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Measurement of 18 Perfluorinated Organic Acids and Amides in Human Serum Using On-Line Solid-Phase Extraction

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We have developed an on-line solid-phase extraction (SPE) method coupled to high-performance liquid chromatography (HPLC)−tandem mass spectrometry (MS/MS) for measuring trace levels of 18 perfluorinated chemicals (3 perfluorosulfonates, 8 perfluorocarboxylates, 7 perfluorosulfonamides) in serum. Without protein precipitation, only dilution with 0.1 M formic acid, one aliquot of 100 μL of serum was injected into a commercial column switching system that allowed for concurrent SPE and HPLC−MS/MS acquisition. First, the analytes were concentrated on a C18 SPE column. Then, this column was placed automatically in front of a C8 analytic HPLC column for chromatographic separation of the analytes. Detection and quantification were done using negative-ion TurboIonSpray ionization, a variant of electrospray ionization, MS/MS. Excellent recovery was achieved for all analytes including the volatile sulfonamide derivatives that could not be determined before using traditional off-line SPE methods. The high throughput and low limits of detection (0.05−0.8 ng/mL) using a small sample volume (100 μL of serum) and isotope dilution quantification make this method suitable for large-scale epidemiologic studies.

Perfluorochemicals (PFCs) are used in many commercial applications, including surfactants, lubricants, paints, polishes, food packaging, and fire-retarding foams. Concerns about the persistent and bioaccumulative nature of PFCs were raised when perfluorooctanesulfonate (PFOS), a widely used surfactant, was found to be ubiquitous in human populations[1−5] and in wildlife[6−13] around the world. In response to these concerns, in 1999, the United States Environmental Protection Agency (EPA) began investigating PFOS. In May 2000, 3M, the sole manufacturer of PFOS in the United States and the principal manufacturer worldwide, announced it was discontinuing its perfluorooctanyl chemistries, including PFOS. Shortly thereafter, in June 2000, EPA identified possible related concerns with respect to perfluorooctanoic acid (PFOA) and fluorinated telomers. PFOA is used primarily in the production of fluoroelastomers and fluoropolymers, such as poly(tetrafluoroethylene) (Teflon) and poly(vinylidine fluoride). These polymers have numerous uses in many industrial and consumer products, including soil-, stain-, grease-, and water-resistant coatings on textiles and carpet; uses in the automotive, mechanical, aerospace, chemical, electrical, medical, building, and construction industries; personal care products; and nonstick coatings on cookware. Since April 2003, EPA released two preliminary risk assessments on PFOA that indicate potential human exposure to low levels of PFOA in the United States.[14,15]

Animal data suggest potential developmental, reproductive, and systemic toxicity[16−18] for selected PFCs. However, to date, no clear association has been established between human exposure to PFCs and adverse health effects, and the environmental sources of PFOS and PFOA have not been clearly identified. PFCs have been measured in biologic[1−6,8−12,19] and environmental[20−23] ma-

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trices mostly by high-performance liquid chromatography−tandem mass spectrometry (HPLC−MS/MS). Gas chromatography−mass spectrometry was used for measuring *N*-alkylperfluorooctane sulfonamides and 2-(perfluorooctane sulfonamido) ethanol derivatives.[24,25] Early methods used liquid−liquid extraction (LLE) to extract the PFCs from the biologic matrix.[1−4,6,8−12,19] Methods using solid-phase extraction (SPE) instead of LLE have been reported for environmental[21,22,26] and biologic samples.[27−30] Modern approaches such as using protein precipitation columns for measuring PFOA in serum[31] and on-line SPE methods for measuring PFOS in river water[23] or PFOS, PFOA, and perfluorooctane sulfonamide (PFOSA) in human plasma[32] had also been reported.

Previously, we developed an automated off-line SPE method for measuring 13 PFCs.[27] Although this method had adequate sensitivity, the recovery and precision for some sulfonamides and short-alkyl chain acids were low. This was mainly due to losses during evaporation and preconcentration processes after SPE. To eliminate these steps, we developed an on-line SPE method. The on-line SPE-HPLC−MS/MS method has a throughput similar to that of our previous off-line SPE method, but has better recovery, sensitivity, and precision for all analytes and uses smaller amounts of sample. The method also allows for the measurement of the relatively volatile *N*-alkyl sulfonamide and sulfonamido ethanol derivatives, potential metabolic precursors of PFOS.[33] Although, at present, toxicologic data on these sulfonamide derivatives are ambiguous, the method reported here may be used for their analysis in other biologic and environmental matrices. We tested the applicability of the method on human serum collected from persons nonoccupationally exposed to PFCs.

MATERIALS AND METHODS

Chemicals. PFOSA, *N*-ethylperfluorooctane sulfonamide (Et-PFOSA), *N*-methylperfluorooctane sulfonamide (Me-PFOSA), 2-(*N*-ethylperfluorooctane sulfonamido) ethanol (Et-PFOSA-EtOH), 2-(*N*-methylperfluorooctane sulfonamido) ethanol (Me-PFOSA-EtOH), 2-(*N*-ethylperfluorooctane sulfonamido) acetic acid (Et-PFOSA-AcOH), 2-(*N*-methylperfluorooctane sulfonamido) acetic acid (Me-PFOSA-AcOH), potassium perfluorobutane sulfonate (PFBuS), potassium perfluorohexanesulfonate (PFHxS), potassium perfluorooctanesulfonate (PFOS), and ammonium perfluorooctanoate (PFOA) were provided by 3M Co. (Saint Paul, MN). Perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDeA), perfluoroundecanoic acid (PFUA), and perfluorododecanoic acid (PFDoA) were purchased from Oakwood Products (West Columbia, SC). 1,2-$^{13}C_2$-Perfluorooctanoic acid ($^{13}C_2$-PFOA) was provided by Dupont Co. (Wilmington, DE). $^{18}O_2$-Perfluorooctanesulfonate ($^{18}O_2$-PFOS) and $^{18}O_2$-ammonium perfluorooctanesulfonamide ($^{18}O_2$-PFOSA) were purchased from Research Triangle Institute (Research Triangle Park, NC). HPLC grade methanol, acetonitrile, and water were purchased from Caledon (Ontario, Canada), acetic acid was (glacial) from Sigma-Aldrich (St. Louis, MO), formic acid (98% min, GR) from EM Science (Gibbstown, NJ), and ammonium hydroxide (30%) from J. T. Baker (Phillipsburg, NJ). All chemicals and solvents were used without further purification.

Preparation of Standard Solutions and Quality-Control Materials. Standard stock solutions (10 mg/mL) were prepared by dissolving solid standards in methanol. Nine working standard solutions containing all analytes were prepared by serial dilutions in methanol to final concentrations such that a 50-μL spike in 200 μL of serum would cover a concentration range from 0.1 to 100 ng/mL for PFOS and 0.025 to 25 ng/mL for the other analytes.

Stock solutions of $^{13}C_2$-PFOA, $^{18}O_2$-PFOS, and $^{18}O_2$-PFOSA (0.1 mg/mL) in methanol were prepared from the solid standards and were diluted to provide working solutions such that a 50-μL spike into 200 μL pf serum provided a final concentration of ∼10 ng/mL. All stock solutions and standards were stored in polypropylene vials at or below −20 °C.

The quality control (QC) materials were prepared from calf serum (Gibco, Grand Island, NY). The serum was mixed uniformly and divided into three subpools. One subpool was used as a matrix blank QC and to prepare the calibration standards, and the other two were enriched with PFCs as needed to afford low-concentration (QCL) and high-concentration (QCH) subpools. The pools were dispensed in aliquots (∼3 mL) into polypropylene vials prerinsed with methanol and stored at −20 °C until used. The QCH and QCL pools were characterized to define the mean and the 95 and 99% control limits by a minimum of 30 measurements in a 3-week period. QC materials extracted and analyzed after the initial characterization showed that the PFCs remained stable at −20 °C for at least 3 months.

Sample Preparation. Standards, QCs, and blanks were prepared and processed using the same procedure. To 500 μL of 0.1 M formic acid, placed in a 0.9-mL polypropylene autosampler vial and spiked with 50 μL of internal standard solution and 50 μL of standard solution (for standards only), 200 μL of serum was added. This solution was vortex-mixed, sonicated for 20 min, and placed on a Symbiosis on-line SPE-HPLC system (Spark Holland, Plainsboro, NJ).

Automated SPE. The Symbiosis system was used in concurrent SPE-HPLC mode (Figure 1, Table 1). The left clamp, left

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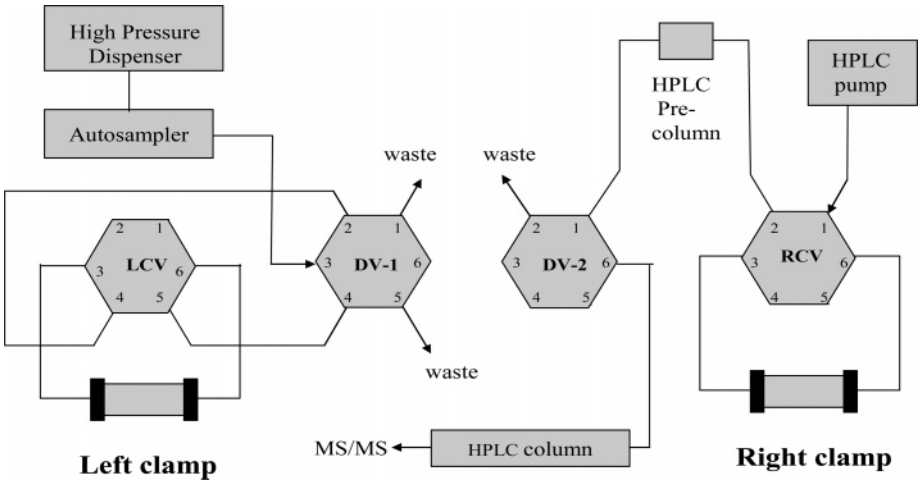

Figure 1. Tubing diagram for the Symbiosis column switching system used in concurrent SPE/HPLC mode. (LCV, left clamp valve; DV-1, divert valve 1; DV-2, divert valve 2; RCV, right clamp valve).

Table 1. Valve Configurations Used for Concurrent SPE and HPLC−MS/MS Acquisition Using a Symbiosis System (Figure 1)

steps[a]	method	LCV	DV-1	DV-2	RCV	duration (min)
1	move cartridge from left clamp to right clamp	6−1	1−2	6−1	1−2	0.1
2	load new cartridge into left clamp	6−1	1−2	6−1	1−2	0.2
3	send contact closure signal to HPLC−MS/MS	6−1	1−2	6−1	1−2	0.1
4	begin HPLC gradient elution, bypass analytical column and MS/MS	6−1	1−2	1−2	6−1	2.5
5	condition left cartridge (2 mL MeOH, 2 mL/min)	1−2	1−2	6−1	6−1	1.2
6	equilibrate left cartridge (2 mL 0.1 M formic acid, 2 mL/min)	1−2	1−2	6−1	6−1	1.2
7	load 400 μL sample on left cartridge (4 mL, 0.1 M formic acid, 1 mL/min)	1−2	1−2	6−1	6−1	4.4
8	forward wash left cartridge (1 mL 80% 0.1 M formic acid/20% MeOH, 1 mL/min)	1−2	1−2	6−1	6−1	1.2
9	backwash left cartridge (0.5 mL 0.2% NH_4OH/water, 1 mL/min)	1−2	6−1	6−1	6−1	0.7
10	wait for HPLC elution to finish	6−1	1−2	6−1	1−2	2.0[b]
11	return right cartridge to tray	6−1	1−2	6−1	1−2	0.1

[a] The method used for the first sample included only steps 2 and 5−9. The method used for the acquisition of the last sample included only steps 1, 3, 4, 10, and 11. [b] For the acquisition of the last sample, the duration of step 10 was 10.5 min.

clamp valve (LCV), and left divert valve (DV-1) were used for SPE separation, and the right clamp, right clamp valve (RCV), and right divert valve (DV-2) were used for the HPLC elution. The SPE run started with the conditioning of a HySphere HD C18 cartridge (7 μm, 10 mm × 1 mm; Spark Holland, Plainsboro, NJ, reused a maximum of 10−12 times), with HPLC grade methanol (2 mL) and 0.1 M formic acid (2 mL). Then, 400 μL of the sample (containing 100 μL of serum) was injected into the 1-mL sample loop and loaded onto the SPE column using 3 mL of 0.1 M formic acid (1 mL/min). Next, the SPE column was washed with 1 mL of 50% 0.1 M formic acid/50% methanol and then with 300 μL of 0.2% NH_4OH/water (flow reversed with DV-1). Time of the SPE cleanup above (including injection time) was 8 min. Before starting the SPE for the next sample, the SPE cartridge containing the extracted analytes was transferred by a robotic gripper (part of the Symbiosis system) from the left clamp into the right clamp. In this way, the right clamp was used for eluting analytes from the SPE cartridge, and the left clamp was used for the SPE of the next sample. Once the SPE column was positioned in the right clamp, the RCV remained in bypass (1-2) position until the HPLC−MS/MS system was ready to begin acquisition.

HPLC. At the beginning of the HPLC−MS/MS acquisition, the RCV turned automatically to the 6-1 position to allow the transfer of the analytes from the SPE column to the HPLC column. The analytes were separated from other extracted components on a Betasil C8 column (3 × 50 mm, 5 μm; ThermoHypersil-Keystone, Bellefonte, PA), preceded by a Betasil C8 precolumn (3 × 10 mm). During the first 2.5 min of the HPLC gradient program, the six-port valve (DV-2, Figure 1) placed between the precolumn and the HPLC column diverted the eluent flow carrying the fast-eluting matrix components to waste. At 2.5 min, before elution of the first analyte from the HPLC precolumn, the flow was diverted toward the HPLC column for the chromatographic separation. At 14 min, after elution of the last analyte from the SPE column, the RCV switched back to the 1-2 position, and the SPE column was returned to the cartridge tray while the HPLC gradient program continued.

The HPLC pump was an Agilent 1100 HPLC binary pump (Agilent Technologies, Wilmington, DE) operating at a 600 μL/min flow rate with 20 mM ammonium acetate (pH 4) in water as mobile-phase A and methanol as mobile-phase B. The HPLC gradient program (17 min) was as follows: started at 20% methanol; next, the methanol content was increased in 4 min to 60%; then from 4 to 14.5 min to 85% methanol. The last-eluting analyte was Et-PFOSA (14.2 min). The HPLC column was regenerated by increasing the methanol content to 90% in 0.5 min and kept for 0.5 min. Finally, methanol content was decreased in

Table 2. Multiple Reaction Monitoring Analysis of Perfluorochemicals (PFCs) Indicating the PFC Precursor[a] and Product[b] Negative-Ion Masses (*m/z*)

analyte	abbreviation	precursor ion $(M - H)^-$ (m/z)	product ion (m/z)
perfluorooctane sulfonamide	PFOSA	498	78
	$^{18}O_2$-PFOSA	502	82
N-methylperfluorooctane sulfonamide	Me-PFOSA	512	169
N-ethylperfluorooctane sulfonamide	Et-PFOSA	526	169
2-(*N*-methylperfluorooctane sulfonamido) ethanol	Me-PFOSA-EtOH	616	59
2-(*N*-ethylperfluorooctane sulfonamido) ethanol	Et-PFOSA-EtOH	630[b]	59
2-(*N*-methylperfluorooctane sulfonamido) acetic acid	Me-PFOSA-AcOH	570	512
2-(*N*-ethylperfluorooctane sulfonamido) acetic acid	Et-PFOSA-AcOH	584	526
perfluorohexane sulfonic acid	PFHxS	399	80[c]/99
perfluorooctane sulfonic acid	PFOS	499	99[c]/130/80
	$^{18}O_2$-PFOS	503	103[c]/84
perfluoropentanoic acid	PFPeA	263	219
perfluorohexanoic acid	PFHxA	313	269
perfluoroheptanoic acid	PFHpA	363	319
perfluorooctanoic acid	PFOA	413	369
	$^{13}C_2$-PFOA	415	370
perfluorononanoic acid	PFNA	463	419
pefluorodecanoic acid	PFDeA	513	469
perfluoroundecanoic acid	PFUA	563	519
perflurododecanoic acid	PFDoA	613	569

[a] $[M-H]^-$ represents the molecular ion; for Me-PFOSA-EtOH and Et-PFOSA-EtOH, the precursor ions were $[M - H + \text{acetate}]^-$ adducts. [b] Product ions were $[M - CO_2H]^-$ for the carboxylic acids; $[M - CH_2CO_2H]^-$ for Me-PFOSA-AcOH and Et-PFOSA-AcOH; $[FSO_3]^-$, $[CF_2SO_3]^-$, and $[SO_3)]^-$ for PFOS; $[SO_3]^-$ and $[FSO_3]^-$ for PFHxS; $[SNO_2]^-$ for PFOSA; and $[CH_3COO]^-$ for Me-PFOSA-EtOH and Et-PFOSA-EtOH [c] Quantitation ion.

0.5 min to 20% and kept for 1.5 min to equilibrate the column. The total HPLC gradient run was 17 min.

MS/MS. Negative-ion TurboIonSpray (TIS), a variant of electrospray, was used to convert liquid-phase ions into gas-phase ions on an API 4000 Q Trap, a hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA). Although the linear ion trap could be useful for qualitative confirmation of the analytes using information-dependent acquisition of enhanced resolution product ion or MS/MS/MS mass spectra, the quantitative analysis can be performed equally on a regular API 4000 triple quadrupole mass spectrometer. The TIS settings were curtain gas (N_2) 20 arbitrary units (au), heated TIS gas (zero air) 40 au, heated TIS gas temperature 500 °C, and ion spray voltage −4500 V. Ionization and collision cell parameters were optimized individually for each analyte (Table 2). Unit resolution was used for both Q1 and Q3 quadrupoles.

Me-PFOSA-EtOH and Et-PFOSA-EtOH molecular ions $[M - 1]^-$ gave very weak m/z signals that could only be observed above pH 7. By contrast, strong $[M + 59]^-$ m/z signals (Me-PFOSA-EtOH, $m/z = 616$ and Et-PFOSA-EtOH, $m/z = 630$) that suggest the formation of strong adducts between these sulfonamido ethanol derivatives and acetate were observed between pH 3 and pH 8. Fragmentation by N_2 in the Q2 collision cell produced exclusively $m/z = 59$ product ions, corresponding to acetate. Under our experimental conditions, this was the only product ion that could be used for MS/MS quantitation. We obtained qualitative confirmation of the presence of the $m/z = 616$ and $m/z = 630$ adducts and their retention times by fragmentation in the Q3 linear ion trap using MS/MS/MS scanning mode during on-line SPE/HPLC. The ion trap fragmentation of both adduct ions produced a dominant product ($m/z = 419$) consistent with the formation of a perfluorooctyl product ion through the loss of the sulfonamide moiety. We also observed 630 → 526 and 616 → 512 m/z ion trap fragments consistent with ethoxyl group loss and weak but distinct 630 → 570 and 616 → 556 m/z transitions consistent with the formation of free sulfonamido ethanol species through acetate loss.

Data Analysis. Data acquisition and analysis for all samples, blanks, standards, and QC materials were performed using the Analyst 1.4 software of the API 4000. The peak area ratio of each analyte to internal standard (i.e., response factor, RF) was used for quantification. $^{18}O_2$-PFOSA internal standard was used for the seven sulfonamides, $^{18}O_2$-PFOS for the three sulfonates, and $^{13}C_2$-PFOA for the eight carboxylates. To correct potential quantitation errors from contributions of the quantitation ions between analytes and isotope-labeled internal standards, we applied the Colby correction method.[34] The deviation of the corrected concentrations in the 1−100 ng/mL range was ±5%. Nine standard analyte concentrations spiked into calf serum diluted with 0.1 M formic acid were used to construct daily calibration curves weighted by the reciprocal of the standard amount ($1/x$). Since standard and unknown samples went through the same extraction procedure, reagent contributions were automatically corrected by the calibration curve intercept. The native amounts of PFCs in calf serum were calculated from average of multiple measurements from calf serum using a calibration curve obtained from standards spiked into 0.1 M formic acid (without serum). The calf serum contained nondetectable concentrations for most analytes. When using the PFOS 499 → 99 m/z transition for quantitation, blank serum concentrations of 0.6 ± 0.3 ng/mL PFOS were measured ($n = 7$). To correct for the endogenous PFOS present in calf serum, the calculated PFOS concentrations obtained from this transition were increased by 0.6 ng/mL. No corrections were applied to the other PFCs.

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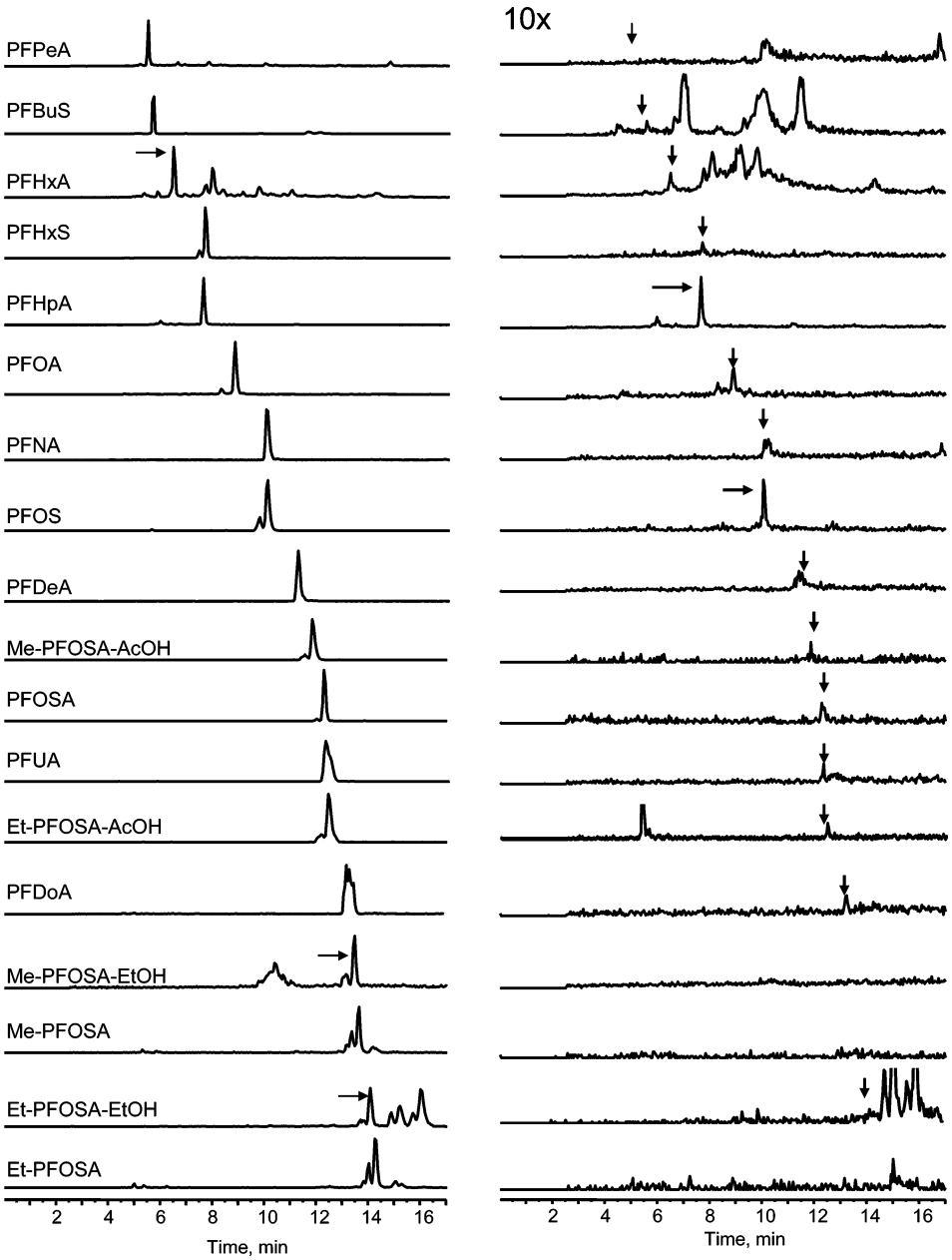

Figure 2. Typical HPLC−MS/MS chromatogram of a 200-μL calf serum extract spiked with PFCs (1.25 ng/mL) (left) and a blank, nonspiked calf serum (right, with the same scale magnified 10×).

SPE recovery of the analytes from serum was obtained with the following procedure: First, 400 μL of sample containing 100 μL of blank calf serum and a known amount of native standards was injected on the SPE column. At the start of the HPLC elution and MS/MS acquisition of the extracted analytes, 50 μL of internal standard was injected into the HPLC gradient flow (using an Agilent 1100 autosampler). Although injected separately, the native and the isotope-labeled compounds eluted from the HPLC column and were acquired by MS/MS at the same time. A response factor (RF_a) was calculated from this experiment. Second, a response factor (RF_b) was calculated by injecting a 400 μL of sample containing 100 μL of blank calf serum on the SPE column, and injecting a 50-μL solution of internal standard and standards into the HPLC flow. The difference between the two experiments was that in the first (RF_a) the same amount of native standards did go through the SPE cleanup but in the second (RF_b) they did not. SPE recovery was calculated from the RF_a/RF_b because the internal standard amount used for both experiments was the same and the matrix effects were equivalent.

RESULTS AND DISCUSSION

The on-line SPE method reported here was developed using information gained from our previous off-line SPE method.[27] PFCs were extracted from serum without protein precipitation by diluting and sonicating the serum with 0.1 M formic acid. Formic acid effectively suppressed interaction of the PFCs with serum macromolecules (e.g., proteins) and facilitated binding to the SPE sorbent.

Although the off-line SPE method performed very well for measuring 13 PFCs, including PFOS and PFOA, the two analytes that have attracted the most attention, it could not be used for measuring some sulfonamides (Me- and Et-PFOSA, Me- and Et-

Table 3. Solid-Phase Extraction Recoveries, Accuracy, and Limits of Detection Using Calibration Standards Extracted from Serum (Compared with Results Obtained by the Off-Line SPE Method[27])[a]

analyte	SPE recovery on-line (off line)	accuracy (%) at LOD/1.25/12.5 ng/mL			LOD ng/mL on-line (off line)
PFOSA	95 ± 6 (90)	87 ± 7	100 ± 6	103 ± 2	0.05 (0.2)
Me-PFOSA	94 ± 12	130 ± 26	84 ± 5	97 ± 2	0.07
Et-PFOSA	90 ± 13	125 ± 27	89 ± 6	96 ± 2	0.05
Me-PFOSA-EtOH	91 ± 12	105 ± 24	80 ± 16	94 ± 8	0.4
Et-PFOSA-EtOH	80 ± 12	63 ± 25	77 ± 12	95 ± 15	0.4
Me-PFOSA-AcOH	91 ± 12 (40)	135 ± 66	89 ± 7	100 ± 3	0.2 (0.6)
Et-PFOSA-AcOH	81 ± 12 (70)	110 ± 8	90 ± 9	102 ± 2	0.2 (0.4)
PFBuS	78 ± 16	103 ± 35	82 ± 9	95 ± 3	0.1
PFHxS	72 ± 4 (88)	108 ± 30	98 ± 8	95 ± 5	0.1 (0.3)
PFOS[b]	90 ± 6 (84)	118 ± 25	93 ± 10	102 ± 3	0.2 (0.4)
PFPeA	114 ± 6 (22)	75 ± 55	75 ± 55	99 ± 14	0.8
PFHxA	114 ± 4 (60)	146 ± 26	90 ± 24	99 ± 14	0.6 (3.2)
PFHpA	127 ± 4 (112)	200 ± 10	130 ± 30	103 ± 15	0.4 (0.3)
PFOA	105 ± 6 (91)	95 ± 60	109 ± 14	99 ± 7	0.2 (0.1)
PFNA	109 ± 8 (82)	95 ± 55	109 ± 20	107 ± 14	0.2 (0.1)
PFDeA	96 ± 4 (70)	123 ± 16	106 ± 9	94 ± 8	0.2 (0.3)
PFUA	95 ± 7 (72)	87 ± 67	102 ± 6	98 ± 11	0.2 (0.3)
PFDoA	75 ± 4 (30)	80 ± 52	86 ± 15	105 ± 7	0.2 (1.0)

[a] The SPE recovery and standard deviation was obtained from average of duplicate measurements at two serum concentrations, 2.5 and 12.5 ng/mL, for all analytes except for PFOS (10 and 50 ng/mL). The standard accuracy and standard deviation were obtained from concentration variation of five replicate measurements. [b] For PFOS, accuracies are reported at LOD, 6.5 and 50 ng/mL.

PFOSA-EtOH) and short-alkyl chain perfluorinated acids (PFPeA and PFHxA). We believe the source of this problem is the partial vaporization of PFCs during evaporation of the SPE extract before HPLC−MS/MS. These evaporation losses were minimized by stopping the evaporator when the extracts' volume was reduced to ~100 μL followed by dilution with 200 μL of aqueous solvent, to a final total volume of ~300 μL, of which 30 μL was injected into the HPLC system. Although with partial evaporation we minimized evaporation losses, only ~1/10 of the original serum was used; the rest was wasted. With on-line SPE, we eliminated the evaporation step, were able to measure all 18 analytes of interest (Figure 2), and used 1/5 (200 μL of serum instead of 1000 μL) of the serum used by our off-line SPE method. Elimination of the evaporation step considerably improved recovery of the short-alkyl chain carboxylic acids and the N-substituted sulfonamides (Table 3). The on-line SPE method also recovered the long-alkyl chain carboxylates (i.e., PFDeA, PFUA, and PFDoA) more effectively than our off-line SPE method.[27]

The key to a successful on-line SPE-HPLC method is choosing the optimal organic content eluent for transferring the analytes from the SPE to the HPLC column. Because of the diverse chromatographic behavior of the PFCs, very different optimal organic content was needed for the transfer of short versus long alkyl chain analytes. At nonoptimal conditions, the analyte signals showed peak tailing, peak doubling, and substantial peak broadening. These peak distortions are suggestive of a mixed (i.e., normal-phase/reversed-phase) partitioning mechanism that is influenced by the availability of free silanol groups in the sorbent, the organic content of the eluent, and the nature of the analytes. We found evidence of this mixed partitioning mechanism during the development of the off-line HPLC method,[27] and it is probably due to the unique dual hydrophobic−hydrophilic surfactant nature of the analytes.

To accommodate the diverse chromatographic behavior of the analytes, we transferred them from the SPE to the HPLC column using a relatively slow 20−80% methanol gradient for 14 min.

Table 4. Precision of Measurements of PFCs Concentrations in Spiked QC Serum Pools (Compared with Results Obtained with the Off-Line SPE Method[27])[a]

analyte	QC low		QC high	
	mean	% CV	mean	% CV
PFOSA	3.0 (2.2)	6 (7)	14.2 (12.0)	5 (6)
Me-PFOSA	3.6 (4.0)	9 (35)	5.9 (8.3)	10 (29)
Et-PFOSA	3.1 (3.1)	11 (30)	8.5 (11.2)	8 (29)
Me-PFOSA-EtOH	1	10	11.7	16
Et-PFOSA-EtOH	0.7	14	7.8	7
Me-PFOSA-AcOH	3.7 (2.7)	9 (11)	9.8 (8.3)	8 (12)
Et-PFOSA-AcOH	4.3 (2.8)	6 (15)	9.2 (6.7)	8 (20)
PFBuS	4.5 (3.5)	6 (19)	14.2 (13.2)	7 (17)
PFHxS	2.8 (2.1)	10 (11)	12.4 (10.7)	5 (10)
PFOS	9.1 (8.3)	10 (7)	33.3 (30.3)	5 (6)
PFPeA	5.4 (4.6)	24 (49)	27.7 (24.0)	31(56)
PFHxA	5.0 (5.1)	16 (34)	15.7 (15.5)	21 (34)
PFHpA	6.7 (7.5)	13 (17)	14.1 (16.3)	18 (13)
PFOA	3.0 (3.2)	10 (16)	13.3 (14.5)	11 (12)
PFNA	2.6 (2.5)	17 (14)	13.7 (12.6)	13 (13)
PFDeA	2.4 (2.1)	15 (24)	9.6 (8.2)	19 (22)
PFUA	1.8 (1.7)	13 (26)	10.6 (10.3)	19 (26)
PFDoA	1.7 (2.2)	15 (21)	7.7 (8.8)	9 (23)

[a] Mean concentrations ($N = 30$) in ng/mL; CV is the coefficient of variation.

Without the HPLC column, the fastest eluting analyte left the SPE column in 0.5 min and the last analyte left at 13 min. Therefore, the SPE column needed to be kept in front of the HPLC column during most of the HPLC−MS/MS acquisition. For a high-throughput method using the HPLC−MS/MS acquisition time period for the injection and cleanup of the next sample, a second SPE column was needed. Fortunately, such concurrent SPE and HPLC operations, using two SPE columns, can be easily performed on the Symbiosis on-line SPE system. We took full advantage of the system by incorporating both column clamps and all four switching valves into our method (Figure 1 and Table 1). In particular, we used DV-1 to reverse the flow going through

Table 5. Concentrations (in ng/mL) of Selected PFCs Measured in Serum Samples Collected in Atlanta, GA, in March 2004 from Five Adults[a]

	PFOSA	Me-PFOSA-AcOH	Et-PFOSA-AcOH	PFHxS	PFOS	PFOA	PFNA	PFDeA
A	<LOD	0.57 (*0.00*)	<LOD	1.02 (*0.2*)	21.0 (*0.1*)	2.7 (*0.08*)	0.55 (*0.08*)	<LOD
B	0.07 (*0.04*)	1.37 (*0.13*)	<LOD	2.86 (*0.2*)	28.5 (*2.6*)	3.3 (*0.4*)	1.28 (*0.16*)	0.43 (*0.06*)
C	0.08 (*0.04*)	0.96 (*0.03*)	0.10 (*0.02*)	1.32 (*0.2*)	24.7 (*1.2*)	4.4 (*0.04*)	1.3 2(*0.28*)	<LOD
D	<LOD	0.16 (*0.02*)	<LOD	1.22 (*0.6*)	29.3 (*2.3*)	3.2 (*0.3*)	0.99 (*0.09*)	0.64 (*0.08*)
E	0.10 (*0.05*)	0.44 (*0.01*)	0.12 (*0.01*)	1.49 (*0.1*)	63.5 (*0.9*)	7.0 (*1.0*)	0.95 (*0.08*)	0.42 (*0.02*)
average	0.06	0.70	0.07	1.6	33.4	4.1	1.02	0.34

[a] Duplicate measurements (*standard deviation*). <LOD indicates that the concentration was below the limit of detection. The other analytes were <LOD in all the samples analyzed.

the SPE column during the wash steps, thus eliminating particles from the loading end of the SPE column. We also used DV-2 to direct the flow to waste after the HPLC precolumn at the beginning of the gradient elution, allowing for an extra cleaning step by "heart cutting" the analytes from the precolumn before they enter into the analytical column.

In the current method, we used three isotope-labeled internal standards, $^{13}C_2$-PFOA, $^{18}O_2$-PFOSA, and $^{18}O_2$-PFOS. To compensate for the lack of isotope-labeled internal standards for the other analytes and account for matrix effects, we spiked the calibration standards into calf serum.

Spiked serum was analyzed repeatedly to determine the limit of detection (LOD), accuracy, and precision for measuring the various analytes included in the method. The LOD was calculated as $3S_0$, where S_0 is the standard deviation as the concentration approaches zero.[35] S_0 was determined from five repeated measurements of low-level standards. As expected, the greatest improvement in sensitivity compared with the off-line SPE method was observed for the sulfonamide derivatives (Table 3).

The calibration curves showed adequate linearity (i.e., correlation coefficients 0.98−0.99). The method accuracy was assessed by five replicate analyses of serum spiked at three different concentrations and expressed as a percentage of the expected value. Above the LOD, the intraday variability, reflected in the standard accuracies (77−109%) and their relative standard deviations (5−24%) was very good (Table 3); the values at the 1.25 ng/mL spike level were somewhat higher for PFPeA (75 ± 55%) and PFHpA (130 ± 30%) because of inaccuracy resulting from lack of isotope-labeled internal standard and reagent contribution. We determined method precision by calculating the coefficient of variation (CV) of 30 repeated measurements of the QCL and QCH materials over a period of 3 weeks (Table 4). These CVs, which reflect the intraday and interday variability of the method, show very good precision (6−11%) for PFOSA, PFOS, and PFOA for which labeled internal standards were available. For the rest of the analytes, CVs were adequate (i.e., 6−24%) for quantification.

Analyte concentrations in QC materials measured after 3 months of storage at −20 °C remained in the CV range established during QC characterization. To check the possibility of analyte losses during sample preparation and analysis for the analytes for which no labeled internal standard was available, we monitored the stability of the analytes in QC samples (nonprepared) kept in closed polypropylene storage containers at around 25 and 11 °C for 5 days. The response factors measured after 1, 2, 3, and 5 days showed no decreasing trend, suggesting that concentrations were essentially constant. We also monitored analyte concentration changes in QCH samples prepared (i.e., 0.1 M formic acid and internal standard added) in polypropylene autosampler vials with intact and punctured septa for 5 days at ~25 °C. In vials with intact septa, even 5 days after preparation, none of the analytes showed a marked decrease in concentration. In vials with punctured septa, concentrations of Me-PFOSA and Et-PFOSA started to decrease after 1 day and were 20% of the original amount after 5 days. In the vials (punctured or intact septum) with only standard spiking solution and 0.1 M formic acid but no serum present, most of the Me-PFOSA and Et-PFOSA vaporized within 3 h.

To demonstrate the performance of our on-line SPE-HPLC method on human serum samples, we analyzed samples collected from five adults in Atlanta, GA during March 2004 (Table 5). We found PFOS (21.0−63.5 ng/mL), PFOA (2.7−7.0 ng/mL), PFHxS (1.0−2.9 ng/mL), PFNA (0.6−1.3 ng/mL), and Me-PFOSA-AcOH (0.4−5.2 ng/mL) in samples from all five subjects. Our method is currently applied to the simultaneous quantitative determination of 18 PFCs for large epidemiologic studies to assess the prevalence of environmental exposure to PFCs.

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