



## Laboratory Procedure Manual

*Analyte:* Polyfluoroalkyl chemicals: Perfluorooctane sulfonamide, 2-(N-methyl-perfluorooctane sulfonamido) acetate, 2-(N-ethyl-perfluorooctane sulfonamido) acetate, perfluorobutane sulfonate, perfluorohexane sulfonate, perfluorooctane sulfonate, perfluoroheptanoate, perfluorooctanoate, perfluorononanoate, perfluorodecanoate, perfluoroundecanoate, and perfluorododecanoate

*Matrix:* Serum

*Method:* Online Solid Phase Extraction-High Performance Liquid Chromatography-Turbo Ion Spray-Tandem Mass Spectrometry (online SPE-HPLC-TIS-MS/MS)

*Method No:* 6304.04

*Revised:* 11/01/2013

*as performed by:*

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### **Important Information for Users**

The Centers for Disease Control and Prevention (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:

File Name	Variable Name	SAS Label (and SI units)
PFC_G	LBXPFOA	Perfluorooctanoic acid (ng/mL)
	LBXPFOS	Perfluorooctane sulfonic acid (ng/mL)
	LBXPFHS	Perfluorohexane sulfonic acid (ng/mL)
	LBXEPAH	2-(N-ethyl-PFOSA) acetic acid (ng/mL)
	LBXMPAH	2-(N-methyl-PFOSA) acetic acid (ng/mL)
	LBXPFDE	Perfluorodecanoic acid (ng/mL)
	LBXPFBS	Perfluorobutane sulfonic acid (ng/mL)
	LBXPFHP	Perfluoroheptanoic acid (ng/mL)
	LBXPFNA	Perfluorononanoic acid (ng/mL)
	LBXPFSA	Perfluorooctane sulfonamide (ng/mL)
	LBXPFUA	Perfluoroundecanoic acid (ng/mL)
	LBXPFDO	Perfluorododecanoic acid (ng/mL)

## 1. Clinical Relevance and Summary of Test Principle

### a. Clinical Relevance

Polyfluoroalkyl chemicals (PFCs) have been used since the 1950s in numerous commercial applications, including surfactants, lubricants, paper and textile coatings, polishes, food packaging, and fire-retarding foams. Some of these PFCs, including perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), persist in humans and the environment and have been detected worldwide in wildlife [1]. Exposure to PFOS and PFOA in the general population is also widespread. In animals, exposure to PFOS and PFOA is associated with adverse health effects albeit at serum concentrations orders of magnitude higher than the concentrations observed in the general population [2-5]. In May 2000, 3M, the sole manufacturer of PFOS in the United States and the principal manufacturer worldwide, announced that it was discontinuing its perfluorooctanesulfonyl fluoride chemistry, including the manufacture of PFOS. Although PFOA and its salts and precursors are still being manufactured by others by a different process, reductions in their manufacturing emissions have been proposed [6-8]. The reductions in serum concentrations of several PFCs, including PFOS, reported in humans and wildlife [9-11], are most likely related to such industrial production changes.

### b. Test Principle

Solid phase extraction coupled to High Performance Liquid Chromatography-Turbo Ion Spray ionization-tandem Mass Spectrometry (online SPE-HPLC-TIS-MS/MS) is used for the quantitative detection of perfluorooctane sulfonamide (PFOSA), 2-(N-methyl-perfluorooctane sulfonamido) acetic acid (Me-PFOSA-AcOH), 2-(N-ethyl-perfluorooctane sulfonamido) acetic acid (Et-PFOSA-AcOH), perfluorobutane sulfonate (PFBuS), perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS), perfluoroheptanoate (PFHpA), perfluorooctanoate (PFOA), perfluorononanoate (PFNA), perfluorodecanoate (PFDeA), perfluoroundecanoate (PFUA), and perfluorododecanoate (PFDoA) [12].

## 2. Safety Precautions

### a. Reagent Toxicity or Carcinogenicity

Formic acid is a known sensitizer. Prolonged or repeated exposure to sensitizers may cause allergic reactions in certain sensitive individuals. Some of the reagents used are toxic. Special care should be taken to: 1) avoid contact with eyes and skin, 2) avoid use of the organic solvents in the vicinity of an open flame, and 3) use solvents only in well-ventilated areas.

**Note:** The Material Safety Data Sheets (MSDS) for the chemicals and solvents used in this procedure can be found on the internet at MSDS Xchange (<http://www.msdsxchange.com/english/index.cfm>) and as a hard copy found in

the laboratory. Laboratory personnel must review the MSDS before using chemicals.

Care should be exercised in the handling of all chemical standards.

**b. Radioactive Hazards**

None.

**c. Microbiological Hazards**

The possibility of being exposed to various microbiological hazards exists. Appropriate measures (i.e., universal precautions) should be taken to avoid any direct contact with the specimens (i.e., utilize gloves, laboratory coats, safety glasses, chemical or biological hoods, etc.). The Hepatitis B vaccination series is recommended for health care and laboratory workers who are exposed to human fluids and tissues. Laboratory personnel handling human fluids and tissues is required to attend the "Bloodborne Pathogens Training" course offered at CDC to insure proper compliance with CDC safe work place requirements. Any residual sample material should be appropriately discarded and prepared for autoclaving after analysis is completed. All disposable laboratory supplies must also be placed in an autoclave bag for disposal.

**d. Mechanical Hazards**

There are only minimal mechanical hazards when performing this procedure using standard safety practices. Laboratorians should avoid any direct contact with the electronics of the mass spectrometer, unless all power to the instrument is off. Generally, only qualified technicians should perform the electronic maintenance and repair of the mass spectrometer. Contact with the heated surfaces of the mass spectrometer (e.g., interface) should be avoided.

**e. Protective Equipment**

Standard safety protective equipment should be utilized when performing this procedure. This includes lab coat, safety glasses, durable gloves (e.g., nitrile or vinyl), and a chemical fume hood (if using primary containers of organic solvents or acids/bases).

**f. Training**

Training and experience in the use of a triple quadrupole mass spectrometer and the on-line SPE extractor should be obtained by anyone using this procedure. Operators are required to read the operation manuals or laboratory standard operating procedure. Formal training is not necessary; however, an experienced user should train all of the operators.

**g. Personal Hygiene**

Care should be taken in handling any biological specimen. Routine use of gloves and proper hand washing should be practiced. No food or drink is allowed in laboratory areas.

#### **h. Disposal of Wastes**

Solvents and reagents are disposed of in an appropriate container clearly marked for waste products and temporarily stored in one of the chemical fume hoods. Containers, glassware, etc., that come in direct contact with the specimen are either autoclaved or decontaminated with 10% bleach. Contaminated analytical glassware is treated with bleach, washed and reused; disposable labware is autoclaved before disposal. To insure proper compliance with CDC requirements, laboratory personnel are required to attend annual hazardous waste disposal courses.

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

#### **a. Software and Knowledge Requirements**

All samples are queued for analysis in a database created using Microsoft Access. Mass spectrometry data are collected and stored using the Analyst Software of the ABI 5500 mass spectrometer workstation. During sample preparation and analysis, samples are identified by their Sample Name and Sample ID. The Sample Name is a number that is unique to each sample during the sample preparation and the mass spectrometry analysis. The unique Sample ID, included on the label of each sample vial, is used to identify each specimen and links the laboratory information with the demographic data recorded by the sample takers. In case of repeated measurements, one specimen in the database may have more than one Sample Name, but only one Sample ID. All raw data files are processed using the ABI 5500 Analyst software and are archived for future reference. The Analyst software selects the appropriate peak based on the precursor/product ion combination and chromatographic retention time and subsequently integrates the peak area. It also allows manual peak selection and area integration. The raw data (peak area, peak height, retention time, analyte name, MRM transition name) are exported to the Access database used for storage and retrieval of data. The Access database is stored on the secure DLS-PC Network as well as in several archive locations. Statistical analysis of the data, programming, and reporting are performed using the Statistical Analysis System (SAS) software (SAS Institute, Cary, NC). Knowledge and experience with these software packages (or their equivalent) are required to utilize and maintain the data management structure.

#### **b. Sample Information**

Sample names and Sample IDs are entered into the Access database before sample preparation. If possible, for unknown samples, the sample IDs are scanned in by a barcode reader directly from the sample vials. Sample IDs for

quality control materials (QCs) may be entered manually. The Sample Log Sheet, containing Sample Names and Sample IDs, is printed from the Access database and is used to record information during the sample preparation. Sample Names and Sample IDs are exported as tab delimited text files from the Access database and imported into the Acquisition Batch table (\*.dab) of the Analyst program on the ABI 5500 mass spectrometer. After MS data collection and peak integration, the data are saved in tab delimited files and imported into the Access database. Further manipulation of the data, including QC evaluation and statistical analyses, are performed using SAS statistical software. After any additional calculations or corrections by the analyst are completed and the reviewing supervisor approves the final values for release, a comma-delimited file of the final data (SAS output) is generated.

**c. Data Maintenance**

Raw files are regularly backed up onto an external hard drive. All sample and analytical data are checked after being entered into the database for transcription errors and overall validity. Additionally, final reported data are saved in a paper copy as an official government record. Documentation for data system maintenance may be contained in copies of data records.

**d. Final Reports**

A final report of the data, generated using a SAS program, is reviewed by the DLS statistician before being transmitted to the Division Director, through the Branch Chief, via an inter-office memorandum for final revision. From the inter-office memorandum, a report is generated to the individual(s) requesting the analysis. Hard copies of data and correspondences are maintained in the office of the Branch Chief of the Organic Analytical Toxicology Branch or his/her designee under the CDC study number assigned by the DLS specimen-receiving personnel. Data are also maintained in the notebook of the analyst, and by the laboratory supervisor and/or his/her designee.

**4. Procedures for Collecting, Storing, and Handling Specimens; Criteria for Specimen Rejection**

**a. Sample Collection and Storage**

Preferably, a minimum of 0.5 mL of serum should be collected in standard collection containers. Samples should be refrigerated as soon as possible and then transferred to labeled specimen containers preferably within 4 hours of collection for storage. Samples should be stored frozen preferably in polypropylene or polyethylene containers. Glass containers may be used if the specimens are to be analyzed for other environmental chemicals for which storage in plastic may be a problem. PTFE/Teflon<sup>®</sup> and polyvinylidene fluoride (PVDF) coated materials should be avoided.

**b. Sample Handling**

Specimen handling conditions are outlined in the Division protocol for serum collection and handling. In the protocol, collection, transport, and special equipment required are discussed. In general, serum specimens should be transported cold (dry ice, ice or blue ice can be used). Special care must be taken in packing to protect collection vials from breakage during shipment.

Before analysis, samples are thawed, vortexed, aliquoted, and the residual specimen is again stored frozen. The integrity of samples thawed and refrozen several times does not appear to be compromised.

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

Not applicable for this procedure.

#### 6. Preparation of Reagents, Calibration (Standards), Controls, and All Other Materials; Equipment and Instrumentation

##### a. Reagents and Sources

Methanol (MeOH), acetonitrile (ACN) and water were of HPLC grade (Honeywell Burdick & Jackson, Muskegon, MI). Formic acid (FA, 98% min, GR) was purchased from EM Science (Gibbstown, NJ). Acetic acid (glacial) was purchased from J.T. Baker (Phillipsburg, NJ). 2-(N-methyl-perfluorooctane sulfonamido) acetic acid (Me-PFOSA-AcOH), 2-(N-ethyl-perfluorooctane sulfonamido) acetic acid (Et-PFOSA-AcOH). Perfluorooctane sulfonate potassium salt (PFOS) were provided by 3M Company (Saint Paul, MN). Perfluorooctane sulfonamide (PFOSA), perfluorohexane sulfonate potassium salt (PFHxS), perfluorobutane sulfonate potassium salt (PFBuS), perfluorooctanoic acid ammonium salt (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDeA), perfluoroundecanoic acid (PFUA), and perfluorododecanoic acid (PFDoA) were purchased from Wellington Laboratories (Guelph, ON, Canada).  $^{18}\text{O}_2$ -perfluorooctane sulfonamide ( $^{18}\text{O}_2$ -PFOSA) was purchased from RTI Laboratories (RTP, NC). Perfluoro-n-[1,2,3,4- $^{13}\text{C}$ ]-octanoic acid ( $^{13}\text{C}_4$ -PFOA), perfluoro-n-[1,2,3,4,5- $^{13}\text{C}$ ]-nonanoic acid ( $^{13}\text{C}_5$ -PFNA), 2-Perfluorooctyl [1,2- $^{13}\text{C}$ ]-ethanoic acid ( $^{13}\text{C}_2$ -PFDeA), 2-perfluorooctyl [1,2- $^{13}\text{C}$ ]-undecanoic acid ( $^{13}\text{C}_2$ -PFUA), perfluoro-n-[1,2- $^{13}\text{C}$ ]-dodecanoic acid ( $^{13}\text{C}_2$ -PFDoA), N-methyl- $\text{d}_3$ -perfluoro-1-octanesulfonamide acetic acid ( $\text{D}_3$ -Me-PFOSA-AcOH), and N-ethyl- $\text{d}_5$ -perfluoro-1-octanesulfonamide acetic acid ( $\text{D}_5$ -Et-PFOSA-AcOH), sodium perfluoro 1-hexane [ $^{18}\text{O}_2$ ]-sulfonate ( $^{18}\text{O}_2$ -PFHxS), sodium perfluoro 1-[1,2,3,4- $^{13}\text{C}$ ]-octanesulfonate ( $^{13}\text{C}_4$ -PFOS) were purchased from Wellington Laboratories. All reagents were used without further purification.

##### b. Working Solutions

(1) HPLC Aqueous Mobile Phase, 20mM Ammonium Acetate Buffer, pH 4.

Dilute 570  $\mu\text{L}$  of concentrated acetic acid to 400 mL 18 M $\Omega$  water in a beaker. Adjust pH to 4 by adding drop-wise 1:10 ammonium hydroxide:water mixture. Transfer it into 500 mL volumetric flask and fill it up to volume with water. Prepare fresh daily and store at room temperature.

**(2) HPLC Organic Mobile Phase, 100% HPLC ACN**

Refill as needed and store at room temperature

**(3) Organic solvent for SPE column regeneration, 100% ACN**

Refill as needed and store at room temperature.

**(4) Solid phase extraction (SPE) Acid Wash Solution, 0.1 M FA**

Dilute 3,930  $\mu\text{L}$  of 96% concentrated FA with 18 M $\Omega$  water to 1000 mL in a volumetric cylinder. Prepare monthly and store at room temperature.

**c. Standards Preparation**

**(1) Stock Solutions**

Standard stock solutions are prepared by dissolving solid standards (20-100 mg) in 10 mL MeOH. Working native standard solutions containing 10  $\mu\text{g}/\text{mL}$  (ppm) of the target analytes are prepared in methanol from the stock solutions and the commercial solution (50  $\mu\text{g}/\text{mL}$  in methanol). Stock solutions of the isotope labeled internal standards are prepared similarly. All stock solutions are divided into 2.0 mL polypropylene cryogenic vials and stored frozen.

**(2) PFCs Analytical Calibration Standards**

Standard spiking solutions are prepared in MeOH from the 10  $\mu\text{g}/\text{mL}$  working native standard solution such as a 25- $\mu\text{L}$  spike into 50  $\mu\text{L}$  calf serum provides concentrations that cover the linear range of the method, from 0.04-215 ng/mL for PFOS, 0.002-10 ng/mL for PFHpA, 0.008-41 ng/mL for PFNA, 0.008-44 ng/mL for Me-PFOSA-AcOH and 0.01-50 ng/mL for all other analytes. The spiking solutions are stored frozen in 1.5 mL aliquots in polypropylene cryogenic vials until use.

**(3) Internal Standard Spiking Solution**

The internal standard spiking solution is prepared by dissolving appropriate amounts of  $^{13}\text{C}_4$ -PFOA,  $^{13}\text{C}_4$ -PFOS,  $^{18}\text{O}_2$ -PFOSA,  $^{18}\text{O}_2$ -PFHxS,  $^{13}\text{C}_5$ -PFNA,  $^{13}\text{C}_2$ -PFDeA,  $^{13}\text{C}_2$ -PFDaA, D<sub>3</sub>-Me-PFOSA-AcOH, and D<sub>5</sub>-Et-PFOSA-AcOH (8-40 ng/mL) in methanol. A 25  $\mu\text{L}$  spike of this solution provides 2 ng/mL of  $^{18}\text{O}_2$ -PFOSA,  $^{18}\text{O}_2$ -PFHxS,  $^{13}\text{C}_2$ -PFDeA,  $^{13}\text{C}_2$ -PFDaA, D<sub>3</sub>-Me-PFOSA-AcOH, and D<sub>5</sub>-Et-PFOSA-AcOH,



5 ng/mL of  $^{13}\text{C}_2$ -PFOA, and 30 ng/mL of  $^{18}\text{O}_2$ -PFOS in 50  $\mu\text{L}$  sample. The spiking solutions are stored frozen in 1.8 mL aliquots in polypropylene cryogenic vials until use.

#### **(4) Instrument Test Standard**

The instrument test sample is prepared by spiking a reagent blank (i.e., formic acid) with 0.1 ng of PFOS and 0.05 ng of other analytes, equivalent to 0.5 and 0.25 ng/mL in serum, respectively.

#### **(5) Proficiency Testing (PT) Standards**

Appropriate aliquots of each stock standard are added to calf serum pools to produce proficiency testing (PT) standards of low-, medium- and high-concentrations. The PT standards are mixed, aliquoted into polypropylene cryogenic vials and frozen until needed. PT standards are characterized by at least 20 repeated analyses to determine the mean and standard deviation of the measurements.

#### **d. Materials**

- 1) Cartridge tray containing 96 Polaris C18 HD, 7  $\mu\text{m}$ , 10 mm x 2 mm, (Spark Holland, Plainsboro, NJ).
- 2) 750  $\mu\text{L}$  polypropylene autosampler vials with polyethylene snap caps (Agilent Tech., Wilmington DE).
- 3) Tip ejector variable volume micropipettes (Wheaton, Millville, NJ) and pipette tips (Rainin Instruments Co., Woburn, MA).
- 4) BETASIL C8 Column (3 mm x 50 mm, 5  $\mu\text{m}$ ) (Thermo Electron Corporation, Bellefonte, PA) and Betasil C8 precolumn (3 x 10 mm).
- 5) Nalgene polypropylene cryogenic vials, various sizes (Thermo Fisher Scientific, Rochester, NY).
- 6) Assorted glass and polypropylene labware.

#### **e. Equipment**

- 1) Symbiosis extractor equipped with Triathlon autosampler run by the SparkLink software program (Spark Holland, Plainsboro, NJ).
- 2) Agilent 1100 binary pump and degasser (Agilent Tech., Wilmington DE).
- 3) Applied Biosystems ABI 5500 mass spectrometer (Applied Biosystems, Foster City, CA).
- 4) Sartorius Genius Series ME models Electronic Analytical & Semi – microbalances (Sartorius AG, Goettingen, Germany).
- 5) Sartorius top – loading balance (Sartorius AG).
- 6) pH meter (Corning pH/ion analyzer 455, Corning, NY).
- 7) Vortex mixer (Type 16700, Barnstead International, Dubuque, IA).
- 8) Magnetic stirrer (Corning).

#### **f. Instrumentation**

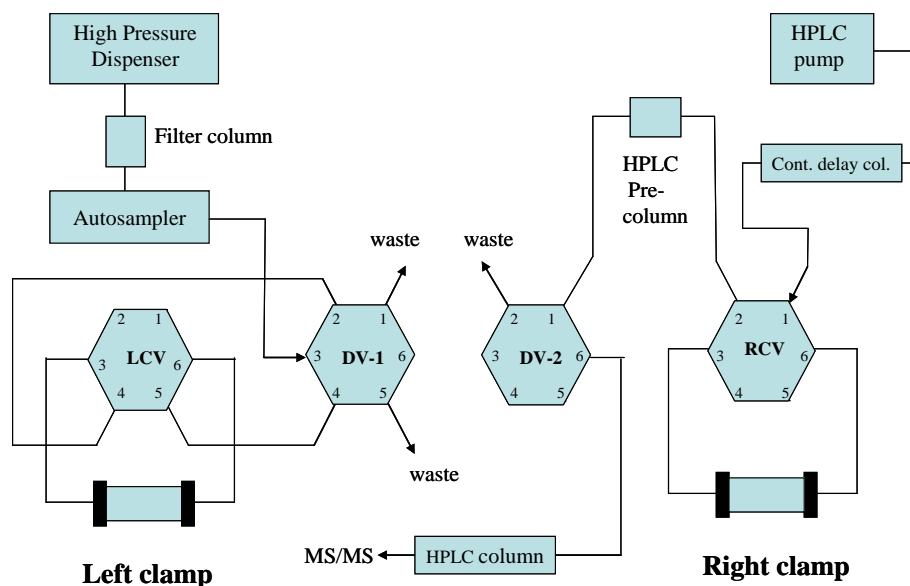
### 1) Automated SPE

The Symbiosis system is used in concurrent SPE/HPLC mode controlled by the SparkLink software (Table 1).

**Table 1.** Valve configurations used for concurrent SPE clean up and HPLC-MS/MS acquisition.

Steps <sup>a</sup>	Method	LCV	DV-1	DV-2	RCV	Time (min)
1	Move cartridge from left clamp to right clamp	6-1	1-2	6-1	1-2	0.1
2	Load new cartridge into left clamp	6-1	1-2	6-1	1-2	0.2
3	Send contact closer signal to HPLC-MS/MS	6-1	1-2	6-1	1-2	0.1
4	Begin HPLC gradient elution by-pass HPLC column and MS/MS	6-1	1-2	1-2	6-1	3.0 <sup>b</sup>
5	Condition left cartridge (2 mL acetonitrile, 2 mL/min)	1-2	1-2	6-1	6-1	1.2
6	Equilibrate left cartridge (2 mL 0.1 M formic acid, 2 mL/min)	1-2	1-2	6-1	6-1	1.2
7	Load 400 µL sample on left cartridge (3 mL, 0.1 M formic acid, 1 mL/min)	1-2	1-2	6-1	6-1	4.4
8	Forward wash left cartridge (1 mL 90% 0.1 M formic acid/10% ACN, 1 mL/min)	1-2	1-2	6-1	6-1	1.2
9	Return right cartridge to tray	6-1	1-2	6-1	1-2	0.1

<sup>a</sup>The method used for the first sample included only steps 2 and 5-9. The method used for the acquisition of the last sample included only steps 1, 3, 4, and 10. <sup>b</sup>For the acquisition of the last sample duration of step 4 was 13 min.



Tubing diagram for the Symbiosis column switching system used in concurrent SPE/HPLC mode. (LCV: left clamp valve; DV-1: divert valve 1; DV-2: divert valve 2; RCV: right clamp valve)

The method uses both left and right cartridge clamps, the four switching valves, and the high pressure dispenser. The left clamp, the left clamp valve (LCV), and left divert valve (DV-1) are used for SPE separation while the right clamp, the right clamp valve (RCV) and right divert valve (DV-2) are used for the HPLC elution. The SPE run of each sample starts with the conditioning of a Polaris HD C18 7  $\mu\text{m}$ , 10 mm x 1 mm (Prospekt 2) cartridge with HPLC-grade acetonitrile (2 mL) and 0.1 M formic acid (2 mL). Afterward, 400  $\mu\text{L}$  of the sample (containing 50  $\mu\text{L}$  serum) injected into the 1 mL sample loop is loaded onto the SPE column using 3 mL 0.1 M formic acid with 1 mL/min flow rate. Next, the SPE column is washed with 1 mL 90% 0.1 M formic acid/10% acetonitrile. The time of the SPE cleanup (including injection time) is 8 min long. Before starting the clean up of the next sample, the cartridge containing the extracted analytes is transferred by a robotic gripper from the left clamp into the right clamp. Therefore, while the right clamp is used for analyte elution and HPLC-MS/MS acquisition, the left clamp could be used for the clean up of the next sample. Once the SPE column is in the right clamp, the right clamp valve remains in by-pass (1-2) position until the HPLC-MS/MS system becomes ready to begin acquisition.

## 2) HPLC configuration

At the beginning of the HPLC-MS/MS acquisition, the right clamp valve is turned into 6-1 position for the first 10 min of the HPLC gradient program to

allow for transferring the analytes from the SPE column to the HPLC column. At 10 min, the right clamp valve is turned back to 1-2 position and the SPE column is returned to the cartridge tray while the HPLC gradient program continues. The HPLC pump is operated at a 600  $\mu\text{L}/\text{min}$  flow rate with 20 mM ammonium acetate (pH 4) in water and acetonitrile as mobile phase A and mobile phase B, respectively. The analytes are separated from other extracted components on a Betasil C8 column (3  $\times$  50 mm, 5  $\mu\text{m}$ ; Thermo Electron Corporation, Bellefonte, PA), preceded by a Betasil C8 precolumn (3  $\times$  10 mm). To delay the elution of the PFC contaminants leaching out from Teflon parts of the HPLC pump, a 3 mm  $\times$  50 mm, 5  $\mu\text{m}$  Betasil C8 column is inserted between the HPLC pump and the right clamp valve. Because the contaminant has to go through twice the column length, its peak elutes 1 min after the main analyte band without interfering with the concentration measurement. To filter out contaminants coming from the 0.1 M formic acid (used for sample clean up), a Betasil C8 3  $\times$  10 mm, 5  $\mu\text{m}$  precolumn is inserted between the high pressure dispenser and the autosampler.

#### HPLC configuration

Parameters	Setting
Mobile Phase A	20 mM ammonium acetate in water, pH = 4
Mobile Phase B	100% acetonitrile
Flow rate	600 $\mu\text{L}/\text{min}$

#### Mobile phase gradient

Time (min)	0	3	4	8	8.5	8.6	10
Mobile phase B%	15	15	55	80	80	15	15

### 3) Mass Spectrometer Configuration

Mass spectrometric analyses are conducted on the API 5500 tandem mass spectrometer in the negative ion Turbo Ion Spray (TIS) mode. The TIS ionization source is a variant of the electrospray source and is used to convert liquid phase ions into gas phase ions. We use laboratory-grade air heated turbo ion spray gas (GS1=45 and GS2=45) gas. The curtain and collision gas (nitrogen) settings are as follows: collision (CAD=2.0), curtain gas (CUR=25). Ionization parameters and collision cell parameters are optimized individually for each analyte (**Table 2**). Unit resolution is used for both Q1 and Q3 quadrupoles.

**Table 2. Mass spectrometric parameters for the analysis of serum for PFCs**

	(M-H) <sup>-</sup> Precursor ion (m/z)	Product ion (m/z)	Dwell Time (ms)	DP (volts)	CE (volts)
PFOSA	498	78	50	-60	-85
<sup>18</sup> O <sub>2</sub> -PFOSA (IS)	503	82	50	-60	-85
Me-PFOSA-AcOH	570	512	50	-45	-30
D <sub>3</sub> -Me-PFOSA- AcOH(IS)	573	515	50	-45	-30
Et-PFOSA-AcOH	584	526	50	-45	-30
D <sub>5</sub> -Et-PFOSA-AcOH (IS)	589	531	50	-45	-30
PFBuS-1	299	99	50	-70	-80
PFBuS-2 <sup>a</sup>	299	80	50	-70	-85
PFHxS-1	399	99	50	-70	-80
PFHxS-2 <sup>a</sup>	399	80	50	-70	-85
PFHxS- <sup>18</sup> O <sub>2</sub> -1 (IS)	403	103	50	-70	-80
PFHxS- <sup>18</sup> O <sub>2</sub> -2 (IS) <sup>a</sup>	403	84	50	-70	-85
PFOS-1 <sup>a</sup>	499	99	50	-70	-90
PFOS-2	499	80	50	-70	-80
PFOS- <sup>13</sup> C <sub>4</sub> -1 (IS)	503	99	50	-70	-85
PFOS- <sup>13</sup> C <sub>4</sub> -2 (IS)	503	80	50	-70	-85
PFHpA	363	319	50	-25	-13
PFOA	413	369	50	-27	-14
PFOA- <sup>13</sup> C <sub>4</sub> (IS)	417	372	50	-30	-15
PFNA	463	419	50	-30	-13
PFNA- <sup>13</sup> C <sub>5</sub> (IS))	468	423	50	-30	-13
PFDeA	513	469	50	--30	-15
PFDeA- <sup>13</sup> C <sub>2</sub> (IS)	515	470	50	-30	-15
PFUA	563	519	50	-30	-17
PFUA - <sup>13</sup> C <sub>2</sub> (IS)	565	520	50	-30	-17
PFDoA	613	569	50	-30	-18
PFDoA- <sup>13</sup> C <sub>2</sub> (IS)	615	570	50	-45	-15

<sup>a</sup> used for confirmation. For PFOS and PFHxS, we monitor more than one ion transition. One transition is used for quantification while the other(s) are for confirmation.

## 7. Calibration and Calibration-Verification Procedures

### a. Calibration Curve

Nine-point calibration curves are calculated daily from the analyte area ratios (i.e., analyte area/internal standard area) obtained from extracted standards in calf serum. A linear regression analysis (weighted by 1/x) of the area ratio versus standard concentration is performed. Correlation coefficients should be greater than 0.97. Samples with values exceeding the highest point in the calibration curve are reanalyzed using less serum.

### b. Mass Spectrometer Calibration

The ABI 5500 mass spectrometer is calibrated and tuned every 3-6 months using a polypropylene glycol (PPG) solution according to the instructions

contained in the ABI 5500 operator's manual. The instrument sensitivity is checked before every run by injecting the Instrument Test sample.

**c. Calibration Verification**

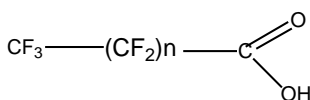
- 1) Calibration verification is not required by the manufacturer. However, it should be performed after any substantive changes in the method or instrumentation (e.g., new internal standard, change in instrumentation), which may lead to changes in instrument response, have occurred.
- 2) Calibration verification must be performed at least once every 6 months.
- 3) All calibration verification runs and results shall be appropriately documented.
- 4) According to the updated Clinical Laboratory Improvement Amendments (CLIA) regulations from 2003 the requirement for calibration verification is met if the test system's calibration procedure includes three or more levels of calibration material, and includes a low, mid, and high value, and is performed at least once every six months.
- 5) All of the conditions above are met with the calibration procedures for this method. Therefore, no additional calibration verification is required by CLIA.

**d. Proficiency Testing (PT)**

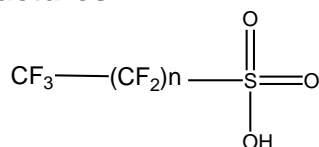
- 1) Three serum pools of PT samples, which encompass the entire linear range of the method, prepared in-house are characterized from at least 20 separate determinations. Once the PT pools are characterized, the mean concentration and standard deviation of the PT materials are forwarded to a DLS representative (PT administrator) responsible for executing the PT program. These PT samples are blind-coded by the PT administrator and returned to the laboratory staff for storage. When proficiency testing is required, the laboratory supervisor or his/her designee will notify the PT administrator, and the PT administrator will provide the blinded reference numbers for the 5 PT samples to be analyzed.
- 2) Proficiency testing should be performed a minimum of once per 6 months. The PT administrator will randomly select five PT materials for analysis. Following analysis, the results will be forwarded directly to the PT administrator for evaluation. A passing score is obtained if at least four of the five samples fall within the prescribed limits established by the PT administrator. The PT administrator will notify the laboratory staff of the PT results (i.e., pass/fail).
- 3) All proficiency results shall be appropriately documented.

- 4) In addition to the in-house PT program, since 2005 we have successfully participated in the international round-robin program organized by Intercal (Sweden) and RIVO (The Netherlands) when it is conducted for human serum/plasma [13,14].
- 5) Also, since 2006, at least once per year, we participate in the ongoing German External Quality Assessment Scheme (G-EQUAS) for PFOS and PFOA in serum, organized and managed by the Institute and Outpatient clinic for Occupational, Social and Environmental Medicine of the University of Erlangen-Nuremberg (Erlangen, Germany). The design, evaluation and certification of G-EQUAS are based on the guidelines of the German Federal Medical Council. Since 2011, three times a year we also participate in the ongoing Arctic Monitoring and Assessment Program (AMAP) Ring Test for several PFCs in human serum, conducted by the Institut National de Santé Publique du Québec (INSP) in Canada.

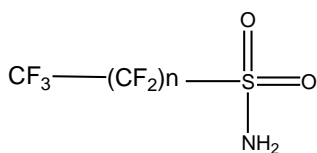
#### f. Analytes nomenclature and structures



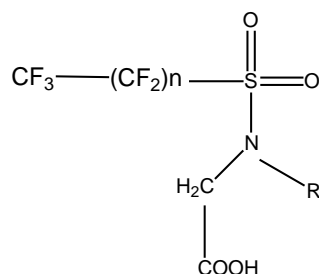
n=3 PFPeA  
n=4 PFHxA  
n=5 PFHpA  
n=6 PFOA  
n=7 PFNA  
n=8 PFDeA  
n=9 PFUA  
n=10 PFDaA



n=3 PFBuS  
n=5 PFHxS  
n=7 PFOS



n=7 PFOSA



n=7, R= Et Et-PFOSA-AcOH  
n=7, R= Me Me-PFOSA-AcOH

## 8. Operating Procedures; Calculations; Interpretation of Results

### a. Sample preparation

- 1) Label polypropylene snap-cap autosampler vials with appropriate Sample Names.
- 2) Remove serum samples, working standard solutions and internal standard solution from the freezer(s). Once thawed, vortex for at least 10 sec and aliquot 50  $\mu\text{L}$  of serum, QCs, or serum blank (SB) into the polypropylene

snap-cap autosampler vials that will contain the sample to be analyzed. For the standards (S1-S9), use 50 µL of blank serum.

- 3) Aliquot 0.1 M formic acid (375 µL for reagent blanks [QCBs], 325 µL for unknown [UNK], QCs, and SBs, 300 µL for STDs) into appropriate vials.
- 4) Add 25 µL of the appropriate native standard solution (S1-S9) into the polypropylene vials designated for standards.
- 5) Dispense 25 µL of internal standard solution (mix of <sup>13</sup>C<sub>4</sub>-PFOA, <sup>13</sup>C<sub>4</sub>-PFOS, <sup>18</sup>O<sub>2</sub>-PFOSA, <sup>18</sup>O<sub>2</sub>-PFHxS, <sup>13</sup>C<sub>5</sub>-PFNA, <sup>13</sup>C<sub>2</sub>-PFDeA, <sup>13</sup>C<sub>2</sub>-PFDoA, D<sub>3</sub>-Me-PFOSA-AcOH, and D<sub>5</sub>-Et-PFOSA-AcOH) into each polypropylene autosampler vial.
- 6) Vortex all vials for five 5 seconds to make sure all the internal standard and standard mixed into the sample.

## b. Analysis

### 1) Check out the LC system

After the column has been conditioned, click on the Equilibrate icon, select the current method, and let the system equilibrate for approximately 30 minutes. Run the Instrument Check sample by opening the batch file named Instrument\_test.dab. Change the date in the Sample Name field. Make sure the proper Acquisition Method and Vial Position are entered, and submit the batch. The file should be saved into the Instrument\_test.wiff file. Open the chromatogram and compare the intensities and peak shape to those obtained a day and a week before. If peaks appear distorted (tailing peaks, broad peaks, etc.) change the column and submit the Instrument Check sample again. If the absolute intensity is too low (peak intensity should not be <70% less intense than before) check with the laboratory supervisor or his/her designee.

### 2) Check out the SPE system

- (a) In SparkLink, put the Triathlon autosampler into load position. Initialize the high pressure dispenser (HPD) and the automated cartridge exchanger (ACE) unit.
- (b) Exchange the cartridge tray as needed (generally after analyzing approximately 500 samples).
- (c) Make sure that the MS remote cable is connected to the ACE unit.

### 3) Check out the LC/MS interface

- (a) Periodically, take off the interface housing (if hot, wait until it cools down), and wipe out the skimmer plate.



- (b) Periodically, open the rough pump cabinet, check for oil leaks and unusual noise. Report anything unusual.

4) **Automated SPE-HPLC-MS/MS Analysis Procedure**

- (a) Place the Triathlon autosampler into load position. Initialize the high pressure dispenser (HPD) and the automated cartridge exchanger (ACE) unit.
- (b) Purge the solvent lines on the HPLC binary pump and equilibrate the HPLC column.
- (c) In the **SparkLink** software, go to RunTables and open and set up the batch table. For the first sample enter xx-method 1 which runs the injection and cleanup of the first sample. For the second and consecutive samples use xx-method 2 which initiates the HPLC/MS acquisition and runs the injection and cleanup of the next sample. For the last sample enter xx-method 3 which initiates the HPLC/MS acquisition of the last sample. For injection volume, enter 400  $\mu$ L. Make sure the right vial positions are entered and there is no sequential duplication of cartridge numbers.
- (d) In the Analyst software, open a new subproject folder for each new run. The subproject should have the same YYYY-MMDD name as the unknowns it includes. Each subproject should have separate Acquisition Methods, Quantitation Methods, Batch, Data, and Results directories. Copy the latest Acquisition Method and Quantitation Method from the previous subfolder.
- (e) Go to **Excel**, open the text file containing the batch table created from Sample Login Table in the Microsoft Access database. This file should not require any editing. Save the table into the text file named import.txt into the Batch directory (overwrite). Remember to CLOSE THE FILE IN EXCEL!!!!
- (f) Go to **Analyst**, open a new batch table and import the import.txt file (Sample pull down, go to gray header and click RMB, then Import From/File, select Agilent 1100 G1313 autosampler). Make sure that the proper Acquisition Method and Quantitation Method are entered. The vial positions entered in Analyst should agree with the vial positions used on the Triathlon autosampler. Submit the batch (highlight and/or click Submit, go to View Queue, and click Start Sample). All samples on the Queue Manager should be in "waiting".
- (g) Start the batch table in **SparkLink**. From this on everything should run automatically. Submit the batch table in Analyst (highlight and/or click Submit, go to View Queue, and click Start Sample). From this on

everything should run automatically. After the SPE cleanup of the first sample, each N+1 sample in the SparkLink batch table will correspond with sample N in the Analyst batch table.

**c. Processing data**

**(a) Quantification**

All raw data files are analyzed using the Quantitation Wizard application in the Analyst software, which allows both automatic and manual peak selection and area integration. The area values and retention times are exported into a tab delimited text file and imported into the Access database with the name YYYY-MMDD.txt.

**(b) Importing Data into the Database**

The tab-delimited file is read into the Access database. No prior editing is required.

**6). Statistical Analysis and Interpretation of Data**

Data are exported from the Access database to a fixed ASCII text file and imported into SAS. SAS programs for standard curve generation, QC analysis, blank analysis, limit of detection determination, unknown calculations, and data distribution have been created and may be executed in SAS when this information is needed.

**d. Replacement and periodic maintenance of key components**

**1) ABI 5500 Mass Spectrometer**

Preventative maintenance is done by a qualified engineer at least once a year. In addition, to ensure proper performance of the system, a periodic maintenance of the system may be required.

(a) When a partial blockage of the vacuum is suspected, the orifice is probed with a syringe-cleaning wire.

(b) Cleaning of the spray shield and the entrance end of the heated capillary is performed weekly as described in the ABI 5500 Hardware Manual. First, wash with a solution of water: methanol (1:1) and then, with 100% methanol. Wipe the area using flake free paper wipes.

(c) The pump oil is changed approximately every six months as part of the periodic maintenance of the system.

**2) Agilent 1100 HPLC**

Preventative maintenance is done by a qualified engineer at least once a year. Additional maintenance may be necessary if there is a general decrease in instrument performance (see below). In general, performance maintenance procedures are performed after detecting a decrease in the system performance (sensitivity and/or S/N ratio) without any other apparent technical reasons.

- (a) The HPLC column is replaced when analyte resolution decreases. Once the analyte peaks start tailing, the HPLC column should be replaced.
- (b) If high pressure error messages are observed, the purge valve frit, the guard column, analytical column frit, HPLC lines, needle seat, or injector components may need to be replaced.
- (c) Reestablishment of performance and calibration. Every time the system is disturbed for cleaning or maintenance, a mass spec instrument test standard is analyzed to assess the HPLC and MS performance. For the mass spectrometer, a retune of the system may or may not be necessary. If the instrument does not pass this test, then the instrument is retuned using PPG as described previously.

### 3) Spark system

Preventative maintenance is done by a qualified engineer at least once a year. Additional maintenance may be necessary if there is a general decrease in instrument performance.

If the SparkLink error "HPD 1 high pressure problem" occurs, check the SPE lines and HPD 6 port valve. The HPD valve stator and/or rotor may need to be replaced.

## 9. Reportable Range of Results

The linear range of the standard calibration curves and the method limit of detection (LOD) determine the reportable range of results. The reportable range must be within the range of the calibration curves. However, samples with concentrations exceeding the highest reportable limit may be diluted, re-extracted, and reanalyzed so that the measured value will be within the range of the calibration.

If a sample needs more than 100 times dilution (which would require using less than 1  $\mu\text{L}$  of specimen) the dilution is performed in at least two steps. For example, first, at least 10  $\mu\text{L}$  serum is diluted up to 1 mL with water in a 2 mL Eppendorf tube (or equivalent), then a second dilution is performed by aliquoting the appropriate fraction of the dilute into an autosampler vial and adding 100  $\mu\text{L}$  0.1 M formic acid. With very concentrated specimens it may be difficult to estimate the dilution that is necessary, and the measured value may be higher than the highest calibration point even after the dilution. To avoid further repeats, the calibration curve can be extended on the high end by measuring additional standards using multiple amounts (100, 150 and 200  $\mu\text{L}$ ) of the highest (S9) spiking solution.

Formula to calculate the dilution factor to be entered into the Analyst batch file:

$$D = (1000 / V_{1st}) \times (100 / V_{2nd}).$$

Formula to calculate the volume of specimen to be entered into the Access database:

$$V = V_{1st} \times (V_{2nd} / 1000)$$

Where  $V_{1st}$  is the volume of the aliquot taken from the original specimen and  $V_{2nd}$  is the volume of the dilute measured into the autosampler vial.

#### a. Analytical Sensitivity

The limits of detection (LOD) are defined for each analyte by replicate analysis of low level standards and the calculation of the standard deviation at zero concentration ( $S_o$ ) [15]. The formal LOD is defined as  $3S_o$ . The limit of quantitation is defined as  $5S_o$ . The functional LOD is equal to the formal LOD since it is higher than the lowest standard concentration used in the calibration curve. **Table 4** summarizes the LOD determined for each analyte in serum.

#### b. Analytical Specificity

This is a highly selective method that requires that the PFCs 1) elute at a specific retention time; 2) have precursor ions with specific mass/charge ratios; 3) have specific product ions formed from the precursor ion with specific mass/charge ratios.

#### c. Linearity Limits

The calibration curve is linear for all analytes ( $R^2 > 0.97$ ). The limit on the linearity is determined by the highest standard analyzed in the method. Due to the wide variation of PFCs concentrations in humans, we set our highest standard near the high end of the linear range (**Table 4**). Unknown samples whose concentrations exceed the highest standard concentration must be re-extracted using a smaller aliquot. The low end of the linear range is limited by the method LOD. Samples whose concentrations are below the method LOD (or the concentration of the lowest standard in the calibration curve) are reported as non-detectable.

**Table 4. Linear range (LOD – highest standard concentration), LOD and LOQ for each PFC measured in serum.**

Analyte	LOD	LOQ	Linear range (ng/mL)
PFOSA	0.1	0.2	0.01-50
Me-PFOSA-AcOH	0.09	0.2	0.009-44
Et-PFOSA-AcOH	0.1	0.2	0.01-50

PFBuS	0.1	0.2	0.01-50
PFHxS	0.1	0.2	0.01-50
PFOS	0.2	0.3	0.04-215
PFHpA	0.1	0.7	0.002-10
PFOA	0.1	0.2	0.01-50
PFNA	0.08	0.3	0.008-41
PFDeA	0.1	0.3	0.01-50
PFUA	0.1	0.4	0.01-50
PFDoA	0.1	0.4	0.01-50

d. **Accuracy**

The accuracy of the method is determined by enriching serum samples with known concentrations of the PFC analytes and comparing the calculated and expected concentrations. To examine their consistency over the range of levels encountered in serum, repeated measurements (N = 5) are taken at 3 different concentrations (Table 5).

**Table 5. Spiked recoveries of extracted standards in calf serum**

Analyte	Accuracy (%) at ~LOQ/1.25/12.5 ng/mL		
	PFOSA	87±7	100±6
Me-PFOSA-AcOH	135±66	89±7	100±3
Et-PFOSA-AcOH	110±8	90±9	102±2
PFBuS	103±35	82±9	95±3
PFHxS	108±30	98±8	95±5
PFOS	118±25	93±10	102±3
PFHpA	200±10	130±30	103±15
PFOA	95±60	109±14	99±7
PFNA	95±55	109±20	107±14
PFDeA	123±16	106±9	94±8
PFUA	87±67	102±6	98±11
PFDoA	80±52	86±15	105±7

e. **Precision**

The precision of this method is reflected in the variance of two serum quality control (QC) pools over time. The coefficient of variation (CV) of repeated measurements of these QC pools, which reflects both inter and intra-day variations, is used to estimate precision (Table 6).

Table 6. Mean QC concentration (ng/mL) and CV%

Analyte	QC Low	CV%	QC High	CV%
PFOSA	2.29	8.0	7.95	9.6
Me-PFOSA-AcOH	2.03	11.7	6.99	10.7
Et-PFOSA-AcOH	1.55	10.5	5.59	12.1
PFBuS	1.66	14.2	6.1	16.5
PFHxS	1.93	11.6	6.85	8.6
PFOS	8.48	10.3	27.77	9.8
PFHpA	1.55	25.4	5.42	25.1
PFOA	3.04	11.6	11.21	12.8
PFNA	2.26	11.9	8.32	12.3
PFDeA	3.29	11.6	11.38	12.20
PFUA	1.78	8.00	7.95	9.6
PFDoA	2.05	12.9	7.19	11.9

## 10. Quality Control (QC) Procedures

### a. QC Materials

QC materials were prepared in bulk from calf serum (Gibco, Grand Island, NY).

### b. Preparation of QC Pools

The calf serum pools were mixed uniformly, divided into three subpools, dispensed into small aliquots (ca. 2 mL) into polypropylene vials, and stored frozen until used. One subpool was used as a blank QC and to prepare the calibration standards, and the other two were enriched with PFCs as needed to afford low concentration (QCL) and high concentration (QCH) subpools (Table 6).

### c. Characterization of QC Materials

For characterization, a minimum of 30 runs of QCL and 30 runs of QCH were measured over 1 month. In each run, one pair of QCL and one pair of QCH materials were analyzed and averaged. Using the pair average value from the 30 runs, the mean, and upper and lower 99% and 95% control limits were established.

### d. Use of QC materials

A maximum of 50 unknown samples are run with randomly placed 2 QCH, 2 QCL, and 2 reagent blank samples. The concentrations of the two QCH and the

two QCL in each batch are averaged to obtain one average measurement of QCH and QCL for each batch per run.

e. **Final evaluation of Quality Control Results**

Standard criteria for run rejection based on statistical probabilities are used to declare a run either in-control or out-of-control [16]. When using 2 QC pool levels per run, the rules are:

For 1 QC result per pool

- 1) If both QC run results are within  $2S_i$  limits, then accept the run.
- 2) If 1 of the 2 QC run results is outside a  $2S_i$  limit - reject run if:
  - Extreme Outlier – Run result is beyond the characterization mean  $\pm 4S_i$
  - 1 3S Rule – Run result is outside a  $3S_i$  limit
  - 2 2S Rule – Both run results are outside the same  $2S_i$  limit
  - 10 X-bar Rule – Current and previous 9 run results are on same side of the characterization mean
  - R 4S Rule – Two consecutive standardized run results differ by more than  $4S_i$  (standardized results are used because different pools have different means). Since runs have single measurements per pool for 2 pools, comparison of results for the R 4S rule will be with the previous result within run or the last result of the previous run.

For 2 or more QC results per pool

- 1) If both QC run means are within  $2S_m$  limits and individual results are within  $2S_i$  limits, then accept the run.
- 2) If 1 of the 2 QC run means is outside a  $2S_m$  limit - reject run if:
  - Extreme Outlier – Run mean is beyond the characterization mean  $\pm 4S_m$
  - 1 3S Rule – Run mean is outside a  $3S_m$  limit
  - 2 2S Rule – Both run means are outside the same  $2S_m$  limit
  - 10 X-bar Rule – Current and previous 9 run means are on same side of the characterization mean
- 3) If one of the 4 QC individual results is outside a  $2S_i$  limit - reject run if:
  - R 4S Rule – Within-run ranges for all pools in the same run exceed  $4S_w$  (i.e., 95% range limit). Since runs have multiple measurements per pool for 2 pools, the R 4S rule is applied within runs only.

Abbreviations:

$S_i$  = Standard deviation of individual results (the limits are not shown on the chart unless run results are actually single measurements).

$S_m$  = Standard deviation of the run means (the limits are shown on the chart).

$S_w$  = Within-run standard deviation (the limits are not shown on the chart).

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

If the QC systems or the calibrations failed to meet acceptable criteria, all operations are suspended until the source or cause of failure is identified and corrected. If the source of failure is easily identifiable (e.g., failure of the mass spectrometer or a pipetting error), the problem is immediately corrected. Otherwise, fresh reagents are prepared and the mass spectrometer is cleaned. Before beginning another analytical run, several QC materials (in the case of QC failure) or calibration standards (in the case of calibration failure) are reanalyzed. After calibration or quality control has been reestablished, analytical runs may be resumed.

## **12. Limitations of Method; Interfering Substances and Conditions**

Occasionally, the concentration of the PFCs in serum may be higher than the highest standard in the calibration curves, and 0,05 mL of sample may be too much to use. This may be evident by the low recovery of the isotope-labeled standard. In this case, a smaller aliquot of serum can be used. Most likely, the LOD is not higher in this case because of the concentrated nature of the specimen.

## **13. Reference Ranges (Normal Values)**

The results from the National Health and Nutrition Examination Survey (NHANES) 1999-2000, 2003-2004, 2005-2006, 2007-2008 and 2009-2010 [17-19] will be used as the reference ranges for the general US population before and after the changes in PFC manufacturing practices (Table 7).



**Table 7. PFCs concentrations in serum (ng/mL): NHANES 1999-2000, 2003-2004, 2005-2006, 2007-2008**

Analyte	Survey years	N	Geometric mean	Median	75 <sup>th</sup> percentile	90 <sup>th</sup> percentile	95 <sup>th</sup> percentile
PFOSA	99-00	1562	0.4	0.3	0.3	1.0	1.4
	03-04	2094	NR	<LOD	<LOD	0.2	0.2
	05-06	2120	NR	<LOD	<LOD	0.2	0.3
	07-08	2100	NR	<LOD	<LOD	<LOD	<LOD
	09-10	2233	NR	<LOD	<LOD	<LOD	<LOD
Me-PFOSA-AcOH	99-00	1562	0.9	0.9	1.4	2.2	2.8
	03-04	2094	<LOD	<LOD	0.6	0.9	1.1
	05-06	2120	0.4	0.4	0.7	1.2	1.6
	07-08	2100	0.3	0.3	0.5	1.0	1.3
	09-10	2233	0.2	0.2	0.4	0.7	1.0
Et-PFOSA-AcOH	99-00	1562	0.6	0.5	1.1	1.6	2.2
	03-04	2094	NR	<LOD	<LOD	<LOD	<LOD
	05-06	2120	NR	<LOD	<LOD	<LOD	0.3
	07-08	2100	NR	<LOD	<LOD	<LOD	<LOD
	09-10	2233	NR	<LOD	<LOD	<LOD	0.1
PFHxS	99-00	1562	2.1	2.1	3.3	5.7	8.7
	03-04	2094	1.9	1.9	3.3	5.9	8.3
	05-06	2120	1.7	1.8	3.2	9.0	11.3
	07-08	2100	2.0	2.0	3.5	5.9	9.8
	09-10	2233	1.7	1.7	3.0	4.8	6.9
PFOS <sup>a</sup>	99-00	1562	30.4	30.2	43.7	57.0	75.7
	03-04	2094	20.7	21.2	30.0	41.3	54.6
	05-06	2120	17.1	17.5	27.2	39.4	47.5
	07-08	2100	13.2	13.6	21.0	32.6	40.5
	9-10	2233	9.3	9.7	14.8	23.7	32.0
PFBuS	99-00	1562	NR	NA	NA	NA	NA
	03-04	2094	NR	<LOD	<LOD	<LOD	<LOD
	05-06	2120	NR	<LOD	<LOD	<LOD	0.1
	07-08	2100	NR	<LOD	<LOD	<LOD	<LOD
	09-10	2233	NR	<LOD	<LOD	<LOD	<LOD

PFHpA	99-00	1562	NR	NR	NR	NR	0.7
	03-04	2094	NR	<LOD	<LOD	<LOD	0.4
	05-06	2120	NR	<LOD	<LOD	<LOD	0.7
	07-08	2100	NR	<LOD	<LOD	<LOD	0.5
	09-10	2233	NR	<LOD	<LOD	0.2	0.2
PFOA <sup>b</sup>	99-00	1562	5.2	5.2	6.9	9.4	11.9
	03-04	2094	4.0	4.1	5.8	7.8	9.8
	05-06	2120	3.9	4.2	6.2	9.0	11.3
	07-08	2100	4.1	4.3	5.9	7.9	9.6
	09-10	2233	3.1	3.2	4.6	6.0	7.5
PFNA	99-00	1562	0.6	0.6	0.9	1.3	1.8
	03-04	2094	1.0	1.0	1.5	2.3	3.2
	05-06	2120	1.1	1.1	1.7	2.6	3.6
	07-08	2100	1.2	1.2	1.7	2.6	3.3
	09-10	2233	1.3	1.2	1.8	2.7	3.8
PFDeA	99-00	1562	NR	0.2	NR	0.4	0.6
	03-04	2094	NR	<LOD	0.3	0.6	0.9
	05-06	2120	0.4	0.4	0.5	0.9	1.5
	07-08	2100	0.3	0.3	0.4	0.7	0.9
	09-10	2233	0.3	0.3	0.4	0.7	0.9
PFUA	99-00	1562	NR	0.2	0.2	0.3	0.4
	03-04	2094	NR	<LOD	<LOD	<LOD	0.6
	05-06	2120	NR	<LOD	0.3	0.5	0.7
	07-08	2100	NR	<LOD	0.2	0.4	0.6
	09-10	2233	0.2	0.2	0.3	0.6	0.9
PFDoA	99-00	1562	NR	<LOD	<LOD	<LOD	<LOD
	03-04	2094	NR	<LOD	<LOD	<LOD	<LOD
	05-06	2120	NR	<LOD	<LOD	<LOD	<LOD
	07-08	2100	NR	<LOD	<LOD	<LOD	<LOD
	09-10	2233	NR	<LOD	<LOD	<LOD	<LOD

NA: Not applicable; NR: Not reported (frequency of detection was <25%)

<sup>a, b</sup>Note: The reported concentrations of PFOS and PFOA included their branched and linear isomers.

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

Insufficient data exist to correlate any PFCs concentrations with serious health effects. Therefore, critical call values have not been established.

#### **15. Specimen Storage and Handling During Testing**

Remove serum samples, working standard solutions and internal standard solution from the freezer, and let the serum thaw. Place solutions back in the freezer after finalizing the aliquoting step.

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

Alternate procedures do not exist in-house for the measurement of PFCs.

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

- a. The Quality Control officer reviews each analytical run, identifies the QC samples within each analytical run and determines whether the analytical run is performed under acceptable QC conditions.
- b. Data from analytical runs of unknowns are reviewed by the laboratory supervisor. The supervisor provides feedback to the analyst and/or his/her designee and requests confirmation of the data as needed.
- c. One of the Division statisticians reviews and approves the quality control charts pertinent to the results being reported.
- d. If the quality control data are acceptable, the laboratory supervisor or his/her designee generates a memorandum to the Branch Chief, and a letter to be signed by the Division Director to the person(s) who requested the analyses reporting the analytical results.
- e. The data are then sent to the person(s) that made the initial request.
- f. All data (chromatograms, etc.) are stored in electronic format in the laboratory.
- g. Final hard copies of correspondence are maintained in the office of the Branch Chief and with the quality control officer.

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

Standard record keeping systems (e.g., notebooks, sample logs, data files) should be employed to keep track of all specimens. One spreadsheet form (CLIA Specimen Tracking Records) with information for receiving/transferring specimens is kept in the

laboratory. In this form, the samples received are logged in when received and when stored/transferred after analysis. For NHANES samples, the person receiving the specimens signs and dates the shipping manifests. The shipping manifests for NHANES and other samples are kept in a binder in the Laboratory.

19. Summary Statistics and QC Graphs

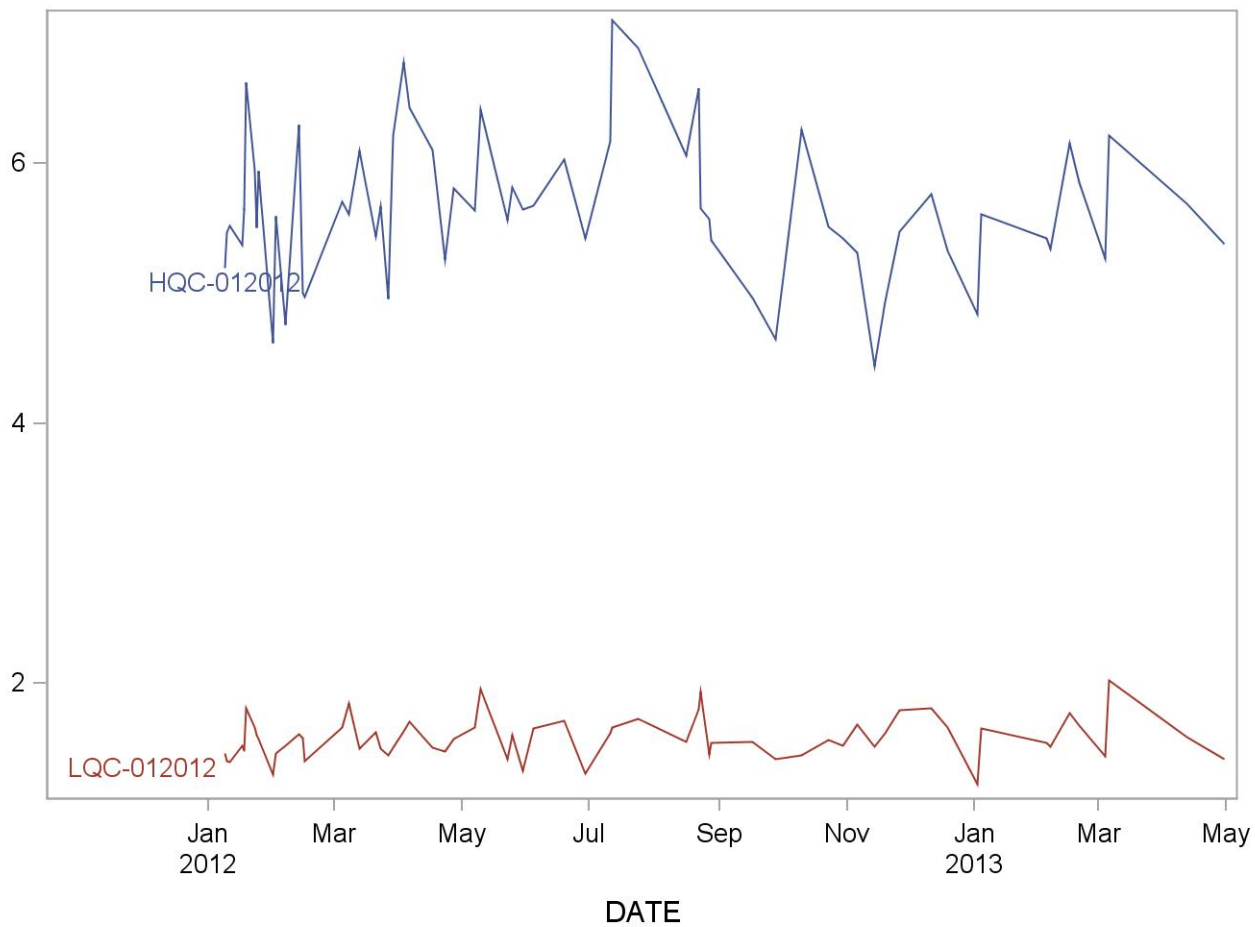
See following pages

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*Use of trade names is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.*

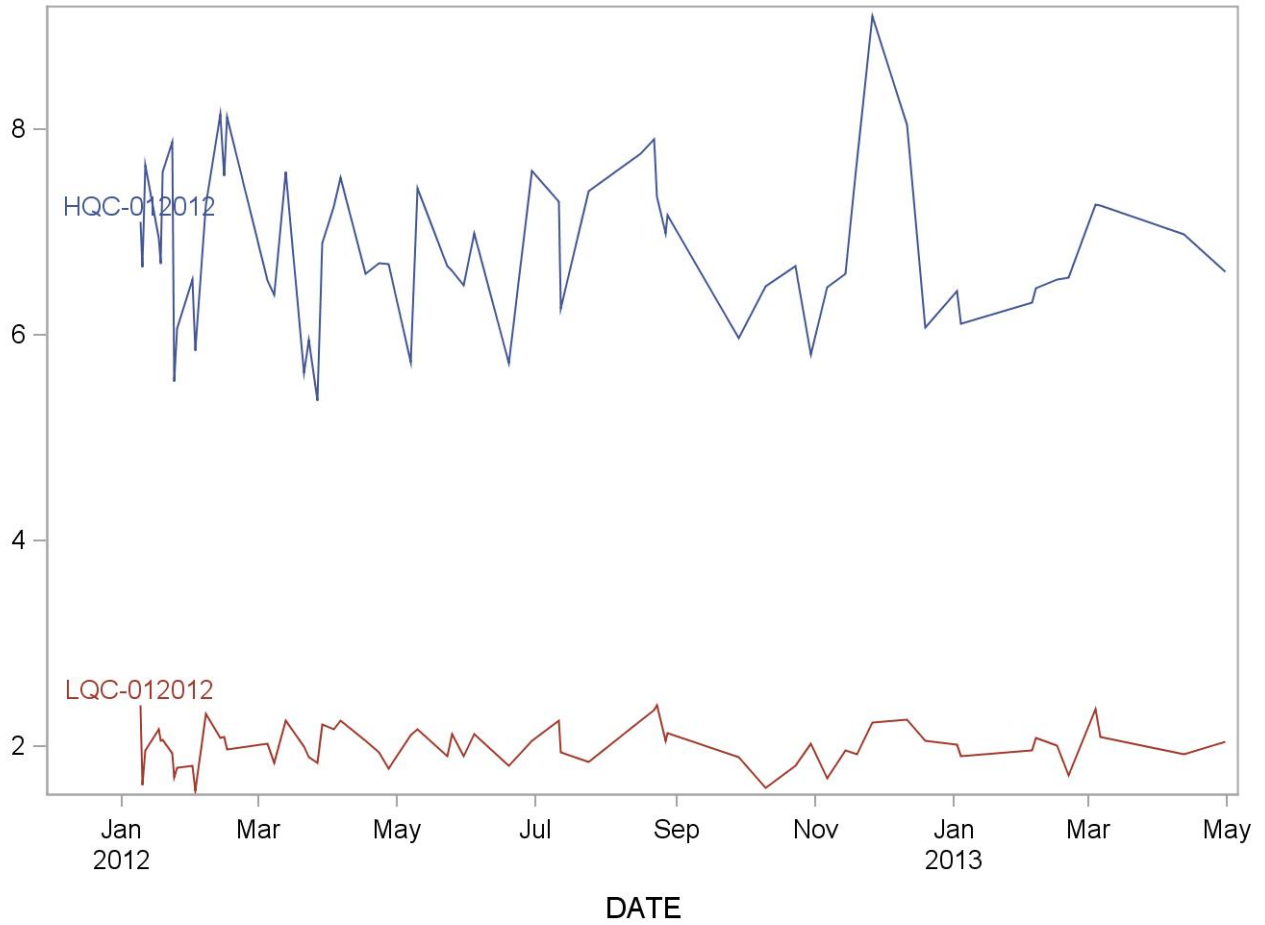
2011-2012 Summary Statistics and QC Chart for Et-PFOSA-AcOH 2-N-ethyl-perfluorooctane

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012012	65	09JAN12	30APR13	5.66	0.55	9.8
LQC-012012	65	09JAN12	30APR13	1.58	0.16	10.1



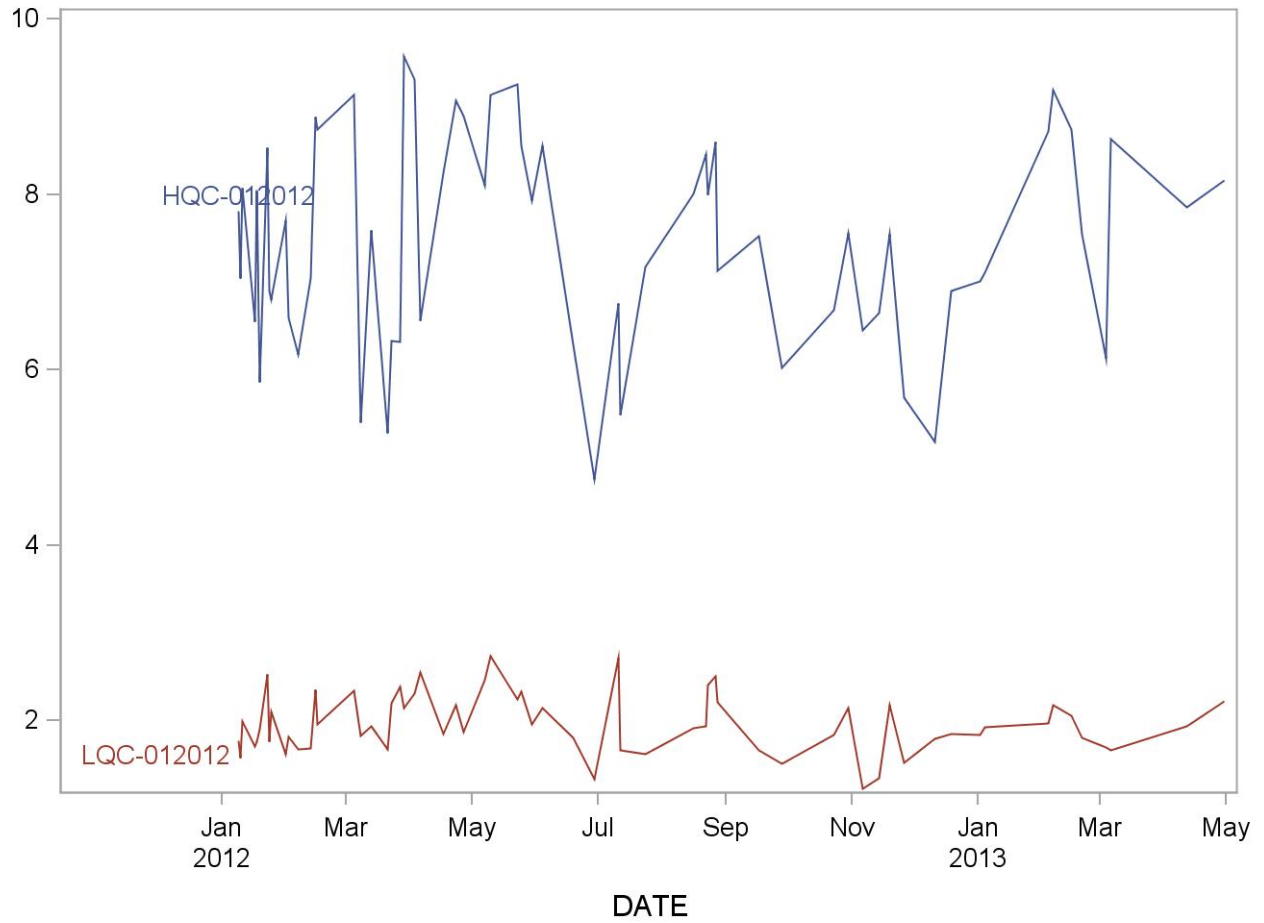
2011-2012 Summary Statistics and QC Chart for Me-PFOSA-AcOH 2-N-methyl-perfluorooctan

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012012	64	09JAN12	30APR13	6.85	0.74	10.9
LQC-012012	64	09JAN12	30APR13	2.02	0.20	9.7



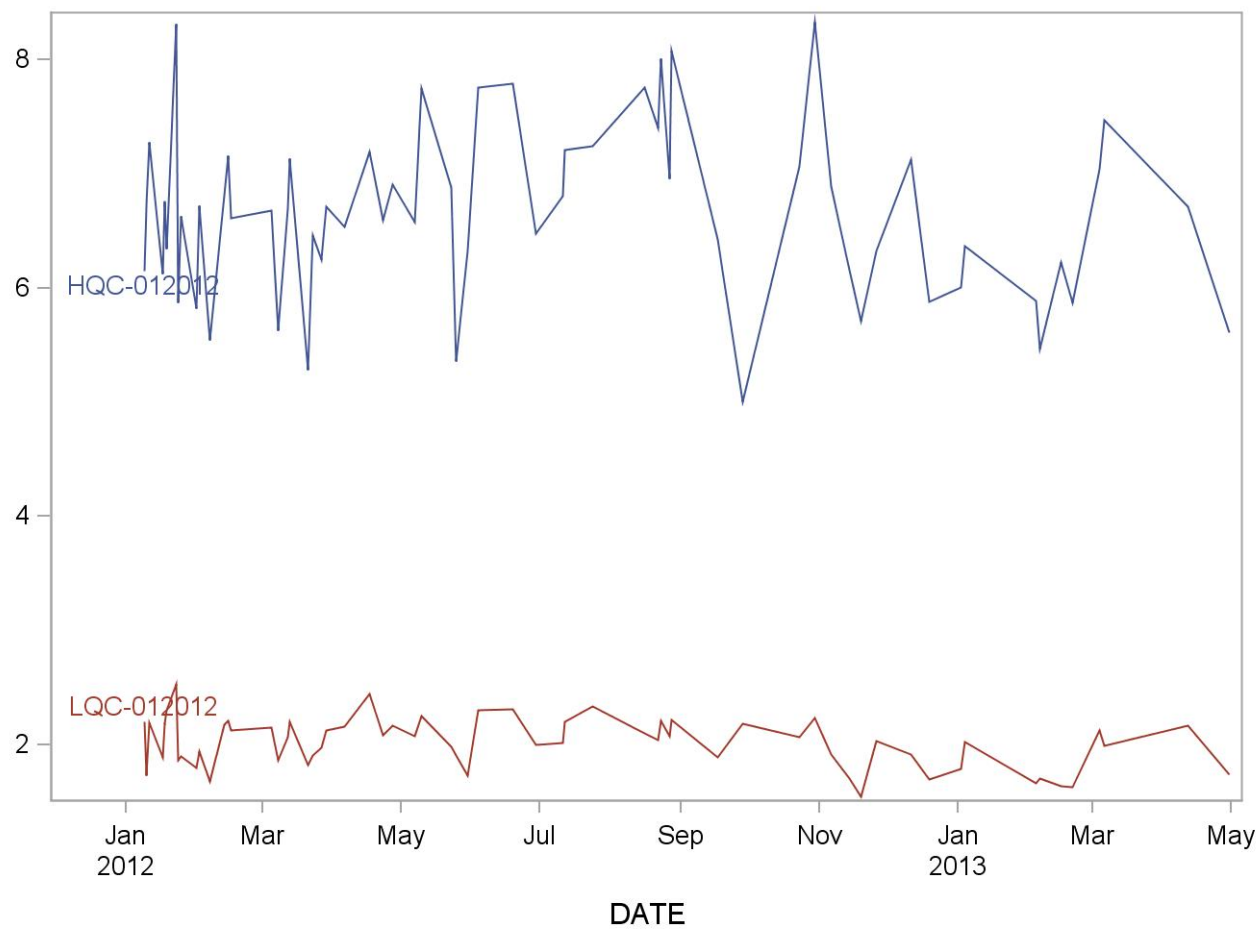
**2011-2012 Summary Statistics and QC Chart for PFBS**

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012012	64	09JAN12	30APR13	7.47	1.20	16.0
LQC-012012	64	09JAN12	30APR13	1.96	0.33	16.9



**2011-2012 Summary Statistics and QC Chart for PFD<sub>o</sub>A perfluorododecanoic acid**

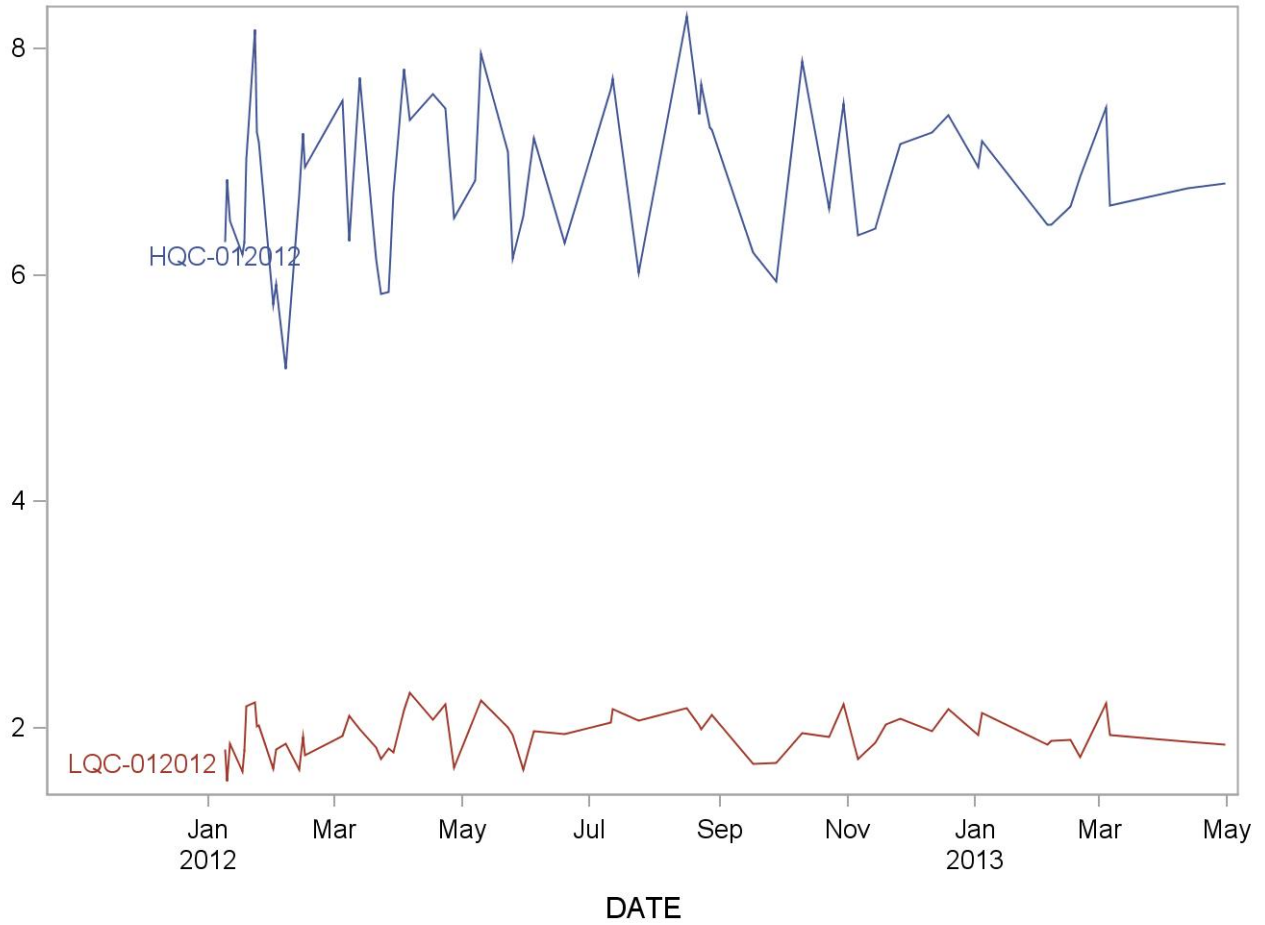
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012012	64	09JAN12	30APR13	6.65	0.75	11.3
LQC-012012	64	09JAN12	30APR13	2.02	0.22	10.8





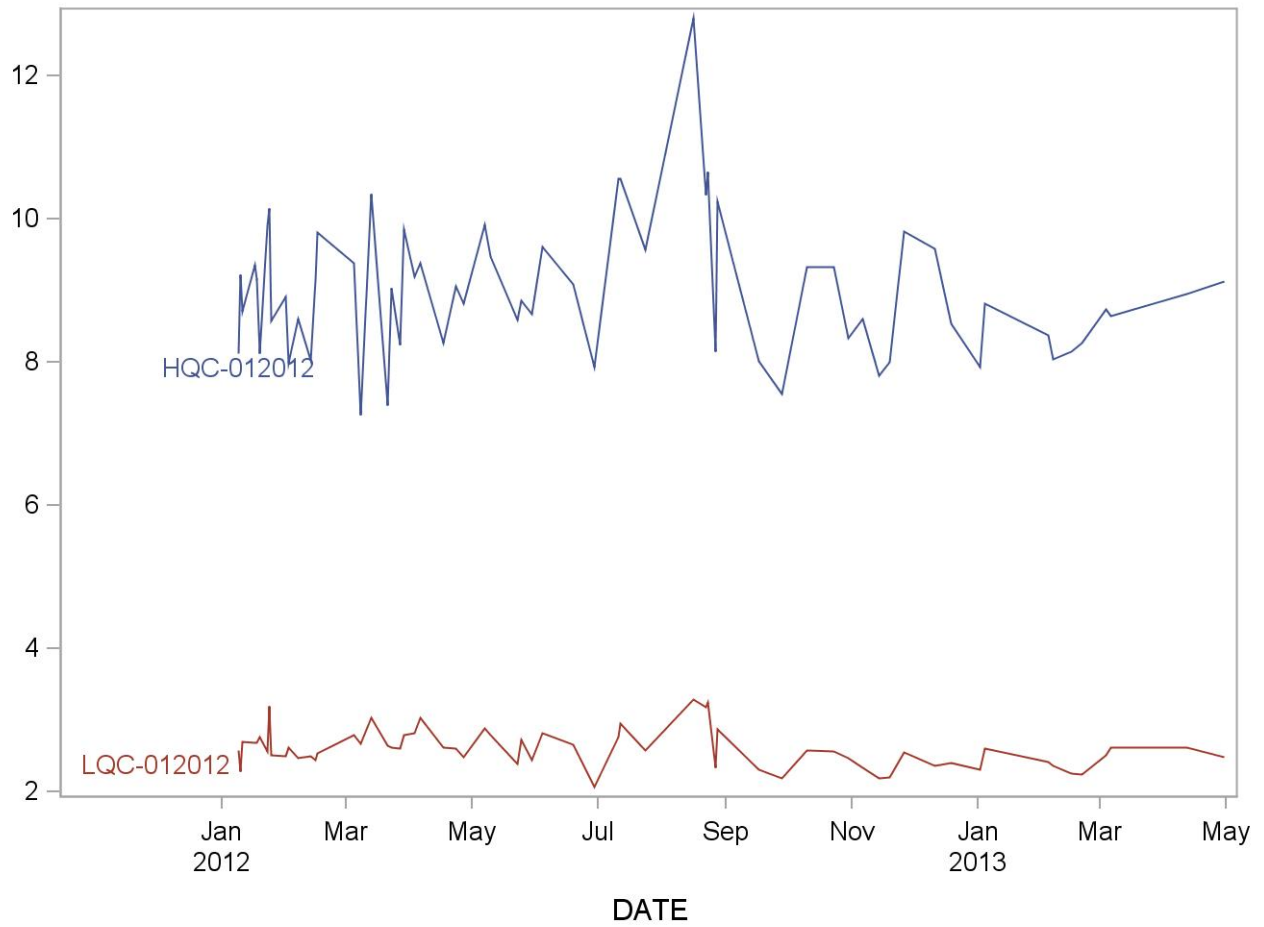
2011-2012 Summary Statistics and QC Chart for PFHS Perfluorohexane sulfonic acid

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012012	64	09JAN12	30APR13	6.87	0.67	9.7
LQC-012012	64	09JAN12	30APR13	1.94	0.19	9.5



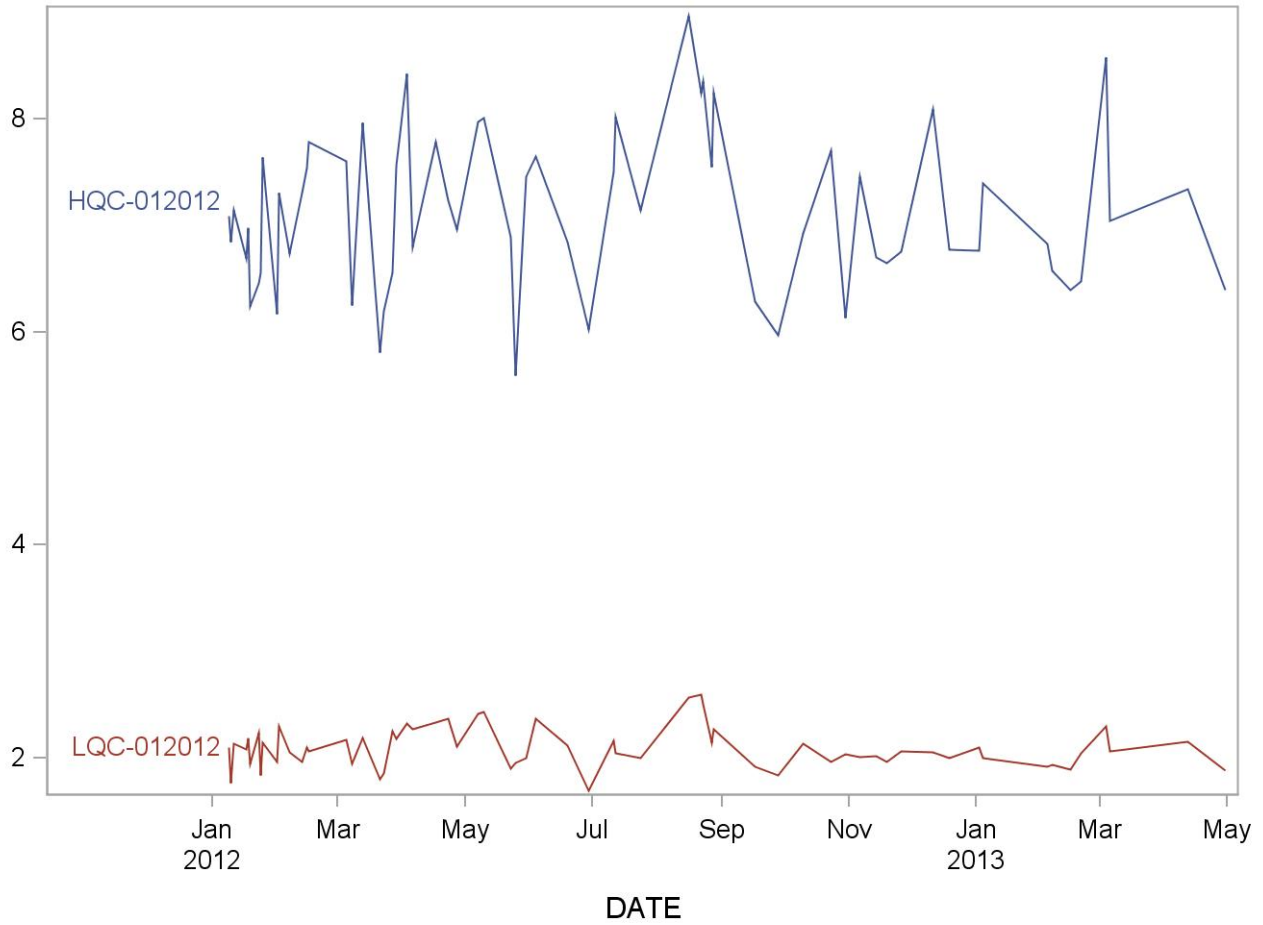
**2011-2012 Summary Statistics and QC Chart for PFNA Perfluorononanoic acid**

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012012	65	09JAN12	30APR13	8.98	0.96	10.7
LQC-012012	65	09JAN12	30APR13	2.60	0.26	10.2



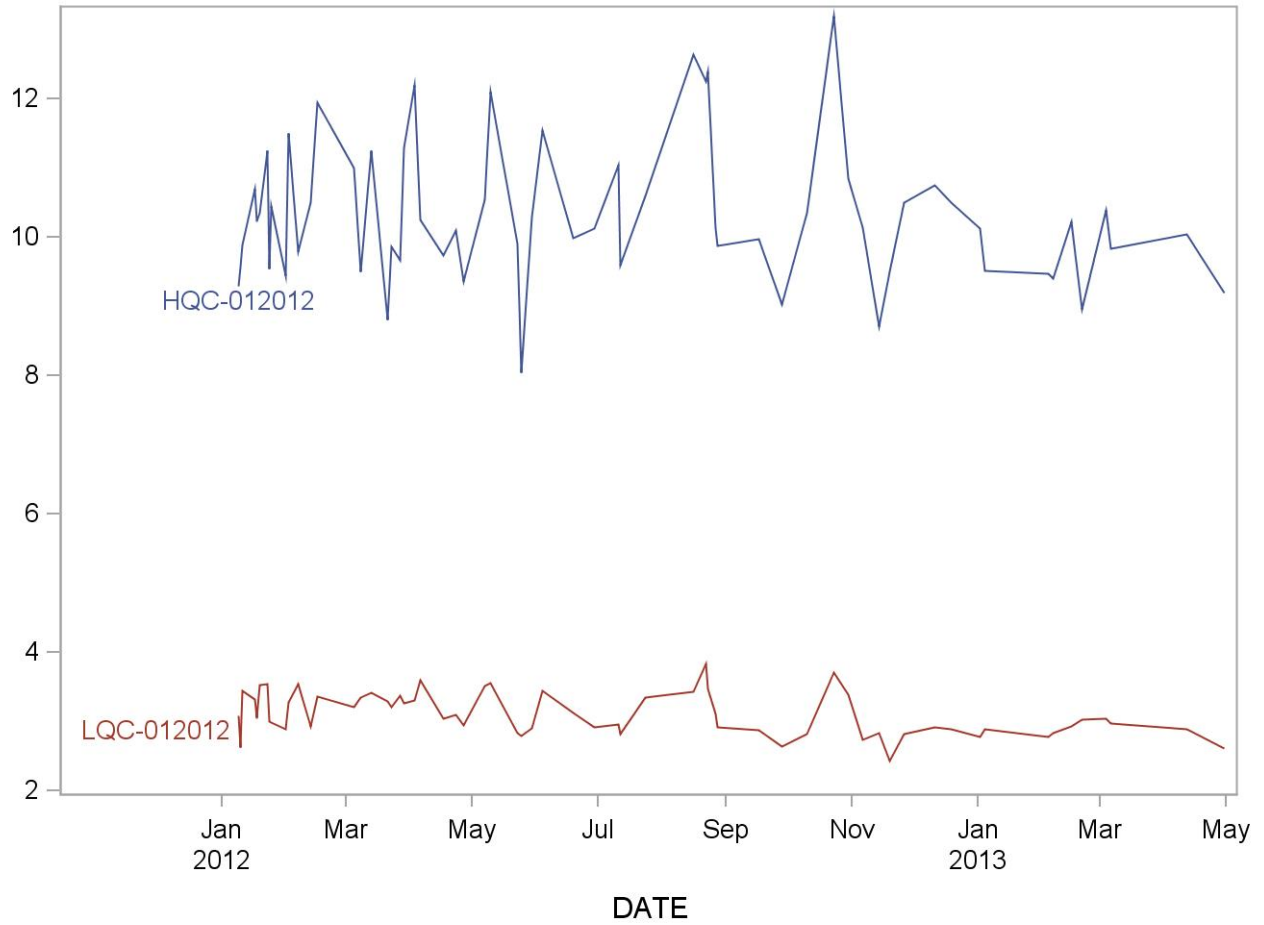
**2011-2012 Summary Statistics and QC Chart for PFUA Perfluoroundecanoic acid**

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012012	65	09JAN12	30APR13	7.11	0.73	10.3
LQC-012012	65	09JAN12	30APR13	2.10	0.19	9.1



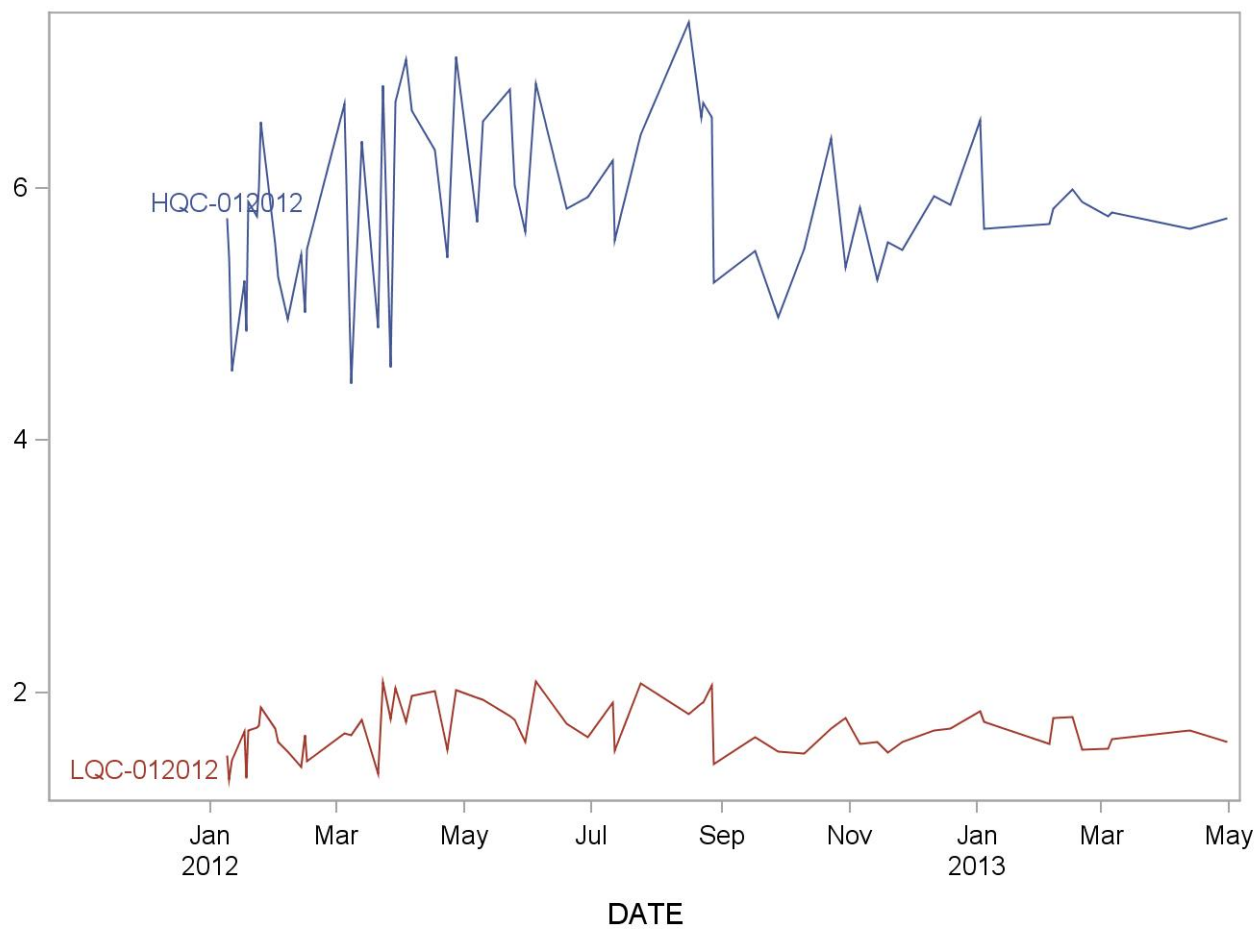
**2011-2012 Summary Statistics and QC Chart for Pefluorodecanoic acid**

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012012	65	09JAN12	30APR13	10.33	1.02	9.8
LQC-012012	65	09JAN12	30APR13	3.11	0.30	9.7



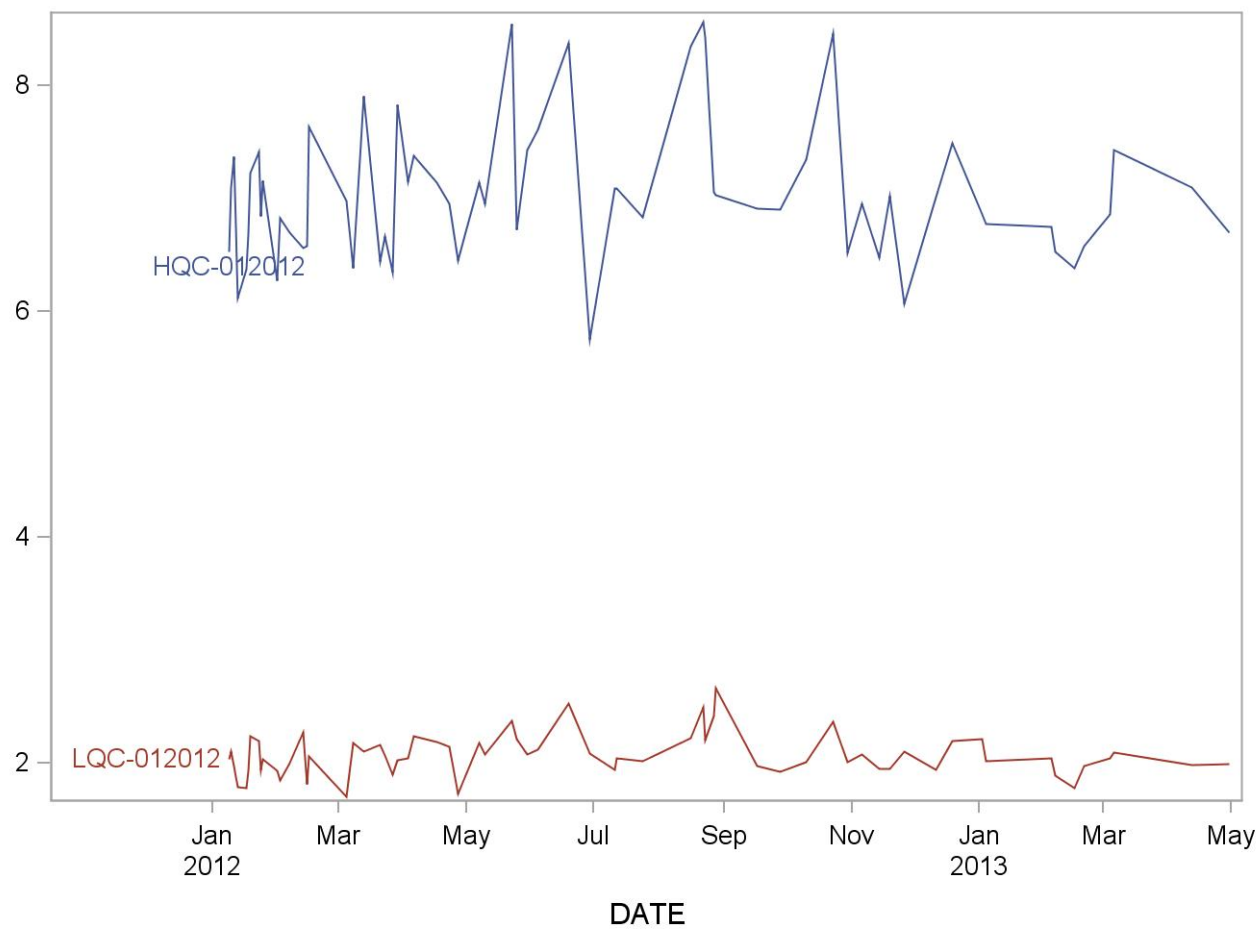
**2011-2012 Summary Statistics and QC Chart for Perfluoroheptanoic acid**

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012012	65	09JAN12	30APR13	5.85	0.66	11.2
LQC-012012	65	09JAN12	30APR13	1.71	0.20	11.6



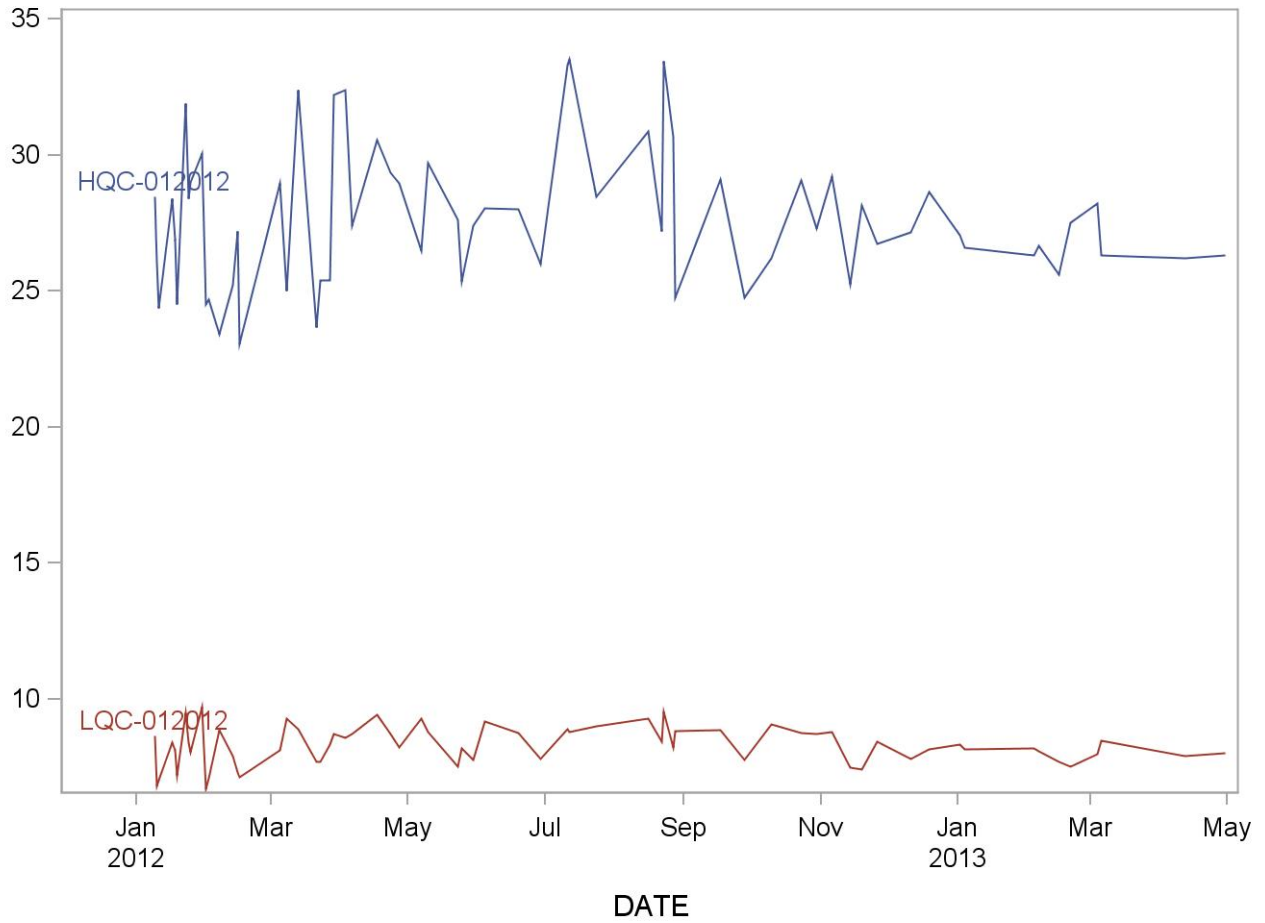
## 2011-2012 Summary Statistics and QC Chart for Perfluorooctane Sulfonamide

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012012	65	09JAN12	30APR13	7.03	0.62	8.8
LQC-012012	65	09JAN12	30APR13	2.07	0.19	9.0



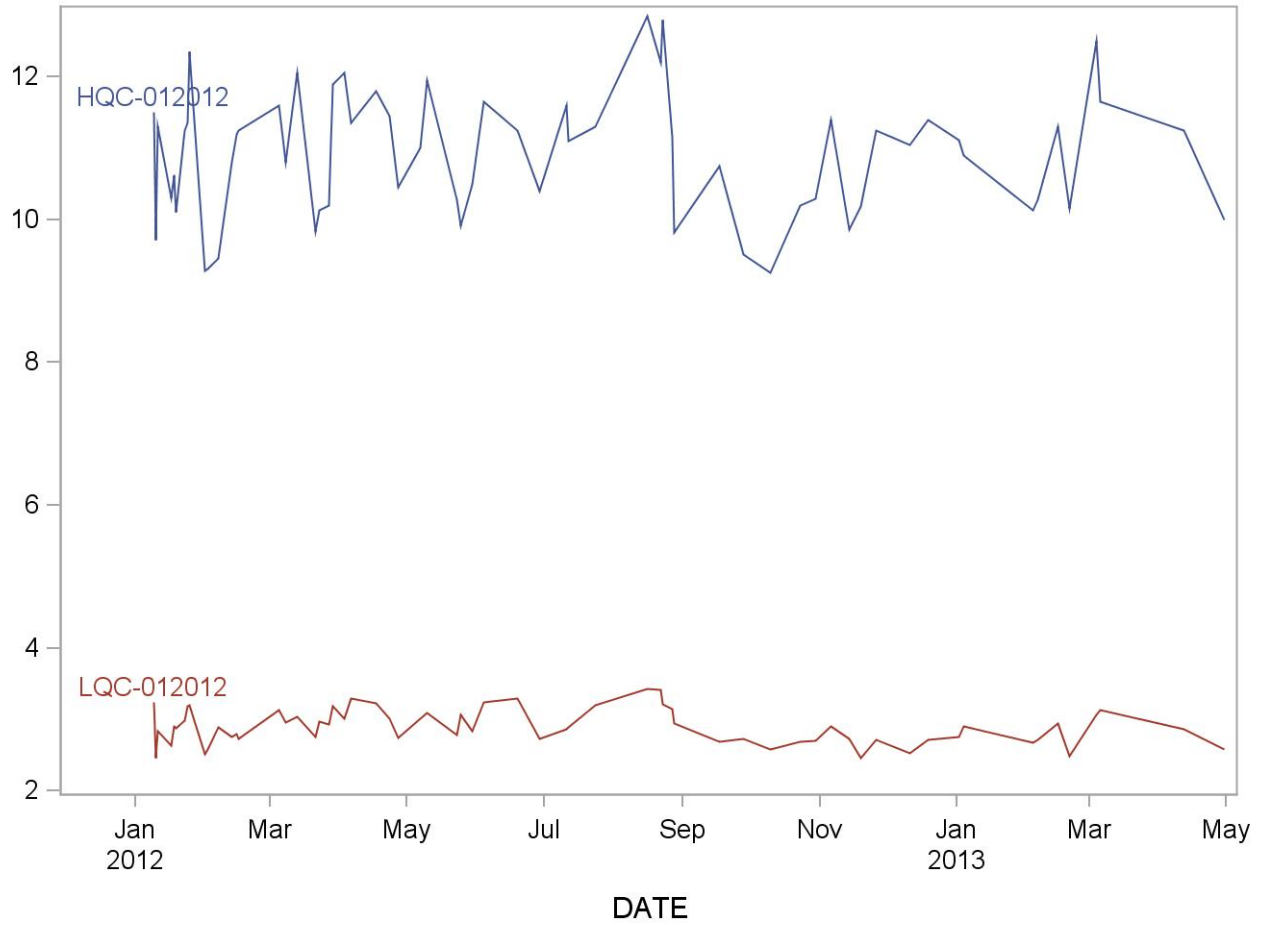
**2011-2012 Summary Statistics and QC Chart for Perfluorooctane Sulfonate**

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012012	66	09JAN12	30APR13	27.63	2.52	9.1
LQC-012012	66	09JAN12	30APR13	8.29	0.70	8.4



**2011-2012 Summary Statistics and QC Chart for Perfluorooctanoic acid**

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
HQC-012012	65	09JAN12	30APR13	10.90	0.88	8.0
LQC-012012	65	09JAN12	30APR13	2.90	0.24	8.3





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