Received: 12 September 2023 | Revised: 29 November 2023 | Accepted: 20 December 2023

DOI: 10.1002/jms.4999

RAPID COMMUNICATION

Nordic Symposium for Mass Spectrometry 2023

Journal of MASS SPECTROMETRY WILEY

Assessing the performance of a targeted absolute quantification isotope dilution liquid chromatograhy tandem mass spectrometry assay versus a commercial nontargeted relative quantification assay for detection of three major perfluoroalkyls in human blood

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Funding information
This study received funding from the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS) under grant number 2015-756.

Abstract

Isotope dilution ultrahigh-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) is commonly used for trace analysis of polyfluoroalkyl and perfluoroalkyl substances (PFAS) in difficult matrices. Commercial nontargeted analysis of major PFAS where relative concentrations are obtained cost effectively is rapidly emerging and is claimed to provide comparable results to that of absolute quantification using matrix matched calibration and isotope dilution UHPLC–MS/MS. However, this remains to be demonstrated on a large scale. We aimed to assess the performance of a targeted absolute quantification isotope dilution LC–MS/MS assay versus a commercial nontargeted relative quantification assay for detection of three major PFAS in human blood. We evaluated a population-based cohort of 503 individuals. Correlations were assessed using Spearman's rank correlation coefficients (rho). Precision and bias were assessed using Bland–Altman plots. For perfluorooctane sulfonic acid, the median concentrations were 5.10 ng/mL (interquartile range [IQR] 3.50–7.24 ng/mL), the two assays correlated with rho 0.83. For perfluorooctanoic acid, the median concentrations were 2.14 ng/mL (IQR 1.60–3.0 ng/mL), the two assays correlated with rho 0.92. For perfluorohexanesulfonate, the median concentrations were 5.5 ng/mL (IQR 2.50–11.61 ng/mL), the two assays correlated with rho 0.96. The Bland–Altman statistical test showed agreement of the mean difference for the majority of samples (97–98%) between the two assays. Absolute plasma concentrations of PFAS obtained using matrix matched calibration and isotope dilution UHPLC–MS/MS show agreement with relative plasma concentrations from a nontargeted commercial platform by Metabolon. We observed striking consistency between the two assays when examining the associations of the three PFAS with cholesterol, offering additional confidence in the validity of utilizing the nontargeted approach for correlations with various health phenotypes.

KEYWORDS
cholesterol, isotope dilution, mass spectrometry, metabolon, nontargeted, PFAS, targeted

1 | INTRODUCTION

Perfluoroalkyl substances (PFAS), consisting of perfluorosulfonates and perfluorocarboxylates, have been manufactured and employed as surfactants and surface protectors in various commercial and industrial products such as textiles, paper packaging, aqueous film-forming foams, mining and oil well surfactants, alkaline cleaners, and cosmetics, since their initial commercialization in the 1940s.[1,2]

The widespread environmental contamination of PFAS has driven extensive research in the context of exposure assessment and environmental health epidemiology.[3–5] The research field has also expanded due to advancements in analytical technologies, particularly within the context of ultrahigh pressure liquid chromatography hyphenated with electrospray ionization (ESI) mass spectrometry (UHPLC–MS/MS).[6,7] The most common methods for PFAS analysis in complex matrices involve targeted methods and isotope dilution using ^{13}C-labeled internal standards for quantification.[8] Isotope ratio measurements of the most intense ions for both native and labeled internal standards ensure the required specificity and support accuracy and precision in PFAS in analysis and subsequent human exposure and health assessment.

Recent advances in analytical techniques and computational methods have provided the opportunity to perform nontargeted high-resolution mass spectrometry semiquantitative analysis of endogenous metabolites and exogenous exposures simultaneously within a domain known as the exposome.[2,7,9–15] Such nontargeted exposome assays for the most part have provided relative concentrations with arbitrary units and are considered less informative because they are contextual and relevant only to the study at hand and therefore difficult to extend to other studies for comparing exposure levels, temporal trend monitoring, and meta-analyses.[16]

Nontargeted analysis of PFAS where relative concentrations are obtained is rapidly emerging and is claimed to provide comparable results to that of isotope dilution UHPLC–MS/MS. However, this remains to be demonstrated on a large scale. Thus, the main aim of the current study is to compare absolute plasma concentrations of PFAS obtained by protein precipitation and matrix matched calibration isotope dilution UHPLC–MS/MS with semiquantitative relative concentration measurements performed on the same plasma samples using a widely used commercial nontargeted platform. Further, numerous prior studies have reported an association between perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) concentrations and cholesterol levels.[17–21] Therefore, we aimed to compare the relationship of PFOA and PFOS obtained from these two assays with total cholesterol levels.

2 | MATERIALS AND METHODS

2.1 | Study participants

The Prospective study on Obesity, Energy, and Metabolism recruited 50-year-old men and women from the general population by a random invitation by mail using public population registers for the municipality of Uppsala, Sweden.[22] The participants received their invitation 1 month after their 50th birthday. A total of 502 individuals took part in the study, a participation rate of 25%. The study was approved by the ethics committee at Uppsala University (No. 2009/057 and No. 2012/143), and the participants gave their informed consent. All participants were requested to fast from midnight before the investigation that took place in the morning between 8 and 10 a.m. All participants had to confirm that they adhered to the fasting request before blood sampling, whereafter serum and plasma were separated and stored in −80°C freezers until analysis.

2.2 | PFAS analysis

2.2.1 | Targeted isotope dilution UHPLC–MS/MS

Target PFAS were determined in 150 µL serum samples using matrix matched isotope dilution ultraperformance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS; Waters Corporation, Milford, USA) equipped with an ACQUITY UPLC PFC isolator column) that is situated between injector and mixer and traps any PFAS coming from the solvents and tubings from the system. Analytes are separated on an ACQUITY UPLC BEH C18 (1.7 µm, 2.1 mm × 100 mm) analytical column coupled to tandem mass spectrometry as previously described.[23] Native calibration standards and corresponding labeled internal and recovery standards (13C) were purchased from Wellington Laboratories (Guelph, Ontario, Canada). The analytical method used to measure PFAS was not isomer specific; thus, concentrations for linear PFOS, PFOA, and perfluorohexanesulfonate (PFHxS) isomers are reported. For quantification, each analytical batch of authentic samples was processed together with an eight-point matrix matched calibration (seven matrix matched calibrations: R^2 > 0.999 and mean percent relative standard deviations (% RSDs): linear PFHxS = 13.4%, linear perfluoro-1-octanesulfonate [PFOS] = 11.0%, linear perfluoro-n-octanoic acid [PFOA] = 7.00%). For quality assurance and quality control, 15 NIST SRM 1957 serum samples were processed throughout the study and found to conform with the certified values (mean values and RSDs; PFHxS = 3.70 ng/mL and 6%; PFOS = 11.77 ng/mL and 7%; PFOA = 4.84 ng/mL and 4%; perfluorodecanoic acid = 0.29 ng/mL and 9%). For quality

precision, 49 in-house reference plasma samples were processed throughout the study (mean RSDs: PFHxS = 11.9%, L-PFOS = 10.5%, PFOA = 8.60%, perfluorodecanoic acid = 14.4%). For method detection limits, 49 water blanks were processed throughout the study, and the established method detection limits were PFHxS = 0.06 ng/mL; PFOS = 0.02 ng/mL; and PFOA = 0.11 ng/mL. Overall, the method produced satisfactory results in terms of linear range, accuracy, precision, and sensitivity throughout the study.

2.2.2 | Commercial nontargeted metabolomics

Nontargeted metabolomics was performed on plasma samples stored at −80°C (Metabolon Inc., USA). Samples were prepared using the automated MicroLab STAR® system from Hamilton Company. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase UPLC–MS/MS methods with positive ion-mode ESI, one for analysis by reverse phase UPLC–MS/MS with negative ion-mode ESI, and one for analysis by hydrophilic interaction UPLC–MS/MS with negative ion-mode ESI, and one sample was reserved for backup. The values were normalized and given in arbitrary units. Only the PFAS PFHxS, PFOA, and PFOS were provided using this commercial platform, and these were detected in >95% of the participants.

2.3 | Statistical analysis

PFAS concentrations were not normally distributed; therefore, we first log normal-transformed the concentrations to achieve normal distributions. The presence of different units evaluated by the two methods hinders straightforward comparison of the estimates. To address this limitation, mean centering and unit variance scaling were applied during the data preprocessing stage. This normalization approach enables the comparison of variables with disparate units or distributions on a standardized basis. By employing this method, all variables are afforded equal inherent importance in subsequent statistical analyses.[24] For comparisons between the absolute concentrations of PFAS measured using the conventional isotope dilution UHPLC–MS/MS method and the nontargeted commercial platform, pairs for all individuals' measurements were plotted on scatter plots and Spearman's rank correlation coefficients (*r*) were calculated. Next, Bland–Altman plots were constructed with 95% confidence intervals (CI) shown[25] based on the log normal-transformed and autoscaled data. The plot displays the differences between two measurements on the *y*-axis and the average of the two measurements or instruments on the *x*-axis. Each point on the plot represents a paired difference between the two measurements. The plot also includes lines for the mean difference and limits of agreement, which were set at 1.96 times the standard deviation of the differences. The limits of agreement represent the range of differences within which 95% of the differences between the two measurements are expected to lie, assuming that there is no systematic bias. Statistical analyses were carried out using STATA 16.1 (StataCorp LLC).

TABLE 1 Basic characteristics of the study participants in the Prospective study on Obesity, Energy, and Metabolism cohort.

N	493
Age, years, mean (standard deviation, [*SD*])	50 (0.1)
Female sex (%)	50
Systolic blood pressure (mmHg), mean (*SD*)	125.6 (16.4)
Diastolic blood pressure (mmHg), mean (*SD*)	77.0 (10.1)
High-density lipoprotein cholesterol (mmol/L), mean (*SD*)	1.3 (0.3)
Triglycerides (mmol/L), mean (*SD*)	1.2 (0.9)
Body mass index (BMI) (kg/m^2), mean (*SD*)	26.4 (4.2)
Waist circumference (cm), mean (*SD*)	92.5 (11.4)
Fasting glucose (mmol/L), mean (*SD*)	4.9 (0.9)
Diabetes medication (%)	0.2
Antihypertensive medication (%)	8.1
Exercise habits, 4-grade scale, mean (*SD*)	2.8 (1.01)
Education, years, (%)	<10 years: 8% 10–12 years: 44% >12 years: 48%
Smokers (%)	9.8

3 | RESULTS

Demographic characteristics of the participants included in the present study are shown in Table 1. In total, 493 participants from Uppsala, Sweden, with available PFAS measurements using isotope dilution UHPLC–MS/MS and nontargeted metabolomics were included at the baseline. The mean age was 50 (standard deviation: 0.1) years, 247 (50%) were female, mean body mass index (BMI) was 26.4 (standard deviation: 4.2), and 9.8% were smokers.

Figure 1 shows a comparison of the targeted method providing absolute concentrations of PFOS versus the nontargeted method providing relative concentrations of PFOS calculated from a total of 493 individuals. The Spearman's rank correlation coefficient was $r = 0.83$. The Bland–Altman plot shows agreement between two methods, with most measurement points falling withing the 95% CI. The Bland–Altman plot also shows that the largest differences between the two methods occurred at higher concentrations. However, the observed bias was found to impact approximately 2–3% (20–30 measurements) of the total samples ($n = 493$) and did not exhibit a systematic pattern, as not all samples with higher exposure

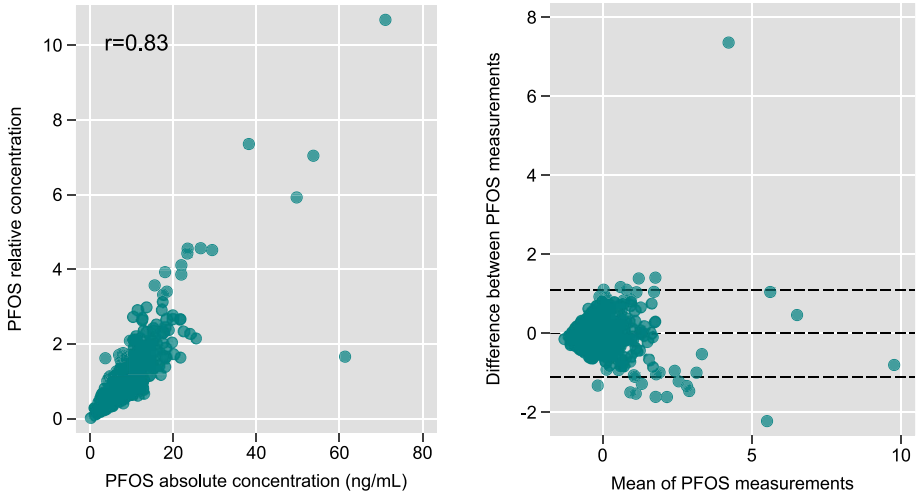

FIGURE 1 Comparison of the targeted method providing absolute concentrations of perfluorooctane sulfonic acid (PFOS) versus the commercial nontargeted method providing relative concentrations of PFOS calculated from a total of 493 individuals.

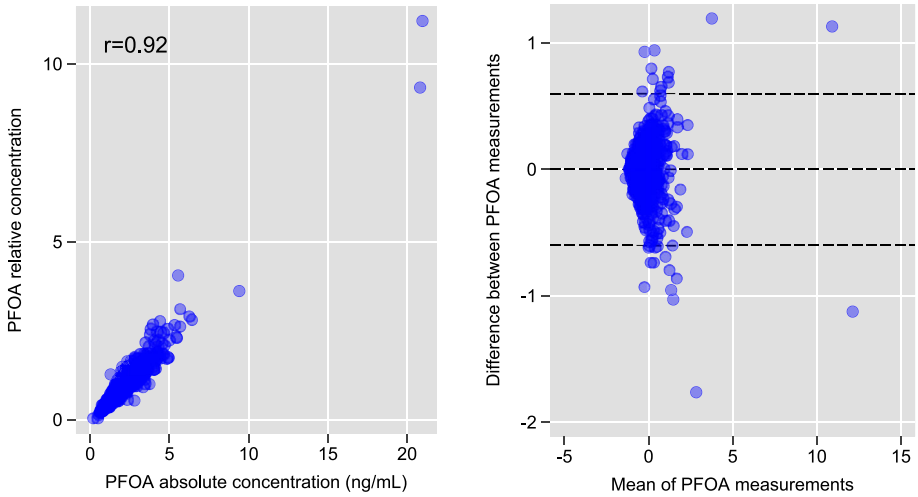

FIGURE 2 Comparison of the targeted method providing absolute concentrations of perfluorooctanoic acid (PFOA) versus the commercial nontargeted method providing relative concentrations of PFOA calculated from a total of 493 individuals.

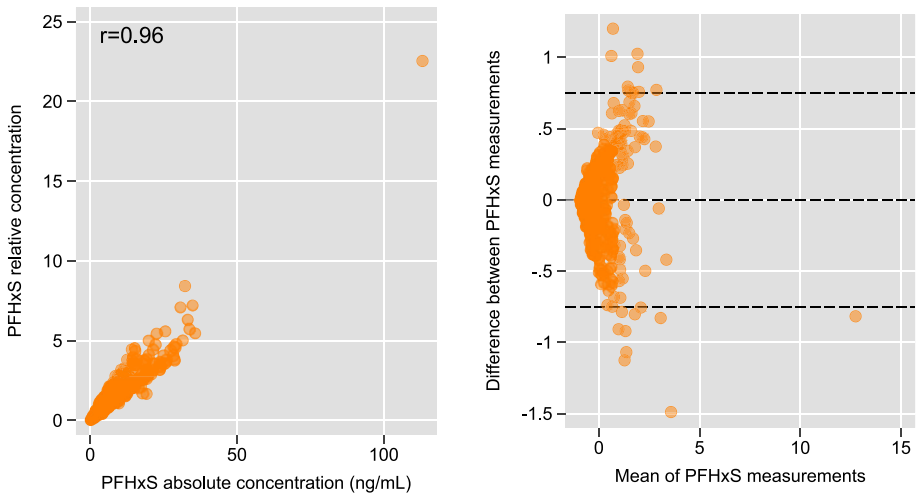

FIGURE 3 Comparison of the targeted method providing absolute concentrations of perfluorohexanesulfonate (PFHxS) versus the commercial nontargeted method providing relative concentrations of PFHxS calculated from a total of 493 individuals.

values exceeded the boundaries of the 95% limits, and the differences between the measurements obtained by the two methods did not consistently skew toward higher or lower values.

Figure 2 shows a comparison of the targeted method providing absolute concentrations of PFOA versus the nontargeted method providing relative concentrations of PFOA calculated from a total of 493 individuals. The Spearman's rank correlation coefficient was $r = 0.92$. The Bland–Altman plot shows agreement between two methods, with most measurement points falling withing the 95% CI. The Bland–Altman plot also shows that the largest differences

between the two methods occur at higher concentrations. However, also for PFOA, the differences between the measurements obtained from the two methods were not consistently skewed toward higher or lower values but occurred for 21 of the total sample size ($n = 493$) in middle and higher concentration range.

Figure 3 shows a comparison of the targeted method providing absolute concentrations of PFHxS versus the nontargeted method providing relative concentrations of PFHxS calculated from a total of 493 individuals. The Spearman's rank correlation coefficient was $r = 0.96$. The Bland–Altman plot shows agreement between two methods, with most measurement points falling withing the 95% CI. Again, the Bland–Altman plot also shows that the largest differences between the two methods occur at higher concentrations, albeit affecting only a small proportion (2%) of the entire sample size.

As demonstrated in Figure 4A, a significant correlation was observed between cholesterol levels and PFOS, with consistent correlation coefficients for both techniques (Spearmans's rho 0.17 and 0.15, respectively, P-value < 0.05). A parallel trend emerged in the analysis of PFOA (Figure 4B), with comparable strength evident in both nontargeted analysis and conventional assays (Spearmans's rho 0.13 and 0.14, respectively, P-value < 0.05). Similarly, for PFHxS (Figure 4C), comparable Spearmans's rho values were observed between the two techniques (Spearmans's rho 0.06 and 0.06, respectively); however, in this instance, none of these associations reached statistical significance.

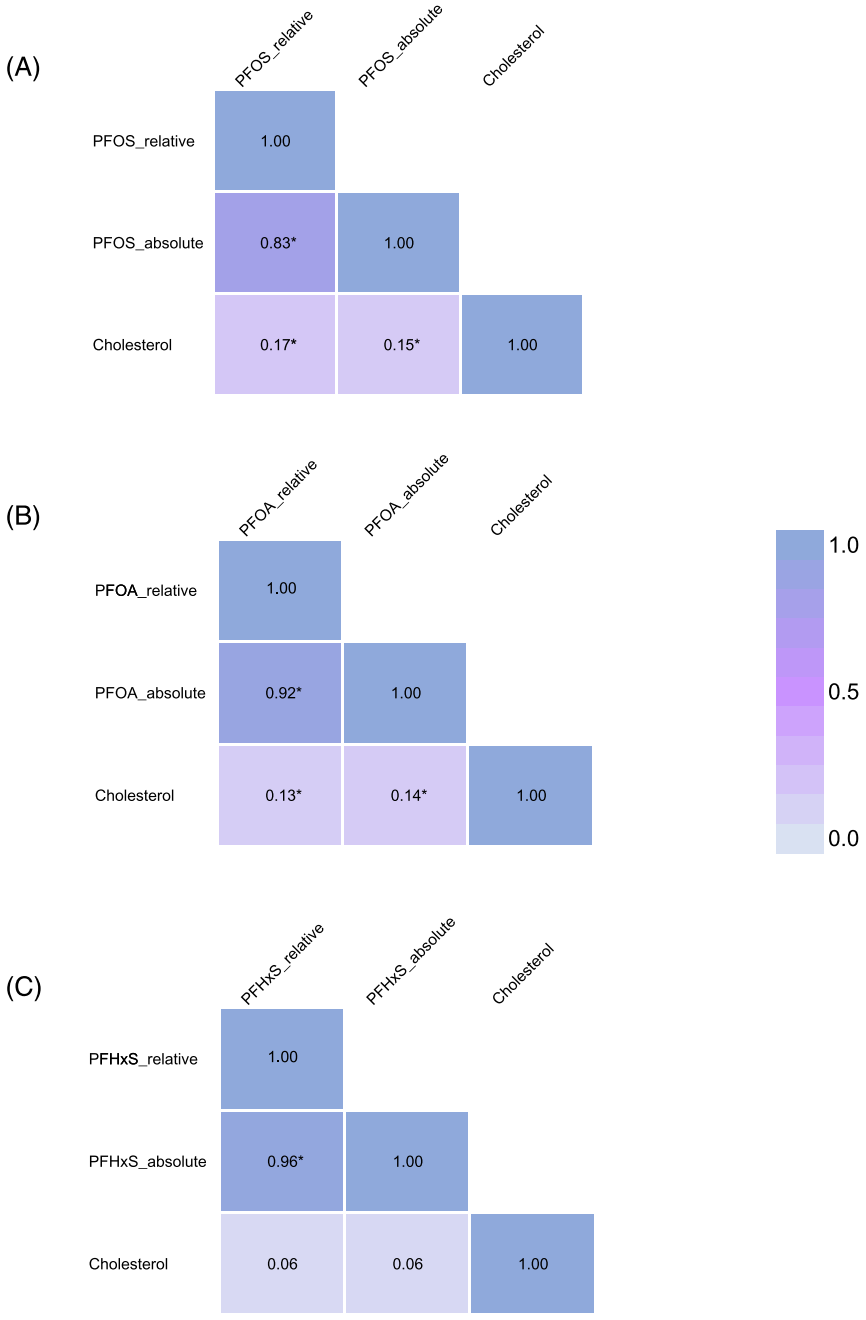

FIGURE 4 Differences in Spearman's rho coefficients for perfluorooctane sulfonic acid (PFOS) (A), perfluorooctanoic acid (PFOA) (B), and perfluorohexanesulfonate (PFHxS) (C) as measured by the two different assays in correlation with cholesterol levels ($N = 493$). *P-value < 0.05.

4 | DISCUSSION

We found that the absolute plasma concentrations of PFOS, PFOA, and PFHxS measured with the conventional targeted isotope dilution UHPLC–MS/MS method correlated well with the relative plasma concentrations obtained with a semiquantitative nontargeted metabolomics platform in a population-based cohort of adults. Notably, when comparing the mean differences between the two methods, we observed the largest differences between the two methods at the higher concentrations of the three evaluated PFAS (PFOS, PFOA, and PFHxS). Observing the Bland–Altman plots, the reason behind the positive and negative directions of deviation occurring at higher concentrations is unknown because these findings suggest that the observed bias does not follow a predictable trend and is not limited to specific measurement ranges or directions. Considering the high correlation observed between the data obtained from the two measurements, our study findings indicate that the data obtained from the conventional method and nontargeted metabolomics are comparable.

Human PFAS exposure has been studied extensively over the past decades, and reports of the widespread distribution have prompted the use of conventional targeted methods based on isotope dilution UHPLC–MS/MS.[8,9] More recently, nontargeted methods provided from commercial companies within the context of the exposome have become increasingly appealing due to the possibility to simultaneously measure environmental exposure and endogenous metabolite markers of health at similar cost. The main advantage of such exposome-wide analysis is that it provides the possibility to not only measure the environmental exposure but also allows for evaluation of associations of environmental exposures and metabolite markers of health.[15] One drawback, however, is that the nontargeted methods provide data that are for the most part expressed in relative concentrations, hampering absolute quantification exposure assessment, temporal trend monitoring, and comparison with other independent studies and meta-analyses.[16]

Even though data from targeted absolute concentration methods and nontargeted relative concentration methods are presented in different units, comparisons between these data are underexplored. In a smaller study comprising 180 girls from New York City, Petrick et al. compared targeted versus nontargeted measures of PFOS, PFOA, PFHxS, and perfluorononanoic acid (PFNA) and found a Spearman rho of PFOS = 0.92, PFOA = 0.81, PFHxS = 0.90, and PFNA = 0.69.[16] In our study, including more participants and different targeted and nontargeted platforms, we also found strong correlations between values of the absolute concentrations and relative concentrations of PFOS, PFOA, and PFHxS. The strongest correlation was observed for PFHxS (rho = 0.96), followed by PFOA (rho = 0.92) and PFOS (rho = 86). The Bland–Altman plot showed agreement between the two methods but also revealed that the largest differences in the evaluated PFAS occurred in samples where PFAS were detected in higher concentrations. Possible explanations for these observations are unknown because typically, the lower PFAS concentration samples would be expected to introduce the greatest variability between the two methods due to different limits of detection. Therefore, these deviations occurring at higher concentrations could potentially be related to matrix-dependent interferences such as suppression or enhancement of the analyte response in the specific samples in question that are not accounted for in the nontargeted metabolomics analysis.

An additional method for assessing two measurement techniques involves examining their relationship to a common third variable. In our study, we opted to use cholesterol levels, as prior research has established connections between at least PFOS and PFOA levels and cholesterol levels.[17–20] The evaluation of both abundances from nontargeted analysis and concentrations from conventional assays in relation to cholesterol yielded strikingly similar Speakmans' rho values. This consistency provides additional assurance in the validity of employing the nontargeted approach for correlations with health outcomes.

4.1 | Strengths and limitations

The strong correlations and low mean difference observed between targeted method providing absolute concentrations of PFAS versus the nontargeted method providing relative concentrations in a relatively large nonoccupationally population-based cohort strengthen the credibility of our results. Although the targeted method provided absolute concentrations for additional PFAS analogs, the present study was limited to the comparison of only three PFAS (PFOS, PFOA, and PFHxS) because these were the PFAS that could be detected using the commercial nontargeted metabolomics platform by Metabolon. This highlights an important difference in sensitivity between the two methods. In addition, limitations of this study include a lack of raw data and information about the potential sources of variation from the commercial platform by Metabolon. One such limitation is the lack of MS/MS data and additional analytical information to confirm PFAS chemicals and isomers. Furthermore, there is a lack of method details regarding the use of analytical hardware typically employed in quantitative PFAS assays to eliminate PFAS contaminants from the instrument and solvents.

5 | CONCLUSION

Nontargeted metabolomics methods where PFAS are measured have been claimed to provide comparable results to the conventional targeted methods. In this study, the performance of conventional targeted isotope dilution UHPLC–MS/MS was compared with that of a commercial nontargeted metabolomics by Metabolon. We observed a strong correlation of absolute plasma concentrations of PFOS, PFOA, and PFHxS measured using targeted isotope dilution UHPLC–MS/MS with the relative concentrations of a widely used nontargeted metabolomics platform. Further, the comparison between the two assays consistently demonstrated a significant correlation between cholesterol levels and PFOS and PFOA, with remarkably consistent correlation coefficients for both techniques. Similarly, for PFHxS,

though lacking statistical significance, a comparable correlation pattern was observed between the two methods. Although the relative concentrations of PFAS obtained from the nontargeted metabolomics are not suitable for absolute quantification exposure assessment, temporal trend monitoring, and exposure comparison with other studies and meta-analysis, our findings suggest that they accurately reflect levels obtained from targeted methods based on isotope dilution and therefore could be used in studies assessing relationships with other phenotypes and health outcomes.

ACKNOWLEDGMENTS

We greatly acknowledge the contribution of the POEM research study participants for their involvement.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed in the current study are not publicly available but are available from the corresponding author upon reasonable request.

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How to cite this article: Salihovic S, Dunder L, Lind M, Lind L. Assessing the performance of a targeted absolute quantification isotope dilution liquid chromatograhy tandem mass spectrometry assay versus a commercial nontargeted relative quantification assay for detection of three major perfluoroalkyls in human blood. *J Mass Spectrom*. 2024;59(2):e4999. doi:10.1002/jms.4999