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Long-term exposure assessment to phthalates: how do nail analyses compare to commonly used measurements in urine

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Highlights

- A novel and fast approach was developed for exposure assessment on PEs
- Internal exposure to 7 metabolites was determined via nails and various urine spots
- Higher PE levels in morning urine spots than in afternoon/evening
- Correlations between PE metabolites measured in the two matrices were performed
- Lower variation of the levels and long-term exposure is given by nail data

Abstract

Phthalate esters (PEs) are easily metabolized and commonly excreted via urine within 24h, therefore their bioaccumulation potential is thought to be rather low. In the present study, we developed a sample preparation combined with a new microextraction method to measure seven PE metabolites in nails. The use of whole nails did not result in significantly different levels compared to powdered nails, which makes the method very fast and user friendly. The method was validated using whole nails showing good accuracy, satisfactory precision and low limits of quantification (2-14 ng/g).

Although method development was the primary aim of the study, the method was also applied to real samples. PEs were measured in nails of 9 individuals collected at 2 distinct time points (15 days apart) and compared to levels in the respective urine samples (daily morning sample for 15 days). Additionally two volunteers have collected two more urine spots (afternoon and evening) per day.

Major metabolites in nails were mono (ethyl hexyl) phthalate (MEHP), monoethyl phthalate (MEP) and sum of mono-*n*-butyl and mono-isobutyl phthalate ($\Sigma(\text{MnBP}, \text{MiBP})$) while MEP and $\Sigma(\text{MnBP}, \text{MiBP})$ were the major ones identified in urine. In urine, first void morning urine reflected higher total excretion (sum of PEs of 7.0 $\mu\text{g/g}$ creatinine) for all individuals than the afternoon/evening voids.

Participants also filled a questionnaire regarding their life-style. The use of hand care products and consumption of pre-packed food was associated with di-(2-ethylhexyl) phthalate (DEHP) oxidative metabolites, while the use of medical devices with butylbenzyl phthalate (BBzP) exposure. Although the metabolism (rate) and other factors that influence the transfer of the analytes from blood or other body compartments into nails needs further investigation, nails can be used to assess exposure to PEs. From our knowledge, urine reflects the excretion of PEs on 'daily basis' while nails show less fluctuation and more stable levels.

Keywords: nails, urine spots, human biomonitoring, phthalate metabolites, exposure, predictors

1. Introduction

Phthalate esters (PEs) have been widely used in a multitude of products. Depending on their number of carbon atoms in the alkyl chain, PEs are classified as short (1 to 6-carbon atoms) to long (>6 carbon atoms) alkyl chain PEs [1,2]. The stability, fluidity and low volatility of long-chain PEs makes them highly suitable as plasticizers (e.g. to soften plastic polymers as polyvinyl chloride (PVC)) in a wide array of products (for example, construction materials, upholstery, medical devices, food contact materials or toys), while short-chain PEs are principally used as solvents and emollients of adhesives, personal care and house cleaning products and pharmaceuticals [1,3,4].

Since PEs are not chemically bound to these products, they are easily released to the environment and enter in the human body via innumerable pathways including inhalation, absorption, ingestion or dermal contact [1].

After intake, PEs are hydrolyzed to their monoesters and may undergo further metabolism to produce (oxidative) secondary metabolites or glucuronide conjugates [5].

Many human biomonitoring (HBM) studies have been conducted to assess human exposure on PEs in several matrices including urine [3,6–20], serum [9,21], plasma [9], saliva [21], breast milk [21], semen [22] and more recently in hair [23].

Although urine is the most common matrix for measuring PE metabolites, there are two main drawbacks in using it, as the urinary concentrations are affected by urine dilution and the time point of the urine collection. Thus, some authors defend that normalizing the levels of the metabolites based on creatinine is a suitable approach to minimize errors on the measurements, to better quantify excretion and evaluate exposures [17,19,24]. In contrast, others defend that normalization is not relevant because several factors such as sex, age, body mass index (BMI), fat-free mass, and even ethnicity can have a significant influence on the renal clearance of PEs [25,26]. Instead, the urine osmolality or the specific gravity determination is suggested as an alternative for correcting the urine dilution [11,18,22]. Another influential factor that determines the concentration is the time point or period of urine collection (24h, spot and morning urine). There are some evidences of a higher variability of the levels for both between and within-persons for 24h urine than for first-morning or spot urine samples [11]. Therefore, some authors consider that it is of considerable importance to collect multiple urine spots [19,24] to accurately classify

exposure to PEs. Still it is noteworthy that the current literature regarding the time sampling (days to two weeks) and the number of spots collected is limited [11,17,19,24].

Although there are disadvantages on sampling and quantifying PE metabolites in urine, it seems that this is a very common matrix used for assessing the exposure on PEs. Thus, in order to overcome some of the drawbacks on sampling and in particular on the PEs' exposure assessment based on urinary levels, other non-invasive matrices such as nails were recently explored [27]. Although from our understanding, exists a lack of knowledge and some skepticism in exploring the potential of this matrix for translating internal exposure, yet we believe that nails can better reflect a wider exposure range (days to months) than urine, for instance.

Taking into account the anatomy of the nail bed, i.e. that it is beneath the nail plate and is in contact with nerves, blood vessels and lymph, then it is possible to consider that many chemicals can get easily the contact with nail via diffusion into the nail matrix (which is the responsible tissue by producing cells that become the nail plate) [28]. Likewise in hair [29], we believe that once the compounds are incorporated into keratin of nails (and are not excreted via urine), the levels remain isolated from other tissues, therefore low (or no) fluctuation in the levels is expected and they are not affected by metabolism activities that may occur in other body regions. Thus, it is possible to evaluate past exposures especially due to the bioaccumulation of chemicals in the nail plate and low growth rate of nails.

Also advantages associated with an easier collection, transport, storage, stability and handling can make this matrix more attractive for HBM purposes [30,31]. As an example, nail clippings can eventually obtained by participants at home and mailed to the researcher, making studies in the remote areas highly feasible. Epidemiological research in large populations, children, babies or vulnerable people is also no issue [31]. For instance, the exposure assessment to diverse types of pollutants (e.g. perfluoroalkyl substances, heavy metals, etc.) [30,32,33] and more recently to PE metabolites [27,34] have shown that nails can be a reliable alternative. Making the sample preparation less time consuming and using an environmental friendly microextraction (i.e. ultrasound assisted extraction combined with dispersive liquid-liquid microextraction (US-DLLME)) can be considered as a step forward in routine HBM/analyses.

Several challenges remain regarding the assessment of PE metabolites in nails (including MEHP (mono (ethylhexyl) phthalate), 5-oxo-MEHP (mono(2-ethyl-5-oxohexyl) phthalate), mono(2-ethyl-5-hydroxyhexyl) phthalate (5-OH-MEHP), mono-*n*-butyl phthalate (MnBP),

mono-isobutyl phthalate (MiBP), monoethyl phthalate (MEP) and mono-benzyl phthalate (MBzP)). This study aims at improving the following issues: 1) improvement of the sample preparation methodology (time reduction) was tested in nails by comparing levels of the metabolites between powdered and whole nail samples; 2) a microextraction method was validated using the best conditions (i.e. for whole nails); 3) the validity of the method was verified by assessing the PE metabolites in nine individuals from who two nail samples were collected 15 days apart. Also, one morning spot urine sample was sampled per day per participant. More, two participants have collected two more urine spot samples/day (one afternoon and one evening) during the sampling period to evaluate the diurnal variation in excretion levels. Finally, the levels in the two matrices were compared (short and long-term exposure) and major predictors of exposure were investigated based on personal/environmental surveys.

One of the major novelties of this study is the optimization of the US-DLLME method using whole nails for assessing human exposure to PEs, which makes the method suitable for large-scale routine analysis.

2. Materials & Methods

2.1 Reagents, standards and equipment

Neat standards of phthalate metabolites, including MEHP (99.9 %), MiBP (97.8 %), MnBP (97.4 %), MBzP (99.8 %) and MEP (100 %) were supplied from Accustandard Inc. (Conneticut, USA). 5-oxo-MEHP (>95 %) and 5-OH-MEHP (>96.5 %) were obtained from BCP instruments (Irigny, France). Mass-labelled internal standard (IS) solutions for all metabolites ($^{13}\text{C}_4$ -MEHP, $^{13}\text{C}_4$ -5-oxo-MEHP, $^{13}\text{C}_4$ -5-OH-MEHP, d_4 -MiBP, $^{13}\text{C}_4$ -MnBP, $^{13}\text{C}_4$ -MBzP and $^{13}\text{C}_4$ -MEP) were acquired from Cambridge Isotope Laboratories (Andover, USA) (95 %). Individual stock standard solutions of the metabolites and IS were prepared on a weight basis in acetonitrile at final concentrations approximately of 20 $\mu\text{g/mL}$. Working, spike and calibration standard solutions of both native and IS were prepared in UPLC acetonitrile and in ultra-pure water.

The organic solvents trichloroethylene, acetic acid (100 %) and nitric acid (65 %) were purchased from Merck (Darmstadt, Germany). The organic solvents such as acetone, acetonitrile (ACN), methanol (MeOH) were of UPLC grade (Fisher Scientific,

Loughborough, UK). Trifluoroacetic acid (TFA, 99 %) was supplied by Sigma-Aldrich (Steinheim, Germany). The ammonium acetate buffer (NH₄Ac) was prepared by dissolving 1.93 g of ammonium acetate (99.99 %, Sigma-Aldrich, Diegem, Belgium) in 200 mL ultra-pure water and acidifying the solution with acetic acid to pH 6.5. The enzyme β -glucuronidase (E. coli K12) was supplied by Roche Applied Sciences (Mannheim, Germany). The creatinine urinary colorimetric kit was supplied by Sanbio (Netherlands). The creatinine measurements were done using the 96-well plate spectrophotometer Tecan Bio M200 PRO (Mechelen, Belgium).

The ultra-pure water was provided by the Millipore S.A (Advantage A10 system, Overijse, Belgium).

Before use, all glassware was decontaminated in the oven at 450°C (overnight) for removal of organic contamination possibly deposited on the glass surface. Also, the contact with plastic materials (e.g. septa/ caps of centrifuge vials) was eliminated by sealing the septa with aluminium foil during sample preparation and extraction.

2.2 Nails and urine collection

Nine adults, five males and four females were recruited for this study in the beginning of 2015 in our institute (VITO NV, Belgium). The participants were duly informed about the purpose of the study giving their consent to participate (Ethical approval register N.° B300201316329). All volunteers collected one morning urine sample per day during 15 days of sampling period and two nail samples (one at the beginning (day 1) and another at end of the campaign (day 15)). During the sampling, the participants did not cut the nails, except at the last day of the sampling.

Furthermore, among the volunteers, two individuals have collected three urine voids per day, i.e. one morning spot urine sample (spot 1), an afternoon spot urine sample (spot 2) and a night/evening spot urine sample (spot 3)). At the end of the sampling (day 15), the participants filled in a short questionnaire. The volunteers were asked for their personal lifestyle (e.g. use of nail polish, hand care products, detergents), daily behavior (e.g. eating with hands, sport activities, consumption of prepacked food, and heating/storing food in plastics, etc.) and environmental exposure (e.g. time spend outdoor, time in car/public transport). The reported answers were regarded to the sampling period (15 days).

The urine samples were collected in 50 mL polypropylene containers initially washed with 10 % HNO₃ (v/v) followed by MeOH and air dried. The urine samples were stored in the dark at -24°C until analysis.

The nails were collected in polypropylene containers and stored at room temperature. Prior analysis, the nails were rinsed (twice) with acetone in order to remove dust particles and residues of nail polish.

For method development, nail samples collected from three individuals were considered. One part of the amount was homogenized, powdered in a mixer mill (30 min, 20 Hz) and further used as a pooled sample for replicate analyses (n=4). Another part of these nails was not powdered (four replicate analyses were done per person).

For method validation, a composite nail (whole) sample was made from some participants who had collected a high amount.

2.3 Phthalate metabolites microextraction from nails (US-DLLME)

The ultrasound extraction combined with dispersive liquid-liquid microextraction (US-DLLME) was previously developed at VITO for extracting PE metabolites from powdered nails [27]. Additionally in the current study, the use of whole nails was tested following the same procedure. Briefly, the extraction conditions of PE metabolites in nails were carried out as follows: 2 mL TFA (2M) in MeOH was added to 30 mg of sample (powdered or whole) spiked with IS (5-6 ng) and the extraction was performed under ultrasonication at 45 °C for 2h. Afterwards, the methanol extracts containing the analytes were mixed with 180 µL of trichloroethylene (extraction solvent) in a clean conical vial. These extracts were further dispersed in water (1 mL) promoting the formation of a ‘cloudy’ solution. The samples were centrifuged (6 min, 2851 RCF) for complete separation of the organic phase. The settled volume was collected with a syringe (~ 140 µL). Finally the extracts were completely evaporated under gentle N₂ flow and the residue was re-suspended in 100 µL of a mixture H₂O:ACN (75:25, v/v).

2.4 US-DLLME method validation for whole nails

The following method parameters were validated: working range, limits of quantification, precision and accuracy.

Calibration curves were constructed with 7-point calibration standards (0.3-490 µg/L) plotting the response (peak area $\times$ (IS concentration/IS area) *versus* the analyte concentration. After assessing the curve fit, the linearity was evaluated based on the calibration fit of repeated calibration standard injection (n=2 per calibration point). Also, repeated extractions for evaluation of the metabolites' levels in procedural blanks (in total 12) were performed.

The instrumental limit of quantification (LOQ_i) was determined based on the signal-to-noise ratio (S/N) of 10:1 extrapolated from the lowest standard in the calibration curve (0.3-0.7 µg/L). In case a method blank value was recorded, the average blank value was subtracted. The method limit of quantification (LOQ_m) was subsequently defined as 3 times the standard deviation (SD) of the blanks (corresponding to 99 % confidence level).

The method precision was evaluated by the intra- (repeatability; n = 6) and inter-day (intermediate precision; n = 6, during 3 consecutive days) variation for two spike levels (low spike level (LQ) of 4-12 ng and high level (HQ) of 7-23 ng in nails, depending of the compound. The relative standard deviations within and between days (% RSD_{within} and % RSD_{between}) were investigated.

Since there is no certified reference material available for the analysis of PE metabolites in nails, the method accuracy was evaluated based on in-house spiked samples. A known amount of target analytes was spiked (at two levels, low and high) to the nail samples and left standing until evaporation of the solvent (ACN) and/or absorption of the target compounds into the nail matrix. Finally, in order to ensure the quality control (QC) during the analyses, the target analytes were analyzed in procedural blanks (4 blanks extracted per day and injected in parallel with the nail extracts. Also new LOQ_m were calculated based on the levels detected in the procedural blanks provided in the same extractions set as the nail samples), blank solvents and a middle range calibration standard (between each series of ten samples) were injected in the LC-MS. The variation of the instrument performance was evaluated by the repeatability (RSD %) and accuracy (% R) of the standard.

2.5 Glucuronide deconjugation in urine

The deglucoronidation of the PE metabolites in urine was done according to Servaes et al. [35]. The metabolite's dilution effect in urine (ng/mL) was corrected by the creatinine

measurement via a colorimetric assay and the results were expressed as μg metabolite/g creatinine.

2.6 UPLC-MS/MS analysis

The LC separation of the PE metabolites was performed by Ultra Performance Liquid Chromatography (UPLC) and their quantification by tandem mass spectrometer (Quattro Premier XE Micromass) from Waters (Mildford, MA, USA). The chromatographic separation was performed in an Acquity UPLC BEH phenyl column (100 mm x 2.1 mm I.D; 1.7 μm particle size) with a Van Guard Acquity UPLC BEH C₁₈ pre-column (5 mm x 2.1 mm I.D; 1.7 μm particle size). The column was pre-heated at 40 °C.

Optimal separation conditions were achieved using a gradient elution program composed by water (solvent A) and ACN (solvent B), both acidified with 0.1 % acetic acid (v/v). The gradient elution program was: 0-0.5 min: 95 % A; 0.5-8 min: 95-10 % A; 8-8.5 min: 10-95 % A (return to initial conditions); 8.5-10 min: equilibration of the column. The mobile phase was discarded in the first 3 min to avoid the interference of proteins or other hydrophilic substances. The flow rate was set in 0.4 mL/min and the injection volume was 10 μL .

The UPLC system was coupled to the mass spectrometer (MS) interfaced with an electrospray ionisation source operating in negative ionization mode (ESI-). The MS operation conditions were as follows: cone voltage was set at 25 V, collision cell energy varied between 14 and 20 eV, dwell time varied between 0.05 and 0.25 sec. The electrospray source block and the desolvation temperature were 120 °C and 350 °C, respectively. The argon collision gas flow was kept constant at 0.25 mL/min. The cone and desolvation nitrogen gas flow were set at 50 L/h and 800 L/h, respectively. The characteristic precursor and daughter ions that were selected for detection of PE metabolites in multiple reaction monitoring mode (MRM) are the following: 197>77 (MEP), 197>79 (¹³C₄-MEP), 293>121 (5-OH-MEHP), 297>124 (¹³C₄-5-OH MEHP), 221>77 (MiBP and MnBP), 225>81 (d₄-MiBP), 225>79 (¹³C₄-MnBP), 291>121 (5-oxo-MEHP), 295>124 (¹³C₄-5-oxo MEHP), 255>183 (MBzP), 259>107 (¹³C₄-MBzP), 277>134 (MEHP), 281>137 (¹³C₄-MEHP) [27,34]. The daughter ions provided from the transitions were used for the quantification. The quantification of the MnBP and MiBP in the samples was done as a sum of the two isomers (Σ MnBP and MiBP) since they were not baseline separated.

2.7 Statistical analysis

All statistical analyses were performed using STATISTICA (version 12.0, StatSoft Inc.). Samples with levels below the LOQ_m were transformed to $LOQ_m/\sqrt{2}$.

Analysis of variance (two-way ANOVA) was performed for estimating differences between the paired nail samples collected from the nine individuals at the beginning and the end of the 15 days of sampling time (intra-person variability). A t-test was performed in order to evaluate the overall differences (inter-day variability) between the two nail sampling points (initial and final collection).

Spearman rank correlation coefficients (r_s) were calculated between concentrations determined in nails and the average concentration in urine collected during 15 days.

A repeated measures analysis of variance (ANOVA) was performed to calculate the intra-class correlation coefficient (ICC) of the ratio of between-individual variance divided by the sum of between-individual and within-individual variance. The ICC gives an indication of the variability of the repeated measurements within one individual. Firstly, the ICC was calculated for the urine spot 1 of all individuals ($n = 9$). Furthermore, the ICC was calculated including urine spots 1, 2 and 3 collected by two individuals (1 male and 1 female) during the 15 days sampling period. ICC values were interpreted as following: < 0.40 weak agreement, $0.40 - 0.75$ fair to good agreement and > 0.75 excellent agreement.

Univariate single pollutant linear regression analysis was performed to evaluate potential sources of exposure (e.g. use hand care products, nail polish, etc.) in relation to the levels of each of the metabolites in nails (average of two samples) and urine (averaged over the 15 days). Only the predictors that were significant in the univariate models ($p < 0.05$) were further examined in a multivariate regression model including all predictors as explanatory variables and each of the PE metabolite as outcome variable.

3. Results and discussion

3.1 Comparison of PE metabolites levels between powdered and whole nails

In our previous studies [27,34], we demonstrated that nails can be good biomarkers of exposure to PEs. In those publications, the nails were powdered before extraction of the PE metabolites using a new extraction method (US-DLLME).

In the current study we tested for the first time if the levels of the target analytes in powdered and ‘whole’ nails (cut into pieces), were significantly different or not (Figure 1). Reducing the sample preparation time was one of the main reasons to compare whole and powdered nail samples, especially if many samples need to be analyzed in a limited time. Seeing the limited number of grinding apparatus available in an average lab, the procedure has to be carried out in a sequential manner, which makes it a laborious and time-consuming procedure. Thus, skipping the grinding step in the sample preparation, can decrease on average 30 min per sample, making the overall workflow more efficient. Hence, levels of PE metabolites were compared between the two sample preparation methodologies.

The major metabolites observed were MEHP, MEP and $\Sigma(\text{MiBP}, \text{MnBP})$ with levels in whole nails ranging from 74 to 477 ng/g *versus* those in powdered nails averaging from 95 to 455 ng/g (Table 1). These results are confirmed by our earlier studies where these analytes were the most relevant ones in (powdered) nails [27,34].

According to our data, powdered nails showed less variation in the replicated measurements per individual which is most probably due to the higher sample homogeneity. Nevertheless, it is noticed that the levels of MEHP, $\Sigma(\text{MnBP}, \text{MiBP})$ and MEP can largely vary among the individuals, independently on the sample preparation method and which can be due to different exposures, instead. However, no significant differences were observed between the two extraction methodologies (Table 1) for paired samples (ANOVA p-values = 0.99). Of course, it is always important to consider factors such as: which metabolites, the amount of nails that can be collected per individual, number of replicate experiments, automation of the sample preparation and its limitations, number of total samples to analyze including the timing and purpose of the study (e.g. non-occupational exposure based on one collection, time trend evaluation).

Due to similar translation of the metabolites’ levels in both nail types, significant time and cost reduction in overall process and limited amount of nails collected per participant, whole nails were used for further analyses.

3.2 US-DLLME method validation for whole nails

In our first study, the US-DLLME was successfully validated using powdered nails [27]. In the present study, we have demonstrated that using whole nails is a good alternative and a huge time saver, especially when the method is especially implemented for routine analyses.

Hence, the US-DLLME protocol was validated using whole nails regarding the working range, accuracy (recovery), intra and inter-day precision and limit of quantification (LOQ_m) (Table 2). The linearity was evaluated by the fit of the calibration curves in the working range 0.3-490 $\mu\text{g/L}$. Thus, good fit of the responses *versus* concentration for each analyte was observed for all analytes ($R^2 > 0.987$).

So far, no certified reference materials are available for the analytes and matrix of interest, thus the recovery from fortified nail samples was measured to assess accuracy. The % R for each analyte was determined based on a curve fit, considering the zero point (non-spiked), a low spike level (LQ, 4-12 ng) and a high spike level (HQ, 7-23 ng). Native levels of PE metabolites in non-spiked nails ranged from 0.03 ng (e.g. 5-oxo-MEHP) to 3 ng (e.g. MEHP and $\Sigma(\text{MnBP}, \text{MiBP})$).

Recoveries ranged between 75 % and 106 %, demonstrating good accuracy. The precision (intra and inter-day) was expressed as relative standard deviation (RSD %) for both LQ and HQ spike levels. Thus, inter-day precision ranged between 3 and 35 % for LQ and varied between 4 and 10 % for HQ. Also the intra-day precision was also acceptable (% RSD values were lower than 20 % for LQ and < 14 % for HQ).

LOQ_i were in low ng/g range (0.02 to 0.92 ng/g) and levels in procedural blanks were used to calculate the LOQ_m . During validation the LOQ_m was estimated between 2 to 14 ng/g, depending on the metabolite. Additionally, due to possible contribution of external contamination and hydrolysis of the diesters into monoesters during the sample preparation, a set of blanks were prepared and analyzed together with the nails, and the levels were subtracted from the ones in the samples. Also, for a more accurate determination of the analytical threshold, the LOQ_m was calculated based on the levels in these blanks. Although, the levels in blanks were relatively low during nails analyses, still a fluctuation of the MEHP and $\text{sum}(\text{MnBP}, \text{MiBP})$ levels was observed from the one obtained in the validation, suggesting that external contamination can occur during extraction/sample preparation.

3.3 Method applicability and human exposure evaluation via nails using two time points

Two nail samples were collected by each participant (one at the start (day 1) and one at the end of the 15 day sampling (see a chromatogram of the analytes in Figure SI-1 in Supplementary Information), except for individual 3 who did not provide the first sample).

In this way, both method applicability and evaluation of the variability in the long-term exposure to PE was investigated.

Figure 2 shows the distribution of the levels among all individuals for the 1st (day 1) and 2nd nail (day 15) collection. Here, it is visible that the levels are more equitably distributed in the 1st collection (range from 57 to 179 ng/g) than in the 2nd collection (range from 54 to 304 ng/g) (see total PE levels for 1st and 2nd collection in Figure SI-2 in Supplementary Information). Yet, median levels were similar for the initial and final sample among the participants (97 and 121 ng/g, respectively), as well as the average levels (Table 3). More, the t-test conducted for assessing differences between the initial and final samples, revealed that there were no significant difference (p-values >0.05) between the two sampling points (Table 4). Also, there were no significant differences between the paired initial and final nail samples grouped per individual (ANOVA, $p = 0.33$), i.e. showing no intra-person variability. Although as expected there was a significant difference between the analytes' levels in detected nails for all participants, leading to two hypotheses: 1) that the bioaccumulation rate (*versus* excretion) is not equal to all target analytes; and/or 2) that the participants had distinct exposure scenarios to the various PEs. As an example and based on our results, 5-oxo-MEHP and 5-OH-MEHP do not tend to accumulate in nails (detection frequency 57-76 %), due to the fast metabolism of the MEHP into secondary metabolites (and/or conjugation) with consequent high excretion in urine (~70 % of the oral dose) [36]. Although this is true, we observed that the 5-OH-MEHP levels in nails (Table 3) were considerable high for one individual (N.º 7). Thus, if both metabolites have similar bioaccumulation rate in nails (but assuming that there are different levels of these metabolites circulating in blood, where 5-OH-MEHP prevails), it is possible to assume that 5-OH-MEHP which was not totally converted into 5-oxo-MEHP (and did not conjugate with glucuronic acid for fast excretion) can still accumulate in nails. Also, other factors such as age, gender diet, state of health, route and extend of exposure may influence the metabolism rate of DEHP into primary and secondary metabolites, also 5-OH-MEHP into 5-oxo-MEHP [37,38].

In contrary, it is suggested that other PEs and in particular the short-chain such as DEP are low excreted in urine in the monoester form (i.e. 24 % of MEP is excreted in 24h, 35 % in 48h and 50 % after 7 days) [39]. Therefore upon metabolism into the monoester their bioaccumulation in nails can be rather high as it happens for MEP once still 50 % is

circulating in our body. Actually, MEP is one of the major metabolites detected in nails which is also confirmed in our previous studies [27,34].

From our knowledge, the mechanisms of the PE metabolites's incorporation (like for many other contaminants) in nails was not yet explored. Still, there are some studies which refer that keratin has a major mediation and/or melanin into chemical's incorporation (e.g. drugs) into keratinized matrices, such as nails or hair, for instance [40]. Additionally, other authors have referred that factors such as age, gender and season [41,42] can influence the nail growth and therefore may provide shorter/longer exposure integration of a certain chemical. Fingernails grow on average 3.47 mm/month according to a recent study involving twenty-two American individuals [41] and at a faster rate (30-50 %) than toe nails [42], still fingernails are considered good indicators of past exposure. Based on this, an assumption can be made regarding the exposure reflected by the two nail samples analyzed in our study, i.e., the levels measured in the initial and final nail sample (day 1 and day 15) will reflect the exposure to PEs on average counting five to six months ago (depending on the nail length), where the difference in exposure between the two collections (approximately 2 mm), will reflect the levels accumulated in nails two weeks later than the 1st sampling. Thus, the reasoning on how reliable are nails to translate past exposure was done by comparing two nail samples from the same individual.

Ideally, a more detailed exposure information could be obtained from the analysis of various nails segments (collected weekly for a long period, for instance). However due to ethical/personal issues regarding the amount that could be collected per participant (i.e. in each time point), also the nail growth and taking into account the sampling duration, unfortunately was only possible to request more than two nail samples per participant collected apart from 15 days.

With regard to our results and based on the presented data we assumed that: 1) the exposure to different sorts of PEs is continuous but not equal to all PEs; 2) the metabolites (mostly the hydrolytic monoesters) which are not prone to oxidation or conjugation for fast elimination, circulate in the blood stream and therefore their bioaccumulation via the nail bed into the nail plate is favorable; 3) the incorporation of some metabolites in nails (replicate analysis) seems relatively high and constant over a time period of ca. 6 months suggesting stability of the matrix in measuring long-term exposure.

With the aim of understanding some of the most pertinent issues, especially to understand the metabolism with consequent bioaccumulation of the different PE metabolites

in nails (i.e. how and to which extend) and investigate which factors most contribute for their non-excretion in urine, we agree that in a near future more investigation is needed.

In this sense, the use of multidisciplinary research fields to cover the highlighted issues is highly recommended.

3.4 Spot urine samples

3.4.1 Human exposure to phthalate esters

A morning spot urine sample (spot 1) was collected by all participants during 15 days. Additionally, two participants collected two more urine samples in the afternoon (spot 2) and evening (spot 3). Table 5 represents the descriptive statistics (mean, geometric mean, media, maximum, minimum, 25th and 95th percentiles) for the measured metabolites in the urinary spot 1 (for all participants), spot 2 and spot 3 (only for two participants).

In overall, the major metabolites present in urine (97-100 % detection frequency (DF)) were Σ (MnBP, MiBP) (range of 0.06-30.5 $\mu\text{g/g}$ creatinine in spot 1; range of 0.53-6.0 $\mu\text{g/g}$ creatinine in spot 2; range of 0.69-5.8 $\mu\text{g/g}$ creatinine in spot 3) and MEP (range of 0.12-17.0 $\mu\text{g/g}$ creatinine in spot 1; range of 0.16-2.1 $\mu\text{g/g}$ creatinine in spot 2; range of 0.21-14.1 $\mu\text{g/g}$ creatinine in spot 3) (see boxplot distributions of the metabolites for the three urinary spots in Figure SI-3, Supplementary Information).

High total levels were observed in morning urine (spot 1) when comparing the levels with the other two spots (mean of 9.2-9.3 $\mu\text{g/g}$ creatinine) (Table 6) that were collected by the two participants (see also Figure SI-4 to Figure SI-6 in Supplementary Information).

Even considering the overall (low) levels excreted by this population, high median levels of short-chain metabolites such as MEP and MBzP were shown in spot 1 (morning) while for the long-chain, spots 2 and 3 seem to better represent their excretion. However it is important to highlight that the levels reported in Table 5 regarding the spot 1, considered the levels excreted during 15 days for nine individuals, while for spots 2 and 3 urine collections only two individuals were considered. Although, in terms of number of individuals who sampled more than one urine spot can be seen as not statistically representative, still the same is not valid in terms of individual spots (morning, afternoon and evening) collected per participant during the 15 days (i.e. spots per person > 40).

Yet, for better understanding an evaluation between levels excreted among the three spot urine samples was conducted. Since the levels were relatively low but still taking into account the two major metabolites (MEP, $\Sigma(\text{MnBP}, \text{MiBP})$), it was possible to evaluate which was the most relevant spot. Significant differences of $\Sigma(\text{MnBP}, \text{MiBP})$ levels between the spots were found in both individuals ($p=0.03$ and $p=0.009$), although for MEP significant differences were only found for the female participant ($p=0.004$). In addition, median values were higher for morning urine (spot 1) in both individuals either for MEP (1.0 and 2.0 $\mu\text{g/g}$ creatinine) and $\Sigma(\text{MnBP}, \text{MiBP})$ (4.6 and 5.1 $\mu\text{g/g}$ creatinine), as well as for minor metabolites.

So far, other authors have reported that the collection of multiple spots over time can provide a more complete assessment of exposure to various PEs [24], whereas morning urine seems to meaningfully represent the exposure to PEs [14]. Although we cannot draw major conclusions about the extent of the exposure (to different PEs) for the two individuals, it was however interesting to observe that the male participant showed a higher clearance of the total PEs, whereas the female participant showed a higher variability in exposure (mainly due to the higher levels of DBP and DEP metabolites excreted) (Table 6). Women may be more likely exposed to PEs than men due to the use of personal care products [1] which can be pointed out as one of the major contributors. Still, the human exposure to PEs via other sources (e.g. food, furniture, upholstery or pharmaceuticals [20,43,44]) can be considered high and not different among genders [12], therefore those sources would most probably not help to differentiate exposures among individuals.

3.4.2 Variability of PE metabolites in repeatedly sampled urine

Intra-class correlation coefficients were used to describe how strongly repeated measurements (of each of the PE metabolites) from different individuals (in different days) resembled each other in relation to the total variation (Table 7). The ICCs were all above 0.70 (ICCs 0.69-0.96) for repeatedly collected spot 1 (morning) urine by the nine participants. This means that the reproducibility of the measurements is considered very good for all analytes.

Morning spot urine samples reflected a rather stable continuous exposure to the parent PEs. Also, when considering all spot urine samples collected on different time points during the day, the ICCs were relatively high for 5-OH-MEHP, 5-oxo-MEHP and MBzP (ICC

from 0.77 to 0.94, i.e., excellent agreement), whereas for MEP and $\Sigma(\text{MnBP}, \text{MiBP})$ they were moderate high (ICC from 0.34 to 0.69, i.e., fair to good agreement) and very low for MEHP (ICC from 0.00 to 0.32, i.e., weak agreement). The low correlation between the levels over the days for MEHP can be explained by the very low detected levels (some levels were even $< \text{LOQ}_m$).

$\Sigma(\text{MnBP}, \text{MiBP})$ and MEP levels were variable over the day, especially higher in morning urine (spot 1) compared to the other time points. This indicates that morning urine does not cover all (peak) exposure variation during the previous day.

Interestingly, Frederiksen et al. [11] observed comparable ICCs for morning urine and 24h urine (collected among Danish individuals) for the major metabolites, i.e. MEP and $\Sigma(\text{MnBP}, \text{MiBP})$. However, their ICCs were lower than ours for spot and first morning urine samples (i.e. high within-person variability in the measured levels) either in unadjusted (ng/mL) or osmolality adjusted samples (ICCs < 0.68).

In fact, morning spot urine is much easier to obtain than 24h (e.g. logistics in sampling, storage, etc.), therefore 24h urine was not studied.

Other previously published ICCs for repeated urinary excretion of PE metabolites showed a high difference in reproducibility of the measurements over the days, depending on the population and the metabolite. Fromme et al. [12] reported high within-subject variability and a low reliability of repeated measures over 8 days for ten PEs metabolites measured in urine (ICCs ranged between 0.2-0.57). Also in a study by Valvi et al. [19] involving Spanish individuals, a high within- and between-individual variability was reported for a total of nine PE metabolites measured in two urine spots; Preau et al. [24] reported that there was a high between-person variability for 5-OH-MEHP levels in morning urine voids (ICC=0.25) while for MEP, the between-person variability was very low (ICC=0.91). Hoppin et al. [14] reported a better reproducibility of the measures between two urine spots collected among 46 African-American women (ICCs 0.53-0.80) while Peck et al. [17] observed that the reproducibility was rather low between 2 morning urine spots measured for 10 PE metabolites in 65 individuals (ICCs ranging from 0.13 to 0.64).

3.4.3 Creatinine measurements

Also, a discussion remains whether the levels of metabolites in urine should be creatinine corrected or not when comparing same sub-populations (e.g. adults, children, pregnant women, etc.) [7,24,45,46] where aside from affecting the dilution factor of the compounds in urine, also other factors such as muscle mass, diet (e.g. red meat consumption), gender and age can influence the creatinine levels [45,47].

Previously, some authors [47] have stated that morning urine contains higher creatinine levels than other spots. Thus, after creatinine correction the creatinine adjusted levels of the metabolites in urine become lower compared with that of an individual with a similar exposure.

In the current study, the morning urine creatinine levels in all participant ranged from 77 mg/dL to 157 mg/dL, which is within the normal range established by WHO (30-300 mg/dL) [48]. In contrast with a study by Barr et al. [47], in our study there was an increase in creatinine levels from spot 2 (afternoon) to spot 3 (evening) observed for the two participants (from 90 to 121 mg/dL and 39 to 94 mg/dL, respectively) who collected more than one spot/day. It is possible to verify that there were significant difference in creatinine levels between the two individuals for each spot (p -values = 0.05; inter-variability), but creatinine levels did not fluctuate significantly over the different days for each individual (p -values = 0.10; intra-variability). Apart from the fact that: 1) muscle mass is an important factor for increasing the levels in urine and 2) differences in creatinine were observed between the two participants (male and female), still the variation of the creatinine can also be due to other factors such as diet, physical exercise, gender or age [45,47]. Yet, since creatinine fluctuation was evaluated in only 2 individuals (considered not statistical significant), no definitive conclusions about the main influential factor can be drawn.

3.4.4. Urinary levels - a study comparison

The urinary levels of PE metabolites in the present study were compared to the levels in other Belgian sub-populations [3,8,49]. The exposure range for these metabolites within those populations was nevertheless very wide between sub-population (differences in the levels reached up to 100-200, 300 and 500 times more for MnBP, MiBP and MEP, respectively) [3,8,49], which was not observed in our small population, i.e. the variability of

the levels per analyte is much lower. Despite the low levels reported in our study (either expressed in ng/mL or either in $\mu\text{g/g}$ creatinine) compared to other Belgian studies, i.e. indicating that these individuals had a relatively low short-term exposure to PEs, is possible to observe that the same happened in other populations. For instance, in a study by Invin et al. [50] involving pregnant women, it was shown that the levels of PE metabolites (including MEP) were very low and especially when data is compared with same sub-population (i.e. pregnant women) from developed countries, for instance. Also other studies have noticed that levels of the major excreted metabolites, for instance MBP levels can be rather low among Japanese individuals (e.g. children and their mothers), sometimes even $< \text{LOQ}$ [6] or very high (range 22.8 to 554 $\mu\text{g/g}$ creatinine) even considering the same country [15].

This discrepancy in levels can be related not only to social-economic factors but also it can be country dependent and related to lifestyle habits (food, cosmetics, smoking), race/ethnicity and commercial purposes of specific consumer products [3,49].

Yet, we can observed that the major metabolites identified in our study (Σ (MnBP, MiBP) and MEP) correspond to the ones identified by most other authors: MnBP (and MiBP) and MEP in urine samples of Belgian populations [3,8,49]; MEP (mean of 183 $\mu\text{g/g}$ creatinine) and MBP (mean of 52.7 $\mu\text{g/g}$ creatinine) in American women [14]; MEP (median of 246-386 $\mu\text{g/g}$ creatinine) and $\Sigma(\text{DEHP})$ metabolites (median of 80- 87.8 $\mu\text{g/g}$ creatinine) in Spanish pregnant women [19]; MnBP and MiBP in a German [12] as well as in a Danish population [11].

3.5 Phthalate metabolite correlations: urine *versus* nails

Levels in urine and nails (representing short to long-term exposure to PEs, respectively) were compared to assess any correlation between matrices. Average levels of the target metabolites measured in morning urine spots were checked for correlation with the levels in the two nail samples collected per participant. In general, the data was not normal distributed (data not shown, but tested by the Shapiro-Wilk test, $p > 0.05$), therefore, Spearman rank correlation (r_s) was performed.

The levels of $\Sigma(\text{MnBP, MiBP})$, MBzP and MEP were good correlated among both matrices (Spearman rank $r = 0.77$, $r = 0.75$, $r = 0.73$, $p < 0.05$) (Table 8).

This was not observed in our previous study [34] and it may not be expected as the time frame of exposure reflected by the two matrices, is quite different. Although, the main reason for observing a correlation between the two matrices might be due to averaging the levels over a larger number of repeated spot urine samples, representing the continuous exposure levels of the individuals. The analysis of repeated (urine and nail) samples allows minimizing the intra-person variability of the levels (even if the levels in urine were rather low), therefore representing a more accurate result on the total individual exposure over a longer sampling period.

In our previous study [34], we found that the levels in one spot urine (morning) do not correlated with the ones in nails (in total of twenty participants), suggesting that each matrix has an individual response on PEs exposure assessment in a certain timeframe (i.e. urine mostly represents 24-48 h exposure, while nails represent past exposure). Also, changes in behavior (e.g. frequency and use of personal care products, contact with detergents/house cleaning products, consumption of food items containing PEs, etc.) can be responsible for increasing/decreasing the intake of PEs and therefore influence the excretion *versus* accumulation rates.

In fact, $\Sigma(\text{MnBP}, \text{MiBP})$ and MEP were the major detected metabolites, in urine and nails, both representing the high exposure to the parent PEs. The good correlation between urine and nails for these analytes may suggest that even if the metabolites are excreted in urine in less than 24h, also due to the continuous exposure they can also be stored partly in the nails. In fact, DEP is one of the major PEs incorporated in a wide range of personal and house cleaning products, including cosmetics, hair spray, body lotions, perfumes, detergents, softeners, etc. [4,51,52]. Besides, the DBP presence in cosmetics is no longer allowed since 2009 [53] it was still detected after the ban in nail polish products for instance, [51] and likewise DEP is being incorporated in house care products [4]. As a result, dermal contact and dermal absorption is highlighted by some authors as the major source of exposure to these PEs [18,54].

In urine, DBP metabolites are excreted within 24 h mostly as MnBP. However from BBzP metabolism, MnBP can be produced together with MBzP (major metabolite) [55]. Still, we observed that the MBzP correlation with $\Sigma(\text{MnBP}, \text{MiBP})$ in urine is weak ($r_s = 0.13$), showing that BBzP probably has a low contribution on $\Sigma(\text{MnBP}, \text{MiBP})$ metabolite's formation.

The lack of correlation between the urine and nails for 5-OH-MEHP and 5-oxo-MEHP indicate that these matrices reflect a different response. Previously, we have seen that the bioaccumulation in nails is rather low and that the clearance via urine is higher (100 % detection). These results are also in agreement with our previous study [34]. Indeed, urinary MEHP was well correlated with the DEHP oxidative metabolites ($r_s = 0.69-0.76$, $p < 0.05$), as was expected [16,34,56]. The high urinary clearance, was also reflected by a moderate correlation between the two oxidative metabolites ($r_s = 0.60$).

3.6 Explaining exposure in nails and urine - Elucidation of personal/environmental predictors

The influence of exposure sources on the levels measured in urine and nails was estimated from questionnaire information collected at the end of the 15 days sampling period. The multivariate regression models did not reveal any predictors of significance to the PE levels in nails and urine.

However, in the single regression models some relevant factors could be identified. Thereby, an association was observed between $\Sigma(\text{MnBP}, \text{MiBP})$ urinary levels that were influenced by the number of hours spend outdoor per day ($p = 0.04$; $R^2 = 0.46$). The same factor, and even more significant was observed in nails ($p = 0.00$; $R^2 = 0.68$). Time spend outdoors describes human contact with possibly a wide range of products via many sources (air, dust, soil, food, etc.), therefore the identification of the specific source without a more detailed survey is difficult to achieve.

In other studies, high levels of MEP in urine were associated with the use of certain household products (i.e., bleach, dish liquids and air fresheners, among others) [19] and with use of sunscreen lotions and cosmetics [30]. In our study, MEP levels in urine, but not in nails, were determined by consumption of prepacked food ($p = 0.03$; $R^2 = 0.49$). Also, in our study there are indications that this factor have contributed but not significantly, especially to explain the long-term DEP exposure (i.e. via nails). Nevertheless as reported by Rudel et al. [57], diet is not considered the major source of DEP, still none of the other predictors seemed to be associated with the levels in urine/nails.

Despite the reported low levels, 5-oxo-MEHP bioaccumulation in nails ($p = 0.03$; $R^2 = 0.49$) were more likely influenced by consumption of prepacked food than for MEP. In fact

its precursor PE, i.e. DEHP has been considered a ‘substance of very high concern’ (SVHC) since 2008 under REACH, thus its incorporation in food contact materials is supposed to not happen. Nevertheless, DEHP association with food packaging and food contact materials was already highlighted by other authors [44,57], making this finding quite interesting. Also this association (translated by a major DEHP metabolite in urine) is not totally impossible to occur, especially if we consider that many food products and plastic containers/packaging are imported outside EU where legislations aspects are not strict.

Furthermore, the use of hand care products and nails polish were associated with 5-OH-MEHP levels in nails ($p = 0.04$; $R^2 = 0.46$ and $p = 0.004$; $R^2 = 0.71$, respectively) and surprisingly not with MEP levels. Likewise DEP, also DEHP can be incorporated in cosmetics (e.g. perfume, nail polish, shampoo, foot cream, body milk) [4,52] therefore this predictor can be identified to predict the long-term exposure to DEHP, instead. Despite MEHP was one of the major metabolites in nails, none of the predictors could explain the high levels.

Also, a good relationship was found between the use of medical devices and MBzP in nails ($p = 0.02$; $R^2 = 0.55$). Despite DEHP is pointed out as the major PE incorporated in PVCs used in medical devices, it is also valid that DBP and BBzP used in other PVC products (e.g. vinyl flooring, adhesives and sealants, etc.) might contribute to elevate levels of the monoesters in urine [33,44]. For example, in studies by Geens et al. [3] and Larsson et al. [58] MnBP and MBzP levels in urine were mostly associated with PVC in flooring or wall covering.

3.7 Study Limitations and Future Perspectives

In order to evaluate the tendency of the metabolite’s bioaccumulation in nails (how and in what extent) and possibly identify influential factors (age, gender, race, season etc.), we suggest that together with a detailed personal survey, more nail (repeated) samples should be collected over a longer period (1- 6 months) from individuals who were preferably high exposed to PEs under controlled conditions (e.g. labelled PEs). Also due to the complexity of this subject, investigation should be conducted: 1) on which are the vehicles/body compartments (blood, tissues) responsible for the transportation of the formed (either primary and secondary) metabolites into the nail plate; 2) which are the main (intrinsic or

external) factors involved in differentiating and controlling the bioaccumulation *versus* excretion (in urine) for each metabolite.

Despite some issues were not yet deeply investigated and/or have a straightforward understanding, with regard to our results we believe that once the metabolites are incorporated in the nail plate, their levels remain unchanged. Thus, PE metabolites can be detected in nails (with less variability than in urine spots), therefore nails can be an alternative matrix in HBM and/or routine analyses on PE's exposure assessment. Still, the inter and intra-individual fluctuations in the levels that were seen among the participants, could be reflected by intrinsic (age, season, etc.) factors plus different exposure scenarios and metabolism rates amongst the individuals.

4. Conclusions

Improvements of the recently optimized US-DLLME method were suggested by reducing the sample preparation time and making the method more efficient and applicable in HBM/routine nail analyses. Levels of seven PE metabolites were determined in nails, either using whole or powdered samples. No statistical differences were found between the two types of samples, therefore in terms of cost-time benefit the method was successfully validated for whole nails extracts. Method applicability and a pilot exposure evaluation was conducted involving nine Belgian individuals that collected two nail samples (both reflecting the exposure window of ~ 6 months) and daily urine spots (short-term exposure) over 15 days.

Relevant metabolites in nails were MEHP, MEP and $\Sigma(\text{MnBP}, \text{MiBP})$. MEP and $\Sigma(\text{MnBP}, \text{MiBP})$ were the major PE metabolites identified in all urine samples. Morning urine spots showed the highest (total and median) levels and reflected a rather stable continuous exposure to the parent PE metabolites (ICC values > 0.70) during 15 days of sampling period. Good correlations between MEP, MBzP and $\Sigma(\text{MnBP}, \text{MiBP})$ between urine and nails were observed for the first time. The fact that multiple samples of both urine and nails were available from each individual, may contribute to significantly reduce the intra-person variability of the results, enabling to find a good correlation among those analytes in the two matrices. The good correlation between matrices for these analytes may also suggest that even if the metabolites are excreted in urine in less than 24h, however due

to the person's continuous exposure to the parent PEs and incomplete total excretion of the metabolites through urine, the metabolites can be partly stored in the nails.

Although there was a limited information in this study about the number of personal/environmental contributors of exposure studied, some associations were disclosed as for instance levels in nails and the use of personal care products or medical devices seems to be associated to the exposure to some relevant PEs (e.g. BBzP or DEHP).

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Figures and Tables

Figure 1. Whole nails (A) and powdered nails' powder (B).

Figure 2. Distribution of the levels of the PE metabolites (ng/g) detected in nails collected in the 1st and 2nd collection.

Table 1. Average levels of PE metabolites in powdered and whole nails for three volunteers.

Table 2. Intra and inter-day precision (n=6), % recoveries, LOQ_m and LOQ_i for the US-DLLME using whole nails.

Table 3. Average levels (ng/g) of the seven PE metabolites collected in two nail samples for nine Belgian individuals.

Table 4. T-test values (p-values) between independent nail sub-samples (initial and final) collected among nine volunteers.

Table 5. Results of 15 days repeated measurement of PE metabolites in spot urine samples of nine individuals (of which two also collected an afternoon and evening sample each over 15 days).

Table 6. Individual levels of PE metabolites in spot urine samples (morning, afternoon and evening) collected by two individuals (1 male and 1 female).

Table 7. Intraclass correlation coefficients (ICCs), between- and within-day variances in urinary levels (µg/g creatinine) for spots 1 (morning), spot 2 (afternoon) and spot 3 (evening) collected during 15 days.

Table 8. Spearman correlation coefficients between the levels of PE metabolites detected in nails averaged over the sampling at the beginning and the end of the 15-days period (ng/g) and in urine levels averaged over the 15-days period ($\mu\text{g/g}$ creatinine).

Supplementary Information

Figure SI-1. Chromatograms of the PE metabolites detected in nails (initial) of individuals 5, 7 and 9.

Figure SI-2. Boxplot distribution for total concentration of PE metabolites in nails (ng/g) for the initial (A) and final nail sample (B). The box hinges represent the 25th percentile and 75th percentile. The median (50th percentile) is represented by the middle line in the box. The whiskers extend 1.5 times the inter-quartile range.

Figure SI-3. Boxplot distribution for PE metabolites in urinary spots ((morning (spot 1), N=132), afternoon (spot 2), N=28), evening (spot 3), N=28)) collected by the nine individuals. Spots 2 and 3 were collected by only two individuals. The box hinges represent the 25th percentile and 75th percentile. The median (50th percentile) is represented by the middle dot in the box. The whiskers extend 1.5 times the inter-quartile range. The outliers are represented as a point (°) and extremes as a star (*).

Figure SI-4. Boxplot of urinary levels of PE metabolites (creatinine adjusted) in spot 1 (morning) collected over 15 days for the male and female participants.

Figure SI-5. Boxplots of urinary levels of PE metabolites (creatinine adjusted) in spot 2 (afternoon) over 15 days for the male and female participants.

Figure SI-6. Boxplots of urinary levels of PE metabolites (creatinine adjusted) in spot 3 (evening) collected over 15 days for the male and female

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Table 1

Average levels (ng/g) $\pm$ SD ^a							
Nails	Individual	MEHP	5-OH-MEHP	5-oxo-MEHP	Σ (MnBP, MiBP)	MBzP	MEP
Whole	1	73 $\pm$ 15	<3	1.2 $\pm$ 0.9	46 $\pm$ 12	<4	32 $\pm$ 14
	2	140 $\pm$ 25	<3	<2	63 $\pm$ 12	4.8*	1319 $\pm$ 365
	3	266 $\pm$ 171	3.6 $\pm$ 1.1	3.8 $\pm$ 4.0	114 $\pm$ 24	5.1*	79 $\pm$ 25
Average		160	3.6	2.5	74	5.0	477
Powdered	1	96 $\pm$ 1	<3	1.8 $\pm$ 0.5	67 $\pm$ 6	<4	46 $\pm$ 4
	2	132 $\pm$ 8	4.3 $\pm$ 0.3	1.4 $\pm$ 0.6	78 $\pm$ 18	8.1*	1252 $\pm$ 236
	3	256 $\pm$ 14	<3	2.5 $\pm$ 0.6	140 $\pm$ 10	<4	65 $\pm$ 5
Average		162	4.3	1.9	95	8.1	455

*detected in only one replicate sample; ^aSD- Standard deviation of four replicates (n=4); LOQ_m was calculated based on 3 times SD of procedural blanks (6 ng/g for MEHP; 7 ng/g for MEP; 3 ng/g for 5-OH-MEHP; 2 ng/g for 5-oxo-MEHP; 14 ng/g for Σ (MnBP, MiBP); 4 ng/g MBzP)

Table 2

Compound	LQ (4-12 ng, n=6)		HQ (7-23 ng, n=6)		% R	LOQ _m (ng/g)	LOQ _i (ng/g)
	RSD %	RSD %	RSD %	RSD %			
	intra-day ^a	inter-day ^b	intra-day ^a	inter-day ^b			
MEHP	20	16	9	8	95	6	0.06
5-OH-MEHP	10	5	9	8	106	3	0.08
5-oxo-MEHP	10	4	7	4	92	2	0.02
Σ(MnBP, MiBP)	18	35	9	10	103	14	0.92
MBzP	9	3	10	5	75	4	0.05
MEP	19	35	14	7	103	7	0.04

^a Intra-day Relative Standard Deviation (n=6); ^b Inter-day Relative Standard Deviation (n=6); %R- Recovery (%); LOQ_m-Method limit of quantification (3× SD blanks (n=10); LOQ_i-Instrumental limit of quantification (S/N=10 of the lowest calibration standard)

Table 3

Individual	Gender	MEHP	5-OH-MEHP	5-oxo-MEHP	Σ (MnBP, MiBP)	MBzP	MEP	Sum PE
1	Male	49.2	3.1	0.5	31.1	1.9	12.8	98.5
2	Male	48.0	1.2	2.5	33.0	1.1	7.0	93.0
3	Male	28.7	1.2	<LOQ _m	26.6	0.3	126.7	183.6
4	Female	44.7	11.8	2.0	69.1	0.7	22.2	150.6
5	Male	28.2	<LOQ _m	2.9	13.2	1.5	10.3	56.1
6	Female	41.3	3.8	0.8	49.5	1.9	4.5	101.9
7	Female	23.8	29.2	<LOQ _m	26.6	3.3	32.8	115.7
8	Female	20.1	3.1	0.7	134.8	1.1	44.4	204.2
9	Female	80.2	2.4	0.4	31.3	0.6	17.1	132.1

LOQ_m was calculated based on 3 times SD of three procedural blanks analyzed together with batch of samples (4 ng/g for MEHP; 1 ng/g for 5-OH-MEHP; 0.3 ng/g for 5-oxo-MEHP; 7 ng/g for Σ (MnBP, MiBP); 0.1 ng/g MBzP; 0.8 ng/g for MEP).

Table 4

Variables	p-values (t- test)					
	(between independent samples, initial and final nail samples)					
	MEHP	5-OH-MeHP	5-oxo-MEHP	Σ (MnBP, MiBP)	MBzP	MEP
Day 1 and Day 15	0.24	0.89	0.62	0.54	0.32	0.42

Table 5

Levels in urine for daily sampling over 15 days ($\mu\text{g/g}$ creatinine)															
Spot 1 (morning, 9 individuals)								Spot 2 (afternoon, 2 individuals)							
Metabolite	Mean	GM	Median	Min	Max	25 th p	95 th p	Mean	GM	Median	Min	Max	25 th p	95 th p	Max
MEHP	0.19	0.14	0.13	<LOQ _m	1.21	<LOQ _m	0.64	0.28	0.23	0.21	<LOQ _m	0.81	0.16	0.78	0.81
5-OH-MEHP	0.8	0.55	0.52	0.03	4.23	0.36	2.47	0.99	0.69	0.83	0.04	4.42	0.51	2.04	0.81
5-oxo-MEHP	0.45	0.36	0.36	0.09	1.96	0.23	0.98	0.57	0.43	0.52	0.06	2.13	0.27	1.05	0.81
$\Sigma(\text{MnBP, MiBP})$	3.02	1.98	2.23	0.06	30.53	1.36	6.97	2.85	2.49	2.79	0.53	5.99	2.18	5.75	3.02
MBzP	0.19	0.09	0.08	<LOQ _m	1.49	0.05	0.71	0.09	0.05	0.08	0.01	0.48	0.02	0.27	0.09
MEP	2.31	1.20	1.05	0.12	16.96	0.46	10.71	0.84	0.72	0.72	0.16	2.11	0.48	1.46	1.05
Σ (7 metabolites)	6.96	4.32	4.37	-	-	-	-	5.62	4.61	5.16	-	-	-	-	6.96

Descriptive statistics: spot 1 (N=132), spot 2 (N=28), spot 3 (N=28); Spot 1 levels are reported for all individuals (n=9) over 15 days; Spots 2 and 3 were only collected by two participants (male and female) over 15 days; GM-Geometric mean; 25th p and 95th p: 25th and 95th percentiles; Min-Minimum; Max-Maximum; Levels below the LOQ_m were represented as LOQ_m/√2 and adjusted for creatinine level.

Table 6

Analytes	Levels in urine from daily sampling over 15 days ($\mu\text{g/g}$)							
	Spot 1 (male)		Spot 1 (female)		Spot 2 (male)		Spot 2 (female)	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
MEHP	0.25	<LOQ _m -0.68	0.20	<LOQ _m -0.64	0.30	0.10-0.78	0.26	<LOQ _m -0.81
5-OH-MEHP	1.51	0.52-3.36	0.66	0.07-2.30	1.44	0.66-4.42	0.54	0.04-1.25
5-oxo-MEHP	0.89	0.39-1.96	0.51	0.20-1.71	0.84	0.38-2.13	0.29	0.06-0.57
$\Sigma(\text{MnBP, MiBP})$	4.60	1.49-6.97	6.52	1.00-30.53	3.40	1.22-5.99	2.30	0.53-4.37
MBzP	0.26	0.05-0.57	0.11	0.02-0.32	0.17	0.04-0.48	0.03	<LOQ _m -0.08
MEP	1.89	0.44-3.24	1.24	0.42-3.31	1.03	0.44-2.11	0.67	0.16-1.26
Σ (7 metabolites)	9.39	-	9.23	-	7.18	-	4.09	-

Levels below the LOQ_m were represented as LOQ_m/ $\sqrt{2}$ and adjusted for creatinine level.

Table 7

	MEHP	5-OH-MEHP	5-oxo-MEHP	Σ (MnBP, MiBP)	MBzP	MEP
Spot 1, 15 days, n=9						
ICCs	0.70	0.74	0.76	0.74	0.96	0.93
between-days σ^2	0.07	1.96	0.61	56.36	1.21	28.86
within-days σ^2	0.03	0.68	0.20	19.62	0.05	2.06
Spot 1, 15 days, n=2						
ICCs	0.23	0.83	0.74	0.34	0.83	0.69
between-days σ^2	0.02	5.36	1.08	27.68	0.17	3.21
within-days σ^2	0.06	1.06	0.38	52.55	0.03	1.45
Spot 2, 12 days^a, n=2						
ICCs	0.00	0.85	0.90	0.66	0.94	0.62
between-days σ^2	0.00	6.01	1.94	5.39	0.08	0.64
within-days σ^2	0.06	1.08	0.22	2.78	0.00	0.40
Spot 3, 13 days^b, n=2						
ICCs	0.32	0.77	0.75	0.43	0.79	0.53
between-days σ^2	1.25	2.50	0.85	2.95	0.85	15.30
within-days σ^2	2.66	0.76	0.29	3.93	0.29	13.58

The ICC is calculated by dividing the between-individual variability by the sum of the between- and within-person variability. ICC values were interpreted as following: < 0.40 weak agreement, 0.40 - 0.75 fair to good agreement and > 0.75 excellent agreement); ^a Day 3, 11 and 12 were excluded because there was only one urine sample; ^b Day 10 and 11 were excluded because there was only one urine sample; spot 1, 9 individuals ; spot 2 and 3, 2 individuals.

Table 8

		Spearman rank correlation coefficients (rs)					
		MEHP	5-OH-MEHP	5-oxo-MEHP	$\Sigma(\text{MnBP, MiBP})$	MBzP	MEP
Urine vs. Nails	MEHP	0.47	-0.02	0.14	0.36	-0.22	-0.12
	5-OH-MEHP	0.3	0.23	-0.3	0.1	-0.02	0.25
	5-oxo-MEHP	0.47	0.57	-0.01	0.38	0.15	-0.17
	$\Sigma(\text{MnBP, MiBP})$	0.33	0.45	0.19	0.77	-0.02	-0.07
	MBzP	-0.38	0.43	0.07	-0.25	0.75	-0.03
	MEP	-0.1	-0.32	-0.45	-0.08	-0.68	0.73

Values statistical significant at p-value < 0.05 are marked by bold script

Figure 1

Figure 2