LABORATORY INFORMATION BULLETIN

Validation of an LC-MS/MS Method for the Determination of 26 Per- and Polyfluoroalkyl Substances (PFAS) in Animal Foods

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ABSTRACT

A method validation for PFAS in animal foods was completed at The US FDA, Office of Regulatory Affairs (ORA), Arkansas Laboratory (ARKL) for twenty-six analytes in six different matrices: wet cat food (WCF), wet dog food (WDF), dry cat food (DCF), dry dog food (DDF), dried pelletized alfalfa (AA), and chicken feed (CF). A QuEChERS liquid extraction technique was used followed with cleanup using a Weak Anion Exchange (WAX) Solid Phase Extraction cartridge. The resulting extract is then analyzed via LC-MS/MS. Acceptance criteria was taken from Guidelines for the Validation of Chemical Methods in Food, Feed, Cosmetics, and Veterinary Products. 3rd Ed.[1] and the ORA Laboratory Manual[2]. An initial eight-point calibration curve showed good linearity ($r^2 \geq 0.995$ for all analytes) and accuracy (calculated concentrations within ±30% of theoretical) for all analytes. The chromatographic system demonstrated the ability to separate PFOS from the known potential interferences taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), and tauroursodeoxycholic acid (TUDCA). Method detection limits (MDL) were calculated per the latest method given in 40 CFR Appendix B to Part 136 with initial MDL values ranging from 18 ppt (ADONA) to 9388 ppt (6:2 FTS). The high value for 6:2 FTS was due to random contamination in one of twelve method blanks analyzed and recalculation with the outlier removed results in a value of 41 ppt. Matrix specific MDLs were also calculated for WCF, DDF, AA, and CF and ranged from 13 ppt (PFBS in CF) to 9699 ppt (PFHxA in AA). PFBA consistently showed a higher background than other analytes with an initial MDL of 390 ppt and matrix MDL values from 301 ppt (CF) to 866 ppt (AA). Analyte recovery was evaluated from duplicate spikes at six different spike levels (0.05, 0.15, 0.5, 1.5, 2, and 5 ng PFAS/g of sample) in six different matrices: wet cat food (WCF), wet dog food (WDF), dry cat food (DCF), dry dog food (DDF), dried pelletized alfalfa (AA), and chicken feed (CF). Matrix blanks were evaluated for co-extracted interferences and background contamination. Most elevated MDL values and failed recovery spikes could be traced to area contribution from integrated signal in the blanks. Accuracy and precision were evaluated from eleven low level spikes done for the MDL study with accuracies ranging from 96.8% (ADONA) to 149.7% (Br_PFOS) and RSDs from 5.2% (PFHxA) to 23.1% (Br_PFOS). Accuracy and precision could not be assessed for PFBA because of background contamination at this level. Measurement uncertainty was also calculated from eleven

low level spikes done for the MDL study and ranged from 0.1 to 0.7. This method, following the current protocol, will support up to 35 sample results per week with one extraction setup and one instrument.

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INTRODUCTION

Per and Poly – Fluorinated Alkyl Substances (PFAS) have become of international interest over the last decade. These substances are used for a multitude of reasons ranging from fire retardant[3] to waterproofing of clothing[4] to non-stick cookware and oil resistant food contact packaging[5]. Health concerns are growing for this family of compounds, known as “Forever Chemicals”, which are becoming ubiquitous in the environment. Potential health issues range from the early age of low birth weights, reduced immune functions, attention deficit disorder, delayed puberty, reduction in fertility to the elderly with elevated cholesterol and triglycerides in addition to thyroid hormone disruption and cancer[6-15]

Levels of PFAS have been detected - some low but should be considered with concern due to bioaccumulation - in human and animal foods and drinking water[16-26] many times with known contamination sources nearby. Within the US, several states and federal agencies have begun analytical testing for these substances. Within FDA, CFSAN has published a method for determinations on a subset of the analytes presented here in human foods seen in the Total Diet Study[27, 28]. In response to discussions with CVM about PFAS testing, the Persistent Organic Pollutants group at the FDA Arkansas Laboratory (ARKL) has developed and validated a method for use in wet dog and cat food, dry dog and cat food, alfalfa, and chicken feed.

The FDA currently has no regulatory limit established for PFAS, however, the EPA has established health advisory levels at 70 part per trillion exposure to PFOA and PFOS from drinking water[29]. The European Union released an exposure limit of 4.4 ng/kg body weight/week for PFOA, PFOS, PFNA and PFHxS combined[30].

EXPERIMENTAL

(Equipment and reagents have been provided for guidance and is not an endorsement of any specific supplier or brand. Equivalent products may be substituted as appropriate.)

Methods & References:

Guidelines for the Validation of Chemical Methods for the FDA Foods Program, 3rd Edition[1]
CVM GFI #118 Mass Spectrometry for Confirmation of Identity of Animal Drug Residues[31]
ORA Laboratory Manual[2]
Investigations Operations Manual (IOM)[32]
40 CFR Appendix B to Part 136[33]

Analytical Standards:
Twenty-six natives (Table 1), thirteen isotopically labeled surrogate internal standards (Table 2), and one recovery internal standard (N-ethyl-d5-perfluoro-1-octanesulfonamidoacetic acid (d5-N-EtFOSAA)) were purchased as individual solutions (50 µg/mL in methanol) from either Wellington Laboratories (Guelph, ON, Canada), or Cambridge Isotope Laboratories, Inc. (Tewksbury, MA).

Table 1 - Native PFAS Standards

Analyte	Abbreviation
Perfluorobutanoic acid	PFBA
Perfluoropentanoic acid	PFPeA
Perfluorohexanoic acid	PFHxA
Perfluoroheptanoic acid	PFHpA
Perfluorooctanoic acid*	PFOA
Perfluorononanoic acid	PFNA
Perfluorodecanoic acid	PFDA
Perfluoroundecanoic acid	PFUdA
Perfluorododecanoic acid	PFDoA
Perfluorotridecanoic acid	PFTrDA
Perfluorotetradecanoic acid	PFTeDA
Sodium Dodecafluoro-3H-4,8-dioxanonoate	ADONA
Hexafluoropropylene oxide dimer acid	HFPO-DA
N-methyl perfluorooctanesulfonamidoacetic acid**	NMeFOSAA
N-ethyl perfluorooctanesulfonamidoacetic acid**	NEtFOSAA
Potassium perfluoro-1-butanesulfonate	PFBS
Sodium perfluoro-1-pentane sulfonate	PFPeS

Potassium Perfluorohexanesulfonate**	PFHxS
Sodium perfluoro-1-heptane sulfonate	PFHpS
Potassium Perfluorooctanesulfonate **	PFOS
Potassium 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate	9Cl-PF3ONS
Potassium 11-chloroeicosafluoro-3-oxaundecane-1-sulfonate	11Cl-PF3OUdS
Sodium 1H,1H,2H,2H-perfluorohexane sulfonate	4:2 FTS
Sodium 1H,1H,2H,2H-perfluorooctane sulfonate	6:2 FTS
Sodium 1H,1H,2H,2H-perfluorodecane sulfonate	8:2 FTS
Sodium 1H,1H,2H,2H-perfluorododecane sulfonate	10:2 FTS
* PFOA was purchased as the linear only isomer for use in preparation of calibration solutions. The PFOA technical mixture containing branched and linear isomers is unsuitable for quantitative analysis but was also purchased for use as a qualitative standard to establish retention times for the branched isomers. ** Native solutions for PFHxS, PFOS, N-MeFOSAA, and N-EtFOSAA were mixtures of branched and linear isomers.	

Table 2 - Individual PFAS Surrogates

Surrogate	Abbreviation
Perfluoro-n-[2,3,4-$^{13}C_3$] butanoic acid	13C3-PFBA
Perfluoro-n-[3,4,5-$^{13}C_3$] pentanoic acid	13C3-PFPeA
Perfluoro-n-[1,2-$^{13}C_2$] hexanoic acid	13C2-PFHxA
Perfluoro-n-[$^{13}C_8$] octanoic acid	13C8-PFOA
2,3,3,3-Tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)-$^{13}C_3$-propanoic acid	13C3-HFPO-DA
N-deuteriomethylperfluoro-1-octanesulfonamidoacetic acid	d3-NMeFOSAA
Sodium perfluoro-1-[2,3,4-$^{13}C_3$] butane sulfonate	13C3-PFBS
Sodium perfluoro-1-hexane[$^{18}O_2$] sulfonate	18O2-PFHxS
Sodium perfluoro-[$^{13}C_8$] octane sulfonate	13C8-PFOS
Sodium $1^2H,1^2H,2^2H,2^2H$-Perfluoro-[1,2-$^{13}C_2$]hexane sulfonate†	d4-13C2-4:2 FTS
Sodium $1^2H,1^2H,2^2H,2^2H$-Perfluoro-[1,2-$^{13}C_2$]octane sulfonate†	d4-13C2-6:2 FTS
Sodium $1^2H,1^2H,2^2H,2^2H$-Perfluoro-[1,2-$^{13}C_2$]decane sulfonate†	d4-13C2-8:2 FTS
Sodium $1^2H,1^2H,2^2H,2^2H$-Perfluoro-[1,2-$^{13}C_2$]decane sulfonate†	d4-13C2-10:2 FTS
† Currently, these four surrogates are only available from Cambridge Isotope Laboratories, Inc.	

Individual PFAS native solutions were used to prepare stock mixtures at 1000 ng/mL (1 µg/mL), 100 ng/mL (0.1 µg/mL), and 10 ng/mL (0.01 µg/mL) in methanol as follows:

- 0.2 mL of each individual 50 µg/mL (50000ng/mL) PFAS analytical standard (26 native compounds in Table 1 – 5.2 mL total) was combined and diluted with 4.8 mL of methanol to produce 10mL of the 1 µg/mL (1000 ng/mL) stock solution.
- A 1 mL aliquot of the 1000 ng/mL stock solution was diluted with 9 mL of methanol to produce 10 mL of the 100 ng/mL stock solution.

- A 0.1 mL aliquot of the 1000 ng/mL stock solution was diluted with 9.9 mL of methanol to produce 10 mL of the 10 ng/mL stock solution.

A PFAS surrogate stock solution (SUR) at 100ng/mL was prepared by combining 0.020 mL of each 50 µg/mL analytical standard (13 isotopically labeled PFAS in Table 2 - 0.26 mL total), then diluting with 9.74 mL methanol to give a final volume of 10mL.

A 10 mL solution of the isotopically labeled recovery internal standard was prepared at 100 ng/mL by diluting 0.02 mL of N-ethyl-d5-perfluoro-1-octanesulfonamidoacetic acid (d5-N-EtFOSAA) analytical stock standard (50 µg/mL or 50000 ng/mL) with 9.98 mL of methanol.

Eight calibration solutions were prepared by combining the volumes of methanol, the appropriate native stock, surrogate stock, and recovery standard stock solutions given in Table 3 below.

Table 3 - Calibration Standard Composition

Level	Final [Native] ($ng \cdot mL^{1}$)	Concentration Native Stock ($\mu g \cdot mL^{1}$)	Volume Native Stock (µL)	Volume SS (µL)	Volume IS (µL)	Volume MeOH (mL)	Final Volume (mL)
CS1	0.01	0.01	10	100	100	9.79	10
CS2	0.05	0.01	50	100	100	9.75	10
CS3	0.1	0.01	100	100	100	9.70	10
CS4	0.5	0.1	50	100	100	9.75	10
CS5	1	0.1	100	100	100	9.70	10
CS6	5	1	50	100	100	9.75	10
CS7	10	1	100	100	100	9.70	10
CS8	25	1	250	100	100	9.55	10

All prepared calibration standard solutions were stored in polypropylene containers, not glass.

Equipment and Consumables:

a. Sample homogenization – blender pre-rinsed with DI water and methanol (Waring Commercial Blender, McConnellsburg, PA)
b. Shaker – 2010 Geno/Grinder (Spex SamplePrep, Metuchen, NJ)
c. Vortexer – Vortex-Genie 2 (Scientific Industries, Inc. Bohemia, NY)
d. Centrifuge – Sorvall legend XFR centrifuge (Thermo Fisher Scientific, Waltham, MA)
e. Nitrogen evaporation system – (Organomation, Berlin, MA)
f. Ultra-high purity (99.9999%) nitrogen gas (Messer LLC, Bridgewater, NJ) [Evaporated head gas from a bulk liquid nitrogen holding tank is fed into a house distribution system, passing through two

dryers. This source is used for both the nitrogen evaporation system and the mass spectrometer gases.]

g. 1L Borosilicate culture (media) storage bottles with polypropylene (PP) lids– (Corning Pyrex, Corning, NY)
h. 4 mL Nalgene Narrow-Mouth Bottles (Thermo Fisher Scientific, Waltham, MA)
i. Falcon 50 mL PP conical centrifuge tubes (Thermo Fisher Scientific, Waltham, MA)
j. Falcon 15 mL PP conical centrifuge tubes (Thermo Fisher Scientific, Waltham, MA)
k. Target DP Vial, PP, 300µL (Thermo Scientific, Waltham, MA)
l. AVCS Clear Membrane Autosampler Caps (Thermo Scientific, Waltham, MA)
m. Nano filter autosampler vials 0.2 µm nylon (Thomson Instrument Company, Oceanside, CA)
n. SPE cartridge – 6mL Strata™-PFAS (Polymeric Weak Anion Exchange 200 mg / Graphitized Carbon Black 50mg) tubes (Phenomenex, Torrance, CA)
o. Advantage Polypropylene Needles for Vacuum Manifold System (Analytical Sales and Services, Inc., Flanders, NJ)
p. 24-port Vacuum Manifold, Visiprep 24 (Supelco, Bellefonte, PA)

Matrices:

Wet and dry cat food, wet and dry dog food, alfalfa/timothy (pelleted), and chicken feed.

Sample Collection, Preservation, and Storage:

Follow the Pesticide Sample Schedule located in Chart 3 of the IOM[32] Chapter 4 for guidance in determining sample size.

When performing sampling, avoid wearing clothing that has been treated with water repellants. If manufacturer containers must be opened, wear clean nitrile gloves while removing samples. Avoid allowing the samples to come into contact with aluminum foil, adhesives (sticky notes), and inks (pens or markers). Samples must be collected in new polypropylene or high-density polyethylene (HDPE) containers with lids that do not use teflon liners. Shelf stable materials may be stored and shipped at ambient temperature. If canned samples must be opened, freeze the materials as soon as possible and ship packed in dry ice.

A homogenate of shelf stable samples should be prepared within 60 days of receipt by the laboratory and the homogenate stored in a freezer. Frozen samples received at the lab should be transferred to a freezer as soon as practicable with homogenates also stored in the freezer.

Instrumental samples will be submitted for instrumental analysis as soon as practicable after preparation. Instrument samples may be held in a freezer for up to 14 days before analysis if necessary. Extra supernatant from the liquid QuEChERS extraction may be held in the freezer for seven days if diluted with 4 mL of LC/MS water for every mL of supernatant. Instrument samples prepared

from the diluted supernatant so held and subjected to SPE cleanup must be analyzed within seven days.

Reagents:

a. Formic acid – Sigma Aldrich, reagent grade (St. Louis, MO)
b. Water – Fisher Scientific, Optima LC/MS grade (Waltham, MA)
c. Acetonitrile - Fisher Scientific, Optima LC/MS grade (Waltham, MA)
d. Methanol - Fisher Scientific, HPLC grade (Waltham, MA)
e. Ammonium acetate – Fisher Scientific, HPLC grade (Waltham, MA)
f. Ammonium hydroxide - Fisher Scientific, certified ACS Plus 14.8N (Waltham, MA)
g. Acetic acid, glacial – Fisher Chemical, HPLC grade (Waltham, MA)

HPLC mobile phases and SPE solutions were prepared as follows:

a. Mobile Phase Stock Solution: 5 M Ammonium Acetate (aq)
   3.85 g of ammonium acetate was weighed and add to a 15 mL conical polypropylene vial. Five milliliters of LC/MS Optima water was added and mixed until the solid dissolved. Once the ammonium acetate appeared to be dissolved, the solution was brought up to 10 mL with additional LC/MS Optima water and vortexed until the solution was mixed well. The solution was stored at 4°C and allowed to come to room temperature before preparing HPLC Mobile Phases A and B.

b. HPLC Mobile Phase A: 5 mM Ammonium Acetate (aq)
   500 µL of the mobile phase stock solution was added to ~500mL of LC/MS Optima water in an appropriate mobile phase reservoir and mixed well.

c. HPLC Mobile Phase B: 5 mM Ammonium Acetate in Methanol
   500 µL of the mobile phase stock solution was added to ~500mL of LC/MS Optima methanol in an appropriate mobile phase reservoir and mixed well.

d. 5% (v/v) Methanolic Ammonium Hydroxide
   50 mL of ammonium hydroxide (14.8 N) was added to 950 mL of LC/MS Optima methanol in a 1L culture bottle and mixed well.

e. 1% (v/v) Acetic Acid (aq)
   10 mL of glacial acetic acid was added to 990 mL of LC/MS Optima water in a 1L culture bottle and mixed well.

Extraction and Cleanup:

The study was performed using the following extraction and cleanup procedure. Method blanks were prepared by pipetting 5 mL of reagent water into a 50 mL PP conical tube in lieu of the sample matrix.

All sample matrices should be thawed, if frozen, and allowed to come to room temperature before sample preparation.

Sample Preparation

1. Weigh out 5g of sample into a 50 mL PP conical tube.
2. Add 100 µL of 1 µg/mL isotopically labeled surrogate standard.

   Note: For spiked samples, the mixed native spike solution of the appropriate concentration was also pipetted onto the matrix or reagent water at this step.
3. Wait 30-60 minutes.
4. Add water based on the commodity type.
   a. Wet pet foods - 5 mL
   b. Dry pet foods and feeds - 15 mL
5. Mix thoroughly and let hydrate for 30-60 minutes. This can be achieved by shaking on the Geno/Grinder at 1500 rpm for 1 minute, then putting on the Glas-Col shaker for 30 minutes at 900 rpm, 100 pulses/min.

Liquid QuEChERS Extraction

6. Add 10 mL of acetonitrile to sample.
7. Add 150 µL formic acid to sample.
8. Cap the samples well and shake on Geno/Grinder at 1500 rpm for 1 minute.
9. Add QuEChERS salt packet (6000 mg $MgSO_4$ and 1500 mg NaCl) to sample.
10. Cap the samples well and shake on the Geno/Grinder at 1500 rpm for 5 minutes.
11. Centrifuge samples for 5 minutes at 4300 rpm and 15°C.
12. Transfer 1 mL of supernatant to a 15 mL polypropylene centrifuge tube.
13. Dilute 1 mL of extract to 5 mL using 4 mL LC/MS Optima Water.

Solid-phase Extraction (SPE) Cleanup

All steps in this procedure use a flow rate of 1-2 drops per second, or 0.5-1 mL per minute

14. Condition each Strata™-PFAS (WAX/GCB) cartridge using the reagents below without letting the cartridge dry between conditioning steps. If the cartridge dries at any point, conditioning must re-start from the beginning.
    a. 6 mL of 5% ammonium hydroxide in methanol
    b. 6 mL of water
    c. 6 mL of 1% acetic acid

15. Load sample to column and let all liquid pass through and save the sample tube.
16. After all liquid has passed through, pass air through for ~1 minute.
17. Wash sample with 6 mL of LC/MS Optima water.
18. After all water has passed through, pass air through the column for ~1 minute.
19. Wash sample with 6 mL of HPLC grade methanol.
20. After all methanol has passed through, pass air through for ~1 minute.
21. Release the vacuum on the manifold. Place a new 15mL polypropylene centrifuge tube under each sample position and reapply vacuum.
22. Rinse each centrifuge tube saved from Step 15 with a 3 mL portion of 5% ammonium hydroxide in methanol and transfer the rinsate to the cartridge.
23. Repeat the tube rinsing with a second 3mL aliquot of the elution solvent, transfer to the cartridge, and elute.

Nitrogen Concentration

24. Remove the tubes from the manifold. Using an N-Evap with a bath temperature of approximately 60°C, evaporate the sample extract to dryness with a gentle stream of nitrogen .
25. Remove dry sample tubes from the N-Evap.
26. Add 0.990 mL of HPLC methanol to the dried tube to reconstitute the sample.
27. Add 10 µL of 1 µg/mL d5-N-EtFOSAA recovery internal standard solution to the reconstituted sample extract.
28. Recap each centrifuge tube and vortex well. Visually verify that nothing is suspended in the solution.
29. Transfer a portion of the sample to a 0.2 µm nylon Nano Filter autosampler vial.
30. Extract is ready for instrument analysis.

Instrumentation:

A Sciex 6500+ triple quadrupole mass spectrometer (MS/MS) coupled with an Agilent 1260 Infinity II high performance liquid chromatography (HPLC) system was used for this study. A copy of the instrument acquisition method can be found in Appendix A. The LC was modified to reduce potential PFAS background before use as follows: all fluorinated ethylene-propylene (FEP) tubing was replaced with polyetheretherketone (PEEK); stock pump seals were replaced with polyethylene normal phase seals; the stock auto-sampler switching rotor was replaced with a PEEK version; and a delay column (Phenomenex Luna C18, 5µm, 30mm x 3mm) was installed between the eluent mixer and the autosampler.

Separation was carried out on a Phenomenex Gemini C18 column (3µm, 100mm x 2mm) with the column compartment at 40°C. Mobile phase A consisted of aqueous 5mM ammonium acetate and mobile phase B was 5mM ammonium acetate in methanol. The elution gradient and flows are shown in Table 4. All injections were 5µL.

Table 4 - LC Gradient Method

Time (min)	[B]	Flow ($mL \cdot min^{1}$)
0	10%	0.300
1	10%	0.300
2	50%	0.300
16	99%	0.350
17	99%	0.400
18	10%	0.400
20	10%	0.300
23	10%	0.300

Instrument acquisition was controlled with Sciex Analyst 1.7.1 software. Electrospray Ionization (ESI) was utilized. The source was operated in negative mode with a capillary voltage of -4500V, and a temperature of 350°C. Ultra-high purity nitrogen was used as the source (GS1, GS2 50 psi), curtain (40 psi), and collision gas (8 psi).

The mass spectrometer was operated in scheduled multiple reaction monitoring (sMRM) mode. Table 5 shows parent and product ions, declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP). The target scan time per MRM was set to 1.1sec with windows set to 120sec except for analytes with branched isomers that were set to 240sec.

Table 5 - MS/MS MRM Parameters

Q1 Mass (Da)	Q3 Mass (Da)	ID	DP (V)	EP (V)	CE (V)	CXP (V)
213	169	PFBA_1*	-15	-10	-14	-15
263	219	PFPeA_1*	-14	-10	-13	-5
313	269	PFHxA_1*	-23	-8	-14	-5
313	119	PFHxA_2	-23	-10	-30	-13
363	319	PFHpA_1*	-20	-10	-16	-9
363	169	PFHpA_2	-20	-10	-25	-10
413	369	PFOA_1*	-43	-7	-16	-25
413	219	PFOA_2	-24	-5.5	-23	-25

463	419	PFNA_1*	-38	-11	-15	-37
463	269	PFNA_2	-40	-5	-24	-13
513	469	PFDA_1*	-15	-10	-16	-29
513	269	PFDA_2	-20	-10	-26	-17
563	519	PFUdA_1*	-25	-10	-18	-15
563	169	PFUdA_2	-25	-10	-28	-15
613	569	PFDoA_1*	-25	-10	-18	-15
613	169	PFDoA_2	-25	-10	-30	-15
663	619	PFTrDA_1*	-25	-10	-20	-15
663	169	PFTrDA_2	-25	-10	-36	-15
713	669	PFTeDA_1*	-25	-10	-22	-15
713	169	PFTeDA_2	-25	-10	-38	-15
377	251	ADONA_1*	-24	-8	-15	-20
377	85	ADONA_2	-20	-7	-39	-10
285	169	HFPO-DA_1*	-20	-6	-11	-27
285	185	HFPO-DA_2	-20	-5	-21	-27
570	419	NMeFOSAA_1*	-75	-10	-28	-12
570	483	NMeFOSAA_2	-75	-10	-22	-12
584	419	NEtFOSAA_1*	-90	-10	-28	-12
584	526	NEtFOSAA_2	-90	-10	-28	-12
299	80	PFBS_1*	-44	-10	-70	-11
299	99	PFBS_2	-35	-4	-36	-15
349	99	PFPeS_1*	-80	-9	-80	-12
349	80	PFPeS_2	-53	-9	-40	-12
399	80	PFHxS_1*	-80	-10	-85	-9
399	99	PFHxS_2	-80	-10	-70	-11
449	99	PFHpS_1*	-58	-8	-84	-24
449	169	PFHpS_2	-68	-8	-41	-27
499	80	PFOS_1*	-150	-4	-120	-10
499	99	PFOS_2	-150	-4	-100	-10
531	351	9Cl-PF3ONS_1*	-75	-9	-41	-37
531	83	9Cl_PF3ONS_2	-69	-11	-60	-29
631	451	11Cl-PF3OUdS_1*	-20	-10	-42	-6
631	199	11Cl-PF3OUdS_2	-20	-10	-36	-11
327	307	4:2 FTS_1*	-50	-6	-28	-24
327	81	4:2 FTS_2	-40	-11	-62	-10
427	407	6:2 FTS_1*	-31	-11	-34	-38
427	81	6:2 FTS_2	-41	-12	-88	-12

527	507	8:2 FTS_1*	-69	-11	-37	-29
527	81	8:2 FTS_2	-70	-11	-109	-36
627	607	10:2 FTS_1*	-54	-6	-46	-48
627	81	10:2 FTS_2	-68	-6	-129	-15
216	172	13C3-PFBA_1*	-17	-8	-12	-14
266	222	13C3-PFPeA _1*	-17	-6	-11	-28
315	270	13C2-PFHxA_1*	-13	-10	-14	-12
315	119	13C2-PFHxA_2	-25	-10	-30	-13
421	376	13C8-PFOA_1*	-36	-8	-13	-20
421	172	13C8-PFOA_2	-19	-5	-25	-7
287	169	13C3-HFPO_1*	-31	-5	-10.6	-25
287	185	13C3-HFPO_2	-28	-4	-22	-17
573	419	d3-NMeFOSAA	-75	-10	-28	-12
302	80	13C3-PFBS_1*	-88	-6	-73	-9
302	99	13C3-PFBS_2	-85	-6	-36	-8
403	84	18O2-PFHxS_1*	-80	-10	-85	-9
403	103	18O2-PFHxS_2	-60	-10	-81	-15
507	80	13C8-PFOS_1*	-100	-5	-125	-15
507	99	13C8-PFOS_2	-100	-5	-100	-15
333	312	d4-13C2-4:2 FTS_1*	-60	-10	-30	-29
333	82	d4-13C2-4:2 FTS_2	-60	-10	-63	-12
433	412	d4-13C2-6:2 FTS_1*	-80	-10	-33	-13
433	82	d4-13C2-6:2 FTS_2	-80	-10	-67	-13
533	512	d4-13C2-8:2 FTS_1*	-100	-10	-49	-23
533	82	d4-13C2-8:2 FTS_2	-100	-10	-81	-34
633	612	d4-13C2-10:2FTS_1*	-100	-10	-45	-19
589	419	RS d5-NEtFOSAA_1	-50	-10	-30	-20
589	219	RS d5-NEtFOSAA_2	-50	-10	-38	-20
499	124	TDCA	-150	-4	-120	-10
* quantitative transition						

Data Processing:

All data processing was performed using Sciex OS (version 1.6.1.29803 and 1.6.2.36627) software. A copy of the data processing method can be found in Appendix B.

Qualitative confirmation of analyte identity is accomplished by monitoring ion ratios (area of qualitative transition/area of quantitative transition), and retention times. Measured ion ratios are evaluated against the expected ratios (an average calculated from all calibration standards) using a variable tolerance scheme shown in Table 6 below. Analyte retention times in validation samples were required to be within 2% of the corresponding retention time in the processing method reference standard. Peaks with signal to noise (S/N) ratios ≥ 10 which also meet ion ratio and retention time tests are considered positive detects.

Table 6 - Ion Ratio (IR) Tolerance

IR Lower Limit	IR Upper Limit	Acceptable % Difference
0	0.1	50
0.101	0.2	40
0.201	0.5	35
0.501	1	30

Eight calibration standard solutions were prepared with native concentrations of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, and 25 ng/mL. For PFBS, PFPeS, PFHxS, PFHpS, PFOS, ADONA, 11-Cl-PF3ONS, 9-Cl-PF3ONS, 4:2 FTS, 6:2 FTS, 8:2 FTS, and 10:2 FTS these concentrations are for salt forms used to prepare the standard. Concentrations in the calibration table were corrected to reflect the concentration of the analytes in their acid form by multiplying the nominal concentration times the ratio of molecular weight of acid form divided by the molecular weight of the salt form. Concentrations of the surrogate internal standards and the recovery internal standard were kept constant at 10 ng/mL.

Calibration standards were injected in order of least to greatest concentration, an instrument blank (methanol injection) was acquired, and then a second injection of each calibration level was acquired in the same least to greatest concentration sequence.

Calibration was accomplished using native peak area data for the two calibration standard sets fit to a linear calibration model weighted at 1/x and the fit forced through the origin. Analyte/surrogate internal standard pairings are given in Table 7. Quantitative transitions were previously noted in the MS/MS parameters (Table 5). For analytes with two monitored transitions the qualitative transition also received a calibration fit but determinations are only reported from quantitative transition data.

Table 7 - Analyte/Internal Standard Pairing

Analyte	Internal Standard
PFBA	13C3-PFBA
PFPeA	13C3-PFPeA
PFHxA	13C2-PFHxA
PFHpA	13C2-PFHxA
PFOA	13C8-PFOA
PFNA	13C8-PFOA
PFDA	13C8-PFOA
PFUdA	13C8-PFOA
PFDoA	13C8-PFOA
PFTrDA	13C8-PFOA
PFTeDA	13C8-PFOA
ADONA	13C8-PFOA
HFPO-DA	13C3-HFPO-DA

Analyte (cont'd)	Internal Standard
NMeFOSAA	d3-NMeFOSAA
NEtFOSAA	d3-NMeFOSAA
PFBS	13C3-PFBS
PFPeS	18O2-PFHxS
PFHxS	18O2-PFHxS
PFHpS	18O2-PFHxS
PFOS	13C8-PFOS
9Cl-PF3ONS	13C8-PFOS
11Cl-PF3OUdS	13C8-PFOS
4:2 FTS	d4-13C2-4:2FTS
6:2 FTS	d4-13C2-6:2FTS
8:2 FTS	d4-13C2-8:2FTS
10:2 FTS	d4-13C2-10:2FTS

Surrogate internal standards are entered twice in the calibration table. The first entry is as an internal standard and used to calculate concentrations of associated natives. The second entry is as a surrogate. Surrogate entries have the recovery standard (d5-NEtFOSAA) assigned as the internal standard and use the mean response ratio of surrogate to recovery standard to calculate surrogate recovery.

If data for an analyte did not pass qualitative requirements in one or both low-level standards (calculated concentrations outside ± 50%, missing peak in a quantitative trace, or failed ion ratio), the data point from both standards was excluded ('Used' box unchecked in the peak table). This evaluation was repeated until the lower limit of quantitation (LLOQ) was established for each analyte.

Both linear and branched alkane isomers of PFAS can be expected in samples. Most of the target analyte determinations are for only the compound's linear alkane isomer. However, PFOA, PFHxS, PFOS, N-MeFOSAA, and N-EtFOSAA are present in the calibration standards as both branched and linear form and determinations are for the sum of all isomers. For these compounds each quantitative and qualitative transition is entered in the calibration twice. One pair of entries is for the linear isomer only (entries prefixed with "L_") while the second pair is the sum of branched plus linear isomers (entries prefixed with "Br_"). For the "L_" entries, only the linear isomer peak is integrated and used to generate the ion ratio for qualitative evaluation.

For the "Br_" entries, integration parameters were adjusted to integrate the branched and linear isomers as a single area. To accomplish this, the Peak Noise setting was decreased from the default 40

to 10, and the Peak Splitting factor was increased from the default 2 to 20. An example chromatogram for PFHxS (left – quantitative transition for target analyte, right – primary transition for the labeled surrogate) with integration shaded in the mid-level calibration standard is shown in Figure 1. Manual integration was still sometimes required for low level standards or samples. Start and stop integration times from the automatic integration were noted from a mid-level calibration standard and used to guide the manual integration. Tracking of manual integrations was automatic by Sciex OS. Compound determinations are only reported from the qualitative transition data of the "Br_" entry and not the "L_".

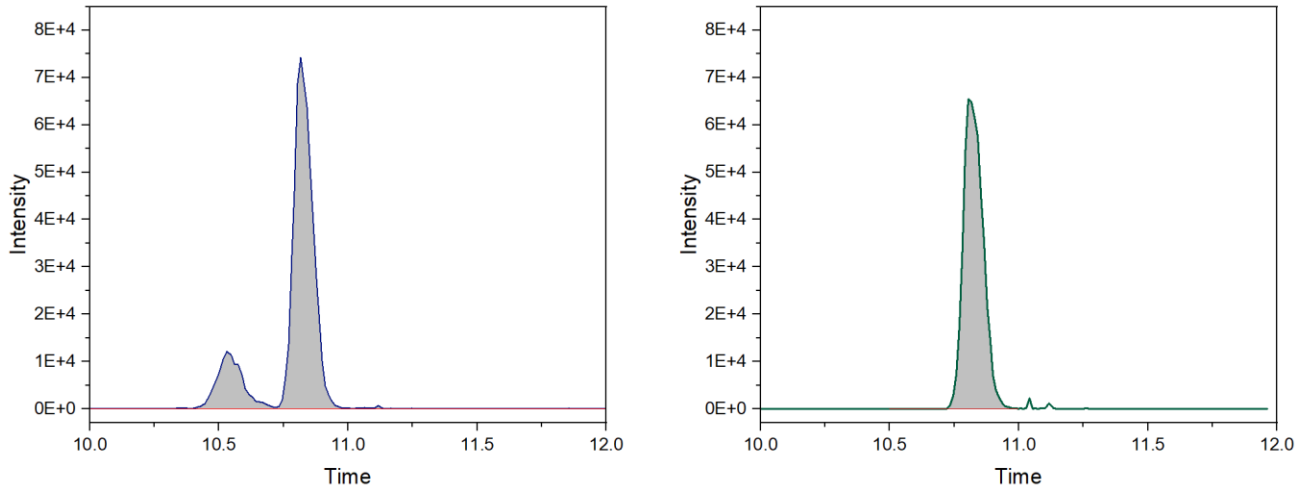


Figure 1 - Integration example for the quantitative transition of target analyte PFHxS encompassing branched and linear isomers (left). The primary transition for the labeled linear isomer only internal standard (right) is shown for reference.

Branched isomers were present in the native solutions for PFHxS, PFOS, N-MeFOSAA, and N-EtFOSAA used to create the calibration standards while PFOA was present only as the linear isomer. A technical PFOA (T-PFOA) mix of branched and linear isomers was acquired separately and used to establish the retention times of branched isomers for this compound.

[Note: **PFBA, and PFPeA** each have only a single MRM transition to monitor, thus ion ratio evaluation was not applicable to these analytes. Because they lack two transitions, these analytes **do not meet the minimum ORA requirements for identification in an LC-MS/MS method**. In addition, because of the summed integration approach to analytes with branched and linear isomers, ion ratios for these targets are not especially useful in real samples as the ratio of isomers may vary from that in the standard. Consequently, the identification of all components included in the integration for these targets may also not meet ORA minimum requirements. However, for purposes of this study

determinations for these target analytes are reported as if they met normal minimum requirements for identification.]

Three cholic acids have been recognized as possible interferents in the determination of PFOS. Taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), and tauroursodeoxycholic acid (TUDCA) have molecular weights <1amu from the PFOS [M-H]- (499 m/z) parent ion. Additionally, each contains a sulfonate group and generates the same sulfite radical ion (80 m/z) monitored as the product for PFOS determinations. If not chromatographically resolved from PFOS, they can generate a false positive or bias high determination. Solutions of certified reference materials for the three cholic acids were acquired to demonstrate their separation from PFOS.

FTS Surrogate Internal Standard Choice:

The isotopically labelled surrogates for the FTS compounds available from Wellington have two ^{13}C atom substitutions creating a 2 Dalton mass offset between the native and surrogate. Because there are two naturally occurring isotopes of sulfur, ^{32}S and ^{34}S, approximately 4.5% of the native molecules contain ^{34}S and have the same nominal molecular weight as their surrogate. If the [M-H]-HF transition is monitored for native and surrogate the native peak areas show an excellent fit to a linear regression. However, instead of being constant over the calibration range the surrogate areas show an increase in area as the concentration of native increases because of the crosstalk between the ^{34}S native molecules and the surrogate[34]. There are two possible ways to deal with this: First, fit the analyte/IS ratio to a quadratic equation instead of a linear one. Second, instead of monitoring the [M-H]-HF transition, monitor the much weaker [M-H] → 80 m/z transition.

For this work a third solution was implemented. Cambridge Isotope Laboratories offers isotopically labeled FTS surrogates that have two ^{13}C atom substitutions like the Wellington standards. In addition, they also feature an additional substitution of four deuterium atoms for ^{1}H on the carbons in positions one and two of the alkyl chain and therefore a mass offset of 6 Daltons compared to the native. Use of these standards to eliminate native/surrogate crosstalk allowed both monitoring the strongest intensity transition for the quantitative signal and a linear fit to the data.

RESULTS AND DISCUSSION

Validation Criteria

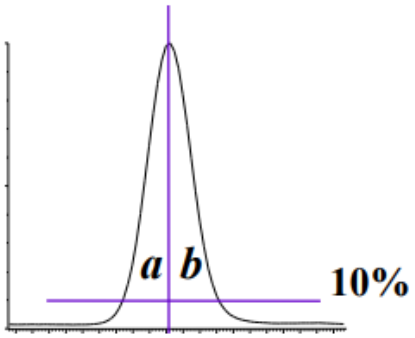
The validation follows requirements of Guidelines for the Validation of Chemical Methods in Food, Feed, Cosmetics, and Veterinary Products, 3rd Edition, U.S. Food and Drug Administration Foods Program, October 2019[1] (FVM Guidelines hereafter), Level Two validation criteria as well as ORA Lab Manual section 5.4.5 - Methods, Method Verification and Validation[2](ORA LM). The FVM Guidelines state that, “Validation of new quantitative methods should include at a minimum evaluation of the following performance characteristics: accuracy, precision, selectivity, limit of detection, limit of

quantitation, linearity (or other calibration model), range, measurement uncertainty, ruggedness, confirmation of identity and spike recovery" and the ORA LM that, "Typical validation characteristics which should be considered are: accuracy, precision, specificity, detection limit, limit of quantitation, linearity, range, and ruggedness and robustness." Spike recovery requirements for a Level Two validation are ≥2 replicates at ≥3 spike levels for ≥3 matrices plus a matrix blank, with acceptable recoveries in the range of 40-120%. Recovery spikes for the validation were duplicates at six different spike levels (0.05, 0.15, 0.5, 1.5, 2, and 5ng PFAS/g of sample) in six different matrices: wet cat food (WCF), wet dog food (WDF), dry cat food (DCF), dry dog food (DDF), dried pelletized alfalfa (AA), and chicken feed (CF).

Chromatography

Peak asymmetries were calculated according to the equation given in section 9.3.9 of EPA method 537.1[35] shown in Figure 2. Acceptable asymmetry values for the EPA method are 0.8-1.5. All analytes in the present study show good symmetry by this evaluation. Retention times and peak asymmetry values from a CS5 standard in the initial calibration are shown in Table 8, and a reference chromatogram displayed in Figure 3. A report showing detail of peak shape for each extracted ion chromatograph is available (supplemental material S11_CS5 XIC Detail.docx).

$$A_s = \frac{b}{a}$$



where:

A_s = peak asymmetry factor

B = width of the back half of the peak measured (at 10% peak height) from the trailing edge of the peak to a line dropped perpendicularly from the peak apex

a = the width of the front half of the peak measured (at 10% peak height) from the leading edge of the peak to a line dropped perpendicularly from the apex.

Figure 2 - EPA 537.1 Peak Asymmetry Calculation

Table 8 - Chromatography Reference Values

Analyte	Retention Time	537.1 Asymmetry Factor
PFBA	5.97	1.10
PFPeA	7.76	1.18
PFBS	8.06	1.19
4:2 FTS	9.20	0.93
PFHxA	9.36	1.11
PFPeS	9.51	1.13
HFPO-DA	9.80	1.16
PFHpA	10.72	1.13
L_PFHxS	10.79	1.19
ADONA	10.87	1.24
6:2 FTS	11.84	1.22
L_PFOA	11.92	1.07
PFHpS	11.94	1.02
L_PFOS	12.94	1.26
PFNA	12.96	1.20
9Cl-PF3ONS	13.45	1.19
8:2 FTS	13.82	1.05
PFDA	13.84	1.10
L_NMeFOSAA	14.23	1.15
PFUdA	14.59	1.08
L_NEtFOSAA	14.61	1.05
11Cl-PF3OUdS	14.92	1.19
PFDoA	15.24	1.11
10:2 FTS	15.26	1.15
PFTrDA	15.81	1.06
PFTeDA	16.29	1.13

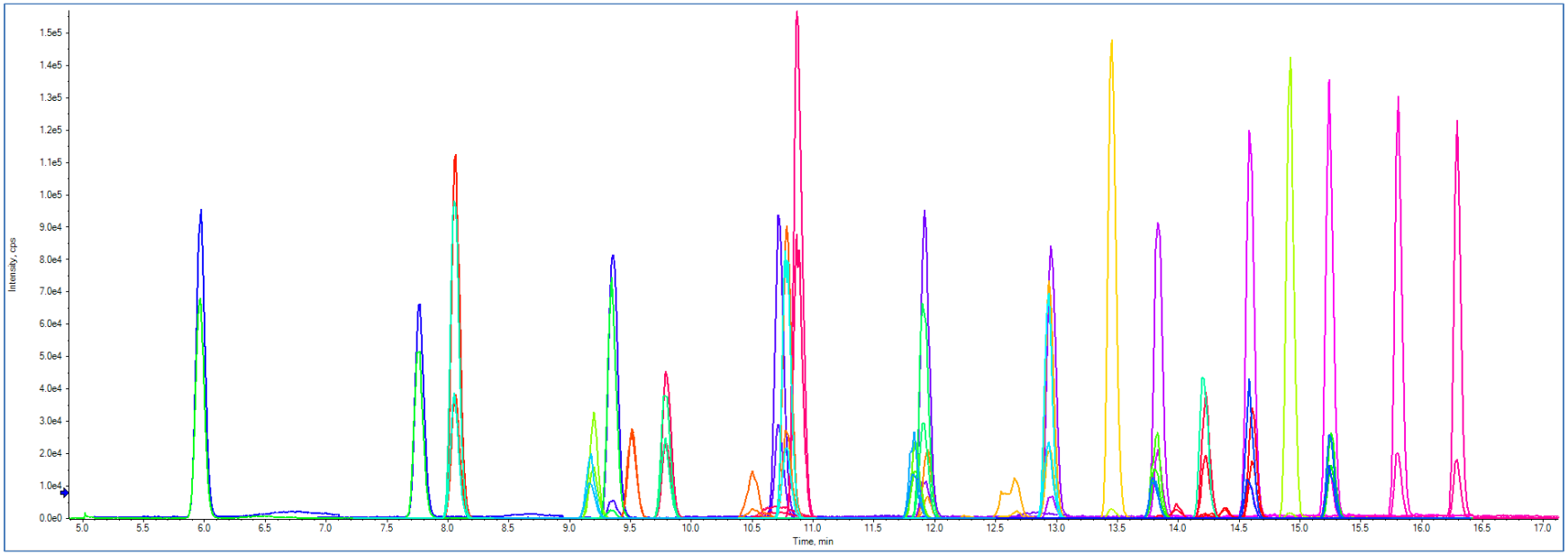


Figure 3- Overlay of extracted ion chromatograms for all MRM transitions monitored. Data is from a CS5 (1ng/mL) calibration standard.

Initial Calibration

A copy of the complete calibration results can be found in Appendix C. After applying qualitative requirements to the standard data, PFBA, PFHxA, Br_PFOA, PFNA, PFUdA, Br_NEtFOSAA, 9-Cl-PF3ONS, 4:2 FTS, and 10:2 FTS had lower limits of quantitation (LLOQ) set to 0.05ng/mL; 11-Cl-PF3ONS had an LLOQ of 0.1ng/mL; all other analytes had LLOQs at the lowest calibration point of 0.01ng/mL.

All qualitative transition data showed excellent linearity over the calibration range (LLOQ-25ng/mL). Correlation coefficients (r) and coefficient of determination (r^2) values for the calibration fit are shown in Table 9. All r^2 values exceed the minimum requirement (≥0.995) in the ORA Laboratory Manual (ORA-LAB.5.4.5 6.3.1.1.E) for single analyte/signal matrix methods.

Table 9 - Regression Statistics for Initial Calibration

Analyte	Equation	r	r^2
PFBA	y = 1.3016x	0.9999	0.9998
PFPeA	y = 1.2171 x	0.9999	0.9997
PFHxA	y = 1.1978 x	0.9998	0.9996
PFHpA	y = 1.2511 x	0.9995	0.9991
Br_PFOA	y = 1.2368 x	0.9997	0.9993
PFNA	y = 1.1627 x	0.9996	0.9991
PFDA	y = 1.2718 x	0.9993	0.9985
PFUdA	y = 1.5210 x	0.9992	0.9983
PFDoA	y = 1.5499 x	0.9999	0.9998
PFTrDA	y = 1.4996 x	0.9996	0.9993
PFTeDA	y = 1.3849 x	0.9997	0.9994
ADONA	y = 2.3519 x	0.9998	0.9995
HFPO-DA	y = 1.0891 x	0.9998	0.9996
Br_NMeFOSAA	y = 0.9640 x	0.9997	0.9995
Br_NEtFOSAA	y = 0.7639 x	0.9994	0.9988
PFBS	y = 1.1912 x	0.9998	0.9996
PFPeS	y = 0.3345 x	0.9994	0.9988
Br_PFHxS	y = 1.3681 x	0.9995	0.9991
PFHpS	y = 0.2457 x	0.9993	0.9985
Br_PFOS	y = 1.4873 x	0.9998	0.9996
9Cl-PF3ONS	y = 2.1761 x	0.9999	0.9997
11Cl-PF3OUdS	y = 1.9089 x	0.9996	0.9992
4:2 FTS	y = 1.5739 x	0.9990	0.9980
6:2 FTS	y = 0.9279 x	0.9984	0.9967
8:2 FTS	y = 2.3192 x	0.9988	0.9976
10:2 FTS	y = 1.0087 x	0.9998	0.9995

The calculated analyte concentration divided by the theoretical concentration (expressed as a percentage) was used to assess the accuracy of the calibration. Results are shown in Table 10 below. CS Group A is the first injection of each calibration level and B is the second. The accuracy of all the calculated values was within ±30% of the true value for all concentrations above the LLOQ.

Table 10 - Accuracy of Calibration Concentrations Calculated from the Regression

Analyte	CS group	0.01 ng·mL^{1}	0.05 ng·mL^{1}	0.1 ng·mL^{1}	0.5 ng·mL^{1}	1 ng·mL^{1}	5 ng·mL^{1}	10 ng·mL^{1}	25 ng·mL^{1}
PFBA	A	N/A	LLQ	113.1	101.3	104.7	98.2	101.3	99.1
	B	N/A	LLQ	115.2	101.8	103.7	100.0	103.1	98.9
PFPeA	A	LLQ	106.1	99.4	102.5	102.8	97.6	103.5	99.2
	B	LLQ	101.4	108.7	103.1	104.6	99.8	101.4	98.9
PFHxA	A	N/A	LLQ	109.3	106.7	101.7	97.2	102.8	100.8
	B	N/A	LLQ	106.9	100.2	102.6	100.9	100.9	97.8
PFHpA	A	LLQ	108.3	114.9	102.5	102.2	99.1	101.8	100.0
	B	LLQ	108.1	108.7	102.4	106.1	104.4	104.6	96.1
Br_PFOA	A	N/A	LLQ	109.6	106.9	104.9	98.7	99.1	103.2
	B	N/A	LLQ	107.9	100.8	101.4	95.8	100.7	97.5
PFNA	A	N/A	LLQ	109.6	107.7	104.5	101.3	103.1	101.7
	B	N/A	LLQ	109.0	102.8	102.8	98.6	101.7	95.7
PFDA	A	LLQ	97.3	106.4	106.1	101.4	98.7	99.5	105.3
	B	LLQ	106.7	97.6	100.6	105.3	96.9	98.6	96.0
PFUdA	A	N/A	LLQ	103.8	103.1	102.0	98.0	96.8	105.3
	B	N/A	LLQ	105.0	102.9	102.7	100.1	101.1	95.5
PFDoA	A	LLQ	104.3	115.3	102.8	103.5	98.5	100.8	100.8
	B	LLQ	98.9	98.0	103.9	101.3	96.2	100.5	99.4
PFTrDA	A	LLQ	111.9	99.1	96.6	101.4	97.0	98.7	103.8
	B	LLQ	112.2	98.4	101.2	100.4	96.9	100.2	97.9
PFTeDA	A	LLQ	97.1	104.9	100.9	100.4	98.4	96.3	103.3
	B	LLQ	108.9	102.1	99.1	101.3	96.3	99.0	99.6
ADONA	A	LLQ	99.7	99.3	97.9	99.5	98.8	99.5	103.0
	B	LLQ	97.1	94.2	97.5	102.5	95.8	100.1	98.3
HFPO-DA	A	LLQ	117.2	108.1	102.1	103.8	101.2	102.5	99.2
	B	LLQ	111.4	107.0	102.7	101.6	100.7	102.6	97.9
Br_NMeFOSAA	A	LLQ	96.5	109.2	106.9	97.6	100.0	104.8	99.7
	B	LLQ	124.5	107.4	100.6	103.9	100.5	100.3	97.8
Br_NEtFOSAA	A	N/A	LLQ	123.8	111.8	110.2	100.4	107.8	98.1
	B	N/A	LLQ	125.8	103.3	106.1	100.8	97.3	98.3
PFBS	A	LLQ	98.8	99.9	101.8	102.8	96.0	102.5	97.9
	B	LLQ	97.3	99.3	102.0	102.0	103.1	102.0	100.3
PFPeS	A	LLQ	100.6	103.0	95.7	99.9	101.2	104.3	100.2
	B	LLQ	98.8	118.1	99.0	107.8	100.8	104.5	95.6
Br_PFHxS	A	LLQ	105.4	107.2	97.1	105.3	96.3	102.1	100.5
	B	LLQ	105.2	106.6	99.1	105.3	100.5	105.5	96.6

PFHpS	A	LLQ	112.9	95.1	99.9	105.5	103.4	103.0	97.4
	B	LLQ	106.7	103.5	102.3	108.2	101.3	107.4	97.0
Br_PFOS	A	LLQ	114.5	109.8	100.2	105.9	99.5	104.1	98.7
	B	LLQ	104.7	107.6	101.1	105.9	98.2	100.2	99.4
9Cl-PF3ONS	A	N/A	LLQ	100.7	104.4	102.7	101.6	102.5	97.9
	B	N/A	LLQ	100.6	104.2	101.1	101.5	101.6	99.5
11Cl-PF3OUdS	A	N/A	N/A	LLQ	105.3	105.5	105.2	100.8	98.9
	B	N/A	N/A	LLQ	102.7	100.2	96.7	104.9	98.1
4:2 FTS	A	N/A	LLQ	115.3	103.1	107.7	102.6	105.3	94.7
	B	N/A	LLQ	122.8	107.7	103.0	95.1	106.7	100.0
6:2 FTS	A	LLQ	96.3	106.3	87.7	103.1	99.6	108.1	95.8
	B	LLQ	105.0	97.8	100.8	101.8	95.7	111.3	97.4
8:2 FTS	A	LLQ	102.3	110.2	103.6	97.0	94.7	107.4	104.1
	B	LLQ	99.8	90.1	95.6	104.2	93.2	92.9	98.2
10:2 FTS	A	N/A	LLQ	102.0	96.1	101.8	104.7	102.8	98.4
	B	N/A	LLQ	95.3	99.4	97.1	95.3	100.3	100.5

Surrogate concentrations in each calibration standard are constant. The relative standard deviation (%RSD) of the surrogate areas across all standards was evaluated as an additional precision check on standard preparation and instrument acquisition and shown below in Table 11.

Table 11 - Precision of Internal Standard Areas from the Initial Calibration

Surrogate	Average Area	σ	%RSD
13C3-PFBA	3.38E+05	1.10E+04	3.25
13C3-PFPeA	2.61E+05	6.80E+03	2.61
13C2-PFHxA	3.48E+05	7.34E+03	2.11
13C8-PFOA	3.42E+05	1.37E+04	4.00
13C3-HFPO	2.03E+05	3.95E+03	1.94
d3-NMeFOSAA	2.11E+05	5.99E+03	2.83
13C3-PFBS	5.00E+05	1.31E+04	2.63
18O2-PFHxS	4.14E+05	1.10E+04	2.67
13C8-PFOS	3.16E+05	5.30E+03	1.68
d4-13C2-4:2 FTS	9.40E+04	3.76E+03	4.00
d4-13C2-6:2 FTS	1.28E+05	4.63E+03	3.62
d4-13C2-8:2 FTS	6.01E+04	3.71E+03	6.17
d4-13C2-10:2 FTS	1.11E+05	7.18E+03	6.49
RS d5-NEtFOSAA	5.87E+04	3.15E+03	5.37

Identify retention times of PFOA branched vs. linear isomers from a qualitative standard

Due to the lack of a quantitative PFOA standard containing branched and linear isomers, the linear PFOA isomer was used in the calibration standards. Identification of the retention times for the branched isomers is necessary to report PFOA determinations as the sum of branched and linear isomers. The retention times of branched PFOA isomers were identified by injecting a technical standard diluted to approximately 1 ng/mL. Figure 4 shows the extracted ion chromatogram for PFOA.

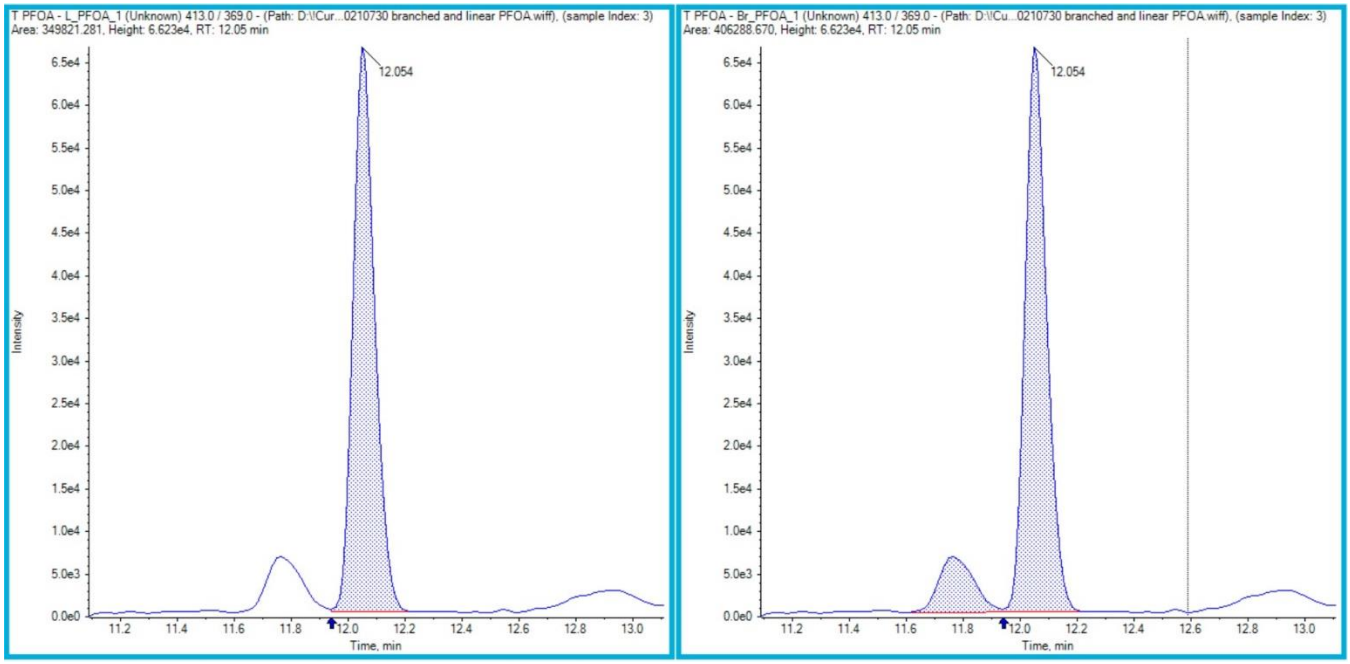


Figure 4 – Extracted ion chromatogram (quantitative transition) for technical PFOA mix. Shaded areas show the integration of the linear isomer only with a retention time of 12.054 min (left) and the total PFOA area including the branched isomer with a retention time of 11.77 min (left).

Selectivity: Resolution between PFOS and cholic acids TCDCA, TDCA, and TUDCA

Three cholic acids have been recognized as possible interferents in the determination of PFOS: Taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), and tauroursodeoxycholic acid (TUDCA). Solutions of the certified reference materials were prepared in methanol and chromatograms of the three compounds were acquired separately. Figure 5 shows an overlay of the three chromatograms.

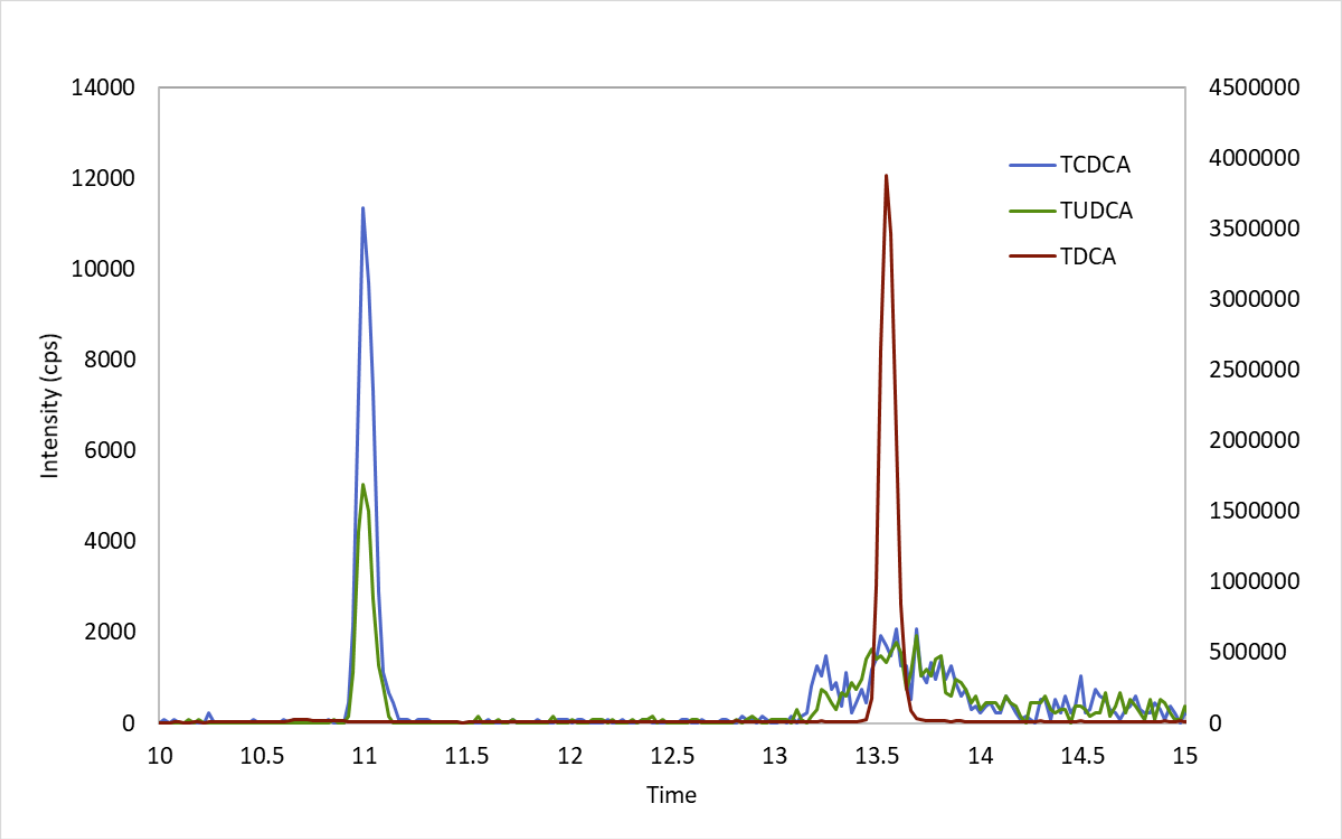


Figure 5 - Overlay of chromatograms for three cholic acids. Vertical scale: TCDCA and TUDCA (left), and TDCA (right).

A chromatogram for PFOS is shown in Figure 6 showing both the quantitative and qualitative transition.

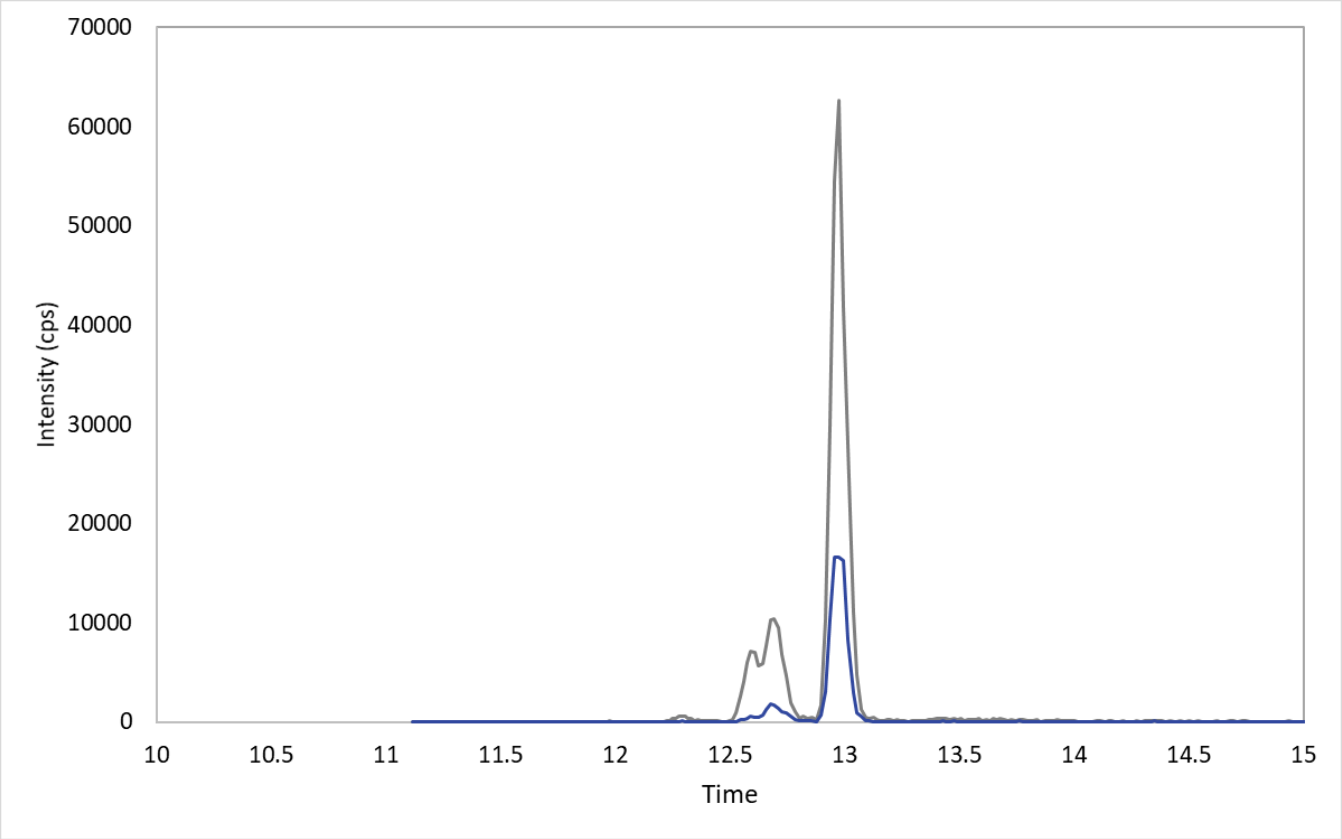


Figure 6 - Chromatogram for PFOS showing the quantitative (grey) and qualitative (blue) transitions. The linear isomer retention time is ~12.9 min.

Figure 7 shows an overlay of the three individual cholic acid chromatograms as well as the PFOS. TCDCA and TUDCA are well resolved from both the branched and linear PFOS peaks. TDCA elutes ~0.5 minutes after the linear PFOS peak.

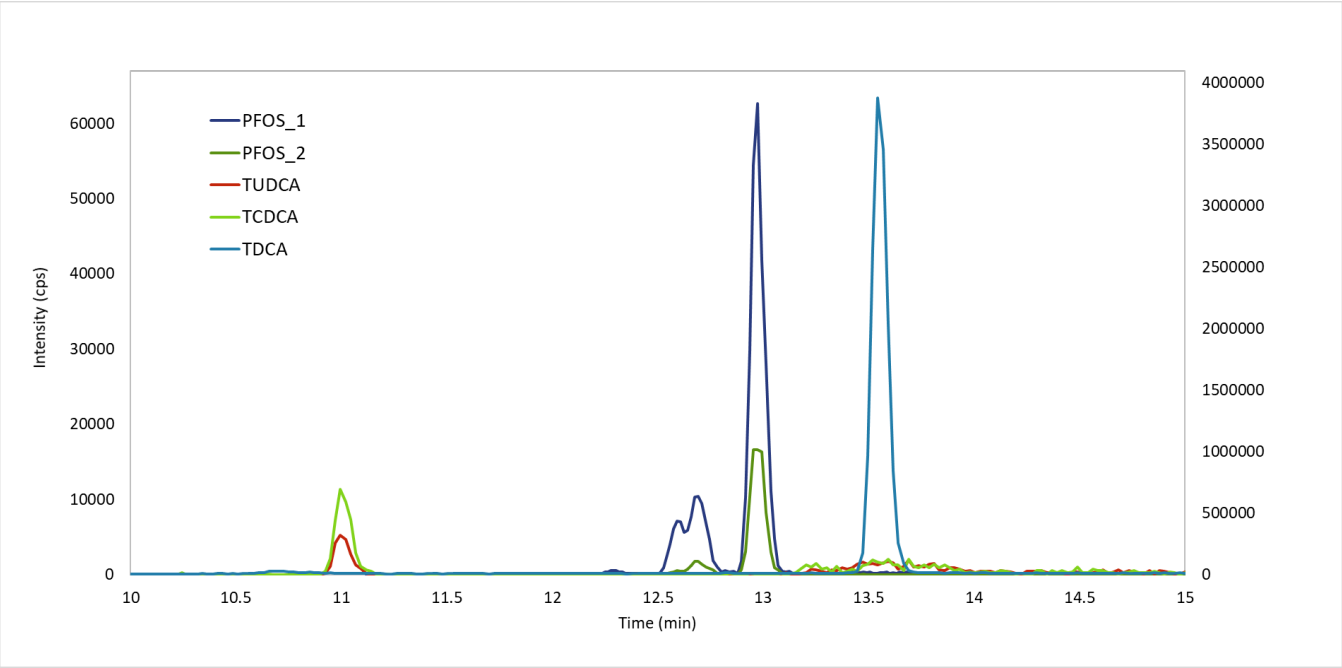


Figure 7 - Overlay of cholic acid and PFOS chromatograms showing relative retention times for the compounds.

Finally, Figure 8 shows a chromatogram obtained for a combined solution of PFOS and the cholic acids which shows that the chromatographic system is indeed capable of resolving these compounds.

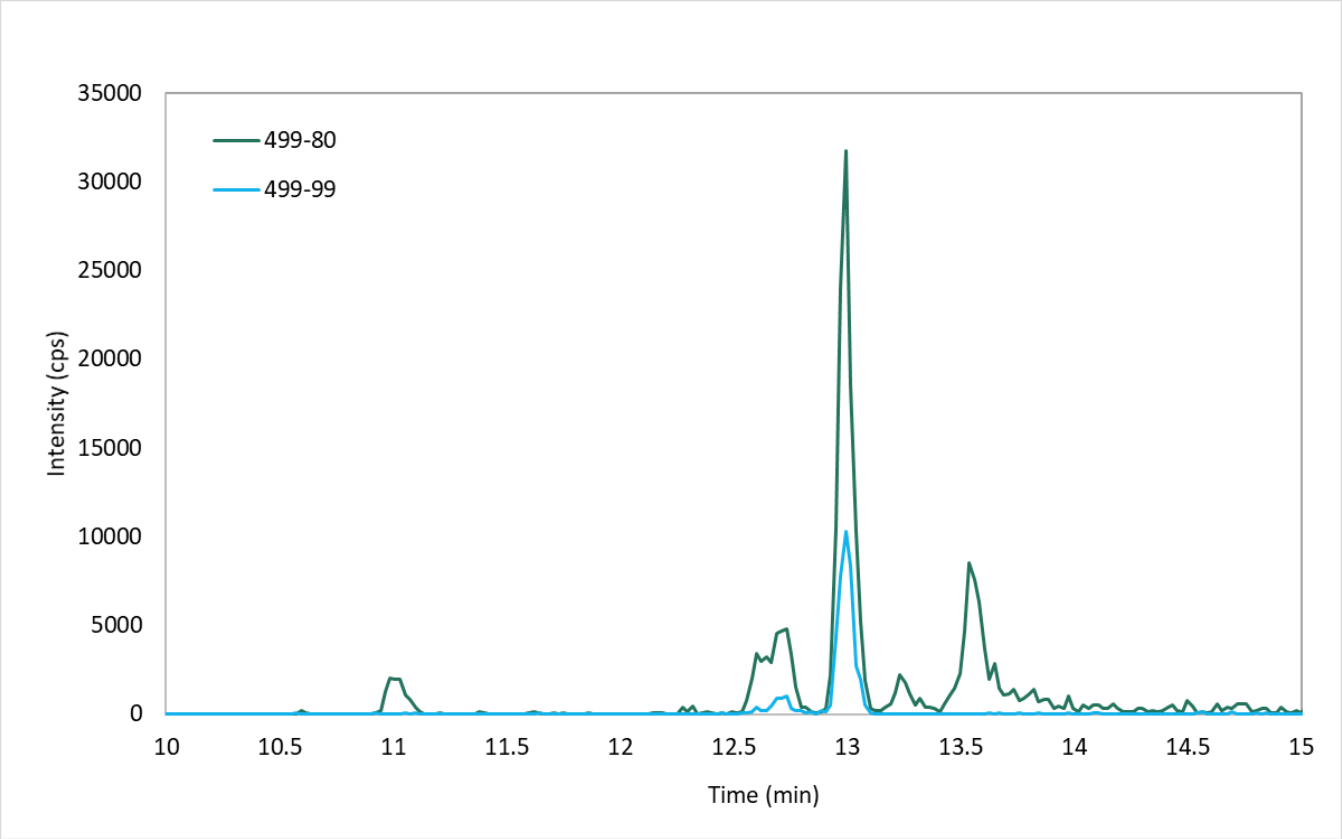


Figure 8 - Chromatogram of mixture of cholic acids and PFOS. While the cholic acids share the 499-80 m/z transition with PFOS quantitative transition, they lack the qualitative transition of 499-99 m/z.

MDL Evaluation

Method detection limits (MDL) were calculated per the current method given in 40 CFR Appendix B to Part 136 where the MDL is defined as "the minimum measured concentration of a substance that can be reported with 99% confidence that the measured concentration is distinguishable from method blank results." Twelve low level spikes (one sample was lost during extraction) and twelve method blanks were extracted over the course of 7 days. Where the calculated concentration of an analyte in all seven blanks is non-zero, a MDL_b value was calculated by multiplying the standard deviation of the concentrations by a coverage factor. Where some but not all concentrations were non-zero, the MDL_b for the analyte is taken to be the max value measured. Where all concentrations were zero, a MDL_b was not applicable. A MDL_s was calculated by multiplying the standard deviation of the analyte's calculated concentration in the spikes by a coverage factor. Finally, the greater of the MDL_s and MDL_b values was taken to be the initial analyte MDL. Sample batches where an analyte's concentration in the method blank exceeds its initial MDL, and the analyte meets the qualitative identification criteria will not be used and the samples re-extracted. Matrix specific MDLs were also calculated from seven spikes in wet cat food, dry dog food, chicken feed, and alfalfa. The results are summarized in Table 12.

Table 12 - Initial and Matrix Specific MDL Values

Analyte	MDL_b (ng/kg)	MDL_s (ng/kg)	Initial Analyte MDL (ng/kg)	WCF MDL (ng/kg)	DDF MDL (ng/kg)	CF MDL (ng/kg)	AA MDL (ng/kg)
PFBA	390	300	390	442	675	301	866
PFPeA	12	21	21	33	119	56	83
PFHxA	26	18	26	59	60	144	9699
PFHpA	13	23	23	41	64	35	107
Br_PFOA	28	26	28	43	44	49	51
PFNA	17	35	35	81	64	62	59
PFDA	26	29	29	123	92	55	53
PFUdA	9	33	33	48	22	24	83
PFDoA	17	33	33	46	41	34	48
PFTrDA	24	22	24	35	19	30	46
PFTeDA	18	38	38	40	31	28	40
ADONA	1	18	18	51	58	28	33
HFPO-DA	16	29	29	50	43	60	58
Br_NMeFOSAA	7	25	25	34	69	50	31
Br_NEtFOSAA	12	47	47	37	66	63	29
PFBS	103	19	103	21	26	13	18
PFPeS	N/A	24	24	54	66	59	31
Br_PFHxS	145	48	145	108	1511	65	333
PFHpS	22	25	25	39	78	53	41
Br_PFOS	92	69	92	33	119	47	525
9Cl-PF3ONS	89	23	89	24	47	35	25
11Cl-PF3OUdS	83	24	83	24	42	29	29
4:2 FTS	60	32	60	34	102	87	99
6:2 FTS	9388	55	9388	37	1389	426	378
8:2 FTS	31	45	45	48	74	49	25
10:2 FTS	19	28	28	75	89	43	34

The MDL_s and MDL_b values for PFBA show that background levels are both much higher and quite variable compared to the other analytes. While not substantially higher than the MDL_s, the MDL_b in this case nevertheless provides a better MDL value by accounting for both the measured value and the standard deviation of those measurements.

The MDL_b value for 6:2 FTS is extremely high due to the calculated concentration from a single extraction. While the method in 40 CFR Appendix B to Part 136 doesn't allow for removal of outliers

from the initial MDL calculation because of small sample size, dropping the outlying point and recalculating results yields an MDL_b value of 41 ng kg^{-1}.

While the MDL for 6:2 FTS in wet cat food was on par with the MDL_s where background contamination was absent, values in dry dog food, chicken feed, and alfalfa were higher. Calculated concentrations compared to theoretical spike level were:

- ~2-13x higher in 5 of 7 DDF matrix spikes
- ~4x higher in 2 of 7 CF matrix spikes
- ~2-5x higher in 2 of 7 AA matrix spikes

Because of the variability in occurrence and calculated amount, the presumption is that the elevated MDL values were due to contamination and not incurred analyte. Out of the 51 total extracts for the MDL study, 10 showed signs of possible 6:2 FTS contamination.

A. Ahmadireskety et al.[36] reported 6:2 FTS as the highest background species present in their extraction blanks using methanol with a concentration of 53.4 ng mL^{-1}. The high background led them to drop the analyte from their study. During our method development work during CY2020, elevated levels of this compound were routinely encountered in extracts. Anecdotal evidence suggests that methanol leaches this compound from some plastic vessels in use at the time. Modifications to supplies and procedures have greatly reduced, but not eliminated, extract contamination by this compound. Procedural control can be maintained for this compound by rejection of sample batches with elevated method blank values or confirming detects through sample replicates.

Matrix Blanks

Blanks for each of the six matrices were prepared and evaluated for co-extracted interferences and background levels of target analytes. Reports for each matrix can be found in the supplemental materials (S1_AA Mtx Blk.pdf; S2_CF Mtx Blk.pdf; S3_DCF Mtx Blk.pdf; S4_DDF Mtx Blk.pdf; S5_WCF Mtx Blk.pdf; S6_WDF Mtx Blk.pdf). Noteworthy interferences in qualitative transition traces are described below and help explain elevated MDL values for certain analyte/matrix combinations and failed low concentration recovery spikes (next section).

WCF

- PFDA – an elevated baseline and a small peak are integrated (calculated concentration 0.026 ng/mL)
- PFHxS – a low, broad set of unresolved peaks are integrated (calculated concentration 0.048 ng/mL)

DDF

- PFHxS – a series of unresolved peaks in quant transition are integrated (calculated concentration 0.876 ng/mL)

CF

- PFHxA – a peak at RT < analyte causing leading shoulder is integrated (calculated concentration 0.174 ng/mL)

AA

- PFHxA – a large peak near analyte RT is integrated (calculated concentration 2.65 ng/mL)
- PFOS – a peak at RT > analyte causing tailing shoulder is integrated (calculated concentration 0.174 ng/mL)

WDF

- PFHxS – a set of broad overlapping peaks is integrated (calculated concentration 0.0186 ng/mL)
- 4:2 FTS – a peak matching the analyte RT is integrated (quant and qual peaks are both present, calculated concentration 0.0126 ng/mL)

DCF

- PFPeA – presence of an elevated baseline, and a peak at a RT later than the analyte RT is integrated (calculated concentration 0.218 ng/mL, peak is unresolved from analyte in spikes)
- PFHxA – one peak closest to the analyte RT is integrated (calculated concentration of the 0.077 ng/mL) but several more peaks are in the vicinity and may be unresolved in the spikes
- PFHxS – a set of broad overlapping peaks (with nearly an identical pattern to the DDF blank) is integrated (calculated concentration 2.336 ng/mL)

In addition, all four cat and dog food matrices show evidence of TDCA being present.

Spike Recoveries

All six matrices were spiked in duplicate at 0.05, 0.15, 0.5, 1.5, 2, and 5ng PFAS/g of sample, extracted, and evaluated for analyte recovery. The FVM Guidelines give 40-120% as an acceptable range of recovery for spikes at method level of 1 part per billion. A summary of the results is found in Table 13 with full treatment of the data in supplementary material S7_CVM SLV Recovery Spikes.xlsx.

Table 13 - Summary of Matrix Recovery Spikes

Analyte	Wet Dog Food	Dry Dog Food	Wet Cat Food	Dry Cat Food	Chicken Feed	Alfalfa
PFBA	1.5 – 5 ng/g	1.5 – 5 ng/g	2 – 5 ng/g	1.5 – 5 ng/g	2 – 5 ng/g	0.5 – 5 ng/g
PFPeA	0.05 – 5 ng/g	1.5 – 5 ng/g	0.05 – 5 ng/g	2 – 5 ng/g	0.5 – 5 ng/g	0.5 – 5 ng/g
PFHxA	0.05 – 5 ng/g	0.15 – 5 ng/g	0.15 – 5 ng/g	0.5 – 5 ng/g	2 – 5 ng/g	Outside Limits
PFHpA	0.05 – 5 ng/g	0.15 – 5 ng/g	0.15 – 5 ng/g	0.15 – 5 ng/g	2 – 5 ng/g	Outside Limits
PFOA	0.15 – 5 ng/g	0.15 – 5 ng/g	0.15 – 5 ng/g	0.05 – 5 ng/g	0.15 – 5 ng/g	0.5 – 5 ng/g
PFNA	0.05 – 5 ng/g	0.15 – 5 ng/g	0.05 – 5 ng/g	0.15 – 5 ng/g	0.15 – 5 ng/g	0.15 – 5 ng/g
PFDA	0.05 – 5 ng/g	0.05 – 5 ng/g	0.15 – 5 ng/g	0.05 – 5 ng/g	0.15 – 5 ng/g	0.15 – 5 ng/g
PFUdA	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.15 – 5 ng/g
PFDoA	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g
PFTrDA	0.05 – 5 ng/g	0.05 – 0.15 ng/g	0.15 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g
PFTeDA	0.05 – 5 ng/g	0.05 – 0.15 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.15 – 5 ng/g	0.05 – 5 ng/g
ADONA	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g
HFPO-DA	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.5 – 5 ng/g	0.05 – 5 ng/g
N-MeFOSAA	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g
N-EtFOSAA	0.05 – 5 ng/g	0.5 – 5 ng/g	0.15 – 5 ng/g	0.15 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g
PFBS	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g
PFPeS	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g
PFHxS	0.15 – 5 ng/g	5 ng/g	0.05 – 5 ng/g	Outside Limits	0.5 – 5 ng/g	0.5 – 5 ng/g
PFHpS	0.05 – 5 ng/g	0.5 – 5 ng/g	0.05 – 5 ng/g	0.5 – 5 ng/g	0.15 – 5 ng/g	0.15 – 5 ng/g
PFOS	0.05 – 5 ng/g	0.5 – 5 ng/g	0.05 – 5 ng/g	0.5 – 5 ng/g	0.15 – 5 ng/g	0.15 – 5 ng/g
9Cl-PF3ONS	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g
11Cl-PF3OUdS	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g
4:2 FTS	0.5 – 5 ng/g	0.15 – 5 ng/g	0.15 – 5 ng/g	0.15 – 5 ng/g	0.5 – 5 ng/g	0.5 – 5 ng/g
6:2 FTS	0.05 – 5 ng/g	0.15 – 5 ng/g	0.05 – 5 ng/g	0.5 – 5 ng/g	0.5 – 5 ng/g	0.15 – 5 ng/g
8:2 FTS	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.15 – 5 ng/g
10:2 FTS	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.05 – 5 ng/g	0.15 – 5 ng/g	0.05 – 5 ng/g

Three analytes in two matrices failed to produce acceptable recoveries at any spike level. In the case of PFHxS in DDF, the calculated concentration of the interference in the matrix blank was approximately equal to the highest spike amount before the addition of analyte. Consequently, recoveries exceeded the upper acceptability limit.

In the case of PFHxA and PFHpA in AA, a combination of factors appears to have led to high recoveries. A matrix effect (ME) study was conducted during the validation and showed significant ionization suppression of the labeled PFHxA surrogate internal standard. Native PFHxA showed strong ionization enhancement (likely from the previously noted matrix interference and not truly ionization related), and native PFHpA showed little effect on its ionization. As both target compounds use the same suppressed surrogate as their internal standard, recoveries exceeded the upper acceptability limit at all spike levels. [Note: The matrix effect study is beyond the normal validation requirements and aside from providing an explanation for this issue will not be fully explained here. A summary of the ME study can be found in the supplemental material (S8_CVM Matrix Effect Study.xlsm and S9_CVM Matrix Effects and Interferences.docx).]

Recoveries for the long chain (C_{11}-C_{14}) carboxylic acid analytes as well as ADONA show a drop in recovery in the dry cat and dog foods. The effect was more pronounced in dog than cat food. By design the C_8-C_{14} carboxylic acids and ADONA use the same internal standard, 13C8-PFOA, analogous to the EPA 537.1 method. It appears that the partitioning coefficients for these compounds versus the PFOA become dissimilar enough to produce the drop in recovery. Presumably this is due to either the dryness or composition of the matrix. We anticipate that the incorporation of additional surrogate internal standards in the future will remedy this issue.

Accuracy & Precision

Neither the FVM Guideline nor the ORA Lab Manual specify a procedure for evaluating accuracy and precision. EPA Method 537.1 requires an initial demonstration of accuracy and precision using four to seven laboratory fortified blanks to be extracted near the midpoint concentration of the calibration. For accuracy, the average recovery must be ±30% of the true value; and for precision, the RSD must be less than 20%. Average recovery and RSD were calculated for the 11 native spike extracts used for the MDL_s calculation and the results are shown in Table 14.

Table 14 - Average Recovery, Standard Deviation and RSD of Native Spike Extracts in MDL Study

Analyte	Average Recovery (%)	SD (%)	RSD (%)
PFBA	332.9	108.6	32.6
PFPeA	110.1	7.4	6.8
PFHxA	124.2	6.5	5.2

PFHpA	119.9	8.4	7.0
Br_PFOA	121.6	9.5	7.8
PFNA	120.3	12.6	10.4
PFDA	113.9	10.4	9.2
PFUdA	104.2	12.0	11.5
PFDoA	107.6	12.1	11.2
PFTrDA	104.6	7.8	7.5
PFTeDA	101.2	13.6	13.4
ADONA	96.8	6.8	7.0
HFPO-DA	107.9	10.5	9.7
Br_NMeFOSAA	111.2	9.0	8.1
Br_NEtFOSAA	127.0	17.1	13.5
PFBS	103.7	7.9	7.6
PFPeS	110.5	9.3	8.4
Br_PFHxS	149.7	19.0	12.7
PFHpS	128.5	9.6	7.5
Br_PFOS	116.6	27.0	23.1
9Cl-PF3ONS	98.9	8.9	9.0
11Cl-PF3OUdS	97.5	9.0	9.3
4:2 FTS	131.8	12.3	9.3
6:2 FTS	126.6	20.8	16.5
8:2 FTS	111.5	17.0	15.3
10:2 FTS	112.4	10.4	9.3

Spikes by this method met the precision requirement from the EPA procedure except for PFBA and Br_PFOS. All but three natives (PFBA, Br_PFHxS, and 4:2 FTS) also pass the accuracy requirement. The accuracy and precision failure by PFBA is due to the background contamination previously described. It should be noted that the natives were spiked at the 0.1 ng/g level (0.05 ng/mL theoretical in the extract; comparable to the CS2 calibration level) and 20x lower than the CS5 level used as the calibration check. This concentration is at, or slightly above, the calibration LLOQ for the target analytes instead of near the calibration midpoint. Considering that the accuracy requirement for a calibration standard at LLOQ is ±50% of the true value, accuracy for these three compounds should be considered adequate. A corresponding increase in precision tolerance at this concentration would be for an RSD < 33%, making the Br_PFOS at 23.1% quite acceptable also.

Average recovery of the surrogate internal standards is shown for the same set of MDL spikes in Figure 9.

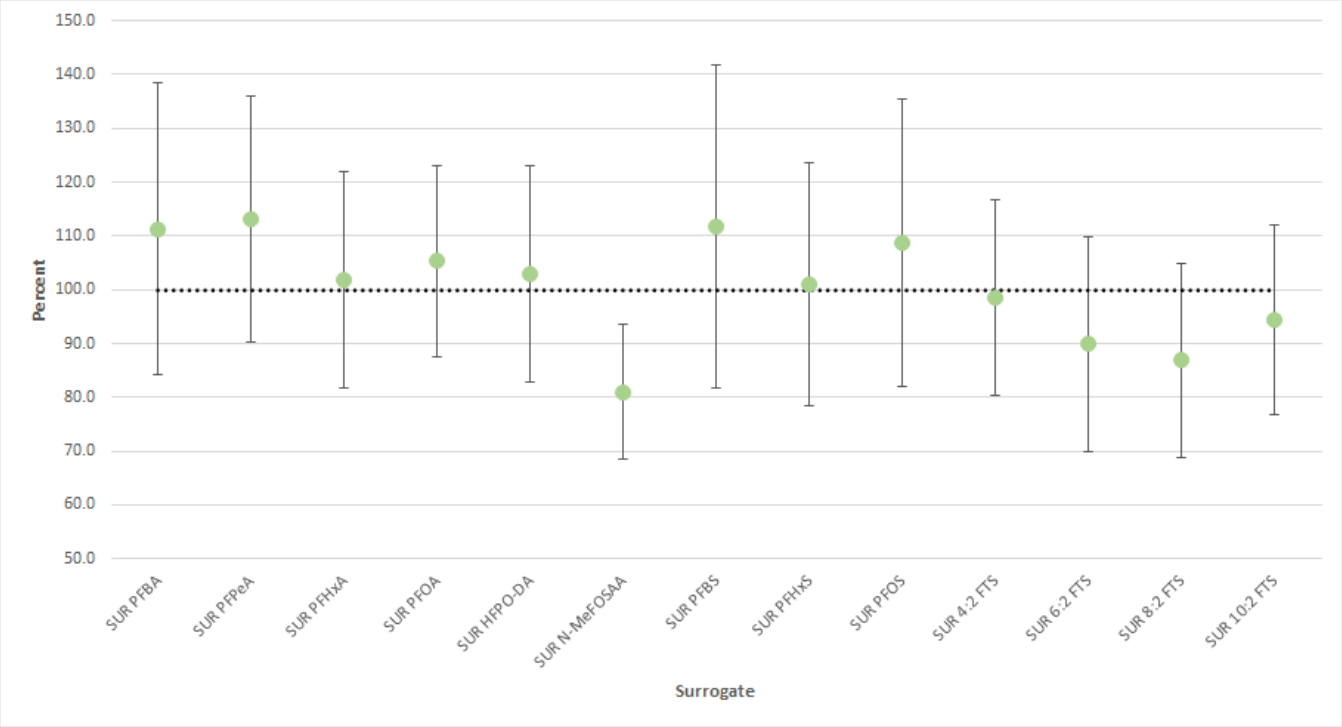


Figure 9 – Average surrogate recovery for the 11 MDL_s spikes. Error bars represent one standard deviation.

Ruggedness

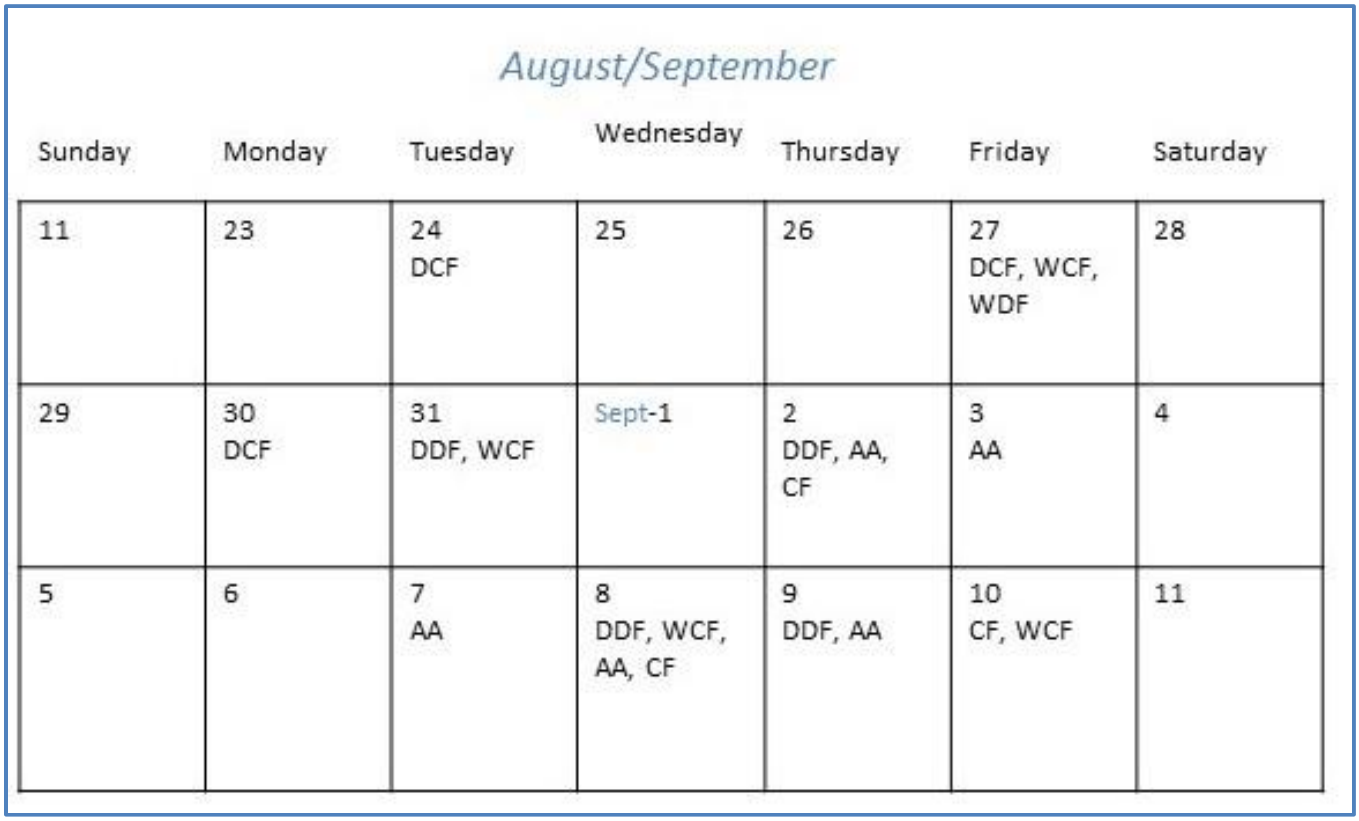
All extractions were carried out in the same location, lab B118, and were performed by two different analysts with different levels of experience. Analyst A had over a year experience with using QuEChERS followed by SPE for extraction, and Analyst B had approximately 2 months experience with this method before beginning the method validation process for animal feed products. Results between analyst A and B are statistically the same for each matrix tested (see supplementary material S10_tTest_Two Sample Unequal Variances.xlxs).

Instrument acquisitions were performed in lab B148 on a single instrument. Two calibrations were performed during the work and used in data processing. Data analysis was carried out by analysts A & B, and an additional analyst.

Furthermore, Table 15 and the accompanying calendar (Figure 8) show that the analyses were performed across multiple days between 08/08/2021 and 09/10/2021.

Table 15 - Commencement Dates for Extraction Batches by Matrix

Matrix	Dates
Dry Cat Food (DCF)	08/19, 08/24, 08/27, 08/30
Dry Dog Food (DDF)	08/19, 08/31, 09/02, 09/08, 09/09
Wet Cat Food (WCF)	08/18, 08/27, 08/28, 08/31, 09/08, 09/10
Wet Dog Food (WDF)	08/18, 08/19, 08/27
Alfalfa/Timothy (AA)	08/19, 09/02, 09/03, 09/07, 09/08, 09/09
Chicken Feed (CF)	08/19, 09/02, 09/08, 09/10



August/September

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
11	23	24 DCF	25	26	27 DCF, WCF, WDF	28
29	30 DCF	31 DDF, WCF	Sept-1	2 DDF, AA, CF	3 AA	4
5	6	7 AA	8 DDF, WCF, AA, CF	9 DDF, AA	10 CF, WCF	11

Figure 10 - Day of the Week View of Extraction Commencement

Circumstances and workflow resulted in data being produced by three separate time sequences:

- QuEChERS extraction, SPE cleanup, and instrumental analysis performed on a single day

- QuEChERS extraction performed on day one, followed by SPE cleanup, and instrumental analysis on day two
- QuECHERS extraction, and SPE cleanup performed on day one, followed by instrumental analysis on day two

Data from the three sequences was indistinguishable from one another.

Finally, all instrumental check data collected during the SLV is presented in supplemental material S12_2021 ARKL PFAS SLV_ICV CCV Filterable.xlsx for both native analyte accuracies and surrogate internal standard recoveries. Over the course of the study, only one analyte – 10:2 FTS exceeded the upper accuracy tolerance of 130% in a CCV. Three surrogates - 13C3-PFBA (twice), 13C3-PFPeA, and 13C2-PFHxA – slightly exceeded the upper recovery tolerance in three CCVs. The surrogate recovery for d4-13C2-6:2 FTS fell below the lower tolerance of 70% once at 67.8% in one CCV. Accuracy/recovery standard deviation calculated from all checks performed ranged from 1.7% for PFBA (native) to 13.6% (d4-13C2-6:2 FTS). The stability of instrumental checks, especially those following matrix extracts, supports the conclusion that there is no rapid buildup of instrument contamination from the extracts.

Measurement Uncertainty

An estimation of the measurement uncertainty was made using the following procedure from the MDL^s spike data generated for the MDL study. The average and standard deviation were calculated for each analyte from the replicate calculated concentration (ng/mL) data. The relative standard deviation (RSD) was then calculated by dividing the standard deviation by the average. Finally, the uncertainty (U) was calculated by multiplying the RSD by a coverage factor (k) of 2 where k=2 is approximately equal to a 95% confidence interval. A summary is given below in Table 16.

Table 16 - Measurement Uncertainty

Analyte	1	2	3	4	5	6	7	8	9	10	11	Avg	SD	RSD	U
PFBA	0.132	0.169	0.114	0.123	0.183	0.155	0.160	0.101	0.208	0.191	0.295	0.166	0.054	0.326	0.7
PFPeA	0.051	0.055	0.053	0.052	0.051	0.053	0.055	0.059	0.059	0.063	0.055	0.055	0.004	0.068	0.1
PFHxA	0.062	0.059	0.067	0.061	0.057	0.064	0.060	0.059	0.066	0.065	0.064	0.062	0.003	0.052	0.1
PFHpA	0.063	0.056	0.058	0.053	0.057	0.063	0.060	0.062	0.068	0.061	0.057	0.060	0.004	0.070	0.1
Br_PFOA	0.068	0.056	0.068	0.060	0.054	0.059	0.059	0.065	0.060	0.056	0.063	0.061	0.005	0.078	0.2
PFNA	0.059	0.054	0.059	0.066	0.059	0.065	0.052	0.075	0.059	0.058	0.055	0.060	0.006	0.104	0.2
PFDA	0.049	0.056	0.061	0.049	0.055	0.062	0.051	0.063	0.059	0.061	0.059	0.057	0.005	0.092	0.2
PFUdA	0.054	0.051	0.050	0.047	0.048	0.054	0.049	0.063	0.048	0.063	0.046	0.052	0.006	0.115	0.2
PFDoA	0.052	0.049	0.059	0.053	0.045	0.049	0.050	0.066	0.053	0.060	0.057	0.054	0.006	0.112	0.2
PFTrDA	0.052	0.046	0.052	0.050	0.048	0.057	0.050	0.056	0.056	0.058	0.050	0.052	0.004	0.075	0.1
PFTeDA	0.046	0.046	0.046	0.050	0.040	0.051	0.050	0.063	0.060	0.056	0.048	0.051	0.007	0.134	0.3
ADONA	0.046	0.043	0.049	0.045	0.041	0.044	0.044	0.052	0.046	0.048	0.043	0.046	0.003	0.070	0.1
HFPO-DA	0.055	0.047	0.060	0.054	0.046	0.058	0.052	0.057	0.052	0.063	0.050	0.054	0.005	0.097	0.2
Br_N-MeFOSAA	0.058	0.051	0.057	0.047	0.057	0.064	0.055	0.056	0.058	0.056	0.054	0.056	0.004	0.081	0.2
Br_N-EtFOSAA	0.059	0.055	0.074	0.059	0.053	0.066	0.057	0.082	0.064	0.064	0.066	0.063	0.009	0.135	0.3
PFBS	0.046	0.045	0.045	0.044	0.043	0.048	0.045	0.047	0.049	0.053	0.040	0.046	0.003	0.076	0.2
PFPeS	0.052	0.048	0.056	0.048	0.047	0.059	0.054	0.048	0.055	0.057	0.048	0.052	0.004	0.084	0.2
Br_PFHxS	0.062	0.055	0.068	0.055	0.076	0.073	0.065	0.072	0.082	0.065	0.075	0.068	0.009	0.127	0.3
PFHpS	0.055	0.066	0.055	0.065	0.060	0.064	0.063	0.064	0.058	0.068	0.056	0.061	0.005	0.075	0.1
Br_PFOS	0.047	0.045	0.055	0.047	0.041	0.048	0.062	0.065	0.085	0.052	0.048	0.054	0.013	0.231	0.5
9Cl-PF3ONS	0.047	0.046	0.049	0.041	0.042	0.043	0.049	0.051	0.048	0.052	0.040	0.046	0.004	0.090	0.2
11Cl-PF3OUdS	0.047	0.044	0.051	0.045	0.037	0.045	0.041	0.050	0.051	0.049	0.045	0.046	0.004	0.093	0.2
4:2 FTS	0.057	0.065	0.067	0.056	0.057	0.067	0.059	0.065	0.057	0.072	0.055	0.062	0.006	0.093	0.2
6:2 FTS	0.053	0.057	0.055	0.067	0.062	0.056	0.052	0.059	0.068	0.084	0.048	0.060	0.010	0.165	0.3
8:2 FTS	0.047	0.050	0.052	0.054	0.048	0.040	0.060	0.057	0.062	0.069	0.048	0.053	0.008	0.153	0.3
10:2 FTS	0.056	0.050	0.061	0.053	0.058	0.047	0.053	0.054	0.057	0.061	0.047	0.054	0.005	0.093	0.2

CONCLUSIONS

The method presented was shown to be valid for use in animal food matrices. A summary of analytes reportable in matrix sub-types is shown below in Table 17. Reportable analytes are indicated by green check mark and unreportable by red x marks.

Table 17 - Reportable Analytes by Matrix

Analyte	Wet Dog Food	Dry Dog Food	Wet Cat Food	Dry Cat Food	Chicken Feed	Alfalfa
PFBA	✗	✗	✗	✓	✗	✓
PFPeA	✓	✓	✓	✗	✓	✓
PFHxA	✓	✓	✓	✓	✗	✗
PFHpA	✓	✓	✓	✓	✗	✗
PFOA	✓	✓	✓	✓	✓	✓
PFNA	✓	✓	✓	✓	✓	✓
PFDA	✓	✓	✓	✓	✓	✓
PFUdA	✓	✓	✓	✓	✓	✓
PFDoA	✓	✓	✓	✓	✓	✓
PFTrDA	✓	✗	✓	✓	✓	✓
PFTeDA	✓	✗	✓	✓	✓	✓
ADONA	✓	✓	✓	✓	✓	✓
HFPO-DA	✓	✓	✓	✓	✓	✓
N-MeFOSAA	✓	✓	✓	✓	✓	✓
N-EtFOSAA	✓	✗	✓	✓	✗	✓
PFBS	✓	✓	✓	✓	✓	✓
PFPeS	✓	✓	✓	✓	✓	✓
PFHxS	✓	✗	✓	✗	✓	✓
PFHpS	✓	✗	✓	✓	✓	✗
PFOS	✓	✓	✓	✓	✓	✓
9Cl-PF3ONS	✓	✓	✓	✓	✓	✓
11Cl-PF3OUdS	✓	✓	✓	✓	✓	✓
4:2 FTS	✓	✓	✓	✓	✓	✓
6:2 FTS	✓	✓	✓	✗	✓	✓
8:2 FTS	✓	✓	✓	✓	✓	✓
10:2 FTS	✓	✓	✓	✓	✓	✓

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Appendix A – Sciex Analyst Instrument Method

Appendix A_LCMS Method_All Sections.p

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Appendix B – Sciex OS Data Processing Method

Appendix B_PFAS Processing Method.xl

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Appendix C – Report of Initial Calibration

Appendix C_20210816_Calibratic

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