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Hair as an alternative matrix to monitor human exposure to plasticizers – Development of a liquid chromatography - tandem mass spectrometry method

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Abstract

The application and production of alternative plasticizers (APs) has been increasing in the last decade in replacement of conventional phthalates due to their toxicity. This calls for the development of non-invasive monitoring approaches to assess human exposure to APs. A method for the simultaneous measurement of exposure biomarkers of di(2-propylheptyl) phthalate (DPHP), di(isononyl)cyclohexane-1,2-dicarboxylate (DINCH), di(2-ethylhexyl) terephthalate (DEHTP) and di-2-ethylhexyl adipate (DEHA) in hair samples was developed and validated in this study. Prior to the analysis, the hair samples were washed in acetone and ultrapure water and pulverized to powder. Further, a solid-liquid and solid-phase extraction, followed by quantification using liquid-chromatography - tandem mass spectrometry (LC-MS/MS) was performed. The method was validated in terms of recovery, matrix effects, carry-over, linearity, limits of quantification, within- and between-run precision and trueness. Satisfying results were obtained for all targeted compounds, except for mono(2-ethylhexyl) adipate (MEHA), which was monitored only qualitatively. The optimized method was implemented in a pilot biomonitoring study with hair samples from 9 healthy volunteers. Detection frequencies of seven metabolites ranged from 11% to 100%. Mono(2-ethylhexyl) terephthalate (MEHTP) and mono(2-ethyl-5-oxohexyl) adipate (oxo-MEHA) were found in all hair samples. More hydrophobic monoester metabolites were found to be incorporated in hair to a greater extent compared to their oxidized counterparts. Obtained results show that the developed method can detect AP metabolites in hair, supporting the use of this alternative matrix in human biomonitoring.

Keywords

Alternative biomatrix, Hair, Alternative plasticizers, metabolites, human biomonitoring, LC–MS/MS

1. Introduction

Plasticizers are the key factor that provides durability, elasticity, and flexibility to polymeric products [1, 2]. Phthalate esters (PEs), the most widely applied plasticizers, exhibit a large variety of physicochemical properties and toxicities. PEs can be released into the environment through volatilization, abrasion, leaching or even direct diffusion from the surface of polyvinyl chloride plastics (PVC) such as flooring, food containers and toys [3]. PEs have been shown to cause adverse health effects in humans [4-6]. Some conventional PEs (i.e. butylbenzyl phthalate (BBP), di(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP) and diisobutyl phthalate (DIBP)), was banned in EU for childcare products and toys since 2015 and completely banned for consumer products have more than 0.1% content of PEs in 2017 [7, 8]. Alternative plasticizers (APs), which are expected to migrate less and have lower toxicity compared to PEs [9], were introduced to meet the similar restriction in EU and United States [10]. Among these, di(2-propylheptyl) phthalate (DPHP), di(isononyl) cyclohexane-1,2-dicarboxylate (DINCH), di(2-ethylhexyl) terephthalate (DEHTP) and di-2-ethylhexyl adipate (DEHA), were introduced as replacements for PEs, in particular for PVC [11-14].

Although a study showed that DINCH and DPHP did not affect testosterone production in an *in vivo* experiment using CD-1 cells [15], exposure to these chemicals and their metabolites could still have adverse health effects in humans. In fact, Eljezi *et al.* found that DINCH and its metabolites (*i.e.*, cyclohexane-1,2-dicarboxylic mono isononyl ester (MINCH)) showed cytotoxicity on L929 cell lines. Similar to mono(2-ethylhexyl)phthalate (MEHP), the bioactive metabolite of di(2-ethylhexyl)phthalate (DEHP), the metabolization of APs seems to increase their toxicity [16]. One of the main metabolites of DINCH, *i.e.* MINCH was found to be a potent PPAR-α agonist and a metabolic disruptor, capable of inducing SVF preadipocyte differentiation, which may interfere with the endocrine system in mammals [17]. An *in silico* approach using Induced Fit Docking method for DEHTP and DINCH against human sex hormone-binding globulin (SHBG), found that these APs fit well into the steroid binding pocket of SHBG, suggesting they might be able to interfere with the homeostatic function of human steroids [18].

Since primary and secondary metabolites of conventional PEs were already evaluated as biomarkers of human exposure [19], several studies have determined the human internal exposure to APs using metabolites. Most human biomonitoring studies use urine as biomatrix [20-22]. While spot urine samples are easy to collect, the low concentration of target analytes and the limited time window for which it is representative, are among its main disadvantages

[23, 24]. Hair has been increasingly used as a complementary matrix to urine since it presents some advantages. Hair is a complex matrix consisting of cylindrical shafts of tightly packed cells with the cuticle forming an outer protective layer and it is predominantly composed of Sulphur-rich proteins [25]. The hair follicle is surrounded by capillary blood vessel systems at the root, which provides material for the hair growth cycle. The latter is composed by the anagen (active growing), catagen (transition) and telogen (resting) stages. During the anagen growing phase of the hair shaft, there is an increase in metabolic activity, cell division and growth. Usually, it is at this stage that chemicals are considered to be incorporated in hair [26]. Consequently, chemicals found in serum can migrate into hair, making it a suitable matrix to assess exposure [27]. External contamination may occur through contact with chemicals in the environment (*e.g.*, air and dust). Hair has been used as a suitable matrix to assess integrated exposure including both internal and external exposure. As it is also a noninvasive sampling method, it can be used to monitor exposure in vulnerable populations such as neonates and thus provide an approach to assess maternal exposure to chemicals. Furthermore, hair samples reflect both short- and long-term exposure, which is a significant advantage in human exposure assessment [23, 28]. Polychlorinated biphenyls (PCBs), per- and polyfluoroalkyl substances (PFASs), organophosphorus flame retardants (PFRs), conventional phthalates and/or their metabolites have been measured in hair to monitor human exposure [29-33].

Owning to the utility of hair to monitor human exposure to environmental contaminants and its advantages when focusing on populations difficult to sample, such as neonates, the aim of this study was to develop and validate a method to assess human exposure to DPHP, DINCH, DEHTP and DEHA by measuring their exposure biomarkers in hair samples. To achieve this, a sample preparation protocol based on solid-liquid extraction and solid phase extraction (SPE) was developed. Conditions for i) hair denaturation, ii) hair wash, and iii) solid-liquid extraction process, were optimized during method development. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was used to analyze and quantify target analytes.

2. Materials and Methods

2.1 Chemicals and reagents

The targeted exposure biomarkers of DEHTP, DEHA, DINCH and DPHP are listed in Table 1. Metabolites of DEHA, DEHTP and isotope labelled standards of OH-MPHP-D_4, Cx-MPHxP-D_4 and oxo-MPHP-D_2 were purchased from Synchem (Elk Grove Village, IL,

USA), DPHP metabolites were obtained from BASF (Ludwigshafen, Germany), while DINCH metabolites and corresponding isotope labelled standards (*i.e.*, MINCH-D_2, OH-MINCH-D_4, Cx-MINCH-D_2) were kindly provided by Dr. Koch (IPA Bochum, Germany).

Stock solutions of both native and isotope labelled internal standards (IS) were prepared using acetonitrile at 1 µg/mL. Analytical grade formic acid (99-100%), sodium hydroxide (NaOH) and nitric acid (HNO_3) were purchased from Sigma-Aldrich (Bornem, Belgium). Dichloromethane (for gas chromatography ECD and FID), acetone (for gas chromatography ECD and FID), methanol (LC-MS grade) and acetonitrile (LC-MS grade) were purchased from Merck (Merck, Darmstadt, Germany). The phosphate buffer saline (PBS, pH=6, 1 M) was prepared according to Dulbecco's method [34] and stored at 4°C before use.

Ultrapure water was obtained from a PURELAB Flex system (ρ = 18.2 MΩ/cm, Elga Veolia, Tienen, Belgium). Oasis HLB extraction cartridges (3 mL, 60 mg) were purchased from Waters (Waters, Zellik, Belgium). A Visiprep SPE vacuum station with 24 ports (Sigma-Aldrich) was used in the SPE steps in this study.

2.2 Hair collection and pretreatment

Hair samples used in this study were collected from adult volunteers (n=9) without known occupational exposure to the selected APs according to Kucharska et al. [31]. Samples were wrapped in aluminium foil and stored at room temperature in the dark. No additional circumstantial information was collected for this study. Prior to analysis, hair samples were washed in both UPW and acetone for 1 min, air-dried and pulverized using a ball mill (Retsch MM 400, Retsch Benelux, Aartselaar, Belgium) for 5 min at 30 Hz.

2.3 Solid-phase extraction

The following protocol was used for SPE: Oasis HLB cartridges (60 mg/3 mL) were preconditioned using 3 mL of dichloromethane, methanol and ultrapure water. Samples were then loaded, sample tubes rinsed twice with ultrapure water and loaded onto the cartridges. These were then washed with 3 mL of 5% methanol in ultrapure water and vacuum dried for 30 min. The target analytes were then eluted with 6 mL of methanol. The eluate was then dried under a gentle stream of nitrogen and reconstituted in 100 µL of acetonitrile/ultrapure water (1:1, v/v) for further analysis.

2.4 Optimized hair extraction process

For each donor, 50 mg of milled hair were weighed in a pre-conditioned glass tube (*i.e.*, baked at 300° C and rinsed with ultrapure water and acetone). IS (5 ng) was subsequently added and the sample was sonicated for 30 min using 2 mL of methanol followed by 2 mL of PBS. After sonication, samples were centrifuged, and the supernatant transferred to a glass tube. Methanol extracts were dried under gentle nitrogen flow and re-dissolved using the PBS extract. The latter was then processed using the detailed SPE protocol. Eluates were finally reconstituted in 100 µL of acetonitrile/ultrapure water (1:1, v/v) for analysis.

2.5 Instrumental analysis and quantification

Analysis of target metabolites was carried out using an Agilent 1290 Infinity liquid chromatography system coupled to an Agilent 6460 Triple Quadrupole mass spectrometer (LC-MS/MS, Agilent, Santa Clara, USA) equipped with an electrospray ionization source in negative mode (ESI-). Method details are described in the Supporting information. Briefly, the separation was achieved using a Phenomenex Kinetex Biphenyl column (2.1 x 100 mm, 2.6 µm, Phenomenex, Torrace, USA) using UPW and acetonitrile, both containing 0.05% formic acid, as mobile phase (Table-S1). The acquisition was carried out in dynamic multiple reaction monitoring (dMRM) (Table-S2). Quantification of target metabolites was performed using 8-point calibration curve ranging from 0.1 ng to 75 ng. The correlation coefficients (R^2) of the calibration curves were all above 0.99. The method can baseline separate the metabolites of DEHTP (MEHTP and 5-OH-MEHTP) with the metabolites of DEHP (MEHP and 5-OH-MEHP), which share similar structures, as well as MRM transitions (Figure-S1) [38]. However, this method is not able to separate the metabolites of diisodecyl phthalate (DIDP) and DPHP. Thus, the results for DPHP metabolites should be interpreted as the contribution of DIDP and DPHP in this study [39].

2.6 Method validation

Sample preparation and instrumental analysis were validated following the protocols established by the European Medicines Agency [40].

Matrix effects were evaluated using a blank matrix spiked with native standards (at 0.4 ng (8 ng/g) and 60 ng (1200 ng/g)) and IS after extraction. Analyte response in procedural blanks was subtracted from the response of analytes in the sample extracted after extraction, which was then compared to the response of analytes in solvent. Samples from four different donors were used.

Calibration curves were analyzed in triplicates to estimate the correlation coefficients (R^2). Carryovers were assessed by injecting solvent blanks immediately after the analysis of the highest calibration point.

Within- and between-run precision and trueness of the method were assessed over the course of three days using hair matrix from a single donor (50 mg) spiked with target analytes at at 0.4 ng (corresponding to 8 ng/g), 6 ng (120 ng/g) and 60 ng (1200 ng/g) concentrations and processed as described above (*i.e.*, sequential extraction with methanol and PBS followed by SPE). On each day, three replicates per spiking level (five for the lowest level on day one), one blank matrix and one procedural blank were processed. All samples and blanks were spiked with IS (5 ng, 100ng/g) prior to processing. As blank matrix is not available, trueness was calculated by subtracting the concentration measured in blank matrix from the concentration measured in low, mid and high spiked samples. Precision and trueness were considered satisfactory if results were < 15% or < 20% (for low spikes). Method detection and quantification limits (MDL and MQL) were determined using blank or low spiked hair samples giving a signal-to-noise ratio (S/N) of 3 and 10, respectively. Recoveries of the extraction process were estimated using hair samples spiked with native and mass labelled reference standards (at low and high concentrations) before and after extraction [41]. Matrix effects were assessed by comparing the signal of reference standards in samples spiked after extraction with calibration standards prepared in acetonitrile/ultrapure water (1:1, v/v) [41]. Analyte signals recorded in non-spiked samples (*i.e.*, background) were subtracted from analyte signals in post-extraction spikes prior to matrix effect calculation.

Hair samples from three different donors were extracted in triplicate, to calculate the within-run precision using different matrices. These samples were only spiked at mid concentration.

2.7 Pilot biomonitoring study

To evaluate the applicability of the developed method, hair samples from healthy volunteers (n=9) were processed and analyzed using the optimized method. The sampled populations were not expected to be occupationally exposed to the targeted chemicals.

3. Results and Discussion

3.1 Method development

3.1.1 Hair denaturation process

Unlike blood, urine or other conventional biological matrices, biomarkers incorporated in hair are expected to be stable for months or even longer [42], could be a result to limited enzymatic activity in the hair segments. Thus, concerns have been raised as to how to release the target compounds from the hair structure and increase the extraction yields [43]. To achieve this, denaturation of hair prior to extraction was often proposed. Most commonly, strong acids or bases have been used [36, 37, 44, 45]. Because in acidic or basic conditions both parent APs and their metabolites are likely hydrolyzed to monoesters or further oxidized, strong acids and bases were not considered here. Therefore, digestion experiments were carried out using diluted acid (10% HNO_3) and base (0.05 M of NaOH). The stability of target metabolites during denaturation was tested by spiking native and IS in 2 mL of the diluted acid/base, adjusting to pH 6 using PBS and extracting them using the described SPE protocol. Recovery relative to native compounds spiked in PBS and extracted using SPE was used to assess the stability of the target analytes. Results showed that both acid and base denaturation substantially decrease the recovery of AP metabolites. The recovery for target analytes decreased with acid (by 11%-45%) and basic (by 11%-85%) denaturation (Figure S2). Based on these results it was decided to avoid denaturating hair samples prior to extraction to avoid degrading the target analytes.

3.1.2 Washing procedure

Although it is difficult to find an ideal procedure which removes only external contamination without affecting compounds embedded in the hair [31], removal of external (environmental) contamination is still necessary to obtain more reliable information about the integral exposure [35].

In our study, a mild washing procedure was adopted based on a method developed by Cappelle *et al* [46]. for the extraction and analysis of illicit drug metabolites in hair. Hair samples (i.e., approximately 2 g) were washed in ultrapure water and acetone, both for 1 min, and then air-dried.

The washing solutions (50 mL for each solution) were tested for potential removal of target analytes from the processed samples. Ultrapure water was extracted using the described SPE protocol, while acetone was dried under a gentle flow of nitrogen and reconstituted in acetonitrile/ultrapure water (1:1, v/v). Three monoester metabolites were found in the washing solution. MEHA (0.7 ng) and MEHTP (0.25 ng) were observed in the acetone, whilst MINCH (0.6 ng) was found after washing hair with ultrapure water. Monoester metabolites measured in washing solutions were also found in procedural blanks. In both cases, however, measured

concentrations were low, considering that approximately 2 g of hair were washed, thus analyte losses due to washing were considered negligible.

3.1.3 Selection of solid-liquid extraction solvents

The selection of the solid-liquid extraction solvents needs to take into account the different polarity of the considered analytes. For instance, hydroxy- and carboxy-metabolites are expected to be more hydrophilic compared to hydrolysis metabolites. In particular, three organic solvents, namely methanol, acetone and acetonitrile and two water-based solvents, ultrapure water and PBS (1 M, pH 6) were chosen. Spiked hair samples from a single donor were sonicated for 1 h in one of the selected solvents and then centrifuged at 2500 g for 10 min. The supernatants were collected, evaporated and analyzed to assess the extraction yields. Results indicate that none of the solvents was suitable for extraction from the hair as most recoveries were below 80% (Figure 1A). For MEHA, OH-MEHA, oxo-MPHP, and MINCH, best results were obtained with methanol, while between ultrapure water and PBS, the latter showed higher recovery except for oxo-MEHA, Cx-MINCH, Cx-MPHxP and oxo-MPHP.

These variable results could be linked to the wide range of metabolites which are being considered in this study, which cannot be efficiently leached from hair using a single solvent. Consequently, as sequential extraction was considered as a possible alternative to increase solid-liquid extraction yield, it was decided to evaluate it using both an organic solvent and an aqueous solution [47, 48]. Based on the obtained results, a sequential solid-liquid extraction process using methanol followed by PBS and vice-versa was investigated. Supernatants were collected from each step. The methanol extract was evaporated using a gentle flow of nitrogen, re-dissolved using the PBS extract and then processed using the described SPE protocol (Figure 1B). Recoveries using PBS followed by methanol were lower, likely due to the precipitation of residual salts from the buffer after Methanol addition. On the other hand, the solid-liquid extraction process using first methanol followed by PBS provided better results, also compared to the solid-liquid extraction process using individual solvents, and was thus selected for the optimized procedure.

3.1.4 Time for solid-liquid extraction process

The time for solid-liquid extraction was optimized for the sequential solid-liquid extraction process (*i.e.*, methanol and PBS) by processing spiked hair samples for 30, 60, and 120 min for each step. Results were shown in Table S3. The optimal solid-liquid extraction time was chosen based on the average recovery, calculated as the ratio of the peak area of

target analytes in samples spiked before and after solid-liquid extraction process. A solid-liquid extraction time of 30 min for both methanol and PBS provided the best compromise in terms of duration and recovery.

3.2 Recovery and matrix effect of the optimized method

As blank hair samples are not available, samples from four different donors were used to evaluate the recovery of the whole procedure. The latter was calculated by comparing the peak areas of native standards spike before and after the extraction in hair from different donors.

The average overall recovery (*i.e.*, solid-liquid extraction followed by SPE) ranged between 78 to 113% (Figure S3), with RSDs < 15% for all compounds.

Matrix effects were evaluated using a blank matrix spiked with native (at 0.4 ng(8 ng/g) and 60ng (1200 ng/g)) and IS after extraction. Analyte response in procedural blanks was subtracted from the response of analytes in the sample extracted after extraction, which was then compared to the response of analytes in neat solvent. Sample from four different donors were used. Matrix effects ranged from -9% to -94%, with RSDs below 15% for all compounds (Figure S4). As it was pointed out by many researches, proper clean-up process is critical in the method development process as matrix effects from complex biological matrices may have strong impact on the accuracy and the precision [49, 50]. In our method, all analytes presented signal suppression in the instrumental analysis, which could lower the instrumental sensitivity and thus lead to higher limit of quantification. Except for MEHTP, which had the lowest matrix effect (9%), the secondary metabolites for APs showed lower matrix effect than the corresponding monoesters. For DPHP metabolites, the matrix effects ranged from -86% to -93%, thus leading to higher limit of detection and lower detection frequency in real sample analysis.

The use of isotope-labelled internal standards is essential to the quantification process [51], however, it was not available for all target chemicals. Best matched isotope-labelled internal standards for quantification were chosen based on the bias of trueness in standard spiked matrix samples based on the quantification, and further used in the quantification (Table S2).

3.3 Method validation

Calibration curves were prepared using a mixture of native standards ranging from 0.1 ng to 75 ng (IS concentration of 5 ng in all calibrators). Calibration curves were computed using

second degree polynomials with a weight factor of 1/x. Correlation coefficients (R^2) were all > 0.99 (Table 2). Instrumental limits of detection (LOD_i) and quantitation (LOQ_i) were estimated from standards giving a signal-to-noise ratio of 3 and 10, respectively. The LOD_i and LOQ_i varied from 9 to 153 pg/mL and 29 and 510 pg/mL, respectively.

Procedural blanks revealed the presence of MEHTP at concentrations ranging from 0.8 to 1.1 ng/g. Therefore, the LOQ_m was calculated using the average value of the procedural blanks (n=6) plus three times of the standard deviation of low spike (n=7) [52].

Solvent blanks were injected right after the highest concentration of calibration curve to assess carry-overs. For MINCH, OH-MINCH and OH-MPHP, carry-over was detected, which was < 20% of the LOQ_m for all analytes. As those metabolites were not detected in solvent blank injections, carry-over was observed for the AP metabolites which were harder to elute from the LC column and at the highest concentrations.

The precision of the method was evaluated using hair samples spiked at three concentration levels, as described in Section 2.6, and results are indicated in Table 3. For low spike (0.4 ng, 8 ng/g), the within- and between-run precision was lower than 20 %, and for mid (6 ng, 120 ng/g) and high spike (60 ng, 1200 ng/g), the precision was lower than 15 % among three days for most target compounds, except for OH-MEHA (22 %), oxo-MEHA (23.5 %), and 5-OH-MEHTP (17.1 %). The inter-individual variation in precision of the method was also assessed using hair samples from three additional donors. The results showed that, except for OH-MEHA (40.4% and 51.6%), oxo-MEHA (28.7%) and oxo-MPHP (25.7%), the precision across different donors was acceptable (<15%). The high variation observed for OH-MEHA, oxo-MEHA and oxo-MPHP in both between-run precision and inter-individual variation could be linked to the lack of corresponding isotope-labelled internal standards for these analytes [53].

Because blank hair samples are not available, the trueness of the method was evaluated using spiked hair samples. Low, mid and high concentrations of target analytes were directly spiked into hair samples from one donor. The nominal concentration was defined as the sum of the background and spiked concentrations. Results were shown in Table 2. The trueness for individual metabolites was acceptable for all concentration levels except for MEHA and its oxidized metabolites. For oxo-MEHA and OH-MEHA, lower between-run accuracies were obtained for low and high spiking levels. Based on the results obtained during method validation as MEHA could not achieve a good trueness for all concentration levels, it was decided not to report any quantitative result for MEHA but only qualitative information.

Overall, the results obtained during method validation indicate that the protocol is adapted for the analysis of targeted AP biomarkers, except for MEHA, which did not provide satisfactory results. Nevertheless, one limitation of the used approach needs to be highlighted. Namely, validation samples were prepared by spiking liquid native and IS onto a solid matrix since reference fortified hair samples are not available and there is no consensus in the literature regarding how these should be prepared in-house [29, 54-57].

3.5 Pilot biomonitoring study

The validated method was implemented to measure analyte concentrations in hair samples from nine different healthy volunteers and results were reported in Table 4. MEHTP and oxo-MEHA were detected in all samples, with average concentrations of 50 ng/g (IQR: 8.5 - 106 ng/g) and 5.4 ng/g (IQR: 3.9 - 18 ng/g), respectively. MINCH and OH-MINCH were detected in most samples, with a detection frequency of 78% and 67%, respectively, yet concentrations were low (*i.e.*, IQR 1.3-3.4 ng/g and 0.3-3.1 ng/g, respectively). Although MEHA could not be quantified accurately, it could be detected in all samples.

Particularly high concentrations of DEHTP and DEHA metabolites were measured in one sample (*i.e.*, 211 ng/g for MEHTP, 2.2 ng/g for 5-OH-MEHTP, 16 ng/g for OH-MEHA and 582 ng/g for oxo-MEHA). The obtained results indicate that 5-OH-MEHTP, OH-MEHA, oxo-MEHA, and OH-MINCH were detected only in samples in which also the corresponding monoester (i.e., MEHTP, MEHA and MINCH) was present. OH-MPHP was detected only in one sample at a concentration of 1.5 ng/g (Figure 2), however, results for OH-MPHP should be interpreted as the contribution of OH-MPHP and OH-MIDP since their metabolites are isomers and cannot be separated with the current method. Detecting less polar compounds at a higher frequency compared to more hydrophilic oxidized metabolites is in agreement with findings from forensic hair analysis. In fact, cocaine was found to be incorporated to a higher extent compared to its more hydrophilic metabolite benzoylecgonine [58]. This might indicate that hair is a better reservoir for more hydrophobic metabolites.

To the best of our knowledge, this is the first study that tackles the measurement of metabolites of APs in hair samples. In the literature, only a limited number of studies have investigated the occurrence of targeted analytes in this study in biological matrices (*i.e.*, urine, pooled urine and nails), though there were some studies about the analysis of phthalate esters [19, 52, 59, 60]. For the metabolites of DEHTP, both 5-OH-MEHTP and Cx-MEHTP were found in urine from pregnant women in Israel, with median concentrations of 1.3 ng/mL and 7.7 ng/mL, respectively [61]. 5-OH-MEHTP and MEHTP were also found in a screening

study using high-resolution mass spectrometry in nails with a detection frequency of 22% and 8%, respectively [62]. Metabolites of DEHA were detected in less than 20% of urine samples (n=144) from adults from the US, with concentrations ranging from <LOD to 23.9 ng/mL for OH-MEHA and <LOD to 10.4 ng/mL of oxo-MEHA [21].

The metabolites of DPHP had low detection frequencies and low concentrations measured in the hair samples. Urinary concentrations ranging from <LOD to 0.64 ng/mL (detection frequency 3.3%) for OH-MPHP and <LOD to 0.96 ng/mL (detection frequency 21.7%) for oxo-MPHP in urine samples from German environmental specimen bank from 2009 and 2012 [22]. However, current LC-MS/MS methods, including the one described here, are not able to separate DPHP metabolites from those of DIDP [63]. MINCH, the hydrolyzed monoester of DINCH, was found in pooled urine samples from Australia with a mean concentration of 3.9 ng/mL (range: 1.8-16.2 ng/mL) [64]. In 208 urine samples from children in daycare centers in Germany, further oxidized metabolites OH-MINCH, Cx-MINCH and oxo-MINCH were measured with high frequency (100%, 100% and 99%, respectively) than MINCH (22.6%), with a median concentration of 1.66, 1.14, 1.54 and <0.1 ng/mL, respectively [20]. A biomonitoring study from a Norwegian population showed similar result, in spot urine, further oxidized metabolites had detection frequency higher than 54% while in nail samples, which have similar contents compare to hair, frequencies around 20% [24]. However, in our study, none of the carboxy-metabolites (namely Cx-MINCH, and Cx-MPHxP) was found in hair samples. Concentrations of DINP metabolites (*i.e.*, MINP, oxo-MINP and OH-MINP) in urine have been reported to increase less after administration compared to hair samples [65]. For DINP metabolites, it was found that concentrations of some metabolites saturate earlier compared to hair. MPHP [66] was also found to be a major metabolite in blood, but a minor one in urine.

In urine samples, further oxidized metabolites (*i.e.*, OH-, Cx-, and oxo-) were more frequently detected compared to their corresponding monoesters, which are more hydrophobic. On the other hand, similar to blood, hair contains more monoesters compared to urine, thus making it a potentially more appropriate matrix for long term monitoring of exposure to plasticizers.

4. Conclusions

In this study, a method to assess human exposure to alternative plasticizers through the analysis of hair samples was developed and optimized. The method was based on a solid-liquid extraction using methanol and phosphate buffer saline, to enhance the recovery of

target biomarkers, followed by solid-phase extraction. Hair was chosen as an alternative non-invasive matrix to assess integrated exposure, which could reflect both short- and long-term exposure to alternative plasticizers. It is both easy to collect and store, and analytes are supposedly more stable compared to other matrices such as urine and serum. The result of our pilot biomonitoring experiment suggests that more hydrophobic monoester metabolites tend to be incorporated to a greater extent compared to their more hydrophilic oxidized counterparts. This could indicate the hair is a better reservoir for more hydrophobic metabolites. Results obtained here illustrate that hair is a viable complementary matrix to monitor human exposure to alternative plasticizers. Its use could prove to be highly useful when investigating exposure in vulnerable or hard to sample populations, such as neonates and toddlers.

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References

[1] P. Handbook, Wilkes, CE, Summers, JW, and Daniels, CA, Eds, Munich: Hanser, DOI (2005).

[2] M.J. Silva, L.-Y. Wong, E. Samandar, J.L. Preau, A.M. Calafat, X. Ye, Exposure to di-2-ethylhexyl terephthalate in a convenience sample of U.S. adults from 2000 to 2016, Archives of Toxicology, 91 (2017) 3287-3291.

[3] P. Wallner, M. Kundi, P. Hohenblum, S. Scharf, H.-P.P. Hutter, Phthalate Metabolites, Consumer Habits and Health Effects, International journal of environmental research and public health, 13 (2016).

[4] H. Gao, Y.-w. Zhang, K. Huang, S.-q. Yan, L.-j. Mao, X. Ge, Y.-q. Xu, Y.-y. Xu, J. Sheng, Z.-x. Jin, P. Zhu, X.-g. Tao, J.-h. Hao, F.-b. Tao, Urinary concentrations of phthalate metabolites in early pregnancy associated with clinical pregnancy loss in Chinese women, Scientific Reports, 7 (2017) 6800.

[5] S.H. Swan, Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans, Environmental Research, 108 (2008) 177-184.

[6] P.J. Lioy, R. Hauser, C. Gennings, H.M. Koch, P.E. Mirkes, B.A. Schwetz, A. Kortenkamp, Assessment of phthalates/phthalate alternatives in children's toys and childcare articles: Review of the report including conclusions and recommendation of the Chronic Hazard Advisory Panel of the Consumer Product Safety Commission, Journal of Exposure Science and Environmental Epidemiology, 25 (2015) 343-353.

[7] Commission Delegated Directive (EU), 2015/863 of 31 March 2015 amending Annex II to Directive 2011/65/EU of the European Parliament and of the Council as regards the list of restricted substances, 2015. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv:OJ.L_.2015.137.01.0010.01.ENG#ntr1-L_2015137EN.01001001-E0001 (accessed, Aug 2018)

[8] B. Erickson, European Union further restricts four phthalates, C&EN Global Enterprise, 95 (2017) 15-15.

[9] Plasticizers: phthalate alternatives, Plastics, Additives and Compounding, 4 (2002) 30-31.

[10] Consumer product safety improvement act of 2008. Public law 110-314, 2008. https://www.cpsc.gov/s3fs-public/pdfs/blk_pdf_cpsia.pdf (accessed, Aug 2018)

[11] K.S. Carlos, L.S. de Jager, T.H. Begley, Investigation of the primary plasticisers present in polyvinyl chloride (PVC) products currently authorised as food contact materials, Food Additives & Contaminants: Part A, DOI 10.1080/19440049.2018.1447695(2018) 1-9.

[12] L. Coltro, J.B. Pitta, E. Madaleno, Performance evaluation of new plasticizers for stretch PVC films, Polymer Testing, 32 (2013) 272-278.

[13] B.L. Wadey, An innovative plasticizer for sensitive applications, Journal of Vinyl and Additive Technology, 9 (2003) 172-176.

[14] L. Coltro, J.B. Pitta, P.A. da Costa, M.Â. Fávaro Perez, V.A. de Araújo, R. Rodrigues, Migration of conventional and new plasticizers from PVC films into food simulants: A comparative study, Food Control, 44 (2014) 118-129.

[15] J.R. Furr, C.S. Lambright, V.S. Wilson, P.M. Foster, L.E. Gray, A Short-term In Vivo Screen Using Fetal Testosterone Production, a Key Event in the Phthalate Adverse Outcome Pathway, to Predict Disruption of Sexual Differentiation, Toxicological Sciences, 140 (2014) 403-424.

[16] T. Eljezi, P. Pinta, D. Richard, J. Pinguet, J.-M. Chezal, M.-C. Chagnon, V. Sautou, G. Grimandi, E. Moreau, In vitro cytotoxic effects of DEHP-alternative plasticizers and their primary metabolites on a L929 cell line, Chemosphere, 173 (2017) 452-459.

[17] E. Campioli, T.B. Duong, F. Deschamps, V. Papadopoulos, Cyclohexane-1,2-dicarboxylic acid diisononyl ester and metabolite effects on rat

epididymal stromal vascular fraction differentiation of adipose tissue, Environmental Research, 140 (2015) 145-156.

[18] I.A. Sheikh, M. Yasir, M. Abu-Elmagd, T.A. Dar, A.M. Abuzenadah, G.A. Damanhouri, M. Al-Qahtani, M.A. Beg, Human sex hormone-binding globulin as a potential target of alternate plasticizers: an in silico study, BMC Structural Biology, 16 (2016) 15.

[19] E.D. Tsochatzis, R. Tzimou-Tsitouridou, H.G. Gika, Analytical Methodologies for the Assessment of Phthalate Exposure in Humans, Critical reviews in analytical chemistry, 47 (2017) 279-297.

[20] H. Fromme, A. Schütze, T. Lahrz, M. Kraft, L. Fembacher, S. Siewering, R. Burkardt, S. Dietrich, H.M. Koch, W. Völkel, Non-phthalate plasticizers in German daycare centers and human biomonitoring of DINCH metabolites in children attending the centers (LUPE 3), International Journal of Hygiene and Environmental Health, 219 (2016) 33-39.

[21] M.J. Silva, E. Samandar, X. Ye, A.M. Calafat, In vitro metabolites of di-2-ethylhexyl adipate (DEHA) as biomarkers of exposure in human biomonitoring applications, Chemical research in toxicology, 26 (2013) 1498-1502.

[22] A. Schütze, W. Gries, M. Kolossa-Gehring, P. Apel, C. Schröter-Kermani, U. Fiddicke, G. Leng, T. Brüning, H.M. Koch, Bis-(2-propylheptyl)phthalate (DPHP) metabolites emerging in 24h urine samples from the German Environmental Specimen Bank (1999-2012), International journal of hygiene and environmental health, 218 (2015) 559-563.

[23] A. Alves, A. Kucharska, C. Erratico, F. Xu, E. Den Hond, G. Koppen, G. Vanermen, A. Covaci, S. Voorspoels, Human biomonitoring of emerging pollutants through non-invasive matrices: state of the art and future potential, Analytical and bioanalytical chemistry, 406 (2014) 4063-4088.

[24] G. Giovanoulis, A. Alves, E. Papadopoulou, A.P. Cousins, A. Schütze, H.M. Koch, L.S. Haug, A. Covaci, J. Magnér, S. Voorspoels, Evaluation of exposure to phthalate esters and DINCH in urine and nails from a Norwegian study population, Environmental research, 151 (2016).

[25] M.R. Harkey, Anatomy and physiology of hair, Forensic Science International, 63 (1993) 9-18.

[26] F. Pragst, M.A. Balikova, State of the art in hair analysis for detection of drug and alcohol abuse, Clinica Chimica Acta, 370 (2006) 17-49.

[27] A. Covaci, M. Tutudaki, A.M. Tsatsakis, P. Schepens, Hair analysis: another approach for the assessment of human exposure to selected persistent organochlorine pollutants, Chemosphere, 46 (2002) 413-418.

[28] R. Wennig, Potential problems with the interpretation of hair analysis results, Forensic Science International, 107 (2000) 5-12.

[29] A. Alves, G. Jacobs, G. Vanermen, A. Covaci, S. Voorspoels, New approach for assessing human perfluoroalkyl exposure via hair, Talanta, 144 (2015) 574-583.

[30] L. Altshul, A. Covaci, R. Hauser, The Relationship between Levels of PCBs and Pesticides in Human Hair and Blood: Preliminary Results, Environmental Health Perspectives, 112 (2004) 1193-1199.

[31] A. Kucharska, A. Covaci, G. Vanermen, S. Voorspoels, Non-invasive biomonitoring for PFRs and PBDEs: new insights in analysis of human hair externally exposed to selected flame retardants, The Science of the total environment, 505 (2015) 1062-1071.

[32] A. Kucharska, E. Cequier, C. Thomsen, G. Becher, A. Covaci, S. Voorspoels, Assessment of human hair as an indicator of exposure to organophosphate flame retardants. Case study on a Norwegian mother-child cohort, Environment international, 83 (2015) 50-57.

[33] M.-J. He, J.-F. Lu, J.-Y. Ma, H. Wang, X.-F. Du, Organophosphate esters and phthalate esters in human hair from rural and urban areas, Chongqing, China: Concentrations, composition profiles and sources in comparison to street dust, Environmental Pollution, 237 (2018) 143-153.

[34] R. Dulbecco, M. Vogt, PLAQUE FORMATION AND ISOLATION OF PURE LINES WITH POLIOMYELITIS VIRUSES, The Journal of Experimental Medicine, 99 (1954) 167-182.

[35] G. Cooper, Hair testing is taking root, Annals of Clinical Biochemistry, 48 (2011) 516-530.

[36] N. Takayama, R. Iio, S. Tanaka, S. Chinaka, K. Hayakawa, Analysis of methamphetamine and its metabolites in hair, Biomedical chromatography : BMC, 17 (2003) 74-82.

[37] H. Zhang, P. Wang, Y. Li, H. Shang, Y. Wang, T. Wang, Q. Zhang, G. Jiang, Assessment on the Occupational Exposure of Manufacturing Workers to Dechlorane Plus through Blood and Hair Analysis, Environmental Science & Technology, 47 (2013) 1427389844.

[38] F. Lessmann, A. Schütze, T. Weiss, T. Brüning, H.M. Koch, Determination of metabolites of di(2-ethylhexyl) terephthalate (DEHTP) in human urine by HPLC-MS/MS with on-line clean-up, Journal of chromatography. B, Analytical technologies in the biomedical and life sciences, 1011 (2016) 196-203.

[39] H.M. Koch, A.M. Calafat, Human body burdens of chemicals used in plastic manufacture, Philosophical Transactions of the Royal Society B: Biological Sciences, 364 (2009) 2063.

[40] European Medicines Agency, 2011. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf (accessed in Aug,2018)

[41] I. Marchi, V. Viette, F. Badoud, M. Fathi, M. Saugy, S. Rudaz, J.-L. Veuthey, Characterization and classification of matrix effects in biological samples analyses, Journal of Chromatography A, 1217 (2010) 4071-4078.

[42] G.A.A. Cooper, Hair testing is taking root, Annals of Clinical Biochemistry, 48 (2011) 516-530.

[43] Y.-J. Chang, K.-L. Lin, Y.-Z. Chang, Determination of Di-(2-ethylhexyl)phthalate (DEHP) metabolites in human hair using liquid chromatography–tandem mass spectrometry, Clinica Chimica Acta, 420 (2013) 155-159.

[44] L.-Y. Liu, A. Salamova, K. He, R.A. Hites, Analysis of polybrominated diphenyl ethers and emerging halogenated and organophosphate flame retardants in human hair and nails, Journal of Chromatography A, 1406 (2015) 251-257.

[45] D. Lu, C. Feng, Y. Lin, D. Wang, H. Ip, X. Qiu, G. Wang, J. She, Determination of organochlorines, polychlorinated biphenyls and polybrominated diphenyl ethers in human hair: Estimation of external and internal exposure, Chemosphere, 114 (2014) 327-336.

[46] D. Cappelle, M. De Doncker, C. Gys, K. Krysiak, S. De Keukeleire, W. Maho, C.L. Crunelle, G. Dom, A. Covaci, A.L. van Nuijs, H. Neels, A straightforward, validated liquid chromatography coupled to tandem mass spectrometry method for the simultaneous detection of nine drugs of abuse and their metabolites in hair and nails, Analytica chimica acta, 960 (2017).

[47] E. Grata, J. Boccard, D. Guillarme, G. Glauser, P.-A. Carrupt, E.E. Farmer, J.-L. Wolfender, S. Rudaz, UPLC–TOF-MS for plant metabolomics: A sequential approach for wound marker analysis in Arabidopsis thaliana, Journal of Chromatography B, 871 (2008) 261-270.

[48] A. Chauvin, D. Caldelari, J.L. Wolfender, E.E. Farmer, Four 13-lipoxygenases contribute to rapid jasmonate synthesis in wounded Arabidopsis thaliana leaves: a role for lipoxygenase 6 in responses to long-distance wound signals, New Phytologist, 197 (2013) 566-575.

[49] T. Mose, G.K. Mortensen, M. Hedegaard, L.E. Knudsen, Phthalate monoesters in perfusate from a dual placenta perfusion system, the placenta tissue and umbilical cord blood, Reproductive toxicology (Elmsford, N.Y.), 23 (2007) 83-91.

[50] N. Rastkari, R. Ahmadkhaniha, Magnetic solid-phase extraction based on magnetic multi-walled carbon nanotubes for the determination of phthalate monoesters in urine samples, Journal of chromatography. A, 1286 (2013) 22-28.

[51] A.M. Calafat, A.R. Slakman, M.J. Silva, A.R. Herbert, L.L. Needham, Automated solid phase extraction and quantitative analysis of human milk for 13 phthalate metabolites, Journal of chromatography. B, Analytical technologies in the biomedical and life sciences, 805 (2004) 49-56.

[52] A. Alves, A. Covaci, S. Voorspoels, Method development for assessing the human exposure to organophosphate flame retardants in hair and nails, Chemosphere, 168 (2017).

[53] Y. Guo, K. Kannan, Challenges encountered in the analysis of phthalate esters in foodstuffs and other biological matrices, Analytical and bioanalytical chemistry, 404 (2012) 2539-2554.

[54] J. Martín, J. Santos, I. Aparicio, E. Alonso, Analytical method for biomonitoring of endocrine-disrupting compounds (bisphenol A, parabens, perfluoroalkyl compounds and a brominated flame retardant) in human hair by liquid chromatography-tandem mass spectrometry, Analytica Chimica Acta, 945 (2016) 95-101.

[55] A. Kucharska, A. Covaci, G. Vanermen, S. Voorspoels, Development of a broad spectrum method for measuring flame retardants - overcoming the challenges of non-invasive human biomonitoring studies, Analytical and bioanalytical chemistry, 406 (2014) 6665-6675.

[56] S. Lee, Y. Park, E. Han, S. Choe, M. Lim, H. Chung, Preparation and application of a fortified hair reference material for the determination of methamphetamine and amphetamine, Forensic Science International, 178 (2008) 207-212.

[57] G.A.A. Cooper, R. Kronstrand, P. Kintz, Society of Hair Testing guidelines for drug testing in hair, Forensic Science International, 218 (2012) 20-24.

[58] T. Franz, F. Scheufler, K. Stein, M. Uhl, T. Dame, G. Schwarz, H. Sachs, G. Skopp, F. Musshoff, Determination of hydroxy metabolites of cocaine from hair samples and comparison with street cocaine samples, Forensic Science International, 288 (2018) 223-226.

[59] A. Alves, G. Koppen, G. Vanermen, A. Covaci, S. Voorspoels, Long-term exposure assessment to phthalates: How do nail analyses compare to commonly used measurements in urine, Journal of chromatography. B, Analytical technologies in the biomedical and life sciences, 1036-1037 (2016).

[60] G. Giovanoulis, T. Bui, F. Xu, E. Papadopoulou, J.A. Padilla-Sanchez, A. Covaci, L.S. Haug, A.P. Cousins, J. Magnér, I.T. Cousins, C.A. de Wit, Multi-pathway human exposure assessment of phthalate esters and DINCH, Environment international, 112 (2017) 115-126.

[61] R. Machtinger, T. Berman, M. Adir, A. Mansur, A.A. Baccarelli, C. Racowsky, A.M. Calafat, R. Hauser, R. Nahum, Urinary concentrations of phthalate metabolites, bisphenols and personal care product chemical biomarkers in pregnant women in Israel, Environment international, 116 (2018) 319-325.

[62] A. Alves, G. Giovanoulis, U. Nilsson, C. Erratico, L. Lucattini, L.S. Haug, G. Jacobs, C.A. de Wit, P.E. Leonards, A. Covaci, J. Magner, S. Voorspoels, Case Study on

Screening Emerging Pollutants in Urine and Nails, Environmental science & technology, 51 (2017) 4046-4053.

[63] W. Gries, D. Ellrich, K. Küpper, B. Ladermann, G. Leng, Analytical method for the sensitive determination of major di-(2-propylheptyl)-phthalate metabolites in human urine, Journal of Chromatography B, 908 (2012) 128-136.

[64] M.J. Ramos, A.L. Heffernan, L.M.L. Toms, A.M. Calafat, X. Ye, P. Hobson, S. Broomhall, J.F. Mueller, Concentrations of phthalates and DINCH metabolites in pooled urine from Queensland, Australia, Environment International, 88 (2016) 179-186.

[65] J.-Y.Y. Hsu, H.-H.H. Ho, P.-C.C. Liao, The potential use of diisononyl phthalate metabolites hair as biomarkers to assess long-term exposure demonstrated by a rat model, Chemosphere, 118 (2015) 219-228.

[66] D. Klein, W. Kessler, C. Pütz, B. Semder, W. Kirchinger, A. Langsch, W. Gries, R. Otter, A.K.E. Gallien, X. Wurzenberger, J.G. Filser, Single ingestion of di-(2-propylheptyl) phthalate (DPHP) by male volunteers: DPHP in blood and its metabolites in blood and urine, Toxicology Letters, 294 (2018) 105-115.

Table 1: List of parent and metabolites of AP (biomarkers) in this study. Only metabolites were measured in the context of this study.

Parent	Metabolite (Biomarkers)	Abbreviation
di(2-ethylhexyl) terephthalate (DEHTP)	mono(2-ethylhexyl) terephthalate	MEHTP
	mono(2-ethyl-5-hydroxyhexyl) terephthalate	5-OH-MEHTP
di-2-ethylhexyl adipate (DEHA)	mono(2-ethylhexyl) adipate	MEHA
	mono(2-ethyl-5-oxohexyl) adipate	oxo-MEHA
	mono(2-ethyl-5-hydroxyhexyl) adipate	OH-MEHA
di(isononyl)cyclohexane-1,2-dicarboxylate (DINCH)	cyclohexane-1,2-dicarboxylic mono isononyl ester	MINCH
	cyclohexane-1,2-dicarboxylic mono hydroxyisononyl ester	OH-MINCH
	cyclohexane-1,2-dicarboxylic mono carboxyisooctyl ester	Cx-MINCH
di(2-propylheptyl) phthalate (DPHP)	mono(2-propyl-6-hydroxyheptyl) phthalate	OH-MPHP
	mono(2-propyl-6-carboxyhexyl) phthalate	Cx-MPHxP
	mono(2-propyl-6-oxoheptyl) phthalate	oxo-MPHP

Table 2: Correlation coefficients (R^2), the instrumental and method limits of detection and quantitation (LOD and LOQ), and carryover in the validation.

Analytes	R^2	LOD_i (pg/mL)	LOQ_i (pg/mL)	LOD_m (ng/g)	LOQ_m (ng/g)	Carry-over
MEHTP	0.991	127	423	4.8	6.5	0%
5-OH-MEHTP	0.994	68	228	0.3	0.9	0%
OH-MEHA	0.993	153	510	0.4	1.3	0%
oxo-MEHA	0.992	133	442	0.9	2.6	0%
MINCH	0.997	14	47	0.1	0.2	6.7%
OH-MINCH	0.991	9.0	29	0.1	0.2	3.4%
Cx-MINCH	0.994	48	160	0.3	1.0	0%
OH-MPHP	0.995	74	247	0.5	1.5	11%
Cx-MPHxP	0.991	59	198	0.4	1.2	0%
oxo-MPHP	0.997	67	223	0.5	1.3	0%

Table 3: Within-run and between-run trueness and precision. RSD% = relative standard deviation. a) For within- and between-run trueness and precision, hair samples of one donor were spiked at three concentration levels were used. b) Hair samples from three different donors (120 ng/g) were extracted in triplicate, to calculate the within-run precision using different matrices.

Compound	Spiking levels (ng/g)	Hair Matrix						
		[a]Trueness (%)		Precision (RSD %)		Within-run precision for additional donors (n=3, RSD %)[b]		
		Within-run	Between-run	Within-run	Between-run			
MEHTP	8	90.9	97.6	9.8	15.8	5.9	1.2	3.5
	120	90.1	94.1	6.6	9.9			
	1200	95.1	97.0	3.7	6.7			
5-OH-MEHTP	8	89.2	88.0	12.1	13.7	9.6	5.7	2.7
	120	84.0	87.3	9.2	17.1			
	1200	88.0	88.7	6.3	9.7			
MEHA	8	80.9	81.1	12.2	18.7	9.0	3.3	10.2
	120	61.4	61.3	7.7	12.7			
	1200	39.6	39.6	12.6	15.0			
OH-MEHA	8	81.4	81.0	13.3	11.8	40.4	4.3	51.6
	120	90.4	46.0	8.4	22.0			
	1200	88.7	90.7	4.1	9.5			
oxo-MEHA	8	82.1	71.7	12.7	17.5	13.1	2.0	28.7
	120	87.4	62.2	11.5	23.5			
	1200	91.9	93.8	4.6	10.8			
MINCH	8	91.5	99.9	7.6	16.2	14.8	5.4	5.8
	120	87.1	87.1	6.3	6.5			
	1200	96.9	96.7	6.0	3.7			
OH-MINCH	8	88.5	94.2	18.5	15.9	13.0	4.1	12.4
	120	89.3	89.3	7.5	11.1			
	1200	87.7	87.7	9.4	12.9			
Cx-MINCH	8	85.2	87.5	12.7	16.3	1.6	13.6	13.6
	120	86.7	89.5	16.4	14.4			
	1200	92.2	87.2	11.8	9.0			
OH-MPHP	8	99.6	93.8	12.1	18.5	13.4	13.3	25.7
	120	86.4	90.8	3.3	14.5			
	1200	88.4	88.2	6.6	6.6			
Cx-MPHxP	8	92.2	99.0	6.5	11.9	1.6	5.1	6.0
	120	96.1	95.1	3.5	9.1			
	1200	90.2	89.6	6.1	12.2			
oxo-MPHP	8	96.2	99.6	10.7	10.5	5.6	5.7	12.1
	120	99.3	99.9	2.9	2.8			
	1200	91.8	91.3	8.2	7.2			

Table 4: Results (in ng/g) from the analysis of hair samples from healthy volunteers (n = 9). Results for OH-MPHP should be interpreted as the contribution of OH-MPHP and OH-MIDP since their metabolites are isomers and cannot be separated with the current method. $>LOD_m$: number of samples higher than LOQ_m; N.A.: not available; N.D.: not detected

Analytes	$>LOQ_m$	Detection frequency (%)	25^{th} percentile (ng/g)	Median (ng/g)	75^{th} percentile (ng/g)
MEHTP	9	(100%)	8.5	50	106
5-OH-MEHTP	2	(22%)	2.2	2.7	N.A.
MEHA	9*	(100%)	-	-	-
OH-MEHA	2	(22%)	1.5	8.6	N.A.
oxo-MEHA	9	(100%)	3.9	5.3	18
MINCH	7	(78%)	1.3	2.9	3.4
OH-MINCH	6	(67%)	0.3	0.6	3.1
Cx-MINCH	0	(0%)	N.D.	N.D.	N.D.
OH-MPHP	1	(11%)	1.5	N.A.	N.A.
Cx-MPHxP	0	(0%)	N.D.	N.D.	N.D.
oxo-MPHP	0	(0%)	N.D.	N.D.	N.D.

* cannot be quantified in this study, all 9 samples have peaks with S/N>10.

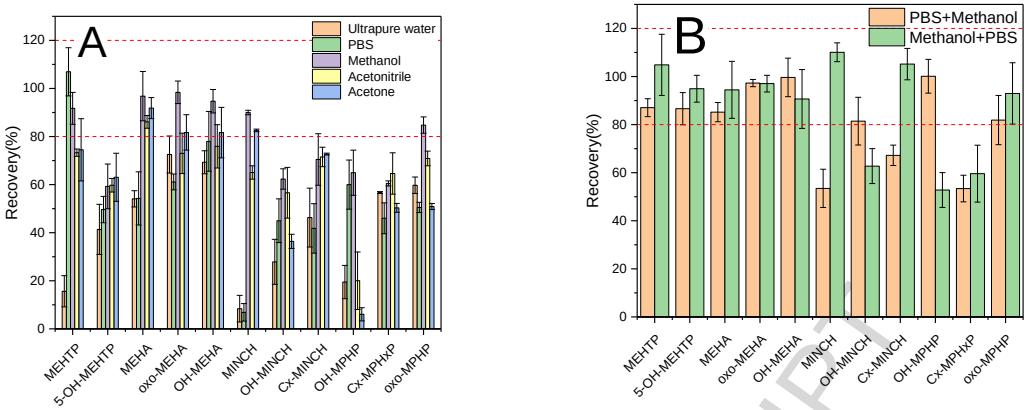

Figure 1. A) Extraction recovery with a single solvent (for 1 h) (n=6). B) Recovery for sequential extraction using methanol and PBS (for 30 min, 30 min).

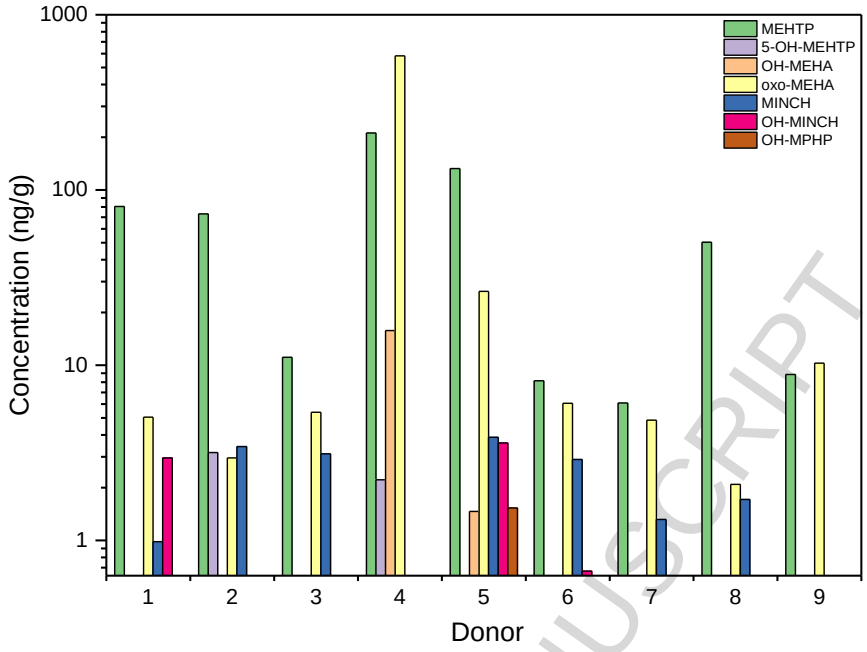

Figure 2: Analyte concentrations (in ng/g) measured in the pilot biomonitoring study (n = 9). Results for OH-MPHP should be interpreted as the contribution of OH-MPHP, OH-MINP and OH-MIDP since their metabolites are isomers and cannot be separated with the current method. The Y-axis is $\log_{10}$ transformed.

Highlights

- A method for the analysis in hair of exposure biomarkers to alternative plasticizers was developed
- The newly developed analytical method was successfully applied to human hair.
- Oxidative metabolites were incorporated less into the hair than monoesters.
- Patterns of plasticizer metabolites were different in hair compared to urine
- Hair can be used to assess short- and long-term exposure