Application Note

Clinical Research

Agilent

Trusted Answers

PFAS Analysis in Human Plasma Using the Agilent Ultivo Triple Quadrupole LC/MS

Authors

Sartaj Ahmad Mir[1,2], Vaitheeswari[1,2], Federico Torta[1,2,3], Anne K. Bendt[1,4], Siji Joseph[5], and Aimei Zou[5]

[1] Singapore Lipidomics Incubator, Life Sciences Institute, National University of Singapore, Singapore

[2] Department of Biochemistry and Precision Medicine Translational Programme, National University of Singapore, Singapore

[3] Signature Research Program in Cardiovascular and Metabolic Disorders, Duke-National University of Singapore (Duke-NUS) Medical School, Singapore

[4] Department of Biochemistry and Cardiovascular-Metabolic Disease Translational Research Programme, National University of Singapore, Singapore

[5] Agilent Technologies, Inc.

Abstract

This application note presents a validated LC/MS/MS method for the targeted quantification of 14 perfluoroalkyl and polyfluoroalkyl substances (PFAS) in human plasma using the stackable, space-saving Agilent Ultivo Triple Quadrupole (TQ) LC/MS. The method is simple and robust, uses relatively small plasma volumes, and relies on fast and effective protein precipitation, facilitating its use for large-scale population studies. Method performance in terms of sensitivity, linearity, carryover, accuracy (recovery), and precision was evaluated. The method enabled baseline separation of analytes and unambiguous PFAS identification. Calibrations showed good linearity with $R^2 > 0.996$ for all compounds. Acceptable carryover and recovery were achieved and complied with US EPA criteria for PFAS analysis in complex tissue matrices. Precision (%CV) of intraday repeatability and interday reproducibility measurements was less than 10%. Method applicability to real-world samples (n = 96) collected for a research study of PFAS in human blood plasma is also shown.

Introduction

Perfluoroalkyl and polyfluoroalkyl substances (PFAS) are a group of synthetic compounds that have been widely used in various industrial and consumer applications, including cosmetics and personal care products. Due to their widespread use, persistence in the environment, and known bioaccumulation with adverse effects, PFAS are a significant concern for human health. For example, PFAS have been linked to several adverse health effects, including cardiometabolic diseases,[1] reproductive effects,[2] and increased risk of certain cancers.[3] PFAS exposure has been shown to lead to alterations in lipid and energy metabolism, contributing to metabolic dysfunction.[4,5] It is also known that PFAS accumulate in liver and other tissues, contributing to age-related morbidities.[6] To increase the understanding the molecular basis of these effects, analysis of PFAS in blood plasma has been combined with lipidomics to identify the lipid biomarkers associated with metabolic risks.[7] Further, the PFAS concentrations in serum have also been shown to differ by gender[7,8] and ethnicity.[9] Considering these and other findings, monitoring PFAS levels in plasma is important to understand their health impacts and develop strategies for their mitigation.

This application note validates an LC/MS/MS method for targeted quantification of fourteen PFAS in plasma using the Agilent Ultivo Triple Quadrupole (TQ) LC/MS. For the method presented, the Ultivo LC/TQ offers performances on par with mid- to high-end TQ systems in a much smaller footprint. The protein-precipitation-based sample preparation method is fast and effective for plasma samples. Method performance in terms of sensitivity, linearity, carryover, accuracy, and precision are described. Stability of targeted PFAS in quality control (QC) samples and analysis of real human plasma samples are also discussed.

Experimental

Standards and reagents

Reference materials (standards) were mass-labeled and non-labeled PFAS supplied by Wellington Laboratories Inc., Ontario, Canada and charcoal-stripped human plasma (CSP) (#HUMANPLK2-0102294) from BIOIVT.

Reagents were LC/MS grade and included water, methanol (Fisher Scientific, Thermo Fisher Scientific, MA, USA), ammonium acetate (Sigma-Aldrich Pte Ltd.), acetic acid, acetonitrile, and MilliQ water. Mobile phase A was 5 mM ammonium acetate in water and mobile phase B was methanol. Needle wash solutions (S1, S2, and S3) for the multiwash functionality were double-distilled water (ddH_2O) for S1, 50% methanol for S2, and 100% methanol for S3. The 50% methanol was a mix of 500 mL methanol and 500 mL of ddH_2O.

Mass-labeled internal standard (ISTD) solution preparation

PFAS ISTD Stock Solution_01 consisted of the labeled compounds at 50 µg/mL in methanol as supplied by Wellington Laboratories Inc. in original stock solution (PFAS_<Compound name> SS_01).

PFAS solutions for each ISTD (PFAS ISTD Stock Solution_02) were prepared based on initial experiments according to the analyte-to-ISTD response in plasma. Solutions were prepared for each PFAS as listed in Table 1.

The PFAS ISTD working solution (PFAS_ISTD_WS_01) was prepared by mixing equal volumes from the individual PFAS ISTD stock solutions, spiking 10 µL of PFAS_ISTD_WS_01 per sample analyzed.

Table 1. Individual PFAS ISTD concentrations and concentrations in the ISTD mix.

ISTD Name	Concentration (ng/mL)	Concentration in ISTD mix (ng/mL)
PFOS-$^{13}C_4$	500	71.42
PFOA-$^{13}C_4$	100	14.2
PFHxS-$^{13}C_3$	100	14.2
N-MeFOSSA-d_3	100	14.2
PFHxA-$^{13}C_2$	20	2.85
PFDA-$^{13}C_2$	20	2.85
PFDoA-$^{13}C_2$	20	2.85

Non-labeled standard preparation for calibration curve determination

Calibration curve solutions were prepared by successive dilution of non-labeled standards from two primary stock solutions to generate a total of 13 levels, ranging from 0.003 to 12.5 ng/mL (Cal01 = 0.003, Cal02 = 0.0061, Cal03 = 0.0122, Cal04 = 0.0244, Cal05 = 0.0488, Cal06 = 0.0976, Cal07 = 0.1953, Cal08 = 0.3906, Cal09 = 0.7812, Cal10 = 1.5625, Cal11 = 3.125, Cal12 = 6.25, Cal13 = 12.5 ng/mL) in the final extract. 50 µL of each calibration level and 10 µL of the mix (PFAS_ISTD_WS_01) were spiked in 100 µL of charcoal-stripped plasma (CSP).

Note: The CSP (equivalent to matrix blank) used in this study does not contain any of the PFAS analytes of interest. These plasma samples were extracted as described in the sample preparation section.

QC samples

QC samples were prepared by spiking an equimolar solution of 14 non-labeled PFAS standards at a concentration of 2.5, 12.5, and 30 ng/mL for the low-quality control (LQC), middle-quality control (MQC), and high-quality control (HQC) in CSP, respectively.

Sample preparation

Aliquots of 100 µL of plasma sample and 10 µL of PFAS working ISTD solution (PFAS_IS_WS_01) were mixed into 1.5 mL microcentrifuge tubes. To precipitate proteins, 400 µL of cold (–20 °C) methanol were added and the contents were vortexed manually for approximately 20 seconds, followed by centrifugation at 14,000 g for 10 minutes at 4 °C. A total of 200 µL of the supernatant was transferred into mass spectrometry vials for LC/MS/MS analysis.

Instrumentation and LC/MS/MS method

Chromatographic conditions were optimized for analyte separation and retention time for each of the PFAS as determined by analyzing the labeled and non-labeled standards. PFAS were separated by reversed-phase LC on an Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 50 mm, 2.7 µm (part number 699775-902) and detected in negative electrospray ionization (ESI) mode using an Agilent 1290 Infinity II LC system coupled with the Ultivo LC/TQ. The liquid chromatograph was equipped with a binary pump (part number G7120A), column compartment (part number G7116B), and Agilent 1290 Infinity II Multisampler (part number G7167B). For each experiment, 5 µL of extract was injected. The LC parameters are listed in Table 2.

The Ultivo LC/TQ was operated in dynamic multiple reaction monitoring (dMRM) mode. The dMRM parameters and retention times were optimized by analyzing each unlabeled PFAS standard. The MS parameters are listed in Table 3. The Agilent MassHunter Acquisition software dMRM parameters per PFAS are in Table 4. Agilent MassHunter Quantitative Analysis software was used for data processing.

Table 2. LC parameters.

Parameter	Value
Analytical Column	Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 50 mm, 2.7 µm (p/n 699775-902)
Delay Column	Agilent Eclipse Plus C18, 4.6 × 50 mm, 3.5 µm (p/n 959943-902)
Column Temperature	50 °C
Mobile Phase A	5 mM ammonium acetate in water
Mobile Phase B	Methanol
Injection Volume	5 µL
Needle Wash Solution (Multi-Wash Function)	Step / Solvent / Time (s) / Seat Back Flush / Needle Wash 1 / S1 / 10 / ✓ / ✓ 2 / S2 / 10 / ✓ / ✓ 3 / S3 / 10 / ✓ / ✓ Start Cond / S1 / / ✓ / ✓ S1: ddH_2O S2: 50% Methanol S3: Methanol
Gradient	Time (min) / MPA (%) / MPB (%) / Flow Rate (mL/min) 0 / 95 / 5 / 0.5 0.5 / 95 / 5 / 0.5 1 / 50 / 50 / 0.5 6.5 / 0 / 100 / 0.5 7.5 / 0 / 100 / 0.5 8 / 95 / 5 / 0.5 10 / 95 / 5 / 0.5

Table 3. MS parameters.

Parameter	Value
Ionization Source and Mode	Agilent Jet Stream Technology ion source, negative mode
Gas Temperature	230 °C
Gas Flow	5 L/min
Nebulizer	15 psi
Shear Gas Temperature	350 °C
Shear Gas Flow	12 L/min
Capillary Voltage	2,000 V

Table 4. Agilent MassHunter Acquisition software dMRM parameters per PFAS.

Compound Name	Precursor (*m/z*)	Product (*m/z*)	RT (min)	Fragmentor (V)	CE (V)	Polarity
N-EtFOSAA	584	525.9	4.85	115	15	Negative
N-EtFOSAA	584	419	4.85	115	15	Negative
N-MeFOSAA	570	482.9	4.67	115	12	Negative
N-MeFOSAA	570	419	4.67	115	15	Negative
PFBS	298.9	98.9	2.63	100	22	Negative
PFBS	298.9	80	2.63	100	34	Negative
PFDA	513	469	4.49	81	5	Negative
PFDA	513	218.7	4.49	100	12	Negative
PFDoA	613	569	5.18	79	4	Negative
PFDoA	613	268.7	5.18	100	15	Negative
PFHpA	362.9	319	3.22	72	5	Negative
PFHpA	362.9	169	3.22	72	9	Negative
PFHxA	313	268.6	2.85	70	6	Negative
PFHxA	313	119	2.85	70	14	Negative
PFHxS	398.9	99	3.26	100	34	Negative
PFHxS	398.9	80	3.26	100	37	Negative
PFNA	462.9	418.9	4.08	66	3	Negative
PFNA	462.9	169	4.08	66	13	Negative
PFOA	412.9	368.9	3.64	69	3	Negative
PFOA	412.9	169	3.64	69	9	Negative
PFOS	498.9	99	4.11	100	38	Negative
PFOS	498.9	80	4.11	100	38	Negative
PFTeDA	712.9	668.5	5.7	100	8	Negative
PFTeDA	712.9	169	5.7	100	23	Negative
PFTrDA	663	618.7	5.45	91	8	Negative
PFTrDA	663	169	5.45	100	23	Negative
PFUdA	563	519	4.85	73	4	Negative
PFUdA	563	218.7	4.85	100	15	Negative
N-MeFOSAA d_3	573	515	4.67	115	15	Negative
N-MeFOSAA d_3	573	486	4.67	115	12	Negative
PFDA-$^{13}C_2$	514.9	469.9	4.49	81	5	Negative
PFDoA-$^{13}C_2$	614.9	570	5.18	79	4	Negative
PFHxA-$^{13}C_2$	314.9	269.9	2.85	70	6	Negative
PFHxS-$^{13}C_3$	401.9	79.8	3.26	100	34	Negative
PFOA-$^{13}C_4$	417	372	3.64	69	3	Negative
PFOA-$^{13}C_4$	417	169	3.64	69	9	Negative
PFOS-$^{13}C_4$	502.9	80	4.1	100	38	Negative

Results and discussion

Because real human plasma already contains most of the PFAS targeted in this study, commercial CSP as matrix blank was spiked with targeted PFAS to create multiple levels of QC samples (LQC, MQC, and HQC) for the evaluation of method sensitivity, linearity, carryover, recovery, and precision.

Method sensitivity and linearity

The method enabled baseline separation of analytes in the 10-minute total runtime (Figure 1).

Limit of detection (LOD), LOQ, and linearity (R^2) were determined by spiking non-labeled standards at various concentrations covering the analytical range of the instrument and fixed concentrations of labeled internal standards in CSP. The LOD and LOQ for the non-labeled standards were then obtained based on signal-to-noise (S/N) ratio. The LOD and LOQ were defined as the concentrations where the S/N ratios were more than or equal to 3:1 and 10:1, respectively. The calibration curves showed good linearity with $R^2 \geq 0.996$ for all compounds. The calculated LODs, LOQs and linearities are listed in Table 5.

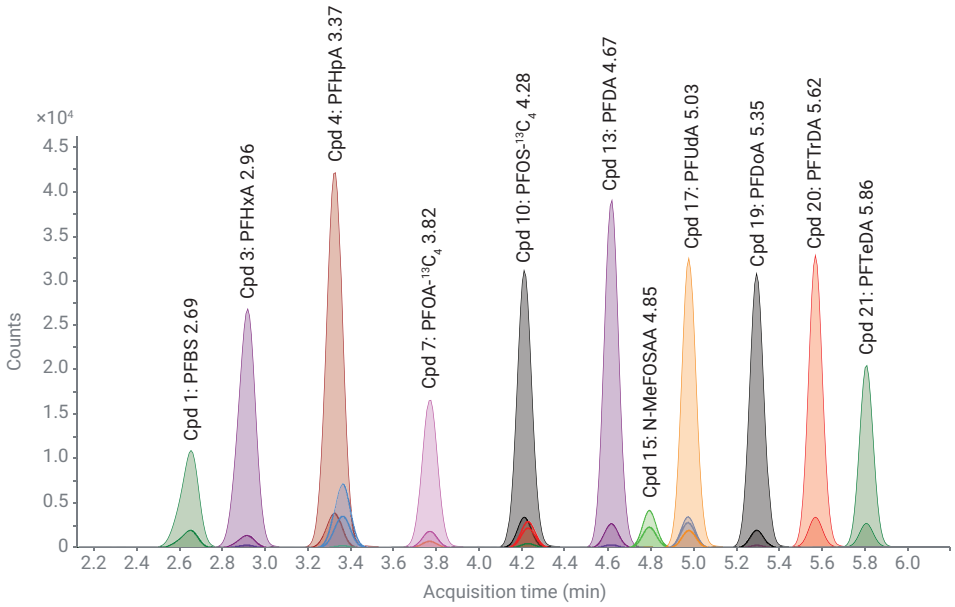

Figure 1. Representative MRM chromatogram of the labeled and non-labeled PFAS standards.

Table 5. Linearity (R^2), limit of detection (LOD), and limit of quantitation (LOQ) for the 14 PFAS analytes.

Compound ID	Analyte	ISTD	LOQ (ng/mL)	LOD (ng/mL)	R^2
1	N-EtFOSAA	N-MeFOSSA-d_3	0.009	0.003	0.996
2	N-MeFOSAA	N-MeFOSSA-d_3	0.009	0.003	0.998
3	PFBS	PFDA-$^{13}C_2$	0.018	0.006	0.999
4	PFDA	PFDA-$^{13}C_2$	0.024	0.006	0.999
5	PFDoA	PFDoA-$^{13}C_2$	0.018	0.006	0.997
6	PFHpA	PFHxA-$^{13}C_2$	0.048	0.006	0.997
7	PFHxA	PFHxA-$^{13}C_2$	0.048	0.006	0.997
8	PFHxS	PFHxS-$^{13}C_3$	0.195	0.048	0.999
9	PFNA	PFDA-$^{13}C_2$	0.048	0.012	0.997
10	PFOA	PFOA-$^{13}C_4$	0.072	0.024	0.999
11	PFOS	PFOS-$^{13}C_4$	0.097	0.024	0.999
12	PFTeDA	PFDoA-$^{13}C_2$	0.024	0.006	0.999
13	PFTrDA	PFDoA-$^{13}C_2$	0.048	0.006	0.999
14	PFUdA	PFDoA-$^{13}C_2$	0.048	0.006	0.999

Carryover evaluation

Carryover was assessed in solvent and matrix blank samples after injection of the 30 ng/mL HQC. Here, carryover was well below 20% for the target PFAS compounds at the LOQ level, and less than 5% for the response of ISTDs (Figures 2 and 3), indicating acceptable carryover.

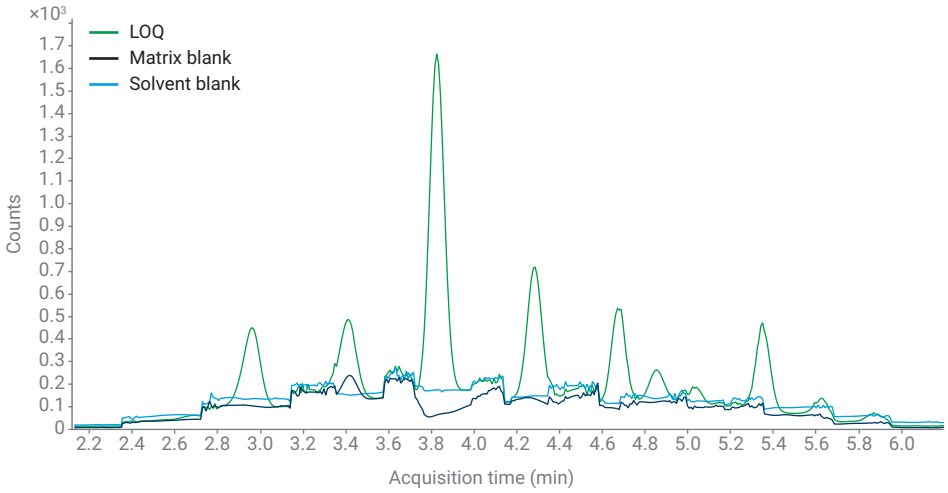

Figure 2. Total ion chromatogram (TIC) of solvent blank, matrix blank, and LOQ.

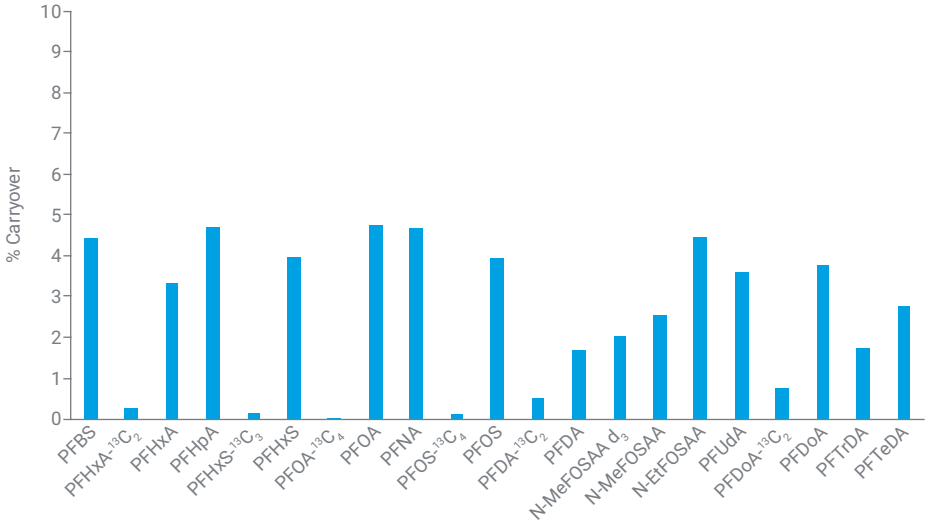

Figure 3. Percentage carryover calculated from injection of solvent blank following injection of the HQC.

Method recovery

The mean recoveries of the LQC (n = 5), MQC (n = 5), and HQC (n = 5) were obtained within the ranges of 67 to 110% (Figure 4), demonstrating the acceptable extraction efficiency of the method developed for PFAS analysis in plasma. Additionally, the results showed compliance with criteria defined in US EPA methods for PFAS analysis in complex tissue matrices.

Method precision

To determine method precision, QC samples were extracted as described above for intraday repeatability (n = 10) measurements and the experiment was repeated on another day to determine interday reproducibility (n = 10). %CV of intra- and interday measurements were less than 10% (Figure 5), demonstrating that the method is robust and reliable for large-scale routine analyses and research studies.

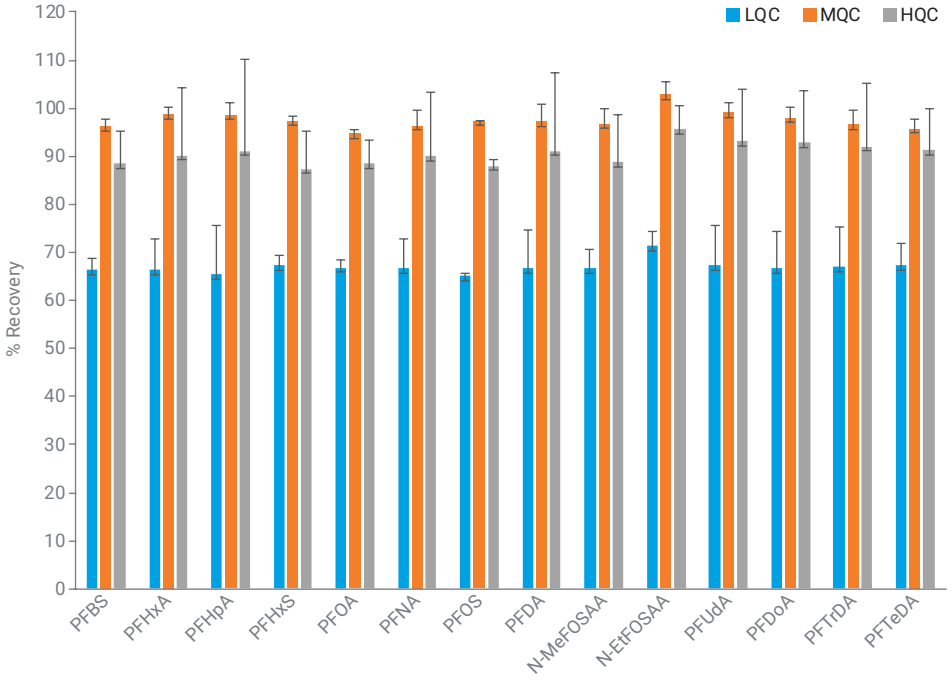

Figure 4. Mean recoveries of the non-labeled PFAS at the spiked QC level of the LQC, MQC and HQC (all n = 5).

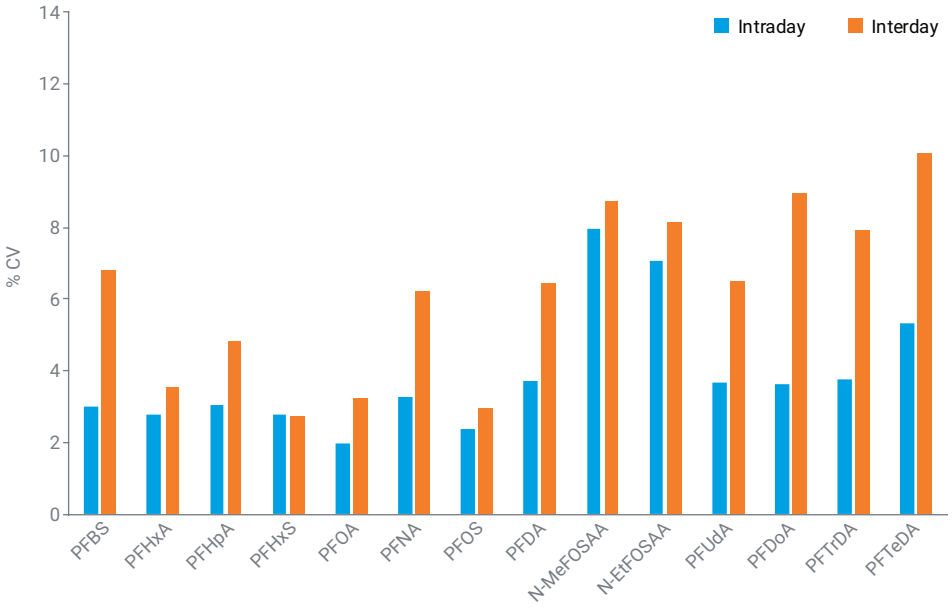

Figure 5. Intra- and interday %CV of the measurements of the targeted PFAS in QC samples.

Stability study for PFAS in QCs

The stability of PFAS in the CSP QC samples under different storage conditions and temperatures, was tested. The %CV of results for PFAS in QC samples was less than 15% over the storage conditions at room temperature and 4 °C (Figure 6).

Quantification of PFAS in a human cohort

Measuring PFAS levels in real plasma samples is necessary for understanding their health impacts, carrying out PFAS monitoring programs and developing strategies for mitigation. Therefore, the method was applied to analyze samples (n = 96) collected for a research study in a multi-ethnic Asian population in which samples were stratified by gender and other factors.[7] Of the 14 targeted PFAS, three were present below the LOQ, and the total concentration of the remaining eleven PFAS were reported as 6.85 ± 3.1 ng/mL in human plasma samples. Linear and branched chain forms of PFAS are reported as the total sum. The results also showed that, in this cohort, women had lower total plasma PFAS concentrations compared to men and, in particular, significant differences were observed for PFHxS ($p < 0.001$) and PFOA ($p < 0.01$).

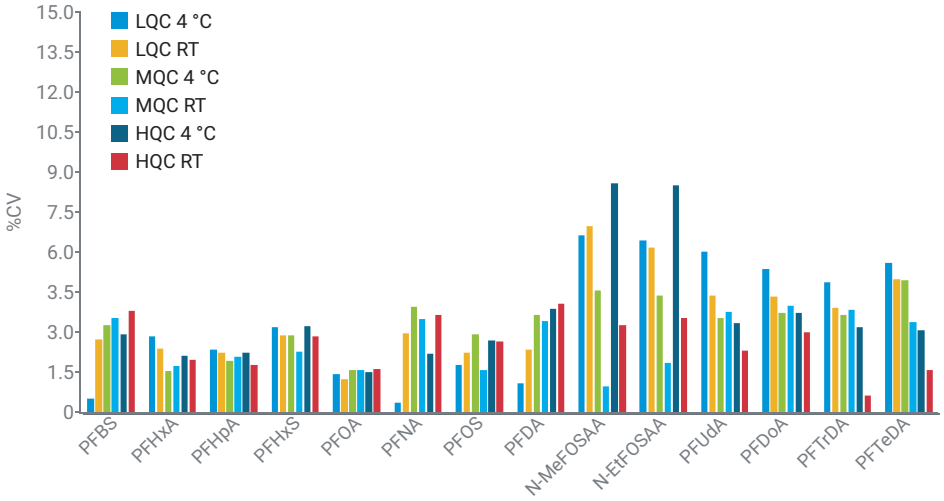

Figure 6. Stability of the targeted PFAS in QC samples stored at room temperature (RT) and 4 °C.

Conclusion

The quantitative method for the analysis of 14 PFAS in human plasma samples that is presented here is simple and robust, uses relatively small plasma volumes, and relies on fast and effective protein precipitation, facilitating its large-scale application. The method's sensitivity, linearity, carryover, recovery, and precision make it scalable to population-based studies and biomonitoring. The compact, stackable Ultivo LC/TQ and MassHunter software, in combination with columns and consumables from Agilent Technologies, offer a fit-for-purpose, end-to-end solution to enable widespread adoption in both routine monitoring and research. The method has already been used in combination with plasma lipidomics to characterize the relationship of circulating PFAS and lipidomic profiles.[7] These types of studies promise to reveal the molecular basis of the adverse effect of PFAS exposure on human health outcomes.

References

1. Donat-Vargas, C.; *et al.* Associations Between Repeated Measure of Plasma Perfluoroalkyl Substances and Cardiometabolic Risk Factors. *Environ. Int.* **2019**, *124*, 58–65.
2. Rickard, B. P.; Rizvi, I.; Fenton, S. E. Per- and Poly-Fluoroalkyl Substances (PFAS) and Female Reproductive Outcomes: PFAS Elimination, Endocrine-Mediated Effects, and Disease. *Toxicology* **2022**, *465*, 153031.
3. Li, S.; *et al.* Associations Between Per- and Polyfluoroalkyl Substances (PFAS) and County-Level Cancer Incidence Between 2016 and 2021 and Incident Cancer Burden Attributable to PFAS in Drinking Water in the United States. *J. Expo. Sci. Environ. Epidemiol.* **2025**, *35(3)*, 425–436.
4. Jacobsen, A. V.; *et al.*, Effects of Perfluorooctane Sulfonate on Genes Controlling Hepatic Fatty Acid Metabolism in Livers of Chicken Embryos. *Environ. Sci. Pollut. Res. Int.* **2018**, *25(23)*, 23074–23081.
5. Khazaee, M.; *et al.* Perfluoroalkyl Acid Binding with Peroxisome Proliferator-Activated Receptors Alpha, Gamma, and Delta, and Fatty Acid Binding Proteins by Equilibrium Dialysis with a Comparison of Methods. *Toxics* **2021**, *9(3)*.
6. Obeng-Gyasi, E.; Factors Associated with elevated Per- and Polyfluoroalkyl Substances Serum Levels in Older Adults. *Aging Health Res.* **2022**, *2(3)*.
7. Narasimhan, K.; *et al.* Integrated Analysis of Per- and Polyfluoroalkyl Substances and Plasma Lipidomics Profiles in Multi-Ethnic Asian Subjects for Exposome Research. *Environ. Health* **2024**, *23(1)*, 105.
8. Jain, R. B.; Ducatman, A. Serum Concentrations of Selected Perfluoroalkyl Substances for US Females Compared to Males as They Age. *Sci. Total Environ.* **2022**, *842*, 156891.
9. Park, S. K.; *et al.* Determinants of Per- and Polyfluoroalkyl Substances (PFAS) in Midlife Women: Evidence of Racial/Ethnic And Geographic Differences in PFAS Exposure. *Environ. Res.* **2019**, *175*, 186–199.

www.agilent.com

RA250612.394

Printed in the USA, July 29, 2025
5994-8487EN

Agilent
Trusted Answers