Comparison of extraction and quantification methods of perfluorinated compounds in human plasma, serum, and whole blood

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ABSTRACT

Perfluorinated compounds are ubiquitous in the environment and have been reported to occur in human blood. Accurate risk assessments require accurate measurements of exposures, but identification and quantification of PFCs in biological matrices can be affected by both ion suppression and enhancement in liquid chromatography–tandem mass spectrometry techniques (LC/MS-MS). A study was conducted to quantify potential biases in LC/MS-MS quantification methods. Using isotopically labeled perfluorooctanoic acid ($[^{13}C_2]$-PFOA), perfluorononanoic acid ($[^{13}C_2]$-PFNA), and ammonium perfluorooctanesulfonate ($[^{18}O_2]$-PFOS) spiked tissues, ion-pairing extraction, solid-phase extraction, and protein precipitation sample preparation techniques were compared. Analytical accuracy was assessed using both solvent calibration and matrix-matched calibration for quantification. Data accuracy and precision of $100 \pm 15\%$ was demonstrated in both human sera and plasma for all three sample preparation techniques when matrix-matched calibration was used in quantification. In contrast, quantification of ion-pairing extraction data using solvent calibration in combination with a surrogate internal standard resulted in significant analytical biases for all target analytes. The accuracy of results, based on solvent calibration was highly variable and dependent on the serum and plasma matrices, the specific target analyte $[^{13}C_2]$-PFOA, $[^{13}C_2]$-PFNA, or $[^{18}O_2]$-PFOS, the target analyte concentration, the LC/MS-MS instrumentation used in data generation, and the specific surrogate internal standard used in quantification. These results suggest that concentrations of PFCs reported for human blood using surrogate internal standards in combination with external solvent calibration can be inaccurate unless biases are accounted for in data quantification.

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1. Introduction

The widespread use, environmental persistence and bioaccumulative potential of some perfluorinated compounds (PFCs) has resulted in the global occurrence of these substances [1]. PFCs have been found in a number of abiotic and biotic matrices including water, sediment and sludge as well as various wildlife species inhabiting not only locations in close proximity to urbanized and industrialized areas, but also in remote areas [2,3]. Exposure of humans to PFCs has been monitored by measures of PFCs in the diet [4]. More direct measures of exposure have included concentrations of PFCs in both human blood [5–8] and milk [7,9,10]. It is important that these analyses are both accurate and precise. Accurate risk assessments require accurate determination of concentrations of PFCs in blood and other tissues.

While PFCs have been used for a long time in a number of products, measurements in the environment have been limited by the availability of accurate and sensitive methods [11]. It was only with the advent of high-performance liquid chromatography (HPLC) coupled with electrospray ionisation–tandem mass spectrometry that PFCs could be accurately identified. However, until recently, quantification was hampered by a lack of standards, especially those labeled with stable isotopes, and standard reference materials. Early efforts at inter-laboratory validation of methods demonstrated that there was a great degree of variation in quantification of PFCs in methanol, water, and blood [12]. A 2003 workshop that investigated the current status of PFC analytical methods identified a number of limitations and issues in quantification of PFCs in various matrices [11]. Since the workshop, a number of advances in quantification of PFCs have been made. There are now more native standards available and standards labeled with ^{18}O and ^{13}C are also available. The availability of these standards has allowed investigation of matrix effects such as ion-suppression and -enhancement and recoveries achieved by different methods of extraction. Both of these types of matrix effects have been identified for different PFCs in different matrices [13]. Thus, it is important to assess the potential effects of matrices on both the extraction and quantification before accurate quantification can be assured. Early analyses have relied upon the use of external standards or surrogate internal standards (IS) for calibration. For instance, in earlier studies, tetrahydroperfluorooctanesulfonate (THPFOS) was often used as a surrogate analyte for assessment of method performance. THPFOS has been spiked into samples to measure recoveries with quantification based on external standards of compounds of interest such as perfluorooctanesulfonate (PFOS) or perfluorooctanoic acid (PFOA) [14]. Now that isotopically labeled standards are available, it is possible to assess the potential bias in using external and internal standards for quantifying PFCs [15,16].

This study was conducted to compare extraction and LC/MS-MS quantification and calibration methods used in determining concentrations of PFCs measured in human whole blood, blood plasma (BP), and blood serum (BS). Ion-pairing liquid extraction, solid-phase extraction (SPE), and protein precipitation extraction (PPE) methods were evaluated. Replicates of pre-extraction, post-extraction, and combined

pre- and post-extraction quality control (QC) samples were prepared using matrix spiked $[^{13}C_2]$-PFOA, $[^{13}C_2]$-PFNA, and $[^{18}O_2]$-PFOS human sera and human plasma. Additionally, perfluorobutanesulfonate (PFBS) and THPFOS were evaluated as surrogate internal standards. Pre-extraction spiked human serum or plasma was extracted by all three methods and quantified with matrix-matched calibration curves prepared with human serum and plasma using the same extraction methods. Matrix spike recoveries of $[^{13}C_2]$-PFOA, $[^{13}C_2]$-PFNA, and $[^{18}O_2]$-PFOS were evaluated over a range of 1.0–40 ng mL^{-1}. In addition, for direct comparison of results using the same extracts, quantification of ion-pairing extraction (IP) method performance was accomplished by both surrogate internal standard calibration and matrix-matched external calibration. Quantification of ion-pairing extraction data by solvent calibration with pre-extraction, post-extraction, and combined pre- and post-extraction internal standard additions was compared to matrix-matched external calibration.

Furthermore, an inter-laboratory comparison was conducted by analyzing human serum, plasma, and whole blood samples that had been previously analyzed using ion-pairing extraction and internal standard solvent calibration. The blood samples were reanalyzed by two laboratories using a validated method of ion-pairing extraction and matrix-matched external calibration [8].

2. Materials and methods

2.1. Experimental design—three extraction methods

The experimental design to determine analytical accuracy for three blood extraction methods was a $2 \times 3 \times 3 \times 3$ orthogonal design. The test system included two human blood test matrices (serum and plasma), three target analytes, ^{18}O-PFOS, ^{13}C-PFOA, and ^{13}C-PFNA, spiked into the blood samples at three different concentrations in triplicate in three individual lots each of human blood serum and human blood plasma, and three extraction methods: [ion-pairing extraction, solid-phase extraction and protein precipitation extraction]. Details of the extraction methods are given below. Three lots of BS and BP were used as test matrices and were spiked with ^{18}O-PFOS, ^{13}C-PFOA, and ^{13}C-PFNA prior to extraction. A fourth lot each of BS and BP was used for extracted calibration in the range of 0.25–50 ng mL^{-1} ^{18}O-PFOS, ^{13}C-PFOA, and ^{13}C-PFNA. The pre-spiked test matrix samples of serum and plasma were spiked in triplicate with ^{18}O-PFOS, ^{13}C-PFOA, and ^{13}C-PFNA at approximately 1.0, 7.5, and 40 ng mL^{-1}. For ion-pairing extraction in BS and BP, sets of pre-extraction, post-extraction, and combined pre- and post-extraction spiked samples of approximately 1.0, 7.5, or 40 ng mL^{-1} THPFOS and PFBS surrogate internal standards, ^{18}O-PFOS, ^{13}C-PFOA, and ^{13}C-PFNA were prepared.

2.2. Experimental design—quantification

Concentrations of ^{18}O-PFOS, ^{13}C-PFOA, and ^{13}C-PFNA were determined by three methods of calibration. The first calibration used PFBS and THPFOS pre-extraction spiked internal standards in combination with external standards in

methanol for calibration, the second was PFBS and THPFOS as post-extraction spiked internal standards in combination with external standards in methanol for calibration, and the third was matrix-matched external calibration standards in human plasma and serum. Matrix-matched calibration was applied to all three extraction methods to quantify method accuracy and precision. All three calibration methods were applied to the ion-pairing extraction method samples to assess analytical bias by the application of surrogate internal standards with external solvent calibration.

To assess the performance of the extraction and quantification methods, pooled samples of commercially available human BP and BS were used. Normal pooled human serum (off the clot) and plasma were purchased from Gemini Bio-Products (Woodland, CA), Rockland (Gilbertsville, PA), and Bioreclamation Inc. (Hicksville, NY) and stored frozen ($\leq -20\,^\circ$C) until used. Chemicals and reagents used in the extraction procedures or in the mobile phase were of reagent grade and were obtained from VWR Scientific (Bridgeport, NJ) and Sigma–Aldrich (St Louis, MO). HPLC grade solvents used for the mobile phase (methanol, water) were purchased from EM Science (Gibbstown, NJ).

2.3. Ion-pairing extraction

^{18}O-labeled perfluorooctanesulfonate (^{18}O$_2$-PFOS), ^{13}C-labeled perfluorooctanoate (^{13}C$_2$-PFOA), ^{13}C-labeled perfluorononanoate (^{13}C$_2$-PFNA), PFBS, and THPFOS were extracted from human BP or BS by use of an ion-pairing method that has been described previously [5,6,14]. Extracted calibration standards and quality control samples in methanol were spiked in 20 μL amounts into control human BP or BS. Analytes of interest were extracted from serum or plasma by adding 1 mL of 0.5 mol L^{-1} tetrabutylammonium hydrogen sulfate (TBAHS) and 2 mL of 0.25 mol L^{-1} sodium carbonate/sodium bicarbonate buffer. The analytes of interest paired with TBAHS were partitioned into 5 mL methyl-tert-butyl ether (MtBE) which was added to the solution. Four milliliters of the MtBE extract were removed and placed into a nitrogen evaporator until dry. The extract was reconstituted in 1 mL of methanol and filtered. The QC samples were used to determine extraction efficiency, matrix effects, method accuracy and precision.

Authentic standards, with stable isotope labels were used in this study so that effects of the endogenous PFOS, PFOA, and PFNA concentrations of analyte present in the samples were removed. To quantify extraction recoveries and biases introduced by both matrix effects and calibration methods, samples were spiked either before or after extraction or both before and after extraction. Pre-spiked QC samples were prepared by spiking PFBS, THPFOS, and/or ^{18}O-PFOS, ^{13}C-PFOA and ^{13}C-PFNA prior to extraction into control human BP or BS. Post-spiked QC samples were prepared by spiking an extract of a matrix blank. In addition, an aliquot of the pre-spiked QC sample extracts was also spiked as post-spiked samples and analyzed as combined pre- and post-spiked samples. The combined spike before and after extraction was used qualitatively as additional confirmation of the individual determinations of extraction efficiency and matrix suppression/enhancements. When calibration was done by standards

pre-spiked into matrices and extracted, concentrations of PFCs were determined (Eq. (1)).

$$\frac{\text{ng}}{\text{mL sample}} = \frac{\text{ng}}{\text{mL extract}} \times \frac{\text{amount of extract (mL)}}{\text{original sample amount (mL)}} \tag{1}$$

When external standards in solvent were used for calibration, concentrations of PFCs expressed as ng mL^{-1} sample were calculated (Eq. (2)).

$$\frac{\text{ng}}{\text{mL sample}} = \frac{\text{ng}}{\text{mL solvent}}$$
$$\times \frac{\text{reconstitution solvent amount (mL)}}{\text{amount of MTBE removed (mL)}}$$
$$\times \frac{\text{amount of MTBE added to sample (mL)}}{\text{original sample amount (mL)}} \tag{2}$$

Concentrations in post-spike samples, expressed as ng mL^{-1} extract were determined by use of an extracted curve were calculated (Eq. (3)).

$$\frac{\text{ng}}{\text{mL extract}} = \frac{\text{ng}}{\text{mL matrix}}$$
$$\times \frac{\text{mL matrix}}{\text{amount of MTBE added to sample (mL)}}$$
$$\times \frac{\text{amount of MTBE removed (mL)}}{\text{mL extract}} \tag{3}$$

2.4. Solid-phase extraction

PFCs were extracted from BP or BS by use of an adaptation of previously published methods [17–19]. Extracted calibration standards and pre-extraction spiked QC samples were prepared in undiluted control BS and BP by spiking analytes of interest in methanol. Five milliliters of acetonitrile were added to 1 mL of sample. The combination of solvent and serum were mixed and subsequently centrifuged to separate the precipitated proteins. The supernatant was transferred to a clean tube and diluted with 40 mL of ASTM Type I water to reduce the percentage of organic solvent. The sample was then passed through a SPE cartridge (1.0 g, Sep-Pak 6 cc tri-functional C$_{18}$; Waters, Milford, MA, USA) which had been preconditioned with methanol and water. After taking the column to dryness, the analytes of interest were eluted with 2 mL of methanol. Concentrations were determined (Eq. (4)). Samples of human blood serum or plasma were spiked with ^{18}O-PFOS, ^{13}C-PFOA, or ^{13}C-PFNA prior to extraction. An extracted calibration curve ranging from 0.25 to 50 ng mL^{-1} serum or plasma was prepared along with the pre-spiked quality control samples. The pre-spiked quality control samples were spiked in triplicate at three concentrations of approximately 1.0, 7.5, or 40 ng mL^{-1} serum or plasma.

$$\frac{\text{ng analyte}}{\text{mL sample}} = \left(\frac{\text{ng analyte}}{1\ \text{mL solvent}} \right)$$
$$\times \left(\frac{\text{amount of elution solvent (mL)}}{\text{actual sample volume (mL)}} \right) \tag{4}$$

2.5. Protein precipitation extraction

Extracted calibration standards and pre-extraction spiked QC samples were prepared in control serum or plasma. Analytes of interest were extracted from serum or plasma by protein precipitation in acetonitrile [16] via a MultiPROBE II HT EX robotic liquid handling system utilizing 96-well plates (PerkinElmer Life and Analytical Sciences, Shelton, CT). One hundred microliter aliquots of sample were added to a 96-well plate containing Argonaut ISOLUTE array protein precipitation wells (2 mL). To each well, 50 μL of a spiking solution in methanol was added and mixed with the sample. After allowing the sample to equilibrate for 20 min, 450 μL of acetonitrile was added to each well. After 5 min, a vacuum was applied to the entire plate to separate the precipitated proteins from the solvent extracts. The extracts were collected below in an Argonaut ISOLUTE array collection plate (1 mL). Following collection, the extracts were transferred to glass micro-vials for analysis. Concentrations were calculated (Eq. (5)).

$$\frac{\text{ng}}{\text{mL sample}} = \frac{\text{ng}}{\text{mL extract}}$$
$$\times \frac{\text{amount of extraction solvent (mL)}}{\text{original sample amount (mL)}} \quad (5)$$

Samples of human blood serum or plasma were spiked with ^{18}O-PFOS, ^{13}C-PFOA, or ^{13}C-PFNA prior to extraction. An extracted calibration curve ranging from 0.25 to 50 ng mL^{-1} in BP or BS was prepared along with the pre-spiked quality control samples. The pre-spiked quality control samples were spiked in triplicate at three concentrations of approximately 1.0, 7.5, or 40 ng mL^{-1} serum or plasma.

2.6. Liquid chromatography

The chromatographic conditions used during the analysis of sample extracts were identical for each of the three sample preparation techniques evaluated. An Agilent 1100 series (Palo Alto, CA) HPLC system consisting of a binary pump, vacuum degasser, auto-sampler, and controlled-temperature column compartment was used. A 2 mm × 50 mm Betasil^{TM} C$_{18}$, 5 μm particle size (Keystone, Bellefonte, PA) guard column was attached in-line after the purge valve and before the sample injector port to trap any residue contaminants that may be in the mobile phase and/or HPLC system. This effectively displaced the retention times of analytes in the background so that they could be separated from the analytical signal obviating the need to make alterations to the instrument, which have been suggested to be necessary by other researchers [20]. Chromatographic separation was achieved using a 2 mm × 100 mm Betasil^{TM} C$_{18}$, 5 μm particle size analytical column operated at a temperature of 30 °C. The mobile-phase consisted of a 2 mM ammonium acetate solution in water (A) and methanol (B). The flow rate was fixed at 0.30 mL min^{-1}. The following solvent gradient was used to elute the analytes of interest: 30% B to 1.0 min, to 65% B at 5.0 min, to 90% B at 11.0 min, remaining at 90% B until 12.0 min, to 30% B at 13.5 min, and ending at 30% B at 16.0 min.

2.7. Mass spectrometric conditions

Samples prepared using protein precipitation and solid-phase extraction were analyzed using a PE SCIEX API 4000 triple quadrupole mass spectrometer (Foster City, CA), equipped with a SCIEX Turbo V ion-spray interface, operated in the negative ion MS/MS mode using multiple reaction monitoring (MRM). Instrument tuning was conducted for each analyte by direct-infusion of a ~1 μg mL^{-1} standard solution at a flow rate of 10 μL min^{-1} introduced via a "T" into a stream of mobile phase containing 60% methanol and 40% 2 mM ammonium acetate in water at a 0.3 mL min^{-1} flow rate. The analyte was initially tuned for the parent ion and then subsequently tuned for the product ions. Typically, the following tune parameters were used: source temperature 350 °C; desolvation temperature (450 °C), desolvation (curtain) gas 35.0 psi; nebulizer gas flow 30 L h^{-1}; ion spray voltage −4500 V; entrance potential −10 V; dwell time 150 ms. The optimal settings for collision energies that were determined for each analyte are presented (Supporting Materials Table S1). For quantitative purposes, the instrument was operated in MRM mode, monitoring the transitions indicated for each analyte. Quantification using these transitions was performed using the Analyst 1.4.1 software provided by PE (Applied Bioscience, Foster City, CA).

Sample extracts prepared using ion-pairing were analyzed using both Quattro II® and Quattro Ultima® triple quadrupole mass spectrometers (Micromass, Waters, Milford, MA), equipped with a Z-spray interface, operated in the negative ion mode using multiple reaction monitoring. Instrument tuning was conducted for each analyte by direct-infusion of a ~1 μg mL^{-1} standard solution at a flow rate of 10 μL min^{-1} introduced via a "T" into a stream of mobile phase containing 60% methanol and 40% 2 mM ammonium acetate in water at a 0.3 mL min^{-1} flow rate. The analyte was initially tuned for the parent ion and then subsequently tuned for the product ions. Typically, the following tune parameters were used: source temperature 130 °C, desolvation temperature 250 °C, capillary voltage 3.15 V, multiplier 650 V, inter-channel delay 0.03 s, low mass resolution 1 = 14.5, high mass resolution 1 = 14.5, low mass resolution 2 = 13.0, high mass resolution 2 = 13.0. For quantitative purposes, the instrument was operated in MRM mode, monitoring the transitions presented (Supporting Materials Table S2). Quantification using these transitions was performed using the MassLynx^{TM} Version 3.4 software provided by Micromass (Waters, Milford, MA).

3. Results and discussion

3.1. Ion-pairing extraction—signal enhancement/suppression and extraction efficiency

Representative % signal enhancement/suppression data in serum and plasma extracts versus methanol and extraction efficiencies by the ion-pairing extraction method are summarized in Table 1 and Fig. 1. Percent signal enhancement or signal suppression in matrix extracts are determined directly from post-extraction spiked matrix recovery data as summarized in Table 2. Percent extraction efficiencies are determined

Table 1 – Ion-pairing extraction—analyte specific % signal enhancement/suppression in serum and plasma extract versus solvent

Analyte	Human serum		Human plasma	
	%Signal	%Extracted	%Signal	%Extracted
^{13}C-PFOA	157	69	160	61
^{18}O-PFOS	126	54	138	49
^{13}C-PFNA	99	95	104	83
PFBS	30	108	46	84
THPFOS	160	105	164	101

Analyte specific ion-pairing extraction efficiency from pre-extraction spiked samples in serum and plasma. Mean ($n = 9$) values over the range of 1–40 ng mL^{-1} on Micromass Quattro II.

Fig. 2 – Ion-pairing extraction. Pre-extraction spiked IS. Percent analytical bias with external methanol calibration curve.

Fig. 1 – Ion-pairing extraction. Analyte specific % signal enhancement/suppression in serum and plasma extract versus solvent. Analyte specific ion-pairing extraction efficiency.

from ratios of pre-extraction/post-extraction spiked sample recoveries with methanol calibration and are summarized in Table 3. Mean ($n = 9$) percent recoveries of pre-extraction spiked analytes in serum and plasma over the range of 1–40 ng mL^{-1} quantified with methanol calibration are available in Supporting Materials Tables S3 and S4. The Micromass Quattro II % recovery data in Table 1 and Fig. 1 represent combinations of both signal enhancement/supression in serum and plasma extracts and the specific analyte ion-pairing extraction efficiency. Substantial analytical bias would result for all target analytes if the differences in percent recoveries were not accounted for in quantitation by external methanol calibration. Values versus methanol ranged from 30% for PFBS in human serum extract to 164% for THPFOS in human plasma extract. Similar independently variable results were obtained from Micromass Quattro Ultima instrumentation (see Supporting Materials Table S5) (Fig. 2).

Table 2 – Analyte % signal enhancement or suppression in ion-pairing serum extracts versus methanol

Analyte	1.0 ng mL^{-1} serum		7.5 ng mL^{-1} serum		40 ng mL^{-1} serum		Precision (%)	
	Quattro II	Ultima	Quattro II	Ultima	Quattro II	Ultima	Quattro II	Ultima
^{13}C-PFOA	122	82.4	155	125	194	171	18	18
^{18}O-PFOS	106	118	124	135	148	151	5.0	9.7
^{13}C-PFNA	97.2	100	101	114	98.7	125	7.0	4.7
PFBS	9.08	365	41.8	156	38.9	123	24	5.3
THPFOS	199	178	149	130	132	116	20	14

Mean ($n = 3$) % recoveries of perfluorochemicals post-extraction spiked into extracts of blood serum and quantified by use of external calibration in methanol. Analyses were conducted on both Micromass Quattro II and Micromass Quattro Ultima mass spectrometers. Precision = % relative standard deviation over the range of 1–40 ng mL^{-1} ($n = 9$).

Table 3 – Calculated percent ion-pairing extraction efficiency determined from ratio of pre-extraction/post-extraction spiked sample recoveries with methanol calibration

Analyte	1.0 ng mL^{-1} serum		7.5 ng mL^{-1} serum		40 ng mL^{-1} serum		Precision (%)	
	Quattro II	Ultima	Quattro II	Ultima	Quattro II	Ultima	Quattro II	Ultima
^{13}C-PFOA	83.4	103	66.3	73.9	57.9	58.4	6.2	7.7
^{18}O-PFOS	61.4	67.3	54.1	53.3	47.5	49.5	4.8	5.5
^{13}C-PFNA	96.7	129	97.9	104	90.4	68.1	11.6	9.2
PFBS	NR	95.9	107	100	109	89.7	14.8	38
THPFOS	124	96.2	93.7	81.3	96.5	83.2	21	24

Analyses were conducted with both Micromass Quattro II and Quattro Ultima mass spectrometers. Precision = % relative standard deviation over the range of 1–40 ng mL^{-1} ($n = 9$). NR: not reportable.
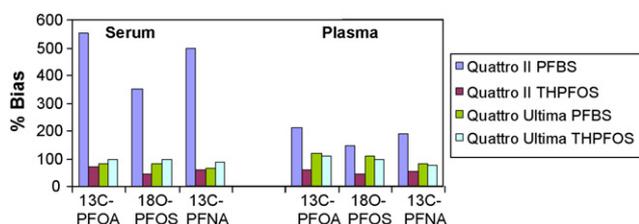
Fig. 3 – Ion-pairing extraction. Post-extraction spiked IS. Percent analytical bias with external methanol calibration curve.

3.2. Ion-pairing extraction. Post-extraction spiked QC samples quantified by external calibration in methanol

Analyte specific mean ($n=9$) percent recoveries of post-extraction spiked analytes in human serum extracts over the range of 1–40 ng mL^{-1} quantified with methanol calibration are presented in Table 2. Micromass Quattro II and Quattro Ultima instrumentation data are presented which represent the percent signal enhancement/supression of each target analyte in serum extracts relative to methanol. Significant analytical bias would result for all target analytes if the differences due to matrix specific signal enhancement/suppression were not accounted for in quantitation by external methanol calibration. Values versus methanol ranged from 39% for PFBS in human serum extract to 199% for THPFOS in human serum extract both with Quattro II instrumentation. Three compounds, ^{18}O-PFOS, ^{13}C-PFOA, and THPFOS were affected by matrix enhancement when quantification was by either Micromass Quattro II or Quattro Ultima. PFBS exhibited matrix enhancement when analyzed by the Micromass Quattro Ultima while on the Micromass Quattro II it showed significant matrix suppression. Similar highly independently variable results were obtained in human plasma extracts (see Supporting Materials Table S6) (Fig. 3).

3.3. Ion-pairing extraction quantitative bias—surrogate IS/external methanol calibration

Analytical bias introduced by using external solvent calibration with surrogate internal standard is summarized in Table 4 for both ion-pairing extracted sera and plasma samples on both Quattro II and Quattro Ultima instrumentation. Bias was calculated using the ratios of target analyte % recoveries to surrogate IS % recoveries with both pre- and post-extraction IS spiking. Mean % recoveries ($n=27$) are quantified with external calibration in methanol and are based on three lots of commercial pooled sera and plasma, spiked with 1, 10, or 40 ng mL^{-1} in triplicate. Bias values above and below 100% represent quantitative bias which would result in both under and over reporting, respectively. Bias introduced with PFBS as the surrogate IS ranged from 144 to 552% with the Quattro II and from 43 to 118% with the Quattro Ultima. In contrast, the bias introduced with THPFOS as the surrogate IS ranged from 41 to 68% with the Quattro II and from 64 to 106% with the Quattro Ultima.

3.4. Protein precipitation, solid-phase extraction, and ion-pairing methods quantified with matrix-matched calibration curves

Comparison of accuracy and precision of extraction methods with quantification by external calibration with matrix-matched standards is shown in Table 5. All samples were spiked with analyte prior to extraction. Mean % recoveries are based on three lots of commercial pooled human blood serum or plasma, spiked at three concentrations, 1.0, 10 or 40 ng mL^{-1} in triplicate ($n=27$). Regardless of the method, ion-pairing, solid-phase extraction, or protein precipitation, if the extracts were quantified versus a matrix-matched external calibration curve, all three methods resulted in highly accurate and precise results (Table 5).

3.5. Inter-laboratory comparison

Concentrations of perfluorinated compounds in whole human blood, serum, and plasma samples determined by use of ion-pairing extraction with quantification by external calibration in methanol and adjusted for internal standard response, were reported in an earlier study [6]. When PFCs were extracted by ion-pairing and quantified using an external calibration curve, significant positive biases were observed for PFOA concentrations, depending on the nature of the samples. The bias was more significant for PFOA than for other perfluorinated acids. PFOS concentrations obtained from the initial analyses were not significantly different than published normal population mean values for PFOS, however, PFOA concentrations reported were biased significantly greater than the normal population mean, for a population in New York, at that time. To demonstrate the advantage of using matrix-matched calibration curves, representative samples in question were split and were re-analyzed by two laboratories using ion-pairing extraction with matrix-matched calibration. As displayed in Figs. 4 and 5, concentrations of PFOS and PFOA in human whole blood, blood serum, and plasma compared well between the two laboratories and also compared well to published mean values of normal populations. When the results for PFOS reported in an earlier study [6] were compared with those quantified using matrix-matched extracted calibration curves, as reported in this study, the bias was not significant (43.5 ng mL^{-1} versus 34.6 ng mL^{-1}).
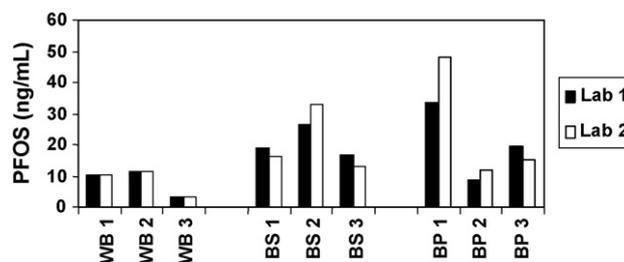

Fig. 4 – Comparison of concentrations of PFOS in three samples of whole human blood (WB), blood plasma (PB) and blood serum (BS) determined by use of ion-pairing extraction with matrix-matched calibration. Each value represents the mean of three replicates.

Table 4 – Ion-pairing extraction analytical bias—introduced by using external methanol calibration corrected for surrogate IS response

	Micromass Quattro II				Micromass Quattro Ultima			
	Pre-extraction IS spike % bias		Post-extraction IS spike % bias		Pre-extraction IS spike % bias		Post-extraction spike % bias	
	PFBS	THPFOS	PFBS	THPFOS	PFBS	THPFOS	PFBS	THPFOS
Serum								
^{13}C-PFOA	237	68	552	68	55	80	81	96
^{18}O-PFOS	151	43	353	44	43	64	81	100
^{13}C-PFNA	211	59	500	60	59	92	67	84
Plasma								
^{13}C-PFOA	254	64	210	61	89	79	118	106
^{18}O-PFOS	173	43	144	41	68	59	110	98
^{13}C-PFNA	223	54	188	53	78	65	83	75

Bias was calculated using the ratios of target analyte % recoveries to surrogate IS % recoveries with both pre- and post-extraction spiking.

Table 5 – Comparison of accuracy and precision of extraction methods with quantification by external calibration with matrix-matched standards

	Protein precipitation		Solid-phase extraction		Ion pairing			
	SCIEX API 4000		SCIEX API 4000		Micromass Quattro Ultima		Micromass Quattro II	
	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision
Serum								
^{13}C-PFOA	99	6	111	9	107	7	102	5
^{18}O-PFOS	100	6	115	8	107	5	98	5
^{13}C-PFNA	102	5	112	9	98	11	92	14
Plasma								
^{13}C-PFOA	108	3	84	15	91	12	92	16
^{18}O-PFOS	102	8	86	19	94	14	89	13
^{13}C-PFNA	102	5	83	16	88	7	92	13

All samples were spiked with analyte prior to extraction. Based on three lots of commercial pooled human blood serum or plasma, spiked at three concentrations, 1.0, 10 or 40 ng mL^{-1} in triplicate ($n = 27$). Accuracy = mean percent matrix spike recovery over the range of 1–40 ng mL^{-1}. Precision = % relative standard deviation over the range of 1–40 ng mL^{-1}.

When the PFOA results reported for selected plasma samples ($n = 9$) from an earlier study [6] were compared to those determined in the same samples using ion-pairing extraction and matrix-matched calibration, concentrations were, on average 4.5-fold (27.8 ng mL^{-1} versus 5.9 ng mL^{-1}) less. The bias in PFOA concentrations was significant for plasma samples, but not significant for whole blood and serum samples reported in the earlier study.
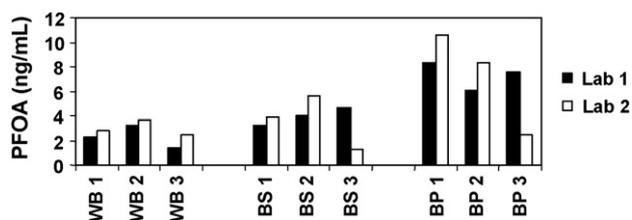

Fig. 5 – Comparison of concentrations of PFOA in three samples of whole human blood (WB), blood plasma (PB) and blood serum (BS) determined by use of ion-pairing extraction with matrix-matched calibration. Each value represents the mean of three replicates.

4. Conclusions

Comparable results were obtained from all three extraction techniques when quantification was by matrix-matched calibration, in which analytes were spiked into representative matrix including human plasma and serum. When matrix-matched calibration was used, accuracy and precision of 100 ($\pm$) 15% was obtained over the concentration range of 1.0–40 ng mL^{-1} in human serum and plasma with all three sample preparation techniques. However, this study demonstrates an inherent variability with the ion-pairing method when results are quantified versus a non-extracted external solvent curve in combination with a surrogate IS, resulting in significant analytical biases for all three target analytes. Analytical bias ranged from 61 to 552%, for ^{13}C-PFOA, from 53 to 500% for ^{13}C-PFNA and from 41 to 353% for ^{18}O-PFOS. Analyte-specific ion-pairing extraction efficiency differences and analyte-specific matrix signal suppression and/or signal enhancement accounted for the observed analytical bias. Significant analytical bias was observed regardless of IS spiking pre- or post-extraction. Solvent calibration bias was highly variable and dependent on both serum and plasma matri-

ces, the specific target analyte $[^{13}C_2]$-PFOA, $[^{13}C_2]$-PFNA, and $[^{18}O_2]$-PFOS, the target analyte concentration, the LC/MS-MS instrumentation used in data generation, and the specific surrogate internal standard used in quantitation.

The use of matrix-matched calibration curves, regardless of extraction procedure, leads to acceptable LC/MS-MS PFC quantitation. Moreover, use of external surrogate solvent standards coupled with a commonly used ion paring extraction technique can give rise to considerable bias and inaccuracy. Much of the existing literature reporting PFC quantitation by LC/MS-MS techniques do not include sufficient experimental calibration detail needed to assess the potential for analytical bias. Without this experimental data it is difficult if not impossible to determine the extent of potentially biased PFC data in the published literature.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2008.09.029.

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Supporting Information

Comparison of Extraction and Quantification Methods of Perfluorinated Compounds in Human Plasma, Serum, and Whole Blood

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6 Tables – 4 pages

The ions monitored and instrument conditions used to quantitate the target analytes are listed for the PE Sciex API 4000 in Table S1 and for the Micromass Quattro II and Quattro Ultima in Table S2.

Table S1. Ions and instrumental conditions for identification and quantification of analytes with the API 4000 (Applied Biosystems, Inc., Foster City, CA).

Compound	Parent Ion (m/z)	Product Ion (m/z)	Dwell (milli-seconds)	Declustering Potential (V)	Collision Energy (V)	Collision Cell Exit Potential (V)
^{18}O-PFOS	503.00	103.00	150.0	-70.00	-54.00	-7.00
	503.00	84.00	150.0	-70.00	-52.00	-5.00
^{13}C-PFOA	414.92	369.80	150.0	-30.00	-14.00	-7.00
^{13}C-PFNA	465.00	420.00	150.0	-30.00	-16.00	-9.00

Table S2. Ions and instrumental conditions for identification and quantification of analytes with the Micromass Quattro II and Micromass Quattro Ultima (Micromass, Waters, Milford MA).

Compound	Parent Ion (m/z)	Product Ion (m/z)	Dwell (seconds)	Coll Energy (eV)	Cone (V)
^{18}O-PFOS	503	103	0.10	50	60
	503	84	0.10	50	60
^{13}C-PFOA	415	370	0.10	10	20
^{13}C-PFNA	465	420	0.25	15	20
PFBS	299	80	0.25	55	35
	299	99	0.25	55	35
THPFOS	427	80	0.25	45	60

Provided in this supporting information are percent recoveries of pre-extraction spiked analytes in serum (Table S3) and plasma (Table S4) over the range of 1 to 40 ng/mL using ion pairing extraction quantified with methanol calibration curves.

Table S3: Mean (n=3) % recoveries of perfluorochemicals pre-extraction spiked into extracts of blood serum and quantified by use of external calibration in methanol. Analyses were conducted on both Micromass Quattro II and Micromass Quattro Ultima mass spectrometers. Precision = % relative standard deviation over the range of 1 to 40 ng/ml (n=9).

Analyte	1.0 ng/mL serum		7.5 ng/mL serum		40 ng/mL serum		Precision	
	Quattro II	Ultima	Quattro II	Ultima	Quattro II	Ultima	Quattro II	Ultima
^{13}C-PFOA	102	84.6	103	92.0	112	100	6.2%	7.7%
^{18}O-PFOS	65.3	79.1	66.9	71.8	70.3	74.6	4.8%	5.5%
^{13}C-PFNA	94.0	129	98.7	119	89.3	85.4	12%	11%
PFBS	46.6	530	44.8	156	42.6	110	15%	10%
THPFOS	246	171	140	106	127	96.7	21%	24%

Table S4: Mean (n=3) % recoveries of perfluorochemicals pre-extraction spiked into extracts of blood plasma and quantified by use of external calibration in methanol. Analyses were conducted on both Micromass Quattro II and Micromass Quattro Ultima mass spectrometers. Precision = % relative standard deviation over the range of 1 to 40 ng/ml (n=9).

Analyte	1.0 ng/mL plasma		7.5 ng/mL plasma		40 ng/mL plasma		Precision	
	Quattro II	Ultima	Quattro II	Ultima	Quattro II	Ultima	Quattro II	Ultima
^{13}C-PFOA	77.4	81.8	107	105	107	110	13%	12%
^{18}O-PFOS	55.7	66.4	76.1	84.7	67.6	75.6	15%	16%
^{13}C-PFNA	79.1	100	100	106	78.3	68.1	4.5%	12%
PFBS	37.0	198	42.3	97.8	35.7	92.3	92%	47%
THPFOS	228	323	151	120	125	88.9	26%	14%

Representative % signal enhancement/suppression data in serum and plasma extracts versus methanol calibration curve and extraction efficiencies by the ion-pairing extraction method as analyzed on the Micromass Quattro Ultima are summarized in Table S5. The recovery data in this table represent combinations of both signal enhancement/suppression in serum and plasma extracts and the specific analyte ion-pairing extraction efficiency. Values versus the methanol calibration curve ranged from 87% for THPFOS in human serum extract to 215% for PFBS in human serum extract.

Table S5: Ion Pairing Extraction – Analyte specific % signal enhancement/suppression in serum and plasma extract versus a solvent calibration curve. Analyte specific ion pairing extraction efficiency from pre-extraction spiked samples in serum and plasma. Mean (n=9) values over the range of 1 to 40 ng/ml on the Micromass Ultima.

	Human Serum	Human Serum	Human Plasma	Human Plasma

Analyte	% Signal	% Extracted	% Signal	% Extracted
13C PFOA	126%	78%	157%	70%
18O PFOS	135%	57%	147%	52%
13C PFNA	113%	100%	110%	83%
PFBS	215%	95%	134%	96%
THPFOS	141%	87%	156%	105%

Values versus an external methanol calibration curve ranged from 36.3% for PFBS in human plasma extract on the Quattro II to 210% for THPFOS in human plasma extract on the Quattro Ultima. As happened with the serum extracts, three compounds, ^{18}O-PFOS, ^{13}C-PFOA, and THPFOS were affected by matrix enhancement when quantification was by either Micromass Quattro II or Quattro Ultima. PFBS exhibited matrix enhancement when analyzed by the Micromass Quattro Ultima while on the Micromass Quattro II it showed significant matrix suppression. The plasma data is presented in Table S6.

Table S6: Mean (n=3) % recoveries of perfluorochemicals post-extraction spiked into extracts of blood serum and quantified by use of external calibration in methanol. Analyses were conducted on both Micromass Quattro II and Micromass Quattro Ultima mass spectrometers. Precision = % relative standard deviation over the range of 1 to 40 ng/ml (n=9).

Analyte	1.0 ng/mL plasma		7.5 ng/mL plasma		40 ng/mL plasma		Precision	
	Quattro II	Ultima	Quattro II	Ultima	Quattro II	Ultima	Quattro II	Ultima
^{13}C-PFOA	154	155	175	167	152	150	7.8%	9.1%
^{18}O-PFOS	146	157	138	151	129	134	7.3%	11%
^{13}C-PFNA	108	106	105	112	98.2	113	4.7%	2.6%
PFBS	36.3	142	51.4	127	51.5	133	30%	17%
THPFOS	183	210	168	138	141	121	6.9%	10%