Supplementary information for

Towards the development of a standardized method for extraction and analysis of PFAS in biological tissues

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S1. Interlaboratory Studies. van Leeuwen et al.[1] organized a 38 participant worldwide per- and polyfluoroalkyl substances (PFAS) interlaboratory study (ILS) targeting 13 analytes in a study standard mixture and multiple environmental and human matrices distributed to all participants. All environmental matrices except water were spiked with most of the target analytes, enabling assignment of known ("assigned") concentrations. Fractions of submitted data exceeding the study's acceptability threshold (z-score <|2|) for each distributed matrix decreased as follows: standard solution (76%) > human blood (67%) > human plasma (63%) > fish liver extract (55%) > surface water (31%) > fish tissue (17%).[1]

A follow-up ILS distributed surface water, fish fillet tissue, a shared standard solution to evaluate variabilities with participants' (21) in-house standards, and three separate solutions containing a total of nine isotopically-labeled (^{13}C or ^{2}H) PFAS.[2] The precision of the submitted data improved relative to the 2006 results. However, precision data for individual laboratories (mean %RSD for all analytes for a single lab; 12% for water and 6.8% for fish) were considerably better than the cumulative precision.[2] Additionally, mean reported values for some analytes fell below the spiked concentrations and the majority of minimum reported concentrations were approximately 50% of the spiked concentration, with a value of 34% for perfluorooctane sulfonate (PFOS).[2] Inefficient extraction was the likely cause of these low values, since losses during sample preparation should have been accounted for via surrogate addition.

Lindström et al.[3] coordinated an additional 15-participant ILS published in 2009 involving a shared study standard and two NIST human blood serum standard reference materials (SRMs; 1589a and 1957). A shared mass-labeled standard containing ^{13}C-labeled PFOS, perfluorooctanoic acid (PFOA), and perfluorononanoic acid (PFNA) was also distributed, and was used by some participants. SRM 1589a contains lower PFAS concentrations than 1957, and had fewer detects and higher uncertainties. Although improvements were realized relative to van Leeuwen et al.,[1] precision remained poor.[3] However, the SRM 1957 data exhibited greater precision than the study standard data, which highlights matrix cleanliness is not necessarily a predictor of method performance. Besides SRM 1957 data having higher precision than that of the study standard, an additional peculiar irregularity emerges when results from Lindström et al.[3] are compared to those of van Leeuwen et al..[2] Since these two studies examined different matrices, the data are not directly comparable. However, both exercises distributed a study reference standard that included three common mass-labeled compounds from the same manufacturer. Interestingly, participants performed better at accurately determining target analytes in the study reference standard in the van Leeuwen et al.[2] study despite avoiding the use of mass-labeled standards in their determinations. Participants in Lindström et al.[3] were allowed the liberty to choose their methods, which likely included mass-labelled standards, although the report is not explicit.

A subsequent six-participant ILS provided the first assigned perfluorinated alkyl acid (PFAA) SRM values for the two human blood serum (NIST SRMs 1957 and 1958) and two human milk SRMs (NIST SRMs 1953 and 1954).[4] SRMs 1954 and 1958

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were spiked with a suite of 172 organohalogen contaminants (no PFAAs) resulting in four SRMs from two initial samples, two spiked (SRMs 1954 and 1958), and two unspiked (SRMs 1953 and 1957). While multiple extraction and cleanup methods were employed by participating analysts, all participants utilized multiple mass-labelled standards spiked prior to extraction in order to quantify the target analytes.[4] In comparison with van Leeuwen et al.,[1] significant precision improvements were apparent for the human serum matrix (SRM 1957). Resultant ILS reference values were in good agreement with previously established consensus values.[3]

Seven laboratories participated in an ILS organized by Reiner et al.[5] lead to establishment of reference values for PFOS and information values for several other PFAAs for three NIST SRMs including 1946 and 1947 (Great Lakes fish tissue) and 1577c (bovine liver). NIST SRM 2974a (mussel tissue) was also distributed, but only PFOS data were reported by two of the three participating laboratories, and these values differed by 40%. Overall, there was good agreement among participants' submitted PFOS data (%RSDs of 3.65% and 6.61% for SRMs 1946 and 1947, respectively) with lower precision for the other analytes (%RSDs >15%). Reiner et al.[6] sought to establish PFAS reference values for less frequently analyzed abiotic matrices by organizing an eight-participant ILS. Poor agreement among the submitted data (analyte-specific %RSDs 10-165%) contrasted with the generally increasing trend in precision over time demonstrated by previously summarized ILSs. Internal standard calibration was universally applied among participants. However, there were inconsistencies in the internal standards used when a corresponding mass-labeled counterpart (i.e. perfluorobutane sulfonate (PFBS) in SRM 2585; house dust) was not commercially available. Furthermore, the use of one sample preparation procedure for all of the matrices was cited as an additional possible contributor to discrepancies in the submitted data.

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While precision generally improved over time, it is important to note that the ILSs that displayed increased precision also generally included fewer participants compared to studies reporting low precision. For instance, data for PFAS in fish muscle tissue was submitted by 27 and 15 participants in the 2006[1] and 2009[2] ILSs documented by van Leeuwen et al., respectively, with corresponding %RSD ranges of 65-236% and 22-47%. This contrasts with the relative errors of 3.7% and 6.6% among seven participants for the PFOS reference values in Great Lakes fish tissue SRMs 1946 and 1947, respectively, reported in 2012 by Reiner et al.[5] Additionally, the number of replicate analyses performed by individual participants was generally low (n=3-6), with a range of 2-26 for any single analytical method (some participants applied multiple methods).[4,5] Since specific guidelines regarding analyzing replicates in multiple batches and/or on multiple days are not supplied in the text of the cited ILSs, participants may have combined replicate analyses into few or even a single batch, which would also contribute to increased precision.

The reviewed ILSs highlight the potential for substantial variability during PFAS quantification, regardless of analyzed matrix. Application of well-defined native standards (purity and isomer composition), appropriate mass-labeled surrogate standards (ideally using isotope dilution), and use of cleanup procedures which adequately remove interferences from environmental sample matrices were commonly identified in these ILSs as keys to producing high quality, reproducible data.

Additionally, use of external calibration methods for PFAS quantitation was illadvised.[1] With the exception of native standard quality, the causes of inconsistency identified by the reviewed ILSs can be generally simplified as differential exchange of analytes between two steps in the analytical method. The availability of isotopically-labeled surrogates for most commonly analyzed PFAS enables the use of isotope dilution methods, which counteracts most of these complications. However, the following section highlights known instances of low and/or variable surrogate recoveries that likely contributed to poor precision of past PFAS analytical methods that necessarily applied a relatively narrow array of surrogates to a broad range of target analytes. Additionally, low and/or variable surrogate recoveries have potential to jeopardize QA/QC requirements in modern methods if unaddressed.

Table S1. Comparison of PFAS interlaboratory studies

Reference	Matrix	Spiked[a]	Participants	Precision[b]
van Leeuwen et al. (2006)[1]	Standard mixture	N/A	38; 13 countries	25-256 %RSD (PFHxS, PFNA)
				80-202% [analyte means; (in-house)/(shared)]
	Human plasma	X	17 submissions	32-64 %RSD (PFOS, PFHxS)
	Human whole blood	X		29-90 %RSD (PFHxS, PFOSA)
	Fish muscle	✓	27 submissions	65-236 %RSD (PFBS, PFNA)
	Fish liver extract	✓		37-202 %RSD (PFHxS, PFHpA)
	Surface water	X		47-250 %RSD (PFBS, PFHxS)
van Leeuwen et al. (2009)[2]	Standard mixture	N/A	21	95-105% [analyte means; (in-house)/(shared)]
	Fish muscle	✓	15 submissions	22-47 %RSD (PFDA, PFOSA)**
	Surface water	✓	17 submissions	16-69 %RSD (PFBA, PFOSA)**
Lindström et al. (2009)[3]	Standard mixture	N/A	15	44-101 %RSD (PFNA, PFDS)
	Human serum (SRM 1957)	X		5-133 %RSD (PFDS, PFDoA)
	Human serum (SRM 1589a)	X		14-139 %RSD (PFOS, PFDoA)
Keller et al. (2010)[4]	Human serum (SRM 1957)	X	6	7-26 %RSD (PFOS, PFDA)
	Human milk (SRM 1954)	X	3	19 %***(PFOS)
Reiner et al. (2012)[5]	Fish tissue (SRM 1946)	X	7	3.65% ***(PFOS only)
	Fish tissue (SRM 1947)	X	7	6.61%*** (PFOS only)
	Bovine liver (SRM 1577c)	X	3	23.8%*** (PFOS only)
	Mussel tissue (SRM 2974a)	X	2 submissions	40% difference (PFOS only)
Reiner et al. (2015)[6]	Sediment (SRM 1941b)	X	6	>60 %RSD (PFOS)
	Sediment (SRM 1944)	X	7	>60 %RSD (PFOS)
	House dust (SRM 2585)	X	3	<10-81 %RSD (PFOS, PFBS)
	Soil (SRM 2586)	X	6	32 %RSD (PFOS)
	Sludge (SRM 2781)	X	5	10-165 %RSD (~30% for PFOS)

[a]describes whether target analytes were added to the matrix prior to distribution to participants (✓ = yes, X = no)

[b]values represent pooled, unreduced data (n occasionally <3) unless otherwise specified. Analyte abbreviations in parentheses correspond to min, max values from cited reference, respectively

**based on solvent-based calibration curve quantification data (standard addition also performed)

*** calculated as (expanded uncertainty)/(reference or consensus value)*100

Table S2. Description of target analytes and mass-labeled standards used

Compound	Full name	Formula	Surrogate
	Native PFCAs		
PFBA	Perfluorobutanoic acid	$CF_3(CF_2)_2$ COOH	PFBA^{13}C
PFPeA	Perfluoropentanoic acid	$CF_3(CF_2)_3$ COOH	PFHxA^{13}C
PFHxA	Perfluorohexanoic acid	$CF_3(CF_2)_4$ COOH	PFHxA^{13}C
PFHpA	Perfluoroheptanoic acid	$CF_3(CF_2)_5$ COOH	PFOA^{13}C
PFOA	Perfluorooctanoic acid	$CF_3(CF_2)_6$ COOH	PFOA^{13}C
PFNA	Perfluorononanoic acid	$CF_3(CF_2)_7$ COOH	PFNA^{13}C
PFDA	Perfluorodecanoic acid	$CF_3(CF_2)_8$ COOH	PFDA^{13}C
PFUnA	Perfluoroundecanoic acid	$CF_3(CF_2)_9$ COOH	PFUnA^{13}C
PFDoA	Perfluorododecanoic acid	$CF_3(CF_2)_{10}$ COOH	PFDoA^{13}C
PFTrA	Perfluorotridecanoic acid	$CF_3(CF_2)_{11}$ COOH	PFDoA^{13}C
PFTeA	Perfluorotetradecanoic acid	$CF_3(CF_2)_{12}$ COOH	M2PFTeA
PFHxDA	Perfluorohexadecanoic acid	$CF_3(CF_2)_{14}$ COOH	M2PFHxDA
	Native PFSAs		
PFBS	Potassium perfluoro-1-butanesulfonate	$CF_3(CF_2)_3SO_3^-$	PFHxS^{18}O
PFHxS	Sodium perfluoro-1-hexanesulfonate	$CF_3(CF_2)_5SO_3$-	PFHxS^{18}O
PFOS	Sodium perfluoro-1-octanesulfonate	$CF_3(CF_2)_7SO_3^-$	PFOS^{13}C
PFDS	Sodium perfluoro-1-decanesulfonate	$CF_3(CF_2)_9SO_3^-$	PFOS^{13}C
	Mass-labeled PFCAs		
PFBA^{13}C	Perfluoro[1,2,3,4-$^{13}C_4$]butanoic acid		
PFHxA^{13}C	Perfluoro[1,2-$^{13}C_2$]hexanoic acid		
M5PFHxA*	Perfluoro[1,2,3,4,6-$^{13}C_5$]hexanoic acid		
PFOA^{13}C	Perfluoro[1,2,3,4-$^{13}C_4$]octanoic acid		
PFNA^{13}C	Perfluoro[1,2,3,4,5-$^{13}C_5$]nonanoic acid		
PFDA^{13}C	Perfluoro[1,2-$^{13}C_2$]decanoic acid		
PFUnA^{13}C	Perfluoro[1,2-$^{13}C_2$]undecanoic acid		
M7PFUnA*	Perfluoro[1,2,3,4,5,6,7-$^{13}C_7$]undecanoic acid		
PFDoA^{13}C	Perfluoro[1,2-$^{13}C_2$]dodecanoic acid		
M2PFTeA**	Perfluoro[1,2-$^{13}C_2$]tetradecanoic acid		
M2PFHxDA**	Perfluoro[1,2-$^{13}C_2$]hexadecanoic acid		
	Mass-labeled PFSAs		
PFHxS^{18}O	Sodium perfluoro-1-hexane[$^{18}O_2$]sulfonate		
PFOS^{13}C	Sodium perfluoro-1-[1,2,3,4-$^{13}C_4$]octanesulfonate		

*Used as UPLC injection standard

**Purchased as individual solution and added to mass-labeled mixture

Table S3. UPLC solvent gradient

Time (min)	Flow (mL/min)	%A	%B	T_2-T_1 Change
0.0	0.4	75	25	
1.0	0.4	75	25	
1.5	0.4	40	60	Linear
9.6	0.4	0	100	Linear
14	0.4	0	100	
14.5	0.4	75	25	Linear
20	0.4	75	25	

Table S4. Compound-specific instrumental parameters

Compound	Quantification transition (m/z)	Confirmation m/z	Retention time[a] (min)	RF lens (V)	Collision Energy[b] (V)
PFBA	213→169		3.05	30	10
PFBA^{13}C	217→172			30	10
PFPeA	263→219		3.57	30	10
PFBS	299→80.2	99.2, 169	3.60	115	50, 50, 25
PFHxA	313→269	119	4.13	40	10, 15
PFHxA^{13}C	315→270			40	10
M5PFHxA	318→273			35	10
PFHxS	399→80.2	99.2, 169.1	4.74	135	45, 35, 29
PFHxS^{18}O	403→103			135	30
PFHpA	363→319	169	4.80	45	15, 20
PFOA	413→369	169	5.54	50	10, 20
PFOA^{13}C	417→372			50	10
PFOS	498.9→80	99	6.15	160	40
PFOS^{13}C	503→99			160	40
PFNA	463→419	219	6.30	50	10, 20
PFNA^{13}C	468→423	422.986		50	10
PFDA	513→469	219	7.02	55	10, 20
PFDA^{13}C	515→470			55	10
PFDS	598.9→80.3	99.1, 230.1	7.52	170	60, 60, 50
PFUnA	563→519	169	7.71	62	20, 25
PFUnA^{13}C	565→520			62	10
M7PFUnA	570→525			62	10
PFDoA	613→569	169	8.35	67	15, 25
PFDoA^{13}C	615→570			67	10
PFTrA	663→619	169	8.95	50	15, 25
PFTeA	713→669	219	9.49	75	15, 25
M2PFTeA	715→670			75	15
PFHxDA	813→769	219	10.51	50	15
M2PFHxDA	815→770			50	15

[a] Identical values for native and mass-labeled analogs

[b] Multiple values correspond to m/z values listed left to right starting with the quantification transition

Table S5. Summary of surrogate recoveries obtained using SOP 10.4

	Tissue samples n=300			Method blanks n=26		
Compound	Mean	St. dev.	%RSD	Mean	St. dev.	%RSD
PFBA^{13}C	72%	13%	18%	87%	12%	13%
PFHxA^{13}C	83%	14%	17%	87%	12%	13%
PFHxS^{18}O	92%	11%	12%	94%	9%	9%
PFOA^{13}C	78%	20%	26%	91%	12%	14%
PFOS^{13}C	93%	12%	13%	92%	8%	9%
PFNA^{13}C	83%	8%	9%	90%	7%	7%
PFDA^{13}C	83%	8%	9%	89%	6%	7%
PFUnA^{13}C	89%	7%	8%	90%	7%	8%
PFDoA13C	88%	11%	13%	87%	8%	9%
M2PFTeA	93%	18%	19%	79%	9%	11%
M2PFHxDA	96%	21%	22%	60%	13%	22%
M7PFUnA[a]	97%	11%	12%	97%	9%	9%
Overall mean[b]:	86%	13%	15%	86%	9%	11%

[a] used as UPLC injection standard

[b] overall means exclude M7PFUnA value

Extracted species for which the surrogate recoveries listed in Table S5 were obtained include:
lake trout (*Salvelinus namaycush*), walleye (*Sander vitreus*), spottail shiner (*Notropis hudsonius*), emerald shiner (*Notropis atherinoides*), yellow perch (*Perca flavescens*), white perch (*Morone Americana*), trout perch (*Percopsis omiscomaycus*), round goby (*Neogobius melanostomus*), gizzard shad (*Dorosoma cepedianum*), rainbow smelt (*Osmerus mordax*), slimy sculpin (*Cottus cognatus*), deepwater sculpin (*Myoxocephalus thompsonii*), bloater (*Coregonus hoyi*), alewife (*Alosa pseudoharengus*), *Mysis* (*Mysis diluviana*), and bulk zooplankton and phytoplankton homogenates.

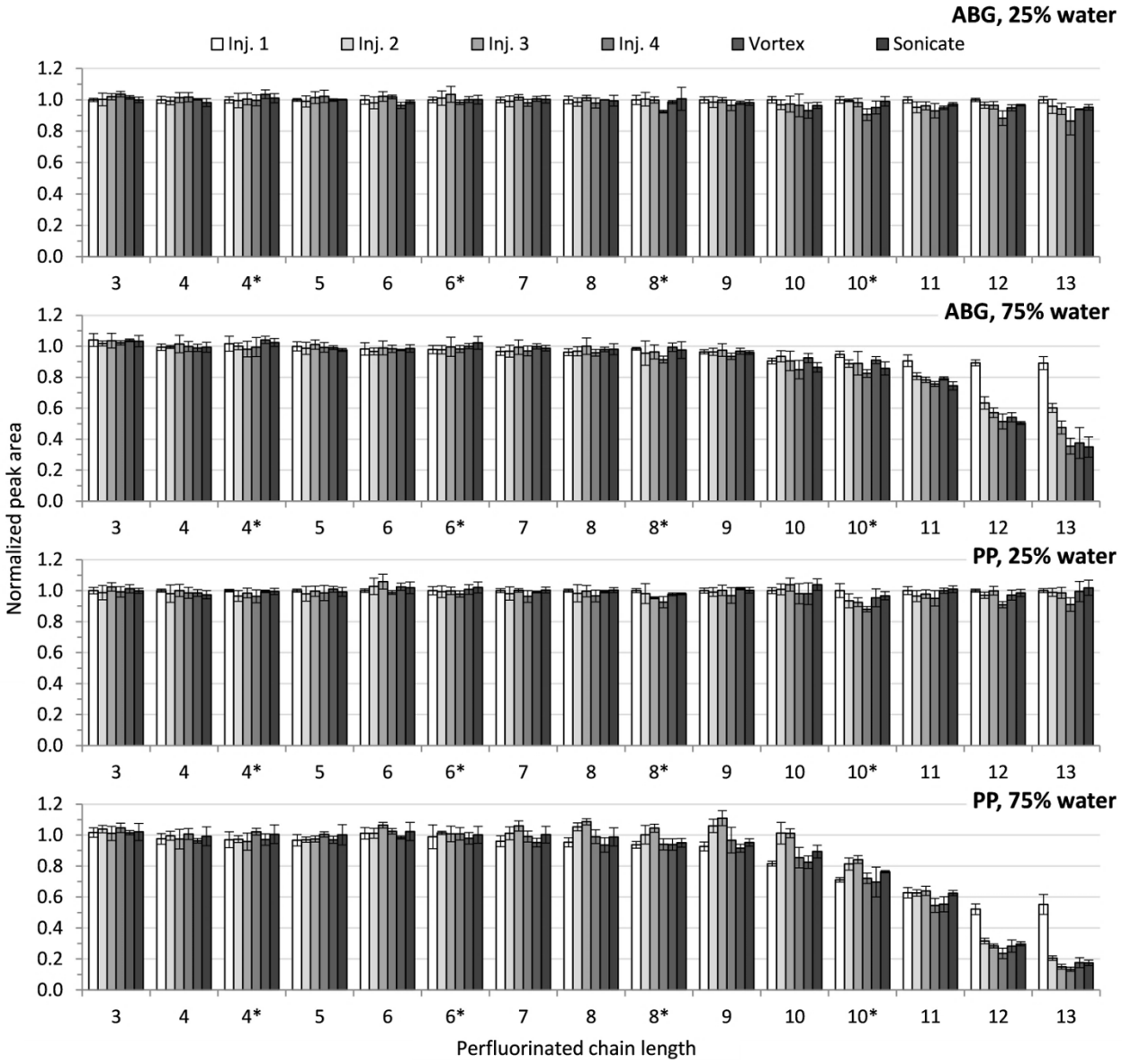

Figure S1. Mean (n=3) normalized peak areas vs. PFAA perfluorinated chain length for each vial material and solvent composition tested. * indicates PFSA. Bars represent sequential injections from day one (Inj. 1-3), day two (Inj. 4), day 2 after vortexing for 2 min (Vortex), and day 2 after sonicating for 2 min (Sonicate). For each vial type, plotted values for each compound are normalized to the mean of the first injections from the solutions containing 25% water. Error bars represent ± std. dev.

S2. Ionization Suppression: Preliminary data suggested that low apparent surrogate recovery for the isotopically labeled surrogate used for PFOA ($PFOA^{13}C$) was likely due to ionization suppression. To test this hypothesis, several matrix spikes were processed alongside samples to test for ionization suppression and/or enhancement during MS analysis. For these tests, a duplicate of one sample in the batch being processed was extracted following SOP 10.4 and spiked with the surrogate mixture and injection standard immediately prior to instrumental analysis. Lake trout (*Salvelinus namaycush*) tissue was used for these tests because lake trout is the predominant species extracted using SOP 10.4. The results of these matrix spikes are provided in Table S6. Note that only one of three matrix blanks (matrix blank 2) yielded low $PFOA^{13}C$ recovery. This is due to the quantity of coextracted matrix interference present. Based on the evidence presented in Figures S2 and S3, this matrix interference is assumed to be taurocholic acid. The sodium salt of taurocholic acid is the primary component of carnivorous animals' bile, and functions as a facilitator of intestinal and liver transport of fats and sterols.[7] Taurocholic acid possesses structural properties similar to that of PFAAs, allowing it to be carried through the weak anion exchange solid phase extraction procedure. Presumably, the variability of this interference's concentration is due to some degree of heterogeneity among tissue homogenates. During full scan MS analysis, this matrix interference is clearly visible as a large hump in the total ion chromatogram which elutes from the LC column starting very near PFOA, and is present in nearly all biological tissue samples extracted using SOP 10.4. However, only when this species is extracted in sufficient quantity does its chromatographic peak overlap with that of PFOA and cause suppression. This result is demonstrated in Figure S2. Peaks in panels A and B (corresponding to matrix blank 1 from Table S6) do not overlap significantly, and no matrix suppression was observed. However, peaks in panels C and D (corresponding to matrix blank 2 from Table S6) overlap and matrix suppression was observed. It should be noted that PFOA is not expected to bioaccumulate in fish due to rapid elimination,[8] and is generally not present above the detection limit in the majority of species that have been analyzed following SOP 10.4 to date. Additionally, isotope dilution is utilized for PFOA quantification in SOP 10.4, which should negate the influence of ionization suppression.

Table S6. Summary of matrix suppression/enhancement encountered using SOP 10.4

Compound	Matrix blank 1	Matrix blank 2	Matrix blank 3
$PFBA^{13}C$	92%	98%	89%
$PFHxA^{13}C$	109%	117%	109%
$PFHxS^{18}O$	91%	93%	90%
$PFOA^{13}C$	113%	54%	103%
$PFOS^{13}C$	105%	91%	90%
$PFNA^{13}C$	99%	97%	92%
$PFDA^{13}C$	98%	101%	99%
$PFUnA^{13}C$	100%	98%	98%
PFDoA13C	98%	94%	96%
M2PFTeA	88%	99%	111%
M2PFHxDA	105%	112%	113%
M7PFUnA[a]	102%	105%	117%

[a] used as UPLC injection standard

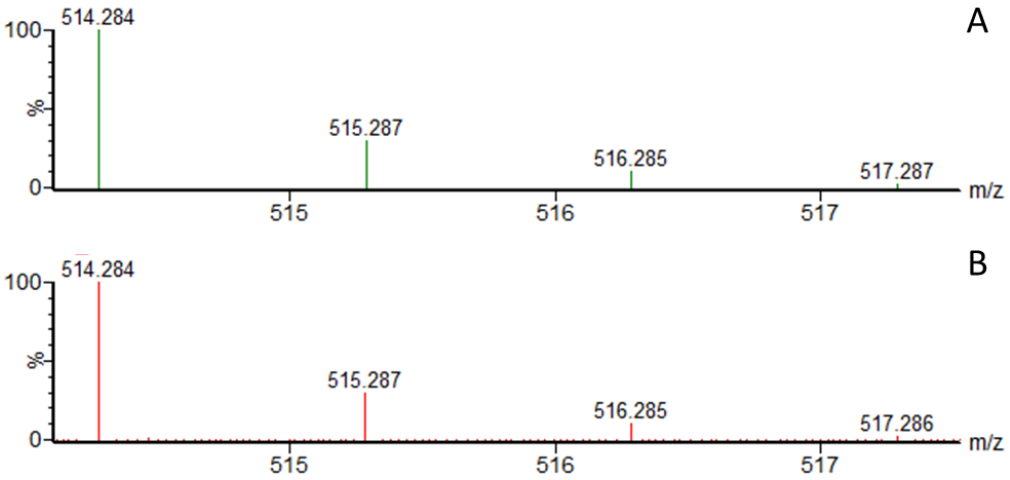

Figure S2. Comparison of theoretical isotope model for taurocholic acid ($C_{26}H_{44}NO_7S$; panel A) and mass spectrum extracted for the chromatographic peak shown in Figure S2, panel D (panel B).

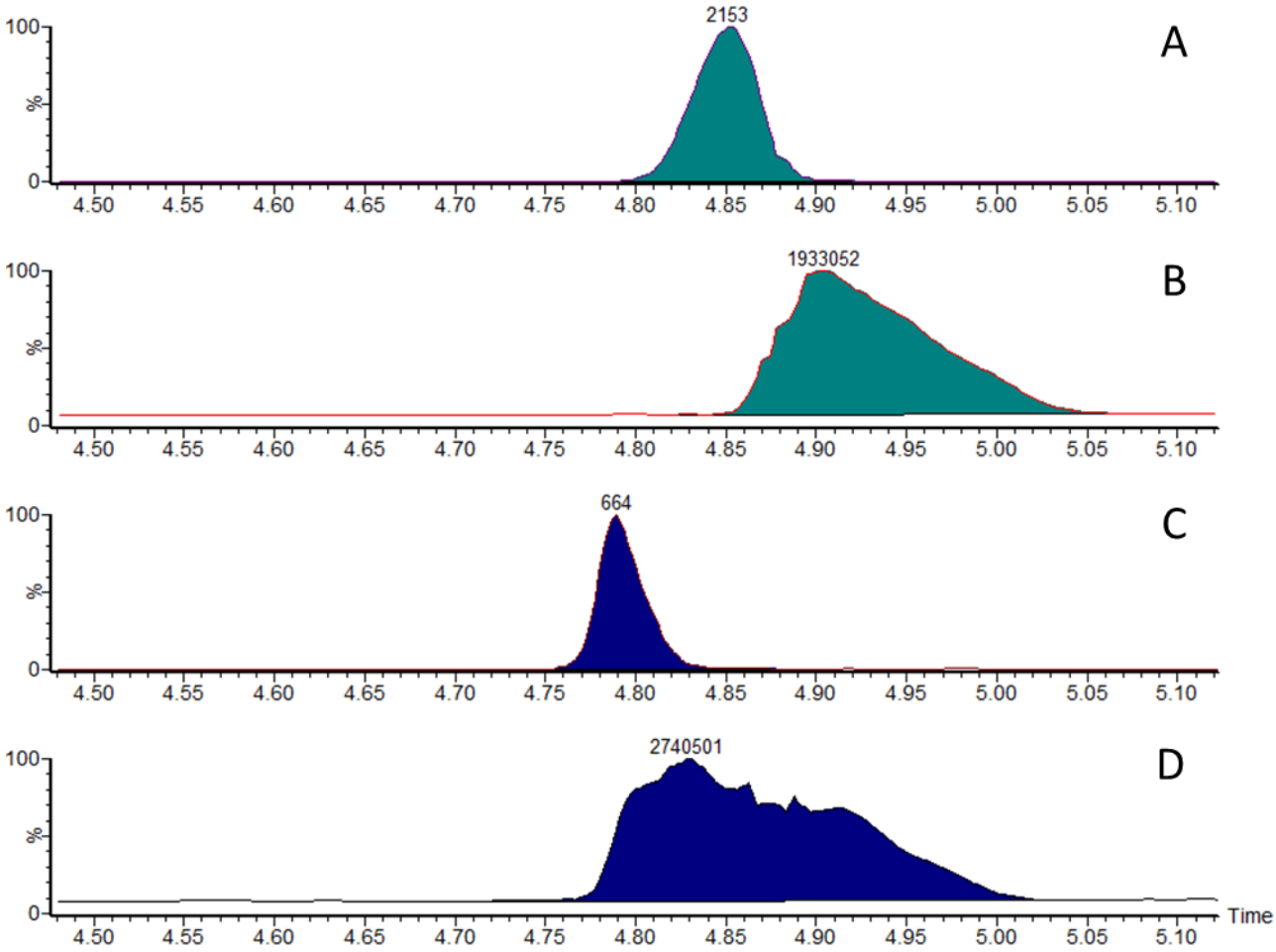

Figure S3. Comparison of peak areas (displayed above each peak) for PFOA^{13}C (panels A and C) and total ion chromatograms (panels B and D) for two matrix blanks processed following SOP 10.4. Panels A and B correspond to matrix blank 1 in Table S6, and panels C and D correspond to matrix blank 2. These matrix blanks were extracts of two different whole lake trout homogenates spiked with surrogate immediately prior to UPLC-MS analysis. Taurocholic acid (mass spectrum available in Figure S1, panel B) contributed virtually the entire signal integrated in the peaks displayed in panels B and D.

Table S7. Reproducibility of duplicate sample analyses using SOP 10.4 (n=16)

Compound	Mean % diff.	95% CI
PFHxS	6.9	3.5 – 10.3
PFNA	9.0	5.9 – 12.0
PFOS	6.1	3.5 – 8.7
PFDS	6.5	2.5 – 10.5
PFDA	7.3	4.5 – 10.2
PFUnA	5.0	3.4 – 6.6
PFDoA	7.1	4.3 – 10.0
PFTrA	10.5	6.4 – 14.6
PFTeA	6.4	3.1 – 9.7

Table S8. Summary of PFAS quantified in NIST SRM 1947 using SOP 10.4 (n=18)

Compound	Reference concentration (ng/g w.w)	**Mean concentration**; *st. dev.* (ng/g w.w.)	%RSD	Mean recovery
PFNA	0.20	**0.29**; *0.03*	10%	143%
PFOS	5.90 ± 0.39	**8.17**; *0.85*	10%	139%
PFDA	0.26	**0.30**; *0.03*	11%	116%
PFUnA	0.28	**0.39**; *0.04*	9%	140%
PFTrA	0.20	**0.32**; *0.05*	15%	161%

References

1 S. P. van Leeuwen, A. Kärrman, B. van Bavel, J. de Boer and G. Lindström, *Environmental science & technology*, 2006, **40**, 7854–7860.

2 S. P. J. van Leeuwen, C. P. Swart, I. van der Veen and J. de Boer, *Journal of Chromatography A*, 2009, **1216**, 401–409.

3 G. Lindström, A. Kärrman and B. van Bavel, *Journal of Chromatography A*, 2009, **1216**, 394–400.

4 J. M. Keller, A. M. Calafat, K. Kato, M. E. Ellefson, W. K. Reagen, M. Strynar, S. O'Connell, C. M. Butt, S. A. Mabury, J. Small, D. C. G. Muir, S. D. Leigh and M. M. Schantz, *Analytical and Bioanalytical Chemistry*, 2010, **397**, 439–451.

5 J. L. Reiner, S. G. O'Connell, C. M. Butt, S. A. Mabury, J. M. Small, A. O. De Silva, D. C. G. Muir, A. D. Delinsky, M. J. Strynar, A. B. Lindstrom, W. K. Reagen, M. Malinsky, S. Schäfer, C. J. A. F. Kwadijk, M. M. Schantz and J. M. Keller, *Analytical and Bioanalytical Chemistry*, 2012, **404**, 2683–2692.

6 J. L. Reiner, A. C. Blaine, C. P. Higgins, C. Huset, T. M. Jenkins, C. J. A. F. Kwadijk, C. C. Lange, D. C. G. Muir, W. K. Reagen, C. Rich, J. M. Small, M. J. Strynar, J. W. Washington, H. Yoo and J. M. Keller, *Analytical and Bioanalytical Chemistry*, 2015, **407**, 2975–2983.

7 National Center for Biotechnology Information. PubChem Database., Taurocholic acid, https://pubchem.ncbi.nlm.nih.gov/compound/6675, (accessed August 15, 2019).

8 D. M. Consoer, A. D. Hoffman, P. N. Fitzsimmons, P. A. Kosian and J. W. Nichols, *Aquatic Toxicology*, 2014, **156**, 65–73.

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Method for the Determination of Classes of Perfluoroalkyl Substances (PFASs), Perfluorinated Carboxylic Acids (PFCAs), Perfluorinated Sulfonic Acids (PFSAs), and Perfluoroalkyl Sulfonamides (FASAs), in Liver, Egg and Serum of Wildlife Species
Method Number: MET-OCRL-EWHD-PFC-Version 4-August 2014
Environmental Chemistry/Organic Contaminants Research Laboratory (OCRL)
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The above cited method was obtained through correspondence with Robert Letcher during the initial phases of method development/validation, and was modified in order to improve performance and/or adapt to materials and resources available.

Purpose:

This procedure outlines the method used to extract perfluoroalkyl acids (PFAAs) from homogenized whole fish tissue and invertebrate samples using an acidic acetonitrile extraction solvent combined with ultrasonication followed by solid phase extraction (SPE) cleanup using a weak anion exchange (WAX) sorbent. This procedure can also be used to analyze samples for fluorotelomer alcohols (FTOHs) and perfluorinated sulfonamides (FOSAs), but these analytes are not currently analyzed in tissue extracts (see step III.II.4).

Read this SOP in its entirety prior to processing any samples.

Materials Needed:

Oasis WAX 3 cc Vac Cartridge, 60 mg Sorbent, 30 µm Particle Size, 100/pk – Waters PN: 186002490
Homogenized fish tissue stored at -20°C in tightly sealed PTFE-free container
Standard reference material 1947 (NIST)
Mass-labeled (^{13}C) PFAA internal standard (IS) mixture (MPFAC-MXA) – Wellington Laboratories
Individual mass-labeled (^{13}C) PFAA standards (M2PFTeDA and M2PFHxDA) – Wellington Laboratories
Individual mass-labeled (^{13}C) PFAA injection standard (M7PFUdA) – Wellington Laboratories
Individual mass-labeled (^{13}C) PFAA injection standard (M5PFHxA) – Wellington Laboratories
PFAA native standard mixture (PFAC-MXB) – Wellington Laboratories
Polypropylene (PP) centrifuge tubes (15 mL) – for extraction process - Fisher (Evergreen) PN: 05-558-33C
1 L glass vacuum flask (for drying invertebrate samples)
Thermo Scientific™ Nalgene™ Polysulfone Filter Holder with Funnel - Fisher PN: 09-745 (for drying invertebrate samples)
Grade 41 ashless filter paper circles (Whatman, PN: 1441-047)
47 mm pitri dish – PALL Corporation (for containing filters when drying invertebrate samples)

Optima LC-MS grade methanol (MeOH) – Fisher PN: A4564
Optima LC-MS grade water – Fisher PN: W64
Optima LC-MS grade acetonitrile (ACN) – Fisher PN: A955-4
Formic acid (98-100%) – Sigma Aldrich PN: 27001-500ML-R
Screw cap borosilicate glass media storage bottles – Fisher PN: 02542355 - for storing solvents
100, 1000, and 5000 µL Eppendorf-style pipettors – for spiking standards and transferring solvents
PP Eppendorf® epT.I.P.S. pipette tips – Fisher
- 2-200 µL – PN: 0540341
- 50-1000 µL – PN: 0540343
- 0.1-5 mL – PN: 05-403-62

PP autosampler vials (ASVs) (700 µL) – Waters PN: 186005219
Septa-less polyethylene ASV screw caps – Waters PN: 186004169
Amber glass ASVs (2 mL) – Agilent Technologies PN: 5182-0716
Double-ended micro-tapered stainless steel spatula – Fisher PN: 21-401-10

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Ultra-high purity nitrogen gas – Airgas (for sample concentration)

Equipment:

Analytical balance
Biologics model 150VT ultrasonic homogenizer – Biologics, Inc.
    Titanium microtip – Biologics PN: 0-120-0005
Centrifuge – Beckman Coulter Allegra™ 25R – Cat No 369434
SPE vacuum manifold equipped for use with small volume vacuum cartridges
Sample concentrator – Zymark TurboVap® LV concentration workstation or similar equipped with rack to accommodate 15 mL
PP centrifuge tubes

Instrumentation:

Waters® Acquity ultra-performance liquid chromatograph (UPLC®) (Waters Corp., Milford, MA)
    PFAS analysis kit (Waters Corp., PN 176001744)
LC isolator column (Waters Corp., PN 186004476)
Acquity UPLC® HSS T3 analytical column (Waters Corp., 2.1 x 100mm, 1.8 µm, PN
    186003539)
Xevo G2-XS quadrupole time-of-flight (QToF) mass spectrometer (Waters Corp.)

I. Solvent and Surrogate Preparation

I.I. Solid-liquid extraction:

1. Acetonitrile + 0.2 % formic acid.....6 mL/sample
    a. 200 µL formic acid per 100 mL
2. MeOH .....4 mL/sample to clean sonicator tip
    + 3x 50 mL PP cent. tubes full for rinsing tip
3. ACN.....50 mL tube full for rinsing sonicator tip

I.II. SPE:

1. 2% aq. formic acid .....1 mL/sample
    a. 2 mL formic acid per 100 mL
2. 1% NH_4OH in MeOH .....6 mL/sample
    a. 1 mL 28% NH_4OH per 100 mL
3. Water .....~13 mL/sample
4. MeOH .....5 mL per sample

Table 1. Quick reference guide for solvent preparation

Solvent	Amount per batch of 12 samples
Extraction	
Methanol	Three 50 mL tubes + 100 mL for rinsing
Acetonitrile	Two 50 mL tubes
Acetonitrile + 0.2% formic acid	80 mL + 160 µL formic acid
SPE	
Water + 2% formic acid	20 mL water + 400 µL formic acid
Water	175 mL
Methanol	75 mL
Methanol + 1% ammonium hydroxide	80 mL + 800 µL ammonium hydroxide

Table 2. Quick reference guide for surrogate preparation for 24 samples (2 batches)

Methanol	2,000 ng/mL C4-C12 surrogate	1,000 ng/mL C14 + C16 surrogate
1408 µL (704 µL x2)	64 µL	128 µL

II. Sample Extraction

1. Defrost samples at room temperature immediately prior to extraction (0.5-4 hrs depending on sample mass) or in refrigerator overnight
2. Obtain clean stainless steel spatulas
    a. Can use each end of a spatula for different samples as long as the unused end does not touch any surface (including your glove) during mixing and weighing of the first sample
    a. Follow cleaning procedure applicable for trace contaminant analysis. Rinse with methanol immediately before use and allow spatula to air dry before touching sample
        i. To dry multiple spatulas at a time, two pieces of methanol rinsed aluminum foil can be folded into triangular strip and laid on the bench top parallel to each other approximately 1-2 inches apart. Place the methanol rinsed spatulas across these pieces of foil to keep them from touching the bench top or other surface while drying.
        ii. These foil drying strips can be placed in a Ziploc bag and reused
3. Obtain two 15 mL PP centrifuge tubes per sample and label 2 tubes for each sample ID
4. Rinse one set of these tubes with methanol by using a 5 mL pipettor to add approximately 1 mL of methanol to each tube, then cap the tube, shake, and discard the methanol
    a. Rinsing with methanol cleans tube and helps keep tissue from sticking to walls
5. Remove as much methanol from the tube as possible prior to weighing samples

II.I Invertebrate samples

Perform the following steps for preparing invertebrate samples (plankton, insect larvae, *Mysis*, amphipods, oligochaetes, etc.)

Invertebrate samples are normally frozen with excess water in the sample jar. This water must be removed by following the steps below prior to weighing the sample

1. Obtain thawed samples to be extracted

2. Ottawa sand should be used as a method blank for invertebrate samples in order to measure any background that may transfer to a sample during the filtering process. To prepare the Ottawa sand:
    a. Transfer several grams of Ottawa sand to a 50 mL PP centrifuge tube, add enough methanol to submerge the sand, sonicate for 5 minutes
    b. Pour the methanol into a waste container and wash the sand several times with LCMS water to rinse off most of the remaining methanol
    c. Pour enough LCMS water into the tube to submerge the sand, sonicate for 5 min
    d. Pour off the excess water, and then add more LCMS water to the tube to submerge the sand
3. Obtain 1 L glass filter flask, polysulfone funnel and filter holder plate, silicone stopper gasket (gasket is placed at the opening of the flask to seal around the funnel), and piece of tubing to connect the filter flask to the fume hood vacuum
    e. The polysulfone filter holder plate, funnel, and gasket should be stored in Ziploc bags to keep dust off
4. If the filter funnel and flask have not been used recently, rinse all surfaces that will contact the sample (inside of flask and funnel and filter holder) 3 times with methanol prior to use. A methanol squirt bottle made of non-fluorinated plastic can be used for this or a 5 mL pipettor
    a. The filter holder plate should be removed from the funnel and wiped thoroughly with a methanol-wetted kimwipe prior to rinsing with methanol to ensure removal of more stubborn contamination (e.g. dust or residue from previous samples if not cleaned properly before storage)
    b. If these parts are suspected to be severely contaminated, after wiping with methanol-wetted kimwipe and rinsing with methanol to remove most of the contamination, place components in a methanol-rinsed beaker, submerge with methanol, and sonicate for 10 minutes.
5. Obtain petri dishes (at least 1 per sample)
6. Obtain 47 mm ashless filters listed in the materials section
7. Use clean (rinse with methanol) forceps to transfer one filter to a pitri dish from step 5 above
8. Use a pipettor to transfer enough methanol to the dish to fully cover the filter
9. Soak the filter for approximately 1 minute. Swirl the dish occasionally to mix the methanol
10. Insert the gasket into the mouth of the clean filter flask and then insert the bottom half of the funnel and place the filter holder plate on top.
    f. Ensure the black rubber gasket is installed on the bottom side of the filter plate
11. Use forceps to remove the filter from the soaking dish and place it on the filter holder plate, making sure the filter is centered and covers all of the holes
12. Turn on the fume hood vacuum by loosening the knob until you hear a hissing sound
13. Allow vacuum to dry the filter of any methanol from soaking
14. Obtain a sample and mix it well
    g. Larger invertebrates like *Mysis* can be mixed using a clean stainless steel spatula
    h. Smaller invertebrates such as plankton are usually suspended in water and can be mixed by swirling the jar in a circular motion
15. Carefully transfer approximately 0.5 g of sample or prepared Ottawa sand (blank) onto the filter
    a. Larger invertebrates like *Mysis* can be transferred using a stainless steel spatula
    b. Smaller invertebrates such as plankton that are suspended in water can be transferred using a 5 mL pipettor. Slowly dispense the sample onto the filter while a small amount of vacuum is used to drain excess water into the filter flask
16. When all of the sample volume has been dried, turn off the vacuum and use forceps to transfer the filter with sample on it to a new petri dish labeled with the sample ID. Place the top on the petri dish to keep the sample covered until weighing
17. Large invertebrates like insect larvae or *Mysis* have hard exteriors that are not easily broken down by the homogenizer during extraction. These samples should be ground with a stainless steel mortar and pestle prior to weighing. Small invertebrates or those without hard exteriors like plankton do not need to be ground and can be weighed after drying
    a. Obtain stainless steel mortar and pestle
    b. If not used recently, follow the procedure for washing equipment for trace organic analysis to wash the mortar and pestle
    c. Rinse the mortar and pestle 3 times with methanol (use a squirt bottle or pipettor)
    d. Allow methanol to air dry before grinding a sample
    e. Use the same spatula as used to transfer the sample to the filter to transfer the dried sample from the filter to the mortar
    f. Grind the sample until well homogenized into a paste
    g. The sample is now ready to be weighed
    h. Repeat this cleaning procedure after each sample
    i. If samples requiring grinding are analyzed, also transfer the dried Ottawa sand to the cleaned mortar prior to weighing
18. Before filtering the next sample, wet a kimwipe with methanol and wipe off the side of the filter plate that contacted the filter and sample and rinse the filter flask and funnel assembly 3 times with methanol as described above

II.II Homogenized Fish Tissue and/or Prepared Invertebrate Samples

6. Mix sample well with clean, methanol-rinsed stainless steel spatula (or reuse the spatula if used to transfer invertebrate sample)
7. Place a tall glass jar on the scale. This will hold the 15 mL tube upright when weighing a sample
8. Place an uncapped, methanol rinsed, labeled 15 mL PP centrifuge tube into the glass jar
9. Tare (zero) the scale
10. Transfer approximately 0.5 g of sample into the tared 15 mL PP centrifuge tube. Try not to let tissue touch the walls of the tube because it will stick and is difficult to get back off
    b. For invertebrate samples that were transferred to the filter using a spatula, use the same spatula for transferring sample to 15 mL tube
    c. After transferring ~0.5 g of tissue into the tube, use the spatula to scrape most of the tissue stuck on the walls of the tube down towards the bottom of the tube
    d. When done transferring tissue and scraping off the tube wall, wipe the end of the spatula off with a kimwipe and use the other end of the spatula for the next sample (as long as the other end did not contact any surface while transferring tissue with the first end)
    e. Weigh approximately 1 g of SRM 1947 (NIST)
        iii. Note SRM mass is greater than sample mass because PFAA concentrations in SRM are low (~0.2 ng/g)
11. Spike 50 µL 80 ng/mL IS mixture (C_4-C_{16}, 4 ng of each compound) on top of tissue
    a. For blanks, add 3 mL of extraction solvent to tube prior to spiking standard
12. Add 3 mL 0.2% formic acid in acetonitrile, tip/rotate tube to rinse down any surrogate spike
13. Obtain one additional 15 mL PP centrifuge tube per sample
    b. Label with permanent ink and record weight (tube + cap) of each tube
14. Extract samples using ultrasonic homogenizer as follows:
    c. Set the homogenizer settings to: 50% pulse time, 20% power (will need to have homogenizer on and running to adjust power based on neon green light bar display)
        i. **DO NOT exceed 50% power or tip will be damaged**
    d. Remove tube cap and use homogenizer tip to scrape any tissue residue down the side of the tube wall into the extraction solvent
    e. Insert the tip about ~1/4 inch below liquid surface and press the start button
    f. Sonicate for minimum of 5 pulses
        i. The sonicator should make minimal noise and there should be considerable turbulence in the liquid when the tip is properly positioned. Experiment with tip position to achieve this and count at least 5 "good" pulses
15. Remove as much tissue residue from sonicator tip as possible before removing it from the sample tube
    g. The best way to do this is to insert the tip until it almost touches the bottom of the tube, then tilt/shake the tube back and forth. Then push the tip down until it contacts the bottom of the tube and turn the power on for one pulse while pulling the tip away from the bottom of the tube about 1/4 inch. This will vibrate the tip, but not cause enough turbulence in the solvent to wash tissue back up onto the tube walls.
16. Clean the homogenizer tip between each sample as follows:
    h. Fold a small kimwipe in half twice in same direction, wet with 1 mL MeOH, and wipe tip thoroughly from starting from the top, wide portion downwards towards the tip to remove tissue residue. Fold the kimwipe in half prior to each wipe to avoid transferring material from kimwipe back onto tip
    i. Place waste container under tip and use a pipette to rinse the rip with 1 mL of MeOH by dispensing starting at the top, wide portion of the tip and allowing it to run downwards into the waste container
    j. Immerse sonicator tip into one 50 mL PP centrifuge tube filled with MeOH
        i. Turn pulse function off, press start, sonicate for approximately 5 sec while moving the tube vertically along the length of the tip to keep the tip exposed to fresh solvent. Do not raise the end of the tip above the solvent surface.
    k. Repeat step 9. c. with a second tube of MeOH followed by one tube of ACN
17. Centrifuge sonicated sample 4 min., 3500 RCF
18. Transfer supernatant to new, weighed (tube + cap), labeled 15 mL PP tube
19. Repeat above steps 4-9 once more
    l. **Loosen pellet from bottom of tube prior to turning homogenizer on.** This can be accomplished by holding the tube in one hand and the wide portion of the sonicator probe with the other for stability. Then insert the tip down the wall of the tube and firmly press down into the tissue pellet. Then use a prying motion to loosen the pellet from the conical tube end.
    m. The pellet can be manually broken up a bit by squishing the tissue against the side wall of the tube before activating sonicator to aid in homogenization
20. Centrifuge (5 min, 5000 RCF), combine supernatants for each respective sample
21. Record weight of tube + cap + combined supernatants

22. Immediately prior to SPE, mix combined supernatants well by vortexing 1 min, 500 rpm and sonication in a bath sonicator for 5 minutes. Centrifuge 5 min, 5525 RCF to settle any solids

III. SPE

If using a 12 port manifold, this can be done in batches of 6 samples for easier regulation of solvent flow through the SPE cartridges. Prior to beginning SPE, each port on the manifold should be disassembled and cleaned by sonicating in methanol. This eliminates carryover contamination from previous batches found to occur if components are simply rinsed with methanol.

III.I. Cleaning SPE Manifold

1. When not in use, cover the SPE manifold with a large Ziploc bag to keep dust off
2. Obtain a cleaned glass beaker large enough to contain all SPE manifold components (≥200 mL). Rinse 3 times with methanol using solvent from media storage bottle or pour straight from 4 L stock bottle (avoid squirt bottles if possible due to higher risk of contamination).
3. Remove bag dust cover from manifold and remove each of the plastic stopcocks by grabbing it and pulling straight up while twisting
4. Disassemble each stopcock by holding the piece that accepts the cartridge with one hand and pulling the rotating cylinder-shaped portion straight out while twisting with the other hand
5. Place disassembled stopcocks into rinsed beaker
6. Remove lid from SPE manifold. Loosen and remove each plastic retaining nut for each port; place in rinsed beaker
7. Replace manifold lid on glass housing and unscrew each port's threaded adapter from top of lid; place into beaker
    a. If not removed with the stopcock, remove the stainless steel solvent guide needles from each threaded adapter by tapping lightly on a hard, clean surface (e.g. top of SPE manifold lid)
8. With all components removed from manifold lid and inside rinsed beaker, fill beaker with enough methanol (from 4 L stock bottle) to submerge all components
9. Sonicate for 10 minutes in bath sonicator
10. Obtain shallow non-fluorinated plastic (e.g. HDPE or PP) container such as a food storage container with lid and rinse inside surfaces 3 times with methanol
    a. This container will hold SPE components once sonicated so they can be more easily grabbed and assembled
    b. If this container is visibly dirty or has not been used for this purpose for a long time, wet a kimwipe with methanol and wipe inner surfaces well to clean prior to rinsing.
    c. After use, this container should be left in a fume hood until residual methanol is evaporated and then covered and stored in a drawer or cabinet (keeps dust off).
11. After sonicating, pour excess methanol into waste container
    a. Put on new, clean pair of gloves and hold components from falling out of beaker while pouring trying to only touch portions of components which don't contact sample
12. Dump components into methanol-rinsed plastic container
13. Reassemble all components in reverse order as disassembly (threaded fitting, nut, stainless needle, stopcock)

III.II. SPE cleanup

Unless otherwise stated, "dry" means apply enough vacuum (~5 in Hg) to remove the majority of solvent retained in the sorbent until dripping stops and solvent sputters/splashes from stainless steel needle

If sample extracts were stored in the refrigerator after extraction, warm them to room temperature and mix them (step 15 in section II Sample Extraction)

Whenever new cartridges are ordered, and the LOT number or sorbent batch number is different from previously used cartridges, they should be tested for abnormally high background levels. To do this, follow step 1.a. below and collect 3 mL of MeOH + 1% NH4OH eluate in a 15 mL PP tube. Collect an additional 1 mL of MeOH + 1% NH4OH eluate in a second tube to ensure the first 3 mL completely cleaned the cartridge. Analyze both of these eluates by UPLC-MS. Typical background levels are <1 ng, but higher levels (30 ng PFOA) have been observed. If high background is detected, contact Waters for replacement of product.

1. Precondition SPE cartridges (2 mL/min or ~1 drop/s):
    a. 1% NH_4OH in MeOH (3 mL)
        i. Load 3 mL of 1% NH_4OH in MeOH to each cartridge
        ii. Allow approximately half of the 3 mL to drain (1 drop/s), then close stopcock
        iii. Soak for 2 minutes
        iv. Open stopcock and drain remaining solvent until dripping stops, dry the cartridge
    b. Methanol (3 mL, dry)
    c. Water (3 mL, **DO NOT dry, keep meniscus just above sorbent**)
2. Load sample onto the cartridge (~1 drop/s):
    a. First, add 2.62 mL water to head of cartridge
    b. Add 0.5 mL sample using the following technique:
        i. Insert the pipette tip (containing sample) into the previously loaded water and dispense approximately half from the pipettor as close to the SPE sorbent as possible without overflowing the top of the cartridge. Then withdraw the tip up towards the surface and dispense the rest of the sample. Sample extracts often appear cloudy when they mix with water in the cartridge. If mixed properly, the liquid should be cloudy from top to bottom. If sample is dispensed too slowly, the sample will not mix with the water and will appear to float, and the top layer will look cloudy and the bottom will be clear.
    c. Once all samples are loaded, open the stopcocks and drain until the liquid is just above the sorbent.
    d. Repeat step 2 two more times (load 1.5 mL of each sample)
    e. Dry the cartridge
3. Wash Cartridge:
    a. 2% aqueous formic acid (1 mL, dry)
    b. Water (2 x1 mL, keep meniscus above sorbent until second mL has drained)
    c. **Dry for several minutes at 20" Hg until entire sorbent bed appears visibly dry based on color change to lighter color**
4. Elute fraction 1 (contains neutral FTOHs and FOSAs) – discard (collect if targeting these analytes)
    a. MeOH (1 mL, drain 1 drop/s until dripping stops, do not dry further)
5. Wash cartridge
    a. MeOH (1 mL, drain 1 drop/s until dripping stops, do not dry further)
6. Elute fraction 2 into new, labeled 15 mL PP centrifuge tube (PFSAs, PFCAs and FTUCAs):
    a. Load 3 mL1% NH_4OH in MeOH
    b. Allow approximately half of the 3 mL to drain (1 drop/s), then close stopcock
    c. Soak for 2 minutes
    d. Open stopcock and drain remaining solvent until dripping stops, do not dry further
7. Record weight of tube + cap + remaining extract. The mass fraction of the extract loaded will be used to calculate analyte concentration:

IV. Final Cleanup and Preparation

IV.I. Fraction 1

NOTE: Fraction 1 is not currently analyzed. These steps were taken from MET-OCRL-EWHD-PFC-Version 4 in case this fraction is analyzed in the future

7.3.7. Weigh about 20 mg of active carbon in a clean disposable glass tube.
7.3.8. Transfer 500µL of the Fraction 1 solution (see 7.3.4) into the tube containing active carbon and mix it with vortex.
7.3.9. Add 0.5 mL of methanol in a VWR centrifugal filter, and centrifuge at 6000 rpm × 5 min to wash the filter. Discard the filtrate.
7.3.10. Transfer the mixture of solution and active carbon (7.3.8.) into the centrifugal filter, and centrifuge at 6000 rpm × 5 min to filter the sample. Transfer the filtrate into a UPLC polypropylene vial (700 µL). The fraction is then ready for analysis of FASAs by UPLC/MS/MS analysis.

IV.II. Fraction 2

1. Evaporate to 0.5 mL (5-10 psi N_2 pressure, 55°C) based on tube graduations (takes ~10-12 min.)
    a. Use a separate tube with 0.5 mL MeOH added as reference if desired
2. Tilt/rotate tube to rinse walls just up to about the 5 mL graduation (where sample contacted)
3. If concentrated below 0.5 mL, dilute with MeOH to 0.5 mL graduation
4. Transfer all sample volume to 700 µL PP ASV
5. If samples are not analyzed by LC-MS on the same day sample prep is completed, store in refrigerator until analyzed. Bring samples to room temperature, vortex, and sonicate 10 minutes in bath sonicator prior to proceeding with addition of injection standard and water
6. Add 25 µL of 40 ng/mL M7PFUdA injection standard (1 ng mass)
7. Add 175 µL water, invert several times to mix by hand, sonicate for 5 minutes in bath sonicator
    a. For sonicating autosampler vials, cut a thin sheet of foam (thick enough to float with weight of vials) to the desired size and use a hole punch to create holes for vials. Insert vials into holes in foam sheet vial holder so cap is above top of foam, float foam with vials in bath sonicator

V. LC-MS Analysis

V.I. Calibration standard preparation (all dilutions done in methanol)

Working Stock Solutions:

NATIVE:

100 $^{ng}/_{mL}$ Native standard PFAA solution (product code PFAC-MXB): *make 400 µL per batch of calibration standards*

- *Can make larger amount (>400 µL and use for multiple batches, **store in refrigerator**)*

Add 50 µL of 2 $^{µg}/_{mL}$ stock solution per 1 mL total volume

Diluted Native Working Solutions:

10 $^{ng}/_{mL}$→ 450 µL MeOH + 50 µL of 100 $^{ng}/_{mL}$ stock

1 $^{ng}/_{mL}$ → 450 µL MeOH + 50 µL of 10 $^{ng}/_{mL}$ stock

0.1 $^{ng}/_{mL}$ → 450 µL MeOH + 50 µL of 1 $^{ng}/_{mL}$ stock

SURROGATE:

1 $^{µg}/_{mL}$ C14, C16 PFAA solution (product codes: M2PFTeDA, M2PFHxDA):

First make a 5 $^{µg}/_{mL}$ mixed stock solution:

400 µL MeOH + 50 µL of 50 $^{µg}/_{mL}$ each M2PFTeDA and M2PFHxDA stocks

Then dilute 5x:

800 µL MeOH + 200 µL of 5 $^{µg}/_{mL}$ mixed stock

80 $^{ng}/_{mL}$ C4-C12 (product code MPFAC-MXA) + C14, C16 combined PFAA solution:

Spike 50 µL (4 ng) per sample at prior to extraction, dilute 8x prior to spiking to calibration standards

Add 40 µL of 2 $^{µg}/_{mL}$ 13C-labelled mixture stock solution per 1 mL total volume

Add 80 µL of 1 $^{µg}/_{mL}$ M2PFTeDA/M2PFHxDA mixture solution per 1 mL total volume

INJECTION STANDARD:

40 $^{ng}/_{mL}$ internal standard solution (product code M7PFUdA):

Spike 25 µL (1 ng) per sample immediately prior to injection

Make a 10x dilution of 50 $^{µg}/_{mL}$ stock:

450 µL MeOH + 50 µL of 50 $^{µg}/_{mL}$ stock

Make a 5x dilution of the above 5 $^{µg}/_{mL}$ stock

800 µL MeOH + 200 µL 5 $^{µg}/_{mL}$ stock

Add 40 µL of above 1 $^{µg}/_{mL}$ stock per 1 mL total volume

Table 3. Calibration standard specifics

Native mass (in 700 µL)	MeOH (µL)	Native stock	Surrogate (µL)	Inj. Std. (µL)	Water (µL)
0.01 ng	350	100 µL 0.1 ng/mL stock	50	25	175
0.05 ng	400	50 µL 1 ng/mL stock			
0.2 ng	250	200 µL 1 ng/mL stock			
1 ng	350	100 µL 10 ng/mL stock			
5 ng	400	50 µL 100 ng/mL stock			
20 ng	250	200 µL 100 ng/mL stock			

Table 4. Description of target analytes and mass-labeled standards used

Compound	Full name	Formula	Surrogate
	Native PFCAs		
PFPeA	Perfluoropentanoic acid	$CF_3(CF_2)_3$ COOH	PFHxA^{13}C
PFHxA	Perfluorohexanoic acid	$CF_3(CF_2)_4$ COOH	PFHxA^{13}C
PFHpA	Perfluoroheptanoic acid	$CF_3(CF_2)_5$ COOH	PFOA^{13}C
PFOA	Perfluorooctanoic acid	$CF_3(CF_2)_6$ COOH	PFOA^{13}C
PFNA	Perfluorononanoic acid	$CF_3(CF_2)_7$ COOH	PFNA^{13}C
PFDA	Perfluorodecanoic acid	$CF_3(CF_2)_8$ COOH	PFDA^{13}C
PFUnA	Perfluoroundecanoic acid	$CF_3(CF_2)_9$ COOH	PFUnA^{13}C
PFDoA	Perfluorododecanoic acid	$CF_3(CF_2)_{10}$ COOH	PFDoA^{13}C
PFTrA	Perfluorotridecanoic acid	$CF_3(CF_2)_{11}$ COOH	PFDoA^{13}C
PFTeA	Perfluorotetradecanoic acid	$CF_3(CF_2)_{12}$ COOH	M2PFTeA
PFHxDA	Perfluorohexadecanoic acid	$CF_3(CF_2)_{14}$ COOH	M2PFHxDA
	Native PFSAs		
PFBS	Potassium perfluoro-1-butanesulfonate	$CF_3(CF_2)_3SO_3^-$	PFHxS^{18}O
PFHxS	Sodium perfluoro-1-hexanesulfonate	$CF_3(CF_2)_5SO_3$-	PFHxS^{18}O
PFOS	Sodium perfluoro-1-octanesulfonate	$CF_3(CF_2)_7SO_3^-$	PFOS^{13}C
PFDS	Sodium perfluoro-1-decanesulfonate	$CF_3(CF_2)_9SO_3^-$	PFOS^{13}C
	Mass-labeled PFCAs		
PFBA^{13}C	Perfluoro[1,2,3,4-$^{13}C_4$]butanoic acid		
PFHxA^{13}C	Perfluoro[1,2-$^{13}C_2$]hexanoic acid		
M5PFHxA*	Perfluoro[1,2,3,4,6-$^{13}C_5$]hexadecanoic acid		
PFOA^{13}C	Perfluoro[1,2,3,4-$^{13}C_4$]octanoic acid		
PFNA^{13}C	Perfluoro[1,2,3,4,5-$^{13}C_5$]nonanoic acid		
PFDA^{13}C	Perfluoro[1,2-$^{13}C_2$]decanoic acid		
PFUnA^{13}C	Perfluoro[1,2-$^{13}C_2$]undecanoic acid		
M7PFUnA*	Perfluoro[1,2,3,4,5,6,7-$^{13}C_7$]undecanoic acid		
PFDoA^{13}C	Perfluoro[1,2-$^{13}C_2$]dodecanoic acid		
M2PFTeA	Perfluoro[1,2-$^{13}C_2$]tetradecanoic acid		
M2PFHxDA	Perfluoro[1,2-$^{13}C_2$]hexadecanoic acid		
	Mass-labeled PFSAs		
PFHxS^{18}O	Sodium perfluoro-1-hexane[$^{18}O_2$]sulfonate		
PFOS^{13}C	Sodium perfluoro-1-[1,2,3,4-$^{13}C_4$]octanesulfonate		

1. Inject 10 µL of standards and samples to UPLC-MS
2. Calculate analyte concentration in each sample as follows:

$$\text{Analyte concentration (ng/g wet weight)} = m_{TL} / (VF_{concentration} * MF_{SPE} * m_{sample})$$

m_{TL} = mass (ng) calculated (TargetLynx software) based on internal standard calibration
$VF_{concentration}$ = volume fraction of sample transferred to ASV after concentration (=1 if entire sample transferred)
MF_{SPE} = mass fraction of combined supernatant loaded to SPE cartridge
m_{sample} = mass (g) of sample extracted

V.II. Instrument parameters

Table 5. UPLC solvent gradient

Time (min)	Flow (mL/min)	%A	%B	T_2-T_1 Change
0.0	0.4	75	25	
1.0	0.4	75	25	
1.5	0.4	40	60	Linear
9.6	0.4	0	100	Linear
13.5	0.4	0	100	
14.5	0.4	75	25	Linear
19.5	0.4	75	25	

A: water +0.1% formic acid
B: methanol +0.1% formic acid

Table 4. Compound-specific instrumental parameters (Waters Xevo G2-XS QToF)

Compound	$[M-H]^-$ m/z	$[M-H-CO2]^-$ m/z	Retention time* (min, **target**, max)	Cone Voltage*[1] (V)	Collision Energy*[1] (eV)
PFBA	212.979	168.988	2.10, **2.55**, 2.80	3	2
PFBA^{13}C	216.994	171.999			
PFPeA	262.975	218.986	2.80, **2.96**, 3.25	5	2
PFBS	298.943	N/A	2.80, **3.00**, 3.25	30	10
PFHxA	312.973	268.983	3.25, **3.47**, 3.80	5	2
PFHxA^{13}C	314.973	269.985			
PFHxS	398.937	N/A	3.80, **4.01**, 4.42	30	15
PFHxS^{18}O	402.937	N/A			
PFHpA	362.970	318.979	3.80, **4.08**, 4.42	5	2
PFOA	412.966	368.976	4.42, **4.79**, 5.15	5	2
PFOA^{13}C	416.978	371.985			
PFOS	498.930	N/A	5.15, **5.39**, 6.00	30	15
PFOS^{13}C	502.943	N/A			
PFNA	462.962	418.973	5.15, **5.54**, 6.00	30	3
PFNA^{13}C	467.980	422.986			
PFDA	512.960	468.971	6.00, **6.28**, 6.60	30	3
PFDA^{13}C	514.966	469.974			
PFDS	598.923	N/A	6.60, **6.77**, 7.40	30	15
PFUnA	562.957	518.967	6.60, **6.98**, 7.40	30	4
PFUnA^{13}C	564.962	519.969			
M7PFUnA	569.981	524.987			
PFDoA	612.954	568.964	7.40, **7.63**, 8.00	30	6
PFDoA^{13}C	614.960	569.968			
PFTrA	662.950	618.960	8.00, **8.22**, 8.55	30	6
PFTeA	712.947	668.956	8.55, **8.77**, 9.30	30	6
M2PFTeA	714.953	669.960			
PFHxDA	812.940	768.950	9.30, **9.74**, 10.40	30	6
M2PFHxDA	814.947	769.954			

VI. Revision History

1. 12/16/2016: added 3 mL of methanol +1% ammonium hydroxide as a first conditioning step. This had no impact on method recovery and eliminated background contamination from the SPE cartridge.
2. 12/19/2016: changed sample loading procedure from diluting a 2 mL aliquot of combined supernatant with 8 mL of water in second tube to diluting sample within the SPE cartridge. This improved recoveries of PFTeA and PFHxDA by roughly 60% and 20%, respectively.
3. 11/26/2019: Alteration of methanol elution/wash steps and basic methanol elution step. Prior to this change, 1 mL of methanol was added to the dried cartridge and allowed to elute until dripping ceased, then vacuum (~5 in Hg for several seconds) was applied to drain residual methanol from the sorbent bed. The second mL of wash methanol was then added, and this process repeated. This was altered to eliminate the use of additional vacuum so that the stopcock is closed once the methanol stops dripping.

   One additional alteration was the addition of a 2 minute soak during elution of fraction 2 and the elimination of cartridge drying after elution of fraction 2. Prior to this change, 3 mL of methanol +1% ammonium hydroxide was added to the cartridge and allowed to drain until dripping stopped. Vacuum was then increased to ~5 in Hg until the residual solvent in the sorbent bed had been drained. Now, 3 mL of elution solvent are added and approximately half is drained. The stopcock is then closed for 2 minutes, then opened and the remaining volume is drained until dripping ceases. These changes were found to increase method recoveries as well as recovery precision for most analytes. Most noticeably, recoveries for method blanks increased after implementing these changes, likely because analytes are bound more strongly to the adsorbent due to lack of matrix components.
4. 7/19/19 Invertebrate drying procedure was added