

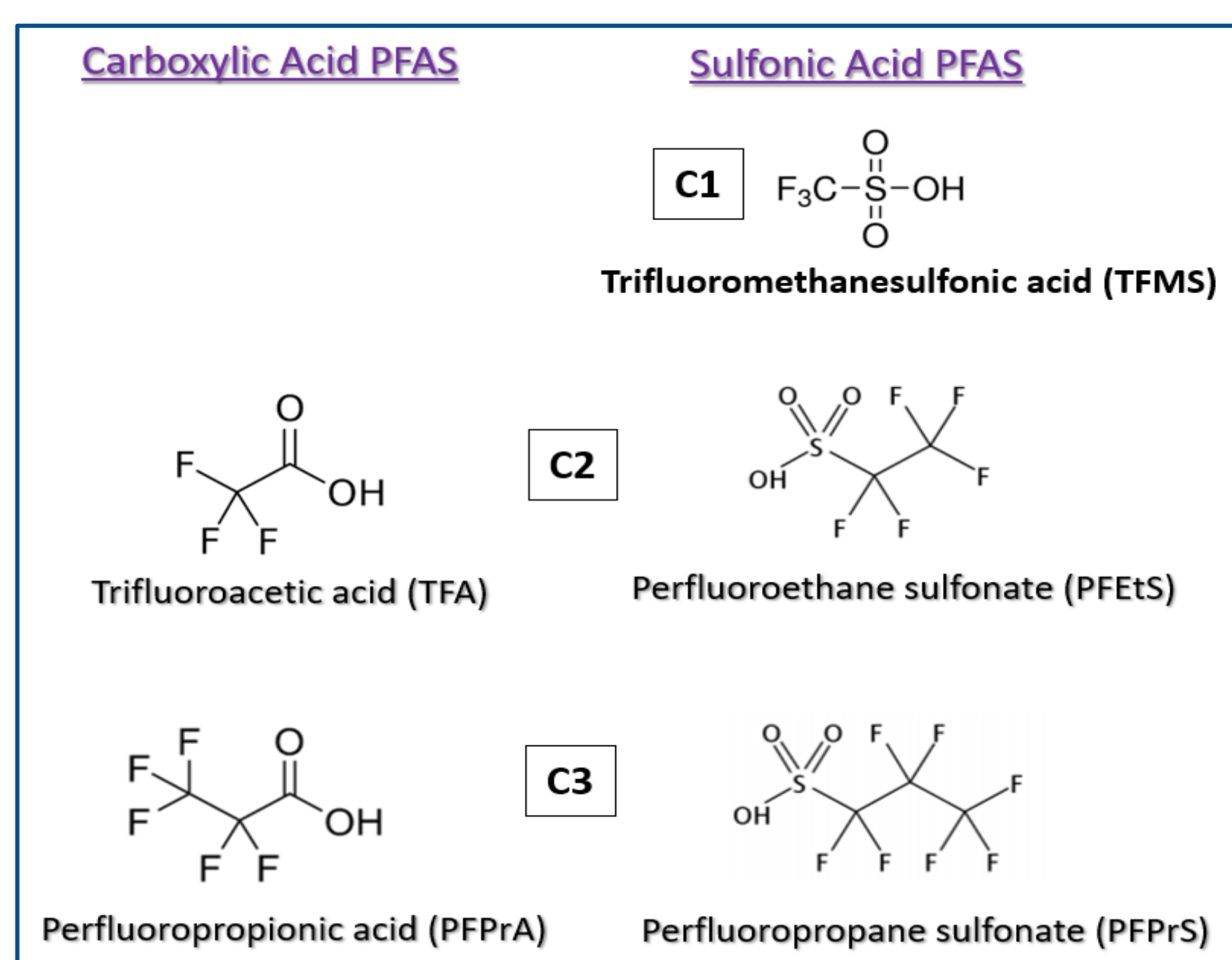
Simultaneous Analysis of Ultrashort-Chain to Long-Chain (C1 to C10) and Alternative PFAS in Human Plasma and Serum

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Introduction

Ultrashort-chain (USC) per- and polyfluoroalkyl substances (PFAS) are small and very polar compounds with carbon chain lengths shorter than C4 (Figure 1). Their ubiquitous and high levels of occurrence in environmental aquatic systems have raised significant concern in conjunction with long-chain PFAS contamination. Measuring USC PFAS in blood can not only monitor human exposure but also serves as a valuable tool for studying the potential risks associated with USC PFAS exposure. The high polarity of USC PFAS poses a challenge to current analytical practices based on the reverse-phased liquid chromatography, primarily due to insufficient chromatographic retention. In this study, a simple and reliable workflow was developed for the simultaneous analysis of C1 to C10 perfluoroalkyl carboxylic and sulfonic acids, along with four alternative PFAS, in human plasma and serum. The samples underwent a single-step protein precipitation procedure and were analyzed with a user-friendly LC-MS/MS method, implementing a polar-embedded LC column. Method accuracy and precision were evaluated with fortified fetal bovine serum. The method's validity was confirmed by accurately measuring targeted PFAS with known concentrations in NIST standard reference human plasma (1950) and serum (1957).

Figure 1: Structures of C1 to C3 PFAS



Methods

Table 1: Analytical Conditions (Waters Xevo TQ-S with Acquity UPLC)

| | | |
|--------------------------|--|-----------|
| Analytical Column | Ultra IBD 100 mm x 2.1 mm, 3 μm (Restek Cat.# 9175312) | |
| Delay Column | PFAS Delay Column (Restek Cat.# 27854) | |
| Mobile Phase A | 5 mM ammonium formate, 0.1% formic acid in water | |
| Mobile Phase B | Acetonitrile | |
| Gradient | Time (min) | %B |
| | 0.00 | 20 |
| | 7.00 | 95 |
| | 9.00 | 95 |
| | 11.00 | 20 |
| Flow Rate | 0.3 mL/min | |
| Injection Volume | 5 μL | |
| Column Temp. | 40°C | |
| Ion Mode | Scheduled MRM with negative ESI | |

Plasma and Serum Samples

Charcoal-stripped fetal bovine serum (FBS) obtained from MilliporeSigma (Burlington, MA) was utilized for method validation due to its absence of all analytes of interest with the exception of TFA. Subsequently, a TFA isotope, 13C-TFA, was employed as a surrogate to assess the method accuracy for TFA in FBS. NIST SRM 1950 metabolites in human plasma and NIST SRM 1957 organic contaminants in non-fortified human serum were purchased from NIST Store (Gaithersburg, MD). These SRMs are characterized by known concentrations of six or seven short-chain and long-chain PFAS.

Standard and Sample Preparation

The calibration standard solutions (100 μL) were prepared in the reverse osmosis water, supplemented with 1x phosphate-buffered saline, at the range of 0.05 to 40 ng/mL (ppb) in polypropylene HPLC vials. Five isotopically labeled PFAS were implemented as non-extracted or quantification internal standards (QIS) (see Table 2). A 5 μL aliquot of QIS working solution containing 40 ng/mL of 13C₃-PFBA, 20 ng/mL of 13C₂-PFHxA and 13C₄-PFOA, and 10 ng/mL of 13C₅-PFNA and 13C₂-PFDA, was added to the standard solution, followed by mixing with 200 μL of methanol containing 1.5% formic acid.

For the assessment of method accuracy and precision, 100 μL of FBS was fortified at 0.4, 2, 10, and 30 ppb with non-labeled analytes and isotopically labeled 13C-TFA, which served as a surrogate for the determination of TFA recovery. The fortified FBS was mixed with 5 μL of QIS working solution and 2 μL of extracted internal standard (EIS) working solution containing 50 ng/mL of 13C₃-PFPrA, 13C₄-PFBA, 13C₅-PFPeA, 13C₅-PFHxA, 13C₄-PFHpA, 13C₈-PFOA, 13C₉-PFNA, 13C₆-PFDA, 13C₃-PFHxS, and 13C₈-PFOS. A 200 μL aliquot of methanol containing 1.5% formic acid was then added to fortified FBS followed by vortexing for 30 seconds at 3000 rpm. After centrifugation at 4200 rpm for 10 minutes, approximately 150 μL of supernatant was transferred to a polypropylene vial insert placed in an HPLC vial and subsequently injected for LC-MS/MS analysis. For the analysis of non-fortified human plasma and serum samples, the same procedure for fortified FBS analysis was followed.

Results & Discussion

- LC-MS/MS Method Development:** A chromatographic method was established (see Table 1) for the analysis of C1 to C10 PFAS (Figure 2). The Ultra IBD column exhibited satisfactory retention for USC PFAS under reverse-phased conditions. More importantly, the extensive retention of USC PFAS, especially for the first-eluted analyte (TFA), led to reduced matrix interferences. The MS/MS transition parameters for each analyte are provided in Table 2.
- Linearity:** With quadratic regression (1/x weighted), all analytes showed acceptable linearities with r² >0.995 and deviations <20%. Table 3 shows differential linearity ranges of analytes, ranging from 0.05 ppb to 40 ppb, with variation at the lowest calibration concentration.
- Accuracy & Precision:** Three batches of analyses were conducted on different days, totaling nine repetitions at each fortified level. The averaged recovery and relative standard deviation (RSD) are presented in Table 3. All analytes exhibited recovery values within the range of 82.3 – 115% across three fortification levels. Satisfactory method precision was demonstrated with %RSD values ranging from 1.51 – 11.3%. Additionally, the results indicated that all EIS had recovery values within 20% of the nominal concentration.
- Measurement of C1 – C10 and Alternative PFAS in NIST 1950 and 1957:** Six preparations of each SRM were subjected to the analysis with the developed LC method. The results indicated that all measured EIS concentrations fell within 20% of the nominal concentration. Moreover, Table 4 illustrates that the averaged experimental concentrations of most PFAS closely matched the reference concentrations, with deviations within 20%. Although the measured PFDA concentration in NIST 1957 exhibited a slightly higher deviation of 26%, it remained within the deviation range of the reference concentration. These results demonstrated that the established method was suitable for accurate measurement of PFAS in both human plasma and serum. In addition to the reference PFAS, other measurable PFAS were also reported in Table 4.

Figure 2: Chromatogram for the Analysis of a 10 ppb Standard Solution

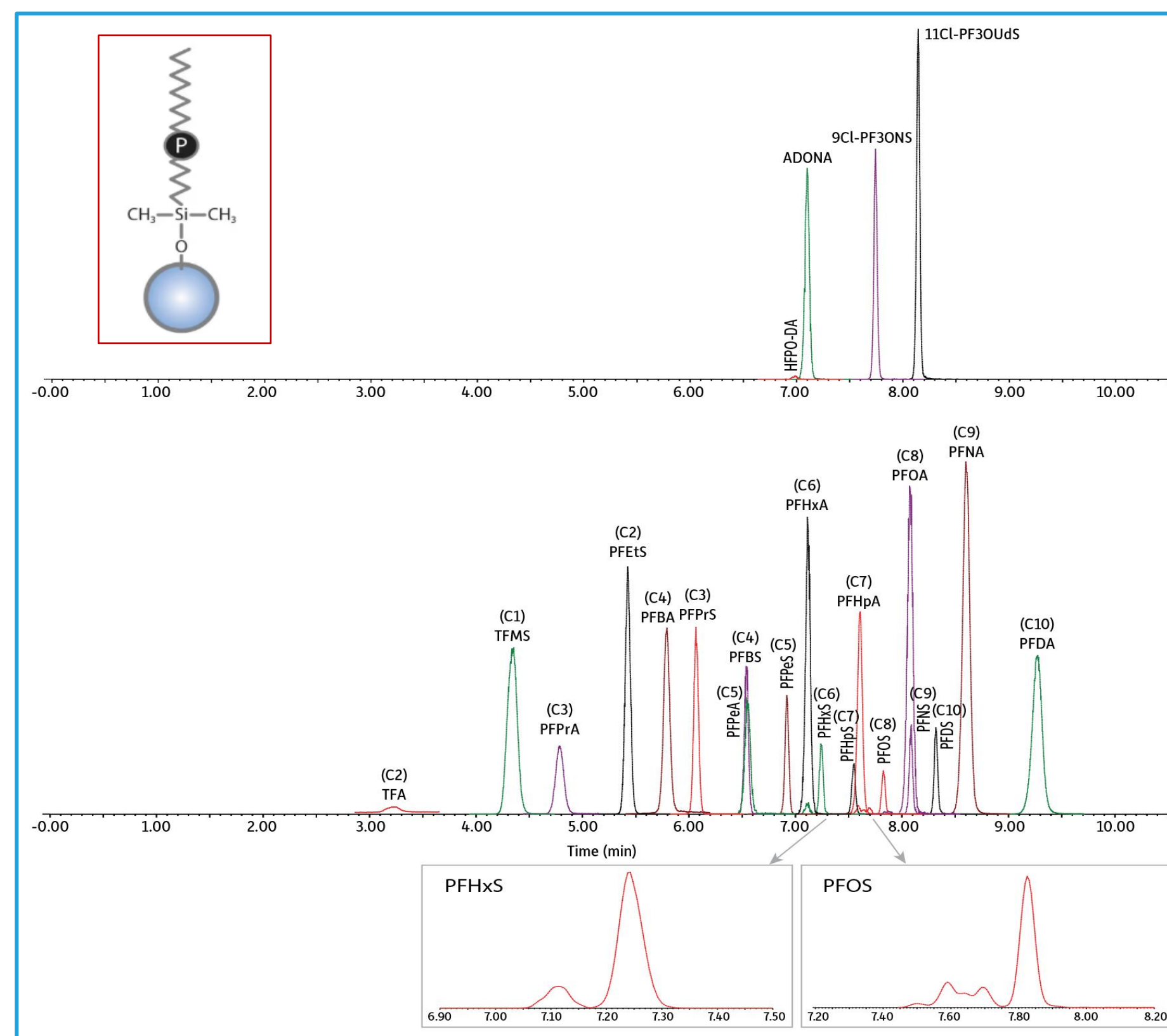


Table 2: MS Transitions and Analytes Retention Times

| Compounds | Retention Time (min) | Precursor Ion | Product ions* | Cone (V) | Collision (V) | Quantification Internal Standard |
|---|----------------------|------------------------------|---------------|----------|---------------|----------------------------------|
| Target Analytes | | | | | | |
| TFA | 3.25 | 113.03 [M-H] ⁻ | 69.01 | 10 | 10 | 13C ₃ -PFBA |
| PFPrA | 4.81 | 162.97 [M-H] ⁻ | 119.02 | 10 | 8 | 13C ₄ -PFBA |
| PFBA | 5.80 | 213.03 [M-H] ⁻ | 168.98 | 14 | 8 | 13C ₅ -PFBA |
| PFPeA | 6.56 | 262.97 [M-H] ⁻ | 218.97 | 2 | 6 | 13C ₅ -PFHxA |
| PFHxA | 7.13 | 313.10 [M-H] ⁻ | 268.97/118.99 | 2 | 8/20 | 13C ₄ -PFHxA |
| PFHpA | 7.62 | 363.16 [M-H] ⁻ | 319.09/169.06 | 8 | 10/18 | 13C ₄ -PFHxA |
| PFOA | 8.10 | 413.10 [M-H] ⁻ | 368.96/168.90 | 2 | 10/16 | 13C ₄ -PFOA |
| PFNA | 8.62 | 463.10 [M-H] ⁻ | 419.01/219.02 | 4 | 10/16 | 13C ₅ -PFNA |
| PFDA | 9.29 | 513.17 [M-H] ⁻ | 469.16/219.06 | 4 | 12/16 | 13C ₆ -PFDA |
| TFMS | 4.37 | 148.97 [M-H] ⁻ | 79.93/98.92 | 62 | 18/18 | 13C ₃ -PFBA |
| PFES | 5.44 | 198.90 [M-H] ⁻ | 79.92/98.91 | 38 | 22/22 | 13C ₃ -PFBA |
| PFPrS | 6.08 | 248.97 [M-H] ⁻ | 79.92/98.91 | 2 | 24/24 | 13C ₄ -PFBA |
| PFBS | 6.55 | 298.97 [M-H] ⁻ | 79.97/98.89 | 2 | 26/26 | 13C ₅ -PFHxA |
| PFPeS | 6.93 | 349.10 [M-H] ⁻ | 79.98/98.98 | 6 | 32/30 | 13C ₅ -PFHxA |
| PFHxS | 7.24 | 398.90 [M-H] ⁻ | 79.97/98.89 | 56 | 32/34 | 13C ₅ -PFHxA |
| PFHpS | 7.56 | 449.17 [M-H] ⁻ | 79.98/98.97 | 4 | 42/38 | 13C ₅ -PFHxA |
| PFOS | 7.82 | 499.03 [M-H] ⁻ | 79.92/98.90 | 8 | 40/40 | 13C ₅ -PFOA |
| PFNS | 8.09 | 549.10 [M-H] ⁻ | 79.92/98.83 | 12 | 42/40 | 13C ₅ -PFOA |
| PFDS | 8.32 | 599.17 [M-H] ⁻ | 79.98/98.83 | 8 | 44/46 | 13C ₅ -PFNA |
| HFPO-DA | 7.01 | 285.03 [M-COOH] ⁻ | 169.02/185.02 | 2 | 6/16 | 13C ₄ -PFHxA |
| ADONA | 7.11 | 376.90 [M-H] ⁻ | 250.93/84.97 | 22 | 12/26 | 13C ₄ -PFHxA |
| 9Cl-PF3ONS | 7.75 | 530.78 [M-H] ⁻ | 350.85/82.96 | 12 | 26/24 | 13C ₄ -PFHxA |
| 11Cl-PF3OUdS | 8.15 | 630.78 [M-H] ⁻ | 450.80/82.95 | 8 | 26/32 | 13C ₄ -PFNA |
| Extracted Internal Standards | | | | | | |
| 13C ₃ -TFA | 3.25 | 114.03 [M-H] ⁻ | 69.03 | 10 | 8 | 13C ₃ -PFBA |
| 13C ₄ -PFPrA | 4.81 | 165.97 [M-H] ⁻ | 120.96 | 10 | 11 | 13C ₄ -PFBA |
| 13C ₅ -PFBA | 5.80 | 217.03 [M-H] ⁻ | 171.98 | 2 | 8 | 13C ₅ -PFBA |
| 13C ₅ -PFPeA | 6.56 | 267.97 [M-H] ⁻ | 222.99 | 2 | 6 | 13C ₅ -PFHxA |
| 13C ₄ -PFHxA | 7.13 | 318.03 [M-H] ⁻ | 272.93 | 2 | 7 | 13C ₄ -PFHxA |
| 13C ₄ -PFHpA | 7.62 | 366.90 [M-H] ⁻ | 321.93 | 2 | 10 | 13C ₄ -PFOA |
| 13C ₅ -PFOA | 8.10 | 420.97 [M-H] ⁻ | 375.94 | 2 | 10 | 13C ₄ -PFOA |
| 13C ₅ -PFNA | 8.62 | 471.97 [M-H] ⁻ | 426.87 | 4 | 8 | 13C ₅ -PFNA |
| 13C ₆ -PFDA | 9.29 | 518.90 [M-H] ⁻ | 473.87 | 4 | 13 | 13C ₆ -PFDA |
| 13C ₃ -PFHxS | 7.24 | 401.90 [M-H] ⁻ | 79.97 | 2 | 32 | 13C ₅ -PFHxA |
| 13C ₈ -PFOS | 7.82 | 506.84 [M-H] ⁻ | 79.97 | 4 | 48 | 13C ₅ -PFOA |
| Non-Extracted Internal Standards | | | | | | |
| 13C ₄ -PFBA | 5.80 | 215.97 [M-H] ⁻ | 171.97 | 10 | 8 | |
| 13C ₅ -PFHxA | 7.13 | 314.97 [M-H] ⁻ | 269.93 | 8 | 8 | |
| 13C ₅ -PFOA | 8.10 | 416.87 [M-H] ⁻ | 371.88 | 2 | 8 | |
| 13C ₅ -PFNA | 8.62 | 467.87 [M-H] ⁻ | 422.89 | 16 | 10 | |
| 13C ₆ -PFDA | 9.29 | 514.87 [M-H] ⁻ | 469.84 | 8 | 10 | |

* Quantifier Ion/Qualifier Ion

Table 3: Accuracy & Precision Analysis of Fortified Fetal Bovine Serum

| Analytes | Linearity Range (ng/mL) | LOD (ng/mL) | Average Recovery (RSD, %) Fortified Concentration (ng/mL) | | | |
|--------------|-------------------------|-------------|---|-------------|-------------|-------------|
| | | | 0.4 | 2 | 10 | 30 |
| 13C-TFA | 0.50 - 40 | 0.1250* | - | 90.6 (9.28) | 98.0 (2.99) | 100 (10.7) |
| PFPrA | 0.25 - 40 | 0.0102 | 108 (2.53) | 115 (0.965) | 105 (6.99) | - |
| PFBA | 0.10 - 40 | 0.0222 | 104 (5.57) | 109 (1.75) | 104 (1.51) | - |
| PFPeA | 0.10 - 40 | 0.0125 | 97.4 (3.25) | 93.3 (4.19) | 89.1 (5.57) | - |
| PFHxA | 0.10 - 40 | 0.0098 | 99.2 (2.46) | 109 (4.65) | 102 (6.31) | - |
| PFHpA | 0.10 - 40 | 0.0050 | 86.7 (5.37) | 99.2 (2.04) | 89.2 (1.80) | - |
| PFOA | 0.10 - 40 | 0.0051 | 94.8 (8.24) | 107 (5.30) | 95.6 (3.35) | - |
| PFNA | 0.10 - 40 | 0.0012 | 96.2 (1.44) | 111 (1.87) | 99.1 (2.62) | - |
| PFDA | 0.10 - 40 | 0.0083 | 93.6 (1.93) | 102 (2.03) | 95.5 (3.64) | - |
| TFMS | 0.05 - 40 | 0.0070 | 89.4 (7.33) | 88.8 (3.80) | 91.9 (3.19) | - |
| PFES | 0.05 - 40 | 0.0020 | 98.0 (2.62) | 103 (1.47) | 99.3 (2.26) | - |
| PFPrS | 0.05 - 40 | 0.0030 | 98.1 (8.18) | 108 (4.23) | 98.9 (4.15) | - |
| PFBS | 0.05 - 40 | 0.0124 | 88.0 (8.84) | 94.1 (4.95) | 86.5 (6.17) | - |
| PFPeS | 0.10 - 40 | 0.0031 | 94.8 (4.57) | 100 (8.05) | 94.3 (5.43) | - |
| PFHxS | 0.10 - 40 | 0.0115 | 85.8 (7.88) | 96.0 (10.0) | 92.2 (8.98) | - |
| PFHpS | 0.10 - 40 | 0.0088 | 92.5 (6.75) | 99.8 (6.45) | 93.4 (5.74) | - |
| PFOS | 0.10 - 40 | 0.0200 | 97.8 (8.66) | 97.9 (7.01) | 95.9 (3.37) | - |
| PFNS | 0.10 - 40 | 0.0129 | 92.1 (7.98) | 94.2 (4.43) | 91.9 (2.78) | - |
| PFDS | 0.10 - 40 | 0.0111 | 92.6 (8.20) | 82.3 (3.48) | 87.6 (3.55) | - |
| HFPO-DA | 0.50 - 40 | 0.1875 | - | 99.9 (11.3) | 91.1 (8.95) | 90.4 (6.13) |
| ADONA | 0.10 - 40 | 0.0035 | 90.4 (7.14) | 106 (4.67) | 95.7 (4.25) | - |
| 9Cl-PF3ONS | 0.10 - 40 | 0.0031 | 95.8 (4.20) | 93.7 (5.12) | 93.8 (6.71) | - |
| 11Cl-PF3OUdS | 0.10 - 40 | 0.0023 | 106 (5.10) | 84.5 (4.20) | 97.2 (4.86) | - |

* For non-labeled TFA

Table 4: The Quantification of PFAS in NIST 1950 and 1957

| Analytes | Reference Concentration (ng/mL) NIST 1950 | Experimental Avg. Concentration (ng/mL) | Experimental Precision (%RSD) | Concentration Ratio (%) |
|------------|---|---|-------------------------------|-------------------------|
| NIST 1950* | | | | |
| PFOA | 3.27 ± 0.06 | 3.12 | 3.70 | 95.4 |
| PFNA | 0.720 ± 0.028 | 0.85 | 0.74 | 117 |
| PFDA | 0.322 ± 0.007 | 0.30 | 3.47 | 91.6 |
| PFHxS | 3.25 ± 0.08 | 2.91 | 6.26 | 89.5 |
| PFOS | 10.64 ± 0.13 | 12.57 | 2.92 | 118 |
| TFA | - | 5.74 | 4.45 | - |
| PFPrA | - | 0.26 | 6.90 | - |
| PFHpA | - | 0.23 | 3.04 | - |
| TFMS | - | 0.08 | 3.95 | - |
| PFPeS | - | 0.15 | 4.31 | - |
| PFHpS | - | 0.36 | 4.90 | - |
| PFDS | - | 0.10 | 4.64 | - |
| NIST 1957* | | | | |
| PFHpA | 0.305 ± 0.051 | 0.28 | 2.16 | 92.1 |
| PFOA | 5.00 ± 0.44 | 4.21 | 1.91 | 84.2 |
| PFNA | 0.878 ± 0.077 | 0.77 | 1.38 | 87.9 |
| PFDA | 0.39 ± 0.12 | 0.29 | 2.69 | 74.4 |
| PFHxS | 4.00 ± 0.83 | 3.35 | 9.88 | 83.8 |
| PFOS | 21.1 ± 1.3 | 20.46 | 3.81 | 97.0 |
| TFA | - | 3.22 | 3.79 | - |
| TFMS | - | 0.07 | 0.10 | - |
| PFPeS | - | 0.10 | 7.97 | - |
| PFHpS | - | 0.48 | 6.20 | - |
| PFDS | - | 0.10 | 4.23 | - |

* The reference concentration for NIST 1957 is present as mass fraction (μg/kg)

Conclusions

A simple and reliable workflow was established in this study to provide a unique solution for the integration of ultrashort-chain compounds into the measurement of PFAS in human plasma and serum. The reported method was rugged, accurate, and precise by implementing a polar-embedded column for chromatographic analysis. Most importantly, this solution can offer a valuable tool for gaining insights into human exposure to these emergent ultrashort-chain PFAS.