FDA Foods Program Compendium of Analytical Laboratory Methods: Chemical Analytical Manual (CAM)

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PROGRAM AREA: Chemical Contaminants

METHOD TITLE: Determination of 16 Per and Polyfluoroalkyl Substances (PFAS) in Processed Food using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

VALIDATION STATUS: Single Laboratory Validation per the Guidelines for the Validation of Chemical Methods for the FDA Foods Program 3rd Edition

AUTHOR(S): Susan Genualdi and Lowri deJager

METHOD SUMMARY/SCOPE:

The method describes a procedure for measuring 16 PFAS in food using LC-MS/MS. The method has been single laboratory validated in the following food matrices:

Matrices	Validation	Date	Analyst
infant formula, strawberry gelatin, pancake syrup, cream cheese, shredded wheat cereal	Single lab validation	2021	Susan Genualdi, Jessica Beekman
lettuce, milk, bread, and salmon	Verification per Guidelines for the Validation of Chemical Methods in Food, Feed, Cosmetics and Veterinary Products (3rd Ed.)	2021	Susan Genualdi

Analytes: Perfluorobutanoic acid, Perfluoropentanoic acid, Perfluorohexanoic acid, Perfluoroheptanoic acid, Perfluorooctanoic Acid, Perfluorononanoic acid, Perfluorodecanoic acid, Perfluorobutanesulfonic acid, Perfluoropentanesulfonic acid,

Perfluorohexanesulfonic acid, Perfluoroheptanesulfonic acid, Perfluorooctanesulfonic acid, Sodium dodecafluoro-3H-4, 8-dioxanonanoate, 2,3,3,3-Tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)propanoic acid (GenX), Potassium 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate, 11-chloroeicosafluoro-3-oxaundecane-1-sulfonic acid,

Matrices: Infant formula, strawberry gelatin, pancake syrup, cream cheese, shredded wheat cereal. These matrices were chosen based on those that have been challenging in the past (infant formula, cream cheese (cheese), shredded wheat cereal (grains/breads) and two other matrices that appeared complex (strawberry gelatin, pancake syrup). Lettuce, milk, bread, and salmon were validated in C-010.01 and have been verified for the updated method.

REVISION HISTORY: This method was updated in December 2021 to version C-010.02. See the other notes section below.

OTHER NOTES:

- This method has been modified from C-010.01 to cover a wider range of food matrices and includes improvements based on recent advances in the literature. The changes are described below.
- M3PFPeA added as a surrogate standard
- Concentration of isotopically labeled internal standard solution (d5NEtFOSAA) changed from 1 to 0.2 µg/mL
- The calibration curve was adjusted to allow for a 5 µL spike of internal standard in the final SPE extract so concentration and volume of d5NEtFOSAA added to calibration curve was changed.
- 50 ng/mL calibration curve point was removed
- Nitrogen evaporation station changed to Biotage Turbovap LV
- Addition of 1-methyl piperidine to mobile phase, which improves ionization of PFAS in negative mode resulting in higher response and lower background.
- The analytical column was changed to a Waters 150 mm x 2.1 mm, 3.5 µm XBridge C18 which can accommodate a higher pH range which is necessary due to the use of 1-MP in the mobile phase
- The amount of water added to each sample for QuEChERS extraction is based on whether the food is high or low water content with a reference to the FDA guidelines for chemical methods
- SPE elution solvent changed from 0.3% NH4OH in acetonitrile to 0.3% NH4OH in methanol due to recent studies showing the poor stability of HFPO-DA in acetonitrile
- Water equilibration step added after conditioning step in SPE
- SPE performed on all samples except liquids, fruits, and vegetables unless there is a detection
- Gradient profile for LC conditions was adjusted to have a 3 minute hold time at 10 %B and a 3 µL injection volume for improved peak shape of early eluting PFAS
- MS/MS transitions improved for HFPO-DA to represent a more stable transition which gives a higher response
- MS/MS transitions quantifier and qualifier ions switched for PFOA
- HRMS confirmation necessary for confirmation of PFBA and PFPeA
- Analyst software updated to Sciex OS for data processing

Title: Determination of 16 Per and Polyfluoroalkyl Substances (PFAS) in Processed Food using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Version 2021 (2021)

Author: Susan Genualdi and Lowri deJager

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2021.1 METHOD TITLE: Determination of 16 Per and Polyfluoroalkyl Substances (PFAS) in Processed Food using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

2021.2 SCOPE OF APPLICATION

The method describes a procedure for measuring 16 PFAS in food using LC-MS/MS. The method has been single laboratory validated in the following food matrices:

Matrices	Validation	Date	Analyst
infant formula, strawberry gelatin, pancake syrup, cream cheese, shredded wheat cereal	Single lab validation	2021	Susan Genualdi, Jessica Beekman
lettuce, milk, bread, and salmon	Verification per Guidelines for the Validation of Chemical Methods in Food, Feed, Cosmetics and Veterinary Products (3rd Ed.)	2021	Susan Genualdi

- A method verification was performed with lettuce, milk, bread, and salmon to capture the matrices originally described in C-010.01. Samples were spiked in duplicate at 0.5 µg/kg and 2 µg/kg. All recoveries were between 70-130% with the exception of 11Cl-PF3OUdS in bread which had recoveries ranging from 26-34%. This analyte has known issues with certain matrices, which may reduce its confidence in certain food types.
- This method should be used by analysts experienced in the use of LC-MS/MS, including but not limited to operation of the instrumentation and software, data analysis and reporting results.
- Analysts should also be able to identify chromatographic and mass spectrometric interferences during sample analysis and take necessary actions following validated procedures for their correction to achieve reliable identification and quantitation.
- The method should be used only by personnel thoroughly trained in the handling and analysis of samples for the determination of trace contaminants in food and beverage products. PFAS chemicals are prevalent in all laboratory environments and special care must be taken to prevent false positives due to accidental and/or routine laboratory contamination.
- Only LC-MS grade solvents should be used unless otherwise noted in the procedure below. All solvents and complete method blanks should be analyzed on the LC-MS/MS instrument prior to sample analysis. If PFAS compounds are determined, complete method blank results should be subtracted from samples. Complete method blanks should be performed and analyzed daily, preferably in the same instrument sequence as the samples. Sources of potential contamination during sample preparation include; solvents, syringe filters, centrifuge tubes, SPE sorbents, septa, and others.
- A delay column should be used between the mobile phase mixer and sample injector to temporarily trap any system related interferences, which results in their elution at a later retention time than the analyte. This eliminates contamination from instrument tubing, mobile phase solvents, and solvent bottles.

- Due to the extreme low concentrations of detection required for this analysis, choice of MS/MS instrumentation is critical. Our analysis has been performed using Sciex 6500 and 6500 plus instrumentation platforms. We have not fully evaluated any Orbitrap MS systems and have not yet demonstrated adequate lower levels of quantitation (LLOQ) for these systems.

2021.3 PRINCIPLE

The test sample is homogenized and fortified with isotopically labeled surrogates prior to the addition of water. The PFAS are extracted from the food samples using acetonitrile and formic acid. Following extraction, a modified QuEChERS extraction technique is performed. For complex samples, further clean-up using solid phase extraction is required. The resulting extract is filtered and fortified with internal standard solution and analyzed using LC-MS/MS. The PFAS compounds are identified by multiple reaction mode (MRM) transitions and retention time matching with the calibration standards. Ion ratios are used to confirm the identity. If two MRM transitions are not available (e.g PFBA and PFPeA), then HR-MS is necessary for confirmation. The concentration of each PFAS is determined using the response ratio of the PFAS quantitation transition to that of the relevant labeled surrogate standard (SS). The concentration is calculated by preparing a calibration curve using response ratios versus concentration ratios for native analytes to that of their labeled-SS. During analysis, quality control samples and method blanks must be analyzed. Analyte response in method blanks must be subtracted from the sample response prior to final quantitation. After determination of the concentration from the curve, the concentration must be adjusted for dilution and starting sample mass. Certain analytes will also need to be corrected based on their salt concentrations and technical PFOS for its actual concentration in the mixture.

2021.4 REAGENTS

The use of trade names in this method constitutes neither endorsement nor recommendation by the U.S. Food and Drug Administration (FDA). Equivalent performance may be achievable using apparatus and materials other than those cited here.

- Formic acid, reagent grade >95% (Sigma Aldrich St. Louis, MO)
- LC/MS grade Optima water (Fisher Scientific, Hampton, NH)
- LC/MS grade Optima acetonitrile (Fisher Scientific, Hampton, NH)
- LC/MS grade Optima methanol (Fisher Scientific, Hampton, NH)
- Acetic acid, ammonium salt, 98% for analysis (Acros Organic, Geel, Belgium)
- Original QuEChERS extraction salt ECMSSCFS-MP with 6000 mg MgSO4 and 1500 mg NaCl (UCT, Bristol, PA)
- QuEChERS dSPE ECMPSCB-MP with 900 mg MgSO4, 300 mg PSA, 150 mg graphitized carbon black (UCT, Bristol, PA) or ECMPSCB15-CT prefilled units
- Ammonium hydroxide, certified ACS Plus 14.8N (Fisher Scientific, Hampton, NH)

2021.5 STANDARDS

- Isotopically labeled PFAS analytical standards (Wellington laboratories, Guelph, ON, Canada)
- Native PFAS analytical standards (Wellington laboratories, Guelph, ON, Canada)

- Both PFOA and PFOS were quantified using technical standards and reported as the sum of linear and branched isomers. All other analytes were reported as the concentration of the linear isomer (if applicable).

Table 1. PFAS native, surrogate, and internal standard compounds

Acronym	Name	CAS	Formula	MW
	Native PFAS			
PFBA	Perfluorobutanoic acid	375-22-4	$C_4F_7O_2$	214
PFPeA	Perfluoropentanoic acid	2706-90-3	$C_5HF_9O_2$	264
PFHxA	Perfluorohexanoic acid	307-24-4	$C_6HF_{11}O_2$	314
PFHpA	Perfluoroheptanoic acid	375-85-9	$C_7HF_{13}O_2$	364
PFOA	Perfluorooctanoic Acid	335-67-1	$C_8HF_{15}O_2$	414
PFNA	Perfluorononanoic acid	375-95-1	$C_9HF_{17}O_2$	464
PFDA	Perfluorodecanoic acid	335-76-2	$C_{10}HF_{19}O_2$	514
PFBS	Perfluorobutanesulfonic acid	375-73-5	$C_4HF_9O_3S$	300
PFPeS	Perfluoropentanesulfonic acid	2706-91-4	$C_5HF_{11}O_3S$	350
PFHxS	Perfluorohexanesulfonic acid	355-46-4	$C_6HF_{13}O_3S$	400
PFHpS	Perfluoroheptanesulfonic acid	375-92-8	$C_7HF_{15}O_3S$	450
PFOS	Perfluorooctanesulfonic acid	1763-23-1	$C_8HF_{17}O_3S$	500
NaDONA	Sodium dodecafluoro-3H-4, 8-dioxanonanoate	958445-44-8	$C_7H_5F_{12}NO_4$	395
HFPO-DA	2,3,3,3-Tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)propanoic acid (GenX)	62037-80-3	$C_6HF_{11}O_3$	330
9Cl-PF3ONS	Potassium 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate	73606-19-6	$C_8ClF_{16}KO_4S$	570
11Cl-PF3OUdS	11-chloroeicosafluoro-3-oxaundecane-1-sulfonic acid	763051-92-9	$C_{10}HClF_{20}O_4S$	632
	Internal Standard/Surrogates			
M3 PFBA	Perfluoro-n-[2,3,4-13C3]butanoic acid			217
M3 PFPeA	Perfluoro-n-[3,4,5-13C3]pentanoic acid			267
MPFHxA	Perfluoro-n-[1,2-13C2]hexanoic acid			316
13C PFOA	Perfluoro-n-[13C8]octanoic acid			422
M3 PFBS	Sodium perfluoro-1-[2,3,4-13C3]butanesulfonate			303
MPFHxS	Sodium perfluoro-1-hexane[18O2]sulfonate			404
13C PFOS	Sodium perfluoro-[13C8]octanesulfonate			508
M3 HFPO	2,3,3,3-Tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)-13C3-propanoic acid			333
d5-N-EtFOSAA	N-ethyl-d5-perfluoro-1-octanesulfonamidoacetic acid			590

2021.6 PREPARATION OF SAMPLES OR TEST PORTIONS

2021.6.1 Prepare native PFAS stock solution at 1000 ng/mL, 100 ng/mL, 10 ng/mL and 1 ng/mL.

(1) Add 0.2 mL of each 50 µg/mL PFAS analytical standard (16 native compounds in Table 1) to 6.8 mL methanol. In the resulting solution, each compound has a concentration of 1000 ng/mL in methanol. Individual PFAS 50 µg/mL methanol standards were purchased from Wellington, but other sources are acceptable. This solution will be used for calibration curve preparation and single lab validation (SLV) spikes.

(2) Add 1 mL of 1000 ng/mL stock solution to 9 mL of methanol to produce a 100 ng/mL stock solution. This solution will be used for calibration curve preparation and SLV spikes.

(3) Add 1 mL of 100 ng/mL stock solution to 9 mL of methanol to produce a 10 ng/mL stock solution. This solution will be used for SLV and method detection limit (MDL) spikes.

(4) Add 1 mL of 10 ng/mL stock solution to 9 mL of methanol to produce a 1 ng/mL stock solution. This solution will be used for calibration curve preparation.

2021.6.2 Prepare isotopically labeled PFAS surrogate stock solution (SS) at 1000 ng/mL

(1) Add 0.2 mL of each 50 µg/mL analytical standard (8 isotopically labeled PFAS in Table 1) to 8.4 mL methanol. Individually labeled PFAS 50 µg/mL methanol standards were purchased from Wellington but other sources are acceptable. This stock solution was used for both sample analysis and calibration curve preparation.

2021.6.3 Prepare isotopically labeled internal standard solution (IS) at 200 ng/mL

(1) Add 0.04 mL of N-ethyl-d5-perfluoro-1-octanesulfonamidoacetic acid (d5-N-EtFOSAA) 50 µg/mL analytical standard to 9.96 mL methanol. The individual *d5*-N-EtFOSAA standard was purchased from Wellington but other sources are acceptable.

2021.6.4 Prepare mobile phase A (5 mM ammonium acetate in water) and 5mM 1-methyl piperidine

(1) Weigh out 0.38 ± 0.01 g of ammonium acetate.
Add to mobile phase bottle with 1000 mL of LC/MS Optima water.
Add 0.5 mL of 1-methyl piperidine.
Invert several times to mix.

2021.6.5 Prepare mobile phase B (100% methanol)

(1) Add ~ 1000 mL of LC/MS Optima methanol to a mobile phase bottle.

2021.6.6 Continuing Calibration Verification (CCV) standard

(1) A duplicate solution was prepared of the 1 ng/mL calibration standard and used as the CCV standard (Table 2).

2021.6.7 Solution for solid phase extraction (SPE) clean-up

(1) Add 6 mL of a 14.8 N ammonium hydroxide solution to 1000 mL volumetric flask and fill to volume with methanol to make up a 0.3 % w/w solution.

2021.6.8 Calibration Standards

(1) Calibration standards are prepared at concentrations of 0.01, 0.05, 0.10, 0.50, 1.0, 5.0, 10, and 25ng/mL according to Table 2 below.

Table 2. Calibration standard preparation

Final concentration	Native stock solution concentration	Volume of stock solution to add	Volume of 1000 ng/mL surrogate stock solution	Methanol	Final volume	Volume of 200 ng/mL IS stock solution
ng/mL	ng/mL	mL	mL	mL	mL	mL
0.01	1	0.1	0.01	9.89	10	0.05
0.05	1	0.5	0.01	9.49	10	0.05
0.1	1	1	0.01	8.99	10	0.05
0.5	100	0.05	0.01	9.94	10	0.05
1	100	0.1	0.01	9.89	10	0.05
5	100	0.5	0.01	9.49	10	0.05
10	100	1	0.01	8.99	10	0.05
25	1000	0.25	0.01	9.74	10	0.05

2021.6.9 Preparation of Samples or Test Portions

(1) The samples used for method development were previously homogenized by FDA's Kansas City lab. The sample size for analysis was 5 grams.

(2) The samples used for method verification (lettuce, milk, bread, and salmon) were homogenized using an IKA tube mill with a disposable 100 mL polypropylene grinding chamber. Samples were ground at 5000 rpm for approximately 2 minutes.

2021.7 APPARATUS/INSTRUMENTATION

(1) Digital pulse mixer/vortexer (Glas-Col, Terre Haute, IN) capable of 1500 rpm with pulse 70

(2) Sorvall legend XTR centrifuge (Thermo Fisher Scientific, Waltham, MA)

(3) Nitrogen evaporation system (Turbovap LV, Biotage, Uppsala, Sweden)

(4) Nexera X2 (Shimadzu, Kyoto, Japan) with binary pump, degasser, autosampler, and thermostatted column compartment

(5) A Sciex 6500 plus QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer with an electrospray ESI ion source (Sciex, Toronto, ON Canada)

(6) Analyst® Software version 1.7.1

(7) ScieX OS Version 2.0.0.45330

(8) Falcon 50 mL polypropylene (PP) conical centrifuge tubes (Thermo Fisher Scientific, Waltham, MA)

(9) Falcon 15 mL polypropylene (PP) conical centrifuge tubes (Thermo Fisher Scientific, Waltham, MA)
(10) 300 µL PP autosampler vials (SUN Sri, Rockwood, TN)
(11) PP autosampler vial caps (SUN Sri, Rockwood, TN)
(12) 0.2 µm Acrodisc nylon syringe filters (Pall Corporation, Port Washington, NY)
(13) 5 mL PP/PE luer lock syringes (Sigma Aldrich, St. Louis, MO)
(14) Nano filter vials 0.2 µm nylon without cap (Thomson Instrument Company, Oceanside, CA)
(15) PP vial caps (Sun Sri, Rockwood, TN)
(16) Analytical column – 150 mm x 2.1 mm, 3.5 µm XBridge C18 (Waters Corp, Milford, MA)
(17) Guard column – 2.1 mm x 5 mm, 1.7 µm Vanguard™ Acquity BEH C18 (Waters Corp, Milford, MA)
(18) Delay column – 2.1 mm x 50 mm, 5 µm Atlantis T3 (Waters Corp, Milford, MA)
(19) SPE cartridge – Strata™-XL-AW 100 µm Polymeric Weak Anion 200 mg / 3 mL, Tubes (Phenomenex, Torrance, CA)

2021.8 METHOD

QuEChERS (Quick, easy, cheap, effective, rugged, safe) is used for the extraction of PFAS from foods. Due to the high variability of the sample matrix, sample preparation steps may vary by food type.

2021.8.1 Sample Preparation

(1) Add 5 grams of sample to a 50 mL polypropylene (PP) centrifuge tube
(2) Add 10 µL of 1000 ng/mL isotopically labeled surrogate standard solution to the sample.
(3) Add 5 mL of LC/MS grade Optima water if the sample is fruit or vegetable based to the 50 mL PP conical centrifuge tube. Dry samples (< 25% water content) will need additional water. Descriptions of low water content commodity groups can be found in Appendix 4 of FDA Foods Program Guidelines for Chemical Methods. For most dry foods, the addition of 15 mL of water is sufficient. In some cases (e.g. protein powder) up to 25 mL of additional water is needed to adequately swell the matrix.
(4) Add 10 mL acetonitrile to the 50 mL PP conical centrifuge tube
(5) Add 150 µL formic acid to the 50 mL PP conical centrifuge tube
(6) Shake vigorously for 1 minute
(7) Add QuEChERS salt packet (Original extraction salt ECMSSCFS-MP from UCT with 6000 mg $MgSO_4$ and 1500 mg NaCl)
(8) Place on Glas-Col shaker at 1500 rpm with pulse set to 70 for 5 minutes
(9) Centrifuge for 5 minutes at 10000 rcf
(10) Add supernatant to 15 mL PP conical centrifuge tube with dSPE sorbent (ECMPSCB-MP from UCT with 900 mg $MgSO_4$, 300 mg PSA, 150 mg graphitized carbon black)
(11) Vortex/shake for 2 minutes
(12) Centrifuge 5 minutes at 10000 rcf
(13) Filter the supernatant with a 0.2 µm nylon syringe filter and transfer to a 15 mL conical centrifuge tube
(14) Fruit, vegetable, and beverage samples do not require SPE clean-up, unless there is a positive detection. SPE clean-up can be performed on every sample if desired.
(15) If the QuEChERS extract is to be analyzed independently, take 1 mL of the filtered supernatant and transfer to a 15 mL centrifuge tube. Then 5 µL of 200 ng/mL *d5* N-EtFOSAA is added to the tube.

(16) Transfer ~ 100 µL to a Thomson nano filter vial with 0.2 µm nylon® filter and a PP screw cap (Sun Sri) to run using LC-MS/MS.

2021.8.2 Clean-up of extract using weak anion exchange solid-phase extraction (SPE) column

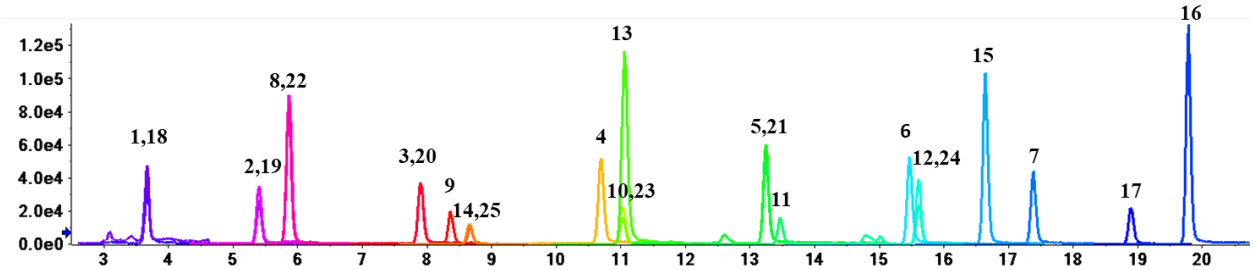
The SPE step is necessary for all samples that are not beverages or fruit/vegetable based and can be performed on all samples if desired.

(1) Take 1 mL of filtered QuEChERS extract and dilute to ~ 15 mL with LC Optima water in a clean 15 mL PP conical centrifuge tube
(2) Condition a Strata™-XL-AW 100 µm column (200 mg/3 mL) with 9 mL of 0.3% ammonium hydroxide in methanol
(3) Add 5 mL of LC Optima water to equilibrate column
(4) Add sample to column and let pass through
(5) Add 5 mL of LC Optima water to wash column
(6) Let column dry 1 minute
(7) Add 4 mL of 0.3% ammonium hydroxide in methanol to elute analytes into a clean 15 mL PP conical centrifuge tube
(8) Blow to near dryness in a 60 °C water bath
(9) Reconstitute to 1 mL with methanol and add 5 µL of 200 ng/mL *d5* N-EtFOSAA to the tube.
(10) Transfer ~ 100 µL to a Thomson nano filter vial with 0.2 µm nylon® filter and a PP screw cap (Sun Sri) to run using LC-MS/MS.

2021.8.3 LC-MS/MS Analysis

All samples were analyzed using a liquid chromatograph (Nexera X2, (Shimadzu, Kyoto, Japan)). The MS/MS data was acquired using scheduled MRM with an AB Sciex 6500 plus QTRAP.

An example chromatogram is included below of a spiked SLV sample (prepared infant formula) with native and labeled PFAS concentrations at 10 ng/mL



1. PFBA, 2. PFPeA, 3. PFHxA, 4. PFHpA, 5. PFOA, 6. PFNA, 7. PFDA, 8. PFBS, 9. PFPeS, 10. PFHxS, 11. PFHpS, 12. PFOS, 13. NaDONA, 14. HFPO-DA, 15. 9Cl-PF3ONS, 16. 11Cl-PF3OUdS, 17. d5 N-EtFOSAA, 18. M3 PFBA, 19. M3 PFPeA 20. MPFHxA, 21. 13C PFOA, 22. M3 PFBS, 23. MPFHxS, 24. 13C PFOS, 25. M3 HFPO-DA

The LC gradient and the MS/MS monitored transitions can be found in Tables 3 and 4.

Table 3. Gradient Profile for the LC Conditions

Time (min)	Concentration of B
0.01	10%
3	10%
3.1	40%
26	90%
26.1	10%
28	10%

Table 4. MS/MS Conditions for the Monitored Transitions on a 6500 plus QTRAP

ID	Retention Time (min)	Q1 mass (m/z)	Q3 mass (m/z)	DP (volts)	EP (volts)	CE (volts)	CXP (volts)
Internal Standard							
d5-N-EtFOSAA [a]	19	589	419	-50	-10	-30	-20
d5-N-EtFOSAA	19	589	219	-50	-10	-38	-20
Surrogates							
M3 PFBA[a]	5.1	216	172	-17	-8	-12	-14
M3 PFPeA[a]	6.8	266	222	-17	-6	-11	-28
MPFHxA[a]	9	315	270	-13	-10	-14	-12
13C PFOA[a]	13.9	421	376	-36	-8	-13	-20
13C PFOA	13.9	421	172	-19	-5	-25	-7
M3 PFBS[a]	7.3	302	80	-88	-6	-73	-9
M3 PFBS	7.3	302	99	-85	-6	-36	-8
MPFHxS[a]	11.9	403	103	-60	-10	-81	-15
MPFHxS	11.9	403	169	-60	-10	-42	-15
13C PFOS[a]	16	507	80	-100	-5	-125	-15
13C PFOS	16	507	99	-100	-5	-100	-15
M3 HFPO[a]	9.7	287	169	-19	-12	-10	-25
M3 HFPO	9.7	287	185	-41	-5	-35	-35
Natives							
PFBA[a]	5.1	213	169	-10	-6	-13	-19
PFPeA[a]	6.8	263	219	-20	-8	-11	-20
PFHxA[a]	9	313	269	-10	-12	-13	-45
PFHxA	9	313	119	-23	-12	-27	-14
PFHpA[a]	11.5	363	319	-17	-10	-14	-26
PFHpA	11.5	363	169	-32	-11	-25	-24
PFOA[a]	13.9	413	369	-43	-7	-16	-25
PFOA	13.9	413	219	-24	-5	-23	-25
PFNA[a]	15.9	463	419	-38	-11	-15	-37
PFNA	15.9	463	269	-40	-5	-24	-13

PFDA[a]	17.6	513	469	-15	-10	-16	-29
PFDA	17.6	513	269	-20	-10	-26	-17
PFBS[a]	7.3	299	80	-44	-10	-70	-11
PFBS	7.3	299	99	-35	-4	-36	-15
PFPeS[a]	9.5	349	99	-80	-9	-80	-12
PFPeS	9.5	349	119	-53	-10	-40	-18
PFHxS[a]	11.9	399	99	-108	-6	-84	-8
PFHxS	11.9	399	169	-66	-5	-42	-20
PFHpS[a]	14.1	449	99	-58	-8	-84	-24
PFHpS	14.1	449	169	-68	-8	-41	-27
PFOS[a]	16	499	80	-150	-4	-120	-10
PFOS	16	499	99	-150	-4	-100	-10
NaDONA[a]	11.9	377	251	-25	-8	-15	-20
NaDONA	11.9	377	85	-20	-7	-39	-10
HFPO-DA[a]	9.7	285	169	-78	-6	-11	-27
HFPO-DA	9.7	285	185	-20	-5	-21	-27
9Cl-PF3ONS[a]	17	531	351	-100	-14	-38	-13
9Cl-PF3ONS	17	531	83	-100	-4	-92	-9
11Cl-PF3OUdS[a]	19.8	631	451	-34	-9	-40	-12
11Cl-PF3OUdS	19.8	631	199	-20	-10	-36	-11

[a] Primary MRM transition used for quantification

The following conditions are for the 6500 plus Q-trap:

- Curtain gas: 40 au
- Collisionally activated dissociation (CAD) gas: medium
- Ion spray voltage: -4500 V
- Source temperature: 350 °C
- Gas 1 pressure: 50 au
- Gas 2 pressure: 50 au
- Injection volume: 3 µL
- Column temperature: 40 °C
- Flow rate: 0.30 mL/min

Run the samples using the following template:

- Blank (MeOH) injection
- Standard curve
- Blank (MeOH) injection
- Samples

For every 6 samples analyzed, a CCV standard (typically 1 ng/mL) is run to check for accuracy. The accuracy of the calculated concentration of the CCV should be statistically evaluated, which can typically be within 70-130 % of the original value. If the accuracy

falls outside this range, the calibration curve is rerun, and any test samples run since the last successful CCV are remeasured.

2021.9 CALCULATIONS

Example calculation for concentration measured on LC-MS/MS to concentration in 5 grams of food with a final extract of 10 mL:

- The lowest calibration curve point is 0.01 ng/mL in 0.5 mL of solution.

$$\frac{0.01\ ng}{mL} * 0.5\ mL = 0.005\ ng$$

- Since there are 0.005 ng in 0.5 mL of extract, there would be 0.1 ng in the total 10 mL extract

$$0.005\ ng * \frac{10\ mL}{0.5\ mL} = 0.1\ ng$$

- With a 5 gram food sample, this is equivalent to 20 ng/kg in foods

$$\frac{0.1\ ng}{5\ g} = 0.02\frac{ng}{g} or\ 20\frac{ng}{kg}$$

Sciex OS software is used to prepare a linear standard curve where x is the concentration ratio (analyte/SS) and y is the instrument response ratio (analyte/SS) with 1/x weighting. Surrogates and their internal standard pairs are listed in Table 5, which are used to calculate absolute recoveries of the surrogate standards over the entire extraction method. Surrogates and their native analyte pairs are also listed in Table 5 with their curve fit. The calibration curve has surrogate and internal standard concentrations of 1 ng/mL.

Table 5. Analytes with calibration curve fit and surrogates used as the internal standard

Surrogates			
M3 PFBA[a]	N-EtFOSAA[a]	mean response factor	none
M3 PFPeA[a]	N-EtFOSAA[a]	mean response factor	none
MPFHxA[a]	N-EtFOSAA[a]	mean response factor	none
13C PFOA[a]	N-EtFOSAA[a]	mean response factor	none
13C PFOA	N-EtFOSAA[a]	mean response factor	none
M3 PFBS[a]	N-EtFOSAA[a]	mean response factor	none
M3 PFBS	N-EtFOSAA[a]	mean response factor	none
MPFHxS[a]	N-EtFOSAA[a]	mean response factor	none
MPFHxS	N-EtFOSAA[a]	mean response factor	none
13C PFOS[a]	N-EtFOSAA[a]	mean response factor	none
13C PFOS	N-EtFOSAA[a]	mean response factor	none
M3 HFPO[a]	N-EtFOSAA[a]	mean response factor	none

M3 HFPO	N-EtFOSAA[a]	mean response factor	none
	Natives		
PFBA[a]	M3 PFBA[a]	Linear	1/x
PFPeA[a]	M3 PFPeA[a]	Linear	1/x
PFHxA[a]	MPFHxA[a]	Linear	1/x
PFHxA	MPFHxA[a]	Linear	1/x
PFHpA[a]	MPFHxA[a]	Linear	1/x
PFOA[a]	13C PFOA[a]	Linear	1/x
PFOA	13C PFOA[a]	Linear	1/x
PFNA[a]	13C PFOA[a]	Linear	1/x
PFNA	13C PFOA[a]	Linear	1/x
PFDA[a]	13C PFOA[a]	Linear	1/x
PFDA	13C PFOA[a]	Linear	1/x
PFBS[a]	M3 PFBS[a]	Linear	1/x
PFBS	M3 PFBS[a]	Linear	1/x
PFPeS[a]	MPFHxS[a]	Linear	1/x
PFPeS	MPFHxS[a]	Linear	1/x
PFHxS[a]	MPFHxS[a]	Linear	1/x
PFHxS	MPFHxS[a]	Linear	1/x
PFHpS[a]	MPFHxS[a]	Linear	1/x
PFHpS	MPFHxS[a]	Linear	1/x
PFOS[a]	13C PFOS[a]	Linear	1/x
PFOS	13C PFOS[a]	Linear	1/x
NaDONA[a]	13C PFOA[a]	Linear	1/x
NaDONA	13C PFOA[a]	Linear	1/x
HFPO-DA[a]	M3 HFPO[a]	Linear	1/x
HFPO-DA	M3 HFPO[a]	Linear	1/x
9Cl-PF3ONS[a]	MPFHxS[a]	Linear	1/x
9Cl-PF3ONS	MPFHxS[a]	Linear	1/x
11Cl-PF3OUdS[a]	MPFHxS[a]	Linear	1/x
11Cl-PF3OUdS	MPFHxS[a]	Linear	1/x

[a] Primary MRM transition used for quantification

2021.9.1 Corrections for salts and technical mixture

In sample analysis, the final positive detections must be corrected for the analytical standard used in the analysis. In this study, the following analytical standards were obtained in salt form with either the sodium or potassium ion; PFBS, PFPeS, PFHxS, PFHpS, PFOS, 9Cl-PF3ONS, 11Cl-PF3OUdS, NaDONA. The certificate of analysis must be examined for either the concentration in anion form or the concentration in salt form. If only the salt form is listed, the corrected concentration can be calculated by multiplying by the ratio below.

$$\frac{Molecular\ weight\ of\ acid\ form}{Molecular\ weight\ of\ salt\ form}$$

For the PFOS technical mixture used in this study, a separate correction needs to be made for PFOS in the technical mixture. The T-PFOS standard is 80% PFOS-K isomers. So, final concentrations need to be multiplied by 0.8 and then by 0.928 to account for both the technical mixture and salt concentration in this standard purchased by Wellington. These values may vary by manufacturer and the certificate of analysis should be consulted.

2021.10 VALIDATION INFORMATION/STATUS

Single lab validation. A level 2 validation was conducted under the Guidelines for the Validation of Chemical Methods for the FDA FVM Program 2nd Ed. A total of 5 different types of foods and beverages were evaluated. These included infant formula, shredded wheat cereal, strawberry gelatin, cream cheese, and pancake syrup. The method was validated at 3 concentrations (0.05, 0.5, 2 ng/mL) in 5 food matrices. Acceptable recovery ranges for these compounds based on the FDA guidelines for the validation of chemical methods is 40-120% for concentrations at a method level of 1 ng/mL. All compounds were within the acceptable range except for 11Cl-PF3OUdS in shredded wheat cereal samples which were on the lower side at 24-32% recovery. Validation information can be obtained from reference (3) and raw data may be examined by contacting the study director.

Table 6. Single Lab validation recovery ranges. "Not validated" indicates that for this compound and matrix, the compound fell below the acceptable recovery range.

	infant formula	shredded wheat cereal	strawberry gelatin	cream cheese	pancake syrup
PFBA	0.05 - 2 ng/mL	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g
PFPeA	0.05 - 2 ng/mL	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g
PFHxA	0.05 - 2 ng/mL	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g
PFHpA	0.05 - 2 ng/mL	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g
PFOA	0.05 - 2 ng/mL	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g
PFNA	0.05 - 2 ng/mL	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g
PFDA	0.05 - 2 ng/mL	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g
PFBS	0.05 - 2 ng/mL	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g
PFPeS	0.05 - 2 ng/mL	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g
PFHxS	0.05 - 2 ng/mL	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g
PFHpS	0.05 - 2 ng/mL	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g
PFOS	0.05 - 2 ng/mL	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g
NaDONA	0.05 - 2 ng/mL	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g
HFPO-DA	0.05 - 2 ng/mL	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g
9Cl-PF3ONs	0.05 - 2 ng/mL	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g
11Cl-PF3OUdS	0.05 - 2 ng/mL	Not Validated	0.05 - 2 ng/g	0.05 - 2 ng/g	0.05 - 2 ng/g

Method detection limits were calculated by performing 7 low-level spikes at 0.05 ng/g for all matrices. The standard deviation of the replicates was multiplied by 3.14 (t-value for seven replicates where 1-α =0.99). The MDL is defined as the statistically calculated minimum concentration that can be measured with 99% confidence that the reported value is greater than 0. This procedure is published in the Code of Federal Regulations, see references. Since MDLs were calculated using challenging matrices and the

MDLs appear consistent among food types, the highest MDL for each analyte was used as the MDL that represents total diet study food samples. MDLs are recalculated yearly and are dependent on instrumental conditions at the time of the study.

Table 7. Method detection limits in ng/kg.

Sample type	PFBA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFBS	PFPeS	PFHxS	PFHpS	PFOS	ADONA	HFPO-DA	9Cl-PF3ONS	11Cl-PF3OUdS
infant formula	58	17	18	12	12	7	12	3	15	14	20	7	11	10	6	4
cereal	67	22	40	43	17	11	12	9	20	13	21	28	7	21	13	3
gelatin	56	29	25	19	17	21	17	21	28	17	28	27	16	34	18	17
cream cheese	63	29	30	11	24	13	22	7	21	22	23	7	9	20	13	16
pancake syrup	31	31	48	31	22	29	15	20	23	35	36	20	25	32	20	17

*These values represent MDLs calculated during the method validation, the UCL reported during the sample set was 344 ng/kg and all PFBA and PFPeA detects must be confirmed using HR-MS

- *Verification of matrices validated in original method C-010.01.* A method verification (per the Guidelines for the Validation of Chemical Methods in Food, Feed, Cosmetics and Veterinary Products, 3rd Ed.) was performed with lettuce, milk, bread, and salmon to capture the matrices originally described in C-010.01. Samples were spiked in duplicate at 0.5 µg/kg and 2 µg/kg. All recoveries were between 70-130% with the exception of 11Cl-PF3OUdS in bread which had recoveries ranging from 26-34%. This analyte has known issues with certain matrices, which may reduce its confidence in certain food types.

2021.11 REFERENCES

(1) FDA Guidelines for the Validation of Chemical Methods for the FDA Foods Program; http://www.fda.gov/downloads/ScienceResearch/FieldScience/UCM298730.pdf.

(2) Definition and procedure for the determination of the method detection limit-revision 1.11. Code of Federal Regulations. 40 CFR Appendix B to Part 136. Washington (DC). https://www.govinfo.gov/app/details/CFR-2011-title40-vol23/CFR-2011-title40-vol23-part136-appB

(3) Susan Genualdi, Jessica Beekman, Katherine Carlos, Christine M. Fisher, Wendy Young, Lowri DeJager, and Timothy Begley, "Analysis of per- and poly-fluoroalkyl substances (PFAS) in processed foods from FDA's Total Diet Study", *Analytical and Bioanalytical Chemistry*, **2021**, https://doi.org/10.1007/s00216-021-03610-2.