研究成果の刊行に関する一覧表

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雑誌

発表者氏名	論文タイトル	発表誌名	巻号	ページ	出版年
1. Amakura, Y., Tsutsumi, T., Nakamura, M., Handa, H., Yoshimura, M., Matsuda, R., Yoshida, T.	Tectochrysin in propolis is a potent natural aryl hydrocarbon receptor ligand	*Planta Medica,*	76	1380	2010
2. Amakura, Y., Tsutsumi, T., Nakamura, M., Handa, H., Yoshimura, M., Matsuda, R., Yoshida, T.	Aryl hydrocarbon receptor ligand activity of commercial health foods	*Food Chemistry*	126	1515-1520	2010
3. Ashizuka, Y. , Nakagawa, R. , Yasutake, D. , Shintani, Y. , Hori, T. , Horie, M. , Tanaka, Y. , Tsutsumi, T.	Daily intake of brominated dioxins, Co-PCBs and brominated flame retardants estimated from market basket study	BFR2010			2010
4. Nakagawa, R. , Murata, S., Ashizuka, Y., Shintani, Y., Hori, T., Tsutsumi, T.	Hexabromocyclododecane determination in seafood samples collected from Japanese coastal areas.	Chemosphere	81	445-452	2010

separate cages throughout the gestational period and were acclimatized and fed in the same conditions. Animals in all group received tape water in whole gestational period. In 20th day of pregnancy, animals were anesthetized and their foetuses were extracted through a cesarean section. The placenta was excised, weighed, and the number and placement of implantation sites and other parameters were recorded. Mean number of live, dead or resorbed foetuses in animals receiving saffron extracts before the mating were dose dependently less than in the control group. A maximum decrease was observed in animals receiving 0.8% saffron solution. Saffron and its components may affect embryonic implantation and may result in contraceptive-like effects. References: 1. Rios J.L. et al. (1996) Phytother Res 10: 189 – 193.

P676 Tectochrysin in propolis is a potent natural aryl hydrocarbon receptor ligand

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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. As part of an investigation to clarify the interaction of foods with AhR, we previously reported that excessive intake of foods containing AhR-activators may be conductive to promote dioxin-like toxicity though there would not be a problem following normal intake. Additionally, it is discussed that the signal transduction of natural AhR ligands, which occurs after AhR activation, should differ from that of dioxins [1]. In this study, we examined the binding ability of 50 extracts prepared from kinds of commercial supplements and health foods to the AhR using reporter gene assay. Though most tested samples did not show any luciferase induction at a high concentration level, propolis product showed activation of luciferase at high concentration range. To characterize the AhR-activating substances in the propolis product, its extract was subjected to fractionation with *n*-hexane, ethyl acetate, and water followed by estimation their AhR activities. HPLC analysis of the *n*-hexane fraction which showed AhR activity detected eight compounds including flavonoids such as tectochrysin and pinocembrin. Among their compounds, tectochrysin showed remarkable AhR activation. Recently, several papers reported that AhR activation may be involved in various immune system [2 – 4]. Therefore it is suggested that natural AhR ligands characterized in the present study may play some beneficial regulatory role in human. Acknowledgements: This work was supported by a Health and Labour Sciences Research Grant from the Ministry of Health, Labour and Welfare of Japan. References: 1. Amakura, Y et al. (2008) Phytochemistry. 69: 3117 – 3130. 2. Quintana, FJ et al. (2008) Nature. 453: 65 – 71. 3. Veldhoen, M et al. (2008), Nature. 453: 106 – 110. 4. Kimura, A et al. (2007), Proc. Natl. Acd. Sci. USA. 105: 9721 – 9726.

P677 Effects of polyphenols: Resveratrol and its natural analogues and tannic acid on DNA oxidative damage and apoptosis in human neutrophils

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Our earlier studies (1,2) showed that tannic acid and naturally occurring stilbenes resveratrol and its analogue pterostilbene, the common ingredients of berries fruits, are involved in the key events of the initiation and promotion stages of carcinogenesis. The latter included the inhibition of transcription factors responsible for the inflammatory response. The aim of this study was to evaluate the effect of these compounds on DNA oxidative damage and apoptosis in human polymorphonuclear neutrophils (PMN). In order to induce oxidative burst PMN were stimulated with 12Otetradecanoylphorbol13acetate. Treatment of PMN with the tested polyphenols at the concentration range which did not show cytotoxicity resulted in the reduced production of reactive oxygen species and subsequently DNA oxidative damage assessed by Single Cell Gel Electrophoresis (comet assay). Tannic acid caused the 50% decrease in DNA oxidative decomposition at the concentration as low as 1 µmol. Resveratrol caused a similar effect at a concentration 100 times higher. All tested polyphenols induced apoptosis by increasing the activity of procaspase3, phosphatidylserine translocation and loss of mitochondrial membrane potential. The highest proapoptotic activity was demonstrated by 3'5,4'trimethoxystilbene and tannic acid. This effect however was dependent on the dose. At the higher concentrations (50µmol) an antiapoptotic effect was observed. Collectively ROS production in activated PMN seems to influence their lifespan and can be modulated by stilbenes and phenolic acids. Such activity might be useful in adjuvant therapy of inflammatory diseases. References: 1. Cichocki, M. et al. (2008) Mol.Nutr.Food Res. 52: S62-S70. 2. Cichocki, M. et al. (2010) Toxicology 268: 118 – 124.

Poster Young Researcher

P678 *In Vitro* multiplication used in preserving the *Arnica montana* L. species in the Romanian Bistrita Mountains

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Arnica montana is considered a rare and vulnerable species and is intensively harvested in Romania for medicinal aims. In the natural areas of the Romanian Bistrita Mountains, the preservation of the species is important by extending in this zone the results of the Conservation of Estern European Medicinal Plants – *Arnica montana* in Romania [3]. In the present paper we intend the possibility of using *in vitro* multiplication as a complementary component of the strategy to initiate and develop the species preservation in the mentioned area. Being an ever rarer species, we studied and elaborated diverse micropropagation techniques [1 – 2]. The final aim of the study is the reintroduction of the species in the montane grasslands where the species has been fully disappeared, and the introduction of new crops (genes) in small, endangered populations (to increase the heterozys), especially because the micropropagation techniques provides the advantage of a better control of the genetic material, and a relative large number of crops in a short period of time. For the *in vitro* regeneration, the biological material used to initiate the experimental cultures, originates from *Arnica montana* plantlets resulted after the germination of seeds from a natural population in the aimed area. Shoot formation was induced from excised shoot tip explants, on the Murashige and Skoog (MS, 1962) multiplication medium, supplemented with BA (1 mg/l) and ANA (0.1 – 0.3 mg/l) and solidified with 0.8% agar, we obtained a number of 2 – 3 neoplantlets/explant. The multiplication of the buds was followed by the rooting phase, the neoplantlets being transferred for *ex vitro* adaptation, 75 – 80% being the survival ratio. The plants obtained will be transferred, after invigoration, to the experimental field from the natural habitat. The succession of the *in situ* preservation techniques and the *ex situ* ones will be accompanied by comparative phytochemical screening of the generatively obtained variants as well as by *in vitro* multiplication. Acknowledgements: The work is sustained in the PNCDI-2 program financed by the Romanian Government – National R&D Agency References: 1. Butiuc-Keul A.L., Deliu C., Clonal propagation of *Arnica montana* L., a medicinal plant, *In Vitro* Cell Dev. Biol. – Plant (2001), 37, 581 – 585. 2. Cassells A.C., Walsh C., Belin M., Cambornac M., Robin J.R., Lubrano C., Establishment of a plantation from micropropagated *Arnica chamissonis* a pharmaceutical substitute from the endangered A. montana, Plant Cell, Tissue and Organ Culture (1999) 56, 139 – 144. 3. Wolfgang Kathe, Conservation of Estern – European Medicinal Plants – *Arnica montana* in Romania, Medicinal and Aromatic Plants (2006), 203 – 211.

P679 Inhibitory effects of *Cissus barbeyana* leaf extracts on α-amylase and α-glucosidase *in vitro*

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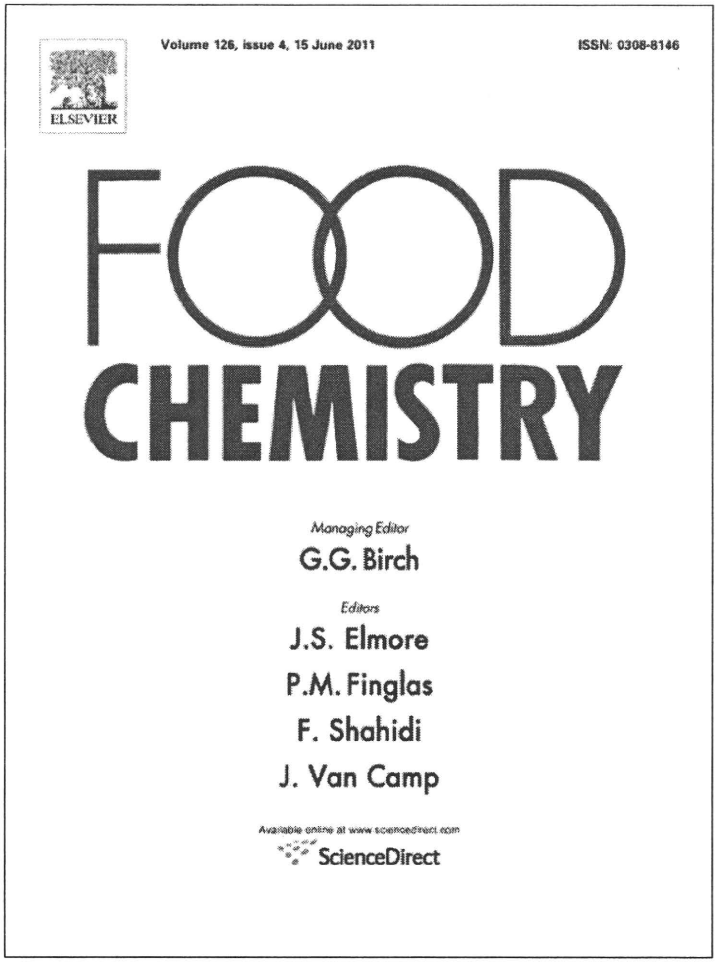
α-amylase and glucosidase inhibitors are used in the therapeutic management of type II diabetes mellitus. Inhibition of pancreatic α-amylase



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Rapid Communication

Aryl hydrocarbon receptor ligand activity of commercial health foods

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ABSTRACT

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates toxicological effects by binding to agonists such as dioxins. We previously reported the presence of natural dioxin-like ligands in foods. To further characterise natural ligands with dioxin-like activity, we examined the influence of 50 kinds of commercial supplement and health food on the AhR, using a reporter gene assay. Some samples, prepared using soybean, sesame, or propolis as an ingredient, were revealed to show AhR-binding activity, similar to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), at high concentrations. To characterise the AhR-activating substances in eight active samples, the respective extracts were subjected to fractionation with *n*-hexane, ethyl acetate, and water, followed by estimating their AhR activities. The *n*-hexane fraction of the propolis extract sample, and the ethyl acetate fractions of the other samples, showed AhR activity similar to that of TCDD, at a high concentration range. HPLC analysis of the active fractions identified isoflavones, such as daidzein and glycitein, and flavones, such as tectochrysin and chrysin, in the samples. Among these compounds, tectochrysin exhibited marked AhR activation. Flavonoids, which are characterised as natural AhR ligands, are known to have representative beneficial effects on human health. The natural AhR ligands identified in this study are known to be useful for human health. Therefore, it is considered that AhR may play a beneficial regulatory role in humans.

1. Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that is present in mammalian cells or tissues. This receptor is also called a dioxin receptor because it binds to environmental pollutants (e.g., dioxins) and is involved in the manifestation of biotoxicity linked to xenobiotic AhR ligand exposure in animals, including cancers, reproductive impairment, and immunological impairment (Fujii-Kuriyama & Mimura, 2005; Machala, Vondráček, Bláha, Ciganek, & Neča, 2001; Nebert & Dalton, 2006). Although numerous xenobiotic ligands for AhR, such as dioxins, have been identified, the essential functions of AhR are largely unknown; therefore, AhR is still regarded as an orphan receptor. Natural AhR agonists and antagonists have been suggested to exist in foods in small amounts (Ashida, Fukuda, Yamashita, & Kanazawa, 2000; Jeuken et al., 2003). Moreover, we previously reported the presence of natural dioxin-like ligands in foods (Amakura et al., 2003, 2004, 2008). These agonists have been demonstrated to upregulate an AhR reporter gene at high concentrations compared with dioxins. Although normal meals are unlikely to represent a problem, the influence on health of natural AhR ligands in foods containing concentrated extracts, such as supplements, is of concern. Therefore, it is necessary to elucidate the actual characteristics of supplements in relation to AhR.

A simple technique to measure dioxins, using a biological assay based on the toxicity mechanism of dioxin (AhR-binding activity), has been established and recognised as the standard method for measuring environmental samples in Japan (JIS K0463, 2009). This biological assay provides a rapid and cost-effective method for screening. In contrast to instrumental analysis by conventional high-resolution GC/MS, which analyses each isomer of dioxins separately and integrates data, the biological assays provide only a comprehensive value. Compared with environmental samples, dioxins can be detected only in small amounts in ordinary food samples (Toyoda et al., 1999; Tsutsumi et al., 2001). Thus, when a biological assay is applied to the analysis of dioxins in foods, reliable data can be ensured by identifying and removing impurities exhibiting dioxin-like activity. However, few substances with such activity have been identified in foods, and further information is required on natural AhR-ligand-containing foods. Therefore,

Abbreviations: AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; CALUX, chemical activated luciferase gene expression.

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Table 1
List of supplements and health foods used for the estimation of AhR binding activity.

No.	Main materials	Type	No.	Main materials	Type
1	Agaricus mushroom	T	26	Propolis extract	C (S)
2	Amino acid/vitamin/mineral	T	27	Royal jelly extract	C
3	Astaxanthin	C (S)	28	Saw palmetto extract	C (S)
4	Barley grass (A)	P	29	Sesame	C (S)
5	Barley grass (B)	P	30	Soy isoflavone (A)	C (S)
6	Bilberry extract	C (S)	31	Soy isoflavone (B)	T
7	β-Carotene	T	32	Soy isoflavone (C)	T
8	Cassis extract	T	33	Soy isoflavone (D)	T
9	Chitosan	T	34	Soybean extract (A)	C (S)
10	Coenzyme Q10	C (S)	35	Soybean extract (B)	T
11	Fish oil (A)	C (S)	36	Tea (Chinese sweet tea)	TM
12	Fish oil (B)	C (S)	37	Tea (Echinacea)	TM
13	Garcinia extract	T	38	Tea (Eucommia)	TM
14	Ginkgo leaf extracts	T	39	Tea (Guava)	TM
15	Gymnema extract	T	40	Tea (Japanese mugwort)	TM
16	*Gymnema sylvestre* leaf extract	T	41	Tea (Loquat leaf)	TM
17	Haematococcus color	C (S)	42	Tea (Perilla herb)	TM
18	Kidachi aloe (pulp of *Aloe arborescens*)	T	43	Tea (Persimmon leaf)	TM
19	Lutein (A)	C (S)	44	Tea (Rooibos)	TM
20	Lutein (B)	C (S)	45	Turmeric	T
21	Maca extract	C	46	Turmeric extract (A)	C (S)
22	Onion extract	T	47	Turmeric extract (B)	T
23	Plant steroid	C (S)	48	Urazirogashi (leaf of *Quercus salicina*)	T
24	Processed vegetable (A)	T	49	Vitamin C	T
25	Processed vegetable (B)	T	50	Vitamins	C (S)

T, Tablet; C, Capsule; C (S), Soft Capsule; P, Powder; TM, Tea material.

accumulation of basic data is indispensable for ensuring the reliability of rapid measurements employing a biological assay.

In order to further characterise AhR ligands present in foods, we herein examined AhR activity, using an *in vitro* reporter gene assay called the chemical activated luciferase gene expression (CALUX) assay (Misaki et al., 2007; Overmeire et al., 2001), which has been applied for the rapid measurement of dioxins in food samples (Tsutsumi et al., 2003, 2006, 2008), for 50 kinds of supplement and health foods containing a high concentrations of their respective ingredients. Active sample extracts were subsequently fractionated, and reversed-phase HPLC analysis was conducted to characterise the AhR active fractions.

Table 2
Relative responses of the CALUX assay for selected supplements and health food samples.

Sample No.	$TCDD_{0.5}$[a] (mg/mL)
26	2.3
29	7.6
30	5.6
31	1.8
32	19.0
33	1.4
34	28.0
35	16.5
41	56.6
42	54.5

Each value is the mean of at least three replicates.
[a] Concentration producing luciferase activity equal to 0.5 ng/mL of TCDD. Calculated from the slope of the linear portion of each dose–response curve near the origin.

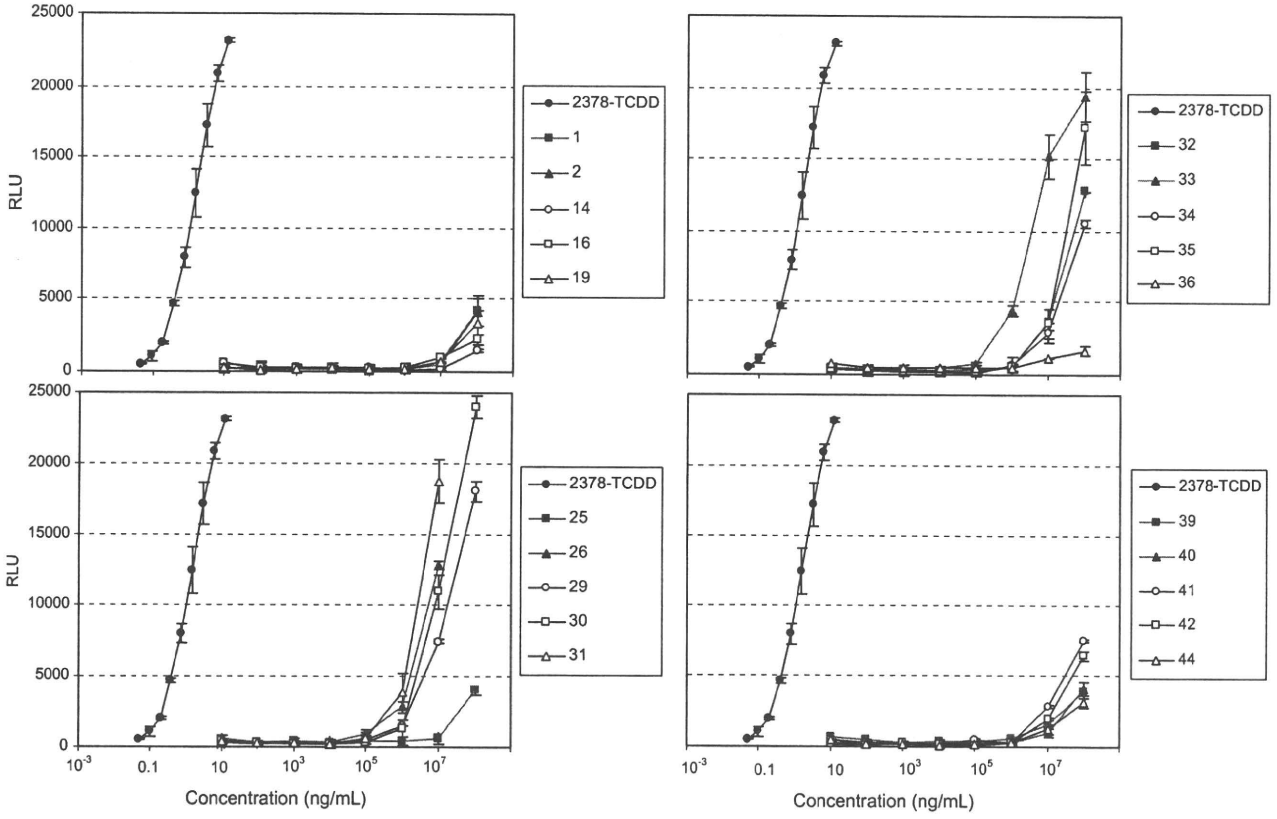

Fig. 1. Concentration–response curve of selected supplement and health food samples and TCDD for the induction of luciferase activity in the CALUX assay. Each point represents the mean of at least three replicate analyses. Results are expressed as means ± SD. All samples showed at several concentrations a statistically increased response in comparison with the blank ($p < 0.05$).

2. Materials and methods

2.1. Samples and reagents

TCDD and reagents used in the present study were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), Funakoshi (Tokyo, Japan), and Tokyo Kasei (Tokyo, Japan), and 50 supplements and health foods, as shown in Table 1, were from drug stores in Japan (in 2007).

2.2. Extraction and isolation

The samples were prepared as follows: Tablets were powdered and the contents of capsules and soft capsules were used for sam-

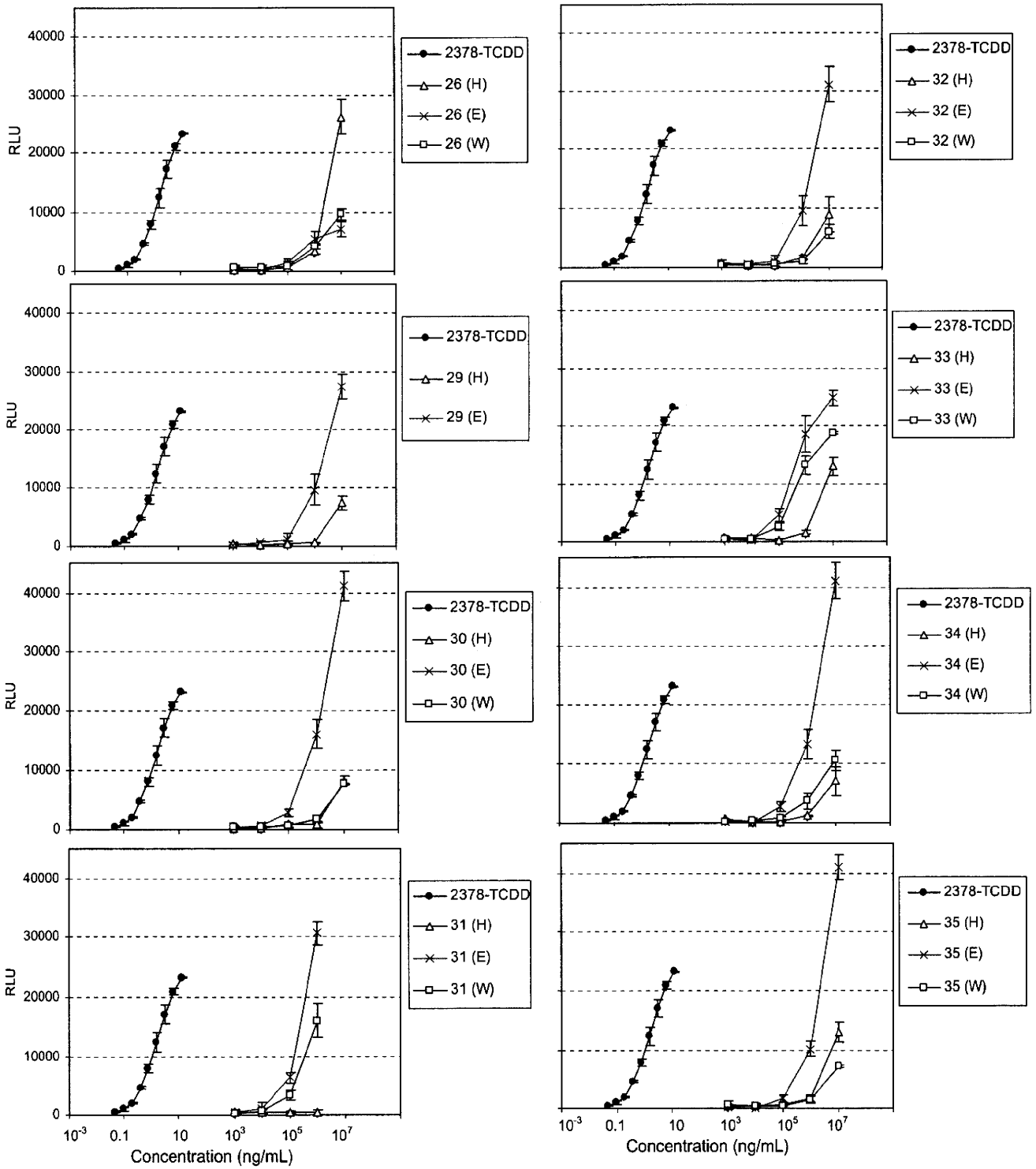

Fig. 2. Concentration–response curve of fractions of active samples and TCDD for the induction of luciferase activity in the CALUX assay. H, *n*-hexane fraction; E, ethyl acetate fraction; W, aqueous fraction. Each point represents the mean of at least two replicate analyses. Results are expressed as means ± SD. Sample 29 did not provide as aqueous fraction for analysis. All samples except 31 (H) showed at several concentrations a statistically increased response in comparison with the blank ($p < 0.1$).

ple preparation. The materials (1 g) were homogenised in aqueous ethanol [ethanol/water (4:1)] (30 ml) for 10 min and filtered. The filtrates were concentrated under reduced pressure and freeze-dried (total extract). Total extracts were added to water (10 ml), and these solutions were subjected to liquid–liquid partitioning (each 30 ml) to give three extracts: *n*-hexane-, ethyl acetate-, and water-soluble portions. Column chromatography for the identification of compounds was conducted using MCI Gel CHP-20P (75–150 μm) (Mitsubishi Chemical, Tokyo, Japan), YMC GEL ODS-AQ (AQ12S50) (YMC, Kyoto, Japan), and Silica Gel 60 (Nacalai Tesque, Kyoto, Japan). Compounds were identified by direct comparison with valid standards or by comparison of their spectral data with those reported in the literature. The extracts were dissolved in dimethyl sulfoxide and evaluated for AhR-binding activity, using a luciferase assay.

2.3. HPLC conditions

HPLC analysis was carried out using a Shimadzu Prominence system (Shimadzu, Kyoto, Japan). Reversed-phase HPLC conditions were as follows: column, L-column ODS (5 μm, 150 × 2.1 mm i.d.; Chemicals Evaluation and Research Institute, Tokyo, Japan); mobile phase, solvent A was 3% acetic acid and solvent B was acetonitrile (0–30 min, 0–50% B in A; 30–35 min, 50–85% B in A; 35–40 min, 85–85% B in A); injection volume, 5 μl; column temperature, 40 °C; flow-rate, 0.3 ml/min; detection, 200–400 nm.

2.4. Estimation of AhR ligand activity

For the identification of AhR-activating materials, a CALUX assay was used. When mouse hepatoma (H1L6.1c2) cells are exposed to environmental ligands, such as dioxins, luciferase protein synthesis is induced. The amount of light emitted by the luciferase protein is directly correlated with the dioxin level, and this system is used as a simple dioxin monitoring method. The CALUX assay for AhR ligand activity was carried out as follows: mouse hepatoma H1L1 cells (*ca.* 1.5×10^5 cells/well) were cultured in 96-well culture plates, and the samples dissolved in DMSO were added at a concentration range of 1×10^{-5}–1×10^2 mg/ml (final concentrations of 1×10^{-4}–1×10^3 μg/ml) in 8-steps (5-steps in fractions). The concentration data express the additional level not the final concentration. The final DMSO concentration was 1% in the cell culture medium. The plates were incubated at 37C in 5% CO_2 for 24 h to produce the optimal expression of luciferase activity. After incubation, cell viability was confirmed using a microscope. Subsequently, the medium was removed and the cells were lysed. After

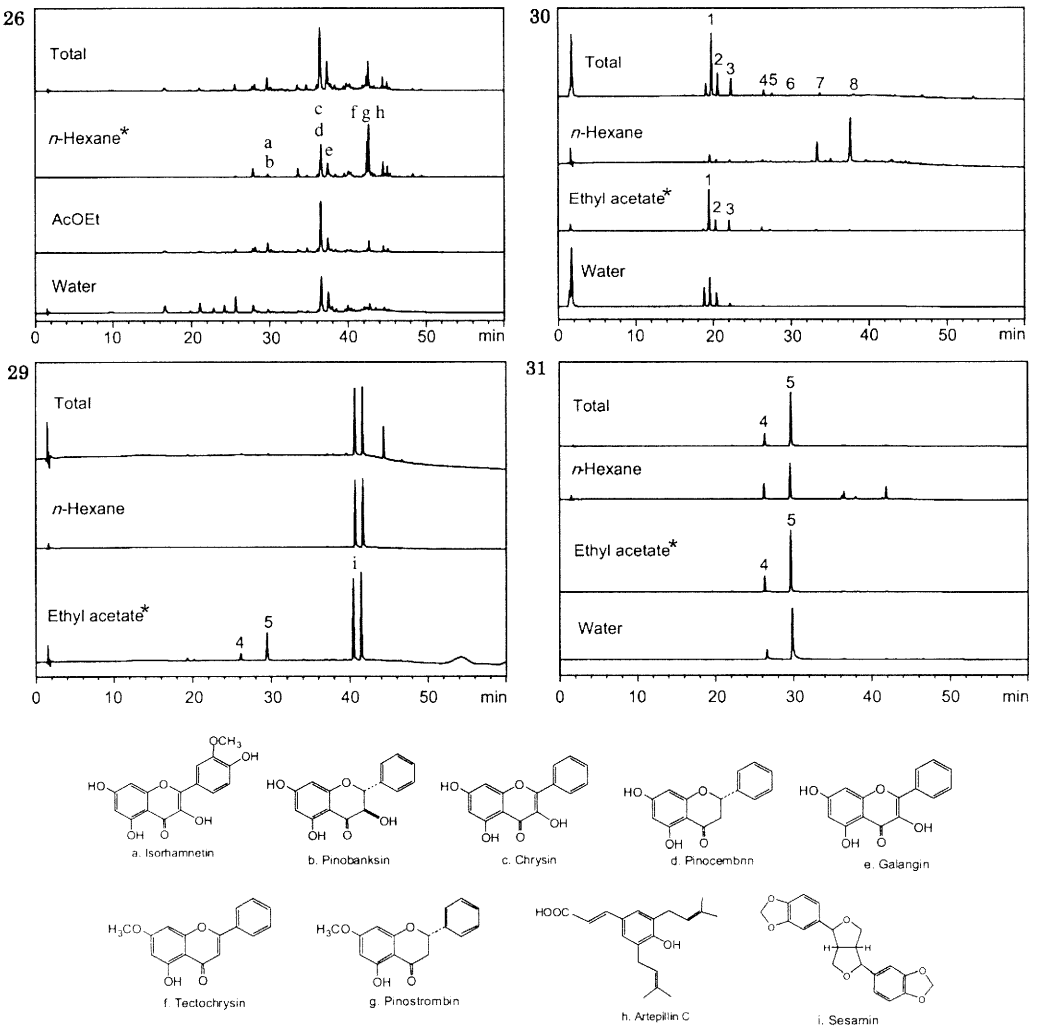

Fig. 3. RP-HPLC profiles of *n*-hexane, ethyl acetate, and aqueous fractions of AhR-activated samples and chemical structures of identified constituents. a, Isorhamnetin; b, pinobanksin; c, chrysin; d, pinocembrin; e, galangin; f, tectochrysin; g, pinostrombin; h, artepillin C; i, sesamin. 1, daidzin; 2, glycitin; 3, genistin; 4. daidzein; 5, glycitein; 6, genistein; 7, formononetin; 8, biochain A. Signals were detected at 254 nm except sample 26 (at 280 nm). Sample 29 did not provide an aqueous fraction for analysis. *AhR activated fraction.

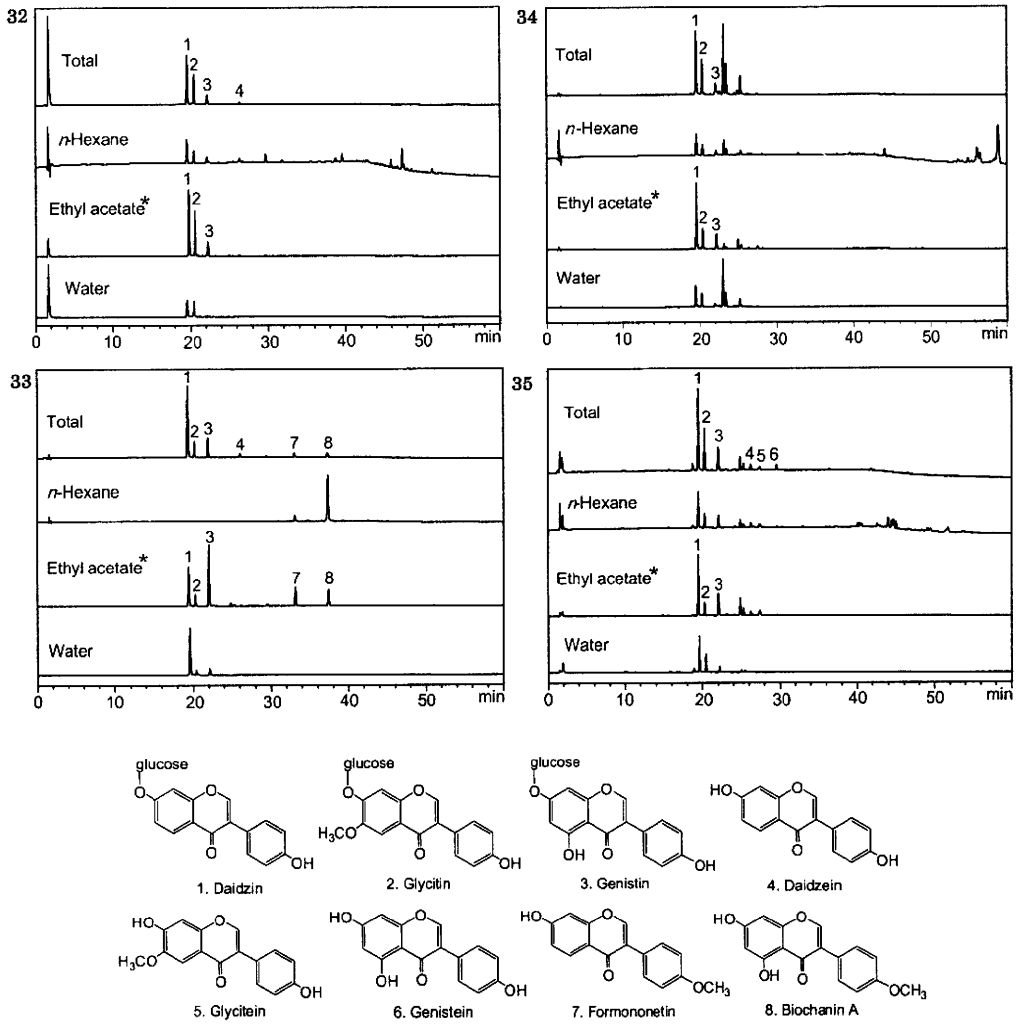

Fig. 3 (*continued*)

the addition of luciferin as the substrate, the luciferase activity was determined using a luminometer (Centro LB960, BERTHOLD, Bad Wildbad, Germany) and reported as relative light units (RLU). Values represent the means ± SD of at least two or three independent determinations for each experiment. Statistical significance was analysed using the Student's *t* test.

3. Results and discussion

AhR activity was estimated for the 50 samples listed in Table 1 using the CALUX assay. Although most of the samples showed no dioxin-like activity even at a high concentration, some samples shown in Fig. 1 exhibited activity at high concentration in a dose-dependent manner. Notably, the active samples included most of the soybean-related foods (samples 30–35). The active samples showed dioxin-like activity similar to that of TCDD at 10–100 mg/ml. Sesame processed food (sample 29) and propolis extract (sample 26) and some teas [loquat leaf (sample 41) and perilla herb (sample 42)] also exhibited an activity similar to that of the soybean extract foods at high concentration. To compare the dioxin-like activity of individual samples with that of TCDD, their relative concentration ($TCDD_{0.5}$) for AhR activity induced by 0.5 ng/ml of TCDD was calculated and the results are given in Table 2. Foods prepared from soybean (samples 30, 31, and 33), sesame (sample 29), and propolis (sample 26), exhibited dioxin-like activity similar to that of TCDD at about a 10^6-fold concentration of TCDD. The following samples showed slight dioxin-like activity of less than the $TCDD_{0.5}$ level at high concentrations: agaricus mushroom processed food (sample 1), amino acid/vitamin/mineral food (sample 2), ginkgo leaf extract (sample 14), *Gymnema sylvestre* extract (sample 16), lutein (sample 19), processed vegetable food (sample 25), and teas [Chinese sweet tea (sample 36), guava (sample 39), Japanese mugwort (sample 40), loquat leaf (sample 41), perilla herb (sample 42), and rooibos (sample 44)].

In order to characterise the active components of the samples from soybean-related samples (30–35), sesame (29), and propolis (26), AhR activity was measured for the respective *n*-hexane, ethyl acetate, and water fractions. The results are given in Fig. 2. The *n*-hexane fraction of the propolis extract sample exhibited AhR activity similar to that of TCDD at a concentration range of 1–10 mg/ml. In addition, marked AhR activity was noted for the ethyl acetate fractions of the other samples (soybean and sesame extract samples) at 0.1–10 mg/ml.

Previously, soy isoflavones, such as daidzein and glycitein, and so-called vegetable estrogens, such as resveratrol, were shown to exhibit dioxin-like activity (Amakura et al., 2003, 2008). The distribution of soy isoflavones and their glycosides in the active fractions was examined by reverse-phase HPLC. As shown in Fig. 3, some soy isoflavones were detected in various combinations in all six soybean extract samples. According to the combination of

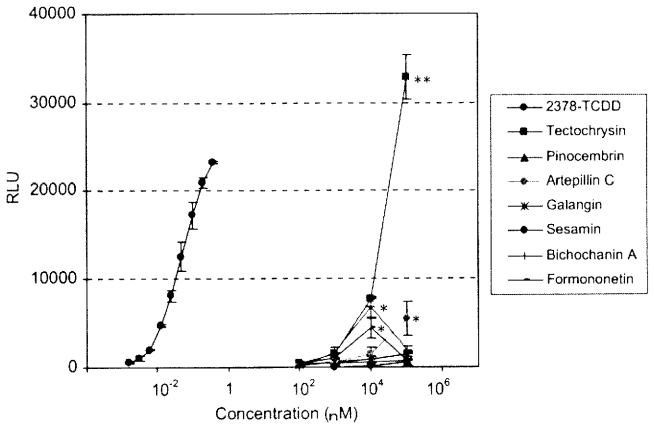

Fig. 4. Concentration–response curve of selected compounds and TCDD for the induction of luciferase activity in the CALUX assay. Each point represents the mean of at least three replicate analyses. Results are expressed as means ± SD, and asterisks indicate statistically significant differences ($^{*}p < 0.05$, $^{**}p < 0.01$).

the six standard isoflavones, they could be roughly classified into three groups [1st group (samples 30, 32, 33, and 35) including both aglycones and glycosides; 2nd group (sample 34) including glycosides; 3rd group (sample 31) including only aglycones]. On the other hand, some of the soybean extract products (samples 30, 31, and 33) also contained other isoflavones, biochanin A, and formononetin, which is also one of components of red clover. Biochanin A and formononetin slightly activated luciferase (Fig. 4). Thus, the methoxy group (–OMe) at C-4 of the B-ring in isoflavones may contribute to weakening the activity (Amakura et al., 2003,2008).

Sesame product (sample 29) was also found to contain daidzin and glycitin, together with sesamin. Sesamin showed no luciferase induction at the level of 10^5 nM (Fig. 4). HPLC analysis of some ethyl acetate fractions (samples 29–34, and 35) suggested the occurrence of components other than the six isoflavones. Thus, the influence of isoflavones and unidentified constituents was also investigated in more detail at the food level, using the biological assay for the determination of dioxins.

The *n*-hexane fraction of the propolis extract product (sample 26) showed the strongest AhR activity, suggesting the existence of dioxin-like active factors other than isoflavones. To clarify the compounds present, the *n*-hexane extract of propolis was subjected to chromatography using MCI-gel CHP 20P and YMC-gel ODS, to give chrysin, pinocembrin, galangin, tectochrysin, pinostrombin, isorhamnetin, pinobanksin, and artepillin C as UV-sensitive constituents. In our previous study, chrysin was reported to show some AhR ligand activity. In addition, the AhR activation of some flavonoids from the propolis extract product was examined by reporter gene assay in the present study. As shown in Fig. 4, tectochrysin showed marked AhR binding activity, followed by artepillin C. These results suggest that tectochrysin might be a natural AhR ligand. Thus, it was suggested that the AhR activity of the propolis extract may be related to flavones.

We herein demonstrated that food isoflavones and flavones are considered to be responsible for the AhR activity observed for several supplements or foods. The determination of dioxins in foods, using biological assays, requires the interpretation of data with regard to the influence of these confounding ingredients. The signal transduction of isoflavones and flavones, which occurs after AhR activation, should differ from that of dioxins. Recently, several papers have reported that the activation of AhR may be involved in various immune system responses; therefore, natural AhR ligands may play some beneficial regulatory role in humans (Kimura, Naka, Nohara, Fujii-Kuriyama, & Kishimoto, 2008; Quintana et al., 2008; Veldhoen et al., 2008). Further studies on the AhR-activating ingredients derived from natural foods might clarify, not only the physiological significance of AhR, but also the risks and benefits from food constituents.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Daily Intake of Brominated Dioxins, Co-PXBs and Brominated Flame Retardants Estimated from Market Basket Study

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Introduction

Brominated flame retardants (BFRs) such as polybrominated diphenyl ethers (PBDEs) and polybrominated biphenyls (PBBs) have been widely used in plastics and textile coatings throughout the world. The major commercial products made with PBDEs were penta-BDE, octa-BDE and deca-BDE products. In Japan, although the use of low-brominated PBDEs has decreased, deca-BDE is currently in use. PBDEs are additives to polymers such as polystyrene and are not chemically bound to the polymer. Therefore, they are easily released into the environment from waste products. For PBBs, the commercial products are mixtures containing hexa-BB, octa-BB, nona-BB, and deca-BB. Products made with PBBs have not been produced in Japan, but PBBs have been detected in environmental samples in Japan (Ishikawa et al. 2004). It is suspected that the contaminants came from imported products or impurities in other BFRs. Furthermore, *de novo* synthetic compounds related to BFRs, such as polybrominated dibenzo-*p*-dioxins and dibenzofurans (PBDD/DFs) have been found in human samples (Choi et al. 2003) and market fish (Ashizuka et al. 2008). In addition, coplanar polychlorinated / brominated biphenyls (Co-PXBs), which are new contaminants, have been detected (Ohta et al. 2008, Ohta et al. 2007). Although the toxicity of these brominated dioxins is unclear, some studies have shown that the toxicity of 2, 3, 7, 8-TBDD is comparable to that of 2, 3, 7, 8-TCDD (WHO 1998). Co-PXBs may also be formed from BFRs and have toxicities similar to those of Co-PCBs due to the similarity of their structures.

It is important that we investigate levels of these brominated organic compounds in foods and estimate their influence on humans. A market basket study is a useful method for estimating the average intake levels in regions, based on a model of the average domestic diet. In the present study, we analyzed brominated dioxins and PBDEs in food mixtures from each of 13 food groups from 2 regions of Kanto and Kansai in Japan and estimated the daily intake levels of brominated dioxins, Co-PXBs and BFRs. The goal of this research was to evaluate the health risk presented by the daily intake of these brominated compounds in Japan.

Materials and Methods

Sampling.

Table 1 shows the food groups analyzed in this study and their mean daily consumption for 2 regions (A region : Kanto region, B region : Kansai region) as calculated from data from the Japanese Nutrition Survey carried out by the Ministry of Health, Labour and Welfare. For a market basket study, 120-200 kinds of foods were purchased from markets in each of 2 regions in 2007. These foods were divided into 13 food groups, and were weighed and cooked based on the daily consumption data of each region. The foods were then, blended in a food processor. The food mixtures were prepared and analyzed for groups 10, 11, and 12 (n=2) and other groups (n=1). The food mixtures were kept below –20°C until analysis.

Analytical Methods and Instrumentation.

The PBDD/DFs (tetra-octa) and Co-PXBs (4'-Br-2,3',4,5-TeCB, 4'-Br-2,3,3',4-TeCB, 4'-Br-3,3',4,5-TeCB, 4'-Br-2,3,3',4,5-PeCB, 4'-Br-3,3',4,5,5'-PeCB, 3',4',5'-Br-3,4-DiCB) analytical standards were purchased from Cambridge Isotope Laboratories (Cambridge, MA). The PBDEs (tri-deca) analytical standards were purchased from Wellington Laboratories (Guelph, Ontario). The PBBs (tri-deca) analytical standards were purchased from Wellington Laboratories and AccuStandard, Inc. (New Haven, CT). The concentrations of brominated compounds were determined using high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS). Further information about instrumentation can be found in our previous article (Ashizuka et al. 2009).

Sample Preparation.

We analyzed the brominated dioxins, Co-PXBs, PBDEs and PBBs simultaneously using accelerated solvent extraction (ASE). Each 50 g sample was freeze-dried using a model AD 2.0ES-BC (Virtis, Gardiner, NY) freeze dryer, and then dried samples were extracted with 10% (v/v) dichloromethane/*n*-hexane using an accelerated solvent extractor ASE300 (Dionex, Sunnyvale, CA). The extraction temperature was 100°C; the time was 10 min. Extracts were treated with sulfuric acid three times and applied to a silica gel column. The mixture for group 4 was dissolved in 100 mL of *n*-hexane and purified with sulfuric acid and the silica gel column in the same way. The column was prewashed with 100 mL of *n*-hexane, and brominated compounds were eluted with 150 mL of 10% (v/v) dichloromethane/*n*-hexane. The eluate was evaporated and dissolved in *n*-hexane. It was then loaded onto a Florisil (5 g) column. The PBDEs, PBBs and Co-PXBs were obtained by elution with 150 mL of *n*-hexane (fraction 1), and the PBDD/DFs fraction was obtained by elution with 200 mL of 60% (v/v) dichloromethane/*n*-hexane (fraction 2). The fraction 1 was treated with a DMSO/*n*-hexane partition to remove the matrix and then concentrated to a final volume of approximately 25 μL. The fraction 2 was loaded onto an active carbon column, which in advance had been washed with 50 mL of 10% (v/v) dichloromethane/*n*-hexane and eluted with 200 mL of toluene. The fractions were concentrated to a final volume of approximately 15 μL, and these samples were analyzed by HRGC/HRMS.

Table 1 Daily food consumption (13 groups) in 2 regions of Japan

No.	Food group	Daily consumption (g)*	
		A region	B region
1	Rice and rice products	332.8	341.4
2	Cereals seeds and potatoes	175.4	174.2
3	Sugars and confectioneries	32.1	35.1
4	Fats and oils	11.0	10.6
5	Pulses	59.6	57.5
6	Fruits	125.4	120.8
7	Green vegetables	100.3	92.8
8	Other vegetables and sea weeds	209.1	184.1
9	Beverages	540.8	616.4
10	Fish and shellfish	84.8	82.2
11	Meat and eggs	111.3	121.4
12	Milk and dairy products	137.7	142.9
13	Other foods (seasoning)	94.5	92.9
	Total	2014.8	2072.3

*The values obtained from the data of Japanese Nutrition Survey (the Ministry of Health, Labour and Welfare of Japan).

Results and Discussion

We analyzed brominated dioxins (a total of 18 congeners of PBDD/DFs and MoBrPCDD/DFs), Co-PXBs (7 congeners), PBDEs (23 congeners) and PBBs (18 congeners) in food mixtures from each of 13 food groups from 2 regions in Japan. In our study, the limits of detection (LODs) of the

PBDD/DFs were 0.01 pg/g wet weight (ww) for tetra and penta, 0.05 pg/g ww for hexa, 0.1 pg/g ww for hepta and 1 pg/g ww for octa. The LODs of PBDEs were 0.1 pg/g ww for tetra-hepta, 0.2 pg/g ww for octa, 0.5 pg/g ww for nona and 1 pg/g ww for deca. The LODs of Co-PXBs were 0.05 pg/g ww. The LODs of PBBs were 0.1 pg/g ww for tri-penta, 0.2 pg/g ww for hepta-nona and 0.5pg/g ww for deca. From the results of analyzing brominated dioxins, only 1,2,3,4,6,7,8-HpBDF was detected in the mixture of group 4 (fats and oils) from the A region at 0.66 pg/g ww. Co-PXB congeners were not detected in any food mixtures. PBDE congeners were detected in all food mixtures. The highest total PBDE (tri-deca) concentrations were found in the food mixture of group 4 (fats and oils) at 1114 - 1729 pg/g ww. PBBs were detected from samples of group 4, 10 and 11. The detected congeners were mainly DeBB (#209) in group 4, 2,2',5,5'-TeBB (#52), 2,2',4,5'-TeBB (#49), 2,2',4,5,5'-PeBB (#101), 2,2',4,4',6,6'-HxBB (#155) and 2,2',4,4',5,5'-HxBB (#153) in group 10, and 2,2',4,4',5,5'-HxBB (#153) in group 11. Table 2 shows data for the daily intakes calculated from the concentrations of PBDEs and PBBs in each food group. The highest value of PBDEs daily intake was group 10 (Fish and shellfish). For PBBs, the highest contribution to daily intake was group 10 (Fish and shellfish), the same as for the PBDEs. The PBB levels, however, were much lower than the total PBDE levels.

Table 2 Daily intakes of PBDEs and PBBs in each food group

	Food group	PBDEs ng/day		PBBs ng/day	
		A region	B region	A region	B region
1	Rice and rice products	5.35	11.9	0	0
2	Cereals seeds and potatoes	3.11	0.796	0	0
3	Sugars and confectioneries	0.399	2.14	0	0
4	Fats and oils	19.0	11.8	0.013	0.008
5	Pulses	1.50	4.27	0	0
6	Fruits	5.76	4.14	0	0
7	Green vegetables	0.872	2.31	0	0
8	Other vegetables and sea weeds	20.7	1.87	0	0
9	Beverages	31.5	3.01	0	0
10	Fish and shellfish	43.4	64.5	0.327	0.141
11	Meat and eggs	19.4	8.91	0.038	0.020
12	Milk and dairy products	3.24	11.9	0	0
13	Other foods (seasoning)	6.30	9.35	0	0
	total	161	137	0.378	0.169

*Daily intake calculated assuming that ND = zero.

Table 3 shows daily intakes of brominated dioxins, Co-PXBs and BFRs in 2 regions of Japan. The WHO has stated that use of the same TEF values for PBDD/PBDF or PXDD/PXDF congeners as the chlorinated analogues appears to be justified. To estimate the influence of brominated dioxins, we calculated the total TEQ per day, using the TEFs of chlorinated dioxins. The daily intake was calculated as 0.00145 pg TEQ /kg body weight (bw)/day for the A region on a 50 kg bw (assuming ND = 0). Due to the small daily consumption of fats and oils, the daily intake of brominated dioxins was at a low level. In the case assuming that ND = 1/2LOD, the mean daily intake was calculated as 1.46 pg TEQ/kg bw/day for the A region and 1.72 pg TEQ/kg bw/day for the B region, which were estimated to be within Japanese TDI (4 pg TEQ/kg bw/day).

For PBDEs, the total daily intake was estimated as 161 ng /day for the A region and 137 ng/day for the B region assuming that ND = 0. In the case of 50 kg of bw, the daily intake was calculated as 3.21 and 2.74 ng/kg bw/day (assuming ND = 0). In the case assuming that ND = 1/2 LOD, the daily intake

was calculated at 3.25 and 2.80 ng/kg bw/day. In a recent report, the lowest observed adverse effect level (LOAEL) value suggested as reasonable for compounds or mixtures belonging to the PBDE group was 1mg/kg bw/day (Darnerud et al. 2001). Since the calculated value in this study was much less than this LOAEL value, the daily intake level of PBDEs was not considered a serious problem.

For PBBs, the daily intake from the A and B regions was estimated to be 0.378 and 0.169 ng/day, respectively. In the case of 50 kg of bw, the daily intake was calculated as 0.00755 ng/kg bw/day for the A region and 0.00337 ng/kg bw/day for the B region (assuming ND = 0). In the case assuming that ND = 1/2 LOD, the daily intake was calculated at 0.0593 ng/kg bw/day for the A region and 0.0647 ng/kg bw/day for the B region. For PBBs, it was suggested that the total daily intake should be less than 0.15 μg/kg bw/day, extrapolating from a no observed adverse effect level (NOAEL) obtained from a positive carcinogenicity study, using an uncertainty (safety) factor of 1000 (WHO 1994). Compared with these values, the levels of these brominated compounds in fish were not considered a serious problem. However, it is important to collect more data about brominated dioxins and BFRs in food, because little information is available regarding the levels of these brominated compounds.

Table 3 Daily intakes of Brominated dioxins, Co-PXBs and BFRs in 2 regions of Japan

	Region	Daily intake***			
		Brominated Dioxins	Co-PXBs	PBDEs	PBBs
ND=0*	A region	0.00145 pgTEQ/kg/day	0 ng/kg/day	3.21 ng/kg/day	0.00755 ng/kg/day
	B region	0 pgTEQ/kg/day	0 ng/kg/day	2.74 ng/kg/day	0.00337 ng/kg/day
ND=1/2LOD**	A region	1.46 pgTEQ/kg/day	0.00629 ng/kg/day	3.25 ng/kg/day	0.0593 ng/kg/day
	B region	1.72 pgTEQ/kg/day	0.00742 ng/kg/day	2.80 ng/kg/day	0.0647 ng/kg/day

*Daily intake calculated assuming that ND = zero. ** Daily intake calculated assuming that ND = 1/2LOD.
*** Daily intake calculated assuming that the average body weight of a Japanese adult is 50 kg.

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Hexabromocyclododecane determination in seafood samples collected from Japanese coastal areas

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ABSTRACT

The levels of three hexabromocyclododecane (HBCD) isomers and ΣHBCDs in 54 wild and 11 farmed seafood samples collected from four regions of Japan were determined by LC/MS/MS. For the fish classified as Anguilliformes, Perciformes, Clupeiformes and farmed Salmoniformes, the medians (ranges) of ΣHBCDs are 2.09 (0.05–36.9), 0.75 (ND–26.2), 0.12 (0.09–77.3) and 1.29 (1.09–1.34) ng g^{-1} ww, respectively. However, HBCDs were not detected in samples classified as Crustacea, Mollusca, Pleuronectiformes and Scorpaeniformes, or if detected, the levels were very low. The rank correlation between ΣHBCDs (or α-HBCD) and fat content could not be found except for the Japanese sea bass of the Tohoku region. In HBCD isomer profiles, for fish samples above 20 ng g^{-1} ww, the trend was found that γ-HBCD was predominant, which suggests the influence of discharge from a nearby industrial plant. In the other wild fish and the farmed fish samples, on the other hand, α-HBCD was mostly predominant, which suggests biomagnification via the food chain. Additionally, to assess the risk to human health, based on the determined HBCD median concentrations for Anguilliformes, farmed Salmoniformes and Perciformes, the daily intake of HBCDs from fish by an average Japanese adult was tentatively calculated to be 3.7, 2.3 and 1.3 ng (kg body weight)$^{-1}$ d^{-1}, respectively.

1. Introduction

The use of flame retardants, with the intent to minimize the human and economic losses due to fire, began in the 1970s. Nowadays, the use of flame retardants has expanded around the world and they are applied to plastics in consumer electronics and computers, textiles of carpets and curtains, as well as to polystyrene and polyurethane products for automotive seats and house insulation. In the classification of flame retardants, there is typically a group of brominated flame retardants (BFRs) with bromine atoms in the chemical structures. In addition to polybrominated diphenyl ethers (PBDEs), tetrabromobisphenol A (TBBPA) and polybrominated biphenyls, hexabromocyclododecanes (HBCDs) are also included in the BFR group. Through the disposal of products to which BFRs have been applied, BFRs have been released into the environment (Watanabe and Sakai, 2003; Morf et al., 2005). As would be naturally expected, concerns regarding the exposure to humans have been raised; therefore, studies on BFRs have begun to be carried out, especially in the northern hemisphere from around 2000. On PBDEs, especially in the early stage, a substantial number of research reports could be referenced (de Wit, 2002). Through eating fish or inhaling dust, the high accumulation of BFRs in human breast milk and subcutaneous fat was revealed (Meironyté et al., 1999; Akutsu et al., 2003; Choi et al., 2003). However, information on HBCDs is still limited. Like PBDEs, HBCDs are also highly lipophilic as expected from the fact that the Log P_{OW} is calculated to be 7.74 (Chemicals Evaluation and Research Institute, Japan, 2001) with the software KowWin ver 1.66 (Syracuse Research Corporation, NY). This means that once emitted into the water environment, HBCDs can be also easily adsorbed on river and sea sludge, and thus HBCD pollution over a long period would allow HBCDs to accumulate in fatty tissue of organisms throughout the food chain. Furthermore, since three major stereoisomers exist in HBCD products, the importance of isomer specific analysis has been emphasized (Tomy et al., 2004; de Wit et al., 2006). Due to the obviously high persistency of HBCD, the possibility of harm to human health is suspected. Therefore, in 2009, the European Chemicals Agency (ECHA) also designated HBCD as a substance of very high concern to be monitored, as well as PBDEs and PBBs as specified substances in RoHS. Similarly, in Japan, under the "Act on the Evaluation of Chemical Substances and Regulation of Their Manufacture, etc." (2004), HBCD also has been specified as substrate to be monitored. The consumption of HBCD in 2003 in Japan was about 2400 tons per year, which is almost equal to that of

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decabromodiphenyl ether (2000 tons per year), which is the only recently used PBDE in Japan, but is far less than that of TBBPA (around 32 000 tons per year) (Japan Environment Government Report, 2006). The consumption amounts of these BFRs have not changed largely for several years.

Due to the possibility of global environmental pollution caused by the BFRs, we began in 2004 to conduct monitoring of BFRs in seafood as well as to develop analytical methods for determining trace levels of BFRs in foods to ensure food safety and security to prevent damage to human health (Ashizuka et al., 2005; Nakagawa et al., 2006; Ashizuka et al., 2008). In the present paper, we report the recent results for α-, β-, and γ-HBCD isomers and ΣHBCDs levels in the seafood of Japan. Additionally, we report the estimated daily intake (EDI) of HBCDs from seafood to assess the risk to human health. This study is the first to report the EDI of ΣHBCDs for the Japanese.

2. Materials and methods

Sixty-five individual marine samples were collected from four regions (Kyushu: Kumamoto City, Nagasaki City and Kagoshima City, which have populations of ca. 730 000, 450 000 and 600 000, respectively; Chugoku/Shikoku: Okayama City (population: ca. 700 000); Chubu: Nagoya City (population: ca. 2 200 000); and Tohoku: Sendai City (population: ca. 1 000 000); see Fig. 1). Samples were purchased at food markets or obtained from the Miyagi Prefectural Institute of Public Health and Environment, between 2004 and 2008. Tables 1-1, 1-2 list the 65 samples, of which 54 are wild and 11 are farmed. A majority of fish samples are classified by order, and the others are classified by phylum or subphylum categories (Mollsca, Crustacea). Edible parts of each sample were pooled and homogenized and then stored below −20 °C until analysis.

2.1. Chemicals

Dichloromethane, *n*-hexane, acetone and anhydrous sodium sulfate were pesticide residue analysis grade (Kanto Chemical Co. Ltd., Tokyo). Sodium chloride was reagent grade. Anhydrous sodium sulfate and sodium chloride were baked at 600 °C before use to reduce contamination. Native and $^{13}C_{12}$-labeled standards of α-, β- and γ-HBCD were purchased from Cambridge Isotope Laboratories (CIL; Andover, MA). Methanol and distilled water were LC/MS analysis grade (Kanto Chemical Co. Ltd., Tokyo).

2.2. Sample preparation

To 5 g of each sample, 20 mL of 10% dichloromethane/*n*-hexane (DCM/HEX) and $^{13}C_{12}$-labeled HBCDs (1 ng) as a clean-up spike were added and homogenized. The mixture was centrifuged at 3000 rpm for 5 min and the upper layer of solution was dried with anhydrous sodium sulfate and transferred into another tube. The residue was re-homogenized with 20 mL of 10% DCM/HEX, similarly centrifuged, and dried and then the extracts were pooled. The extract solution was gently mixed once with conc. sulfuric acid and stored overnight. After centrifugation, the upper layer of

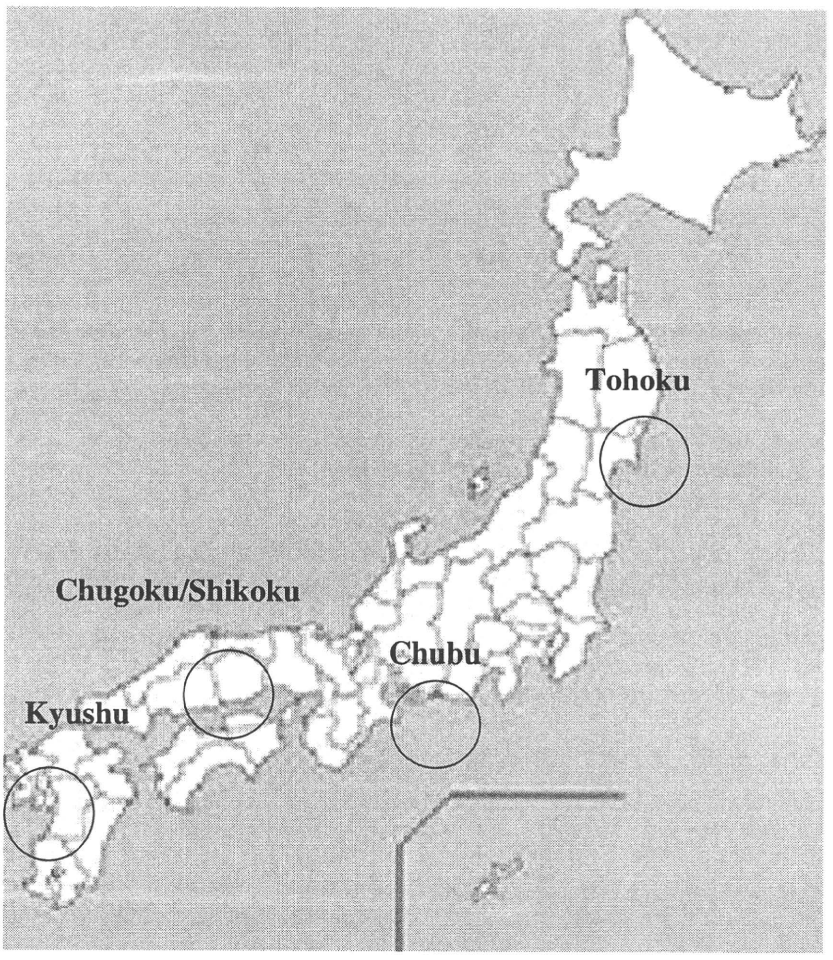

Fig. 1. Location of sampling sites.

Table 1-1
Details and HBCD concentrations of seafood samples (wild) caught in Japan coast.

Class and name of sample	Sampling location	No. of fish	Length of fish (cm)	Weight of fish (g)	Sampling date	Fat (%)	α-HBCD (ng g^{-1}, ww)	β-HBCD (ng g^{-1}, ww)	γ-HBCD (ng g^{-1}, ww)	ΣHBCD (ng g^{-1}, ww)
Perciformes										
Barracuda	Kyushu	5	30	237	2004	9.88	0.73	<0.01	0.27	1.01
Barracuda	Chubu	5	31	234	2004	4.50	3.85	<0.01	1.45	5.29
Butter fish	Kyushu	7	20	156	2005	3.93	<0.02	<0.01	<0.02	0.00
Horse mackerel	Kyushu	3	30	314	2004	5.67	0.17	<0.01	<0.02	0.17
Horse mackerel	Kyushu	4	32.3	360	2007	4.90	0.10	<0.01	0.02	0.12
Horse mackerel	Chubu	5	23	226	2004	4.72	3.78	<0.01	1.10	4.88
Horse mackerel	Chugoku/Shikoku	11	19	64	2004	2.28	0.25	<0.01	<0.02	0.25
Japanese seabass	Tohoku	5	52.2	1896	2003	3.40	2.31	0.02	1.86	4.19
Japanese seabass	Tohoku	5	48.8	1524	2003	2.50	2.04	0.02	1.62	3.68
Japanese seabass	Tohoku	5	49.5	1534	2003	2.40	3.25	0.08	4.37	7.69
Japanese seabass	Tohoku	5	37.6	680	2003	1.40	1.40	0.02	1.17	2.59
Japanese seabass	Tohoku	5	36.1	728	2003	1.30	1.15	<0.01	1.03	2.18
Japanese seabass	Chubu	2	41	775	2004	0.98	7.75	<0.01	3.76	11.5
Japanese seabass	Chubu	1	45	1230	2004	0.72	9.06	0.36	16.5	25.9
Japanese Spanish mackerel	Chubu	1	67	2680	2004	11.3	6.52	0.25	19.4	26.2
Japanese Spanish mackerel	Chubu	2	40	555	2004	1.30	2.52	0.10	4.17	6.79
Japanese Spanish mackerel	Chugoku/Shikoku	1	52	750	2004	1.91	0.16	<0.01	0.33	0.49
Largehead hairtail	Kyushu	–[a]	–[a]	–[a]	2004	0.33	0.13	<0.01	<0.02	0.13
Pacific mackerel	Kyushu	2	30	376	2004	20.4	0.17	<0.01	0.18	0.35
Pacific mackerel	Kyushu	3	34.1	573	2007	12.0	2.86	<0.01	0.95	3.81
Pacific mackerel	Chubu	11	34	638	2004	13.7	15.7	<0.01	7.74	23.4
Sea bream	Chugoku/Shikoku	1	39	1000	2004	1.10	<0.02	<0.01	<0.02	0.00
Sea bream	Chugoku/Shikoku	1	35.1	216	2007	0.60	0.05	<0.01	0.03	0.08
Sea bream	Kyushu	4	23	265	2004	1.01	<0.02	<0.01	<0.02	0.00
Sea bream	Kyushu	2	32.6	664	2007	0.19	<0.02	<0.01	<0.02	0.00
Sea bream	Chubu	1	42	1250	2008	0.48	0.21	<0.01	0.03	0.24
Sea bream	Chubu	1	43	1300	2008	2.77	5.28	0.04	2.21	7.53
Sillago	Chubu	15	17	46	2004	0.46	0.26	<0.01	0.10	0.36
Sillago	Chugoku/Shikoku	10	21.7	89	2007	0.42	0.23	<0.01	0.05	0.28
Tuna	Chugoku/Shikoku	–	–	602/slice	2004	0.51	<0.02	<0.01	<0.02	0.00
Average						3.90	2.33	0.03	2.28	4.64
Median						2.10	0.50	0.00	0.30	0.75
Min.						0.19	<0.02	<0.01	<0.02	0.00
Max.						20.4	15.7	0.36	19.4	26.2
Anguilliformes										
Conger myriaster	Kyushu	4	52	233	2004	7.52	0.09	<0.01	<0.02	0.09
Conger myriaster	Chugoku/Shikoku	7	38	100	2005	12.65	5.80	<0.01	3.23	9.03
Conger myriaster	Chugoku/Shikoku	9	43	120	2007	9.90	1.36	0.04	0.70	2.09
Muraenesox Cinereus	Chugoku/Shikoku	–[a]	–[a]	771/slice	2005	3.40	0.05	<0.01	<0.02	0.05
Conger myriaster	Chubu	7	35	102	2008	11.80	17.7	0.40	18.8	36.9
Average						9.05	5.00	0.09	4.55	9.63
Median						9.90	1.36	0.00	0.70	2.09
Min.						3.40	0.05	<0.01	<0.02	0.05
Max.						12.65	17.7	0.40	18.8	36.9
Scorpaeniformes										
Marbled rockfish	Kyushu	8	19	128	2004	0.37	<0.02	<0.01	<0.02	0.00
Sebastes inermis	Chugoku/Shikoku	7	24	214	2005	0.50	0.20	<0.01	0.42	0.62
Average						0.43	0.10	0.00	0.21	0.31
Min.						0.37	<0.02	<0.01	<0.02	0.00
Max.						0.50	0.20	<0.01	0.42	0.62
Pleuronectiformes										
Cynoglossus joyneri	Kyushu	2	44	464	2004	1.42	0.04	<0.01	<0.02	0.04
Olive flounder	Kyushu	2	39	632	2004	0.30	<0.02	<0.01	<0.02	0.00
Cynoglossus joyneri	Chugoku/Shikoku	4	35	253	2005	0.35	<0.02	<0.01	<0.02	0.00
Righteye flounder	Chugoku/Shikoku	6	26	159	2005	0.35	<0.02	<0.01	<0.02	0.00
Righteye flounder	Chugoku/Shikoku	3	28	313	2007	1.10	<0.02	<0.01	<0.02	0.00
Average						0.70	0.01	0.00	0.00	0.01
Median						0.35	0.00	0.00	0.00	0.00
Min.						0.30	<0.02	<0.01	<0.02	0.00
Max.						1.42	0.04	<0.01	<0.02	0.04
Mollusca										
Ocellated octopus	Chugoku/Shikoku	3	28	209	2004	0.26	<0.02	<0.01	<0.02	0.00
Pacific Flying Squid	Chubu	2	40	300	2004	1.19	0.08	<0.01	0.09	0.16

(*continued on next page*)

Table 1-1 (*continued*)

Class and name of sample	Sampling location	No. of fish	Length of fish (cm)	Weight of fish (g)	Sampling date	Fat (%)	α-HBCD (ng g^{-1}, ww)	β-HBCD (ng g^{-1}, ww)	γ-HBCD (ng g^{-1}, ww)	ΣHBCD (ng g^{-1}, ww)
Octopus	Chubu	2	-	436	2004	0.35	0.25	0.04	0.65	0.93
Spear squid	Kyushu	2	37	247	2007	0.38	<0.02	<0.01	<0.02	0.00
Average						0.54	0.08	0.01	0.18	0.27
Median						0.36	0.04	0.00	0.04	0.08
Min.						0.26	<0.02	<0.01	<0.02	0.00
Max.						1.19	0.25	0.04	0.65	0.93
Clupeiformes										
Silver-stripe round herring	Kyushu	191	9	5	2004	1.82	0.11	<0.01	0.02	0.13
Sardine	Kyushu	20	15	31	2004	0.74	0.09	<0.01	<0.02	0.09
Sardine	Kyushu	28	16	48	2007	1.70	0.08	<0.01	0.02	0.10
Sardinella zunasi	Chugoku/Shikoku	35	11	11	2005	4.53	18.3	2.44	56.6	77.3
Average						2.20	4.63	0.61	14.2	19.4
Median						1.76	0.10	0.00	0.02	0.12
Min.						0.74	0.08	<0.01	<0.02	0.09
Max.						4.53	18.3	2.44	56.6	77.3
Beloniformes										
Japanese halfbeak	Chugoku/Shikoku	14	32	68	2005	0.92	0.22	<0.01	0.05	0.27
Mugiliformes										
Flathead mullet	Chubu	2	46	1350	2004	1.69	0.38	0.00	0.37	0.75
Crustacea										
Shrimp	Kyushu	16	16	37	2004	0.19	<0.02	<0.01	<0.02	0.00
Shrimp	Kyushu	58	9	10	2007	0.12	<0.02	<0.01	<0.02	0.00
Shrimp	Chugoku/Shikoku	34	15	18	2005	0.49	<0.02	<0.01	<0.02	0.00
Average						0.27	0.00	0.00	0.00	0.00
Median						0.19	0.00	0.00	0.00	0.00
Min.						0.12	<0.02	<0.01	<0.02	0.00
Max.						0.49	<0.02	<0.01	<0.02	0.00

LODs were 0.02 ng g^{-1}, ww for α- and γ- HBCD, and 0.01 ng g^{-1}, ww for β-HBCD. Less than LOD was treated as 0 for calculation of average, median and ΣHBCDs.
[a] Means not known because in almost cases sample was a part of one individual.

Table 1-2
Details and HBCD concentrations of seafood samples (farmed) caught in Japan coast.

Class and name of sample	Sampling location	No. of fish	Length of fish (cm)	Weight of fish (g)	Sampling date	Fat (%)	α-HBCD (ng g^{-1}, ww)	β-HBCD (ng g^{-1}, ww)	γ-HBCD (ng g^{-1}, ww)	ΣHBCD (ng g^{-1}, ww)
Salmoniformes										
Salmon1	Tohoku	5	51	2376	2003	14.30	1.06	<0.01	0.20	1.26
Salmon2	Tohoku	5	49	2232	2003	10.80	1.05	<0.01	0.24	1.29
Salmon3	Tohoku	5	51	2278	2003	10.20	0.86	<0.01	0.23	1.09
Salmon4	Tohoku	3	55	2900	2004	10.80	1.12	<0.01	0.22	1.34
Salmon5	Tohoku	3	56	3317	2004	14.50	1.11	<0.01	0.18	1.30
Average						12.12	1.04	0.00	0.21	1.26
Median						10.80	1.06	0.00	0.22	1.29
Min.						10.20	0.86	<0.01	0.18	1.09
Max.						14.50	1.12	<0.01	0.24	1.34
Perciformes										
Sea bream	Chugoku/Shikoku	2	37	750	2005	7.11	0.22	<0.01	<0.02	0.22
Sea bream1	Chubu	2	31	1070	2004	8.12	0.31	<0.01	<0.02	0.31
Sea bream2	Chubu	1	37	918	2004	9.36	0.71	<0.01	<0.02	0.71
Sea bream3	Chubu	1	38	1073	2004	4.10	0.26	<0.01	0.08	0.34
Yellow tail	Chubu	1	73	3000/half	2004	17.28	0.33	<0.01	<0.02	0.33
Average						9.19	0.37	0.00	0.08	0.38
Median						8.12	0.31	0.00	0.08	0.33
Min.						4.10	0.22	<0.01	0.08	0.22
Max.						17.28	0.71	<0.01	0.08	0.71
Mollusca										
Oyster	Chugoku/Shikoku	46	7.5	15.8	2005	2.26	0.19	<0.01	<0.02	0.19

LODs were 0.02 ng g^{-1}, ww for α- and γ- HBCD, and 0.01 ng g^{-1}, ww for β-HBCD.
Less than LOD was treated as 0 for calculation of average, median and ΣHBCDs.

solution was transferred to another round-bottom vessel, concentrated under vacuum and dissolved in 0.2 mL of acetone. Subsequently, a portion (less than 0.1 mL) of the prepared sample was injected onto a gel permeation chromatography column (8 mm i.d. × 300 mm, Shodex CLNpak PAE800AC, Showa Denko Co. Ltd., Tokyo) using acetone as the mobile phase at 0.8 mL/min, and the HBCD fractions eluted between 15 and 16 min were collected. The fractions were then re-dissolved into a small amounts of

acetonitrile, passed through a solid mini column (PSA, Spelco, CA) using 20 mL of acetonitrile/toluene (3:1, v/v), and the eluates were concentrated and re-dissolved in 25–50 μL of methanol. Fat in each fish sample was gravimetrically determined using separately prepared extract solution by an accelerated solvent extractor (ASE 300, Dionex, CA) with 10% DCM/HEX.

2.3. LC/MS/MS analysis

Determination of HBCDs was performed by LC/MS/MS using a Quattro Ultima (Waters, Milford, MA) equipped with a Waters Alliance 2695 detector. The MS/MS was operated in electrospray ionization negative ion mode using multiple reaction monitoring (MRM) for [M–H] (m/z 641 and 639) → Br (m/z 79). The following parameters were used: capillary voltage, 3.0 kV; source temperature, 130 °C; desolvation temperature, 300 °C; desolvation nitrogen gas flow rate, 600 L/h; cone voltage, 35 V; collision energy, 10 eV. HBCD isomers were separated by reversed-phase chromatography using an Inertsil ODS column (5 μm, 2.1 mm × 150 mm; GL Science Co. Ltd., Tokyo): column temperature, 40 °C; injection volume, 5 μL; mobile phase, 20% 10 mM ammonium acetate (A), 50% methanol (B) and 30% acetonitrile (C) for 2 min, and then gradually changed to 0% A, 70% B and 30% C; flow rate, 0.2 mL/min.

2.4. Quality assurance

Quality and sensitivity controls for the LC/MS/MS analyses were carried out by repeated injections of solvent blanks (methanol) and a mixed HBCD standard solution (0.02 ppm of each isomer) consisting of α-, β-, and γ-native isomers and the corresponding ^{13}C-labeled isomers. In addition, laboratory blanks were simultaneously analyzed in parallel to the samples and the signals of each native HBCD isomer of laboratory blank were checked to avoid contamination throughout the whole analysis procedure. The concentrations of the native α-HBCD, β-HBCD and γ-HBCD isomers in the laboratory blank were respectively 1/10, 1/5 and 1/20 of the lowest concentration in the fish samples in this study. As a reference sample, a fish sample that was once analyzed in our laboratory was also included in a set of samples to assure the repeatability. The recoveries of fortified HBCDs ($^{13}C_{12}$-labeled HBCDs) were above 71% for α-HBCD, 95% for β-HBCD and 77% for γ-HBCD.

3. Results and discussion

3.1. Levels of HBCDs in seafood samples

Tables 1-1, 1-2 show concentrations of α-HBCD, β-HBCD and γ-HBCD isomers, as well as ΣHBCDs in a total of 65 marine samples that consisted of 54 naturally fed (wild) fish and 11 farmed fish, accompanied by the classification of the seafood samples and the sampling locations. As seen in Table 1-1, the number of wild fish classified into the order of Perciformes was the largest: 30. Sea bream, mackerel and horse mackerel, which are popular foodstuffs in Japan, were classified as Perciformes. The HBCD concentrations ranged from not detected (ND) to 26.2 ng g^{-1} ww. The median value for each region decreased stepwise in the order of Chubu (7.16 ng g^{-1} ww, $n = 10$), Tohoku (3.14 ng g^{-1} ww, $n = 5$), Chugoku/Shikoku (0.16 ng g^{-1} ww, $n = 6$) and Kyushu (0.14 ng g^{-1} ww, $n = 9$). Although there is not a full set of the same fish family for all regions, the same trend that the HBCD pollution in fish from the Chubu region is heavier than that in fish from the other regions could be proved by taking together the data for sea bream, sea bass and horse mackerel in Table 1-1. For tuna, only one sample from Chugoku/Shikoku ($n = 1$) was analyzed (ND, fat, 0.51%) in this study; however, it was interesting that HBCDs were detected in skipjack tuna (32–45 ng g^{-1} lipid weight, fat: 4.8–4.9%) caught between 1997 and 2001 off the Japan coast, likely near the Chubu and Tohoku regions (Ueno et al., 2006). These facts might also support the trend of higher ΣHBCDs in fish samples from Chubu and Tohoku. In the coastal area of the Chubu region (mainly Nagoya City), there are larger-scale industrial and consumption sites, compared to the other regions of Japan. The Chugoku/Shikoku region faces the Seto Inland Sea, where various sizes of industrial sites are also scattered. Tohoku and Kyushu have also some industrial sites in the confined coastal area. The results are thought to show the status of HBCD pollution in the coastal sea of each region. The difference between the maximum and the minimum of median values in the order Perciformes was up to 50-fold.

In addition, in four fish classified to the order Clupeiformes, the ΣHBCDs concentrations ranged from 0.10 to 77.3 ng g^{-1} ww. The highest concentration of 77.3 ng g^{-1} ww (corresponds to 1706 ng g^{-1} lw) in the present study was observed in mamakari (*Sardinella zunasi*) from Chugoku/Shikoku. This level considerably exceeds reported levels in herring of the Baltic Sea (34–180 ng g^{-1}lw, Remberger et al., 2004). In eels (order Anguilliformes), the ΣHBCDs concentration ranged from 0.05 to 36.9 ng g^{-1} ww. The two higher concentrations (36.9 and 9.03 ng g^{-1} ww) were observed in eels (*Conger myriaster*) from Chubu and Chugoku/Shikoku, respectively. For eel in Belgium, the ΣHBCDs levels have been recently reported to be 394 (average), 73 (median), and 16–4397 ng g^{-1} lipid weight (range) (Roosens et al., 2010), these levels are several times higher than that of our data converted to lipid basis concentration: 81 (average), 21 (median) and 1.2–312 ng g^{-1} lipid weight (range). Therefore, the pollution of HBCDs may be also spreading rapidly in Europe. On the other hand, in shrimp classified as Crustacea, HBCDs were not detected. In octopus and squid classified as Mollusca, the ΣHBCDs concentrations ranged from ND to 0.55 ng g^{-1} ww. The ΣHBCDs concentrations ranged from ND to 0.04 in flounder (order Pleuronectiformes) and from ND to 0.62 ng g^{-1} ww in marbled rockfish (order Scorpaeniformes). The fish of Anguilliformes and a part of the fish of Perciformes have higher fat content, while the fish of Crustacea, Mollusca, Pleuronectiformes and Scorpaeniformes have lower fat content. Subsequently, the observations in this study would support the hypothesis that fat-rich fish can accumulate lipophilic pollutant HBCDs like PCBs (Thomann, 1995; Elskus et al., 2005). However, a positive relationship between ΣHBCDs and fat content for all the wild samples was not found except for the Japanese sea bass of Tohoku region, even when statistically analyzed using Spearman's rank correlation method (data not shown). From this, in addition to fat content of fish, various conditions, which are fish species-specific accumulation and metabolism, how much prey associated with pollutants are available, and how heavily sediment is polluted, are considered to affect the pollution of fish (predator) (Melwani et al., 2009). On the other hand, in farmed fish of salmon (Salmoniformes), sea bream (Perciformes) and oyster (Mollusca), the ΣHBCDs concentrations (median) were 1.29, 0.33 and 0.19 ng g^{-1} ww (see Table 1-2). Although it is difficult to comment on the state of HBCD pollution in the farmed fish due to the lack of details such as environment and feed, our data at least suggest that farmed fish are not free from HBCD pollution. Therefore, it will be necessary to monitor the pollution of farmed fish, taking into consideration future increase of the demand for farmed fish.

3.2. HBCD isomer profile of seafood samples

HBCD isomer contribution profile was examined in fish with ΣHBCDs concentrations higher than 20 ng g^{-1} ww and in fish with ΣHBCDs concentrations lower than 20 ng g^{-1} ww, using mainly

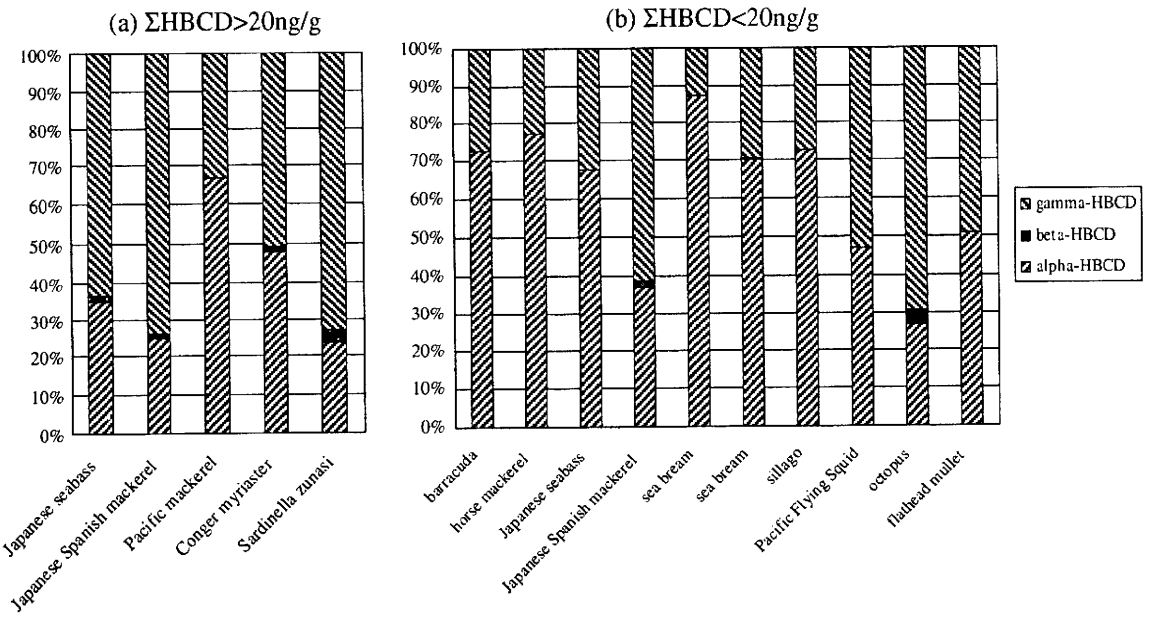

Fig. 2. HBCD isomer profile in the sea food samples of the Chubu region and one sample (*Sardinella zunas*) of the Chugoku/Shikoku region.

the results for fish of Chubu region. Fig. 2a and b shows the resulting HBCD isomer profiles in the two groups of fish, respectively. In fish with higher ΣHBCDs, the profiles showed a generally larger contribution of γ-HBCD as well as of α-HBCD, while in fish with lower ΣHBCDs, the profiles often showed a larger contribution of α-HBCD than other isomers except for octopus. The β-isomer was seldom found in fish samples except for extremely polluted samples. It was suggested that these trend of HBCD isomer profile could be found in the results of fish in the other three regions. In this study, we happened to detect high concentrations of γ-HBCD in mamakari (*S. zunasi*); which was 56.6 ng g^{-1} ww and the contributing ratio to ΣHBCDs (77.1 ng g^{-1} ww) was 73.2%. Considering that γ-HBCD is the major HBCD isomer in a flame-retardant HBCD product and much lower water-soluble than the other isomers (Hunziker et al., 2004), the fish in which γ-HBCD is dominantly found was thought to be strongly affected by near point pollution sources of HBCD. For example, the primary exposure to HBCDs adsorbed to sediment and/or the secondary exposure via prey can be speculated. The octopus, a benthos, of Chubu with γ-isomer predominant would be the just pollution case, although the ΣHBCDs level was lower (0.93 ng g^{-1} ww) (Fig. 2). On the other hand, α-HBCD tends to be also predominantly found in the farmed fish, as shown in Table 2. Concerning the stability of HBCDs, it has been already reported that γ-HBCD in HBCD products is eliminated by not only decomposition (Köppen et al., 2007; Larsen and Ecker, 1988) and/or rearrangement to the α-isomer upon being subjected to heating stress (Heeba et al., 2008), but also possible biodegradation under anaerobic conditions such as in sludge (Nordic Council

Table 2
List of estimated daily intakes of ΣHBCDs from food by various countries' populations.

Population	Intake of ΣHBCD (ng $kgbw^{-1}d^{-1}$)	Samples from which daily intake was estimated	Reference	Mainly contributing food	Estimation methods
Sweden	1.9/2.15	Animal food samples based on a food frequency	Lind et al. (2002)	Fish	Median intake (female/male)
UK	<5.9	Total diet study samples	FSA, UK (2006)	Fruit, milk	Upper bound estimation
UK	5.9–7.9	Total diet study samples	Driffield et al. (2008)	Meat, fish, vegetable	Upper bound estimation (divided by 60 kg bw)
Japan	3.7	Five fish samples (wild Anguilliformes, two species)	Present study	–	Median intake (divided by 50 kg bw)
	2.3	Five fish samples (farmed Salmoniformes)	Present study	–	Median intake (divided by 50 kg bw)
	1.3	30 fish samples (wild Perciformes, 10 species)	Present study	–	Median intake (divided by 50 kg bw)
Japan	0.45–34	Oysters and mussels	Ueno et al. (2010)	–	Minimum and maximum intakes (divided by 50 kg bw)
Netherlands	0.12	44 fish samples (10 species)	van Leeuwen and de Boer (2008)	–	Medium bound estimation (mean)
Netherlands	1.5/2.9	Total diet study samples	de Winter-Sorkina et al. (2003)	Meat	Lower bound/medium bound (mean)
Norway	0.2/0.3	Individual food samples based on a food frequency questionaire	Knutsen et al. (2008)	Fish	Lower bound/medium bound (mean)
Belgium	0.30	Eel	Roosens et al. (2010)	–	Median intake (divided by 60 kg bw)
Belgium	0.09/0.12	Duplicated diet	Roosens et al. (2009)	Meat, fish	Median/mean intake(divided by 60 kg bw)
USA	0.50	Total diet study samples	Schecter et al. (2010)	Meat	Lower bound estimation (mean)
China	0.432	Total diet study samples	Shi et al. (2009)	Meat	Medium bound estimation (mean)