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Development of HPLC-MS/MS method for the simultaneous determination of environmental phenols in human urine

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We present a sensitive method for simultaneous determination of bisphenol A (BPA), benzophenone-3 (BP-3), 4-*tert*-octylphenol (*t*-OP), *ortho*-phenylphenol (OPP), four parabens (methyl, ethyl, propyl, butyl parabens) and five chlorophenols (2,4-dichlorophenol (2,4-DCP), 2,5-dichlorophenol (2,5-DCP), 2,4,5-trichlorophenol (2,4,5-trichlorophenol), 2,4,6-trichlorophenol (2,4,6-TCP), and triclosan (TCS)), in human urine by high-pressure liquid chromatography (HPLC) mass spectrometry (MS). Samples were processed using enzymatic deconjugation of glucuronides followed by solid phase extraction (SPE) on a C18 cartridge and the eluate was concentrated. Analytes were separated by reversed-phase HPLC and then detected by atmospheric pressure chemical ionisation (APCI) MS and quantified by isotope dilution method. We describe details for optimisation of each step of the procedure. The sample treatment steps are straightforward and not labour-intensive and, therefore, permit a high sample throughput with excellent prospects for automation. This method shows low inter-day variation, and detection limits for most of the compounds are below 1 ng/mL in 1 mL of urine. The method accuracy was also verified by the analysis of proficiency testing urine samples.

Keywords: environmental phenols; HPLC-MS/MS; human urine; solid-phase extraction

1. Introduction

Exposure to certain phenols in the environment is a concern for human health, particularly because of the known or suspected endocrine activity of these compounds [1–3]. Other health concerns of some phenols include potential effects on development and reproduction [4,5]. Bisphenol A (BPA) is used in the manufacture of food and beverage can linings, thermal paper, polycarbonate plastic, dental sealants, and medical devices [6]. Benzophenone-3 (BP-3) is used as sunscreen agent, and as UV filter in cosmetic products and plastics to improve stability [7,8]. Triclosan (TCS) is a widely used antimicrobial agent in liquid soaps, personal care products, and numerous consumer products, such as cutting boards, toys and garden hoses [9,10]. Parabens are widely used as preservatives in cosmetics and personal care products [11]. Chlorophenols have been widely used in the wood preservation industry and as intermediates in the production of herbicides. Chlorophenols can also be metabolites and environmental breakdown products of other chemicals of concern [12]. *Ortho*-phenylphenol is used as a commercial disinfectant and as a fungicide on some citrus fruit [13]. 4-*tert*-Octylphenol is an industrial chemical used primarily to make rubber products [14].

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In the human body, these phenols have relatively short biological half-lives and are generally excreted in urine as glucuronide or sulfate conjugates [15,16]. Therefore, human exposure to these phenols can be assessed by measuring the conjugated or free metabolites in urine.

Gas chromatography (GC) coupled with mass spectrometry (MS) has been extensively used for measuring phenols in urine [17–19]. GC-MS methods offer the highest sensitivity. However, these methods require time-consuming cleanup and derivatisation steps. Liquid chromatography (LC-MS) methods do not require a derivatisation step, which greatly simplifies sample preparation [20–22] and results in a higher sample throughput. An on-line SPE sample cleanup with a column-switching and peak focusing HPLC-MS/MS method for phenols in human urine, serum, and milk was recently described [23–25]. On-line HPLC-MS/MS offers full automation. However, its drawback is that a dedicated instrument is required.

The present study describes a sensitive method for the simultaneous determination of 13 environmental phenols (see Figure 1) in human urine using off-line solid phase extraction (SPE) sample cleanup and HPLC-MS/MS sample analysis with a QTRAP 5500 (AB Sciex, Foster City, CA). Off-line SPE-HPLC-MS/MS was used in the previous works for a subset of the analytes. Compared with the on-line method, the off-line method generally provides better peak shape, and separation. In addition, the method does not require a dedicated instrument, thus the instrument may be shared for other applications, which is the case for most environmental analytical laboratories. This paper details sample preparation, analysis, method optimisation, validation and performance. Furthermore, proficiency testing (PT) was performed to verify the method accuracy.

2. Experimental

2.1 Standards, chemicals, and supplies

Methanol, water, formic acid, and other solvents were of analytical or HPLC grade. Methanol and formic acid (98%) were purchased from Fisher Scientific (Pittsburgh, PA). Water was purchased from EMD (Billerica, MA). Bisphenol A (BPA), benzophenone-3 (BP-3), triclosan (TCS), *ortho*-phenylphenol (OPP), ethyl paraben (EP), propyl paraben (PP), butyl paraben (BP), 2,4-dichlorophenol (2,4-DCP), 2,5-dichlorophenol (2,5-DCP), 2,4,5-trichlorophenol (2,4,5-TCP), 2,4,6-trichlorophenol (2,4,6-TCP), β -glucuronidase ($\geq 300,000$ units/gm)/sulfatase ($\geq 10,000$ units/gm) (*Helix pomatia*, H1), ammonium acetate, and acetic acid were purchased from Sigma Aldrich Laboratories, Inc. (St. Louis, MO). Methyl paraben (MP) and 4-*tert*-Octyl phenol (4-*t*-OP) were purchased from Supelco Analytical (Bellefonte, PA). $^{13}\text{C}_{12}$ -BPA, $^{13}\text{C}_6$ -BP-3, $^{13}\text{C}_6$ -triclosan, $^{13}\text{C}_6$ -OPP, $^{13}\text{C}_6$ -2,4-DCP, D_3 -2,5-DCP, $^{13}\text{C}_6$ -2,4,5-TCP, $^{13}\text{C}_6$ -2,4,6-TCP, $^{13}\text{C}_6$ -MP, $^{13}\text{C}_6$ -BP were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). $^{13}\text{C}_6$ -*t*-OP, $^{13}\text{C}_6$ -EP, and $^{13}\text{C}_6$ -PP were purchased from Sigma-Aldrich Laboratories, Inc..

We tested four different sorbents for optimising the SPE. Two polymeric sorbents were Oasis HLB (3cc, 60mg) from Waters (Milford, MA) and Strata-X (3cc, 60mg) from Phenomenex (Torrance, CA). Two C18 sorbents were Bakerbond (6cc, 500mg) from J.T. Baker (Phillipsburg, NJ) and Bond Elut C18 (3cc, 100mg) from Varian Inc. (Palo Alto, CA).

2.2 Preparation of standards and quality control materials

We prepared native stock solutions (1.0 mg/mL) from neat materials by accurately weighing approximately 10 mg of each compound and dissolving the weighed compound in an appropriate volume of methanol to yield a solution containing 1 mg of compound per mL. The

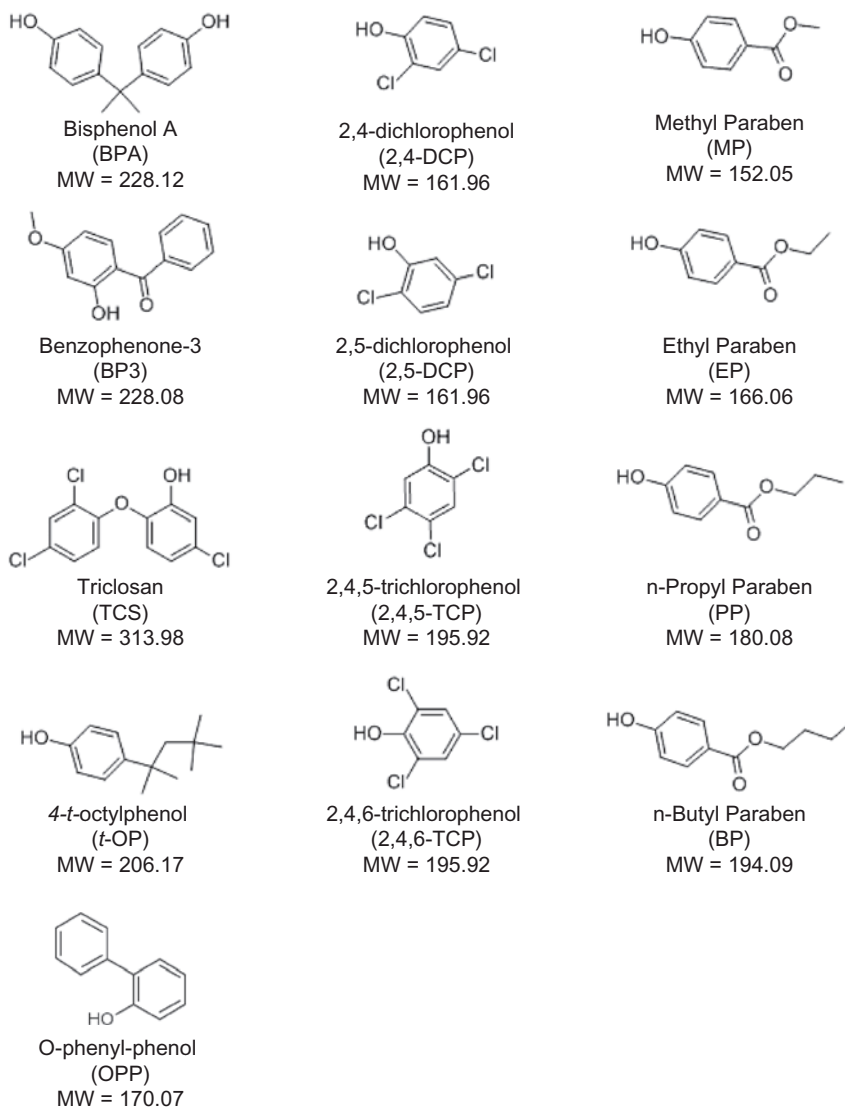


Figure 1. Chemical structures of 13 environmental phenols.

internal standard spiking solution contained one D₃ and twelve of ¹³C-labelled phenols. They were prepared at 10 µg/mL for MP, EP, PP, BP, BPA, OPP, *t*-OP and at 100 µg/mL for TCS, BP-3, 2,4-DCP, 2,5-DCP, 2,4,5-TCP, 2,4,6-TCP in methanol. A mixture of 13 target compounds was prepared in methanol at a concentration of 10 µg/mL from stock solution (1.0 mg/mL). This solution was then diluted in methanol to 12 levels at 1.0, 2.0, 5.0, 10, 20, 50, 100, 500, 1000, 2000, 5000 ng/mL, and 10 µg/mL of working standard solutions. To prepare the calibration curve, 100 µL of each of the calibration standards was spiked to 1 mL of the diluted human urine pool to yield the concentrations ranging from 0.1 ng/mL to 1000 ng/mL. We prepared 50 mL working internal standard solution in methanol at 50 ng/mL for MP, EP, PP, BP, BPA, OPP, 4-*t*-OP; 500 ng/mL for BP-3, 2,4-DCP, 2,5-DCP, 2,4,5-TCP; and 1.0 µg/mL for TCS, 2,4,6-TCP. All standard stock solutions were stored at -20°C until use.

Quality control (QC) material consisted of anonymously collected human urine from multiple volunteers that was pooled and filtered through a 0.45 μm SuperCap-100 Capsule (Pall Corp., Ann Arbor, MI). QC samples were prepared by spiking the appropriate amount of spiking solutions into the 100 mL pooled human urine to yield concentrations of 5.0 ng/mL (Low Quality Control or LQC), 20 ng/mL (Medium Quality Control or MQC), and 50 ng/mL (High Quality Control or HQC) with the exception of BPA having a concentration of 1.0 ng/mL, 4.0 ng/mL, and 10 ng/mL. These QC samples were vortexed at room temperature and subsequently aliquoted (2.5 mL) into 4 mL screw cap tubes, and stored at -70°C until use. One aliquot from each of the LQC, MQC, and HQC was analysed along with real urine samples to monitor accuracy and precision throughout the analytical runs. The enzyme solution was freshly prepared at 4 mg/mL for each run by dissolving 0.2 g of glucuronidase/sulfatase (*Helix pomatia*, H1) to 50 mL of 1 M ammonium acetate buffer (pH 5.0 ± 0.1) solution.

2.3 Off-line sample preparation

To measure the total amount of the free and conjugated forms of environmental phenols, all unknown, blank, standard, and QC samples were enzymatically hydrolysed by deconjugation of glucuronidated and sulfatated metabolites. A 1.0 mL aliquot of urine was mixed with 100 μL of internal standard mixture. Immediately prior to the incubation, 500 μL of the enzyme mixture containing β -glucuronidase/sulfatase (freshly prepared) were added to all samples, mixed, and incubated at 37°C overnight. The reaction was terminated by addition of 0.5 mL of 1 M formic acid.

All samples were purified by SPE procedure using a VBond Elut-C18 column (100 mg 3 mL, Varian, Inc.) on a vacuum manifold (Supelco Visiprep 24TM DL). The SPE cartridges were preconditioned by the use of 2.0 mL methanol and 1.0 mL water. Subsequently, samples (2.1 mL) were applied to the columns. Prior to elution, the cartridges were washed with 1.0 mL of 10% methanol in water. The compounds were eluted with 2.0 mL methanol. The eluates were evaporated to dryness under a stream of dry nitrogen (10–12 psi, UHP grade) with a Turbo Vap LV evaporator from Caliper Life Sciences (Hopkinton, MA) at 40°C for about 30 min. The residues of total phenols were reconstituted with 200 μL of methanol:water (1:1, v:v) and transferred to inserts in auto sampler vials.

2.4 HPLC separation and mass spectrometry detection

The chromatographic separation was performed using a Shimadzu Prominence LC system (LC-20AD, Columbia, MD), composed of two binary pumps with degasser, mixer tee, column, and auto-sampler with temperature control. A trap column was connected before the mixer tee using a 4.6×50 mm, 4 μm column from Phenomenex. The injection volume was 10 μL on an ACE Excel C18-PFP (4.6×100 mm, 3.0 μm , Chadds Ford, PA) column using a gradient elution program. The total flow rate was 500 $\mu\text{L}/\text{min}$. Solvent A was water and solvent B was methanol. Gradient programming was 0.0–2.0 min, 60% B; 2–10 min, 60% to 100% B; 10–15 min, 100% B; 15–15.1 min, 100% to 60% B; and 15.1 min –20 min, 60% B.

The 5500 QTRAP mass spectrometer with the Analyst 1.5.1 software program was used for acquisition and quantification (AB Sciex, Foster City, CA). Negative ion atmospheric pressure chemical ionisation (APCI) was used to form negatively charged analyte ions with the following settings: curtain gas (N_2) 20 psi, heated ion source gas 30 psi, heated gas temperature 450°C , nebuliser current -3.5 μA . Declustering potential, entrance potential, and collision energy were optimised for each analyte. All channels were monitored with a different dwell time adding up to 1.4-s cycle time. Data acquisition was performed in the MRM (Multiple Reaction Monitoring) mode. Table 1 summarises the precursor/product ion transitions for each analyte

Table 1. MS/MS parameters, retention time (RT), calibration parameters monitored on QTRAP 5500 for 13 environmental phenols in urine.

Analyte Names	MRM		DP (V)	CE (V)	MRM		Labeled Analyte	MRM Transitions (IS)	RT (min)	Calibration Range (ng/ mL)	Correlation ^b (R ²)	Intercept ^b	Slope ^b	CV% of Slope ^b
	Quantitation Transitions	Confirmation Transitions			Confirmation Transitions	Transitions								
Bisphenol A	227–133	227–212	-105	-35	227–212	239–139	¹³ C ₁₂ -BPA	239–139	9.23	0.1–100	0.998	0.031	1.16	4.37
Benzophenone-3	227–183	227–211	-95	-35	227–211	233–190	¹³ C ₆ -BP3	233–190	12.9	0.2–1000	0.999	0.025	0.427	7.74
Triclosan	161–125	287–142	-110	-25	287–142	167–131	¹³ C ₁₂ -TCS	167–131	13.1	0.1–1000	0.999	0.006	1.03	6.40
4- <i>tert</i> -octylphenol	205–133	169–141	-140	-20	169–141	211–139	¹³ C ₆ -t-OP	211–139	12.7	0.5–500	0.997	0.065	0.344	6.75
<i>Ortho</i> -phenylphenol	169–115	163–125	-125	-30	163–125	175–121	¹³ C ₆ -OPP	175–121	10.3	0.5–500	0.999	0.015	0.201	4.75
2,4-dichlorophenol	161–125	125–100	-90	-23	125–100	167–131	¹³ C ₆ -2,4-DCP	167–131	10.6	0.5–500	0.999	0.035	0.708	3.10
2,5-dichlorophenol	161–125	197–161	-85	-22	197–161	164–127	D ₃ -2,5-DCP	164–127	10.0	1.0–1000	0.999	0.059	1.38	7.99
2,4,5-trichlorophenol	195–159	197–161	-95	-25	197–161	201–165	¹³ C ₆ -2,4,5-TCP	201–165	12.4	0.5–1000	0.999	0.003	0.899	3.91
2,4,6-trichlorophenol	195–159	197–161	-70	-32	197–161	201–165	¹³ C ₆ -2,4,6-TCP	201–165	11.8	1.0–500	0.997	0.017	1.27	3.87
Methyl paraben	151–92	151–136	-30	-12, (-30) ^a	151–136	157–98	¹³ C ₆ -MP	157–98	5.95	0.5–1000	0.999	0.080	0.066	8.92
Ethyl paraben	165–92	165–137	-80	-12, (-30) ^a	165–137	171–98	¹³ C ₆ -EP	171–98	7.83	0.5–500	0.998	0.002	0.006	5.95
Propyl paraben	176–92	179–136	-55	-12, (-24) ^a	179–136	185–98	¹³ C ₆ -PP	185–98	9.42	0.5–1000	0.999	0.001	0.005	4.27
Butyl paraben	193–92	193–136	-100	-18, (-36) ^a	193–136	199–98	¹³ C ₆ -BP	199–98	10.7	0.2–500	0.999	0.006	0.131	2.94

^aDe-tuned CE.^b10 repeated measurements between days.

and labeled analyte, declustering potential and collision energies used for measurement of each analyte. Entrance potential is always 10 volt for all the analytes. These parameters were selected to maximise the specificity, sensitivity, and linear range of the assay.

3. Results and discussion

3.1 Method optimisation

In humans, phenols are excreted as glucuronide or sulfate conjugates. We conducted deconjugation studies to optimise both the amount of enzyme and pH for completing the deconjugation reaction. A study reported that 0.5 mg of β -glucuronidase (*Helix pomatia*, H1) at pH 5 is able to deconjugate both glucuronide and sulfate metabolites [26]. Our study confirmed that these conditions are effective, and these conditions were used in our method.

SPE was used to clean up samples in order to concentrate the analytes and remove the interference. We conducted initial studies to optimise the extraction steps by examining several different factors. These factors included type of sorbent, sorbent volume, wash solution, elution solvent/volume, and sample volume. First, we tested four different sorbents according to each manufacturer's recommended method by loading spiked samples and calculating recovery of each analyte. These cartridges were two polymeric sorbents, Oasis HLB (3 cc, 60 mg) from Waters (Milford, MA) and Strata-X (3 cc, 60 mg) from Phenomenex (Torrance, CA) and two C18 sorbents, Bakerbond from J.T. Baker (Phillipsburg, NJ) and a Bond Elut C18 (3 cc, 100 mg) from Varian Inc. (Palo Alto, CA). The best results were achieved using the Bond Elut C18 packed cartridges (3 cc, 100 mg) in Figure 2.

Once the Bond Elut C18 cartridge was chosen, its conditioning, wash and elution steps were further optimised. To condition and clean the cartridge, 2 mL methanol was applied to the cartridge. To investigate the retention property of the cartridge, different pHs (pH3 and pH5) were tested. We found that pH has no significant effects on the retention of the analytes. After loading urine samples, we tested acid and neutral wash solutions with different percentages of methanol (0%, 5% 10%, 20%) to remove a maximum of co-extracted interfering substances from the urine and at the same time to maintain high recovery of the analytes. 10% methanol

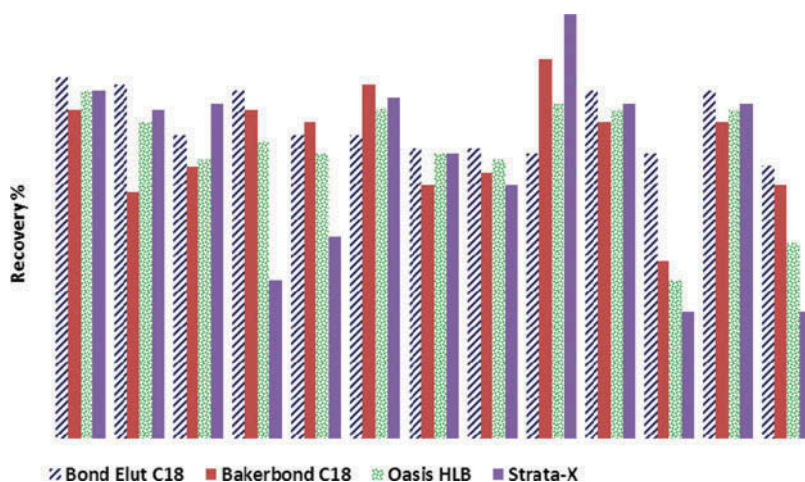


Figure 2. Comparison of recovery from spiked 13 compounds in the urine on four different sorbents ($n = 2$).

was found to be effective. Finally, the most commonly used elution solvents such as acetonitrile, methanol, and ethyl acetate, were tested to elute analytes off the cartridges. The optimum elution solvent was found to be methanol (2.0 mL). SPE absolute recoveries were calculated by using the ratio of the amount of analytes recovered after SPE to the amounts originally added. The internal standard mixture containing ^{13}C -labeled analytes was added after the drying step to account for any instrumental variation among the injected samples and to calculate the recovered amount. As showed in Table 2, the SPE recoveries were between 70% and 101% for most analytes.

For quantitative MS/MS analysis, a QTRAP system operating in multiple reaction monitoring (MRM) was used. To choose the proper ionisation technique for detecting all analytes, both an electrospray ionisation (ESI) source and an atmospheric pressure chemical ionisation (APCI) source were used in the initial experiments. In order to gain maximum analytical sensitivity and instrument precision, we optimised parameters such as declustering potential (DP) and collision energy (CE) for each compound by infusing compounds at concentrations between 1 and 100 ng/mL into the ion source. Although some compounds showed a slightly higher intensity with the ESI source (e.g., BPA), triclosan (TCS) had poor ionisation with the ESI probe. In the APCI mode, owing to the in-source fragmentation, TCS broke down into dichlorophenol (DCP). DCP further fragments in the collision cell demonstrating a typical transition of m/z 161–125. This transition was more sensitive than the m/z 287–142 (TCS molecular ion-production ion) transition. Therefore, we used the m/z 161–125 DCP transition at the retention time of TCS for quantification of TCS.

APCI also improved the ionisation efficiency of 2,4-DCP, 2,5-DCP and 2,4,6-TCP, and had better sensitivities for them. Moreover, APCI ionisation was known to be less susceptible to matrix interference than ESI ionisation [27]. Therefore, APCI was chosen as the ionisation technique for this method. During method development with the APCI probe, we also observed that parabens generated too strong signals. In order to be able to measure these compounds together in a reasonable linearity range, de-tuned CE parameters were selected for the detection of these four parabens (Table 1). In addition, we injected all the compounds individually at concentrations between 10 and 100 ng/mL into the HPLC with MS detection to optimise the ion source parameters such as curtain gas flow, collision gas flow, nebuliser gas flow, nebuliser gas temperature, and corona needle voltage.

We tested the effect of the probe temperature and curtain gas flow on ionisation efficiency. We found that 2,4-DCP, 2,5-DCP, 2,4,5-TCP and 2,4,6-TCP gave a higher signal at lower curtain gas flow and high probe temperature. The probe temperature of 450°C and curtain gas flow of 20 psi were selected as the best compromise for the detection of these 13 compounds. We also tested the corona needle voltage. Our experiment showed that corona needle voltages for these compounds are between 2.5 to 4.5V. Other source parameters such as collision gas flow, and nebuliser gas flow had less effect on the MS signal.

The influence of mobile phase composition on ionisation efficiency was also evaluated. Acetonitrile gave the higher baseline. Methanol produced the lower baseline while generating the best signal for all compounds. Therefore, methanol was selected as the organic solvent of choice for further LC method development. Several different sources of methanol were evaluated to select the best brand that produces the lowest baseline.

It is difficult to use an isocratic method for simultaneous determination of 13 analytes in a single LC run. Specifically, there are two pairs of isomers which have the same mass transition, so we needed a baseline separation in order to perform reliable quantification. Thus, a gradient elution method was developed to ensure that all the isomers could be well separated and determined in one analytical run (Figure 3). The gradient program was initiated with solvent

Table 2. Solid-phase extraction (SPE) recoveries, method accuracy ($n = 20$), and limit of detection (LOD) for 13 environmental phenols in human urine.

Analytes	SPE Absolute Recovery (%)	LQC			MQC			HQC		
		Spiked conc. (ng/mL)	Spiked recovery (%)	Spiked conc. (ng/mL)	Spiked recovery (%)	Spiked conc. (ng/mL)	Spiked recovery (%)	Spiked conc. (ng/mL)	Spiked recovery (%)	LOD (ng/mL)
Bisphenol A	87	1.00	100	4.00	102	10.0	99.4	10.0	99.4	0.1
Benzophenone-3	96	5.00	99.1	20.0	95.8	50.0	101	50.0	101	0.2
Triclosan	78	5.00	91.7	20.0	98.4	50.0	101	50.0	101	0.1
4- <i>tert</i> -octylphenol	88	5.00	98.1	20.0	101	50.0	96.4	50.0	96.4	0.5
<i>Ortho</i> -phenylphenol	87	5.00	96.6	20.0	90.7	50.0	93.5	50.0	93.5	0.5
2,4-DCP	90	5.00	102	20.0	99.3	50.0	104	50.0	104	0.5
2,5-DCP	82	5.00	98.0	20.0	100	50.0	100	50.0	100	1.0
2,4,5-TCP	86	5.00	98.7	20.0	98.6	50.0	104	50.0	104	0.5
2,4,6-TCP	70	5.00	90.1	20.0	93.7	50.0	97.6	50.0	97.6	1.0
Methyl Paraben	75	5.00	98.3	20.0	100	50.0	102	50.0	102	0.5
Ethyl Paraben	75	5.00	97.4	20.0	94.6	50.0	100	50.0	100	0.5
Propyl Paraben	101	5.00	91.3	20.0	90.5	50.0	93.2	50.0	93.2	0.5
Butyl Paraben	97	5.00	98.2	20.0	98.0	50.0	102	50.0	102	0.2

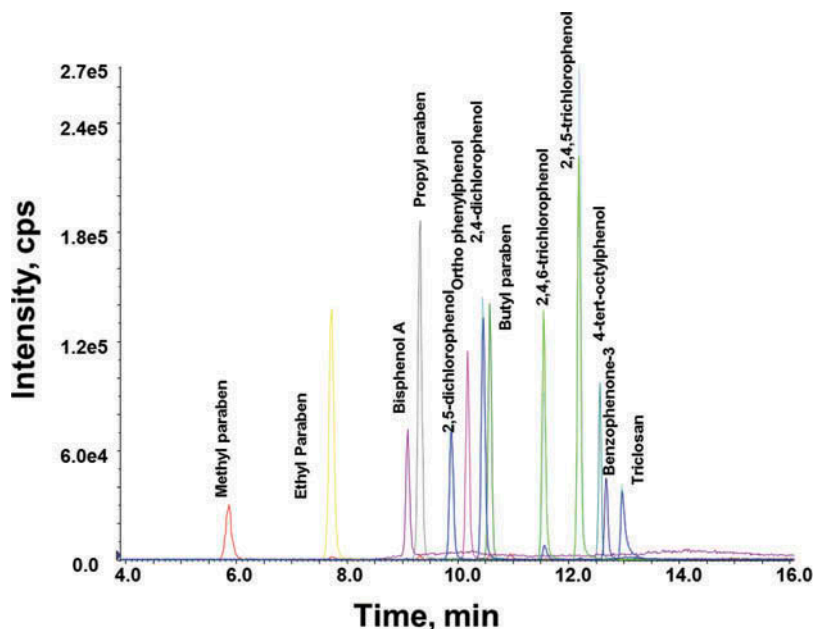


Figure 3. HPLC-APCI-MS/MS total ion chromatogram of a standard mixture (concentration are between 2 to 50 ng/mL) in solvent.

A (water) and solvent B (methanol) with a ratio of 80:20. Two pairs of isomers could not be separated, and the peak of methyl paraben eluted too early. With a ratio of 40:60 of solvent A (water) to solvent B (methanol), we achieved our goal of good separation of the isomer pairs (2,4-DCP/2,5-DCP and 2,4,5-TCP/2,4,6-TCP) as well as reasonable retention of methyl paraben. An ACE Excel C18-PFP column with a gradient elution program could achieve baseline separation for all 13 compounds in 20 min. We further evaluated the impact of mobile phase additives such as formic acid with respect to retention and ionisation efficiency. The results showed there is no change in retention times and no increase in ionisation efficiency. Therefore, formic acid was not used in the mobile phase.

3.2 Method validation

Matrix effects may affect assay reproducibility and accuracy [28]. We took several precautionary steps to eliminate or reduce matrix effects. First, we used SPE to remove co-eluting matrix components in diluted urine to decrease the amount of matrix injected onto the column and hence reduce its effect [29]. Second, we used a stable isotope labeled (SIL) internal standard because its chemical and physical properties are nearly identical to the target analyte. The SIL internal standard not only helped to correct for the loss of the target analyte during extraction and cleanup, but also compensated for variability in MS detection [30]. Last, we used an APCI probe as mentioned before. APCI is known to associate with a smaller degree of ion suppression owing to the fact that the ionisation occurs in the gas phase which is almost free of analytes precipitation [27].

In our method, matrix effects on the calibration curve with isotope dilution method were evaluated by analysing standards prepared in water, synthetic urine, diluted urine, and urine. Standards spiked in urine generated calibration curves with a slope not significantly different

from the slope generated by standards prepared in water and synthetic urine. Therefore, no significant matrix effect was observed for the concentration range of analytes measured. Calibration curves were generated using data collected by analysing standards and the internal in diluted urine.

Our calibration curves were generated by plotting the peak area-ratio (analytes/internal standard) versus the concentration-ratio (analytes/internal standard) by least-squares regression analysis. The resulting slope and y -intercept values for each analyte were used to interpolate analyte values in unknown specimens. Table 1 shows that the calibration curves cover a broad linearity range for all analytes with correlation coefficients greater than 0.99. The variability of the slopes of calibration curves was less than 10% for all analytes.

The limits of detection (LOD) for this method ranged from 0.1 to 1 ng/mL (Table 2). The LOD for each of the 13 analytes is defined as higher value of either the lowest measurable

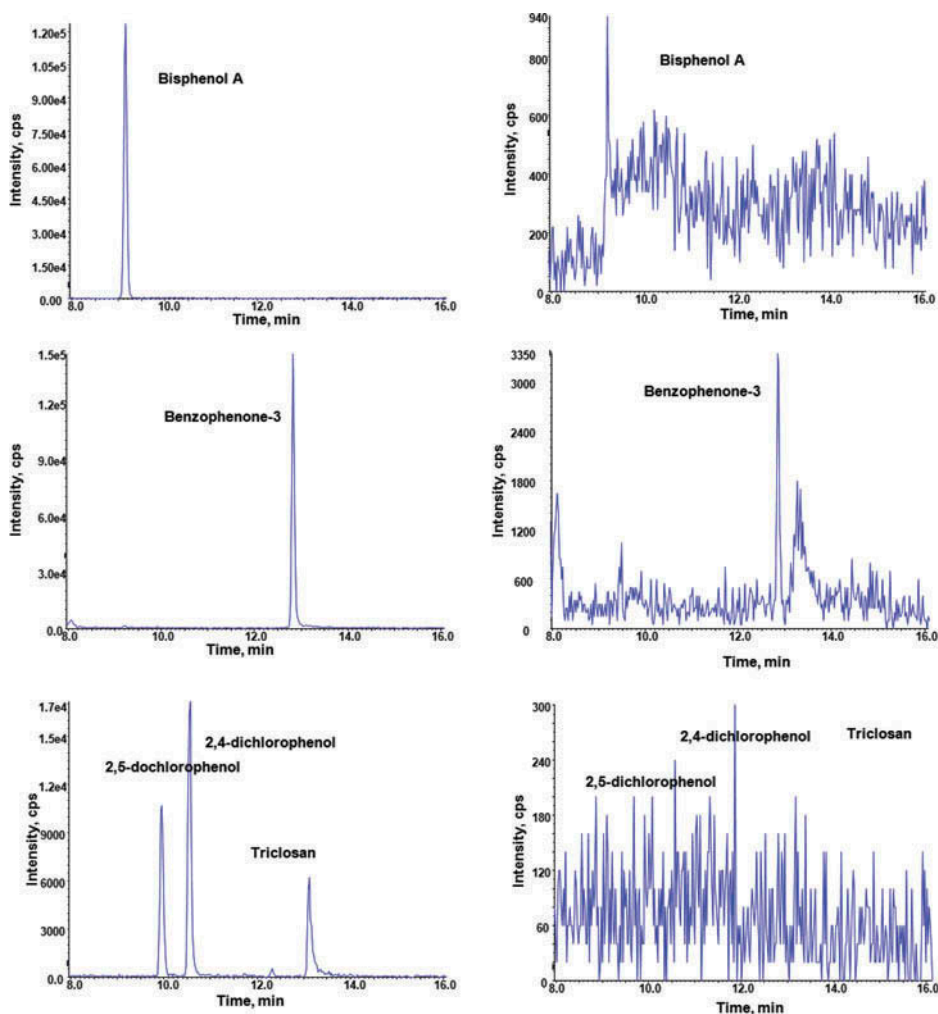


Figure 4. HPLC-APCI-MS/MS extracted ion chromatograms for a low concentration calibration standard (left: BPA, BP-3, TSC, OPP, t -OP, 2,4-DCP, 2,5-DCP, 2,4,5-TCP at 5ng/mL, 2,4,6-TCP at 100ng/mL, and MP, EP, PP, BP at 10ng/mL and right: a urine blank). The calculated concentrations of the urine blank were <LOD for all analytes.

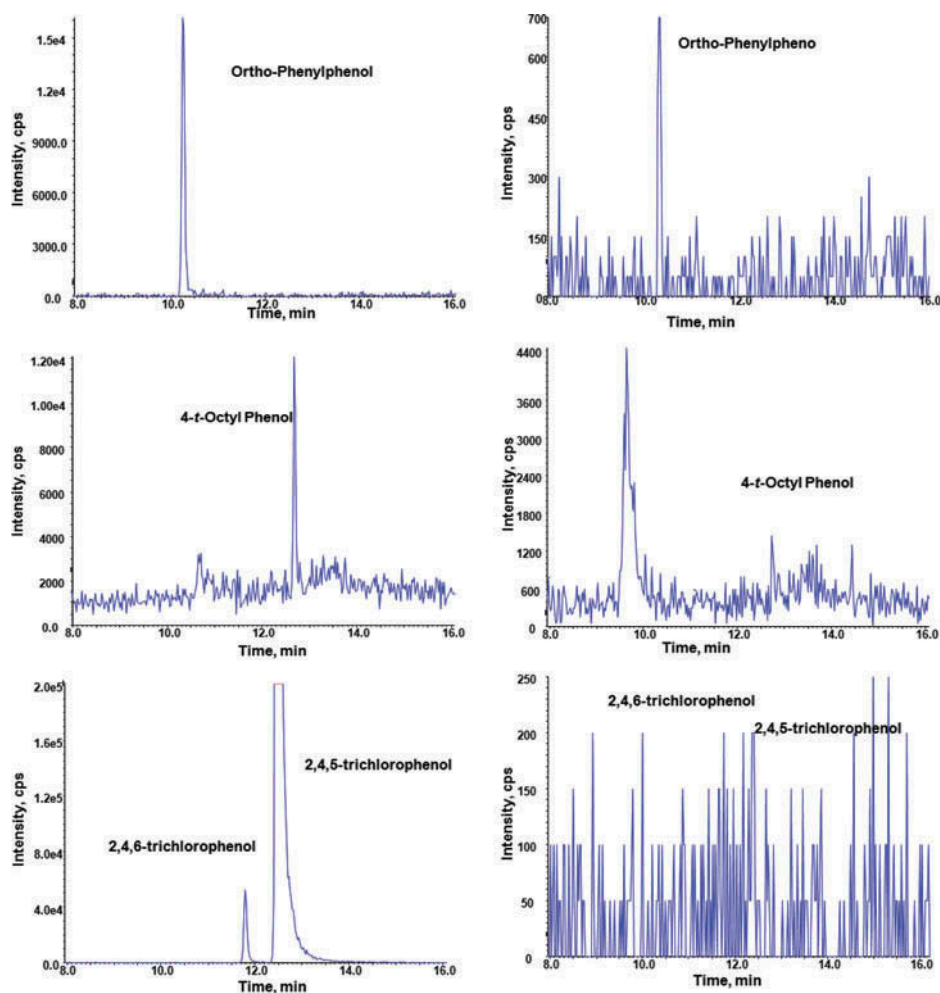


Figure 4. Continued.

standard (with a S/N equal to or greater than 3) or three times the standard deviation at concentrations approaching zero ($3 \cdot S_0$) [31]. S_0 is the standard deviation as the concentration approached zero and is determined from five repeated analyses for the low level standard. The calculated LODs were verified by the analysis of the similar concentration of analytes spiked into urine. LOD values show good sensitivity of the method and demonstrate that the method could be used to evaluate the exposure of environmental phenols for the general population. Figure 4 shows the extracted ion chromatograms of blank urine and low concentration calibration standards. From the chromatograms, one can see that our method is almost free of background contamination which allows us to reach a very low LOD.

The method accuracy and precision were assessed with twenty replicate analyses of urine spiked at three different concentrations (LQC, MQC, and HQC) and expressed as a percentage of the expected value. The relative recoveries (accuracy) were very good, between 90.1% and 104% for all 13 analytes at all spike levels (Table 3). The inter-day variability (method precision) was determined by calculating the coefficients of variation (CVs) of twenty repeated

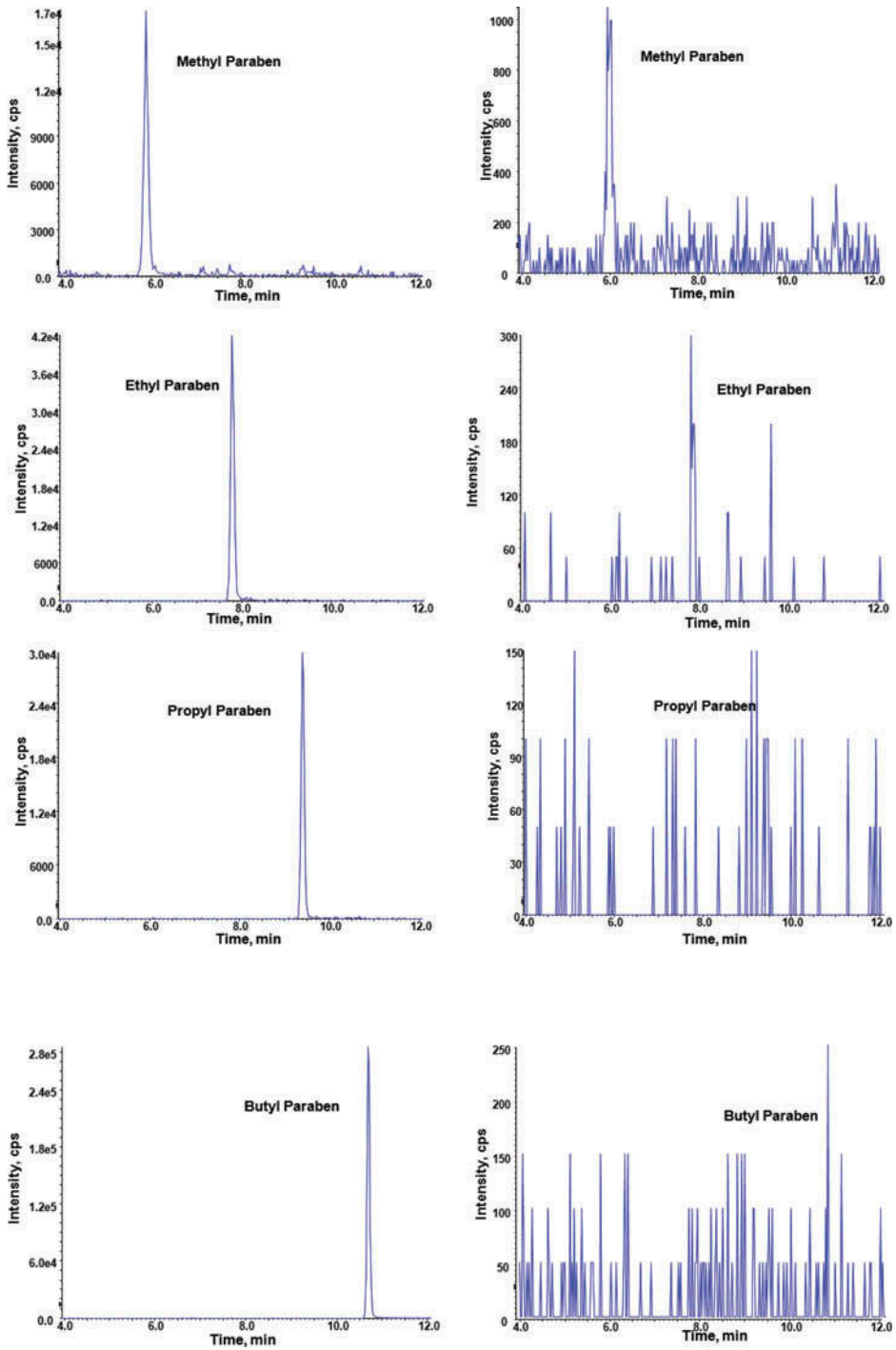


Figure 4. Continued.

Table 3. Precision of concentration measurements of 13 environmental phenols in urine in spiked low quality control (LQC), medium quality control (MQC) and high quality control (HQC) samples ($n = 20$).

Analytes	LQC ($n = 20$)			MQC ($n = 20$)			HQC ($n = 20$)		
	Spiked Conc. (ng/mL)	Measured mean (ng/mL)	CV%	Spiked Conc. (ng/mL)	Measured mean (ng/mL)	CV%	Spiked Conc. (ng/mL)	Measured mean (ng/mL)	CV%
Bisphenol A	1.00	1.00	4.93	4.00	4.08	8.39	10.0	10.0	7.72
Benzophenone-3	5.00	4.96	10.9	20.0	19.2	9.03	50.0	50.3	11.7
Triclosan	5.00	4.59	9.48	20.0	19.7	10.4	50.0	50.7	9.73
4- <i>tert</i> -octylphenol	5.00	4.90	11.2	20.0	20.2	12.5	50.0	48.2	12.9
<i>Ortho</i> -phenylphenol	5.00	4.83	9.41	20.0	18.1	8.42	50.0	46.8	7.46
2,4-DCP	5.00	5.08	9.69	20.0	19.9	8.51	50.0	51.9	8.37
2,5-DCP	5.00	4.90	11.9	20.0	20.1	13.8	50.0	50.1	9.84
2,4,5-TCP	5.00	4.93	8.95	20.0	19.7	8.65	50.0	52.2	6.17
2,4,6-TCP	5.00	4.51	9.51	20.0	18.7	13.2	50.0	48.8	13.1
Methyl Paraben	5.00	4.92	11.3	20.0	20.0	11.2	50.0	50.8	14.0
Ethyl Paraben	5.00	4.87	9.57	20.0	18.9	9.53	50.0	50.2	14.3
Propyl Paraben	5.00	4.57	10.7	20.0	18.1	9.78	50.0	46.6	11.5
Butyl Paraben	5.00	4.91	5.24	20.0	19.6	10.5	50.0	50.9	8.98

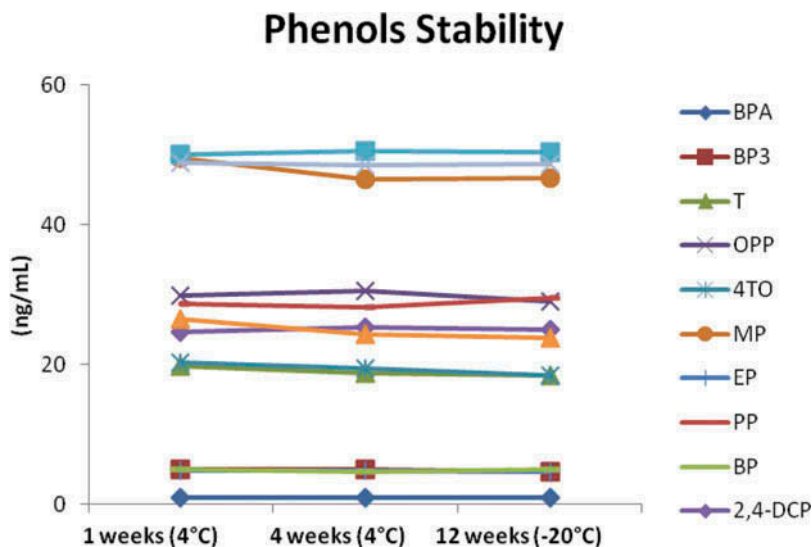


Figure 5. Post-preparation stability of 13 compounds in urine samples at refrigerator temperature (4°C) for 1 and 4 weeks and at freezer temperature (-20°C) for 12 weeks.

measurements at three different concentrations of the LQC, MQC, and HQC. The CVs between 5.24% and 14.3% reflect low variability or excellent precision of the method.

The post-preparation stability of samples was examined to determine the valid hold time of the prepared samples under different conditions, such as an analysis delay due to instrument malfunction or other uncontrolled event. We examined the stability of analytes using extracted samples at room temperature (25°C) for 24 h and one week, refrigerator temperature (4°C) for one week and one month, and freezer temperature (-20°C) for 3 months. Figure 5 shows the stability of these extracted 13 phenols in urine samples at refrigerator temperature (4°C) for one week and one month, and freezer temperature (-20°C) for 3 months. The mean relative recoveries were between 91.8% and 106%. We also conducted three cycles of freeze-thaw study. We observed that phenols were stable without noticeable degradation under these conditions.

3.3 Method verification

This method accuracy was further evaluated by the analysis of external performance assessment samples at low, medium, and high concentrations. These performance assessment samples were blind analysed and the results were reported to an external QC provider for evaluation. For the evaluation, our measured values were compared with the target values. According to the acceptance criteria, except for 2,4-DCP at low concentration, the results of the other eight tested chemicals at all concentration levels successfully passed external performance tests, which verified our method is reliable and the results generated by it are comparable with other laboratories.

4. Conclusions

We have developed and validated a sensitive HPLC-MS/MS method on QTRAP 5500 for the simultaneous measurement of 13 environmental phenols in human urine. This method shows

good reproducibility and accuracy with low detection limits for most compounds without significant matrix effects. Because the sample treatment steps in the method described are not labour-intensive and the method does not need a dedicated analysis instrument, they allow for a high sample throughput and a flexible workflow. The accuracy of the method was confirmed by blind analysis of PT samples. This method is suitable for epidemiologic studies to assess human exposures to environmental phenols.

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