Comprehensive suspect screening for the identification of contaminants of emerging concern in urine of Flemish adolescents by liquid chromatography high-resolution mass spectrometry

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ABSTRACT

The increasing human exposure to contaminants of emerging concern (CECs) cannot be fully assessed by targeted biomonitoring methods alone as these are limited to a subset of known analytes. On the contrary, suspect screening approaches based on liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) allow the simultaneous detection of a high number of CECs and/or their (predicted) metabolites leading to a more comprehensive assessment of possible human exposure to these compounds. Within this study, 83 urine samples of Flemish adolescents (47 males, 36 females) collected in the frame of the 4th cycle of the Flemish Environment and Health Study (FLEHS IV) were selected with the aim of including a high and a low exposure group based on the overall exposure of 45 known contaminants. Samples were analyzed using a previously developed method involving a suspect screening approach to annotate CECs and their metabolites. The applied suspect list contained a total of > 12,500 CECs and their known and predicted metabolites resulting from metabolization reactions, such as hydroxylation, glucuronidation and methylation. In total, 63 compounds were annotated at a confidence level of 3 or better, with most of the detected compounds not included in current biomonitoring programs. 5 out of the 63 compounds could be assigned with confidence level 2. Five compounds could unequivocally be identified (confidence level 1) through the comparison with reference standards. Personal care products were the main detected compound class (42% of detected compounds). Additionally, a detailed literature search indicated potential toxic effects for several of the detected CECs. Lastly, in the urine samples, a significantly higher number ($p < 0.05$) of compounds was detected in the high exposure group as opposed to the low exposure group. This difference could only be observed between high and low exposure load samples of female participants ($p < 0.01$).

KEYWORDS

Metabolite prediction; Exposure load; Organophosphate flame retardants; Personal care products; Urine analysis; Flemish Environment and Health Study (FLEHS)

1. INTRODUCTION

Human biomonitoring (Hbm) studies, such as the 4th cycle of the Flemish Environment and Health Study (FLEHS IV, 2016-2020) (Schoeters et al., 2017), aim to assess human exposure to environmental chemicals. These studies are of high importance for the collection of quantitative data on internal exposure to known contaminants. Such chemicals can be monitored using targeted analytical approaches (Smolders et al., 2009) given that precise information about the chemical identity of the analytes and their corresponding reference standards are available. In the scope of the FLEHS IV study, several targeted studies reported biomonitoring results for known biomarkers from various classes such as phthalates, alternative plasticizers (APs), organophosphate flame retardants (PFRs), polycyclic aromatic hydrocarbons (PAHs), and others (Bastiaensen et al., 2021a; Bastiaensen et al., 2021b; Gys et al., 2021; Verheyen et al., 2021).

While these studies are indispensable to obtain quantitative biomonitoring data and eventually link the data with health effects and potential exposure pathways, targeted approaches leave many unknown or recently discovered chemicals, commonly referred to as contaminants of emerging concern (CECs) (Sauve and Desrosiers, 2014), undetected. Since the toxicity of many CECs or their influence on the environment and humans are not yet well understood, they are not comprehensively included in (bio)monitoring programs. Therefore, complementary analytical approaches are needed to document the occurrence of CECs in humans.

Suspect screening approaches based on liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) are valuable tools for the identification of CECs and their metabolites in human samples (Pourchet et al., 2020). LC-HRMS allows the simultaneous acquisition of accurate-mass data for a high number of analytes. Additionally, the acquisition of MS/MS fragmentation spectra can provide additional spectral information for compound annotation (Zedda and Zwiener, 2012). The acquired accurate-mass data can subsequently be matched against a predefined list containing CECs suspected to be present in the samples (suspect list). The suspect list can also include metabolites of CECs predicted based on modifications of known contaminants or known metabolization pathways. Additionally, this analytical approach acknowledges that environmental contaminants are often present in human samples in a metabolized form. Thus, the inclusion of the parent compounds alone could potentially lead to a high number of false negative detects (del Mar Gómez-Ramos et al., 2011; Huntscha et al., 2014).

In addition to matching accurate-mass data, the acquired MS/MS spectra can be compared with mass spectral libraries or predicted MS/MS spectra derived from *in silico* prediction tools (Djoumbou-Feunang et al., 2019; Kind et al., 2018; Ruttkies et al., 2016) to further increase identification confidence. Optimally,

within suspect screening studies, confidence levels of up to 2 can be reached based on the principles of reporting identification confidence proposed by Schymanski et al. (Schymanski et al., 2014), if experimental MS/MS spectra can unequivocally be matched with reference data. Despite the high relevance of suspect and non-target analysis of human biological samples using HRMS, research works in this field are still limited (González-Gaya et al., 2021). For example, only 7 studies on suspect screening of contaminants in urine samples have been published so far, three of them focused on pesticides(Bonvallot et al., 2021; López-García et al., 2019; López et al., 2016), three studying different CECs (Caballero-Casero et al., 2021a; Dolios et al., 2019; Plassmann et al., 2015) and another one investigating occupational exposure to PAHs (Tang et al., 2016).

Even though the described techniques show high potential for the identification of CECs and their metabolites, several limiting factors must be considered within the development of suspect screening approaches. Despite continuous developments and expansion of mass spectral libraries, the availability of reference MS/MS spectra of novel CECs and their metabolites is limited, hampering compound identification at high confidence levels (Oberacher et al., 2020; Stein, 2012). The analysis of complex human matrices, such as urine, blood, or serum, can be accompanied by considerable matrix effects leading to signal suppression and limiting the detection of exogenous compounds. This is especially challenging since the latter are present at low concentration levels (sub ng/mL range) and can additionally be suppressed by the presence of endogenous compounds, which normally show higher concentrations (Hu et al., 2019; Raposo and Barceló, 2021). These limitations indicate that an extensive optimization of each analysis step is crucial to obtain reliable suspect screening results. This issue has been addressed by a previous study conducted by Caballero-Casero et al. (Caballero-Casero et al., 2021b) in which a comprehensive suspect screening approach for the detection of CECs and their metabolites in urine samples has been described.

The present study involved additional optimization steps to the method developed by Caballero-Casero et al. The modified method was then applied to biobanked urine samples of 83 Flemish adolescents participating in the FLEHS IV (2016-2020) aiming to identify additional CECs and their metabolites not included in previous target FLEHS biomonitoring studies. A suspect list previously proposed by Caballero-Casero et al. was further expanded and finally it included > 3,200 CECs from several compound classes, such as traditional phthalate-based and new non-phthalate alternative plasticizers, organophosphate flame retardants, synthetic antioxidants, UV-light stabilizers, pesticides, and others (Caballero-Casero et al., 2021a). As the study of Caballero-Casero et al. had shown, most CECs were present in urine samples

in a metabolized form. However, the inclusion of only the parent compounds in the suspect screening workflow would leave potential metabolites undetected. Consequently, metabolites of all parent compounds corresponding to most commonly observed metabolization reactions (Ballesteros-Gomez et al., 2015; Caballero-Casero et al., 2021a; Gys et al., 2018; Testa and Krämer, 2008a; Testa and Krämer, 2008b), namely hydroxylation (Phase I), glucuronidation and methylation (Phase II) were predicted, which resulted in a suspect list containing > 12,500 compounds. In particular, the focus of this study was on CECs and metabolites which were not included in the list of targeted analytes available from the FLEHS IV study. The obtained results revealed the complementary value of suspect screening for the analysis of human exposure to environmental contaminants by reporting a high number of CECs and their metabolites which would have remained undetected if targeted screening methods alone are applied. The reported compounds could subsequently be added to the list of targeted analytes of, among others, upcoming FLEHS cycles.

2. MATERIALS AND METHODS

2.1 Chemicals

Methanol (MeOH), acetonitrile (ACN), and formic acid (FA) were purchased from Biosolve BV (Valkenswaard, the Netherlands) (≥ 99.9%). All organic solvents were of LC grade. A PURELAB Flexsystem was used to obtain ultrapure water (18.2 MΩ cm, Milli-Q, Millipore). Ammonium acetate was purchased from Sigma-Aldrich (eluent additive for LC-MS). A set of 30 native standards of organophosphate and alternative plasticizer metabolites was used for the optimization and the quality control of the sample preparation and the LC-HRMS. Additionally, 13 standards were purchased in order to confirm the identity of compounds assigned within the suspect screening approach. The name, formula, and further identifiers of both sets of compounds are summarized in Table S1. Samples were spiked with nine isotopically labelled internal standards (IS) which are summarized in Table S2. Working solutions of IS were prepared at a concentration of 300ng/mL in methanol.

2.2 Sample collection

The spot urine samples investigated in this study were selected from the biobanked samples stored at - 20°C. Samples were collected between September 2017 and June 2018 as part of the FLEHS IV reference biomonitoring study (2016-2020). The study was approved by the Ethical Committee of the University Hospital of Antwerp, Belgium (Belgian Registry Number: B300201732753). For the participants of the FLEHS IV reference population (428 adolescents, 14-15 years), quantitative data on the exposure to a set

of known contaminants was available since the samples had already been investigated within previous targeted biomonitoring studies (Bastiaensen et al., 2021a; Bastiaensen et al., 2021b; Gys et al., 2021; Verheyen et al., 2021). Based on the 45 quantified chemicals studied in these targeted biomonitoring studies, Buekers et al. calculated the exposure load of a participant (Buekers et al., 2021). Participants were scored based on their exposure to each chemical as opposed to a threshold, placed at the 50th percentile (P50) of the FLEHS IV cohort. A value of 0 was assigned if the exposure was below the P50, and 1 if the concentration was above P50. This exposure load, therefore, summarizes the overall exposure above the threshold (P50) for 45 known contaminants belonging to the phthalates, APs, PFRs, PAHs, bisphenols and others. In total 83 urine samples were selected according to the selection procedure described in figure S1. The exposure load sum (EL) was used to select the samples for analysis with the primary objective of having high and low exposure load groups. The high exposure group consisted of 43 samples with the highest exposure load, which was (≥27). The low exposure group consisted of the 39 lowest exposure load samples (≤17). The second objective for sample selection was to have a balanced distribution across sexes. Based on the first objective of the exposure load, urine samples of 47 male and 36 female participants were selected. Since this distribution was considered balanced, no further intervention was made to ensure the maximal potential of the EL. The distribution stayed balanced when we included the EL, we had 19 female and 20 male participants in the low exposure group, and 17 female and 27 male participants in the high exposure group. Specific gravity was measured on the selected urine samples by employing the hand refractometer (RF .5612) from EUROMEX microscopes (Holland).

2.3 Sample preparation

Glass tubes were thoroughly cleaned (rinsed with water, acetone and baked at 300 °C before usage). Urine spot samples were collected in clean metal-free polyethylene containers; they were kept at 4°C and processed within 24 h. Samples were divided into aliquots in glass vials and kept at -20°C until analysis. A 750 µL aliquot of urine was transferred to the precleaned tubes and centrifuged for 5 min at 3,500 rpm. Then, 500 µL of the supernatant were transferred to a clean glass tube and spiked with the IS working solution at 30 ng/mL (final concentration in urine) and vortexed. Captiva® non-drip lipid cartridges (3 mL, Agilent Technologies, Santa Clara, USA) were used for sample clean-up. One milliliter of ACN (with 0.1% formic acid, v/v) was added to the cartridge, immediately followed by the addition of the spiked urine. The solution in the cartridge was then carefully mixed and collected by push out. The obtained eluate was stored overnight at -20 °C. Then, 500 µL of the solution were filtered through a centrifugal nylon filter of 0.2 µm (VWR, Leuven, Belgium) for 5 min at 3,500 rpm, to ensure filtration of solids and precipitated

material. Optimization of the applied sample preparation method can be found in the supplementary information.

2.4 Instrumental analysis

All measurements were conducted on an Agilent 6560 quadrupole time-of-flight high-resolution mass spectrometer (QTOF-MS) coupled to an Agilent Infinity II UPLC (ultra-high performance liquid chromatography; Agilent Technologies, Santa Clara, USA). The instrument was equipped with a Dual Jet Stream electrospray ionization (ESI) source.

For chromatographic separation, an InfinityLab Poroshell 120 EC-C18 column (3.0 x 100 mm, particle size 2.7 µm) equipped with a guard column (3.0 x 5 mm) of the same stationary phase was used. Column temperature was maintained at 35 °C. The mobile phases consisted of ultrapure water (A) and MeOH (B). As modifiers, 0.1% FA (v/v) and 5 mM ammonium acetate were added for positive and negative ionization modes, respectively. The flow was maintained at 0.3 mL/min with an injection volume of 3 µL. For both ionization polarities, the following gradient was applied: 5% B - 50% B (0-3 min), 50% B - 80% B (3-5 min), 80% B - 100% B (5-16 min), 100% B – 5% B (16-16.5 min), 5% B (16.6-21 min).

The mass spectrometer was operated in 2 GHz, extended dynamic range mode. The ESI source parameters of the Agilent 6560 were based on the optimized values proposed by Caballero-Casero et al. (Caballero-Casero et al., 2021b) with slight modifications given in Table S3.

Both MS and MS/MS spectra were acquired in a mass range ranging from *m*/*z* 50 to 1,500. Data dependent acquisition mode (referred to as 'AutoMS/MS' by the vendor's software) was used whereby four precursors per acquisition cycle were automatically selected for fragmentation based on their abundance. The quadrupole isolation width was set to 'narrow (~1.3 *m*/*z*)', and collision energies of 10, 20 and 40 eV were applied.

2.5 Quality control (QC) and quality assurance (QA)

The quality of the analyses was assured by several measures to obtain reliable results. Samples were prepared in batches consisting of 20 samples and one batch of 3 samples, two QC samples of which one consisted of Milli-Q water spiked with native standards (table S1) (30 ng/mL) and IS (table S2), and one of pooled urine spiked with IS were added to each batch. Additionally, two procedural blanks (water) were included per batch. Each QC sample was prepared applying the same workflow as for real urine samples (see section 2.3). Standards of native compounds (10 ng/mL) (table S1) solubilized in methanol were

directly injected into the LC at the beginning and end of the sequence to monitor the stability of retention times (RT) and instrument sensitivity. Pooled urine samples spiked with IS (table S2) were prepared to ensure the detectability of the IS in a pooled matrix, as well as ensuring instrument sensitivity. Procedural blanks were used to monitor potential background contamination during batch preparation or analysis.

Additionally, a solvent blank (MeOH) was injected (every 5 samples) to monitor potential carryover during the sequence. All urine samples were spiked with the IS working solution to monitor potential analyte losses during sample preparation. During analysis, a reference mass solution was continuously infused to ensure automatic mass calibration. The mass calibration was based on ions with *m*/*z* 121.0509 and 922.0098, as well as *m*/*z* 119.0363 and 980.0164 for positive and negative ionization modes, respectively. The intensity of the reference mass ions was also monitored as an additional indicator for potential signal suppression due to matrix effects and instrumental variation.

2.6 Data analysis

2.6.1 Data processing

First, an in-house suspect list containing chemical information (Name, molecular formula, exact monoisotopic mass, and canonical SMILES) of different classes of CECs was built (supplementary information). Canonical SMILES have only been provided if their were no isomers for the compound. Chemical information was extracted from Caballero-Casero et al. (Caballero-Casero et al., 2021b), the NORMAN Suspect List Exchange (Meijer et al., 2021), the HBM4EU suspect screening lists (Govarts et al., 2020), and PubChem (Kim et al., 2021). A total number of 3,221 compounds, including synthetic antioxidants, plasticizers, organophosphate flame retardants, personal care products, UV filters, food additives, and pesticides, were included in the in-house suspect list. For the prediction of biotransformation products, hydroxylation (Phase I), as well as O- and N-glucuronidation and methylation (both Phase II) were selected. On the molecular level, hydroxylation, O- or N-glucuronidation and methylation correspond to the addition of oxygen (O), $C_6H_8O_6$ and CH_2 to the molecular formula of the parent conpound, respectively. To predict each of the three considered metabolization reactions for each compound included in the suspect list, the corresponding amounts of C, O and/or H atoms were added to the molecular formulae through an in-house developed R script (RStudio, version 2021.09.1). At this stage, the predicted molecular formula have not been accessed on the probability of their occurrence. This step was performed after matching the suspect list as described below. Molecular formulae and exact monoisotopic masses of the generated metabolites were incorporated in the suspect list, containing >

12,500 compounds in total. The complete suspect list in Excel format is available in the Supporting information.

The suspect screening workflow was based on a previously developed approach (Caballero-Casero et al., 2021b) with some modifications (Figure 1). Two HRMS datasets (one in positive and one in negative ionization polarity) were analyzed applying the same suspect screening workflow.

As a preliminary step, mass accuracy, isotopic pattern, and stability of RT and intensities (area and height) for IS (table S2) in all samples (with the exception of solvent blanks) were checked using the Find By Formula (FBF) algorithm in MassHunter Qualitative Analysis (version 10.0, Agilent Technologies, Santa Clara, USA). In accordance, the native standards (table S1) were analysed in the spiked miliQ standards and the standards that were directly injected into the LC system. Then, molecular feature extraction (peak picking and deconvolution) and alignment of the batch data files were performed using the "Batch recursive feature extraction" algorithm for small organic molecules in MassHunter Profinder (version 10.0, Agilent). The following settings were applied: i) considered ion species: $[M+H]^+$ and $[M+Na]^+$ in ESI+, and $[M-H]^-$ in ESI-; ii) a peak height above 2000 counts; iii) a mass tolerance of 20 and 25 ppm, for parent and product ions, respectively; iv) a maximal RT variation of ± 0.3 min; and v) a match score above 70. A match score has a range from 0-100 and takes into account accurate mass, isotope abundance, isotope spacing and retention time.

After performing a principal component analysis to investigate the general grouping of the different sample types, features were filtered by fold change (FC) analysis applying an FC > 5 between samples and procedural blanks, performed using the Mass Profiler Professional software (version 15.0, Agilent). Next, MassHunter ID Browser (version 8.0, Agilent) was used for compound annotation. The filtered molecular features were screened against the in-house suspect list. The criteria for screening were based on Caballero-Casero et al. and were as followed: i) a mass tolerance of 7 ppm for parent ions, to account for instrument deviation; ii) an isotope abundance score (measured vs predicted) of at least 80, strengthening the match of a feature to a suspected molecular formula; and iii) a match score above 75, including a strict general score for features that are borderline accepted for both accurate mass and isotope abundance (Caballero-Casero et al., 2021b). Which in addition takes into account isotope spacing.

Finally, a manual inspection of each annotated compound in each urine sample was performed using the FBF algorithm in MassHunter Qualitative Analysis. When no fragmentation spectra were available, if only one molecular formula satisfactorily explained the MS spectra of a tentative annotation according to the

abovementioned criteria (mass tolerance: 7 ppm, isotope score > 80, and match score > 75), it was directly assigned as CL4. Otherwise, a combination of *in silico* fragmentation tools, such as ACD/MS Fragmenter (version 2019.1.3, Advanced Chemistry Development Inc., Toronto, Canada) and CFM-ID 4.0 (Wang et al., 2021), and mass spectral databases, such as mzCloud (HighChem Ltd., Bratislava, Slovakia) and MassBank (Helmholtz-Zentrum für Umweltforschung GmbH, 2006), were used to check all fragmentation spectra of tentatively identified compounds. A fragmentation spectrum was considered as matched if at least two fragments matched the reference data at all applied collision energies or when at least three fragments matched the reference data for 2 applied collision energies. In addition, The identification of compounds was based on the confidence level system introduced by Schymanski et al. (Schymanski et al., 2014) With the addition of confidence level (CL) 2C. We defined CL 2C as a feature for which no fragmentation spectra were available but for which the retention time was within 0.2 min. of a reference standard. A diagram of the criteria for the assignment of an identification CL is presented in Figure 1.

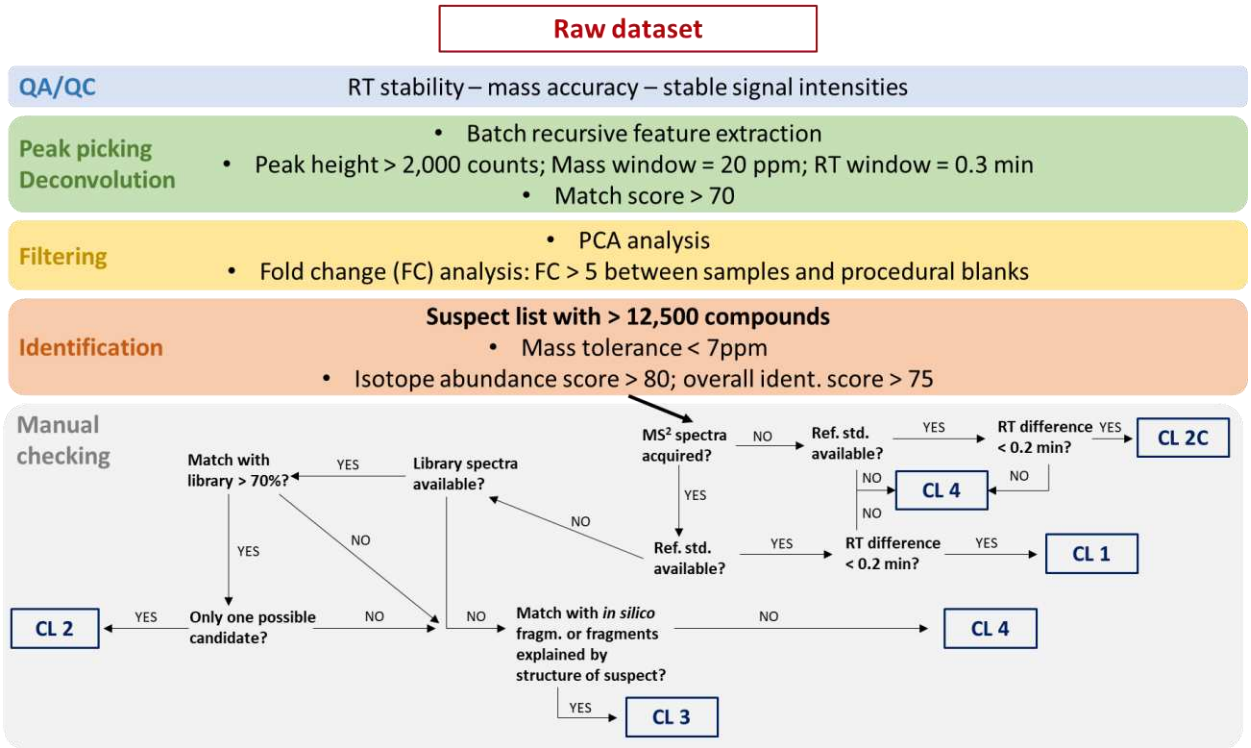

Figure 1: Diagram containing the different steps, cut-off values and criteria used in the suspect screening workflow for the detection and identification of CECs in human urine. CL – confidence level

When a predicted metabolite was tentatively identified, the feasibility of its occurrence in the human body was evaluated considering its structure and the functional groups in which metabolism reactions

could take place (Testa and Krämer, 2006). In addition, annotated endogenous compounds that were not classified under any CEC group were removed from the final results. If more than one isomer could be potentially assigned to a feature, and the experimental data did not allow a distinguishment, all possible isomers are reported. Ultimately, commercially available reference standards were purchased for the compounds assigned with CL 2. The standards were injected applying the same chromatographic conditions (see section 2.4). The data obtained from the standard injection was used for the confirmation of compound assignment (CL 1) applying the same cut-offs as mentioned above. Thereby, CL 1 was assigned if all experimental data (exact mass, isotopic pattern, RT and MS/MS spectra) could be matched with the reference standards. In case, no fragmentation spectra were acquired, CL 2C was assigned to the corresponding samples.

Statistical analysis

For each sample, the total number of detected compounds was submitted to R (RStudio, version 2021.09.1) indicating the assigned CL of identification. From the submitted data, the number of compounds detected at CL 3 and CL 4 or better was calculated.

For all statistical analysis, an in-house R script (RStudio, version 2021.09.1) was applied. The ggplot2 package (version 3.3.5) was used for data visualization. The density plots of both the number of compounds annotated at CL 1-3 and CL 1-4, were visually investigated to ensure the normal distribution of the data. Subsequently, numbers of annotated compounds were compared between low and high exposure groups through a two-sample t-test ($p < 0.05$). For the comparison between high and low exposure groups, the dataset was additionally split in two groups based on sex. The statistical analysis aimed at testing the hypothesis that the exposure to CECs is expected to be significantly higher in the high exposure load group in comparison to the low exposure load group.

3. RESULTS AND DISCUSSION

3.1 Quality control and quality assurance results

All urine samples were spiked at 30 ng/mL with the mixture of labelled IS. The detectability of the IS in the samples was on average 95%, ranging from 83% for chlorpyrifos-d_{10} to 100% for diphenyl phosphate-d_{10} (or DPHP-d_{10}), $^{13}C_4$-2-(((2-ethylhexyl)oxy)carbonyl)benzoic acid (or $^{13}C_4$-oxo-MEHP), $^{13}C_6$-methyl 4-hydroxybenzoate (or ^{13}C-methylparaben) and $^{13}C_3$-3,5,6-trichloro-2-pyridinol (or ^{13}C-TCPY). Detection frequencies for each individual IS can be found in Table S4.

The lower intensities or non-detectability for some IS in some samples can be due to several factors. For example, the ionization sensitivity of a specific IS could influence its detectability, though this was not reflected in the observed trends. The detection frequency (DF) was significantly lower ($p < 0.01$) for the first half of the randomly injected samples. This could point to a variable sensitivity of the QTOF-MS or to analyte losses during sample preparation. However, both polarities, which were separately injected, showed the same trend, which excludes QTOF-MS sensitivity issues. In addition, this trend was not reflected in QC samples and is not maintained within batches, making the loss of sensitivity during sample preparation unlikely. Moreover, specific gravities and exposure loads for samples that had undetected IS were similar to those observed in samples that had all IS detected. Therefore, a complex combination of the abovementioned factors is assumed to contribute to the DF for IS.

The RTs of IS in the samples, which can be found in Table S4, were recorded to estimate the stability of the LC system. The RTs were stable with a standard deviation between 0.01 and 0.03 min. A FC analysis was applied to subtract the background features present in the samples. A feature was eliminated if it had an abundance less than 5 times higher than the average abundance of the feature in the procedural blanks. This allows the analysis of compounds such as, for example, the low molecular weight plasticizers that are present as a contamination in the blanks but show a more than 5-fold higher abundance in urine. This is caused by their presence in the indoor and laboratory environment leading to low-level contamination in the procedural blanks (Christia et al., 2019; Reid et al., 2007). For the blank control samples, the number of features that matched the suspect list is reported. For solvent blanks, the number of detected features was 175 and 135 in positive and negative ionization modes, respectively. For procedural blanks, 543 and 1011 features were detected in positive and negative ionization polarities, respectively. The high number of features detected could be caused by the low abundance cut-off in data analysis, necessary for the detection of low abundant metabolites. For standards of native compounds injected at the beginning and end of the sequence, variance stayed within expected values. All compounds were detected, RT variation was below 1%, area variation of (alternative) plasticizers was between 0.02-23.1% for 6-Hydroxy Monopropylheptylphthalate (6OH-MPHP) and Mono(2-ethyl-5-hydroxyhexyl) adipate (5OH-MEHA), respectively, and area variation of organophosphate plasticizers was between 0.21-43.7% for 3-Hydroxyphenyl diphenyl phosphate (3OH-TPHP) and 5-Hydroxy-2-ethylhexyl diphenyl phosphate (5OH-EHDPHP), respectively.

Ten compounds detected in the FLESH IV target studies that had DF close to 100% (Bastiaensen et al., 2021a; Bastiaensen et al., 2021b; Gys et al., 2021; Verheyen et al., 2021) were selected as positive controls

for the suspect screening approach (Table S5). The DF was between 15% for mono-carboxy isodecyl phthalate and 100% for mono-n-butyl phthalate. A lower DF was expected due to the lower sensitivity of the instrumental method, the less selective sample preparation and chromatographic method. Additionally, mentioned target studies used deconjugation steps resulting in measurements of aglycons only which can contribute to higher sensitivity. Moreover, annotation at a CL better than 4 was not feasible for most of the compounds, due to the absence of MS/MS spectra.

3.2 Suspect screening results

After method optimization and the evaluation of QA/QC results, the samples were analyzed following the procedure described in section 2.6. The matching of the created suspect list against the filtered set of features resulted in a total of 1,806 and 1,677 hits in positive and negative ionization polarities, respectively. However, the number of the matched compounds was lower, as several compounds appeared in the reported list of hits several times at different RTs. Each compound was manually investigated aiming to assign a confidence level of identification following the considerations described in Figure 1. Within this study, only compounds assigned with a CL 3 or better (thus lower) in at least one sample are reported, since the assignment of CL 4 (throughout all samples) allows only a proposal of a tentative molecular formula without any additional information about the structure of the (potential) contaminant. Such tentative reporting was outside the scope of this study and would not allow the interpretation of potential adverse effects of the equivocally annotated contaminants.

Additionally, the assignment of CLs for the annotation of glucuronidated metabolites was challenging. As for all other compounds annotated at CL 3, this level was assigned to a glucuronidated metabolite if fragmentation spectra were obtained which provided additional experimental evidence for the compound's identity. Most of the glucuronidated conjugates included in the suspect list are derived from *in silico* prediction of metabolites, none of the annotated glucuronidated metabolites could be assigned with CL 2 as no library spectra were available. Furthermore, the observed fragmentation spectra only allowed the unequivocal identification of the glucuronide moiety since in most cases no fragments or only one fragment corresponding to the molecular ion of the parent compound could be assigned, not allowing to draw structural conclusions. As an example of this limitation, the fragmentation spectrum of the glucuronidated form of mono(2-ethyl-5-hydroxyhexyl) adipate is shown in Figure 2. The structure of the glucuronide moiety is confirmed by the corresponding fragment ($[C_6H_9O_7]^-$; theoretical *m*/*z* 193.0354),

derived from the glucuronide moiety and not from the parent compound, since the same fragments appeared in several library spectra of other known glucuronides. Only a few other characteristic fragments deriving from this moiety were observed in the mass range between *m/z* 50 and 200, providing limited information about the structure of the parent compound. It can only be confirmed by the observed molecular ion ($[C_{14}H_{25}O_5]^-$; theoretical *m/z* 273.1707) and two losses of water. None of the fragments below *m/z* 200 could be assigned to the parent compound. It was suspected that the fragmentation spectrum of the parent compound was suppressed by the fragments of the presumably better ionizing glucuronide moiety. The same effect was observed for most other glucuronides reported in this study and must be considered within the interpretation of the results. Nevertheless, the assignment of CL 3 was considered to be acceptable in these cases, since the observed fragments confirmed the presence of a glucuronide moiety, and the molecular ion of the parent compounds was observed.

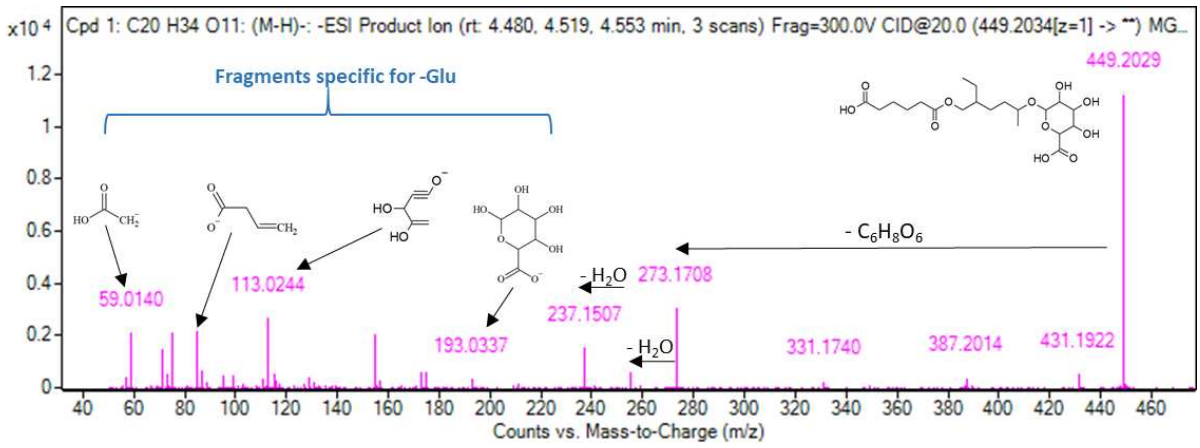

Figure 2: Example of a fragmentation spectrum of a glucuronidated metabolite. The fragmentation spectrum and the proposed structure of the glucuronide of mono(2-ethyl-5-hydroxyhexyl) are shown. For selected fragments specific for the glucuronide moiety proposed structures are indicated.

After manual investigation of all matched candidates, 63 compounds were reported with a CL 3 or better. These compounds belonged to eight different compound classes as summarized in Figure 3. These classes included personal care products (PCPs) (42%), food related compounds (21%), (alternative) plasticizers (11%), organophosphate flame retardants (PFRs) (6%), synthetic antioxidants (5%), parabens (5%), UV filters (2%) and other (8%). All the compounds that are a part of the class of food related compounds have additional uses as personal care products. The different metabolization reactions (Figure 3b) show the high fraction of metabolites found, especially the glucuronidation metabolites. The distribution of the CLs (figure 3C) shows that higher identification levels are most likely for classes of parent compounds for

which, reference spectra and standards are available. Additionally, reference spectra or standards for metabolites of CECs are not readily available, limiting identifications with high confidence.

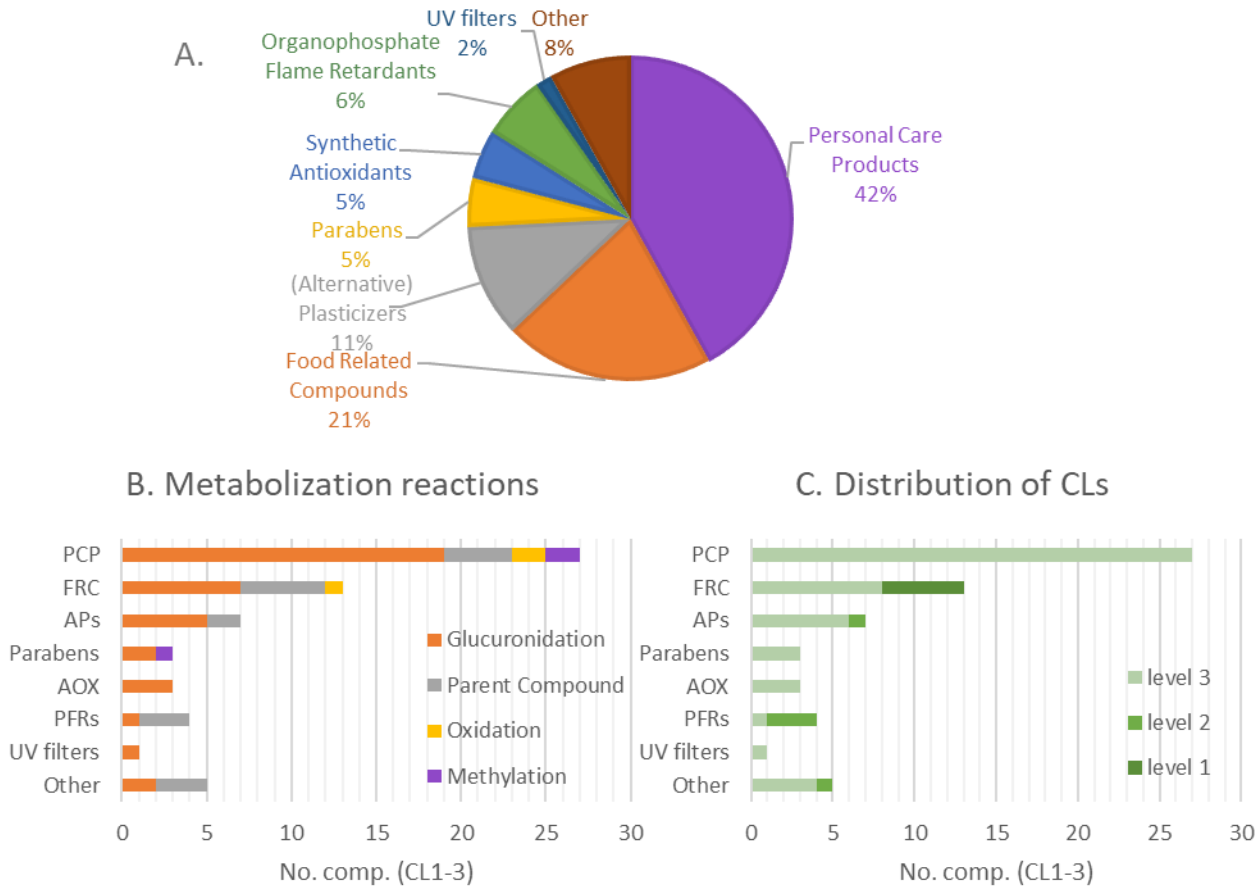

Figure 3: Overview of the compound classes which were included in the 63 compounds detected in urine samples. A) pie chart showing the distribution of the different classes. B) the distribution of the different metabolization products and the parent compounds for each class. C) distribution of the Confidence levels for each class. Abbreviations: PCP; personal care product, FRP; food related compounds, APs; (Alternative) plasticizers, AOX; Synthetic antioxidants, PFRs; phosphate flame retardants

After completing the suspect screening data analysis workflow, from the 63 annotated compounds, 13 compounds were assigned with CL 2. For all CL2 compounds, commercially available reference standards were purchased in order to confirm the annotations. For five compounds, all experimental data (exact mass, isotopic pattern, RT and MS/MS spectra) could be matched with the reference standards using the same mass tolerance window as described above resulting in five CL1 identifications (Table S6). For four compounds, no MS/MS spectra were acquired within sample analysis resulting in fewer identifiers available for compound confirmation. Therefore, level 2C was assigned.

Ultimately, the purchase of reference standards revealed three false positive annotations: Based on the comparison with in silico predicted MS/MS spectra, three compounds (Catechol, Benzyl alcohol and 8-Hydroxyquinoline) were initially assigned with CL2. However, the RTs observed for the corresponding reference standards did not match the samples' data which led to the removal of the mentioned compounds from the results.

All results of CL1 and CL2 assignments are summarized in Table 1, indicating their name, formula, RT, compound class, CL and DF. For each PCP, the subcategory was retrieved from the Chemical and Products Database (CPDat) (Williams et al., 2017). Of the summarized compounds (n = 10), six were assigned to the class of food components/additives, although they are also used as PCPs. For example, theobromine, theophylline, riboflavin (or vitamin B_2) and pantothenate (or vitamin B_3), identified at CL1 in 84, 61, 18 and 69% of the urine samples, respectively, are more likely to originate from food (plants) (Kim et al., 2021) than from PCP exposure. This may provide an explanation for their detection in most samples. Moreover, theophylline is a prescription drug as a bronchodilator for asthma and chronic obstructive pulmonary disease (COPD) (National Institute for Health and Care Excellence [NICE], 2017; National Institute for Health and Care Excellence [NICE], 2018). Theobromine and theophylline have been also identified in a previous suspect screening study on breast milk samples (Baduel et al., 2015). Another compound commonly present in food but as a flavoring agent, named isoquinoline, was detected with a DF of 25% at CL 2 and 35% at CL 4. Apart from dietary intake, exposure to this compound may occur through cigarette smoke and it is also used in the chemical industry as an intermediate (National Library of Medicine USA, 2019).

Among the PFRs investigated in this research, 2-ethyl hexyl phenyl phosphate (EHPHP), a specific metabolite of ethyl hexyl diphenyl phosphate (EHDPHP), diphenyl hydrogen phosphate (DPHP), a non-specific biomarker of EHDPHP and TPHP (Van den Eede et al., 2016), and bis(1,3-dichloro-isopropyl) phosphate (BDCIPP), a specific metabolite of tris(1,3-dichloro-isopropyl) phosphate (TDCIPP), were detected at CL 2, with DFs of 1%, 43%, and 35%, respectively. EHDPHP is an organophosphate used as a plasticizer in food-contact materials and other consumer products (Poma et al., 2017), and TDCIPP, which has been associated with reproductive, dermal and endocrine effects in humans (Meeker and Stapleton, 2010), is used in upholstered furniture and decorative materials. Human exposure to these compounds is predominantly caused by the ingestion of contaminated food and indoor dust, and to a lesser extent by dermal contact (Cequier et al., 2014; Poma et al., 2017; Poma et al., 2018, Xu et al., 2015). The detection of these PFR metabolites is in agreement with previous results of target studies on Flemish adolescents

(Bastiaensen et al., 2021a). In addition, other PFRs and their metabolites, mainly tris-chloro-organophosphates, have also been identified in two previous suspect screening studies on urine (Dolios et al., 2019) and breast milk samples (Baduel et al., 2015), confirming the ubiquitous human exposure to this compound class.

Table 1: Summary of compounds detected at confidence level 1 or 2. For each compound the name, formula, retention time (RT), detection polarity, confidence level (CL), compound class and detection frequency (DF) are indicated.

Name	Formula	RT [min]	Polarity	CL	Compound Class	DF [%]
L-/D-Pantothenate	$C_9H_{17}NO_5$	2.75	-	1	Food, Personal care products (hair and skin care products)	68.7 (CL 1); 16.9 (CL 2C)
4-hydroxy-benzaldehyde	$C_7H_6O_2$	4.99	-	1	Food, Personal care products (fragrance)	36.1 (CL 1); 49.4 (CL 2C)
Diphenyl hydrogen phosphate	$C_{12}H_{11}O_4P$	6.02	-	2C	Organophosphate flame retardants – metabolite	43.4 (CL 2C)
Bis(1,3-dichloro-isopropyl) phosphate	$C_6H_{11}Cl_4O_4P$	6.56	-	2C	Organophosphate flame retardants – metabolite	25.3 (CL 2C)
2-ethyl hexyl phenyl phosphate	$C_{14}H_{23}O_4P$	7.67	-	2C	Organophosphate flame retardants – metabolite	1.2 (CL 2C)
Theobromine	$C_7H_8N_4O_2$	3.78	+	1	Food, Personal care products (skin conditioning)	84.3
Theophylline	$C_7H_8N_4O_2$	4.17	+	1	Food, Personal care products (skin conditioning)	61.4 (CL 1) 2.4 (CL 2C)
Phthalic anhydride	$C_8H_4O_3$	4.88	+	2	Plasticizers - metabolite	6.0 (CL 2); 74.7 (CL 4)
Riboflavin	$C_{17}H_{20}N_4O_6$	4.92	+	1	Food, Personal care products (cosmetic colorant)	18.1 (CL 1); 39.8 (CL 2C)
Isoquinoline	C_9H_7N	4.99	+	2C	Food, Other	74.7 (CL 2C)

[b]: RT in samples matched with RT of the corresponding native standard, but no fragments were observed in samples for further confirmation.

Table S7 shows the name, formula, RT, compound class, CL, and DF of the 53 compounds annotated at level 3 in the 83 urine samples. Out of the 53 compounds, 39 were PCPs, 6 alternative plasticizers, 3 antioxidants, 3 parabens, 1 UV-filter, 1 PFR, among others. Among the potential candidates, 15 were also food components/additives. Due to the lack of libraries with reference MS/MS spectra of metabolites, most compounds with CL 3 were predicted metabolites (85%), with glucuronides being the most abundant

ones (77%), followed by methylated (6%) and hydroxylated (4%) compounds. The most relevant findings and compounds annotated at CL 3 with a high DF are discussed in the following paragraphs.

Among PCPs, the most frequently detected compounds were L-/D-Pyroglutamic acid (DF = 98.8% at CL3), an (uncommon) amino acid derivative that is naturally present in some plants (Wishart et al., 2022) and is also used in cosmetic products, benzyl alcohol (DF = 79.5% at CL3 and 20.5% at CL4), which is a flavoring agent also used as a solvent in the production of perfumes, naphthylamine (DF = 44.6% at CL3 and 55.4% at CL4), a urinary biomarker of exposure to amino and nitro PAHs (He et al., 2021; Niu et al., 2018; Yu et al., 2020), two metabolites of nail conditioning products, i.e. the oxidation product of 1-N-(2-methoxyethyl)-benzene-1,4-diamine (DF = 75.9 % at CL3 and 24.1% at CL4) and the glucuronide of 2-carboxyethyl acrylate (DF = 94% at CL3) (Dionisio et al., 2018), and the glucuronide of (4Z)-hept-4-en-2-yl salicylate (DF = 18.1% at CL3 and 78.3% at CL4), normally used in fragrances. Most of these compounds can cause harmful effects on human health (Wishart et al., 2022) (U.S. Food & Drug Administration, 2012) (Dionisio et al., 2018; Williams et al., 2017), and have not been extensively addressed yet in HBM studies.

In the case of parabens, the most abundant metabolites were the methylated products of butyl paraben (DF = 80.7% at CL3 and 19.3% at CL4), although no information about the methylation of parabens in the human body has been published in the literature yet. Glucuronides of benzyl paraben isomers or benzophenone-3 (both have the same molecular formula), as well as isomers of propyl paraben were also detected in more than 40% of the samples, but less than 4% could be assigned with a CL 3. Baduel et al. (2015) and Tran et al. (2020), and Gerona et al. (2018) have also identified parent compounds of these and other parabens in breast milk and human serum samples, respectively, by suspect screening strategies.

In the case of APs, mono(2-ethylhexyl) adipate (Gakidou et al.) derivatives (Gluc-MEHA, 5-OH-MEHA and Gluc-5-OH-MEHA), which are metabolites of bis(2-ethylhexyl) adipate (Hermabessiere et al.), were annotated at CL 3 with DFs between 6 – 24%, which are in line with the targeted results of the FLEHS study (Bastiaensen et al., 2021a; Buekers et al., 2021). In addition, glucuronidated conjugates of phthalates, i.e. MEHP, MnBP, MiDP and MiNP, were found at CL 3 with DFs between 2 – 17%, although these compounds were detected in more than 30% of the samples in the targeted FLEHS study. This difference is assumed to be caused by the lower sensitivity of suspect screening approaches compared to the targeted methods (Bastiaensen et al., 2021a). Unconjugated compounds of these phthalates have been previously reported by a suspect screening study on human serum with DFs up to 90% (Gerona et al., 2018).

For antioxidants, the glucuronidated Irganox 1135 was identified at a CL3 in 92.8% of the urine samples. Despite having been previously detected in environmental and consumer products, such as in house dust, air particles, and car seats for children (Wu et al., 2019), this is the first study reporting the presence of this compound in human samples. Among the UV-filters included in the suspect list, only the glucuronide of homosalate, commonly used in sunscreen formulations, was detected with a DF > 80% (CL 3 = 1.2% / CL 4 = 83.1%). This is in agreement with a recent HBM study conducted in Eastern China, which showed a high DF > 75% of unconjugated homosalate at median concentrations of 0.16 ng/mL in urine (Ao et al., 2018).

3.3 Comparison with literature

Several compounds identified/annotated here, such as Irganox 1135, methylated products of parabens and some glucuronides, have not been previously determined in HBM studies on urine (Bonvallot et al., 2021; González-Gaya et al., 2021; López-García et al., 2019; López et al., 2016; Tang et al., 2016). As an example, Plassman et al. (2015) performed a suspect screening study on CECs, which were also included in the present research, but they only tentatively identified less than 10 compounds, most of them food items. The study did not report any of the compounds identified/annotated here, which may be due to the differences in the applied methodologies, since they used pooled samples, a different sample preparation method (deconjugation), and only included 1,500 compounds in their suspect list. Other compounds, i.e. metabolites of pesticides (Bonvallot et al., 2021; López-García et al., 2019; López et al., 2016), PFRs (Dolios et al., 2019), and PAHs (Tang et al., 2016) have been previously identified in urine samples using other suspect and non-target strategies. However, the chemicals and metabolites found in those studies were not detected in the present study, probably because the sample preparation approach was not optimized for these specific contaminant groups and/or since these groups were not included in the applied suspect list. In other studies, some parabens and phthalates, that were annotated here as conjugated metabolites, were identified in breast milk (Baduel et al., 2015; Tran et al., 2020) (Baduel et al., 2015) and serum samples (Gerona et al., 2018) as unmetabolized compounds.

Compared to a previous suspect screening approach which also aimed to identify CECs in urines of the FLEHS IV (Caballero-Casero et al., 2021b) a higher number of CECs were annotated in the present study (63 compounds compared to 45 for Caballero-Casero et al. at CL 3 or better). This is assumed to be caused by the larger suspect list (10,000 vs 12,500 entries) and the higher number of analyzed samples (50 vs 83). In addition, some differences were observed in the classes of annotated compounds. For example, most of the features reported in the present study were matched with PCPs (42%) and no pesticides were

detected, while Caballero-Casero et al. (2021) found more frequently plasticizers (40%) than PCPs (31%), and 7% of the detected compounds were matched with pesticides and/or their metabolites (Caballero-Casero et al., 2021b). However, similar findings were observed when comparing parent compounds vs metabolites, since in both studies more than 60% of the tentatively identified compounds were predicted metabolites, predominantly glucuronides. Due to the lack of native standards of glucuronide conjugates, a deconjugation step would be necessary if targeted methods are used to quantify these compounds in urine samples.

Compared to the study conducted by Caballero-Casero et al., a higher number (n = 63) of annotated compounds is reported here which indicates that the applied suspect screening approach is a valuable tool for the detection of unknown CECs and their metabolites. These reported compounds would remain undetected if only targeted biomonitoring approaches would have been applied. Nevertheless, the annotation of only 63 compounds at CL 3 or better using a suspect list of >12,500 entries indicates limitations of the applied workflow. Firstly, by including possible metabolization products we strongly decrease the false negatives as opposed to only using the parent compound. However, adding metabolization products based on molecular formula is efficient but largely increased the entries in the suspect list making it unfeasible to include all possible metabolization reactions. In addition, it should also be noted that in this study only 3 metabolization reactions have been included in the predictions. Although these are the most frequent, other metabolization reactions are not negligible. Moreover, in an *in vivo* scenario metabolization products can be based on several metabolization reactions, of special mention is the combination of hydroxylation and glucuronidation. It is recommended to include these in future studies. Alternatively, predictions software can be used resulting in a higher chance of including realistic metabolites in the suspect list. However, this approach is currently not feasible with large amounts of entries. Secondly, the applied acquisition approach (Auto MS/MS) fragments the 4 highest features at a given time, resulting in a limit of fragmentation spectra generated. Other techniques such as iterative MS/MS expand on the number of fragmentation spectra generated but increase the analysis time by at least 3-fold (Koelmel et al., 2017). For an increase in the annotation of compounds at CL2 or better further improvements of the available reference mass spectral libraries or of the available standards are needed (Picardo et al., 2021). Furthermore, the application of novel approaches in data processing, such as in silico deconjugation methods, could allow resolving the above-described challenges within the identification of glucuronidated metabolites (Huber et al., 2022).

3.4 Results of the statistical analysis

The numbers of assigned compounds were compared between high and low exposure groups in order to investigate whether a significant difference could be observed (Figure 4).

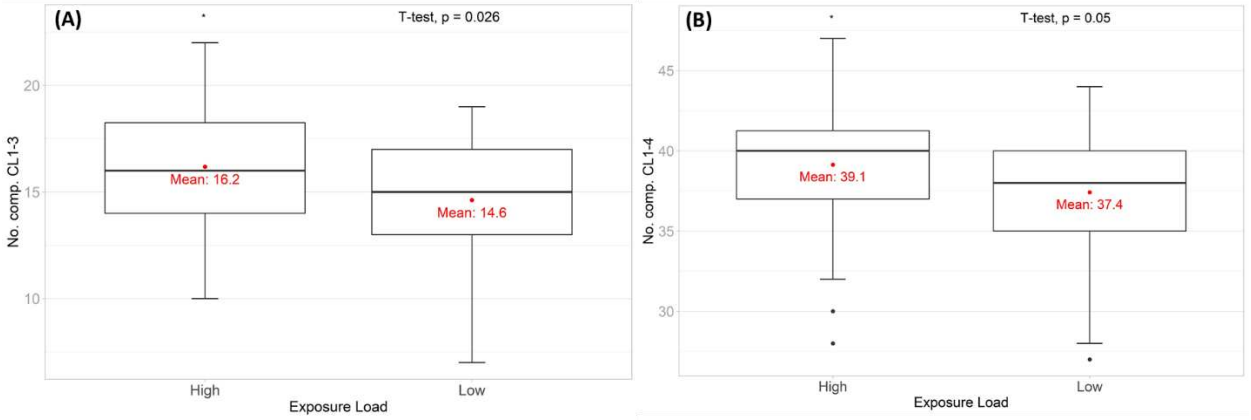

Figure 4: Boxplots representing the number of annotated compounds in the low and high exposure load groups. (A) Only compounds annotated at CL 3 or lower are considered. (B) All compounds reported in this study (i.e. CL 1-4) are considered. (*) Significant difference between mean values ($p < 0.05$).

Thereby, the comparison was made including only compounds assigned at CLs 1-3 as well as all compounds reported (i.e., assigned CLs 1-4). In both cases, the number of assigned compounds differed significantly ($p < 0.05$) between the high and low exposure groups. When considering only compounds assigned with CLs 1-3 mean values of 15.2 and 13.4 were observed for the high and low exposure groups, respectively. To further investigate which compounds contribute to the observed significant difference, the number of detections at CL 1-3 was compared between the high and low exposure groups for each compound separately. Of the 63 reported compounds, for 41 compounds the number of detections was higher in the high exposure group. However, it should be noted that the total number of samples in the high exposure group was 44, while the low exposure group contained 39 samples. Therefore, for compounds whose DFs differed by less than five detects, the observations might be biased by the slight differences in the sample size. Therefore, only compounds which differed by at least five detects between the high and low exposure groups (n = 13) are listed in Table S8 as they are assumed to have an unbiased influence on the observed statistical differences.

Ten out of the 13 compounds belong to the class of PCPs which is in line with the fact that most compounds reported in this study belong to this group.

Figure 5 shows the comparison of high and low exposure load groups divided by sex. Only compounds assigned with CL 1-3 were considered. This approach showed that the observed significant differences were caused by the significantly different numbers of detected compounds in high and low exposure load

samples from female participants (p = 0.0038). For samples from male participants, no significant differences could be observed. It is assumed that PCPs, which were the most frequently detected compound group in this study, are used more often or more extensively among females. Yet, no significant differences for neither of the sexes could be observed between high and low exposure load groups when CL 4 compounds were considered (Figure S2).

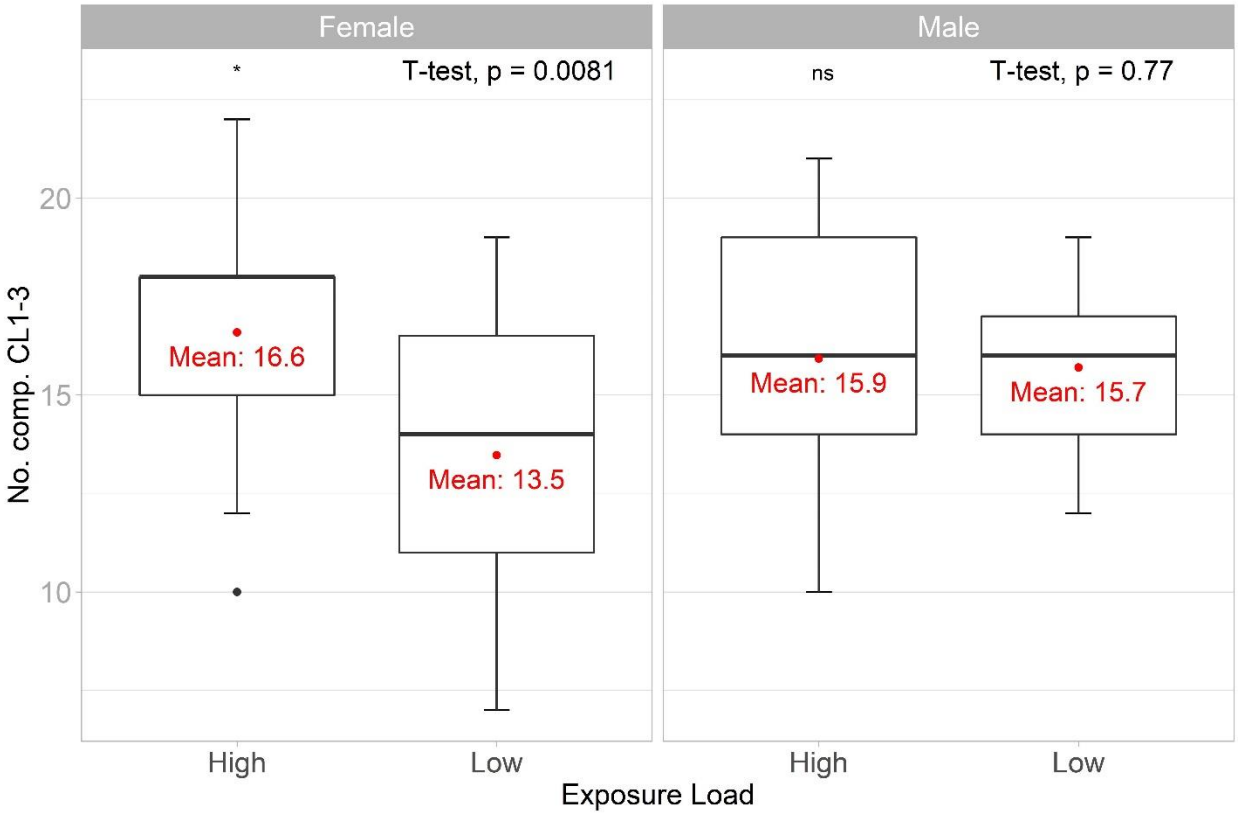

Figure 5: Boxplots representing the number of annotated compounds in the low and high exposure load groups. Each group was divided based on sex. Only compounds annotated at CL 3 or lower are considered. (**) significant difference between mean values (p<0.01); ns: not significant.

In conclusion, the number of detected compounds differed significantly between high and low exposure groups for samples from female participants. However, a few factors have to be considered in the interpretation of results. The size of the suspect list and the high number of included compound classes do not allow a full optimization of the sample preparation and chromatographic methods for all compounds equally. Therefore, it cannot be excluded that the applied method favored a particular compound class resulting in higher DFs and ultimately leading to the observed significant differences. In addition, when the deviation between sex is made the number of participants in the high and low exposure groups was rather small.

4. CONCLUSIONS

The present study describes the analysis of 83 urine samples from Flemish adolescents by applying a suspect screening workflow and suspect list containing > 12,500 CECs and their metabolites. The screening yielded the identification of 5 compounds (CL1) and the tentative identification of 63 compounds (CL2-3) of which several have not been previously reported in urine. This clearly indicates the added value of suspect screening as a complementary tool to common targeted approaches in HBM. Due to the high number of hits (most of them unknowns) obtained using the suspect screening approach, the need for risk assessment of exposure to mixtures is evidenced. Several possibly toxic compounds that are not currently quantified in HBM programs have been tentatively identified. For example, several PCPs (e.g., benzyl alcohol) and Irganox 1135 , were detected at high detection frequencies, showing a need to include them in targeted HBM studies. The comparison of the number of detected compounds between high and low exposure groups revealed a significant difference ($p < 0.05$). When differentiating between sexes, this difference could only be observed between high and low exposure groups of females ($p < 0.01$). In comparison with target HBM studies, this study shows that higher exposure to targeted contaminants also encompasses higher exposure to the newly identified CECs, especially for female participants, which points towards a higher exposure of personal care product related compounds for female participants as opposed to male participants. Consequently, more investment in suspect screening as a tool to support, enhance, and complement quantitative targeted studies is necessary. Apart from suspect screening, a full non-targeted approach could be applied to further identify new CECs.

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