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Determination of perfluorochemicals in biological, environmental and food samples by an automated on-line solid phase extraction ultra high performance liquid chromatography tandem mass spectrometry method

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ABSTRACT

A rapid on-line solid phase extraction ultra high performance liquid chromatography tandem mass spectrometry method was developed for the identification and quantitation of nine perfluorinated compounds in matrices of environmental, biological and food interest. Pre-treatment, solid phase extraction, chromatographic and mass detection conditions were optimised, in order to apply the whole methodology to the analysis of different matrices. Particular attention was devoted to the evaluation of matrix effect and the correlated phenomena of ion enhancement or suppression in mass spectrometry detection. LOD and LOQ range from 3 to 15 ng L^{-1} and from 10 to 50 ng L^{-1}, respectively. Method detection limits (MDLs) were also calculated for each kind of matrix. The recovery, evaluated for each analyte, does not depend on analyte concentration in the explored concentration range: average $\bar{R}$% values are always greater than 82.9%. In the whole, the results obtained for samples of river waters, blood serum, blood plasma, and fish confirm the ubiquitous presence of perfluorinated compounds, as recently denounced by many sources.

1. Introduction

Recent alarms concern the potential toxicity of polyfluorinated compounds, mainly in consideration of their ubiquitous presence in the environment, their persistence and potential bioaccumulation and biomagnification. Polyfluorinated compounds comprise different chemicals, that present an alkyl chain partially or fully fluorinated (as the perfluorinated compounds PFCs) containing different functional groups. While persistence and volatility depend on these functional groups, the strong carbon–fluorine bond and the physicochemical properties of the perfluorinated moieties confer to PFC characteristics of rigidity, low chemical reactivity and stability to thermal and biological degradation, associated with both oleophobic and hydrophobic properties. In a recent past all these characteristics have made PFCs excellent products for a great variety of industrial applications and uses. In 2000 the global production of perfluorooctylsulfonate (PFOS) and perfluorooctanoic acid (PFOA), the most common PFCs, was estimated around 3500 and 500 metric tons, respectively [1]. PFOA is primarily used in the production of fluoroelastomers and fluoropolymers as polytetrafluoroethylene (PTFE or Teflon®) [2–4], that finds applications ranging from coating of cookware to material used for labware, and analytical instrumentation [1]. PFOS is the precursor in several PFC products. Over the past 50 years PFCs have been used in the production of surfactants, lubricants, paints, polishes, adhesives, food packaging, fire retarding foams, refrigerants, components of pharmaceuticals, cosmetics and personal care products, insecticides, grease-, water- and stain-resistant paper and textiles coatings; they find large use also in automotive, mechanical, aerospace, chemical, electrical and medical fields, as well as in building and construction industries.

An unexpected consequence of PCF widespread use and stability is the release to the environment: presence of PFCs has been found in surface waters, aquatic environments, sediments, soils, sludges, aerosol [1,5–24], fish, herring gull eggs, seal liver [2,14,16,25,26] and in human blood, milk and many tissues [2,5,12–15,17,19,20,22,27,28]. Already in 2000 the 3M company, the major producer of PFOS voluntarily phased out the production but PFOS and many related PFCs are still produced by other manufactures [29]. Since April 2003 the US Environmental Protection Agency (US EPA) released two risk assessments about potential human exposure to PFOA and in 2006 launched a voluntary stew-

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ardship program to reduce by 95% PFOA and related chemicals in the environment by 2010 and to work towards their elimination by 2015 [30]. In October 2006 the European Union EU issued a Directive that prohibited from June 2008 the general use of PFOS and derivatives [31].

To date, toxicological information is available only for PFOS and PFOA. Animal studies indicate that ionic PFCs are absorbed when orally ingested [26]: the amphiphilic species do not preferentially accumulate in adipose tissues but bind to blood proteins influencing hormone feedback systems [11,32]. PFOS and PFOA are also absorbed by inhalation, dermal contact and can cause in rats and mice hepatic diseases and hepatocarcinogenesis [3]. Based on laboratory animal feeding studies, the NOAEL (no observed adverse effect level) value of PFOS was estimated as 0.1 mg kg^{-1} day^{-1} and the LOAEL (the lowest observed adverse effect level) value as 0.4 mg kg^{-1} day^{-1} [33].

Humans can assume PFCs through dermal contact, air-bone transport, drinking water and food, in particular fish, crops and livestock. Even if EPA suggested PFOA possible carcinogenity, a final risk assessment is not yet completed. In January 2009 US EPA established the Provisional Health Advisories (PHA) limits for PFOS (0.2 μg L^{-1}) and for PFOA (0.4 μg L^{-1}) in drinking water [30,34] but PHA values are still subjected to possible changes as a function of new information [30]. In 2008 the EFSA (European Food Safety Authority) Journal reported that the data available allow only indicative values [35]. From 17th March 2010 the European Commission suggested to the Member States to monitor along 2010 and 2011 the presence of PFCs in food in order to define exposure levels [36]. The UK Health Protection Agency fixed PFOS and PFOA maxima acceptable concentrations in drinking water as 0.3 μg L^{-1} and 10 μg L^{-1}, respectively.

Literature methodologies for the identification and determination of PFCs take into account only one kind of matrix (biological, food, environmental) and consist in the pre-treatment step of the sample, followed by the extraction process of the analytes and then their quantification.

Pre-treatment and extraction conditions mainly depend on the kind of the matrix. The most used analytical techniques are gas chromatography (GC) and high performance liquid chromatography (HPLC) hyphenated with mass spectrometry (MS) detection. GC–MS methods have been applied for volatile neutral per- and poly-fluorinated compounds and for ionic PFCs after a derivatisation reaction [1]. While in general capillary zone electrophoresis (CZE) [3] and GC–MS methods allow detection limits only of the order of mg L^{-1} and μg L^{-1}, respectively, HPLC–MS and HPLC–MS/MS methods are more sensitive, with detection limits of the order of ng L^{-1}. In addition HPLC–MS/MS also offers better selectivities. HPLC–MS and HPLC–MS/MS make use of both atmospheric pressure chemical ionisation (APCI) [5] and electrospray ionisation (ESI) in negative ion (NI) mode [1,5,9,10,12,15,16,22–25].

When analysing complex matrices, co-elution of matrix components is easy to occur and, when using MS detection, to give phenomena of ion suppression or enhancement [20,37]. To overcome matrix effects the steps of pre-treatment and extraction of the analytes must be optimised. For the quantitation process, isotopic labelled standard (I.S.) [5,12,13,22] or the standard addition method [11,12,20,22,38,39] has been used. Calibration in surrogate matrix (as for example rabbit serum for analysing human serum) has also been employed [12]. Different pre-treatments have been employed, as the use of activated carbon, protein precipitation [12], filtration [18] or dilution with formic acid [2]. To extract PFCs from biological samples, techniques such as liquid–liquid extraction (LLE) [5,12] or ion pairing LLE were used [5,14,27,40,41]. With respect to the LLE, solid phase micro-extraction (SPME) technique is a solvent free process that includes simultaneous extraction and preconcentration of analytes from aqueous samples or from the headspace of the samples. SPME followed by GC–MS [42] or LC–MS [3] analysis was also employed. But SPME is a slow and expensive technique, its fibre is fragile with limited lifetime and sometimes sample carry-over could be a problem. On the contrary, solid phase extraction (SPE) has the advantage such as simplicity, high speed, low consumption of organic solvent and high reproducibility. The use of SPE was largely applied and the performances of different solvents, extraction mixtures and sorbent packing materials were compared [5,9,26,43]. Automated off-line extraction methods were also developed, in which the eluting agent is chosen as a function of the matrix (milk or serum) [20].

Recently, on-line SPE HPLC–MS methodologies have been optimised, that comprise the steps of extraction and analysis [1,2,22,44]. To our knowledge, all the methodologies present in literature have been optimised in order to separate and determinate mixtures of PFCs in a specific matrix. On the other hand, in routine analysis it should be very advantageous that the availability of a methodology to be applied to different kinds of matrix in which the presence and the amount of PFCs must be controlled.

Aim of the present study is the development of an automated on-line SPE ultra high performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS) methodology that can be applied in the determination of PFCs in matrices of environmental, biological and food interest, as river water, human plasma, human serum and fish. In addition, the UHPLC technique allows shorter analysis times, very advantageous in routine analysis.

2. Experimental

2.1. Reagents

Perfluorooctanesulfonamide (FOSA), sodium perfluorobutane sulfonate (PFBS), perfluoroheptanoic acid (PFHpA), sodium perfluorohexansulfonate (PFHxS), perfluorooctanoic acid (PFOA), perfluorooctadecanoic acid (PFODA), sodium perfluorooctansulfonate (PFOS), perfluoropentanoic acid (PFPeA), and perflurotetradecanoic acid (PFTeDA) were purchased from Wellington Laboratories (Guelph, ON, Canada), as also the two labelled standards, ^{13}C mass labelled PFOA ($^{13}C_4$-PFOA) and ^{13}C mass labelled PFOS ($^{13}C_4$-PFOS).

HPLC grade acetonitrile (CH_3CN) was purchased from Merck (Darmstadt, Germany) and methanol (CH_3OH) Chromasolv (>99.9%) from Sigma–Aldrich (Milwaukee, WI, USA). Formic acid (HCOOH) (99%), ammonium acetate (99%) and ammonium hydroxide (NH_4OH) (30%) were acquired from Fluka (Buchs, Switzerland). Ultrapure water was produced by a Millipore Milli-Q system (Milford, MA, USA).

The stock solutions of the standards were prepared in methanol and diluted as required with a mixture 80/20 (v/v) of 0.1% HCOOH solution in ultrapure water and 0.1% HCOOH solution in CH_3OH. All the solutions were prepared every week and preserved at 4 °C in polypropylene (PP) vials. In order to avoid PFC contaminations, all the autosampler vials used in the UHPLC–MS/MS analyses were in PP and without the vials septum.

2.2. Apparatus

The chromatographic analyses were performed using a Dionex (Sunnyvale, CA, USA) Ultimate 3000 UHPLC system equipped by a Ultimate 3000 Degasser, a Ultimate 3000 Pump, a Ultimate 3000 RS Autosampler and a Ultimate 3000 RS column compartment. The system was interfaced with a 3200 QTrap™ LC–MS/MS system (Applied Biosystems, Foster City, CA, USA) by a Turbo V™ interface equipped with an ESI probe. The data were processed by Analyst 1.5 software (Toronto, Canada). A homogeniser Ultra-Turrax T25 (IKA-Werke, Staufen, Germany) and an IEC CL31R multispeed cen-

trifuge (Thermo Electron Corporation, Milford, MA) were employed in sample pre-treatment.

2.3. Sample pre-treatments

2.3.1. Biological samples

The blood samples were kindly obtained from laboratory analysts. For serum analysis, a volume of 1 mL of blood was centrifuged at 14,000 rpm for 10 min; 100 μL of the supernatant was added to 300 μL of acetonitrile. The mixture was centrifuged at 14,000 rpm for 10 min and the supernatant was diluted 1/4 (v/v) in a 80/20 (v/v) mixture of 0.1% HCOOH solution in ultrapure water and 0.1% HCOOH solution in CH_3OH and then subjected to on-line SPE UHPLC–MS/MS analysis. For plasma analysis, 100 μL of blood was added to 300 μL of acetonitrile and centrifuged at 14,000 rpm for 10 min. The liquid fraction was diluted 1/4 (v/v) in a mixture 80/20 (v/v) of 0.1% HCOOH solution in ultrapure water and 0.1% HCOOH solution in CH_3OH and then subjected to on-line SPE UHPLC–MS/MS analysis.

2.3.2. River samples

The samples of river water were collected in Sesia, Po and Tanaro rivers (Piedmont, Italy) in 1 L PP bottles and stored in dark and at 4 °C until further processing. The samples were filtered through 0.22 μm PP filters before on-line SPE UHPLC–MS/MS analysis.

2.3.3. Fish sample

A sample of *Morone saxatilis* was purchased in retail fish market.

About 10 g of fish fillet were cut into small pieces, homogenised with 25 mL of methanol at 13,500 rpm for 5 min in a Falcon tube and then centrifuged at 14,000 rpm for 10 min. The supernatant was diluted 1/10 (v/v) in a 80/20 (v/v) mixture of 0.1% HCOOH solution in ultrapure water and 0.1% HCOOH solution in CH_3OH and then subjected to on-line SPE UHPLC–MS/MS analysis.

In order to simulate in laboratory the possible effects of the cooking process on PFC content, weighed amounts (around 3 g) of fish were cooked at 180 °C and for 30 min in two non-stick pans, the first characterised by a new and integral coating and the second by a damaged one. The cooked fish samples were then transferred into a Falcon tube, homogenised with 6.5 mL of methanol at 13,500 rpm for 5 min and centrifuged at 14,000 rpm for 10 min. The supernatant was then treated as the supernatant from fresh fish.

Table 1
Mobile phase gradient for UHPLC and on-line SPE. (a) UHPLC conditions, percentage of: (A) 0.01% NH_4OH solution in 5 mM ammonium acetate; (B) 0.01% NH_4OH solution in acetonitrile. (b) On-line SPE conditions, percentage of: (C) 0.1% HCOOH solution in ultrapure water; (D) 0.1% HCOOH solution in CH_3OH; (E) CH_3CN/CH_3OH 60/40 (v/v).

(a) Right pump UHPLC

Time (min)	Flow (mL min^{-1})	A%	B%
0.0	1.000	80	20
0.8	1.000	80	20
4.7	1.000	0	100
7.0	1.000	0	100
7.1	1.000	80	20

(a) Left pump on-line SPE

Time (min)	Flow (mL min^{-1})	Valve position	C%	D%	E%
0.0	2.000	Loading	95	5	0
0.6	2.000	Injection	95	5	0
0.9	1.000	Injection	0	0	100
6.0	1.000	Injection	0	0	100
7.0	2.000	Loading	95	5	0
7.1	2.000	Loading	95	5	0

2.4. UHPLC–MS/MS conditions

The stationary phase was a Zorbax Eclipse XDB-C18 (4.6 mm × 50 mm, 1.8 μm) purchased from Agilent (Milan, Italy). The mobile phase was a mixture of 0.01% NH_4OH solution in 5 mM ammonium acetate (component A) and of 0.01% NH_4OH solution in acetonitrile (component B), eluting at flow rate 1 mL min^{-1} and under the UHPLC gradient conditions shown in Table 1a. The injection volume was 350 μL.

Temperatures of autosampler and of column oven were set at 5 °C and 55 °C, respectively.

The turbo ion spray (TIS) ionisation was obtained using the Turbo V™ interface working in negative polarity ion (NI) mode. The instrumental parameters were set as follows: curtain gas (N_2) at 30 psig, nebulizer gas GS1 and GS2 at 45 and 60 psig, respectively, desolvation temperature (TEM) at 600 °C, collision activated dissociation gas (CAD) at 6 units of the arbitrary scale of the instrument and ionspray voltage (IS) at −4500 V. The 3200 QTrap™ was used in scheduled multiple reaction monitoring (sMRM) considering the transitions of each species at a prefixed retention time. Unit mass resolution was established and maintained in each mass-resolving quadrupole by keeping a full width at half maximum (FWHM) of about 0.7 u.

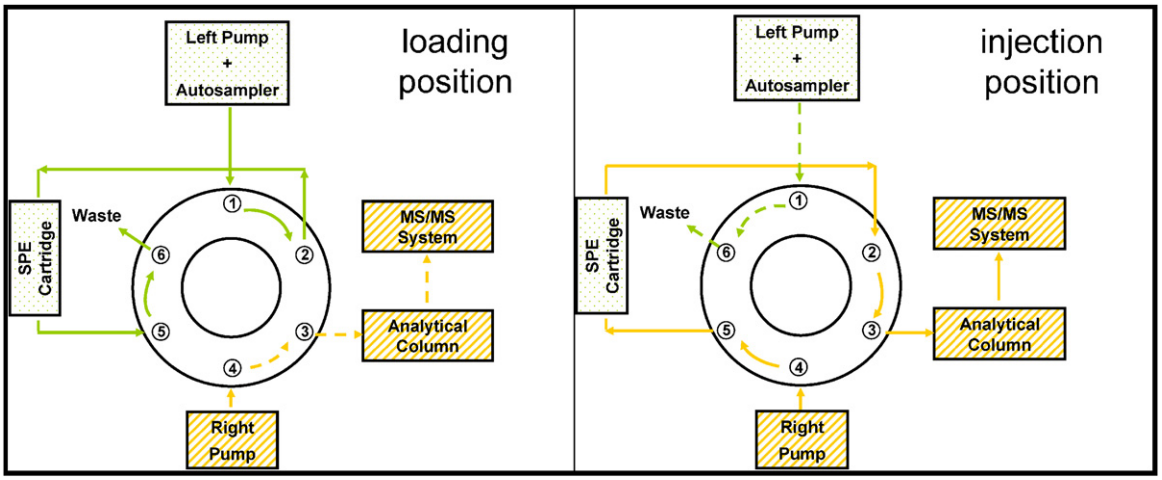

Fig. 1. Instrumental configuration of on-line SPE.

Table 2
Molecular weight (all the sulfonates are sodium salts), sMRM transitions (Q1 and Q3 masses) and mass spectrometry parameters: DP (declustering potential), EP (entrance potential), CEP (collision cell entrance potential), CE (collision energy), CXP (collision cell exit potential). For each species, the most sensitive transition, marked as "*", was used for quantitation (*quantifier*) and the second one was used for confirmation (*qualifier*).

Analyte	Molecular weight (a.m.u.)	Q1 mass (m/z)	Q3 mass (m/z)	Time (min)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
*FOSA	499.14	497.90	78.00	3.8	−75.00	−4.00	−30.60	−70.00	0.00
FOSA	499.14	497.90	478.00	3.8	−75.00	−4.00	−30.60	−34.00	−4.20
*PFBS	322.08	299.00	80.00	2.6	−45.00	−9.00	−23.24	−45.00	−1.00
PFBS	322.08	299.00	99.00	2.6	−45.00	−9.00	−23.24	−53.00	−1.00
*PFHpA	364.06	362.90	318.90	2.9	−14.00	−4.00	−25.61	−18.00	−3.00
PFHpA	364.06	362.90	169.00	2.9	−14.00	−4.00	−25.61	−24.00	−1.00
*PFHxS	422.10	398.90	79.90	3.1	−63.00	−10.00	−26.94	−68.00	−1.00
PFHxS	422.10	398.90	98.90	3.1	−63.00	−10.00	−26.94	−57.00	−1.80
*PFOA	414.07	412.90	368.90	3.1	−12.00	−4.30	−27.46	−14.00	−2.00
PFOA	414.07	412.90	218.90	3.1	−12.00	−4.30	−27.46	−23.00	−2.00
*$^{13}C_4$-PFOA	418.04	417.00	168.90	3.1	−11.00	−4.80	−27.61	−26.00	−0.80
$^{13}C_4$-PFOA	418.04	417.00	371.90	3.1	−11.00	−4.80	−27.61	−17.00	−3.55
*PFDOA	914.15	913.00	869.00	4.8	−36.00	−8.00	−45.96	−27.00	−6.20
PFDOA	914.15	913.00	218.90	4.8	−36.00	−8.00	−45.96	−40.00	−2.00
*PFOS	522.11	499.10	80.00	3.6	−74.00	−9.00	−30.64	−85.00	−1.00
PFOS	522.11	499.10	99.10	3.6	−74.00	−9.00	−30.64	−67.00	−1.00
*$^{13}C_4$-PFOS	526.11	502.90	79.90	3.6	−80.00	−9.00	−30.79	−88.00	−1.00
$^{13}C_4$-PFOS	526.11	502.90	98.90	3.6	−80.00	−9.00	−30.79	−71.00	−1.00
*PFPeA	264.05	262.90	218.90	2.1	−11.00	−4.00	−21.91	−15.00	−1.70
PFPeA	264.05	262.90	68.90	2.1	−11.00	−4.00	−21.91	−53.00	0.00
*PFTeDA	714.12	713.00	169.00	4.2	−30.00	−5.70	−38.56	−39.00	−2.00
PFTeDA	714.12	713.00	219.00	4.2	−30.00	−5.70	−38.56	−36.00	−2.00

2.5. On-line SPE conditions

The purification of the samples was performed through an on-line SPE method: the SPE column was a Poros HQ column (2.1 mm × 30 mm, 10 μm) (Applied Biosystems, Foster City CA, USA).

A mixture 95/5 (v/v) of 0.1% HCOOH solution in ultrapure water (indicated as C in Table 1b) and of 0.1% HCOOH solution in CH_3OH (indicated as D in Table 1b) was the loading solution, while the eluting agent had the same initial composition (time = 0 min) of the mobile phase used in the chromatographic separation. To reduce the carryover effects, the autosampler loop and the autosampler needle were washed for 5.1 min with a mixture of CH_3CN/CH_3OH 60/40 (v/v), indicated as E in Table 1b.

The system setup for the on-line SPE consists of three steps. In the loading step 350 μL of water or of the extracts of the real samples are loaded onto the cartridge through the Dionex 3000 autosampler. The trap cartridge is fitted into the loading position of the Valco 6-port switching valve (Fig. 1). Through the Dionex Ultimate 3000 RS Dual pump the sample is loaded at flow rate of 2 mL min^{-1} onto the trapping cartridge (left pump). While the analytes are retained on the SPE column and the matrix is flushed to waste, the analytical LC column is equilibrated with the chromatographic pump (right pump). In the injection step (Fig. 1), at 0.6 min, the valve is switched to injection position that couples the SPE cartridge with the chromatographic column, into which the analytes are transferred. The Dionex right pump is used to provide the gradient elution. In the separation step the analytes are separated in the analytical column. After 7 min the valve is switched back to the loading position to equilibrate the on-line SPE cartridge with the loading phase flowing at 2 mL min^{-1} for 4.5 min, prior to injecting the next sample. The on-line SPE conditions are reported in detail in Table 1b.

3. Results and discussion

3.1. Development and optimisation of the UHPLC–MS/MS method

The investigated PFCs were previously subjected to a MS/MS characterisation study both in ESI NI mode and in APCI NI sources, in order to identify the successive fragments formed under increasing collision energy. The experiments were carried out for direct infusion of 300 μg L^{-1} solutions of each PFC in CH_3OH (syringe flow rate: 50 μL min^{-1}, chromatographic pump flow rate 200 μL min^{-1}, connecting it through a T valve to the syringe pump). As expected, ESI source offers a better sensitivity due to the presence of ionisable groups (sulfonic and carboxylic groups) in the molecular structure of the analytes studied. All PFCs presented many transitions: for each of them the most intense one was used for the quantitative analysis and referred as "quantifier" transition, while the second one (the "qualifier" transition) was employed to confirm the identification. The "quantifier" and "qualifier" transitions are reported in Table 2, together with the instrumental potential values.

In order to increase peak symmetry and chromatographic resolution, two SPE sorbents and different elution phases were tested. While the Dionex HRGP (2 mm × 10 mm, 20 μm) SPE cartridge gave a poor sorption, the Poros HQ sorbent, working in perfusion mode, permitted good performances in terms of both sorption and chromatographic separation. The composition of the UHPLC mobile phase was optimised in order to reach the complete elution of the analytes from the sorbent together with a good chromatographic separation. The use of different buffers (ammonium acetate, ammonium formate and ammonium carbonate) at different concentrations (1, 5 and 10 mM) and of different organic solvents (methanol, acetonitrile) was tested and compared. The use of 5 mM ammonium acetate solution brought to pH 8.2 for 0.01% NH_4OH solution favored the desorption of PFCs from the SPE cartridge and gave the best separation. The best chromatographic peak symmetry was obtained with the use in the mobile phase of acetonitrile at the indicated percentage.

Fig. 2 reports a typical chromatogram of the standard mixture of the analytes (1000 ng L^{-1} each), recorded under the optimised conditions.

3.2. Validation of the analytical method

For each PFC a calibration plot reporting the peak area of the "quantifier" transition (y) versus standard concentration (x) was built: six concentration levels in the range between the LOQ value and 1000 ng L^{-1} were considered. To overcome possible memory effects, the standard solutions were injected in randomised order.

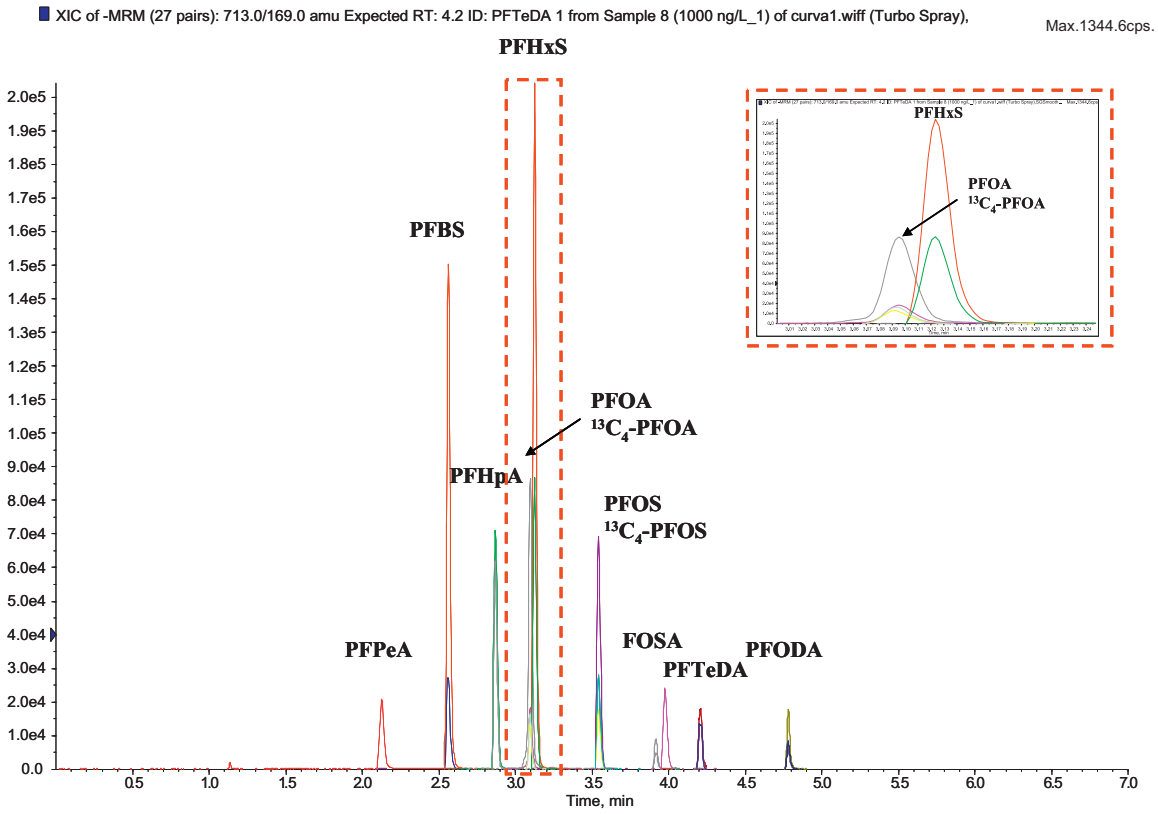

Fig. 2. A typical on-line SPE UHPLC–MS/MS chromatogram of a standard mixture of the eleven analytes at 1000 ng L^{-1}. The chromatographic conditions are reported in Section 2.

For all the analytes a linearity regression fit was used with a weighting factor $1/x$. A good linearity of the response with regression coefficients (R^2) always greater than 0.9910 was obtained. A t-test at 95% confidence level was applied to verify the significance of the intercept of each calibration equation [45].

The limit of detection (LOD) was calculated as the concentration of the analyte that gives a signal (peak area) equal to the average background (S_{blank}) plus three times the standard deviation s_{blank} of the blank (LOD = $S_{blank} + 3s_{blank}$), while the limit of quantification LOQ is given as LOQ = $S_{blank} + 10s_{blank}$ [46]. Linearity ranges, R^2, LOD and LOQ are reported in Table 3 for all the analytes. LOD values range from 3 to 15 ng L^{-1} and are lower or of the same magnitude order of those obtained in other studies that employ on-line SPE HPLC–MS methods [1,20,44].

Method detection limit (MDL) of each analyte was determined by using a statistical approach [47]. The procedure involves spiking seven replicates of each blank matrix with each analyte at a concentration resulting in an instrumental signal to noise ratio between 2.5 and 5. The MDL was then calculated as MDL = $t_{(n-1,1-\alpha=0.99)} \times S_d$ where $t = 3.14$ that corresponds to the Student's value appropriate for a 99% confidence level and 6 degrees of freedom, whereas S_d is the standard deviation of the replicate analyses. The MDL values were always lower than 75 ng L^{-1} (Table 3).

The intra- and inter-day precision on the retention time and on the concentration were evaluated by analysing a standard mixture of all PFCs (100 ng L^{-1} each) every day for a week and repeating the analysis five times. The results (Table 3) show that intra-day precision of retention time range from 0.1% to 0.2% and inter-day precision from 0.3% to 5%. The intra-day and inter-day relative standard deviation (RSD%) of concentration range from 1.2% to 3.3% and from 3% to 9.3%, respectively.

To check the stability of the system as it concerns the quantitative response, at random intervals along the analyses, standard quality control (QC) solutions (at concentrations of 500 ng L^{-1}) of

Table 3
Regression coefficient (R^2), linearity range, LOD, LOQ, method detection limit (MDL), intra- and inter-day RSD (%) on concentration for the 11 analytes considered.

Analyte	R^2	Linearity range (ng L^{-1})	LOD (ng L^{-1})	LOQ (ng L^{-1})	MDL in fish sample (ng kg^{-1})	MDL in serum sample (ng L^{-1})	MDL in plasma sample (ng L^{-1})	MDL in river water sample (ng L^{-1})	Intra-day RSD (%)	Inter-day RSD (%)
FOSA	0.9910	50–1000	15	50	60	75	70	42	1.9	7.5
PFBS	0.9982	25–1000	8	25	40	29	21	17	2.3	6.2
PFHpA	0.9956	10–1000	3	10	15	13	10	12	1.9	9.3
PFHxS	0.9970	25–1000	8	25	24	26	35	25	2.6	7.9
PFOA	0.9942	10–1000	3	10	10	10	9	12	3.2	5.2
$^{13}C_4$-PFOA	0.9930	10–1000	3	10	10	10	9	12	3.1	5.1
PFODA	0.9948	10–1000	3	10	13	12	13	9	1.7	6.6
PFOS	0.9974	50–1000	15	50	60	75	65	41	3.2	7.5
$^{13}C_4$-PFOS	0.9988	50–1000	15	50	60	65	75	41	3.3	7.6
PFPeA	0.9954	50–1000	15	50	60	65	75	41	3.2	6.9
PFTeDA	0.9948	50–1000	15	50	50	38	35	49	1.2	3.0

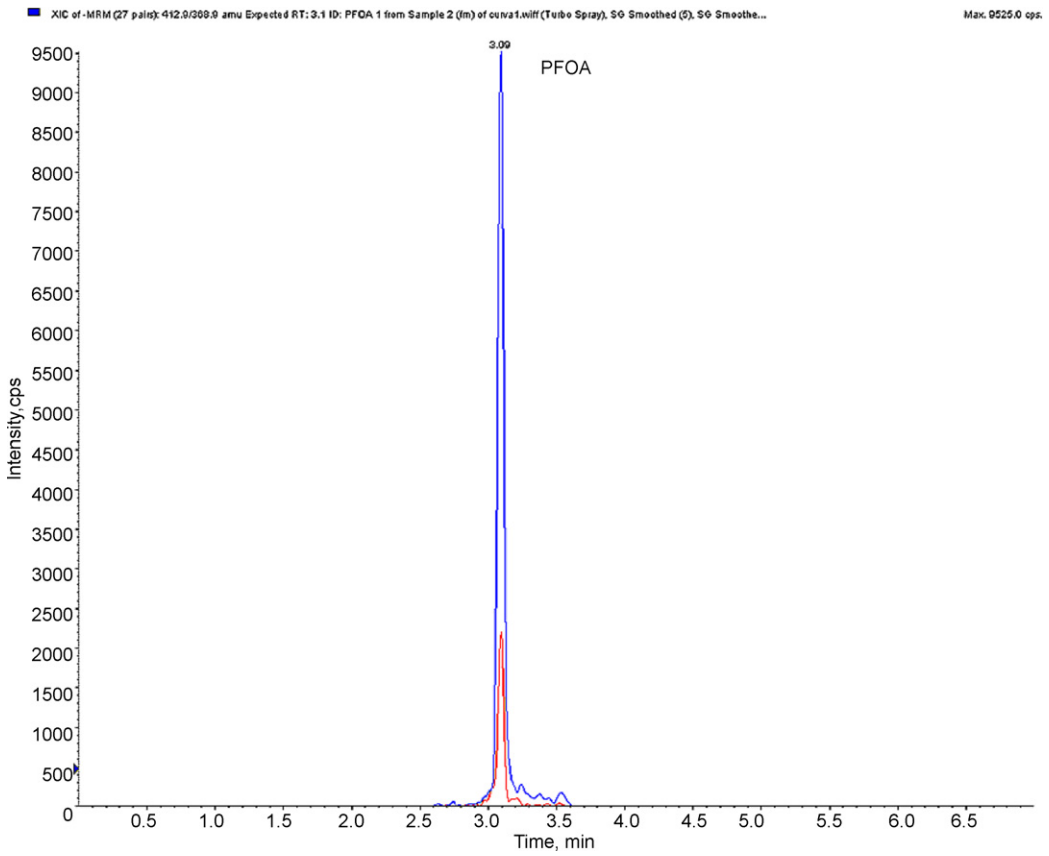

Fig. 3. A typical on-line SPE UHPLC–MS/MS blank chromatogram obtained for injection of mobile phase. The chromatographic conditions are reported in Section 2.

the labelled standards $^{13}C_4$-PFOA and $^{13}C_4$-PFOS were injected. All the results obtained for the QC solutions lay within the $\pm 3\sigma$ control limits of the calibration plots.

No memory effect was evidenced, likely due to the optimised washing process of the SPE sorbent during the loading step.

Particular attention was devoted to the quantification of PFOA, that can be released from Teflon® junctures, capillaries and connections (total length of about 1 m) of the chromatographic system and that can be cumulated in the SPE cartridge during the chromatographic run [5,20,48]. The concentration of PFOA released was evaluated through the standard addition method, by injecting mobile phase and standard solutions of PFOA at concentration of 100, 250 and 400 ng L^{-1}. The analyses were repeated for three times and the quantified PFOA resulted to be 418 (±17) ng L^{-1}: this contribution was taken into consideration in the quantification of PFOA, by subtracting this value in each PFOA quantification data. Fig. 3 shows a typical on-line SPE UHPLC–MS/MS blank chromatogram.

To evaluate for each analyte in each sample matrix the recovery R and to verify its possible dependence on concentration, a mixture of PCF standard solutions at three different concentration levels was added to the samples. The solutions were prepared taking into account that samples are diluted 1/20 (v/v) in a 80/20 (v/v) mixture of 0.1% HCOOH solution in ultrapure water and 0.1% HCOOH solution in CH_3OH prior to injection, in order to obtain final concentration values laying in the linearity range (200, 500 and 800 ng L^{-1}). Each analysis was repeated three times. The recovery values were calculated as C_{obs}/C_{ref} where C_{obs} is the difference between the concentration determined for the spiked sample and the native concentration of the same sample, whereas C_{ref} is the spiked concentration. A t-test at 95% confidence level showed that for all the analytes the difference among the R values obtained for the three concentration levels was not statistically significant and indicated that in the explored concentration range, recovery does not depend on analyte concentration. For all the analytes and for

Table 4
Average recovery yields $\bar{R}$ (%) in the different matrices. The values marked as "*" represent the $\bar{R}$ (%) values not statistically different from 100%.

Analyte	$\bar{R}$ (%) in fish sample	$\bar{R}$ (%) in serum sample	$\bar{R}$ (%) in plasma sample	$\bar{R}$ (%) in Po river sample	$\bar{R}$ (%) in Tanaro river sample	$\bar{R}$ (%) in Sesia river sample
FOSA	89.3 (±1.9)	91.9 (±1.9)	91.9 (±5.9)	98.9 (±2.0)	99.7 (±2.0)*	98.7 (±2.0)
PFBS	83.8 (±2.5)	90.5 (±8.4)	92.8 (±8.3)	98.3 (±8.7)	96.1 (±8.5)	97.2 (±8.8)
PFHpA	90.0 (±2.0)	96.0 (±6.4)	93.5 (±6.3)	98.2 (±2.2)	98.0 (±2.2)	100.6 (±2.4)*
PFHxS	92.6 (±2.7)	96.0 (±2.8)	94.3 (±8.1)	98.9 (±8.6)	99.9 (±8.8)*	99.7 (±8.7)*
PFOA	89.1 (±3.4)	96.6 (±3.5)	93.6 (±3.4)	101.9 (±3.7)*	98.8 (±3.7)	100.1 (±3.6)*
$^{13}C_4$-PFOA	87.7 (±1.9)	95.9 (±3.0)	89.3 (±3.0)	99.3 (±3.0)	99.6 (±3.2)	98.8 (±3.0)
PFODA	85.4 (±1.8)	92.3 (±5.9)	94.6 (±5.9)	97.8 (±2.0)	98.7 (±2.1)	100.3 (±2.0)*
PFOS	84.8 (±4.9)	93.5 (±5.2)	92.5 (±5.2)	91.2 (±5.5)	91.7 (±5.5)	91.7 (±5.5)
$^{13}C_4$-PFOS	85.3 (±5.1)	94.4 (±5.4)	93.4 (±5.4)	93.7 (±5.9)*	93.1 (±5.8)*	92.9 (±5.8)
PFPeA	89.2 (±3.7)	94.6 (±6.9)	97.2 (±3.9)	101.2 (±4.2)*	100.9 (±4.1)*	101.4 (±4.3)*
PFTeDA	82.9 (±5.8)	92.9 (±2.1)	91.8 (±6.1)*	96.5 (±2.1)	96.4 (±2.2)	95.7 (±2.1)

Table 5
Equations of the external calibration plot (standard solution) and of the standard addition calibration plot (built on the fish samples).

Analyte	External calibration plot	Standard addition calibration plot
FOSA	$y=(50\pm6)x$	$y=(44\pm2)x$
PFBS	$y=(232\pm3)x$	$y=(180\pm10)x$
PFHpA	$y=(113\pm1)x+(14\pm1)\times10^2$	$y=(88\pm6)x+(15\pm1)\times10^3$
PFHxS	$y=(312\pm5)x$	$y=(220\pm15)x$
PFOA	$y=(103\pm3)x+(42\pm1)\times10^3$	$y=(79\pm9)x+(71\pm6)\times10^3$
PFODA	$y=(20.2\pm0.4)x$	$y=(15\pm1)x$
PFOS	$y=(47\pm1)x$	$y=(41\pm1)x$
PFPeA	$y=(42\pm1)x$	$y=(34\pm1)x$
PFTeDA	$y=(2.9\pm0.1)x$	$y=(3.7\pm0.5)x$

each sample matrix an average percentage of recovery $\overline{R}$ (%) was therefore calculated and reported in Table 4: as it can be observed all the $\overline{R}$ (%) values are greater than 82.9%. Before using the calculated $\overline{R}$ values to correct the quantitative data measured for the analytes investigated, the statistical significance of the difference of the $\overline{R}$ (%) data obtained with respect to $\overline{R}$ 100% was checked. The *t*-test at 95% confidence level indicated (Table 4) that 13 values out of 66 are not significantly different, so that for these analytes no correction on the amount experimentally evaluated was performed.

3.3. Matrix effect evaluation

To evaluate the presence of the matrix effect ME, a *t*-test at 95% confidence level was applied to compare the slopes of the external calibration plot and of the standard addition plot built for the real samples (fish, serum, plasma and the three river samples).

The samples prepared by adding a mixture of the standard analytes (at concentrations 100, 500 and 1000 ng L^{-1}) to the extracts or to the filtered water samples, were undergone to the on-line SPE UHPLC–MS/MS analysis and the analysis was repeated three times. A *t*-test (at 95% confidence level) was performed on the intercepts of the standard addition calibration plots to evaluate if they were statistically different from zero (Table 5) [45].

The average matrix effect (ME) was estimated for each analyte and for all the concentrations through the ratio A_{add}/A_{ex}, where A_{add} is the average peak area of the analyte added to the real sample and A_{ex} is the average peak area of the analyte in the standard solution [38,39,49].

For the samples of river water and blood, the slopes of the two plots are not statistically different so indicating the absence of matrix effect: this result can be likely ascribed to the optimised washing step of SPE sorbent, that employs a washing volume of about 20-folds the column volume.

On the contrary matrix effect was shown to be present for the fish sample, as reported in Table 5 and in Fig. 4. Fig. 4 also shows that matrix effect in fish is different for different PCFs, ranging from a signal suppression of around −29% for PFHxS to a signal enhancement around +29% for PFTeDA. No matrix effect was instead observed for FOSA and PFOA.

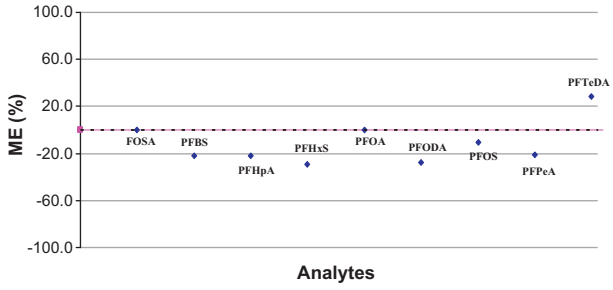

Fig. 4. Matrix effect trend in the fish sample for all the analytes considered.

Table 6
Quantification data in the real samples (n.d. for not detected).

Analyte	Fish sample (ng kg^{-1})	Fish sample cooked in a non-stick pan with integral coating (ng kg^{-1})	Fish sample cooked in a non-stick pan with damaged coating (ng kg^{-1})	Serum (ng L^{-1})	Plasma (ng L^{-1})	Po river sample (ng L^{-1})	Tanaro river sample (ng L^{-1})	Sesia river sample (ng L^{-1})
FOSA	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ	<LOQ	n.d.
PFBS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.
PFHpA	3118 (±29)	3106 (±20)	3108 (±26)	718 (±7)	n.d.	<LOQ	23 (±1)	73 (±3)
PFHxS	n.d.	n.d.	n.d.	641 (±5)	3150 (±25)	n.d.	n.d.	n.d.
PFOA	9082 (±47)	9071 (±29)	9069 (±32)	n.d.	n.d.	<LOQ	<LOQ	<LOQ
PFODA	n.d.	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.
PFOS	n.d.	n.d.	n.d.	4892 (±37)	5530 (±42)	<LOQ	n.d.	n.d.
PFPeA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PFTeDA	n.d.	n.d.	n.d.	n.d.	897 (±5)	n.d.	n.d.	n.d.

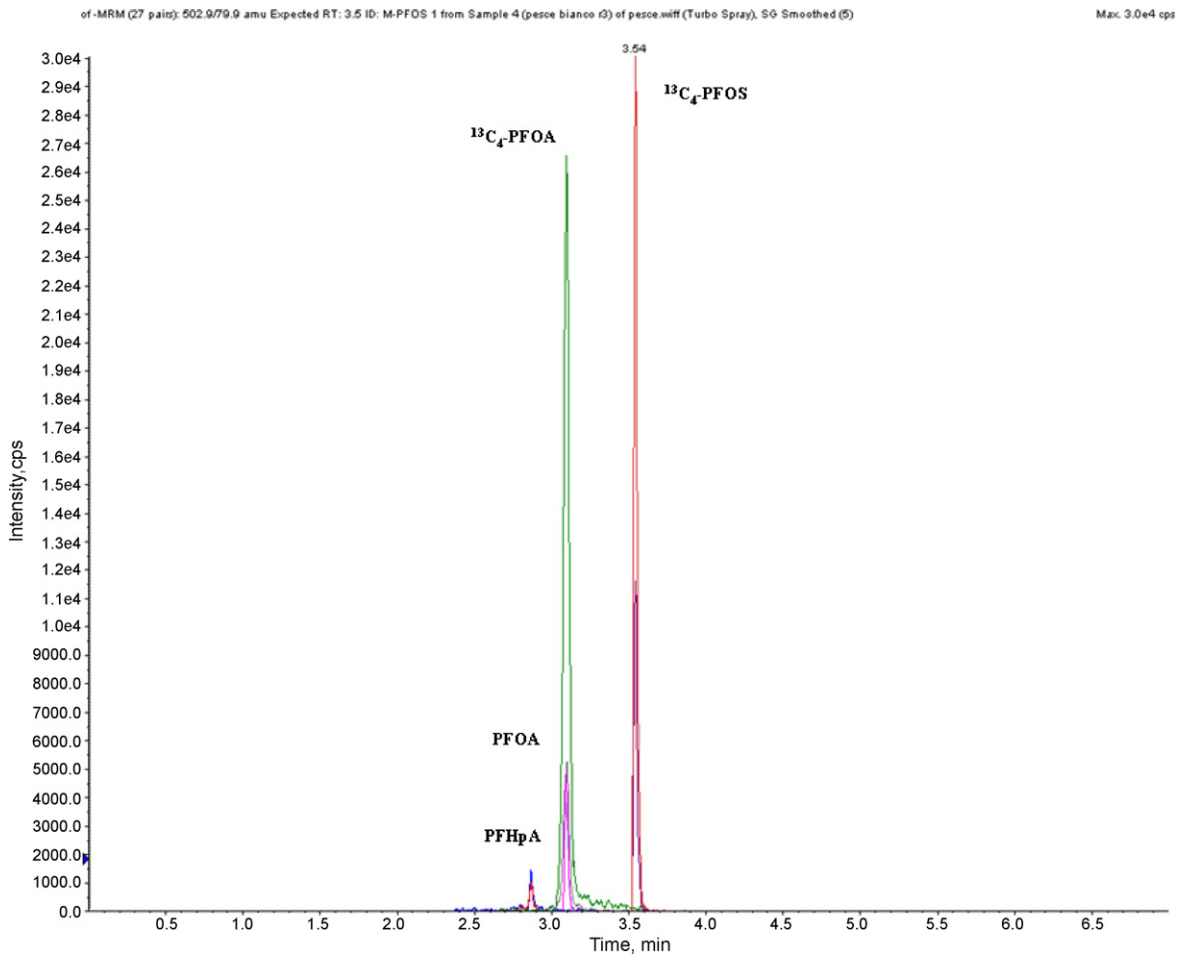

Fig. 5. On-line SPE UHPLC–MS/MS chromatogram of a fish sample. The sample pre-treatment and the chromatographic conditions are reported in Section 2.

3.4. UHPLC–MS/MS analysis of real samples

The whole UHPLC–MS/MS methodology was applied in the analysis of six different matrices, namely water samples collected in Po, Sesia and Tanaro rivers, human serum and plasma, fish.

Taking into account the performance criteria of the EU Commission, also the MRM ratio between the abundances of the two selected precursor and product ion transitions (qualifier transition to quantifier transition) was used to confirm analyte identification [50]: in the presence of the analyte the calculated MRM ratio for the real sample must be within ±20% of the average MRM ratio calculated for the standard.

For each concentration level considered in the calibration plot, the MRM ratio was calculated. The quantification data obtained by standard addition method are reported in Table 6. In Po river waters PFCs were detected but not quantified because the amounts laid between LOD and LOQ values. In Tanaro river water PFBS and PFHpA were quantified, while in Sesia river water only PFHpA. The maximum value was found for PFHpA in Sesia river: its concentration is anyway lower than the legal threshold concentration established by EPA for the two more toxic PFOA and PFOS. In all the river samples analysed, no presence of PFOA and PFOS was found.

In the biological samples investigated, while PFOA resulted always absent, the presence of PFOS was observed both in serum and in plasma, at concentration of the order of 5000 ng L^{-1}, that is comparable with those found in this matrix by other authors [1,44].

The highest concentrations of PFHpA and PFOA, of the order of 3118 and 9082 ng kg^{-1}, respectively, were found in fish (Table 6). Fig. 5 reports a typical chromatogram of a fish sample. As mentioned, comparison experiments performed on fresh fish and two fish samples cooked at 180 °C for 30 min in two non-stick pans (the first characterised by an integral coating and the other by a damaged one) all showed similar results. It can therefore be assumed that the high PFC amounts found are not due to the cooking process or to release of PFCs from the cooking material but they are more likely associated to bioaccumulation and biomagnification effects in fish tissues [51–54], associated to PFC binding to proteins [32].

4. Conclusions

An automated on-line SPE UHPLC–MS/MS method for the identification and determination of nine PFCs in 7 min was developed: the innovative aspect of the method proposed consists, together with the short analysis time, in its applicability in the analysis of environmental, biological and food matrices.

The method was validated by evaluating linearity range, precision, LOD, LOQ, MDL and recovery.

The occurrence of matrix effect was evaluated and overcome.

In the whole, the results confirmed the ubiquity of PFCs. With the exception of FOSA, PFBS, PFODA and PFPeA, the other five PFCs considered were found in all the investigated samples. PFOS and PFOA were quantified in human serum and in fish, respectively. The high amount in cooked fish cannot be ascribed to the release of PFCs from the non-stick pans or to the cooking process.

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