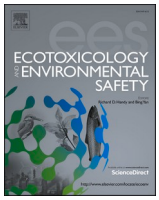
Ecotoxicology and Environmental Safety 309 (2026) 119698

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Quantification of phthalate and DINCH metabolites in human urine and maternal breast milk: Assessing maternal body burden and infant exposure

Agneta A. Runkel [a,d,*,1], Žan Rekar [a,b,1], Neja Kosirnik [c], Darja Mazej [a], Milena Horvat [a,b], Janja Snoj Tratnik [a,b], Tina Kosjek [a,b]

[a] Jožef Stefan Institute, Department of Environmental Sciences, Jamova cesta 39, Ljubljana 1000, Slovenia
[b] Jožef Stefan International Postgraduate School, Jamova cesta 39, Ljubljana 1000, Slovenia
[c] Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, Ljubljana 1000, Slovenia
[d] Lund University, Division of Occupational and Environmental Medicine, Scheelevägen 8, Lund 22363, Sweden

ARTICLE INFO

Edited by Richard Handy

Keywords:
Phthalate metabolites
DINCH
Urine
Maternal milk
LC-MS/MS
Human biomonitoring

ABSTRACT

Based on toxicological evidence, human exposure to phthalates (PHs) and diisononylcyclohexane-1,2-dicarboxylate (DINCH) may contribute to adverse health effects, especially during vulnerable developmental stages. To support the exposure assessment for this group of endocrine disruptors, we developed and validated a method for the analysis of 14 PH and 3 DINCH metabolites in human urine and maternal milk, applied the method in a pilot study, and identified crucial obstacles in the path of establishing maternal milk as a routine matrix in human biomonitoring. Urine and milk samples were extracted with solid-phase extraction (SPE) and QuEChERS salts, respectively, and analysed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The method accuracy was confirmed for urine samples via a certified standard reference material and the G-EQUAS intercomparison programme. We identified a need for sampling protocols, reference materials, and external method verification schemes in order to establish maternal milk as a routine matrix. Finally, the method was tested for its applicability in a pilot biomonitoring study on 30 paired urine and milk samples from lactating mothers, with medians ranging from <LLOQ – 15 μg/L in urine and <LLOQ – 16 μg/L in maternal milk and generally higher detection rates in urine. Furthermore, the results indicate extensive monoester formation under cooled storage conditions, resulting in potentially high infantile exposure to phthalate monoesters for which, to date, no guidance values exist despite their demonstrated toxicity.

1. Introduction

Di-esters of phthalic acid, otherwise known as phthalates (PHs), are a group of industrial chemicals with a common chemical structure. They find numerous applications in plastic and personal care products, such as polyvinyl chloride (PVC) plastics, toys, food packaging, cosmetics, and pharmaceuticals (Gao and Kannan, 2020; Gkrillas et al., 2021). Due to their widespread applications and tendency to leach into the environment, PHs can be detected in nearly all environmental and food matrices (Sioen et al., 2012).

Abbreviations: ACN, Acetonitrile; BBzP, Butyl-benzyl phthalate; Cx-MEPP, Mono2-ethyl-5-carboxypentyl phthalate; Cx-MINCH, Cyclohexane-1,2-dicarboxylate-mono-7-carboxylate-4-methylheptylester; Cx-MINP, mono4-methyl-7-carboxyheptyl phthalate; DEHP, di-2-ethylhexyl phthalate; DiBP, di-iso-butyl phthalate; DINCH, Diisononylcyclohexane-1,2-dicarboxylate; DEP, di-ethyl phthalate; DnBP, di-n-butyl phthalate; ECHA, European Chemicals Agency; GC, Gas chromatography; G-EQUAS, German External Quality Assessment Scheme; HBM, Human biomonitoring; HMW, High molecular weight; HPLC-MS, High performance liquid chromatography mass spectrometry; LMW, Low molecular weight; MBzP, Monobenzyl phthalate; MCHP, Monocyclohexyl phthalate; MEHP, Mono2-ethylhexyl phthalate; MeOH, Methanol; MEP, Monoethyl phthalate; MiBP, Mono-iso-butyl phthalate; MnBP, Mono-n-butyl phthalate; NIST, National Institute of Standards and Technology; OH-MEHP, Mono2-ethyl-5-hydroxyhexyl phthalate; OH-MIDP, Mono6-hydroxy-2-propylheptyl phthalate; OH-MINCH, Cyclohexane-1,2-dicarboxylate-mono-7-hydroxy-4-methyloctyl ester; OH-MINP, mono4-methyl-7-hydroxyoctyl phthalate; Oxo-MEHP, mono2-ethyl-5-oxohexyl phthalate; Oxo-MIDP, mono6-oxo-2-propylheptyl phthalate; Oxo-MINCH, Cyclohexane-1,2-dicarboxylate-monooxo-isononyl ester; Oxo-MINP, mono4-methyl-7-oxooctyl phthalate; PHs, Phthalates; PVC, Polyvinyl chloride; QC, Quality control; REACH, Registration, Evaluation, Authorisation and Restriction of Chemicals.

* Corresponding author at: Jožef Stefan Institute, Department of Environmental Sciences, Jamova cesta 39, Ljubljana 1000, Slovenia.
E-mail address: agneta.runkel@ijs.si (A.A. Runkel).

[1] These two authors contributed equally to this work.

https://doi.org/10.1016/j.ecoenv.2026.119698
Received 6 October 2025; Received in revised form 25 December 2025; Accepted 7 January 2026
Available online 10 January 2026
0147-6513/© 2026 Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Thus, di-2-ethylhexyl phthalate (DEHP) was detected in food from the Belgian market at maximum concentrations of 11–2154 μg/kg fresh weight (Fierens et al., 2012), and close to 400 ng/g of diethyl phthalate (DEP), di-n-butyl phthalate (DnBP), and di-iso-butyl phthalate (DiBP) were detected in hygiene products (Gao and Kannan, 2020). Butyl-benzyl phthalate (BBzP) was measured in face masks (486 ng/g) in the United States (Vimalkumar et al., 2022), DEP, DEHP, DiBP, DnBP, BBzP, di-cyclohexyl phthalate (DCHP), di-iso-decyl phthalate (DIDP), and di-iso-nonyl phthalate (DINP) were detected in food and beverages from Northwest China at median concentrations between 0.0002 and 0.1 μg/g (Ma et al., 2022). Low molecular weight (LMW) PHs (3 – 6 carbon atoms) were detected in indoor air in Norway at concentrations of 785 ng/m^3 (Sakhi et al., 2019). These data demonstrate that humans are exposed to multiple PHs via inhalation, ingestion, and dermal adsorption.

Many studies have reported on the endocrine activity of PHs, listing altered hormone levels, reproductive toxicity, developmental effects, and a potential risk for cancer among others (Martino-Andrade and Chahoud, 2010; Ventrice et al., 2013; Xu et al., 2020; Zhu et al., 2018; Zuccarello et al., 2018). Five phthalates, DiBP, DnBP, BBzP, DEHP, and DCHP are classified by the European Chemicals Agency (ECHA) as substances of very high concern (ECHA, 2025). Therefore, ECHA proposed and enacted the restriction of four PHs, namely DEHP, BBzP, DnBP, and DiBP in consumer products, leading to an increase in the utilisation of alternative plasticisers over the last few years. Dominating the market is di(isononyl)cyclohexane-1,2-dicarboxylate, alias Hexamoll® DINCH (DINCH) (Bui et al., 2016), that is structurally similar to high molecular weight (HMW) PHs but supposedly less toxic (David et al., 2015).

In the human body, PHs and DINCH undergo rapid biotransformation and urinary excretion (half-life $<$ 24 h), though interindividual differences apply due to variations in (non-) genetic susceptibility (Choi et al., 2012; Nehring et al., 2020; Stajnko et al., 2022). The diesters are enzymatically cleaved by esterases and lipases to the respective monoesters. LMW PH monoesters can be excreted in either free form or as conjugates. HMW PH monoesters, however, can undergo further biotransformation in the liver via cytochrome P450 enzymes, predominantly leading to the formation of hydroxylated, oxidised, and carboxylated metabolites that are excreted in urine predominantly in conjugated form. Thus, human exposure assessment is mainly achieved via the measurement of metabolites (free + conjugated) in urine. This approach allows the assessment of exposure via all pathways and avoids over- or underestimation of exposure that would occur if only free or only conjugated metabolite levels were assessed (Silva et al., 2003).

Besides feasibility, however, the assessment of metabolite concentrations can yield further information important for health risk assessment, as the toxicological profiles of the monoester metabolites often differ from their di-ester parent compounds. For instance, the acute toxicity of MEHP exceeds the toxicity of DEHP by a factor of 10 (Zhou et al., 2023), and has been associated with oxidative stress, neurotoxicity, and endocrine disruption (Liu et al., 2023; Wang et al., 2012; Zhou et al., 2023), among others. However, exposure is rarely assessed at the metabolite level, as evaluations more commonly focus on diester exposure. This approach is also typically assumed for dietary exposure in infants via maternal milk consumption, even though both diesters and monoesters have been detected in maternal milk (Calafat et al., 2004). This has been confirmed in a Swedish study that investigated the distribution of DEHP and its metabolites in the human body by analysing 42 matched urine, serum, and maternal milk samples (Högberg et al., 2008). Their findings revealed that the diester can be predominantly detected in maternal milk (9 μg/L) and to a lesser extent in blood (0.5 μg/L). The monoester MEHP, however, is predominantly detected in urine (15 μg/g creatinine) and present as a fraction in blood and milk (0.49 μg/L). The findings from Calafat et al. (2004) add to this that monoesters are mainly present in glucuronidated form in blood and in free form in maternal milk. Secondary metabolites of MEHP - formed via cytochrome P450 enzymes - could only be detected in urine and at equal or higher concentrations than MEHP (15–24 μg/g creatinine, respectively) (Högberg et al., 2008). Similarly, Brucker-Davis et al. (2008) determined DnBP at median concentrations of 44.5 ng/mL and 81.2 ng/g in cord blood and maternal milk, respectively, with 18- and 7-fold lower concentrations of its metabolite MnBP (2.5 ng/mL in cord blood and 12 ng/g in maternal milk). Those results demonstrate that the diester can be transferred unchanged from blood to maternal milk.

Thus, the characteristic distribution of parent compounds and metabolites can serve as a quality control factor when assessing the potential for sample contamination if the enzymatic activity in blood and maternal milk samples is inhibited immediately after sample collection (Calafat et al., 2004; Koch et al., 2004). Previous studies suggest that esterases and lipases remain fully active in stored samples even under frozen conditions (Berkow et al., 1984; Wardell et al., 1984). This has implications for future exposure assessment studies as it is becoming more and more common for mothers to pump their milk and store it under cooled conditions (Rasmussen et al., 2017) where monoester formation can occur rapidly.

Human biomonitoring (HBM) has become a popular tool in assessing the chemical body burden of populations, and many laboratories conduct routine measurements of phthalates in urine. However, maternal milk monitoring has become of increasing interest as an exposure route for infants, a highly vulnerable population, and monoester monitoring has been scarcely reported despite reason to suspect high levels in stored maternal milk. The aim of our study was to develop a UHPLC-MS/MS method for 14 metabolites of common phthalates —DEP, DiBP, DnBP, BBzP, DCHP, DIDP, DINP, and DEHP— and three DINCH metabolites applicable to urine and maternal milk samples to 1) derive a dietary monoester exposure dose for infants under consideration of the maternal phthalate body burden, and to 2) reveal knowledge gaps and analytical needs that hinder standardisation and applicability in routine HBM. The method was thoroughly validated and applied to urine and maternal milk samples from the first national HBM study in Slovenia.

2. Materials and methods

2.1. Materials and chemicals

The list of materials and chemicals can be found in the Supplementary Material (SM), Chapter 1. Importantly, not all calibration standards were compliant with ISO/IEC 17034, while NIST Standard Reference Material® 3672 (SRM), which was used for quality control and bias correction, where needed, is the highest level metrological standard traceable to the International System of Units (SI).

2.2. Preparation of working solutions and quality control samples

The working solutions of PH and DINCH metabolites were prepared by diluting each reference standard solution (0.1 g/L) in methanol (MeOH) to a final concentration of 1000 μg/L. For the internal standard (IS) working solution, the IS reference standard solutions (0.1 g/L) were diluted in MeOH to achieve a final concentration of 100 μg/L. All solutions were stored in a glass flask at −20 °C until use. Synthetic urine was prepared following the published procedure (CDC, 2013). A fresh 3 M ammonium acetate buffer, used for the deconjugation step, was prepared by dissolving ammonium acetate in HPLC-grade water and adjusting the pH to 6.5 with formic acid (FA). Quality control (QC) standards were prepared for each batch by spiking synthetic urine (5 μg/L and 50 μg/L) and goat milk (0.19 μg/L and 19 μg/L) with PH and DINCH metabolites at two concentration levels.

To minimise external contamination, only thermally treated glassware was used in the analytical workflow, except for specific plastic components like Oasis-HLB 96-well plates, collection plates, and filter vials.

2.3. Sample preparation for urine and maternal milk

2.3.1. Urine sample preparation

To 0.5 mL of urine sample, 300 μL of HPLC water, 150 μL of 3 M ammonium acetate buffer (pH 6.5), 10 μL of β-glucuronidase, and 20 μL of the internal standard mixture (final concentration 4 μg/L) were added. The mixture was then incubated at 37 °C for 1.5 h on an orbital thermo-shaker to achieve the deconjugation of metabolites. The samples were enriched by SPE, where a 60 mg Oasis-HLB sorbent packed in a 96-well plate was conditioned with 3.6 mL of MeOH and equilibrated with 1.8 mL of HPLC-grade water. The sample was then loaded and passed through the SPE plate, followed by a washing step with 1.5 mL of 0.1 M FA and 1.5 mL of water-MeOH mixture (9:1) with 0.1 % FA. After 20 min of drying under full vacuum, the target analytes were eluted from the plate with 1.8 mL MeOH and transferred to HPLC vials. The eluates were then evaporated to almost dryness under a gentle flow of nitrogen gas, and the residues were reconstituted in 0.5 mL of water-MeOH mixture (9:1) with 0.1 % FA for UHPLC-MS/MS analysis. The same protocol was applied to the procedural blanks, calibration, and QCs with synthetic urine to match the urine matrix.

2.3.2. Milk sample preparation

2 mL of maternal milk is transferred into a conical tube. 70 μL of internal standard at a final concentration of 3.5 μg/L and 10 mL of acetonitrile (ACN) are added to the sample. After vortex-mixing for 1 min, the QuEChERS salts are added, and the mixture is shaken for 1 min prior to 10 min of centrifugation at 5511 ×g to achieve phase separation. The supernatant is then transferred into a new vial, 10 mL of n-hexane is added, and the mixture is vortexed-mixed for 1 min and centrifuged for 10 min at 5511 ×g. After centrifugation, the supernatant was discarded, and the lower layer was evaporated to dryness under a stream of nitrogen gas in a new vial at 40 °C. The residue was then reconstituted with 750 μL of 10 % ACN and filtered through a 0.20 μm filter (Regenerated Cellulose) prior to injection. The same protocol was applied to the procedural blanks, calibration, and QCs with goat milk to match the milk matrix.

2.4. Instrumental analysis

The instrumental analyses were carried out by ultra-high-performance liquid chromatography (UHPLC, Shimadzu, Kyoto, Japan) coupled to a hybrid quadrupole-linear ion trap mass spectrometry analyser QTRAP 4500 (Sciex, Framingham, MA, USA). The Analyst software from Sciex was used for data acquisition and analysis.

Separation of the MEP, MiBP, MnBP, MCHP, MBzP, MEHP, oxo-MEHP, OH-MEHP, oxo-MINP, OH-MINP, oxo-MINCH, OH-MINCH, oxo-MIDP, and OH-MIDP was achieved by "METHOD 1" on a Kinetex Phenyl-Hexyl column (2.6 μm, 2.1 × 150 mm, Phenomenex, Torrance, CA, USA), whereas a shorter, i.e., 10 cm Kinetex Phenyl-Hexyl column with the same stationary phase was used for the separation of carboxy metabolites cx-MEPP, cx-MINP and cx-MINCH ("METHOD 2"). The total flow rate was 0.2 mL/min for method 1 with an analysis time of 18 min, whereas the flow rate of method 2 (cx-MEPP, cx-MINP and cx-MINCH) was 0.3 mL/min with a total analysis time of 10 min.

The mobile phase for method 1 was 0.1 % FA in LC-MS grade water (B) and 0.1 % FA in LC-MS grade MeOH (A) with a gradient from 10 % A to 15 % A in 30 s, followed by a gradient increase to 60 % A over 4.5 min and isocratic elution for 5 min, followed by a steep gradient to 100 % A in one minute and isocratic elution for four minutes. Afterwards, the concentration of A was reduced to 10 % and this level was kept for column equilibration for three minutes. The gradient for method 2 was as follows: A gradient from 10 % A (ACN) and 90 % B (0.5 mM ammonium fluoride in water) to 100 % A within 6 min, followed by isocratic elution for one minute. Afterwards, the concentration of A decreased to 10 % and was kept for three minutes. The injection volume was 10 μL for both separation methods.

Table 1
Optimised mass spectrometric and chromatographic parameters. Rt: retention time; DP: Declustering Potential; CE: Collision Energy; CXP: Collision Cell Exit Potential.

Compound	Rt (min)	Precursor Ion (*m/z*)	Product Ion(s) (*m/z*)	DP (V)	CE (V)	CXP (V)
MEP	8.05	193	**77**; 121	-15	-18; −16	-5; −11
IS-MEP		197	**79**	-15	-20	-7
MiBP	11.18	221	**77**; 134	-55; −5	-22; −18	-11; −11
IS-MiBP		225	**79**	-5	-18	-7
MnBP	11.67	221	**77**; 149	-55	-22; −14	-11; −13
IS-MnBP		225	**79**	-5	-28	-11
MCHP	12.90	247	**95**; 147	-60	-46; −20	-7; −9
IS-MCHP		251	**79**	-60	-28	-5
MBzP	12.72	255	**77**; 107	-30	-26; −18	-5; −7
IS-MBzP		259	**77**	-30	-30	-7
MEHP	13.39	277	**134**; 77	-55	-20; −36	-7; −7
IS-MEHP		281	**137**	-55	-20	-9
OH-MEHP	12.09	293	**121**; 145	-70	-22; −18	-15; −5
IS-OH-MEHP		297	**124**	-70	-20	-3
Oxo-MEHP	12.07	291	**143**; 121	-70	-18; −26	-7; −9
IS-oxo-MEHP		295	**124**	-70	-18	-1
cx-MEPP	2.87	307	**159**; 113	-45	-16; −40	-11; −9
IS-cx-MEPP		311	**159**	-5	-16	-9
OH-MIDP	13.08	321	**121**; 173	-57	-24; −20	-11; −11
IS-OH-MIDP		325	**124**	-57	-24	-11
Oxo-MIDP	13.12	319	**171**; 121	-65	-20; −24	-7; −9
IS-oxo-MIDP		323	**124**	-65	-22	-9
OH-MINP	12.87	307	**121**; 159	-70	-24; −20	-11; −15
IS-OH-MINP		311	**124**	-70	-22	-17
Oxo-MINP	12.88	305	**121**; 157	-75	-24; −20	-11; −13
IS-oxo-MINP		309	**124**	-75	-24	-11
cx-MINP	3.03	321	**173**; 121	-10	-22; −24	-13; −1
IS-cx-MINP		325	**173**	-10	-22	-13
OH-MINCH	13.15	313	**153**; 109	-60	-20; −36	-13; −5
Oxo-MINCH	13.20	311	**153**; 109	-75	-20; −40	-13; −7
IS-oxo-MINCH		315	**157**	-75	-24	-11
cx-MINCH	3.79	327	**173**; 153	-45	-22; −30	-13; −11
IS-cx-MINCH		331	**173**	-45	-22	-17

The ion source parameters were maintained as follows: ion spray voltage (IS) −4500 V; source temperature (TEM) 450 °C; curtain gas (CUR) 35 psi; ion source gases 1 and 2 (GS1 and GS2) 20 psi. The analytes were monitored in multiple reaction monitoring (MRM) mode under electrospray negative ionisation. Two transitions were selected for each native analyte, whereas one transition, along with Rt match, was used for the corresponding isotopically labelled IS. The first transition (in bold, Table 1) was used as the quantification ion and the second one for the identity confirmation. The retention times, monitored transitions, and respective MS analyser settings are summarised in Table 1. The identity of the PH metabolites was further confirmed by matching retention times with their respective internal standards, except for OH-MINCH, where IS-oxo-MINCH was employed. For analytes such as OH-MINP, oxo-MINP, and cx-MINP, which encompass various isomeric forms, the entire cluster of signals was integrated for their quantitative evaluation.

2.4.1. Analytical method validation

The analytical method was validated based on the guidelines established by the European Medicines Agency (European Medicines Agency, 2022) and the U.S. Food and Drug Administration (FDA, 2018). Each analyte underwent rigorous evaluation for sensitivity, method linearity, within-run accuracy and precision, instrumental repeatability, carryover, recovery, and matrix effect. The validation protocol involved analysing five replicates of synthetic urine and three replicates of goat milk for each parameter. The suitability of these substitute matrices was assessed and verified using real samples.

The method's sensitivity was assessed by determining the lower limit of quantification (LLOQ), defined as the lowest analyte concentration with a signal-to-noise (S/N) of at least 10, at an accuracy error and a coefficient of variation (% CV), both within ± 20 %.

Linearity was evaluated based on the determination coefficient (R^2) of the matrix-matched calibration curve within the defined concentration range (1/x weighted). Calibration curves were prepared by fortifying synthetic urine in triplicate at concentrations ranging from the LLOQ to 100 μg/L, and goat milk at concentrations ranging from the LLOQ to 60 μg/L. The acceptance criterion was for the calibration standards to fall within ± 15 % of their nominal values, and ± 20 % in the case of LLOQ levels. The use of surrogate matrices for calibration curve preparation was necessitated by the lack of blank samples and validated through participation in multiple German External Quality Assessment Scheme (G-EQUAS) and the analysis of SRM.

Within-run accuracy and precision were assessed by analysing synthetic urine spiked at LLOQ, 5 μg/L, and 50 μg/L, as well as goat milk spiked at LLOQ and 19 μg/L. Accuracy was considered satisfactory if the mean concentrations were within ± 15 % of the nominal value (± 20 % at LLOQ). Precision was deemed acceptable if the coefficient of variation (% CV) did not exceed 15 % or 20 % at LLOQ. Instrumental repeatability was evaluated as the % CV of five consecutive injections of the same sample at two concentration levels.

Carryover was examined by analysing solvent blanks following the acquisition of the highest calibration standard. The analyte peak area in the solvent blanks should not surpass 20 % and 5 % of the LLOQ for native and labelled reference standards.

For the method recovery and matrix effect (ME), replicates at two concentration levels (5 μg/L and 50 μg/L) for urine and at two concentration levels (0.19 μg/L and 19 μg/L) for milk were analysed. For each level, we compared synthetic urine and goat milk spiked before and after sample preparation, whereas IS were added before the analysis to cover for instrumental variability. Recovery was calculated by comparison of the mean peak area ratios of the analyte and the IS in samples spiked before and after sample preparation. The matrix effect was evaluated by comparing the mean peak area ratios of the analyte to the IS in matrix-matched samples spiked after extraction with those in the reconstitution solvent at specified concentration levels. Procedural blanks and zero calibration were prepared in triplicate and were used to assess potential background contamination. The procedural blank for the calibration curve consisted of synthetic urine and goat milk spiked with isotopically labelled internal standards, while LC-MS purity grade water spiked with labelled internal standards was used to correct levels in real samples.

2.5. Quality assurance and control

QC samples were injected every ten samples through analytical batch to continuously monitor instrumental performance, injection repeatability and signal stability. Long-term method performance was assessed only for urine by evaluating between-run precision, accuracy, and measurement uncertainty (MU). This was accomplished by recording quantification results for QCs at two different concentration levels (5 μg/L and 50 μg/L), both in duplicates. We established a control chart for each analyte (SM, Chapter 2) and calculated the limits based on the first 6 measurements of QC samples. The central line represents the average of all measurements as well as the upper (UWL) and lower warning (LWL) and action (upper: UAL, lower: LAL) limits. These were established using Eqs. 1 and 2, respectively:

$$WL = AVER \pm (2 \times STDEV) \quad (1)$$

$$WL = AVER \pm (3 \times STDEV) \quad (2)$$

According to common practice in quality control, if a QC exceeds two consecutive warning limits (WL), or a single point exceeds an action limit (AL), the batch would be rejected and flagged for investigation. Additionally, if a positive or negative trend of 7 consecutive points in one direction is observed, this would also trigger a review. In these cases, the batch would not be accepted until the reason for the deviation has been identified and corrected. The MU was calculated based on the control charts of the analytes and individual error contributions throughout the workflow. A detailed description of the MU calculation is provided in SM, Chapter 3.

To ensure the high quality of the analytical process and results, we constantly utilised SRM for urine and periodically participated in the interlaboratory comparison investigation programme G-EQUAS. Unfortunately, no similar external validation schemes are available for maternal milk. 8 PH metabolites (MEP, MnBP, MiBP, MBzP, MEHP, oxo-MEHP, OH-MEHP, and cx-MEPP were determined in Standard Reference Material® 3672 (SRM3672) and compared with the certified levels to ensure the accuracy and reliability of our results. The SRM has been regularly used to ensure the validity of our results. By participating in a nonoccupational G-EQUAS study, we determined levels of 13 PH and DINCH metabolites (MEP, MnBP, MiBP, MBzP, MEHP, oxo-MEHP, OH-MEHP, cx-MEPP, OH-MINP, cx-MINP, oxo-MINCH, OH-MINCH, and cx-MINCH) in urine and compared them to the reference values for the above-stated analytes.

2.6. Method application in biomonitoring studies

2.6.1. Study population description

Thirty women were randomly selected among 448 female participants of the first national HBM study in Slovenia to verify the applicability of the developed methods in HBM. Urine and maternal milk samples (among other sample types) were collected by the regional health care centres, mainly 6 – 8 weeks after delivery. Details on the study population are published elsewhere (Snoj Tratnik et al., 2019). The aim of the original study design was to investigate exposure to trace elements across twelve geographical areas in Slovenia. The study was approved by the Republic of Slovenia National Medical Ethics Committee, with numbers of accordance 42/12/07 and 53/07/09. Additional ethical approval was obtained for the use of biobanked samples (number of accordance 0120–431/2018/4). Informed written consent was obtained from all participants.

2.6.2. Sampling procedures

Spot urine samples were obtained approximately 6–8 weeks after delivery. Urine samples were stored and transported under cooled conditions (2 – 8 °C) for a maximum of six hours prior to aliquoting and storage at −80 °C. The participants collected the maternal milk samples themselves and stored them at 2 – 8 °C for a maximum period of six days. To decrease the influence of individual differences in excretion and milk composition, the samples were pooled at the end of the collection period, yielding one pooled sample per participant. Due to the aim of the original study design, the samples were not acidified after collection. The samples were stored at −80°C at our laboratory at the Jozef Stefan Institute.

2.7. Normalisation and statistical analyses

To account for urine sample dilution, the concentrations of PHs were normalised using specific gravity (SG) adjustment. SG was determined using a PAL-10S refractometer (Atago®, Japan), with a measurement range of 1.000–1.060. SG-corrected concentrations were calculated by the formula:

$$C_{adjusted}\ (\mu g/L) = C_{measured} \times (SG_s - 1) / (SG_i - 1)$$

where $C_{adjusted}$ is the adjusted concentration, $C_{measured}$ is the measured concentration, SG_s is standard specific gravity, which was calculated as average specific gravity ($SG_s = 1.013$), and SG_i is the measured specific gravity of an individual sample.

The statistical analyses and graphical illustrations were carried out in RStudio (Version 4.3.3). All graphs were created with ggplot2 (Wickham, 2016). The descriptive statistics were calculated in Excel.

3. Results and discussion

3.1. Optimisation of urine sample preparation

3.1.1. Sample pre-treatment

The predominant excretion of PH and DINCH metabolites as glucuronides has been repeatedly reported in the literature (Koch et al., 2004). Thus, the optimal performance of the deconjugation is crucial for the reliable determination of PH and DINCH metabolites in urine. It is widely known that β-glucuronidase from *Escherichia coli* is preferred over the β-glucuronidase/sulfatase mixture derived from *Helix pomatia* due to increased background effects, PHs contamination, and non-specific activity of the latter that leads to analytical challenges and artificially increased background levels (Blount et al., 2000; Fareed et al., 2022; Koch et al., 2003). The enzymatic deconjugation process for the urine matrix was optimised by varying the enzyme volume (10 μL and 100 μL) and deconjugation time (1.5 h and 16 h). To assess the effectiveness of different deconjugation procedures, we compared them using pooled urine samples and SRM 3672, the latter contains certified concentrations of 8 phthalate glucuronides (NIST, 2022). In pooled urine samples, we did not observe a statistically significant difference in the concentrations of metabolites between adding 10 μL or 100 μL of β-glucuronidase. Moreover, as illustrated in Fig. 1, no significant differences were found in SRM metabolite concentrations between deconjugation times of 1.5 h and 16 h. The concentrations obtained were well aligned with the mass fraction values provided in the SRM certificate (NIST, 2022). Therefore, the final method for deconjugating PH metabolites involves adding 150 μL of 3 M ammonium acetate buffer (pH 6.5), 10 μL of β-glucuronidase, and 300 μL of HPLC water to 0.5 mL of urine sample, followed by a 1.5-hour incubation at 37 °C on a thermo-shaker.

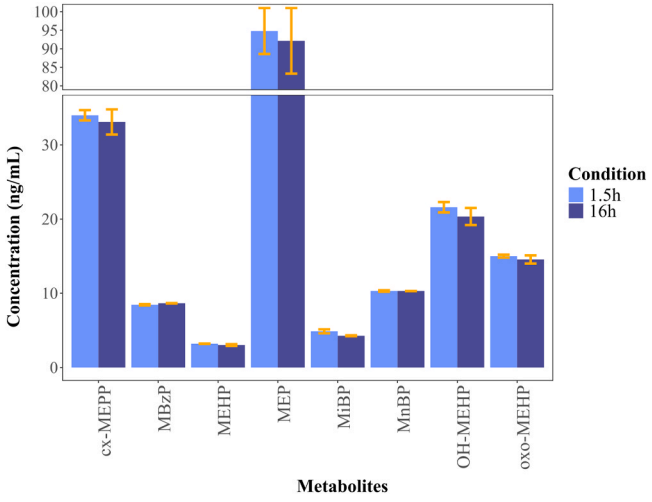


Fig. 1. Metabolite concentration in NIST certified Standard Reference Material (SRM) after 1.5 h and 16 h of deconjugation time.

3.1.2. Solid-phase extraction

The solid-phase extraction with a 60 mg Oasis-HLB sorbent was optimised with regard to the pre-washing and washing steps to achieve the highest analyte recovery while minimising interferences. The washing procedure was optimised in a systematic stepwise approach. 10 %, 25 %, and 40 % of MeOH in acidified (0.1 % FA) and non-acidified HPLC grade water were evaluated without and after a pre-wash step with non-acidified and acidified (0.1 M and 0.2 M) 1.5 mL of water. The analytes responded well to the acidified solution as it assures the analytes remain in a non-dissociated state, enhancing their binding to the sorbent prior to elution with MeOH. However, 0.2 M FA significantly worsened the matrix effect and negatively affected the chromatographic peak shape and signal integration; hence, the final concentration of 0.1 M FA was selected for sample pre-wash. The optimal water/MeOH ratio for the washing step was 9:1, with the addition of 0.1 % FA. A higher MeOH content resulted in analyte loss and lower recoveries, while using only a pre-wash solution was inefficient in reducing the background effect and led to higher detection limits. The addition of 0.1 % FA improved recoveries and reduced matrix effects of the analytes compared to the non-acidified mixture. Optimal results were obtained for a combination of 1.5 mL 0.1 M HPLC-grade water followed by 1.5 mL of a 9:1 water/MeOH mixture, acidified with 0.1 % FA.

3.2. Optimisation of milk sample preparation

3.2.1. Sample pre-treatment

Unlike urine, maternal milk predominantly contains metabolites in a non-conjugated form, a characteristic attributed to differences in metabolic processes and matrix composition. Phase II metabolic (conjugation) reactions primarily occur in the liver, where they detoxify compounds and facilitate their urinary excretion. Maternal milk, however, has a higher lipid content, which promotes the distribution of lipophilic compounds and reduces the likelihood of conjugated metabolites (Fromme, 2011). To verify the absence of conjugated monoesters in maternal milk, we compared deconjugated and non-deconjugated samples. Maternal milk was treated with 75 μL of β-glucuronidase and 2 mL of 3 M ammonium acetate buffer (pH 6.5), while the second replicate was left untreated, receiving only 2 mL of the buffer. Both samples were incubated for 16 h at 37 °C. No significant differences in PH and DINCH metabolite levels were observed between the deconjugated and non-deconjugated samples. Consequently, the deconjugation step can be omitted from the sample preparation process.

Subsequent experiments were conducted to evaluate potential contamination with phthalate diesters during the sample preparation. Since the maternal milk samples were not acidified immediately after collection, a subset of five randomly selected samples was pooled, divided, and analysed with and without the addition of 0.2 mL of 1 M

phosphoric acid prior to extraction. Consistent with findings by (Mortensen et al., 2005), no difference in metabolite concentrations was observed between acidified and non-acidified samples. This suggests that contamination with phthalate diesters did not influence the measured metabolite levels.

3.2.2. Solid-phase and QuEChERS extraction

The suitability of SPE for maternal milk samples was assessed for 1 mL of undiluted sample as well as for a 1:1 (v/v) dilution with 1 mL of 0.1 M FA. Oasis HLB cartridges (3 cc, 60 mg) were used in this experiment. Our SPE protocol from the urine method proved to be unsuitable for the preparation of maternal milk samples due to the high-fat content of the matrix. Dilution of the samples did not facilitate the passing of the matrix through the SPE sorbent either. Therefore, we evaluated the QuEChERS approach with a slight modification presented by An et al. (2020). The method, while thoroughly optimised by An et al. (2020), might not be easily applicable in large-scale HBM studies, due to temporal requirements, especially about the described total of 20 min of manual vortex mixing of each sample. Thus, we limited the shaking time to one minute during both mixing steps and verified the suitability of this approach by closely monitoring the achieved recoveries. Furthermore, to lower the detection limit, we reduced the reconstitution volume to 0.75 mL.

3.3. Instrumental analysis

A phenyl-hexyl stationary phase of the chromatographic column was chosen to overcome the challenge of analysing multiple analytes with different polarities and consequently different affinities for the stationary phase by utilising the π-π interactions between the phenyl group and the analyte (Croes et al., 2005; Marchand et al., 2005). The hydrophobic hexyl group has a strengthening effect on this interaction, further increasing analyte retention. Additionally, π-π interactions are intensified by using MeOH in the mobile phase, whereas studies report that ACN-containing mobile phases can decrease the retention of aromatic analytes in phenyl columns by up to 50 % compared to MeOH (Croes et al., 2005). The suitability of phenyl-hexyl columns for PH metabolite analysis has been demonstrated (Koch et al., 2003) and our results confirm its suitability for these compounds, even in the presence of isomers (MiBP and MnBP) that can be challenging to chromatographically separate (Figure S-5) (Alves et al., 2016; Frederiksen et al., 2010). Representative extracted ion chromatograms obtained using method 1 are shown in Figure S-6.

While all phthalate and DINCH monoesters contain a carboxyl group, some metabolites (cx-MEPP, cx-MINP and cx-MINCH) possess an additional carboxyl functional group, which change polarity and alters chromatographic behaviour. This additional carboxyl group enhances the hydrogen bonding with either the stationary phase or the solvent molecules, dipole-dipole interactions, and pH-dependent electrostatic effects. Additionally, its electron-withdrawing nature reduces the electron density of the aromatic ring, thereby weakening π–π interactions and reducing retention. Through careful adjustment of mobile phase composition and pH, these effects can be managed. Decreasing the pH of the mobile phase prevents the deprotonation of the carboxyl group, which increases π-π interactions and reduces electrostatic interactions. In its non-dissociated state, the carboxyl group can form strong hydrogen bonds with the mobile phase if it acts as a hydrogen donor (FA and MeOH), which can lead to insufficient retention and compromised peak shape.

Therefore, cx-MEPP, cx-MINP and cx-MINCH were analysed using a separate chromatographic method employing ACN as the organic solvent and ammonium fluoride as additive in the aqueous phase. As a polar aprotic solvent, ACN exhibits weak hydrogen-bonding capacity, reducing solvation of carboxyl groups and improving retention. The addition of ammonium fluoride further enhances ionisation efficiency in negative electrospray ionisation mode, resulting in increased sensitivity (McFadden and Ames, 2023). Extracted ion chromatograms obtained under these conditions are provided in Figure S-7.

This chromatographic approach helped to achieve LLOQs that are sufficient for the monitoring of common PHs and DINCH, but might pose challenges for less common xenobiotics.

3.3.1. Analytical method validation, quality assurance and control

The validation results for method sensitivity, accuracy, method and instrumental repeatability, recovery, matrix effect, and corrected matrix effect for urine samples are presented in Table 2, and for milk samples in Table 3. Additionally, combined and expanded measurement uncertainties for urine samples are reported in Table 2.

3.3.1.1. Lower limit of quantification for urine and maternal milk samples. The LLOQ in urine was in the range of 0.05 – 0.25 μg/L (see Table 2), except for MCHP (0.50 μg/L). These levels are sufficiently low to determine exposure to DINCH and common PHs in non-occupational HBM (Koch et al., 2017; Vogel et al., 2023). For milk, LLOQ levels range from 0.09 μg/L to 0.19 μg/L (Table 3), except for MCHP. Its signal was found to be suppressed in milk, and the compound was therefore excluded from the validation.

3.3.1.2. Calibration curve linearity. The calibration linearity results demonstrated an acceptable correlation between the measured PH metabolite concentrations in synthetic urine and their corresponding reference standard, with R^2 values ranging from 0.9969 to 0.9996 for all metabolites. Similarly, in goat milk, the R^2 was between 0.9950 and 0.9999.

3.3.1.3. Procedural blanks and investigation of carry-over. We investigated procedural blanks for possible contamination with monoester metabolites, finding traces of MEHP, MiBP, MnBP, oxo-MINCH, OH-MINCH, and cx-MINCH, but their peak area did not exceed 40 % of the mean LLOQ peak area. Since these background levels were stable and they were always accompanying the preparation of batches, together with other QCs, we were able to correct the samples for their levels. Injection of a solvent blank immediately after analysis of the highest calibration standard did not highlight any carry-over.

3.3.1.4. Measurement accuracy. Accuracy errors for both milk and urine were within 16 % of the nominal concentration for each metabolite, while the acceptance criteria were set to < 15 %, or < 20 % at LLOQ levels, which deems the method accuracy suitable. Accuracy at two concentration levels was further confirmed at the G-EQUAS interlaboratory scheme, with the analytes being between 69 % and 117 % relative to the reference values (Table S-1, Table S-2, Table S-3). All the measurements were within the respective tolerance range for each analyte. Additionally, a comparison with NIST SRM3672 (NIST, 2022) revealed measurement accuracies ranging from 76 % to 99 % of the reference concentrations (Table S-4). Specifically, the accuracies for oxo-MEHP and cx-MINCH were 99 %, while MnBP and cx-MINCH demonstrated 95 % accuracy. The accuracies for MEP and OH-MEHP were 98 % and 85 %, respectively, whereas MiBP and MEHP had accuracies below 80 % (78 % and 76 %).

By combining three independent approaches to evaluate the method's accuracy, we enhanced the reliability of our results and confirmed its suitability for application in HBM. Considering the critical role of maternal milk as an exposure source for infants, we highlight the need for interlaboratory comparison schemes and the development of SRMs to support its integration into monitoring studies.

3.3.1.5. Repeatability, recoveries, and matrix effect (ME). The method proved to be repeatable, with instrumental repeatability at CV lower than 9 % and method repeatability within 12 %. As shown in Table 2, metabolites in urine showed recoveries ranging from 71 % to 91 %, most

Table 2
Validation results for urine samples: LLOQ, accuracy error, method and instrumental repeatability, recovery, matrix effect, corrected matrix effect, and measurement uncertainty.

Metabolite	LLOQ (µg/L)	Accuracy error (%)			Method repeatability (CV %)			Instr. repeatability (CV %)		Recovery (%)		Matrix effect (%)		Corr. matrix effect (%)		u_c (%)		U (%)	
		LLOQ	Mid	High	LLOQ	Mid	High	Mid	High	Mid	High	Mid	High	Mid	High	Mid	High	Mid	High
MEP	0.25	1.8	5.6	5.8	4.6	4.0	8.8	5.4	2.0	71	82	98	101	110	99	6.9	12	14	24
MiBP	0.10	4.8	5.3	4.0	9.4	9.5	5.8	1.0	3.5	91	78	102	100	99	99	9.7	10	19	21
MnBP	0.10	3.3	3.5	1.3	12	6.1	5.2	3.0	6.1	88	80	101	99	103	105	11	8.7	21	17
MCHP	0.50	3.9	5.2	4.6	9.1	4.1	4.0	1.8	4.7	82	82	96	90	100	94	15	13	30	26
MBzP	0.25	1.6	1.0	5.2	9.9	7.6	6.8	3.1	6.1	77	84	89	94	108	98	8.2	4.5	16	9.0
MEHP	0.25	11	4.4	2.2	3.7	7.0	5.9	4.7	3.7	75	79	85	90	99	101	13	11	26	23
OH-MEHP	0.10	16	15	10	4.2	2.1	3.5	4.9	2.5	81	80	105	101	107	103	11	7.3	22	15
oxo-MEHP	0.10	9.5	9.1	9.2	8.7	3.5	4.5	2.9	4.1	82	90	96	98	100	95	11	11	22	21
cx-MEPP	0.05	0.44	7.9	8.0	6.9	4.2	3.8	2.5	1.7	81	82	93	94	100	97	1.7	5.3	3.5	11
OH-MIDP	0.05	0.12	7.8	3.3	5.8	2.4	3.9	3.0	2.7	82	81	89	97	94	101	11	11	23	22
oxo-MIDP	0.05	5.2	8.9	1.3	6.3	6.0	4.9	3.7	4.0	84	83	87	106	91	104	12	9.9	24	20
OH-MINP	0.25	0.72	5.4	5.7	8.9	7.0	2.8	4.5	3.5	88	85	88	99	91	99	11	12	23	24
oxo-MINP	0.05	3.3	4.6	4.2	3.5	4.3	3.2	3.6	1.3	79	90	95	95	97	99	11	8.7	22	17
cx-MINP	0.05	5.6	11	9.2	8.1	2.4	1.1	2.8	0.84	84	80	85	92	102	102	9.9	6.2	20	12
OH-MINCH	0.25	6.4	10	5.8	8.6	4.1	4.0	2.9	2.4	80	80	94	99	99	99	11	8.1	22	16
oxo-MINCH	0.05	2.4	9.4	6.9	7.4	2.0	5.9	3.2	3.7	83	82	92	98	98	99	10	10	20	20
cx-MINCH	0.05	7.7	10	9.8	6.1	2.0	3.2	0.94	1.4	80	84	93	94	102	96	3.9	5.1	7.7	10

LLOQ: lower limit of quantification; Mid: 5 µg/L; High: 50 µg/L; CV: coefficient of variation; u_c: combined uncertainty; U: expanded uncertainty

exceeding 80 %. In contrast, recoveries from maternal milk after QuEChERS extraction were lower, between 48 % and 87 %, likely due to the matrix complexity and additional n-hexane extraction step. The ME was evaluated before and after IS correction. For urine (Table 2), uncorrected ME ranged from 85 % to 106 %, improving to 91–110 % after IS correction. For milk, the ME was more pronounced, particularly for MEHP, demonstrating 59 % at low and 22 % at high concentrations. However, after IS correction, ME in milk improved significantly, ranging from 77 % to 130 %, highlighting the effectiveness of IS adjustment in reducing matrix effects (see Table 3).

3.3.1.6. Measurement uncertainty and long-term method performance for urine analysis. To monitor the method's long-term performance, including possible trends, systematic error and between-run precision, we set up a control chart for each metabolite (SM, Chapter 2). Data retrieved from the control charts were subsequently used to determine the method's expanded MU. The MU encompasses bias and within-laboratory reproducibility (between-run precision) of QCs, as well as uncertainties arising from individual contributors such as pipettes, volumetric flasks, and standard preparation, alongside uncertainties introduced by different analysts, the impact of long-term analysis (e.g., day-to-day mass spectrometer response, different batches of reagents, materials, and solvents) (Magnusson et al., 2017). It can be determined by identifying and evaluating individual sources of uncertainty (GUM approach - Joint Committee for Guides in Metrology, 2008) or by calculating uncertainty from validation data (ISO, 2017; Magnusson et al., 2017). The expanded measurement uncertainty for two concentration levels of 17 metabolites in urine samples was calculated by following the Guide to the Expression of Uncertainty in Measurement (Joint Committee for Guides in Metrology, 2008). The MUs were mostly within ± 24 % of the reported value at a 95 % confidence interval (Table 2). The exceptions were MCHP and MEHP, where the MUs were within ± 30 %. Overall, MUs within the above-stated ranges are a reasonable outcome given the levels, matrix complexity, and complexity of sample preparation. Additionally, the control charts (see SM, Chapter 2) showed consistent performance without any notable trends or significant deviations.

3.4. Phthalate and DINCH metabolites in paired urine/milk samples

Thirty paired human urine and milk samples donated by lactating mothers 6 – 8 weeks postpartum were processed and analysed using the above-described method. The results are presented in Table 4 and compared with findings from other studies, as illustrated in Fig. 2.

As indicated by Table 4, 17 PH and DINCH metabolites could be quantified in urine and 11 in maternal milk. The majority of urinary metabolites could be detected in 80–100 % of samples, except for MCHP, which was detected in 10 % of samples. The detection rates in maternal milk were lower. While OH-MEHP, oxo-MEHP, cx-MEPP, cx-MINP, and cx-MINCH were not detected in milk samples, the detection rate for the remaining ones ranged from 7 % to 100 %. The concentrations of metabolites detectable in urine and human milk did not correlate significantly between matrices. The results are presented in Table S5.

For most metabolites in urine, the concentrations are comparable with those for adults reported by other studies, however, it is noteworthy that OH-MINCH is remarkably high compared to other reported values in the literature (Fig. 2), especially considering that the population was sampled between 2008 and 2014, and that the use of traditional phthalates experienced the largest decrease toward 2011 (Bui et al., 2016).

Given the absence of field blanks, contamination is a factor of uncertainty in our study. To approximate the level of contamination, we calculated the ratios between the urinary metabolites of DEHP and DINCH and compared them with the literature. The median ratios of OH-

Table 3
Validation results for milk samples: LLOQ, accuracy error, method and instrumental repeatability, recovery, matrix effect, corrected matrix effect.

Metabolite	LLOQ (µg/L)	Accuracy error (%)		Method repeatability (CV %)		Instrumental repeatability (CV %)		Recovery (%)		Matrix effect (%)		Corr. matrix effect (%)	
		LLOQ	19 µg/L	LLOQ	19 µg/L	0.19 µg/L	19 µg/L	0.19 µg/L	19 µg/L	0.19 µg/L	19 µg/L	0.19 µg/L	19 µg/L
MEP	0.19	3.4	6.7	4.7	2.5	6.2	8.2	49	49	126	76	130	100
MiBP	0.09	1.2	9.5	6.2	3.1	6.4	6.1	68	58	97	95	108	102
MnBP	0.19	5.1	5.3	1.6	5.4	6.9	6.9	77	52	119	95	122	104
MBzP	0.19	5.9	6.8	5.2	4.5	3.8	3.7	62	63	70	81	78	100
MEHP	0.09	4.3	9.5	11.2	7.4	1.0	5.4	84	55	59	22	128	101
OH-MEHP	0.19	15	1.5	3.8	3.4	2.4	2.8	54	57	90	89	97	95
oxo-MEHP	0.19	4.7	3.3	8.1	4.4	6.4	1.3	48	51	104	96	116	100
cx-MEPP	0.09	6.3	5.0	3.4	1.7	3.5	2.2	47	48	67	127	101	104
OH-MIDP	0.09	6.0	0.40	6.6	1.6	5.8	1.1	59	54	86	93	90	100
oxo-MIDP	0.09	14	3.6	1.7	9.1	7.5	8.6	62	52	80	89	95	105
OH-MINP	0.19	10.0	5.7	3.5	6.2	4.8	3.3	60	58	82	89	87	104
oxo-MINP	0.09	3.2	8.9	7.4	4.9	3.4	2.7	57	53	88	91	103	107
cx-MINP	0.09	4.0	12.0	6.8	4.0	2.0	1.2	53	48	66	110	90	99
OH-MINCH	0.09	3.2	4.3	7.4	3.5	5.7	2.0	87	52	82	85	88	103
oxo-MINCH	0.09	4.3	4.7	2.4	6.3	2.1	3.6	71	53	73	87	77	106
cx-MINCH	0.09	5.2	11.0	5.5	3.6	7.4	3.1	61	53	63	91	93	101

LLOQ: lower limit of quantification; CV: coefficient of variation

Table 4
Results from the biomonitoring study of urine and milk samples (n = 30). MCHP, OH-MEHP, oxo-MEHP, cx-MEPP, cx-MINP, and cx-MINCH were not detected in maternal milk samples.

Metabolite	Urine (µg/L)					Milk (µg/L)				
	QR (%)	AM	Median	Range	P90	QR (%)	AM	Median	Range	P90
MEP	100	32	14	4.9–316	80	83	3	0.84	<LLOQ-36	5.2
MiBP	100	28	15	2.3–281	52	100	18	16	1.0–45	34
MnBP	100	18	9.5	0.39–104	38	100	1.3	1.2	0.29–3.4	2.5
MCHP	10	0.28	< LLOQ	<LLOQ-0.95	0.28	/	/	/	/	/
MBzP	100	4.7	2.4	0.18–37	14	30	0.42	<LLOQ	<LLOQ-1.1	0.98
MEHP	97	3.0	1.3	<LLOQ-28	6	100	6.3	5.0	1.7–29	9.5
OH-MEHP	100	12	5.2	0.39–132	26	0	/	/	/	/
oxo-MEHP	97	7.7	3.5	<LLOQ-87	16	0	/	/	/	/
cx-MEPP	100	18	6.8	1.3–233	41	0	/	/	/	/
OH-MIDP	100	1.3	0.71	0.08–7.5	2.5	57	0.15	0.13	<LLOQ-0.44	0.29
oxo-MIDP	100	0.63	0.47	0.14–2.7	1.2	7	0.19	0.19	<LLOQ-0.24	0.23
OH-MINP	100	4.6	2.4	0.52–41	12	100	1.9	1.4	0.32–7.2	4.2
oxo-MINP	100	2.0	1.2	0.14–14	5.5	67	0.11	0.11	<LLOQ-0.39	0.16
cx-MINP	100	5.9	3.1	0.49–62	7.6	0	/	/	/	/
OH-MINCH	80	2.5	1.8	<LLOQ-8.7	8.5	67	0.14	0.15	<LLOQ-0.36	0.23
oxo-MINCH	87	0.92	0.67	<LLOQ-3.6	2.5	100	0.46	0.47	0.08–1.0	0.74
cx-MINCH	100	1.4	1.0	0.08–5.6	4.7	0	/	/	/	/

QR: Quantification rate; AM: arithmetic mean; P: percentile

MEHP, oxo-MEHP, and cx-MEPP to MEHP are 2.95, 1.98, and 3.94, and the ratios between OH-MEHP and oxo-MEHP and between cx-MEPP and OH-MEHP are both 1.48, indicating that DEHP is predominantly excreted as cx-MEPP, followed by OH-MEHP, oxo-MEHP, and MEHP. Those results are in agreement with the literature (Koch et al., 2003; Stajnko et al., 2022). In the absence of the primary metabolite, MINCH, the ratios for the DINCH metabolites were 3.09, 0.59, and 1.86 or OH-MINCH to oxo-MINCH, cx-MINCH to OH-MINCH, and cx-MINCH to oxo-MINCH, respectively. The difference in DINCH excretion patterns (MINCH < cx-MINCH = oxo-MINCH < OH-MINCH) has been observed by other studies as well (Koch et al., 2013). The levels of the LMW PHs MEP, MiBP, and MnBP are higher compared to other metabolites, which have been repeatedly reported in the literature and attributed to the use of personal care products. However, they are only roughly a third of those reported for Slovenian female DEMOCOPHES participants of similar age and sampled in 2011 (Runkel et al., 2020). It should be noted that, given the substantially smaller sample size (30 individuals), the metabolite levels observed in this study do not reflect the population-level data, as reported in the larger-scale DEMOCOPHES study (Runkel et al., 2020).

The metabolite concentrations determined in maternal milk samples are largely in the range of those reported in the literature (Fig. 2). However, two monoester metabolites, MEHP and MiBP, were unexpectedly high compared to the concentrations in paired urine samples (Fig. 3) with maternal milk/urine ratios of 3.9 and 1.1, respectively. This is noteworthy, as metabolite levels in milk are typically lower than in urine and the ratios between maternal milk and urine for MEHP and MiBP are generally range from 0.05 to 0.3 and from 0.03 to 0.07, respectively (Arbuckle et al., 2016; Högberg et al., 2008; Kim et al., 2018; Lin et al., 2011). The maternal milk/urine ratios can be further calculated for MEP and MnBP for four studies (Arbuckle et al., 2016; Högberg et al., 2008; Kim et al., 2018; Lin et al., 2011), and range from 0.0003 to 0.03 and from 0.01 to 0.07, respectively. In comparison, the observed ratios in our study are 0.06 for MEP and 0.1 for MnBP. Thus, we observe that our ratios exceed the calculated average ratios of the four studies (MEP 0.02, MiBP 0.05, MnBP 0.04, and MEHP 0.29) by magnitudes of 4, 20, 3, and 13. Due to the limited number of studies reporting metabolite concentrations in paired urine and maternal milk, this observation needs verification; however, it is likely the result of enzymatic activity in the samples that were not acidified after collection, not immediately frozen, and relocated between freezers of different temperatures.

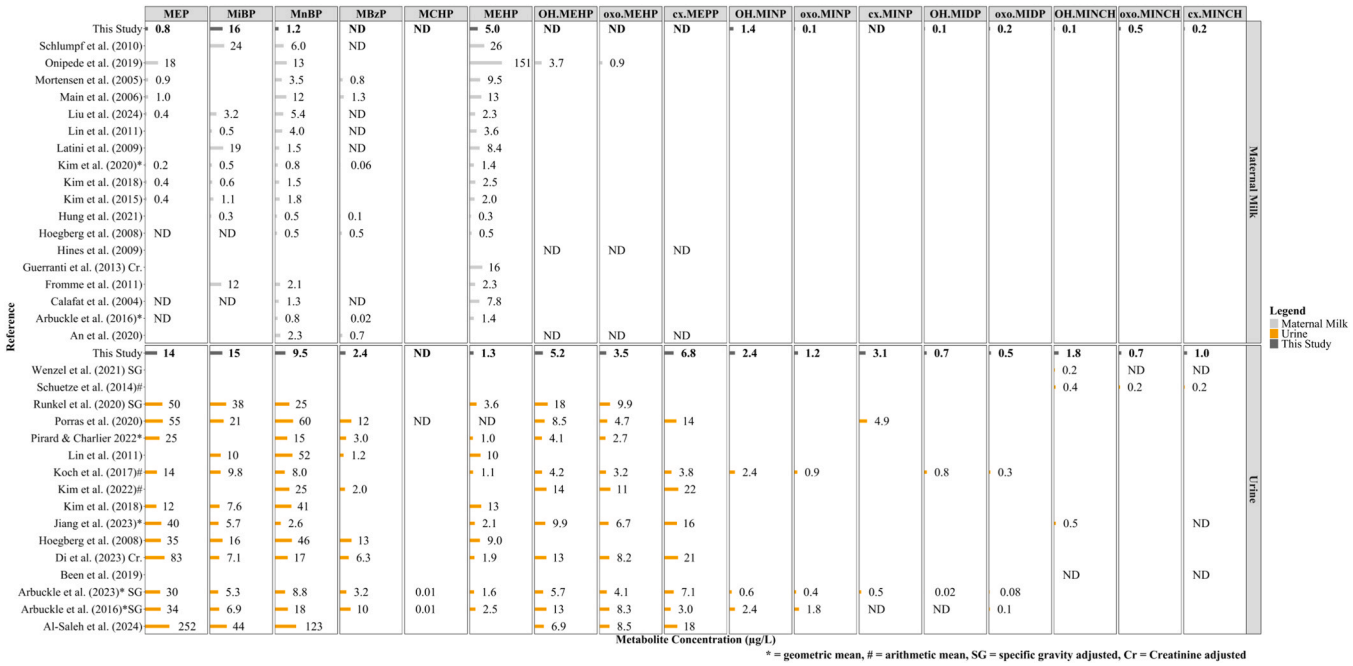


Fig. 2. Phthalate metabolite concentrations in urine and maternal milk of non-occupationally exposed adults reported in the literature. Presented are median concentrations, unless indicated otherwise (* = geometric mean, # = arithmetic mean). The reported concentrations are indicated as numbers behind the bars and are in µg/L unless the reference is labelled "SG" = specific gravity or "Cr." = Creatinine. ND = not detected. Blank fields = metabolite was not analysed.

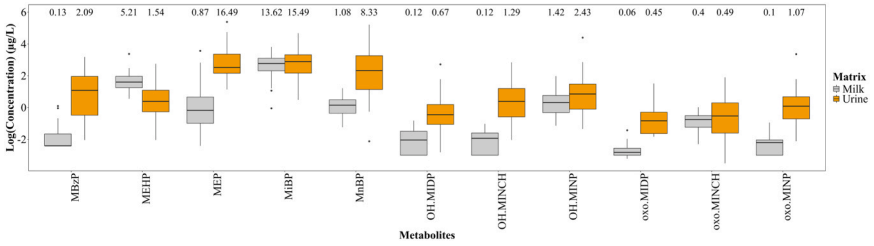


Fig. 3. Metabolite concentrations (µg/L) in urine and maternal milk from lactating primiparous women from Slovenia. Excluded were MCHP, cx-MINCH, cx-MINCH, OH-MEHP, cx-MEPP, and oxo-MEHP, which were not sufficiently quantified in maternal milk. The geometric means (µg/L) are indicated above the boxplots.

The presence of secondary metabolites in maternal milk is somewhat surprising, especially considering the low concentrations of some of them in urine (oxo-MIDP and oxo-MINCH). Secondary metabolites are formed largely in the liver and only to a small degree in the intestines, and are more hydrophilic than primary metabolites to facilitate urinary excretion. Thus, they are expected and confirmed by other studies to be present, if detectable at all, only at very low levels in maternal milk and serum (Calafat et al., 2004; Högberg et al., 2008). At low exposure levels, such as indicated by the urinary concentrations of oxo-MIDP and oxo-MINCH, their detection in maternal milk would generally be unlikely and not expected to reach as high as 70 % of the urinary concentration. While secondary metabolites of PH and DINCH can leach into the environment from bio-based fertilisers (Estoppey et al., 2024) and not sufficiently efficient wastewater treatment plants (Kusk et al., 2011), their environmental concentrations are generally negligible for contamination considerations. The metabolite levels observed in maternal milk likely reflect the transfer of PH and DINCH metabolites from the maternal bloodstream, with potential contributions from enzymatic activity occurring during sample storage, particularly in the case of MEHP. Few studies have thoroughly investigated the change in lipase and esterase activities in human milk in cooled conditions, however, Berkow et al. (1984) and Wardell et al. (1984) investigated the effects of cooling, freezing, and heating on enzyme activities in human maternal milk and reported that esterases and lipases remain fully active with hydrolysation occurring even at temperatures as low as – 20 °C. Considering the rapid transformation from DEHP to MEHP in the human body (approximately 2 h after oral exposure) (Li et al., 2022), it can be assumed that DEHP hydrolysis to MEHP continues in the milk at a fast rate and under cooled conditions. MEHP is the most toxic DEHP metabolite and is associated with increased oxidative stress (Liu et al., 2023; Wang et al., 2012), neurotoxicity (Liu et al., 2023), decreased antioxidant capacity (Wang et al., 2012), and endocrine disruption (Wang et al., 2012; Zhou et al., 2023), among others, and overall toxicity potency that exceeds that of DEHP by a factor of 10 (Zhou et al., 2023). The Tolerable Daily Intake value of 0.05 mg/kgbw/d for DEHP is not reached when assuming mean and high daily intakes of maternal milk of 150 and 217 mL /kgbw/d (concentration in milk (µg/L) x volume of milk intake (mL/kgbw/d; (Dualde et al., 2020; U.S. EPA, 2011)), however one sample exceeds the ten times lower threshold in the high-intake scenario (0.0064 mg/kgbw/d).

Although this study does not directly support exposure assessment, the results, however, confirm the applicability of the methods in HBM studies and illustrate the importance of study design, sample handling, and enzyme deactivation in PH and DINCH monitoring, and provide insights into the potential intake dose of phthalate metabolites from stored maternal milk. Thus, we would like to highlight the need to systematically evaluate the formation of phthalate metabolites during short-term storage in the fridge and, if necessary, to account for this

exposure pathway in future health risk assessments for children.

4. Identified research needs and knowledge gaps for the establishment of maternal milk as an alternative HBM matrix

Maternal milk is unique as a matrix in HBM insofar as it reflects maternal exposure status and excretion, as well as fetal exposure doses of different chemicals. Thus, maternal milk monitoring has been employed for decades, especially for persistent organic pollutants (Colles et al., 2008). However, the research area suffers from a lack of standardisation that hampers comparability across studies (LaKind et al., 2004). Specifically, studies would benefit from the availability of reference materials and external verification schemes, such as inter-laboratory comparisons. Additionally, a straightforward standard operating procedure for milk collection and handling prior to its arrival at a laboratory is essential to reduce uncertainties related to potential contamination during milk pumping. Furthermore, the focus of phthalate monitoring in maternal milk often lies on the diesters due to their lipophilicity and increased transfer into maternal milk compared to the metabolites (Fromme et al., 2011a, 2011b; Hanberg et al., 2005; Högberg et al., 2008; Zhu et al., 2006). Independent studies, however, suggest that primary monoester metabolites can form endogenously in the milk also under cooled conditions (Berkow et al., 1984; Wardell et al., 1984). Considering the increasing trend in Europe for both parents to share parental obligations and for mothers to return to work early, it is common for the mother to pump maternal milk and store it in the fridge for later use (Rasmussen et al., 2017). Therefore, 1) more research is needed on the endogenous formation of monoester metabolites under different storage conditions, 2) an attention shift from diester exposure to a mixed exposure to diesters and monoesters may be necessary, 3) intensified focus is needed on the toxicological profile of metabolites as exogenous chemicals, and 4) more attention has to be given to the study design in maternal milk monitoring, as previously highlighted by (Rasmussen et al., 2017).

5. Conclusions

In this manuscript, we present a fast, sensitive, robust, and streamlined analytical method for the determination of phthalate and DINCH metabolites in human urine and maternal milk. The method was meticulously validated for sensitivity, accuracy and precision, carry-over, recovery, matrix effect, and measurement uncertainty, demonstrating its suitability for human biomonitoring (HBM) applications, even in laboratories without the online sample preparation setup. Furthermore, the external validation using standard reference material and interlaboratory schemes significantly reinforces the reliability of results derived from urine samples. The application of the method to paired urine and maternal milk samples revealed valuable insights into metabolite distribution, emphasising the importance of stringent sample handling to avoid enzymatic degradation and contamination on one hand and the extent of endogenous metabolite formation during cooled storage on the other hand. While the absence of standard reference materials for maternal milk limits interlaboratory comparability, our findings highlight the critical need for standardisation in maternal milk sampling and analysis to support infant exposure assessments.

This validated method contributes meaningfully to advancing HBM studies by providing an effective tool for monitoring exposure to endocrine disruptors. Its adaptability for large-scale studies underscores its potential to inform regulatory policies and public health strategies aimed at reducing exposure to PHs and DINCH, especially among vulnerable populations.

CRediT authorship contribution statement

Runkel Agneta Annika: Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Žan Rekar:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Neja Kosirnik:** Investigation. **Tina Kosjek:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Milena Horvat:** Writing – review & editing, Supervision, Funding acquisition. **Janja Snoj Tratnik:** Project administration. **Darja Mazej:** Project administration.

Funding

The funding for the method development and biomonitoring study was obtained within the frame of national and international human biomonitoring studies, namely the European Union's Horizon 2020 research and innovation programme NEUROSOME (H2020-MSCA-ITN-2017 SEP-210411486 under Grant Agreement No. 766251, the Chemicals Office of the Republic of Slovenia (contract numbers C2715–07Y000042, C2715–11–634801, C2715–13–634801, C2715–13–634802, C2715–14–634801, C2715–11–000005, C2715–18–634803, C2715–19–634801), and by the Agency for Research and Innovation of Slovenia – ARIS (Program group P1–0143). Agneta A. Runkel has been funded by the European Union's Horizon 2020 research and innovation programme NEUROSOME (H2020-MSCA-ITN-2017 SEP-210411486) under Grant Agreement No. 766251. Žan Rekar is a recipient of the Young Researcher Grant of the Slovenian Research and Innovation Agency – ARIS. The work of Tina Kosjek, Milena Horvat, Janja Snoj Tratnik, and Darja Mazej has been supported by ARIS and by the Chemicals Office of the Republic of Slovenia. These fundings do not create a conflict of interests.

Declaration of Competing Interest

Employment and funding of authors

The authors Agneta A. Runkel, Žan Rekar, Darja Mazej, Milena Horvat, Janja Snoj Tratnik, and Tina Kosjek are employed at the Jožef Stefan Institute, Slovenia. Agneta A. Runkel is additionally employed at Lund University, Sweden. At the time of the laboratory work, Neja Kosirnik was a student enrolled at the Faculty of Pharmacy of the University of Ljubljana. The authors declare no conflict of interest regarding the authors' employments and affiliations.

Agneta A. Runkel has been funded by the European Union's Horizon 2020 research and innovation programme NEUROSOME (H2020-MSCA-ITN-2017 SEP-210411486) under Grant Agreement No. 766251. Žan Rekar is a recipient of the Young Researcher Grant of the Slovenian Research and Innovation Agency – ARIS. The work of Tina Kosjek, Milena Horvat, Janja Snoj Tratnik, and Darja Mazej has been supported by ARIS and by the Chemicals Office of the Republic of Slovenia. These fundings do not create a conflict of interests.

Acknowledgments

The authors would like to thank the participants of the national human biomonitoring campaign for their involvement.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2026.119698.

Data availability

Individual HBM results cannot be shared to protect privacy. Data supporting method development are in the paper and Supplementary Information.

References

Alves, A., Covaci, A., Voorspoels, S., 2016. Are nails a valuable non-invasive alternative for estimating human exposure to phthalate esters? Environ. Res 151, 184–194. https://doi.org/10.1016/j.envres.2016.07.023.

An, J., Kim, Y.Y., Cho, H.D., Kim, J., Lee, J.Y., Lee, Y., Jo, E., Lee, J., Cha, S., Han, S.B., 2020. Development and investigation of a QuEChERS-based method for determination of phthalate metabolites in human milk. J. Pharm. Biomed. Anal. 181. https://doi.org/10.1016/j.jpba.2019.113092.

Arbuckle, T.E., Fisher, M., MacPherson, S., Lang, C., Provencher, G., LeBlanc, A., Hauser, R., Feeley, M., Ayotte, P., Neisa, A., Ramsay, T., Tawagi, G., 2016. Maternal and early life exposure to phthalates: the plastics and personal-care products use in pregnancy (P4) study. Sci. Total Environ. 551–552, 344–356. https://doi.org/10.1016/j.scitotenv.2016.02.022.

Berkow, S.E., Freed, L.M., Hamosh, M., Bitman, J., Larry Wood, D., Happ, B., Hamosh, P., 1984. Lipases and lipids in human milk: effect of freeze-thawing and storage. Pedia Res 18, 1257–1262. https://doi.org/10.1203/00006450-198412000-00006.

Blount, B.C., Silva, M.J., Caudill, S.P., Needham, L.L., Pirkle, J.L., Sampson, E.J., Lucier, G.W., Jackson, R.J., Brock, J.W., 2000. Levels of seven urinary phthalate metabolites in a human reference population. Environ. Health Perspect. 108, 979–982. https://doi.org/10.1289/ehp.00108979.

Brucker-Davis, F., Wagner-Mahler, K., Delattre, I., Ducot, B., Ferrari, P., Bongain, A., Kurzenne, J.-Y., Mas, J.-C., Fenichel, P., 2008. Cryptorchidism at birth in Nice area (France) is associated with higher prenatal exposure to PCBs and DDE, as assessed by colostrum concentrations. Hum. Reprod. 23, 1708–1718. https://doi.org/10.1093/humrep/den186.

Bui, T.T., Giovanoulis, G., Cousins, A.P., Magnér, J., Cousins, I.T., de Wit, C.A., 2016. Human exposure, hazard and risk of alternative plasticizers to phthalate esters. Sci. Total Environ. https://doi.org/10.1016/j.scitotenv.2015.09.036.

Calafat, A.M., Slakman, A.R., Silva, M.J., Herbert, A.R., Needham, L.L., 2004. Automated solid phase extraction and quantitative analysis of human milk for 13 phthalate metabolites. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 805, 49–56. https://doi.org/10.1016/j.jchromb.2004.02.006.

CDC, T.C. for D.C. and P., 2013. Laboratory Procedure Manual. Method No. 6301.02: On line SPE-HPLC-Isotope dilution-MS/MS.

Choi, K., Joo, H., Campbell, J.L., Clewell, R.A., Andersen, M.E., Clewell, H.J., 2012. In vitro metabolism of di(2-ethylhexyl) phthalate (DEHP) by various tissues and cytochrome P450s of human and rat. Toxicol. Vitr. 26, 315–322. https://doi.org/10.1016/j.tiv.2011.12.002.

Colles, A., Koppen, G., Hanot, V., Nelen, V., Dewolf, M.-C., Noël, E., Malisch, R., Kotz, A., Kypke, K., Biot, P., Vinkx, C., Schoeters, G., 2008. Fourth WHO-coordinated survey of human milk for persistent organic pollutants (POPs): Belgian results. Chemosphere 73, 907–914. https://doi.org/10.1016/j.chemosphere.2008.07.002.

Croes, K., Steffens, A., Marchand, D.H., Snyder, L.R., 2005. Relevance of π–π and dipole–dipole interactions for retention on cyano and phenyl columns in reversed-phase liquid chromatography. J. Chromatogr. A 1098, 123–130. https://doi.org/10.1016/j.chroma.2005.08.090.

David, R.M., White, R.D., Larson, M.J., Herman, J.K., Otter, R., 2015. Toxicity of Hexamoll® DINCH® following intravenous administration. Toxicol. Lett. 238, 100–109. https://doi.org/10.1016/j.toxlet.2015.07.013.

Dualde, P., Pardo, O., Corpas-Burgos, F., Kuligowski, J., Gormaz, M., Vento, M., Pastor, A., Yusà, V., 2020. Biomonitoring of parabens in human milk and estimated daily intake for breastfed infants. Chemosphere 240, 124829. https://doi.org/10.1016/j.chemosphere.2019.124829.

ECHA, E.C.A., 2025. Phthalates [WWW Document]. ⟨https://echa.europa.eu/hot-topics/phthalates⟩. URL https://echa.europa.eu/hot-topics/phthalates (accessed 12.22.25).

Estoppey, N., Castro, G., Slinde, G.A., Hansen, C.B., Løseth, M.E., Krahn, K.M., Demmer, V., Svenni, J., Tran, T.-V.-A.T., Asimakopoulos, A.G., Arp, H.P.H., Cornelissen, G., 2024. Exposure assessment of plastics, phthalate plasticizers and their transformation products in diverse bio-based fertilizers. Sci. Total Environ. 918, 170501. https://doi.org/10.1016/j.scitotenv.2024.170501.

European Medicines Agency, 2022. ICH guideline M10 on bioanalytical method validation and study sample analysis - EMA/CHMP/ICH/172948/2019. Amsterdam.

Fareed, Y., Braun, D., Flasch, M., Globisch, D., Warth, B., 2022. A broad, exposome-type evaluation of xenobiotic phase II biotransformation in human biofluids by LC-MS/MS. Exposome 2. https://doi.org/10.1093/exposome/osac008.

FDA, F. and D.A., 2018. Bioanalytical Method Validation: Guidance for Industry.

Fierens, T., Servaes, K., Van Holderbeke, M., Geerts, L., De Henauw, S., Sioen, I., Vanermen, G., 2012. Analysis of phthalates in food products and packaging materials sold on the Belgian market. Food Chem. Toxicol. 50, 2575–2583. https://doi.org/10.1016/j.fct.2012.04.029.

Frederiksen, H., Jorgensen, N., Andersson, A.-M., 2010. 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, 400–410. https://doi.org/10.1093/jat/34.7.400.

Fromme, H., 2011. Phthalates: Human Exposure. Encyclopedia of Environmental Health. Elsevier, pp. 498–510. https://doi.org/10.1016/B978-0-444-52272-6.00607-3.

Fromme, H., Gruber, L., Seckin, E., Raab, U., Zimmermann, S., Kiranoglu, M., Schlummer, M., Schwegler, U., Smolic, S., Völkel, W., 2011a. Phthalates and their metabolites in breast milk - results from the bavarian monitoring of breast milk (BAMBI). Environ. Int 37, 715–722. https://doi.org/10.1016/j.envint.2011.02.008.

Fromme, H., Raab, U., Fürst, P., Vieth, B., Völkel, W., Albrecht, M., Schwegler, U., 2011b. Vorkommen und gesundheitliche Bedeutung von persistenten organischen Substanzen und Phthalaten in der Muttermilch. Das. Gesundh. 73, e27–e43. https://doi.org/10.1055/s-0030-1268452.

Gao, C.J., Kannan, K., 2020. Phthalates, bisphenols, parabens, and triclocarban in feminine hygiene products from the United States and their implications for human exposure. Environ. Int 136. https://doi.org/10.1016/j.envint.2020.105465.

Gkrillas, A., Dirven, H., Papadopoulou, E., Andreassen, M., Husøy, T., 2021. Exposure estimates of phthalates and DINCH from foods and personal care products in comparison with biomonitoring data in 24-hour urine from the Norwegian EuroMix biomonitoring study. Environ. Int. 155. https://doi.org/10.1016/j.envint.2021.106598.

Hanberg, A., Högberg, J., Berglund, M., Bensryd, I., Skerfving, S., Remberger, M., Calafat, A., Appelgren, M., Falk Filipsson, A., Jansson, B., Håkansson, H., 2005. Phthalates and their metabolites in human breast milk, blood and urine as measures for monitoring exposure in human risk groups.

Högberg, J., Hanberg, A., Berglund, M., Skerfving, S., Remberger, M., Calafat, A.M., Filipsson, A.F., Jansson, B., Johansson, N., Appelgren, M., Håkansson, H., 2008. Phthalate diesters and their metabolites in human breast milk, blood or serum, and urine as biomarkers of exposure in vulnerable populations. Environ. Health Perspect. 116, 334–339. https://doi.org/10.1289/ehp.10788.

ISO, I.O. for S., 2017. Guidance for the use of repeatability, reproducibility and trueness estimates in measurement uncertainty evaluation. ISO 21748:2017.

Joint Committee for Guides in Metrology, 2008. Evaluation of measurement data — Guide to the expression of uncertainty in measurement. https://doi.org/10.59161/JCGM100-2008E.

Kim, Sunmi, Eom, S., Kim, H.-J., Lee, J.J., Choi, G., Choi, S., Kim, Sungjoo, Kim, S.Y., Cho, G., Kim, Y.D., Suh, E., Kim, S.K., Kim, Seunghyo, Kim, G.-H., Moon, H.-B., Park, J., Kim, Sungkyoon, Choi, K., Eun, S.-H., 2018. Association between maternal exposure to major phthalates, heavy metals, and persistent organic pollutants, and the neurodevelopmental performances of their children at 1–2 years of age- CHECK cohort study. Sci. Total Environ. 624, 377–384. https://doi.org/10.1016/j.scitotenv.2017.12.058.

Koch, H.M., Bolt, H.M., Angerer, J., 2004. Di(2-ethylhexyl)phthalate (DEHP) metabolites in human urine and serum after a single oral dose of deuterium-labelled DEHP. Arch. Toxicol. 78, 123–130. https://doi.org/10.1007/s00204-003-0522-3.

Koch, H.M., Gonzalez-Reche, L.M., Angerer, J., 2003. 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, 169–182. https://doi.org/10.1016/S1570-0232(02)00785-7.

Koch, H.M., Rüther, M., Schütze, A., Conrad, A., Pälmke, C., Apel, P., Brüning, T., Kolossa-Gehring, M., 2017. Phthalate metabolites in 24-h urine samples of the German Environmental Specimen Bank (ESB) from 1988 to 2015 and a comparison with US NHANES data from 1999 to 2012. Int J. Hyg. Environ. Health 220, 130–141. https://doi.org/10.1016/j.ijheh.2016.11.003.

Koch, H.M., Schütze, A., Pälmke, C., Angerer, J., Brüning, T., 2013. Metabolism of the plasticizer and phthalate substitute diisononyl-cyclohexane-1,2-dicarboxylate (DINCH®) in humans after single oral doses. Arch. Toxicol. 87, 799–806. https://doi.org/10.1007/s00204-012-0990-4.

Kusk, K.O., Krüger, T., Long, M., Taxvig, C., Lykkesfeldt, A.E., Frederiksen, H., Andersson, A., Andersen, H.R., Hansen, K.M.S., Nellemann, C., Bonefeld-Jørgensen, E.C., 2011. Endocrine potency of wastewater: contents of endocrine disrupting chemicals and effects measured by in vivo and in vitro assays. Environ. Toxicol. Chem. 30, 413–426. https://doi.org/10.1002/etc.385.

LaKind, J.S., Amina Wilkins, A., Berlin, C.M., 2004. Environmental chemicals in human milk: a review of levels, infant exposures and health, and guidance for future research. Toxicol. Appl. Pharm. 198, 184–208. https://doi.org/10.1016/j.taap.2003.08.021.

Li, A., Kang, L., Li, R., Wu, S., Liu, K., Wang, X., 2022. Modeling di (2-ethylhexyl) Phthalate (DEHP) and its metabolism in a body's organs and tissues through different intake pathways into human body. Int J. Environ. Res Public Health 19, 5742. https://doi.org/10.3390/ijerph19095742.

Lin, S., Ku, H.-Y., Su, P.-H., Chen, J.-W., Huang, P.-C., Angerer, J., Wang, S.-L., 2011. Phthalate exposure in pregnant women and their children in central Taiwan. Chemosphere 82, 947–955. https://doi.org/10.1016/j.chemosphere.2010.10.073.

Liu, Y., Huo, W.-B., Deng, J.-Y., Tang, Q.-P., Wang, J.-X., Liao, Y.-L., Gou, D., Pei, D.-S., 2023. Neurotoxicity and the potential molecular mechanisms of mono-2-ethylhexyl phthalic acid (MEHP) in zebrafish. Ecotoxicol. Environ. Saf. 265, 115516. https://doi.org/10.1016/j.ecoenv.2023.115516.

Ma, G., Ma, B., Wang, L., Tao, W., 2022. Occurrence and dietary exposure risks of phthalate esters in food in the typical valley city Xi'an, Northwest China. Environ. Sci. Pollut. Res. 29, 31426–31440. https://doi.org/10.1007/s11356-022-18592-6.

Magnusson, B., Näykki, T., Hovind, H., Krysell, M., Sahlin, E., 2017. Handbook for calculation of measurement uncertainty in environmental laboratories, Nordtest Report TR 537 (ed. 4).

Marchand, D.H., Croes, K., Dolan, J.W., Snyder, L.R., Henry, R.A., Kallury, K.M.R., Waite, S., Carr, P.W., 2005. Column selectivity in reversed-phase liquid chromatography. J. Chromatogr. A 1062, 65–78. https://doi.org/10.1016/j.chroma.2004.11.014.

Martino-Andrade, A.J., Chahoud, I., 2010. Reproductive toxicity of phthalate esters. Mol. Nutr. Food Res. https://doi.org/10.1002/mnfr.200800312.

McFadden, J.R., Ames, D.M., 2023. Assessment of ammonium fluoride as a mobile phase additive for sensitivity gains in electrospray ionization. Anal. Sci. Adv. 4, 347–354. https://doi.org/10.1002/ansa.202300031.

Mortensen, G.K., Main, K.M., Andersson, A.-M., Leffers, H., Skakkebæk, N.E., 2005. Determination of phthalate monoesters in human milk, consumer milk, and infant formula by tandem mass spectrometry (LC–MS–MS). Anal. Bioanal. Chem. 382, 1084–1092. https://doi.org/10.1007/s00216-005-3218-0.

Nehring, A., Bury, D., Ringbeck, B., Kling, H.-W., Otter, R., Weiss, T., Brüning, T., Koch, H.M., 2020. Metabolism and urinary excretion kinetics of di(2-ethylhexyl) adipate (DEHA) in four human volunteers after a single oral dose. Toxicol. Lett. 321, 95–102. https://doi.org/10.1016/j.toxlet.2019.12.006.

NIST, N.I. of S. and T., 2022. Standard Reference Material 3672. Organic Contaminants in Smokers' Urine (Frozen). Certificate of Analysis.

Rasmussen, K.M., Felice, J.P., O'Sullivan, E.J., Garner, C.D., Geraghty, S.R., 2017. The meaning of "Breastfeeding" is changing and so must our language about it. Breastfeed. Med. 12, 510–514. https://doi.org/10.1089/bfm.2017.0073.

Runkel, A.A., Snoj-Tratnik, J., Mazej, D., Horvat, M., 2020. Urinary phthalate concentrations in the slovenian population: an attempt to exposure assessment of family units. Environ. Res 186. https://doi.org/10.1016/j.envres.2020.109548.

Sakhi, A.K., Cequier, E., Becher, R., Bølling, A.K., Borgen, A.R., Schlabach, M., Schmidbauer, N., Becher, G., Schwarze, P., Thomsen, C., 2019. Concentrations of selected chemicals in indoor air from Norwegian homes and schools. Sci. Total Environ. 674, 1–8. https://doi.org/10.1016/j.scitotenv.2019.04.086.

Silva, M.J., Barr, D.B., Reidy, J.A., Kato, K., Malek, N.A., Hodge, C.C., Hurtz, D., Calafat, A.M., Needham, L.L., Brock, J.W., 2003. Glucuronidation patterns of common urinary and serum monoester phthalate metabolites. Arch. Toxicol. 77, 561–567. https://doi.org/10.1007/s00204-003-0486-3.

Sioen, I., Fierens, T., Van Holderbeke, M., Geerts, L., Bellemans, M., De Maeyer, M., Servaes, K., Vanermen, G., Boon, P.E., De Henauw, S., 2012. Phthalates dietary exposure and food sources for Belgian preschool children and adults. Environ. Int. 48, 102–108. https://doi.org/10.1016/j.envint.2012.07.004.

Snoj Tratnik, J., Falnoga, I., Mazej, D., Kocman, D., Fajon, V., Jagodic, M., Stajnko, A., Trdin, A., Šlejkovec, Z., Jeran, Z., Osredkar, J., Sešek-Briški, A., Krsnik, M., Kobal, A. B., Kononenko, L., Horvat, M., 2019. Results of the first national human biomonitoring in Slovenia: trace elements in men and lactating women, predictors of exposure and reference values. Int. J. Hyg. Environ. Health 222, 563–582. https://doi.org/10.1016/j.ijheh.2019.02.008.

Stajnko, A., Runkel, A.A., Kosjek, T., Snoj Tratnik, J., Mazej, D., Falnoga, I., Horvat, M., 2022. Assessment of susceptibility to phthalate and DINCH exposure through CYP and UGT single nucleotide polymorphisms. Environ. Int. 159, 107046. https://doi.org/10.1016/j.envint.2021.107046.

U.S. Environmental Protection Agency (EPA), 2011. Exposure Factors Handbook: 2011 Edition. EPA/600/R-09/052F. Available from the National Technical Information Service, Springfield, VA, and online at http://www.epa.gov/ncea/efh.

Ventrice, P., Ventrice, D., Russo, E., De Sarro, G., 2013. Phthalates: European regulation, chemistry, pharmacokinetic and related toxicity. Environ. Toxicol. Pharm. https://doi.org/10.1016/j.etap.2013.03.014.

Vimalkumar, K., Zhu, H., Kannan, K., 2022. Widespread occurrence of phthalate and non-phthalate plasticizers in single-use facemasks collected in the United States. Environ. Int 158, 106967. https://doi.org/10.1016/j.envint.2021.106967.

Vogel, N., Schmidt, P., Lange, R., Gerofke, A., Sakhi, A.K., Haug, L.S., Jensen, T.K., Frederiksen, H., Szigeti, T., Csákó, Z., Murinova, L.P., Sidlovska, M., Janasik, B., Wasowicz, W., Tratnik, J.S., Mazej, D., Gabriel, C., Karakitsios, S., Barbone, F., Rosolen, V., Rambaud, L., Riou, M., Murawski, A., Leseman, D., Koppen, G., Covaci, A., Lignell, S., Lindroos, A.K., Zvonar, M., Andryskova, L., Fabelova, L., Richterova, D., Horvat, M., Kosjek, T., Sarigiannis, D., Maroulis, M., Pedraza-Diaz, S., Cañas, A., Verheyen, V.J., Bastiaensen, M., Gilles, L., Schoeters, G., Esteban-López, M., Castaño, A., Govarts, E., Koch, H.M., Kolossa-Gehring, M., 2023. Current exposure to phthalates and DINCH in European children and adolescents - Results from the HBM4EU Aligned Studies 2014–2021. Int J. Hyg. Environ. Health 249, 114101. https://doi.org/10.1016/j.ijheh.2022.114101.

Wang, W., Craig, Z.R., Basavarajappa, M.S., Hafner, K.S., Flaws, J.A., 2012. Mono-(2-Ethylhexyl) phthalate induces oxidative stress and inhibits growth of mouse ovarian antral follicles1. Biol. Reprod. 87. https://doi.org/10.1095/biolreprod.112.102467.

Wardell, J.M., Wright, A.J., Bardsley, W.G., D'souza, S.W., 1984. Bile salt-stimulated lipase and esterase activity in human milk after collection, storage, and heating: nutritional implications. Pedia Res 18, 382–386. https://doi.org/10.1203/00006450-198404000-00017.

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Xu, S., Zhang, H., Pao, P.C., Lee, A., Wang, J., Suen Chan, Y., Manno, F.A.M., Wan Chan, S., Cheng, S.H., Chen, X., 2020. Exposure to phthalates impaired neurodevelopment through estrogenic effects and induced DNA damage in neurons. Aquat. Toxicol. 222. https://doi.org/10.1016/j.aquatox.2020.105469.

Zhou, F., Guo, C., Wang, L., Zhang, G., Wang, J., Chen, W., Cui, K., Tan, Y., Zhou, Z., 2023. Mono-(2-ethylhexyl) Phthalate (MEHP)-induced telomere structure and function disorder mediates cell cycle dysregulation and apoptosis via c-Myc and its upstream transcription factors in a mouse spermatogonia-derived (GC-1) cell line. Toxics 11, 448. https://doi.org/10.3390/toxics11050448.

Zhu, M., Huang, C., Ma, X., Wu, R., Zhu, W., Li, X., Liang, Z., Deng, F., Wu, J., Geng, S., Xie, C., Zhong, C., 2018. Phthalates promote prostate cancer cell proliferation through activation of ERK5 and p38. Environ. Toxicol. Pharm. 63, 29–33. https://doi.org/10.1016/j.etap.2018.08.007.

Zhu, J., Phillips, S.P., Feng, Y.-L., Yang, X., 2006. Phthalate Esters in human milk: concentration variations over a 6-month postpartum time. Environ. Sci. Technol. 40, 5276–5281. https://doi.org/10.1021/es060356w.

Zuccarello, P., Oliveri Conti, G., Cavallaro, F., Copat, C., Cristaldi, A., Fiore, M., Ferrante, M., 2018. Implication of dietary phthalates in breast cancer. A systematic review. Food Chem. Toxicol. 118, 667–674. https://doi.org/10.1016/j.fct.2018.06.011.