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Simple, high throughput ultra-high performance liquid chromatography/tandem mass spectrometry trace analysis of perfluorinated alkylated substances in food of animal origin: Milk and fish

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ABSTRACT

The present study documents development and validation of a novel approach for determination of 23 perfluorinated alkylated substances (PFASs) in food of animal origin represented by milk and fish. The list of target analytes comprises four classes of PFASs, both ionic and non-ionic: 11 perfluorocarboxylic acids (PFCAs), 4 perfluorosulphonic acids (PFSAs), 5 perfluorosulphonamides (FOSAs) and 3 perfluorophosphonic acids (PFPAs). Fast sample preparation procedure is based on an extraction of target analytes with acetonitrile (MeCN) and their transfer (supported by inorganic salts and acidification) into the organic phase. Removing of matrix co-extracts by a simple dispersive solid phase extraction (SPE) employing ENVI-Carb and C18 sorbents is followed by an efficient sample pre-concentration performed by acetonitrile evaporation and subsequent dilution of residue in a small volume of methanol (matrix equivalent in the final extracts was 16 and 8 g mL^{-1}, for milk and fish respectively). Using modern instrumentation consisting of ultra-high performance liquid chromatography (UHPLC) hyphenated with a tandem mass spectrometer (MS/MS), limits of quantification (LOQs) as low as 0.001–0.006 $\mu g\,kg^{-1}$ for milk and 0.002–0.013 $\mu g\,kg^{-1}$ for fish can be achieved. Under these conditions, a wide spectrum of PFASs, including minor representatives, can be determined which enables collecting data required for human exposure studies. The pilot study employing the new method for examination of milk and canned fish samples was realized. Whereas in majority of canned fish products a wide spectrum of PFCAs, perfluorooctanesulphonic acid (PFOS) and perfluoro-1-octanesulphonamide (PFOSA) was detected, only in a few milk samples very low concentrations (LOQ levels) of PFOS and perfluorooctansulphonic acid (PFDS) were found.

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1. Introduction

Perfluorinated alkylated substances (PFASs), a broad group of anthropogenic chemicals, are widely used in various industrial and consumer applications, mainly thanks to their unique ability to repel both water and oil [1]. Within the last decade, PFASs have been identified as “emerging” food and environmental contaminants, due to their presence in various types of abiotic and biotic matrices, including human tissues and fluids [2,3]. In order to enable a risk assessment associated with dietary exposure to PFASs, EFSA (the European Food Safety Authority) recommended that further data on their levels in foods and in humans would be desirable, particularly with respect to the human exposure assessment [4]. Therefore, an additional monitoring focused not only on perfluorooctanesulphonic acid (PFOS) and perfluorooctanoic acid (PFOA), which are the most known representatives of this group, but also on other PFASs is needed. On this account, in March 2010, Commission Recommendation 2010/161/EU invited the Member States to monitor the presence of PFOS and PFOA, compounds similar to PFOS and PFOA but with different chain length (C4–C15) and their precursors (perfluorooctane sulphonamide (PFOSA), N-ethyl perfluorooctane sulfon-amidoethanol (NEtFOSE) and 8:2 fluorotelomer alcohol), in order to estimate the relevance of their presence in food [5]. For this purpose, it is required to use a method of analysis that has been proven to generate reliable results. Ideally, the recovery rates should be in the range 70–120%, with limits of quantification (LOQs) of 1 $\mu g\,kg^{-1}$.

Currently, due to its high sensitivity and selectivity, liquid chromatography hyphenated with tandem mass spectrometry (LC–MS/MS) operated in the multiple reaction monitoring mode (MRM) is the preferred technique for a quantitation of PFASs trace levels. While more or less general agreement exists on an optimal determinative step, it is rather difficult to find an extraction strategy that would enable a rapid analysis of a wide range of both ionic and

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non-ionic PFASs in complex matrices such as food. One of the first sample processing procedures, developed by Hansen et al. [6] for trace analysis of PFASs in biotic matrices, was an ion-pair extraction into methyl *tert*-butyl ether (MTBE) employing tetrabutylammonium (TBA) as an ion-pairing agent. This approach was then used in various environmental studies; nevertheless, the robustness of such extraction strategy is questionable, since very variable recoveries of PFASs, ranging from <50 to >200%, were reported [7]. For instance, PFOS recoveries reported by Kannan et al. [8] for tuna, swordfish and dolphin livers were in the range 66–140%, for tuna blood 37–47%. In any case, ion-pair method is relatively laborious; and suffers from drawbacks such as co-extraction of lipids and other lipophilic matrix components, which significantly complicates PFASs analysis in fatty matrices. As regards the reported LOQs, when employing the ion-pair extraction method, these were typically in the range 0.1–1 $\mu g\,kg^{-1}$.

As a less laborious, faster alternative, solid phase extraction (SPE) represents the option for isolation and/or pre-concentration of PFASs from different biotic and abiotic samples. In the study by Taniyasu et al. [9], who tested HLB (hydrophilic–lipophilic balanced sorbents) and WAX (weak anion exchanger) cartridges in water analysis, it was observed that the latter ones were found more effective, because almost all tested PFASs were retained from water and recoveries ranged from 50 to 90%. The exceptions were neutral perfluorosulphonamides (PFOSAs) and perfluorotelomer alcohols (FTOHs), recoveries of which were lower, varying between 35 and 55%. Contrary to these results, Fromme et al. [10] reported mean recoveries on WAX cartridge only 12% for PFOSA and 63% for PFOA. Kärrman et al. [11], who also used Taniyasu's WAX SPE method for milk and serum obtained relatively low recoveries (not exceeding 50%) for all long chain perfluorocarboxylic acids (PFCAs) (>C11), perfluorosulphonic acids (PFSAs) (C10) and PFOSA.

As regards HLB SPE cartridge-based sample preparation, the limitation is low recoveries of the most polar, short chain (C4–C6) ionic PFCAs (typically less than 30%). It should be noted, that LOQs in published methods employing SPE for the analysis of biotic matrices, were significantly lower (varied from 0.01 to 0.2 $\mu g\,kg^{-1}$) compared to those achieved by ion pair methods. Unfortunately, none of the cartridges available at the market allows simultaneous retaining of all commonly monitored representatives of PFASs with acceptable recoveries.

To avoid the above limitations, sample preparation strategies consisting of a simple homogenization of a sample with respective extraction solvent, (optionally) followed by a simple clean-up and centrifugation/filtration and direct LC–MS/MS analysis were developed. Powley et al. [12] used methanol (MeOH) for extraction of environmental matrices and dispersive SPE with ENVI-Carb graphitized carbon for treatment of crude extract to remove matrix interferences. Hradkova et al. [13] used a similar approach to analyse PFASs in canned fish and seafood; MeOH was employed as an extraction solvent and activated charcoal for clean-up, thus replacing more expensive ENVI-Carb. Berger and Haukås [14], who analysed PFASs in animal livers, used a MeOH/2 mM aqueous ammonium acetate mixture (50:50, *v*/*v*), but this method provided lower recoveries (<50%) of long chain carboxylic acids (>C10) and non-ionic PFOSA. Recently, a micro-extraction method was developed by Luque et al. [15] for analysis of PFASs in biota; it employs a mixture consisting of tetrahydrofuran (THF) water (75:25, *v*/*v*). The main advantages of this approach were not only low sample amount required for analysis, but also rapid extraction and good recoveries of tested analytes. It should be emphasized, that although the methods mentioned in this paragraph are simple, fast and provide acceptable recoveries for a wide range of analyte/matrix combinations, due to the absence of any enrichment step, the achievable LOQs are similar to those of the ion-pair method.

The aim of the present study was to implement an innovative solution that would enable not only high throughput sample handling, but also accurate determination of entire set of analytes of concern at the ultra-trace level. For this purpose, QuEChERS (Quick, Easy, Cheap, Rugged and Safe) approach, originally developed by Anastassiades et al. [16] for determination of a wide range of pesticide residues in fruits and vegetables and then modified by Lehotay et al. for analysis of fatty matrices [17], was selected for a feasibility testing and follow-up validation. The benefits resulting from integrating of a new rapid sample processing QuEChERS strategy with a well-established LC–MS/MS determinative step were demonstrated. The key requirement for performance characteristics of this new analytical procedure was to achieve LOQs $\approx 0.01\,\mu g\,kg^{-1}$, which would enable generation of data needed for the dietary exposure assessment.

2. Experimental

2.1. Chemicals and reagents

Certified standards of PFAS in methanol and their isotopically labelled internal standards (see Table 1) were purchased from Wellington Laboratories (Canada). The purity of each standard was >98%. Anhydrous magnesium sulphate ($MgSO_4$) was obtained from Fluka (Germany), formic acid (95%), ammonium acetate for LC–MS and HPLC grade acetonitrile and methanol were purchased from Sigma–Aldrich (Germany). Sodium chloride, sodium hydroxide, hydrochloric acid and sulphuric acid were bought from Penta (Czech Republic), Bondesil C18 sorbent (40 μm) from Varian (USA) and Supelclean ENVI-Carb (particle size: 120–400 mesh) was obtained from Sigma–Aldrich (Germany).

2.2. Samples

The analytical method was developed and validated using samples of fresh fish (salmon, trout) and milk purchased in a local store. The method was also used for preliminary PFASs screening in 12 samples of milk (pasteurized and UHT, fat content varied from 0.5 to 3.5%) from different Czech producers and 16 canned fish products (7 mackerels, 3 sardines, and 6 cod livers). The canned fish were from Poland, Latvia and Sweden, thus most likely all fish were from Baltic Sea.

From the fresh fish (salmon, trout) only edible parts were homogenized and stored in freezer until its use. All canned fish products were in the vegetable or olive oil. Whole content of cans was homogenized and stored in freezer.

2.3. Sample preparation

7.5 g of homogenized fish muscle tissue and 10 g water were weighted into a 50 mL polypropylene (PP) centrifuge tube and mixed by shaking for 1 min. It should be noted that added water was 18 MΩ MiliQ water from apparatus without parts made from PFASs containing polymers and water was tested for contamination by target analytes. In the case of milk samples, 15 g was weighted and no water was added.

The following steps of sample preparation were the same for both matrices, fish and milk. Isotopically labelled internal standards were added to the sample and sample was mixed. 0.2 mL formic acid and 15 mL acetonitrile (MeCN) were added and the tube was vigorously shaken for 1 min by hand. In the next step, 6 g of $MgSO_4$ and 1.5 g NaCl were added and the tube was immediately shaken to prevent coagulation of $MgSO_4$. The tube was then centrifuged (Hettich, Germany) for 5 min at 11 000 rpm and 12 mL aliquot of the upper acetonitrile phase was transferred to a new 50 mL PP

Table 1
List of analytes and parameters of LC–MS/MS detection.

Compound	Abbreviation	Group	Chain length	Retention time (min)	Parent ion	Product ion 1				Product ion 2			
					(m/z)	(m/z)	DP (V)	CE (V)	CXP (V)	(m/z)	DP (V)	CE (V)	CXP (V)
Perfluoro-n-butanoic acid	PFBA		C4	2.4	213	169	−45	−14	−7				
Perfluoro-n-pentanoic acid	PFPeA		C5	3.1	263	219	−45	−12	−9				
Perfluoro-n-hexanoic acid	PFHxA		C6	3.7	313	269	−55	−14	−11				
Perfluoro-n-heptanoic acid	PFHpA		C7	4.2	363	319	−30	−14	−21	169	−30	−24	−7
Perfluoro-n-octanoic acid	PFOA		C8	4.6	413	369	−40	−14	−23	169	−40	−24	−15
Perfluoro-n-nonanoic acid	PFNA	PFCAs	C9	5.0	463	419	−35	−16	−19	219	−35	−24	−9
Perfluoro-n-decanoic acid	PFDA		C10	5.3	513	469	−40	−18	−19	219	−40	−26	−9
Perfluoro-n-undecanoic acid	PFUdA		C11	5.6	563	519	−70	−16	−19	269	−70	−28	−11
Perfluoro-n-dodecanoic acid	PFDoA		C12	5.9	613	569	−70	−18	−22	169	−70	−36	−7
Perfluoro-n-tridecanoic acid	PFTrDA		C13	6.1	663	619	−65	−20	−25	169	−65	−38	−7
Perfluoro-n-tetradecanoic acid	PFTeDA		C14	6.2	713	669	−85	−20	−27	169	−85	−38	−7
Perfluoro-1-butanesulfonic acid	PFBS		C4	3.2	299	79.9	−90	−64	−13	99	−90	−38	−9
Perfluoro-1-hexanesulfonic acid	PFHxS		C6	4.2	399	79.9	−90	−88	−11	99	−90	−72	−9
Perfluoro-1-octanesulfonic acid	PFOS	PFSAs	C8	5.0	499	79.9	−105	−106	−11	99	−105	−98	−9
Perfluoro-1-decanesulfonic acid	PFDS		C10	5.6	599	79.9	−120	−124	−11	99	−120	−110	−17
Perfluorohexylphosphonic acid	PFHxPA		C6	3.2	399	78.9	−105	−106	−9				
Perfluorooctylphosphonic acid	PFOPA	PFPAs	C8	4.2	499	78.9	−115	−106	−11				
Perfluorodecylphosphonic acid	PFDPA		C10	5.1	599	78.9	−140	−112	−9				
Perfluoro-1-octansulphonamide	PFOSA		C8	5.7	498	78	−85	−88	−7				
N-methylperfluoro-1-octanesulphonamide	N-MeFOSA		C8	6.3	512	169	−110	−38	−7	219	−110	−34	−9
N-ethylperfluoro-1-octanesulphonamide	N-EtFOSA	FOSAs,	C8	6.5	526	169	−110	−38	−7	219	−110	−36	−9
N-methylperfluorooctanesulphonamidoethanol	N-MeFOSE	FOSEs	C8	6.3	616	59	−65	−84	−7				
N-ethylperfluorooctanesulphonamidoethanol	N-EtFOSE		C8	6.5	630	59	−65	−80	−29				
Perfluoro-n-[1,2,3,4-13C4]butanoic acid	13C4 PFBA		C4	2.4	217	172	−35	−12	−9				
Perfluoro-n-[1,2,3,4-13C4]hexanoic acid	13C2 PFHxA		C6	3.7	315	270	−35	−12	−13				
Perfluoro-n-[1,2,3,4-13C4]octanoic acid	13C4 PFOA		C8	4.6	417	372	−55	−16	−21				
Perfluoro-n-[1,2,3,4-13C4]decanoic acid	13C2 PFDA		C10	5.3	515	470	−50	−16	−21				
Perfluoro-n-[1,2,3,4-13C4]dodecanoic acid	13C2 PFDoA	Labelled internal	C12	5.9	615	570	−80	−18	−29				
Perfluoro-1-[1,2,3,4-13C4]octasulphonic acid	13C4 PFOS	standards	C8	5.0	503	79.9	−120	−96	−9				
Perfluoro-n-[13C8]octanesulphonamide	13C8 FOSA		C8	5.7	506	77.9	−80	−90	−9				
N-ethyl-d5-perfluoro-1-octanesulphonamide	d5-N-EtFOSA		C8	6.5	531	169	−110	−38	−9				
2-(N-deuteriomethylperfluoro-1-octanesulphonamido)-1,1,2,2,-tetradeuterioethanol	d7-N-MeFOSE		C8	6.3	623	59	−65	−84	−7				
2-(N-deuterioethylperfluoro-1-octanesulphonamido)-1,1,2,2,-tetradeuterioethanol	d9-N-EtFOSE		C8	6.5	639	59	−65	−80	−29				

Table 2
Recoveries, repeatabilities and LOQs of extraction process achieved during validation study on spiked milk and fish.

Analyte	Internal standard	Milk			Trout		
		Spike 0.015 μg kg^{-1} (n = 6)		LOQ (μg kg^{-1})	Spike 0.03 μg kg^{-1} (n = 6)		LOQ (μg kg^{-1})
		Recovery (%)	RSD (%)		Recovery (%)	RSD (%)	
PFBA	13C4 PFBA	101	4	0.006	107	8	0.013
PFPeA	13C4 PFBA	109	6	0.006	101	7	0.013
PFHxA	13C2 PFHxA	102	3	0.003	98	3	0.006
PFHpA	13C2 PFHxA	106	2	0.003	95	4	0.006
PFOA	13C4 PFOA	98	4	0.003	99	3	0.006
PFNA	13C4 PFOA	103	3	0.003	101	3	0.006
PFDA	13C2 PFDA	101	3	0.003	100	2	0.006
PFUdA	13C2 PFDA	101	4	0.003	99	5	0.006
PFDoA	13C2 PFDoA	100	5	0.003	89	4	0.006
PFTrDA	13C2 PFDoA	97	3	0.003	98	8	0.006
PFTeDA	13C2 PFDoA	92	5	0.003	91	7	0.006
PFBS	13C4 PFOS	101	3	0.003	97	4	0.003
PFHxS	13C4 PFOS	98	4	0.003	96	2	0.003
PFOS	13C4 PFOS	96	3	0.003	97	3	0.003
PFDS	13C4 PFOS	97	3	0.003	97	4	0.003
PFHxPA	13C4 PFOS	118	14	0.006	92	18	0.006
PFOPA	13C4 PFOS	112	18	0.006	102	15	0.006
PFDPA	13C4 PFOS	117	16	0.006	103	17	0.006
PFOSA	13C8 FOSA	98	4	0.001	102	5	0.001
N-MeFOSA	d5-N-EtFOSA	96	5	0.003	88	11	0.006
N-EtFOSA	d5-N-EtFOSA	100	9	0.003	99	2	0.006
N-MeFOSE	d7-N-MeFOSE	95	6	0.003	86	7	0.006
N-EtFOSE	d9-N-EtFOSE	102	8	0.003	92	10	0.006

tube containing 1.8 g $MgSO_4$, 0.18 g C18 sorbent and 0.09 g ENVI-Carb sorbent. The tube was shaken again (20 s) and centrifuged. The purified extract (8 mL) was then evaporated near to dryness and reconstituted in 0.5 mL methanol. To support dissolution of evaporated sample, the flask with methanol was placed into an ultrasonic bath for 1 min. The reconstituted extract was filtered through the 0.2 μm PVDF filter (National Scientific, USA) and transferred into the autosampler vial prior the analysis.

2.4. Method validation

To demonstrate the applicability of the present analytical method, a validation study on milk and fish was conducted. The influence of fat content on the extraction efficiency and repeatability of measurement was tested on milk samples containing different amount of fat: (i) skimmed milk (0.5% fat), (ii) whole milk (3.5% fat) and (iii) whole milk enriched by the addition of 33% cream (5% fat). The procedural recoveries of isolation/partitioning, purification and enrichment steps were examined separately. Finally, the whole optimized procedure was validated employing isotopically labelled surrogates.

2.5. Calibration

External matrix matched calibration was used for quantification and estimation of limits of quantitation in most cases. If extracts with low PFASs contamination (up to 0.01 μg kg^{-1}) were used for calibration preparation, the blank signals were subtracted from the standards.

Solvent calibration was used for PFOS quantification, since it was present in all fish samples examined in our study at concentration >0.1 μg kg^{-1}.

Calibration was prepared by mixing 270 μL solvent (methanol) or blank matrix extract prepared as described above (without addition of internal standards) with 30 μL of particular working standard mixture to obtain matrix-matched standards corresponding to the relevant concentration level 0.010; 0.025; 0.05; 0.1; 0.5; 1; 5 and 10 ng mL^{-1}. The concentration of matrix in the extract was 8 g mL^{-1} for fish or 16 g mL^{-1} for milk respectively.

2.6. Determination of LOQs

The LOQs were estimated as the lowest matrix matched calibration standard which provided signal-to-noise ratio (S/N) higher than 10 and the second MS/MS transition (if available) had to provide S/N > 3. The S/N was determined as the peak-to-peak. The LOQs for particular matrix/analyte combinations are summarized in Table 2.

2.7. Liquid chromatography–tandem mass spectrometry

The UHPLC analyses were performed using an Acquity UltraPerformance LC system (Waters, USA) equipped with an Acquity UPLC HSS T3 column (100 mm × 2.1 mm I.D., 1.8 μm particle size, Waters, USA) maintained at 40 °C and 10 μL sample loop. Between the mixer and the sample valve HPLC column (Atlantis T3, 50 mm × 2.1 mm I.D., 5 μm, Waters, USA) was placed, which retained the PFASs interferences coming from the mobile phase and the UHPLC system, and as a consequence their retention times were higher than those of the injected analytes. The mobile phase consisted of methanol (A) and 0.005 M ammonium acetate in Mili-Q water (B). The starting mobile phase composition was 10% A (flow 0.3 mL min^{-1}), which linearly changed to 40% A in 0.5 min. A slower linear gradient from 40% A to 100% A in 7 min followed, simultaneously with flow rate change from 0.3 to 0.4 mL min^{-1}. The column was washed for 2 min (flow 0.7 mL min^{-1}) of 100% A and reconditioned for 2.5 min in the starting composition of 10% A (0.45 mL min^{-1}). Typical chromatograms of 23 PFASs included in this study are shown in Fig. 1. Sample volume of 5 μL with the partial loop injection mode was used in all experiments. Autosampler temperature was maintained at 10 °C.

The UHPLC system was connected to a 5500 QTRAP tandem mass spectrometer (AB SCIEX, Canada), equipped with a Turbo V^{TM} ion source operated in negative mode. The ion source parameters were as follows: needle voltage −4500 V, curtain gas 25 psi, nebulizer

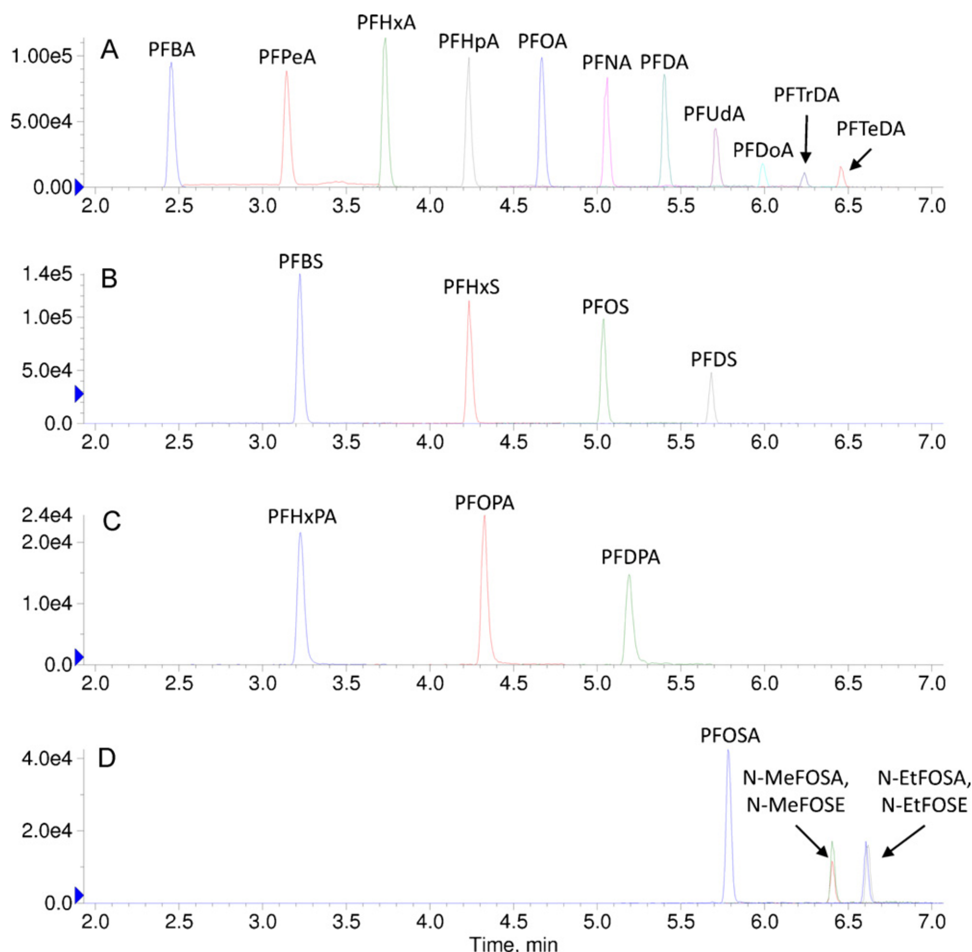

Fig. 1. Extracted ion chromatograms of PFCAs (A), PFSAs (B), PFPAs (C) and non-ionic FOSAs/FOSEs (D) in methanol at concentration 0.5 ng mL^{-1}.

(Gas 1) and Turbo gas (Gas 2) 55 psi, temperature of Turbo gas 650 °C. Declustering potential (DP), collision (CE) and collision cell exit potential (CXP) were optimized during infusion of mixture of analytes (10–100 ng mL^{-1}) employing an automatic function of Analyst software. All analyte dependent parameters are summarized in Table 1.

3. Results and discussion

In the following paragraphs, all sections performed within the development of an alternative, simple and sensitive method for PFASs analysis in milk and fish matrices are described and discussed in detail.

3.1. Background contamination control

Due to a wide use of fluoropolymers, background contamination originated from various sources in laboratory environment might represent a serious problem in (ultra)trace analysis of PFASs [18]. Investigation of instrumental blanks performed in the first phase of method development confirmed these concerns. In particular case, a tubing of UHPLC instrument supplying solvents to the degasser was identified as a source of contamination. Besides PFOA, which was the major PFCA released into mobile phase with intensity equal to 0.05 ng mL^{-1} standard, almost all other targeted PFCAs (C4–C14) could be detected in blanks (their intensity decreased with number of carbons). To overcome this problem, original tubing was replaced by another one made from PEEK and, in addition, a short C18 HPLC column was inserted between the mixer and the sample loop, which delayed compounds coming from the LC system, thus injected analytes were eluted earlier than the contamination. By this set-up, practically all interferences coming from the prior part of LC were baseline separated from analytes contained in sample, thus overestimation of their levels can be avoided. The only exception was the most polar PFBA, therefore higher LOQs were taken into consideration.

No contamination by target PFASs was found by testing apparatus such as polypropylene centrifugation tubes, various glasswares, rotary evaporators and also examined organic solvents, salts and sorbents were free of detectable amounts of PFASs. Special attention was paid to microfilters, since they were identified as a source of PFASs contamination by Schultz et al. [19]. For this purpose, each microfilter was rinsed three times by 0.5 mL MeOH portions which were then separately analysed. Unknown bulk compound strongly interfering with PFHxS at transition m/z 399 > 79.9 was eluted from cellulose acetate and regenerated cellulose filters. Although the amount of this interfering compound was successively reduced by repeated washing, cellulose-based filters should be avoided, because the intensity of the interference does not allow detection of PFHxS at its most sensitive transition, even after three washes. When using nylon filters, only traces of PFBS, close to the LOD, were detected, however, contrary to previous case, its signal increased with filtered volume of MeOH. Rather surprisingly, no detectable perfluorinated interferences were released from polyvinylidene fluoride (PVDF) filters, nevertheless, it is highly recommended to check each batch of filters before its use.

In any case, PFASs background levels were monitored regularly, the entire procedure blank was prepared at least each day of

Table 3
Results of pilot screening of PFASs contamination in canned fish products.

Analyte	Canned mackerel ($n = 7$)			Canned sardine ($n = 3$)			Canned cod liver ($n = 6$)		
	AVG[a] ($\mu g\,kg^{-1}$)	Min–max ($\mu g\,kg^{-1}$)	Positive samples	AVG[a] ($\mu g\,kg^{-1}$)	Min–max ($\mu g\,kg^{-1}$)	Positive samples	AVG[a] ($\mu g\,kg^{-1}$)	Min–max ($\mu g\,kg^{-1}$)	Positive samples
PFBA	0.023	<LOQ–0.111	2	0.058	<LOQ–0.133	2		<LOQ	
PFPeA		<LOQ			<LOQ			<LOQ	
PFHxA	0.011	<LOQ–0.078	1		<LOQ			<LOQ	
PFHpA	0.014	<LOQ–0.099	1	0.012	0.008–0.016	2	0.006	<LOQ–0.030	2
PFOA	0.015	<LOQ–0.102	1	0.057	0.022–0.092	3	0.015	<LOQ–0.056	3
PFNA	0.035	<LOQ–0.142	4	0.194	0.175–0.213	3	0.460	0.035–0.583	6
PFDA	0.052	<LOQ–0.169	5	0.098	0.063–0.133	3	0.394	0.052–0.481	6
PFUdA	0.206	<LOQ–0.888	4	0.187	0.082–0.292	3	0.803	0.082–1.097	6
PFDoA	0.068	<LOQ–0.220	5	0.062	0.052–0.072	3	0.261	0.052–0.306	6
PFTrDA	0.245	<LOQ–1.105	4	0.126	0.097–0.155	3	0.530	0.097–0.713	6
PFTeDA	0.050	<LOQ–0.172	5	0.027	0.020–0.033	3	0.077	0.020–0.087	6
PFBS		<LOQ			<LOQ			<LOQ	
PFHxS		<LOQ		0.083	0.051–0.114	3	0.044	<LOQ–0.076	5
PFOS	0.215	0.125–0.447	7	3.164	2.828–3.500	3	5.401	0.215–7.063	6
PFDS		<LOQ			<LOQ			<LOQ	
PFHxPA		<LOQ			<LOQ		0.030	0.000–0.037	
PFOPA		<LOQ			<LOQ			<LOQ	
PFDPA	0.016	<LOQ–0.115	1		<LOQ			<LOQ	
PFOSA	0.049	<LOQ–0.306	2	0.079	0.076–0.131	3	0.211	0.049–0.281	6
N-MeFOSA		<LOQ	0		<LOQ			<LOQ	
N-EtFOSA		<LOQ	0		<LOQ			<LOQ	
N-MeFOSE		<LOQ	0		<LOQ			<LOQ	
N-EtFOSE		<LOQ	0		<LOQ			<LOQ	

[a] Average concentration of all tested samples.

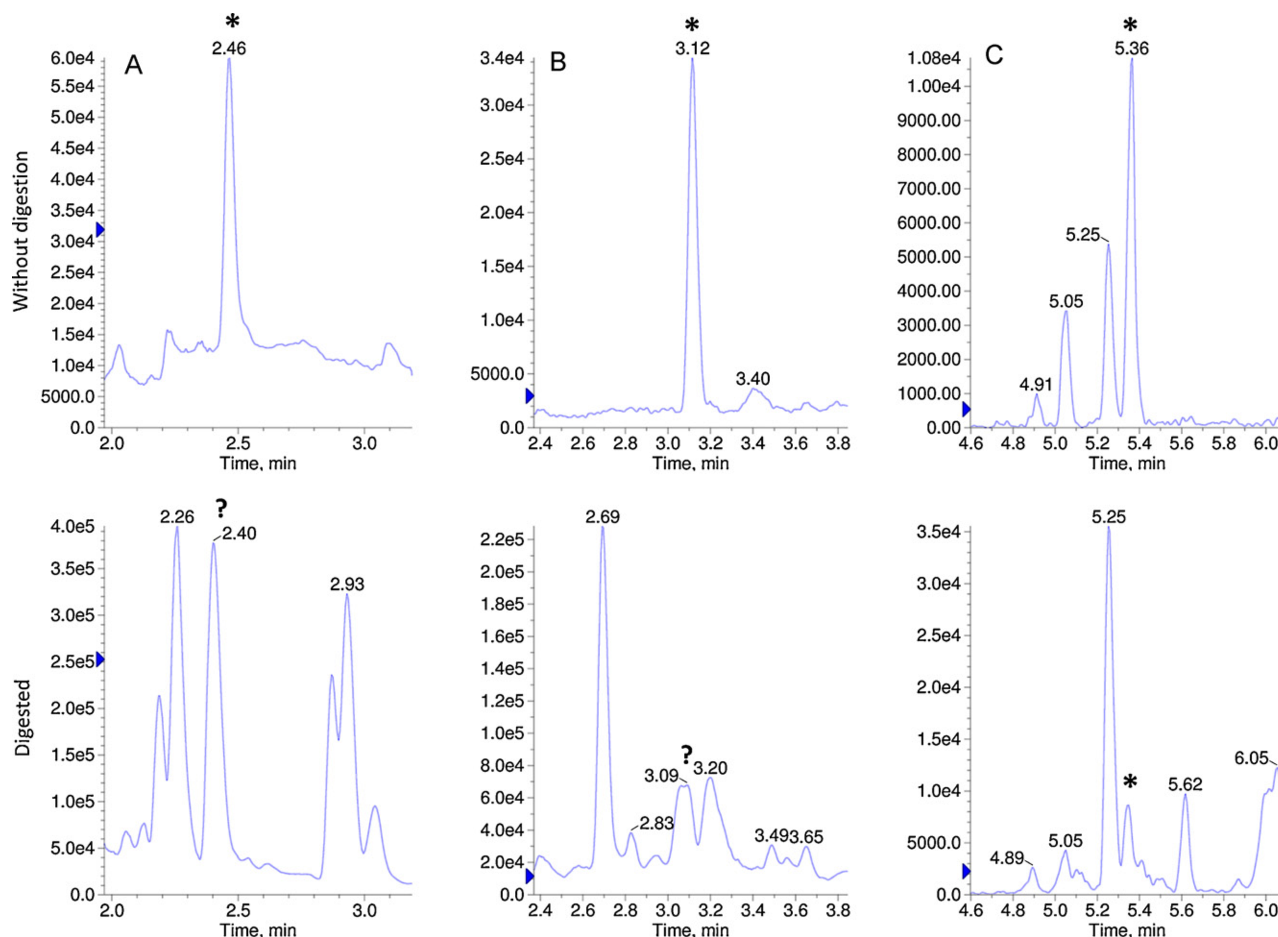

Fig. 2. Comparison of selected chromatograms of fish spiked at 0.06 μg kg^{-1} with and without alkaline digestion. In the case of (A) PFBA (*m*/*z* 213 > 169), (B) PFPeA (*m*/*z* 263 > 213) and (C) PFDA (*m*/*z* 513 > 213), co-eluting interferences released after the alkaline digestion overlaid peaks of analytes and made their analysis impossible. Peaks of analytes are labelled by (*), the interference in the similar retention time is marked by (?).

extraction and whenever a new bottle of solvent or other chemical was used.

3.2. Method development

3.2.1. Extraction method optimization

The QuEChERS procedure is based on extraction of target analytes from the sample by H_2O:MeCN mixture and their subsequent transfer (induced by added inorganic salts) into the acetonitrile layer. The known weakness of this step is a limited transfer of charged (ionic) analytes into the organic phase, so later introduced modifications overcame this problem by employing either acetate or citrate buffers [20,21] to adjust optimal pH in the extract and to achieve sufficient transfer of both, bases and acids. Since PFASs are only acidic and/or neutral compounds, low pH should be achieved.

In the early state of the method development, the influence of different parameters on extraction efficacy, including H_2O:MeCN ratio, addition of formic or sulphuric acids, and amount of NaCl used in partition were tested. The experiments were performed using model mixtures of H_2O:MeCN and fish tissue. The water content in a sample was recognized as an important parameter, which significantly influences recovery of the partition step. This fact is also noted in the European standard EN 15662:2008 [21], which deals with pesticide residue analysis employing QuEChERS method. Generally, for samples containing less than 80% moisture, the portion taken for analysis had to be reduced and water was added to achieve more than 80% of volume of added MeCN.

In the study of acidification impact on the efficacy of the extraction, addition of formic acid significantly improves efficiency of the partition step for perfluorophosphonic acids (PFPAs), C4–C6 PFCAs and PFBS. Contrary to formic acid, sulphuric acid has a negative effect on the recovery of these short chain acidic PFASs. It is probably due to a partial transfer of formic acid into the MeCN layer, which increased its polarity, whereas sulphuric acid remains in the water.

Different amounts of NaCl used for phase separation were also tested, because as Mastovska and Lehotay [22] observed, that lowered NaCl leads to a higher content of water in MeCN phase and consequently increased recovery of polar analytes. However in our study, lower amount of NaCl had no effect.

As a result of the experiments, the optimal extraction approach for isolation of PFASs from fish and milk was identified as follows: prior to the addition of acetonitrile, formic acid should be added to the sample in the amount 2–3% of the volume of later added MeCN. In the case of matrices containing less than 80% moisture (data about the moisture content were obtained from [23]) water should be added. Matrix concentration in samples containing more than 80% water was 1 g mL^{-1} (milk) and in samples with added water it was 0.5 g mL^{-1} (fish) due to the lowered weight of analysed portion. At this stage, supposing ultra-low detection limits were not required, acetonitrile extract could be directly

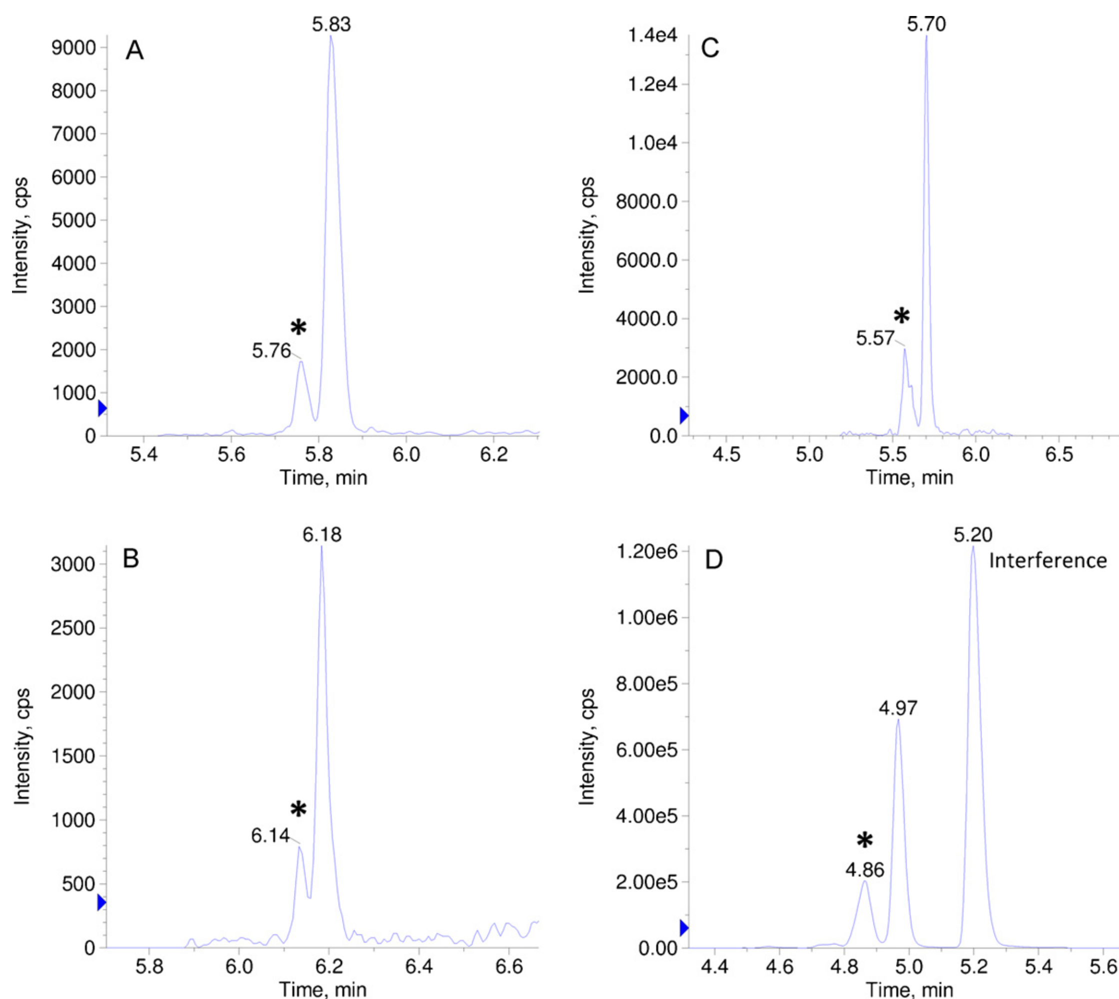

Fig. 3. Example of selected branched and linear PFASs in sample of canned mackerel from Poland. Branched isomers are marked by asterisk (*). The concentration level of linear PFDoA (m/z 613 > 569) (A) was 0.22 μg kg^{-1}, PFTeDA (m/z 713 > 669) (B) was 0.1 μg kg^{-1}, PFOSA (m/z 498 > 78) (C) was 0.076 μg kg^{-1} and PFOS (m/z 499 > 79) (D) was 1.7 μg kg^{-1}. In the PFOS chromatogram is present also baseline separated peak of interference.

analysed by LC–MS/MS, the achievable LOQs were in the range 0.025–0.2 μg kg^{-1} depending on analyte/matrix combination.

3.2.2. Dispersive SPE clean-up and sample concentration

Although the final extract was suitable for the direct LC–MS/MS analysis, due to the matrix concentration of 0.5 or 1 g mL^{-1}, the achieved LOQs were not low enough for the human exposure study and did not enable detection of minor PFASs in food. Higher injected volume or sample enrichment was possible way to decrease LOQs. It should be noted, however, that the final QuEChERS extract, which contains over 85% MeCN, is unsuitable for injection on reversed-phase column, and injection volume higher than 5 μL resulted in significant band broadening of the first eluting analytes PFBA and PFPeA.

The method of choice to concentrate analytes in extract is usually SPE; however, as mentioned in Section 1, none of the available sorbents was able to provide sufficient recovery for all PFASs. Pre-concentration by means of simple solvent evaporation without any purification is also associated with a risk of loss of analytes due to sorption on precipitated matrix. Bearing in mind our objective to keep the sample preparation as simple as possible, the final enrichment procedure consists of the dispersive SPE purification followed by solvent evaporation and its reconstitution in a small volume of methanol.

The use of primary–secondary amine (PSA) sorbent, a weak anion exchanger, which is commonly used for clean-up of QuEChERS extracts, was not suitable with regard to an acidic nature of most PFASs. The strong cation exchanger (SCX) was also ruled out, since impurities of basic nature were not foreseen to be present in crude QuEChERS extract in notable amount, hence purification effect was minimal.

Although sorption of PFASs to activated carbon sorbents is referred in several studies concerning water treatment [24,25], these sorbents are also used for clean-up in PFASs analysis of both, biotic and abiotic samples [11,13] to decrease matrix effects. Because the extracts are in organic solvent, no sorption of target analytes on activated charcoal is observed. When employed for purification of QuEChERS plant extracts [20], ENVI-Carb was shown to remove planar co-extracts such as chlorophylls or sterols.

Worth to notice, that although bulk fats represented by triacylglycerols (TAGs) are not soluble in acetonitrile, some TAGs and other lipophilic compounds move to the organic layer during a partitioning step in the form of micro-micelles. Under these conditions, they can cause clogging of an analytical column and/or sorption of non-ionic PFASs. To eliminate these non-polar co-extracts, besides ENVI-Carb, also C18 sorbent was added, alike in the study by Lehotay et al. [17]. It should be emphasized that no sorption of significantly less hydrophobic target analytes from acetonitrile QuEChERS extract was observed.

In addition to the ENVI-Carb and C18 sorbents, also $MgSO_4$ was added during the dispersive SPE clean-up phase as a desiccant, because the QuEChERS extract contains up to 14% of residual water, which complicated sample evaporation and concentration process.

3.2.3. Alkaline digestion

To assess comprehensively all conceivable approaches in PFASs analysis, alkaline digestion which is believed to release analytes bound to matrix components thus improve overall recovery was also tested [9,12,26]. Maintaining the solvent partitioning step as the key principle of target analyte separation from the matrix, only a minor modification of extraction procedure was needed for incorporation of a digestion step: 1 mL of 10 M NaOH solution was added into the centrifugation tube with a sample, the content of the tube was mixed and the suspension was incubated for 30 min. The hydrolysed sample was neutralised by hydrochloric acid to pH ≈ 6 and subsequent extraction procedure was the same as described in Section 2.3. To keep similar ion strength during the partition, the amount of added NaCl was reduced from 1.5 g to 1 g.

The naturally contaminated fish samples were used for alkaline digestion testing. In all cases the analyte concentrations were comparable to those determined in non-digested samples. However, due to the hydrolysis, a number of intensive co-eluting interferences were observed, especially for PFBA and PFPeA. Because of these analytes provided only one MRM transition, their identification was after digestion impossible (see Fig. 2). In general, the matrix effects were higher in alkaline digested samples, obviously due to the presence of 'new co-extracts', compounds originated from lipids hydrolysis (fatty acids and glycerol are typically released under alkaline conditions). Due to their more polar nature, they were not removed by C18 sorbent anymore, contrary to parent triacylglycerols. On this account, alkaline digestion was not implemented in the final procedure.

3.2.4. Method validation

The developed method was validated by analysis of spiked blank or very low contaminated fish (trout) and milk samples. To learn more on uncertainties of individual sample preparation steps, each of them was evaluated separately. To overcome matrix effects, matrix matched calibration was used for quantification of each analyte. The volume changes (especially in the case of the concentration step) were corrected by addition of 13C labelled PFOS standard.

The recovery of extraction/partitioning step was tested by spiking the mixture of analytes directly onto the matrix and leaving for 1 h to equilibrate. Then extraction procedure without purification and sample enrichment was carried out. The spiking levels for milk and fish were 0.25 and 0.50 $\mu g\,kg^{-1}$ (wet weight), respectively. Relatively lower recoveries (between 50 and 70%) were obtained for the most polar, short chain PFCAs (C4–C6) and PFPAs, which were not completely transferred into organic phase during the partitioning. The recovery of a purification step was determined by spiking (crude) extracts of a blank sample at the same levels as in previous experiments and the purified extracts were analysed directly (without concentration). When matrix effects were compensated by matrix matched calibration, no significant decrease of recoveries (more than 5%) was observed.

The sample concentration step was tested by spiking purified extract at levels 0.015 and 0.030 $\mu g\,kg^{-1}$ (wet weight) in milk and fish, respectively. Obtained recoveries for the non-ionic N-MeFOSA and N-EtFOSA were in some cases, when pressure about 100 mbar was applied in a rotary evaporator during solvent reduction, less than 70%. No other analyte losses were observed during the sample enrichment and to avoid loss of the most volatile analytes, minimal pressure of 150 mbar was applied during evaporation.

The final validation experiments were performed with spiking levels 0.015 and 0.030 $\mu g\,kg^{-1}$ in milk and fish respectively, and employing all isotopically labelled surrogates. The performance characteristics obtained within this part of study are summarized in Table 2. The PFCAs were corrected by their analogues or the nearest analogue with one carbon shorter chain, except of PFTrDA and PFTeDA, which were corrected by 13C2 PFDoA (the commercially available labelled PFCA with the longest chain). The results of validation demonstrated that this approach provided reliable results and satisfactory trueness was achieved even for those analytes, which due to their high polarity provided lower recovery during partition. The recoveries of all PFSAs were corrected by 13C labelled PFOS and, the experimental data shown, that the influence of the chain length on recovery is lower than in the case of PFCAs. To correct recoveries of PFOSA, FOSAs and FOSEs, 13C8 FOSA and deuterated analogues of FOSAs and FOSEs were employed. The most problematic analytes were PFPAs. Physico-chemical properties of PFPAs are fairly different from other PFASs and, due to their high polarity and known affinity to metallic surfaces, the chromatographic peaks were prone to tail, more than other PFASs involved in this study. The Acquity HSS T3 was chosen as the best for PFPAs separation; nevertheless, the chromatography was still sensitive on column ageing. In addition to differences in chromatographic behaviour, the signal intensity under optimal ESI(−) conditions was approximately twice lower compared to equimolar concentrations of PFCAs. Worth to notice, also worse PFPAs' reproducibility was observed during the method validation study. As mentioned above, the recovery of the partition step in some cases drop below 70%, but during most experiments recovery about 100% was achieved. Because of 13C labelled PFPAs internal standards, which could correct this recovery fluctuation were not available, 13C labelled PFOS was used.

Although labelled surrogates were used, the quantification of the analytes was in most cases carried out on matrix matched calibrations, which helped to compensate matrix effects (ion suppression/enhancement). The labelled internal standards could compensate matrix effects only for analyte with the same similar retention time. However, in the case of PFOS in fish, for which any blank matrix was not available, solvent standards were always used for quantitation.

3.3. Pilot screening study: PFASs in milk and fish

In the final phase of our experiments, the developed method was employed for a pilot screening of occurrence of 23 PFASs in real life samples of animal origin. For this purpose 12 milk samples differing in fat content (0.5–3.5%) and 16 canned fish products (mackerel, sardine and cod liver) available at the Czech retail market were collected.

No significant contamination was found in any of examined milk samples. Only traces at the LOQ level of PFOS and, surprisingly, also PFDS were found in four milk samples. The presence of PFSAs, which might be biased when detecting only less selective fragment *m*/*z* 80 $[SO_3]^-$ (it is originated also from other compounds containing a sulphonic group), was confirmed by the presence of a second transition (fragment *m*/*z* 99 $[SO_3F]^-$) at the respective retention time. No relationship between the level of particular PFAS in milk and its fat content was found.

Compared to milk, a fairly broader spectrum of PFASs was determined in canned fish products; the overview of obtained results is shown in Table 3. Alike in similar studies concerned with PFASs in fish [27–29], PFOS was the dominating representative of these contaminants. Unfortunately, at the time of study, only linear PFOS was in the calibration mixture, therefore branched forms could not be quantified nevertheless they were detected in all samples.

Also representatives of PFCAs were found in all examined samples and their concentration generally increased with the chain length. While C4–C6 PFCAs were present in only a few samples, C7–C14 PFCAs were detected in most of samples and at higher concentration levels. It should be noted, that PFOA (C8), monitoring of which is recommended by EC [5], was found at lower levels compared to the longer chain PFCAs (C9–C14). The long chain PFCAs with odd number of carbons (C9, C11 and C13) were found at higher concentrations than those with even number of carbons (C8, C10, C12, and C14). This trend was also mentioned by Van Leeuwen et al. [27] and the similar pattern of PFCAs was found also in other studies [29]. In a few samples with overall higher PFASs contamination, branched forms of C11–C14 PFCAs were detected (see Fig. 3). Regarding the group of neutral PFASs, only PFOSA was detected above LOQ, in addition to the linear form, also the branched PFOSA was detected in a few samples.

4. Conclusions

The present study documents in detail the development of a new simple and fast LC–MS/MS method for simultaneous analysis of 23 PFASs in milk and fish products. The main outcomes of the analytical research can be summarized as follows:

- Within the validation study, good performance characteristics were achieved at concentrations 0.015 and 0.030 $\mu g\,kg^{-1}$, for milk and fish respectively, for both ionic and non-ionic PFASs: recoveries between 70 and 120% and repeatability less than 20% (expressed as RSDs). The ultra-trace LOQs in the range 0.002–0.013 $\mu g\,kg^{-1}$ were determined as S/N (peak-to-peak) >10.
- The sample preparation procedure based on the QuEChERS approach was relatively fast (5 samples per hour) and only a basic laboratory equipment (centrifuge, rotary evaporator) is needed.
- Alkaline sample digestion of naturally contaminated samples recommended in some studies does not improve the overall recovery of PFASs, moreover, interfering substances released from examined matrix could not be removed by sorbents employed in purification step.
- Due to the use of sub 2 μm UHPLC column and optimized separation conditions, the chromatographic run took only 11 min (from injection to injection), without any compromise of chromatographic resolution and peak shape.
- Broad spectrum of PFASs could be found in examined canned fish products as a result of very low LOQs. In addition to PFOS and PFOSA, occurrence of which was commonly reported in fish, many long chain PFCAs (C9–C14) were documented to be present. The information of PFASs pattern might be helpful in identification of contamination sources.

It should be emphasized that, to our best knowledge, none of until now published LC–MS/MS methods enables such rapid and accurate analysis of a wide range of PFASs, including problematic PFPAs, at ultra-trace level as that introduced in this study. We are convinced that it can be generically employed for many other matrices of animal origin such as seafood, meat, egg and liver.

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