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Short communication

Perfluorocarboxylic acids in cell growth media and technologically treated waters: Determination with GC and GC–MS

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ABSTRACT

The present paper reports on an analytical method for the routine analysis of perfluorinated carboxylic acids (PFCAs). A rapid method for the derivatization, extraction and determination of PFCAs was developed. Technological samples were extracted with ethyl acetate and the anilides obtained were determined by gas chromatography–mass spectrometry (GC–MS) and gas chromatography with flame ionization detector (GC-FID). Residue levels in cell growth incubation media were determined by GC-FID. Confirmation analysis of PFCAs was carried out by GC–MS in selected ion monitoring (SIM) and total ion current (TIC) modes. The compounds were identified on the basis of retention time and comparison of primary and secondary ions. The results showed that this method provided a simple, rapid and sensitive way of analyzing PFCAs in different matrices.

1. Introduction

PFCAs and their salts have been recognized as an important class of contaminants in the global environment. The strong fluorine-carbon bond is responsible for their extreme chemical stability and is one of the main reasons for their resistance to hydrolysis, photolysis, microbial degradation and living cell metabolism [1]. They are increasingly applied in the household, in polymer production for the aviation and electronics industries, and in many other branches of technology as wetting agents, lubricants, corrosion inhibitors and foam fire extinguishers. For this reason, determining the level of these compounds, as well as specifying their influence on the human body and the environment, has become necessary. Perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) have been detected in human serum and blood [2], surface water [3] and freshwater and marine biota [4,5]; they have also reached such geographically remote areas as Alaska, Spitzbergen and the Canadian Arctic [6]. PFCAs with chain lengths from 9 to 15 carbon atoms have been detected in several animal species collected at various locations in the circumpolar region [6] and in harbor porpoises (*Phocoena phocoena*) from Northern Europe [7]. It is not surprising that only PFCAs with longer carbon chains are found in marine animals: this is due to their bioaccumulative potency that tends to increase with the perfluoroalkyl chain length of the acid, a property that influences the compound's lipophilicity [8].

Although PFCAs were earlier considered to be metabolically inert and therefore presumed to be non-toxic, these compounds turned out to be biologically active and to have a certain toxicological impact. Diverse studies have demonstrated the toxicity of PFCAs, which concern mortality, carcinogenicity, as well as adverse effects on development, thyroid functions, pancreatic functions, and PFOS reproduction. Various PFCAs have been shown to induce peroxisome proliferation [9] and to alter lipid metabolism [10] in the livers of rodents. Recently, it was demonstrated that PFOA altered the function of Leydig cells [11] and could be a cancer promoter [12].

HPLC and HPLC/MS are the methods used most frequently for the qualitative and quantitative analysis of PFCAs, because of their properties like low volatility and good solubility in organic solvents. The application of HPLC–MS/MS to determine PFOA and PFOS in water using solid-phase extraction (SPE) with a LOD of 25 ng/l was first reported by Hansen et al. [5]. Yamashita et al. [3] reported a modified method for PFOA and PFOS in seawater at concentrations of 0.001 ng/l using SPE, paying careful attention to procedural and instrumental blank contamination. Although LC/MS/MS with electrospray ionization has exhibited excellent sensitivity and specificity for PFCA analysis in a wide variety of matrices without the need for chemical derivatization, there are still problems that must be overcome in the quantitative and qualitative determination of PFCAs. The main problems are matrix suppression

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effects because of co-extractive material particularly in complex effluents. There are also difficulties concerning LC–MS systems consisting of fluorinated polymers from which PFCAs, most notably PFOA, can be leached, causing background problems. Use of GC–MS to determine PFCAs and PFOA as methyl esters derivatives was described by Scott et al. [13].

In this work we tested the applicability of perfluorocarboxylic acid anilides as GC-separable volatile derivatives. The aim of this study was first to develop the method and then to apply it in an assessment of PFOAs in a Fenton-like degradation system, as well as in a growth medium used in the toxicological evaluation of selected PFOAs towards eukaryotic cells. These two matrices are quite complex and difficult to analyze mainly because of the various interferents present either in technological waters or in biological media.

2. Experimental

2.1. Chemicals and reagents

PFCAs (PFHxA, CAS number: 307-24-4), perfluoroheptanoic acid (PFHpA, CAS number: 375-85-9), perfluorooctanoic acid (PFOA, CAS number: 335-67-1), perfluorononanoic acid (PFNA, CAS number: 375-95-1) and perfluorodecanoic acid (PFDA, CAS number: 335-76-2) were purchased from ABCR GmbH (Karlsruhe, Germany), and 2,4-difluoroaniline (2,4-DFA) and *N,N′*-dicyclohexylcarbodiimide from Sigma–Aldrich (Steinheim, Germany). Dichloromethane (HPLC grade) was obtained from Chempur (Piekary Śląskie, Poland); ethyl acetate p.a. and sodium sulfate p.a. were purchased from Eurochem BGD (Tarnów, Poland); toluene p.a. from POCH S. A. (Gliwice, Poland); and sodium hydrogen carbonate from Lach – Ner. (Radom, Poland).

2.2. Synthesis of perfluorocarboxylic acid anilides

The analytical procedure for the synthesis of PFCA derivatives was based on the one described by Scott and Alaee [14]. The aim of the modifications was to reduce the time of analysis and to improve the efficiency of the synthesis. Extraction time, the quantity of substrates and the kind and quantity of solvents were optimized. The best procedure is described below.

PFCA (0.2–1.5 mg) was dissolved in 10 ml H_2O, after which 72 μl 2,4-DFA and 1.5 ml ethyl acetate were added. If necessary (undivided layers: water and organic) 1–2 ml ethyl acetate were added to the reaction mixture. Next, 120 mg DCC (*N,N′*-dicyclohexylcarbodiimide) and 50 mg sodium chloride were added and stirred at 20 °C for 1 min. After this time, a further 1–2 ml ethyl acetate was added. The mixture was then treated consecutively with 1 ml saturated aq $NaHCO_3$, 1 ml saturated aq Na_2SO_4 and 1 ml HCl (50:50, v/v) and left for 20 h. After the layers were separated, the organic phase was filtered off and dried over sodium sulfate. The remaining solution was extracted twice with ethyl acetate and the extracts dried over sodium sulfate. The solvent was removed from the reaction mixture in a stream of nitrogen, and the residues were dissolved in dichloromethane, then analyzed by GC and GC–MS.

Since the PFCAs derivatives were synthesized in various matrices, the separation of organic and water layers was difficult. The quantities of ethyl acetate added were different each time: this depended on the kind of matrix and the phase separation. The quantities of ethyl acetate added to the medium were changed from 2 to 10 ml. However, after degradation of PFCAs in ionic liquids, from 2 to 5 ml of ethyl acetate were added to the samples. The crude product was dissolved in toluene and analyzed by GC and GC–MS. Fig. 1 shows the scheme for preparing the sample for analysis.

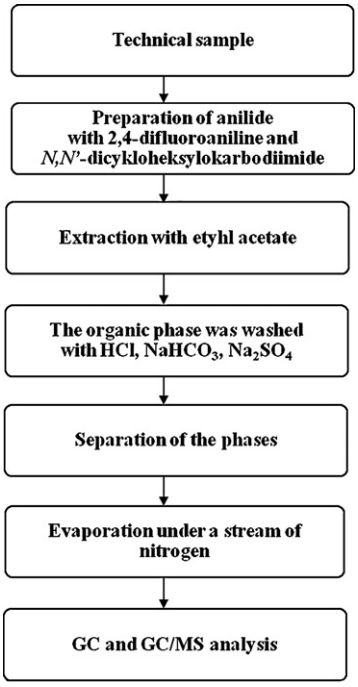

Fig. 1. Preparation of perfluorocarboxylic acid anilides.

2.3. Culture of human cells with the addition of perfluorodecanoic acid

Normal human dermal fibroblasts (N-HDF) and human colon carcinoma (HCT116) cell lines were obtained from the Intercollegiate Faculty of Biotechnology. Cultures were grown in suitable incubation media according to the protocol described by Kleszczyński et al. [15].

The stock solutions of PFDA (10 mM) were prepared in 1% DMSO to increase their solubility. Next, 1.0 μg PFOA was added as an internal standard to the sample of medium, after which the anilides were prepared in accordance with the procedure given above. The concentrations of both acid (PFDA and PFOA) derivatives were then measured using GC and GC–MS.

2.4. Degradation of perfluorocarboxylic acids with ionic liquids in a Fenton-like system

The degradation of PFCAs with ionic liquids was conducted according to the procedure described by Siedlecka et al. [16].

Reaction mixtures were obtained by mixing a dilute aqueous solution of 1-butyl-3-methylimidazolium chloride (1 mM) or 1-butyl-3-methylpyrrolidinium chloride (1 mM) with PFDA (0.2–1.3 μg), adding Fe^{3+}, and adjusting the pH with perchloric acid to 3.0 or 3.5. During the experiment, samples were collected after various reaction times and immediately stopped with 0.05 N NaOH. After that, a range of concentrations (0.2–1.4 mg) of internal standard (PFOA) was added to the sample (10 ml) after the degradation. Then, in accordance with the procedure described in Section 2.2, anilides were prepared and measured by GC.

2.5. Gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS)

The analyses were carried out on a Clarus 500 (Perkin Elmer, Waltham, MA, USA) gas chromatograph equipped with a split ratio of 1:30 for the injection port and direct connection to a FID. The RTX 5 column (Restek, Bellefonte, PA, USA) (30 m × 0.25 mm i.d.,

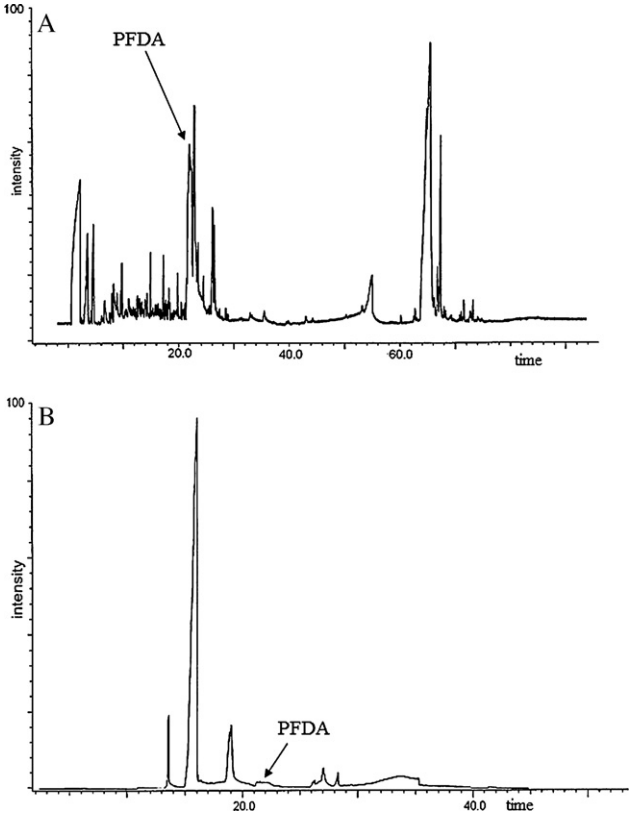

Fig. 2. Total ion current (TIC). (A) The extract from a Fenton-like system with PFDA and (B) the extract from incubation media with PFDA. The ion source was maintained at 220 °C, injector – 310 °C, temperature program: 60–130 °C, rate 2 °C/min and then 130–310 °C, rate 8 °C/min. The RTX 5 column, 30 m × 0.25 mm i.d., film thickness 0.1 μm.

film thickness 0.1 μm) was used. The carrier gas was argon. For reliable analysis, the injector and detector temperatures were 310 °C. The following temperature programs were applied: 60–130 °C, rate 2 °C/min and then 130–310 °C, rate 8 °C/min.

Mass spectra (70 eV) were recorded on an SSQ 710 mass spectrometer (Finnigan). The samples were injected into a Hewlett-Packard 5890 gas chromatograph equipped with a column and under the chromatographic conditions described above but with helium as carrier gas. The ion source was maintained at 220 °C. Confirmation analysis of PFCA derivatives was carried out by GC/MS in the selected ion monitoring (SIM) and total ion current (TIC) modes.

3. Results and discussion

3.1. Development of GC and GC–MS techniques for the analysis of perfluorinated carboxylic acids

In view of the very low volatility of PFCAs it was prerequisite to convert them to more volatile derivatives. The perfluorinated carboxylic acid anilides were synthesized in incubation media and a Fenton-like system. The derivatives were then extracted with ethyl acetate. The extracts were analyzed by GC–MS. A full scan mode acquisition was used for qualitative analysis. Afterwards, molecular ions and other peculiar ions were extracted from TIC in order to identify the retention time of the compounds under investigation. Fig. 2A and B shows the total ion current (TIC) of ethyl acetate extracts obtained from the Fenton-like system and incubation media, respectively. Quantitative analysis was impossible in both cases, because the retention times of the derivatized PFCAs were in the same range as the other compounds extracted from the

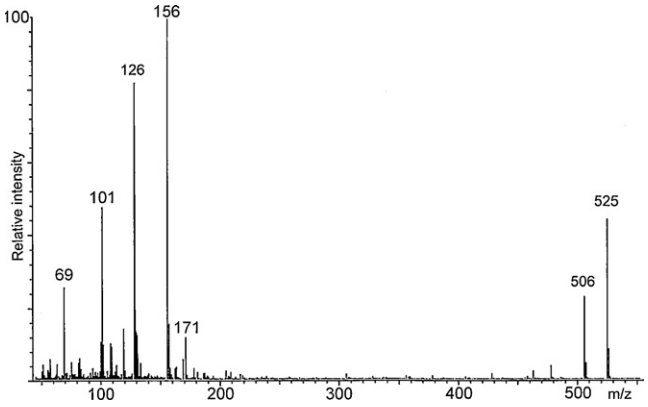

Fig. 3. Mass spectrum of perfluorooctanoic acid anilide.

reaction mixture (Fig. 2A and B). Fig. 3 shows the mass spectra of the PFOA derivative. For qualitative purposes, the instrument was operated in single ion monitoring (SIM) mode, monitoring the molecular ion: $m/z = 525$. In order to obtain a larger amount of the derivative we modified the procedure somewhat in order to obtain an extract that could be analyzed by GC–MS. These modifications resulted in a shorter analysis time, reduced quantity of catalyst, and a larger amount of derivative in the final extract. The last-mentioned effect of these changes is the most important. The largest anilide contents were obtained from the analysis of the toluene extract. Fig. 4 shows the optimal result of the chromatographic analysis of the toluene extract. The analysis was also carried out in SIM mode, monitoring the molecular ion: $m/z = 525$. The synthesis, separation and GC–MS analysis were prepared for quantitative analyses of PFCAs.

3.2. Qualitative analysis

PFCAs were identified on the basis of characteristic anilide derivative ions. GC–MS and then GC analyses of the same samples were used to identify each PFCA signal in the GC chromatograms. To confirm the correct identification of the PFCA signals in the GC chromatograms we analyzed the following samples: PFHpA, PFOA and PFDA. Each acid was identified on the basis of the presence or absence of signals in the chromatograms.

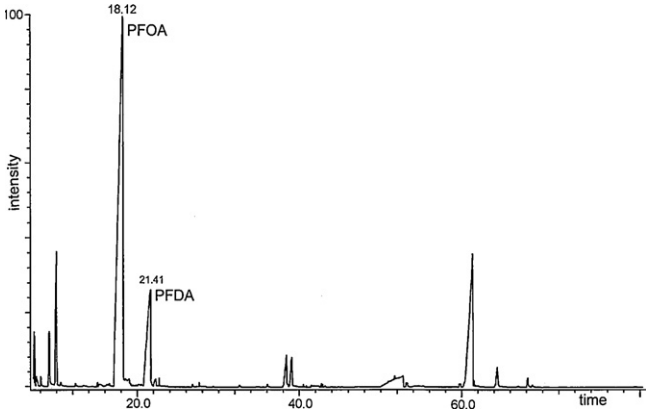

Fig. 4. Total ion current (TIC) of the toluene extract from incubation media with PFOA and PFDA after modification of the derivatization reaction. The ion source was maintained at 220 °C, injector – 310 °C, temperature program: 60–130 °C, rate 2 °C/min and then 130–310 °C, rate 8 °C/min. The RTX 5 column, 30 m × 0.25 mm i.d., film thickness 0.1 μm.

Table 1
Summary of validation data for PFDA.

Property	PFDA	
	Incubation media	Fenton like system
Linear range (μg/ml)	0.1–1.5	0.1–1.5
Correlation coefficient (r)	0.997 ± 0.046	0.998 ± 0.051
Accuracy	102.4 ± 5.3	101.4 ± 4.3
Precision (%)		
Inter-day	3.41–5.76	2.55–4.79
Intra-day	3.60–5.54	3.66–4.51
Specificity	Specific	Specific

3.3. Method validation

The method was validated for linearity, sensitivity, specificity, inter-day and intra-day precision and accuracy; the results are listed in Table 1. The calibration curve was established by plotting the ratio of the peak areas versus concentrations in the working range (0.1–1.5 μg/ml). Good linearity was achieved in the investigated range for PFDA in the incubation media and Fenton-like system. The linear regression equations were $y = 68.37x + 26.74$ and $y = 77.45x + 29.54$ (correlation coefficients, $r = 0.997$, and $r = 0.998$), respectively, where y is the peak area and x is the concentration (μg/ml). The limit of detection (LOD S/N = 3) and the limit of quantification (LOQ S/N = 10) for the incubation media were found to be 0.127 and 0.422 μg/ml, respectively. Both the accuracy and the precision of these values lay within the proposed criteria (RSD% < 20%).

The specificity was investigated in regard to the other co-eluting components. It was found that under optimized chromatographic conditions (both matrices), closely eluting peaks did not interfere with PFCAs.

Intra-day (repeatability) was evaluated for six replicates of PFCA during the same day. Inter-day (intermediate precision) was evaluated by triplicate quantitation of PFCA in the incubation media extract and the Fenton-like system extract on five different days. Relative errors for both the intra-day and inter-day accuracy were <7%, which is considered acceptable.

The accuracy of the analysis was evaluated by determining the recovery at different PFCA concentrations. The mean recoveries for recovery studies ranged from 82.0 to 110.0% for the incubation media and from 81.5 to 103.4 for the Fenton-like system. The respective CV (%) values ranged from 3.09 to 6.68% and from 3.06 to 4.04% for the two matrices. The recoveries with % CV values for PFHxA, PFOA and PFCA are presented in Table 2.

The results indicated that the method enabled the accurate estimation of PFCAs. The validation parameters of this work compare very well with those reported recently for the GC analysis of these compounds.

3.4. Comparison of this method with others

Other authors have usually used GC, HPLC, zone electrophoresis and LC–ESI-MS to analyze the PFCA content in plasma, urine and in rat liver as native compounds or after derivatization.

Table 2
Recovery of PFCAs ($n = 3$).

Amount added (μg/ml)	Incubation media		Fenton like system	
	Recovery %	CV %	Recovery %	CV %
PFDA				
0.1	82.0	6.45	81.5	3.87
0.2	99.5	4.24	92.5	4.04
0.5	110.0	3.11	102.0	3.06
1.0	102.3	3.09	103.4	3.61
1.5	98.1	6.68	102.3	3.54
PFOA				
0.1	85.1	4.87	91.3	3.32
0.2	86.1	3.27	93.3	4.23
0.5	95.5	2.45	95.9	3.76
1.0	95.9	2.12	100.4	4.21
1.5	103.7	3.97	102.4	2.54
PFHxA				
0.1	81.6	4.63	87.9	4.98
0.2	88.5	3.75	90.7	4.43
0.5	101.5	2.65	101.4	4.63
1.0	108.2	4.17	102.2	6.58
1.5	111.9	3.46	102.5	5.24

Table 3
Concentration of perfluorodecanoic acid in incubation media.

Experiment time [h]	Concentration of perfluorodecanoic acid [μg/ml] ($n = 3$)
0	0.93 ± 0.05
1	0.82 ± 0.05
4	0.81 ± 0.05
12	0.52 ± 0.03
24	0.46 ± 0.02
48	0.45 ± 0.04
72	0.40 ± 0.02

In other research, PFOA was analyzed by GC-FID in plasma and urine as benzylperfluorooctanoate. The precision obtained for the determination of 5 μg/ml of PFOA in plasma and urine was 3.3% and 3.0%, respectively; LOD was 1 ng (2.4 pmol) [17].

PFCAs have been investigated by GC-ECD: that analysis yielded LODs of 3–5 μg of PFCA per sample. The concentration of PFDA in rat liver 24 h after intraperitoneal administration at a dose of 20 mg/kg body weight was 113.99 ± 11.4 μg/g of liver [18].

Zone electrophoresis on a chip with conductivity detection has also been used to determine PFCAs. In this case, LODs of 0.3–6.5 μmol/l were achieved [19].

Liquid chromatography coupled with electrospray ionization mass spectrometry was used to determine PFOA in human plasma. The method was validated over the 1–200 ng/ml concentration range for PFOA, yielding correlation coefficients of 0.997. The within-assay and between-assay precisions ranged from 2.1 to 9.2. The concentration limit of detection (cLOD) of PFOA was 0.5 ng/ml in untreated plasma [20].

Table 4
Concentration of perfluorodecanoic acid in a Fenton-like system.

Time [min]	Concentration of perfluorodecanoic acid [μg/ml] ($n = 3$)					
	(BMIMCl), Fe^{2+}	(BMIMCl), Fe^{3+}	(BMPyrCl), Fe^{3+}	(BMIMCl), Fe^{2+}	(BMIMCl), Fe^{3+}	(BMIMCl), Fe^{2+}
0	0.54 ± 0.03	0.54 ± 0.03	0.47 ± 0.02	0.18 ± 0.01	0.18 ± 0.01	1.28 ± 0.06
60	0.50 ± 0.02	0.44 ± 0.02	0.44 ± 0.02	–	–	–
150	0.55 ± 0.03	0.42 ± 0.02	0.36 ± 0.02	0.11 ± 0.01	0.16 ± 0.01	0.60 ± 0.03
1440	0.53 ± 0.03	0.44 ± 0.02	0.24 ± 0.01	–	–	–

BMIMCl: 1-butyl-3-methylimidazolium chloride; BMPyrCl: 1-butyl-3-methylpyrrolidinium chloride.

3.5. Applicability of the method to PFCA analysis

The present method was applied to determine the concentrations of PFCAs in technologically treated water and cell incubation media.

Seven media samples (10 ml) (after 0, 1, 4, 12, 24, 48 and 72 h of the experiment) with the addition of 1 μg/ml PFDA were investigated. The quantities of PFCAs (as anilides) ranged from 0.93 ± 0.05 μg/ml to 0.40 ± 0.02 μg/ml of the mixture (72 h of the experiment) (Table 3).

PFDA was determined in a Fenton-like system (different time of oxidation with BMPyrCl or BMIMCl). Table 4 shows the concentrations (μg/ml) of PFDA: they ranged from 0.11 ± 0.01 to 1.28 ± 0.06 μg/ml of the mixture.

4. Conclusions

PFCAs have been the object of a large number of toxicity investigations. For this, suitable methods of quantitative and qualitative analysis are necessary. Although analytical methods using HPLC are routinely used for different samples, no GC method for determining PFCAs in incubation media and a Fenton-like system has yet been developed. The method for measuring PFCAs developed in this study is specific: it is capable of measuring the target compounds. It is applicable in the analysis of water, biological and technological matrices, giving a better understanding of the fate of perfluorinated compounds in the environment. The method developed in the present study consisted of three steps: (1) derivatization reaction with 2,4-difluoroaniline and dicyclohexylcarbodiimide as catalyst; (2) extraction of PFCAs into an organic solvent; (3) determination of PFCAs by GC and GC–MS. Several samples from different matrices were analyzed using the method developed in this study; the results were excellent.

There are many and diverse analytical problems associated with the determination of PFCAs, such as their unique physicochemical properties, the lack of reliable standards, impurities, and contamination during derivatization. Initially, the determination of PFCAs in incubation media and in a Fenton-like system was impossible. Some compounds were found to interfere with the PFCAs under the given experimental conditions. In addition, those compounds had the same retention times as the analyzed PFCAs. After certain modifications (reduction in the quantity of reagents, increase in the quantity of ethyl acetate, extraction with toluene) had been made to the method, no interferents were found.

The present paper reports on a GC method for determining PFCAs in incubation media and a Fenton-like system. This method was successfully developed and partially validated. The extraction method of perfluorocarboxylic acid anilides has a high recovery and excellent reproducibility. The results show that the method is sensitive and selective. The combination of GC and GC–MS techniques allows positive identification and quantitation of PFCAs in two different samples. Selective ion monitoring (SIM) was utilized to achieve high selectivity and relative sensitivity.

Summing up, this method (derivatization reaction, separation and applied techniques) is simple and successful for the determination of PFCAs. Perfluorinated compounds (PFCs) are widely distributed in aquatic ecosystems. This method permits comprehensive evaluation of aqueous samples for the presence of perfluorinated surfactants and have applicability to other sample matrixes. A method can be used for the determination of PFCs in various biological tissue and environmental samples, in sediments and benthic organisms. The results can be used to assess biomagnification of PFCs via the food chain.

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