

An automated high-throughput SPE micro-elution method for perfluoroalkyl substances in human serum

Sandra Huber · Jan Brox

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Abstract An automated high-throughput solid phase extraction (SPE) micro-elution method for 8 perfluorosulfonic acids, 11 perfluorocarboxylic acids and fluorooctane sulfonamide in human serum was developed. Importance was attached to the application of small volumes of reagents and solvents in addition to low sample volumes (50 μ L) in order to save the highly valuable sample material for follow-up and other studies. Instrumental analysis was performed by ultra high pressure liquid chromatography coupled to triple quadrupole mass spectrometry (UHPLC-MS/MS). The recoveries of perfluoroalkyl substances (PFASs) were satisfactory and between 70 and 120 % for most of the compounds at the three investigated spike concentrations. Perfluorobutane sulfonate (PFBS) was slightly underestimated at high concentrations (20 ng/mL; 67 %), whereas perfluoropentanoate (PFPA), perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS) were overestimated with 134, 131 and 133 % respectively, at low spike concentrations (0.2 ng/mL). The precision of the method was below 20 % coefficient of variation (CV%)

for all target compounds with the only exception of PFOS (22 %) at low spike concentrations. Method detection limits ranged from 0.006 to 0.34 ng/mL. High sensitivity, accuracy, repeatability and robustness have been demonstrated for an appropriate concentration range. The applicability for real samples was satisfactory demonstrated by analysis of 40 sera samples from the general population from Tromsø, Norway.

Keywords Automated sample preparation · High-throughput · Human · Micro-elution · PFAS · Serum · Solid-phase extraction

Introduction

Perfluoroalkyl substances (PFASs) are compounds with unique physicochemical properties generated by their fully fluorinated carbon chain of varying length attached to a functional end group. PFASs have been used in many different industrial and commercial applications since the 1950s (e.g. fire fighting foams, impregnation agents and surface coatings for furniture, textiles and paper products).

Among the PFASs, the groups of perfluoroalkyl sulfonic acids (PFASs) and perfluoroalkyl carboxylic acids (PFCAs) have gained most attention in the last decade due to their persistence to environmental and biological degradation with some analogues demonstrating bioaccumulative and toxic properties [1–3] and global distribution [4–7]. This induced the announcement of perfluorooctane sulfonic acid (PFOS) as a candidate persistent organic pollutant (POP) under the Stockholm convention 2009 [8], which followed a restricted use of the compound in Europe from June 2008 [9] and regulated use in the USA [10]. In the USA, the “Perfluorooctane carboxylic acid (PFOA) Stewardship Program” [11] was

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S. Huber (✉) · J. Brox
Department of Laboratory Medicine, Division of Diagnostic Services, University Hospital of Northern Norway, Sykehusveien 38, 9038 Tromsø, Norway
e-mail: sandra.huber@unn.no

S. Huber
Department of Environmental Chemistry, NILU- Norwegian Institute for Air Research, Fram Centre, Hjalmar Johansens gate 14, 9007 Tromsø, Norway

J. Brox
Department of Medical Biology, Faculty of Health Sciences, University of Tromsø, Sykehusveien 44, 9037 Tromsø, Norway

launched, where eight of the major PFOA-producing companies committed to reduce emissions of PFOA and related chemicals by 95 % by 2010. In Norway, the ban of PFOA in Norwegian consumer products by 1st of June 2014 with the exception of food packaging, contact material and medical equipment was recently announced [12].

PFASs are lifestyle-related substances and rather present in industrialized than less-developed areas. But due to long-range transport, ubiquitously distribution in the environment around the globe is reported in previous studies [4, 5, 7, 13–15]. Uptake and accumulation of PFAS in mammals (inclusive humans) and other top predator species were demonstrated [15–18]. Effects of PFAS on the general human population are little studied. However, the following exposure associations were observed: between PFOA and PFOS and lowered female fecundity and altered menstrual cyclicity [19]; in utero exposure to PFOA and birth weight [20] and delay in age of menarche [21]; negative associations between PFOS and testosterone levels in men [22], perfluorononanoate (PFNA) and free T4 in adolescents and young adults [23]; positive associations between PFOS and PFOA and total cholesterol [24–26] and low-density lipoprotein cholesterol [25].

According to several published studies and the World Health Organization report on State of the Science of Endocrine Disrupting Chemicals [27], further research on PFAS and their effects on humans are needed. Conduction of large epidemiological studies with preferable high number of samples is necessary in order to demonstrate and proof correlations and their significance between investigated compounds and their impacts on humans. For this purpose, high-throughput sample preparation methods are needed, since sample preparation steps are time consuming parts within analysis. There are some interesting approaches like online SPE [28, 29], automated SPE [30] and 96-well [31] extraction methods reported, which are either partly automated or adopted for higher sample throughput. However, at present, no fully automated high-throughput sample preparation method for environmental contaminants (including PFAS) in serum is available.

By automatization of sample transfer, addition of solvents and internal standards as well as solid-phase extraction (SPE) and following steps, inter- and intra-day variations and variations between operators can be minimised. In the present study, a fully validated method for automated high-throughput sample preparation of PFAS in human serum is presented.

Materials and methods

Chemicals and standards

For details on chemicals and standards, see Electronic Supplementary Material.

Samples

Newborn bovine serum was purchased from Sigma-Aldrich (Steinheim, Germany). Standard reference material sera SRM 1957 and SRM 1958 were purchased from the National Institute of Standard and Technology (NIST, Gaithersburg, MD, USA) and prepared according to the instructions. The quality of the analysis was additionally verified by samples with known PFAS concentrations from previous round-robins of the international interlaboratory comparison programme, namely the AMAP Ring Test for Persistent Organic Pollutants in Human Serum (organized by the Laboratoire de toxicologie, Institut National de Sante Publique du Quebec, Canada; AMAP, 2013). Real sera samples stemming from blood donors were prepared by the University Hospital of North Norway (UNN) according to the internal procedure (Tekniskblod PR27709; TS1, TS2). Forty sera samples of the general population from Tromsø, Norway were randomly collected in February 2014 at UNN and anonymised before sample preparation. The procedure was accepted by the Regional Board of Ethics. The samples consisted of 20 female and 20 male persons aged from teenager to retiree. All sample material was stored frozen at <-20 °C until analysis.

Sample preparation

Samples were thawed in the fridge over night at <8 °C, placed at room temperature the following morning for at least 30 min and then put to the ultrasonic bath (Bransonic 5800, Branson Ultrasonics, Danbury, USA) for 15 min at 25 °C. Samples were prepared by an automated liquid handler Tecan Freedom Evo 200 (Männedorf, Switzerland) equipped with an eight-channel liquid handler arm (LiHa) for conductive disposable tips, a robotic manipulator arm (RoMa) for transport of microtiterplates and a Te-Vac station for solid-phase extraction. Disposable tips (50, 200 and 1000 μ L) were of Tecan pure quality. Ninety-six samples per batch (including 4 MilliQ-water blanks, 3 bovine sera blanks, 4 SRM 157 and 4 SRM 158) and a 1.2 mL Sarsted Megablock 96-well plate (Sarsted, Nümbrecht-Rommelsdorf, Germany) were placed onto the carriers of the liquid handler (Electronic Supplementary Material Fig. S1) and a 96-well- μ Elution SPE-plate (Oasis WAX μ Elution Plate, 30 μ m, Waters, Milford, USA) was put onto the Te-Vac station (rear position).

Standard solutions and reagents were placed to the liquid handler right before their use and standard solutions were removed immediately after application for avoiding evaporation. Extraction of the samples was performed as follows (Electronic Supplementary Material Fig. S2): 50 μ L serum/sample was transferred to a 1.2 mL 96-well plate (Sarsted, Nümbrecht-Rommelsdorf, Germany) and 25 μ L internal standard mixture (1 pg/ μ L in methanol for all analytes except [13 C $_4$]-PFOA 4 pg/ μ L and [13 C $_4$]-PFOS 20 pg/ μ L) and

150 μ L formic acid (50 % p.a.) were added, respectively, and mixed by resuspending ten times. The μ -SPE-plate was conditioned with 200 μ L of a 0.1 % methanolic ammonium hydroxide solution, 200 μ L MeOH and 200 μ L MilliQ-water, respectively. After the different pipetting steps in general, a 1 min timer was set before applying the vacuum (700 mbar) at the Te-Vac station. The samples were loaded onto the μ -SPE-plate. Then 150 μ L of 2 % formic acid were transferred to the 1.2 mL well plate and resuspended six times before transfer to the μ -SPE-plate. After this washing step, the μ -SPE-plate was centrifuged for 6 min at 1600 rpm (Hettich Universal 320, Tuttlingen, Germany) and replaced to the Te-Vac station. The vacuum box was moved by the RoMa together with the μ -SPE-plate to the front position of the Te-Vac station onto a 1 mL round 96-well collection plate (Waters, Milford, MA, USA).

The analytes were eluted with 50 μ L MeOH and 2×50 μ L of a 2 % methanolic ammonium hydroxide solution, respectively. The vacuum box together with the μ -SPE-plate were moved to the rear position of the Te-Vac station, and the 1 mL round well plate was transferred to a carrier where 10 μ L of a performance standard (10.5 pg/ μ L in MeOH) and 150 μ L MilliQ-water were added followed by ten times resuspending for mixing of the extract. A RAPID slit seal (Bio chromat, Honcho, Japan) was attached for sealing of the 96-well plate. Sample preparation of 96 samples was achieved within <1 h 45 min (not including pre- and post-analytical preparations).

Instrumental analysis

PFASs were analysed by ultrahigh pressure liquid chromatography triple-quadrupole mass spectrometry (UHPLC-MS/MS). Analysis was performed on a Waters Acquity UPLC system (Milford, MA, USA) consisting of a binary solvent manager, an autosampler and a column manager coupled to a Xevo TQ-S MS (Waters, Milford, MA, USA) with an atmospheric electrospray interface. The autosampler temperature was set to 10 °C and column oven to 50 °C. A volume of 10 μ L was injected on an Acquity UPLC HSS 3T column (2.1 \times 100 mm, 1.8 μ m) (Waters, Milford, MA, USA) equipped with an inlet filter (0.2 μ m, 2.1 mm, Waters, Milford, MA, USA). Separation of the target analytes was achieved by using a programmed gradient of 2 mM NH₄OAc in MilliQ-water (A) and 2 mM NH₄OAc in MeOH (B) as eluents. The following gradient programme was applied: initial conditions with 0.3 mL/min, 90 %A/10 %B; gradient to 0.5 min and 60 %A/40 %B; gradient to 7.5 min, 0.4 mL/min and 100 %B; gradient to 7.9 min and 0.7 mL/min, 100 %B; isocratic column wash step to 9.5 min at 100 %B; back to initial conditions to 9.7 min, 0.45 mL/min and 90 %A/10 %B; equilibration to 11.50 min, 0.3 mL/min and 90 %A/10 %B. In order to minimize the PFAS background coming from the LC-system, a Waters PFC-kit was installed. In order to be able to distinguish between the PFCAs leaching from the

pump and the degasser from the PFCAs originating from the standard solutions or sample extracts, a Waters PFC-isolator column or a XBridge C₁₈ column (2.1 \times 50 mm, 5 μ m, Waters, Milford, MA, USA) was mounted after the pump and before the injector. This set up resulted in different retention times of the PFCAs according their origin. The electrospray interface was operated in negative mode (ESI⁻) with a capillary voltage of 0.8 kV, source temperature of 150 °C, desolvation temperature of 500 °C, cone gas flow of 150 L/h, desolvation gas flow of 1000 L/h, collision gas flow of 0.15 mL/min and nebuliser gas at 7 bar. Details on the parent ions, monitored transitions and collision energies are provided in the Electronic Supplementary Material Table S1.

Quantification

Quantification was conducted using the Masslynx and Targetlynx software (Version 4.1, Waters, Milford, MA, USA) and achieved by the internal-standard method with isotope-labelled PFASs. An eight-point calibration curve with concentrations ranging from 0.01 to 10 pg/ μ L was applied for quantification. The corresponding labelled compound was used as the internal standard (IS) for ¹²C native compounds, with the following exceptions: [¹³C₄]-perfluorohexane sulfonate (PFHxS) for perfluorobutane sulfonate (PFBS) and perfluoropentane sulfonate (PFPS); [¹³C₄]-PFHxS and [¹³C₄]-PFOS for perfluoroheptane sulfonate (PFHpS); [¹³C₄]-PFOS for perfluorodecane sulfonate (PFDS) and perfluorododecane sulfonate (PFDoDS); and [¹³C₂]-perfluorododecanoate (PFDoDA) and [¹³C₂]-perfluorotetradecanoate (PFTeDA) for perfluorotridecanoate (PFTTrDA). The linear isomers of PFASs were used for calculating the contribution of the branched compounds. For integration, the mean smoothing method and one iteration was applied. During analysis, solvent injections were done regularly in order to monitor instrument background and carry-over effects.

Method validation

The developed method was validated using solvent standards, bovine serum, spiked bovine serum, standard reference material (SRM) 1957 and 1958 and real samples in order to evaluate selectivity, linearity, recovery, precision, method detection (MDL) and method quantification limits (MQLs). The selectivity of the method was evaluated by verification of the absence of interfering peaks at the retention time of each compound in blank samples for the acquired MS/MS transitions. Additionally, potential co-eluting compounds, as for example endogenous steroid sulphates and bile acids, were taken into consideration when choosing the analytical column for chromatographic separation. The linearity was studied by injecting standards in methanol/MilliQ-water 1:1 at eight concentration levels (0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 5, 10 pg/

μL) and was considered acceptable when regression coefficients were >0.99 . Accuracy of the established method was estimated from recovery experiments by analyzing replicates of eight spiked bovine sera at low (0.2 ng/mL; $N=3 \times 8$), medium (2 ng/mL; $N=3 \times 8$) and high (20 ng/mL; $N=3 \times 8$) concentrations at three different days.

The precision was evaluated by analysis of certified standard reference material and samples stemming from the AMAP interlaboratory comparison. Batches were assayed to assess the precision and accuracy of the method over one batch (96 samples) and 8 months. The intrabatch accuracy and precision and the inter-day precisions were defined as relative standard deviation (RSD).

Accuracies and precisions were assessed by comparing measured concentrations with the spiked concentrations and the assigned concentrations of the reference material.

The presence of matrix effects during the ionization process in the ESI-source were investigated by comparing the response of the individual analytes in solvent standards with the response of analytes in extracts of blank ($N=3$), bovine serum ($N=3$) and six different real samples spiked with mass-labelled, native and performance standards prior to LC-MS analysis. The matrix effect (percent) was calculated by analyte response solvent standard/analyte response spiked extract $\times 100$. One hundred percent indicates no matrix effects, $>100\%$ indicates signal enhancement and $<100\%$ signal suppression present during the LC-MS/MS analysis.

MDL was set as a concentration calculated by the Quanlynx-software for each individual sample with a S/N of 3 divided by the related sample amount. Where blank contamination was detected, MDLs were estimated as an average of the blanks multiplied by three times of their standard deviation. If the MDL calculated from the blank contamination was higher as the individual MDL of the sample, MDL calculated based on blank sample was used. MQL was defined as ten times the signal-to-noise ratio.

Statistical methods

Statistical tests were performed in SPSS version 22 (IBM Corporation, New York, USA). The t test with a confidence interval of 95 % was applied for estimation of significant differences between genders. The two-tailed Spearman correlation coefficients were calculated between age and detected PFAS compounds at a 0.01 % significance level.

Results and discussion

Development of the analytical procedure

During method development, the following instrument and sample preparation parameters were tested, optimised and

modified: On the UHPLC, a PFC-kit consisting of PTFE-free solvent-frits, solvent tubings and a PFC-isolator column was installed to minimize LC-system background contamination.

In the first period, glass vials were used for analysis of calibration standards which resulted in excellent regression coefficients ($R^2 > 0.99$) over short batches (~60 injections, 12 h) but a drop was observed over longer batches (~230 injections, 46 h) which was not with polypropylene vials. This phenomenon related to glass vials was not observed during earlier studies at other instruments with a different autosampler set up [16, 32] or same autosampler but different injector mechanism [33].

Background contamination of the extracts, probably coming from the cooling system, was observed when placing an unsealed 1 mL round 96-well collection plate into the Acquity sample manager. Different seal brands (Waters, Sarsted, Eppendorf, Bio chromate) and seal types (different self-adhesive seals and foils, as well as heating seals and foils) were tested, where the Bio chromate seals showed to be the most suitable ones for LC-analysis as well as for long-time storage in the fridge and even in the freezer without accounting for PFAS contamination.

For the sample preparation steps, different mixtures and amounts of reagents were tested. Sera samples were diluted with different concentrations of formic acid (e.g. 0.1 M up to 1 M and 15 % up to 50 % formic acid) with initial starting points described in the literature [28, 30, 34]. A dilution with 50 % formic acid gave the best recoveries for long chain mass labelled PFASs, whereas lower percentage of formic acid is favoring shorter chain PFAS compounds and perfluorooctane sulfonamide (PFOSA). The addition of 50 % formic acid was chosen for further work focusing on longer chain PFASs and due to uncertainties in reliable detection of perfluorobutanoate (PFBA) and perfluoropentanoate (PFPA) (details later in the chapter of "Selectivity"). In the case of PFOSA, the compromise was justified with the fact that PFOSA was not detected in high frequencies in earlier studies [35–38] and might not be detected in future studies on the general Norwegian population.

Washing steps in the extraction method were tested by using, e.g. 2 % formic acid, MeOH and MeOH/water mixtures as a single step or successively in combinations. Methanolic wash steps caused a partly elution of PFOSA, which were not observed with aqueous 2 % formic acid.

Drying step of the SPE-material by either applying negative vacuum for a certain period or centrifugation was investigated. The centrifugation step was preferred in order to confine contamination coming from dust particles in the air.

For elution reagents as MeOH, methanolic ammonium hydroxide and aqueous formic acid as single solution or subsequently in different combinations were tested. MeOH with subsequently two equal portions of 2 %

methanolic ammonium hydroxide were suitable and gave the best recoveries and accuracy.

The extraction efficiency was evaluated by applying a second elution round. Only 0.1–0.2 % of the spiked analytes was found in the second eluate. This was defined as negligible. After the elution step, nitrogen blow down of the solvent was considered, but dismissed since a negative effect on the accuracy of PFDCS and PFOSA was observed. However, there was neither signal suppression nor enhancement or negative effect on the LC-MS/MS system noticed when analyzing ammonium hydroxide containing extracts and standards.

Validation of the developed method

Selectivity

For each analyte, two specific multiple reaction monitoring (MRM) transitions were evaluated with respect to selectivity. Three MRM transitions were implemented for PFHxS and PFOS in order to monitor separation of the potentially interfering endogenous steroid sulphates [34, 39] and bile acids [39]. In addition, a chromatographic separation of the PFAS compounds and the mentioned interferences of 0.3 min at least were set as criteria (Figs. 1, 2 and Electronic Supplementary Material Fig. S3). The selectivity was satisfactory for 18 of the 20 compounds analysed: For PFBA and PFPA, only one MRM transition was available. The criteria of only one MRM transition is not enough for a reliable identification of these analytes, especially in the case of PFBA, where also matrix interferences are co-eluting in relation to ageing of the filter-inlet and the analytical column.

Linear range

Linearity was studied in the range from 0.01 to 10 $\mu\text{g}/\mu\text{L}$ using solvent standard solutions and a linear polynomial type with a $1/x$ weighing. Calibration curves were injected in triplicates and exhibited excellent linearity with correlation coefficients >0.99 for each analyte. Concentrations of PFASs were within both the linear range of the instrument and the calibration curve in all samples and experiments.

Accuracy

Trueness and accuracy of intra-day and inter-day performance were evaluated by arithmetic means of recovery experiments at three concentration levels (0.2, 2 and 20 ng/mL) in fortified foetal bovine sera. Intra-day performance was investigated by preparation of eight parallel samples at three fortification levels on the same day ($N=3 \times 8$), whereas inter-day performance was tested at three different days over a period of 8 months ($N=(3 \times 8) \times 3$). The intra-day recoveries ranged from 66 to 129 % with the majority of the analytes presenting

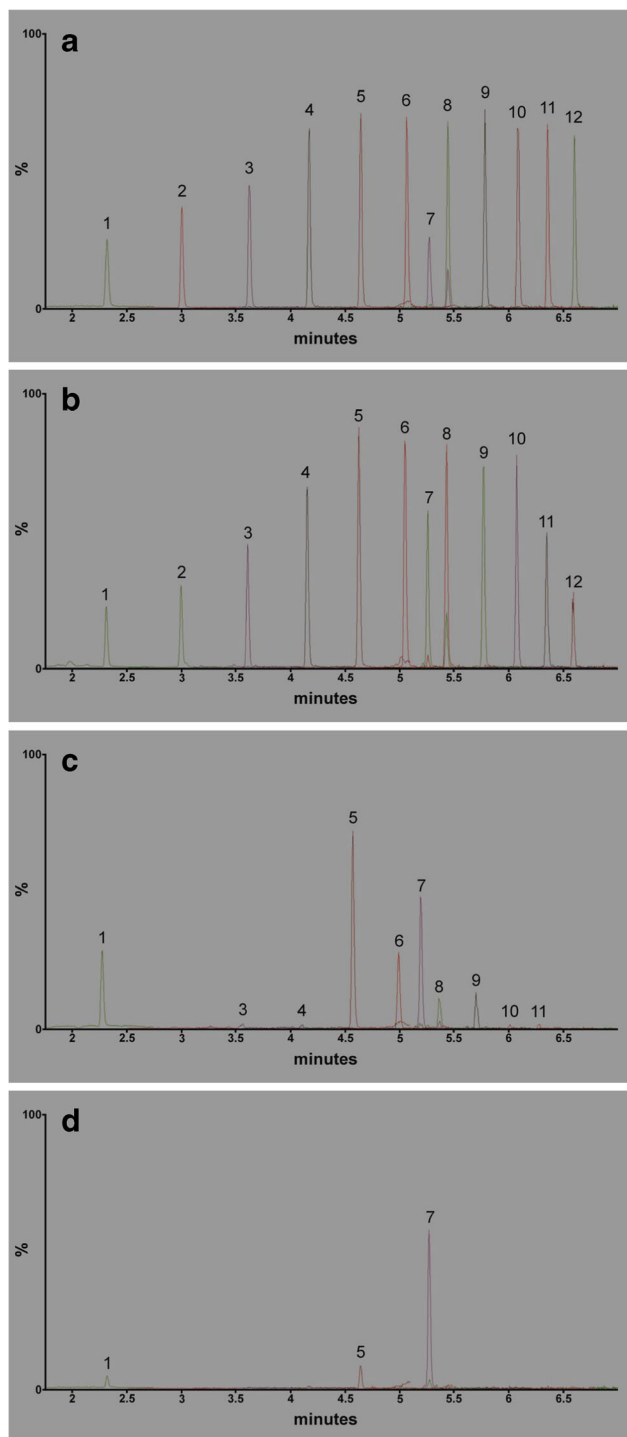


Fig. 1 Chromatograms showing native perfluoroalkyl carboxylic acids in a standard solution (a), spiked bovine serum (b), real sample (c) and blank sample (d). 1 PFBA, 2 PFPA, 3 PFHxA, 4 PFHpA, 5 PFOA, 6 PFNA, 7 performance standard, 8 PFDA, 9 PFUnDA, 10 PFDoDA, 11 PFTTrDA, 12 PFTeDA

recoveries between 70 and 120 %. Figure 3 and Electronic Supplementary Material Table S2 show satisfactory recoveries between 70 and 120 % at all investigated concentrations for the majority of the analytes. PFPA, PFOA and PFOS are

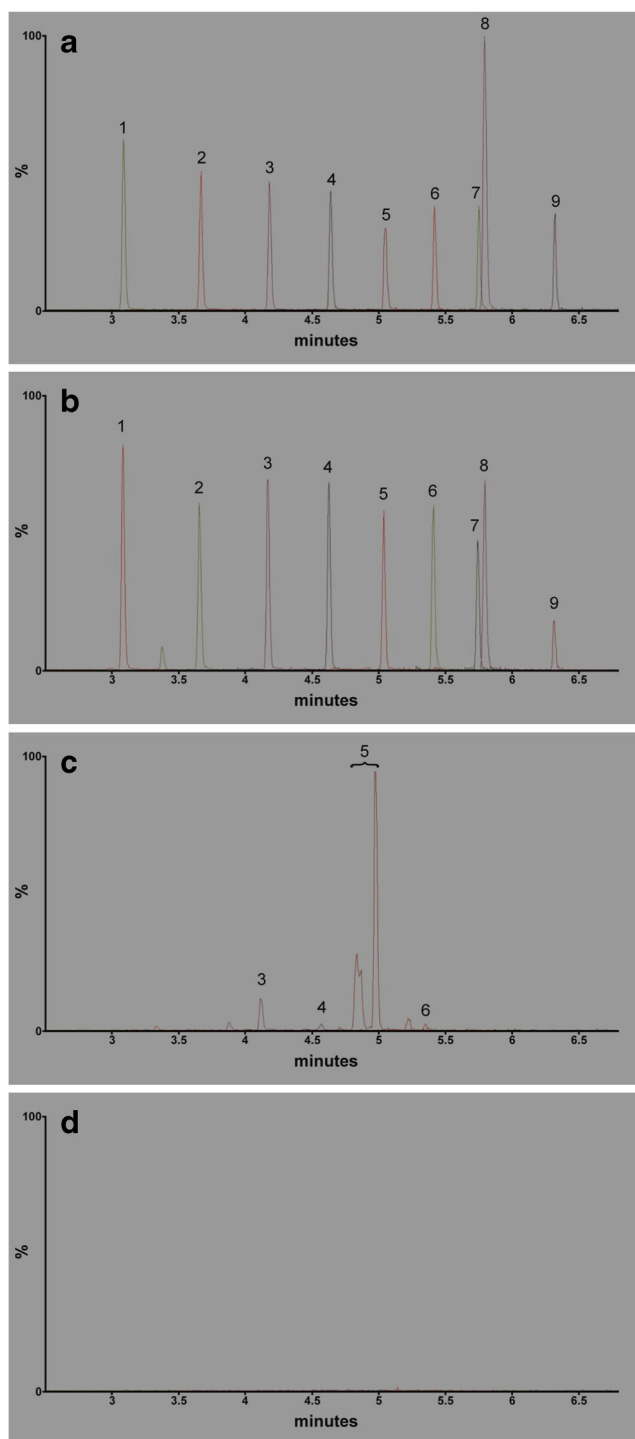


Fig. 2 Chromatograms showing native perfluoroalkyl sulfonic acids and PFOSA in a standard solution (a), spiked bovine serum (b), real sample (c) and blank sample (d). 1 PFBS, 2 PFPS, 3 PFHxS, 4 PFHpS, 5 PFOS, 6 PFNS, 7 PFDS, 8 PFOSA, 9 PFDoDS

slightly overestimated at the low fortification level. Concentrations of these species cannot be measured with satisfactory accuracy at very low concentrations. This is only problematic for PFPA since this analyte could be found in very low concentrations if detected, but it will not be of concern for PFOA

and PFOS which are usually measured in higher concentrations in the general population (Figs. 1 and 2). For the high concentration spikes, PFBS show slightly low recoveries of 67 %, which might not be of concern when studying real samples since PFBS concentrations reported in previous studies were found to be close to or below the MDL [35–38].

Precision

The precision of the developed method was evaluated through the overall relative standard deviation (RSD) of the spike experiments and was satisfactory for both the analytes and the three different concentration levels investigated (Fig. 3 and Electronic Supplementary Material Table S2). RSDs were <20 % with the exception of PFOA at the low concentration experiments. The slightly higher RSD for PFOA (22 %) may be influenced by the presence of PFOA in MilliQ-water blanks as well as in the bovine serum used for the spike experiments (Fig. 1).

The instrumental repeatability was investigated by injecting extracts of one real sample and two SRMs six times in a row (Electronic Supplementary Material Table S3). RSDs ranged from 2 to 12 % depending on the concentrations measured in the samples; the variations were higher for concentrations close to the MDL (Electronic Supplementary Material Table S3). The repeatability of the whole method within one batch of 96 samples (one 96-well plate) was tested for different samples. The RSD for the within-batch repeatability of spiked bovine serum ($N=22$) at medium concentration level and for a real serum sample (TS1, $N=24$) reached from 5.2 to 10.9 % and 2.1 to 17.4 %, respectively.

The reproducibility of the method and its precision were evaluated over a period of several months for two different samples of each: real serum sample (TS1; TS2), AMAP ringtest and SRM (Table 1). The RSDs were satisfactory and below 20 % for each sample with the only exception of perfluoroundecanoate (PFUnDA) in the NIST SRM 1957 (RSD=28 %). Differences from the assigned mean reference concentrations were between 2 and 15 % for the AMAP samples and 3 to 10 % for SRM 1958. SRM 1957 showed higher variation in differences ranging from 3 to 41 % which could be related to the matrix since SRM 1958 and the AMAP samples performed much better. On the other hand, also other working groups observed a mismatch for these specific analytes (personal communication, data not published). In order to investigate the reproducibility of the application further and on matrix-related individual differences, triplicates of 40 real samples of the general population from Tromsø, Norway, were prepared over a period of 4 months. The mean concentrations were plotted against the RSD for the following analytes: PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, L-PFHxS, Σ -PFHxS, L-PFHpS, Σ -PFHpS, L-PFOS and Σ -PFOS (Electronic Supplementary Material

Fig. 3 Accuracy and reproducibility of the developed method displaying recovery versus overall relative standard deviation of spiked bovine serum ($N=3 \times (3 \times 8)$)

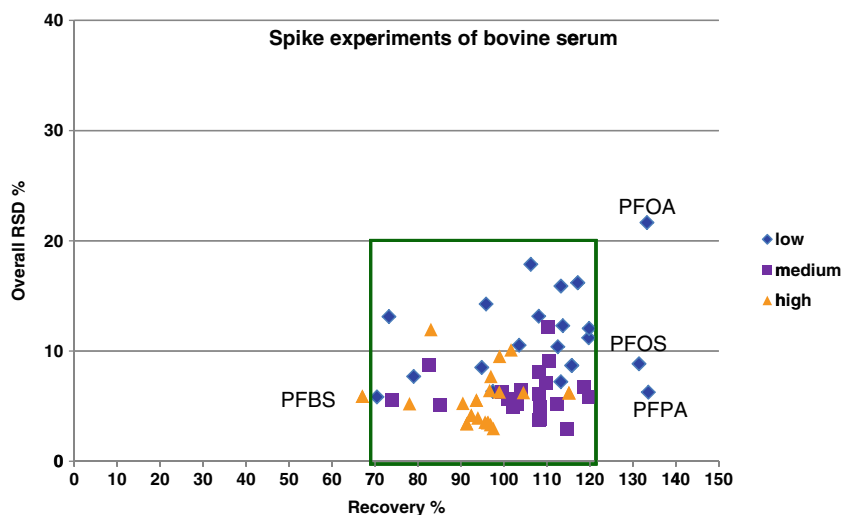


Fig. S4). Majority of the compounds as well as samples were below a RSD of 20 %. A correlation between high RSDs and low concentrations is clearly shown.

Matrix effects

Matrix effects, like suppression or enhancement of the analyte response, were investigated in solvent standards compared to three different extract types (blank, bovine serum and human serum). For the majority of the compounds, matrix effects were negligible and within the accepted range of $\pm 15\%$ (Electronic Supplementary Material Table S4). Only the isotope-labelled internal standard for PFTeDA showed signal suppression in the three different extracts and the native PFTeDA in the blank extract as well as PFDoDS in the bovine serum extract. However, the use of isotope-labelled internal standards together with the performance standard will correct for matrix effects during sample preparation and LC-MS analysis.

Detection limit

Low levels of PFBA and PFOA were detected in MilliQ-water blanks and PFBA, PFHxA, PFOA and PFOS in foetal bovine serum samples. For serum samples, MilliQ-water blanks were taken into account when adjusting MDLs for background contamination. For spike experiments, the foetal bovine sera blanks were applied for blank correction. MDLs were generally low and ranged from 0.006 to 0.339 ng/mL (Electronic Supplementary Material Table S5). Mean MDLs are comparable with MDLs from previous published studies where state of the art instrumentation or high sample volumes were applied and when blank concentrations of the different methods were taken into account [28, 31, 40].

Robustness and throughput

The robustness of the analytical method was tested during a period of 8 months where different batches and qualities of solvents (MeOH) and reagents (formic acid, ammonium hydroxide), different batches of extraction plates, conductive pipette-tips, 96-well plates and other equipment were used. Additionally, different conditions of the instrument were taken into account since weekly, monthly and periodical maintenance were performed. The accuracy and precision shows that the method is robust over time and suitable for human sera samples.

High-throughput of the method was evaluated with real samples, SRMs, bovine serum and MilliQ-water blanks. Sample preparation of 96 samples was achieved within <1 h 45 min (not including pre- and post-analytical preparations) which is very fast compared to other published methods which managed to prepare between 15 and 48 samples per working day [16, 20, 22, 28, 30, 31, 36, 37, 41]. With the presented method, a preparation of 96 samples including QA/QC samples (blanks and SRMs) and a start of the instrumental analysis on the UHPLC-MS/MS system is feasible within a 7.5 working hour day. Our method demonstrates the suitability for large epidemiological studies on PFAS where high-throughput methods are a huge advantage in terms of time management during sample preparation.

Application to real samples

The developed and validated method was applied to 40 sera samples of the general population from Tromsø, Norway. PFHpA, PFOA, PFNA, PFDA, PFUnDA and PFOS had a detection frequency of 100 % while PFHxS and PFHpS of 98 % (Electronic Supplementary Material Table S5). The highest mean concentrations were measured for Σ -PFOS (13.6 ng/mL) followed by PFOA (1.71 ng/mL), Σ -PFHxS

Table 1 Precision, repeatability and reproducibility of different serum samples

Sample	TS 1		TS 2		AM-Y-S1303		AM-Y-S1308		SRM 1957		SRM 1958	
	Present method	Difference [%]	Present method	Difference [%]	Present method	Difference [%]	Present method	Difference [%]	Present method	Difference [%]	Present method	Difference [%]
<i>N</i>	13		14		6		5		28		28	
Months	8		8		4		4		9		9	
PFHxA	<MDL		<MDL	9	2.58 (0.30/12)	2.83 (1.65–4.01)	7.35 (1.13/15)	7.51 (4.5–10.5)	0.225 (0.02/8)	0.305 (±0.036)	0.210 (0.02/10)	
PFHpA	<MDL		<MDL		0.095 (0.01/16)		0.122 (0.01/9)		5.00 (0.13/3)		3.68 (0.26/7)	10
PFOA	1.42 (0.14/10)	1.47 (0.12/8)	1.47 (0.12/8)	9	5.76 (0.10/2)	6.30 (4.53–8.07)	12.8 (0.41/3)	14.0 (9.83–18.2)	4.02 (0.683)	5.00 (±0.15)	3.68 (0.609)	
PFNA	0.568 (0.05/8)	0.903 (0.06/7)	0.903 (0.06/7)	12	0.628 (0.05/8)	0.716 (0.425–1.01)	3.91 (0.30/8)	4.53 (2.62–6.28)	0.683 (0.04/5)	0.880 (±0.068)	0.609 (0.05/9)	3
PFDA	0.335 (0.04/13)	0.501 (0.04/9)	0.501 (0.04/9)		0.140 (0.02/14)		0.199 (0.02/9)		0.230 (0.03/12)	0.390 (±0.1)		41
PFUnDA	0.377 (0.05/13)	0.735 (0.05/7)	0.735 (0.05/7)	15	1.18 (0.08/6)	1.38 (0.812–1.95)	3.06 (0.18/6)	3.44 (2.05–4.83)	0.114 (0.03/28)	0.174 (±0.031)		35
PFPS	0.020 (0.004/18)	<MDL	<MDL		0.035 (0.01/16)		<MDL					
L-PFHxS	0.518 (0.05/11)	0.800 (0.05/6)	0.800 (0.05/6)		14.5 (0.67/5)		3.65 (0.17/5)		2.98 (0.19/6)		2.50 (0.20/8)	
Σ-PFHxS	0.659 (0.05/7)	1.07 (0.09/8)	1.07 (0.09/8)	15	16.2 (0.80/5)	19.0 (10.9–27.1)	3.90 (0.18/4)	4.2 (2.47–5.93)	3.32 (0.24/7)	4.0 (±0.75)	2.77 (0.32/12)	4
L-PFHpS	0.099 (0.01/11)	0.223 (0.02/10)	0.223 (0.02/10)		<MDL		0.192 (0.02/12)		0.346 (0.03/8)			
Σ-PFHpS	0.121 (0.01/12)	0.271 (0.02/9)	0.271 (0.02/9)		<MDL		0.231 (0.03/14)		0.434 (0.03/8)			
L-PFOS	4.35 (0.37/9)	8.72 (0.45/5)	8.72 (0.45/5)	6	4.09 (0.10/2)	5.61 (1.26–9.96)	24.4 (1.15/5)	35.2 (20.8–49.6)	9.56 (0.61/6)	21.1 (±1.2)	8.04 (1.22/15)	8
Σ-PFOS	6.81 (0.75/11)	14.3 (0.61/4)	14.3 (0.61/4)		5.92 (0.15/3)		35.8 (1.55/4)		20.4 (0.98/5)		17.8 (1.13/6)	

Standard deviation and relative standard deviation (%) in parenthesis below the measured concentrations of the present method. Accepted reference concentration ranges for AMAP samples in parenthesis below the assigned reference concentrations. Concentrations in ng/mL. Calculation on differences based on the whole period (months)

TS technical serum, AM AMAP ringtest samples, SRM NIST standard reference material



Fig. 4 Sum-concentrations (a) and distribution patterns (b) of PFAS in sera samples of the general population from Tromsø, Norway

(1.47 ng/mL) and PFNA (0.85 ng/mL) with ranges from 0.13 to 118 ng/mL, 0.53 to 3.44 ng/mL, 0.18 to 11.6 ng/mL and 0.03 to 2.06 ng/mL, respectively. For PFHxS, PFOS and PFHpS, branched isomers were detected in up to 39, 21 and 63 % of the peak-areas of linear and branched species quantified together.

Concentrations of females and males (Fig. 4a) were not statistically different ($p > 0.05$). Distribution patterns were similar and dominated by PFOS (>50 %), PFOA and PFHxS as well as PFDA and PFUnDA in varying amounts (Fig. 4b). Previous published studies on the Norwegian population showed similar concentration ranges and distribution patterns [28, 35–38, 42, 43].

A significant correlation between age and measured concentrations was demonstrated for PFHpS ($p = 0.67$) and PFOS ($p = 0.66$) in females and for PFNA ($p = 0.57$), PFDA ($p = 0.63$ at 0.01 level), PFUnDA ($p = 0.65$) and PFOS ($p = 0.67$) for males. This is not surprising since the samples were taken cross-sectional and within the same time period. Exposure windows related to lifetime of the individuals have to be taken into account when interpreting correlations between age and concentration. In the case of PFAS, younger persons were not exposed to the same periods and probably amount of PFASs as older persons. Additionally, age-related lifestyle factors as different preferences for food, outdoor equipment and interior/furniture will influence the individual PFAS concentrations. Males are not transferring parts of their body burden to their offsprings like females during pregnancy and nursing, and an increasing concentration and correlation with age could therefore be considered in cross-sectional studies [41, 42]. However, Nøst et al. demonstrated clearly in a well-designed longitudinal study that PFASs do not correlate with age, instead the sampling year is the dominating factor on PFAS concentrations [36].

Conclusion

To our knowledge, an automated high-throughput SPE micro-elution method for environmental contaminants was presented for the first time. The method for analysis of PFAS, as PFSA, PFCAs and PFOSA, demonstrates the suitability for large-scale epidemiological studies where innovative high-throughput methods are a huge advantage in terms of time management during sample preparation for fast generation of high-quality data on PFAS in human sera. However, the bottleneck in time consumption is the quantification work where still man power has to be used for validation and control of each single chromatogram. The low sample volumes applied meets the demand and requirement to save the highly valuable sample material for follow-up and other studies. Application of the micro-elution technique minimises solvent consumption which is more environmental friendly and also cost effective. High sensitivity, accuracy, repeatability and robustness have been demonstrated for an appropriate

concentration range. The applicability for real samples was satisfactory, demonstrated by analysis of sera samples from the general population.

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