



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: **6701.04**

Revised: **June 17, 2016**

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.

Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table:

Lab Name	Analyte	SAS label
PSTPOL_F	LBCBHC	Beta-hexachlorocyclohexane (ng/g)
	LBCGHC	Gamma-hexachlorocyclohexane (ng/g)
	LBCHCB	Hexachlorobenzene (ng/g)
	LBCMIR	Mirex (ng/g)
	LBCOXY	Oxychlorane (ng/g)
	LBCPDE	p,p'-DDE (ng/g)
	LBCPDT	p,p'-DDT (ng/g)
	LBCTNA	trans-Nonachlor (ng/g)

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- A Ion Volume Cleaning Procedure for DFS instruments
- B Typical accurate masses, target isotopic ratios, ¹³C label standard used, selected ion monitoring window (SIM) and lock and calibration masses used for high resolution isotope dilution measurements of polychlorinated (PCDD/F) and coplanar polychlorinated biphenyls (cPCBs). Also given are calibration curve range and sample quality control (QC) criteria, i.e, relative retention time and recovery.

1. Clinical Relevance and Summary of Test Principle

1.1. Clinical Relevance

Organohalogen compounds may be characterized as halogen substituted hydrocarbons, neutral and lipophilic organic compounds that are only very slowly degraded or transformed under environmental conditions. According to the United Nations Environmental Program, 12 polychlorinated compounds or compound groups have been defined as persistent organic pollutants (POPs), including polychlorinated biphenyls (PCBs) and 2,2-bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT) [1]. The physicochemical properties of such man-made chemicals have led to their accumulation in fatty tissues of wildlife and humans. This behavior of POPs was basically unknown at the time of World War II, when the chemical industry developed these substances and made them available in increasing quantities. Organohalogen compounds were commercially produced for use in agricultural, industrial and/or household applications, while others were formed unintentionally during municipal waste incineration, in other combustion and thermal processes or as by-products in the chemical industry. For example, PCB products are industrial chemicals that were used as dielectric and heat-exchange fluids, as sealants and much more [2]. DDT was applied as a pesticide, in agriculture and household applications [3].

The environmental implications first of DDT and later of PCB were not realized until the 1960s, when DDT and also PCBs were detected at high concentrations (several hundred to a few thousand ppm) in wildlife from the Baltic Sea region [3;4]. These high concentrations of DDT; 2,2-bis(4-chlorophenyl)-1,1-dichloroethene (DDE), and PCB were later found to correlate with toxicological effects observed in e.g. white-tailed sea eagles [5] and seals living in the Baltic Sea region [6-8]. However, 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. This illustrates the research needs about environmental issues and persistent pollutants to hopefully avoid future problems similar to those caused by PCB and DDT including bioaccumulation and biomagnifications in fatty tissues.

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 [9;10]. Hence humans may be exposed through food and/or through contact with flame retarded products [11-13]. Increasing PBDE levels have been observed in mothers' milk from Sweden [14] as well as in blood from Germany [15] and Norway [16]. The PBDE levels are in general lower than that of polychlorinated biphenyls (PCBs) in Europe [13;17]. However, the PBDE concentrations found in the North Americans are considerably higher compared to European subjects [11;13;17;18]. The PBDEs are dominated by 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) [11;13;17;18]. 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 BDE-209 has a fairly short half-life of approximately two weeks [19].

PBDEs have in pregnant mice been shown to cause neurodevelopmental disorders in the offspring, as measured by behavioral test systems [20;21]. Neurodevelopmental disorders in relation to exposure to PBDEs in humans has to date not been assessed, although, such investigations are currently ongoing

Polybrominated biphenyls (PBBs) are another type of chemicals that in the past has been used and applied for similar application areas as PBDEs [9;22]. No known commercial production of PBBs currently exists. HexaBB has in humans been shown to have a half-life of approximately 30 years [23].

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 automated liquid/liquid extraction 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 concentration 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 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) methanol 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; 10 mL) of the extract through a column containing from the top 0.25 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.

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.

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 to 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 (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

3.1. Data Entry and Transfer

Sample analysis results generated by this method are stored in SAS and/or 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.

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.
- Minimum preferred serum amount is 0.5grams and the minimum acceptable amount is 0.125 grams.
- Acceptable containers for storage are thick-walled glass vials with Teflon™-lined caps or cryovials or equivalent container. 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 (< 0.125 g) or suspected contamination due to improper collection procedures or collection devices. In all such cases, request a second serum specimen. The limit of detection for the minimum acceptable serum amount 0.125 to 2 g of serum is given in Table 1.
- Transport and ship frozen serum specimens on dry ice. Upon receipt, they must be kept frozen at ≤ -60 °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.

Table 1. Method limit of detection (LOD, pg/gram of serum) by target analyte and used sample amount (gram). The method LOD corresponding to the minimum preferred sample amount of 0.5 grams are colored in blue, method LODs between the minimum preferred sample amount and the minimum acceptable sample size are colored in red. Method LODs two and four fold higher than the minimum preferred sample amount are colored in green. A sample amount greater than the minimum preferred sample amount may be used to lower the method LOD. Any sample for which the available serum amount for measurement is less than the minimum acceptable serum amount of 0.125grams will be reported as QNS (Quantify Not Sufficient) in reportable data tables.

Class	Analyte	Serum	Method LOD	Class	Analyte	Serum	Method LOD
		Weight (g)	(pg/g serum) ^a			Weight (g)	(pg/g serum) ^a
BFR	PBDE17	0.125	8.8	BFR	PBDE99	0.125	40
		0.25	4.4			0.25	20
		0.375	2.9			0.375	13
		0.5	2.2			0.5	10
		1	1.1			1	5
		2	0.55			2	2.5
BFR	PBDE28	0.125	14	BFR	PBDE100	0.125	21
		0.25	6.8			0.25	10
		0.375	4.5			0.375	6.9
		0.5	3.4			0.5	5.2
		1	1.7			1	2.6
		2	0.85			2	1.3
BFR	PBDE47	0.125	88	BFR	PBDE153	0.125	96
		0.25	44			0.25	48
		0.375	29			0.375	32
		0.5	22			0.5	24
		1	11			1	12
		2	5.5			2	6
BFR	PBDE66	0.125	29	BFR	PBDE154	0.125	22
		0.25	14			0.25	11
		0.375	9.6			0.375	7.2
		0.5	7.2			0.5	5.4
		1	3.6			1	2.7
		2	1.8			2	1.4
BFR	PBDE85	0.125	17	BFR	PBDE183	0.125	1000
		0.25	8.4			0.25	520
		0.375	5.6			0.375	350
		0.5	4.2			0.5	260
		1	2.1			1	130
		2	1.1			2	65

^a Method LOD defined the higher value of S_0 (Taylor, K. T. (1987) In Quality Assurance of Chemical Measurements, pp 79-82, Lewis Publishers, Washington, DC) and three times the standard deviation of blank samples. Method LOD determination based on generated measurements during 2015 and 1st and 2nd quarter of 2016.

Table 1 (Continued). Method limit of detection (LOD, pg/gram of serum) by target analyte and used sample amount (gram). The method LOD corresponding to the minimum preferred sample amount of 0.5 grams are colored in blue, method LODs between the minimum preferred sample amount and the minimum acceptable sample size are colored in red. Method LODs two and four fold higher than the minimum preferred sample amount are colored in green. A sample amount greater than the minimum preferred sample amount may be used to lower the method LOD. Any sample for which the available serum amount for measurement is less than the minimum acceptable serum amount of 0.125grams will be reported as QNS (Quantify Not Sufficient) in reportable data tables.

Class	Analyte	Serum	Method LOD	Class	Analyte	Serum	Method LOD
		Weight (g)	(pg/g serum) ^a			Weight (g)	(pg/g serum) ^a
BFR	PBDE209	0.125	180	PCB	PCB99	0.125	56
		0.25	92			0.25	28
		0.375	61			0.375	19
		0.5	46			0.5	14
		1	23			1	7
		2	12			2	3.5
BFR	PBB153	0.125	11	PCB	PCB105	0.125	60
		0.25	5.6			0.25	30
		0.375	3.7			0.375	20
		0.5	2.8			0.5	15
		1	1.4			1	7.5
		2	0.7			2	3.8
PCB	PCB28	0.125	75	PCB	PCB114	0.125	19
		0.25	38			0.25	9.6
		0.375	25			0.375	6.4
		0.5	19			0.5	4.8
		1	9.4			1	2.4
		2	4.7			2	1.2
PCB	PCB66	0.125	68	PCB	PCB118	0.125	69
		0.25	34			0.25	34
		0.375	23			0.375	23
		0.5	17			0.5	17
		1	8.5			1	8.6
		2	4.3			2	4.3
PCB	PCB74	0.125	68	PCB	PCB138-158	0.125	120
		0.25	34			0.25	60
		0.375	23			0.375	40
		0.5	17			0.5	30
		1	8.5			1	15
		2	4.3			2	7.5

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Class	Analyte	Serum	Method LOD	Class	Analyte	Serum	Method LOD
		Weight (g)	(pg/g serum) ^a			Weight (g)	(pg/g serum) ^a
PCB	PCB146	0.125	51	PCB	PCB170	0.125	53
		0.25	26			0.25	26
		0.375	17			0.375	18
		0.5	13			0.5	13
		1	6.4			1	6.6
		2	3.2			2	3.3
PCB	PCB153	0.125	70	PCB	PCB172	0.125	14
		0.25	35			0.25	7.2
		0.375	23			0.375	4.8
		0.5	18			0.5	3.6
		1	8.8			1	1.8
		2	4.4			2	0.9
PCB	PCB156	0.125	62	PCB	PCB177	0.125	17
		0.25	31			0.25	8.4
		0.375	21			0.375	5.6
		0.5	15			0.5	4.2
		1	7.7			1	2.1
		2	3.9			2	1.1
PCB	PCB157	0.125	56	PCB	PCB178	0.125	51
		0.25	28			0.25	26
		0.375	19			0.375	17
		0.5	14			0.5	13
		1	7			1	6.4
		2	3.5			2	3.2
PCB	PCB167	0.125	54	PCB	PCB180	0.125	59
		0.25	27			0.25	30
		0.375	18			0.375	20
		0.5	14			0.5	15
		1	6.8			1	7.4
		2	3.4			2	3.7

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Table 1 (Continued). Method limit of detection (LOD, pg/gram of serum) by target analyte and used sample amount (gram). The method LOD corresponding to the minimum preferred sample amount of 0.5 grams are colored in blue, method LODs between the minimum preferred sample amount and the minimum acceptable sample size are colored in red. Method LODs two and four fold higher than the minimum preferred sample amount are colored in green. A sample amount greater than the minimum preferred sample amount may be used to lower the method LOD. Any sample for which the available serum amount for measurement is less than the minimum acceptable serum amount of 0.125grams will be reported as QNS (Quantify Not Sufficient) in reportable data tables.

Class	Analyte	Serum	Method LOD	Class	Analyte	Serum	Method LOD
		Weight (g)	(pg/g serum) ^a			Weight (g)	(pg/g serum) ^a
PCB	PCB183	0.125	54	PCB	PCB199	0.125	54
		0.25	27			0.25	27
		0.375	18			0.375	18
		0.5	14			0.5	14
		1	6.8			1	6.8
		2	3.4			2	3.4
PCB	PCB187	0.125	54	PCB	PCB206	0.125	80
		0.25	27			0.25	40
		0.375	18			0.375	27
		0.5	13			0.5	20
		1	6.7			1	10
		2	3.4			2	5
PCB	PCB189	0.125	58	PCB	PCB209	0.125	52
		0.25	29			0.25	26
		0.375	19			0.375	17
		0.5	15			0.5	13
		1	7.3			1	6.5
		2	3.7			2	3.3
PCB	PCB194	0.125	55	PST	HCB	0.125	110
		0.25	28			0.25	56
		0.375	18			0.375	37
		0.5	14			0.5	28
		1	6.9			1	14
		2	3.5			2	7
PCB	PCB196-203	0.125	100	PST	B-HCCH	0.125	220
		0.25	52			0.25	110
		0.375	35			0.375	75
		0.5	26			0.5	56
		1	13			1	28
		2	6.5			2	14

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Class	Analyte	Serum	Method LOD	Class	Analyte	Serum	Method LOD
		Weight (g)	(pg/g serum) ^a			Weight (g)	(pg/g serum) ^a
PST	G-HCCH	0.125	63	PST	PP-DDT	0.125	140
		0.25	32			0.25	68
		0.375	21			0.375	45
		0.5	16			0.5	34
		1	7.9			1	17
		2	4			2	8.5
PST	OXYCHLOR	0.125	58	PST	MIREX	0.125	60
		0.25	29			0.25	30
		0.375	19			0.375	20
		0.5	14			0.5	15
		1	7.2			1	7.5
		2	3.6			2	3.8
PST	T-NONA	0.125	71				
		0.25	36				
		0.375	24				
		0.5	18				
		1	8.9				
		2	4.5				
PST	PP-DDE	0.125	260				
		0.25	130				
		0.375	85				
		0.5	64				
		1	32				
		2	16				
PST	OP-DDT	0.125	48				
		0.25	24				
		0.375	16				
		0.5	12				
		1	6				
		2	3				

^a Method LOD defined the higher value of S_0 (Taylor, K. T. (1987) In Quality Assurance of Chemical Measurements, pp 79-82, Lewis Publishers, Washington, DC) and three times the standard deviation of blank samples. Method LOD determination based on generated measurements during 2015 and 1st and 2nd quarter of 2016.

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 consumables listed in Table 2 and 3. Other manufacturer's products of equivalent purity can be used after verification of chemicals purity.

Table 2. 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 / LABSOLV	Pesticide
Dodecane	EM Science	min 99%
Hexane	TEDIA / LABSOLV	Pesticide
Methyl <i>tert</i> -Butyl Ether (MTBE)	TEDIA / LABSOLV	Pesticide
Methanol	TEDIA / LABSOLV	Pesticide
n-Nonane	Sigma	99%
Water	TEDIA / LABSOLV	Pesticide
SPE sorbents		
Silica gel	UCT	100-200 mesh

Table 3. 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 Glass Pasteur 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 Consumables 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 or equivalent dish washer). 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**”.

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 septa: 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 cannot 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 (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 aluminum foil. The caps for GC vials are cleaned by Soxhlet 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 °C overnight. After completing the heating cycle for at least 12 hours, the pipets are ready to be used.

6.1.2 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 ¹³C₁₂-labeled PBDE and PBB congeners. The PCB standard contains 7.5 pg/μL of 21 different PCB congeners and the PP standard contains 11 ¹³C-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)

6.1.3 Recovery standard (RS)

Use one recovery standard for measurement of recovery. This standard contains 1234-¹³C₆-TCDD (2.5 pg/μL), ¹³C₁₂-CB-208 (10.0pg/μL) and ¹³C₁₂-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.

6.1.4 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 ampoules.

When opening a new ampoule, aliquot the standard into GC vials (5-10uL in each vial). Label the vials BFRX, PCBX, and PSTX where X corresponds to the calibration point 1through 10 using a computer-generated label. Replace the standards used for calibration of the DFS 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, methanol 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 in Figure 1. 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.



Figure 1. Gilson 215 Liquid Handler used for automated additions of internal surrogate standards and water to the serum samples with mixing by rotation in-between the additions. This equipment also adds recovery standard to GC vials.

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.

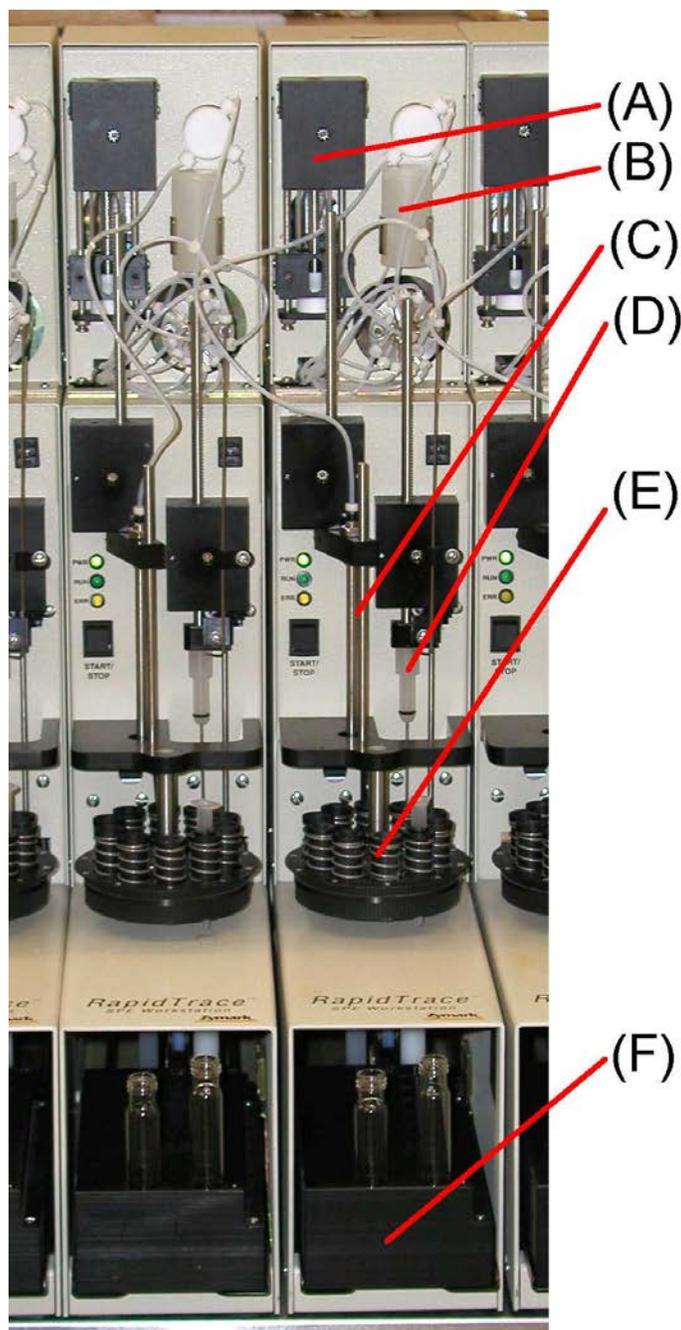


Figure 2. Rapid Trace modular SPE work station up to 10 modules controlled by one computer. Instrument includes (A) syringe pump, (B) mixing chamber [not used in current method], (C) plunger, (D) cannula and (F) sample and fraction collection rack.

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. Example QC rules are below. All QC rules are checked by the DLS QC program.

- (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 $\pm 2SD$. 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 °C) until the serum is completely thawed. 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

7.1. Calibration of Mass Spectrometer

Calibrate and tune the Thermo 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.

Sensitivity Check prior to analytical run:

- **BFRs:** After tuning the instrument to 10,000 resolution, a greater than 10:1 signal to noise ratio for the native ions is required for an injected CS1 standard (0.2pg/uL) except PBDE209 which needs to meet a signal to noise ratio of 10:1 for the CS4 standard (5pg/uL).

- **PCB/PST:** After tuning the instrument to 10,000 resolution a 100:1 signal to noise ratio for the injection of 0.01pg/ul of 2378-tetrachloro-p-dibenzodioxin (TCDD) with a 2ul injection (20fg on-column).

The GC program used for S/N function check are:

- Start at 140 °C with a hold of 2 minute
- Then ramp to 220°C (30°C/minute) and hold for 2 minutes
- Then ramp to 240°C (15°C/minute) and hold for 5 minutes

Mass Spectrometer gain checks are performed when the multiplier is replaced or as needed. A Magnetic Calibration (MCAL) is performed during routine PMs (preventative maintenance) and/or as needed. An Electric Calibration (ECAL) is performed during routine PMs and/or as needed. Routine PM are to be performed once or more per year.

7.2. Creation of Calibration Curve

A linear calibration curve, consisting of at least five 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 0.995. 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 ¹³C-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.

7.4. Standard concentrations and target isotopic ratios

The specified concentration for analytical standards and target isotopic ratios for all measured analytes are given in Appendix A.

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 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 (100 μ l) using enzymatic methods by the Clinical Chemistry Branch (CCB). Aliquot 100 μ l 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. A lipid aliquot may have been drawn upon arrival of the samples to CDC and prior to the samples being sent to the POPs laboratory in which case no additional lipid aliquot needs to be made prior to analysis.

If the available sample amount is low (<1mL of serum) then the entire sample may be sent to CCB for lipids measurements to minimize losses of serum during aliquoting. In this case the sample is returned to the POPs lab upon completion of the lipids measurements.

8.2. Thawing and weighing samples

Store samples in a -70 °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 spanning the range 1.000 g and 10.000 g before weighing each batch of samples and document recorded weights on the run sheet in the "Balance Calibration" section. 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. Adjust the volume of the sample to 2mL if less serum was available for the measurement.
- B. Place new internal standards vials containing the internal standards in the rack on the Gilson 215.
- C. Begin the Gilson Spiking Application in Trilution LH. During the procedure all samples are fortified with the internal surrogate standards (Approximately 20 minutes).
- D. After completion the Gilson spiking application is complete, the samples are removed from the Gilson and vortexed manually for at least 10 seconds each.
- E. Next 0.5mL of 6M hydrochloric acid is added to each sample. All samples are then vortexed again for at least 10 seconds each.
- F. To each sample, add 2.5mL methanol 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 (Figure 3). 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 Methanol 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.



Figure 3. Detail of the Trilution Main Menu. A: The Application Menu button.

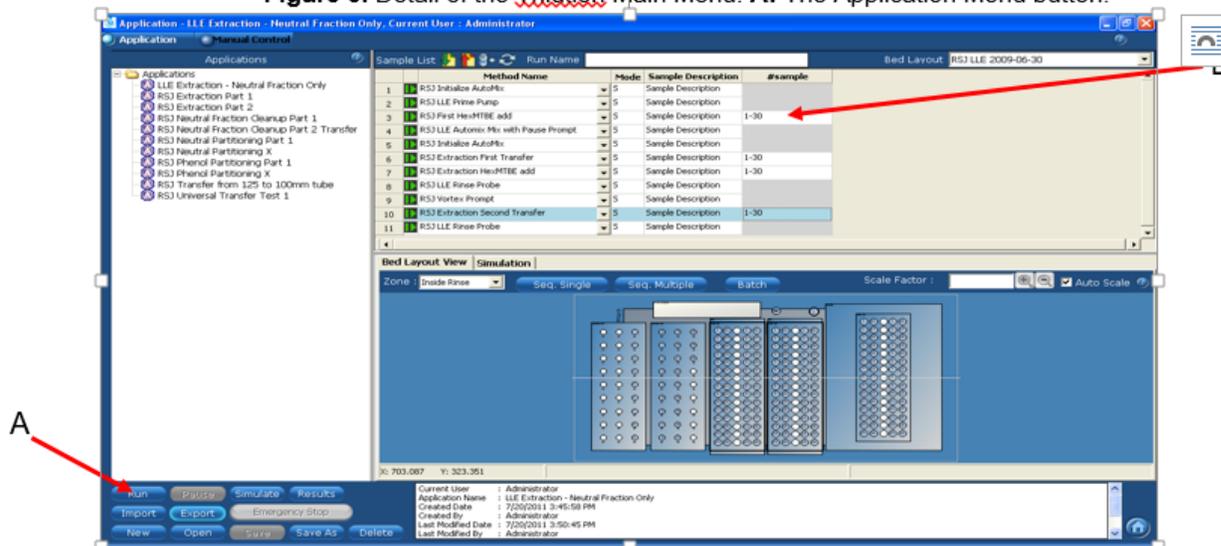


Figure 4. Detail of the Application Menu in Trilution LH. A: The Application Run Button. B: The column where the number of samples to be extracted is entered.

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 16x100mm tubes in positions 1-30 in the "Sample Extract" rack on the tray.
- E. Select the application named "LLE Methanol Extraction – Neutral Fraction Only – Std Rinse Port". Click on the "Run" button.
- F. The Gilson will add the hexane/MTBE solution to each sample and then mix the samples automatically by rotation via the 818 AutoMix for 10 minutes.
- G. After mixing the Gilson will prompt the user to remove the samples and centrifuge them.
- H. 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.

- I. The Gilson will then transfer the organic phase from the original sample tube to the corresponding 16x100mm tube.
- J. 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.
- K. Remove the samples from the AutoMix and mix by vortexing for at least 10 seconds each.
- L. Place the samples back in the rack in the AutoMix and click the "OK" button to continue the Application.
- M. The Gilson will then transfer the organic phase from the original sample tube to the corresponding 16x100mm 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 when stored in plastic sealable bag (Ziploc) 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 >200 °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 gloves. 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 confirm that no lumps are 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 0.25 g activated Silica gel (>200 °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 all unknowns and QC samples to dryness and blank samples to approximately 0.2-0.5mL by placing samples in the Caliper TurboVap evaporator and using the settings 50deg C water bath temperature and ~5psi.
- B. 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.
- C. If necessary, empty waste containers by replacing the container with an empty one.
- D. Place extracts in racks (one rack per module) on the right hand side of the racks, and remove screw caps.
- E. Place collection tubes on the left hand side of the racks.
- F. Place racks in tray at the bottom of each module.
- G. 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.
- H. Exit the setup racks menu by pressing OK.
- I. 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.
- J. 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 <10uL remains using the Caliper TurboVap evaporator. Start the evaporization with the following settings as a guide: ~5-10psi line pressure. Adjust the final volume to 10uL with nonane.
- 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 DFS (ThermoFisher, Bremen, Germany) instrument. The chromatographic separations are carried out on an Trace 1310 gas chromatograph (GC) (ThermoFisher, Bremen, Germany) fitted with a Rxi-5HT [(15-m length, 0.25 mm I.D. and 0.10- μ m film thickness); Restek, Bellfonte, PA] capillary column - or an equivalent 5% phenyl GC column from a different manufacturer such as the Phenomenex ZB5.

The GC is set up to use Splitless injection with the following GC inlet programming:

Constant Flow = 0.8 mL/min	
Temperature = 260 °C	
Split Flow = 50 mL/min	Gas Saver Flow = 15 mL/min
Split Time = 1.00 min	Gas Saver Time = 10 min
Septum Purge Flow = 3.0 mL/min	
Aux/Transfer Line Temperature = 260 °C	

The GC oven temperature is programmed as follows:

#	Rate (°C/min)	Temperature (°C)	Hold Time (min)
Initial		140	1.00
1	10	300	7.00

Injections (2 μ L) are performed using the TriPlus RSH (ThermoFisher, Bremen, Germany) autosampler. All wash solutions should be changed at least weekly. The autosampler is programmed with the following settings:

Sampling	
Sample Volume = 2.3 μ L	Pre-Injection Dwell Time = 3.0sec
Air Volume = 1.0 μ L	Post-Injection Dwell Time = 2.0sec
Sample Type = Viscous	
Washing/Rinsing Parameters	
Pre-Injection:	Nonane; 5 Cycles; 7 μ L each
Post-Injection:	Toluene/Nonane; 10 Cycles; 7 μ L each

The DFS source temperature is set to 290°C \pm 5°C in the electron impact mode using a filament bias of 45 eV. Refer to the MS_PARAM file for all monitored masses to be used in MID setup.

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 (ThermoFinnigan, Bremen, Germany) instrument. The chromatographic separations are carried out on an Trace 1310 gas chromatograph (GC) (ThermoFisher, Bremen, Germany) fitted with a Rxi-5sil MS [(30-m length, 0.25 mm I.D. and 0.25- μ m film thickness); Restek, Bellfonte, PA] capillary column – or an equivalent 5% phenyl GC column from a different manufacturer such as the Phenomenex ZB5.

The GC is set up to use Splitless injection with the following GC inlet programming:

Constant Flow = 1.0 mL/min	
Temperature = 275 °C	
Split Flow = 70 mL/min	Gas Saver Flow = 15 mL/min
Split Time = 2.00 min	Gas Saver Time = 10 min
Septum Purge Flow = 3.0 mL/min	
Aux/Transfer Line Temperature = 275 °C	

The GC oven temperature is programmed as follows:

#	Rate (°C/min)	Temperature (°C)	Hold Time (min)
Initial		100	1.00
1	30	200	5.00
2	4	250	0.00
3	45	320	1.00

Injections (1 μ L) are performed using the TriPlus RSH (ThermoFisher, Bremen, Germany) autosampler. All wash solutions should be changed at least weekly. The autosampler is programmed with the following settings:

Sampling	
Sample Volume = 1.0 μ L	Pre-Injection Dwell Time = 3.3sec
Air Volume = 0.5 μ L	Post-Injection Dwell Time = 1.0sec
Sample Type = Viscous	
Washing/Rinsing Parameters	
Pre-Injection:	Nonane; 5 Cycles; 7 μ L each
Post-Injection:	Toluene/Nonane; 10 Cycles; 7 μ L each

The DFS source temperature is set to 275°C \pm 5°C in the electron impact mode using a filament bias of 45 eV. Refer to the MS_PARAM file for all monitored masses to be used in MID setup.

9. Reportable Range of Results

The linear range 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 or the extract may be diluted so that the native area counts are less than the corresponding area count for the highest calibration standard.

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/ μ L, 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. 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 3 below.

Table 3. 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 +/- 26% 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 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.

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 as verified by the Division QC program available in StarLIMS:

- (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 outlier:** 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 ^{13}C -labelled internal standard, must not deviate more than 26% from the theoretical value.
- (ii) The ratio of the retention time of the analyte over its corresponding ^{13}C -labeled internal standard must be within the range 0.99 – 1.01. For analytes that do not have an identical ^{13}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 10-150%.

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. In addition, our lab uses an in house PT program (as specified in the Division Policy and Procedures manual) where 5 blinded PT samples are measured twice per year.

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 been reported for BFRs in the NHANES survey and are available at www.cdc.gov/exposurereport

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 <-40 °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 report is created. These data and a cover letter will be routed through the appropriate channels for approval (i.e. supervisor and/or branch chief, DLS statistician, 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 typically in an email.

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

If greater than 0.1 mL of sample remains following successful completion of analysis, this material must be returned to storage at <-40 °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).

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Appendix A: Ion Volume Cleaning Procedure for DFS instruments

General:

- Keep parts from each ion volume separate from parts from other ion volumes during the disassembly, cleaning and reassembly.
- Use gloves while handling ion volumes

1. Disassemble the ion volume:

- a. Keep parts from each ion volume separate throughout cleaning procedure and replace any damaged parts.

2. Cleaning of Sapphire rings and ceramic rods:

- a. Place the large and small sapphire rings in one ceramic crucible and the rods in a separate ceramic crucible. Keep the rods and rings specific to a particular ion volume together by using separate crucibles for each ion volume.
- b. Heat parts to 600C in muffle furnace for 2 hours then let the furnace cool before removing ion volume parts.
- c. Inspect all rods and rings for breaks, cracks, spots or discoloration. Replace any damaged parts.

3. Cleaning of metal ion volume parts:

- a. Remove any heavy deposit spots using a Fiberglass pen or the Dremel polisher using metal polish and a cotton swab inserted in the Dremel polisher.
- b. Metal parts will then be cleaned by tumbling in a mixture of walnut and metal polish using the Lortone Tumblers. Tumble metal parts for 1.5-2 days.
 - i. Use one tumbler for the block and another tumbler for the small parts. Do not tumble the block with other small parts.
 - ii. Note, part #1062810 (Spring PE) should not be tumbled as it is fragile and does not usually have any deposit build up.
- c. **IMPORTANT:** Remove any residual walnut residues from all metal parts ***including inside treads***

4. Sonication of metal parts cleaned by tumbling:

- a. Sonicate for 15 minutes using a beaker that has 2 drops of Dawn dish soap added per 50mL of water. The tap water/soap should cover the metal parts.
- b. Pour out the soapy water and rinse the parts under the faucet for several minutes to remove the soap.
- c. Sonicate for 15 minutes in ***Deionized Water***.
- d. Sonicate for 15 minutes in ***Methanol***.
- e. Sonicate for an additional 15 minutes in fresh ***Methanol***.
- f. Sonicate for 15 minutes in ***Hexane*** and repeat with fresh hexane.
- g. After residual solvent has evaporated in fume hood, heat to 150°C in the GC oven or muffle furnace using crucible
 - i. Discard all solvents in appropriate waste container

5. Reassemble the ion volume:

- a. Replace any parts that appears to be bent, broken or scratched.

Appendix B: Typical accurate masses, target isotopic ratios, ¹³C label standard used, selected ion monitoring window (SIM) and lock and calibration masses used for high resolution isotope dilution measurements of polychlorinated (PCDD/F) and coplanar polychlorinated biphenyls (cPCBs). Also given are calibration curve range and sample quality control (QC) criteria, i.e, relative retention time and recovery.

Analyte	Accurate Masses				Actual Label used	SIM ¹	Lock / Cali Mass	Sample QC criteria			Calibration standard	
	¹² C-masses		¹³ C-masses					¹² C Isotope	¹³ C Isotope	RRT Limit ²	Recovery Limits	range (low / high)
	Quan mass	Ratio Mass	Quan mass	Ratio Mass				Ratio Limits	Ratio Limits	(%)	(pg/ul) ³	
Polybrominated diphenyl ethers (PBDEs) and 2,2',4,4',5,5'-hexabromobiphenyl (PBB-153)												
PBDE17	405.8021	407.8001	417.8424	419.8403	PBDE28	1	404.9755 / 430.9723	0.72 - 1.23	0.72 - 1.23	+/-0.004	10 - 150	0.2 - 2000
PBDE28	405.8021	407.8001	417.8424	419.8403	PBDE28	1	404.9755 / 430.9723	0.72 - 1.23	0.72 - 1.23	+/-0.004	10 - 150	0.2 - 2000
PBDE47	483.7126	485.7106	495.7529	497.7508	PBDE47	3	480.9691 / 504.9691	1.08 - 1.84	1.08 - 1.84	+/-0.004	10 - 150	0.2 - 2000
PBDE66	483.7126	485.7106	495.7529	497.7508	PBDE47	3	480.9691 / 504.9691	1.08 - 1.84	1.08 - 1.84	+/-0.004	10 - 150	0.2 - 2000
PBDE99	403.7865	405.7844	415.8267	417.8247	PBDE99	4	392.9755 / 430.9723	0.72 - 1.23	0.72 - 1.23	+/-0.004	10 - 150	0.2 - 2000
PBDE100	403.7865	405.7844	415.8267	417.8247	PBDE100	4	392.9755 / 430.9723	0.72 - 1.23	0.72 - 1.23	+/-0.004	10 - 150	0.2 - 2000
PBDE85	403.7865	405.7844	415.8267	417.8247	PBDE99	4	392.9755 / 430.9723	0.72 - 1.23	0.72 - 1.23	+/-0.004	10 - 150	0.2 - 2000
PBDE154	483.6949	485.6934	493.7372	495.7352	PBDE154	5	442.9723 / 492.9691	0.48 - 0.82	1.08 - 1.84	+/-0.004	10 - 150	0.2 - 2000
PBDE153	483.6950	485.6930	493.7372	495.7352	PBDE153	5	442.9723 / 492.9691	0.48 - 0.82	1.08 - 1.84	+/-0.004	10 - 150	0.2 - 2000
PBDE183	721.4406	723.4380	733.4809	735.4783	PBDE183	6	704.9563 / 754.9531	0.72 - 1.23	0.72 - 1.23	+/-0.004	10 - 150	0.2 - 2000
PBDE209	797.3355	799.3329	809.3757	811.3731	PBDE209	9	754.9531 / 792.95	0.9 - 1.53	0.9 - 1.53	+/-0.004	10 - 300	0.2 - 2000
PBB153	465.7021	467.7000	477.7423	479.7403	PBB153	5	442.9723 / 492.9691	1.08 - 1.84	1.08 - 1.84	+/-0.004	10 - 150	0.2 - 2000
Polychlorinated biphenyls (PCBs)												
PCB28	255.9613	257.9584	268.0016	269.9986	PCB28	1	213.9903 - 264.9905	0.96 - 0.71	0.96 - 0.71	+/- 0.004	10 - 150	0.2 - 500
PCB74	289.9224	291.9194	335.9236	337.9207	PCB101	3	264.9905 - 413.977	1.6 - 1.18	1.6 - 1.18	+/- 0.004	10 - 150	0.2 - 500
PCB66	289.9224	291.9194	335.9236	337.9207	PCB101	3	264.9905 - 413.977	1.28 - 0.95	1.6 - 1.18	+/- 0.004	10 - 150	0.2 - 500
PCB99	323.8834	325.8804	335.9236	337.9207	PCB101	3	264.9905 - 413.977	1.6 - 1.18	1.6 - 1.18	+/- 0.004	10 - 150	0.2 - 500
PCB118	323.8834	325.8804	335.9236	337.9207	PCB118	4	213.9903 - 313.9839	1.28 - 0.95	1.6 - 1.18	+/- 0.004	10 - 150	0.2 - 7500
PCB114	323.8834	325.8804	335.9236	337.9207	PCB114	4	213.9903 - 313.9839	1.6 - 1.18	1.6 - 1.18	+/- 0.004	10 - 150	0.2 - 500
PCB105	323.8834	325.8804	335.9236	337.9207	PCB105	4	213.9903 - 313.9839	1.6 - 1.18	1.6 - 1.18	+/- 0.004	10 - 150	0.2 - 500
PCB146	289.9038	291.9008	301.9440	303.9411	PCB153	4	213.9903 - 313.9839	1.6 - 1.18	0.48 - 0.36	+/- 0.004	10 - 150	0.2 - 500
PCB153	289.9038	291.9008	301.9440	303.9411	PCB153	4	213.9903 - 313.9839	1.6 - 1.18	0.48 - 0.36	+/- 0.004	10 - 150	0.2 - 7500
PCB138-158	289.9038	291.9008	301.9440	303.9411	PCB138-158	5	213.9903 - 313.9839	1.6 - 1.18	0.48 - 0.36	+/- 0.004	10 - 150	0.2 - 7500
PCB167	289.9038	291.9008	301.9440	303.9411	PCB167	5	213.9903 - 313.9839	1.6 - 1.18	0.48 - 0.36	+/- 0.004	10 - 150	0.2 - 500
PCB156	289.9038	291.9008	301.9440	303.9411	PCB156	6	264.9905 - 313.9839	1.6 - 1.18	0.48 - 0.36	+/- 0.004	10 - 150	0.2 - 500
PCB157	289.9038	291.9008	301.9440	303.9411	PCB157	6	264.9905 - 313.9839	1.6 - 1.18	0.48 - 0.36	+/- 0.004	10 - 150	0.2 - 500

¹ Selected Ion Monitoring Window; ² Relative retention time deviation limit. Calculated against ¹³C-labeled standard; ³ Standard part number EDF-5524 obtained from Cambridge Isotope Laboratories (www.isotope.com)

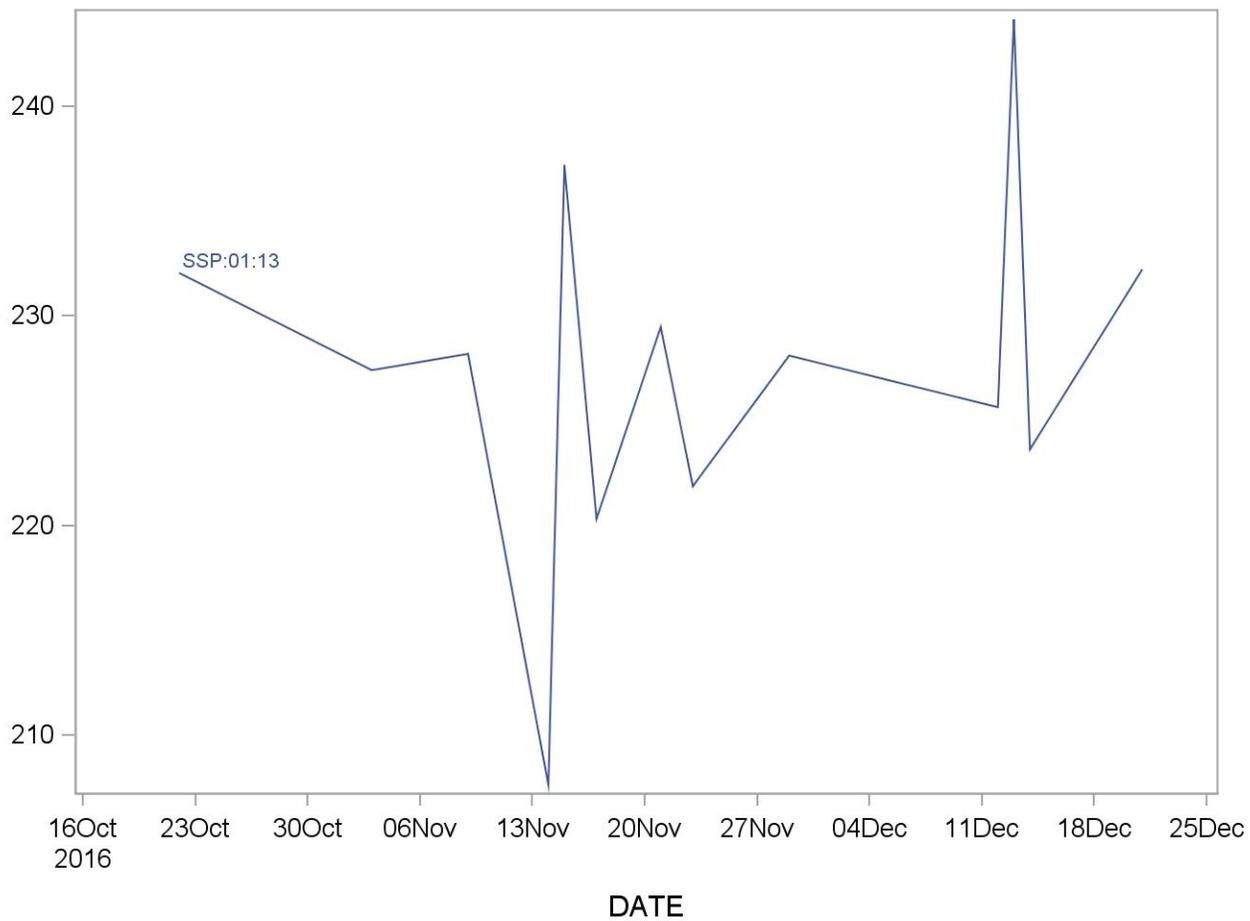
Appendix B (Continued): Typical accurate masses, target isotopic ratios, ¹³C label standard used, selected ion monitoring window (SIM) and lock and calibration masses used for high resolution isotope dilution measurements of polychlorinated (PCDD/F) and coplanar polychlorinated biphenyls (cPCBs). Also given are calibration curve range and sample quality control (QC) criteria, i.e, relative retention time and recovery.

Analyte	Accurate Masses				Actual Label used	SIM ¹	Lock / Cali Mass	Sample QC criteria			Calibration standard	
	¹² C-masses		¹³ C-masses					¹² C Isotope	¹³ C Isotope	RRT Limit ²	Recovery Limits	range (low / high)
	Quan mass	Ratio Mass	Quan mass	Ratio Mass				Ratio Limits	Ratio Limits	(%)	(pg/ul) ³	
<i>Polybrominated diphenyl ethers (PBDEs) and 2,2',4,4',5,5'-hexabromobiphenyl (PBB-153)</i>												
PCB178	323.8648	325.8618	335.9050	337.9021	PCB178	5	213.9903 - 313.9839	0.48 - 0.36	0.64 - 0.47	+/- 0.004	10 - 150	0.2 - 500
PCB187	323.8648	325.8618	335.9050	337.9021	PCB178	5	213.9903 - 313.9839	0.48 - 0.36	0.64 - 0.47	+/- 0.004	10 - 150	0.2 - 7500
PCB183	323.8648	325.8618	335.9050	337.9021	PCB178	5	213.9903 - 313.9839	0.48 - 0.36	0.64 - 0.47	+/- 0.004	10 - 150	0.2 - 500
PCB180	323.8648	325.8618	335.9050	337.9021	PCB180	6	264.9905 - 313.9839	0.48 - 0.36	0.64 - 0.47	+/- 0.004	10 - 150	0.2 - 7500
PCB170	323.8648	325.8618	335.9050	337.9021	PCB170	7	264.9905 - 413.977	0.48 - 0.36	0.64 - 0.47	+/- 0.004	10 - 150	0.2 - 7500
PCB189	323.8648	325.8618	335.9050	337.9021	PCB189	7	264.9905 - 413.977	0.48 - 0.36	0.64 - 0.47	+/- 0.004	10 - 150	0.2 - 500
PCB199	357.8258	359.8229	335.9050	337.9021	PCB170	7	264.9905 - 413.977	0.48 - 0.36	0.64 - 0.47	+/- 0.004	10 - 150	0.2 - 500
PCB196-203	357.8258	359.8229	335.9050	337.9021	PCB170	7	264.9905 - 413.977	0.48 - 0.36	0.64 - 0.47	+/- 0.004	10 - 150	0.2 - 500
PCB194	357.8258	359.8229	369.8661	371.8631	PCB194	7	264.9905 - 413.977	0.48 - 0.36	0.8 - 0.59	+/- 0.004	10 - 150	0.2 - 500
PCB206	463.7216	465.7186	475.7619	477.7589	PCB206	8	463.9743 - 502.9745	0.48 - 0.36	0.75 - 0.55	+/- 0.004	10 - 150	0.2 - 500
PCB209	497.6826	499.6797	509.7229	511.7199	PCB209	8	463.9743 - 502.9745	0.48 - 0.36	0.85 - 0.63	+/- 0.004	10 - 150	0.2 - 500
<i>Persistent Pesticides</i>												
HCB	283.8102	285.8072	289.8303	291.8273	HCB	1	313.9839 / 351.9802	0.95 - 1.61	0.95 - 1.61	+/- 0.004	10 - 150	1 - 1000
B-HCCH	182.9349	184.932	188.955	190.9521	B-HCCH	2	313.9839 / 375.9807	1.18 - 2.02	1.18 - 2.02	+/- 0.004	10 - 150	1 - 1000
G-HCCH	182.9349	184.932	188.955	190.9521	G-HCCH	3	351.9802 / 413.977	0.59 - 1.01	0.59 - 1.01	+/- 0.004	10 - 150	1 - 1000
OXYCHLOR	386.8052	388.8023	396.8388	398.8358	OXYCHLOR	6	351.9802 / 413.977	0.59 - 1.01	0.59 - 1.01	+/- 0.004	10 - 150	1 - 1000
T-NONA	406.787	408.784	416.8205	418.8176	t-Nona	7	351.9802 / 413.977	0.59 - 1.01	0.59 - 1.01	+/- 0.004	10 - 150	1 - 1000
PP-DDE	246.0003	247.9974	258.0406	260.0376	pp-DDE	8	413.977 / 463.9743	0.71 - 1.21	0.71 - 1.21	+/- 0.004	10 - 150	1 - 6000
OP-DDT	235.0081	237.0052	247.0484	249.0454	op-DDT	11	351.9802 / 413.977	0.71 - 1.21	0.71 - 1.21	+/- 0.004	10 - 150	1 - 1000
PP-DDT	235.0081	237.0052	247.0484	249.0454	op-DDT	12	413.977 / 463.9743	0.83 - 1.41	0.83 - 1.41	+/- 0.004	10 - 150	1 - 1000
MIREX	271.8102	273.8072	276.8269	278.824	Mirex	13	313.9839 / 351.9802	0.95 - 1.61	0.95 - 1.61	+/- 0.004	10 - 150	1 - 1000

¹ Selected Ion Monitoring Window; ² Relative retention time deviation limit. Calculated against ¹³C-labeled standard; ³ Standard part number EDF-5524 obtained from Cambridge Isotope Laboratories (www.isotope.com)

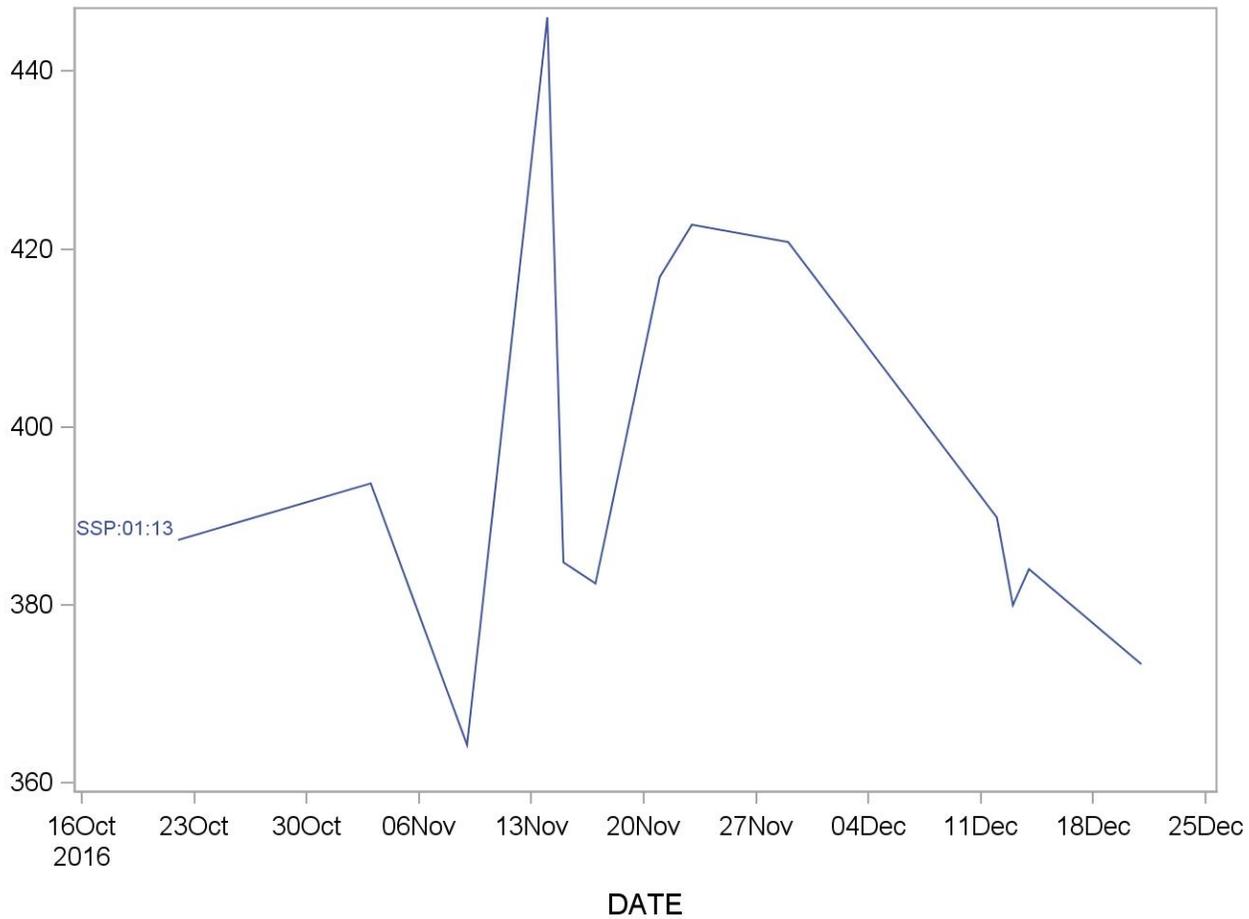
2009-2010 Summary Statistics and QC Chart for Beta-hexachlorocyclohexane (ng/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP:01:13	13	22OCT16	21DEC16	227.5279	8.746172	3.8



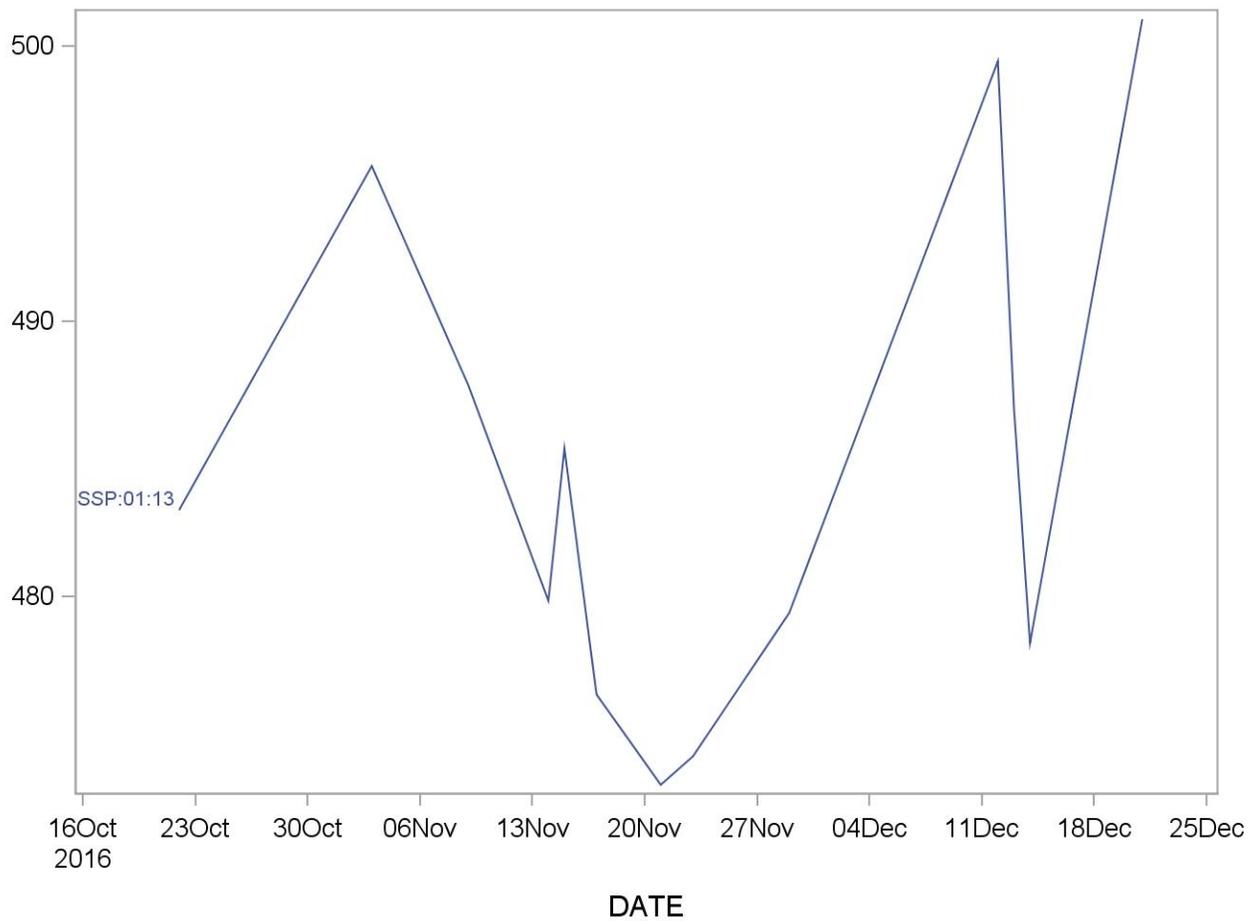
2009-2010 Summary Statistics and QC Chart for Gamma-hexachlorocyclohexane (ng/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP:01:13	13	22OCT16	21DEC16	395.8421	23.48974	5.9



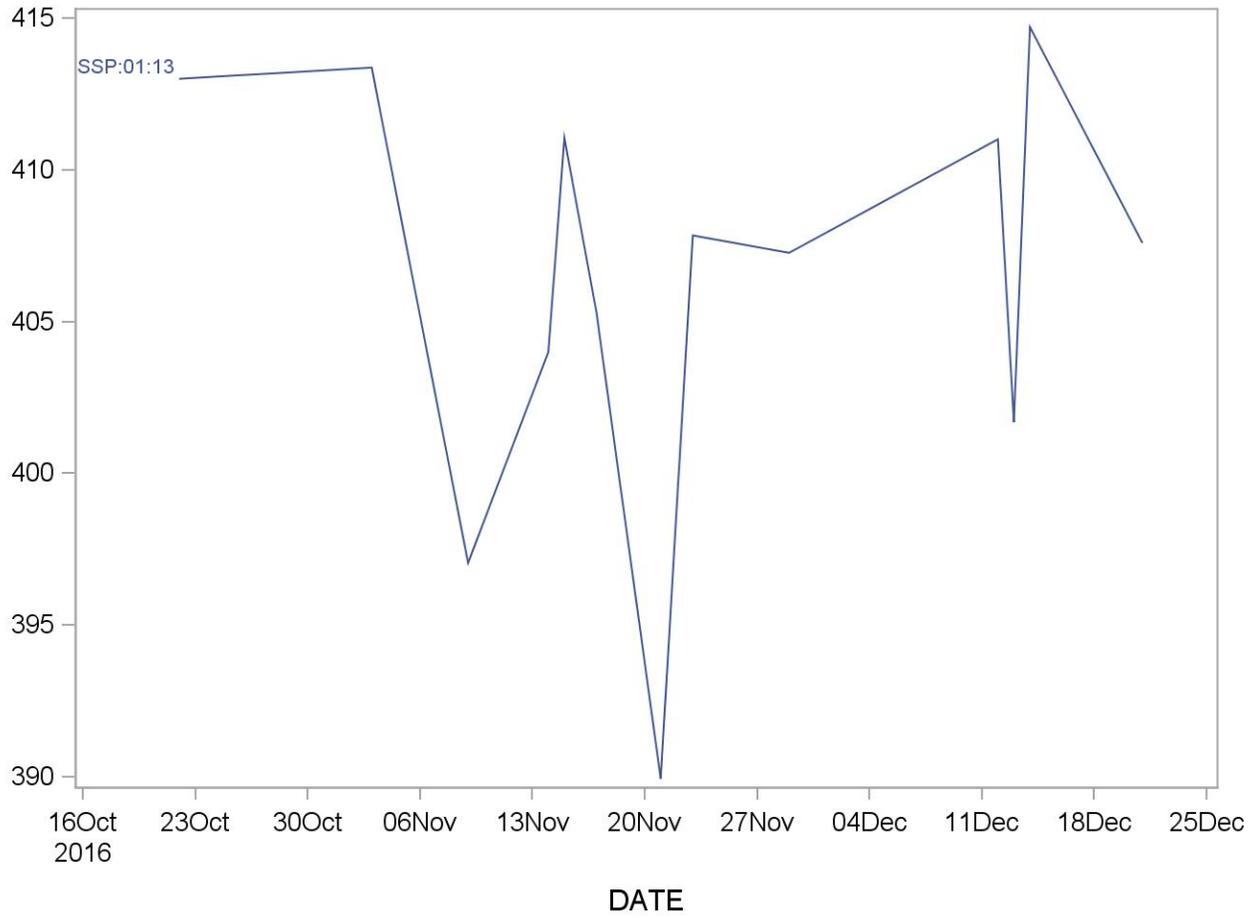
2009-2010 Summary Statistics and QC Chart for Hexachlorobenzene (ng/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP:01:13	13	22OCT16	21DEC16	484.6566	9.241143	1.9



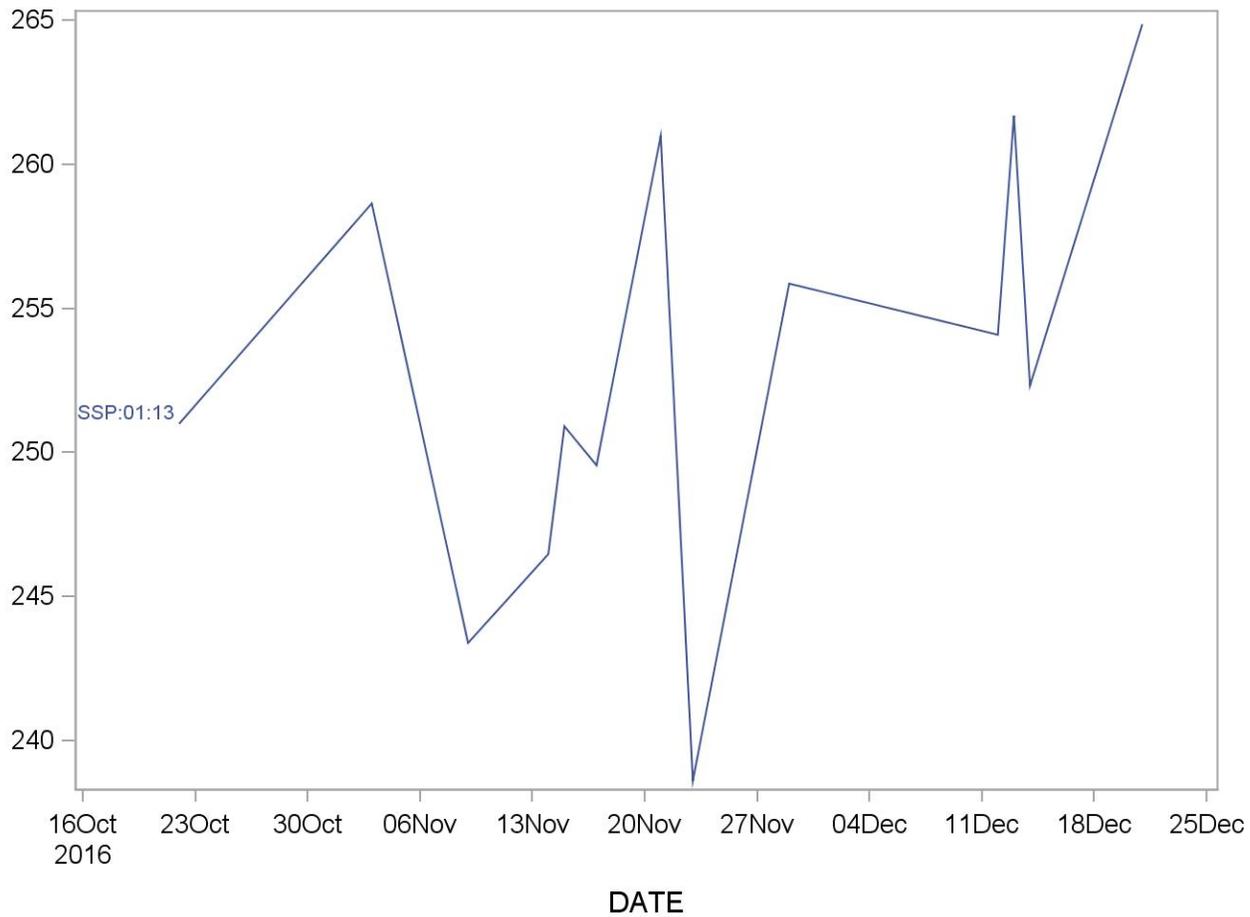
2009-2010 Summary Statistics and QC Chart for Mirex (ng/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP:01:13	13	22OCT16	21DEC16	406.4398	7.047935	1.7



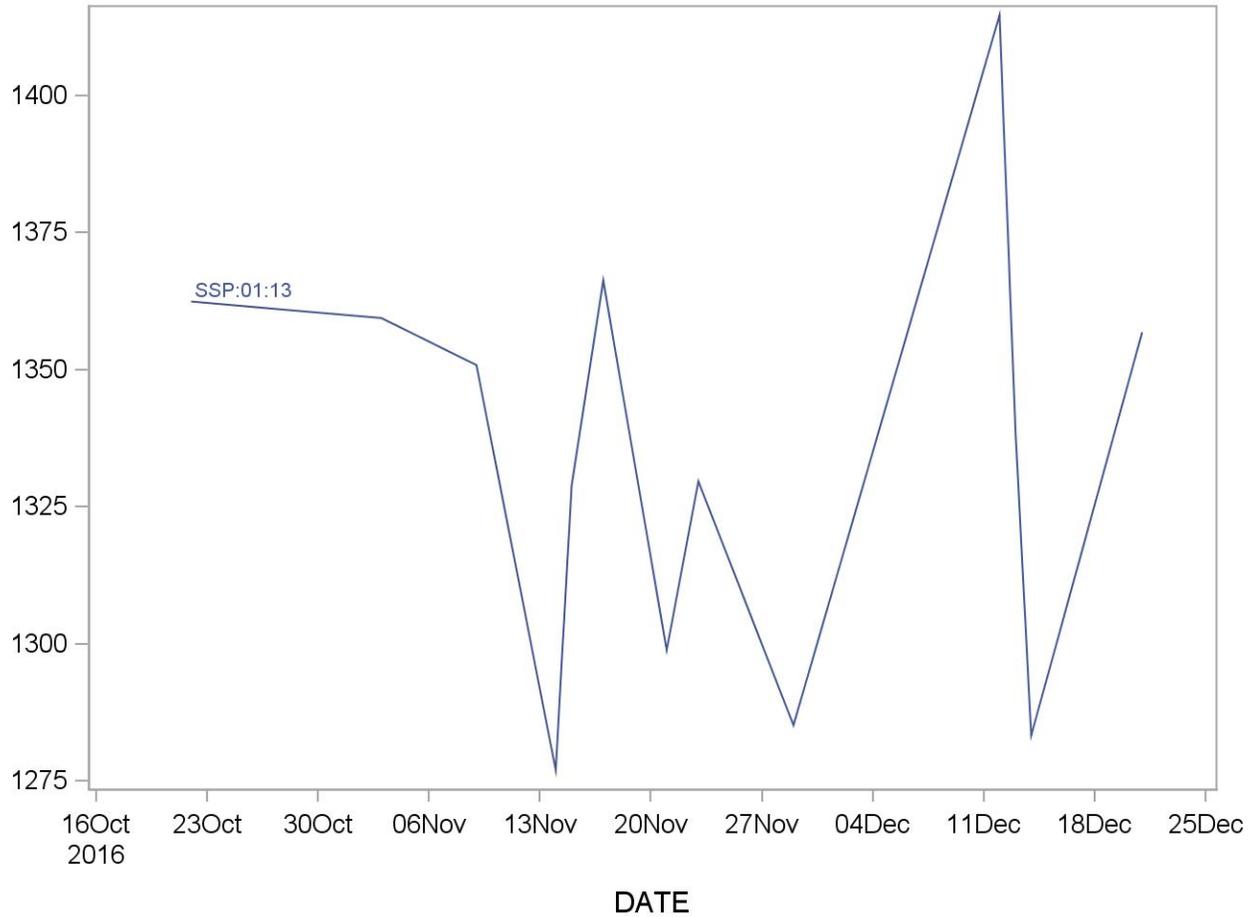
2009-2010 Summary Statistics and QC Chart for Oxychlordane (ng/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP:01:13	13	22OCT16	21DEC16	252.9448	7.548544	3.0



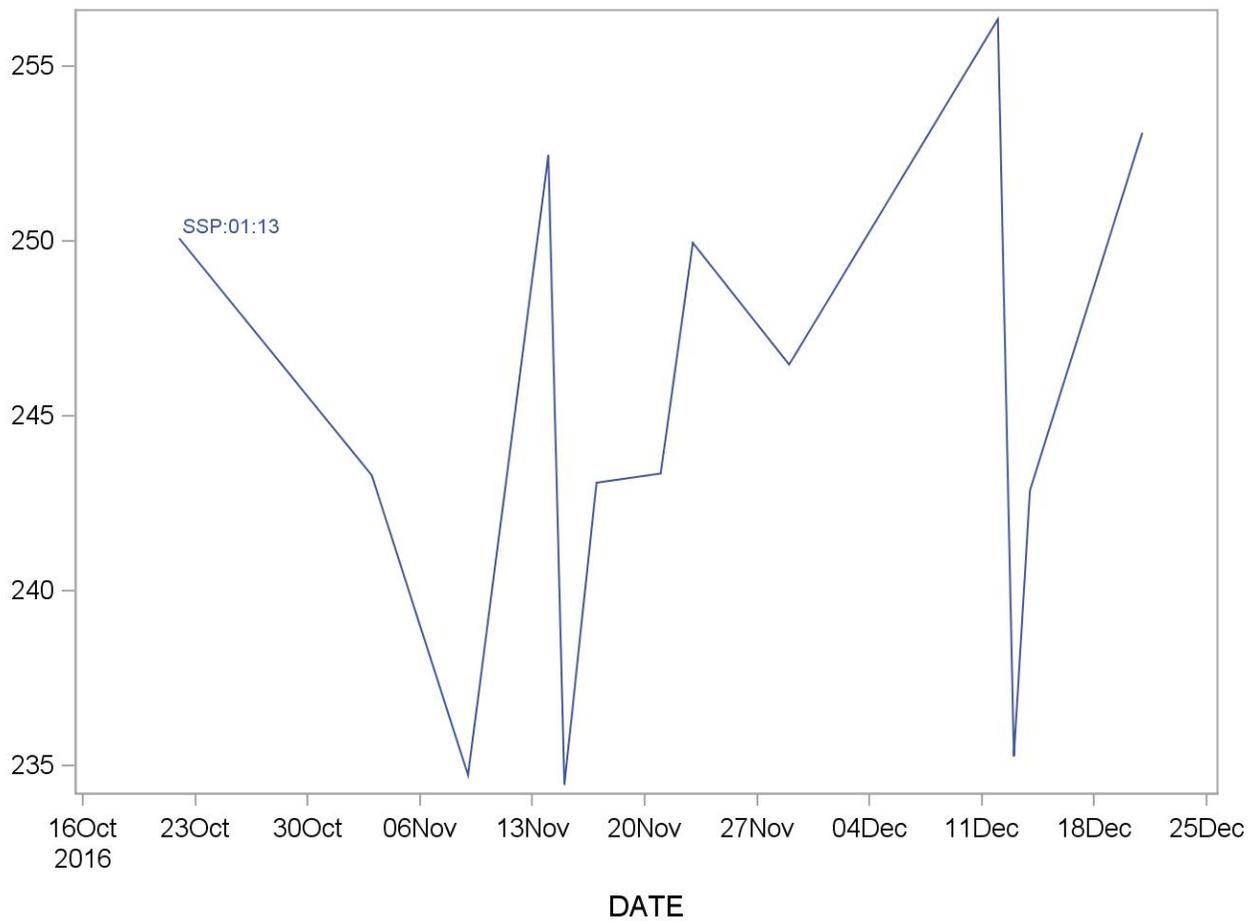
2009-2010 Summary Statistics and QC Chart for p,p'-DDE (ng/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP:01:13	13	22OCT16	21DEC16	1334.790	40.12912	3.0



2009-2010 Summary Statistics and QC Chart for p,p'-DDT (ng/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP:01:13	13	22OCT16	21DEC16	245.0442	7.232789	3.0



2009-2010 Summary Statistics and QC Chart for trans-Nonachlor (ng/g)

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
SSP:01:13	13	22OCT16	21DEC16	489.0787	9.017720	1.8

