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A green analytical method for the simultaneous determination of 17 perfluoroalkyl substances (PFAS) in human serum and semen by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)

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ABSTRACT

The ubiquity of perfluoroalkyl substances has raised concerns about the unintended consequences of PFAS exposure on human health. In the present study, an eco-friendly ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method was developed for the simultaneous determination of 17 PFAS in human serum and semen samples. QuEChERS salts $MgSO_4$:NaCl 4:1 (w/w) were used for the extraction. The separation of analytes was performed on an ACQUITY BEH C_{18} column (100 × 2.1 mm, 1.7 µm), using water: methanol 95:5 and methanol as mobile phases A and B, respectively, both containing 2 mM ammonium acetate. Multiple reaction monitoring (MRM) in negative ion mode was used, selecting two transitions for each analyte, except for perfluorobutanoic acid (PFBA) and perfluoropentanoic acid (PFPeA). The analytical method was validated according to the Organization of Scientific Area Committees (OSAC) for Forensic Sciences guidelines and AGREE approach software was used to evaluate the greenness of the method. The developed procedure was applied to the analysis of 10 paired human serum and semen samples, proving the suitability in high throughput laboratories due to the easy preparation and the reduced volume of toxic solvents. Moreover, it allows to perform further investigation on the correlation between serum and semen PFAS concentration, focusing on male reproductive system correlated pathologies, such as male infertility.

1. Introduction

Per- and polyfluoroalkyl Substances (PFAS) represent a class of synthetic chemicals structurally characterized by a tail of fluorinated carbon chains and a head of carboxylic or sulfonic group (Fig. 1). Specifically, the tail is responsible for the unique physicochemical properties of these compounds, such as impermeability to water and greases, resistance to heat and abrasion; differently, the head determines the high solubility in water. In this concern, the C—F single bond is the most inert and one of the strongest bonds in organic chemistry [1]. This explains the high stability to degradation and the accumulation of PFAS in the environment, especially in soil, air and water [2]. Due to their characteristics, these chemicals have become popular in many industrial applications, such as non-stick cookware, water-repellent textiles, firefighting foams, paints and detergents [3]. However, this ubiquity has also raised concerns about the unintended consequences of PFAS exposure. In particular, long-chain PFAS ($C>7$) have been classified as bioaccumulative, while short- chain PFAS ($C<7$) share similar resistance to degradation but a reduced binding to solid materials, resulting in an increased mobility in the environment [4,5]. This bioaccumulation can occur through the ingestion of contaminated food and water or inhalation of airborne particles. Specifically, occupational exposure is a notable route in industries such as firefighting, manufacturing, and construction where PFAS-containing products are prevalent [6]. For

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instance, high serum concentrations in Australian firefighters were reported, due to the perfluorooctane sulfonate (PFOS) and perfluorohexane sulfonate (PFHxS) based aqueous film forming foam (AFFF) [7]. Moreover, PFAS can accumulate in the human body over time; previous biomonitoring studies estimated half-lives of 3–5 years for PFOS and 2–4 years for perfluorooctanoic acid (PFOA) [8–10]. This accumulation may lead to adverse health effects, such as disruptions in hormone regulation, adverse reproductive outcomes [3], dyslipidemia [11], increased cholesterol levels, hypertension, obesity [12], gestational and post-natal lower birth weight [13]. For this reason, the development of analytical methodologies for the determination of PFAS in biological matrices is essential for assessing human exposure levels, potential health risks and for understand their distribution over time.

Several analytical strategies are described in literature for PFAS determination in conventional and unconventional biological matrices, such as serum [14–16], urine [15], hair [17], semen [18] and placenta [19].

In recent years, Green Chemistry has gained significant attention of scientists from different areas of chemistry, which aims to reduce the negative impacts of the used chemical products on human health. In analytical chemistry, the promotion of sustainability and the reduction of the environmental impact of chemical processes is constantly increasing, especially in high throughput laboratories, which produce a large amount of chemical wastes. In this concern, Green Analytical

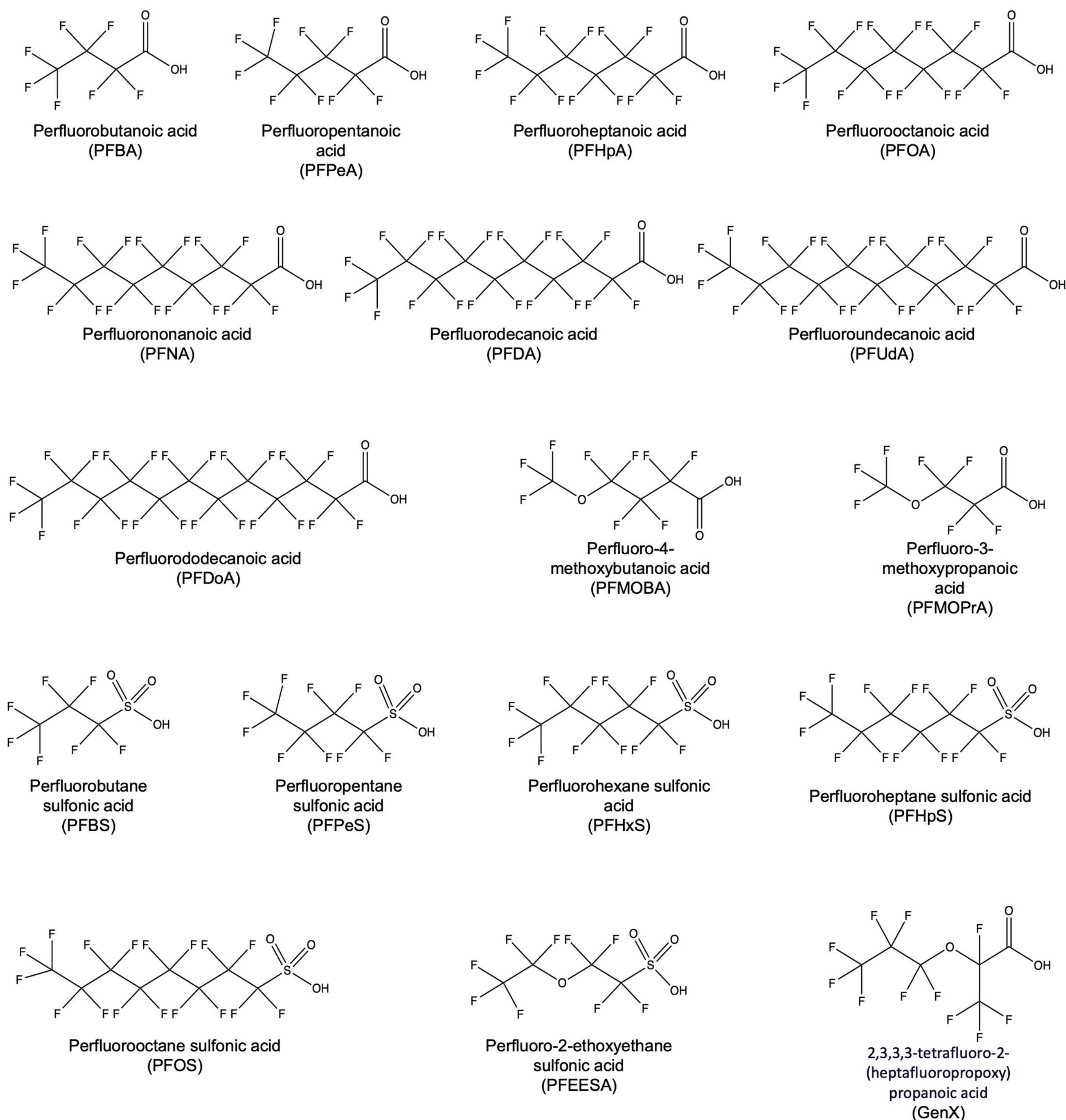

Fig. 1. PFAS chemical structures.

Chemistry (GAC) plays a crucial role in minimizing the waste generation and the use of hazardous reagents enhancing the analytical technique efficiency. For this reason, the aim of our study was to develop and validate a green analytical method for the simultaneous determination of 17 PFAS in serum and semen by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), following the Green Chemistry principles.

2. Materials and methods

2.1. Chemicals

Perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUdA), perfluorododecanoic acid (PFDoA), PFOS, PFHxS, perfluoro-4-methoxybutanoic acid (PFMOBA), perfluoro-2-ethoxyethane-sulfonic acid (PFEESA) and GenX were purchased from LGC (Queens Road, Teddington, Middlesex, UK). Perfluorobutane sulfonic acid (PFBS), perfluoropentane sulfonic acid (PFPeS), perfluoroheptane sulfonic acid (PFHpS), perfluoro-3-methoxypropanoic acid (PFMOPrA) and the internal standards (IS) $^{13}C_6$-PFHxS, $^{13}C_8$-PFOA, $^{13}C_8$-PFOS, $^{13}C_9$-PFNA were supplied from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Standards were stored according to the information provided by the supplier. LC-MS grade water, methanol and acetonitrile were supplied from Carlo Erba (Cornaredo, Italy). Ammonium acetate was purchased from Agilent Technologies (Palo Alto, CA, USA). Fetal bovine serum (FBS), $MgSO_4$, NaCl, sodium citrate 2 H_2O, KCl, K_2HPO_4, sodium pyruvate, sodium lactate, glucose 1 H_2O, fructose, $NaHCO_3$, urate, urea, $ZnSO_4$ 7 H_2O and $CaCl_2$ were obtained from Sigma-Aldrich (Milano, Italy).

2.2. Calibrators and quality control (QC) solutions

Working standard solutions at 10 µg/mL and 1 µg/mL containing all analytes under investigation were prepared by appropriate methanolic dilution of stock solutions. IS solution of $^{13}C_6$- PFHxS, $^{13}C_8$-PFOA, $^{13}C_8$-PFOS and $^{13}C_9$-PFNA was prepared at 1 µg/mL by dilution of stock solution. Serum calibrators were prepared adding the appropriate working standard solution volumes in FBS at 0.5, 10, 50, 200, 500, 1000 ng/mL. Semen calibrators were prepared adding the adequate working standard solution volumes in artificial seminal fluid at 0.5, 1.0, 2.5, 5.0, 10, 20 ng/mL. Low-, medium- and high- quality control samples were set at 1.5, 400 and 800 ng/mL for serum, respectively, and 1.5, 8.0 and 16 ng/mL for semen, respectively.

2.3. Human samples

Real human paired serum and semen samples were collected and donated by "International Society of Doctors for the Environment (ISDE)" (Vicenza, Italy). All volunteers gave written informed consent before their inclusion in the project. The study was carried out according to the Declaration of Helsinki and approved by the local ethical committee for human research (protocol no. 113421).

2.4. Serum and semen sample preparation

Serum and semen underwent the same treatment protocol. A 200 µL aliquot was fortified with internal standard (IS) and 600 µL acetonitrile was added for protein precipitation. The supernatant was collected and 200 mg QuEChERS salts $MgSO_4$:NaCl 4:1 (w/w) was added. Then, samples were vortexed and centrifuged at 4000 rpm for 5 min. The supernatant layer was collected and dried under nitrogen stream. Samples were reconstituted in 100 µL water:methanol 80:20 (v/v), before the injection of 10 µL in the UPLC-MS/MS system.

2.5. Instrumental analysis

The analysis of PFAS was performed with an ultra-high performance liquid chromatography system (Waters Acquity UPLC, Waters Corporation, Milan, Italy) coupled to a triple quadrupole mass spectrometer (Waters Xevo TQ, Waters Corporation) equipped with an electrospray ionization source operating in negative mode (ESI-). The separation of analytes was carried out using an ACQUITY UPLC BEH C_{18} column (2.1 mm×100 mm, 1.7 µm, Waters Corporation). Mobile phase A consisted in 2 mM ammonium acetate in water:methanol 95:5, while 2 mM ammonium acetate in methanol was mobile phase B. The linear elution gradient and the flow were reported in Table 1. The autosampler temperature was set to 10°C and the column oven temperature was 35°C.

The mass spectrometer (MS) operated in multiple reaction monitoring (MRM) acquisition mode, selecting two transitions for each analyte and IS, where possible, as reported in Table 2. MS parameter setting was optimized by the individual infusion of neat standards (50 ng/mL in methanol) and by ramping cone voltage and collision energy. The capillary voltage was 3.0 kV, cone gas flow rate was set to 150 L/h, source temperature was 150°C, desolvation gas flow rate 850 L/h.

2.6. Method validation

The analytical method was validated according to Organization of Scientific Area Committees (OSAC) for Forensic Sciences guidelines. In particular, linearity, sensitivity, accuracy and precision, carryover, dilution integrity and stability were evaluated. Moreover, recovery and matrix effect were assessed following the scheme proposed by Matuszewski et al. [20].

Considering the difficulty to obtain blank human serum and semen, FBS was used as blank serum and artificial seminal fluid was prepared according to Gholizadeh et al. [21] and was used as blank semen. Both matrices were screened, and the absence of contamination was confirmed.

2.6.1. Linearity

Linearity was assessed by preparing 5 calibration curves on 5 different days. Each calibrator was required to be quantified within 15% of the target concentration and the coefficient of determination was required to be ≥0.99. Moreover, the acceptable quantifying/confirming transition ratios (for analytes where two transitions where chosen) was within ±20% of the average ratio in calibrators. Mandel test was also performed to assess linearity.

2.6.2. Sensitivity

Sensitivity was assessed in terms of limit of detection (LOD) and limit of quantification (LOQ). Specifically, the LOD determination was performed by spiking blank matrix at the LOQ and diluting 5-, 10-, 20-fold. The LOD for each analyte was defined as the lowest concentration at which a peak eluted within ±0.1 min of the average calibrator retention

Table 1
Gradient elution and flow rate.

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.3	100	0	6
1.0	0.3	90	10	6
2.0	0.3	80	20	6
5.0	0.3	55	45	6
8.0	0.3	30	70	6
9.0	0.3	20	80	6
10.0	0.4	5	95	6
13.0	0.4	5	95	6
13.5	0.3	100	0	6
20.0	0.3	100	0	6

Table 2
MRM acquisition mode parameters.

Analyte	Parent (m/z)	Daughter (m/z)	Dwell (s)	Cone (V)	Collision (V)	RT (min)	IS
PFBA	212.9	169*	0.009	10	10	4.08	2
PFPeA	262.9	219*	0.009	10	5	6.39	
PFHpA	362.9	169	0.009	15	15	8.67	
	362.9	319*	0.009	15	10		
PFOA	412.9	169	0.009	10	10	9.30	
	412.9	369*	0.009	10	15		
PFNA	462.9	219	0.009	10	15	9.73	3
	462.9	419*	0.009	10	10		
PFDA	512.9	219	0.009	15	10	10.04	
	512.9	469*	0.009	25	20		
PFUdA	562.9	269	0.009	25	20	10.17	
	562.9	519*	0.009	25	10		
PFDoA	612.9	169	0.009	30	25	10.32	
	612.9	569*	0.009	30	10		
PFBS	298.9	80.1*	0.009	15	30	6.81	1
	298.9	99.1	0.009	15	30		
PFPeS	348.9	80.1*	0.009	10	10	7.95	
	348.9	99.1	0.009	30	30		
PFHxS	398.9	80.1*	0.009	10	35	8.74	
	398.9	99.1	0.009	10	30		
PFHpS	448.9	80.1*	0.009	15	35	9.33	
	448.9	99.1	0.009	15	35		
PFOS	498.9	80.1*	0.009	15	40	9.74	4
	498.9	99.1	0.009	15	40		
PFEESA	314.8	82.7*	0.009	25	25	7.32	1
	314.8	134.8	0.009	25	25		
PFMOPrA	229	85*	0.009	10	10	5.05	2
	229	185	0.009	10	3		
PFMOBA	278.7	84.8*	0.009	10	10	6.89	
	278.7	234.8	0.009	10	10		
GenX	285	119	0.009	5	35	8.05	
	285	169*	0.009	5	7		
$^{13}C_6$-PFHxS	405	80.1*	0.009	10	40	8.72	1
	405	99.1	0.009	10	35		
$^{13}C_8$-PFOA	421	172	0.009	5	15	9.28	2
	421	375.9*	0.009	5	10		
$^{13}C_9$-PFNA	472	223	0.009	10	15	9.70	3
	472	426.9*	0.009	10	10		
$^{13}C_8$-PFOS	507	80.1*	0.009	15	40	9.71	4
	507	99.1	0.009	15	40		

Abbreviations: GenX, 2,3,3,3-tetrafluoro-2-heptafluoropropoxy-propanoic acid; PFBA, perfluorobutanoic acid; PFBS, perfluorobutanesulfonic acid; PFDA, perfluorodecanoic acid; PFDoA, perfluorododecanoic acid; PFEESA, perfluoro-2-ethoxyethane sulfonic acid; PFHpA, perfluoroheptanoic acid; PFHpS, perfluoroheptane sulfonic acid; PFHxS, perfluorohexanesulfonic acid; PFMOBA, perfluoro-4-methoxybutanoic acid; PFMOPrA, perfluoro-3-methoxypropanoic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid; PFPeA, perfluoropentanoic acid; PFPeS, perfluoropentanesulfonic acid; PFUdA, perfluoroundecanoic acid.

* Daughter ion used for quantification

time with a signal-to-noise ratio ≥3.

LOQ was assessed by spiking blank matrix at the lowest non-zero calibrator. The acceptable criteria were the retention time within ±0.1 min of the average calibrator retention time and the quantification ±20% of the target concentration.

2.6.3. Accuracy and precision

Accuracy was assessed by fortifying three separate blank matrices for each QC sample (low, medium, high) over five different runs. The maximum acceptable bias was ±20% of the target concentration.

Precision was evaluated by analyzing in triplicate each QC sample (low, medium, high) over five different runs performed in the same day and in 5 different days. Precision was expressed as percent coefficient of variation (%CV) and acceptable criteria was ±20%.

2.6.4. Carryover

The carryover was assessed by analyzing in triplicate blank samples after the highest calibrator. Specifically, the peaks eluting within ±0.1 min of the average calibrator retention time were evaluated. In this case, carryover was negligible if no peaks were present with a signal-to-noise ratio ≥3.

2.6.5. Dilution integrity

Dilution integrity was evaluated by spiking blank matrices at 2-fold the highest point of the calibration curve and analyzing in triplicates. The performed dilutions were 2, 5, 10 and 20 with blank matrix. Analytes were required to be quantified within ±20% of the target concentration.

2.6.6. Stability

The stability of the analytes was evaluated at +4°C, at room temperature for 24 h, after 3 freeze/thaw cycles (-20°C) and in water: methanol 80:20 (reconstitution solvents) after extraction in the LC autosampler. The procedure was performed considering 4 replicates of QC samples (low, medium, high). Analytes were stable if the quantification was within ±20% the target concentration.

The processed sample stability was assessed spiking 3 blank matrices at low and high QC. Samples were extracted and the reconstituted solvents were combined and mixed. Then, samples were divided in different vials and analyzed. Analytes were considered stable until the average ratio analyte/internal standard area compared to the time zero response exceed ±20%.

2.6.7. Recovery and matrix effect

Recovery and matrix effect were assessed by spiking blank matrices at low, medium and high QC concentration. Three different sets of samples were prepared. In set A, the internal standard was added before the extraction; in set B, the internal standard was added after the extraction and before the evaporation; set C was the neat standards reconstituted in water:methanol 80:20. For the calculations, the mean chromatographic peak area of each analyte was considered. In particular, recovery was assessed by dividing set B by set A; differently, matrix effect was calculated dividing set B by set C. Acceptable criteria were ±30% target concentration.

2.7. Method greenness evaluation

The Analytical GREEnness (AGREE) calculator was applied to assess the environmentally friendly index of this novel analytical procedure [20]. The AGREE final score was recorded using the web app version of the tool. Each criterion refers to a Green Chemistry principle. The weight, from 1 to 4, was assigned depending on the relevance of the criterion for the improvement of analytical toxicology procedures and the differences with other published methods. More specifically, a weight of 2 was applied to criteria 1, 3, 4, 5, 8, 9, 10, 11, 12, corresponding to direct analytical techniques, position of the analytical device, distinct steps in the sample preparation procedure, miniaturized and automated methods, number of analytes detected in the method, total power consumption, reagents obtained from renewable sources, toxic reagents used and operator's safety, respectively. A weight of 3 was assigned to criteria 2 (small amount of sample size), 6 (absence of derivatizaton step), and 7 (reduced volume of generated analytical waste).

3. Results and discussion

3.1. Method optimization

The UPLC conditions were mainly established by the selection of chromatographic column, the optimization of the mobile phases' composition and elution gradient for the effective separation of PFAS under investigation. In particular, the ACQUITY BEH C_{18} column (100 × 2.1 mm, 1.7 μm, Waters Corporation) allowed the baseline resolution

of analytes. Moreover, different mobile phases were investigated during the preliminary tests. Water:acetonitrile 95:5 (v/v) and acetonitrile were considered as mobile phases A and B, respectively. However, this composition was discarded due to the toxicity of acetonitrile and the not satisfying separation of short-chain PFAS. Thus, acetonitrile was replaced by methanol, obtaining baseline resolution of analytes and a solvent with less environmental impact [21,22]. These considerations were supported by the use of Analytical GREEnness (AGREE) calculator.

The chromatographic gradient was optimized in order to obtain the baseline separation of all analytes, enhancing the peak resolution. Indeed, the long chromatographic run-time facilitated the re-equilibration following the gradient elution and ensured method reproducibility and robustness in analytical sets including a high number of samples. During the method development, several tests were performed to reduce the chromatographic run-time; among these, the reduction of the re-equilibration affected the retention times of PFBA, PFMOPrA and PFPeA and the reproducibility. For these reasons, the reported gradient represented the best compromise. Figs. 2 and 3 show representative chromatograms in overlay of all analytes under investigation at LOQ concentrations in serum and semen, respectively; moreover, chromatograms of serum and semen medium QC levels are reported in Supplementary Material Fig. S3 and S4, respectively.

Methanol and acetonitrile were investigated also as protein precipitation solvents. In this case, the use of acetonitrile could not be avoided, thus the smallest possible volume was used (600 μL).

Different extraction techniques were tested, such as liquid-liquid extraction (LLE) or solid-phase extraction (SPE). However, LLE was discarded due to the required organic solvents, while SPE was not ideal for large number of samples. Consequently, QuEChERS (acronym for Quick, Easy, CHeap, Effective, Rugged, and Safe) demonstrated to be the optimal compromise for the method greenness, high recoveries and suitability for high-throughput laboratories [23]. Several salts compositions were tested for this extraction and $MgSO_4$:NaCl 4:1 (w/w) allowed the highest recovery percentages. Moreover, the QuEChERS purification step did not increase recovery rates. Indeed, the primary secondary amine (PSA) used for the sample clean-up yielded percentages in the range 90–111%; considering the obtained acceptable values without this step, the choice was to perform only the extraction step saving time and costs.

Finally, different reconstitution solvents were tested, such as water, methanol and mixtures with different percentages of these latter. In particular, water:methanol 50:50 (v/v) gave peak splitting for perfluorobutanoic acid (PFBA); for this reason, the increase of the water percentage to water:methanol 80:20 (v/v) resulted in the best peak shape for all the analytes.

During the method development, particular attention was given to the elimination of PFAS contamination from inner instrument components and from laboratory tools, such as vials and tubes. Waters PFAS Solution Kit (Waters Corporation) was installed to the UPLC system in order to delay the retention time of PFAS from solvents and tube lines avoiding the co-elution with PFAS from samples. Polypropylene material was the choice to ensure the absence of PFAS contamination from laboratory tools.

3.2. Evaluation of the method greenness

Several approaches were developed for the evaluation of the Green Analytical Chemistry metrics, such as the Analytical Eco-Scale [24], the Green Analytical Procedures Index [25], the National Environmental Methods Index [26] or the abovementioned AGREE approach. The main advantage of this latter is the inclusion of all the GAC principles, providing an overall score, which considers the weight of each criterion.

While the default weight of 2 was applied to the majority of criteria, the weight of 3 was applied to criterion 2, considering that a small volume of sample is important in a green analytical technique to use less volume of solvents and, consequently, to reduce the amount of waste. The same weight was applied to criterion 6 due to the choice of UPLC-MS/MS also to avoid derivatization step of gas-chromatographic techniques, which usually involves toxic and polluting reagents. Finally, the criterion 7 was a consequence of criterion 2, considering that a reduced amount of waste is crucial especially in laboratories performing high throughput analyses. For this reason, the weight of 3 was assigned.

The final score was 0.72 and the pictogram highlighted some environmentally friendly characters and some hazardous subsections (Fig. 4). Specifically, the absence of acetonitrile in the mobile phases and the reduced volume used in the sample preparation, the small sample volume and the reduced amount of waste represented the greenest aspects of the procedure. Contrarily, criteria 3 and 9 highlighted the most hazardous subsections, corresponding to the off-line sampling and the energy consumption by the LC-MS/MS instrument, respectively. However, considering the nature of biological matrices and the field of application, a different sampling procedure could not be performed, as

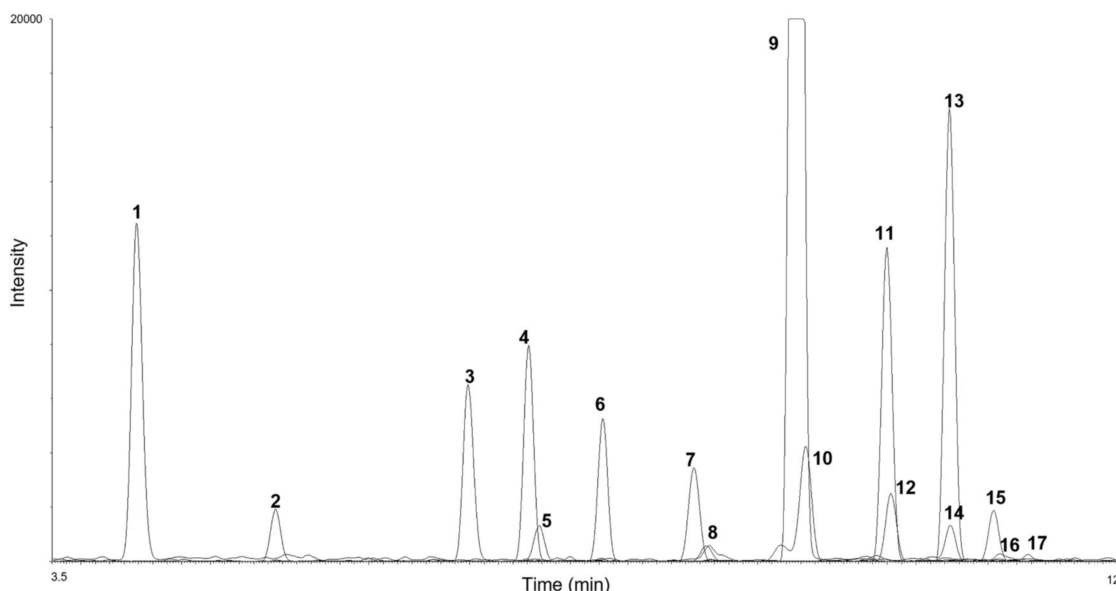

Fig. 2. Overlay chromatogram of the quantifying transitions of all analytes spiked at medium QC in semen. PFBA (1), PFMOPrA (2), PFPeA (3), PFBS (4), PFMOBA (5), PFEESA (6), PFPeS (7), GenX (8), PFHpA (9), PFHxS (10), PFOA (11), PFHpS (12), PFNA (13), PFOS (14), PFDA (15), PFUdA (16), PFDoA (17).

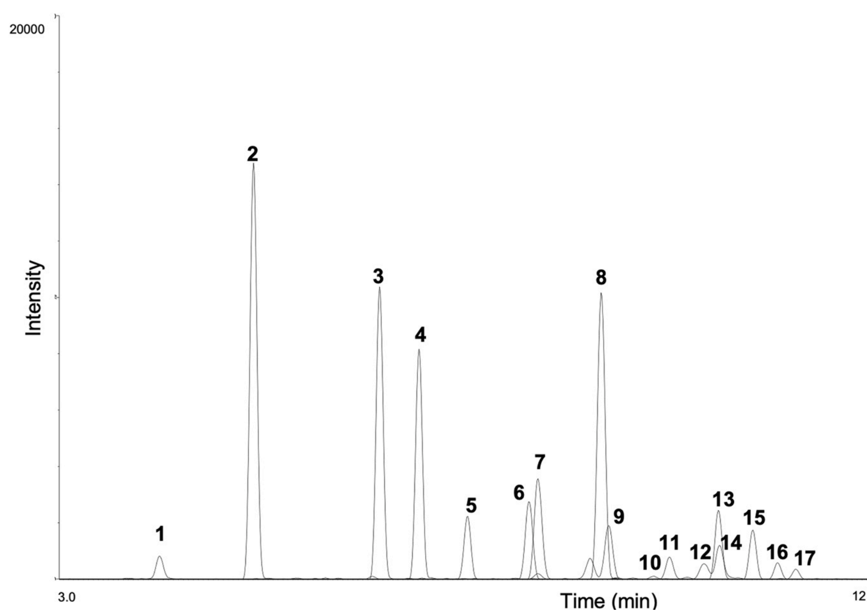

Fig. 3. Overlay chromatogram of the quantifying transitions of all analytes spiked at medium QC in serum. PFBA (1), PFMOPrA (2), PFPeA (3), PFBS (4), PFMOBA (5), PFEESA (6), PFPeS (7), GenX (8), PFHpA (9), PFHxS (10), PFOA (11), PFHpS (12), PFNA (13), PFOS (14), PFDA (15), PFUdA (16), PFDoA (17).

Fig. 4. AGREE score.

well as the analytical technique that still represents the gold standard for toxicological analysis. Thus, the optimized conditions are a satisfying compromise between the greenness and the desirable performances. Indeed, the use of toxic solvents was reduced and only 600 μL acetonitrile were used for protein precipitation. Contrarily, several methods available in literature presented acetonitrile as organic solvent in the mobile phases [27,28]. Overall, QuEChERS extraction proved to be the greenest aspect of this method; other extraction procedures available in literature were based on SPE or LLE. Specifically, one of the most used organic solvents used for LLE is methyl tertiary butyl ether (MTBE) [29–31], a persistent groundwater and surface water pollutant [32]. The resulting concern is due to the use of this organic solvent in high-throughput laboratories, which generates a large amount of polluting waste. The large number of analytes simultaneously detected was also an advantage.

3.3. Method validation

Validation parameters for serum and semen are reported in Tables 3 and 4, respectively, and satisfied the OSAC validation requirements. In particular, the method resulted linear for all analytes under investigation with a determination coefficient (r^2) always higher than 0.99. LOD and LOQ were 0.1 and 0.5 ng/mL. The method presented an intra-day precision in the range 86–115% and 81–120% for serum and semen, respectively; the inter-day precision was in the range 86–115% for serum and 81–119% for semen. Moreover, the accuracy was in the range 80–109% and 80–120% for serum and semen, respectively. No carry-over was observed in blank samples after the injection of the highest point of the calibration curve. Samples analyzed for dilution integrity tests were quantified ±20% target concentration for all analytes. All analytes were stable at room temperature for 24 h, refrigerated (4°C) for 24 h, after three/thaw cycles, 24 h post-extraction in the autosampler, and up to 6 months when stored at −20°C with respect to time zero response. No interfering peaks due to contaminants from the extraction procedure were detected at the retention time of analytes under investigation and internal standards, suggesting that laboratory tools made of polypropylene were suitable for PFAS analysis. In this concern, the chromatograms of blank serum and semen samples are provided in Supplementary Material Fig. S1 and S2, respectively. Recovery percentages were 80–120% and 81–120% for serum and semen, respectively, while matrix effect was in the range 80–119% for serum and 87–115% for semen.

3.4. Analysis of paired serum and semen samples

The newly developed and validated analytical method was applied for the determination of PFAS in 10 paired serum and semen samples. These biological matrices were donated by Italian volunteers from “zona rossa” of Veneto region, a highly exposed territory [33].

As shown in Tables 5 and 6, PFOA, PFDA, PFHxS, PFHpS and PFOS were the most detected compounds in serum, with average values of 37.0, 1.0, 9.5, 1.1 and 7.7 ng/mL, respectively. PFOA was the only compound detected in 100% semen samples with an average concentration of 1.4 ng/mL. The overlay chromatograms of real serum and semen samples are provided in Figs. 5 and 6, respectively. Considering the obtained results, a limitation of the analytical method may be represented by the LOQ values for those analytes which were not quantified in real semen samples. However, few studies were available on the PFAS concentrations in this matrix; indeed, most of the study correlated the serum PFAS concentration to semen quality parameters. Also, only PFOA was found at high levels in serum and was determined in all semen samples, suggesting that the PFAS accumulation in semen occurs in subject with a high exposure level. The possibility to monitor a wide

Table 3
Validation parameters in serum. Low-, medium- and high- quality control samples (QC) at 1.5 ng/mL, 400 ng/mL and 800 ng/mL, respectively.

Analyte	Linear Range (ng/mL)	r^2	$F_{crit\ 95\%}$	LOD (ng/mL)	LOQ (ng/mL)	Accuracy (%)			Intra-day precision CV (%)			Inter-day precision CV (%)			Recovery (%)			Matrix Effect (%)		
						Low QC	Mid QC	High QC	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC
PFBA	0.5–1000	0.997	2.2	0.1	0.5	87	94	81	110	95	95	110	87	96	114	111	120	108	119	111
PFPeA	0.5–1000	0.991	1.9	0.1	0.5	106	107	86	115	115	108	106	112	110	83	88	109	102	99	85
PFHpA	0.5–1000	0.993	0.6	0.1	0.5	101	83	96	86	106	110	91	95	102	120	88	118	94	102	99
PFOA	0.5–1000	0.999	1.3	0.1	0.5	97	88	85	103	109	108	103	98	115	115	102	85	119	96	102
PFNA	0.5–1000	0.999	3.4	0.1	0.5	100	82	105	90	104	113	100	115	98	110	108	104	113	90	108
PFDA	0.5–1000	0.992	2.9	0.1	0.5	108	82	87	111	88	92	103	97	90	115	112	89	114	111	107
PFUdA	0.5–1000	0.994	0.9	0.1	0.5	88	108	109	94	115	102	110	86	92	113	108	115	101	82	114
PFDoA	0.5–1000	0.996	0.8	0.1	0.5	107	105	101	93	105	103	90	101	94	88	93	109	82	114	86
PFBS	0.5–1000	0.998	1.2	0.1	0.5	96	87	82	91	87	89	115	94	112	96	115	89	109	84	102
PFPeS	0.5–1000	0.991	1.4	0.1	0.5	96	80	89	98	99	115	103	115	113	94	86	80	105	113	89
PFHxS	0.5–1000	0.999	2.6	0.1	0.5	103	86	106	86	110	91	100	90	113	112	102	108	102	104	118
PFHpS	0.5–1000	0.997	3.5	0.1	0.5	98	96	82	93	91	98	97	92	103	85	81	82	84	108	119
PFOS	0.5–1000	0.998	2.9	0.1	0.5	102	80	99	106	108	91	104	104	110	116	87	102	95	102	82
PFEESA	0.5–1000	0.994	1.8	0.1	0.5	95	98	86	114	100	89	114	90	113	103	110	113	81	98	80
PFMOPrA	0.5–1000	0.992	1.0	0.1	0.5	108	87	108	98	93	91	92	87	86	99	85	118	84	112	111
PFMOBA	0.5–1000	0.995	2.5	0.1	0.5	108	86	85	107	100	113	97	98	113	87	101	94	102	83	92
GenX	0.5–1000	0.992	0.7	0.1	0.5	107	103	104	97	86	109	99	105	86	95	98	81	85	117	81

Abbreviations: CV, coefficient of variation; GenX, 2,3,3,3-tetrafluoro-2-heptafluoropropoxy-propanoic acid; LOD, limit of detection; LOQ, limit of quantification; PFBA, perfluorobutanoic acid; PFBS, perfluorobutanesulfonic acid; PFDA, perfluorodecanoic acid; PFDoA, perfluorododecanoic acid; PFEESA, perfluoro-2-ethoxyethane sulfonic acid; PFHpA, perfluoroheptanoic acid; PFHpS, perfluoroheptane sulfonic acid; PFHxS, perfluorohexanesulfonic acid; PFMOBA, perfluoro-4-methoxybutanoic acid; PFMOPrA, perfluoro-3-methoxypropanoic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid; PFPeA, perfluoropentanoic acid; PFPeS, perfluoropentanesulfonic acid; PFUdA, perfluoroundecanoic acid; QC, quality control; r^2, correlation coefficient.

Table 4
Validation parameters in semen. Low-, medium- and high-quality control (QC) samples at 1.5 ng/mL, 8.0 ng/mL and 16 ng/mL, respectively.

Analyte	Linear Range (ng/mL)	r^2	$F_{crit\ 95\%}$	LOD (ng/mL)	LOQ (ng/mL)	Accuracy (%)			Intra-day precision CV (%)			Inter-day precision CV (%)			Recovery (%)			Matrix Effect (%)		
						Low QC	Mid QC	High QC	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC
PFBA	0.5–20	0.991	0.6	0.1	0.5	88	108	89	84	100	104	85	115	102	107	111	92	103	95	109
PFPeA	0.5–20	0.993	1.5	0.1	0.5	90	88	120	115	116	113	98	115	119	115	85	113	107	111	106
PFHpA	0.5–20	0.997	1.3	0.1	0.5	85	119	82	109	99	120	106	105	102	83	109	93	114	92	98
PFOA	0.5–20	0.999	2.4	0.1	0.5	111	113	100	81	84	108	106	81	89	106	83	113	112	110	105
PFNA	0.5–20	0.998	1.9	0.1	0.5	90	119	109	102	90	100	103	94	87	101	91	81	108	104	96
PFDA	0.5–20	0.995	3.1	0.1	0.5	101	110	97	108	108	109	112	116	95	92	117	85	114	101	111
PFUdA	0.5–20	0.992	0.9	0.1	0.5	118	115	81	86	108	90	88	95	94	89	102	108	110	91	111
PFDoA	0.5–20	0.993	1.5	0.1	0.5	119	109	120	114	112	102	104	117	107	87	104	99	87	114	95
PFBS	0.5–20	0.991	0.6	0.1	0.5	111	88	115	108	86	92	94	83	102	88	88	93	110	105	100
PFPeS	0.5–20	0.998	3.1	0.1	0.5	106	108	95	82	119	91	88	92	90	92	117	104	87	114	101
PFHxS	0.5–20	0.999	1.4	0.1	0.5	101	95	86	89	94	113	119	90	91	119	116	93	99	101	115
PFHpS	0.5–20	0.996	1.7	0.1	0.5	82	111	82	113	105	88	108	85	104	98	93	81	107	103	90
PFOS	0.5–20	0.999	2.2	0.1	0.5	105	111	108	84	113	100	118	84	90	120	85	110	111	112	113
PFEESA	0.5–20	0.997	3.6	0.1	0.5	97	112	91	94	97	112	89	100	92	82	120	114	111	99	109
PFMOPrA	0.5–20	0.991	0.8	0.1	0.5	113	109	84	101	91	101	88	92	115	100	85	115	93	89	112
PFMOBA	0.5–20	0.998	0.7	0.1	0.5	103	96	80	98	98	81	119	83	91	120	100	109	108	91	103
GenX	0.5–20	0.993	1.1	0.1	0.5	97	110	82	81	97	117	103	107	112	96	101	106	115	95	111

Abbreviations: CV, coefficient of variation; GenX, 2,3,3,3-tetrafluoro-2-heptafluoropropoxy-propanoic acid; LOD, limit of detection; LOQ, limit of quantification; PFBA, perfluorobutanoic acid; PFBS, perfluorobutanesulfonic acid; PFDA, perfluorodecanoic acid; PFDoA, perfluorododecanoic acid; PFEESA, perfluoro-2-ethoxyethane sulfonic acid; PFHpA, perfluoroheptanoic acid; PFHpS, perfluoroheptane sulfonic acid; PFHxS, perfluorohexanesulfonic acid; PFMOBA, perfluoro-4-methoxybutanoic acid; PFMOPrA, perfluoro-3-methoxypropanoic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid; PFPeA, perfluoropentanoic acid; PFPeS, perfluoropentanesulfonic acid; PFUdA, perfluoroundecanoic acid; QC, quality control; r^2, coefficient of determination.

Table 5
PFAS concentrations in real serum samples.

Sample	Concentration (ng/mL)																
	PFBA	PFPeA	PFHpA	PFOA	PFNA	PFDA	PFUdA	PFDoA	PFBS	PFPeS	PFHxS	PFHpS	PFOS	PFEESA	PFMOPrA	PFMOBA	GenX
1	0.6	<LOD	<LOQ	37.8	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	4.6	1.1	3.5	<LOD	<LOD	<LOD	<LOD
2	0.8	<LOD	<LOQ	72.3	0.68	<LOQ	<LOD	<LOD	<LOD	<LOD	4.4	1.0	3.6	<LOD	<LOD	<LOD	<LOD
3	1.0	<LOD	<LOQ	15.2	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	2.4	0.8	2.7	<LOD	<LOD	<LOD	<LOD
4	<LOQ	<LOD	<LOQ	22.8	<LOQ	0.8	<LOD	<LOD	<LOD	<LOD	13.7	1.7	6.2	<LOD	<LOD	<LOD	<LOD
5	<LOQ	<LOD	<LOQ	26.9	<LOQ	0.5	<LOD	<LOD	<LOD	<LOD	11.5	1.1	2.8	<LOD	<LOD	<LOD	<LOD
6	<LOQ	<LOD	<LOQ	69.3	<LOQ	2.5	<LOD	<LOD	<LOD	<LOD	33.3	0.8	41.8	<LOD	<LOD	<LOD	<LOD
7	<LOQ	<LOD	<LOQ	45.1	<LOQ	0.5	<LOD	<LOD	<LOD	<LOD	9.4	1.4	9.1	<LOD	<LOD	<LOD	<LOD
8	<LOQ	<LOD	<LOQ	44.2	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	6.1	1.4	1.1	<LOD	<LOD	<LOD	<LOD
9	<LOQ	<LOD	<LOQ	6.1	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	1.6	0.7	2.8	<LOD	<LOD	<LOD	<LOD
10	<LOQ	<LOD	<LOQ	29.9	<LOQ	0.6	<LOD	<LOD	<LOD	<LOD	8.5	0.8	3.7	<LOD	<LOD	<LOD	<LOD

Abbreviations: GenX, 2,3,3,3-tetrafluoro-2-heptafluoropropoxy-propanoic acid; LOD, limit of detection; LOQ, limit of quantification; PFBA, perfluorobutanoic acid; PFBS, perfluorobutane sulfonic acid; PFDA, perfluorodecanoic acid; PFDoA, perfluorododecanoic acid; PFEESA, perfluoro-2-ethoxyethane sulfonic acid; PFHpA, perfluoroheptanoic acid; PFHpS, perfluoroheptane sulfonic acid; PFHxS, perfluorohexane sulfonic acid; PFMOBA, perfluoro-4-methoxybutanoic acid; PFMOPrA, perfluoro-3-methoxypropanoic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid; PFPeA, perfluoropentanoic acid; PFPeS, perfluoropentane sulfonic acid; PFUdA, perfluoroundecanoic acid.

Table 6
PFAS concentrations in real semen samples.

Sample	Concentration (ng/mL)																
	PFBA	PFPeA	PFHpA	PFOA	PFNA	PFDA	PFUdA	PFDoA	PFBS	PFPeS	PFHxS	PFHpS	PFOS	PFEESA	PFMOPrA	PFMOBA	GenX
1	<LOD	<LOD	<LOD	1.8	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
2	<LOD	<LOD	<LOD	2.8	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
3	<LOD	<LOD	<LOD	0.6	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
4	<LOD	<LOD	<LOD	1.2	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
5	<LOD	<LOD	<LOD	0.9	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ	0.7	<LOD	<LOD	<LOD	<LOD
6	<LOD	<LOD	<LOD	1.3	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
7	<LOD	<LOD	<LOD	1.6	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
8	<LOD	<LOD	<LOD	1.8	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
9	<LOD	<LOD	<LOD	0.6	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
10	<LOD	<LOD	<LOD	1.9	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD

Abbreviations: GenX, 2,3,3,3-tetrafluoro-2-heptafluoropropoxy-propanoic acid; LOD, limit of detection; LOQ, limit of quantification; PFBA, perfluorobutanoic acid; PFBS, perfluorobutane sulfonic acid; PFDA, perfluorodecanoic acid; PFDoA, perfluorododecanoic acid; PFEESA, perfluoro-2-ethoxyethane sulfonic acid; PFHpA, perfluoroheptanoic acid; PFHpS, perfluoroheptane sulfonic acid; PFHxS, perfluorohexane sulfonic acid; PFMOBA, perfluoro-4-methoxybutanoic acid; PFMOPrA, perfluoro-3-methoxypropanoic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid; PFPeA, perfluoropentanoic acid; PFPeS, perfluoropentane sulfonic acid; PFUdA, perfluoroundecanoic acid.

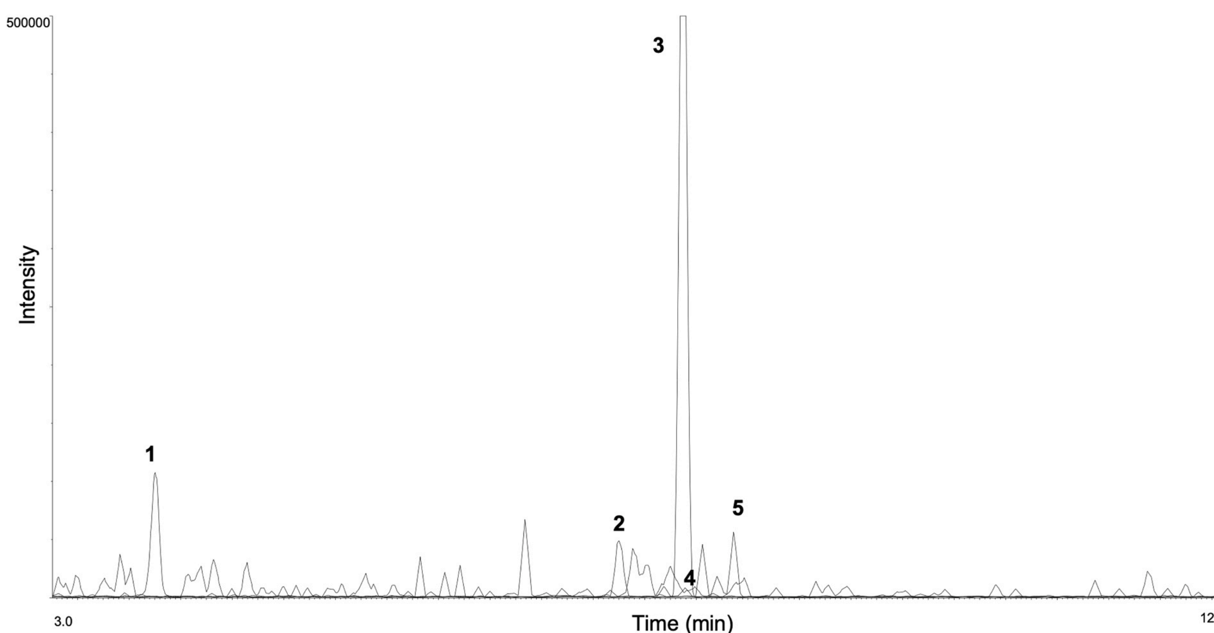

Fig. 5. Overlay chromatogram of the quantifying transitions of all analytes in a real serum sample. PFBA (1), PFHxS (2), PFOA (3), PFHpS (4), PFOS (5).

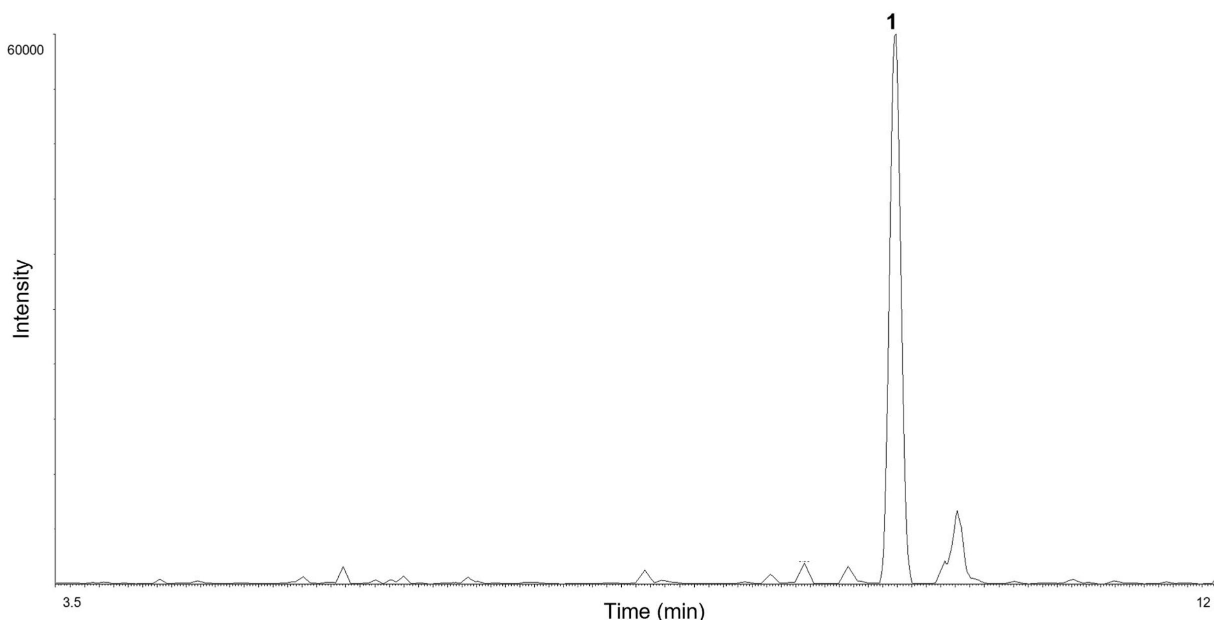

Fig. 6. Overlay chromatogram of the quantifying transitions of all analytes in a real semen sample. PFOA (1).

spectrum of these chemicals plays a crucial role to evaluate the risk related to the exposure.

As expected, the only analyte detected in both matrices was PFOA. The ratio between PFOA serum and semen concentrations was 25.4, in agreement with results from a previous study [34]. In addition, the application of the procedure to serum and semen matrices may provide a useful tool to study the correlation between PFAS exposure and human pathologies related to the male reproductive system, focusing on male infertility.

4. Conclusion

The green analytical method presented in this study not only proved to be effective for the determination of 17 PFAS in human serum and semen, but also suggested the significance of following the Green Chemistry principles. The analysis of real serum and semen sample proved that it is a valuable tool for the biomonitoring of human exposure to PFAS, where further investigations are needed to evaluate a possible correlation between the concentration of these chemicals in serum, semen and male reproductive health, especially male infertility.

Analytical laboratories can decrease their environmental impact and improve the cost-effectiveness by adopting the Green Chemistry principles. In this concern, the reduced volume of toxic solvents for the protein precipitation, the QuEChERS extraction and the acetonitrile-free mobile phases contributed to the method greenness, as highlighted by the AGREE score. Indeed, it is crucial to highlight that analytical performance should not only focus on the analytes' determination but also on the sustainability of procedures.

CRediT authorship contribution statement

Alessandro Di Giorgi: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Data curation, Conceptualization. **Giuseppe Basile:** Validation, Methodology, Investigation, Data curation. **Francesco Bertola:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Francesco Tavoletta:** Validation, Methodology, Investigation, Data curation. **Francesco Paolo Busardò:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization. **Anastasio Tini:** Writing – review & editing, Validation, Supervision, Methodology, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2024.116203.

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