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Abstract

The bioaccumulation and toxicity of per- and polyfluoroalkyl substances (PFAS) have raised scientific and public concern in recent decades, leading to regulatory measures for some PFAS (e.g. perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA)). In addition, the discovery of new PFAS alternatives in the environment has led to growing concern about the presence of numerous other PFAS that are used unrestricted. Feathers have been successfully applied as non-destructive indicators for various contaminants, mostly metals and persistent organic pollutants (POPs), whereas their suitability as an indicator for PFAS is still discussed. Previous studies on PFAS in feathers have focused primarily on perfluoroalkyl sulfonic acids (PFSAs) and perfluoroalkyl carboxylic acids (PFCAs); analytical methods for other groups of PFAS or PFAS alternatives in feathers are still lacking. Hence, this study aimed to develop a rapid, sensitive and reliable analytical method for determining a broad range of PFAS (N = 32) in feathers, using liquid chromatography-tandem mass spectrometry (LC-MS/MS). An extraction duration of 24 h was found to be sufficient to extract the majority of PFAS from the feathers. The extraction recovery of the internal standards ranged on average from 68 % (PFBA) to 97 % (PFOS). The spike recovery was within an acceptable range of at least 70% for most of the target analytes and the precision was often > 80%. A further extract clean-up using weak anion exchange (WAX) solid phase extraction (SPE), was proven unnecessary, as it resulted in a similar or lower spike recovery, and, as a consequence, a lower precision and higher quantification limit. The analytical method allows detection of low PFAS concentrations in a low quantity of matrix (i.e. small feathers). The developed LC-MS/MS method was validated and shown to be a fast, sensitive and reliable method for determining a broad range of legacy and emerging PFAS in feathers.

Keywords: PFAS, ESI-MS/MS, UPLC, Feathers, Birds

1. Introduction

Environmental pollution co-evolved with the appearance of humans. Harmful activities of ancient civilizations caused long-lasting changes in the environment [1]. Since the last century, the development of organic chemical industries has led to an increased production of numerous anthropogenic chemicals. Many of these chemicals, especially persistent organic pollutants (POPs) have received worldwide scientific and public attention after their global detection in nature [2]. However, much less is known about the more recently produced (since the 1940s) and globally detected per- and polyfluoroalkyl substances (PFAS) [3,4].

Due to the unique physicochemical properties of PFAS, they have been produced and used for over 60 years in numerous industrial applications and consumer products, including food packaging material, surface coatings for carpets and fire-fighting foams [5,6]. As a result of their wide application, as well as degradation of precursor compounds [6,7], PFAS have been detected globally in the environment and in biota including humans [8-11].

Long-chain perfluoroalkyl carboxylic acids (PFCAs, ≥ C8) and perfluoroalkyl sulfonic acids (PFSAs, ≥ C6) have been the main focus of regulatory agencies and researchers over the past decades, due to their high bioaccumulative potential and toxicity [6]. More specifically, the main attention of researchers has been on perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS). The global distribution, strong persistence, and toxicity to the environment and humans have led to the phase-out or ban of PFOA and PFOS in several countries, mainly in the Northern Hemisphere. For example, the 3M company voluntarily phased-out the production of PFOS and related products in the early 2000s [12]. In addition, PFOS and PFOA have been included in the Stockholm Convention on POPs in 2009 and 2019 [13,14], respectively. Due to these regulatory measures, the concentrations of these two PFAS in the environment appear to be decreasing in most cases (reviewed in Land et al. [15]). However, the concentrations of PFOS and PFOA are often still very high in the environment and wildlife [16,17]. Furthermore, the synthesis of new PFAS

alternatives by manufacturers has led to a growing concern about the presence of numerous other PFAS, as for example GenX and ADONA, that still are used unrestricted [18].

Wild birds have been proven useful in the biomonitoring of environmental contaminants, such as metals and POPs [19,20]. Initially, studies that used birds as sentinels for environmental pollution, including PFAS, used primarily destructive matrices, such as eggs, carcasses and organs [21-23]. However, due to both ethical and practical aspects, the use of non- or less-destructive sampling methods, such as feathers, is increasing in biomonitoring studies. Feathers are easy to collect, store and transport compared to invasive matrices, such as serum, muscle and liver [24]. As feathers consist mainly of the protein keratin [25], PFAS could accumulate easily in feathers due to their strong affinity for proteins [26,27]. Nonetheless, studies on PFAS in feathers are still quite scarce [17,28]. Furthermore, the suitability of feathers for biomonitoring of PFAS is still the subject of debate because correlations between internal PFAS concentrations and those in feathers have been reported for some PFAS, but not for others [29]. These associations may be influenced by different ways of exposure among feather types and among bird species [29]. In addition, feather concentrations may not always resemble the concentrations detected in the environment. For example, birds with a large foraging area, such as raptors, may not be appropriate for monitoring local contamination close to point sources [30]. Nonetheless, the usefulness of archived bird of prey feathers in monitoring spatiotemporal PFAS trends has been underlined by Sun et al. [31].

PFAS have been detected in bird feathers in previous studies (reviewed by Jaspers et al. [29]). Current analytical methods to extract PFAS from feathers rely either on acid/base digestion with organic solvent extraction, followed by a clean-up with granular activated carbon [31-37], or on organic solvent extraction followed by a solid phase extraction (SPE) clean-up [38]. As these studies focused primarily on legacy PFSAs and PFCAs, analytical methods for the determination of other groups of PFAS or PFAS alternatives in feathers are still absent.

Here, we aimed to develop a rapid, sensitive and reliable method for simultaneously analyzing a broad range of legacy and emerging PFAS (N = 32) in feather samples using negative electrospray ionization (ESI(-)) mode operating on an ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS).

2. Materials and methods

2.1 Chemicals and reagents

The analyzed PFAS are abbreviated according to Buck et al. [6]. A mixture (EPA-533 PAR: chemical purity > 98% for all PFAS), containing nine native (i.e. unlabeled) linear PFCAs (C4–C12), five native PFSAs (C4–C8), three native fluorotelomer sulfonates (4:2, 6:2 and 8:2 FTS), sodium dodecafluoro-3H-4,8-dioxanonanoate (NaDONA), the major and minor components of F-53B (9Cl-PF3ONS and 11Cl-PF3OUdS), GenX (HFPO-DA), three perfluoroether/polyether-carboxylic acids (PF4OPeA, PF5OHxA and 3,6-OPFHpA) and a perfluoroethersulfonate (PFEESA) was purchased from Wellington Laboratories (Guelph, Canada). Furthermore, a mixture (FTA-MXA, chemical purity > 98% for all PFAS) of three native fluorotelomer acids (6:2, 8:2 and 10:2 FTA) and a mixture of isotopically mass-labeled PFAS (MPFAC-MXA, chemical purity > 98%, isotopic purities ≥ 99% or > 94% per ^{13}C or ^{18}O respectively), containing seven PFCAs (C4, C6, C8, C9, C10, C11 and C12) and two PFSAs (C6 and C8) were purchased from Wellington Laboratories. Finally, a mixture (PERFOOD PFAPA mix, chemical purity > 98% for all PFAS), containing four unlabeled perfluorophosphonic acids (PFPAs; Cl-C6, C6, C8 and C10) was used. Table 1 displays the full name and abbreviation of each analyte. All solvents, including methanol (MeOH, VWR International, Belgium), acetonitrile (ACN, LiChrosolv, Merck Chemicals, Belgium), ammonium acetate (VWR International, Belgium), ammonium hydroxide (Filter Service N.V., Belgium), and Milli-Q (MQ, 18.2 mΩ; TOC: 2.0 ppb; Merck Millipore, Belgium) were HPLC grade.

2.2 Feather collection

In July 2019, feathers (at least 50 mg), shed by two domestic free-ranging chickens in a garden in Zwijndrecht, Belgium, were collected from the ground. This site is located approximately 1 km south-west from a fluorochemical plant, which is a well-known PFAS hotspot [17]. These feathers were expected to be contaminated because home-produced chicken eggs in the same study area in Zwijndrecht contained high PFOS concentrations [39]. Presumably uncontaminated feathers (at least 50 mg) of domestic free-ranging chickens (N ≥ 2 per location) were collected by volunteers across the province of Antwerp and a minimum of 12 km away from the 3M fluorochemical plant in Zwijndrecht). These feathers were collected during the winter of 2019-2020, by picking them up from the ground. In addition, uncontaminated feathers were collected at an organic farm in Westmalle, a site with low PFAS contamination [39,40]. The feathers were stored in 50 mL PFAS-free polypropylene (PP) tubes under dark condition at room temperature, to protect them from UV radiation.

2.3 Extraction: MeOH only

For extraction, only methanol 100% was used as extraction solvent, because it has been proven equally successful in the extraction of PFOS from feathers as compared to alkaline and acid digestion [38]. Feathers were washed thoroughly with MQ to remove soil and dust particles. In agreement with previous studies in feathers, the feathers were cut into small pieces of approximately 1 mm, using stainless-steel scissors [35,36]. To the cut feathers (50–100 mg), 10 mL of 100% MeOH and 10 ng of the mass-labeled internal standard (ISTD) solution (diluted in 1:1 MeOH:MQ) were added. Hereafter, the samples were vortex-mixed for at least 1 min and left in the dark for 24 h at room temperature. This time period was experimentally selected based on the time needed to extract the majority of PFAS from feathers (more details in sections 2.6 and 3.2). After centrifugation (4 °C, 10 min, 1037 x g, Eppendorf centrifuge 5804R), the supernatant was transferred into a 15 mL PP tube and dried completely using a rotational-vacuum concentrator (Martin Christ, RVC 2-25, Osterode am Harz, Germany). Finally, the samples were reconstituted with 2 mL of a 2% ammonium hydroxide solution in ACN, vortex-mixed for at least 1 min

and filtered through an Ion Chromatography Acrodisc 13 mm Syringe Filter with 0.2 μm Supor polyethersulfone (PES) Membrane (VWR International, Leuven, Belgium). The final extracts were collected in a PP auto-injector vial.

2.4 Extraction: MeOH + SPE

In order to validate whether an extract clean-up using SPE is required, we added a clean-up procedure to the extraction described in 2.3. The same procedure as described in 2.3, including the use of 100% MeOH as extraction solvent, was followed until centrifugation. After centrifugation (4 °C, 10 min, 1037 x g, Eppendorf centrifuge 5804R), the supernatant was vortex-mixed for 1 min, and loaded onto a preconditioned (5 mL of ACN) and equilibrated (5 mL of MQ) Chromabond HR-XAW SPE cartridge (Application No 305200, SPE department, Macherey-Nagel, Germany, 2009, 3 mL, sorbent content: 200 mg), which was washed with 5 mL of ammonium acetate buffer (25 mM) in MQ and 2 mL of ACN. Finally, these cartridges were eluted two times with 1 mL of a 2% ammonium hydroxide solution in ACN, dried completely in a rotational-vacuum concentrator and reconstituted with 200 μL of 2% ammonium hydroxide in ACN. The extract was filtered through the previously described syringe filter into a PP auto-injector vial.

2.5 UPLC-TQD analysis

Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS, ACQUITY, TQD, Waters, Milford, MA, USA) with negative electrospray ionization mode was used to analyze the PFAS (N = 32). An ACQUITY BEH C18 column (2.1 x 50 mm; 1.7 μm, Waters, USA) was used to separate the analytes. An ACQUITY BEH C18-pre-column (2.1 x 30 mm; 1.7 μm, Waters, USA), inserted between the injector and the solvent mixer, was used to retain any PFAS contamination from the system. The mobile phase solvent gradient started at 65% of 0.1% formic acid in water, changed to 100% of 0.1% formic acid in ACN in 3.4 min, and returned to 65% of 0.1% formic acid in water at 4.7 min. The flow rate was set at 450 μL/min

with an injection volume of 6 µL (partial loop). Target analytes were quantified using multiple reaction monitoring (MRM) of two diagnostic transitions per analyte. The cone voltages, collision energy and MRM transitions of the target analytes and ISTDs are displayed in Table 1. Specific details on the MRM transitions (reactions to obtain specific product ions) are presented in the supporting information (SI). Target analytes for which corresponding ISTDs were available in the MPFAC-MXA mixture were quantified using these corresponding ISTDs. The analytes, for which no corresponding ISTD was present, were quantified with the ISTD that resulted in the lowest variation in area units of the peak signals, as described by Groffen et al.[41]. Chromatograms of the target analytes are displayed in Figure S1-S7 for the standard mixtures and Figures S8-S14 for spiked feather samples.

2.6 Method validation

Seven-step calibration curves were prepared in quintuplicate by adding a constant amount of the ISTD ($concentration_{ix,}$ hereafter C_{ix}), to different concentrations of the unlabeled PFAS mixtures ($concentration_x$, hereafter C_x). Dilutions of the unlabeled mixture were performed in MeOH. C_x/C_{ix} ratios varied from 1:1000 to 1000:1. After logarithmic transformation, the ratio of the concentrations (C_x/C_{ix}) was plotted against the ratio of the areas of the unlabeled ($Area_x$) and labeled ($Area_{ix}$) compounds. Linearity was assessed by observing the coefficient of determination (R^2) of these linearity plots. The method sensitivity was determined as the gradient of the calibration functions.

The method exactness was determined by the calculation of the spike recovery in uncontaminated feathers, as described below. In addition, as an additional measure of method exactness, certified reference material (N = 5; sterilized fish muscle tissue of pike perch (*Stizostedion lucioperca*), QUASIMEME Laboratory Performance Studies[42]) was analyzed. The closeness between the test results and accepted reference values was determined. Fish muscle tissue was used as reference material as, to the best of our knowledge, no reference material is commercially available that resembles the structure of feathers.

A schematic overview of the steps taken to determine the method selectivity, precision, limit of quantification (LOQ), limit of detection (LOD) and recovery is displayed in Figure 1. The required time for extraction was investigated by analyzing a pool of the contaminated feathers and dividing this pool into seven groups (N = 5 per group, 25 ± 9 mg ww). These groups were then extracted using the protocol described in section 2.3 and left in the dark for 24, 48, 72, 96, 120, 144 and 168 h at room temperature. In the sample batch, five procedural blanks (10 mL of MeOH) were included and these contained minor contamination with PFOA (8.8 ± 2.6 pg/g), PFDA (11.8 ± 6.8 pg/g) and PFUnDA (10.6 ± 3.6 pg/g), which was subtracted from the concentrations in the samples. The extraction recovery of the ISTDs was calculated based on the Area of these ISTDs ($Area_{ix}$) and the $Area_{ix}$ of a non-extracted ISTD solution.

The method selectivity and precision were validated by dividing a pool of uncontaminated feathers into subsamples (N = 10). These samples were spiked with a native (unlabeled) standard mix (containing 25 ng of each analyte diluted in 5 mL of MQ and 5 mL of MeOH), and left under vacuum (400–500 Mbar) until dryness to enable maximum adsorption of the native standards. Hereafter, five of these samples followed the extraction procedure with MeOH only (as described in section 2.3), whereas the other five were further cleaned-up using the XAW-SPE cartridges (as described in section 2.4), in order to investigate the effect of the clean-up on the spike recovery. Background concentrations in the pool of uncontaminated feathers were subtracted from the concentrations in the spiked samples. The selectivity was examined by investigating deviations between the spiked concentrations and measured concentrations. The method's precision was examined by calculating the standard deviation as well as the standard deviation based on the spike recoveries in the samples. The method limit of quantification (LOQ) and limit of detection (LOD) of the target analytes were determined based on spiked samples, as the concentrations at a signal-to-noise (S/N) ratio of 10 and 3, respectively.

2.7 Statistical analyses

Statistical analyses were performed in R Studio (version 3.2.2.) and the level of significance was set at $p \leq 0.05$ (adjusted p-values). The normality assumptions of the used statistical models were examined using the Shapiro-Wilk test and running diagnostic plots. The data were log-transformed, when needed, to meet the normality assumptions of the residuals. Simple linear regression functions were used to test the linearity of the calibration curves. The influence of the extraction time on the total PFAS (ΣPFAS) concentrations was investigated using one-way ANOVA, with extraction duration (h) as a factor, followed by Tukey's Honestly Significant Difference test for post-hoc analysis.

3. Results and discussion

3.1 Linearity

Linear regression functions (Figures S15–S46) described the relationship between C_x/C_{ix} and $Area_x/Area_{ix}$ for all target analytes. All compounds showed a highly significant linear fit ($R^2 > 0.98$, $p < 0.001$), although the linear range may slightly differ depending on the target analyte. The sensitivity of the method (i.e. ranges of the calibration curves) was similar for the majority of the target analytes, as the gradient of the calibration curves for most compounds ranged from 100:1 to 1:100 C_x/C_{ix} ratios. Only the calibration graphs for PFBA, PFPeA, PFOA and NaDONA remained linear in a range from C_x/C_{ix} ratios of 1:1000 to 1000:1. The sensitivity of the PFPAs was much lower, as their linear range varies between C_x/C_{ix} ratios of 100:1 and 1:1, with the exception of PFHxPA where the linearity ranged between ratios of 100:1 and 1:10. This is likely the result of high LOQ (section 3.3), resulting in no detection of the native (unlabeled) solutions at a ratio lower than 1:1. Finally, there were multiple compounds for which a linearity was not guaranteed when internal tracer concentrations were 1000x lower than the concentrations of the unlabeled compounds, or vice versa in case of PFDA.

3.2 Extraction duration

The ΣPFAS concentration in the contaminated feathers (collected at Zwijndrecht, close to the 3M plant) after an extraction duration of 24, 48, 72, 96, 120, 144 and 168 h is displayed in Figure 2. No significant

differences were observed among the different extraction periods ($F_{6,28}$ = 1.35, p = 0.271), showing that a 24 h extraction period is sufficient to extract the majority of the PFAS from the feathers. Although a 48 h extraction seems to be more efficient (Figure 2), the relative standard deviation (RSD, %) for day 2 (50%) was much higher than for the other days (20%).

3.3 Exactness, precision, quantification limits and detection limits (LOQs/LODs)

The PFAS concentrations in the reference materials, as well as the assigned values [42], are displayed in Table S1. Although measured PFUnDA and PFOS concentrations were, on average, lower than the assigned values, the measurements were within the range of the interlaboratory study on the reference material. PFNA and PFDoDA concentrations were not detected in any of the samples, most likely due to assigned values being close to or below the LOQ for fish muscle tissue (i.e. the reference material matrix).

Average values of the spike recovery, the standard deviation (SD) and precision (expressed as imprecision, RSD, %) for the target analytes, extracted using MeOH only, are displayed in Table 2. We aimed for a method with a spike recovery not lower than 70%, and an imprecision (RSD, %, as proxy for precision) of maximum 10% (i.e. a precision of 90%). The spike recovery was within the acceptable range for 29 out of 32 compounds, with a recovery less than 70% for PFPeS (67.3%), PFHxS (68.8%) and PFHxPA (52.3%). Similarly, for the majority (20 out of 32) of the target analytes, the imprecision (%RSD) was < 10%, showing a high precision of the method. With the exception of PFHxPA and PFDPA, the RSD (%) was < 20% for all analytes. In general, a higher imprecision (RSD, %) was reported for PFAS containing sulphur (S) atoms, the FTAs and the PFPAs. The spike recovery of PFCAs, compared to S-containing PFAS, could be higher due to the formation of hydrogen bonds between individual PFCA molecules [41]. These bonds result in larger non-polar molecules, which dissolve better in a non-polar medium such as MeOH than the more polar PFSAs, which are mainly present as their salts [41]. Regarding the FTAs and PFPAs, the LOQs and LODs were also particularly high (Table 2), suggesting suboptimal extraction conditions (e.g. in terms of pH or

extraction solvent) for these analytes. Different extraction techniques for different groups of PFAS have been suggested for other matrices [41,43].

3.4 ISTD recovery

The mean extraction recovery (%) of the isotopically-labeled ISTDs, calculated on the contaminated feathers varied between 68% (PFBA) and 97% (PFOS) (Figure 3), with the only recovery below 84% for $^{13}C_4$-PFBA and $^{13}C_2$-PFNA. The extraction recovery was therefore considered good for the majority of the ISTDs, and the variation in extraction recovery, depicted in Figure 3, is mainly the result of occasional outliers, as the vast majority of recovery values are between Q1 and Q3 values. The extraction recovery of the method described in the present study was similar or higher than those reported for the method using an acid/base extraction, followed by an extract clean-up using granular activated carbon powder [33, 35-37].

3.5 Necessity of extract clean-up

The values of spike recovery, imprecision (RSD,%) and LOQ for the non-cleaned-up feather extracts were compared with the recovery of feathers which were cleaned-up using XAW SPE cartridges (Table 3). The spike recovery after SPE clean-up was below the 70% value for 14 out of 32 analytes. Similarly, the imprecision (%RSD) was higher after clean-up, compared to the MeOH extraction method (described in 2.3), as only 2 compounds showed a RSD below 10% and only 3 PFAS had a RSD below 20%. This means that after clean-up using WAX SPE cartridges, the variation in exactness and precision is larger than the variation observed for the MeOH extraction. Furthermore, the LOQs after clean-up were higher than without clean-up, which was the result of an increased noise in the cleaned-up extracts rather than a reduced signal (the $Area_x$ of both the MeOH and the MeOH+SPE extracts did not differ). The main negative interferences in ESI (-) mode results from interfering background ions, cluster ions or additives and degradation products [44]. Therefore, it is likely that the higher LOQs in the cleaned-up extracts are the

result of interference due to the presence of these ions or additives in the XAW SPE cartridges. Hence, further extract clean-up using WAX SPE is not recommended in the extraction of PFAS from feathers.

3.6 Application on real samples

Especially regarding small bird species, including many passerines, their small body mass makes it difficult to obtain a sufficient amount of matrix to be analyzed with the analytical instruments currently available in most laboratories. However, the analytical technique developed here opens up the possibility of a non-invasive sampling technique for collecting a small amount of tissue (e.g. small feathers) to trace very low concentrations of PFAS (in the order of pg/g) in birds. The method we developed has been successfully applied on tail feathers (N = 75) from great tits (*Parus major*), a terrestrial songbird model species, collected at a fluorochemical site and at four other sites representing a distance gradient [30]. The recovery of the ISTDs for PFCAs and PFSAs in these feathers varied between 60% (PFBA) and 95% (PFOS) which was very similar to those reported in the present study [30]. However, in this study only legacy PFCAs and PFSAs were targeted, and the applicability of this method for the other groups of compounds still needs to be examined further. Although unexpected, based on the similarity in recovery and LOQ between great tits [30] and chicken feathers (present study), it should be mentioned that species-specific differences in extraction efficiency, for example caused by differences in feather protein content [45,46], require further examination.

4. Conclusion

The developed analytical method was validated and shown to be a fast, sensitive and reliable method for determining a broad range of PFAS in feathers. The majority of PFAS were extracted after an extraction duration of 24 h. A further clean-up of the extract using granular activated carbon or WAX SPE is not recommended as this does not improve the exactness and precision of the method, whereas the costs and time of extraction increases. Especially regarding the use of WAX SPE, the exactness and precision of

the method decreases, which is likely due to background interference and the formation of cluster ions or additives possibly leaking from the prepacked cartridges used. The LOQs of the target analytes were also higher after clean-up using SPE as compared to direct analysis of a crude MeOH extract. The developed method opens up the possibility of analyzing small amounts of tissues (e.g. small feathers) to trace very low PFAS concentrations.

Acknowledgements

This work was funded by the research projects G038615N and 12ZZQ21N of the Research Foundation Flanders (FWO). TG is funded by a post-doctoral grant of the FWO. The authors would like to thank all volunteers, i.e. the families Bervoets, D'Hollander, Van Eyken, Van den Brande, Gernaey, Schaerlaeken, Heirbaut, Van den Broeck, De Feyter and Bosman for providing chicken feathers. Furthermore, we would like to thank Raewyn Town and Mieke Goris for proofreading the revised manuscript. The authors declare no conflict of interest.

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Tables and Figures

Table 1. Full name, abbreviation, MRM transition (precursor and product ion), internal standard (ISTD) used for quantification, cone voltage (V) and collision energy (eV) for the target PFAS and the ISTDs.

Analyte		ISTD used for quantification	Precursor ion (m/z)	Product ion (m/z)		Collision energy (eV)		Cone voltage (V)	
Full name	**Abbreviation**			**Diagnostic product ion 1**	**Diagnostic product ion 2**	**Diagnostic product ion 1**	**Diagnostic product ion 2**	**Diagnostic product ion 1**	**Diagnostic product ion 2**
Perfluorobutanoic acid	PFBA	$^{13}C_4$-PFBA	213	169	169	19	50	19	
Perfluoropentanoic acid	PFPeA	$^{13}C_4$-PFBA	263	219	219	10	45	15	
Perfluorohexanoic acid	PFHxA	[1,2-$^{13}C_2$]PFHxA	313	269	119	21	65	19	
Perfluoroheptanoic acid	PFHpA	[1,2-$^{13}C_2$]PFHxA	363	319	169	40	30	24	
Perfluorooctanoic acid	PFOA	[1,2,3,4-$^{13}C_2$]PFOA	413	369	169	13	60	22	
Perfluorononanoic acid	PFNA	[1,2,3,4,5-$^{13}C_2$]PFNA	463	419	169	17	20	28	
Perfluorodecanoic acid	PFDA	[1,2-$^{13}C_2$]PFDA	513	469	219	29	29	25	
Perfluoroundecanoic acid	PFUnDA	[1,2-$^{13}C_2$]PFUnDA	563	519	169	30	35	18	
Perfluorododedanoic acid	PFDoDA	[1,2-$^{13}C_2$]PFDoDA	613	569	319	21	30	22	
Perfluorobutane sulfonate	PFBS	$^{18}O_2$-PFHxS	299	80	99	65	45	40	
Perfluoropentane sulfonate	PFPeS	[1,2,3,4-$^{13}C_4$]PFOS	349	80	99	40	40	40	35
Perfluorohexane sulfonate	PFHxS	$^{18}O_2$-PFHxS	399	80	99	30	60	22	
Perfluoroheptane sulfonate	PFHpS	[1,2,3,4-$^{13}C_2$]PFOA	449	80	98.5	47	45	40	
Perfluorooctane sulfonate	PFOS	[1,2,3,4-$^{13}C_4$]PFOS	499	80	99	58	58	60	

Table 1 (continued). Full name, abbreviation, MRM transition (precursor and product ion), internal standard (ISTD) used for quantification, cone voltage (V) and collision energy (eV) for the target PFAS and the ISTDs.

Analyte		**ISTD used for quantification**	**Precursor ion (m/z)**	**Product ion (m/z)**		**Collision energy (eV)**		**Cone voltage (V)**	
Full name	**Abbreviation**			**Diagnostic product ion 1**	**Diagnostic product ion 2**	**Diagnostic product ion 1**	**Diagnostic product ion 2**	**Diagnostic product ion 1**	**Diagnostic product ion 2**
1H,1H,2H,2H-perfluoro-1-hexanesulfonate	4:2 FTS	[1,2,3,4-$^{13}C_4$]PFOS	327	307	80	25	33	20	
1H,1H,2H,2H-perfluoro-1-octanesulfonate	6:2 FTS	[1,2,3,4-$^{13}C_4$]PFOS	427	407	80	25	33	20	
1H,1H,2H,2H-perfluoro-1-decanesulfonate	8:2 FTS	[1,2,3,4-$^{13}C_4$]PFOS	527	507	81	40	40	36	
Sodium dodecafluoro-3H-4,8-dioxanonanoate	NaDONA	[1,2,3,4-$^{13}C_2$]PFOA	376.8	250.7	84.8	35	32	23	
9-chlorohexadecafluoro-3-oxanonane-1-sulfonate	9CL-PF3ONS	[1,2,3,4,5-$^{13}C_2$]PFNA	531	350.5	83	32	37	46	40
11-chloroeicosafluoro-3-oxaundecane-1-sulfonate	11CL-PF3OUdS	[1,2-$^{13}C_2$]PFUnDA	631	451	83	40	35	50	40
2,3,3,3-Tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy)-propanoic acid	HFPO-DA (GenX)	[1,2-$^{13}C_2$]PFHxA	285	169		20		30	
Perfluoro-4-oxapentanoic acid	PF4OPeA	[1,2,3,4-$^{13}C_2$]PFOA	228.8	85		20		20	
Perfluoro-5-oxahexanoic acid	PF5OHxA	[1,2-$^{13}C_2$]PFHxA	279	85		20		20	
Perfluoro-3,6,dioxaheptanoic acid	3,6-OPFHpA	[1,2-$^{13}C_2$]PFHxA	201	85		25		30	

Table 1 (continued). Full name, abbreviation, MRM transition (precursor and product ion), internal standard (ISTD) used for quantification, cone voltage (V) and collision energy (eV) for the target PFAS and the ISTDs.

Analyte		**ISTD used for quantification**	**Precursor ion (m/z)**	**Product ion (m/z)**		**Collision energy (eV)**		**Cone voltage (V)**	
Full name	**Abbreviation**			**Diagnostic product ion 1**	**Diagnostic product ion 2**	**Diagnostic product ion 1**	**Diagnostic product ion 2**	**Diagnostic product ion 1**	**Diagnostic product ion 2**
Perfluoro(2-ethoxyethane)sulfonate	PFEESA	[1,2-$^{13}C_2$]PFDA	315	135	69	20	55	30	35
2-Perfluorohexyl ethanoic acid	FHEA	[1,2-$^{13}C_2$]PFHxA	376.7	293	312.9	16	16	28	
2-Perfluorooctyl ethanoic acid	FOEA	$^{13}C_4$-PFBA	476.8	392.7	243	14	21	32	30
2-Perfluorodecyl ethanoic acid	FDEA	$^{13}C_4$-PFBA	577	493	513	37	27	17	10
Chloro-perfluorohexane phosphonic acid	Cl-PFHxPA	[1,2,3,4-$^{13}C_2$]PFOA	415	79		35		45	
Perfluorohexane phosphonic acid	PFHxPA	[1,2,3,4-$^{13}C_2$]PFOA	399	79		30		40	
Perfluorooctane phosphonic acid	PFOPA	[1,2,3,4-$^{13}C_2$]PFOA	499	79		30		40	
Perfluorodecane phosphonic acid	PFDPA	[1,2,3,4-$^{13}C_2$]PFOA	599	79		30		40	
	$^{13}C_4$-PFBA		217	172	172	19	50	19	
	[1,2-$^{13}C_2$]PFHxA		315	269	119	21	65	19	
	[1,2,3,4-$^{13}C_2$]PFOA		417	372	172	13	60	22	
	[1,2,3,4,5-$^{13}C_2$]PFNA		468	423	172	17	20	28	

Table 1 (continued). Full name, abbreviation, MRM transition (precursor and product ion), internal standard (ISTD) used for quantification, cone voltage (V) and collision energy (eV) for the target PFAS and the ISTDs.

Analyte		**ISTD used for quantification**	**Precursor ion (m/z)**	**Product ion (m/z)**		**Collision energy (eV)**		**Cone voltage (V)**	
Full name	**Abbreviation**			**Diagnostic product ion 1**	**Diagnostic product ion 2**	**Diagnostic product ion 1**	**Diagnostic product ion 2**	**Diagnostic product ion 1**	**Diagnostic product ion 2**
	[1,2-$^{13}C_2$]PFDA		515	470	220	29	29	25	
	[1,2-$^{13}C_2$]PFUnDA		565	520	170	32	35	18	
	[1,2-$^{13}C_2$]PFDoDA		615	570	320	21	30	22	
	$^{18}O_2$-PFHxS		403	84	103	30	60	22	
	[1,2,3,4-$^{13}C_4$]PFOS		503	80	99	58	58	60	

Table 2. Spike recovery (averages), standard deviation (SD), imprecision (i.e. relative standard deviation, RSD; %), limit of quantification (LOQ; ng/g ww), and limit of detection (LOD; ng/g ww) determined in spiked feathers (N = 5) extracted using MeOH only (as described in section 2.3).

Analyte	Spike recovery (%)	SD	RSD (%)	LOQ (ng/g ww)	LOD (ng/g ww)
PFBA	91.6	3.85	4.20	2.79	0.84
PFPeA	95.7	5.02	5.24	0.53	1.36
PFHxA	97.5	2.47	2.54	4.53	1.36
PFHpA	86.2	7.68	8.91	5.55	1.67
PFOA	97.2	7.34	7.55	0.86	0.26
PFNA	89.7	2.86	3.19	1.42	0.43
PFDA	93.8	2.60	2.77	1.49	0.45
PFUnDA	89.6	5.81	6.49	1.98	0.59
PFDoDA	92.0	3.58	3.89	2.20	0.66
PFBS	81.4	5.71	7.01	7.48	2.24
PFPeS	67.3	6.07	9.02	2.42	0.73
PFHxS	68.8	8.24	12.0	3.93	1.18
PFHpS	93.0	4.38	4.71	8.32	2.50
PFOS	84.4	9.18	10.9	1.48	0.44
4:2 FTS	86.2	16.4	19.1	10.0	3.00
6:2 FTS	78.6	6.29	8.00	6.27	1.88
8:2 FTS	77.1	4.54	5.90	5.37	1.61
NaDONA	93.7	10.9	11.6	0.29	0.09
9CL-PF3ONS	72.8	4.56	6.26	1.67	0.50
11CL-PF3OUdS	96.7	1.06	1.09	1.08	0.32
HFPO-DA	92.0	8.52	9.27	18.0	5.40
PF4OPeA	94.5	13.1	13.9	1.73	0.519
PF5OHxA	107	6.92	6.49	1.99	0.597
3,6-OPFHpA	100	10.7	10.6	2.06	0.62
PFEESA	87.2	12.9	14.8	2.03	0.61
FHEA	95.2	9.95	10.4	12.0	3.60
FOEA	107	11.8	11.0	40.0	12.0
FDEA	90.5	14.4	15.9	51.0	15.3
Cl-PFHxPA	72.6	3.51	4.84	14.0	4.20
PFHxPA	52.3	11.7	22.4	20.0	6.00
PFOPA	108	9.86	9.10	27.0	8.10
PFDPA	112	34.3	30.7	86.0	25.8

Table 3. Spike recovery (averages), standard deviation (SD), imprecision (i.e relative standard deviations, RSD; %), limit of quantification (LOQ; ng/g ww), and limit of detection (LOD; ng/g ww) determined in spiked feathers (N = 5) extracted using MeOH and cleaned-up using WAX-SPE (as described in section 2.4).

Analyte	Spike recovery (%)	SD	RSD (%)	LOQ (ng/g ww)	LOD (ng/g ww)
PFBA	83.7	33.2	39.7	4.26	1.28
PFPeA	37.3	12.1	32.3	2.16	0.65
PFHxA	89.5	38.5	43.1	11.0	3.30
PFHpA	99.5	52.4	52.7	10.0	3.00
PFOA	96.2	42.9	44.6	2.61	0.78
PFNA	93.2	47.0	50.4	1.94	0.58
PFDA	99.0	60.7	61.3	4.66	1.40
PFUnDA	100	41.2	41.1	3.39	1.02
PFDoDA	110	58.4	53.0	11.0	3.30
PFBS	90.0	37.2	41.4	11.0	3.30
PFPeS	53.3	25.9	48.5	6.11	1.83
PFHxS	74.9	41.9	56.0	23.0	6.90
PFHpS	124	49.5	50.0	16.0	4.80
PFOS	61.9	39.6	64.0	1.93	0.58
4:2 FTS	27.7	11.3	41.0	23.0	6.90
6:2 FTS	54.8	22.8	41.5	11.0	3.30
8:2 FTS	62.9	5.94	9.44	18.0	5.40
NaDONA	22.5	14.0	62.3	0.73	0.22
9CL-PF3ONS	68.5	5.24	7.66	48.0	14.4
11CL-PF3OUdS	64.9	7.94	12.3	3.86	1.16
HFPO-DA	72.7	30.6	42.2	4.10	1.23
PF4OPeA	102	48.9	48.0	4.75	1.43
PF5OHxA	61.3	17.4	28.3	7.41	2.22
3,6-OPFHpA	74.0	15.5	20.9	6.46	1.94
PFEESA	65.0	35.0	53.8	4.69	1.41
FHEA	58.4	16.2	27.8	48.0	14.4
FOEA	81.4	43.0	52.8	133	39.9
FDEA	34.4	10.4	30.3	157	47.1
Cl-PFHxPA	73.4	32.8	44.7	12.0	3.60
PFHxPA	42.9	9.67	22.5	33.0	9.90
PFOPA	118	25.7	21.8	43.0	12.9
PFDPA	114	45.3	39.6	327	98.1

Figures

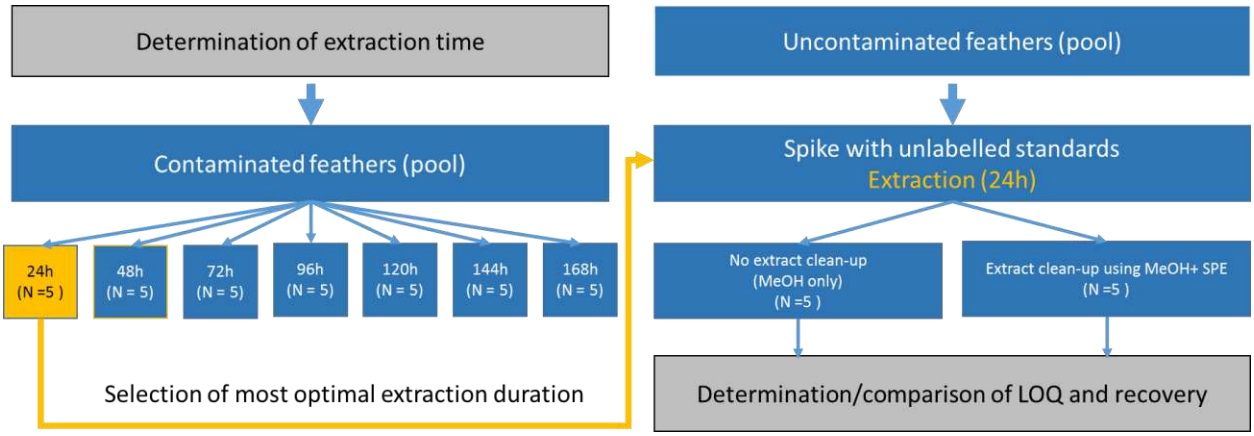

Figure 1. Flow chart showing the steps taken in order to determine the method selectivity, precision, limit of quantification (LOQ), limit of detection (LOD) and recovery. Firstly, the most optimal extraction duration was selected based on a test on subsamples from a pool of PFAS contaminated feathers. Hereafter, subsamples from a pool of presumably uncontaminated feathers were spiked with native (unlabeled) standards, extracted for the most optimal extraction duration. Extracts were either cleaned-up using XAW SPE cartridges, or not cleaned-up at all (MeOH only). In these samples, the LOQ and spike recovery were determined and compared between the different methods.

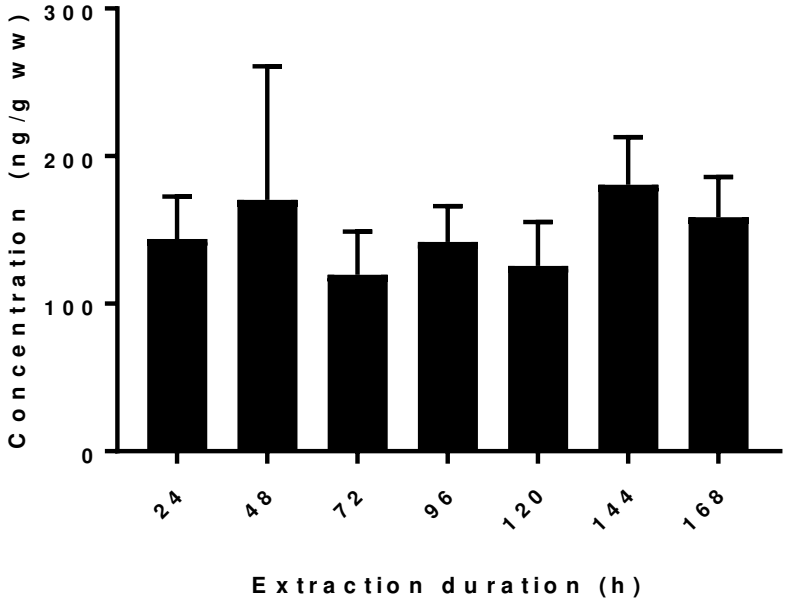

Figure 2. ΣPFAS concentration in the contaminated feathers used to investigate the time (24, 48, 72, 96, 120, 144 and 168h; N = 5 per time period) needed to extract the majority of PFAS from feathers. This was investigated by making a pool of contaminated feathers and dividing this pool into seven groups, representing the different extraction durations. No significant differences were observed among the different extraction periods ($F_{6,28}$ = 1.35, p = 0.271).

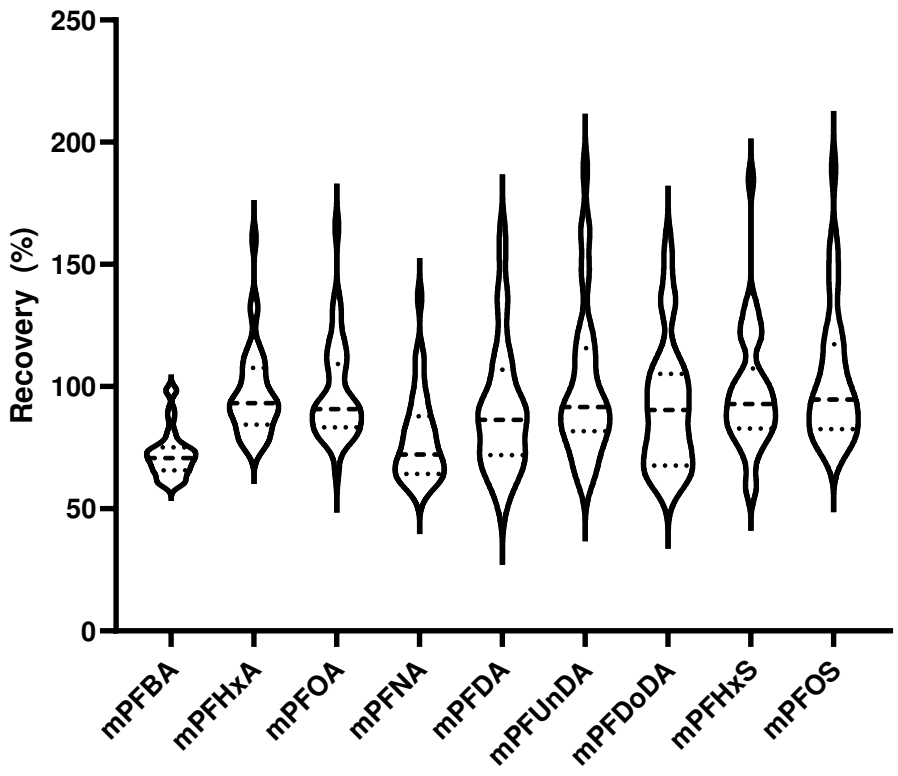

Figure 3. Extraction recovery (%) of the isotopically-labeled ISTDs (mPFAS) that were determined in extracts of contaminated feathers (N = 35) based on the Area of these ISTDs ($Area_{ix}$) in the feather extracts and the $Area_{ix}$ of a non-extracted ISTD solution.