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Short communication

Improved selectivity for the analysis of maternal serum and cord serum for polyfluoroalkyl chemicals☆

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ABSTRACT

Perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid, two of the most widely studied polyfluoroalkyl chemicals (PFCs), can cross the placenta. Therefore, data on the exposure to PFCs of the very young are needed to evaluate the potential health effects associated with such exposure. Human serum, especially serum collected from pregnant women and cord serum, may contain endogenous components that can interfere in the separation by high performance liquid chromatography (HPLC) of PFOS and another PFC of interest, perfluorohexane sulfonic acid (PFHxS), from other serum biomolecules. The presence of such interferences may prevent the adequate quantification of PFOS and PFHxS in cord serum or serum collected from pregnant women, and potentially hinder the assessment of gestational exposure to these important PFCs using biomonitoring. We have modified our on-line solid phase extraction–HPLC–isotope dilution–tandem mass spectrometry analytical method for measuring PFCs in serum and developed an approach that allows for the elimination of these potential interferences without compromising analytical sensitivity and throughput. The combination of acetonitrile as the HPLC mobile phase organic solvent and a Betasil C8 HPLC column provided the best separation of PFOS and PFHxS from interferent peaks. In addition to eliminating these interferences, the acetonitrile method has a shorter runtime and is more sensitive for most PFCs (limits of detection were 0.1 ng/mL except for PFOS (0.2 ng/mL)) than our previous method that used methanol for the HPLC separation. The present method should improve the precise and selective analysis of maternal and cord serum for PFCs.

1. Introduction

Polyfluoroalkyl chemicals (PFCs) have been used in a variety of commercial applications [1]. Some PFCs resist degradation in the environment and are detected at concentrations in the low parts-per-billion range in wildlife and humans [2,3].

Some PFCs have demonstrated various toxicities in animal studies [3,4]. Because two of the most widely studied PFCs, perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) can cross the placenta [5–10], developmental and reproductive toxicities are of concern from the perspective of fetal exposure to PFCs [11–16]. Therefore, additional research, including biomonitoring studies, is needed to evaluate the potential health effects associated with exposure to PFCs.

Biomonitoring studies have relied on mass spectrometry for the quantification of PFCs in biological specimens, mainly serum [2,3]. Several investigators have reported that the precursor/product ion m/z transitions commonly used for the quantification of PFOS ($499 \rightarrow 99$ and $499 \rightarrow 80$) and of another PFC perfluorohexane sulfonic acid (PFHxS, $399 \rightarrow 80$ and $399 \rightarrow 99$) in serum, especially cord serum or serum from pregnant women, can display interferences [5,11,17–19]. We observed the presence of these interferences when we attempted to quantify PFOS and PFHxS in cord serum [11] using our previous analytical method [20]. Because of these interferences, we were unable to quantify the cord serum concentrations of PFHxS, and for PFOS we had to use the ion transition m/z 499/130 [11], which displayed fewer interferences than the other monitored transitions. These observations support the need to develop alternative approaches to separate these interferences from the target PFCs without compromising the analytical sensitivity and increasing considerably the analytical run time. We describe the development and application of an on-line solid phase extraction–high performance liquid chromatography–isotope dilution–tandem mass spectrometry (on-line SPE–HPLC–MS/MS) method that allows for a selective analysis of serum, including cord serum, for 13 PFCs, including PFOS and PFHxS. The updated on-line SPE–HPLC–MS/MS method used

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the same SPE and HPLC columns than our previous method [20], but the SPE sample preparation steps were simpler, the total usage of organic solvents was reduced by 55% and the HPLC run time was shortened from 18 min to 12 min.

2. Experimental

The commercial sources of reagents and analytical standards, detailed descriptions of the preparation of standard solutions, and quality control (QC) materials, sample and standards preparation, and the mass spectrometry-related parameters for the quantification of the PFCs are available in Supplementary Material.

2.1. On-line SPE–HPLC–MS/MS

We measured the concentrations of PFCs following a modification of our previously reported method [20]. A detailed description of the design of the on-line SPE–HPLC–MS/MS system and schematic diagram (Fig. S1) are available in Supplementary Material. For each sample, the SPE run starts with the conditioning of a Polaris C18 HD 10 mm × 2 mm (Spark Holland, Plainsboro, NJ, USA) cartridge with HPLC-grade methanol (MeOH) (2 mL) and 0.1 M formic acid (2 mL). Afterward, 400 μL of sample (containing 100 μL serum) injected into the 1 mL sample loop is loaded onto the SPE column by use of 2 mL 0.1 M formic acid at 2 mL/min. Next, the SPE column is washed (2 mL/min) with 2 mL 80% 0.1 M formic acid/20% MeOH. SPE cleanup duration (including injection time) is 10 min. Before the start of the clean-up of the next sample, the cartridge containing the extracted analytes is transferred by a robotic gripper of the Spark Holland Symbiosis extractor from the left clamp into the right clamp. Therefore, while the right clamp is used for analyte elution and HPLC–MS/MS acquisition, the left clamp is used for the clean-up of the next sample. Once the SPE column is in the right clamp, the right clamp valve (RCV) remains in by-pass (1–2) position until the HPLC–MS/MS system becomes ready to begin acquisition. At the beginning of the HPLC–MS/MS acquisition, the RCV is turned into 6–1 position of the HPLC gradient program to transfer the analytes from the SPE column to a Betasil C8 HPLC precolumn (3 mm × 10 mm, 5 μm; ThermoHypersil-Keystone, Bellefonte, PA, USA). In addition, the right divert valve (DV-2) is turned into 1–2 position for the first 2 min to send to waste any foreign substances from the HPLC precolumn. At 2 min, the DV-2 is turned into 6–1 position to transfer the analytes from the HPLC precolumn to a Betasil C8 HPLC column (3 mm × 50 mm, 5 μm). At 10 min, the RCV is turned back to 1–2 position and the SPE column is returned to the cartridge tray, while the HPLC gradient program continues. The HPLC pump operates at a 600 μL/min flow rate with 20 mM ammonium acetate (pH 4) in water and acetonitrile as mobile phase A and mobile phase B, respectively. The HPLC gradient program (12 min) is as follows: start at 10% B (1 min), B content increases from 10% to 55% (1–4.0 min), from 55% to 80% (4–10 min), then B content 90% hold (0.5 min), decreases to 10% and hold 10% (10.5–12 min). The Agilent valve connecting the HPLC column and the MS/MS is turned into waste position at 5.5 min and after the 12 min, to discard foreign substances from the HPLC column and avoid contamination of the MS/MS interface. To delay the elution of the PFC contaminants potentially leaching out from Teflon parts of the HPLC pump, another 3 mm × 50 mm, 5 μm Betasil C8 column is inserted between the HPLC pump and the RCV. Because a contaminant would have to go through twice the filter column length, its peak would elute after the main analyte peak without interfering with the quantification of the main analyte signal.

2.2. Data analysis

Data acquisition and analysis for all samples, blanks, standards, and QC materials were performed by use of the Analyst 1.4 software of the API 4000. The data analysis program automatically selected and integrated the signals for each target transition in the chromatogram. We manually corrected the integrations, if necessary. For quantification, we used a response factor (RF), calculated as the peak area of each analyte ion divided by the peak area of its internal standard. We used 9 standard PFCs concentrations, spiked into calf serum diluted with 0.1 M formic acid, encompassing the entire linear range of the method (0.04–215 ng/mL [PFOS], 0.002–10 ng/mL [PFHpA], and 0.01–50 ng/mL [other analytes]) to construct daily calibration curves, weighted by the reciprocal of the standard amount ($1/x$), of RF vs the standard amount. The calibration curves were linear over three orders of magnitude and had correlation coefficients exceeding 0.99. Because standards and unknown samples went through the same extraction procedure, reagent contributions were automatically corrected by the calibration curve intercept. The calf serum contained nondetectable concentrations of the target ana-

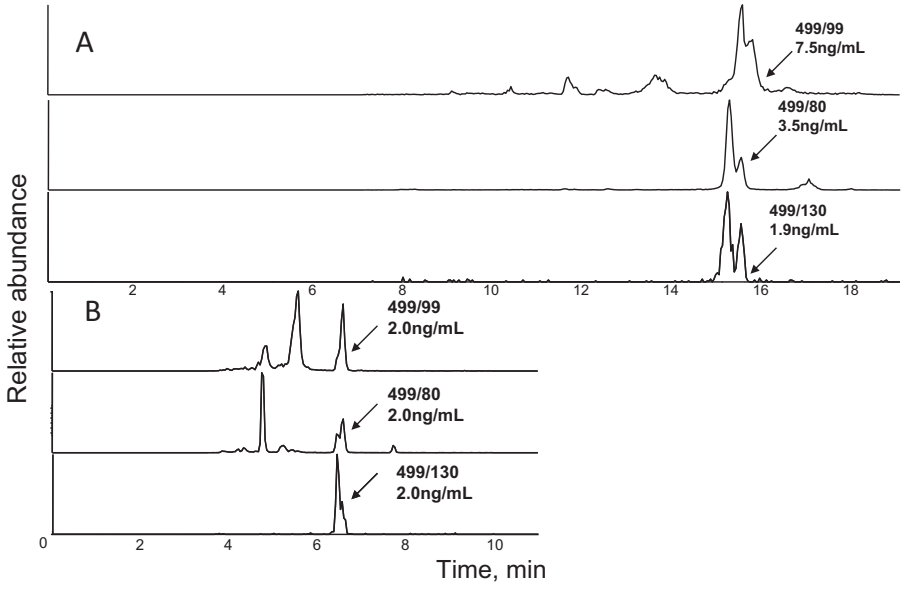

Fig. 1. Example chromatograms of PFOS in one cord serum specimen analyzed by use of the MeOH method (A) and the ACN method (B).

lytes, calculated from the average of multiple measurements using calibration curves obtained from standards spiked into 0.1 M formic acid (without serum).

Two low-concentration QCs (QCL), two high-concentration QCs (QCH), three serum blanks, and two reagent blanks were analyzed concurrently with 30 samples and nine calibration standards. The concentrations of the QCs were evaluated by use of standard statistical probability rules [21].

3. Results and discussion

3.1. Optimization of HPLC conditions

First, we evaluated seven HPLC columns with various flow rates and gradient programs, using 20 mM ammonium acetate (pH 4.0) as mobile phase A and methanol as mobile phase B. We evaluated each combination for the separation of PFHxS and PFOS from their interferences in cord serum. We examined: Betasil C8 (3 mm × 50 mm, 5 μm), Betasil C8 (2.1 mm × 50 mm, 3 μm), Inertsil C8-3 (3 mm × 50 mm, 3 μm; GL Science Inc., Torrance, CA, USA), Inertsil ODS 4 (3 mm × 50 mm, 3 μm; GL Science Inc.), Eclipse Plus C8 (2.1 mm × 50 mm, 3.5 μm; Agilent Tech., Wilmington, DE, USA) and Polaris C8-A 5 μ (3.0 mm × 50 mm, 5 μm, Varian Inc., Walnut Creek, CA, USA). None of the columns under the conditions examined provided sufficient separation between the target analytes and their interferences in cord serum. We also changed the SPE column conditioning solvent from acetonitrile (2 mL) to MeOH (2 mL) and eliminated the 0.2% ammonia backwash step; these changes did not affect the HPLC separation of the target analytes and the ruggedness of the SPE procedure.

Next, we examined the separation of PFOS and PFHxS from the cord serum interferences, using MeOH/acetonitrile (data not shown) or acetonitrile as the HPLC organic mobile phase and a Betasil C8 column (3 mm × 50 mm, 5 μm). By use of MeOH, the PFOS concentrations calculated from the three ion transitions monitored (i.e., m/z: 499/99, 499/80, and 499/130) differed (Fig. 1A). By contrast, analysis of the same cord serum specimen using acetonitrile as the HPLC mobile phase B yielded the same PFOS concentration regardless of the ion transition used (Fig. 1B). Similar results were observed for PFHxS (Fig. 2). The MeOH results for PFOS and PFHxS were not unexpected. In a previous study for which we analyzed cord serum for PFCs [11], we were unable to report results for PFHxS, and for PFOS we had to use the ion transition m/z 499/130, which we normally used for confirmation because it was less sensitive than the transition we used for the quantification of PFOS [20]. Of interest, a previous report [18] suggested that the interferences for PFHxS were a problem in all human serum samples. However, even with our previous method [20], we achieved the separation of PFHxS from its interferences for most sera specimens (Supplementary Material, Fig. S2), with the exception of cord serum.

The comparison of the results obtained with MeOH and with acetonitrile suggests that we were able to separate PFOS and PFHxS from their interferences in cord serum using a combination of 20 mM ammonium acetate and 100% acetonitrile as the HPLC mobile phases A and B, respectively. Furthermore, compared to our previous method that used MeOH as the HPLC mobile phase B [20], using acetonitrile, we reduced the total usage of organic solvents by 55% and we also reduced by one third the HPLC run time from 18 to 12 min (Fig. 3). These modifications will reduce the amount

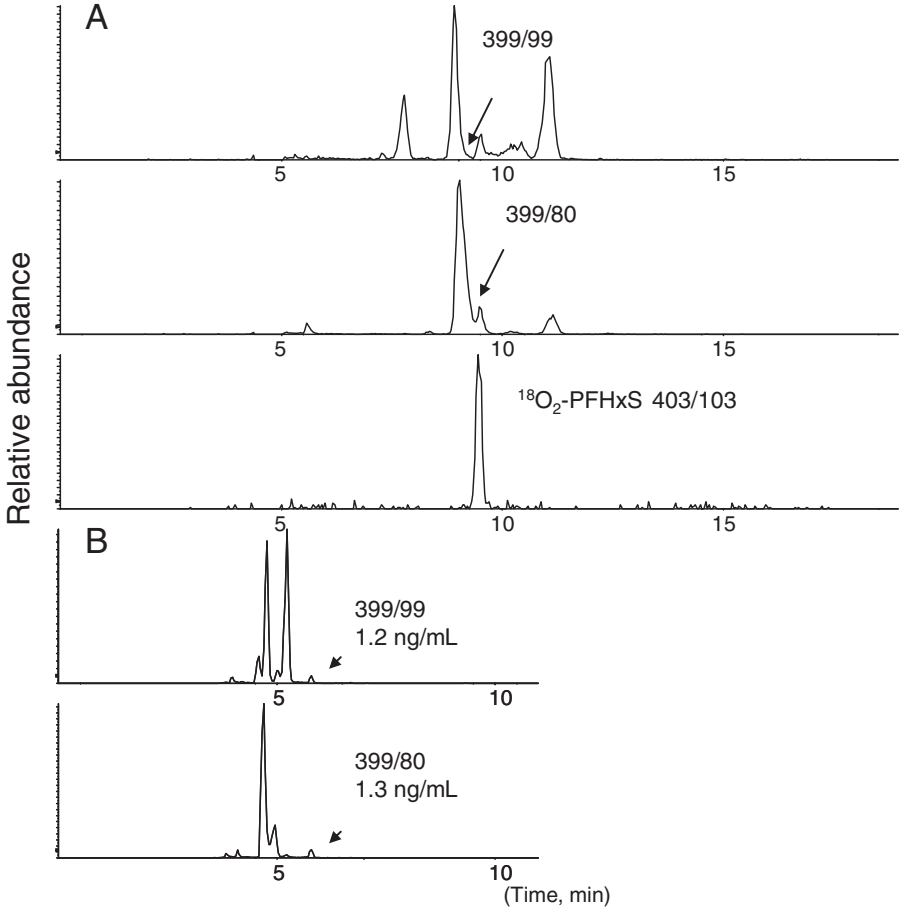

Fig. 2. Example chromatograms of PFHxS in one cord serum specimen analyzed by use of the MeOH method (A) and the ACN method (B).

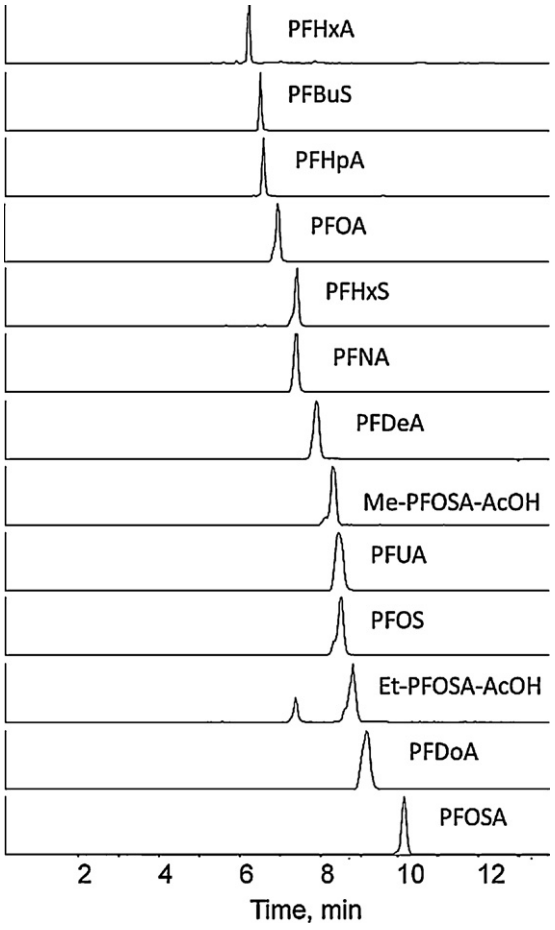

Fig. 3. Typical HPLC–MS/MS chromatogram of 13 PFCs spiked in calf serum at concentrations ranging from 0.5 to 2.5 ng/mL.

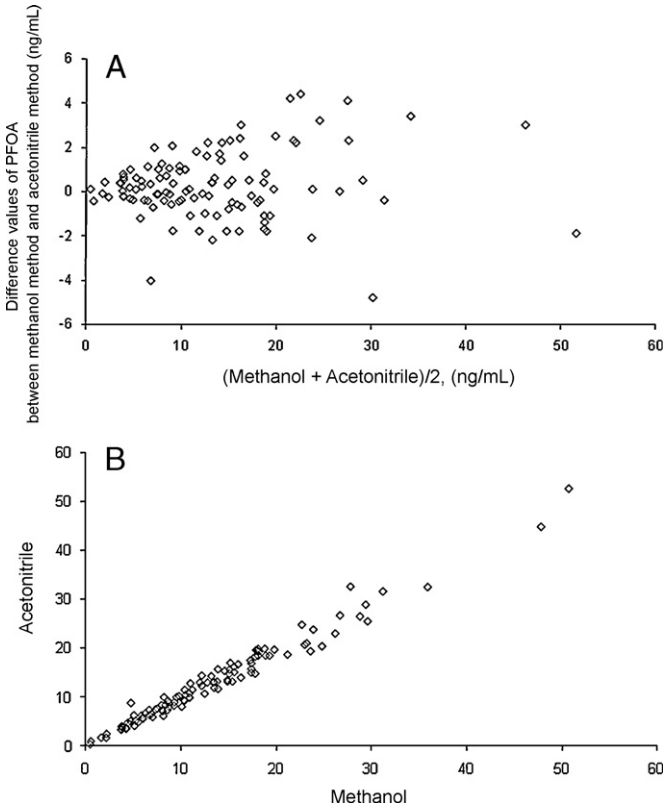

Fig. 4. Repeat analysis of 127 serum samples for PFOS using the present and previous (Kuklenyik et al. [20]) method approaches (ACN vs MeOH). (A) The Bland–Altman plot (difference in concentrations vs average of concentrations using both methods) illustrates the random variation around zero on the *y*-axis across the entire concentration range on the *x*-axis. (B) Correlation plot between the concentrations of 127 samples analyzed with the previous method (Kuklenyik et al. [20]) and present method ($R^2 = 0.9712$, $y = 0.9625x - 0.2011$).

of waste for disposal and may also translate in a reduced cost per sample. Furthermore, these features are particularly advantageous if the method is to be used in support of large biomonitoring programs such as the National Health and Nutrition Examination Survey [22–24].

3.2. Limits of detection, accuracy, and precision of the analytical method

A detailed description of the procedures for determining accuracy and precision is available in Supplementary Material. The limits of detection (LODs) were calculated as $3S_0$, where S_0 is the standard deviation as the concentration approaches zero. S_0 was determined from 5 repeated measurements of low-level standards spiked onto calf serum [25]. The LOD values (0.1 ng/mL, except 0.2 ng/mL for PFOS) are comparable to or even lower for most analytes (Table 1) than the LODs we achieved using our previous method [20]. Similarly, the method accuracy (87–108%) was acceptable for all target analytes (Table 1). Interday precision, calculated as the RSD% of 60 repeated measurements in a 6-month period, ranged from 6.3 to 15.4% for QCL (1.5–8.2 ng/mL) and from

Table 1
Accuracy, limits of detection (LOD), and precision of the analytical method.

Analyte	Accuracy		LOD	QCL			QCH		
	ng/mL	%	ng/mL	ng/mL	Intraday CV%	Interday CV%	ng/mL	Intraday CV%	Interday CV%
PFBuS	2.5	87	0.1	1.9	6.2	10.2	6.8	5.9	13.1
PFHxS	2.5	105	0.1	1.9	4.9	7.1	6.9	1.4	6.1
PFOS	10.0	101	0.2	8.2	5.8	7.3	27.5	4.9	7.6
PFHxA	1.0	96	0.1	1.7	1.4	15.4	5.9	2.3	10.5
PFHpA	1.0	92	0.1	1.5	3.3	8.0	5.3	2.9	9.8
PFOA	5.0	94	0.1	3.1	2.8	7.6	11.7	1.7	5.8
PFNA	5.0	103	0.1	2.7	3.2	7.9	9.6	1.9	7.1
PFDeA	3.5	107	0.1	3.2	4.2	8.2	10.9	3.7	6.5
PFUA	3.5	103	0.1	2.1	2.9	9.6	7.5	1.9	11.7
PFDoA	3.5	108	0.1	2.0	3.3	8.9	7.0	2.9	7.3
PFOSA	5.0	105	0.1	2.2	4.3	6.3	7.6	2.3	5.1
Me-PFOSA-AcOH	5.0	98	0.1	2.0	1.5	13.1	6.9	1.9	9.8
Et-PFOSA-AcOH	5.0	104	0.1	1.6	1.8	7.7	5.5	2.1	7.9

Accuracy and LOD: $n = 5$.
Intraday precision: $n = 5$.
Interday precision: $n = 60$.

5.1 to 13.1% for QCH (5.3–27.5 ng/mL) (Table 1). Intraday precision, calculated as the RSD% of 5 repeated measurements within one day, ranged from 1.4 to 6.2% for QCL and from 1.4 to 5.9% for QCH (Table 1). These data suggest that the sensitivity, accuracy, and precision of the method are adequate for biomonitoring purposes.

3.3. Ruggedness and reliability of the method

We analyzed 28 human serum samples using our previous [20] and the present method. We also analyzed the same 28 samples using the present method on two separate instruments (API 4000 and API 4000 Q trap) and by two different analysts using the API 4000. Spearman's rank correlation coefficients between our previous and the present method, and between two instruments, and between two analysts for the present method for select PFCs were all above 0.976 (Supplementary Material, Table S2). In addition, we analyzed 127 serum samples using our previous analytical method [20] and the current method and found a good agreement between the results (Fig. 4A and B and Supplementary Material, Fig. S3A and B). Of interest, our previous method was deemed to be adequate on the basis of inter-laboratory comparisons [19,26]. Therefore, the good agreement between the two methods, as well as between two different HPLC–MS/MS systems and between multiple analysts for the present method strongly suggests that the present method is also rugged and reliable.

4. Conclusions

We have developed a selective, sensitive, accurate, and precise analytical method for the separation and quantification of PFCs in maternal and cord serum. Furthermore, this method is also adequate for the analysis of general human serum for PFCs, and it can be used in large epidemiologic studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.10.051.

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