Rapid LC-MS/MS method for monitoring bio-relevant levels of per- and polyfluoroalkyl substances (PFAS) in serum

Using Scheduled MRM™ Algorithm on the SCIEX QTRAP® 6500+ LC-MS/MS System

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Per- and polyfluoroalkyl substances (PFAS) are pervasive compounds used in a variety of industrial applications and found in a wide range of consumer products such as cookware, stain repellant, flame-retardant and coatings.[1] PFAS are considered environmental factors due to their persistent and bioaccumulating nature. A recent biomonitoring study conducted by the National Health and Nutrition Examination Survey (NHANES) on a nationally representative sample of the U.S. population found that more than 98% of the people tested had multiple congeners of PFAS present in their bodies.[2]

Bioaccumulation of PFAS in the human body resulting from environmental exposure is a growing public health concern. Recent studies have linked PFAS exposure to adverse health outcomes including childhood health complications, reduction in kidney functions, thyroid disease, hormone suppression, decreased fertility, increased cholesterol levels and diabetes, among others.[3,4,5,6] Given the prevalence and ubiquitous nature of PFAS in the environment and every-day consumer products (including our drinking water supply), there is a critical need to develop quantitative tools capable of accurately and precisely detecting low-levels of PFAS in biological fluids to inform the extent of their bioaccumulation and overall impact on the human body. Close biomonitoring of PFAS levels will help evaluate their toxicity and further understand the health consequences associated with their bioaccumulation over time in exposed human populations.

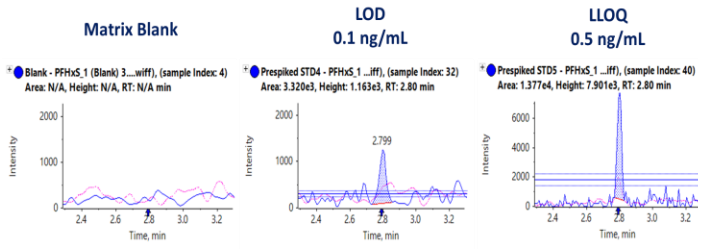

Figure 1: Accurate and sensitive detection of PFAS extracted from serum samples. Representative extracted ion chromatograms (XICs) of the qualifier ion for PFHxS, one of the most persistent PFAS measured at the highest levels in exposed human population. From left to right: in matrix blank, at 0.1 ng/mL (LOD) and 0.5 ng/mL (LLOQ).

In this technical note, a quantitative workflow for the analysis of 22 PFAS in serum was developed using the SCIEX QTRAP 6500+ System. This targeted screening workflow provides a fast analytical method capable of accurately quantifying sub-nanogram per mL levels of PFAS in the human body.

Key features of LC-MS/MS method for accurate quantification of PFAS in serum

- Simple sample preparation procedure enables fast and efficient extraction of PFAS from serum samples
- Modifications to the LC system components and consumables were made to reduce the risk of system-related PFAs interferences and minimize the impact of background contamination
- Rapid (6.5 minutes) and high-throughput acquisition method using the Scheduled MRM Algorithm Pro in SCIEX OS Software enables accurate quantification of 22 PFAS at bio-relevant concentrations in serum
- Method demonstrated excellent reproducibility and linearity for PFAS concentrations ranging from 0.5 to 100 ng/mL, with R^2 values greater than 0.99, while maintaining accuracy and precision across the calibration range
- Presented workflow provides a fast and easily implemented solution to accurately measure bioaccumulation levels of PFAS in serum
- Method is adaptable for high-throughput biomonitoring studies aimed at determining the potential toxic effects of PFAS bioaccumulation associated with human exposure

Experimental details

Standards and internal standards: A total of 22 PFAS and 15 mass-labeled internal standards were purchased from Wellington Laboratories (Guelph, ON, Canada) and prepared using Baker's HPLC-grade methanol. A list of all the PFAS included in this panel is summarized in Table 1.

Calibrator preparation: A 1 µg/mL stock standard solution mixture containing the 22 PFAS was prepared by diluting the stock standard solutions with methanol. The resulting 1 µg/mL stock standard solution mixture was used to spike 50 µL of serum in order to prepare a series of 9 calibrator solutions covering concentations ranging from 0.01 to 100 ng/mL. A 1 µg/mL stock internal standard solution mixture containing the mass-labeled internal standards was prepared similarly by diluting the stock standard solutions with methanol. This solution mixture was used to prepared a 5 ng/mL mass-labeled internal standard solution in 0.1 M formic acid. High density polyethylene or polypropylene consumables (Eppendorf tubes, pipette tips, HPLC vials, etc) were used to minimize PFAS contamination from external sources.

Sample preparation: PFAS were extracted from 50 µL serum samples by using a protein precipitation procedure summarized in Figure 2.

Liquid chromatography: UHPLC separation was performed on a Phenomenex Gemini® C18 column (50 x 2 mm, 3 µm, 00B-4439-B0) at 25ºC on a SCIEX ExionLC™ AC System. A Phenomenex Luna C18(2) column (30 x 2 mm, 5 µm, 00A-4252-Y0) was installed between the pump mixing chamber and the analytical column used for separation. This additional column served as a delay column to isolate PFAS contamination leaching from the LC system components and minimize the risk of system-related PFAS interfering with real signals from the sample during the analytical run.

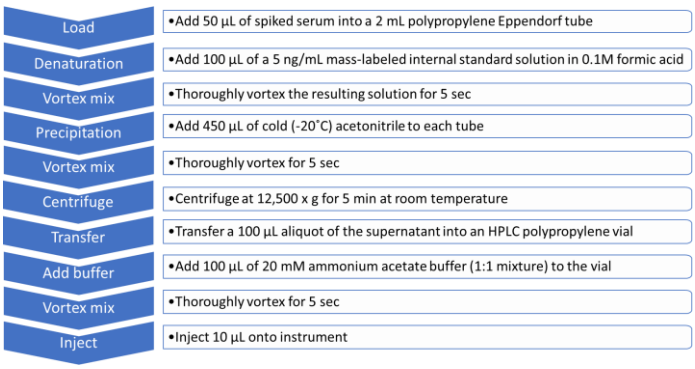

Figure 2. Protein precipitation procedure for serum samples. A 10-step protein precipitation procedure was used for extracting the PFAS from serum samples for MS analysis.

In addition, the fluoroethylene and Teflon tubing on the ExionLC AC System pumps and degasser was replaced with PEEK tubing to minimized the impact of PFAS background contamination and leaching. The FEB and PTFE tubings from the rinse solvent lines, the needle seal, the sample holding loop, pump seal, pump lining and degasser unit were also replaced with PEEK tubing to avoid system-related interferences. Additionally, the PTFE frits and rotor seals were replaced with stainless-steel parts. Mobile phases used for analytical separation were ammonium acetate in water (mobile phase A) and formic acid in methanol (mobile phase B) prepared from HPLC and LC-MS/MS grade solvents. The LC flow rate was 0.6 mL/min and the total run time was 6.5 min. The injection volume was 10 µL. Each extracted sample was injected in triplicate over the course of two consecutive days.

Mass spectrometry: Data were collected using a SCIEX QTRAP 6500+ System with a IonDrive™Turbo V Ion Source, operated with electrospray ionization (ESI) in negative mode. The ion source parameters were optimized for the LC conditions using the compound optimization (FIA) feature in Analyst® Software. A single acquisition method consisting of 57 MRM transitions (42 for the PFAS and 15 for the mass-labeled internal standards) was created in Analyst Software. The Scheduled MRM Algorithm was used to optimize data sampling across each peak and maximize the dwell times used, ensuring reliable integration, quantification and confirmation for each of the PFAS in the panel.

Data analysis: Data processing was performed using SCIEX OS Software. Detection and integration of the peaks from the background was achieved using the MQ4 algorithm. Quantitative analysis was performed in the Analytics module of the software where calibration curves, concentration calculations, assay precision and accuracy statistics were automatically generated.

LC system optimization and use of appropriate laboratory consumables leads to reduction in PFAS contamination

PFAS are pervasive and persistent compounds that have the potential to accumulate and contaminate the LC systems used in analytical testing. As a result, measures were implemented to reduce the risk of outside, ambient and system-related PFAS contamination. The most critical measure was the inclusion of a delay column between the autosampler and LC pumps to trap ambient and system-related PFAS and ensure they will be retained away from the sample signals.

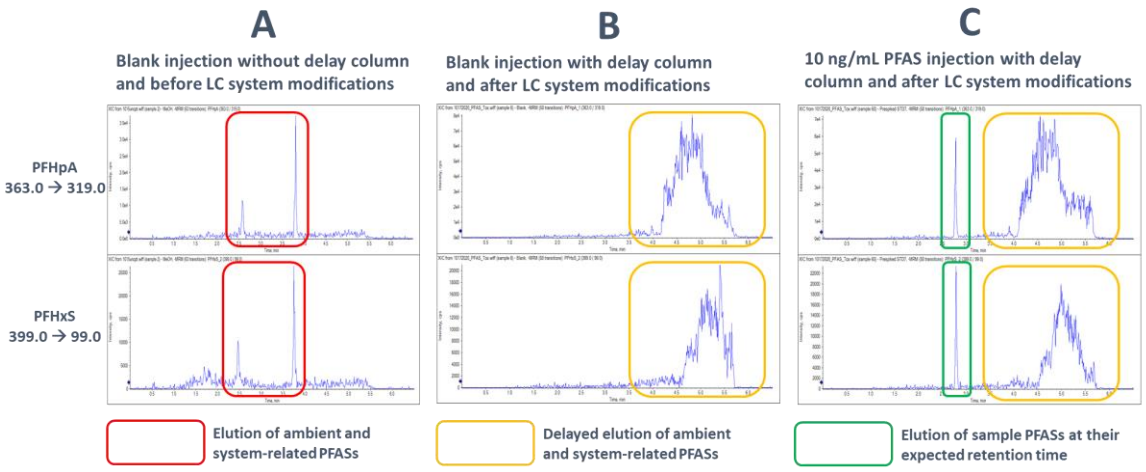

Figure 3. Benefits of using a delay column for PFAS analysis. XICs for PFHpA (top row) and PFHxS (bottom) showing (A) background and system-related contamination signals resulting from a blank sample injection before the system modifications were made showing, (B) broader and delayed contaminant peaks caused by the system-related PFAS that were held up following the addition of the delay column, and (C) sharp peaks resulting from the PFAS in the samples followed by the delayed and broad contamination peaks caused by the delayed column. The modifications made to the LC components significantly reduced the impact of system-related PFAS interferences and enabled accurate quantification of PFAS in serum samples.

Figure 3 summarizes the chromatographic response following the addition of the delay column and shows the extracted ion chromatograms (XICs) for PFHpA (top row) and PFHxS (bottom row) before and after the hardware modifications were made on the LC system.

Figure 3A shows the background signal resulting from a blank sample injection before the system modifications were made. Sharp PFAS peaks resulting from the ambient LC system contamination were observed throughout the chromatograms, including at the retention times at which the sample peaks were expected.

Figure 3B shows the background signal of the same blank sample injection after the system modifications were made (including the addition of the delay column). This configuration eliminated the PFAS interference peaks at the expected analyte retention times and produced a much broader and delayed contaminant peak caused by the system-related PFAS that were held up by the delay column.

Figure 3C shows the XICs resulting from a 10 ng/mL injection of a sample containing all the PFAS in the panel with the system modification. The XICs show a sharp peak resulting from the PFAS in the samples followed by the same broad and delayed contamination peak shown in Figure 3B. The addition of the delay column and the modifications made to the LC system components together minimized the impact of system-related PFAS contamination and ensured the analytical integrity of this quantitative workflow.

Minimizing system-related interference results in accurate measurements of bio-relevant PFAS levels from serum samples

Chromatographic separation of PFAS, including the two compounds that contain both branched and linear isotopes (PFOS and PFHxS), is critical to ensure reliable and accurate quantification. Figure 4 shows the chromatographic profile on an injection of the neat, 10 ng/mL standard solution containing the 22 PFAS. The choice of column, gradient and optimized mobile phase composition resulted in the baseline separation that was needed to correctly distinguish all isomers. As seen in Figure 4, the delayed contamination peaks caused by the delayed column did not interfere with the PFAS sample peaks.

Blank serum samples were spiked with the standard mixture containing the 22 PFAS at concentrations ranging from 0.01 to 100 ng/mL. These standard solutions were extracted using the protein precipitation procedure and injected in triplicate. Data were processed in the Analytics module in SCIEX OS Software 2.0 using the MQ4 Algorithm.

Calibration curves were generated for each of the PFAS in the panel and plotted across 9 calibration levels ranging from 0.1 to 100 ng/mL to evaluate the linearity of the method for the serum-spiked control samples. Figure 5 shows the resulting calibration curves for the native linear perfluoroalkylsulfonates (A) and the native linear perfluoroalkyl carboxylic acids (B). These calibration curves demonstrated excellent linearity covering 3 orders of magnitude with R^2 greater than 0.99 for all of the PFAS in the panel with the exception of PFODA. The system modifications implemented in this method were critical in attaining linearity achieved in this workflow.

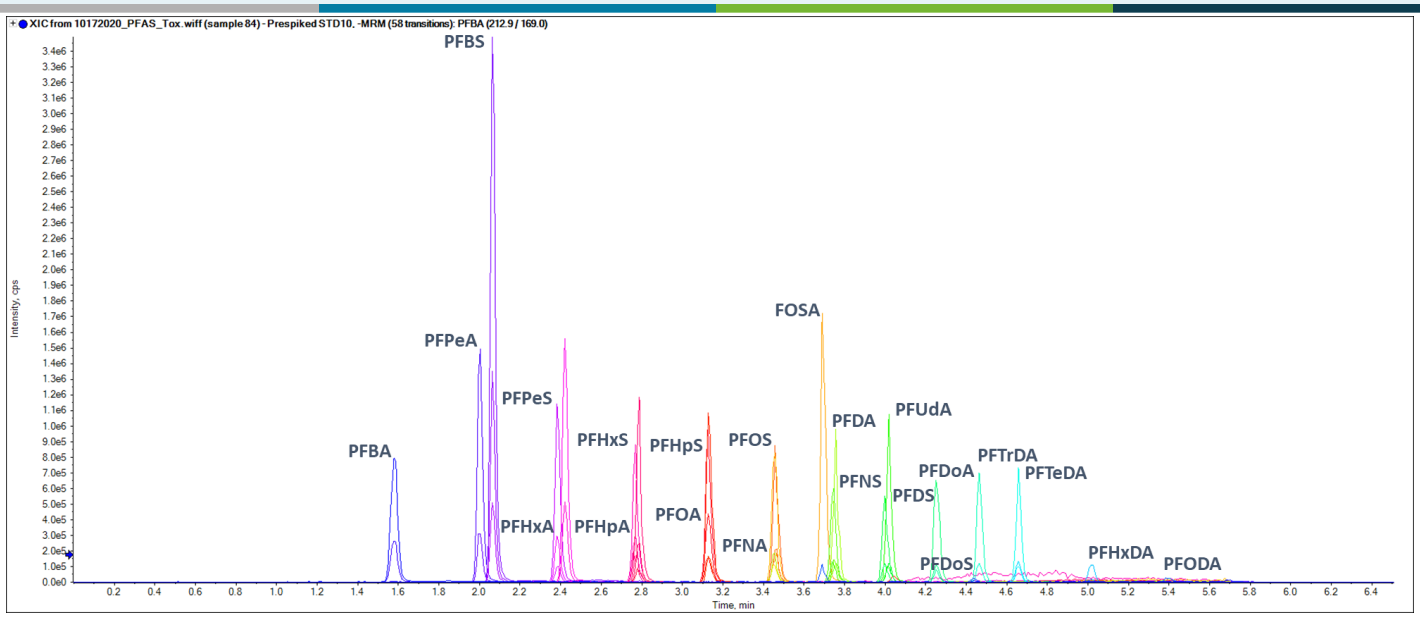

Figure 4. Chromatographic profile of the 22 PFAS monitored in this study. Extracted ion chromatograms (XICs) resulting from the optimized data acquisition method using a 10 ng/mL neat standard mixture. The combination of the optimized mobile phase composition and column choice enabled baseline separation of the PFAS from the injected sample, including isomers such as PFOS and PFHxS.

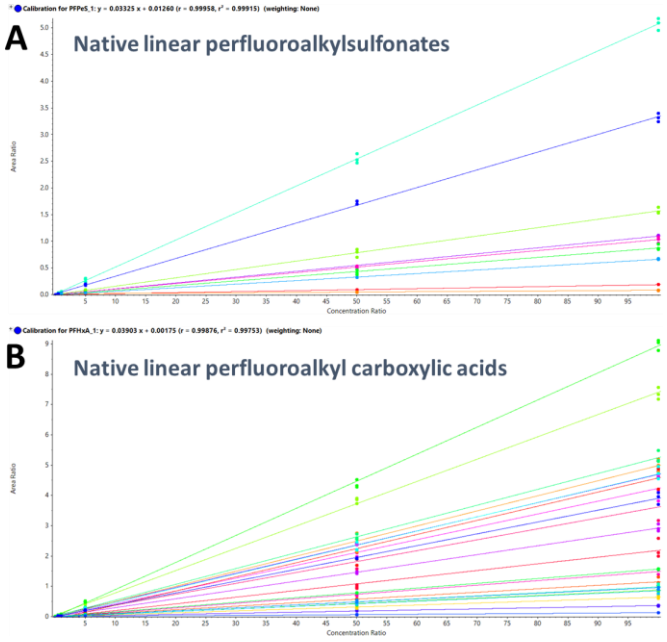

Figure 5. Excellent linearity for the 22 PFAS. Regression curves resulting from the calibration series from 0.5 to 100 ng/mL for A) native linear perfluoroalkylsulfonates and B) native linear perfluoroalkyl carboxylic acid extracted from serum samples. R^2 values greater than 0.99 were observed for all of the PFAS in the panel.

Detection method enables reproducible and accurate quantification of PFAS in serum

The ability to accurately detect low levels of PFAS extracted from serum samples is critical to inform the extent of their accumulation in the human body. The series of calibrators was injected to evaluate the ability to quantify PFAS across a wide range of concentrations. Figure 6 shows representative extracted ion chromatograms (XIC) for the two MRM transitions monitored for PFHxS and PFOA, two PFAS commonly measured in the exposed population. Five levels of calibrators were used to determine the ion ratio criteria for the quantifier and qualifier ions of PFHxS and PFOA. The series of XIC traces for the two compounds showed a high level of consistency and precision, as evidenced by the acceptance criteria (20% or less) of all the ion ratios across the calibration series ranging from 0.5 (LLOQ) to 100 ng/mL. However, the majority of the PFAS showed detectable signal below the LLOQ. Figure 1 shows the XICs for PFHxS for the matrix blank (left), at 0.1 ng/mL (middle) and 0.5 ng/mL (right). The signal at 0.1 ng/mL is the limit of detection (LOD) for PFHxS as it is well above the blank signal. Similar peaks were observed below the LLOQ for the PFAS in the panel. In addition, the assay showed great reproducibility over the course of the two consecutive days the data were acquired (inter-day peak area variations of 5% or less were observed for the 22 PFAS across the calibration range).

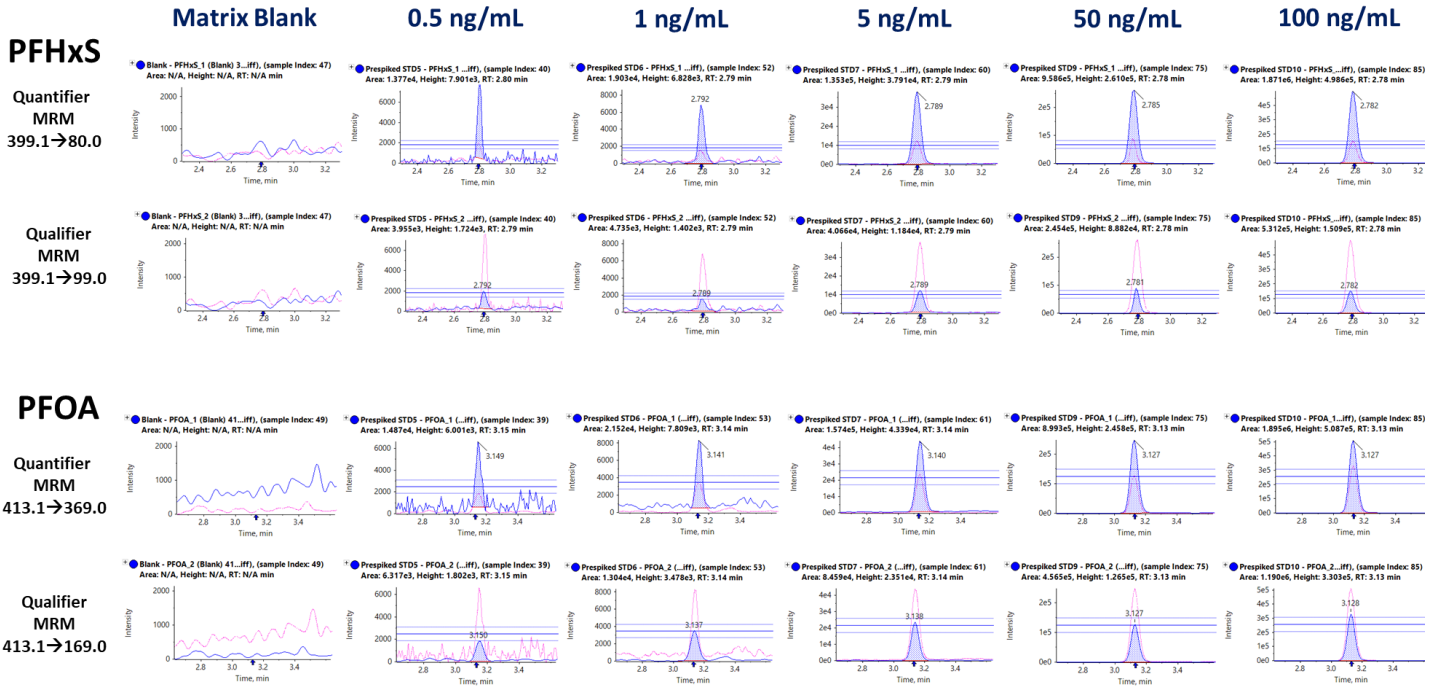

Figure 6. Accurate quantification of bioaccumulating PFAS in serum. Extracted ion chromatograms (XICs) for A) PFHxS and B) PFOA, two of the most persistent and common PFAS measured in the exposed human population. The XICs display overlays of both the quantifier and qualifier ions from 0.5 to 100 ng/mL as well as the tolerance in the form of the ion ratio line overlay that help in visualizing the confidence levels for the ion ratios. All ion ratios passed the ion ratio acceptance criteria (20% or less) across the calibration range (0.5 to 100 ng/mL).

The lower limit of quantification (LLOQ) for each of the PFAS included in the panel was determined based on the lowest concentration at which the integrated peak area of the analyte was quantifiable, with calculated concentration accuracy between 80 and 120%, precision (%bias) below 20%, ion ratio acceptance criteria of less than 20% and maintaining a linear calibration curve with an R^2 value of at least 0.99.

Table 1 summarizes the quantitative performance of the workflow and includes the calibration range, linear correlation coefficient (R^2 value), LLOQ, as well as the accuracy and precision values at the LLOQ for each of the 22 PFAS monitored in this workflow. PFBS and PFODA are the only two PFAS in the panel that proved challenging to quantify accurately due to their ubiquitous presence in the analytical system. This was evidenced by their detection in blank and solvent injections and as a result, their pervasiveness impacted their detection limits in this workflow. PFBS was quantified accurately from 5 to 100 ng/mL however PFODA could not be quantified.

The assay as a whole demonstrated excellent reproducibility, linearity, precision and accuracy for all the other PFAS in the panel. This method achieved the required levels of robustness and qualitative performance necessary to accurately measure sub ng/mL levels of PFAS from serum samples.

Conclusions

A robust and sensitive workflow for the detection of PFAS in serum samples using the SCIEX QTRAP 6500+ System was successfully developed. The addition of a delay column and the modifications made to the LC system components reduced the risk of system-related PFA interferences. The combination of a simple sample preparation procedure with a fast LC separation enabled accurate and sensitive detection of 22 PFAS down to sub ng/mL levels. The assay showed excellent reproducibility, precision, accuracy and linearity, with an LLOQ of 0.5 ng/mL, LOD of 0.1 ng/mL and an R^2 of greater than 0.99 for the vast majority of the PFAS in the panel with the exception of PFBS and PFODA. The excellent precision and accuracy observed at the LLOQ is highlighted in Table 1.

Overall, the developed method provides a robust and accurate method for bio-monitoring of low-levels of PFAS in biological fluids. Therefore, the presented workflow is readily adaptable for high-throughput toxicology investigations aimed at determining the extent of PFAS bio-accumulation and its broader impact on human health.

References

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Table 1. Statistical results for the 22 PFAS monitored in this workflow. The table includes calibration range, linear correlation coefficient (R^2 Value), and LLOQ, as well as the accuracy and precision at the LLOQ for each of the PFAS MRM transitions monitored.

Compound	Calibration Range (ng/mL)	Linear Correlation (R^2)	LLOQ (ng/mL)	Accuracy at LLOQ (%)	Precision at LLOQ (%)
PFBS 1	*0.5-100*	*0.99876*	*0.5*	*101.70*	*10.16*
PFPeA 1	*0.1-100*	*0.99896*	*0.1*	*99.41*	*12.21*
PFBS 1	*5-100*	*0.99647*	*5*	*99.55*	*5.51*
PFBS 2	*5-100*	*0.99689*	*5*	*101.50*	*3.68*
PFHxA 1	*0.5-100*	*0.99753*	*0.5*	*109.93*	*19.61*
PFHxA 2	*0.5-100*	*0.99791*	*0.5*	*102.34*	*11.19*
PFPeS 1	*0.5-100*	*0.99793*	*0.5*	*90.54*	*8.97*
PFPeS 2	*0.5-100*	*0.99846*	*0.5*	*106.62*	*6.88*
PFHpA 1	*0.5-100*	*0.99331*	*0.5*	*114.43*	*6.60*
PFHpA 2	*0.5-100*	*0.99685*	*0.5*	*111.71*	*6.76*
PFHxS 1	*0.5-100*	*0.99780*	*0.5*	*84.15*	*5.96*
PFHxS 2	*0.5-100*	*0.99741*	*0.5*	*80.98*	*1.33*
PFOA 1	*0.5-200*	*0.99885*	*0.5*	*96.41*	*10.31*
PFOA 2	*0.5-200*	*0.99570*	*0.5*	*104.74*	*3.12*
PFHpS 1	*0.5-100*	*0.99359*	*0.5*	*116.49*	*1.59*
PFHpS 2	*0.5-100*	*0.99386*	*0.5*	*115.21*	*3.58*
PFNA 1	*0.5-100*	*0.99151*	*0.5*	*110.17*	*0.88*
PFNA 2	*0.5-100*	*0.99237*	*0.5*	*99.73*	*9.95*
FOSA 1	*0.5-100*	*0.99702*	*0.5*	*89.92*	*1.35*
PFOS 1	*0.5-100*	*0.99294*	*0.5*	*104.67*	*16.54*
PFOS 2	*0.5-100*	*0.99932*	*0.5*	*108.33*	*14.24*
PFDA 1	*0.5-100*	*0.99557*	*0.5*	*100.17*	*7.09*
PFDA 2	*0.5-100*	*0.99002*	*0.5*	*91.69*	*2.00*
PFNS 1	*0.5-100*	*0.99214*	*0.5*	*101.09*	*11.33*
PFNS 2	*0.5-100*	*0.99721*	*0.5*	*99.06*	*15.41*
PFUdA 1	*0.5-100*	*0.99835*	*0.5*	*94.08*	*9.71*
PFUdA 2	*0.5-100*	*0.99524*	*0.5*	*107.37*	*3.33*
PFDS 1	*0.5-100*	*0.99477*	*0.5*	*94.23*	*1.89*
PFDS 2	*0.5-100*	*0.99651*	*0.5*	*111.42*	*0.23*
PFDoA 1	*0.5-100*	*0.99634*	*0.5*	*107.00*	*3.59*
PFDoA 2	*0.5-100*	*0.99950*	*0.5*	*99.49*	*16.56*
PFTrDA 1	*0.5-100*	*0.99732*	*0.5*	*93.24*	*3.47*
PFTrDA 1	*0.5-100*	*0.99495*	*0.5*	*102.89*	*16.07*

Table 1. Statistical results for the 22 PFAS monitored in this workflow. Continued.

Compound	Calibration Range (ng/mL)	Linear Correlation (R^2)	LLOQ (ng/mL)	Accuracy at LLOQ (%)	Precision at LLOQ (%)
PFTeDA 1	*0.5-100*	*0.99761*	*0.5*	*96.96*	*19.47*
PFTeDA 2	*1-100*	*0.99443*	*1*	*90.17*	*11.51*
PFHxDA 1	*0.5-100*	*0.99465*	*0.5*	*111.05*	*2.38*
PFHxDA 2	*10-100*	*0.99481*	*1*	*95.32*	*19.95*
PFODA 1	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*
PFODA 2	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*
PFDoS 1	*1-100*	*0.99263*	*1*	*96.55*	*7.40*
PFDoS 2	*5-100*	*0.99680*	*5*	*100.08*	*16.05*

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