



Laboratory Procedure Manual

Analyte: **Polyfluoroalkyl Substances: 2-(N-methyl-perfluorooctane sulfonamido) acetate, perfluorobutane sulfonate, perfluorohexane sulfonate, n-perfluorooctane sulfonate, sum of perfluoromethylheptane sulfonate isomers, sum of perfluorodimethylhexane sulfonate isomers, perfluoroheptanoate, n-perfluorooctanoate, sum of branched perfluorooctanoate isomers, 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: **6304.06**

As performed by:

Organic Analytical Toxicology Branch
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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) |
|-----------|---------------|--|
| PFAS_H | LBXPFDE | Perfluorodecanoic acid (ng/mL) |
| | LBXPFHS | Perfluorohexane sulfonic acid (ng/mL) |
| | LBXMPAH | 2-(N-methyl-PFOSA) acetic acid (ng/mL) |
| | LBXPFBS | Perfluorobutane sulfonic acid (ng/mL) |
| | LBXPFHP | Perfluoroheptanoic acid (ng/mL) |
| | LBXPFNA | Perfluorononanoic acid (ng/mL) |
| | LBXPFUA | Perfluoroundecanoic acid (ng/mL) |
| | LBXPFDO | Perfluorododecanoic acid (ng/mL) |
| SSPFAS_H | SSNPFOA | Linear perfluorooctanoate (ng/mL) |
| | SSBPFOA | Branched isomers of perfluorooctanoate (ng/mL) |
| | SSNPFOS | Linear perfluorooctane sulfonate (ng/mL) |
| | SSMPFOS | Monomethyl branched isomers of PFOS (ng/mL) |

1. Clinical Relevance and Summary of Test Principle

a. Clinical Relevance

Some per- and polyfluoroalkyl substances (PFASs), including perfluorooctane sulfonate (PFOS) and perfluorooctanoate (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, although demographic, geographic, and temporal differences exist ²⁻¹⁴. In animals, exposure to PFOS and PFOA is associated with adverse health effects ¹⁵⁻¹⁷ albeit at serum concentrations orders of magnitude higher than the concentrations observed in the general population ^{18,19}. PFOS was used in a wide variety of industrial and consumer products including protective coatings for carpets and apparel, paper coatings, insecticide formulations, and surfactants. In 2000, 3M, the sole manufacturer of PFOS in the United States and the principal manufacturer worldwide, announced that it was discontinuing its perfluorooctanyl chemistries, including PFOS. Shortly after, EPA also identified possible related concerns with respect to PFOA and fluorinated telomers. PFOA has been used primarily to produce its salts which are used in the production of fluoropolymers and fluoroelastomers. These polymers are used in many industrial and consumer products, including soil, stain, grease, and water resistant coatings on textiles and carpet; uses in the automotive, mechanical, aerospace, chemical, electrical, medical, and building/construction industries; personal care products; and non-stick coatings on cookware.

The electrochemical fluorination (ECF) manufacturing method used from the 1950s until the early 2000s to produce PFASs including PFOA, and PFOS and its precursors yielded branched and linear isomers. By contrast, another method, telomerization, produces almost exclusively linear compounds ²⁰. The structural isomer patterns of PFOA and PFOS in humans may be useful for understanding routes and sources of exposure ²⁰.

b. Test Principle

Online solid phase extraction coupled to high performance liquid chromatography-turboionspray ionization-tandem mass spectrometry (online SPE-HPLC-TIS-MS/MS) is used for the quantitative detection of PFASs: 2-(N-methyl-perfluorooctane sulfonamido) acetate (Me-PFOSA-AcOH), perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS), n-perfluorooctane sulfonate (n-PFOS), sum of perfluoromethylheptane sulfonate isomers (Sm-PFOS, monomethyl branched isomers of PFOS), sum of perfluorodimethylhexane sulfonate isomers (Sm2-PFOS, dimethyl branched isomers of PFOS), perfluoroheptanoate (PFHpA), n-perfluorooctanoate (n-PFOA), sum of branched perfluorooctanoate isomers (Sb-PFOA, branched PFOA isomers), perfluorononanoate (PFNA), perfluorodecanoate (PFDeA), perfluoroundecanoate (PFUA), and perfluorododecanoate (PFDoA)²¹. Briefly, after dilution with formic acid, one aliquot of 50 μ L of serum is injected into a commercial column switching system allowing for concentration of the analytes on solid-phase extraction column. Separation of the analytes from each other and from other serum components is achieved with high-performance liquid

chromatography. Detection and quantification are done using negative-ion TurbolonSpray ionization, a variant of electrospray ionization, tandem mass spectrometry. This method allows for rapid detection of these PFASs in human serum with limits of detection in the low parts per billion (ppb or ng/mL) range.

2. Safety Precautions

a. Reagent Toxicity or Carcinogenicity

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: Material Safety Data Sheets (MSDS) for the chemicals and solvents used in this procedure can be found at www.ilpi.com/msds/index.html; some of them may be found in a binder in the laboratory. Laboratory personnel are advised to 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 biological specimens (i.e., use gloves, laboratory coats, safety glasses, chemical or biological hoods). Any residual biological 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. The Hepatitis B vaccination series is recommended for health care and laboratory workers who are exposed to human fluids and tissues. Laboratory personnel who handles human fluids and tissues is required to take the "Bloodborne Pathogens Training" course offered at CDC to insure proper compliance with CDC safe work place requirements.

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/or a chemical fume hood or biological safety cabinet.

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 SOP. 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 and ABI 6500 Qtrap mass spectrometers. During sample preparation and analysis, samples are identified by their Sample Name and Sample ID. The Sample Name is used to identify each specimen and links the laboratory information with the demographic data recorded by the sample takers. The Sample ID 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 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 a network drive; it may also be backed up in additional 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, sample study IDs are read in by a barcode reader directly from the vials labels. Sample names for Standards, and Blanks (SBs, HSBs, QCBs) are entered manually. The Sample Log Sheet, containing Sample Names, Sample IDs, and sample study IDs is printed from the Access database and is used to record information during sample preparation. Sample Names, Sample IDs, and sample study 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 mass spectrometer. After MS data collection and peak integration, data are saved as a tab delimited file 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 (SAS output) is generated.

c. Data Maintenance

Raw files are regularly backed up onto an external hard drive. Sample and analytical data are checked after being entered into the database for transcription errors and overall validity. The database is routinely backed up onto a computer hard drive and onto a network drive. Data from completed studies are saved on an external hard drive and/or a network drive. Additionally, paper copies of signed final report memos are scanned and saved as official government records.

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

a. Sample Collection and Storage

Follow recommended phlebotomy practices for the collection of blood and separation of blood serum. Preferably, a minimum of 0.5 mL of serum (plasma may also be used) should be placed in standard collection containers, refrigerated as soon as possible, and transferred to labeled containers for storage. Sera 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. Teflon® coated materials should be avoided.

b. Sample Handling

In general, serum specimens should be shipped or transported cold (dry ice, ice or blue ice can be used). Special care must be taken in packing to protect vials from breakage during shipment.

Before analysis, samples are thawed, vortexed, aliquoted, and the residual specimen is refrozen and stored. The integrity of samples thawed and refrozen several times doesn't appear to be compromised.

c. Criteria for Specimen Rejection

Specimens can be rejected if tubes/vials leaked, are broken, appear compromised or tampered with, or hold inadequate volume for analysis.

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, and water were HPLC grade purchased from Honeywell Burdick & Jackson (Muskegon, MI). Formic acid (99%) was purchased from EM Science (Gibbstown, NJ). Acetic acid (glacial) was purchased from J.T. Baker (Phillipsburg, NJ). The following PFASs were purchased from Wellington Laboratories (Guelph, ON, Canada): N-methylperfluoro-1-octanesulfonamidoacetic acid (Me-PFOSA-AcOH), sodium perfluoro-1-hexanesulfonate (PFHxS), potassium perfluoro 1-butanefluorobutanesulfonate (PFBuS), sodium perfluoro 1-octanesulfonate (n-PFOS), mixture of sodium perfluoro-5-methylheptane sulfonate (P5MHpS) and perfluoro-5-methylheptanoic acid (P5MHpA), mixture of sodium perfluoro-5,5-dimethylhexane sulfonate (P55DMHxS) and perfluoro-5,5-dimethylhexanoic acid (P55DMHxA), perfluoroheptanoic acid (PFHpA), ammonium perfluorooctanoate (n-PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDeA), perfluoroundecanoic acid (PFUA), and perfluorododecanoic acid (PFDoA). Perfluoro-n-[1,2,3,4,5-¹³C]-heptanoic acid (¹³C₅-PFHpA), perfluoro-n-[1,2,3,4-¹³C]-octanoic acid (¹³C₄-PFOA), perfluoro-n-[1,2,3,4,5-¹³C]-nonanoic acid (¹³C₅-PFNA), 2-Perfluorooctyl [1,2-¹³C]-ethanoic acid (¹³C₂-PFDeA), 2-perfluorooctyl [1,2-¹³C]-undecanoic acid (¹³C₂-PFUA), perfluoro-n-[1,2-¹³C]-dodecanoic acid (¹³C₂-PFDoA), N-methyl-d₃-perfluoro-1-octanesulfonamide acetic acid (D₃-Me-PFOSA-AcOH), and sodium perfluoro 1-hexane [¹⁸O₂]-sulfonate (¹⁸O₂-PFHxS), sodium perfluoro 1-[1,2,3,4-¹³C]-octanesulfonate (¹³C₄-PFOS) were purchased from Wellington Laboratories. All reagents were used without further purification. Other standards and reagents with similar specifications may be used.

b. Working Solutions

(1) HPLC Mobile Phase, 20mM Ammonium Acetate Buffer/acetonitrile (95:5), pH 4.

To prepare 20 mM Ammonium acetate buffer (pH4.0), dilute 1140 µL of concentrated acetic acid with approximately 800 mL water in a beaker. Adjust pH to 4±0.1 by adding drop-wise 1:10 ammonium hydroxide:water mixture. Transfer into a 1 L volumetric flask and fill up to volume with deionized water.

Mix 950 mL of ammonium acetate buffer with 50 mL of acetonitrile in a glass bottle. Prepare as needed and store at room temperature.

(2) HPLC Organic Mobile Phase, 100% HPLC acetonitrile

Refill as needed and store at room temperature.

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

Refill as needed and store at room temperature.

(4) Solid phase extraction (SPE) Acid Wash Solution, 0.1M formic acid

Dilute 3810 μL of 99% concentrated formic acid with water to 1000 mL in a volumetric cylinder. Prepare monthly and store at room temperature.

c. Standards Preparation

(1) Analytical Calibration Standards

The native standard stock solutions of all the analytes are prepared in methanol from the commercial solutions. The concentrations of the commercial solutions are: 50 $\mu\text{g}/\text{mL}$ for Me-PFOSA-AcOH and n-PFOS; 2 $\mu\text{g}/\text{mL}$ for PFHxS, PFBuS, n-PFOS, PFHpA, n-PFOA, PFNA, PFDeA, PFUA, and PFD_oA; 1 $\mu\text{g}/\text{mL}$ for P5MHpS and P55DMHxS; 1.96 $\mu\text{g}/\text{mL}$ for P5MHpA and P55DMHxA. We used P5MHpS to quantify Sm-PFOS and P55DMHxS to quantify Sm₂-PFOS. We used the combined response of the P5MHpA and P55DMHxA standards for the quantitation of Sb-PFOA. The PFOA isomers known to be included in Sb-PFOA are perfluoro-3-methylheptanoic acid, perfluoro-4-methylheptanoic acid, perfluoro-5-methylheptanoic acid, perfluoro-6-methylheptanoic acid, perfluoro-4,4-dimethylhexanoic acid, perfluoro-5,5-dimethylhexanoic acid, perfluoro-3,5-dimethylhexanoic acid, and perfluoro-4,5-dimethylhexanoic acid. Similarly, the PFOS isomers known to be included in Sm-PFOS are perfluoro-3-methylheptane sulfonate, perfluoro-4-methylheptane sulfonate, perfluoro-5-methylheptane sulfonate, perfluoro-6-methylheptane sulfonate. The PFOS isomers known to be included in Sm₂-PFOS are perfluoro-4,4-dimethylhexane sulfonate, perfluoro-5,5-dimethylhexane sulfonate, perfluoro-4,5-dimethylhexane sulfonate, and perfluoro-3,5-dimethylhexane sulfonate.

The spiking standard solutions are prepared in MeOH from native standard stock solutions such as a 50- μL spike into 50 μL serum provides concentrations that cover the linear range of the method (Table 1). The spiking solutions are stored frozen in 1.0 mL aliquots in polypropylene cryogenic vials until use.

Table 1. Concentrations of standards #1-9 (in µg/mL)

| Standard No | n-PFOS | Sm-PFOS (P5MHpS) | Sm2-PFOS (P55DMHxS) | PFHxS | PFBuS | Sb-PFOA (P5MHpA+ P55DMHxA) | All other analytes |
|-------------|--------|------------------|---------------------|-------|-------|----------------------------|--------------------|
| Standard 1 | 0.015 | 0.01 | 0.005 | 0.005 | 0.004 | 0.0294 | 0.005 |
| Standard 2 | 0.06 | 0.05 | 0.01 | 0.009 | 0.009 | 0.118 | 0.01 |
| Standard 3 | 0.15 | 0.1 | 0.05 | 0.05 | 0.043 | 0.294 | 0.05 |
| Standard 4 | 0.60 | 0.25 | 0.1 | 0.095 | 0.086 | 0.686 | 0.1 |
| Standard 5 | 1.5 | 0.50 | 0.2 | 0.47 | 0.428 | 1.37 | 0.5 |
| Standard 6 | 6.0 | 1.0 | 0.2 | 0.945 | 0.855 | 2.45 | 1.0 |
| Standard 7 | 15.0 | 2.5 | 0.5 | 4.70 | 4.28 | 5.88 | 5.0 |
| Standard 8 | 60.0 | 5.0 | 1.00 | 9.45 | 8.55 | 11.8 | 10.0 |
| Standard 9 | 115. | 10.0 | 2.50 | 18.9 | 17.1 | 24.5 | 20.0 |

(2) Internal Standard Spiking Solution

The internal standard spiking solution is prepared by dissolving appropriate amounts of ¹³C₂-PFHxA, ¹³C₅-PFHpA, ¹³C₄-PFOA, ¹³C₄-PFOS, ¹⁸O₂-PFHxS, ¹³C₅-PFNA, ¹³C₂-PFDeA, ¹³C₂-PFDoA, and D₃-Me-PFOSA-AcOH (4-6 ng/mL) in water/methanol (50/50). A 50 µL spike of this solution provides concentrations of 4-6 ng/mL in 50 µL serum. Spiking solutions are stored frozen in 2.0 mL aliquots in polypropylene cryogenic vials until use.

(3) Mass-Spec Operational Check Standard

The instrument test sample is prepared by spiking the reagent blank with all analytes to final concentrations of 0.3-0.5 ng/mL.

(4) In-house Proficiency Testing (PT) Standards

Appropriate aliquots of each stock standard are added to calf serum pools to produce 3 sets of in-house proficiency testing (PT) standards. The PT standards are mixed, aliquoted into polypropylene 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) HySphere C8-SE (7µM) cartridge (i-Chrome solutions, Plainsboro, NJ)
- 2) Chromolith® HighResolution RP-18e column (4.6 × 100 mm) (Merck KGaA, Germany).
- 3) Chromolith® HighResolution RP-18e Guard column (5 X 4.6 mm) (Merck KGaA, Germany).
- 4) Chromolith® HighResolution RP-18e column (4.6 × 25 mm) (Merck KGaA, Germany).
- 5) 750 µL polypropylene autosampler vials with polyethylene snap caps (National Scientific Company, Rockwood, TN).
- 6) Tip ejector variable volume micropipettes (Wheaton, Millville, NJ) and pipette tips (Rainin Instruments Co., Woburn, MA).

- 7) 5.0 mL and 2.0 mL polypropylene cryovials (National Scientific Company, Rockwood, TN).
- 8) Assorted glass and polypropylene labware.

e. Equipment

- 1) Symbiosys extractor equipped with an Alias autosampler run by the SparkLink software program (Spark Holland Inc. dba iChrom Solutions, Plainsboro, NJ).
- 2) Agilent 1200 binary pump and degasser (Agilent Technologies).
- 3) Applied Biosystems ABI 5500 or ABI 6500 Qtrap 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, Goettingen, Germany).
- 6) pH meter (AB 15 pH Meter, Fisher Scientific).
- 7) Vortex mixer (Type 16700, Barnstead International, Dubuque, Iowa).

f. Instrumentation

(1) Automated SPE

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 HySphere C8-SE (7 μ M) cartridge with HPLC-grade acetonitrile (2 mL) and 0.1 M formic acid (2 mL). Afterward, 500 μ L of the sample (containing 50 μ L serum) injected into the 1 mL sample loop is loaded onto the SPE column using 2 mL 0.1 M formic acid with 1 mL/min flow rate. Next, the SPE column is washed with 2 mL 90% 0.1 M formic acid/10% Acetonitrile. The time of the SPE cleanup (including injection time) is 10 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 bypass (1-2) position until the HPLC-MS/MS system becomes ready to begin acquisition.

Table 2. Valve configurations used for concurrent SPE clean up and HPLC-MS/MS acquisition.

| Steps ^a | 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 ^b |
| 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 500 µL sample on left cartridge (2 mL, 0.1 M formic acid, 1 mL/min) | 1-2 | 1-2 | 6-1 | 6-1 | 4.4 |
| 8 | Forward wash left cartridge (2 mL 90% 0.1 M formic acid/10% acetonitrile, 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 |

^a The method used for the first sample included only steps 2 and 5-8. The method used for the acquisition of the last sample included only steps 1, 3, 4, and 9.

^b For the acquisition of the last sample duration of step 4 was 13 min.

(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 transfer the analytes from the SPE column to the HPLC column. At 10 min, the right clamp valve turns 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 1000 µL/min flow rate with 95% of 20 mM ammonium acetate (pH 4) and 5% of acetonitrile as mobile phase A and 100% acetonitrile as mobile phase B. The analytes are separated from each other and other extracted components on two Chromolith® HighResolution RP-18e columns (4.6 × 100 mm) preceded by a Chromolith® HighResolution RP-18e (5 X4.6 mm) guard column and a Chromolith® HighResolution RP-18e (4.6 × 25 mm) column. To delay the elution of the PFAS contaminants leaching out

from Teflon parts of the HPLC pump, a 4.6 mm x 25 mm Chromolith® HighResolution RP-18e column is inserted between the HPLC pump and the right clamp valve. Because contaminants have to go through twice the column length, their peaks elute 1 min after the main analytes bands without interfering with the measured concentration.

Table 3. HPLC configuration

| Parameters | Setting |
|----------------|--|
| Mobile Phase A | 95% 20 mM ammonium acetate, pH = 4/5% acetonitrile |
| Mobile Phase B | 100% acetonitrile |
| Flow rate | 1000 μ L/min |

Table 4. Mobile phase gradient

| | | | | | | | | | |
|--------------------|------|------|------|------|------|------|------|-----|------|
| Time (min) | 0 | 1 | 2 | 8.5 | 8.51 | 12 | 12.1 | 13 | 13.2 |
| Mobile phase B% | 25 | 25 | 45 | 49 | 60 | 60 | 80 | 80 | 80 |
| Flow rate (mL/min) | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Time (min) | 13.3 | 13.5 | 13.6 | 15.5 | 15.6 | 16.5 | 16.6 | 18 | 18.1 |
| Mobile phase B% | 80 | 95 | 95 | 95 | 95 | 95 | 25 | 25 | 25 |
| Flow rate (mL/min) | 1.5 | 1.5 | 1.8 | 1.8 | 2.0 | 2.0 | 1.5 | 1.5 | 1.0 |

(3) Mass Spectrometer Configuration

Detection of the target analytes is conducted on the ABI 5500 or ABI 6500 Qtrap 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=50 and GS2=50) gas. The heated turbo ion spray gas temperature is set at 400 °C. The curtain and collision gas (nitrogen) settings are as follows: collision (medium), curtain gas (CUR=30 [ABI 5500], CUR=45 [ABI 6500]). Ionization parameters and collision cell parameters are optimized individually for each analyte (**Table 5**). Unit resolution is used for both Q1 and Q3 quadrupoles. The dwell time is 50 msec for all compounds.

Table 5. Mass spectrometric parameters for measuring PFASs

| | (M-H) ⁺ Precursor ion (m/z) | Product ion (m/z) | DP (volts) | CE (volts) |
|--|--|-------------------|------------|------------|
| Me-PFOSA-AcOH | 570 | 512 | -45 | -30 |
| D ₃ -Me-PFOSA-AcOH(IS) | 573 | 515 | -45 | -30 |
| PFBuS-1 | 299 | 99 | -70 | -80 |
| PFBuS-2 ^a | 299 | 80 | -70 | -85 |
| PFHxS-1 | 399 | 99 | -70 | -80 |
| PFHxS-2 ^a | 399 | 80 | -70 | -85 |
| PFHxS- ¹⁸ O ₂ -1 (IS) ^b | 403 | 103 | -70 | -80 |
| PFHxS- ¹⁸ O ₂ -2 (IS) ^a | 403 | 84 | -70 | -85 |
| n-PFOS-1 ^a | 499 | 80 | -70 | -90 |
| n-PFOS-2 | 499 | 99 | -70 | -80 |
| PFOS- ¹³ C ₄ -1 (IS) ^c | 503 | 80 | -70 | -85 |
| PFOS- ¹³ C ₄ -2 (IS) | 503 | 99 | -70 | -85 |
| Sm-PFOS | 499 | 80 | -70 | -90 |
| PFHpA | 363 | 319 | -25 | -13 |
| PFHpA- ¹³ C ₅ (IS) | 368 | 323 | -25 | -13 |
| n-PFOA | 413 | 369 | -27 | -14 |
| Sb-PFOA | 413 | 369 | -27 | -14 |
| PFOA- ¹³ C ₄ (IS) ^d | 417 | 372 | -30 | -15 |
| PFNA | 463 | 419 | -30 | -13 |
| PFNA- ¹³ C ₅ (IS) | 468 | 423 | -30 | -13 |
| PFDeA | 513 | 469 | -30 | -15 |
| PFDeA- ¹³ C ₂ (IS) | 515 | 470 | -30 | -15 |
| PFUA | 563 | 519 | -30 | -17 |
| PFUA - ¹³ C ₂ (IS) | 565 | 520 | -30 | -17 |
| PFD _o A | 613 | 569 | -30 | -18 |
| PFD _o A- ¹³ C ₂ (IS) | 615 | 570 | -45 | -15 |

^a used only as confirmation ion

^b PFHxS-¹⁸O₂-1 was used as IS for PFBuS.

^c PFOS-¹³C₄ was used as IS for n-PFOS, Sm-PFOS, and Sm₂-PFOS.

^d PFOA-¹³C₄ was used as IS for both n-PFOA and Sb-PFOA.

1. Calibration and Calibration-Verification Procedures

a. Calibration Curve

Nine-point calibration curves are normally constructed with each quantitative run 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. The area of the Q1 ion for each analyte is used for quantification (for PFOS we use Q2). Correlation coefficients are generally 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 or ABI 6500 Qtrap mass spectrometer is calibrated and tuned at least once per year using a polypropylene glycol (PPG) solution according to the instructions contained in the operator's manual. The instrument sensitivity is checked periodically 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 CLIA regulations from 2003 (<http://www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/downloads/6065bk.pdf>), 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.

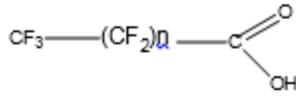
d. Proficiency Testing (PT)

- (1) Three pools of PT samples, which encompass the entire linear range of the method, are prepared in-house as described in the standard preparation section. Characterization of PT materials requires 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.
- (2) Proficiency testing should be performed a minimum of once per 6 months. When proficiency testing is required, the laboratory supervisor or his/her designee will notify the PT administrator, and 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 supervisor and/or his/her designee 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

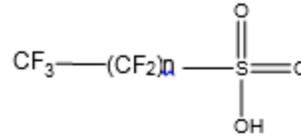
(Sweden) and RIVO (The Netherlands) when it is conducted for human serum/plasma^{22,23}.

- (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.
- (6) Since 2011, three times a year we also participate in the ongoing Arctic Monitoring and Assessment Program (AMAP) Ring Test for several PFASs in human serum, conducted by the Institut National de Santé Publique du Québec (INSP) in Canada.

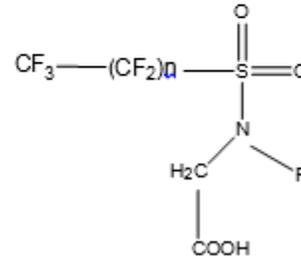
(7) Analytes nomenclature and structures



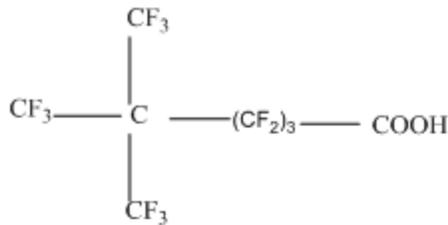
n=5 PFHpA
n=6 n-PFOA
n=7 PFNA
n=8 PFDpA
n=9 PFUA
n=10 PFDpA



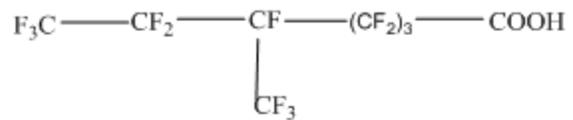
n=3 PFBuS
n=5 PFHxS
n=7 n-PFOs



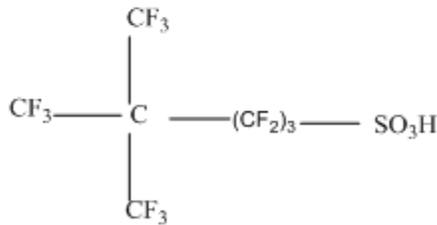
n=7, R=Me, Me-PFOsA-AcOH



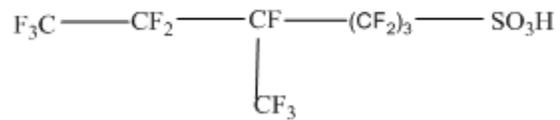
perfluoro-5,5-dimethylhexanoic Acid



perfluoro-5-methylheptanoic Acid



perfluoro-5,5-dimethylhexane sulfonic acid



perfluoro-5-methylheptane sulfonic acid

8. Operating Procedures; Calculations; Interpretation of Results

a. Sample preparation

(1) Unknown, QC, Blank, and Standard Preparation

- (a) Remove serum samples, working standard solutions and internal standard solution from the freezer, and let them thaw. Label polypropylene snap-cap autosampler vials with appropriate Sample Names. Aliquot 0.1 M formic acid (500 μ L for QCBs; 450 μ L for UNKs, QCs, serum blanks (SBs), and human serum blanks (HSBs); 400 μ L for STDs) into appropriate vials.
- (b) Dispense 50 μ L of internal standard into each polypropylene autosampler snap cap vials. In specific cases, the method can be performed using a smaller volume of matrix; the applied dilution factor must be noted appropriately.
- (c) Add 50 μ L of the appropriate native standard solution (S1-S9) into the polypropylene vials designated for standards.
- (d) Aliquot 50 μ L of UNKs, QCs, SBs and HSBs into the designated autosampler vials. For standards, aliquot 50 μ L of blank serum. Analysis may also be conducted with a smaller amount of serum; in these circumstances, the volume used must be noted appropriately throughout the analytical procedure.
- (e) Vortex all vials for at least 10 seconds to make sure all the internal standard and standard mixed into the sample.

(2) Automated SPE-HPLC-MS/MS Analysis Procedure

- (a) Put the Alias into load position. Initialize the high pressure dispenser (HPD) and the automated cartridge exchanger (ACE) unit.
- (b) Exchange the cartridge tray after every 500 samples.
- (c) Purge the solvent lines on the HPLC binary pump and equilibrate the HPLC column.
- (d) 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 500 μ L. Make sure the

right vial positions are entered and there is no sequential duplication of cartridge numbers. The sample names and sample IDs do not matter, since they will not be part of the acquired data.

- (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** and import the import.txt file (Sample pull down, go to gray header and click RMB, then Import From/File, select Alias autosampler). Make sure that the proper Acquisition Method and Quantitation Method are entered. Then, 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.

b. Analysis

(1) Check out the LC/MS interface

- (a) If the instrument is in ready mode, wait until the interface cools down. When the interface is cool enough, take out the capillary from the MS interface. Rinse the capillary with MeOH, sonicate in MeOH for 20 min if necessary. Periodically, take off the interface housing, and wipe out the skimmer plate.
- (b) Open the rough pump cabinet, check for oil leaks and unusual noise. Report anything unusual.

(2) 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.

(3) 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 approximately 500 samples).
- c) Make sure that the MS remote cable is connected ACE unit.

(4) Building batch files

- (a) 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 500 μ L. Make sure that the proper vial positions are entered and there is no sequential duplication of cartridge numbers. The sample names and sample IDs do not matter, since they will not be part of the acquired data.
- (b) In the Analyst software, open a new the 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.
- (c) From Excel, open the text file containing the batch table created from the Access database using Microsoft Access. 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!!!!** 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 Alias autosampler).
- (d) Make sure that the proper Acquisition Method and Quantitation Method are entered. Although the vial positions entered in Analyst will not be used they should agree with the vial positions used on the Triathlon autosampler.

(5) Starting the SPE-HPLC-MS/MS run

- a) Start the batch table in **SparkLink**.
- b) 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

(1) 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.

(2) Importing Data into the Database

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

(3) 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 or ABI 6500 Qtrap 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 Sciex ABI 5500 or ABI 6500 Qtrap 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 1200 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 (>250 bar) 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. See also section 8b.
- (c) Reestablishment of performance and calibration. Every time the system is disturbed for cleaning or maintenance, a mass spec operational check 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.

The instrumentation used is serviced according to the manufacturer’s guidance included in the instrument manuals or based on the recommendation of experienced analysts/operators after following appropriate procedures to determine that the instrument performs adequately for the intended purposes of the method.

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 µL of specimen) the dilution can be performed in at least two steps. For example, first, at least 10 µL specimen 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 µL blank calf serum. 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.

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

$$D = (1000 / V_{1st}) \times (200 / V_{2nd}).$$

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.

1) Analytical Sensitivity

The limits of detection (LOD) for each analyte are listed in **Table 6**.

2) Analytical Specificity

This is a highly selective method that requires that the PFASs 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.

3) Linearity Limits

The calibration curve is linear for all analytes (generally $R^2 > 0.95$). The limit on the linearity is determined by the highest standard analyzed in the method. Due to the wide variation of PFASs levels in humans, we set our highest standard near the high end of the linear range (**Table 6**). 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. Concentrations below the method LOD (or the concentration of the lowest standard in the calibration curve) are reported as non-detectable.

Table 6. Linear range (lowest – highest standard concentration) and LOD for each PFAS measured in serum.

| Analyte | LOD | Linear range (ng/mL) |
|---------------|-----|----------------------|
| Me-PFOSA-AcOH | 0.1 | 0.005-20 |
| PFBuS | 0.1 | 0.004-17.1 |
| PFHxS | 0.1 | 0.005-18.9 |
| n-PFOS | 0.1 | 0.015-115 |
| Sm-PFOS | 0.1 | 0.01-10 |
| PFHpA | 0.1 | 0.01-20 |
| n-PFOA | 0.1 | 0.01-20 |
| Sb-PFOA | 0.1 | 0.029-24.5 |
| PFNA | 0.1 | 0.01-20 |
| PFDeA | 0.1 | 0.01-20 |
| PFUA | 0.1 | 0.01-20 |
| PFDoA | 0.1 | 0.01-20 |

1) Accuracy

The accuracy of the method is determined by enriching serum samples with known concentrations of PFASs and comparing the calculated and expected concentrations. To examine their consistency over the range of levels encountered in serum, the measurements are taken at 3 different concentrations, namely using standards near 3*LOD, middle level (~1.0 ng/mL, except n-PFOS (6 ng/mL), Sm-PFOS and Sm2-PFOS (0.5 ng/mL), and Sb-PFOA (0.7 ng/mL)), and high level (~10.0 ng/mL, except n-PFOS (60 ng/mL), Sm-PFOS and Sm2-PFOS (1.0 ng/mL), and Sb-PFOA (2.5 ng/mL)). The accuracy is calculated from 5 independent measurements (Table 7).

Table 7. Spiked recoveries of extracted standards in serum

| Analyte | Accuracy (%) at ~3*LOD/middle/high | | |
|---------------|---------------------------------------|--------|--------|
| | | | |
| Me-PFOSA-AcOH | 105±20 | 93±5 | 101±3 |
| PFBuS | 113±25 | 105±19 | 110±12 |
| PFHxS | 106±12 | 98±5 | 98±6 |
| n-PFOS | 110±14 | 99.7±6 | 97.0±2 |
| Sm-PFOS | 105±23 | 92±4 | 90±4 |
| PFHpA | 115±12 | 110±11 | 103±10 |
| n-PFOA | 92±14 | 105±5 | 101±2 |
| Sb-PFOA | 112±15 | 106±8 | 102±5 |
| PFNA | 95±13 | 103±7 | 102±3 |
| PFDeA | 103±13 | 96±6 | 98±4 |
| PFUA | 92±17 | 102±3 | 101±3 |
| PFDoA | 110±22 | 96±10 | 101±3 |

1) Precision

The precision of this method is reflected in the variance of two quality control (QC) pools over a period of three weeks. 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 8).

Table 8. Mean QC concentrations (ng/mL) and CV%

| Analyte | VQC | CV% | QCH | CV% |
|---------------|-----|------|------|------|
| Me-PFOSA-AcOH | 0.4 | 14.3 | 6.5 | 10.5 |
| PFBuS | 0.4 | 18.5 | 6.7 | 15.7 |
| PFHxS | 0.4 | 9.3 | 6.3 | 7.0 |
| n-PFOS | 1.0 | 10.6 | 15.8 | 9.8 |
| Sm-PFOS | 0.4 | 10.5 | 2.3 | 10.3 |
| PFHpA | 0.5 | 6.5 | 6.3 | 9.4 |
| n-PFOA | 0.4 | 13.6 | 7.4 | 12.0 |
| Sb-PFOA | 0.8 | 15.5 | 6.5 | 11.0 |
| PFNA | 0.4 | 10.6 | 8.1 | 9.4 |
| PFDeA | 0.5 | 10.9 | 6.3 | 9.7 |
| PFUA | 0.5 | 12.5 | 6.4 | 9.7 |
| PFDoA | 0.5 | 20.0 | 6.4 | 9.1 |

10. Quality Control (QC) Procedures

a. Individual samples (i.e., standards, unknown samples, serum blanks, and quality control (QC) materials) QC procedures

- 1) For each analyte, the relative retention time (RT) (ratio of RT_{analyte} and RT_{IS}) of standards, unknowns, and QCs should be checked. If the relative RT falls outside the range, check the integration to make sure the analyte or IS peak was properly picked up.
- 2) For each analyte, the IS area counts should meet minimum area count requirements. Low IS area counts suggest a) strong ion suppression from the matrix, or b) missing of IS. Depending on the findings, either re-extract the sample as usual or re-extract the sample after dilution.
- 3) For each analyte, the calculated concentration of the calf serum blanks (SB) should be less than three times the LOD. Using the current method, all standards, blanks and unknown samples are prepared following the same procedure, thus background blank values (reflected in the intercept of the calibration curve) are automatically subtracted from the concentrations of unknown samples. If background levels are above the threshold above, the reagents used for sample preparation and (or) mobile phases need to be checked for potential contamination.
- 4) For each analyte, if the concentration in an unknown sample is above the highest calibration standard, the sample needs to be re-extracted with a smaller volume of serum.

b. Quality control of the QC materials

1) QC Materials

The QC materials were prepared in bulk from calf serum (Gibco, Grand Island, NY). The target ranges for the pools were set to encompass the expected concentration ranges in human populations.

2) Preparation of QC Pools

The calf serum purchased was pooled and the QC pools were mixed uniformly, divided into four subpools and stored frozen. One subpool was used as a blank QC and to prepare the calibration standards, and the other three were enriched with PFASs as needed to afford very low concentration (VQC, ~0.3-1.0 ng/mL), low concentration (QCL, ~2.0 ng/mL) and high concentration (QCH, ~0.8-15.8 ng/mL) subpools. The QC pools were characterized to define the mean and the 95% and 99% control limits of PFASs concentrations by a minimum of 30 repeated measurements in a three week period. QC materials reextracted and analyzed after the initial characterization showed that the PFASs remained stable frozen for at least 3 months ²¹.

3) Characterization of QC Materials

For characterization, a minimum of 30 runs of QCL and QCH were measured over 1 month. In each run, one pair of QCL and 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.

QC samples are analyzed along with unknown samples to monitor for accuracy and precision throughout the analysis batch. Maximum 50 unknown samples are run with randomly placed 2 QCL, 2 QCH, and 2 reagent blank samples. The concentrations of the two QCL and two QCH in each batch are averaged to obtain one average measurement of QCL and QCH.

4) 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-control²⁴.

QC rules for: Analytical run with 1 QC pool per run (must also include a blank QC specimen):

One QC pool per run with one QC result per pool

1) If QC run result is within 2Si limits, then accept the run.

2) If QC run result is outside a 2Si limit - reject run if:

- a) Extreme Outlier – Run result is beyond the characterization mean +/- 4Si
 - b) 1 3S Rule - Run result is outside a 3Si limit
 - c) 2 2S Rule - Current and previous run results are outside the same 2Si limit
 - d) 10 X-bar Rule – Current and previous 9 run results are on same side of the characterization mean
 - e) R 4S Rule – The current and the previous run results differ by more than 4Si.
- Note: Since runs have a single result per pool and only 1 pool, the R 4S rule is applied across runs only.

One QC pool per run with two or more QC results per pool

1) If QC run mean is within 2Sm limits and individual results are within 2Si limits, then accept the run.

2) If QC run mean is outside a 2Sm limit - reject run if:

- a) Extreme Outlier – Run mean is beyond the characterization mean +/- 4Sm
- b) 3S Rule - Run mean is outside a 3Sm limit
- c) 2 2S Rule – Current and previous run means are outside the same 2Sm limit
- d) 10 X-bar Rule – Current and previous 9 run means are on same side of the characterization mean

3) If one of the two QC individual results is outside a 2Si limit - reject run if:

- a) R 4S Rule – Within-run range for the current run and the previous run exceeds $4S_w$ (i.e., 95% range limit)

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).

QC rules for: Analytical run with 2 QC pools per run:

Two QC pools per run with one 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:

a) Extreme Outlier – Run result is beyond the characterization mean $\pm 4S_i$

b) 3S Rule - Run result is outside a $3S_i$ limit

c) 2S Rule - Both run results are outside the same $2S_i$ limit

d) 10 X-bar Rule – Current and previous 9 run results are on same side of the characterization mean

e) R 4S Rule – Two consecutive standardized run results differ by more than $4S_i$. Note: Since runs have a single result 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. Standardized results are used because different pools have different means.

Two QC pools per run with two 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:

a) Extreme Outlier – Run mean is beyond the characterization mean $\pm 4S_m$

b) 3S Rule - Run mean is outside a $3S_m$ limit

c) 2S Rule - Both run means are outside the same $2S_m$ limit

d) 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:

a) R 4S Rule – Within-run ranges for all pools in the same run exceed $4S_w$ (i.e., 95% range limit). Note: Since runs have multiple results per pool for 2 pools, the R 4S rule is applied within runs only.

QC rules for: Analytical run with 3 QC pools per run:

Three QC pools per run with one QC result per pool

- 1) If all 3 QC run results are within 2Si limits, then accept the run.
- 2) If 1 of the 3 QC run results is outside a 2Si limit - reject run if:
 - a) Extreme Outlier – Run result is beyond the characterization mean +/- 4Si
 - b) 3S Rule - Run result is outside a 3Si limit
 - c) 2S Rule - 2 or more of the 3 run results are outside the same 2Si limit
 - d) 10 X-bar Rule – Current and previous 9 run results are on same side of the characterization mean
 - e) R 4S Rule – Two consecutive standardized run results differ by more than 4Si. Note: Since runs have a single result per pool for 3 pools, comparison of results for the R 4S rule will be with the previous result within the current run or with the last result of the previous run. Standardized results are used because different pools have different means.

Three QC pools per run with two or more QC results per pool

- 1) If all 3 QC run means are within 2Sm limits and individual results are within 2Si limits, then accept the run.
- 2) If 1 of the 3 QC run means is outside a 2Sm limit - reject run if:
 - a) Extreme Outlier – Run mean is beyond the characterization mean +/- 4Sm
 - b) 3S Rule - Run mean is outside a 3Sm limit
 - c) 2S Rule - 2 or more of the 3 run means are outside the same 2Sm limit
 - d) 10 X-bar Rule – Current and previous 9 run means are on same side of the characterization mean
- 3) If one of the QC individual results is outside a 2Si limit - reject run if:
 - a) R 4S Rule - 2 or more of the within-run ranges in the same run exceed 4Sw (i.e., 95% range limit). Note: Since runs have multiple results per pool for 3 pools, the R 4S rule is applied within runs only.

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, 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 PFASs in serum may be higher than the highest standard in the calibration curves, and 0.1 mL of sample may be too much to use. This

is evident by the low recovery of the isotope-labeled standard after the SPE extraction. 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)

Results (<http://www.cdc.gov/exposurereport>) from the National Health and Nutrition Examination Survey (NHANES) can be used as reference ranges for the general US population ²⁵.

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

Critical call values have not been established for any PFAS concentrations.

15. Specimen Storage and Handling During Testing

Specimens are stored in the laboratory frozen prior to analysis. Frozen samples are allowed to thaw completely at room temperature prior to the initiation of the analytical procedure.

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

Alternate procedures do not exist in-house for the measurement of PFASs. If the analytical system fails, storage of samples refrigerated is recommended until the system is operational again.

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

- a. The Quality Control officer reviews each analytical run, identifies the quality control samples within each analytical run and determines whether the analytical run is performed under acceptable quality control conditions.
- b. The data from analytical runs of unknowns are initially reviewed by the laboratory supervisor.
- c. If the quality control data and results are acceptable the laboratory supervisor generates a memorandum to the Branch Chief reporting the results.
- d. These data are then sent to the person(s) that made the initial request.
- e. Final hard copies of correspondence are maintained in the office of the Branch Chief and with the quality control officer.

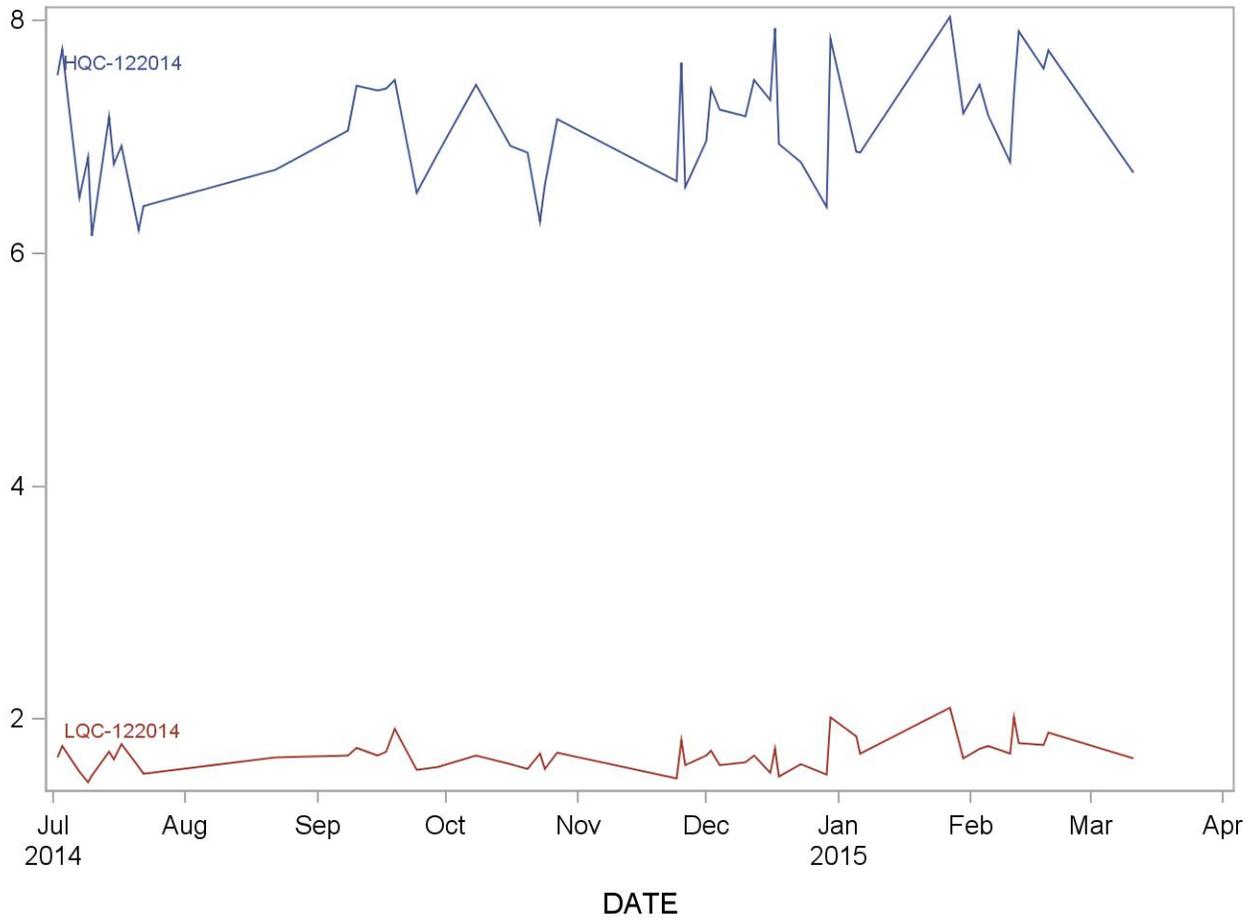
18. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

Standard record keeping systems (e.g., notebooks, sample logs, data files) should be employed to keep track of all specimens. One spreadsheet form 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.

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.

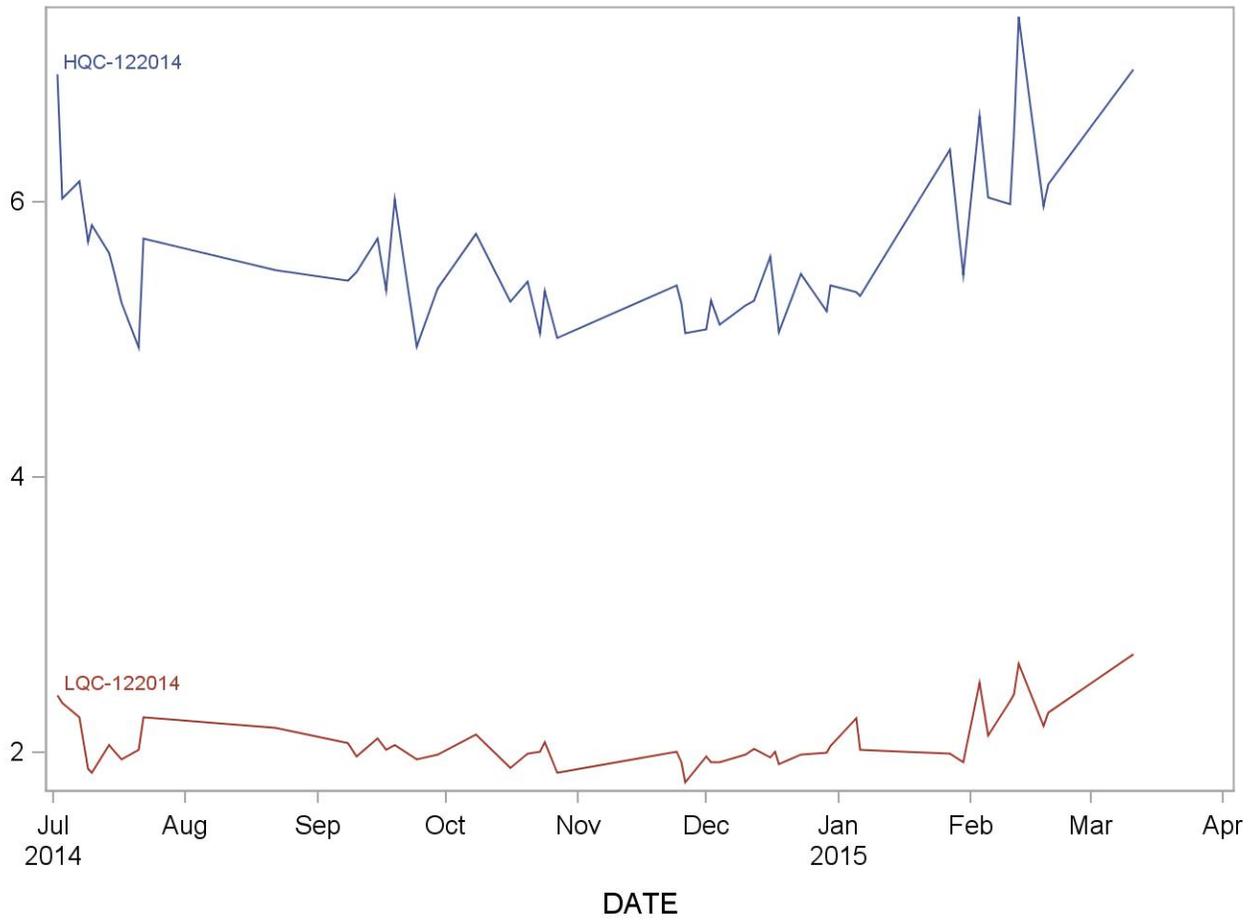
2013-2014 Summary Statistics and QC Chart for 2-(N-methyl-PFOSA) acetate (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|------------|----|------------|----------|---------|--------------------|--------------------------|
| HQC-122014 | 50 | 02JUL14 | 11MAR15 | 7.09100 | 0.48089 | 6.8 |
| LQC-122014 | 50 | 02JUL14 | 11MAR15 | 1.69470 | 0.13635 | 8.0 |



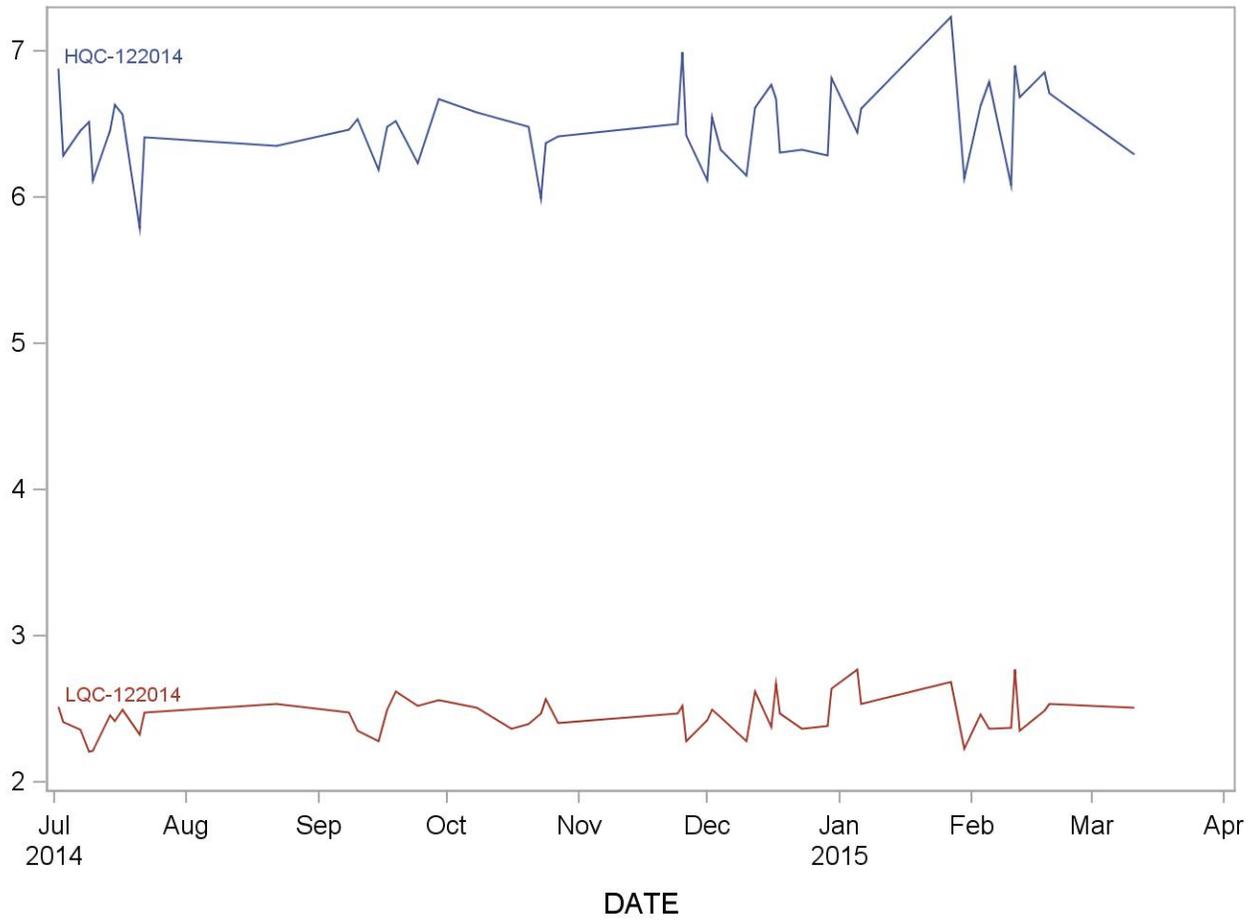
2013-2014 Summary Statistics and QC Chart for Perfluorobutane sulfonic acid (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|------------|----|------------|----------|-------|--------------------|--------------------------|
| HQC-122014 | 50 | 02JUL14 | 11MAR15 | 5.629 | 0.548 | 9.7 |
| LQC-122014 | 50 | 02JUL14 | 11MAR15 | 2.088 | 0.202 | 9.7 |



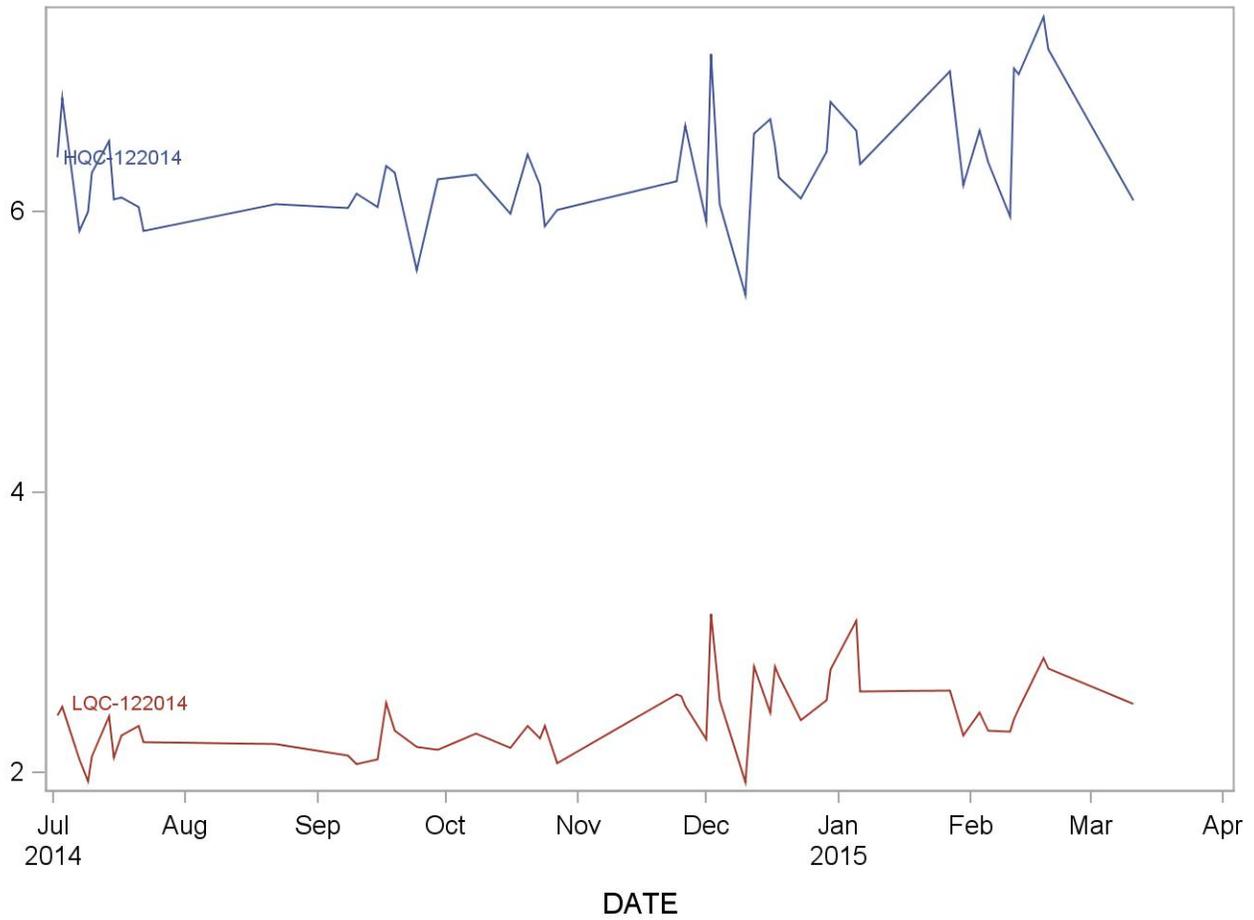
2013-2014 Summary Statistics and QC Chart for Perfluorodecanoic acid (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|------------|----|------------|----------|-------|--------------------|--------------------------|
| HQC-122014 | 50 | 02JUL14 | 11MAR15 | 6.484 | 0.272 | 4.2 |
| LQC-122014 | 50 | 02JUL14 | 11MAR15 | 2.461 | 0.129 | 5.3 |



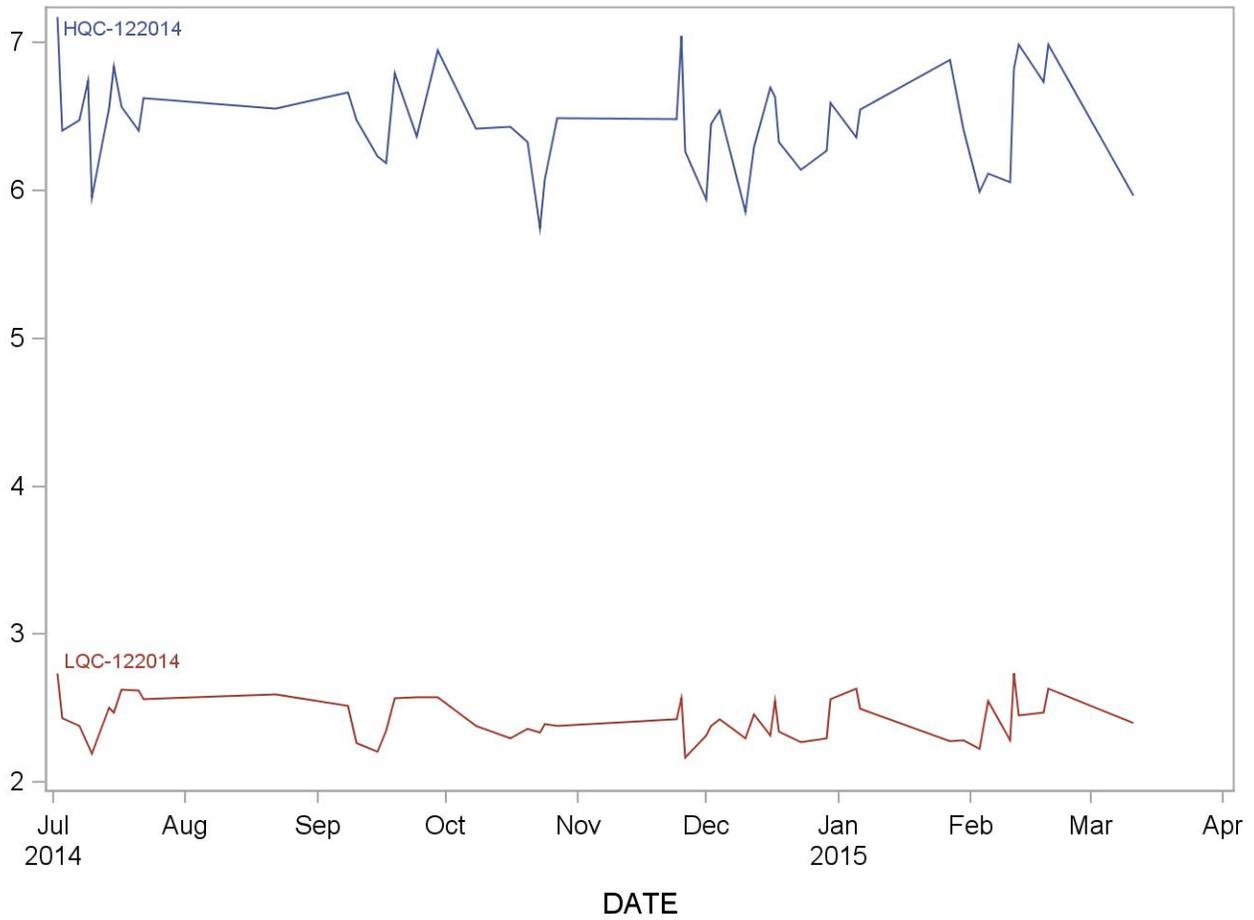
2013-2014 Summary Statistics and QC Chart for Perfluorododecanoic acid (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|------------|----|------------|----------|-------|--------------------|--------------------------|
| HQC-122014 | 50 | 02JUL14 | 11MAR15 | 6.324 | 0.406 | 6.4 |
| LQC-122014 | 50 | 02JUL14 | 11MAR15 | 2.390 | 0.263 | 11.0 |



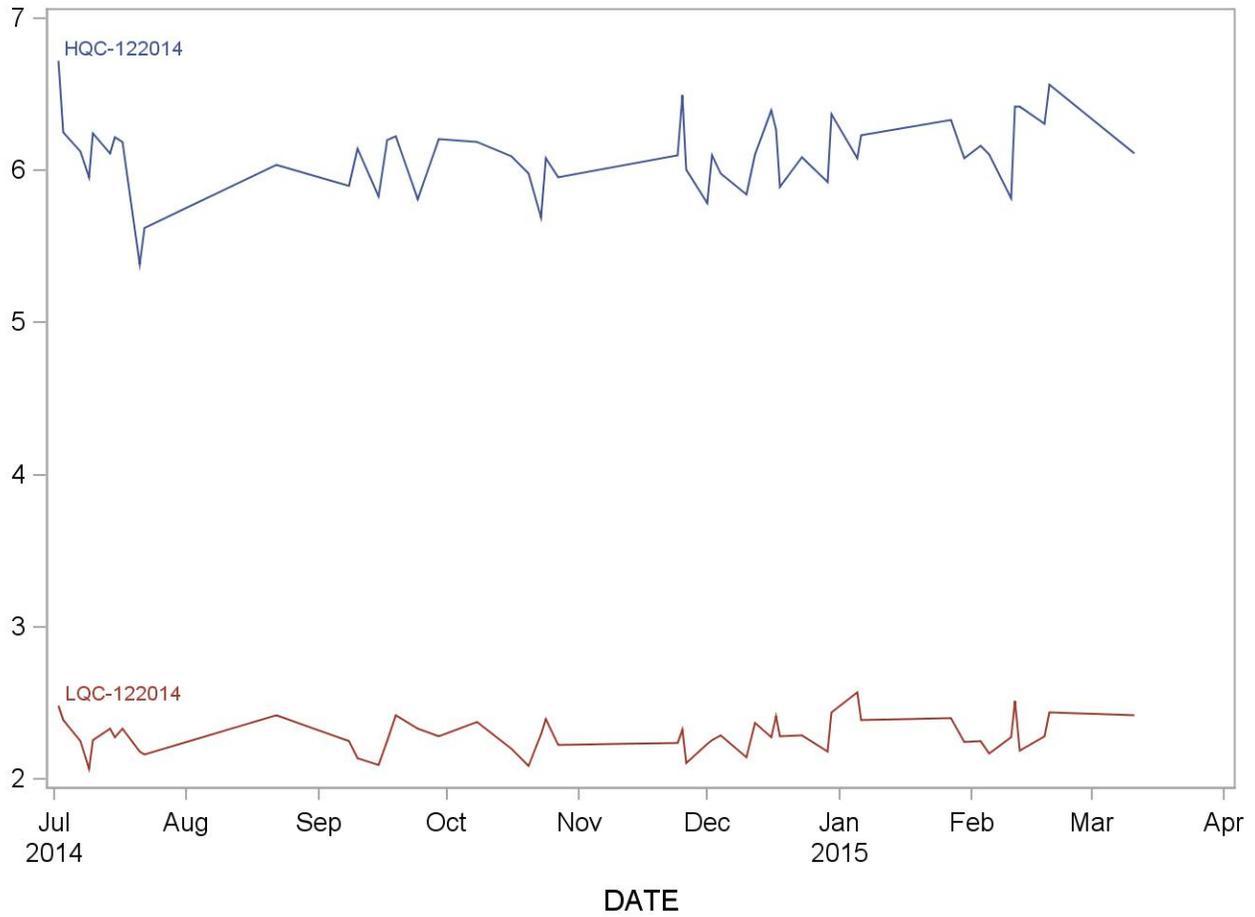
2013-2014 Summary Statistics and QC Chart for Perfluoroheptanoic acid (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|------------|----|------------|----------|-------|--------------------|--------------------------|
| HQC-122014 | 50 | 02JUL14 | 11MAR15 | 6.458 | 0.328 | 5.1 |
| LQC-122014 | 50 | 02JUL14 | 11MAR15 | 2.429 | 0.144 | 5.9 |



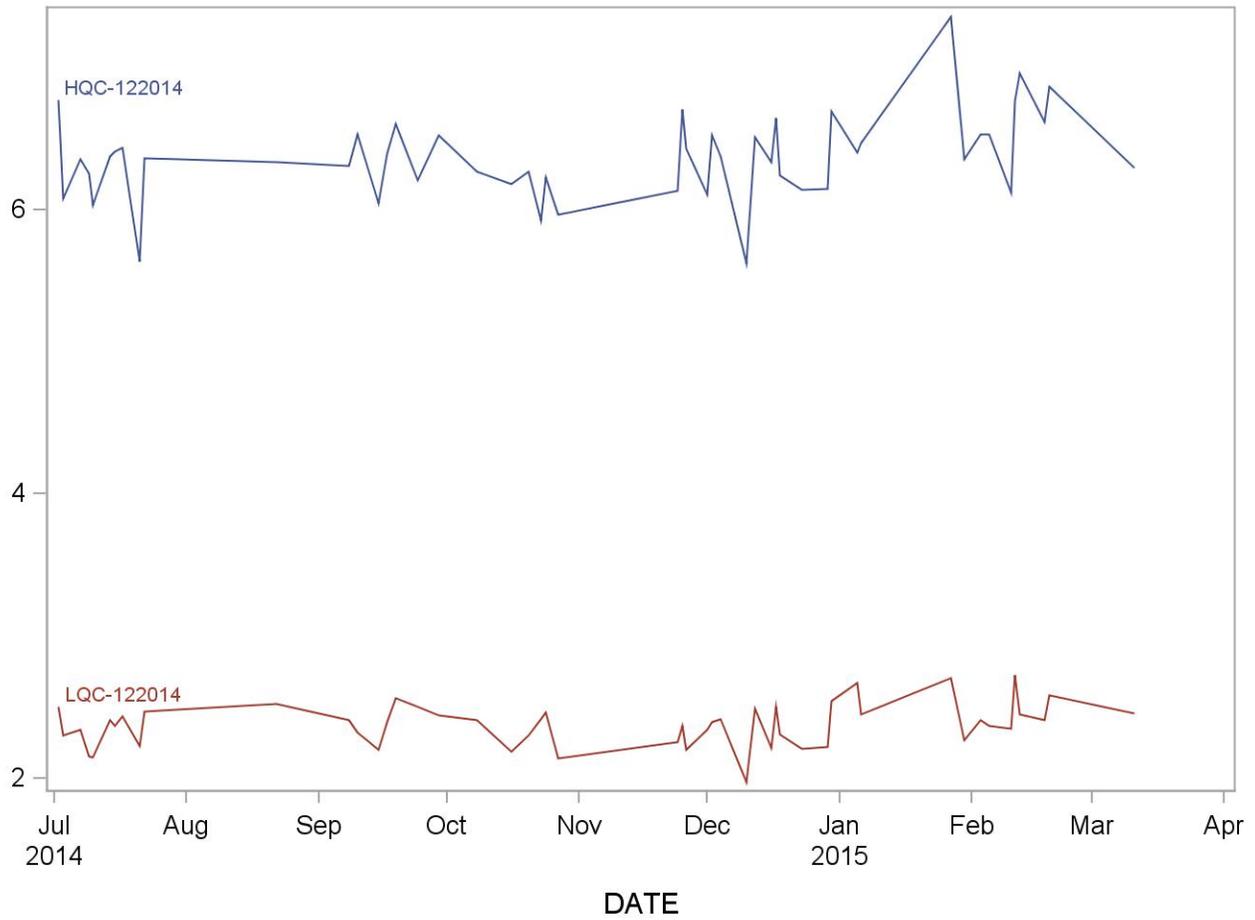
2013-2014 Summary Statistics and QC Chart for Perfluorohexane sulfonic acid (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|------------|----|------------|----------|-------|--------------------|--------------------------|
| HQC-122014 | 50 | 02JUL14 | 11MAR15 | 6.105 | 0.243 | 4.0 |
| LQC-122014 | 50 | 02JUL14 | 11MAR15 | 2.291 | 0.114 | 5.0 |



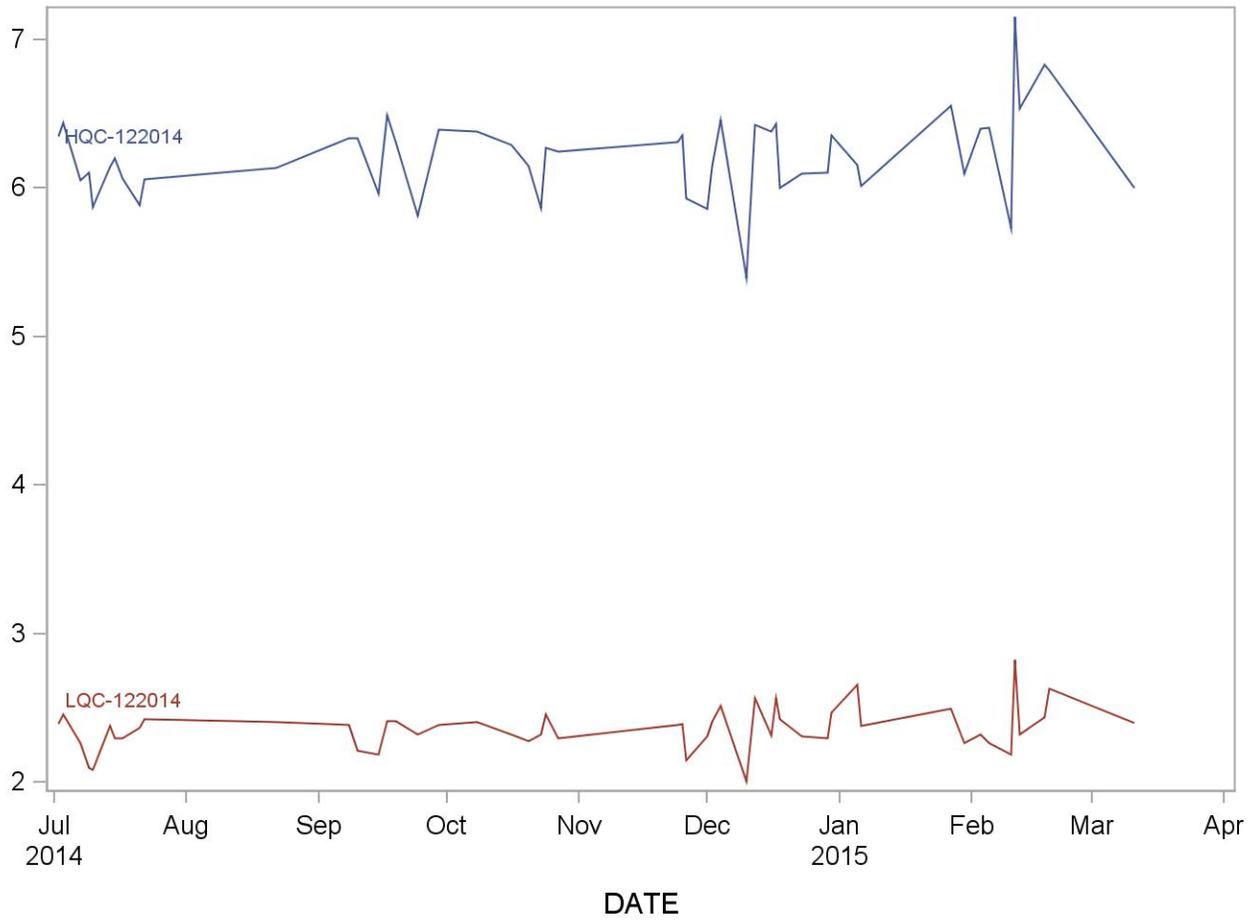
2013-2014 Summary Statistics and QC Chart for Perfluorononanoic acid (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|------------|----|------------|----------|--------|--------------------|--------------------------|
| HQC-122014 | 50 | 02JUL14 | 11MAR15 | 6.3701 | 0.3081 | 4.8 |
| LQC-122014 | 50 | 02JUL14 | 11MAR15 | 2.3770 | 0.1517 | 6.4 |



2013-2014 Summary Statistics and QC Chart for Perfluoroundecanoic acid (ng/mL)

| Lot | N | Start Date | End Date | Mean | Standard Deviation | Coefficient of Variation |
|------------|----|------------|----------|-------|--------------------|--------------------------|
| HQC-122014 | 50 | 02JUL14 | 11MAR15 | 6.223 | 0.297 | 4.8 |
| LQC-122014 | 50 | 02JUL14 | 11MAR15 | 2.364 | 0.145 | 6.1 |



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