



Assessment of baby disposable diapers application for urine collection and determination of phthalate metabolites

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ABSTRACT

The baby disposable diapers were investigated as a sampling material for urine collection and validated for the evaluation of the exposure of children to xenobiotics. Phthalate metabolites detected in urine samples were chosen as proof-of-concept analytes. For the determination of phthalate metabolites in children's urine samples, high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) was used. Two sampling approaches were compared, namely sterile containers and baby disposable diapers. Thirty urine samples from infants and toddlers were analyzed by both methods in parallel and the results were compared. It was found that for diaper sampling, lower concentrations of the metabolites were observed, however, the general distribution for particular metabolites remains the same for both methods. For most of the metabolites high determination coefficients were obtained, namely 0.9929 for MEHHP, 0.9836 for MMP, 0.9796 for MECPP, and 0.9784 for 2-cx-MMHP. For MEOHP the determination correlation coefficient was 0.9154, while for MBP was – 0.7771 and MEHP was – 0.5228. In general, for diaper sampling an underestimation for 2-cx-MMHP and MEOHP was observed, while for MMP diaper-based approach provides overestimation. However, the proposed procedure confirms the possibility of using baby disposable diapers as a material for the collection of urine samples for biomonitoring purposes and fast screening of phthalates exposure.

1. Introduction

Exposure to environmental xenobiotics poses a threat to human health at every stage of life, starting in fetal life and ending in adulthood. Their presence implies the deterioration of overall well-being, developmental disorders early in life, organ diseases or reproductive problems (Calatayud Arroyo et al., 2021). As toddlers are particularly susceptible to harmful substances, and the effects of some xenobiotics are still not fully explored, the determination of children's exposure to them is being pursued (Treviño et al., 2023). Due to their omnipresence, it is extremely difficult to establish all sources of many xenobiotics. Phthalates are a typical group of harmful xenobiotics, which are commonly used in the production of polymers and are delivered to the

body by ingestion, inhalation, or through the skin (Kasper-Sonnenberg et al., 2012), e.g. via baby care products (Sathyanarayana et al., 2008). Exposure may be higher for toddlers because of their hand-to-mouth behaviors (Huang et al., 2021; Langer et al., 2014). The results of toxicological and epidemiological studies indicate that some phthalic acid esters have oestrogenic and/or anti-androgenic properties that affect the reproductive system (Hlisenfková et al., 2020). Due to their harmful nature, some of them, such as bis(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), diisobutyl phthalate (DiBP), and benzyl butyl phthalate (BBP) are banned in European Union (EU) from cosmetics application (The Commission of the European Communities, 2004). Furthermore, recent EU regulations also pay attention to the toxic effect of phthalates, and great effort was put into toy safety as well as other

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articles made for children (The European Commission, 2018). The regulation applies to DEHP, DBP, BBP and, from 2020, DiBP, whose combined content in children's products must not exceed 0.1% of the weight of the polymeric material.

The common way to estimate the exposure of children to xenobiotics is to monitor the concentration of the metabolites of these compounds that are excreted from the body with urine (Patterson et al., 2010). Thus, an appropriate procedure for collecting a urine sample that both allows a representative sample to be obtained and does not involve discomfort for the child and its caregivers, especially if it is a non-toilet-trained toddler, is necessary (Liu et al., 2012). Common methods include clean-catch, sterile urine bag collectors, catheterization or suprapubic aspiration, which are cumbersome to perform and/or invasive.

Recently, disposable diapers have been studied as an alternative way of urine sampling and has been used for monitoring of pesticides (Hu et al., 2004; Oerlemans et al., 2018; Ueyama et al., 2020), bisphenols (Lucarini et al., 2020), and phthalate metabolites (Liu et al., 2012; Lucarini et al., 2021a). The authors of these studies point to the non-invasive and comfortable procedure for the child, the lack of special care required from doctors or caregivers, and the ubiquity of disposable diapers. In addition, diaper-based sampling, unlike other methods, makes overnight collection of urine possible. An important issue for this kind of sampling is urine release from the superabsorbent polymer which is used in diapers to ensure liquid absorption and guarantee the dryness of the diaper and the safety of the baby. The most frequently used urine release procedures are based on the addition of calcium chloride (CaCl_2) to the gel absorbent of diapers. In this case, calcium ions replace the water molecules in the polyacrylate polymer backbone and the urine is subsequently released from the gel (Oerlemans et al., 2018). Also, a saturated potassium chloride (KCl) solution could be applied for urine release due to the osmotic effect (Shiotsuki et al., 2012). Another procedure involves the application of two syringes connected head-to-head. A piece of wetted diaper is inserted into one of them and acetone into the other. Then, the urine sample is eluted with acetone by manually transferring the solvent between both syringes. Extraction of the urine by acetone (Ueyama et al., 2020) directly in the solid phase extraction (SPE) column, as well as with methanol (Sivan et al., 2001), has also been utilized. Although the proposed methods promote sufficient urine release, analytes could remain adsorbed on the polymeric sorbent or in other diaper components and salt or polar solvent used for urine release may interfere with subsequent sample preparation steps.

This study aimed to develop a strategy for urine sample release from disposable diapers for the determination of seven phthalate metabolites (ESM Table 1). Phthalate metabolites were chosen as proof-of-concept analytes since phthalates are ubiquitous and absorbed by various routes as well as have a short half-life in the body, thus their metabolites could be present in urine between 4 and 48 h after exposure (Huang et al., 2021; Sprowles et al., 2022). The relatively short half-life in the human body indicates that there is little accumulation, and all measured

levels of metabolites describe daily intake of phthalates and are thus directly related to exposure. Phthalate metabolites can act as agonist ligands or antagonists to hormone receptors, thus interfering with the endocrine hormone-mediated pathways (Lucarini et al., 2021b). The efficiency of phthalate metabolism differs greatly between children and adults, and toxicokinetics depends on several factors, including the size of lipid and tissue compartments, organ blood flow and protein binding capacity, and renal and hepatic system function. Therefore, children are likely to be much more affected than adults after prolonged exposure, which may lead to more adverse effects due to the not yet developed detoxification functions (Lucarini et al., 2021b).

Previously, the researches described the possibility of diaper application for the determination of phthalate metabolites (Liu et al., 2012; Lucarini et al., 2021a; Mohanto et al., 2023). Liu et al. first proposed the use of a disposable diaper in the determination of phthalate metabolites in children's urine (Liu et al., 2012). They used CaCl_2 to release the urine and developed an in-house system to filter and centrifuge the urine from the diaper. However, the study focused only on the polyacrylate gel that fills the diaper, although there is a possibility that other diaper components may capture phthalate metabolites. Other studies also used CaCl_2 ; however, it was used in the form of a solution, and the volume of the resulting urine was calculated as the difference in volume between the added salt solution and the resulting liquid (Lucarini et al., 2021a). In our opinion, this approach does not take into account the possibility of adsorption of the CaCl_2 solution or volume losses throughout the extraction. In addition, it results in the dilution of the sample. The recently available research on using diapers for urine sampling for phthalate metabolites determination uses a protocol that applies two syringes connected head-to-head and acetone as an extraction agent (Mohanto et al., 2023). The validation of the suggested procedure was performed using so-called diaper-extracted synthetic urine (DESU) spiked with standards.

In the current research, to validate the diaper-based urine sampling and provide a comprehensive investigation of urine release from the diapers, the developed procedure was compared with standard sampling using sterile containers. The metabolites under study were monomethyl phthalate (MMP), mono-ethyl phthalate (MEP), monobutyl phthalate (MBP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono[2(carboxymethyl)hexyl] phthalate (2-CX-MMHP), mono[(2RS)-2-ethyl-5-oxohexyl] phthalate (MEOHP), mono(2-ethylhexyl) phthalate (MEHP), which are primary metabolites of dimethyl phthalate, diethyl phthalate, and dibutyl phthalate as well as secondary and oxidative metabolites of di-2-ethylhexyl phthalate (Lucarini et al., 2021b).

2. Materials and Methods

2.1. Reagents and solutions

All chemicals and reagents used were of analytical grade. Unlabeled

Table 1
Analytical results for phthalate metabolites in urine samples.

	MMP	MEP	MECPP	MEHHP	2-cx-MMHP	MEOHP	MBP	MEHP
Direct urine analysis								
Mean [ng mL ⁻¹]	9.0	53.7	31.8	33.1	15.7	18.4	147.5	7.5
Range [ng mL ⁻¹]	1.0-31.5	2.9-218.9	1.2-120	1.3-259	1.4-57.4	1.7-64.2	6.7-637	1.8-25.9
Detection frequency ^a [%]	87	97	93	100	87	90	100	93
Diaper analysis								
Mean [ng mL ⁻¹]	11.3	39.3	21.9	22.5	8.1	9.4	96.3	5.0
Range [ng mL ⁻¹]	1.8-41.4	1.6-189	1.1-84.3	1.1-178.3	1.2-27.9	1.0-37.0	9.9-379	1.3-13.8
Detection frequency ^a [%]	87	100	97	100	87	93	100	100
Mean recovery ^b [%]	126	73	69	68	52	51	65	67

^a Samples with the metabolites' levels less than the LOQ were calculated as non-detected and were not used for calculation.

^b Mean recovery was calculated as follows: Recovery, % = $100 \times C_{\text{diaper}}/C_{\text{direct}}$, where C_{diaper} is a concentration of metabolite measured according to the diaper-based approach, while C_{direct} is a concentration of metabolite received by direct urine analysis.

standards of monomethyl phthalate (MMP), mono-ethyl phthalate (MEP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono[2(carboxymethyl)hexyl] phthalate (2-CX-MMHP), mono[(2RS)-2-ethyl-5-oxohexyl] phthalate (MEOHP), monobutyl phthalate (MBP), mono(2-ethylhexyl) phthalate (MEHP) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Isotope-labeled internal standards of MEP (ring-1,2- $^{13}\text{C}_2$, dicarboxyl- $^{13}\text{C}_2$, 99%), MEOHP ($^{13}\text{C}_4$, 99%), MECPP ($^{13}\text{C}_4$, 99%) and MEHP ($^{13}\text{C}_4$, 99%) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Sodium chloride (NaCl) and formic acid were purchased from POCH S.A. (Gliwice, Poland). Ammonium acetate was purchased from Sigma-Aldrich® (St. Louis, USA). β -Glucuronidase (from *E. coli*, solution) was purchased from Roche (Mannheim, Germany). Acetonitrile for LC-MS LiChrosolv® was purchased from Merck (Darmstadt, Germany). Diethyl ether for HPLC, n-hexane and magnesium sulphate (dried) were purchased from VWR Chemicals BDH® (Radnor, USA). Deionized water was obtained from HLP 5 water purification system (Hydrolab, Poland).

2.2. Instrumentation

HPLC-MS/MS Agilent 1200 Series with binary pump G1312B, column thermostat G1316A, HiP sampler G1367C and degasser G1322A & Ultivo LC/TQ (USA) was used for the determination of the analytes. Column ThermoFisher Scientific®: Accucore™ Phenyl-Hexyl, 150 mm \times 4.6 mm \times 2.6 μm , (USA) was applied for the separation of analytes. Vortexing was performed with Vortex VWR, (USA). Centrifugation of the extraction mixture was provided with Centrifuge Eppendorf® 5804 R, (Germany). Freeze Dryer Christ Alpha 2-4 with Rotational-vacuum-concentrator RVC 2-18 CD plus Christ® (Germany) was used to evaporate the extraction solvent. Shaking was performed with the use of a homemade wrist-action shaker set at 120 rpm.

2.3. Description of investigated population based on application forms received from parents (age, gender, habits)

The present study was conducted in accordance with the Ethics Policies of the Gdańsk University of Technology, University Clinical Centre and other nationally valid regulations, and guardians of all participants had provided written informed consent for inclusion in the study. The Independent Bioethics Committee for Scientific Research at the Medical University of Gdansk approved the study protocol (Declaration no. NKBBN/130/2022). Before enrollment into the study, written informed consent with no remuneration for guardians of children was obtained.

Children participating in the study were living in Poland and their guardians, as legally acceptable representatives, were asked to participate in this study, and a total of 40 children participated in the current study. The age of the children was between 1 month and 6 years, the gender distribution was 19 females and 21 males (ESM Fig. 1). Thirty urine samples from infants and toddlers were collected into the sterile containers and used both for direct urine analysis and the diaper-based approach. Additionally, 10 diaper samples with adsorbed urine were collected from newborn children and infants.

The children's guardians were asked to answer a questionnaire about exposure to phthalates. The questions concerned pacifier usage, baby bottle-feeding, playing with plastic toys, application of plastic tableware for baby feeding, type of baby feeding (breastfeeding, home-made food, infant formula feeding), type of fabric for baby clothes, and place of residence.

According to the questionnaire, infants and toddlers could potentially be exposed to phthalates through the use of plastic tableware, eating products packaged in plastic, drinking water from plastic bottles and playing with plastic toys. In the case of newborns, the questionnaire referred to the child's mother and her habits regarding the use of plastics in everyday life.

2.4. Sample collection including diaper-based urine sampling

Urine samples were collected from healthy children with the permission of their guardians according to the Ethics policy.

Two urine collection approaches, i.e. sterile containers and baby disposable diapers, were used for urine sampling from newborns (0 - 2 months), infants (2 months - 1 year) and toddlers (1 - 6 years). The first approach was used for toddlers in a non-clinical setting by placing a 50 mL sterile polymer container into the urine stream. The spot urine samples collected at one time point were divided into two parts and frozen at -40°C . Before analysis, one part was thawed, while the other remained frozen for archiving purposes. The volume of the collected samples varied, depending on how much urine the child urinated.

The other approach was based on the application of baby disposable diapers. Commercially available disposable diapers were used for sampling. The diapers with superabsorbent polymer and plant-based diaper made with bamboo material were purchased either in supermarkets, department stores or pharmacies (Gdansk, Poland). They were different brands, both internationally recognized and local brands. This procedure was used for newborns and infants. After overnight urine collection, each wetted diaper was inserted into a sterile sealed bag and frozen at -40°C before analysis. Such an approach does not require any special effort from parents or clinical staff; however, the release of urine from

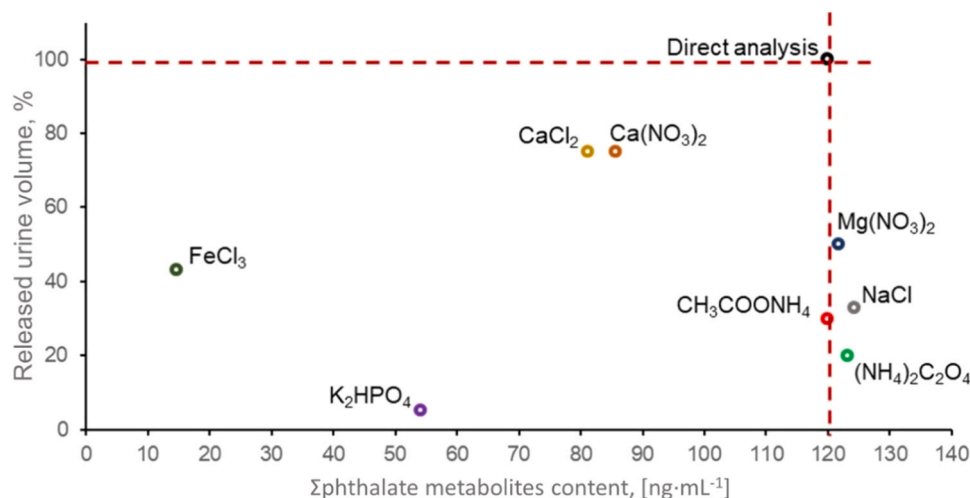


Fig. 1. Effectiveness of different salts in urine released from diaper and determination of phthalate metabolites (investigation performed using pooled urine; n = 3).

the disposable absorbent diaper is necessary. It should be noted that diapers contaminated with feces were excluded.

The pooled urine sample used for validation of the diaper-based approach was prepared by blending the urine samples. The pooled sample was provided by mixing the same infants' and toddlers' urine as it was in the main investigation.

2.5. Diaper-based approach for determination of phthalate metabolites in urine

The diaper-based approach for the determination of phthalate metabolites in urine consisted of two stages. In the first stage, the urine was released from a diaper. For this, a wetted part of the diaper after urine sampling was cut out, then the bottom waterproof layer was removed and the gel absorbent particles and the cotton layer containing urine were carefully cut out into smaller pieces (~5 g each) and transferred into a mortar. Then, 1.5 g of NaCl was added and thoroughly mixed with the gel absorbent particles and the cotton layer. After that, incubation at room temperature for 10 min was performed, and the urine was released from the diaper due to the osmotic effect. For further analysis, the released urine, together with diaper residue, was carefully transferred using stainless steel spatula to a sterile single-use syringe and then filtered through a syringe filter (polyethersulfone (PES), 0.45 μm). At least 6 mL of the urine was necessary for performing the analysis in 3 replications.

In the second stage, sample preparation based on liquid-liquid extraction was performed according to the modified procedure presented by Kim et al. (Kim et al., 2011). Two milliliter aliquot of the urine sample was spiked with 50 μL of each internal standard (IS) (1 $\mu\text{g L}^{-1}$). Then, 700 μL of 1 mol L^{-1} of aqueous ammonium acetate (pH 7.0) and 30 μL of β -glucuronidase were added to the mixture. After that, the mixture was thoroughly mixed by vortex and enzymatic hydrolysis at 37 $^{\circ}\text{C}$ for 2 h took place (ESM Fig. 2).

After the enzymatic hydrolysis was completed, liquid-liquid extraction was performed. For this, 1 mL of the saturated magnesium sulfate solution (to improve the extraction efficiency) and 0.3 mL of

concentrated formic acid (for receiving acidic pH under which the neutral form of mono-phthalate ester predominates) was added to the mixture. Two milliliters of the extracting solvent, a mixture of n-hexane and ethyl ether in a ratio 8:2 (v/v), were added and the mixture was thoroughly mixed during 10 min in the shaker and then centrifuged for 6 min at 20 $^{\circ}\text{C}$ at 4200 rpm. The procedure was repeated three times for one sample and the obtained extracts were mixed. A total volume of approx. 6 mL of the extract was then evaporated under vacuum for 30 min at 45 $^{\circ}\text{C}$ and reconstitution with 500 μL of the mobile phase (phase A: B as 65:35 (v/v)) was performed.

2.6. RP-HPLC-MS/MS determination of phthalate metabolites

The chromatographic separation was achieved with a ThermoFisher Scientific®: Accucore™ Phenyl-Hexyl column (150 mm \times 4.6 mm \times 2.6 μm) at 40 $^{\circ}\text{C}$. The mobile phase consisted of 0.1% acetic acid in water used as phase A and 0.1% acetic acid in acetonitrile used as phase B. The gradient elution program was carried out as follows: the concentration of solvent B was linearly increased from 35% to 75% in 8 min, and then increased to 99% B till 8.5 min and kept constant at 99% B to 12.0 min, afterwards back to 35% B from 12.0 to 12.1 min and kept constant to 17 min. The gradient elution program is presented in ESM Table 2. The flow rate was 0.7 mL min^{-1} , injection volume was 10 μL . Analyses were performed in negative polarity. Parameters of ESI source were established as follows: gas temperature 200 $^{\circ}\text{C}$, gas flow 6 L min^{-1} , nebulizer pressure 35 psi, capillary voltage 4000 V, sheath gas temperature 350 $^{\circ}\text{C}$, sheath gas flow: 11 L min^{-1} . Multiple reaction monitoring (MRM) mode parameters are summarized in ESM Table 3. All of the obtained HPLC-MS/MS data were preprocessed using Agilent MassHunter Workstation software. Later, data presentation and data statistics, including box-plots were performed in MS Excel.

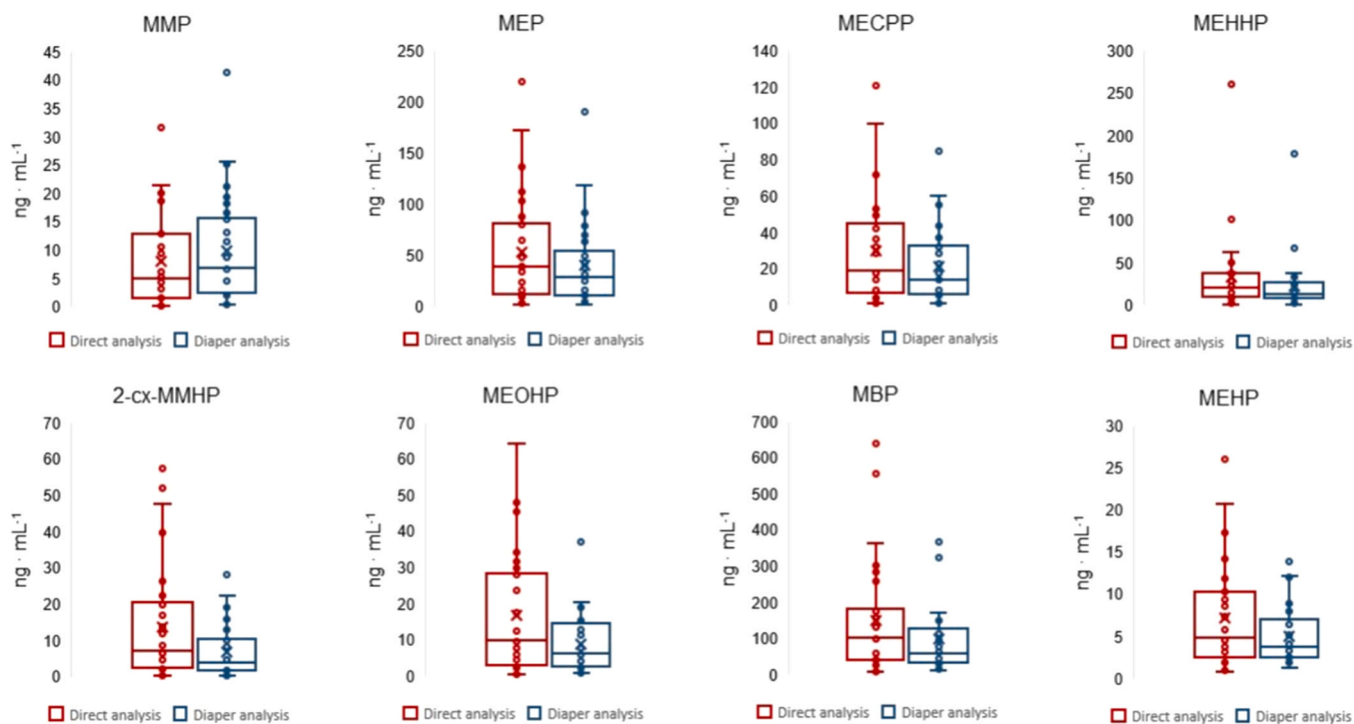


Fig. 2. The distribution of phthalate metabolite concentrations in urine samples collected using sterile containers (red) and baby disposable diapers (blue) for each box plot, the solid line represents the median while x indicates the mean value.

Table 2
Analytical characteristics of the developed procedure.

Analyte	Internal standard	Linear range [ng·mL ⁻¹]	Calibration curve equation	S _a	S _b	LOD ^a [ng·mL ⁻¹]	LOQ ^a [ng·mL ⁻¹]
MMP	MEP-IS	1 – 250	$y = 106.2 \times 10^{-2} x + 40 \times 10^{-4}$	2.5×10^{-2}	4.1×10^{-4}	0.37	1.1
MEP	MEP-IS	1 – 250	$y = 139.4 \times 10^{-2} x + 13.4 \times 10^{-3}$	1.1×10^{-2}	1.7×10^{-3}	0.35	1.1
MECPP	MECPP-IS	1 – 250	$y = 547.0 \times 10^{-2} x - 31.1 \times 10^{-4}$	5.5×10^{-2}	7.2×10^{-4}	0.33	0.98
MEHHP	MEOHP-IS	1 – 250	$y = 133.2 \times 10^{-2} x - 4.4 \times 10^{-3}$	1.2×10^{-2}	1.7×10^{-3}	0.34	1.0
2-cx-MMHP	MEOHP-IS	1 – 100	$y = 48 \times 10 x - 0.3 \times 10^{-3}$	1.1×10	4.2×10^{-3}	0.33	0.98
MEOHP	MEOHP-IS	1 – 250	$y = 1442.9 \times 10^{-3} x - 47.8 \times 10^{-4}$	7.5×10^{-3}	6.3×10^{-4}	0.32	0.95
MBP	MEOHP-IS	1 – 250	$y = 97.5 \times 10^{-2} x + 266.1 \times 10^{-4}$	1.7×10^{-2}	2.6×10^{-4}	0.32	0.97
MEHP	MEHP-IS	1 – 250	$y = 147.0 \times 10^{-2} x + 17.3 \times 10^{-3}$	2.7×10^{-2}	4.7×10^{-3}	0.37	1.1

S_a - is a standard deviation of slope.

S_b - is a standard deviation of intercept.

^a The limits of detection (LOD) and quantification (LOQ) were 3 and 10 fold the signal-to-noise ratio, respectively.

Table 3
Analytical results for phthalate metabolites in urine samples received after diaper sampling.

Sample	Concentration of phthalates metabolites [ng·mL ⁻¹]							
	MMP	MEP	MECPP	MEHHP	2-cx-MMHP	MEOHP	MBP	MEHP
Sample 1	1.470 ± 0.090	33.0 ± 1.9	5.17 ± 0.50	4.50 ± 0.49	< LOQ	2.06 ± 0.15	186 ± 19	3.43 ± 0.12
Sample 2	2.210 ± 0.070	33.21 ± 0.63	5.46 ± 0.36	5.45 ± 0.17	< LOQ	2.150 ± 0.060	205 ± 12	1.86 ± 0.33
Sample 3	1.590 ± 0.010	20.01 ± 0.68	2.500 ± 0.080	2.040 ± 0.080	< LOD	1.210 ± 0.040	124.1 ± 4.8	2.36 ± 0.30
Sample 4	1.680 ± 0.060	1.850 ± 0.080	4.19 ± 0.11	3.04 ± 0.16	< LOQ	< LOQ	16.6 ± 1.2	2.22 ± 0.15
Sample 5	< LOD	1.460 ± 0.040	4.330 ± 0.040	1.850 ± 0.080	< LOD	< LOQ	3.48 ± 0.37	1.490 ± 0.020
Sample 6	3.070 ± 0.110	38.0 ± 1.6	8.620 ± 0.040	6.93 ± 0.17	< LOQ	1.06 ± 0.19	218.2 ± 15.5	3.17 ± 0.32
Sample 7 ^a	1.27	2.31	3.42	2.29	< LOD	< LOQ	21.83	1.42
Sample 8	1.170 ± 0.010	4.33 ± 0.17	3.450 ± 0.060	1.28 ± 0.10	< LOD	< LOQ	6.55 ± 0.26	2.41 ± 0.20
Sample 9	2.40 ± 0.10	2.780 ± 0.050	1.110 ± 0.010	1.000 ± 0.040	< LOQ	< LOQ	24.30 ± 0.32	3.140 ± 0.060
Sample 10	< LOD	1.470 ± 0.050	3.97 ± 0.10	1.930 ± 0.030	< LOD	< LOQ	2.98 ± 0.10	2.16 ± 0.11

^a Received volume enables 1 analysis only

3. Results and Discussion

3.1. Urine release procedure

Using salt for releasing urine from the diaper could lead to collapse or 'shrink' of polyacrylate adsorbent due to the presence of metal cations (Hu et al., 2004). Additionally, the osmotic effect could take place.

In the present research, different salts were studied for urine release: NaCl, iron (III) chloride (FeCl₃), CaCl₂, calcium nitrate (Ca(NO₃)₂), magnesium nitrate (Mg(NO₃)₂), ammonium acetate (CH₃COONH₄), ammonium oxalate (NH₄)₂C₂O₄ and dipotassium phosphate (K₂HPO₄). In each case, 1 g of the dry diaper piece was spiked with 10 mL of the pooled urine and incubated for 30 min. Then, 1 g of each salt was added and mixed with the diaper piece to evaluate both the effectiveness of urine release from the diaper as well as the phthalate metabolites' total concentration. According to the results (Fig. 1), the lowest effectiveness was observed for K₂HPO₄. For FeCl₃ the lowest sensitivity was observed. The highest urine release was observed for CaCl₂ and Ca(NO₃)₂, the volume of the received urine was more than 75%. Satisfactory results were also received for Mg(NO₃)₂. Volumes of the released urine for CH₃COONH₄ were slightly below 30% and (NH₄)₂C₂O₄ close to 20%, however, the total content of phthalate metabolites was similar to Mg(NO₃)₂, (NH₄)₂C₂O₄ and NaCl. The differences between particular metabolites are presented in ESM Table 4. For further experiments NaCl was used due to the following considerations: i) it gives the highest sensitivity; ii) this salt was used for extraction of the phthalates in the liquid-liquid extraction step; iii) no precipitation was observed when NaCl was used in contrary to calcium and magnesium salts where sediment formed.

The minimum NaCl amount required for urine release was evaluated. For this purpose, experiments were provided as follows. Thirty grams of gel absorbent was isolated from the diaper, spiked with 60 mL of the pooled urine and homogenized. After that, five portions of 5 g of the spiked gel absorbent were weighed and mixed with the NaCl portions in the range from 0.5 to 2.5 g. It was found that the volume of the released

urine increases till 1.5 g of salt and after that remains constant at approximately 3.0 g. Thus, 1.5 g of the salt was applied for further urine analysis. However, salt addition is preferable only for diapers with gel absorbent. When plant-based diapers (for example, with bamboo material) were applied the urine release was not satisfactory.

3.2. Validity of diaper-based sampling in the determination of phthalate metabolites

In the present research, two methods to urine sampling were compared: sterile containers and the diaper-based approach. In this case, 30 urine samples received by voiding into a sterile container were firstly analysed and then the same urine samples were used for spiking of model diapers.

The first procedure was based on the application of urine samples received in sterile containers. In this case, urine samples which were received for analysis and frozen were thawed to room temperature and centrifuged to exclude solid particles. Next, 2 mL of the sample was placed into a clean vial and 0.5 g of NaCl was added, followed by a liquid-liquid extraction procedure including the enzymatic hydrolysis step as it is described in Section 2.5. The results obtained for each sample are presented in ESM Table 5.

The second procedure consisted of the application of baby disposable diapers. To improve the applicability of baby disposable diapers for urine sampling, in the present research model diapers were prepared by spiking a piece of the diaper (5 × 5 cm) with the urine received by voiding into a sterile container. The piece of the diaper was wetted with 10 mL of urine (the same as used for direct urine analysis) and left for 30 min. After that, the urine release was performed according to the procedure presented in Section 2.5. The results received for each sample are presented in ESM Table 5.

Thirty urine samples were analyzed by both methods and were juxtaposed. The obtained results are presented first as a box plot to illustrate the global distribution of the data (Fig. 2).

The results showed that there were no significant differences

between the results obtained using direct urine sampling and a diaper-based approach. In addition, a noticeable narrowing of the spread of the data from diaper sampling could be observed for seven of the eight metabolites (except MMP). This indicates that in the next step, it is necessary to pairwise compare individual metabolite concentrations for each sample to estimate the direct effect of the diaper-based approach on the obtained measurement results. Fig. 3 represents the results received for the individual metabolites and particular urine samples showing also the differences between direct and diaper-based sampling procedures. The summarized results of individual phthalates metabolites concentration are presented in Table 1. It can be seen that the highest mean concentration was observed for MBP, while the lowest was for MEHP. MBP and MEHHP were observed in 100% of samples (Table 1).

Correlations for the determination of phthalate metabolites by the diaper-based approach versus direct urine analysis are shown in Fig. 4. For most of the metabolites high determination coefficients (R^2) were obtained, namely 0.9929 for MEHHP, 0.9836 for MMP, 0.9796 for MECPP, and 0.9784 for 2-cx-MMHP. For MEOHP the determination coefficient is 0.9154, while for MBP was -0.7771 and MEHP was -0.5228 . To determine whether a diaper-based approach generates a good estimation of phthalates exposure, the slope was depicted in Fig. 4. The slope values greater than 1 suggest underestimation and below 1 overestimation of the diaper-based approach. Thus, we can conclude that for most cases measured concentrations were lower than in direct urine sampling with a strong underestimation for 2-cx-MMHP and MEOHP. Only in the case of the MMP diaper-based approach provides overestimation. This led to the conclusion that using diapers as a sampling material provides an overestimation of dimethyl phthalate exposure (DMP, MMP metabolite). Additionally, it is not suitable for DBP (MBP metabolite) exposure monitoring due to the low determination correlation coefficient. For DEHP exposure studies it is more justified to monitor MEHHP and MECPP rather than MEHP concentration, but it can result in underestimation. Moreover, considering 2-cx-MMHP and MEOHP as DEHP exposure estimators can lead to approximately two times lower concentrations than in direct urine analysis, thus diaper-based approach is not recommended for quantification of these metabolites. However, determining levels of such metabolites could give a rough snapshot of the current exposure to DEHP.

3.3. Analytical performance of the extraction procedure followed by HPLC-MS/MS detection

For the HPLC-MS/MS determination of phthalate metabolites, isotope-labelled internal standards were used. To improve the reproducibility of results, 4 internal standards were applied (MEP-IS, MECPP-IS, MEOHP-IS, MEHP-IS). The analytical parameters such as the linear ranges, limits of detection (LODs), limits of quantification (LOQs) and repeatability were determined. For all analytes except 2-cx-MMHP, the linear ranges were between 1 to 250 ng mL⁻¹. The LOD and LOQ were calculated as 3 and 10-fold the signal-to-noise ratio, respectively. The repeatability of the developed procedure was determined by analyzing of 5 replicate of analytes solution at two concentration levels. Interday precision calculated was less than 11%, while intraday – was less than 4%. The validation results are shown in Table 2. To verify the accuracy of the procedure, the added-found method was used with a spiked urine sample containing 10 and 25 ng mL⁻¹ of phthalate metabolites. It should be mentioned that non-conjugated metabolites were used in this study. The relative recoveries for the metabolites ranged from 62.2 to 150.6% (ESM Table 6). The reason for such a distribution of relative recoveries could be that conjugated metabolites may act differently throughout the extraction procedure. However, glucuronic acid esters are more soluble and therefore recoveries may be higher. The verified procedure was used both for sterile containers and baby disposable diapers sampling methods comparison.

3.4. Application of baby disposable diapers for determination of phthalate metabolites

After the procedure optimization, baby disposable diapers were used for urine collection and the determination of phthalate metabolites. The diapers were received from 10 children (newborns and infants) after overnight sampling. The results are presented in Table 3. It was found that 2-cx-MMHP as one of the DEHP metabolites was not detected in the urine samples. Rather high content was observed for MBP (mean value 80.7 ng mL⁻¹) and MEP (mean value 13.8 ng mL⁻¹). The received results are in good correspondence to the previously reported investigations where a rather high concentration of both metabolites is indicated (Carlstedt et al., 2013; Frederiksen et al., 2014; Kim et al., 2017; Liu et al., 2020; Lucarini et al., 2021b; Navaranjan et al., 2020). This could be explained by rather high exposure to diethyl phthalate and dibutyl phthalate which are commonly present in personal care products, cosmetics, and washing and cleaning products, as well as polyvinyl chloride (PVC) items (Lucarini et al., 2021a). However, the content of dibutyl phthalate is strongly regulated by European Commission in children's care products and toys. The variation in the results might be as well due to the different urine dilution rates. To perform proper biomonitoring studies it is recommended to normalize obtained data to creatinine concentration or standard gravity (Sauvé et al., 2015).

4. Conclusions

The comprehensive investigation of baby disposable diapers for biomonitoring of xenobiotics in children's urine samples has been evaluated. It was shown that NaCl is preferable for urine release and could further improve the liquid-liquid extraction of analytes. Two sampling approaches were compared and it was found that for diaper sampling lower concentrations of the metabolites were observed, however, the general distribution for particular metabolites remains the same for both methods. It was found that for MBP and MEHP determination coefficient is lower than for other metabolites, which could lead to underestimation of dibutyl phthalate and di-2-ethylhexyl phthalate exposure. Nevertheless, the developed procedure could be used for biomonitoring purposes and rapid screening of phthalate exposure, as the non-invasive sampling minimizes the effort for urine collection for both parents and medical staff. In addition, the urine sample could be collected overnight in the desired amount for biomonitoring without causing discomfort to the child. Among diaper types only diapers with superabsorbent polymer could be used for urine collection due to sufficient urine release for subsequent analyte determination.

CRedit authorship contribution statement

Młynarczyk Michał: Writing – review & editing, Validation, Investigation. **Bagińska Ewa:** Writing – review & editing, Resources. **Majchrzak Tomasz:** Writing – original draft, Writing – review & editing, Visualization, Methodology. **Glinka Marta:** Writing – review & editing, Methodology, Investigation. **Jążdżewska Katarzyna:** Validation, Writing – original draft, Investigation. **Vakh Christina:** Writing – original draft, Validation, Methodology, Writing – original draft, Investigation. **Drązkowska Izabela:** Writing – review & editing, Resources. **Rachoń Dominik:** Writing – review & editing, Resources, Conceptualization. **Wasik Andrzej:** Writing – review & editing, Conceptualization. **Płotka-Wasyłka Justyna:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

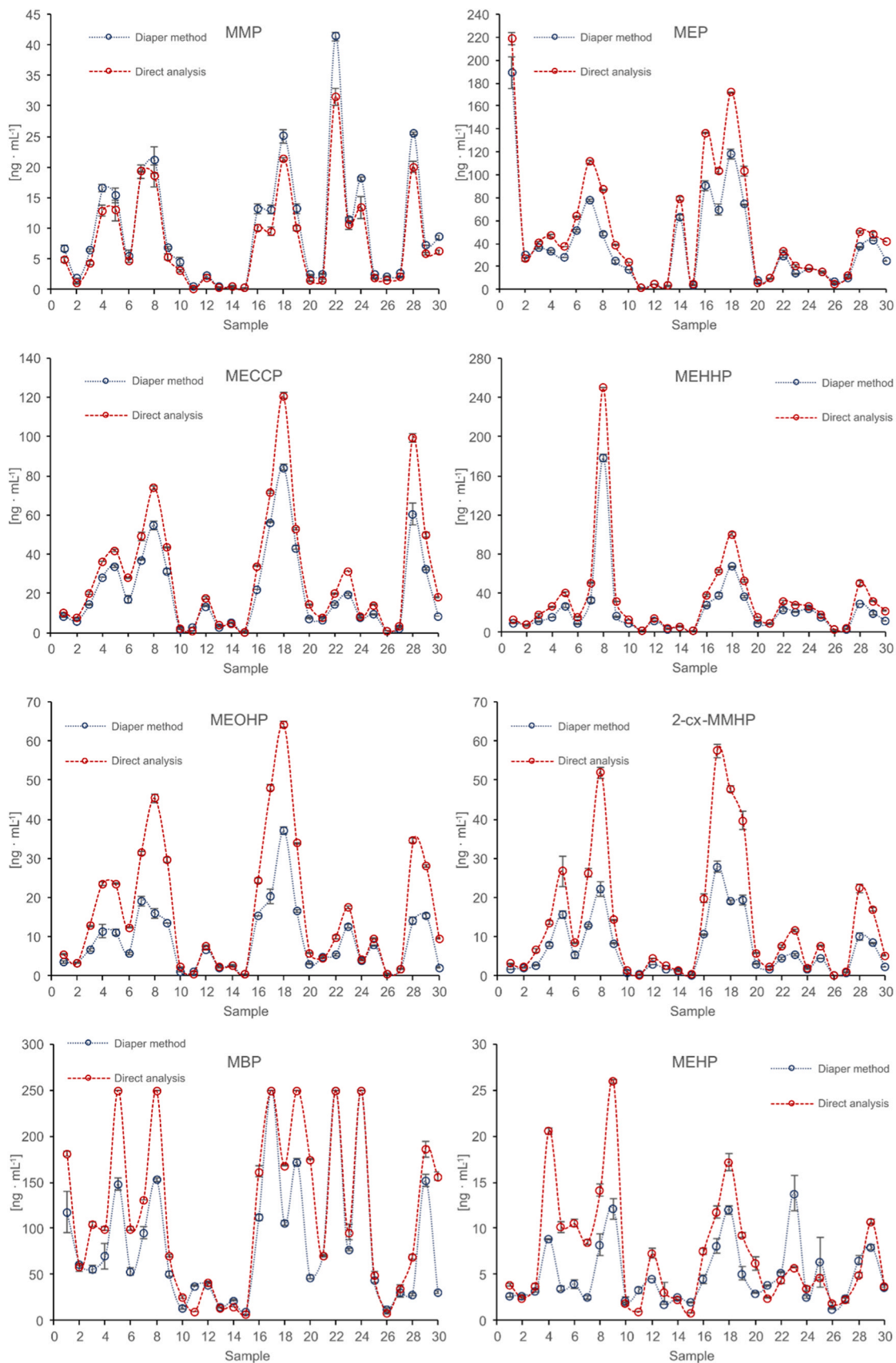


Fig. 3. The determination of phthalate metabolites in urine samples collected using two different sampling methods for each participant.

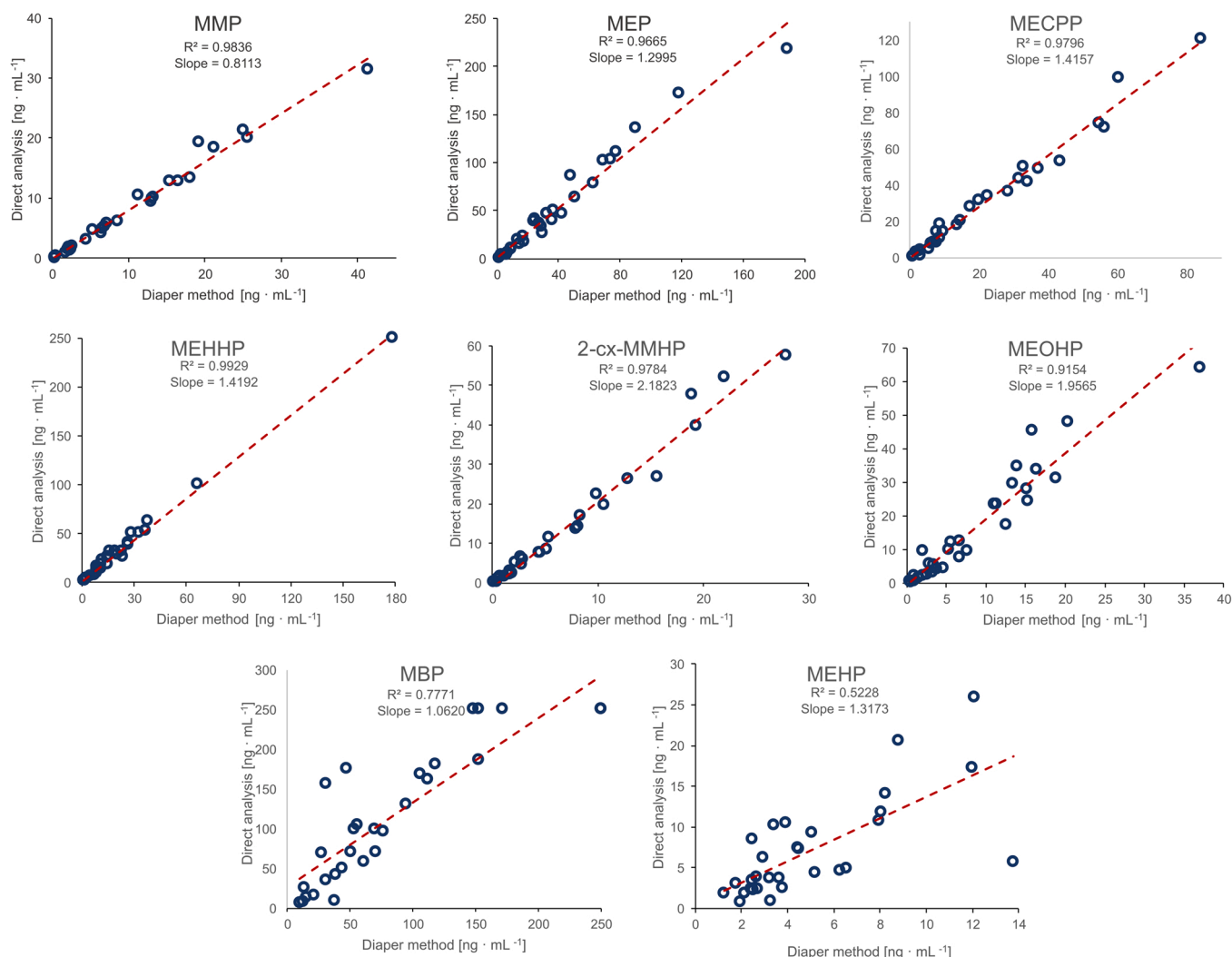


Fig. 4. Correlations of each urinary metabolite determined by diaper-based approach (X-axis) versus direct urine analysis (Y-axis).

Data Availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2024.116033](https://doi.org/10.1016/j.ecoenv.2024.116033).

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