3M Environmental Laboratory

Method

Determination of Perfluorinated Alkyl Acids (PFAAs) via Protein Precipitation of Fish Tissues (Fillet or Whole Body) and Analysis by High Performance Liquid Chromatography with Tandem Mass Spectrometry

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William K. Reagen, Ph.D. Date
Laboratory Manager

1 Scope and Application

This performance-based method describes the extraction of perfluorinated alkyl acids (PFAAs) from fish tissues using cryogenic protein precipitation followed by separation, identification, and quantification using high performance liquid chromatography tandem mass spectrometry (LC/MS -MS).

These procedures are applicable for perfluoroalkyl sulfonates (PFASs), including perfluorooctane sulfonate (PFOS), and the perfluorocarboxylic acids (PFCAs), including perfluorooctanoic acid (PFOA) and perfluorooctanesulfonamide (FOSA) in fish fillet and whole body tissues. 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.

2 Method Summary

This analytical method is based on a full analytical method validation and describes the procedure for the extraction and quantification of PFAAs in fish fillet and whole body tissues.[1] Initially, whole fish or fish fillets are processed before extraction by placing a frozen sample in a food processor and homogenizing with dry ice. The dry ice is allowed to sublimate overnight at -20°C. A weighed aliquot of frozen, homogenized tissue to which internal standards (ISs), surrogates, and target analytes are added, if appropriate, is extracted by homogenizing in acetonitrile (protein precipitation), cooled for at least one hour at -20°C, and centrifuged cold (approximately –5 °C.) at approximately 3000 rpm for 20 minutes. An aliquot of the acetonitrile supernatant is transferred to an autovial, acidified, and analyzed using high performance liquid chromatography tandem mass spectrometry. Quantitation is performed with stable isotope internal standard calibration using either (1) extracted “matrix-matched” or “surrogate matrix-matched” tissue calibration or (2) unextracted acetonitrile solvent calibration.

3 Warnings and Cautions

3.1 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 Environmental Laboratory’s Chemical Hazard Review, the 3M Guide to Laboratory Practices or other information as appropriate.

3.2 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-002-5), tissue homogenizers/processors, centrifuges, solvents, high temperatures, pressurized gas and solvent lines, and high voltage. Refer to the appropriate equipment procedures SOPs or the appropriate operator manuals for additional information and cautions.

4 Interferences

To minimize interferences, polytetrafluoroethylene (PTFE, e.g. Teflon) should be avoided for sample 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.[2] Manipulation of the instrument parameters (LC gradient conditions) can minimize the co-elutions 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.

5 Instrumentation, Supplies, and Materials

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

5.1 Instrumentation

Balance, analytical (display at least 0.0001 g)

Homogenizer, POLYTRON®, Robot Coupe, OmniPrep or similar

Agilent HPLC 1100 or 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
- Column Compartment (Temperature Controlled), Agilent®, Model G1316A
- Controller, Hand Held, Agilent®, Model # G1323A

Applied Biosystems MDS SCIEX API 4000 or 5000 Biomolecular Mass Analyzer

- SCIEX Turbo Ion Spray Liquid Introduction Interface

Other instrumentation as needed, document as appropriate

5.2 Supplies and Materials

Hamilton gas tight syringes (capable of dispensing at least 50 µL)

Class A volumetric flasks

Eppendorf or plastic disposable pipettes

Thermo Fischer Scientific Betasil C_{18}, 2.1 x 100 mm, 5 µm particle size

Thermo Fischer Scientific Prism™ RP 2x50 mm, 5 µm particle size

HPLC vials capable of containing approximately 2 mL extract

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

Centrifuge tubes, polypropylene, various sizes

Disposable scoops

Other supplies and materials as needed, document as appropriate

6 Reagents and Standards

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

6.1 Reagents

Acetonitrile, HPLC grade or equivalent

Methanol, HPLC grade or equivalent

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

Ammonium acetate, Mallinckrodt 98% pure or equivalent

Formic acid, 98% or equivalent

Various fish tissues from supplier or study sponsor for use as control matrix

Note: The concentrations of all acid and base reagent solutions are not corrected for the reagent strength. For example, a 10% Formic Acid solution might be prepared by adding 2 mL of the 98% acid to water and brought up to volume of 20 mL.

6.2 Calibration Standards

Due to the likely possibility of ester formation between carboxylic acids and alcohols, when preparing stock solutions in methanol, it is important that the methanol be treated with an excess of base. In this method, when preparing the primary stock solution of 1 ppm perfluorocarboxylic acid in methanol, the methanol needs to be treated with base 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, as the base will still be present in excess to the acids. Alternatively, stock standards of perfluorocarboxylic acids can be prepared in acetonitrile to avoid ester formation. Standards purchased from Wellington now come prepared in a base-treated methanol solution and do not require further treatment. Wellington 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 LCSs include both linear isomer QCs and combined linear and branched isomer QCs.

6.2.1 Matrix-matched or Surrogate Matrix-Matched Calibration.

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 0.0025 ng/mL in extract (0.025 ng/g tissue) following the extraction procedure described in section 11. NOTE: For stable isotope internal standard quantitation, extracted matrix-matched calibration or extracted surrogate matrix-matched calibration or unextracted acetonitrile calibration may be used.

Prepare individual stock solutions at approximate concentrations of 1 μg/mL (1 ppm) as needed [3]. For example, accurately weigh out 10 mg of the standard reference material corrected for purity and anion formula weight into a 100-mL volumetric flask and bring up to volume using the appropriate solvent (base-treated methanol or acetonitrile). Store appropriately at room temperature or refrigerated and assign an expiration date of a maximum six month period from the date of preparation (unless determined otherwise – do not exceed the expiration date of any of the individual components). Prepare the necessary mixed stock solutions of the target and surrogate analytes (if applicable) by combining known amounts of the individual stock solution and bringing to a final volume with the appropriate solvent. Dilute the mixed stock solution as necessary to prepare calibration standard and QC spiking solutions. In a similar fashion, prepare any additional internal standard and surrogate spiking solutions as required.

Matrix-matched or surrogate matrix-matched extracted calibration standards are prepared by spiking known amounts of the calibration spiking solution and the separate internal standard spiking solution (if included) into an accurately weighed aliquot of frozen fish homogenate. The spike is allowed to sit and equilibrate for a minimum of 15 minutes before the acetonitrile extraction solvent is added and the solvent/tissue mixture is homogenized. The final calibration curve must consist of at least six calibration points after analysis. If the analyst suspects that some curve points may need to be deactivated to meet method criteria for LLOQ, more calibration standards should be prepared. The following table provides examples of spike concentrations and volumes used to achieve a multi-point extracted calibration curve

with internal standard.

Table 1. [(1)]Example Preparation of Extracted Calibration Curve with Internal Standard.

Sample Description	*Weight of Fish Tissue Used for Extraction (g)*	*Internal Standard Spike Volume (μL)*	*Internal Standard Spike Conc. (ng/mL)*	*Internal Standard Tissue Conc. (ng/g)*	*Curve Point Spike Volume (μL)*	*Curve Point Spike Solution Conc. (ng/mL)*	*Target Analyte Tissue Conc. (ng/g)*
Extracted Cal. Curve Point 1	0.5	25	20	1	63	0.2	0.025
Extracted Cal. Curve Point 2	0.5	25	20	1	88	0.2	0.035
Extracted Cal. Curve Point 3	0.5	25	20	1	13	2	0.05
Extracted Cal. Curve Point 4	0.5	25	20	1	25	2	0.1
Extracted Cal. Curve Point 5	0.5	25	20	1	63	2	0.25
Extracted Cal. Curve Point 6	0.5	25	20	1	13	20	0.5
Extracted Cal. Curve Point 7	0.5	25	20	1	25	20	1
Extracted Cal. Curve Point 8	0.5	25	20	1	63	20	2.5
Extracted Cal. Curve Point 9	0.5	25	20	1	13	200	5
Extracted Cal. Curve Point 10	0.5	25	20	1	19	200	7.5
Extracted Cal. Curve Point 11	0.5	25	20	1	25	200	10
Extracted Cal. Curve Point 12	0.5	25	20	1	63	200	25

The number of calibration standards, the mass of fish tissue, spike volumes, and spike concentrations can all be adjusted or altered accordingly to meet the needs of the project and for preparatory feasibility (i.e. quantity of available fish tissues, available standards, required limit of quantitation, etc.) The concentrations shown in the table above will usually suffice for analysis of the PFCAs (C4-C12), PFBS, and PFHS. Higher concentrations are usually required for PFOS and occasionally FOSA.

6.2.2 Unextracted acetonitrile (solvent) calibration

Alternatively, if matrix-matched or surrogate matrix-matched extracted calibration is not going to be used, then prepare solvent calibration standards in acetonitrile with the appropriate internal standards at solution concentrations equivalent to the tissue concentrations listed above, or higher if needed. (PFOS tissue concentrations in the range of 100-1000 ng/g are possible.) Solution concentration in ng/mL is a factor of ten less than the ng/g tissue concentration. i.e. 25 ng/g tissue concentrations are equivalent to 2.5 ng/mL in extract solution (0.5 g of tissue extracted with 5 mL acetonitrile). Prior to analysis, acetonitrile (solvent) calibration standards should be acidified in a similar fashion as the sample extracts: a 1 mL aliquot of solvent standard is transferred to an autovial pre-spiked with 10 μL of 10% formic acid (see Section 10.2 for additional information).

7 Sample Handling

After the initial processing outlined in Section 10.1, additional sample processing is usually needed for fish fillet or whole body samples. All aliquoting and accurate weighing of tissue samples should be done while ensuring that the bulk tissue homogenate samples remain frozen. After accurate weighing of the tissue samples, the samples must be allowed to completely thaw, un-aided, at room temperature. Sample aliquots may be pre-weighed the day before and stored refrigerated overnight until time of extraction. If this is done, the aliquots should also be allowed to equilibrate to room temperature prior to extraction.

If at any time, the bulk tissue samples thaw, or appear to be non-homogenous, they should be reprocessed by regrinding with dry ice in a sample processor.

8 Quality Control

Refer to Section 12 for acceptance criteria. Each extraction batch (i.e. each day samples are extracted) should include the following:

8.1 Blanks

8.1.1 Matrix Procedural Blanks

For extracted matrix-matched or surrogate matrix-matched calibration, at least two, preferably four, blank matrix or surrogate matrix controls should be extracted and analyzed with each batch. At least half of the matrix blanks should be spiked with the internal standard(s)/surrogate(s), and the other half without.

8.1.2 Method Procedural Blanks

At least two, preferably four, method controls (e.g. water blanks) should be extracted with each batch. This is accomplished by extracting an equivalent mass of laboratory reagent water instead of the sample matrix. For example, if 0.5 g of tissue is extracted then 0.5 mL (0.5 g) of water should be used for the method blank. At least half of the method blanks should be spiked with the internal standards/surrogates and the other half without.

8.1.3 Acetonitrile Blanks

Acetonitrile blanks should be run after calibration standards and CCVs. At least two acetonitrile blanks, acidified in the same manner as the sample extracts, should also be analyzed. A minimum of two acetonitrile blanks should be fortified with internal standard and surrogate at the same level as the sample extracts.

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 blank after this sample to evaluate instrument carry-over. If consecutive blanks are run, use the last blank in the series to determine if it passes method performance criteria.

8.2 Laboratory Control Samples (LCSs)

Prepare triplicate spikes using the same control fish tissue that was used for the extracted matrix-matched calibration standards. If unextracted solvent calibration is going to be used for quantitation, then select a control fish matrix that is the same species and tissue type as the study samples or a majority of the study samples. Alternatively, a surrogate control fish matrix may be used if a matched species is not available. It is recommended that three levels of LCSs be prepared at the following targeted levels: 2-3 times the anticipated lower limit of quantitation (LLOQ), mid-calibration range, and mid-upper calibration range (greater than the mid-level spikes but approximately 80% the upper limit of quantitation ULOQ).

As it can be hard to predict LLOQs in a given species or a previously unevaluated control matrix, it is recommended to add additional sets of QC samples near the LLOQ if multiple analytes are extracted and significantly different LLOQs are anticipated. This will allow a set of LCSs to be excluded for a particular analyte when the resulting low level spike is not at an appropriate level. Alternatively, if anticipated LLOQs are known, the individual analyte concentrations for the LCS spiking solutions can be varied to meet the low-level spiking requirement.

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

calibration.

8.3 Laboratory Matrix Spikes (LMSs)

8.3.1 Surrogate Analyte Laboratory Matrix Spikes

Isotopically labeled surrogate standard(s) should be spiked into test samples, test sample replicates, procedural blanks, and LCSs. Surrogate calibration curves of the surrogate standards shall be prepared (i.e. the surrogates should be included in the calibration curve).The surrogate is spiked at a level approximately 10-20 x LOQs into tissue homogenates prior to extraction. $^{13}C_8$-Perfluorooctane sulfonate [$^{13}C_8F_{17}SO_3$]$^-$ or equivalent stable isotope labeled PFOS should be used for assessment of method quantitation of all PFSAs and FOSA. $^{13}C_8$-Perfluorooctanoic acid [$^{13}CF_3(^{13}CF_2)_6\ ^{13}COOH$] or equivalent stable isotope labeled PFOA should be used for assessment of method quantitation of all PFCAs.

8.3.2 Target Analyte Laboratory Matrix Spikes (optional)

For each study sample, a separate aliquot will be separately fortified at a known concentration of each target analyte and carried through the procedure to verify 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 quantitated.

For large studies, or if sample quantity is limited, the frequency of LMSs may be reduced (e.g., every 10 samples or each species).

8.4 Sample Duplicate

At least one sample will be prepared in duplicate to evaluate sample quantitation precision.

8.5 Internal Standard (IS)

If available, stable isotope internal standards (isotopically labeled target analytes) of each target analyte should be spiked at the same level in all extracted or solvent calibration standards, test samples, sample replicates, procedural blanks, LCSs, and LMSs (typically around 1 ng/g).

Table 2 lists recommended stable isotope internal standards 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, [1,2,3,4-$^{13}C_4$]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-$^{13}C_4$]PFOS (Wellington Laboratories, Guelph, ON) in combination with added [$^{18}O_2$]PFBS and [$^{18}O_2$]FOSA 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 isotope ISs applied in the method. Other stable isotope ISs of target analytes not listed in the table may 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 surrogates 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 and FOSA.

Table 2. Stable Isotope PFSAAs used for Internal Standards and Surrogates.

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, *PFPeA	Wellington Labs (Mix or Individual)
$^{13}C_2$-Perfluorohexanoic acid	[1,2 -$^{13}C_2$]PFHxA	$CF_3(CF_2)_3(^{13}CF_2)^{13}COOH$	IS for *PFPeA, PFHxA	Wellington Labs (Mix or Individual)
$^{13}C_4$-Perfluorooctanoic acid	[1,2,3,4-$^{13}C_4$]PFOA	$CF_3(CF_2)_3(^{13}CF_2)_3{}^{13}COOH$	IS for *PFHpA, PFOA, [1,2 $^{13}C_2$]PFOA	Wellington Labs (Mix or Individual)
$^{13}C_5$-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 (Mix or Individual)
$^{13}C_2$-Perfluorodecanoic acid	[1,2 -$^{13}C_2$]PFDA	$CF_3(CF_2)_7(^{13}CF_2)^{13}COOH$	IS for PFDA	Wellington Labs (Mix or Individual)
$^{13}C_2$-Perfluoroundecanoic acid	[1,2 -$^{13}C_2$]PFUnA	$CF_3(CF_2)_8(^{13}CF_2)^{13}COOH$	IS for PFUnA	Wellington Labs (Mix or Individual)
$^{13}C_2$-Perfluorododecanoic acid	[1,2 -$^{13}C_2$]PFDoA	$CF_3(CF_2)_9(^{13}CF_2)^{13}COOH$	IS for PFDoA	Wellington Labs (Mix or Individual)
$^{18}O_2$-Ammonium Perfluorobutane sulfonate	[$^{18}O_2$]PFBS	$[C_4F_9S^{18}O_2O]^- NH_4^+$	IS for PFBS	RTI International (Individual)
$^{18}O_2$-Ammonium Perfluorohexane sulfonate	[$^{18}O_2$]PFHS	$[C_6F_{13}S^{18}O_2O]^- NH_4^+$	IS for PFHS	Wellington Labs (Mix or Individual)
$^{13}C_4$-Sodium Perfluorooctane sulfonate	[1,2,3,4-$^{13}C_4$]PFOS	$CF_3(CF_2)_3(^{13}CF_2)_3{}^{13}CF_2SO_3^-$ Na^+	IS for PFOS, [$^{18}O_2$]PFOS	Wellington Labs (Mix or Individual)
$^{18}O_2$-Perfluorooctanesulfonamide	[$^{18}O_2$]FOSA	$[C_8F_{17}S^{18}O_2NH_2]$	IS for FOSA	RTI International
$^{13}C_2$-Perfluorooctanoic acid	[1,2-$^{13}C_2$]PFOA	$CF_3(CF_2)_5(^{13}CF_2)\,^{13}COOH$	**Surrogate for all Acids	Perkin Elmer, Wellington
$^{13}C_8$-Perfluorooctanoic acid	[$^{13}C_8$]PFOA	$^{13}CF_3(^{13}CF_2)_6\,^{13}COOH$	**Surrogate for all Acids	Wellington
$^{13}C_8$-Perfluorooctane sulfonate	[$^{13}C_8$]PFOS	$[^{13}C_8F_{17}SO_3]^- Na^+$	Surrogate for all Sulfonates, FOSA	Wellington
$^{18}O_2$-Ammonium Perfluorooctane sulfonate	[$^{18}O_2$]PFOS	$[C_6F_{13}S^{18}O_2O]^- NH_4^+$	***Alternate Surrogate for PFOS	RTI International

*No isotopically labeled counterpart for PFPeA or PFHpA is currently commercially available. The labeled PFBA or PFHxA can be used as the surrogate IS for PFPeA, but either compound may exhibit some analytical bias with unextracted solvent quantitation. Isotopically labeled PFOA has exhibited minimal bias when used as a surrogate IS for PFHpA.

**Either the [1,2-$^{13}C_2$]PFOA or [$^{13}C_8$]PFOA may be used as the PFCA surrogate.

***[$^{18}O_2$]PFOS may be used as a surrogate; however, the [$^{13}C_8$]PFOS is the preferred.

8.6 Sample Dilution

Any sample with an analyte area greater than that of the highest acceptable standard will need to be diluted and reanalyzed. The final sample extract can be diluted at a known ratio with additional extraction solvent (acetonitrile). Internal standards should be respiked in the final dilution to produce an internal standard concentration consistent with the rest of the samples.

9 Calibration and Standardization

9.1 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 tissue or unextracted acetonitrile

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 LCSs include both linear isomer QCs and combined linear and branched isomer QCs.

9.1.1 Matrix-Matched or Surrogate Matrix-Matched Calibration

Analyze the matrix-matched or surrogate matrix-matched calibration curve using internal standard quantitation for each target analyte and surrogate. Target analyte, surrogate and internal standard concentrations of the calibration standards are entered in units of ng/mL. The standard curve may be plotted by linear regression ($y = mx + b$) or a quadratic fit ($y = ax^2 + bx + c$); weighted $1/x$, $1/x^2$, 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 ±25% of the known concentration, except for the lowest calibration standard, which can be within ±30% of the expected value. Calibration standards that fall outside the accuracy limits of 100±25% (30% at the low end) must be discarded. A minimum of six standards must be included that meet the accuracy requirement.

The area counts of the target analyte in the lowest calibration standard(s) should be compared to the area counts in the extracted control matrix blanks to determine if endogenous levels are present in the control matrix. Deactivate any low level standards whose area counts are not at least twice those of the control 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 in the control tissue.

9.1.1.1 Method of Standard Addition

If endogenous levels of analyte are present 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 present analytes. Compare the area counts of the target analyte in the control matrix blanks to those of the water blanks. If the area counts in the matrix blank are at least twice those of the water blanks, this is an indication that endogenous levels are present in the control tissue and the Method of Standard Addition should be performed.

To calculate the endogenous amount of analyte in the matrix, create a calibration curve where the target analyte concentration and internal standard concentration in units of ng/g using the theoretical values are plotted. Include all calibration points that meet the accuracy requirements as per the method (See Section 9.1.1) and that have area counts that are at least twice those of the matrix 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 ng/g concentration of endogenous analyte in the matrix used. This value should be used to correct the theoretical value for each standard level. Reconstruct the calibration curve using the new values plus adding the matrix blanks (for now the endogenous value is known) as standards also. Apply the acceptance criteria from Section 9.1.1 as would be used for a standard curve not using the Method of Standard Addition.

A minimum of six calibration points should be used that produces the best fit curve at the low end of the acceptable calibration range.

9.1.2 Unextracted Acetonitrile (Solvent) Calibration

Analyze the acetonitrile solvent calibration curve using internal standard quantitation for each target analyte and surrogate. Target analyte, surrogate, and internal standard concentrations of the calibration standards are entered in units of ng/mL. The standard curve may be plotted by

linear regression ($y = mx + b$) or a quadratic fit ($y = ax^2 + bx + c$); weighted $1/x$, $1/x^2$, 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 ±25% of the known concentration, except for the lowest calibration standard, which can be within ±30% of the expected value. Calibration standards who fall outside the accuracy limits of 100±25% (30% at the low end) must be discarded. A minimum of six standards must be included that meet the accuracy requirement.

The area counts of the target analyte in the lowest calibration standard(s) should be compared to the area counts in the extracted water blanks. The area counts of the target analyte in the lowest passing calibration curve point must be at least twice those of the extracted water blanks. Deactivate any low level calibration standards that do not meet this requirement.

If solvent calibration is used, determination of endogenous levels in the control matrix is accomplished by direct quantitation of the control matrix blanks spiked with internal standard. This value should be used to correct LCS spike concentrations.

9.2 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 CCV per sample set. Samples must be bracketed by passing CCVs or by the standard curve and a CCV. Multiple CCV levels may be used if abbreviated curve ranges are anticipated.

CCV recoveries should be within 75% -125%. Samples not bracketed by passing CCVs or the standard curve and a CCV shall be rerun.

9.3 System Suitability Check

A minimum of three injections of a mid-range standard are analyzed prior to the calibration curve to verify the system stability. This standard may be one of the extracted standards or a standard prepared in solvent. The peak area and retention time are monitored.

The system suitability injections must have peak area count ratios (target analyte area counts/IS area counts with an RSD ≤10% 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 sample analysis

10 Procedures

10.1 Sample Preparation – Initial Fish Processing

The initial fish tissue (fillets or whole bodies) must be processed before extraction. Place the frozen samples in a food processor and homogenize with dry ice. Place the samples in polyethylene containers or bags and leave open in frozen storage (-20°C) overnight to allow for carbon dioxide sublimation. Once the carbon dioxide has sublimated, seal the containers and place the samples in frozen storage until the time of analysis.

Note: While an optimum size distribution for fish particle size has not been determined, practical experience suggests that processing the fish tissue until it resembles a fine powder or dust provides sufficient homogenization with the acetonitrile in later steps of the method.

10.2 Sample Preparation

Other weights or volumes than the ones provided here may be used as long as adequate QC is performed and a 1g fish/10 mL acetonitrile ratio is maintained.

Accurately weigh approximately 0.5 gram of pre-ground frozen fish tissue into an appropriate container for homogenization (50 mL high density polyethylene centrifuge tube). Sample duplicates or replicates will be prepared by weighing out a separate aliquot(s) of the processed fish tissue and then carrying the aliquot through all the extraction steps outlined below.

Spike tissue aliquots with an appropriate calibration standard, surrogate, and internal standard solution. The spike should be allowed to sit and equilibrate for at least 15 minutes on the thawed fish homogenate aliquot prior to extraction.

Add 5 mL of acetonitrile, homogenize for 2 minutes using an Omniprep multiplace homogenizer or equivalent.

Place homogenate in *freezer (approximately -20 °C.) for a minimum of 1 hour.

*Note: The freezer incubation step may not be necessary for all fish species and tissue types. It may be excluded on a case-by-case basis. Exclusion will be documented appropriately with justification in the prep sheets.

Centrifuge homogenized sample solution at -5°C for 20 minutes at approximately 3000 rpm. The sample must be stored at approximately -20 °C and re-centrifuged if there are delays.

Add 10 μL of 10% formic acid to each 2 mL autovial. Add 1 mL of homogenate supernatant to each vial. Cap autovial and vortex mix.

All sample preparation steps should be recorded appropriately.

10.3 Sample Analysis

Two sets of instrument parameters are given below. The first set provides conditions that are suited for analysis of longer perfluorinated carboxylic acids (C7-C12), sulfonates, and FOSA. The second set of conditions has been optimized for analysis of the smaller acids (C4-C6). Depending on the fish species, tissue type (whole body vs. fillet) and endogenous compounds, LC gradient conditions or column stationary phases may need to be modified to separate the analyte peak of interest from other matrix interferences. Additionally, inclusion of an analyte within an LC method group may be changed depending on matrix interferences.

Because this method is applicable to a wide range of perfluorinated compounds, the total number of transitions that need to be monitored can become quite large depending on the needs of a 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 (dwell time and cycle time effect the number of scans across the chromatographic peak). Therefore, it is recommended that 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 perfluorinated acids, C4, C6, C8 perfluorinated sulfonates, and FOSA may be broken into two or more separate injections to optimize the overall performance if a large number of transitions are required.

The conditions below are intended to be a general guideline and a starting point for method performance optimization. The LC gradient given below will separate PFHS, PFOS, PFHpA, PFOA , PFNA, PFDA, PFUnA, and PFDoA branched isomers from the linear isomer. Gradients may be lengthened or shortened to meet the overall needs of the given project (time constraints, additional isomer separation, separation of matrix interferences, etc..) However, the final LC conditions used should be consistent throughout the study/project for a given fish species and target analyte as much as possible.

10.3.1 Instrument Parameters

10.3.1.1 Conditions Recommended for C7-C12 Carboxylic Acids, Sulfonates, and FOSA

LC Conditions:

Column Temperature (ºC):	30
Injection Volume (µL):	5 to 25*
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 2x50 mm, 5 µm particle size
Extraction Column (placed prior the analytical column with the outlet directed to the column switching valve)	Waters Corporation, Oasis HLB Online Column, 3 x 20 mm, 25 µm particle size
Analytical Column (inlet coming from the column switching valve)	Thermo Fischer Scientific Betasil C_{18}, 2.1 x 100 mm, 5 µm particle size
Column Switching Valve	0 to 5 minutes Position: Left (to waste) 5 minutes to 21 minutes Position: Right (to analytical column)

*If greater sensitivity is needed for lower LOQs, injection volumes up to 50 µL may be used as long as chromatographic integrity is maintained (peak shape, retention time, isomer separation, etc…)

Gradient for Multiperiod Analysis:

Step	Time	A (%)	B (%)	Flow (mL/min)
0	0.00	97	3	0.4
1	3.00	97	3	0.4
2	3.50	70	30	0.4
3	13.5	40	60	0.4
4	15.5	40	60	0.4
5	16.0	10	90	0.4
6	18.0	10	90	0.4
7	18.3	97	3	0.4
8	21.0	97	3	0.4

The gradient shown above will provide an approximate 1.0 minute retention time separation between PFNA and PFDA. A multi-period acquisition method may be constructed where the MRM transitions for PFBS, PFHpA, PFOA, [1,2-$^{13}C_2$]PFOA, PFHS, and PFNA and their respective ISs are collected in the first time period (approximately 0-*11 minutes). The MRM transitions for PFDA, PFOS, [$^{18}O_2$]PFOS, PFUnA, PFDoA, and FOSA and their respective ISs are collected in the second time period (approximately *11-21 minutes).

*It should be noted that the exact location of the period break should be evaluated each time a new analytical column is installed and/or LC tubing is replaced as variability in the columns and tubing lengths may change the absolute retention time of the analytes. Additionally, as the columns age, retention times may begin to drift to shorter times. Care should be taken that the period break does not clip any of the peaks of interest.

10.3.1.2 Conditions Recommended for Shorter Acids (C_4-C_6)

LC Conditions:

Column Temperature (ºC):	30
Injection Volume (µL):	5 to 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 2x50 mm, 5 µm particle size
Analytical Column	Thermo Fischer Scientific Prism™ RP 2x50 mm, 5 µm particle size

Gradient:

Time	A (%)	B (%)	Flow (mL/min)
0.00	90	10	0.3
3.00	90	10	0.3
3.50	30	70	0.3
9.00	5.0	95	0.3
15.0	5.0	95	0.3
15.1	90	10	0.3
19.0	90	10	0.3

10.3.2 Mass Spectrometer Parameters

Samples are to be analyzed via electrospray ionization in the negative-ion MRM mode. The table below provides a list of several PFAAs that may be analyzed using this method and the corresponding mass transitions, and suggested dwell time.

Table 3. MRM Transitions for Target Analytes, Surrogates, and Internal Standards

Compound	***Analyte Description***	***[1]MRM Transition(s)***	***Dwell Time (ms)***
PFBA (C4 Acid)	Target	213>169	100
PFPeA (C5 Acid)	Target	263>219	100
PFHxA (C6 Acid)	Target	313>269 313>119	100 100
PFHpA (C7 Acid)	Target	363>319 363>169	50 50
PFOA (C8 Acid)	Target	413>369 413>219 413>169	50 50 50
PFNA (C9 Acid)	Target	463>419 463>219 463>169	50 50 50
PFDA (C10 Acid)	Target	513>469 513>269 513>219	50 50 50
PFUnA (C11 Acid)	Target	563>519 563>269 563>219	50 50 50
PFDoA (C12 Acid)	Target	613>569 613>319 613>169	50 50 50
PFBS (C4 Sulfonate)	Target	299>80 299>99	50 50
PFHS (C6 Sulfonate)	Target	399>80 399>99	50 50
PFOS (C8 Sulfonate)	Target	499>80 499>99 499>130	50 50 50
FOSA (C8 Sulfonamide)	Target	498>78	50
[1,2,3,4 -$^{13}C_4$]PFBA	IS for PFBA and PFPeA	217>172	100
[1,2 -$^{13}C_2$]PFHxA	IS for PFHxA	315>270	100
[1,2,3,4-$^{13}C_4$]PFOA	IS for PFHpA and PFOA	417>372	50
[1,2,3,4,5-$^{13}C_5$]PFNA	IS for PFNA	468>423	50
[1,2 -$^{13}C_2$]PFDA	IS for PFDA	515>470	50
[1,2 -$^{13}C_2$]PFUnA	IS for PFUnA	565>520	50
[1,2 -$^{13}C_2$]PFDoA	IS for PFDoA	615>570	50
[$^{18}O_2$]PFBS	IS for PFBS	303>84	50
[$^{18}O_2$]PFHS	IS for PFHS	403>84	50
[1,2,3,4- $^{13}C_4$]PFOS	IS for PFOS	503>80	50
[$^{18}O_2$]FOSA	IS for FOSA	502>82	50
[1,2 - $^{13}C_2$]PFOA	Surrogate (Acids)	415>370	50
[$^{18}O_2$]PFOS	Surrogate(Sulfonates, FOSA)	503>84	50

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. 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 been documented in literature as interferences in some biological tissues arising from the presence of 5-pregnan-3,20-diol-3-sulfate isomers and taurodeoxycholate isomers (bile salt), respectively.[2]

Each time a new species is analyzed, the area count ratios of the individual MRM transitions in the sample extracts should be compared to those of a solvent standard as a verification of analyte specificity and matrix interference, especially for PFOS and PFHS where known interferences with the 499>80, 499>99, 399>80 and 399>99 transitions could be present [2,4]. Relative percent differences of area count ratios of the MRM transitions between sample extracts and solvent within ±25% indicate that the measured sample results are largely free from unidentified matrix interferences. This should be performed, at a minimum, for PFOS and PFHS, for one sample for each new species evaluated.

11 Sample Data Analysis and Calculations

Calculate the amount of analyte in fish tissue extract (ng/mL) using the appropriate calibration curve and applying all necessary conversion factors.

Calculate the amount of analyte in fish tissue as follows:

$$\text{Fish Tissue Conc.}\left(\frac{ng}{g}\right) = \frac{\text{Analytical Result}\left(\frac{ng}{mL}\right) * \text{Final Extract Volume(mL)}}{\text{Tissue Mass Extracted (g)}}$$

Include other dilution factors as appropriate.

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

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

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:

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

Assuming the control matrix is clean (endogenous concentration < LLOQ), 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, the percent recovery is calculated as:

$$\text{Percent recovery} = \frac{[A] - [B]}{\text{Amount of analyte spiked (ng)}} \times 100\%$$

Where

$$[A] = \text{ng detected in matrix spiked sample} = \text{Detected LMS Conc.}(\frac{ng}{mL}) * \text{Final Extract Volume}(mL)$$

and

$$[B] = \text{ng equivalence of non} - \text{spiked sample} = \text{LMS fish tissue mass}(g) * \text{Endogenous Conc. of tissue}(\frac{ng}{g})$$

12 Method Performance

12.1 System Suitability

The system suitability injections must have peak area count ratios (analyte area counts/IS area counts) with an RSD ≤10% 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 sample analysis.

12.2 Calibration

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). If the extracted calibration curve does not contain at least a minimum of six passing standards, the batch will be reanalyzed. Passing standards, when back-calculated (including ULOQ) shall fall within ±25%, except for the LLOQ, which can be within ±30% of the nominal value. Values falling outside these limits must be discarded with proper technical justification.

12.3 Continuing Calibration Verification (CCV)

CCVs should be within 75% -125%. Samples not bracketed by passing CCVs or the standard curve and a CCV shall be rerun.

12.4 Sensitivity

If extracted matrix-matched or surrogate matrix-matched calibration is used, the analyte response (peak area counts) of the limit of quantitation (LOQ) standard for each analyte shall be at least two times the analyte response in the highest control matrix blank. If the control matrix blank responses produce a %RSD ≤ 30%, then the average matrix blank response may be used to evaluate the LOQ. However, if the %RSD is greater than 30%, the largest matrix blank response will be used for the comparison, i.e., the response of the LLOQ must be greater than two times the highest matrix blank response.

If unextracted acetonitrile solvent calibration is used, the same criteria described above shall be used except the extracted aqueous blanks will be used for evaluation instead of the control matrix blanks. Endogenous levels in the control matrix (matrix blanks spiked with IS) may be quantitated using the solvent calibration if method accuracy is met. (See next section.)

12.5 Accuracy

12.5.1 LCS Samples

At least two-thirds of the LCS samples shall be within ± 30% of their respective nominal value. One-third of the LCS samples may be outside the ± 30% of their respective nominal value, but not more than one at any given spike level. LCSs not meeting the criteria stated above will be considered on an individual basis as data quality objectives for individual projects may vary.

As stated in Section 8.2, three levels of LCS are recommended; however, it is likely that one level may need to be disregarded because the prepared spike level did not fall within the needed range. Ideally, each preparation batch should have two usable levels of LCSs. If this is not achieved, the project lead will consider what action is needed on an individual basis.

If the control matrix has measurable levels of target analytes, the LCS concentrations should be adjusted to account for the endogenous levels present in the tissue. Endogenous concentrations may be determined by method of standard addition (extracted calibration) or by quantitation of the matrix blanks spiked with ISs (solvent calibration).

12.5.2 Matrix Spikes

Target analyte and/or surrogate matrix spike recoveries shall be within ± 30% of their respective spiked value. Samples producing matrix spike recoveries exceeding ± 30% will be considered on an individual basis. However, the following approaches may be taken to handle such cases:

- The affected samples results will be flagged and footnoted appropriately in the final report and assigned a sample-specific analytical uncertainty adjusted to reflect the matrix spike recovery.
- Sample results will not be reported. A footnote in the data table in the final report will indicate the results are not reported due to sample QC failures.
- Samples and laboratory matrix spikes will be re-prepared, possibly at different spike levels if necessary, and analyzed. This approach should be considered if the original lab matrix spikes were prepared at levels inappropriate for the resulting endogenous concentration (i.e. spike level was less than one-half the endogenous level). Depending on the results of the reanalysis, sample results may or may not be reported.

12.5.3 Analytical Method Uncertainty

The analytical uncertainty should be determined based on historical QC data to evaluate method accuracy and precision. The method uncertainty is determined by statistical evaluation of the individual analyte recovery in laboratory control spike (LCS) samples. The standard deviation is calculated for the set of recovery results (in %). The expanded uncertainty is calculated by multiplying the standard deviation by a factor of 2, corresponding to a confidence limit of 95%. A minimum of twenty data points, but no more than a maximum of fifty points, should be used to determine method uncertainty by this method for a given project or study [5]. The analytical method uncertainty will be determined separately for fillets and whole-body fish analyses. If evidence strongly suggests that there is a species-specific dependence on method uncertainty, separate control charts will be developed for a given species.

If there are fewer than twenty data points for a given species and/or tissue type, the analytical uncertainty is determined using lab control spike results generated for the given project, until a total of twenty points have been compiled.

12.6 Precision

Method precision will be evaluated by calculating the relative standard deviation (%RSD) for LCS samples. The percent RSD, for each analyte, shall be within ± 20% for all LCSs (collectively). Precision data outside ± 20% may be flagged and reported as outside method acceptance criteria. The report will address this departure from method criteria.

12.7 Sample Duplicates

The sample and sample duplicate should have a relative percent difference within 20%. If the relative percent difference is not within 20% for these two samples, the results will be reported and footnoted as having a RPD of greater than 20%.

13 Pollution Prevention and Waste Management

Biological 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.

14 Records

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

15 Revisions

Version 1.0

Verbage updates throughout to reflect current practices. Specific changes include the following:

- List of internal standards and surrogates updated to reflect current availability.
- The use of solvent calibration can be used and is discussed throughout.
- Updated the LC gradient used for multi-period acquisition method.
- Updated the MRM transition table.
- Added MRM transition area count ratio analysis for specificity

16 References

[1] 3M Environmental Laboratory Study E08-0261 “Method Validation of ETS 8-45 – Determination of Fluorochemicals via Protein Precipitation of Fish Tissues (Fillet or Whole Body) and Analysis by High Performance Liquid Chromatography with Tandem Mass Spectrometry”.

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

[3] ETS 4-013 “Documentation of Solutions and Standards Preparation”.

[4] Keller, J. M. et. al, **Anal. Bioanal. Chem** 2009, DOI 10.1007/s00216-009-322-x. Published online 28 October 2009.

[5] ETS 12-012 “Estimation of Uncertainty of Measurements”.