A rapid and fully automatic method for the accurate determination of a wide carbon-chain range of per- and polyfluoroalkyl substances (C4–C18) in human serum

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ABSTRACT

A rapid and fully automatic method for determining 21 per- and polyfluoroalkyl substances (with carbon chains ranging from C4 to C18, including 13 PFCAs, 5 PFASs, 2 Cl-PFESAs, and PFOSA) in human serum samples was developed. The HPLC parameters, Turboflow column, mobile phase, sample injection volume, loading flow rate, and sample cleanup and elution time were optimized. 25 μ L serum sample was directly injected into the developed on-line Turboflow SPE HPLC–MS/MS system for analysis after dilution. Matrix effects were corrected due to the matrix removal efficiency of the Turboflow column and sufficient types of internal isotope standards that were used. The established method showed a good linearity ($r^2 > 0.99$), rapid processing time (20 min per sample), satisfactory recoveries (matrix spiked recoveries range from 84.6% to 114%) and precision (intra-day and inter-day RSDs ranged from 1.5% to 9.2% and from 1.1% to 7.0%, respectively). The limits of detection (LODs) of the 21 analyzed PFASs were between 0.008 and 0.19 ng mL^{-1} . The LODs of short- and long-chain PFASs, such as PFBA, PFPeA, PFHxDA, and PFODA, were 0.008, 0.022, 0.15 and 0.19 ng mL^{-1} , respectively; the spiked recoveries of these PFASs were 101, 105, 87.1, and 85.8%, respectively. Both the LODs and recoveries were better than previous studies. Further, serum PFASs concentrations detected by the presented on-line SPE method were consistent with the traditional off-line SPE method (r : 0.98–0.99), which verified the accuracy and applicability of the present method. The method shows good practical prospects in the analysis of trace per- and polyfluoroalkyl substances in human serum.

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1. Introduction

Per- and polyfluoroalkyl substances (PFASs) are compounds with unique physicochemical properties. These compounds have been produced and used for many different industrial and commercial applications, such as textiles, paper packaging, carpets, pesticides, surfactants, polymer additives and fire-fighting foams, since the 1950s [1–3]. PFASs have been detected in human serum [4–6], milk [7], and urine [8]. Their potential risks, such as reproductive and developmental toxicity to humans, have caused extensive public concern [9,10]. Although most leading global fluorochemical manufacturing companies have agreed to phase out perfluorooctanoate (PFOA), perfluorooctane sulfonate (PFOS) and related chemicals and the concentrations of PFOS in humans are apparently

decreasing over time [6,11], numerous nonregulated alternative PFASs are being produced in increasing quantities [12,13].

For example, short-chain PFASs, such as perfluorobutanoic acid (PFBA) (C4), perfluorobutanesulfonate (PFBS) (C4), have been used as alternatives for PFOA and PFOS by several manufacturers [12]. Pérez et al. found a high concentration of short-chain PFASs in human lungs, which indicated that more attention should be paid to these compounds [17]. Additionally, polyfluoroalkyl substances, such as 6:2 chlorinated polyfluorinated ether sulfonate (6:2 Cl-PFESA, with the trade name F-53B), have been applied as a mist suppressant in hard chrome plating for more than 30 years in China [14,15]. F-53B may have bioaccumulation potential and toxic effects because of its structural similarities with PFOS [2,14,16]; however, there was only one study regarding the concentrations of F-53B in human serum to date [16] and more data were needed.

Several studies found the increasing temporal trends of long-chain PFASs in Chinese sturgeon and human blood [18,19]. Moreover, the greater bioaccumulative potential and toxicities of

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long-chain PFASs were also reported [20,21]. The carbon chain length of long-chain PFASs in these studies were generally less than 12, more data for PFASs with carbon chain length longer than 12 (such as perfluorotetradecanoic acid (PFTeA) (C14), perfluorohexadecanoic acid (PFHxDA) (C16) and perfluorooctadecanoic acid (PFODA) (C18)) was needed to further clarify their toxic potencies [22].

High performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS) is the most common method for analyzing these compounds [4,5,16,23–26], which has good sensitivity and low detection limits for PFASs. Liquid-liquid extraction (LLE) was performed with an ion-pairing agent, such as tetra-*n*-butylammonium hydrogen sulfate (TBA), and then extracted using methyl *tert*-butyl ether (MTBE), followed by solid phase extraction (SPE); the extracted liquid is generally used as a pre-treatment to extract and pre-concentrate PFASs from serum samples [4–6,16,23] and was regarded as an off-line SPE method for the determination of PFASs in human serum. However, the off-line SPE method was time-consuming and solvent-intensive and may introduce errors during the manual pre-treatment process. On-line SPE methods could overcome these shortcomings mentioned above, till now, several on-line SPE coupled with LC–MS methods have been applied to the analysis of PFAS in serum. At present, the reported on-line SPE methods require the precipitation of the protein in serum samples before injecting the sample into the analytical instruments [24,25,27], and no fully automatic on-line method for the analysis of PFASs in serum was available. Moreover, previous studies usually focused on PFASs of a moderate carbon chain length (C6–C10). The polarity and hydrophilicity of the PFASs varied with the carbon chain length and the different functional groups; therefore, simultaneously detecting a wide range of PFASs (C4–C18) was a challenge, especially for shorter (PFBA, perfluoropentanoic acid (PFPeA), and PFBS) and longer chain PFASs (PFHxDA and PFODA). PFBA, PFPeA and PFBS usually showed poor chromatographic peak intensities or unsatisfactory recoveries during the analytical process; PFHxDA and PFODA usually had low spiked recoveries (less than 50%), which made them difficult to determine [1,23,28].

Investigations of PFAS contamination involving humans usually involve a large number of samples, and therefore, a rapid, accurate and fully automatic method, which has the capacity for direct injection, is urgently needed. Ideally, this method could simultaneously detect the wide range of PFASs mentioned above. Turboflow column is a novel type of column for on-line extraction and pre-concentration. The turbulent fluid in the column can provide an efficient separation of large matrix components from smaller molecules and has been applied to the analysis of PFASs in urine and water samples [26,29]. However, it has not yet been used for the analysis of PFASs in human serum. The matrix of human serum is far more complex than that of water and urine. Serum samples contain compounds with a wide range of molecular weights, such as proteins, peptides, fats, carbohydrates, hormones and inorganics [30]. If the complex matrix in serum can be removed by optimizing the on-line Turboflow SPE parameters, the Turboflow SPE technology could be used to establish a rapid and fully automatic on-line method.

The aim of the present study is to develop a rapid, accurate, and fully automatic method without any sample pre-treatment for analyzing a wide range of PFASs in human serum. The Turboflow SPE column, sample volume, loading flow rate, sample cleanup and elution time were carefully optimized in this study. In addition to optimizing these on-line Turboflow SPE HPLC parameters, we employed as many internal isotope standards as possible to correct the matrix effects; a total of 13 types of isotope-labeled PFASs were used in the present study, including a new commercial internal standard, [$^{13}\text{C}_2$]-perfluorobutane sulfonate (MPFBS). Finally,

the established method was then applied to human serum samples ($n = 35$) and verified using the traditional off-line SPE method.

2. Experimental section

2.1. Chemicals and materials

PFAC-MXB (98% purity in methanol) containing PFBA, PFPeA, perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUDA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTeA), PFHxDA, PFODA, PFBS, perfluorohexanesulfonate (PFHxS), PFOS, and perfluorodecanesulfonate (PFDS); perfluorohexanesulfonate (PFHpS); perfluorooctane sulphonic acid (PFOSA); MPFAC-MXA (>98%) containing [$^{13}\text{C}_4$]-perfluorobutanoic acid (MPFBA ($^{13}\text{C}_4$)), [$^{18}\text{O}_2$]-perfluorohexanesulfonate (MPFHxS ($^{18}\text{O}_2$)), [$^{13}\text{C}_2$]-perfluorohexanoic acid (MPFHxA ($^{13}\text{C}_2$)), [$^{13}\text{C}_4$]-perfluorooctanesulfonate (MPFOS ($^{13}\text{C}_4$)), [$^{13}\text{C}_4$]-perfluorooctanoic acid (MPFOA ($^{13}\text{C}_4$)), [$^{13}\text{C}_5$]-perfluorononanoic acid (MPFNA ($^{13}\text{C}_5$)), [$^{13}\text{C}_2$]-perfluorododecanoic acid (MPFDoA ($^{13}\text{C}_2$)), [$^{13}\text{C}_2$]-perfluorodecanoic acid (MPFDA ($^{13}\text{C}_2$)), and [$^{13}\text{C}_2$]-perfluoroundecanoic acid (MPFUDA ($^{13}\text{C}_2$)); [$^{13}\text{C}_4$]-perfluoropentanoic acid (MPFPeA); [$^{13}\text{C}_2$]-perfluoroheptanoic acid (MPFHpA); MPFBS; and [$^{13}\text{C}_8$]-perfluorooctanesulfonate (MPFOS ($^{13}\text{C}_8$)) were supplied by Wellington Laboratories, Canada. 6:2 CI-PFESA and 8:2 CI-PFESA were purified from the commercial F-53 B product purchased from Shanghai Synica Co., Ltd. LC–MS-grade solvents, acetonitrile, methanol, ammonium hydroxide (28%) were purchased from Fisher Scientific (Ottawa, ON, Canada). Ammonium acetate (NH_4OAc , >97%), acetic acid (>99.8%, HPLC grade), and formic acid (>98%, HPLC grade) were obtained from Alfa Aesar (Ward Hill, MA, USA). Water (>18.2 MV cm) used in the present study was purified on a Milli-Q Advantage A10 system (Millipore, USA).

2.2. Instrumentation

The UltiMate™ 3000 system (Thermo Scientific, USA) consisted of a dual- gradient rapid separation pump DGLC-3600RS, a TCC-3200 column oven with a two-position, six-port (2P-6P) valve, and a WPS-3000 TLS auto-sampler. The entire analysis process is controlled by a Chromeleon 6.70 chromatography workstation. A TSQ Quantiva triple quadrupole mass spectrometer (Thermo Scientific, USA) was used to measure the target compounds. The data was recorded using the Xcalibur 3.0 software. The schematic of the on-line Turboflow-SPE-HPLC–MS/MS system is shown in Fig. S1 in Supplementary materials.

2.3. On-line SPE procedure, HPLC analysis and MS detection

The on-line SPE procedure included sample pre-concentration, cleanup, elution, SPE column regeneration, and HPLC separation steps. The mobile phase consisted of 0.1% formic acid in water (A), acetonitrile (B), distilled water (C), delivered by left pump; 30 mmol L $^{-1}$ ammonium formate buffer solution in water (A) (pH adjusted to 4) and acetonitrile (B), distilled water (C), delivered by right pump. Initially, the valve of on-line SPE liquid chromatography was switched to the loading position, shown in Fig. S1(a) in Supplementary materials. Due to the high viscosity, serum was diluted with distilled water firstly, then each 25 μL serum dilution was drawn by a syringe from a 250 μL liner pipe and injected into the large-volume loop, which was carried over by the mobile phase from left pump (loading pump) and then delivered to the on-line SPE column (Turboflow Cyclone-P, 1.0 $\times$ 50 mm, Thermo Scientific) at a high mobile-phase flow rate (2 mL min $^{-1}$, 100% A).

Initial mobile phase of loading pump (2 mL min^{-1} , 100% A) was kept for 1.0 min to clean up the matrix components that concentrated on

PFASs concentrations below LOD in unspiked serum were set as LOD/2 when calculated spiked recoveries.

$$\text{Recoveries \%} = \frac{\text{PFASs concentrations detected in spiked serum sample} - \text{PFASs concentrations detected in unspiked serum sample}}{\text{Theoretical PFASs concentration in spiked serum sample}} \times 100\% \quad (1)$$

the SPE column. Noteworthy that to on-line Turboflow SPE column was needed to be regenerated after the elution step, thus loading pump kept at 1 mL min^{-1} 95% acetonitrile for 10 min to remove the residual contamination and ensure method reproducibility. And then the mobile phase of loading pump was returned to its original composition for the next sample.

After sample cleanup, the valve was switched to the injecting position (Fig. S1(b) in Supplementary materials) to elute the analytes from the SPE column to the analytical column and maintained for 5.0 min. The trapped analytes were desorbed from the Turboflow column and eluted on to the analytical column for further separation and detection. Then the valve returned to the loading position (Fig. S1(a) in Supplementary materials), while the eluted analytes were further separated on the analytical column (Acclaim 120 C18, $4.6 \times 150 \text{ mm}$, $5.0 \mu\text{m}$, Thermo Scientific) at a flow rate of 0.8 mL min^{-1} . The analytical column temperature was set at 40°C . The on-line SPE procedure and HPLC gradient elution conditions for the left and right pumps and the timing of the valve switching are listed in Table 1.

The mass spectrometer was operated in the negative ion ESI-MS/MS using the Multiple Reaction Monitoring (MRM) mode. The MS related parameters were set as follows: sheath gas, 40 units; aux gas, 12 units; source voltage, 2500 V; vaporizer temperature, 350°C ; capillary temperature, 400°C ; and scan time, 0.01 s. Other parameters and analyzed compounds are listed in Table 2 in Supplementary materials.

2.4. Method validation, quality control and quality assurance

The data acquisition and analysis for all of the samples, blanks, standards, and QC materials were performed using the analysis software of the Xcalibur 3.0, Foundation 3.0 (Thermo Scientific, USA). The developed method was validated using standard solutions, internal isotope standards and real serum samples to evaluate the linearity, selectivity, accuracy, precision, recovery and matrix effects, limits of detection (LODs) and limits of quantification (LOQs).

2.5. Serum samples collection and preparation

Serum samples ($n=35$) were collected from workers of a fluorochemical manufacturing facility ($n=20$) and ordinary people ($n=15$) in Wuhan, Hubei Province in 2013. After sampling, serum was separated from red blood cells and other components by centrifugation at 3000 rpm for 10 min. Then, the serum samples were transferred as soon as possible to the laboratory in polypropylene containers and stored at -20°C until analysis. Because the human serum was too thick to draw accurately and might clog the syringe, the serum samples were diluted with distilled water before injection. Briefly, $25 \mu\text{L}$ serum sample was diluted by $175 \mu\text{L}$ mixture of distilled water and isotope internal standards solution, and then $25 \mu\text{L}$ serum dilution was directly injected to LC-MS/MS by the auto-sampler. To verify the results of the present study, the traditional off-line SPE method was also used to analyze the serum samples. Detailed information about the off-line SPE method was included in Supplementary materials. In the present study, spiked recoveries of detected PFASs were calculated by Eq. (1), and the

Two serum samples were selected for the calculation of LODs in the present study: one sample did not contain target analytes except for PFBS (0.18 ng mL^{-1}), and another serum sample did not contain PFBS. The LODs of PFASs were obtained by spiking a series of low concentrations standard solutions (ranging from 0.005 ng mL^{-1} to 0.5 ng mL^{-1}) into the serum samples and expressed as a concentration at signal-to-noise ratio equal 3:1 in the present study.

3. Results and discussion

3.1. Optimization of the on-line Turboflow SPE HPLC procedure

To obtain the optimized chromatographic efficiency, the trapped analytes should be eluted and refocused onto the analytical column when the extraction column was switched to the analytical flow path. Several related conditions of on-line Turboflow SPE HPLC procedure, such as the HPLC parameters, selection of the on-line SPE column (Turboflow Cyclone-P column), sample volume ($25 \mu\text{L}$), sample loading flow rate (2 mL min^{-1}), and sample cleanup (1 min) and elution time (5 min), were carefully optimized in the present study.

3.1.1. HPLC parameters

The HPLC parameters were optimized to ensure the identification and quantification of all selected analytes. The Acclaim 120 C18 column ($4.6 \times 150 \text{ mm}$, $5.0 \mu\text{m}$, Thermo Scientific) was selected as the analytical column which has been applied to the separation and detection of PFASs in our previous studies [4,5]. A guard column ($4.6 \times 50 \text{ mm}$, $5.0 \mu\text{m}$, Thermo Scientific) was placed between the analytical pump and injection valve to decrease the interfering background peaks of the PFASs. The mobile phase was then optimized using the above selected columns. Better peak shapes for the analytes, especially PFBA and PFPeA, were obtained using the acetonitrile rather than methanol (Fig. 1(a)). Ammonium formate buffer (pH adjusted to 4) were optimized and applied to detect PFASs in previous studies [4,5,24,34]. Five different concentrations (5, 10, 20, 30, 40 mmol L^{-1}) of ammonium formate buffer were tested on the separation, peak shape, and sensitivity for PFASs determination (pH maintained at 4) in the present study. Increasing the concentration of ammonium formate from 5 to 30 mmol L^{-1} resulted in sharper, more symmetrical peaks, and higher resolution and sensitivity for the PFASs. However, when the concentration of ammonium formate was increased to 40 mmol L^{-1} , the detection sensitivities for most of the PFASs did not improve and some even worse, it might due to high mobile phase buffer concentration exhibited effects on ionization efficiency (or sensitivity), and thus resulted in a severe suppression of the analyte signal intensity. Therefore, 30 mmol L^{-1} ammonium formate was selected for the further study. Different percentages of acetonitrile in initial mobile phase were also optimized. More attention was paid to short-chain PFASs (PFBA, PFPeA) in the present study, they were higher water solubility and weaker sorption potentials than long-chain PFASs [31]. Peak shapes and intensities of short-chain PFASs were improved when the percentage of acetonitrile was increased from 5% to 10% (Fig. 1(b)). The low percentage of acetonitrile (5%) resulted in long retention time and poor peak shape due to its weak elution ability. However, the peak shape and intensity of PFBA became worse when the percentage of acetonitrile increased from

Table 1
On-line SPE procedure, HPLC gradient elution and valve switching program.

Step	Time (min)	left pump				Analyzing pump				Valve position
		Flow rate (mL min ⁻¹)	%A	%B	%C	Flow rate (mL min ⁻¹)	%A	%B	%C	
1	0.0	2	100	0		0.8	90	10		1–2 ^a
2	1.0	2	100	0		0.8	90	10		6–1 ^b
3	6.0	1	0	95	5	0.8	90	10		1–2
4	7.0	1	0	95	5	0.8	55	45		1–2
5	11.0	1	0	95	5	0.8	23	77		1–2
6	14.0	1	0	95	5	0.8	0	98	2	1–2
7	16.0	1	0	95	5	0.8	0	98	2	1–2
8	17.0	2	100	0		0.8	0	98	2	1–2
9	17.2	2	100	0		0.8	90	10		1–2
10	20.0	2	100	0		0.8	90	10		1–2

Mobile phase for loading pump: 0.1% formic acid in water (A), acetonitrile (B), distilled water (C). Mobile phase for analyzing pump: 30 mmol L⁻¹ ammonium formate buffer solution in water (A) (pH adjusted to 4), acetonitrile (B), distilled water (C)

^a 1–2: loading position.

^b 1–6: injecting position.

Table 2
Target analytes measured in the present study with QA/QC information including monitoring parent ion, product ion. Quantifying ions are highlighted in bold.

Compound	Precursor (<i>m/z</i>)	Product (<i>m/z</i>)	Collision Energy (V)	RF Lens (V)	Internal Standard
PFBA	213	169	8	30	¹³ C ₄ -PFBA
PFPeA	263	219	10	30	¹³ C ₄ -PFPeA
PFHxA	313	269	5	30	¹³ C ₂ -PFHxA
PFHpA	363	319	10	47	¹³ C ₂ -PFHpA
PFOA	413	369	8	39	¹³ C ₄ -PFOA
PFNA	463	419	18	44	¹³ C ₅ -PFNA
PFDA	513	469	17	40	¹³ C ₂ -PFDA
PFUnA	563	519	16	52	¹³ C ₂ -PFUnA
PFDoA	613	569	10	53	¹³ C ₂ -PFDoA
PFTTrDA	663	619	11	61	¹³ C ₂ -PFTTrDA
PFTeDA	713	669	13	71	¹³ C ₂ -PFTeDA
PFUnDA	813	769	13	70	¹³ C ₂ -PFUnDA
PFODA	913	869	13	67	¹³ C ₂ -PFODA
PFBS	299	80,99	33	113	¹³ C ₂ -PFBS
PFHxS	399	80,99	39	141	¹⁸ O ₂ -PFHxS
PFOS	499	80,99	48	169	¹³ C ₂ -PFOS
PFOSA	498	78	42	160	¹³ C ₂ -PFOS
6:2Cl-PFAES	531	83	26	112	¹³ C ₂ -PFOS
8:2Cl-PFAES	631	451	28	164	¹³ C ₂ -PFOS
¹³ C ₄ -PFBA	217	172	8	30	
¹³ C ₄ -PFPeA	266	222	5	30	
¹³ C ₂ -PFHxA	315	270	10	30	
¹³ C ₂ -PFHpA	367	322	24	47	
¹³ C ₄ -PFOA	417	372	7	39	
¹³ C ₅ -PFNA	468	219	16	45	
¹³ C ₂ -PFDA	515	470	17	40	
¹³ C ₂ -PFUnA	565	520	10	53	
¹³ C ₂ -PFDoA	615	570	10	53	
¹³ C ₂ -PFBS	302	80,99	31	113	
¹⁸ O ₂ -PFHxS	403	103	36	111	
¹³ C ₂ -PFOS	503	80,99	49	188	
¹³ C ₈ -PFOS	503	80,99	49	188	

10% to 15%. It might cause by the strong elution ability of high percentage acetonitrile, which decreased the resolution of analytical column. Thus, the percentage of acetonitrile at 10% was set as the optimized condition. A relatively satisfactory separation was obtained when the composition of acetonitrile increased from 10% to 45% in 1 min, then increased to 77% in 4 min and finally increased to 98% in 3 min and was maintained for 3 min to allow the separations of all perfluoroalkyl acids and the elution of the longer chain homologues (such as PFHxDA and PFODA). Then, the mobile phase was rapidly returned to 10% A in 0.2 min, followed by a conditioning step of 3.8 min at the starting composition before the next analysis. The final gradient elution and optimized parameters were shown in Table 1.

3.1.2. Selection of turboflow SPE column and sample volume

Turboflow columns provide an efficient separation of small molecules from large matrix components by creating turbulence inside the column, and macro molecules, such as proteins, can be excluded from the turboflow column [32,33]. Two types of commercial Turboflow SPE columns were compared, including silica-based Turboflow C18-P and polymeric-based Turboflow Cyclone-P columns. Both of them can retain polar and nonpolar compounds. The online analysis of a 10 ng mL⁻¹ standard solution was performed on duplicate batches (n = 5). No peaks for PFBA or PFPeA were found using a C18-P column, and the intensities of the long chain PFAS, PFHxDA and PFODA, which were often ignored in previous literature, were strong using the Cyclone-P column, as shown in Fig. 1(c). Therefore, the Cyclone-P column was selected for further study. A large sample volume made the identification of analytes at a low concentration possible; therefore, in the present

Fig. 1. (a) Chromatograms of PFBA and PFPeA (methanol or acetonitrile as the mobile phase); (b) chromatograms of PFBA and PFPeA using different initial ratios of acetonitrile (5%, 10%, 15%); (c) chromatograms of PFBA, PFPeA, PFHxDA and PFODA using the Turboflow Cyclone-P and Turboflow C18-P columns; injected samples were the spiked 5 ng mL⁻¹ standard solutions.

study, the injected sample volume was increased from 10 to 50 μ L. Increasing the injected sample volume resulted in sharper, higher and more symmetrical peaks for the PFASs. However, when the volume was increased to 30 μ L, the pump pressure was obviously higher than the initial value after analysis. A sample volume of 25 μ L was finally selected in the present study.

3.1.3. Loading flow rate, sample cleanup and elution time

The flow rate during sample loading, which could affect the efficiency of the extraction and matrix elimination, was a critical parameter for the on-line Turboflow SPE procedure. Four different loading flow rates (1.0, 2.0, 3.0 and 4 mL min⁻¹) were employed to investigate the extraction efficiency (Fig. S2 in Supplementary materials). The peak intensities were highest while loading flow

Fig. 2. Typical chromatogram of the standard mixture of 21 analytes (5 ng mL⁻¹ each) 1 PFBA, 2 PFPeA, 3 PFHxA, 4 PFBS, 5 PFHpA, 6 PFOA, 7 PFHxS, 8 PFNA, 9 PFHpS, 10 PFDA, 11 PFOS, 12 PFUnDA, 13 F-53B, 14 PFDoA, 15 PFDS, 16 PFTeDA, 17 8:2 Cl-PFESA, 18 PFOSA, 19 PFTeDA, 20 PFHxDA, 21 PFODA.

Table 3

The linearity, LODs, spike recoveries and RSDs for the 21 PFASs in the serum samples (ng mL⁻¹).

Analytes	r^2	Linear range	LOD	LOQ	Spike recovery (%) $\pm$ SD			RSD (% , n = 5)	
					0.5	5	50	Intra-day	Inter-day
PFCAs									
PFBA	0.9976	0.1–100	0.008	0.025	114 $\pm$ 4.3	101 $\pm$ 4.1	96.9 $\pm$ 3.5	4.8	2.6
PFPeA	0.9969	0.1–100	0.022	0.066	103 $\pm$ 4.6	105 $\pm$ 5.3	101 $\pm$ 7.3	2.8	1.4
PFHxA	0.9943	0.1–100	0.032	0.097	110 $\pm$ 5.6	96.6 $\pm$ 3.7	88.3 $\pm$ 5.2	3.8	2.4
PFHpA	0.9934	0.1–100	0.031	0.094	113 $\pm$ 2.7	96.0 $\pm$ 2.6	86.3 $\pm$ 5.5	7.2	6.0
PFOA	0.9906	0.1–100	0.023	0.068	105 $\pm$ 5.7	97.4 $\pm$ 5.4	92.5 $\pm$ 6.0	2.6	3.1
PFNA	0.9961	0.1–100	0.036	0.06	108 $\pm$ 5.2	90.6 $\pm$ 6.1	93.5 $\pm$ 3.6	4.4	4.4
PFDA	0.9919	0.1–100	0.025	0.076	97.6 $\pm$ 6.7	103 $\pm$ 2.7	104 $\pm$ 3.4	6.5	1.1
PFUnDA	0.9985	0.1–100	0.033	0.070	95.4 $\pm$ 7.2	93.5 $\pm$ 5.5	96.7 $\pm$ 5.9	2.2	4.6
PFDoA	0.9935	0.1–100	0.031	0.092	98.7 $\pm$ 7.5	92.1 $\pm$ 8.3	98.6 $\pm$ 7.8	8.9	4.8
PFTeA	0.9977	0.1–100	0.045	0.046	86.7 $\pm$ 9.6	94.7 $\pm$ 6.9	93.4 $\pm$ 8.2	5.5	2.4
PFTeA	0.9916	0.1–100	0.058	0.083	87.4 $\pm$ 5.4	93.5 $\pm$ 9.2	106 $\pm$ 2.8	1.5	4.2
PFHxDA	0.9945	0.1–100	0.15	0.45	89.7 $\pm$ 3.6	87.4 $\pm$ 7.3	87.1 $\pm$ 5.7	4.4	2.4
PFODA	0.9909	0.1–100	0.19	0.57	84.6 $\pm$ 0.9	85.8 $\pm$ 2.6	91.6 $\pm$ 3.5	2.6	1.6
PFASs									
PFBS	0.9986	0.1–100	0.011	0.032	106 $\pm$ 7.2	94.9 $\pm$ 2.7	90.5 $\pm$ 0.9	3.4	7.0
PFHxS	0.9924	0.1–100	0.053	0.16	101 $\pm$ 8.2	103 $\pm$ 7.1	94.7 $\pm$ 7.6	4.8	1.7
PFHpS	0.9934	0.1–100	0.062	0.186	95.2 $\pm$ 1.1	93.4 $\pm$ 6.7	98.1 $\pm$ 3.5	9.2	3.1
PFOS	0.9922	0.1–100	0.056	0.168	93.1 $\pm$ 6.8	102 $\pm$ 2.7	94.7 $\pm$ 5.4	2.7	1.3
PFDS	0.9975	0.1–100	0.053	0.159	89.4 $\pm$ 8.8	87.3 $\pm$ 9.5	93.5 $\pm$ 4.0	3.0	2.4
PFOSA									
PFOSA	0.9941	0.1–100	0.034	0.102	93.1 $\pm$ 7.1	94.2 $\pm$ 3.3	91.5 $\pm$ 4.6	4.3	6.1
CI-PFAESs									
F-53B	0.9967	0.1–100	0.037	0.11	89.3 $\pm$ 9.2	93.5 $\pm$ 5.9	96.2 $\pm$ 6.8	6.3	2.8
8:2 Cl-PFESA	0.9903	0.1–100	0.061	0.093	87.1 $\pm$ 5.4	90.4 $\pm$ 4.1	89.5 $\pm$ 4.3	3.0	4.0

rate was set at 2 mL min⁻¹, and decreased with the increasing to a higher flow rate, thus a flow rate of 2 mL min⁻¹ was chosen.

An appropriate sample cleanup procedure can ensure optimal analytes responses as well as the elimination of the matrix components. 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B) were used to precipitate proteins [27]. In present study, 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B) with different proportions as loading solution were tested, and the peak intensity and shape of short-chain PFASs was not satisfactory, even there was no apparent peak for PFBA. Eventually, 0.1% formic acid as the only loading solution was selected as the optimized condition. And according to our subsequent monitoring data, the column pressure had no obviously change after 50

serum samples analysis, which indicated that protein was precipitated and removed efficiently. Sample cleanup times of 0.5, 1.0, and 2 min were compared (Fig. S3 in Supplementary materials). The peak response of the detected PFASs increased apparently when the cleanup time was 1.0 min; however, the peak response of the detected PFASs decreased sharply when the time was 2 min. Therefore, the cleanup time of 1 min was used.

After cleanup, the valve was switched to elute the analytes from the SPE column to the analytical column for a specified time. The effect of the elution time was determined for a range from 1 to 6 min (Fig. S4 in Supplementary materials). As the elution time increased to 5 min, the peak intensity of the detected PFASs increased significantly, and there was no obvious improvement when the elution

Table 4The linearity, LODs, spike recoveries and RSDs for the 21 PFASs in the serum samples (ng mL⁻¹).

Analytes	present study		our previous off-line method		Glynn 2012 (off-line) [11]		Llorca, 2012 (off-line) [27]		Hanssen 2013 (off-line) [23]		Kuklenyik 2005 (on-line) [25]		Gosetti 2010. (on-line) [35]		Huber 2015 (on-line) [1]	
	LOD	Recovery	LOD	Recovery	LOD	Recovery	LOD	Recovery	LOD	Recovery	LOD	Recovery	LOD	Recovery	LOD	Recovery
PFASs																
PFBA	0.008	101	0.035	87.6			0.35	55							0.128	108
PFPeA	0.022	105	0.031	96.0			0.76	94					0.065	94.6	0.15	115
PFHxA	0.032	96.6	0.036	88.4	0.05	80	0.14	120			0.8	114			0.055	108
PFHpA	0.031	96.0	0.039	91.8	0.05	78	0.11	126			0.4	127	0.013	96	0.108	108
PFOA	0.023	97.4	0.031	97.1	0.035	84	0.11	83	0.05	93	0.2	105	0.01	96.6	0.339	112
PFNA	0.036	90.6	0.042	98.4	0.03	90	0.2	83	0.08	76	0.2	109			0.023	110
PFDA	0.025	103	0.055	92.7	0.07	93	0.087	116			0.2	96	0.012	92.3	0.028	108
PFUdA	0.033	93.5	0.057	87.3	0.05	92	0.5	63	0.05	62	0.2	95	0.038	92.9	0.032	108
PFDoA	0.031	92.1	0.064	80.0	0.1	90	0.22	60			0.2	75			0.031	119
PFTTrA	0.045	94.7	0.19	53.2	0.15	81	0.32	56							0.034	120
PFTeA	0.058	93.5	0.23	66.1	0.25	72	0.22	50							0.064	111
PFHxDA	0.15	87.1	0.27	50.7			0.53	57								
PFODA	0.19	85.8	0.32	51.0			0.42	54								
PFOSAs																
PFBS	0.011	94.9	0.029	88.5	0.013	77	0.064	104			0.1	78	0.029	90.5	0.004	74
PFHxS	0.053	95.6	0.045	96.6	0.007	81	0.031	97	0.03	66	0.1	72	0.026	96	0.015	101
PFHpS	0.062	93.4	0.053	78.3											0.033	102
PFOS	0.056	102	0.044	87.0	0.008	83	0.018	100	0.08	87	0.2	90	0.075	93.5	0.035	103
PFDS	0.053	87.3	0.056	77.6	0.01	76	0.094	119							0.027	104
PFOSA																
PFOSA	0.034	94.2	0.061	68.9	0.04	77	0.026	112	0.1	66	0.05	95	0.075	91.9	0.006	110
CI-PFAES																
F-53B	0.037	93.5	0.057	83.6												
8:2 CI-PFAES	0.061	90.4	0.066	77.8

Fig. 3. Concentrations and profiles of PFASs in human serum samples (ng mL⁻¹).

time was longer than 5 min. Therefore, an elution time of 5 min was used. Fig. 2 showed a typical chromatogram of the standard mixture of 21 analytes (5 ng mL⁻¹, each) under the optimized conditions.

3.2. Validation of the method and performance with serum samples

One prominent advantage of this method was the minimization of manual sample handling, which could eliminate several procedural errors and significantly decrease the analysis time. However,

in contrast with the analysis of the standard solutions in acetonitrile or water, the serum samples always showed matrix effects. In present study, 13 isotope-labeled internal standards have been used to correct matrix effects on ionization efficiency as well as to compensate for variation in injection, signal variation due to ion suppression or signal enhancement [4,5,14,16,23,26].

Performance standard (M₈PFOS) was used to verify the daily dynamic range of the instrument response, namely the ionization efficiency on ESI-ion source. Blank water (N = 3), real serum samples (N = 3) were spiked with standard solutions prior to LC-MS/MS

analysis. Absolute recoveries of different PFASs were shown in Table S2 in Supplementary materials. The absolute recoveries of 21 detected PFASs ranged from 87.3 to 103%, with an average of 95.4%, which indicated the low magnitude for ionization suppression. Matrix effects of most target PFASs analytes were also within the accepted range except for PFHxS, PFHpS and PFDS (<85%) in the spiked human serum samples. However, the internal standard method were adopted for quantitative analysis in present study, the signals of isotope internal standards were suppressed/enhanced as well, thus the matrix effects were well corrected during the analysis process.

The linearity, LODs, spike recoveries and RSDs for the 21 PFASs in the serum samples (shown in Table 3), were calculated on the basis of the dilution serum. The present method displayed good linearity ($r^2 > 0.99$) over the concentration range of 0.1–100 ng mL⁻¹ for the 21 detected PFASs.

The LODs of 13 PFCAs, 5 PFASs, PFOSA, and 2 Cl-PFESAs were 0.008 to 0.19, 0.011 to 0.062, 0.054, and 0.037 to 0.061 ng mL⁻¹, respectively, which are considerably lower than the off-line or other on-line SPE LC–MS methods for PFASs in human serum reported in the literature (Table 4), e.g., Llorca et al. [27] (0.018–0.76 ng mL⁻¹, off-line SPE) and Gosetti et al. [35] (0.012–0.075 ng mL⁻¹, on-line SPE). Short-chain (PFBA and PFPeA) and long-chain PFASs (PFTrA, PFTeA, PFHxDA and PFODA) were not all detected in several previous studies due to poor peak shape or blank contamination, which resulted in unsatisfactory LODs or recoveries. In the present study, the LODs of short-chain PFASs (PFBA and PFPeA) and long-chain PFASs (PFTrA, PFTeA, PFHxDA and PFODA) were 0.008, 0.022, 0.045, 0.058, 0.15 and 0.19 ng mL⁻¹, respectively, which were much lower than those of previous studies [1,11,23,25,27,35], the LODs of PFBA, PFPeA, PFTrA, PFTeA, PFHxDA and PFODA were 0.35, 0.76, 0.32, 0.22, 0.53 and 0.42 ng mL⁻¹, respectively, in Llorca et al.'s study [27], and the LODs of PFBA, PFPeA, PFTrA, PFTeA, PFHxDA and PFODA in our traditional MTBE method were 0.035, 0.031, 0.19, 0.23, 0.27 and 0.32 ng mL⁻¹, respectively. The low LODs of short- and long-chain PFASs in the present study might result from our efforts to improve the peak shape of analytes and blank contamination control during the detection process.

The matrix spiked recoveries were obtained by spiking the serum samples of ordinary people with three levels of PFASs (0.5 ng mL⁻¹, 5 ng mL⁻¹ and 50 ng mL⁻¹), and each level of recovery experiments was repeated for three times. We have incubated the spiked standards with serum samples for one hour, keeping consistent with our previous off-line MTBE method. The spiked recoveries (spiked 5 ng mL⁻¹) of 13 PFCAs, 5 PFASs, PFOSA, and 2 Cl-PFESAs were 83.8–115%, 85.3–104%, 104%, and 87.6–90.7%, respectively, which were better than those of previous studies [1,11,23,25,27,35]. In particular, the recoveries of short-chain PFASs (PFBA and PFPeA) and long-chain PFASs (PFTrA, PFTeA, PFHxDA and PFODA) were 92.0, 88.5, 114, 104, 85.8, and 84.4%, respectively, which were much better than those of previous studies [1,11,23,25,27,35], recoveries of PFBA, PFPeA, PFTrA, PFTeA, PFHxDA and PFODA were 55, 94, 56, 50, 57 and 54%, respectively, in Llorca et al.'s study [27], and recoveries of PFBA, PFPeA, PFTrA, PFTeA, PFHxDA and PFODA in our previous off-line MTBE method were 87.6, 96.0, 66.1, 50.7 and 51.0%, respectively.

The method precision was evaluated by measuring the intra-day and inter-day RSDs of replicate analyses ($n = 5$) of the 5 ng mL⁻¹ standard solution under optimum conditions. The RSDs of intra-day injections (repeatability) and inter-day injections (reproducibility) ranged from 1.5% to 9.2% and from 1.1% to 7.0%, respectively. All of the RSDs were below 10%, which are lower than those of previous studies, including from off-line (RSDs, 5–23%) [11,23,27] and on-line methods (RSDs, 2–17%) [1,25,35]. The satisfactory precision was obtained due to the minimal sample manipulation, fewer

Fig. 4. Box plots of the concentrations for the main PFASs that were detected in serum samples.

sources of error, and lack of sample pretreatment. Additionally, the large particle size of the solid phase effectively eliminated the matrix components.

3.3. Analysis of serum samples

The present method was then applied to a population survey. Human serum samples were collected from workers of a fluorochemical manufacturing plant ($n = 20$) and ordinary adults ($n = 15$) who lived around this plant. The analytical results are listed in Table S3, S4, S5 in Supplementary materials.

The concentrations and profiles of the PFASs that were detected in the human serum samples are shown in Fig. 3. Because PFPeA, PFHxA, PFDA, PFDoDA, PFUdA, PFDoA, PFTrA, PFTeA, PFHxDA, PFODA, PFOSA, PFHpS and PFDS had low detection frequencies in all serum samples, they were not included in the following data analysis and discussion. PFBA, PFOA, PFBS, PFHxS, PFOS and F-53B were the principal PFASs in all of the serum samples, with detection frequencies above 70% and average concentrations of 19.2, 266, 148, 1266, 1417 and 7.6 ng mL⁻¹, respectively. Serum PFBA, PFBS, PFHxS and PFOS were statistically higher in occupational workers than those in ordinary people ($p < 0.01$) (Fig. 4). Particularly, the average concentrations of F-53B in ordinary people and occupational workers were 1.8 and 11.9 ng mL⁻¹, respectively, and the detection frequencies were 80% and 100%, respectively. To our knowledge, there is only one previous report of F-53B in human serum [16]. In this study, the mean concentration of F-53B in workers was lower than in high fish consumers (93.7 ng mL⁻¹) and metal plating workers (51.5 ng mL⁻¹), however, it was higher than that of the background control group (4.78 ng mL⁻¹) [16]. Spearman's rank correlation analysis was used to test the correlation between PFOS and F-53B, and a significant positive correlation between PFOS and F-53B (Spearman's $\rho = 0.68$; $p < 0.01$) was found, which showed that they may have similar sources.

Additionally, we detected the concentration of PFASs using the traditional MTBE off-line SPE method, and the results are listed in Table S6 in Supplementary materials. The concentrations of PFBA, PFBS, PFHxS, PFOS, F-53B and PFOA that were detected using on-line SPE and off-line SPE were in good agreement (Fig. 5), with all of the r values above 0.98 ($p < 0.01$). The results indicated that the on-

Fig. 5. Consistency comparison of the concentrations of the PFASs that were detected by on-line and off-line SPE.

line SPE method established in present study can obtain accurate data and can be applied in large scale surveys.

4. Conclusions

A rapid, accurate, and fully automatic method was established to simultaneously analyze 21 PFASs (with carbon chains that ranged from C4 to C18, including 13 PFCAs, 5 PFSA, 2 Cl-PFESAs, and PFOA) in human serum. The serum samples were directly ana-

lyzed without any pre-treatment, and the LODs, accuracy, and spiked recoveries of the method satisfied the requirements for determining PFASs well. In particular, the method showed lower LODs, higher spiked recoveries for the analysis of short-chain (PFBA, PFPeA) and long-chain (PFHxDA, PFODA) PFASs than both off-line and on-line methods from previous studies. Moreover, the concentrations of the PFASs that were detected by the present on-line SPE method were consistent with the traditional off-line SPE method (r : 0.98–0.99). The procedures of the present method were greatly

simplified and achieved a fully automatic detection of PFASs, with an analysis time of only 20 min per sample. The method has good practical prospects for the large scale investigation of trace PFASs in human serum in a rapid, accurate, and facile manner.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.chroma.2016.09.050>.

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