New SPE-LC-MS/MS method for the simultaneous determination in urine of 22 metabolites of DEHP and alternative plasticizers from PVC medical devices

Jérémy Pinguet, Nicolas Kerckhove, Teuta Eljezi, Celine Lambert, Emmanuel Moreau, Lise Bernard, Benoit Boeuf, Bertrand Décaudin, Stéphanie Genay, Morgane Masse, et al.

TITLE PAGE

Title

New SPE-LC-MS/MS method for the simultaneous determination in urine of 22 metabolites of DEHP and alternative plasticizers from PVC medical devices.

Jérémy PINGUET[1], Nicolas KERCKHOVE[1,2], Teuta ELJEZI[3,8], Céline LAMBERT[4], Emmanuel MOREAU[6], Lise BERNARD[3]; Benoit BOEUF[5], Bertrand DECAUDIN[7,8]; Stéphanie GENAY[7,8]; Morgane MASSE[7,8]; Laurent STORME[9,10], Valérie SAUTOU[3] and Damien RICHARD[1], for the ARMED Study Group.

Author affiliations

1 CHU Clermont-Ferrand, Université Clermont-Auvergne, service de Pharmacologie médicale, UMR INSERM 1107 Neuro-Dol, F-63000 Clermont-Ferrand, France

2 CHU Clermont-Ferrand, Délégation Recherche Clinique & Innovation, F-63000 Clermont-Ferrand, France

3 CHU Clermont-Ferrand, CNRS, SIGMA Clermont, Institut de Chimie de Clermont-Ferrand, Université Clermont Auvergne, F-63000 Clermont-Ferrand, France

4 CHU Clermont-Ferrand, Unité de Biostatistiques, Délégation Recherche Clinique & Innovation, F-63000 Clermont-Ferrand, France

5 CHU Clermont-Ferrand, service de réanimation pédiatrique et périnatalogie, F-63000 Clermont-Ferrand, France

6 Université Clermont-Auvergne, INSERM U1240 Imagerie Moléculaire et Stratégies Théranostiques, F-63000 Clermont Ferrand, France

7 Université Lille II, EA 7365 - GRITA, F-59000 Lille, France

8 CHRU Lille, Institut de Pharmacie, F-59000 Lille, France

9 CHRU Lille, Service de Médecine Néonatale, F-59000 Lille, France

10 Université Lille I, UPRES EA 4489, Laboratoire de Périnatalité et croissance, F-59000 Lille, France

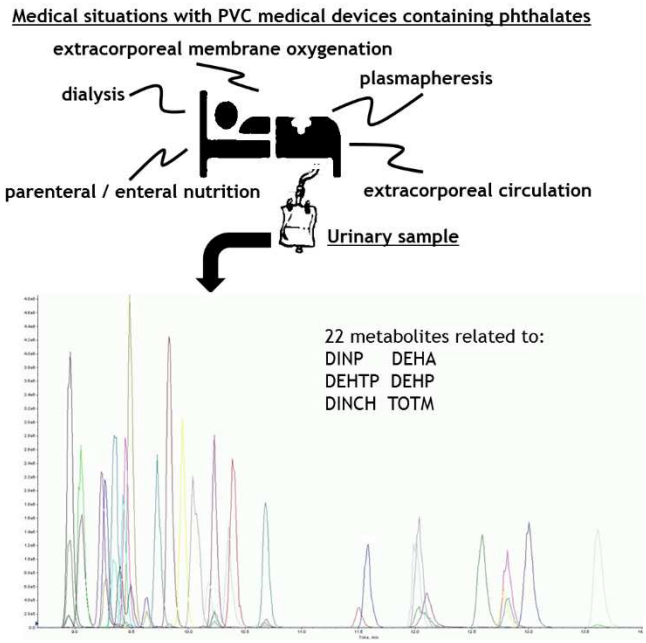
Corresponding author

Jérémy PINGUET, +33(0)4 73 751 822, jpinguet@chu-clermontferrand.fr, CHU Clermont-Ferrand, service de Pharmacologie médicale, 58 rue Montalembert, F-63000 Clermont-Ferrand, France

ABSTRACT

DiEthylHexylPhthalate (DEHP) can leach out of plasticized PVC medical devices (MD) and may enter into contact with patients. This phthalate is known for its reprotoxic and endocrine disrupting effects. Its use in medical devices (MD) has been restricted and alternative plasticizers have been developed. Nevertheless, no published clinical studies exist concerning patient exposure to these alternative plasticizers during medical care. This is particularly worrisome when high-risk populations, such as newborns, are exposed to these new plasticizers in intensive care units. Our study aimed to develop a novel sensitive and selective method to simultaneously identify and quantify DEHP and 17 other plasticizer metabolites (free or glucuronide conjugates), which are specific biomarkers of DEHTP, TOTM, DINP, DINCH and DEHA exposure in human urine. This robust method uses turbulent-flow online extraction technology coupled to high performance liquid chromatography – tandem mass spectrometry. Special care was taken to address two major problems in plasticizer analysis: contamination and chromatographic separation of interfering analogue structures. The validation was assessed in synthetic urine and the linearity of response was demonstrated for all compounds (R^2 > 0.99), with limits of quantification from 0.01 to 0.1 ng/ml. Accuracies ranged from 86% to 117% and inter- and intra-day precisions were < 20%. The clinical applicability and suitability of our new method was assessed in patients in a neonatal intensive care unit to measure urinary concentrations of DEHP and alternative plasticizer metabolites. These metabolites were found in the majority of urine samples, with a median detection frequency of 95.2% (ranging from 12.5% to 100%). The high sensitivity, selectivity and ruggedness make the method suitable for large-scale biomonitoring studies of high-risk and general populations.

Graphical abstract



Highlights

1 A new analytical method including primary and secondary metabolites of new DEHP-alternative plasticizers.

2 Highly sensitive, specific and fast on-line turbulent flow liquid-chromatography technology coupled to tandem mass spectrometry for routine human biomonitoring.

3 First study assessing exposure to phthalates and alternative plasticizers derived from medical devices in neonatal intensive care unit patients.

KEYWORDS

Alternative plasticizers, Medical devices, SPE-LC-MS/MS, Human biomonitoring, urinary metabolite

Abbreviations

1-MEHTM	1-mono-(2-ethylhexyl) trimellitate
2cx-MMHP	mono-(2-ethyl-2-carboxypentyl) phthalate
2cx-MMHTP	1-mono-(2-carboxylmethylhexyl) benzene-1,4-dicarboxylate
2-MEHTM	2-mono-(2-ethylhexyl) trimellitate
4-MEHTM	4-mono-(2-ethylhexyl) trimellitate
5cx-MEPP	mono-(2-ethyl-5-carboxypentyl) phthalate
5cx-MEPTP	1-mono-(2-ethyl-5-carboxylpentyl) benzene-1,4-dicarboxylate
5OH-MEHTP	1-mono-(2-ethyl-5-hydroxyhexyl) benzene-1,4-dicarboxylate
5oxo-MEHTP	1-mono-(2-ethyl-5-oxohexyl) benzene-1,4-dicarboxylate
ACN	acetonitrile
ARMED	assessment and risk management of medical devices in plasticized polyvinyl chloride
CHDA	cyclohexane-1,2-dicarboxylic acid
CMR1b	carcinogenic, mutagenic, or toxic for reproduction 1b
CPP	comité de protection des personnes
CV	coefficient of variation
cx-MINCH	cyclohexane-1,2-dicarboxylate-mono-(7-carboxylate-4-methyl)heptyl ester
cx-MiNP	mono-(4-methyl-7-carboxy-heptyl) phthalate
DEHA	di-2-ethylhexyl adipate
DEHP	diethylhexylphthalate
DEHTP	di-2-ethylhexyl terephthalate
DINCH	diisononyl cyclohexane-1,2-dicarboxylate
DiNP	diisononyl phthalate
EMA	european medicines agency
FIA	flow injection analysis
HPLC	high-performance liquid chromatography
IPA	isopropanol
IS	internal standard
LC-MS	liquid chromatography mass spectrometry
LLOQ	lower limit of quantification
LOD	limit of detection
LOQ	limit of quantification
MCOP	monocarboxyoctyl phthalate
MD	medical devices
MEHA	mono-(2-ethylhexyl) adipate
MEHHP	mono-(2-ethyl-5-hydroxyhexyl) phthalate
MEHP	mono-(2-ethylhexyl) phthalate
MEHTP	mono-(2-ethylhexyl) terephthalate
MeOH	methanol

MEOHP	mono (2-ethyl-5-oxohexyl) phthalate
MHNCH	mono hydroxyisononyl ester
MINCH	monoisononyl cyclohexane-1,2-dicarboxylate
D_{17}-a-MINCH	2-(((octyl-D17)oxy)carbonyl) cyclohexane-1-carboxylic acid
MINP	monoisononylphthalate
MRM	multiple reaction monitoring mode
MTBE	tert-butyl methyl ether
NICU	neonatal intensive care unit patients
OH-MINCH	cyclohexane-1,2-dicarboxylate-mono-(7-hydroxy-4-methyl) octyl ester
OH-MINP	mono-(4-methyl-7-hydroxy-octyl) phthalate
oxo-MINCH	cyclohexane-1,2-dicarboxylate-mono-(7-oxo-4-methyl) octyl ester
oxo-MINP	mono-(4-methyl-7-oxo-octyl) phthalate
PVC	plasticized polyvinyl chloride
QC	quality control
SCENIRH	scientific committee on emerging and newly-identified health risks
SIL	stable isotopically-labelled
SPE	solid phase extraction
TOTM	tri-2-ethylhexyl trimellitate
ULOQ	upper limit of quantification
UMR	unité mixte de recherche

Acknowledgements & Funding sources

This study is a part of the ARMED (Assessment and Risk Management of MEdical Devices in plasticized polyvinylchloride) project and received financial support from the French National Agency for the Safety of Medicines and Health Products (ANSM).

We thank Dr. Belov (Max Planck Institute for Biophysical Chemistry, Germany) for the synthetization of Mono-(2-ethyl-2-carboxypentyl) phthalate (2cx-MMHP) and 1-mono-(2-carboxylmethylhexyl) benzene-1,4-dicarboxylate (2cx-MMHTP).

INTRODUCTION

The report from the Scientific Committee on Emerging and Newly-Identified Health Risks (SCENIRH, https://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_047.pdf) highlighted the risks associated with the use of medical devices (MD) containing phthalates, DiEthylHexylPhthalate (DEHP) plasticized PolyVinylChloride (PVC), notably in certain medical situations (e.g. haemodialysis, transfusions, extracorporeal membrane oxygenation, enteral and parenteral nutrition, lipophilic drug infusions) and populations (e.g. newborns, prepubescent adolescents and pregnant or lactating women). DEHP is a chemical compound classified as CMR 1B (carcinogenic, mutagenic or toxic for reproduction) according to European Regulation (EU) 1272/2008. DEHP is able to spread from the MD into contacting liquids, such as nutritional admixtures, lipophilic drugs solutions or blood[1], and expose the patient to potential toxic doses. In this context, a pressing need to find alternatives to DEHP plasticized PVC has emerged. Currently, alternative plasticizers (e.g. DINP, DEHTP, DEHA, DINCH or TOTM) have already been incorporated into MDs. However, there remains insufficient data in terms of population exposure and toxicity to draw conclusions on the safety of these plasticizers and their metabolites. With the potential harm induced by endocrine disruptors (phthalates, Bisphenol A), it is important to improve the risk assessment for these plasticizers in medical situations, which has not yet been studied in the food-processing and environmental sectors. European regulation 2017/745 from 5 April 2017 indicates that DEHP (as CMR compounds) is limited to 0.1% (mass/mass) in medical devices. However, there is no recommendation concerning the alternative plasticizers. To our knowledge, with the exception of our previous work[2], there are no published studies concerning the release of these new plasticizers from the MD and into the patient. Four studies on the leaching of TOTM from tubings, dialysis or ECMO lines suggest that the release of this alternative plasticizer remains lower than DEHP[3–6]. The only available study on DINCH release, investigated leaching from PVC tubes into enteral feeding solutions, was funded by Nutricia (for review [7]). The study by Wirnitzer *et al.* showed evidence of DEHTP release from tubings into a lipid solution [8], while Haishima *et al*.

analysed the amounts of alternative plasticizer (including DINCH, DEHP and TOTM) eluted from a PVC blood container[9].

In order to evaluate the patient exposure to plasticizers from MDs, it is necessary to analyse the amount of plasticizer metabolites (biomarkers) in biological fluids. This requires specific and sensitive analytical methods.

Online-solid phase extraction (SPE) coupled with liquid-chromatography tandem mass spectrometry (LC–MS/MS) with electrospray ionization (ESI) is mainly used to dose plasticizers [10–12]. One-line SPE has many advantages, such as reduced analysis time, manual handling and sample volume. It also increases sensitivity by concentrating the sample and increases selectivity through rinsing methods. To our knowledge, this is the first time that TurboFlow® technology (Thermo Fisher Scientific, Illkirch, Fance) has been used for the quantification of plasticizer metabolites in urine. TurboFlow® technology combines diffusion, chemistry and steric exclusion to perform selective online purification, allowing direct injection of biological fluids and reducing ion suppression [13].

In the literature, metabolites of DINP and DEHP have been extensively studied by various methods [11,14–16]. Few methods exist for investigating the others metabolites of alternative plasticizers that have been examined in our study. Some problems are recurrent in these analysis methods for alternative plasticizers: the standards of the molecules analysed are not always available and some are therefore analysed semi-quantitatively; the methods are not always validated; and sometimes the sensitivity is not sufficient for biomonitoring in a human population. The analytical methods for DINCH metabolites were developed by Schütze *et al.* [17] and Silva *et al.* [18]. Two methods have also been used to identified MEHA in urine [19,20]. More recently, the analysis of DEHTP [20,21] and TOTM [22] metabolites in urine has been described in study reports. Until now, no analytical method has been published for the simultaneous analysis of DEHP, DINP, DEHTP, DEHA, DINCH and TOTM metabolites.

We have therefore developed and validated a new analytical method to evaluate the concentration of their metabolites in biological fluids, and to supplement the partial biomonitoring data present in the literature. In the present article, we elaborate a rapid and automated online SPE-LC-MS/MS method

for the analysis of 8 primary metabolites (MEHP, MEHTP, MEHA, MINP, MINCH and 1-MEHTM/2-MEHTM/4-MEHTM) of DEHP, DEHTP, DEHA, DINP, DINCH and TOTM respectively, and 14 secondary metabolites (MEHHP/MEOHP/5cx-MEPP/2cx-MMHP; oxo-MINP/OH-MINP/cx-MINP; oxo-MINCH/OH-MINCH/cx-MINCH and 5OH-MEHTP/5oxo-MEHTP/5cx-MEHTP/2cx-MMHTP) of DEHP, DINP, DINCH and DEHTP respectively, found in human urine. Future data acquired using this method will also be used for the determination of acceptable doses and the prioritization of the risks associated with the different plasticizers currently integrated in MDs.

MATERIAL AND METHODS

Chemicals and reagents

Liquid Chromatography Mass Spectrometry (LC-MS) grade acetonitrile (ACN), acetic acid, methanol (MeOH), isopropanol (IPA), water and High-Performance Liquid Chromatography (HPLC) gradient grade acetone were purchased from Carlo Erba (Lyon, France). Analytical grade ammonium acetate, 4-methylumbelliferone and 4-methylumbelliferyl-β-D-glucuronide were purchased from Sigma-Aldrich (Lyon, France). β-Glucuronidase (Escherichia coli-K12) was purchased from Roche Diagnostics Gmbh (Mannheim, Germany). The following were synthesized by the UMR INSERM U1240 Imagerie Moléculaire et Stratégies Théranostiques (Clermont-Ferrand, France): mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethylhexyl) terephthalate (MEHTP), monoisononylphthalate (MINP), monoisononyl cyclohexane-1,2-dicarboxylate (MINCH), mono(2-ethylhexyl) adipate (MEHA), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono-(2-ethyl-5-carboxypentyl) phthalate (5cx-MEPP), mono-(4-methyl-7-hydroxy-octyl) phthalate (OH-MINP), mono-(4-methyl-7-oxo-octyl) phthalate (oxo-MINP), mono-(4-methyl-7-carboxy-heptyl) phthalate (cx-MINP), cyclohexane-1,2-dicarboxylate-mono-(7-hydroxy-4-methyl)octyl ester (OH-MINCH), cyclohexane-1,2-dicarboxylate-mono-(7-oxo-4-methyl)octyl ester (oxo-MINCH), cyclohexane-1,2-dicarboxylate-mono-(7-carboxylate-4-methyl)heptyl ester (cx-MINCH), 1-mono-(2-ethyl-5-hydroxyhexyl)benzene-1,4-dicarboxylate (5OH-MEHTP), 1-mono-(2-ethyl-5-oxohexyl)benzene-1,4-dicarboxylate (5oxo-MEHTP), 1-mono-(2-ethyl-5-carboxylpentyl)benzene-1,4-dicarboxylate (5cx-MEPTP), 1-mono-(2-ethylhexyl) Trimellitate (1-MEHTM), 2-mono-(2-ethylhexyl) Trimellitate (2-MEHTM),), 2-(((octyl-D_{17})oxy)carbonyl)cyclohexane-1-carboxylic acid (analog of D_{17}-MINCH, D_{17}-a-MINCH), mono-D_{17}-(2-ethylhexyl) adipate (D_{17}-MEHA), 1-mono-D_{17}-(2-ethylhexyl) Trimellitate (D_{17}-1-MEHTM), 2-mono-D_{17}-(2-ethylhexyl) Trimellitate (D_{17}-2-MEHTM), 4-mono-D_{17}-(2-ethylhexyl) Trimellitate (D_{17}-4-MEHTM). D_4-mono-2-ethylhexyl phthalate (D_4-MEHP), D_4- monoisononylphthalate (D_4-MINP), mono-D_4-(2-ethyl-5-hydroxyhexyl) phthalate (D_4-MEHHP), $^{13}C_4$-mono-(2-ethyl-5-oxohexyl) phthalate ($^{13}C_4$-MEOHP), $^{13}C_4$-

mono-(2-ethyl-5-carboxypentyl) phthalate ($^{13}C_4$-5cx-MEPP), mono-D4-(4-methyl-7-carboxy-heptyl)phthalate (D_4-cx-MINP), cyclohexane-1,2-dicarboxylate-mono-D_8-(7-oxo-4-methyl)octyl ester (D_8-oxo-MINCH), 4-mono-(2-ethylhexyl) Trimellitate (4-MEHTM), $^{13}C_4$-4-methylumbelliferone and synthetic urine were purchased from LGC Standards (Molsheim, France). Mono-(2-ethyl-2-carboxypentyl) phthalate (2cx-MMHP) and 1-mono-(2-carboxylmethylhexyl) benzene-1,4-dicarboxylate (2cx-MMHTP) were synthesized by Dr. Belov, Max Planck Institute for Biophysical Chemistry, Germany (**Supplementary table 1**).

Instruments

The chromatographic system consisted of a Prominence UFLC (Shimadzu, Marne la Vallée, France) equipped with a SIL-20AC XR autosampler, a LC-20AB module, two LC-20 AD XR pumps, two FCV-11AL reservoir switching valves, two FCV-12AH 6-port switching valves, a CTO-20AC column oven, two DGU-20A3 on-line solvent degasser and a CBM-20A system controller. A precolumn in-line filter (0.5 µm, Thermo Fisher Scientific, Illkirch, Fance) was inserted between the injector and the switching valve to extend the lifetime of the columns. Two TurboFlow® Cyclone 0.5 x 50 mm columns (Thermo Fisher Scientific) were placed at the pump outlet to avoid contamination between the LC-20AB pump outlet and the switching valve and between the LC-20AD XR pump outlet (pump A) and the solvent mixer. The mass spectrometer was a hybrid triple quadripole/linear ion trap QTRAP 5500 (Sciex, Framingham, MA, USA) equipped with a Turbo V IonSpray ionization source.

LC method

Fifty microliters of processed sample solution were injected into the LC system. The online extraction and purification were performed on a TurboFlow® Cyclone™ 0.5 x 50 mm column. A Betasil® phenyl/hexyl 100 x 3 mm 3 µm column (Thermo Fisher Scientific) was used for chromatographic separation. Both columns were maintained at 30°C. The composition of the mobile phase was A: H_2O+0.1% acetic acid (v/v) and B: ACN+0.1% acetic acid (v/v) for both loading and eluting pumps.

During the charging step, the flow rate of the loading pump was set to 1.5 ml/min for 0.61 min (100% A). The loading column was then rinsed for 0.9 min (25% B, 0.5 ml/min) before switching into backflush mode, which allowed the eluting pump to transfer the compound to the analytical column. The eluting pump was programmed as follows: 25% B (0.5 ml/min) for 5 min, linear gradient to 55% B over 4 min, linear gradient to 75% B in 5 min, linear gradient to 99% B in 1 min, 99% B for 9 min, linear gradient to 25% B in 0.1 min, and equilibrated for 0.9 min. In parallel, after 16 min of analysis, the valve was switched and the TurboFlow® column was flushed consecutively for 4 min with 100% B (1.5 ml/min); 100% A for 1 min and with a solvent mixture (Acetone / Acetonitrile / isopropanol 50/30/20 (v/v/v)) for 2 min. Finally, the loading column was equilibrated for 2.5 min (1.5 ml/min, 100% A) before the next injection. The chromatographic run lasted 25 min. During the first 8.5 min, the sample was sent to waste through the divert valve. Acquisition by the mass detector was performed over the following 16 min. A schematic diagram illustrating the configuration of the online SPE-LC-MS/MS method is shown in **Figure 1**.

MS parameters

The mass spectrometer was operated in negative ion mode using Electrospray with the following optimized parameters: curtain gas: 25 psi; collision gas: medium; ion spray voltage: -4000 V; ion source temperature: 500°C; ion source gas 1: 50; ion source gas 2: 60. The ion source parameters were optimized by flow injection analysis (FIA), using the same mobile phase composition as the chromatographic method. Analyses were performed in multiple reaction monitoring mode (MRM) with a dwell time of 10 ms per MRM channel, a 5 ms pause between scans and an in-unit resolution for both Q1 and Q3. For all analytes, two ion transitions were chosen for the quantification (quantifier) and the confirmation (qualifier). Instrumental parameters of each analyte were optimized manually by continually infusing a standard solution diluted in H_2O/ACN 50/50 (v/v) at a concentration ranged of 10 ng/ml to 100 ng/ml (**Table 1**). Data acquisition and processing were monitored by Analyst 1.6.2 and Multiquant 2.1 (Sciex), respectively.

Preparation of standards and quality control material

The initial standard stock solution and the internal standard stock solution were prepared by dissolving each compound, in powder form or an oil, in ACN or MeOH according to their solubility, and stored at -20°C in amber glass vials with Teflon caps. D_4-MEOHP and D_4-5cx-MEPP were conditioned in an ampoule with tert-Butyl methyl ether (MTBE) and stored at +4°C. The intermediate standard stock solution containing all plasticizer metabolites was prepared at 15 µg/ml in ACN. The working standard solution was prepared daily by diluting the appropriate quantity of intermediate stock solution in ACN. The calibration standard curve was carried out using 9 to 11 calibration standards, depending on the compound, by spiking synthetic urine with working standard solutions from 0.01 ng/ml to 50 ng/ml. Separate stock solutions were prepared and used for the calibration standards and quality control (QC) samples. Four levels of QC were defined and prepared in synthetic urine, with the concentrations used depending on the compound and its LLOQ and ULOQ: lower limit of quantification (LLOQ or QC1 at 0.01, 0.025, 0.05 or 0.1 ng/ml), upper limit of quantification (ULOQ or QC4 at 10, 25 or 50 ng/ml) and two intermediate concentrations (QC2 at 0.4 ng/ml and QC3 at 4 ng/ml). Each analytical batch included a blank solvent (mobile phase), a double blank sample (synthetic urine without internal standard), a blank sample (synthetic urine with internal standard), 12 calibration standards and a QC before and after a set of unknown urine samples.

Sample preparation

Urine samples were stored in 6.5 ml Vacuette® Z urine tubes with no additives (Greiner Bio-One, Courtaboeuf, France) and kept frozen at -20°C until analysis. Samples were thawed to room temperature and vortexed to ensure homogeneity. An aliquot of 300 µl was dispensed directly into a 1.5 ml amber glass vial with Teflon lined screw-cap with 100 µl of ammonium acetate buffer (1 M, pH 6.5), 10 µl internal standard (IS) solution and 10 µl enzyme solution, which was prepared daily (β -Glucuronidase from E.coli K12 diluted 1:1 with ammonium acetate buffer). Samples were gently mixed and incubated for 2h at 37°C for the enzymatic hydrolysis of plasticizer metabolites. Then, 150 µl of

10% acetic acid were added to lower the pH and stop the enzyme activity. After mixing, 50 µl were injected into the TurboFlow®-LC-MS/MS system. The calibration standards, QC and reagent blanks were processed in the same way. This sample preparation, using β-Glucuronidase, led to the quantification of the total concentration of plasticizer metabolites (free and conjugated). The amount of 4-methylumbelliferone formed from the deglucuronidation of 4-methyl-umbelliferryl-glucuronide was monitored to confirm the β-Glucuronidase enzymatic activity. To determine the free metabolite concentration in urine, 10 µl of enzyme solution was replace by 10 µl of acetate buffer. Urinary creatinine concentrations were determined using the Flex® reagent cartridge CRE2 on a Dimension Xpand plus Automate (Siemens Healthcare Diagnostics, Saint-Denis, France).

Validation

The method has been validated with reference to the EMA Guideline on bioanalytical method validation and the French COFRAC (ISO 15189) standards. The following parameters were evaluated: linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), matrix effects, carryover, extraction recovery, dilution integrity, stability and specificity.

Linearity, accuracy, precision

Calibration curves were calculated using the ratio of the peak area of the analyte to IS with a 1/x weighted quadratic regression. The linearity was validated by repeating the calibration range for five consecutive days, with r^2 greater than 0.995. Accuracy and precision were assessed by replicate analysis of five (intra-day and inter-day) QCs for the four levels of control. To validate the precision, the coefficient of variation (CV) was expected to be ≤15% (20% for the LLOQ) and accuracies were expected to be between 85 and 115% (80% and 120% for the LLOQ) of the nominal values.

Limit of detection and limit of quantification

The theoretical LOD and LOQ for each molecule were subsequently determined by analysing five spiked matrix samples prepared at the LLOQ concentration. LLOQ was determined to be the lowest compound concentration that could be quantified with acceptable accuracy and precision (≤20%) and the chromatographic peak was required to be equal to ten times the baseline noise. The theoretical LOD was determined to be three times the standard deviation of repeatability of LLOQ and the theoretical LOQ to be ten times the standard deviation of repeatability of LLOQ.

Selectivity: Matrix effects, recovery and specificity

To evaluate the matrix effect, 2 controls (low and high concentrations) were processed in 6 different urinary samples. The calculated CV should not be greater than 15%. The extraction recovery was calculated for the 2 water-spiked controls by comparing the peak area of the analyte, directly injected on to the analytical column, with that obtained with TurboFlow® injection. The specificity of the analytical method was determined by analysing a spiked control (QC2) in a complex matrix with 26 molecules (DAU LC 2 urine toxicology control, UTAK Laboratories, Valencia, CA, USA). The acceptance criterion for precision was between 85 and 115% of the nominal value.

Carryover, stability and dilution integrity

To study carryover, blank samples were injected after the calibration standard at the ULOQ on 4 consecutive days. Carryover into the blank sample after the high-concentration standard should not exceed 20% of the LLOQ. The matrix stability of the plasticizer metabolites was tested at +4°C and -20°C (after two freeze-thaw cycles) for 7 and 14 days at two control levels (low and high). Dilution integrity was demonstrated by spiking the matrix with working solution at 50 ng/ml and diluting this sample in the matrix on 5 replicates. The dilution ratios tested were 1:10 and 1:100. Accuracy and precision were validated if CV values did not exceed 15 %.

Clinical validation

The clinical applicability and suitability of our method were demonstrated by the analysis of urine samples from 104 newborns. Urine samples were collected from patients in neonatal intensive care units (NICU) in the university hospitals of Clermont-Ferrand and Lille (France) after 24 h of exposure to medical devices. NICU patients were participating in a larger study examining their exposure to new alternative plasticizers (ARMED project). This study was approved by the national ethics committee (CPP Sud-Est VI, approval number AU-1082) and registered in clinicaltrials.gov (NCT03477409).

RESULTS AND DISCUSSION

Selection of analysed plasticizers metabolites

Human biomonitoring studies allow the exposure to plasticizers to be assessed by measuring the levels of these chemicals, their metabolites, and/or their reaction products, in human fluids like blood (and components), urine, saliva, or expired air. Hence, a better understanding of the human metabolism and excretion kinetics of these plasticizers is crucial for identifying metabolites that are specific to plasticizer exposure. Literature data shows a similar metabolism process for DEHP and several of the alternative plasticizers, i.e. DINP, DINCH, DEHA, and DEHTP [18,21,23–26]. They are metabolized very quickly and do not bioaccumulate, leading to a negligible remaining dose after 48 h. After an initial presystemic and rapid ester hydrolysis to the corresponding monoesters, which appear in the gastrointestinal tract, they undergo further oxidation in the liver to produce secondary metabolites. These metabolites could also undergo conjugation with glucuronic acid and sulfonic acid to form the respective conjugates before being eliminated via the urine. In most cases, these secondary metabolites have been identified as specific biomarkers of plasticizer exposure.

Metabolites of DEHP have been characterized for long time. Especially Koch *et al.* showed that 5OH-MEHP, 5oxo-MEHP and 5cx-MEPP present the major share of DEHP metabolites excreted in urine (about 70% for these three oxidized metabolites vs. about 6% for MEHP)[27,28]. They also show that premature neonates were exposed to DEHP up to 100 times above the limit values depending on the intensity of medical. A similar distribution was found for DINP metabolites in urine samples, with 20.2% as OH-MINP, 10.7% as carboxy-MINP, 10.6% as oxo-MINP, thus considered as specific biomarkers of DINP exposure, and only 2.2% as MINP[29,30]. For DINCH, Schütze *et al.* developed a HPLC-MS/MS allowing the determination of DINCH metabolites[31], which was applied in the work of Koch *et al.*[32]. They found that the 14.8 % (11.3-16.7 %) of the dose administrated orally was excreted as monoesters with oxidative modifications, in particular OH-MINCH 10.7 % (7.7-12.9 %), oxo-MINCH 2.0 % (1.5-2.6 %) and carboxy-MINCH 2.0 % (1.8-2.3 %). Less than 1 % was excreted as the simple monoester MINCH

Metabolites of DEHTP were determinate in human liver microsomes by Silva *et al.*[33]. Although a major excretion of terephthalic acid (TPA), oxidized metabolites are considered as specific metabolites of DEHTP and may be suitable biomarkers for assessing exposure to DEHTP. Höllerer *et al.* identified hydrolysed di-2-(ethylhexyl) trimellitates (1,2-DEHTM, 2,4-DEHTM) diesters and its monoester isomers di-2-(ethylhexyl) trimellitates (1-MEHTM, 2-MEHTM) and 2-MEHTM was found to be the most dominant urinary biomarker, followed by several specific secondary metabolites[34]. For DEHA, the work of Silva *et al.* identified MEHA and only two oxidized specific metabolites, mono-2ethylhydroxyhexyl adipate (MEHHA) and mono-2-ethyloxohexyl adipate (MEOHA), and also a large urinary amount of adipic acid, not considered as relevant biomarker[19]. Moreover, MEHA has been shown *in vivo* to be cytotoxic on L929 cells, suggesting toxicity and therefore clinical interest for population exposure assessment[35].

Based on these literature data, we selected the major secondary oxidized metabolites of DEHP, DINP, DINCH and DEHT and monoesters of TEHTM to be relevant for the assessment of the exposure of neonates in our study. Their rate in patients' urinary samples actually reflects patients' exposure to the plasticizers from medical devices, whereas the hydrolysed monoesters, found at low rates, do not because of a potential contamination. Nevertheless, thanks to the improvement of the sensitivity of the analytical techniques and all the measures taken for the sample preparation to prevent from contamination, we can obtain interesting overcomes with monoesters. Indeed, the analysis of these primary metabolites could be relevant for the neonates and premature population, because of its enzymatic and metabolic immaturity, leading to higher rates in urines.

LC-method development & enhancement

The first challenge was to retain analytes with different physicochemical properties on SPE cartridges and to transfer them to the analytical column. We tested different types of SPE columns: Strata X 20 x 2.0 mm, 25 µm (Phenomenex, Le Pecq, France), Oasis® HLB 20 mm x 2.1, 25 µm (Waters, Guyancourt, France) and TurboFlow® technology columns (Cyclone™, Cyclone P™, Cyclone MAX™, Cyclone MCX-

2™ and C8) (Thermo Fisher Scientific). The TurboFlow® Cyclone™, Strata X and Oasis® HLB SPE columns had good retention and peak shapes after transfer, but the TurboFlow® technology was the most robust and allowed us to inject more samples before changing the column. Loading parameters were optimized (volume injected, sample flow rate, mobile phase composition, elution gradient and washing step) to obtain better resolution and sensitivity. An important parameter was the mobile phase composition of the loading step. As a first step, we chose to charge with 10% ACN, which gave a better extraction recovery for a compound that was highly retained on the SPE column and difficult to elute, in particularly primary metabolites. However, the use of this concentration of organic solvent affects the chromatographic resolution for the secondary metabolites of DEHP, DINP and DINCH, which are retained less by the column. We also decided to focus on chromatographic separation, with an optimum result obtained with a 100% water charge at 1.5 ml/min. The recovery loss of extraction was negligible in relation to the sensitivity of the method.

We found many isobaric compounds with similar structures to the plasticizers. These molecules can share common fragments in MRM mode and it is not always possible to differentiate specific fragments (differentiation is often associated with a loss of sensitivity). This is in line with Lessmann *et al.* [36] who recently showed that DEHTP metabolites can interfere with isobaric metabolites of DEHP. They also noted that the carboxy-metabolites of these two phthalates could interfere with OH-MINP. We have therefore taken this problem into account in our method of analysing these phthalates. We focused on optimizing the chromatographic separation in order to integrate these isobaric compounds and did not attempt to obtain a shorter analysis. To do this, the following analytical columns were tested: Hypersil GOLD C18 50 x 2.1 mm, 1.9 µm; Hypersil GOLD PFP 50 x 2.1 mm, 1.9 µm; Betasil® phenyl/hexyl 100 x 3 mm, 3 µm (Thermo Fisher Scientific). The best result was obtained with the Betasil® phenyl/hexyl column, which provided a good peak shape, resolution and sensitivity (**Figure 2**). This column gave the baseline resolution of the three MEHTM isomers due to its selectivity for aromatic compounds (interaction of the aromatic ring with phenyl group π-electrons). The separation

of DEHTP from DEHP metabolites was thus possible, as was the isolation of isobaric compounds like the isomers cx-MINP and MEHTM, which share the same mass spectrometric transition (*m/z* 121). However, significant chromatographic separation was required for the baseline resolution of OH-MINP and the DEHTP/DEHP carboxylic metabolites, which have a very similar retention profile. Adequate separation was achieved with the blank matrix standard but we noted a peak overlap with OH-MINP in urinary samples with very high concentrations of 2cx-MMHP and 5cx-MEPTP. Therefore, manual integration and verification was required for this compound. This is especially true for non-hydrolysed samples, since the OH-MINP is mainly in a glucuroconjugated form compared to 2cx-MMHP and 5cx-MEPTP, and there is therefore a higher probability that its signal is low (**Figure 3**). Other studies have already noted that conjugation with glucuronic acid is preferred for the hydroxy- and oxo-metabolite compared to the carboxy metabolite[19,20,29,36].

MS optimization and quantification

In order to minimize possible interactions between the metabolites themselves and other molecules present in the analytical method, we chose the most specific transitions and verified possible interactions. Each metabolite and IS were infused separately during the optimization of the mass spectrometry parameters and injected separately during chromatography to evaluate whether the detected signal interfered with another molecule present in our method. The choice to have the most specific transitions was made at the expense of sensitivity.

Initially, mass acquisition was used with Scheduled MRM pro algorithmic mode for a better peak detection and reproducibility. This acquisition was configured to use the base width of the chromatographic peak and the advanced mode to define acquisition windows for each transition. As a first step, this method of acquisition was used because it is particularly suitable for the metabolites of plasticizers which may have very different peak widths. As mentioned above, metabolites may have analogous structures, which result in superimposed chromatographic signals due to the limited selectivity for their mass fragments. This is particularly true for secondary metabolites DINCH and

DINP, which have a characteristic cluster chromatographic peak due to the presence of different isomers in human urine. These isomers were very difficult to separate. So, as previously published [17,29,37], we decided to integrate the whole area under the cluster of peaks for DINP and DINCH metabolites. Despite the potential interest of this method, we were confronted with a limitation of the Scheduled MRM mode during the development of our analytical method. This method can only detect the signal response for a short period of time, which makes it impossible to detect the elution signal of the isobaric compound. Consequently, the non- Scheduled MRM mode was used in the method presented in this article. Despite this, the 5500 Qtrap used for our analysis provided the data acquisition points necessary for reliable quantification and excellent sensitivity.

During the development and validation of our method, the DEHTP and the 2cx-MMHP secondary metabolite standards were not commercially available. Therefore, the mass spectrometry parameters in the literature [36] were used to evaluate the possible coelution with these isobaric compounds. Also, the observed transitions of common ions between the DEHTP and DEHP metabolites allowed a retrospective and quantitative study of the secondary metabolite of DEHTP using the calibration curve of the corresponding secondary metabolite of DEHP and their stable isotopically-labelled (SIL) IS. The 5cx-MEPP calibration curve was also used for the 2cx-MMHP. As a result, the reported concentrations for this analyte are only semi-quantitative. Unfortunately, these standards were finally be synthesized at the end of our study. QCs were prepared from the standards for DEHTP and 2cx-MMHP metabolites and calculated from the calibration curve of the DEHP secondary metabolite. The expected ratio to observed concentration was applied to all previously calculated patient urine concentration values for these compounds. We consider that this approach provided the best possible estimates of the concentrations of these metabolites.

It should be noted that SIL was not commercially available for all metabolites. Some were synthesized by INSERM U1240 (Imagerie Moléculaire et Stratégies Théranostiques, Clermont Ferrand, France). For MINCH, the IS used was an analogue (2-(octyl-D_{17})oxycarbonyl) cyclohexane-1-carboxylic acid) which differs in the absence of a methyl group on its side chain. For the metabolites for which the SIL was

not available, the IS of the structure and the retention time closest to the metabolite was chosen (**Table 1**).

Contamination

Because phthalates are ubiquitous, contamination control is essential, especially when the analytical methods used are very sensitive to detecting very small quantities of molecules. Exogenous contaminations can be diverse: ambient air, components of the HPLC-MS/MS system, mobile phases of the HPLC and materials and solutions used during sample preparation. It is therefore imperative not to find any in the work environment in order to provide reliable results. In addition, it has been described that mono-ester phthalates can be generated from a simple chemical or microbiological ester cleavage [23]. To prevent this contamination, numerous measures were taken: i) solutions were prepared in glass vials that had been rinsed with LC-MS grade methanol; ii) solvents, water and eluent additives were LC-MS quality (or HPLC was if not available); iii) the caps are made of cotton or Teflon; iv) the automatic rinsing system at the rear of the pistons was disconnected; v) two pre-columns were placed directly at the pump outlet to collect possible mono-ester contaminants in the mobile phase and in the HPLC system and were washed at the end of each sample analysis by inverting the selector solvent with the organic phase; vi) all tubing used in the analytical system was inert stainless steel or contained PEEK; vii) E. coli β -glucuronidase (K12, RocheB) without non-specific lipase activity was used to prevent the generation of mono-ester from di-esters or tri-ester[24] and viii) solutions used for sample preparation were filtered on a TurboFlow® c18 column. Despite all these precautions, traces of MEHP and MEHA were found, but at concentrations below our LLOQ (Supplementary Figure 1). A blank solvent was injected before any batch to control this contamination.

Analytical validation

Linearity, accuracy, precision, LOQ and LOD

Calibration curves were linear with average correlation coefficients (r^2) greater than 0.9988. The intra- and inter-day precisions (CV) were between 2.00 to 9.82% and between 1.91 to 17.25%, respectively. Intra-day and inter-day mean calculated accuracies were in the range of 86.1 to 117.2% and 88.2 to 114.7%, respectively. The theorical LOQ ranged from 0.004 to 0.048 ng/ml depending on the metabolite studied (**Table 2)**.

In the literature, the metabolites of DINP and DEHP have been extensively studied by various methods. Few methods exist for the evaluation of the other metabolites of alternative plasticizers investigated in our study. Two methods by Silva *et al*. and Alves *et al.* identified MEHA in urine [19,20]. Only in the study by Silva *et al.* was MEHA quantified but not with a fully validated method and without information on LOQ [19].

DEHTP-related urine metabolites have already been evaluated by two methods. MEHTP and other metabolites were identified by Alves *et al.* while Lessmann *et al.* quantified DEHTP-related metabolites, but without any information on MEHTP. This is in contrast to our method [20,21]. Recently, an analytical method for DINCH metabolites has been developed by Schütze *et al.* with an LOQ of 0.05 ng/ml for cx-MINCH and OH-MINCH and 0.1 ng/ml for MINCH, but oxo-MINCH was only analysed by a semi-quantitative method [17]. To date, few biomonitoring methods for the quantification of TEHTM metabolites are available. There are two methods for blood [25,38] and one recently published method by Höllerer *et al*. for urine [22]. In the latter, the quantification method was developed for the determination of both specific primary metabolites of TEHTM and selected side chain oxidized monoester metabolites present in human urine. This method enables an individual determination of regioisomeric TEHTM metabolites. However, we consider that its sensitivity is not really compatible with the levels observed in the general population or even in some exposed patients. In our application, the median concentration observed for the main metabolite, 2-MEHTM, was 0.4 ng/ml and was below the LOQ of the above method.

Matrix effects, recovery and specificity

Matuszewski *et al.*[39] defined the absolute matrix effect as the ratio of the peak area of the analyte in a blank extracted matrix that has been spiked post-extraction with a standard compared to the peak area of the same standard concentration in a neat solution. However, in the case of online solid phase extraction, the impact of the sample on extraction recovery cannot be assessed separately from the matrix effects on ionization efficiency. Thus, in this study, matrix effects were evaluated by measuring QCs prepared with batches of urine from different hospitalized patients. All sample were analysed in native condition (non-spiked) and spiked at different levels of low and high concentrations depending on the calibration range of the analyte and native concentration (**Table 3**). These urine samples were selected to represent the variable creatinine content (0.1-17.3 mmol/L) and the variable concentrations of plasticizer metabolites in order to reflect the different urine composition found in hospitalized patients.

The coefficient of variation and the accuracy ranged from 3.13 to 14.92% and 86.16 to 111.22% respectively, which were within the acceptance criteria. This suggests that the accuracy of the method was not compromised by the matrix (**Table 3**).

The extraction recovery was higher than 84% for the secondary DEHP, DINP and DINCH metabolites. For MEHA, MINP, MEHP and 1-MEHTM, a decrease in recovery was observed when the concentration increased. This could be explained by the fact that these metabolites are more hydrophobic and so more difficult to elute from the TurboFlow® column, as discussed earlier in HPLC optimization. Consequently, MEHTP, MINCH and 4-MEHTM, which eluted later, have an extraction recovery below 40% (**Table 3**). However, the use of specific IS for these molecules prevented a loss of robustness.

For specificity analysis, a QC2 was added in a complex urine solution (see Section *Selectivity: Matrix effects, recovery and specificity*). No interference was observed and accuracy ranged from 85.32 to 112.40% (**Supplementary table 2**). These results suggest that our method is able to differentiate and quantify the analyte in the presence of other molecules in the sample.

Carryover, stability and dilution integrity

We have optimized the washing conditions in order to avoid the carryover of too retained compounds. To do this, the loading and analysis column was washed with a mixture of solvents (acetone/ACN/isopropanol, 50/30/20) and the rinsing conditions of the autosampler were improved. Carryover in the blank sample following the high concentration standard was less than 20% of the LLOQ for all compounds, with the exception of 4-MEHTM for which, despite our precaution, a blank sample had to be injected between two patients when the concentration exceeded 10 ng/mL. However, in our application, we observed that the maximum value measured did not exceed 4.14 ng/mL for this compound.

For the sample stability test, we found that the variation of the low and high QC concentration values was less than 15% compared to their nominal concentrations after storage at +4°C and -20°C (after two freeze-thaw cycles) for 7 and 14 days. The results showed that the analytes seem to be stable under these conditions and that repeated freeze-thawing did not affect the concentration in human urine during this period (**Supplementary table 2**).

Metabolites of plasticizers are present at different concentrations in human urine. The metabolites of DEHP are generally found at much higher concentrations than those of other plasticizers analysed (see following results), although this compound should have been replaced by alternative plasticizers. We tested the dilution integrity in order to be able to reliably quantify samples with concentrations above the calibration range. CVs of precision (2.45-14.66%) and accuracy (85.42-114.21%) were within the acceptance range, so our analytical method allowed us to dilute the samples 10-fold and 100-fold (**Table 3**).

Method utility

The usefulness of the method was assessed by analysing 104 samples from newborns exposed to plasticizers during 24h through one or multiple MDs in university hospitals in Clermont-Ferrand and Lille (FRANCE). The births of the newborns were at 36.2±4.3 weeks and 61% were boys. Hospitalization

and exposure to phthalates were potentially due to the following medical conditions: parenteral nutrition, enteral nutrition, extracorporeal membrane oxygenation and plasmapheresis (**Supplementary Table 3**). The mean/median and range concentration of creatinine in neonate samples were 0.90/0.73 mmol/l and 0.02-9.29 mmol/l. MEHP, MEHHP, MEOHP, 5cx-MEPP, 2cx-MMHP, MINP, OH-MINP, oxo-MINP, cx-MINP were the phthalate metabolites that were detected in all urine samples. The highest concentrations were detected for metabolites of DEHP (MEHP, MEHHP, MEOHP, 5cx-MEPP, 2cx-MMHP), with a median concentration range of 38.184 to 206.976 ng/ml, respectively (**Table 4 and Supplementary Table 4**). Different patient chromatograms are presented for all metabolites studied (**Figure 4**). In this study, DEHP metabolites are 100- to 2000-fold more concentrated than alternative plasticizer metabolites. Other studies have evaluated the concentration of various plasticizers and phthalate derivatives in newborns and their mothers, with or without a MD [40]. These studies highlighted a strong relationship between maternal and neonatal plasticizer concentrations. The DEHP metabolites concentrations obtained in our study are consistent with other recent studies which assessed neonates and prematurates exposure to DEHP via MDs [41–43]. In all these studies, the monoester MEHP, when measured, is the minor metabolite whereas carboxy metabolite is the by far major metabolite, with a rate above 1000ng/mL in the work of Strommen *et al.*. Moreover, as in our study, the concentrations of hydroxyl- and oxo- secondary DEHP metabolites are close. However, it seems that the ratio between carboxy and hydroxy varies widely according the intensity of the exposure [44], especially by at risk medical procedure, such as cardiac surgery [45] and the vulnerability of the population, especially in terms of prematurity [41]. Indeed, this difference also exists in the adults' population, as demonstrated by Huygh *et al.*, who showed that an ECMO procedure may increase the level of 5-cx-MEPP by more than 100 times [46]. Finally, it is interesting to note that the general levels of DEHP metabolites (monoesters and oxidized ones) have decreased over time since the use of DEHP in MDs has been restricted. For examples, the urinary rates of MEHP, MEHHP and MEOHP in the work of Calafat *et al.* in 2004 [47] were respectively more than 3 times, 33 times and 10

times higher than the rates found in our study. This observation is also found by comparison with the study of Green *et al.* and Weuve *et al.* [44,48].

Thus, it is important to note that our study aims to validate a new analytical method and not to evaluate exposure to alternative plasticizers contained in MDs and the relevance of biomarkers. Therefore, our results should be used with caution in terms of exposure to these plasticizers as the absence of comparisons between unexposed and exposed neonate urinary samples, as well as the absence of data in the literature on exposure of a healthy population, do not allow us to draw conclusions on exposure levels related to MDs. Urinary sampling of each newborn prior to any use of a MD or of unexposed newborns would help to estimate environmental exposure. We cannot rule out that the presence of these plasticizers and their metabolites in the newborns is not due to the mother's exposure during pregnancy. A maternal exposure assessment is therefore necessary to determine the real exposure of newborns when using a MD.

Conclusion

This new LC-MS/MS sensitive method has been developed for the rapid and simultaneous detection and quantification of plasticizer metabolites in human urine. Except for DEHA, these metabolites are specific biomarkers of the exposure to DEHP-alternative plasticizers that are contained in MD. This validated method is characterized by a fast and robust online extraction procedure using TurboFlow® technology combined with an excellent chromatographic selectivity which allows individual determination of problematic structural isomers. Finally, another limit of our method is that oxidized MEHTP metabolites are not validated (semi-quantitative data).

Compared to previously published methods for the analysis of plasticizer contaminants in urine, this method includes the simultaneously analysis of more analytes of interest. The LOQ for each DEHP-alternative plasticizer metabolite has also been significantly improved. The majority of metabolites were quantified in the urine samples, which demonstrates that our method can be used to reliably and accurately monitor human exposure to several plasticizers (detection of a median frequency of 95.2%).

Ultimately, these biological results, combined with migration and toxicity studies, will make it possible to achieve the objectives of the ARMED project, or even to define a maximum threshold of exposure of high-risk patients to these alternative plasticizers during their hospitalization.

REFERENCES

[1] U. Heudorf, V. Mersch-Sundermann, J. Angerer, Phthalates: toxicology and exposure, Int. J. Hyg. Environ. Health. 210 (2007) 623–634. doi:10.1016/j.ijheh.2007.07.011.

[2] C. Marie, S. Hamlaoui, L. Bernard, D. Bourdeaux, V. Sautou, D. Lémery, F. Vendittelli, M.-P. Sauvant-Rochat, Exposure of hospitalised pregnant women to plasticizers contained in medical devices, BMC Womens Health. 17 (2017) 45. doi:10.1186/s12905-017-0398-7.

[3] K. Kambia, T. Dine, R. Azar, B. Gressier, M. Luyckx, C. Brunet, Comparative study of the leachability of di(2-ethylhexyl) phthalate and tri(2-ethylhexyl) trimellitate from haemodialysis tubing, Int. J. Pharm. 229 (2001) 139–146.

[4] R. Ito, N. Miura, H. Iguchi, H. Nakamura, M. Ushiro, N. Wakui, K. Nakahashi, Y. Iwasaki, K. Saito, T. Suzuki, H. Nakazawa, Determination of tris(2-ethylhexyl)trimellitate released from PVC tube by LC-MS/MS, Int. J. Pharm. 360 (2008) 91–95. doi:10.1016/j.ijpharm.2008.04.020.

[5] E. Eckert, F. Münch, T. Göen, A. Purbojo, J. Müller, R. Cesnjevar, Comparative study on the migration of di-2-ethylhexyl phthalate (DEHP) and tri-2-ethylhexyl trimellitate (TOTM) into blood from PVC tubing material of a heart-lung machine, Chemosphere. 145 (2016) 10–16. doi:10.1016/j.chemosphere.2015.11.067.

[6] C. Fernandez-Canal, P.-G. Pinta, T. Eljezi, V. Larbre, S. Kauffmann, L. Camilleri, B. Cosserant, L. Bernard, B. Pereira, J.-M. Constantin, G. Grimandi, V. Sautou, for A.S. Group, Patients' exposure to PVC plasticizers from ECMO circuits, Expert Rev. Med. Devices. 15 (2018) 377–383. doi:10.1080/17434440.2018.1462698.

[7] E. Van Vliet, E. Reitano, J. Chhabra, G. Bergen, R. Whyatt, A review of alternatives to di (2-ethylhexyl) phthalate-containing medical devices in the neonatal intensive care unit, J. Perinatol. 31 (2011) 551–560. doi:10.1038/jp.2010.208.

[8] U. Wirnitzer, U. Rickenbacher, A. Katerkamp, A. Schachtrupp, Systemic toxicity of di-2-ethylhexyl terephthalate (DEHT) in rodents following four weeks of intravenous exposure, Toxicol. Lett. 205 (2011) 8–14. doi:10.1016/j.toxlet.2011.04.020.

[9] Y. Haishima, T. Kawakami, C. Hasegawa, A. Tanoue, T. Yuba, K. Isama, A. Matsuoka, S. Niimi, Screening study on hemolysis suppression effect of an alternative plasticizer for the development of a novel blood container made of polyvinyl chloride, J. Biomed. Mater. Res. B Appl. Biomater. 102 (2014) 721–728. doi:10.1002/jbm.b.33052.

[10] M.J. Silva, E. Samandar, J.L. Preau, J.A. Reidy, L.L. Needham, A.M. Calafat, Quantification of 22 phthalate metabolites in human urine, J. Chromatogr. B. 860 (2007) 106–112. doi:10.1016/j.jchromb.2007.10.023.

[11] H. Frederiksen, N. Jørgensen, A.-M. Andersson, Correlations between phthalate metabolites in urine, serum, and seminal plasma from young Danish men determined by isotope dilution liquid chromatography tandem mass spectrometry, J. Anal. Toxicol. 34 (2010) 400–410.

[12] H.M. Koch, L.M. Gonzalez-Reche, J. Angerer, On-line clean-up by multidimensional liquid chromatography–electrospray ionization tandem mass spectrometry for high throughput quantification of primary and secondary phthalate metabolites in human urine, J. Chromatogr. B. 784 (2003) 169–182. doi:10.1016/S1570-0232(02)00785-7.

[13] A. Liesener, U. Karst, Turbulent flow chromatography for the reduction of matrix effects in electrospray ionization mass spectrometry-based enzyme assays, J. Sep. Sci. 28 (2005) 1658–1665. doi:10.1002/jssc.200500090.

[14] A. Sabaredzovic, A.K. Sakhi, A.L. Brantsæter, C. Thomsen, Determination of 12 urinary phthalate metabolites in Norwegian pregnant women by core–shell high performance liquid chromatography with on-line solid-phase extraction, column switching and tandem mass spectrometry, J. Chromatogr. B. 1002 (2015) 343–352. doi:10.1016/j.jchromb.2015.08.040.

[15] A.L. Heffernan, K. Thompson, G. Eaglesham, S. Vijayasarathy, J.F. Mueller, P.D. Sly, M.J. Gomez, Rapid, automated online SPE-LC-QTRAP-MS/MS method for the simultaneous analysis of 14 phthalate metabolites and 5 bisphenol analogues in human urine, Talanta. 151 (2016) 224–233. doi:10.1016/j.talanta.2016.01.037.

[16] R. Preuss, H.M. Koch, J. Angerer, Biological monitoring of the five major metabolites of di-(2-ethylhexyl)phthalate (DEHP) in human urine using column-switching liquid chromatography–tandem mass spectrometry, J. Chromatogr. B. 816 (2005) 269–280. doi:10.1016/j.jchromb.2004.11.048.

[17] A. Schütze, C. Pälmke, J. Angerer, T. Weiss, T. Brüning, H.M. Koch, Quantification of biomarkers of environmental exposure to di(isononyl)cyclohexane-1,2-dicarboxylate (DINCH) in urine via HPLC–MS/MS, J. Chromatogr. B. 895–896 (2012) 123–130. doi:10.1016/j.jchromb.2012.03.030.

[18] M.J. Silva, T. Jia, E. Samandar, J.L. Preau, A.M. Calafat, Environmental exposure to the plasticizer 1,2-cyclohexane dicarboxylic acid, diisononyl ester (DINCH) in US adults (2000—2012), Environ. Res. 126 (2013) 159–163. doi:10.1016/j.envres.2013.05.007.
[19] M.J. Silva, E. Samandar, X. Ye, A.M. Calafat, In Vitro Metabolites of Di-2-ethylhexyl Adipate (DEHA) as Biomarkers of Exposure in Human Biomonitoring Applications, Chem. Res. Toxicol. 26 (2013) 1498–1502. doi:10.1021/tx400215z.
[20] A. Alves, G. Giovanoulis, U. Nilsson, C. Erratico, L. Lucattini, L.S. Haug, G. Jacobs, C.A. de Wit, P.E.G. Leonards, A. Covaci, J. Magner, S. Voorspoels, Case Study on Screening Emerging Pollutants in Urine and Nails, Environ. Sci. Technol. 51 (2017) 4046–4053. doi:10.1021/acs.est.6b05661.
[21] F. Lessmann, A. Schütze, T. Weiss, T. Brüning, H.M. Koch, Determination of metabolites of di(2-ethylhexyl) terephthalate (DEHTP) in human urine by HPLC-MS/MS with on-line clean-up, J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci. 1011 (2016) 196–203. doi:10.1016/j.jchromb.2015.12.042.
[22] C. Höllerer, T. Göen, E. Eckert, Comprehensive monitoring of specific metabolites of tri-(2-ethylhexyl) trimellitate (TEHTM) in urine by column-switching liquid chromatography-tandem mass spectrometry, Anal. Bioanal. Chem. (2018). doi:10.1007/s00216-018-1086-7.
[23] H.M. Koch, L.M. Gonzalez-Reche, J. Angerer, On-line clean-up by multidimensional liquid chromatography-electrospray ionization tandem mass spectrometry for high throughput quantification of primary and secondary phthalate metabolites in human urine, J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci. 784 (2003) 169–182.
[24] Benjamin C. Blount, † K. Eric Milgram, Manori J. Silva, Nicole A. Malek, John A. Reidy, and Larry L. Needham, J.W. Brock*, Quantitative Detection of Eight Phthalate Metabolites in Human Urine Using HPLC−APCI-MS/MS, (2000). doi:10.1021/ac000422r.
[25] C. Höllerer, J. Müller, T. Göen, E. Eckert, Isomeric separation and quantitation of di-(2-ethylhexyl) trimellitates and mono-(2-ethylhexyl) trimellitates in blood by LC–MS/MS, J. Chromatogr. B. 1061 (2017) 153–162. doi:10.1016/j.jchromb.2017.07.014.
[26] E. Eckert, J. Müller, T. Göen, Simultaneous determination of polyvinylchloride plasticizers di(2-ethylhexyl) phthalate and tri(2-ethylhexyl) trimellitate and its degradation products in blood by liquid chromatography-tandem mass spectrometry, J. Chromatogr. A. 1410 (2015) 173–180. doi:10.1016/j.chroma.2015.07.083.
[27] H.M. Koch, H.M. Bolt, J. Angerer, Di(2-ethylhexyl)phthalate (DEHP) metabolites in human urine and serum after a single oral dose of deuterium-labelled DEHP, Arch. Toxicol. 78 (2004) 123–130. doi:10.1007/s00204-003-0522-3.
[28] H.M. Koch, R. Preuss, J. Angerer, Di(2-ethylhexyl)phthalate (DEHP): human metabolism and internal exposure-- an update and latest results, Int. J. Androl. 29 (2006) 155–165; discussion 181-185. doi:10.1111/j.1365-2605.2005.00607.x.
[29] H.M. Koch, J. Müller, J. Angerer, Determination of secondary, oxidised di-iso-nonylphthalate (DINP) metabolites in human urine representative for the exposure to commercial DINP plasticizers, J. Chromatogr. B. 847 (2007) 114–125. doi:10.1016/j.jchromb.2006.09.044.
[30] M.J. Silva, J.A. Reidy, J.L. Preau, L.L. Needham, A.M. Calafat, Oxidative metabolites of diisononyl phthalate as biomarkers for human exposure assessment, Environ. Health Perspect. 114 (2006) 1158–1161. doi:10.1289/ehp.8865.
[31] A. Schütze, C. Pälmke, J. Angerer, T. Weiss, T. Brüning, H.M. Koch, Quantification of biomarkers of environmental exposure to di(isononyl)cyclohexane-1,2-dicarboxylate (DINCH) in urine via HPLC–MS/MS, J. Chromatogr. B. 895–896 (2012) 123–130. doi:10.1016/j.jchromb.2012.03.030.
[32] H.M. Koch, A. Schütze, C. Pälmke, J. Angerer, T. Brüning, Metabolism of the plasticizer and phthalate substitute diisononyl-cyclohexane-1,2-dicarboxylate (DINCH(®)) in humans after single oral doses, Arch. Toxicol. 87 (2013) 799–806. doi:10.1007/s00204-012-0990-4.
[33] M.J. Silva, E. Samandar, A.M. Calafat, X. Ye, Identification of di-2-ethylhexyl terephthalate (DEHTP) metabolites using human liver microsomes for biomonitoring applications, Toxicol. In Vitro. 29 (2015) 716–721. doi:10.1016/j.tiv.2015.02.002.
[34] C. Höllerer, G. Becker, T. Göen, E. Eckert, Human metabolism and kinetics of tri-(2-ethylhexyl) trimellitate (TEHTM) after oral administration, Arch. Toxicol. 92 (2018) 2793–2807. doi:10.1007/s00204-018-2264-2.
[35] T. Eljezi, P. Pinta, D. Richard, J. Pinguet, J.-M. Chezal, M.-C. Chagnon, V. Sautou, G. Grimandi, E. Moreau, In vitro cytotoxic effects of DEHP-alternative plasticizers and their primary metabolites on a L929 cell line, Chemosphere. 173 (2017) 452–459. doi:10.1016/j.chemosphere.2017.01.026.

[36] F. Lessmann, A. Schütze, T. Weiss, T. Brüning, H.M. Koch, Determination of metabolites of di(2-ethylhexyl) terephthalate (DEHTP) in human urine by HPLC-MS/MS with on-line clean-up, J. Chromatogr. B. 1011 (2016) 196–203. doi:10.1016/j.jchromb.2015.12.042.
[37] M.J. Silva, T. Jia, E. Samandar, J.L. Preau, A.M. Calafat, Environmental exposure to the plasticizer 1,2-cyclohexane dicarboxylic acid, diisononyl ester (DINCH) in US adults (2000—2012), Environ. Res. 126 (2013) 159–163. doi:10.1016/j.envres.2013.05.007.
[38] E. Eckert, J. Müller, T. Göen, Simultaneous determination of polyvinylchloride plasticizers di(2-ethylhexyl) phthalate and tri(2-ethylhexyl) trimellitate and its degradation products in blood by liquid chromatography-tandem mass spectrometry, J. Chromatogr. A. 1410 (2015) 173–180. doi:10.1016/j.chroma.2015.07.083.
[39] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 75 (2003) 3019–3030.
[40] S. Sathyanarayana, R. Grady, E.S. Barrett, B. Redmon, R.H.N. Nguyen, J.S. Barthold, N.R. Bush, S.H. Swan, First trimester phthalate exposure and male newborn genital anomalies, Environ. Res. 151 (2016) 777–782. doi:10.1016/j.envres.2016.07.043.
[41] A. Demirel, A. Çoban, Ş. Yıldırım, C. Doğan, R. Sancı, Z. İnce, Hidden Toxicity in Neonatal Intensive Care Units: Phthalate Exposure in Very Low Birth Weight Infants, J. Clin. Res. Pediatr. Endocrinol. 8 (2016) 298–304. doi:10.4274/jcrpe.3027.
[42] A. Stroustrup, J.B. Bragg, S.A. Busgang, S.S. Andra, P. Curtin, E.A. Spear, A.C. Just, M. Arora, C. Gennings, Sources of clinically significant neonatal intensive care unit phthalate exposure, J. Expo. Sci. Environ. Epidemiol. (2018). doi:10.1038/s41370-018-0069-2.
[43] K. Strømmen, J.L. Lyche, E.W. Blakstad, S.J. Moltu, M.B. Veierød, A.N. Almaas, A.K. Sakhi, C. Thomsen, B. Nakstad, K. Brække, A.E. Rønnestad, C.A. Drevon, P.O. Iversen, Increased levels of phthalates in very low birth weight infants with septicemia and bronchopulmonary dysplasia, Environ. Int. 89–90 (2016) 228–234. doi:10.1016/j.envint.2016.01.024.
[44] J. Weuve, B.N. Sánchez, A.M. Calafat, T. Schettler, R.A. Green, H. Hu, R. Hauser, Exposure to Phthalates in Neonatal Intensive Care Unit Infants: Urinary Concentrations of Monoesters and Oxidative Metabolites, Environ. Health Perspect. 114 (2006) 1424–1431. doi:10.1289/ehp.8926.
[45] J.W. Gaynor, R.F. Ittenbach, A.M. Calafat, N.B. Burnham, A. Bradman, D.C. Bellinger, F.M. Henretig, E.E. Wehrung, J.L. Ward, W.W. Russell, T.L. Spray, Perioperative Exposure to Suspect Neurotoxicants from Medical Devices in Newborns with Congenital Heart Defects, Ann. Thorac. Surg. (2018). doi:10.1016/j.athoracsur.2018.06.035.
[46] J. Huygh, K. Clotman, G. Malarvannan, A. Covaci, T. Schepens, W. Verbrugghe, E. Dirinck, L. Van Gaal, P.G. Jorens, Considerable exposure to the endocrine disrupting chemicals phthalates and bisphenol-A in intensive care unit (ICU) patients, Environ. Int. 81 (2015) 64–72. doi:10.1016/j.envint.2015.04.008.
[47] A.M. Calafat, L.L. Needham, M.J. Silva, G. Lambert, Exposure to di-(2-ethylhexyl) phthalate among premature neonates in a neonatal intensive care unit, Pediatrics. 113 (2004) e429-434.
[48] R. Green, R. Hauser, A.M. Calafat, J. Weuve, T. Schettler, S. Ringer, K. Huttner, H. Hu, Use of di(2-ethylhexyl) phthalate-containing medical products and urinary levels of mono(2-ethylhexyl) phthalate in neonatal intensive care unit infants, Environ. Health Perspect. 113 (2005) 1222–1225. doi:10.1289/ehp.7932.

FIGURE CAPTIONS

Figure 1: Schematic diagram illustrating the configuration of the online SPE-LC-MS/MS method using two six-port valves

Figure 2: Total ion chromatogram of the 10 ng/mL analytical standard, showing the separation of 22 metabolites of plasticizers. 1, 5cx-MEPP; 2, MEHHP; 3, 5OH-MEHTP; 4, 5cx-MEPTP; 5, MEOHP; 6, 2cx-MMHP; 7, cx-MINP; 8, OH-MINP; 9, 2cx-MMHTP; 10, 5oxo-MEHTP; 11, oxo-MINP; 12, cx-MINCH; 13, OH-MINCH; 14, 2-MEHTM; 15, oxo-MINCH; 16, 1-MEHTM; 17, MEHA; 18, MEHP; 19, 4-MEHTM; 20, MEHTP; 21, MINP; 22, MINCH.

Figure 3: Chromatograms of a non-hydrolysed (A) and hydrolysed (B) processed urine sample showing overlapping between 5cx-MEPTP, 2cx-MMHP and OH-MINP peaks.

Figure 4: Chromatograms of processed urine samples from neonate intensive care unit patients. Example of 22 medical device derived plasticizer metabolites. **A)** DEHP and DEHTP metabolites; **B)** DINP metabolites; **C)** TOTM and DEHA metabolites; **D)** DINCH metabolites. Concentrations are given in ng/mL; 5cx-MEPP = 319.89 ; MEHHP = 20.27 ; 5OH-MEHTP = 2.33 ; 5cx-MEPTP = 9.24 ; MEOHP = 19.92 ; 2cx-MMHP* = 1.71 ; cx-MINP = 1.45 ; OH-MINP = 2.26 ; 2cx-MMHTP* = 0.63 ; 5oxo-MEHTP = 13.86 ; oxo-MINP = 1.52 ; cx-MINCH = 0.88 ; OH-MINCH = 1.78 ; 2-MEHTM = 50.60; oxo-MINCH = 0.48 ; 1-MEHTM = 1.82 ; MEHA = 0.45 ; MEHP = 41.68 ; 4-MEHTM = 0.24 ; MEHTP = 0,06 ; MINP = 0.27 ; MINCH = 0.15.
* The signal for these metabolites was too weak compared to the others; the peaks were therefore hidden and are not indicated.

Supplementary Figure 1: MRM chromatograms of the quantifier transition in blank samples and in spiked samples at the LLOQ concentration.

Table 1: Mass transitions for each compound analysed.

Table 2: Method performance results - Linearity, accuracy, precision; limit of detection and limit of quantification.

Table 3: Method performance results - Dilution integrity; Matrix effect and Recovery.

Table 4: Total urinary concentrations of plasticizer metabolites (ng/mL) detected in neonatal intensive care unit patients (n=104).

Supplementary Table 1: List of plasticizers and abbreviations, including phthalates, alternative plasticizers and their primary and secondary metabolites.

Supplementary Table 2: Method performance results - Stability and Specificity.

Supplementary Table 3: Demographic data and medical devices of patients.

Supplementary Table 4: Free urinary concentrations of plasticizer metabolites (ng/mL) detected in neonatal intensive care unit patients (n=104).

FIGURES AND TABLES

Figure 1

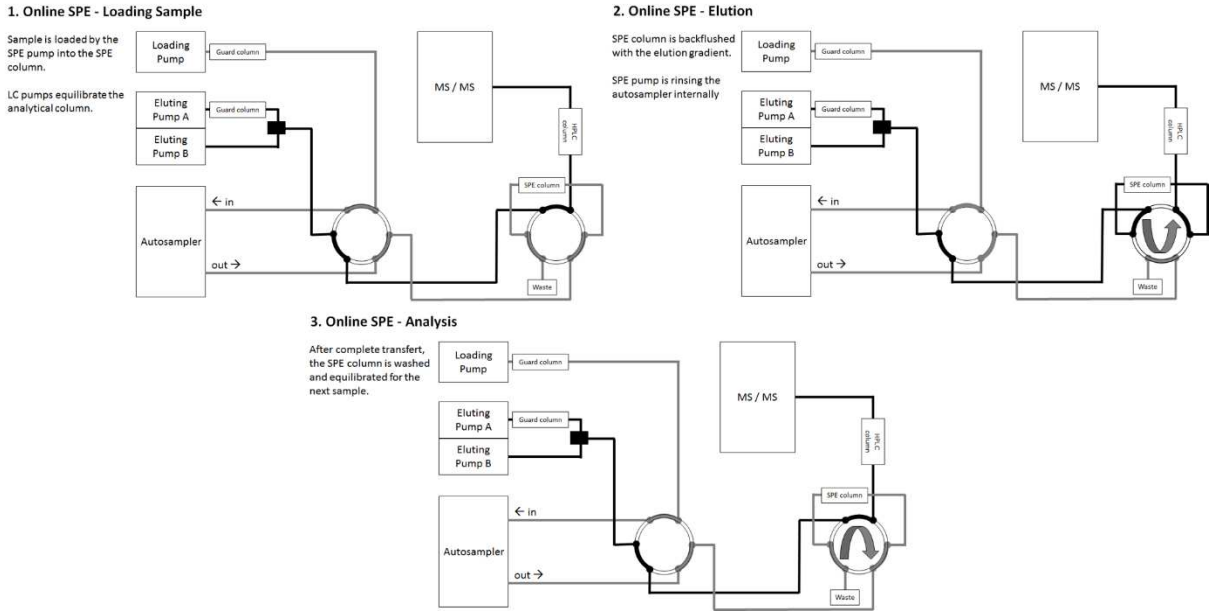


Figure 2

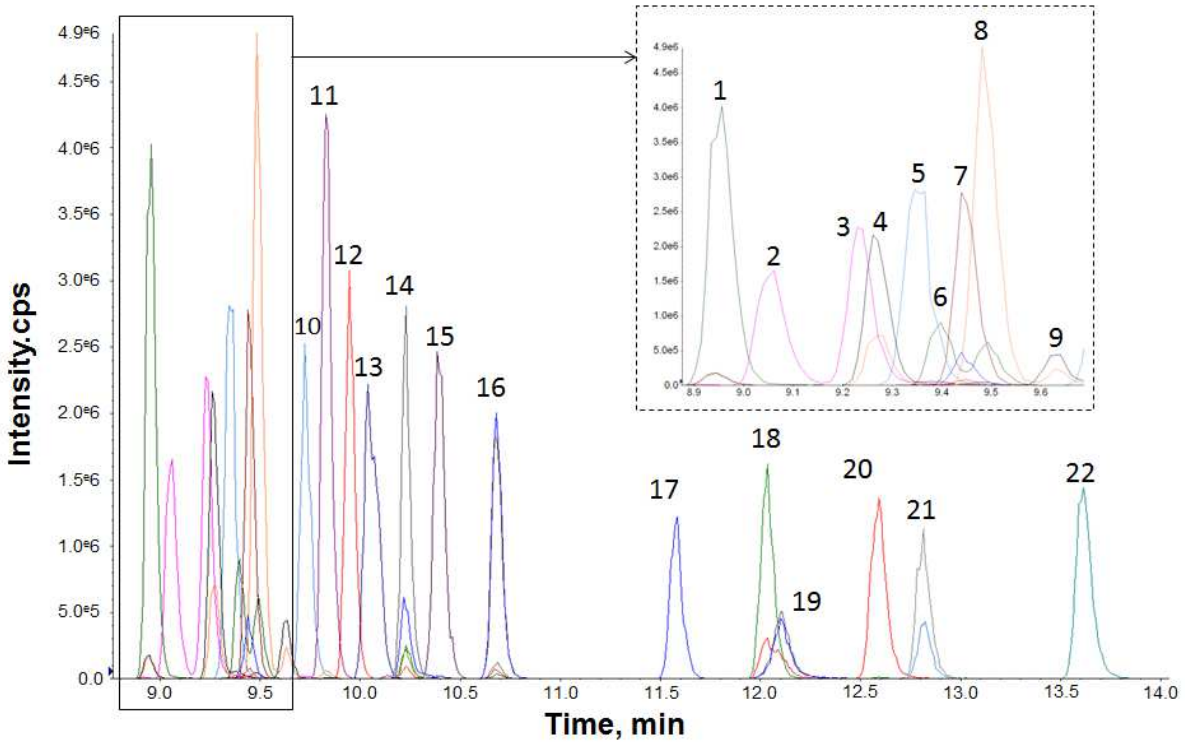

Intensity.cps
Time, min
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22

Figure 3

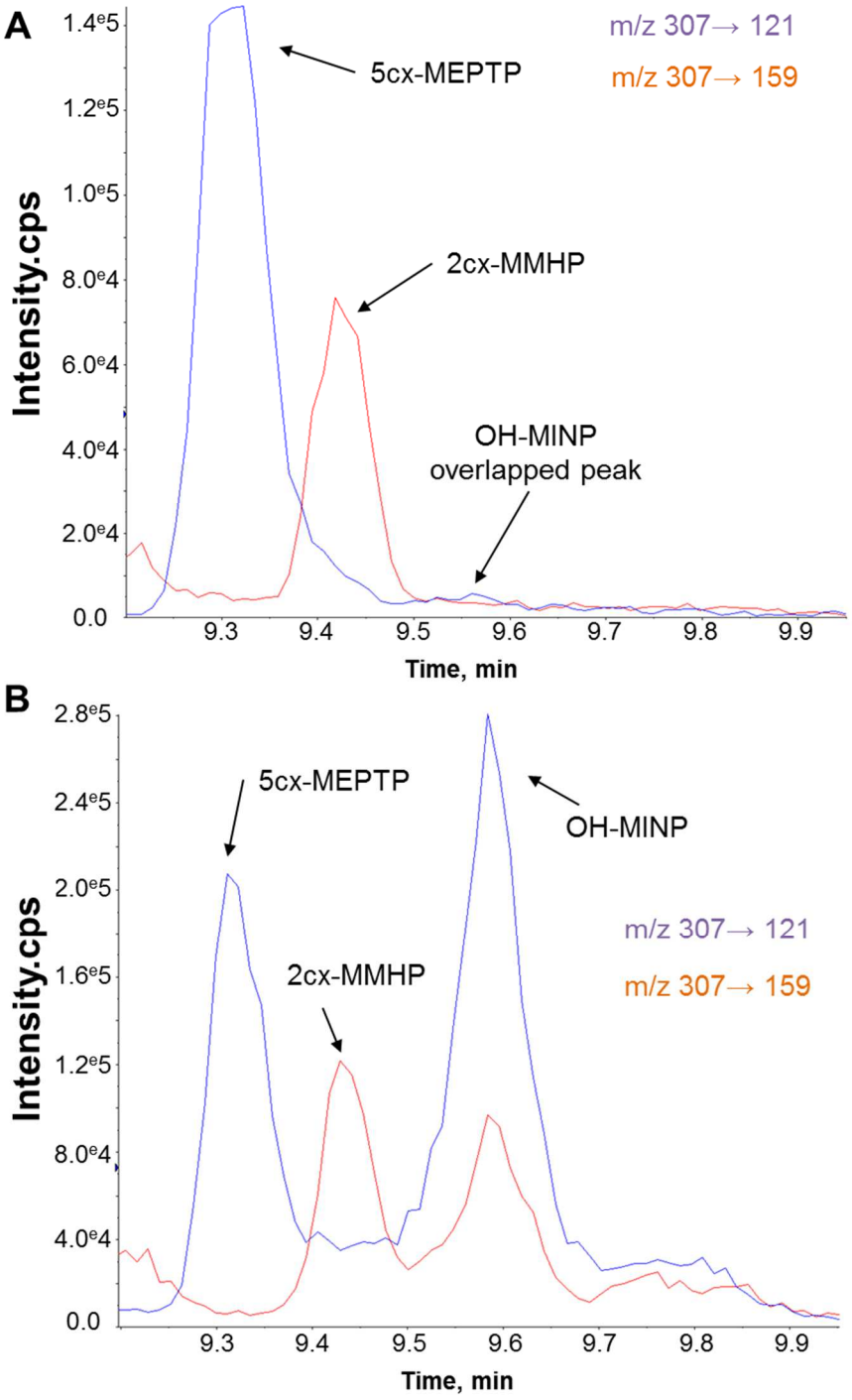
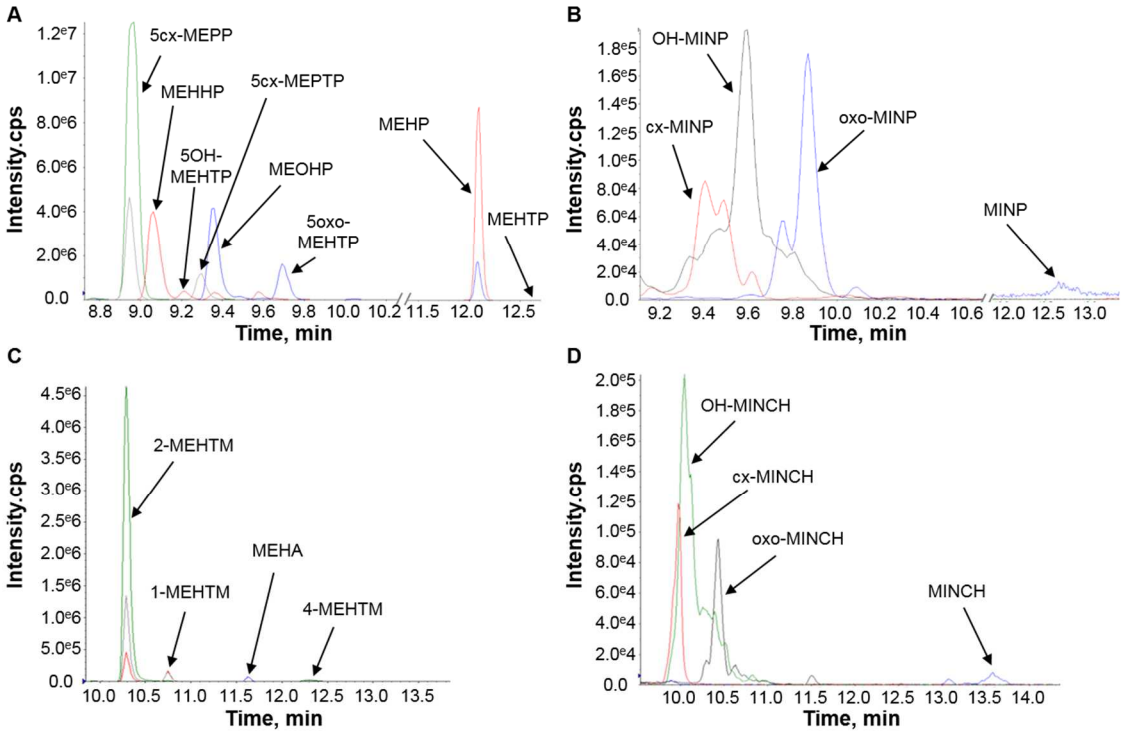

A
1.4e5
1.2e5
1.0e5
8.0e4
6.0e4
4.0e4
2.0e4
0.0
Intensity.cps
5cx-MEPTP
2cx-MMHP
OH-MINP
overlapped peak
m/z 307→ 121
m/z 307→ 159
9.3
9.4
9.5
9.6
9.7
9.8
9.9
Time, min
B
2.8e5
2.4e5
2.0e5
1.6e5
1.2e5
8.0e4
4.0e4
0.0
Intensity.cps
5cx-MEPTP
2cx-MMHP
OH-MINP
m/z 307→ 121
m/z 307→ 159
9.3
9.4
9.5
9.6
9.7
9.8
9.9
Time, min

Figure 4


A
Intensity.cps
Time, min
5cx-MEPP
MEHHP
5cx-MEPTP
5OH-MEHTP
MEOHP
5oxo-MEHTP
MEHP
MEHTP
B
OH-MINP
cx-MINP
oxo-MINP
MINP
C
2-MEHTM
MEHA
1-MEHTM
4-MEHTM
D
OH-MINCH
cx-MINCH
oxo-MINCH
MINCH

Supplementary Figure 1

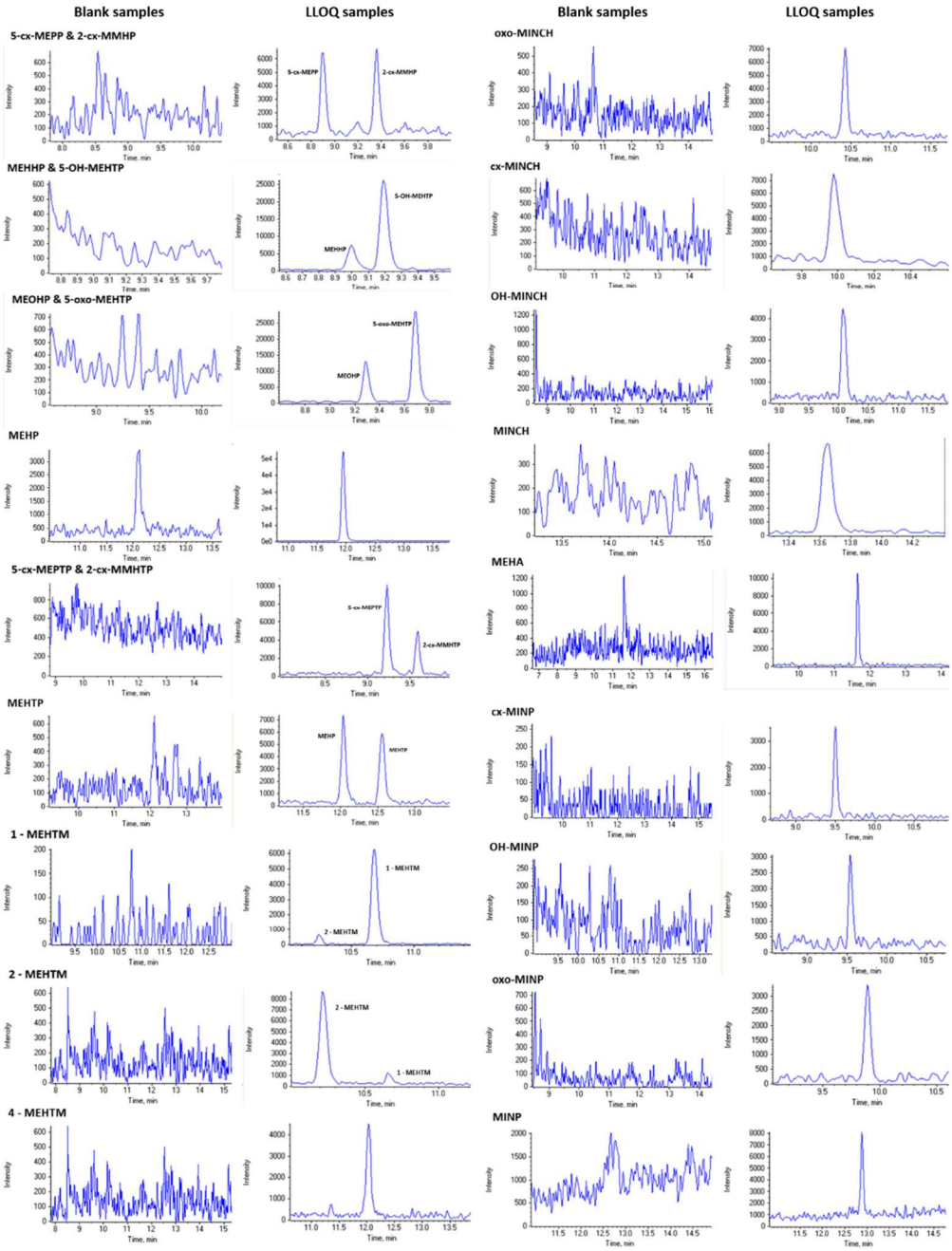


Table 1

Compound	RT (min)	Q1/Q3 (m/z)	Type	DP (V)	EP (V)	CE (V)	CXP (V)
5cx-MEPP / 2cx-MMHP	8.98 / 9.45	307.0/113.1	quantifier	-70	-10	-38	-11
		307.0/159.0	qualifier	-70	-10	-18	-13
5cx-MEPTP / 2cx-MMHTP	9.31 / 9.69	307.0/165.0	quantifier	-70	-10	-20	-10
		307.0/121.0	qualifier	-70	-10	-36	-12
$^{13}C_4$-5cx-MEPP	8.98	311.1/159.0	internal standard	-70	-10	-18	-13
MEHHP	9.08	293.1/121.0	quantifier	-100	-10	-22	-15
		293.1/145.1	qualifier	-100	-10	-20	-7
5OH-MEHTP	9.26	293.1/121.0	quantifier	-100	-10	-22	-15
		293.1/77.0	qualifier	-100	-10	-42	-10
D_4-MEHHP	9.08	297.0/124.9	internal standard	-100	-10	-26	-13
MEOHP	9.38	291.0/120.9	quantifier	-95	-10	-22	-11
		291.0/143.0	qualifier	-95	-10	-18	-9
5-oxo-MEHTP	9.77	291.0/121.0	quantifier	-95	-10	-22	-11
		291.0/77.0	qualifier	-95	-10	-41	-5
$^{13}C_4$-MEOHP	9.38	295.0/143.0	internal standard	-95	-10	-18	-11
MEHP	12.08	277.0/133.9	quantifier	-110	-10	-20	-9
		277.0/127.1	qualifier	-110	-10	-22	-11
MEHTP	12.62	277.0/121.0	quantifier	-120	-10	-22	-7
			qualifier	-120	-10	-24	-11
D_4-MEHP	12.05	281.0/138.0	internal standard	-110	-10	-20	-11
cx-MINCH	10.00	327.0/172.9	quantifier	-100	-10	-22	-15
		327.0/153.2	qualifier	-100	-10	-29	-7
OH-MINCH	10.10	313.1/153.0	quantifier	-90	-10	-24	-11
		313.1/109.1	qualifier	-90	-10	-40	-7
oxo-MINCH	10.45	311.1/153.0	quantifier	-110	-10	-24	-16
		311.1/109.2	qualifier	-110	-10	-38	-7
D_8-oxo-MINCH	10.41	319.1/160.9	internal standard	-110	-10	-22	-5
MINCH	13.64	297.0/153.0	quantifier	-130	-10	-21	-13
		297.0/109.1	qualifier	-130	-10	-34	-13
D_{17}-a-MINCH	13.02	300.3/153.0	internal standard	-90	-10	-22	-11
cx-MINP	9.48	321.0/173.2	quantifier	-115	-10	-20	-5
		321.0/121.0	qualifier	-115	-10	-32	-7
OH-MINP	9.54	307.0/120.9	quantifier	-105	-10	-24	-13
		307.0/159.1	qualifier	-105	-10	-20	-13
oxo-MINP	9.88	305.0/120.9	quantifier	-115	-10	-24	-7
		305.0/157.1	qualifier	-115	-10	-20	-17
D4-cx-MiNP	9.48	325.1/173.1	internal standard	-115	-10	-20	-9
MINP	12.83	291.0/141.0	quantifier	-115	-10	-24	-7
		291.0/120.9	qualifier	-115	-10	-24	-7
D_4-MINP	12,82	295.1/141.0	internal standard	-115	-10	-24	-13
MEHA	11.60	257.0/126.9	quantifier	-120	-10	-14	-9
		257.0/83.0	qualifier	-120	-10	-22	-11
D_{17}-MEHA	11.55	274.2/83.0	internal standard	-120	-10	-22	-7
1-MEHTM	10.73	321.1/150.0	quantifier	-110	-10	-20	-13
		321.1/121.0	qualifier		-10		
D_{17}-1-MEHTM	10.65	338.1/250.3	internal standard	-90	-10	-26	-5
2-MEHTM	10.28	321.1/233.3	quantifier	-65	-10	-26	-13
		321.1/121.0	qualifier		-10		
D_{17}-2-MEHTM	10.20	338.1/250.3	internal standard	-90	-10	-26	-5
4-MEHTM	11.93	321.1/233.3	quantifier	-65	-10	-26	-13
		321.1/121.0	qualifier	-65	-10	-26	-13
D_{17}-4-MEHTM	11.85	338.1/250.3	internal standard	-90	-10	-26	-5

DP:declustering potential; EP:entry potential; CE:collision energy; CXP:collision exit potential; RT:retention time.

Table 2

Compound	**Theoritical LOD** (ng/mL)	**Theoritical LOQ** (ng/mL)	**Quantitation range** (ng/mL)	**Linearity** (r2)	**Intra-day** (n=5)								**Inter-day** (n=5)							
					QC1		QC2		QC3		QC4		QC1		QC2		QC3		QC4	
					CV (%)	Accuracy(%)	CV (%)	Accuracy(%)	CV (%)	Accuracy(%)	CV (%)	Accuracy(%)	CV (%)	Accuracy(%)	CV (%)	Accuracy(%)	CV (%)	Accuracy(%)	CV (%)	Accuracy(%)
MEHP	0.014	0.048	0.1-25	0.9998	5.21	92.6	2.00	112	4.24	106	7.70	108	3.47	88.2	4.88	96.0	5.63	107	2.92	103
5cx-MEPP	0.004	0.012	0.025-50	0.9995	4.34	111	4.75	106	7.11	105	3.07	114	11.4	102	4.43	95.9	8.19	101	6.08	105
MEHHP	0.004	0.013	0.025-25	0.9997	5.31	95.3	3.14	106	6.87	110	9.42	108	10.6	103	4.74	98.0	6.31	107	3.69	101
MEOHP	0.007	0.023	0.025-25	0.9997	8.11	111	4.88	104	6.41	107	8.54	113	11.0	99.0	3.59	95.8	7.64	105	4.42	100
MINCH	0.005	0.017	0.025-10	0.9992	7.46	92.7	4.88	103	4.63	104	6.69	114	17.2	94.0	7.14	99.2	5.59	106	9.41	106
cx-MINCH	0.002	0.008	0.01-10	0.9993	7.77	106	3.02	110	5.30	109	5.31	109	15.1	107	4.16	99.6	8.03	107	3.84	103
OH-MINCH	0.001	0.005	0.01-10	0.9994	4.15	117	5.98	111	5.99	108	4.28	112	2.94	114	4.86	99.7	6.58	107	2.90	108
oxo-MINCH	0.001	0.004	0.01-10	0.9994	4.19	101	3.88	115	6.08	111	2.78	107	7.58	102	5.11	100	6.15	108	2.90	105
MINP	0.008	0.028	0.1-50	0.9989	3.04	91.2	4.63	110	8.16	97.0	3.11	109	13.0	92.8	3.59	94.6	5.83	102	3.78	101
cx-MINP	0.001	0.005	0.01-10	0.9993	4.44	109	4.93	108	5.77	113	3.16	110	10.8	108	4.41	97.7	5.04	109	3.82	109
OH-MINP	0.001	0.005	0.01-10	0.9995	4.59	108	2.73	115	8.57	112	5.33	109	8.92	103	2.34	99.7	7.18	107	2.62	105
oxo-MINP	0.002	0.006	0.01-10	0.9994	5.21	116	3.03	111	6.19	110	4.77	108	3.03	112	3.32	104	6.70	108	1.91	104
MEHA	0.013	0.044	0.05-25	0.9997	9.39	93.0	3.49	111	8.81	105	9.25	110	12.9	92.2	5.24	98.6	6.75	104	3.42	99.9
MEHTP	0.005	0.018	0.05-25	0.9988	3.76	96.0	2.90	95.7	3.90	88.0	8.91	91.9	5.26	89.3	8.28	95.2	14.4	93.4	12.8	103
1-MEHTM	0.003	0.012	0.025-50	0.9995	5.42	86.2	7.36	86.1	8.78	103	4.70	114	17.0	101	5.12	93.8	6.95	102	11.0	104
2-MEHTM	0.013	0.044	0.05-50	0.9996	9.82	90.3	3.27	103	9.00	109	3.22	114	10.4	100	8.57	95.8	6.84	100	4.74	103
4-MEHTM	0.004	0.012	0.025-50	0.9991	5.29	92.7	9.73	109	4.54	113	5.42	113	17.2	115	3.00	105	2.43	109	5.73	109

QC1-2-3-4: quality controls concentration (0.01-0.1 ng/L; 0.4ng/mL; 4 ng/mL; 10-50ng/mL respectively); CV: coefficient of variation; LOD: limit of detection; LOQ: limit of quantification

Table 3

Compound	**Dilution integrity** (ng/ml)				**Matrix effect**							**Extraction recovery** (%)				
	1/10		1/100		QC low			QC high			Native conc. measured (ng/ml)	QC1	QC2	QC3	QC4	Mean
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Conc. (ng/ml)	Accuracy (%)	CV (%)	Conc. (ng/ml)	Accuracy (%)	CV (%)						
MEHP	96.9	10.5	89.7	7.42	4	97.1	5.09	20	96.5	5.74	<LOQ-17.1	93.0	90.1	68.6	60.0	77.9
5cx-MEPP	88.9	12.9	86.7	5.69	4	98.3	13.1	40	104	13.1	1.95-17.7	96.3	100	101	74.0	92.9
MEHHP	91.9	12.1	86.9	10.0	4	92.6	3.62	20	89.1	6.25	0.17-6.47	84.7	88.1	92.0	71.3	84.0
MEOHP	93.5	12.6	87.4	6.27	4	93.6	3.13	20	89.8	8.02	0.45-9.02	97.8	90.1	96.3	75.8	90.0
MINCH	110	14.0	86.5	9.01	0.4	90.3	12.2	8	91.1	7.70	<LOQ-0.06	43.0	38.5	37.5	38.2	39.3
cx-MINCH	92.9	13.3	86.4	2.45	0.4	101	8.98	8	96.8	8.22	<LOQ-1.38	88.5	100	101	101	97.7
OH-MINCH	96.8	12.6	86.6	4.21	0.4	103	12.0	8	103	13.4	<LOQ-1.32	118	93.3	93.4	97.6	101
oxo-MINCH	93.4	12.5	87.6	5.09	0.4	95.6	8.63	8	92.6	5.91	<LOQ-0.64	127	90.7	92.9	92.8	101
MINP	102	13.8	90.0	5.62	0.4	107	13.4	40	107	9.05	<LOQ-0.84	65.1	40.2	29.7	22.1	39.3
cx-MINP	92.7	14.0	89.0	6.60	0.4	104	10.1	8	99.9	10.0	0.08-2.41	105	103	105	103	104
OH-MINP	96.0	11.5	86.9	4.02	0.4	104	8.70	8	102	10.5	0.01-0.57	119	107	103	102	108
oxo-MINP	99.0	14.6	85.4	5.09	0.4	111	9.62	8	88.5	9.52	<LOQ-0.77	120	99.6	104	104	107
MEHA	99.9	13.0	89.5	5.15	0.4	96.5	10.0	20	89.8	6.57	<LOQ-0.05	99.7	70.0	69.6	59.0	74.6
MEHTP	114	14.3	106	3.17	0.4	96.2	14.9	20	86.2	8.24	<LOQ-17.1	26.3	21.4	21.8	19.8	22.3
1-MEHTM	87.7	13.3	85.8	7.27	0.4	106	14.5	40	99.1	8.19	<LOQ-1.20	85.3	50.8	47.3	35.3	54.7
2-MEHTM	87.3	14.7	86.0	5.84	0.4	90.8	4.24	40	95.9	8.36	<LOQ-0.27	74.5	68.7	61.0	62.9	66.8
4-MEHTM	97.0	11.8	86.1	12.4	0.4	92.9	8.02	40	99.1	11.6	<LOQ-0.04	19.4	25.1	24.2	25.5	23.6

QC: quality controls; CV: coefficient of variation

Table 4

Compound	Mean (ng/ml)	Median (ng/ml)	Range (ng/ml)	95% CI (ng/ml)	Detection Frequency (%)
MEHP	190	38.2	3.76 - 13339	256	100
MEHHP	162	49.6	1.45 - 5731	113	100
MEOHP	127	36.2	0.64 - 4977	96.8	100
5cx-MEPP	785	207	0.96 - 22988	461	100
2cx-MMHP	20.4	2.15	0.07 - 1593	27.6	100
MINP	1.73	0.13	<LOQ - 97.4	1.89	60.6
OH-MINP	8.30	2.25	0.06 - 121	3.56	100
oxo-MINP	4.23	0.78	0.02 - 82.5	2.05	100
cx-MINP	18.5	3.06	0.17 - 397	10.0	100
MINCH	0.35	<LOQ	<LOQ - 19.7	0.44	12.5
OH-MINCH	25.6	0.04	<LOQ - 2227	43,0	70.2
oxo-MINCH	11.0	0.01	<LOQ - 971	18.7	51.0
cx-MINCH	31.1	0.10	<LOQ - 2007	42.6	93.3
MEHA	0.38	0.08	<LOQ - 7.92	0.19	76.0
MEHTP	0.21	<LOQ	<LOQ - 9.90	0.20	29.8
5OH-MEHTP*	2.82	<LOQ	<LOQ - 116	2.06	97.3
5oxo-MEHTP*	4.87	1.58	0.06 - 192	3.37	100
5cx-MEPTP*	14.8	5.02	0.07 - 201	5.28	100
2cx-MMHTP*	0.82	0.19	<LOQ - 13.0	0.30	76.1
1-MEHTM	0.52	0.10	<LOQ - 13.1	0.28	97.1
2-MEHTM	25.6	0.47	<LOQ - 925	25.0	97.1
4-MEHTM	0.19	0.04	<LOQ - 4.14	0.11	72.1

LOQ: limit of quantification; CI: confidence interval

* Semi-quantitative data

Supplementary Table 1

Parent compound	Abbreviation	Monitored biomarker	Abbreviation
di(ethylhexyl) phthalate	DEHP	Mono(2-ethylhexyl) phthalate	MEHP
		mono(2-ethyl-5-hydroxyhexyl) phthalate	MEHHP[a]
		mono(2-ethyl-5-oxohexyl) phthalate	MEOHP[a]
		mono(2-ethyl-5-carboxypentyl) phthalate	5cx-MEPP[a]
		mono(2-ethyl-2-carboxypentyl) phthalate	2cx-MMHP[a]
di(isononyl) phthalate	DINP	monoisononylphthalate	MiNP
		mono-(4-methyl-7-hydroxy-octyl) phthalate	OH-MiNP[a]
		mono-(4-methyl-7-oxo-octyl) phthalate	oxo-MiNP[a]
		mono-(4-methyl-7-carboxy-heptyl) phthalate	cx-MiNP[a]
di(isononyl)cyclohexane-1,2-dicarboxylate	DINCH	monoisononyl cyclohexane-1,2-dicarboxylate	MINCH
		cyclohexane-1,2-dicarboxylate-mono-(7-hydroxy-4-methyl)octyl ester	OH-MINCH[a]
		cyclohexane-1,2-dicarboxylate-mono-(7-oxo-4-methyl)octyl ester	oxo-MINCH[a]
		cyclohexane-1,2-dicarboxylate-mono-(7-carboxylate-4-methyl)heptyl ester	cx-MINCH[a]
di-2-ethylhexyl Adipate	DEHA	mono(2-ethylhexyl) adipate	MEHA
di-2-ethylhexyl terephthalate	DEHTP	mono(2-ethylhexyl) terephthalate	MEHTP
		1-mono(2-ethyl-5-hydroxyhexyl)benzene-1,4-dicarboxylate	5OH-MEHTP[a]
		1-mono(2-ethyl-5-oxohexyl)benzene-1,4-dicarboxylate	5oxo-MEHTP[a]
		1-mono(2-ethyl-5-carboxylpentyl)benzene-1,4-dicarboxylate	5cx-MEPTP[a]
		1-mono(2-carboxylmethylhexyl)benzene-1,4-dicarboxylate	2cx-MMHTP[a]
tri-2-ethylhexyl trimellitate	TOTM	1-mono(2-ethylhexyl)trimellitate	1-MEHTM
		2-mono(2-ethylhexyl)trimellitate	2-MEHTM
		4-mono(2-ethylhexyl)trimellitate	4-MEHTM

[a] Secondary metabolites

Supplementary Table 2

Compound	Stability								Specificity
	+4°C				-20°C				
	7 days		14 days		7 days (1 freeze-thaw cycle)		14 days (2 freeze-thaw cycles)		
	Accuracy (%bias)		Accuracy (%bias)		Accuracy (%bias)		Accuracy (%bias)		Accuracy (%)
	QC 1	QC 4	QC 1	QC 4	QC 1	QC 4	QC 1	QC 4	QC 2
MEHP	6.04	4.84	6.85	-5.70	7.48	4.77	5.32	-6.63	88.2
5cx-MEPP	-1.55	8.72	14.0	-4.03	3.88	7.38	-3.49	0.56	105
MEHHP	7.22	5.19	-10.3	0.33	12.5	2.96	10.6	3.60	93.5
MEOHP	7.22	3.94	12.5	3.85	10.6	4.63	12.5	0.98	100
MINCH	14.7	-7.36	-14.0	-6.63	6.20	5.80	-7.36	1.33	103
cx-MINCH	6.31	-5.24	5.41	-7.76	-12.6	-0.35	-1.80	-3.85	93.5
OH-MINCH	0.85	-11.5	0.85	-11.2	-10.2	-3.64	-0.85	-4.06	94.2
oxo-MINCH	-3.36	-8.55	-9.24	-9.88	-11.8	-4.90	-5.04	-3.42	101
MINP	11.5	5.58	13.0	-13.2	8.50	-1.18	-12.1	-9.02	97.5
cx-MINP	0.85	8.57	-8.47	-1.24	0.85	9.31	-1.70	-2.64	111
OH-MINP	1.70	3.41	-0.85	-3.27	-0.85	7.60	-2.54	0.70	106
oxo-MINP	1.75	1.00	-7.89	-3.34	-1.75	7.60	2.63	-0.03	86.3
MEHA	4.56	-8.96	-11.0	-10.1	12.0	-0.78	-10.1	-11.6	112
MEHTP	0.22	13.8	-2.88	11.7	8.85	12.9	8.19	4.42	93.3
1-MEHTM	1.46	9.36	9.12	8.13	1.82	10.5	-12.0	3.42	85.3
2-MEHTM	9.98	9.09	0.20	0.84	2.20	4.71	-7.19	1.71	98.8
4-MEHTM	12.7	-1.71	-6.36	-2.31	7.63	-4.41	12.3	-9.05	103

%Bias with respect to nominal concentration. QC1-2-4: quality controls concentration (0.01-0.1 ng/L; 0.4ng/mL; 10-50ng/mL respectively)

Supplementary Table 3

Population n (newborns)	104
Female %	39.4
Term birth weeks (SD)	36.2 (4.3)
Medical device n (% population)	
dialysis	0 (0)
extracorporeal circulation	0 (0)
parenteral nutrition	103 (99)
enteral nutrition	19 (18)
extracorporeal membrane oxygenation	2 (2)
plasmapheresis	1 (1)

SD: standard deviation

Supplementary Table 4

Compound	**Mean** (ng/ml)	**Median** (ng/ml)	**Range** (ng/ml)	**95% CI** (ng/ml)	**Detection Frequency** (%)
MEHP	121	22.9	2.72 - 7902	153	100
MEHHP	20.0	3.35	0.12 - 733	17.2	100
MEOHP	13.6	2.72	<LOQ - 408	10.4	99.0
5cx-MEPP	715	193	0.53 - 20903	421	100
2cx-MMHP	9.29	1.31	<LOQ - 686	11.8	100
MINP	1.29	<LOQ	<LOQ – 90.7	1.76	26.9
OH-MINP	5.00	0.24	0.01 – 86.7	8.46	19.2
oxo-MINP	0.45	0.06	<LOQ – 23.9	0.46	93.3
cx-MINP	14.3	1.99	0.10 - 382	9.30	100
MINCH	0.01	<LOQ	<LOQ – 0.90	0.02	5.77
OH-MINCH	0.20	<LOQ	<LOQ – 13.9	0.28	11.5
oxo-MINCH	0.13	<LOQ	<LOQ – 8.73	0.18	7.69
cx-MINCH	2.72	<LOQ	<LOQ - 167	3.49	50.0
MEHA	0.32	0.05	<LOQ – 8.17	0.19	56.7
MEHTP	0.17	<LOQ	<LOQ – 6.17	0.14	22.1
5OH-MEHTP*	0.58	0.11	<LOQ – 15.5	0.36	94.8
5oxo-MEHTP*	0.72	0.17	<LOQ – 21.2	0.47	92.0
5cx-MEPTP*	15.0	3.54	0.06 - 162	5.16	100
2cx-MMHTP*	0.94	0.18	<LOQ – 10.4	0.32	71.9
1-MEHTM	0.45	0.07	<LOQ – 10.7	0,26	93.3
2-MEHTM	21.1	0.48	<LOQ - 818	19.6	95.2
4-MEHTM	0.20	0.05	<LOQ - 4.65	0.11	71.2

LOQ: limit of quantification; CI: confidence interval

* Semi-quantitative data