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Study on Determining Perfluorooctanoic Acid and Perfluorooctane Sulfonic Acid in Fishery Products

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Abstract

In the study, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for simultaneous determining perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) in fishery products. QuEChERS method (Quick, Easy, Cheap, Effective, Rugged and Safe), originally developed by Anastassiades, was modified to extract samples. The samples were hydrated and extracted with acetonitrile and formic acid, supported by inorganic salts, matrix co-extracts were removed by C18 and the extracts were passed through OASIS PRiME HLB catridges.The limit of detection (LOD) and limit of quantification (LOQ) of PFOA and PFOS were 0.016 ng/g, 0.05 ng/g and 0.05 ng/g, 0.15 ng/g, respectively. The recovery ranged from 96.2 - 120%, all repeatability is satisfactory (RSD < 21%). 10 fishery samples were analyzed, in which PFOA was detected in 2 samples and PFOS was not detected.

Keywords: *PFOA, PFOS, fishery products, LC-MS/MS, QuEChERS.*

1. Introduction

In 2008, the European Food Safety Authority (EFSA) described perfluoroalkyl and polyfluoroalkyl substances (PFAS) as common names for a large group of fluorine substances, including oligomers and polymers. Their structure consisted of a hydrophobic alkyl chain of varying lengths (usually C4-C16) linked to a hydrophilic end group. The hydrophobic part could be completely fluoridated or partially fluoridated. When fully fluoridated, the molecules were called perfluoroalkyls, while when partially fluoridated, they were called polyfluoroalkyls [1]. The two most common compounds of PFAS are perfluorooctanoic acid (PFOA) and perfluorooctane-sulfonic acid (PFOS).

PFOA is an 8-carbon PFAS, all hydrogen atoms in the alkyl chain are replaced by fluorine and the polar end group was carboxylic acid, so PFOA belongs to the large group of perfluoroalkyl carboxylic acids (PFCA). Due to its composition consisting of a hydrophobic alkyl end and a hydrophilic end group, PFOA has been commonly produced and used as a surfactant and as a production material for some commercial products [2-4].

PFOS is a PFAS that also has 8 carbons and all the hydrogen atoms in the alkyl chain are replaced by fluorine like PFOA but the hydrophilic group of PFOS was sulfonic acid, so PFOS belongs to the large group of PFSA. Similar to PFOA, PFOS is also used as a surfactant due to the construction of a hydrophobic end and a hydrophilic end [2-4].

Food contamination with these compounds was mainly through the use of PFAS-containing materials in direct contact with foods and as a result of bioaccumulation in the food chain through PFAS-containing water and soil sources. PFOS and its salts were listed under Appendix B of the Stockholm Convention on Persistent Organic Pollutants [5], while PFOA, salts and PFOA-related compounds were added to Annex A in 2019 [6]. In 2022, the European Union published Commission Regulation (EU) 2022/2388 amending Regulation (EC) No. 1881/2006 concerning the maximum levels of perfluoroalkyl substances in certain foods to ensure the protection of human health, which the content of PFOA and PFOS were stipulated from 2 to 35 ng/g and from 0.2 to 8 ng/g in the muscles of fishery products not used for the production of baby food [7].

Due to high sensitivity and selectivity, liquid chromatography-tandem mass spectrometry (LC-MS/MS) with the multiple reaction monitoring (MRM) mode was the preferred technique for determining PFASs at parts-per-billion (ppb) levels. The study of PFASs extraction method in biotic matrices was initially performed many years ago. In 2001, Hansen *et al*. developed an ion-pair extraction

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method to extract PFASs in biotic matrices by using methyl tert-butyl ether (MTBE) employing tetrabutylammonium (TBA) as an ion-pairing agent [8]. This approach was used in several studies but the results showed that the robustness of the method was not stable. Kannan *et al*. used this method to analyze PFASs in tuna, swordfish and dolphin livers [9], in which PFOS recoveries ranged from 66 - 140%. On the other hand, ion-pair extraction was relatively laborious and in high-fat matrices such as fishery products, lipid and other lipophilic matrix components could be co-extracted. Therefore, the extract was complicated and the determination of analytes could be affected.

The QuEChERS method (Quick, Easy, Cheap, Effective, Rugged, and Safe) originally developed by Anastassiades *et al*. [10] for determining a wide range of pesticide residues in vegetables and later modified by Lehotay *et al*. for analyzing pesticide residues in fatty matrices [11], was chosen for method development. To increase the efficiency of co-extract cleaning, the modified QuEChERS method was combined with solid-phase extraction (SPE) prior to analysis by LC-MS/MS.

The aim of this study was to develop a fast sample preparation that would enable not only multiple sample handling in short time, but also accurate determination of analytes. Besides, more information about the contamination of PFOA and PFOS in fishery products in Vietnam could be achieved.

2. Materials and Method

2.1. Chemicals and Reagents

PFOA and PFOS were purchased from Dr Ehenstorfer (UK). Formic acid (HCOOH), methanol (CH_3OH), acetonitrile (CH_3CN), ammonium formate ($HCOONH_4$), ammonium acetate (CH_3COONH_4), sodium acetate (CH_3COONa), anhydrous magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) were obtained from Merck (Germany), bondesil C18 sorbent was from Agilent technologies (USA) and OASIS PRiME HLB 3cc cartridges were obtained from Waters (USA).

2.2. Samples

A fresh fish sample was determined to be free of analytes, purchased at a local store for method development and validation. 10 fishery samples (squids, fishes, clam, shrimps) were collected at different local markets (as shown later in Table 5) for analysis. The fish samples were removed from the skin, head, bones and internal organs. The shrimps were peeled and removed from heads. The squids were removed from heads and organs. The clams were removed from shells. These edible parts were homogenized, coded from M1 to M10 and stored in a freezer until analysis.

2.3. Sample Preparation

About 5.0 g of homogenized sample was weighted into a 50 mL polypropylene (PP) centrifuge tube. 5.0 mL of MiliQ water was added and mixed by shaking for 1 min. 10 mL acetonitrile and 0.15 mL formic acid were added and the tube was vigorously shaken by hand for 1 min. 6.0 g $MgSO_4$ and 1.5 g NaCl were added to the tube. To avoid the coagulation of $MgSO_4$, the tube must be shaken immediately by hand for 1 min after adding two inorganic salts. In the next step, the tube was centrifuged (Hermle Z326K, Germany) for 5 min at 6,000 rpm and 1.0 mL of the extract of the upper acetonitrile phase was transferred to a 2.0 mL dispersive solid phase extraction (d-SPE) tube containing 0.18 g $MgSO_4$ and 0.02 g Bondesil C18 sorbent. The tube was shaken by a vortex mixer for 2 mins and then centrifuged (Hettich, UK) for 2 mins at 13,000 rpm. At the end of the sample preparation, 0.6 mL of the extract was passed through an OASIS PRiME HLB 3 cc cartridge, transferred into an auto-injector vial and analyzed by LC-MS/MS.

2.4. Liquid Chromatography-Tandem Mass Spectrometry

The analyses were performed using a SCIEX 6500 Triple Quad instrument with negative electrospray ionization (ESI) and multiple reaction monitoring (MRM) mode. Symmetry C18 column (3.0 x 150 mm, 3.5 µm particle size, Waters, USA) was used to separate the analytes. The mobile phase consisted of ammonium acetate 2 mM/methanol (9:1) (A) and methanol (B). The gradient program was: starting with 50% B in 2 mins, increased from 50 to 95% B in 16 mins, 95% B was maintained in 4 mins, then decreased to 50% B in 6 secs, and finally decreased to 0% B in 5 mins. The injection volume was 5 µL and the flow rate was 0.25 mL/min. The ion source parameters of the tandem mass spectrometer were: ion spray voltage 4,500 V, collision gas (CAD) 8 psi, curtain gas (CUR) 35 psi and capillary temperature (TEM) 350°C. The MS/MS parameters including the mass-to-charge (m/z) ratio, collision energy (CE) and collision cell exit potential (CXP) of PFOA and PFOS were summarized in Table 1.

The contents of PFOA and PFOS in the samples were determined by using the matrix-match calibration technique. The blank samples were analyzed similar to the sample and then the extract was spiked at different concentration to make a calibration curve which was used for quantification.

Table 1. MS/MS parameters of PFOA and PFOS

Analyte	Parention (m/z)	Production 1			Production 2		
		m/z	CE (volts)	CXP (volts)	m/z	CE (volts)	CXP (volts)
PFOA	413	369.0	-7.5	-25	169.0	-16.5	-16
PFOS	499	98.9	-84	-11	79.9	-106	-9

3. Results and Discussion

3.1. Method Development

3.1.1. Chromatography conditions optimization

PFOS and PFOA were separated by a Symmetry C18 column (3.0 x 150 mm, 3.5 µm particle size, Waters, USA). Some mobile phases were tested including (1) ammonium acetate 2 mM/methanol (9:1) and methanol; (2) 0.1% formic acid and acetonitrile; and (3) ammonium acetate 20 mM and methanol. The concentration of PFOA and PFOS standard solutions was 20 ng/g. The peak heights of PFOA and PFOS obtained by three mobile phases are shown in Table 2. Mobile phase (1) and (3) were both given good signals for analytes. However, the retention time of PFOA and PFOS with mobile phase (3) were 12.0 min and 12.6 min; longer time with mobile phase (1) were 10.7 min and 11.3 min, respectively. Therefore, the mixture of ammonium acetate 2 mM/methanol (9:1) and methanol was selected for further experiments.

Table *2*. Peak heights of PFOS and PFOA with three mobile phases

Mobile phase	PFOA	PFOS
Amonium acetate 2 mM/methanol (9:1) and methanol	258000	437000
0.1% formic acid and acetonitrile	No peak	No peak
Amonium acetate 20 mM and methanol	248000	447000

3.1.2. Sample extraction optimization

In this study, the QuEChERS method was selected for extracting analytes from fishery samples. The analytes were extracted from the matrix by water and acetonitrile mixture with the support of inorganic salts. PFASs are acidic and neutral compounds, so the pH of the solvent affects the extraction efficiency. Therefore, low pH should be achieved by adding a small amount of formic acid to acetonitrile. Besides, inorganic salts were important to support the transfer of analytes into the organic phase because the pH of the extract could be adjusted to the optimum. Some buffers were tested including $MgSO_4$-NaCl and $MgSO_4$-CH_3COONa. The result showed that better extraction performance was obtained by the neutral buffer ($MgSO_4$-NaCl).

For the high-fat matrix, Bondesil C18 was used to eliminate matrix effects. Different amount of C18 in dispersive SPE clean-up was tested with the $MgSO_4$ amount remaining unchanged. The weight levels of C18 were 0.02 g; 0.04 g; 0.06 g; 0.08 g. At these levels, the recovery of two analytes were relatively good, in the range of 80-118%. Therefore, it could be seen that the loss of analytes due to adsorption by C18 was not much. The C18 weight was selected as 0.02 g for subsequent experiments due to the best mean recovery for both PFOA (99.05%) and PFOS (107%).

After being cleaned with d-SPE, the extract was transferred through the OASIS PRiME HLB 3cc cartridge to remove fat and phospholipid components. The OASIS PRiME HLB cartridge could be eluted in two modes: pass-through or conventional elution. Both modes were tested to compare the signal of PFOA and PFOS in each procedure. With the pass-through elution, the extract was allowed to move through the cartridge to the vial by gravity and analyzed by LC-MS/MS. With the conventional elution, the following procedure was applied and performed on a SPE vacuum manifold (Supelco, USA): (1) 0.6 mL of the extract after cleaning with d-SPE was diluted to ~9 mL with MiliQ water in a 15 mL centrifuge tube; (2) the cartridge was activated with 9 mL of 0.3% ammonium hydroxide in methanol and (3) balanced with 5 mL of MiliQ water; (4) the extract was added and passed through the cartridge: (5) 5 mL of MiliQ water was added to wash the column; (6) the cartridge was dried for about1 minute; (7) 3 mL of 0.3% ammonium hydroxide in methanol was added to elute the analyte into a 15 mL centrifuge tube; (8) the elution was dried under nitrogen flow in a water bath at 60°C (Organomation nitrogen evaporator, USA); (9) 0.6 mL of methanol containing 1% formic acid was added to the centrifuge tube and the tube was mixed with a vortex mixer and (10) LC-MS/MS analysis. The chromatograms of the two processes are shown in Fig. 1 and Fig. 2.

Accordingly, the signal of PFOA in the pass-through mode is much better than the conventional elution mode. Besides, for PFOS, the noise signal was reduced significantly compared to the conventional elution. The sensitivity of the method was increased so that the analytes could be detected at lower concentrations. Therefore, the pass-through mode was chosen for the next experiments.

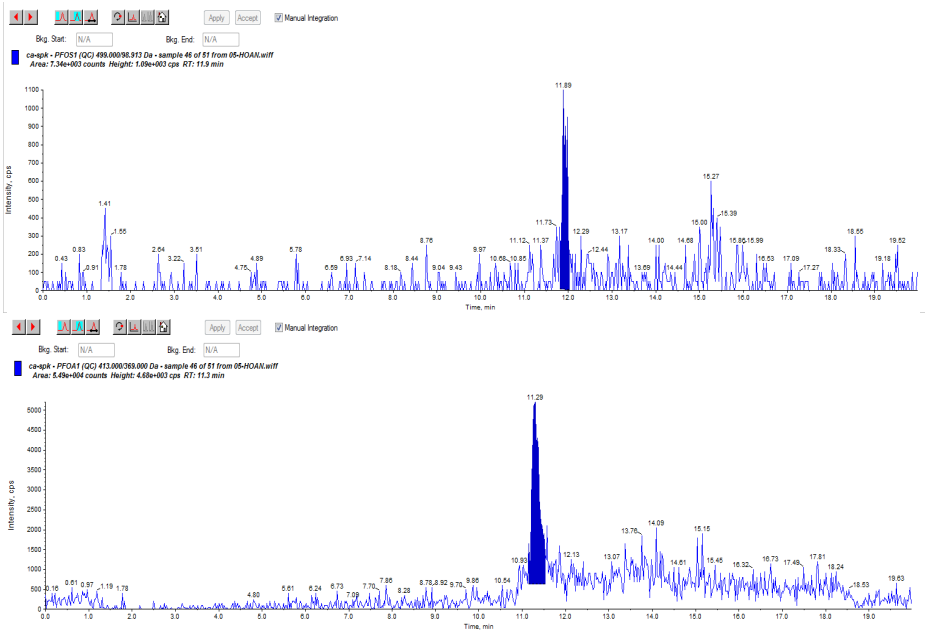

Fig. 1. Chromatograms of PFOS (upper) and PFOA (lower) with the pass-through mode

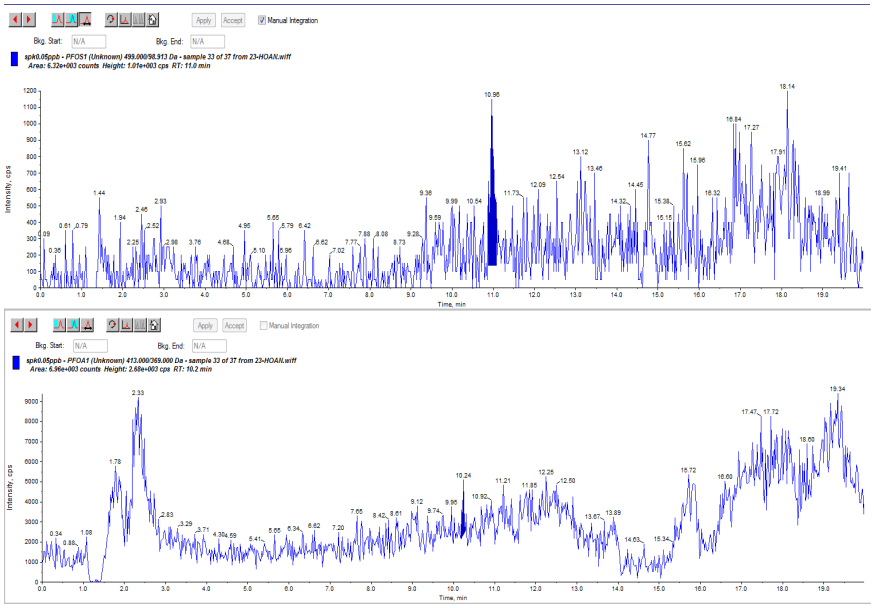

Fig. 2. Chromatograms of PFOS (upper) and PFOA (lower) with the conventional elution mode

3.1.3. Matrix effect

The influence of one or more components in the sample matrix on the qualitation and quantitation of the analytes was the matrix effect (*ME*). The signal of the analytes could be affected by the matrix effect (increase or decrease). The matrix effect could be calculated by comparing two slopes of the two standard curves according to the following formula. The first standard curve was built on the solvent and the second standard curve was built on the blank matrix.

$$ME = ((a_m - a_s)/a_s) \times 100 \quad (1)$$

where, *ME*: matrix effect (%), a_m: the slope of the standard curve on the blank matrix, a_s: the slope of the standard curve on the solvent.

Six concentration levels were used to assess the matrix effect, with PFOA concentrations ranged from 0.050 to 2.000 ng/g and with PFOS concentrations ranged from 0.150 to 6 ng/g. The matrix effect (Table 3) did not exceeded ± 20% of the AOAC requirement, so the determination of PFOA and PFOS content was not affected by the sample matrix. Therefore, both the standard curves on the solvent and on the blank matrix could be used for further experiments.

Table 3. Evaluation of the matrix effect

Analyte	Slope of the standard curve on the solvent	Slope of the standard curve on the blank matrix	Matrix effect (%)
PFOS	253239	250531	-1%
PFOA	19379	21917	+13%

3.2. Method Validation

3.2.1. Specificity

Blank samples, standard solutions, and spiked samples were analyzed to evaluate the method specificity. The European Council (EU 2021/808) regulated the calculation of identification points (IPs) for each method to confirm the certainty of the presence of an analyte. 1 parent ion and 2 product ions were identified for each compound, 1 IP of the LC-MS/MS method was added, and a total of 5 IPs was satisfactory.

In addition, there were no peaks of the blank sample that had the same retention time as the peaks of PFOS (12.0 min) and PFOA (11.4 min) in standard solutions. The spiked sample had the retention times of PFOS and PFOA were 12.0 min and 11.4 min, which exactly coincided with the retention time of the standard solutions (Fig. 3 and Fig. 4). These results indicated that the LC-MS/MS method had adequate specificity for analyzing PFOA and PFOS.

3.2.2. Limit of detection, limit of quantitation, linearity

The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the signal-to-noise ratio (S/N), S/N at LOD and LOQ should be at least 3 and 10, respectively. The lowest concentration of PFOA and PFOS was spiked into the blank samples and S/N was evaluated. Accordingly, the LOQs of PFOS and PFOA were 0.150 ng/g and 0.050 ng/g. The LODs of PFOS and PFOA were 0.050 ng/g and 0.016 ng/g (Fig. 5).

Six standard solutions in different concentrations were used to test the linearity, starting from the LOQ of each analyte (from 0.050 to 2.000 ng/mL of PFOA and 0.150 to 6.000 ng/mL of PFOS). The coefficients of determination for both PFOS and PFOA were satisfactory (greater than 0.990) (Fig. 1). The result showed that the linear relationships were good between concentrations and peak areas of the target analytes.

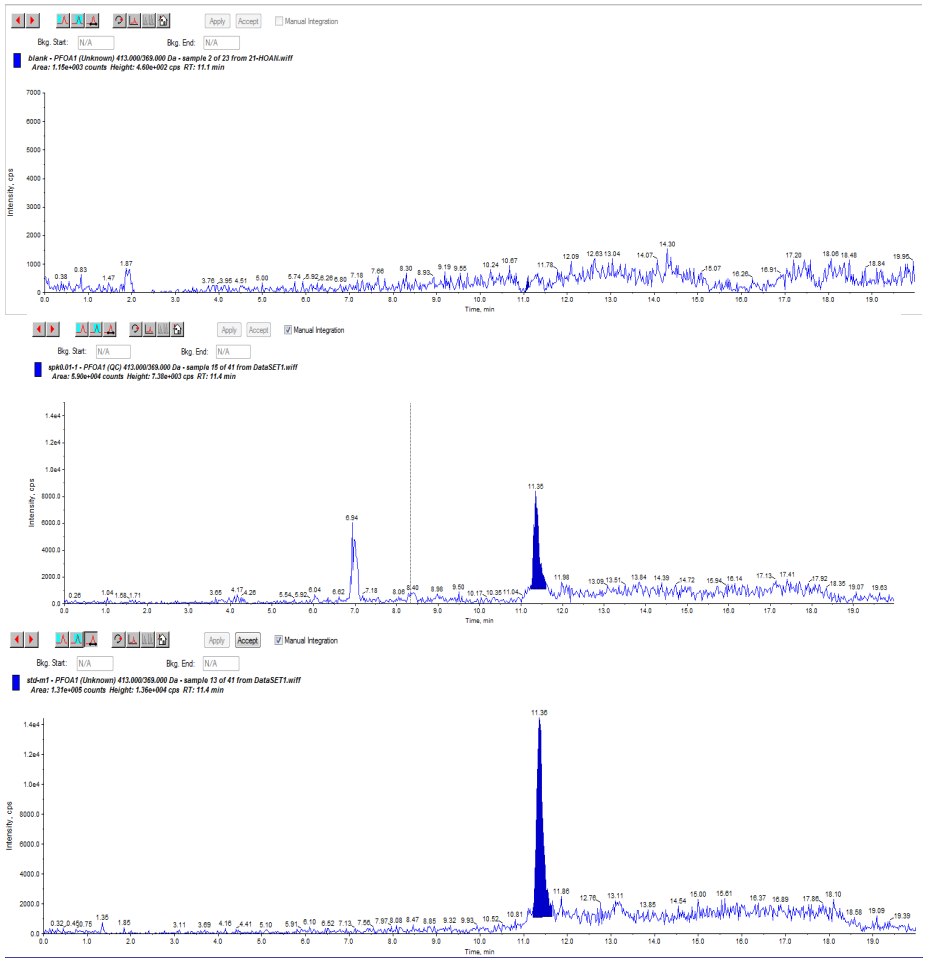

Fig. 3. Chromatograms of blank samples, spiked samples, and standard solutions of PFOA

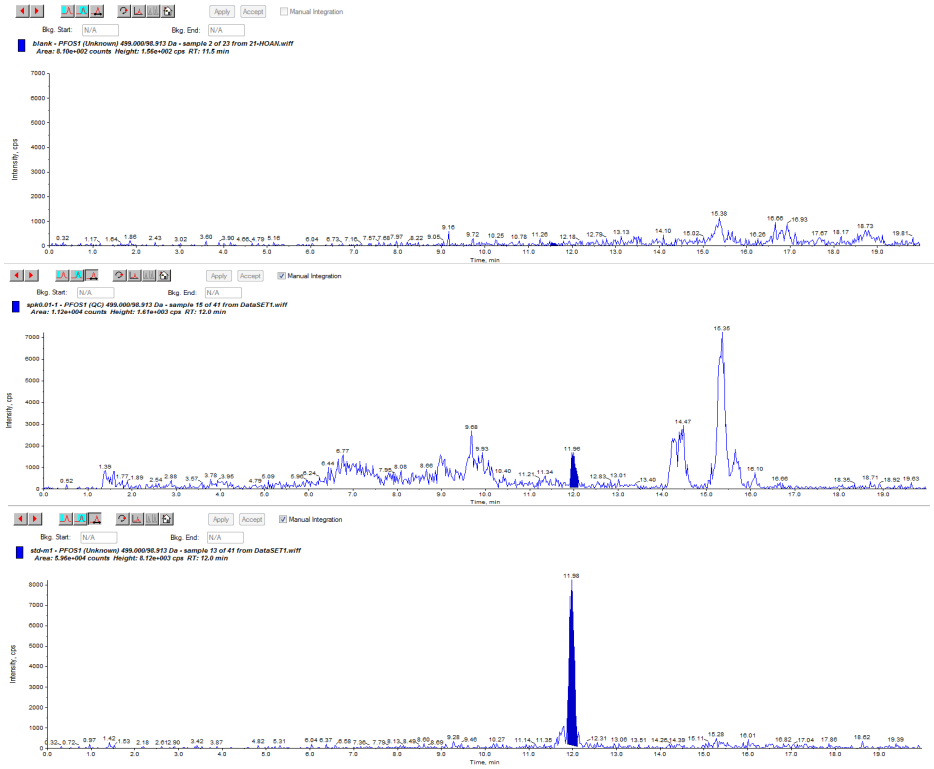

Fig. 4. Chromatograms of blank samples, spiked samples, and standard solutions of PF

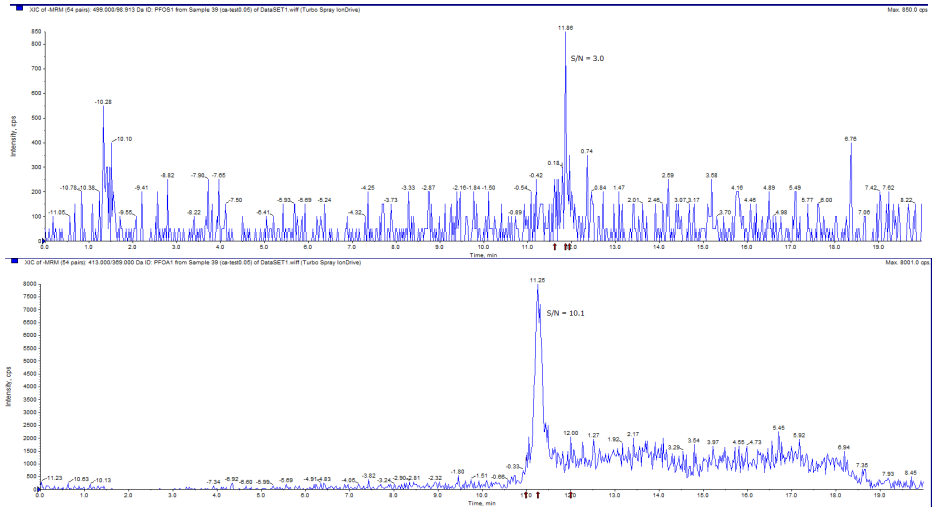

Fig. 5. Signal-to-noise ratio of PFOS (upper) and PFOA (lower) at 0.05 ppb

3.2.3. Repeatability and recovery

PFOA and PFOS were spiked to the blank samples at three concentration levels of LOQ, 3LOQ and 10 LOQ (0.050, 0.150 and 0.500 ng/g for PFOA and 0.150, 0.450, 1.500 ng/g for PFOS).

The experiment was repeated 6 times for each concentration level. The recoveries and relative standard deviations (RSD) of PFOA and PFOS are shown in Table 4. The RSD values of PFOA ranged from 4.55 to 6.90%, while with PFOS they were from 3.87 to 7.66%. The mean recoveries ranged from 107 to 116% with PFOA and 109 to 115% with PFOS.

Table 4. Relative standard deviations and mean recoveries of PFOA and PFOS

Analyte	Spiking concentration (ng/g)	Relative standard deviation (RSD%)	Mean recovery (%)
PFOS	0.15	5.60	113
	0.45	3.87	115
	1.50	7.66	109
PFOA	0.05	6.90	107
	0.15	4.55	108
	0.50	4.78	116

According to AOAC International, at ppb levels, the recovery and relative standard deviation (RSD) ranged from 40 to 120% and less than 21%, respectively [12]. Therefore, the recovery and repeatability of the method were all satisfactory according to the AOAC standard.

Although an internal standard was not used in this study, the method performance meets the requirements of AOAC International in terms of precision and trueness. The matrix-match calibration technique was used for application in real samples.

10 fishery samples were randomly collected from local toad markets (Bach Khoa district), including 1 sample of clam, 1 sample of tilapia, 1 sample of bombay duck, 1 sample of tuna, 1 sample of caridina flavilineata, 2 samples of penaeus merguiensis, 1 sample of black tiger shrimp, 1 sample of squid and 1 sample of bigfin reef squid. The collected samples were homogenized by a grinder, coded from M1 to M10 and stored in a freezer until analysis. Analytes were extracted from samples by the validated procedure and analyzed on the LC-MS/MS system with defined conditions. The concentration of PFOA and PFOS were calculated by the calibration curves on the solvent (Fig. 6).

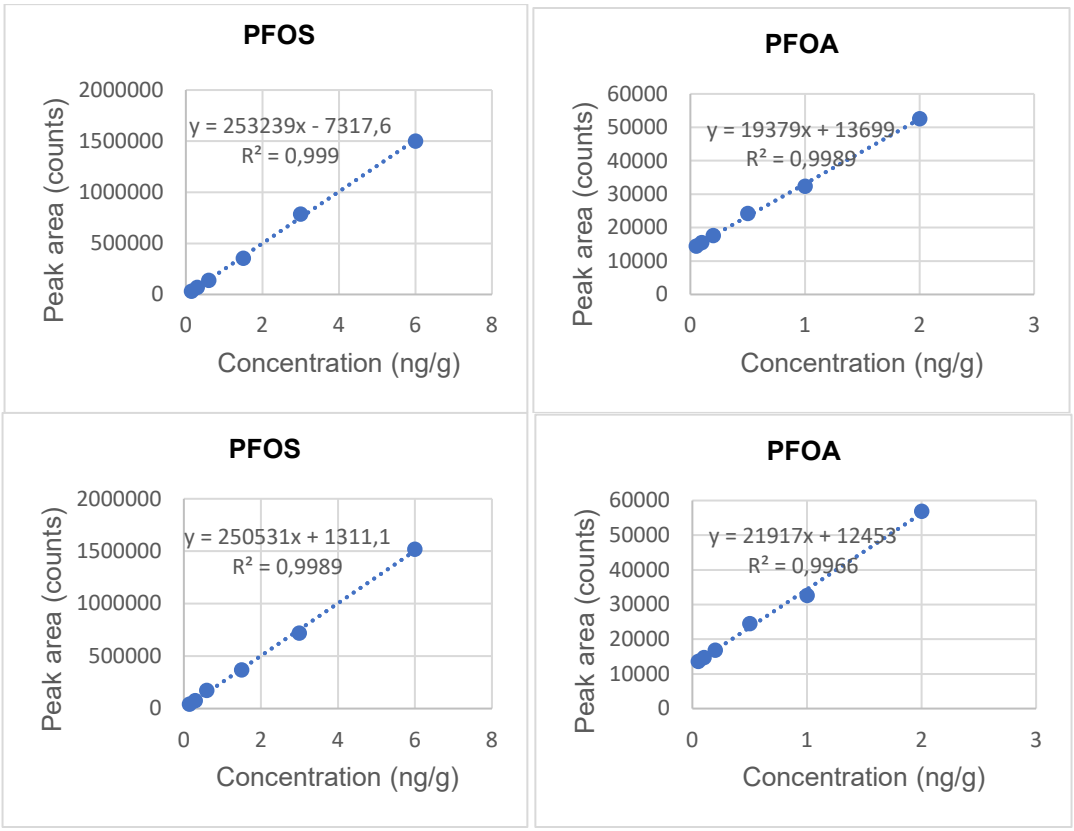

Fig. 6. Calibration curves, regression equations and coefficients of determination of PFOS and PFOA on the solvent (upper) and on the blank matrix (lower)

Table 5. Concentrations of PFOA and PFOS found in several fishery samples

Sample code	Market	Sample type	PFOA concentration	PFOS concentration
M1	Bach Khoa	Clam	0.29 ng/g	Not detected
M2	Bach Khoa	Tilapia	Not detected	Not detected
M3	Bach Khoa	Bombay duck	Not detected	Not detected
M4	Bach Khoa	Tuna	Not detected	Not detected
M5	Bach Khoa	Caridina flavilineata	Not detected	Not detected
M6	Bach Khoa	Penaeus merguiensis	< 0,05 ng/g	Not detected
M7	Ta Quang Buu	Penaeus merguiensis	Not detected	Not detected
M8	Ta Quang Buu	Black tiger shrimp	Not detected	Not detected
M9	Ta Quang Buu	Squid	Not detected	Not detected
M10	Ta Quang Buu	Bigfin reef squid	Not detected	Not detected

Analytical results of PFOA and PFOS in 10 fishery were summarized in Table 5. PFOS was not detected in all the fishery samples. Meanwhile, PFOA was detected in clam (0.29 ng/g) and penaeus merguiensis (lower than LOQ). The PFOS and PFOA concentrations in fishery samples were comparable with a number of studies in the world and Vietnam. In a study by Tran Thi Lieu *et al*. on the assessment of pollution levels and exposure hazards of PFOS and PFOA in some fish species collected in West Lake and Yen So Lake (Hanoi, Vietnam), PFOS and PFOA were detected in tilapia (mean 0.040 and 0.048 ng/g) [13]. PFOS and PFOA were also detected in golden freshwater clams (mean 0.1 and 0.04 ng/g) and in tilapias (mean 0.02 and less than 0.20 ng/g) in Da Rang River (Kon Tum, Vietnam), respectively [14]. The surface water quality of the Da Rang River was also detected PFOS (average 0.02 ng/L) and PFOA (0.07 ng/L) [14]. In China, high concentration of PFOS was detected in some aquatic species in Taihu Lake, most commonly in crucican carp with an average content of 20 ng/g [15]. In South Korea, several fish species taken from Namhan River, Yeongsan River and Nakdong River were analyzed and the highest concentration of PFOS was found in the muscle of the crucican carp with 28.3 ng/g [16]. Through a number of studies, PFOA and PFOS have been present in some aquatic species in Vietnam. However, the content in the muscle is basically lower than in some countries in the region. Thus, there may be an impact of the habitat on the bioaccumulation of these two analytes in aquatic species. Therefore, more specific studies on the environment and other PFASs are needed, as well as their pathways of penetration.

4. Conclusion

In the study, PFOA and PFOS were determined by a liquid chromatography-tandem mass spectrometry method in several fishery samples. The method was optimized and validated to have adequate specificity, linearity, sensitivity, and accuracy, complied with the requirements of AOAC International. 10 fishery samples randomly collected from local toad markets in Hanoi, Vietnam were successfully analyzed to determine PFOA and PFOS. In particular, PFOA was detected in clam and penaeus merguiensis samples, while PFOS was not detected in any samples. The content of these two compounds in fishery samples in Vietnam is generally lower than that of some countries in the region. However, further studies on the occurrence of other PFASs in fishery products and in the environment should be performed to fully assess the level of contamination as well as the pathway of penetration.

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