



Comprehensive LC-MS/MS method combined with tandem hybrid hydrolysis for multiple exposure assessment of multiclass environmental pollutants

Seunghwa Lee^a, Kang Mi Lee^a, Sang Moon Han^a, Hyeon-Jeong Lee^a, Changmin Sung^a, Hophil Min^a, Hosub Im^b, Sang Beom Han^c, Sangwon Cha^d, Jaeick Lee^{a,*}

^a Doping Control Center, Korea Institute of Science and Technology, Hwarang-ro 14-gil 5, Seongbuk-gu, Seoul, 02792, South Korea

^b Institute for Life & Environmental Technology, Smartive Corporation, 155, Misagangbyeon-hangang-ro, Hanam-si, Gyeonggi-do, South Korea

^c Department of Pharmaceutical Analysis, College of Pharmacy, Chung-Ang University, 84 Heukseok-ro, Dongjak-gu, Seoul, 06974, South Korea

^d Department of Chemistry, Dongguk University, 30, Pildong-ro 1-gil, Jung-gu, Seoul, 04620, South Korea

ARTICLE INFO

Keywords:

Environmental pollutants (EPOs)
Tandem hybrid hydrolysis
Comprehensive mobile phase
Liquid chromatography-mass spectrometry (LC-MS)

ABSTRACT

Environmental pollutants (EPOs), such as phthalates, volatile organic compounds, phenols, parabens, polycyclic aromatic hydrocarbons, pyrethroids, and environmental tobacco smoke, are highly heterogeneous compounds. Recently, attention has been drawn to the assessment of the combinatory effects of multiple EPs. To correlate multiple exposures with potential health implications, advanced comprehensive analytical methods covering multiclass EPOs are essential. However, because of several technical problems associated with enzyme hydrolysis, simultaneous extraction, and multiresidue liquid chromatography–tandem mass spectrometry analysis, it is difficult to establish a comprehensive method covering a number of EPOs in a single sample preparation and analytical run. We developed tandem hybrid hydrolysis, modified direct injection, and a comprehensive mobile phase to overcome these technical problems and established a comprehensive analytical method for simultaneous biomonitoring of multiclass EPOs. Tandem hybrid hydrolysis using β -glucuronidase and consecutive acid hydrolysis allowed selective hydrolysis of glucuronide- and sulfate-conjugated metabolites without phthalate degradation. The comprehensive mobile phase composed of 0.01% acetic acid and acetonitrile enabled us to simultaneously analyze 86 EPOs, with good chromatographic behavior and ionization efficiency. Modified direct injection allowed a small amount of sample and simultaneous urinary extraction. The method was validated and applied to 39 urine samples from 19 mother–newborn pairs for multiple exposure assessment. Results showed that BP-3, a general component in sunblock products, and monoethyl phthalate, a metabolite of diethyl phthalate, exhibit a clear positive correlation between mothers and newborns. Therefore, the developed method has potential as a novel analytical tool for long-term, large-scale, and data-rich human biomonitoring of EPOs.

1. Introduction

Among diverse environmental pollutants (EPOs) such as metals and organic pollutants, phthalates, volatile organic compounds (VOCs), phenols, polycyclic aromatic hydrocarbons (PAHs), pyrethroids and environmental tobacco smoke (ETS) are the major EPO classes excreted through urine (Azzouz et al., 2016; Gao et al., 2021). These are present everywhere, and exposure is through various routes, such as inhalation, ingestion, and absorption through the skin (Diamanti-Kandarakis et al., 2009). Many of these chemicals are associated with diverse human

diseases (Han et al., 2008; Heudorf et al., 2007; Lang et al., 2008; Levine et al., 2015; Schnatter et al., 2005). Furthermore, recently, there has been an increasing interest in human multiple exposure assessment and the combinatory effects, which involve multiclass EPOs. Therefore, many countries have passed regulations on these chemicals and monitor them using environmental exposure assessments, such as the National Health and Nutrition Examination Survey (NHANES), the Canadian Health Measures Survey (CHMS), and the Korean National Environmental Health Survey (KoNEHS) (Kassotis et al., 2020). To monitor multiclass EPOs and investigate potential health implications, they

* Corresponding author. Korea Institute of Science and Technology, Hwarang-ro 14-gil 5, Seongbuk-gu, Seoul, 02792, South Korea.

E-mail address: jaeicklee@kist.re.kr (J. Lee).

<https://doi.org/10.1016/j.envres.2022.113053>

Received 26 October 2021; Received in revised form 25 February 2022; Accepted 27 February 2022

Available online 1 March 2022

0013-9351/© 2022 The Authors.

Published by Elsevier Inc.

This is an open access article under the CC BY-NC license

(<http://creativecommons.org/licenses/by-nc/4.0/>).

determine multiclass EPOLs in urine via class-specific individual Laboratory Procedure Manuals with different sample preparations, instruments, and analytical conditions. Thus, current analytical methods for multiclass EPOLs have typically focused on a single EPOL class (Alwis et al., 2012; Chen et al., 2012b; Chiang et al., 2015; Onyemauwa et al., 2009; Silva et al., 2007; Xu et al., 2004). However, EPOL analysis using class-specific individual methods is not practical in large-scale and long-term epidemiological studies involving the measurement of thousands of samples because of the excessive demand for analytical instruments, manpower, sample preparation time, and cost (Preindl et al., 2019). Considering these points, an advanced comprehensive and practical method covering multiclass EPOLs in a single sample preparation and analytical run is essential for successful large-scale and long-term multiple exposure assessment (Li et al., 2021; Preindl et al., 2019; Warth et al., 2017). However, to date, there is no comprehensive and practical method available for multiclass EPOLs. Consequently, the establishment of a comprehensive method is a current key challenge in environmental research fields.

Despite the efforts to integrate diverse class-specific sample preparation and analytical conditions, several methods that integrate no more than two different classes (phthalates and phenols) were reported due to several technical limitations (Azzouz et al., 2016; Heffernan et al., 2016; Myridakis et al., 2015; Yao et al., 2018). Many laboratories have trouble in overcoming these technical limitations. First, integrating enzymatic hydrolysis procedures to convert glucuronide- or sulfate-conjugated metabolites into their free forms is challenging. The Centers for Disease Control and Prevention National Health and Nutrition Examination Survey (CDC NHANES) and related references used H-1 or H-2 type of β -glucuronidase/sulfatase enzymes from *Helix pomatia* for enzymatic hydrolysis of phenols, parabens, and PAHs (Chen et al., 2012b; Onyemauwa et al., 2009; Wang et al., 2013; Yao et al., 2018). Some studies applied β -glucuronidase extracted from *Escherichia coli* K12 to phthalates (Heffernan et al., 2016; Myridakis et al., 2015; Silva et al., 2007) because sulfatase-containing enzymes include lipase and esterase and cause an error in quantitation results due to degradation of phthalate diesters in biological samples and containers (Blount et al., 2000; Heffernan et al., 2016). Thus, these two hydrolysis conditions are mutually incompatible in a single sample preparation. The second concern is simultaneous urinary extraction of all the EPOLs in a single sample preparation. Although online solid phase extraction or column switching can be used for simultaneous extraction (Chen et al., 2012a; Chiang et al., 2015; Heffernan et al., 2016; Mortensen et al., 2014; Ye et al., 2005), these methods are not suitable for hydrophilic EPOLs, such as VOCs. Thus, it is not easy to simultaneously extract all the EPOLs with diverse hydrophilic, hydrophobic, acidic, neutral, and basic properties from a biological matrix. The third challenge is to establish a comprehensive mobile phase satisfying both good chromatographic behavior and ionization efficiency for all the EPOLs. Because of the structural characteristics of EPOLs, it is difficult to optimize the mobile-phase composition involved in the type and concentration of diverse additives and organic modifiers (Hua and Jenke, 2012). Consequently, it is critical to solve these technical problems for the successful development of a comprehensive analytical method.

This study aims to develop a comprehensive and practical analytical method for multiple exposure assessment of EPOLs in human urine using liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS). To the best of our knowledge, there is no method to assess most of the EPOLs from seven different classes excreted through urine, including phthalates, VOCs, environmental phenols, parabens, PAHs, pyrethroids, and ETS. Hence, we developed tandem hybrid hydrolysis for conversion of conjugated metabolites, modified direct injection without sample preparation for simultaneous extraction, and a comprehensive mobile phase for superior ionization efficiency and chromatographic behavior. Finally, we applied the developed method to 39 urine samples from 19 mother–newborn pairs to prove the method's feasibility to simultaneously identify and quantify multiple EPOLs for

multiple exposure assessment.

2. Experimental section

2.1. Chemicals and materials

Detailed information about compound names, abbreviations, chemical formulas, molar weights, and suppliers of analytes and internal standards (ISTDs) are provided in Supplementary Table 1. Sodium phosphate monobasic, sodium phosphate dibasic, and acetic acid (99.99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA); analytical-grade water from Merck (Darmstadt, Germany); high-performance liquid chromatography (HPLC)-grade solvent acetonitrile and acetone from J. T. Baker (Center Valley, PA, USA); 6N hydrochloric acid from Daejung (Seoul, South Korea); *E. coli* β -glucuronidase (140 U/mL) from Roche (Mannheim, Germany); artificial urine from BIOZOA (Seoul, South Korea); and standard reference material (SRM) 3672 (organic contaminants in smoker's urine) and SRM 3673 (organic contaminants in nonsmoker's urine) from the National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA).

2.2. Instruments

For validation and real sample analysis, LC-MS/MS was used. A Thermo Scientific™ Vanquish™ ultrahigh-performance liquid chromatography (UHPLC) system (Thermo Finnigan, San Jose, CA, USA) with an ACE Excel 2 C18-AR column (150 mm $\times$ 2.1 mm I.D.; Advanced Chromatography Technology, Scotland) was connected to a TSQ Altis triple quadrupole mass spectrometer (Thermo Finnigan) equipped with a heated electrospray ionization (H-ESI) source. The spray voltage was set to 4500 V in positive mode and 3500 V in negative mode. The ion transfer tube temperature was 320°C, and the vaporizer temperature was 340°C in both modes. The column temperature was fixed at 35°C. Nitrogen gas was used as a sheath and an auxiliary gas at a flow rate of 60 arbitrary units (arb) and 15 arb, respectively. The mobile phase comprised 0.01% acetic acid in water (A) and 0.01% acetic acid in acetonitrile (B). The initial gradient composition (2% B) was maintained for 0.1 min, linearly increased up to 95% B over 12 min, and then maintained for 3.5 min before being decreased to 2% B over 0.1 min. Then, equilibration was performed for 2.4 min. The flow rate was set to 0.4 mL/min. All experiments were performed in time-dependent selected reaction monitoring mode for simultaneous analysis.

Optimization of the mobile-phase composition was performed using an LC-20AD XR ultrafast liquid chromatography (UFLC) system (Shimadzu, Kyoto, Japan) coupled to a Q Exactive quadrupole orbitrap mass spectrometer (Thermo Finnigan) under the same analytical condition and scan mode.

2.3. Method validation

The optimized analytical method was validated for linearity, limit of detection (LOD), limit of quantitation (LOQ), intra- and inter-day precision, and accuracy. The concentration range of the calibration curve was 0.05–500 ng/mL. Linearity was assessed by the correlation coefficient (R^2). Validation results were evaluated as per guidelines established by the Bioanalytical Method Validation Guidance for Industry (Brodie and Hill, 2002). LOD and LOQ were obtained using the equation from the ICH Q2B guidelines:

$$\text{LOD} = 3.3(\sigma/S)$$

$$\text{LOQ} = 10(\sigma/S)$$

Where, σ is the standard deviation of the response and S is the slope of the calibration curve (Walfish, 2006).

Precision and accuracy were evaluated by analyzing low, medium, and high concentration levels of quality control (LQC, MQC, and HQC,

respectively) samples with five replicates in the same run (intra-day precision) and three separate runs (inter-day precision). Results were represented as the percentage coefficient of variation of the peak area ratio for the analyte to the ISTD. Accuracy was expressed as the bias of the measured concentration to the expected value. As part of the method validation, SRM 3672 and SRM 3673 were analyzed for 3 days. Their accuracy was evaluated by comparing the NIST certified values, and precision was determined by the relative standard deviation derived from the accuracy assay results. All SRM samples were stored at -80°C before analysis.

2.4. Sample collection

To demonstrate the feasibility of the developed method, 39 samples of human urine were obtained from mothers and their newborns (birth within 1 year): 19 mother and 20 newborn (including a pair of twins) urine samples. The urine samples were stored in a deep freezer at -70°C until analysis.

2.5. Sample preparation

Urine (0.2 mL) was taken into a tube, and ISTD solution (10 μL), phosphate buffer (280 μL , pH 7.2), and β -glucuronidase (20 μL) were added. For enzymatic hydrolysis of glucuronide conjugates, the mixture was incubated at 55°C for 2 h. The reaction was quenched by adding 24 μL of HCl, and the mixture was again incubated at 90°C for 60 min to hydrolyze sulfate conjugates. Finally, the sample was centrifuged at 13,200 rpm for 10 min, the supernatant was transferred to a vial, and 10 μL of the supernatant was injected into the LC-MS/MS system.

2.6. Ethics

A research ethics review was conducted by the institutional review board at Chung-Ang University (Seoul, Korea) to supply human-derived samples (IRB no. 1,041,078–201,709-BR-179–01).

2.7. Statistical analysis

According to a modified 80% rule, EPOLs that had more than 80% nonzero values in any group of samples were preserved for further analysis. Urinary concentrations of EPOLs below the LOQ for each compound were replaced by LOQ/2 and transformed into the natural-log (Ln) scale. The Kolmogorov–Smirnov test was performed to assess data normality. In the case of a normal distribution, the paired *t*-test and Pearson correlation were conducted. If the EPOLs shows no normal distribution, nonparametric tests (Wilcoxon signed-rank test and Spearman's rank correlation) were performed. Through statistical analysis, the correlation of the urinary EPOLs between mothers and newborns was evaluated. $P < 0.05$ was considered statistically significant. All statistical comparisons were carried out using Medcalc version 12 (Medcalc, Belgium).

3. Results and discussion

3.1. Optimization of mass spectrometry condition

Based on the CDC, NIST SRMs, and related references, 86 EPOLs from 7 classes were selected: 18 phthalates, 28 VOCs, 22 environmental phenols, 4 parabens, 10 PAHs, 3 pyrethroids, and 1 ETS. Most of the EPOLs were ionized in negative mode, except for seven (AMCC, CEMA, 2,4-DPMA, 2,5/3,4-DPMA, BP-3, 9-FLU, and COT), which were ionized in positive mode. The selected reaction monitoring conditions for the 86 EPOLs and 32 isotope-labeled ISTDs are summarized in Supplementary Table 2. Protonated or deprotonated molecular ions ($[\text{M}+\text{H}]^{+}$ or $[\text{M}-\text{H}]^{-}$) were used as the Q1 ion, except for 9-Flu generating a $[\text{M}+\text{H}-\text{H}_2\text{O}]^{+}$ ion by in-source dissociation in positive mode. In the

selected reaction monitoring transitions of the EPOLs, product ions with the highest intensity were selected as the Q3 ion. However, considering interference, sensitivity, and the signal-to-noise ratio in urine samples, 2-Flu, 1-OHP, and D9-1-OHP were optimized for selected reaction monitoring transitions of m/z 181.2 $\rightarrow$ 181, 217.2 $\rightarrow$ 217, and 226.2 $\rightarrow$ 226, respectively (Onyemauwa et al., 2009). In addition, we employed the time-dependent selected reaction monitoring and polarity-switching method. All substances were monitored during a retention time ± 12 s, except for several hydrophilic VOCs, and the cycle time was optimized to 0.25 s (Supplementary Table 2). All chromatographic peaks were obtained with over 12 data points at least, and the dwell time ranged from 10.66 to 60.63 ms.

3.2. Optimization of comprehensive mobile phase

The analytical methods for EPOLs have typically focused on monitoring an individual EPOL class such as phthalates, phenols and parabens, VOCs, PAHs, and cotinine (Alwis et al., 2012; Chen et al., 2012b; Chiang et al., 2015; Onyemauwa et al., 2009; Silva et al., 2007; Xu et al., 2004). To integrate diverse class-specific methods into a single method, it is a prime challenge to establish a comprehensive mobile phase satisfying both chromatographic behavior and ionization efficiency of all the EPOLs (Hua and Jenke, 2012). We investigated the effect of the mobile-phase composition on the chromatographic behavior and ionization efficiency of EPOLs because the mobile-phase composition, such as additives and organic modifiers, is a key parameter for achieving the best ionization efficiency and chromatographic behavior of analytes. Ammonium fluoride, ammonium acetate, ammonium formate, acetic acid, formic acid, and no additive were evaluated as mobile-phase additives under methanol or acetonitrile as an organic modifier. First, we investigated the optimal concentration of each additive to exactly compare the relative chromatographic behavior and ionization efficiency of EPOLs in diverse mobile-phase compositions. The optimal concentration of each additive was determined at 0.5 mM for ammonium fluoride, 1.0 mM for ammonium acetate, 0.2 mM for ammonium formate, 0.01% for formic acid, and 0.01% for acetic acid by considering the overall chromatographic behavior and ionization efficiency of EPOLs (our unpublished data). On the basis of optimized additive concentrations, we investigated the influence of organic modifiers and additives on the chromatographic behavior of EPOLs because chromatography is a factor prior to ionization.

Overall, EPOLs showed better chromatographic behavior in acetonitrile than in methanol regardless of additives. Methanol gave rise to incomplete separation in several phthalates and PAHs, and the VOCs peaks tended to broaden. Therefore, even though methanol exhibited a higher ionization efficiency than acetonitrile, we selected acetonitrile as the organic modifier compatible with all the EPOLs based on chromatographic behavior. Fig. 1A shows the heat map for the relative chromatographic behavior of EPOLs with diverse additives in an optimized concentration under acetonitrile. The result provided important information for the selection of additives in terms of chromatography. In acetic acid and formic acid at low pH, overall, all the EPOLs showed good chromatographic behavior. Conversely, in ammonium salts and no additive at around neutral pH, phthalates, phenols, parabens, PAHs, and pyrethroids exhibited good or moderate chromatographic behavior, while VOCs showed poor chromatographic behavior (Box A in Fig. 1A). These results suggest that ammonium salts are not suitable as additives for an integrated mobile phase, because VOCs prefer a low-pH mobile phase for chromatography. Therefore, acetic acid and formic acid as mobile-phase additive seem to be the best candidates to achieve satisfactory chromatographic behavior of all the EPOLs. Fig. 1B illustrates the relative ionization efficiency of EPOLs with formic acid and acetic acid as mobile-phase additives. In phthalates and VOCs, formic acid and acetic acid exhibited a similar ionization efficiency. However, in phenols, parabens, PAHs, and pyrethroids, formic acid showed a lower ionization efficiency than acetic acid. Moreover, when using formic acid

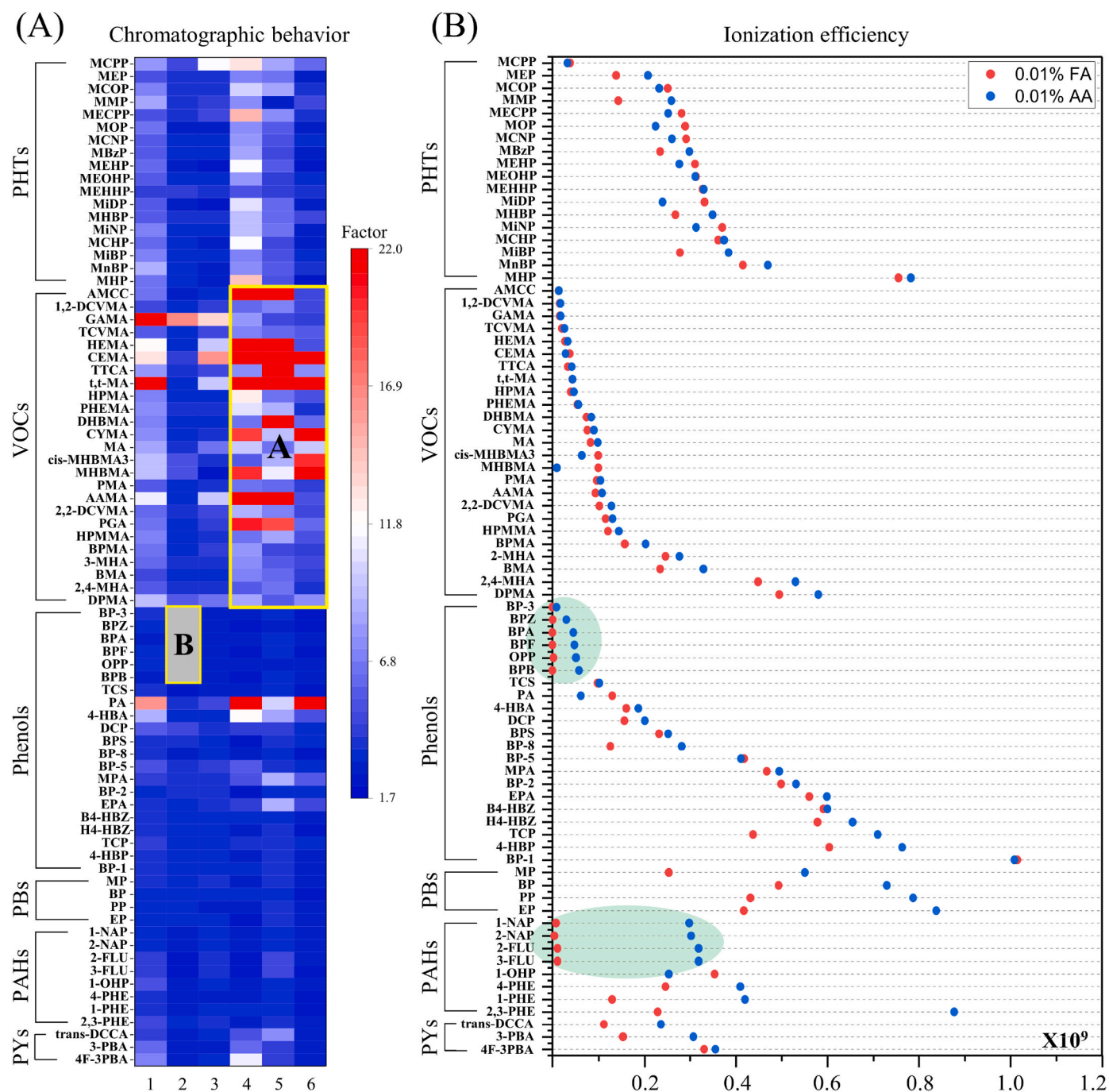


Fig. 1. Chromatographic behavior and ionization efficiency of EPOLs with different additives at optimized concentrations and acetonitrile (A) Heat map for relative chromatographic behavior (chromatographic factor: area/height ratio; Box A: chromatographic behavior of VOCs in ammonium salt additives; Box B: chromatographic factor not obtained due to poor ionization efficiency; 1 = no additive, 2 = 0.01% formic acid, 3 = 0.01% acetic acid, 4 = 0.2 mM ammonium formate, 5 = 1.0 mM ammonium acetate, 6 = 0.5 mM ammonium fluoride) (B) Relative ionization efficiency in negative ESI mode. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

as an additive, BP-3, BPZ, BPA, OPP, BPB, 1-NAP, 2-NAP, 2-FLU, and 3-FLU in phenols and PAHs were barely detected. This result indicates that formic acid is not suitable as an additive for a comprehensive mobile phase because of low ionization efficiency. Therefore, the final composition of the integrated mobile phase was set to 0.01% acetic acid in distilled water and acetonitrile. Fig. 2 illustrates the selected reaction monitoring chromatograms of 86 EPOLs and the chromatographic separation of representative isomers under the optimized mobile-phase condition, and they were satisfactorily separated. Although the present mobile-phase composition is simple and common, to the best of our

knowledge, it is a unique mobile phase satisfying both chromatographic behavior and ionization efficiency of all the EPOLs and enabling the establishment of a comprehensive method for multiple exposure assessment.

3.3. Optimization of tandem hybrid hydrolysis

Most of the EPOLs in the body are excreted in the urine as a parent, phase I, or phase II metabolites. In EPOL quantification, conjugated metabolites, such as glucuronide and sulfate, were converted into free

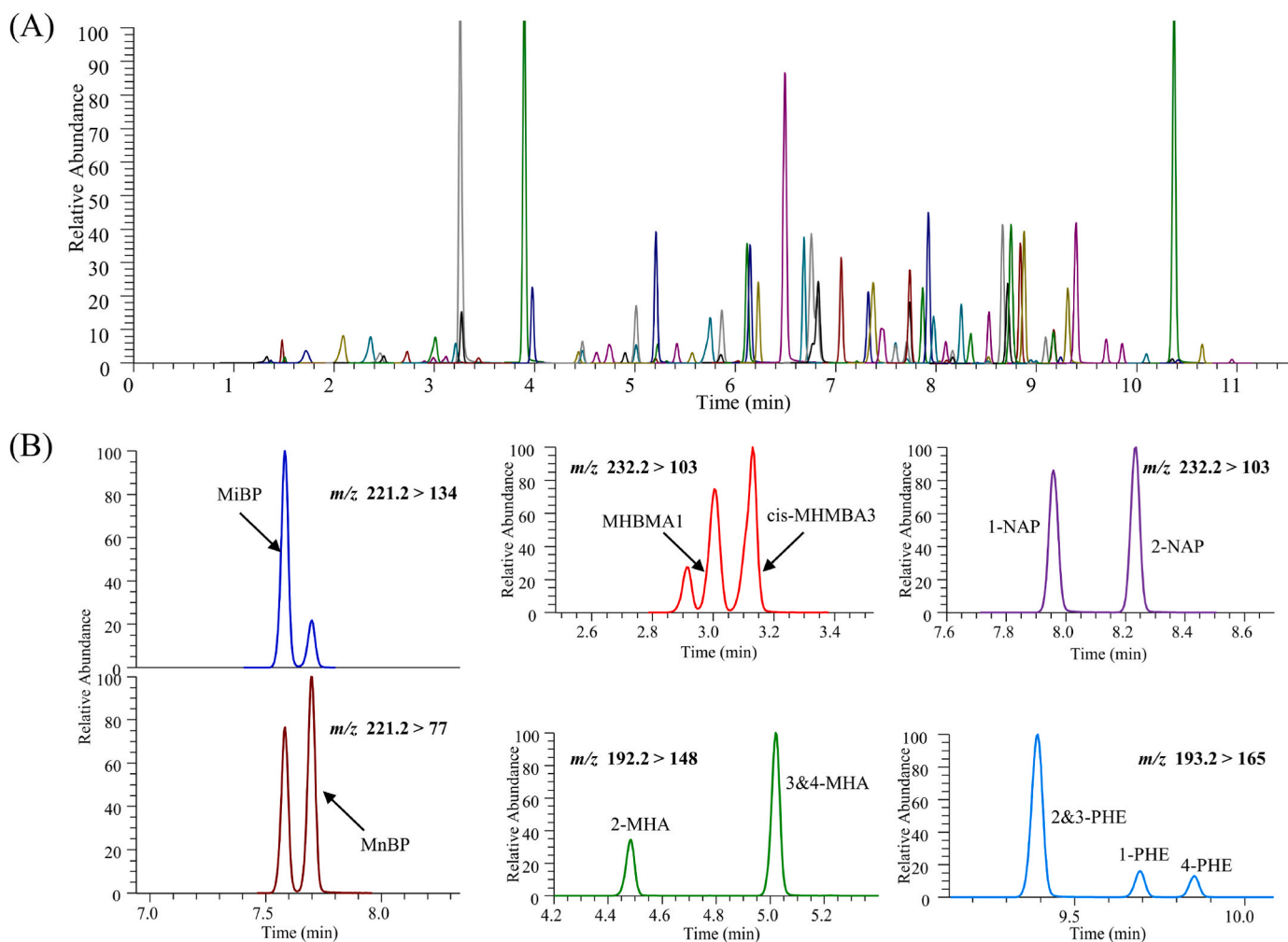


Fig. 2. (A) Representative selected reaction monitoring chromatograms of EPOLs (B) Chromatographic separation of MiBP and MnBP; MHBMA1 and cis-MHBMA3; 1-NAP and 2-NAP; 2-MHA and 3&4-MHA; and 2&3-PHE, 1-PHE, and 4-PHE. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

forms via enzymatic hydrolysis, and the total amount of free forms was measured (Dewalque et al., 2014). The different enzyme hydrolysis processes between phthalates (β -glucuronidase) (Heffernan et al., 2016; Myridakis et al., 2015; Silva et al., 2007) and phenols, parabens, and PAHs (β -glucuronidase/sulfatase) are mutually incompatible in a single sample preparation (Chen et al., 2012b; Onyemauwa et al., 2009; Wang et al., 2013; Yao et al., 2018). First, β -glucuronidase/sulfatase is not suitable as a hydrolysis enzyme for the simultaneous hydrolysis of multiclass EPOLs because of the degradation of phthalates by lipase and esterase activity (Blount et al., 2000). In contrast, although β -glucuronidase lacks sulfatase activity, it can be a preferable candidate for simultaneous enzyme hydrolysis because sulfate-conjugated metabolites of phenols (BPA) and PAHs are present in small amounts (<3%–5%, general error range) in human urine (Buratti et al., 2000; Heffernan et al., 2016). Therefore, β -glucuronidase can be applied for phthalates, phenols, and PAHs, including a small amount of sulfate- or only glucuronide-conjugated metabolites. However, while phthalates are metabolized to unconjugated (~12%) metabolites and glucuronide-conjugated (~88%) metabolites, parabens (MP, EP, and PP) except for BP (unconjugated: 22%; glucuronide: 74%; sulfate: 4%) are metabolized to unconjugated (~8%), glucuronide-conjugated (~50%), and sulfate-conjugated (~42%) metabolites (Dewalque et al., 2014; Ye et al., 2006). Eventually, single-enzyme hydrolysis using β -glucuronidase can result in serious errors in quantitation results of parabens with a large amount of sulfate-conjugated metabolites (~40%)

(Dewalque et al., 2014).

To overcome this problem, we developed tandem hybrid hydrolysis allowing the selective hydrolysis of glucuronide- and sulfate-conjugated metabolites without phthalate degradation. Primary hydrolysis using β -glucuronidase (55°C, 2 h) was performed to hydrolyze glucuronide-conjugated metabolites, and secondary acid hydrolysis (90°C, 60 min) was performed to selectively hydrolyze sulfate-conjugated metabolites. HCl was used for both enzyme activity quenching and secondary acid hydrolysis (Lu et al., 2015). For optimization of secondary acid hydrolysis, diverse incubation conditions, such as temperature (50°C, 70°C, 90°C) and time (15, 30, 45, 60, 90, 120, 180, 240 min) were investigated. Fig. 3A illustrates the intensity change of ethylparaben (EP) and its sulfate-conjugated metabolite (EP sulfate) after incubation at 90°C. As shown in Fig. 3B, EP sulfate was completely hydrolyzed within 60 min at 90°C. However, incubation at 50°C and 70°C needed a longer time for complete hydrolysis of EP sulfate. Fig. 3C shows the accuracy change of EP in SRM samples by secondary acid hydrolysis. The accuracy of EP at 0 min with only enzyme hydrolysis was ~53% (EP + EP glucuronide) and at 60 min with tandem hybrid hydrolysis was ~97% (EP + EP glucuronide + EP sulfate), indicating that ~44% of EP sulfate is present in the sample. With no enzyme and only acid hydrolysis (Fig. 3C), the accuracy of EP at 0 min without hydrolysis was ~6% (EP) and at 60 min was ~40% (EP + EP sulfate), indicating that EP has ~54% of EP glucuronide.

The proportion of unconjugated and conjugated metabolites using

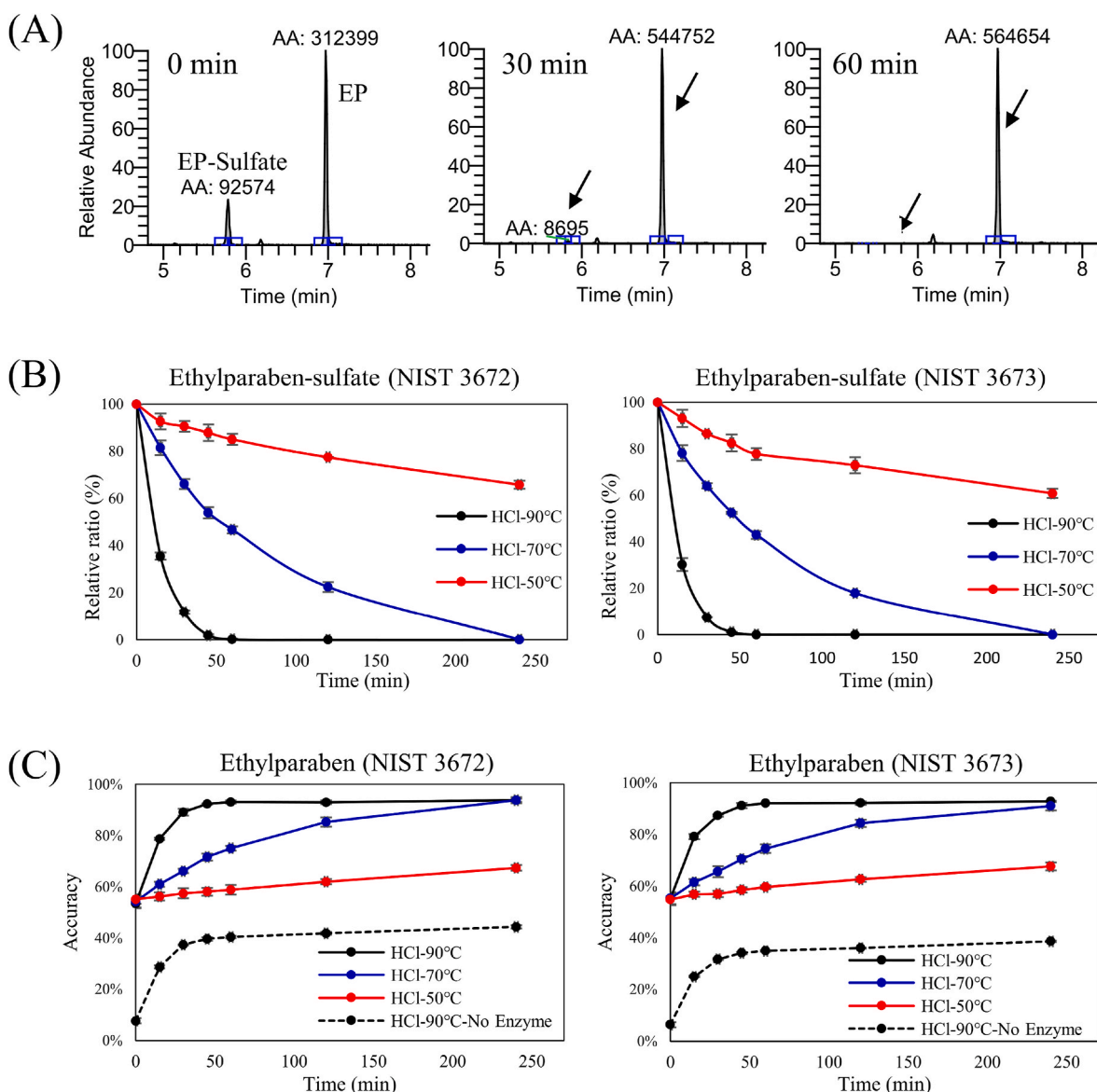


Fig. 3. Tandem hybrid hydrolysis (A) Chromatograms of EP and EP sulfate during acid hydrolysis (B) Temperature and incubation time effect on hydrolysis of EP sulfate (C) Accuracies of NIST SRM samples by tandem hybrid hydrolysis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

tandem hybrid hydrolysis are consistent with previous reports (Dewalque et al., 2014). Methylparaben (MP) and propylparaben (PP) showed similar results as EP, and accuracies of phthalates in the SRM sample were the same at 0 and 60 min and 90°C. These results indicate that selective hydrolysis of sulfate-conjugated metabolites was achieved without phthalate degradation. Therefore, tandem hybrid hydrolysis is a novel and integrated method allowing the hydrolysis of glucuronide- and sulfate-conjugated metabolites without phthalate degradation in a single sample preparation. The combination of an integrated mobile phase and tandem hybrid hydrolysis is a key technique for comprehensive analysis of EPOLs.

3.4. Optimization of sample preparation

In research dealing with a vast number of analytes, a key concern is to simultaneously extract target analytes from a biological matrix in a single sample preparation. In this study, we focused on developing a simple, rapid, and cost effective sample preparation method without time-consuming and laborious steps. If sensitivity and selectivity are

satisfied, direct injection is the best alternative. Furthermore, direct injection allows simultaneous extraction without loss of analytes and does not depend on the sample volume. A small amount of urine can be used for sample preparation, which is especially effective for infant urine samples. Therefore, we employed direct injection, and results showed satisfactory selectivity and LOD values for simultaneous EPOL quantitation.

3.5. Influence of the internal standard in accuracy

In EPOL quantitation, even if the accuracy and precision results of QC in validation are satisfied, the concentration measured in a real urine sample may not be accurate because of the heterogeneous nature of artificial urine for calibration and real urine. To solve this problem and accurately quantitate in real urine, the use of EPOLs' own isotope-labeled ISTD is the best choice. Table 1 shows representative two cases of variable accuracy by using different ISTDs in SRM samples. In the case of propylparaben (PP) with its isotope-labeled ISTD, the use of $^{13}\text{C}_6$ -PP showed good accuracy in both QC and NIST SRM samples.

Table 1
Variation of accuracy in NIST SRM samples by use of different ISTD.

STD	ISTD	Accuracy (%)	
		NIST SRM 3672	NIST SRM 3673
PP	¹³ C ₆ -PP	103.00	103.01
	¹³ C ₆ -EP	137.20	131.17
	¹³ C ₆ -BP	84.81	94.26
MCNP	¹³ C ₁₂ -MECPP	104.55	101.96
	¹³ C ₁₂ -MnBP	117.38	110.04
	¹³ C ₄ -MEHP	50.89	64.03

However, although the use of ¹³C₆-EP and ¹³C₆-butylparaben (BP) as a structural analog showed good accuracy in QC samples, NIST samples had over- or underestimated accuracy results. In the case of monocarboxy-isononyl phthalate (MCNP) without its isotope-labeled ISTD, the use of ¹³C₁₂-mono(2-ethyl-5-carboxypentyl) phthalate (MECPP) as a structural analog showed good accuracy in both QC and NIST SRM samples, while the use of ¹³C₁₂-mono-n-butyl phthalate (MnBP) and ¹³C₄-mono(2-ethylhexyl) phthalate (MEHP) as structural analogs showed over- or underestimated accuracy results in NIST SRM samples. These results indicate that it is important to use the EPOLs' own isotope-labeled ISTDs for accurate quantitation in urine and, if impossible, to select optimal isotope-labeled ISTDs within structural analogs. In addition, even if validation results have been satisfied, accuracy in NIST SRM samples plays a more important indicator role for accurate quantitation in urine samples. We used 32 isotope-labeled ISTDs, and Supplementary Table 3 summarizes the individual ISTDs used for the quantitation of 86 EPs.

3.6. Method validation and accuracy in NIST SRM samples

The validation results are summarized in Supplementary Table 3. Calibration curves were obtained using over seven different concentrations, and the linearity (R^2) of all the calibration curves for 86 EPOLs was more than 0.99. The intra- and inter-day accuracies for the target analytes were obtained by evaluating three different concentrations of QC samples (LQC, MQC, and HQC). Except for the intra-day accuracy for the LQC of AAMA and BP-2, the accuracy for all the EPs was between 85% and 115%, and the precision was less than 15%. The method detection limit was between 0.003 and 0.838 ng/mL, and the LOQ was 0.009–2.794 ng/mL.

Supplementary Table 4 summarizes the accuracies and precisions of 40 EPOLs in NIST SRM samples, providing the certified concentration values. In SRM 3673, 3-FLU and 4-PHE in PAHs were not detected due to a concentration lower than the LOQ. In the case of cotinine, the NIST protocol measured only the free form without any hydrolysis, but our method measured both the free and the glucuronide-conjugated form. Nicotine is metabolized to cotinine, and the relative proportions of cotinine and cotinine-glucuronide are approximately 46% and 54%, respectively (Hukkanen et al., 2005). The measured concentration and accuracy of cotinine in SRM 3672 were 2293 ng/mL and 206%, respectively. When considering their metabolic ratio, the concentration of cotinine and cotinine-glucuronide were revaluated to ~1055 and ~1238 ng/mL, respectively, and the NIST accuracy of cotinine was ~95%. This result is completely consistent with the certified value of SRM 3672. Except for cotinine, the accuracy of 39 EPOLs in SRM 3672 was between 87.34% and 111.14% and the precision was 0.99%–10.13%. The accuracy in SRM 3673 was 85.39%–111.26%, and the precision was between 0.31% and 8.80%. The accuracy results of parabens and phthalates indicate that the secondary acid hydrolysis of sulfate-conjugated metabolites of parabens was successfully carried out without the degradation of phthalate diesters. Therefore, of the 86 EPOLs, the accuracies of 40 EPOLs were verified in SRM samples and their concentrations measured in real urine samples could be verified as true values. However, SRM samples did not include the certified values

of remaining 46 EPOLs and their concentrations measured in real urine samples could not be verified as true values.

3.7. Applications

Recently, Preindl et al. (Preindl et al., 2019) reported a generic LC-MS/MS exposome method for assessment of the combinatory effects of multiple xenoestrogens. They simultaneously analyzed 75 xenoestrogens (benzophenones, parabens, PAHs, bisphenols, and phthalates) in urine, serum, and milk samples obtained from mothers and infants. Results showed that it is possible to simultaneously analyze multiclass estrogenic compounds and estimate the total estrogenic burden at an individual level. The study was the first to demonstrate the general importance of multiple exposure assessment in the context of the exposome paradigm and first proof-of-principle experiments. We compared the results of our multiple exposure assessment of EPOLs in 39 urine samples from 19 mother–newborn pairs with Preindl et al.'s result (Preindl et al., 2019). Supplementary Table 5 summarizes the urinary concentrations and detection frequencies of EPOLs determined in our study. Preindl et al. found much lower detection frequencies and urinary concentrations compared with our results. As shown in Supplementary Table 5, among the 15 EPOLs overlapping with our target EPOLs, BPF, BPS, TCS, BP-1, BP-2, and BPB were not detected, and MnBP, MEHP, and BPA were detected in only one of six samples (four mothers and two infants). Moreover, our urinary concentrations of parabens were higher. Preindl et al. explained that the no-deconjugation step results in a small number of positive samples and there are limitations in this respect, such as low detection frequency and sensitivity. This indicates that our tandem hybrid hydrolysis and sensitivity by mobile-phase composition play an important role in the comprehensive analysis of EPOLs.

On the basis of the urinary concentrations of EPOLs obtained from our multiple exposure assessment, we selected EPOLs showing a positive correlation between mothers and newborns. After excluding EPOLs that showed nonzero values in <80% of samples, 39 EPOLs were selected for statistical analysis. The correlation coefficients of EPOLs between mothers and newborns were 0.60 ($P = 0.0047$, 95% confidence interval [CI] = 0.2216–0.8262) for MEP, 0.69 ($P = 0.0007$, 95% CI = 0.3590–0.8680) for BP-3, -0.46 ($P = 0.0436$, 95% CI = -0.7473 to -0.0161) for DHBMA, -0.46 ($P = 0.0415$, 95% CI = -0.7497 to -0.0214) for AAMA, and 0.53 ($P = 0.0172$, 95% CI = 0.1088–0.7857) for PA. Among five EPOLs that had statistically significant correlation coefficients, BP-3 showed $P = 0.3118$ (Wilcoxon signed-rank test) and MEP showed $P = 0.3613$ (paired t -test). As illustrated in Fig. 4, both BP-3 and MEP exhibited a clear positive correlation between mothers and newborns.

BP-3 is a general component in sunblock products and has slight estrogenic or anti-androgenic properties (Chang et al., 2017). Overall, the mean concentration of BP-3 was higher in mothers than in newborns. Chang et al. (Chang et al., 2017) reported that the frequency of applying BP-3 lotion to avoid sun exposure is relatively high in adult women. In addition, we found a prominent mother–newborn pair with a high concentration of BP-3 at 4068.56 and 260.03 ng/mL, respectively; the concentrations of BP-1 and BP-8 were also relatively high in this pair. This was reasonable because BP-1 and BP-8 are metabolites of BP-3 as well as ingredients of sunblock products (Bae et al., 2016). These results imply that BP-3 exposure in newborns may be transferred from the mothers. The mean concentration of MEP, which is a metabolite of diethyl phthalate (DEP) and is widely used in cosmetic products (e.g., lotions, creams, and fragrances) and beverage bottles, was higher in newborns than in mothers and showed not only a significant correlation between mothers and newborns but also high variation within an individual, ranging from 3.03 to 872.03 ng/mL. This variable range of MEP has also been observed in other studies (Frederiksen et al., 2010; Koppen et al., 2019). These results imply individually exposure of MEP in mothers and newborns.

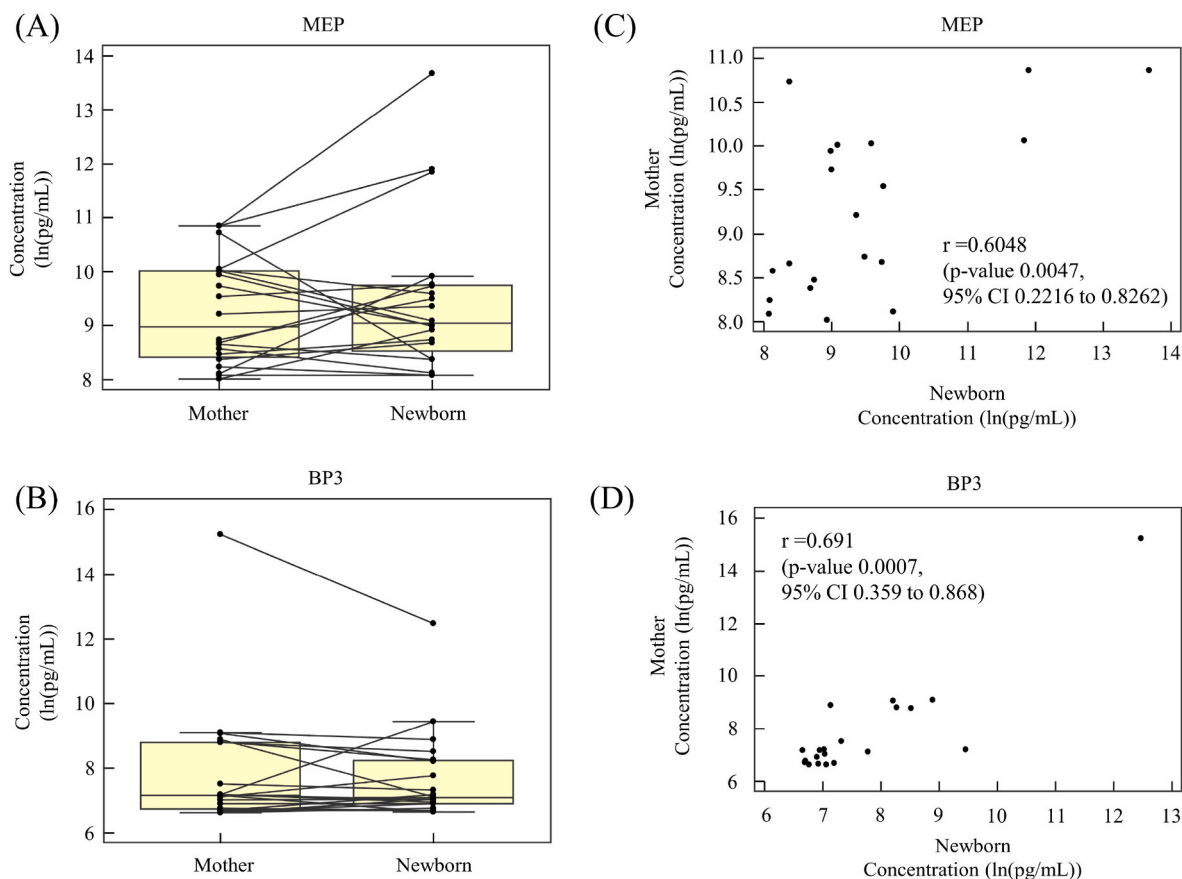


Fig. 4. Box-and-whisker plots showing MEP (A) and BP-3 (B) natural logarithm concentrations from mother and newborn groups. The plots illustrate the 5th and 95th percentiles (whiskers), 25th and 75th percentiles (box), and the median marked by a horizontal line in the box. Mother–newborn pairs are shown by the line connecting single dots. Scatter plots show the correlation of natural logarithm concentrations of MEP (C) and BP-3 (D) between mothers and newborns.

4. Conclusions

This work presents the development of a novel comprehensive method for large-scale and long-term multiple exposure assessment. We developed tandem hybrid hydrolysis, modified direct injection, and a comprehensive mobile phase to overcome the technical problems in simultaneous biomonitoring of multiclass EPOLs in multiple exposure assessment. To the best of our knowledge, this is the first study to simultaneously quantify 86 EPOLs from 7 different classes excreted through urine, including phthalates, VOCs, phenols, parabens, PAHs, pyrethroids, and ETS. The main strength of this novel approach are: (1) the ability to simultaneously quantify most of multiclass EPOLs, (2) the use of small-sample volumes, (3) simple, cost-effective, and time-efficient sample preparation, and (4) reducing analytical instruments and manpower. In conclusion, our comprehensive method is effective for multiple exposure assessment of EPOLs in urine samples and has potential as a novel analytical platform for large-scale human biomonitoring of multiclass EPOLs in environmental research fields.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This work was supported by an intramural grant (2V09270) from Korea Institute of Science and Technology (KIST).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2022.113053>.

References

- Alwis, K.U., Blount, B.C., Britt, A.S., Patel, D., Ashley, D.L., 2012. Simultaneous analysis of 28 urinary VOC metabolites using ultra high performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC-ESI/MS/MS). *Anal. Chim. Acta* 750, 152–160.
- Azzouz, A., Rascon, A.J., Ballesteros, E., 2016. Simultaneous determination of parabens, alkylphenols, phenylphenols, bisphenol A and triclosan in human urine, blood and breast milk by continuous solid-phase extraction and gas chromatography-mass spectrometry. *J. Pharmaceut Biomed* 119, 16–26.
- Bae, J., Kim, S., Kannan, K., Louis, G.M.B., 2016. Couples' urinary concentrations of benzophenone-type ultraviolet filters and the secondary sex ratio. *Sci. Total Environ.* 543, 28–36.
- Blount, B.C., Milgram, K.E., Silva, M.J., Malek, N.A., Reidy, J.A., Needham, L.L., Brock, J. W., 2000. Quantitative detection of eight phthalate metabolites in human urine using HPLC-APCI-MS/MS. *Anal. Chem.* 72, 4127–4134.
- Brodie, R.R., Hill, H.M., 2002. Validation issues arising from the new FDA guidance for industry on bioanalytical method validation. *Chromatographia* 55, S91–S94.
- Buratti, M., Pellegrino, O., Brambilla, G., Colombi, A., 2000. Urinary excretion of 1-hydroxypyrene as a biomarker of exposure to polycyclic aromatic hydrocarbons from different sources. *Biomarkers* 5, 368–381.
- Chang, F.K., Shiea, J., Tsai, H.J., 2017. Urinary concentrations of triclosan, benzophenone-3, and bisphenol A in Taiwanese children and adolescents. *Int. J. Environ. Res. Publ. Health* 14.
- Chen, M., Tao, L., Collins, E.M., Austin, C., Lu, C.S., 2012a. Simultaneous determination of multiple phthalate metabolites and bisphenol-A in human urine by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B* 904, 73–80.
- Chen, M.J., Zhu, P.F., Xu, B., Zhao, R.C., Qiao, S.L., Chen, X.J., Tang, R., Wu, D., Song, L., Wang, S.L., Xia, Y.K., Wang, X.R., 2012b. Determination of nine environmental phenols in urine by ultra-high-performance liquid ChromatographyTandem mass spectrometry. *J. Anal. Toxicol.* 36, 608–615.

- Chiang, W.C., Chen, C.Y., Lee, T.C., Lee, H.L., Lin, Y.W., 2015. Fast and simple screening for the simultaneous analysis of seven metabolites derived from five volatile organic compounds in human urine using on-line solid-phase extraction coupled with liquid chromatography-tandem mass spectrometry. *Talanta* 132, 469–478.
- Dewalque, L., Pirard, C., Dubois, N., Charlier, C., 2014. Simultaneous determination of some phthalate metabolites, parabens and benzophenone-3 in urine by ultra high pressure liquid chromatography tandem mass spectrometry. *J. Chromatogr. B* 949, 37–47.
- Diamanti-Kandarakis, E., Bourguignon, J.P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, R.T., Gore, A.C., 2009. Endocrine-Disrupting chemicals: an endocrine society scientific statement. *Endocr. Rev.* 30, 293–342.
- Frederiksen, H., Jorgensen, N., Andersson, A.M., 2010. Correlations between phthalate metabolites in urine, serum, and seminal plasma from young Danish men determined by isotope dilution liquid chromatography tandem mass spectrometry. *J. Anal. Toxicol.* 34, 400–410.
- Gao, C.Z., He, H.H., Qiu, W.H., Zheng, Y., Chen, Y.Y., Hu, S.Y., Zhao, X., 2021. Oxidative stress, endocrine disturbance, and immune interference in humans showed relationships to serum bisphenol concentrations in a dense industrial area. *Environ. Sci. Technol.* 55, 1953–1963.
- Han, Y., Xia, Y.K., Han, J.Y., Zhou, J.P., Wang, S.L., Zhu, P.F., Zhao, R.C., Jin, N.Z., Song, L., Wang, X.R., 2008. The relationship of 3-PBA pyrethroids metabolite and male reproductive hormones among non-occupational exposure males. *Chemosphere* 72, 785–790.
- Heffernan, A.L., Thompson, K., Eaglesham, G., Vijayasathay, S., Mueller, J.F., Sly, P.D., Gomez, M.J., 2016. Rapid, automated online SPE-LC-QTRAP-MS/MS method for the simultaneous analysis of 14 phthalate metabolites and 5 bisphenol analogues in human urine. *Talanta* 151, 224–233.
- Heudorf, U., Mersch-Sundermann, V., Angerer, E. Phthalates, 2007. Toxicology and exposure. *Int. J. Hyg Environ. Health* 210, 623–634.
- Hua, Y.S., Jenke, D., 2012. Increasing the sensitivity of an LC-MS method for screening material extracts for organic extractables via mobile phase optimization. *J. Chromatogr. Sci.* 50, 213–227.
- Hukkanen, J., Jacob, P., Benowitz, N.L., 2005. Metabolism and disposition kinetics of nicotine. *Pharmacol. Rev.* 57, 79–115.
- Kassotis, C.D., Vandenberg, L.N., Demeneix, B.A., Porta, M., Slama, R., Trasande, L., 2020. Endocrine-disrupting chemicals: economic, regulatory, and policy implications. *Lancet Diabetes Endocrinol.* 8, 719–730.
- Koppen, G., Govarts, E., Vanermen, G., Voorspoels, S., Govindan, M., Dewolf, M.C., Den Hond, E., Biot, P., Casteleyn, L., Kolossa-Gehring, M., Schwedler, G., Angerer, J., Koch, H.M., Schindler, B.K., Castano, A., Lopez, M.E., Sepai, O., Exley, K., Bloemen, L., Knudsen, L.E., Joas, R., Joas, A., Schoeters, G., Covaci, A., 2019. Mothers and children are related, even in exposure to chemicals present in common consumer products. *Environ. Res.* 175, 297–307.
- Lang, I.A., Galloway, T.S., Scarlett, A., Henley, W.E., Depledge, M., Wallace, R.B., Melzer, D., 2008. Association of urinary bisphenol A concentration with medical disorders and laboratory abnormalities in adults. *JAMA, J. Am. Med. Assoc.* 300, 1303–1310.
- Levine, H., Berman, T., Goldsmith, R., Goen, T., Spungen, J., Novack, L., Amitai, Y., Shohat, T., Grotto, I., 2015. Urinary concentrations of polycyclic aromatic hydrocarbons in Israeli adults: demographic and life-style predictors. *Int. J. Hyg Environ. Health* 218, 123–131.
- Li, X.J., Zhong, Y., He, W.Y., Huang, S.Y., Li, Q., Guo, C.S., Ma, S.T., Li, G.Y., Yu, Y.X., 2021. Co-exposure and health risks of parabens, bisphenols, triclosan, phthalate metabolites and hydroxyl polycyclic aromatic hydrocarbons based on simultaneous detection in urine samples from guangzhou, south China. *Environ. Pollut.* 272.
- Lu, D.S., Feng, C., Wang, D.L., Lin, Y.J., Ip, H.S.S., She, J.W., Xu, Q., Wu, C.H., Wang, G. Q., Zhou, Z.J., 2015. Analysis of twenty phenolic compounds in human urine: hydrochloric acid hydrolysis, solid-phase extraction based on K₂CO₃-treated silica, and gas chromatography tandem mass spectrometry. *Anal. Bioanal. Chem.* 407, 4131–4141.
- Mortensen, M.E., Calafat, A.M., Ye, X.Y., Wong, L.Y., Wright, D.J., Pirkle, J.L., Merrill, L. S., Moye, J., 2014. Urinary concentrations of environmental phenols in pregnant women in a pilot study of the National Children's Study. *Environ. Res.* 129, 32–38.
- Myridakis, A., Balaska, E., Gkaitatzi, C., Kouvarakis, A., Stephanou, E.G., 2015. Determination and separation of bisphenol A, phthalate metabolites and structural isomers of parabens in human urine with conventional high-pressure liquid chromatography combined with electrospray ionisation tandem mass spectrometry. *Anal. Bioanal. Chem.* 407, 2509–2518.
- Onyemauwa, F., Rappaport, S.M., Sobus, J.R., Gajdosova, D., Wu, R.A., Waidyanatha, S., 2009. Using liquid chromatography-tandem mass spectrometry to quantify mono hydroxylated metabolites of polycyclic aromatic hydrocarbons in urine. *J. Chromatogr. B* 877, 1117–1125.
- Preindl, K., Braun, D., Aichinger, G., Sieri, S., Fang, M.L., Marko, D., Warth, B., 2019. A generic liquid chromatography-tandem mass spectrometry exposome method for the determination of xenoestrogens in biological matrices. *Anal. Chem.* 91, 11334–11342.
- Schnatter, A.R., Rosamilia, K., Wojcik, N.C., 2005. Review of the literature on benzene exposure and leukemia subtypes. *Chem. Biol. Interact.* 153, 9–21.
- Silva, M.J., Samandar, E., Preau, J.L., Reidy, J.A., Needham, L.L., Calafat, A.M., 2007. Quantification of 22 phthalate metabolites in human urine. *J. Chromatogr. B* 860, 106–112.
- Walfish, S., 2006. A statistical perspective on the ICH Q2A and Q2B guidelines for validation of analytical methods. *Biopharm. Int.* 19, 28(– +).
- Wang, H.X., Wang, B., Zhou, Y., Jiang, Q.W., 2013. Rapid and sensitive analysis of phthalate metabolites, bisphenol A, and endogenous steroid hormones in human urine by mixed-mode solid-phase extraction, dansylation, and ultra-performance liquid chromatography coupled with triple quadrupole mass spectrometry. *Anal. Bioanal. Chem.* 405, 4313–4319.
- Warth, B., Spangler, S., Fang, M.L., Johnson, C.H., Forsberg, E.M., Granados, A., Martin, R.L., Domingo-Almenara, X., Huan, T., Rinehart, D., Montenegro-Burke, J.R., Hilmers, B., Aisporna, A., Hoang, L.T., Uritboonthai, W., Benton, H.P., Richardson, S. D., Williams, A.J., Siuzdak, G., 2017. Exposome-scale investigations guided by global metabolomics, pathway analysis, and cognitive computing. *Anal. Chem.* 89, 11505–11513.
- Xu, X., Zhang, J.F., Zhang, L., Liu, W.L., Weisel, C.P., 2004. Selective detection of monohydroxy metabolites of polycyclic aromatic hydrocarbons in urine using liquid chromatography/triple quadrupole tandem mass spectrometry. *Rapid Commun Mass Sp* 18, 2299–2308.
- Yao, Y., Shao, Y.J., Zhan, M., Zou, X.L., Qu, W.D., Zhou, Y., 2018. Rapid and sensitive determination of nine bisphenol analogues, three amphenicol antibiotics, and six phthalate metabolites in human urine samples using UHPLC-MS/MS. *Anal. Bioanal. Chem.* 410, 3871–3883.
- Ye, X.Y., Bishop, A.M., Reidy, J.A., Needham, L.L., Calafat, A.M., 2006. Parabens as urinary biomarkers of exposure in humans. *Environ Health Persp* 114, 1843–1846.
- Ye, X.Y., Kuklenyik, Z., Needham, L.L., Calafat, A.M., 2005. Automated on-line column-switching HPLC-MS/MS method with peak focusing for the determination of nine environmental phenols in urine. *Anal. Chem.* 77, 5407–5413.