Analytical strategies to confirm the presence of emerging PFASs in blood samples without analytical standards

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1 Introduction

Since the 1950s, per- and polyfluoroalkyl substances (PFASs) have been extensively used in industrial and commercial applications due to their attractive properties, such as their thermal and chemical stabilities and their amphiphilic nature[1]. Due to their inherent stability and widespread use, these compounds are found and prevail in all environmental matrices. However, the presence of these compounds in the environment, water, and food is of concern as toxicological studies have demonstrated that PFASs may be related to several health issues such as thyroid disorders or cancers[2]. Nevertheless, only a few of these substances are regulated, such as perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), which have been included in the annexes of the Stockholm convention. In response to growing concerns about PFOS and PFOA, major manufacturers voluntarily discontinued production of these and related substances, in the early 2000s[1]. As a result, alternative PFASs have emerged and have been introduced to the market. Chloro-perfluoropolyether carboxylates (Cl-PFPECA) are one of the classes of emerging compounds[3-6]. They have been detected in New-Jersey soil samples downwind of two PFAS manufacturing facilities (Solvay and DuPont/Chemours)[3], and in surface water and groundwater surrounding them[4]. Both facilities have been participating in the PFOA Stewardship program[3] and a mixture of Cl-PFPECA oligomers, also called congeners in the related literature (ClC_3F_6O-$[CF(CF_3)O]_e$-$[CF_2CF(CF_3)O]_p$-CF_2H-COO, e = 0-2, p = 0-4) was implemented as processing aid by Solvay[5] and reported in a product evaluation by the European Food Safety Authority (EFSA), at their request[3,6], (CAS No. 329238-24-6). The objective of our study was to determine the presence of Cl-PFPECA congeners in the discharge water of the Solvay facility located in Spinetta Marengo, Italy, and in the blood of some inhabitants of this region. Five congeners have already been identified in water sample from the Bormida River, downstream of this Solvay facility[3,7]. At the time we performed this study during summer 2022, as in the articles mentioned[3,4], no Cl-PFPECA analytical standard was available. The major challenge of this work was therefore to conclusively identify the congeners with sufficient confidence. The target confidence level was at least 3 (i.e., tentative candidate structure or class of structures), as defined by conventional HRMS identification confidence context[8,9]. To this end, liquid chromatography coupled with high-resolution mass spectrometry was used for the analyses of water and blood samples and the results were supported by comparison with the literature data. This work is one of the first report published to assess the presence of Cl-PFPECA in human blood of non-occupational worker from the plant, except for a few retired former workers [10].

2 Materials and Methods

As a reference, a mixture of native standards (PFAC-MXC) was purchased from Wellington Laboratories, Inc (Ontario, Canada), and consisted of C_4-C_{14}, C_{16}, and C_{18} perfluoroalkyl carboxylic acids (PFCAs), and C_4-C_{10} and C_{12} perfluoroalkane sulfonic acids (PFSAs). The water sample analyzed in this study was collected directly from the discharge channel of the Solvay facility at Spinetta Marengo, Alessandria, Italy in March 2022, and diluted with groundwater. The study was performed on a cohort of 30 volunteers, living in Spinetta Marengo, close to the Solvay plant, some of them being former employees of this factory. Blood samples were collected in 10mL clot activator tubes (BD Vacutainer^{MD}) in Italy. Four water samples were prepared: the discharge water sample, a blank of unspiked Milli-Q water, another blank of unspiked tap water, and a reference sample with spiked tap water. Tap water was used for spiking, as it was expected to provide similar matrix effects as the wastewater. The water preparation and extraction protocol were adapted from the literature[11]. For the four water samples, 500 mL were firstly filtered through a Büchner with a high-porosity paper filter (4-7 µm, grade MN 640md, Macherey-Nagel, Düren, Germany), followed by a 0.3 µm-porosity glass microfiber filter (Euro-scientific, Lint, Belgium). For the spiked tap water, 25 µL of the 2000ng/mL PFAC-MXC solution were added, after the filtration step. The filtered water was loaded onto an Oasis WAX SPE cartridge (6 cc, 150 mg, 30 µm), previously conditioned with successively 4 ml of 0.2% ammonium hydroxide in methanol, 4 mL of methanol and 4 mL of water. The column was then washed with 4 mL of 20 mM ammonium acetate, followed by 4 mL of methanol. The compounds were

eluted with 4ml of 0.2% ammonium hydroxide in methanol. The eluate was filtered with a 0.2 µm nylon syringe filter (Fischer Scientific, Hampton, NH, USA) and evaporated to dryness at 30 °C under a gentle flow of nitrogen. The residue was finally reconstituted in 250 µL of a 95/5 (v/v) methanol/water mixture and transferred to an injection vial. For the blood samples, in addition to the 30 blood samples from Italy, unspiked and spiked control sera were prepared and analyzed. The protocol used for the preparation of blood samples was based on a paper by the toxicology laboratory of the University of Liege[12], with some adaptations. First, sera were obtained after centrifugation of coagulated blood samples. Two milliliters of 5% formic acid in water were added to 1 ml of serum. The acidified sera were sonicated for 15 minutes and centrifugated at 5000 rpm for 10 minutes. The supernatant was loaded onto an Oasis WAX SPE cartridge (3 cc, 60 mg, 30 µm) previously conditioned with 2 ml of methanol and 2 mL of water. The cartridge was then washed with 1 mL of 2% formic acid in water, followed by 1.5 mL of methanol. Analytes were eluted with 2 × 2 mL of ammonium hydroxide 2% in methanol. The same steps as for the water samples were applied to the eluate except that it was reconstituted in 80 µL of a 95/5 (v/v) methanol/water mixture. Unspiked and spiked control sera (8 µL of PFAC-MXC in 1 mL) were also analyzed. No internal standard was added to the samples, the main objective being to confirm or deny the presence of Cl-PFPECA congeners. Chromatographic separation was performed on an Acquity I-Class UPLC system (Waters, Milford, MA, USA) using a Acquity BEH C_{18} column heated to 45 °C (2.1 × 150 mm × 1.7 µm particles) (Waters, Milford, MA, USA). Chromatographic separation was conducted on an injected volume of 5 µL for the water samples, and 10 µL for the blood samples. The flow rate was 0.2 mL/min with a binary mobile phase gradient of solvent A (water + 0.1% formic acid) and solvent B (acetonitrile). The gradient was adapted from the literature[13] and started at 20% B and increased linearly from 20% to 40% (0-0.5 min); remained constant for 1 min (0.5-1.5 min); increased linearly from 40% to 100% for 10 min (1.5-11.5 min); remained constant at 100% until 19.5 min; decreased from 100% to 20% (19.5-20 min) and was kept constant at 20% during 9 min to recondition the column for the next analysis. After each sample injection, 1 µL of a blank mixture of water/MeOH (95/5 V.Vv) was injected to prevent carryover. The LC was coupled to a Q-Exactive Orbitrap high-resolution mass analyzer (Thermo Fisher Scientific, Waltham, M, USA) equipped with an electrospray source operating in the negative mode. The MS/MS analyses were performed using the data-dependent acquisition mode and scan settings were based on the literature[4]. For data processing, Xcalibur (Thermo Fisher Scientific, Waltham, M, USA) and Skyline[14] software were used. Data were analyzed manually, and structures were tentatively identified based on MS/MS data and comparison with literature data[3,4,16].

3 Results

Some homologs of the legacy PFCAs (C_4-C_{11}) and PFSAs (C_4-C_6, C_8) were identified in the discharge water at the same retention times as in the tap water spiked with the PFAC-MXC mixture containing these analytes. For each PFCA homolog, $[M-H]^-$ and $[M-H-COO]^-$ ions were coeluting and the mass accuracy on both ions was less than 3 ppm. In addition, MS/MS spectra obtained from PFCA $[M-H]^-$ and $[M-H-COO]^-$ ions displayed peaks compatible with $[CF_3[CF_2]_x]^-$ ions within a mass accuracy range of 5 ppm. Five Cl-PFPECA congeners (Figure 1) were tentatively identified in the discharge water sample, whereas they were not detected in the tap water and milli-Q water blanks.

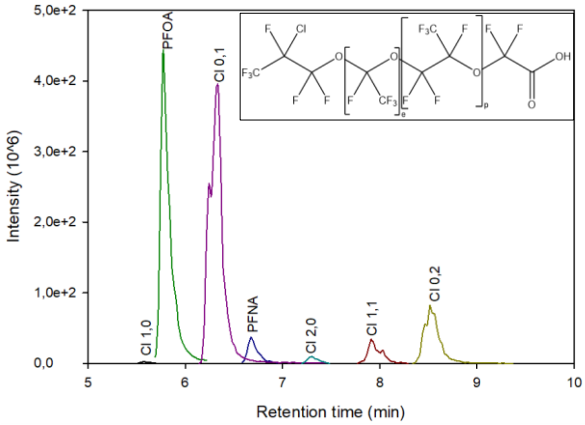

Figure 1: Chromatogram of the Solvay plant discharge water sample representing the probable presence of 5 Cl-PFPECA congeners (identified as Cl e,p; where e and p are the numbers of fluoroether ethyl and propyl units, respectively.) Also represented is a general structure of Cl-PFPECAs.

The identified congeners corresponded to: e,p = 1,0; e,p = 0,1; e,p = 2,0; e,p = 1,1; and e,p = 0,2; and were eluted in the same retention time range as C_8 and C_9 PFCAs (PFOA and PFNA). As for PFCAs, for each Cl-PFPECA

congener, another ion was found to co-elute with the $[M-H]^-$ ion. This ion could correspond to the loss of one CF_2CO_2 unit from the $[M-H]^-$ ion and was the predominantly observed ion. The mass accuracy on both $[M-H]^-$ and $[M-H-CF_2CO_2]^-$ ions was below 2 ppm for each congener. The natural abundance of chlorine was verified by evaluating the M+2/M isotopic ratio of the $[M-H-CF_2CO_2]^-$ ion (i.e., most intense ion) for each congener. For all five congeners, this ratio was compatible with the expected value of 32%.

Moreover, an ion with *m/z* equal to 200.9547, within a mass accuracy range of 5 ppm, was found at the same retention time as all five congeners (Figure 2). This mass-to-charge ratio is consistent with a compositional formula of $[ClC_3F_6O]^-$, which could correspond to the $[M-H-CF_2CO_2]^-$ fragment of Cl-PFPECA e,p = 0,0. It is therefore coherent that this ion is a common ion between all congeners. Figure 2 also illustrates the chromatographic trace of the M+2 isotope due to the presence of the chlorine atom on this common ion. Finally, the MS/MS spectra of the $[M-H]^-$ and $[M-H-CF_2CO_2]^-$ ions of the e,p = 0,1 and e,p = 0,2 congeners were recorded in DDA mode, as well as the MS/MS spectrum of the $[M-H-CF_2CO_2]^-$ ion of the e,p = 1,1 congener. The MS/MS spectra of the deprotonated e,p = 0,1 and e,p = 0,2 ions displayed one feature consistent with the loss of the CF_2CO_2 unit and another with the common $[ClC_3F_6O]^-$ fragment within a mass accuracy range of 5 ppm. This common fragment was also detected in the MS/MS spectra of the $[M-H-CF_2CO_2]^-$ ions of these two congeners and of the e,p = 1,1 congener. Finally, the MS/MS spectra of the two ions of congener e,p = 0,2 displayed a peak coherent with the $[M-H-CF_2CO_2]^-$ ion that lost a fluoroether propyl unit ($CF_2CF(CF_3)O$).

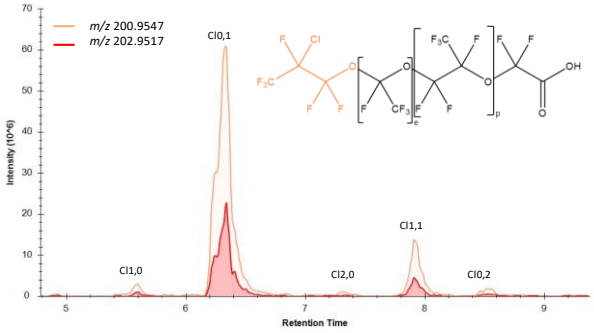

Figure 2: Chromatogram of the Solvay plant discharge water sample representing the traces of the ^{35}Cl (*m/z* 200.9547) and ^{37}Cl ions (*m/z* 202.9517) of the common Cl-PFPECA ion ($[ClC_3F_6O]^-$, outlined in orange on the general schematic Cl-PFPECA structure).

Legacy PFCAs (C_4 and C_{7-10}) and PFSAs (C_{4-6} and C_8) were detected in the analyzed blood samples, with a mass accuracy of 2 ppm on the deprotonated ions of PFCAs and PFSAs and on the $[M-H-COO]^-$ ions of PFCAs. MS/MS spectra of the most abundant homologs were recorded in DDA and compatible with the identified PFCAs and PFSAs. A slight retention shift was observed between the blood samples and the discharge water sample for these compounds. Therefore, based on the retention times observed for Cl-PFPECA congeners in the discharge water sample, their retention times in the blood sample analyses could be predicted. The coeluting $[M-H]^-$ and $[M-H-CF_2CO_2]^-$ ions of congener e,p = 0,1 were detected in all 30 blood samples, within a mass accuracy range of 2 ppm. These ions were detected at the same retention time in all blood samples tested and their retention time were consistent with the ones observed in the discharge water sample. For the other four congeners identified in the discharge water, depending on their intensities, the $[M-H]^-$ ion could not be detected in the blood samples, but the more abundant $[M-H-CF_2CO_2]^-$ ion could be identified at retention time consistent with the discharge water sample. Furthermore, the expected 32% value of the ^{37}Cl and ^{35}Cl isotope ratio of this ion was observed for these four congeners in the samples with the highest Cl-PFPECA signal intensities. In addition, MS/MS spectra were acquired for all five congeners in these blood samples and the peaks identified were consistent with the fragments of these compounds in the 5-ppm mass accuracy range. With respect to these considerations, the e,p = 0,1 congener was identified in all the 30 blood samples, while e,p = 0,2; e,p = 1,0; e,p = 1,1 and e,p = 2,0 congeners were identified in 20, 6, 27 and 27 samples, respectively. No Cl-PFPECA congeners were identified in the control serum samples.

4 Discussion

The presence of the legacy PFOA and PFNA in the discharge water sample was used as a reference for the retention times. Indeed, the relative retention times of Cl-PFPECA congeners to PFOA and PFNA were available in the literature[3]. Using a mobile phase gradient that went linearly from 20/80 ACN/H_2O with 0.1% formic acid to 90/10 ACN/H_2O with 0.1% formic acid, the observed elution order was as follows: Cl 1,0 < PFOA < Cl 0,1 < PFNA < Cl 2,0 < Cl 1,1 < Cl 0,2[3]. This elution order is the same as the one observed in Figure 1, increasing the confidence

in the identification of these congeners in the discharge water sample. Furthermore, the five congeners identified in the discharge water are the same as those identified in the Bormida River[3,7], downstream of the Solvay plant of Spinetta Marengo, where the analyzed discharge water was collected. Furthermore, the relative intensities of the five congeners are similar in the discharge water sample and the river water sample[3]. Therefore, even though no analytical standard were available for the Cl-PFPECA congeners, consistency with literature data increases the confidence level in their identification, in addition to verification of the isotopic pattern due to the chlorine atom and identification of several distinctive fragments in the MS spectra (in-source fragmentation) and DDA MS/MS spectra. In the literature, the $[M\text{-}H\text{-}CF_2CO_2]^-$ has also been reported as the major observed ion[3,4,16] and the common $[ClC_3F_6O]^-$ fragment is observed in the MS/MS spectra of each congener[3]. In conclusion, confidence level 3 (i.e. tentative structure[8,9]) in the identification of the 5 congeners of Cl-PFPECA in the discharge water sample is reached, and the decisional tree used to achieve this confidence level is represented in Figure 3. However, the exact structure of the congeners cannot be determined as some Cl-PFPECA elute as split peaks, which may reflect the presence of structural isomers. For instance, the chlorine atom could be on the ultimate or penultimate carbon atom, isomerization within the fluoroether propyl unit(s), and group regioisomerism between the ethyl and propyl groups could be likely[3,4]. For the blood samples, confidence level 3 is also reached for the identification of the same five congeners as in the discharge water sample. Indeed, although the deprotonated ions could not be identified for all five congeners in each sample, the detection of the $[M\text{-}H\text{-}CF_2CO_2]^-$ ions at consistent retention times between the blood and the water samples was sufficient for identification. The latter was confirmed by verifying the isotopic pattern due to the chlorine atom on the $[M\text{-}H\text{-}CF_2CO_2]^-$ ions. Moreover, the same distinctive ions as in the discharge water sample could be identified in the MS (probably in-source fragmentation) and DDA MS/MS spectra of the most contaminated samples.

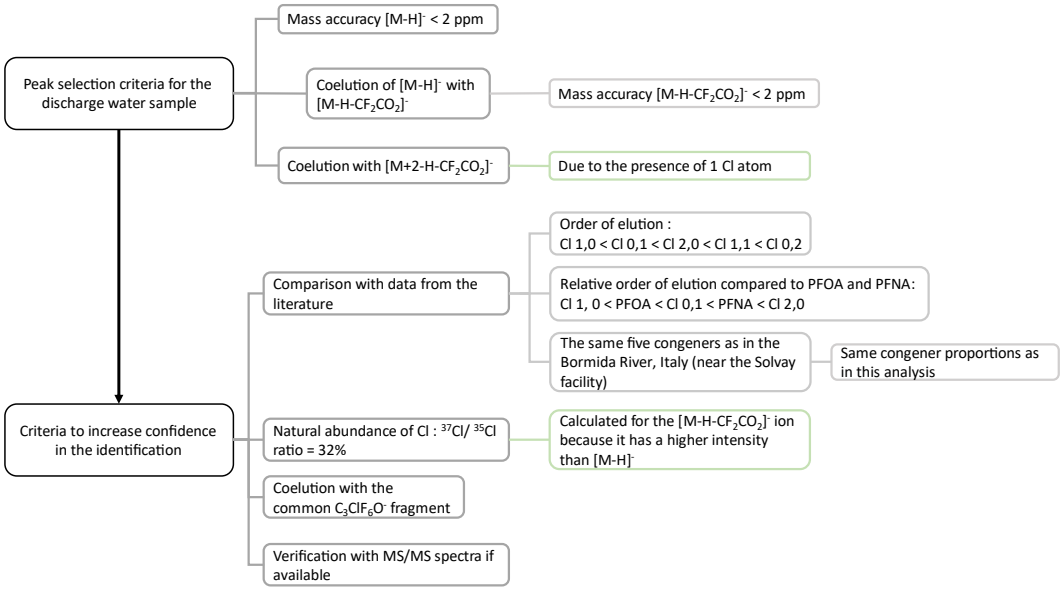

Figure 3: Decisional tree summarizing the selection and verification criteria that were used to assess the presence of the Cl-PFPECA congeners in the discharge water sample.

5 Conclusions

The focus of this study was to identify emerging Cl-PFPECA congeners in a sample of wastewater from the Solvay plant in Spinetta Marengo, Italy, and in blood samples from residents of the area near this facility. Despite the unavailability of their analytical standards, five Cl-PFPECA congeners could be identified within the third confidence level as described in the conventional HRMS identification confidence context. This could be achieved by performing UPLC-HRMS/MS analyses in data-dependent acquisition mode. Congener identification was supported by the detection of distinctive ions in the MS and MS/MS spectra and by the observation of the isotopic pattern due to the presence of a chlorine atom. The identification was also supported by comparison with data available in the literature (i.e., elution order relative to legacy PFCAs) of the identification of Cl-PFPECA in soil and water samples. Nevertheless, the exact structure of the congener could not be accurately determined, and the split chromatographic peaks could indicate the presence of several isomers. Coupling an ion mobility spectrometry method with LC-MS could increase the separation power and assist in the separation of potential isomers of Cl-PFPECA congeners. In addition, degradation products (e.g. hydrohalogenated H-PFPECA[4,16]) of identified Cl-PFPECA congeners could be sought in the blood samples to monitor their degradation pathways.

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