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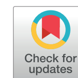
Simple and fast method for the measurement of legacy and novel brominated flame retardants in human serum



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HIGHLIGHTS

- Simultaneous measurement of 15 BFRs including BDE-209 within a single injection.
- Results obtained from interlaboratory test material were close to the assigned values.
- The analytical technique successfully passed the validation process.
- The user-friendly method could be easily implemented for high throughput analyses.
- Provides clean extracts avoiding usual solvent and time consuming purification steps.

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ABSTRACT

Recent and reliable human biomonitoring data on brominated flame retardants (BFRs), either legacy or new BFRs, are still needed to assess human exposure. The aim of this work was therefore to develop and validate an accurate, fast and user-friendly analytical strategy for the determination of 15 legacy and novel BFRs in human serum namely 8 polybrominated diphenylethers (BDE-28, -47, −99, −100, −153, −154, −183, and −209), 1 hexabromobiphenyl (PBB-153), and 6 novel BFRs (pentabromotoluene, hexabromobenzene, pentabromoethylbenzene, 2-ethylhexy-2,3,4,5-tetrabromobenzoate, 1,2-bis(2,4,6-tribromophenoxy) ethane, and decabromodiphenylethane). This analytical procedure consisted in a simple liquid-liquid extraction followed by elution on a PHREE cartridge avoiding further laborious purification steps. The final determination was performed by gas chromatography coupled to mass spectrometry in electron capture negative ionization mode (GC-ECNI-MS). The 15 m long RTX-1614 allowed the simultaneous measurement of the 15 BFRs including low and high brominated species within a single injection on a single column. Except for 2-ethylhexy-2,3,4,5-tetrabromobenzoate (EHTBB) which showed very high response variations resulting in poor linearity, trueness and precision, and decabromodiphenylethane for which very low sensitivity was achieved, the 13 other BFRs passed the validation process with recoveries varying between 56 and 82%, and limits of quantification (LOQs) ranging from 2.5 to 6.0 pg/ml (34.5 pg/ml for BDE-209). Within the validated range of concentrations, the relative bias from the introduced levels were below 20% while the intra and inter precisions were maintained below 15%. The reliability of the technique was confirmed by successfully analyzing interlaboratory test materials (AMAP ring test for POPs in human serum).

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1. Introduction

Brominated flame retardants (BFRs) are used in a wide range of consumer products such like textiles, plastics, electric or electronic equipments, insulation foams, building materials, to prevent or reduce the ignition and rate of combustion (de Wit, 2002; Sjodjin et al., 2003). Among them, polybrominated diphenylethers (PBDEs) manufactured as mixtures, mainly the penta-, octa- and deca-formulations, represented an important class extensively used as additive flame retardants since the 1970s. Because not chemically bound to the polymer, they can migrate from the final

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product during lifetime, disposal or recycling. Due to their persistence and lipophilicity, they were able to bioaccumulate in the environment and reach the human organism through the food chain. Indoor environment has also been suggested as an important source of exposure for humans through the inhalation, ingestion and/or dermal contact of domestic dust however all exposure pathways are still not fully determined. Their widespread occurrence and health concerns led to the ban of use and production in Europe and North America of the penta- and octa-formulations in 2003 and 2004, whereas phase out and restrictions on the deca-brominated formulation occurred 5–10 years later (Covaci et al., 2011; de Wit et al., 2010; Gramatica et al., 2016). Moreover, tetra-, penta-, hexa-, hepta- and deca-PBDEs were progressively added to the list of the Persistent Organic Pollutants (POPs) from 2009 to 2017 (UNEP, 2007). Nevertheless, their ubiquity in all compartments of environment and subsequent human exposure still are occurring.

On the other hand, other brominated compounds have been gradually used as substitutes to these legacy BFRs. This is the case for instance for decabromodiphenylethane (DBDPE) or 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE), used now as replacement of deca-BDE and octa-BDE respectively in various plastics and textiles (Covaci et al., 2011), or 2-ethylhexy-2,3,4,5-tetrabromobenzoate (EHTBB) contained in Firemaster 550 which is added to polyurethane foam instead of penta-BDE (Stapleton et al., 2012). These “novel BFRs” (NBFRs) although defined as new to the market or newly or recently observed in the environment (Covaci et al., 2011), have been produced most of the time since several decades, but their increasing production led to increasing interest and consequently to increasing recent data in the environment (Arp et al., 2011; Covaci et al., 2011). The reason for the apparent re-emergence in recent studies of other NBFRs such like hexabromobenzene (HBBz), pentabromotoluene (PBT), or pentabromoethylbenzene (PBEB) which is not longer manufactured to date, remains unclear but definitively reflects a re-emergence in concern for non-PBDE BFRs (Arp et al., 2011). However, reliable human exposure or body burden data are still scarce likely due to the analytical difficulties encountered for instance for high brominated compounds such like DBDPE, the few availability of commercial reference materials or standards, or the very few analytical methods specifically dedicated to NBFRs (Covaci et al., 2011). Indeed although several times reviewed (Covaci et al., 2011; Kierkegaard et al., 2009; Papachlimitzou et al., 2012), their determinations were usually based on retrospective analyses from extracts initially intended to PBDE measurements, or in the best case included in legacy BFR analyses but without specific optimization and validation (Covaci et al., 2011; Papachlimitzou et al., 2012).

Both novel and legacy BFRs used to be detected in the serum of the general population at the ppb or ppt level resulting in a challenge for the analytical chemists. Historically the PBDE's analytical methods were mainly derived from the complex dioxin analyses, requiring huge volumes of serum sample (up to 10 ml), involving laborious extraction and multistage purification steps and the use of high resolution mass spec (HRMS) for final determination (Guvenius et al., 2003; Papke et al., 2004; Pirard et al., 2003; Sjodin et al., 2004). Nevertheless efforts were expended to move from HRMS to low-cost and user-friendly low resolution mass spec (LRMS) using preferentially electron capture negative ionization mode (ECNI) because of its high sensitivity toward brominated compounds despite its lower selectivity (Eljarrat et al., 2003; Gomara et al., 2007; Thomsen et al., 2002; Vizcaino et al., 2009), and to develop the sample preparation in order to reduce solvent and matrix consumption. Nowadays, 3–5 ml of serum are typically needed to achieve sufficient sensitivity, and extraction and cleanup procedures are simplified or partially automated in well-equipped labs (reviewed in Covaci et al., 2007; Gao et al., 2016; Lu et al., 2017; Ramos et al., 2007; Thomsen et al., 2007). Nevertheless, due to the high lipophilicity of the analytes, the sample preparation usually still requires multistep purification for lipid and other interference removals. These latter include subsequently to solid phase (SPE) or liquid-liquid extraction (LLE), additional steps such like gel permeation chromatography (GCP), acidic treatment, elution through multilayer silicagel, alumina, or Florisil, etc (Covaci et al., 2007). All these fastidious steps could be solvent and time consuming, and increase the risk of external contamination of the sample (Gao et al., 2016; Kierkegaard et al., 2009; Papke et al., 2004). Moreover, the determination of high brominated compounds (i.e. BDE-209, DBDPE) used to be touchy due to their thermal instability and low volatility, inducing frequently separate GC injections on shorter columns (Kierkegaard et al., 2009), increasing the analysis duration.

Therefore due to the need to still monitor historical BFRs including BDE-209 for which human data remain scarce, as well as to collect reliable recent data on NBFRs, the aim of this work was to develop and validate a robust, accurate, fast and user-friendly analytical strategy for the measurement of 15 legacy and novel BFRs in human serum allowing high throughput analyses for large scale epidemiological studies. The BFRs targeted were 8 PBDEs (BDE-28, -47, −99, −100, −153, −154, −183, and −209), 1 hexabromobiphenyl (PBB-153), and 6 novel BFRs namely PBT, HBBz, PBEB, EHTBB, BTBPE, and DBDPE.

2. Material and methods

2.1. Chemicals and materials

The individual standard solutions of PBEB, BTBPE, DBDPE, HBBz, PBT, EHTBB, BDE-51, BDE-156 and BDE-181 (50 μg/ml) as well as the $^{13}C_{12}$-labeled internal standard MBDE-209 (25 μg/ml) were purchased from Wellington Laboratories (Ontario, Canada), while the BDE-CM containing BDE-28, -47, −99, −100, −153, −154, −183, and −209 (at 2.5 μg/ml for all congeners except for BDE-209 which was at 25 μg/ml) was come from AccuStandard Inc (New Haven, CT, USA). Water was obtained from a Milli-Q® UltrapureWater Purification Systems (Millipore, Brussels, Belgium). Glacial acetic acid (99.5%) and acetone (AR®) were bought from Macron Fine Chemicals (Gliwice, Poland), hexane (ultra resi-analyzed grade) and 2-propanol (LC-MS reagent grade) from J.T. Baker (Pennsylvania, USA), and nonane anhydrous (≥99%), sodium sulfate anhydrous (≥99%) and fetal bovine serum (research grade) from Sigma Aldrich (St Louis, USA). PHREE phospholipid removal cartridges (1 ml) were purchased from Phenomenex (Torrance, CA, USA).

2.2. Stock standard solutions

The native stock solution was prepared in isopropanol to obtain 50 ng/ml for all compounds except for BDE-209 and DBDPE which were set at 500 ng/ml. The BDE-51, -156, −181 and the $^{13}C_{12}$-labeled BDE-209 were used as internal standard and were diluted in isopropanol to obtain respectively 10 ng/ml and 100 ng/ml. All standard solutions were stored in amber vials at 4 °C.

2.3. Sample preparation

Internal standard (20 μl) was added to 1 ml of serum, 0.3 ml of glacial acetic acid and 0.7 ml of water and let equilibrated using multi-tube vortex at 2500 rpm for 1 h, before being extracted twice with 4 ml of a hexane/acetone mixture (95/5) by vortexing at 2500 rpm for 10 min. The combined organic fractions were

collected and then concentrated till 0.5 ml using SuperVap® 12 positions concentration system from Fluid Management System (Watertown, MA, USA), and loaded on 1 ml PHREE cartridge top filled with 0.3 g of anhydrous sodium sulfate. The eluate obtained by centrifugation (5 min at 3000 rpm) was transferred to silanized GC vial with 25 μl of nonane as keeper, and let evaporate at room temperature till 25 μl. Only disposable glassware was used except the evaporation tubes which were previously rinsed with acetone and hexane, and heated at 300 °C overnight. All standard solutions, solvents, vessels, pipette tips, vials, etc were sheltered from dust (stored in closed boxes, or packed in aluminum foils).

2.4. Instrumental analysis

Final determination was performed using a gas chromatograph (GC) coupled to a mass spectrometer (7890A GC/7000A Triple Quad MS, Agilent Technologies, California, USA), and equipped with a Rtx-1614 column (15 m × 0.25 mm IDx0.10 μm df, Restek, Bellefonte, PA, USA). Helium (He N60, Air Liquide, France) was used as carrier gas at a constant flow of 2 ml/min. The injection (2 μl) was performed in pulsed splitless mode with an additional pressure of 65 psi for 4 min, and at a constant temperature of 325 °C (split vent: 4.05 min). The initial oven temperature was set at 140 °C held for 1 min, then successively increased to 180 °C at 10 °C/min, to 195 °C at 3 °C/min, to 240 °C at 10 °C/min, to 250 °C at 5 °C/min and finally increased at 100 °C/min to 315 °C held for 8 min. The MS operated in electron capture negative ionization mode (ECNI) using methane as reagent gas. The transfer line, the source and the quadrupoles were set respectively at 300 °C, 200 °C and 150 °C. Both 79.2 and 81.2 masses were selected in Single Ion Monitoring for all target BFRs except for native BDE-209 and $^{13}C_{12}$-labeled BDE-209 for which 486.8, 484.8 (for native), and 494.8 (for labeled) were monitored. Besides decabrominated BFRs (BDE-209 and DBDPE) quantified using $^{13}C_{12}$-labeled BDE-209, BDE-51, -156 and −181 were used for the quantification of all other BFRs depending on their brominated degree and retention times.

2.5. Validation

The whole analytical method was validated according to the total error approach (Dubois et al., 2012; Hubert et al., 2007) using E-noval software V4.0 (Arlenda, Liege, Belgium), and based on the standard addition method. For this purpose, fetal bovine serum previously checked for initial contamination was spiked to elaborate the calibration curve (7 points with concentrations ranging from 20 to 5000 pg/ml for BDE-209 and DBDPE, and from 2 to 500 pg/ml for all other BFRs) and the validation samples (concentrations ranging from 1 to 500 pg/ml excepted for BDE-209 and DBDPE for which concentrations varying from 10 to 5000 pg/ml). These validation samples were run in triplicate within a single day, and replicated on two other days to determine the trueness (as relative bias), intra and inter essay precisions (as repeatability and intermediate precision), uncertainty, and sensitivity (in terms of limits of detection and quantification) (Dubois et al., 2012; Hubert et al., 2007).

2.6. Analysis of unknown samples

The measurement of unknown samples was performed using a 7-point calibration curves prepared by spiking fetal bovine serum with native solutions from to 1–500 pg/ml of serum (10–5000 pg/ml for BDE-209 and DBDPE) and extracted as real samples. Additionally each sequence included 1 procedural blank, 1 fetal bovine serum blank, 2 home-made quality controls (QC) (low and high levels) and 1 reference material obtained from the AMAP interlaboratory ring test for POPs in human serum (National Institute of Public Health, Quebec, Canada). The quality of the solvent was tested for each new batch to control external contamination.

3. Results and discussion

3.1. GC/MS determination

Since the determination of BFRs was predominantly carried out using GC-ECNI-MS by monitoring only bromine atom, efficient GC separation on a 30 m column is needed to avoid interferences (Eljarrat et al., 2003). On the other hand, the highly brominated compounds such like BDE-209 and DBDPE have been determined on a shorter column with thin stationary phase, typically a 15 m long column with a film of 0.1 μm to minimize debromination occurring during the instrumental analysis (Kierkegaard et al., 2009). In the present study, the aim was to include within a single injection both low and high brominated PBDEs and NBFRs. To our knowledge, only one study (Bending and Vetter, 2013) reported the separation of tetra-to decabrominated BDEs using of a 30 m column with a 60 min run time, by increasing the carrier gas flow for BDE-209 elution. The present separation was achieved in 26 min by using the 15 m Rtx-1614 column which according to the manufacturer was specifically designed for PBDE analysis, providing fair separation and higher response for BDE-209 compared to classical column, likely due to its high inertness. Fig. 1 shows the chromatograms obtained with the Rtx-1614 and with a traditional HP-5ms from Agilent (15 m × 0.25 mm i.d. × 0.25 μm film thickness). When using the Rtx-1614, the separation was achieved in 26 min, and as reported by the manufacturer, significantly higher signal was obtained for PBDE-209 and DBDPE compared to the HP-5ms. Unfortunately, only partial separations were obtained for PTB and BDE-28 on one hand, and PBB-153 and BDE-154 on the other hand, making sometimes difficult the quantification. Nevertheless the use of the Rtx-1614 improved the resolution for PBB-153 and BDE-154 because perfect coelution occurs on the short HP-5ms column, but also on a 30 m column (Eljarrat et al., 2003; Vetter and Rosenfelder, 2008). More generally, one should be conscious that all potential interferences coming from complex samples like biological matrices could not be entirely eliminated however the detection technique used or the GC conditions (Eljarrat et al., 2003).

Although Programmable Temperature Vaporizer (PTV) was reported to be the most suitable injector for BDE-209, traditional split/splitless injector (SSL) is still the most often used for PBDE determination likely due to the ease of use and optimization, knowing that very small bias in injector set up could lead to the complete loss of nona- and decabrominated BDEs (Bjorklund et al., 2004; Vizcaino et al., 2009). In the present study, pulsed splitless mode appeared to be the most powerful injection technique and the high efficiency obtained for highly brominated compounds by Bjorklund et al. (2004) and Vizcaino et al. (2009) using PTV could not be achieved on our instrument despite the optimization carried out. As expected, the injection temperature, the surge pressure applied in the injector, and the time splitless were the major parameters impacting the response of high brominated compounds. However a residual degradation of BDE-209 could not be totally avoided as observed in Fig. 1. These degradations which seemed to be independent of the GC run duration would occur in the inlet during the sample vaporization (Gomara et al., 2007), and seemed to increase with the increasing number of injections using the same liner likely due to activation of internal liner surfaces (Tollback et al., 2003). In order to decrease its frequency of replacement, highly inert liner should be used, but also extracts of the highest cleanliness as possible should be injected to avoid fast activation.

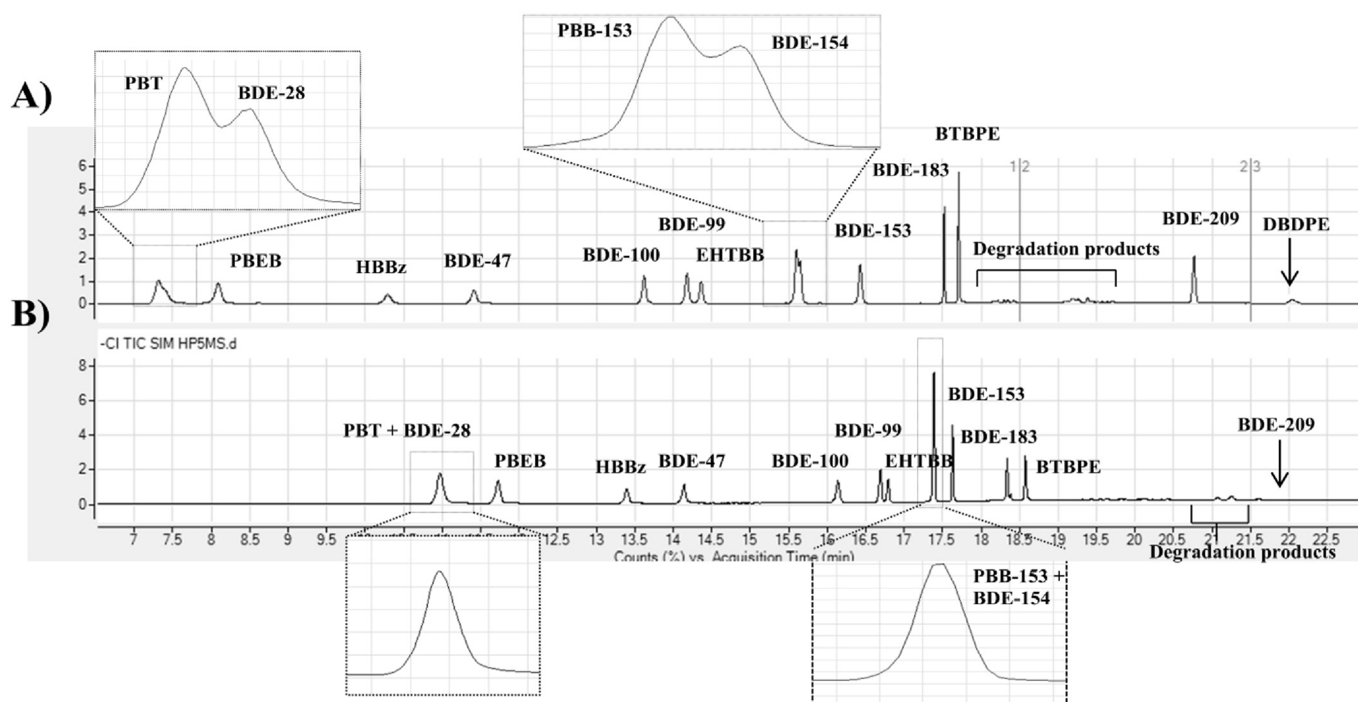

Fig. 1. Chromatograms obtained from a standard solution (2 pg injected) on a RTX-1614(A) versus HP-5MS (B) both 15 m length.

This highlighted that the performance of the sample cleanup steps is crucial for highly brominated compound determination. Among the different liners tested, the highly deactivated Sky Liners double "Gooseneck splitless" (from Restek) provided the lower degradation rate and the longer span life.

Because thought to be more resistant to thermal degradation than BDE-209 (Covaci et al., 2011; Kierkegaard et al., 2009), the poor sensitivity observed for DBDPE compared to BDE-209 would be likely inherent to its very low volatility resulting in very late elution when the bleeding of the GC column used to be maximal, rather than higher thermal instability although degradations are also expected.

3.2. Extraction procedure

The sample prep dedicated to BFR analysis in biological materials used to be laborious, time and solvent consuming, involving several extraction and purification steps to remove lipids and other interferences. Throughput could be improved by semi-automation of the cleanup (Ramos et al., 2007; Sjodin et al., 2004; Thomsen et al., 2007), but requires sophisticated and high-cost instruments not particularly compatible with budget of small- or medium size labs. In the present strategy, the aims were to reduce time, sample and solvent volumes, handling, and cost, to achieve high throughput analysis on a routine basis. Only 2×4 ml of organic solvent (mixture of hexane/acetone 95/5 vv) added to the hydrolyzed sample in disposable glass test tube (10 ml) were sufficient to efficiently extract BFRs from serum after agitation for 10 min using a multi-tube vortexer widespread in analytical labs and allowing the simultaneous vortex of 50 tubes. Due to the lipid content of such extract, further purification steps used to be needed. Again the goal was to avoid time and solvent consuming lipid removal steps using for instance GPC or elution on home-made multilayer silica columns or other adsorbents. Thenceforth, the organic layer was evaporated to 500 μl, and loaded on a solid-phase phospholipids removal tube (PHREE). These cartridges, consisting of sorbent with extremely high affinity for phospholipids, were initially intended by the manufacturer to remove proteins and phospholipids directly from plasma samples. Proteins are precipitated with organic solvent on the tube and left above the frits when the sample and precipitating solvents are pulled by centrifugation or vacuum through the sorbent retaining phospholipids and leading to very clean extract. The main advantages of this sample preparation are that no conditioning, washing, nor elution steps are required saving a lot of time and solvent consumption. Moreover, high recoveries were obtained for a wide range of compounds however their acidic, basic or neutral properties without the need for method development. In the present use, the procedure has to be adapted to start from higher sample volumes because maximum of 200 μl were recommended by the manufacturer for 1-ml cartridge. Thus 1 ml serum sample was firstly extracted with organic solvent, slightly evaporated and then loaded on the PHREE tube. An additional elution of 500 μl of hexane/acetone (95/5) was tested to eventually improve the recovery but this second fraction contained only very few amounts of target compounds and thus did not justify this further step. Fig. 2 shows the chromatograms obtained from 1 ml of serum sample extracted by LLE (chromatogram a) and 1 ml of serum sample extracted by LLE with subsequent PHREE cleanup (chromatogram b), both spiked after extraction at 100 pg/ml (1000 pg/ml for BDE-209 and DBDPE). Both chromatograms are strictly superposed, demonstrating the drastic decrease of the baseline and the practically absence of interference when using the small cleanup step. Moreover, the higher extract quality would prevent the GC column phase degradation, and the activation of the inlet liner known to be critical for highly brominated compounds such like BDE-209. The recovery rates gathered in Table 1 were evaluated by comparing serum samples ($N = 3$) spiked (at 100 pg/ml) before and after the extraction procedure and using PCB-198 as surrogate standard. They ranged between 70 and 82% excepted for BDE-209 and EHTBB (with 56 and 55% respectively). Higher initial serum volumes were also tested (from 2 to 5 ml) but resulted in lower quality of extract with significant higher background and too

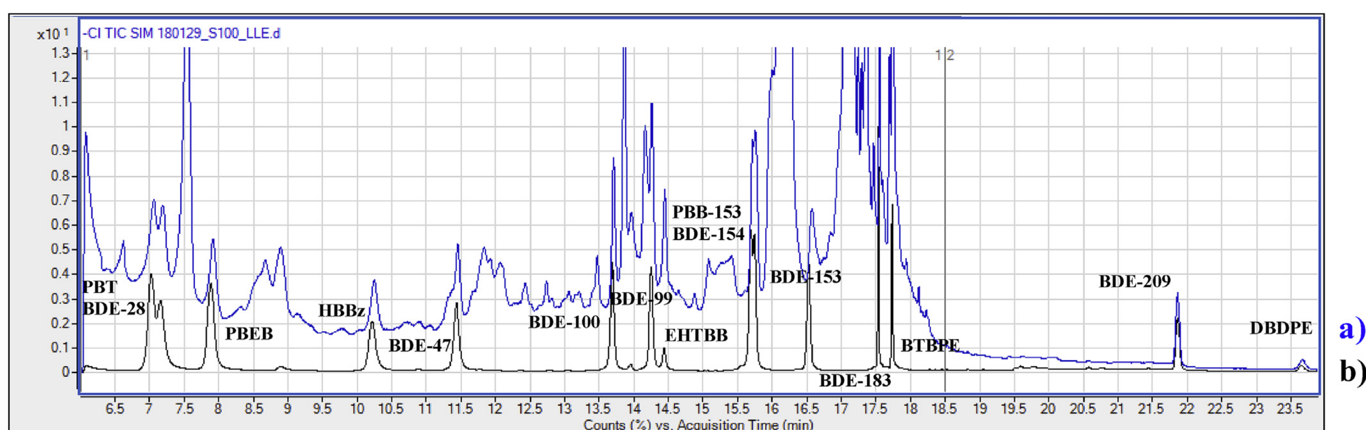

Fig. 2. Chromatograms obtained from 1 ml serum sample extracted by LLE only (a) and extracted by LLE with subsequent PHREE cleanup (b), both spiked after extraction at 100 pg/ml.

Table 1
Recovery percentages (mean and standard deviation) and matrix effects (ME) evaluated on serum samples spiked at 1000 ng/ml for BDE-209 and DBDPE, and 100 ng/ml for all other BFRs (N = 3). The matrix effect was assessed by comparing the area measured in a serum extract spiked after extraction and the corresponding standard solution.

	Recovery (%)		ME (%)
	Mean	SD	
BDE-28	80	7	90
BDE-47	81	7	101
BDE 99	78	9	99
BDE-100	75	8	98
BDE-153	74	11	92
BDE-154	70	12	97
BDE-183	80	14	99
BDE-209	56	19	73
PBT	82	1	104
PBEB	70	6	100
HBBz	77	5	103
EHTBB	55	13	98
PBB-153	81	13	94
BTBPE	76	17	95
DBDPE	82	18	97

many interference.

Thomsen et al. (2001) reported matrix effect occurring when analyzing brominated compounds in biological matrices. They therefore recommended to use matrix-matched calibration curve instead of calibration standard solutions, although initial plasma or serum used for calibration could contain brominated flame retardants and thus also affect the quantification. In GC determination, the matrix effect is related to the residual matrix components remaining in the extract despite the purification steps, enhancing or lowering the transfer from the injector to the column, and/or affecting the analyte behavior in the column or the detector (Hajslova et al., 1998; Yarita et al., 2015). If the addition of isotope-labeled analytes for the quantification could help to provide accurate results although not entirely ensure absence of bias (Yarita et al., 2015), this determination by isotope dilution is not compatible with ECNI mode for brominated compound analysis. Therefore, the quality of extract and consequently the efficiency of the cleanup are substantial to minimize matrix effect and avoid as possible the loss of sensitivity, accuracy and precision. The matrix effect resulting from the present analytical method was assessed by comparing the area measured in a serum extract spiked at 100 pg/ml after extraction and a corresponding standard solution, and are also gathered in Table 1. If a signal slightly lower was obtained for BDE-209, likely due to a little higher background noise and/or bleeding of the GC column, matrix effects seemed to be nearly fully avoided, demonstrating the high efficiency of the fast and simple purification step. Because the quantification relied on internal standards differing from the target analytes (BDE-51, -156, and-181), matrix-matched calibration was nevertheless used to maintain highest accuracy as possible.

3.3. Method validation

The validation parameters determined at each concentration tested are reported in Table 2. Values obtained for concentrations outside the validated range defined as the concentrations between Lower Limit of Quantification (LLOQ) and Upper Limit of Quantification (ULOQ) are grayed and italic written. The concentrations are expressed in pg/ml of serum while the validation parameters are percentages. EHTBB showed very high response variations during the validation process, resulting in poor linearity, trueness and precision, and therefore did not meet fair validation criteria as described below. Since not successfully passing the validation, EHTBB was not included in Table 2.

3.3.1. Calibration curves and linearity

The 7 point calibration curves consisted in fetal bovine serum samples spiked with increasing concentrations of BFR ranging from 2 to 500 pg/ml (20–5000 pg/ml for BDE-209 and DBDPE). The 1/x weighted quadratic regression model was selected as response function for all targets. The linearity was defined as the ability of the method to obtain measured results directly proportional to the concentration of the analyte introduced in the sample within a defined range (Hubert et al., 2007). The coefficient of determination R^2 of the linear regression, fitted on the results as a function of the introduced concentrations, ranged between 0.9684 and 0.9985 depending of the analyte.

3.3.2. Trueness, precision and uncertainty of measurements

The trueness is the relative bias between the value introduced in the sample and thus considered as true, and the mean value really obtained when measured (Hubert et al., 2007). The intra- and inter assay precisions were determined as the relative standard deviation (RSD) within the analyses carried out in triplicate respectively the same day and during 3 different days. The bias for trueness determination in the validated range was lower than 15% however

Table 2
Trueness, intra and inter assay precision, uncertainty, limit of detection (LOD), lower and upper limit of quantification (LLOQ and ULOQ) and correlation coefficient (R^2) obtained for each analyte during the validation process.

	Target conc. (pg/ml)	BDE-28	BDE-47	BDE-99	BDE-100	BDE-153	BDE-154	BDE-183	PBT	PBEB	HBBz	PBB-153	BTBPE	Target conc. (pg/ml)	BDE-209	DBDPE
Trueness	1	–	–	–	–	–	–	–	–	–	–	–	–	10	*40.1*	–
Relative bias (%)	2	*−52.1*	*27.0*	*−69.4*	*10.7*	*−19.5*	*11.3*	*−3.5*	*−66.5*	*0.4*	*0.5*	*−10.9*	–	20	*29.4*	–
	5	*−0.4*	2.4	*2.4*	0.3	5.0	7.9	2.5	−10.0	5.8	4.8	3.3	−22.4	50	3.3	–
	10	−0.5	2.3	−1.0	−1.1	−1.5	2.8	4.2	1.8	1.1	−0.1	3.6	−1.0	100	3.9	−11.9
	200	5.9	−6.3	−5.6	−3.4	11.7	1.3	−2.9	5.7	5.4	0.03	13.6	−11.4	2000	−4.8	−13.2
	500	0.7	−1.8	2.9	−6.9	0.1	1.7	0.0	−2.3	1.5	−1.6	−1.5	−5.2	5000	0.3	−17.1
Intra and inter assay precision																
Repeatability (RSD%)	1	–	–	–	–	–	–	–	–	–	–	–	–	10	*6.4*	–
	2	*3.7*	*35.7*	*18.2*	*6.8*	*13.8*	*2.5*	*12.0*	*31.1*	*13.8*	*71.3*	*13.0*	–	20	*25.4*	–
	5	*20.4*	8.8	*6.9*	5.3	4.7	6.9	3.0	11.0	5.1	5.2	8.6	12.9	50	9.5	–
	10	11.9	10.3	4.8	6.0	10.7	1.8	3.8	7.2	6.1	3.6	9.7	7.9	100	9.5	6.1
	200	5.2	4.6	8.3	4.2	3.5	6.7	3.9	3.5	3.8	6.1	6.6	9.3	2000	2.8	7.3
	500	2.3	3.1	3.1	5.0	6.2	4.4	2.0	1.6	2.8	3.8	2.6	4.0	5000	2.6	6.0
Intermediate precision	1	–	–	–	–	–	–	–	–	–	–	–	–	10	*52.6*	–
(RSD%)	2	*66.5*	*35.7*	*46.3*	*21.7*	*57.7*	*45.4*	*44.2*	*56.3*	*30.6*	*93.6*	*59.3*	–	20	*25.4*	–
	5	*28.8*	8.8	*22.2*	5.3	9.7	21.6	7.3	12.8	7.6	5.2	10.6	18.0	50	12.5	–
	10	12.5	10.3	9.3	6.0	10.7	9.4	5.5	7.2	6.1	3.6	9.7	10.1	100	9.5	6.1
	200	5.2	5.7	9.7	6.1	8.2	12.6	7.1	9.4	8.4	6.1	7.7	9.3	2000	3.5	7.3
	500	2.3	3.1	5.0	13.6	12.4	6.9	3.3	3.0	3.8	3.8	7.8	4.7	5000	2.6	10.9
Uncertainty																
Relative Expanded	1	–	–	–	–	–	–	–	–	–	–	–	–	10	*128.8*	–
Uncert. (%)	2	*153.5*	*78.1*	*105.6*	*49.7*	*132.6*	*111.1*	*101.3*	*126.5*	*69.5*	*205.4*	*136.3*	–	20	*54.9*	–
	5	*63.8*	18.7	*50.9*	11.1	21.9	49.5	16.6	27.6	16.8	10.9	23.1	39.8	50	27.3	–
	10	26.5	21.8	21.0	12.8	22.6	21.6	12.3	15.2	12.9	7.6	20.6	22.2	100	20.1	13.1
	200	11.1	12.5	21.1	13.5	18.6	28.5	16.1	21.4	19.2	12.8	16.7	20.2	2000	7.6	15.6
	500	4.9	6.4	11.2	31.0	28.1	15.4	7.5	6.8	8.3	8.1	17.9	10.2	5000	5.4	24.5
LOD (pg/ml)		1.6	1.1	1.6	0.8	1.3	1.8	1.1	1.4	0.9	1.3	1.3	1.7		10.4	–
LLOQ (pg/ml)		5.4	3.7	5.3	2.5	4.2	6.0	3.8	4.6	3.0	4.3	4.2	5.7		34.5	100.0
ULOQ (pg/ml)		500.0	500.0	500.0	412.7	500.0	500.0	500.0	500.0	500.0	500.0	500.0	500.0		5000.0	3056.0
R^2		0.9984	0.9977	0.9948	0.9798	0.9838	0.9928	0.9979	0.9965	0.9973	0.9974	0.9894	0.9949		0.9985	0.9684

the levels. Similarly, the RSD for the intra and inter precision were maintained below 15% even close to the LOQs. The expanded uncertainty is associated with the dispersion of the values measured that could reasonably be attributed to the measurand, and represents the 95% interval confidence around the results where the unknown real value is expected (Dubois et al., 2012). The relative expanded uncertainty estimated ranges between 4.9 and 28.1% excepted for BDE-154 at 5 pg/ml (49.5%).

3.3.3. Accuracy, limits of detection and quantification

According to the total error approach, the accuracy is defined as the sum of the precision and the trueness, and is estimated from the accuracy profile. This latter is obtained by joining the extreme bounds (lower and upper) of the β-expectation tolerance intervals calculated at each concentration and set at 17.5% level (Dubois et al., 2012; Hubert et al., 2007). The acceptance limits were ±30% and ±50% respectively for concentrations above and below 10 pg/ml except for PBDE-209 (threshold set at 50 pg/ml). The method is considered as valid within the range of concentrations for which the accuracy profile is within the acceptance limits. Therefore the LLOQ and ULOQ defining the validated dosing range are obtained from the intersection of both β-expectation tolerance intervals and acceptance limits. LLOQ provided by this statistical method were estimated at 34.5 and 100 pg/ml for BDE-209 and DBDPE, and ranged between 2.5 and 6 pg/ml for all other BFR. The Limits of Detection (LOD) defined as one third of the LOQ were therefore ranging from 0.8 to 1.8 pg/ml, except for BDE-209 (10.4 pg/ml). LOD was not evaluated for DBDPE because when dividing the LOQ value by 3, the LOD obtained (30.3 pg/ml) seemed to be not realistic, the chromatographic peak observed for concentrations below 100 pg/ml being such spread that difficult to discern.

These limits of detection are close to those obtained with analytical procedure starting from 5 to 10 times higher serum volume (Cariou et al., 2005; Covaci and Voorspoels, 2005; Gao et al., 2016; Sales et al., 2017; Thomsen et al., 2007). This very fair sensitivity is likely due to the high quality of extract obtained with our very simple and fast cleanup, but also due to the increasing performance of analytical instruments, GC columns and other GC consumables, and the very low and controlled background contamination of the lab, justifying all precautions implemented including reduction of solvent and consumable's uses, reduction of handling, and reduction of the non-disposable vessel use.

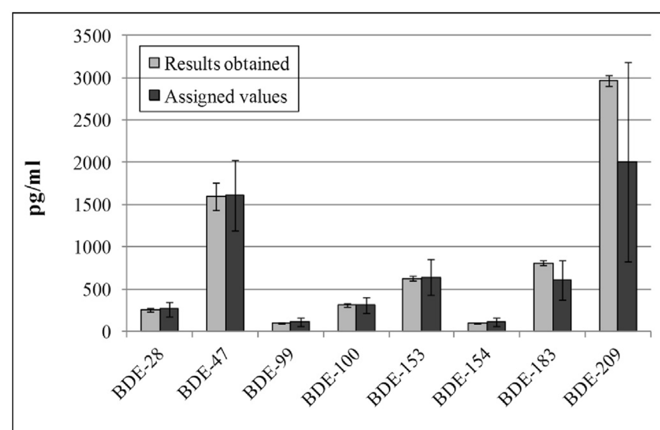

Fig. 3. Comparisons between results obtained for AMAP materials (AM-S-W-1402) analyzed in triplicate (SD as error bars) and assigned values (tolerable range as error bars).

3.4. Analysis of reference materials

Materials from the AMAP interlaboratory ring test for POPs in human serum (AM-S-W-1402, Institut National de Santé Public du Québec, Quebec, Canada) were analyzed in triplicate, and results obtained are shown on Fig. 3. Excellent correlations were observed between results obtained and assigned values except for BDE-209 for which higher levels were measured although still included in the tolerable range. This difference would likely be due to a small degradation of the old native BDE-CM solution initially stored in transparent vials and used to build the calibration curve. Other interlaboratory ring test materials (AM-S-W-1705 and AM-S-W-1706) were analyzed afterwards using a new native solution properly stored, and very fair results were then obtained for BDE-209 (779 vs 1040 pg/ml and 630 vs 606 pg/ml for respectively AM-S-W-1705 and -1706).

4. Conclusion

A simple and fast analytical procedure was developed for the determination of the 8 main legacy PBDEs and 6 NBFRs in human serum. The simple liquid-liquid extraction followed by the elution on PHREE cartridge provide very clean extracts and good recovery rates avoiding usual solvent and time consuming purification steps for lipid removal for instance. The very low matrix effect and interference levels generated by the sample prep, and the "under control" laboratory background contamination allow to start from low sample volumes while achieving usual sensitivity. The GC conditions and the 15 m long column enable the measurement of all targeted BFRs including high brominated species like BDE-209 in a single 26 min's GC run. Results obtained from interlaboratory test materials were very close to the assigned values confirming the high reliability of the analytical procedure. Moreover, this user-friendly method successfully passed a rigorous validation process based on the total error approach, and could be easily implemented in routine laboratories for high throughput analyses for instance within large scale epidemiological studies.

Declarations of interest

None.

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