



## Laboratory Procedure Manual

*Analyte:* **Polybrominated diphenyl ethers (PBDEs), Polybrominated Biphenyls (PBBs), Polychlorinated biphenyls and Persistent Pesticides (PPs)**

*Matrix:* **Serum**

*Method:* **Isotope dilution High resolution Mass Spectrometry (IDHR-MS)**

*Method No:*

*Revised:* **August, 2007**

*as performed by:*

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### **Important Information for Users**

CDC periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

This document details the Lab Protocol for NHANES 2003–2004 data.

A tabular list of the released analytes follows:

<b>Lab Number</b>	<b>Analyte</b>	<b>SAS Label</b>
I28PBE_c	LBXBB1	2,2',4,4',5,5'-hexabromobiphenyl
	LBXBR1	2,2',4-tribromodiphenyl ether
	LBXBR2	2,4,4'-tribromodiphenyl ether
	LBXBR3	2,2',4,4'-tetrabromodiphenyl ether
	LBXBR4	2,2',3,4,4'-pentabromodiphenyl ether
	LBXBR5	2,2',4,4',5-pentabromodiphenyl ether
	LBXBR6	2,2',4,4',6-pentabromodiphenyl ether
	LBXBR7	2,2',4,4',5,5'-hexabromodiphenyl ether
	LBXBR8	2,2',4,4',5,6'-hexabromodiphenyl ether
	LBXBR9	2,2',3,4,4',5',6-heptabromodiphenyl
	LBXBR66	2,3',4,4'-tetrabromodiphenyl ether

## **1. Clinical Relevance and Summary of Test Principle**

### Clinical Relevance

The list of organohalogen compounds present in the environment is long today, including chemicals such as toxaphene, polychlorinated paraffins (CPs), polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), polychlorinated naphthalenes (PCNs), bis(4-chlorophenyl) sulfone (BCPS) and numerous other pesticides and technically applied substances

Polybrominated diphenyl ethers (PBDEs), included in the group of chemicals known as Brominated Flame Retardants (BFRs), have been and are still heavily used as additive chemicals in polymers and textiles [1;2]. The PBDEs are dominated by 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) [3;5;6;7]. Decabromodiphenyl ether (BDE-209) is reported both in the general population and in occupationally exposed persons showing the bioavailability of this high molecular weight compound [11;18;19]; while the lower and medium brominated diphenyl ethers are persistent Polybrominated biphenyls (PBBs) are another type of chemicals that in the past has been used and applied for similar application areas as PBDEs [1;8]. No known commercial production of PBBs currently exists. HexaBB has in humans been shown to have a half-life of approximately 30 years [9].

### Test Principle

The method described in this manual assesses human body burden of BFRs, specifically PBDEs and PBBs, as well as polychlorinated biphenyls (PCBs) and persistent pesticides (PPs) in serum and/or plasma. This is done by measuring the concentration in serum/plasma through the use of solid-phase extraction (SPE) and subsequent sample clean-up. Final determination of target analytes is performed by isotope dilution gas chromatography high-resolution mass spectrometry GC/IDHRMS.

Concentrations of target analytes are reported on two different bases, i.e., (i) fresh weight basis (i.e., pg/g serum) and (ii) lipid weight basis (i.e., ng/g lipid).

Lipid adjusted concentration values are preferable because (i) organohalogen compounds are lipophilic and hence distribute in the body mainly according to the tissues lipid content. Lipid adjusted concentrations correlates with the adipose tissue concentrations of the chemical. Normalization according to lipid content further reduces variability since differences in individuals serum lipid concentrations are cancelled out.

The samples are extracted using SPE, employing an automated SPE workstation (Rapid Trace®, Caliper Life Sciences). Required sample pretreatment prior to extraction is performed on the Gilson 215 liquid handler, including automated addition of (i) internal standards, (ii) formic acid (denaturant) and (iii) water (diluent) with mixing in-between each addition by rotation. Formic acid is added to denature proteins in the

sample enabling extraction of target compounds. During the extraction step the target analytes are transferred from a water medium to an organic solvent.

Sample cleanup, i.e., removal of co-extracted lipids, is obtained by elution (hexane; 8 mL) of the extract through a column containing from the top 0.1 g of silica and 1 g of silica/sulfuric acid (33% by weight). Serum lipids are during this procedure degraded in the sulfuric acid layer while cholesterol is removed in the top layer consisting of activated silica gel. Without the activated silica gel layer cholesterol would eliminate water forming cholestene when coming in contact with the sulfuric acid. Cholestene would not be removed in the silica gel/sulfuric acid layer and interfere in the final HR-MS analyses, i.e., causing an ion suppression in the region of 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) and 2,2',4,4',6-pentabromodiphenyl ether (BDE-100).

This sample cleanup is automated using the Rapid Trace® (Caliper Life Sciences) also used for sample extraction. The samples are evaporated and transferred to GC vials. Evaporization is performed on the RapidVap® (LabConco), using vacuum, increased temperature and vortex action to aid the evaporization.

Serum concentrations are determined using gas chromatography isotope dilution high resolution mass spectrometry (GC/IDHRMS), which minimizes or eliminates much interference associated with low-resolution measurement of organohalogen compounds. Splitless injection is used employing a short GC column (DB-5HT; 15 m length, 0.1 µm film thickness, 0.25 mm ID) enabling the determination of high molecular weight compounds such as decabromodiphenyl ether (BDE-209) having a molecular weight close to 1000 amu. Electron impact ionization (EI) is used. The two most abundant ions in the isotopic cluster (fragment or molecular ion) are monitored for the target analyte as well as for the <sup>13</sup>C-labeled internal-surrogate standard. Quantification is made against a calibration curve covering the full concentration range of the target analytes. Serum PCB/PP concentration is also determined using GC/IDHRMS but using a longer column (DB-5MS, 30 m length, 0.25 µm film thickness, 0.25 mm ID).

## **2. Safety Precautions**

### Biohazards

Follow Universal Precautions. Wear appropriate gloves, lab coat, and protective eye glasses while handling human serum. Serum may be contaminated with pathogens such as hepatitis or HIV; hence all safety precautions must be followed as outlined in the laboratory hazardous chemicals exposure plan. Wear gloves, lab coat and glasses at all times, and conduct all work in fume hood or biological safety cabinets (BSCs).

Place disposable plastic, glass, and paper (e.g., pipette tips, autosampler tubes, and gloves) that come in contact with serum in a biohazard autoclave bag. Keep these

## Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum NHANES 2003-2004

bags in appropriate containers until they are sealed and autoclaved. When work is finished, wipe down all work surfaces where serum was handled with a 10% (v/v) sodium hypochlorite solution or equivalent.

**After an accident the CDC/ATSDR Incident Report must be filed according to hazardous exposure control plan by supervisor.**

### Chemical hazards

**Acids and Bases:** Exercise caution when handling and dispensing concentrated sulfuric acid, formic acid and nitric acid. Always remember to add acid to water. Acids and bases are capable of causing severe eye and skin damage. Wear powder-free gloves, a lab coat and safety glasses. If acids or bases come in contact with any part of the body, quickly wash the exposed area with copious quantities of water for at least 15 minutes. Use safety shower if exposed area is not limited to hands and/or arms. Use eye wash station in the event of eye exposure to acids and/or bases. In the event of an accident, lab colleagues will contact the clinic by phone or emergency medical response by dialing 9-911.

Formic acid used in the extraction step is an organic acid. A common misperception is that “weak organic acids” are less damaging than “strong inorganic acid”. This is not correct since organic acids can efficiently penetrate the skin. In the event of skin contact with this chemical every effort needs to be made to rinse the acid off. Wash the exposed area with water for at least 15 minutes according to above.

**Solvents:** Solvents may penetrate skin causing long-term adverse health effects. Exercise caution and always use gloves when handling solvents and other chemicals. In the event of spill on gloves immediately change to a new glove since solvents do penetrate many gloves with time.

**After an accident the CDC/ATSDR Incident Report must be filed according to hazardous exposure control plan by supervisor.**

### Hazardous waste handling

**Solvent waste:** Collect solvent waste in waste bottles (empty solvent bottles may be used). Clearly write **WASTE** on bottles, and list the solvent(s) the waste bottle contains. If possible, always keep different solvents separated in different waste bottles, since this will make the final disposal of the different solvent wastes easier. When a bottle is filled, arrange for waste pickup according the Chemical Hygiene Plan.

**Serum waste:** Dispose of serum waste originating as a waste fraction in the extraction step on the Rapid Traces (Caliper Life Sciences) by completing the forms as outlined by Chemical Hygiene Plan. Also attach a Memorandum stating that the

contents of the bottle are a mixture of formic acid, water, and serum that is considered to be biologically inactivated by the acid present.

**Solid wastes:** Sort solid waste in three fractions and placed in metal boxes with lid according to below and Chemical Hygiene Plan:

- **Non-Biogenic Contaminated Reusable Glassware** (e.g. beakers, cylinders and other reusable glassware). When the container is filled, label and return to Glassware Services according to CDC protocol.
- **Broken glass** includes used Pasteur pipets contaminated with biogenic materials, or serum bottles and vials that are not reused. When this container is filled (i) add approximately 1 L water to container, (ii) place sticker with your name, room and building number on container, (iii) place autoclave tape over lid and down the side of the box and (iv) bring the container to autoclave located in the loading dock, building 103.
- **Gloves and other plastic parts contaminated with biogenic material** - Place biohazard bag in metal container before placing any waste in container. When container is filled (i) add approximately 1 L water to container, (ii) place sticker with your name, room and building number on container, (iii) place autoclave tape over lid and down the side of the box, (iv) place autoclave sticker on container and (v) bring the container to DLS designated handling area.

### **3. Computerization; Data System Management**

#### Data Entry and Transfer

Sample analysis results generated by this method are stored in Microsoft Excel™ software. The analytical results should include at least the analysis date; analytical run number, quality-control (QC) results for the run, results of specimen analysis by specimen identification (ID), and method identifier.

#### Routine Computer Hard-Drive Maintenance

Defragment the computer hard drive regularly by using software such as Norton Utilities™ to maximize computer performance and maintain data integrity for files on the hard drive.

#### Data Backup and Schedule of Back-ups

**GC/IDHRMS:** Monthly full-data back-up onto recordable CD or DVD.

#### **4. Procedures for Collecting, Storage and Handling of Specimens; Criteria for Specimen Rejection**

- No special instructions for fasting or special diets are required, although, preferably the sample has been drawn in the morning before breakfast (i.e. fasting).
- The specimen type is serum or plasma.
- Optimal amount of specimen is 4.0 mL; minimum is about 0.5 mL.
- Acceptable containers for storage are thick-walled glass vials with Teflon™-lined caps. Rinse containers using the same procedure as for other glassware used in the current method (see section 6.A). Preferred container is a 10 mL Wheaton glass serum vial.
- The criteria for an unacceptable specimen are either a low volume (< 0.5 mL) or suspected contamination due to improper collection procedures or collection devices. In all such cases, request a second serum specimen. Contamination of specimen could occur from contact with indoor dust from improper handling. If a specimen is determined to have a concentration ratio of 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) over 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) that is greater than 1.0, a replacement specimen should be requested if available for reanalysis. In case a replacement specimen is not available, the recipient of the analytical data will be informed that this particular sample may have been contaminated during sample collection.
- Transport and ship frozen serum specimens on dry ice. Upon receipt, they must be kept frozen at  $\leq -60$  °C until time for analysis. Refreeze at  $\leq -60$  °C any portions of the sample that remain after analytical aliquots are withdrawn. Samples thawed and refrozen several times are not compromised.

#### **5. Procedures for Microscopic Examinations; Criteria for Rejecting Inadequately Prepared Slides**

Not Applicable

#### **6. Preparation of Reagents, Calibration Materials, Control Materials, and all Other Materials; Equipments and Instrumentation**

##### **A. Reagents and consumables**

The method has been validated using the chemicals, solvents and expendables listed in Table 1 and 2. Other manufacturer's products of equivalent purity can be used after verification of chemicals purity.

**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

**Table 1.** Solvents and chemicals used for development of current methodology, equivalent products from other manufacturer may be used with exception to the SPE sorbent.

<b>Chemical/Solvent</b>	<b>Manufacturer</b>	<b>Grade</b>
<b>Acids</b>		
Formic acid	EM Science	ACS, 88%
Hydrochloric acid	Aldrich	37%
Sulfuric acid	Aldrich	99.999%
<b>Solvents</b>		
Dichloromethane	TEDIA	Pesticide
Dodecane	EM Science	min 99%
Hexane	TEDIA	Pesticide
Methanol	TEDIA	Pesticide
n-Nonane	Sigma	99%
Water	TEDIA	Pesticide
<b>SPE sorbents</b>		
OASIS HLB®	Waters	n/a
Silica gel	Sigma	63-230 µm

**Table 2.** Expendables used for development of current methodology, equivalent products from other manufacturer may be used.

<b>Item</b>	<b>Manufacturer/Source</b>
<b>Glassware and caps</b>	
Test tube 16 x 100 mm	Fisher Scientific
Septum for test tube	Fisher Scientific
Open top cap for test tube	Fisher Scientific
Pasteur pipet	CDC Glassware
Boston Round (amber glass bottle)	Fisher Scientific
V-vial (3 mL) with septum-cap	Fisher Scientific
GC vials and caps	Fisher Scientific
<b>Others</b>	
Label printer (Brady TLS PCLink)	Fisher Scientific
Magnetic stirrer (heavy duty, large)	Fisher Scientific
Pipet dispenser	VWR

**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

Preparation of rinse solution used at extraction

The rinse solution is eluted through the SPE cartridge, after the serum, during the extraction step and contains 0.1M hydrochloric acid and 5% methanol by volume. This solution is prepared by adding specified amounts, to an amber 4 L solvent bottle according to below.

**Table 3.** Preparation of 0.1M hydrochloric acid in 5% (v/v) methanol solution.

<b>Volume to prepare (mL)</b>	<b>Water (mL)</b>	<b>Hydrochloric acid (mL)</b>	<b>Methanol (mL)</b>
500	470	5	25
1000	940	10	50
2000	1880	20	100
3000	2820	30	150
4000	3760	40	200

Rinsing of Expendables Prior to Use

PBDEs and other brominated flame retardants are common indoor pollutants. Clean all glassware including new glassware according to following procedure to eliminate risk of sample contamination.

**Culture tubes and other glassware:** Rinse glassware first in dishwasher (Labconco, Steam Scrubber). Place test tubes in racks and insert them in the dishwasher. Place detergent in reservoir in the door, and start the dishwasher using program “**Scientific**” with the “**steam option checked**”.

After completion of the program, transfer the glassware to the oven located next to the dishwasher. After a heat cycle of at least 12 hours at >200 °C, the glassware is ready to be used.

**Caps and septums:** Rinse caps and septums for test tubes prior to use to remove contaminants. This is done by Soxhlet extraction for five hours using methanol as the extraction solvent. Alternatively, if the Soxhlet apparatus can not be used it is also acceptable to sonicate the items in methanol (20 min x 3 times). After cleaning the items, allow them to dry on aluminum foil. After the caps are completely dry, place them in a large glass beaker or in plastic resealable bags (not in cardboard boxes) for safe storage until used.

**Gas Chromatography Vials:** Heat GC vials in an oven at >200 °C overnight prior to use. Store vials in a beaker covered with aluminium foil. The caps for GC vials are cleaned by Soxhlet extraction, using the same procedure as for caps and septums.

## Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum NHANES 2003-2004

**Pasteur Pipets:** Place glass Pasteur pipets in oven on aluminum foil and heat the oven to  $>200\text{ }^{\circ}\text{C}$  overnight. After completing the heating cycle for at least 12 hours, the pipets are ready to be used.

### Internal standards (IS)

The current method is validated for BFRs, PCBs, and acid stable PP. Use three internal standard spiking solutions for quantification of the three compound classes included. Order these standards pre-made from Cambridge Isotope Laboratory (CIL). The PBDE standard contains  $7.5\text{ pg}/\mu\text{L}$  of 10 different  $^{13}\text{C}_{12}$ -labeled PBDE and PBB congeners. The PCB standard contains  $7.5\text{ pg}/\mu\text{L}$  of 21 different PCB congeners and the PP standard contains 11  $^{13}\text{C}$ -labeled PPs. CIL supplies the spiking standard, in 10-mL ampoules.

When opening a new ampoule transfer the standard to a Wheaton 3-mL vial. Label the vial with "BFR IS", "PCB IS" or "PP IS" using a computer-generated label. Note the weight of the container, and the date the ampoule was opened. (The weight is used to detect any potential evaporation of the standard during storage) One vial of each standard is consumed in each analytical run on the automated liquid handler. (See 8.3)

File the certificate of analysis from CIL for each internal standard solution in the SOP binder located in building 103, room 2401.

### Recovery standard (RS)

Use one recovery standard for measurement of recovery. This standard contains  $1234\text{-}^{13}\text{C}_6\text{-TCDD}$  ( $2.5\text{ pg}/\mu\text{L}$ ),  $^{13}\text{C}_{12}\text{-CB-208}$  ( $10.0\text{pg}/\mu\text{L}$ ) and  $^{13}\text{C}_{12}\text{-BDE-139}$  ( $10.0\text{pg}/\mu\text{L}$ ) in hexane containing 10% nonane and 2% dodecane by volume. Add the standard ( $100\mu\text{L}$ ) to the GC vial during initial liquid handling. Transfer and mix the final extracted and purified sample with the recovery standard at the end of the procedure. Nonane and dodecane is present in the standard to act as a "keeper" (solvent that will not evaporate or evaporate to a lesser degree during subsequent evaporation steps) to reduce evaporation losses during the final evaporation step. (This recovery standard is ordered pre-made from CIL)

When opening a new ampoule the standard is transferred to a Wheaton 3-mL vial, and the vial is labeled using a computer-generated label. The weight of the container is noted as well as the date the ampoule was opened. The weight is used to detect any potential evaporation of the standard during storage. One vial of recovery standard is consumed in each analytical run on the automated liquid handler. See 8.C.

File the certificate of analysis from CIL for the recovery standard solution in the SOP binder located in building 103, room 2401.

## GC/IDHRMS Calibration Standard (CS)

The calibration standards includes several calibration levels denoted CSX (X=1 through 10). This standard is prepared by CIL and delivered in amber ampoules.

When opening a new ampoule, aliquot the standard into 3 GC vials. Label the vials BFR/PCB CS1 through CS10 using a computer-generated label. Replace the standards used for calibration of the MAT95 at least every two months. Hence, one set of standards aliquoted into 3 GC vials will last at the most for 6 months, and are then replaced with new standards aliquoted from ampoules.

## B. Instrumentation

- 1. Gilson 215 liquid handler:** Liquid handling is automated using the Gilson 215 Liquid handler, 1. Place the samples in the auto-mix to the far right. The probe (moving arm) picks up and dispenses reagents (internal standards, formic acid and water) to the samples according to a predefined sequence with mixing in-between each type of addition.

Recovery of the internal standards, as a percentage, is an important quality measurement of the analytical run. In order to enable recovery measurements, in this automated procedure, recovery standard will be added to empty GC vials located in a rack at the far left These GC vials will be stored capped until the last step of the sample preparation method in which the purified extract will be transferred to the GC vials and mixed with the recovery standard.

- 2. Rapid Trace®, SPE work station:** The Rapid Trace® SPE workstation (Caliper Life Sciences) includes (A) syringe pump for drawing and dispensing solvents and sample (B) mixing chamber (not used in this method), (C) plunger, compressing SPE cartage and dispensing liquids through cartridge, (D) cannula used for drawing serum sample from test tube and (E) rack containing serum samples and collected fractions. The Rapid Trace® instrument processes the samples in sequence. Up to 5 samples can be loaded in any one module for unattended extraction, and 10 samples per module for unattended cleanup. Six modules are used for the default batch size of 30 samples, resulting in simultaneous processing of six samples at any one time.

### **Instrumental operation and steps in automated extraction method**

Load serum samples into positions 1, 3, 5, 7 and 9 on the right-hand side of the rack. Load collection tubes into positions 2, 4, 6, 8, and 10 on the left-hand side. Load tubes for collecting serum waste into position 1, 3, 5, 7 and 9 on the left-hand side of the rack. Load SPE cartridges containing OASIS HLB sorbent (540 mg, Waters) into the cartridge carousel at positions 1, 3, 5, 7 and 9.

## Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum NHANES 2003-2004

Process the first sample run on each module with a method that includes purge steps (Method: EXT1ONLY.spe) (see below for instructions on setting-up the software), including a complete purge of all solvent lines as well as the extraction of the sample. Purge the system sequentially by drawing 6 mL of each: water, rinse solution (0.1M hydrochloric acid in 5% methanol), methanol, dichloromethane and methanol and rinse solution. Dispensing these liquids through the cannula seated in the rinse port. (After this purge, no air remains in the instruments solvent lines) The method used for remaining samples (EXT3TO9.SPE) is identical to EXT1ONLY.spe except for the initial purge steps included only in the method EXT1ONLY.spe.

The extraction includes the following steps:

- Rinse the SPE cartridge with 3 mL each of methanol, dichloromethane, methanol and rinse solution at a flow rate of 3 mL/min.
- There after, draw the serum from the test tube through the cannula into the syringe pump and dispense through the plunger and the SPE cartridge into the serum waste tube on the left hand side adjacent to the original serum tube.
- Rinse the SPE cartridge first with 1.5 mL of the rinse solution into the same waste tube located in the rack. After this initial rinse removing almost all serum residues from the SPE cartridge, send an additional 1.5mL of rinse solution through the cartridge into the biological waste line. (This procedure is used to avoid the biological waste line clogging-up with time. In the current method, only clean water is passed through this waste line)
- Initiate the drying step with the SPE cartridge still pressed into the biological waste port, forcing compressed nitrogen (40 psi  $\pm$  10) through the cartridge.
- Elute the sample into the collection tube located behind the serum waste tube with dichloromethane.
- Processing of the next sample in the sequence is then automatically initiated.

### C. Procedures for preparing quality control materials

The QC material for this assay is bovine serum in which the concentration of the target analytes has been certified. One QC sample is analyzed in every set of 10 samples to ensure comparability and reliability between different sets of samples over time. In addition to the QC sample, a bovine blank is analyzed in every set of 10 samples. The method is designed to include several sets of 10 samples to be analyzed in parallel in one batch. (See Sample preparation below).

Specific predefined rules are applied in order to determine if the QC sample analyzed in one set is in agreement with previously analyzed QC samples. If the QC sample is found to be an outlier that set has to be reanalyzed. These rules are:

- (i) The QC determination must not deviate more than 3 times the standard deviation from the mean value of previous determinations of the same QC pool, and

**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

- (ii) No more than ten consecutive QC samples may fall either above or below the mean value of previous determinations of the same pool. If the QC sample fails any of these tests the set of unknown study samples must be reanalyzed.

For further details, see data handling section below.

**Day 1:** Rinse the vials (including caps in which the serum will be aliquoted) according to the procedure outlined in glassware rinsing procedures before use (see section 6.A.2. Label the vials with computer-generated labels.

This label should contain a unique name, constructed from the page number in the pool note book. For example SERUM: 02:03 where 02 is the notebook number and 03 is the page number. State the date of the pool preparation on the label.

Thaw the serum by submerging the container in water (37 °C) until the serum is completely unfrozen. Pour the serum into a large beaker (4 L) containing a heavy-duty stir bar (45-mm length). Spike with native analytes to appropriate concentration level, e.g., 500 pg/mL, and stir solution overnight using a magnetic stirrer.

**Day 2:** While still stirring the solution, transfer serum in 6.0 mL aliquots to each of the vials. Cap the vials and place them in cardboard boxes (e.g., a lid for Xerox paper boxes) for simple freezer shelf organization. Place one identifying label on the edge of the cardboard box and place in freezer (-70 °C).

## 7. Calibration and Calibration Verification

### A. Calibration of Mass Spectrometer

Calibrate and tune the Finnigan MAT95XL mass spectrometer using high boiling PFK (perfluorokerosine) according to the instructions in the operator's manual. After tuning the instrument to 10,000 resolution, a greater than 3:1 signal to noise ratio is required for a manually injected CS1 standard (0.2pg/uL).

### B. Creation of Calibration Curve

A linear calibration curve, consisting of six CS standards with concentrations ranging from 0.5 to 1000 pg/ $\mu$ L, is generated using the ratio of the peak area of the analyte to the labeled internal standard.

The R-squared value of the curve must be equal or greater than 0.990. Linearity of the standard curve must extend over the entire standard range.

The lowest point in the calibration curve is the lowest reportable level and the highest point is highest reportable value. The remainder of the points is equally distributed between the two extreme concentrations.

Generate a new calibration curve with every new set of samples to be analyzed, using the certified calibration standards from CIL. Before using a new batch of standards with the current method, verify that the new standards agree with in 20% of the old standard, this is accomplished by quantifying the new standard using the old standard. The certified value (pg/μl) of the new standard must be within 20% of the in-house quantified value (pg/μL). The tolerance of 20% between new and older standard is derived from the certificate of analysis giving a 10% tolerance of each standard released by CIL. Due to the fact that the response ratio between a native and <sup>13</sup>C-labeled internal standard is measured, a maximum deviation of 20% is used. This is accomplished by quantifying the new standard using the old standard. The certified value of the new must be within 20% of the in-house quantified value.

#### B. Calibration Verification

Perform calibration verification is performed once every 6 months. During this procedure, the current calibration standards will be analyzed by the HR-MS operator followed by, at a minimum, 3 calibration standards from a different lot (identity of each standard is unknown to the operator). The additional calibration standards are then quantified using the current calibration standards. The quantified result (pg/μL) has to fall within 20% of the by CIL certified value.

### 8. Procedure Operation Instructions; Calculations; Interpretation of Results

Formal training in the use of a high resolution mass spectrometer is necessary for all GC/HRMS operators. Users are required to read the operation manuals and must demonstrate safe techniques in performing the method. New operators must be evaluated after 6 months of initial training by the supervisor to certify that they are appropriately qualified to perform the assay.

Anyone involved in sample preparation must be trained in for all sample preparation equipment, chemical handling, and have basic chemistry laboratory skills. The training may be delegated to more experienced analyst.

#### A. Sending aliquot of serum for lipid determination

Serum lipid concentration in serum is determined in an aliquot of the sample (250 μl) using enzymatic methods by the Clinical Chemistry Branch (CCB). Aliquot 250 μl of each sample into polypropylene vials after mixing the thawed serum samples; use a new pipette tip for every sample to avoid cross contamination. Label vials for lipid weight determination with Study name, Study Number and notebook number. An lipid aliquot may have been drawn upon arrival of the samples to CDC and prior to the samples being sent to the CPPBL laboratory in which case no lipid aliquot needs to drawn prior to analysis

B. Thawing and weighing samples

Store samples in  $-70^{\circ}\text{C}$  freezer awaiting analysis. Samples are taken out from the freezer to thaw completely; this can be done the day before analysis and the samples placed in a refrigerator over night. Thoroughly mix the samples by vortex. For each batch of 30 samples, complete a run sheet. On the run sheet, enter ALL requested information under heading "Contact Information", e.g., analyst's name or initials, the date and run number.

Print four complete sets of labels for the samples to be used during the cleanup procedure.

To ensure optimum performance of the balance (Ohaus Adventure) used for weighing serum samples, verify the balance calibration using NIST calibration weights (1.000 g and 10.000 g) before weighing each batch of samples. Calibration weights are placed on the balance after taring, and the reading is recorded on the run sheet. The difference from true value may not exceed  $\pm 0.01$  g. If this limit is exceeded, any problems must be resolved, such as cleaning the balance tray, recalibration of balance and/or calling for service of balance. After verifying the balance calibration, weigh serum samples into 16 x 100 mm test tubes with septum-equipped open-top screw caps. Record all sample weights on the run sheet.

C. Sample pretreatment, using Gilson 215 - Liquid handler

1. Procedure

- a. Place new internal standards vials in the rack containing the internal standards on the Gilson 215.
- b. Place the serum samples (weighed into 16x100mm test tubes fitted with septum-equipped screw caps) in the auto-mix. Mark the locations for the tubes in the auto-mix. Place the lid for the auto-mix on the auto-mix. Make certain that all locks in the four corners of the lid snapped into place. **(Failure to secure lid properly will adversely affect the function of the auto-mix)**
- c. Refill formic acid and water containers in rack (Boston-round flasks).
- d. Place GC vials labeled with run number in rack for GC vials according to labeling on rack, cf. section 9.C.3.
- e. Go to the application screen in the 735 Software (Gilson), select application "Complete 30 sample run including references" and press the green play bottom.
- f. During the procedure all samples are spiked with the internal surrogate standards (approximately 20 minutes). After completion of this part of the sequence, the samples will be mixed and fortified with formic acid and water with mixing in-between each addition. During this time, prepare the Rapid Traces, Caliper Life Sciences for extraction, cf. section 9.E.

D. Solid Phase Extraction, using Caliper Life Sciences, Rapid Trace SPE workstation

The extraction procedure is automated using the Rapid Trace® modular SPE system, cf. section 6.

The software controlling the workstation is initiated by the Rapid Trace Development icon located on the desktop. After launching the software, the main menu is displayed. For setting up the software for extraction, click on "Setup Racks", the menu is displayed. Select the modules to be used in lower left corner in this menu, and transfer method "EXT1ONLY.spe" to position "one" and transfer method "EXT3to9.spe" to positions 3, 5, 7 and 9. Exit this menu by pressing "OK". Enter the "Run Monitor Menu", and launch the modules to be used for extraction.

**Check List - Extraction**

1. Ensure that sufficient quantities of all solvents and reagents are present in containers under the Rapid Trace instrument and that all solvent lines are kept at the bottom of each container by an attached weight at the end of the solvent line.
2. If necessary, empty waste containers by replacing the container with an empty one.
3. Place the SPE cartridges in position 1, 3, 5, 7 and 9 in the cartridge carousel for each module used.
4. Place samples in racks (one rack per module) in position 1, 3, 5, 7 and 9 on the right hand side of the racks and remove screw cap fitted with septum.
5. Place collection tubes in position 1, 3, 5, 7 and 9 on the left hand side of the racks.
6. Place waste tubes in position 2, 4, 6, 8 and 10 on the left-hand side of the racks.
7. Place racks in moving tray at the bottom of each module.
8. Assign method to each module by clicking "Setup racks" in the main menu of the Rapid Trace software and placing method EXT1ONLY.spe as sample one for each module used and method EXT3to9.spe for the samples 3, 5, 7 and 9.
9. Exit the setup racks menu by pressing OK.
10. Enter the Run Monitor Screen. Wait a few seconds after entering the Run Monitor Screen to allow the software time to detect all modules present. Press start on modules to be run.
11. Watch the instrument for a few minutes to ensure that all modules has been initiated and inspect the modules running during the initial purge to ensure that all solvents lines are connected properly.

E. Cleanup, using Caliper Life Sciences, Rapid Trace SPE workstation

The cleanup procedure is automated using the Rapid Trace® modular SPE system, cf. section 6.B.2).

### **Preparation of Silica gel / Silica gel: Sulfuric acid and packing of SPE cartridges**

The SPE cartridges packed with Silica and Silica: Sulfuric acid have a shelf life of 2 days and hence must be prepared directly prior to use.

#### **1. Procedure for preparation of cartridges**

- a. See section 6.A for Manufacturer, grade and brand for all chemicals used
- b. Activate silica gel in oven at 250 °C overnight
- c. Using laboratory balance add 6.6 g Silica gel to 50-mL glass tube fitted with Teflon lined cap and add 3.3 g of concentrated sulfuric acid to the tube. After adding the acid, vigorously shake mixture to break up large lumps. Standard laboratory Personal Protective Equipment must be used, such as lab coat, safety glasses and gloved. See section 2.B for additional safety precautions when handling concentrated acids.
- d. Allow the mixture to rotate overnight using rotating mixer. After overnight rotation, no lumps should be present in mixture.
- e. Press frit to bottom of empty 3-mL SPE
- f. Add 1.0 Silica/Sulfuric acid mixture to the cartridge, and place another frit on top
- g. Add 0.1 g activated Silica gel (250 °C) overnight and place another frit on top of the silica
- h. Store packed cartridges in resealable plastic bag in dessicator until just prior to use

#### **2. Setting up the Equipment for Processing Samples (Cleanup)**

The software controlling the workstation is launched by the Rapid Trace™ Development Icon on the desk top. After launching the software the main menu is displayed. For setting up the software for cleanup click on "Setup Racks", the menu is displayed. Select the modules to be used in lower left corner in this menu and transfer method CL#1ONLY.spe to position "one". Transfer method CL2 to10.spe to positions 3, 5, 7 and 9. Exit this menu by pressing "OK". Enter the "Run Monitor Menu" and launch the modules to be used for cleanup.

#### **3. Check List - Cleanup**

- a. Evaporate samples from extraction step by placing samples in Labconco Rapidvap evaporator and starting the evaporation with the following settings as a guide: Pressure 550 mBar, Temperature 40 °C and Vortex 40%. Evaporate the samples to dryness.
- b. Reconstitute the samples in approximately 0.5 mL hexane
- c. Make certain that sufficient quantities of hexane are present in the solvent bottle under the Rapid Trace™ instrument and that all solvent lines are kept at the bottom of the container by an attached weight at the end of the solvent line.
- d. If necessary, empty waste containers by replacing the container with an empty one.

**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

- e. Place extracts in racks (one rack per module) on the right hand side of the racks, and remove screw caps.
- f. Place collection tubes on the left hand side of the racks.
- g. Place racks in tray at the bottom of each module.
- h. Assign method to each module by clicking "Setup racks" in the main menu of the Rapid Trace™ software and placing method "CL#1ONLY.spe" as sample one for each module used and method "EXT3to9.spe" for remaining positions.
- i. Exit the setup racks menu by pressing OK.
- j. Enter the Run Monitor Screen. Wait a few seconds after entering the Run Monitor Screen to allow the software time to detect all modules present. Press start on modules to be run.
- k. Watch the instrument for a few minutes to ensure that all modules has been initiated and inspect the modules running during the initial purge to ensure that all solvents lines are connected properly.

4. Evaporization and transfer to final GC-vial

- a. Conduct all in a fume hood or BSC or at the Labconco™ evaporator.
- b. Samples from cleanup step are evaporated to approximately 0.5 mL using the Labconco RapidVap™ with the following settings as a guide, Pressure 230 mBar, Temperature 40 °C and Vortex 40%. ***It is essential that the samples are not evaporated to dryness at this step, since all volatile analytes would be lost.***
- c. Transfer the sample to the GC vial that was spiked with recovery standard in section 8.C. **MAKES CERTAIN THAT THE SAMPLES ARE TRANSFERRED TO THE CORRECT VIAL!!!**
- d. Evaporate samples until only the nonane remains (10 µL) using the LabConco RapidVap™.
- e. Complete any lab notes, and bring samples to HR-MS operator.

F. GC/IDHRMS analysis of PBDEs and PBB

GC/IDHRMS analysis is performed on a MAT95XP (ThermoFinnigan MAT™, Bremen, Germany) instrument. The chromatographic separations are carried out on an 6890N gas chromatograph (GC) (Agilent Technologies, Atlanta, GA) fitted with a DB5HT [(15-m length, 0.25 mm I.D. and 0.10-µm film thickness); Agilent Technologies, Atlanta, GA] capillary column. Splitless injection is used with an injector temperature of 280 °C, the oven is programmed to increase from 140 °C (1 min) to 320°C (0 min) with a ramp rate of 10 oC/min. The source temperature is 280 °C in the electron impact mode using a filament bias of 40 eV. The descriptors used for the GC/IDHR-MS analyses are given in Table 1.

**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

**Table 1.** Selected ion monitoring (SIM) descriptors for the high-resolution mass spectrometer (ThermoFinnigan MAT<sup>TM</sup>; Bremen, Germany)

Retention time Window		Ions monitored		Dwell	Theoretical	Acceptable	
Minutes	Compounds	Quantification mass	Isotope Ratio mass	time (ms)	isotopic ratio	range (20%)	
Window 1	<sup>12</sup> C <sub>12</sub> -triBDE	405.8021 [M+2]	407.8001	168	1.03	0.82	- 1.24
5:30-6:50	<sup>13</sup> C <sub>12</sub> -triBDE	417.8424 [M+2]	419.8403	42	1.03	0.82	- 1.24
	PFK	404.9755 <sup>a</sup>	430.9723 <sup>b</sup>	6			
Window 2	<sup>12</sup> C <sub>12</sub> -hexaCB	289.9037 [M+2]-2Cl	291.9008	136	0.49	0.39	- 0.59
6:50-8:00	<sup>13</sup> C <sub>12</sub> -hexaCB	301.9440 [M+2]-2Cl	303.9411	34	0.49	0.39	- 0.59
	<sup>13</sup> C <sub>6</sub> -tetraCDD	327.9137 [M+2]	327.9465 <sup>c</sup>	34	<sup>c</sup>		
	PFK	268.9819 <sup>a</sup>	318.9787 <sup>b</sup>	5			
Window 3	<sup>12</sup> C <sub>12</sub> -tetraBDE	483.7126 [M+2]	485.7106	161	1.46	1.17	- 1.75
8:00-9:40	<sup>13</sup> C <sub>12</sub> -tetraBDE	495.7529 [M+2]	497.7508	40	1.46	1.17	- 1.75
	PFK	480.9691 <sup>a</sup>	504.9691 <sup>b</sup>	10			
Window 4	<sup>13</sup> C <sub>12</sub> -nonaCB	403.8271 [M+2]-2Cl	405.8241	32	0.90	0.72	- 1.08
9:40-11:24	<sup>12</sup> C <sub>12</sub> -pentaBDE	403.7870 [M+2]-2Br	405.7850	131	1.01	0.81	- 1.21
	<sup>13</sup> C <sub>12</sub> -pentaBDE	415.8273 [M+2]-2Br	417.8252	32	1.01	0.81	- 1.21
	PFK	392.9755 <sup>a</sup>	430.9723 <sup>b</sup>	8			
Window 5	<sup>12</sup> C <sub>12</sub> -hexaBDE	481.6975 [M+2]-2Br	483.6955	78	1.47	1.18	- 1.76
11:24-13:00	<sup>13</sup> C <sub>12</sub> -hexaBDE	493.7378 [M+2]-2Br	495.7357	19	1.47	1.18	- 1.76
	<sup>12</sup> C <sub>12</sub> -hexaBB	546.6189 [M+4]-Br	548.6168	78	1.02	0.82	- 1.22
	<sup>13</sup> C <sub>12</sub> -hexaBB	558.6591 [M+4]-Br	560.6571	19	1.01	0.81	- 1.21
	PFK	480.9691 <sup>a</sup>	554.9659 <sup>b</sup>	5			
Window 6	<sup>12</sup> C <sub>12</sub> -heptaBDE	561.6060 [M+4]-2Br	563.6039	90	1.02	0.82	- 1.22
13:00-17:00	<sup>13</sup> C <sub>12</sub> -heptaBDE	573.6462 [M+4]-2Br	575.6442	22	1.02	0.82	- 1.22
	<sup>12</sup> C <sub>12</sub> -octaBDE	639.5165 [M+4]-2Br	641.5144	90	1.32	1.06	- 1.58
	PFK	554.9659 <sup>a</sup>	642.9595 <sup>b</sup>	6			
Window 7	<sup>12</sup> C <sub>12</sub> -decaBDE	797.3355 [M+6]-2Br	799.3334	90	1.23	0.98	- 1.48
17:00-20:00	<sup>13</sup> C <sub>12</sub> -decaBDE	809.3757 [M+6]-2Br	811.3737	22	1.23	0.98	- 1.48
	PFK	704.9563 <sup>a</sup>	754.9531 <sup>b</sup>	6			

<sup>a</sup> Lock mass using PFK; <sup>b</sup> Calibration mass using PFK; <sup>c</sup> Resolution check mass, ratio of mass 327.9137/327.9465 must be below 5% for all analytical runs, used for verification of operation of instrument at 10,000 or above in resolution. Abbreviations: BDE, bromodiphenyl ether; PFK; perfluorokerosine; CB, chlorobiphenyl; CDD, chlorodibenzo-*p*-dioxin; BB, bromobiphenyl.

## 9. Reportable Range of Results

The linear range (0.2 - 2000 pg/uL) of the standard calibration curves determines the highest and lowest analytical values of an analyte that are reportable. However, samples with a concentration exceeding the highest reportable limit may be re-extracted using a smaller volume and re-analyzed, so that the result is in the reportable range.

**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

A. Linearity Limits

Calibration standards are linear for all analytes through the range of concentrations evaluated. The linear range for all analytes except p, p'-DDE were 0.5 to 1000 pg/uL. Calibration curves for p, p'-DDE were extended to 6,000 pg/uL, due to higher concentrations in unknown specimens. Samples exceeding the calibration curve must be diluted or analyzed using a smaller volume of serum.

Certificate of analysis for all standards used are stated in the certificate of analysis as provided by the manufacturer, Cambridge Isotope Laboratory (CIL).

B. Limit of detection

The limit of detection (LOD) for this method is defined as the higher of LODs calculated by two methods: (i) in direct relation to method blanks prepared in parallel with the unknown samples, as 3 times the standard deviation of the method blanks, and (ii) according to the instrumental detection limit defined as the lowest point in the calibration curve (0.5 pg/uL or 5 pg/uL) verified to give a signal with a signal to noise equal to or greater than 3.0. Typical detection limits are shown in Table 2.

**Table 2.** Typical Limits of Detection (LOD).

Analyte	LOD (pg/mL)	Analyte	LOD (pg/mL)	Analyte	LOD (pg/mL)
BB-153	2.5	PCB149	2.5	PCB28	14.1
BDE-017	2.5	PCB151	2.5	PCB44	4.0
BDE-028	2.5	PCB153	2.5	PCB49	2.5
BDE-047	6.2	PCB156	2.5	PCB52	4.4
BDE-066	2.8	PCB157	2.5	PCB66	2.5
BDE-085	16.4	PCB167	2.5	PCB74	2.8
BDE-099	7.0	PCB170	2.5	PCB87	2.5
BDE-100	2.5	PCB172	2.5	PCB99	2.5
BDE-153	17.0	PCB177	2.5	HCB	12.8
BDE-154	2.5	PCB178	2.5	b-HCCH	2.5
BDE-183	4.1	PCB180	2.5	g-HCCH	8.1
BDE-203	12.5	PCB183	2.5	OXYCHLOR	2.5
BDE-209	10.7	PCB187	2.5	t-NONA	2.5
PCB101	3.4	PCB189	2.5	pp-DDE	7.9
PCB105	2.5	PCB194	2.5	op-DDT	2.5
PCB110	3.0	PCB195	2.5	pp-DDT	2.5
PCB118	2.5	PCB196-203	2.5	MIREX	2.5
PCB128	2.5	PCB201	2.5		
PCB138-158	2.7	PCB206	2.5		
PCB146	2.5	PCB209	2.5		

C. Precision

The precision of the method is reflected in the variance of quality control samples analyzed over time. The coefficients of variance (CV) of the method over analyses of 24 batches of samples (N=72) are listed in Table 4 below. These QC samples were prepared over 4 months.

**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

**Table 3.** Mean Concentration and CV for 72 QC samples (QC identifier SSP: 01:004).

Analyte	Mean	CV	Analyte	Mean	CV
BB-153	83.0	4.6	PCB180	80.8	3.3
BDE-017	98.0	17.9	PCB183	80.0	4.1
BDE-028	90.9	6.3	PCB187	79.9	3.8
BDE-047	198.4	10.6	PCB189	78.3	3.8
BDE-066	106.7	7.6	PCB194	78.8	3.8
BDE-085	125.3	11.5	PCB195	81.3	4.8
BDE-099	127.0	7.6	PCB196-203	145.6	12.2
BDE-100	96.4	5.1	PCB201	75.4	5.8
BDE-153	325.8	17.2	PCB206	73.1	4.5
BDE-154	95.0	5.5	PCB209	75.4	3.2
BDE-183	100.8	8.1	PCB28	86.2	4.1
PCB101	79.7	3.5	PCB44	83.7	6.5
PCB105	76.0	3.4	PCB49	81.7	4.6
PCB110	82.4	6.3	PCB52	81.2	4.1
PCB118	79.3	3.6	PCB66	73.1	4.2
PCB128	67.0	6.1	PCB74	73.9	3.9
PCB138-158	175.0	8.1	PCB87	82.4	5.6
PCB146	84.1	3.8	PCB99	77.8	3.8
PCB149	77.0	6.0	HCB	105.8	3.9
PCB151	78.6	6.9	b-HCCH	59.1	4.3
PCB153	81.8	3.8	g-HCCH	85.1	5.9
PCB156	76.6	3.6	OXYCHLOR	75.7	5.4
PCB157	78.7	3.1	t-NONA	78.1	3.9
PCB167	78.0	4.5	pp-DDE	76.2	3.7
PCB170	91.0	6.1	op-DDT	78.9	3.3
PCB172	81.1	3.8	pp-DDT	84.2	4.0
PCB177	74.9	4.9	MIREX	81.5	3.6
PCB178	79.1	3.2			

**D. Analytical specificity**

Isotope Dilution High Resolution Mass Spectrometry (ID-HRMS) coupled with gas chromatography is used for sample analysis. This instrumentation provides a high resolution (10,000 resolution) measurement providing excellent specificity in the measurement performed. In addition, for all measurements performed, two ions are monitored for each native and <sup>13</sup>C-labeled internal standard. For each measurement the ratio between these two ions are verified to be with +/- 20% from the theoretical isotope ratio, giving additional confirmation of the identity of the target analyte.

In addition, the relative retention time of native compound divided with its <sup>13</sup>C-internal standard is verified for each measurement to eliminate the risk of mistakes during integration.

**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

E. Accuracy

Presently no established standard reference material exists for polybrominated diphenyl ethers in serum or milk. However, we are currently in the process of establishing standard reference materials (SRM) for serum and milk in collaboration with the National Institute of Standards and Technology (NIST). Once these SRMs are available these specimens will be included as blind QC specimens at a minimum of every six months to provide ongoing estimates of the methods accuracy over time.

In absence of an SRM material certified for PBDEs we have analyzed the SRM# 1589a and compared our data to that generated by Heather Stapleton at the time working at NIST, cf. Table 4. The absolute difference in concentration between NIST and CDC was 2.1% for BDE-47 while larger differences were observed for compounds present at lower levels in this serum.

**Table 4.** Comparison of the analysis of SRM 1589a between NIST and CDC. BDE-183 was below the limit of detection (LOD) for the NIST methodology, and BB-153 was not determined by NIST.

Analyte	CDC			NIST			Absolute difference
	pg/mL	SD	CV	pg/mL	SD	CV	
BDE28	9.6	0.44	4.6	8.9	1.3	14	7.4
BDE47	169	4.4	2.6	173	12	7.0	-2.1
BDE100	23	0.93	4.1	20	0.63	3.1	12
BDE99	34	1.6	4.8	29	2.8	9.6	17
BB153	36	1.2	3.4	ND			
BDE153	16	0.20	1.3	12	1.3	10	32
BDE183	4.3	0.76	17.8	<7			

Abbreviations: SD, standard deviation and CV, coefficient of variance.

## 10. Quality Assessment and Proficiency Testing

### A. Quality Assessment

In this method a set of samples is defined as 24 unknown samples, prepared and analyzed together with 3 analytical blanks and 3 QC sample. Quality control limits are established by characterizing assay precision with repeated analyses of the QC pool. Typical QC-limits are given in Table 4.

For QA/QC purposes measurement of a target analyte in a set of samples is considered valid only after the QA/QC sample have fulfilled the following criteria:

- (i) If all of the QC samples are within  $2\sigma$  limits, then accept the run.
- (ii) If one or more QC results are outside the  $2\sigma$  limits, then apply the rules below and reject the run if any conditions are met.

**Extreme outlier:** the results are outside the characterization mean by more than  $4\sigma$ .

- **$1_{3\sigma}$** , Average of three QCs is outside of the  $3\sigma$  limit.
- **$2_{2\sigma}$** , QC results from two consecutive runs are outside of  $2\sigma$  limit on the same side of the mean.
- **$R_{4\sigma}$  sequential**, QC results from two consecutive runs are outside of  $2\sigma$  limit on opposite sides of mean.
- **$10_x$  sequential**, QC results from ten consecutive runs are on the same side of the mean.

If the QC result for an analyte is declared "out of control", the results of that analyte for all patient samples analyzed during that run are invalid for reporting.

Further, every measurement of a set of samples must fulfill the following criteria to be considered a valid measurement:

- (i) The ratio of the two ions monitored for every analyte and  $^{13}\text{C}$ -labelled internal standard, must not deviate more than 20% from the theoretical value.
- (ii) The ratio of the retention time of the analyte over its corresponding  $^{13}\text{C}$ -labeled internal standard must be within the range 0.99 – 1.01. For analytes that do not have an identical  $^{13}\text{C}$ -labeled IS the ratio to the IS used may not deviate more than 1% from the average of the same ratio of the calibration standards analyzed in the same analytical run
- (iii) The measured recovery of the IS must be within the range 25-150%.

**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

B. Proficiency testing (PT)

Currently the only established PT program for this assay is the Artic Monitoring and Assessment program (AMAP) in which our lab participates. In this program 3 serum samples are received three to four times per year and analyzed with respect to PCB/PP/PBDEs. The program provides a report after each set of PT samples has been reported.

**Table 4.** QC Characterization statistics.

Analyte	Mean -3 $\sigma$	Mean -2 $\sigma$	Mean	Mean +2 $\sigma$	Mean +3 $\sigma$
BB-153	69.1	73.8	83.0	92.2	96.8
BDE-017	44.2	62.1	98.0	133.8	151.7
BDE-028	71.9	78.2	90.9	103.6	109.9
BDE-047	166.6	177.2	198.4	219.6	230.2
BDE-066	83.8	91.4	106.7	121.9	129.6
BDE-085	90.6	102.2	125.3	148.4	159.9
BDE-099	104.1	111.7	127.0	142.3	149.9
BDE-100	81.1	86.2	96.4	106.5	111.6
BDE-153	274.3	291.5	325.8	360.2	377.4
BDE-154	78.6	84.0	95.0	105.9	111.4
BDE-183	76.5	84.6	100.8	117.0	125.1
PCB101	69.2	72.7	79.7	86.7	90.2
PCB105	65.7	69.1	76.0	82.8	86.2
PCB110	63.4	69.7	82.4	95.0	101.4
PCB118	68.6	72.2	79.3	86.4	90.0
PCB128	48.8	54.9	67.0	79.2	85.2
PCB138-158	150.6	158.7	175.0	191.2	199.3
PCB146	72.8	76.6	84.1	91.6	95.4
PCB149	58.9	64.9	77.0	89.1	95.2
PCB151	57.8	64.7	78.6	92.5	99.4
PCB153	70.4	74.2	81.8	89.5	93.3
PCB156	65.9	69.4	76.6	83.7	87.3
PCB157	69.4	72.5	78.7	85.0	88.1
PCB167	64.5	69.0	78.0	87.0	91.5
PCB170	72.7	78.8	91.0	103.3	109.4
PCB172	69.9	73.6	81.1	88.7	92.4
PCB177	60.2	65.1	74.9	84.6	89.5
PCB178	69.5	72.7	79.1	85.4	88.6
PCB180	70.9	74.2	80.8	87.4	90.7
PCB183	67.8	71.9	80.0	88.2	92.2
PCB187	68.5	72.3	79.9	87.5	91.3
PCB189	66.8	70.6	78.3	86.0	89.9
PCB194	67.4	71.2	78.8	86.4	90.2
PCB195	66.8	71.6	81.3	90.9	95.7
PCB196-203	109.0	121.2	145.6	170.0	182.2
PCB201	58.1	63.9	75.4	86.9	92.7
PCB206	59.7	64.2	73.1	82.1	86.6

**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

PCB209	65.7	69.0	75.4	81.9	85.1
PCB28	73.8	77.9	86.2	94.4	98.6
PCB44	64.1	70.7	83.7	96.8	103.3
PCB49	68.0	72.5	81.7	90.9	95.5
PCB52	68.9	73.0	81.2	89.4	93.5
PCB66	60.6	64.7	73.1	81.4	85.5
PCB74	62.3	66.1	73.9	81.7	85.5
PCB87	65.5	71.1	82.4	93.7	99.3
PCB99	66.3	70.1	77.8	85.4	89.3
HCB	94.1	98.0	105.8	113.7	117.6
b-HCCH	46.1	50.4	59.1	67.8	72.1
g-HCCH	67.6	73.4	85.1	96.8	102.7
OXYCHLOR	59.6	65.0	75.7	86.5	91.9
t-NONA	66.2	70.2	78.1	86.0	89.9
pp-DDE	65.0	68.8	76.2	83.7	87.4
op-DDT	69.1	72.4	78.9	85.4	88.7
pp-DDT	72.3	76.3	84.2	92.1	96.0
MIREX	70.6	74.3	81.5	88.7	92.4

### 11. Remedial Action if Calibration or QC Systems Fail to Meet Acceptable Criteria

If the calibration or QC systems fail to meet acceptable criteria, suspend all operations until the source or cause of failure is identified and corrected. If the source of failure is easily identifiable for instance, failure of the mass spectrometer or a pipeting error corrects the problem immediately. Otherwise, prepare fresh reagents and clean the mass spectrometer system. Before beginning another analytical run, re-analyze several QC materials (in the case of QC failure) or calibration standards (in the case of calibration failure). After re-establishing calibration or quality control, resume analytical runs. Document the QC failures, review the cases with supervisor to determine the source(s) of problem, and take measures to prevent re-occurrence of the same problem.

### 12. Limitations of Method, Interfering Substances and Conditions

This method is an isotope dilution mass spectrometry method, widely regarded as the definitive method for the measurement of organic toxicants in human body fluids. By using high resolution mass spectrometry, most interferences are eliminated. Due to the matrix used in this procedure, occasional unknown interfering substances have been encountered. If chromatographic interference with the internal standards occurs, reject that analysis. If repeat analysis still results in an interference with the internal standard, the results for that analyte are not reportable.

### 13. Reference Ranges (Normal Values)

## Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum NHANES 2003-2004

Reference ranges have not been reported for BFRs in the NHANES survey, but are to be included in the NHANES 2003-04 report. Normal concentration ranges of BFRs are given in Table 5 below, data from reference [24].

**Table 5.** Normal concentration range (ng/g lipid) of Brominated flame retardants (BFRs) in human serum [24]

<b>Compound</b>	<b>Median</b>	<b>Quartile Range</b>	<b>&lt;LOD</b>
BDE-47	8.4	5.4 - 18	10%
BDE-100	1.7	1.0 - 3.5	6%
BDE-99	1.7	1.4 - 2.5	16%
BB-153	0.33	0.27 - 1.3	23%
BDE-154	0.21	0.21 - 0.28	71%
BDE-85	0.21	0.21 - 0.37	56%
BDE-153	2.2	1.2 - 3.8	3%
BDE-183	0.21	0.21 - 0.21	65%

### 14. Critical Call Results (“Panic Values”)

It is unlikely that any result would be a "critical call", which would only be observed in acute poisonings. There are no established “critical call” values. Application of this method to NHANES studies will assist in determining levels of BFRs normally found in the US populations. Test results in this laboratory are reported in support of epidemiological studies, not clinical assessments. Data will help determine critical exposures.

### 15. Specimen Storage and Handling During Testing

Store serum samples in -70 °C freezer before and after analysis. Keep extracts at room temperature covered with aluminum foil for storage, due to documented UV-sensitivity of target analytes.

After analysis, keep GC vials in Styrofoam boxes for storage at room temperature until the final analytical data have been reported.

### 16. Alternate Methods for Performing Test or Storing Specimens if Test System Fails

Alternate validated methods have not been evaluated for measuring BFRs in human serum. If the analytical system fails, refrigerate the samples (at 4 - 8 °C) until the analytical system is restored to functionality. If long-term interruption (greater than one day) is anticipated, then store serum specimens at -70 ± 10 °C.

The method is designed to run on a GC/IDHRMS instrument, and is not generally transferable to other instrumentation. If the system fails, store sample extracts at room temperature covered with aluminum foil until the analytical system is restored to functionality.

### **17. Test Result Reporting System; Protocol for Reporting Critical Calls (if Applicable)**

Study subject data is reported in both concentration units (pg/mL serum) and adjusted based on serum lipids (ng/g lipid).

Once the validity of the data is established by the QC/QA system outlined above, these results are verified by a DLS statistician, and the data are reported in both hard copy and electronic copy. These data and a cover letter will be routed through the appropriate channels for approval (i.e. supervisor, branch chief, division director) as outlined in the DLS Policy and Procedure Manual. After approval at the division level, the report will be sent to the contact person or principal investigator who requested the analyses.

### **18. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking**

If greater than 0.2 mL of sample remains following successful completion of analysis, this material must be returned to storage at  $-70 \pm 5$  °C in case reanalysis is required. These samples shall be retained until valid results have been obtained and reported and sufficient time has passed for review of the results.

Standard record keeping formats (e.g., database, notebooks, and data files) are used to track specimens. Specimens will only be transferred or referred to other DLS Branch laboratories or, if required, to CLIA certified laboratories. Specimens may be stored at the CDC specimen handling and storage facility (CASPIR).

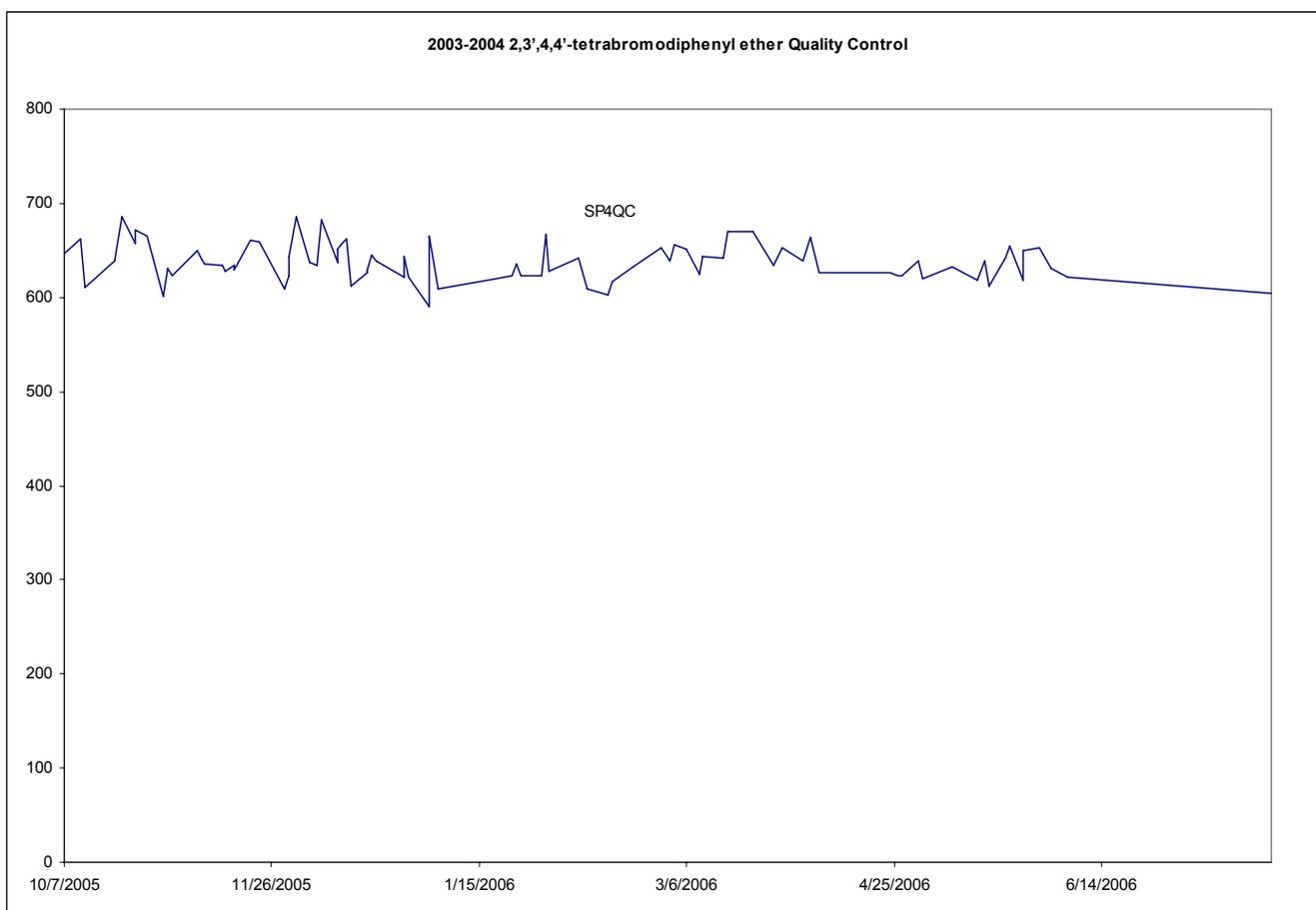
**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

**19. Summary Statistics and QC Graphs**

A. 2, 3', 4, 4'-tetrabromodiphenyl ether

Summary Statistics for 2,3',4,4'-tetrabromodiphenyl ether by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SP4QC	87	10/7/2005	7/25/2006	637.69	20.11	3.2

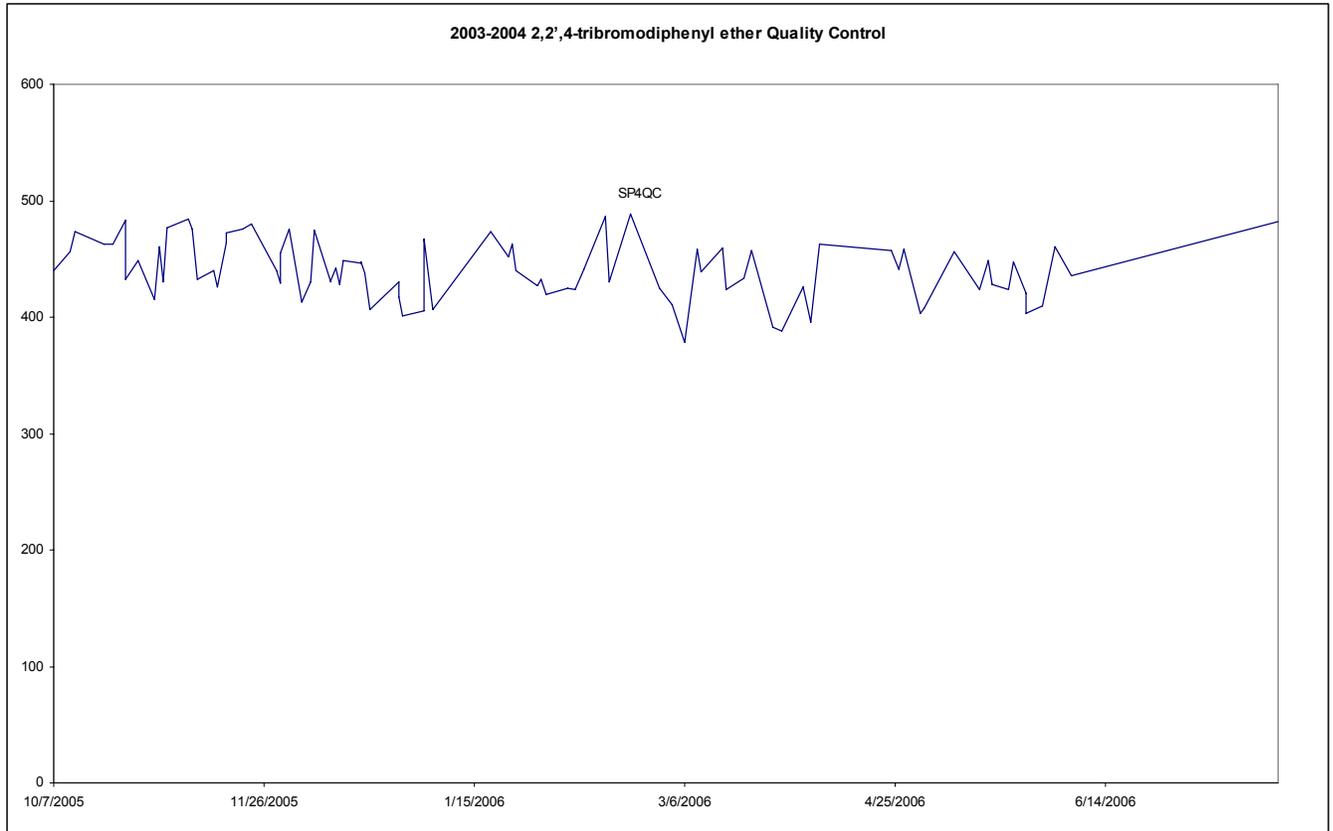


**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

**B. 2,2',4-tribromodiphenyl ether**

**Summary Statistics for 2,2',4-tribromodiphenyl ether by Lot**

<b>Lot</b>	<b>N</b>	<b>Start Date</b>	<b>End Date</b>	<b>Mean</b>	<b>Standard Deviation</b>	<b>Coefficient of Variation</b>
SP4QC	87	10/7/2005	7/25/2006	440.37	25.41	5.8

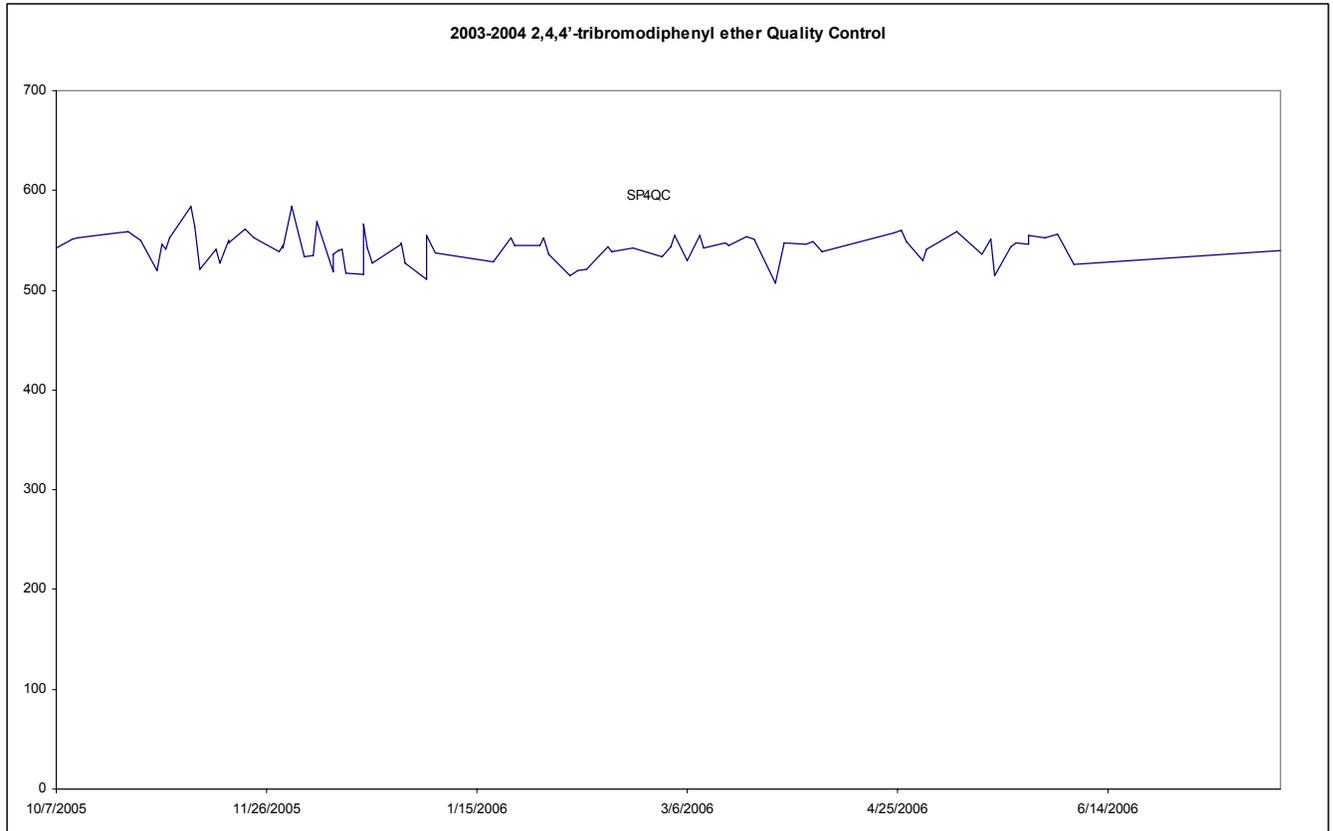


**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

C. 2,4,4'-tribromodiphenyl ether

Summary Statistics for 2,4,4'-tribromodiphenyl ether by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SP4QC	86	10/7/2005	7/25/2006	542.79	14.78	2.7

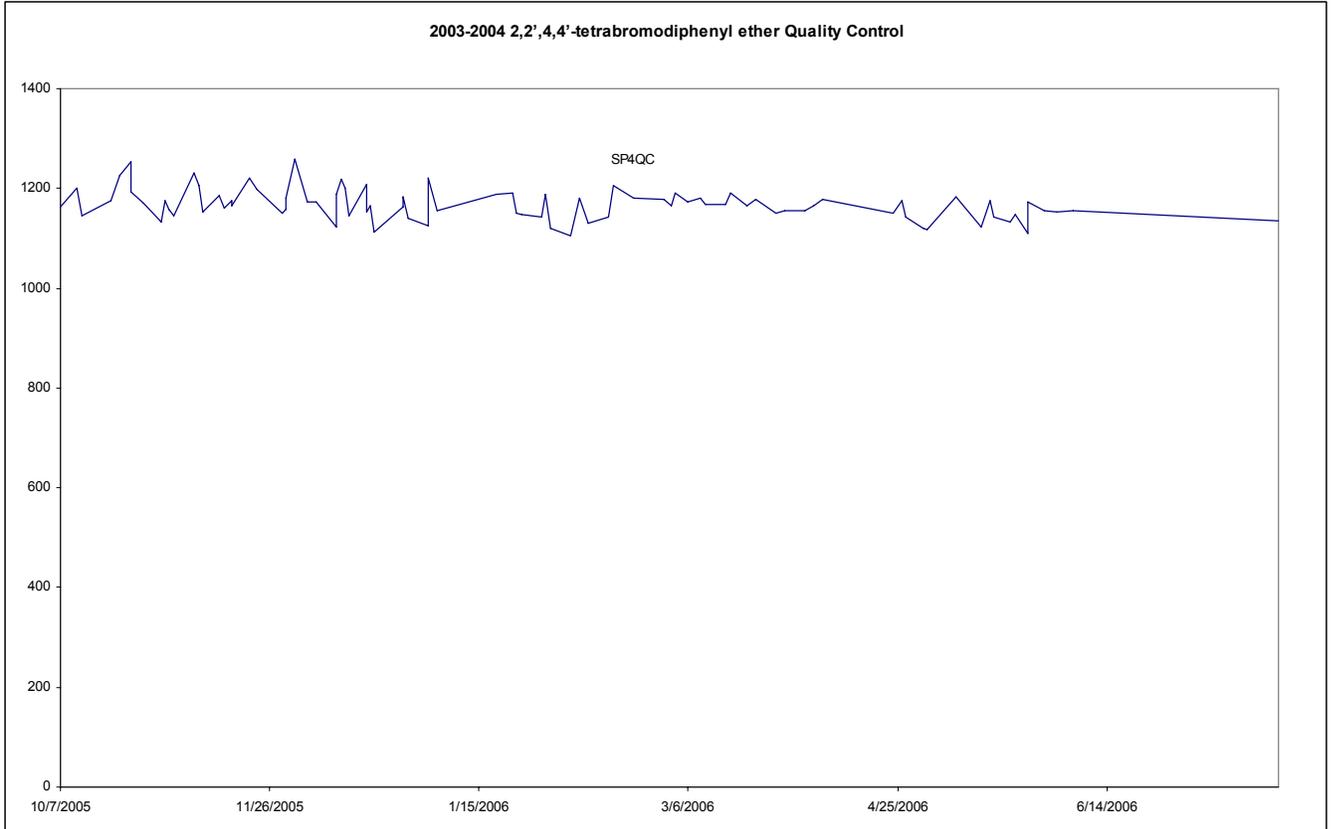


**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

**D. 2,2',4,4'-tetrabromodiphenyl ether**

**Summary Statistics for 2,2',4,4'-tetrabromodiphenyl ether by Lot**

<b>Lot</b>	<b>N</b>	<b>Start Date</b>	<b>End Date</b>	<b>Mean</b>	<b>Standard Deviation</b>	<b>Coefficient of Variation</b>
SP4QC	87	10/7/2005	7/25/2006	1167.24	30.69	2.6

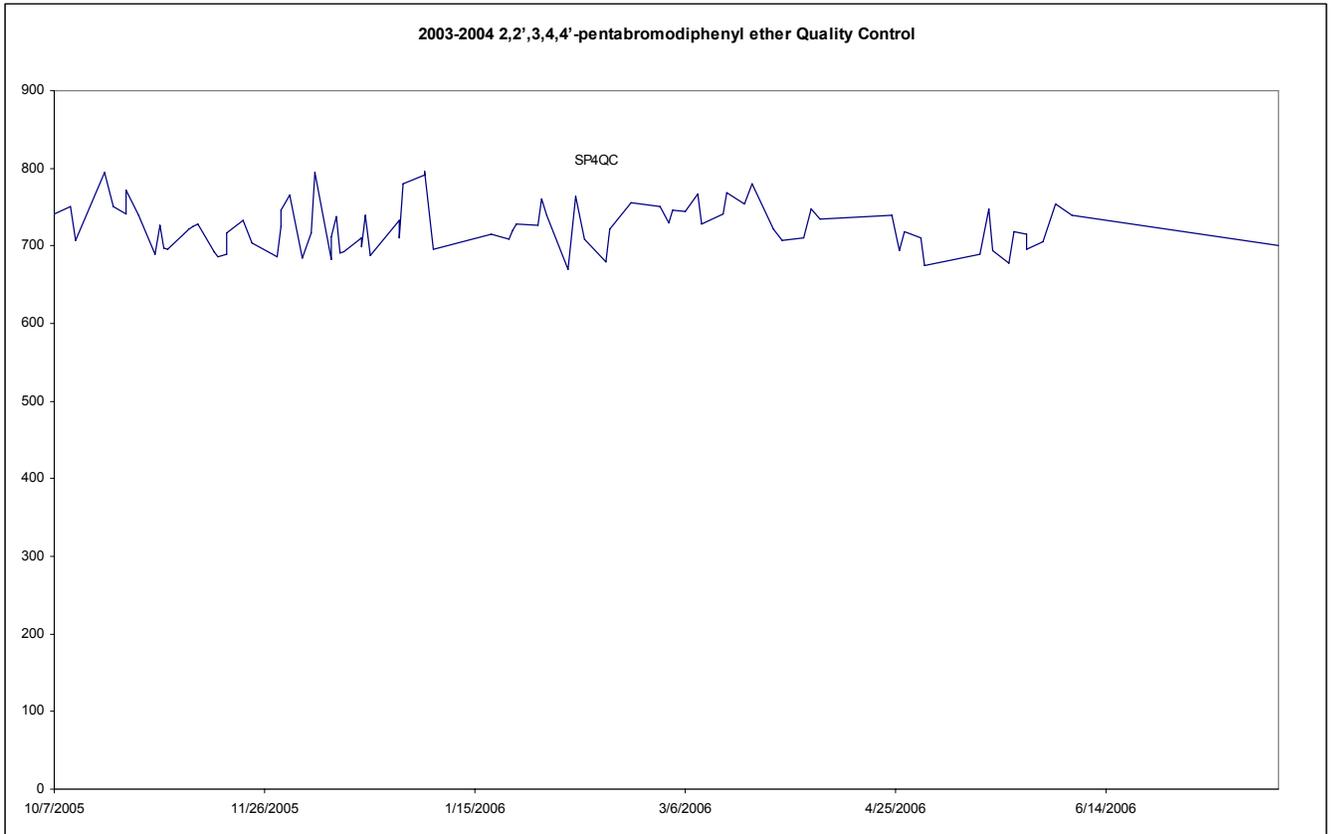


**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

**E. 2,2',3,4,4'-pentabromodiphenyl ether**

**Summary Statistics for 2,2',3,4,4'-pentabromodiphenyl ether by Lot**

<b>Lot</b>	<b>N</b>	<b>Start Date</b>	<b>End Date</b>	<b>Mean</b>	<b>Standard Deviation</b>	<b>Coefficient of Variation</b>
SP4QC	87	10/7/2005	7/25/2006	725.06	30.4	4.2

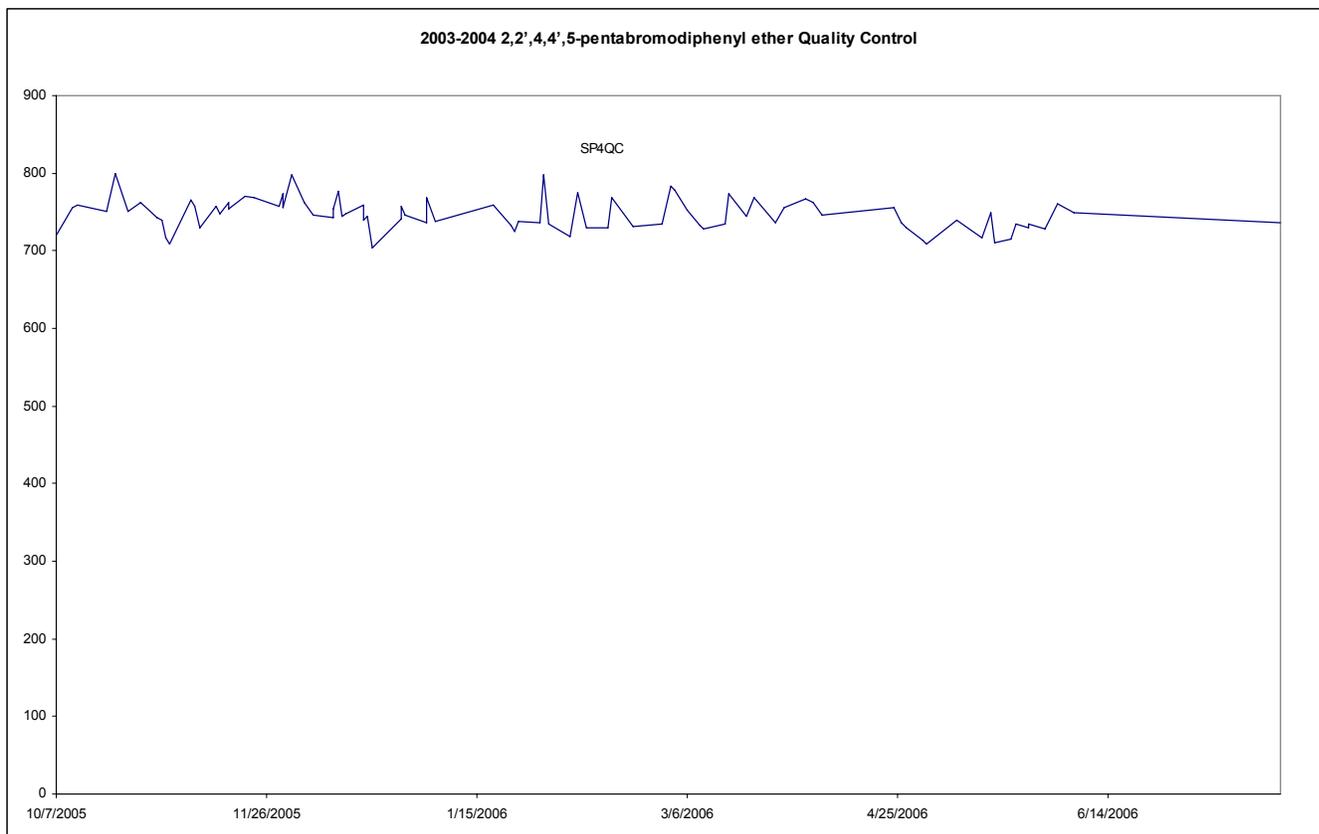


**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

**F. 2,2',4,4',5-pentabromodiphenyl ether**

**Summary Statistics for 2,2',4,4',5-pentabromodiphenyl ether by Lot**

<b>Lot</b>	<b>N</b>	<b>Start Date</b>	<b>End Date</b>	<b>Mean</b>	<b>Standard Deviation</b>	<b>Coefficient of Variation</b>
SP4QC	86	10/7/2005	7/25/2006	746.9	20.46	2.7

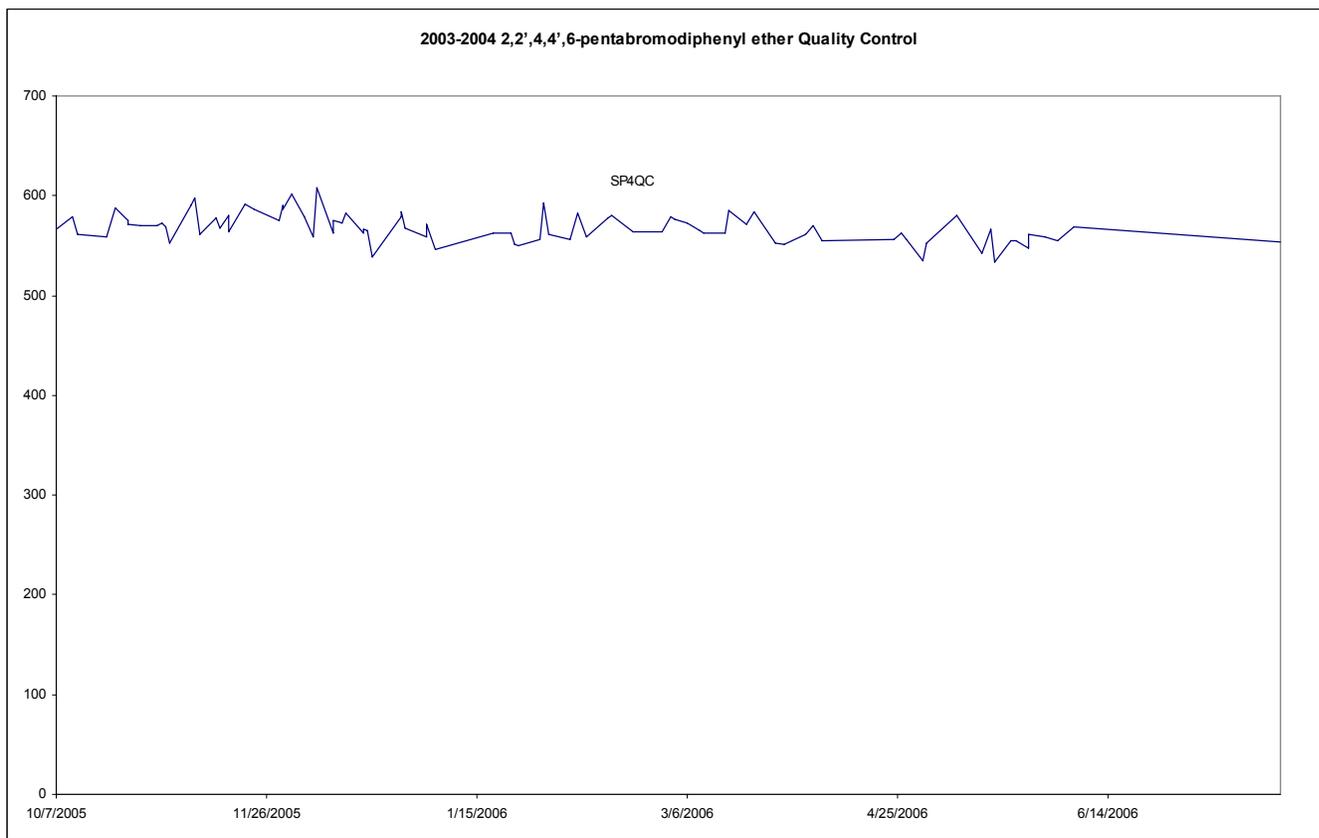


**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

G. 2,2',4,4',6-pentabromodiphenyl ether

Summary Statistics for 2,2',4,4',6-pentabromodiphenyl ether by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SP4QC	88	10/7/2005	7/25/2006	567.77	14.51	2.6

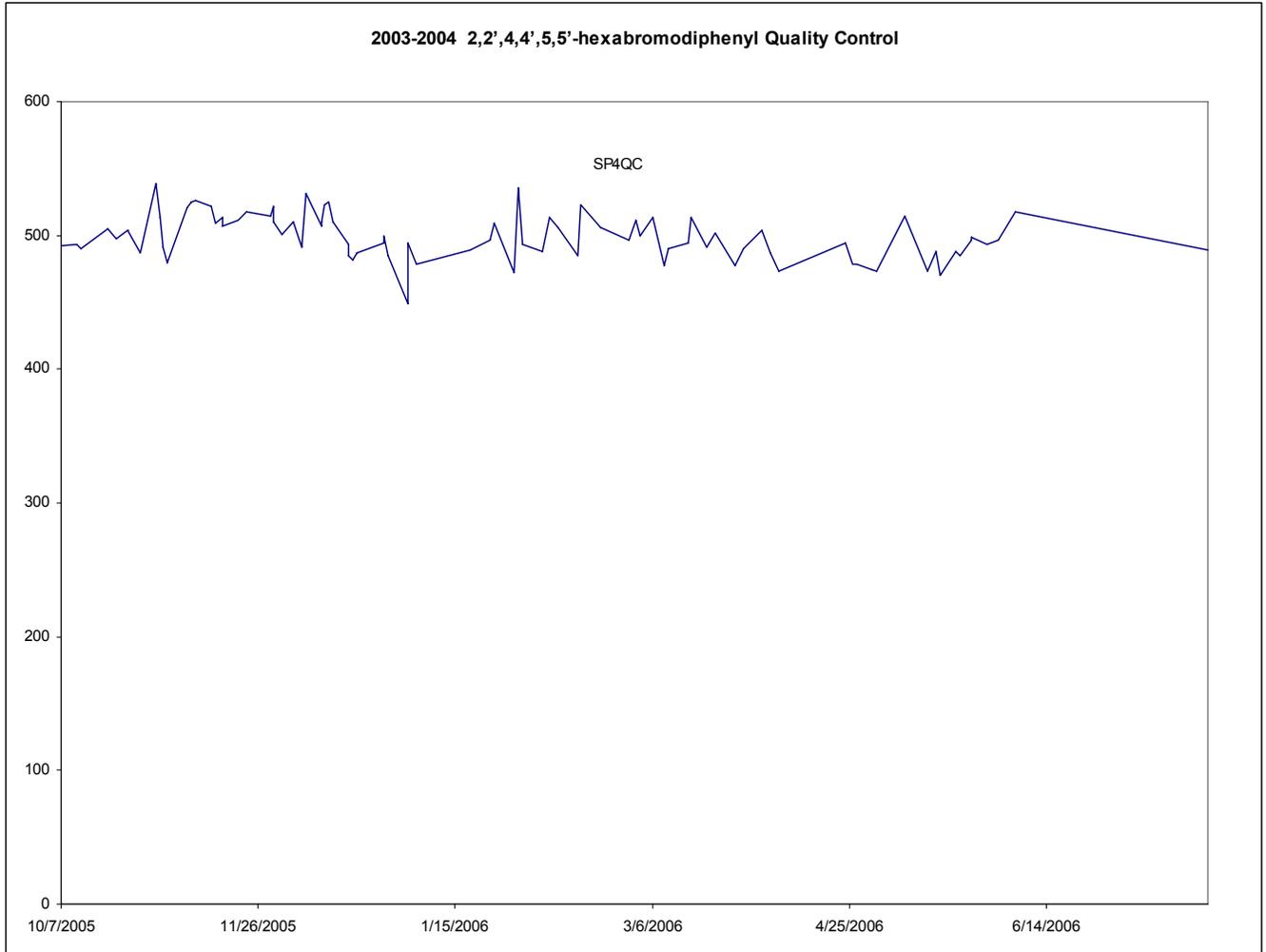


**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

H. 2,2',4,4',5,5'-hexabromodiphenyl ether

Summary Statistics for 2,2',4,4',5,5'-hexabromodiphenyl ether by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SP4QC	88	10/7/2005	7/25/2006	1928.8	71.07	3.7

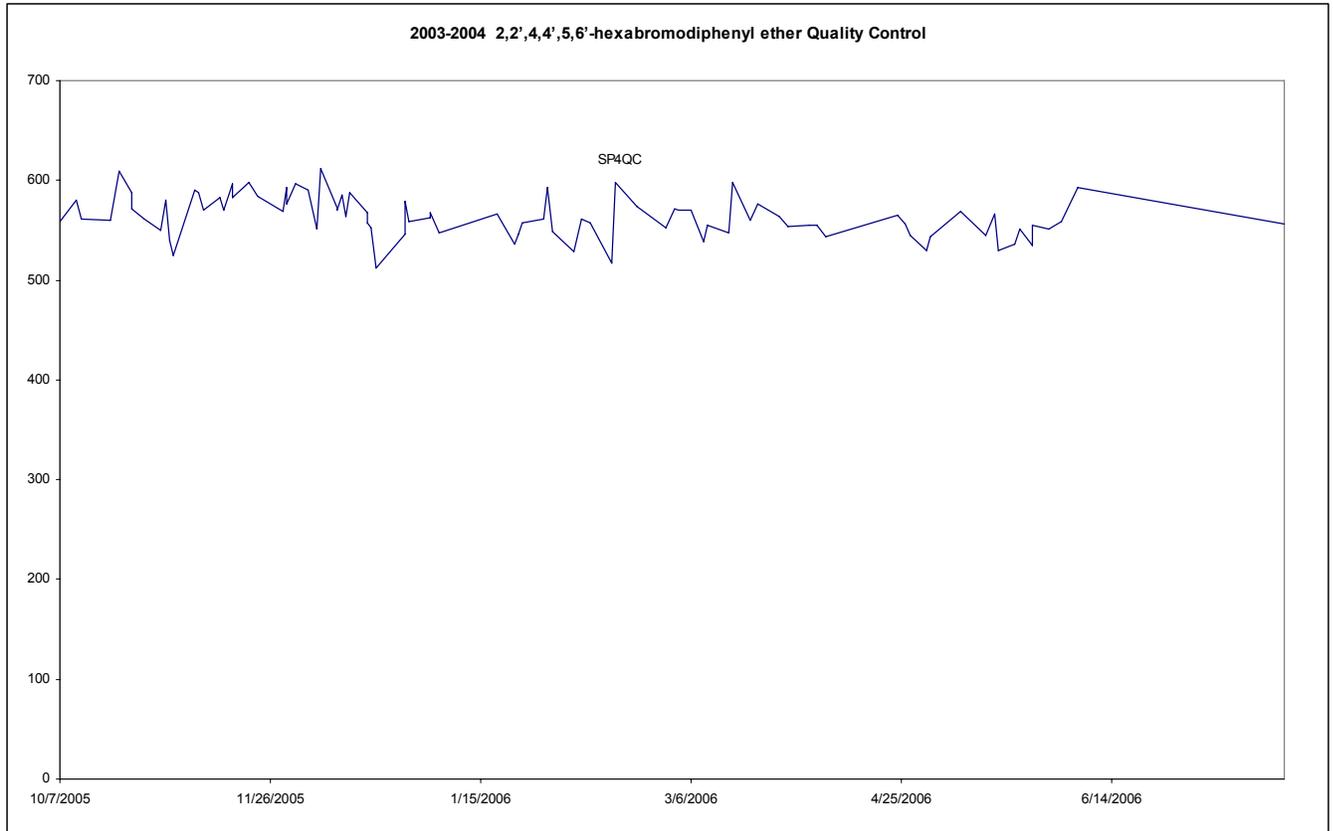


**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

I. 2,2',4,4',5,6'-hexabromodiphenyl ether

Summary Statistics for 2,2',4,4',5,6'-hexabromodiphenyl ether by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SP4QC	88	10/7/2005	7/25/2006	563.62	20.64	3.7

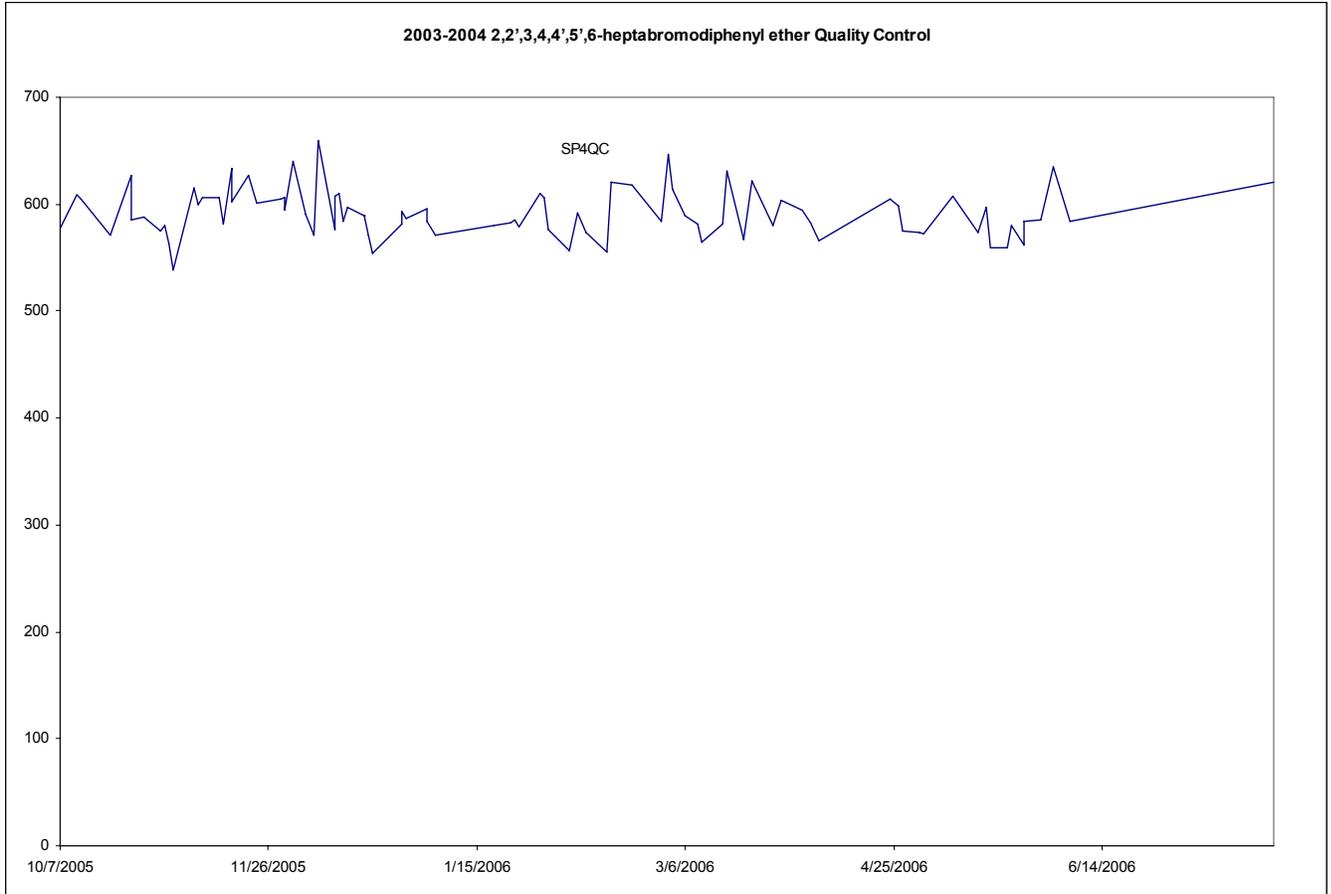


**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

J. 2,2',3,4,4',5',6-heptabromodiphenyl ether

Summary Statistics for 2,2',3,4,4',5',6-heptabromodiphenyl ether by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SP4QC	87	10/7/2005	7/25/2006	591.44	22.63	3.8

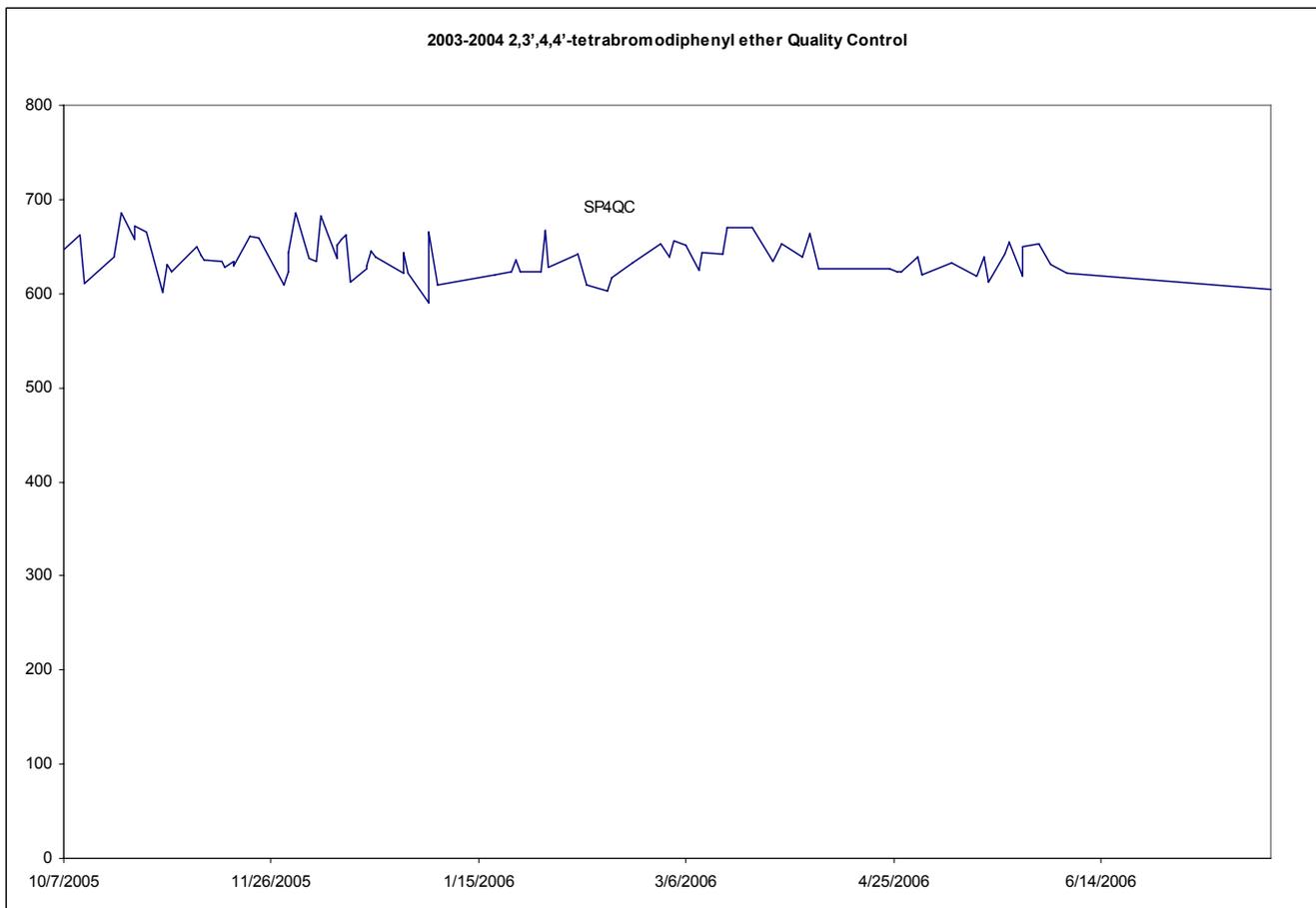


**Polybrominated diphenyl ethers (PBDEs) or BFRs in Human Serum  
NHANES 2003-2004**

I. 2,3',4,4'-tetrabromodiphenyl ether

Summary Statistics for 2,3',4,4'-tetrabromodiphenyl ether by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SP4QC	87	10/7/2005	7/25/2006	637.69	20.11	3.2



## **20. References**

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9. H. M. Blanck, M. Marcus, V. Hertzberg, P. E. Tolbert, C. Rubin, A. K. Henderson and R. H. Zhang, Determinants of polybrominated biphenyl serum decay among women in the michigan PBB cohort, *Environ Health Perspect* **108**, pp. 147-152 (2000).