# Ultrasound assisted extraction combined with dispersive liquid-liquid microextraction (US-DLLME)-a fast new approach to measure phthalate metabolites in nails

# Ultrasound Extraction Combined with Dispersive Liquid-Liquid microextraction (US-DLLME) for biomonitoring of phthalate metabolites in nails

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**Abstract**

A new, fast and environmental friendly approach, ultrasound extraction combined with dispersive liquid-liquid microextraction (US-DLLME) was developed for assessing the levels of seven phthalate metabolites (including the mono(ethyl hexyl) phthalate (MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (5-OH-MEHP), mono(2-ethyl-5-oxohexyl) phthalate (5-oxo-MEHP), mono-*n*-butyl phthalate (MnBP), mono-isobutyl phthalate (MiBP), monoethyl phthalate (MEP) and mono-benzyl phthalate (MBzP)) in human nails by UPLC-MS/MS. The optimization of the US-DLLME method was developed according to the Taguchi combinatorial design ($L_9$ array). Several parameters such as extraction solvent nature, extraction solvent volume, extraction time, acid media, acid concentration and vortex time were studied. The optimal extraction conditions achieved were 180 µL of trichloroethylene (extraction solvent), 2 mL trifluoroacetic acid in methanol (2M), 2 h extraction and 3 min vortex. Under the most favorable conditions, good intra-day (RSD % between 6 % and 14 %) and inter-day (RSD % between 2 % and 17 %) precisions were obtained. The accuracy ranged from 79 % to 108 % and the limit of method quantification ($LOQ_m$) was below 14 ng/g for all compounds. The developed US-DLLME method was applied to determine the target metabolites in ten Belgian individuals. Average levels of the analytes measured in nails ranged between 3.6 ng/g and 918 ng/g. The MEHP, MBP isomers and MEP are the major metabolites (100 % detection). Despite the miniaturization (low volumes of organic solvents), low costs and simplicity on extraction are main advantages of the proposed US-DLLME.

**Keywords:** nails, US-DLLME, UPLC-MS/MS, biomonitoring, non-invasive, phthalate metabolites

## 1. Introduction

Since 1920s, the phthalate esters (PEs) have been used worldwide in several consumer products (e.g. personal care products, perfumes, upholstery, food containers, toys, medical devices, insects repellents, antifoaming agents, building products, etc.) mainly as plasticizers, additives, emollients, humectants or sealants [1–6]. PEs are composed by a group of aromatic chemicals containing a phenyl ring with two attached and extended ester groups (i.e., alkyl chain). Depending on the length of the alkyl chain, PEs can be classified as high molecular weight (> 7 carbons) or low molecular weight (3 to 6 carbons) compounds [1, 7]. In addition, toxicity of PEs is associated with the length and branching of alkyl side-chain [8, 9], however several studies reported that the high molecular weight PEs are quickly metabolized and excreted than low molecular weight (short chain), therefore high molecular PEs have rather low bioaccumulation in human body [10, 11]. Yet, the PEs (and in some cases their metabolites) are considered toxic for animals and/or humans, where the most common effects include hepatoxicity, carcinogenicity, endocrine disruption, fertility decrease (reproductive system), low birth weight and decreased anogenital distance in children among other effects [5, 12-15].

However, the ubiquitous presence of PEs combined with the unexpected external contamination (e.g. for food during processing, handling, packaging or storage) makes the identification of the main routes (ingestion, dermal contact, hand-mouth contact) and factors (food, water, environment) that contribute for the total human exposure much more difficult [1, 5]. Also, it is known that PEs are constantly released into the environment by direct release, migration, evaporation, leaching and abrasion of and from the products they are used in, therefore they can be detected in different types of environmental samples such as indoor dust [16, 17] and/or (personal) ambient air [3].

Still, after all (preventive) regulation aspects imposed by law for controlling and/or reducing human exposure to PEs [18-22], there has been an increasing attention for human biomonitoring (HBM) and in particular for these substances and/or metabolites in target populations (e.g. pregnant/breastfeeding women, young/adolescents, etc.) [3, 6, 23, 24].

Thus, due to the fast metabolization (short half-life) of PEs in human body, urine is seen as a great matrix for assessing PEs, where the measurements in this matrix are considerable easy and fast [2, 4, 6, 7, 12, 23, 27-30]. Nevertheless, the dilution effect of certain metabolites even after creatinine-adjustment in urine (e.g. mono (ethyl hexyl) phthalate, MEHP) [1, 4] can be a considerable disadvantage [30] when a study comparison within participants and between matrices (blood, plasma) is needed. Thus, to overcome this gap alternative non-invasive samples like hair [31], saliva [12, 32] or nails (explored in this study), seems to be promising alternatives

in HBM studies [1], although not yet very explored due to the high frequency of non-detects or low capacity to translate significant internal exposure (> LOQ).

In fact, the major advantages of non-invasive over the invasive samples are remarked by the broader detection time window for human exposure (days to years) and by the easier, faster sampling and handling, long term storage and high stability [1].

Another point of concern which has been slightly overlooked is the lack of novel extraction/analytical methods able to detect PE metabolites at trace levels in biological samples. Most of the methods presented in the current literature [2, 6, 23, 28, 29, 31] for assessing PE metabolites either in liquid or solid matrices, involve a considerable time-consuming, tedious and an expensive approach based on solid phase extraction (SPE) or liquid- liquid extraction (LLE) followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

From our knowledge, the miniaturization of sample preparation and/or extraction can lead to reach the low (expected) detection levels in ng/g or pg/µL range, where there is a little even foreseen and applied to the solid non-invasive samples such as nails. The main feature of DLLME is the miniaturization of the extraction, leading therefore to enormous advantages including fastness, easiness, use of an environmental friendly approach, less waste of organic (and toxic) solvents, the use of low extraction solvent volumes (µL), combination of ultra-concentration and clean-up, low costs, simplicity of operation [33]. Due to these advantages, DLLME and/or its combination with other techniques (solid phase extraction, ultrasonication, solidification of single drop, for example) has gained an increasingly importance in distinct fields of application (environmental, food, pharmaceutical, inorganic, etc.) [34-38]. In particular, DLLME-based techniques have been also widely used to determine phthalate esters in water, wine, food, cosmetics, etc [39-42]. In addition, the use of microextraction techniques for assessing their metabolites seems very rare. So far, only one study by Sun et al. (2013) [43] that assessed the DEHP metabolites in urine using a DLLME-based approach was disclosed.

Thus, in the present study we proposed the use of the dispersive liquid-liquid microextraction (DLLME) in combination with an ultrasound extraction (US) for assessing low ng/g levels of seven PE metabolites (including, MEHP, 5-oxo-MEHP, 5-OH-MEHP, mono-*n*-butyl phthalate (MnBP), mono-isobutyl phthalate (MiBP), monoethyl phthalate (MEP) and mono-benzyl phthalate (MBzP)) in human nails. For the first time, the fast and more environmental friendly US-DLLME approach based on a combinatorial Taguchi design is developed.

## 2. Experimental section

### 2.1 Chemicals, material and Equipment

The neat phthalate metabolites including MEHP (99.9 %), MiBP (97.8 %), MnBP (97.4 %), MBzP (99.8 %) and MEP (100 %) were supplied from Accustandard Inc. (Connecticut, USA). The neat MEHP oxidative metabolites (5-oxo-MEHP (>95 %) and 5-OH-MEHP (>96.5 %)) were provided by BCP instruments (Irigny, France). Mass-labelled internal standard (IS) solutions for all metabolites ($^{13}$C-MEHP, $^{13}$C-5-oxo-MEHP, $^{13}$C-5-OH-MEHP, $^{4}$d-MiBP, $^{13}$C-MnBP, $^{13}$C-MBzP and $^{13}$C-MEP) were obtained from Cambridge Isotope Laboratories (Andover, USA) (purity 95 %). Individual stock standard solutions of PE metabolites and IS were prepared on a weight basis in acetonitrile for final concentrations approximately of 20 µg/mL. Working standard solutions of both native and IS were prepared separately by mixing the individual standards in Ultra Performance Liquid Chromatography (UPLC)-grade acetonitrile. The spike solutions (PEs and IS) and the standard used for the standard calibration curves were prepared in ultra-pure water by appropriated dilution of the working solutions.

The organic solvents such as trichloroethylene, chloroform, toluene, nitric acid (65% purity) and the acetic acid (100 %) were all purchased from Merck (Darmstadt, Germany). Acetone, acetonitrile (ACN) and methanol (MeOH) by UPLC-grade were purchased from Fisher Scientific (Loughborough, UK). Ttrifluoroacetic acid (TFA, 99% purity) and hydrochloric acid (HCl, 37% purity) were supplied by Sigma-Aldrich (Steinheim, Germany).

The ultra-pure water was provided by the Millipore S.A (Advantage A10 system, Overijse, Belgium). Before use, all glassware was prior washed and heated in the oven at 450°C (overnight) in order to reduce the environmental contamination deposited in the glass surface. Afterwards, the materials were stored and protected with aluminium foil against the contact with air and dust. The use (and contact) with plastic materials was minimized or eliminated (e.g. septa and caps of centrifuge vials were covered with aluminum foil prior use).

## 2.2 Samples collection and preparation

Nail samples were collected among volunteers in our institute (VITO) during 2014. All participants were duly informed about the purpose of this study giving their consent to participate (register N. ° B300201316329). Before the extraction, all nails were rinsed twice with acetone in order to remove dust particles and some residues of polish material. Then the samples were grinded and homogenized in a mixer mill (30 min, 20 Hz) and stored afterwards in a glass vial. For the method development, a pool sample was prepared mixing nails from different individuals, since the amount collected from an single participant was not enough for performing the assigned experiments (development and validation). After validation of the US-

DLLME, the evaluation of method and its applicability was performed in Belgian individuals (n= 10).

For analysis, 30 mg of powder nails were weighted into glass vials and spiked with IS (5-6 ng) (and PE metabolites, i.e., 8 ng of MEP, 9 ng of 5-oxo-MEHP and MBzP, 10 ng of MEHP and 5-OH-MEHP and 18 ng of MBP isomers in the method development and validation).

### 2.3 UPLC-MS/MS analysis

The instrumental analysis was performed by Ultra Performance Liquid Chromatography (UPLC) coupled to 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 $C_{18}$ pre-column (5 mm x 2.1 mm I.D; 1.7 µm particle size). The column temperature was kept at 40 °C.

Optimal separation was obtained using a gradient elution program: 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 into the analyte's ionization. The flow rate was set in 0.4 mL/min and the injection volume was 10 µL. The total run time was 10.5 min.

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: the cone voltage was set at 25 V. The collision cell energy varied between 14 and 20 eV (Table 1). The 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 presented in Table 1. The daughter ions provided from the transitions were used for the quantification.

### 2.4 Method development

#### 2.4.1 Extraction solvent nature selection for the DLLME

The extraction conditions of PE metabolites were carried out as follows: 2 mL of TFA:MeOH solution (1M) were added to 30 mg nails spiked with IS (5-6 ng) and native PE metabolites (8-18 ng). Then, the extraction was performed by US at 45°C. After 2h, the extracts were collected and together with 120 µL of each extraction solvent (trichloroethylene, chloroform, tetrachloroethylene, chlorobenzene or toluene were tested) were dispersed in 1 mL of water prior placed in a conic screw-cap vial. Then, the samples were centrifuged (6 min, 3500 rpm) and the settled organic phase was collected. The volume was completely evaporated under a gentle nitrogen flow and the residue was resuspended in 100 µL $H_2O$: ACN (75:25, *v/v*). The selection criteria for the suitable extraction condition was based on the instrumental response factor (Area analyte × (IS Concentration/IS Area) and in the extraction efficiency (% recoveries).

### 2.4.2 Optimization of the extraction parameters based on the Taguchi design

After the selection of the extraction solvent for the DLLME, other parameters such as the extraction time, acid media, vortex time and trichloroethylene volume (µL) were tested based on an orthogonal experimental array, namely the Taguchi design [44].

Thus, a Taguchi design composed by 4 factors and 3 levels summarized in the Table 2 was tested (1st and 2nd design). A total of nine experiments were carried out by following the $L_9$ orthogonal array. Each experiment was conducted in triplicate and the averages of the peak areas were analyzed. Also, the S/N ratio was calculated according with the following equation:

$$\frac{S}{N} = -log_{10}\frac{1}{n}\left(\sum y^2\right) \quad \textbf{(Equation 1)}$$

Where n is the number of observations and y the data (average of peak area).

Moreover, in the method optimization two sets of combinatorial designs were tested (1st and 2nd Taguchi design) which the main goal in the first set of experiments was to find the best condition, leading us afterwards to the fine tuning and achievement of the optimal (steady state) set of extraction in the 2nd design model.

## 2.5 Final US-DLLME procedure for extraction of PE metabolites from human nails

Two mL of TFA:MeOH mixture (2M) was added to the reaction vials containing the powder nails (30 mg) and 5-6 ng of IS. The samples were extracted in the US during 2h at 45°C. After, the methanolic extract (~ 2 mL) was collected to a clean vial and used as dispersion solvent in DLLME. The extraction solvent, i.e., trichloroethylene (180 µL) was mixed with the dispersion

solvent and both were simultaneously transferred at once to 5 mL screw cap conic vials containing 1 mL of ultra-pure water. Afterwards, a cloudy (triphasic phase) solution was formed by the fine organic droplets dispersed in the aqueous solution and the samples were vortex during 3 min for promoting a better enrichment of the analytes in the organic phase. After, the samples were centrifuged (6 min, 3500 rpm) for separation of the small µL drop deposited at the bottom. The organic phase was collected using a µL syringe (Hamilton, Bonaduz, Switzerland) into 300 µL conical glass inserts (Supelco, Bellefonte, USA). Further, the organic solvent was completely evaporated under a gentle nitrogen flow, the extracts were reconstituted in 100 µL of $H_2O$: ACN (75:25, *v/v*) and 10 µL was injected in the UPLC system (Figure 1).

### 2.6 US-DLLME method validation and nail samples analyses

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 determined.

Since there is no certified reference material available for the analysis of PE metabolites in nails, the method accuracy was evaluated based on the recoveries (%) calculated for non-spiked pool nail sample, LQ and HQ spike levels. The uncertainty was expressed as the expanded uncertainty U using a coverage factor $k = 2$, corresponding to a confidence level of 95 %.

The quality control (QC) was ensured by analyzing the levels of the target analytes in procedure blanks (4 blanks extracted per day and injected in parallel with the nail extracts) that were spiked with same amount of IS used for spiking the nails. The variation of the instrument performance (RSD %) during the injection of individual samples was evaluated by the injection a middle range standard (µg/L) in each ten samples.

The instrumental limit of quantification ($LOQ_i$) was defined as the concentration of analyte in the lowest calibration standard (0.3-0.7 µg/L) at which the signal-to-noise ratio of the quantification ion is greater than 10 times. The method limit of quantification ($LOQ_m$) was defined as 3 times the standard deviation (SD) of the PE metabolites's concentration that could be measured in procedure blanks (99 % confidence level). If the analytes were not detected in the blanks, thus the LOQ was defined as the $LOQ_i$.

In the end, the levels of the analytes detected in the procedure blanks were afterwards subtracted from those detected in the nail samples of ten volunteers.

## 3. Results and Discussion

In the US-DLLME method development, a set of parameters like the extraction solvent nature, extraction solvent volume, vortex time as well as acid media nature, its concentration and extraction time were optimized. The first parameter that was optimized is the one which has more significance on the DLLME step, i.e., extraction solvent nature. This step is crucial once only ternary mixtures that form stable emulsions and that are separable into two distinct phases after centrifugation, are a result of a successful DLLME. If no stable cloudy state and no sedimented phase is observed, it means that the triphasic phase in DLLME was not successfully reached, therefore the analyte's transfer did not occur.

### 3.1 Optimization of the extraction solvent nature in DLLME

In DLLME, halogenated and non-halogenated solvents can be used depending on the nature of the target analytes [33-36]. Thus, before choosing the suitable extraction solvent, it is important to consider in advance its chemical properties which should comply with the essential DLLME requirements [33].

Based on this criteria, a set of five halogenated organic solvents were selected for the DLLME in this study (see Table SI-1 in Supporting Information) using the mixture TFA:MEOH (1M) as dispersion solvent (see experimental conditions in section 2.4.1).

Stable cloudy solutions were formed immediately after mixing both extraction and dispersion solvents (containing the metabolites) in the aqueous phase. Nevertheless, when using chloroform (120 µL) a bad dispersion was observed and consequently a small and instable settle phase was formed (8-28 µL) after centrifugation. In fact, chloroform has the highest water solubility among the extraction solvents selected, thus a higher volume was needed for better dispersion and subsequent drop formation. However, the addition of a higher volume was not tested here because: 1) in DLLME the use of organic solvent should be minimized as much as possible depending on the overall conditions (in µL range); 2) add more chloroform would promote less enrichment of the analytes in the final extract resulting in a formation of higher drop volume (not desirable) and less enrichment of the analytes. The dispersion (cloudy state) using other solvents was well performed, therefore the settle phase volume ranged from 80 to 100 µL (except for toluene which volumes between the replicates were more variable and around 62 to 100 µL).

According to the results (Figure 2), high and low response factors were obtained for trichloroethylene and toluene, respectively. Additionally, the recoveries of the metabolites were

better for trichloroethylene, i.e., the % R ranged between 80 and 108 %. Due to the low response factor and non-reproducible replicates observed for chloroform, then this solvent was excluded from further method development. The trichloroethylene was considered the best extraction solvent in the US-DLLME.

### 3.2 Outputs of the Taguchi design during method development

#### 3.2.1 First Taguchi design

The analysis of data resulted from a combinatorial design such as the analysis of (mean) peak areas, the S/N ratio or the analyses of the variance (ANOVA), are the recommended parameters to identify the influence of each factor in the Taguchi model [44]. Nevertheless, the conceptual approach of analyzing the output by plotting the factors (and levels) and analyzing their effect based on the increase of peak area/response, seems to lead to a fast and easy visual identification of the most significant factors in the extraction.

In this study the analysis of the results provided by the Taguchi method were firstly based on plotting the peak areas of each significant factor for the first and second models (i.e., extraction time, acid media type and concentration, vortex time and trichloroethylene volume) against each level. Later on, the S/N was also evaluated in both models for confirmation of the chosen optimal conditions.

Firstly, the results presented in Figure 3, show that extraction time, acid media and the TC volume are the most significant factors in the first design. The best peak areas were represented for 2h of extraction, TFA in the acid media and 140 µL of TC. Accordingly, there is on one hand a more predominant increase of the peak areas after increasing the extraction time and TC volume, mostly for MEHP and for MBP isomers. On the other hand, for MEP, 5-oxo-MEHP, 5-OH-MEHP and MBzP, there was only a slightly increase of the peak areas observed for these two parameters. Thereby, it is noticed that the optimal conditions were not yet reached, therefore further optimization is needed for extraction time and TC volume, as well as for the vortex time which no clear trend was observed for most of the analytes (except for MEHP).

In addition, the use of different acids (i.e. the acid media) were tested in the 1$^{st}$ Taguchi design. Here, strong acid such as the TFA (pKa of -0.25) favored the protonation of the analytes before the ionization in negative mode in the LC-MS/MS. The other two acids tested, HCl and $HNO_3$ (pKa -6 and -1.45, respectively) although are considered strong acids, do not have the same dissociation degree that TFA has, making it a much more powerful acid [45]. Also, some authors have already reported that TFA promotes an efficient extraction of the PE

metabolites from a similar matrix, i.e. the hair [31]. Nevertheless, any other extraction method (and/or condition) were so far explored for these PE metabolites in human nails.

Thus, 2h extraction, 140 µL TC volume, 2 min vortex and TFA: MeOH (1M) were the conditions selected as starting point for further optimization (in the 2nd Taguchi design).

### 3.2.2 Second Taguchi design

The 2nd Taguchi was performed for fine-tuning the method extraction. Therefore, the best conditions extracted from the first model were used as initial optimization conditions. In addition, two more levels (i.e., 2h30 and 3 h (extraction time); 0.5 M and 2 M (TFA:MeOH concentration); 3 and 4 min (vortex time); 160 µL and 180 µL (TC volume)) for each parameter were tested.

With regard to the results (Figure 3), there was in general a significant increase from the level 1 to level 3 for each factor (parameter), except for the extraction time where there was no significant contribution to carry on the extraction for more than 2h. The same was observed for the vortex time, i.e. 3 min was considered enough time to promote interaction between the TC and the analytes in the cloudy system. This step is important to accelerate the transfer of the analytes and subsequent the drop formation. However after 3 min, we observed that the peak areas did not improve and beyond that it would make the method undesirable more time-consuming.

The responses based on the acid media concentration increase were substantially high for all metabolites, except for MEHP. From our knowledge this metabolite is one of the major (among the studied PE metabolites) present in nails, therefore its detection in nails using TFA:MeOH (2M) will promote the release of relatively high amounts still.

The increase from 140 to 180 µL promoted better enrichment of the analytes in the final extracts (high areas, except for MBP isomers where a slightly decreased is noticed), thereby the optimal condition selected was 180 µL.

### 3.2.3 The S/N evaluation-Taguchi design

Another factor which can be representative of the extraction efficiency in the Taguchi design, is the S/N ratio (Equation 1) which results are presented in Figure 4.

Here we explained that for the 1st model (section 3.2.1), the level 3 represents the lowest S/N values for the extraction time (120 min), vortex time (3 min) and TC volume (140 µL) while for the acid media level 1 (TFA) shows better S/N (i.e. lower value).

Further, the optimal conditions (2nd Taguchi, section 3.2.2) are confirmed by the level 3 which is visibly the best for TFA concentration (i.e., 2M) and also for the TC volume (180 µL); the level 2 showed lower S/N than level 3 for vortex time (i.e. for 3 min), therefore again increasing the vortex time seems do not contribute positively for the extraction of the analytes to the organic phase (as shown before in Figure 3).

The steady-state condition resulting from the variation of the extraction time, showed that extending the extraction time more than 2 h is not beneficial. Also, the peak areas for some compounds (Figure 3) represented by 2.5 h and 3 h of extraction had quite similar response. Therefore, the final extraction conditions for the US-DLLME are set as 2h extraction time, TFA:MeOH (2M), 3 min vortex and 180 µL of TC.

### 3.3 US-DLLME method validation

The linearity method was determined for each compound by injecting seven standard solutions in the concentration range of 0.3-490 µg/L. The concentration of the IS was constant in all standard solutions, however dependent on the analyte (13-18 µg/L) once the standard solutions were made on mass weight basis (and not volume). The calibration curves fulfilled the requirements of a linear fit, where the square regression coefficients ($R^2$) were higher than 0.99 for all calibration curves.

Since there are no existing certified reference materials (CRM) for the analysis of PEs in nails, the accuracy of the method (% recoveries) was assessed based on spiking the nails with two levels of the target compounds (high (HQ) and low (LQ) spike levels) and evaluating the levels of PE metabolites in a non-spiked pool sample. After, the accuracy levels for each analyte was determined based on a curve fit of the corrected concentrations (difference between detected concentration and theoretical spike in nails) considering as points: the zero (non-spiked sample), LQ (4-12 ng) and the HQ (7-23 ng) levels. Therefore, good accuracy was achieved in the present method for all compounds (% R ranged between 79 % and 108 %). The precision (intra and inter-day) was expressed as relative standard deviation (RSD %) for both LQ and HQ spike levels. The intra-day variation did not exceeded 14 % and 9 % for LQ and HQ, respectively. Also, a good inter-day precision was determined (RSD % < 17 %). The uncertainty for these experiments were acceptable (ranging from 14 % to 24 % (LQ) and between 13 % to 26 % (HQ)), except for MEHP (Table 3).

Although, all efforts were done for reducing or eliminating the analyte levels' from the procedure blanks, thereby this was not completely possible and some metabolites were still detected. Albeit, we are analyzing metabolites and not the phthalate precursor compounds, the

probability of detecting false positives provided by external contamination is minimized in this study. As preventive actions, all glassware was decontaminated in the over (450 °C) prior extraction, reducing the amount of PEs that could come from environmental contamination (being deposited in glass surface and which by chemical hydrolysis in TFA could form some monoesters that are not exclusively enzymatically formed (e.g. MEP)). Thereby, a set of procedure blanks was done in the same day as the sample's extraction, subtracting the direct external contamination effect possibly occurred during sample preparation. The concentration of the analytes in the procedure blanks was relatively low (0.03 to 0.18 ng/g). In the US-DLLME, the $LOQ_m$ was determined based on the levels in procedure blanks, therefore $LOQ_m$ varied between 2 and 14 ng/g (Table 3). Also, QC standards (middle point calibration standard) and solvent blanks were analyzed for instrumental quality control and assessment of external contamination. The RSD % between standard replicates did not exceed 11 %, thus good analytical quality on the measurements was ensured.

### 3.4 Method applicability: Nail Analyses

The US-DLLME method was applied to the determination of the PE metabolites in nails of ten Belgian individuals. The average levels ranged between 3.6 ng/g and 918 ng/g (Table 4) showing the good applicability of the developed method and consequently the importance of nails as matrix of choice in HBM field. The major metabolites were the MEHP, the MBP isomers and the MEP (detected in 100 % of the studied individuals) (see chromatograms in Supporting Information). The MEHP oxidative metabolites were minority detected in nails (<LOQ-3.8 ng/g) and their detection seems not relevant in this matrix, instead MEHP seems a better biomarker of DEHP if nails are considered in HBM studies. Although, MBzP was not the major metabolite detected, their exposure in nails should not be neglected (40 % of detection frequency).

The comparison of the present method with other extraction methods for nails was not possible due to the lack of literature in this field. Thus, the only possible comparison was done between methods that determined sorts of PE metabolites in matrices that are not usually explored (e.g. hair, saliva, serum, plasma). The methods reported in Table 5, suggest that in other matrices (instead urine), enzymatic (for serum and plasma, for instance) or acid (for hair) hydrolysis [7, 23, 31] followed by solid phase extraction (SPE) or liquid-liquid extraction (LLE) need to be carried out, firstly to destroy the matrix and release the target metabolites and then to concentrate them (and remove interferences) in the final extracts. Nevertheless, we pointed out some disadvantages of those methods such as the extraction time exceeds largely

2h, the extraction (and clean-up) is labour extensive (time consuming), an higher volume of solvents (organic, acids/bases, etc.) is used especially for SPE where a higher volume of waste solvents, increase of overall costs.

Instead, the optimized US-DLLME method presents good potential in the determination of PE metabolites in nails (high detection in ng/g for some metabolites), comparable or lower extraction time, low costs because less solvent are used in the extraction (µL) and less waste is produced, so in this sense this method can be consider more environmental friendly. Therefore, the proposed method seems of high relevance in HBM for determining and quantifying PE metabolites via nails.

## 4. Conclusions

For the first time a new, fast and more environmental friendly extraction approach than the conventional solid-phase extraction (SPE) or liquid-liquid extraction (LLE) was developed, combining the US and the DLLME for assessing seven PE metabolites in human nails. Taking advantage of the well-known miniaturization features in DLLME, thus less organic solvents were used (µL of trichloroethylene), high preconcentration of the target analytes in final µL drop was successfully obtained and the extraction efficiency was increased by the vortex association upon the cloudy phase formation.

The Taguchi design enabled the simplification of the number of experiments ensured by the linear combination of the levels and factors, which therefore reduces the workflow, solvents and sample consumption, etc. Also, the US-DLLME optimal conditions were achieved for a reduced amount of nail samples (30 mg). The good precision, accuracy and low quantification (instrumental or method) limits of the method and its applicability for determining the levels of the metabolites in ten individuals is successfully demonstrated. Also, the first insights of this study showed that nails can be a valuable alternative matrix for measuring human exposure to certain chemicals.

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448 **Figure Captions**

449

450 **Figure 1**. US-DLLME scheme for analysis of PE metabolites in human nails.

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452 **Figure 2.** Optimization of the extraction solvent nature in DLLME.

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454 **Figure 3.** Output from 1st and 2nd Taguchi models during method optimization (parameters
455 tested: extraction time, acid media and concentration, vortex time, trichloroethylene volume).

456

457 **Figure 4.** S/N values for the 1st and 2nd Taguchi designs.

458

459 **Table 1.** MRM transitions, collision energy, cone voltage and retention times for the target
460 metabolites and respective IS.

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462 **Table 2.** Selected factors and levels in the 1st and 2nd Taguchi design for the US-DLLME.

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464 **Table 3.** Analytical performance of the US-DLLME in nails.

465

466 **Table 4.** Levels of the 7 PE metabolites in ten individuals.

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468

469 **Table 5.** Comparison between the US-DLLME method and other extraction methods for
470 determining PE metabolites in non-conventional matrices (nails, hair, serum and seminal
471 plasma).

472

473 **Supporting Information**

474

475 **Table SI-1.** Density, solubility in water and polarity index for five halogenated solvents.

476

477 **Figure SI-1.** Chromatograms of Σ(MnBP, MiBP), MEHP, MEP and MBzP detected in nails of
478 two participants (participant 8 and 3).

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