Journal of Chromatography A, 1217 (2010) 5913–5921

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Tetrahydrofuran–water extraction, in-line clean-up and selective liquid chromatography/tandem mass spectrometry for the quantitation of perfluorinated compounds in food at the low picogram per gram level

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ARTICLE INFO

Article history:
Received 1 April 2010
Received in revised form 15 June 2010
Accepted 13 July 2010
Available online 21 July 2010

Keywords:
PFOS
PFOA
Human dietary exposure
Teas plot
Hildebrand solubility parameter

ABSTRACT

A new solvent extraction system was developed for extraction of PFCs from food. The extraction is carried out with 75:25 (v/v) tetrahydrofuran:water, a solvent mixture that provides an appropriate balance of hydrogen bonding, dispersion and dipole–dipole interactions to efficiently extract PFCs with chains containing 4–14 carbon atoms from foods. This mixture provided recoveries above 85% from foods including vegetables, fruits, fish, meat and bread; and above 75% from cheese. Clean-up with a weak anion exchange resin and Envi-carb SPE, which were coupled in line for simplicity, was found to minimize matrix effects (viz. enhancement or suppression of electrospray ionization). The target analytes (PFCs) were resolved on a perfluorooctyl phase column that proved effective in separating mass interferences for perfluorooctane sulfonate (PFOS) in fish and meat samples. The mass spectrometer was operated in the negative electrospray ionization mode and used to record two transitions per analyte and one per mass-labeled method internal standard. The target PFCs were quantified from solvent based calibration curves. The limits of detection (LODs) were as low as 1–5 pg analyte g^{-1} food; by exception, those for C_4 and C_5 PFCs were somewhat higher (25–30 pg g^{-1}) owing to their less favourable mass response. To the best of our knowledge these are among the best LODs for PFCs in foods reported to date. The analysis of a variety of foods revealed contamination with PFCs at levels from 4.5 to 75 pg g^{-1} in 25% of samples (fish and packaged spinach). C_{10}–C_{14} PFCs were found in fish, which testifies to the need to control long-chain PFCs in this type of food. The proposed method is a useful tool for the development of a large-scale database for the presence of PFCs in foods.

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1. Introduction

Human exposure to perfluorinated compounds (PFCs) is currently receiving considerable attention from scientists and policy makers owing to the ubiquity of these substances in human blood and tissue samples worldwide, but particularly in industrialized areas [1,2]. The most abundant PFC in human samples is perfluorooctane sulfonate (PFOS), which was widely used; however, other perfluoroalkyl sulfonates (PFASs) and carboxylic acids (PFACs) are also frequently detected [1–3]. PFCs are toxic, highly persistent and bioaccumulative; this has led the European Union [4], North America [5] and major manufacturers such as 3M [6] and DuPont [7] to impose stringent restrictions on the production and use of compounds such as PFOS and perfluorooctanoic acid (PFOA).

Although humans are exposed to PFCs from a number of sources, food (drinking water included) could be the dominant intake pathway. PFCs can contaminate food by bioaccumulation of, especially, longer chain members in fish and shellfish (a result of oceans acting as contaminant sinks) [8] or contact with packaging material. Few systematic investigations on PFC levels in food are conducted to date mostly in North America and Western Europe [9–15]. The European Food Safety Authority (EFSA) has completed a risk assessment on PFOS and PFOA in the food chain and established a tolerable daily intake (TDI) of 150 and 1500 ng kg^{-1} body weight day^{-1}, respectively [16]. EFSA has noted an urgent need for data on PFC levels in various food items in order to better understand contamination routes and monitor trends in exposure levels.

Analysing PFCs in such complex and variable matrices as foods is a rather challenging task in many ways. The PFCs typically encountered in food include ionic, water-soluble short-chain and non-polar long-chain compounds (*viz.* C_4–C_{14} PFACs and C_4–C_8 PFASs), the extraction efficiency of which is strongly dependent on solvent polarity. The PFC concentrations measured so far suggest their presence at low levels (pg g^{-1} to low ng g^{-1} range) in

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doi:10.1016/j.chroma.2010.07.032

primary foods such as meat, milk, cereals, oil, fruits and vegetables, but higher levels ($ng\,g^{-1}$ to $\mu g\,g^{-1}$) for some specific compounds in fish and offal foods [1]. Whereas the quantitation of PFCs in fish is generally straightforward and has improved considerably in recent years [17–19], scaling down to the $pg\,g^{-1}$ level requires using highly efficient extraction methods in addition to extensive, complex clean-up and time-consuming solvent reduction procedures.

Only a limited number of methods are currently available to investigate dietary exposure to PFCs [10,11,20,21]. Food samples (1–10 g, wet weight) are usually serially extracted with medium-polar solvents such as methanol or acetonitrile, whether directly or following alkaline digestion. Commonly, a clean-up procedure involving successive treatment with dispersive graphitized carbon (ENVI-carb) and/or filtration through a weak anion exchange (WAX) SPE material is needed. Liquid chromatography (LC)/negative electrospray ionization (ESI)/triple-quadrupole mass spectrometry (MS/MS) has become the *de facto* standard for quantifying PFCs inasmuch as it provides detection limits in the range 1–100 pg. Most LC separation procedures for this purpose use standard C_{18} or C_8 phases; however, use of fluorinated stationary phases to separate PFCs by fluorine content and conventional reversed phase mechanisms is being fostered to prevent co-elution of known biological mass interferences with PFOS [22] and PFHxS [23].

These methods, however, are still confronted with many problems. Thus, they provide low recoveries which are strongly dependent on the chain length and polar groups present in the particular PFC, as well as on the sample matrix components. For example, the absolute recoveries from lamb liver provided by the most sensitive method reported so far (detection limits 1–650 $pg\,g^{-1}$) [11] are in the range 83–72% for C_{4-8} PFASs and 65–17% for C_{6-12} PFACs; these recoveries are matrix-dependent and differ from those for other foods (*e.g.* 30–70% for $^{13}C_4$-PFOS and 60–133% for $^{13}C_4$-PFOA). In addition, little information is available about the concentrations of the shorter- (C_{4-5}) and longer-chain (C_{13-14}) PFACs in foods owing to their poor extraction by medium-polar solvents.

In this work, we developed a simple, fast, and efficient method for the quantitative extraction of C_4–C_{14} PFACs and C_4–C_8 PFASs from a variety of representative food items prior to their LC–ESI–MS/MS determination. Mixtures of tetrahydrofuran (THF) and water were used for this purpose on the grounds of their large differences in dielectric constant (ε) and Hildebrand solubility parameter (δ), and hence of the ability to prepare mixed solvents spanning a wide range of dispersion, dipole–dipole and hydrogen bonding forces [24] which were examined with a view to facilitating solubilization of all PFCs. Sample clean-up was done by using an in-line coupled anion exchange resin and graphitized carbon SPE; and LC was done on a perfluorooctyl stationary phase to prevent matrix mass interferences for PFASs. The results are discussed below.

2. Material and methods

2.1. Chemicals

All chemicals were analytical reagent-grade and used as supplied. Both target and mass-labeled PFCs were supplied by Wellington Laboratories, in 50 $\mu g\,mL^{-1}$ solutions. The 14 target PFCs studied were as follows: perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA); perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUdA), perfluorododecanoic acid (PFDoA), perfluorotridecanoic acid (PFTrDA), perfluorotetradecanoic acid (PFTeDA), potassium perfluoro-1-butanesulfonate (PFBS), potassium perfluoro-1-hexanesulfonate (PFHxS) and PFOS. The stable isotope analogues $^{13}C_4$ PFBA, $^{13}C_2$ PFHxA, $^{18}O_2$ PFHxS, $^{13}C_4$ PFOA, $^{13}C_5$ PFNA, $^{13}C_2$ PFDA, $^{13}C_4$ PFOS and $^{13}C_2$ PFUdA were used as method standards (ISs) to control for potential losses during extraction and clean-up and MS performance (incl. ion suppression and enhancement). $^{13}C_8$ PFOS, $^{13}C_8$ PFOA and $^{13}C_7$ PFUdA were used as injection ISs and added just prior to injection. The injection ISs were only used to *monitor* MS performance and were not used for correction of the results. Sodium taurodeoxycholate hydrate (TDCA) and ammonium formate were supplied by Sigma. Tetrahydrofuran (THF) was obtained from Sigma–Aldrich (Steinheim, Germany) and methanol (MeOH) and LC-grade water were supplied by JT Baker (Deventer, The Netherlands). Stock standard solutions each containing a mixture of target PFCs, method ISs or injection ISs, at a 100 $ng\,mL^{-1}$ concentration each were prepared separately in methanol and stored in closed polypropylene bottles at room temperature.

2.2. Determination of PFCs in foods

2.2.1. Sample preparation and preservation

Fillets (muscle tissue) of raw fish (herring, pangasius, salmon and flounder) and meat (pork and chicken), whole-grain bread, vegetables (spinach and carrot), fruits (orange and apple), cheese (*Gouda*) and sunflower oil samples were bought at local supermarkets in Amsterdam (The Netherlands) in August–September 2009. An amount of 50 g of fish, meat or fruit was homogenized in an Ultra-Turrax homogenizer T25 equipped with S25N-8g and S25N-25g dispersing elements (Ika Werke, Germany); on the other hand, cheese and vegetables were homogenized in a crushing machine. About 10 g of each homogenized sample (2.5 g for cheese) was weighed in a 50 mL polypropylene (PP) tube, freeze-dried for 24 h (freeze-drier Lyph lock 1 L, Labconco, Kansas City, MO, USA) and stored frozen at −20 °C until analysis. Sunflower oil samples (5 g) were used untreated.

2.2.2. Tetrahydrofuran–water extraction

Freeze-dried vegetable, fruit, meat and fish samples were fortified at a 125 $pg\,g^{-1}$ wet weight (w.w.) concentration level with method ISs and extracted with 20 mL of 75:25 (v/v) THF:water by shaking in 50 mL PP tubes with an orbital shaker (SM 30, Edmund Buhler Gmbh, Hechingen, Germany) at 500 rpm for 10 min. Cheese samples were spiked with 500 $pg\,g^{-1}$ concentrations of the method ISs. The volume of THF:water mixture used to extract bread was 30 mL. Sunflower oil samples (5 g fresh weight, 125 $pg\,g^{-1}$ method IS) were cleaned up directly. After extraction, samples were centrifuged (centrifuge SW 12, Firlabo, Meyzieu, France) at 3500 rpm for 10 min and 10 mL of their clear supernatant (15 mL for bread) was transferred to 15 mL PP tubes and allowed to evaporate down to 6 mL at 50 °C under a nitrogen stream; because only THF evaporated, the concentrated solution contained 42% water. The samples were then diluted to 15 mL with distilled water and centrifuged again at 3500 rpm for 5 min to facilitate precipitation of solids and phase separation of lipids.

2.2.3. Clean-up by anion exchange and in-line coupled graphitized carbon SPE

Oasis WAX (6 cm^3, 150 mg) and Supelclean ENVI-carb (6 cm^3, 250 mg) cartridges supplied by Waters and Supelco (Zwijndrecht, The Netherlands), respectively, were used for sample clean-up. Diluted extracts (15 mL, solid material and lipids discarded) or sunflower oil (5 g) were transferred onto preconditioned [25] weak anion exchange (WAX) SPE cartridges at a rate of 1 drop/s. After loading, the cartridges were washed with 4 mL of 25 mM acetate buffer at pH 4 and 8 mL of a 50:50 (v/v) THF:acetonitrile mixture at a rate of 2 drop/s. Further cleaning was achieved by using larger volumes of THF:acetonitrile mixture (12 mL for vegetables and fruits, and 16 mL for sunflower oil). Then, the SPE WAX cartridge

Table 1
Figures of merit of the quantitation of PFCs with the proposed method.

PFCs	Calibration range ($ng\,mL^{-1}$)	[a]Coefficient of determination (r^2)	Retention time (min)	[b]Method LOQ ($pg\,g^{-1}$ w.w.)	[b]Method LOD ($pg\,g^{-1}$ w.w.)
PFBA	0.7–20	0.995	14.4	60	30
PFBS	0.7–20	0.994	25.0	50	25
PFPeA	0.7–20	0.994	25.7	60	30
PFHxA	0.05–30	0.995	33.1	15	5
PFHxS	0.5–20	0.996	36.6	25	10
PFHpA	0.05–20	0.993	38.7	15	5
PFOA	0.15–20	0.994	42.8	10	5
PFOS	0.05–20	0.997	43.8	3.5	2
PFNA	0.05–20	0.996	45.8	5	3
PFDA	0.05–20	0.997	47.8	5	3
PFUdA	0.15–20	0.998	49.2	10	5
PFDoA	0.025–20	0.998	50.4	2.5	1
PFTrA	0.025–20	0.997	51.6	2.5	1
PFTeA	0.025–20	0.993	52.8	2.5	1

[a] $n = 8$.
[b] Calculated on the basis of 70% recovery for each PFCs (a "worst-case scenario").

was coupled to the ENVI-carb cartridge via a suitable polyethylene (PE) adaptor cap and a volume of 6 mL of methanol containing 0.1% NH_4OH passed through both SPE materials. Cartridges were dried under vacuum to ensure maximal recovery of the eluates, which were evaporated to dryness (50 °C, N_2) and reconstituted with 250 μL of a mixture of 1:1 methanol and aqueous ammonium formate (6.3 mM, pH 4) containing a 5 $ng\,mL^{-1}$ concentration of injection ISs. Finally, the extracts were transferred to 1.5 mL PP Eppendorf tubes and centrifuged (ultracentrifuge Biofuge 28RS, Heraeus Sepatech, Lelystad, Netherlands) at 13,000 rpm for 10 min, after which a 200 μL aliquot of supernatant was transferred to a PP LC vial.

2.2.4. Quantitation of PFCs by LC–ESI–MS/MS

The target PFCs were separated and quantified by using Agilent 1200 Series LC system (Palo Alto, CA, USA) coupled with an Agilent 6410 electrospray interface (ESI) operating in the negative ion mode prior to triple-quadrupole mass spectrometric detection. A Fluorosep RP Octyl column (particle size 5 μm, i.d. 2.1 mm, length 15 cm) supplied by ES Industries (West Berlin, NJ, USA) was used as stationary phase. A Waters Symmetry C_{18} guard column (particle size 5 μm, i.d. 3.9 mm, length 20 mm) obtained from Waters (Milford, MA, USA) was inserted before the LC column. A Water Symmetry C_{18} column (particle size 5 μm, i.d. 2.1 mm, length 50 mm) also supplied by Waters was used to assess the advantages of the fluorinated column in terms of selectivity. The injection volume used was 20 μL. The mobile phase consisted of 6.3 mM aqueous ammonium formate at pH 4 and methanol, and was passed at a flow rate of 0.3 $mL\,min^{-1}$. The column temperature was set at 25 °C. The gradient elution program was 65% water during the first 2 min, a linear gradient from 65% to 5% water over the next 53 min and 100% methanol for another 10 min. Reconditioning the column took about 10 min. As recommended by the supplier, the Fluorosep column was cleaned after each batch of runs. For this purpose, the column was flushed with water for 15 min to remove the buffer and then with a 30:70 THF:ACN (v/v) mixture for 2 h to remove highly hydrophobic compounds. The operating conditions for the ESI source were as follows: capillary voltage 1000 V, source temperature 325 °C, gas flow rate 6 $L\,min^{-1}$ and nebulizer gas pressure 25 psi. Table 1 of Supplementary Data shows the quantifier and qualifier ions coming from two selected transitions used for each target PFC, the internal standards together with their corresponding quantifier ions, and the associated values for the fragmentor voltage and collision energy. The quantifier and qualifier ions for TDCA (a common interference for PFOS) are also given. The selected reaction monitoring transitions for each analyte and internal standard were acquired by using a dwell time of 0.02 s for each. Solvent based calibration curves were constructed from standard solutions containing the target PFCs at concentrations over the ranges stated in Table 1 and 5 $ng\,mL^{-1}$ concentrations of method and injection ISs and were prepared by appropriate dilution of methanolic stock solutions with 1:1 methanol/aqueous ammonium formate (6.3 mM, pH 4). Instrument control, file acquisition and peak integration were done with the software Mass Hunter (Agilent). PFC concentrations in sample extracts (containing the method IS at 5 $ng\,mL^{-1}$ that is added before extraction) were calculated from the calibration curve obtained by plotting the ratio of analyte peak area to method IS peak area against the analyte concentration.

3. Results and discussion

3.1. Control of background contamination

One typical problem encountered in determining PFCs is background contamination arising from the presence of a variety of fluoropolymer materials in the components of LC equipment or labware [21,26]. Inlet solvent polytetrafluoroethylene (PTFE) tubes were identified as the main source of contamination with PFCs (at the low $ng\,mL^{-1}$ level) in our LC system. Its effect, however, was effectively suppressed by replacing the PTFE tubes with PEEK tubes. As a precautionary measure, an additional column (Water Symmetry 5 μm, 2.1 mm × 50 mm) was inserted between the pump and injector in order to trap PFCs (mainly PFOA) released from the instrument. Contamination arising from labware was prevented by using disposable PP tubes, vials and pipettes. Eluates were evaporated with 99.999% pure nitrogen. No contaminating PFC was detected above its detection limit. In any case, appropriate blanks were routinely injected into the instrument during sample processing sequences in order to check for potential procedural or instrumental contamination.

3.2. Solvent extraction method

Efficient extraction of amphiphilic molecules requires the use of solvents capable of establishing properly balanced polar and non-polar interactions. The hydrocarbon chains of PFCs investigated here contain 4–14 carbon atoms and are highly hydrophobic owing to the presence of fluorine. The polar groups in PFACs include hydrogen donors and acceptors, whereas those in PFASs include anions and hydrogen acceptors. It is difficult to obtain strong enough polar (hydrogen bonding, dipole–dipole) and non-polar (dispersive) interactions with all PFCs to ensure efficient extraction from food by using an individual solvent. A solvent mixture must thus be used instead.

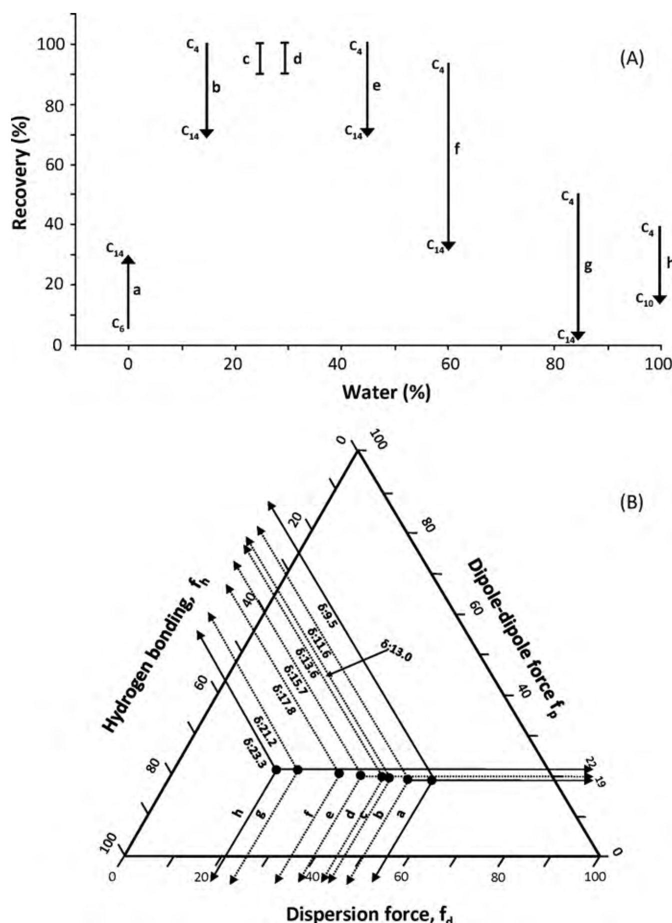

Fig. 1. (A) Recovery ranges obtained by extracting of frozen-dried pangasius fillet samples (10 g, wet weight, blank material) fortified with C_4–C_{14} PFCs (10 ng g^{-1} w.w.), using water:THF mixtures in variable (v/v) ratios: (*a*) 0:100, (*b*) 15:85, (*c*) 25:75, (*d*) 30:70, (*e*) 45:55, (*f*) 60:40, (*g*) 85:15 and (*h*) 0:100. (B) Teas graph showing the Hildebrand solubility (δ) and Hansen (f_d, f_p and f_h) parameters for each THF:water mixture.

In this work, we used mixtures of THF and water on the grounds of their substantial difference in solvation capability this being given by their different Hildebrand solubility parameter values (δ_{water} = 23.3 $cal^{1/2}$ $cm^{-3/2}$, δ_{THF} = 9.5 $cal^{1/2}$ $cm^{-3/2}$). The Hildebrand parameter (δ_T) provides a measure of the overall intermolecular forces resulting from the additive effect of dispersion (δ_d), dipole–dipole (δ_p) and hydrogen bonding (δ_h) forces. Individual values for these forces (Hansen parameters, $cal^{1/2}$ $cm^{-3/2}$) are easily available from the literature [24]; based on their values for water (δ_d = 7.6, δ_p = 7.8, δ_h = 20.6) and THF (δ_d = 8.2, δ_p = 2.8, δ_h = 3.9), hydrogen bonding and dispersion forces are the major components in water and THF, respectively. Hansen parameters can be used to calculate fractional Teas parameters in order to construct a Teas diagram, i.e. a triangular plot that graphically represents the solubility of a solvent in terms of these three forces [24]. Solvents spanning a wide range of solvation can be obtained simply by changing the composition of the THF:water mixture as shown in Fig. 1B.

The ability of THF:water mixtures in v/v ratios from 100:0 to 0:100 to extract C_4–C_{14} PFACs and C_4–C_8 PFASs from food was assessed by using freeze-dried pangasius fillet samples (10 g, wet weight, blank material) fortified at a 10 ng g^{-1} w.w. concentration of target PFCs. After extraction, the target compounds were directly measured in the untreated solvent extract. Before development of the method was completed, matrix-matched calibration was used in all tests to ensure accurate quantitation. Fig. 1A shows the PFC recoveries obtained with the different THF:water mixtures studied (solvent volume = 40 mL).

No extraction of the most polar (<C_6) and non-polar (>C_{10}) PFCs was achieved with water; also, the recoveries obtained with pure THF never exceeded 40% (Fig. 1A,a,h). The balance between polar (dipole–dipole and hydrogen bonding) and non-polar (dispersion) forces for both solvents (Fig. 1B,a,h) resulted in inadequate solubilization of PFCs. The recoveries obtained with most of the solvent mixtures were strongly dependent on the length of the hydrocarbon chain of the PFCs (Fig. 1A,b,e–g), the lowest values invariably being those for the most hydrophobic compounds. Using a 75:25 (v/v) THF:water mixture (Fig. 1A,c) suppressed the dependence of recoveries on the PFC structure and raised them above 94%. Similar results were obtained with THF:water mixture compositions around the previous value (e.g. Fig. 1A,d).

Based on these results, the ideal solvent for extracting C_4–C_{14} PFCs is one with a Hildebrand parameter value around 12–14 (Fig. 1B); and Teas parameters with (f_h + f_p)/non-polar (f_d) force ratios of about 1.2. For example, a 25:75 mixture of water and THF has f_h = 34, f_p = 20 and f_d = 46 (Fig. 1B,c). A solvent mixture consisting of 75:25 (v/v) THF:water was finally chosen as optimal for extraction.

Because water content varies among food types (*e.g.* ∼70–75% for fish and meat, but ∼90–95% for fruits and vegetables), obtaining quantitative recoveries and reproducible results entails freeze-drying food samples for analysis. The effectiveness of this procedure was assessed by freeze-drying 10 g (wet weight) of pangasius fillets fortified at a 10 ng g^{-1} w.w. concentration of target C_4–C_{14} PFACs and C_4–C_8 PFASs for 24 h, and extracting the freeze-dried samples with 40 mL of 75:25 (v/v) THF:water. These tests were conducted in triplicate. The recoveries obtained from the freeze-dried samples exceeded 95% for all PFCs, and standard deviations were 2–5%. Therefore, freeze-drying the samples caused no significant PFC losses.

Since water is a major component of food and can be expected to influence the solvation behaviour of common solvents for PFC extraction (methanol, acetonitrile), it is advisable to estimate its influence via a Teas diagram; irrespective of the particular solvent used for extraction, freeze-dried samples can be expected to give more reproducible, less matrix-dependent results in PFC quantitation.

The optimal ratio of solvent volume to sample amount was determined by extracting a variety of foods containing variable proportions of proteins, carbohydrates and lipids (*e.g.* herring and flounder fillets, cheese, pork, green pepper and bread). Freeze-dried samples (10 g, wet weight, fortified at a 10 ng g^{-1} w.w. concentration of method ISs) were extracted with 10, 20, 30 and 40 mL of 75:25 (v/v) THF:water in 50 mL PP tubes with shaking at 500 rpm for 10 min and subsequent centrifugation at 3500 rpm for 10 min. An aliquot of each extract was used for analysis. The recoveries thus obtained were independent of the solvent volume within the studied range. A 2:1 ratio of solvent (mL) to sample (g) was selected as optimal for most samples in order to be able to analyse an aliquot of the extract representing half the solvent volume used for extraction. Higher solvent (mL)/sample (g) ratios were selected for cheese and bread (4:1 and 3:1) owing to the high fat content of cheese (∼27%) which resulted in phase separation and precluded the use of a homogeneous solution at a 2:1 ratio—and the high porosity of bread, which hindered sample dispersion and solvent recovery after extraction.

The influence of matrix components on PFC recoveries was investigated by extracting representative food samples fortified at a 10 ng g^{-1} w.w. concentration of method ISs. Fig. 2 shows the results. Matrix-matched calibration was used to correct recoveries for potential suppression or enhancement of IS ionization. As can be seen from Fig. 2, recoveries were matrix-independent and exceeded

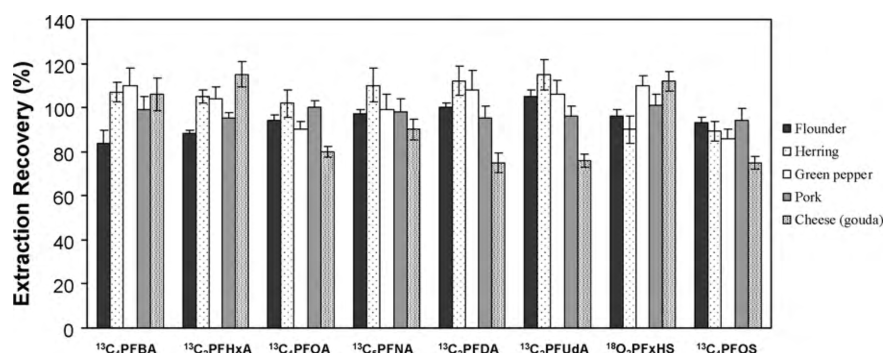

Fig. 2. Recoveries and standard deviations ($n = 2$) obtained by extraction with 20 mL of 75:25 (v/v) THF:water of a variety of food samples (10 g, wet weight, and 2.5 g for Gouda cheese) fortified with method internal standards at a 10 ng g^{-1} concentration. Matrix-matched calibration was used to calculate recoveries.

85% in all samples except cheese, where they were around 75% for some ISs (*e.g.* $^{13}C_4$ PFOA, $^{13}C_2$ PFDA and $^{13}C_2$ PFUdA). The high extraction efficiency of this method for PFCs spanning a wide polarity range in any type of food is one clear advantage over existing methods [11,17].

The extraction method was validated by analysing flounder fillets previously used in the Second International Interlaboratory Study on PFCs [20] and containing the following concentrations of the target compounds, expressed as mean values and their standard deviations: 18.0 ± 4.1, 17.5 ± 4.6, 21.1 ± 4.6, 15.9 ± 4.1, 17.3 ± 5.2 and 150 ± 44 ng g^{-1} for PFOA, PFNA, PFDA, PFUdA, PFDoA and PFOS, respectively. Sample aliquots (10 g, wet weight) were freeze-dried and spiked with the corresponding mass-labeled PFCs (see Table 1 of Supplementary Data). The mean values and standard deviations thus obtained with the proposed method were 18.5 ± 0.8, 19.2 ± 0.3, 21.7 ± 0.3, 18.0 ± 0.9, 21.9 ± 0.8 and 166 ± 7 ng g^{-1} for PFOA, PFNA, PFDA, PFUdA, PFDoA and PFOS, respectively. These results are all within the range of reported mean $\pm$ *s* values for the reference material.

3.3. *Sample clean-up and concentration*

Individual or serial weak anion exchange (WAX) SPE and dispersive graphitized carbon are currently the most widely used clean-up choices for quantifying PFCs in food [11,17,18]. Because the amount of sample used for analysis was 10 g, the use of both SPE materials was highly advisable in order to have clean enough sample extracts—and avoid MS signal suppression or ionization caused by matrix interferences and sensitivities at the pg g^{-1} level as a result. In this work, two serial clean-up steps involving WAX and graphitized carbon materials were applied to the THF:water extracts; investigations were intended for getting quantitative recovery of the target PFCs and simplify the overall procedure.

Similarly to the methanol extracts, dilution with water was required to obtain good recoveries [11,23]; in fact, direct filtration of 20 mL of the 75:25 (v/v) THF:water extracts through WAX SPE resulted in very low retention of PFCs (Table 2). Retention greatly improved by effect of partially or completely evaporating the THF in the extract with nitrogen at 50 °C and adding water to obtain a final THF:water volume of 20 mL—at this point, centrifugation was needed to remove matrix components which had thus been rendered insoluble. Table 2 shows the most salient results. Quantitative retention of PFCs on WAX was only achieved with THF proportions below 25%, which was thus chosen as optimal—evaporation of the required volume of THF took only around 5–10 min by virtue of the high volatility of the solvent. Retention of PFCs was also favoured by filtering lower sample volumes [*e.g.* 10 mL of sample extract in 75:25 (v/v) THF:water], but was still inadequate for most PFCAs (Table 2). Samples of sunflower oil (5 g, fresh weight) were directly filtered through WAX SPE cartridges.

The cartridges were initially eluted with 4 mL of acetate buffer (25 mM, pH 4), which is allegedly required to remove interfering biomolecules (lipids, proteins) and improve adsorption of the target anions [25], and then with 8 mL of a 50:50 (v/v) mixture of THF and acetonitrile. This hydrophobic solvent mixture ($\varepsilon_{THF} = 7.5$, $\varepsilon_{ACN} = 36$) proved more efficient than another commonly used solvent—methanol [11,25]—in removing highly hydrophobic compounds such as dyes or lipids, and rendered the PFC extracts less coloured. The low solubility of PFCs in pure THF and ACN—neither is a hydrogen donor, so neither can solubilize the typical anionic polar groups of PFCs—ensured the absence of PFC losses during the washing step. In order to obtain cleaner extracts, a volume of THF:ACN mixture of 12 mL was used with vegetables and fruits; as a result, the internal standard recoveries for $^{13}C_8$ PFOA, $^{13}C_7$ PFUdA and $^{13}C_8$ PFOS from final orange sample extracts rose from 67%, 64% and 80% to 110%, 94% and 105%, respectively, as the amount of washing solvent was increased from 8 to 12 mL. A volume of 16 mL of solvent mixture was used for sunflower oil samples, which were subjected to no pretreatment.

Elution of PFCs from the WAX material with 4 mL of methanol containing 0.1% NH_4OH was quantitative and independent of the particular food type; however, the most hydrophobic PFCs were strongly adsorbed to the insoluble matrix components produced during evaporation to dryness of the eluates and could not be completely recovered by reconstitution with 250 μL of a 50:50 (v/v) mixture of 6.3 mM ammonium formate and methanol; for example, salmon extracts exhibited losses of 50–60% for

Table 2
Performance of the WAX SPE clean-up step as a function of the THF content of the sample extracts (expressed as recoveries of method ISs).

THF (%)	$^{13}C_4$ PFBA	$^{13}C_2$ PFHxA	$^{13}C_4$ PFOA	$^{13}C_5$ PFNA	$^{13}C_2$ PFDA	$^{13}C_2$ PFUdA	$^{13}C_4$ PFHxS	$^{13}C_4$ PFOS
75	6.4 ± 0.2	4.8 ± 0.6	2.3 ± 0.2	1.9 ± 0.1	1.51 ± 0.04	1.3 ± 0.1	5.9 ± 0.4	1.5 ± 0.4
35	90 ± 4	97 ± 4	85 ± 14	87 ± 18	79 ± 25	75 ± 31	96 ± 4	94 ± 2
25	103 ± 6	99 ± 3	95 ± 5	102 ± 5	99 ± 3	102 ± 4	97 ± 4	98 ± 2
15	100 ± 4	99 ± 1	105 ± 3	105 ± 6	104 ± 5	102 ± 3	95 ± 4	105 ± 3
7.5	107 ± 7	100 ± 3	101 ± 4	105 ± 1	103 ± 1	102 ± 2	105 ± 1	105 ± 5
0	108 ± 6	98 ± 3	95 ± 2	98 ± 4	90 ± 5	89 ± 5	100 ± 3	91 ± 4
[a]75	91 ± 5	50 ± 7	44 ± 7	36 ± 6	33 ± 6	28 ± 7	76 ± 20	73 ± 17

Blank herring sample extract (20 mL, except [a]10 mL) fortified with a 20 ng g^{-1} concentration of method IS PFCs before SPE; $n = 3$.

Table 3
Method recoveries of the whole sample treatment (expressed as recovery for the method ISs).

	Fruits and vegetables				Meat		Fish			Cereal	Fat-rich	
	Carrot	Spinach	Apple	Orange	Chicken	Pork	Salmon	Panga	Herring	Bread	Cheese	Oil
Recovery ± s[a] (%)												
$^{13}C_4$ PFBA	111 ± 5	104 ± 8	111 ± 13	82 ± 9	102 ± 7	90 ± 8	104 ± 3	96 ± 7	100 ± 4	91 ± 10	79 ± 4	60 ± 2
$^{13}C_2$ PFHxA	116 ± 2	61 ± 3	103 ± 7	93 ± 9	105 ± 5	94 ± 7	105 ± 3	92 ± 5	86 ± 4	110 ± 12	69 ± 3	63 ± 4
$^{13}C_4$ PFOA	82 ± 6	90 ± 6	98 ± 7	82 ± 4	97 ± 5	105 ± 4	102 ± 3	84 ± 4	78 ± 1	108 ± 9	75 ± 4	70 ± 5
$^{13}C_2$ PFNA	103 ± 6	78 ± 3	103 ± 5	82 ± 6	91 ± 6	93 ± 4	75 ± 3	90 ± 1	73 ± 6	100 ± 4	76 ± 7	73 ± 5
$^{13}C_2$ PFDA	87 ± 6	70 ± 5	96 ± 4	84 ± 6	89 ± 5	103 ± 7	70 ± 5	92 ± 1	77 ± 3	114 ± 7	70 ± 7	75 ± 4
$^{13}C_2$ PFUdA	90 ± 7	72 ± 6	106 ± 8	82 ± 5	82 ± 4	97 ± 9	83 ± 3	89 ± 3	74 ± 3	108 ± 4	67 ± 4	74 ± 4
$^{13}C_4$ PFHxS	110 ± 13	65 ± 5	120 ± 13	81 ± 4	115 ± 5	104 ± 7	105 ± 6	104 ± 1	106 ± 12	104 ± 6	96 ± 6	98 ± 7
$^{13}C_4$ PFOS	97 ± 1	70 ± 5	120 ± 12	81 ± 4	104 ± 6	111 ± 5	107 ± 1	111 ± 4	105 ± 12	100 ± 2	87 ± 5	89 ± 7

[a] Standard deviation (n = 2).

C_{13} and C_{14} acids. This led us to subject the extracts to additional clean-up with ENVI-carb as proposed by other authors elsewhere [11,12].

In order to simplify the clean-up procedure with ENVI-carb, which is usually applied in a dispersive format [11,12,18]—and thus requires transfer and centrifugation of the extracts 2–4 times and causes the loss of some solvent as a result, we chose to conduct this step with Supelclean ENVI-carb cartridges (6 cm^3, 250 mg). The cartridges were previously conditioned with 4 mL of methanol and connected at the bottom with Oasis WAX cartridges through a suitable PE adaptor cap at the time of elution of PFCs—using 6 mL of methanol containing 0.1% NH_4OH is recommended to completely elute PFCs from both types of cartridges. The addition of acetic acid when using ENVI-carb [27] is recommended to avoid potential losses of anionic compounds by effect of the slightly basic nature of graphitized carbon. However, we checked that no losses of PFCs occurred in methanolic solutions containing 0.1% NH_4OH (fortified with 5 ng mL^{-1} concentrations of the PFCs) on passage thorough cartridges containing 250 mg of ENVI-carb. After elution, cartridges were vacuum-dried in order to maximize recovery of the eluate, which was evaporated to dryness (50 °C, N_2) and reconstituted with 250 μL of a mixture of aqueous 50:50 (v/v) ammonium formate (6.3 mM, pH4):methanol containing a 5 ng mL^{-1} concentration of injection internal standards (Table 1 of Supplementary Data). Ultracentrifugation of this extract is recommended because, although suitable for standard solutions, filtration causes losses of the most hydrophobic PFCs in some foods (*e.g.* around 20% for $^{13}C_2$ PFUdA in pork sample extracts passed through Waters GHP syringe filters, 0.2 μm, 13 mm).

3.4. Total method recoveries

The lack of mass-labeled homologs for some PFCs at the time of this study (namely for PFPeA, PFHpA, PFTrDA, PFTeA and PFBS) led us to examine the suitability of the proposed sample treatment for efficiently recovering the target PFCs throughout the procedure (extraction, WAX/ENVI-carb SPE, evaporation, reconstitution). Pangasius fillets samples—which were previously found to be free of detectable levels of PFCs; were fortified with target C_4–C_{14} PFACs and C_4, C_6 and C_8 PFASs at two different concentration levels (100 and 2000 pg g^{-1}) prior to freeze-drying. The recoveries ranged from 88% to 110%, and their relative standard deviations from 5% to 10% (n = 3). No significant differences in recovery between PFCs were found. This justifies using the recommended method ISs for PFCs having no commercially available mass-labeled homologs (Table 1 of Supplementary Data).

The ability of the proposed sample treatment to effectively extract PFCs from a variety of foods was assessed by spiking representative samples of each of the major food groups (*viz.* fruit and vegetables, meat, fish, cereals and fat-rich foods) following freeze-drying with a 125 pg g^{-1} concentration of method ISs, and calculating the total method recoveries. Table 3 shows the results. Recoveries exceeded 80% for fruits and vegetables—spinach excepted, and also for meat, fish and cereals. Therefore, the proposed method is the first reported method capable of quantitatively recovering PFCs at the low picogram per gram level from a variety of foods. Recoveries were lower for PFACs in fat-rich foods, but were still above 60% in all instances. Losses of PFACs from these matrices occurred mainly in their extraction with the THF:water mixture (*e.g.* in the longest chain PFACs from cheese, Fig. 2) or during treatment with WAX/ENVI-carb SPE for oil directly subjected to SPE. The decreased recoveries for spinach were ascribed to matrix effects on the signals for the injection ISs. An increase in the volume of solvent used for the washing SPE WAX step (maybe up to 16 mL as used for sunflower oil) could help to reduce matrix effects in this more complex matrix.

3.5. Analytical performance

3.5.1. Sensitivity

Table 1 lists the analytical figures of merit of the proposed method. The instrumental limits of quantitation (LOQs) and detection (LODs) were calculated from 50:50 (v/v) MeOH:aqueous ammonium formate (6.3 mM, pH 4) blanks containing a 5 ng mL^{-1} concentration of method and injection ISs at a signal-to-noise ratio of 10 and 3, respectively. The LOQs and LODs were 0.7 and 0.5 ng mL^{-1} for short-chain PFCs (PFBA, PFPeA and PFBS); 0.025 and 0.01 ng mL^{-1} for long-chain acids (PFDoA, PFTrA and PFTeA); and 0.05 and 0.02 ng mL^{-1} for all other compounds except PFOA and PFUdA (LOQ = 0.2 ng mL^{-1}, LOD = 0.12 ng mL^{-1}) and PFHxS (LOQ = 0.5 ng mL^{-1}, LOD = 0.3 ng mL^{-1}). The increased LODs and LOQs for the latter compounds were a result of their presence as impurities in proportions below 1% in the mass-labeled reagents used as internal standards and could be improved by decreasing the amount of ISs added to samples.

Method LOQs and LODs were calculated on the basis of sample blanks taking into account a signal-to-noise ratio of 3 and 10, respectively, and the concentration factor obtained with the method. Carrot, orange, pork, bread and sunflower oil sample extracts were used for this purpose. No significant differences in background noise between matrices were observed. This led us to adopt general LOQs and LODs for all types of foods based on worst-case calculations assuming 70% total average recovery for each PFC for the concentration factor since total method recoveries ranged from about 60–120% in all samples. Table 1 shows the estimated LOQs and LODs, which are among the lowest reported so far to the best of our knowledge for this type of determination [11,12,17]. Values of LOQs and LODs for cheese are four times higher due to the use of a smaller sample size (2.5 g). A smaller volume of solvent at reconstitution step could be used to compensate this loss of sensitivity.

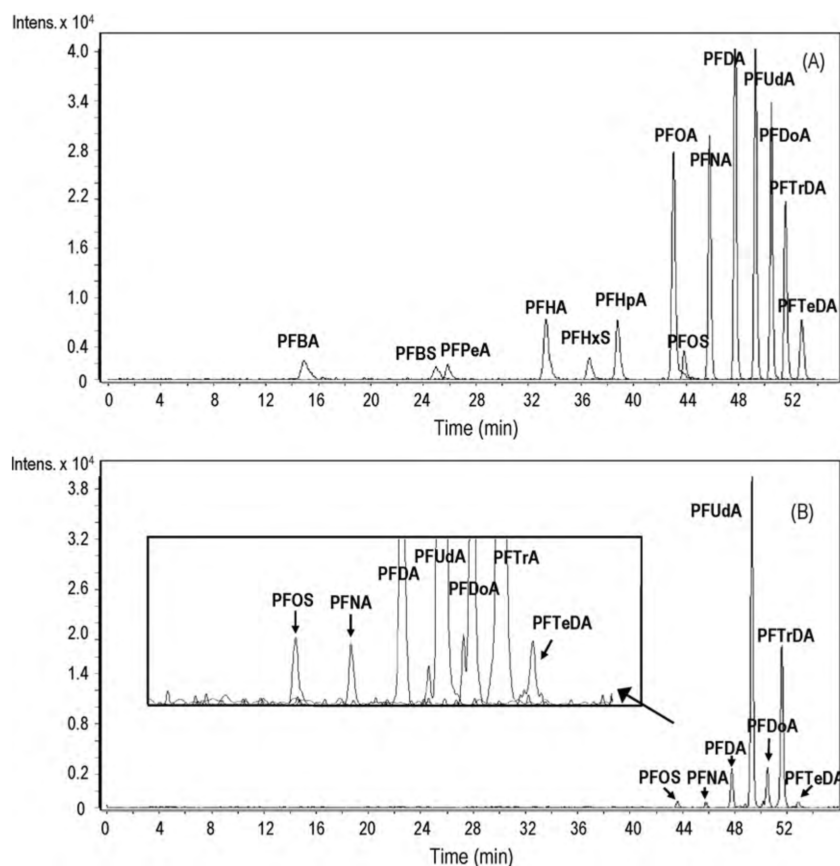

Fig. 3. LC–ESI–MS/MS selected ion chromatograms for PFCs extracted from (A) a standard solution containing a 1 ng mL^{-1} concentration and (B) a salmon muscle sample.

3.5.2. Selectivity

PFC quantitation is reportedly subject to substantial matrix effects even after thorough SPE clean-up [11,12,17]. Such effects, which result from co-eluting matrix components, are compound- and food type-dependent, and may cause signal enhancement or suppression, or even PFAS peak misidentification by effect of mass interferences in samples of animal origin. The effect of these interferences on the quantitation of PFCs with the proposed method were investigated in depth by using both the proposed column (Fluorosep RP Octyl), which provides selective retention, and the most widely used stationary phase (C_{18}).

Signal enhancement or suppression was estimated by comparing the response for injection ISs (5 ng mL^{-1}) in the final reconstituted extracts with the average response of these standards in the calibration solutions on the assumption that signal changes due to instrument fluctuations would be negligible. Table 2 of Supplementary Data shows the results, expressed as recoveries for the three injection ISs and two stationary phases used. Most of the samples analysed with the Fluorosep column (~85%) exhibited little signal suppression or enhancement (≤10%); the greatest effects in this respect were those on $^{13}C_8$ PFOS in spinach (25% suppression) and $^{13}C_7$ PFUdA in bread (15% enhancement). Matrix effects were generally more marked with the C_{18} column; thus, signal suppression was 36% and 57% for $^{13}C_8$ PFOA in spinach and herring, respectively, and 37% for $^{13}C_8$ PFOS in spinach, whereas signal enhancement amounted to 40% for $^{13}C_8$ PFOS in apple and cheese. Therefore, the combination of the proposed sample treatment and the selective chromatographic retention mechanism for PFCs is effective towards preventing signal enhancement or suppression by co-eluting matrix components.

Overestimating the proportion of PFOS by misreporting co-eluting cholic acids is a major problem here and continues to detract from accuracy in the determination of PFCs in samples of animal origin. For example, TDCA bile salts, which contain a sulfonate group, have been found to co-elute with PFOS on C_{18} columns [22,28,29]. The mass difference of these compounds (498.2968) is not large enough from that of PFOS (498.9297) and compromise accuracy in their QQQ tandem mass spectrometry determination; therefore, co-elution, especially in the presence of high levels of the interferents, can lead to overestimation at the 499–80 transition. This problem has been addressed in various ways including the use of other column types providing more selective retention mechanisms (*e.g.* perfluorooctyl [17] or a ion-exchange phase column [29]), the use of a more selective—but also less sensitive—transition (the 499–99 transition, mainly [11]) or that of time-of-flight (TOF) mass spectrometry, which affords accurate mass discrimination between PFOS and interferents [17].

PFOS peak misidentification was investigated by analysing a number of samples (n = 10) of animal origin including muscle meat from pork and chicken, and fish fillets from herring, salmon and pangasius, all of which were fortified at a 125 pg g^{-1} w.w. concentration of method ISs. To this end, the chromatographic separation of PFOS and TDCA on the Fluorosep column was optimized, the retention times being 43.8 and 38.0 min, respectively. Although the C_{18} column also provided well-resolved peaks for the TDCA reagent (37 min) and PFOS (36 min), TDCA isomers are commonly present in these samples, and co-elute with PFOS. Characteristic transitions for TDCA (498.3–80, 498.3–107 and 498.3–124) were also optimized, the fragmentor voltage and collision energy values being quite different from those used for PFOS (see Table 1 of Supplementary Data); this rendered TDCA undetected at the 499–80 transition used to quantify PFOS at concentrations below ~100 ng mL^{-1} (equivalent to 5 ng g^{-1} in the samples at an arbitrary extraction efficiency of 100% for TDCA). This had no effect on the Fluo-

rosep column, but increased the tolerance of TCDA by the C_{18} columns.

TCDA was present in all meat (chicken and pork) and fish samples (herring, salmon and pangasius), its estimated concentrations in them ranging from 2.5 to 350 ng g^{-1}. Quantifying PFOS with the Fluorosep column (499–80 transition) provided concentrations of 57 ± 5 and 16 ± 1 pg g^{-1} in herring and salmon, respectively, but undetectable levels in the other samples (including chicken). These values are consistent with those obtained by using the C_{18} column with the 499–99 selective transition, namely: *viz.* 61 ± 6 and 14 ± 1 pg g^{-1} for herring and salmon, respectively, and undetectable levels for the other samples. However, using the more sensitive 499–80 transition provided PFOS concentrations of 251 ± 50, 17 ± 1 and 33 ± 2 pg g^{-1} in herring, salmon and chicken, respectively, thus revealing that TCDA concentrations above 100 ng mL^{-1} in the extracts led to PFOS overestimation (~440% in herring) and misidentification (*e.g.* in chicken) with the C_{18} column. Although both the Fluorosep column and the C_{18} column can be used to quantify PFOS with accuracy, use of the former is recommended because it affords measurement at the 499–80 transition, which is more sensitive than the 499–99 transition. By way of example, Fig. 1 of Supplementary Data shows the chromatographic peaks for TDCA, PFOS and the method IS $^{13}C_4$ PFOS in the herring sample as obtained with the two columns. As can be seen, the cholic acid signal at the transition of interest for PFOS (499–80) was rather low and the measurement of PFOS with the C_{18} column was interfered with by this transition.

3.6. Analysis of food samples

The proposed method was used to analyse a variety of food samples ($n = 12$) including fish (herring, pangasius and salmon) and meat (pork and chicken) muscle fillets, whole-grain bread, vegetables (spinach and carrot), fruits (orange and apple), cheese (*Gouda*) and sunflower oil, all in duplicate.

Fig. 3 shows the selected ion chromatograms for PFCs extracted from (A) a standard solution containing a 1 ng mL^{-1} concentration and (B) a salmon muscle sample. Only three samples (25% of the foods studied) were found to be contaminated with PFCs (salmon, herring and spinach). Among PFASs, only PFOS was present in fish (at 57 ± 5 pg g^{-1} in herring and 16 ± 1 pg g^{-1} in salmon); also, PFHxS was detected in spinach, albeit at levels below its LOQ. As noted earlier, PFOS was clearly distinguished from the bile salts present in fish. Long-chain PFACs were present in fish, which confirms that bioaccumulation of these compounds involves mainly those with the longest carbon chains [30] and the need for simple methods to quantify C_{12}–C_{14} PFACs. The concentrations found were 27 ± 2, 7.7 ± 0.5, 31 ± 2, 11.3 ± 0.5 and 27 ± 6 pg g^{-1} for PFOA, PFNA, PFUdA, PFDoA and PFTrDA, respectively, in herring; and 5.6 ± 0.6, 10.3 ± 0.7, 75 ± 4, 16 ± 1, 31.3 ± 2 and 4.5 ± 0.6 pg g^{-1} for PFNA, PFDA, PFUdA, PFDoA, PFTrDA and PFTeDA, respectively, in salmon. Spinach samples, which were bought packaged, were also contaminated with PFACs: PFHpA at 8.8 ± 0.4 pg g^{-1} and PFOA at 31 ± 2 pg g^{-1}. PFC contamination in packaged lettuce was previously reported by other authors [12]. Transfer from soil to crops [31] is a possible source of contamination with PFCs in these foods. In addition, contamination may also originate from processing the foods (e.g. washing) and packaging. The generally low levels of PFCs found in this study are consistent with previous results of other authors [9,11,12].

4. Conclusions

The following method is proposed for PFCs in food: freeze-drying of the samples; extraction using THF–water 75:25%; SPE enrichment and clean-up using combined Oasis WAX and graphitised carbon followed by LC–ESI–MS/MS using a fluorinated stationary phase column. The main assets of the proposed method for determining PFCs in a variety of dietary foods include (a) very low detection limits (1–5 pg g^{-1} except for C_4 and C_5 PFCs), which should enable accurate estimation of current food contamination levels; (b) the ability to quantitatively extract PFCs spanning a wider polarity range (C_4–C_{14}) relative to most existing methods (C_6–C_{12}); (c) the matrix-independence of recoveries for a variety of samples (lipid-, protein- and carbohydrate-rich food) by effect of the high extraction efficiency and minimal matrix effects—a result of a smart combination of efficient extraction and clean-up with the use of a highly selective perfluorooctyl phase column for PFC separation; and (d) a high accuracy and precision.

Acknowledgments

The authors gratefully acknowledge funding from Spain's MCyT (Project CTQ2008-01068), from the EU (research project PERFOOD, ref. nr. 227525) and from the Dutch Food and Consumer Product Safety Authority (VWA, Office for Risk Assessment). A. Ballesteros-Gómez is also grateful to Spain's MEC for award of a doctoral fellowship (Grant AP2005-4275) which covered a short stay at the VU University of Amsterdam to conduct this research.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.07.032.

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