OPTIMIZATION OF MATRIX SOLID PHASE DISPERSION EXTRACTION PROCEDURE FOR THE ANALYSIS OF POLYBROMINATED DIPHENYL ETHERS IN HUMAN PLACENTA

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Abstract
Characterization of pre natal exposure to hazardous chemicals most often rely upon the analysis of cord blood. However, human placenta is an appropriate alternative with noteworthy advantages. Owing to the analytical challenges, reports on placental levels of toxic chemicals are limited. The purpose of this study was to establish a reliable, cost effective and a relatively fast and easy method to extract polybrominated diphenyl ethers (PBDEs) from human placenta. The matrix solid phase dispersion (MSPD) method was optimized. Different sorbents, sample conditions, grinding methods, elution solvents, and single and repeated extractions were compared for their effects on the extraction efficiency. With the optimized method, the recovery of PBDEs was 91- 114% for the spiked placenta samples and 89-115% for a standard reference material. The extraction efficiency of the MSPD method was found to be comparable with the Soxhlet method and superior to that using a liquid extraction method.

Introduction
During their development, humans may be exposed to toxicants via maternal circulation as fetuses, via breast milk as infants and by involuntary ingestion as children and adults. In-utero exposure to environmental toxicants such as pesticides, polychlorinated biphenyls (PCBs), lead and mercury and the consequence on fetal development have been studied and documented[1, 2]. As an emerging pollutant, polybrominated diphenyl ethers (PBDEs) have been found ubiquitously in the human environment. Similar to polychlorinated biphenyls (PCBs), one of the major concerns on PBDEs is their developmental toxicity to human. Studies have found correlations between elevated PBDE levels and health outcomes such as low birth weight, length, chest circumference, cryptorchidism, in newborns[3, 4].

In prenatal exposure assessment, cord blood is the most widely used matrix. Though not extensively used thus far, human placenta can be valuable substitute and may provide additional information on the exposure. Placenta is an ephemeral organ that starts growing with the developing fetus. It acts as mediator in selective exchange of materials between maternal and fatal blood. Placenta acts as a transfer barrier to some xenobiotics thereby preventing or reducing the fetal exposure[5]. Therefore, the analyte levels in placenta may proportionally reflect the exposure during the entire pregnancy, especially for bioaccumulative chemicals. The use of placenta has remarkable advantages over using cord blood. The non invasiveness with no risk to the mother and the infant, as well as the trouble-free sample collection where professional expertise is not required, are attractive to researchers. Furthermore, the bulk sample size allows multiple chemical analyses, permitting the investigation of chemical synergy. However, placenta is a unique and complex tissue. In addition to the common difficulties associated with environmental analysis, treatment of placenta samples is rather tedious compared to those for homogeneous matrices like plasma, serum, or breast milk. It is important to apply an extraction technique which is vigorous enough to surface the analytes which may be buried deeply in the tissue in order to reliably determine the concentration levels.

Matrix solid phase dispersion (MSPD) is an efficient solid phase extraction method for solid and semisolid samples. The principle of this method is to disrupt and disperse the sample in a solid phase sorbent which is present in excess quantity. The basic procedure, which comprises three major steps, is extensively illustrated in Baker, 2000[6]. The first step involves sample grinding in the presence of excess amounts of sorbent in a pestle with a mortar. Then the ground mixture of sample and sorbent is loaded on to a chromatographic column followed by elution with a suitable solvent. The quality of the MSPD performance depends on several factors,

particularly the selection of the sorbent type and extraction solvents. MSPD extraction has been the method of choice in several studies owing to its simplicity and efficacy[6]. It has been successfully used to extract trace level organic pollutants, including PBDEs, from different environmental and biological matrices[7, 8, 9, 10].

To date, only limited data on PBDEs in placenta are available[10, 4, 11, 12], and to our knowledge, no study has reported PBDE concentrations in human placenta in the United States. The purpose of this study was to find out a reliable, cost effective, and a relatively fast and easy method to extract PBDEs from human placenta. The MSPD method was optimized and compared with two conventional extraction methods. Validation of the MSPD method was carried out by analyzing matrix spike samples and a fish tissue standard reference material (SRM) with certified PBDE concentrations.

Materials and Method

Chemicals. A mixture of 39 PBDEs (BDEs 1, 2, 3, 7, 8, 10, 11, 12, 13, 15, 17, 25, 28, 30, 32, 33, 35, 37, 47, 49, 66, 71, 75, 77, 85, 99, 100, 116, 118, 119, 126, 138, 153, 154, 155, 166, 181, 183, 190), individual PBDE standards (196, 206, 207, 209) and $^{13}C_{12}$-labeled BDE118 (BDE 118L) and decabromobiphenyl (BB209) were purchased from Cambridge Isotope Laboratories (Andover, MA) and AccuStandard (New Haven, CT). Bio-beads S-X3 (200 – 400 mesh) were purchased from Bio-Rad Laboratory (Richmond, CA). Bondesil (C18, 40μm) was purchased from Varian Inc (Palo Alto, Ca). Solvents, anhydrous sodium sulfate, silica gel (100 – 200 mesh, Davisil Grade 644) and florisil (60-100 mesh) were purchased from Fisher Scientific. Hexane, dichloromethane and acetone were GC grade. The standard reference material (Lake Michigan fish tissue SRM 1947) was purchased from National Institute of Standards and Technology (NIST, Gaithersburg, MD). It was stored in a -80 °C freezer upon receiving.

Placenta Collection and Pretreatment. Full term human placenta samples were collected from the university medical center with signed consent form by the mothers. Collected placentas were immediately placed in a freezer at -20 °C. Before extraction, excess blood from the placenta was wiped and the umbilical cord and other connective tissues were removed before it was cut and homogenized in a commercial blender. Homogenate was freeze dried using a lypholizer (Freezone 4.6L, Labconco, Kansas City, MO) and kept in a dessicator.

General MSPD Extraction Procedure. A known amount of freeze dried sample was weighed into a solvent washed aluminum pan. To the same pan, extraction sorbent was added in a 1:2 sample to sorbent mass ratio and transferred into a glass pestle. After adding a known amount of surrogate (BDE118L), the sample was thoroughly mixed with the sorbent by grinding *ca* 5 min using a glass mortar until a fine powder was resulted. A glass chromatographic column (13 mm ID and 30 cm length) was packed from bottom to top with a blob of glass wool, pre-washed anhydrous sodium sulfate (~10 g), sorbent (~4 g) and prepared mixture of the sample and extraction sorbent. The packed column was gently tapped to remove the trapped air. After loading the sample, the column was eluted with 100 mL of extraction solvent. The flow was controlled at 1-2 drops/second. When the gravitational flow ceased, vacuum was applied to collect the remainder of solvent in the column.

Cleanup. Sample cleanup procedure is similar for all extraction methods. The extract was first transferred into a K-D concentrator flask for the solvent evaporation. Further volume reduction (down to ~2 mL) was achieved by gentle N_2 blow. Co-extracted lipid was determined gravimetrically before the solvent evaporation. Sample clean up was accomplished by gel permeation chromatography (GPC) followed by silica gel chromatography. The GPC column was manually prepared glass column (25 mm ID x 400 mm L) with S-X3 bio beads. Sample was eluted with 140 mL hexane and DCM (1:1) mixture. A multilayer silica gel column was prepared using (from bottom to top) 1 g neutral silica, 1 g basic silica, 4 g acidic silica, 1 g neutral silica and 5 g anhydrous sodium sulfate. 50 mL hexane was used as the elution solvent. Final volume of the sample was reduced to 1 mL by K-D evaporation and N_2 blow before instrumental analysis.

Instrumental Analysis. Instrumental analyses of PBDEs were performed on an Agilent Model 6890 gas chromatograph (GC) with a Model 5973 mass spectrometer (MS) detector. Rtx1614 capillary column (15 m × 0.25 mm i.d., 0.1 μm film thickness; Restek Inc.) was used for separation with helium as the carrier gas.

Injection standard CB204 was added to each sample before a GC injection to normalize the peak areas in the quantification of targeted PBDEs. Each sample was introduced into GC/MS through a programmable temperature evaporation (PTV) injection port. A total of 120 µL was injected in each run using solvent vent mode. The operational parameter of the PTV inlet was described in details elsewhere[13]. The initial oven temperature was 90°C, which lasted for 3 min, and then increased to 140°C at 10°C/min and further to 300°C at 5°C /min. The final temperature was kept for 15 min until the run was completed. Selected ion monitoring (SIM) was used in electron capture negative chemical ionization (ECNI) MS. The m/z values for the monitored ions of individual BDEs and BB209 were 79 and 81. CB204 was monitored using 428, 430 and 432 and BDE209 with 484 and 486. PCB204 was the internal standard for mono- to hepta- congeners while BB209 was used to correct the concentration of heavy congeners.

Quality Control and Method Validation. Procedural blanks were analyzed with each batch of extraction. The concentrations found in the blanks were deducted from those of the samples in that batch before reporting the final concentration. Known amount of C-13 labeled surrogate BDE118L was added in all the samples before extraction. The recovery of the surrogate was used to indicate the analytical accuracy and to correct the concentration values for each sample. Three placenta samples were spiked with known amounts of PBDE standards and kept overnight in a refrigerator to allow proper penetration and mixing. They were extracted along with their respective non-spiked samples. PBDE concentration of the non-spiked sample was subtracted from results of the corresponding spiked sample to calculate the recovery of the spiked PBDEs. SRM 1947 was analyzed in duplicates. Using a clean spatula, 1.00 g of the fish tissue was transferred to a pre cleaned aluminum pan. After adding the surrogates, each sample was mixed with 5 g sodium sulfate and kept covered in the refrigerator for 12 hours. Extraction of the matrix spike and the SRM samples was using the optimized MSPD method, and the cleanup and instrumental analysis were carried out as described above. All samples were analyzed in duplicates.

Results and Discussion

MSPD Method Development. The MSPD method for PBDE extraction was optimized by comparing different sorbents, sample conditions, grinding methods, elution solvents, and single or repeated extractions. The results of the comparison are illustrated in Figure 1.

One of the major steps in developing the MSPD method is to select the extraction sorbent. In this study, three sorbents including silica gel, C18, and florisil were compared, all at a sorbent-to-sample ratio of 1:2 was in the dispersion. In the reported studies 1:1 to 1: 4 have been used[7], and Valsamaki et al. found no improvement upon increasing in the sorbent amount higher than twice the sample[14]. PBDE extraction was the highest using C18, while the extraction efficiency using florisil and silica gel was 73% and 67%, respectively, of that using C18. Surrogate recovery for both C18 and florisil was similar (> 92%) but it was lower (60%) using silica gel. However, co-extracted lipid was also higher using C18, making sample cleanup more difficult. Method blanks revealed that C18 has the highest level of contamination even after intensive solvent wash before use. Based on these findings and a comparison of the cost, it was decided to use florisil as the MSPD sorbent.

Fresh and freeze-dried placenta samples were analyzed simultaneously to examine whether the sample condition affect the MSPD extraction efficiency. The difference was not significantly different (Figure 1). The major advantages of using freeze dried samples were the handing convenience and the ability to use a large quantity of sample. Loss of any congener during freeze drying was not detected.

Dry grinding of the sample sorbent mixture was tested against the wet grinding in which 20 to 40 mL of acetone-hexane (3:1) mixture was added. The effect of grinding method appears to be insignificant (Figure 1). However, the lipid extracted was five-fold higher using the wet grinding, probably due to the addition of acetone which can denature the tissue structure and thereby enhance the lipid extraction. Nevertheless, higher lipid extraction was not found to associate with higher PBDE extraction efficiency. The addition of polar solvent acetone promotes the extraction of polar lipids (phospholipids), which may not be the major depository of PBDEs in placenta.

Selection of a suitable elution solvent is critical in MSPD[6]. The results of this work indicated clearly that the mixtures of hexane and DCM gave the highest extraction efficiency (Figure 1). The additional advantage of this mixture was that it extracted lower amounts of lipids than the other two mixtures making the cleanup less tedious. It was also noted that every time acetone is used in the extraction the elution of polar lipids was higher but without improving the PBDE extraction efficiency.

Repeated extraction was tested against single extraction. In repeated extraction, the collected eluate was reintroduced into the column and recycled 2 to 4 times. A significant difference was not found; in fact the PBDE concentration was higher using the single extraction.

Based on these results, the optimized MSPD method includes freeze drying the sample, dispersing the dried sample on sorbent florisil in 1:2 sample-to-sorbent ratio, dry grinding without wetting solvent, packing the extraction column, and eluting with 100 mL of hexane-DCM (8:2) mixture in a single extraction.

Method Validation. The extraction efficiency of the optimized MSPD method was found to be comparable with those using the conventional Soxhlet extraction, and superior to that obtained with a liquid extraction method, as shown in Figure 2. The reliability of the optimized MSPD method was further examined by analyzing spiked placenta samples and NIST SRM 1947. Tables 1 and 2 demonstrate the analysis results for the spiked samples and the SRM, respectively. Excellent recoveries of 91% - 114% were achieved for the spiked placenta samples, and 89% - 115% for the SRM with the exception of BDE28, which had a low recovery (49%).

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Table 1. PBDE recovery from the spiked placenta samples (ng/mL)

Congener	Spike level	Sp1	Sp2	Sp3	Un-sp	Recovery (%)	SD (%)
BDE28+33	5.29	4.87	4.65	4.88	0.01	91	2.71
BDE47	6.08	6.19	5.86	6.28	0.31	95	3.62
BDE66	5.71	6.06	5.23	5.67	ND	99.0	7.35
BDE100	6.38	6.28	5.72	6.08	0.06	94.0	4.71
BDE99	6.21	6.95	5.87	6.27	0.13	100.0	8.58
BDE118L	5.57	6.86	5.45	6.03	ND	110.0	11.59
BDE85	5.75	7.16	5.89	6.82	0.06	114.0	9.93
BDE154	6.27	6.87	5.7	6.21	0.03	99.0	9.37
BDE153	6.16	7.13	6.05	6.47	0.14	104.0	8.31
BDE183	6.32	7.48	6.76	7.22	0.04	113.0	5.10
BDE209	6.11	7.92	8.22	7.35	1.91	97.0	5.64

Table 2. Analytical results of SRM 1947 using optimized MSPD extraction (μg/kg)

BDE	(A)	(B)	Mean ± SD	RSD %	Certified	% Recovery
BDE28	1.26	0.94	1.10±0.23	20.8	2.26 ±0.46	49
BDE47	70.17	67.38	68.77±1.98	2.9	73.30 ±2.9	94
BDE66	1.84	1.46	1.65±0.27	16.4	1.85 ±0.13	89
BDE100	14.82	15.50	15.16±0.48	3.2	17.10 ±0.6	89
BDE99	23.15	20.91	22.03±1.58	7.2	19.20 ±0.8	115
BDE154	6.49	6.66	6.57±0.12	1.8	6.88 ±0.52	96
BDE153	4.16	4.27	4.22±0.07	1.7	3.83 ±0.04	110

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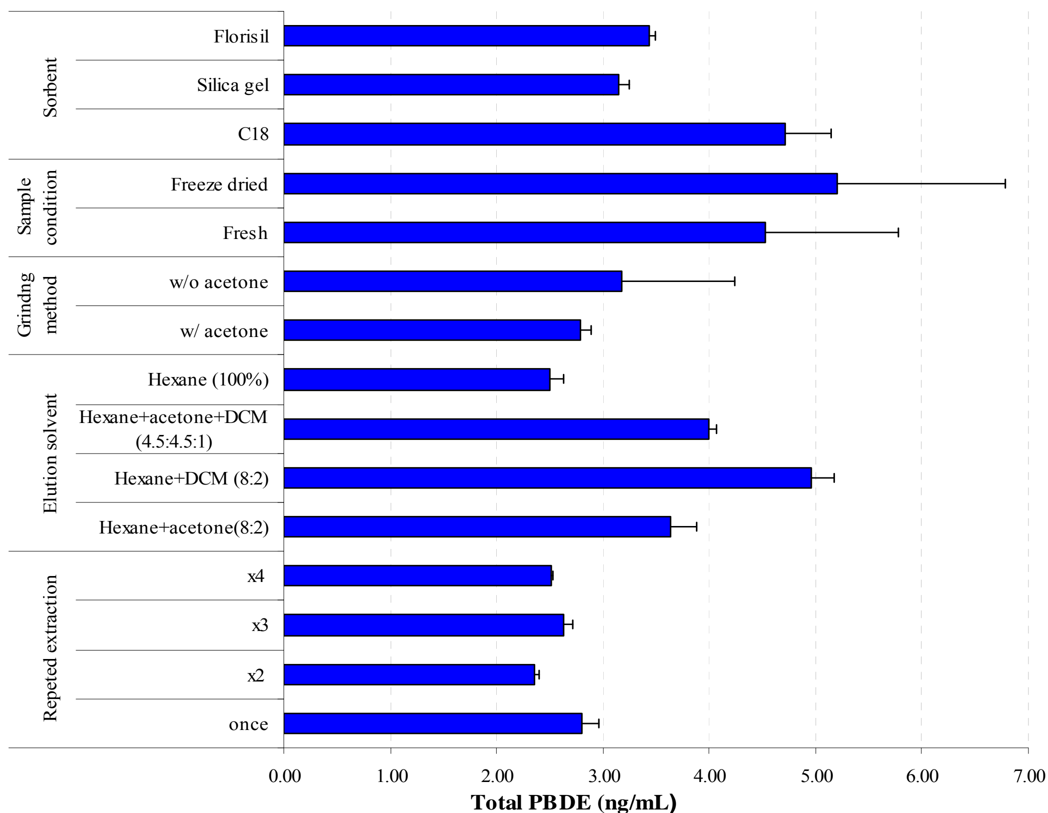

Figure 1. Total PBDE extraction under different conditions used during MSPD optimization (N=2)

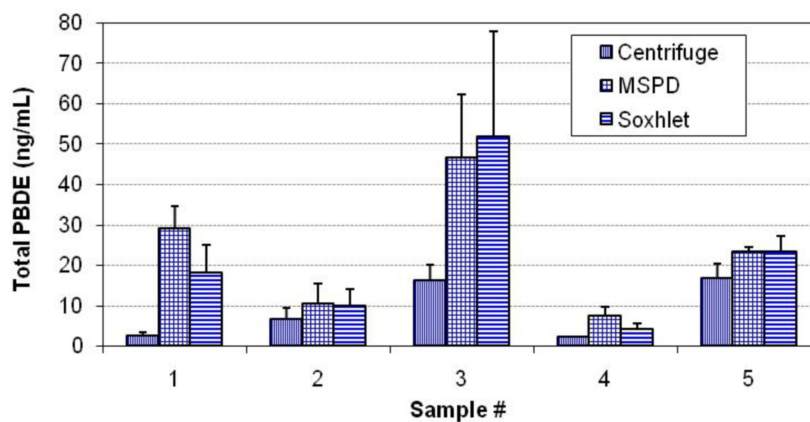

Figure 2. Total PBDE concentration from 5 individual placenta samples extracted using, MSPD, liquid and soxhlet extraction methods. The error bars represent one standard deviation of duplicated analyses (N = 2).