Measurement of 7 Critical PFAS Compounds in Human Serum Utilizing Dispersive In-Pipette SPE prior to LC-MS/MS Analysis

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Abstract

This study analyzes seven per- and polyfluoroalkyl substances (PFAS) identified as highly persistent in the environment and in biological systems. A methodology for the analysis of human serum was developed to support improved understanding of human exposure to PFAS. Sample preparation and cleanup were performed using hybrid solid phase extraction (HybridSPE®) in a DPX tip (dispersive pipette) format to enable efficient automation. Subsequent analysis was conducted by ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS). Reproducibility testing of matrix-prepared samples showed good accuracy and precision. The accurate and reliable detection achieved by this method can support future studies on PFAS exposure and associated health implications.

Introduction

PFAS (per- and polyfluoroalkyl substances) testing in clinical research is crucial and has gained increasing attention due to their occurrence as environmental contaminants and their associated health risks. The "Guidance on PFAS Testing and Health Outcomes" report[1] issued by the National Academies of Sciences, Engineering, and Medicine (NASEM) highlights the need for biomonitoring of seven key PFAS compounds, namely perfluorooctanoic acid (PFOA), perfluorooctanesulfonic acid (PFOS), perfluorononanoic acid (PFNA), perfluorohexanesulfonic acid (PFHxS), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), and *N*-methylperfluorooctanesulfonamidoacetic acid (MeFOSAA). These substances are persistent and bioaccumulative, which necessitates rigorous testing to investigate exposure pathways and potential health implications. Understanding these aspects is vital for evaluating strategies to reduce PFAS exposure and for supporting public health and regulatory decisions aimed at mitigating their impact on human health and the environment.

Human serum samples can be an appropriate matrix of choice for evaluation the presence of PFAS in the body. But the complex composition of serum necessitates sample cleanup prior to LC-MS analysis. One of the compound classes that interfere with LC-MS quantitation through disruption of compound ionization in the mass spectrometric source are phospholipids. Several technologies are available for selective removal phospholipids from serum and plasma. HybridSPE® technology is one such approach which has been widely applied for phospholipid removal from biological matrices.[2-11]

Unlike the "bind-elute" method used in other solid phase extraction processes, the HybridSPE® approach facilitates "interference removal" or "chemical filtration" by specifically targeting and removing phospholipids based on their chemical properties, as illustrated in **Figure 1**. This strategy provides a simpler and faster workflow compared to "bind-elute" processes and supports higher sample throughput with reduced method complexity.

In this study, the HybridSPE® adsorbent was used in a dispersive SPE pipette tip (DPX tip) format, in which the loose sorbent is contained within the tip (**Figure 2**) to allow mixing and interaction with the aspirated sample. The DPX tips are typically arranged in a 96-well plate format, allowing straightforward integration into automated liquid handling systems.

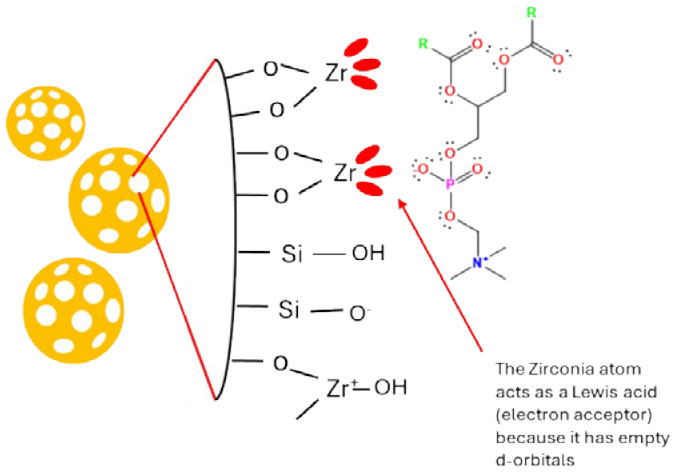

Figure 1. Retention mechanism for phospholipid removal based on a Lewis acid-base interaction between the zirconia coated silica HybridSPE® material and the phosphate group of the phospholipids.

Experimental

Standards and Sample preparation

Standards

Standards of PFOA, PFOS, PFNA, PFHxS, PFDA, PFUnA, and MeFOSAA were prepared in methanol at concentrations ranging from 0.02 to 2.5 ng/mL for quantitation of the spiked samples. Isotopically labelled internal standards (listed in **Table 3**) were used to construct calibration curves at concentrations of 1.25 and 5.0 ng/mL, depending on the compound.

Spiked samples

A blank processed serum extract was spiked at 0.5 ng/mL with each compound to assess reproducibility and remaining matrix interference.

Serum samples were spiked with the seven PFAS compounds each at 1.25 ng/mL for recovery determination.

Sample Preparation

Samples were prepared/cleaned-up using protein precipitation followed by HybridSPE® DPX tips on a liquid handler system (**Table 1**). Binding, or chemical filtration, when using HybridSPE® technology, refers to the selective removal of phospholipids from the sample. Conditioning decreases affinity of the analytes-of-interest for the SPE material, thereby ensuring improved recovery.

Table 1. Sample preparation conditions using HybridSPE® DPX tips

Sample pretreatment conditions	
Protein precipitation	
1. To 250 µL of serum, add 700 µL of cold 1% ammonium formate in methanol. 2. Vortex/shake the mixture for 10 seconds 3. Centrifuge at 10,000 g for 3 minutes at 4 °C. ** 4. Transfer 400 µL of the supernatant into a 2 mL polypropylene 96-well plate.	
** Alternatively, for automated processing, serum samples may be handled without centrifugation by allowing the samples to settle for a minimum of 15 minutes before transferring them to a new well plate.	
Dispersive in-pipette SPE conditions (phospholipid binding/ removal, Figure 2)[12]	
Instrument:	Hamilton MICROLAB STARlet
Sample/matrix:	Human serum
SPE tube/cartridge:	HybridSPE® DPX Tip, weight 50 mg (bed), Hamilton, tip volume 1 mL (**52978-U**)
Conditioning:	• 750 µL of 1% ammonium formate in methanol • Pipette volume set to 1000 µL to ensure complete mixing via aspiration • Two aspirate/dispense cycles
Sample processing:	• 400 µL supernatant • Pipette volume set to 1000 µL • Three aspirate/dispense cycles

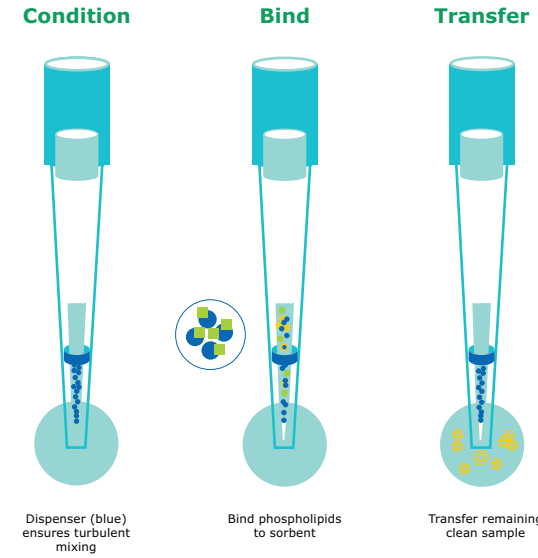

Figure 2. Schematic of the automated removal of phospholipids using hybrid solid phase extraction (HybridSPE®) in DPX format.

LC-MS Analysis

Standards and spiked samples were analyzed by LC-MS/MS using Ascentis® Express PFAS analytical and delay columns (**Table 2**).

Table 2. LC-MS/MS conditions

LC Conditions			
Instrument:	Agilent 1290 Infinity II LC		
Column:	Ascentis® Express 90 Å PFAS 2.7 µm, 10 cm x 2.1 mm I.D (**53559-U**)		
Delay column:	Ascentis® Express 160 Å PFAS Delay 2.7 µm, 5 cm x 3.0 mm I.D. (**53572-U**) (Installed after the pump and before the injector)		
Mobile phase:	[A] 10 mM Ammonium acetate in water; [B] Methanol		
Gradient:	**Time (min)**	**A%**	**B%**
	0.0	95	5
	2.0	60	40
	11.0	0	100
	13.0	0	100
	13.1	95	5
	16.0	95	5
Flow rate:	0.40 mL/min		
Column temp.:	35 °C		
Detector:	MS/MS, ESI(-), MRM (**Table 3**)		
Injection:	5 µL		
MS Conditions			
Instrument:	SCIEX Triple Quad 6500+		
Ion source gas 1:	30 psi		
Ion source gas 2:	50 psi		
Curtain gas:	30 psi		
CAD gas:	9 psi		
Source temperature:	450 °C		
Polarity:	negative		
Spray voltage:	4500 V		

Table 3. MS Conditions and Transitions monitored by MS/MS

Analyte	Q1 mass (Da)	Q3 mass (Da)	DP (V)	EP (V)	CE (V)	CXP (V)	Conc. of IS (ng/mL)
PFOA	413	369	-50	-10	-14	-12	
EIS-PFOA-$^{13}C_8$	421	376	-50	-10	-14	-12	2.50
PFNA	463	419	-60	-10	-14	-12	
EIS-PFNA-$^{13}C_9$	472	427	-50	-10	-14	-12	1.25
PFDA	513	469	-70	-10	-16	-12	
EIS-PFDA-$^{13}C_6$	519	474	-60	-10	-16	-12	1.25
PFUnA	563	519	-70	-10	-18	-12	
EIS-PFUnA-$^{13}C_7$	570	525	-60	-10	-18	-12	1.25
PFHxS	399	99	-80	-10	-75	-12	
EIS-PFHxS-$^{13}C_3$	402	80	-80	-10	-74	-12	2.50
PFOS	499	80	-80	-10	-108	-12	
EIS-PFOS-$^{13}C_8$	507	99	-80	-10	-85	-12	2.50
N-MeFOSAA	570	419	-70	-10	-28	-12	
EIS-*N*-MeFOSAA-D3	573	419	-50	-10	-28	-12	5.00

Results & Discussion

Serum samples spiked with seven PFAS compounds were analyzed using protein precipitation and phospholipid removal by dispersive SPE, followed by LC-MS/MS. Automation of the sample preparation was achieved using a liquid handler, enhancing reproducibility, and allowing simultaneous preparation of up to 96 samples using in-pipette-tip dispersive adsorbent. This approach eliminated the need for positive or negative pressure SPE devices, resulting in a more economical workflow in terms of instrument design and simplified programming.

A calibration method assessment was performed by comparing standards at 0.5 ng/mL of each compound prepared as matrix-matched and solvent-based solutions to evaluate accuracy and matrix interference (**Figure 3**). Concentrations were determined using external calibration curves (0.02–2.25 ng/mL) in methanol. Both methods showed similar results across all seven analytes, with only a single concentration for one analyte (PFDA), differing by 10% (**Table 4**). These findings demonstrate the effectiveness of the developed sample preparation in mitigating matrix effects for the selected analytes, allowing future studies to utilize solvent-prepared calibration curves.

Reproducibility tests for the matrix matched samples spiked at 0.5 ng/mL showed relative standard deviation (RSDs) below 6% for all analytes, except for PFUnA, which showed a 15% deviation (**Table 4**).

Background evaluation of the serum samples identified some of the studied compounds at very low levels. In all cases, the compound background was at or below the lowest calibration point of 0.02 ng/mL and therefore is not reported here.

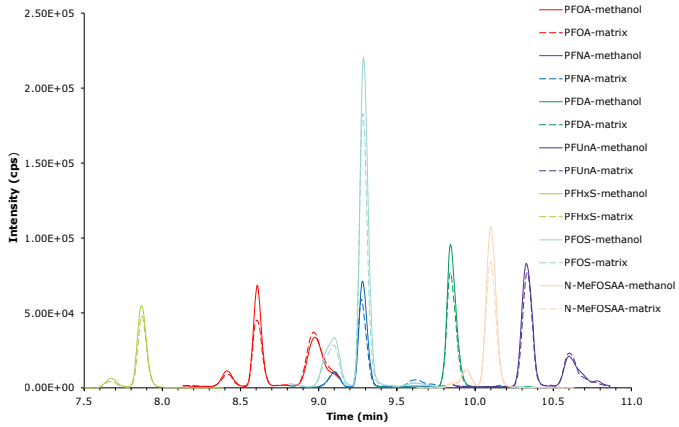

Figure 3. Comparison of a 0.5 ng/mL standard prepared in methanol to an equivalent standard prepared in blank serum, cleaned-up by HybridSPE® and subsequently spiked.

Table 4. Recovery and reproducibility of methanol based solutions and matrix matched solutions spiked after HybridSPE® sample clean-up, each at 0.5 ng/mL for all compounds

Analyte	Concentration (ng/mL)	Standard solvent	n	Mean concentration determined (ng/mL)	Recovery mean (%)	Reproducibility (%RSD)
PFOA	0.5	Methanol	2	0.53	106.6	9.3
	0.5	Matrix match	3	0.54	108.2	5.4
PFNA	0.5	Methanol	2	0.50	101.0	4.0
	0.5	Matrix match	3	0.53	106.6	3.3
PFDA	0.5	Methanol	2	0.57	113.3	8.7
	0.5	Matrix match	3	0.52	103.4	0.4
PFUnA	0.5	Methanol	2	0.57	113.5	25.6
	0.5	Matrix match	3	0.59	117.4	15.4
PFHxS	0.5	Methanol	2	0.50	100.7	6.0
	0.5	Matrix match	3	0.52	104.0	1.6
PFOS	0.5	Methanol	2	0.50	100.7	0.5
	0.5	Matrix match	3	0.52	103.0	4.4
N-MeFOSAA	0.5	Methanol	2	0.48	95.1	2.8
	0.5	Matrix match	3	0.48	96.1	3.7

Recovery Spiked Serum

For serum samples spiked at 1.25 ng/mL with each analyte (PFOA, PFOS, PFNA, PFHxS, PFDA, PFUnA, and *N*-MeFOSAA), and processed by protein precipitation and HybridSPE® clean-up, relative recoveries (quantified using IS) ranged from 77.2% to 101.8%, while absolute recoveries varied from 72.8% to 92.6% (**Table 6**).

Table 6. Relative and absolute recoveries of spiked serum samples (1.25 ng/mL) following HybridSPE® sample cleanup (n=6)

Analyte	Relative recovery (%)	Relative recovery (%RSD)	Absolute recovery (%)	Absolute recovery (%RSD)
PFOA	90.4	9.0	72.8	7.4
PFNA	97.0	3.6	92.6	4.6
PFDA	100.5	8.4	88.0	9.7
PFUnA	88.3	17.1	75.2	7.0
PFHxS	83.4	3.3	73.5	5.4
PFOS	77.2	2.8	80.3	3.7
N-MeFOSAA	101.8	5.0	85.2	6.2

Conclusion

As PFAS exposure and monitoring continue to receive the attention of government agencies for their long-lasting health impacts, a growing number of body fluid samples will require analysis in the future. Here an LC-MS/MS method was developed to assess the seven PFAS analytes identified in the NASEM report in human serum, using a sample preparation approach that can be readily automated on a liquid handling system. The use of HybridSPE® sorbents in a DPX tip format enable accurate and reliable detection of PFAS compounds in complex biological matrices, supporting efficient future research on PFAS exposure and associated health implications.

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HybridSPE® DPX Tip Hamilton, tip volume 1 mL, bed weight 50 mg, Pk. 96	**52978-U**
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Water, tested for PFAS Methods LiChrosolv® (or alternaively tapfresh water from a suitable Milli-Q® system)	**1.04735**
Ammonium acetate LiChropur™, eluent additive for LC-MS	**73594**
Nunc® 96 DeepWell™ plate, non-treated	**Z717266**
Certified Reference Materials, *Trace*Cert® (if not otherwise indicated)	
Pentadecafluorooctanoic acid (PFOA), 25 mg	**93973**
Perfluorodecanoic acid (PFDA), 10 mg	**91367**
Perfluorononanoic acid (PFNA), 10 mg	**05167**
Perfluoroundecanoic acid (PFUnDA), 10 mg	**89988**
Perfluorooctanesulfonic acid (PFOS), 100 µg/mL in methanol, analytical standard, 1 mL	**33607**
2-(*N*-Methylperfluorooctanesulfoamido)acetic acid (*N*-MeFOSAA), 25 mg	**94706**

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