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# Identification of Di-isononyl Phthalate Metabolites for Exposure Marker Discovery Using In Vitro/In Vivo Metabolism and Signal Mining Strategy with LC-MS Data

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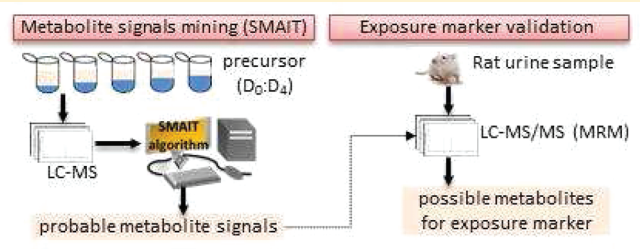
S *Supporting Information*

**ABSTRACT:** Di-isononyl phthalate esters (DINPs) are endocrine-disrupting chemicals and have replaced di(2-ethylhexyl) phthalate (DEHP) as the major plasticizer for poly(vinyl chloride) (PVC) products in recent years. Exposure marker discovery of DINPs is crucial, because of their high potential for human exposure and toxicity. Here, we propose an alternative approach for tracing signals derived from stable isotope-labeled precursors with varied labeling ratios to efficiently filter probable metabolite signals. The statistical process, which involves a signal mining algorithm with isotope tracing (SMAIT), has effectively filtered 13 probable DINP metabolite signals out of the 8867 peaks in the LC-MS data obtained from incubated stable isotope-labeled precursors with liver enzymes. Seven of the 13 probable metabolite signals were confirmed as DINP structure-related metabolites by preliminary MS/MS analyses. These 7 structure-related metabolite signals were validated as effective DINP exposure markers, using urine samples collected from DINP-administered rats without time-consuming comprehensive structure identification. We propose that the 7 identified possible DINP metabolite signals of $m/z$ 279.1, 293.1, 305.1, 307.1, 321.1, 365.1, and 375.1 are potential markers for DINP exposure and should be investigated further. The integrated approach described here can efficiently, and systematically, filter probable metabolite signals from a complex LC-MS dataset for toxic exposure marker discovery. It is a relatively low-cost/rapid workflow for exposure marker discovery.




Phthalates are a group of synthetic and high-production volume industrial chemicals. They are widely used in plastics, building materials, and personal care products. Phthalates are endocrine disruptors and have an effect on reproductive outcomes, development of the male reproductive tract,[1−3] and sexual differentiation in male rats.[1,4,5] Epidemiological studies have also revealed that exposure to several commonly used phthalates can alter sex steroid hormone levels in human subjects.[6−8] Phthalates are considered to be ubiquitous compounds to which humans are frequently exposed. Moreover, the illegal use of di(2-ethylhexyl) phthalate (DEHP) and di-isononyl phthalates (DINPs) as clouding agents in food and beverages was reported in Taiwan in May 2011.[9] Therefore, the evaluation of phthalate exposure in humans is a critical issue.[10]

Understanding the metabolism of an environmental pollutant or drug is important for exposure assessments of pollutants and pharmaceuticals.[11] However, the detection and identification of metabolites is often very difficult and laborious, requiring the synthesis of standard compounds and months of investigation. It is not an easy task, because of the extremely complex metabolism of an environmental pollutant or drug within an organism, which involves multiple enzymatic pathways and leads to a range of compounds with varying concentrations.[12] The use of a mass spectrometer (MS) coupled with online liquid chromatography (LC) has emerged as an ideal approach for the screening and identification of diverse metabolites.[13,14] Various methods have been used to filter the metabolite signals in complex LC-MS data, such as stable-isotope tracing,[15] mass defect filtering,[16] neutral loss filtering,[17] isotope pattern filtering,[18] and knowledge-based metabolic predictions.[19]

The stable isotope-labeled tracing strategy was developed to detect or mine metabolite signals in LC-MS data.[15,20,21] Using this strategy, equal amounts of native and stable isotope-labeled compounds are used as a tracer, and the user-defined intensity ratio of native to isotope-labeled signal response is traced.[15,20] However, the isotopic intensity ratios of metabolite signals from equal amounts of spiked native and isotope-labeled compounds are not always close to 1. Yam and Caldwell indicated that the error ranges of the intensity ratios could be determined by the purity of the natural and isotope-labeled compounds.[15]

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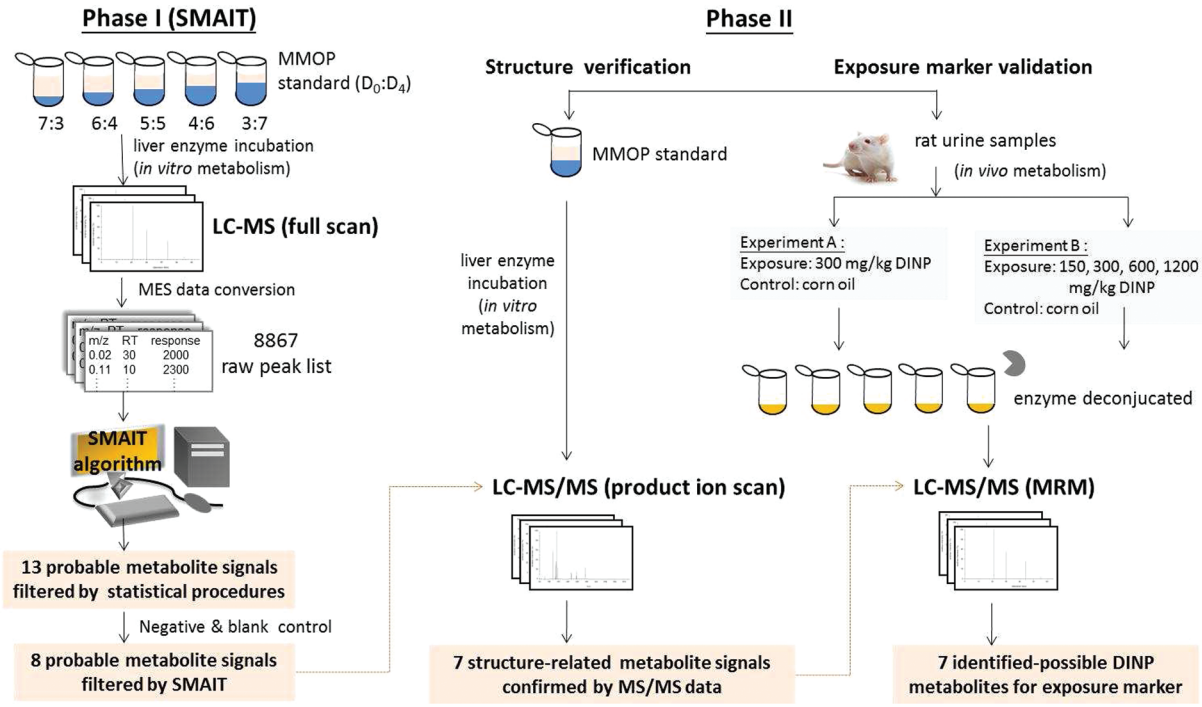



**Figure 1.** Experimental scheme of DINP metabolite identifications for exposure marker discovery using in vitro/in vivo metabolism.

To address the problem of error ranges of the isotope intensity ratios, Ling et al. proposed a statistical procedure, termed signal mining algorithm with isotope tracing (SMAIT), to selectively detect metabolite signals in LC-MS data based on the use of variable isotope ratios.[10] In SMAIT, varying isotope concentration ratios of native to stable isotope-labeled compounds among five mixtures are used as tracers, and a linear composition model is applied to filter out probable metabolite signals. The efficiency and confidence of the SMAIT statistical procedure has previously been demonstrated by mining metabolite signals in complex LC-MS datasets using the model compound DEHP.

We propose here an alternative approach to tracing stable isotope-labeled metabolite signals for DINP exposure marker discovery. It is a relatively low-cost/rapid workflow for exposure marker discovery, unlike the traditional metabolite identification workflow that has been adopted by the pharmaceutical industry. DINPs have replaced DEHP as the major plasticizer for poly(vinyl chloride) (PVC) in Europe.[22] DINPs consist of a complex mixture of isomeric nine-carbon branch-chain dialkyl phthalates,[23] and little is known about the metabolism of DINPs. Here, an alternative approach of tracing signals from stable isotope-labeled precursors with varied labeling ratios to efficiently filter probable DINP metabolite signals was demonstrated. Furthermore, validations of the filtered signals as effective DINP exposure markers using in vitro incubation samples, and urine samples collected from DINP-administered rats (in vivo metabolism) were also demonstrated.

## EXPERIMENTAL SECTION

Because of space considerations, experimental methods included Chemicals, Rat Liver Enzyme Incubation Sample (in vitro metabolism), Rat Urine Sample (in vivo metabolism), LC-MS analysis, Peak defining in LC-MS spectra, and LC-MS/MS analysis are provided in the Supporting Information.

## RESULTS AND DISCUSSION

The goal of this study was to investigate DINP exposure markers using an alternate approach for tracing signals derived from stable isotope-labeled precursors with varied labeling ratios to efficiently filter probable metabolite signals, preliminary verify the structures of the metabolite signals, and validate the metabolite signals as DINP exposure markers. A statistical procedure to selectively detect metabolite signals in LC-MS data based on the concept of SMAIT by variable isotope ratios was developed in our previous work.[10] In this study, the SMAIT concept was used to mine DINP metabolite signals in LC-MS data. The overall strategy for the discovery of DINP exposure markers applying the SMAIT concept is shown in Figure 1. The following details of the strategy for discovering DINP exposure markers will help illustrate the approach, which include phase I, which is DINP metabolite signal mining by SMAIT, and phase II, which is preliminary structure verification and DINP exposure marker validation.

**Mining DINP Metabolite Signals in LC-MS Data (Phase I).** DINPs are a complex mixture containing predominantly $C_8$- and $C_9$-branched isomers. The phthalates with methyl octanol side chains account for 35%−40% of the commercial DINP mixture and are first metabolized to mono side-chain phthalates.[24,25] Therefore, mono(4-methyloctyl) phthalate ($D_0$-MMOP) and four deuterium-labeled MMOP ($D_4$-MMOP) were synthesized as the parent compounds to generate the DINP metabolites. The chemical structures and positions of the deuterium labels for the MMOP parent compounds are shown in Figure 2. As shown in phase I of the experimental scheme (see Figure 1), five MMOP mixtures with different isotope-labeled concentration ratios (3:7, 4:6, 5:5, 6:4, and 7:3) were incubated with liver enzymes to generate DINP metabolites. The full-scan LC-MS data for the five incubation mixtures was obtained by LC-Q-TOF MS analysis. The Analyst QS files of the five incubation mixtures

from the LC-Q-TOF analysis were subjected to the MES program, to convert the data to peak list files containing $m/z$, intensity, and retention time (RT). After the peak stratification step, 8867 peaks in the LC-MS data were found when an isotope-labeled concentration ratio of 5:5 was used (see Figure S1 in the Supporting Information). The five peak lists from the five mixtures with different isotope-labeled concentration ratios were obtained and applied to a SMAIT in-house computational algorithm to filter probable DINP metabolite signals. Briefly, the intensity ratios of any two peaks that fulfilled the following parameters were identified from the peak lists of the five mixtures: RT difference of <0.25 min and mass difference ($m/z$) of 4.0 ± 0.2 amu. The user-defined parameters of RT and mass difference were determined empirically, based on the instrumental conditions. A linear composition model was used to carry out the correlation coefficients of any five intensity ratios in the five mixtures, where the RT shift among the five samples was <0.5 min. It was assumed that the intensity ratios with high correlation coefficients had a higher probability of containing probable metabolite signals. The correlation coefficients of any five intensity ratios above 0.9 were subjectively determined to contain probable metabolite signals. Also, the natural isotopic abundance signal pairs were excluded if the signals meet the following criteria: the RT difference of the signals was <0.1 min, and the difference in isotope abundance ratios between theoretical and experimental values was <20%. After the statistical procedure of SMAIT, using five DINP mixtures with different isotope-labeled concentration ratios, 13 probable DINP metabolite signals were filtered, including $m/z$ 247.1, 263.1, 279.1, 293.1, 305.1, 307.1, 321.1, 359.1, 365.1, 421.1, 495.1, 375.1, and 511.1.

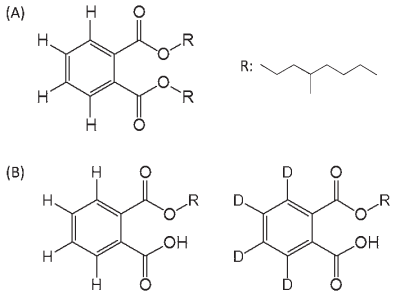
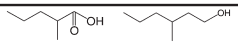
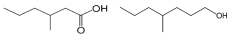
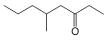
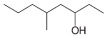
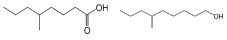



**Figure 2.** (A) Chemical structure of di(4-methyloctyl) phthalate, which is an isomer of di-isononyl phthalate. (B) Chemical structures of the parent compounds: mono(4-methyloctyl) phthalate ($D_0$-MMOP, left) and deuterium-labeled mono(4-methyloctyl) phthalate ($D_4$-MMOP, right).

One blank and two negative control samples were used to confirm the 13 probable DINP metabolite signals filtered by SMAIT. The results from the blank control sample indicated that there were no identical signals within the 13 probable DINP metabolite signals filtered by SMAIT. There are five signals generated from the procedure and impurity negative control samples, which included $m/z$ 247.1, 263.1, 359.1, 421.1, and 495.1. These signals, generated from the blank and two negative control samples, were excluded from the signal list of the 13 metabolite signals filtered by SMAIT. The remaining 8 signals listed in Table 1 were considered as probable metabolite signals that contain probable DINP metabolites with high confidence and were further evaluated as DINP exposure markers. The details of performing the blank and two negative control samples have been described in the Supporting Information.

Using the metabolite signal $m/z$ 307.1, which indicates a probable metabolite in this study, as an example, Figure 3A presents the extracted ion chromatograms (XICs) of the probable DINP metabolite. The $D_4$-labled isotopic peak ($m/z$ 311.1)

**Table 1. Characteristics of the Eight Probable DINP Metabolite Signals Filtered by SMAIT Strategy**[abcdefg]

| NO | $m/z$ for metabolite signals (isotopic pair) | RT (min) [a] | number of peaks [b] | resolution of peaks | accurate mass [c] | product ions identical to MMOP product ions (relative intensity, %) [d] | | | literature [e] | speculated chemical structure (side chain R) [f] | MRM transition [g] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| I | 279.1 (283.1) | 27.66 | 2 | 1.01 | 280.11 | 121.0 (100) | | | * |  | 279.1/121.1 |
| II | 293.1 (297.1) | 29.04 | 1 | | 294.19 | 247.1 (100) | 121.0 (32) | 147.0 (15) | * |  | 293.1/121.0 |
| III | 305.1 (309.1) | 29.84 | 2 | 1.41 | 306.15 | 121.0 (93) | 146.9 (45) | | * |  | 305.1/121.0 |
| IV | 307.1 (311.1) | 30.43 | 2 | 0.74 | 308.17 | 121.0 (100) | | | * |  | 307.1/121.0 |
| V | 321.1 (325.1) | 31.39 | 2 | 1.01 | 322.16 | 121.0 (100) | | | * |  | 321.1/121.0 |
| VI | 365.1 (369.1) | 30.44 | 2 | 0.92 | 366.10 | 217.0 (62) | 121.0 (24) | | | derivative of 307.1 | 365.1/307.1 |
| VII | 375.1 (379.1) | 30.43 | 2 | 0.70 | 376.15 | 121.0 (19) | | | | derivative of 307.1 | 375.1/307.1 |
| VIII | 511.1 (515.1) | 30.43 | 2 | 0.79 | 512.15 | | | | | | |

[a] Average retention time (RT) of metabolite signals among five samples. [b] Total number of chromatographic peaks present in XIC of the probable DINP metabolite signal. [c] The accurate mass data was obtained from the molecule ion $m/z$ information and the data derived by Q-TOF. [d] The overlapping fragments with MMOP product ion profile shown in Figure 4A. [e] The metabolites were reported in the literature (ref 13). [f] The speculated chemical structure, according to the exact mass and MS/MS fragment profile. The side chain R of chemical structures of phthalate monoesters in Figure 2B. [g] The MRM transition for determining the metabolite signals in rat urine samples by LC-MS/MS.

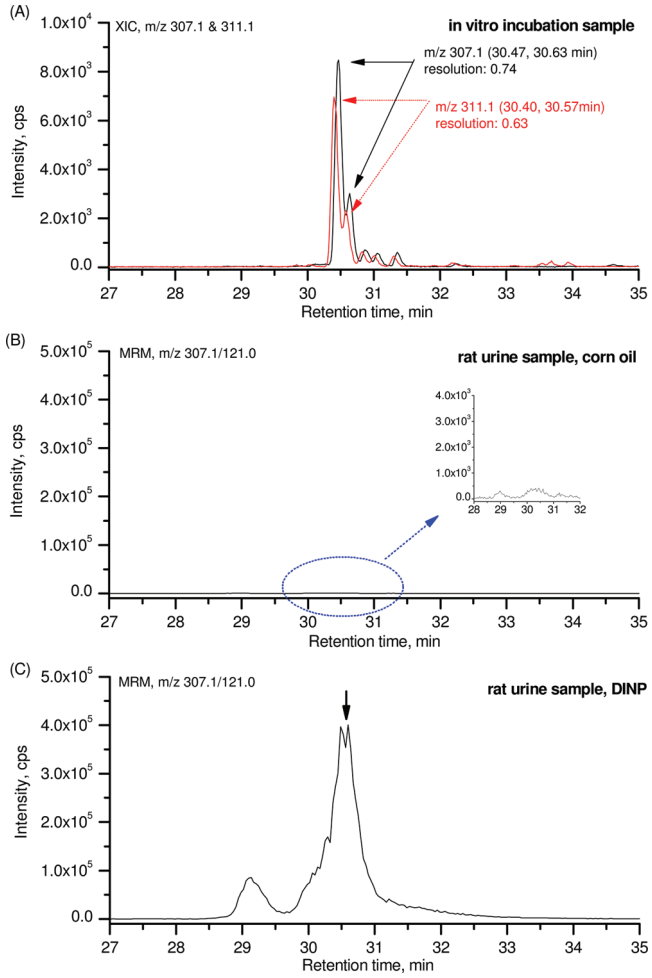



**Figure 3.** Extracted ion chromatograms (XICs) of the probable DINP metabolite signal $m/z$ 307.1: (A) XIC from an in vitro incubation sample with $D_0$-MMOP and $D_4$-MMOP (5:5), (B) XIC from a urine sample from a rat administered corn oil (placebo), and (C) XIC from a urine sample from a rat administered 300 mg/kg commercial DINP.

eluted slightly earlier than its native peak ($m/z$ 307.1), and this phenomenon appeared with all 8 probable metabolite signals filtered by SMAIT. The RT differences between the native and isotopic peaks for the 8 probable DINP metabolites were all within 6 s. As shown in Figure 3A, the probable metabolite signal $m/z$ 307.1 contained more than one chromatographic peak. However, the SMAIT program reported the chromatographic peak cluster as one signal, which could be attributed to the signal stratification process by MES. The chromatographic peak cluster was recognized as one peak in MES and therefore was reported as one signal by the SMAIT program. In addition, the resolutions of the other chromatographic peak clusters of these signals were manually calculated and are shown in Figure S2 in the Supporting Information. According to the resolution calculations, the chromatographic peak clusters with resolutions of >0.7 were defined as containing more than one peak. After the resolution calculations, 8 probable metabolite signals containing 15 probable metabolite peaks were defined. The peak numbers and resolutions for the chromatographic peak clusters in the 8 probable metabolite signals are listed in Table 1.

**Verification of the Eight Probable DINP Metabolite Signals by Preliminary Structure Information (Phase II).** For new

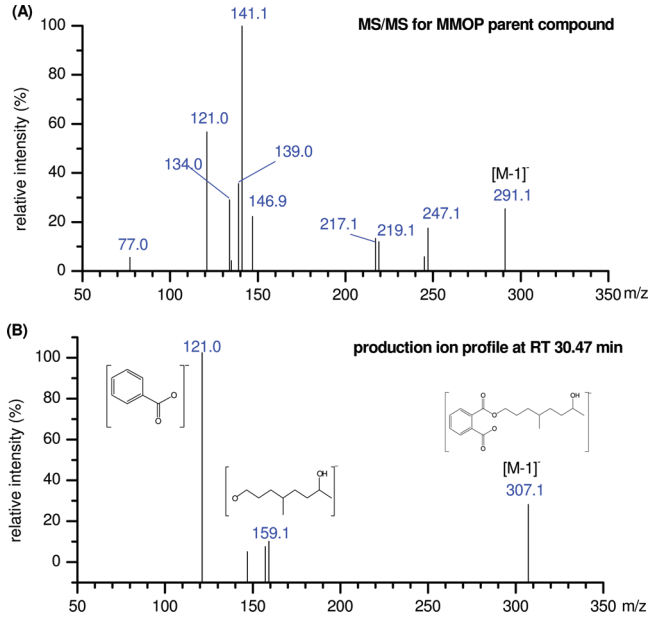



**Figure 4.** MS/MS product ion profiles of (A) the $D_0$-MMOP parent compound and (B) the probable DINP metabolite signal $m/z$ 307.1, product ion profile of precursor ion 307.1 at RT = 30.47 min.

metabolite discovery, it is often difficult to obtain pure standards for chemical structure confirmations. An alternate approach using structural information was used to initially confirm that the eight probable metabolite signals filtered by SMAIT were actually DINP metabolites. An assumption was made that a metabolite and the metabolite parent compound would have one or more identical product ions in their MS/MS product ion profiles. The LC-MS/MS product ion profiles of the eight probable metabolite signals were obtained and were compared with the product ion profiles of the MMOP parent compound. As shown in Figure 4A, the MMOP parent compound was a deprotonated ion with a single charge $[M-H]^-$ in negative ionization mode, and it generated product ions at $m/z$ 121.0 (57%, the proportion of relative abundance to the most intense product ion), 134.0 (29%), 139.1 (36%), 141.1 (100%), 147.0 (22%), 217.1 (13%), 219.1 (12%), 247.1 (17%), and 291.1 (25%). The product ion profiles of the eight probable metabolite signals (shown in Figure S3 in the Supporting Information) were compared to the product ion profile of the MMOP parent compound. Using metabolite signal $m/z$ 307.1 as an example, Figure 4B presents the product ion profile of the most-abundant isomer peak in the $m/z$ 307.1 chromatographic peak cluster. The product ion profile generated by precursor ion $m/z$ 307.1 at RT = 30.47 min included $m/z$ 121.0 (100%), 159.1 (7.5%), and 307.1 (26.8%). Comparison with the product ion profile of the MMOP standard indicates that the product ion $m/z$ 121.1 generated by precursor ion $m/z$ 307.1 was identical to an MMOP product ion. As presented in Table 1, each of the probable metabolite signals shared at least one identical product ion with the MMOP parent compound, with the exception of metabolite signal $m/z$ 511.1. In addition, the product ion at $m/z$ 121.1 was present in the product ion profiles of all probable metabolite signals, with the exception of metabolite signal $m/z$ 511.1. As a result, the seven probable metabolite signals $m/z$ 279.1, 293.1, 305.1, 307.1, 321.1, 365.1, and 375.1 were most likely DINP structure-related metabolite signals, as confirmed by MS/MS data.

In addition, the information derived from the accurate mass and product ion profiles of the eight DINP probable metabolite signals (shown in Figure S3 in the Supporting Information) was used to speculate the probable structures of these signals. The speculated chemical structures of the eight DINP probable metabolite signals have been summarized in Table 1. The probable metabolite signals *m/z* 279.1, 293.1, and 321.1 were tentatively identified as hydroxyl metabolite or carbonyl metabolite; signal *m/z* 305.1 was mono(oxo-isononyl) phthalate; signal *m/z* 307.1 was mono(hydroxyl-isononyl) phthalate; and signals *m/z* 365.1 and 375.1 were derivatives of mono(hydroxyl-isononyl) phthalate. The metabolite signal *m/z* 511.1 did not have enough information to speculate about the probable structure. According to the molecule ion *m/z* information, five metabolite signals (*m/z* 279.1, 293.1, 305.1, 307.1, and 321.1) have previously been reported in the literature[13,26] and three metabolites signals (*m/z* 365.1, 375.1, and 511.1) were identified as novel metabolites. As shown in Table 1, the metabolite signal *m/z* 279.1 was identified as two potential chemical structures: mono(hydroxy-isoheptyl) phthalate and mono(carbonyl-isopentyl) phthalate. The accurate mass of metabolite signal *m/z* 279.1 was 280.11, based on the information of molecule ion *m/z* 279.11. The calculated exact masses for the mono(hydroxy-isoheptyl) phthalate and mono(carbonyl-isopentyl) phthalate are 280.1238 and 280.0874, respectively. However, the precision of the accurate mass data is 0.1 amu, based on the Q-TOF MS resolution in this application experiment (7000 at *m/z* 514). The Q-TOF MS resolution is not sufficient to distinguish the mass difference of the two phthalates (mono(hydroxy-isoheptyl) phthalate and mono(carbonyl-isopentyl) phthalate). The product ion profile of the metabolite signal *m/z* 279.1 is shown in Figure S3A in the Supporting Information. The most abundant product ion at *m/z* 121.0 was assigned to a benzoate ion ($[C_6H_5CO_2]^-$), and the fragment *m/z* of 131.0 was assigned to a hydroxyl-substituted alkyl side chain ($[O(CH_2)_7OH]^-$) or carboxylic acid-substituted alkyl side chains ($[O(CH_2)_5COOH]^-$). The calculated exact mass for the hydroxyl-substituted alkyl side chain ($[O(CH_2)_7OH]^-$) and carboxylic acid-substituted alkyl side chains ($[O(CH_2)_5COOH]^-$) are 131.1072 and 131.0708, respectively. The Q-TOF MS resolution is not sufficient to distinguish the mass difference of these two possible structures for side chains at *m/z* 131.0. Therefore, the probable metabolite signal *m/z* 279.1 was tentatively identified as two potential chemical structures: mono(hydroxy-isoheptyl) phthalate and mono(carbonyl-isopentyl) phthalate. A mass spectrometry with higher mass accuracy can be used to distinguish mass difference between the two chemical compounds in a further study. For the metabolite signals *m/z* 293.1 and 321.1, the condition was similar to the metabolite signal *m/z* 279.1. The metabolite signal *m/z* 293.1 was tentatively identified as two potential chemical structures: mono(hydroxy-isooctyl) phthalate and mono(carbonyl-isohexyl) phthalate. The metabolite signal *m/z* 321.1 was tentatively identified as two potential chemical structures: mono(hydroxy-isodecyl) phthalate and mono(carbonyl-isooctyl) phthalate. The chemical structures of the metabolite signals are shown in Table 1. For metabolite signal *m/z* 305.1, Figure S3C in the Supporting Information presents the product ion profile and it was tentatively identified as mono(oxo-isononyl) phthalate (calculated exact mass: 306.1394), which has been identified as a DINP metabolite in previous reports.[13,26] Figure 4B presents the product ion profile of metabolite signal *m/z* 307.1. The metabolite with a precursor ion *m/z* of 307.1 was a deprotonated ion with a single charge $[M-H]^-$. The accurate mass was 308.17, based on the information of molecule ion *m/z* 307.17. The most abundant product ion at *m/z* 121.1 was assigned to a benzoate ion ($[C_6H_5CO_2]^-$), and the fragment *m/z* of 159.1 was assigned to a hydroxyl-substituted alkyl side chain ($[C_9H_{19}O_2]^-$). Therefore, the probable metabolite signal *m/z* 307.1 was tentatively identified as mono(hydroxyl-isononyl) phthalate (exact mass: 308.1551), which has been identified as a DINP metabolite in previous reports.[13,26] For the probable metabolite signals *m/z* 365.1 and 375.1, the most abundant product ion was 307.1, and the fragment *m/z* of 121.0 was assigned to a benzoate ion ($[C_6H_5CO_2]^-$) (shown in Figures S3E and S3F in the Supporting Information). By comparing with the product ion profile of the metabolite signal *m/z* 307.1 (shown in Figure 4B), two identical product ions *m/z* 121.1 and 159.1 were presented in the product ion profile of the metabolite signal *m/z* 365.1 and 375.1. Furthermore, the product ions *m/z* 121.1 and 159.1 are the major product ions for the metabolite signal *m/z* 307.1. It is speculated that the metabolite signals *m/z* 365.1 and 375.1 are derivatives of metabolite signal *m/z* 307.1. Therefore, the probable metabolite signals *m/z* 365.1 and 375.1 were tentatively identified as derivatives of mono(hydroxyl-isononyl) phthalate.

Among the seven structure-related metabolites identified in this study, two metabolites have previously been reported in the literature,[13,26] and two metabolites (derivatives of mono(hydroxyl-isononyl) phthalate at *m/z* 365.1 and 375.1) were identified as novel metabolites. Silva et al. reported that the first-stage metabolite, monoisononyl phthalate, is only a minor urinary metabolite of DINP, and the oxidative metabolites, such as mono(carboxy-isooctyl) phthalate and mono(hydroxyl-isononyl) phthalate, are the major urinary metabolites in DINP-dosed rats.[13] The oxidative metabolites have been suggested to be better biomarkers for DINP exposure assessment than monoisononyl phthalate.[27] Unfortunately, a systematic study of DINP metabolism is currently lacking, and an evaluation of suitable biomarkers for the exposure assessment of DINPs cannot be carried out. Using the isotopic signal tracing method to study the DINP metabolites systematically can provide valuable information for understanding DINP metabolism and can directly assist with DINP exposure marker discovery.

**Validation of the Seven Structure-Related Metabolite Signals as DINP Exposure Markers (Phase II).** The seven structure-related metabolite signals verified by MS/MS data were candidates for DINP exposure markers and were validated as DINP exposure markers using a rat model. As mentioned in the above section, DINPs are complex mixtures containing mainly $C_8$- and $C_9$-branched isomers, and humans are exposed to commercial DINP, not a pure compound. Therefore, the commercial DINP mixture was used for the rat exposure study. The seven structure-related metabolite signals were measured in rat urine collected from the rats administered corn oil (placebo) and with a 300 mg/kg DINP mixture using LC-MS/MS in MRM mode. The structure-related metabolite signals were directly measured in the urine samples but the structures were not identified, because of the advantage of using LC-MS and LC-MS/MS for metabolite discovery and validation. Using the metabolite signal *m/z* 307.1 as an example to explain the determination of the metabolite signal in rat urine sample by LC-MS/MS in MRM mode, certain parameters, including the RT of the signal and the precursor/product ion transitions, were necessary. An advantage of using LC-MS and LC-MS/MS for

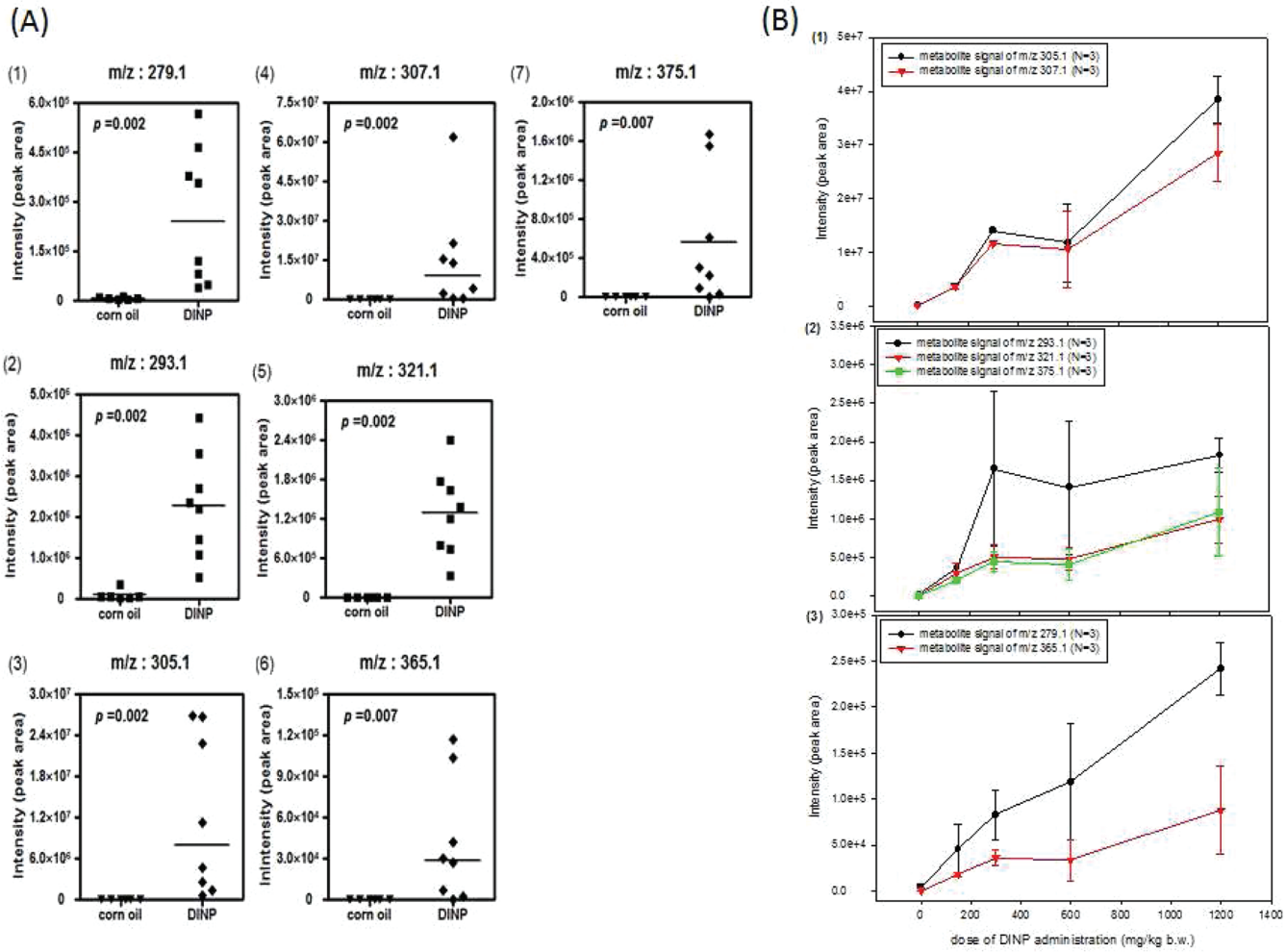



**Figure 5.** The signal responses of probable DINP metabolite signals in urine samples collected from rats administered (A) 300 mg/kg DINP or corn oil and (B) varied doses of DINP.

metabolite discovery was that the RT for the metabolite signal $m/z$ 307.1 was already identified when the signal was filtered by the SMAIT procedure.

For the precursor/product ion transitions for MRM mode, the LC-MS/MS product ion profiles of the metabolite signals were obtained when preliminary characterizing the structure information, and the most abundance product ion was chosen for monitoring. The transitions for determining the seven structure-related metabolite signals are listed in Table 1. As an example, the XICs of the probable metabolite signal $m/z$ 307.1 in rat urine samples collected from rats administered corn oil and 300 mg/kg commercial DINP mixture are shown in Figures 3B and 2C. For the rat urine sample collected from rat administered corn oil, no peaks (signal-to-noise ratio of S/N < 3) were found after 30−31 min in the XIC of MRM transition 307.1/121.0 (shown in Figure 3B). For the rat urine samples collected from rats administered 300 mg/kg commercial DINP mixture, a peak was found at RT = 30.50 min in the XIC of MRM transition 307.1/121.0 (shown in Figure 3B). Unfortunately, only one wide peak was found, not two separate peaks, as shown in Figure 3A for the in vitro incubation samples. The poor resolution of the peaks in the urine samples could be attributed to the complex matrix of urine, and more efforts in sample cleanup most likely would improve the resolution in further studies. On the other hand, the wide peaks could be peak clusters formed by the myriad signals of DINP metabolite isomers generated from the dosed compound, commercial DINP containing mainly $C_8$- and $C_9$-branched phthalate isomers. Poor resolution was observed for metabolite signals $m/z$ 279.1, 305.1, 307.1, 321.1, 365.1, and 375.1. Therefore, the signal responses of these six metabolite signals were calculated by the wide peaks and regarded as the total response of a metabolite signal in the rat urine sample. The signal response results for the seven structure-related DINP metabolite signals in the rat urine samples are shown in Figure 5A. The signal responses for the seven structure-related DINP metabolites in urine collected from rats administered 300 mg/kg DINP mixture were statistically higher than those from rats administered corn oil (placebo) (Wilcoxon rank-sum test, $p < 0.01$). As a consequence, the seven structure-related metabolite signals that were potential DINP exposure markers did, in fact, represent exposure to DINPs.

The relationship between the DINP exposure dose in rats and the responses of the seven structure-related metabolite signals in rat urine samples was investigated. The seven structure-related metabolite signals were measured in the rat urine samples collected from rats orally administered by gavaging 0, 150, 300, 600, or 1200 mg/kg with commercial DINPs, using LC-MS/MS in MRM mode. As Figure 5B shows, the plots reveal the

relationship between varied administered doses with varied metabolite signal intensities in rat urine. These plots indicate that all seven metabolites had a positive correlation with increasing administered dose. Metabolite signals $m/z$ 305.1 and 307.1 especially had higher signal intensities, compared to the other metabolite signals, and the increase in intensity as a function of dose was significant. It was assumed that metabolite signals $m/z$ 305.1 and 307.1 were exceptional DINP exposure markers and were worth further investigation.

## CONCLUSIONS

This study is the first example demonstrating that the signal mining strategy SMAIT (signal mining algorithm with isotope tracing) can effectively and systematically discover urinary exposure makers for toxic di-isononyl phthalate esters (DINPs). Seven metabolite signals were identified as potential markers for DINP exposure assessment. The seven possible DINP metabolite signals might be more suitable DINP exposure markers, because of all of the metabolite signals that were second-stage oxidative metabolites derived from monoisononyl phthalate (MINP), which has been reported to be an insensitive exposure marker.[28] These potential DINP exposure markers should be further applied to human DINP exposure assessments. Although the chemical structure of the filtered metabolite signals still must be identified, our strategy substantially reduced the number of potential metabolite signals. It increased the efficiency of metabolite identification, as a result of focusing on the metabolite signals with high confidence. In contrast to pharmaceutical research, relatively low-cost/rapid workflow for exposure marker discovery is needed. But the traditional metabolite identification workflow that has been adopted by the pharmaceutical industry is costly and time-consuming. Comprehensive structure elucidation of all possible metabolites that is required for drug evaluation may not be essential for toxic exposure assessment. Our strategy is a mass spectral signal-centered approach, rather than a metabolite structure elucidation-centered approach. Moreover, the metabolite signals in LC-MS data can be used not only for marker discovery but also routine marker measurement. It can be a useful tool for investigating the metabolism of environmental toxicants and drugs, discovering exposure markers for environmental toxicants, and assessing human metabolites in preclinical safety testing.[29]

## ASSOCIATED CONTENT

**Supporting Information.** Experimental details and additional information as noted in the text. (PDF) This material is available free of charge via the Internet at http://pubs.acs.org.

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