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The Lipid Content of Serum Affects the Extraction Efficiencies of Highly Lipophilic Flame Retardants

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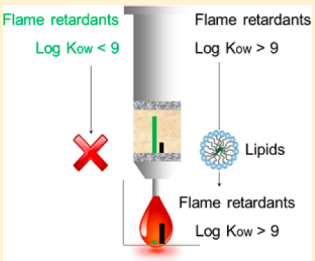
Supporting Information

ABSTRACT: This work investigates the recoveries from human serum of eight halogenated flame retardants of emerging concern: hexabromobenzene, hexachlorocyclopentenyl-dibromocyclooctane, 1,2-bis(2,4,6-tribromophenoxy)ethane, Dechlorane 602, Dechlorane 603, Dechlorane Plus, decabromodiphenyl ether (BDE-209), and decabromodiphenyl ethane (DBDPE). Extraction efficiencies were assessed using solid phase extraction (Oasis HLB) at two spiking levels with recoveries ranging from 18 to 84% [relative standard deviations (RSDs) of 4−25% (n = 8)]. Recoveries for DBDPE, BDE-209, and Dechlorane Plus averaged 24% (RSD of 18%), 38% (RSD of 20%), and 49% (RSD of 12%), respectively. These low recoveries were negatively associated with the lipid content of the serum, and Pearson correlations ranged from −0.798 to −0.839 (p < 0.002). This fact indicates that interactions between highly lipophilic flame retardants and lipids affect the extraction efficiencies. Therefore, even with thoroughly optimized SPE procedures, studies conducted without a proper internal standard (similar recovery) might result in erroneous calculated concentrations of the highly lipophilic halogenated flame retardants in serum.



INTRODUCTION

Polybrominated diphenyl ethers (PBDEs) have been used as flame retardants in a wide range of consumer products but have been restricted or banned because of environmental and health concerns.[1] This has increased the frequency of use of a number of replacements often termed emerging flame retardants.[2,3] Biomonitoring using serum samples has frequently been used to assess human exposure to halogenated flame retardants (HFRs) such as PBDEs. However, data of emerging HFRs in human serum are scarce in the literature. Techniques such as solid phase extraction (SPE) and liquid−liquid extraction (LLE) have been widely used in sensitive methods for the analysis of common HFRs in human body fluids,[4,5] although for some HFRs recoveries are insufficient regardless of the extraction technique.[6,7]

When using SPE, highly lipophilic compounds are not always satisfactorily extracted from serum. For instance, in the analysis of the most abundant polybrominated diphenyl ethers, the majority is well-extracted, but recoveries for BDE-183 and BDE-209 are usually lower than for the less brominated PBDEs. For BDE-183, recoveries reported from sheep[8] and humans[9,10] serum ranged from 43 to 89%, while recoveries for BDE-209 from human serum ranged from 13 to 64%.[10−13] It has been suggested that these poor recoveries are due to irreversible adsorption to the stationary phase[10] and/or other surfaces[13] as well as incomplete protein denaturation,[14] but this has not been experimentally proven. Therefore, this study focuses on the use of SPE for the extraction of eight HFRs of emerging concern from human serum: hexabromobenzene (HBB), hexachlorocyclopentenyl-dibromocyclooctane (HCDBCO), 1,2-bis-(2,4,6-tribromophenoxy)ethane (BTBPE), decabromodiphenyl ethane (DBDPE), Dechlorane 602 (Dec602), Dechlorane 603 (Dec603), Dechlorane Plus (DPs, *syn* and *anti*), and decabromodiphenyl ether (BDE-209) (Table 1). The objective is to investigate the extraction efficiencies of these HFRs and sources of losses during the extraction process.

MATERIALS AND METHODS

Spiking Solutions of Flame Retardants. Spiking solution A containing seven emerging HFRs ([^{13}C]HBB, HCDBCO, Dec602, BTBPE, Dec603, [^{13}C]DPs, and DBDPE) and [^{13}C]BDE-209 were prepared in toluene at two concentrations: a low level (LL) of 4 pg/μL, except for [^{13}C]BDE-209 and DBDPE, which were at 18 and 89 pg/μL, respectively, and a high level (HL) of 81 pg/μL, except for [^{13}C]BDE-209 and DBDPE, which were at 161 and 806 pg/μL, respectively. A second spiking solution (B) containing BDE-18, BDE-103, BDE-181, [^{13}C]HBB, [^{13}C]DPs, and [^{13}C]BDE-209 at 10 pg/μL was also prepared in toluene. Recovery standards (RS; [^{13}C]BDE-205 and [^{13}C]BDE-139) were diluted to 100 pg/μL in toluene. See the Supporting Information for further information about standards and solvents.

Sample Pretreatment. Horse serum (Sigma-Aldrich, St. Louis, MO; for further details, see the Supporting Information) and an in-house quality control sample of pooled human sera

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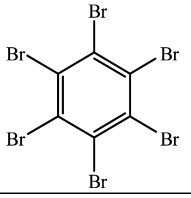
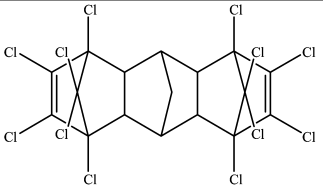
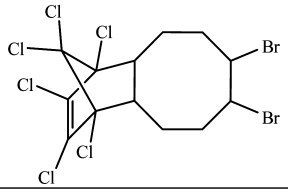
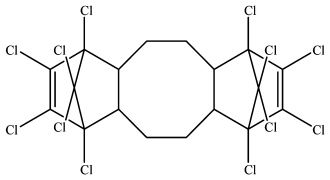
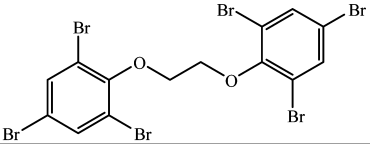
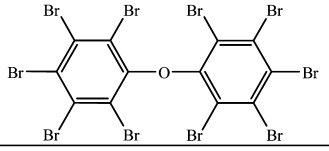
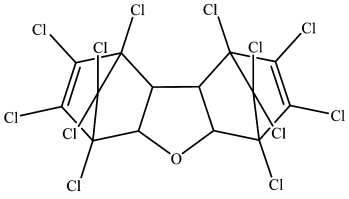
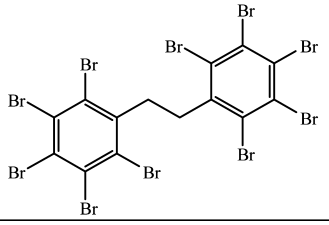
Table 1. Chemical Structures, Chemical Abstracts Service Registry Numbers, and Octanol Water Partition Coefficients (K_{ow})[a] for the Emerging HFRs and BDE-209

molecular structure	compound	molecular structure	compound
	HBB CAS 87-82-1 Log K_{ow} 7.3		Dec603 CAS 13560-92-4 Log K_{ow} 11.2
	HCDBCO CAS 51936-55-1 Log K_{ow} 7.9		DPs CAS 13560-89-9 Log K_{ow} 11.3
	BTBPE CAS 37853-59-1 Log K_{ow} 9.1		BDE-209 CAS 1163-19-5 Log K_{ow} 12.1
	Dec602 CAS 31107-44-5 Log K_{ow} 8.1		DBDPE CAS 84852-53-9 Log K_{ow} 13.6

[a]The log K_{ow} values were obtained from EPI Suite version 4.1 (see the Supporting Information for further details).

were used to develop the analytical method and assess recoveries of emerging HFRs and [^{13}C]BDE-209. Ten individual serum samples from nonoccupationally exposed persons that participated in studies at the Norwegian Institute of Public Health were used to assess extractions on samples with different lipid content. Informed consent was obtained from all participants, and the Regional Committee for Medical Research Ethics approved the study. All sera were stored at −20 °C. Serum concentrations of triglycerides, phospholipids, and total cholesterol were determined enzymatically at the Oslo University Hospital, and the total lipid content was calculated according to the summation method described by Grimvall et al.[15]

Horse and human sera were thawed overnight. Two milliliters of serum was spiked with 30 μL of solution A (LL or HL) or B. After being briefly manually whirl mixed (Heidolph REAX top, Schwabach, Germany) and sonicated for 10 min (Branson 2510, Sigma-Aldrich), samples were placed in the refrigerator overnight. Denaturation of proteins was conducted in 20 mL glass tubes at room temperature using 2 mL of formic acid followed by whirl mixing and sonication for 10 min. Prior to SPE, samples were diluted with water to a final volume of 6 mL.

Extractions and Cleanup. Labeled compounds, if available, were used in the recovery experiments to avoid the possible contamination of native HFRs from the environment. SPE was performed on Oasis HLB columns [500 mg (Waters, Milford, MA)] applying the following method in four steps: (1) conditioning of the column using consecutively 4 mL each of dichloromethane (DCM), methanol, and water, (2) loading denaturized serum, (3) washing of the column in two steps with 4 mL of water and 1 mL of methanol, and (4) before elution of target analytes with 8 mL of DCM, drying of the column by suction (30 min). The eluate was concentrated to approximately 1 mL using a Rapidvap (Labconco, Kansas City, MO). Subsequently, further cleanup was conducted using SPE cartridges packed with silica (∼150 mg), sulfuric acid/silica (33%, v/w) (∼1 g), silica (∼150 mg), and sodium sulfate (∼600 mg) (from bottom to top, respectively). The cleanup cartridges were conditioned by 4 mL of a heptane/DCM mixture (3:1, v/v). After the extract had been loaded via SPE, elution was conducted by gravity with 8 mL of a heptane/DCM mixture (3:1, v/v). The eluate was evaporated to approximately 0.5 mL in the Rapidvap instrument. This volume was transferred to inserts and reduced to a few microliters with a gentle stream of nitrogen. Finally, 20 μL of RS was added and the volume adjusted to 50 μL with toluene.

For comparison, 2 mL of serum, denaturized with 2 mL of formic acid and spiked with solution A at LL, was liquid−liquid extracted (whirl mixing for 1 min) using 3 × 7 mL of either toluene or a toluene/heptane mixture (1:1, v/v) or a heptane/methyl *tert*-butyl ether mixture (1:1, v/v). A heptane/DCM mixture (6:1, v/v) was used for LLE of the eluted aqueous phase from the SPE to look for nonretained HFRs. After the aqueous phase (serum) and organic phase had been whirl mixed for 1 min and following centrifugation at room temperature (*g* force = 2163 RCF for 2 min) (Rotina 46, Hettich Lab Technology, Tuttlingen, Germany), the organic phases were collected and reduced to approximately 1 mL. Cleanup was performed using the procedure described above.

In addition, an experiment to assess the efficiency of the former LLE for the highly lipophilic HFRs was conducted using

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an ultrasonic bath (Transonic TS 540, Elma, Singen, Germany). First, 1 mL of DCM was added to 2 mL of denaturized horse serum, and then 4 mL of a heptane/DCM/2-propanol mixture (85:5:10, v/v) was added to obtain three separated phases. The rest of the process followed the previous description of LLE except that ultrasonication was applied for 45 min.

To check for losses due to adsorption of HFRs onto the walls of the original sample tubes, these were dried by a flow of nitrogen and rinsed repeatedly with DCM. The combined volumes were reduced and subjected to gas chromatography–mass spectrometry (GC–MS) analysis (see the Supporting Information for instrumental analysis, Tables S1 and S2, and chromatograms in Figures S1–S3).

RESULTS AND DISCUSSION

Extraction Efficiency of SPE. For method development, horse serum was used as it was unlikely to contain HFRs and was expected to result in matrix effects similar to those of human serum. Procedural blanks (5% 2-propanol in water) contained BTBPE, Dec603, and BDE-209 (Figure S3 of the Supporting Information). Because ^{13}C-labeled standards were not available for BTBPE and Dec603, blank subtraction was performed on serum samples.

Emerging HFRs and [^{13}C]BDE-209 were extracted from horse and human serum using Oasis HLB as previous studies have demonstrated better performance for halogenated compounds such as PBDEs than for other sorbents (C_{18} Empore from 3M Co., Strata-X from Phenomenex, and Isolute-phenyl, 101, and ENV+ from International Sorbent Technology).[10,11] A tendency toward a decreasing recovery with an increasing K_{ow} was observed (Figure 1; see Table S3 of the Supporting Information for numerical data).

In general, the SPE recoveries were lower for human than for horse serum (e.g., 35% for DPs), which might be ascribed to the different percentage of lipid content (0.21 and 0.57% for horse and human serum, respectively) and/or composition. Ren et al. also reported lower recoveries for DPs from human and bovine serum using LLE (~20%).[16] Recoveries obtained by LLE of horse and human serum were in the same range as those obtained by SPE (Table S4 of the Supporting Information), indicating similar extraction efficiencies for the two methods. Consequently, either SPE or LLE has been demonstrated to be a suitable technique for extracting HFRs with log K_{ow} values of less than 8–9 from human serum. For the SPE of the more lipophilic HFRs, it was possible to increase the recoveries of dechloranes by optimizing the SPE procedure, e.g., by thorough drying of the SPE sorbent prior to elution.[17]

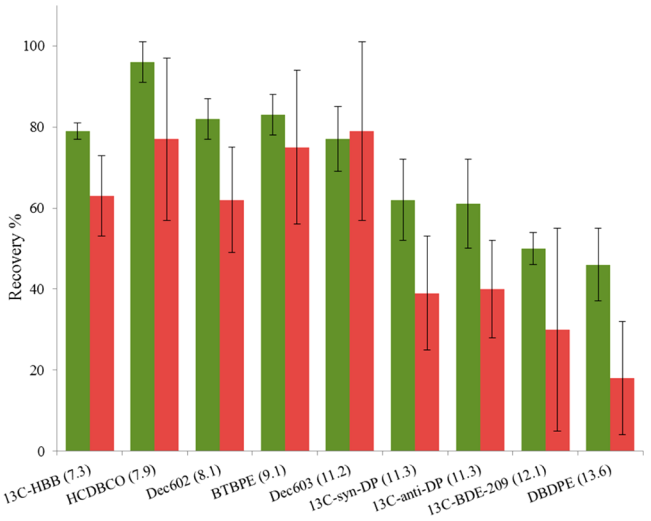

Figure 1. SPE recoveries of emerging HFRs and [^{13}C]BDE-209 from human (red; n = 4) and horse (green; n = 3) serum spiked with solution A at LL. Error bars show the relative standard deviations. The log K_{ow} values are given in parentheses.

Breakthrough of HFRs during SPE. To date, obtaining good extraction efficiencies for compounds with log K_{ow} values of >9 is still a challenge. To investigate the loss during SPE, the aqueous eluent from the SPE column was subjected to LLE. Only HFRs with very high K_{ow} values were found in the extract: Dec602, Dec603, [^{13}C]DPs, [^{13}C]BDE-209, and DBDPE. Their recoveries ranged from 1 to 6% [relative standard deviation (RSD) of 27–67% (n = 3)] and from 1 to 8% [RSD of 18–42% (n = 3)] at LL and HL spiking, respectively (Figure S4 of the Supporting Information). Strikingly, the most lipophilic flame retardants ([^{13}C]BDE-209 and DBDPE) had the highest percentages in the aqueous eluent representing ~20% of their total SPE recoveries. A possible explanation for the breakthrough might be that the highly lipophilic HFRs are trapped in micellar type structures formed by surface active serum constituents such as phospholipids. Measured concentrations of phospholipids in serum were within the range of 2.0–3.3 mM. Because the critical micellar concentration for typical phospholipids is between 0.05 and 0.77 mM,[18] this phenomenon is therefore likely to occur during both SPE and LLE. Then, HFRs would be trapped in the inner lipophilic core of the amphipathic substance, while the outer polar surface would have little interaction with the hydrophobic stationary phase from SPE or the organic liquid phase from LLE, thereby reducing the extraction efficiency. Such encapsulation or trapping is well-known in analytical chemistry, e.g., in investigations of macromolecules by nuclear magnetic resonance[19] and separation of persistent pollutants by cloud point extraction.[20] Figure 2 visualizes the increasing breakthrough of emerging HFRs and [^{13}C]BDE-209 with increasing K_{ow} values at both spiking levels.

The pattern in Figure 2 was tested and confirmed by repeating the extraction experiments with human and horse serum after 10 and 30 days, respectively (Figure S5 of the Supporting Information). However, the detected breakthrough does not explain the total loss of highly lipophilic HFRs in the SPE recoveries. An exhaustive extraction of the sample tubes and SPE sorbent did not reveal any residues adsorbed to the surfaces. Consequently, the loss of analytes might still remain in the aqueous phase as suggested by the breakthrough. Horse serum was spiked with solution B and DBDPE (100 pg/μL), and HFRs were extracted in the three-phase system using ultrasonication. Significantly higher recoveries for [^{13}C]BDE-209 (83%; RSD of 22%; n = 4) and DBDPE (65%; RSD of 23%; n = 4) compared to SPE recoveries of [^{13}C]BDE-209 (50%; RSD of 4%; n = 3) and DBDPE (46%; RSD of 9%; n = 3) were obtained (Figure S6 of the Supporting Information). The ultrasonic treatment in LLE seems to extract the highly HFRs more efficiently than SPE, possibly by disrupting their interactions with the active serum constituents. This supports the hypothesis that micelle formation of highly lipophilic HFRs with serum constituents is responsible for their low recoveries in SPE.

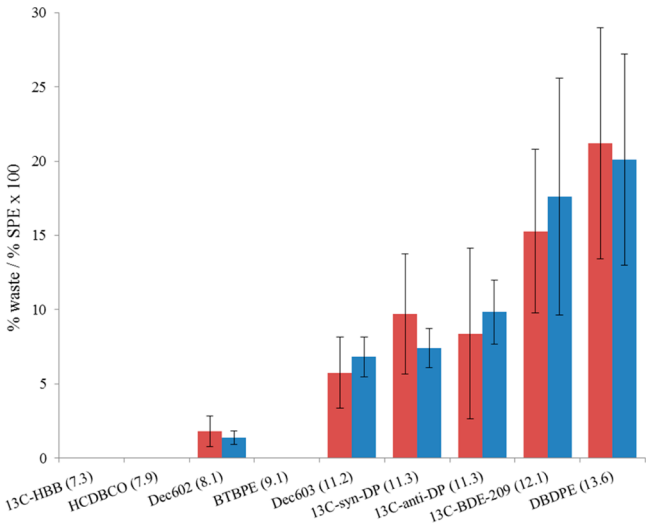

Figure 2. Amounts of emerging HFRs and [^{13}C]BDE-209 in the aqueous phase expressed as a percentage of SPE recovery. Human serum spiked with solution A at LL (red) and HL (blue). The log K_{ow} values are given in parentheses. Error bars show the RSD.

Association between SPE Recoveries of HFRs and Lipid Content of Serum. Recoveries from matrices such as dust are considerably higher for BDE-209 and DBDPE than those from serum.[21,22] Although dust also may contain lipids, these cannot form micelles because HFRs are extracted using nonpolar organic solvents; i.e., there is no aqueous phase in which the lipids can form micelles with lipophilic HFRs. Because lipids are suspected to take part in the SPE breakthrough and therefore in the low recoveries, a new set of extractions of samples with differing lipid content, i.e., procedural blanks, horse serum, and 10 human sera, was conducted. Sera and procedural blanks were spiked with 30 μL of solution B and solid phase extracted as described above. Table 2 shows that compounds with log K_{ow} values of >9.4 have in general lower recoveries (red) than the rest of the analytes (green), indicating the expected different behavior in their extraction efficiencies. Recoveries obtained from BDE-18 were somewhat lower than expected, possibly because of the partial evaporation during sample preparation.

To increase the statistical power, procedural blank recoveries were included in the statistics (see the calculation without the procedural blank in Table S5 of the Supporting Information). SPE recoveries of ^{13}C-labeled *anti*-DP, *syn*-DP, and BDE-209 were significantly and highly correlated with the total lipid content of the sample ($p < 0.01$). Strong correlations were also seen with triglycerides, cholesterol, and phospholipids individually (Table S6 of the Supporting Information). These tendencies suggest that the lipid content negatively influences the recoveries of HFRs, but especially those with high K_{ow} values.

This fact could affect the determination of the levels of emerging HFRs and [^{13}C]BDE-209 in serum samples if the internal standards (IS) used for their quantification have different K_{ow} values. This is, for example, the case in some published studies that did not use ^{13}C-labeled IS for the determination of DPs[23,24] and for DBDPE, which is usually determined against [^{13}C]BDE-209.[12,17] As an example from our study, if BDE-181 (GC retention time close to that of *syn*-DP) is used as IS for quantification of DPs, the reported concentrations would be underestimated by ~30% (based on the recoveries) when an additional correction factor is not applied. The choice of an IS based merely on GC retention times can lead to errors in quantification for compounds with different K_{ow} values. Therefore, use of isotopically labeled analogues as IS, whenever available or possible, is strongly recommended.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Technical details regarding GC–MS, chromatograms, and extraction efficiencies using LLE and SPE. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

Table 2. SPE Recoveries (percent) and Statistical Associations with the Total Lipid Content (percent) in Serum Spiked with Solution B

Matrix	Total lipid	BDE-18	^{13}C-HBB	BDE-103	BDE-181	^{13}C-syn-DP	^{13}C-anti-DP	^{13}C-BDE-209
[a]procedural blank	0.00	70	82	85	73	81	79	86
[b]horse serum	0.21	59	75	75	67	65	61	55
human A	0,44	53	68	68	59	46	44	39
human B	0.45	63	78	74	59	38	39	34
human C	0.49	64	79	64	64	44	42	51
human D	0.49	66	75	80	70	45	43	42
human E	0.53	79	89	93	74	53	51	45
human F	0,59	50	62	67	62	35	33	38
human G	0.60	76	87	85	63	37	37	33
human H	0,62	64	74	79	71	57	54	57
human I	0,72	49	56	59	53	38	36	37
human J	0.79	65	82	77	60	38	37	31
	Log K_{ow}	5.9	7.3	7.7	9.4	11.3	11.3	12.1
	[c]Pearson (r)	-0.162	-0.224	-0.273	-0.495	-0.823	-0.839	-0.798
	p-value	0.615	0.484	0.391	0.102	0.001	0.001	0.002

[a]Procedural blank (5% 2-propanol in water); $n = 4$; RSD of 2–6%. [b]$n = 4$; RSD of 3–10%. [c]Performed using SPSS version 20.

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■ ACKNOWLEDGMENTS

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