A High-Throughput SPE Method to Support the Biomonitoring of Phthalate Metabolites in Human Urine Using ISOLUTE® ENV+ Columns Prior to LC-MS/MS

Tested on Patient Samples

Roy Gerona[1], Frank Kero[2] Matthew Friesen[1], Victor Vandell[2] , Michael Yu[2]

1. *UCSF Department of Laboratory Medicine, 513 Parnassus Ave, Medical Sciences Bldg S 864 San Francisco, CA, USA 94143*
2. *Biotage, 10430 Harris Oaks Blvd., Suite C, Charlotte, NC USA 28269*

This application note describes the extraction of nine phthalate metabolites from human urine using ISOLUTE® ENV+ solid phase extraction columns.

Introduction

Phthalates are plasticizers used in industry to adjust the mechanical (and sometimes barrier) properties of plastics in consumer products and packaging. Their ubiquitous presence in our everyday lives constantly presents threats of low level exposure through inhalation or ingestion. Thus, large biomonitoring studies including the US National Health and Nutrition Examination Survey (NHANES) have screened for phthalates since 1999. Because phthalates themselves are difficult to eliminate from sampling and processing materials, including laboratory ware and instruments, analysis of phthalates in human samples have focused on their monoester metabolites. Monoethyl phthalate (MEP), monobutyl phthalate (MBP), monobenzyl phthalate (MBzP) and mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) have been constantly detected in human urine since the first NHANES survey of phthalates in 1999. Phthalates exposure has been associated with decreased anogenital distance, lower sperm count, cryptorchidism and hypospadias among other clinical endpoints in humans.

To facilitate the high throughput population screening of phthalate metabolites, sample preparation methods need to be simple, sensitive and robust to mitigate matrix suppression and instrument down time common to many dilute and shoot approaches to mass spectrometry. Thus, a solid phase extraction procedure was developed for these analytes. This application note details the optimization strategy for nine phthalate metabolites. Proof-of-concept for this sample preparation method was determined on a set of real patient samples (n=5). The results were in general agreement with previously reported concentration ranges for these compounds. It is anticipated that this method will have significant impact in environmental biomonitoring strategies for these analytes.

Analytes

Monomethyl phthalate (MMP); Monoethyl phthalate (MEP); Monobutyl phthalate (MBP); Monobenzyl phthalate (MBzP); Monohexyl phthalate (MHxP); Mono (2-ethylhexyl) phthalate (MEHP); Mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP); Mono (2-ethyl-5-carboxypentyl) phthalate (MECPP); Monoisononyl phthalate (MiNP)

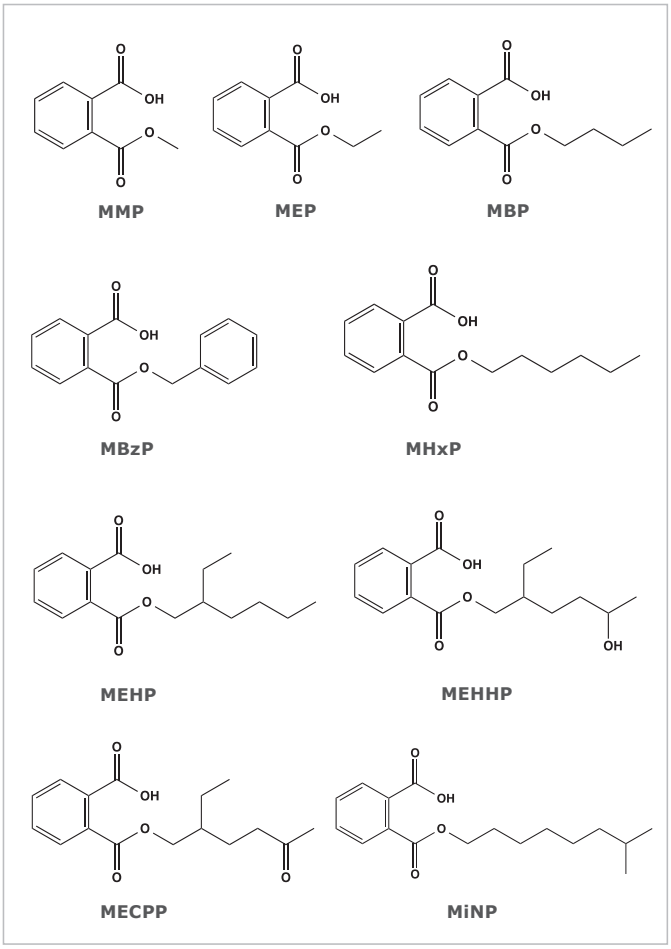

Figure 1. Structures of the target analytes in the phthalate metabolites panel.

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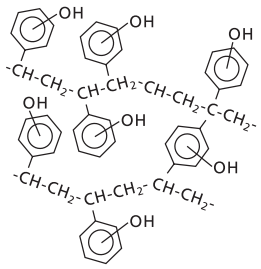
Sample Preparation Procedure

Format: **ISOLUTE® ENV+ 50 mg/ 3 mL columns, p/n 915-0005-B**



The SPE sorbent chemistry is a hyper crosslinked hydroxylated polystyrene-divinylbenzene copolymer with very high surface area that is ideal for extracting polar analytes from urine samples (**Figure 2**).

Figure 2. ISOLUTE® ENV+ sorbent chemistry

Sample pre-treatment:	Urine (500 µL) was deconjugated using 450 U of H pomatia glucuronidase Type H3 and incubation at 37°C and pH 5.5 for 2 hours. **C^{13}**-labeled internal standard (5 ng/mL) for each analyte was added after deconjugation.
Conditioning:	Condition each column with methanol (1 mL)
Equilibration:	Equilibrate each column with water (1 mL)
Sample Loading:	Load all pretreated sample at a flow rate of 1 mL /min
Interference Wash:	Elute interferences with H_2O/MeOH (90/10, v/v, 1 mL*)
Analyte Elution:	Elute analytes with MeOH (2 X 1 mL)
Post Extraction:	Extracts were evaporated to dryness using a Biotage TurboVap (37 °C, 40 psi) and reconstituted prior to injection.

*suggested variable to try on implementation – increase interference wash volume to 2 mL

HPLC Conditions

Instrument:	Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA)
Column:	Phenomenex Kinetex C18 (100 x 4.6 mm, 2.6 µm)
Column Temperature	40 °C
Injection Volume	25 µL
Mobile Phase:	Solvent A: 0.05% Ammonium acetate, pH 7.8 (aq)
	Solvent B: 0.05% Ammonium acetate, pH 7.8 (methanol)
Gradient:	

Table 1. Gradient parameters for the sepration phthalate metabolites.

Step #	Time (min)	Flow Rate (µL/min)	A (%)	B (%)
0	0	500	50	50
1	0.5	500	50	50
2	6.5	500	0	100
3	10.0	500	0	100
4	10.5	500	50	50
5	14.0	500	50	50

Mass Spectrometry Conditions

Instrument: AB Sciex 5500 triple quadrupole equipped with a Turbo Ionspray® interface operated in negative ion mode (Applied Biosystems, Foster City, CA.)

Ion Source Temperature: 700 °C

Spray Voltage: -4500V

The MRM transitions used are detailed in **Table 2**.

Table 2. MS/MS details for the selected phthalate analyte panel.

Multiple Reaction Monitoring (MRM) transitions								
Q1	**Q3**	**Time**	**Analyte**	**DP**	**EP**	**CE**	**CXP**	**Type**
179.1	77.0	15	MMP-1	-25	-10	-24	-11	Quant
179.1	107.0	15	MMP-2	-25	-10	-14	-15	Qual
193.1	76.9	15	MEP-1	-40	-10	-22	-13	Quant
193.1	43.0	15	MEP-2	-40	-10	-24	-19	Qual
221.2	71.0	15	MBP-1	-30	-10	-16	-11	Quant
221.2	68.9	15	MBP-2	-30	-10	-16	-33	Qual
255.2	77.0	15	MBzP-1	-35	-10	-24	-13	Quant
255.2	183.0	15	MBzP-2	-35	-10	-14	-17	Qual
249.2	99.0	15	MHxP-1	-40	-10	-20	-13	Quant
249.2	77.0	15	MHxP-2	-40	-10	-30	-17	Qual
277.2	133.9	15	MEHP-1	-45	-10	-18	-13	Quant
277.2	77.0	15	MEHP-2	-45	-10	-24	-11	Qual
293.3	120.9	15	MEHHP-1	-40	-10	-26	-15	Quant
293.3	76.9	15	MEHHP-2	-40	-10	-50	-33	Qual
307.2	159.1	15	MECPP-1	-40	-10	-20	-13	Quant
307.2	120.8	15	MECPP-2	-40	-10	-30	-17	Qual
291.2	76.9	15	MiNP-1	-55	-10	-28	-17	Quant
291.2	247.1	15	MiNP-2	-55	-10	-20	-25	Qual

Results and Discussion

Following a comprehensive sorbent screening and method optimization process, the solid phase extraction method described in this application note was applied to spiked synthetic urine samples.

Method repeatability (%RSD) and relative recovery was determined in five replicates of synthetic human urine specimen spiked with 20 ng/mL of each analyte. A representative chromatogram of spiked samples processed by the optimized method is given in **Figure 3**. The method repeatability measured for all analytes is ‹ 10% RSD (n=5). **Figure 4** presents the average relative recoveries obtained for each analyte (n=5). Error bars represent +/- 1 standard deviation of the mean. Actual measurements of phthalate metabolites in five representative subjects using the optimized method were also completed. Data obtained from the analysis are presented in **Table 3**.

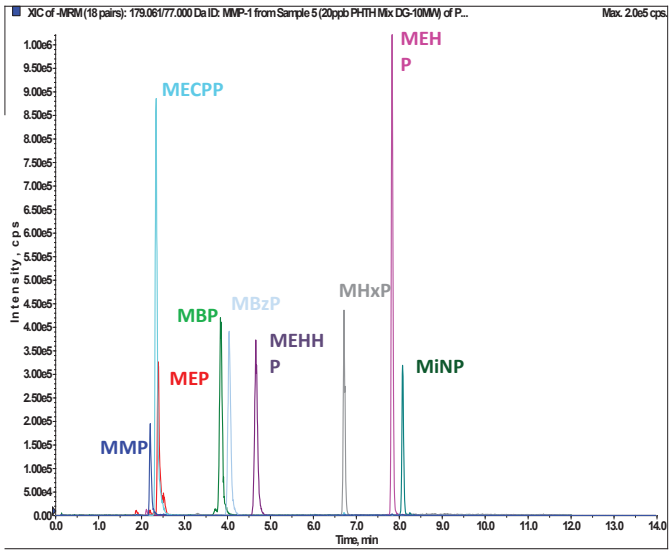


Figure 2. Representative chromatogram for extracted phthalate metabolites from synthetic urine (20 ng/mL).

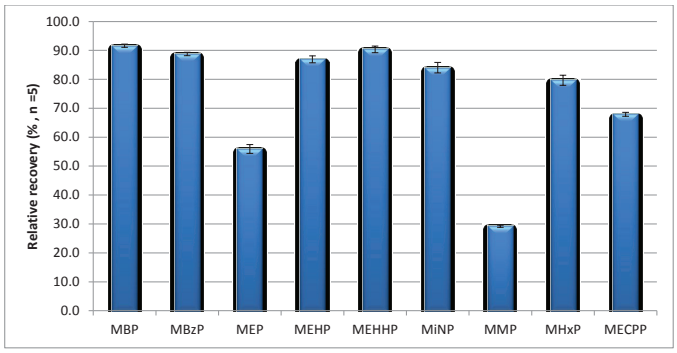


Figure 4. Relative recovery of the selected phthalate panel from human urine.

Table 3. Phthalate metabolite levels (ng/mL) measured from five pregnant women in a Northern California cohort.

Subject	MMP	MEP	MBP	MBzP	MHxP	MEHP	MEHHP	MECPP	MiNP
1	<LOD	50.4	14.5	0.5	1.2	0.4	12.5	12.5	<LOD
2	1.0	75.5	15.0	7.2	3.5	1.6	15.0	15.0	1.4
3	10.5	150.5	13.4	10.5	10.5	10.5	55.6	55.6	2.5
4	11.0	203.5	6.5	4.5	2.5	2.0	12.6	12.6	<LOD
5	<LOD	35.5	3.5	3.6	<LOD	0.5	6.4	6.4	<LOD

Conclusions

ISOLUTE® ENV+ 50mg / 3 mL SPE cartidges were successful in providing quantitative analyte recovery, repeatable method precision and minimal matrix suppression for nine phthalate metabolites in urine.

Acknowledgment

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Ordering Information

Part Number	Description	Quantity
915-0005-B	ISOLUTE® ENV+ 50 mg/3 mL	50
PPM-48	Biotage® PRESSURE+ 48 Positive Pressure Manifold	1
C103198	TurboVap® LV, 100/120V	1
C103199	TurboVap® LV, 220/240V	1

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EUROPE
Main Office: +46 18 565900
Toll Free: +800 18 565710
Fax: +46 18 591922
Order Tel: +46 18 565710
Order Fax: +46 18 565705
order@biotage.com
Support Tel: +46 18 56 59 11
Support Fax: + 46 18 56 57 11
eu-1-pointsupport@biotage.com

NORTH & LATIN AMERICA
Main Office: +1 704 654 4900
Toll Free: +1 800 446 4752
Fax: +1 704 654 4917
Order Tel: +1 704 654 4900
Order Fax: +1 434 296 8217
ordermailbox@biotage.com
Support Tel: +1 800 446 4752
Outside US: +1 704 654 4900
us-1-pointsupport@biotage.com

JAPAN
Tel: +81 3 5627 3123
Fax: +81 3 5627 3121
jp_order@biotage.com
jp-1-pointsupport@biotage.com

CHINA
Tel: +86 21 2898 6655
Fax: +86 21 2898 6153
cn_order@biotage.com
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