3M EHS Laboratory

Method

Determination of Fluorochemicals via Protein Precipitation in Serum and Plasma by Large Volume Injection High Performance Liquid Chromatography with Tandem Mass Spectrometry

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Quality Assurance

1 Scope and Application

This method describes the extraction of fluorochemicals from serum and plasma using protein precipitation followed by separation, identification, and stable isotope internal standard quantification using high performance liquid chromatography tandem mass spectrometry (LC/MS/MS).

These procedures are applicable for perfluoroalkyl sulfonates (PFSAs), including perfluorooctane sulfonate (PFOS), and the perfluorocarboxylic acids (PFCAs), including perfluorooctanoic acid (PFOA) and perfluorooctanesulfonamide (FOSA) in whole blood, serum, and plasma. Application of this method to other per- and polyfluorinated alkyl substances (PFAS) and other biological tissues must meet the quality control acceptance criteria in this method.

Table 1. Representative Target Analytes

Acronym	Analyte	Chemical Abstract Services Registry Number (CASRN)
PFBA (C4 Acid)	Perfluorobutanoic acid	375-22-4
PFPeA (C5 Acid)	Perfluoropentanoic acid	2706-90-3
PFHxA (C6 Acid)	Perfluorohexanoic acid	307-24-4
PFHpA (C7 Acid)	Perfluoroheptanoic acid	375-85-9
PFOA (C8 Acid)	Perfluorooctanoic acid	335-67-1
PFNA (C9 Acid)	Perfluorononanoic acid	375-95-1
PFDA (C10 Acid)	Perfluorodecanoic acid	335-76-2
PFUnA (C11 Acid)	Perfluoroundecanoic acid	2058-94-8
PFDoA (C12 Acid)	Perfluorododecanoic acid	307-55-1
PFTrDA (C13 Acid)	Perfluorotridecanoic acid	72629-94-8
PFBS (C4 Sulfonate)	Perfluorobutanesulfonic acid	375-73-5
PFHS (C6 Sulfonate)	Perfluorohexanesulfonic acid	355-46-4
PFOS (C8 Sulfonate)	Perfluorooctanesulfonic acid	1763-23-1
FBSA (C4 Sulfonamide)	Perfluorobutanesulfonamide	30334-69-1
FOSA (C8 Sulfonamide)	Perfluorooctanesulfonamide	754-91-6
N-MeFOSAA	N-methylperfluoro-1-octanesulfonamidoacetic acid	2355-31-9
N-EtFOSAA	N-ethylperfluoro-1-octanesulfonamidoacetic acid	NA

The Minimum Reporting Level (MRL) is a batch specific Limit of Quantitation (LOQ) that meets defined criteria based on method blanks and curve points.

Method Flexibility- This is a published validated performance-based method[1] that relies on sample specific surrogate recovery standard (SRS) performance and may be generally applied to the determination of

[1] The method is supported by validation with internal standard calibration for C4-C13 PFCAs, C4, C6, and C8 PFSAs, and C8 perfluoroalkane sulfonamide in laboratory control samples under 3M method validation E08-0706 and Cross Validation of Method ETS-8-230.1 For Single Injection Analysis of All Analytes under 3M method validation E13-0136. Also see Harrington et.al.

perfluorinated compounds in serum, plasma, whole blood or other biological tissues when analytical batch quality control (QC) criteria are met.[2] Each set of samples are prepared in an analytical batch in conjunction with calibration standards, LCSs, QCs, blanks, and continuing calibration check standards analyzed on the same instrument during a time period that begins and ends with the analysis of the appropriate continuing calibration check standards. The laboratory is permitted to modify the LC column, mobile phase composition, LC conditions, and MS/MS conditions. Method modifications should be considered to improve method performance or to meet data quality objectives for the study. In all cases where method modifications are implemented, the batch analytical QCs (section 9) must be completed and pass QC acceptance criteria (section 13) if the data from the analytical batch are to be reported.

2 Method Summary

This method describes the procedures for the extraction and quantification of fluorochemicals. Analytes of interest are extracted from serum, blood and/or plasma by protein precipitation via a robotic liquid handling system or calibrated micropipettes.

Quantitation is by stable isotope internal standard calibration in control plasma or serum or solvent. All perfluorinated compounds (PFCs) target analyte concentrations of perfluorosulfonic acids (PFSAs) and perfluorocarboxylic acids (PFCAs) are reported as anions and corrected for their salt or free acid forms.

This is a SRS performance-based method. Method uncertainty for each SRS is determined for each analytical batch using a constant SRS concentration spiked in each sample. Quantification is accomplished with stable isotope internal standard calibration by high performance liquid chromatography tandem mass spectrometry.

3 Definitions

3.1 Protein Precipitation

A sample preparation technique that employs organic solvent for denaturation of proteins from biological matrices followed by removal of solid denatured protein from the extract by centrifugation and analysis of the clarified supernatant.

3.2 Analysis Batch

A set of study samples that are prepared with calibration standards, laboratory control samples, quality control samples, and procedural blanks, and analyzed on the same instrument during a time period that begins and ends with the analysis of the appropriate continuing calibration check standards.

3.3 External Solvent Calibration Standard

A solution prepared by spiking a known volume of the Working Standard (WS), including analytes, IS and SRSs, into a predetermined amount of acetonitrile and analyzed according to this method. External solvent calibration standards are used to calibrate the instrument response with respect to analyte concentration in acetontrile. This standard is used to achieve a lower limit of quantitation.

3.4 Matrix-Matched Calibration Standard

A solution prepared by spiking a known volume of the Working Standard (WS), including analytes, IS and SRSs, into a predetermined amount of serum or plasma and analyzed according to this method. Matrix-matched calibration standards are used to calibrate the instrument response with respect to analyte concentration in matrix samples.

[2] Guidance for establishing method QC Criteria based on a.) FDA May 2001, "Guidance for Industry, Bioanalytical Method Validation", b.) EPA Method 537, and c.) European Commission: Guidance for Generating and Reporting Methods of Analysis in Support of Pre-registration Data Requirements for Annex II (Part A, section 4) and Annex III (Part A,section 5) of Directive 91/414, SANCO/3029/99 rev. 4 (11/07/00).

3.5 Surrogate-Matched Calibration Standard

A solution prepared by spiking a known volume of the Working Standard (WS), including analytes, IS and SRSs, into a predetermined amount of laboratory reagent water and analyzed according to this method. Surrogate-matched calibration standards are used to calibrate the instrument response with respect to analyte concentration in samples containing water.

3.6 Surrogate Recovery Standard (SRS)

An isotopically labeled standard, not used as an internal standard, that is added to each sample and appropriate QC samples as a means to evaluate the method performance for a chemical class of compounds (e.g., PFSAs, PFCAs).

3.7 Internal Standard (IS)

A compound added to each study sample, calibration standard, laboratory control samples, and procedural blanks at a consistent level (typically around 1 ng/mL). The internal standard(s) are stable isotope labeled versions of the target analytes. Surrogate ISs are applied when stable isotope ISs of target analytes are unavailable. A surrogate IS is not necessarily a stable isotope labeled version of the target analyte, but is treated as an internal standard for quantitation.

3.8 Surrogate Internal Standard

An IS that is not necessarily a stable isotopically labeled target analyte, but is treated as an internal standard for quantitation. Surrogate ISs are used when isotopically labeled counterparts of the target analyte are not commercially or readily available.

3.9 Stock Standard Solution (SSS)

A concentrated solution of a single-analyte prepared in the laboratory with an assayed reference compound.

3.10 Working Standard (WS)

A solution of several analytes prepared in the laboratory from SSSs and diluted as needed to prepare calibration standards and other required analyte solutions.

3.11 Quality Control (QC)

A quality control is a term used for all samples used to ensure quality requirements are met. These include and are not limited to laboratory control samples, system suitability samples, laboratory matrix spikes and continuing calibration verification values.

3.12 Laboratory Duplicate Sample (LDS, or Lab Dup)

A laboratory duplicate sample is a separate aliquot of a sample taken in the analytical laboratory that is analyzed separately with identical procedures. Analysis of LDSs compared to that of the first aliquot give a measure of the precision associated with laboratory procedures, but not with sample collection, preservation, or storage procedures.

3.13 Laboratory Control Sample (LCS)

An aliquot of control matrix to which known quantities of the target analytes, ISs and SRSs (when applicable) are added in the laboratory at the time when samples are aliquotted. At least three levels (two levels for SRSs) in triplicate are included, one generally at the low end of the calibration curve and one near the mid range and the upper end of the curve. The LCSs are analyzed exactly like a laboratory sample as a measure of the method accuracy, precision, and uncertainty. LCSs should be prepared each day with samples.

3.14 Limit of Quantitation (LOQ)

The lower limit of quantitation (LLOQ) for an analytical batch is the lowest concentration that can be reliably quantitated within the specified limits of precision and accuracy. The LLOQ is generally selected as the lowest non-zero standard in the calibration curve that meets method acceptance criteria. The LLOQ for each target analyte is established for each analysis batch as the lowest calibration standard with area ratios at least twice that of the average area ratios of the procedural blanks. The upper limit of quantitation (ULOQ) for an analytical batch is the highest concentration that can be reliably quantitated within the specified limits of precision and accuracy. The highest standard in the calibration curve that meets method acceptance criteria is defined as the ULOQ.

3.15 Procedural Blank

An aliquot of control matrix or solvent that is treated exactly like a laboratory sample including exposure to all glassware, equipment, solvents, and reagents that are used with other laboratory samples. The procedural blank is used to determine if test substances or other interferences are present in the laboratory environment, the reagents, or the apparatus. Common procedural blanks include: human matrix match blanks, surrogate bovine matrix match blanks, solvent extraction blanks.

3.15 Solvent Extraction Blank

A blank prepared the same way as samples, but with water instead of human or bovine matrix. Used to determine procedural contamination. If contamination occurs for a particular analyte during solvent calibration, must be used instead of calibration solvent blank for that particular analyte to determine the LLOQ.

3.16 Calibration Solvent Blank

A solvent blank prepared at the same time as the solvent calibration standards containing IS and SRSs. Used to determine LLOQ in analytical batches using solvent calibration unless contamination of an analyte occurs.

4 Warnings and Cautions

4.1 Health and Safety Warnings

Always wear appropriate personal protective equipment such as protective gloves, eye protection, and appropriate clothing when working with biological matrices, solvents, chemicals and instrumentation. For potential hazard information refer to material safety data sheets, packing material, the 3M EHS Laboratory's Chemical Hazard Review, the 3M Guide to Laboratory Practices or other information as appropriate.

4.2 Cautions

The analyst must be familiar with the laboratory equipment and potential hazards including, but not limited to, the use of biological materials (refer to ETS-2-005), solvents, high temperatures, pressurized gas and solvent lines, high voltage, and vacuum systems. Refer to the appropriate equipment procedure or operator manual for additional information and cautions.

5 Interference

To minimize potential interference, polytetrafluoroethylene (PTFE, e.g. Teflon®) should not be used for sample collection, preparation, and storage or any part of instrumentation that comes in contact with the sample or extract.

Co-extracted matrix components may enhance or suppress the measured analyte signal in the mass spectrometer. Additionally, other matrix components may produce the same multiple reaction monitoring (MRM) mass spectrometer transitions that may result in false positives. Manipulation of the instrument parameters (LC gradient conditions) can minimize the co-elution of matrix components with common MRM transitions, but the precision and accuracy of spike results must be evaluated for possible effects of co-extracted matrix interferences that may be present.

Some components present in blood may mimic similar MS/MS transitions used for certain target analytes, and therefore should be carefully assessed during review of the data for inconsistencies such as severely altered MRM ratios when multiple MRMs are employed for an analyte.

6 Instrumentation, Supplies, and Materials

The following instrumentation, supplies, and materials are used while performing this method. Equivalent instrumentation, supplies and materials may be used in place of those listed.

6.1 Instrumentation

Balance, analytical (display at least 0.0001 g)

Packard MultiPROBE II Plus HT EX robotic liquid handling system

CAVRO 500 µL syringes

Packard Smart Actuator Six Way Valve Control

Microcentrifuge Adapter (1.5 mL) Labware, part number 7601651

Flush wash rack with 120 mL reagent trough, part number 5079598

4 place 60 mL reagent trough rack

Tip chute

Agilent HPLC 1200 system:

Pump: Quaternary Pump, Agilent®, Model G1311A; or Binary Pump, Agilent®, Model 1312

Solvent Degasser, Agilent®, Model G1322A

Autosampler, Agilent®, Model G1313A, or Thermostated Autosampler, Agilent®, Model G1329A

Autosampler thermostat (optional), Agilent®, Model G1330A

Column Compartment (Temperature Controlled), Agilent®, Model G1316A

Diode Array Detector, (DAD) Agilent®, Model # G1315A

Controller, Hand Held, Agilent®, Model # G1323A

Applied Biosystems MDS SCIEX API 5000 Biomolecular Mass Analyzer

SCIEX Turbo Ion Spray Liquid Introduction Interface

Centrifuge

Other instrumentation as needed, document as appropriate.

Note: Other brands/models of LC/MS/MS instruments may be used as long as the method criteria are met.

6.2 Supplies and Materials

Hamilton gas tight syringes (capable of dispensing at least 10 µL)

Class A volumetric flasks

Eppendorf variable volume pipettor with disposable tips

Thermo Fischer Scientific Betasil C_{18}, 2 x 100 mm, 5 µm particle size

Thermo Fischer Scientific Prism™ RP 2 x 50 mm, 5 µm particle size

Waters Corporation, Oasis HLB Online Column, 3 x 20 mm 25 um particle size

Thermo Fischer Scientific Betasil C_{18}, 4.1 x 100 mm, 5 µm particle size

Clear HPLC vials

Packard MBP Disposable Conductive Tips (1 mL), part number 6000655

1.5 mL microcentrifuge tubes, MaxyClear Microtubes, Axygen Scientific, part number 10011-722

Other supplies and materials as needed, document as appropriate

7 Reagents and Standards

The procedures for preparing the solutions described below are provided for demonstrative purposes only. If preparing different amounts or concentrations than those described, adjust accordingly.

7.1 Reagents

Acetonitrile, HPLC grade or equivalent

Methanol, HPLC grade or equivalent

Purified water provided by a Milli-Q Element system or equivalent vendor's system. Water purified with a reverse-osmosis system may also be used.

Ammonium acetate, Mallinckrodt 98% pure or equivalent

Acetic Acid, glacial, or equivalent

Phosphoric acid, 85% or equivalent

Various biological matrices from supplier or study sponsor for use as control matrix

Additional reagents as needed

7.2 Calibration Standards

Due to the likely possibility of ester formation between carboxylic acids and alcohols (ie. PFCAs), when preparing stock PFCA solutions in methanol, it is important that the methanol be treated with an excess of base (ie. Sodium hydroxide). In this method, when preparing the primary stock solution of 1 ppm PFCA in methanol, the methanol needs to be treated with sodium hydroxide to an approximate concentration of 10 μM. If stock solutions are created at a higher concentration, then the concentration of NaOH should be scaled up appropriately. Upon making dilutions of the primary stock solutions, it is not necessary to use caustic methanol. Alternatively, stock standards of PFCA can be prepared in acetonitrile to avoid ester formation. Standards purchased from Wellington Laboratories now come prepared in a base-treated methanol solution and do not require further treatment. Wellington Laboratories standards purchased before 2006 were not treated in this manner. The analyst should review the certificate of analysis before proceeding to confirm the presence of base in the solution ampule. If available, certified linear isomer calibration standards of all target analytes are applied in the method. Alternatively, certified technical grade calibration standards of mixed linear and branched isomers may be applied if they include both linear and branched isomer and combined linear and branched isomer LCSs are included in the analytical batch.

Calibration curves can be constructed from either an external solvent curve, an extracted matrix-matched or surrogate matrix-matched calibration curve. For external acetonitrile solvent curves the curve must be prepared in acetonitrile with a lower limit of quantitation of 0.00500 ng/mL solvent to then be diluted 50:50 in reagent grade water for a final lower limit of quantitation of 0.00250 ng/mL. The external solvent curve must be prepared using both IS and SRSs.

The following is an example for preparing spiking solutions for an extracted matrix-matched or surrogate matrix-matched calibration curve with a lower limit of quantitation of 0.0250 ng/mL in serum following the extraction procedure described in section 11.

NOTE: For stable isotope internal standard quantitation, extracted matrix-matched calibration, extracted surrogate matrix-matched calibration or unextracted acetonitrile solvent calibration may be used. When performing extractions with different volumes, diluting for analysis using one injection or conducting analyses

with a different required lower limit of quantitation (LLOQ), adjust accordingly.

Prepare individual stock solutions of 100 µg/mL (stock solution A) by weighing out 10 mg of analytical standard (corrected for purity) and dilute to 100 mL with acetonitrile or other appropriate solvent in a 100 mL volumetric flask. Each stock solution may be stored in a refrigerator at 2 ºC to 6 ºC and has an expiration date of a maximum period of six months from the date of preparation (unless determined otherwise).

Prepare the following solutions in acetonitrile or other appropriate solvent.

Stock Solution I.D	Concentration (µg/mL)	Volume taken from Stock (µL)	Final Volume (mL)	Final Solution Concentration (ng/mL)	New Solution I.D.
A	100	500	25.0	2000	B
B	2.00	2500	25.0	200	C

Next, prepare the following solutions in acetonitrile or other appropriate solvent. At least six calibration standards should be prepared (nine are recommended). Standards may be prepared in alternative disposable glassware (e.g. 40 mL I-chem vials) to avoid possible cross contamination. The final matrix- matched calibration curve must consist of at least six calibration points after analysis. If the analyst suspects that some curve points may be deactivated, more calibration standards should be prepared.

Stock Solution I.D	Concentration (ng/mL)	Volume taken from Stock (µL)	Final Volume (mL)	Final Solution Concentration (ng/mL)	New Solution I.D.
B	2000	100	25.0	8.00	D
B	2000	50.0	25.0	4.00	E
C	200	250	25.0	2.00	F
C	200	100	25.0	0.800	G
C	200	50.0	25.0	0.400	H
C	200	25.0	25.0	0.200	I

Following the procedure as described in section 11, the final concentration in extract would be:

$$\frac{\text{ng}}{\text{mL PPT extract}} = \text{spike concentration}\,\frac{\text{ng}}{\text{mL}} \times \frac{25\,\text{uL spike solution}}{1000\,\text{uL total extract volume}} \times \frac{1000\,\text{uL extract}}{1060\,\text{uL total extract (extract} + 60\,\text{uL 5\% } H_3PO_4)}$$

8 Sample Handling

No sample processing is needed for serum or plasma samples. However, frozen samples must be allowed to completely thaw, un-aided, at room temperature. Samples stored refrigerated should also be allowed to equilibrate to room temperature. All samples must be thoroughly mixed before being sampled for extraction.

9 Quality Control

Refer to section 13 for acceptance criteria. Each analytical batch (i.e. each day samples are extracted) must include the following:

9.1 Procedural Blanks

9.1.1 Solvent Blanks

Solvent blanks made using acetonitrile and acidified in the same fashion as the sample extracts should be run after calibration standards and CCVs.

If a sample or calibration standard is known or suspected to contain a high concentration of analyte(s), it is recommended to run at least one solvent blank after this sample to evaluate instrument carry-over.

9.1.2 Calibration Solvent Blanks

At least six solvent blanks, acetonitrile with IS and SRSs and acidified in the same manner as the sample extracts, should also be analyzed throughout the analytical batch. These blanks are to be used to determine the LLOQ when using solvent calibration. The mean area ratio for each analyte in the calibration solvent blanks must be less than 50% of the area ratio counts of the LOQ standard.

9.1.3 Method Blanks

At least two, preferably four, solvent extraction blanks, matrix-matched and matrix-matched with SRS blanks should be extracted with each batch of each matrix used (i.e. human serum, bovine serum, solvent blank). Solvent extraction blanks are accomplished by extracting an equivalent volume of laboratory reagent water instead of the sample matrix. For example, if 0.500 g of serum/plasma is extracted then 0.500 mL (0.500 g) of water should be used for the method blank. At least half of the method blanks should be spiked with the IS/SRSs and the other half without. Blanks must be run throughout the analytical batch. For solvent calibration if contamination of spiking solutions, bovine, or human serum/plasma occurs these blanks must be used to determine the LLOQ for the contaminated analyte, otherwise calibration solvent blanks will be used to determine LLOQ. If using matrix-matched calibration a minimum of six bovine serum blanks (3 with IS and SRSs, 3 without) must be prepared, run throughout the analytical batch and analyzed to determine the LLOQ based on two times the average area ratio.

The mean area ratio for each analyte in the procedural blanks must be less than 50% of the area ratio counts of the LLOQ standard. The standard deviation of the area ratio of these procedural blanks should be calculated. A specific %RSD acceptance criteria is not specified but is assessed on an analytical batch basis. If the mean area ratio of the procedural blanks exceeds 50% of the LOQ standard, then the LOQ must be raised to the first standard level in the curve that meets criteria. Method blanks may be eliminated if technical justification can be provided (e.g. the method blank was analyzed after an unexpectedly high level sample). If procedural blanks are removed from the LOQ determination, document in the raw data and report as appropriate.

9.2 Laboratory Control Spike (LCS) Samples

Prepare triplicate spikes using the same control serum/plasma that was used for the extracted matrix-matched calibration standards. For solvent calibration use a control serum/plasma for LCSs. At least 3 levels (levels at approximately 3- to 10-times lower limit of quantitation (LLOQ), mid range, and 65% to 80% of the upper limit of quantitation (ULOQ)) should be prepared. The total number of LCS samples should be at least 5% of the total number of samples extracted with each extraction batch or at least 9 total; whichever is greater. Additional LCS samples near the LLOQ may be added if multiple analytes are extracted and significantly different LLOQs are anticipated. Additional LCS samples may be prepared in another similar matrix (e.g. actual matrix of samples if another matrix is used for calibration curve, bovine or human plasma/serum) at levels determined for each project/study.

At least one level of triplicate LCS samples should be prepared using technical grade PFOS/PFOA which contains a mix of linear and branched isomers. These LCSs will be used to demonstrate quantitative equivalency (or quantitative bias) of the isomeric mix when using a predominantly linear standard for calibration.

9.3 Matrix Spike Samples

9.3.1 Surrogate Recovery Standards (SRSs)

Isotopically labeled SRSs should be spiked into test samples, test sample replicates, procedural blanks, and LCS samples. Calibration curves of the SRSs shall be prepared (i.e. the SRSs should be included in the calibration curve). The SRS is spiked at a level approximately 10-20 x the LLOQs into the samples prior to extraction. $^{13}C_4$-Perfluorooctane sulfonate [$^{13}CF_3(^{13}CF_2)_3(CF_2)_4SO_3^-$] or equivalent stable isotope labeled PFOS should be used for assessment of method quantitation of all PFSAs, FOSA, EtFOSAA and MeFOSAA. $^{13}C_4$-Perfluorooctanoic acid [$^{13}CF_3(^{13}CF_2)_3(CF_2)_3COOH$] or equivalent stable isotope labeled PFOA should be used for assessment of method quantitation of C9-PFCAs and smaller, and $^{13}C_2$-Perfluoroundecanoic acid used for C10-PFCAs and larger. The SRSs are used to demonstrate data accuracy of 100% $\pm$ 30% for individual samples. In lieu of target analyte specific laboratory matrix spikes, SRSs are used to establish sample data accuracy of PFCA, PFSA, FBSA, FOSA, MeFOSAA and EtFOSAA target analytes.

Compound Name	Synonym or Acronym	Formula
$^{13}C_4$-Perfluorooctanoic acid	[$^{13}C_4$]PFOA	$^{13}CF_3(^{13}CF_2)_3(CF_2)_3COOH$
$^{13}C_4$-Sodium Perfluorooctane sulfonate	[$^{13}C_4$]PFOS	$^{13}CF_3(^{13}CF_2)_3(CF_2)_4SO_3^-Na^+$
$^{13}C_2$-Perfluoroundecanoic acid	[1,2-$^{13}C_2$]PFUnA	$CF_3(CF_2)_7(^{13}CF_2)^{13}CF_2COOH$

9.3.2 Target Analyte Laboratory Matrix spikes (Optional QCs)

When sample volume permits, at least one sample may be separately fortified at a known concentration and carried through the procedure to verify sample-specific analyte recovery. The fortification level will be determined on an individual basis depending on the amount of analyte that is suspected in the sample. The endogenous level of each analyte in that specific sample will be subtracted from the determined concentration of the fortified sample prior to calculation of recovery. If the endogenous sample levels are unknown or expected to vary over a wide range, it is suggested that multiple LMSs be prepared at different levels so the recovery of at least one sample matrix spike can be accurately quantified.

9.4 Sample Duplicate

At least one sample may be prepared in duplicate to evaluate sample quantitative precision.

9.5 Internal Standard

If available, stable isotope ISs (isotopically labeled target analytes) of each target analyte should be used. All calibration standards, QC samples, samples and half of the procedural blanks should be spiked at the same concentration (typically at one ng/mL matrix).

The following table lists recommended stable isotope ISs for several PFSA and PFCA target compounds. A commercially available mix of isotopically labeled target analytes in a methanolic solution containing [1,2,3,4-$^{13}C_4$]PFBA, [1,2-$^{13}C_2$]PFHxA, [$^{13}C_8$]PFOA, [1,2,3,4,5-$^{13}C_5$]PFNA, [1,2-$^{13}C_2$]PFDA, [1,2-$^{13}C_2$]PFUnA, [1,2-$^{13}C_2$]PFDoA, [$^{18}O_2$]PFHS, [1,2,3,4,5-$^{13}C_5$]PFPeA, [1,2,3,4]-$^{13}C_2$]PFHpA, [$^{13}C_8$]PFOS (Wellington Laboratories, Guelph, ON) in combination with added [$^{18}O_2$]PFBS can be used to prepare a stock IS solution. Alternative sources of certified stable isotope labeled target analytes are applicable. Alternatively, individual stable isotope ISs can be used to prepare a stock IS mixture. The table below lists the recommended stable ISs applied in the method. Other stable isotope ISs of target analytes not listed in the table may also be used. The same internal standard should be used for a given analyte throughout the entire project/study. Note: some of the compounds listed below are appropriate to use as surrogate ISs when a stable isotope IS of a target analyte is not available. Generally, isotopically labeled PFCAs are used for PFCAs, and isotopically labeled PFSAs are used for PFSAs, FOSA, MeFOSAA and EtFOSAA.

Compound Name	Synonym or Acronym	Formula	Analytical Purpose	Reference Standard Source
$^{13}C_4$-Perfluorobutanoic acid	[1,2,3,4-$^{13}C_4$]PFBA	$^{13}CF_3(^{13}CF_2)_2{}^{13}COOH$	IS for PFBA	Wellington Labs
$^{13}C_2$-Perfluorohexanoic acid	[1,2 -$^{13}C_2$]PFHxA	$CF_3(CF_2)_3(^{13}CF_2)^{13}COOH$	IS for PFHxA	Wellington Labs
$^{13}C_8$-Perfluorooctanoic acid	[$^{13}C_8$]PFOA	$^{13}CF_3(^{13}CF_2)_6\ ^{13}COOH$	IS for PFOA	Wellington Labs
$^{13}C_5$-Perfluorononanoic acid	[1,2,3,4,5-$^{13}C_5$]PFNA	$CF_3(CF_2)_3(^{13}CF_2)_4{}^{13}COOH$	IS for PFNA	Wellington Labs
$^{13}C_2$-Perfluorodecanoic acid	[1,2 -$^{13}C_2$]PFDA	$CF_3(CF_2)_7(^{13}CF_2)^{13}COOH$	IS for PFDA	Wellington Labs
$^{13}C_2$-Perfluoroundecanoic acid	[1,2 -$^{13}C_2$]PFUnA	$CF_3(CF_2)_8(^{13}CF_2)^{13}COOH$	IS for PFUnA	Wellington Labs
$^{13}C_2$-Perfluorododecanoic acid	[1,2 -$^{13}C_2$]PFDoA	$CF_3(CF_2)_9(^{13}CF_2)^{13}COOH$	IS for PFDoA	Wellington Labs
$^{18}O_2$-Ammonium Perfluorobutane sulfonate	[$^{18}O_2$]PFBS	$[C_4F_9S^{18}O_2O]^-\ NH_4^+$	IS for PFBS	Wellington Labs
$^{18}O_2$-Ammonium Perfluorohexane sulfonate	[$^{18}O_2$]PFHS	$[C_6F_{13}S^{18}O_2O]^-\ NH_4^+$	IS for PFHS	Wellington Labs
$^{13}C_8$-Sodium Perfluorooctane sulfonate	[$^{13}C_8$]PFOS	$^{13}CF_3(^{13}CF_2)_7SO_3^-\ Na^+$	IS for PFOS, [$^{13}C_4$]PFOS	Wellington Labs
$^{18}O_2$-Perfluorooctanesulfonamide	[$^{18}O_2$]FOSA	$[C_8F_{17}S^{18}O_2NH_2]$	IS for FOSA	RTI International
$^{13}C_4$-Perfluorooctanoic acid	[$^{13}C_4$]PFOA	$^{13}CF_3(^{13}CF_2)_3(CF2)_3COOH$	*SRS for all Acids	Wellington Labs
$^{13}C_4$-Perfluorooctane sulfonate	[$^{13}C_4$]PFOS	$[^{13}C_4F_{17}SO_3]^-\ Na^+$	**SRS for all sulfonates and FOSA	Wellington Labs
d3-methyl-d3-perfluoro-1-octanesulfonamidoacetic acid	d3-N-MeFOSAA	$C_{11}D_3H_3F_{17}NO_4S$	IS for MeFOSAA	Wellington Labs
N-deuterioethylperfluoro-1-octanesulfonamidoacetic acid	d5-N-EtFOSAA	$C_{12}D_5H_3F_{17}NO_4S$	IS for EtFOSAA	Wellington Labs
$^{13}C_5$-Perfluoropentanoic acid	[1,2,3,4,5-$^{13}C_5$]PFPeA	$^{13}CF_3(^{13}CF_2)_3{}^{13}COOH$	IS for PFPeA	Wellington Labs (Mix or Individual)
$^{13}C_4$-Perfluoroheptanoic acid	[1,2.3.4]-$^{13}C_2$]PFHpA	$CF_3(CF_2)_2(^{13}CF_2)_3{}^{13}COOH$	IS for PFHpA	Wellington Labs (Mix or Individual)

* The [$^{13}C_4$]PFOA may be used as the PFCA SRS.

** [$^{13}C_4$]PFOS may be used as the PFSA SRS.

9.6 Sample Dilution

Any sample with a calculated concentration that is greater than the known concentration of the highest acceptable calibration standard will need to be diluted and reanalyzed. This may be performed by either diluting the sample with additional blank matrix prior to extraction or to dilute the final extract with a procedural blank extract. The second procedure consists of diluting the extract with solvent post-extraction.

10 Calibration and Standardization

10.1 Instrument Calibration

Analyze the standard curves prior to each set of samples. Stable isotope internal standard calibration with extracted matrix-matched or surrogate matrix-matched control serum/plasma or unextracted acetonitrile solvent calibrations are applicable by the method. If available, certified linear isomer calibration standards of all target analytes are applied in the method. Alternatively, certified technical grade calibration standards of mixed linear and branched isomers may be applied if QCs [LCSs and LMSs (optional)] include both linear isomer QCs and combined linear and branched isomer QCs.

10.1.1 External Solvent Calibration

Analyze an external solvent calibration curve using internal standard quantitation for each target analyte and SRS. Target analyte, SRS and IS concentrations of the calibration standards are entered in units of weight per volume in the serum/plasma (e.g. ng/mL). The standard curve may be plotted using linear regression ($y = mx + b$) or quadratic fit ($y = ax^2 + bx + c$); weighted 1/x or unweighted, using suitable software. The coefficient of determination (r^2) value for the calibration curve must be greater than or equal to 0.990 (or a correlation coefficient (r) of 0.995). Each calibration standard must be verified for accuracy. A standard is considered acceptable if the calculated concentration falls within $\pm$ 30% of the expected value. Calibration standards that fall outside the accuracy limits must be discarded. A minimum of six standards must be included that meet the accuracy requirement, however, it is recommended that more calibration standards be included to allow flexibility when adjusting LOQs during data analysis. Additional analytes not listed in this method may be analyzed if proper quality controls pass within the LLOQ desired.

The area ratio of the target analyte in the lowest calibration standard(s) should be compared to the area ratio in the calibration solvent blanks. Deactivate any low level standards whose area ratios are not at least twice those of the calibration solvent blanks (i.e. raise the limit of quantitation). If contamination of a particular analyte is observed in any spiking solutions, solvents or matrices, the solvent extraction blank must be used to determine the LLOQ and the area ratios in the lowest activated standard must be at least twice that the area ratios of the solvent extraction blank. All blanks used to determine the LLOQ must be run throughout the analytical batch.

10.1.2 Matrix-Matched Calibration

Analyze a matrix-matched calibration curve using internal standard quantitation for each target analyte and SRS. Target analyte, SRS and IS concentrations of the calibration standards are entered in units of weight per volume (e.g. ng/mL). The standard curve may be plotted using linear regression ($y = mx + b$) or quadratic fit ($y = ax^2 + bx + c$); weighted 1/x or unweighted, using suitable software. The coefficient of determination (r^2) value for the calibration curve must be greater than or equal to 0.990 (or a correlation coefficient (r) of 0.995). Each calibration standard must be verified for accuracy. A standard is considered acceptable if the calculated concentration falls within $\pm$ 30% of the expected value. Calibration standards that fall outside the accuracy limits must be discarded. A minimum of six standards must be included that meet the accuracy requirement, however, it is recommended that more calibration standards be included to allow flexibility when adjusting LOQs during data analysis

The area ratios of the target analyte in the lowest calibration standard(s) included in the curve should be compared to the area ratios in the extracted method blanks to determine if endogenous levels are present in the control matrix. Deactivate any low level standards whose area ratios are not at least twice those of the method blanks (i.e. raise the limit of quantitation) or apply the Method of Standard addition (see next section) to determine the endogenous level present and then correct the concentration of the calibration standard to reflect the contribution from the endogenous control serum/plasma. All blanks used to determine the LLOQ must be run throughout the analytical batch.

10.1.2.1 Method of Standard Addition

If endogenous levels of analyte are detected in the control matrix used to prepare the matrix-matched or surrogate matrix-matched calibration curve, the Method of Standard Addition should be used to determine the endogenous level of analytes present. Compare the area ratios of the target analyte in the method blanks to those of the solvent blanks. If the area ratios in the method blank are at least twice those of the water procedural blanks, this is an indication that endogenous levels are present in the control serum/plasma and the Method of Standard Addition should be performed.

To calculate the endogenous amount of analyte in the matrix, create a calibration curve using the theoretical values including the method blanks (with a concentration of zero). Keep all calibration points that meet the accuracy requirements as per the method and are at least twice the solvent blanks. Calculate the value of the calibration curve for the x intercept (when $y = 0$), this should be a negative number. The absolute value of this number is the amount of endogenous analyte in the matrix used and this result should be added to the theoretical value for each standard level. Reconstruct the calibration curve using the new values plus adding the method blanks (for now the endogenous value is known) as standards also. Apply the acceptance criteria from section 13.2 as would be used for a standard curve not using the Method of Standard Addition.

10.1.3 Surrogate Matrix-Matched Calibration with Matrix-Matched Calibration

Analyze the surrogate matrix-matched and matrix-matched calibration curve using internal standard quantitation for each target analyte and SRS. Target analyte, SRS, and IS concentrations of the calibration standards are entered in units of ng/mL. The standard curve may be plotted using linear regression ($y = mx + b$) or quadratic fit ($y = ax^2 + bx + c$); weighted 1/x or unweighted, using suitable software. The coefficient of determination (r^2) value for the calibration curve must be greater than or equal to 0.990 (or a correlation coefficient (r) of 0.995). Each calibration standard must be verified for accuracy. A standard is considered acceptable if the calculated concentration falls within 100% $\pm$ 30% of the known concentration for all calibration standards. Calibration standards that fall outside the accuracy limits must be discarded. A minimum of six standards must be included that meet the accuracy requirement; if the curve fails a new surrogate matrix-matched and matrix matched curve should be prepared and analyzed. The use of surrogate matrix-matched calibration is to extend the LLOQ of the matrix-matched calibration. Such as in instances where endogenous levels of target analyte prevent the use of the lower curve points in the matrix-matched curve the surrogate matrix-matched curve points may be included in the overall calibration curve to help lower the LLOQ.

The area ratios of the target analyte in the lowest calibration standard(s) should be compared to the area ratios in the solvent extraction blanks. Deactivate any low level standards whose area ratios are not at least twice those of the solvent extraction blanks. When using Matrix-Matched Calibration, the low level standard's area ratio must be at least twice those of the matrix blanks. Any low level standards whose area ratios are not twice those of the endogenous levels in the method blanks must be deactivated. All blanks used to determine the LLOQ must be run throughout the analytical batch.

10.2 Continuing Calibration Verification (CCV)

Continuing calibration verifications (CCVs) are analyzed to verify the continued accuracy of the calibration curve.

Analyze a mid-range calibration standard (at a minimum) after every tenth sample, not including solvent blanks, with a minimum of one per sample set. Samples must be bracketed by passing CCVs (or the calibration curve and one CCV). Multiple CCV levels may be used. LCS samples may be used instead of CCVs, provided that the LCS samples have recoveries within 100% $\pm$ 30%. Samples not bracketed by passing CCVs or the standard curve and a CCV must be rerun.

10.3 System Suitability Check

At least three injections of a mid range standard should be run before the calibration curve and samples to evaluate that the analytical system's performance is adequate for analysis so as to minimize lost time in the event the system was not operating properly and used to analyze an analytical batch and likely resulting in a run that may fail to meet passing criteria. This standard may be one of the extracted standards or a standard prepared in solvent. The peak area and retention time are monitored at the beginning of the run. Additional system suitability checks may be added at the analyst's discretion.

The system suitability injections must have peak area ratio with and RSD $\leq$ 5% and a retention time RSD $\leq$ 2%. If the system suitability fails, the sample set must be reanalyzed. It is recommended to verify that the system suitability passes prior to the start of sample analysis.

10.4 Solvent Blanks

Solvent blanks should be run after calibration standards and CCVs. It is also recommended to run a solvent blank after samples if the analyst suspects a high concentration of the analyte.

11 Procedures

Other volumes may be used other than those listed provided adequate QC is provided. This method may be performed with the aid of a robotic sample prep station or may be performed manually.

11.1 Sample Preparation

Transfer 200 µL of serum or plasma to a microcentrifuge tube.

As necessary, add 25 µL of a spiking solution to each microcentrifuge tube. Additional dilution solutions (e.g. water) may be added.

To each microcentrifuge tube, add 775 µL of acetonitrile. Centrifuge at approximately 10000 rpm (approx 9300 RCF) for 20 minutes.

If analyzing using three different injections for chromatography add 60 µL of a 5% phosphoric acid solution and extract to an autovial for a final volume of 1060 µL.

If analyzing using one injection for chromatography aliquot 450 µL extract, 387 µL MilliQ water and 30 µL of 5% phosphoric acid solution to each autovial for a final volume of 867 µL.

11.2 Sample Analysis

Two sets of instrument parameters are given below. The first set provides conditions that are suited for analysis of longer chain-length PFCA (C7-C12), PFSAs, FOSA, EtFOSAA and MeFOSAA. The second set of conditions has been optimized for analysis of the shorter-chain perfluorinated carboxylic acids (C4-C6).

Because this method is applicable to a wide range of perfluorinated and partially fluorinated compounds, the total number of transitions that need to be monitored can become quite large depending on the needs of the given project. Care should be taken to use a reasonable number of transitions within a given time period of the mass spectrometer acquisition method as the accuracy and precision of the results can be affected by the overall number of transitions being monitored. A multi-period mass spectrometer method may be used if sufficient chromatographic separation of the target analytes is achieved. Alternatively, the analyte list for C7-C12 PFCAs and C4, C6, and C8 PFSAs, FOSA, EtFOSAA and MeFOSAA may be divided into two or more separate injections to optimize the overall performance of the instrument if a large number of transitions are required.

Instrument configuration: Seek reference from the equipment procedure or manual that pertains to the specific instrument, as appropriate. Conditions may be adjusted provided adequate QC is used. Conditions should be consistent throughout the study/project as much as possible.

11.2.1 Liquid Chromatograph Parameters

For analysis using one injection for all analytes:

Recommended for primary analysis of all analytes including long-chain and short-chain analytes.

Column Temperature (ºC):	30
Injection Volume (µL):	25
Mobile Phase A:	5 mmol/L ammonium acetate with 0.01% acetic acid in water
Mobile Phase B:	50:50 Methanol and Acetonitrile
Guard Column (placed after purge valve)	Thermo Fischer Scientific Betasil C_{18}, 2.1 x 100 mm, 5 µm particle size
Analytical Column	Thermo Fischer Scientific Betasil C_{18}, 4.6 x 100 mm, 5 µm particle size

Gradient (one-injection method)			
Time	A (%)	B (%)	Flow (mL/min)
0.0	70	30	750
0.3	70	30	750
11.0	20	80	750

13.0	20	80	750
13.5	10	90	750
16.0	10	90	750
16.5	70	30	750
19.0	70	30	750

Note: Other HPLC gradients may be used as long as the method criteria and project data quality objectives are met.

It may be necessary to adjust the HPLC gradient in order to optimize instrument performance. Columns with different dimensions (e.g. 2.1 mm X 150 mm) and columns from different manufacturers (Keystone Betasil C18 etc.) may be used.

To analyze using two injections, or more, and to acquire data with better resolution of analyte peaks or use of longer dwell times for increased sensitivity, the following optional two-injection method described below may be used: **Injection #1:** Recommended for analysis of long-chain analytes (i.e. PFHS, PFOS, FOSA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, etc.)

Column Temperature (ºC):	30
Injection Volume (µL):	50
Mobile Phase A:	2 mmol/L ammonium acetate in water
Mobile Phase B:	Acetonitrile
Guard Column (placed after purge valve)	Thermo Fischer Scientific Prism™ RP 2 x 50 mm, 5 µm particle size
Analytical Online Column (placed before analytical column)	Waters Corporation, Oasis HLB Online Column, 3 x 20 mm 25 um particle size
Analytical Column	Thermo Fischer Scientific Betasil C_{18}, 2 x 100 mm, 5 µm particle size

Gradient (Two-injection method, long-chain analytes)			
Time	A (%)	B (%)	Flow (mL/min)
0.00	95	5.0	0.3
1.00	95	5.0	0.3
11.0	10	90	0.3
13.5	10	90	0.3
14.0	95	5.0	0.3
17.0	95	5.0	0.3

Injection #2: Recommended for analysis of short-chain analytes: (PFBS, PFBA, PFPeA, PFHA, etc.)

Column Temperature (ºC):	30
Injection Volume (µL):	20
Mobile Phase A:	5 mmol/L ammonium acetate plus 0.01% Acetic acid in water
Mobile Phase B:	Methanol
Guard Column (placed after purge valve)	Thermo Fischer Scientific Prism™ RP 2 x 50 mm, 5 µm particle size
Analytical Column	Thermo Fischer Scientific Prism™ RP 2 x 50 mm, 5 µm particle size

Gradient (Two-injection method, short-chain analytes)			
Time	A (%)	B (%)	Flow (mL/min)
0.00	95	5	0.3
3.00	95	5	0.3
8.00	5.0	95	0.3
14.0	5.0	95	0.3
15.0	95	5	0.3
19.0	95	5	0.3

Note: Other HPLC gradients may be used as long as the method criteria and project data quality objectives are met.

It may be necessary to adjust the HPLC gradient in order to optimize instrument performance. Columns with different dimensions (e.g. 2.1 mm X 150 mm) and columns from different manufacturers (Keystone Betasil C18 etc.) may be used.

11.2.2 Mass Spectrometer Parameters

Document in the raw data the mass spectrometer parameters used for each analyte. The following table lists recommended transitions that may be used for several fluorinated compounds and their associated ISs that may be analyzed using this method. Transitions may be added or deleted as necessary (e.g. if interferences are observed).

Compound	*Parent Ion*	*Product Ion*	*Internal Standard*	*IS Parent Ion*	*IS Product Ion*
PFBA (C4 Acid)	213	169	[1,2,3,4-$^{13}C_4$]PFBA	217	172
(1,2,3-$^{13}C_3$)PFBA (M3PFBA; SRS)	216	172			
PFPeA (C5 Acid)	263	219	[1,2,3,4,5-$^{13}C_5$]PFPeA	268	223
PFHxA (C6 Acid)	313	269*	[1,2 -$^{13}C_2$]PFHxA	315	270
	313	119			
PFHpA (C7 Acid)	363	319	[1,2,3,4]-$^{13}C_2$]PFHpA	367	322
	363	169			
PFOA (C8 Acid)	413	369	[$^{13}C_8$]PFOA	421	376
	413	219			
	413	169			
[1,2,3,4-$^{13}C_4$]PFOA (M4PFOA; SRS)	417	372			
PFNA (C9 Acid)	463	419	[1,2,3,4,5-$^{13}C_9$]PFNA	472	427
	463	219			
	463	169			
PFDA (C10 Acid)	513	469	[1,2,3,4,5,6 - $^{13}C_6$]PFDA	519	474
	513	219			

Compound	*Parent Ion*	*Product Ion*	*Internal Standard*	*IS Parent Ion*	*IS Product Ion*
PFUnA (C11 Acid)	563	519	$^{13}C_7$–PFUnA	570	525
	563	269			
	563	219			
PFDoA (C12 Acid)	613	569	[1,2 -$^{13}C_2$]PFDoA	615	570
	613	219			
	613	169			
PFBS (C4 Sulfonate)	299	99	[$^{18}O_2$]PFBS	303	84
	299	80			
PFHS (C6 Sulfonate)	399	99	[$^{13}C_3$]PFHS	402	80
	399	80			
PFOS (C8 Sulfonate)	499	130	[$^{13}C_8$]PFOS	507	80
	499	99			
	499	80			
[1,2,3,4-$^{13}C_4$]PFOS (M4PFOS; SRS)	503	80			
PFDS (C10 Sulfonate)	599	80			
	599	99			
	599	130			
FOSA (C8 Sulfonamide)	498	78	[$^{13}C_2$]FOSA	506	78
[1,2 -$^{13}C_2$]PFUnA (M2PFUnA; SRS)	565	520	$^{13}C_7$–PFUnA	565	520
MeFOSAA	570	169	*d3*-MeFOSAA	573	169
	570	219		573	219
	570	83		573	83
EtFOSAA	584	169	*d5*-EtFOSAA	589	169
	584	219		589	219
	584	83		589	83
FBSA	297.7	78	[$^{18}O_2$]PFBS	303	103

The 313>269 transition is not recommended when using newborn calf serum due to possible interference in that matrix.

Multiple MRMs are typically summed for each analyte

Monitoring multiple transitions is a desirable option because summing multiple transitions may provide quantitation of isomers that more closely matches NMR data and may have the added benefit of increased analytical signal for improved sensitivity. However, the use of one daughter ion is acceptable if method sensitivity requirements are achieved, provided that retention time criteria are met to assure adequate specificity.

When creating the final quantitation method, the analyst should carefully consider each transition as several of the above transitions are also present in biological matrix interferences. Specifically, the MRMs of 399 to 80 and 99 (PFHS) and 499 to 80 (PFOS) have interferences in some biological tissues arising from the presence of 5-pregnan-3,20-diol-3-sulfate isomers and taurodeoxycholate isomers (bile salt), respectively. [3]

12 Data Analysis and Calculations

Calculate the amount of analyte in the serum or plasma extract using the appropriate calibration curve generated.

Calculate the amount of analyte in serum or plasma as follows:

$$\frac{\text{ng}}{\text{mL serum}} = \frac{\text{ng}}{\text{mL extract}} \times \frac{\text{total extract (transferred extract} + 5\%\ H_3PO_4\text{), mL}}{\text{transferred volume of extract , mL}} \times \frac{\text{total extract volume from PPT step, mL}}{\text{volume of serum used, mL}}$$

Include other dilution factors as appropriate.

A common dilution factor used in external solvent calibration is 9.63 calculated from the following:

$$9.63 = \frac{\text{total extract (transferred extract} + 5\%\ H_3PO_4\text{), mL}}{\text{transferred volume of extract , mL}} \times \frac{\text{total extract volume from PPT step, mL}}{\text{volume of serum used, mL}}$$

Calculate the percent relative standard deviation (%RSD) for the system suitability as follows:

$$\%\text{RSD} = \frac{\text{Standard Deviation}}{\text{Average}} \times 100$$

Where the standard deviation (s) is: $s = \sqrt{\frac{n\sum x^2 - \left(\sum x\right)^2}{n(n-1)}}$ and average is: $\text{average} = \frac{1}{n}\sum x$

The relative percent difference should be calculated as follows:

$$\text{Relative Percent Difference} = \frac{\text{Absolute Value}\left(X_2 - X_1\right)}{\text{Average of } X_2 \text{ and } X_1} \times 100$$

Calculate the percent recovery for the QC samples as follows:

$$\text{Percent recovery} = \frac{\text{Amount of analyte detected (ng/mL)}}{\text{Amount of analyte spiked (ng/mL)}} \times 100$$

For the matrix spike (or QC samples with endogenous amounts), the percent recovery is calculated as:

$$\text{Percent recovery} = \frac{\text{Amount of analyte detected (ng/mL) - Amount of analyte in non spiked sample (ng/mL)}}{\text{Amount of analyte spiked (ng/mL)}} \times 100$$

13 Analysis Batch Method Performance Criteria

Any method performance parameters that are not achieved must be considered in the evaluation of the data. Nonconformance to any specified parameters must be described and discussed in the final report if the Technical Director (non-GLP study) or Study Director (GLP study) chooses to report the data.

If criteria listed in this method performance section are not met, maintenance may be performed on the system and samples reanalyzed, or other actions taken as appropriate. Document all actions in the raw data.

If data are to be reported when performance criteria have not been met, the data must be footnoted on tables and discussed in the text of the report.

13.1 System Suitability

The system suitability injections must have area ratios with an RSD ≤5% and a retention time RSD ≤2%. If the

system suitability fails, the sample set must be reanalyzed. It is recommended to verify that the system suitability passes prior to the start of the analysis. There is no defined acceptability limit on these results as the %RSDs are dependent on the number of MRM transitions being monitored in the LC/MS/MS run or time period. Any effect of system suitability is incorporated in the QC acceptance criteria.

13.2 Calibration and Limit of Quantitation (LOQ)-Analysis Batch

Calibration Curve: The coefficient of determination (r^2) value for the calibration curve must be greater than or equal to 0.990 or a correlation coefficient (r) of 0.995. A minimum of six standards will be included in the calibration curve, and when back-calculated (including ULOQ) the determined standards concentrations shall fall within 100% ±30% for the LLOQ and 100% ±25% for all other points of the nominal value. Values falling outside these limits shall be technically justified for their exclusion from the curve (i.e. determined preparation error, such as vials in the wrong position and incorrect final extract volumes or instrument error).

13.3 Continuing Calibration Verification (CCV)

CCVs should be within 100% $\pm$ 30%. Samples not bracketed by passing CCVs shall be reanalyzed.

13.4 Sensitivity

If external solvent, extracted matrix-matched or surrogate matrix-matched calibration is used, the analyte response (area ratio) of the LLOQ standard for each analyte shall be at least two times the analyte response of the average area ratio of all calibration blanks. The analyte response shall be at least two times the response as compared to the calibration blank response.

13.5 Accuracy

13.5.1 LCS Samples

At least six of every nine (i.e. two-thirds) LCS samples shall be within 100% ± 30% of their respective nominal value. Three of the nine LCS samples may be outside the 100% ± 30% of their respective nominal value, but not all at the same concentration. If more than three levels of LCS samples are prepared, a level that is less than three times the LLOQ may be disregarded provided that at least 5% of the total number of samples extracted are kept and there are three levels at higher concentrations that meet the required spiking levels in section 9.2. All LCS samples will be control charted as per ETS-4-026. If the average recovery of one of the spiking levels exceeded the analytical method uncertainty as determined by ETS-12-012, that analytical batch uncertainty will be expanded for that particular study.

If more than 67% of the LCS samples fail to meet method acceptance criteria, the data will not be reported. The average recovery at each LCS level for mixed branched/linear isomer PFOA and PFOS should be within 70-130%.

13.5.2 Matrix Spike Samples

Matrix spikes shall be within 100% ± 30% of their respective nominal value.

13.6 Analytical Method Uncertainty

Analytical method uncertainty for each target analyte and the SRS is determined with control charted historical analysis batch LCS data for the method and reported with each analysis batch.[4] Uncertainty determinations are based on INTERNATIONAL ANS/ISO/IED STANDARD 17025 reference (GUM, Guide to the Expression of Uncertainty in Measurement) and described in ETS-12-012.

13.7 Quantitation of Linear + Branched PFOA/PFOS- Analysis Batch

If the calibration standards are comprised of predominantly linear isomers only, the method requires the addition of LCSs of mixed branched/linear isomer PFOS/PFOA to demonstrate quantitative equivalency (or quantitative bias) of the isomeric mix when using a predominantly linear PFOS or PFOA standard for calibration. The

average recovery at each LCS level for the mixed branched/linear isomer PFOA and PFOS should be within 70-130%.

13.8 Precision

QC samples shall be within ≤ 25% relative standard deviation (RSD).

Sample duplicates shall be within ≤ 30% relative percent difference (RPD).

13.9 Sample Dilution

If duplicate dilutions are made, the two samples should have a RPD within 30%. If the RPD is not within 30% for these two samples, additional testing may be performed to determine which value is a correct representation of the sample concentration as determined by the analyst and documented in the raw data.

14 Pollution Prevention and Waste Management

Human and primate sample waste is disposed of in infectious biohazard waste containers.

Flammable solvent waste is disposed of in high BTU containers.

Glass pipette waste is disposed of in broken glass containers located in the laboratory.

15 Records

Use the appropriate prepsheet or equivalent to record the pertinent data. Other information should be documented as appropriate.

16 References

[1] Harrington, L.M., **Anal. Methods**, 2017, 9, 473-481.

[2] Guidance for Industry: Bioanalytical Method Validation; U.S. Department of Health and Human Services, Food and Drug Administration Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM); May 2001.

[3] Benskin, J.P., Bataineh, M, Martin, J.W., **Anal. Chem** 2007, 79, 6455-6464.

[4] Method uncertainty based on INTERNATIONAL ANS/ISO/IED STANDARD 17025 reference (GUM, Guide to the Expression of Uncertainty in Measurement).

17 Revisions

Revision Number	Summary of Changes
1	Section 1: Added quantification was using stable isotope internal standards. Section 2: Added that quantitation was with internal standard stable isotope standards. Section 7.1: Updated water requirements. Section 7.2: Added if perfluorocarboxylic acid stocks are made in methanol, the methanol should have base added to it. Section 9.5: Added more information regarding what surrogates to use. Section 9.6: Added more information regarding what internal standards to use. Section 10.1: Clarified that the solvent curve is a water extracted curve. Section 10.4: Clarified that solvent blanks are acidified acetonitrile. Section 11.3.2: Added more information regarding mass transitions for specific analytes and their recommended internal standards. Section 16: Added Benskin reference.
2	Section 1 added Table 1 and method guidelines. Section 3 added definitions. Section 6.2

	added supplies. Section 7.2: Added external solvent calibration criteria and preparation. Section 9: added isotope internal standards. Section 9.1.2: Added blank criteria and clarified blank definitions. Section 10.1: Added external solvent calibration criteria. Section 11.2: Added sample prep for one injection chromatography analysis. Section 11.3.1: Added chromatography parameters for analysis using one injection. Section 11.3.2: Added and edited analytes and transitions. Also, edited the dilution procedure to simplify and clarify the dilution procedures and removed discussion around unnecessary dilution validation procedures, etc. Section 13 added criteria. Section 13.5.2 to add reference for determining analytical method uncertainty.
3	Added Analytical Methods reference. Added definitions for blanks and external solvent calibration standard in section 3. Section 6.1: added clause to allow for the use of different instrumentation. Updated Section 9.1 with new blank nomenclature, additional blank analysis and criteria. Updated Section 10.1.1 with proper blank nomenclature and requirements. Updated Section 10.1.2 with blank requirements. Updated Section 10.1.3 with blank nomenclature and requirements and removed irrelevant requirements. Section 11.3.1: added wording to allow for HPLC method variance. Removed section 11.1 MultiProbe Setup, and updated Section IDs for other sections to reflect section deletion. Updated Section 12 with common dilution factor calculation. Removed criteria not used in the method from Section 13.4. Updated PFNA IS ID throughout report. Updated the use of surrogate to SRS nomenclature throughout the SOP. Updated basic grammar throughout the SOP.