Supporting information

Environmental Exposure to the plasticizer 1,2-cyclohexane dicarboxylic acid, diisononyl ester (DINCH) in US adults (2000–2012)

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Quantification of urinary phthalates and DINCH metabolites using online solid phase extraction with Chromolith RP-18e guard columns and HPLC-MS/MS

Chemicals and Reagents

Phthalate metabolites and their stable isotope labeled internal standards were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA), Los Alamos Laboratories. Sigma Aldrich Laboratories, Inc. (St. Louis, MO, USA), or CanSyn LLC (Ontario, CA). Cyclohexane-1,2-dicarboxylic acid- monohydroxyisononyl ester (MHNCH) and D_4-MHNCH were generous gifts from Dr. Holger Koch (Germany). 4-methylumbelliferone (4-MeUmb), its glucuronide, and ammonium acetate (>98%) were purchased from Sigma Aldrich Laboratories, Inc. (St. Louis, MO, USA). Acetonitrile and water (HPLC grade) were purchased from Honeywell Burdick and Jackson, and acetic acid (98% min., GR) was purchased from EM Science (Gibbstown, NJ, USA). β-Glucuronidase (*Escherichia coli*-K12) was purchased from Roche Biomedical (Mannheim, Germany). All chemicals and reagents were used without further purification.

Solution preparation and daily operation

Stock solutions of phthalate metabolites, MHNCH, 4-MeUmb, and isotopically-labeled metabolites and 4-MeUmb were prepared in acetonitrile and stored at 4 °C in Teflon-capped glass bottles. The calibration standards, containing phthalate,

MHNCH and 4-MeUmb and were prepared in 10% aqueous acetonitrile from the stock solutions to create 10 unique standard solutions and stored at 4 °C in Teflon-capped glass vials until use. QC materials were prepared from a base urine pool obtained from multiple anonymous donors and divided into two subpools enriched with native phthalate metabolites to create low-concentration and high-concentration.

Urine is thawed, sonicated, vortex-mixed, and dispensed (100 μL) into a 1.5 mL conical bottom autosampler vial. Using a Surveyor HPLC autosampler (ThermoFinnigan, Bellefonte, PA, USA) the urine sample is spiked with the internal standard containing a mixture of isotopically labeled analytes (100 μL), 2% β-glucuronidase in ammonium acetate buffer (25 μL, pH 6.5/1M), and 4-MeUmb-glu solution (25 μL) using a Xcalibur sample preparation program and incubated at 37^{o}C for at least 120 minutes for the enzymatic hydrolysis of the glucuronidated phthalate metabolites followed by the addition of acetic acid (25 μL) and 5% aqueous acetonitrile (200 μL).

Instrumental analysis

The pretreated urine sample was loaded onto a Chromolith high resolution RP-18e guard cartridge (4.6 mm x 5 mm, Merck KGaA, Germany) placed in double female end cartridge holder (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 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% water) to 100% mobile phase B (0.1% acetic acid in acetonitrile) at a flow rate of 0.35 mL/minute (Table S1) and analyzed on a ThermoFinnigan TSQ Vantage triple quadrupole mass spectrometer equipped with an electrospray ionization interface using multiple reaction monitoring mode. Precursor and product-ion combinations specific to each phthalate metabolite were monitored and presented elsewhere. For MHNCH, we used m/z of 313/153, whereas for D_4-MHNCH, we used 317/153 transitions. Data acquisition and analysis were performed using the Xcalibur® software (ThermoFinnigan, Bellefonte, PA, USA). Similar to phthalate metabolites representing many isomers (i.e., MHNP, MCNP, MCOP, MONP, MNP), the whole cluster of peaks was integrated for MHNCH quantification. Area ratio of 4-MeUmb to its glucuronide was assessed to monitor the completion of deglucuronidation (Figure S1). Analytes were quantified using calibration curves constructed with peak area ratio of each analyte to its isotopically labeled internal standard versus standard concentration.

Method validation

We have modified our previously developed online SPE-HPLC-MS/MS method to measure the concentrations of urinary phthalate metabolites to include MHNCH, a DINCH metabolite. We replaced our Chromolith HPLC column with a Chromolith RP-18e guard column for SPE. The method uses less solvent and is cost effective. Replacement of the Chromolith RP-18e analytical column with Chromolith RP-18e guard column improved the HPLC column life. With our gradient program (Table S1) we achieved adequate separation of all analytes (Figure S2). The fragmentation and

relative abundance of the product ion fragments for MHNCH and their isotopically labeled internal standard, D_4-MHNCH were examined to select the best precursor/product ion combinations for quantification.

Most of the phthalate and DINCH metabolites excrete glucuronidated. Thus for accurate analysis, complete hydrolysis of the conjugate is required. Phthalates and MHNCH did not form sulfate conjugates, and MHNCH required longer hydrolysis time (120 min) compared with phthalate metabolites (90 min). The glucuronidase activity is monitored throughout the analytical run by measuring the area ratio of the free 4-MeUmb released to its internal standard. 4-MeUmb released from its glucuronide adequately monitored the enzyme activity (Figure S1). Analysis of spiked QC materials over a period of 3 months indicated excellent long-term reproducibility. We evaluated the method accuracy; we achieved excellent accuracy with good agreement between the calculated and actual spiked analyte concentrations (Table S2). The limits of detection (LOD) estimated were in the low ng/mL levels (Table S2) for all analytes—adequate for general population biomonitoring.

Table S1. SPE wash and HPLC run gradient programs.

Pump 1 SPE Wash Gradient				Pump 2 HPLC Run Gradient			
Time min	Flow rate ml/min	Composition		Time min	Flow rate ml/min	Composition	
		A	B			A	B
0	0.3	100	0	0	0.3	77	23
0.4	1.2	100	0	3.0	0.3	75	25
0.5	1.2	80	20	5.0	0.3	75	25
1.1	1.2	80	20	10.0	0.35	67	33
1.2	0.1	100	0	17.0	0.325	70	30
3.5	0.1	100	0	20.0	0.35	66	34
4.0	1.0	100	0	21.0	0.35	60	40
8.1	1.0	100	0	23.0	0.35	45	55
8.2	1.0	0	100	25.1	0.35	20	80
10.2	1.0	0	100	25.2	0.35	0	100
14.0	0.5	0	100	25.6	0.35	0	100
22.5	0.5	0	100	26.0	0.4	0	100
23.0	0.3	100	0	26.5	0.4	77	23
27.0	0.3	100	0	27.0	0.35	77	23

Table S2. Accuracy and LOD of phthalate and DINCH metabolites (in ng/mL).

Analyte	Accuracy		LOD
	Expected	Observed	
MMP	7.0	7.2	0.2
MCPP	3.5	3.6	0.2
MEP	50.0	48.0	0.6
MEHHP	12.5	12.7	0.2
MiBP	5.0	4.6	0.2
MECPP	12.5	12.7	0.2
MBP	13.1	11.8	0.4
MEOHP	13.8	12.8	0.2
MHNP	5.0	5.1	0.7
MCOP	5.0	5.0	0.2
MBzP	11.0	11.3	0.3
MCHP	5.0	4.5	0.2
MONP	5.0	5.2	0.4
MHNCH	2.5	2.5	0.4
MCNP	5.0	5.2	0.2
MEHP	5.0	5.1	0.5
MOP	5.0	4.9	0.5
MNP	5.0	4.6	0.5

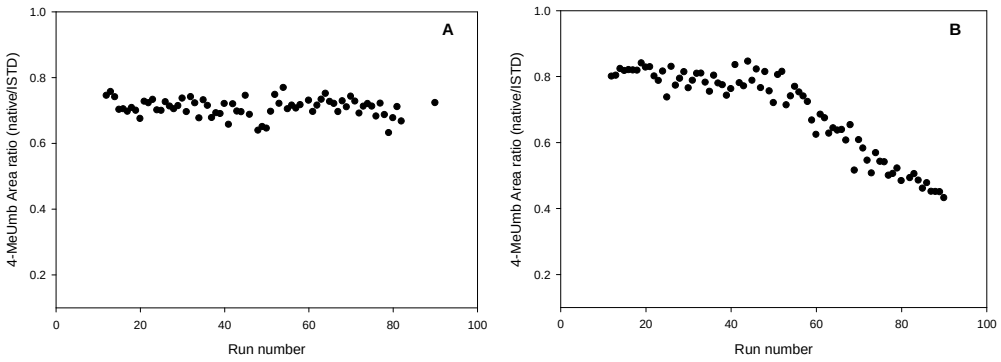

Figure S1. Evaluation of the glucuronidase enzyme activity during the sample preparation. Constant area ratio of the 4-MeUmb released from its glucuronide to its internal standard (A) shows proper working of the enzyme, whereas the declining area ratio (B) shows the deactivation of the enzyme during sample preparation.

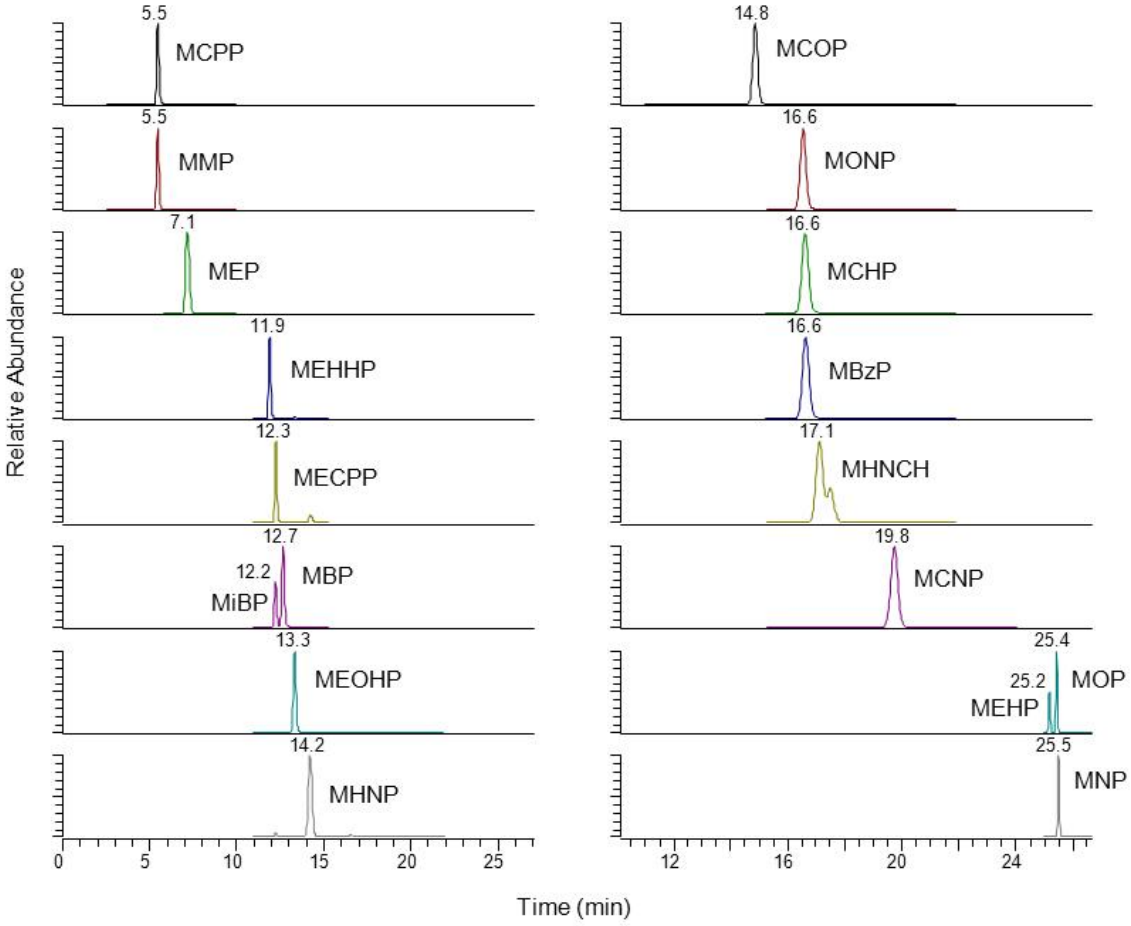

Figure S2: Chromatographic separation of phthalate and DINCH metabolites. Mono-ethyl phthalate (MEP), mono-butyl phthalate (MBP), Mono-cyclohexyl phthalate (MCHP), mono benzyl phthalate (MBzP), mono-isobutyl phthalate (MiBP), mono-2-ethylhexyl phthalate (MEHP), mono-isooctyl phthalate (MOP), mono-isononyl phthalate (MNP), mono-2-ethyl-5-oxohexyl phthalate (MEOHP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-3-carboxypropyl phthalate (MCPP), Mono-methyl phthalate (MMP), mono-oxoisononly phthalate (MONP) and mono-hydroxyisononyl phthalate (MHNP), cyclohexane-1,2-dicarboxylic acid- monohydroxyisononyl ester (MHNCH),

Mono-carboxy isooctyl phthalate (MCOP), mono-carboxy isononyl phthalate (MCNP), mono-2-ethyl-5-carboxypentyl phthalate (MECPP).