Laboratory Procedure Manual

Analyte: **Polybrominated diphenyl ethers (PBDEs)**

Matrix: **Serum**

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

Method No: **6701.02**

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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.

BFRs in Human Serum
NHANES 2007-2008

This document details the Lab Protocol for NHANES 2005–2006 data.
A tabular list of the released analytes follows

: **Lab Number**	**Analyte**	**SAS Label**
BFRPOL_E	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
	LBXBR11	2,2',3,3',4,4',5,5',6,6'-decabromo ether
	LBXBR66	2,3',4,4'-tetrabromodiphenyl ether

1. Clinical Relevance and Summary of Test Principle

1.1. Clinical Relevance

Polybrominated diphenyl ethers (PBDEs) are included in a larger group of chemicals known as brominated flame retardants and they are added to products such as foam padding, textiles, or plastics to retard combustion. PBDEs are not chemically bound to the flame-retarded material, so they can enter the environment from volatilization, leaching, or degradation of PBDE-containing products. PBDEs are generally persistent in the environment and have been measured in aquatic sediments, house dust, and aquatic and terrestrial animals, especially in fish where PBDEs are known to bioconcentrate. Hence humans may be exposed though the diet, including breast feeding, and by contact with flame retarded products and contaminated house dust. The rate of elimination of PDBEs from the human body depends on the specific PDBE, with elimination half-lives that can vary from a couple weeks to several months.

1.2. 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 lipophillic 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 LLE, employing an automated Liquid Handling instrument (Gilson 215 Liquid Handler®, Gilson, Inc.). Required sample pretreatment prior to extraction is performed on the Gilson 215 liquid handler, including automated addition of (*i*) internal standards, (*ii*) isopropanol (diluent) with a manual vortexing step in-between each addition. Hydrochloric acid is added manually to denature proteins in the sample enabling efficient 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 (5% DCM in 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 is not removed in the silica gel/sulfuric acid layer and would then interfere in the final HR-MS analyses. The presence of cholestene causes 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).

The lipid removal is automated using the Rapid Trace® (Caliper Life Sciences). The samples are evaporated and transferred to GC vials. Evaporization is performed on the Caliper TurboVap using increased temperature and a stream of nitrogen to aid evaporization.

Serum concentrations are determined using gas chromatography isotope dilution high resolution mass spectrometry (GC/IDHRMS), which minimizes or eliminates many interferences 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 13C-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

2.1 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 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.

2.2. 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.

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.

2.3. Hazardous waste handling

Solvent waste: Collect solvent waste in waste bottles (empty solvent bottles may be used). Clearly write **WASTE** on bottles, and 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 Gilson Liquid Handler 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 hydrochloric 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 (i*v*) 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

3.1. 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.

3.2. 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.

3.3. 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^{TM}-lined caps. Rinse containers using the same procedure as for other glassware used in the current method (see section 6.1). Preferred container is a 10 mL Wheaton glass serum vial.
- The criteria for an unacceptable specimen are either a low volume (< 0.1 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. 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 ≤ -60 ^{o}C until time for analysis. Refreeze at ≤ -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

6.1 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.

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.

Chemical/Solvent	Manufacturer	Grade
Acids		
Hydrochloric acid	Aldrich	37%
Sulfuric acid	Aldrich	95-97%
Solvents		
Dichloromethane	TEDIA	Pesticide
Dodecane	EM Science	min 99%
Hexane	TEDIA	Pesticide
Isopropanol	TEDIA	Pesticide
Methyl *tert*-Butyl Ether (MTBE)	TEDIA	Pesticide
Methanol	TEDIA	Pesticide
n-Nonane	Sigma	99%
Water	TEDIA	Pesticide
SPE sorbents		
OASIS HLB®	Waters	n/a
Silica gel	Sigma	100-200 mesh

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

Item	Manufacturer/Source
Glassware and caps	
Test tube 16 x 100 mm	Fisher Scientific
Septum for test tube	Fisher Scientific
Open top cap for test tube	Fisher Scientific
Borosilicate GlassPasteur pipette	Fisher Scientific
Boston Round (amber glass bottle)	Fisher Scientific
V-vial (3 mL) with septum-cap	Fisher Scientific
GC vials and caps	Fisher Scientific
Others	
Label printer (Brady TLS PClink)	Fisher Scientific
Magnetic stirrer (heavy duty, large)	Fisher Scientific

Pipette dispenser	VWR

6.1.1 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 ^{o}C, the glassware is ready to be used.

For satellite bottles such as glass tapered-stopper bottles intended for storing for small volume, everyday use in the BSC the normal large labels are not to be used because it would interfere with the proper procedure for re-cleaning them. Instead label by hand using a "Sharpie" pen and if necessary affix a small "flammables" sticker to the bottle.

Caps and septums: Rinse caps and septums for test tubes prior to use to remove contaminants. This is done by Soxhelet extraction for five hours using methanol as the extraction solvent. Alternatively, if the Soxhelet 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 re-sealable bags (<u>not</u> in cardboard boxes) for safe storage until used.

Gas Chromatography Vials: Heat GC vials in an oven at >200 ^{o}C overnight prior to use. Store vials in a beaker covered with aluminum foil. The caps for GC vials are cleaned by Soxhelet extraction, using the same procedure as for caps and septum's.

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

6.1.3 Internal standards (IS)

The current method is validated for BFRs, PCBs, and acid stable persistent pesticides (PPs). 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 pg/µL of 10 different $^{13}C_{12}$-labeled PBDE and PBB congeners. The PCB standard contains 7.5 pg/µL of 21

different PCB congeners and the PP standard contains 11 ^{13}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 2103.

6.1.4 Recovery standard (RS)

Use one recovery standard for measurement of recovery. This standard contains 1234-$^{13}C_6$-TCDD (2.5 pg/µL), $^{13}C_{12}$-CB-208 (10.0pg/µL) and $^{13}C_{12}$-BDE-139 (10.0pg/µL) in hexane containing 10% nonane and 2% dodecane by volume. Add the standard (100µL) 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 step) 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 8.3.

File the certificate of analysis from CIL for the recovery standard solution in the SOP binder located in building 103, room 2103.

6.1.5 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 GC vials (5uL in each vial). Label the vials BFR/PCB CS1 through CS10 using a computer-generated label. Replace the standards used for calibration of the MAT95 after completion of every run.

6.2 Instrumentation

6.2.1 Gilson 215 liquid handler: Liquid handling is automated using the Gilson 215 Liquid handler, cf. Figure 1. Place the samples in the auto-mix to the far right in Figure 1. 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. 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.

6.2.2 Rapid Trace®, SPE work station: The Rapid Trace® SPE workstation (Caliper Life Sciences) (Figure 2) 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 (F) rack containing serum samples and collected fractions. The Rapid Trace® instrument processes the samples in sequence. Up to 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.

6.3 Procedures for preparing quality control materials

The QC material for this assay is bovine serum in which the concentrations of the target analytes have 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

(ii) No more than ten consecutive QC samples may fall either above or below the mean value of previous determinations of the same pool after one data point has fallen outside of +/- 2SD. 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 6.1.2. 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 ^{o}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 ^{o}C).

7. Calibration and Calibration Verification

7.1. Calibration of Mass Spectrometer

Calibrate and tune the Thermo MAT95XL or DFS mass spectrometer using the appropriate calibration gas (either high boiling PFK (perfluorokerosene) for BFR analysis or FC43 for PCB/PP analysis) 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).

7.2. Creation of Calibration Curve

A linear calibration curve, consisting of six CS standards with concentrations ranging from 0.5 to 500 pg/µL, 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 are equally distributed between the two extreme concentrations (on a log scale).

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 ^{13}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.

7.3. Calibration Verification

Calibration verification of the test system is done by the inclusion of quality control samples with a determined concentration in every run of unknown specimens and by the analysis of Proficiency Testing (PT) samples at least twice per year. See section 10 for further information on PT procedures.

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.

8.1 Sending aliquot of serum for lipid determination

Serum lipid concentration in serum is determined in an aliquot of the sample (50 µl) using enzymatic methods by the Clinical Chemistry Branch (CCB). Aliquot 50 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.

8.2. Thawing and weighing samples

Store samples in a –70 ^{o}C freezer before starting 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 overnight. 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 +/- 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.

8.3. Sample pretreatment, using Gilson 215 - Liquid handler

Procedure

A. Place new internal standards vials containing the internal standards in the rack on the Gilson 215.
B. Begin the Gilson Spiking Application in Trilution LH. During the procedure all samples are fortified with the internal surrogate standards (Approximately 20 minutes).
C. After completion the Gilson spiking application is complete, the samples are removed from the Gilson and vortexed manually for at least 10 seconds each.
D. Next 0.5mL of hydrochloric acid is added to each sample. All samples are then vortexed again for at least 10 seconds each.
E. To each sample, add 2.5mL isopropanol and vortex for at least 10 seconds each.

8.4. Liquid-Liquid Extraction, using the Gilson 215 Liquid Handler

The extraction procedure is automated using the Gilson 215 Liquid Handler®

The software controlling the Gilson Liquid Handler is called Trilution LH and a shortcut/icon is located on the desktop. After launching the software, the main menu is displayed . For setting up the software for extraction, first click on “Applications” button in the menu. In the Application Menu (Figure 4) select the application named “LLE Extraction – Neutral Fraction Only”. Make sure that number of samples to be extracted is correct for each method in the application. Then click the “Run” button to begin the extraction procedure outlined below. After the first sample transfer step, the samples will be removed from the 818 AutoMix, vortexed, and centrifuged (3min, @2000rpm) to separate the organic/aqueous phases. Then, the samples are placed back in the 818 AutoMix and the Application proceeds with the second transfer of the organic phase.

Check List - Extraction

A. Ensure that sufficient quantities of all solvents and reagents are present in containers under the Gilson 215 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.
B. If necessary, empty waste containers by replacing the container with an empty one.
C. Place the sample tubes in positions 1-30 in the rack in the 818 AutoMix.
D. Place empty 16x125mm tubes in positions 1-30 in the "Sample Extract" rack on the tray.
E. Ensure that the GX Rinse Port is primed with hexane. If necessary, prime the GX Rinse Port using the Manual Control tab in the Application Window.
F. Select the application named "LLE Extraction – Neutral Fraction Only". Click on the "Run" button.
G. The Gilson will add the hexane/MTBE solution to each sample and then mix the samples automatically by rotation via the 818 AutoMix for 20 minutes.
H. After mixing the Gilson will prompt the user to remove the samples and centrifuge them.
I. After centrifuging, the samples are placed back in the rack in the AutoMix and click on the "OK" button on the prompt window in the software.
J. The Gilson will then transfer the organic phase from the original sample tube to the corresponding 16x125mm tube.
K. After transferring all samples, the Gilson will add more hexane/MTBE solution to each original sample tube. The Application will then pause and prompt the user to vortex the samples for at least 10 seconds each.
L. Remove the samples from the AutoMix and mix by vortexing.
M. Place the samples back in the rack in the AutoMix and click the "OK" button to continue the Application.
N. The Gilson will then transfer the organic phase from the original sample tube to the corresponding 16x125mm tube. Then the Application will end.

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

The cleanup procedure is automated using the Rapid Trace® modular SPE system, cf. section 6.2.2).

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

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

Procedure for preparation of cartridges

A. See section 6.1 for Manufacturer, grade and brand for all chemicals used
B. Activate silica gel in oven at 250 ^{o}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 with. 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 2.2 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 0.2 g activated Silica gel (250 ^{o}C) overnight and place another frit on top of the silica
H. Store packed cartridges in a reseal-able plastic bag in dessicator until just prior to use

Setting up the Equipment for Processing Samples (Cleanup)

The software controlling the workstation is launched by the RapidTrace™ Development Icon on the desk top. After launching the software the main menu is displayed (Figure 3). For setting up the software for cleanup click on “Setup Racks”, the menu given in Figure 4 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 CL2to10.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, cf. Figure 5.

Check List - Cleanup

A. Evaporate samples from extraction step to approximately 4mL by placing samples in the Caliper TurboVap evaporator and starting the evaporization with the following settings as a guide: 50deg C water bath temperature and ~5psi line pressure.
B. Using a Pasteur pipette, transfer each sample from the 16x125mm test tube to a 16x100mm test tube. Avoid transferring any water from the 125mm tube to the 100mm tube.
C. Rinse each 125mm tube with approximately 2mL of hexane and transfer this to the 100mm tubes.
D. Evaporate samples to approximately 0.5-1mL by placing samples in the Caliper TurboVap evaporator and using the settings listed above in Step A.
E. Add approximately 2mL DCM to each sample.
F. Evaporate the samples to dryness using the settings listed above in Step A.
G. Make certain that sufficient quantities of the 5% DCM in Hexane solution are present in the solvent bottle under the RapidTrace™ 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.

H. If necessary, empty waste containers by replacing the container with an empty one.
I. Place extracts in racks (one rack per module) on the right hand side of the racks, and remove screw caps.
J. Place collection tubes on the left hand side of the racks.
K. Place racks in tray at the bottom of each module.
L. Assign method to each module by clicking “Setup racks” in the main menu of the RapidTrace™ software and placing method "CL#1ONLY.spe" as sample one for each module used and method "CL2to10.spe" for remaining positions.
M. Exit the setup racks menu by pressing OK.
N. 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.
O. 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.

8.6. Evaporization and transfer to final GC-vial

A. Conduct all in a fume hood or BSC or at the Caliper TurboVap evaporator.
B. Samples from cleanup step are evaporated to approximately 0.5 mL using the Caliper TurboVap evaporator and starting the evaporization with the following settings as a guide: 50deg C water bath temperature and ~5psi line pressure. ***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 8.3. **MAKE CERTAIN THAT THE SAMPLES ARE TRANSFERRED TO THE CORRECT VIAL !!!**
D. Rinse the sample test tube with ~0.5mL of hexane and transfer to the GC-vial
E. Evaporate samples until <1uL remains using the Caliper TurboVap evaporator. Start the evaporization with the following settings as a guide: ~5-10psi line pressure.
F. Complete any lab notes, and bring samples to HR-MS operator.

8.7 GC/IDHRMS analysis of BFRs

GC/IDHRMS analysis is performed on a MAT95XP or DFS (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 260^{o}C, the oven is programmed to increase from 140 ^{o}C (1 min) to 320^{o}C (0 min) with a ramp rate of 10 ^{o}C/min. The source temperature is 290^{o}C in the electron impact mode using a filament bias of 45 eV.

8.8 Final Preparation of GC Vials for PCB Analysis

A. After analysis for BFRs the samples are returned to the Controlled-Air Environment Clean Room. If necessary, reconstitute the samples with nonane to bring the volume back to 10uL.
B. Recap the samples.
C. Bring samples to HR-MS operator for PCB/PP analysis.

8.9 GC/IDHRMS analysis of PCBs/PPs

GC/IDHRMS analysis is performed on a Thermo DFS or MAT95XP (ThermoFinnigan MAT^{TM}, Bremen, Germany) instrument. The chromatographic separations are carried out on an 6890N gas chromatograph (GC) (Agilent Technologies, Atlanta, GA) fitted with a DB5MS [(30-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 260^{o}C, the oven is programmed to increase from 140 ^{o}C (1 min) to 320^{o}C (0 min) with a ramp rate of 10 ^{o}C/min. The source temperature is 300^{o}C in the electron impact mode using a filament bias of 40 eV.

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.

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/µL, 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 3 times the standard deviation of the method blanks prepared with each set of unknown samples. Typical detection limits are shown in Table 3.

Table 3. Typical Limits of Detection (LOD).

Analyte	Average Blank (pg/uL)	LOD (pg/uL)	Analyte	Average Blank (pg/uL)	LOD (pg/uL)
PBDE17	0.1	5.0	PCB153	0.6	5
PBDE28	0.1	5.0	PCB138/158	0.9	5
PBDE47	6.7	26.2	PCB128	0.1	5
PBDE66	0.0	5.0	PCB167	0.3	5
PBDE100	0.1	5.0	PCB178	0.1	5
PBDE99	1.2	9.3	PCB187	0.2	5
PBDE85	0.2	5.0	PCB183	0.0	5
BB153	0.0	5.0	PCB177	0.2	5
PBDE154	0.0	5.0	PCB172	0.1	5
PBDE153	0.1	5.0	PCB180	0.3	5
PBDE183	0.5	5.0	PCB170	0.4	5
PBDE203	0.0	5.0	PCB189	1.1	5
PBDE209	2.3	50.0	PCB199	0.5	5
PCB018	6.5	5	PCB196/203	1.2	5
PCB028	7.0	5	PCB195	0.3	5
PCB052	3.0	5	PCB194	0.9	5
PCB049	1.6	5	PCB206	0.4	5
PCB044	1.6	5	PCB209	1.4	5
PCB074	1.1	5	PCB114	0.2	5
PCB066	1.3	5	PCB123	0.2	5
PCB101	0.8	5	HCB	19.2	28.6
PCB099	0.6	5	B-HCCH	0.1	25.0
PCB087	0.3	5	G-HCCH	0.1	25.0
PCB110	0.9	5	OXYCHLOR	0.1	25.0
PCB118	1.2	5	T-NONA	2.0	25.0
PCB105	1.2	5	PP-DDE	2.4	25.0
PCB151	0.2	5	OP-DDT	0.1	25.0
PCB149	0.2	5	PP-DDT	0.3	25.0
PCB146	0.4	5	MIREX	0.4	25.0

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 are listed in Table 4 below.

Table 4. Mean Concentration and CV for QC samples (QC identifier SSP:01:08).

Analyte	Mean (pg/g fw)	CV	N	Analyte	Mean (pg/g fw)	CV	N
PBDE17	462.7	5.0	39	PCB138/158	839.0	2.7	36
PBDE28	455.7	3.9	39	PCB128	415.8	1.9	36
PBDE47	643.7	6.2	39	PCB167	401.6	1.9	36
PBDE66	448.4	13.9	39	PCB156	412.2	1.9	36
PBDE100	474.6	5.0	39	PCB157	417.3	1.7	36
PBDE99	486.0	4.7	39	PCB178	396.1	3	36
PBDE85	512.4	13.0	39	PCB187	396.3	4.4	36
BB153	425.0	5.2	29	PCB183	393.7	3.7	36
PBDE154	427.5	3.7	29	PCB177	399.1	3.2	36
PBDE153	470.3	3.6	29	PCB172	391.5	2.2	36
PBDE183	413.7	4.3	39	PCB180	429.3	1.7	36
PBDE203	409.0	16.4	39	PCB170	419.5	1.8	36
PBDE209	417.0	3.3	29	PCB189	392.1	2	36
PCB018	399.0	7	36	PCB199	393.2	1.5	36
PCB028	401.9	1.7	36	PCB196/203	753.9	2.3	36
PCB052	408.9	1.9	36	PCB195	414.0	11	36
PCB049	429.8	4.5	36	PCB194	383.0	2.8	36
PCB044	453.8	5.2	36	PCB206	365.4	3.5	36
PCB074	415.4	3.8	36	PCB209	341.2	2.3	36
PCB066	426.2	3.4	36	PCB114	.	.	0
PCB101	410.7	1.8	36	PCB123	.	.	0
PCB099	400.7	1.8	36	HCB	438.8	1.3	36
PCB087	426.0	3.4	36	BHCCH	209.1	3.2	36
PCB110	430.3	3.6	36	GHCCH	374.4	2.7	36
PCB118	426.5	1.9	36	OXYCHLOR	243.2	5.5	36
PCB105	418.1	2.1	36	TNONA	476.6	3.1	36
PCB151	399.6	6.7	36	PPDDE	1265.2	4	36
PCB149	378.8	8.8	36	OPDDT	345.6	4.5	36
PCB146	398.8	2.2	36	PPDDT	248.0	2.7	36
PCB153	443.9	2.4	36	MIREX	399.5	1.3	36

d. Analytical specificity

Isotope Dilution High Resolution Mass Spectrometry (ID-HRMS) coupled with gas chromatography is used for sample analysis. This instrumentation offers a high mass resolution (10,000 resolution) measurement which provides excellent specificity. In addition, two ions are monitored for each native analyte and 13C-labeled internal

standard. For each measurement, the ratio between these two ions is verified to be with +/- 20% from the theoretical isotope ratio. This provides additional confirmation of the identity of the target analyte.

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

10. Quality Assessment and Proficiency Testing

a. Quality Assessment

In this method, a set of samples is defined as 21 unknown samples, prepared and analyzed together with 6 analytical blanks and 3 QC sample. Quality control limits are established by characterizing assay precision with repeated analyses of the QC pool.

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 2σ limits, then accept the run.

(ii) If one or more QC results is outside the 2σ limits, then apply the rules below and reject the run if any conditions are met.

- **Extreme outliner:** the result is outside the characterization mean by more than 4σ.

- **$1_{3\sigma}$**, Average of three QCs is outside of the 3σ limit.

- **$2_{2\sigma}$**, QC results from two consecutive runs are outside of 2σ limit on the same side of the mean.

- **$R_{4\sigma}$ sequential**, QC results from two consecutive runs are outside of 2σ 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", then the results of that analyte for all samples analyzed during that run are considered 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 13C-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 13C-labeled internal standard must be within the range 0.99 – 1.01. For analytes that do not have an identical 13C -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%.

b. Proficiency testing (PT): Currently the only established PT program for this assay is the Arctic 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.

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 a failure of the mass spectrometer or a pipetting error, correct 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 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)

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]

Compound	Median	Quartile Range	<LOD
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 ^{o}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 ^{o}C) until the analytical system is restored to functionality. If long-term interruption (greater that one day) is anticipated, then store serum specimens at -70 $\pm$ 10 ^{o}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.

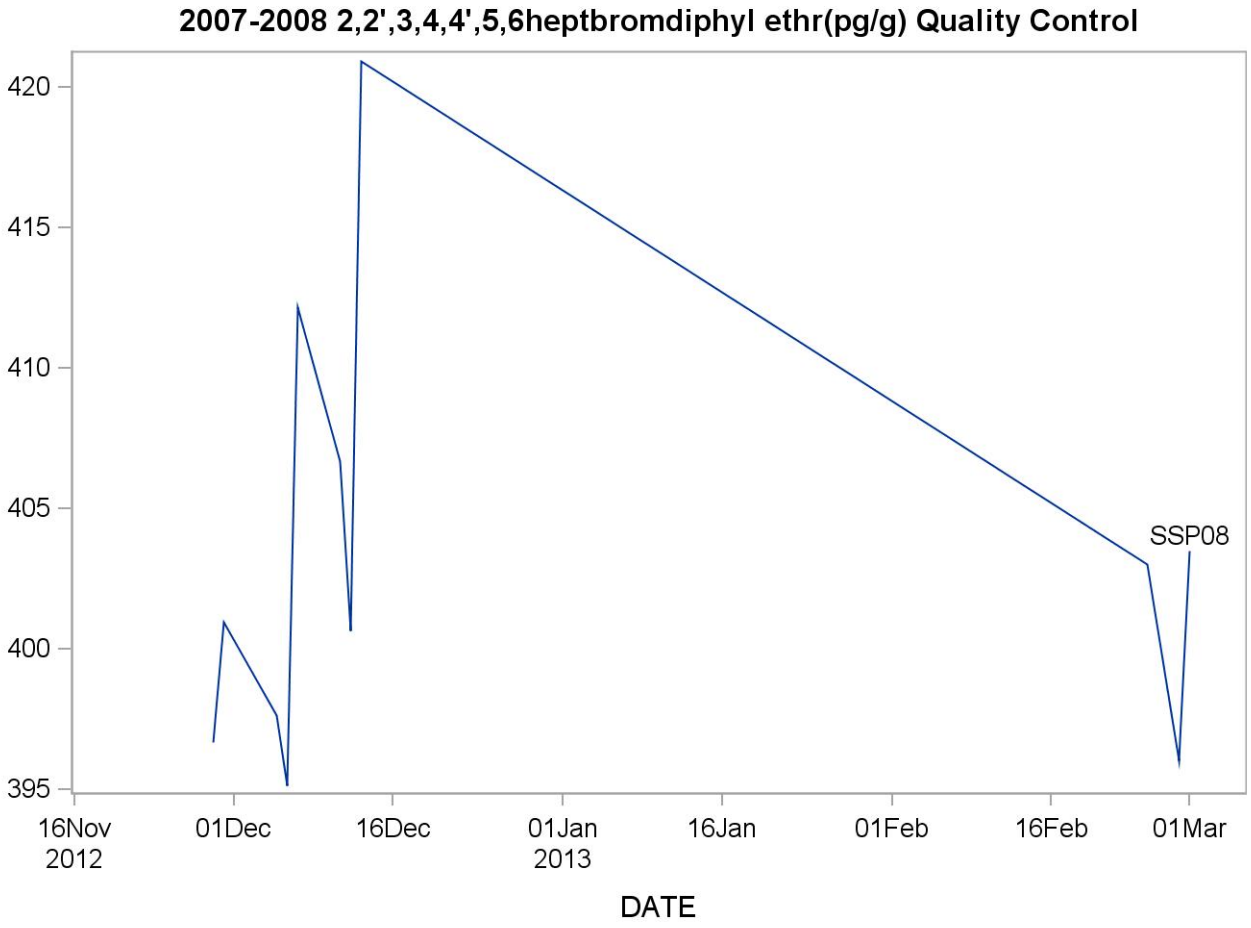
Standard record keeping formats (e.g., database, notebooks, data files) are used to track specimens. Specimens will only be transferred or referred to other DLS Branch laboratories or, if required, to CLIA certified laboratories. Specimens may be stored at the CDC specimen handling and storage facility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

19. Summary Statistics and QC Graphs

See following pages.

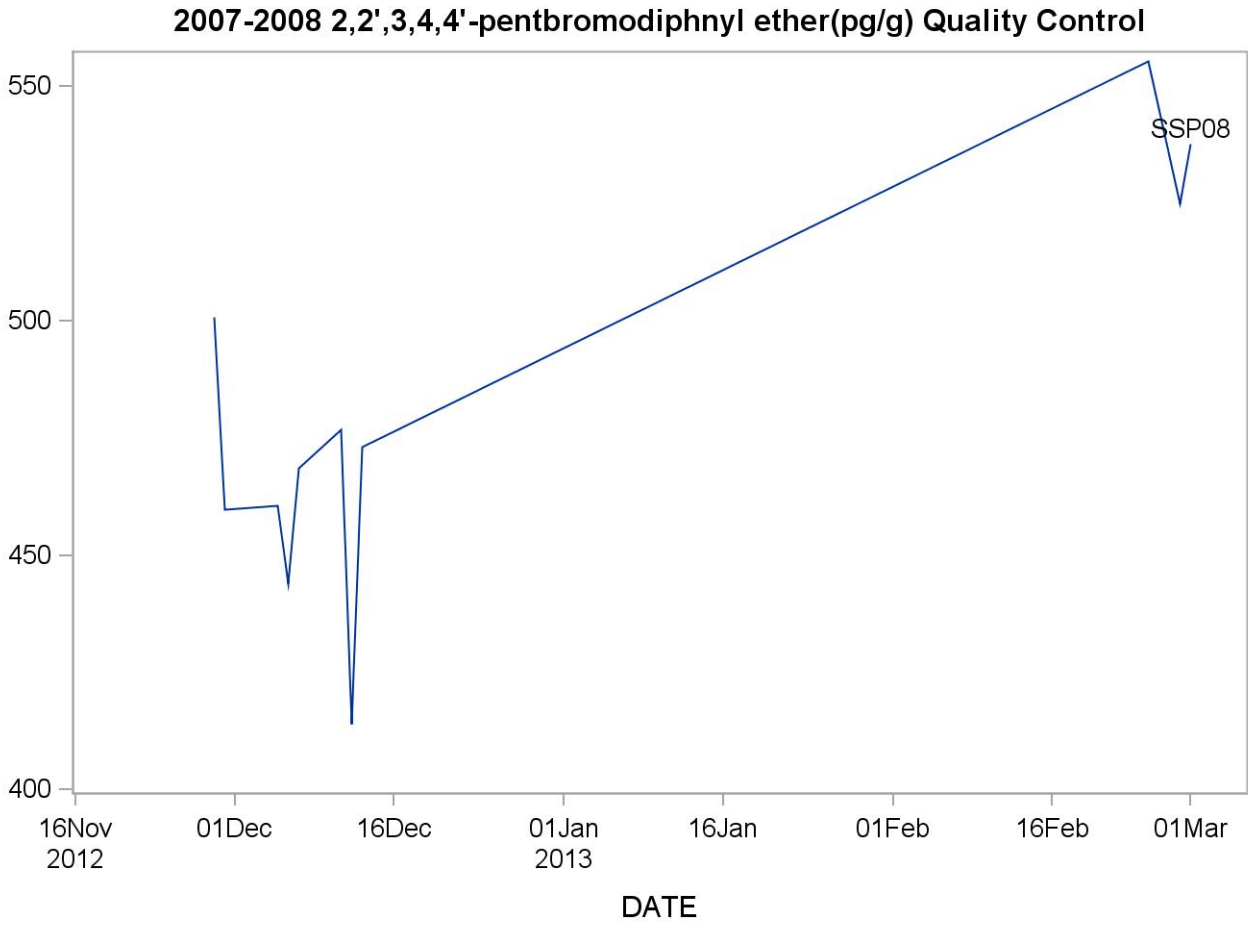
Summary Statistics for 2,2',3,4,4',5,6heptbromdiphyl ethr(pg/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP08	11	29NOV12	01MAR13	403.04	7.80	1.9



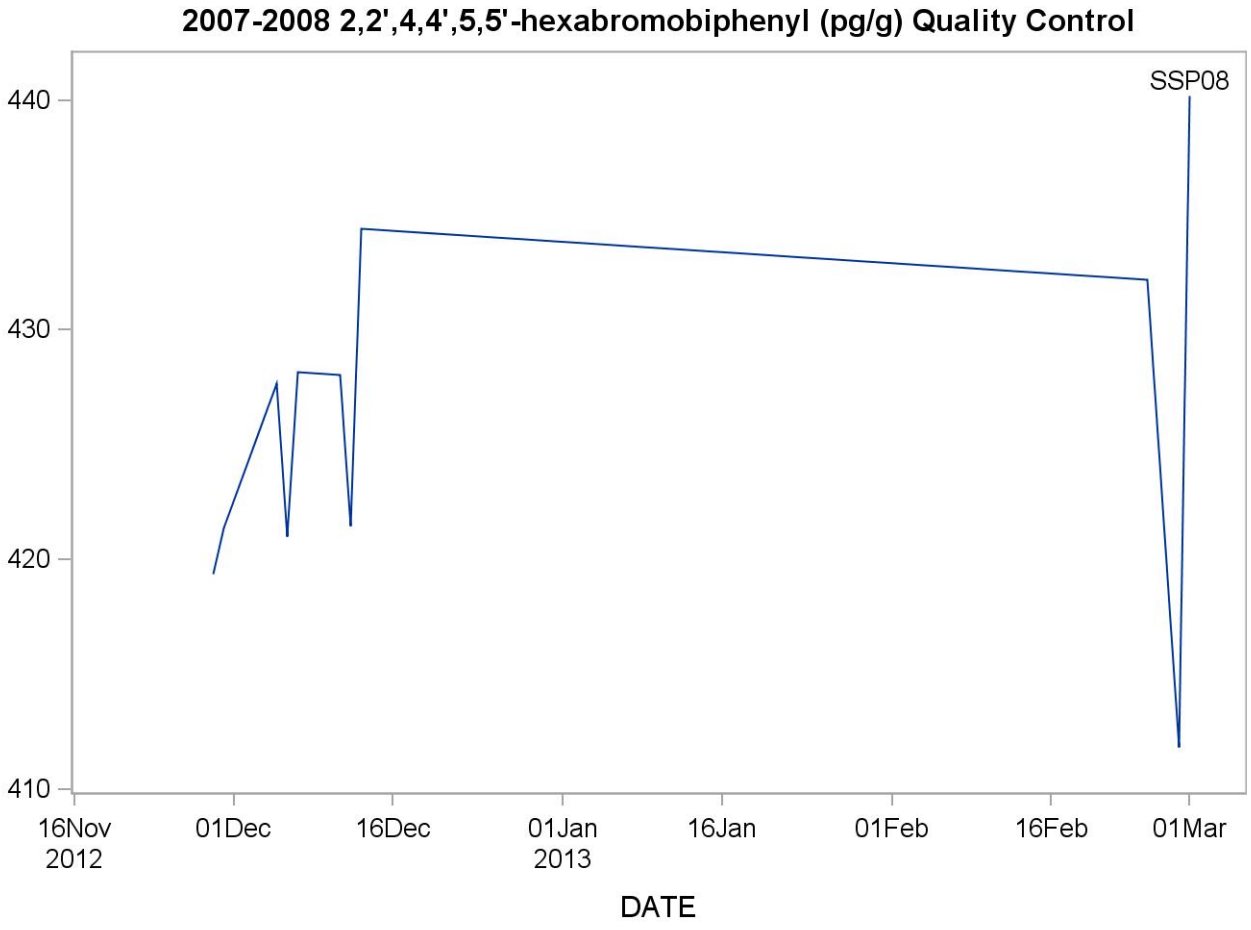
Summary Statistics for 2,2',3,4,4'-pentbromodiphnyl ether(pg/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP08	11	29NOV12	01MAR13	483.24	42.44	8.8



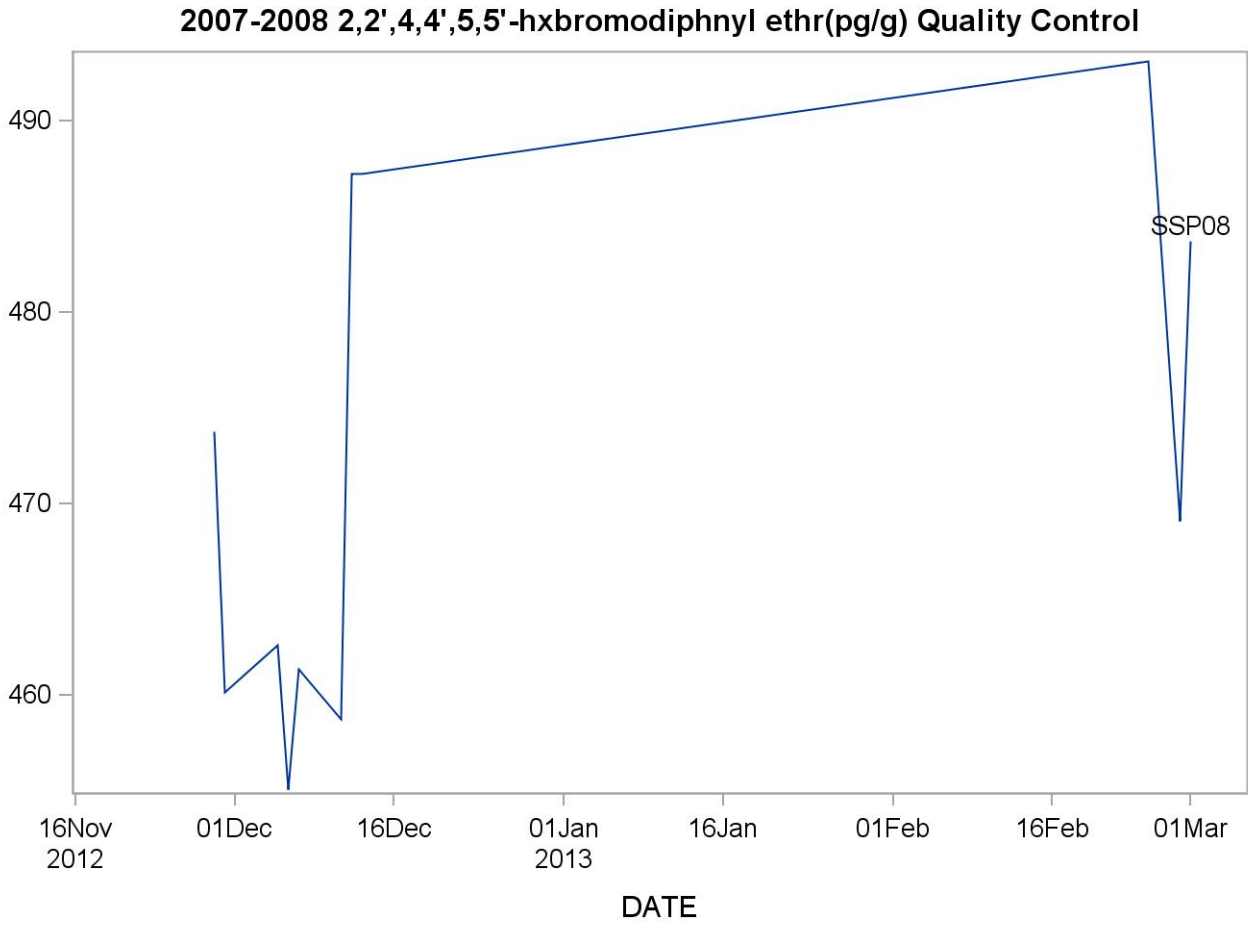
Summary Statistics for 2,2',4,4',5,5'-hexabromobiphenyl (pg/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP08	11	29NOV12	01MAR13	425.99	7.95	1.9



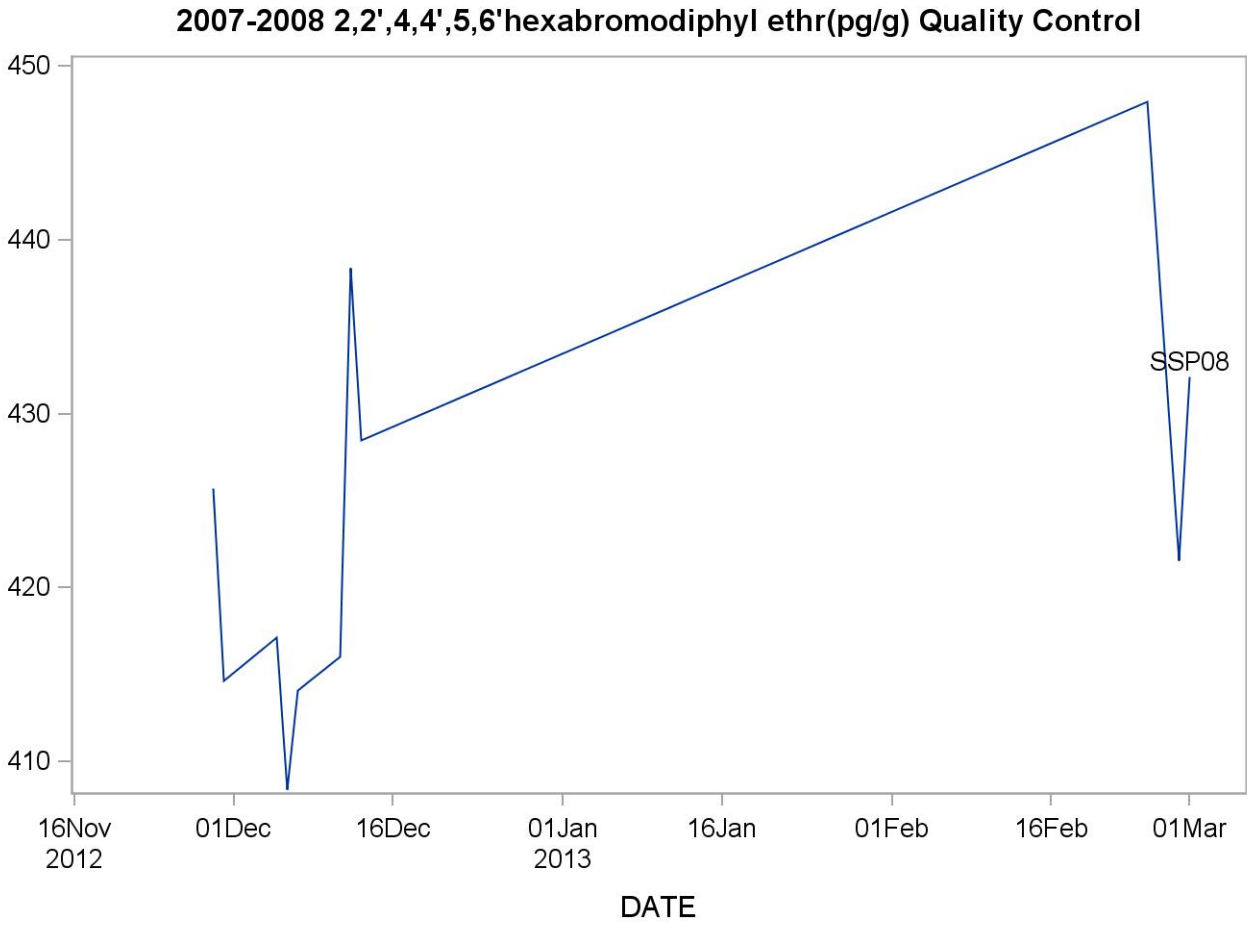
Summary Statistics for 2,2',4,4',5,5'-hxbromodiphnyl ethr(pg/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP08	11	29NOV12	01MAR13	472.02	13.65	2.9



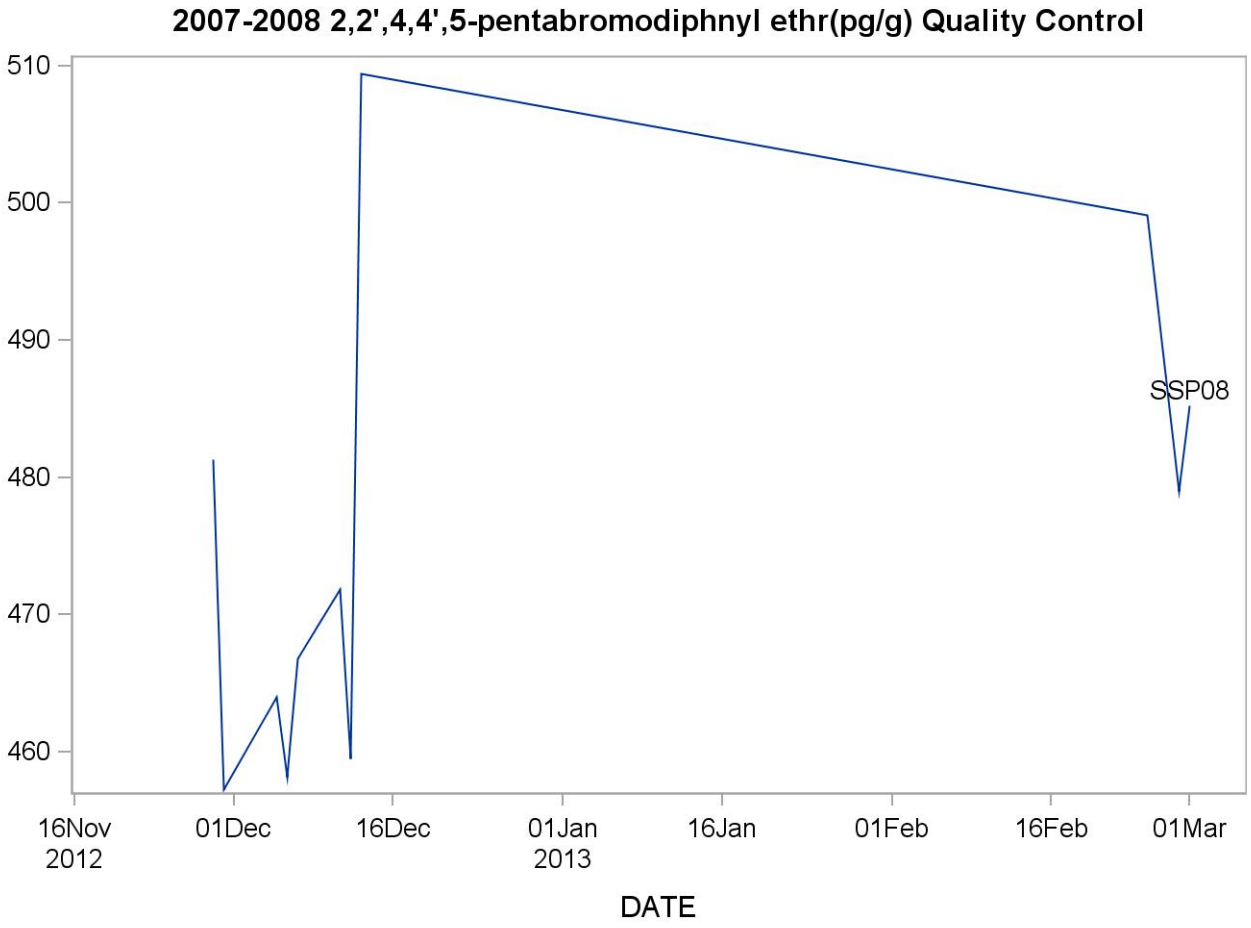
Summary Statistics for 2,2',4,4',5,6'hexabromodiphyl ethr(pg/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP08	11	29NOV12	01MAR13	424.06	11.90	2.8



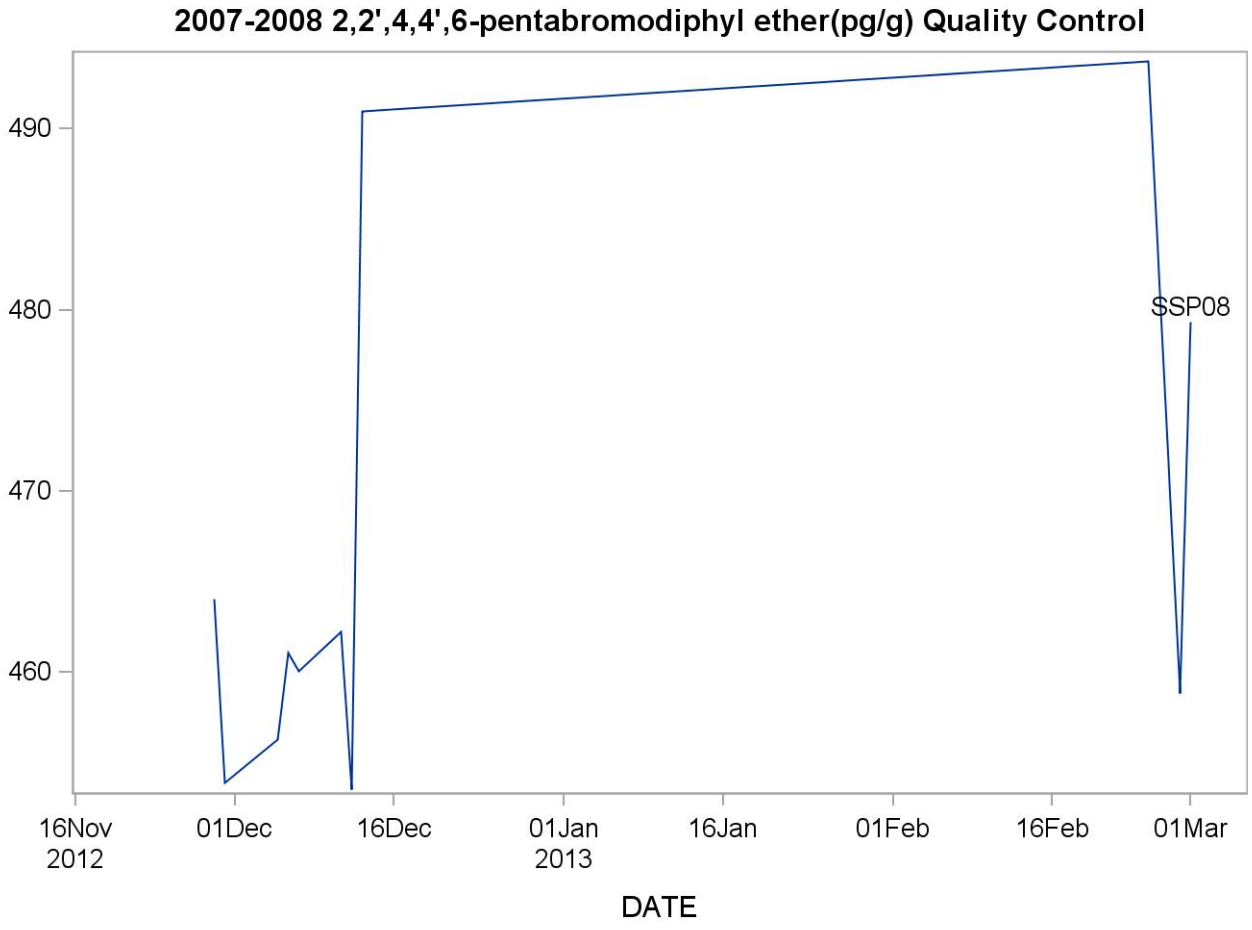
Summary Statistics for 2,2',4,4',5-pentabromodiphnyl ethr(pg/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP08	11	29NOV12	01MAR13	475.60	17.21	3.6



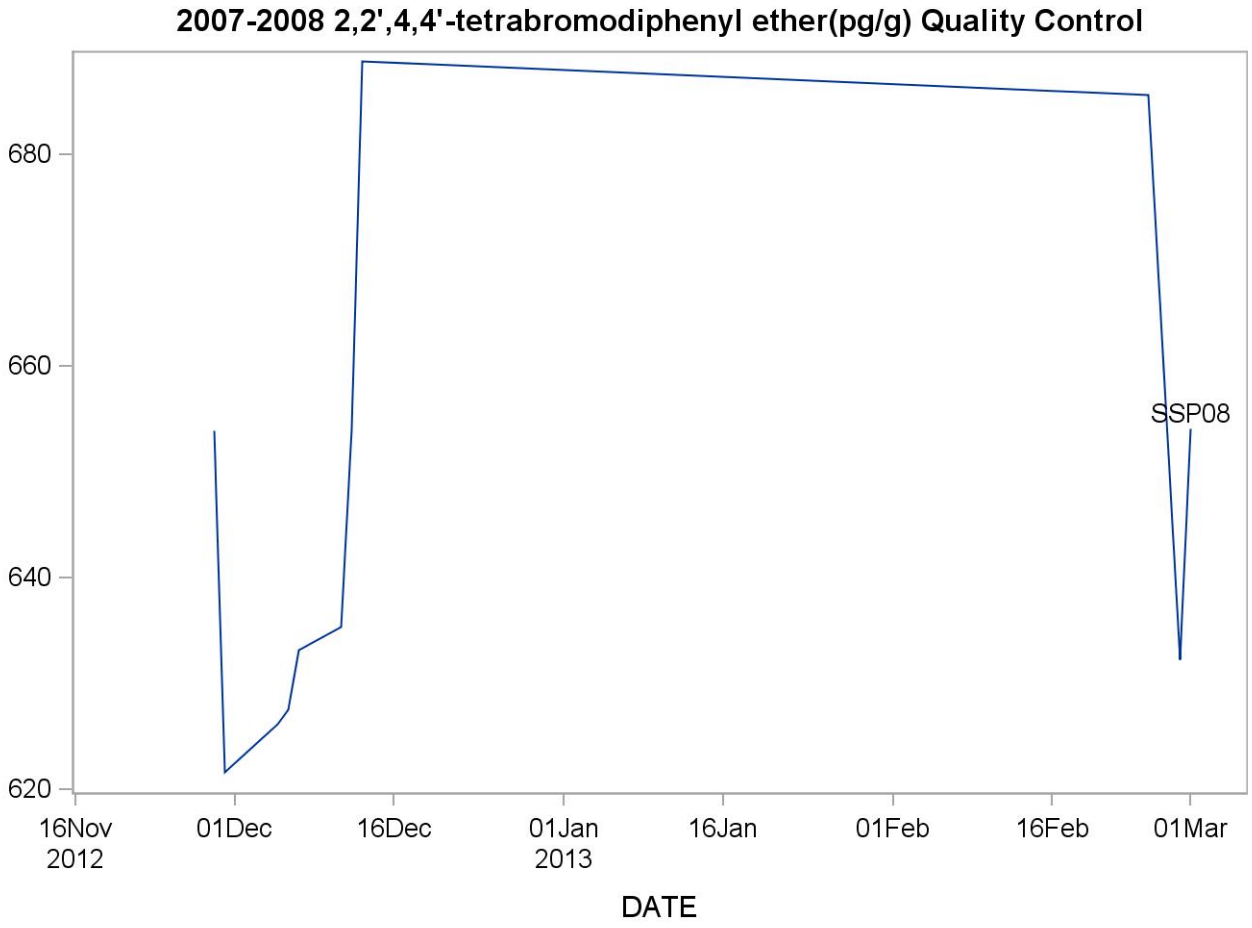
Summary Statistics for 2,2',4,4',6-pentabromodiphyl ether(pg/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP08	11	29NOV12	01MAR13	466.74	14.45	3.1



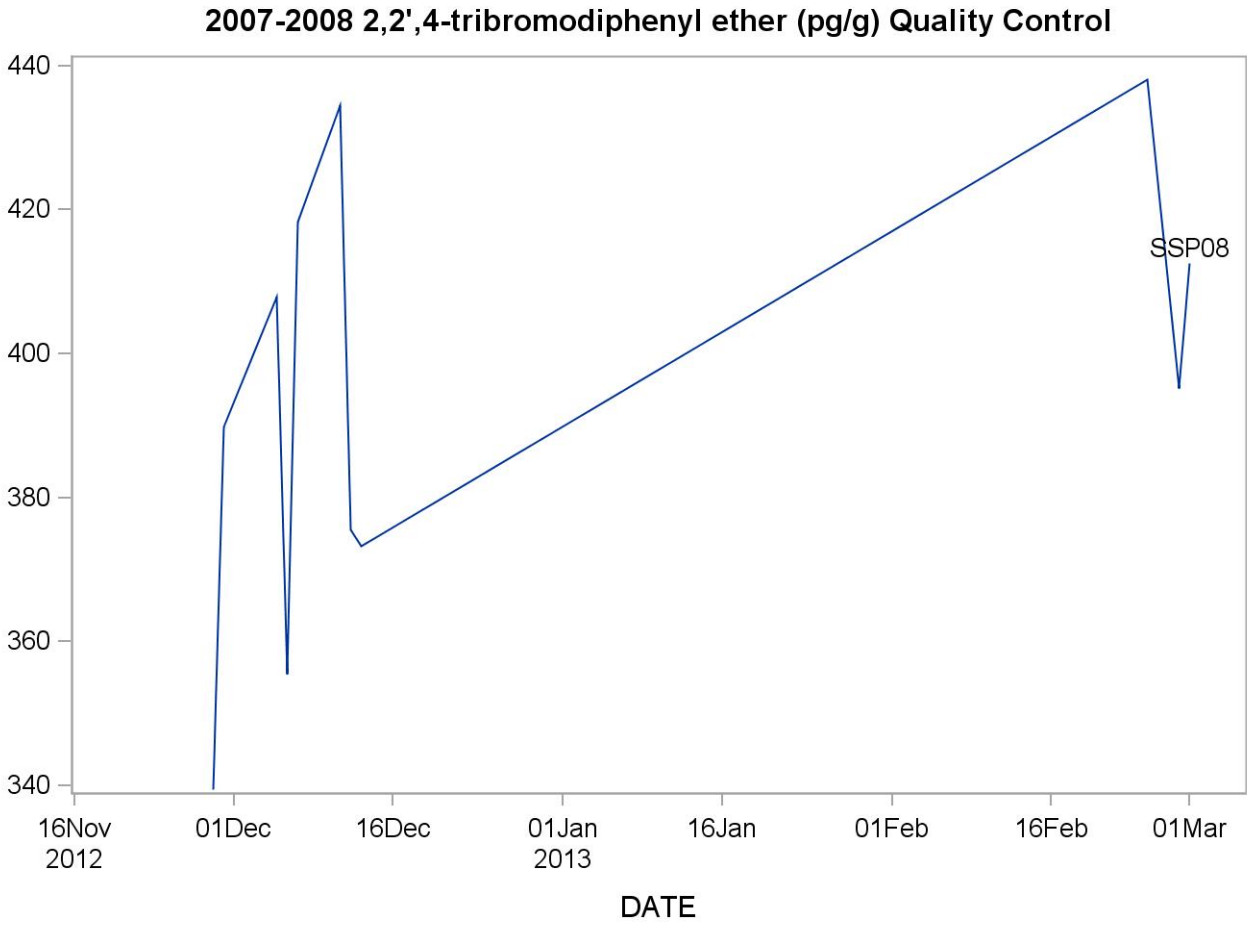
Summary Statistics for 2,2',4,4'-tetrabromodiphenyl ether(pg/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP08	11	29NOV12	01MAR13	646.62	23.20	3.6



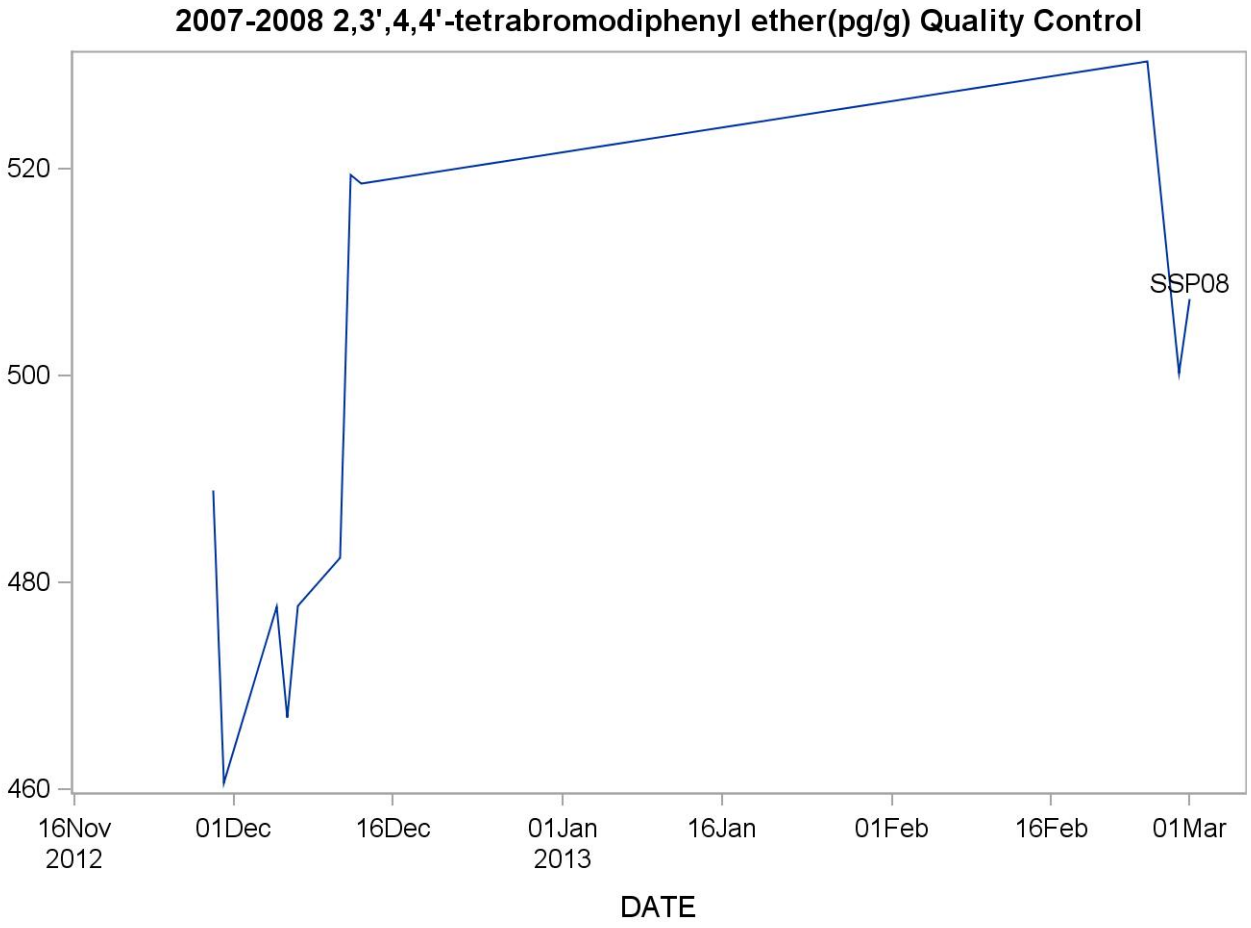
Summary Statistics for 2,2',4-tribromodiphenyl ether (pg/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP08	11	29NOV12	01MAR13	394.57	31.58	8.0



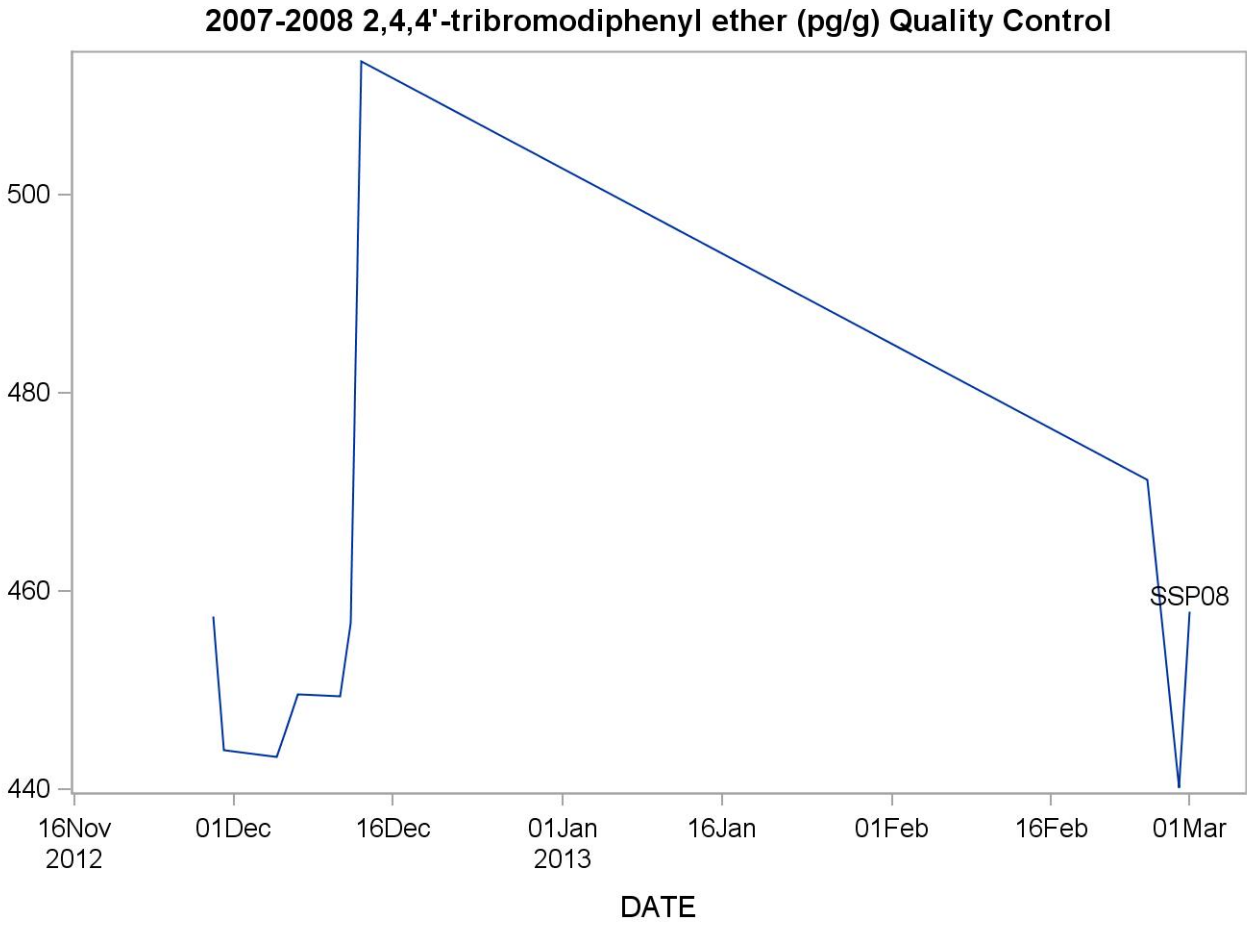
Summary Statistics for 2,3',4,4'-tetrabromodiphenyl ether(pg/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP08	11	29NOV12	01MAR13	493.73	23.10	4.7



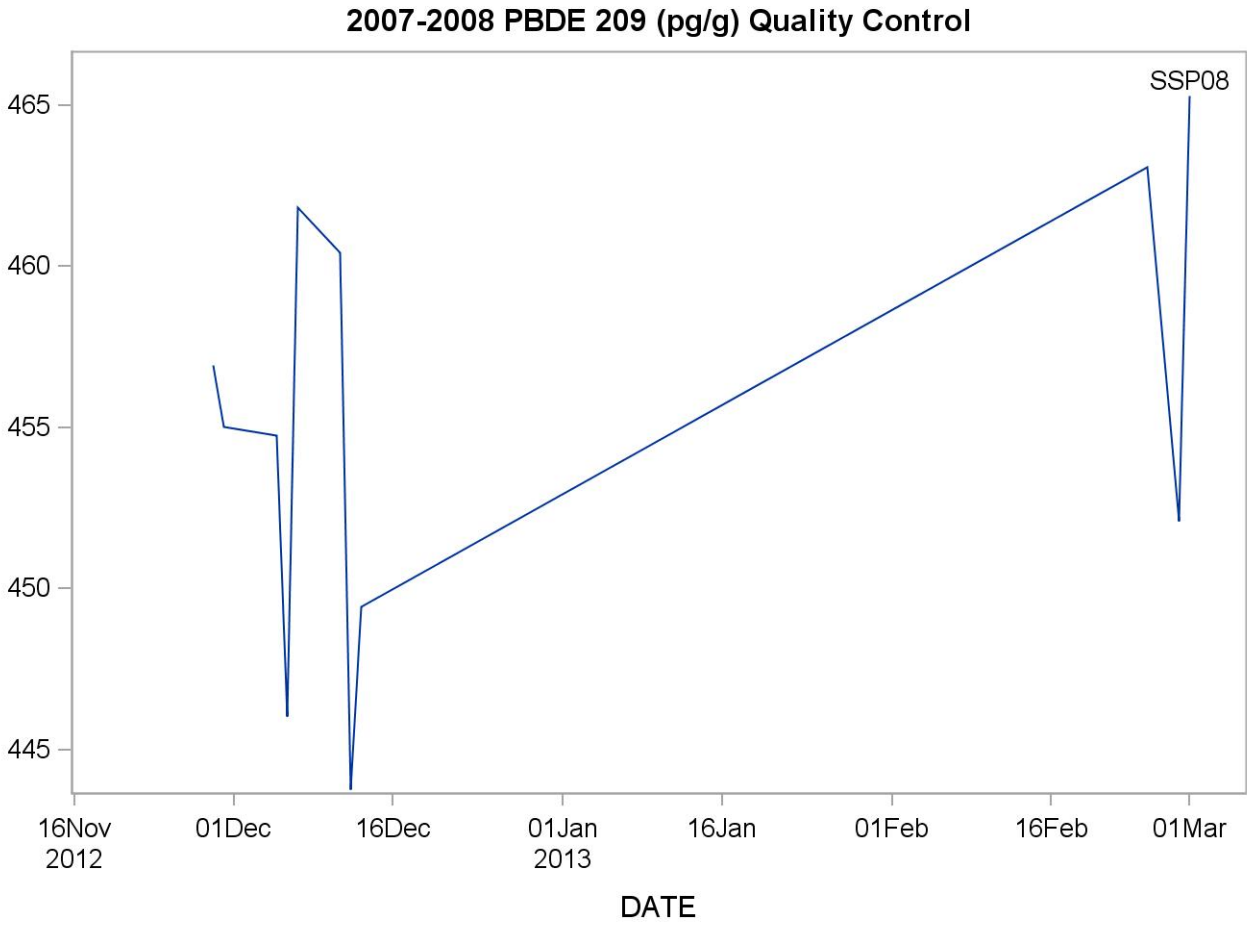
Summary Statistics for 2,4,4'-tribromodiphenyl ether (pg/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP08	11	29NOV12	01MAR13	457.25	20.63	4.5



Summary Statistics for PBDE 209 (pg/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP08	11	29NOV12	01MAR13	455.34	7.05	1.5



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