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Dummy molecularly imprinted polymer-based solid-phase extraction method for the determination of some phthalate monoesters in urine by gas chromatography–mass spectrometry analysis

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ABSTRACT

A dummy molecularly imprinted polymer-based solid-phase extraction (SPE) sorbent was used for the selective extraction of some phthalate monoesters, monoethyl phthalate (MEP), monobutyl phthalate (MnBP) and mono-(2-ethylhexyl) phthalate (MEHP) in urine prior to gas-chromatography-mass spectrometry (GC-MS) analysis. Diethyl phthalate (DEP), a phthalate ester, was successfully used as a dummy template to prepare selective sorbent for MEP, MnBP, and MEHP extraction. DEP-imprinted poly(ethylene glycol dimethacrylate N-methacryloyl-L-tryptophan methyl ester) (DPEMT) microbeads were synthesized by suspension polymerization and characterized by Fourier Transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and Brunauer Emmet Teller (BET) analysis. The critical parameters (i.e., pH, sorbent amount, ionic strength, sample volume, elution solvent) affecting the extraction performance of the DPEMT-SPE sorbent were optimized. Under optimum conditions, good linearities were obtained in the concentration range of 4 to 60 ng/mL with determination coefficients (R^2) of greater than 0.9959. The developed SPE method provided low limits of detection (LOD) of 0.05–1.20 ng/mL and limits of quantification (LOQ) of 0.18–4.01 ng/mL with relative standard deviations (RSDs) of less than 8.95 % for intra- and inter-day analyses. The proposed SPE method was used to analyze phthalate monoesters in spiked urine samples, and recoveries of 97.45–109.26 % were obtained. DPEMT-SPE sorbent was reused for 15 times without any losses of performance. Consequently, a highly selective and sensitive SPE method based on a dummy molecularly imprinted polymer combined with GC-MS was successfully developed to monitor human phthalate exposure via urine samples.

1. Introduction

The concept of exposome was first introduced by Chris Wild in 2005, and he defined the exposome as all of the factors that are exposed throughout life from the prenatal period [1]. The exposome includes the cumulative measure of exposures to chemical and non-chemical agents, such as diet, stress, and socio-behavioral factors [2]. The exposome is an important approach that includes the traditional biomonitoring measurement that aims to identify all exposures that potentially affect health and cause diseases [2,3]. A group of exposome compounds called endocrine disruptors are exogenous substances or mixtures of substances that alter the development and function of the endocrine system [4]. These substances affect the production, release, binding, transport, activity, and secretion of hormones in the body [5]. Endocrine disruptors are chemicals frequently used in many fields, such as perfluorochemicals, pesticides, bisphenol A, and phthalates.

Phthalates (PAEs) are dialkyl or alkyl/aryl esters of 1,2-benzenedicarboxylic acid [6]. PAEs are organic synthetic chemicals often added as plasticizers to provide flexibility, softness, and durability to polyvinyl chloride (PVC) [7,8]. PAEs are widely used in many products, such as building materials, paints, household goods, clothing, cosmetics, personal care products, pharmaceuticals, medical devices, children's toys, food packaging, cleaning materials, and pesticides [9,10]. Due to the widespread use of these substances, the risk of exposure exists in all life aspects. Since PAEs can not bond chemically to matrices, their migration into the environment occurs easily. PAEs are plasticizers ubiquitously detected in the indoor environment and food as well as in humans. Humans are exposed to PAEs by inhalation, ingestion, dermal absorption, and even intravenous route [6,9]. PAEs, which are abundant in the environment, are reported to cause cardiovascular, thyroid, respiratory,

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Table 1
Retention times and selected ions for the analysis of the analytes.

Compound name	Retention time (t_R) (min)	*m/z*
MEP	6.419	74.9*, 75, 102.9, 103, 118.9, 119, 148.9, 162.9, 163, 176.9, 177, 22.9, 223, 251*, 266, 266.1
BBz (IS)	7.515	76.9, 77, 90.9, 91, 104.9*, 193.9, 194, 211.9*, 212
MnBP	7.942	73, 74.9*, 75, 103, 103.1, 118.9, 119, 148.9, 162.9, 220.9, 222.9*, 239, 278.9, 279
MEHP	10.560	73, 148.9, 162.9, 178.9, 179, 220.9, 221*, 222.9*, 238.9, 239

* Ouantification ions.

and reproductive system-related diseases in addition to diabetes and obesity [11].

PAEs are rapidly metabolized (within 6–24 h) in the human body and are generally excreted through urine and feces after exposure [12,13]. The conversion of diester phthalates to monoester metabolites occurs by phase I (hydrolysis) and phase II (hydroxylation and oxidation) biotransformation process, which is catalyzed by lipases and esterases found in the intestine and parenchyma. Generally, low molecular weight phthalates, such as diethyl phthalate (DEP), can be excreted directly from the body. In contrast, high molecular weight phthalates, such as diethyl hexyl phthalate (DEHP), undergo phase II to increase their water solubility so they can be later excreted as glucuronide conjugates [12–15]. Phthalate esters and their metabolites have been detected in various biological matrices such as urine [16], serum [17], saliva [18], breast milk [19], amniotic fluid [20], and semen [21] in humans. Selection of the biological matrix is essential to monitor phthalates exposure and distribution in humans. The most frequently preferred matrix for monitoring phthalate exposure is urine because it is relatively easy to collect thanks to non-invasive sampling and has higher metabolite concentrations than other matrices [22].

There are many analytical methodologies for determining phthalate monoesters in biological matrices. The determination of these metabolites is usually carried out using mass spectrometric methods combined with gas and liquid chromatography [13]. However, due to the complexity of the urine matrix and its low phthalate metabolites concentrations, it is required to apply a series of sample preparation steps (sample pretreatment, enrichment, extraction, cleaning, etc.) prior to instrumental analysis. Several sample pretreatment techniques have been performed to enrich and detect phthalate monoesters in urine, including online/off-line solid-phase extraction (SPE) [16,19,23,24], liquid-liquid extraction (LLE) [25,26], solid-phase microextraction (SPME) [22], magnetic solid-phase extraction (MSPE) [27], dispersive solid-phase extraction (DSPE) [28], dispersive liquid-liquid microextraction [29,30] and disposable pipette extraction (DPX) [31].

SPE is the most widely used sample pre-treatment method for determining phthalate monoesters in urine. Most generally used solid phases in SPE methods are silica, reversed-phase, hydrophobic-hydrophilic balanced (HLB), polyamide, poly[*n-vinylpyrrolidone-divinylbenzene* (DVB)], methacrylate DVB, and hydroxylated polystyrene DVB sorbents (OASIS HLB, OASIS MAX, C18-SampliQ, Strata XL and Nexus SPE commercial cartridges) [13,16,17,32,33]. Recently, studies using micro- or nano-sized sorbents with more selectivity, higher affinity, increased surface area, and magnetically superior as SPE materials have been reported instead of commercial sorbents [24,27,28,34,35]. Molecularly imprinted polymers (MIPs) which are based on the creation of specific recognition sites in crosslinked polymer networks that have proper shape, size, and functional groups toward the template molecule, have attracted increasing interest as selective adsorbents for separation and enrichment of small organic molecules. In the SPE procedure, MIPs are considerably attractive compared to commercial sorbents, because they provide high selectivity and affinity to target analytes, as well as physical durability, significant reusability, ease of preparation, low cost, long storage life, inertness to chemicals, resistance to high pressure and temperatures [36,37]. Instead of using toxic target compounds as templates in synthesizing MIPs, environmentally friendly dummy templates can be used. The MIPs prepared via a dummy template can also used to enrich a group of target analytes simultaneously. In recent years, the dummy imprinting strategy has been increasingly applied by using structurally analogous analytes of the target compounds as template molecules, which offers an attractive alternative.

In this study, a dummy molecularly imprinted polymer-based SPE method combined with GC-MS analysis was developed for the determination of phthalates monoesters (monoethyl phthalate (MEP), monobutyl phthalate (MnBP) and mono-(2-ethylhexyl) phthalate (MEHP)) in urine. A dummy molecularly imprinted polymer (DMIP) was synthesized by suspension polymerization technique using DEP as a dummy template for the selective recognition of targeted phthalates monoesters. DEP-imprinted poly(ethylene glycol dimethacrylate-N-methacryloyl-L-tryptophan methyl ester) (DPEMT) microbeads were prepared using methacryloyl-amido-L-tryptophan methyl ester (MATrp) as a functional monomer, ethylene glycol dimethacrylate (EGDMA) as a crosslinker. The specific surface area, surface morphology, and chemical structure of the DPEMT microbeads were characterized by Brunauer Emmet Teller (BET), scanning electron microscopy (SEM), and Fourier Transform infrared spectroscopy (FTIR) analysis, respectively. DPEMT microbeads were used as a sorbent for the preparation of DMIP-SPE cartridges, and the parameters (pH, sorbent amount, ionic strength, sample volume, elution solvent) affecting the extraction performance of the SPE method were carefully optimized. Additionally, method validation studies were conducted, as well as real sample studies.

2. Experiments

2.1. Chemicals and materials

MEP, MnBP, MEHP, and benzyl benzoate (BBz, Internal standard (IS)) were purchased from AccuStandard (New Haven, USA). DEP, EGDMA, 2,2′-azobisisobutyronitrile (AIBN) and dichloromethane (DCM) were obtained from Merck (Taufkırchen, Germany). L-tryptophan methyl ester, methacryloyl chloride, and β-glucuronidase (from Escherichia coli, 25,000 Unit) were supplied from Sigma (Taufkırchen, Germany). The polyvinyl alcohol (PVA; Mw: 100.000, 98 % hydrolyzed) was obtained from Aldrich (USA). N-methyl–N-(trimethylsilyl) trifluoroacetamide (MSTFA) was supplied from Sigma-Aldrich (Taufkırchen, Germany). 60 mg, 3 mL OASIS HLB cartridge was purchased from Waters (Milford, USA). The empty commercial SPE cartridges (3 mL) and SPE frits were purchased from Agilent Technologies (California, USA). Millipore Millex HV Hydrophilic 0.45 μm (PVDF) filters were supplied from Merck (Taufkırchen, Germany). Ultrapure water was provided from the Elga Flex3 water purification system (Veloia Water Solutions & Technologies, France). All solvents and other using chemicals were analytical grade and purchased from Merck (Taufkırchen, Germany).

Synthetic urine was prepared by modifying the preparation process that was used in the work of Sarigul et al. (2019) [38]. 0.1700 g Na_2SO_4, 0.0250 g $C_5H_4N_4O_3$, 0.0720 g $Na_3C_6H_5O_7{\cdot}2H_2O$, 0.0881 g $C_4H_7N_3O$, 1.500 g CH_4N_2O, 0.2308 g KCl, 0.1756 g NaCl, 0.0245 g $CaCl_2{\cdot}2H_2O$, 0.1266 g NH_4Cl, 0.0028 g $Na_2C_2O_4$, 0.1082 g $MgSO_4{\cdot}7H_2O$, 0.2912 g $NaH_2PO_4{\cdot}2H_2O$ and 0.0831 g $Na_2HPO_4{\cdot}2H_2O$ were weighed, dissolved in some ultrapure water with the help of ultrasonic bath. The final volume of the solution was made up to 100 mL with ultrapure water and stirred overnight at 37 °C with a magnetic stirrer. Prepared synthetic urine was used in method validation to mimic the real urine matrix.

2.2. Apparatus and instrument conditions

Agilent 7890A model gas chromatography coupled with a 5975C

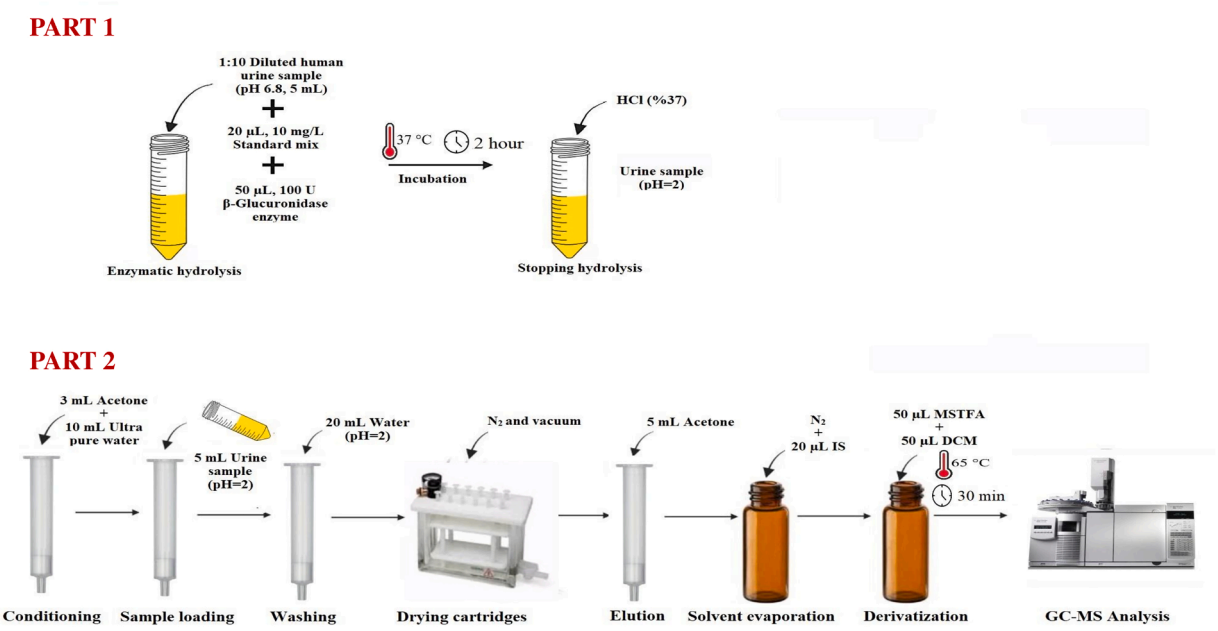

Fig. 1. Real sample analysis process.

model mass spectrometer was used to analyze phthalate monoesters. Chromatographic separation of the analytes was performed in an HP-5MS fused silica capillary column (0.25 µm, 0.25 mm × 30 m). Helium (99.999 %) was used as carrier gas with a constant flow rate of 1.0 mL min^{-1}. The injection volume was 1 µL, injection temperature was maintained at 280 °C with a splitless inlet. The oven temperature programmed was set as follows: 100 °C (1 min), 30 °C min^{-1} to 180 °C (1 min), and 10 °C min^{-1} to 280 °C. The detector temperature was 230 °C, which worked in selective ion mode. Table 1, gives the retention times of the target analytes and the mass-to-charge ratio (*m/z*) of the characteristic ions in the selective ion mode (SIM).

A Mettler Toledo pH meter (Mettler-Toledo AG, Schwerzenbach, Switzerland) was used to adjust the pH of the solutions. Agilent Vac Elut 20 Position SPE Manifold and a pump system were used in the SPE procedure (Brazil).

2.3. Synthesis and characterization of DPEMT microbeads

The DPEMT microbeads used as SPE sorbent for the determination of phthalates monoesters in urine were synthesized using the procedure in our previous study [39]. A suspension polymerization technique was used in the synthesis and DEP, EGDMA, MATrp, AIBN, and PVA was used as a dummy template, crosslinker, functional monomer, initiator, and stabilizer, respectively. The MATrp (functional monomer) to DEP (template molecule) ratio was 2:1 to prepare the selective microbeads for phthalates, which was optimized previously [39]. The pre-organization complex was prepared by treating the functional monomer (MATrp) and template molecule (DEP) for 2 h. The aqueous phase was prepared by dissolving 200 mg of PVA in 50 mL of distilled water. Then, the organic phase was prepared by adding toluene (pore former), EGDMA, and 100 mg of AIBN onto the pre-organization complex. The organic phase was dispersed in the aqueous phase and mixed in the polymerization reactor at a stirring speed of 400 rpm. The reactor was mixed at 65 °C for 4 h at a mixing speed of 400 rpm and 85 °C for 8 h at a mixing speed of 600 rpm. The resulting DMIP was washed with excess ethanol and water to remove unreacted chemicals (MATrp and toluene) and then dried in an oven at 50 °C. After that, the DMIP was washed repeatedly with a mixture of methanol: acetic acid (9:1) to remove the template molecule DEP from the structure completely; thus, polymeric microbeads containing binding sites that could specifically recognize phthalate monoesters were obtained. Whether or not the DEP molecule was removed entirely from the DMIP was spectrophotometrically followed by absorbance measurements at a wavelength of 239 nm. Non-imprinted polymer (NIP) was synthesized using the same method without a template molecule.

The chemical structure of DPEMT microbeads was studied by FTIR spectrometer (Perkin Elmer, Spectrum 100, USA). The physical morphology of DMIP was examined using a scanning electron microscope (Jeol, JEM 1200EX, Tokyo, Japan). The specific surface area, pore size, and pore volume of the DPEMT microbeads were determined by Brunauer Emmet Teller (BET) analysis (ASAP2000 instrument, Micromeritics). The microbeads' average size and size distribution were determined by screen analysis performed with Tyler standard sieves (Retsch Gmbh; Haan, Germany).

2.4. Analytical procedures

2.4.1. Optimization of SPE procedure in aqueous media

In the SPE procedure, parameters such as sample pH, elution solvent type, sorbent amount, sample loading volume, and salt concentration must be optimized to obtain the highest extraction efficiency. For this pupose, commercially purchased empty SPE cartridges (3 mL) were filled with the DPEMT microbeads. The upper and lower parts of the cartridges were closed with frits to prevent sorbent escape. The prepared cartridges were placed in the vacuum manifold (20′s) assembly and conditioned with 3 mL of acetone and 10 mL of ultrapure water. The desired volume of a standard solution (containing 0.2 µg of each standard (MEP, MnBP, and MEHP)) was prepared with distilled water and passed through the cartridge at a constant rate (0.2 mL/min) through a vacuum manifold system. The cartridges were dried by passing nitrogen (N_2) under vacuum. Then, 5 mL of solvent was passed through the cartridge at a certain speed (1 mL/min) to elute the phthalate monoesters adsorbed onto the solid phase. An 20 µL of 10 mg/L internal standard (BBz) solution was added to the eluted analytes and evaporated in N_2 atmosphere. 50 µL of MSTFA and 50 µL of DCM were added to the residue and derivatized at 65 °C for 30 min, and then the samples were analyzed by GC-MS. Optimum SPE conditions were determined considering the peak areas obtained from the GC-MS analysis.

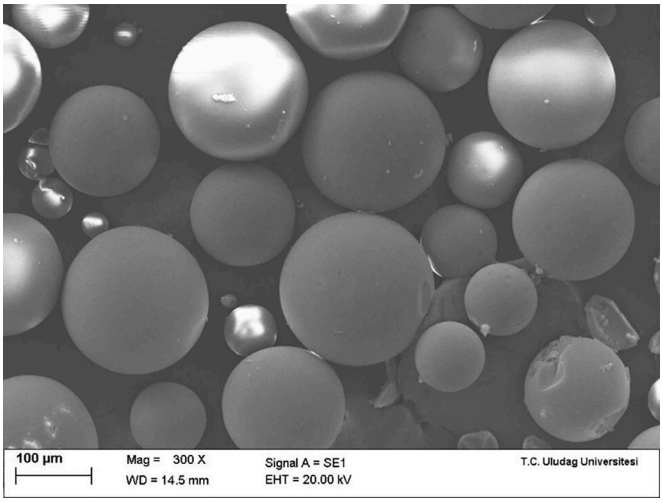

Fig. 2. SEM image of the DPEMT microbeads.

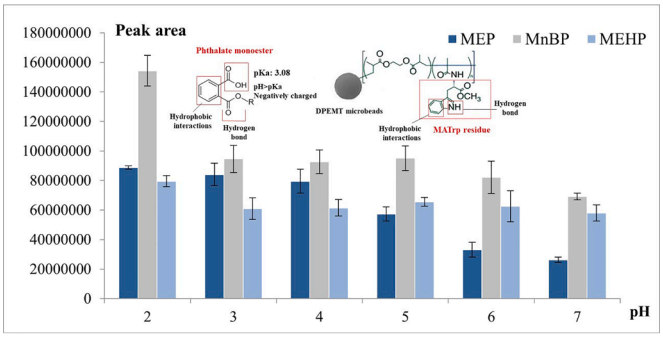

Fig. 3. Effect of medium pH on the extraction efficiency (100 mg sorbent; 2 mL water containing 0.2 µg of each standard (MEP, MnBP and MEHP) adjusted to different pHs; elution with 5 mL acetone; eluent evaporation in N_2 atmosphere and derivatization).

2.4.2. *Optimization of SPE procedure in synthetic urine*

The DPEMT-SPE cartridge washing procedure and synthetic urine dilution ratio were optimized to remove some interfering matrix compounds from the cartridge. For this purpose, synthetic urine was used to mimic the real matrix. As mentioned above, the conditioning procedure (3 mL of acetone and 10 mL of ultrapure water) was applied to the cartridges filled with 60 mg of DPEMT microbeads. A standard mixture (containing 0.1 µg of each standard (MEP, MnBP, and MEHP)) was added into 5 mL of synthetic urine (pH 2.0) diluted at different ratios (1:1, 1:5, 1:3, and 1:10), and the synthetic urine sample was passed through the cartridge at a constant rate (0.2 mL/min) with the help of vacuum manifold system. Subsequently, the cartridges were washed with different volumes of water (3, 5, 10, and 20 mL) adjusted to pH 2.0. The DPEMT -SPE cartridges were dried under vacuum and with N_2. The elution, internal standard addition, N_2 atmosphere evaporation, and derivatization procedures were performed as mentioned above, and then the samples were analyzed by GC-MS. The optimum SPE conditions for synthetic urine were determined considering the peak areas obtained from GC-MS analysis.

2.5. *Real sample analysis*

A 24-hour human urine sample was collected from a healthy volunteer. It was filtered through 0.45 µm filters and stored at −20 °C. Before the analysis, the urine sample reached room temperature and was homogenized by vortexing for 2 min. Firstly, the pH value of the 5 mL urine sample diluted in the ratio of 1:10 was adjusted to 6.8 with ammonium acetate buffer. A standard mixture (containing 0.2 µg of each standard (MEP, MnBP, and MEHP)) and 50 µL (100 units) of β-glucuronidase enzyme were added to the urine sample (pH 6.8) and glucuronide conjugate of monoester phthalates was hydrolyzed at 37 °C for 2 h. At the end of this period, hydrolysis was stopped by adding concentrated HCl (37 %, w/w) solution, and the pH of the sample was adjusted to 2.0. The samples were analyzed by GC-MS after passing through the DPEMT-SPE cartridge under the optimum SPE conditions. The applied procedure is summarized in Fig. 1.

3. Results and discussion

3.1. *Properties of the DPEMT microbeads*

The DPEMT microbeads were synthesized using DEP as a dummy template via the procedure developed in our previous study [39]. Because, in the previous study, the DMIP were successfully used as SPE sorbent for the enrichment of different PAEs [dimethyl phthalate (DMP), DEP, di-n-butyl phthalate (DBP), benzylbutyl phthalate (BBP), DEHP and di-n-octyl phthalate (DnOP)] from aqueous media prior to GC-MS analysis. The developed SPE method was successful because the microbead binding sites represented high affinity for the aromatic group of the PAEs. The aromatic group of the PAEs tended to interact with functional monomer (MATrp) via mainly hydrophobic interaction besides hydrogen bonds. The selective recognition capacity of the DPEMT microbeads used in this study (functional monomer to template ratio is 2:1) was previously determined using vanillic acid and styrene as competitor molecules. The relative selectivity coefficients for styrene

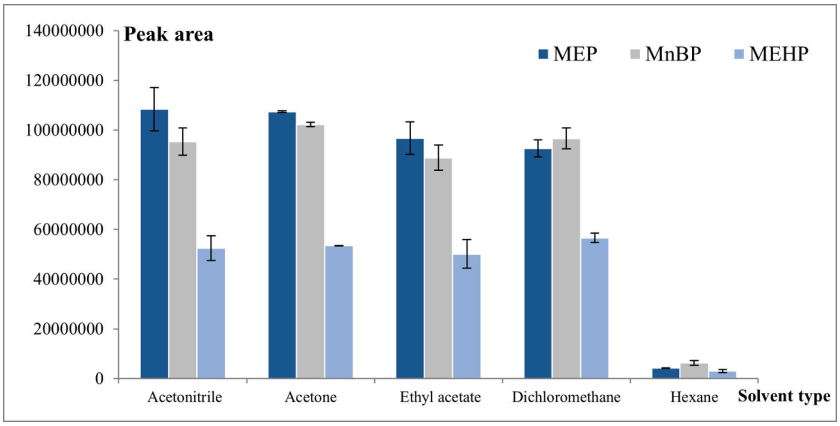

Fig. 4. Effects of elution solvents on the extraction efficiency (100 mg sorbent; 2 mL water containing 0.2 µg of each standard (MEP, MnBP and MEHP) adjusted to pH 2; elution with 5 mL organic solvent; eluent evaporation in N_2 atmosphere and derivatization).

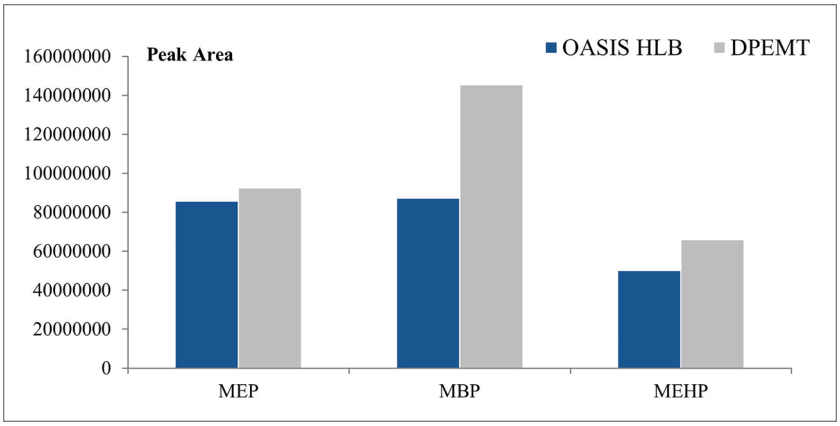

Fig. 5. Extraction efficiencies of DPEMT and commercial OASIS HLB SPE cartridges (60 mg sorbent; 5 mL water containing 0.2 μg of each standard (MEP, MnBP and MEHP) adjusted to pH 2; elution with 5 mL acetone; eluent evaporation in N_2 atmosphere and derivatization).

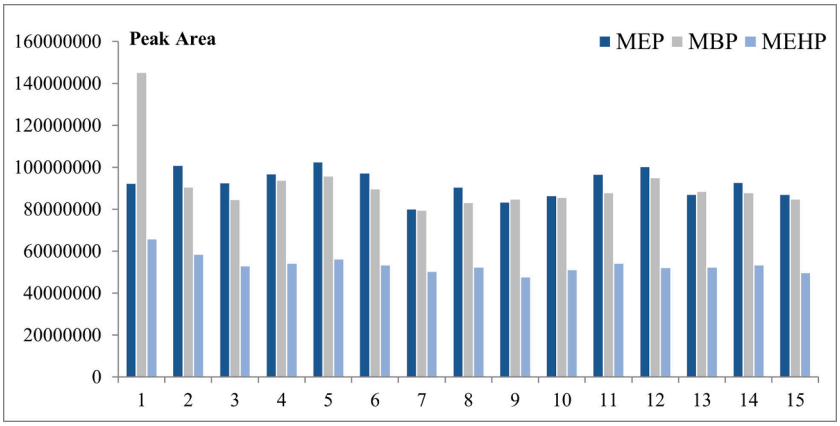

Fig. 6. Reusability of the DMIP-SPE cartridge for phthalate monoesters (60 mg sorbent; 5 mL water containing 0.2 μg of each standard (MEP, MnBP and MEHP) adjusted to pH 2; elution with 5 mL acetone; eluent evaporation in N_2 atmosphere and derivatization).

Table 2
Quantitative characteristic of DPEMT-SPE coupled with GC-MS detection method for the analysis of phthalate monoesters.

Analytes	Linear Range (ng/mL)	R^2	LOD (ng/mL)	LOQ (ng/mL)	Intra-day R (%)	Inter-day R (%)	Intra-day RSD (%)	Inter-day RSD (%)
MEP	4–60	0.9993	0.29	0.97	97.14	101.12	1.56	4.49
MnBP	4–60	0.9959	1.20	4.01	93.52	96.38	1.10	7.14
MEHP	4–40	0.9980	0.05	0.18	98.47	99.70	1.78	8.95

and vanillic acid were 2.02 and 1.86, respectively [39]. The imprinting factor (IF), the ratio of the maximum template molecule adsorption capacity (Qmax, mg/g) of the DMIP to that of NIP, was 1.28, verifying the selectivity of the template molecule. In the presented study, the main approach was to use the DPEMT microbeads, including selective binding sites for the aromatic group of phthalates, to enrich phthalates monoesters (MEP, MnBP, and MEHP) containing the same aromatic group. The chemical structures of the phthalates monoesters, DEP, and DPEMT microbeads are given in Fig. SI 1. The blue dashed lines show the repeating aromatic groups in the dummy template (DEP) and phthalates monoesters.

The morphological properties of DPEMT microbeads were examined using the SEM analysis (Fig. 2). SEM images of the DPEMT microbeads revealed that the polymeric structure was spherical. The surface of the microbeads were very rough, increasing the surface area and consequently enabling high adsorption capacity for phthalate monoesters. The microbeads in the diameter range of 150–300 μm were used as SPE sorbent.

FTIR analysis was performed for the chemical characterization of the DPEMT microbeads. In the FTIR spectrum (Fig SI 2), an amide carbonyl group (C=O) stretch at 1662 cm^{-1} and an ester carbonyl group (C=O) stretch at 1715 cm^{-1} were clearly observed. The absorption band observed at 2955 cm^{-1} was due to the stretch of the aliphatic C—H bonds repeating in the polymeric structure. At 3403 cm^{-1}, the absorption band resulting from the N—H stretching in the structure of the MATrp monomer appeared. These results showed that the functional monomer MATrp was successfully incorporated into the polymeric structure [39].

The specific surface area, pore size, and pore volume of the DPEMT microbeads were determined by BET analysis. P/P_o-V(cc/g) and pore

Table 3
Comparison of the developed DPEMT -SPE method with other methods reported in the literature for determination of phthalate monoesters in human urine.

Analytes	Chromatographic method	Sample preparation	Eluent	LOD (ng/mL)	Refs.
Monoethyl phthalate (MEP) Monobutyl phthalate (MnBP) Monocyclohexyl phthalate (MCHP) Monobenzyl phthalate (MBzP) Mono-2-ethylhexyl phthalate (MEHP) Mono-n-octyl phthalate (MnOP) Mono-isononyl phthalate (MiNP) Mono-isodecyl phthalate (MiDP)	HPLC-MS/MS Column: Betasil phenyl (5 μm, 50 mm × 3 mm)	SPE Oasis HLB Cartridge, 60 mg/3 mL and 60 mg/10 mL	Basic and acidic buffer	**MEP: 1.0** **MnBP: 0.6** MCHP: 0.7 MBzP: 0.8 **MEHP: 1.2** MnOP: 0.9 MiNP: 0.8 MiDP: 1.5	[16]
MEP MBzP MnBP MEHP MnOP MiNP Mono-isobutyl phthalate (MiBP)	GC-MS/MS Column: Restek Rxi-5MS (0.25 μm, 0.25 mm × 30 m)	SPME Divinylbenzene/carboxen /polydimethyl-siloxane fiber, 50/30 μm	Thermally desorbed	-	[22]
MEP MnBP MBzP MEHP Monomethyl phthalate (MMP)	HPLC-DAD Column: ZORBAX SB-C18 (5 μm, 250 mm × 4.6 mm)	SPE A mixed-mode strong anion-exchange (MAX) sorbent	Methanol (1 % formic acid)	**MEP: -** **MnBP: 6** MBzP: 6 **MEHP: 9** MMP: -	[24]
MMP MEP MnBP MEHP MBzP	GC-MS Column: BPX5 5 % phenyl (25 μm, 0.25 mm × 30 mm)	MSPE Magnetic multi-walled carbon nanotubes	Isopropanol	MMP: 0.03 **MEP: 0.025** **MnBP: 0.04** **MEHP: 0.05** MBzP: 0.05	[27]
MMP MEP MnBP MBzP MEHP	HPLC-UV Column: Acchrom (5 μm, 3.0 mm × 150 mm)	DSPME Magnetic dummy molecularly imprinted polymer	0.1 % Formic acid in H_2O and 0.1 % formic acid in methanol	MMP: 77 **MEP: 57** **MnBP: 96** MBzP: 194 MEHP: 404	[28]
MMP MBzP MnBP MEHP	HPLC-DAD Column: C18 Phenomenex (5 μm, 250 mm × 4.6 mm)	DPX	Acetonitrile	MMP: 7.6 MBzP: 7.6 **MnBP: 3.0** **MEHP: 7.6**	[31]
MEP MEHP Mono[2-(ethyl-5-carboxypentyl) hexyl] phthalate (MECPP) Mono-[2-(carboxymethyl)hexyl] phthalate (MCMHP) Mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) Mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP)	UHPLCMS/MS Column: Kinetex Phenyl- Hexyl (2.6 μm, 2.1 × 150 mm)	SPE OasisMAX cartridge, 150 mg/6 mL	Methanol (1 % formic acid)	**MEP: 0.14** **MEHP: 0.03** MECPP:0.03 MCMHP:0.02 MEOHP: 0.01 MEHHP: 0.02	[33]
MnBP MBzP MEHP	HPLC–MS/MS Column: Inertsil ODS-3 (5 μm, 50 mm × 2.1 mm)	On-line SPE HySphere-C HD (3 μm, 10 × 2 mm)	0.1 % Acetic acid and acetonitrile (75:25 v/v)	**MnBP: 1.3** MBzP: 1.7 **MEHP: 0.7**	[43]
MMP MEP MnBP MCHP MBzP MEHP MnOP MiNP MiDP MEOHP MEHHP	HPLC–MS/MS Column: Betasil phenyl (5 μm, 50 mm × 2 mm)	SPE Nexus, 60 mg and 200 mg	Acetonitrile and ethyl acetate	MMP: 0.7 **MEP: 1.2** **MnBP: 0.94** MCHP: 0.93 MBzP: 0.47 **MEHP: 0.86** MnOP: 0.77 MiNP: 0.79 MiDP: 0.5 MEOHP: 1.2 MEHHP: 1.6	[44]
MBzP MEP MnBP MEHP MEHHP	UPLCMS/MS Column: Kinetex Phenyl-Hexyl (1.7 μm, 2.1 mm × 100 mm)	SPE Bond Elut Certify LRC Cartridge, 130 mg/10 mL	Acetonitrile	MBzP: 0.19 **MEP: 0.28** **MnBP: 0.3** **MEHP: 0.19** MEHHP: 0.13	[45]

(*continued on next page*)

Table 3 (*continued*)

Analytes	Chromatographic method	Sample preparation	Eluent	LOD (ng/mL)	Refs.
MEOHP MiBP				MEOHP: 0.16 MiBP: 0.37	
MnBP MiBP MBzP MEHP	UPLC-MS/MS Column: Waters Acquity UPLC BEH Phenyl (1.7 μm, 2.1 mm × 100 mm)	SPE ABS ELUT-Nexus Cartridge, 60 mg/ 3 mL	Acetonitrile and methanol	**MnBP: 0.3** MiBP: 0.3 MBzP: 0.3 **MEHP: 0.3**	[46]
MMP MEP MnBP MiBP MBzP MEHP MEOHP MECPP MEHHP MCMHP	LC/MS/MS Column: AQUASIL C18 (4.6 × 150 mm)	SPE PLS cartridge, 60 mg/3 mL	Methanol	MMP: 0.02 **MEP: 0.2** **MnBP: 0.04** MiBP: 0.04 MBzP: 0.2 **MEHP: 0.6** MEOHP: 0.1 MECPP: 0.2 MEHHP: 0.03 MCMHP: 0.5	[47]
MEP MnBP MEHP	GC-MS Column: HP-5MS fused silica capillary column (0.25 μm, 0.25 mm × 30 m)	SPE DPEMT	Acetone	**MEP: 0.29** **MnBP: 1.2** **MEHP: 0.05**	This study

diameter (A^o)-dV(d)($cm^3/A^o/g$) graphs were prepared by using N_2 adsorption-desorption data obtained under vacuum at 77 K (Fig. SI 3). The specific surface area of the DPEMT was determined as 651.2 m^2/g. Type IV standard isotherms were obtained. According to the definition of IUPAC, adsorbent pores are divided into three groups; micropore (diameter < 2 nm), mesopore (diameter < 2–50 nm), and macropore (> 50 nm) [40]. The DPEMT microbeads' pore size was in the range of 0.996–6.52 nm, containing micro- and mesopores. The pore volume was 0.746 cm^3/g. The DPEMT microbeads have a high surface area increasing the adsorption capacity of the microbeads.

3.2. SPE efficiency of the DPEMT microbeads in aqueous media

3.2.1. Effect of pH

When the nature of functional groups of DPEMT microbeads and the chemical structure of phthalate monoesters are considered, the pH of the sample solutions has a critical role in extraction efficiency. Because the interactions (ionic interactions, hydrogen bonding, and hydrophobic interactions) between the DPEMT-SPE sorbent and the target analytes in the aqueous medium strongly depend on the pH of the sorption medium. Thus, the SPE procedure was performed using standard solutions at different pHs (2.0 to 7.0). The relationship between the extraction efficiency and the pH of the medium was determined by considering the chromatographic peak areas and is presented in Fig. 3. It can be seen that the peak areas of three phthalate monoesters decreases with increasing pH, demonstrating that the adsorbed amount of the phthalate monoesters decreased under basic conditions. At high pHs, phthalate monoesters ionized (pKa=3.08) and turned into negatively charged form; thus, the hydrophobic interaction between MATrp residues and phthalate monoesters decreased. In contrast, phthalate monoesters (MEP, MnBP, and MEHP) maintained their molecular form at pH 2.0, enabling the most effective adsorption via hydrophobic interaction with indole ring of MATrp monomer residues (Fig. 3). It should also be noted that the peak area of MEHP was less affected by the pH change, probably because of the existence of the nonpolar hexyl group, which interacts MATrp residues of DPEMT microbeads via hydrophobic interactions also at higher pHs.

3.2.3. Effect of elution solvent

The elution solvent used in an SPE procedure is crucial since it significantly affects the extraction efficiency of the targeted analytes. Volatility, price, reliability, stability, and toxicity of a solvent are determinative parameters to decide the solvent type to be used. In this study, elution of phthalate monoesters was carried out using organic solvents with various polarity indexes, such as acetonitrile (5.8), acetone (5.1), ethyl acetate (4.4), dichloromethane (3.1) and hexane (0.1) and the peak areas are shown in Fig 4. The higher the solvent polarity, the higher the peak area and the better the elution efficiency. Moreover, more polar solvents, such as acetonitrile and acetone, represented better elution performance and higher peak areas due to the polar nature of phthalate monoesters. Among the solvents, acetone was preferred as an elution solvent due to its high volatility compared to acetonitrile.

3.2.4. Effect of sorbent amount

The amount of the SPE sorbent must be sufficient for the complete and quantitative extraction of the target molecules. Therefore, the effect of sorbent amount on the extraction efficiency was investigated by varying the DPEMT microbeads' amount in the range of 10.0 to 100.0 mg, and the peak areas obtained for the targeted phthalate monoesters are given in Fig. SI 4. The results demonstrated that 60.0 mg of the sorbent was adequate for quantitatively extracting all analytes. Using a higher amount of sorbent had no significant effect on the peak area of the analytes. Therefore, 60.0 mg of the DPEMT microbeads were used for subsequent studies.

3.2.5. Effect of sample loading volume

The enrichment factor is the ratio of the analyte concentration in the collected phase to the analyte concentration in the sample. The sample volume loaded into the SPE cartridge is essential to obtain a high enrichment factor for the applied SPE procedure. Low-loading sample volumes are beneficial in reducing potential matrix effects and extraction times. However, the extraction efficiency and enrichment factor increase as the sample volume increases. In this study, the extraction efficiencies were evaluated by loading different sample volumes, 2, 3, 5, and 10 mL, into the SPE cartridges. When the peak areas obtained for the analytes by GC-MS analysis were considered, 5 mL was determined as the appropriate sample volume (Fig. SI 5).

3.2.6. Effect of salt concentration

Addition of a salt into the medium can either increase the extraction efficiency via the salting-out effect by decreasing the solubility of the analyte in the aqueous solution or lower the extraction due to mass

transfer difficulty from the aqueous solution to the sorbent [41]. In this study, the effect of ion strength on the extraction efficiency of phthalate monoesters was investigated by adding different concentrations of KCl solutions (0.01, 0.03, 0.06, and 0.1 M). The extraction efficiency decreased sharply with the increase in the concentration of KCl from 0.01 to 0.1 M (Fig. SI 6). As a result, the increase in the ionic strength in the environment caused an increase in the affinity of the targeted analytes to the DPEMT microbeads, and the extraction efficiency decreased.

3.2.7. Comparison of the extraction efficieny of DPEMT and OASIS HLB SPE cartridges

The commercial OASIS HLB cartridge is a preferred SPE cartridge for determining phthalate monoesters in urine [16,33]. Therefore, the extraction efficiency of the commercial OASIS HLB cartridge (60 mg, 3 mL) and the DPEMT-SPE cartridge (60 mg, 3 mL) were compared under optimum conditions. 40 ng/mL concentration of the standard mixture (MEP, MnBP, and MEHP) (5 mL) was used for SPE processes, and the peak areas obtained are represented in Fig 5. The results demonstrated that the DPEMT-SPE cartridge obtained higher peak areas for MEP, MnBP, and MEHP than the OASIS HLB SPE cartridge.

3.2.8. Reusability

The same DMIP-SPE cartridge, including DPEMT microbeads (60 mg, 3 mL), was used to extract a 40 ng/mL standard mixture solution 15 times under optimum conditions. The reusability of the DPEMT -SPE cartridge was evaluated by the peak areas (Fig. 6). The results of GC-MS analysis revealed that the RSDs of the peak areas of MEP, MBP, and MEHP were 6.6, 10.6 and 6.5 %, respectively. The DPEMT-SPE cartridge was reusable for phthalate monoester extraction before GC-MS analysis.

3.3. Effect of synthetic urine matrix on the SPE efficiency of DPEMT microbeads

Biological matrices are much more complex than many other matrices due to the presence of proteins, salts, and various organic compounds with chemistry similar to the related analytes [42]. Therefore, many compounds in SPE can interfere with targeted analytes. In addition, the high salt concentration in the urine reduces the extraction efficiency of phthalate monoesters. For these reasons, it is crucial to optimize the washing procedure before the elution of analytes from urine-loaded DPEMT-SPE cartridges to increase extraction efficiency, improve the purification effect and achieve maximum recovery of analytes in SPE. The washing procedure was optimized using synthetic urine to mimic real urine. For this purpose, the synthetic urine-loaded cartridges were washed with 3, 5, 10, and 20 mL water adjusted to pH 2.0. The peak areas obtained from GC-MS analysis were evaluated and it was determined that citric acid, 4-trimethyl silane (TMS) derivative, and 9H-Purine, 9-(trimethylsilyl)−2,6,8-tri[(*trimethylsilyl*)*oxy*]-species interfere with the retention times of the targeted analytes MBP and MEHP. These species were successfully removed by washing the SPE cartridge with 20 mL of water adjusted to pH 2.0.

Another parameter that needs to be optimized to eliminate the matrix effect is the dilution ratio of synthetic urine. For this purpose, 1:1, 1:5, 1:3, and 1:10 dilution ratios were studied. When the peak areas of the analytes were evaluated, it was seen that MEP had the same peak area at all dilution ratios (Fig. SI 7). It showed that peak areas did not change even as the dilution ratio increased for MEP. For MnBP and MEHP, on the other hand, an increase was observed in the peak areas with decreasing dilution ratio. However, the increase in the peak areas did not result from the analytes but appeared due to interfering species in the matrix. When the peak areas of all analytes were wholly evaluated, a dilution ratio of 1:10 was preferred since the matrix effect was minimized.

3.4. Method validation

Method validation parameters such as linearity, the limit of detection, the limit of quantification, and intra-day and inter-day reproducibility were investigated under optimum SPE conditions. SPE procedure was applied for the calibration solutions prepared by adding increasing concentrations (4–60 ng/mL) of phthalate monoester standards (MEP, MnBP, and MEHP) to the synthetic urine (5 mL) solution. Matrix-matching calibration solutions were analyzed by GC-MS, and calibration curves were plotted for each phthalate monoester using the peak areas. Linearity was obtained for MEP and MnBP analytes in the 4–60 ng/mL and for MEHP in the 4–40 ng/mL concentration range, and the regression (R^2) coefficients ranged from 0.9959 to 0.9993. Three and ten times standard deviations of calibration curves divided by their slopes to calculate the detection limits (LOD) and quantification limits (LOQ). The LOD and LOQ values for phthalate monoesters (MEP, MnBP, and MEHP) were found in the range of 0.05–1.20 ng/mL and 0.18–4.01 ng/mL, respectively. To determine the accuracy and precision of the developed method, a standard mixture was added to the synthetic urine in the concentration of 20 ng/mL during the day and analyzed by GC-MS by applying the optimized SPE procedure. These procedures were carried out with five repetitions during the day. In the inter-day study, the SPE procedure was applied with the addition of a standard to the synthetic urine (20 ng/mL), and intra-day and inter-day reproducibility were determined. Intra-day and inter-day relative standard deviation (RSD %) data ranged from 1.10 % to 1.78 % and 4.49 % to 8.95 %, respectively (Table 2). Intra-day and inter-day accuracy expressed as percentage recovery (R %) was calculated from the found mass of the analytes on the spiked urine samples toward added mass after replicate analysis of spiked samples at 20 ng/mL concentration.

The developed DPEMT-SPE–GC-MS method was compared with previously reported methods. Table 3, compares DPEMT-SPE–GC-MS with other SPE methods for determining phthalate monoesters reported in previous studies. It is quite apparent that the extraction efficiency of the proposed method is higher than the magnetic dummy MIP-SPE-based method [28], and the LOD of the proposed DMIP-SPE method is also superior or equal to the previous SPE or SPME-based methods in the literature. Therefore, it can be applied to analyzing phthalate monoesters in urine samples.

3.5. Real sample analysis

The DPEMT-SPE method, in which the sample preparation steps were optimized, was used to determine phthalate monoesters in a real urine sample. A mixture of standards (MEP, MnBP, and MEHP) at 40 ng/mL concentration was added to the 24-hour urine samples collected from a healthy volunteer and analyzed under optimum conditions to demonstrate the method's applicability. When the method was applied, the MEP, MnBP, and MEHP concentrations in real urine were below the detection limits. In the recovery studies, the recovery values of the analytes were found to be 97.45 %, 98.03 %, and 109.26 % for MEP, MnBP, and MEHP, respectively.

4. Conclusion

In conclusion, a novel SPE method utilizing dummy molecularly imprinted polymers was successfully developed and optimized for determining phthalate monoesters in urine samples by GC-MS analysis. DEP molecule, a phthalate ester, was used as a dummy template to prepare DMIP to extract MEP, MnBP, and MEHP. The hydrophobicity of the MATrp functional monomer used in the molecular imprinting procedure increased the affinity of the phthalate monoesters containing mainly hydrophobic groups to the binding sites. The DPEMT microbeads showed high selectivity for phthalate monoesters. The developed SPE method exhibited high selectivity, sensitivity, linearity, and repeatability with low LOD and LOQ values. The method was validated by

analyzing real urine samples, where the analytes were detected below the detection limits and showed satisfactory recovery rates (MEP: 97.45 % MnBP: 98.03 %, and MEHP:109.26 %). The DPEMT-SPE cartridge showed superior performance compared to commercial OASIS HLB cartridge. Sample preparation steps, including loading, washing and extraction procedures, and derivatization of the analytes, are the limitations of the developed method; however, they are inevitable due to the chemical structure of the analytes, composition of the urine, and the nature of the analysis method. Overall, the developed SPE method shows promise as a reliable and efficient approach for assessing phthalate exposure in real urine samples.

CRediT authorship contribution statement

Melike Küçük: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Bilgen Osman:** Conceptualization, Validation, Writing – review & editing. **Elif Tümay Özer:** Conceptualization, Project administration, Supervision, Validation, Writing – review & editing.

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.

Data availability

The data that has been used is confidential.

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Supplementary materials

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