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Abstract

Persistent and mobile chemicals (PMs) and per- and polyfluoroalkyl substances (PFAS) are groups of chemicals that have received recent global attention due to their potential health effects on the environment and humans. In this study, exposure to a broad range of PMs and PFAS was investigated in Flemish adolescents' urine samples (*n* = 83) using a suspect screening approach. For this purpose, three sample preparation methods were evaluated, and a basic liquid-liquid extraction was optimised for urine analysis based on the extraction efficiency of PMs (53–80%) and PFAS (> 70%). In total, 9 PMs were identified in urine samples at confidence levels (CL) 1–3 and, among them, acetaminophen, 4-aminophenol, 2,2,6,6-tetramethyl-4-piperidone, trifluoroacetic acid (TFAA), sulisobenzone, ethyl sulfate, and 1,2-benzisothiazol-3(2H)-one 1,1-dioxide were confirmed at CL 1 and 2. In addition, the detection and identification of 2,2,6,6-tetramethyl-4-piperidone, 4-aminophenol, TFAA, and m-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl) aniline (CL 3), has been reported for the first time in human urine in this study. For PFAS, only 2 compounds were identified at CL 4, implying that urine is not a suitable matrix for suspect screening of such compounds. A significant difference between sexes was observed in the detection rate of identified PMs, in particular for acetaminophen, 4-aminophenol, and sulisobenzone. The findings of this study can be used in future human biomonitoring programs, such as by including the newly identified compounds in quantitative methods or monitoring in other human matrices (e.g., serum).

Keywords

high-resolution mass spectrometry; persistent and mobile chemicals; per- and polyfluoroalkyl substances; Flemish Environment and Health Studies; urine; adolescents

1. Introduction

Over the past few decades, rapid industrial growth and improvements in living standards have led to the production of several new chemicals. Among the numerous chemicals marketed today, chemicals with two intrinsic properties, persistence and mobility, have recently been paid attention to and the need for their regulation and management has been identified (UBA, 2019). They are defined as persistent and mobile organic compounds (PMs) (Knepper et al., 2020; Montes et al., 2019; Reemtsma et al., 2016; Rüdel et al., 2020). PMs are not a specific class of compounds, but any substance characterized by persistency, mobility, high aqueous solubility, and polarity can be defined as PM. Recently, the European Chemicals Agency (ECHA) has paid attention to the management of PMs and, in 2019, the German Environment Agency (UBA) made a substantial effort to propose criteria for classifying PMs. These include the mobility criterion based on the organic carbon partition coefficient (K_{oc}), the octanol-water partition coefficient (K_{ow}) or the pH-dependent K_{ow}, and the degradation half-life under specific conditions. A tentative list of compounds that fulfil these criteria includes chemicals such as perfluorobutane sulfonic acid (PFBS) and trifluoroacetic acid (TFAA), tris[2-chloro-1-(chloromethyl)ethyl] phosphate, ibuprofen, and propazine (UBA, 2019).

Due to the specific combination of such intrinsic properties, PMs are less prone to adsorption onto soil and sediments, making them poorly removable by sorption processes in the environment and during water treatment (Jin et al., 2020; Knepper et al., 2020; Reemtsma et al., 2016; Rüdel et al., 2020). Thus, PMs are potentially recirculated within the water cycle and can end up in drinking water, posing a potential threat to the environment and human health. The toxicity of PMs was evaluated under the UBA assessment and they defined PMs that fulfil the REACH criteria for toxicity, including carcinogenic, reproductive toxicity and specific target organ toxicity as PMT (persistent, mobile and toxic chemicals) (UBA, 2019). However, potential toxicity has been reported even for PMs not classified as PMT. For example, cyanuric acid is one of the PMs, but not classified as PMT and some studies reported its adverse effect on the kidney (Suchý et al., 2009; Wang et al., 2012) and bladder (WHO, 2004). This implied that it is needed to keep paying attention to the occurrence and human exposure to PM compounds.

Despite the possibility of their presence in the environment and adverse effect on humans, research on PMs has been rarely carried out compared to other substances including persistent, bioaccumulative, and toxic chemicals due to the lack of specific, sensitive, and reliable analytical methods. So far, only a few studies have been conducted and focused on the development of analytical methods and monitoring of water samples. Most of them used solid-phase extraction (SPE) with different sorbents for the suspect screening of PMs in water samples and reported the detection of

some PMs, such as trifluoromethanesulfonic acid, acesulfame, saccharin, and tris(2-butoxyethyl) phosphate (Boulard et al., 2018; Montes et al., 2019, 2017; Schulze et al., 2019). Research on human exposure to PMs remains however still very limited.

Per- and polyfluoroalkyl substances (PFAS) are a group of synthetic chemicals that have been widely used in various industrial and consumer products (e.g., surfactants, mist suppressants, aqueous firefighting foams, cosmetics, and non-stick cookware) since the 1940s (Lindstrom et al., 2002; Paul et al., 2008). Over the past years, concerns about the presence of PFAS in the environment have increased due to their strong persistence, bioaccumulation potential, and toxicity, resulting in the inclusion of perfluorooctanoic acid (PFOS) and perfluorooctanoic acid (PFOA), and their salts, in the list of persistent organic pollutants (POPs) (UNEP, 2019, 2009). As a response to these restrictions, industries have attempted to produce less bioaccumulative and more degradable PFAS, such as perfluorobutanoic acid (PFBA), PFBS, and fluoroether alternatives. However, recent studies have reported that these substitutes are still recalcitrant and can have comparable toxicity to PFOS and PFOA (Gaballah et al., 2020; Gordon, 2011; Wang et al., 2013). In addition, fluorine mass balance studies indicated that routinely monitored PFAS accounts for only a small percentage of extractable organofluorine in human blood (Yeung et al., 2008), implying that the current human exposure to PFAS is probably underestimated. These findings highlight the need for a broad screening of PFAS in human matrices. So far, human exposure to PFAS has been assessed using various matrices, such as serum, hair, or urine, however, those studies analysed a limited number (typically 25 to 40) of PFAS with targeted analytical methods (Calafat et al., 2019; Yi Wang et al., 2018; Worley et al., 2017; Zhang et al., 2015).

Suspect screening by liquid chromatography and high-resolution mass spectrometry (HRMS) allows to obtain structural information on substances present in the sample through matching of exact masses and MS/MS spectra and to create an extensive dataset so that numerous substances in environmental and biological samples can be identified (Menger et al., 2020; Cortéjade et al., 2016; Pourchet et al., 2020; Caballero-Casero et al., 2021). In a suspect screening approach, it is crucial to use appropriate sample preparation methods that can minimize the interferences potentially derived from the matrix, while keeping the loss of suspects to a minimum and obtaining data for a large number of substances (Guo et al., 2020). Urine is a suitable matrix for human exposure to environmental chemicals because it reflects the internal chemical exposure, and it is easy and cheap to collect, transfer, and store (Esteban and Castaño, 2009; Smolders et al., 2009).

This study aimed at a comprehensive suspect screening of PMs and PFAS in urine samples from Flemish adolescents obtained within the 4th cycle of the Flemish Environment and Health Study (FLEHS IV,

2016–2020). To accomplish this goal, a sample preparation method was optimized and two suspect lists encompassing more than 200 PMs and 8,700 PFAS were used to screen urine samples by LC-HRMS. In addition, the detection rate of the identified compounds was compared between sexes and between two exposure load categories (high and low) calculated based on earlier quantitative analysis of 45 chemicals from FLEHS IV (Buekers et al., 2021). To the best of our knowledge, this is the first application of suspect screening in human urine using suspect lists containing a large number of PMs and PFAS advancing thus our knowledge on human exposure to these compounds.

2. Methods and materials

2.1 Sample collection

The Flemish adolescents' spot urine samples investigated in this study were selected from the biobanked samples (*n* = 428, 14–15 years old adolescents; kept at -20°C) collected between September 2017 and June 2018 in the frame of the FLEHS IV reference biomonitoring study (2016–2020; approved by the Ethical Committee of the University Hospital of Antwerp, Belgium; Belgian Registry Number: B300201732753). The subset of samples (*n* = 83) were selected on the exposure load (high and low) calculated based on earlier quantitative analysis of 45 environmental chemicals (Buekers et al., 2021; Schoeters et al., 2022), such as phthalates and alternative plasticizers (Bastiaensen et al., 2021a), organophosphorus flame retardants (OPFRs) and plasticizers (Bastiaensen et al., 2021b), bisphenol analogues (Gys et al., 2021), and polycyclic aromatic hydrocarbon metabolites (Verheyen et al., 2021). Detailed information on exposure load calculation method is described in Roggeman et al. (2022; submitted). Final selection of urine samples of this study consisted of 44 and 39 participants from high and low exposure load groups, respectively, with inclusion of 17 female and 27 male in the high exposure load group and 19 females and 20 males in the low exposure load group.

2.2 Sample preparation

Information on chemicals and reagents used in this study is presented in the Supporting Information (SI). In this study, different sample preparation methods such as solid-phase extraction (SPE; method 1), dilute-and-shoot (method 2), and liquid-liquid extraction (LLE; method 3) were tested to investigate the most suitable method for analyzing PMs and PFAS in urine. Detailed sample preparation protocols for each method are provided in the SI. Based on the comparison of extraction efficiency and the number of detected PMs and PFAS (described in section 3.1), the LLE method with the addition of ammonia was selected for the suspect screening of PMs and PFAS in urine samples.

An aliquot of 500 µL urine was transferred to a 15 mL polypropylene tube and spiked with internal

standards (ISTDs) of PMs (25 ng) and PFAS (3 ng). Detailed information on the used ISTDs of PMs and PFAS is presented in **Table S1 and S2**, respectively. After vortexing for 30 s, 100 µL of 5% ammonia in water (v/v) and 2 mL of ethyl acetate/isopropanol (95:5, v/v) were added. Urine samples were extracted by vortexing for 5 min and centrifugation at 4000 rpm for 10 min. The supernatant was transferred into a clean 15 mL tube. The extraction step was repeated twice, and the combined supernatant was concentrated to near dryness under a gentle nitrogen stream. After adding 125 µL of a mixture of methanol (MeOH) and water (1:1, v/v), samples were filtered with 0.2 µm centrifugal nylon filters prior to instrumental analysis.

2.3 Instrumental analysis

An Agilent 1290 Infinity ultra-performance liquid chromatography (UHPLC) coupled with Agilent 6530 quadrupole time-of-flight mass spectrometry (QTOF-MS; Agilent Technologies) was applied.

For PMs, chromatographic separation was achieved by an InfinityLab Poroshell 120 EC-C18 column (100 mm x 4.6 mm; 2.7 µm) fitted with a guard column (5 mm x 2.1 mm; 1.8 µm). The mobile phases were (A) 0.1% formic acid in H_2O (v/v) and (B) 0.1% formic acid in MeOH (v/v) with a flow rate of 0.4 mL/min for both ESI+ and ESI-. The LC gradient started from 5% B (2 min) and changed to 95% B (in 20 min), maintained for 5 min at 95% B, and then changed to 5% B (25.1 min). The total run time was 30 min. The QTOF-MS was operated with ESI positive and negative modes at a 2 GHz extended dynamic range mode (1700 *m/z*). The source parameters for PM analysis were as following: gas (nitrogen) temperature 325 °C, gas flow 10 mL/min, nebulizer pressure 45 psi, sheath gas temperature 350 °C, sheath gas flow 12 L/min, fragmentor voltage 120 V, capillary voltage 3500 V, and nozzle voltage 500 V. The acquisition mode applied was data-dependent acquision (DDA; Auto MS/MS) in which the precursor ions were automatically selected by the software based on the observed abundances. The acquired mass range was *m*/*z* 50–1200. Three different collision energies were used in the collision-induced dissociations (CID) at 10, 20, and 40 eV. The generated data were stored in centroid mode before being exported for further data analysis.

Chromatographic separation of PFAS was conducted on a Zorbax Eclipse Plus RRHD C18 column (2.1 × 100 mm, 1.8 µm) connected with a guard column (Eclipse Plus C18, 2.1 × 5 mm, 1.8 µm). The gradient program was employed using (A) 2 mM ammonium acetate in water and (B) MeOH with a flow rate of 0.25 mL/min. The gradient of LC started from 10% B, increased to 90% B in 8 min, maintained for 5 min (13 min), changed to 100% B at 17 min, held for 8 min (25 min) and then changed to 10% B at 26 min (total run time: 35 min). Data acquisition was accomplished using ESI negative data-dependent acquisition (DDA; Auto MS/MS) mode operated at a 2 GHz extended dynamic range mode (1700 *m/z*) and collision energies were set to 10 and 30 eV. The source parameters for PFAS analysis were as

following: gas (nitrogen) temperature 300 °C, gas flow 8 mL/min, nebulizer pressure 40 psi, sheath gas temperature 350 °C, sheath gas flow 12 L/min, fragmentor voltage 350 V, capillary voltage 3500 V, and nozzle voltage 1000 V. Mass range for MS and MS/MS were 50–1200 *m/z* and 40–1000 *m/z*, respectively with acquisition rate 4 spectra/s. Isolation width setting was narrow (~1.3 *m/z*).

During the analytical run of PMs and PFAS, real-time calibration was performed by monitoring reference mass ions (*m/z* 121.0508 and 922.0098 for ESI+ and 119.036 and 966.0007 for ESI-) for PMs and 119.0363 and 980.0163 for PFAS analysis (ESI-).

2.4 Quality assurance and quality control

Procedural blanks prepared with ultrapure water was included (n = 8) in every sample batch (n = 10) to examine the contamination during the sample preparation. During LC-QTOF-MS analysis, the injection needle was washed with MeOH prior to each injection for 30 s. Every 5 to 10 injections, a MeOH solvent blank was injected to check the carryover. All procedural and solvent blanks did not show any PMs and PFAS which were identified at confidence level (CL) 4 or above. A standard mixture containing known amounts of native and ISTDs of PMs and PFAS in MeOH was injected every 15 samples to monitor the instrumental stability (e.g., retention time (RT), peak abundances, and mass accuracy). The ISTDs of PMs and PFAS fortified to urine samples were identified in all samples with consistent retention times, and an isotopic pattern match of over 90 was selected as a cut-off using the Find by Formula algorithm in Agilent MassHunter Qualitative Analysis software (versions 7.0 and 10.0). The recoveries of ISTDs were calculated by comparing peak abundances between analyzed urine samples and ISTD standard mixture, which ranged from 12% to 71% for PMs (average: 42%), and from 41% ($^{13}C_2$-perfluorotetradecanoic acid; PFTeDA) to 106% ($^{13}C_9$-perfluorononanoic acid; PFNA), with an average value of 87% for PFAS.

2.5 Suspect screening workflow

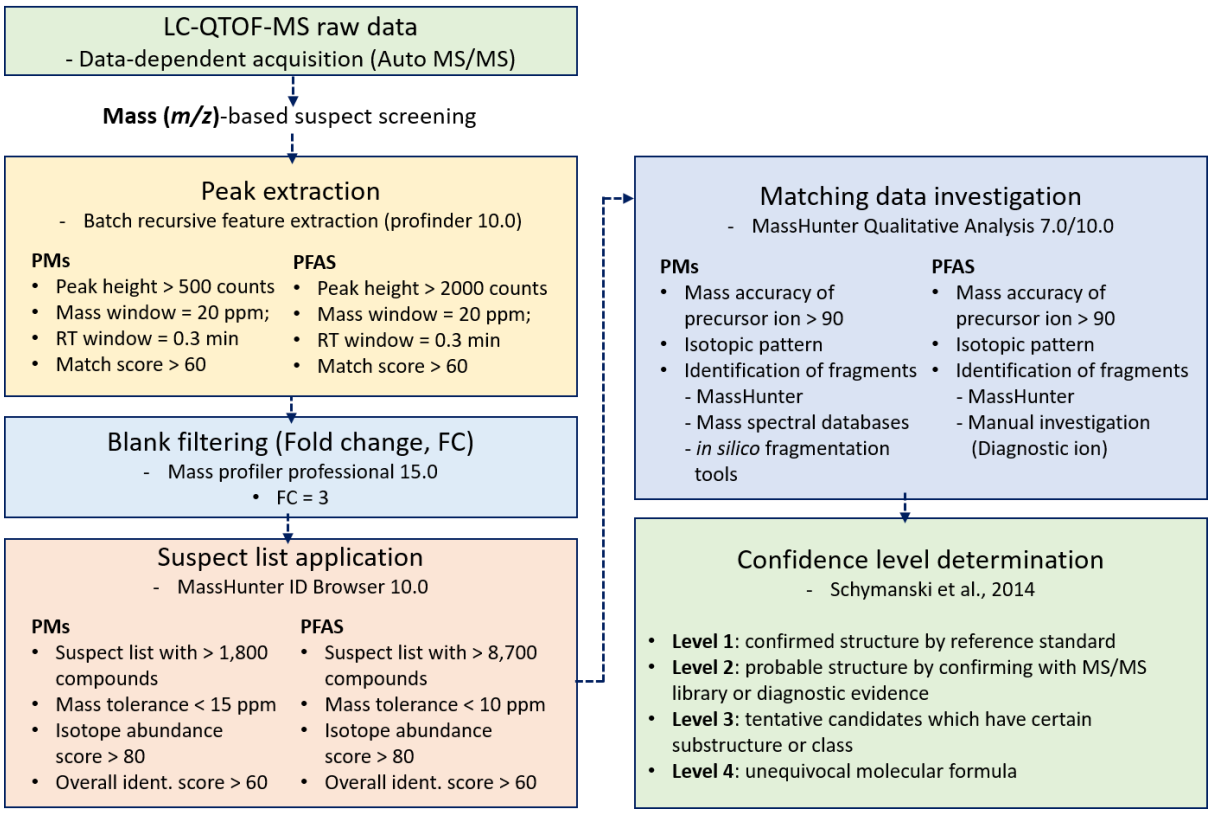
The PM suspect list applied in this study was generated by including the PMs listed by UBA (UBA, 2019) and previously published literature (Montes et al., 2019; Schulze et al., 2019). Additionally, since some PMs are possibly metabolized and excreted in urine, potential metabolites of all PMs were predicted and added to the suspect list. Prediction of PM metabolites was performed manually based on Phase I and Phase II metabolic pathways. For the prediction of Phase I metabolites, oxidation, reduction of H, OH, SH, and NH_2, and hydrolysis were selected. The prediction of Phase II metabolites was performed by adding $C_6H_8O_6$ (glucuronidation), CH_2 (methylation), SO_3 (sulfatation), and $C_{10}H_{16}N_3O_5S$ (conjugation with glutathione). As a result, 201 PMs and 1517 predicted metabolites were included in the PM suspect list used in the current study.

For suspect screening of PFAS, two suspect lists were merged and applied to urine samples including PFAS Master List provided by United States Environmental Protection Agency (US EPA) CompTox (*n* > 9,000) and a list of PFAS reported in previous non-target studies (PFASNTREV19; *n* > 1,000) (Liu et al., 2019). In the combined suspect list, compounds without molecular formula or monoisotopic mass were excluded. In total, approximately 8,700 PFAS were screened in Flemish adolescents' urine samples. Additionally, frequently detected PFAS in human and environmental matrices, including PFBS, PFOS, PFBA, PFOA, and several fluoroether alternatives, were investigated individually with 'Find by Formula' algorithm.

The schematic diagram of the data analysis workflow for suspect screening of PMs and PFAS is presented in **Figure 1**. Initially, the data acquisition files obtained from QTOF-MS were introduced to Profinder 10.0 (Agilent Technologies), and a batch recursive feature extraction was performed. After exporting the data files (.cef) from Profinder, files were processed with Agilent Mass Profiler Professional (version 15.0) and Agilent MassHunter ID Browser (version 8.0) for statistical analysis and suspect list application. Data files were grouped as blanks (*n* = 8) and urine samples (*n* = 83) and a fold change analysis was performed. For PMs and PFAS, a fold change of 3 was used to remain features at least 3 times higher in the urine samples than in the procedural blanks. The filtered results were matched with the suspect list for compound annotation using Agilent MassHunter ID Browser 10.0, with a match tolerance of ± 15 ppm + 2 mDa for PMs, and ± 10 ppm + 2 mDa for PFAS. Adduct of $[M+H]^+$, $[M+Na]^+$, and $[M+NH_4]^+$ for ESI+ and $[M-H]^-$ for ESI- were considered. The resulting files containing *m/z*, RT, matching formula, annotation, and score were exported and examined with Agilent MassHunter Qualitative Analysis (versions 7.0 and 10.0). The MS/MS spectra of each annotated PMs and PFAS was manually investigated by and compared with the fragment ions of native standards of annotated compounds, or the MS/MS spectra obtained from mass spectral databases, such as MassBank, Human Metabolome Database and mzCloud library, and *in silico* fragmentation tools, such as CFM-ID 3.0 predictor and ACD/MS Fragmenter. For emerging PFAS, the MS/MS spectra were manually inspected based on diagnostic fragment ions (i.e., CF_3, C_2F_3, C_3F_7, SO_2F, SO_3F, etc.).

For the assignment of CLs, the scale from Schymanski et al. (Schymanski et al., 2014) was followed. A detailed description of each level assignment is presented as follows: confirmed structure (MS, MS/MS spectra, and RT matching) by reference standard (CL 1); probable structure by confirming MS/MS spectra with library (i.e, MassBank), previous literature or diagnostic evidence (CL 2); tentative structure confirmation with a substructure or class (CL 3); unequivocal molecular formula matching without sufficient evidence to propose possible structure (CL 4).

Figure 1. Schematic diagram of data analysis workflow applied for suspect screening of persistent and mobile chemicals (PMs) and per- and polyfluoroalkyl substances (PFAS).



2.6. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 28.0 (IBM Corp., Armonk, NY, USA). The total number of compounds identified at CL 1–3 in each participant were counted and used for statistical analysis. To increase the reliability of the statistical analysis results, the detection rate of compounds identified at CL 4 was not considered for the statistical analysis. The dataset was divided into two categories, high (n = 39) and low (n = 44) exposure loads, and in sexes, females (n = 36) and males (n = 47). The difference between groups (exposure loads and sexes) was determined by using an independent sample t-test with the significance set at $p < 0.05$.

3. Results and discussion

3.1 Method optimization

For the suspect screening of PMs and PFAS, three different sample preparation methods (method 1: SPE; method 2: dilute-and-shoot; method 3: LLE) were applied to pooled urine samples to investigate the sample preparation method showing the best extraction efficiencies for PM and PFAS compounds listed in **Table S1 and S2**, respectively. For method 1, three SPE sorbents including Oasis WAX, Strata X-CW, and Captiva ND Lipids were compared. In method 2, urine samples were diluted by the addition of the same volume of ACN (1:1 dilution, v/v). For method 3, formic acid (method 3-1) or ammonia (method 3-2) were added to the urine, and then samples were extracted with ethyl acetate:isopropanol (95:5, v/v). Detailed information on sample preparation methods and the results are described in the **SI**.

Extracting all PMs using one sample preparation method is challenging, owing to a wide range of PMs' physio-chemical properties. Thus, the optimization of sample preparation for PMs was aimed at obtaining a method capable of extracting the maximum number of PMs, rather than obtaining 100% of extraction efficiency. Thus, both the number of extracted PMs fortified to pooled urine samples and their extraction efficiencies were investigated and considered for the selection of the sample preparation method for the suspect screening of PMs. The extraction efficiencies of PMs were calculated as the ratio percentage (%) of the peak abundances between pure native standard and fortified samples. Results are presented in **Figure S1a**. Among 11 PMs fortified to urine samples, less than six PMs were detected in urine samples extracted by methods 1, 2 and 3-1, whereas 8 of 11 PMs were detected in samples treated by method 3-2. There was no PM detected in methods 1 and 2. In contrast, cyanuric acid, melamine, and 2-morpholinoethanol were detected only in samples extracted by method 3-1 and 3-2. The extraction efficiencies of detected PMs varied among the methods. Considering the extraction efficiencies of PMs observed in all methods, method 3-2 showed better results (53–80%) than the other methods (method 1: 8.3–128%; method 2: 30–65%; method 3-1: 22–330%). Given the number of detected PMs and their extraction efficiencies, method 3-2 was selected for the suspect screening of PMs in human urine.

The extraction efficiency (%) of PFAS was calculated by comparing the ISTD abundances in the urine sample to those of a standard mixture (prepared in MeOH) (**Figure S1b**). In method 1, all tested sorbents (Captiva ND, Oasis WAX, and Strata X-CW) showed a low efficiency (< 20%) for all ISTDs spiked. The extraction efficiency of method 2 was substantially higher (> 200%) for lower fluorinated compounds (< C6) and decreased with the length of the carbon chain (> C10). The LLE method with the addition of formic acid or ammonia showed acceptable efficiencies (> 50% for method 3-1; > 68%

for method 3-2) for all ISTDs. With these results, LLE with the addition of ammonia was regarded as the most suitable extraction method for analyzing PFAS in urine samples.

Based on the results of this experiment, the LLE method with the addition of ammonia (method 3-2) was selected for the sample preparation method for the simultaneous analysis of PMs and PFAS in Flemish adolescent urine samples.

3.2 Suspect screening of urine samples

3.2.1 Suspect screening of PMs

In the 83 analyzed urine samples, more than 5,000 features met the criteria for the peak extraction step of the suspect screening workflow, of which 71 features in ESI+, and 42 features in ESI- matched the PM suspect list with a score > 60. Since some features were matched with the same compound at different RTs, the number of the matching compounds for 113 features was lower, namely 30 and 15 in ESI+ and ESI-, respectively. Each compound was manually checked using Agilent MassHunter to assign a CL. Out of 45 compounds that matched the suspect list, 22 compounds were excluded due to their poor peak shape and incorrect MS/MS fragments. In total, 14 compounds including $C_6H_4F_{11}NO_3$ (potential name: ammonium 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propanoate) and $C_8H_{10}O_3$ (Cyclohexane-1,2-dicarboxylic anhydride) were assigned with CL 4 in all urine samples. Although tentative molecular formulas of them were available, there was only insufficient evidence to suggest a certain structure of them. Thus, these 14 compounds were also excluded from the list of matched compounds. As a result, 5 compounds for ESI+ and 4 compounds for ESI- were identified at CL 1–3 in at least one urine sample and were, therefore, assigned as tentatively identified PMs. In ESI+, acetaminophen, 4-aminophenol, 2,2,6,6-tetramethyl-4-piperidone, and m-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline were tentatively identified, and in ESI-, TFAA, sulisobenzone, ethyl sulfate (EtS), and 1,2-benzisothiazol-3(2H)-one 1,1-dioxide were detected. Among them, acetaminophen, 2,2,6,6-tetramethyl-4-piperidone, TFAA and EtS were assigned with CL 1, and their fragmentation spectra are shown in **Figure S2**. 4-aminophenol, sulisobenzone, and 1,2-benzisothiazol-3(2H)-one 1,1-dioxide were identified at CL 2, and m-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline was assigned with CL 3. In some urine samples, the peaks suspected as PMs were tentatively identified without MS/MS spectra. If these peaks had the same RTs and isotopic patterns as that of the chromatogram of PMs identified as CL 1–3 in other urine samples, they were reported as CL 4. Details of identified PMs are presented in **Table 1**.

Most of the identified PMs are included in the list of PMs classified by UBA (UBA, 2019). Their uses cover many different fields of application, including pharmaceuticals and personal care products,

paints, coating products, dyeing textiles, laboratory reagents, chelating agents, chemical intermediate, and household products. In addition, their production and/or import volumes into the European Union vary widely, at ≥ 100–10000 tons per year, although the related data for some identified PMs is unavailable (ECHA, 2022). The UBA reported the REACH Emission Likelihood, which is a simplistic screening approach to carry out an emission characterization for PMs considering the Emission-Score (Schulze et al., 2018), tonnage of each PM registered in REACH, and monitoring data (UBA, 2019). For example, if a chemical has a high Emission-Score and full registration type in REACH and was detected in raw water, drinking water or groundwater, it is classified as 'very high' REACH Emission Likelihood. According to their report, all PMs identified in urine samples of this study, except ethyl sulfate, are considered to have at least 'medium' Emission Likelihood. There is no report about the REACH Emission likelihood of ethyl sulfate. Detection frequencies of PMs identified at CL 1–4 and CL 1–3 ranged from 19-98% and 10-98%, respectively. Each PM tentatively identified in urine samples is briefly discussed below.

Acetaminophen ($C_8H_9NO_2$) is a commonly used over-the-counter antipyretic analgesic, known also as paracetamol, (Jensen et al., 2004; Wu et al., 2012; Yeung et al., 2008) and is considered a PM under the UBA. It is known to be primarily metabolized by conjugation with glucuronic acid and sulfate when used and is subsequently excreted as parent compound and its metabolites in urine (Jensen et al., 2004; Modick et al., 2013). Acetaminophen was identified in 16% of the urine samples at CL 1 and 50% at CL 4, while the major metabolites of acetaminophen, acetaminophen glucuronide and sulfate, were not detected in any urine sample. Monitoring of acetaminophen in human urine was conducted in previous studies (Modick et al., 2014, 2013). Among them, one study showed the detection of acetaminophen in urine samples from people who declared never to have taken acetaminophen in their life (Modick et al., 2013). This indicates the possibility of exposure to acetaminophen from sources other than taking acetaminophen directly. Aniline which is used in the synthesis of rubber and pesticides and metabolized in to acetaminophen, was suggested as a possible source of acetaminophen (Modick et al., 2014). In addition, acetaminophen was detected in the environment, particularly in drinking and surface water, and groundwater that could be used as drinking water sources at levels up to mg/L (Behera et al., 2011; Gros et al., 2012; Kleywegt et al., 2011; Pedrouzo et al., 2007; Rabiet et al., 2006; Santos et al., 2013; Stackelberg et al., 2007; Vulliet et al., 2011). Considering these results, it is possible that individuals participating in this study were indirectly exposed to acetaminophen (e.g. exposure to aniline) from their surroundings in addition to actively taking acetaminophen.

4-aminophenol (C_6H_7NO) is a mutagen category 2 substance and one of PMTs classified under UBA. It was tentatively identified in 16% of urine samples at CL 2, and in 50% at CL 4. 4-aminophenol has been

used for various purposes, such as household products and cosmetics, indicating that it can be released into the environment and be exposed to the general population. It is also one of the primary degradation products of acetaminophen (Santos et al., 2013; Dejaegher et al., 2008). The detection of 4-aminophenol in wastewater effluents (Gómez-Ramos et al., 2011) and freshwater was previously reported (Santos et al., 2013), whereas there is no previous study reporting the detection of 4-aminophenol in human samples.

2,2,6,6-tetramethyl-4-piperidone ($C_9H_{17}NO$) is a potential PMT and was detected in 12% of urine samples at CL 1 and 28% at CL 4. It is widely used as an intermediate in the production of antioxidants and stabilizers for polymeric materials (Du et al., 2014). The information on exposure of 2,2,6,6-tetramethyl-4-piperidone to the environment and human body is very limited and, to our knowledge, there is no previous report of its detection in human urine.

Trifluoroacetic acid ($C_2HF_3O_2$) is a ubiquitous chemical with a wide range of uses including catalysts and reagents (López and Salazar, 2013; Solomon et al., 2016; Xie et al., 2020), and one of the metabolites of several chemicals, such as hydrochlorofluorocarbons and hydrofluorocarbons, and halothane (Solomon et al., 2016). Since it is a perfluorocarboxylic acid with the shortest carbon chain, it is classified as PFAS, as well as PM. Applying the suspect screening workflow of PMs, TFAA was detected in 30% of urine samples at CL 1 and 63% at CL 4. Its presence at levels up to mg/L or ng/g in the environment has already been shown in several studies (Berg et al., 2000; Björnsdotter et al., 2019; Janda et al., 2019; Xie et al., 2020; Zhai et al., 2015), suggesting that it has a high potential for human exposure. Urinary concentration of TFAA was reported in some previous studies, but those studies included patients treated with halothane (Hankins and Kharasch, 1997; Kawahara et al., 1988). To the best of our knowledge, this is the first report showing the detection of TFAA in urine from the general population.

m-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl) aniline ($C_{15}H_{19}NO_4$) was found in over 70% of urine samples at CL 3–4. It has been used in various industries, such as computer and electronic product manufacturing and was classified as a chemical that has a very high emission likelihood under UBA (UBA, 2019). There is no previous report on environmental fate and human biomonitoring.

Sulisobenzone ($C_{14}H_{12}O_6S$), also known as benzophenone-4, is a UV-filter used as an ingredient in cosmetics and personal care products, especially sunscreens (González, 2014; Kang et al., 2016; Zucchi et al., 2011), and is identified as one of the most widespread UV-filters in the aquatic environment (Zenker et al., 2008; Zucchi et al., 2011). It was classified as PMT under the UBA and its potency as an estrogenic disruptor has been reported (Ruszkiewicz et al., 2017; Zucchi et al., 2011). In this study, sulisobenzone was tentatively identified in 43% of urine samples at CL 2. So far, there is only one study

that showed the detection of sulisobenzone in urine samples from volunteers who applied sunscreen containing sulisobenzone (González, 2014).

Ethyl sulfate ($C_2H_6O_4S$, EtS) is one of the potential PMs identified in 6% of urine samples at CL 1 and in 6% of the samples at CL 4. It is used in industrial and consumer products and is also well-known as a direct ethanol metabolite (Andrés-Costa et al., 2016; Wurst et al., 2006). Since EtS can be used as a biomarker for recent alcohol consumption of people, urine monitoring of EtS has been conducted for the general population, patients and also in postmortem investigations (Alsayed et al., 2022; Graham et al., 2017; Helander and Beck, 2004; Thierauf-Emberger et al., 2016; Wurst et al., 2006). Among study participants with EtS in urine samples, 30% responded to the questionnaire from FLEHS IV reference biomonitoring study that they occasionally drink alcohol. But other 70% answered to have drunk alcohol only once or never, indicating that there is another source of ethyl sulfate. In addition to alcohol consumption, previous studies found the urinary excretion of EtS following the consumption of non-alcoholic beer, grape juice, bananas, and sauerkraut (Musshoff et al., 2010) and the intensive use of high ethanol content mouth wash (Reisfield et al., 2011). This supports the detection of EtS in urine from participants who are non-drinkers.

1,2-benzisothiazol-3(2H)-one 1,1-dioxide ($C_7H_5NO_3S$), well-known as saccharin, is one of the most popular artificial sweeteners and millions of people consume it through several foods and beverages, especially calorie-free drinks (Pang et al., 2020; Uçar and Yilmaz, 2015). It is also widely used in pharmaceutical and personal care products, preservatives, adhesive removal, etc. According to UBA, saccharin was classified as a PM and a medium release chemical to the environment. It was found in 68% of Flemish adolescents' urine samples at CL 2, and in 4.8% of the samples at CL 4. There is one study showing the detection of saccharin in human urine, and it was performed using urine samples from volunteers given a single saccharin dose (McChesney and Golberg, 1973). Thus, the information on 1,2-benzisothiazol-3(2H)-one 1,1-dioxide exposure to the general population is very limited. However, its occurrence at ng/L to µg/L has been confirmed in several studies, mostly about water samples including wastewater, surface water, groundwater, tap water, and seawater (Berset and Ochsenbein, 2012; Buerge et al., 2011; Gan et al., 2013; Ordóñez et al., 2012; Schulze et al., 2020, 2019; Stefania et al., 2019), which suggests its wide presence in the environment.

Because mobility and bioaccumulation are not inherently exclusive, some persistent and bioaccumulative chemicals would be also PMs (Arp et al., 2017). This suggests that other human matrices, such as blood and breast milk can also be suitable matrices to investigate the human exposure of those PMs with bioaccumulation potential (e.g. melamine and PFBS). Thus, suspect screening of PMs using different human matrices should be considered in further research to examine

PMs which are not detected in urine samples.

Table 1. Description of identified persistent and mobile chemicals (PMs) in Flemish urine samples by suspect screening analysis. Classification of PM and toxic PM (PMT) is based on the report of UBA (UBA, 2019). Confidence level (CL) for each PM was determined according to Schymanski et al. (2014). Tonnage data (Ton/yr: tons per year) for each PM is from REACH. RT: retention time; DF: detection frequency (%); LOD: Limit of detection; PFAS: per- and polyfluoroalkyl substances.

Identified PMs	Classification	CAS number	Molecular formula	Adduct type	Lowest CL	RT (min)	% DF	Fragment ions (*m/z*)	Ton/yr	LOD (ng/mL)	Usage
					ESI+						
Acetaminophen	Potential PMT/PM	103-90-2	$C_8H_9NO_2$	$[M+H]^+$	1	8.60	16 (CL 1) 50 (CL 4)	43.0186, 65.0387, 93.0331, 110.0591, 134.0589	10–100	3.7	Pharmaceutical
4-aminophenol	PMT	123-30-8	C_6H_7NO	$[M+H]^+$	2	2.68	10 (CL 2) 9.6 (CL 4)	65.0403, 67.0428, 82.0636, 92.0452	10–100		Various (e.g., cosmetics)
2,2,6,6-tetramethyl-4-piperidone	Potential PMT/PM	826-36-8	$C_9H_{17}NO$	$[M+H]^+$	1	5.34	12 (CL 1) 28 (CL 4)	41.0401, 58.0659, 156.1401		7.1	Intermediate for the synthesis of pharmaceuticals and pesticides
m-(2,3-epoxypropoxy)-N,N-bis(2,3-epoxypropyl)aniline	PM/PMT	71604-74-5	$C_{15}H_{19}NO_4$	$[M+NH_4]^+$	3	10.1	77 (CL 3) 2.4 (CL 4)	57.0325, 161.1057	100–1000		Various (e.g., intermediate)
					ESI-						
Trifluoroacetic acid	PM	76-05-1	$C_2HF_3O_2$	$[M-H]^-$	1	3.43	30 (CL 1) 63 (CL 4)	44.9997, 68.9963	100–1000	20	PFAS
Sulisobenzone	PMT	4065-45-6	$C_{14}H_{12}O_6S$	$[M-H]^-$	2	16.5	43 (CL 2) 26 (CL 4)	121.0321, 227.0707	100–1000		Personal care product
Ethyl sulfate	Potential PM	540-82-9	$C_2H_6O_4S$	$[M-H]^-$	1	3.23	6.0 (CL 1) 6.0 (CL 4)	79.9572, 96.9603, 124.9910	≥100	0.1	Various (e.g., Household product)
1,2-benzisothiazol-3(2H)-one 1,1-dioxide	PM/PMT	81-07-2	$C_7H_5NO_3S$	$[M-H]^-$	2	8.82	68 (CL 2) 4.8 (CL 4)	41.9989, 105.9601, 181.9900	100–1000		Various (e.g., personal care product)

3.2.2 Suspect screening of PFAS

In total, 581 features were matched with compounds in the applied suspect list and 163 features showing a compound matching score > 60 were sorted. Out of 163 features examined with Agilent MassHunter Qualitative 7.0, only 2 features were annotated as CL 4, which are $C_{13}H_{18}F_8O_2$ (potential name: 2,2,3,3,4,4,5,5-octafluorooctyl pentanoate; average *m/z* = 357.1085; average mass error = -8.4 ppm) and $C_{17}H_{13}F_7N_4O$ (3,4-dihydro-3-[(3-pyridinylmethyl)amino]-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)-ethyl]-2(1H)-quinazolinone; *m/z* = 421.0914; mass error: 2.2 ppm) (**Table S3**). It should be noted that there might be other formulae to be matched with these features, but MS/MS spectra were not available to confirm the molecular structures. Other features (*n* = 161) were excluded from further investigation due to poor peak shape, low matching score derived by unclear MS isotopic pattern, or unmatched MS/MS fragmentation patterns.

The compound with formula $C_{13}H_{18}F_8O_2$ (2,2,3,3,4,4,5,5-octafluorooctyl pentanoate), which was assigned as CL 4, was detected in 90% of Flemish adolescents' urine samples with an average matching score of 75 (highest: 88). The chemicals of emerging concern (CEC) screening list provided by HBM4EU project listed this compound as a CEC metabolite, but detailed information on the parent compound was not provided. To the best of our knowledge, there is no previous report on the environmental fate, exposure, and toxicity data of this compound.

The compound with formula $C_{17}H_{13}F_7N_4O$ (3,4-dihydro-3-[(3-pyridinylmethyl)amino]-6-[1,2,2,2-tetrafluoro-1-(trifluoromethyl) ethyl]-2(1H)-quinazolinone) was assigned as CL 4 and observed in 65% of urine samples with an average matching score of 84 (highest: 97). According to US EPA Comptox Chemical Dashboard data qualification, this compound is classified as a compound with the highest confidence in accuracy and consistency and is a known metabolite of pyrifluquinazon (You et al., 2017). Pyrifluquinazon belongs to Insecticide Resistance Action Committee (IRAC) group 9 insecticide which is used for controlling sucking and chewing insects on vegetables (i.e., whiteflies) (Wilson et al., 2019). Given that the parent compound has been used as an insecticide, the occurrence of this metabolite in urine may be of concern for human exposure.

In this study, several representative PFAS which have been routinely monitored in human samples (i.e., perfluoroalkyl carboxylic acids, perfluoroalkyl sulfonic acids, and their precursors) and well-known alternatives (i.e., Gen-X, ADONA, and F-53B) were manually screened using Find by Formula algorithm, and none of these compounds were observed in the urine samples. In previous target analysis studies, a lower concentration of shorter-chain PFAS in urine of US general population (Calafat et al., 2019) and lower detection rate and levels of urinary PFAS compared to other matrices, such as serum, hair, nails (Li et al., 2021; Yuan Wang et al., 2018) were reported. Given that the aforementioned studies have

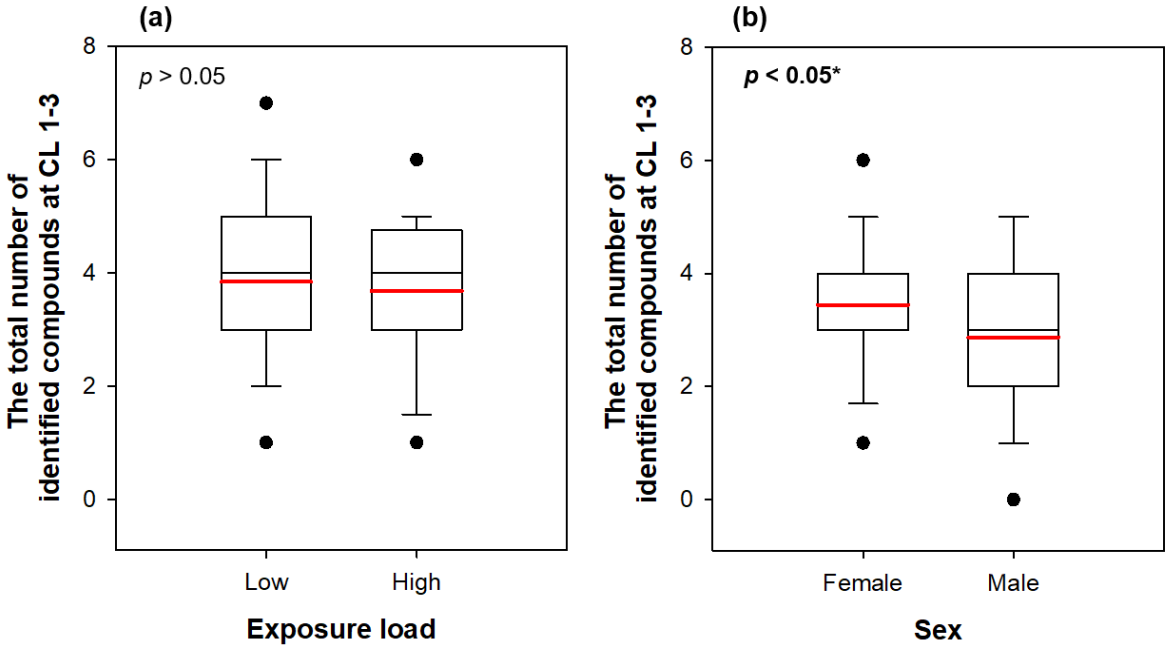
conducted targeted analysis by LC-MS/MS, which has a higher instrumental sensitivity than Q-TOF, the urinary levels of PFAS in Flemish adolescents might not be high enough to be analyzed with a suspect screening approach. Considering that suspect screening has a great advantage to investigate a broad range of chemicals, more suitable matrices, such as serum, need to be chosen to investigate human exposure to emerging PFAS.

3.3 Statistical analysis

The number of detected compounds identified at CL 1–3 in each participant was compared between 2 different categories, exposure loads (low and high) and sexes (females and males). Since all PFAS identified in urine samples were annotated as CL 4, the total number of PFAS detected in each participant was not used for the statistical analysis.

No significant difference was found between the number of identified PMs in high and low exposure load groups ($p > 0.05$; **Figure 2a**). In contrast, the number of identified PMs at CL 1–3 in females (median: 3; mean: 3.4) was significantly higher than those in males (median: 2.8; mean: 3) ($p = 0.033$; **Figure 2b**). The DFs of identified PMs were overall similar between females (17–81%) and males (4–74%). However, the DFs of acetaminophen (19% at CL 1), 4-aminophenol (17% at CL 2) and sulisobenzone (56% at CL 2) in females were slightly higher than in males (acetaminophen: 13% at CL 1; 4-aminophenol: 4% at CL 2; sulisobenzone: 34% at CL 2), suggesting that acetaminophen, 4-aminophenol and sulisobenzone contributed to the significant difference between the sexes. In particular, the higher DF of acetaminophen and sulisobenzone in females compared to males is consistent with literature surveys showing that women tend to use pain relievers and sunscreen more frequently than men (Cho et al., 2017; Lee et al., 2015; Memon et al., 2019; Richardson and Holdcroft, 2009). Since 4-aminophenol is one of acetaminophen metabolites, the higher DF of 4-aminophenol in females might be related to the detection of acetaminophen in urine.

Figure 2. Number of identified PMs at CL 1–3 in urine samples by (a) exposure load groups (low and high exposure loads) and (b) sexes (females and males). Red lines and black dots in the figure indicate arithmetic mean values and outliers, respectively.



4. Conclusions

In this study, a sample preparation method for PMs and PFAS was optimized and successfully applied for the suspect screening of PMs and PFAS in urine samples from Flemish adolescents. To the best of our knowledge, 4 PMs identified at CL 1–3 in this study have not been previously reported in human urine. This indicates that humans are exposed to unexplored PMs and that suspect screening approaches can be successfully used to identify these chemicals. Within suspect screening of PFAS, two compounds were assigned as CL 4 indicating that urine might not be a suitable matrix to investigate human exposure to PFAS applying suspect screening methods. PMs identified in this study require further investigation on toxicity and environmental fate to fill the current knowledge gap and develop a quantitative biomonitoring strategy for emerging contaminants. In addition, target analysis and non-target screening methods could be applied in further research along with the suspect screening to obtain a more comprehensive overview of human exposure to PMs.

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