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Development and validation of a pressurized liquid extraction liquid chromatography–tandem mass spectrometry method for perfluorinated compounds determination in fish

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ABSTRACT

This paper describes the development and validation of an analytical methodology to determine eight perfluorinated compounds (PFCs) in edible fish using pressurized liquid extraction (PLE) with water and solid-phase extraction (SPE) with an ion-exchanger as extraction and pre-concentration procedures, followed by liquid chromatography–quadrupole-linear ion trap mass spectrometry (LC–QqLIT–MS). The rapidity and effectiveness of the proposed extraction procedure were compared with those most commonly used to isolate PFCs from fish (ion-pairing and alkaline digestion). The average recoveries of the different fish samples, spiked with the eight PFCs at three levels (the LOQ, 10 and 100 $\mu g\,kg^{-1}$ of each PFC), were always higher than 85% with relative standard deviation (RSD) lower than 17%. A good linearity was established for the eight PFCs in the range from 0.003–0.05 to 100 $\mu g\,kg^{-1}$, with $r > 0.9994$. The limits of quantification (LOQs) were between 0.003 and 0.05 $\mu g\,kg^{-1}$, which are well below those previously reported for this type of samples. Compared with previous methods, sample preparation time and/or LOQs are reduced. The method demonstrated its successful application for the analysis of different parts of several fish species. Most of the samples tested positive, mainly for perfluoropentanoic acid (PFPA), perfluorobutane sulfonate (PFBS) and perfluorooctanoic acid (PFOA) but other of the eight studied PFCs were also present.

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1. Introduction

Perfluorinated compounds (PFCs) have been and are used in a wide variety of industrial applications, such as stain repellents, textile, paints, waxes, polishes, electronics, adhesives and food packaging [1,2]. They have been manufactured for more than 50 years, having been estimated that from 1951 to 2004 up to 7300 tons were released into the environment following production and use [1]. As a consequence, these compounds show a global distribution all over the world and have been detected not only in environmental samples but also in human blood and liver. PFCs show persistence in the environment and some of them are related to different carcinogenic actions, for example perfluorooctanoic acid (PFOA) has been identified as a potent hepatocarcinogen in rodents [3,4]. Meanwhile PFCs have been recognized as emerging contaminants in the food chain by the European Food Safety Authority (EFSA), which have recently finalized its opinion on perfluorooctane sulfonate (PFOS), PFOA and its salts establishing tolerable daily intakes (TDI) of 150 $ng\,kg^{-1}$ b.w. day^{-1} for PFOS and 1500 $ng\,kg^{-1}$ b.w. day^{-1} for PFOA [5]. The opinion of the EFSA on these compounds also highlights that concentration levels, contamination pathways, and toxicological potency should be assessed in the food chain and expresses its concern by the lack of available data [5].

A growing but still insufficient number of studies report on the occurrence of PFCs in food and drink [6–9]. In these papers, bioaccumulation in fish has been shown to be the main influence of PFCs in dietary exposure [10]. Some reports have also found a positive correlation between PFCs concentrations in plasma and consumption of fish, corroborating the importance of this exposure route [11]. Accordingly, these compounds have been widely analyzed in blood, bile and liver [12–16] but not so often in the edible part (muscle) of fish [17,18]. Levels of PFOS and PFOA have been reported in mussels, oysters, shrimp and fish from different countries [19–22]. However, it is often impossible to give details of the other PFCs homologues present in this matrix.

So far, most of the analysis methods to determine PFCs are based on liquid chromatography coupled to mass spectrometry

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or tandem mass spectrometry approaches (LC–MS or LC–MS/MS) [1,2]. Among them, triple quadrupole (QqQ) MS is the most widely employed analyzer because of their high dynamic range and good performance when working in selected reaction monitoring (SRM) mode [1]. In the recent LC–MS/MS methods, ion paired, potassium hydroxide or solvent extractions were applied, for which the reported limits of quantification (LOQ) for PFOA and PFOS were as low as 1 $\mu g\,kg^{-1}$ [2]. However, many challenges still remain for either LC–MS/MS or the sample preparation protocols. Hybrid MS instruments have proved to be powerful tools to achieve high sensitivity, specificity and selectivity, as they combine the main advantages of the two analyzers (i.e. quadrupole and time of flight in case of QqTOF or quadrupole and liner ion trap in case of QqLIT) [23,24]. The main advantage of the hybrid QqLIT over other LC–tandem MS equipments relies on that it achieves unequivocal identification and confirmation of target compounds at highly sensitive levels [23,25]. Its unique feature is that the second mass analyzer, Q3, can be run in two different modes, retaining the classical QqQ scan functions such as SRM, product ion, neutral loss, and precursor ion while providing access to sensitive ion trap scans. This allows very powerful scan combinations when performing information-dependent data acquisition (IDA), enhanced product ion (EPI) or MS^3 experiments obtaining concomitantly both quantitative and qualitative information. Simultaneously, modern extraction and clean-up techniques, such as pressurized liquid extraction (PLE), microwave assisted extraction (MAE) or solid-phase microextraction (SPME), have not been applied to the determination of PFCs yet. These techniques provide rapidly and accurately clean extracts for sensitive analysis [24].

Consequently, the aim of this study was the development and validation of a simple, sensitive and selective analytical methodology to determine eight PFCs, using PLE with water and SPE on ion-exchanger for the extraction and pre-concentration of target compounds from various fish samples including liver, muscle and roe. To our knowledge, this work is the first example of the application of PLE for the determination of PFCs from food. Target compounds were perfluorobutanesulfonate (PFBS), perfluoropentanoic acid (PFPA), PFOA, PFOS, perfluoro-7-methyloctanoic acid (i,p-PFNA), perfluorononaoic acid (PFNA), perfluorodecanoic acid (PFDA) and perfluoro-1-decanesulfonate (L-PFDS). Validation comprised the assessment of linearity, limit of quantification, recovery and precision. To prove the potential of this method, a comparison with ion-pairing and alkaline digestion extractions, – the two most widely employed procedures to extract PFCs from fish – was also included in this study. The ion-pairing forms neutral species of the anionic surface-active FFCs making them extractable from food samples by organic solvents. The use of alkaline digestion helps to extract bound PFC residues by removing lipids and proteins before extraction. Analyte identification and confirmation was performed using a LC–QqLIT–MS/MS in compliance with the EU regulations (EU Commission Decision 2002/657/EC). Finally, PFC residues were determined in different fishes taken in several markets of Valencia and Barcelona cities.

2. Experimental

2.1. Chemicals

The isotope-labelled internal standards (ISs) perfluoro-n-[1,2,3,4$^{13}C_4$]octanoic acid (MPFOA), perfluoro-1-[1,2,3,4$^{13}C_4$] octanesulfonate potassium salt (MPFOS), and perfluoro-n-[1,2$^{13}C_2$]decanoic acid (MPFDA) as well as sodium L-PFDS, PFNA and i,p-PFNA were purchased from Wellington Laboratories (Guelph, Ontario, Canada) as 50 $\mu g\,ml^{-1}$ methanolic solutions (1.2 ml). Tetrabutylammonium PFBS (purum ≥ 98%), PFOS sodium salt (98%), PFPA (97%) PFOA (96%), PFDA (97%), were purchased from Aldrich (Steinheim, Germany). Separate stock solutions of the analytes were prepared in methanol at a concentration of 1.0 $mg\,ml^{-1}$ of free compound or salt. A standard mixture containing the 8 analytes was made from the stock solutions (commercial or laboratory made) to provide different concentrations of the analytes depending on their expected concentrations in fish and on the sensitivity of the method. Concentrations of the analytes in the standard mixture were calculated as free compounds. Working mixtures were diluted from the standard mixture in methanol/water both 20 mM ammonium acetate (10/90, v/v). Solutions of ISs were diluted to a concentration of 2 $\mu g\,ml^{-1}$ with methanol/water both 20 mM ammonium acetate (10/90, v/v), and appropriate volumes of the ISs were added to fish samples so as to obtain concentrations of 1.5 $\mu g\,kg^{-1}$ in the sample material.

LC-grade 'suprasolv' water, methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Deionized water (<18 MΩ cm resistivity) was from a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). All the solvents were passed through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain) before use. Analytical grade reagent sodium sulfate anhydrous and glacial acetic acid were also from Scharlau. Ammonium acetate (99%, pa for HPLC) and sea sand were from (Sigma–Aldrich, Steinheim, Germany). Ammonium hydroxide (25% in water), sodium hydroxide (analytical grade), tetrabutyl ammonium hydrogen sulfate and methyl-ter-butyl ether were from Merck (Darmstadt, Germany).

Oasis Wax cartridges of 60 mg (3 cm^3), particle size 30 μg and pore size 80 Å used were from Waters (Milford, MA, USA). Oasis WAX is a polymeric reversed-phase, Weak Anion Exchange mixed-mode sorbent that allows for the retention and release of strong acidic compounds (e.g. such as sulfonates).

2.2. Sampling

The following fish species were purchased in retail fish markets and supermarkets as a whole fish: young hake (*Merluccius bilinearis*, *n* = 5), anchovy (*Engraulis encrasicolus*, *n* = 5) and striped mullet (*Mujil cephalus*, *n* = 3). Each sample of young hake and anchovy weighted around 2 kg (ca. 16 specimens/sample and 100 specimens/sample, respectively) and each sample of striped mullet consisted of only one specimen (weights between 180 and 520 g). Furthermore, hake roe (*n* = 2) and swordfish fillets (*Xiphias gladius*, *n* = 3) were also taken in these markets. All the samples were sent in fresh conditions (on ice) to the laboratory. Whole fishes were dissected, taken the liver and the entire right dorsal lateral fillet with the skin. The liver was completely and carefully separated. The livers corresponding to each sample were homogenized together. The right dorsal lateral fillets, swordfish fillets and hake roe were cut in small pieces. Subsamples of 200 g were homogenized using a bapitaurus food chopped (Taurus, Berlin, Germany), placed into polyethylene (PP) bags and stored at −80 °C prior to analysis.

2.3. Sample preparation

2.3.1. Pressurized liquid extraction

The muscle and liver samples (ca. 2 g, fresh weight) were weighted into a porcelain mortar, added with the ISs and homogenized with approximately 25 g of sea sand using a pestle. The advantages of homogenizing the tissue with sea sand were to disrupt the cell membranes (the great pore and particle sizes of this solid support in comparison with others helps to gridding the sample) and to disperse the sample over a large surface area to obtain better extraction. This mixture was put into a 22 ml extraction cell then, this cell was filled up with washed sea sand. Whatman glass fiber filters were placed at the bottom and top of the extraction cell

to avoid the obstruction of metal filters by solid particles. Samples were extracted by PLE using an ASE 200 system (Dionex, Sunnyvale, CA, USA). The sample was heated to 110 °C with a static period of 7 min and extracted by a flush volume of 100% in 3 cycles using water. Pressure was set to 1500 psi and purge time to 1 min. The final extracts had a volume of 42 ml.

The process SPE/clean-up used in this work was based on that reported by Ye et al. [26] for the analysis of perfluorinated compounds in carp fillets. Briefly, Oasis WAX 3 cartridges were conditioned by passing 4 ml of 0.1% ammonium hydroxide in methanol, 4 ml of methanol, and 4 ml of deionized water through the cartridge. The PLE extract was passed through the cartridge, that was then washed with 4 ml of 25 mM acetate buffer (pH 4) followed by 4 ml of methanol. The PFCs were eluted with 4 ml of 0.1% ammonium hydroxide in methanol. SPE extracts were concentrated to 0.5 ml under nitrogen (60 °C) using a Zymark TurboVap concentrator.

2.3.2. Alkaline digestion

Samples were analyzed using a modification according to Ye et al. [26] of a method described by Taniyasu et al. [27]. About 1 g of liver and muscle (fresh weight) was homogenized with 5 ml of water using Ultraturrax T-25 digital homogenizer and added with the IS. The homogenate was combined with 8 ml of 10 mM sodium hydroxide in methanol. Each sample was digested by shaking on an orbital shaker table at room temperature for 16 h. After digestion, samples were centrifuged at 2000 × g for 5 min, and 3 ml of the supernatant was diluted with 27 ml of deionized water prior to solid phase extraction (SPE) cleanup as described for the previous procedure.

2.3.3. Ionic-pair extraction

The sample pre-treatment procedure was similar to that described by Hansen et al. [28] except for some modifications. Briefly, 5 ml of distilled water were added to the homogenized tissue sample (about 1 g, fresh weight) and spiked with the ISs. After homogenization on an Ultra Turrax homogenizer, 1 ml of TBA (0.5 M, pH 10) and 2 ml of sodium carbonate solution (0.25 M) were added to the homogenized tissue sample. The sample solution was agitated on a vortex mixer for 20 s and 5 ml MTBE was added. After agitation on an orbital shaker for 20 min, the sample solution was centrifuged at 3000 rpm for 10 min (at 25 °C). The organic and aqueous layers were separated by centrifugation, and an exact volume of MTBE (4.0 ml) was removed from the solution. The aqueous phase was again extracted twice with two fresh portions of MTBE solution (4 ml); all rinses were combined in a second polypropylene tube. The solvent was allowed to evaporate under nitrogen using the Zymark TurboVap before being reconstituted in 0.5 ml of methanol–water both 20 mM ammonium acetate (10/90, v/v).

2.4. Liquid chromatography–quadrupole-linear ion-trap mass spectrometry (QTRAP)

Perfluorinated compounds were analyzed on a 4000 Q TRAP™ MS/MS system from Applied Biosystems/MDS Sciex (Concord, Ontario, Canada) coupled to a Symbiosis™ Pico system (Spark Holland, Emmen, The Netherlands). The latter integrated HPLC and Online SPE system but only the HPLC system was used that basically consist of an Alias™ autosampler and two high pressure gradient LC pumps with a 4-channel solvent selector for each pump. Separations were accomplished on a LiChroCART-LiChrospher 100 RP-18 analytical column of 250 mm × 4 mm and 5 μm particle diameter from Merck at room temperature. The mobile phase consisted of 20 mM ammonium acetate in water (solvent A) and 20 mM ammonium acetate in methanol (solvent B) and was delivered at a flow rate of 0.5 ml min^{-1}. The linear gradient elution program was as follows: 10–80% B over 5 min, then 80–90% B over other 5 min followed by an isocratic hold at 90% B for 8 min. At 18 min, B was returned to 10% in 2 min. The total run time for each injection was 20 min and the injection volume 20 μl. The mass spectrometer was operated in the negative ion mode with a TurboIonSpray source. The selected reaction monitoring (SRM) conditions and the retention time of each analyte are listed in Table 1. The other ionization parameters were as follows: curtain gas (CUR), 30 (arbitrary units); ion source gas 1 (GS1), 25 (arbitrary units); ion source gas 2 (GS2), 25 (arbitrary units); source temperature (TEM), 350 °C; ionspray (IS), −4500 V; entrance potential (EP), −10 V, collision cell exit potential (CXP) −10 C and declustering potential (DP) −25 V.

The dwell time of each MRM transition was 150 ms. The mass spectrometer was controlled by Analyst 1.4.2 software from Applied Biosystems/MDS Sciex and the Symbiosis from the Symbiosis Pico for Analyst software.

2.5. Quality assurance

Validation of the method included determination of linearity range, intra-assay precision, accuracy, matrix effects, limit of detection (LOD) and LOQ. With the exception of linearity, the validation experiments were performed by spiking muscle and liver samples of young hake and anchovy with all 8 compounds. For spiking the sample, 1 or 2 g portions of chopped fish were placed in the appropriate container according to the further extraction method and spiked with the PFCs standard solution, taken care to uniformly spread them on the sample. The spiked sample was left for 10 min at room temperature to ensure the appropriate distribution in the matrix. Then, the sample was processed, as reported in Section 2.3, depending on the extraction procedure. Five replicates of sample preparation and analysis were performed at each level. For the assessment of all the mentioned parameters, the analyte response was always related to the IS response (1.5 μg kg^{-1} of each, MPFOA, MPFOS and MPFDA) to compensate for undesirable matrix effects and losses during the extraction procedure. The ISs were selected because at the beginning of this study they were the only available ones.

Procedural blanks were carried out by the three extraction procedures and they did not show contamination by PFCs for the entire method. Blank tests were also carried out on all the sample containers to rule out possible contamination from the sampling, storage and shipment contained. Of the 18 fish samples analyzed, only two samples, one of young hake and other of anchovy did not show PFCs contamination. They were used for the matrix effect and recovery studies as well as, for LOD and LOQ assessment.

Finally, in order to comply with internal quality control (IQC) procedures, two control samples (spiked materials), two solvent injections and two procedural blanks were inserted into each analytical batch made up of six samples. The individual values obtained for control samples were plotted on a process-behaviour chart during the entire duration of the study to establish if the analysis is in a state of statistical control or not.

2.5.1. Selectivity

For identification purposes, retention times of PFCs in the standards and in the samples were compared at a tolerance of ±2.5%. Moreover, in accordance with the 2002/657/EC Decision [29], the relative ion intensities (each product ion area signal versus the base product ion area signal) of the spiked samples were compared with the relative ion intensities of PFCs standard solutions, at the same concentration levels as used for the construction of the calibration curve.

Selectivity in fish samples was demonstrated by analyzing 10 young hake and 10 anchovy extracts. These test samples, were analyzed by the three methods, being negative for PFCs.

Table 1
IS deprotonated molecules $[M-H]^-$, monitored transitions (including the tentative identification of the product ion), retention times (RT), collision energies (CE) of the PFCs in the order of retention time.

Analyte	IS	t_R (min)	Transitions m/z	Tentative ion identification	Transitions ratio	CE (V)
PFPA	MPFOA	9.36	263 > 219	$[M-H-CO_2]^-$	1.00	−15
PFBS	MPFOS		299 > 80	$[SO_3]^-$	1.00	−80
			299 > 99	$[FSO_3]^-$	0.11	−80
PFOA	MPFOA	11.02	413 > 169	$[C_3F_7]^-$	1.00	−25
			413 > 369	$[M-H-CO_2]^-$	0.42	−25
			413 > 219	$[C_4F_9]^-$	0.33	−25
MPFOA		11.02	417 > 372	$[M-H-CO_2]^-$		−25
i,p-PFNA	MPFDA	11.54	463 > 169	$[C_3F_7]^-$	1.00	−15
			463 > 219	$[C_4F_9]^-$	0.80	−15
PFNA	MPFDA	11.83	463 > 219	$[C_4F_9]^-$	1.00	−15
			463 > 169	$[C_3F_7]^-$	0.90	−15
PFOS	MPFOS	11.77	499 > 80	$[C_3F_7]^-$	1.00	−15
			499 > 99	$[FSO_3]^-$	0.20	−100
			499 > 280		0.01	−100
MPFOS		11.77	503 > 80	$[C_3F_7]^-$		−100
PFDA	MPFDA	12.85	513 > 119	$[C_2F_5]^-$	1.00	−100
			513 > 469	$[M-H-CO_2]^-$	0.40	−35
			513 > 268		0.02	−35
MPFDA		12.85	515 > 471	$[M-H-CO_2]^-$		−35
L-PFDS	MPFOS	13.61	599 > 80	$[SO_3]^-$	1.00	−100
			599 > 99	$[FSO_3]^-$	0.20	−100

A majority of the PFCs were separated chromatographically from each other during the LC run, as demonstrated in Table 1. Selectivity was assured by utilizing a QqLIT system in MS/MS mode, in which single chromatographic peaks were observed for all SRM transitions, except for i,p-PFNA and PFNA.

2.5.2. Limits of detection and quantification

The LOD was defined as the lowest concentration for which the peak area was at least three times larger than the background noise. Criteria for the LOQ were established as the lowest concentration fulfilling all of the following criteria: (1) bias from the calibration curve less than 25%, (2) relative standard deviation of four replicates below 19%, (3) peak shapes acceptable, and (4) signal-to-noise ratio at least 10. The LOQs obtained served as the lower limits of the linear range.

2.5.3. Linearity

Linearity range was defined by plotting the peak area ratio of the PFC to the IS versus PFC concentration. The following criteria for linearity range were applied: linear regression through zero with a correlation coefficient better than 0.990, bias from the calibration line less than 25% for all individual calibration points, and RSD of four replicates less than 25%. The lower limit of the linear range was at LOQ.

2.5.4. Matrix effect

The matrix effects were assessed by comparing the response of the analytes at 10 $\mu g\,kg^{-1}$ concentration in 20 mM ammonium acetate methanol/water (10/90, v/v) solution to the response of the analytes spiked at the same concentration into an extract of a blank matrix sample extract (young hake or anchovy) obtained through the sample preparation process.

2.5.5. Recovery and precision

According to the 2002/657/EC Decision [29], since no certified reference materials were available for the analytes and matrices of interest, the recovery from fortified negative samples was measured as an alternative to trueness. Briefly, negative samples of tissue and liver of anchovy and young hake (previously analyzed and found to be not contaminated) were spiked in quintuplicate as previously described with the eight PFCs at three different levels (LOQ, 10.0, 100.0 $\mu g\,kg^{-1}$). Precision, expressed as repeatability, was calculated by repeated analyses on the same sample sets as used for recovery tests, with the only difference that independent samples were re-extracted and analyzed on two other occasions for calculating inter-day repeatability.

3. Results and discussion

3.1. LC–MS/MS optimization

Many earlier studies have reported the use of conventional C_{18} LC columns for the separation of PFCs [7,9,10,30]. Under the mobile phase flow rates and gradient described above, PFCs were well resolved at retention times in the range of 9–14 min. The presence of a volatile salt (ammonium acetate) in the mobile phase is essential to obtain a proper peak shape. This salt could cause a suppressing effect on the analyte signal. Several ammonium acetate concentrations (5, 10, 20 and 30 mM) were evaluated to determine the mobile phase that offers short retention time and sufficient resolution for the PFCs with little or no suppression in signal-to-noise ration of the analytes. Up to 20 mM the possible reduction of the MS response of the analyte is compensated for by the improvement in peak shape providing a negligible reduction in the intensity of the signal observed.

Identification of the compounds was based on (1) precursor ion (deprotonated molecule), (2) two or three (when possible) selective product ions, and (3) retention time (Table 1). The most intense product ion of each compound was used for quantification. The only exception was PFPA, for which only one product ion was obtained at reasonable intensity. For the isotope-labelled ISs, only one fragment ion was monitored.

SRM transitions were chosen after optimization of the conditions considering both, sensitivity and selectivity. The precursor → product ion transitions reported in Table 1 are the same reported in earlier studies using LC–QqQ–MS/MS [17,19–21,31–34].

Table 2
Instrumental parameters of the LC–QqLIT–MS method developed for the analysis of PFCs (values obtained by injection of standard solutions in methanol–water 20 mM ammonium acetate (10:90, v/v)).

Compound	Calibration range (ng l^{-1})	R^2	ILOD (pg)	ILOQ (pg)	Repeatability (RSD 50 pg l^{-1})
PFPA	0.05–1000	0.9994	0.0003	0.001	10
PFBS	0.05–1000	0.9996	0.0003	0.001	12
PFOA	0.005–50	0.9994	0.00003	0.0001	13
PFNA	0.01–100	0.9994	0.0006	0.002	15
i,p-PFNA	0.01–100	0.9999	0.0006	0.002	9
PFOS	0.003–300	0.9996	0.0002	0.0006	7
PFDA	0.05–500	0.9996	0.0003	0.001	11
PFDS	0.05–500	0.9999	0.0003	0.001	12

However, those studies have used different precursor → product ion transition for quantification of the perfluorinated carboxylic acids (PFCAs) [19,31–33], e.g. product ions m/z 369, 419, 446 instead of the m/z 169, 169 and 119 used here for PFOA, PFNA and PFDA, respectively. Apparently, the lower mass fragments are more intense using the QqLIT mass analyzer than the QqQ, at least, in the chosen conditions. For perfluorinated sulfonates (PFSs) the product ions m/z 80 and 99, were used previously [19,31–33].

The ion ratios were calculated from calibration samples at five concentration levels between LOQ and 150 LOQ, and did not show dependence on the concentration. The variation of the ion ratios was below 10%, except for PFPS that shows higher variation up to 15%. The evaluated stability of the ion ratios was thereby in agreement with previous studies, in which the ion ratio tolerance between 20% and 50% was used.

The two stereo-isomers, PFNA and i,p-PFNA, are not separable by precursor or product ions, but with the LC-conditions used they were separated to baseline with a 0.2 min difference. Because of the high sensitivity of the QqLIT system, all transitions were acquired simultaneously at a relatively high flow rate for mass spectrometry without loss sensitivity.

The calibration curves obtained for both the quantification and the confirmation SRMs were linear for all compounds in a wide range of concentrations, typically from LOQ to 100 ng ml^{-1} with correlation coefficients (R^2) higher than 0.9994 for all compounds (see Table 2). It has been recently demonstrated that a quite wide dynamic measuring range of the analytical method is needed in order to quantify levels of PFCs in fish because they highly vary between samples [17,19].

In this study, the QqLIT instrument was used to perform the LC–MS/MS analyses in the SRM mode. As an additional feature, in this instrument, the SRM mode can be combined with attractive working modes (EPI and MS^3 modes) for the unambiguous confirmation of compounds. However, these modes have a limitation because the isolation and fragmentation steps are both occurring in the LIT, only fragment ions produced with m/z values of 30% of the parent mass and higher are stable in the ion trap. This drawback is difficult to overcome for perfluorinated sulfonates. On the contrary, a shortcoming of the use of the SRM mode is that at low concentrations of analyte, the second SRM transition is not detected, which is solved because of the higher sensitivity of this system.

3.2. Optimization of the PLE procedure

All parameters affecting the PLE extraction efficiency, such as temperature, pressure, static time, cell size, number of extraction cycles and flush volume, were carefully evaluated by the absolute recovery obtained by external standard calibration (without adding the IS). The optimum conditions were those reported in Section 2.3.1. The parameters with stronger influence on the recovery were the temperature, number of cycles and flush volume. The reported values were considered optimum because lower values provided considerable low recoveries whereas higher ones did not provide an increase in the recovery that justifies the longer time required. PLE using water as a solvent has already been reported to determine other contaminants and residues in food [35,36].

These reports [35,36] also check different dispersing agent and solvents. In this study, the sea sand was directly selected because it has thicker particle diameter that the other sorbents favoring the dispersion of the sample [35]. Some procedures treat the sand with EDTA to deactivated metal impurities present in the sorbent surface and, probably, chelates also present in the matrix facilitating decomplexation of analytes. PFCs recoveries does not show differences between EDTA treated and non-treated sand probably because they do not strongly bind to metals.

Methanol, water and methanol–water (50:50, v/v) were tested as extracts at different temperatures, to establish the better conditions. The best results were obtained with water at 110 °C and the coupling of the extraction with the clean-up by SPE is much easier with water. The mixtures of methanol required an additional step to evaporate the sample or to dilute the sample, which is detrimental for the LOD.

3.3. Validation

Selectivity of the method may be deteriorated by presence of endogenous species in biological extracts. It was difficult to find fishes without PFCs, especially when the LOQs are as low as those reported in the present study. However analysis of two blank sam-

Table 3
Recovery and RSD obtained at three concentration levels in liver and muscle fish using the PLE method.

Compound	Liver						Muscle					
	Conc.[a] µg kg^{-1}	Rec., % (x ± RSD)	Conc. µg kg^{-1}	Rec., % (x ± RSD)	Conc. µg kg^{-1}	Rec., % (x ± RSD)	Conc.[a] µg kg^{-1}	Rec., % (x ± RSD)	Conc. µg kg^{-1}	Rec., % (x ± RSD)	Conc. µg kg^{-1}	Rec., % (x ± RSD)
PFPA	0.05	85 ± 17	10	86 ± 9	100	85 ± 9	0.025	89 ± 9	10	88 ± 8	100	89 ± 7
PFBS	0.05	87 ± 12	10	88 ± 10	100	89 ± 15	0.025	90 ± 15	10	89 ± 11	100	89 ± 8
PFOA	0.005	92 ± 15	10	99 ± 11	100	101 ± 12	0.0025	97 ± 12	10	93 ± 10	100	93 ± 7
i,p-PFNA	0.01	93 ± 11	10	90 ± 10	100	95 ± 10	0.005	99 ± 10	10	100 ± 7	100	100 ± 9
PFNA	0.01	92 ± 10	10	95 ± 9	100	97 ± 9	0.005	100 ± 9	10	102 ± 10	100	92 ± 6
PFOS	0.003	101 ± 8	10	94 ± 8	100	100 ± 10	0.0015	102 ± 10	10	99 ± 9	100	99 ± 8
PFDA	0.05	101 ± 9	10	102 ± 9	100	93 ± 9	0.025	101 ± 9	10	95 ± 7	100	95 ± 6
PFDS	0.05	101 ± 7	10	96 ± 8	100	97 ± 9	0.025	99 ± 9	10	102 ± 8	100	102 ± 5

[a] Concentration corresponding to the LOQ.

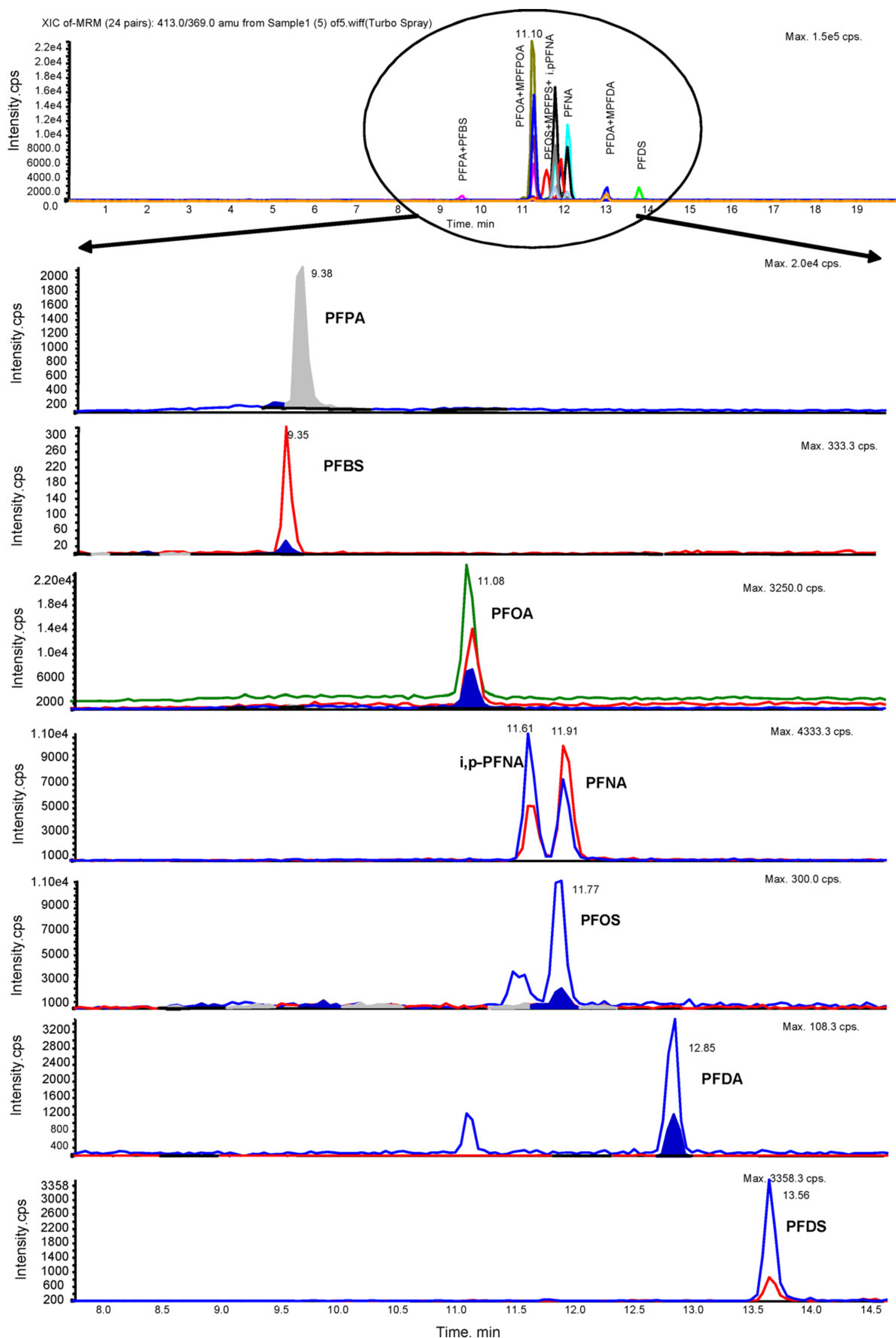

Fig. 1. Extracted ion chromatograms showing the monitored SRM transitions for the studied PFCs in a spiked anchovy sample at 0.05 μg kg^{-1}. The most intense is the one used for quantification, the other for confirmation of the compound.

ples, one of young hake and other of anchovy showed that no peaks were detected at the retention time of none of the 8 PFCs.

The calibration lines showed good linearity over the concentration range selected for the majority of the compounds (Table 2). Quantification was performed on standard in solvent, since matrix-matched standards are not very viable due to the considerable number of different fishes analyzed that have a great different fat content (3–33%). The differences in samples composition can raise recovery problems. For example, the extraction recoveries were >82% for fish muscle but they dropped to approximately 75% for liver samples. The use of isotopically labelled ISs normalized the recovery to an acceptable level, i.e. >85% in liver samples (see Table 3) achieving an improvement in recovery when it was below an acceptable level.

Even through matrix-matched standards were not used, matrix effects on LC–MS/MS were estimated comparing the analytical response given by a neat standard at 10 μg kg^{-1} and the same solution added to a fish extract. These effects did not contribute seriously to dismiss the process efficiency values because they were comprised between 80% and 96% with repeatability RSD < 15%. The use of ISs compensated for the matrix effects totally, in the case of PFOA, i,p-PFNA, PFNA, PFOS, PFDA and PFDS, and partially for PFPA and PFBS.

The LOQs obtained in muscle and liver (Table 3) were far below the concentrations of PFCs reported in fish. For PFOA, i,p-PFNA, PFNA and PFOS, the LOQ was lower than 0.01 μg kg^{-1} for both muscle and liver samples. Markedly higher LOQs were obtained for PFPA, PFBS, PFDA and PFDS (0.05 μg kg^{-1}). Nevertheless, these LOQs were below the reported concentrations in fish [17,19,34] and were thereby low enough to allow use of this method in routine screening and quantitation of PFCs in marketed samples. Comparison of this method to earlier LC–MS/MS methods revealed that the LOQs obtained here for most of the compounds were essentially better [6,7,20,21]. This improvement is mainly because of the application of LC–QqLIT–MS instrument, which allows an increase in sensitivity of more than 100 times over those using conventional QqQ instruments. However, the PLE procedure has also a minor influence in the better sensitivity because it provides high concentration factor and appropriate recoveries. Fig. 1 displays typical extracted ion chromatogram of the PFCs from an extract of spiked anchovy muscle at 0.05 μg kg^{-1} of each compound.

Precision and accuracy are summarized in Table 3. The RSD in liver and fish was lower than 15% for PCFs in fish and lower than (17%) in liver. In fish, the recovery was for the majority of the compounds higher than 88%. In liver samples, recovery was usually higher than 85%. It becomes clear that with the ISs acceptable relative PFCs recoveries (>85–89%) were obtained even in situations when the differences in sample matrix provided lower absolute PFCs recoveries. Precision and recovery were essentially at the equal level as

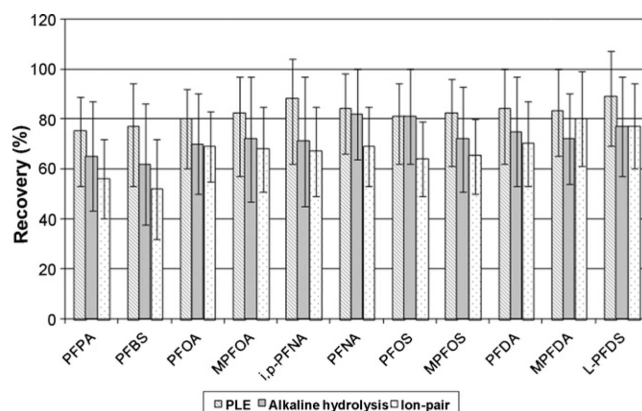

Fig. 2. Histogram of the absolute recoveries (calculated by the external standard method) and RSDs obtained from young hake spiked at 1 μg kg^{-1}.

in other PFCs LC–MS/MS methods, which also employs ISs [19–21].

3.4. Comparison to other methods

Results obtained by the present method were compared to those obtained by the commonly used ion-pair and alkaline hydrolysis methods described in Sections 2.3.2 and 2.3.3. The summarized results are presented in Table 4, in which the average percentage of recovery at the LOQ level is shown. No systematic difference existed between the results, except for the LOQs that are higher using the alkaline digestion than the ion-pair and those are higher than those obtained by the proposed PLE procedure. The accurate determination of PFCs was achieved by employing commercial isotopically labelled ISs, which compensated for target analyte losses and enhanced or suppression matrix effects.

Then, external standard calibration (the analyte response was not related to the IS) was evaluated. The rationale for evaluating external calibration is that neither recovery nor matrix effects, e.g. ion suppression, was accounted for quantitation. The recoveries and the RSDs of the method showed that PLE provides better recoveries and lower RSDs (Fig. 2). Furthermore, the PLE method is much more rapid than the alkaline digestion and provides cleaned extracts than that based on ion pairing. PLE allows to process up to 24 samples and extract them automatically, which saves time and personnel.

3.5. Application to fish samples taken from the market

The applicability of the method was assessed through the analysis of the selected PFCs in several fish samples. Table 5 shows the mean values of PFCs for each type of sample. The highest PFCs con-

Table 4
Recovery and RSD obtained using ion-pair and alkaline hydrolysis in samples of anchovy liver and fish spiked at the LOQ.

Compound	Alkaline hydrolysis				Ion-pairing			
	Liver		Muscle		Liver		Muscle	
	Conc. [a] μg kg^{-1}	Rec., % ($x \pm$ RSD)	Conc. [a] μg kg^{-1}	Rec., % ($x \pm$ RSD)	Conc. [a] μg kg^{-1}	Rec., % ($x \pm$ RSD)	Conc. [a] μg kg^{-1}	Rec., % ($x \pm$ RSD)
PFPA	0.15	80 ± 17	0.15	85 ± 17	0.07	79 ± 19	0.05	75 ± 18
PFBS	0.15	85 ± 12	0.15	87 ± 12	0.08	75 ± 17	0.05	82 ± 17
PFOA	0.015	92 ± 15	0.015	92 ± 15	0.001	95 ± 14	0.005	96 ± 15
i,p-PFNA	0.03	90 ± 11	0.03	93 ± 11	0.02	92 ± 16	0.015	95 ± 14
PFNA	0.03	95 ± 10	0.03	92 ± 10	0.02	97 ± 15	0.015	99 ± 16
PFOS	0.01	101 ± 8	0.01	93 ± 8	0.004	102 ± 13	0.005	93 ± 12
PFDA	0.15	101 ± 9	0.15	100 ± 9	0.06	93 ± 15	0.05	102 ± 10
PFDS	0.15	99 ± 7	0.15	99 ± 7	0.06	92 ± 17	0.05	94 ± 9

[a] Concentration corresponding to the LOQ.

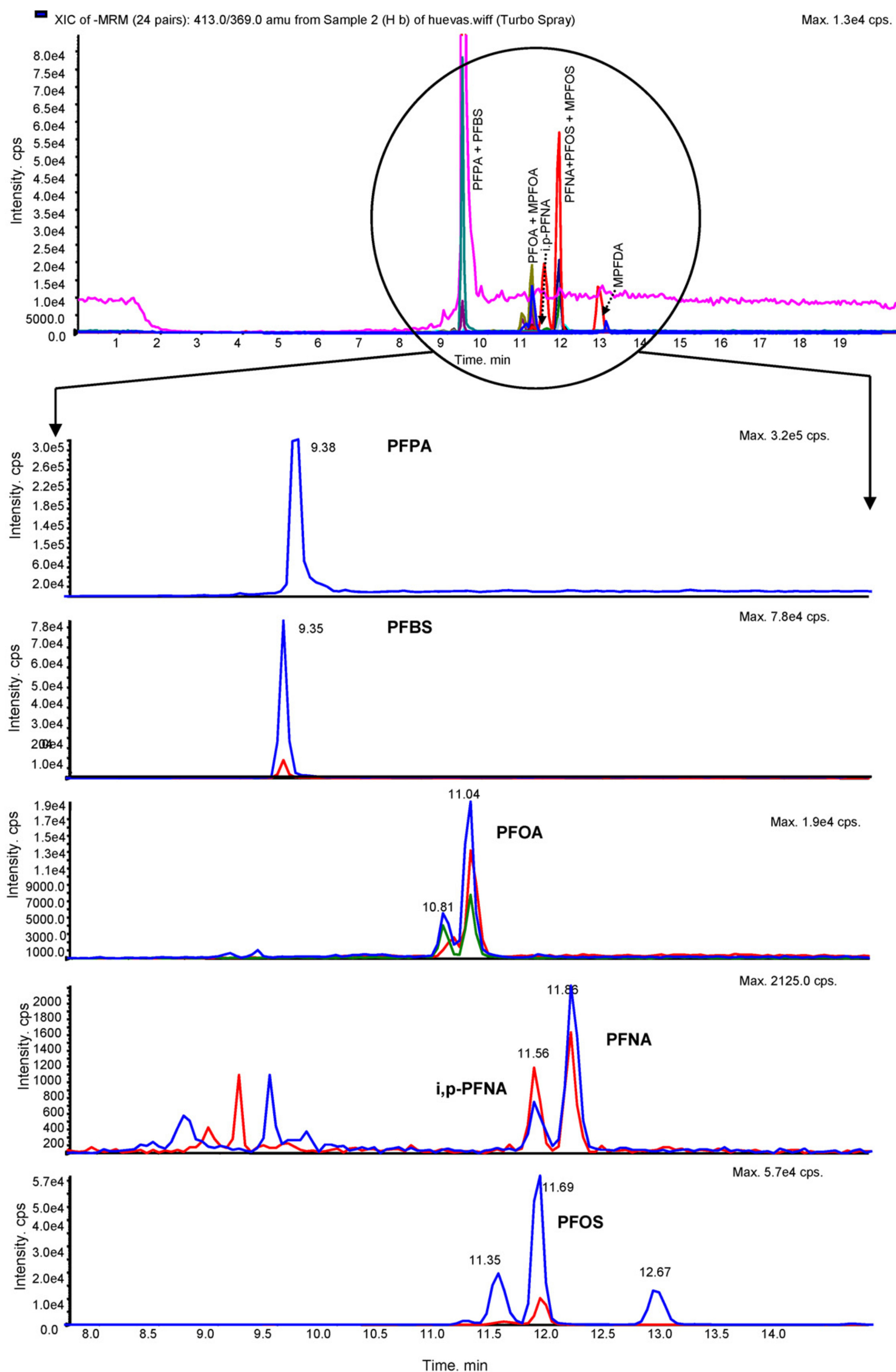

Fig. 3. Extracted ion chromatograms corresponding to the PLE extraction and LC–QqLIT–MS analysis of one hake roe sample. Concentrations calculated were: PFPA, 46.82 μg kg^{-1}; PFBS, 12.82 μg kg^{-1}; PFOA, 1.75 μg kg^{-1}; i,p-PFNA, 0.41 μg kg^{-1}; PFNA, 0.63 μg kg^{-1}; PFOS, 24.35 μg kg^{-1}.

Table 5
Mean PFCs concentrations ($\mu g\,kg^{-1}$) detected in the fish samples analyzed.

Compound	Hake Roe	Swordfish	Stripped Mullet		Young Hake		Anchovy	
			Muscle	Liver	Muscle	Liver	Muscle	Liver
PFPA	50.00	12.84	42.03	12.32	0.52	0.71	0.09	0.12
PFBS	10.00	13.45	<LOQ	2.04	<LOQ	1.24	0.83	2.23
PFOA	2.50	1.25	2.43	2.83	3.25	5.21	0.21	1.03
i,p-PFNA	0.44	3.24	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
PFNA	0.58	1.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
PFOS	23.04	8.24	<LOQ	<LOQ	1.25	3.54	0.23	0.94
PFDA	<LOQ	0.24	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
PFDS	<LOQ	1.02	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

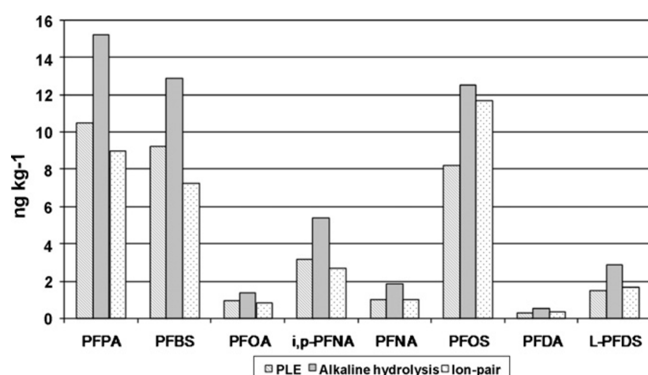

Fig. 4. Histogram of PFCs average concentrations in swordfish muscle by the three extraction methods.

centrations were those corresponding to hake roe. Fig. 3 shows the chromatograms corresponding to one of the three replicates of the hake roe sample, in which PFPA, PFBS, PFOA, PFNA and PFOS were detected. It should be noted that the transition for quantification corresponding to PFOA (413 → 219) shows two additional peaks that cannot be fully confirmed by the second and third confirmatory transitions. These peaks are probably caused by the presence of branched isomers in natural contaminated samples.

The sample of swordfish, which contains all the studied PFCs, was analyzed by the three extraction methods, the results are summarized in Fig. 4. According to Taniyasu et al. [27], the alkaline digestion provided three-to-five higher concentration levels of several PFCs in liver samples than the ion pairing. These authors attributed the greater concentrations of PFCs obtained by the alkaline digestion method to the effective digestion of the matrix and the release of these compounds from the sample. In our study, slightly higher levels in the sample obtained by alkaline digestion were also observed but not so markedly higher as those reported by Taniyasu et al. [27]. Statistical comparison by one-way analysis of variance showed that the differences were not significative, and cannot be considered conclusive.

Analyzing the monitoring results presented in this study, a general conclusion is that PFOA and PFOS levels were in the same range as those found in previous studies carried out in different geographic areas [19–22].

4. Conclusions

The present study demonstrates that LC–MS/MS using a QqLIT mass analyzer was applicable to the simultaneous analysis of 8 PFCs in liver, roe and muscle fish. PLE extraction was chosen for the pretreatment because it was more suitable than par ionic and alkaline digestion for liver and fish samples. It was more rapid and automatic achieving the simultaneous process of up to 24 samples. The proposed method demonstrates to improve LOQs, marginally enhance method recoveries, and decrease analysis times, which will be likely of high value to industry and research laboratories interested in quantitation of PFCs in aquatic organisms.

The high sensitivity of the method provided by the use of QqLIT and the optimized fragmentation conditions, attained reliable quantification at trace level in muscle and liver samples. Separation of i,p-PFNA and PFNA was possible with the LC gradient that combined proper resolution and not too long chromatographic run. The method has shown its feasibility in a study of several edible fish samples from the market. Since the method was developed and validated, it has been routinely used in both laboratories for the screening, quantification and confirmation of PFCs in food as part of a monitoring program.

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