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8EHQ-80-373
AR-226-3889

NO CBI

3M

CERTIFIED MAIL

April 9, 2009

Document Processing Center
EPA East – Room 6428 Attn: Section 8(e)
Office of Pollution Prevention and Toxics, U.S. EPA
1200 Pennsylvania Avenue NW
Washington, DC 20460-0001

Re: TSCA 8(e) Substantial Risk Notice: Supplemental to Docket No. 8EHQ-0598-373; Sulfonate-based and Carboxylate-based Fluorochemicals

To whom it may concern:

3M is submitting this notice to supplement its previous submissions on sulfonate and carboxylate-based fluorochemicals.

3M recently received data from a fluorochemical (FC) analytical method validation study in which fish tissues, purchased from Osage Catfisheries, Inc. (Osage Beach, MO), were used as control samples. As detailed in the following table, endogenous levels of various FCs were detected in these tissues. All levels were in the ng/g range and are provided in the enclosed report.

whole-body largemouth bass (Micropterus salmoides)	**whole-body channel catfish** (Ictalurus punctatus)	**whole-body bluegill sunfish** (Lepomis macrochirus) and **fillets of rainbow trout** (Oncorhynchus mykiss)
perfluorooctane sulfonate (PFOS) perfluorobutanoic acid (PFBA) perfluorodecanoic acid (PFDA) perfluoroundecanoic acid (PFUnA) perfluorododecanoic acid (PFDoA)	PFOS PFBA perfluoropentanoic acid (PFPeA) perfluorononanoic acid (PFNA) PFDA PFUnA PFDoA	PFOS PFBA PFUnA

While 3M does not believe that any of these data taken alone or cumulatively meet the "substantial risk" reporting threshold, we nevertheless recognize the ongoing work by U.S. EPA to assess fluorochemical exposure pathways. Therefore, we are placing these results in the 8(e) docket as a supplement to previous submissions.

If you have any questions or would like any additional information, please contact Deanna Luebker at (651) 737-1374 or djluebker@mmm.com.

Sincerely,

Jean B. Sweeney (DL)

Jean B. Sweeney
Staff Vice President, 3M Environmental, Health and Safety Operations

Enclosure

CONTAINS NO CBI

318509

Final Analytical Report

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"

Laboratory Request Number: E08-0261

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Analytical Report 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"

Report Date: March 12, 2009

1 Introduction

This report summarizes the method validation of 3M Environmental Laboratory analytical method 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". The method validation protocol was detailed in the general project outline (Attachment 1) and was developed using the 2001 FDA Bioanalytical Method Validation Guidance for Industry as an analytical reference which requires the use of matrix-matched calibration.(1) The extraction procedure employs acetonitrile protein precipitation and incorporates a cryogenic incubation step. Extracts were analyzed using liquid chromatography tandem mass spectrometry (LC/MS/MS) for perfluoroalkane carboxylic acids (PFCAs) ranging from C4 to C12, perfluoroalkane sulfonates (C4, C6, and C8), and perfluorooctanesulfonamide. A comprehensive validation is presented for whole-body homogenates of largemouth bass (*Micropterus salmoides*) and method cross-validation results are shown for whole-body channel catfish (*Ictalurus punctatus*), whole-body bluegill sunfish (*Lepomis macrochirus*), and rainbow trout fillets (*Oncorhynchus mykiss*). All data presented here were generated using fish from a supplier for scientific studies. No environmental samples were used.

For the full method validation, linearity, precision, and accuracy were determined in three separate extraction batches prepared over the course of two separate days which provided inter-day and intra-day statistics. Each preparatory batch consisted of the following samples: thirteen point matrix-matched calibration curve ranging from 0.025 ng/g to 25 ng/g spiked tissue concentrations, four matrix blanks (two with internal standards (ISs) and surrogates, two without ISs and surrogates), four method (aqueous) blanks (two with ISs and surrogates, two without ISs and surrogates), two acetonitrile solvent blanks with ISs and surrogates, triplicate lab control matrix spikes at three levels, and triplicate lab control matrix spikes of perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS) from 3M electrochemical fluorination (ECF) production lots. On the first extraction day, additional PFOS "dilution" QC samples were also prepared where the PFOS concentration (ppm range) was two to three orders of magnitude higher than the rest of the target analytes (ppb range). Additionally, an alternate approach to PFOS quantitation was explored using a species specific matrix-matched calibration curve prepared using [1,2,3,4- $^{13}C_4$]PFOS. The cross validation procedures for the three other fish species represented an abbreviated approach to the full validation performed for the whole-body largemouth bass. Finally, this study explored the use of a solvent (unextracted) calibration curve for quantitation of the fish extracts.

2 Study Information

2.1 Target Analytes.

Table 1 below provides pertinent information regarding the target analytes, internal standards, and surrogate compounds investigated in this method validation. Table 2 lists the 3M Environmental Laboratory identification numbers for the control tissues used.

Table 1. Target Analyte Summary.

Compound Name	*Synonym or Acronym*	*Analytical Purpose*	*Formula*	*Reference Standard Source*
Perfluorobutanoic acid	PFBA (C4 Acid)	Target	C_3F_7COOH	Aldrich
Perfluoropentanoic acid	PFPeA (C5 Acid)	Target	C_4F_9COOH	Alfa Aesar
Perfluorohexanoic acid	PFHxA (C6 Acid)	Target	$C_5F_{11}COOH$	Oakwood Products
Perfluoroheptanoic acid	PFHpA (C7 Acid)	Target	$C_6F_{13}COOH$	Aldrich
Perfluorooctanoic acid	PFOA (C8 Acid)	Target	$C_7F_{15}COOH$	L-PFOA (linear) Wellington Labs
			$[C_7F_{15}COO^-][NH_4^+]$	ECF PFOA (branched) 3M Production Lot 332
Perfluorononanoic acid	PFNA (C9 Acid)	Target	$C_8F_{17}COOH$	Oakwood Products
Perfluorodecanoic acid	PFDA (C10 Acid)	Target	$C_9F_{19}COOH$	Oakwood Products
Perfluoroundecanoic acid	PFUnA (C11 Acid)	Target	$C_{10}F_{21}COOH$	Oakwood Products
Perfluorododecanoic acid	PFDoA (C12 Acid)	Target	$C_{11}F_{23}COOH$	Oakwood Products
Perfluorobutane sulfonate	PFBS (C4 Sulfonate)	Target	$[C_4F_9SO_3^-][K^+]$	3M Production Lot 2
Perfluorohexane sulfonate	PFHS (C6 Sulfonate)	Target	$[C_6F_{13}SO_3^-][Na^+]$	L-PFHS (linear)- Wellington Labs
Perfluorooctane sulfonate	PFOS (C8 Sulfonate)	Target	$[C_8F_{17}SO_3^-][K^+]$	L-PFOS (linear)- Wellington Labs
			$[C_8F_{17}SO_3^-][K^+]$	ECF PFOS (branched) 3M Production Lot 217
Perfluorooctanesulfonamide	FOSA (C8 Sulfonamide)	Target	$C_8F_{17}SO_2NH_2$	3M (L-15709)

Table 1 Continued.

Compound Name	Synonym or Acronym	Analytical Purpose	Formula	Reference Standard Source
$^{13}C_4$-Perfluorobutanoic acid	[1,2,3,4-$^{13}C_4$]PFBA	Surrogate	$^{13}CF_3(^{13}CF_2)_2{}^{13}COOH$	Wellington Labs
$^{13}C_4$-Perfluorooctanoic acid	[1,2,3,4-$^{13}C_4$]PFOA	Internal Standard	$CF_3(CF_2)_5(^{13}CF_2)_3{}^{13}COOH$	Wellington Labs
$^{13}C_2$-Perfluorodecanoic acid	[1,2-$^{13}C_2$]PFDA	Surrogate	$CF_3(CF_2)_7(^{13}CF_2){}^{13}COOH$	Wellington Labs
$^{18}O_2$-Ammonium Perfluorobutane sulfonate	[$^{18}O_2$]PFBS	Surrogate	$[C_4F_9S^{18}O_2O^-][NH_4^+]$	RTI International
$^{18}O_2$-Ammonium Perfluorohexane sulfonate	[$^{18}O_2$]PFHS	Surrogate	$[C_6F_{13}S^{18}O_2O^-][NH_4^+]$	Wellington Labs
$^{18}O_2$-Ammonium Perfluorooctane sulfonate	[$^{18}O_2$]PFOS	Internal Standard	$[C_8F_{17}S^{18}O_2O][NH_4^+]$	RTI International
$^{13}C_4$-Sodium Perfluorooctane sulfonate	[1,2,3,4-$^{13}C_4$]PFOS	Calibration	$[CF_3(CF_2)_5(^{13}CF_2)_3{}^{13}CF_2SO_3^-][Na^+]$	Wellington Labs

Table 2. Control Tissue Summary

Species	3M Environmental Lab ID Number
Whole-body largemouth bass	TN08-0194-1/1
Whole-body catfish	TN08-0199-1/1
Whole-body bluegill	TN08-0203-1/1
Rainbow trout fillet	TN08-0196-1/1

3 Methods - Analytical and Preparatory

3.1 Tissue Preparation

Whole-body largemouth bass (*Micropterus salmoides*), whole-body channel catfish (*Ictalurus punctatus*), whole-body bluegill sunfish (*Lepomis macrochirus*), and rainbow trout fillets (*Oncorhynchus mykiss*) were purchased from Osage Catfisheries, Inc. (Osage Beach, MO). Whole-body and fillet tissues were homogenized frozen in a two-step process. The initial homogenization step occurred at MPI Research (State College, PA). Frozen fish tissue was homogenized with dry ice until a course-meal consistency was reached. After the initial homogenization, the ground fish was transferred to a polyethylene bag which was placed in the freezer unsealed overnight to allow the residual carbon dioxide to sublime. After sublimation, the bag was sealed and homogenates were kept frozen and shipped to the 3M Environmental Laboratory for sample preparation and analysis. Approaching the time of extraction, the frozen course-ground fish tissue was added to a pre-chilled stainless steel bowl of a Robot Coupe RSI 2Y-1 vertical batch processor (Joliet, IL) and re-homogenized with dry ice until a powder-like consistency was reached. Again, the tissue homogenates were transferred to a pre-chilled polyethylene bag and the residual dry ice was allowed to sublime overnight.

3.2 Sample Extraction

Polyethylene centrifuge tubes (50 mL) were chilled in a cooler with dry ice for approximately thirty minutes prior to weighing homogenate aliquots to prevent the tissue powder from thawing and congealing during the weighing process. A 0.5 g sub-sample of the fish tissue homogenate was accurately weighed in a pre-chilled 50 mL polyethylene centrifuge tube. Tissue aliquots were then spiked with internal standard (IS) to produce an approximate concentration of 1 ng/g. Samples designated as matrix-matched calibration standards were additionally spiked with the target and surrogate analytes at appropriate levels to produce the desired tissue concentrations. Samples

intended for laboratory QC (matrix blanks and laboratory control matrix spikes) were spiked with surrogates at 1 ng/g and the other target analytes at appropriate levels. After spiking, the tissue homogenates were allowed to sit for 30 minutes before 5 mL of acetonitrile was added. The samples were then thoroughly homogenized with the acetonitrile using an Omni Prep multi-place homogenizer with disposable plastic probes (15,000 rpm for 2 minutes). The acetonitrile/tissue extracts were then placed in a freezer at -20°C for at least one hour. Upon removal from the freezer, sample extracts were centrifuged at -5°C for 20 minutes at 3000 rpm. If any delays occurred, samples were returned to the -20°C freezer and then re-centrifuged prior to resumption of preparation and analysis.

After centrifugation, 1 mL of clarified supernatant was transferred to a 2 mL autovial spiked with 10 µL of 10% formic acid. Autovials were then capped and vortex mixed.

3.3 Analysis

Analysis for the suite of PFCs listed above was performed using high performance liquid chromatography-tandem mass spectrometry (HPLC/MS/MS). For this investigation, samples were analyzed using an Agilent 1100 series (Palo Alto, CA) HPLC system interfaced to either a PE SCIEX API 4000 triple quadrupole or SCIEX API 4000 Q-Trap mass spectrometer (Foster City, CA). Both instruments were equipped with a SCIEX Turbo V ion-spray interface operating in the negative ion MS/MS mode using multiple reaction monitoring (MRM). Analyst™ 1.4.2 software was used for all data collection and reduction. Table 3 lists the MRM transitions monitored for each analyte. A Thermo Scientific PRISM RP guard column (2.1mm × 50 mm; 5µ particle size) was placed in-line after the purge valve and before the sample injection port to trap any PFC contaminants coming from the HPLC instrument and/or the mobile phases. This sufficiently separated the elution of the "system" PFC peaks from those present in the sample extracts.

Table 3. MRM Transition Summary.

Compound	Analyte Description	[1]MRM Transition(s)	Dwell Time (ms)
PFBA (C4 Acid)	Target	213>169	100
PFPeA (C5 Acid)	Target	263>219	100
		313>269	100
PFHxA (C6 Acid)	Target	313>119	100
		363>319	50
PFHpA (C7 Acid)	Target	363>169	50
		413>369	50
		413>219	50
PFOA (C8 Acid)	Target	413>169	50
		463>419	50
		463>219	50
PFNA (C9 Acid)	Target	463>169	50
		513>469	50
		513>269	50
PFDA (C10 Acid)	Target	513>219	50
		563>519	50
		563>269	50
PFUnA (C11 Acid)	Target	563>219	50
		613>569	50
		613>319	50
PFDoA (C12 Acid)	Target	613>169	50
		299>80	50
PFBS (C4 Sulfonate)	Target	299>99	50

Table 3 Continued.

Compound	Analyte Description	(1)MRM Transition(s)	Dwell Time (ms)
PFHS (C6 Sulfonate)	Target	399>80	50
		399>99	50
		499>80	50
		499>99	50
PFOS (C8 Sulfonate)	Target	499>130	50
FOSA (C8 Sulfonamide)	Target	498>78	50
[1,2,3,4 -$^{13}C_4$]PFBA	Surrogate (Small PFCAs: C4-C6)	217>172	100
[1,2- $^{13}C_2$]PFDA	Surrogate (Large PFCAs: C7-C12)	515>470	50
[$^{18}O_2$]PFBS	Surrogate (Sulfonates, FOSA)	303>84	50
[1,2,3,4- $^{13}C_4$]PFOA	Internal Standard (All PFCAs)	417>372	50
[$^{18}O_2$]PFOS	Internal Standard (Sulfonates, FOSA)	503>84	50

(1) Individual transitions were summed to produce a "total ion chromatogram" (TIC). The TICs were used for quantitation.

Analysis of the small acids (C4 through C6) was performed using a Thermo Scientific PRISM RP column (2.1mm × 50 mm; 5 µ particle size) held at 30°C with the mobile phase system consisting of 5 mM ammonium acetate with 0.01% acetic acid in water (A) and methanol (B). The gradient used to elute the analytes of interest is presented in Table 4. The flow rate was held at 300 µL min^{-1} throughout the run and a 20 µL injection volume was used. The outlet of the analytical column was directed to a column switching valve where the first three minutes of the run were diverted to waste. After three minutes, the valve was switched to direct the effluent to the mass spectrometer for analysis.

The large acids (C7 through C12), the sulfonates, and FOSA were analyzed using a Thermo Scientific Betasil™ C_{18} analytical column (2.1mm × 100 mm; 5µ particle size) held at 30°C with the mobile phase system consisting of 2 mM ammonium acetate in water (A) and acetonitrile (B). The gradient used is also provided in Table 4. The flow rate was held at 400 µL min^{-1} throughout the run and a 25 µL injection volume was used. Samples were injected onto a Waters (Milford, MA) Oasis® HLB on-line extraction column (20 mm × 3.0 mm, 25 µ particle size) with the outlet directed to a column switching valve where the first five minutes were diverted to waste. After five minutes, the valve was switched and the effluent was directed to the analytical column for separation of the target analytes and analysis by the mass spectrometer.

Table 4. LC Gradient Parameters.

Small Acids: C4-C6				Large Acids: C7-C12; Sulfonates, FOSA			
Step	Total Time (min)	%A	%B	Step	Total Time (min)	%A	%B
0	0.0	90	10	0	0.0	97	3.0
1	3.0	90	10	1	3.0	97	3.0
2	3.5	30	70	2	3.5	75	25
3	9.0	5.0	95	3	15.0	10	90
4	15.0	5.0	95	4	17.0	10	90
5	15.1	90	10	5	17.1	97	3.0
6	19.0	90	10	6	20.0	97	3.0
A	5 mM ammonium acetate with 0.01% acetic acid (aq)			A	2 mM ammonium acetate (aq)		
B	Methanol			B	Acetonitrile		

To manage the large number of transitions required for the large acids, sulfonates, and FOSA, two separate injections/analyses were acquired. Inclusion of all transitions in one chromatographic run made it challenging to get a sufficient number of scans across the chromatographic peak while maintaining a long enough dwell time to achieve the sensitivity needed for accurate and reproducible quantitation. The first injection acquired the MRM transitions for the C8-C12 acids (17 transitions) and the second injection acquired the MRM transitions needed for the anlaysis of C7 acid, the sulfonates, and FOSA (13 transitions). Chromatographic conditions were identical for both injections. For analytes where more than one transition was acquired, a total ion chromatogram (TIC) which summed the respective transitions for that analyte was used analyze the data.

A table summarizing the extraction and analysis dates is provided in the supplemental information.

4. Data Analysis

4.1 Calibration

Species-specific matrix-matched calibration standards were prepared by spiking known amounts of target analytes, surrogates, and internal standards into individual 0.5 g aliquots of fish tissue homogenate. Each spiked fish aliquot was then extracted using the procedures outlined in Section 3.2. A total of thirteen spiked standards ranging from 0.025 ng/g to 25 ng/g (nominal) were prepared. A quadratic, 1/x weighted, calibration curve was used to fit the data for each analyte. Internal standard quantitation was used. The data was not forced through zero during the fitting process. The accuracy of each curve point was verified by back-calculating the concentration using the area count ratio of the target analyte to the internal standard. Each extracted calibration standard used to generate the final calibration curve met the method calibration accuracy requirement of 100±25%, except the LOQ standard (100±30%). The coefficients of determination (r^2) were greater than 0.990 for all analytes. As required by ETS 8-45, a minimum of six calibration points were used to generate the final calibration curve.

4.2 Method of Standard Addition

Some target analytes, most notably PFBA and PFOS, had significant levels present in the control matrix. This resulted in the exclusion of several low-level points from the final calibration curve. When this occurred, the method of standard addition was used to determine the endogenous amount in the matrix, which was then used to correct the calibration and QC spiked concentrations. The two matrix blanks that were spiked with IS were included in the final calibration curve with concentration values assigned as the determined endogenous level. The calibration curve, with the adjusted concentrations, was then used to quantitate the QC samples.

4.3 Limit of Quantitation (LOQ)

The LOQ as defined in ETS 8-45 is the lowest non-zero calibration standard in the curve in which the area counts of the target analyte are at least twice those of the matrix blank(s). The limit of quantitation for each analyte varied from extraction date and instrument batch. If the percent relative standard deviation (RSD) of the matrix blanks area counts was less than 30%, then the average area counts was used to determine the LOQ. If the RSD was greater than 30%, then the matrix blank with the largest area counts was used for the LOQ determination. The resulting LOQs are provided in the Data Summary and Discussion section.

4.4 System Suitability

Five replicate injections of the solvent calibration standard were analyzed at the beginning of the analytical sequence to demonstrate overall system suitability. Method criteria states that system suitability injections shall produce a RSD of less than 7% for the ratio of target analyte area counts to internal standard area counts and an RSD of less than 2% for the retention time. In general, method

acceptance criteria were met for both area counts and retention times. Method deviations have been issued for the instances of non-compliant system suitabilities and are documented in the Supplemental Information.

4.5 Continuing Calibration

During the course of the analytical sequence, several continuing calibration verification samples (CCVs) were analyzed to confirm that the instrument response from initial calibration curve was still in control. In general, CCV injections produced recoveries within 100%±25%, which met method criteria. Non-compliant CCV recoveries were documented in method deviations in the raw data package and are provided in the Supplemental Information.

4.6 Blanks

Five types of blanks were prepared and analyzed with the samples: matrix blanks (two with IS and surrogate, two without IS and surrogate), aqueous method blanks (two with IS and surrogate, two without IS and surrogate), acidified acetonitrile solvent blanks, acetonitrile blanks with internal standards and surrogates, and straight acetonitrile blanks (no acid, IS, or surrogate). Each blank result was reviewed and used to evaluate method performance to determine the LOQ for each analyte. Surrogate recoveries of spiked blanks are provided in the Supplemental Information.

4.7 Lab Control Spikes (LCSs)

Triplicate lab control spikes at three different levels were prepared each extraction day. For PFHS, PFOS, and PFOA, the standard reference material used for the LCS spikes was the linear isomer. Separate ECF spikes of branched PFOS and PFOA were prepared to evaluate any potential bias from quantitation against a linear standard. For Day 1 only, triplicate PFOS dilution QC samples were prepared where PFOS was spiked at ppm levels whereas the rest of the analytes were at low ppb levels. Table 5 provides the approximate spike levels for the prepared LCS samples. The lab control spikes were prepared to evaluate method accuracy and precision. LCS recoveries will be presented and discussed in the following section.

Table 5. Validation QC Spike Levels.

	Approximate Spike Concentration (ng/g)				
QC Sample Description	[1]PFBA	[2]PFOS	PFOA	All Other Target Analytes	Surrogates
LCS Low	4	4	0.3	0.3	1
LCS Mid	12	12	1.5	1.5	1
LCS High	20	20	8	8	1
ECF LCS	NA	5	5	NA	1
[2]PFOS Dilution QC	12	4000	1.5	1.5	1

(1) Initial screening of the largemouth bass control matrix indicated that PFBA and PFOS were at levels higher than the rest of the target analytes. Spike levels were adjusted accordingly based on the endogenous level so that the low level spike was approximately twice that of the endogenous level. The spike concentration listed reflects the amount of analyte spiked into the tissue and does not account for the endogenous level.

(2) PFOS dilution QC samples were only prepared on Day 1.

5 Data Summary and Discussion

5.1 Whole body Largemouth Bass Validation Results

5.1.1 Accuracy and Precision

Table 6 below displays the accuracy (average percent recovery) and precision (%RSD) results for the three separate levels of LCSs (N=3) prepared in the whole-body largemouth bass control matrix for each preparation batch of the method validation. Internal standard, species-specific matrix-matched calibration was used for quantitation. Table 7 provides the average batch accuracy and precision when all three spike levels are considered collectively (N=9) as well as the LOQ, inter-day, and intra-day statistics. Additionally, the validation results are summarized graphically in Figure 1. For simplicity, the results of the ECF QC spikes are provided separately (N=3) for a given batch.

In general, almost all of the analytes demonstrated excellent accuracy and precision with average percent recoveries within arbitrary method acceptance criteria of 100±30% and %RSDs less than 20% when individual batch, inter-day, intra-day, and all data collectively were considered. With the exception of the small PFCAs(PFBA, PFPeA, and PFHxA), all analytes demonstrated average accuracies within 100±15% with %RSDs less than 10%. Endogenous levels were detected in the control matrix for the following analytes: PFBA, PFHpA (Day 2 Analyst B only), PFDA, PFUnA, PFDoA, and PFOS. For these analytes, the method of standard addition was performed to determine the endogenous concentration. The resulting concentration was then used to correct the spiked amount for the calibration standards and QC samples.

Table 6. Whole-body Largemouth Bass Accuracy and Precision Results Summary By Spike Level.

Accuracy (Average % Recovery) ± Precision (%RSD)									
	Day 1 Analyst A			Day 2 Analyst A			Day 2 Analyst B		
Analyte	Low	Mid	High	Low	Mid	High	Low	Mid	High
(1)PFBA	124 ± 4.9	125 ± 4.8	121 ± 0.83	110 ± 3.3	117 ± 2.7	116 ± 1.3	103 ± 5.1	103 ± 3.9	107 ± 0.93
PFPeA	151 ± 5.4	137 ± 3.6	128 ± 3.7	134 ± 6.8	124 ± 3.7	120 ± 2.2	85.0 ± 9.6	103 ± 5.3	102 ± 2.2
PFHxA	115 ± 31	118 ± 5.5	121 ± 3.3	129 ± 2.7	128 ± 2.8	119 ± 3.5	114 ± 18	106 ± 8.2	96.8 ± 4.4
PFHpA	126 ± 6.4	107 ± 2.2	103 ± 4.4	90.8 ± 1.7	104 ± 0.44	109 ± 1.1	112 ± 5.1	106 ± 4.4	104 ± 2.9
PFOA	86.3 ± 13	95.5 ± 3.6	101 ± 7.7	82.1 ± 8.2	95.6 ± 2.4	92.6 ± 1.6	(2)<0.920	102 ± 2.2	89.4 ± 1.5
PFNA	111 ± 1.7	114 ± 2.8	113 ± 10	114 ± 4.6	111 ± 3.4	107 ± 2.0	112 ± 3.7	111 ± 2.0	108 ± 5.2
(1)PFDA	111 ± 5.8	100 ± 5.4	94.8 ± 6.3	104 ± 8.0	94.5 ± 2.5	91.7 ± 1.9	102 ± 2.0	96.7 ± 0.45	93.9 ± 1.6
(1)PFUnA	100 ± 8.1	102 ± 2.1	110 ± 1.0	100 ± 9.4	103 ± 5.9	97.9 ± 1.9	106 ± 4.8	110 ± 1.6	103 ± 3.5
(1)PFDoA	100 ± 10	110 ± 5.1	104 ± 10	105 ± 4.4	105 ± 0.55	100 ± 1.2	105 ± 1.7	108 ± 2.4	101 ± 2.2
PFBS	113 ± 0.0	106 ± 7.8	102 ± 11	122 ± 3.2	110 ± 3.0	98.5 ± 2.1	106 ± 11	97.5 ± 1.1	92.4 ± 1.6
PFHS	99.6 ± 3.8	104 ± 3.8	100 ± 9.6	103 ± 5.6	104 ± 1.5	95.9 ± 2.5	104 ± 1.0	100 ± 2.6	94.5 ± 0.92
(1)PFOS	95.5 ± 3.6	102 ± 6.8	104 ± 5.6	98.2 ± 2.9	98.7 ± 2.4	96.7 ± 5.5	95.9 ± 1.3	96.0 ± 3.2	96.4 ± 4.1
FOSA	101 ± 4.6	109 ± 4.5	109 ± 11	120 ± 4.7	118 ± 0.43	112 ± 3.9	98.6 ± 4.4	103 ± 4.0	97.4 ± 2.6
[1,2,3,4-$^{13}C_4$]-PFBA	114 ± 2.5	113 ± 0.54	108 ± 1.1	106 ± 4.7	110 ± 1.4	108 ± 0.93	95.3 ± 4.2	94.2 ± 3.3	91.3 ± 1.7
[1,2,-$^{13}C_2$]-PFDA	106 ± 6.8	110 ± 3.2	109 ± 2.5	105 ± 7.5	97.6 ± 1.9	102 ± 3.5	101 ± 2.3	100 ± 1.8	104 ± 2.7
[$^{18}O_2$]-PFBS	96.2 ± 6.1	99.8 ± 3.7	102 ± 4.2	100 ± 1.3	102 ± 2.1	98.3 ± 2.1	101 ± 5.2	96.1 ± 6.6	100 ± 8.0

(1) Endogenous levels detected in the control matrix. Method of standard addition was used to determine endogenous concentration. All QC spike concentrations were corrected for endogenous values.

(2) Matrix blanks exhibited contamination and several low-level calibration standards were deactivated. Consequently, the low-level LCSs were below the resultant LOQ.

No.

(L) If the notice includes a health and safety study concerning the new chemical substance, the submitter must also answer the questions in Sec. 720.90(b)(2).

See below.

720.90 (b) (2)

(i) Would disclosure of the chemical identity disclose processes used in the manufacture or processing of a chemical substance or mixture? Describe how this would occur.

Yes. Disclosure of the chemical identity would enable a chemist to identify feedstock chemicals and the manufacturing process. Because of the type of chemical, its identity would also enable a chemist to identify processing and use information.

(ii) Would disclosure of the chemical identity disclose the portion of a mixture comprised by any of the substances in the mixture? Describe how this would occur.

The notified substance is not a mixture.

(iii) Do you assert that disclosure of the chemical identity is not necessary to interpret any of the health and safety studies you have submitted? If so, explain how a less specific identity would be sufficient to interpret the studies.

The health and safety studies stand by themselves. The generic name and the toxicology studies should enable any toxicologist to comment on the safety of the chemical.

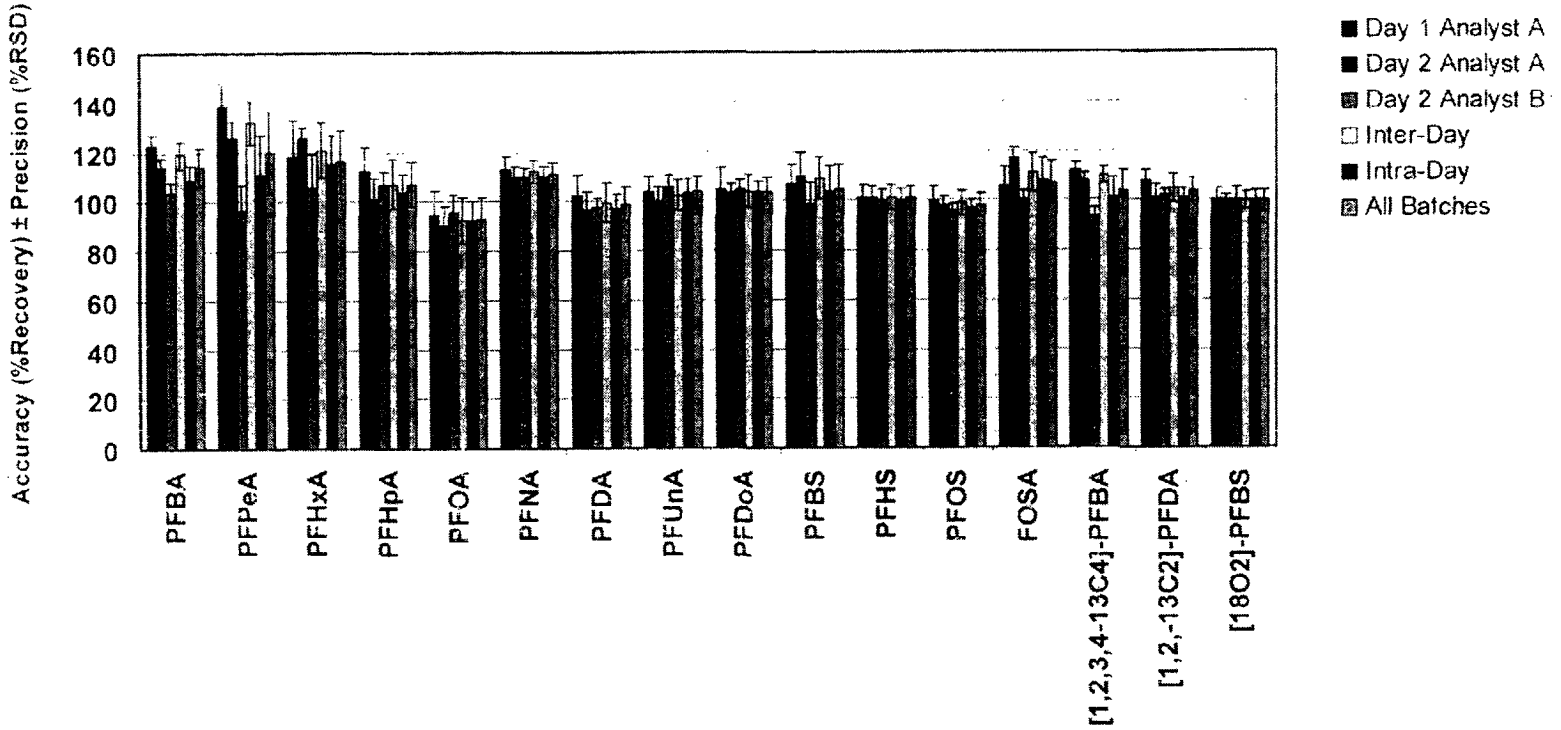

Figure 1. Method ETS 8-45.0 Whole-body Largemouth Bass Validation Results Summary.

Average percent recovery and %RSD reflect all LCS spike levels collectively.

Table 7. Whole-body Largemouth Bass Results Summary: LOQ, Batch, Inter-Day, and Intra-Day Statistics.

	Day 1 Analyst A		Day 2 Analyst A		Day 2 Analyst B		(1) Accuracy ±Precision Inter-Day	(1) Accuracy ±Precision Intra-Day	(1) Accuracy ±Precision All B atches
Analyte	(1) Accuracy ±Precision (N=9)	LOQ (ng/g)	(1) Accuracy ±Precision (N=9)	LOQ (ng/g)	(1) Accuracy ±Precision (N=9)	LOQ (ng/g)	(N=18)	(N=18)	(N=27)
PFBA	123 ± 3.8	(2)E=2.01	114 ± 3.7	(2)E=3.43	104 ± 3.7	(2)E=2.37	119 ± 5.4	109 ± 5.8	114 ± 7.8
PFPeA	139 ± 8.4	0.248	126 ± 6.4	0.259	96.6 ± 10	0.246	132 ± 8.9	111 ± 16	120 ± 17
PFHxA	118 ± 15	0.248	126 ± 4.3	0.259	106 ± 13	0.347	121 ± 11	115 ± 12	116 ± 13
PFHpA	112 ± 10	0.0969	101 ± 8.2	0.102	107 ± 4.9	(2)E=0.0332	107 ± 10	104 ± 7.1	107 ± 8.9
PFOA	94.4 ± 10	0.0504	90.1 ± 7.9	0.0493	95.5 ± 7.2	(3)0.920	92.3 ± 9.2	92.2 ± 7.9	93.1 ± 8.7
PFNA	113 ± 5.4	0.0512	110 ± 4.2	0.0501	110 ± 3.7	0.0490	112 ± 4.8	110 ± 3.9	111 ± 4.5
PFDA	102 ± 8.5	(2)E=0.184	96.7 ± 7.3	(2)E=0.224	97.4 ± 3.7	(2)E=0.155	99.3 ± 8.2	97.0 ± 5.6	98.7 ± 7.0
PFUnA	104 ± 6.1	(2)E=0.285	100 ± 6.0	(2)E=0.392	106 ± 4.2	(2)E=0.393	102 ± 6.2	103 ± 5.8	104 ± 5.8
PFDoA	105 ± 8.5	(2)E=0.0747	103 ± 3.8	(2)E=0.0693	105 ± 3.6	(2)E=0.0537	104 ± 6.5	104 ± 3.7	104 ± 5.6
PFBS	107 ± 7.8	0.0994	110 ± 9.4	0.105	98.7 ± 8.6	0.246	109 ± 8.5	104 ± 10	105 ± 9.5
PFHS	101 ± 5.2	0.0509	101 ± 4.9	0.0468	100 ± 4.5	0.0457	101 ± 5.2	100 ± 4.6	101 ± 5.0
PFOS	100 ± 6.	(2)E=1.89	97.9 ± 3.4	(2)E=2.77	96.1 ± 2.7	(2)E=1.96	99.0 ± 4.9	97.0 ± 3.1	98.1 ± 4.5
FOSA	106 ± 7.4	0.0511	117 ± 4.4	0.105	100 ± 4.0	0.049	111 ± 7.6	108 ± 9.1	107 ± 8.5
[1,2,3,4-$^{13}C_4$]-PFBA	112 ± 3.0	0.0245	108 ± 2.9	0.0257	93.6 ± 3.4	0.0221	110 ± 3.4	101 ± 7.9	104 ± 8.1
[1,2,-$^{13}C_2$]-PFDA	108 ± 4.1	0.0504	101 ± 5.3	0.0257	102 ± 2.5	0.0221	105 ± 5.7	101 ± 4.0	104 ± 5.1
[$^{18}O_2$]-PFBS	99.9 ± 4.9	0.0471	100 ± 2.2	0.0241	99.7 ± 5.6	0.0207	99.6 ± 3.6	99.5 ± 4.5	99.4 ± 4.5

(1) Accuracy = average percent recovery all three spike levels combined. Precision = percent relative standard deviation all three levels combined.
(2) E = endogenous. Analyte present in the control matrix. Endogenous concentration determined by method of standard addition. Endogenous value = LOQ.
(3) Matrix blanks exhibited contamination and several low level calibration standards were deactivated resulting in an elevated LOQ for this analysis.

5.1.2 ECF PFOA and PFOS QC Spikes

Table 8 provides the accuracy (percent recovery) and precision (%RSD) results of the 3M ECF QC spikes of PFOS and PFOA quantitated against the linear reference standard. For PFOS, the mean recovery of all three batches collectively was 95.9% with a RSD of 3.4% demonstrating that no measurable bias could be attributed to the use of the linear reference material. Alternatively, the average PFOA recovery of the ECF spike was consistently approximately 87% for all three batches indicating that a potential bias may exist when branched PFOA isomers are quantitated using a linear reference material. Potential explanations of the observed decrease in recovery may be differences in the response factor from the linear to the branched isomers or co-extracted matrix components may suppress the signal of the branched isomers relative to the linear. Even though the PFOA demonstrated some decrease in recovery, the results were still within 100±15%.

Table 8. Results Summary: ECF (branched) PFOA and PFOS QC Spikes Quantitated Against a Linear Reference Standard.

	Accuracy (Average Percent Recovery) ± Precision (%RSD)					
Analyte	*Day 1 Analyst A*	*Day 2 Analyst A*	*Day 2 Analyst B*	*Inter-Day*	*Intra-Day*	*All Batches*
PFOS	97.6 ± 2.0	95.7 ± 2.6	94.5 ± 5.3	96.6 ± 2.3	95.1 ± 3.8	95.9 ± 3.4
PFOA	87.4 ± 4.0	87.0 ± 1.1	86.6 ± 4.1	87.2 ± 2.7	86.8 ± 2.7	87.0 ± 3.0

5.1.3 PFOS Dilution QC Spikes

On Day 1, additional QC spikes identified as "PFOS dilution QC" were also prepared. These QC samples were prepared with PFOS at approximate levels of 4000 ng/g with all other analytes at 1.5 ng/g, with the exception of PFBA at 12 ng/g. The undiluted extract was analyzed for all analytes except PFOS. A 1:1000 acetonitrile dilution of the final extract was prepared for the PFOS analysis. The PFOS analysis of the diluted extract was performed using external standard calibration as the internal standard spiked into the tissue prior to extraction was diluted below the limit of detection. Spiking these QC samples with IS at a level that could be diluted into a usable range was not feasible as the isotopically labeled standards come from the vendor already in solution at a concentration of 50 µg/mL. Table 9 lists the results of the PFOS dilution QC. With the exception of PFBA and PFPeA, the average recoveries were within 100±20% with RSDs less than 15%. The internal standard for PFBS, PFHS, FOSA and [$^{18}O_2$]PFBS surrogate was switched from [$^{18}O_2$]PFOS to [1,2,3,4-$^{13}C_4$]PFOA for these samples only. When [$^{18}O_2$]PFOS was used as the internal standard, recoveries ranged from approximately 65-75% because the internal standard area counts increased by about 32% in these samples. The IS signal increase was attributed to the co-elution of the [$^{18}O_2$]PFOS spiked at 1 ng/g with the unlabeled PFOS spiked at 4000 ng/g. The combination of ^{34}S and a single ^{18}O in the unlabeled PFOS would also produce the same 503 m/z parent ion as the [$^{18}O_2$]PFOS. Based on the natural abundance of ^{34}S (4.4%) and ^{18}O (0.2%), the probability of both ^{34}S and a single ^{18}O present in the unlabeled PFOS spike is 0.0088%.(2) With the PFOS spiked at 4000 ng/g, this contribution would be equivalent to approximately 0.35 ng/g or 35% of the spiked IS and explains the observed increase in IS area counts. When the IS was switched to [1,2,3,4-$^{13}C_4$]PFOA, which does not co-elute with PFOS, the recoveries improved dramatically demonstrating that the extraction procedures were not responsible for the low recoveries when the [$^{18}O_2$]PFOS IS was used. The average recovery of the post-extraction dilutions for PFOS was lower than the other analytes at 84.4%. Possible explanations include dilution of matrix components along with the PFOS that alter the signal enhancement against the matrix-matched curve or simple variability in dilution technique.

Table 9. PFOS Dilution QC Results.

Analyte	(1)Accuracy ± Precision
PFBA	129 ± 7.4
PFPeA	144 ± 9.6
PFHxA	115 ± 12
PFHpA	93.8 ± 6.6
PFOA	93.2 ± 7.9
PFNA	106 ± 13
PFDA	105 ± 5.3
PFUnA	96.4 ± 6.7
PFDoA	106 ± 10
(2)PFBS	99.1 ± 1.9
(2)PFHS	91.7 ± 7.6
(3)PFOS	84.4 ± 12
(2)FOSA	96.4 ± 5.0
$^{13}C_4$-PFBA	120 ± 6.2
$^{13}C_2$-PFDA	117 ± 12
(2)$^{18}O_2$-PFBS	98.0 ± 6.1

(1) Accuracy = average percent recovery. Precision = %RSD. (N=3)
(2) [1,2,3, 4-$^{13}C_4$]PFOA was used as the internal standard.
(3) PFOS concentration determined by external standard calibration of 1:1000 dilution of the final extract.

5.1.4 Alternate PFOS Quantitation

In a separate preparation batch, a whole-body largemouth bass matrix-matched calibration curve was prepared using [1,2,3,4-$^{13}C_4$]PFOS as the calibrant, [$^{18}O_2$]PFBS as the internal standard, and [$^{18}O_2$]PFHS as the surrogate. Thirteen calibration standards ranging from 0.025 ng/g to 25 ng/g spiked tissue concentrations were generated along with four matrix blanks (two with IS and surrogate and two without IS and surrogate). Triplicate lab control matrix spikes were also prepared by spiking unlabeled linear PFOS at approximate levels of 4 ng/g (low), 12 ng/g (mid), and 20 ng/g (high) along with triplicate ECF PFOS spikes at 5 ng/g. Consistent with previous validation QC samples, the surrogate was spiked at an approximate level of 1 ng/g for all samples. The three [1,2,3,4-$^{13}C_4$]PFOS transitions (503>131, 503>99, 503>80) as well as the corresponding unlabeled PFOS transitions (499>130, 499>99, and 499>80) were monitored. The calibration curve was constructed using the total ion chromatogram (summation) of the three [1,2,3,4-$^{13}C_4$]PFOS transitions. The three unlabeled PFOS transitions were also summed and the total area counts were plugged into the resulting regression equation for [1,2,3,4-$^{13}C_4$]PFOS. The average endogenous PFOS concentration in the control matrix was calculated for the thirteen matrix-matched calibration standards and the two matrix blanks spiked with IS. The mean endogenous PFOS concentration in the whole-body largemouth bass control matrix was 2.76 ng/g ± 10%(RSD) (N=15). This concentration was then used to correct the spiked values of the QC spikes. Table 10 provides the accuracy and precision data of the QC spikes. All QC results met method acceptance criteria for accuracy (100±30%) and precision (RSD<20%).

Table 10. QC Results for Alternate PFOS Quantitation using [1,2,3,4-$^{13}C_4$]PFOS as a Calibrant.

	Accuracy (Average % Recovery) ± Precision (%RSD)				
Analyte	Low (N=3)	Mid (N=3)	High (N=3)	ECF (N=3)	Batch Average (N=12)
PFOS (Linear)	107 ± 5.6	114 ± 3.8	111 ± 5.1	105 ± 2.4	109 ± 5.0
(1) $^{18}O_2$ PFHS surrogate	111 ± 1.8	117 ± 2.5	112 ± 4.4	112 ± 4.8	113 ± 3.8

(1) $^{18}O_2$ PFHS surrogate was spiked at approximately 1 ng/g for all QC levels.

5.2 Multi-Species Cross Validation

The method presented here was subsequently cross-validated for three additional fish species: whole-body channel catfish (*Ictalurus punctatus*), whole-body bluegill sunfish (*Lepomis macrochirus*), and rainbow trout fillets (*Oncorhynchus mykiss*) using species-specific matrix-matched calibration curves. The cross-validation procedures represented an abbreviated approach to the full validation performed for the whole-body largemouth bass. The cross-validation for the three additional species included species-specific matrix-matched calibration with triplicate lab control matrix spikes at three levels to assess accuracy and precision. Additional ECF spikes of PFOS and PFOA were prepared and analyzed against predominantly linear reference materials. PFOS dilution QC and inter- and intra-day evaluations were not included.

The results from the multi-species cross-validation are summarized below (whole-body catfish: Table 11, whole-body bluegill: Table 12, and rainbow trout fillet: Table 13). The average percent recovery and %RSD for all spike levels combined is presented graphically in Figure 2. For all three species, the average percent recovery (all levels combined) was within 100±30% with a %RSD less than 20% for all analytes, with the exception of PFBA for rainbow trout fillet. Rainbow trout fillet PFBA results were not reported because several of the calibration curve points did not meet method acceptance criteria once the concentrations were adjusted for endogenous levels. Rainbow trout cross validation samples were re-extracted and analyzed for the small PFCAs to see if a better calibration curve could be achieved for PFBA. For the re-prepared trout samples, the area counts of the [1,2,3,4-$^{13}C_4$]PFOA internal standard in the LCS samples dropped by approximately 30% when compared to the IS area counts of the curve resulting in high recoveries. No results were reported from the reanalysis; however, external standard quantitation of the samples produced acceptable recoveries in general. The variability of the [1,2,3,4-$^{13}C_4$]PFOA IS during the small acid analysis was observed throughout the cross validation analyses and was more pronounced as the PRISM analytical column aged. This variability was not observed during the analysis of the large PFCAs using the Betasil C18 column, therefore, the signal degradation and variability over the course of an instrument batch is largely attributed to the IS's lack of robustness on the analytical column and not to extraction issues. Analysis of the smaller PFCAs may benefit by selecting a different isotopically labeled internal standard with a smaller chain length, more representative of the target analytes.

As observed with whole-body largemouth bass, the ECF spikes of PFOS did not exhibit any measurable bias when quantitated against a linear reference standard for the three additional species (average recoveries were greater than 95%). The ECF PFOA LCSs exhibited lower recoveries for the whole body catfish and bluegill species, 90.9% ± 0.82% and 83.0% ± 3.0%, respectively; however, the rainbow trout fillets produced an average recovery of 96.4% ± 3.0%. This may suggest that a co-extracted matrix component present in whole-body tissues, but not in fillets, is affecting the PFOA isomer response. Because quantitation of the ECF materials against a linear reference standard produced variable recoveries in the different species and tissue types, it is recommended that this QC component be evaluated at a minimum each time this method is applied to a new species and ideally with every preparation batch.

Table 11. Method Cross-Validation Results for Whole-Body Catfish.

	Accuracy (Average %Recovery) ± Precision (%RSD)				
Analyte	Low	Mid	High	All Levels Combined	LOQ (ng/g)
PFBA	121 ± 1.0	120 ± 2.1	123 ± 4.6	121 ± 2.9	E=2.29
PFPeA	102 ± 3.3	108 ± 3.0	115 ± 3.5	109 ± 5.9	E=0.376
PFHxA	113 ± 3.7	117 ± 2.8	111 ± 5.0	113 ± 4.2	0.103
PFHpA	109 ± 2.1	114 ± 1.6	110 ± 3.2	111 ± 2.9	0.0486
PFOA	(1)126 ± 15	111 ± 8.4	102 ± 1.2	113 ± 13	0.265
PFNA	101 ± 1.7	114 ±4.2	113 ± 3.2	109 ± 6.3	E=0.332
PFDA	107 ± 3.1	109 ± 3.4	96.0 ± 1.4	104 ± 6.3	E=0.0784
PFUnA	(1)124 ± 14	120 ± 6.3	106 ± 3.8	117 ± 11	E=0.142
PFDoA	116 ± 4.4	112 ± 6.9	104 ± 4.2	111 ± 6.5	E=0.0376
PFBS	94.3 ± 10	99.9 ± 1.4	95.1 ± 2.2	96.5 ± 5.7	0.103
PFHS	94.4 ± 7.7	106 ± 3.3	95.1 ± 3.2	98.6 ± 7.3	0.0468
PFOS	97.6 ± 3.8	103 ± 2.9	98.7 ± 3.1	99.7 ± 3.7	E=0.166
FOSA	118 ± 2.9	117 ± 3.7	105 ± 3.1	113 ± 6.4	0.268
ECF PFOA	NA	NA	NA	90.9 ± 0.82	NA
ECF PFOS	NA	NA	NA	99.1 ± 2.1	NA
[1,2,3,4-$^{13}C_4$]-PFBA	116 ± 1.1	102 ± 2.9	114 ± 2.7	111 ± 6.2	0.0244
[1,2,-$^{13}C_2$]-PFDA	109 ± 2.6	107 ± 6.8	111 ± 5.7	109 ± 5.6	0.0245
[$^{18}O_2$]-PFBS	101 ± 6.2	98.7 ± 3.4	101 ± 1.7	100 ± 3.8	0.0229

(1) One LCS exceeded 130% recovery.
E= endogenous. The endogenous concentration listed is the LOQ for the control matrix studied.
NA = Not applicable.

Table 12. Method Cross-Validation Results for Whole-Body Bluegill.

	Accuracy (Average %Recovery) ± Precision (%RSD)				
Analyte	*Low*	*Mid*	*High*	*All Levels Combined*	*LOQ (ng/g)*
PFBA	121 ± 11	111 ± 3.7	105 ± 7.5	112 ± 9.4	E=1.45
PFPeA	(1)NA	109 ± 7.0	100 ± 4.5	104 ± 7.1	0.359
PFHxA	(1)NA	133 ± 3.2	109 ± 3.9	121 ± 11	0.505
PFHpA	104 ± 5.3	100 ± 2.2	95.8 ± 2.5	100 ± 4.6	0.251
PFOA	93.5 ± 8.9	99.8 ± 7.4	(2)84.0 ± 0.46	93.5 ± 9.4	0.255
PFNA	112 ± 9.4	111 ± 3.0	(2)96.8 ± 2.1	108 ± 8.5	0.259
PFDA	93.5 ± 8.0	97.2 ± 8.9	(2)80.6 ± 0.60	91.7 ± 10	0.248
PFUnA	99.1 ± 2.6	107 ± 13	(2)87.1 ± 3.9	99.1 ± 11	E=0.382
PFDoA	103 ± 12	116 ± 3.8	(2)98.1 ± 8.9	107 ± 10	0.251
PFBS	112 ± 3.8	105 ± 7.0	99.0 ± 2.3	105 ± 6.8	0.258
PFHS	93.8 ± 9.9	101 ± 2.1	94.5 ± 1.6	96.5 ± 6.2	0.0977
PFOS	99.7 ± 0.93	98.3 ± 0.85	96.2 ± 3.5	98.0 ± 2.4	E=1.99
FOSA	122 ± 8.7	114 ± 3.9	106 ± 8.8	114 ± 8.9	0.105
ECF PFOA	NA	NA	NA	83.0 ± 3.0	NA
ECF PFOS	NA	NA	NA	98.1 ± 0.46	NA
[1,2,3,4-$^{13}C_4$]-PFBA	110 ± 11	99.2 ± 5.4	93.3 ± 7.8	101 ± 10	0.0247
[1,2,-$^{13}C_2$]-PFDA	92.2 ± 4.0	100 ± 5.8	(2)93.6 ± 5.2	95.7 ± 5.9	0.051
[$^{18}O_2$]-PFBS	98.8 ± 3.0	98.0 ± 3.4	99.0 ± 3.2	98.6 ± 2.8	0.0231

(1) Spike concentration less than the resultant LOQ, recoveries not reported.
(2) Bad instrument injection for one of the replicates, data not generated. Precision evaluated as percent relative difference (N=2).
NA= Not applicable.

Table 13. Method Cross-Validation Results for Rainbow Trout Fillets.

	Accuracy (Average %Recovery) ± Precision (%RSD)				
Analyte	*Low*	*Mid*	*High*	*All Levels Combined*	*LOQ (ng/g)*
PFBA	NR	NR	NR	NR	NR
PFPeA	(1)131 ± 19	(1)127 ± 6.7	(1)131 ± 14	129 ± 13	0.252
PFHxA	124 ± 7.8	122 ± 1.8	115 ± 12	120 ± 7.8	0.252
PFHpA	109 ± 0.27	110 ± 4.5	104 ± 5.9	108 ± 4.4	0.0243
PFOA	105 ± 4.0	108 ± 5.6	103 ± 6.5	106 ± 5.2	0.0501
PFNA	110 ± 2.2	117 ± 2.1	114 ± 4.9	114 ± 3.9	E=0.110
PFDA	102 ± 1.6	102 ± 2.8	100 ± 3.1	101 ± 2.5	0.0487
PFUnA	108 ± 7.5	112 ± 3.6	111 ± 1.1	110 ± 4.5	0.0495
PFDoA	108 ± 6.9	112 ± 1.8	109 ± 4.5	110 ± 4.5	0.0493
PFBS	102 ± 6.7	106 ± 4.9	93.2 ± 16	101 ± 10	0.0249
PFHS	99.0 ± 3.3	102 ± 3.5	93.8 ± 8.4	98.1 ± 5.9	0.0234
PFOS	103 ± 1.7	102 ± 3.6	104 ± 3.8	103 ± 2.8	E=0.287
FOSA	124 ± 1.5	121 ± 5.7	124 ± 9.8	123 ± 5.8	0.104
ECF PFOA	NA	NA	NA	96.4 ± 3.0	NA
ECF PFOS	NA	NA	NA	96.5 ± 2.5	NA
[1,2,3,4-$^{13}C_4$]-PFBA	113 ± 12	114 ± 2.2	107 ± 2.1	111 ± 7.0	0.0246
[1,2,-$^{13}C_2$]-PFDA	105 ± 3.2	107 ± 2.6	113 ± 4.1	108 ± 4.5	0.0247
[$^{18}O_2$]-PFBS	103 ± 2.7	103 ± 3.2	96.8 ± 13	101 ± 7.1	0.0231

(1) One or more of the LCS replicates produced a recovery greater than 130%
NR = Not reported. The calibration curve once adjusted for the endogenous concentration did not meet method acceptance criteria for several points.
NA = Not applicable.

5.3 Solvent Curve Analysis.

For each of the four species studied here, triplicate matrix blanks along with triplicate laboratory matrix spikes at three levels were prepared in a separate preparation batch and analyzed against an acetonitrile solvent (un-extracted) calibration curve that was acid adjusted in a similar fashion to the sample extracts. The results from the solvent (un-extracted) calibration are presented in Table 14 and graphically in Figure 3 for all the target analytes and surrogates except for the small PFCAs (PFBA, PFPeA, PFHxA, and [1,2,3,4 -$^{13}C_4$]PFBA.

Results for the small acids were not reported as the [1,2,3,4 -$^{13}C_4$]PFOA IS signal response was significantly suppressed when compared to IS signal in the solvent curve. Furthermore, the small acid target analyte signals were also suppressed when compared to the solvent response, but at different percentages than the IS. The combined target analyte and IS signal suppression observed in the extracted samples for the small acids resulted in recoveries that greatly exceeded method acceptance criteria of 100±30% for PFBA, PFPeA, and [1,2,3,4 -$^{13}C_4$]PFBA. Acceptable results were observed for PFHxA as it was the largest acid analyzed and structurally most resembles the C8 internal standard. For simplicity, none of the PFHxA results are reported. Samples were prepared and analyzed twice for the small acids using newer PRISM columns to see if improved results could be achieved. Varying degrees of IS signal suppression relative to the solvent curve were observed during both analyses.

The [1,2,3,4 -$^{13}C_4$]PFOA IS signal response did not demonstrate near the level of suppression in the large acid and sulfonate analyses for most species which indicates that the low IS response was not an extraction efficiency issue. These results again suggest that the PRISM analytical column may not be suitable for analysis of the larger PFCAs and selection of a different internal standard for the smaller acids may improve the analysis. Although the ISs for the large PFCAs and sulfonates exhibited some suppression in some species, the target analyte signal was suppressed by approximately the same amount. This produced a "self-correcting" result and most recoveries for the target analytes were within 100±30% (Table 14). However, it should be noted that the average overall recovery of FOSA in the whole-body bluegill samples was less than 40% when quantitated against the solvent curve. This result emphasizes the criticality of inclusion of appropriate QC samples to verify the method applicability and quantitation approach for each species for each analyte.

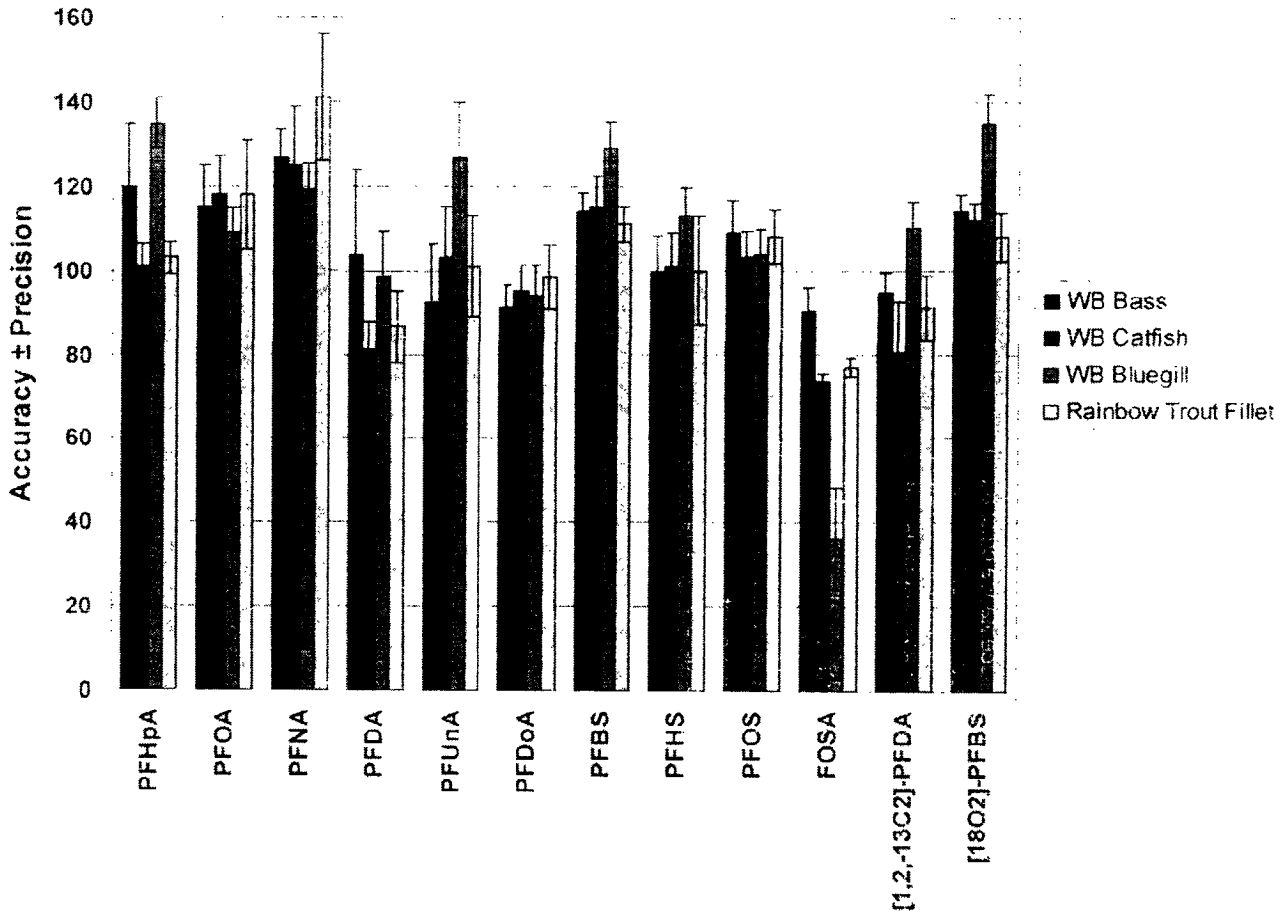

Figure 3. Solvent Curve Analysis Results Summary.

Table 14. Solvent Curve Analysis Results Summary.

	Whole-Body Largemouth Bass		*Whole-Body Catfish*		*[1]Whole-Body Bluegill*		*Rainbow Trout Fillet*	
	[2]Accuracy±Precision	*N*	*[2]Accuracy±Precision*	*N*	*[2]Accuracy±Precision*	*N*	*[2]Accuracy±Precision*	*N*
PFBA	NR		NR		NR		NR	
PFPeA	NR		NR		NR		NR	
PFHxA	NR		NR		NR		NR	
PFHpA	120 ± 15	9	101 ± 5.3	9	135 ± 5.9	9	103 ± 3.9	9
PFOA	115 ± 10	[3]8	118 ± 9.2	[4]8	109 ± 5.7	[4]8	118 ± 13	9
PFNA	127 ± 6.4	[3]8	125 ± 14	9	119 ± 6.4	9	141 ± 15	9
PFDA	104 ± 20	[3]8	81.3 ± 6.4	[4]7	98.5 ± 11	9	86.7 ± 8.6	[4]7
PFUnA	92.5 ± 14	[3]8	103 ± 12	9	127 ± 13	9	101 ± 12	8
PFDoA	91.1 ± 5.6	[4]7	95.0 ± 6.5	[4]8	94.1 ± 7.2	9	98.4 ± 7.5	[4]7
PFBS	114 ± 4.6	9	115 ± 7.6	9	129 ± 6.2	9	111 ± 4.3	9
PFHS	99.4 ± 9.0	[4]7	101 ± 8.1	[4]8	113 ± 6.4	9	100 ± 13	9
PFOS	109 ± 7.5	9	103 ± 6.2	9	104 ± 5.8	9	108 ± 6.4	9
FOSA	90.3 ± 5.5	9	73.8 ± 1.6	[4]6	36.1 ± 12	9	76.9 ± 2.2	[4]6
[1,2,3,4-$^{13}C_4$]-PFBA	NR		NR		NR		NR	
[1,2,-$^{13}C_2$]-PFDA	94.9 ± 4.6	[3]11	80.7 ± 12	12	110 ± 6.2	12	91.1 ± 7.7	[5]11
[$^{18}O_2$]-PFBS	114 ± 4.2	12	112 ± 3.8	12	135 ± 6.7	12	108 ± 5.8	[5]11

(1) Whole-body bluegill were reprepared and analyzed in a separate batch as four of the LCSs were not spiked with IS.
(2) Accuracy = average percent recovery (all levels combined). Precision = percent relative standard deviation (all levels combined).
(3) Instrument injection error observed for one low-level LCS.
(4) One or more of the low-level LCSs produced a final concentration below the limit of quantitation and were excluded from statistical calculations.
(5) Sample preparation error. One of the matrix blanks was not spiked with internal standard/surrogate prior to extraction
NR=Not reportable.

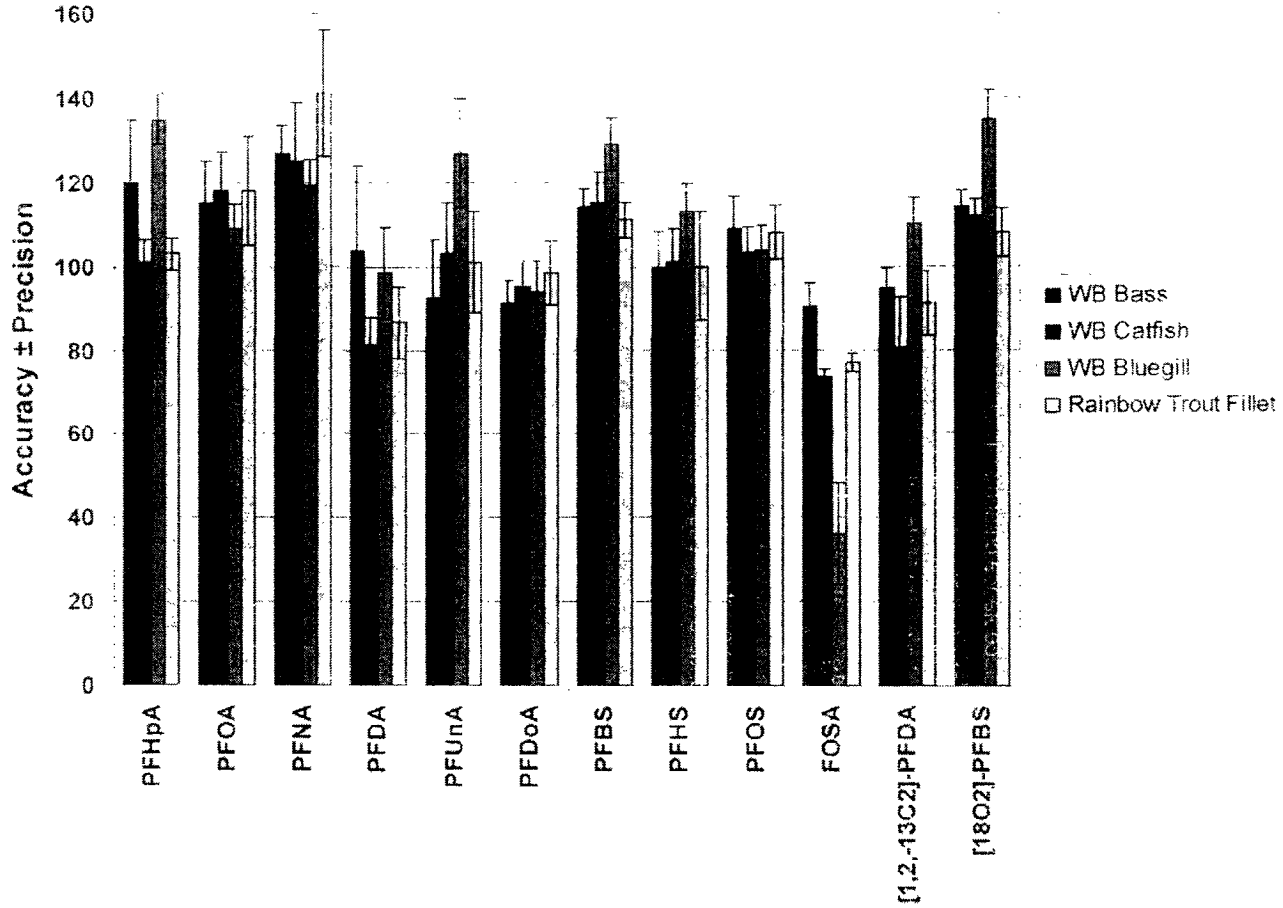

Figure 3. Solvent Curve Analysis Results Summary.

6 Conclusions

The method presented here produces excellent accuracy (average percent recovery) and precision (%RSD) when species-specific, matrix-matched calibration is used for the C7 through C12 perfluorocarboxylic acids, PFBS, PFHS, PFOS, and FOSA. In general, the method used here produces acceptable results (accuracy 100±30%, precision <20% RSD) for the small PFCAs (C4-C6); however, it is hypothesized that improved results can be achieved if a different internal standard is selected for the small PFCA analysis in future studies.

Analysis against a solvent curve produced more varied results than the species-specific matrix-matched calibration. The solvent curve analysis may be an acceptable approach for screening level analyses or when a suitable control matrix is not available. If the solvent curve calibration is used for generating quantitative values, enough QC elements (lab control and lab matrix spikes) should be included to verify that matrix effects are not artificially biasing the sample results.

When quantitated against a linear reference standard, QC spikes of 3M ECF PFOS and PFOA produced recoveries within method acceptance criteria of 100±30%, although the recoveries for the ECF PFOA exhibited a small, but measurable, bias in the whole body largemouth bass, catfish, and bluegill tissues (average recoveries ranging from 83.0-90.9%). No bias was observed for the ECF PFOS (all species) and PFOA in rainbow trout fillets with average recoveries greater than 95%.

7 Data Archival Retention

All remaining sample and associated project data (hardcopy and electronic) will be archived according to 3M Environmental Laboratory standard operating procedures.

8 Signatures

Michelle D. Malinsky, Ph.D., 3M Research Specialist, Principal Analytical Investigator — Date 3/13/2009

William K. Reagen, Ph.D., Environmental Laboratory Management — Date 12 March 2009

Cliffton B. Jacoby, Ph.D., 3M Technical Reviewer, Project Coordinator — Date 12 March 2009

The 3M Environmental Laboratory's Quality Assurance Unit has audited the data and report for this project.

Casey Howell — 3-13-09

QAI Representative — Date

9 Analytical Personnel

9.1 3M Environmental Laboratory

Michelle D. Malinsky, Ph.D Research Specialist

9.2 Pace Lab Ops

Jonathan Steege

10 References

[1] 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.

[2] F. W. McLafferty, Interpretation of Mass Spectra, Third Edition 1980, University Science Books.

Attachments

1.1 General Project Outline

3M Environmental Health & Safety Operations, Environmental Laboratory

Amended General Project Outline

To: Bill Reagen, 3M EHS&Opns; Environmental Lab

From: Michelle Malinsky, 3M EHS&Opns; Environmental Lab

cc: Cliff Jacoby, 3M EHS&Opns; Environmental Lab

Date: January 14, 2009

Subject: Method Validation - Determination of Fluorochemicals via Protein Precipitation of Fish Tissues (Fillet and Whole Body) and analysis by High Performance Liquid Chromatography with Tandem Mass Spectrometry

1 General Project Information

Project Requester	William Reagen 3M EHS Opns - Environmental Laboratory 260-5N-17 St. Paul, MN 55144-1000 651-733-9739 wkreagen@mmm.com
Project Coordinator	Cliff B. Jacoby 3M EHS&Opns, Environmental Laboratory 260-5N-17 St. Paul, MN 55144-1000 651-733-2533 cbjacoby2@mmm.com
Principal Analytical Investigator	Michelle D. Malinsky 3M EHS&Opns, Environmental Laboratory 260-5N-17 St. Paul, MN 55144-1000 651-733-9859 mmalinsky@mmm.com
Contract Facility/Laboratory	3M Environmental Health and Safety Operations, Environmental Laboratories
Lab Request Number	**E08-0261**
Six Digit Department Number	832202
Project Schedule/Test Dates	Starting August, 2008

All verbal and written correspondence will be directed to Cliff Jacoby.

2 Background Information and Project Objective(s)

The purpose of this project is to validate method ETS-08-045. The validation of this method will generally follow the FDA Guidance for Industry: Bioanalytical Method Validation, May 2001.

Method ETS-08-045 is used to extract perfluorochemicals (PFCs) from fish fillet and whole body sample homogenates, followed by quantitation by LC/MS/MS methodology.

In general, this method involves the homogenization of fish fillet or whole body fish samples with acetonitrile at a 1:10 fish mass to solvent volume ratio (i.e. 0.5 g of tissue to 5 mL acetonitrile). Note: all fish fillets and whole body samples will be pre-homogenized before weighing out aliquots for extraction. The centrifuge tubes containing the protein precipitated fish tissue and solvent are then placed in a freezer for at least one hour. After removal from the freezer, samples are centrifuged at -5°C to pelletize the fish solids. Then, a measured volume of the acetonitrile supernatant is removed to an autovial and acidified with a known volume of 10% formic acid (necessary for the analysis of small acids). PFCs in the acetonitrile protein precipitate extract are analyzed using LC/MS/MS where the mass transitions appropriate for the analytes of interest are monitored. Stable isotopes of various analytes will be incorporated into the method for use as internal standards and surrogates to allow for matrix internal standard quantitation and monitoring of method performance via surrogate recoveries.

The method validation proposed here will explore PFC quantitation in fish tissues via the following approaches:

(1) internal standard calibration against a matrix-matched calibration curve. (If quantifiable levels of target analytes are present in the control matrix, method of standard addition will be used to determine the endogenous concentration.)

(2) internal standard quantitation of endogenous PFC using a matrix-matched calibration curve of the target analyte's isotopically labeled counterpart. (This approach will be performed for quantitation of PFOS only using a matrix-matched calibration curve of [1,2,3,4 ^{13}C]PFOS.)

(3) internal standard calibration against a solvent (unextracted) calibration curve.

The target quantitative range of this method validation will be 0.2 ng/g (0.2 ppb) to 10 ng/g (10 ppb), with selected QC samples spiked at levels up to 4000 ng/g of fish tissue for PFOS. However, if the endogenous concentrations of the test matrices are sufficiently low, quantitation down to 0.025 ng/g will be attempted.

The validation of this method will proceed utilizing whole body homogenates from largemouth bass samples purchased from Osage Catfisheries (Osage Beach, MO), a supplier of fish for scientific testing purposes. These samples have been sent to MPI (State College, PA) for initial processing, then returned to the 3M Environmental Lab for further processing, storage and utilization.

Following the full validation of this method for whole body largemouth bass homogenates, tissues from other species will be cross validated using abbreviated procedures. The planned species cross validation includes fillet and/or whole body homogenates of channel catfish, bluegill, and rainbow trout.

The application of this method for additional sample types, ie. shellfish, may be appropriate, providing sufficient quality control components are included in the analysis of those sample types. This project will not include any type of shellfish as part of the validation.

This validation project does not address the process of the initial whole body or fillet homogenization, only the preparation and analysis of individual aliquots of the homogenized whole body or fillet.

3 Project Schedule

The project is scheduled to start in August 2008. The validation of this method utilizing largemouth bass is projected to take 1 month. Cross validation of this method to other species will depend on the number of species analyzed.

4 Test Parameters

The goal of this project is to validate analytical method ETS-8-45 for whole body largemouth bass homogenates. This method can be used to quantitate the levels of perfluorochemical analytes in both fish fillet and whole body fish homogenates. This method utilizes solvent extraction of the PFCs via protein precipitation, followed by LC/MS/MS analysis.

The analytes to include in this method validation are the C_4 to C_{12} perfluorocarboxylic acids, PFBS, PFHS, PFOS and FOSA. A list of the formulae and molecular weights of these analytes can be found in Table 1. The stable isotope labeled compounds [1,2,3,4-$^{13}C_4$]PFBA, [1,2,3,4-$^{13}C_4$]PFOA, [1,2 -$^{13}C_2$]PFDA, [$^{18}O_2$]PFOS, [$^{18}O_2$]PFHS, and [$^{18}O_2$]PFBS will be spiked for potential use as internal standards (IS) and surrogates. The sample spike level for each IS and surrogate will be approximately 1.0 ng/g (1.0 ppb).

The reference materials to be used in this project are all commercially available. This method validation will incorporate the commercially available linear forms of PFOA, PFHS, and PFOS. However, select quality control samples spiked with 3M electrochemical fluorination (ECF) production lots of PFOA and PFOS will be analyzed with the intent to show that these branched materials may be accurately quantitated against the linear standard.

The target quantitation range of this validation for all analytes is 0.2 ng/g (200 ppt) to 10 ng/g (10 ppb) for each analyte, or as defined/limited by the endogenous levels of any of these analytes in the blank fish tissues. If the endogenous levels of any analytes are below the target level of 0.2 ng/g, the actual LOQ will be the lowest standard point of the curve. In this case, the next target LOQ will be 0.025 ng/g (25 ppt).

The ability of this method to quantitate high levels of PFOS in the presence of lower levels of other PFC analytes will be evaluated via dilution QC. The dilution QC samples will be spiked with PFOS at levels up to 4000 ng/g (4 ppm) in the presence of the other analytes spiked at lower levels.

Because PFOS may be present in the endogenous fish samples at levels above 0.2 ng/g, a separate analysis will be performed where a matrix calibration curve of [1,2,3,4-$^{13}C_4$]PFOS will be prepared using [$^{18}O_2$]PFBS as an internal standard and [$^{18}O_2$]PFHS as the surrogate. The purpose of this analysis will be to investigate if a lower quantitation limit of PFOS can be achieved using a calibration curve prepared from a stable isotopically substituted analog of PFOS. Area counts of the unlabeled PFOS transitions will be monitored and entered into the calibration equation of [1,2,3,4-$^{13}C_4$]PFOS to quantify the endogenous and spiked levels.

Table 1. Target Analytes.

Name	*Synonym or Acronym*	*Formula*	*Molecular Weight (Anionic Form)*
Perfluorobutanoic acid	PFBA (C4 Acid)	C_3F_7COOH	213 $[M-H]^-$
Perfluoropentanoic acid	PFPeA (C5 Acid)	C_4F_9COOH	263 $[M-H]^-$
Perfluorohexanoic acid	PFHxA (C6 Acid)	$C_5F_{11}COOH$	313 $[M-H]^-$
Perfluoroheptanoic acid	PFHpA (C7 Acid)	$C_6F_{13}COOH$	363 $[M-H]^-$
Perfluorooctanoic acid	PFOA (C8 Acid)	$C_7F_{15}COOH$	413 $[M-H]^-$
Perfluorononanoic acid	PFNA (C9 Acid)	$C_8F_{17}COOH$	463 $[M-H]^-$
Perfluorodecanoic acid	PFDA (C10 Acid)	$C_9F_{19}COOH$	513 $[M-H]^-$
Perfluoroundecanoic acid	PFUnA (C11 Acid)	$C_{10}F_{21}COOH$	563 $[M-H]^-$
Perfluorododecanoic acid	PFDoA (C12 Acid)	$C_{11}F_{23}COOH$	613 $[M-H]^-$
Perfluorobutane sulfonic acid	PFBS (C4 Sulfonate)	$C_4F_9SO_3H$	299 $[M-H]^-$
Perfluorohexane sulfonic acid	PFHS (C6 Sulfonate)	$C_6F_{13}SO_3H$	399 $[M-H]^-$
Perfluorooctane sulfonic acid	PFOS (C8 Sulfonate)	$C_8F_{17}SO_3H$	499 $[M-H]^-$
Perfluorooctanesulfonamide	FOSA (C8 Sulfonamide)	$C_8F_{17}SO_2NH_2$	498 $[M-H]^-$
$^{13}C_4$-Perfluorobutanoic acid	[1,2,3,4-$^{13}C_4$]PFBA	$^{13}CF_3(^{13}CF_2)_2{}^{13}COOH$	217 $[M-H]^-$
$^{13}C_4$-Perfluorooctanoic acid	[1,2,3,4-$^{13}C_4$]PFOA	$CF_3(CF_2)_3(^{13}CF_2)_3{}^{13}COOH$	417 $[M-H]^-$
$^{13}C_2$-Perfluorodecanoic acid	[1,2-$^{13}C_2$]PFDA	$CF_3(CF_2)_7(^{13}CF_2)^{13}COOH$	515 $[M-H]^-$
$^{18}O_2$-Ammonium Perfluorobutane sulfonate	[$^{18}O_2$]PFBS	$C_4F_9S^{18}O_2O^-NH_4^+$	303 $[M-H]^-$
$^{18}O_2$-Ammonium Perfluorohexane sulfonate	[$^{18}O_2$]PFHS	$[C_6F_{13}S^{18}O_2O]^-NH_4^+$	403 $[M-H]^-$
$^{18}O_2$-Ammonium Perfluorooctane sulfonate	[$^{18}O_2$]PFOS	$[C_8F_{17}S^{18}O_2O]^-NH_4^+$	503 $[M-H]^-$
$^{13}C_4$-Sodium Perfluorooctane sulfonate	[1,2,3,4-$^{13}C_4$]PFOS	$CF_3(CF_2)_3(^{13}CF_2)_3{}^{13}CF_2SO_3^-Na^+$	503 $[M-H]^-$

Table 2 lists the mass transitions (MRMs) typically monitored for these analytes. Multiple MRM transitions can be summed for an individual analyte to improve the sensitivity for that analyte. However, care should be taken to use a reasonable number of transitions within any one time period, as the inclusion of a large number of transitions can affect the precision and accuracy of all results obtained during that time period. For this reason, it is recommended that the analyte list be split between two or more injections to optimize the overall performance of the method when analysis of all the listed analytes is required. Division of the analyte list also allows the analyst to vary chromatographic conditions to separate matrix interferences from the target analyte

when present, which often cannot be done for simultaneous analysis of all compounds in a single run. For this validation, three separate injections of each sample, QC, blank, etc. are planned. Table 3 provides a summary of the analytes to be monitored in each injection and a brief description of the chromatographic conditions.

Table 2 Mass Transitions of Analytes.

Compound	*Q1*	*Q3*
PFBA (C4 Acid)	213	169
PFPeA (C5 Acid)	263	219
PFHxA (C6 Acid)	313	269, 119
PFHpA (C7 Acid)	363	319, 169
PFOA (C8 Acid)	413	369, 219, 169
PFNA (C9 Acid)	463	419, 219, 169
PFDA (C10 Acid)	513	469, 269, 219
PFUnA (C11 Acid)	563	519, 269, 219
PFDoA (C12 Acid)	613	569, 319, 169
PFBS (C4 Sulfonate)	299	99, 80
PFHS (C6 Sulfonate)	399	99*, 80*
PFOS (C8 Sulfonate)	499	130, 99, 80**
FOSA (C8 Sulfonamide)	498	78
$^{13}C_4$-PFBA	217	172
$^{13}C_4$-PFOA	417	372
$^{13}C_2$-PFDA	515	470
$^{18}O_2$-PFBS	303	84
$^{18}O_2$-PFHS	403	84
$^{18}O_2$-PFOS	503	84
$^{13}C_4$-PFOS	503	131, 99, 80

* The MRMs of 399 to 80 and 99 have been documented in literature to result in interferences in some biological tissues, arising from the presence of 5-pregnan-3,20-diol-3-sulfate isomers[2].

**The MRM of 499 to 80 for PFOS has been documented in literature to result in interferences in some biological tissues, arising from the presence of taurodeoxycholate isomers (bile salt)[2].

Table 3. Analysis Summary.

Method	Transitions Monitored	Chromatographic Conditions
Small Acids (6 transitions)	213>169 (PFBA) 217>172 ([1,2,3,4-$^{13}C_4$]PFBA surrogate) 263>219 (PFPeA) 313>269, 313>119 (PFHxA) 417>372 ([1,2,3,4-$^{13}C_4$]PFOA internal standard)	**Analytical Column:** Prism RP (2 x 50 mm, 5µm particle size) **Mobile Phases** A: 5 mM ammonium acetate in 0.01% acetic acid B: Methanol
PFHpA, Sulfonates, and FOSA (13 transitions)	*363>319, 363>169 (PFHpA) 299>99, 299>80 (PFBS) 399>99, 399>80 (PFHS) 499>99, 499>80, 499>130 (PFOS) 498>78 (FOSA) 304>84 ([$^{18}O_2$]PFBS surrogate) 504>84 ([$^{18}O_2$]PFOS internal standard for sulfonates, FOSA) 417>372 ([1,2,3,4-$^{13}C_4$]PFOA internal standard for PFHpA)	**Extraction Pre-Column:** Waters HLB Online Column (3x20mm, 25µm particle size) **Analytical Column:** Betasil C18 (2.1x100 mm, 5µm particle size **Mobile Phases** A: 2mM ammonium acetate B: Acetonitrile
Large Acids (17 transitions)	413>369, 413>219, 413>169 (PFOA) 463>419, 463>219, 463>169 (PFNA) 513>469, 513>269, 513>219 (PFDA) 563>519, 563>269, 563>219 (PFUnA) 613>569, 613>319, 613>169 (PFDoA) 417>372 ([1,2,3,4-$^{13}C_4$]PFOA internal standard) 515>470 ([1,2-$^{13}C_2$]PFDA surrogate)	**Extraction Pre-Column:** Waters HLB Online Column (3x20mm, 25µm particle size) **Analytical Column:** Betasil C18 (2.1x100 mm, 5µm particle size **Mobile Phases** A: 2mM ammonium acetate B: Acetonitrile

*([1,2-$^{13}C_2$]PFDA surrogate recovery for PFHpA will be recorded from the large acid analysis. The ([1,2-$^{13}C_2$]PFDA transition will not be monitored in the sulfonate analysis.

5 Method Validation

The individual components of this method validation are listed below. It is anticipated that all of these aspects will be evaluated, addressed and discussed in the method validation report.

5.1 Species and Sample Types

The method validation will include analysis of whole body homogenates of largemouth bass (*Micropterus salmoides*). Method cross validation may include whole body and/or fillet homogenates from the following species: channel catfish (*Ictalurus punctatus*), bluegill (*Lepomis macrochirus*), and rainbow trout (*Oncorhynchus mykiss*).

5.2 Analytes

The analytes to be evaluated in this validation are PFBA, PFPeA, PFHxA, PFHpA, linear and branched PFOA, PFNA, PFDA, PFUnA, PFDoA, PFBS, linear PFHS, linear and branched PFOS and FOSA (C_4 to C_{12} PFCAs, C_4, C_6 and C_8 sulfonates, and FOSA).

5.3 Internal Standards and Surrogates

The isotopically substituted compounds [1,2,3,4-$^{13}C_4$]PFBA, [1,2,3,4-$^{13}C_4$]PFOA, [1,2 -$^{13}C_2$]PFDA, [$^{18}O_2$]PFOS, [$^{18}O_2$]PFHS, and [$^{18}O_2$]PFBS will be used as internal standards and surrogates. Internal standards, surrogates, and calibration spikes (where appropriate) will be spiked into individual aliquots of homogenized fish tissue prior to extraction. The surrogate concentration in extracted calibration standards will be at the same level as the other target analytes, while the internal standard concentration will be 1 ng/g for each individual standard (i.e. a multi-level surrogate calibration curve will be generated for accurate quantification of surrogate recovery). QC samples, appropriate blanks, and "samples" will have surrogate spiked at 1 ng/g.

- [1,2,3,4-$^{13}C_4$]PFOA will be used as the internal standard for the quantitation of all the target carboxylic acids.
- [1,2,3,4-$^{13}C_4$]PFBA will be used as a surrogate to estimate the recoveries of the C4-C6 carboxylic acids
- [1,2-$^{13}C_2$]PFDA will be used as a surrogate to estimate the recoveries of the C7-12 carboxylic acids.
- [$^{18}O_2$]PFOS will be used as the internal standard for the quantitation of the target sulfonates and FOSA.
- [$^{18}O_2$]PFBS will be used as a surrogate to estimate the recoveries of the target sulfonates and FOSA.
- For the separate PFOS analysis, [1,2,3,4-$^{13}C_4$]PFOS will be used to construct the calibration curve with [$^{18}O_2$]PFBS serving as the internal standard and [$^{18}O_2$]PFHS as the surrogate.

5.4 Endogenous Levels of Target Analytes in Control Tissues

The endogenous levels of the target analytes will be determined in the whole body largemouth bass in triplicate (x3) by quantitation against a solvent curve and by the method of standard addition. These data can be collected as part of the evaluation of Linearity, Precision and Accuracy (Section 5.5).

The endogenous levels of each analyte in the tissue will be evaluated. These values, if significant, may need to be accounted for in the rest of this validation project, and in future quantitative studies. The endogenous values will be considered specific to the validation tissue used for the study (ie. date of sample receipt, lot#).

5.5 Linearity, Precision and Accuracy

The intended quantitative range for this method is 0.25 ng/g (0.25 ppb) to 10 ng/g (10 ppb) comprised of at least six standard points in the final calibration curve. If the endogenous level of the target analyte is significant, then the endogenous level of that analyte in that sample will be the LOQ. If the endogenous level of the analyte is sufficiently low, then the lowest calibration standard satisfying the method criteria for LOQ will be the LOQ (area counts > two times the area counts of the blank samples and accuracy of 100±30%). Quantitation down to 0.025 ng/g will be attempted when endogenous levels are sufficiently low.

Using whole body largemouth bass homogenates, three curves will be prepared over the range of 0.025 ng/g (25 ppt) to 25 ng/g (25 ppb). The final LOQ for each analyte will largely depend on the endogenous amount detected in the control tissue, and for some analytes will be significantly greater than 0.025 ng/g. Each curve point will include internal standards and surrogates. (IS concentration will be constant at 1 ng/g, surrogate concentrations will be at the same levels as the other target analytes.)

Each run will also include duplicates of the following control samples:

- control matrix blanks with the ISs and surrogates
- control matrix blanks without the ISs and surrogates,
- aqueous blanks with the ISs and surrogates,
- aqueous blanks without the ISs and surrogates

- acid adjusted acetonitrile extraction solvent blanks with the IS and surrogates
- acid adjusted acetonitrile extraction solvent blanks without the ISs and surrogates.

The full validation of whole-body bass will include the following preparations to assess linearity, precision, and accuracy:

- Duplicate matrix-matched curves prepared on a single day to evaluate the intra-day precision and accuracy. (Two separate analysts may prepare the curves; however, each analyst must have his or her own set of accompanying QCs as outlined in Section 5.6.)
- A single matrix-matched curve prepared on a different day to evaluate the inter-day precision and accuracy.

For each target analyte and surrogate, the data will be reduced according to ETS 8-45 using the following approaches:

(1) internal standard calibration against a matrix-matched calibration curve using the method of standard addition when appropriate.

(2) internal standard quantitation of PFOS using a matrix-matched calibration curve of [1,2,3,4-$^{13}C_4$]PFOS for a separate single preparation batch

5.6 Quality Control Samples

Each day an extracted curve is prepared, four levels of QC samples should be prepared for the whole body bass. An additional fifth level of QC will be included on the extraction day in which the single extracted curve is prepared for inter-day comparisons. For PFOA, PFHS, and PFOS, the standard curves will use the linear reference materials. Aspects of the QC samples will utilize both the linear and 3M ECF production lot branched reference materials. Each QC sample will be analyzed against the relevant species-specific matrix matched curve. As discussed previously, samples will include appropriate internal standards and surrogates spiked at 1 ng/g. Internal standard quantitation will be performed. The QC spike levels should be at different concentrations than the curve points. The first four QC levels need to be prepared each time an extracted curve is generated. Dilution QC need to only be prepared once during the validation.

(1) The low-level spiked QC should be approximately 200% of the LLOQ or approximately 0.3 to 0.4 ng/g if the LLOQ is ≤ 0.5 ng/g. If the LLOQ is >0.5 ng/g, then the low-level spikes will be adjusted accordingly for the affected analytes. (PFOA, PFHS, and PFOS in these QC samples will be the linear reference materials).

(2) The mid-level spiked QC should be in the middle part of the curve range. (PFOA, PFHS, and PFOS in these QC samples will be the linear reference materials.)

(3) The high-level spiked QC should be greater than the mid-level spike but still within 80% of the ULOQ. (PFOA, PFHS, and PFOS in these QC samples will be the linear reference materials.)

(4) Separate triplicate mid-level QCs at approximately 5 ng/g will be prepared that contain the 3M ECF PFOA and PFOS only (no other target analytes). These samples will only be quantitated for PFOA and PFOS (i.e. endogenous levels and impurities of other analytes will not be evaluated).

(5) The dilution QC should be spiked with PFOS at a level of approximately 4000 ng/g (4 ppm). The spike levels of the other analytes should be approximately 1ng/g. These QCs will be analyzed without dilution for all analytes excluding PFOS and then post-extraction dilutions will be performed to bring the PFOS levels within calibration range. These high-level QCs are intended to show that low levels of PFCs can be accurately quantitated in the presence of high levels of PFOS. Note: the quantitation of the high levels of PFOS will be done via external standard calibration only. Spiking the internal standard at a comparable ppm level is not practical given that the laboratory's labeled PFOS reference material is only available as a 50 ppm solution. The dilution QC only needs to be prepared once during the validation. Care should be taken to run the dilution QC near the end of the run after any low-level samples. Additionally, several blanks should be analyzed after the dilution QC to verify that PFOS instrument carryover from the high level sample is not present.

For the alternate PFOS analysis using the [1,2,3,4-$^{13}C_4$]PFOS calibration curve, a minimum of triplicate LCSs of non-labeled PFOS at a level ≥200% the endogenous concentration will be included.

5.7 Solvent Curve Analysis

In a separate preparation batch, triplicate matrix blanks (with IS and surrogate) and triplicate lab control spikes at the low, mid, and high levels will be prepared and analyzed against an acid-adjusted solvent curve in acetonitrile. The preparation and analysis batch will include the whole body largemouth bass and all the species/tissue types selected for the cross-validation discussed below. Internal standard quantitation will be performed.

6 Cross Validation of Other Species

Cross validation procedures of this method to other tissue matrices will depend on the results obtained as part of this formal validation of whole body largemouth bass. The nine additional matrices proposed for cross validation include the following: largemouth bass fillet, whole body catfish, catfish fillet, whole body bluegill, bluegill fillet, whole body carp, carp fillet, whole body rainbow trout, and rainbow trout fillet. The priority and inclusion/exclusion of some matrices may be reassessed after the full validation of the whole body bass. The cross validation procedures will include all preparation, analysis, and quantitation aspects of the full validation described in Sections 5 with the following exceptions:

(1) Only a single species-specific, matrix-matched calibration curve will be prepared on a single day (no intra-day or inter-day comparisons will be performed).

(2) Dilution QC of PFOS will not be included.

(3) Alternate PFOS quantitation via calibration with [1,2,3,4-$^{13}C_4$]PFOS will not be included.

7 Method Validation Acceptance Criteria

The applicability and acceptability of the method towards each species and tissue type will be determined by evaluation of the results from this project.

The aspects to be evaluated and their *proposed* acceptance criteria are listed below.

(1) Precision - The precision of each analyte in each control tissue will be a %RSD of ≤ ±20%.

(2) Accuracy - The accuracy of each analyte in each control tissue will be 100±25% (100±30% at the LLOQ).

(3) Correlation Coefficient - The correlation coefficient (r) of each analyte in each control tissue will be ≥0.995.

(4) QC Samples - The precision and accuracy of the QC samples will be 100±30% for each analyte and the %RSD of ≤ ±20% for each analyte, respectively.

Based on the results of the method validation results, the acceptance criteria of this method may be modified based on the analytical needs of a project. Because the intent of the method validation is to determine what acceptance criteria is plausible for this method, method deviations will not be issued for QC samples not meeting the arbitrary requirements listed above.

8 Attachments

None

9 References

1. FDA Guidance for Industry, Bioanalytical Method Validation, May 2001.
2. Benskin, J.P.. Bataineh, M, Martin, J.W.. Anal. Chem 2007, 79, 6455-6464.

10 Revisions

The original GPO stated that method ETS 8-49 would be validated. While writing the validation report, management decided that the method validated was sufficiently different from ETS 8-49 and requested that a new method number be assigned (ETS 8-45).

The original GPO included four different aspects of quantitation (internal standard quantitation with a matrix-matched calibration curve, external standard quantitation against a matrix-matched curve, internal standard quantitation against a solvent curve and external standard quantitation against a solvent curve). As the project progressed, it became apparent that the amount of instrument analysis time and data reduction needed to fulfill the original proposal was not going to be feasible in the time allotted. After discussions with management, it was decided to eliminate all external standard and solvent curve quantitation requirements. After analysis was completed for the three selected species for cross-validation, management reconsidered the need for internal standard solvent curve analysis. The samples outlined in Section 5.7 were then prepared.

PFOS dilution QC was intended to be 10,000 ng/g (10 ppm). Due to a calculation error, the PFOS dilution QC were prepared at 4,000 ng/g. It was decided that this concentration was sufficiently high. The GPO was updated to reflect the concentration prepared.

The original GPO detailed proposed analyses for NIST SRMs of Lake Michigan and Lake Superior trout fillets. Analyses of these samples was moved to a separate project with its own GPO (E07-0295).

Supplemental Information

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"

Laboratory Request Number: E08-0261

Testing Laboratory

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Requester

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1. Extraction and Analysis Dates

Table 1. Extraction and Analysis Dates and Instrument Information.

Prep Batch Description	Prep Analyst	Prep/Extraction Dates	Analyte Group	Instrument	Batch
WB Largemouth Bass Day 1	A	8/20-8/21/2008	C7 Acid/Sulfonates/FOSA	Stan	o080821a
			Large Acids	Ollie	s080824a
			Small Acids	Ollie	o080910a
WB Largemouth Bass Day 2	A	8/27-8/28/2008	C7 Acid/Sulfonates/FOSA	Ollie	o080828a
			Large Acids	Ollie	o080830a
			Small Acids	Ollie	o080905a
WB Largemouth Bass Day 2	B	8/27-8/28/2008	C7 Acid/Sulfonates/FOSA	Ollie	o080829a
			Large Acids	Ollie	o080831a
			Small Acids	Ollie	o080906a
WB Largemouth Bass - Alternate PFOS Quantitaion	A	9/3-9/4/2008	PFOS only	Stan	s080919a
WB Catfish	A	9/9-9/10/2008	C7 Acid/Sulfonates/FOSA	Ollie	o080912a
			Large Acids	Ollie	o080913a
			Small Acids	Ollie	o080911a
WB Bluegill	A	9/26/2008 & 9/29/2008	C7 Acid/Sulfonates/FOSA	Ollie	o080930a
			Large Acids	Stan	s080929a
			Small Acids (re-analysis)	Ollie	o081031a
Rainbow Trout Fillet	A	9/16-9/17/2008	C7 Acid/Sulfonates/FOSA	Ollie	o080918a
			Large Acids	Ollie	o080919a
			Small Acids (re-analysis)	Ollie	o081110a
		11/17-11/18/2008 (small acid analysis only)	Small Acids	Ollie	o081118a

Table 1 Continued.

Prep Batch Description	Prep Analyst	Prep/Extraction Dates	Analyte Group	Instrument	Batch
Solvent Curve Analysis	A	10/1-10/2/2008 (all species)	C7 Acid/Sulfonates/FOSA	Ollie	o081006a
			Large Acids	Stan	s081003b
			Small Acids	Ollie	o081016a
			Small Acids (re-analysis)	Ollie	o081111a
		10/26-10/27/2008 WB Bluegill only	C7 Acid/Sulfonates/FOSA	Ollie	o081105a
			Large Acids	Ollie	o081105b
			Small Acids	Ollie	o081030a
		11/20/2008-11/21/2208 (small acids only)	Small Acids (re-prep)	Ollie	o081121a

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Table 2 System Suitability Non-Compliances.

Prep Batch Description	*Analyte Group*	*Instrument*	*Batch*	*Non-compliance Description Area Counts %RSD*
WB Largemouth Bass Day 1	Large Acids	Stan	s080824a	PFNA: 8.8%
Rainbow Trout Fillet	C7 Acid/Sulfonates/FOSA	Ollie	o080918a	FOSA: 14%
Rainbow Trout Fillet	Small Acids	Ollie	o081110a	PFBA: 12%, PFPeA: 9.0%, [1,2,3,4-$^{13}C_4$]PFBA: 12%
	C7 Acid/Sulfonates/FOSA	Ollie	o081006a	FOSA: 8.6%
	Large Acids	Stan	s081003b	PFNA: 7.6%, PFUnA: 7.9%
	C7 Acid/Sulfonates/FOSA (WB Bluegill reprep)	Ollie	o081105a	FOSA: 10%
Solvent Curve Analysis	Large Acids (WB Bluegill reprep)	Ollie	o081105b	PFDA: 8.1%

Table 3. Continuing Calibration Verification (CCV) Non-Compliances.

Prep Batch Description	*Analyte Group*	*Instrument*	*Batch*	*Non-compliance Description CCV Recovery*
WB Largemouth Bass Day 1	Small Acids	Ollie	o080910a	PFPeA 127%, 13C PFBA 127%

3 Surrogate Recoveries in Spiked Blanks

Table 4. Surrogate Recoveries: Small Acids.

[1,2,3,4-$^{13}C_4$]PFBA Surrogate Recoveries in Spiked Blank Samples (Small Acids)

	Matrix Blank		Aqueous Blank		Acetonitrile Blank	
Prep Batch Description	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
WB Largemouth Bass Day 1	112	112	89.6	89.9	78.6	80.9
WB Largemouth Bass Day 2A	101	102	113	104	110	101
WB Largemouth Bass Day 2B	104	101	98.6	95.4	92.2	86.4
WB Catfish	108	105	85.7	86.6	83.2	84.0
WB Bluegill	131	107	132	110	95.5	98.8
Rainbow Trout Fillet	124	114	140	156	136	120

Table 5. Surrogate Recoveries: Large Acids.

[1,2-$^{13}C_2$]PFDA Surrogate Recoveries in Spiked Blank Samples (Large Acids)

	Matrix Blank		Aqueous Blank		Acetonitrile Blank	
Prep Batch Description	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
WB Largemouth Bass Day 1	121	109	90.3	131	132	121
WB Largemouth Bass Day 2A	106	110	104	101	106	95.0
WB Largemouth Bass Day 2B	103	100	103	99.4	106	99.2
WB Catfish	105	104	100	102	103	96.8
WB Bluegill	92.6	82.6	109	114	120	115
Rainbow Trout Fillet	106	104	103	109	114	108

Table 6. Surrogate Recoveries: Sulfonates, FOSA.

[$^{18}O_2$]PFBS Surrogate Recoveries in Spiked Blank Samples (Sulfonates, FOSA)

	Matrix Blank		Aqueous Blank		Acetonitrile Blank	
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
WB Largemouth Bass Day 1	97.5	99.0	96.6	106	94.7	90.4
WB Largemouth Bass Day 2A	98.3	106	104	95.5	103	96.1
WB Largemouth Bass Day 2B	104	102	97.4	103	91.9	90.6
WB Catfish	102	99.2	103	94.9	101	93.5
WB Bluegill	97.5	101	95.9	98.9	88.5	90.7
Rainbow Trout Fillet	92.4	114	107	98.3	104	98.8

Table 7. Surrogate Recoveries: Solvent Curve Analysis.

	Aqueous Blank				*Acetonitrile Blank*	
Solvent Curve Analysis	*Rep 1*	*Rep 2*	*Rep 3*	*Rep 4*	*Rep 1*	*Rep 2*
Large Acids	89.6	101	100	108	109	98.2
Large Acids WB Bluegill Reprep	99.3	105	NA	NA	101	100
C7 Acid, Sulfonates, FOSA	106	98.3	97.1	105	98.4	92.5
C7 Acid, Sulfonates, FOSA WB Bluegill Reprep	110	109	NA	NA	110	101