

SEMI-AUTOMATED HIGH THROUGHPUT EXTRACTION AND CLEANUP METHOD FOR THE ANALYSIS OF POLYBROMINATED DIPHENYL ETHERS, POLYBROMINATED, AND POLYCHLORINATED BIPHENYLS IN HUMAN SERUM

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Introduction

Brominated flame retardants (BFRs) have been and are still heavily used as additive and/or reactive chemicals in polymers and textiles ¹. Only a few of the BFRs have been assessed in human subjects with a major data set on internal exposures to polybrominated diphenyl ethers (PBDEs). Increasing PBDE levels have been observed in mothers' milk from Sweden ² and in whole blood from Germany ³. The levels of PBDEs are, in general, lower than that of PCB levels in biological samples. However, the PBDE concentrations found in North Americans are considerably higher compared to European subjects. This paper reports on the development of a semi automated high throughput method for the analysis of human serum for polychlorinated biphenyls (PCBs) and BFRs, such as PBDEs, and polybrominated biphenyls (PBBs).

Methods and Materials

Certified reference standards: Two internal standard spiking solutions are used for BFRs and PCBs, respectively. The spiking standard for BFRs contained eight ¹³C₁₂-labeled PBDEs (tri- to decaBDE) congeners and ¹³C₁₂-2,2',4,4',5,5'-hexabromobiphenyl (BB-153) at a concentration of 7.5pg/μl in methanol. The spiking standard for PCBs contained thirty-eight ¹³C₁₂-labeled PCB congeners at a concentration of 7.5pg/μl in methanol. The recovery spiking standard contained 2,2',3,4,4',6-hexaBDE (¹³C₁₂-BDE-139; 7.5pg/μl) and 2,2',3,3',4,5,5',6,6'-nonaCB (¹³C₁₂-CB208; 10pg/μl). The solvent for the recovery standard is n-hexane containing 10% and 2% by volume of nonane and dodecane, respectively. A seven-point calibration standard (0.2 to 1000pg/μl) in nonane is used with a ¹³C-labeled internal standard concentration of 75pg/μl.

Chemicals: Reagents and solvents used in the current inventory are of the highest possible grade available and/or intended for pesticide residue analysis and are only used after verification of the batch by HR-MS analysis monitoring for the BFRs listed above. Acid silica (silica/sulfuric acid 2:1 by weight) is prepared in house by adding concentrated sulfuric acid to activated silica gel (activated at 250°C over night). The mixture is then shaken vigorously and mixed by rotation over night prior to use.

Cleaning of glassware and other consumables: All glassware is cleaned in a Steam Scrubber dishwasher (Labconco; Kansas City, MO) and heated in a laboratory oven at 250°C overnight prior to use. Pasteur pipettes are not washed in the dishwasher but heated directly in the same oven

over night. Teflon-lined screw caps as well as Teflon-lined silicone septa are sonicated in methanol and air-dried prior to use.

Serum used for method development: Pooled serum used for development of the analytical method was obtained from Inter State Blood Bank (Memphis, TN). The serum pool contained serum from fifteen blood donors from three U.S. cities (Philadelphia, PA; Memphis, TN and Miami, FL), collected in 2002. Upon arrival the serum was transferred to a -70°C freezer for storage. To minimize freezing and refreezing the serum was aliquoted into 10 and 100mL glass-flasks equipped with Teflon-lined screw caps.

Automated sample pretreatment: Addition of internal standards (100 μl ; 7.5pg/ μl), denaturation of the sample (4mL) using formic acid (4mL), and dilution with water (4mL) and spiking of recovery standard (100 μl ; 10pg/ μl) to empty GC-vials, prior to extraction was automated with a 215 Liquid Handler (Gilson; Middleton, WI) fitted with a 402 syringe pump (Gilson). The syringe pump is equipped with a 250 μl syringe as well as a 10mL syringe. The syringe pump is connected via 10.5mL coiled-transfer tubing to a septum-piercing probe (Gilson). A methanol bottle is connected to the syringe pump and used for purging the transfer tubing and rinsing the septum-piercing probe used for drawing and dispensing standard solutions and reagents. Mixing of samples by rotation is obtained by an automix (Gilson). The above instrumentation is controlled and operated under the UniPoint v5.5 (Gilson) software.

Automated SPE extraction and cleanup: Extraction and cleanup steps are automated using the Rapid Trace[®] SPE workstation (Zymark; Hopkinton, MA). The Rapid Trace[®] is a modular SPE system. The above instrumentation is controlled and operated under the Rapid Trace[®] workstation software v1.20 (Zymark). The samples are extracted unattended by the Rapid Trace[®] SPE workstation. The method used for extraction of serum has previously been reported⁴ and used in the present method with modifications. Custom SPE cartridges packed with Oasis HLB sorbent (540mg; Waters Corp.; Milford, MA) are used. The cartridges are first conditioned with methanol (3mL) then 0.1M hydrochloric acid in 5% aqueous methanol (3mL; rinse solution), then dichloromethane (3mL), and finally with rinse solution (3mL). The sample is then loaded at a flow rate of 0.4mL/minute and eluted into a test tube. An additional 1.5mL of rinse solution is then eluted through the cartridge to wash out any serum residues. The instrument is equipped with three separate rinse ports; however, if serum is passed through these ports they clog-up with time. Eluting the serum into a test tube eliminated this problem. An additional 1.5ml of rinse solution is eluted through the cartridge into the waste port, followed by drying of the cartridge under a constant nitrogen flow (40 minutes at 20 psi). The sample is then eluted with dichloromethane (12mL) into a test tube. Unattended processing of thirty samples for extraction using six modules (5 samples/module) takes approximately 7.5 hours of overnight processing time. The resulting extracts are then concentrated to dryness using co-extracted lipids as a keeper. The extracts are then applied to a two layered cleanup column packed with (i) activated silica on top (0.1g; activated 250°C) and (ii) a silica: sulfuric acid (1g; 2:1 by weight) layer in the bottom of the SPE cartridge; the two layers are separated by a polypropylene frit. The samples and blanks are processed for cleanup in an unattended fashion using the Rapid Trace[®]. The cartridge used for sample cleanup is first conditioned with hexane (10mL). The syringe pump on the Rapid Trace[®] instrument delivers hexane (1mL) to the tube containing the sample extract. Drawing the hexane just delivered to the sample tube back into the syringe pump and back into the test tube ensures a complete reconstitution of the sample. Next, the syringe pump delivers the sample to the cleanup cartridge. This procedure is repeated twice with additional hexane (2x1mL) to ensure a quantitative transfer. Elution

of the sample is accomplished by passing an additional 7mL of hexane through the cartridge; a total volume of 10mL of hexane is collected into a 16x100mm test tube. Simultaneous processing of five samples per module gave a processing time of 2.5 hours (for a set of 30 samples). After evaporation, the extracts are transferred to the GC-vials that were spiked with recovery standard, cf. above, and subsequently evaporated to a 10 μ l volume. The nonane and dodecane in the recovery standard act as a keeper.

Quality Control and Quality Assurance (QA/QC): One set of samples is defined in this method as eight unknown samples, prepared and analyzed together with one analytical blank and one certified reference (QA/QC) sample. Due to the high degree of automation in the current method, three sets are prepared and analyzed at the same time as one batch. The measurement of a target analyte in a set of samples is considered valid only after the QA/QC sample has fulfilled the following criteria: (i) the measurement of the target analyte in the QA/QC sample must fall within the interval defined as +/- three standard deviations of the mean of QA/QC samples analyzed, and (ii) seven or more consecutive measurements of the QA/QC sample cannot fall above or below the mean of QA/QC samples analyzed. Further, every measurement of the set of samples must fulfill the following criteria to be considered valid: (i) the ratio of the two ions monitored for every analyte and ¹³C-labeled internal standard must be within 20% of the theoretical value, (ii) the ratio of the retention time of the analyte compared to its ¹³C-labeled internal standard must be within the range 0.99 – 1.01, and (iii) measured recovery of the IS must be within the range 25-150%.

High Resolution Mass Spectrometry (HR-MS): HR-MS analysis was performed on a MAT95 (Finnigan; Bremen, Germany) instrument. The chromatographic separations were carried out on an Agilent 6890N gas chromatograph (GC) in the split-less mode, fitted with a DB5HT [(15m, 0.25mm I.D. and 0.10 μ m film thickness); Agilent, Atlanta, GA] capillary column.

Evaluation of spiking reproducibility and accuracy of liquid handler: The reproducibility of the liquid handler was determined by spiking ¹³C₁₂-BDE-28 (100 μ l; 200pg/ μ l) along with an exact amount of BDE-28 (1mL; 40pg/ μ l) standard, as determined by an analytical balance. A total of ten replicates were made and analyzed by HR-MS. The area ratio of ¹³C₁₂-BDE-28 over BDE-28 (adjusted for any deviations in amount of native BDE-28 added as determined by the analytical balance) was used to estimate the reproducibility of the liquid handler as a relative standard deviation (RSD). Precision of the liquid handler was estimated by comparing the average of the 10 samples generated by the liquid handler, cf. above, with 10 samples containing ¹³C₁₂-BDE-28 (100 μ l; 200pg/ μ l) and BDE-28 (1mL; 40pg/ μ l), as determined by an analytical balance.

Results and Discussion

The current methodology allows the processing of up to 30 samples (incl. 3 analytical blanks and 3 QA/QC samples) per person per day due to a high degree of automatization. A minimal use of solvents has been achieved which is not only environmentally friendly and decreases the overall cost per sample, but also decreases analytical background originating from the concentration of contaminants during evaporation. Analytical background is a major concern in trace level analysis of BFRs since the contaminants are ubiquitous in the indoor environment⁵ due to current use. The total solvent volume currently used per sample (excluding purging of solvent lines) for extraction and cleanup is 22mL. The background is further reduced by the application of septum-equipped test tubes which further reduces contamination during spiking of internal standards and denaturation with formic acid. An analytical background of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) in blank samples of 20-40pg/sample has been obtained. This background is expected to

be reduced further with the addition of a clean room to be constructed and implemented for these analyses during 2003.

The 215-liquid handler (Gilson) used for spiking internal standards was found to have a reproducibility of 2%-RSD and a precision of 4% as compared with an analytical balance, cf. above. The reproducibility and precision is well within what may be considered as normal variability of the GC-MS system used.

Replicate analyses of a serum pool collected in three U.S. cities in 2002 from fifteen blood donors gave a reproducibility of 2, 4 and 3%-RSD for BDE-47, 2,2',4,4',5-pentaBDE (BDE-99) and 2,2',4,4',6-pentaBDE (BDE-100), respectively (Figure 1).

The linearity of the response of BFRs in serum samples were further verified by spiking calibration standard (10 μ l) directly into extracted and cleaned-up serum samples. Three replicates were made for every point in a 7-point calibration curve covering the concentration range 0.2 to 1000 pg/ μ l (100pg/ μ l of ¹³C₁₂-labeled IS). All spiked samples were analyzed together with the calibration curve containing no sample matrix. The slope and intercept for the matrix spiked calibration curve was in good agreement to the calibration curve containing no matrix (data not shown), illustrating that no detectable matrix effect exists. It must however be emphasized that the use of a two layered cleanup column containing activated silica (0.1g) and silica: sulfuric acid (2:1 by weight;1g) is of utmost importance. Removal of the activated silica results in the break through of what has been tentatively identified by time-of-flight (TOF) MS as cholestene. Cholestene is an elimination product of cholesterol present in serum and is believed to be a byproduct of passing through the silica/sulfuric acid. The top layer of activated silica in the cleanup cartridge removes any co-extracted polar compounds prior to them reaching the sulfuric acid layer; thereby eliminating any interferences originating from co-extracted cholesterol.

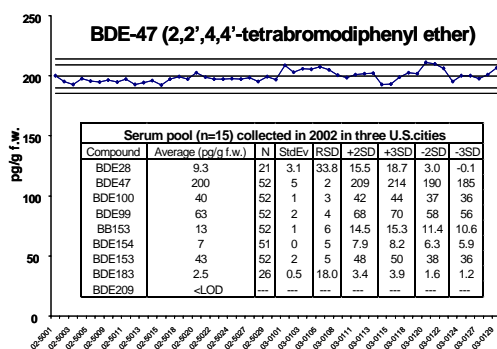


Figure 1. Quality assurance / quality control (QA/QC) chart for 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) (n=52) and in table format QA/QC data for eight polybrominated diphenyl ethers and 2,2',4,4',5,5'-hexabromodiphenyl (BB-153). Data expressed on a fresh weight (f.w.) basis.

References

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