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A simple and rapid extraction method for sensitive determination of perfluoroalkyl substances in blood serum suitable for exposure evaluation

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ABSTRACT

In this work, we propose a microextraction method based on a new supramolecular solvent (SUPRAS) made up of reverse aggregates of hexanoic acid, combined with liquid chromatography/triple quadrupole mass spectrometry (LC/QQQ MS–MS) for the determination of the perfluoroalkyl substances (PFASs) in blood serum. A SUPRAS is a nano-structured liquid made up of surfactant aggregates synthesized through a self-assembly process. The method involved the acidification of 765 μL of blood serum (600 μmol of hydrochloric acid per mL of serum) followed by the addition of hexanoic acid (97 μL) and tetrahydrofuran (THF) (600 μL), conditions under which the supramolecular solvent (~360 μL) formed in situ after vortex-shaking and centrifugation. Parameters affecting extraction efficiency and concentration factors were studied. The overall sample treatment took only 20 min and several samples (20–30) can be simultaneously analyzed using conventional lab equipments, making additional investments unnecessary. Recoveries for the internal standards in samples ranged from 75 to 89% with relative standard deviations between 1 and 15%. Calibration was based on the use of internal standards. The method was very sensitive with detection limits ranging from 2 to 20 pg mL^{-1} for PFASs. The approach developed was successfully applied to the determination of PFASs in different blood serum samples. The concentration of PFASs found in samples of animal origin ranged between 17 and 197.3 pg mL^{-1} and between 84 and 5168 pg mL^{-1} in samples of human origin. Both the analytical and operational features of this method make it suitable for the evaluation of exposure to PFASs.

1. Introduction

Per- and polyfluoroalkyl substances (PFASs) are emerging contaminants that comprise a large group of chemicals [1]. Attention has been focused on these chemicals due to the discovery of their global biospheric distribution explained by their persistence and bioaccumulation in biota [2,3].

The marketing and use of perfluorooctane sulfonate (PFOS) were restricted by the European directive 2006/122/EC [4]. PFOS has been included in the annex III of the Directive 2008/105/EC as part of the list of substances subject to review for identification as priority hazardous compounds in the framework of the European water policies [5]. PFASs have been also listed as contaminants of relevance to be monitored in fish and other seafood for human consumption for determining good environmental status of marine waters in the Marine Strategy Framework Directive [6]. In addition, PFOS, its salts and perfluorooctane sulfonyl fluoride (PFOSF) have been included in Annex B to the Stockholm Convention on persistent organic pollutants (POPs) [7].

The presence of organic fluoride in humans was first reported by over 30 years ago [8] but studies to determine the concentration of PFASs in serum of workers with an occupational exposure (in the order of 1000–2000 ng mL^{-1}) and general population (about 100 times lower) did not begin until the 1990s and 2000s, respectively [9,10]. The exposure to PFASs is likely to occur via several routes e.g. ingestion (e.g. food, drinking water, dust, etc.), dermal contact and inhalation [11] and they are widely distributed in the body and especially in blood, liver and kidney [12]. Major PFASs found in human serum are PFOS, perfluorooctanoic acid (PFOA) and perfluorohexane sulfonate (PFHxS), although long chain perfluoroalkyl carboxylates (e.g. C_9–C_{14}) are also found in these biological matrices at relevant concentrations [13–16].

The determination of PFASs in blood samples (i.e. whole blood, serum and plasma) is mostly made by liquid chromatography (LC)–tandem mass spectrometry (MS/MS) with negative electrospray ionization (ESI-) interfaces [16,17]. Comparison of extraction and quantitation methods for PFASs in human plasma, serum and whole blood has been reported [18]. Current sample

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treatments for PFASs analysis are mostly based on ion-pair extraction employing methyl-tert-butyl-ether after reaction with tetrabutylammonium [10], solvent extraction with acetonitrile [3,19] or solid phase extraction (SPE), both off-line [15,20,21] or column switching [16,22,23]. Organic solvent volumes around 10–15 mL [3,13,18] and times between 15 min and 16 h are spent by extraction [13,18,20,21]. Acidification [20,22], alkaline digestion [21] or addition of organic solvent to samples [3,16,18,23] are usually carried out prior SPE in order to prevent the clogging of pre/columns caused by the precipitation of blood proteins. Clean-up strategies include column washing [15,20,22], filtration [10,20], centrifugation [22], dispersive graphitized carbon with glacial acetic acid [3], and Wax SPE of extracts [19], being common the use of multiple-steps methods. Main drawbacks of these strategies are much hands-on time due to the need for repetitive washes [10], low recoveries for long-chain PFASs due to co-precipitation with serum protein [16,19], thorough washings of SPE cartridges after sample percolation [15,19], problems of contamination in the different procedural steps, lack of enrichment or even dilution of the samples (only online SPE techniques permit to concentrate them) or the need for additional column switching equipment investments [16,22,23]. In addition most of the developed methodologies require matrix-matched calibration for accurate quantitation of PFASs. Usual detection limits (LODs) are in the range 0.1–3 ng mL^{-1} [17] although a method allowing PFAS quantification at concentrations as low as 0.05 ng PFAS mL^{-1} serum has been reported [16]. According to the above statements, the development of new methods for exact quantitation of PFASs, which feature straightforward and reduced sample handling (thus reducing risk of contamination and loss of analytes) is necessary for large-scale human biomonitoring. With this aim, this article investigates the potential of amphiphile-based supramolecular solvents.

Supramolecular solvents (SUPRASs) are water-immiscible liquids made up of supramolecular assemblies dispersed in a continuous phase. The outstanding properties of SUPRASs for extractions derive from their special structure and the high concentration of the ordered aggregates that constitute them, which provide a huge amount of binding sites. SUPRASs have regions of different polarities that offer a variety of interactions (e.g. hydrophobic, hydrogen bonds, ion-dipole, and π-cation) for the extraction of analytes with a wide polarity range. These properties permit the development of simple and robust sample treatment methods for most of the solutes, especially for amphiphilic compounds which form mixed aggregates with the ordered structures in the solvents. To date, SUPRASs based on non-ionic [24], zwitterionic [25], cationic [26] and anionic [27] aqueous micelles, reverse micelles [28] and vesicles [29] have been successfully used for the extraction of pollutants from biological, food and environmental samples. A review covering progress on both theoretical and practical aspects related to the use of supramolecular solvents for analytical extractions has been recently reported [30]. Applications have mainly focus on the extraction of polycyclic aromatic hydrocarbons, pesticides, surfactants, pharmaceuticals, vitamins, drugs, dyes, hormones, bisphenol A, phenols and toxins mainly in liquids (e.g. [28,31–34]) and more recently in solid samples [35–37]. Supramolecular solvents are compatible with LC but they have to be removed before injection in gas chromatography or capillary electrophoresis.

Against conventional organic solvents, SUPRASs are specially suited for the extraction of amphiphilic contaminants such as PFASs since analyte-extractant mixed aggregates are formed through both interactions between fluorocarbon chains and polar groups. The use of SUPRASs for the extraction of these emerging contaminants is here presented for the first time. With this aim, a SUPRAS made up of hexanoic acid reverse micelles was here synthesized for the first time through a self-assembly process, and then characterized and assessed for the extraction/concentration of PFASs from serum samples prior to LC/triple quadrupole (QQQ) MS–MS determination. The selection of hexanoic acid (HA) was based on both the dispersion and hydrogen bonding interactions they can establish with PFASs and its short hydrocarbon chain length that allows an early elution from the chromatographic column thus preventing coelution with the PFASs usually found in blood (i.e. PFHxS, PFOS, PFOA and C_9–C_{14} perfluoroalkyl carboxylates). Carboxylic acids with chain lengths shorter than hexanoic acid were not employed because of their high water solubility and accordingly low yield of SUPRAS production under the proper self-assembly conditions. Parameters affecting extraction efficiency and concentration factors were optimized and the applicability of the proposed method to the determination of PFASs in different sera was assessed.

2. Experimental

2.1. Chemicals

The seven target PFASs studied were as follows: PFOA, perfluorononanoic acid (PFNA); perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA); perfluorotetradecanoic acid (PFTeDA), potassium perfluoro-1-hexanesulfonate (PFHxS) and PFOS. PFOA, PFNA, PFDA, PFHxS and PFOS were supplied by Wellington Laboratories and PFUnDA and PFTeDA were obtained from Aldrich (Schenelldorpf, Germany). Stable isotope analogues, $^{18}O_2$PFHxS (MPFHxS), $^{13}C_4$PFOA (MPFOA), $^{13}C_5$PFNA (MPFNA), $^{13}C_2$PFDA (MPFDA), $^{13}C_4$PFOS (MPFOS), $^{13}C_2$PFUnDA (MPFUnDA) and $^{13}C_2$-perfluododecanoic acid (MPFDoDA), supplied by Wellington Laboratories (Ontario, Canada), were used as internal standards (ISs) to control potential losses of PFASs during extraction and MS performance (e.g. ion suppression and enhancement).

Hexanoic acid, sodium taurodeoxycholate hydrate (TDCA) and ammonium acetate were purchased from Sigma–Aldrich (Steinheim, Germany) and LC-grade methanol, tetrahydrofuran (THF) and hydrochloric acid were obtained from Panreac (Madrid, Spain). Ultra-high-quality water was obtained from a Milli-Q water purification system (Millipore, Madrid, Spain). Stock standard solutions, each containing a mixture of target PFASs or method ISs at 100 ng mL^{-1}, were prepared in methanol and stored in closed polypropylene tubes at 4 °C. Standards were prepared by dilution of the stock solution with a 75:25 THF:water (v/v) mixture solution.

2.2. Apparatus and materials

The LC–MS system used was an AB Sciex 4000 Qtrap® mass spectrometer (Foster City, CA, USA), with a negative-ion TurboSpray interface coupled to an Agilent 1200 Series LC system (Palo Alto, CA, USA). The stationary phase was a SymmetryShield™ RP 18 column (particle size 3.5 μm, i.d. 2.1 mm, length 50 mm) from Waters (Milford, MA, USA). A SymmetryShield™ RP 8 guard column (particle size 3.5 μm, i.d. 3.9 mm, length 20 mm) was inserted before the analytical column. Coloumetric Karl Fischer titrator from Metrohm (Herisau, Suize) was used for determination of water content in the SUPRAS. Two mL-microtubes Safe-Lock from Eppendorf Iberica (Madrid, Spain), glass balls (3 mm diameter) from Albus (Córdoba, Spain), a Reax Heidolph vortex (Schwabach, Germany), with an attachment for 10 test tubes, a High Speed Brushless centrifuge MPW-350R (Warsaw, Poland), and a 50-μL microsyringe 750 NR from Hamilton (Bonaduz, Switzerland) were used for sample preparation and extraction. The volume obtained of SUPRASs under different experimental conditions was measured with a digital calliper from Medid Precision, S.A. (Barcelona, Spain).

2.3. SUPRAS characterization

The composition and volume produced of hexanoic acid-based SUPRASs, as synthesized through a process of self-assembly of the amphipile in mixtures of THF and water, were determined. Non-linear regression was used to fit a model for the prediction of the volume of SUPRAS after separation from the equilibrium solution ($n = 70$). The statistic program Statgraphic plus 3.0 was used for this purpose. Regarding SUPRAS composition, a variety of solvents were prepared from bulk solutions with concentrations of HA and THF in the ranges of 2–10% (v/v) and of 1–50% (v/v), respectively. Water content was determined by injecting aliquots of the SUPRASs in a coloumetric Karl Fisher titrator and THF was calculated by weighing after removing water and THF from a certain amount of SUPRASs (~1 g) by just allowing to stand at room temperature in open tubes for 7 days, period during which THF and water were completely evaporated. Water evaporation was checked by coulometric titration.

2.4. Determination of PFASs in serum samples

2.4.1. Sample collection and preservation

Animal serum samples (newborn calf, sheep, bovine, and horse) were purchased from Invitrogen (Barcelona, Spain) and human serum samples were kindly provided by the laboratory of clinical analyses "José Ortega Cruz" (Córdoba, Spain) from anonymous donors. Samples were stored at −20 °C until their analysis.

2.4.2. Sample preparation and SUPRAS-based microextraction

The frozen serum samples were allowed to thaw at room temperature and shaken until homogenization. Extraction of PFASs was performed by mixing a sample aliquot of 765 μL of serum, 38 μL of HCl (12 M), 97 μL of hexanoic acid, 600 μL of THF and 18 μL of ISs (100 ng mL^{-1} in methanol) into a 2 mL polypropylene centrifuge microtube with 3–4 glass balls (3 mm diameter) at the bottom. Glass balls were added to accelerate and enhance sample mixing and extraction. The mixtures were vortex-shaken for 7 min and then centrifuged (15,000 rpm, 15 °C, 13 min) to achieve the complete separation and clarification of the SUPRAS (i.e. the supernatant) formed into the bulk solution. The volume of SUPRAS (~360 μL) was calculated by measuring its height with a digital caliper. More information regarding synthesis of SUPRASs is detailed in Section 3.2.1. Finally, an aliquot of SUPRAS was withdrawn with a microsyringe and transferred to autosampler glass vials with inserts. Samples were capped with aluminum/silicone septum barriers (Supelco, Bellefonte, USA) and subjected to LC/MS/MS analysis.

2.4.3. Quantitation of PFASs by LC (ESI-) QQQ-MS^2

The target PFASs were separated and quantified by using LC coupled with a turbo spray interface operating in the negative ion mode prior to the Qtrap mass spectrometer. Quantitative analyses were performed on the Scheduled MRM mode recording the transitions between the precursor ion and the two most abundant products ions. Table 1 shows the quantifier and qualifier ions used for each native PFAS and the quantifier ion for the ISs. The quantifier and qualifier ions for TDCA (a common interference for PFOS) are also given. The injection volume used was 10 μL. The mobile phase consisted of 2 mM aqueous ammonium acetate (A) and methanol (B) at a flow rate of 0.3 mL min^{-1}. The temperature for the analytical column was set at 30 °C and the gradient elution was programmed as follows: linear gradient from 0 to 100% of methanol for 50 min, and then reverting to initial conditions allowing 10 min for stabilization. The eluates from the analytical column were diverted by switching valve to waste from 0 to 30.5 min in order to avoid the entry of hexanoic acid and the most polar matrix compounds in the mass spectrometer. The turbo spray settings were as follows: curtain gas (N_2) 30 psi; ion spray voltage −4500 V; temperature 450 °C; nebulizer and turbo gas 70 psi. Declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) parameters were optimized for each analyte (Table 1). Unit resolution was used for both Q_1 and Q_3 quadrupoles. Calibration curves were constructed at concentrations of PFASs over the ranges stated in Table 2 with a concentration of the method ISs of 5 ng mL^{-1}.

3. Results and discussion

3.1. Sources and control of background contamination

One typical problem in determining PFASs is the background contamination arising from the presence of a variety of fluoropolymer materials in the components of LC equipment or labware [38]. As a precautionary measure, an additional column (Water Symmetry® 3.5 μm, 4.6 mm × 75 mm) was inserted between the pump and injector in order to trap PFASs potentially released from the instrument. Contamination arising from labware was prevented by using disposable polypropylene tubes, aluminum/silicone septa, a glass microsyringe, nylon syringe filters and glass coated round magnetic stirring bars. Procedural contamination was investigated by adding distilled water at different hydrochloric acid concentrations (150–1500 mM) into polypropylene centrifuge microtubes, instead of serum samples, and subjecting them to different supramolecular microextraction conditions (i.e. several hexanoic acid and tetrahydrofuran concentrations and vortex-shaken and centrifugation at variable times). No contamination of PFASs was observed above the method detection limits. Appropriate control reagent blanks were routinely

Table 1
Quantifier and qualifier ion transitions and MS parameters used to determine PFASs in serum samples.

Target compound			Method ISs		MS parameters		
	Quantifier transition	Qualifier transition			Declustering potential (V)	Collision energy (V)	Collision cell exit potential (V)
PFHxS	399 > 119	399 > 119	$^{18}O_2$PFHxS	403 > 119	−95	−48	−1
PFOA	413 > 369	413 > 169	$^{13}C_4$PFOA	417 > 372	−45	−16	−9
PFOS	499 > 99	499 > 99	$^{13}C_4$PFOS	503 > 99	−100	−72	−15
PFNA	463 > 419	463 > 219	$^{13}C_5$PFNA	468 > 423	−40	−16	−21
PFDA	513 > 469	513 > 219	$^{13}C_2$PFDA	515 > 470	−40	−16	−21
PFUnDA	563 > 519	563 > 269	$^{13}C_2$PFUdDA	565 > 520	−50	−22	−37
PFTeDA	713 > 669	713 > 369	$^{13}C_2$PFDoDA	615 > 570	−75	−16	−11
TDCA (PFOS interference)	498.3 > 107	498.3 > 80498.3 > 124	–	–	−100	−72	−15

PFCAs precursor ion $[M-H]^-$, quantifier product ion $[M-COOH]^-$.
PFHxS precursor ion $[M-K]^-$, quantifier product ion $[CF_3CF_2]^-$.
PFOS precursor ion $[M-K]^-$, quantifier product ion $[FSO_3]^-$.

Table 2
Figures of merit of the quantitation of PFASs with the proposed method.

PFASs	Solvent calibration linear range (ng mL^{-1})	[a]Correlation coefficient (r)	Retention time (min)	[b]Method LOQ (pg mL^{-1})	[b]Method LOD (pg mL^{-1})
PFHxS	0.1–30	0.9997	31.6	59	20
PFOA	0.01–30	0.9998	33.8	6	2
PFOS	0.05–30	0.9995	36.3	29	10
PFNA	0.01–30	0.9996	35.4	6	2
PFDA	0.05–30	0.9997	37.7	29	10
PFUdDA	0.05–30	0.9991	39.1	29	10
PFTeDA	0.05–30	0.9994	42.7	29	10

[a] $n = 8$.
[b] Calculated on the basis of 80% recoveries for PFASs (mean recoveries from all analyzed serum samples).

injected into the instrument during sample processing sequences in order to check for potential procedural or instrumental contamination.

3.2. Description and characterization of hexanoic acid-based SUPRASs

3.2.1. Synthesis

SUPRASs were synthesized by adding water to solutions containing hexanoic acid in THF. Water promoted the self-assembly of HA and caused the spontaneous formation of oily droplets (i.e. coacervate droplets) that flocculated through the formation of conglomerates of individual droplets. The overall density of such conglomerates was slightly lower than that of the solution in which they were formed, leading to creaming and phase separation (coacervate phase or SUPRAS) from the bulk solution. The term creaming is defined as the macroscopic separation of a dilute emulsion into a highly concentrated emulsion, in which interglobular contact is important, and a continuous phase, under the action of gravity or a centrifugal field. This separation usually occurs upward, but the term may still be applied if the relative densities of the dispersed and continuous phases are such that the concentrated emulsion settles downward [39]. The process occurred from the protonated hexanoic acid form (pK_a 4.8 ± 0.1), so pH values below 4 were required for the formation of the SUPRAS.

Fig. 1 shows the relative concentrations of THF and hexanoic acid at which coacervation occurred leading to the formation of the SUPRAS. Only concentrations of HA within the range of analytical interest were investigated (e.g. below ~10%). Water, the third component, is not represented in this figure but its concentration can be easily calculated as the difference between 100 and the sum of the percentages of HA and THF. Above the coacervation region, the SUPRAS solubilized in the THF:water bulk solution, the boundary being dependent on the amphiphile (i.e. hexanoic acid) concentration. On the other hand, the SUPRAS was only partially formed at percentages of THF below 1% where a miscible mixture of hexanoic acid and SUPRAS was obtained. It is worth mentioning the behavior found for the hexanoic acid in mixtures at relative concentrations of 0.5% for HA and 0.5–4.5% for THF. In these solutions HA was completely solubilized; however, the SUPRAS started to form at a concentration of 5% of THF, despite the fact of the higher solubility of HA in this medium.

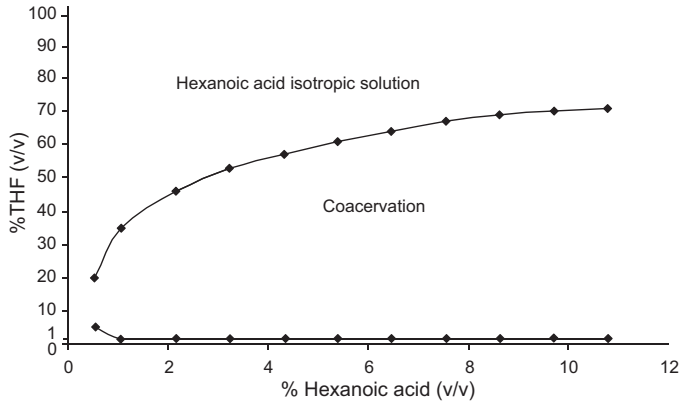

Fig. 1. Phase diagram for the hexanoic acid in mixtures of tetrahydrofuran and water.

3.2.2. Composition

Table 3 shows the composition of the SUPRASs obtained from bulk solutions containing THF, water and HA at different proportions. The percentage of HA incorporated in the SUPRAS from the amount initially present in the bulk solution is also included.

The composition of the SUPRAS was THF dependent; the higher the content of THF in the bulk solution the higher the percentages of THF and water in the SUPRAS, this leading to the synthesis of solvents progressively containing decreased concentrations of HA. On the other hand, the amount of hexanoic acid remaining in the bulk solution after SUPRAS separation was both THF and HA dependent. Thus, at HA concentrations above approximately 3%, the self-assembly of the amphiphile was favored as the THF in the bulk solution, and consequently in the SUPRAS, increased. Incorporation of HA to the coacervate phase was practically quantitative as the percentage of THF in the bulk solution ranged from 30 to 50%. The opposite was found for SUPRAS concentrations nearly and below 2%; solubilization of the amphiphile in the equilibrium solution was favored with increasing concentrations of THF and its incorporation into the SUPRAS did not exceed the 60% over the range investigated. This behavior, as it was expected, negatively influenced the extraction processes since solutes partitioned between the amphiphile in the SUPRAS and the equilibrium solution. As an example, recoveries for PFASs ranged from 39 to 67% with SUPRASs synthesized from 2% of HA and 40% of THF and from 80 to 94% with SUPRAS synthesized from 5% of HA and 50% of THF despite the SUPRAS composition was practically the same (see Table 3). So, only SUPRASs synthesized at hexanoic acid concentrations above 3% were considered in this study.

3.2.3. Volume prediction

The volume of SUPRAS obtained through the self-assembly of hexanoic acid was both THF and amphiphile dependent. So, a series of experiments were carried out to develop an equation for the prediction of the volume of SUPRAS as a function of these components. The aim was to be able to predict the maximum concentration factor that could be obtained under given experimental conditions. For this purpose, a set of SUPRASs was prepared using a variety of amphiphile (3–10%) and THF (1–50%) concentrations within the range of analytical interest according to the results obtained in the above section.

Nonlinear regression was used to fit a model to the data obtained ($n = 60$). This procedure uses the algorithm Marquardt as an

Table 3
SUPRAS compositions and percentage of hexanoic acid (HA) incorporated into SUPRASs at different initial percentages of THF and HA in bulk solutions.

% THF (v/v)	% HA (v/v)											
	2				5				10			
	SUPRAS composition (w/w, %)			% of HA incorporated in SUPRAS	SUPRAS composition (w/w, %)			% of HA incorporated in SUPRAS	SUPRAS composition (w/w, %)			% of HA incorporated in SUPRAS
	THF	Water	HA		THF	Water	HA		THF	Water	HA	
0	–	5	95	–	–	6	94	–	–	5	95	–
1	9	6	85	48	6	6	88	78	5	6	89	72
2	14	7	79	50	10	7	83	76	9	6	85	75
3	25	8	67	53	13	8	79	81	10	7	83	84
5	27	8	65	56	21	9	70	85	20	8	72	86
10	36	9	55	67	33	9	58	82	31	9	60	83
20	57	10	33	56	53	10	37	81	48	10	42	91
30	67	14	19	39	61	12	27	98	58	11	31	98
40	70	20	10	25	69	15	16	91	64	12	24	104
50					70	19	11	95	67	14	19	110

iterative approach to minimize the sum-of-squares of the vertical distances of the experimental points to a proposed curve based on preliminary estimates [40]. The proposed model for predicting the volume of SUPRASs (y, μL) was $y = \theta_1\ \mathrm{HA} + \theta_2\ \mathrm{HA} + e^{\theta_3\ \mathrm{THF}}$, being the amount of hexanoic acid (HA, mg) and the percentage of THF (v/v) in the bulk solution, the independent variables. The units of the parameters θ_1 and θ_2 were μL mg^{-1}, while the parameter θ_3 was dimensionless. The resultant equation was:

$$y = 0.69\ \ \mathrm{HA} + 0.0761\ \ \mathrm{HA}\ \ \mathrm{THF} + e^{0.104\ \ \mathrm{THF}} \quad (1)$$

The asymptotic standard errors of θ_1, θ_2 and θ_3 were 0.05, 0.004 and 0.0006, respectively and the determination coefficient was 99.126%, thus indicating a good capability of prediction of this equation. So, the maximum concentration factors that can be achieved with hexanoic acid-based SUPRASs under given conditions can be known a priori and this makes easier method selection and optimization.

3.2.4. Handling of supramolecular solvents

Application of supramolecular solvents to the extraction of analytes from liquid samples (e.g. urine) always involves their spontaneous formation into the target sample after the addition of solvent ingredients (e.g. THF and hexanoic acid). Practical aspects related to the use of SUPRAS for the extraction of contaminants on liquid food have been reviewed [30,32]. As extraction of solid samples is of interest, supramolecular solvents made up of reverse micelles can be previously synthesized and then aliquots used for extraction [37]. In this case, the synthesis of SUPRASs is usually carried out in 100 mL-glass centrifuge tube which permits to obtain enough volume to treat 10–20 samples. The SUPRAS is then withdrawn using a 1 mL-syringe, transferred to a hermetically closed storage glass vial to avoid THF losses and then stored at 4 °C. Under these conditions, reverse micelle-based SUPRAS are stable for at least one month.

3.3. Optimization

Optimization of the microextraction process was carried out by extracting 622–979 μL of distilled water or newborn calf serum fortified with 5 μg L^{-1} of the target PFASs under a variety of experimental conditions: 4.3–7.5% (v/v) of hexanoic acid, 15–50% (v/v) of THF, 69–1800 mM of hydrochloric acid (μmoles of HCl mL^{-1} of serum); stirring time 0–40 min. The final volume was 1.5 mL. Extractions were carried out according to the procedure specified in Section 2.4.2 and varying each variable in turn while keeping the other constant. ISs were added just prior injection in order to correct MS performance and matrix effects and ensure accurate quantitation during optimization. Experiments were made in triplicate. Selection of the optimal conditions was based on the recoveries (R) and actual concentration factors [ACF = 0.01R (%) × phase volume ratio (PVR)] obtained for PFASs. Phase volume ratios were calculated as the ratio of sample volume over the SUPRAS volume, so they represented the maximum ACFs that could be obtained under given experimental conditions. Two criteria were considered for variable selection, namely the highest possible ACF and recoveries above 75%. The variables investigated were THF and HA concentrations, pH, analyte concentration and stirring time.

Because of the water content of serum, SUPRASs were spontaneously synthesized in these matrices, in a similar way to that occurring in aqueous solutions, as hexanoic acid and THF were added to the samples. The SUPRAS was clearly visible as an immiscible liquid after centrifugation according to the procedure in Section 2.4.2. The volume of SUPRAS thus obtained fit to the Eq. (1) in the range of hexanoic acid and THF concentrations specified in Section 3.2.3. Proteins in the serum agglutinated at percentages of THF below 20% (v/v) and stood as a gelatinous layer between the SUPRAS and the serum after centrifugation. Proteins did not flocculate at percentages of THF in the range 25–50% (v/v) and remained as a dense precipitate at the bottom.

The acidity of the medium was found essential to efficiently extract PFASs from serum. On the contrary, extraction efficiency for these solutes from water (R = 90–100%) was rather independent of the pH in the range of existence of hexanoic acid-based SUPRASs (i.e. below 4). Interaction between PFASs and matrix components were expected to be the reason for the different behavior observed in serum and water. Table 4 shows the recoveries obtained for PFAS-spiked serum as a function of the concentration of hydrochloric acid; they were maximal and above 75% from around 300 mM and then they kept constant up to 1800 mM. Increased acidity possibly favored the break of PFAS-matrix bonds since the extraction efficiency for PFASs increased when HCl concentration did. A concentration of HCl of 600 mM (μmol of HCl/mL of serum) was selected as optimal in order to make the method robust against variations in the amount of matrix components present in the samples.

Table 5 shows the recoveries obtained for PFASs in serum as a function of both THF and hexanoic acid concentrations. The volume of SUPRAS, and so concentration factors, also varied with these reagents according to Eq. (1). Recoveries above 75% and fluorocarbon chain length independent were obtained with SUPRASs made from 35% (v/v) of THF and 5.4% (v/v) of HA. Recoveries increased with the concentration of THF because this solvent facilitated the rupture of PFAS-protein interactions, however SUPRAS volume also

Table 4
Mean PFAS recoveries and standard deviation ($R \pm S$, %) from spiked serum samples as a function of the concentration of hydrochloric acid.

μmol of HCl/mL of serum	$R \pm S$[a]						
	PFHxS	PFOA	PFOS	PFNA	PFDA	PFUnDA	PFTeDA
69	51 ± 3	45 ± 2	64 ± 3	48 ± 1	54 ± 2	54 ± 3	70 ± 1
100	50 ± 2	44 ± 1	55 ± 4	47 ± 2	52 ± 1	55 ± 1	67 ± 4
140	52 ± 1	47 ± 4	65 ± 2	50 ± 5	55 ± 3	60 ± 3	68 ± 3
164	52 ± 4	52 ± 5	65 ± 4	57 ± 3	61 ± 2	66 ± 3	74 ± 2
267	70 ± 3	82 ± 4	79 ± 4	81 ± 2	83 ± 2	81 ± 1	84 ± 2
398	81 ± 5	86 ± 3	78 ± 3	84 ± 1	84 ± 2	82 ± 2	84 ± 2
792	73 ± 4	86 ± 1	83 ± 1	83 ± 1	86 ± 3	83 ± 1	80 ± 1
1500	77 ± 5	82 ± 2	84 ± 4	82 ± 2	81 ± 1	80 ± 1	80 ± 2
1800	73 ± 5	81 ± 3	78 ± 1	80 ± 1	83 ± 2	82 ± 2	83 ± 1

[a] Hexanoic acid = 6.5% and THF = 40%; $n = 3$; spiked PFAS concentration = 5 μg L^{-1}.

Table 5
Mean recoveries and standard deviation ($R \pm S$, %) of PFASs from spiked serum samples at 600 μmoles of HCl/mL of serum as function of the concentration of hexanoic acid and THF.

% (v/v)	Analytes ($R \pm S$[c])						
	PFHxS	PFOA	PFOS	PFNA	PFDA	PFUnDA	PFTeDA
THF[a]							
25	51 ± 7	67 ± 7	55 ± 1	64 ± 2	64 ± 5	66 ± 7	71 ± 2
30	61 ± 2	78 ± 2	73 ± 4	74 ± 2	75 ± 2	75 ± 3	77 ± 2
35	68 ± 1	83 ± 1	81 ± 1	78 ± 1	80 ± 1	81 ± 1	81 ± 1
40	83 ± 6	83 ± 3	79 ± 4	81 ± 1	83 ± 2	82 ± 1	83 ± 2
45	79 ± 1	88 ± 4	82 ± 2	84 ± 2	83 ± 2	87 ± 2	85 ± 4
50	80 ± 1	97 ± 5	92 ± 5	92 ± 4	86 ± 2	92 ± 2	94 ± 5
HA[b]							
4.3	61 ± 1	75 ± 1	78 ± 1	78 ± 1	80 ± 6	81 ± 6	87 ± 6
5.4	75 ± 3	89 ± 2	86 ± 1	82 ± 4	84 ± 3	82 ± 2	82 ± 1
6.5	83 ± 6	83 ± 3	79 ± 4	81 ± 1	83 ± 2	82 ± 1	83 ± 2
7.5	77 ± 3	86 ± 1	86 ± 1	86 ± 1	88 ± 3	88 ± 3	86 ± 4

[a] Hexanoic acid = 6.5% (v/v).
[b] THF = 40% (v/v).
[c] $n = 3$; spiked PFAS concentration = 5 μg L^{-1}.

increased and consequently concentration factors diminished. So, values of 40% of THF and 6.5% (v/v) of HA were selected as optimal, which provided recoveries of 79–83% and ACFs of 1.68–1.76. Simultaneous extraction/concentration of PFASs was based on the formation of mixed aggregates by dispersion and hydrogen bond analyte:extractant interactions. Recoveries were in the intervals 73–90% for all the analytes in the whole range of concentration assessed (i.e. between 0.1 and 15 ng mL^{-1}).

The time for extraction (vortex-shaking, 2.300 rpm) was investigated in the interval 0–40 min. Equilibrium conditions were achieved after 5 min owing to the high solubilizing capability of the SUPRAS. A time of 7 min was fixed for further experiments in order to get maximal reproducibility.

The lack of a mass-labeled homologue for PFTeDA led us to examine the suitability of MPFDoDA as a method IS for this PFAS. Calf and human-1 sera samples were subjected to extraction and analysis after fortification with 5 ng mL^{-1} of both mass-labeled and target PFASs. PFTeDA was accurately quantified with MPFDoDA (recoveries in the range 104–106%) due to the proximity in their retention time, similar extraction efficiency and lack of significantly different matrix effects in the elution window. The rest of PFASs recoveries were in the range 95–107%.

3.4. Analytical performance

3.4.1. Sensitivity

Table 2 shows the analytical figures of merit for the proposed method. The instrumental limits of quantitation (LOQs) and detection (LODs) were determined from standards prepared in 75:25 THF:water (v/v), containing a 5 ng mL^{-1} of the method IS, and using a signal-to-noise ratio of 10 and 3, respectively. Each curve point was quantified using the overall calibration curve and reviewed for accuracy. Method calibration accuracy requirements of 100 ± 25% were met for all analytes. The LOQs and LODs thus obtained were 0.1 and 0.03 ng mL^{-1} for PFHxS; 0.05 and 0.02 ng mL^{-1} for PFOS, PFDA, PFUnDA and PFTeDA; and 0.01 and 0.003 ng mL^{-1} for PFOA and PFNA; respectively. Correlation between peak areas and PFAS concentrations was determined by linear regression and $1/x$ weighted calibration. The correlation coefficients were in the range 0.9991–0.9998 for all analytes indicating good fits.

Due to the lack of blank serum samples, method LOQs and LODs were estimated from the respective instrumental quantitation and detection limits and the mean actual concentration factor obtained (1.7) considering 80% as mean recovery for all PFASs (see Table 2). No significant differences in background noise were observed for serum samples and standards. A typical chromatogram obtained for a standard solution containing 5 ng mL^{-1} of each PFAS is shown in Fig. 2a.

3.4.2. Selectivity

Analysis of PFASs in serum is commonly confronted with the presence of co-eluting matrix components which can cause ionization suppression or enhancement in the ESI source and even perfluoroalkyl sulfonic acids (PFSAs) peak misidentification. Thus, PFSAs have been over-reported in serum due to co-eluting with TDCA and steriod sulfates when using C_{18} columns [19,41]. The masses of these compounds are too close to those of PFHxS and PFOS for accurate analysis by the one-unit resolution QQQ-MS and can lead to overestimation at the 499 > 80, 399 > 80 and 399 > 99 transitions. This problem has been addressed in various ways including the use of other columns providing more selective retention mechanisms (e.g. perfluorooctyl, Synergi hydro-RP or ion-exchange phase columns [19,41,42]) or the use of more selective—but also less sensitive—transitions (mainly 499–99 and 399–119 [41,42]). In this work we quantified PFASs in serum using a C_{18} column and the more selective transitions 399 > 119

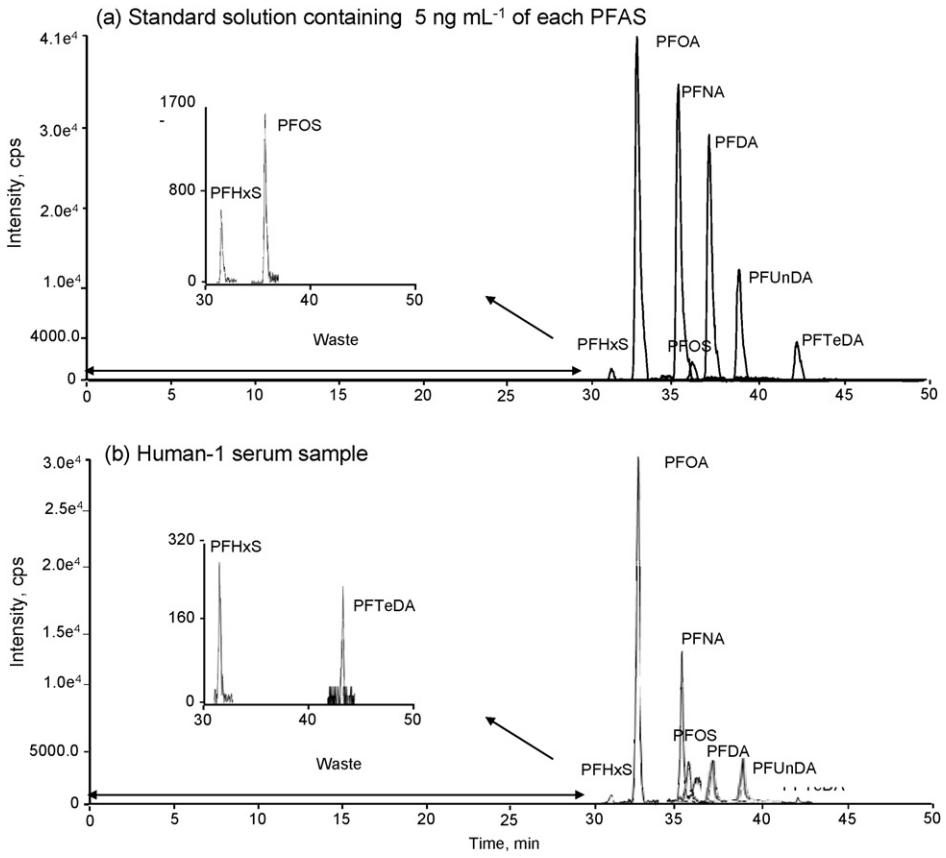

Fig. 2. LC (ESI)–QQQ MS^2 selected ion chromatograms of PFASs obtained from: (a) a standard solution containing 5 ng mL^{-1} of each PFAS (inset; selected ion chromatogram of PFHxS and PFOS), and (b) a human-1 serum sample (inset; selected ion chromatogram of PFHxS and PFTeDA).

and 499 > 99 to prevent PFAS misidentification. The presence of steroid sulfates in the human sera analyzed was confirmed by the transition 399 > 99 while TDCA isomers were detected in all the human and animal serum samples at the transitions 498.3 > 107 and 498.3 > 80. These interferences co-eluted, therefore using the 399 > 119 and 499 > 99 transitions were fundamental to quantify PFHxS and PFOS properly. Although the use of these transitions resulted in a somewhat decreased sensitivity, the overall method sensitivity was sufficient for analysis of human samples. Signal enhancement or suppression was estimated by comparing the response for ISs added to the SUPRAS extracts obtained after serum treatment, just prior injection (5 ng mL^{-1}), to the average response of ISs in the calibration solutions. The serum samples analyzed exhibited low signal suppression (≤12%) or enhancement (≤6%) for all PFASs, including PFHxS and PFOS. Therefore, matrix effects on the ionization of PFASs were negligible in this method and further clean-up steps were not necessary.

3.4.3. Precision

The precision of the method was evaluated by extracting 11 independent fortified (5 ng mL^{-1}) serum samples (calf, horse and bovine). PFAS recoveries ranged between 95 and 111%. The values, expressed as relative standard deviation (RSD), were between 1 and 6% for all the PFASs.

3.5. Analysis of serum samples

Serum samples from animals and humans ($n = 6$) were analyzed in duplicate to prove the suitability of the proposed method to determine PFASs. Table 6 lists the concentrations found for the target compounds. Method recoveries expressed as recovery for the method ISs (concentration level; 5 ng mL^{-1}) ranged between 75 and 89%. Fig. 2b shows the selected ion chromatogram for PFASs extracted from the sample of human serum 1.

No PFHxS neither interferences coming from steroid sulfates were detected in the samples of animal origin; however, interferences co-eluting with PFHxS were detected in all the human samples analyzed at the transitions 399 > 80 and 399 > 99, so the selective transition 399 > 119 was employed for quantitation. Bile salts interfering with PFOS were detected in bovine, horse and human sera at the transition 499 > 80 and so the daughter ion 99 was used for quantitation. The concentrations of PFASs in animal

Table 6
Mean concentrations (pg mL^{-1}) ($n = 2$) obtained for the target compounds in the analysis of serum samples using the proposed method.

Samples	PFHxS	PFOA	PFOS	PFNA	PFDA	PFUnDA	PFTeDA
Calf serum	<LOD	14.3 ± 0.7	197.3 ± 0.6	23 ± 3	<LOQ	69 ± 3	70 ± 10
Bovine serum	<LOD	17 ± 1	156 ± 2	52 ± 1	37 ± 4	72 ± 4	108 ± 13
Horse serum	<LOD	35 ± 2	<LOQ	45 ± 2	<LOQ	71 ± 5	<LOQ
Human serum 1	746 ± 116	1735 ± 35	5168 ± 63	908 ± 33	376 ± 32	624 ± 3	99 ± 3
Human serum 2	678 ± 66	1242 ± 47	4597 ± 171	565 ± 33	261 ± 5	548 ± 23	86 ± 5
Human serum 3	794 ± 41	1219 ± 20	4670 ± 10	567 ± 17	258 ± 5	560 ± 6	84 ± 4

samples ranged between 17 and 197.3 pg mL^{-1} and between 84 and 5168 pg mL^{-1} in human sera. RSDs were between 1 and 15%. The highest concentrations were found for PFOS and PFOA and both branched and linear PFOS were clearly observed in the chromatograms for human samples. The presence of long-chain PFCAs (PFDA, PFUnDA and PFTeDA) in human sera highlights the need for their routine determination along with the most commonly found PFOA and PFOS.

4. Conclusions

A supramolecular solvent consisting of reverse aggregates of hexanoic acid in THF:water mixtures was here described and characterized for the first time and its suitability as a solvent for the microextraction of PFASs in serum was proved. The formation of the supramolecular solvent is spontaneous and requires minute volumes of THF (~600 μL per sample) that additionally provokes the breaking of PFAS-protein interactions. The method offers a valuable alternative to current approaches for the evaluation of human and animal exposure to these contaminants. The major benefits of this method compared to other previously reported are: (a) lower volume of organic solvent per sample extraction (e.g. 600 μL of THF); (b) shorter extraction times (e.g. 7 min); (c) no clean-up or evaporation of extracts required; (d) no need for additional steps or reagents different from those used for the extraction system itself for protein precipitation and no contamination or analyte losses produced during this step; (e) accurate quantitation obtained using IS solvent-based calibration; (f) high sensitivity (e.g. LODs 2–20 pg mL^{-1} and LOQs 6–59 pg mL^{-1}). The method meets the analytical and operational requirements to be used in large-scale epidemiological studies.

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