Original Article

Method Development of Di-(2-Ethylhexyl) Phthalate Metabolites Detection by Dispersive Liquid–liquid Microextraction Gas Chromatography–mass Spectrometry from Urine

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Abstract

Aims: Phthalates (e.g., diethylhexyl phthalate) act as one of the endocrine disruptive compounds and can have adverse effects on different biological pathways including hormonal receptors, affecting the synthesis, secretion, or metabolism of hormones. The aim of this study was, method development of the dispersive liquid-liquid microextraction (DLLME), to determine the monoesters of diethylhexyl phthalate in children and adolescents in Isfahan city, Iran. **Materials and Methods:** This study was conducted in 2016 (during a year) on 242 children and adolescents, aged 6–18 years, living in Isfahan, Iran. In this method, acetonitrile and chlorobenzene were used as disperser and extractant, respectively, to extract the investigated monoesters. The analytes were determined by gas chromatography–mass spectrometry. **Results:** Acetonitrile (as disperser) and chlorobenzene (as extractant) were used for monoester phthalates extraction (750 µl of acetonitrile and 80 µl of chlorobenzene). The results indicated that the recovery and relative standard deviation (RSD) of the utilized method were 55–109 and 6.3%–13.2%, respectively. The limit of detection and limit of quantification (LOQ) of the method were 0.024–0.088 and 0.05–0.48 µg/L, respectively. Monoethylhexyl phthalate (MEHP), monoethyl oxo-hexyl-phthalate (MEOHP), and monoethyl hydroxyl-hexyl-phthalate (MEHHP) were observed in 99.6%, 95.87%, and 96.28% of the studied samples, respectively. The mean concentration of MEHP, MEOHP, and MEHHP in the study population was 151.7 ± 143.8, 258.26 ± 143.07, and 194.17 ± 147.3 µg/L, respectively. **Conclusion:** Acetonitrile (as disperser) and chlorobenzene (as extractant) were suitable solvents for phthalate monoesters extraction in method development of DLLME. On the other hand, the mean concentrations were very high values in comparison with other similar studies that require the attention and legislation regarding limitation of the application of phthalate compounds.

Keywords: Biomonitoring, Isfahan, metabolite, urinary phthalates

Introduction

Phthalates are industrial chemicals used as a plasticizer to develop resilience and stability in plastic products,[1-5] personal care products (i.e., cosmetics, perfume, and lotions), colors, and glaze. Low molecular weight phthalates (e.g., diethyl phthalate, di-n-butyl phthalate, di-n-octyl phthalate, and di-n-isobutyl phthalate) are mostly used in shampoos, cosmetics, lotions, and other personal care products to preserve perfume and scent. On the other hand, high molecular weight phthalate (e.g., di-2-ethylhexyl phthalate and butyl benzyl phthalate [BBzP]) are utilized for production of plastic to be used in floor covering and light packaging of foods.[6-8] Over the past decade, concerns over the health risks associated with exposure to the phthalate compounds, especially sensitive population such as pregnant mothers and children, have increased.[9] Phthalate acts as an endocrine glands' disruptor and can affect hormonal receptors, synthesis, secretion, or metabolism of hormones through biological pathways.[10,11]

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Exposure to phthalate alters the hormonal level and brings about adverse effects to men fertility, precocious puberty in girls (exposure to phthalates causes girls to reach puberty earlier and have a shorter stature), increased incidence of chronic diseases, and possible role in the development of cancer.[6]

Due to the toxic properties of phthalates, the European Commission has inhibited the use of phthalate compounds such as di-2-ethyl hexyl phthalate (DEHP), dibutyl phthalate (DBP), and BBzP in the production of toys and goods associated with children. Phthalates such as DEHP, DBP, and BBzP should not be used in cosmetics and consumer products such as colors and resins. Moreover, their usage in materials that are in contact with food products is not allowable either. Usage of DEHP in medical polyvinyl chloride devices is limited for cases when it causes exposure of newborn infants.[12] Metabolism of phthalates develops monoester phthalate compounds, which can be metabolized into oxidative compounds. Phthalates are rapidly metabolized in the body through hydrolysis mechanism (over 24–48 h after exposure) and are converted to monoester metabolites. They are then re-metabolized or removed from the body in urine in the form of glucuronides. Figure 1 represents the metabolism pathway of DEHP decomposition in the human body.

Biomonitoring is used for evaluation of human exposure to chemicals. Hines *et al*. investigated the concentration of metabolites in different matrices (blood, urine, milk, saliva, and serum).[13] To use biomonitoring in the evaluation of human exposure to nonresistant chemicals, urine is usually a suitable choice, as the levels of metabolites are higher in urine than in blood, and its collection is also easier. Various studies have been conducted regarding biomonitoring of urinary metabolites of phthalate including biomonitoring of phthalate compounds across pregnant women in Rotterdam City in the Netherlands,[14] Danish mothers and children,[15] Canadian people (6–49 years),[16] 8–13 year-old Mexican children,[17] and 3–14 year-old German children.[12] The most extensive biomonitoring study has been conducted by Centers for Disease Control and Prevention (CDC) in the US in the form of a plan called National Health and Nutrition Examination Survey (NHANES). This plan has taken a representative statistical sample out of the entire US population and evaluated the status of health and nutrition of this population and their exposure to a wide range of chemicals including phthalate metabolites.[18] CDC has reported measurable concentrations of the phthalate metabolites including mono ethyl phthalate, mono-butyl phthalate, and mono-benzyl phthalate in over 97%, and mono-2-ethyl hexyl phthalate (MEHP) and monomethyl phthalate in over 75% of the US population.[19]

As the biomonitoring program is essential to investigate the presence of contaminants in human samples, in this research, attempts were made to investigate the concentration of three major metabolites of di-2-ethyl hexyl phthalate (DEHP) including mono (2-ethyl-5-exohexyl) phthalate, mono (2-ethyl-5-hydroxy hexyl) phthalate, and mono-(2-ethyl hexyl) phthalate in children and adolescents of Isfahan city through method development of dispersive liquid–liquid microextraction (DLLME).

Materials and Methods

The study population

A total of 242 people of children and teenagers (6–18 years of age) in Isfahan city, Iran, who were referred to developmental disorder clinics in Amin Hospital in Isfahan city were chosen randomly. Then, by getting their informed consent, their fasting urine sample was collected in a polyethylene container. Two milliliters of the urine sample was separated for determination of creatinine, and the rest of it was kept at −20°C until use.

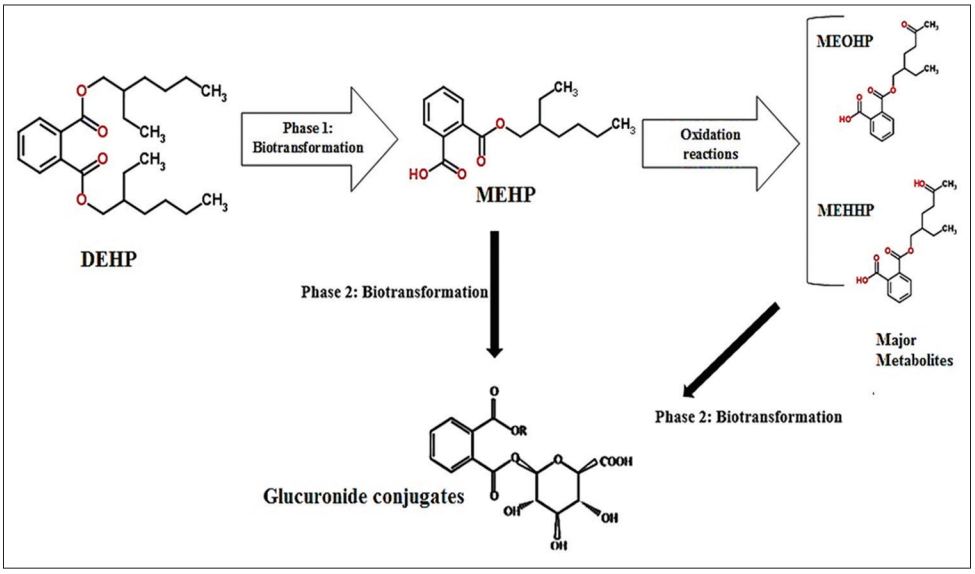


Figure 1: The metabolism pathway of DEHP decomposition

Chemicals

The monoesters of MEHP, 5OH-MEHP, 5oxo-MEHP (analytical standard), β-glucuronidase enzyme (from *Escherichia coli*), and N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Sigma-Aldrich Co., whereas the other materials (methanol, HCl, acetonitrile, and chlorobenzene) were purchased from Merck Co., Germany.

Preparation of the standards

All of the utilized laboratory glasses were washed with acetone and hexane and exposed to 200°C in an oven for 24 h after being rinsed with distilled water so that the absence of phthalate compounds was ensured. Following withdrawal from oven and cooling down, the containers were rewashed with distilled water. Standard solutions of metabolites were prepared in methanol using standard experimental methods and stored in Teflon-capped amber glass bottles at −20°C until use. The concentrations of 0.001, 0.005, 0.01, 0.05, 0.1, and 0.5 μg/mL of the investigated metabolites were employed to plot calibration curve.

Preparation of the sample and extraction

The urine samples (242 persons) were kept at −20°C and before use, they were melted at room temperature. A total of 5 ml of the urine sample was poured into a glass test tube made of borosilicate, and for digesting the samples and changing the state of compounds from glucuronide state, 20 μl of the β-glucuronidase enzyme was added to it. The samples were placed inside incubator shaker device (37°C for 18 h). Once the samples were withdrawn from the incubator shaker device, 2.5 ml of the urine sample was diluted and its pH was adjusted at 2 using sulfuric acid 10% (using AD1020 pH meter). To extract the phthalate metabolites from the liquid phase, acetonitrile (as disperser) and chlorobenzene (as extractant) were used with different values. Following several times of trial and error, the best extraction was obtained at 750 μl of acetonitrile and 80 μl of chlorobenzene. Chlorobenzene and acetonitrile were injected to the samples quickly to obtain a cloudy solution. The samples were then placed in centrifuge device (for 5 min at 6000 rpm). The sediment in the bottom of the tube (20 ml) was extracted by syringe and dried by dry nitrogen gas, and following the addition of 20 μl of MSTFA for derivatization, it was injected into gas chromatography–mass spectrometry (GC/MS) device. The obtained data were analyzed by SPSS (version 16.0, IL, USA) using statistical tests. All tests were evaluated at the error value of 5%.

Instrumental analysis

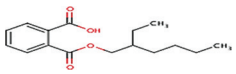
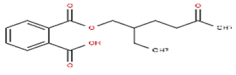
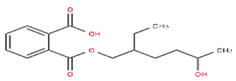
To measure the value of phthalate urinary metabolites, GC (Agilent, model: 7890A) equipped with MSD detector (Agilent, model: 5975C) made by Agilent Tech Co., USA, was used. Chemstation software (Win XP) was utilized to employ GC/MSD device. DB5-MS column (Agilent Co., length = 60 m, column diameter = 0.25 mm, and internal film diameter = 25 μm) was used. Helium (Grade 5.5) was used as the carrier gas with a flow rate of 1 ml/min. The injector was used at 270°C in split form with a ratio of 2:1 to inject the sample. The oven temperature began with 100°C (initial temperature), then remained at this temperature for 3 min, and after that was brought to 300°C with a rate of 20°C/min, and remained at this temperature for 7 min. The ion source temperature and transfer line temperature were adjusted at 230°C and 290°C, respectively. MSD detector was put in selected ion monitoring (SIM) mode. The ions used for SIM, retention time, and time window for the investigated metabolites are presented in Table 1. Calibration curves for all of the three phthalate monoesters were plotted with a coefficient of determination over 0.97 (r^2 = 0.97). Figure 2 represents a typical sample of the calibration curve for MEHP compound. To normalize the diversity of urine density, creatinine (Cr) was measured by Jaffe method using Hitachi 704 autoanalyzer.[20]

To extract phthalate monoesters in biological samples (urine matrix), DLLME method was used. In DLLME, factors including the type and volume of the extractant and disperser as well as extraction time influence the extraction efficiency. To select optimal extraction conditions, a preliminary study was performed to find the suitable extractant and disperser solvent. The selected solvents were acetonitrile (disperser) and chlorobenzene (extractant). The typical chromatograms from GC/MS device for the three phthalate monoesters are shown in Figure 3a. In Figure 3b, mono ethyl phthalate of selected ions are indicated for MEHP analyte

Validation

The limit of detection (LOD) and limit of quantification (LOQ) were 3 and 10 times the concentration of the signal-to-noise

Table 1: Chemical structure, retention time, selected ions, and time window for investigated analytes (monoethylhexyl phthalate, MEOHP, monoethyl hydroxyl-hexyl-phthalate)

Metabolites	Chemical structure	Retention time (min)	Selected ions	Time window
MEHP		14.7	149, 221, 239	14-15.1
MEOHP		15.8	108, 127, 149, 221, 239	15.6-15.95
MEHHP		16.05	117, 147, 221, 265, 295	15.95-20

MEHP: Mono ethylhexyl-phthalate, MEHHP: Monoethyl hydroxyl-hexyl-phthalate

ratio. Trueness can be assessed in large concentration ranges with recovery studies. Recovery is defined as the "proportion of the amount of analyte, present or added to the analytical portion of test material, which is extracted and presented for measurement."

The "apparent recovery" is calculated as:

$$R = \frac{C}{C_{ref}} \times 100 \quad (1)$$

Where C is the concentration found with the method to be validated and C_{ref} is the reference concentration.

A generally accepted definition of relative standard deviation (RSD) is "the standard deviation (S) of a set of data, divided by the mean (X_{avg}) of the data set, expressed in units of percent." Thus, the formula is:

$$RSD\% = \frac{S}{X_{ave}} \times 100 \quad (2)$$

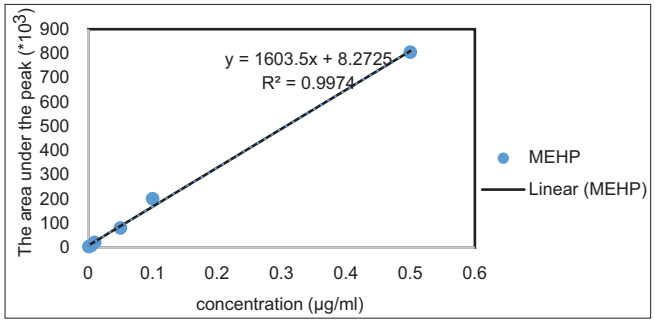


Figure 2: A calibration curve for monoethylhexyl phthalate representing all analytes

Results

Validation

The results of validation are provided in Table 2. The results indicate that RSD lies between 6.2% and 13.2%, suggesting the high accuracy of the experiments. The recovery of studied metabolites was obtained through calculation of the ratio of the value of a measured analyte to the real value of the metabolite. The recovery was investigated across three concentrations of low, medium, and high. Based on Table 2, the recovery of MEHP, monoethyl oxo-hexyl-phthalate (MEOHP), and MEHHP metabolites was 55–90, 68–96, and 69%–109%, respectively. LOD and LOQ were examined for every phthalate monoester, using $3S_0$ and $10S_0$, respectively. S_0 is the standard deviation obtained from the blank analysis (in this study: 15 blank analyses). LOD and LOQ are presented in Table 2 for each studied monoester. LOD and LOQ for the studied monoesters were around 0.02–0.09 and 0.05–0.5 µg/L, respectively. Based on Table 3 and comparison with other studies, the results show that the detection limit, detection value, RSD, and recovery of the utilized method are desirable.

Monitoring the phthalate monoesters in the studied population

From the 242 people under investigation, 140 (57.9%) and 102 (42.1%) participants were female and male, respectively. MEHP, MEOHP, and MEHHP were observed in 99.6%, 95.87%, and 96.28% of the studied samples, respectively. According to Table 3, the mean concentration of MEOHP is greater than that of MEHHP and MEHP. Furthermore, in this table, the mean concentration adjusted with creatinine has also been provided.

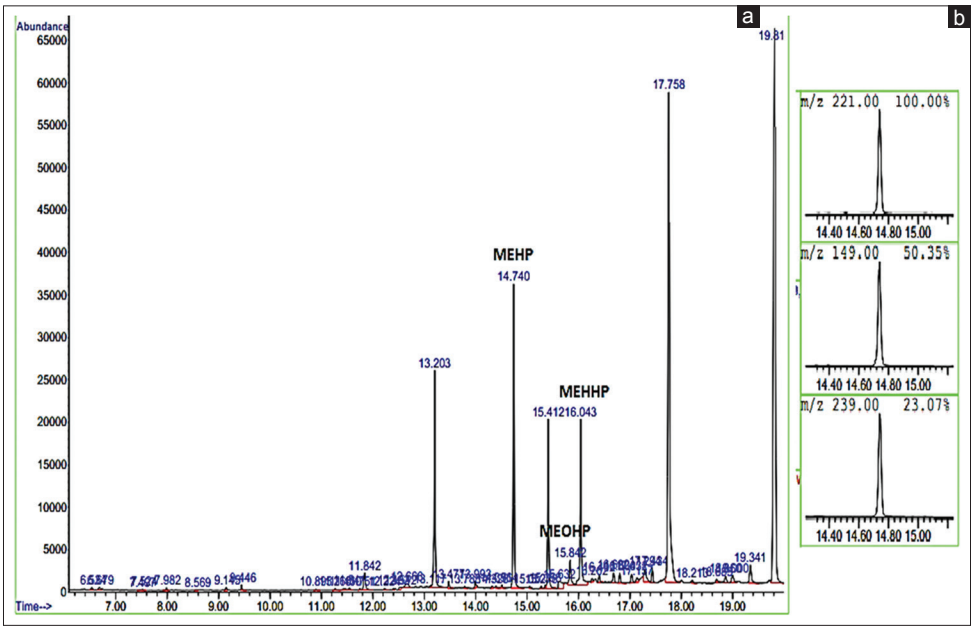


Figure 3: A typical chromatogram for a standard mixture of monoethylhexyl phthalate, MEOHP, and monoethyl hydroxyl-hexyl-phthalate phthalate monoesters (a), monitored ions (m/z) of monoethylhexyl phthalate (b)

Table 2: The results of validation of dispersive liquid-liquid microextraction/gas chromatography-mass spectrometry method for monoester phthalate analytes

Metabolites	LOD	LOQ	Recovery%			RSD (%)	R^2
			Low concentration (1 μ/L)	Medium concentration (10 μ/L)	High concentration (100 μ/L)		
MEHP	0.024	0.05	90	55	69	13.2	0.997
MEOHP	0.23	0.48	80	68	96	8.5	0.977
MEHHP	0.088	0.18	69	109	105	6.2	0.995

LOD: Limit of detection, LOQ: Limit of quantification, MEHP: Mono ethylhexyl-phthalate, MEHHP: Monoethyl hydroxyl-hexyl-phthalate, RSD: Relative standard deviation

Table 3: Mean concentration, creatinine-adjusted concentration, and median of mono ethylhexyl-phthalate, MEOHP, and monoethyl hydroxyl-hexyl-phthalate analytes in investigated population

Metabolite	Number of positives	Concentration			
		Mean±SD (μg/l)	Median (μg/l)	Creatinine-adjusted (μg/g creatinine)	Median (μg/g creatinine)
MEHP	241 (1< LOD)	151.69±143.8	101.97	499.02±475.8	372.85
MEOHP	232 (10< LOD)	257.9±143.4	270.9	845.6±559.8	786.04
MEHHP	233 (9< LOD)	193.8±147.67	177.56	682.07±603.4	536.4

SD: Standard deviation, LOD: Limit of detection, MEHP: Mono ethylhexyl-phthalate, MEHHP: Monoethyl hydroxyl-hexyl-phthalate

Discussion

LOD and LOQ are presented in Table 2 for each studied monoester. LOD and LOQ for the studied monoesters were around 0.02–0.09 and 0.05–0.5 μg/L, respectively. Based on Table 4 and comparison with other studies, the results show that the LOD, limit of quantification, RSD, and recovery of the utilized method are desirable.

According to Table 4, and based on a study of Herrero *et al*., determination of primary and secondary phthalate metabolites in urine samples was conducted by GC/MS. Extraction was done by hexane (2 mL) and sodium chloride (0.3 g). For derivatization, dichloromethane (80 mL), (TMSDM) trimethylsilyldiazomethane (1.5 mL), and methanol (15 mL) were added.[21] Comparison of the current study showed that LOD and LOQ in this study were lower than this shows the higher accuracy of method development by acetonitrile and chlorobenzene. On the other hand, RSD in this study (6.2–13.2) was higher than the study of Herrero *et al*.[25] Based on Table 3, the highest analyte concentration in the studied population is related to MEOHP. The concentration median and median adjusted concentration for MEOHP were 257.9 ± 143.4 and 845.6 ± 559.8 μg/L, respectively. On the other hand, the geometric mean of MEHP, MEOHP, and MEHHP in the male gender was 83.7, 191.9, and 132.2 μg/L, respectively. The geometric mean of these compounds in the female gender was 75, 169.6, and 102.6 μg/L, respectively. According to independent *t*-test analysis, no significant difference was observed between the male and female groups in terms of the level of studied metabolites (*P* value [MEHP] = 0.55,9 *P* value [MEOHP] = 0.269, and*P* value [MEHHP] = 0.231). These results have a significant difference with the investigations by NHANES in 2012. According to the investigation by this institute, the geometric mean of MEHP, MEOHP, and MEHHP in the male gender was 1.51, 5.5, and 8.71 μg/L, respectively, whereas in the female gender, the values were 1.24, 4.71, and 7.2 μg/L, respectively.[18] In spite of the limited number of samples in this study, the obtained results indicate a significant difference with the uses of phthalate diesters in Iran (Isfahan) and the USA. According to NHANES investigation, from 1999 to 2012, the level of DEHP metabolites has had a significant decrease in the studied groups, which can be due to the strict rules that have been enforced in some countries including American and European countries. DEHP is one of the most widely used phthalate esters, especially in the packaging industry. As chemically DEHP does not attach to the polymer, and during the production and application, it is easily detached off the polymer; thus, it can transfer through in air, water, food, and medical equipment (e.g., blood bags and injection instruments). However, the most important source of exposure to phthalate compounds for a human is through swallowing. According to the investigations in this study, questions were asked about usage of plastic packaging for storage of foods and usage of plastic toys by children, where 64.9% of the households used plastic packaging. Nearly 71.5% of the children used plastic toys, which can be the main reason for the higher level of DEHP metabolites in the urine of the studied group. In the study by Song *et al*. on biomonitoring of DEHP metabolites, the results indicated that the geometric mean of MEHP, MEOHP, and MEHHP metabolites in South Korean children was 18.7, 111.1, and 109.1 μ/g of creatinine, which are lower values than those obtained in this study.[24] The geometric mean of MEOHP and MEHHP in the study of Meeker *et al*. on male population of Boston city in Massachusetts state was 36.2 and 55.6 μg/L, respectively, with these values in this study around five and two times as large as Meeker's study, respectively.[29]

Table 4: Comparison of the current study with other studies about biomonitoring phthalate metabolites

Methods	Matrix	Analytes	Extraction and derivatization	LOD (µ/L)	LOQ (µ/L)	RSD (%)	Reference
LLS-GC/MS*	Urine	MEHP, MEOHP, MEHHP	ethyl ether/n-hexane	0.05-0.2	0.10.5	4.76-16.3	21
LLS-GC/MS	Urine	MEHP, MEOHP, MEHHP	LLE with diethyl ether, derivatization with TEOTFB in DCM. LLE with hexane	13-25	-	6-16	22
LLS-GC/MS	Urine	MEP, MBP, MEHP, MBzP	LLE with hexane and derivatization with diazomethane. Florisil cleanup	0.05-0.1	-	0.6-6.1	23
LLS-GC/MS	Urine	MEHP, 5-OH-MEHP, 5-oxo-MEHP	LLE with hexane: ether, derivatization with TMCS + BSTFA	-	-	-	24
GC/MS	Urine	MEHP, 5oxo-MEHP, 5OH-MEHP	hexane (2 mL) and sodium chloride (0.3 g)/for derivatization dichloromethane (80 mL), TMSDM (1.5 m L), and methanol (15 mL) add	0.23-8.89	0.77-29.6	2-14	25
SPE/UHPLC- MS**	Urine	MEHP, 5oxo-MEHP, 5OH-MEHP	SPE cartridge (20 0 mg) was packed on a glass column and equilibrated with acetonitrile (5 mL), water (1 mL), and sodium phosphate buffer	0.07-0.1	0.23-0.35	1.5-1.7	25
SPE/GC-MS	Urine	MEHP, 5oxo-MEHP, MBzP	hydrochloric acid/ethyl acetate/ derivatization by MTBSTFA	-	-	16.7-26.8	26
SPE/(LC-MS/MS)	Urine	MEHP, 5oxo-MEHP, MBzP, mEOHP	ammonium hydroxide buffer (0.15% w/v NH4 OH in 1:1 acetonitrile/water) was added to the samples, which were loaded onto the 60 mg solid phase extraction cartridge	-	-	2.8-7.9	27
HPLC -APCI-MS/MS***	Urine	MEHP, MBzP	SPE	0.8-1.2	-	9.6-10.6	28
DLLME-GC/MS	Urine	MEHP, MEOHP, MEHHP	For extraction, acetonitrile (as disperser), and chlorobenzene (as extractant)/20 µl of MSTFA for derivatization	0.02-0.09	0.05-0.5	6.2-13.2	This study

*LLE-GC/MS, ****SPE/UHPLC-MS, ***APCI-tandem mass spectrometry. GC/MS: Gas chromatography-mass spectrometry, APCI: Atmospheric pressure chemical ionization, SPE/UHPLC-MS: Solid-phase extraction/Ultra high-performance liquid chromatography-mass spectrometry, MEHP: Mono ethylhexyl-phthalate, MEHHP: Monoethyl hydroxyl-hexyl-phthalate, LLE: Liquid-liquid extraction, RSD: Relative standard deviation, DLLME: Dispersive liquid-liquid microextraction, MSTFA: N-Methyl-N- (trimethylsilyl) trifluoroacetamide, TEOTFB: triethyloxonium tetrafluoroborate, TMCS: trimethyl chloro silane, BSTFA: bis(trimethylsilyl)-triflouracetamide, TMSDM: trimethylsilyldiazomethane, MTBSTFA: N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide, HPLC: high-performance liquid chromatography

In the study conducted by Kim *et al*. in Korea, the mean concentration of MEHP, MEOHP, and MEHHP metabolites was 14.4, 34.77, and 26.75 µg/g of creatinine, respectively. The results of the present study indicate that the concentration of these metabolites in Isfahan (Iran) is 25.9, 22.6, and 20 times more than their concentration in Korea.[21] The concentrations of the three DEHP metabolites in this study were significantly correlated with each other according to Pearson correlation ($P < 0.001$), suggesting that these metabolites originate from a single mother compound (DEHP).

Conclusion

In this study, DLLME method was employed for extracting diethyl hexyl phthalate monoesters in urine matrix. Validation of the data indicates that the studied method is reliable (RSD = 6.2%–13.2%). On the other hand, LOD = 0.024–0.088 and LOQ = 0.05–0.48 in this method in comparison with other methods reveal the accuracy of the studied method. The mean concentration of MEHP, MEOHP, and MEHHP monoesters in the studied population was 151.7 ± 143.8, 258.143 ± 26.07, and 194.17 ± 147.3 µg/L, respectively, which are very high values in comparison with other similar studies that requires the attention and legislation regarding limitation of the application of phthalate compounds. It also highlights the necessity of biomonitoring in human samples more than ever.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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