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Title: Determination and Confirmation of PFOA and PFOS by UPLC/MS/MS		
Revision: 01	Replaces: CLG-PFC1.00	Effective: 11/05/2009

Contents

A. INTRODUCTION

1. Theory

Perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) are extracted from serum with acidified acetonitrile and from muscle with a base digestion followed by a solid phase extraction. Quantitation is performed by LC/MS/MS with Ultra Performance Liquid Chromatography (UPLC) and triple quadruple mass spectrometer in electrospray negative ion (ESI-) mode. Confirmation is based on comparison of sample LC retention time and product ion abundance ratios against those obtained for a positive control (recovery).

2. Applicability

This method is applicable for the determination and confirmation of PFOA and PFOS in bovine serum at levels ≥ 10 ppb and bovine muscle at levels ≥ 20 ppb.

3. Structures

PFOA	$CF_3(CF_2)_6COOH$	CAS No. 335-67-1
PFOS	$CF_3(CF_2)_7SO_3H$	CAS No. 1763-23-1

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

1. Apparatus

a. Analytical Balance - Readable to 0.1 mg, Model AE 160, Mettler.

b. Centrifuge Tubes - 15 mL (polypropylene) Falcon tubes, Cat. No. 352096, Becton Dickinson.

c. Variable volume pipettors - Automatic pipettors capable of accurately delivering 2 – 5,000 µL.

d. Vortex Mixer - Vortexer-2, Model G-560, VWR.

e. Centrifuge - Sorvall RC-4, Kendro laboratory Products, Inc., Asheville, NC.

f. Glass volumetric flasks, Class A.

g. Food Homogenizer – Robot Coupe model RSI-2Y-1, Robot Coupe USA.

h. SPE Cartridges – OASIS WAX, Part Number 186002490, Waters Corp.

i. 0.45 µm PTFE Membrane Filter – Part Number 4422T, Pall Life Sciences.

j. Sonicator – Branson 8510, Part Number CPN-952-818, Branson Ultrasonics.

k. Disposable Culture Tubes – 16 x 100 mm glass tubes, Part Number 73500 16100, Kimble Glass Inc.

l. 50 mL Glass Centrifuge Tubes – Part Number 89002-222, VWR Scientific.

m. Homogenizer – Polytron model PT 10-35 GT, Kinematica Inc.

2. Instrumentation

a. LC/MS/MS - Waters Acquity UPLC equipped with Waters Acquity TQD triple quadrupole mass spectrometer.

b. UPLC column - Waters Acquity UPLC BEH C18 1.7 µm, 2.1 x 50 mm.

c. PFC Isolator Column - Waters Acquity UPLC PFC Isolator Column (if necessary)

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted.

1. Reagents

a. Acetonitrile - HPLC grade, Cat. No. 015-4, Burdick & Jackson.

b. Methanol - HPLC grade, Cat. No. 230-4, Burdick & Jackson.

c. Formic Acid - 98%, Cat. No. 94318, Fluka Analytical.

d. Water - Deionized, HPLC grade, Millipore Milli-Q System.

e. Ammonium Acetate - Mass Spectrometric grade, Cat. No. 73594-25G-F, Sigma-Aldrich.

f. Serum Control - Fetal Bovine Serum, Certified, Cat. No. 16000-044, Invitrogen Corporation.

g. Ammonium Hydroxide - 28-30 wt % Solution of NH_3 in H_2O, Cat. No. 205840025, ACROS Organics.

h. Sodium Hydroxide - ≥ 95%, Cat. No. 567530, EMD Chemicals.

i. Sodium Acetate Trihydrate - ≥ 99.0%, Cat. No. 71193, Fluka.

j. Glacial Acetic Acid - Reagent grade, Cat. No. A38-212, Fisher.

2. Solutions

a. Mobile Phase A (2 mM ammonium acetate aqueous with 5% methanol):

Weigh 0.154 g ammonium acetate and place into a 1 L volumetric flask. Add 50 mL of methanol and bring to 1 L volume with fresh Milli-Q water.

b. Mobile Phase B:

100% Methanol

c. 0.1 M Formic Acid:

Add 4.6 mL formic acid to a 1 L volumetric flask and dilute to volume with Milli-Q water.

d. 2 mM Ammonium Acetate aqueous solution:

Weigh 0.154 g ammonium acetate and place into a 1 L volumetric flask and bring to 1 L volume with fresh Milli-Q water.

e. 2 mM Ammonium Acetate/2 mM Ammonium Hydroxide:

Weigh 0.0154 g ammonium acetate and place into a 100 mL volumetric flask, add 26.92 µL of 29% ammonium hydroxide and bring to volume with fresh Milli-Q water.

f. 0.1% Ammonium Hydroxide:

Pipette 1.0 mL of ammonium hydroxide into a 1 L volumetric flask. Bring to volume with methanol.

g. 10 mM Sodium Hydroxide in Methanol:

Weigh 0.4 g sodium hydroxide into a 1 L volumetric flask, bring to volume with methanol.

h. 25 mM Sodium Acetate Buffer:

Weigh 3.402 g sodium acetate trihydrate into a 1 L volumetric flask, bring to volume with fresh Milli-Q water. Check pH and bring to 4.0 ± 0.1 with glacial acetic acid if necessary.

D. STANDARDS

Note: Equivalent standards may be substituted.

1. Source

For Serum:

Reference standard solution containing PFOA and PFOS in methanol with a concentration of 2.0 µg/mL, approximately 98% pure, stored in sealed ampoule. Cat. No. PFC-MXB, Wellington Laboratories (Guelph, Ontario, Canada).

For Muscle:

mPFOA IS: Internal Reference standard solution containing mPFOA in methanol with a concentration of 50.0 µg/mL, approximately 98% pure, stored in sealed ampoule. Cat. No. MPFOA, Wellington Laboratories (Guelph, Ontario, Canada).

mPFOS IS: Internal Reference standard solution containing mPFOS in methanol with a

concentration of 50.0 µg/mL, approximately 98% pure, stored in a sealed ampoule. Cat. No. MPFOS, Wellington Laboratories (Guelph Ontario, Canada).

Perfluorooctanoic Acid (PFOA) – 96%, Cat. No. 171468, Aldrich Chemical Co.

Heptadecafluorooctanesulfonic Acid (PFOS) – 98%, Cat. No. HO781, TCI America.

2. Preparation

Note: Different perfluorinated compound (PFC) solution concentrations may be prepared as long as fortification volumes are adjusted accordingly.

a. Serum

i. PFC Intermediate solution (0.5 µg/mL):

Pipet 250 µL of the stock standard solution (2.0 µg/mL) into a 1.0 mL volumetric flask and bring to volume with methanol.

ii. PFC Intermediate solution (0.05 µg/mL):

Pipet 250 µL of the stock standard solution (2.0 µg/mL) into a 10 mL volumetric flask and bring it to volume with methanol.

iii. LC/MS/MS Matrix-Matched Standard Curve Solutions for Serum:

Each of the calibration curve standards is prepared and extracted by following the procedure for serum samples as described in F.1.a.

b. Muscle

i. PFOA Stock Standard (1.0 mg/mL):

Weigh 10 mg of neat PFOA standard into a 10 mL volumetric flask. Bring to volume with methanol.

ii. PFOS Stock Standard (1.0 mg/mL):

Weigh 10 mg of neat PFOS standard into a 10 mL volumetric flask. Bring to volume with methanol.

iii. PFOA and PFOS Working Standards:

(a) PFOA and PFOS Working Standard (2.0 µg/mL):

Pipet 200 µL of the PFOA stock standard and 200 µL of the PFOS stock standard into a 100 mL volumetric flask. Bring to volume with methanol.

(b) PFOA and PFOS Working Standard (1.0 µg/mL):

Pipet 5.0 mL of the PFOA and PFOS working standard (2.0 µg/mL) into a 10.0 mL volumetric flask. Bring to volume with

methanol.

(c) PFOA and PFOS Working Standard (0.5 µg/mL):

Pipet 5.0 mL of the PFOA and PFOS working standard (1.0 µg/mL) into a 10.0 mL volumetric flask. Bring to volume with methanol.

(d) PFOA and PFOS Working Standard (0.25 µg/mL):

Pipet 5.0 mL of the PFOA and PFOS working standard (0.5 µg/mL) into a 10.0 mL volumetric flask. Bring to volume with methanol.

iv. mPFOA and mPFOS Working Standard (0.5 µg/mL):

Pipet 100 µL of the Wellington mPFOA IS (50.0 µg/mL) and 100 µL of the Wellington mPFOS IS (50.0 µg/mL) into a 10 mL volumetric flask and bring to volume with methanol.

v. LC/MS/MS Matrix-Matched Standard Curve Solutions for Muscle:

Each of the calibration curve standards is prepared and extracted by following the procedure for muscle samples as described in F.2.d.

3. Storage and Stability

The standard mixture in D.1 is to be stored refrigerated (2 - 8 °C) and is stable for 2 years.

The working standard solutions are to be stored refrigerated (2 - 8 °C) and are stable for 1 year.

E. SAMPLE PREPARATION

1. Muscle

Allow sample to thaw and trim sample of extraneous connective tissue. If only a sub-sample is to be prepared, assure the sub-sample is representative of the entire sample. Using a food processor, thoroughly blend tissue to homogenize. Immediately transfer mixture to a suitable container and store in a freezer at ≤ -10 °C. Avoid any unnecessary freeze/thaw cycles.

2. Serum

Thaw serum sample and mix well until homogenous before use.

Store samples at ≤ - 10 ºC when not in use.

F. ANALYTICAL PROCEDURE

1. Extraction for Serum

   a. Pipet 50 µL of thawed serum sample into 15 mL Falcon centrifuge tube. Pipet two 50 µL aliquots of blank serum into separate 15 mL Falcon centrifuge tubes. Use the first tube as negative blank and fortify the second tube as a 40 ppb positive control (recovery) by adding a 4 µL aliquot of working standard (500 ng/mL).

   At this time calibration curve standards are prepared. Pipet 50 µL of thawed serum matrix control blank sample into a 15 mL Falcon centrifuge tube for each standard. Fortify as follows:

Standard concentration in serum (ppb)	Fortification solution used (µL)	Conc. of fortification solution (ng/mL)
10	10	50
20	20	50
40	4	500
80	8	500
160	16	500

   b. Add 100 µL 0.1 M formic acid to each tube. Cap and vortex mix for 30 seconds.

   c. Add 1000 µL of cold acetonitrile to each tube. Cap and vortex mix for 30 seconds.

   d. Centrifuge at 2000 *g* for 3 minutes.

   e. Pipet 250 µL of the clear supernatant and transfer to an auto-sampler vial.

   f. Add 750 µL of the 2 mM ammonium acetate aqueous solution to the extract in the auto-sampler vial.

   g. Cap the vial and vortex mix briefly for ~1 second. The final sample extract is ready for LC/MS/MS analysis.

2. Extraction for Muscle

   a. Weigh 2.0 ± 0.05 g of thawed tissue into 50.0 mL glass centrifuge tubes.

   b. Pipet 2.0 mL of fresh Milli-Q water into each sample, vortex until well mixed. Homogenize samples with a Polytron homogenizer until sample is completely

blended with the water.

c. Place a 1.0 ± 0.05 g subsample of the homogenate (equivalent to 0.5 g muscle) into a 15.0 mL polypropylene tube. Use one tube as a negative blank and another as the 40 ppb positive control. To make the 40 ppb positive control add 40 µL of the 0.5 µg/mL PFOA and PFOS working standard (D.2.b.iii.(c)).

d. At this time calibration curve standards for muscle are prepared. Fortify as follows:

Standard concentration in tissue matrix (ppb)	µL of fortification solution used	Concentration of fortification solution (µg/mL)
20	40	0.25
40	40	0.50
80	40	1.0

e. Add the internal standard to all samples by pipetting 160 µL of the mPFOA and mPFOS Working Standard solution (0.5 µg/mL).

f. Add 9.0 mL of 10 mM sodium hydroxide in methanol solution to the 15.0 mL polypropylene tube, invert several times to mix.

g. Allow samples to digest for 30 minutes in a room temperature ultrasonic bath.

h. Centrifuge samples at 2,000 x *g* for 5 minutes.

i. WAX Cartridge Clean Up: Attach 60.0 mL reservoirs to WAX column cartridges on a vacuum manifold. Condition the cartridges with 4.0 mL 0.1% ammonium hydroxide in methanol followed by 4.0 mL methanol followed by 4.0 mL Milli-Q water. After conditioning is complete, close all stopcocks. Pipet 1.0 mL of the supernatant (equivalent to 50 mg muscle) and 9.0 mL of fresh Milli-Q water into the 60.0 mL reservoir. Open stopcocks and allow sample to load onto the column ~ 1 drop/second. Rinse the cartridge with 4.0 mL of 25 mM sodium acetate buffer followed by 4.0 mL of methanol.

j. Elute the residue with 4.0 mL 0.1% ammonium hydroxide in methanol (elute into a 16x100 mm disposable glass tube).

k. Evaporate the 0.1% ammonium hydroxide in methanol eluate to dryness under a stream of nitrogen in a < 70 °C water bath.

l. Reconstitute the residue with 200 µL 2 mM ammonium acetate / 2 mM ammonium hydroxide and 800 µL of methanol to yield the final extract.

m. Vortex for approximately 10 seconds.

n. Filter the extract into an autosampler vial for LC/MS/MS analysis using 0.45 µm PTFE filters.

3. LC/MS/MS Analysis

Note: Frequent instrument cleaning is recommended.

a. UPLC Instrumental Settings

To minimize possible PFC background levels, sonicate mobile phases and bypass instrument degasser. If needed, install and use PFC isolator column (B.2.c).

Note: The following instrument parameters may be optimized:

Column temperature: 50 °C

Injection volume: 20 µL

For serum use the following conditions:

Time (min)	Flow Rate (mL/min)	(A) Aqueous (%)	(B) Organic (%)
0.0	0.5	75	25
0.5	0.5	75	25
3.5	0.5	10	90
3.6	0.5	0	100
4.5	0.5	0	100
4.6	0.5	75	25
6.0	0.5	75	25

For muscle use the following isocratic conditions:

Column temperature: 50 °C

Injection volume: 15 µL

Time (min)	Flow Rate (mL/min)	(A) Aqueous (%)	(B) Organic (%)
0.0	0.3	20	80
2.0	0.3	20	80

b. MS/MS Parameters

Ion Mode: Negative ion ESI

Source Temperature: 150 ºC
Desolvation Temperature: 450 ºC

Dwell Time (s): 0.05
Capillary Voltage: -3.0 kV

Note: Tune the instrument as needed.

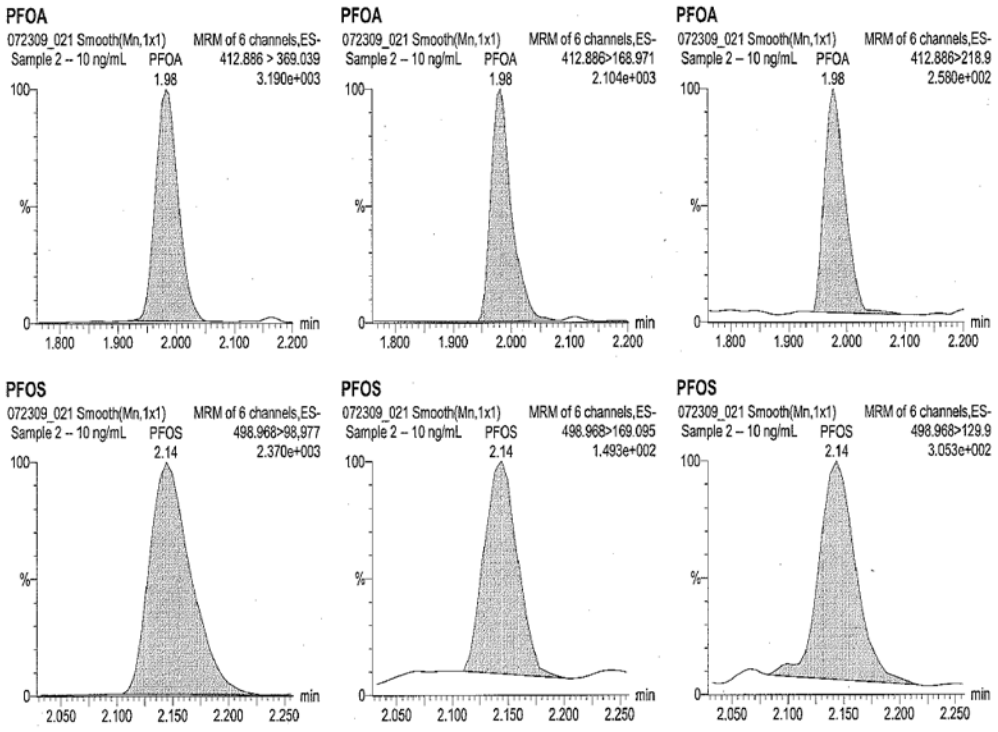
c. Summary of Multiple Reaction Monitoring (MRM) transitions and parameters selected for each Compound:

Compound	Serum RT (min)	Muscle RT (min)	Precursor Ion (m/z)	Cone (V)	Product ions (m/z)	Collision (eV)
PFOA	2.0	0.62	412.9	20	168.9	16
				20	218.9	20
				20	**369.0**	12
PFOS	2.2	0.65	498.9	70	**98.9**	40
				70	129.9	40
				70	169.1	38
mPFOA	NA	0.62	417.0	20	372.0	10
mPFOS	NA	0.65	503.0	70	80.0	40

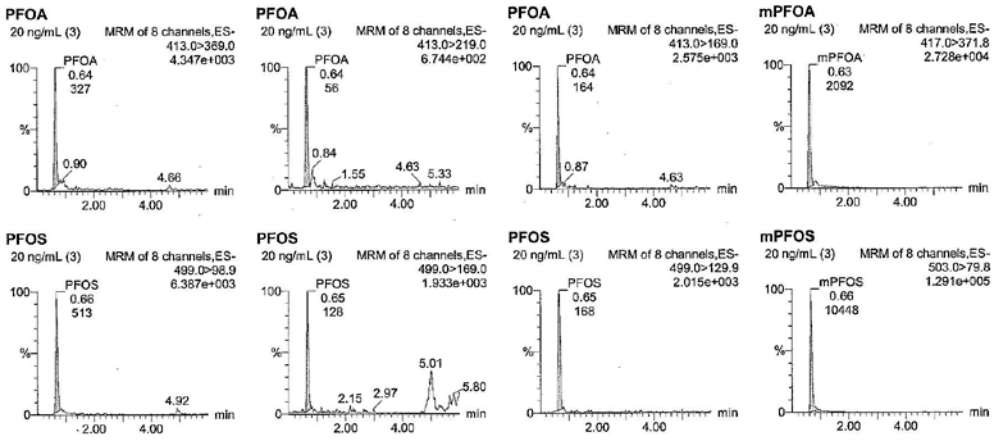
Note: Quan ion (most abundant product ion, used for quantitation) is in **Bold**

4. Sample Chromatograms

   a. PFOA and PFOS, 10 ppb positive control (recovery) in serum



   b. PFOA and PFOS, with internal standards mPFOA and mPFOS, 20 ppb positive control (recovery) in muscle



G. DETERMINATION AND CONFIRMATION

1. Determinative instructions and criteria:

   a. Ensure that all quantitation ions used for constructing the standard curve are present at a signal to noise ratio > 5.

   b. Using linear regression analysis, calculate the slope, intercept, and correlation coefficient of a standard curve for each compound (PFOA and PFOS). This is constructed by plotting peak areas versus concentration (ng/mL) for the matrix-matched injection standards listed in F.1.a for serum or F.2.d for muscle.

   c. For serum, the concentration of each analyte can be calculated using the following equation:

      ng/mL PFC analyte = (A – B) / C where

      A = PFC analyte peak area

      C = slope of the calibration curve

      B = intercept from the calibration curve

      Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

      For muscle, each calibration curve is created using the ratio (PFOS/mPFOS) and (PFOA/mPFOA) plotted against the concentration of the respective analyte. The concentration of the analyte in the sample is calculated using the following equation:

      ng/mL PFC analyte = (A – B) / C where

      A = (Peak area of quan ion of PFC)/(Peak area of mPFC)

      C = slope of the calibration curve

      B = intercept from the calibration curve

   d. Calculate results when the following conditions are met:

      i. The correlation coefficient for the standard curve is greater than 0.99

      ii. The recovery of the positive control falls within the limits specified in section I.1.

2. Confirmation criteria

   a. Retention time of the sample must be ± 5% of the fortified control.

   b. Monitored ions for PFOA and PFOS will be assessed as follows:

      Product ion abundance ratios must meet that of the reference within ± 20% absolute difference.

The reference is the 40 ppb calibration curve point or 40 ppb positive control.

c. The negative control (tissue blank):

i. must confirm negative for both compounds following G.2.a and G.2.b, or

ii. the area count of the quan ion must be less than the area count of the quan ion for the lowest calibration curve point (10 ppb for bovine serum; 20 ppb for bovine muscle).

d. The positive control:

i. Serum

(a) must confirm positive for both compounds following G.2.a and G.2.b, and

(b) the area count of the quan ion for each native compound (PFOA and PFOS) must lie within the range of the curve.

ii. Muscle

(a) must confirm positive for both compounds following G.2.a and G.2.b, and

(b) the monitored ratio used for quantitation for each native compound (PFOA and PFOS) must lie within the range of the curve.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment: protective clothing, eyewear, and gloves.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Organic Solvents (Acetonitrile, Methanol)	Flammable, vapors are corrosive to the skin, eyes, and respiratory system.	Use only in an efficient chemical fume hood, away from any electrical or heating devices.
Concentrated Formic acid	Acid burns	Prepare acid solutions with care in a well-ventilated area. Wear protective eyewear, gloves, and clothing when handling
PFOA	Persistent, bioaccumulative and toxic to mammalian species	Handle with care in a well-ventilated chemical fume hood. Avoid contact with skin, eyes.

PFOS	Persistent, bioaccumulative and toxic to mammalian species.	Handle with care in a well-ventilated chemical fume hood. Avoid contact with skin, eyes.

3. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Organic Solvents (see above)	Flammable, vapors are corrosive to the skin, eyes, and respiratory system.	Collect waste in tightly sealed container. Store away from non-compatibles in a cool, well ventilated, flammable liquid storage area for disposal in accordance with local, state, and Federal regulations.
Concentrated Formic acid	Acid Burns	Neutralize solutions to meet local, state, and Federal guidelines
PFOA	Persistent, bioaccumulative, and toxic to mammalian species	Collect waste and store in a tightly sealed container. Store away from non-compatibles in a cool, well ventilated storage area for disposal in accordance with local, state and Federal regulations
PFOS	Persistent, bioaccumulative, and toxic to mammalian species	Collect waste and store in a tightly sealed container. Store away from non-compatibles in a cool, well ventilated storage area for disposal in accordance with local, state and Federal regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Analytes	*Analytical Range*		*Acceptable Recovery*	*Acceptable Repeatability (CV)*
	Serum (ppb)	*Muscle (ppb)*		
PFOA, PFOS	10 – 160	20 – 80	70 – 120%	20%

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	Sample volume for serum	50 µL, using properly calibrated pipette
b.	Sample weight for muscle	2.0 ± 0.05 g
c.	Weight of subsample homogenate	1.0 ± 0.05 g

3. Readiness To Perform

   a. Familiarization

      i. Phase I: Matrix-based standards - Create a standard curve on each of 3 consecutive days, which will include the following:

         (a) 10 ppb (for serum only)

         (b) 20 ppb

         (c) 40 ppb

         (d) 80 ppb

         (e) 160 ppb (for serum only)

      ii. Phase II: Fortified samples for serum- 3 replicates at 10 ppb, 40 ppb, and 160 ppb over a period of 3 different days.

         Fortified samples for muscle- 3 replicates at 20 ppb, 40 ppb, and 80 ppb over a period of 3 different days.

         NOTE: Phase I and Phase II may be performed concurrently.

      iii. Phase III: Check samples for analyst accreditation.

(a) A minimum of eight samples are to be quantitated and confirmed at levels unknown to the analyst. One sample is to be unfortified. The remaining samples are fortified from a mixed standard within the analytical range for each analyte.

(b) Report analytical findings to Supervisor and Quality Assurance Manager (QAM).

(c) Authorization from QAM and Supervisor is required to commence official analysis.

b. Acceptability criteria.

Refer to I. 1.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: Once per week per analyst when samples are analyzed.

ii. Records are to be maintained.

b. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Take corrective action.

5. Sample Acceptability and Stability

a. Matrix : Bovine serum and muscle

b. Sample storage:

i. Condition: Frozen, -10 ºC or lower.

ii. Time: 5 years.

6. Sample Set

a. Solvent blank

b. Blank (negative control)

c. Check sample (as needed)

d. Recovery (positive control)

e. Samples

Note: Solvent blanks may be distributed between controls and samples as needed to minimize carryover.

7. Sensitivity
   a. Minimum proficiency level (MPL):
      i. For bovine serum: 10 ppb.
      ii. For bovine muscle: 20 ppb.

J. WORKSHEET

[RESERVED]

K. APPENDIX

1. References
   a. Reiner JL, Nakayama SF, Delinsky AD, Stanko JP, Fenton SE, Lindstrom AB, Strynar MJ. Analysis of PFOA in dosed CD1 mice: Part 1. Methods development for the analysis of tissues and fluids from pregnant and lactating mice and their pups. *Reproductive Toxicology* **27** (2009) 360-364.
   b. PFC Analysis Kit for Acquity UPLC System Guide – Document 71500183002/Revision A. Waters Corporation 2009
   c. Ye X, Strynar MJ, Nakayama SF, Varns J, Helfant L, Lazorchak J, Lindstrom AB. Perflourinated compounds in whole fish homogenates from the Ohio, Missouri, and Upper Mississippi Rivers, USA. Environmental Pollution 156 (2008) 1227-1232.

L. APPROVALS AND AUTHORITIES

1. Approvals on file.
2. Issuing Authority: Director, Laboratory Quality Assurance Division.