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Quantification of 22 phthalate metabolites in human urine☆

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Abstract

Phthalates are ubiquitous industrial chemicals with high potential for human exposure. Validated analytical methods to measure trace concentrations of phthalate metabolites in humans are essential for assessing exposure to phthalates. Previously, we developed a sensitive and accurate automated analytical method for measuring up to 16 phthalate metabolites in human urine by using on-line solid phase extraction coupled with isotope dilution–high performance liquid chromatography (HPLC)–electrospray ionization-tandem mass spectrometry. To include the measurement of seven additional analytes, including oxidative metabolites of diisononyl and diisodecyl phthalates, two chemicals used extensively in numerous consumer products, we used a novel nontraditional HPLC solvent gradient program. With this approach, we achieved adequate resolution and sensitivity for all 22 analytes with limits of detection in the low ng/mL range, without increasing the analytical run time. The method also has high accuracy with automatic recovery correction, high precision, and excellent sample throughput with minimal matrix effects. Although it is possible to measure these 22 phthalate metabolites with adequate precision and accuracy at sub-parts-per-billion levels, additional information, including toxicokinetic data, is needed to demonstrate the usefulness of these phthalate metabolites for exposure assessment purposes .
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Keywords: Reverse HPLC gradient; Phthalate metabolites; Phthalates; Biomonitoring; Exposure assessment; Diisodecyl phthalate; Diisononyl phthalate; Phthalate analysis

1. Introduction

Phthalates are a group of industrial chemicals widely used in consumer products as solvents, additives, and plasticizers [1]. Several phthalates are known to cause carcinogenic, reproductive, and development toxicities in animals [2–8]. Despite an emerging number of studies in this field [9–12], data on the effects of phthalate exposure in humans are still limited.

Humans can be exposed to phthalates through food, water, air, and using phthalate-containing consumer products. After exposure, phthalates are rapidly metabolized to their respective hydrolytic monoesters. For some phthalates, the monoesters can be further metabolized to their oxidative products [13–19] before excretion in urine or feces either as free or conjugated species [2,20–23]. These metabolites have been used as biomarkers of exposure to phthalates.

A reliable and sensitive method to measure the concentration of phthalates in humans is essential for exposure assessment [24–26], and eventually for understanding how human health might be affected by exposure to phthalates. Recently, we developed a method based on on-line solid phase extraction coupled with isotope dilution–high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) to measure the urinary concentrations of 16 phthalate metabolites at low parts-per-billion levels [25]. Here, we modified our previous analytical method to quantify 22 phthalate metabolites in human urine: phthalic acid (PA), monomethyl phthalate (MMP), monoethyl phthalate (MEP), mono-3-carboxypropyl phthalate (MCPP), mono-*n*-butyl phthalate (MBP), mono-isobutyl phthalate (MiBP),

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monocyclohexyl phthalate (MCHP), monobenzyl phthalate (MBzP), mono-(2-ethylhexyl) phthalate (MEHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-*n*-octyl phthalate (MOP), mono-isononyl phthalate (MNP), mono-isodecyl phthalate (MDP), mono-*n*-hexyl phthalate (MHxP), mono-*n*-heptyl phthalate (MHpP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP), mono-carboxy-*n*-heptyl phthalate (MCHpP), mono-carboxy-isooctyl phthalate (MCOP), mono-hydroxyisononyl phthalate (MHNP), mono-oxoisononyl phthalate (MONP), and mono-carboxyisononyl phthalate (MCNP), in one analytical run, without compromising the sensitivity or the analytical run time.

2. Experimental

2.1. Reagents

MMP, MEP, MBP, MCHP, MBzP, MEHP, MOP, MNP, MDP, MEOHP, and MEHHP (>99.9%), their $^{13}C_4$-labeled internal standards (>99.9%), and $^{13}C_4$-4-methyl-umbelliferone were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). MCPP and $^{13}C_4$-MCPP were obtained from Los Alamos National Laboratory (Los Alamos, NM, USA) and from Cambridge Isotope Laboratories, Inc. PA and $^{13}C_2$-PA, 4-methylumbelliferone (4-MeUmb) and its glucuronide, and ammonium acetate (>98%) were purchased from Sigma Aldrich Laboratories, Inc. (St. Louis, MO, USA). MECPP, MiBP, D_4-MiBP, MONP, and MHNP were generous gifts from Prof. Jürgen Angerer (University of Erlangen-Nuremberg, Germany). MHxP, MHpP, MCHpP, MCOP, and MCNP were purchased from Cansyn LLC (Ontario, Canada). Acetonitrile and water (HPLC grade) were purchased from Tedia (Fairfield, OH, USA), and formic acid (98% min, GR) was purchased from EM Science (Gibbstown, NJ, USA). β-Glucuronidase (*Escherichia coli*-K12) was purchased from Roche Biomedical (Mannheim, Germany).

2.2. Standards preparation

Reagent solutions were prepared in acetonitrile and water using standard laboratory procedures. Stock solutions of phthalate metabolites, 4-MeUmb, and isotopically-labeled metabolites and 4-MeUmb were prepared in acetonitrile and stored at −20 °C in Teflon-capped amber glass bottles until used as described before [24]. The intermediate stock standards, containing phthalate metabolites and 4-MeUmb and their labeled internal standards, were prepared in acetonitrile:water (1:9) from serial dilutions of the stock solutions to create 10 standard solutions [24]. The working standards were prepared, as needed, by diluting 10-fold each intermediate stock standard solution with 0.1% acetic acid in 1:9 acetonitrile:water (1 mL acetic acid in 100 mL acetonitrile and 900 mL water), and stored at 4 °C in Teflon-capped glass vials until use. A standard solution of 4-MeUmb glucuronide (0.16 μg/mL) was prepared in water. The calibration curves were prepared directly from the working standard solutions. The range of analyte concentrations in the calibration standards varied depending on the analyte (Table 1).

Table 1
Phthalate metabolites, native and labeled precursor and product ion transitions monitored, collision energies, retention times (RT), and calibration range

Analyte	Precursor/product ions (*m*/*z*)	Isotope-labeled precursor/product ions (*m*/*z*)	Collision energy (V)	RT (min)		Calibration range (ng/mL)
				Previous method[a]	Updated method	
PA	165/77	167/77	21	4.5	4.9	0.2–800
MMP	179/77	183/79	24	6.8	6.7	0.2–800
MCPP	251/103	255/103	10	6.7	6.7	0.1–400
MEP	193/77	197/79	25	8.4	8.5	0.2–800
MEHHP	291/121	295/124	27	16.8	13.6	0.2–800
MiBP	221/77	225/81	26	16.4	14.1	0.1–400
MECPP	307/159	311/159	22	17.4	14.0	0.1–400
MBP	221/77	225/79	26	17.2	14.6	0.2–800
MCHpP	307/159	311/159	22	–	14.6	0.1–400
MEOHP	293/121	297/124	26	18.8	15.3	0.2–800
MHNP	307/121	311/125	27	–	16.7	0.2–800
MCOP	321/173	325/173	19	–	17.8	0.1–400
MBzP	255/183	259/186	16	21.9	19.7	0.1–400
MCHP	247/77	251/79	27	22.0	20.0	0.05–200
MONP	305/121	309/125	27	–	20.3	0.1–400
MCNP	335/187	339/187	21	–	23.0	0.1–400
MHxP	249/77	253/81	21	–	24.4	0.1–400
MHpP	263/77	267/81	27	–	25.4	0.1–400
MEHP	277/134	281/137	23	26.2	25.6	0.1–400
MOP	277/125	281/127	21	26.4	25.8	0.1–400
MNP	291/247	295/124	26	26.4	25.9	0.1–400
MDP	305/260	309/264	16	26.8	26.3	0.1–400

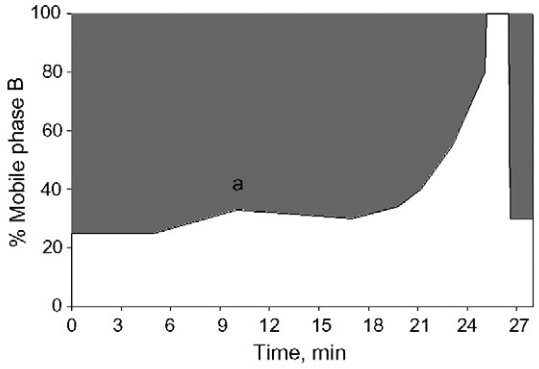
[a] Ref. [25].

2.3. Sample preparation and on-line SPE

Thawed, sonicated, and vortex mixed human urine (0.1 mL) was dispensed into a 1.5 mL silanized conical bottom autosampler vial, and the vial was placed on a sample tray on a Surveyor HPLC autosampler (ThermoFinnigan, Bellefonte, PA, USA). A 16 mL glass reservoir bottle (ThermoFinnigan, Bellefonte, PA, USA), used to contain the spiking internal standard solution, was silanized before use to eliminate active adsorption of MECPP, MCOP and MCNP onto the glass surface. We used a customized Xcalibur program to both spike the sample with the internal standard and 4-MeUmb solution and to incubate the sample at 37 °C. Specifically, the sample was automatically spiked with 25 μL 4-MeUmb glucuronide (0.16 μg/mL) to assess activity of the β-glucuronidase, 100 μL of a solution containing isotope-labeled phthalate metabolite analogs and 4-MeUmb, and 25 μL β-glucuronidase in acetate buffer (pH 6.5, 1 M); the spiked sample was kept at 37 °C for at least 90 min for the enzymatic hydrolysis of the phthalate metabolites conjugates. After completing a sequence of 100 samples containing unknown reagent blank and quality control (QC) samples, a solution containing 20% acetic acid, 5% acetonitrile, and 75% water (200 μL) was added to each sample. The temperature of the autosampler tray holding the samples was set to 0 °C. The following morning, the sample tray was moved to the analytical system and the temperature of the tray compartment was set to 10 °C for the duration of the on-line SPE–HPLC–MS/MS analysis [25]. We used an analogous procedure without β-glucuronidase in the acetate buffer for measuring the concentrations of the free species of phthalate metabolites.

2.4. Instrumental analysis

The pretreated urine sample (final volume was 500 μL) was loaded onto a Chromolith Flash RP-18e column (2 μm, 4.6 mm × 25 mm, Merck KGaA, Germany) for the preconcentration of the analytes. A gradient of 0.1% acetic acid in water and 0.1% acetic acid in acetonitrile [25] was used to transfer the analytes onto a Betasil Phenyl analytical column (3 μm, 150 mm × 2.1 mm, ThermoHypersil-Keystone, Bellefonte, PA, USA) which was preceded by inline filters (2 μm and 0.5 μm, Upchurch Scientific, Oak Harbor, WA, USA). The analytes were chromatographically resolved using a nonlinear solvent gradient from 100% mobile phase A (0.1% acetic acid in water) to 100% mobile phase B (0.1% acetic acid in acetonitrile) at a flow rate of 0.35 mL/min (Fig. 1). The mass specific detection was achieved using a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface in the multiple reaction monitoring (MRM) mode. Precursor and product ion combinations specific to each analyte were monitored (Table 1). The analytical run was segmented to achieve an optimum number of scans for each analyte. The source collision induced dissociation voltage was set to 10 V to break down acetate clusters. Data acquisition and analysis were performed using the Xcalibur® software (ThermoFinnigan, Bellefonte, PA, USA) on a PC-based data system. The data analysis program automatically selected and



Time, min	0	3.0	5.0	10.0	17.0	19.8	21.0	23.0	25.0	25.2	25.6	26.5	26.6
%A[a]	75	75	75	67	70	66	60	45	20	0	0	0	75
Flow rate, μL/min	300	300	350	350	350	350	350	350	350	350	400	400	400

[a] Mobile phase A: 1-mL acetic acid in 1 L of water; mobile phase B: 1-mL acetic acid in 1 L acetonitrile. %B=100-%A

Fig. 1. HPLC gradient program used to separate 22 phthalate metabolites including three pairs of structural isomers. 'a' indicates the start of the reverse gradient to accelerate the elution of less water-soluble analytes while keeping adequate separation of the compounds.

integrated each ion of interest in the chromatogram. The identity of the phthalate metabolites was confirmed by matching retention times with the isotopically-labeled internal standard. For the analytes that represented one of the many possible isomers (i.e., MHNP, MCNP, MCOP, MONP, and MNP), the whole cluster of peaks was integrated. 4-MeUmb was measured to monitor the completion of deglucuronidation. The peak integrations were corrected manually, if necessary. For quantification, calibration curves, weighted by the reciprocal of the standard concentration ($1/x$) of the peak area of each analyte ion divided by the peak area of its isotope-labeled standard versus standard concentration, were constructed.

2.5. Daily operation and quality control procedure

QC materials were prepared from a base urine pool obtained from multiple anonymous donors. The phthalate concentrations in the pool urine were measured to assess endogenous levels. The pool was divided into two sub-pools enriched with native phthalate metabolites to create low-concentration (QCL, 4–62 ng/mL depending upon the analyte) and high-concentration (QCH, 26–485 ng/mL depending upon the analyte) QC materials. The QC pools were dispensed in 1.5 mL aliquots in polypropylene cryovials and stored at −70 °C. Both QC materials were characterized by repeated measurements with and without enzyme treatment to define for each metabolite the mean concentrations and the 95% and 99% control limits (Fig. 2).

Each analytical batch included two QCL, two QCH, five reagent blanks, and 40 unknown samples. The concentrations of the reagent blanks were averaged, and the average was subtracted from the concentrations of phthalate metabolites in the QCs and unknown samples. The concentrations of phthalate metabolites in the two QCH and two QCL samples were averaged, and the QC data were evaluated using modified Westgard statistical probability rules. Specifically, if both QC means

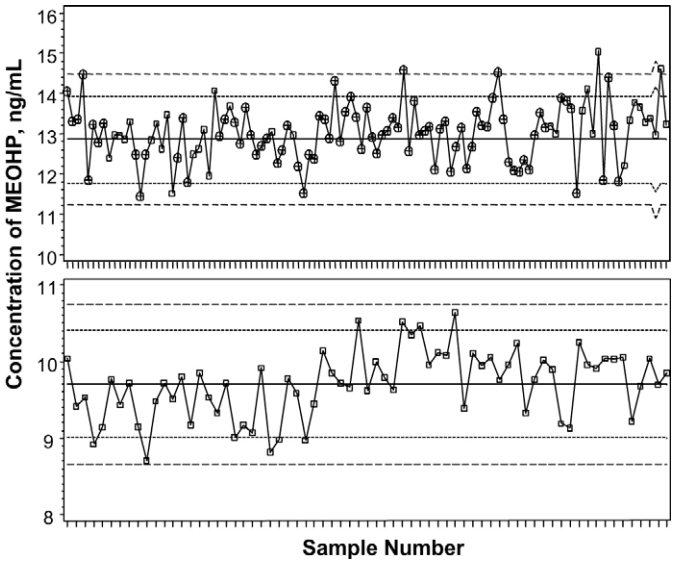


Fig. 2. Shewhart charts for total (above) and free (below) concentrations of MEOHP in the low-concentration quality control (QC) pool. The QC pools were characterized by repeat measurements spanning over 3 months. The lines shown represent the mean, and the 95% and 99% upper and lower control limits. The difference in mean levels reflects endogenous MEOHP in its glucuronidated form in the pooled urine. MEOHP is used to represent all phthalate metabolites.

were within $2S_m$ (standard deviation of the run means) limits and individual QC results were within $2S_i$ (standard deviation of individual results) limits, the analytical run was considered in-control. If one of the QC means was outside the $2S_m$ limit, the run was considered out-of-control, if one or more of the following occurred: (a) the run mean was $\pm 4S_m$ beyond the characterization mean, (b) the run mean was outside a $3S_m$ limit, (c) both run means were outside the same $2S_m$ limit, and (d) the current and previous nine run means were on the same side of the characterization mean. If one of the four QC individual results was outside a $2S_i$ limit, the run was considered out-of-control if within-run ranges for both pools exceed the 95% range limit. Since runs have multiple measurements per pool for two pools, this rule was applied within runs only. All unknown samples in the batch were re-extracted if the analytical run was out-of-control for a particular analyte. The calibration curves for all analytes, derived daily from two full set of standards, were linear over three orders of magnitude and had correlation coefficients exceeding 0.99. If concentrations were above the linear range for any analyte, the sample was re-extracted with less urine and the concentration was calculated after applying the appropriate dilution factor. For each analyte, calibration data, peak areas, and retention times were saved in a Microsoft Excel format, exported to a Microsoft Access database, and processed using SAS statistical software (SAS Institute, Cary, NC).

2.6. Method validation

2.6.1. Method comparison

We analyzed 39 unknown urine samples using both the current and previous methods [25] to compare the concentrations of analytes common to both methods (Fig. 3).

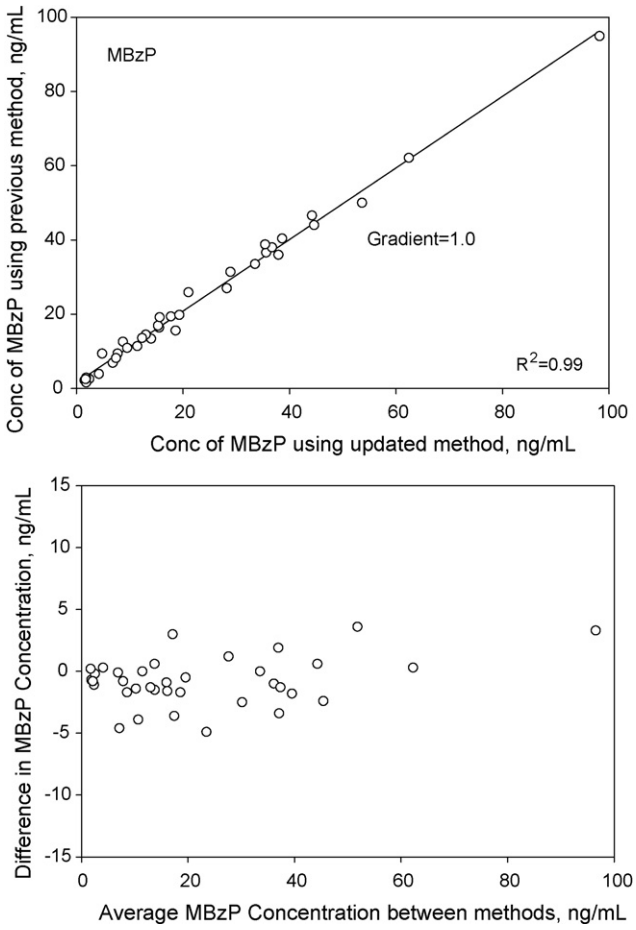


Fig. 3. Repeat analysis of MBzP using the previous analytical method [25] and the upgraded method. The gradient of 1.0 indicates excellent agreement between two methods (above). The difference plot or Bland–Altman plot (below) illustrates the random variation around zero on the *y*-axis across the entire concentration range.

2.6.2. Cross analyst/instrument validation

To estimate interindividual and interinstrument variability, we compared the concentrations of QC samples analyzed by two analysts and on two different on-line SPE–HPLC–MS/MS systems (Table 2).

2.6.3. Method accuracy

The accuracy of the method was assessed by measuring the concentration of known standard solutions spiked in synthetic urine (UriSub, Technologies Inc., NJ) at two different levels (Table 2).

2.6.4. Method precision

Inter day and intra day precision over 3 months were estimated by calculating the coefficients of variation (CVs) of repeated analysis of QCL and QCH samples (Table 2). Precision was further assessed by repeat analysis, with and without enzyme treatment, of a urine sample pooled from five anonymous donors with no known exposure to phthalates.

Table 2
Accuracy, precision, and the limits of detection of selected phthalate metabolites

Analyte	Mean QCL (ng/mL)				Mean QCH (ng/mL)				%CV (QCH)		Accuracy (ng/mL)				LOD (ng/mL)
	Analyst		Instrument		Analyst		Instrument		Intra day	Inter day	Theoretical	Actual	Theoretical	Actual	
	1	2	1	2	1	2	1	2							
MMP	5.3	5.8	5.4	5.2	48.2	48.2	47.7	48.7	4.2	4.0	4.0	3.3	32.0	32.6	1.1
MEP	62.2	59.9	60.3	62.8	484.8	484.7	482.7	483.3	1.8	2.7	4.8	4.8	153.6	151.2	0.7
MCPP	4.1	4.6	4.3	4.5	26.5	28.9	27.1	24.7	10.0	14.0	2.4	2.0	19.2	19.3	0.2
MBP	13.8	13.7	14.0	13.4	67.9	67.3	66.5	67.9	6.7	7.5	5.2	4.9	41.6	43.1	0.6
MiBP	13.0	14.0	13.9	12.5	63.3	66.6	64.2	64.9	7.6	7.3	2.6	2.6	20.8	22.7	0.3
MCHP	4.0	4.1	4.1	4.0	35.6	36.4	35.7	36.1	4.8	7.5	1.2	0.8	16.0	15.0	0.3
MBzP	8.5	8.8	8.6	8.5	72.4	74.0	72.9	73.1	5.4	6.8	3.6	3.0	28.8	27.3	0.3
MEHP	13.8	14.5	14.3	14.3	76.1	78.1	77.7	76.1	8.1	6.3	3.6	3.2	28.8	29.6	1.1
MEOHP	12.9	12.7	12.9	12.8	51.3	50.4	50.9	51.0	2.8	3.3	3.2	2.5	25.6	24.9	0.6
MEHHP	14.0	14.3	14.2	13.7	56.9	57.7	56.9	56.8	4.7	3.8	2.8	1.7	22.4	20.6	0.7
MECPP	12.8	13.0	12.9	12.9	43.0	43.2	43.2	43.2	2.9	5.3	2.0	1.6	16.0	15.5	0.5
MONP	6.9	6.1	6.7	6.8	45.4	41.5	44.9	45.5	10.2	13.5	2.0	1.3	16.0	15.5	0.4
MHNP	5.2	4.9	4.9	5.3	34.5	34.2	34.2	34.6	2.6	9.0	2.0	2.0	16.0	16.4	0.9
MCOP	7.1	6.6	7.1	6.8	40.7	38.9	40.5	40.1	5.8	6.2	2.0	1.5	16.0	15.4	0.7
MCNP	8.8	nd	9.1	8.4	57.5	nd	59.4	54.6	12.6	10.2	2.0	1.5	16.0	15.5	0.5
MHxP	6.3	nd	nd	6.2	56.7	nd	56.7	56.7	8.2	5.4	2.0	1.4	16.0	15.0	0.7
MHpP	9.5	nd	9.4	9.5	54.2	nd	53.2	54.2	12.5	13.8	2.0	2.1	16.0	16.4	0.5
MNP	8.3	7.7	8.2	8.1	36.2	34.7	35.9	35.1	13.5	6.9	2.0	1.8	16.0	15.4	0.8

nd, not determined.

2.6.5. *Proficiency testing (PT)*

Method accuracy was further verified by the analysis of PT samples in three concentration ranges (low, medium, and high). PT materials were prepared and characterized using a procedure similar to the one described above for QCs. Twice per year, five PT samples were blind analyzed and the results were reported to an external QC officer for evaluation.

2.6.6. *Matrix effects*

Different amounts (5 μL, 10 μL, 50 μL, and 100 μL) of QCL and QCH, adjusted, if needed, to 100 μL with deionized water, were repeatedly extracted ($N = 3$), and the concentrations were compared (Table 3).

3. Results and discussion

Previously, we developed a sensitive on-line SPE–HPLC–MS/MS method to measure the concentrations of 16 phthalate metabolites in human urine [25]. We have expanded the analytical capabilities of the method to measure seven additional phthalate metabolites. Recent research suggests that for high molecular weight phthalates (e.g., di(2-ethylhexyl)

Table 3
Matrix effects on selected phthalate metabolite levels representing all phthalates analyzed using the current method

Analyte	Final concentration ± S.D., QCL (ng/mL)				Final concentration ± S.D., QCH (ng/mL)			
	Volume added (μL)				Volume added (μL)			
	Expected	5	10	50	Expected	5	10	50
MMP	5.5 ± 0.1	5.9 ± 1.1	6.2 ± 1.9	5.7 ± 0.2	50.1 ± 0.8	54.1 ± 3.6	53.0 ± 0.8	50.6 ± 0.8
MCPP	5.4 ± 0.1	6.2 ± 0.4	5.3 ± 0.1	5.5 ± 0.2	33.5 ± 0.1	39.9 ± 6.3	37.5 ± 2.4	34.0 ± 0.7
MEP	61.2 ± 0.5	72.5 ± 13.6	66.2 ± 3.5	60.6 ± 1.8	493.0 ± 3.0	499.0 ± 36.0	499.0 ± 36.0	490.6 ± 6.1
MEHHP	14.9 ± 0.2	15.4 ± 2.7	15.2 ± 0.6	13.9 ± 0.2	59.7 ± 1.1	65.2 ± 2.6	64.9 ± 2.3	57.8 ± 1.6
MECPP	13.1 ± 0.3	18.1 ± 0.8	15.6 ± 0.3	13.2 ± 0.2	43.7 ± 0.4	52.0 ± 7.0	51.2 ± 1.6	43.0 ± 0.2
MiBP	13.6 ± 0.7	14.4 ± 1.0	17.7 ± 2.8	14.2 ± 1.3	64.1 ± 3.5	65.8 ± 19.2	69.4 ± 6.3	62.5 ± 0.9
MBP	14.8 ± 1.1	13.6 ± 2.5	16.7 ± 1.5	16.9 ± 0.5	68.0 ± 2.3	68.7 ± 9.8	70.0 ± 0.9	72.4 ± 1.0
MEOHP	11.8 ± 0.3	13.9 ± 0.5	15.3 ± 0.3	12.6 ± 0.8	47.9 ± 0.8	56.6 ± 3.8	55.5 ± 1.4	48.6 ± 0.2
MHNP	6.2 ± 0.3	6.8 ± 0.2	5.5 ± 0.1	6.3 ± 0.6	39.5 ± 0.6	41.4 ± 2.3	41.0 ± 0.2	37.0 ± 0.4
MCOP	7.5 ± 0.3	7.9 ± 0.2	8.0 ± 0.3	7.8 ± 0.2	39.7 ± 0.1	46.0 ± 1.8	44.9 ± 1.7	39.9 ± 0.7
MBzP	9.0 ± 0.3	8.2 ± 0.4	6.9 ± 0.3	8.8 ± 0.2	75.2 ± 1.7	89.2 ± 2.9	78.8 ± 8.1	76.0 ± 5.1
MCHP	4.5 ± 0.2	3.3 ± 0.9	3.0 ± 0.1	4.1 ± 0.4	38.7 ± 0.7	38.4 ± 1.0	37.0 ± 1.4	37.2 ± 1.3
MONP	5.8 ± 0.1	4.5 ± 0.4	3.9 ± 0.8	5.7 ± 0.2	38.7 ± 0.4	42.0 ± 3.1	44.5 ± 1.6	39.1 ± 1.3
MCNP	8.3 ± 0.2	9.4 ± 0.1	8.9 ± 0.2	8.5 ± 0.2	54.6 ± 0.2	58.7 ± 3.5	57.9 ± 0.9	52.6 ± 0.3
MEHP	15.0 ± 1.0	11.0 ± 6.3	12.5 ± 4.9	13.5 ± 1.6	76.5 ± 1.2	90.0 ± 16.5	78.9 ± 8.1	75.5 ± 7.0
MOP	11.8 ± 0.7	15.4 ± 12.2	12.3 ± 0.4	11.6 ± 1.0	63.2 ± 2.5	82.1 ± 24.9	71.2 ± 6.5	64.3 ± 1.2
MNP	8.0 ± 0.7	7.8 ± 0.1	9.4 ± 3.2	8.6 ± 0.7	37.1 ± 1.4	41.2 ± 13.8	38.0 ± 2.2	36.0 ± 4.0

The expected concentrations used as reference were the concentrations obtained with 100 μL urine, the amount normally used for analysis. S.D., standard deviation.

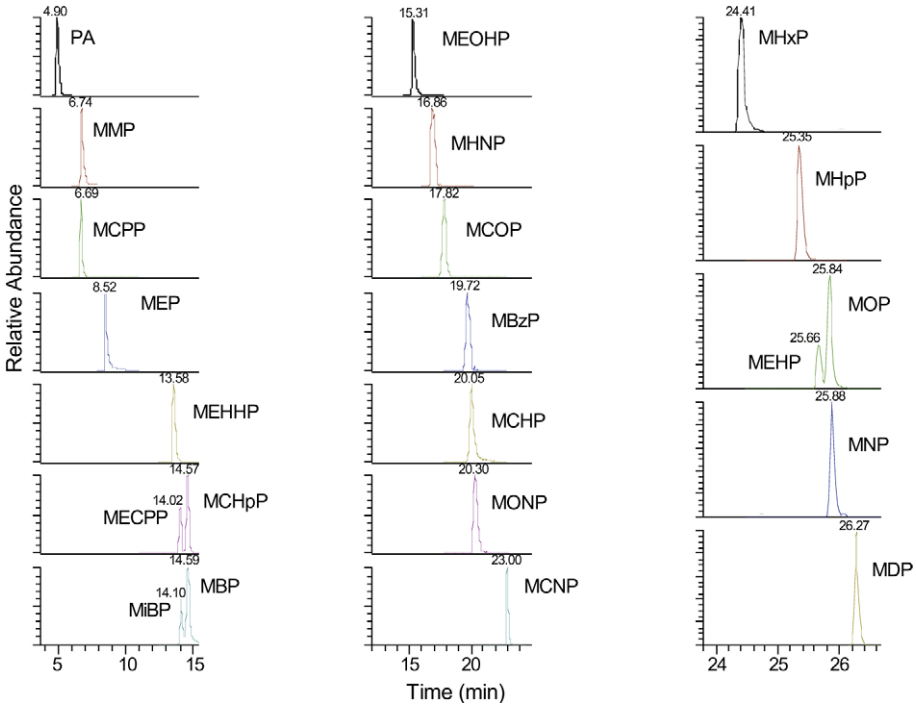


Fig. 4. HPLC–ESI–MS/MS chromatogram of a standard solution containing 22 phthalate metabolites at levels ranging from 16 ng/mL to 32 ng/mL.

phthalate, di-*n*-octyl phthalate [DnOP], di-isononyl phthalate [DiNP], and di-isodecyl phthalate [DiDP]), oxidative metabolites are better biomarkers of exposure to the parent phthalate than the hydrolytic monoesters [13–19]. Therefore, we incorporated additional oxidative metabolites: MCHpP (DnOP metabolite), MHNP, MONP, and MCOP (DiNP metabolites) and MCNP (DiDP metabolite) to our upgraded method. We also included the hydrolytic monoester metabolites of di-*n*-hexyl phthalate and di-*n*-heptyl phthalate, two phthalates which may be used in consumer products. Because monomethyl isophthalate, one of the analytes included in our previous method, was repeatedly undetectable in human urine, it was not included in the current method. The fragmentation and relative abundance of the product ion fragments for the seven new analytes and their isotopically labeled internal standards were examined to select the best precursor/product ion combinations for quantification (Table 1).

With our previous solvent gradient, increasing the number of analytes was not possible without compromising chromatographic resolution or lengthening the HPLC run time. To address the challenge of measuring simultaneously 22 structurally-related analytes, including three pairs of structural isomers (MBP/MiBP, MEHP/MOP, and MECPP/MCHpP) within 29 min, we used a novel nontraditional HPLC gradient that consists of reversing the solvent gradient to improve the separation of closely eluting pairs of compounds (Fig. 1). After an initial isocratic stage, the proportion of the organic mobile phase was slowly and gradually increased to facilitate the elution of more water-soluble analytes (Fig. 1). Before the elution of the two isomeric pairs MiBP/MBP and MECPP/MCHpP, organic mobile phase proportion was rapidly increased and then the gradient was reversed ('a' in Fig. 1), followed by a rapid increase in the organic mobile phase to accelerate the movement of less water-soluble analytes in the HPLC column (Fig. 1). With this approach, we achieved adequate separation of all analytes including the three isomeric pairs MiBP/MBP, MECPP/MCHpP, and MOP/MEHP, which is required because the structural isomers in each pair were quantified using the same *m/z* scan transition (Table 1) and/or similar fragmentation patterns of structural isomers. Interestingly, this unique organic phase gradient (Fig. 1) accelerated the elution of all of the analytes while keeping adequate peak separation (Fig. 4). Finally, a gradual increase in the organic mobile phase completed the elution of all analytes. At the end of the gradient, the HPLC column was rinsed with the organic mobile phase to remove residual contaminants (Fig. 1).

Because of the expected large interindividual variability in the composition of the urine and in the range of phthalate metabolites concentrations in epidemiologic studies, we examined the matrix effects on the analytes concentrations. The concentrations calculated after repeat dilutions of QCH and QCL samples were in excellent agreement, suggesting limited or no matrix effects and the versatility of the calibration curve over a wide concentration range (Table 3).

To further validate our method, we analyzed 39 unknown urine samples using both our updated and the previously developed methods [25]. For the common analytes, the agreement between concentrations obtained using both approaches was very good throughout the entire concentration range ($R^2 = 0.99$, Fig. 3). Furthermore, the fact that no significant variability between analysts or instruments was observed provided additional proof of the ruggedness of the updated approach (Table 2).

Analysis of QC materials over a period of 3 months indicated excellent long-term reproducibility for most analytes (Fig. 2,

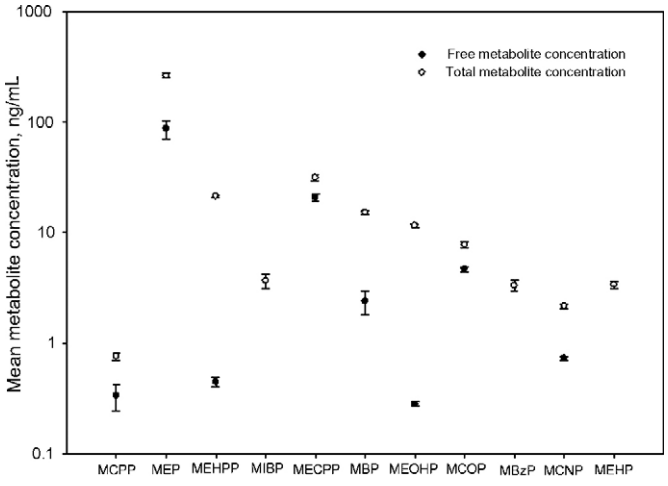


Fig. 5. Variability of the free and total concentrations of commonly found phthalate metabolites in a pooled urine sample. Error bars indicate standard deviation of the mean of the repeated measurements. Concentration of free species was <LOD for MiBP, MBzP, and MEHP. MCPP, MEP, MECPP, MCOP, and MCNP exhibit lower degree of glucuronidation than MiBP, MBzP, and MEHP.

Table 2). High precision of the measurements also was observed in an unspiked pooled urine sample analyzed repeatedly for both total and free phthalate levels (Fig. 5). Method accuracy was evaluated using a synthetic urine substitute spiked with phthalate metabolites at two different concentrations. Excellent accuracy was reflected both in the good agreement between the calculated and expected concentrations (Table 2) and during the blind analysis of PT materials (data not shown). The limits of detection (LOD), estimated from six replicate measurements of the five lowest standards, were in the low ng/mL levels for all analytes (Table 2) and thus adequate for biomonitoring purposes of the general population. The LODs were calculated as $3S_0$ where S_0 is the value of the standard deviation as the concentration approaches zero [27].

In summary, we modified our previous on-line SPE–HPLC–MS/MS method by incorporating seven additional metabolites, changing the mobile phase gradient to achieve adequate HPLC separation of the analytes without compromising either analytical run time or chromatographic resolution. Our research shows that analytically it is possible to measure these 22 phthalate metabolites in one analytical run with the precision and accuracy at the sub-parts-per-billion levels required for biomonitoring purposes. However, some of these metabolites (e.g., MOP, MNP, and MDP) may not be optimal urinary biomarkers to assess environmental exposure to their parent phthalates. Additional information, including metabolism and toxicokinetic data, is needed to demonstrate the utility of these analytes for exposure assessment purposes.

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