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

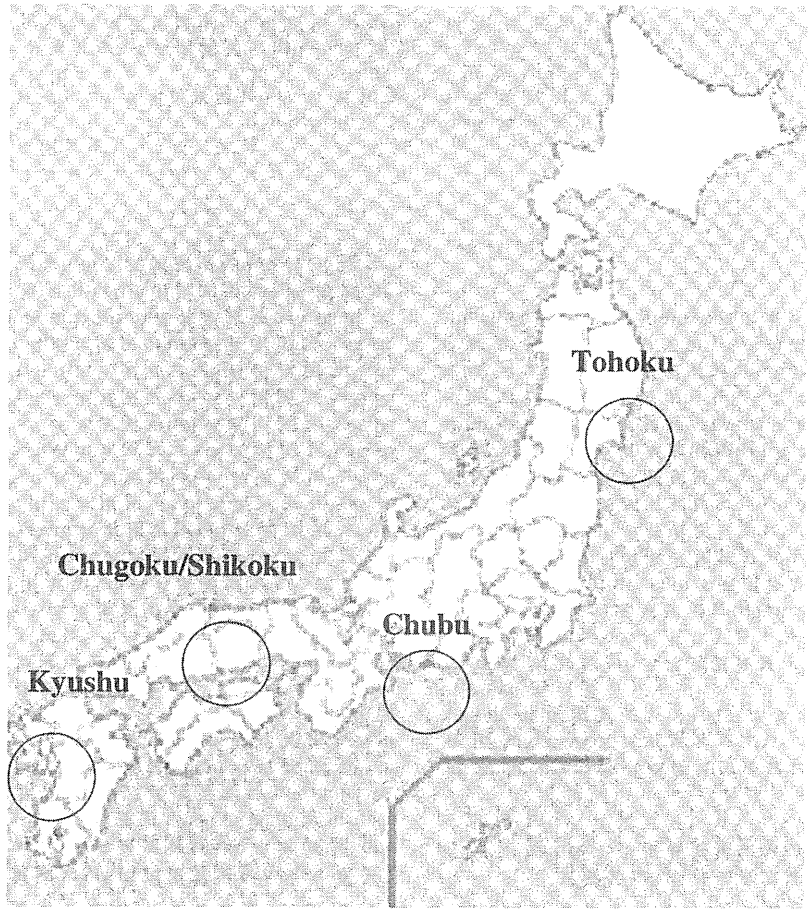

Fig. 1. Location of sampling sites.

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

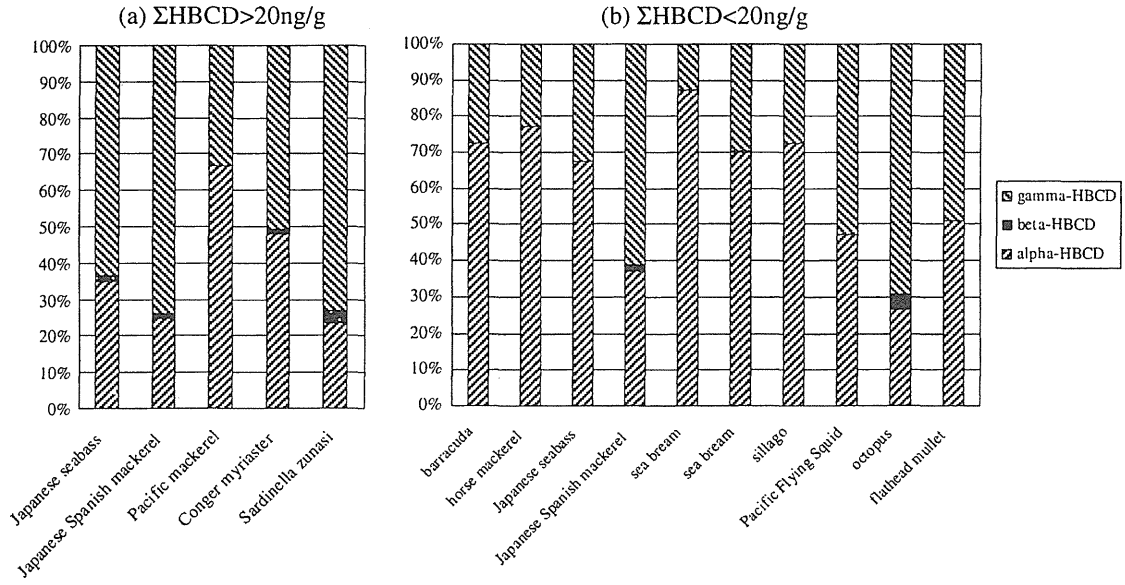

Fig. 2. HBCD isomer profile in the sea food samples of the Chubu region and one sample (*Sardinella zunas*) of the Chugoku/Shikoku region.

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)

of Ministers, 2008). As a result, it is considered that only stable α-HBCD possibly finally remains in the environment; therefore, wild and farmed fish inhabiting far from the pollution sources in coastal would naturally have profiles dominated by α-HBCD due to less uptake of prey much polluted with γ-HBCD. In fact, certain fish in deep sea have also been reported to be α-isomer dominant (Takahashi et al., 2010). Hence, correlation between stable α-HBCD but not ΣHBCDs and fat content was re-examined for the wild fish of the order Perciformes on the both cases of each region and all the regions; however, also here, no Spearman's rank correlation was found except for Japanese sea bass of the Tohoku region. The reason would be the same as that for the less correlation between ΣHBCDs and fat content as described in Section 3.1. From these results, it is speculated that the HBCD pollution in Japanese coastal fish at present may be ubiquitous, but uneven in concentration and isomer profiles among regions and fish species.

3.3. Estimation of daily intake of HBCDs

The daily intake of ΣHBCDs from fish was provisionally estimated. According to the Japan Nutrition Survey (The National Nutrition Survey in Japan, 2002), an average Japanese adult consumes 87.8 g of fish and fish products per day. Therefore, the intake of ΣHBCDs from fish can be simply calculated to be from 0 ng person^{-1} d^{-1} for both Pleuronectiformes and Crustacea to 184, 113 and 65.9 ng person^{-1} d^{-1} for Anguilliformes, farmed Salmoniformes and Perciformes, respectively, by multiplying median values of each category in Table 1 with the amount of consumed fish and fish products. However, it can range from 0 ng person^{-1} d^{-1} for Crustacea to 1700, 846 and 407 ng person^{-1} d^{-1} for Clupeiformes, Anguilliformes and Perciformes, respectively, when using the mean values instead of the median. To avoid overestimation and underestimation, it is considered proper to use median values of ΣHBCDs to calculate the representative daily intake of ΣHBCDs. On the assumption that the body weight of an average Japanese adult is 50 kg, the intakes from fish will become about 3.7, 2.3 and 1.3 ng (kg body weight)$^{-1}$ d^{-1} for Anguilliformes, farmed Salmoniformes and Perciformes, respectively, as the fish that result in the top three EDI values. These EDIs correspond to 0.000036%~0.000013% of the no-observed-adverse-effect level (NOAEL, 10.2 mg (kg body weight)$^{-1}$ derived from the two-generation reproductive toxicity study (Ema et al., 2008), and thus we can conclude that the EDI for the Japanese populace is not a serious amount.

In comparison with other studies carried out around the world, as shown in Table 2, the top three EDIs of our study (3.7, 2.3 and 1.3 ng (kg body weight)$^{-1}$ d^{-1}) were within the range of another Japanese EDI derived from oysters and mussels (Ueno et al., 2010). Furthermore, the EDIs of this study were below the EDI of the United Kingdom (Driffield et al., 2008; Fernandes et al., 2008) (5.9–7.9 ng (kg body weight)$^{-1}$ d^{-1}) and comparable to that of Sweden and the Netherlands (1.9/2.15 and 1.5–2.9 ng (kg body weight)$^{-1}$ d^{-1}) (Lind et al., 2002; de Winter-Sorkina et al., 2003), however, the EDI of Japan was above that of the United States (Schecter et al., 2010), Norway (Knutsen et al., 2008), Belgium (Roosens et al., 2009) and China (Shi et al., 2009) (0.50, 0.2/0.3, 0.09/0.12 and 0.432 ng (kg body weight)$^{-1}$ d^{-1}, respectively). Here, these intakes should be carefully interpreted, because there are differences in the samples from which intakes were estimated and calculation methods as shown in Table 2. In particular, daily intakes were calculated using concentrations in fish in the four studies of Japan, Netherlands and Belgium and the rest were calculated using total diet samples or wide range of food items. Ideally speaking, dietary intakes should be estimated using total diet samples; accordingly, this work is now underway in our laboratory. Because HBCDs are hydrophobic like PCBs, fish with high biomagnifications factors are considered very important among all foodstuffs as the source of human exposure to HBCDs, particularly for Japan, it will be allowed to consider intake from fish as provisional daily intake until total diet study is completed. However, when EDI is calculated using concentrations of highly contaminated fish species, sometimes it may exceed EDI derived using total diet samples. Therefore, it will be better that provisional intakes from fish seen in Table 2, which is Japanese intake from Anguilliformes (eel) in this study and Belgian intake from eel (Roosens et al., 2010) should be considered as the possible maximum estimates. In the studies of Sweden and Norway, the intake from fish was reported to be predominant. However, it was reported that as a HBCD contributor to EDI, meat and dairy food were important for the populations of USA and the other European countries. For China, meat and meat products were also important, except for Shanghai City, where is supposed to consume much fish as same as Japan. The daily intake from fish was recently reported to be 0.12 ng (kg body weight)$^{-1}$ d^{-1} in a Dutch study (van Leeuwen and de Boer, 2008). Taken together with the results of another Dutch study (de Winter-Sorkina et al., 2003), the intake from fish would account for at most 4% of the total EDI for the Dutch people. In the studies of UK and USA, the intake from fish accounted for at most ca.10% (FSA, 2006; Schecter et al., 2010). Thus, excluding several countries, the contribution of fish to total EDI may be now small for ΣHBCDs, compared with that for ΣPCBs (51%) (Schecter et al., 2001). However, if the consumption of HBCD products increases or continues hereafter, fish may become a bigger contributor in food for the intake of ΣHBCDs as same as the case for ΣPCBs.

In summary, from this study, it was suggested that the EDI of ΣHBCDs by the Japanese people is higher compared with foreign peoples, because fish is a favorite foodstuff for the Japanese people, however, it is not yet especially serious level right now, judged from the value of LOAEL for HBCD.

4. Conclusion

The HBCD isomer-specific monitoring of food is so far insufficient and therefore is needed now in order to assess the status of HBCD pollution accurately (Covaci et al., 2006; Kakimoto et al., 2008a). Through this study, for the first time it was discovered that ubiquitous HBCD (mainly α-HBCD) pollution exists in various types of fish collected at markets near the coast of four regions of Japan, and it was also suggested that the concentrations and HBCD isomer profiles of fish would differ depending on how much prey associated with HBCDs in each region is available and what species fish is. In some fish samples, extremely high ΣHBCDs concentrations with the γ-HBCD isomer dominant were observed, suggesting strongly influence by HBCD discharged from industrial plants. Moreover, the daily intakes in Japan from fish of the three categories were calculated to be higher in this study, compared with the reported dietary daily intake in several other countries. It has been recently reported that dust can be a greater source of exposure to HBCDs like polybrominated diphenyl ether (Roosens et al., 2009), we also should pay attention to such other sources. However, when considering the frequency and probability of exposure, the importance of food as an exposure source remains. From the viewpoint of prevention of adverse health effects due to unintended body burden of HBCDs detected in fisherman's serum (Weiss et al., 2006) and human milk (Johnson-Restrepo et al., 2008; Kakimoto et al., 2008b), it will be necessary, as long as HBCD products are used, to monitor the time course variation (trend) of the contents of HBCD pollutants in food, especially in fish, which is well-known to accumulate and magnify chemical pollutants via the food chain.

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技術論文

食品中メチル水銀の定量分析のためのフェニル誘導体化GC-MS法の開発

坂本　智徳[R1]，赤木　浩一[1]，渡邉　敬浩[2]，松田りえ子[2]，樋脇　弘[1]

フェニル誘導体化-ガスクロマトグラフィー-質量分析（GC-MS）法による食品中メチル水銀の分析法を検討した．臭化カリウム・硫酸銅(II)飽和硫酸混液によってメチル水銀を試料から分離し，トルエンに抽出したのちL-システイン溶液に逆抽出した．抽出したメチル水銀をテトラフェニルホウ酸ナトリウムによってフェニル誘導体化し，*n*-ヘプタンに抽出した．誘導体化したメチルフェニル水銀を，1級-2級アミン（PSA）ミニカラムを用いて精製し，GC-MS（SIM）により測定した．5種の認証標準試料（CRM-7402a，CRM-7403a，BCR-463，ERMCE-464及びDOLT-4）を用いた分析法の性能評価の結果，真度（%）98～108，併行精度（RSD%）10未満，室内精度（RSD%）15未満であり，厚生労働省によって示された性能基準を満たす分析法であることが確認された．

1　緒　　言

メチル水銀は，有機水銀化合物の一つであり，多量に摂取すると水俣病のような中毒症状を引き起こすほか，胎児における脳の成長にも影響を及ぼすことが報告されている[1]．またメチル水銀は，生物濃縮のため食物連鎖の上位に位置するマグロやクジラ類のような大型水産動物に特に多く含まれている．これに対し厚生労働省は，該当する水産動物の大量摂取を介した健康危害の未然防止の観点から，妊婦らを対象とし，摂食に関する注意を喚起している[2]．

食品に含まれるメチル水銀の分析法としては，厚生省環境衛生局長通知[3]にガスクロマトグラフィー-電子捕獲型検出（GC-ECD）法が示されている．しかしこの分析法では，発がん性が指摘されているベンゼンを使用することに加え，バックドカラムの使用が規定されているために分解能が低く，さらに検出器の選択性及び安定性にも問題が認められる．

上記のGC-ECD法のほかにも，メチル水銀の定量を目的とする分析法には，魚介類や血液等を対象としたジチゾン抽出法[4]，塩酸酸性・トルエン抽出法[5]，アルカリ分解抽出法[6]，臭化カリウム・硫酸銅(II)飽和硫酸混液添加トルエン抽出法[7]及び超臨界流体抽出法[8]などにより抽出し，GC-ECDや高速液体クロマトグラフィー-誘導結合プラズマ質量分析法（HPLC-ICP-MS）[9]により測定する方法のほか，メチル水銀をエチル，プロピルまたはフェニル誘導体化することで揮発性を高め，注入装置に固相マイクロ抽出（SPME）等を用いてガスクロマトグラフィー-原子蛍光分析法（GC-AFS）[6][10]またはGC-ICP-MS[11]で測定する方法が報告されている．しかし，これらの方法はいずれも，感度及び選択性に優れるものの，高額かつ目的に特化した仕様の測定機器を必要とするため汎用性が低い．より汎用性の高いメチル水銀分析法として，抽出には溶媒抽出法を，測定にはGC-MSを用いる方法が適当と考えられたが，食品分析の分野においては報告がない．

本研究では，食品中のメチル水銀定量を目指した高感度分析法の開発を目的とし，フェニル誘導体化を介して，農薬等の一斉分析法における測定機器としても採用されているGC-MSにより測定する方法について検討し，その性能を明らかにしたので報告する．

2　実　　験

2・1　試薬等

メチル水銀標準品はジーエルサイエンス製塩化メチル水銀を使用した．標準原液は標準品58.2 mgをトルエンで溶解し50 mLに定容した（メチル水銀として1000 mg L^{-1}）．分液漏斗に標準原液1 mL及び1 %システイン溶液100 mLを採り，15分間振とうしたのち水層が清澄になるまで静置し，採取した水層を標準溶液とした（メチル水銀として10 mg L^{-1}）．検量線用標準溶液は標準溶液を1 %システイン溶液で希釈し，1～100 ng mL^{-1}の範囲で5濃度の溶液を調製した．

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Table 1 Analytical conditions for the determination of phenylated methylmercury by GC-MS

Gas chromatograph	Thermo Scientific TRACE GC ULTRA
Inlet temperature	250 ℃
Column	GL Science InertCap 5MS/NP PROG 10M + TL (30 m, 0.25 mm I.D., 0.25 μm film thickness)
Carrier gas	He 1.0 mL min^{-1}
Oven temperature program	50 ℃ (1 min), 10 ℃ min^{-1} to 200 ℃ (0 min)
Mass spectrometer	Thermo Scientific TSQ QuantumGC
Transfer line temperature	280 ℃
Ion source temperature	280 ℃
Mode	SIM
Monitor ions	m/z 292[a], 294, 277

a) Quantitative monitor ion.

Walpole 緩衝液 (pH 1.0)は 1 mol L^{-1} 酢酸ナトリウム溶液 250 mL に 1 mol L^{-1} 塩酸を加え pH を 1.0 に調整したのち，水で 1 L に定容して使用した．

2 % テトラフェニルホウ酸ナトリウム溶液はテトラフェニルホウ酸ナトリウム 2.0 g を水で溶解し 100 mL に定容した．

エチレンジアミン-*N*-プロピルシリル化シリカゲルミニカラム (PSA) はジーエルサイエンス製 InertSep PSA (200 mg/3 mL) を *n*-ヘプタン 2 mL でコンディショニングして使用した．

n-ヘプタンは和光純薬工業製 *n*-ヘプタン (環境分析用) を使用した．

その他の試薬は残留農薬試験用あるいは特級品を使用した．

2・2 試 料

試料には 5 種の認証標準試料 [CRM-7402a (タラ魚肉粉末)，CRM-7403a (メカジキ魚肉粉末)，BCR-463 (マグロ魚肉粉末)，ERMCE-464 (マグロ魚肉粉末) 及び DOLT-4 (ツノザメ肝臓粉末)] を用いた．

2・3 装 置

ホモジナイザーは KINEMATICA 製ポリトロン PT3100 を，遠心分離機は久保田商事製 6200 を，ガスクロマトグラフは Thermo Scientific 製 TRACE GC ULTRA を，質量分析計は Thermo Scientific 製 TSQ Quantum GC を使用した．GC-MS の測定条件は Table 1 に示した．

2・4 操 作

2・4・1 抽 出 試料約 0.3 g を 50 mL 容ポリプロピレン製チューブに精確に秤量し，1 mol L^{-1} 臭化カリウム溶液 10 mL を加え混合したのち，硫酸銅(II) 飽和 4 mol L^{-1} 硫酸 10 mL 及びトルエン 15 mL を加え 45 分間振とうし，3000 rpm で 15 分間遠心分離した．1 % システイン溶液 4 mL を入れた容器にトルエン層 10 mL を分取して 15 分間振とうし，3000 rpm で 15 分間遠心分離したのち，水層を分取し抽出液を得た．エマルジョンが生じた試料については，トルエン層をピペットで除去したのち，*n*-ヘキサン 15 mL を加えて 5 分間振とうし，3000 rpm で 15 分間遠心分離してヘキサンを除去し抽出液を得た．

2・4・2 フェニル誘導体化 抽出液及び各濃度の標準溶液各 1 mL に Walpole 緩衝液 (pH 1.0)5 mL を加え混合したのち，*n*-ヘプタン 2.5 mL 及び 2 % テトラフェニルホウ酸ナトリウム溶液 1 mL を加え混合し，30 ℃ に設定したウォーターバス中で 10 分間ごとに混和しながら 1 時間静置した．ヘプタン層を PSA に全量負荷し，初流 1 mL を廃棄して通過液を採取し測定溶液とした．

2・5 定 量

2・4・2 に従い調製した溶液を GC-MS に注入し測定することにより得られたクロマトグラムのピーク面積から，絶対検量線法により試験溶液中のメチル水銀の濃度を求め，さらに下記の算術式に従って試料の含有量を算出した．

メチル水銀含有量(mg kg^{-1}) = 検量線より求めた試験溶液濃度(ng mL^{-1}) × 抽出液量(4 mL) × 15/10 × 希釈倍率/試料採取量(0.3 g)/1000

検量線の濃度範囲を超過するピーク面積が得られた試料については，抽出液を 1 % システイン溶液で適宜希釈することで測定溶液を再調製した．なお，CRM-7402a，CRM-7403a 及び DOLT-4 については，得られたメチル水銀含有量に 0.93 (水銀とメチル水銀との平均分子量の比) を乗じて，水銀としての含有量もあわせて算出した．

2・6 分析法の性能評価

食品中の化学物質分析法を性能評価するためのガイドラインとして，厚生労働省より“食品中の金属に関する試験法の妥当性評価ガイドライン[15]”及び“食品中に残留する農薬等に関する試験法の妥当性評価ガイドライン[16]”が示されている．これらガイドラインを参考に，分析法の性能を評価することとした．性能評価を目的とする分析結果を

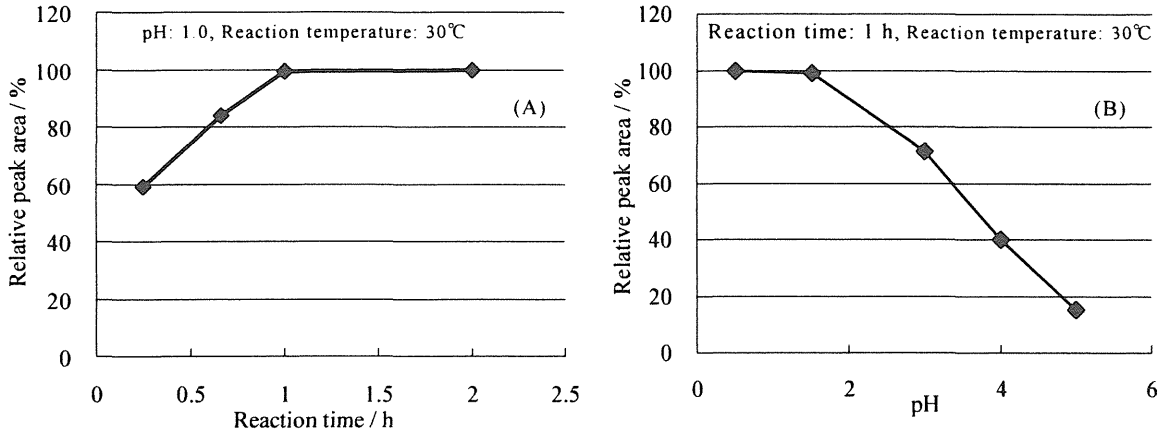

Fig. 1　Relationships between the relative peak area and (A) reaction time and (B) pH

得るための実験計画には，分析者 1 名による枝分かれ実験（2 併行，5 日間）を採用した．本実験計画に従い得られた分析値を一元配置の分散分析により解析し，算出された分散から併行精度及び室内精度を推定した．また真度は各標準試料に付与された認証値に対する平均値（$n = 10$）の割合として算出した．推定された真度及び精度は，濃度範囲が対応しているガイドライン[15]中に示された目標値と比較することで評価した．なお，性能評価に先立ち，試薬のみを操作した試薬ブランクを測定した結果，メチル水銀定量の妨げとなるピークが現れなかったことにより選択性を確認した．また，1 ng mL^{-1} の標準溶液を測定した結果得られたシグナル・ノイズ比（S/N）が 10 以上であることにより，マトリクス非共存下での定量下限を確認した．

3　結果及び考察

3・1　誘導体化条件の検討

メチル水銀の誘導体化には，エチル[11]，プロピル[10]及びフェニル誘導体化法[6)12)~14)]が報告されている．エチル及びプロピル誘導体化法では得られる誘導体の揮発性が非常に高く，GC への注入には SPME 等の装置が必要であった．一方，環境分析の分野において，フェニル誘導体化ののち n-ヘキサンに抽出し GC-MS により測定する，SPME 等を必要としない分析法[14]が報告されている．しかし，この分析法は標準溶液の定量下限が 100 ng mL^{-1} と高く，環境水の分析に対応するため濃縮操作を行っている．食品中のメチル水銀分析においては，定量下限をより下げる必要があると考えられたため，食品中のメチル水銀分析に用いられている GC-ECD 法[5]の定量下限（5 ng mL^{-1}）の 1/5～1/10 を目標としてフェニル誘導体化反応条件を検討した．また，反応条件の最適化とあわせ，測定のノイズを減少させ，より低濃度のメチル水銀の定量を可能とする効果を期待し，誘導体化反応により生じる副生成物の除去について検討した．なお，本精製により，より夾雑物の少ない測定液が注入されることになるため，GC カラムの劣化抑制効果も期待されると考えた．

標準溶液を 1 % システイン溶液で希釈し調製したメチル水銀 100 ng mL^{-1} 溶液を用いて，フェニル誘導体化反応の至適 pH，温度及び時間を検討した．Cai ら[12]は，pH 4～10 の間ではフェニル化の進行と pH との間に大きな相関はないことを報告している．本研究では，メチル水銀をシステイン錯体としてトルエンから逆抽出していることから，フェニル誘導体化の際にメチル水銀-システイン錯体を解離させる必要があると考えられた．そこで，至適 pH については，より酸性な条件下で，フェニル誘導体化の効率に対する影響を検討した．検討結果の一部を Fig. 1 に示す．結果は，検討した反応条件の範囲内で得られた最大のピーク面積値に対するその他のピーク面積値の比を百分率で示した．反応時間を 1 時間以上，反応時の pH を 0.5～1.5 に調整することで，フェニル誘導体化の効率が最大となることが示唆された．反応温度の検討結果については示していないが，同様の検討の結果から，30 ℃ とすることで誘導体化効率が最大となることが明らかとなった．これらの結果に基づき，以後，pH 0.5～1.0 の範囲内で 30 ℃，1 時間の誘導体化を行うこととした．

一方，誘導体化反応の進行に伴い，ヘプタン層に橙色の色素が生成した．そこでグラファイトカーボン及び PSA をそれぞれ充填した 2 種のカートリッジカラムを用いた精製について検討した．その結果，いずれのカラムでも着色成分の除去は可能であったが，分析対象となるメチルフェニル水銀の回収率は，いずれのカラムを用いるかにより大きく異なり，グラファイトカーボンカラムでは 14 %，PSA カラムでは 105 % であった．

以上の結果から，誘導体化反応終了後に PSA カラムによる精製を行うこととした．

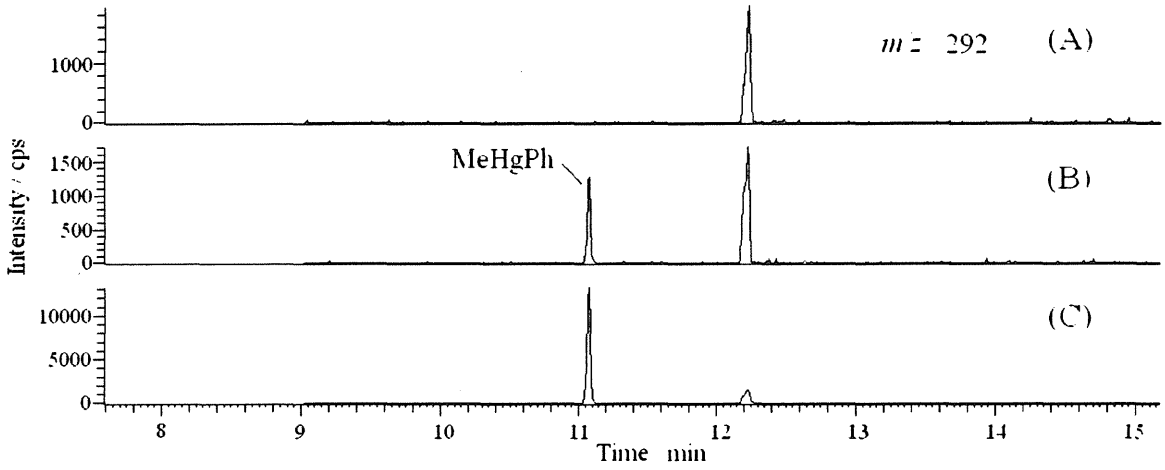

Fig. 2 Mass chromatograms of phenylated methylmercury (m/z = 292) in (A) blank solution (0 ng mL^{-1}), (B) standard solution (5 ng mL^{-1}) and (C) extract of CRM-7402a

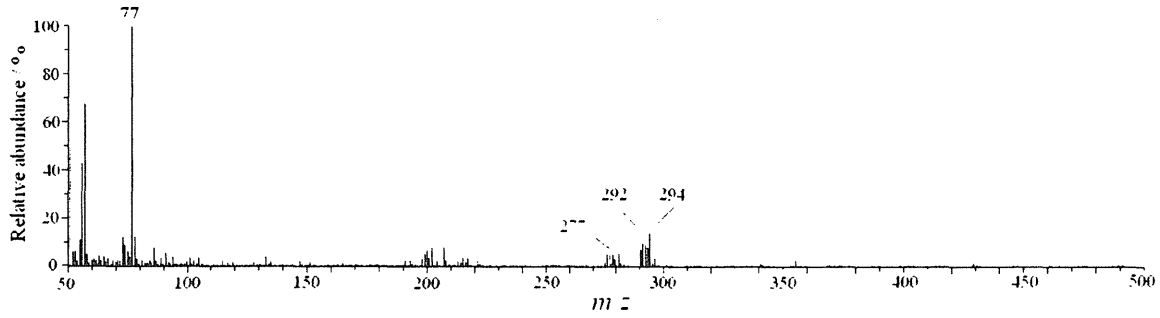

Fig. 3 Mass spectrum of phenylated methylmercury

3・2 GC-MS 測定条件の検討

誘導体化を行わずにガスクロマトグラフによりメチル水銀を分析するためのキャピラリーカラムとして，液相にアルキレングリコールフタル酸エステルポリマーを用いたカラムが市販されているが，最高使用温度が160 ℃ と低くカラムブリードが大きく，MS への接続には適さなかった．また，一般的な微極性（5 % フェニル−95 % メチルポリシロキサン），中極性（トリフルオロプロピルメチルポリシロキサン）及び高極性（ポリエチレングリコール）カラムを用いた分離についても検討したが，いずれのカラムを用いた場合でもピークの対称性が十分とは言えなかった．さらに，一定濃度範囲におけるフェニル化メチル水銀量とピーク面積値に十分な相関（直線性）が得られなかった．そこで，至適化した反応条件下でメチル水銀をフェニル化メチル水銀へと誘導したのち，5 % フェニル−95 % メチルポリシロキサンカラムを用いて分離することについて検討した．その結果，Fig. 2 に示すように対称性のよいピークが観察された．この結果に基づき，本カラムの使用を決定し，さらに定量性及び感度の向上を目的に，GC-MS 条件を検討した．100 ng mL^{-1} メチル水銀標準溶液を試料としてフェニル誘導体化及び精製した後に GC-MS に注入し，SCAN 測定（m/z 50～500, EI）して得られたマススペクトル（Fig. 3）をもとに，m/z 277 (^{200}HgPh$^+$)，292 (Me^{200}HgPh$^+$) 及び 294 (Me^{202}HgPh$^+$) をモニターイオンとして，SIM モードにより定量する測定条件を設定した．モニターイオンのうち，最も高い S/N が得られた m/z 292 を定量イオンに，m/z 277 及び 294 を定性イオンとした．

続いて検量線のダイナミックレンジについて検討した結果，1～100 ng mL^{-1} の濃度範囲で作成した代表的な検量線の相関係数は 0.998，傾きは 916，切片は −778 であった．

また，標準溶液の定量下限（$S/N \geqq 10$）は，1 ng mL^{-1} であった．

3・3 前処理方法の検討

魚介類に分類される食品群には，メチル水銀を含む食品が高頻度に含まれており，またその濃度は個々の食品において大きく異なるものと考えられる[17]．一方，前処理方法を含む分析法の検討には明らかな濃度のメチル水銀を含む均質な試料が不可欠である．しかし実際には，メチル水銀を含まない試料の入手あるいは，一定濃度を含む均質な試

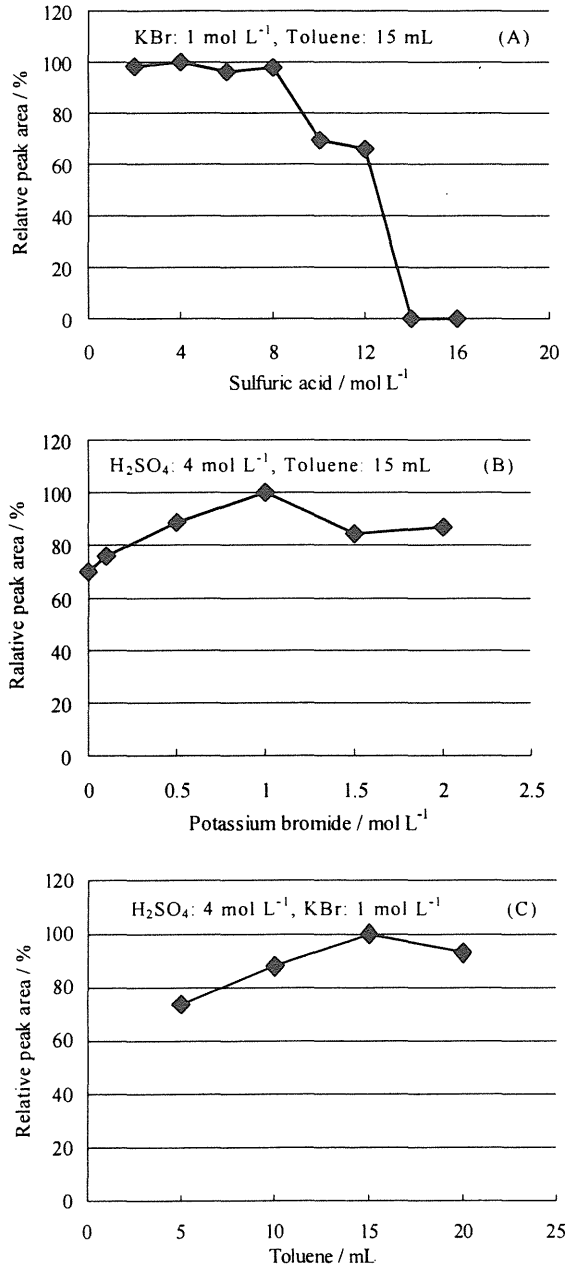

Fig. 4 Relationships between the relative peak area and (A) concentration of sulfuric acid, (B) concentration of potassium bromide and (C) volume of toluene

料の調製は極めて困難である．分析法の真度推定に使用する試料として，第一に認証標準試料を選択することが推奨されていることも勘案し，本研究では，メチル水銀含有量が明らかな認証標準試料を用いることとした．

産業総合研究所が制作した認証標準試料 CRM-7402a（基材：タラ）を用いて，試料の前処理法を検討した．本試料を対象に，塩酸酸性−ベンゼン抽出[11]を試みたところ，強固なエマルジョンを生じ，メチル水銀を効率よく抽出するためには 3 回以上抽出操作を繰り返すことが必要であった．また，ベンゼンをトルエンに変更しても同様の結果であったことから，塩酸の添加がエマルジョン形成の一因と考えられた．そこで，臭化カリウム・硫酸銅(II) 飽和硫酸混液添加トルエン抽出法[7]を用いてエマルジョン形成の抑制及び抽出率の向上を検討した．

硫酸ならびに臭化カリウムの濃度，トルエン量，振とう時間及びシステイン濃度について最適化を行った．検討の結果，硫酸ならびに臭化カリウム濃度及びトルエン量が抽出率に大きく影響することが明らかとなった．結果を Fig. 4 に示す．硫酸濃度を 4 mol L^{-1}，臭化カリウム濃度を 1 mol L^{-1}，トルエン量を 15 mL，振とう時間を 45 分間，システイン濃度を 1 % とすることで，1 回の抽出操作で 95 % 以上の抽出率が得られたことから，上記の条件を抽出条件として採用した．

採用した条件に従い，メカジキ，マグロ及びツノザメを基材とする他の認証試料（CRM-7403a，ERMCE-464 及び DOLT-4）からの抽出を試みた．その結果，トルエン抽出時にエマルジョンは生じなかったが，1 % システイン溶液に逆抽出する際に CRM-7403a，ERMCE-464 及び DOLT-4 において，界面に若干のエマルジョンが生じた．試料に由来する両親媒性物質の影響が考えられたため，トルエンを除去したのち水層を *n*-ヘキサンで洗浄したところ，エマルジョンを消失させることが可能であった．

3･4　分析法の性能評価

タラ，メカジキ，マグロ及びツノザメを基材とする計 5 種の認証標準試料（CRM-7402a，CRM-7403a，BCR-463，ERMCE-464，DOLT-4）を用いて，開発した分析法の性能を評価した．実験計画及び性能評価の目標値は，厚生労働省により通知されたガイドライン[15]に準じた［2 併行 5 日間，目標値：真度（%）70～120，併行精度（RSD%）10 未満，室内精度（RSD%）15 未満］．

性能評価の結果は Table 2 に示すように，真度（%）98～108，併行精度（RSD%）10 未満，室内精度（RSD%）15 未満であり，試料によって若干の違いはあるものの，いずれの認証標準試料についても，ガイドラインに示された性能の目標値を満たす結果が得られた．また，魚種や部位が多岐にわたる認証標準試料を性能評価に使用したが，これらの試料間に明確な差異は認められなかった．以上の結果から，本分析法をメチル水銀分析法として用いることの基本的な妥当性が確認されたと判断できる．今後，適用する食品や濃度ごとに適宜性能を評価し，運用することが可能であると考える．

4　結　　言

フェニル誘導体化を介した GC-MS 法による食品中メチル水銀の分析法の開発を検討し，その性能評価を行った．

Table 2 Evaluation of the performance characteristics of the method

Sample	Certified value [a] (mg kg^{-1})		Measured value (mg kg^{-1}) Days 1st	2nd	3rd	4th	5th	Mean [b] (mg kg^{-1})	Trueness (%)	Repeatability (RSD%)	Reproducibility within laboratory (RSD%)
CRM-7402a (cod fish tissue)	0.58 ± 0.02 (as Hg)	Portion 1	0.675	0.507	0.662	0.610	0.524	0.571	98	9.3	13.4
		Portion 2	0.570	0.484	0.655	0.483	0.543				
CRM-7403a (swordfish tissue)	5.00 ± 0.22 (as Hg)	Portion 1	4.975	4.112	5.382	5.378	5.365	4.952	100	9.9	11.7
		Portion 2	5.425	4.444	4.697	5.627	4.113				
BCR-463 (tuna fish tissue)	3.04 ± 0.16 (as CH_3Hg)	Portion 1	2.564	3.381	3.734	3.246	3.527	3.280	108	9.6	11.1
		Portion 2	3.157	3.675	3.426	3.243	2.851				
ERMCE-464 (tuna fish tissue)	5.50 ± 0.17 (as CH_3Hg)	Portion 1	6.394	4.963	5.351	6.592	5.243	5.694	104	4.1	14.9
		Portion 2	6.707	4.932	5.538	6.622	4.598				
DOLT-4 (dogfish liver)	1.33 ± 0.12 (as Hg)	Portion 1	1.441	1.265	1.435	1.344	1.273	1.364	103	7.1	10.0
		Portion 2	1.498	1.302	1.358	1.595	1.128				

a) Contain their expanded uncertainly (k = 2 is the coverage factor). b) n = 10.

食品試料からの抽出方法を臭化カリウム・硫酸銅(II)飽和硫酸混液添加トルエンを用いた方法とすることで，発がん性が指摘されているベンゼンを使用することなく，効率よくメチル水銀を抽出することが可能となった．抽出したメチル水銀をフェニル化することで，一般的な5％フェニル・95％メチルシロキサンカラムにより良好に分離することが可能となった．

本法の性能は，平成22年12月24日厚生労働省医薬食品局食品安全部通知食安発1224第1号「食品中の金属に関する試験法の妥当性評価ガイドラインについて」に示された性能の目標値を満たしていた．この結果により，本法を食品中のメチル水銀分析法とすることの妥当性が確認されたと判断できる．今後，試料の採取重量等も含め，本法の適用可能性について生鮮食品等を用いた検討を進めたい．

本研究は，平成22年度厚生労働科学研究費補助金・食品の安心・安全確保推進研究事業（課題名　食品を介したダイオキシン類等有害物質摂取量の評価とその手法開発に関する研究）の一部として実施した．

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Development of the GC-MS Method Following Phenylation to Quantify Methylmercury in Foods

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A method for the determination of methylmercury in foods by GC-MS method following phenylation was investigated. Methylmercury was isolated by acid leaching (mixture of potassium bromide solution and sulfuric acid saturated with copper sulfate), the extraction of methylmercury into toluene, then back-extraction into L-cysteine solution. Methylmercury was phenylated with sodium tetraphenylborate, and extracted into *n*-heptane. Phenylated methylmercury was purified by PSA mini-column, then analyzed by GC-MS (SIM). As a result of the performance evaluation using five certified reference materials (CRM-7402a, CRM-7403a, BCR-463, ERMCE-464 and DOLT-4), trueness (%), repeatability (RSD%) and reproducibility within laboratory (RSD%) was 98-108, less than 10 and less than 15, respectively. It was shown that the method satisfy the performance satisfied criteria set by Ministry of Health, Labor and Welfare.

Keywords : methylmercury ; GC-MS ; phenylation.

CHARACTERIZATION OF NATURAL AHR LIGANDS IN HEALTH FOODS ESTIMATED BY IN VITRO REPORTER GENE ASSAY

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Introduction

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.[1)] Additionally, it is discussed that the signal transduction of natural AhR ligands, which occurs after AhR activation, should differ from that of dioxins. In this study, we examined the binding ability of fifty extracts prepared from kinds of commercial supplements and health foods containing high concentrations of their respective ingredients to the AhR using reporter gene assay. At the same time, the active sample extracts were fractionated to characterize the AhR active substances, and reversed-phase HPLC analysis was conducted for the active fractions

Materials and methods

1. Extraction and isolation

Fifty supplements and health foods were from drug stores in Japan. The samples were prepared as follows: Tablets were powdered and the contents of capsules and soft capsules were used for sample preparation. The materials (1 g) were homogenized in 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 partition (each 30 mL) to give three extracts: *n*-hexane, ethyl acetate, and water soluble portions.

2. HPLC conditions

HPLC analysis was carried out using a Shimadzu Prominence system. Conditions were as follows: column, L-column ODS (5 μm, 150 × 2.1 mm i.d.); 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.

3. Estimation for AhR ligand activity

For the identification of AhR-activating materials, a CALUX assay was used.[2)] When mouse hepatoma (H1L6.1c2) cells are exposed to ligands such as dioxins, luciferase protein synthesis is inducted. The amount of light emitted by the luciferase protein is correlated directly with the dioxin level, and this system is used as a simple dioxin monitoring method (Figure 1).

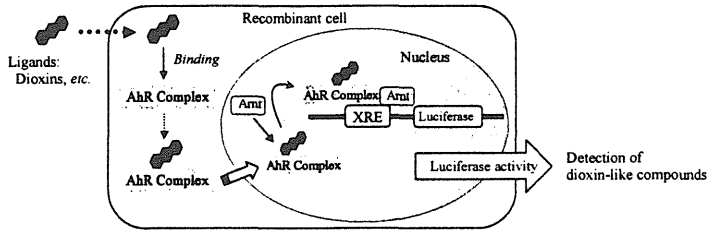

Figure 1. AhR-mediated *in vitro* bioassay (CALUX assay)

Results and discussion

Although most of the samples showed no dioxin-like activity even at a high concentration, some samples exhibited activity at high concentration in the order of mg/mL in dose-dependent manner. In order to characterize the active components of each sample from soybean-related samples (No. 30 and 31), sesame (No. 29), and propolis (No. 26), AhR activity was measured for the respective *n*-hexane, ethyl acetate, and water fractions. The *n*-hexane fraction of the propolis extract sample exhibited AhR activity, and 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 (Figure 2).

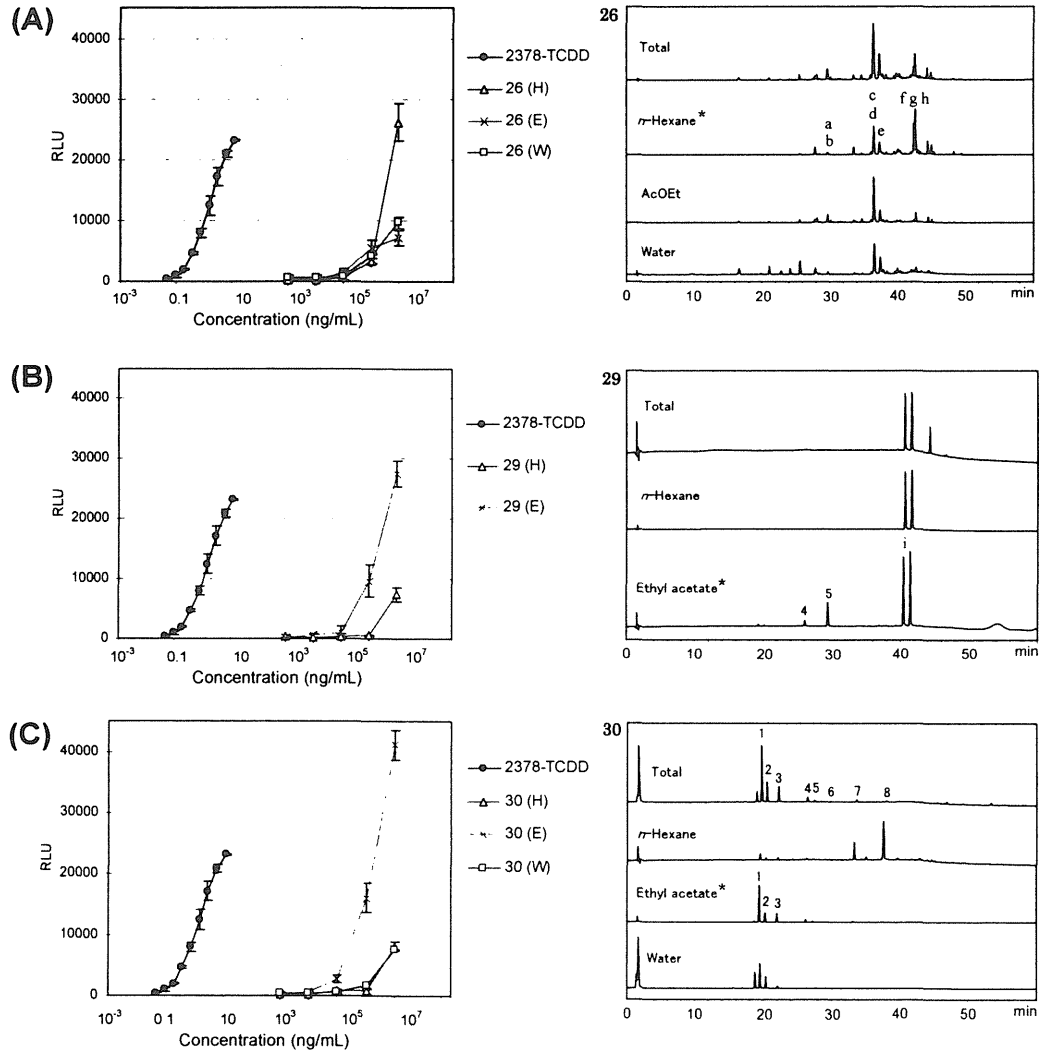

Figure 2 (continued on the following page).

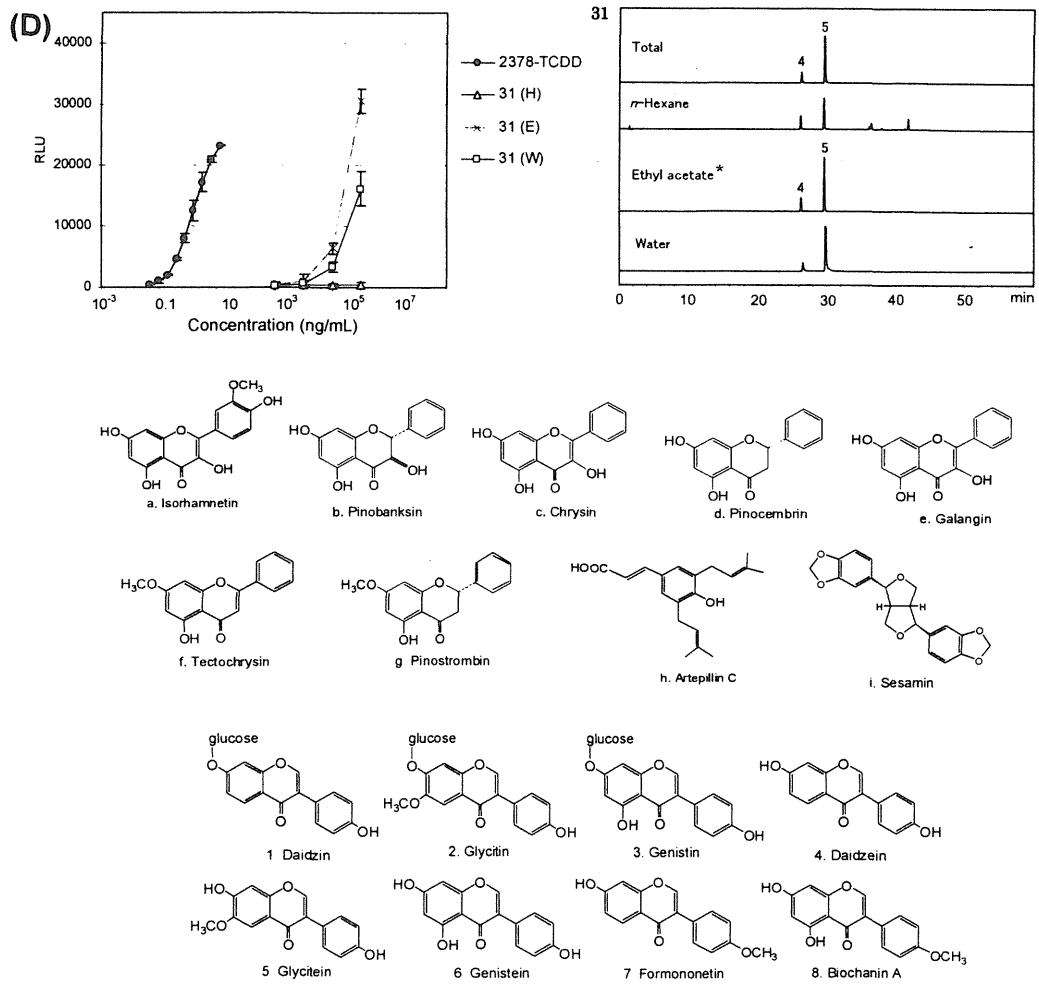

Figure 2. Concentration-response curve and RP-HPLC profiles of active samples and TCDD for the induction of luciferase activity in the CALUX assay.
(A) Propolis (No. 26), (B) Sesame (No. 29), (C) Soy-bean related samples (No. 30), (D) Soy-bean related samples (No. 31)
H, *n*-Hexane fraction; E, Ethyl acetate fraction; W, aqueous fraction
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 in HPLC were detected at 254 nm except sample 26 (at 280 nm). Sample 29 did not provide an aqueous fraction for analysis. * AhR activated fraction

HPLC analysis of the active fractions of sesame and soybean-related samples identified isoflavones, such as daidzein and glycitein. The *n*-hexane fraction of the propolis product which showed AhR activity contained eight compounds such as tectochrysin and pinocembrin. Among these compounds, tectochrysin showed remarkable