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# Contamination trends and factors affecting the transfer of hexabromocyclododecane diastereomers, tetrabromobisphenol A, and 2,4,6-tribromophenol to breast milk in Japan<sup>☆</sup>

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## ABSTRACT

This study investigated contamination trends and factors affecting the levels of brominated flame retardants (BFRs), including hexabromocyclododecane (HBCD) diastereomers, tetrabromobisphenol A (TBBP-A), and 2,4,6-tribromophenol (2,4,6-TBP), in breast milk in Japan. Breast milk samples ( $n = 64$ ) were collected from mothers living in six prefectures in Japan. The mean concentrations were 2.2, 0.19, 0.29, 3.0, and 0.59 ng/g lipid weight for  $\alpha$ -HBCD,  $\beta$ -HBCD,  $\gamma$ -HBCD, TBBP-A, and 2,4,6-TBP, respectively. Based on the previous studies, the levels of  $\Sigma$ HBCD in Japanese women's milk appear to be increasing, and the levels of TBBP-A are higher than those in other Asian countries. Although  $\Sigma$ HBCD were not correlated to phenolic BFRs, the concentration of  $\beta$ -HBCD was significantly correlated to the concentrations of TBBP-A ( $r = 0.440$ ,  $p < 0.01$ ) and 2,4,6-TBP ( $r = 0.320$ ,  $p < 0.01$ ). The concentration of  $\gamma$ -HBCD increased significantly with maternal age ( $r = 0.378$ ,  $p < 0.01$ ), but the concentrations of the other analytes were not dependent on age. The concentration of  $\alpha$ -HBCD was higher in primiparae than in multiparae ( $p < 0.05$ ), while TBBP-A was higher in multiparae. No significant correlation was found between the phenolic BFR levels in milk and mothers' age, working place, and drinking/smoking habits. These results suggest that exposure to  $\alpha$ - and  $\gamma$ -HBCD diastereomers could be affected by maternal age and parity, respectively, because of their different kinetics and sources. Therefore, these factors should be considered when conducting infant risk assessments.

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## 1. Introduction

Over the last two decades, the study of brominated flame retardants (BFRs) has expanded in scope with increased regulation of polybrominated diphenyl ethers (PBDEs). Recent concern may be human exposure to hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBP-A) and 2,4,6-tribromophenol (2,4,6-TBP) (Covaci et al., 2009; Kim and Oh, 2014; Lam et al., 2009; Watanabe and Sakai, 2003).

HBCD is an additive flame retardant that is used primarily in expanded and extruded polystyrene for thermal insulation in

buildings. Worldwide production of HBCD totaled 16 700 tons in 2001 (BSEF, 2006), and most of HBCD was used in Asia (Watanabe and Sakai, 2003). Production of HBCD is restricted by the Stockholm Convention on Persistent Organic Pollutants (POPRC, 2010). In Japan, the market demand for HBCD was gradually increased until 2006 and estimated to be 2800 tons in 2011 (Ministry of the Environment of Japan, 2013). Concentrations of HBCD detected in the environment vary among areas and countries depending on the consumption and use of HBCD. HBCD has been shown to bioconcentrate and biomagnify in fish and wildlife (Covaci et al., 2006; Harrad and Abdallah, 2015; Johnson-Restrepo et al., 2008). In a study with rodents, prenatal and neonatal exposure to HBCDs decreased levels of thyroid-stimulating hormone and altered spontaneous behavior even in the lowest dose (Ema et al., 2008; Eriksson et al., 2006; Saegusa et al., 2009).

TBBP-A is widely used as an additive and reactive flame

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retardant (European Union Risk Assessment Report, 2006). In additive applications, it can be transferred from the product to indoor air or dust. In Japan, the market demand in 2011 was estimated to be 16,200 tons (Ministry of the Environment of Japan, 2013). Even when used as a reactive flame retardant, excessive non-polymerized TBBP-A is always present which can be emitted to the environment. Due to low water solubility and low vapour pressure, TBBP-A is likely to be associated with suspended particulate matter, following release (Abdallah et al., 2016). Because TBBP-A is structurally similar to the thyroid hormone thyroxine, it is a suspected endocrine disruptor. In fact, in multiple *in vitro* assays (Meerts et al., 2000; Riu et al., 2011), TBBP-A has shown competitive binding of transthyretin, and activation of estrogen and peroxisome proliferator-activated receptors (Ghisari and Bonfeld-Jorgensen, 2005; Hamers et al., 2006; Kitamura et al., 2002). *In vitro* studies have also suggested it has immune and neurotoxic effects (Mariussen and Fonnum, 2003; Pullen et al., 2003).

2,4,6-TBP principally enters the environment through its use as a BFR or as a preservative in the timber industry (Howe et al., 2005). BFR intermediates used as wood preservatives may lead to release of 2,4,6-TBP into the environment. The annual consumption of 2,4,6-TBP as a flame retardant in 2011 were reported to be 2400 tons (Ministry of the Environment of Japan, 2013). It has also been detected as impurities in some technical BFR product (Suzuki et al., 2008). Indoor dust is an important exposure route for 2,4,6-TBP, which is of concern because of its thyroxine-disrupting activity (Suzuki et al., 2008). It also occurs naturally in marine biota, such as marine polychaetes, and is a flavor component in seafish and/or prawns (shrimp) (Whitfield et al., 1999). In addition, 2,4,6-TBP has been considered as a possible source of hydroxylated PBDEs or brominated dioxins (Arnoldsson et al., 2012).

Whereas the levels and profiles of PBDEs in serum and breast milk in Japan have been investigated intensively (Haraguchi et al., 2009; Kajiwara et al., 2008), less information is available on the contamination trends and sources of HBCDs and phenolic BFRs in the human body. The aim of present study was to investigate temporal trends, correlations, and potential sources of HBCD diastereomers, TBBP-A and 2,4,6-TBP in breast milk, as well as to evaluate possible factors affecting the transfer of these BFRs to breast milk.

## 2. Materials and methods

### 2.1. Sample collection

Breast milk samples were obtained from the Kyoto University Human Specimen Bank (Kyoto, Japan) using a standardized protocol (Koizumi et al., 2009). A total of 64 breast milk samples were randomly collected between 2008 and 2010 from healthy mothers living in various prefectures in Japan (Miyagi  $n = 19$ , Hyogo  $n = 17$ , Nagasaki  $n = 20$ , Tokyo  $n = 2$ , Gifu  $n = 3$  and Kyoto  $n = 3$ ) (Fig. 1, Table 1). The Ethics Committee of Kyoto University approved the protocol for this study (E25), and written informed consent was obtained from all participants with questionnaire. The milk samples were collected manually during breastfeeding at 4–8 weeks postpartum either with assistance from a midwife or by the subjects themselves. The breast milk was frozen ( $-20^{\circ}\text{C}$ ) prior to analysis. Three tubes of distilled water were prepared as procedural blanks and examined for possible contamination.

### 2.2. Chemicals

Three  $^{13}\text{C}_{12}$ -labeled HBCD diastereomers ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -),  $^{13}\text{C}_{12}$ -labeled TBBP-A,  $^{13}\text{C}_6$ -labeled 2,4,6-TBP, and their native compounds were purchased from Cambridge Isotope Laboratories (Andover,



Fig. 1. Sampling sites for collection of breast milk in Japan.

MA). These standards were used for method validation, including calibration, recovery, and quantification. All solvents (*n*-hexane, ethyl acetate, dichloromethane and methanol) were of pesticide grade.

### 2.3. Extraction and cleanup procedure

The method used for extraction and cleanup of HBCDs, 2,4,6-TBP, and TBBP-A from breast milk was a modification of an established lipid extraction and gel permeation chromatography (GPC) method (Haraguchi et al., 2009). Briefly, 5 g of breast milk spiked with  $^{13}\text{C}$ -labeled internal standards was acidified with 0.1% formic acid (10 mL) and extracted with ethyl acetate/*n*-hexane (1:4, *v/v*). The extracted lipid (50–300 mg) was dissolved in 1.5 mL of dichloromethane/*n*-hexane (1:1, *v/v*) and purified with a GPC column (Bio-Beads S-X3, Bio-Rad Laboratories, Hercules, CA) with the same eluent at a flow rate of 4 mL/min. The first 90-mL fraction of the eluent, which contained lipids, was discarded. The next 60-mL fraction was collected, and then evaporated to dryness under a gentle stream of  $\text{N}_2$ . The residue was reconstituted in 200  $\mu\text{L}$  of  $d_{18}$ - $\beta$ -HBCD (50  $\text{pg}/\mu\text{L}$  in methanol) and was subjected to liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) to determine the recoveries of the internal standards.

### 2.4. LC-ESI-MS/MS

LC-ESI-MS/MS was performed according to a modification of an established method (Abdallah et al., 2008). Briefly, 2,4,6-TBP, TBBP-A, and three HBCD diastereomers was separated using a Shimadzu LC-20AD Prominence liquid chromatograph (Kyoto, Japan) equipped with a Varian Pursuit XRS C18 column (150  $\times$  2 mm I.D., 3  $\mu\text{m}$  particle size, Agilent Technologies, Santa Clara, CA). The LC conditions are given in the Supplementary Fig. S2. 2,4,6-TBP and TBBP-A were eluted as single peaks at 7.9 min and 8.8 min, respectively. The three HBCD diastereomers were baseline separated with retention times of 10.2, 10.6, and 10.9 min for  $\alpha$ -,  $\beta$ -, and  $\gamma$ -HBCD, respectively (Table S1). Mass spectrometric analysis was performed using a tandem mass spectrometer (3200 QTRAP-MS/MS system; AB SCIEX, Tokyo, Japan) operated in negative ESI mode. The MS/MS

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