Supporting Information

Historical Blood Serum Samples from Wilmington, North Carolina: The Importance of Ultrashort-Chain Per- and Polyfluoroalkyl Substances

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Text S1. Sample preparation procedures for analyzing 11 shorter chain PFAS in serum.

A 10-point external calibration curve, ranging in concentration from 0.1 ng/mL to 100 ng/mL (10 ng/mL to 10,000 ng/mL for TFA), was prepared with newborn calf serum (Cytiva) by spiking 10 µL of PFAS standard mix solutions into 50 µL newborn calf serum in 1.5-mL polypropylene microcentrifuge tubes. TFA concentrations in the PFAS standard mix solutions were 100 times higher than the other 10 compounds to account for its higher method reporting limit (MRL). Quality control samples (QCs) with concentrations of 2 ng/mL and 20 ng/mL (200 ng/mL and 2,000 ng/mL for TFA) were prepared using PFAS standard mix solutions from secondary sources. Method blanks and double blanks were prepared by spiking 10 µL deionized water into 50 µL newborn calf serum. Matrix spikes were prepared by spiking PFAS standard mix solutions into National Institutes of Standards and Technology (NIST) standard reference material (SRM) 1957 human serum to achieve PFAS concentrations of 2 ng/mL and 20 ng/mL (200 ng/mL and 2,000 ng/mL for TFA). Serum samples were prepared by spiking 10 µL of deionized water into 50 µL of human serum.

After spiking with PFAS standards or deionized water, all the samples were vortexed. Calibration samples, QCs, method blanks, matrix spikes, and serum samples were then denatured with 150 µL cold (-20 ℃) acetonitrile containing mass labeled internal standards (6 ng/mL). Double blanks were denatured with cold acetonitrile without mass labeled internal standards. After denaturation, the samples were vortex mixed again and centrifuged at 10,000 × g for 5 min in a microcentrifuge (Sorvall Legend Micro 21, Thermo Scientific) at room temperature. Finally, 50µL of the supernatant was transferred into a 250 µL polypropylene liquid chromatography (LC) vial with 100 µL deionized water for the analysis of TFA, PFPrA, PFBA, TFMS, PFEtS, PFPrS, PFBS, MTP, and PFMOAA. Another 50 µL aliquot of the supernatant was transferred into a 250 µL

polypropylene LC vial containing 100 µL of 7.5 mM ammonium acetate for DFSA and MMF analysis.

Duplicates and continuing calibration verifications (CCVs) were analyzed through the analysis to ensure instrument performance. The MRL, reproducibility of duplicates, and recoveries of matrix spikes, CCVs, and QCs are summarized in Tables S7 to S11 in the Excel supplemental information document.

Text S2. Check for analytical interferences

In this study, eleven ultrashort- and short-chain PFAS in serum were quantified using low-resolution mass spectrometry (LRMS). Five compounds (PFBS, PFPrA, PFBA, TFA, MMF) had matching isotopically labeled internal standards. For the compounds without matching internal standards (TFMS, PFEtS, PFPrS, PFMOAA, MTP, DFSA), internal standards that provided the closest retention time match and most accurate matrix spike recoveries were used (Table S6). Six of the eleven compounds (PFBS, PFPrA, PFPrS, PFEtS, TFMS, MTP) had two MS/MS transitions, one of which was used to determine the response of the quantifier ion and the other to determine the response of the qualifier ion. The ratio of the two responses (qualifier ratio) was taken as a measure to confirm analyte presence (Figure S1). The acceptance criterion was that the qualifier ratio was within 30% of the mean value obtained for the calibration standards. The remaining five compounds (PFBA, TFA, PFMOAA, DFSA, MMF) only have one MS/MS transition (Figure S2). Studies found metabolites and fatty acids in biological samples can have the same mass transitions as PFAS, therefore, causing over-reporting for PFAS with only one transition when using LRMS instrumentation.[1, 2] To prevent false positive results, blank samples were prepared with calf serum. In addition, NIST SRM 1957 human serum was analyzed. Resulting chromatograms were carefully examined for interferences at retention times of the targeted compounds. Moreover, the existence of PFMOAA and PFPrA was observed when serum samples were analyzed by high-resolution mass spectrometry (HRMS) by METRIC at NC State University.

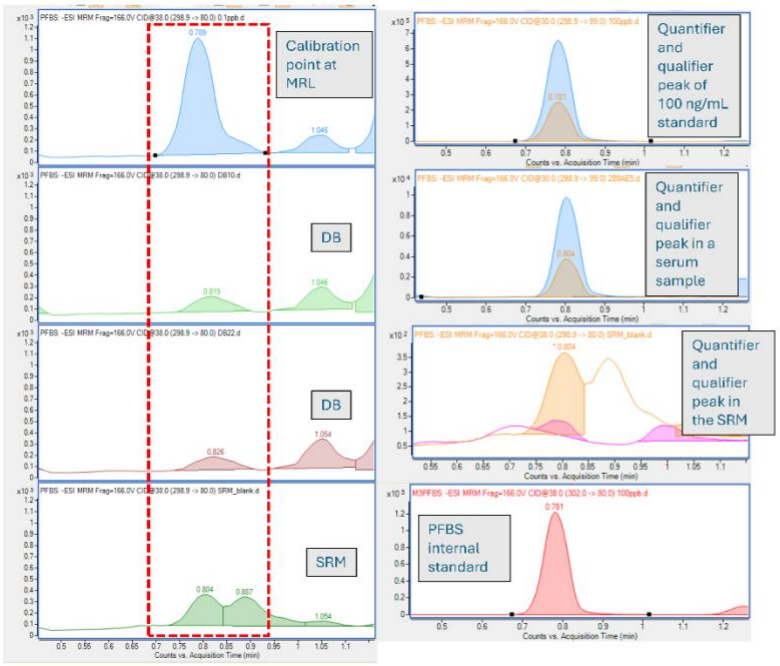
More than 30 double blank samples (calf serum without added PFAS or internal standard) were analyzed throughout this study. The responses of all double blanks were lower than one-half of the MRL responses, indicating no matrix interferences from calf serum. The chromatograms of two randomly selected double blanks are shown in Figures S1 and S2.

Among the six compounds with two MS/MS transitions, TFMS was detected at < MRL and PFPrA at 0.1 ng/mL in the SRM (Table S9). Peaks were also observed in the SRM for what appeared to be PFBS, PFPrS, MTP, and PFEtS, but they were determined to be analytical interferences because the qualifier peaks were absent or the qualifier ratio did not match the analytical standards. These interferences did not affect the quantification because the interference responses were much lower than the analyte responses obtained at the MRL.

Of the five compounds with only one MS/MS transition, no peak was observed in the SRM at the determined retention times for DFSA and MMF (S/N<10), indicating there was no analytical interference for these two compounds. Small peaks that could either be an interference or the targeted PFAS were observed in the SRM for PFBA and TFA, but the responses were much lower than those for the calibration point at the MRL. Pan et al.[2] investigated possible interferences for TFA in a few natural products, including human serum, and found interferences only in the muscle tissue of carp. A peak with a response near 0.1 ng/mL was observed for PFMOAA in the SRM. Based on HRMS data, the peak was not an interference.

The presence of PFMOAA and PFPrA in serum samples was confirmed by HRMS (Figures S3 and S4). PFMOAA was identified by HRMS in nearly every sample. For almost every identification the following evidence of presence occurred: (1) exact precursor mass matching the theoretical mass of PFMOAA (178.9773 Da, [M-H]-), (2) presence and appropriate ratio for multiple isotopes ([M-H]- and [M+1-H]-), (3) retention time matching to a standard, (4) exact mass product ion matching (ions 44.9982 Da, 84.9907 Da, 90.9982 Da, and 134.9881 Da, etc) to a standard, and (5) product ion ratio matches between several product ions (e.g., 84.9907 Da vs 90.9982 Da) when compared to a standard. PFPrA was identified by HRMS in several samples. For almost every identification the following evidence of presence occurred: (1) exact precursor

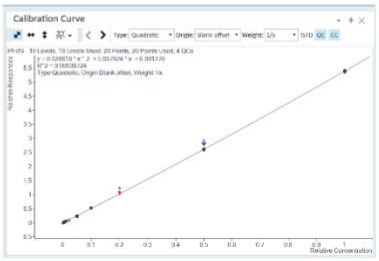
mass matching the theoretical mass of PFPrA (162.9824 Da, [M-H]-), (2) presence and appropriate ratio for multiple isotopes ([M-H]- and [M+1-H]-), (3) retention time matching to a standard from Chemours (run previously), and (4) exact mass product ion matching a C_2F_5 radical product ion.

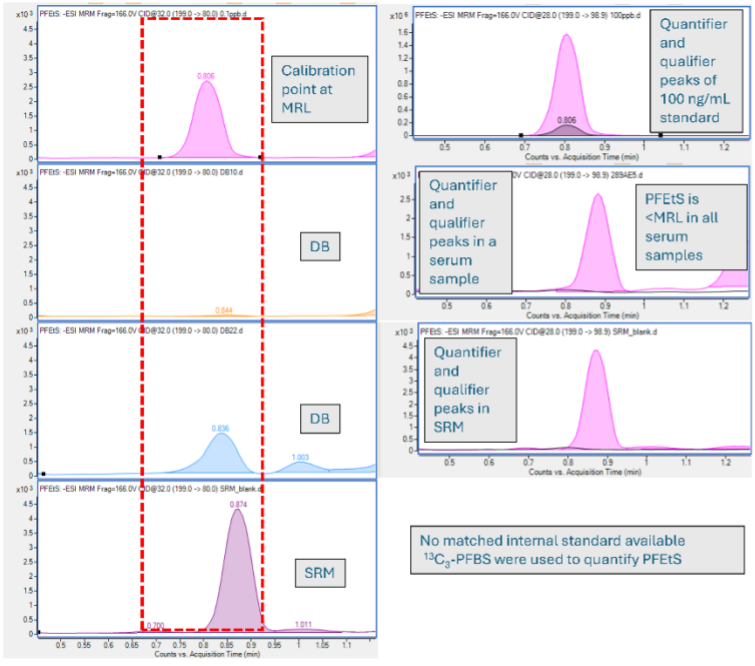


Compound: PFBS
MRL:0.1 ng/mL
Retention time: 0.781 min
Average MRL response:4843
Average DB response:672

Peak in SRM:
response: 1154
retention time match standard? True
Qualifier match standard? False

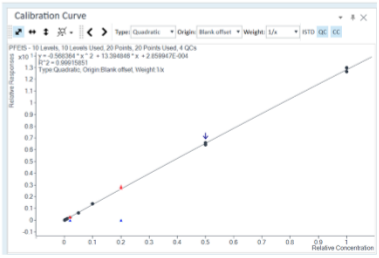
SRM response << MRL response

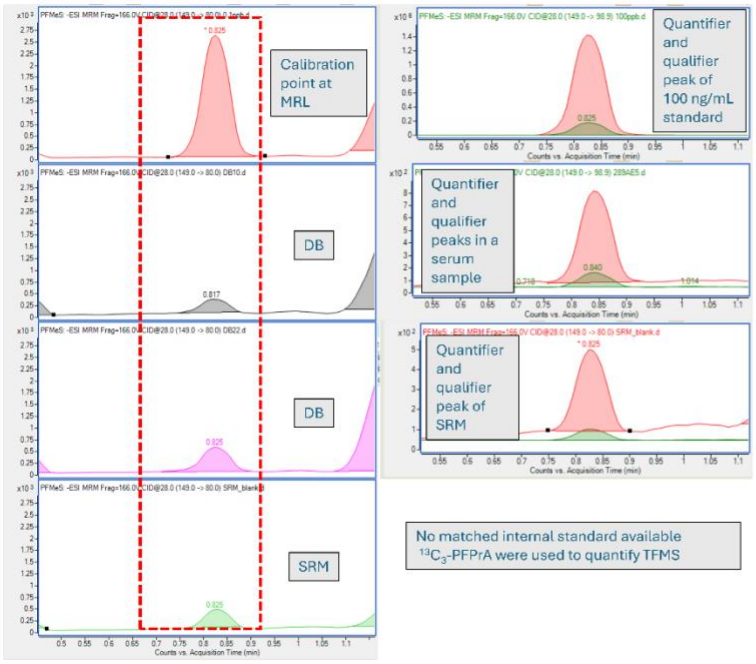




Compound: PFEtS
MRL:0.1 ng/mL
Retention time: 0.807 min
Average MRL response:10836
Average DB response: 3474

Peak in SRM:
response: no peak
retention time match standard? False
Qualifier match standard? False

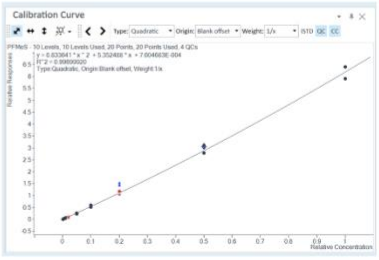


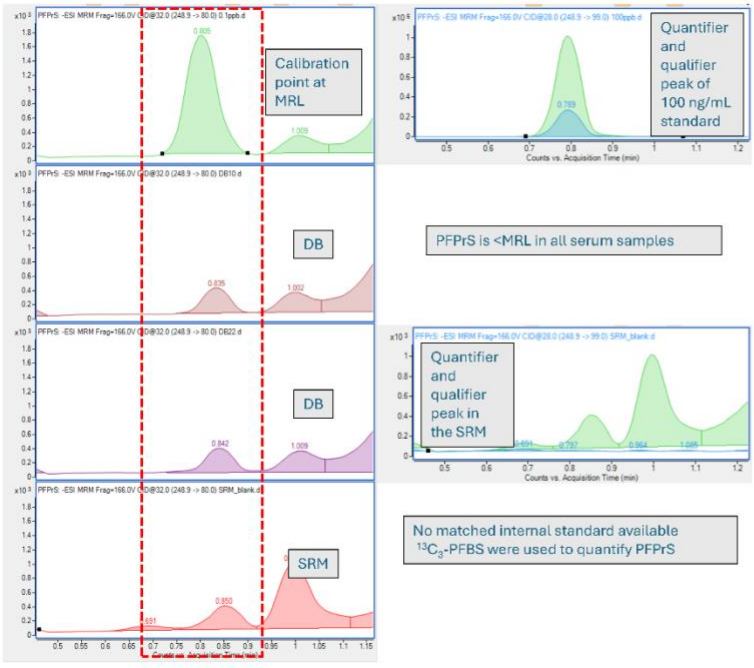


Compound: TFMS
MRL:0.1 ng/mL
Retention time: 0.828 min
Average MRL response:10197
Average DB response:1918

Peak in SRM:
response: 1594
retention time match standard? True
Qualifier match standard? True

SRM response << MRL response

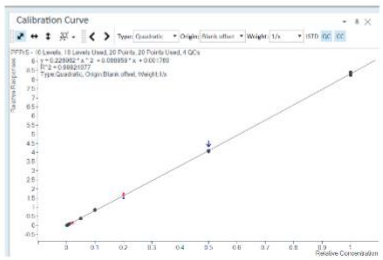




Compound: PFPrS
MRL:0.1 ng/mL
Retention time: 0.789 min
Average MRL response:7653
Average DB response:1405

Peak in SRM:
response: 1283
retention time match standard? False
Qualifier match standard? False

SRM response << MRL response



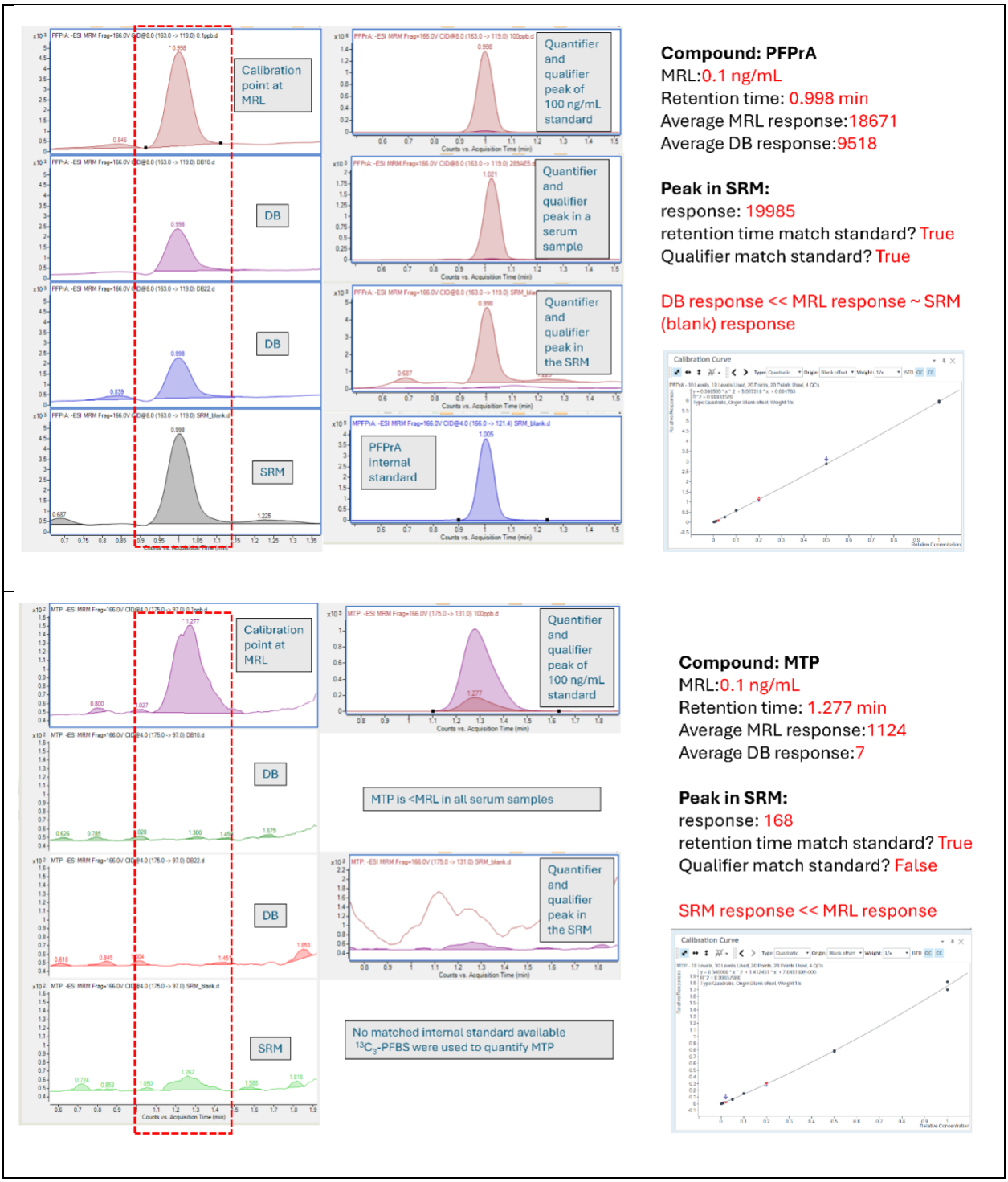
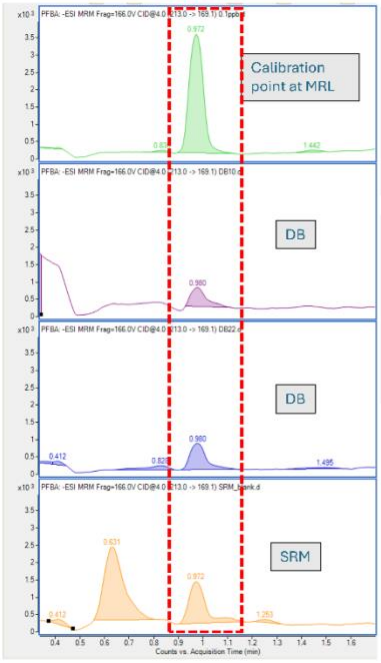
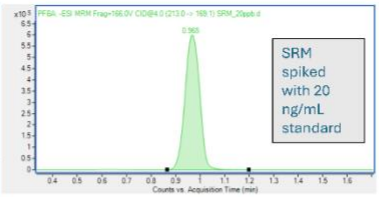
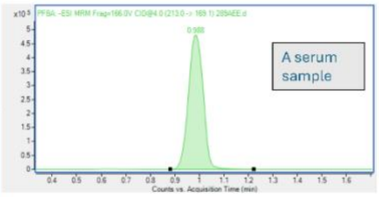
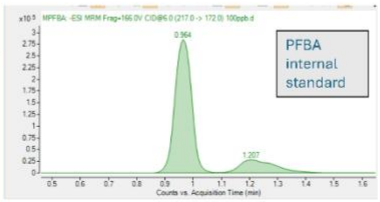


Figure S1. LC-MS chromatograms of ultrashort- and short-chain PFAS with two MS/MS transitions in serum samples. DB: double blank samples prepared with calf serum. SRM: blank samples prepared with NIST SRM 1957 human serum.





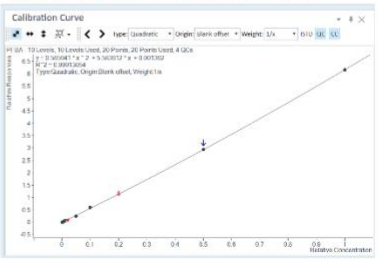


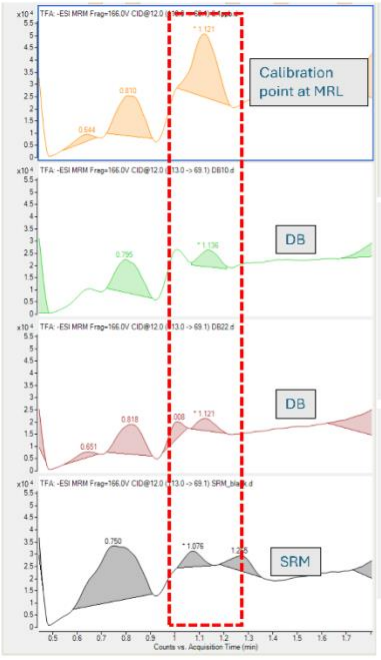


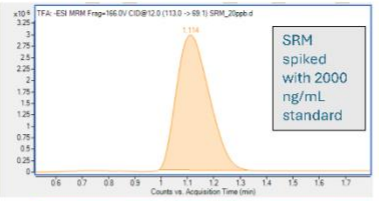
Compound:PFBA
MRL:0.1 ng/mL
Retention time: 0.965 min
Average MRL response:13107
Average DB response:3459

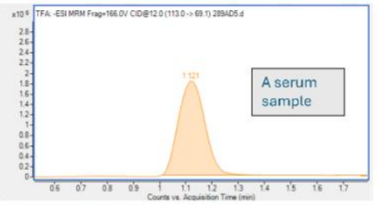
Peak in SRM:
response:5890
retention time match standard? True
Qualifier match standard? No qualifier

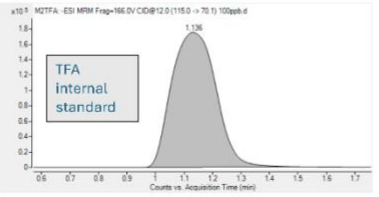
SRM response << MRL response







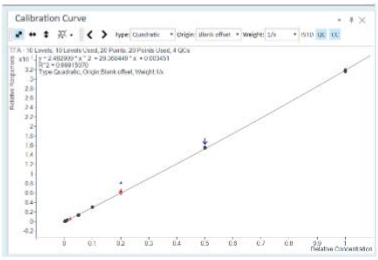


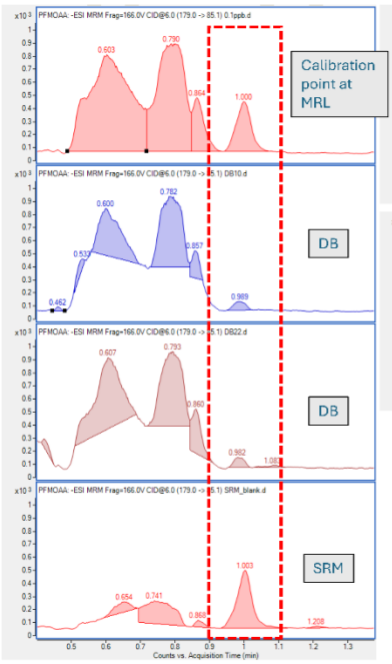


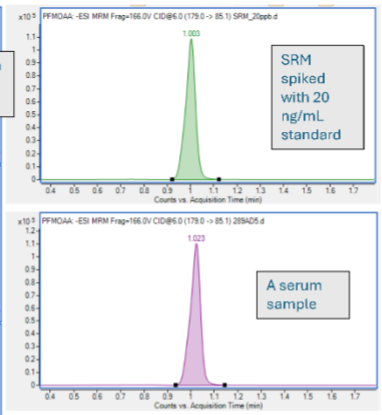
Compound: TFA
MRL:10 ng/mL
Retention time: 1.129 min
Average MRL response:188603
Average DB response:22583

Peak in SRM:
response:36706
retention time match standard? True
Qualifier match standard? No qualifier

SRM response << MRL response





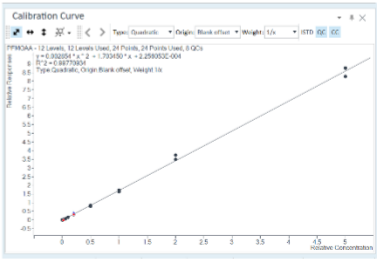


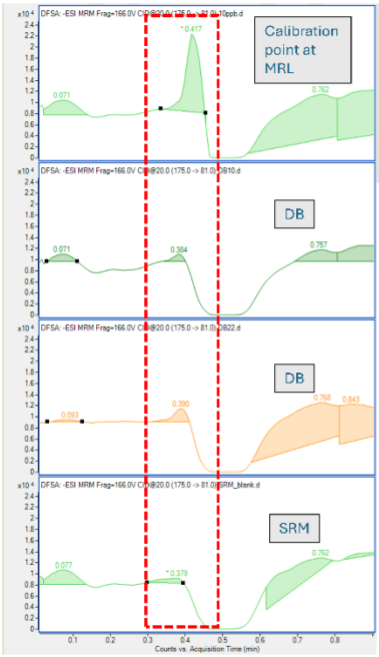
No matched internal standard available
$^{13}C_3$-PFPrA were used to quantify PFMOAA

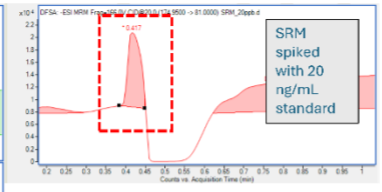
Compound: PFMOAA
MRL:0.1 ng/mL
Retention time: 0.989 min
Average MRL response:1046
Average DB response:165

Peak in SRM:
response: 1315
retention time match standard? True
Qualifier match standard? No qualifier

DB response << MRL response < SRM (blank) response







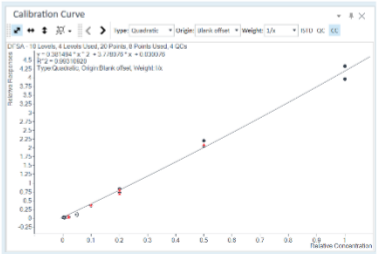
DFSA is <MRL in all serum samples

No matched internal standard available
$^{13}C_3$-MMF were used to quantify DFSA

Compound: DFSA
MRL:10 ng/mL
Retention time: 0.428 min
Average MRL response:40515
Average DB response:3185

Peak in SRM:
response: No peak
retention time match standard? No peak
Qualifier match standard? No qualifier

SRM response << MRL response



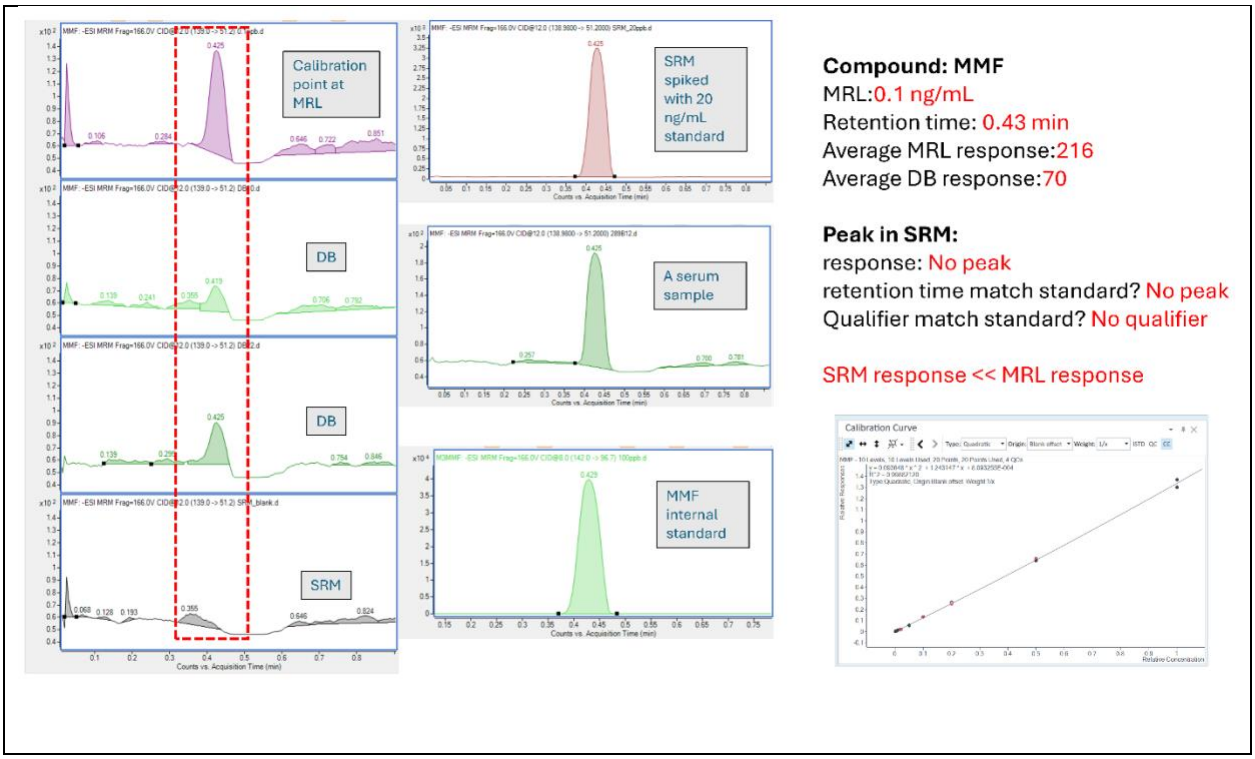
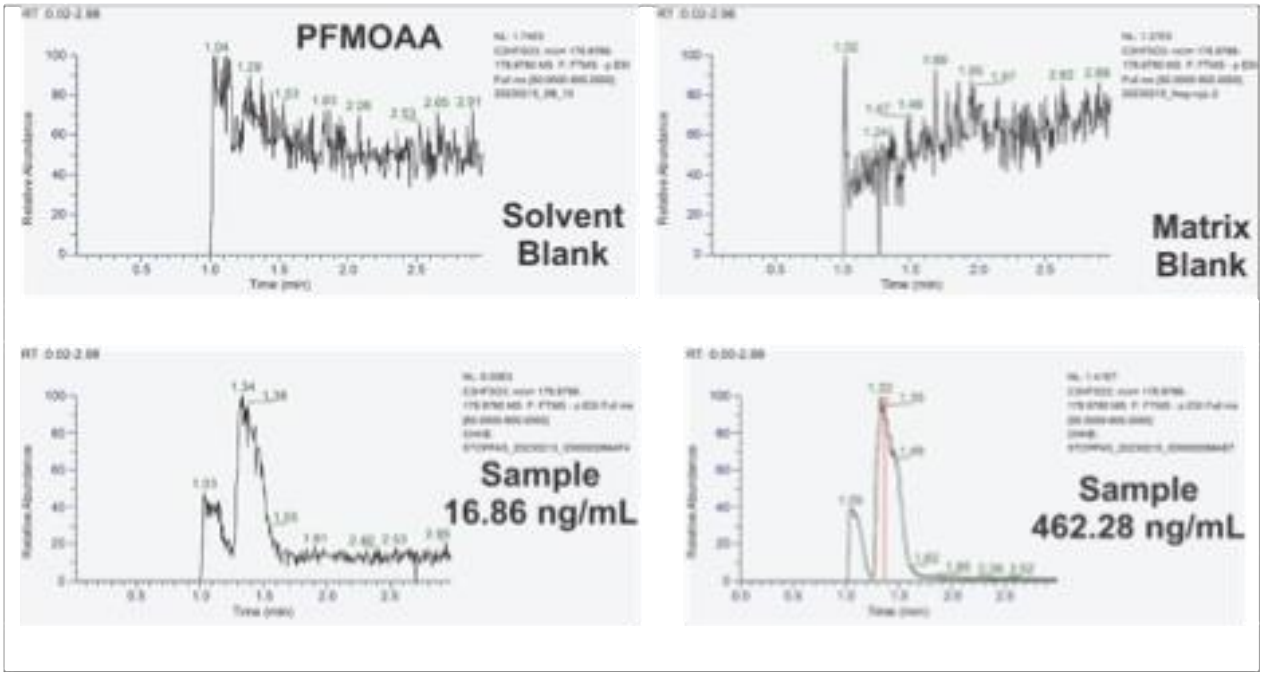


Figure S2. LC-MS chromatograms of ultrashort- and short-chain PFAS with only one MS/MS transition in serum samples. DB: double blank samples prepared with calf serum. SRM: blank samples prepared with NIST SRM 1957 human serum.



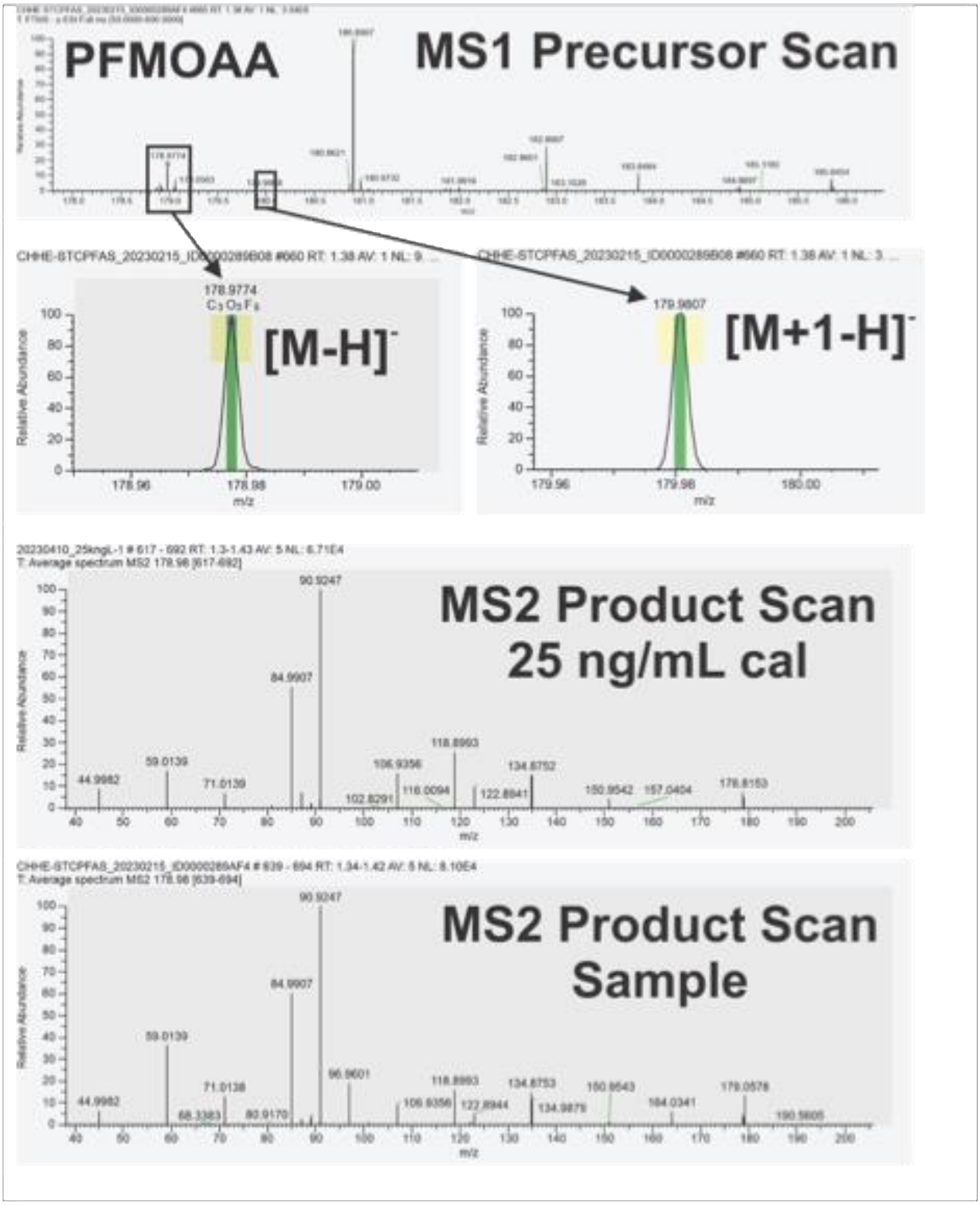
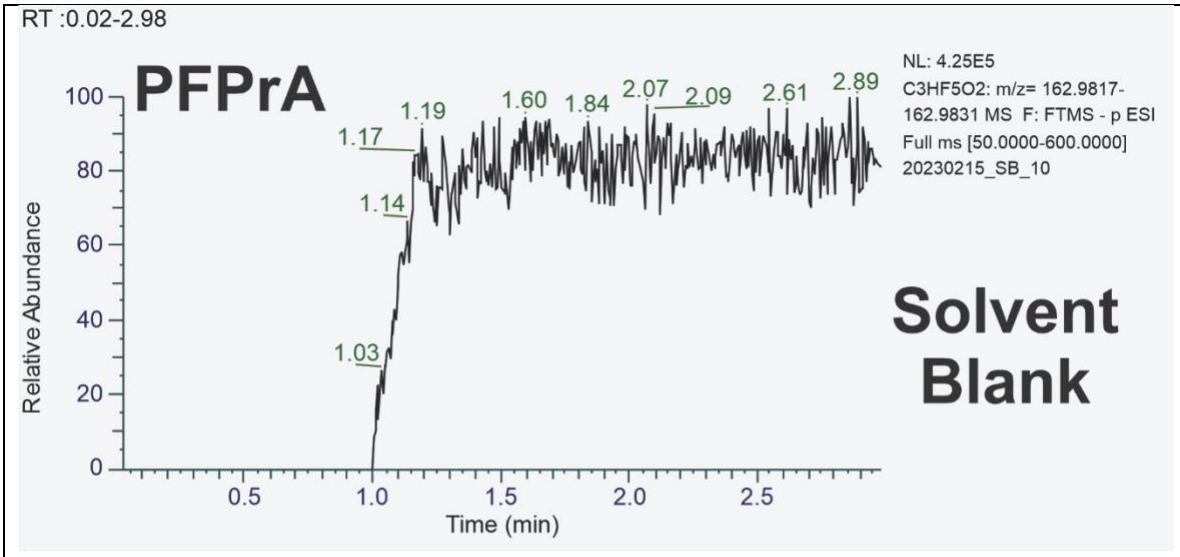
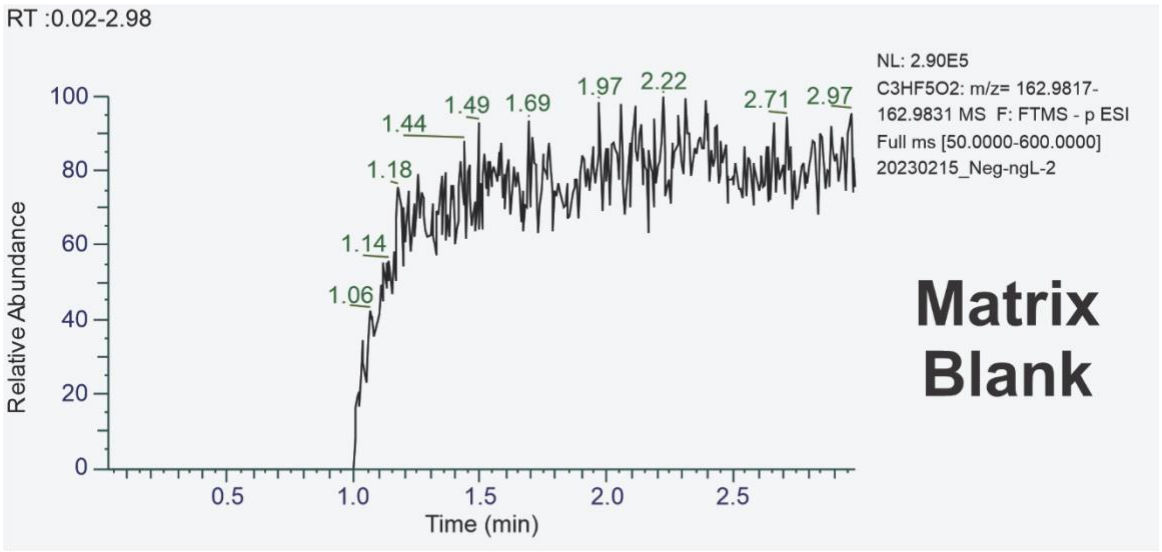
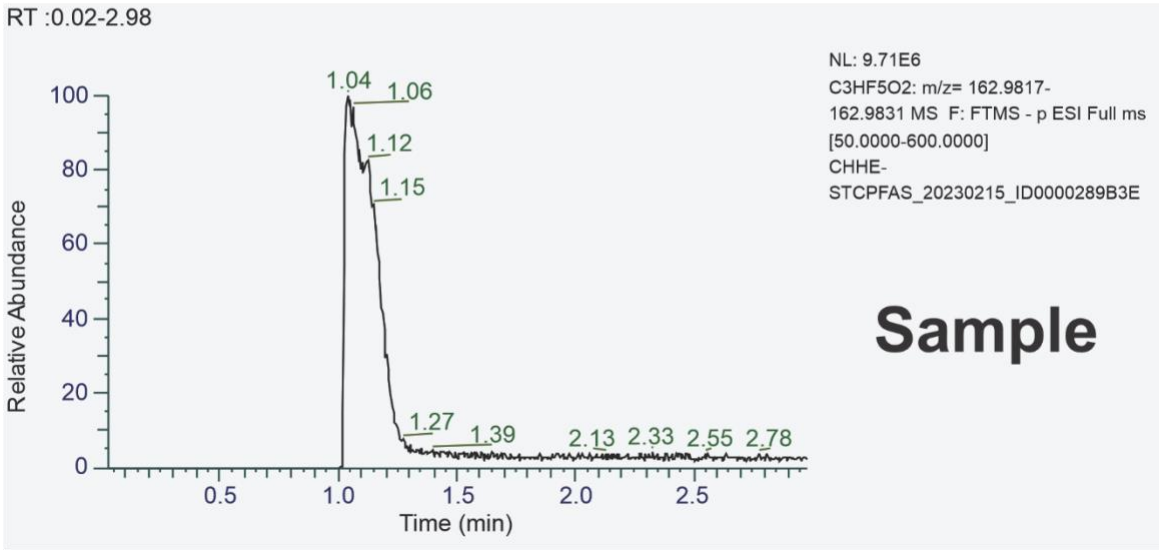


Figure S3. HRMS chromatograms and mass spectra of PFMOAA.


RT :0.02-2.98
PFPrA
NL: 4.25E5
C3HF5O2: m/z= 162.9817-
162.9831 MS F: FTMS - p ESI
Full ms [50.0000-600.0000]
20230215_SB_10
Solvent
Blank
Relative Abundance
Time (min)


RT :0.02-2.98
NL: 2.90E5
C3HF5O2: m/z= 162.9817-
162.9831 MS F: FTMS - p ESI
Full ms [50.0000-600.0000]
20230215_Neg-ngL-2
Matrix
Blank
Relative Abundance
Time (min)


RT :0.02-2.98
NL: 9.71E6
C3HF5O2: m/z= 162.9817-
162.9831 MS F: FTMS - p ESI Full ms
[50.0000-600.0000]
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STCPFAS_20230215_ID0000289B3E
Sample
Relative Abundance
Time (min)

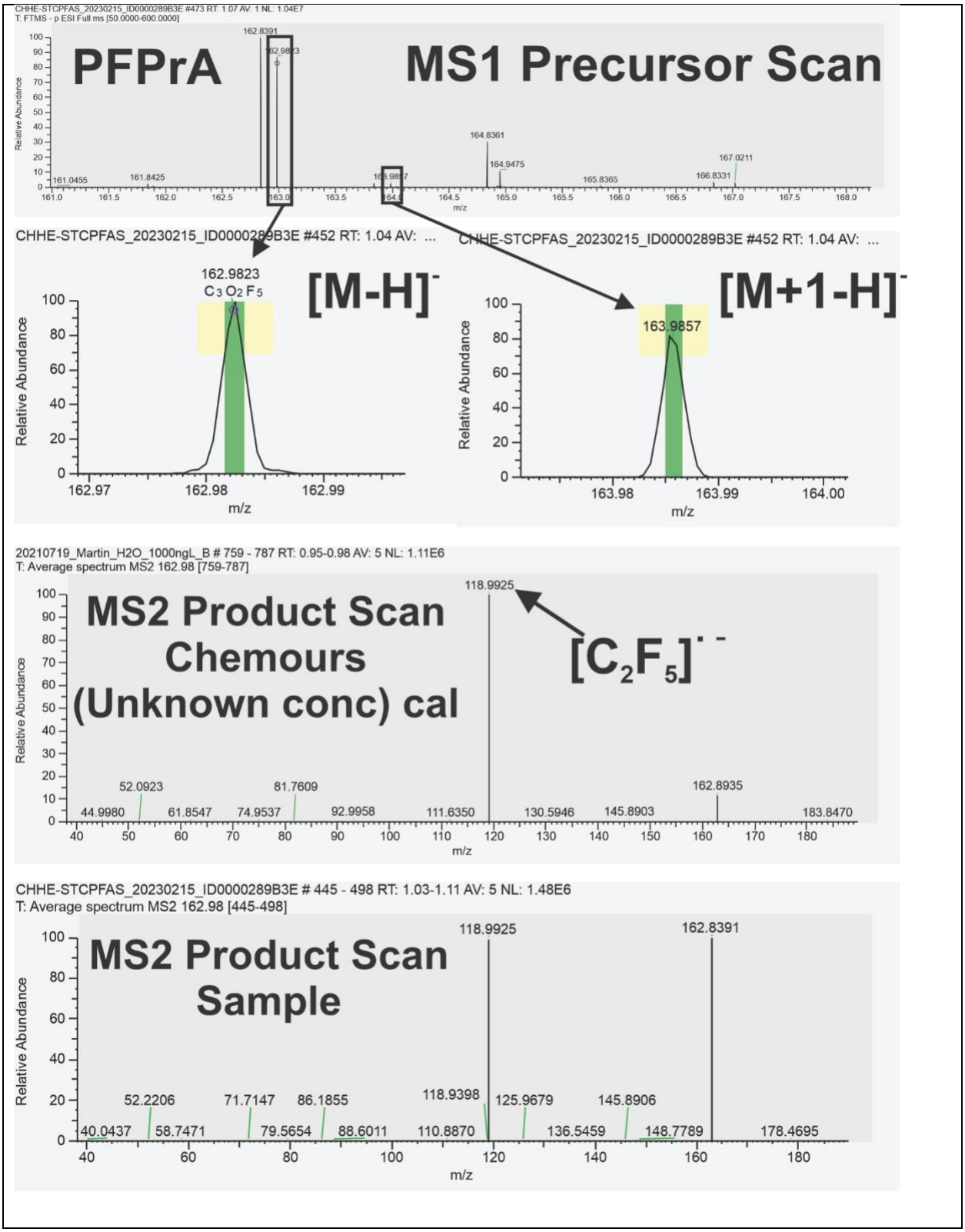

Figure S4. HRMS chromatograms and mass spectra of PFPrA

Text S3. Sample preparation procedures for analyzing 45 longer chain PFAS in serum.

An 8-point calibration curve, ranging in concentration from 0.05 ng/mL to 25 ng/mL, was prepared with newborn calf serum by spiking 5 μL of PFAS standard mix solutions (0.5 ng/mL to 250 ng/mL) into 45 μL newborn calf serum in 1.5-mL polypropylene microcentrifuge tubes. QCs with concentrations of 8 ng/mL were prepared. Method blanks were prepared by spiking 5 μL deionized water into 45 μL newborn calf serum. 50 μL of human serum, calibration standards, QCs, and method blanks were mixed with 150 μL of cold methanol containing internal standards (4.00 ng/mL). The mixture was vortexed and centrifuged at 10,000 × g for 5 min. 100 μL of the supernatant was transferred to an LC vial containing 50 μL of deionized water to produce a final sample containing 50% methanol by volume.

Text S4. Sample preparation procedure for analyzing PFAS in water samples.

Water samples were analyzed with four different analytical methods (D, E, F, and G) to achieve the lowest MRL (Tables S15 to S18 in the Excel supplemental information document).

For all four analytical methods, a 12-point calibration curve, ranging in concentration from 0.5 ng/L to 2000 ng/L were prepared with deionized water and PFAS standard mix solutions (0.0005 ng/µL, 0.005 ng/µL, 0.05 ng/µL, and 0.5 ng/µL) in 15 mL polypropylene centrifuge tubes. Two QCs with concentrations of 20 ng/L and 200 ng/L were prepared with PFAS standards from secondary sources, and their accuracy was ensured to be within the range of 70% to 130%. Matrix spikes were prepared by spiking PFAS standard mix solutions into an aliquot of water samples to account for matrix effects.

For analytical methods D and E, 1620 µL of water sample or calibration standard was transferred into a 2-mL polypropylene LC vial (Thermo Scientific) containing 90 µL of 100 mM ammonium acetate in methanol and 90 µL of methanol containing mass-labeled internal standards (2000 ng/L). The final solution in the LC vial has 5mM ammonium acetate and 10% methanol.

For analytical method F, 5 mL of water sample or calibration standard was mixed with 4 mL of methanol in a 15 mL polypropylene centrifuge tube. 1620 µL of the mixed solution and 180 µL of methanol containing 1000 ng/L mass-labeled internal standards and 50 mM of ammonium acetate were added into a 2-mL polypropylene LC vial and vortexed. The final solution in the LC vial has 5mM ammonium acetate and 50% methanol.

For analytical method G, 810 µL of water sample or calibration standard and 90 µL of 90 % acetonitrile in deionized water containing 1000 ng/L mass-labeled internal standards and 15 mM ammonium formate were transferred into a 2 mL polypropylene LC vial (Thermo Scientific) and vortexed. The final solution in the LC vial has 1.5 mM ammonium formate and 9% acetonitrile.

The MRL, reproducibility of duplicates, and recoveries of matrix spikes are summarized in Table S19 in the Excel supplemental information document. The matrix spike recoveries for DFSA in water samples were significantly higher than 100%. Therefore, DFSA concentrations in water sample results were divided by 3.8 to accommodate matrix effect of water samples. DFSA in serum samples showed 107% matrix spike recoveries (See Table S9). Therefore, DFSA in serum data were used directly.

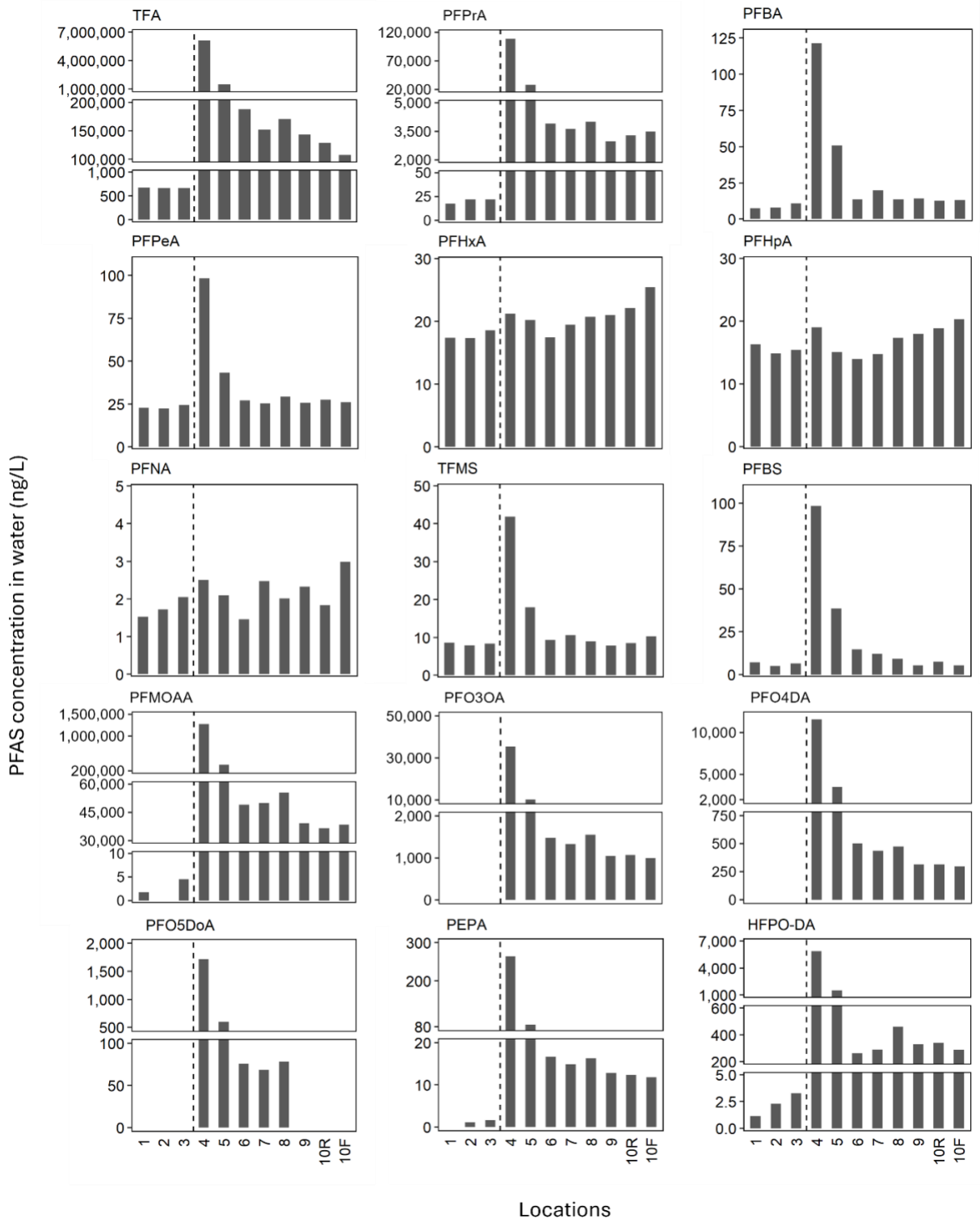

Figure S5. Concentrations of 19 PFAS with concentrations increased significantly (t-test p-value < 0.05) after the fluorochemical facility effluent site located between Location 3 and Location 4 (vertical dashed line) in water samples collected from the Cape Fear River. Sample size =11.

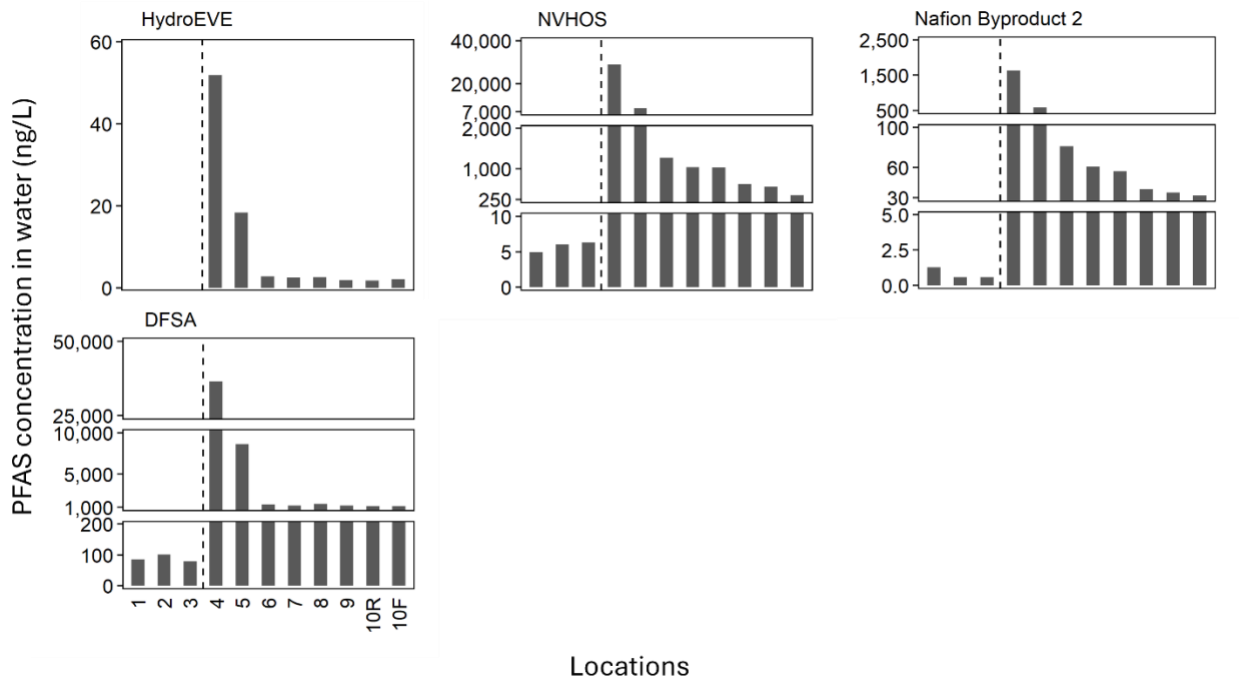

Figure S5. (Continued)

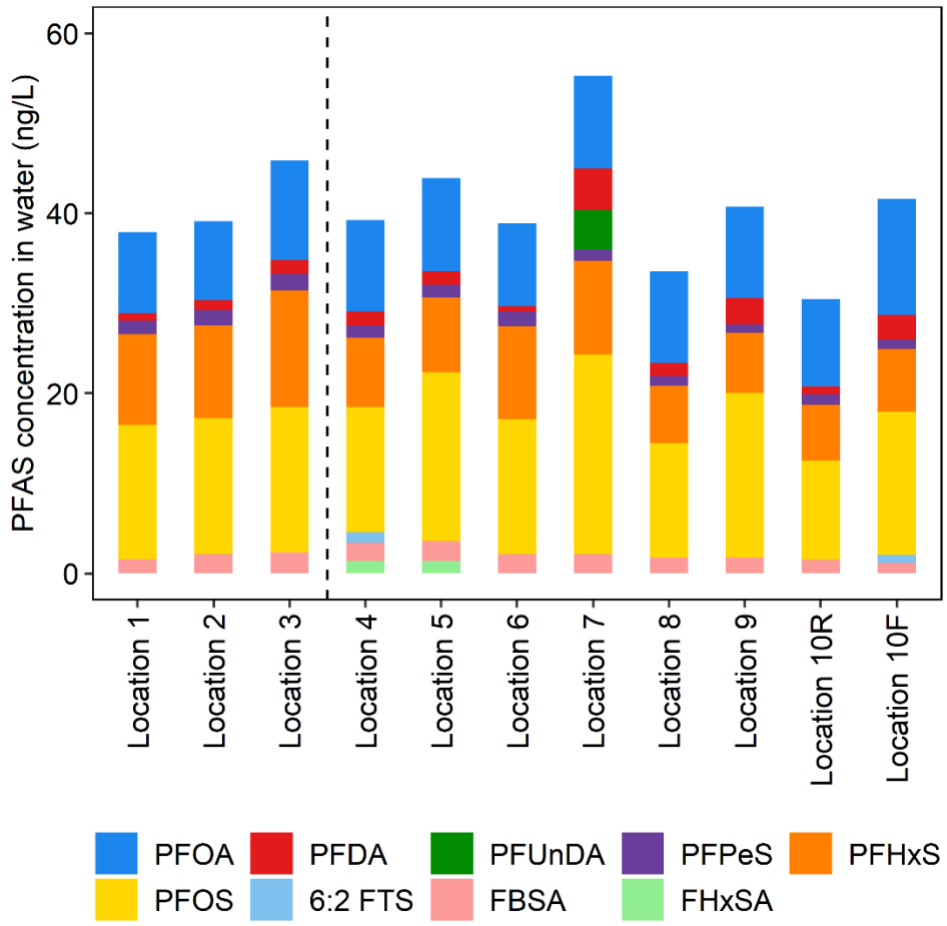

Figure S6. Concentrations of 9 PFAS with no significant change in concentration after the fluorochemical facility effluent site (vertical dashed line) in water samples collected from the Cape Fear River. Sample size =11. 10R and 10F represent raw and finished drinking water collected from a water treatment plant at Location 10.

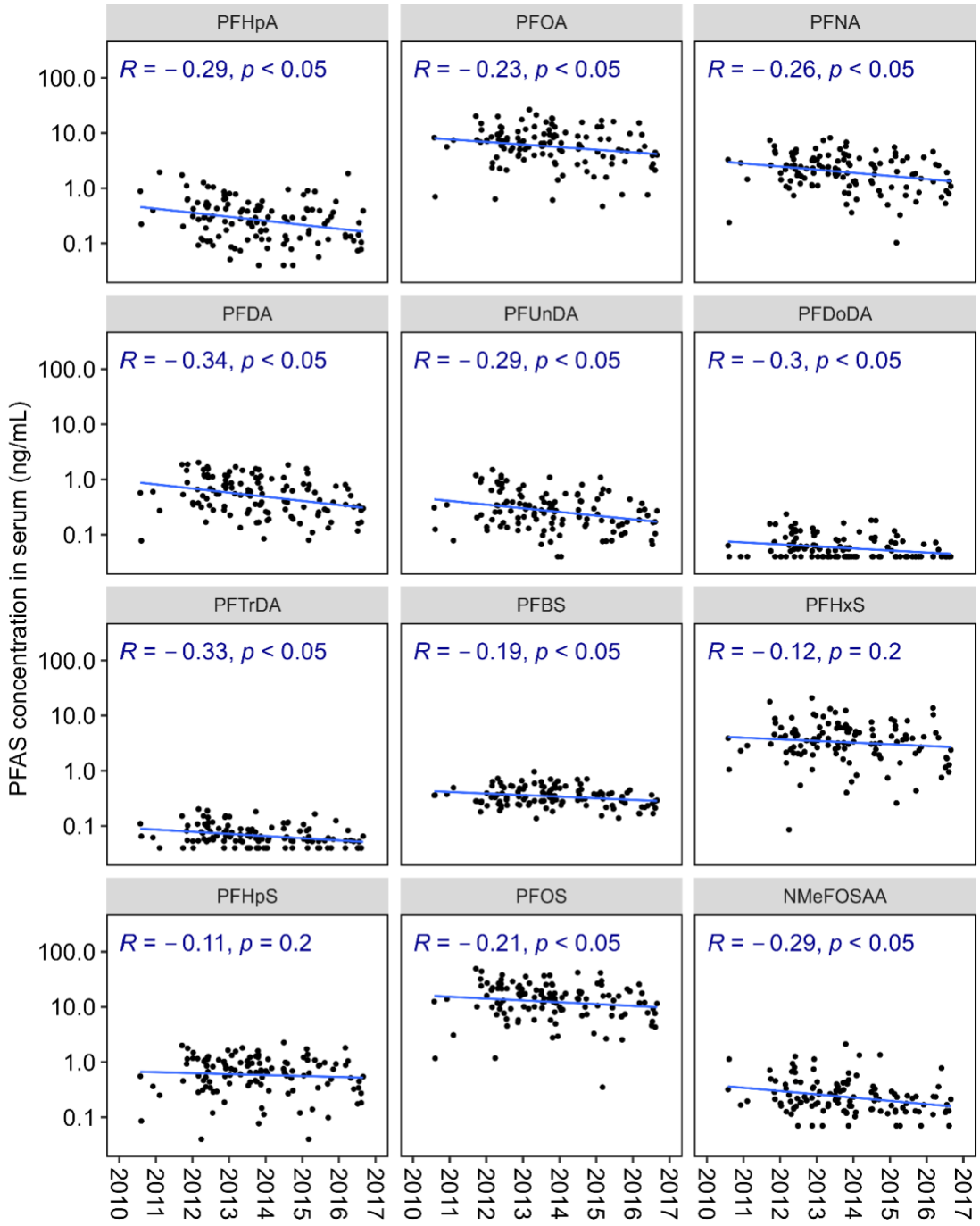

Figure S7. Temporal trend of PFAAs and PFAA precursors in serum over the study period (2010-2016). Sample size = 119. Only PFAS detected in over 50% of the serum samples are presented. Blue text on each plot represents the coefficient and p-value of the spearman correlation. Blue straight lines are linear trend lines.

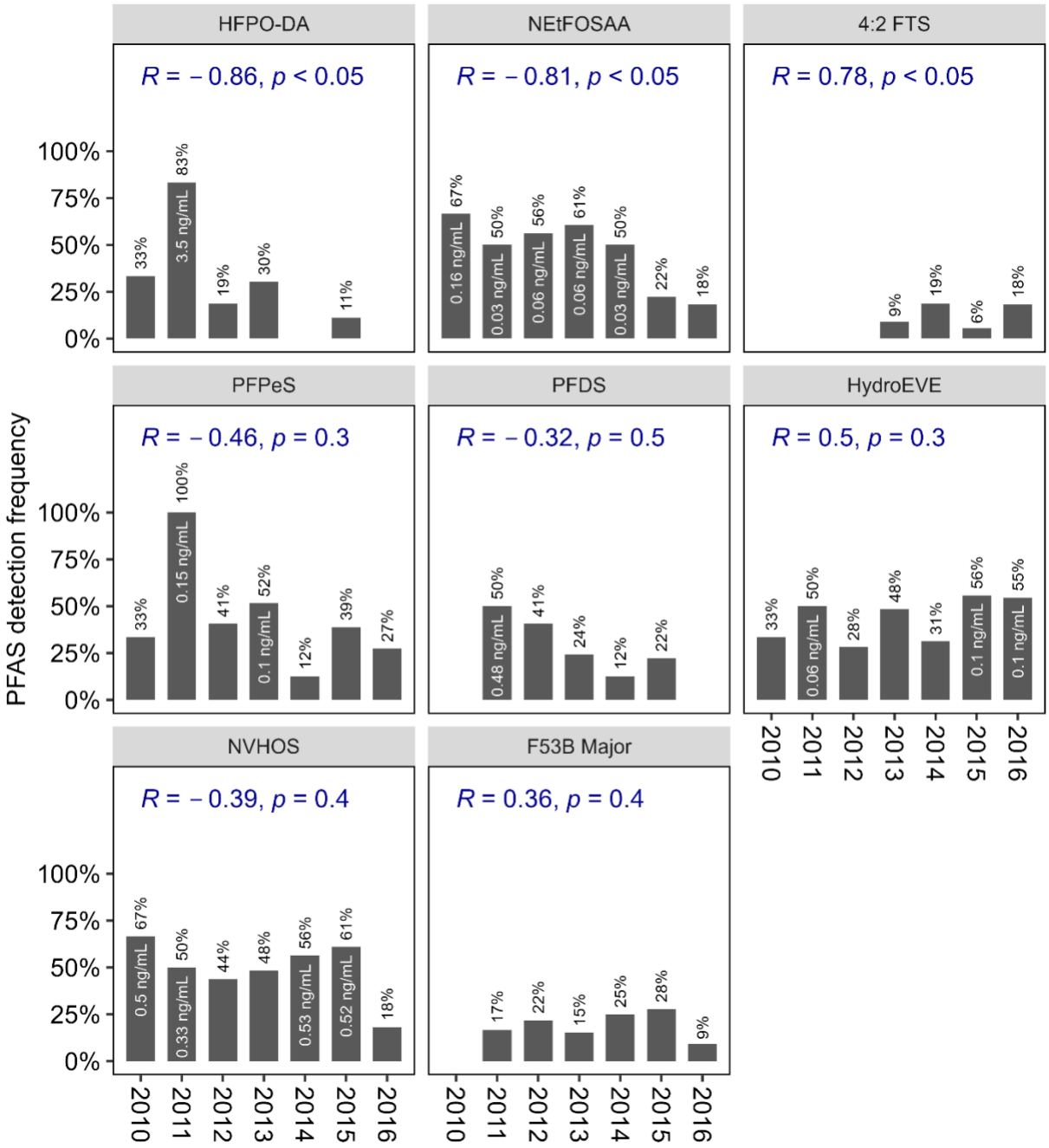

Figure S8. Temporal trend of PFAS with overall detection frequencies in serum samples between 8% to 50% from 2010 to 2016. Sample size = 119. Blue text on each plot represents coefficient and p-value of the spearman correlation.

Figure S9. Comparison of PFAS concentrations in serum between this study and Kotlarz et al.[3, 4]

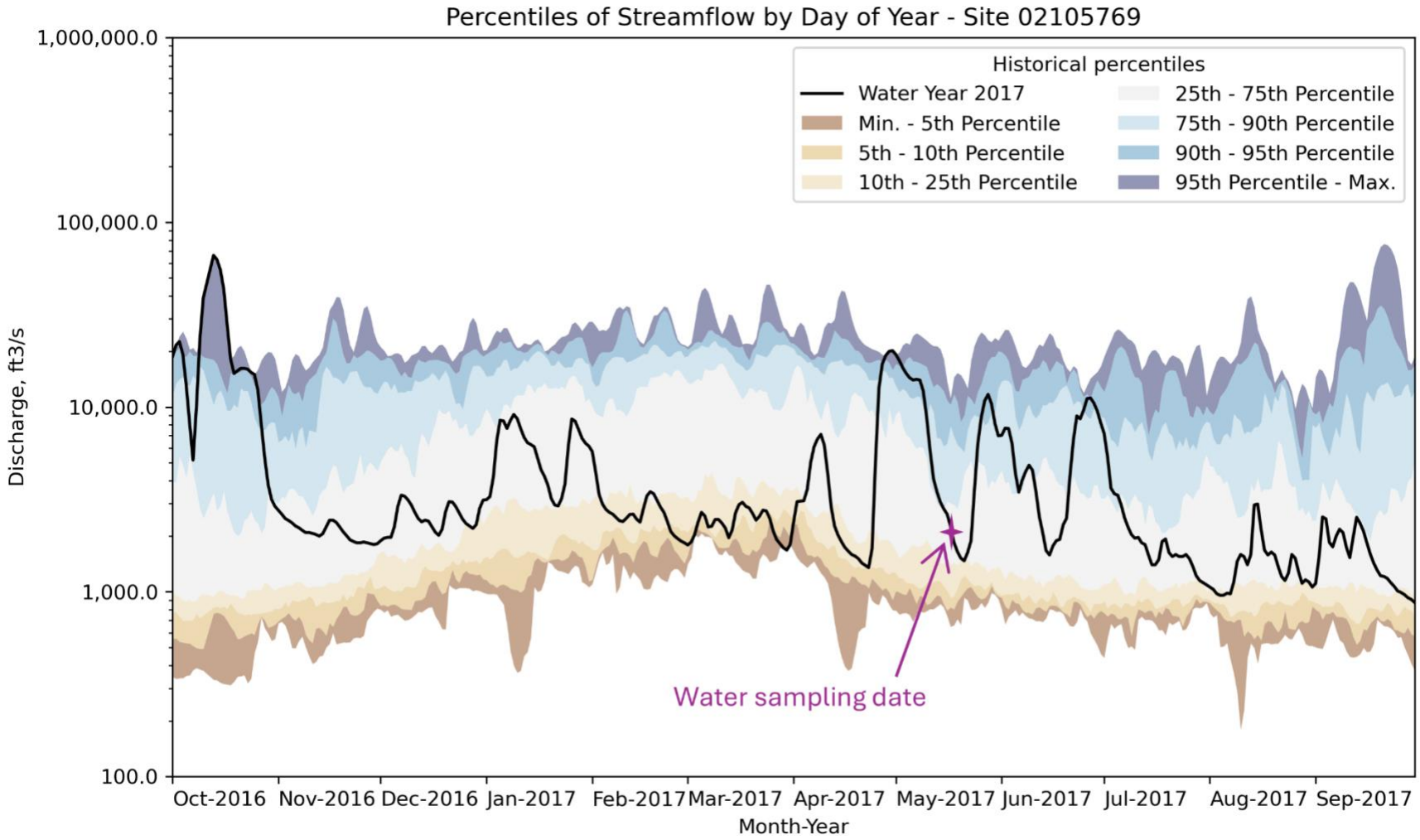

Figure S10.Streamflow of Cape Fear River at intake of the drinking water treatment plant for Wilmington, NC (Location 9) from October 2016 to September 2017 (Black line). Color shaded area: historical streamflow from 1980 to 2024.

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(3) Kotlarz, N.; Mccord, J.; Collier, D.; Lea, C. S.; Strynar, M.; Lindstrom, A. B.; Wilkie, A. A.; Islam, J. Y.; Matney, K.; Tarte, P.; et al. Measurement of Novel, Drinking Water-Associated PFAS in Blood from Adults and Children in Wilmington, North Carolina. *Environmental Health Perspectives* **2020**, *128* (7), 077005. DOI: 10.1289/ehp6837 (acccessed 2022-01-25T03:48:11).

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