Application Note

Extracting and Analyzing PFAS from Human Serum

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Abstract

Due to the bioaccumulative nature of per- and polyfluorinated alkyl substances (PFAS), monitoring levels in human biofluids can help gain understanding into exposure levels and pathways. Since PFAS are not rapidly metabolized or removed from the body, we can get an understanding of these levels of exposure by studying serum content. This work developed an optimized solid phase extraction (SPE) method for the extraction of PFAS from human serum utilizing Oasis WAX µElution 96-well plates. Analysis was performed on Xevo TQ-S micro coupled with an ACQUITY UPLC I-Class PLUS modified with PFAS kit components. An ACQUITY HSS T3 Column provided crucial separations of PFAS from serum steroid sulfate interferences. Method verification was performed using six different pooled lots of human serum. In addition, the optimized extraction method was verified using a standard reference material from NIST (SRM 1957) resulting in consistently accurate results. The final method proved to be accurate and robust for the extraction and analysis of 30 PFAS from a variety of different common PFAS chemistry classes.

Benefits

- An optimized method for studying and understanding human exposure levels to PFAS compounds
- Confidence in results with the utilization of the PFAS Kit for LC modification to isolate possible system and solvent contaminants
- Robust method for accurate analysis of PFAS in human serum using the Xevo TQ-S micro
- Efficient sample clean up using Oasis WAX 96-well plates, an optimal chemistry for extraction of PFAS

Introduction

Per- and polyfluorinated alkyl substances (PFAS) have been used for decades in manufacturing processes, consumer products, and firefighting foams. Their extensive use has led to the persistent release of these pollutants into the environment. Subsequently, humans are persistently exposed to PFAS through consumer product use, contaminated drinking water and food consumption, and air pollution. Thousands of PFAS are known to exist, with limited knowledge on their toxicology. Studies on perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) have implicated that these types of compounds have significant health effects in humans including impacts on immunological, reproductive, and developmental pathways as well as liver and kidney damage.[1,2] PFOA is designated as a possible carcinogen with links to kidney and testicular cancer.[3,4] The strong carbon-fluorine bonds that make PFAS great coatings also make them highly resistant to metabolism. Therefore, they are highly likely to bioaccumulate in humans. Numerous biomonitoring studies have concluded that most humans have detectable amounts of various PFAS compounds in their blood.[5] Therefore, reliable and accurate methods are necessary to continue our understanding of the burden of PFAS in humans for purposes such as occupational health monitoring, environmental exposure monitoring, and toxicology studies.

Serum is a complex matrix to work with so removal of matrix compounds is critical to reproducible and accurate PFAS detection. This is especially true as studies continue to expand to include larger panels of PFAS as the focus shifts away from only studying PFOA and PFOS to a broader range of compounds. Protein precipitation and/or dilution are insufficient strategies for sample preparation on their own. Solid phase extraction (SPE) is a more practical solution for sample preparation. Liquid chromatography coupled to tandem quadrupole mass spectrometry (LC-MS/MS) provides the most sensitive and selective analysis option. The following work will outline a complete SPE method for extraction of PFAS from human serum using Oasis WAX 96-well µElution plates with analysis on Xevo TQ-S micro.

Experimental

Sample Preparation

Human serum samples were extracted by solid phase extraction using Oasis WAX 96-well µElution plates

containing 2 mg sorbent per well. Fetal Bovine Serum (FBS) was extracted as the matrix for the calibration curve, as a suitable PFAS free matrix substitute. The full protocol for SPE extraction can be seen in Figure 1. NIST Standard Reference Material (SRM) 1957 (non-fortified human serum) was extracted with every batch of samples to ensure successful sample preparation. A suite of 30 PFAS compounds was used in this study representing a range of PFAS groups such as carboxylic acids, sulfonic acids, sulfonamides, sulfonamidoacetic acids, fluorotelomer sulfonates, and perfluoroethers. Isotopically labelled internal standards were used for each compound where available.

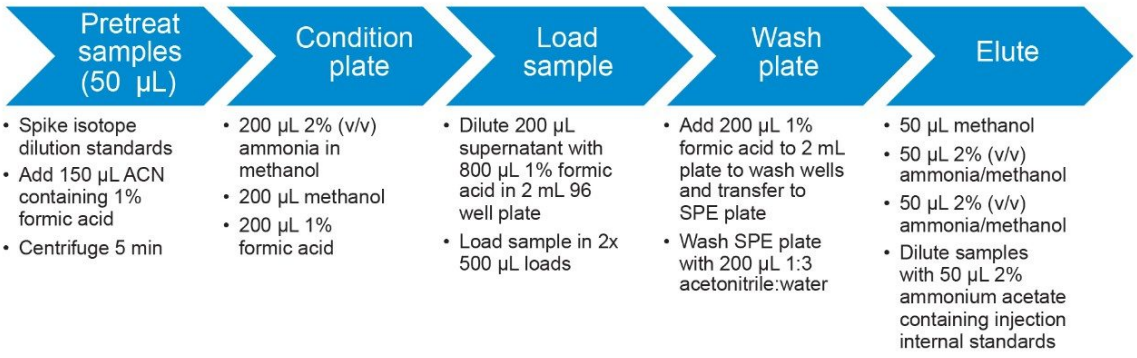

Figure 1. SPE protocol for PFAS in human serum using Oasis WAX 96-well µElution plates.

LC Conditions

LC system:	ACQUITY UPLC I-Class PLUS modified with PFAS Kit
Dilution plate:	96-well sample collection plate, 2 mL square well
Collection plate:	96-well sample well collection plate, 700 µL round well
Column:	ACQUITY UPLC HSS T3 2.1 mm x 100 mm, 1.8 µm
Column temp.:	35 °C

Sample temp.:	4 °C
Injection volume:	5 µL
Flow rate:	Varied (see gradient table)
Mobile phase A:	95:5 water:methanol + 2 mM ammonium acetate
Mobile phase B:	Methanol + 2 mM ammonium acetate

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
0	0.3	100	0	6
1	0.3	80	20	6
6	0.3	55	45	6
13	0.3	20	80	6
14	0.4	5	95	6
17	0.4	5	95	6
18	0.3	100	0	6
22	0.3	100	0	6

MS Conditions

MS system:	Xevo TQ-S micro
Ionization mode:	ESI-
Capillary voltage:	0.5 kV
Desolvation temp.:	300 °C
Desolvation flow:	900 L/hr
Cone flow:	100 L/hr
Collision energy:	Compound dependent (see Appendix table for conditions)
Cone voltage:	Compound dependent (see Appendix table for conditions)
Divert flow to waste:	0–3 and 16–22 min

Data Management

Chromatography software:	MassLynx 4.2 SCN1001
MS software:	MassLynx 4.2 SCN1001
Informatics:	TargetLynx XS

Results and Discussion

Method Optimization

Oasis Weak Anion Exchange (WAX) was chosen as the SPE chemistry for extraction as it is widely used for PFAS extraction from water and other environmental samples. WAX provides both anion exchange and reverse phase retention mechanisms allowing WAX to retain a wide range of PFAS chemistries. Therefore, a similar basic approach to the protocol used for PFAS extraction from water was adapted for serum adjusting the sample pretreatment and plate washing steps accordingly for serum.

Sample pretreatment proved to be the most essential step that needed to be optimized in order to disrupt PFAS protein binding in the serum samples. A dilution in 50% formic acid was compared to protein precipitation followed by dilution using 1% formic acid. Protein precipitation ratios tested included 1:1 (serum:solvent) acetonitrile, 1:3 acetonitrile, and 1:1 methanol. Dilution of the organic phase following protein precipitation is necessary to retain PFAS on the WAX chemistry. To determine the efficiency of each pretreatment option tested, the treated samples were loaded onto the WAX plate and the load waste was collected and analyzed. Detection of PFAS in the load waste indicates the PFAS were not being retained during sample loading and were still most likely bound to protein or other components in the serum matrix. Figure 2 shows selected chromatograms of the analysis of the load waste across the range of PFAS chemistries in the method. From this figure, it is clear that protein precipitation is required, since the formic acid diluted serum caused a significant amount of breakthrough during the sample load. Of the three protein precipitation steps tested, the 1:3 serum:acetonitrile method resulted in almost no PFAS breakthrough on the load.

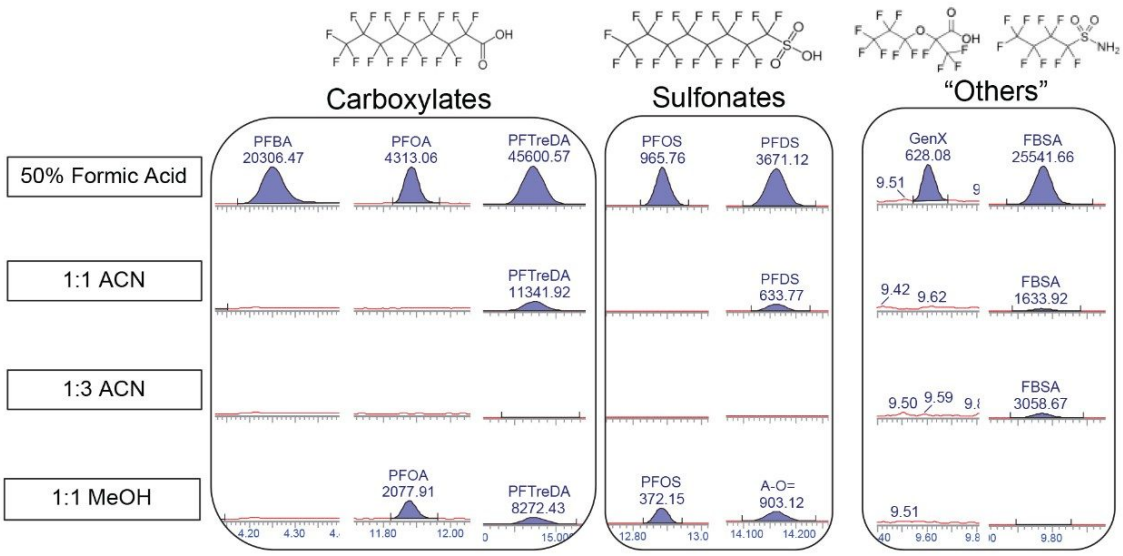

Figure 2. Waste portion collected during sample loading after each sample pretreatment condition tested.

Column optimization also had to be performed due to serum specific interferences that cannot be effectively removed with SPE. Various steroid sulfates, which are metabolites of steroid precursors and degradation products, have been known to coelute with PFAS such as PFOS and PFHxS.[6,7] Initially, an ACQUITY BEH C_{18} Column was utilized to be consistent with previous PFAS applications, but this resulted in interferent coelutions.

Figure 3 demonstrates this issue comparing chromatography of PFHxS on both the BEH C_{18} and HSS T3 Columns. Using the BEH C_{18} Column, the matrix interferent could easily be missed or mistaken for one of the branched PFHxS isomers. Additionally, when the peaks are smoothed during normal automated data processing procedures, this interferent would most likely be smoothed and integrated into the linear PFHxS peak. This would overestimate the concentration of PFHxS in samples. Resolution of the matrix interferent peaks was achieved using the HSS T3 Column, allowing for much more accurate and reliable results. Therefore, the ACQUITY HSS T3 Column was utilized for all serum sample analysis.

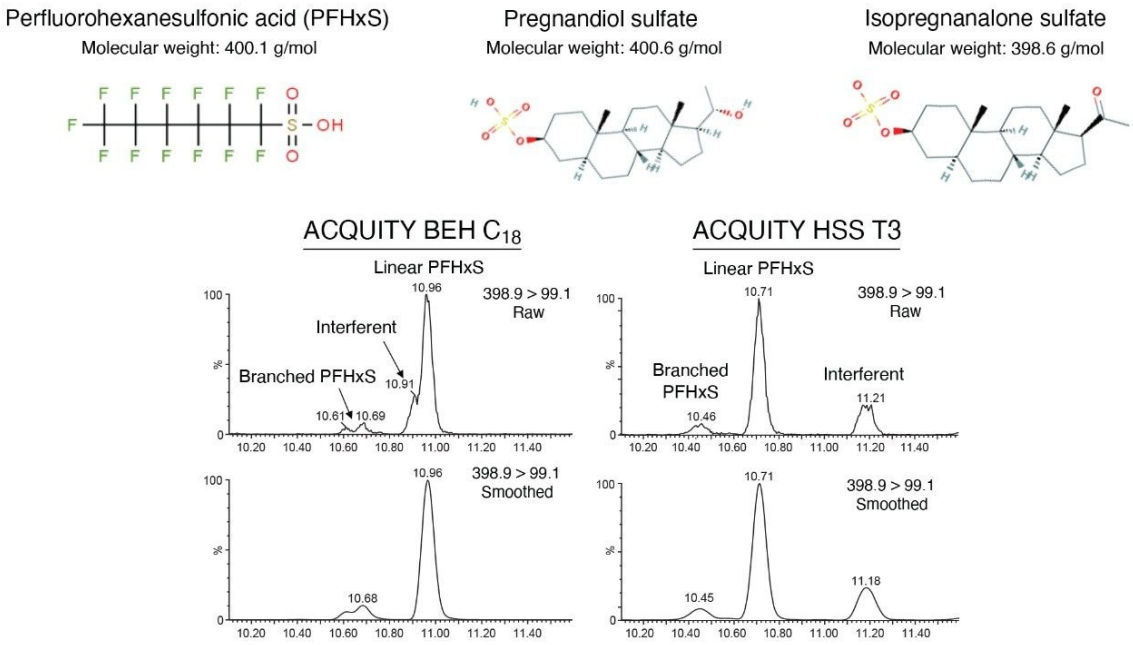

Figure 3. (top) Perfluorohexanesulfonic acid (PFHxS) and two human serum steroid sulfate compounds that have been determined to be potential interferents when using LC-MS/MS. (bottom) Comparison of the BEH C_{18} and HSS T3 Columns for the chromatographic resolution of PFHxS from human serum specific interference compounds.

Method Performance

Linearity and accuracy of calibration curves generated using FBS as the matrix were evaluated and results across the PFAS chemistry range are shown in Figure 4. In general, FBS proved to be a satisfactory PFAS-free matrix replacement for this application and behaved similar to human serum as far as analyte recovery and response. Calibration curves were linear in the tested range of 0.05–20 ng/mL and all compounds exhibited R^2 values of ≥0.996. The only exception was 6:2 FTS, producing an R^2 value of 0.992. Residual values for each compound across the calibration range were also evaluated as a demonstration of accuracy and reproducibility. The residual percentage is a measure of the percentage difference of the calculated concentration of the standard from the theoretical response expected from the calibration data. The smaller the residual range, the more accurate and reproducible the data. Residuals for the native PFAS were ≤15% at the limit of quantitation (LOQ) and ≤10% across the rest of the curve. Additionally, residuals were observed to be ≤10% for isotope labelled standards. QC samples of varying concentrations were also injected throughout the duration of sample

analysis and accuracy of QCs were within 20% around the LOQ and 15% at all other concentrations.

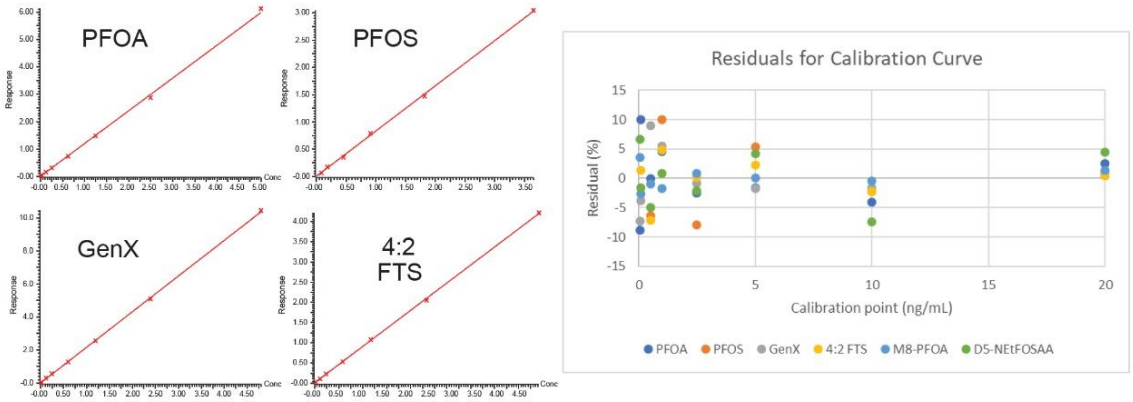

Figure 4. Representative calibration curves of four PFAS compounds in method (left) and the residual percentages for representative native and isotopically labelled standards (right).

Recovery of the 30 PFAS from human serum matrix was evaluated using the internal standards to correct for any matrix effects. Figure 5 shows the recovery of each compound from six different lots of pooled human serum fortified across a range of 0.1 to 10 ng/mL.

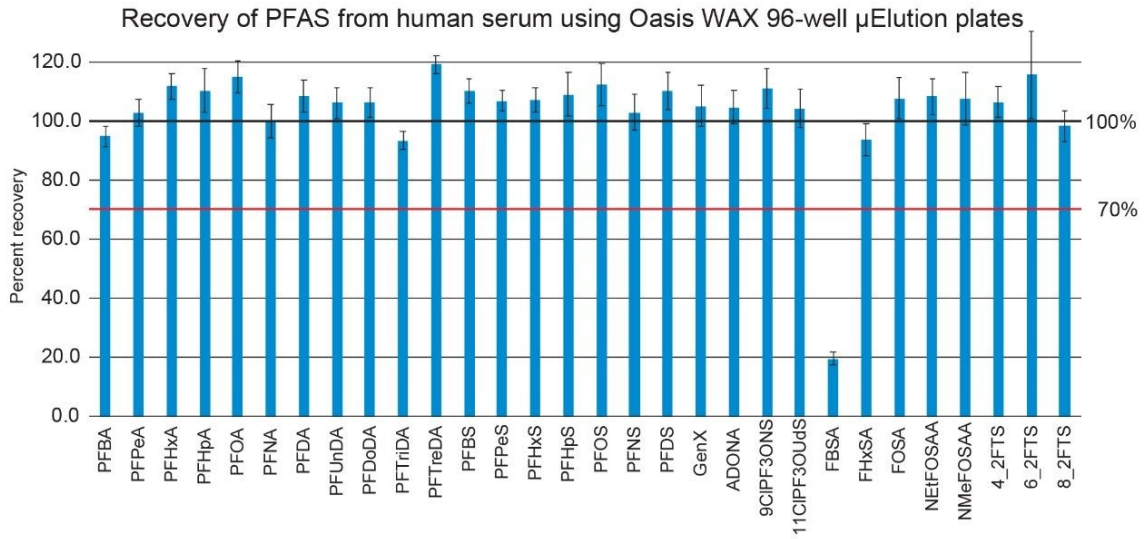

Figure 5. Average adjusted recovery of 30 PFAS included in method extracted from six different lots of pooled human serum fortified across a range of 0.1 to 10 ng/mL. Error bars represent the percent RSD for each compound across these 18 data points.

With the exception of the C4 sulfonamide (FBSA), recoveries were within an acceptable range of 85 to 120%. Recovery of FBSA was approximately 20% and is due to this compound being a low molecular weight, neutral PFAS compound that may not be best suited to a multiresidue extraction. This compound also did not have a suitable internal standard available to aid in isotope dilution correction. Although FBSA recovery was low, it still was detectable allowing for identification, if present, in a sample. The SPE procedure could be slightly altered if increased recovery of this compound was crucial. For example, the second SPE plate wash using 1:3 acetonitrile:water could be eliminated to recover more FBSA. Alternately, if neutral compounds were the only PFAS of interest, a reverse phase chemistry, such as Oasis HLB, could be utilized instead of WAX. However, this would affect the performance and recovery of the ionic PFAS compounds.

In addition to recovery, method performance was continually evaluated through the use of a Standard Reference Material (SRM) from NIST. Replicates of NIST SRM 1957, non-fortified human serum, were extracted with every batch of samples to ensure consistent accurate results. This reference material contains certified levels of seven PFAS naturally occurring in pooled human serum, ranging in concentration from 0.172 to 21.1 µg/kg (or 0.043 to 5.27 ng/L). Since this SRM covers a concentration range of parts per trillion (ppt) through parts per billion (ppb), it is a robust test of the methodology. The accuracy and robustness of the method is demonstrated in Figure 6.

The bar graph shows the experimentally calculated concentration of eight replicates of SRM 1957 extraction and analysis compared to the certified NIST values. The scatter plot demonstrates the percent difference of the experimental results from the certified range, with all measurements within ±10% of certified values. PFOS is the only compound that consistently produced slightly higher experimental results, but this is most likely due to a difference in the way the branched and linear isomers were handled during data processing. Additionally, percent RSD of the eight replicates were all below 4%, indicating the sample extraction and analysis is extremely robust.

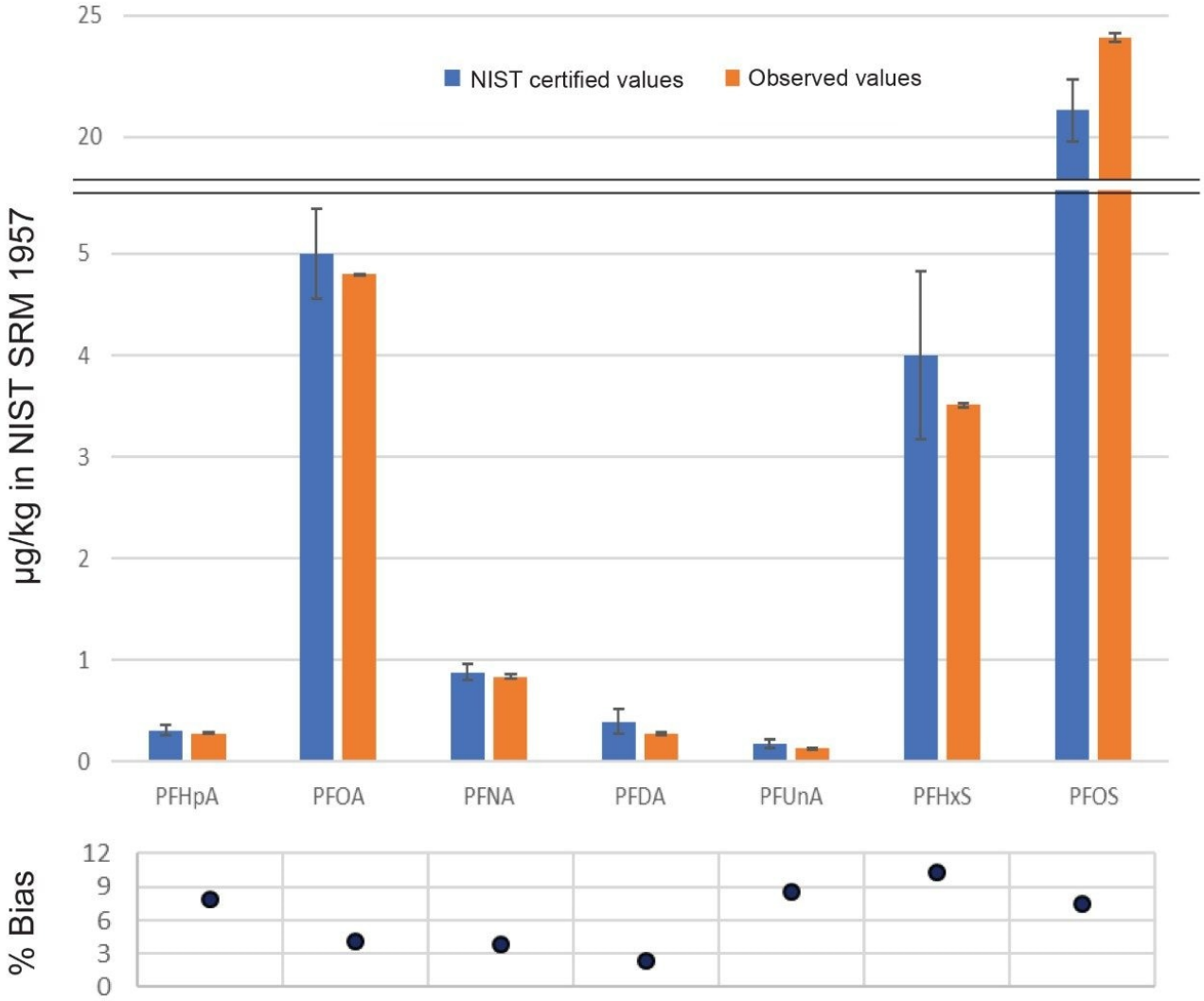

Figure 6. Analysis results for 8 replicates of the NIST 1957 standard reference material. (top) Concentration values determined in the SRM sample. Blue bars represent the certified levels of each PFAS in the SRM. Orange bars represent the observed, or experimentally determined values. (bottom) Calculated percent difference of the observed values from the NIST certified values.

Sample Analysis

The methodology outlined above was used to determine the PFAS levels of six different lots of pooled human serum, containing a mix of genders, ages, and ethnicities. All six lots contained identifiable levels of PFAS. Figure

7 demonstrates examples of the major PFAS identified in 3 of the 6 lots tested, representing individual PFAS identified, the total PFAS level, and ratios of individual PFAS that make up the total PFAS level. Even though very similar PFAS were identified in all human serum lots, they were present at strikingly different concentrations. Also, the ratio of each PFAS to total PFAS was different between each lot. Additionally, the ratio of branched to linear isomers detected in human samples (Figure 8) was quite different than the ratio present in analytical standards. The branched to linear ratio can indicate the PFAS synthesis mechanism. Together, isomer ratios and individual PFAS identifications can be used to create a "PFAS fingerprint" that could potentially help understand exposure pathways or sources.

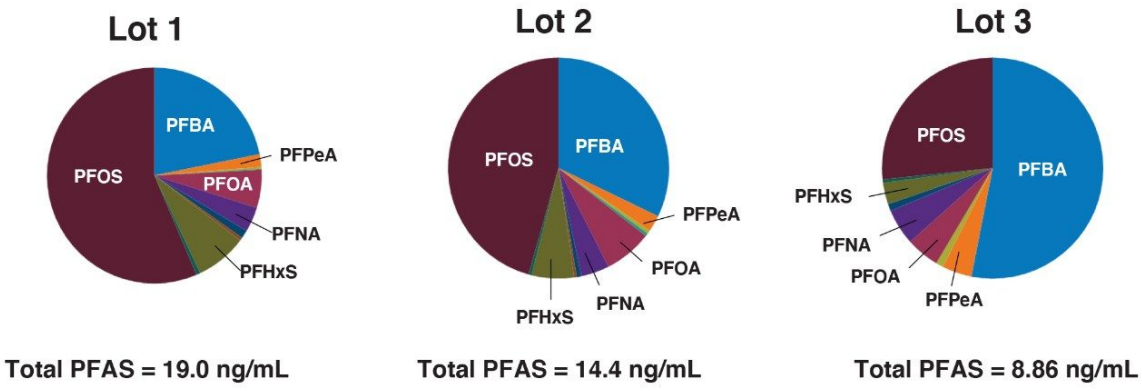

Figure 7. Identifications of PFAS in three lots of pooled human serum demonstrating differences in total PFAS levels as well as differences in ratios of individual PFAS across different samples.

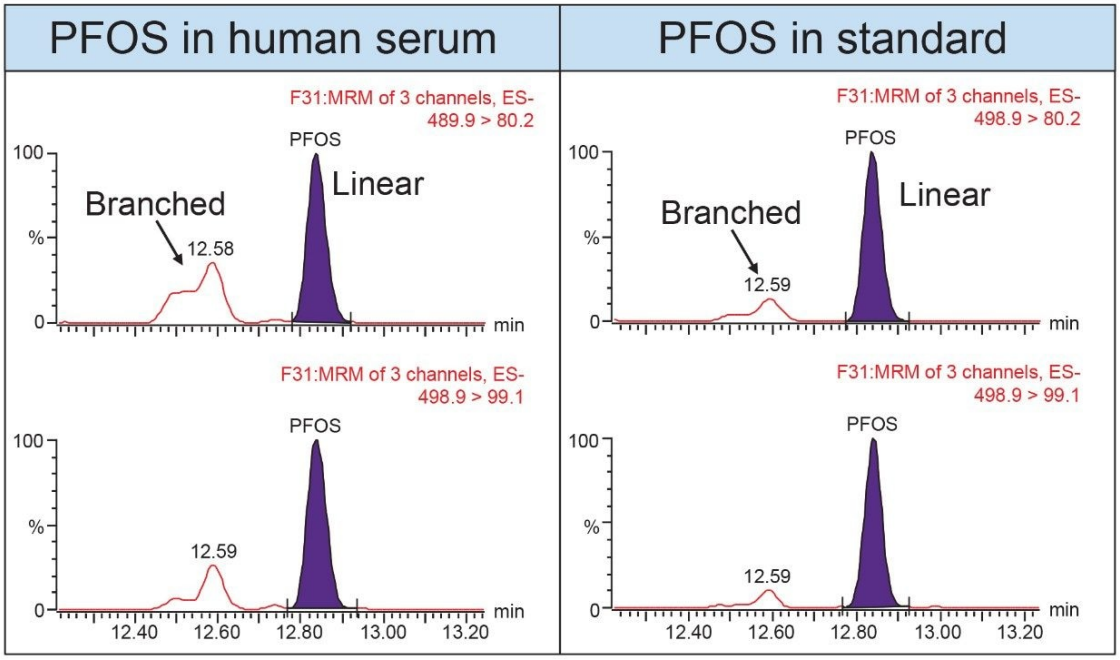

Figure 8. Comparison of the ratio of branched and linear PFOS isomers in human serum (left) and analytical standard (right). Isomer ratios can indicate the type of synthesis protocol used to create PFAS which could potentially indicate source of PFAS exposure.

Conclusion

A robust extraction method was optimized for PFAS in human serum using Oasis WAX µElution 96-well SPE plates. The method was optimized for a selection of 30 PFAS relevant to human exposure covering a range of different PFAS chemistry groups. Analysis was performed using Xevo TQ-S micro coupled to an ACQUITY UPLC I-Class PLUS modified with PFAS Kit components for accurate and reliable results. Utilization of sample preparation for a complex matrix like serum not only allows for easier detection of the PFAS analytes of interest, but also reduces instrument maintenance, allowing longer instrument up time. Method performance was evaluated and accuracy was confirmed using NIST standard reference material 1957. This methodology can be implemented in various types of laboratories to understand levels and trends of PFAS in serum samples for purposes such as occupational health monitoring, understanding human exposure levels, source fingerprinting,

exposure pathways, and understanding impacts on humans based on regional and/or epidemiological differences.

References

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Appendix

Compound	Parent	Fragment	CV	CE
PFBA	213.0	169.0	10	10
PFPeA	262.9	219.0	10	5
PFHxA	312.9	269.0	5	10
		119.0	5	20
PFHpA	362.9	319.0	15	10
		169.0	15	15
PFOA	412.9	369.0	10	10
		169.0	10	15
PFNA	462.9	418.9	10	10
		219.0	10	15
PFDA	512.9	468.9	15	10
		219.0	15	15
PFUnDA	562.9	518.9	25	10
		269.0	25	20
PFDoDA	612.9	568.9	30	10
		169.0	30	25
PFTriDA	662.9	618.9	5	10
		169.0	5	30
PFTreDA	712.9	668.9	10	15
		169.0	10	25
PFBS	298.9	80.1	15	30
		99.1	15	30
PFPeS	348.9	80.1	10	30
		99.1	10	30
PFHxS	398.9	80.1	10	35
		99.1	10	30
PFHpS	448.9	80.2	15	35
		99.1	15	35
PFOS	498.9	80.2	15	40
		99.1	15	40
PFNS	548.9	80.2	20	40
		99.2	20	40
PFDS	598.9	80.2	25	40
		99.1	25	40
FBSA	297.9	78.0	25	25
		118.9	25	15
FHxSA	398.0	78.1	30	25
		169.0	30	25
FOSA	498.0	77.9	40	30
N-MeFOSAA	569.9	418.9	35	20
		219.1	35	25
N-EtFOSAA	584.0	418.8	15	20
		525.9	15	20
4:2 FTS	326.9	307.0	15	15
		327.3	15	35
6:2 FTS	427.0	407.0	15	20
		427.3	15	35

Compound	Parent	Fragment	CV	CE
8:2 FTS	526.9	506.8	15	25
		527.3	15	40
ADONA	376.9	251.0	10	10
		377.3	10	25
9Cl-PF3ONS	531.0	350.9	15	25
		83.0	15	25
11Cl-PF3OUdS	631.0	450.8	30	30
		631.2	30	30
GenX	285.0	169.0	5	7
		GenX	5	35
^{13}C-PFBA	217	172.0	10	10
$^{13}C_5$-PFPeA	268	223.0	10	5
$^{13}C_5$-PFHxA	318	272.9	10	5
		119.9	10	20
$^{13}C_4$-PFHpA	367	321.9	15	10
		172.0	15	15
$^{13}C_8$-PFOA	421	375.9	5	10
		172.0	5	15
$^{13}C_9$-PFNA	472	426.9	10	10
		223.0	10	15
$^{13}C_6$-PFDA	519	473.9	5	10
		223.0	5	15
$^{13}C_7$-PFUnDA	569.9	524.9	5	10
		274.0	5	15
^{13}C-PFDoDA	615	569.9	10	10
		169.0	10	25
$^{13}C_2$-PFTreDA	715	669.9	25	10
		169.0	25	35
$^{13}C_3$-PFBS	301.9	80.0	10	30
		99.0	10	25
$^{13}C_3$-PFHxS	402	80.1	10	40
		99.1	10	35
$^{13}C_8$-PFOS	507	80.1	15	40
		99.1	15	40
$^{13}C_8$-FOSA	506	78.1	35	25
D_3-N-MeFOSAA	573	418.9	35	20
		482.7	35	15
D_5-N-EtFOSAA	589	418.9	30	20
$^{13}C_2$-4:2 FTS	329	308.9	40	15
		81.0	40	25
$^{13}C_2$-6:2 FTS	429	367.0	10	10
		408.8	10	20
$^{13}C_2$-8:2 FTS	529	508.9	10	20
		81.0	10	35
$^{13}C_3$-GenX	287	169	5	12
		119	5	12

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