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Analysis of human urine for fifteen phthalate metabolites using automated solid-phase extraction

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Abstract

We improved our previous analytical method to measure phthalate metabolites in urine as biomarkers for phthalate exposure by automating the solid-phase extraction (SPE) procedure and expanding the analytical capability to quantify four additional metabolites: phthalic acid, mono-3-carboxypropyl phthalate, mono-isobutyl phthalate (miBP), and monomethyl isophthalate. The method, which involves automated SPE followed by isotope dilution-high performance liquid chromatography (HPLC)-electrospray ionization (ESI)-tandem mass spectrometry (MS), allows for the quantitative measurement of 15 phthalate metabolites in urine with detection limits in the low ng/ml range. SPE automation allowed for the unattended sequential extraction of up to 100 samples at a time, and resulted in an increased sample throughput, lower solvent use, and better reproducibility than the manual SPE. Furthermore, the modified method permitted for the first time, the separation and quantification of mono-*n*-butyl phthalate (mBP) and its structural isomer miBP. The method was validated on spiked pooled urine samples and on pooled urine samples from persons with no known exposure to phthalates.

Keyword: Phthalate metabolites

1. Introduction

Phthalates are a group of industrial chemicals widely used in consumer products and as solvents, additives, and plasticizers [1]. Humans are potentially exposed to many products containing phthalates. Phthalates are rapidly metabolized in humans to their respective monoesters, which depending on the phthalate can be further metabolized to their oxidative products. Monoesters and the oxidative metabolites of phthalates may be glucuronidated, and these conjugates excreted in the urine and feces [2–6].

Several phthalates are carcinogenic in animal models [5,7,8]. Some phthalates and their monoester metabolites can cause reproductive and developmental toxicities in animals [9–12], but little is known about the effects of phthalate exposure on humans. Information on the concentration of phthalates in people is essential to understand the human exposure to phthalates. We previously developed a solid-phase extraction (SPE) followed by isotope dilution-high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method to quantify 11 urinary phthalate metabolites in humans: monomethyl phthalate (mMP), monoethyl phthalate (mEP), mono-*n*-butyl phthalate (mBP), 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-3-methyl-5-dimethylhexyl phthalate (*iso*-nonyl, mNP), and mono-3-methyl-7-methyloctyl phthalate (*iso*-decyl, mDP) [13,14]. We used the measurement of these phthalate monoester metabolites in several populations to assess exposure to phthalates [15–18].

Although our previous extraction method has adequate recovery, the manual extraction step is labor-intensive and time-consuming. To address these limitations, we automated the extraction process. Furthermore, we modified and expanded the analytical method to measure four additional metabolites: phthalic acid (PA); monomethyl

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isophthalate (mMiP), a metabolite of dimethyl isophthalate; mono-isobutyl phthalate (miBP), a metabolite of di-isobutyl phthalate; and mono-3-carboxypropyl phthalate (mCPP), a major metabolite of dioctyl phthalate (DOP) and a minor metabolite of dibutyl phthalate (DBP) [3,19]. The modified method permitted for the first time the separation and quantification of mBP and its structural isomer miBP.

We applied our novel method to analyze urine samples from adults with no known exposure to phthalates and found frequently miBP, PA and mCPP along with previously reported mEP, mBP, mBzP, mEHP [17,18], and mEHHP and mEOHP [20–24].

2. Experimental

2.1. Reagents

mMP, mMiP, 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-methylumbelliferone 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 and its glucuronide, and ammonium acetate (>98%) were purchased from Sigma Aldrich Laboratories, Inc (St. Louis, MO, USA). miBP and D_4-miBP were generous gifts from Professor Jürgen Angerer (University of Erlangen-Nuremberg, Germany). Acetonitrile and water (HPLC grade) were purchased from Tedia (Fairfield, OH, USA), phosphoric acid (85%) was purchased from Fisher Scientific (Pittsburgh, PA, USA), formic acid (98% min, GR) was purchased from EM Science (Gibbstown, NJ, USA), ethyl acetate (99.8%) was purchased from Caledon (Ontario, Canada), and monosodium phosphate monohydrate (ultrapure bioreagent) and ammonium hydroxide (30%) were purchased from J.T. Baker (Phillipsburg, 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 monoester metabolites, 4-methylumbelliferone, and isotopically-labeled phthalates and 4-methylumbelliferone internal standards were prepared in acetonitrile and stored at −20 °C in Teflon-capped amber glass bottles until use [13]. The working standards were prepared in 1:9 acetonitrile:water from serial dilutions of the stock solutions to create eleven standard solutions, containing phthalate metabolites, 4-methylumbelliferone and their isotopically-labeled internal standards, whose concentrations encompassed the entire linear range of the method. The working standards were stored at 4 °C in Teflon-capped glass vials until use [13]. The calibration curves were prepared directly from the working standard solutions.

A standard solution of 4-methylumbelliferone glucuronide (0.8 ng/ml) was prepared in water. This standard solution was added to all samples. The 4-methylumbelliferone/$^{13}C_4$-methylumbelliferone peak area ratio was monitored to check the extent of the deconjugation reaction with β-glucuronidase [13].

2.3. Sample preparation and automated SPE

Human urine (1 ml) was buffered with ammonium acetate [13], and spiked with a mixture of labeled internal standards of phthalate monoesters and 4-methylumbelliferone, 4-methylumbelliferyl glucuronide (50 μl, 0.8 ng/ml), and β-glucuronidase enzyme. The sample was then incubated at 37 °C to deconjugate the glucuronidated phthalate metabolites [13]. To measure the concentration of free phthalate metabolites, the enzyme deconjugation step was omitted. After enzymatic cleavage of monoester glucuronides, the urine sample was placed on the Zymark RapidTrace Station (Zymark Corporation, Hopkinton, MA, USA) for the automated SPE procedure. The solvent lines were purged with methanol before each extraction. Commercially available 60 mg/3 ml styrene-divinylbenzene methacrylate copolymer SPE cartridges (NEXUS ABS ELUT from Varian Sample Preparation Products, Harbor City, CA, USA) were conditioned with acetonitrile (1 ml) and with pH 2 phosphate buffer (1 ml, 0.14 M NaH_2PO_4 in 0.85% H_3PO_4). The urine was diluted with pH 2 phosphate buffer (1 ml), and loaded onto the SPE cartridge at a rate of 1 ml/min. The column was rinsed with 0.1 M formic acid (2 ml) and then, water (1 ml). The SPE cartridges were dried by passing air through the column (0.5 min). The analytes were then eluted with acetonitrile (1 ml) followed by ethyl acetate (1 ml) at 1 ml/min. The eluate was evaporated to dryness under a stream of dry nitrogen (UHP grade) in a Turbovap evaporator (Zymark Corporation, Hopkinton, MA, USA) at 55 °C. The residue was resuspended in 200 μl of water for analysis.

2.4. Instrumental analysis

The chromatographic separation was achieved using a Waters Alliance 2690 liquid chromatograph (Waters Corporation, Milford, MA, USA) or a ThermoFinnigan Surveyor liquid chromatograph (ThermoFinnigan, San Jose, CA, USA), each equipped with a Betasil phenyl column (3 μm, 100 mm × 2 mm, ThermoHypersil-Keystone, Bellefonte, PA, USA), preceded by inline filters (2 and 0.5 μm, Upchurch Scientific, Oak Harbor, WA, USA), with 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 (Table 1). The mass specific detection was achieved using a ThermoFinnigan TSQ 7000 or a ThermoFinnigan TSQ Quantum

Table 1
HPLC gradient program[a]

	Time (min)								
	0	0.1	1.0	14.0	21.0	22.0	22.5	23.0	25
%A	96	96	85	75	35	0	0	96	96
%B	4	4	15	25	65	100	100	4	4

[a] Flow rate: 0.35 ml/min; mobile phase A: 1 ml acetic acid in 1 l HPLC-grade water; mobile phase B: 1 ml acetic acid in 1 l acetonitrile.

triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface. The ThermoFinnigan Surveyor liquid chromatograph was coupled with a ThermoFinnigan TSQ Quantum mass spectrometer, and the Waters Alliance 2690 liquid chromatograph was coupled with a ThermoFinnigan TSQ 7000. Each sample (20 μl) was injected using the HPLC autosampler, configured with syringe washes between injections to eliminate carryover. During the first 3 min after the injection, a switching valve directed the post column flow to waste; then the automatic switching valve directed the post column flow to the mass spectrometer. ESI in the negative ion mode was used to form negatively charged analyte ions at the interface under fixed instrument settings (Table 2). The mass spectrometers were tuned and optimized for each analyte at 0.35 ml/min flow rate (Table 2). The instruments were set in multiple reaction monitoring mode, and the precursor and product ion combinations specific to the eluting analyte were monitored (Table 2). The source collision induced dissociation voltage was set to 10 V to break down acetate clusters. The identity of the phthalate metabolites was confirmed by matching retention times (±5%) with the isotopically labeled internal standard. Data acquisition and analysis were performed using the Xcalibur® software on a PC-based data system.

2.5. Daily operation and quality control (QC) procedure

Quality control materials were prepared from a base urine pool obtained from multiple anonymous donors. The pool was divided in two subpools that were enriched with native phthalate metabolites to create low-concentration (QCL, 10–150 ng/ml) and high-concentration (QCH, 50–500 ng/ml) QC materials. The two pools were dispensed in 5-ml portions in prerinsed glass vials and stored at −20 °C. Each QC material was characterized by repeated measurements to define the mean concentrations and the 95% and 99% control limits of each phthalate metabolite.

QCL and QCH were analyzed during each analytical run to ensure proper operation of the method and the validation of the resulting data. For each analytical run, the 10 module RapidTrace SPE system was divided into two extraction batches, each one consisting of five modules. Each batch included five reagent blanks and five QC materials, one in each rapid trace extraction module, and 40 unknown samples. The concentrations of phthalate monoesters in the QCs and unknown samples were corrected for the reagent blank. QC data were evaluated using modified Westgard QC rules. All 40 unknown samples were re-extracted if the QCs failed for a particular analyte. If an individual sample failed the 4-methylumbelliferone QC check (vide supra), only that unknown sample was re-extracted. The calibration curves for all analytes, derived daily from two full sets of 11 standards, were linear over three orders of magnitude and had correlation coefficients exceeding 0.99. The calibration data, along with the integrated peak areas for each analyte and retention

Table 2
Phthalate metabolites and their native and labeled precursor and product ion transitions, collision energies, retention times (RT), limits of detection (LOD), solid-phase extraction (SPE) recovery, and total recovery

Analyte	Precursor/product ions (*m/z*)		Collision energy (V)		RT (min)		LOD (ng/ml)	Mean SPE recovery	Mean total recovery[a]
	Native	Labeled	Quantum	TSQ	Waters	Surveyor			
PA	165/77	167/77	25	25	4.6	3.1	1.59	45	41
mMP	179/77	183/79	20	20	7.4	5.4	0.23	86	59
mMiP	179/135	185/141	20	18	10.6	8.2	0.37	92	66
mEP	193/77	197/152	22	22	10.2	8.1	0.89	101	77
mCPP	251/103	255/103	10	10	9.2	7.0	0.37	99	97
mBP	221/77	225/79	26	26	19.0	17.5	1.07	106	87
miBP	221/77	225/81	26	26	19.4	17.1	1.04	104	86
mCHP	247/77	251/79	27	27	21.2	19.6	0.28	99	93
mBzP	255/183	259/186	16	16	21.1	19.5	0.26	103	102
mEHP	277/134	281/137	21	21	23.3	21.8	0.98	98	90
mEOHP	293/121	297/124	26	26	20.0	18.5	1.07	99	99
mEHHP	291/121	295/124	27	27	19.5	18.0	0.95	97	97
mOP	277/125	281/127	19	19	23.5	22.1	1.00	100	95
mNP	291/247	295/250	17	17	23.6	22.1	0.85	87	88
mDP	305/260	309/264	16	16	23.9	22.7	nd	nd	nd

nd: not determined.

[a] Mean total recoveries were calculated as $100 \times [\text{conc}]_e/[\text{conc}]_b$, where $[\text{conc}]_e$ and $[\text{conc}]_b$ are the concentrations of phthalate metabolites obtained from spiking the sample with the isotopically labeled standards after evaporating the SPE extract and before SPE, respectively.

times, saved in a Microsoft Excel file, were exported to a Microsoft Access database, and the data were statistically analyzed using SAS statistical software (SAS Institute, Cary, NC).

3. Results and discussion

We modified our previous analytical method to measure phthalate metabolites in urine by automating the SPE procedure, changing the dimensions of the HPLC column and HPLC solvent gradient, changing the MS ionization mode, and expanding the analytical capabilities to measure four additional metabolites.

The automated SPE allowed for the unattended extraction of 100 urine samples in 6 h. Although a comparable sample throughput could be achieved with our previous manual extraction procedure, manual SPE was labor-intensive and required constant attention from the analyst performing the extraction. The automated SPE reduces the human intervention during the extraction, thus minimizing the interday and intraday variation associated with the process and therefore resulting in better reproducibility. Furthermore, our manual SPE method involved the use of two SPE cartridges per sample (200 mg/12 ml and 60 mg/3 ml), but only the 60 mg/3 ml cartridge is used for the automated SPE. We eliminated the first step of our SPE manual method, used to remove compounds hydrophopic at basic pH [14], and included two wash steps with diluted formic acid and water in the automated SPE method. The first wash was to remove urine components less acidic than the phthalate metabolites. The purpose of the water wash was to elute the remaining formic acid and to increase the pH. The combination of wash steps, the controlled flow rate provided with the automated extractor, and the fact that basic compounds present in the SPE extract did not seem to interfere with the formation of negatively-charged gas-phase ions of the acidic phthalate metabolites by ESI, allowed us to use only one cartridge for the automated SPE. This resulted in lower solvent use and reduced cost per sample for the automated SPE than for the manual SPE.

We analyzed the fragmentation and the relative abundance of product ion fragments for PA, miBP, mMiP, and mCPP and their isotopically labeled internal standards before selecting the best precursor/product ion combinations for the quantification of these analytes. The mass spectrometer parameters were optimized for the selected combinations to achieve maximum sensitivity for all 15 analytes.

We modified the HPLC parameters and used a lower flow rate (0.35 ml/min versus 0.8 ml/min) and a longer HPLC column with smaller particle size (3 μm, 100 mm × 2 mm versus 5 μm, 50 mm × 2 mm) than we used in our previous method [14]. One advantage of the reduced HPLC flow rate and concomitant use of HPLC solvents was the reduced stress on the mass spectrometer vacuum pumps, thus potentially extending the pumps optimal performance. The

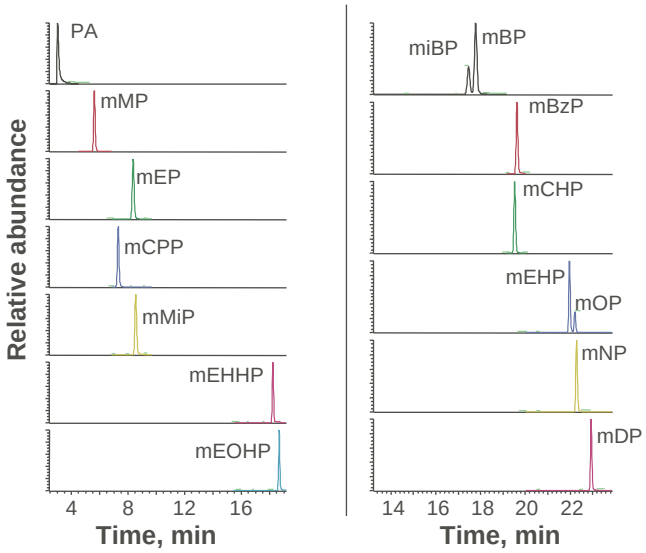

Fig. 1. HPLC-ESI-MS/MS chromatogram of a standard mixture of 15 phthalate metabolites.

changes in HPLC parameters, although resulted in longer run times per sample than before [14] (from 15 to 25 min), were necessary for adequate chromatographic resolution of all 15 analytes, including the isomeric phthalate monoesters mBP and miBP, and mEHP and mOP (Fig. 1). The simultaneous HPLC-MS/MS quantitative determination of mBP and miBP in human urine had not been accomplished before [22]. When we used our previous HPLC experimental conditions [14], mBP and miBP coeluted during the HPLC separation. Because they are structurally close, miBP and mBP produced a very similar fragmentation pattern (Fig. 2) and had to be quantified using the same precursor/product ion transition (Table 2).

Our previous HPLC-MS/MS methods for measuring phthalate metabolites in urine used atmospheric pressure chemical ionization (APCI) [13,14]. APCI provided adequate sensitivity for the phthalate monoesters miBP and miMP. However, PA and mCPP, the only metabolites of the 15 measured with two carboxylic acid groups, did not ionize well using APCI. Therefore, we chose ESI. ESI is a softer ionization technique than APCI. ESI converts liquid-phase ions into gas-phase ions and has been used before to measure phthalate metabolites in urine [22] and serum [25].

The SPE recoveries of the phthalate metabolites from urine were calculated as $100 \times [\text{conc}]_a/[\text{conc}]_b$, where $[\text{conc}]_a$ and $[\text{conc}]_b$ are the concentrations obtained from spiking the urine sample with the isotopically labeled standards after and before the SPE separation, respectively (Table 2). The SPE recoveries were very good and compared well with the recoveries obtained using the manual SPE methods [13,14]. Although PA had a lower SPE recovery than the other metabolites (Table 2), probably because of its increased hydrophilicity, it is acceptable because we use the isotope dilution technique with isotopically labeled PA as the internal standard,

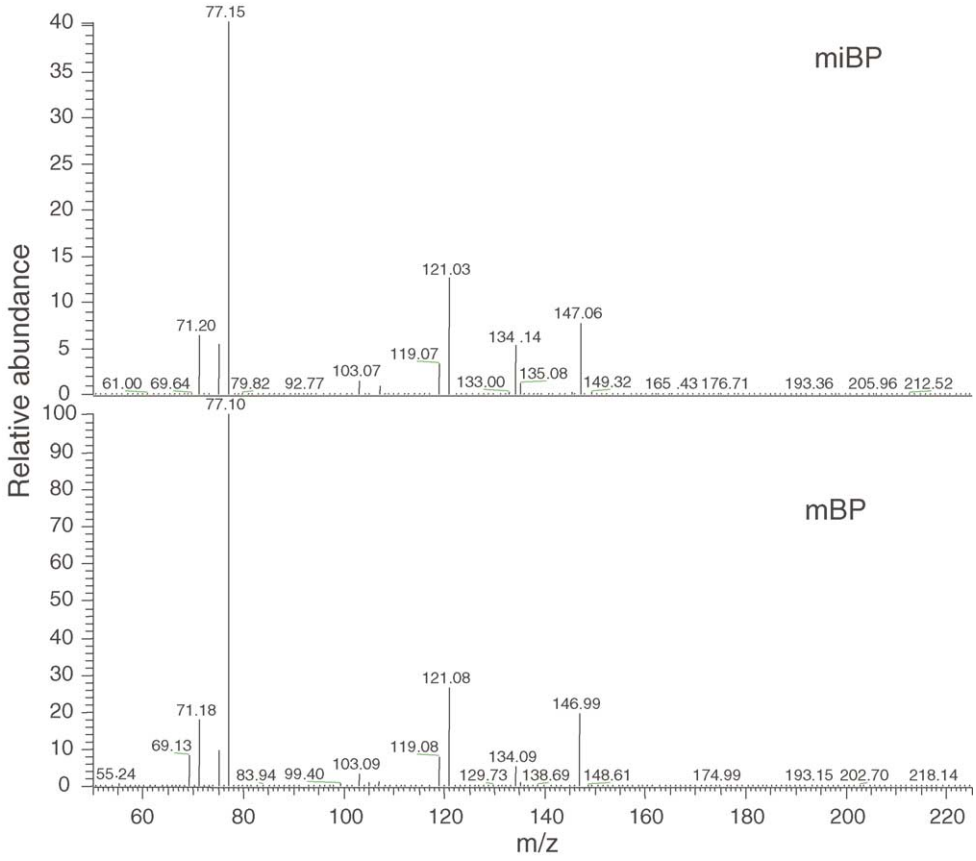

Fig. 2. Full scan mass spectra of miBP and mBP showing the fragmentation pattern.

which allows for automatic recovery correction for each sample.

The limits of detection (LODs), calculated from repeated analyses of urine spiked with phthalate metabolites as previously described [13,14], are shown in Table 2. The LOD values compare well with the LODs achieved before in urine [13,14]. We determined the precision of the method by calculating the average coefficient of variation (CV) of repeated measurements of the QC materials over a 5-week period. This value, which reflects both the intraday and interday variability of the assay, indicates the excellent reproducibility of this method (Table 3). In addition, the agreement between results obtained from the two HPLC-MS/MS systems and between multiple analysts was excellent (Table 3).

We applied this modified, automated SPE-HPLC-MS/MS method to analyze urine samples collected to make urine pools from a demographically diverse group of anonymous adult donors with no known exposure to phthalates. We detected PA, mCPP, and miBP in addition to other previously found phthalate monoesters [15–18,20,22,23] in most of the individual urine samples (Fig. 3). Interestingly, mCPP, a metabolite of DOP [3,19], was detected more frequently and at higher concentrations than mOP, the monoester metabolite of DOP. mCPP is also a minor metabolite of DBP [19]. Research is undergoing in our laboratory to determine the specificity of mCPP as a metabolite of DOP.

In summary, we developed an analytical method for the quantitative determination of 15 phthalate metabolites in urine The method is sensitive and accurate, uses a small amount of urine (1 ml), is not labor-intensive, involves minimal manual sample preparation, and uses an automated SPE system commercially available. This method is suitable for

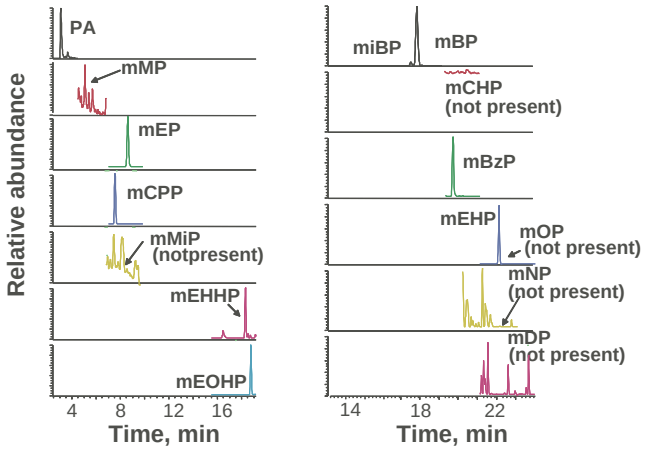

Fig. 3. Example of a HPLC-ESI-MS/MS chromatogram for a non-spiked human urine extract. Concentrations (in ng/ml) were as follows: PA (3.2), mMP (1.5), mEP (131.5), mCPP (3.4), mEHHP (17.5), mEOHP (12.3), miBP (2.3), mBP (29.4), mBzP (14.7), and mEHP (2.4).

Table 3
Precision of the concentration measurements of selected analytes in spiked quality control (QC) pools between analysts and between instruments[a]

Analyte	QC high (mean CV)			QC low (mean CV)			QC high (mean CV)			QC low (mean CV)		
	Analyst A	Analyst B	Analyst C	Analyst A	Analyst B	Analyst C	Quantum 1	Quantum 2	TSQ 7000	Quantum 1	Quantum 2	TSQ 7000
mMP	187.6 (4.2)	178.1 (4.4)	171.3 (6.0)	60.1 (7.3)	56.4 (3.7)	54.3 (6.2)	181.1 (4.7)	182.5 (6.1)	180.0 (5.1)	56.7 (5.1)	56.9 (5.9)	58.3 (9.7)
mCPP	201.1 (3.5)	204.1 (3.3)	199.1 (7.3)	38.4 (5.5)	37.4 (4.6)	37.4 (7.2)	203.9 (4.0)	204.2 (5.0)	200.9 (5.1)	37.7 (5.5)	38.0 (5.9)	38.2 (5.8)
mEHHP	92.8 (5.5)	87.7 (4.6)	84.1 (8.4)	42.2 (12.7)	38.5 (9.8)	36.7 (11.7)	90.2 (6.3)	87.8 (6.1)	87.9 (8.3)	38.9 (13.1)	38.7 (10.9)	39.6 (12.6)
mEOHP	126.4 (8.0)	120.3 (7.8)	129.8 (4.2)	25.9 (12.2)	24.2 (9.6)	23.7 (12.1)	125.3 (5.6)	126.1 (5.2)	125.5 (5.7)	25.0 (12.6)	24.8 (11.8)	25.0 (12.9)
mCHP	48.0 (2.1)	49.1 (2.9)	47.2 (7.2)	11.2 (3.6)	11.4 (4.6)	11.2 (4.4)	49.1 (4.3)	48.7 (6.2)	47.9 (5.6)	11.5 (4.6)	11.3 (5.0)	11.5 (4.6)

[a] Mean concentrations in ng/ml (precision expressed as the average coefficient of variation, %CV).

large epidemiologic studies to assess the relevance of human exposure to phthalates.

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