dissolved in 0.2mL of acetone, and half of this solution was subjected to GPC. HBCD was fractionated over 12 to 14 min after large molecules such as crude fatty acids were eluted in 10 to 12 min. The fraction was re-purified with a cartridge mini-column (Varian BOND ELUT-PSA, 500mg) and then was reconstituted to 50μL using methanol. For determination of HBCDs, a Waters Quatro Micro API was used in the ESI negative operation mode. The HPLC column was a 150mm x 2.1mm i.d. 5μ Inertsil ODS-3 (GL-Science, Tokyo). The detection limit of both α- and γ-HBCD was 0.02 ng/g ww. That for β-HBCD was 0.01 ng/g ww.

Results

HBCD levels in fish collected from four regions of Japan

In this study, fish that have natural feeding habitats and are available at market stores were investigated for HBCD levels. Most of the fish samples in this study belonged to a relatively small fish category, and the others were mollusks (cuttlefish and octopus). From the view point of guaranteeing a precise analysis, the recoveries of

the objectives and the negative controls were checked though the protocol. The recoveries of spiked ^{13}C-labeled HBCDs were obtained at above ca 40 %.

The fish species differed among the regions. However, every fish monitored in this study was familiar to the Japanese populace.

Figure 1 showed the distribution of the levels of ΣHBCDs and ΣPBDEs in the samples for each region.

The median levels of detected ΣHBCDs were 0.04ng/g ww (1.4ng/g lw) for the Kyushu (K) region < 0.06 ng/g ww, (11 ng/g lw) for the Seto (S) region <2.9 ng/g ww (150 ng/g lw) for the Nagoya (N) region<3.6 ng/g ww (170 ng/g lw) for the Tohoku (T) region (Table 1). In the K region, the extent of HBCD pollution is generally considered to be small. In the S region, one extremely polluted sample (small herring) was found and in the N region, three polluted samples (two seer fish and sea bass) were found. In those fish samples, γ-HBCD was dominantly or highly detected more than α- and β-HBCDs. Γ-HBCD is reported to be the main ingredient in HBCD formula (83.9 %)[3], while α- and β-HBCD are reported to be minor ingredients at 8.5% and 7.9%, respectively. Therefore, the highly polluted fish sample would have been intensively influenced by a neighboring heavy pollution source. For the N and T regions, the detection rates of ΣHBCDs in fish samples were both 100 %, and the level range of ΣHBCDs were higher than in the K and S regions.

On the other hand, referring to our previous report, the median levels of ΣPBDEs were 0.10 ng/g ww for the K region < or = 0.11 ng/g ww for the S region <0.33 ng/g ww for the N region and <0.37 ng/g ww for the T region. With the exception of the T region of which the sample number (n=5) was small, the difference in the medians of ΣHBCDs and ΣPBDEs between the K and N regions were 73 and 3.3 times, respectively. Between the S and N regions, these differences were 48 and 3 times. Therefore, on near coast of Japan, HBCD pollution of fish is considered to be increasing, particularly in the N region, which is a commercialized and industrial area. Due to their large bioconcentration factor, HBCDs should be received more attention than PBDEs and TBPPA.

Statistical analysis of ΣHBCDs in all the fish

Figure 2 shows the correlations among ΣHBCDs, ΣPBDEs and fat content in 39 samples excepting 4 samples considered to be affected by a nearby pollution source. The Spearman's rank correlation coefficients were 0.9809 between ΣHBCDs and ΣPBDEs and 0.9432 between ΣHBCDs and fat content. By a one-side test of both coefficients, it was estimated that positive correlations would exist among them at $P<0.01$. However, between TBBPA and ΣHBCDs there was no correlation (data not shown).

Discussions

An U.K. research report on the levels of HBCDs in the blubber of harbor porpoises demonstrated that there is a time trend of pollution from PBDEs to HBCDs[4]. However, there are few data on the pollution trend in Japan, where the domestic consumption of ΣHBCDs was announced as 2,600 tons in 2004 exceeding that of DeBDE (2,000 tons). On the other hand, PBDEs have been identified as related chemicals of polybrominated dioxins (PBDDs/DFs), because they could be generated unintentionally during the formulation of PBDEs or the burning of plastics and textiles to which PBDEs are added. The transfer of PBDEs to HBCDs in BFR use might contribute to the decrease of the emission of PBDDs/DFs into the environment. For that purpose, HBCDs would be acceptable. Recently, the non-observed adverse effect level (NOAEL) of HBCD has been reported to be 10.2 mg/kg/day, which means that the use of HBCDs is safe at present. However, HBCDs are too accumulative to allow entering the environment without further attention. Considering the matter of epigenesis in humans, intensive HBCD pollution in fish should also be avoided and it is necessary to continue the monitoring of

HBCDs in fish and food as is done for other harmful residual chemicals.

Acknowledgements

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Table 1 HBCD levels of marine products from four regions of Japan

Region		Fat (%)	α-HBCD ng/g ww	β-HBCD ng/g ww	γ-HBCD ng/g ww	ΣHBCDs ng/g ww	ΣHBCDs ng/g lw
K	Median	1.2	0.04	ND	ND	0.04	1.4
	Max	20.4	0.73	ND	0.27	1.0	40
	Min	0.2	0.00	ND	ND	ND	ND
S	Median	0.9	0.05	ND	ND	0.06	11
	Max	12.7	18.3	2.4	57	77	1700
	Min	0.3	0.00	ND	ND	ND	ND
N	Median	1.3	1.94	ND	1.1	2.9	153
	Max	13.7	14	0.35	18	24	3300
	Min	0.3	0.07	ND	0.08	0.15	12
T	Median	2.4	2.1	ND	1.5	3.6	170
	Max	3.4	3.2	ND	2.3	5.5	230
	Min	1.3	1.2	ND	0.80	2.2	110

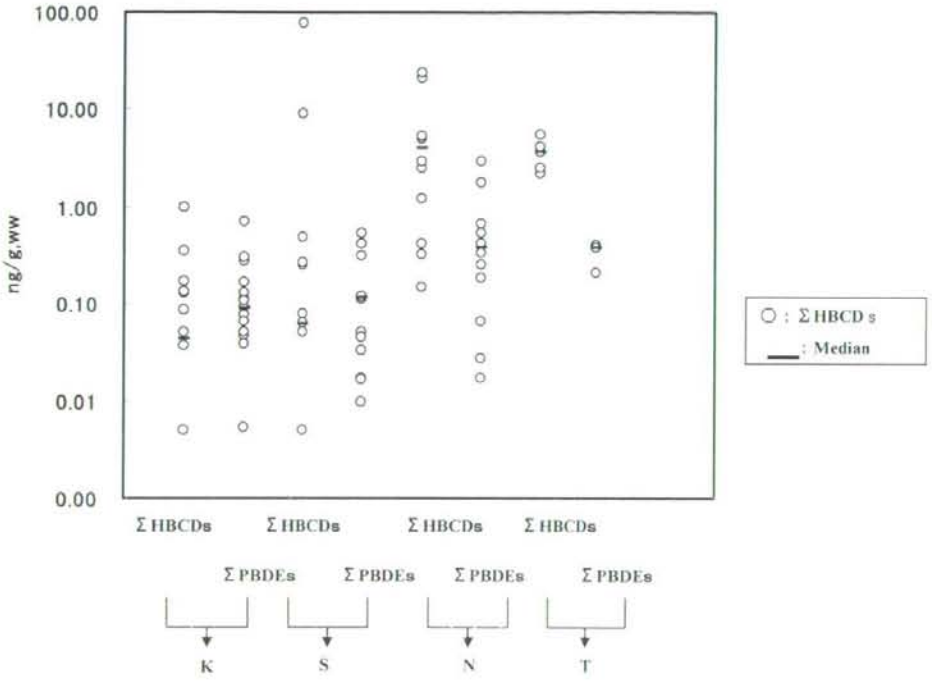

Fig.1 Distribution of ΣHBCDs and ΣPBDEs in marine products from four regions of Japan

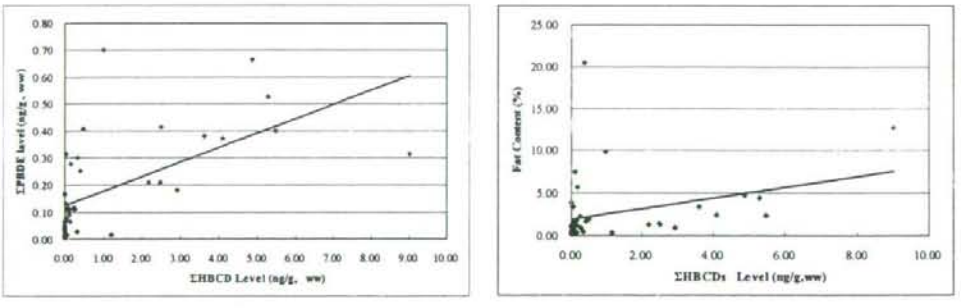

Fig.2 Correlations between ΣHBCDs and ΣPBDEs and between ΣHBCDs and fat content (%)in marine products in Japan

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Review

Influence of food polyphenols on aryl hydrocarbon receptor-signaling pathway estimated by *in vitro* bioassay

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Abstract

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates the toxic and biological actions of many aromatic environmental pollutants such as dioxins. We investigated AhR activation by some vegetable constituents, including flavonoids, tannins, and related polyphenols, using an AhR-based *in vitro* bioassay for dioxins. Among the compounds tested, marked AhR activation was exhibited by isoflavones such as daidzein, resveratrol (a stilbene) structure, some flavanones such as naringenin, and flavones such as baicalein. On the other hand, some flavones such as apigenin, flavonols such as quercetin, and anthraquinones such as emodin, showed notable inhibitory effects on the *in vitro* activation of AhR induced by the dioxin [2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)]. In addition, AhR-mediated interactions between AhR and some plant extracts, including those from vegetables, fruits, herbs, and teas, were tested by using the AhR-based bioassay. Of the samples tested, some leafy green vegetables, citrus fruits, and herbs that contain food polyphenolics showed AhR-based interactions at high concentrations. On the basis of these finding, we discuss the implications of polyphenols on the AhR-signaling pathway.

Keywords: Polyphenol; Aryl hydrocarbon receptor; Vegetable food; *In vitro* bioassay; Dioxin

Contents

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1. Introduction

The aryl hydrocarbon receptor (AhR), which is also referred to as a dioxin receptor, is a basic helix–loop–helix (bHLH)- and Per-Arnt-Sim (PAS)-containing transcription factor. It is present in numerous animal species, including humans and tissues, and activates gene expression in a ligand-dependent manner (Schmidt and Bradfield, 1996; Ma, 2001; Denison et al., 2002; Mimura and Fujii-Kuriyama, 2003) (Fig. 1a). The prototype ligand is known as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an archetypal dioxin known as one of the most potent congeners. Other known ligands are environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs) and persistent organochlorine pollutants (POPs), including polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) (Safe, 1986, 1990; Whitlock, 1993; Denison et al., 2002). Unliganded AhR is present in the cytosol of most cells, forming a complex with a dimer of 90-kDa heat shock protein (Hsp90), an X-associated protein 2 (XAP2), and a 23-kDa co-chaperone protein (p23). Ligand binding to AhR leads to nuclear translocation, followed by release of its associated protein subunits (Hsp90, XAP2, and p23) and heterodimerization with the AhR nuclear translocator protein (Arnt). This AhR/Arnt heterodimer binds to DNA sequences, called xenobiotic responsive elements (XRE), which are distributed in the enhancer regions of dioxin-responsive genes, and regulate the expression of target genes including drug-metabolizing enzymes, such as cytochrome P450 (CYP) 1A1. Accordingly, this DNA interaction is highly correlated with the initial step of subsequent toxicity events including carcinogenicity, developmental and reproductive toxicity, and immunological impairment that are known as dioxin toxicity effects (Landers and Bunce, 1991; Poellinger, 2000; Denison and Nagy, 2003; Mimura and Fujii-Kuriyama, 2003; Mandal, 2005; Schwarz and Appel, 2005) (Fig. 1b).

The lack of TCDD toxicity in AhR knockout mice, along with the ability of AhR to act as a ligand-dependent transcription factor, indicated that AhR mediates the toxic and biological effects of TCDD (Mimura et al., 1997). Recently, research on the structure and physiological functions of AhR cell cycle regulation has been reported (Bock and Köhle, 2006; Harper et al., 2006; Pandini et al., 2007; Goryo et al., 2007), and the characterization of AhR has gradually been clarified. However, AhR is still relatively poorly understood, because its physiological ligand, mechanisms, and functions remain largely unknown. The present functional role of AhR was derived mainly based on studies using environmental contaminants such as dioxins. Environmental contaminants that are prototype AhR ligands are artificial products that have appeared recently. Therefore, AhR might primarily function in human health

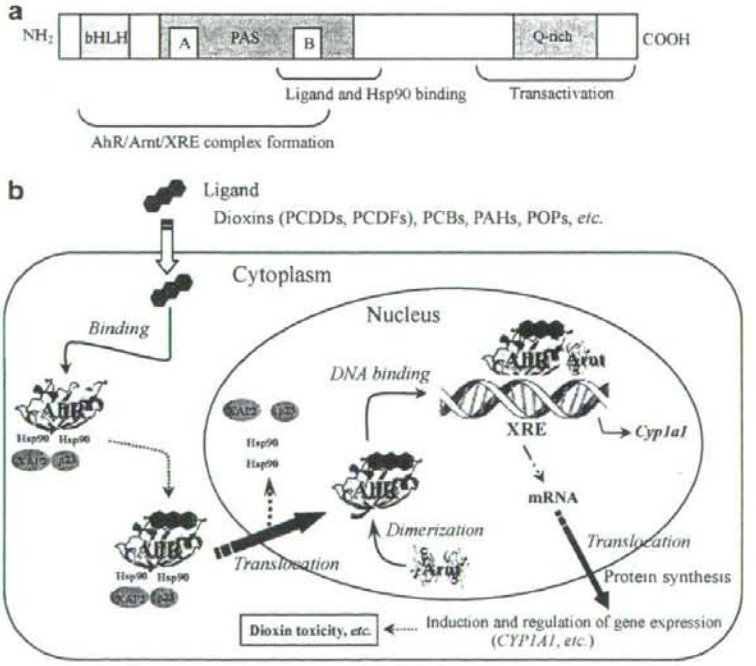

Fig. 1. (a) Domain structure of the aryl hydrocarbon receptor (AhR). bHLH, basic helix–loop–helix; PAS, Per-Arnt-Sim domain; Q-rich, glutamine rich region; Arnt, AhR nuclear translocator protein; XRE, xenobiotic-response element. The PAS domain contains two structural repeats (PAS A and PAS B). (b) Mechanistic model of the AhR signaling pathway. See text for detailed description.

as a regulatory receptor for exogenous natural products such as food constituents.

To better understand AhR's inherent physiological role and significance, information on the interactions between it and compounds such as food constituents related to people's lives is needed, and more fundamental data is required. Recently, numerous investigations have been carried out to search for the agonists and antagonists of AhR contained in natural products rather than in environmental contaminants (Denison et al., 2002; Denison and Nagy, 2003). Regarding AhR agonists, AhR transformation has reportedly been induced by the following: tryptophan and its metabolites (Heath-Pagliuso et al., 1998), carotenoids (β-apo-8′-carotenal, canthaxanthin, and astaxanthin) (Gradelet et al., 1997), berberine (Vrzal et al., 2005), indole-3-carbinol (Bjeldanes et al., 1991; Chen et al., 1996), bilirubin, biliverdin (Phelan et al., 1998), flavonoids (Ashida et al., 2000), and others. Recently, tunicamycin, a well-known antibiotic, has been identified as an activator of the AhR-XRE signal pathway (Horikawa et al., 2006). Indigo and indirubin were also shown to be endogeneous AhR agonists present in human urine (Adachi et al., 2001; Sugihara et al., 2004). The AhR agonist activity of the extracts of dietary herbal supplements, vegetables, and fruits were also assessed (Jeuken et al., 2003). AhR agonists are noted as physiological regulatory factors, while the toxicity of a dioxin-like compound is doubtful.

The following have been reported to function as AhR antagonists that involve inhibition of the AhR-signaling pathway: flavonoids (apigenin, luteolin, kaempferol, quercetin, galangin, etc.) (Reiners et al., 1999; Ciolino et al., 1999; Ciolino and Yeh, 1999; Ashida et al., 2000; Fukuda et al., 2004; Ishida et al., 2005; Hamada et al., 2006), catechins (Williams et al., 2000; Ashida et al., 2000; Fukuda et al., 2004), curcumin (Ciolino et al., 1998a), resveratrol (Ciolino et al., 1998b; Casper et al., 1999), and lutein (Fukuda et al., 2004). Recently, the suppressive effects of anthocyanidins and/or anthocyanins and extracts of black tea, molokhia, and propolis on the dioxin-induced transcription of AhR have also been investigated (Park et al., 2004, 2005; Fukuda et al., 2005; Mukai et al., 2005; Nishiumi et al., 2006). These studies suggest that AhR antagonists might protect against dioxin toxicity.

This review summarizes our recent investigations, which include the interaction between polyphenol constituents and AhR as determined by *in vitro* bioassay. The influence of polyphenols on the AhR-signaling pathway with regard to human health are also discussed.

2. AhR activation by polyphenol constituents determined using *in vitro* bioassay

The *in vitro* AhR-inducing potencies of plant constituents, mainly polyphenol compounds, that are present in vegetables, fruits, teas, and herbs (Amakura et al., 2003a) were investigated. For identification of AhR-activating compounds, an *in vitro* reporter gene assay, called the chemical activated luciferase gene expression (CALUX) assay (Denison et al., 1998), was used. The mechanistic outline of the assay is depicted in Fig. 2a. This assay, which uses mouse hepatoma cells (Hepa lclc7) containing a stably transfected AhR-responsive luciferase reporter gene, detects dioxin-like compounds based on their ability to activate AhR. Since the response for a sample containing dioxin-like compounds can be correlated with dioxin levels in the CALUX assay, this assay has recently been applied as an alternative screening method to determine dioxin levels (Tsutsumi et al., 2003). With this assay, TCDD showed an appreciable, dose-dependent increase in luciferase activity (Fig. 3). The concentrations of a test compound producing luciferase activity equal to 25% and 50% of the maximal response to TCDD were calculated and expressed as EC_{TCDD25} and EC_{TCDD50}, respectively. EC_{25} and EC_{50} of TCDD were determined to be 1.3×10^{-5} and 3.0×10^{-5} μM, respectively. Dose–response curves plotted

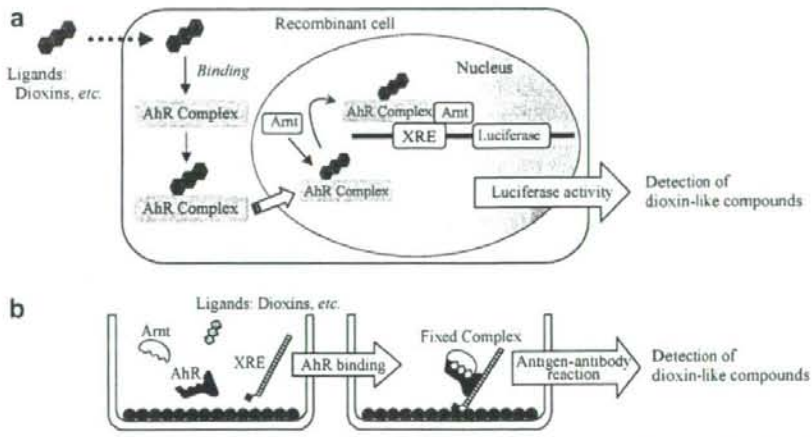

Fig. 2. Outline of mechanistic models of AhR-mediated *in vitro* bioassay used in this study. (a) CALUX assay, (b) Ah-immunoassay.