those reported in the UK (91 ng·d^{-1})[6], USA (88 ng·d^{-1})[7], Spain (82 ng·d^{-1})[8], Japan (68 ng·d^{-1})[4], Sweden (51 ng·d^{-1}) (middle bound)[9], and Belgium (23 ng·d^{-1})[10]. Assuming that a typical Japanese adult weighs merely 50 kg, the lower, middle, and upper bound intakes were estimated to be 0.0009, 0.007, and 0.012 μg·kg^{-1}·d^{-1} for pentaBDE and 0.0004, 0.006, and 0.012 μg·kg^{-1}·d^{-1} for decaBDE, respectively. These values were 2–5 orders of magnitude lower than the reference doses of penta- and decaBDE (2 and 10 μg·kg^{-1}·d^{-1}, respectively), both of which were

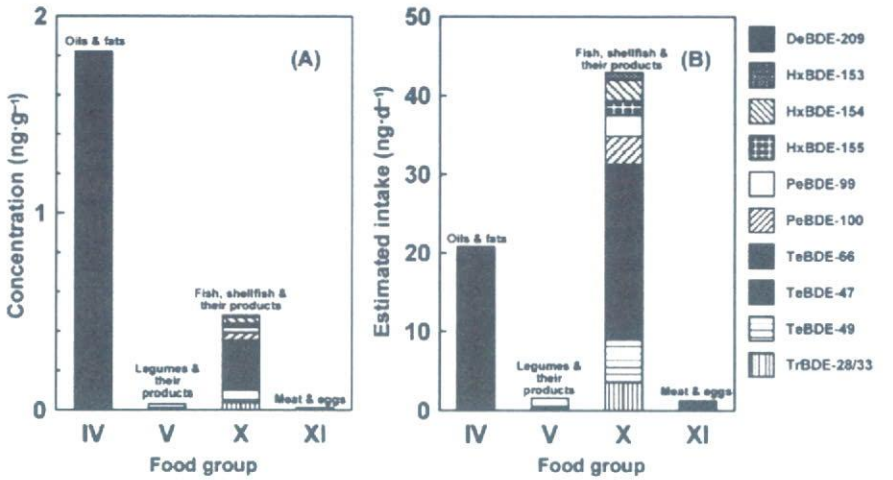

Fig. 2 Concentrations of PBDEs in foods (A) and estimated daily intakes of PBDEs from foods (B)

Table 2. Information of vegetable oil samples

No.	Oil composition	Country of origin	Bottle material	JAS certified
1	Rapeseed oil	NA	PET	No
2	Rapeseed oil	NA	PET	Yes
3	Rapeseed oil	NA	PE, EVOH	Yes
4	Rapeseed oil	Japan	PET	No
5	Rapeseed oil	Japan	Glass	No
6	Rapeseed oil	Japan	Glass	No
7	Brended oil (rapeseed, soybean)	NA	PE, EVOH	Yes
8	Brended oil (rapeseed, soybean)	NA	PE, EVOH	Yes
9	Corn oil	NA	PET	Yes
10	Corn oil	NA	PET	No
11	Corn oil	NA	PE, EVOH	No
12	Safflower oil	NA	PET	Yes
13	Safflower oil	NA	PET	Yes
14	Sesame oil	NA	Glass	No
15	Sesame oil	NA	Glass	Yes
16	Brended oil (perilla, sesame)	NA	Glass	No
17	Olive oil	Spain	Glass	No
18	Brended oil (unspecified)	NA	PET	No

Abbreviation: NA, not available; PET, polyethylene terephthalate; PE, polyethylene; EVOH, ethylene-vinyl alchol copolymer; JAS, Japanese Agricultural Standard

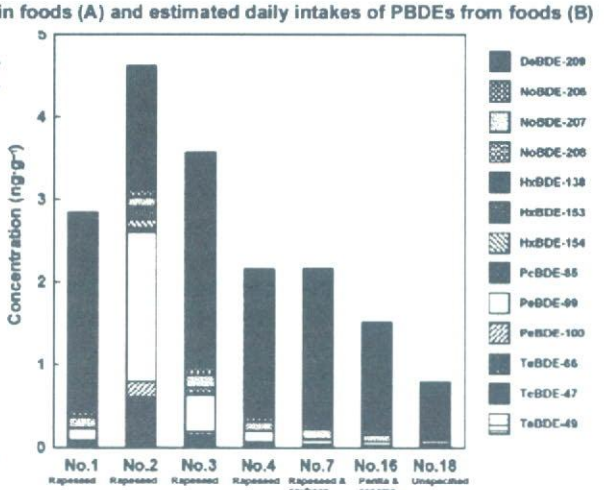

Fig 3. Concentrations of PBDEs in vegetable oil samples

Table 3. Comparison of estimated dietary intake of PBDEs in defferent countries

Country	Daily intake per capita (ng·d^{-1})*			Sampling year	Target congeners	Reference
	Lowerbound	Middlebound	Upperbound			
UK	91	–	–	1999–2000	47, 99, 100, 153, 154	Harrad et al., 2004
USA	88**	–	–	2003–2004	17, 28, 47, 66, 77, 85, 99, 100, 138, 153, 154, 183, and 209	Schecter et al., 2006
Spain	82	97		2000	Tetra- through octabrominated congeners	Bocio et al., 2003
Japan	–	94	–	1995	47, 99, 100, and 153	Wada et al., 2005
Japan	68	–	–	–	47, 49, 66, 71, 77, 85, 99, 100, 119, 126, 138, 153, 154, and 183	Ashizuka et al., 2004
Sweden		51		1999	47, 99, 100, 153, 154	Darnerud et al., 2001
Belgium	23	35	48	2005	28, 47, 99, 100, 153, 154, and 183	Voorspoels et al., 2007
Japan	46	330	610	2006	PentaBDE (17, 25, 28, 30, 32, 33, 35, 37, 47, 49, 66, 71, 75, 77, 85, 99, 100, 116, 118, 119, 126, 138, 153, 154, 155, and 166)	This study
Japan	21	310	600	2006	DecaBDE (206, 207, 208, and 209)	This study

*Lower, middle, and upper bound intakes were estimated by assuming the nondetect values as zero, one half of the detection limit, and the detection limit, respectively. **The intakes were estimated for 70 kg males aged 20–39 years.

proposed by the US Environmental Protection Agency. These results suggested that the dietary exposure to PBDEs was not serious in Japan as well as in the other reported countries.

PentaBDE constituents such as TeBDE-47 and PeBDE-99 were dominant in food groups V, X, and XI. In contrast, a high proportion of DeBDE-209, a major constituent of decaBDE, was observed in the group IV food samples, which mainly consisted of vegetable oils (Fig. 2A). To confirm the presence of DeBDE-209 in vegetable oils, we performed an additional analysis using individual oil samples obtained from rapeseed, corn, safflower, sesame, olive, and soybean. We observed that 7 out of the 18 oil samples contained DeBDE-209 as a major or secondary dominant congener at approximately the ppb level (0.7–2.6 $ng \cdot g^{-1}$, Fig. 3). These results partially explained the reason for the high proportion of DeBDE-209 found in the group IV food samples. Sample No. 2 was the most contaminated rapeseed oil, and it contained TeBDE-47, PeBDE-99, and DeBDE-209 at the concentrations of 0.59, 1.8, and 1.5 $ng \cdot g^{-1}$, respectively. The results indicated that this vegetable oil sample was contaminated with both decaBDE and pentaBDE. The contamination may have occurred during the oil manufacturing processes. Another possible pathway of contamination involved the absorption and adsorption of PBDEs by the original farm plants during their growth processes. Mueller et al. reported that both radish (*Raphanus sativus L.*) and summer squash (*Cucurbita pepo L.*) absorbed pentaBDE from contaminated soil in a model experiment[11]. Thus, farm plants probably absorb a part of the PBDEs from contaminated soil. Hale et al. reported that 11 biosolid fertilizer (recycled sewage sludge) samples that were collected from different regions in the US all contained high concentrations of penta- and decaBDE (1100–2290 and 84.8–4890 $ng \cdot g^{-1}$ dry weight, respectively)[12]. The land application of biosolids may increase PBDE levels in farm plants and their products. However, the relationship between PBDE levels in plants and those in soils has not been sufficiently documented. In addition, it is known that considerable amounts of polybrominated dibenzo-p-dioxins/dibenzofurans (PBDDs/PBDFs) can be formed from PBDEs under thermal stress conditions[13]. Further studies are required to reveal the pathways of oil contamination and to examine the formation of toxic PBDDs/PBDFs from PBDEs in heated vegetable oils under specific cooking conditions.

Acknowledgments

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POLYBROMINATED DIPHENYL ETHERS IN HUMAN SERUM AND SPERM QUALITY

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Abstract

Polybrominated diphenyl ethers (PBDEs) are widely used as flame retardants in different types of consumer products. PBDEs are now ubiquitous environmental contaminants. Several studies have indicated that PBDEs may affect male fertility. We present the results of a pilot study on the relationship between human serum PBDEs and sperm quality. Serum and sperm samples from 10 healthy Japanese males aged 18–22 years were obtained in St. Marianna University. The PBDE concentrations in the serum samples were determined using gas chromatography/mass spectrometry. Four PBDE congeners (2,2′,4,4′-tetrabromodiphenyl ether (TeBDE-47), 2,2′,4,4′,5-pentabromodiphenyl ether (PeBDE-99), 2,2′,4,4′,6-pentabromodiphenyl ether (PeBDE-100), and 2,2′,4,4′,5,5′-hexabromodiphenyl ether (HxBDE-153)) were quantified in all serum samples. The median levels of the individual PBDE congeners were 1.4 $ng \cdot g^{-1}$ lipid weight (lw), TeBDE-47; 0.21 $ng \cdot g^{-1}$ lw, PeBDE-99; 0.24 $ng \cdot g^{-1}$ lw, PeBDE-100; and 0.72 $ng \cdot g^{-1}$ lw, HxBDE-153. These levels are comparable to those found in European countries. Clear inverse correlations were observed between the serum HxBDE-153 concentration and sperm concentration ($r = -0.838$, $p = 0.002$) and testis size ($r = -0.764$, $p = 0.01$). However, the serum concentrations of the other 3 congeners did not correlate with sperm concentration or testis size. Extensive studies on the relationship between PBDEs and sperm quality are required.

Introduction

Polybrominated diphenyl ethers (PBDEs) are used as flame retardants in the production of common consumer products. PBDEs are now ubiquitous and persistent environmental contaminants, and they have been detected in human tissues. Because PBDEs have some structural similarity to thyroid hormones such as thyroxine (T4), it was speculated that PBDEs may mimic thyroid hormones and may disrupt thyroid homeostasis. Several studies indicate that exposure to PBDEs can reduce circulating levels of T4 in laboratory animals[1] and can cause permanent neurological effects similar to those associated with thyroid hormone deficiencies[2]. In addition, several PBDEs possess weak estrogenic/antiestrogenic activities[3]. The proliferation and differentiation of Sertoli cells and sperm production are regulated by thyroid and sexual hormones. Thus, PBDEs may affect male reproductive health by interfering with thyroid and sexual hormone function. Kuriyama et al. have reported that developmental exposure to a single low dose (60 $\mu g \cdot kg^{-1}$ body weight) of 2,2′,4,4′,5-pentabromodiphenyl ether (PeBDE-99) decreased sperm counts in male Wistar rats[4]. However, no previous studies have examined the relationship between human PBDE levels and sperm quality. We participated in the international project examining the sperm quality of fertile men and found that the sperm concentration of Japanese men was lower than that of European men[5]. The examination of sperm quality and the estimation of the concentration of chemicals in the serum would be required to reveal the correlation between the sperm quality of Japanese men and their exposure to chemicals. The aim of this pilot study was to measure PBDEs in serum samples from Japanese young males and to examine the relationship between serum PBDE levels and sperm quality.

Materials and Methods

Sample collection: Blood serum and sperm samples were collected monthly from 45 young Japanese males at the Department of Urology, St. Marianna University School of Medicine, in 2003. The men were asked to remain abstinent for at least 48 h before sperm collection. The blood samples were collected in vacuum tubes, and the serum fractions were separated by centrifugation. Serum samples were stored at –80°C until analysis. Of the 45 sample sets, 10 sample sets were randomly chosen for this study. For PBDE analysis, 10 pooled serum

samples (0.5 g × 12 months; total, 6 g per person) were prepared, and each pool was regarded as a representative sample of each set. In addition, 2 brands of commercially pooled human serum ("L-Consera N" and "L-Suitrol I," both purchased from Nissui Pharmaceutical, Tokyo, Japan) were used as in-house reference materials. The mean ± standard deviation (SD) age of the 10 participants was 22 ± 1 years (range, 18–22 years). The mean ± SD abstinence time was 3.1 ± 0.4 days (range, 2.6–3.8 days).

Chemicals: Standard mixture solutions of native PBDEs (BDE-AAP-A-15X) were purchased from AccuStandard (New Haven, CT, USA), and $^{13}C_{12}$-labeled PBDEs (MBDE-MXC) were purchased from Wellington Laboratories (Ontario, Canada). In this study, 29 PBDE congeners having 3 to 7 bromine atoms were monitored. The PBDE numbers are assigned according to the IUPAC PCB nomenclature. Acetone, acetonitrile, and *n*-hexane of pesticide analysis grade; ammonium sulfate of biochemistry grade; and 44% sulfuric acid-impregnated silica gel and *n*-nonane of dioxin analysis grade were purchased from Wako Pure Chemicals (Osaka, Japan). Water was deionized and purified using a Milli-Q cartridge system (Millipore, Bedford, MA, USA).

Sperm analysis: Sperm analyses were performed at the Department of Urology, St. Marianna University School of Medicine, according to the World Health Organization's recommendations as described elsewhere[5].

Serum PBDE measurements: Serum samples were analyzed at Osaka Prefectural Institute of Public Health. The serum sample (6 g) was extracted using ethanol/*n*-hexane (1:3 v/v, 14 mL) in a 50 mL test tube, after adding $^{13}C_{12}$-labeled surrogate standards ($^{13}C_{12}$-2,4,4′-tribromodiphenyl ether ($^{13}C_{12}$-TrBDE-28), $^{13}C_{12}$-2,2′,4,4′-tetrabromodiphenyl ether ($^{13}C_{12}$-TeBDE-47), $^{13}C_{12}$-2,2′,4,4′,5-pentabromodiphenyl ether ($^{13}C_{12}$-PeBDE-99), $^{13}C_{12}$-2,2′,4,4′,5,5′-hexabromodiphenyl ether ($^{13}C_{12}$-HxBDE-153), $^{13}C_{12}$-2,2′,4,4′,5,6′-HxBDE ($^{13}C_{12}$-HxBDE-154), and $^{13}C_{12}$-2,2′,3,4,4′,5′,6-heptabromodiphenyl ether ($^{13}C_{12}$-HpBDE-183); 10 pg for each congener) and 3.6 mL saturated ammonium sulfate solution. The test tube was shaken for 30 min and then centrifuged for 10 min at 3000 rpm. The *n*-hexane phase was collected, and the aqueous phase was re-extracted twice with 12 mL *n*-hexane. The 3 *n*-hexane phases were combined and washed with 12 mL water. After evaporation of the solvent, the lipid content was determined gravimetrically with a semimicro balance (Sartorius RC210P, Goettingen, Germany). The lipid was dissolved in *n*-hexane and was transferred to a column of 44% sulfuric acid-impregnated silica gel (3 g). The column was eluted with 30 mL *n*-hexane, and the eluate was evaporated to 2 mL. The *n*-hexane solution was transferred to a test tube and partitioned with *n*-hexane-saturated acetonitrile (4 mL) 3 times by shaking the test tube for 10 min and then centrifuging for 10 min at 3000 rpm. The acetonitrile phase was combined and then evaporated to dryness. The residue was redissolved in *n*-hexane and was transferred to a microconcentration tube. After addition of the injection standard ($^{13}C_{12}$-3,3′,4,4′,5-PeBDE) and keeper solvent (10 μL *n*-nonane), the extract was finally evaporated to approximately 10 μL under a gentle stream of nitrogen. The serum extract was assayed by a gas chromatography/mass spectrometry (GC/MS) system (Agilent 6890A GC coupled with JEOL JMS-GCmateII, Tokyo, Japan) with a fused silica capillary column (Rtx-1MS, 15 m, 0.25 mm i.d., 0.1 μm; Restek, Bellefonte, PA, USA). For each compound, 2 ions of the molecular ion or fragment ion cluster were monitored. Quantitation was based on the isotope dilution method using $^{13}C_{12}$-labeled internal standards. The PBDE concentrations were adjusted for total serum lipids and are expressed in units of nanogram per gram lipid weight ($ng \cdot g^{-1}$ lw). TeBDE-47, PeBDE-99, PeBDE-100, and HxBDE-153 were of interest because they are dominant in human serum.

Quality assurance and quality control: We validated the serum extraction procedure before beginning sample analysis by analyzing 4 replicate samples of pooled serum fortified with target analytes at 0.04–0.1 $ng \cdot g^{-1}$ serum. The mean percent recovery of 7 representative PBDE congeners (TrBDE-28, TeBDE-47, PeBDE-99, PeBDE-100, HxBDE-153, HxBDE-154, and HpBDE-183) ranged from 91% to 107%, and the relative standard deviation (RSD) ranged from 2% to 10%. The limit of detection (LOD) and limit of quantification (LOQ) were defined as 3 times and 10 times of the SD values obtained from the analysis of the 7 blank samples. However, for congeners that could not be detected in the blanks, values that were 3 times and 10 times of the SD values that were obtained from the analysis of 5 replicates of the lowest calibration standard were used as LOD and LOQ. The LOD values for all the PBDE congeners were below 0.3 $ng \cdot g^{-1}$ lw. In the analysis of 3 split unfortified serum samples, the RSD values for all the detected congeners were below 10%.

Results and Discussion

Of the 29 PBDE congeners monitored, 4 congeners (TeBDE-47, PeBDE-99, PeBDE-100, and HxBDE-153) were mainly detected in human serum samples (Figure 1). The concentrations of the detected PBDE congeners in the serum samples (n = 10) are shown in Table 1. The median levels of the individual PBDE congeners were as follows: BDE-47, 1.4 ng·g^{-1} lw; BDE-99, 0.21 ng·g^{-1} lw; BDE-100, 0.24 ng·g^{-1} lw; and BDE-153, 0.72 ng·g^{-1} lw. The levels of total PBDEs in Japanese human serum samples were almost the same as those reported in European countries but were one order of magnitude lower than those reported in USA[6]. Significant positive correlations were observed between the concentrations of TeBDE-47 and PeBDE-99 (r = 0.988, p < 0.001), TeBDE-47 and PeBDE-100 (r = 0.938, p < 0.001), and between PeBDE-99 and PeBDE-100 (r = 0.915, p < 0.001). In contrast, no significant correlations were observed between the concentration of HxBDE-153 and those of the other 3 congeners (r = 0.306–0.390, p = 0.26–0.39). The absence of a significant correlation between HxBDE-153 and the other dominant 3 congeners (TeBDE-47, PeBDE-99, and PeBDE-100) means that the main sources and/or biological properties of HxBDE-153 were different from those of the other 3 congeners. It has been reported that the technical mixtures of pentaBDE (DE-71 and Bromkal 70-5DE) and octaBDE (DE-79 and Bromkal 79-8DE) both contained HxBDE-153 in the range 5.32–5.44% w/w and 0.15–8.66% w/w, respectively[7]. The congeners TeBDE-47, PeBDE-99, and PeBDE-100 have been found in pentaBDE as the major components, but they have not been found in octaBDE[7]. These 3 congeners and HxBDE-153 have never been found in a technical decaBDE mixture (Saytex 102E and Bromkal 82-0DE)[7]. Therefore, TeBDE-47, PeBDE-99, and PeBDE-100 are mainly sourced from pentaBDE, although HxBDE-153 is sourced from both pentaBDE and octaBDE. In the early 1990s, Japanese manufacturers

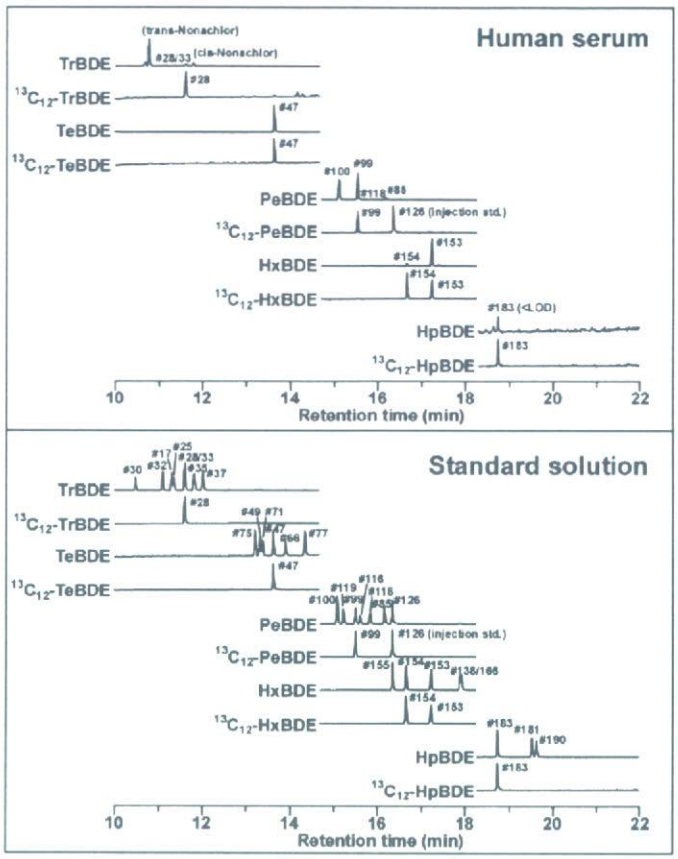

Fig.1 Chromatograms of PBDEs in human serum (participant No.2) and standard solution (1 to 2.5 ng·mL^{-1} each)

Table 1 Concentrations of PBDEs in serum samples from 10 Japanese males (ng·g^{-1} lw)

	Participant No.									
Congener	1	2	3	4	5	6	7	8	9	10
TrBDE-17	tr <0.04	tr <0.05	nd <0.01	nd <0.01	nd <0.02	nd <0.01	nd <0.02	nd <0.01	nd <0.02	nd <0.02
TrBDE-28/33	tr <0.2	0.37	0.16	tr <0.2	0.16	0.24	tr <0.2	0.17	tr <0.2	tr <0.2
TrBDE-37	tr <0.02	tr <0.03	nd <0.01	nd <0.01	nd <0.01	nd <0.01	nd <0.01	nd <0.01	nd <0.01	nd <0.01
TeBDE-49	nd <0.02	nd <0.03	0.09	tr <0.07	tr <0.08	0.07	nd <0.02	0.09	nd <0.03	tr <0.09
TeBDE-47	1.3	5.9	1.5	0.96	1.6	1.8	0.54	2.9	0.93	0.81
TeBDE-66	nd <0.04	nd <0.05	nd <0.04	nd <0.04	nd <0.04	nd <0.04	nd <0.04	tr <0.2	nd <0.05	nd <0.05
PeBDE-100	0.23	0.67	0.24	0.21	0.24	0.40	0.13	0.31	0.21	0.25
PeBDE-99	0.21	1.1	0.21	0.16	0.25	0.21	0.10	0.49	0.15	0.20
PeBDE-118	0.02	0.03	tr <0.02	tr <0.02	0.02	0.03	tr <0.02	tr <0.02	0.03	0.03
PeBDE-85	tr <0.07	tr <0.09	tr <0.07	nd <0.02	tr <0.08	nd <0.02	nd <0.02	tr <0.07	nd <0.03	nd <0.02
HxBDE-155	nd <0.02	tr <0.07	tr <0.05	tr <0.05	nd <0.02	tr <0.06	nd <0.02	nd <0.02	tr <0.07	nd <0.02
HxBDE-154	tr <0.06	0.08	0.05	0.05	tr <0.06	0.06	tr <0.06	tr <0.06	tr <0.07	tr <0.07
HxBDE-153	0.76	0.96	1.1	0.56	0.58	0.68	0.37	0.52	0.91	0.79
HpBDE-183	nd <0.1	nd <0.2	tr <0.4	tr <0.4	tr <0.4	tr <0.4	nd <0.1	nd <0.1	tr <0.5	nd <0.2
Sum of 4 PBDEs*	2.5	8.6	3.1	1.9	2.7	3.1	1.1	4.2	2.2	2.1

Abbreviations: tr, trace; nd, not detected. *Sum of TeBDE-47, PeBDE-100, PeBDE-99, and HxBDE-153.

voluntarily stopped the production and use of pentaBDE because of concern for its potency to accumulate in biota and to produce toxic polybrominated dibenzofurans/dioxins under thermal stresses. However, the production and use of octaBDE were continued in Japan until the early 2000s. There may still be a large number of consumer products that contain octaBDE in the Japanese indoor environment. Thus, with regard to octaBDE components such as HxBDE-153 and HpBDE-183, inhalation and dermal exposure may be important exposure routes for the Japanese people.

Table 2 Sperm concentration and testis size of 10 Japanese males

	Participant No.									
	1	2	3	4	5	6	7	8	9	10
Sperm concentration (million·mL^{-1})*	49	55	38	108	83	74	115	78	25	30
Testis size (mL)	36	36	40	50	46	42	51	54	29	33

*Annual average of monthly data.

The sperm concentration and testis size of the 10 participants are shown in Table 2. The sperm concentration of these participants ranged from 25 to 115 million·mL^{-1}. No participant had a sperm concentration below 20 million·mL^{-1}, a preliminary diagnostic value of male infertility. Clear inverse correlations were observed between serum HxBDE-153 concentration and sperm concentration (r = –0.841, p = 0.002, Fig.2) and testis size (r = –0.764, p = 0.01). However, no significant relationships were observed between the serum concentrations of any of the other congeners and the sperm concentration or testis size. Researchers have hypothesized that endocrine disrupting chemicals with thyroid hormonal or sexual hormonal activities may adversely affect male fertility. The thyroid-disrupting and estrogenic/antiestrogenic activities of PBDEs have been reported in several studies[1, 3]. In addition, considerable evidence is available for the reproductive effects of PBDEs from in vivo studies. Kuriyama et al. have reported that developmental exposure to a single low dose (60 μg·kg^{-1} body weight) of PeBDE-99 decreased sperm counts in male Wistar rats[4]. Although the levels of PBDEs found in our study are relatively low, we observed significant inverse associations between the serum concentration of HxBDE-153 and sperm concentration and testis size; this suggests an association between serum HxBDE-153 concentration and human sperm quality. The lack of a significant relationship among other individual PBDE congeners and sperm parameters may indicate a difference in bioactivity between congeners. The relationship between PBDEs and sperm quality is a complicated problem and needs further study.

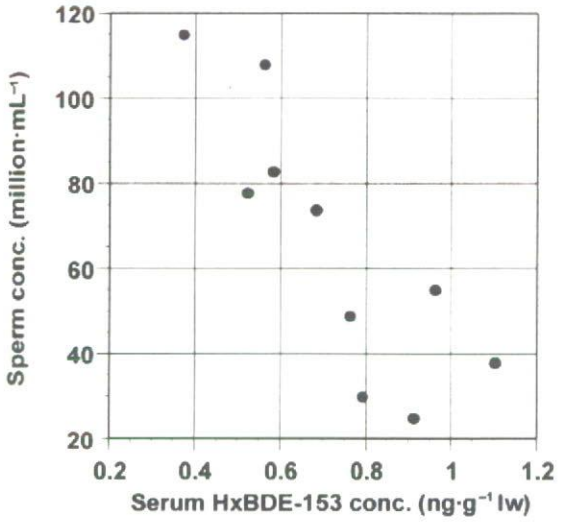

Fig.2 Relationship between serum HxBDE-153 concentration and sperm concentration

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Occurrence of polybrominated diphenyl ethers and polychlorinated biphenyls in shark liver oil supplements

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Abstract
Results are reported of a pilot survey of concentrations of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) in shark liver oil supplements. Eleven brands of dietary supplements were analysed using an isotope dilution GC/MS method. Total concentrations of 10 PBDE congeners (BDE-28, −47, −49, −66, −99, −100, −153, −154, −155 and −183) ranged from 0.1 to 53 ng g^{-1} oil weight and total concentrations of six PCB congeners (CB-28/31, −52, −118, −153 and −180) in the samples ranged from 16 to 340 ng g^{-1} oil weight (undetected values are not included). Two brands of Japanese deep-sea shark liver oil contained the highest levels of PBDEs (49–53 ng g^{-1} oil weight) and PCBs (290–340 ng g^{-1} oil weight). These results indicate that PBDEs may have entered Japanese deep-sea waters.

Keywords: *Dietary supplements, shark liver oil, polybrominated diphenyl ethers, polychlorinated biphenyls*

Introduction

In recent years, deep-sea shark liver oil has become popular as a dietary supplement. This oil contains some therapeutic ingredients, such as squalene, alkylglycerols, squalamine and $n-3$ polyunsaturated fatty acids. Squalene and alkylglycerols have been shown to enhance immune functions in animal models (Pugliese et al. 1998; Kelly 1999), and squalamine has been shown to have some antimicrobial and antitumor activities (Moore et al. 1993; Sills et al. 1998). In addition, it is generally accepted that the consumption of $n-3$ polyunsaturated fatty acids reduces the risk of cardiovascular and certain allergic diseases (Arab 2003; Calder 2003). Owing to these potential bioactivities, deep-sea shark liver oil has been claimed to be effective against cancers and infectious/inflammatory diseases, and its use has been promoted for different age groups, including children.

However, it should be noted that raw shark liver oil may contain considerable amounts of persistent organic pollutants, such as polychlorinated biphenyls (PCBs). Berg et al. (1998) have reported the concentrations of total PCBs (a sum of 31 congeners) in 10 liver samples of velvet belly sharks collected from a depth of about 400 m in Nordfjord, Norway (Berg et al. 1998). Mean concentration of total PCBs was 2390 ng g^{-1} lipid weight with a range of 1470–3870 ng g^{-1} lipid weight. Takahashi et al. (1998) have determined the concentrations of PCBs in the pooled liver samples of dogfish sharks collected from the bathyal zone (220–540 m depth) of Suruga Bay, Japan. This concentration was 1000 ng g^{-1} lipid weight.

De Boer et al. (1998) found relatively high concentrations of polybrominated diphenyl ethers (PBDEs) in sperm whales, indicating the contamination of deep-sea ecosystems by PBDEs. PBDEs are used as flame retardants in the production of common consumer products, such as electronics, furniture and textiles. As PBDEs are not chemically

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bound to the materials, they can leak into the environment during the production, use and disposal of the product. They are persistent and bioaccumulative, and have been shown to alter thyroid homeostasis in animal studies (Zhou et al. 2002). PBDEs have been recognized as environmental pollutants of global concern because their levels in the environment and in humans have increased markedly over the past several decades (Meironyté et al. 1999; Ikonomou et al. 2002; Akutsu et al. 2003; Sjödin et al. 2004). PBDEs have been detected in edible fish and other foodstuffs (Akutsu et al. 2001; Ohta et al. 2002; Bocio et al. 2003; Domingo 2004). It has been reported that fish and shellfish are the major contributors to human intake of PBDEs in European countries and Japan (Joint FAO/WHO Expert Committee on Food Additives (JECFA) 2005). Processed fishery products may also be significant contributors to the total intake of PBDEs in humans. A recent study by Jacobs et al. (2004) reported that 16 of 17 dietary fish oil supplements contain PBDEs, in addition to traditional organochlorine pollutants, such as PCBs. However, the levels of PBDEs in shark liver oil supplements remain unclear. To the best of our knowledge, there has only been one analytical result reported for PBDEs in a UK brand shark liver oil (UK Food Standards Agency 2006).

The aim of this study was to determine PBDE and PCB concentrations in dietary shark liver oil supplements. Determination of these concentrations enabled a comparison of the two classes of contaminants.

Materials and methods

Chemicals

Ten PBDE congeners (BDE-28, −47, −49, −66, −99, −100, −153, −154, −155 and −183) and six PCB congeners (CB-28/31, −52, −118, −153 and −180) were measured in this study. Single congener solutions of each native PBDE (BDE-28, −47, −49, −66, −99, −100, −153, −154, −155 and −183) and $^{13}C_{12}$-labeled PBDE ($^{13}C_{12}$-BDE-28, −47, −77, −99, −153, −154 and −183) were purchased from Cambridge Isotope Laboratories (Cambridge, MA, USA) and Wellington Laboratories (Ontario, Canada). Standard mixture solutions of native PCBs (CB-28, −52, −118, −153 and −180) and $^{13}C_{12}$-labeled PCBs ($^{13}C_{12}$-CB-28, −52, −118, −153 and −180) were purchased from Cambridge Isotope Laboratories. The CB and BDE numbers are assigned according to the IUPAC PCB nomenclature (US Environmental Protection Agency Webpage). Acetone, cyclohexane, hexane and anhydrous sodium sulfate of pesticide analysis grade, dimethyl sulfoxide (DMSO) of biochemistry grade and 44% sulfuric acid-impregnated silica gel of dioxin analysis grade were purchased from Wako (Osaka, Japan). Water was deionized and purified using a Milli-Q cartridge system (Millipore, Bedford, MA, USA) and was then washed with hexane.

Sample collection and preparation

A total of 11 brands of dietary shark liver oil supplement were purchased from two Japanese retailers between January and June, 2004 (Table I). All the oil supplements were in capsule form. The capsule shells were removed and the oils combined to form a single batch sample for each product. For comparison, two different batches (manufactured on two different dates) of the same brand of Japanese shark liver oil (SLO1A and SLO1B) were separately analysed. Since the samples considered included only the leading brands available in the Japanese market, this was not a comprehensive survey of all the available brands.

Cleanup procedure

A previously developed method for analysing PBDEs in fish (Akutsu et al. 2001) was modified and employed in this study. In brief, a sample (0.75 g) was spiked with a surrogate standard solution containing $^{13}C_{12}$-PBDEs ($^{13}C_{12}$-BDE-28, −47, −99, −153, −154 and −183; 0.75 ng each) and $^{13}C_{12}$-PCBs ($^{13}C_{12}$-CB-28, −52, −118, −153, and −180; 0.75 ng each) and was then purified by gel permeation chromatography (GPC) using the AS-2000 system (ABC Laboratories, Columbia, MO, USA) equipped with a Shodex (Tokyo, Japan) GPC column EV-G (guard column, 100×20 mm I.D.) and EV-2000 (300 × 20 mm I.D.). The column was eluted with acetone/cyclohexane (3:7, v/v) at 40°C. The first 70 ml of the eluate was discarded to remove the bulk of lipids and the next 70 ml was collected. This fraction was evaporated to dryness and additional cleanup was done using a 44% sulfuric acid-impregnated silica gel column (3 g) using 30 ml of hexane as the eluent. The hexane solution was concentrated to a volume of 4 ml and then extracted three times with 10 ml of DMSO. Water (30 ml) was added to the combined DMSO phase and back extraction was performed three times with 30 ml of hexane. The combined hexane phase was concentrated to 50 µl of nonane with an injection standard ($^{13}C_{12}$-BDE-77, 0.5 ng).

GC/MS condition

The analysis was performed using an Agilent gas chromatograph (model 6890A) equipped with a

Table I. Sample information.

Sample code	Oil composition	Country of origin	Indicated concentrations of SQ, AG and $n-3$ PUFAs	Proposed dose ($g\,day^{-1}$)[1]
SLO1A*	SLO (cold processed)	Japan	SQ 82%–89%, AG 6%–11%, $n-3$ PUFAs 3%–7%	0.75–1.75
SLO1B*	SLO (cold processed)	Japan	SQ 82%–89%, AG 6%–11%, $n-3$ PUFAs 3%–7%	0.75–1.75
SLO2	SLO (cold processed)	Japan	SQ 82%–89%, AG 6%–11%, $n-3$ PUFAs 3%–7%	0.75–1.75
SLO3	SLO (cold processed)	New Zealand	SQ 44.4%, AG 35.4%, $n-3$ PUFAs 6.7%	1.8
SLO4	SLO (cold processed)	New Zealand	SQ 30%–55%, AG 20%–45%	1.5
SLO5	SLO (cold processed), VE	New Zealand	NA	0.6–1.2
SLO6	SLO	NA	SQ > 99.9%	2.6
SLO7	SLO	NA	SQ > 99%	1.8–2.7
SLO8	SLO	NA	SQ 100%	0.5
SLO9	SLO	NA	NA	0.9–1.2
SLO10	SLO, VE	NA	NA	0.6–1.5
SLO11	SLO (92.5%) + FO (7.1%), VE, VA	NA	SQ 92%	2.2–2.8

SLO, deep-sea shark liver oil; FO, fish oil; VE, vitamin E; VA, vitamin A; PUFAs, polyunsaturated fatty acids; SQ, squalene; AG, alkylglycerol; NA, not available.
*SLO1A and SLO 1B are of the same brand but from different batches.
[1]Proposed doses are based on the information provided by the product manufacturers/suppliers.

JMS-GC mate II mass spectrometer (JEOL, Tokyo, Japan). The separation was carried out in an Rtx-1MS fused-silica capillary column (15 m × 0.25 mm I.D., 0.1 μm film thickness with a 3-m Integra-Guard column; Restek, Bellefonte, PA, USA) using helium as the carrier gas (flow rate 1 ml min^{-1}). The injector temperature was 250°C and the injection volume was 2 μl (splitless). The column temperature was programmed from 100°C (2 min) to 310°C (3 min) at a rate of 10°C min^{-1}. The mass spectrometer was operated in electron ionization mode with selected ion monitoring (35 eV, resolution 1000). The ion source and interface temperatures were 280 and 310°C, respectively. The PBDE and PCB congeners were monitored at the two most intensive ions of the molecular ion cluster and were quantified by the isotope dilution method using the corresponding $^{13}C_{12}$-labelled congeners.

Quality assurance and quality control

Spiked sample recoveries of PBDEs and PCBs were in the range of 80–110% with relative standard deviation of less than 5% (spiking level: each 1 $ng\,g^{-1}$ oil weight). The overall recoveries of the $^{13}C_{12}$-labelled congeners ranged from 65 to 97%. The detection limits of the individual PBDE and PCB congeners varied from 0.05 to 0.5 $ng\,g^{-1}$ oil weight.

Results and discussion

Concentrations of the PBDEs and PCBs are summarized in Tables II and III. In 11 of the 12 analysed samples, BDE-47 was observed to be the most abundant PBDE congener; BDE-183 was not detected in any samples. In 10 of the 12 analysed samples, CB-153 was the most abundant congener. The predominance of both BDE-47 and CB-153, and the absence of BDE-183, are in accordance with previously reported findings on cod liver oil (Jacobs et al. 2004). The total concentrations of the 10 PBDEs (sum of BDE-28, −47, −49, −66, −99, −100, −153, −154, −155 and 183) in the 11 samples ranged from 0.1 to 53 $ng\,g^{-1}$ oil weight, and the total concentrations of the six indicator PCBs (sum of CB-28/31, −52, −118, −153 and −180) ranged from 16 to 340 $ng\,g^{-1}$ oil weight (undetected values are not included). Total concentrations of the 10 PBDEs were lower than those of the six PCBs in all samples. The lowest value of BDE-47 in our study (0.1 $ng\,g^{-1}$ oil weight) is similar to the value measured in the UK brand shark liver oil supplement (0.17 $ng\,g^{-1}$ whole weight) (UK Food Standards Agency 2006). The three samples from the two brands of Japanese deep-sea shark liver oil (SLO1A, SLO1B and SLO2) exhibited the highest values for the sum of the 10 PBDEs (49–53 $ng\,g^{-1}$ oil weight) and the six PCBs (290–340 $ng\,g^{-1}$ oil weight). These levels were close to those found in pure cod liver oil in the UK market (sum of seven PBDEs, 20–34 $ng\,g^{-1}$ lipid weight; sum of seven PCBs, 97–202 $ng\,g^{-1}$ lipid weight) (Jacobs et al. 2004).

The two brands of Japanese shark liver oil (SLO1A, SLO1B and SLO2) were manufactured using the same special method (Japanese patent application no. 2002-84971). This method comprises the following four steps: (i) cold centrifugal extraction, (ii) mesh filtration, (iii) deoxygenation by vacuum, and (iv) deodorization using nitrogen flow. In this method, there was no heating step and only

Table II. Concentrations of PBDEs in deep-sea shark liver oil.

	PBDE ($ng\,g^{-1}$ oil weight)											
Sample code	28	49	47	66	100	99	155	154	153	183	Sum[1] (lower bound)	Sum[2] (upper bound)
SLO1A*	0.9	2.3	23	2.6	5.2	8.0	1.5	2.5	2.9	ND	49	49
SLO1B*	1.0	2.5	28	2.8	3.6	8.2	1.3	2.7	2.4	ND	53	53
SLO2	1.1	2.6	28	2.3	4.3	7.5	1.2	2.4	2.1	ND	52	52
SLO3	ND	0.1	0.6	ND	ND	ND	ND	ND	ND	ND	0.7	1.9
SLO4	ND	0.1	0.5	ND	ND	ND	ND	ND	ND	ND	0.6	1.8
SLO5	ND	0.1	0.1	ND	ND	ND	ND	ND	ND	ND	0.2	1.4
SLO6	ND	ND	0.1	ND	ND	ND	ND	ND	ND	ND	0.1	1.4
SLO7	ND	ND	0.3	0.2	ND	ND	ND	ND	ND	ND	0.5	1.7
SLO8	ND	ND	1.3	0.1	1.8	5.6	2	2.5	1.6	ND	15	16
SLO9	ND	ND	0.1	ND	ND	ND	ND	ND	ND	ND	0.1	1.4
SLO10	ND	ND	0.3	ND	ND	ND	ND	ND	ND	ND	0.3	1.6
SLO11	ND	ND	0.1	ND	ND	ND	ND	ND	ND	ND	0.1	1.4

ND, not detected (<0.5 $ng\,g^{-1}$ oil weight for BDE-183 and <0.1 $ng\,g^{-1}$ oil weight for the other nine PBDE congeners).
*SLO1A and SLO1B are of the same brand but from different batches.
[1]The concentrations of "ND" congeners were assumed to be zero.
[2]The concentrations of "ND" congeners were assumed to be the detection limit.

Table III. Concentrations of PCBs in deep-sea shark liver oil.

	PCB ($ng\,g^{-1}$ oil weight)					
Sample code	28/31	52	118	153	180	Sum
SLO1A*	5.6	18	68	170	82	340
SLO1B*	5.5	20	63	150	81	320
SLO2	4.1	17	57	140	69	290
SLO3	0.2	0.8	7.6	20	14	43
SLO4	0.2	0.6	5.2	4.9	11	22
SLO5	0.3	1.0	3.6	9.1	4.9	19
SLO6	0.5	3.4	8.9	9.3	6.7	29
SLO7	0.2	0.7	1.6	16	12	31
SLO8	0.1	0.1	0.2	1.1	16	18
SLO9	0.2	0.3	0.9	9.0	7.4	18
SLO10	0.3	0.5	2.1	10	6.1	19
SLO11	0.3	0.2	0.7	8.6	6.2	16

*SLO1A and SLO1B are of the same brand but from different batches.

gentle, non-chemical refining steps were adopted to prevent the destruction of unstable ingredients in the shark liver oil. The present study demonstrated that such "cold processed" (virtually unrefined) products may contain considerable amounts of PBDEs and PCBs. Similar cold processed products derived from the deep-sea sharks in New Zealand (SLO3, SLO4 and SLO5) were also contaminated with PBDEs and PCBs. However, the levels of these compounds were one or two orders of magnitude lower than those found in the Japanese shark products (Tables II and III). This result suggests that the Japanese sharks are more contaminated than those from New Zealand. Several studies have shown that the concentrations of PCBs and other organochlorine pollutants are higher in marine mammals from the Northern Hemisphere than those from the Southern Hemisphere (Tanabe et al. 1994; Schröder and Castle 1998). More recently, Ueno et al. (2003, 2004) have reported the geographical distribution of PCB and PBDE concentrations in skipjack tuna collected from the offshore waters of Asia and have speculated that Japan and other countries around the East China Sea are "hot spots" that release PCBs and PBDEs into the marine environment. In contrast, Scobie et al. (1999) reported that the estuarine environment in New Zealand is relatively free of organochlorine contaminants; for example, PCB concentrations in the estuarine sediments of New Zealand (0.12–8.8 $\mu g\,kg^{-1}$ dry weight) were lower than those reported in Japan (63–240 $\mu g\,kg^{-1}$ dry weight) and Sweden (23–262 $\mu g\,kg^{-1}$ dry weight). Thus, it is likely that deep-sea sharks in the Northern Hemisphere tend to possess higher levels of these pollutants than those in the Southern Hemisphere; however, further studies are required to confirm this fact. According to the product specifications, the two brands of Japanese shark liver oil were obtained from selected bathyal sharks (*Centrophoridae* and *Squalidae*) captured using long-line fishing in waters approximately 600–800 m deep around the Goto Islands (50 km off the west coast of Kyushu, Japan). Our results suggest that PBDEs may have entered Japanese deep-sea waters. In the marine environment, the deep sea and its ecosystems will be the ultimate sinks for PBDEs.

PBDE and PCB congener profiles of the three samples from Japanese shark liver oil (SLO1A, SLO1B and SLO2) were almost identical. For PBDEs, the contributions of the nine PBDEs to the total concentration were as follows: BDE-47: 47–54%; BDE-99: 15–16%; BDE-100: 8–11%;

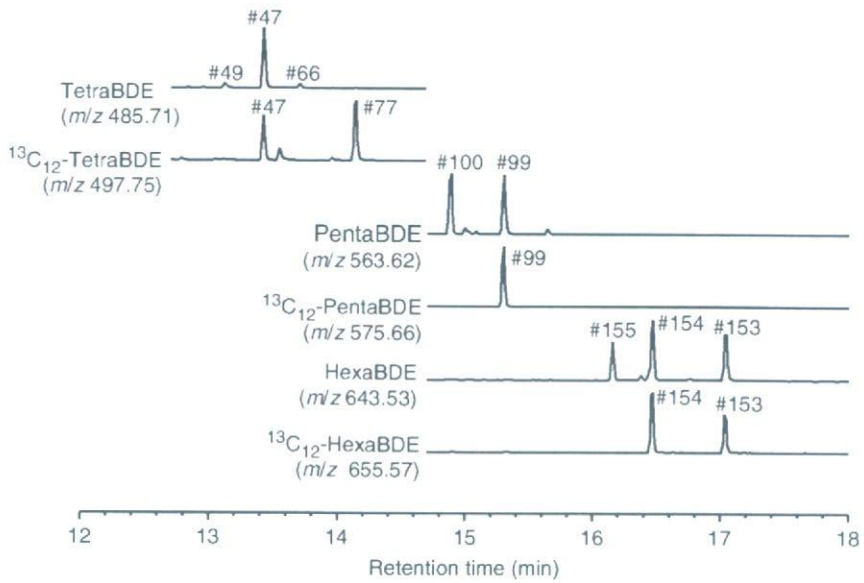

Figure 1. Chromatograms of PBDEs in deep-sea shark liver oil using GC/MS based on the isotope dilution method.

BDE-66: 4.5–5.3%; BDE-154: 4.7–5.1%; BDE-49: 4.7–5.0%; BDE-153: 4.1–5.9%; BDE-155: 2.3–3.1%; BDE-28: 1.8–2.1%. For PCBs, the contributions of the six PCBs to the total concentration were as follows: CB-28/31: 1.4–1.7%; CB-52: 5.2–6.3%; CB-118: 20%; CB-153: 47–49%; CB-180: 24–25%. Example chromatograms of tetra- to hexa-BDEs in Japanese shark liver oil (SLO1A) are shown in Figure 1. Although the known contributions of BDE-28, −49, −66 and −155 in chemical PBDE mixtures (e.g. Bromkal 70-5DE and 79-8DE) are almost negligible (Ikonomou et al. 2002), we observed these minor congeners in clear proportions together with five major congeners (BDE-47, −99, −100, −153 and −154) in the Japanese shark liver oil samples (Table II and Figure 1). Similar proportions have also been reported in salmon (Hites et al. 2004). BDE-28, −49, −66 and −155 are likely to be efficiently biomagnified in these predatory species through the aquatic food chain.

The estimated daily intakes of PBDEs (10 congeners) and PCBs (six congeners) from the shark liver oil supplements according to the daily doses proposed by the product manufacturers/suppliers (Table I) are shown in Table IV. Intake of PBDEs was calculated using the upper bound concentrations for each of the products. Intake of the 10 PBDEs ranged from less than 0.001 to 0.09 μg day^{-1}. The maximum intake of the 10 PBDEs estimated in this study (0.09 μg day^{-1}) was comparable to or higher than the total dietary intake of PBDEs reported in Spain (0.097 μg day^{-1}), Sweden (0.051 μg day^{-1}) and Finland (0.044 μg day^{-1}) (Domingo 2004). The maximum intake of the 10 PBDEs (0.09 μg day^{-1}) was 0.075% of the US EPA reference dose for penta-BDE (120 μg day^{-1} for a 60-kg person) (Wenning et al. 2003). The intake of the six indicator PCBs ranged from 0.01 to 0.60 μg day^{-1}. Based on the data of the six indicator PCBs comprising 23–27% of the total PCBs (sum of all tri- to hepta-congeners) in the typical Japanese diet samples (Akutsu et al. 2005), it can be calculated that the maximum intake of total PCBs from shark liver oil supplements was 2 μg day^{-1} (four times the intake of the six indicator PCBs). This level is less than 1% of the provisional tolerable daily intake of PCBs set by the Japanese government (300 μg day^{-1} for a 60-kg person). Our data indicate that the frequent consumption of shark liver oil supplements will increase human dietary

Table IV. Estimated daily intake (μg day^{-1}) of PBDEs and PCBs from deep-sea shark liver oil.

Sample code	Sum of ten PBDEs[1]	Sum of six PCBs
SLO1A*	0.04–0.09	0.26–0.60
SLO1B*	0.04–0.09	0.24–0.56
SLO2	0.04–0.09	0.22–0.51
SLO3	0.003	0.08
SLO4	0.003	0.03
SLO5	<0.001–0.002	0.01–0.02
SLO6	0.004	0.08
SLO7	0.003–0.005	0.06–0.08
SLO8	0.008	0.01
SLO9	0.001–0.002	0.02
SLO10	0.001–0.002	0.01–0.03
SLO11	0.003–0.004	0.04

*SLO1A and SLO1B are of the same brand but from different batches.
[1]Intakes of PBDEs were calculated using the upper bound concentrations for each product.

exposure to PBDEs and PCBs, although the safety margins between the proposed toxic levels and the estimated intake of these pollutants are considerable. In addition, data regarding the safety of long-term use of shark liver oil supplements do not exist. Further research is needed to evaluate the potential risks and benefits of shark liver oil supplements.

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報　文

液体クロマトグラフィー/質量分析法による魚介類中のノニルフェノール及びオクチルフェノールの定量

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高速液体クロマトグラフィー/質量分析法（LC/MS）を用いた魚介類及び食肉中のノニルフェノール（4-NP）及びオクチルフェノール（4-OP）の簡易かつ迅速な定量法を検討した．前処理は，メタノールで抽出し，マルチモードカートリッジを用いて試験溶液を調製した．LC/MS条件は，4-NP，4-OP共ネガティブモードを用い，測定は脱プロトン化分子（m/z 205.1，219.1）を用いた選択イオン検出（selected ion monitoring，SIM）法を採用した．移動相は揮発性の酢酸（0.005%）及び酢酸アンモニウム（1 mM）を用いることにより，感度良く検出できた．SIM法による検量線はいずれも0.5～50 ng/mLの範囲で良好な直線性を示した．本法による10 ng/g添加時の回収率は72.1～89.6%，標準偏差は10% 以内であった．本法による検出限界は0.5～2 ng/gであった．本法を用いて市販魚介類及び食肉についてNP及びOPの汚染実態調査を実施したところ，シジミ，ツブ貝等から微量の4-NPが検出された．

1　緒　　言

ノニルフェノール（NP），オクチルフェノール（OP）にはエストロゲン様作用があるとされ，内分泌系に対する撹乱作用が疑われている[1)2)]．NP，OPは非イオン性界面活性剤｛nonylphenolethoxylate（NPEO），octylphenolethoxylate（OPEO）｝や樹脂の安定剤や抗酸化剤の原材料として汎用されている．2000年におけるNP，OPの生産量は約16500トン及び10000トンである．環境中へ放出されたNPEO，OPEOは，生態系において容易に分解され，NP，OPを生成する[3)4)]．このことから，NP，OPの河川や下水処理水への汚染が問題視され，水生生物への影響が懸念されている．NP，OPの魚類に対するエストロゲン様作用は強いとされてきたが[5)6)]，NPについては平成13年8月に，OPについても平成14年6月，魚類に対して内分泌撹乱作用が確認された[7)8)]．

我々は，NP，OPに汚染された魚介類や容器包装から移行した食品を経由して，NP，OPを日常的に摂取している可能性がある．したがって，実際にヒトがどの程度NP，OPに暴露されているか知ることは極めて重要である．現在まで，河川水，工場排水，土壌等の環境試料中のNP，OP濃度については，液体クロマトグラフィー（LC），高速液体クロマトグラフィー/質量分析法（LC/MS），ガスクロマトグラフィー/質量分析法（GC/MS）法等を中心に数多くの報告がなされている．しかし，市販されている魚介類，食肉や食事中の分析例は少ない[9)～11)]．そこで今回，LC/MSを用いた魚介類，食肉中のNP，OPの高感度分析法を検討したので報告する．なお，NP，OPには分岐タイプと直鎖タイプの構造異性体があるが，工業用として使用され，環境中から検出されているものは専ら分岐タイプなので[12)13)]，分岐タイプの4-NP及び4-*t*-OPを中心に検討した．

2　実　　験

2・1　試料及び試薬

魚介類及び食肉は埼玉県内で市販されているものを用いた．

標準品：4-ノニルフェノール（4-NP，異性体混合物，分岐型），4-*n*-ノニルフェノール（4-*n*-NP，直鎖型）及び重水素化4-*t*-オクチルフェノール4-*t*-OP(d)は林純薬工業製の環境分析用試薬，4-*t*-オクチルフェノール（4-*t*-OP，分岐型）及び4-*n*-オクチルフェノール（4-*n*-OP，直鎖型）は関東化学製の環境分析用試薬を用いた．

β-グルクロニダーゼ：Sigma製 Type H-2（*β*-glucuronidase 115000 units/mL，sulfatase 4500 units/mL）．

標準溶液：各標準品20 mgを精秤し，メタノール100 mLに溶解して標準原液を調製し，適宜70% メタノールで希釈して標準溶液とした．

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Table 1 Operating conditions of LC/MS for octylphenol and nonylphenol

MS conditions		HPLC conditions	
Ionization	ESI, Negative	Column	Cadenza CD-C18 (100 × 2 mm)
Fragmentor	100 V	Eluent	Gradient
Nebulizer	N_2 (30 psi)	Flow rate	0.2 mL/min
Drying gas	N_2 (10 L/min, 350℃)	Oven temp.	40℃
V-cap	4500 V	Injection size	20 μL
SIM ion	*m/z* 205.1, 210.1, 219.1		

A = 50% acetonitrile (containing 0.005% acetic acid, 1 mM ammonium acetate); B = acetonitrile

Time/min	A, %	B, %
0	80	20
10	40	60
20	30	70

Isolute multimode カートリッジ（500 mg）: International Sorbent Technology Ltd. 製，カートリッジはあらかじめメタノール 10 mL 及び水 5 mL の順で洗浄した後使用した．

その他の試薬はすべて特級品あるいは HPLC 用を用いた．精製水の調製にはオルガノ製超純粋製造装置 Model-S を使用した．

2・2 装置及び測定条件

高速液体クロマトグラフ/質量分析計: Agilent 製 1100 series LC/MSD を使用した．測定条件は Table 1 に示した．

2・3 検量線の作成

安定同位体標識内部標準物質 4-*t*-OP(d_5) を 10 ng 含んだ 4-NP，4-*n*-NP，4-*t*-OP 及び 4-*n*-OP の 1.0，2.0，5.0，10 及び 50 ng/mL の混合標準溶液を調製し，その 20 μL を高速液体クロマトグラフ/質量分析計に注入する．検出には選択イオン検出（selected ion monitoring，SIM）法を採用し，それぞれモニターイオンにより得られた SIM クロマトグラムよりピーク面積を求め，4-NP，4-*n*-NP，4-*t*-OP 及び 4-*n*-OP と 4-*t*-OP(d_5) の面積比より検量線を作成した．

2・4 試験溶液の調製

試料 5 g を取り，メタノール 15 mL 及び内部標準 4-*t*-OP(d_5) 50 ng を加えてホモジナイズ抽出後，遠心分離し，上澄みを分取した．必要に応じてメタノールを加え，上澄みの全量を 15 mL とした．上澄み 3 mL（試料 1 g 相当）を取り，精製水 7 mL を加えた後，Isolute multimode カートリッジに負荷し，カートリッジを 50% メタノール 3 mL で洗浄後，メタノール 5 mL で溶出した．溶出液に 0.1 M KOH 溶液 0.1 mL を加え，窒素気流下で約 0.1 mL に減圧濃縮後，70% メタノールで 1.0 mL とした．抱合体の分析は，上澄み 3 mL に 0.2 M 酢酸緩衝液（pH 5.0）2 mL，精製水 5 mL 及び β-グルクロニダーゼ 30 μL を加え，十分混合した後，37℃ で 10 時間インキュベートした．その後の操作は上記に示した遊離体と同様に行った．

2・5 包装材の溶出試験

市販魚介類の包装材に使用されていたラップフィルム及びトレイからの NP 及び OP の溶出試験は次のように行った．ラップフィルム，トレイを 5 × 5 cm に切り取ったものを内径 9 cm のガラス製シャーレに入れ，*n*-ヘプタン 25 mL ｛4-*t*-OP(d_5)，20 ng 含む｝を加えて時々振とうしながら室温で 60 分間浸漬した．*n*-ヘプタン溶液に KOH 溶液 0.1 mL を加え，窒素気流下で約 0.1 mL に減圧濃縮後，70% メタノールで 2.0 mL とし，試験溶液とした．

3 結果及び考察

3・1 LC/MS 測定条件の検討

一般に，工業用としては NP 及び OP 共に分岐タイプの 4-NP 及び 4-*t*-OP が用いられている．したがって，環境中から検出される NP，OP は分岐タイプのものである[12)13)]．そこで，分岐タイプの 4-NP 及び 4-*t*-OP を中心に，試薬標準品として市販されている直鎖タイプの 4-*n*-NP 及び 4-*n*-OP も含めて LC/MS 測定条件を検討した．

NP は疎水性が高いことから，インターフェースには大気圧化学イオン化（atmospheric pressure chemical ionization，APCI）法が利用されている．一方，最近では操作性に優れているエレクトロスプレーイオン化（electrospray ionization，ESI）法を用いた NP，OP の分析法も数多く報告されている．そこで，APCI 法及び ESI 法を用いて検出感度を比較した結果，いずれの成分も感度的に同程度であった．そこで，インターフェースには操作性に優れている ESI 法を選択した．次に，イオン化モードを検討した結果，いずれもフェノール性水酸基を有していることから negative mode が適していた．また，移動相に微量の酢

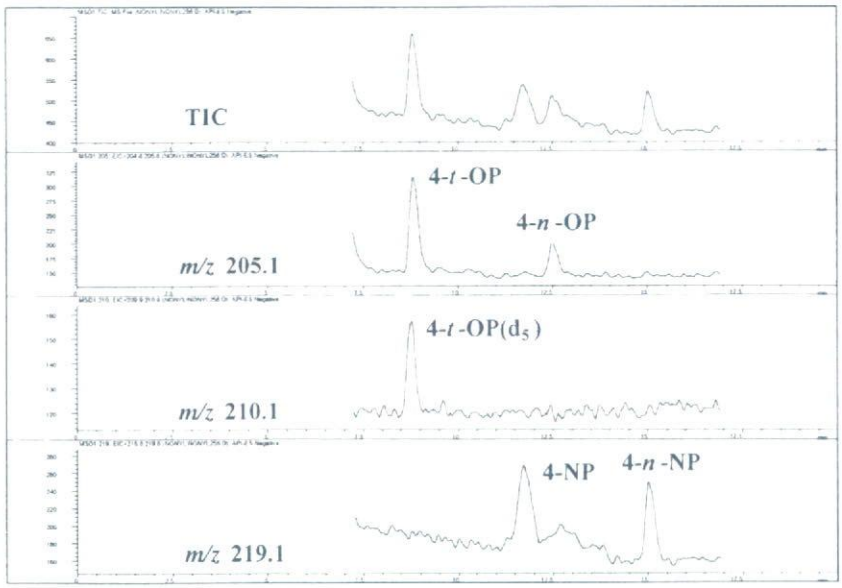

Fig. 1　Typical LC/ESI/MS-SIM chromatograms of standard mixture (1 ng/mL)

酸及び酢酸アンモニウムを加えることにより，各成分共より高感度に検出された[14]．しかし，酢酸及び酢酸アンモニウムの濃度が高くなるに従い検出感度は逆に低下することから，酢酸濃度は 0.005%，酢酸アンモニウム濃度は 1 mM とした．更に，分析対象物質間の疎水性が大きく異なることから，グラジエント溶出法を採用した．なお，検出感度に及ぼすカラムの影響も見られ，検討した中では Cadenza CD-C18（インタクト製）が最も優れていた．

次にイオン強度に及ぼすフラグメンター電圧の影響を検討した結果，各成分の脱プロトン化分子 $[M-H]^-$（m/z 205.1，219.1，210.1）を効率良く生成する 100 V に設定した．更に，他のパラメーターの最適測定条件を検討し，Table 1 に示す条件を設定した．本条件によって得られた 4-NP，4-*n*-NP，4-*t*-OP，4-*n*-OP の検出限界は，SIM モードで 0.5 ng/mL（絶対量として 10 pg）であった．本法による検量線はいずれも 0.5～50 ng/mL の範囲で良好な直線性を示した．混合標準溶液 1 ng/mL の代表的な LC/ESI/MS-SIM クロマトグラムを Fig. 1 に示す．

3・2　前処理法の検討

3・2・1　抽出及びクリーンアップ　試料の前処理法の開発に当たっては実験器材や試薬からの汚染を極力少なくすることが求められる[15]．実際，前処理法を構築するに当たり，実験環境や実験器材・試薬から 4-NP の微量汚染が問題となった．そこで，より信頼性の高い前処理法を構築するため，4-NP の溶出量が少なくかつクリーンアップ効果に優れたカートリッジを検討した．その結果，Isolute multimode が最も夾雑成分の除去効果に優れ，かつ 4-NP の溶出量が少なかった（0.2 ng/cartrigde 以下）．なお，ガラスバイアル瓶のキャップに用いるセプタムからも NP の汚染がみられるものもあった．セプタムには NP の溶出のない両面テフロン用を選択した．本法により得られた代表的なシジミ及びツブ貝抽出液のクロマトグラムを Fig. 2，3 に示す．なお，試料によっては 4-*n*-NP の溶出位置に妨害ピークが出現し，分析が困難な場合があった．工業用としては専ら分岐タイプの 4-NP が使用されていることから，これ以降直鎖タイプの 4-*n*-NP は分析対象から除外することにした．

3・2・2　酵素による加水分解　魚介類や哺乳類に摂取された NP，OP は，グルクロン酸抱合体又は硫酸抱合体として存在する可能性が高いことから[16)~19)]，抱合体も分析対象とした．4-*t*-OP 及び 4-NP 抱合体（約 500 ng/mL）を含む溶液 100 μL を 2・4 に記載した試料上澄み 3 mL に添加して加水分解条件を検討した．β-グルクロニダーゼ量を 10，30 及び 50 μL と変えて加水分解率に及ぼす影響を調べた結果，いずれの量においても 37℃，5 時間インキュベートすることによりほぼ完全に加水分解された．そこで，本法では酵素量は 30 μL，インキュベーション時間は 10 時間とした．

3・2・3　酵素反応に及ぼす MeOH 含量の影響　次に，試料抽出液がメタノールであることから，酵素反応に及ぼす MeOH 含量の影響を検討した．酵素反応溶液中のメタノール含量を 0，10，20，30，40，50，60% と変えて β-グルクロニダーゼの酵素活性に及ぼす影響を調べた．その結果，メタノール含量が 50% までは酵素活性はほとんど阻害されず，抱合体の 90% 以上が加水分解された．そこで，上澄みであるメタノール抽出液 3 mL に 0.2 M 酢酸緩衝液 2 mL 及び精製水 5 mL を加え，インキュベートする

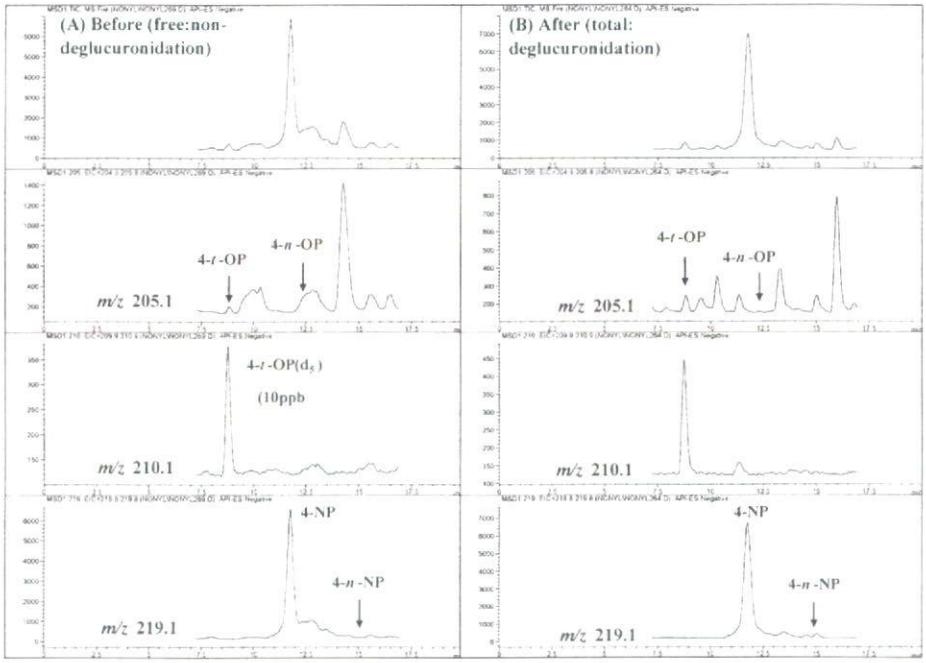

Fig. 2 Typical LC/ESI/MS-SIM chromatograms of (A) before and (B) after deglucuronidation of freshwater clam extracs

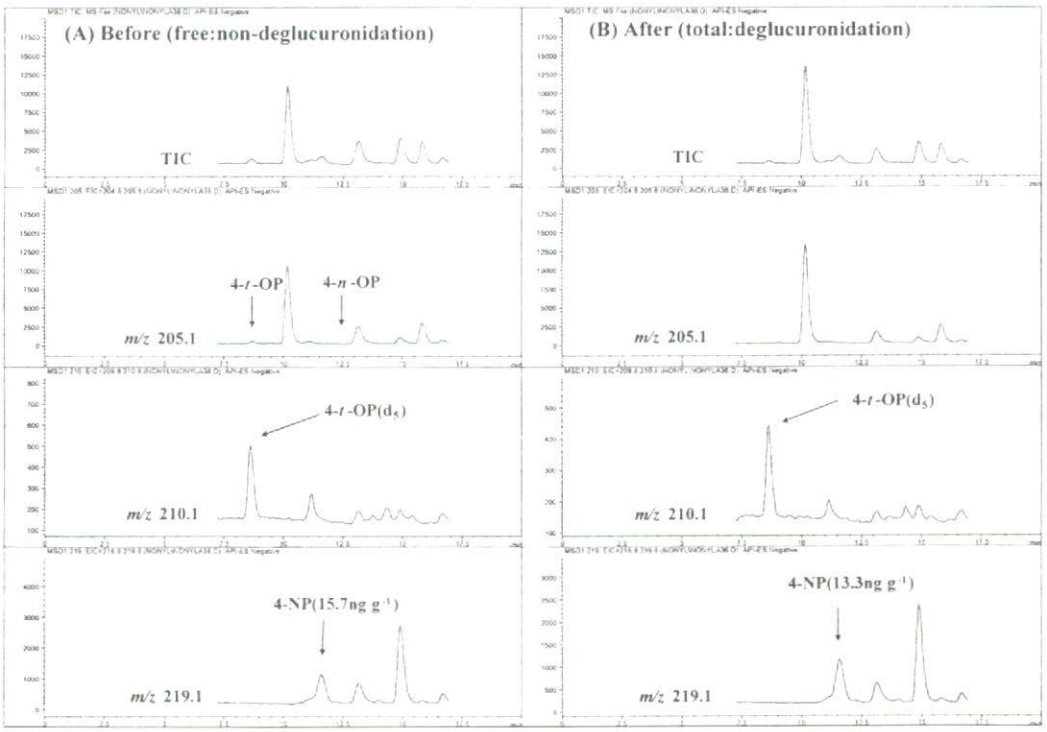

Fig. 3 Typical LC/ESI/MS-SIM chromatograms of (A) before and (B) after deglucuronidation of grain shellfish extract

ことにした．

3・2・4 0.1 M KOH による揮散防止効果 NP，OP は減圧乾固時に揮散し，特に分岐タイプの多くが損失した[メタノールで調製した 10 ng/mL 混合標準溶液 1 mL を 40℃，10 分間減圧乾固したときの残存量は，4-NP = 16%，4-*n*-NP = 82%，4-*t*-OP = 9%，4-*n*-OP = 52%，4-*t*-OP(d_5) = 10% であった]．そこで，NP，OP が弱酸性を有するフェノール化合物であることから，0.1 M KOH 溶液 0.1 mL を乾固時に加えることにより揮散を抑制可能か検討した．0.1 M KOH 溶液 0.1 mL を加えることにより，

Table 2　Recoveries of octylphenol and nonylphenol from fish, shellfish and meat

Sample	Recovery (mean ± S.D., n = 5), %			
	4-*t*-OP	4-*n*-OP	4-NP	4-*t*-OP(d_5)
Yellowtail	85.6 ± 7.6	73.3 ± 6.2	84.2 ± 9.1	86.3 ± 5.7
Freshwater clam	89.6 ± 5.7	81.3 ± 5.6	86.3 ± 7.8	84.7 ± 4.8
Pork	81.3 ± 7.2	72.1 ± 6.6	79.5 ± 8.3	83.3 ± 6.7

Samples were spiked with 10 ng/g of each drug.

Table 3　Concentration of OP and NP in fish, shellfish and meat

Sample	Inspection number	Free (non-deglucuronidation)			Total (deglucuronidation)		
		4-*t*-OP	4-*n*-OP	4-NP	4-*t*-OP	4-*n*-OP	4-NP
Horse mackerel	3	ND	ND	ND	ND	ND	ND
Yellowtail	2	ND	ND	ND	ND	ND	ND
Sardine	2	ND	ND	ND	ND	ND	ND
Rainbow trout	2	ND	ND	ND	ND	ND	ND
Flounder	1	ND	ND	ND	ND	ND	ND
Sweet fish	2	ND	ND	ND	ND	ND	ND
Mackerel	1	ND	ND	ND	ND	ND	ND
Pacific saury	1	ND	ND	ND	ND	ND	ND
Shad	1	ND	ND	ND	ND	ND	ND
Striped pigfish	1	ND	ND	ND	ND	ND	ND
Freshwater clam	7	Tr	ND	ND～64.1	1.0	ND	ND～88.9
Littleneck clam	3	ND	ND	ND～2.0	ND	ND	ND～2.7
Clam	3	ND	ND	ND	ND	ND	ND
Grain shellfish	2	ND	ND	4.3～15.7	ND	ND	5.4～13.3
Scallop	2	ND	ND	ND	ND	ND	ND
Turban shell	1	ND	ND	ND	ND	ND	ND
Sea snail	1	ND	ND	ND	ND	ND	ND
Surf clam	1	ND	ND	ND	ND	ND	ND
Sea squirt	1	ND	ND	ND	ND	ND	ND
Wakame seaweed	6	ND	ND	ND～14.3	ND	ND	ND～11.0
Chicken	3	ND	ND	ND	ND	ND	ND
Pork	3	ND	ND	ND	ND	ND	ND
Beef	3	ND	ND	ND	ND	ND	ND

ND: 4-*t*-OP < 0.5 ppb, 4-*n*-OP < 0.5 ppb, 4-NP < 2.0 ppb

いずれの成分も 95% 以上残存した．なお，減圧濃縮時のロータリエバポレーターからの 4-NP の汚染を防ぐために，エバポレーター内はよく洗浄し，減圧濃縮は窒素気流下で行った．

3・3　添加回収実験

4-NP，4-*t*-OP，4-*n*-OP 及び内部標準 4-*t*-OP(d_5) を，ハマチ，シジミ及び豚肉に 10 ng/g の濃度で添加し，回収率を求めた．その結果，ハマチ，シジミ及び豚肉はいずれの成分も 70% 以上の回収率であった［Table 2，4-*t*-OP(d_5) での補正なし］．本法による 4-*t*-OP，4-*n*-OP の検出限界は 0.5 ng/g (S/N 3) であった．一方，4-NP はハマチ及びシジミ共に操作空試験値（最終試験溶液 1 mL に対して 0.32 ± 0.18 ng/mL）が観測された．一般に操作空試験値が観測された場合の検出限界 LOD は，操作空試験値の平均値プラス標準偏差の 3 倍と定義されている．したがって，試料中の 4-NP の検出下限値は信頼性を考慮して 2 ng/g とした．

3・4　魚介類，食肉中の NP，OP 濃度

本法により，埼玉県内で市販されていた魚介類，食肉等，計 52 検体を分析した．今回分析した中では，一部のシジミ（7 検体中 2 検体），ツブ貝（2 検体中 2 検体）及びワカメ（6 検体中 1 検体）から微量の 4-NP が検出された．また，4-NP が 88.9 ng/g 検出されたシジミからは，同時に 1 ng/g の 4-*t*-OP も検出された．

ヒトやラット，マウス等では NP，OP は経口摂取後，速やかにグルクロン酸抱合体に代謝され，血中や尿中には主にグルクロン酸抱合体として存在するとされている[16)～19)]．一方，貝類からの NP 検出例に関する報告はなされているが，遊離体のみ測定しており，代謝に関する報告は見られない[20)21)]．そこで，今回の調査で微量の 4-NP が検出されたシジミ及びツブ貝について，遊離体と抱合体を含めた総量を測定した．Table 3 に示すとおり，シジミ及びツブ貝

Table 4 Thermal stability of residual 4-nonylphenol in freshwater clam and grain shellfish

Sample		NP	Rate, %
Freshwater clam	Boil liquid	3.4 ng/100 mL	1.0
	tissues	337.1 ng (61.4 ng g^{-1})	99.0
Grain shellfish	Boil liquid	2.5 ng/100mL	2.2
	tissues	108.7 ng (15.1 ng g^{-1})	97.8

Ten pieces of freshwater clam and one piece of grain shellfish were boiled in 100 mL of water for 5 minutes, respecrively.

中から検出された4-NPは，その多くが遊離体であり，抱合体はほとんど検出されなかった．このことから，シジミ，ツブ貝のグルクロン酸抱合化活性は低いと考えられる．

次に，シジミ及びツブ貝中含まれる4-NPの加熱調理における安定性を調べた．シジミ10粒，ツブ貝1粒をそれぞれ100 mLの沸騰水中で5分間加熱調理後，沸騰水中への移行量及びシジミ，ツブ貝中の濃度を調べた．貝中に含まれる4-NPは沸騰水中にはほとんど移行せず，貝中に残存していた（Table 4）．また，加熱処理後においてもその濃度に変化はほとんど見られず，安定であった．なお，Fig. 2，3に市販魚介類の代表例として4-NPが検出されたシジミ及びツブ貝抽出液のLC/ESI/MS-SIMクロマトグラムを示した．

3・5 魚介類中のNP濃度と包装材の影響

魚介類のNP及びOP汚染の原因として，環境汚染由来によるものと，魚介類が包装されていた包装材由来によるものが報告されている[9)10)]．そこで，今回の調査でNPが検出されたシジミ（2検体）及びツブ貝（2検体）について，これらの検体が包装されていた包装材（ラップフィルム，トレイ）について，NP及びOPの溶出試験を行った．その結果，今回NPが検出されたシジミ及びツブ貝に用いられていた包装材からのNP及びOPの溶出量は，いずれも1 ng/cm^2以下であった．更に，シジミ及びツブ貝は，殻は包装材と接触しているが，貝の中身は接触していない．以上のことから，今回NPが微量検出された貝類の汚染原因は，包装材からの影響は極めて少なく，環境汚染由来によるものと考えられる．

4 結　　語

最近，NP，OPは魚類に対して内分泌撹乱作用を示すことが明らかにされた．しかし，魚類と異なりラットやマウス等の哺乳類に対するエストロゲン様作用は非常に弱いとされている．更にその代謝体であるグルクロン酸抱合体は，エストロゲン様作用をほとんど示さないとされている．今回，市販魚介類，食肉等計52検体を分析した結果，一部のシジミ等を除き，4-NP，4-*t*-OPは検出限界以下であった．分析数が少なく，今回の調査から我々が食している多くの食品中のNP，OPレベルを評価することは困難である．しかし，NP，OPの界面活性剤への使用が年々大きく減少しており，NP，OPの河川水や水道源水中の汚染レベルは年々低下している．また，食品への移行が考えられる容器包装への使用も少なくなっている．したがって，我々が暴露されているOP，NPの量は極めて少ないレベルであると推定される．今後，我々が食品等を経由して摂取するOP，NP量は更に減少していくと考えられる．

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Determination of Nonylphenol and Octylphenol in Fish and Shellfish by High-Performance Liquid Chromatography/Electrospray Mass Spectrometry

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A simple and reliable method using liquid chromatography-electrospray ionization-mass spectrometry (LC/ESI-MS) has been developed for the determination of 4-nonylphenol (4-NP) and 4-octylphenol (4-OP) in seafood and meat. LC separation was performed on a Cadenza CD-C18 column (100 × 2 mm i.d.) with a gradient system of 50% acetonitrile (containing 1 mM ammonium acetate and 0.005% acetic acid)-acetonitrile as the mobile phase at a flow rate of 0.2 mL/min. The negative ionization produced molecular related ions: $(M-H)^-$, at m/z 205.1 and 219.1 for 4-OP and 4-NP, respectively. The calibration graphs for 4-NP and 4-OP were rectilinear from 0.5 to 50 ng/mL with selected ion monitoring (SIM). The compounds were extracted with methanol, and the extracts were cleaned up on a Isolute Multimode cartridge (500 mg). The method involves enzymatic deconjugation by β-glucuronidase and correction of the stable isotopically labeled internal standard, 4-octylphenol-d type. The recoveries of the compounds from seafood fortified at a level 10 ng/g was 72.1～89.6%, with high precision. The limits of detection of the compounds in seafood were 0.5～2 ng/g.

Keywords : nonylphenol; octylphenol; alkylphenol; seafood; fish; LC; mass spectrometry; LC/MS.