

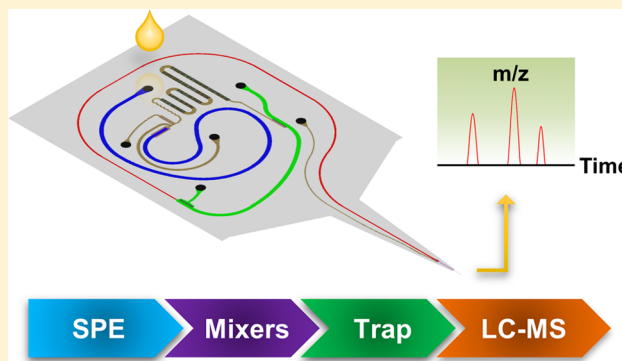
# Biomonitoring of Perfluorinated Compounds in a Drop of Blood

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## S Supporting Information

**ABSTRACT:** Biomonitoring of pollutants and their metabolites and derivatives using biofluids provides new opportunities for spatiotemporal assessment of human risks to environmental exposures. Perfluorinated compounds (PFCs) have been used widely in industry and pose significant environmental concerns due to their stability and bioaccumulation in humans and animals. However, current methods for extraction and measurement of PFCs require relatively large volumes (over one hundred microliters) of blood samples, and therefore, are not suitable for frequent blood sampling and longitudinal biomonitoring of PFCs. We have developed a new microassay, enabled by our silicon microfluidic chip platform, for analyzing PFCs in small volumes (less than five microliters) of blood. Our assay integrates on-chip solid-phase extraction (SPE) with online nanoflow liquid chromatography-electrospray ionization-mass spectrometry (nanoLC-ESI-MS) detection. We demonstrated high sample recovery, excellent interday and intraday accuracy and precision, and a limit of detection down to 50 femtogram of PFCs, in one microliter of human plasma. We validated our assay performance using pooled human plasma and NIST SRM 1950 samples. Our microfluidic chip-based assay may enable frequent longitudinal biomonitoring of PFCs and other environmental toxins using a finger prick of blood, thereby providing new insights into their bioaccumulation, bioavailability, and toxicity.



## INTRODUCTION

Compared to the traditional exposure assessment using ambient monitoring of environmental pollutants, rapid and sensitive detection of large sets of analytes including exogenous chemicals, their metabolites, and other derivatives such as protein adducts ("exposome", e.g., metabolome and adductome) using human specimens such as blood and urine, which reflect the complexity of exposure in the personal environment, has opened up new opportunities for epidemiologic studies of human exposure.<sup>1–4</sup> However, two key challenges remain for "exposomics": (1) rapid and efficient processing of small volumes (1–50  $\mu\text{L}$ ) of biospecimens, especially human blood samples; and (2) detecting multiple (and multiclass) analytes simultaneously with high-sensitivity and high-specificity from such small volumes of biospecimens. One solution is to directly couple sample processing with nanoflow LC-MS in order to minimize sample loss and increase detection sensitivity. Solid-phase extraction (SPE) has been used widely for analyte cleanup, enrichment and extraction.<sup>5,6</sup> There are multiple platforms on the market that take advantage of SPE and the subsequent LC/GC-MS analysis including RapidFire SPE-MS system (Agilent Inc.) and SPE-LC-MS systems from Thermo-Fisher Scientific and Waters Corp.. However, in these conventional systems, SPE is generally online coupled with high-flow LC-MS because the large SPE column volume is required for efficient SPE of target analytes from complex biological samples, and therefore it is incompatible with nanoflow LC-MS and may result in substantial peak broadening

and poor chromatographic separation because of the volume mismatch. Therefore, relatively large sample volumes ( $\geq 100 \mu\text{L}$ ) of blood and serum are required for analyzing low concentrations of target analytes. This renders them impractical for population studies of multiple (multiclass) analytes where very small volumes of blood are collected. Microfluidic devices are ideal for handling small volumes of samples and have been utilized for on-chip SPE, SPE-MS, and LC-MS separately.<sup>7–15</sup> However, a monolithic interface between SPE and nanoLC-MS (i.e., SPE-nanoLC-MS) on a microfluidic chip, particularly on a silicon-based chip, has not been previously achieved. Such a device would potentially facilitate a major improvement in the ability to monitor bioaccumulation of perfluorinated compounds (PFCs) from small sample volumes.

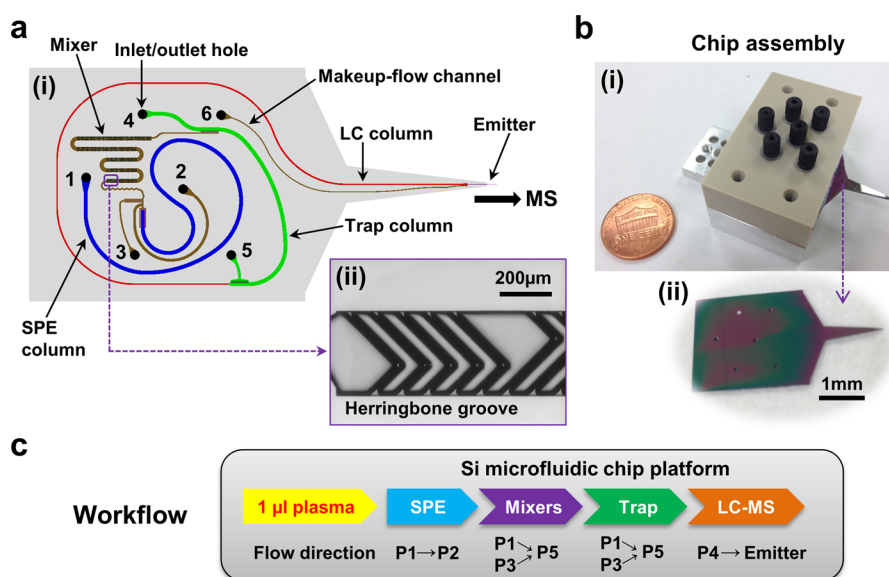
Here we report the development and proof-of-principle application of an integrated SPE-nanoLC-MS chip for on-chip and online SPE-nanoflow LC-nanoESI-MS analysis of PFCs in small volumes of human blood samples. The chip is built on our silicon-based microfabricated monolithic multinozzle emitters ( $M^3$  emitters) and the multinozzle emitter array chips (MEA chips) for nanoflow LC-ESI-MS.<sup>9,16</sup> These devices collectively offer a straightforward yet novel solution to the longstanding problem of the efficient coupling between silicon

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**Figure 1.** A silicon microfluidic chip for biomonitoring of PFCs. (a) Key functional modules on the chip include a SPE column (blue), a herringbone mixer (brown), a trap column (green), a LC column (red), an emitter, and multiple inlet/outlet holes 1–6 (i). The zoom-in view of the mixer (ii) shows herringbone grooves. (b) High-resolution photographs showing the chip and its assembly with a custom-built manifold and fittings, relative to a U.S. penny. (c) Overall workflow for on-chip and online SPE-nanoLC-MS analysis of PFCs in small volumes of human plasma. The flow direction for each major step is specified with fluidic inlet and outlet port positions P1–P6.

microfluidic chips and LC-ESI-MS, and pave the way for large-scale integration on microfluidic chips for application-specific platforms and solutions. In this work, we demonstrate a flexible three-column system including a SPE, a guard (trap), and a LC column on a single silicon microfluidic chip, for efficient extraction and sensitive detection of PFCs from microliters of human blood samples.

## MATERIALS AND METHODS

**Chemicals.** All PFCs were obtained from commercial sources. Perfluoro-*n*-heptanoic acid (PFHpA), perfluoro-*n*-octanoic acid (PFOA), perfluoro-*n*-nonanoic (PFNA), perfluoro-*n*-decanoic acid (PFDeA), sodium perfluoro-1-hexanesulfonate (PFHxS), sodium perfluoro-1-octanesulfonate (PFOS), sodium perfluoro-1-hexane[ $^{18}\text{O}_2$ ]sulfonate ( $^{18}\text{O}_2$ -PFHxS), sodium perfluoro-1-[1,2,3,4- $^{13}\text{C}_4$ ]octanesulfonate ( $^{13}\text{C}_4$ -PFOS), perfluoro-*n*-[1,2,3,4- $^{13}\text{C}_4$ ]octanoic acid ( $^{13}\text{C}_4$ -PFOA), perfluoro-*n*-[1,2,3,4,5- $^{13}\text{C}_5$ ]nonanoic ( $^{13}\text{C}_5$ -PFNA), perfluoro-*n*-[1,2- $^{13}\text{C}_2$ ]decanoic acid ( $^{13}\text{O}_2$ -PFDeA), perfluoro-*n*-[1,2- $^{13}\text{C}_2$ ]undecanoic acid ( $^{13}\text{C}_2$ -PFUnA), and perfluoro-*n*-[1,2- $^{13}\text{C}_2$ ]dodecanoic acid ( $^{13}\text{C}_2$ -PFDoA) were purchased from Wellington Laboratories (Guelph ON, Canada). Sodium  $^{13}\text{C}_8$ -perfluorooctanesulfonate ( $^{13}\text{C}_8$ -PFOS) and  $^{13}\text{C}_8$ -perfluorooctanoic acid ( $^{13}\text{C}_8$ -PFOA) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). Perfluorooctanesulfonamide (PFOSA), ammonium acetate, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade methanol and water were from VWR (Radnor, PA). All chemicals and solvents were used without further purification.

**Sample Preparation.** Standard stock PFCs solutions were prepared by diluting PFC standards in methanol in polypropylene vials. All glassware was rinsed with methanol before use. For plasma samples spiked with PFCs, frozen pooled human plasma was obtained from Innovative Research Inc. (Novi, MI). A 5  $\mu\text{L}$  aliquot of human plasma was thawed and mixed with 2.5  $\mu\text{L}$  of internal standards ( $^{13}\text{C}_8$ -PFOA and

$^{13}\text{C}_8$ -PFOS, 50 ng/mL), 2.5  $\mu\text{L}$  of PFC standards with desired concentration, and 20  $\mu\text{L}$  of cold acetonitrile with 0.1% formic acid. After vortexing for 2 min, the mixture was sonicated for 10 min and then centrifuged at 14 000g for 15 min. Twenty-four  $\mu\text{L}$  of the supernatant was taken out and allowed to evaporate at room temperature until the volume was reduced to  $\sim 16$   $\mu\text{L}$ . All samples were stored in polypropylene vials at  $-20^\circ\text{C}$ . National Institute of Standards and Technology (NIST) Standard Reference Materials (SRMs) 1950 Metabolites in Human Plasma was ordered from NIST and stored at  $-80^\circ\text{C}$ . NIST SRM 1950 was thawed at room temperature and prepared by the same procedure as pooled plasma samples for validation of the chip performance.

**SPE-nanoLC-MS Chip Fabrication and On-Chip Column Packing.** The detailed design and fabrication procedures are similar to what we had described for our MEA chip.<sup>11,12</sup> The single-plex chip consisted of a two-layer Si–Si structure that monolithically integrated several functional modules on a single chip as shown in Figure 1a. The chip included a LC column of 64 mm (length)  $\times$  100  $\mu\text{m}$  (width)  $\times$  100  $\mu\text{m}$  (depth), a trap/enrichment column of 16 mm (length)  $\times$  300  $\mu\text{m}$  (width)  $\times$  100  $\mu\text{m}$  (depth), and a SPE column of 44 mm (length)  $\times$  300  $\mu\text{m}$  (width)  $\times$  100  $\mu\text{m}$  (depth). In addition, a herringbone mixer was created between the SPE column and trap column. In order to fit a sufficient amount of herringbone grooves within the chip geometry, a convoluted path (switchbacks rather than a straight line) was used to fit them within a small footprint. The herringbone grooves were staggered periodically with the groove height of 70  $\mu\text{m}$  and channel height of 100  $\mu\text{m}$ . The microfabricated emitter had nozzles with a cross-section of 25  $\mu\text{m}$   $\times$  25  $\mu\text{m}$  and a protruding length of 250  $\mu\text{m}$ . A makeup-flow channel between the exit of the LC column and the emitter was employed to improve spray and signal stability.<sup>17</sup> Through-holes (labeled 1–6) were produced as the fluid inlet or outlet.

We have built a robust manifold to mechanically assemble the microfluidic chip with external flow sources (Figure 1b).

Built-in frits (micropillar array) were designed for packing beads inside columns. The SPE, trap, and LC columns were packed with ZORBAX SB-C18 beads (5  $\mu\text{m}$ , pore size of 80 Å, Agilent) by an in-house column packing station. The backend of packed channels was sealed by fabricating sol–gel frits to prevent beads from retreating during experiments. Packed columns and sol–gel frits were examined using an Olympus IX83 microscope by infrared microscopy.

**On-Chip and Online SPE-nanoLC-MS Analysis.** The overall workflow and system configuration for fluidic control for performing the on-chip and online SPE-LC-MS/MS analysis on the chip are shown in Figure 1c, Supporting Information Figure S1, and Table S1. The SPE run started with conditioning a C18-packed SPE column by flowing 20  $\mu\text{L}$  of LC solvent B (95%/5% methanol/ $\text{H}_2\text{O}$  with 2 mM ammonium acetate) at 10  $\mu\text{L}/\text{min}$  through the inlet port P1. The column then was equilibrated with 20  $\mu\text{L}$  of LC solvent A (5%/95% methanol/ $\text{H}_2\text{O}$  with 2 mM ammonium acetate) at 10  $\mu\text{L}/\text{min}$ . Four  $\mu\text{L}$  of the sample (containing 1  $\mu\text{L}$  of plasma) was injected into a 20  $\mu\text{L}$  sample loop and loaded onto the SPE column, followed by washing with 20  $\mu\text{L}$  of LC solvent A at 10  $\mu\text{L}/\text{min}$ . Next, the analytes were eluted from the SPE column with LC solvent B at 1  $\mu\text{L}/\text{min}$  and were then transferred to the trap column by mixing it with another stream of LC solvent A through the port P3 (20  $\mu\text{L}/\text{min}$ ). The passive microfluidic mixer (i.e., herringbone structures) enables efficient mixing of two streams and thus prevents heterogeneous streaming of organic solvent from the SPE eluent, allowing the analytes to be enriched on the trap column efficiently. Finally the HPLC gradient elution at flow rate of 600 nL/min was run to separate the analytes that were analyzed by nanoESI-MS. The LC solvent A consisted of 5%/95% methanol/ $\text{H}_2\text{O}$  with 2 mM ammonium acetate, and solvent B was 95%/5% methanol/ $\text{H}_2\text{O}$  with 2 mM ammonium acetate. The LC gradient started at 10% B, was linearly increased to 55% B in 3 min, and then ramped to 75% in 10 min. After that, it was increased to 95% B in 5 min and held at 95% B for 5 min. Finally it returned to the initial condition (10% B) in 2 min and was held for 5 min. The total time for each SPE-LC-MS run was about 1 h. Negative-ion-mode MS detection was performed on a hybrid quadrupole/orthogonal Q-TOF API US mass spectrometer (Waters Corp.) with similar MS and MS/MS settings as we described previously.<sup>11,12</sup> The capillary voltage was set to be  $-2.4$  kV, cone voltage was 25 V, and collision energy was fixed at 10 eV. The nanoelectrospray process on emitters was visualized and monitored using a Waters nanoflow camera kit equipped with a MLH-10 Zoom lenses (Computar).

## ■ DATA ANALYSIS

**(1). MS Data Processing.** The raw LC-MS data were processed using MassLynx 4.0 software package provided with the Q-TOF API US instrument (Waters Corp.). Extracted ion chromatograms (EIC) for all target analytes were generated using their corresponding precursor/product ions with a mass window of 0.10 Da. The peak area ratio (defined as response RF) of each analyte relative to its corresponding isotope-labeled internal standard was used for quantification. For perfluorocarboxylate compounds, their product ions were used for quantitation, because their product ions are more abundant (3–5 times) than precursor ions under our MS settings. Interestingly, the abundance ratio between the product ion and precursor ion for each perfluorocarboxylate compound was found to be consistent over the wide concentration range (1–

100 ng/mL) (data not shown). For the four perfluorosulfonates and one perfluorosulfonamide, their precursor ions were used for quantitation. We found that solvent composition had a much larger impact on ionization efficiency of our target PFC compounds than did their molecular structures. Thus, we chose the internal standard for each compound based on retention time rather than their type.  $^{13}\text{C}_8$ –PFOA was used for the five early eluting compounds (PFHpA, PFHxS,  $^{18}\text{O}_2$ –PFHxS, PFOA, and  $^{13}\text{C}_4$ –PFOA), whereas  $^{13}\text{C}_8$ –PFOS for the other compounds that eluted late in the gradient.

### (2). Calibration Curves, LOD, Accuracy, and Precision.

The calibration curves were obtained by calculating the responses for eight concentrations of PFC standards spiked in 1  $\mu\text{L}$  of pooled human plasma (0, 0.05, 0.2, 0.5, 2, 10, 30, 100 ng/mL). The limit of detection (LOD) was determined with the lowest concentration (0.05–0.2 ng/mL) in the calibration curve and defined as the signal-to-noise (S/N) ratio was equal to 3. S/N ratios were calculated by Masslynx software as their peak-to-peak values. The method accuracy was obtained by analyzing 1  $\mu\text{L}$  of pooled human plasma spiked with PFCs at three different concentrations (1, 5, 25 ng/mL). The PFC concentration was determined according to the calibration equation. Then the method accuracy and precision were expressed as the average value and the relative standard deviations of the ratio of the calculated concentration relative to the expected concentration for each spiking concentration. To assess the reproducibility of the method, we measured both the interday and intraday variation by analyzing pooled plasma with spiking concentration of 5 ng/mL. To obtain statistical values, at least three replicates were measured. All error bars shown are standard deviations.

**(3). Sample Recovery.** Because the overall sample preparation included both protein precipitation and SPE extraction steps, we determined the overall sample recovery of the PFCs from plasma by two separate experiments. In the first experiment, 5  $\mu\text{L}$  of plasma spiked with 125 pg each of PFCs but without the internal standards was prepared. An aliquot ( $1/5$ ) of the above sample containing 1  $\mu\text{L}$  plasma and 25 pg each of PFCs was injected and went through the SPE procedure until the analytes were enriched on the trap column. Then 25 pg each of the internal standards was injected into the trap column (using an autosampler). Although injected separately, the PFCs and the internal standards were enriched on the same trap column. Subsequently, a HPLC gradient flow was run and LC-MS/MS data were acquired. A response ( $\text{RF}_a$ ) was obtained from this experiment for each PFCs. In the second experiment, 5  $\mu\text{L}$  of plasma without spiked PFCs nor spiked internal standards was prepared. An aliquot ( $1/5$ ) of the above sample containing 1  $\mu\text{L}$  plasma only was loaded to SPE column and went through the SPE procedure until the analytes were enriched on the trap column. Then 25 pg each of internal standards plus PFCs was injected into the trap column (using an autosampler) and analyzed by LC-MS. Another response ( $\text{RF}_b$ ) was obtained from this experiment. The main difference between the two experiments was that the PFCs went through the protein precipitation and SPE procedure in the first one, but did not in the second one. Finally the overall sample recovery was calculated by the ratio of  $\text{RF}_a$ :  $\text{RF}_b$ , because the internal standards used for both experiments underwent the same procedures and the matrix effects were equivalent.



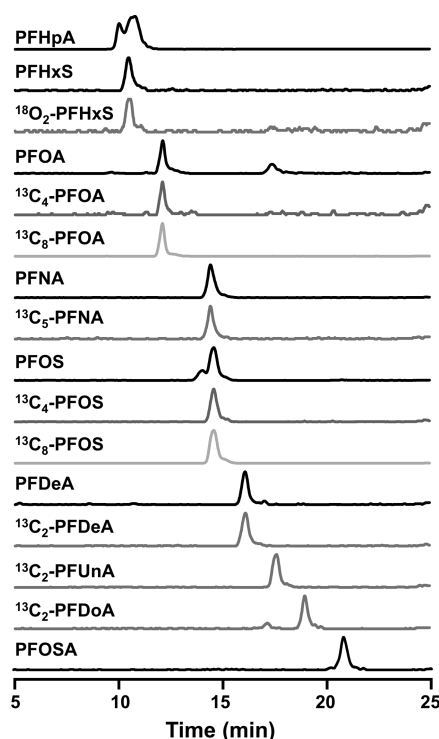
## ■ RESULTS AND DISCUSSION

In order to enable monitoring of PFC levels in small volumes of biological material, and for other future applications, we developed a silicon microfluidic chip for SPE-nanoflow LC-ESI-MS. As shown in Figure 1a (i), our one-plex SPE-nanoLC-MS chip consists of five key functional modules including a SPE column (blue), a herringbone mixer (brown), a trap column (green), a LC column (red), and an emitter interfaced to a mass spectrometer for nanoelectrospray mass spectrometry. The connection between the SPE column and trap column included a herringbone mixer (Figure 1a (ii)) for efficient solvent mixing to allow for the titration of organic content to avoid solvent incompatibility between different columns.<sup>18</sup> A makeup-flow channel between the exit of LC column and the emitter was employed to improve spray and signal stability.<sup>17</sup> Our chip was assembled with a custom-made manifold with screws and alignment pins (Figure 1b) to provide high-pressure leak-free fluidic connections. In contrast to the conventional SPE-LC-MS systems that connect SPE and LC columns directly, our chip platform included an additional trap column in-between for preconcentration of target analytes after they were released from the SPE column and before the nanoLC-ESI analysis. We achieved this by incorporating a on-chip mixer between the SPE and trap columns. This strategy allowed us to employ a large-volume, high-capacity SPE column for maximum capture of target analytes from the complex sample matrix, without the constraint of the potential volume mismatch between the SPE and nanoLC columns.

We implemented the system configuration and fluidic control for the SPE-nanoLC-MS chip (Supporting Information Figure S1). The fluidic system includes an autosampler, three pneumatic pumps (a single HPLC pump (1) and one binary HPLC pump (2 and 3)), three 2-position switching valves (VA-VC), and six inlet/outlet ports (P1–P6) on the chip. The overall workflow included SPE extraction, mixing, trapping, and LC-MS (Figure 1c). This was achieved by six major steps, each with desired fluidic and valve controls at the inlet/outlet holes (Supporting Information Table S1). In brief, the SPE column was washed with LC solvent B and equilibrated with LC solvent A. Then deproteinated plasma was loaded onto the SPE column from P1 to P2. After washing with the LC solvent A, target analytes were eluted from the SPE column by LC solvent B, moved toward the mixers and trap column, and exited to P5 (with P2 and P4 plugged). During the SPE elution process, another solvent stream containing LC solvent A was introduced simultaneously into P3 to titrate the eluent. This reduced the organic contents in the SPE eluent so that target analytes were efficiently captured in the following trap column. After enrichment in the trap column, LC gradients were introduced to P4 with all the other ports closed to perform nanoflow-LC, nanoelectrospray ESI-MS. The full procedure from sample loading to completion of MS acquisition took approximately 1 h with SPE and nanoLC-MS each taking ~30 min. The key to the success of our method is to choose the titrating stream and optimize the mixing ratio between the SPE elution buffer and titration stream, in order to efficiently transfer the analytes eluted from the SPE column and subsequently enrich them in the trap column. We experimented with different mixing ratios to achieve the optimal results on the current chip. We will investigate the effects of herringbone geometries on mixing in the future to reduce the assay run time.

We demonstrated the utilities of our SPE-nanoLC-MS chip for biomonitoring of PFCs using small volumes (~1  $\mu\text{L}$ ) of plasma samples. PFCs have been a major environment concern due to their toxicity and long half-life for bioconcentration.<sup>19,20</sup> Despite restriction of their use in recent years, they are persistent in the environment and human body due to their chemical stability as well as due to retention of their eventual breakdown products. Therefore, it is important to develop an assay that could perform longitudinal biomonitoring of PFCs using small volumes of blood, preferably using a finger prick. A method combining online SPE and normal flow LC-MS has been developed to measure 18 PFCs in human serum.<sup>21,22</sup> However, it was based on conventional HPLC systems and used much larger sample volumes ( $\geq 100 \mu\text{L}$  serum). Here we demonstrate that our new SPE-LC-MS chip can simultaneously analyze different classes of PFCs using 1  $\mu\text{L}$  of plasma through online SPE and nanoflow LC-ESI-MS.

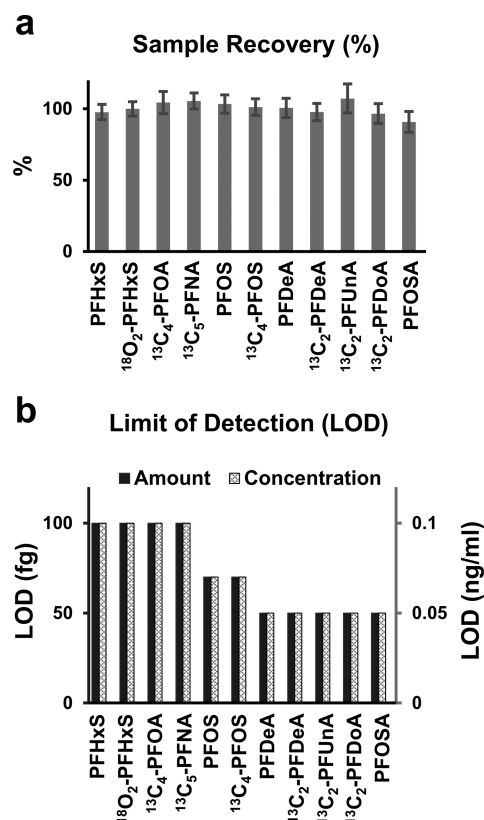
We first confirmed the performance of SPE, LC-separation, and nanoESI-MS analysis of pooled human plasma spiked with 16 standard PFCs at concentrations ranging from ng/ $\mu\text{L}$  to pg/ $\mu\text{L}$  (Supporting Information Table S2). We performed protein precipitation before the on-chip SPE to minimize the nonspecific binding of the mostly hydrophobic PFCs to plasma proteins, thereby maximizing their extraction efficiency. For example, the number of PFOA binding sites to human serum albumin (HSA) was found to be over 6 and more than 90% of PFOA would be bound to HSA in human blood.<sup>23</sup> Therefore, we used acetonitrile/formic acid to effectively suppress the interactions of PFCs with plasma proteins and make them free from protein binding for downstream SPE extraction. We also found that hydrophobic PFCs prepared in low organic solvents tended to stick on tubing and get lost during transfer in the low flow conditions. On the other hand, high organic solvents could reduce the capture of PFCs due to the low capacity of micro-sized SPE columns. To reduce sample loss, PFCs were prepared in 50% methanol during the sample injection for maximum extraction and recovery. Figure 2 shows typical EICs for PFCs spiked in 1  $\mu\text{L}$  human plasma, including 1 pg of each PFC compound and 25 pg of each internal standard. The peaks for PFCs were identified and assigned according to the pair of their precursor and product ions ( $m/z$ ) for each PFC compound and the corresponding retention time. The high mass accuracy of our QTOF mass spectrometer simplified the compound identification by setting a narrow mass window (0.1 Da) for EIC, in contrast to typical multiple reaction monitoring (MRM) analyses using wider mass windows. We observed a shoulder on the PFOS peak which was consistent with its two chromatographic isoforms (branched vs linear),<sup>24</sup> further confirming the high separation efficiency of our on-chip LC column. We generated calibration curves for PFCs (Supporting Information Figure S2). We calculated response (peak area ratios) for eight concentrations of analyte standards (0.05–100 ng/mL) spiked in pooled human plasma relative to their assigned isotope-labeled internal standard. The calibration curves showed excellent linearity with all correlation coefficients  $R^2 > 0.99$ , except PFHpA, PFOA, and PFNA which had strong background signals in our system. We had attempted to pinpoint the sources of background PFCs by replacement of all Teflon-related tubing, fitting, and solvent filters, and carefully rinsed all tubes and bottles. These efforts did significantly reduce contaminants but could not completely eliminate them. To reduce the influence of background contamination, we purposely added and quantified their isotope



**Figure 2.** Separation of PFCs on the SPE-nanoLC-MS chip. Representative extracted ion chromatograms are shown for online SPE-nanoLC-MS analysis of 1  $\mu$ L pooled human plasma spiked with PFCs of 1 pg each.

counterparts ( $^{13}\text{C}_4$ -PFOA and  $^{13}\text{C}_5$ -PFNA) spiked in plasma in this study. We determined the sample recovery for the whole process including both the protein precipitation and SPE extraction steps (Figure 3a). We achieved high recovery ( $\geq 90.8\%$ ) for all analytes. Further optimization of SPE column and mixers as well as solvent conditions will likely result in further improvement of the sample recovery.

We next determined the accuracy, precision, reproducibility, and limit of detection (LOD) of SPE-LC-MS analysis of PFCs in human plasma using our chip (Figure 3b and Table 1). We obtained the method accuracy and precision by replicate analyses ( $n \geq 3$ ) of 1  $\mu$ L plasma spiked at three different concentrations (1, 5, and 25 ng/mL each). For the case of 5 ng/mL, we performed interday and intraday comparison. We expressed the accuracy as the average percentage of the expected amount for each concentration. The method precision was determined by calculating the relative standard deviations (RSD). Good accuracy ( $\geq 88.22\%$ ) and precision (RSD = 1.47%–16.77%) were achieved. Slightly larger fluctuation at the low concentration (1 ng/mL) was presumably due to the sample loss during transfer from the HPLC system to the chip. The reproducibility of our method was confirmed by the intraday and interday accuracy and precision at 5 ng/mL. We determined LOD using the lowest concentration (0.05–0.2 ng/mL) in the calibration curve, with the signal-to-noise (S/N) ratio equal to 3.0. We obtained the LODs for PFCs at 0.05–0.2 ng/mL (corresponded to 50–200 fg in absolute amounts) (Figure 3b), which is significantly better than those reported in the previous study using much larger sample volumes ( $\geq 100$   $\mu$ L serum) in terms of the absolute amount of PFCs ( $\sim 10$  pg).<sup>21</sup>



**Figure 3.** Validation of PFC analysis on the SPE-nanoLC-MS chip. (a) Sample recovery rate was obtained for 1  $\mu$ L of pooled human plasma spiked with PFCs of 25 pg each. Error bars, s.d. ( $n \geq 3$ ). (b) Limit of detection (LOD) of PFCs was measured as the smallest total amount (fg) and the lowest concentration (ng/mL) achieving a S/N of 3.

**Table 1.** Accuracy and Precision at the Different Concentrations of PFCs

analytes	accuracy $\pm$ precision (%)			
	5 ng/mL		1 ng/mL	25 ng/mL
	interday	intraday		
PFHxS	88.7 $\pm$ 3.8	92.2 $\pm$ 6.8	112 $\pm$ 15	102 $\pm$ 12
$^{18}\text{O}_2$ -PFHxS	91.9 $\pm$ 4.4	95.2 $\pm$ 6.9	99.9 $\pm$ 10	95.2 $\pm$ 12
$^{13}\text{C}_4$ -PFOA	97.5 $\pm$ 6.0	98.0 $\pm$ 5.6	97.2 $\pm$ 12	95.2 $\pm$ 3.6
$^{13}\text{C}_5$ -PFNA	96.5 $\pm$ 10	99.7 $\pm$ 10	97.0 $\pm$ 6.2	103 $\pm$ 6.7
PFOS	107 $\pm$ 2.5	106 $\pm$ 7.8	107 $\pm$ 11	97.5 $\pm$ 4.9
$^{13}\text{C}_4$ -PFOS	96.6 $\pm$ 1.5	100 $\pm$ 6.8	95.9 $\pm$ 5.7	103 $\pm$ 4.6
PFDeA	93.7 $\pm$ 8.7	95.8 $\pm$ 9.6	106 $\pm$ 5.1	107 $\pm$ 8.9
$^{13}\text{C}_2$ -PFDeA	90.8 $\pm$ 8.4	94.9 $\pm$ 9.7	101 $\pm$ 4.7	104 $\pm$ 7.3
$^{13}\text{C}_2$ -PFUnA	102 $\pm$ 8.6	96.6 $\pm$ 8.5	101 $\pm$ 2.6	97.1 $\pm$ 8.4
$^{13}\text{C}_2$ -PFDoA	93.0 $\pm$ 4.3	96.1 $\pm$ 3.7	104 $\pm$ 12	97.0 $\pm$ 4.8
PFOSA	88.2 $\pm$ 14.9	91.8 $\pm$ 13	103 $\pm$ 17	119 $\pm$ 14

We last validated the performance of our microfluidic chip and its associated SPE-LC-MS assay using pooled human plasma and NIST SRM 1950 samples (Table 2). SRM 1950 is the first reference material developed specifically for metabolomics research with concentrations of approximately 100 analytes determined, including several PFCs.<sup>25,26</sup> The measured values of PFHxS, PFOS, and PFDeA by our assays were strikingly consistent with the reference values provided for NIST SRM 1950. We also measured the endogenous concentrations of these four PFCs in the pooled plasma used

Table 2. Concentration of PFCs in Human Blood Samples

analytes	NIST SRM 1950		human blood samples	
	measured value (ng/mL)	reference value (ng/mL)	pooled plasma	individual serum
			measured value (ng/mL)	National Exposure Report by CDC (ng/mL) <sup>a</sup>
PFHxS	3.53 ± 0.38	3.25 ± 0.080	1.18	1.61 (1.45–1.79); 1.28 (1.14–1.43)
PFOS	11.1 ± 0.36	10.6 ± 0.13	4.76	9.72 (8.45–11.2); 6.71 (6.24–7.20)
PFDeA	0.364 ± 0.033	0.322 ± 0.007	0.279	0.289 (0.265–0.314); 0.209 (0.189–0.230)
PFOSA	0.172 ± 0.078	N/A	0.094	<0.1; <0.1

<sup>a</sup>Geometric mean (95% confidence interval) of serum concentration for the U.S. population of 20 years and older (survey year 2009–2010; survey year 2011–2012), adapted from the Fourth National Report on Human Exposure to Environmental Chemicals, Updated Tables, February 2015, by Centers for Diseases Control and Prevention (CDC).

for our assay development. Interestingly, the values we obtained were consistently lower than the corresponding values from the geometric mean of serum concentration for the U.S. population of 20 years and older (survey year 2009–2010 and 2011–2012, respectively) (adapted from *The Fourth National Report on Human Exposure to Environmental Chemicals, Updated Tables, February 2015*, by Centers for Diseases Control and Prevention (CDC)). Although we cannot exclude the possibility that the decrease was due to the difference between plasma and serum samples used, or the difference between the assay methods employed, our results might suggest that the slight decrease of PFC concentrations in blood among the general population was the result of the decreased industrial usage of PFCs in recent years, consistent with the lower values obtained by CDC in survey year 2011–2012 than in 2009–2010, as well as the progressive decreases from 1999 to 2012. A future epidemiologic study using a large cohort of retrospective or prospective plasma or serum samples is needed to confirm these interesting findings.

In summary, we have developed a silicon-based, scalable, and integrated microfluidic chip that has the potential to become an enabling platform for small-volume online SPE-nanoLC-MS, which in turn will enable longitudinal biomonitoring of PFCs and other environmental toxins using small volumes of biofluids, for example, a fraction of a drop of blood, as could be obtained by finger pricks from human subjects. Our three-column chip platform includes a SPE column, a guard (trap) column, and a LC column on a single silicon microfluidic chip, and thus takes advantage of the maximum flexibility in coupling SPE with nanoLC-MS while maintaining the high efficiency in both SPE and LC separation. We used C18 beads for all of the three columns in this study in order to achieve maximum extraction, separation, and detection of PFCs because of their high hydrophobicity. Given the diverse classes of environmental chemicals in blood samples, we can optimize the dimensions of the three columns as well as the characteristics of their packing materials (e.g., surface chemistry and size of the beads) for other target analytes as desired in future studies. For example, one could use an ion-exchange SPE column and reverse-phase trap and nanoLC columns on the same chip. We can implement an optimal on-chip mixer between the SPE and trap columns which is capable of titrating the solvent compositions and tackling solvent incompatibility between different chromatographic conditions. Our chip is robust and has gone through over 50 SPE-nanoLC-MS runs without clogging of columns or nozzles. Future work will systematically evaluate the lifetime of SPE columns, LC columns, and the system as a whole, under different SPE and LC conditions for diverse analytes from various biological samples including plasma, serum, and whole blood samples. In

this work, plasma sample was pretreated with protein precipitation in order to maximize the extraction of PFCs. By employing appropriate packing media in the SPE column, we may directly process plasma or serum sample on chip without any additional offline sample preparation for other analytes. For example, restricted access media utilizing a combination of size exclusion and conventional phase partitioning can separate proteins from small molecules that are retained in the inner layer. We could not quantify unlabeled PFHpA, PFOA, and PFNA in human samples because of their high backgrounds in our aged HPLC system. Future work will pinpoint the contamination sources, including usage of highest-purity solvents and reagents, replacement of consumable parts (tubing, frits, etc.), or addition of trap/guard columns in the solvent line right after the HPLC pumps to capture or delay the elution of contaminated compounds to reduce their interference. Finally, our current SPE-nanoLC-MS chip is a single-plex device. We can multiplex both the SPE and LC columns, for example, to a 24-plex format as demonstrated on our 24-plex MEA chip,<sup>11</sup> for high-throughput and multiomics including metabolomics, bottom-up proteomics, and top-down proteomics analysis of biofluids.<sup>12,27</sup> We expect that our microfluidic chip will find wide applications in environmental biomonitoring and biomarker discovery.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

Additional information available. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b01442.

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### Notes

The authors declare the following competing financial interest(s): Daojing Wang is the founder and Pan Mao is the co-founder of Newomics Inc., which is commercializing some of the technologies described in this work.

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