Division of Laboratory Sciences
Laboratory Protocol

Analyte:	**Polyfluorochemicals: 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:	**Solid Phase Extraction-High Performance Liquid Chromatography-Turbo Ion Spray-Tandem Mass Spectrometry (SPE-HPLC-TIS-MS/MS)**
Method Code:	
Branch:	**Organic Analytical Toxicology**

Prepared By:	Susan Kuklenyik Author's name	_____ signature	_____ date
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Branch Chief:	Larry L. Needham Branch Chief's name	_____ signature	_____ date
Adopted:	11/15/2004 date		

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.

Polyfluorochemicals in Serum
NHANES 2003-2004

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

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
l24pfc_c	LBXPFOA	Perfluorooctanoic acid (ng/mL)
	LBXPFOS	Perfluorooctane sulfonic acid (ng/mL)
	LBXPFHS	Perfluorohexane sulfonic acid (ng/mL)
	LBXEPAH	2-(N-ethyl-PFOSA) acetate(ng/mL)
	LBXMPAH	2-(N-methyl-PFOSA) acetate(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. Test Principle

The test principle utilizes solid phase extraction-high performance liquid chromatography-turboionspray ionization-tandem mass spectrometry (SPE-HPLC-TCI-MS/MS) for the quantitative detection of PFCs: perfluorooctane sulfonamide (PFOSA), 2-(N-methyl-perfluorooctane sulfonamido) acetic acid (Me-PFOSA-AcOH), 2-(N-ethyl-perfluorooctane sulfonamido) acetic acid (Et-PFOSA-AcOH), perfluorohexane sulfonate (PFHxS), PFOS, perfluoropentanoate (PFPeA), perfluorohexanoate (PFHxA), perfluoroheptanoate (PFHpA), PFOA, perfluorononanoate (PFNA), perfluorodecanoate (PFDeA), perfluoroundecanoate (PFUA), and perfluorododecanoate (PFDoA) (16). The PFCs are extracted from serum using an automated SPE procedure, separated from other extracted components in the SPE eluate by reversed phase HPLC, and detected by negative ion TIS-MS/MS using a multiple reaction monitoring experiment. This method allows for rapid detection of these fluorochemicals 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:
Avoid contact with eyes and skin.
Avoid use of the organic solvents in the vicinity of an open flame.
Use solvents only in well-ventilated areas.

Note: The Material Safety Data Sheets (MSDS) for the chemicals and solvents used in this procedure are maintained on the PC network (www.ilpi.com/msds/index.html), and as a hard copy found in the Chemical Hygiene Plan present 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.

f. Training

Training and experience in the use of a triple quadrupole mass spectrometer and the automated 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 API 4000 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 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 API 4000 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 DLS-PC Network (Q:\Phthalates\PFOS) 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 read in by a barcode reader directly from the sample vials. Sample IDs for 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 API 4000 mass spectrometer. After MS data collection and peak integration, the 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. 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 a DVD. All sample and analytical data are checked after being entered into the database for transcription errors and overall validity. The Access database is located on the Local Area Network (LAN) and is automatically backed up nightly to a tape by the DLS LAN support staff. In addition, the database is routinely (at least once weekly) backed up onto a computer hard drive and onto a DVD. Data from completed studies are saved on CD-R/RW or DVD, and on the hard drive. Additionally, final reported data are saved as paper copy as an official government record. Documentation for data system maintenance is contained in copies of data records.

d. Final Reports

Final reports are generated using a SAS program, and are reviewed by the DLS statistician before being transmitted to the Branch Chief via an inter-office memorandum. Then, the report is finally reviewed by the DLS Division Director. 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 its designee under the miscellaneous project/case number assigned by the DLS specimen-receiving personnel. Data from the case 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

A minimum of three milliliters of serum should be collected in standard collection containers. Samples should be refrigerated as soon as possible and transferred to labeled specimen containers within 4 hours of collection for storage. Samples should be stored frozen at or below -20 °C in polypropylene or polyethylene containers. Glass containers and Teflon coated materials should not be used.

b. Sample Handling

Specimen handling conditions are outlined in the Division protocol for serum collection and handling (copies are available in the laboratory). In the protocol, collection, transport, and special equipment required are discussed. In general, serum specimens should be transported and stored 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, sonicated, aliquoted, and the residual specimen is again stored at or below -20 °C until needed. The integrity of samples thawed and refrozen several times is not 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 HPLC grade purchased from Caledon (Georgetown, Ont., Canada). Acetic acid (glacial), and 4-methylumbelliferone were purchased from Sigma Aldrich Laboratories, Inc (St.

Louis, MO). Formic acid (FA, 96% min, GR) was purchased from EM Science (Gibbstown, NJ). Ammonium hydroxide (30%) was purchased from J.T. Baker (Phillipsburg, NJ. 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 potassium salt (PFBuS), perfluorohexane sulfonate potassium salt (PFHxS), perfluorooctane sulfonate potassium salt (PFOS), and perfluorooctanoic acid ammonium salt (PFOA), were provided by 3M Company (Saint Paul, MN). Perfluoroheptanoic acid (PFHpA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDeA), perfluoroundecanoic acid (PFUA), and perfluorododecanoic acid (PFDoA) were purchased from Oakwood Products (West Columbia, SC). 1,2-^{13}C-perfluorooctanoic acid ($^{13}C_2$-PFOA) was provided by Dupont Company (Wilmington, DE). $^{18}O_2$-perfluorooctane sulfonate ammonium salt ($^{18}O_2$-PFOS) and $^{18}O_2$-perfluorooctane sulfonamide ($^{18}O_2$-PFOSA) were purchased from RTI Laboratories (RTP, NC). All chemicals and solvents were used without further purification.

b. Working Solutions

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

Dilute 570 µL of concentrated acetic acid to 500 mL in a volumetric flask with water. Adjust pH to 4 by adding drop-wise 1:10 ammonium hydroxide:water mixture. Prepare fresh daily and store at room temperature.

(2) HPLC Organic Mobile Phase for Serum Analyses, 100% HPLC MeOH

Refill as needed and store at room temperature.

(3) Reconstitution Solution for Serum Analyses

To 250 mL 20 mM acetic acid add 25 µL of 100 µg/mL 4-methylumbilleferone and shake vigorously. Prepare monthly and store at or below 10 °C

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

Dilute 3,930 µL of 96% concentrated FA to 1000 mL in a volumetric flask with water and shake vigorously. Prepare monthly and store at room temperature.

(5) SPE Aqueous / Organic Wash Solution, 50% FA / 50% MeOH

Mix 500 mL 0.1M FA and 500 mL MeOH while shaking vigorously. Prepare monthly and store at room temperature.

(6) SPE Basic Wash, 1% Ammonium Hydroxide in Water

Dilute 17 mL of concentrated (30%) ammonium hydroxide to 500 mL in a volumetric flask with water and shake vigorously. Prepare monthly and store at room temperature.

(7) SPE Eluent, 1% Ammonium Hydroxide in Acetonitrile

Dilute 17 mL of concentrated (30%) ammonium hydroxide to 500 mL in a volumetric flask with acetonitrile. Prepare monthly and store in the refrigerator.

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 100 µg/mL (ppm) of all the analytes are prepared in methanol from the stock solutions. Stock solutions of the labeled internal standards, $^{13}C_2$-PFOA, $^{18}O_2$-PFOS, and $^{18}O_2$-PFOSA are prepared similarly. All stock solutions are stored in polypropylene vials at or below -40 °C.

(2) Fluorochemical Analytical Calibration Standards

Standard spiking solutions are prepared in MeOH from the 100 µg/mL working native standard solution such as a 50-µL spike into 1 mL serum provides concentrations that cover the linear range of the method. The spiking solutions are stored in 1 mL aliquots in polypropylene vials kept at or below -40 °C until use.

(3) Internal Standard Spiking Solution

The $^{13}C_2$-PFOA, $^{18}O_2$-PFOSA and $^{18}O_2$-PFOS internal standard spiking solution of 200 ng/mL is prepared in methanol. A 50-µL spike of this solution provides 10 ng/mL of labeled internal standard analytes in 1 mL sample. Three mL aliquots of this spiking solution are dispensed into polypropylene cryovials and stored at or below -40 ºC until use.

(4) Mass-Spec Operational Check Standard

50 µL of 50 ng/mL (Standard 4) is diluted to 5 mL with a 20 mM acetic acid in 10/90 water/MeOH solution in a polypropylene test tube. 200 µL aliquots are dispensed into polypropylene autosampler vials and stored at or below 10 ºC.

(5) Proficiency Testing (PT) Standards

Appropriate aliquots of each stock standard are added to calf serum pools to produce 3 sets of 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) Oasis HLB cartridges (60 mg/3 cc, Waters Corporation, Milford, MA).
(2) Disposable round bottom polypropylene tubes (8 mL, 13 x 100 mm, VWR, West Chester, PA).
(3) Disposable round bottom polypropylene tubes (5 mL, 12 x 75 mm, VWR, West Chester, PA).
(4) 300 µL polypropylene autosampler vials with polyethylene snap caps (Agilent Tech., Wilmington DE).
(5) Tip ejector variable volume micropipettes (Wheaton, Millville, NJ) and pipette tips (Rainin Instruments Co., Woburn, MA).
(6) BETASIL C8 column (3 mm × 50 mm, 5 µm) (Keystone Scientific, Bellefonte, PA).
(7) 3.6 mL Nunc polypropylene cryovials (Krackler Scientific, Albany, NY).
(8) Assorted glass and polypropylene labware.

e. Equipment

(1) Rapid Trace automated solid phase extractor, 10 units (Zymark, Hopkinton, MA).
(2) Agilent 1100 HPLC system (Agilent Tech., Wilmington DE).
(3) Applied Biosystems API 4000 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 (Corning pH/ion analyzer 455, Corning, New York).
(7) Sonicating waterbath (Branson 5510).
(8) Vortex mixer (Type 16700, Barnstead International, Dubuque, Iowa).
(9) Magnetic stirrer (Corning).

f. Instrumentation

(1) Mass Spectrometer Configuration

Mass spectrometric analyses are conducted on the API 4000 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. The TIS source incorporates a heated probe that blows hot dry gas at the spray produced by the electrospray. The electrospray effluent and the heated gas intersect at an angle of approximately 90 degrees near the orifice. We use laboratory-grade air heated turbo ion spray gas (GS1=35 and GS2=35) gas. The heated turbo ion spray gas temperature is set at 400 °C. The curtain and collision gas (nitrogen) settings are as follows: collision (CAD=9.0), curtain gas (CUR=20). Ionization parameters and collision cell parameters are optimized individually for each analyte (**Table 1**). Unit resolution is used for both Q1 and Q3 quadrupoles.

Table 1. Mass spectrometric parameters for the analysis of polyfluorochemicals in serum

	$(M-H)^-$ Precursor ion (m/z)	Product ion (m/z)	Dwell Time (ms)	DP (volts)	CE (volts)
PFOSA	498	78	50	-60	-85
PFHxS-Q1	399	99	50	-70	-80
PFHxS-Q2	399	80	50	-70	-85
PFOS-Q1	499	99	50	-70	-80
PFOS-Q2	499	80	50	-70	-90
PFOS-Q3	499	130	50	-70	-90
PFOS-$^{18}O_2$ (IS)	503	84	50	-70	-85
PFOSA-$^{18}O_2$ (IS)	503	82	50	-60	-85
PFBuS-Q1	299	80	50	-70	-80
PFBuS-Q2	299	99	50	-70	-85
Me-PFOSA-AcOH-Q1	570.2	570	50	-50	-10
Me-PFOSA-AcOH-Q2	570.2	512	50	-50	-26
Et-PFOSA-AcOH-Q1	584.1	584	50	-45	-10
Et-PFOSA-AcOH-Q2	584.1	526	50	-45	-27
PFHpA	363.1	319	50	-25	-13
PFOA	413.1	369	50	-27	-14
PFOA-$^{13}C_2$ (IS)	415.1	370	50	-30	-15
PFNA	463.1	419	50	-30	-13
PFDeA	513.2	469	50	-30	-15
PFUA	563.3	519	50	-30	-17
PFDoA	613.2	569	50	-30	-18

Q1 and IS are the analyte quantitation ion and internal standard quantitation ion, respectively. The Q2 and Q3 transitions are used for confirmation. $^{18}O_2$-PFOSA internal standard was used for the three sulfonamides, $^{18}O_2$-PFOS for the three sulfonates, and $^{13}C_2$-PFOA for the six carboxylates.

(2) LC/MS synchronization

The entire system from sample injection to data acquisition is controlled by the Analyst software using the "LCsync" synchronization mode. The acquisition method (*.dam) file contains both the mass spectral settings and the HPLC parameters. After the HPLC column pressure stabilizes, the program turns on the HPLC autosampler, and with the turn of the injector valve, the autosampler triggers the mass spectrometer for data collection. At the same time, the start HPLC pump gradient program is triggered from the Analyst software.

(3) HPLC Configuration

The analytes are separated from other components of the serum extract using a Betasil C8 column (3 mm × 50 mm, 5 µm). The Agilent 1100 liquid chromatograph operates at 300 µL/min flow rate using mobile phase A (aqueous), and mobile phase B (organic) (**Table 2**) to produce the gradient described in **Table 3.**

Table 2. HPLC configuration for serum analyses

Parameters	Setting
Column Type	Betasil C8 reversed phase (3 mm × 50 mm, 5 µm)
Mobile Phase A	20 mM ammonium acetate in water, pH = 4
Mobile Phase B	100% methanol
Injection Volume	12.5 µL
Flow rate	300 µL/min

(4) HPLC Mobile Phase Gradient

Table 3. Mobile phase gradient for serum analyses

Time (min) →	0.00	1.00	10.00	10.50	14
Solvent					
20 mM ammonium acetate in water, pH=4	40%	20%	20%	40%	40%
100% methanol	60%	80%	80%	60%	60%

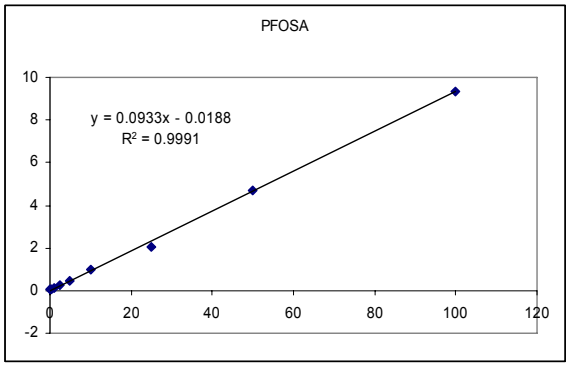
7. Calibration and Calibration-Verification Procedures

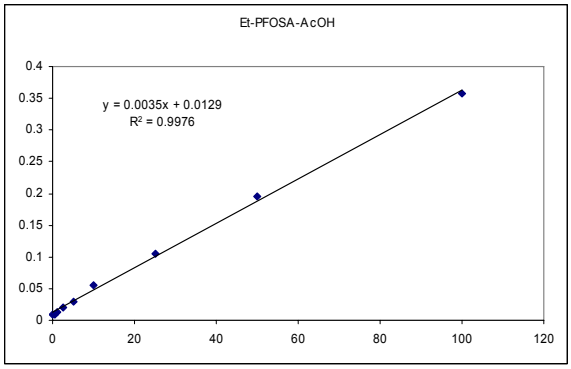
a. Calibration Curve

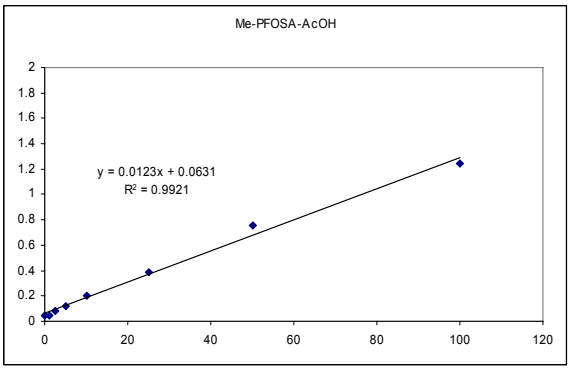
Calibration curves are calculated daily from the analyte area ratios (i.e., analyte area/internal standard area) measured using freshly extracted standards in calf serum. $^{18}O_2$-PFOSA internal standard was used for the three sulfonamides, $^{18}O_2$-PFOS for the three sulfonates, and $^{13}C_2$-PFOA for the six carboxylates. 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 quantitation. Correlation coefficients must be greater than 0.97. Samples with values exceeding the highest point in the calibration curve are reanalyzed using less serum.

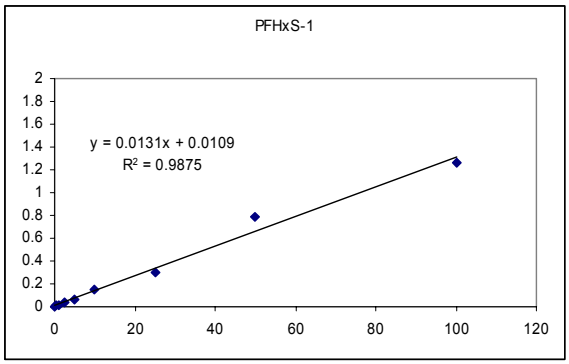
b. Examples of calibration curves

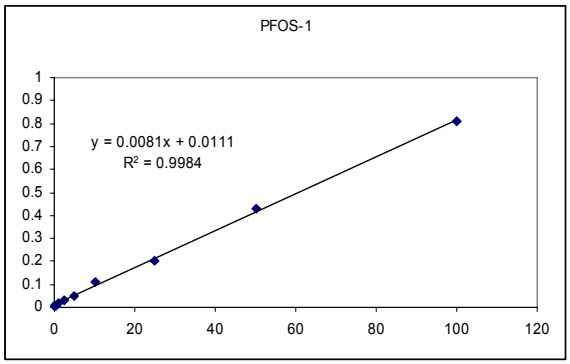
The following are examples of the calibration curves:

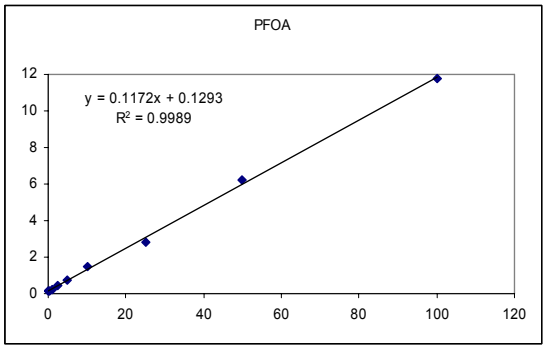


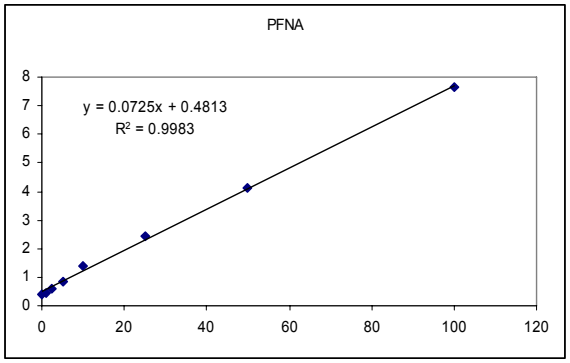


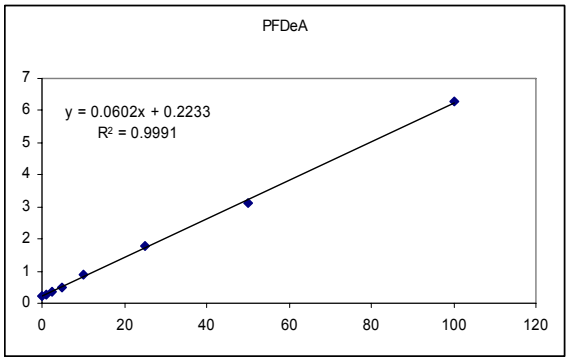


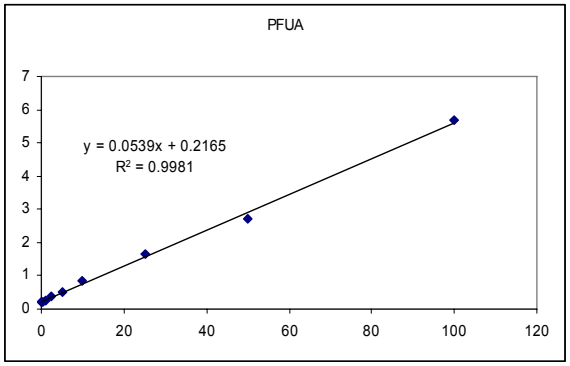


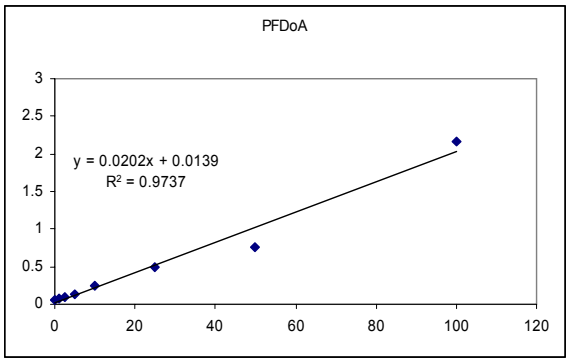












c. Mass Spectrometer Calibration

The API 4000 mass spectrometer is calibrated and tuned every 3-6 months using a polypropylene glycol (PPG) solution according to the instructions contained in the API 4000 operator's manual. The instrument sensitivity is checked before every run by injecting the Instrument Test sample.

d. 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.

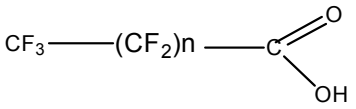
(2) Calibration verification must be performed at least once every 6 months.

(3) All calibration verification runs and results shall be appropriately documented.

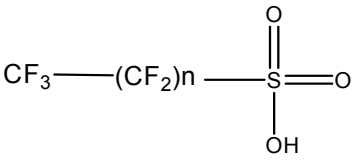
(4) According to the updated CLIA regulations from 2003 (http://www.cms.hhs.gov/CLIA/downloads/6065bk.pdf), the requirement for calibration verification is met if the test system's calibration procedure includes three or more levels of calibration material, and includes a low, mid, and high value, and 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.

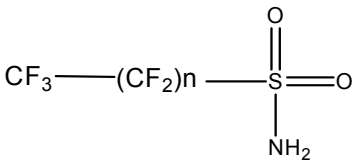
e. Analytes nomenclature and structures



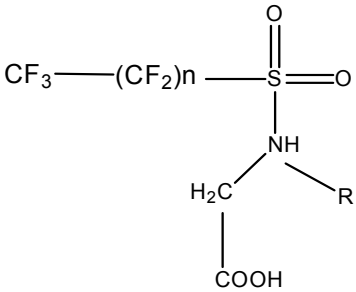
n=5 PFHpA
n=6 PFOA
n=7 PFNA
n=8 PFDeA
n=9 PFUA
n=10 PFDoA



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

f. Proficiency Testing (PT)

(1) Three pools of PT samples, which encompass the entire linear range of the method, are prepared in-house as described before. Characterization of PT materials requires at least 20 separate determinations. Once the PT pools are characterized, the mean concentration and standard deviation 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. 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.

8. Operating Procedures; Calculations; Interpretation of Results

a. Sample preparation

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

(a) Remove serum samples from the freezer and let them thaw. Remove working standard solutions and internal standard solution from the freezer.

(b) Dispense 50 µL of each internal standard solution (^{18}O-PFOSA, ^{18}O-PFOS and ^{13}C-PFOA) into all the polypropylene test tubes that will contain sample to be extracted. *Add the ISTDs to the bottom half of the test tube so the ISTDs can be easily homogenized into the sample when added.*

(c) Add 50 µL of the appropriate native standard solution (S1-S9) into the polypropylene test tubes designated for standards. *Add standard mix to the bottom half of the test tube so the standards can be easily homogenized into the sample when added.*

(d) Add 3 mL 0.1 M formic acid into all tubes.

(e) Add 1 mL of serum sample, QC, serum blank or water to each tube. For the standards (S1-S9), 1 mL of blank serum is used.

(f) Vortex all tubes for five 5 seconds to make sure all the internal standard and standard mixed into the sample.

(g) Sonicate all tubes for 20 min then add 1 mL of 0.1 M formic acid.

(2) Automated Cleanup Procedure

(a) Turn on all Rapid Trace modules.

(b) Choose “Rapid Trace Development” on the Desktop.

(c) Go to “Module Setup” and choose program PURGE.spe. Select this program for 1 position in each module to be used. Place test SPE cartridges in those positions.

(d) Go to “Run”.

(e) Once PURGE.spe program has finished, remove the test cartridges.

(f) Go to “Module Setup” and choose program SERUM_53.spe (**Table 4**). Choose the appropriate positions for your samples.

(g) Place the OASIS HLB cartridges in the appropriate modules. Place 8 mL polypropylene tubes containing the samples to be extracted and the 5 mL polypropylene tubes, used to collect the extracted samples, in the appropriate positions.

(h) Go to “Run.”

(i) Remove the 5 mL tubes and place in a TurboVap rack. Allow the extracted samples to dry until about 150 µL of solution remains. The TurboVap should be set to 55 °C for 10 minutes and about 10 psi nitrogen.

(j) Resuspend the extracted samples in 200 µL of reconstitution solution (10 ng/mL methylumbilleferone in 20mM acetic acid). The methylumbilleferone is used to monitor deviations caused by injection volume changes. In case of low internal standard peak area count, it helps to determine whether the low area count was caused by low SPE recovery or injector problem such as a plug in the sampling needle.

(k) Vortex mix for about 10 seconds and transfer to labeled polypropylene autosampler vials. Cap the vials.

(l) Label autosampler vials for samples and QC as follows: “YYYY-MMDD-4yxx” (YYYY for year; MM for month; DD for day; y=1,2, etc, for number of run; xx=00, 02, …, 99 for tube or vial number).

Table 4. Program used for the automated SPE of polyfluorochemicals in serum.

	Step	Source	Destination	ml	ml/min	Explanation
1	Purge-Cannula	MeOH	Cannula	4	40	Cleans cannula with organic solvent.
2	Purge-Cannula	H_20	Cannula	4	40	Cleans cannula with aqueous solvent.
3	Rinse	MeOH	Org	2	2	Cleans/conditions cartridge with organic solvent.
4	Rinse	F.A.	Bio	2	2	Cleans/conditions cartridge with aqueous solvent.
5	Load	Sample	Bio	4.5	1	Loads the sample onto the cartridge.
6	Rinse	Vent	Bio	5	20	Pushes remaining sample through cartridge into waste.
7	Rinse	F.A.	Bio.	3	2	Further cleans up sample.
8	Rinse	50/50	Org/Aq	6	2	Further cleans up sample with greater organic percentage wash.
9	Rinse	B.H20	Bio	1	1	Uses basic water wash to further cleanup cartridge.
11	Rinse	Vent	Bio	5	20	Dries cartridge with air.
12	Collect	B.ACN	Fract1	1	1	Elutes analytes with basic organic solvent.
13	Add to Sample	H20	Cannula	2	40	Rinses cannula and needle completely.

Misc. Parameters:
LoadCannulaDepth 0
Mix CannulaDepth 0
MixCycles 3
MixVolume 5
MixSpeed 30

Reagent Name	Abbreviation	SipSpeed
Vent	Vent	30
Methanol	MeOH	30
PLC Water	H_20	30
0.1 M Formic Acid	F.A.	30
50% F.A. 50% MeOH	50/50	30
1% NH_4OH/ water	B.H2O	30
1% NH_4OH/Acetonitrile	B.ACN	30

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. Once a week, 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

(a) If it was not done after the last run, wash the HPLC column with 100% organic mobile phase at 0.3 mL/min for at least ~1 hour.

(b) Prepare fresh aqueous mobile phase. Open the purge valve. Purge the degasser with the fresh aqueous mobile phase for 10 min at 3 mL/min. Change the flow rate back to 0.3 mL/min and close the purge valve. Condition the column with 10% organic mobile phase/90% aqueous mobile phase until the pressure reaches 45-65 bar. If the pressure is too high, check the pressure with the purge valve open first. If the pressure is over 10 bar with the purge valve open, the purge valve frit needs to be replaced according to the procedure in the manual.

(c) 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) Building batch files

(a) In the subproject folder, there are separate batch (.dab) files for standards and unknowns. To collect a standard curve open the latest Standards_[date].dab file.

(b) Make sure that the Sample Name, Vial Position and Data File fields are correct. The Data File should have the same YYYY-MMDD name as the unknowns it includes. Submit standard data collection (highlight and/or click Submit, go to View Queue, and click Start Sample).

(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 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).

(d) 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).

(e) Place an autosampler vial with MeOH on the sample tray where the sample which has been already injected was. Open the MeOH_wash.dab batch file. Check the Vial Position. The Acquisition method should be MeOH_wash.dam. Submit this MeOH sample as the last sample on the Queue. After running this last sample, the instrument will automatically go into standby mode in 20 min.

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) Isotope Dilution Calculations

In some cases, native analytes have some response at the mass used for the quantitation of the isotope-labeled analog. Likewise, the isotope-labeled analog may contribute some at the mass being used for analyte quantitation. In these cases, corrections must be made for the contribution of isotope-labeled analyte ion to the native ion and vice versa. To properly determine response factors between the analyte and its isotope-labeled analog, these effects must be taken into account. This is the basis for the use of isotope dilution calculations.

(a) Determine R_x and R_y by adding enough of the solutions of interest to overwhelm any contribution from contamination. These ratios are a measure of the degree to which the native analyte ions contribute to the isotope-labeled analog signal and vice versa. If measured correctly, R_y will also account for the presence of native analyte in the labeled spiking solution.

For the native analytes:

$$R_x = \frac{\text{area of the analyte quantitation ion at analyte retention time}}{\text{area of the analyte contribution to labeled analog quantitation ion at analog retention time}}$$

For the isotope-labeled analogs:

$$R_y = \frac{\text{area of the labeled analog contribution to the analyte quantitation ion at analyte retention time}}{\text{area of the labeled analog quantitation ion at analog retention time}}$$

(b) If no area is detected in the denominator of the R_x calculation, set R_x to a number substantially larger than 1 (e.g.,1000000). If no area is detected in the numerator of the R_y calculation, set R_y to a number substantially smaller than 1 (e.g., 0.000001). This will allow the use of the same calculation in cases in which there is no contribution of labeled analog to native analyte signal or vice versa.

(c) The ratio of the analyte signal area to the labeled analog signal area will be determined for each unknown sample, standard, blank or QC material using the Analyst software as:

$$R_m = \frac{\text{area of the analyte quantitation ion at the analyte retention time}}{\text{area of the labeled analog quantitation ion at analog retention time}}$$

The corrected relative response (RR) is calculated from the above ratios as:

$$RR = \frac{(R_y - R_m)(R_x + 1)}{(R_m - R_x)(R_y + 1)}$$

(d) For this calculation to remain valid, R_m must be between $2R_y$ and $0.5\ R_x$. Otherwise large deviations occur for small errors. For standards, RR is plotted versus standard concentration to create calibration curves. Slopes and intercepts from these calibration curves are determined using linear regression analysis. The following equation is used in SAS to determine unknown concentrations:

$$Conc_{unknown} = \frac{(RR_{unknown} - \text{intercept})}{\text{Slope} \times \text{volume}_{unknown}}$$

Table 5. Measured (R_m) vs. corrected (RR) response factors at 1, 10 and 100 ng/L concentrations

Analyte	R_x	R_y	R_m/RR		
			1 ng/mL	10 ng/mL	100 ng/mL
PFOSA	1771	0.001	100%	100%	99%
PFHxS-Q2	179	0.001	100%	99%	92%
PFHxS-Q1	648	0.001	100%	99%	99%
PFOS-Q2	1421	0.066	103%	106%	96%
PFOS-Q1	1364	0.083	115%	112%	107%
PFOS-Q3	1939	0.089	111%	110%	104%
Me-PFOSA-AcOH-Q1	1184	0.002	100%	100%	99%
Me-PFOSA-AcOH-Q2	112	0.001	101%	101%	98%

Et-PFOSAAcOH-Q1	1226	0.002	100%	100%	99%
Et-PFOSAAcOH-Q2	191	0.001	101%	101%	99%
PFHpA	392	0.024	124%	105%	102%
PFOA	669	0.007	104%	101%	100%
PFNA	533	0.007	106%	101%	100%
PFDeA	543	0.003	104%	101%	100%
PFUA	612	0.001	102%	100%	100%
PFDoA	590	0.001	103%	101%	100%

(3) Importing Data into the Database

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

(4) 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) API 4000 Sciex Mass Spectrometer

Maintenance of the API interface, L1x lenses and the analyzer assembly requires venting the system; maintenance of the ion probe does not. Usually after venting, the system will require about 4-6 hours to reestablish high vacuum.

(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 API 4000 Hardware Manual (page 100). 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

No routine maintenance is necessary. 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 (>150 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.

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.

a. Linearity Limits

The calibration curve is linear for all analytes (R^2>0.95). The limit on the linearity is determined by the highest standard analyzed. Due to the wide variation of polyfluorochemicals 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 (LOD – highest standard concentration) of measurement for each polyfluorochemical measured in serum.

Analyte	Linear range (ng/mL)
PFOSA	0.2-100
Me-PFOSA-AcOH	0.6-100
Et-PFOSA-AcOH	0.4-100
PFHxS	0.3-100
PFOS	0.4-100
PFBuS	0.4-100
PFHpA	0.3-100
PFOA	0.1-100
PFNA	0.1-100
PFDeA	0.3-100
PFUA	0.3-100
PFDoA	1-100

b. 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) (17). The formal LOD is defined as $3S_o$. **Table 7** summarizes the LOD determined for each analyte in serum.

Table 7. LODs (in ng/mL) for polyfluorinated compounds in serum

Analyte	LOD
PFOSA	0.2
Me-PFOSA-AcOH	0.6
Et-PFOSA-AcOH	0.4
PFHxS	0.3
PFOS	0.4
PFBuS	0.4
PFHpA	0.3
PFOA	0.1
PFNA	0.1
PFDeA	0.3
PFUA	0.3
PFDoA	1

c. Accuracy

The accuracy of the method is determined by enriching serum samples with known concentrations of the polyfluorochemical analytes 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 2, 5 and 8, and calculated from 5 independent measurements (Table 8).

Table 8. Spiked recoveries of extracted standards in serum

Analyte	**Standard 2**	**Standard 5**	**Standard 8**
PFOSA	94	87	99
Me-PFOSA-AcOH	123	102	102
Et-PFOSA-AcOH	135	100	101
PFHxS	120	99	98
PFOS	123	86	96
PFHpA	115	108	99
PFOA	106	111	96
PFNA	105	113	101
PFDeA	117	196	96

PFUA	127	110	94
PFDoA	133	100	84

d. Precision

The precision of this method is reflected in the variance of two quality control (QC) pools over time (at least 20 independent runs over a period of two weeks). The coefficient of variation (CV) of repeated measurements which reflects both inter and intra-day variations, is used to estimate precision (Table 9).

Table 9. Mean QC concentration (ng/mL) confidence limits in serum

Analyte	QC Low	CV%	QC High	CV%
PFOSA	2.7	15	13.0	16
Et-PFOSA-AcOH	3.8	17	8.3	19
Me-PFOSA-AcOH	3.4	16	9.1	17
PFBuS	4.4	18	14.6	15
PFHxS	2.5	16	11.9	13
PFOS	8.9	10	31.4	10
PFHpA	7.6	17	15.8	14
PFOA	3.2	10	14.7	11
PFNA	2.5	15	12.7	13
PFDeA	2.4	18	8.5	18
PFUA	1.9	22	9.9	20
PFDoA	2.2	26	8.5	26

e. Analytical Specificity

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

Table 10. PFCs precursor and product ion combinations for MS/MS specificity

Compound	Precursor	Q1*	Q2*	Q3*
PFOSA	498	78		
Et-PFOSA-AcOH	570	512		
Et-PFOSA-AcOH	584	526		
PFHxS	399	99	80	
PFOS	499	99	80	130
PFBuS	299	80	99	
PFHpA	363	319		
PFOA	413	369		

PFNA	463	419		
PFDeA	513	469		
PFUA	563	519		
PFDoA	613	569		

*Q1 ions are used for quantitation, and Q2 and Q3 ions for confirmation. The precursor ion is M-1 (where M is the molecular ion). Q1 were $(M\text{-}CO_2H)^-$ for the carboxylic acids; $(M\text{-}SO_3H)^-$ for Me-PFOSA-AcOH and Et-PFOSA-AcOH; $(FSO_3)^-$ for PFOS and PFHxS; $(SO_3)^-$ for PFBuS; and $(SNO_2)^-$ for PFOSA. Q2 was $(SO_3)^-$ for PFOS and PFHxS; $(FSO_3)^-$ for PFBuS. Q3 was $(CF_2SO_3)^-$ for PFOS

10. Quality Control (QC) Procedures

a. QC Materials

The QC were prepared in bulk from calf serum (Gibco, Grand Island, NY). Preliminary human quantification was used to set target ranges for baseline levels.

b. Preparation of QC Pools

The QC pools were mixed uniformly, divided into three subpools, dispensed into small aliquots (ca. 2-7 mL) into prerinsed polypropylene vials, and stored at – 20 °C 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, ~7 ng/mL) and high concentration (QCH, ~35 ng/mL) subpools. The QC pools were characterized to define the mean and the 95% and 99% control limits of PFCs 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 PFCs remained stable at – 20 °C for at least 3 months.

c. Characterization of QC Materials

For characterization, a minimum of 30 runs of QC low and 30 runs of QC high were measured over 1 month. In each run, one pair of QC low and one pair of QC high 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 Quality Control Samples

For each analytical run, the ten-module RapidTrace SPE system is divided into two extraction batches, each one consisting of 5 modules. The first five modules (Modules 0-4) are defined as Batch 1 and the second five modules (Modules 5-9) as Batch 2. Each batch consists of 50 samples (2 QC high, 2 QC low, 5 reagent blank samples, one matrix blank and 40 unknown samples). Therefore, each module in the batch contains one reagent blank sample; four of the modules also contain one QC sample, and one of the modules contains no QC. The concentrations of the two QC high and the two QC low in each 5-unit module are

averaged to obtain one average measurement of QC high and QC low for each batch per run. These QC samples are analyzed along with unknown samples to monitor for accuracy and precision throughout the analysis batch.

e. Final evaluation of Quality Control Results

Separate QC charts (both QC high and QC low) are maintained for samples analyzed on RapidTrace modules 0-4 and Modules 5-9. Standard criteria for run rejection based on statistical probabilities are used to declare a run either in-control or out-of-control. 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

a. Specimen volume

Occasionally, the concentration of the PFCs in serum may be higher than the highest standard in the calibration curves, and 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.

b. Internal Standard purity

The $^{18}O_2$-PFOS and $^{18}O_2$-PFOSA internal standards did not contribute significantly to any of the analytes, and $^{13}C_2$-PFOA appears to be very pure. See Section 8.C.2.

13. Reference Ranges (Normal Values)

No reference range values are currently available.

14. Critical-Call Results ("Panic" Values)

Insufficient data exist to correlate any of the PFCs values with serious health effects. Therefore, critical call values have not been established. The method described here is designed to measure low level exposure to PFCs; panic values will not be measured with this method.

15. Specimen Storage and Handling During Testing

Serum samples may be stored overnight in the refrigerator to expedite thawing prior to aliquoting the sample. The serum extracts are stored in autosampler vials at -20 °C or below after analysis. Results from stability studies suggest that the concentrations of all of the analytes in the extracts remain stable at room temperature for up to five days. After five days PFOSA, Me-PFOSA-AcOH, Et-PFOSA-AcOH, and PFHpA concentrations show significant decrease due to evaporation. The concentrations of the other analytes in the extracts remain stable up to two weeks at room temperature.

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. If the analytical system fails, storage of samples at 4 °C is recommended until the system is operational again. Stability studies suggest that the extracts remain stable at room temperature for up to five days for all analytes.

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. All data (chromatograms, etc.,) are stored in electronic format in the laboratory.

f. 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.

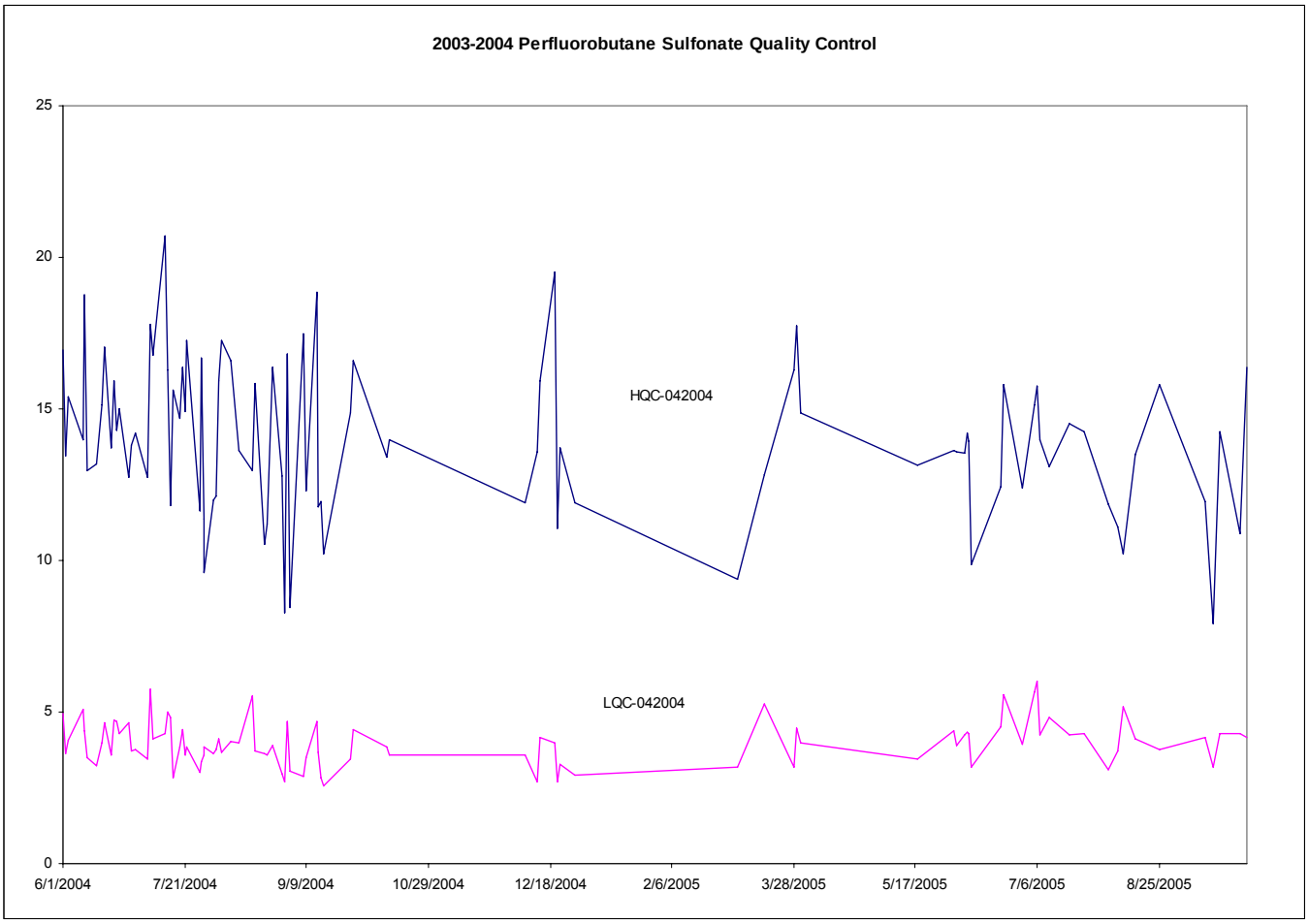
Use of trade names is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

19. Summary Statistics and QC Graphs

A. Perfluorobutane Sulfonate

Summary Statistics for Perfluorobutane Sulfonate by Lot

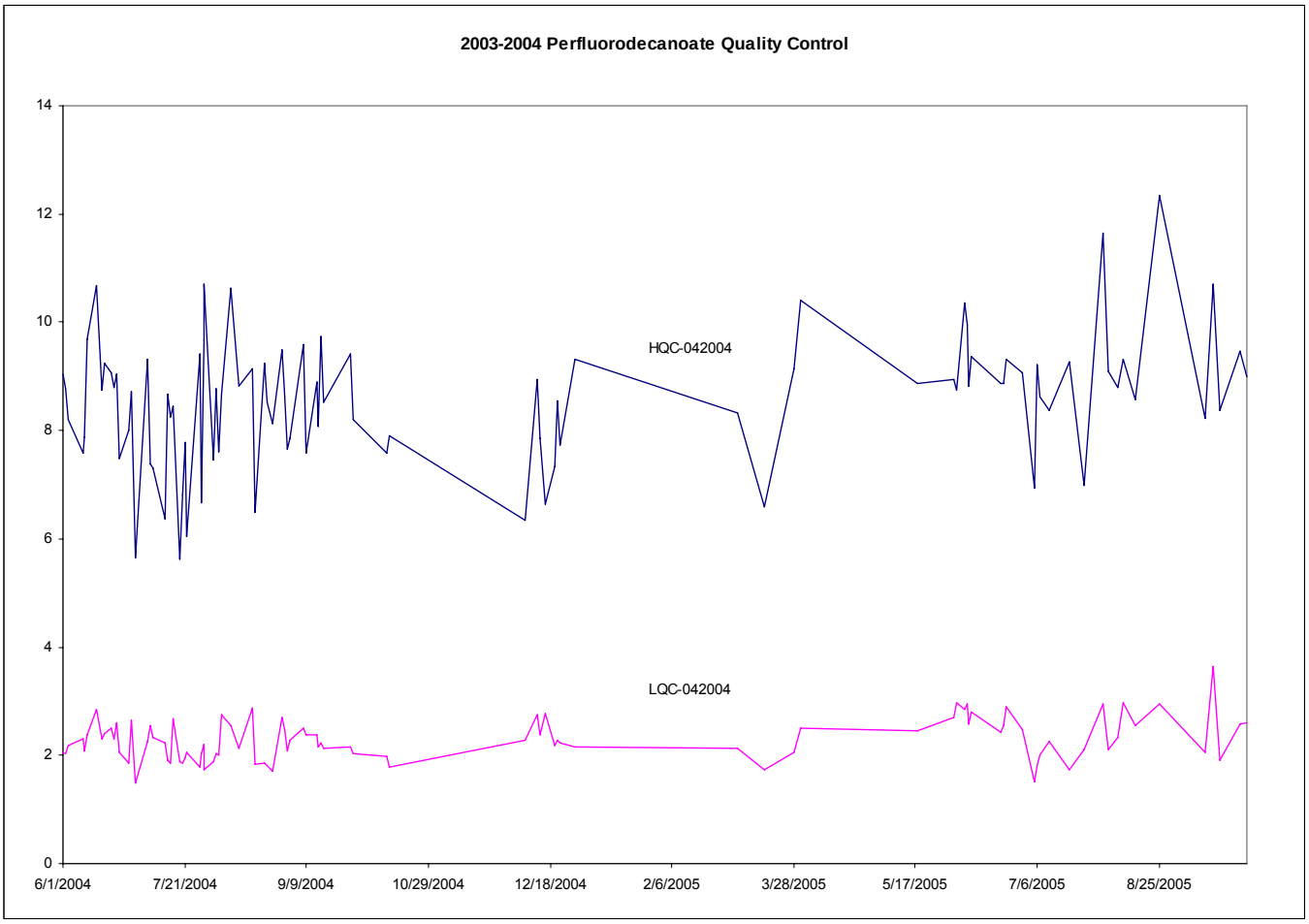
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-042004	94	6/1/2004	9/30/2005	3.97	0.74	18.6
HQC-042004	94	6/1/2004	9/30/2005	13.99	2.55	18.2



B. Perfluorodecanoate

Summary Statistics for Perfluorodecanoate by Lot

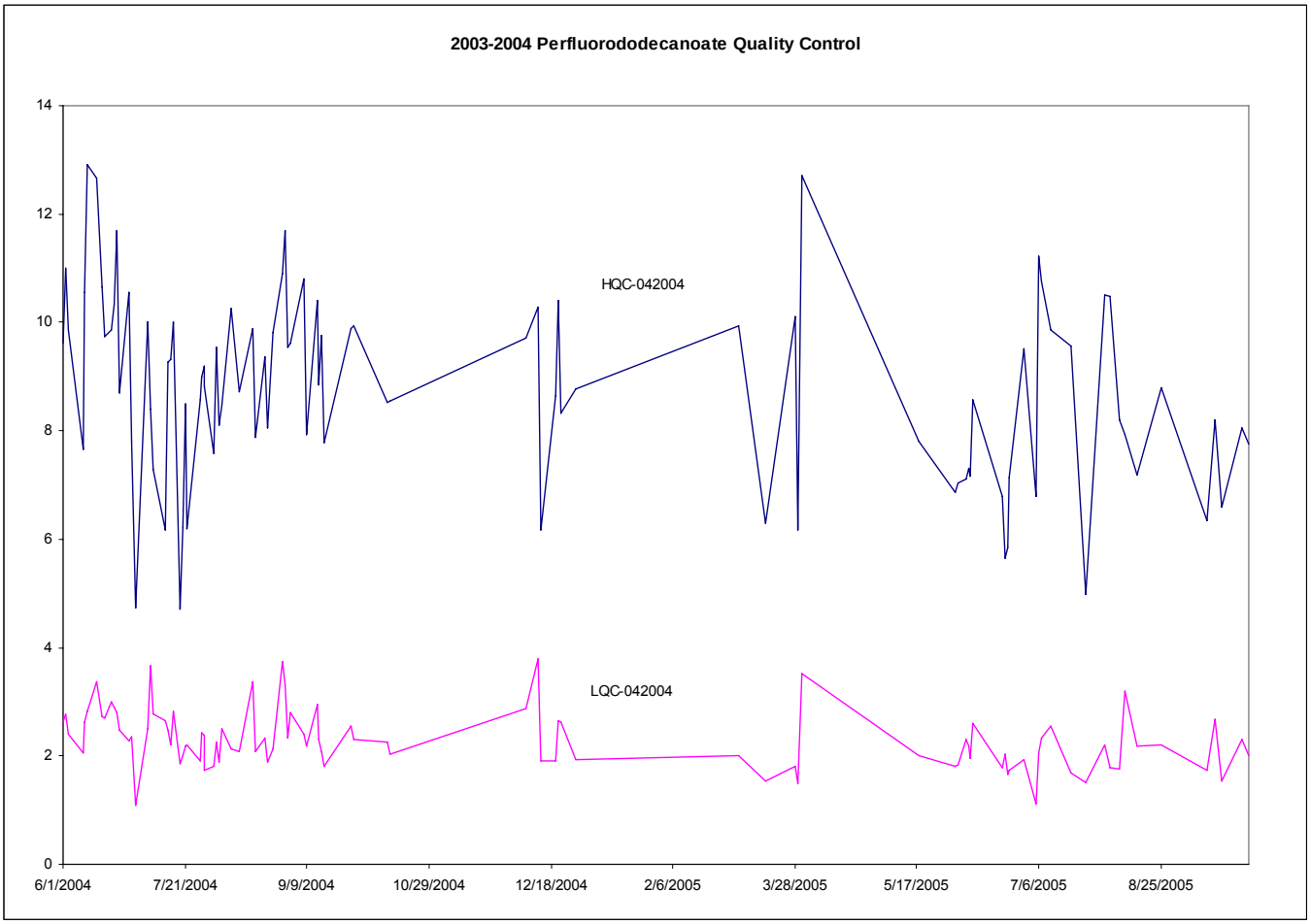
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-042004	96	6/1/2004	9/30/2005	2.28	0.39	16.9
HQC-042004	96	6/1/2004	9/30/2005	8.55	1.21	14.2



C. Perfluorododecanoate

Summary Statistics for Perfluorododecanoate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-042004	97	6/1/2004	9/30/2005	2.30	0.54	23.4
HQC-042004	97	6/1/2004	9/30/2005	8.76	1.75	20.0



D. Perfluoroheptanoate

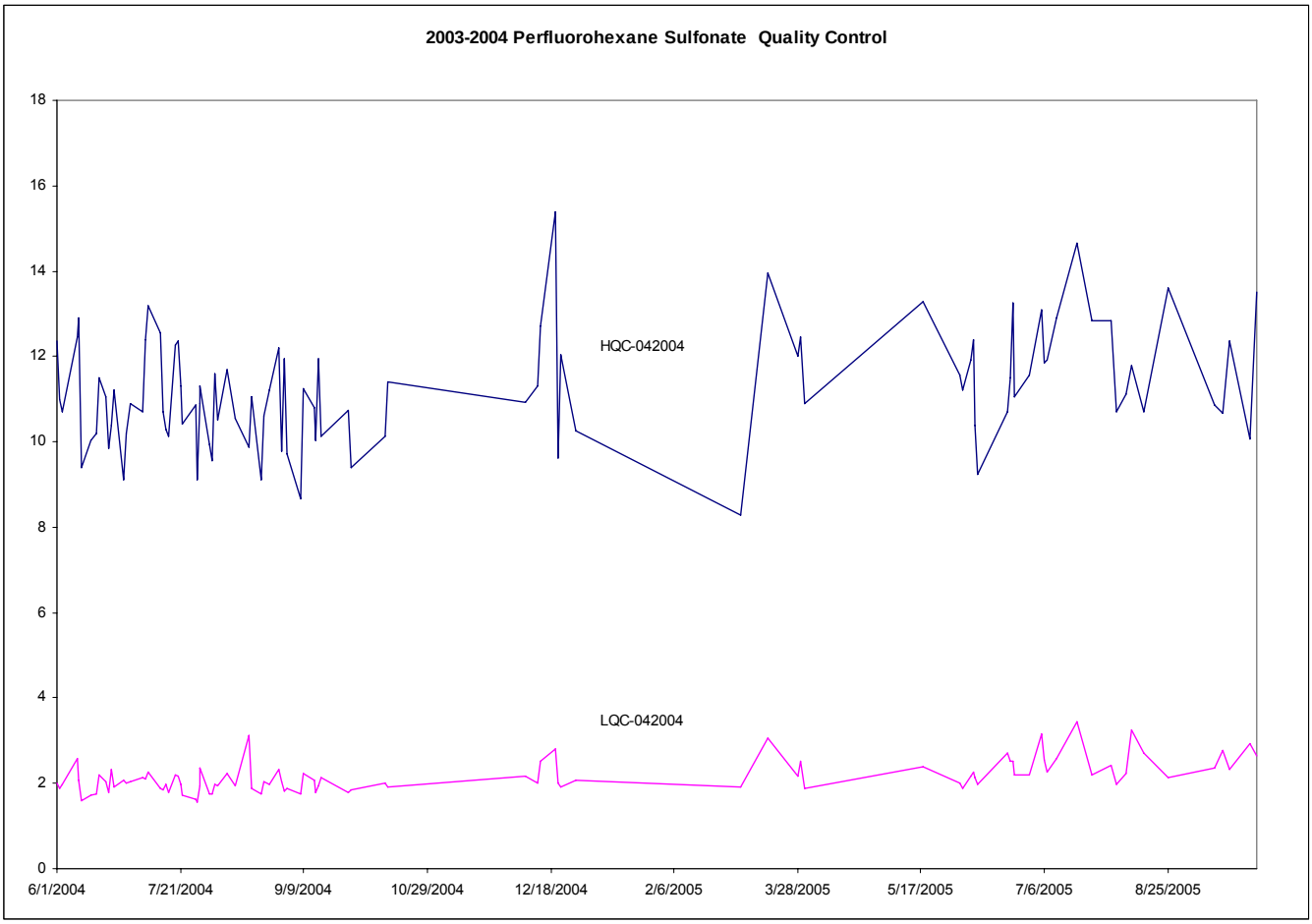
Summary Statistics for Perfluoroheptanoate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-042004	96	6/1/2004	9/27/2005	7.10	1.16	16.3
HQC-042004	96	6/1/2004	9/27/2005	15.43	2.04	13.2

E. Perfluorohexane Sulfonate

Summary Statistics for Perfluorohexane Sulfonate by Lot

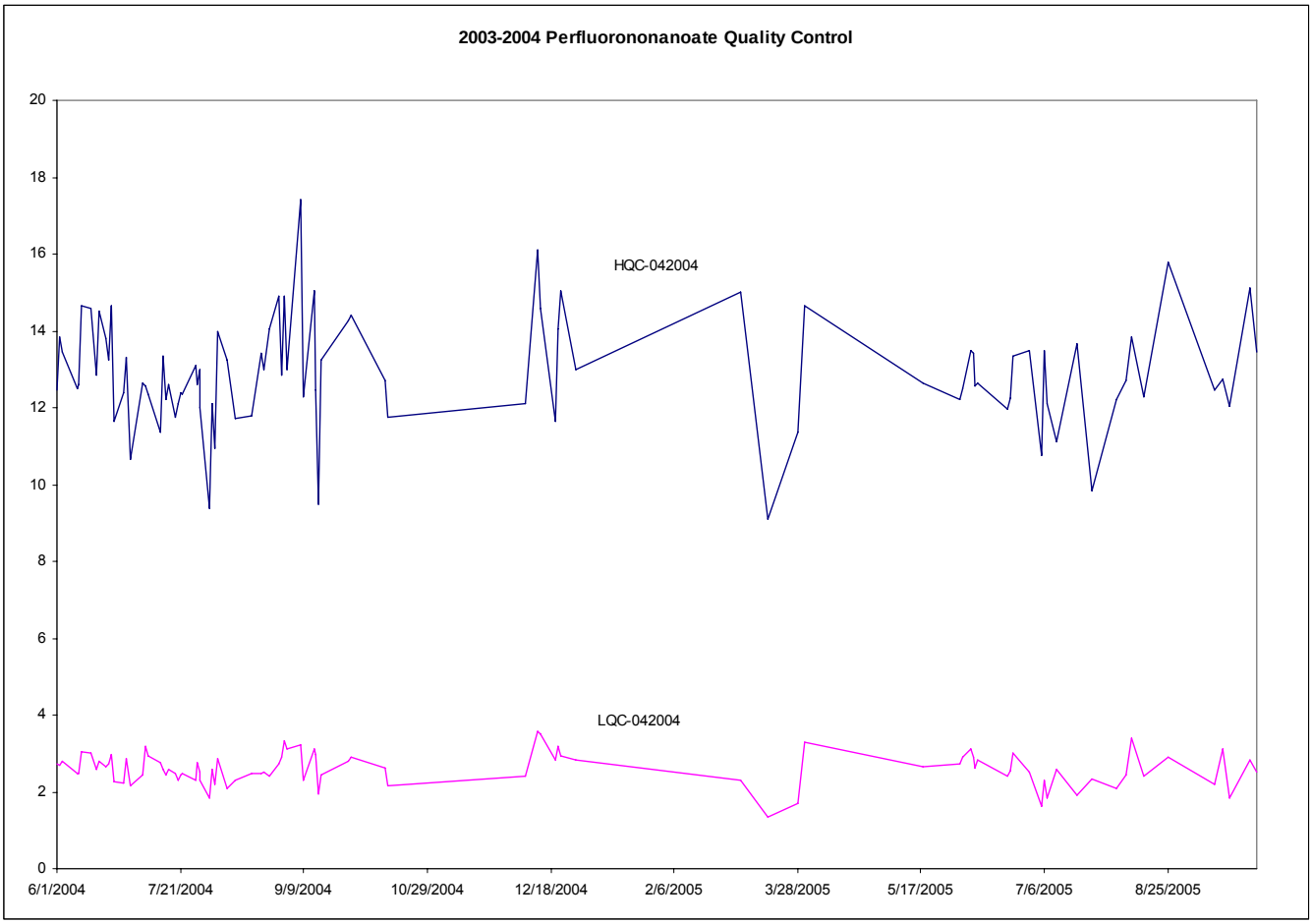
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-042004	97	6/1/2004	9/30/2005	2.16	0.38	17.5
HQC-042004	97	6/1/2004	9/30/2005	11.23	1.32	11.8



F. Perfluorononanoate

Summary Statistics for Perfluorononanoate by Lot

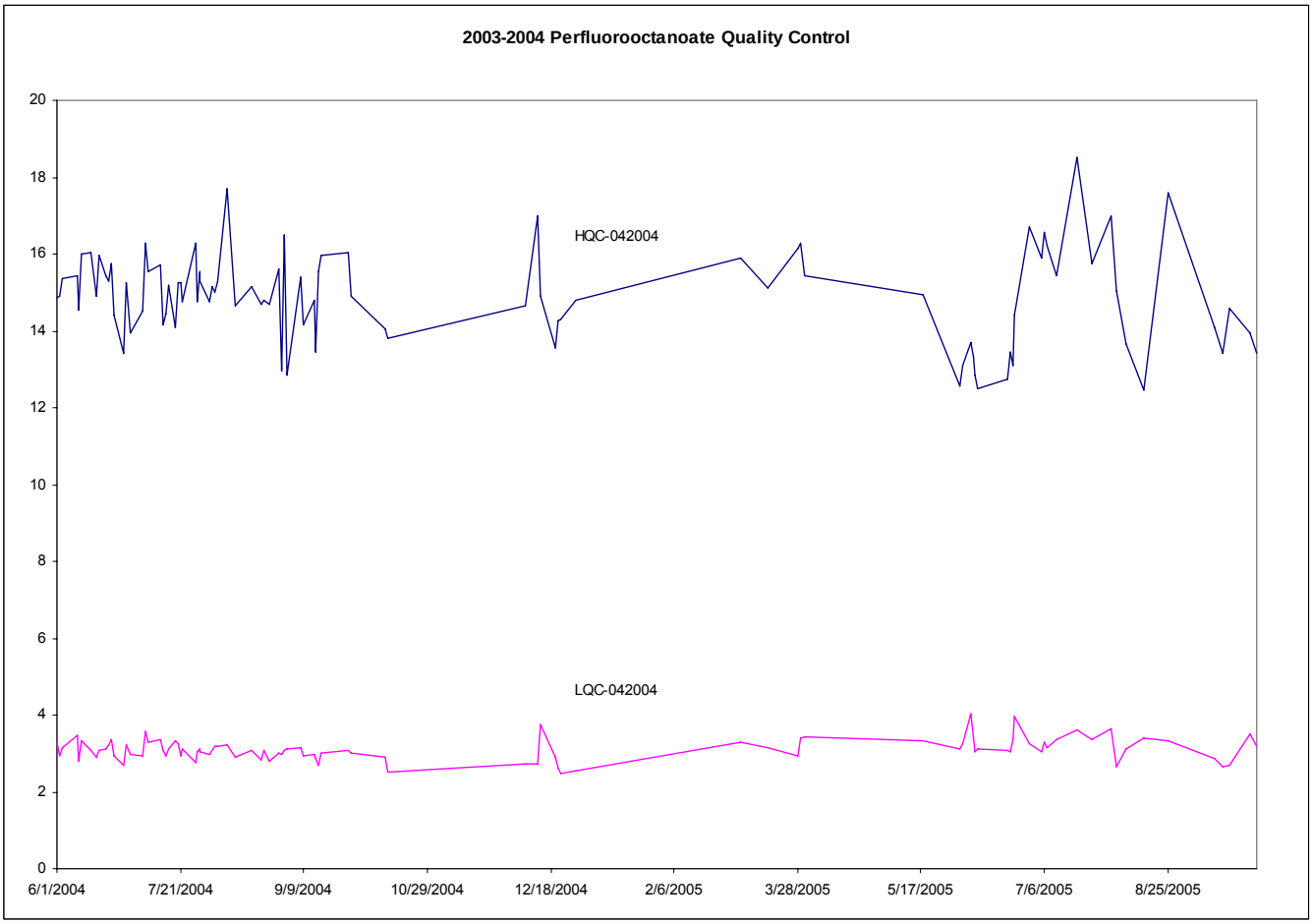
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-042004	93	6/1/2004	9/30/2005	2.60	0.42	16.1
HQC-042004	93	6/1/2004	9/30/2005	12.91	1.42	11.0



G. Perfluorooctanoate

Summary Statistics for Perfluorooctanoate by Lot

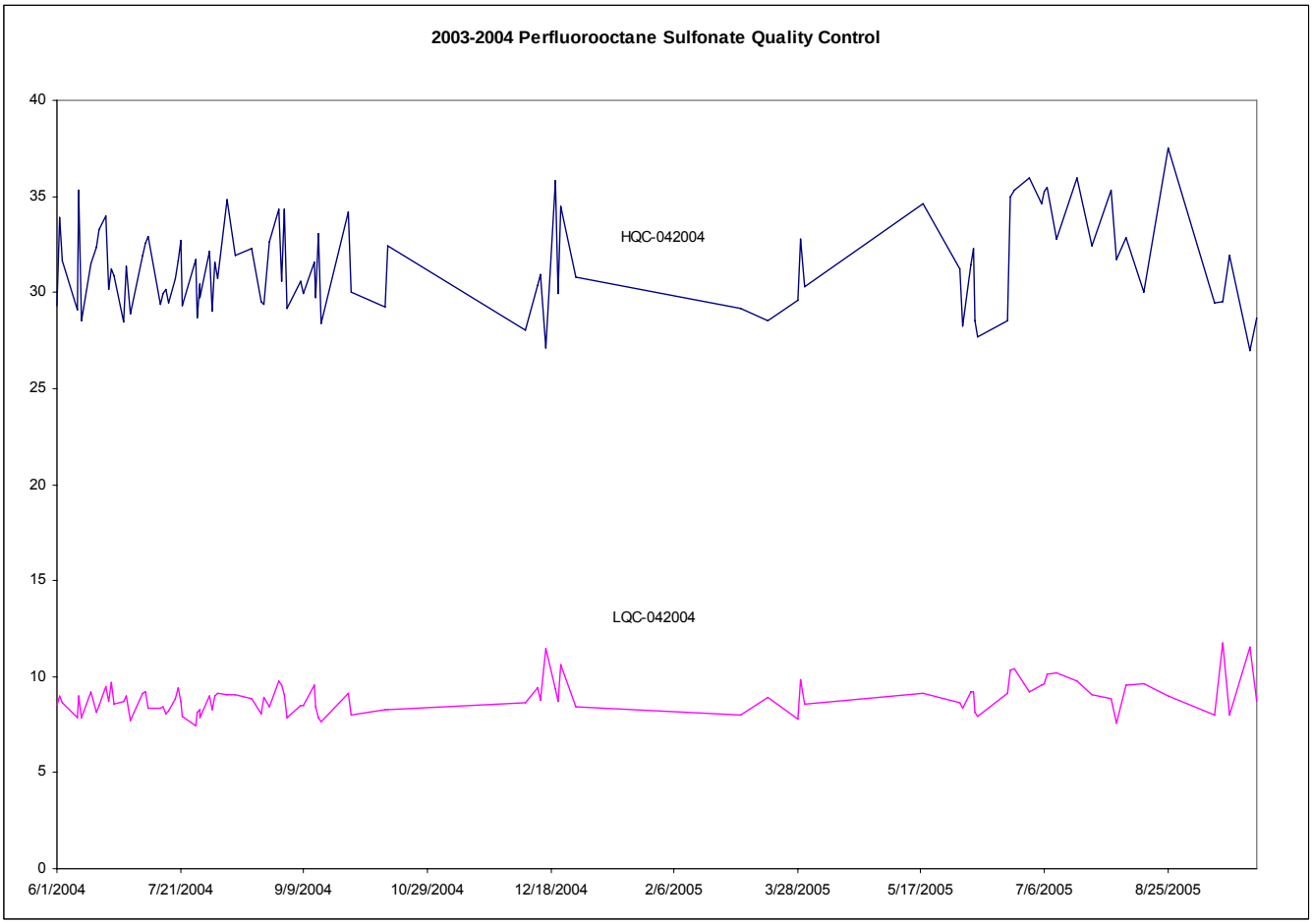
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-042004	95	6/1/2004	9/30/2005	3.10	0.29	9.2
HQC-042004	95	6/1/2004	9/30/2005	14.90	1.21	8.1



H. Perfluorooctane Sulfonate

Summary Statistics for Perfluorooctane Sulfonate by Lot

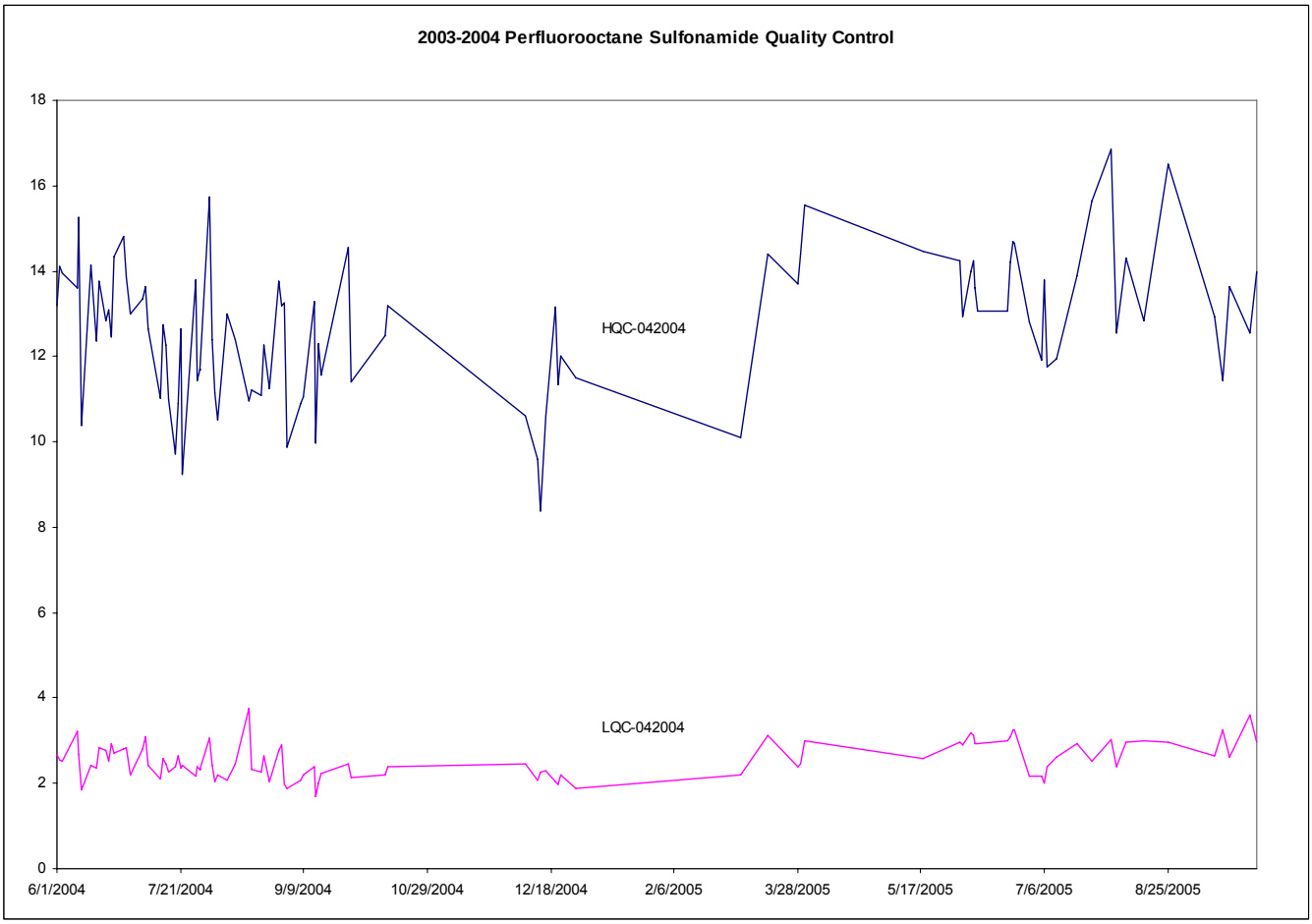
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-042004	95	6/1/2004	9/30/2005	8.87	0.84	9.5
HQC-042004	95	6/1/2004	9/30/2005	31.37	2.36	7.5



I. Perfluorooctane Sulfonamide

Summary Statistics for Perfluorooctane Sulfonamide by Lot

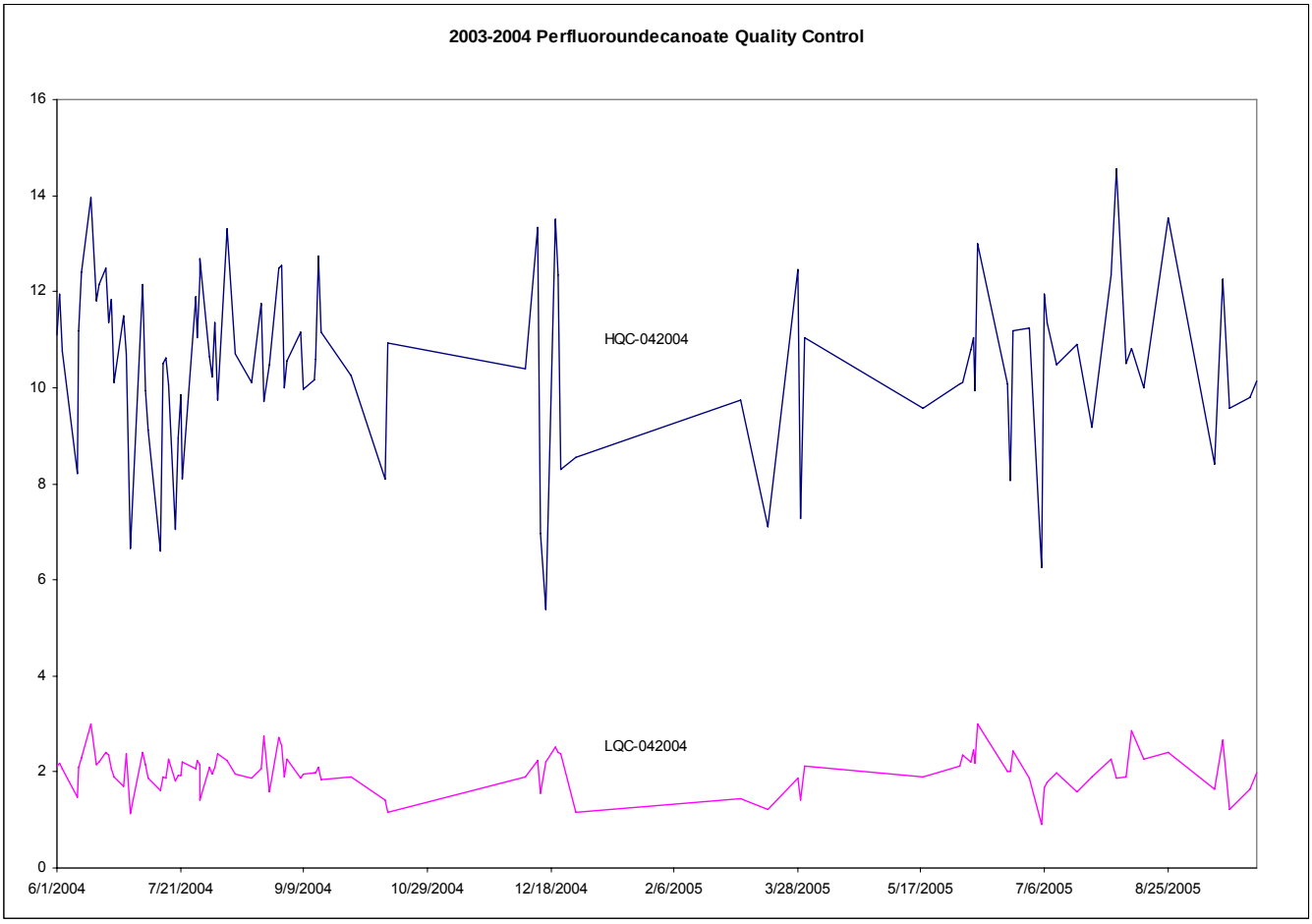
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-042004	97	6/1/2004	9/30/2005	2.54	0.41	16.3
HQC-042004	97	6/1/2004	9/30/2005	12.72	1.64	12.9



a. Perfluoroundecanoate

Summary Statistics for Perfluoroundecanoate by Lot

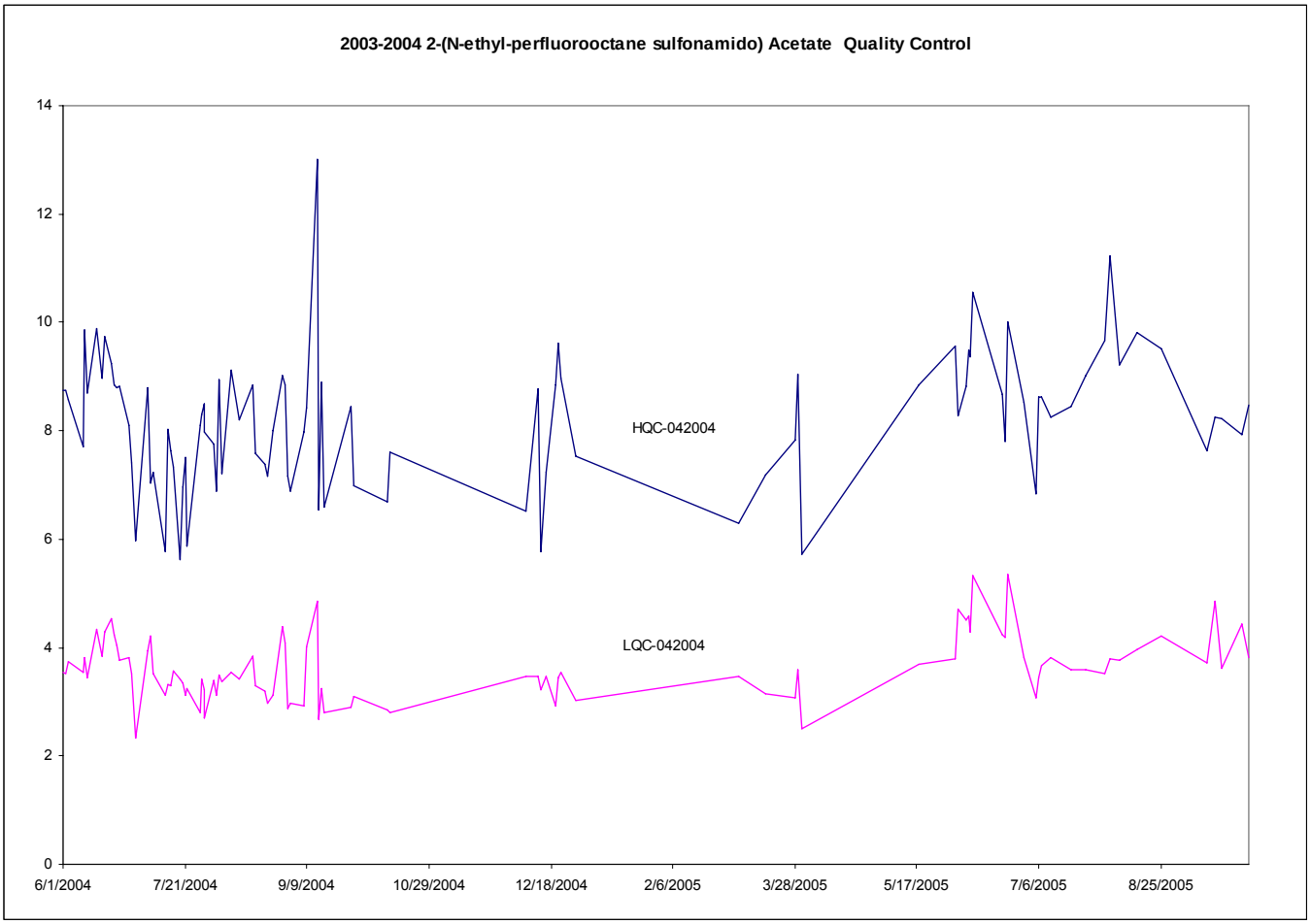
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-042004	95	6/1/2004	9/30/2005	2.02	0.4	19.9
HQC-042004	95	6/1/2004	9/30/2005	10.54	1.8	17.1



K. 2-(N-ethyl-perfluorooctane sulfonamido)

Summary Statistics for 2-(N-ethyl-perfluorooctane sulfonamido) Acetate by Lot

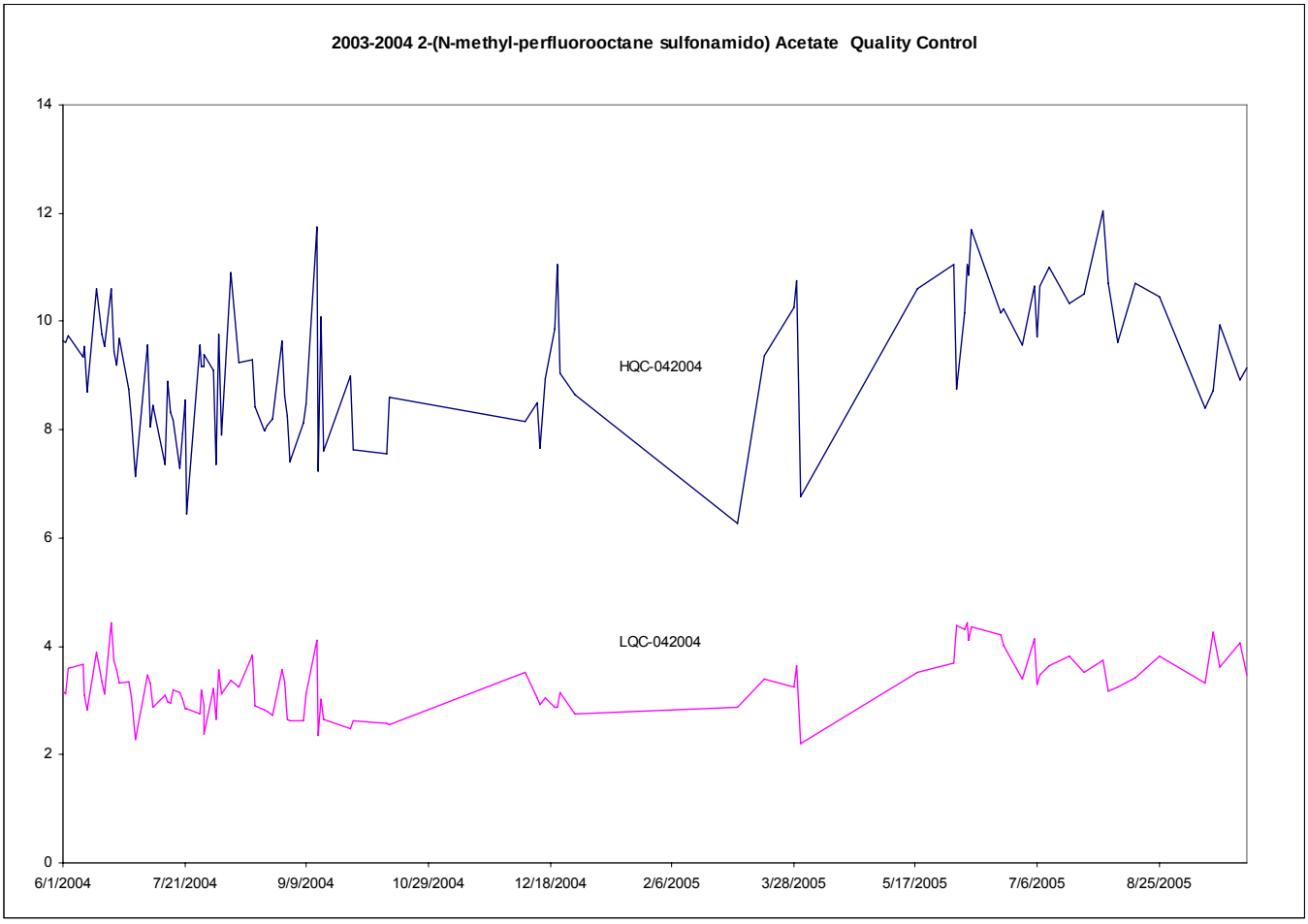
Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-042004	96	6/1/2004	9/30/2005	3.60	0.59	16.3
HQC-042004	96	6/1/2004	9/30/2005	8.21	1.24	15.1



b. 2-(N-methyl-perfluorooctane sulfonamido)

Summary Statistics for 2-(N-methyl-perfluorooctane sulfonamido) Acetate by Lot

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
LQC-042004	95	6/1/2004	9/30/2005	3.27	0.53	16.1
HQC-042004	95	6/1/2004	9/30/2005	9.19	1.24	13.5



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