metabolites have been determined in breast milk (Cerrillo et al., 2005; Shen et al., 2008). Widespread environmental contamination by endosulfan has been reported in China (Li et al., 2007) and Korea (Yeo et al., 2004). Among legacy POPs, toxaphene has been used in China (Wong et al., 2005) and Korea (de Geus et al., 1999) but is not registered in Japan. Exposure to toxaphene in seafood from Hong Kong is reported (Guo et al., 2007). Historical endosulfan and toxaphene trends showed elevated exposure levels via food intake in Korea and China (Desalegn et al., 2011). Other chlorinated cyclodienes such as dieldrin and endrin have been used as pesticides in Japan and Korea, but not in China. However, the recent levels and their temporal trends of chlorinated cyclodienes have never been determined in Asian breast milk.

The present study was conducted to clarify regional differences in recent trends of contamination by chlorinated cyclodienes, including endosulfan, toxaphene and dieldrin, and chlordane-related compounds in human milk from China, Korea and Japan. We also quantified the levels of PCBs, HCHs and HCB for comparison, but not DDT levels, which were described previously in breast milk from the same populations (Fujii et al., 2011). In this study, we improved the gas chromatography/mass spectrometry (GC/MS) methods using electron capture negative ionization (ECNI) monitoring for all analytes except DDTs, compared with electron ionization (EI) monitoring described in previous studies (Haraguchi et al., 2009; Fujii et al., 2011). Our data are compared with other results worldwide to understand the magnitude of contamination.

2. Materials and methods

2.1. Sample collection

Human milk samples were obtained from the Kyoto University Human Specimen Bank using a standardized protocol (Koizumi et al., 2005, 2009). Three individual breast milk samples (5 mL each) were pooled to obtain 15-mL samples. Overall, 70 pooled samples were prepared from 210 human breast milk samples (Supplementary Table S1), which is the same population sample as analyzed by Fujii et al. (2011). The samples were collected from volunteers living in China ($n = 60$ from Beijing, 2007–2008), Korea ($n = 30$ from Seoul in 2007; $n = 30$ from Busan, 2008–2009) and Japan ($n = 30$ from Sendai, 2009; $n = 30$ from Takarazuka, 2008; $n = 30$ from Takayama, 2008). The Ethics Committee of Kyoto University approved the protocol of the present study (E25) and appropriate written informed consent was obtained from all the participants.

2.2. Chemicals

Two internal standards, $^{13}C_{12}$-labeled *cis*-chlordane and $^{13}C_{12}$-labeled 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB-153), were used for determination of OCPs and PCBs. The pesticide standard solution (unlabeled pesticide mix #1037; 2 μg mL^{-1}) was purchased from Kanto Chemical Co., Tokyo. The standards of PCBs (11 isomers: PCB-74, 99, 105, 118, 138, 153, 156, 170, 180, 183, and 187), toxaphenes (Parlar #26, and #50), and endosulfan (α- and β-forms) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The standards were used for the calibration, recovery and quantification of target compounds. Silica-gel (Wako gel S-1) used for purification was obtained from Wako Pure Industries (Osaka, Japan), and was heated at 130 °C for 3 h prior to use. All solvents used were of pesticide-grade quality.

2.3. Clean-up procedure

The methodology used to analyze OCPs in the breast milk samples was based on lipid extraction, gel permeation chromatography (GPC) and silica-gel column cleanup, and GC/MS/ECNI. Briefly, each 15 mL pooled breast milk sample was spiked with two internal standards, namely $^{13}C_{12}$-*cis*-chlordane (2 ng) and 4′-MeO-BDE121 (0.2 ng). We extracted the sample with *n*-hexane, after adding potassium oxalate solution, ethanol and diethyl ether. An aliquot of lipid (300 mg) was dissolved in dichloromethane (DCM):*n*-hexane (1:1), and then subjected to GPC with a Bio-Beads S-X3 column (Bio-Rad Laboratories, CA, USA). The gel material (35 g) was packed in 55 cm × 27 m i.d., glass column with DCM/hexane as the eluting solvent at a flow-rate of 4 mL min^{-1}. The first 90 mL fraction of the eluate containing lipids was discarded, then the next 80 mL fraction was collected. The fraction was purified with a silica-gel column (0.2 g Wako gel S-1), by elution with 15 mL DCM:*n*-hexane (12:88, v/v). The fraction was concentrated to 200 μL prior to GC/MS/ECNI analysis.

2.4. Instruments and quantification

Twenty-three analytes were measured by GC/MS/ECNI using an Agilent GC/MSD 5973i (Agilent Technologies, CA, USA) coupled with a 6890 N gas chromatograph. The GC/MS conditions and target ions for determination of POPs are summarized in Supplementary Table S2. Quantification of the compounds was based on signals in the mass chromatograms and on comparison with ^{13}C-PCB153 used as a syringe spike. The concentrations of chemicals are reported as nanogram per gram of milk fat (ng g^{-1} lipid) using three significant figures.

2.5. Quality control and quality assurance

The extraction, cleanup, and fractionation steps were evaluated by measurement of the absolute recoveries of the compounds ($^{13}C_{12}$-labeled internal and native surrogate standards) spiked and passed through the entire analytical procedure. Procedural blanks were analyzed simultaneously with every batch of ten samples to check for interference or contamination from solvents and glassware. For recovery tests, two levels (2.0 and 10.0 ng g^{-1}) of 14 analytes were spiked to cow milk samples based on GC/MS-selected ion monitoring (GC/MS-SIM). Recoveries were between 87% and 94% with the relative standard deviations of <10% ($n = 5$). The limits of quantification (LOQ), defined as five-times that of the noise, ranged from 0.002 to 0.30 ng g^{-1} lipid (Supplementary Table S2). When the levels of the target chemicals were less than their LOQs, we allocated half of the LOQ as the value for analysis. The calibration (0.1–5 ng mL^{-1} of each analyte) was linear and characterized by good correlation coefficients (>0.99) for all compounds studied. The quality of the method under validation was verified by two Standard Reference Materials (cod liver oil, SRM1588b and non-fortified human milk, SRM1953, NIST) for selected pesticides and PCBs. Data from our laboratory were in good agreement with the certified values (within 15% difference for SRM1953).

2.6. Statistical analysis

The data were analyzed using SPSS version 16.0 for Windows 2007 (SPSS Inc., Chicago, IL, USA). Kruskal–Wallis one-way analysis of variance and the Steel–Dwass test were used to examine differences in the target chemical concentrations among the three countries. Spearman's rank correlation coefficients were used to test the relationship between pesticide levels and characteristics of mothers. Probability values of less than 0.05 were considered to indicate statistical significance.

3. Results and discussion

3.1. ECNI-SIM profiles and overall trends

In this study, we measured 10 pesticides (19 isomers) and 11 PCB congeners in the ECNI mode. ECNI-SIM of all analytes showed higher sensitivity (lower LOQ) and selectivity (Supplementary Table S2) than in the EI-SIM mode. ECNI showed one or two orders of magnitude higher detection response (lower LOQ) for endosulfans (α- and β-isomers), toxaphenes (Parlars 26 and 50) and chlordane-related compounds (lower LOQs) than EI mode. The sensitivity to dieldrin and endrin was lower and no DDT ions were detected in ECNI mode. Some chlorinated components such as HCHs, chlordanes and toxaphenes were measured at *m/z* 71 $[HCl + Cl]^-$ ion as qualified or confirmation ions. As shown in Supplementary Table S2, GC retention times of two pairs (*cis*-HCE versus oxy-chlordane and *cis*-chlordane versus α-endosulfan) were close on the HP-5MS column (30 m). Although separation can be improved using a longer capillary column, the selectivity of these pairs on the present column was improved by using selected ions at *m/z* 388 for *cis*-HCE and *m/z* 424 for oxy-chlordane, and at *m/z* 404 for α-endosulfan and at *m/z* 412 for *cis*-chlordane, without the exchange of the present column.

The mean concentrations of 19 pesticide isomers and PCBs (sum of 11 isomers) in the breast milk samples are listed in Table 1. The profiles of major contaminants were consistent with the previous results from Japan (Haraguchi et al., 2009; Nakai et al., 2009) and from southern China (Hedley et al., 2010). The levels of chlorinated cyclodienes such as CHLs, HCE, dieldrin, and toxaphenes ranged from 0.12 to 0.96 ng g^{-1} lipid in China, from 0.2 to 4.7 ng g^{-1} lipid in Korea and from 0.8 to 4.5 ng g^{-1} lipid in Japan. These results indicate that, among the three countries, the levels of cyclodiene pesticides are highest in Japan. No significant difference in concentrations was observed within domestic regions. Contamination trends in this study are comparable with previous studies from Asian countries including Taiwan (Chao et al., 2006), Philippines (Malarvannan et al., 2009), and Vietnam (Minh et al., 2004), as well as European countries (Shen et al., 2008) (see Supplementary Table S3). The accumulation profiles and levels in Korean breast milk were correlated to those in serum from Korean residents (Kang et al., 2008); the source may be attributed to consumption of such POPs by fish in Korean coastal zones (Yim et al., 2005).

3.1.1. Endosulfans

To our knowledge, this is the first report assessing the levels of α- and β-endosulfans in breast milk from Asian countries. Endosulfan was detected as the α-form in the range of 0.85–1.4 ng g^{-1} lipid and as the β-form in the range of 0.05–0.11 ng g^{-1} lipid in all breast milk samples from China, Korea and Japan. The levels from Korea were significantly higher ($p < 0.05$) than those from China. The present levels in these three countries appear to be lower than those from European countries (Cerrillo et al., 2005; Shen et al., 2008). A recent dietary exposure study reported that endosulfan is present at similar ratios of α- and β-forms in the diet (Desalegn et al., 2011), while technical endosulfan consists of 70% α-form and 30% β-form (Jia et al., 2009). Higher ratios (>10) of α-form to β-form in human milk might be explained by their different physicochemical properties. The α-endosulfan has a higher Henry's Law constant (Rice et al., 1997; Weber et al., 2010) and, as a result, air samples are dominated by the α-form, which is easily transported in the atmosphere (Jia et al., 2009; Weber et al., 2010). In contrast, β-endosulfan has markedly higher aqueous solubility than the α-form and it will therefore partition aqueous phases more readily (Rice et al., 1997; Cetin et al., 2006; Weber et al., 2010). Furthermore, β-endosulfan is possibly converted to the α-form or endosulfan sulfate in the human body (Weber et al., 2010). These findings support the hypothesis that the source of α-endosulfan in breast milk from Asian countries is attributable to inhalation of endosulfan from the atmosphere rather than dietary intake. Endosulfan has been used widely to control a number of insects on crops and fruits in China (Li et al., 2007) and Korea (Yeo et al., 2004), although exposure to endosulfan via seafood products in southern China in 2005 was reported (Guo et al., 2007). A recent survey of endosulfan levels in the diet showed an exponentially increasing trend in China and Korea (Desalegn et al., 2011). No such historical trends of endosulfan levels have been observed in Japan. As its agricultural registration expired in 2010, exposure from local usage would be expected to decrease in future. However, endosulfan is one of the most abundant pesticides in the Arctic air (Halsall et al., 1998) and has a propensity to undergo atmospheric long-range transport (Yeo et al., 2004). Once α- and β-endosulfans enter the human body, it is presumed that the both forms are oxidized to endosulfan sulfate or other metabolites (Casabar et al., 2006). Although the occurrence of endosulfan sulfate was not investigated in the present study, the survey for endosulfan and its metabolites in human specimen samples is ongoing in our laboratories.

3.1.2. Toxaphenes

The mean concentration of toxaphene in breast milk from Japan was 2.5 ng g^{-1} lipid, which was significantly higher than those from Korea (0.73 ng g^{-1} lipid) and China (0.36 ng g^{-1} lipid). These levels were comparable to recent reports from eastern Asia (Nakai et al., 2009; Hedley et al., 2010), but appears to be much lower than those in mothers from Germany (Skopp et al., 2002), Russia (Polder et al., 1998) and southern Canada (Newsome and Ryan, 1999). Technical toxaphene was used until 1999 in Korea (de Geus et al., 1999) and until 1982 in China (Wong et al., 2005), but has never been registered in Japan. The source of toxaphene in breast milk from Japan might be through dietary intake of imported foods, or long-distant transported samples from the Arctic air, since the Arctic environment contains higher levels of toxaphene than the temperate regional environment (Van Oostdam et al., 1999). Due to differences in the persistency of congeners, a much smaller number of toxaphene congeners are found in biota and only two (Parlar #26 and #50) are present in humans (Skopp et al., 2002). Recently, the trend for increasing levels of toxaphenes in the diet in China and Korea has been reported (Desalegn et al., 2011), therefore future monitoring of toxaphenes in human samples is required.

3.1.3. Drins

The mean level of dieldrin in breast milk was 2.9 ng g^{-1} lipid in Japan, significantly higher than those in China (0.34 ng g^{-1} lipid) and Korea (1.3 ng g^{-1} lipid). The trends of dieldrin contamination are comparable with recent reports from China (Hedley et al., 2010) and Japan (Nakai et al., 2009), and lower than those from Europe (Shen et al., 2008). Aldrin, dieldrin and endrin have never been used in China and there is no industrial production of these pesticides (Wong et al., 2005). In Japan, however, these pesticides were used until the mid 1970s principally for soil treatment (Takazawa et al., 2008; Snedeker, 2001). The temporal trend indicates that exposure to dieldrin in Japan has declined during the past decade (Konishi et al., 2001). Endrin was also detected at lower levels than dieldrin in most samples from Japan (detection frequency 77%), and in only a few samples from China (5%) and Korea (25%). The presence of both dieldrin and endrin in breast milk reflects their historical use. Aldrin was not detected in all samples, probably because it has been degraded to dieldrin (Takazawa et al., 2008).

Table 1
Mean concentrations (ng g^{-1} lipid) of POPs in pooled breast milk samples from China, Korea and Japan ($n = 70$).

	China					Korea					Japan					
	Beijing (2007) $n = 10$	Beijing (2008) $n = 10$	Overall mean[b]		$n > LOQ$[a]	Seoul $n = 10$	Busan $n = 10$	Overall mean[b]		$n > LOQ$[a]	Sendai $n = 10$	Takayama $n = 10$	Takarazuka $n = 10$	Overall mean[b]		$n > LOQ$[a]
∑PCB[c]	48	42	46	B	20	62	63	63	B	20	129	89	119	112	A	30
α-HCH	2.5	8.6	5.5	C	20	0.22	0.10	0.16	B	18	0.26	0.27	0.17	0.23	A	30
β-HCH	481	881	681	B	20	62	39	50	A	20	89	22	76	63	A	30
γ-HCH	1.9	1.1	1.5	B	20	0.14	0.09	0.11	A	12	0.11	0.14	0.06	0.10	A	13
ΣHCH	485	890	688	B	20	62	39	50	A	20	89	23	77	63	A	30
HCB	70	43	57	B	20	15	11	13	A	20	19	12	16	16	A	30
Oxy-chlordane	2.3	2.1	2.2	C	20	5.9	3.9	4.9	B	20	14	6.9	13	11	A	30
Trans-chlordane	0.07	0.07	0.07	B	20	0.12	0.09	0.10	B	20	0.21	0.25	0.18	0.22	A	30
Cis-chlordane	0.36	0.17	0.27	n.s.	20	0.25	0.27	0.26	n.s.	20	0.25	0.30	0.27	0.27	n.s.	30
Trans-nonachlor	3.3	4.5	3.9	C	20	7.8	7.5	7.6	B	20	37	18	35	30	A	30
Cis-nonachlor	0.35	1.0	0.69	C	20	1.4	1.6	1.5	B	20	5.6	3.5	5.2	4.8	A	30
ΣChlordane	6.4	7.9	7.2	C	20	15	13	14	B	20	58	29	53	47	A	30
Heptachlor	n.d.	n.d.	n.d.	n.s.	0	n.d.	n.d.	n.d.	n.s.	0	n.d.	n.d.	n.d.	n.d.	n.s.	0
Heptachlor epoxide	0.95	0.97	0.96	B	18	5.6	3.9	4.7	A	20	5.1	3.8	4.7	4.5	A	30
Aldrin	0.11	n.d.	0.08	n.s.	3	0.07	n.d.	0.06	n.s.	1	0.08	n.d.	n.d.	0.06	n.s.	1
Dieldrin	0.34	0.34	0.34	C	13	1.6	0.97	1.3	B	19	3.3	2.9	2.7	2.9	A	30
Endrin	n.d.	0.13	0.12	B	1	0.24	0.12	0.18	B	5	1.1	0.54	0.82	0.82	A	23
Σdrin	0.45	0.47	0.54	C	15	1.9	1.09	1.5	B	19	4.4	3.4	3.5	3.8	A	30
α-Endosulfan	1.0	0.85	0.95	n.s.	20	1.4	1.3	1.3	n.s.	20	0.87	1.2	1.1	1.1	n.s.	30
β-Endosulfan	0.07	0.05	0.06	B	19	0.10	0.08	0.09	A	20	0.06	0.11	0.10	0.09	AB	30
ΣEndosulfan	1.1	0.90	1.0	B	20	1.5	1.4	1.4	A	20	0.93	1.3	1.2	1.2	AB	30
Mirex	0.31	0.42	0.37	B	18	0.40	0.30	0.35	B	17	1.1	1.0	0.94	1.0	A	30
BDE-47	0.83	1.6	1.2	B	20	1.9	1.3	1.6	B	20	1.8	0.71	0.53	1.0	A	30
Toxaphene (Parlar26)	0.07	0.27	0.17	C	11	0.34	0.31	0.32	B	19	1.1	0.80	1.0	0.99	A	30
Toxaphene (Parlar50)	0.08	0.30	0.19	C	8	0.43	0.38	0.41	B	19	2.1	1.1	1.5	1.6	A	30
ΣToxaphene	0.15	0.57	0.36	C	11	0.77	0.69	0.73	B	19	3.2	1.9	2.5	2.5	A	30

n.s.: not significant, n.d.: not detected.
[a] Numbers quantified.
[b] Significant difference ($p < 0.05$) according to the Steel–Dwass test. Means followed by different letters differed significantly from other countries ($p < 0.05$).
[c] Sum of 11 isomers: PCB-74, 99, 105, 118, 138, 153, 156, 170, 180, 183, and 187.

3.1.4. Chlordanes and heptachlors

The mean CHL concentrations in breast milk samples were highest in Japan (47 ng g^{-1} lipid), followed by Korea (14 ng g^{-1} lipid) and lowest in China (7.2 ng g^{-1} lipid). This could reflect the extensive use of CHLs in Japan (Konishi et al., 2001; Taguchi and Yakushiji, 1988). In fact, CHLs have been widely used for termite control in China, Korea and Japan (Li et al., 2007; Liu et al., 2009). Technical chlordane is composed of *trans*-chlordane (24%), *cis*-chlordane (22%), heptachlor (10%), and *trans*-nonachlor (7%) (Hinckley et al., 1990). The CHL contaminants in breast milk were dominated by *trans*-nonachlor and oxy-chlordane (Table 1). Higher levels of *trans*-nonachlor indicate that it is more resistant to metabolism and elimination in the human body (Taguchi and Yakushiji, 1988). Oxychlordane is degraded from other CHLs. Thus the higher ratio of oxychlordane to *trans*-nonachlor implies no recent input of technical CHLs. In a 1984–1985 survey, the mean concentration of *trans*-nonachlor in breast milk was 26 ng g^{-1} lipid in randomly selected populations ($n = 7$) (Taguchi and Yakushiji, 1988), whereas the present levels remains similar (30 ng g^{-1} lipid), indicating that exposure to CHLs has continued during the last 20 years in Japan.

Heptachlor is a constituent of technical CHLs, and also has been used as an insecticide for agricultural purposes in Asian countries (Taguchi and Yakushiji, 1988). Therefore, we determined heptachlor levels separately from CHLs. Heptachlor was not detected in any breast milk samples, probably because it was metabolized to *cis*-HCE in soils and/or biological systems (Bidleman et al., 1998). The mean concentration of HCE in breast milk from Japan (4.5 ng g^{-1} lipid) was within the same range as that from Korea (4.7 ng g^{-1} lipid), and significantly higher than that from China (0.96 ng g^{-1} lipid). The ratio of *cis*-HCE/*trans*-nonachlor was higher in Korea (0.62) than in Japan (0.15). The higher residue of *cis*-HCE in Korean breast milk may reflect the relatively high usage of technical heptachlor in Korea. In fact, heptachlor has been used as a pesticide in Korea (Yeo et al., 2004), but the levels of total CHLs in the atmosphere in Korea are much lower than those in Japan and China (Park et al., 2011). In Japan, a 1984 survey showed that the HCE level in breast milk was 21 ng g^{-1} lipid (Konishi et al., 2001) but decreased to 4.5 ng g^{-1} lipid in 2008 (this study). The difference in temporal trends between CHLs and HCE is probably because of their different half-lives (Park et al., 2011) and different sources such as dietary intake, inhalation in the home of a contaminated atmosphere and partly absorption through the skin (Taguchi and Yakushiji, 1988).

3.1.5. Other pesticides

In our previous report (Fujii et al., 2011), the concentration of DDTs was highest in China, followed by Japan and lowest in Korea. The present study showed that the mean concentration of HCHs in breast milk from China was one order of magnitude higher than those from Japan and Korea ($p < 0.05$). β-HCH accounted for 95% of the total HCHs and α- and γ-HCHs were detected only at trace levels (Table 1). Apart from lindane production, which comprises approximately 99% of γ-HCH, technical HCH consists of 65–70% of α-HCH (Qu et al., 2010), which can be converted to β-HCH in the human body (Wu et al., 2010). The higher ratio of β-HCH/ΣHCH indicated no recent exposure of participants to technical HCH. The concentrations of α- and γ-HCHs in breast milk from China are also one order of magnitude higher than those from Japan and Korea. Their possible source might be inhalation from the atmosphere above Tianjin, near Beijing, where α- and γ-HCHs are dominant (Zheng et al., 2010).

The mean HCB concentration in breast milk was highest in China (57 ng g^{-1} lipid), although it has never been registered as a pesticide. It likely reflects unintentional formation of HCB by industrial activities such as an intermediate for the synthesis of chlorinated solvents and waste incineration (Wong et al., 2005). HCB has been detected also in agricultural products such as tea leaves in China (Nakata et al., 2002), indicating that it has been used illegally as a pesticide or has contaminated widely as an impurity.

3.1.6. Correlation between OCP levels in human milk and characteristics of mothers

The correlations between the OCP concentrations and lipid contents in human milk or characteristics of mothers, including age, parity and BMI are shown in Supplementary Table S4. Endosulfan was correlated with HCB, oxy-chlordane in Korea, but not in Japan and China. Toxaphenes were correlated with chlordanes in the three countries. Lipid content was negatively correlated with the concentration of α-endolsufan in Korea ($r = -0.521$, $p < 0.05$) and Japan ($r = -0.725$, $p < 0.01$), whereas it was positively correlated with the concentration of t-nonachlor in China ($r = 0.574$, $p < 0.01$). The levels of endosulfans in China and chlordanes in Korea were age-dependent, but there was no age relationship for other pesticides. BMI was correlated with the levels of toxaphene and oxy-chlordane in Japan. However, these trends had some limitations due to the pooled milk sample from three individuals that would mask significant correlations between each pesticide and associated characteristics of mothers.

4. Conclusion

To our knowledge, this is the most extensive study on contamination of human breast milk by chlorinated cyclodiene pesticides such as chlordanes, heptachlor, dieldrin, endrin, toxaphenes, mirex and endosulfans in China, Korea and Japan. The results indicate that the levels of α- and β-endosulfans are relatively higher in breast milk from Korea, whereas the other chlorinated cyclodiene congeners are still contaminants in samples from Japan. Therefore, such compounds need further monitoring in the future.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2012.05.098.

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Regional variation and possible sources of brominated contaminants in breast milk from Japan

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ABSTRACT

This study focuses on the regional trends and possible sources of brominated organic contaminants accumulated in breast milk from mothers in southeastern (Okinawa) and northwestern (Hokkaido) areas of Japan. For persistent brominated flame retardants, polybrominated diphenyl ethers (PBDEs; major components, BDE-47 and BDE-153) were distributed at higher levels in mothers from Okinawa (mean, 2.1 ng/g lipid), while hexabromobenzene (HeBB) and its metabolite 1,2,4,5-tetrabromobenzene were more abundantly detected in mothers from Hokkaido (0.86 and 2.6 ng/g lipid), suggesting that there are regional differences in their exposure in Japan. We also detected naturally produced brominated compounds, one of which was identified as 2′-methoxy-2,3′,4,5′-tetrabromodiphenyl ether (2′-MeO-BDE68) at higher levels in mothers from Okinawa (0.39 ng/g lipid), while the other was identified as 3,3′,4,4′-tetrabromo-5,5′-dichloro-2,2′-dimethyl-1,1′-bipyrrole in mothers from Hokkaido (0.45 ng/g lipid). The regional variation may be caused by source differences, i.e. southern seafood for MeO-PBDEs and northern biota for halogenated bipyrroles in the Japanese coastal water.

1. Introduction

Persistent organic pollutants (POPs) are biomagnified in the food chain (Borgå et al., 2001). Irrespective of the nature of their source, they are widespread and probably undergo extensive transport and fates that are governed by their physicochemical properties such as vapor pressure, aqueous solubility, Henry's Law constant and octanol/water partition coefficient (K_{ow}) (Hackenberg et al., 2003; Tittlemier et al., 2004; Vetter et al., 2004). As a result, their residues accumulate in the human body by way of dietary intake or inhalation throughout a person's lifetime. Therefore, regular monitoring of POP contamination in human milk can help to identify specific sources of pollutants, exposure trends and potential risks of exposure to mothers and infants.

It seems likely that bioaccumulative brominated flame retardants (BFRs), such as polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane and hexabromobenzene (HeBB) are globally spreading throughout the marine biosphere. Some of these compounds have been reported to transfer via the placenta and breast milk from mothers to offspring in humans and exhibit endocrine-disrupting effects (Kawashiro et al., 2008) or developmental neurotoxic effects (Costa and Giordano, 2007). In Japan, PBDEs have been used to prevent combustion in consumer products, such as electronics, construction materials and textiles (Ueno et al., 2004), but have leveled off in recent years after voluntary phasing out of penta- and octa-PBDE formulations in the 1990s (Ueno et al., 2010). The residue levels of PBDEs have recently been reported in human milk (Eslami et al., 2006; Haraguchi et al., 2009c) and blood (Kawashiro et al., 2008) as well as in seafood from Japanese coastal water (Ueno et al., 2004). The sources are probably house dust and/or electric waste (Fromme et al., 2009; Thomsen et al., 2010) as well as seafood (Ueno et al., 2004). Although the temporal trends in human exposure to PBDEs are steadily decreasing in Japan, the current status of BFR use seems to differ from region to region and from country to country (Watanabe and Sakai, 2003). Similar to PBDEs, HeBB has been used as an additive flame retardant for paper, plastic and electronic goods and is still used at low volumes in Japan (350 tons per year between 1994 and 2001) (Watanabe and Sakai, 2003). Thus far, the levels of HeBB in adipose tissues of Japanese people have been reported (Yamaguchi et al., 1988), but no recent trends for HeBB levels in breast milk are available.

Regarding related organobromine residues, methoxylated PBDEs (MeO-PBDEs) and halogenated bipyrroles of natural origin have been found in biota from Japanese coastal water (Haraguchi et al., 2009b; Marsh et al., 2005). MeO-PBDEs can biomagnify in higher-trophic

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organisms via the food chain from the Pacific Ocean (Haraguchi et al., 2010; Vetter et al., 2009). A series of mixed halogenated bipyrroles, i.e. 1,1′-dimethyl-3,3′,4,4′-tetrabromo-5,5′-dichloro-2,2′-bipyrrole (DBP-Br_4Cl_2) and 2,3,3′,4,4′,5,5′-heptachloro-1′-methyl-1,2′-bipyrrole (MBP-Cl_7), have also been found to biomagnify at higher-trophic levels via the food chain to similar extents to recalcitrant POPs. In fact, these two bipyrroles have been found in fish, seabirds and marine mammals from the North Pacific (Gribble et al., 1999; Tittlemier et al., 2002; Tittlemier, 2004) and Oceania (Vetter et al., 2001, 2009), owing to their similar physical properties to PBDEs (Hackenberg et al., 2003; Tittlemier et al. 2004; Vetter et al., 2004). Therefore, human exposure to these brominated compounds is of concern for the health of mothers and infants, because DBP-Br_4Cl_2, for example, has displayed some *in vitro* dioxin-like ability (Tittlemier et al., 2003). However, the regional trends in the contamination status of MeO-PBDEs and halogenated bipyrroles in human breast milk are poorly understood.

The aim of this study was to investigate the trends and sources of anthropogenic PBDEs and HeBB, as well as naturally occurring MeO-PBDEs and halogenated bipyrroles, in human breast milk from Japan. To investigate the regional trends in these brominated contaminants, we selected human milk samples from the most northeast area (Hokkaido) and the most southwest area (Okinawa) of Japan (Fig. 1).

2. Materials and methods

2.1. Sample collection

Human milk samples were obtained from the Kyoto University Human Specimen Bank using a standardized protocol (Koizumi et al., 2005, 2009). A total of 40 samples were collected during 2005–2006 from volunteers living in Hokkaido ($n = 20$) and Okinawa ($n = 20$) as shown in Table 1. Milk samples (30–50 mL) were collected manually during breastfeeding at 4–8 weeks after childbirth, either by the subjects themselves or with the assistance of midwifes. The breast milk was kept frozen (−20 °C) prior to analysis. The Ethics Committee of Kyoto University approved the protocol of the present study (E25) and appropriate written informed consent was obtained from all the participants.

Table 1
Information regarding the participants and lipid contents of milk samples from Hokkaido and Okinawa.

Region	Location		Year	*n*	Mean age	Lipid (%)
	Latitude	Longitude				
Hokkaido	42–90′N	140–99′E	2005	20	30.5	2.30
Okinawa	26–20′N	127–69′E	2005–2006	20	30.3	2.63
All				40	30.4	2.45

2.2. Chemicals

Two standards, 4′-methoxy-2,3′,4,5′,6-pentachlorodiphenyl ether (4′-MeO-BDE121), as an internal standard for the determination of all brominated contaminants, and 2,2′-dimethoxy-3,3′,4,4′-tetrabromobiphenyl (2,2′-diMeO-BB80) were donated by Dr. G. Marsh (Stockholm University). Native BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, hexabromobenzene (HeBB), 1,2,4,5-tetrabromobenzene (TeBB), 2′-hydroxy-2,3′,4,5′-tetrabromodiphenyl ether (2′-MeO-BDE68) and 6-methoxy-2,2′,4,4′-tetrabromodiphenyl ether (6-MeO-BDE-47) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Two bipyrrole standards, 1,1′-dimethyl-3,3′,4,4′-tetrabromo-5,5′-dichloro-2,2′-bipyrrole (DBP-Br_4Cl_2) and 2,3,3′,4,4′,5,5′-heptachloro-1′-methyl-1,2′-bipyrrole (MBP-Cl_7), were synthesized according to the methods outlined in Gribble et al. (1999) and Wu et al. (2002), respectively. The purities of the compounds were >99% by gas chromatography. The standards were used for the calibration, recovery and quantification of target compounds. All solvents of pesticide grade quality were purchased

Fig. 1. Sampling sites of breast milk in Japan (Hokkaido and Okinawa Prefecture).

from Kanto Chemical Co. Ltd. (Tokyo, Japan). Silica-gel (Wako gel S-1) was used for purification (Wako Pure Industries Ltd., Osaka, Japan) and heated at 130 °C for 3 h prior to use. The chemical structures of the target analytes of natural origin are shown in Fig. 2.

2.3. Clean-up procedure

The methodology used to analyze brominated contaminants in the breast milk samples was based on lipid extraction, gel permeation chromatography (GPC) and silica-gel column cleanup, and gas chromatography–negative chemical ionization–mass spectrometry (GC–NCI–MS). Briefly, 5 mL of each breast milk was spiked with 4′-MeO-BDE121 (0.2 ng) and extracted with n-hexane, after adding potassium oxalate solution, ethanol and ethylether (1:1:1, v/v/v). The extract was washed with water and dried over sodium sulfate. After solvent evaporation, lipid was determined gravimetrically.

An aliquot of lipid (50–300 mg) was dissolved in 1.5 mL of dichloromethane (DCM)/n-hexane (1:1, v/v), and subjected to GPC with a Bio-Beads S-X3 column (35 g of gel material; Bio-Rad Laboratories, Hercules, CA, USA) with DCM/hexane as the eluting solvent at a flow rate of 4 mL/min. The first 90-mL fraction of the eluate containing lipid was discarded, and the subsequent 80-mL fraction was collected. To remove the remaining trace amount of lipid, the residue was loaded onto a silica-gel column (0.2 g of Wako gel S-1). The fraction was eluted with 15 mL of 12% DCM/n-hexane, and concentrated to 200 μL for GC/MS analysis.

2.4. Instruments and quantification

Thirteen analytes were measured by GC–NCI–MS using an Agilent HP5973MSD 5973i (Agilent Technologies, Palo Alto, CA, USA) coupled with a 6890N gas chromatograph. The GC/MS conditions and target ions for determination of POPs are summarized in Table 2. Quantification of the compounds was based on the signals in the mass chromatograms and on comparisons with the internal standard (4′-MeO-BDE121). PBDEs were analyzed by scanning for the negative bromine ion (isotopes m/z 79 and 81) formed by electron capture reactions at chemical ionization (ECNI) with methane as the reagent gas.

2.5. Quality control and quality assurance

Procedural blanks were analyzed simultaneously with every batch of ten samples to check for interference or contamination from solvents and glassware. For recovery tests, a matrix (cow milk) spiking test was conducted with two spiked levels (2.0 and 10.0 ng/g) of 13 analytes and an internal standard. Based on GC/MS-selected ion monitoring (SIM), their recoveries were 84–91% with relative standard deviations (RSDs) of <10% ($n = 5$). The limits of quantification (LOQs) were defined as five times the noise value and ranged from 0.01 to 0.2 ng/g lipid (Table 3). When the level of the target chemical was less than the LOQ, we allocated one-half of the LOQ as the value for the calculation. The calibrations (0.1–5.0 ng/mL of each analyte) were linear and characterized by good correlation coefficients (>0.99) for all compounds studied. The quality of the method under validation was verified by analysis of a Standard Reference Material (cod liver oil, SRM1588b, NIST) (Stapleton et al., 2007). The data from our laboratory were in good agreement with the certified values (<11% of RSD, $n = 5$) for PBDEs.

Table 2
GC/MS conditions for analysis of brominated compounds in human breast milk.

Carrier gas	Helium (head pressure of 3 psi)
Injection mode	Splitless
Column	HP-5MS (30% dimethylpolysiloxane, 30 m × 0.25 mm i.d. and 0.25 μm film thickness, J&W Scientific, CA, USA)
Oven	70 °C (1.5 min), then 20 °C/min to 230 °C (0.5 min), and then 4 °C/min to 280 °C (5 min)
Temperature	Injector (250 °C), transfer line (280 °C), and ion source (230 °C for EI, 150 °C for ECNI)
Ionization mode	ECNI (electron capture negative ionization)
Reagent gas	Methane
Target ions, (confirmed ions), m/z	79 (81) for brominated contaminants, 386 (388) for MBP-Cl_7

2.6. Statistical analysis

The obtained data were analyzed statistically using SPSS software version 18.0 for Windows 2007 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance was used to examine differences in the target chemical concentrations between regions. Pearson's correlation coefficient was used to examine the strength of the associations between the mothers' ages and the organobromine concentrations. Probability values of less than 0.05 were considered to indicate statistical significance.

3. Results

We detected six PBDE congeners, HeBB and TeBB in breast milk samples from Hokkaido and Okinawa. The major components of the PBDEs were BDE-47 and BDE-153, which were detected at higher frequencies in Okinawa. The congener levels are shown in Table 3. The levels of ΣPBDE ranged from <0.2 to 69 ng/g lipid (median, 1.5 ng/g lipid) and were higher in mothers from Okinawa, although one sample from Hokkaido was considerably highly contaminated with PBDEs (i.e. 46 ng/g lipid for BDE-47 and 4.0 ng/g lipid for BDE-153). HeBB and TeBB were found at ranges of <0.05–2.5 (mean, 0.53) ng/g lipid and 0.76 to 6.6 (mean, 2.6) ng/g lipid, respectively. The HeBB levels were significantly higher in breast milk from Hokkaido ($p < 0.01$), whereas no regional

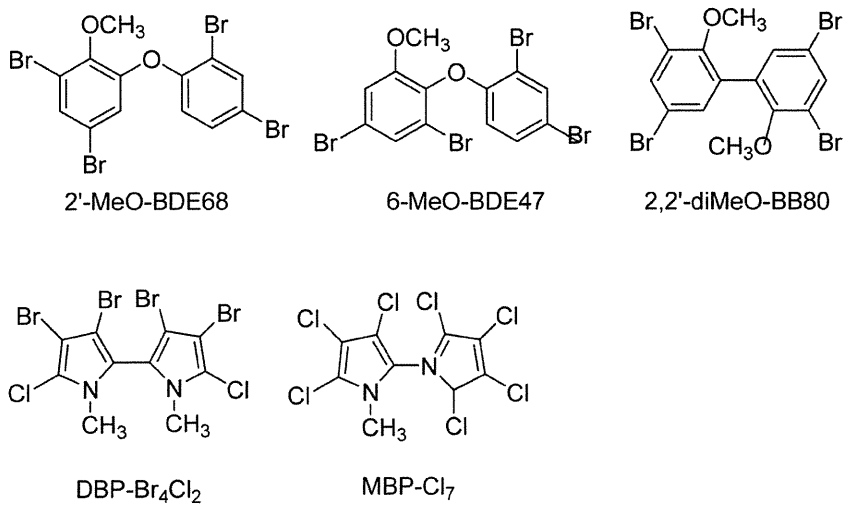

Fig. 2. Structures of naturally produced brominated contaminants. 2′-MeO-BDE68: 4,6-dibromo-2-(2′,4′-dibromo)phenoxyanisole; 6-MeO-BDE-47: 3,5-dibromo-2-(2′,4′-dibromo) phenoxyanisole; 2,2′-diMeO-BB80: 2,2′-dimethoxy-3,3′5,5′-tetrabromobiphenyl; DBP-Br_4Cl_2: 1,1′-dimethyl-2,2′-bipyrrole; MBP-Cl_7: 2,3,3′,4,4′,5,5′-heptachloro-1′-methyl-1,2′-bipyrrole.

Table 3
Concentrations of polybrominated diphenyl ethers and related compounds in breast milk collected from Okinawa and Hokkaido.

	Okinawa $n = 20$				Hokkaido $n = 20$				Overall		LOQ (ng/g lipid)
	Freq ($n >$ LOQ)	Mean	Median	Range	Freq ($n >$ LOQ)	Mean	Median	Range	Mean	Median	
Concentration (ng/g lipid)											
BFRs											
BDE-28	16	0.12	0.12	<0.06–0.38	6	0.16	0.030	<0.06–1.9	0.14	0.040	0.06
BDE-47	20	0.97	0.87	0.10–2.2	16	2.7	0.40	<0.08–46	1.9	0.56	0.08
BDE-99	14	0.20	0.16	<0.1–0.48	4	0.62	0.050	<0.1–10	0.41	0.050	0.1
BDE-100	11	0.16	0.080	<0.1–0.56	4	0.41	0.050	<0.1–6.7	0.29	0.050	0.1
BDE-153	20	0.60	0.56	<0.2–1.6	10	0.54	0.19	<0.2–4.0	0.57	0.48	0.2
BDE-154	14	0.19	0.16	<0.2–0.41	3	0.13	0.10	<0.2–0.57	0.16	0.10	0.2
ΣPBDE	20	2.1	2.1	0.55–5.1	16	4.3	1.0	<0.2–69	3.4	1.5	–
TeBB	20	2.4	2.0	0.83–6.0	20	2.6	2.6	0.76–6.6	2.5	2.1	0.01
HeBB	19	0.19	0.20	<0.05–0.46	20	0.86**	0.71	0.20–2.5	0.53	0.32	0.05
Natural products											
2′-MeO-BDE68	18	0.39*	0.28	<0.06–1.6	12	0.17	0.070	<0.06–0.69	0.28	0.14	0.06
6-MeO-BDE-47	8	0.050*	0.030	<0.05–0.13	0	<0.05	<0.05	<0.05	0.040	0.030	0.05
2,2′-diMeO-BB80	17	0.20**	0.22	<0.04–0.45	7	0.040	0.020	<0.04–0.12	0.12	0.070	0.04
MBP-Cl_7	19	0.19	0.11	<0.01–0.94	17	0.090	0.070	<0.01–0.43	0.14	0.080	0.01
DBP-Br_4Cl_2	17	0.23	0.20	<0.04–0.062	18	0.45	0.28	<0.04–2.7	0.34	0.25	0.04
Ratio											
BDE-47/BDE-153		1.6	1.6			5.0	2.1		3.3	1.2	
TeBB/HeBB		12	9.8			3.1	3.7		4.7	6.6	
2′-MeO-BDE68/BDE-47		0.40	0.32			0.06	0.18		0.15	0.25	

All data were calculated by assuming that values below the LOQ were equal to one-half of the LOQ. *$p < 0.05$, **$p < 0.01$.

difference was found in the TeBB levels. Regarding other brominated contaminants, we detected three methoxylated analogs of tetra-BDEs and two halogenated bipyrroles (Fig. 2). The levels of 2′-MeO-BDE68 and 2,2′-diMeO-BB80 were significantly higher in mothers from Okinawa (0.39 and 0.20 ng/g lipid, respectively, $p < 0.01$ for each) than in mothers from Hokkaido. The levels of MBP-Cl_7 and DBP-Br_4Cl_2 ranged from <0.01 to 0.94 ng/g lipid and <0.01–2.7 ng/g lipid, respectively. No regional differences in the levels of these two bipyrroles were observed between the two areas.

The correlations between the concentrations of individual contaminants in Okinawa ($n = 20$) and Hokkaido ($n = 20$) are shown in Table 4. BDE-47 was correlated with BDE-153 in Hokkaido ($r = 0.927$, $p < 0.01$), but not in Okinawa. In accordance, HeBB was correlated with TeBB in Hokkaido ($r = 0.628$, $p < 0.01$), but not in Okinawa. 2′-MeO-BDE68 was positively correlated with 2,2′-diMeO-BB80 in Okinawa ($r = 0.522$, $p < 0.05$), but not in Hokkaido. DBP-Br_4Cl_2 was not correlated with MBP-Cl_7 in both areas, but well correlated with 2′-MeO-BDE68 ($r = 0.478$, $p < 0.05$) and 2,2′-diMeO-BB80 ($r = 0.767$, $p < 0.01$) in Okinawa. No age dependency was found for any of the congeners investigated in both areas.

4. Discussion

4.1. PBDEs

The contamination trends of PBDEs in this study were of similar magnitude to recent results in Japan (Haraguchi et al., 2009c; Kawashiro et al., 2008) and Europe (Thomsen et al., 2010). The present study showed regional differences in the concentrations of PBDEs in breast milk. These trends were also observed in a recent large-scale survey of PBDEs in Japanese breast milk (Eslami et al., 2006). The variation of PBDE levels in Japanese people may be caused by factors related to food culture. However, one milk sample from Hokkaido contained considerably high levels of PBDEs (69 ng/g lipid), despite the other samples from the same area showing lower levels (median, 1.0 ng/g lipid) of PBDEs. It is assumed that the high concentration of PBDEs may be attributed to occupational exposure via house dust or electric waste consumption (Fromme et al., 2009; Thomsen et al., 2010), rather than food sources and habitual dietary intake. A previous survey using tuna fish as biomarker in the Asia-Pacific region revealed that the highest concentrations of PBDEs were detected in fish from off-Taiwan coastal water, near the Okinawa area (Ueno et al., 2004). The levels of congeners were higher in the order of BDE-47 > BDE-153 > BDE-100 in most samples, although BDE-47 was not correlated with BDE-153 in Okinawa, indicating their different sources. The relative contribution of lower brominated PBDEs (i.e. ratio of BDE-47 to BDE-153) was higher in Hokkaido (5.0) than in Okinawa

Table 4
Peason's correlation coefficients between the levels of the major brominated contaminants in breast milk from Okinawa ($n = 20$) and Hokkaido ($n = 20$).

	BDE-47	BDE-153	TeBB	HeBB	2′-MeO-BDE68	2,2′-diMeO-BB80	MBP-Cl_7
Okinawa							
BDE-153	0.348						
TeBB	−0.202	0.107					
HeBB	0.364	0.775**	0.053				
2′-MeO-BDE68	0.070	−0.189	−0.199	−0.078			
2,2′-diMeO-BB80	0.299	−0.188	−0.104	0.074	0.522*		
MBP-Cl_7	0.432	0.540*	−0.168	0.490*	0.029	0.021	
DBP-Br_4Cl_2	0.284	−0.059*	−0.137	0.158	0.478*	0.767**	0.279
Hokkaido							
BDE-153	0.927**						
TeBB	−0.214	−0.088					
HeBB	−0.117	−0.031	0.628**				
2′-MeO-BDE68	0.054	0.197	−0.077	0.069			
2,2′-diMeO-BB80	0.004	0.071	0.049	−0.273	0.221		
MBP-Cl_7	0.268	0.298	0.054	0.069	0.183	−0.090	
DBP-Br_4Cl_2	−0.064	−0.108	0.301	−0.024	0.408	0.480*	0.129

*$p < 0.05$, **$p < 0.01$.

(1.6) (Table 3). The results may be related to the finding that the percentage contributions of lower brominated congeners (BDE-28 and BDE-47) increased with increasing latitude and the highest ratio of lower PBDEs was found in seafood from the northern colder region in the North Pacific (Ueno et al., 2004).

4.2. HeBB and its metabolite

Although HeBB has been used as one of the BFRs at low volumes in Japan (350 tons per year between 1994 and 2001) (Watanabe and Sakai, 2003), recent contamination trends of HeBB have not been available. This study revealed that, as well as HeBB, debrominated TeBB was present at higher levels than HeBB in most samples, indicating that these compounds are widely distributed as persistent brominated contaminants in the Japanese environment. The HeBB levels were significantly higher in mothers from Hokkaido than in mothers from Okinawa, while no regional difference was observed for the TeBB levels (Table 3). The HeBB levels were not significantly correlated with the TeBB and BDE-47 levels, but were positively correlated with the BDE-153 levels (Table 4), indicating that HeBB may be exposed via the same route as BDE-153. Miyazaki et al. (1986) first detected TeBB in human milk, but not HeBB. Although we have no information that TeBB is contained as a byproduct in agricultural and/or industrial chemicals, the source of TeBB may be partly different from that of HeBB. In a 1988 survey, similar levels of HeBB and TeBB were determined in human adipose tissues (range, 2.1–4.1 ng wet weight) (Yamaguchi et al., 1988) and rat experiments showed that TeBB may be a metabolite (debrominated product) of HeBB. The HeBB levels were positively correlated with the TeBB levels in Hokkaido, but not in Okinawa, suggesting that there may be other factors affecting the variation of HeBB levels.

4.3. MeO-PBDE analogs

Regarding PBDE-related products detected in this study, three methoxylated PBDE analogs, 2′-MeO-BDE68, 6-MeO-BDE-47 and 2,2′-diMeO-BB80, are considered to be of natural origin. The levels of both 2′-MeO-BDE68 and 2,2′-diMeO-BB80 were slightly lower than those of BDE-47. The ratios of 2′-MeO-BDE68 to BDE-47 were higher in samples from Okinawa (0.40) than in samples from Hokkaido (0.06) (Table 3), and the levels of 2′-MeO-BDE68 were not correlated to those of BFRs (Table 4), indicating a specific source via a different exposure pathway. Recent studies have shown that whale blubber, shark liver and seafood (grouper, bluefin tuna etc.) from Okinawa coastal water have accumulated these MeO-PBDE analogs (Haraguchi et al., 2009b; Hisamichi et al., 2007; Marsh et al., 2005). Therefore, the source of MeO-PBDEs in breast milk may be seafood contaminated with naturally produced brominated analogs. The regional difference may be attributed to the extent of occurrence of MeO-PBDEs in nature. For example, these compounds could be produced by specific seaweeds inhabiting the tropical seashore (Haraguchi et al., 2010). MeO-PBDEs and the corresponding OH-PBDEs have also been found in human milk from Italy (Lacorte and Ikonomou, 2009) and Nicaragua (Athanasiadou et al., 2008), although their profiles in breast milk were different from our results. The toxicity of MeO-PBDEs is still unknown but the corresponding OH-PBDEs are known to have endocrine-disrupting properties that allow transfer from mothers to infants via the placenta or breastfeeding (Kawashiro et al., 2008). Wan et al. (2009) reported that OH-PBDEs formed in the livers of marine mammals and fish are demethylation products of MeO-PBDEs rather than hydroxylated metabolites of PBDEs. It is therefore possible that MeO-PBDEs are converted to more toxic OH-PBDEs in the human body. The levels of 2,2′-diMeO-BB80 were positively correlated with those of 2′-MeO-BDE68, indicating that both compounds had the same exposure route. The 2,2′-diMeO-BB80 detected in human milk has also accumulated in whales and sharks (Haraguchi et al., 2009a, 2009b; Marsh et al., 2005). The source may be derived from 2,2′-diOH-BB80 that can be isolated from a marine bacterium (Isnansetyo and Kamei, 2003).

4.4. Halogenated bipyrroles

The present study further showed that two types of halogenated bipyrroles, DBP-Br_4Cl_2 (2,2′-bipyrrole) and MBP-Cl_7 (1′,2-bipyrrole), were distributed at similar levels to 2′-MeO-BDE68 in Japanese breast milk. The greater abundance of DBP-Br_4Cl_2 in mothers from Hokkaido suggests that the source may be biota (foodweb) in the northern latitude of the North Pacific area. In fact, killer whales stranded in Hokkaido had accumulated DBP-Br_4Cl_2 at much higher levels (Haraguchi et al., 2009a). However, DBP-Br_4Cl_2 was also found in the liver of tiger sharks in Okinawa coastal water (Haraguchi et al., 2009b), whale products in the Japanese market (Haraguchi et al., 2006) and Canadian seafood (Tittlemier, 2004), indicating the widespread distribution of DBP-Br_4Cl_2 in the Pacific. In Okinawa breast milk, the levels of DBP-Br_4Cl_2 were significantly correlated with those of the other natural contaminants, such as 2′-MeO-BDE68 and 2,2′-diMeO-BB80 (Table 3), but were not correlated with the levels of MBP-Cl_7. These findings suggest that these bipyrroles may be derived from different biogenic sources. In fact, MBP-Cl_7 has been detected in mammals from Oceania (Vetter et al., 2001), while DBP-Br_4Cl_2 has not. Nevertheless, both bipyrroles appear to have similar physicochemical properties to BDE-47 and 2′-MeO-BDE68 in their potential for global distribution (Hackenberg et al., 2003; Tittlemier et al., 2004). Although the toxicological significance of these bipyrroles is unknown, some reports have shown hepatic enzyme induction by DBP-Br_4Cl_2 (Tittlemier et al., 2003) and moderate biological activity of MBP-Cl_7 (Vetter et al., 2004).

4.5. Daily intake estimates for infants

The estimation of daily intake (EDI) for the brominated contaminants for infants was assessed based on average breast milk consumption by infants (Van Oostdam et al., 1999) (Supplemental Table 1). In this study, the EDIs of PBDEs were less than one-thousand of the No Observed Adverse Effect Level (NOAEL) of Penta-BDEs (NOAEL:0.4 mg/kg body weight/day) (Viberg et al., 2004), indicating that the health risks for PBDEs intake from breast milk are limited. However, infants have different susceptibilities to adults with regard to their dynamic growth and developmental processes (Sly and Flack, 2008). In addition, the toxicokinetics and toxicities of HeBB, naturally occurring MeO-PBDEs and halogenated bipyrroles are still unclear. These uncertainties necessitate more comprehensive toxicological studies on those compounds.

5. Conclusions

The present study showed that Japanese breast milk samples were contaminated with anthropogenic (PBDEs and HeBB) and natural origin (MeO-PBDEs and bipyrroles) compounds The levels of PBDEs (BDE-47 and BDE-153) tended to be higher in mothers from Okinawa, while the levels of HeBB were significantly higher in mothers from Hokkaido. These findings indicate that PBDEs and HeBB have different exposure pathways. Two MeO-PBDEs (2′-MeO-BDE68 and 2,2′-diMeO-BB80) showed higher concentrations in mothers from Okinawa, whereas two bipyrroles (DBP-Br_4Cl_2 and MBP-Cl_7) may be derived from different biota in the Japanese coastal waters. To clarify the exposure pathways and health effects of these brominated contaminants, the spatial trends of these contaminants need to be further investigated.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.envpol.2011.11.022.

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Levels and profiles of long-chain perfluorinated carboxylic acids in human breast milk and infant formulas in East Asia

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ABSTRACT

In this study, 90 human breast milk samples collected from Japan, Korea, and China were analyzed for perfluorooctanoic acid (PFOA) (C8), perfluorononanoic acid (PFNA) (C9), perfluorodecanoic acid (PFDA) (C10), perfluoroundecanoic acid (PFUnDA) (C11), perfluorododecanoic acid (PFDoDA) (C12), and perfluorotridecanoic acid (PFTrDA) (C13). In addition, infant formulas ($n = 9$) obtained from retail stores in China and Japan were analyzed. PFOA was the predominant compound and was detected in more than 60% of samples in all three countries. The PFOA, PFNA, PFDA, and PFUnDA levels in Japan were significantly higher than those in Korea and China ($p < 0.05$). The PFTrDA level was highest in Korea ($p < 0.05$). The median PFOA concentrations were 89 pg mL^{-1} (48% of total perfluorinated carboxylic acids (PFCAs) (C8–C13)) in Japan, 62 pg mL^{-1} (54%) in Korea, and 51 pg mL^{-1} (61%) in China. The remaining $\sum$PFCAs (C9–C13) were 95 pg mL^{-1} in Japan, 52 pg mL^{-1} in Korea, and 33 pg mL^{-1} in China. Among the long-chain PFCAs, odd-numbered PFCAs were more frequently detected than even-numbered PFCAs, except for PFDA in Japan. There were no evident correlations between the mother's demographic factors and the PFCA concentrations. PFOA, PFNA, and PFDA were frequently detected in both Japan and China, but there were no significant differences between the two countries. The total PFCA concentrations in the infant formulas were lower than those in the breast milk samples in Japan ($p < 0.05$), but not in China ($p > 0.05$). In conclusion, various PFCAs were detected in human breast milk samples from East Asian countries.

1. Introduction

Perfluorinated compounds (PFCs) comprise a large group of man-made fluorinated organic chemicals. They have been produced since the 1950s and are used for various industrial and consumer-related applications, such as food packaging materials, protective coatings for textiles, carpets, papers, and surfactants (Key et al., 1997). During the last decade, PFCs such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) have been found at considerable levels in various biota samples including the liver and tissues, and especially human blood and serum, worldwide (Fromme et al., 2009).

The toxic effects of PFOS and PFOA have been investigated in animal studies. Prenatal as well as postnatal toxic effects of PFOA and PFOS were observed in rats and mice, including increased liver weights, growth lags, and delayed development. The reproductive and developmental toxicities of these chemicals toward humans are of particular concern (Lau et al., 2004). Several epidemiological investigations have raised concerns regarding the developmental effects of PFOS and PFOA on children, such as low birth weights (Steenland et al., 2010).

In the Stockholm Convention on Persistent Organic Pollutants, PFOS is listed in Annex B (Wang et al., 2009). Fluoropolymer manufacturers have also committed themselves to voluntarily reducing PFOA emissions under a stewardship program by the US EPA (EPA, 2006). The temporal trends in serum levels have revealed decreases in the serum levels of both PFOA and PFOS in the United States, Norway, and Japan since 2000 (Olsen et al., 2007; Harada and Koizumi, 2009; Haug et al., 2009; Harada et al., 2010).

In contrast to PFOS and PFOA, little information is available for perfluorinated carboxylic acids (PFCAs) with longer chains than PFOA. The emissions of perfluorononanoic acid (PFNA) and perfluoroundecanoic acid (PFUnDA) were 25 and 7 metric tons, respectively, in 2000 (Prevedouros et al., 2006). A modeling study indicated that these PFCAs could also have been emitted from precursor compounds, such as fluorotelomer alcohols (FTOHs), for

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decades (Van Zelm et al., 2008). Recent evidence suggests that the toxicological effects of PFCAs are strongly correlated with their chain lengths and functional groups (Upham et al., 1998; Matsubara et al., 2006; Wolf et al., 2008; Liao et al., 2009). Therefore, the effects of exposure to long-chain PFCAs need to be clarified, especially in infants.

Human breast milk and infant formulas are considered to be the main PFC exposure sources for infants during the lactation period. Indeed, contamination of PFCs in human breast milk has been reported in various studies from Asia (So et al., 2006; Tao et al., 2008b; Nakata et al., 2009; Liu et al., 2010, 2011; Kim et al., 2011), the United States (Kuklenyik et al., 2004; Tao et al., 2008a; von Ehrenstein et al., 2009), and Europe (Kärrman et al., 2007; Bernsmann and Furst, 2008). However, the available data for PFCAs with longer chains than PFNA in human breast milk are limited, because of the low recoveries of long-chain PFCAs from human breast milk samples (Kärrman et al., 2007).

The aim of the present study was to investigate the current levels of long-chain PFCAs in human breast milk in East Asian countries, which were reported to show increasing trends for long-chain PFCAs in serum (Kärrman et al., 2009; Harada et al., 2011). Human breast milk samples collected from Japan, Korea, and China were analyzed for PFOA, PFNA, perfluorodecanoic acid (PFDA), PFUnDA, perfluorododecanoic acid (PFDoDA), and perfluorotridecanoic acid (PFTrDA) using an ion-pair extraction method (Hansen et al., 2001) with modifications. In addition, infant formulas from representative manufacturers in the Japanese and Chinese markets were analyzed for comparison with the PFCA concentrations in the breast milk samples from the same regions.

2. Methods and materials

2.1. Study population and sample information

To evaluate the geographical differences in the PFCA levels in human breast milk, we selected 30 samples each from Japan, Korea, and China that were stored in the Human Specimen Bank of Kyoto University (Koizumi et al., 2005, 2009). For infant formulas, we obtained five products from five different companies in the Japanese market and four products from four different companies in the Chinese market. The main ingredients of these infant formulas were cow milk, cow milk-related products (milk whey protein, lactose, and casein), and edible oils (palm olein and soybean oil). A summary of the sample information is provided in Table 1.

Written informed consent was obtained from all the participants. The research protocol for the present study was reviewed and approved by the Ethics Committee of the Kyoto University Graduate School of Medicine on 14 November 2003 (E25).

2.2. Standards and reagents

Analytical standards for the PFCAs, $^{13}C_4$-labeled PFOA and $^{13}C_5$-labeled PFNA, were obtained from Wellington Laboratories (PFC-MXA, MPFOA, and MPFNA; Guelph, Ontario, Canada).

Methanol, acetone, dichloromethane (DCM), and hexane (purity: >99%, pesticide analysis grade) were obtained from Kanto Chemicals (Tokyo, Japan). Ethyl acetate (pesticide analysis grade), methyl *t*-butyl ether (MTBE, pesticide analysis grade), tetrabutylammonium hydrogen sulfate (TBA), sodium carbonate, sodium bicarbonate, and benzyl bromide were purchased from Wako Pure Chemicals (Osaka, Japan). Ultrapure water (Milli-Q™ Reference; Millipore, Billerica, MA) was used for all solutions. MTBE, DCM, and hexane were prefiltered through silica gel (Presep-C silica gel; Wako Pure Chemicals). Methanol, ethyl acetate, and acetone were distilled before use. Milli-Q water was filtered through an Oasis WAX column (Waters, Milford, MA).

2.3. Sample preparation and extraction

Frozen human breast milk samples were thawed and returned to room temperature before extraction. A liquid–liquid and solid–phase extraction method was used to extract the PFCAs in the samples. Aliquots of breast milk (2 mL) together with an internal standard ($^{13}C_4$-PFOA, 1 ng) were placed in 15-mL polypropylene sample tubes. Next, 2 mL of 0.5 M TBA/0.25 M sodium carbonate buffer (pH adjusted to 10 using NaOH) and 2 mL of methanol were added to the samples and vortexed for 15 s. After addition of 3 mL of MTBE, the samples were mixed again and centrifuged at 10000 rpm for 5 min. The supernatants were separated into new glass tubes. Another 3 mL of MTBE was added and the extraction was performed again. The combined sample extracts were dried under a gentle stream of nitrogen. Subsequently, each extract was dissolved in 4 mL of 1:1 MTBE/DCM and loaded onto a Presep-C silica gel column preconditioned with 45 mL of methanol and 4 mL of 1:1 MTBE/DCM on a vacuum manifold. The silica gel column was washed with 10 mL of hexane and 30 mL of ethyl acetate that had been prefiltered through another Presep-C silica gel column. The target fraction was eluted using 12 mL of acetone that had been prefiltered through an alumina column (Sep-Pak plus alumina N; Waters). The eluate was dried under a gentle stream of dry nitrogen. The residue was then redissolved in 100 μL of 0.1 M benzyl bromide/acetone solution and derivatized at 60 °C for 1 h. No further clean-up was conducted.

The infant formulas were dissolved in Milli-Q water according to the guidelines on the packages. Cow milk (4 mL), Milli-Q water (2 mL, procedural blank), and infant formulas (2 mL) were treated by the same procedure used for the human breast milk samples.

2.4. Instrumental analysis

The extracts were analyzed by gas chromatography–mass spectrometry (Agilent 6890GC/5973MSD; Agilent Technologies Japan Ltd., Tokyo, Japan) in the electron impact ionization mode. The PFCAs were separated on a J&W DB-5MS column with a helium carrier gas (1.5 mL min^{-1}). The splitless injection volume was 2 μL. The oven temperature was 70 °C for 2 min initially, and then ramped up to 280 °C at 20 °C min^{-1}. The monitored ions are listed in Table 2. Standard stock solutions (2 μg mL^{-1}) were diluted to seven working standard solutions (4, 2, 1, 0.8, 0.4, 0.2, and 0.1 ng mL^{-1}) by serial dilutions in acetone. All the standard solutions were stored in a refrigerator at 4 ± 2 °C for a maximum period of 3 months from the date of preparation.

The instrumental detection limits (IDLs) were defined as the mass of analyte producing a peak with a signal-to-noise ratio of 3, and ranged from 0.5 pg (PFUnDA, PFDoDA, and PFTrDA) to 0.2 pg (other PFCAs).

2.5. Quality assurance

We used Milli-Q water as the procedural blank control. The average blank values ($n = 6$) were 20.5 pg mL^{-1} (PFOA), 5.2 pg mL^{-1} (PFNA), and 7.1 pg mL^{-1} (PFDA). In the case of blank levels, the mean blank signal was subtracted from the calculated sample concentration only if the calculated sample concentration was three times higher than the blank concentration. If no signal was detected in the blank samples, the method detection limits (MDLs) were based on the IDLs and 2-mL milk samples. Using this method, we established that the MDLs ranged from 40 to 10 pg mL^{-1} (Table 2).

Table 1
Study areas and sample information.

Sampling site	n	Year	Age (year)[a]	(range)	Parity (n)	Smoking[b,c]	Drinking[c]	Lactation period (week)
A. Human milk								
Japan Kyoto	30	2010	27.8 ± 3.4	(21–33)	1(30)	Ex (7), non (23)	Ex (18), non (12)	3.0 ± 0.5
Korea Seoul	30	2010	30.9 ± 2.3	(26–36)	1(22), 2(8)	Ex (3), non (27)	Curr (3), ex (2), non (25)	1.6 + 1.1
China Beijing	30	2008, 2009	27.0 ± 1.7	(23–30)	1(30)	Non (30)	Curr (2), ex (27), non (1)	NA
B. Infant formula								
			Targeted infant age (month)					
Japan Kyoto	5	2010	0–12					
China Beijing	4	2010	0–12					

[a] Data are presented as the mean ± standard deviation.
[b] Including second-hand tobacco smoke.
[c] Curr: current; ex: experienced; non: never.

Table 2
Recoveries and detection limits for the PFCA analyses in human serum samples.

Compound	Quantification (confirmation)	Instrument detection limit[a] (pg)	Blank (pg mL^{-1}) range (mean)	Detection limit[b] (pg mL^{-1})	Recovery and (reproducibility) mean percentage (SD) (n = 9)	Standard reference material 1954[c]		
						This study (pg g^{-1}) U	Toronto[d] (pg g^{-1})	Env. Canada[d] (pg g^{-1}
PFOA	504 (485)	0.2	12.0–32.1(20.5)	40	104 (14)	117	149	116
$^{13}C_4$ PFOA	508 (489)	–	–	–	99 (12)	–	–	–
PFNA	554 (535)	0.2	<5–14.7(5.2)	10	84 (44)	24	22	<16
$^{13}C_5$ PFNA	559 (540)	–	–	–	–	–	–	–
PFDA	604 (585)	0.2	<5–25.8(7.1)	15	109 (32)	16	14	<6
PFUnDA	654 (635)	0.5	<10	10	95 (45)	12	7	<14
PFDoDA	704 (685)	0.5	<10	10	92 (25)	<10	3	<8
PFTrDA	754 (735)	0.5	<10	10	97 (27)	<10		

[a] Injection of 2 μL.
[b] Milk sample of 2 mL (the mean blank signal was subtracted from the calculated sample concentration only if the calculated sample concentration was three times higher than the blank concentration).
[c] Milk standard reference material from the National Institute of Standards and Technology, 1954.
[d] Analyzed by the University of Toronto and Environment Canada (Keller et al., 2010).

$^{13}C_4$-PFOA was used as an internal standard for the PFCAs. $^{13}C_5$-PFNA was used to monitor the recovery of the internal standard. The recoveries of the PFCAs were examined by spiking 500 pg of each standard compound into cow milk. The mean recoveries of PFOA, PFNA, PFDA, PFUnDA, PFDoDA, and PFTrDA were 104%, 84%, 109%, 95%, 92%, and 97%, respectively. Typical chromatograms of PFCAs obtained in this study are shown in Supplemental Fig. 1.

For quality assurance and quality control of our analytical methods and procedures in the analysis of PFCAs in the breast milk samples, we measured PFCAs in standard reference materials from the National Institute of Standards and Technology (Table 2). The PFCA values were comparable to those reported previously (Keller et al., 2010).

2.6. Statistical analysis

We calculated the percentages of detection of the PFCAs in each country, and determined the range, median, mean, standard deviation, geometric mean, and 90th percentile concentration. Concentrations below the MDL were replaced by half of the MDL for statistical analyses. Nonparametric statistical tests were applied to assess the statistical significance of differences between values. The Steel–Dwass test was used to compare differences in the PFCA concentrations among different countries after the Kruskal–Wallis test. Spearman's rank correlation analysis was used to examine the relationships between the PFCA levels and the mother's age and child's birth weight. The Mann–Whitney test was used to examine the relationships between the PFCA levels and alcohol drinking and cigarette smoking. The level of statistical significance was set at $p < 0.05$. A factor analysis was used to elucidate the number of potential factors of sources. The analyses were conducted via a correlation matrix. Eigenvectors were employed for the analysis when the eigenvalues were greater than 1. Normalized varimax rotation was applied to these eigenvectors. The statistical analyses were carried out using the software JMP® 4 (SAS Institute Inc., Cary, NC) or R Ver. 2.12.1. (Ihaka and Gentleman, 1996) for the Steel–Dwass test.

3. Results

3.1. PFCA concentrations in breast milk in Japan, Korea, and China

The demographic characteristics of the participants are shown in Table 1. The participants in Korea were, on average, about 3 years older than those in Japan and China. The descriptive statistical data are summarized in Table 3. PFOA was the predominant compound and was detected in more than 60% of samples in all three Asian countries. The median concentration of PFOA ranged from 51 pg mL^{-1} in China to 89 pg mL^{-1} in Japan. The PFOA levels in Japan were significantly higher than those in Korea and China ($p < 0.05$, Steel–Dwass test).

PFNA and PFUnDA were detected at comparable rates to PFOA in the three countries. The levels of PFNA and PFUnDA were higher in Japan than in Korea and China ($p < 0.05$, Steel–Dwass test). PFDA was frequently detected in Japan (67%), but rarely detected in Korea (13%) and China (13%). In Korea, half of the milk samples contained detectable levels of PFTrDA, which was the highest among the three countries ($p < 0.05$, Steel–Dwass test). PFDoDA was detected in few samples in the three Asian countries and there

Table 3
Concentrations of PFCAs in breast milk samples.

Sampling site		Concentration (pg mL^{-1})						
		PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	∑PFCAs
Japan	n > MDL(%)	28(93.3)	27(90.0)	20(66.7)	28(93.3)	5(16.7)	10(33.3)	30(100.0)
Kyoto	Median	89(<40–194)A*	31(<10–72)A*	17(<15–65)A*	35(<10–100)A*	<10(<10–29) n.s.	<10(<10–91)AB*	184(50.3–413.5)A*
	Mean	93.5 ± 43.7	32.1 ± 17.2	21.3 ± 15.0	36.6 ± 21.8	<10	15.2 ± 20.6	194.5 ± 83.6
	GM(GSD)	82.7(1.7)	26.5(2.0)	16.9(2.0)	30.4(2.0)	<10	<10	176.7(1.6)
	P90	173	62	44	65	22	36	315
Korea	n > MDL(%)	24(80.0)	20(66.7)	4(13.3)	22(73.3)	4(13.3)	15(50.0)	28(93.3)
Seoul	Median	62(<40–173)B*	15(<10–41)B*	<15(<15–19)B*	19(<10–51)B*	<10(<10–41) n.s.	10(<10–43)A*	114(<10–283.9)B*
	Mean	64.5 ± 33.7	14.7 ± 9.3	<15	19.6 ± 13.1	<10	16.8 ± 13.5	118.8 ± 50.9
	GM(GSD)	55.5(1.8)	11.9(2.0)	<15	15.3(2.2)	<10	11.7(2.4)	109.7(1.5)
	P90	106	29	15	42	11	40	189
China	n > MDL(%)	19(63.3)	21(70.0)	4(13.3)	17(56.7)	3(10.0)	7(23.3)	28(93.3)
Beijing	Median	51(<40–122)B*	15(<10–47)B*	<15(<15–29)B*	15(<10–47)B*	<10(<10–25) n.s.	<10(<10–43)B*	84(<10–200.8)B*
	Mean	51.6 ± 30.6	15.3 ± 9.6	<15	16.0 ± 12.9	<10	<10	87.8 ± 54.9
	GM(GSD)	43.0(1.9)	12.6(2.0)	<15	11.7(2.3)	<10	<10	68.8(2.2)
	P90	103	27	18	42	10	22	164

MDL: method detection limit; GM: geometric mean; GSD: geometric standard deviation; P90: 90th percentile.
* Medians among different sites differ significantly ($p < 0.05$, Steel–Dwass test). For example, the letters A and B indicate that the corresponding values differ significantly at $p < 0.05$, while A and A or B and B indicate that the corresponding values do not differ significantly.

Table 4
Factor analysis among PFCAs.

	Initial solution		Varimax rotated	
	F1	F2	F1	F2
Eigenvalue	2.60	1.14		
Cumulative contribution (%)	43.3	62.3		
Eigenvector				
PFOA	0.387	−0.511	**0.818**	−0.135
PFNA	0.472	−0.375	**0.857**	0.060
PFDA	0.480	−0.020	**0.668**	0.390
PFUnDA	0.518	0.261	**0.563**	**0.677**
PFDoDA	0.114	0.430	−0.086	0.488
PFTrDA	0.340	0.587	0.135	**0.822**
*Factor score (mean ± SD)**				
		Beijing	−0.5 ± 0.6^{B}	−0.2 ± 0.7
		Kyoto	0.9 ± 1.1^{A}	0.2 ± 1.4
		Seoul	−0.4 ± 0.6^{B}	0.1 ± 0.8

* Means among countries differ significantly ($p < 0.05$, Steel–Dwass test). For example, the letters A and B indicate that the corresponding values differ significantly at $p < 0.05$, while A and A or B and B indicate that the corresponding values do not differ significantly.

were no significant differences ($p > 0.05$). Regarding the total PFCAs in the milk samples, PFOA accounted for 48%, 54%, and 61% in Japan, Korea, and China, respectively. Among the long-chain PFCAs, odd-numbered PFCAs were more frequently detected than even-numbered PFCAs, except for PFDA in Japan.

PFOA was only significantly correlated with PFNA (ρ coefficient:>0.4) (Supplemental Table 1). There were also significant correlations between PFNA and PFUnDA, PFDA and PFUnDA, and PFUnDA and PFTrDA (ρ coefficients: >0.4). In general, the PFCA concentrations showed strong correlations between PFCAs of similar (i.e. adjacent) chain lengths.

The factor analysis revealed that two potential factors, F1 and F2, accounted for 43.3% and 19.0% of the total variance (with eigenvalues of >1), respectively (Table 4). After varimax rotation, F1 indicated higher eigenvectors for PFOA, PFNA, PFDA, and PFUnDA, while F2 had positive eigenvectors for PFUnDA and PFTrDA. The mean factor scores of each sampling site are also shown in Table 4. Although the F1 score was higher in Kyoto than in the other two sites ($p < 0.05$, Steel–Dwass test), there were no significant differences in the F2 scores among all the sampling sites ($p > 0.05$, Kruskal–Wallis test).

3.2. PFCA concentrations in commercially available infant formulas in Japan and China

The PFCA concentrations in the infant formulas are shown in Table 5. PFOA, PFNA, and PFDA were frequently detected in both Japan and China, but there were no significant differences between the two countries. PFUnDA was detected at 40.7 pg mL^{-1} in one sample in Japan. PFDoDA and PFTrDA were not detected in any of the formula samples. Compared with the breast milk samples,

Table 5
Concentrations of PFCAs in infant formulas.

Sampling site	Sample No.	Concentration (pg mL^{-1})[a]						
		PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	∑PFCAs
Japan	1	<20	<5	<7	<5	<5	<5	<5
	2	35.8	27.0	<7	<5	<5	<5	62.8
	3	30.8	8.0	12.1	<5	<5	<5	50.9
	4	<20	8.6	11.5	<5	<5	<5	20.1
	5	22.5	92.0	19.8	40.7	<5	<5	175.0
	Meant ± SD	21.8 ± 11.8	27.6 ± 37.2	10.1 ± 6.9	10.1 ± 17.1	<5	<5	66.4 ± 65.6
China	1	35.4	50.4	14.0	<5	<5	<5	99.7
	2	<20	15.2	<7	<5	<5	<5	15.2
	3	37.1	12.2	12.9	<5	<5	<5	62.2
	4	29.9	11.6	13.9	<5	<5	<5	55.4
	Meant ± SD	28.1 ± 12.4	22.4 ± 18.8	11.1 ± 5.1	<5	<5	<5	61.5 ± 29.3

[a] A 4-mL aliquot of each infant formula was analyzed.

the PFOA levels were 4-fold and 2-fold lower in the formula samples in Japan and China, respectively. The total PFCA concentrations in the infant formulas were lower than those in the breast milk samples in Japan ($p < 0.05$, Kruskal–Wallis test), but not in China ($p > 0.05$, Kruskal–Wallis test).

3.3. Relationships between the PFCA levels and the participants' characteristics

To evaluate the influence of the participants' characteristics on the PFCA concentrations in the human breast milk samples, Spearman's correlation analyses were performed (Supplemental Table 2). PFDoDA was positively correlated with the mother's age in Korea ($p < 0.05$) and PFNA was negatively correlated the mother's age in China ($p < 0.05$). However, these correlations were not consistent among the three countries. In several epidemiological studies (Steenland et al., 2010), the PFC concentrations in the cord blood or maternal pregnancy serum were reported to be associated with the child birth weight. In our study subjects, the correlations between the PFCA concentrations and the child birth weights were not significant. The lactation period was also examined for correlations with PFCAs in the milk samples. PFDA was correlated with the lactation period in Japan ($p < 0.05$), but not in Korea. Among the PFCAs, there were no clear trends in the correlation coefficients. Although consumption of fish was one of the sources of exposure to PFCAs, no significant associations were observed between the PFCA levels in the milk samples and the fish intake ($p > 0.05$). Non-smoking mothers in Japan had relatively higher PFCAs levels than other mothers, but the difference was not significant ($p > 0.05$). The PFCA levels in the milk samples were compared between non-drinking mothers and other mothers. The PFTrDA and PFNA levels were lower in non-drinking mothers in Japan and Korea ($p < 0.05$, Mann–Whitney test).

3.4. Daily intake estimation and hazard assessment for infants

The tolerable daily intake (TDI) for PFOA was established to be 1500 ng kg body weight^{-1} d^{-1} by the Scientific Panel on Contaminants in the Food Chain requested by the European Food Safety Authority (EFSA, 2008). The average breast milk consumption rate and body weight for 1-year-old infants were assumed to be 600 g d^{-1} and 7.3 kg, respectively (Schecter, 1994). Based on these assumptions, the daily intakes of PFCAs by 1-year-old infants were estimated (Supplemental Table 3). For the infant formulas, the calculated mean levels were only 0.1–0.2% of the TDI. Meanwhile, the calculated levels for the human breast milk samples (means: 0.3–

Table 6
Comparisons of the PFCA concentrations in human breast milk with reported data (pg mL^{-1}).

Country	Region	Year	*n*		PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	Reference
Japan	Kyoto	2010	30	Mean	93.5	32.1	21.3	36.6	<10	15.2	This study
				Range	<40–194	<10–72	<15–65	<10–100	<10–29	<10–91	
	Hokkaido	NA	51	Mean	89	35					Nakata et al. (2009)
				Range	<12–339	<4–150					
	Ehime	1999	24	Mean	77.7						Tao et al. (2008b)
				Range	<42.5–170	<8.82–23.9					
Korea	Seoul	2010	30	Mean	64.5	14.7	<15	19.6	<10	16.8	This study
				Range	<40–173	<10–41	<15–19	<10–51	<10–41	<10–43	
		2007	17	Mean	41						Kim et al. (2011)
				Range	<43–77	<8.8	<18	<24	<13		
China	Beijing	2008–2009	30	Mean	51.6	15.3	<15	16.0	<10	<10	This study
				Range	<40–122	<10–47	<15–29	<10–47	<10–25	<10–43	
	Zhoushan	2004	19	Mean	106.3	18.1	7.2	19.1			So et al. (2006)
				Range	47–210	6.3–62	3.8–15	7.6–56			
	12 provinces	2007	1237	Mean	116.0	16.2	9.9	37.6			Liu et al. (2010)
				Range (24 pooled samples)	<14.15–814	6–76	<1.44–63	<1.30–196			
Vietnam	Hanoi, Ho Chi Minh	2000, 2001	40	Range	<42.5–89.2	<8.82–10.9					Tao et al. (2008b)
Cambodia	Phnom Penh	2000	24	Range	<42.5–132	<8.82–12.3					Tao et al. (2008b)
Philippines	Quezon	2000, 2004	24	Range	<42.5–183	<8.82–25.0					Tao et al. (2008b)
Malaysia	Penang	2003	13	Range	<42.5–90.4	<8.82–14.9					Tao et al. (2008b)
Indonesia	Jakarta, Purwakarta	2001	20	Range	<42.5	<8.82–135					Tao et al. (2008b)
India	Chidambaram Kolkata, Chennai	2002, 2004, 2005	39	Range	<42.5–335	<8.82					Tao et al. (2008b)
USA	Unknown	2003	2	Range	<200						Kuklenyik et al. (2004)
	Massachusett	2004	45	Mean	43.8	7.26					Tao et al. (2008a)
				Range	<30.1–161	<5.2–18.4					
Sweden	Uppsala	2004	12	Range	<209–492	<5–20	<8	<5			Kärrman et al. (2007)
		1996–2004	9	Range (Pooled annual composite milk sample)	<209	<5–28	<8	<5			
Germany	NA	2006	38	Range (Archived samples + 19 fresh samples)	201–460						Völkel et al. (2008)
	North Rhine Westphalian	NA	203	Range	25–610						Bernsmann and Furst (2008)
Spain	Tarragona	2007	10	Range	<500	<30	<60	<30	<30		Kärrman et al. (2010)
	Barcelona	2008	20		<15.2–907	<11.5	<85.5–1095				Llorca et al. (2010)

0.5% of the TDI; 90th percentiles: 0.6–0.9% of the TDI) were higher than those for the infant formulas. As of 2011, there is no established TDI for PFCAs that are longer than PFOA.

4. Discussion

In the present study, we first demonstrated contamination of human breast milk with PFDoDA and PFTrDA in Asian countries. Simultaneously, we confirmed similar long-chain PFCA profiles in East Asian breast milk samples, as previously reported (Liu et al., 2010, 2011; Kim et al., 2011). A characteristic PFCA composition was observed for PFUnDA and PFTrDA (both odd-numbered PFCAs) with residual PFDoDA and PFDA (both even-numbered PFCAs). These findings indicated that odd-numbered PFCAs predominated over even-numbered PFCAs in East Asian breast milk samples. The PFCAs with longer chains than PFOA reached 47% of the total PFCAs for the average of the three countries. This finding suggests that infants are exposed to not only classical PFOA but also long-chain PFCAs in East Asia. Indeed, a factor analysis demonstrated two potential factors, F1 and F2, as sources of PFCAs. F1 had loading on medium-chain PFCAs, of which the factor score was significantly higher in Kyoto than in Beijing or Seoul. Kyoto is located in the Hanshin area, where there is a large emission source of PFOA and its related by-products (Niisoe et al., 2010). Thus, F1 may represent a local emission source of PFCAs. On the other hand, F2 had strong associations with long-chain PFCAs. The factor scores for F2 in the three large cities did not differ, suggesting that there are similar sources of long-chain PFCAs (>C10) in the three counties. Therefore, PFCA (C10–C13) exposure through the breast milk is likely to commonly occur in East Asian countries. We are the first to document this possibility.

The sources of long-chain PFCAs are still unknown. Odd-numbered PFCAs predominated in the PFCAs in this study. As previously reported (Harada et al., 2011), odd-numbered PFCAs also predominated in serum samples collected from Asian women. A review by Prevedouros et al. (2006) indicated that odd-numbered PFCAs have been manufactured in Japan via oxidation of fluorotelomer olefins. Industrial application of these odd-numbered PFCAs might contribute to the pattern of PFCAs in breast milk samples collected from East Asian women. Although FTOHs are possible precursors of PFCAs, biodegradation of FTOHs preferentially yields even-numbered PFCAs (Fasano et al., 2009). Therefore, FTOHs are unlikely to be the main exposure source for Asian populations. Further investigations into the sources and exposure routes are needed to predict the future trajectory of these PFCA levels.

Although data concerning the PFC levels in human breast milk are not as abundant as those in blood samples, we can still find several reports for PFCs in human breast milk from Asia, the United States, and Europe. The related data are summarized in Table 6. In Japan, the PFOA levels in three regions were comparable (Tao et al., 2008b; Nakata et al., 2009). In Korea, PFOA had a higher value in the present study compared with earlier research in Seoul (Kim et al., 2011) (mean: 63.8 *vs.* 41 pg mL^{-1}, range: 14.7–172.1 *vs.* 21–77 pg mL^{-1}). This increase may be consistent with the increasing trend in the PFOA level in serum samples by 1.27-fold from 2000 to 2007 in Korea (Harada et al., 2010).

In China, the concentrations of PFOA in Zhoushan ranged from 47 to 210 pg mL^{-1} (So et al., 2006) and in 12 different provinces of China, the mean PFOA level was 116 pg mL^{-1} (Liu et al., 2010). The PFOA levels showed large variations within China, although the other PFCAs were comparable among two previous studies and this study. In Southeast Asian developing countries, most of the milk samples did not contain detectable PFCAs (Tao et al., 2008b), which might result from differences in industrialization. In the United States and European countries, PFOA and PFNA were detected in human breast milk samples, but long-chain PFCAs were not observed (Kuklenyik et al., 2004; Kärrman et al., 2007, 2010; Bernsmann and Furst, 2008; Tao et al., 2008a; Völkel et al., 2008; Llorca et al., 2010). The occurrence of long-chain PFCAs in East Asian countries is likely to be a fingerprint of the sources of exposure.

Infant formulas were also evaluated in this study. The compositions of PFCAs in the infant formulas were different from those in the breast milk samples. In Japan, the levels of PFCAs in the infant formulas were lower than those in the breast milk samples. These findings probably reflect differences in the bioaccumulation potential between humans and cows.

In our study, we found no evident relationships between the mother's characteristics and the PFCA concentrations. Although there were statistically significant differences for some of the PFCAs, no consistent trends were observed among the three countries.

The estimated daily intakes of PFOA were much lower than the TDI in this study. These observations may indicate that the health risks for PFOA intake from breast milk and infant formulas are limited. However, infants have different susceptibilities to adults with regard to their dynamic growth and developmental processes (Sly and Flack, 2008). In addition, the toxicokinetics and toxicities of long-chain PFCAs are still unclear, although these PFCAs comprised 48% of the total PFCAs in this study. These uncertainties necessitate more comprehensive toxicological studies on long-chain PFCAs, including PFOA.

The limitations of this study are the sample sizes and the sample selection method. It should be noted that these findings were based on a relatively small number of non-randomly selected volunteer samples. Moreover, the sampling times for the Chinese donors were uncertain, although it is known that the profiles of chemicals may change during the lactation period. Considering these limitations, a future extended study is required for confirmation of these findings,

In conclusion, various PFCAs were detected in human breast milk samples from East Asian countries. Further studies are needed to evaluate the exposure to long-chain PFCAs and the health risks in infants.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2011.10.035.

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Occurrence of perfluorinated carboxylic acids (PFCAs) in personal care products and compounding agents

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HIGHLIGHTS

- Perfluorinated carboxylic acids (PFCAs) were in personal care products (PCPs).
- The samples list polyfluoroalkyl phosphate esters (PAPs) in their ingredients.
- The concentrations of total PFCAs range from not detected to 19 $\mu g\ g^{-1}$ in PCPs.
- Compounding agents contain high concentrations of PFCAs (35 $\mu g\ g^{-1}$).
- PFCAs are detected in PCPs and compounding agents that use PAPs.

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ABSTRACT

Perfluorinated carboxylic acids (PFCAs), including perfluorooctanoic acid (PFOA), are persistent organic pollutants that pose human health risks. However, sources of contamination and exposure pathways of PFCAs have not been explored. In this study, PFCA concentrations were quantified in personal care products. Among 24 samples that listed fluorinated compounds, such as polyfluoroalkyl phosphate esters (PAPs), in their international nomenclature of cosmetic ingredients (INCI) labels, 21contained PFCAs (13 of 15 cosmetic samples, and 8 of 9 sunscreen samples). The concentrations of total PFCAs ranged from not detected to 5.9 $\mu g\ g^{-1}$ for cosmetics and from not detected to 19 $\mu g\ g^{-1}$ for sunscreens. We also investigated components of PFCAs in cosmetics and sunscreens. Commercially available compounding agents, mica and talc, which were treated with PAPs were analyzed and high concentrations of PFCAs were detected (total PFCAs 2.5 $\mu g\ g^{-1}$ for talc treated with PAPs, 35.0 $\mu g\ g^{-1}$ for mica treated with PAPs). To the best of our knowledge, this is the first report on contamination of end consumer products containing PAPs with high concentrations of PFCAs.

1. Introduction

Perfluorinated compounds are a large group of man-made fluorinated organic chemicals. Perfluorinated alkyl acids such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA, C8 carbon chain) have been detected in various environmental samples, wildlife, and humans (Houde et al., 2006). In the Stockholm Convention on Persistent Organic Pollutants, PFOS is listed in Annex B (Wang et al., 2009). Fluoropolymer manufacturers have also committed themselves to voluntarily reducing PFOA emissions under a stewardship program of the US Environmental Protection Agency (EPA US, 2006). Temporal trends have revealed decreases in the serum levels of both PFOA and PFOS in the United States, Sweden, Norway, and Japan since 2000 (Olsen et al., 2007; Harada and Koizumi, 2009; Haug et al., 2009; Harada et al., 2010, 2011; Glynn et al., 2012). By contrast, perfluoroalkyl carboxyl acids (PFCAs) with longer carbon chains than PFOA (>C9) have continued to increase in human serum in East Asia and Sweden (Harada et al., 2011; Glynn et al., 2012) and have been detected in human breast milk (Fujii et al., 2012b). Although dietary intakes are generally considered as a major exposure route of long chain PFCAs to humans, the contribution of diet to total human PFCA exposure is still uncertain (D'Hollander et al., 2010; Fujii et al., 2012a). Perfluorinated chemicals have been used in a number of products and significant elevations of serum PFCA levels of those product users were observed in recent studies (Freberg et al., 2010; Nilsson et al., 2010). Several *in vitro* studies have suggested that long chain PFCAs are biologically more toxic than PFOA (Upham et al., 1998; Matsubara et al., 2006; Liao et al., 2009). Therefore, possible exposure routes in humans should be elucidated to establish a rational management policy.

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The direct emissions of long chain PFCAs have been reported for perfluorononanoic acid (PFNA) and perfluoroundecanoic acid (PFUnDA) at 25 t (1 t = 1000 kg) and 7 t, respectively, in 2000 (Prevedouros et al., 2006). In addition, indirect emission of PFCAs could have occurred from precursor compounds, such as fluorotelomer alcohols (FTOHs) and fluoropolymers, for decades (Ellis et al., 2004); (van Zelm et al., 2008). FTOHs are widely produced and used as intermediates for the synthesis of coatings, polymers, and paints (Kissa, 2001). For example, fluorotelomer-based acrylic polymers are used as oil and water repellents. The production of fluorotelomer alcohols was estimated to be approximately 11–14 $\times 10^6$ kg yr^{-1} in 2004 (DuPont Company, 2005).

Recently, polyfluoroalkyl phosphate esters (PAPs), manufactured by a condensation reaction of FTOHs and phosphate, have been widely used in personal care products (PCPs), such as sunscreen and cosmetics for oil and water repellency (Daito Kasei Kogyo., 1993). Biotransformation of PAPs to PFCA has been observed in rats (D'eon and Mabury, 2007) and in a microbial system in wastewater treatment plants (Lee et al., 2010). In addition, technical-grade FTOHs could contain PFCAs as impurities (DuPont Company, 2006). Because of their fate in the ecological system and the manufacturing process, products that use PAPs could contain PFCAs as impurities and/or produce them after degradation. Their application to PCPs could be an exposure source of long chain PFCAs in human serum.

In this study, we investigated the concentrations of PFCAs in PCPs that contained PAPs and some other fluorinated compounds. We also examined commercially available compounding agents of PCPs. The target chemicals included perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid, PFOA, PFNA, perfluorodecanoic acid (PFDA), PFUnDA, perfluorododecanoic acid (PFDoDA), perfluorotridecanoic acid, and perfluorotetradecanoic acid (PFTeDA).

2. Material and methods

2.1. Sample information

We collected PCP samples (sunscreens and cosmetics) that listed PAPs and other fluorinated compounds (e.g. polyfluoroalkyl silylated mica) in their International Nomenclature of Cosmetic Ingredients (INCI) labels. As control samples, a cosmetic (manicure) sample and a sunscreen sample that did not list any fluorinated compounds were collected. A summary of the sample information is provided in Table 1. We obtained 16 commercially available cosmetic samples and 10 sunscreen products distributed by eight different companies. In addition to these end consumer products, we also obtained commercially available compounding agents, including mica and talc, which were treated with PAPs.

2.2. Chemicals

Acetone (LC-MS grade), methanol (LC-MS grade), sodium carbonate (>99.5% pure) and distilled water (LC-MS grade) were obtained from Kanto Chemicals (Tokyo, Japan). Benzyl bromide, tetrabutylammonium hydrogen sulfate, 11H-perfluoroundecanoic acid (11H-PFUnDA) and methyl *tert*-butyl ether (HPLC grade) were purchased from Wako Pure Chemical Industries (Osaka, Japan). A mixture of $^{13}C_2$-labeled PFHxA, $^{13}C_4$-labeled PFOA, $^{13}C_5$-labeled PFNA, $^{13}C_2$-labeled PFDA, $^{13}C_2$-labeled PFUnDA and $^{13}C_2$-labeled PFDoDA were obtained from Wellington Labs (Guelph, Canada). $^{13}C_{12}$- 2,3,3′,5,5′-pentachlorobiphenyl (CB-111) was obtained from Cambridge Isotope Laboratories (Andover, MA).

2.3. Extraction of the PCP samples

Each of the PCPs was subjected to an ion-pair extraction. Approximately 1–200 mg of each PCP, depending on the PFCA concentration, and 500 μL of methanol were placed in a 15 mL polypropylene tube and mixed for 3 h. A recovery surrogate mixture (1 ng each of $^{13}C_2$-labeled PFHxA, $^{13}C_4$-labeled PFOA, $^{13}C_5$-labeled PFNA, $^{13}C_2$-labeled PFDA, $^{13}C_2$-labeled PFUnDA, and $^{13}C_2$-labeled PFDoDA) was added to the tube. Next, 1 mL of 0.5 M tetrabutylammonium/0.25 M sodium carbonate buffer (pH adjusted to 10 using NaOH) and 1 mL of methyl *tert*-butyl ether were added to the samples, and the tubes were vortex mixed for 60 s. The samples were then centrifuged at 9840*g* for 5 min and the organic layer was removed. This step was repeated and the organic layers were combined in a clean glass tube, and then evaporated to dryness under a gentle stream of nitrogen. The residue was redissolved in 100 μL of a 0.1 M benzyl bromide/acetone solution containing 1 ng of 11H-PFUnDA as external calibration standard. The solution was then derivatized at 60 °C for 1 h. No further clean-up was conducted. Derivatized samples were analyzed within 24 h.

2.4. Instrumentation and quantification

Derivatized PFHxA, perfluoroheptanoic acid, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, perfluorotridecanoic acid and PFTeDA were analyzed by gas chromatography-mass spectrometry with electron-capture negative ionization (GC/ECNI/MS) in selected ion monitoring mode (Agilent 6890GC/5973MSD inert, Agilent Technologies Japan, Ltd., Tokyo, Japan). PFCA benzyl esters were separated on a VF-200 ms column (30 m × 0.25 mm i.d., 1 m film thickness; Agilent Technologies Japan, Ltd.) with a helium carrier gas (99.9999% purity; Air Liquide Japan Ltd., Tokyo, Japan). Splitless injections (1 L) were performed with an injector temperature of 220 °C, and the split vent was opened after 1.5 min. The initial oven temperature was 90 °C for 2.25 min, after which it was increased to 135 °C at 1.5 °C min^{-1}, then to 220 °C at 10 °C min^{-1}, and then to 300 °C at 40 °C min^{-1}, and then held at 300 °C for 1 min. Methane (99.9999% purity; Air Liquide Japan Ltd.) was used as the reagent gas (2 mL min^{-1}). The target ions for determination of PFCAs in ECNI are summarized in Table 2.

Standard stock solutions (2 μg mL^{-1}) of native PFCAs (C6 to C14) were diluted to seven working standard solutions (100, 50, 10, 5, 1, 0.5, and 0.1 ng mL^{-1}) using 100 μL of a 0.1 M benzyl bromide/acetone solution with 1 ng of underivatized 11H-PFUnDA and 10 ng of $^{13}C_{12}$-labeled CB111 to monitor the derivatization efficiency. Standard stock solutions (2 μg mL^{-1}) of isotope-labeled PFCAs ($^{13}C_2$-labeled PFHxA, $^{13}C_4$-labeled PFOA, $^{13}C_5$-labeled PFNA, $^{13}C_2$-labeled PFDA, $^{13}C_2$-labeled PFUnDA and $^{13}C_2$-labeled PFDoDA) were also diluted to four working standard solutions (14, 10, 8 and 4 ng mL^{-1}) by 100 μL of a 0.1 M benzyl bromide/acetone solution with 1 ng of underivatized 11H-PFUnDA and 10 ng of $^{13}C_{12}$-labeled CB111. The derivatization efficiency for all PFCA and the 11H-PFUnDA were assumed to be the same. The calibrations were linear and characterized by good correlation coefficients (>0.99). The coefficient of variation of the response ratio (11H-PFUnDA/CB111) was 20% ($N = 10$). The instrumental detection limit was defined as the mass of the analyte producing a peak with a signal-to-noise ratio of three. There is a decrease in detector response of GC–MS measurement depending on chain length of PFCAs. Longer chain PFCAs have smaller detector responses. However, because blank levels were higher for shorter-chain PFCAs than for longer chain PFCAs, the final net instrument detection limits of the shorter and longer chain PFCAs were nearly equivalent (Table 2).