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Andreia Alves[ab*], Griet Jacobs[a], Guido Vanermen[a], Adrian Covaci[b], Stefan Voorspoels[a]

[a]Flemish Institute for Technological Research (VITO NV), Boeretang 200, 2400 Mol, Belgium

[b]Toxicological Centre, Department of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium

*corresponding author: Andreia Alves

Tel.+32 14 33 50 41, Fax.+ 32 14 31 94 72

E-mail address: andreia.alves@vito.be

Abstract

In the recent years hair has been increasingly used as alternative matrix in human biomonitoring (HBM) of environmental pollutants. Sampling advantages and time integration of exposure assessment seems the most attractive features of hair matrix. In the current study, a novel miniaturized method was developed and validated for measuring fifteen perfluoroalkyl substances (PFAS), including perfluoro *n*-butanoic acid (PFBA), perfluoro *n*-pentanoic acid (PFPeA), perfluoro *n*-hexanoic acid (PFHxA), perfluoro *n*-heptanoic acid (PFHpA), perfluor *n*-octanoic acid (PFOA), perfluoro *n*-nonanoic acid (PFNA), perfluoro tetradecanoic acid (PFTeDA), perfluorobutane sulfonic acid (PFBS), perfluoro pentane sulfonic acid (PFPeS), perfluorohexane sulfonic acid (PFHxS), perfluoroheptane sulfonic acid (PFHpS), perfluorooctane sulfonic acid (PFOS), perfluorononane sulfonic acid (PFNS), perfluorodecane sulfonic acid (PFDS) and perfluorododecane sulfonic acid (PFDoS) in human hair by liquid chromatography tandem mass spectrometry (LC-MS/MS). After extraction using ethyl acetate, dispersive ENVI-Carb was used for clean-up. Good intra and inter-day precision for low (LQ 5 ng/g hair) and high spike (HQ 15 ng/g) levels were achieved (in general < 10 %). The accuracy was assessed using recoveries (%), which ranged between 68-118 % (LQ) and 70-121 % (HQ). The instrumental limit of detection (LOD_i) and limit of quantification (LOQ_i) were between 1-4 pg/g hair and 3-13 pg/g hair, respectively. The method limit of quantification (LOQ_m) ranged between 6 and 301 pg/g hair. The PFAS levels were measured in thirty human hair samples indicating that the levels are low (14 to 1534 pg/g hair). Some PFAS were not present in any hair sample (e.g. PFHpA, PFTeDA, PFPeS, PFHpS and PFNS), while most were frequently detected (PFBA, PFHxA, PFOA, PFNA, PFBS, PFHxS, PFOS, PFDS and PFDoS). Although levels in general were low, there is evidence of higher human exposure to some analytes, such as PFBA, PFPeA, PFHxA, PFOA, PFBS, PFHxS, and PFDoS. The current study shows that hair is a suitable alternative non-invasive matrix for exposure assessment of PFAS.

Keyworks: Perfluoroalkyl substances, human biomoritoring, hair, LC-ESI-MS/MS

1. Introduction

Health authorities around the world try to monitor and control the presence of the most toxic environmental pollutants in environment, wildlife and humans, aiming at reducing their exposure. Among such chemicals are the perfluoroalkyl substances (PFAS), which are included in the list of harmful compounds of the Stockholm Convention since 2009 [1]. These substances are used in diverse everyday consumer and chemical products, such as e.g. cookware, foams, paints, cosmetics, paper, leather and textile coatings, pesticides, electronic devices, adhesives [2–4], consequently leading to a constant and unavoidable human exposure to these chemicals.

Due to their high persistency and bioaccumulation potential [2], human biomonitoring (HBM) tools of PFAS have been widely explored during the last decade, but mainly for invasive samples such as (cord) blood, serum and breast milk [5–10]. Blood perfuses several tissues and organs, so this matrix can be used as an indicator for human exposure to PFAS. However collecting blood holds practical and ethical constraints, especially for children or vulnerable populations [11]. Sampling breast milk and/or cord blood has the same implications as for blood or serum, in addition to the limited exposure assessment (i.e., only mothers who are breastfeeding and who have a newborn child are able to participate).

Meanwhile, the exploitation of alternative non-invasive matrices such as hair brought new insights in HBM of PFAS [12–14] likewise was done in the past for measuring other environmental pollutants [15,16], alcohol, drugs of abuse and their metabolites in hair [17–19]. The main advantages of using hair as matrix in HBM are mostly related to the non-invasive sampling, which is easy, fast and not painful. Moreover collection is possible for both children/babies and elderly and/or sick people. Also, storage is easy, usually done at room temperature (if the target compounds are not volatile) and for long time period as the matrix stability is higher than for liquid matrices [20]. Further, hair can mirror both the short to long-term exposure (months to years depending on the hair length), which is considered the major advantage in human exposure assessment.

The main challenge of HBM of PFAS in human hair is the availability of a sufficiently sensitive analytical technique combined with suitable extraction methods. In past studies PFAS extraction from hair was performed by accelerated solvent extraction (ASE) or ultrasound extraction using different organic solvents (e.g. acetonitrile, methanol and acid/basic digestion). Then solid-phase extraction (SPE) where weak anion-exchange sorbent combined with a reverse phase (Oasis WAX) is highly suggested as clean-up for strong acidic compounds [12–14]. Instrumental measurement is commonly done using liquid chromatography tandem mass spectrometry (LC-MS/MS) which seems to be a common and more direct analytical approach for both invasive

[7,21–23] and non-invasive matrices [12–14]. Although gas chromatography (GC) can be used, there are limitations for PFAS measurement due to their low volatility (especially for the long-chain compounds), leading to a need for derivatization prior to analysis [24]. Capillary electrophoresis (CE) methods could be another choice for detecting polar and ionized PFAS, however the most important drawback is the poor detection sensitivity of this analytical technique, which is mainly due to the low UV/Vis absorption of PFAS [23].

The aim of this study is to overcome pending drawbacks on current extraction methods for HBM of PFAS in human hair [12–14]. The main challenges comprise the development of a sensitive and accurate analytical method for measuring fifteen short to long-chain PFAS in hair at pg/g levels by LC-MS/MS. An alternative clean-up method (dispersive ENVI-Carb) is proposed here as easier, faster and more environmental friendly than the conventional SPE methods presented in the literature. In addition, other factors such as hair type and matrix effect influence in the PFAS detection was evaluated for the first time. The assessment of the human exposure to other perfluoroalkyl sulfonic acids than PFOS (until now the most studied), including the perfluoroalkyl pentane sulfonate (PFPeS), perfluoroalkyl heptane sulfonate (PFHpS), perfluoroalkyl nonane sulfonate (PFNS) and perfluoroalkyl dodecane sulfonates (PFDoS) is another innovative aspect of this research. Finally, advantages and drawbacks associated to the hair analysis for HBM of PFAS in general population is discussed.

2. Materials and Methods

2.1 Chemicals & Equipment

Fifteen PFAS (> 98 % purity) including perfluoro *n*-butanoic acid (PFBA), perfluoro *n*-pentanoic acid (PFPeA), perfluoro *n*-hexanoic acid (PFHxA), perfluoro *n*-heptanoic acid (PFHpA), perfluor *n*-octanoic acid (PFOA), perfluoro *n*-nonanoic acid (PFNA), perfluoro tetradecanoic acid (PFTeDA), perfluorobutane sulfonic acid (PFBS), perfluoro pentane sulfonic acid (PFPeS), perfluorohexane sulfonic acid (PFHxS), perfluoroheptane sulfonic acid (PFHpS), perfluorooctane sulfonic acid (PFOS), perfluorononane sulfonic acid (PFNS), perfluorodecane sulfonic acid (PFDS) and perfluorododecane sulfonic acid (PFDoS) and mass-labelled internal standards (IS) were purchased from Wellington Laboratories (Guelph, Ontario, Canada). The mixtures of PFAS and mass-labelled (^{13}C and ^{18}O) internal standard solutions were prepared in methanol (UPLC grade, Biosolve, Netherlands) from individual stock solutions. Internal standard calibration curves for all analytes considered the concentration and chromatographic area of the respective internal

standard (IS), however the PFAS without a corresponding labelled IS were corrected with the antecedent alkyl chain IS (Table 1).

The ultra-pure water was produced using a Millipore Advantage A10 system (Millipore S.A., Overijse, Belgium. Pure reagents ammonium acetate (NH_4AC) and ammonium hydroxide (NH_4OH) were purchased from Sigma-Aldrich (Diegem, Belgium and Steinheim, Germany, respectively). UPLC grade organic solvents: acetonitrile, methanol and ethyl acetate (Fisher Scientific, Loughborough, UK), 2-propanol (99.8 %), formic acid (98-100 %) and tetrahydrofuran (TFH) (99.9 %) were supplied by Merck (Darmstadt, Germany). The Envi-Carb 120/400 mesh was obtained from Supelco (Bellefonte, USA) and the Oasis WAX cartridges (150 mg, 3cc) were supplied by Waters (Massachusetts, USA).

All glass material was washed and baked out in the oven at 450 °C overnight. It was then stored wrapped in aluminum foil to avoid contact with air and dust particles.

2.2 Sample collection and decontamination

Hair samples (n = 30) were collected during 2013 from a general (non-exposed) population. The sampling campaign was approved by the Ethical Committee of the University of Antwerp (reg. B300201316329). The volunteers were duly informed about the purpose of this study giving their consent to participate. In this study, no personal or lifestyle information was collected through questionnaires.

All hair samples were firstly rinsed with ultra-pure water and acetone. Consecutively the samples were dried at room temperature and then cut into small pieces with stainless steel scissors as described in previous studies [12,14]. The samples were stored in aluminium foil at room temperature since the PFAS are stable compounds and non-volatile.

2.3 Method description

2.3.1 Extraction solvent test followed by dispersive ENVI-Carb clean-up

The samples were weighted (100 mg) in the extraction vials and spiked with 1.5 ng of IS and 1.5 ng of PFAS. Then, 2 mL of each extraction solvent, i.e., ethyl acetate (EA) or 2-propanol or 50/50 % (*v/v*) of THF/2-propanol were added to hair samples. The extraction was performed in an ultrasonic bath (15 min, room temperature) followed by a clean-up step with dispersive ENVI-Carb (~ 30 mg). After extraction, the extracts were transferred and dispersed by the sorbent added in the conical vials. Afterwards, the samples were centrifuged (6 min, 3500 rpm) for complete

organic phase separation. The supernatant was collected in decontaminated glass tubes and evaporated at 37 °C until dryness under a gentle nitrogen stream. The residue was reconstituted in 50 µL of MeOH:H_2O (50:50; *v/v*) and 10 µL was injected in LC-ESI-MS/MS system.

2.3.2 Ethyl acetate extraction followed by OASIS WAX clean-up

The same extraction procedure as described above using ethyl acetate as extraction solvent was applied to hair samples before clean-up by Oasis WAX. The clean-up process was based on the methods described in the literature but with few modifications [12,13]: Firstly the stationary phase was conditioned with 2 mL of 2 % NH_4OH in MeOH, 2 mL of MeOH and 2 mL of water under vacuum. The samples were loaded onto the cartridges, washed with 2 mL of formic acid/H_2O solution (2 %; *v/v*) and 2 mL of formic acid/MeOH (50:50; *v/v*). The analytes were eluted by passing through the cartridges 2 mL of 2 % NH_4OH in MeOH. The extracts were collected in clean vials, evaporated until dryness and reconstituted in 50 µL of MeOH:H_2O (50:50; *v/v*).

2.3.3 Hair extraction for preparing pool sample (matrix matched calibration standards)

The optimized extraction procedure (followed by dispersive ENVI-Carb) was applied for a proportional amount of decontaminated hair (and volume of ethyl acetate) necessary to prepare eight matrix matched calibration standards in MeOH/H2O (50:50; *v/v*). Thus, 245 µL of aqueous hair pool extract was added to the same volume of PFAS in methanol and 10 µL of IS stock solution. The 8-point matrix matched calibration curves for PFAS ranged from 0.02 to 25 ng/g. Only one sample collected from the same individual (dark brown mixed with gray hair) was used for preparing the standards and to perform the method validation.

2.4 LC-ESI(-)MS/MS analysis

The chromatographic conditions were optimized for PFAS in the LC coupled with a tandem mass spectrometer and interfaced with an electrospray ionisation source in negative-ion mode (LC-(-)ESI-MS/MS) from Waters (UPLC system coupled to a XEVO Triple Quad tandem mass spectrometer). The chromatographic separation was performed in a UPLC BEH Shield RP_{18} (1.7 µm, 2.1 x 100 mm, Waters Acquity) column heated at 40 °C. A gradient elution program was performed using Water: MeOH (95:5; *v/v*) in 2 mM NH_4AC (solvent A) and MeOH in 2 mM

NH_4AC (solvent B). The flow was set in 0.3 mL/min and the injection volume was 10 µL. The total run time was 25 min.

The cone voltage ranged between 14 and 65 V for native compounds and between 14 and 59 V for the IS. The collision cell energy ranged between 8 and 50 eV and the dwell time varied between 75 and 175 ms. Two multiple reaction monitoring (MRM) transitions were monitored for each compound (MRM 1 for identification and MRM 2 for quantification), except for PFBA and PFPeA (only one transition). The main monitored product ions for the perfluorocarboxylic acids (PFCA) resulted from the loss of $[M\text{-}COOH]^-$ (-44 a.m.u.) and the loss of $[C_2F_5]$ (-119 a.m.u.) or loss of $[C_3F_7]^-$ (-169 a.m.u.) or loss of $[C_4F_9]^-$ (-219 a.m.u.) or loss of $[C_5F_{11}]$ (-269 a.m.u) or loss of $[C_6F_{13}]$ (-319 a.m.u.) from the precursor ion $[M\text{-}H]^-$ depending on the alkyl chain length; for the perfluorosulfonic acids (PFSA) the loss of $[SO3]^-$ (-80 a.m.u.) and $[FSO3]^-$ (-99 a.m.u.) from the precursor ion $[M]^-$ are the most common detected transitions. The Table 1 shows the list of studied compounds, MRM1 and MRM2 transitions, retention times and internal standards.

2.5 Method validation

The method precision was evaluated by the intra- (repeatability; n = 6) and inter-day (intermediate precision; n = 6, 3 consecutive days) variation for two spike levels (low spike level (LQ) of 5 ng/g hair and high level (HQ) of 15 ng/g hair. The relative standard deviations within and between days (% RSD_{within} and % $RSD_{between}$) were determined. Since there is no certified reference material available for PFAS analysis in hair, the method accuracy was evaluated based on the recoveries (%) calculated for both LQ and HQ spike levels in hair. The uncertainty was expressed as the expanded uncertainty U for both spike levels using a coverage factor k = 2, corresponding to a confidence level of 95 %.

The quality control (QC) was ensured by analyzing PFAS levels in procedural blanks that were spiked with 5 ng/g IS (3 blanks extracted per day and injected in parallel with hair extracts) and system performance was monitored through analysis of a quality control standard (analysis of the variation (RSD %) of the middle concentration in matrix matched calibration curve) that was injected every ten samples.

The instrumental limit of detection (LOD_i) and instrumental limit of quantification (LOQ_i) were determined based on 3 times signal-to-noise ratio and 10 times signal-to-noise ratio of the lowest standard in the calibration curve (0.02 ng/g), respectively. For PFAS quantification in hair samples (n = 30), the method limit of quantification (LOQ_m) was defined 2 times the standard deviation

(SD) of the blanks. For those compounds which were not detected in blanks, LOQ was defined as LOQ_i and the levels in hair were reported based on the LOQ_i.

3. Results and Discussion

3.1 Method Development

3.1.1 Optimization of extraction-selection of organic solvent

The extraction of PFAS from hair samples was optimized by testing different solvents based on boiling point (bp) and polarity index (pi): ethyl acetate (bp 77 °C, pi 4.4), 2-propanol (bp 82 °C, pi 3.9) and the mixture of 50/50 % (*v/v*) of tetrahydrofuran (THF) (bp 65 °C, pi 4.0) and 2-propanol. The detection of PFAS in hair is very challenging due to matrix effects and to their amphiphilic properties, e.g. hydrophobic fluorine chain (tail) and hydrophilic functional group (head) [25]. Additionally, short to long chain PFAS have different polarities, i.e., short-chain PFAS are more polar and water soluble than long-chain PFAS [25].

In this work, organic solvents with polarities ranging from 3.9 to 4.4 were tested and differences in the extraction efficiency (% recoveries) of PFAS (Table 2) were assessed. The results suggested that EA is the best extraction solvent (% R from 69 to 141 %). Because not all PFAS had the same extraction yield, it was important to find a good compromise between the method extraction efficiency and the number and/or the levels of PFAS that could be detected in hair. Thus, EA was selected as extraction solvent with higher extraction recoveries among the PFAS (98 %) and with extraction efficiency of more than 50 % of analyzed analytes with good accuracy and reproducible levels. Still, EA has the lowest boiling point (77 °C). Therefore, solvents with low boiling point are preferable to minimize losses of PFAS during evaporation [26].

3.1.2 Clean-up

Clean-up is an essential step to remove interferences present in the extracts, which may cause matrix effects (ion suppression/enhancement) during LC-MS/MS analysis. Therefore, dispersive ENVI-Carb clean-up was compared with SPE Oasis WAX, previously reported by Li et al. as efficient to remove interferences from hair [12,13]. In this study, the performance of each clean-up was evaluated after extraction with EA by analyzing the recoveries (%).

Oasis WAX is composed by reversed-phase/weak anion-exchange polymer and it is mainly used to retain and release strong acids. Although, there is evidence of good clean-up performance of Oasis WAX for the detection of short-chain PFAS in environmental [26] and biological samples [12,13], the same does not happen for the long-chain PFAS (C > 6), where a non-polar stationary phase is more suitable (e.g. C_{18} or OASIS HLB) [21,27].

In addition, ENVI-Carb is a nonporous fine graphitized carbon that has a high affinity for organic compounds from both polar and non-polar matrices. It is commercially available as packed (SPE cartridges) and in powder (dispersive). In this study, dispersive ENVI-Carb instead of packed was preferable used mainly to avoid contamination from the PTFE frits present in the SPE cartridges. As a result, this technique can be considered more environmentally friendly than the conventional SPE, since no washing/elution solvents are required for the elution step and therefore no organic solvent is wasted. Moreover, this clean-up is easy and fast. After dispersion of the extract containing the PFAS, the samples are centrifuged for a complete and clean separation between sorbent (at bottom) and organic layer (upper phase). The centrifugation promotes also the precipitation of residues or other non-soluble material (e.g. hair, lipids). This clean-up is highly convenient for the removal of different types of non-perfluorinated compounds which have any aromaticity (π electrons) and therefore are suitable for interaction with the hexagonal ring structure (π-π interactions) of the ENVI-Carb [28,29].

Previous studies had reported the high efficiency of dispersive ENVI-Carb for the detection of PFAS in environmental matrices such as dust, soil, sediments, sludge [28,30] or in biological matrices like blood, serum, plasma or milk [4,31]. However, there are no reports for applications in non-invasive samples such as hair.

The results show that OASIS WAX had better recoveries for the short-chain than for the long-chain PFAS (poor recoveries of 58, 56 and 35 % were assessed for PFNS, PFDS and PFDoS, respectively). Dispersive ENVI-Carb resulted in superior clean-up which recoveries were > 87 % for the studied PFAS (Table 3). Therefore, this clean-up was selected for the further experiments.

3.1.3 Extraction and dispersive ENVI-Carb performance

The sensitivity of PFAS measurement in LC-MS/MS can be affected by inadequate extraction/clean-up and/or due to interferences present in hair (matrix effects), which may change the MS response. Firstly, both extraction and clean-up steps were studied for assessing losses of PFAS during method development, therefore the spiking was done at different moments throughout the procedure: before extraction, before clean-up and finally after clean-up. The

experiments were done in triplicate and the results are shown in Figure 1. The best recoveries were reported after clean-up (recoveries ranged between 74 % and 152 %). Still, significant matrix effects were observed for PFHpA and PFTeDA (% R > 120 %). In general, clean-up helps to remove some compounds that interfere with PFAS detection, except for PFBA where the clean-up seems not be beneficial and some losses can occur during the procedure (ratio before extraction and after clean-up is > 1).

Secondly, the matrix effect, ion suppression or enhancement, was also assessed for all compounds. The same extraction and clean-up procedures were applied to the hair samples where the spike of IS and PFAS (1.5 ng) were only added at the end prior to injection in LC. Thus, matrix effect was assessed based on the ratio between the response of extracted samples containing the analytes and the response of a standard injected in the LC at same concentration [32,33]. These experiments showed that ion enhancement was predominant for PFHxA, PFHpA, PFNA, PFHpS, PFOS, PFNS, PFDoS, while PFBA, PFPeA, PFOA, PFTeDA, PFBS, PFPeS, PFHxS and PFDS were subjected to ion suppression.

3.1.4 Influence of hair type

This study helps to assess if a specific type of hair has a higher influence on PFAS retention or release during extraction. Thus, the experiments were conducted for six different types of human hair (brown, dark brown mixed with gray, black, blond, ginger (dyed) and gray).

From our knowledge, there are some factors like melanin content (not measured here), which usually play an important role in drug's binding affinities to the hair structure and not neglecting of course, the chemical properties of the target compounds [20,34–36]. For instance, brown and black hairs have more eumelanin than blond or red hair which is more rich in pheomelanin [37]. Still, higher total melanin content (eumelanin and pheomelanin) was found in human black hair which contains approximately 99 % of eumelanin and 1 % pheomelanin [38]. At the same time, brown and blond hair seems to have identical proportion of eumelanin (95 %) and pheomelanin (5 %). However, Ito et al. [39] concluded that eumelanin content is dependent on the intensity of color, i.e., black hair has more eumelanin than light brown or even blond hair (ranged between 22.2 and 4.7 µg/mg hair).

Hair is mainly composed of 65-95 % proteins including keratin, 15-35 % water, 1-9 % lipids, residual amounts of minerals and polysaccharides [20,40]. The use of hair in HBM studies is truly complex due to several intrinsic factors (i.e. type of hair, color, amount of melanin and keratin), external factors like dietary or smoking habits [15] and mechanistic/kinetic factors related to the

incorporation of chemicals (e.g. passive/active diffusion, pH, lipophilicity, external sources) which play an important role for explaining the incorporation route and the levels of the chemicals detected in human hair [16,18,41]. Previous studies for incorporation of drugs and their metabolites (e.g. cocaine and morphine) in this matrix suggested that higher binding effect occurs in pigmented hair [42], while *in vitro* studies confirm that basic drugs bind more easily to melanin structure [43,44] than the acidic [18]. Nevertheless, studies to assess the total melanin content or either differentiation methods for eumelanin and/or pheomelanin for these samples were not yet conducted.

If hair as an active role on drug's or environmental pollutants incorporation, the pharmacological properties like the basicity/acidity and lipophilic properties [18] of these substances are also relevant for the incorporation process through the blood. Besides, some studies revealed that PFAS bind to serum proteins [45,46], these substances are not basic (high pKa) and are not lipophilic, like other persistent organic pollutants (POPs) (e.g. organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs) or polybrominated diphenyl ethers (PBDEs)) [47,48] for which their detection in hair is easier. In this sense, polar compounds (or metabolites) enter to the hair to a lesser extent than their lipophilic precursors. Since PFAS are not metabolized into less polar compounds, their incorporation in hair seems more difficult to occur either through external (e.g. air, dust) exposure and further deposition in hair surface or either by internal exposure (food, drinking water) through circulation in the blood stream. Their special amphiphilic properties hamper the study of the incorporation mechanisms in hair [49]. Also in hair, there are multiple sites (or body compartments like skin tissues that surround the hair follicle) where drugs can in, via diverse mechanisms (i.e., diffusion from blood to the actively growing follicle, from secretions of the apocrine and sebaceous glands (sweat and sebum) or from the external environment) and various times during the hair growth cycle [50] which can determine how and in what extend chemicals interact with this matrix. In the case of drugs of abuse including amphetamines, cocaine or alcohol, the transfer via sweat and sebum (sebaceous and apocrine gland secretions) may be important vehicles for their incorporation in hair [50]. Nevertheless, for PFAS, there are no studies reporting possible diffusion pathways/mechanisms to the hair. The use of hair as non-invasive matrix in HBM of environmental pollutants is considered a new approach, so more investigation about the binding mechanisms into hair is needed.

The results showed that the extraction efficiency of PFAS from different types of hair samples (brown, dark brown mixed with gray, black, blond, ginger (dyed) and gray) ranged from 56 to 146 % (Figure 2). Accordingly, there is in general a similar profile of extraction between samples ($r = 0.057$ by ANOVA analysis for 95 % confidence level), i.e., the extraction of PFAS is not

dependent of type of hair. However, blond hair had the highest variability and black and black mixed with gray hair contributed for the lowest variance among the studied PFAS. Therefore, these results can leading us to two hypotheses: 1) the binding process of PFAS to the hair is not regulated by melanin content (i.e. assuming that there are different melanin content per type of hair as already described in the literature); 2) if the matrix was not completely destroyed during extraction, hindering thus the analyte' release from the hair shaft, only a certain amount of PFAS deposited onto the hair surface was measured. It is important to highlight that solvent extraction without acid or basic digestion is the most common technique to measure PFAS in hair. The use of strong extraction conditions might promote problems in MS response, once other components (non-volatile) are released [13].

3.1.5 Assessment of inter-individual hair variation

Further investigation of extraction efficiency of PFAS from similar type of hair, i.e. different brown hair samples (collected from distinct individuals) was conducted in triplicate to assess whether the matrix effect for brown hair (the major type of collected hair from the population) is higher than the other tested hair types and if there are even significant differences between individuals with same type of hair (Figure 3).

The PFAS extraction recoveries were evaluated in brown hair for six individuals, evidencing high variability among the recoveries for PFNS, PFDS and PFDoS. This was true, especially for hair samples 2, 3 and 6 (RSD % between 8.8 and 13 % for PFNS, 12 and 24 % for PFDS and 27 and 53 % for PFDoS) and lower recoveries (especially for PFDoS in brown hair 2 where recovery was < 10 %). However, after performing an ANOVA test for 95 % confidence interval ($p < 0.05$), significant differences ($r = 0.0093$) between these six hair samples were found. Sample 2 contributed for the highest variance and sample 4 for the lowest variance among the studied PFAS in brown hair.

Most often, the amount and availability to collect hair from the same individual is limited which oblige to mix different types of samples in a pool sample, increasing indubitably the uncertainty and reproducibility level of the results depending on the analytes. Nevertheless, whenever possible, it is advised to either develop and validate the analytical method using the same sample or at least a pool of homogenized samples from same type of hair (e.g. brown) which helps to reduce or exclude variability factors inherent to the hair matrix (e.g. gender, age, type/color, anatomical region, growth rate, ethnicity) [41]. Other authors, also suggest that when the desirable amount of head hair is not available, the collection of hair from other parts of the body (e.g. pubic,

underarm and beard) is still possible, although more difficult to perform considering important aspects like the personal privacy and the collection integrity [51].
The aim of both experiments (using different and similar types of hair) was to evaluate the PFAS extraction by the inter- and intra-dependency of type of hair for further decision on which hair type to use in method validation.

3.2 Method Validation

3.2.1 Precision (intra-day and inter-day) and accuracy

The method precision (% RSD) of the method was evaluated by the intra (repeatability; n = 6) and inter-day (intermediate precision; n = 6, 3 consecutive days) variation for two spiking levels (low spike level of 5 ng/g hair and high level of 15 ng/g hair). The % RSD_{within} and % $RSD_{between}$ for intra-day and inter-day, respectively were assessed by ANOVA analysis (Table 4). The intra-day precision ranged between 3.2 and 19 % and the inter-day precision ranged between 0.8 and 6.9 % for the two spiking levels. The uncertainty was below 26 % (HQ, 15 ng/g) and 40 % (LQ, 5ng/g).
The accuracy was assessed by the % recoveries of PFAS in hair and was determined for the two spiking levels (5 and 15 ng/g). For the lowest spike level recoveries ranged between 68 % and 114 % and for the highest level ranged between 70 % and 121 % (Table 4).

3.2.2 Linearity, limits of method quantification (LOQ_m), instrumental limit of detection (LOD_i) and quantification (LOQ_i)

Eight concentration levels of PFAS standards were used to perform the matrix-matched calibration curves (0.02-25 ng/g). The calibration curve showed a linear fit with a correlation (r^2) above 0.99 (Table 4).
The instrumental limits of quantification (LOQ_i) and limits of detection (LOD_i) were calculated based on signal-to-noise ratio (10 × S/N and 3 × S/N, respectively) of the lowest matrix-matched calibration standard. The compounds which quantification was not possible in hair samples (non-detects) have their values reported as below LOQ_m or LOQ_i (for PFAS which were detected in procedure blank procedure, i.e., PFPeS, PFHpS, PFDs and PFDoS). The LOQ_m ranged between 6 and 301 pg/g, the LOQ_i ranged between 3 and 13 pg/g and the LOD_i ranged between 1 and 4 pg/g (Table 4).

The matrix effect was further evaluated comparing the slopes of both matrix-matched and solvent standard calibrations [52]. Differences between the slopes for matrix matched and solvent based calibration differed up to 63 %, which confirms the need for using matrix matched calibration. Thus, all deviations for PFAS detection in hair are corrected by the matrix-matched calibration.

3.2.3 Quality Control (QC)

The QC was verified by the injection of matrix-matched standard every ten samples where the variation (%) between injections was far below than 5 %. Also, the levels of PFAS in procedural blanks (spiked with 5 ng/g of IS) were determined for examination of external contamination during sample preparation and/or instrumental analysis. Some PFAS were not detected in procedure blanks, i.e., PFPeS, PFHpS, PFDS and PFDoS. However, other PFAS were detected in blanks, but the levels were till up 2 times the LOQ_m values.

3.3 Applicability to hair samples

In total, PFAS levels were determined in 30 hair samples (blank corrected) collected from different individuals and the levels varied between 14 and 1534 pg/g (above LOQm) (Table 5). The variability in the detection frequency shows that in general more perfluorosulfonates than perfluorocarboxylic acids were detected in hair (17 % against 15 %, respectively for the overall studied samples). The most detected PFAS (>LOD_i) were the PFBA, PFPeA, PFHxA, PFOA, PFBS, PFHxS, PFOS and PFDoS (> 30 %), whilst PFHpA, PFTeDA, PFPeS, PFHpS and PFNS were not detected in any sample. However many of the detected compounds were not quantified, as the levels present in hair were below the LOQ_m (e.g. for PFNA and PFOS) for this population. On contrary, PFBA, PFHxA, PFOA, PFBS, PFHxS and PFDoS were quantified in more than 60 % of the samples. More, PFBA, PFPeA, PFHxA, PFOA, PFBS, and PFDoS are detected and quantified at the same % rate (Table 5), showing that is possible to measure PFAS in human hair (Figure 4). Still, in this study average values of PFAS in hair are not high, ranging between 46 and 214 pg/g.

The present method allows a higher method sensitivity and lower quantification levels (pg/g hair) compared to previously published methods. Also, less variability of detected PFAS among the studied individuals was reported, where levels in hair are shown in the same order of magnitude or

up to 10 times higher for the same compound, except for PFBA in which the minimum and the maximum levels varied from 1 to 100 times of magnitude difference (pg/g).

Although the levels reported for human hair in the present study are not high compared with previous HBM studies, we clearly demonstrated that more than half of the analyzed PFAS (8 PFAS in total of 15) can be measured in hair (Table 5). Previous studies have reported the presence of up 8 PFAS in hair [12–14].

Moreover, this study shows that particularly the long-chain PFAS (eg. PFHxS or PFDoS were quantified in more than 60 % of the studied population) can be monitored in hair, which is not possible in urine samples, in which they are not easily detected due to the low excretion rate [53]. Here, the proposed method demonstrates that both short and long-chain PFAS are efficiently extracted from hair.

In the literature, PFAS can be detected in human hair [12–14], although at extremely high variable concentrations (from < LOQm to approximately 46 ng/g level, depending on the studied compound). For instance, samples collected in the general population in China [12,13] revealed that most of the PFAS were not detected at high levels or with a high detection frequency when compared to serum or urine. Only PFOS and PFOA showed representatively detection frequency (near or equal to 100 %) within the studied population (levels in hair ranged between < LOQ and 6.7 ng/g). Also, in a study by Li et al. [13] it was highlighted that hair, when compared with other non-invasive samples like nails, does not reflect so significantly the human exposure to overall PFAS, although reported in another study [12] that for both matrices high correlation for PFOS and PFOA with serum ($r = 0.545$ and $r = 0.786$) were observed.

On the other hand, Perez et al. [14] analyzed a more exposed population in Barcelona, where 8 PFAS were studied in 24 hair samples, nevertheless only 20 % of overall detects (levels ranged between 0.4 to 45.9 ng/g) were reported. Again, PFOA and PFOS were the most detected PFAS, although present in only 8 and 11 hair samples, respectively.

On the contrary, the least detected was PFDA (1 sample) and PFBS (2 samples), which the highest level of this study was reported for PFDA (45.9 ng/g), therefore not representing at all the human exposure to these two analytes over hair analysis.

These studies show that human exposure to PFAS can be assessed through hair, albeit at variable levels. Therefore, more studies should be conducted for different general (non-exposed) and exposed (e.g. ski wax technicians, people working and/or living in highly industrialized areas) sub-populations. An evaluation of those sub-populations could help to identify more significantly and in what extend is the importance of measuring PFAS in hair.

4. Concluding remarks

In this study a new, faster and more environmental friendly extraction method to measure PFAS in small amount of hair (100 mg) was developed using ethyl acetate followed by dispersive ENVI-Carb as clean-up. The high sensitivity of the method resulted in high detection frequency and low levels of quantification of PFAS in hair, although reported in a non-exposed population where the levels were lower (averages from 46 to 214 pg/g) than usually reported for serum, blood or urine samples. Still, several of the analytes, i.e., six PFAS can be quantified in more than half of the samples (PFBA, PFHxA, PFOA, PFBS, PFHxS, PFDoS) indicating that humans are exposed to these substances. This also indicates that hair is a suitable alternative matrix among the non-invasive approaches for exposure assessment, for example, in babies, children or vulnerable people.

Yet, this is the first study where some emerging perfluorosulfonates, i.e. PFHpS, PFDS and especially PFDoS were studied in human hair, which there is a special evidence of human exposure in general population to PFDoS.

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Figure and Tables Caption

Figure 1. Isolation of extraction and clean-up steps for the evaluation of the PFAS extraction from hair.

Figure 2. Extraction recoveries of PFAS for six types of human hair.

Figure 3. Extraction efficiency (%R) for six brown hair samples.

Figure 4 Chromatograms of PFAS detected and quantified at same (%) rate in human hair.

Table 1. List of PFAS and IS, MRM transitions and retention time.

Table 2. Extraction recoveries (%) of PFAS from hair samples.

Table 3. Clean-up method comparison between dispersive ENVI-Carb and Oasis WAX.

Table 4. Intra-day and inter-day precision (% RSD) for hair samples (n=6).

Table 5. PFAS levels in human hair (n=30).

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