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RESEARCH PAPER

Analysis of twenty phenolic compounds in human urine: hydrochloric acid hydrolysis, solid-phase extraction based on K_2CO_3-treated silica, and gas chromatography tandem mass spectrometry

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Abstract This study developed a new method for the analysis of 20 phenolic compounds in human urine. The urine samples were prepared by hydrochloric acid (HCl) hydrolysis, liquid–liquid extraction (LLE), and solid-phase extraction (SPE) cleanup. We found that HCl hydrolysis is of similar effectiveness to, and much cheaper than, the traditional enzymatic method. Vanillic acid was co-eluted with butyl paraben and interfered with the determination of butyl paraben in urine. K_2CO_3-treated-silica-gel SPE was designed to efficiently eliminate interference from the endogenous organic acids (especially vanillic acid) in urine. After derivatization, the samples were analyzed by large-volume-injection gas chromatography–tandem mass spectrometry (LVI-GC–MS–MS). Good linearity ($R^2 \geq 0.996$) was established in the range 0.1–100 ng mL^{-1} for all analytes. Method detection limits (MDLs) were 0.7–9.8 pg mL^{-1}. Intraday ($n=5$) and interday ($n=5$ days) validation was performed, with satisfactory accuracy (recovery: 70–126 % and 73–107 %, respectively) and precision (RSD≤19 %) at two levels (low: 0.1 and 0.5 ng mL^{-1}; high: 5 and 10 ng mL^{-1}). The method was used in a population study and achieved more than 85 % detection for most analytes; mean analyte concentrations were in the range 0.01–185 ng mL^{-1}. The method is suitable for the analysis of multiple phenolic metabolites in human urine.

Keywords EDCs · LVI-GC–MS–MS · Phenolic biomarker · Human biomonitoring · Acid hydrolysis · K_2CO_3-treated-silica-gel SPE

Introduction

Chemicals (30 listed in Table 1) are widely used in farming, aquaculture, and daily life. There has been increasing concern regarding their ubiquity in the environment and their potential to have undesirable effects on human health [20]. These chemicals might enter the human body and be excreted as phenolic metabolites in urine. A total of 20 metabolites were listed from a literature review (Table 1), and were measured analytically.

Traditionally, gas or liquid chromatography tandem mass spectrometry (GC–MS–MS and LC–MS–MS) have been used for determination of phenolic metabolites in human urine [21, 22]. As shown in Table 1, up to 11 phenols have been measured by GC–MS–MS [1, 8, 9, 16] and 17 compounds by LC–MS–MS [2–7, 10–15, 17–19]. GC–MS–MS has not been reported for analysis of the other nine compounds in our list, namely methyl paraben, ethyl paraben, propyl paraben, butyl paraben, benzyl paraben, triclosan, benzophenone-3 (BP-3), 4-*tert*-octylphenol (tOP), and 4-nonylpheonl (4-NP) (Table 1). Because of similarity in their chemical structures, all 20 phenolic metabolites could be measured using only one analytical

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Table 1 Parent chemicals, their phenolic metabolites in urine, and analytical methods available

Parent compounds and their use	Metabolic analytes	Analytical methods based on tandem MS	
		GC–MS–MS	LC–MS–MS
Metabolite of 1,3-dichlorobenzene, nitrofen and dichlofenthion, or as raw materials for synthesis of 2,4-D and 2,4,5-trichlorophenoxyacetic acid, or degradation of 2,4-D in the environment	2,4-Dichlorophenol (24-DCP)	[1]	[2–5]
Metabolite of 1,4-dichlorobenzene	2,5-Dichlorophenol (25-DCP)	[1]	[2–6]
Metabolite of chlorinated benzenes and chlorinated phenols	2,4,5-Trichlorophenol (245-TCP)	[1]	[2–5, 7]
Metabolite of chlorinated benzenes and chlorinated phenols	2,4,6-Trichlorophenol (246-TCP)	[1]	[3–5]
As pentachlorophenol pesticides or as a metabolite of hexachlorobenzene, other polychlorinated benzenes, and lindane	Pentachlorophenol (PCP)	[1]	[7]
Metabolite of insecticides methyl parathion, ethyl parathion, and *O*-ethyl-*O*-(4-nitrophenyl) phenylphosphonothioate, and of the chemical nitrobenzene	*p*-Nitrophenol (PNP)	[1]	–
Metabolite of pesticides benfuracarb, carbofuran, carbosulfan, and furathiocarb	Carbofuran phenol (CFP) (2,3-Dihydro-2,2-dimethy-7-hydroxybenzofuran)	[1, 8]	–
Metabolite of propoxur pesticide.	2-Isopropoxyphenol (2-IPP)	[1, 9]	–
Metabolite of pesticides chlorpyrifos and chlorpyrifos methyl	3,5,6-Trichloro-2-pyridinol (TCPY)	[1, 9]	[10]
Parent pesticide of 2-phenylphenol and sodium *ortho*-phenylphenate (SOPP)	2-Phenylphenol (OPP)	[1]	[2–5]
Widely used as preservatives in cosmetics and in personal-care products including shampoos, hair and shaving products, facial and skin cleansers, and lotions	Methyl paraben	NA	[3, 4, 6, 11–15]
	Ethyl paraben	NA	[3, 4, 6, 11–15]
	Propyl paraben	NA	[3, 4, 6, 11–15]
	Butyl paraben	NA	[3, 4, 6, 11–15]
	Benzyl paraben	NA	[12–15]
Used as antibacterials in consumer products (for example detergents) and other materials (for example textiles)	Triclosan	NA	[2–7, 12, 14]
Used as an additive in the manufacture of polycarbonate plastics (used as packaging materials and toys)	Bisphenol A (BPA)	[9, 16]	[2–7, 17, 18]
Used as sunscreen in lotions, conditioners, and cosmetics	Benzophenone-3 (BP-3)	NA	[2–7, 13]
Used as raw materials in the manufacture of alkylphenolethoxylates, or environmental degradation of alkylphenolethoxylates and plasticizers and antioxidants in plastics and resins	4-*tert*-Octylphenol (tOP)	NA	[4, 5, 7, 17, 19]
	4-Nonylphenol (4-NP)	NA	[7, 17, 19]

NA: no analytical method reported

method. It would be possible and very interesting for us to develop such a method using GC–MS–MS or LC–MS–MS. Compared with LC–MS–MS, GC–MS–MS is more time-consuming because it requires an extra derivatization step [4]. However, GC–MS–MS has higher separation efficiency because it has a capillary column, lower instrument maintenance costs, and fewer matrix effects [23], and the derivatization step greatly improves its sensitivity [24]. It was reported that GC–MS–MS methods offered higher sensitivity than LC–MS–MS methods for the determination of a few phenolic metabolites (for example 24-DCP and 25-DCP) in urine [1, 4]. Also, some target biomarkers are ubiquitous environmental chemicals and therefore could potentially exist in HPLC mobile phases, resulting in contamination of the LC–MS–MS analysis [25]. Such contamination would not occur with GC–MS–MS. In short, GC–MS–MS is a suitable and more sensitive method for the simultaneous determination of these phenolic metabolites.

Two relevant GC–MS–MS methods [1, 9] have been reported. In Bravo's method [1], ten analytes were measured in 1 mL urine with limits of detection (LODs) in the range 0.1–1.3 μg L^{-1}. In Schmidt's method [9], three analytes were measured in 2 mL urine with LODs comparable to those of Bravo's method. Bravo's method included multiple sample-preparation steps including solid-phase extraction (SPE) enrichment, liquid–liquid extraction (LLE) cleanup, phase-transfer-catalyzed derivatization, and sorbent-immobilized LLE for further cleanup. Schmidt's method simplified

sample-preparation steps and was used for the measurement of phenolic metabolites in many urine samples [9]. However, in these methods matrix effects were not assessed and sample-preparation steps were not well optimized.

The objective of our study was to develop a GC–MS–MS method for simultaneously measuring 20 phenolic metabolites (free and conjugated forms) in human-urine samples. As stated above, the nine compounds which have not been measured by GC–MS–MS (Table 1) were included in our study. Additionally, a new sample-preparation method was designed, developed, and optimized.

Experimental

Chemicals and materials

2,4-Dichlorophenol (24-DCP), 2,5-dichlorophenol (25-DCP), 2,4,5-trichlorophenol (245-TCP), 2,4,6-trichlorophenol (246-TCP), pentachlorophenol (PCP), 3,5,6-trichloropyridinol (TCPY), carbofuran phenol (CAF), *para*-nitrophenol (NTP), 2-isopropoxyphenol (2-IPP), bisphenol A (BPA), 4-*tert*-octylphenol (tOP), 4-nonylphenol (4-NP), *ortho*-phenylphenol (OPP), benzophenone-3 (BP-3), triclosan, and methyl, ethyl, propyl, butyl, and benzyl parabens were native standards (Dr. Ehrenstorfer GmbH, Augsburg, Germany). ^{13}C-labelled standards were $^{13}C_{12}$-BPA, $^{13}C_6$-OPP, $^{13}C_6$-tOP, $^{13}C_6$-24-DCP, $^{13}C_6$-25-DCP, $^{13}C_6$-245-TCP, $^{13}C_6$-246-TCP, $^{13}C_5$-TCPY, $^{13}C_6$-PCP, $^{13}C_6$-BP-3, and $^{13}C_{12}$-triclosan (Cambridge Isotope Laboratories, Inc., Andover, MA, USA). Deuterated standards were D_4-NTP and D_4-methyl, D_4-ethyl, D_4-propyl, D_4-butyl, and D_4-benzyl paraben (CDN Isotopes, Quebec, Canada).

Hexane (Hex, Optima® grade), ethyl ether (HPLC grade), butyl chloride (BuCl, analytical grade), and acetonitrile (HPLC grade) were from Fisher Scientific (NJ, USA). Methyl *tert*-butyl ether (MTBE, HPLC grade) was from Tedia Company, Inc. (Fairfield, Ohio, USA). Hydrochloric acid (36.5 %, analytical grade) and potassium carbonate (99.5 %, analytical grade) were from Shanghai Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1 % trimethylchlorosilane was used for derivatization (Regis Technologies, Inc., Illinois, USA). Silica gel (0.063–0.100 mm, 100–200 mesh) was from Merck (Darmstadt, Germany). Oasis® HLB (3 cc, 60 mg) was from Waters (Milford, MA, USA). β-Glucuronidase and sulfatase (*Helix pomatia*, H1, 463,000 U g^{-1} solid) were from Sigma–Aldrich Laboratories (St Louis, MO, USA). The enzyme solution was prepared daily as described in US CDC's method [3]. To avoid contamination from NP and BPA, hydrochloric acid was extracted with DCM and all glassware was baked for 2 h at 180 °C before use.

Preparation of standard solutions and K_2CO_3-treated-silica-gel SPE cartridges

The calibration standard solutions were prepared in acetonitrile in the range 0.1–100 ng mL^{-1} for each analyte and at 20 ng mL^{-1} for each internal standard. A working solution was prepared in acetonitrile at a concentration of 1 μg mL^{-1} for each native analyte. Another working solution was prepared in acetone at 1 μg mL^{-1} for each isotopically-labelled analyte. All standard solutions were stored at 4 °C.

Silica gel (100 g) was mixed with 50 mL potassium carbonate–water solution (3 mol L^{-1}), dried at 105 °C, washed with 300 mL acetonitrile, and activated at 300 °C for 2 h after evaporating acetonitrile residue in a fume hood. The well-prepared basic silica gel (0.15 or 0.2 g) was packed into a 3 mL SPE cartridge by vacuum.

Sample collection

Eighteen adults and 11 children aged 4 to 51 months were recruited. The adults and the parents of the children were clearly informed of the objectives of the study. After informed consent was obtained, 29 urine samples were collected: one from each of the children and adults. Additional urine samples were donated by our laboratory members, and used to make a combined urine sample for the method development and validation. After collection, all urine samples were stored at −40 °C until analysis.

Sample preparation

Hydrolysis

A flowchart depicting sample preparation and analysis is shown in Fig. 1. This study compared hydrochloric acid (HCl) and enzymatic hydrolysis methods. HCl hydrolysis was performed following the method reported by Lu et al. [26]. In brief: urine samples were thawed, equilibrated to room temperature, and vortex-mixed. From each urine sample, an aliquot (0.5 mL) was spiked with 20 μL internal standards (50 ng mL^{-1}) and 125 μL HCl (12 mol L^{-1}). After mixing, the sample was hydrolyzed at 90 °C for 1 h. The enzymatic method was performed as reported in Refs. [3, 4]. In brief: 0.5 μL urine was added with 250 μL β-glucuronidase or sulfatase (4 mg mL^{-1} in 1 mol L^{-1} ammonium acetate buffer, pH=5), and incubated at 37 °C for 4 h. For comparison, 125 μL HCl (12 mol L^{-1}) was added to the enzymatically hydrolyzed samples before extraction.

Extraction

For comparison, both LLE and SPE methods were used to extract the hydrolyzed urine samples. For LLE, the samples

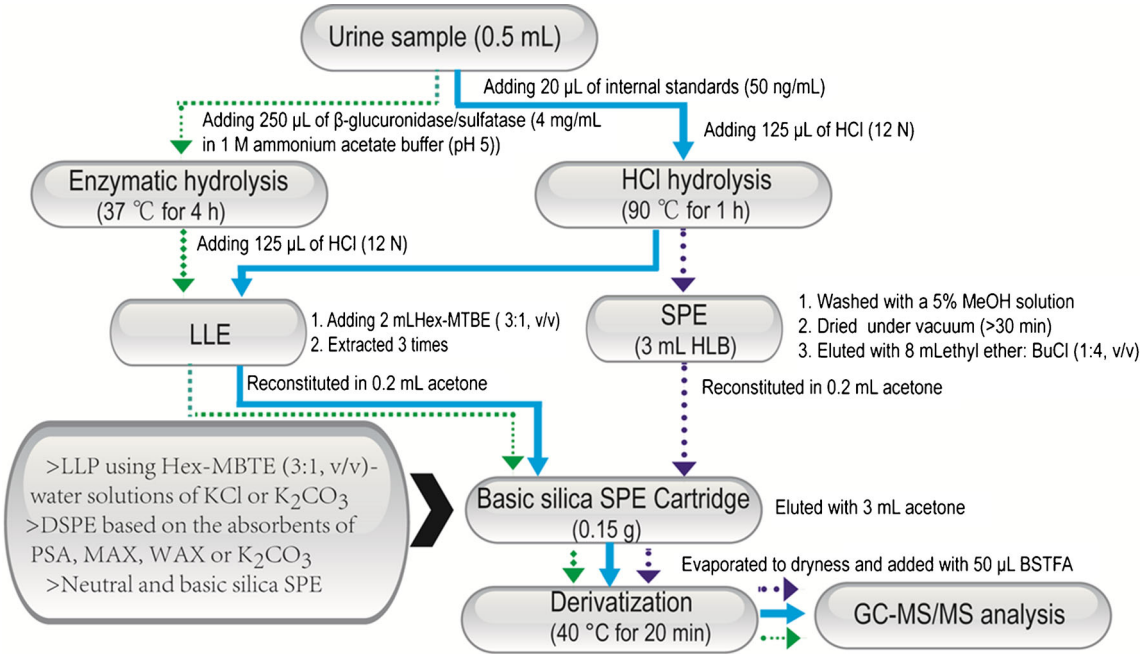

Fig. 1 Flowchart depicting sample preparation and analysis

were extracted three times with 2 mL of a mixture of hexane and methyl *tert*-butyl ether (Hex–MTBE, 3:1, *v*/*v*). The SPE method was as described in Ref. [1] with a slight modification. In brief: the sample (0.5 mL) was applied to a 3 mL Oasis® cartridge and the cartridge was washed with a 5 % MeOH solution and then dried for 40 min under vacuum. After drying, the sample was eluted with 8 mL ethyl ether–BuCl (1:4, *v*/*v*). The eluents or extracts from both extraction methods were concentrated to dryness for cleanup.

Cleanup

Three cleanup techniques were investigated: LLP, dispersed solid-phase extraction (DSPE), and SPE with cartridges filled with neutral and K_2CO_3-treated silica gel (Table S1 of Electronic Supplementary Material, ESM). By examining each method for elimination of matrix interferences and recovery of all analytes, the K_2CO_3-treated-silica-gel SPE cartridge was confirmed as the optimum method. The method details were: the extract (reconstituted with 0.2 mL acetone) was applied in the SPE cartridges and eluted with 3 mL acetone, and the eluent was collected.

Derivatization

The cleaned samples and the calibration standards were evaporated to dryness, and added with 50 μL BSTFA for derivatization at 40 °C for 20 min.

LVI-GC–MS–MS analysis

An Agilent 7890 gas chromatography (GC)–Agilent 7000B mass spectrometry system was used. The GC system was equipped with an Agilent 7693A autosampler and a PTV injector. Mid-column backflushing was used, with a purged ultimate union connecting to two HP-5 ms UI columns (15 m × 0.25 mm i.d. × 0.25 μm, J&W Scientific, Folsom, CA, USA). A fused-silica capillary column was used as a guard column (5 m × 0.25 mm i.d., J&W Scientific, Folsom, CA, US) for the first HP-5 ms UI column. Helium was used as the carrier gas, at a constant flow of 1.1 mL min^{-1} and 1.2 mL min^{-1} for the 1st and 2nd columns, respectively. The PTV was operated in solvent-vent mode with an initial temperature of 70 °C (hold for 0.25 min) and a solvent venting speed of 20 mL min^{-1} (hold for 0.2 min). After the solvent venting, the temperature was ramped to 280 °C at 600 °C min^{-1}, and held for 5.0 min. The column oven-temperature program was set as follows: initial temperature of 60 °C (hold for 2 min), ramped to 250 °C at 10 °C min^{-1}, and finally to 310 °C at 30 °C min^{-1} for sample run, holding at 310 °C for additional 2 min for backflushing. Auxiliary pressure was set at 4 psi during the run and 75 psi during backflushing. The injection volume was 5 μL.

The EI voltage (nominal) was 70 eV. Nitrogen and helium, respectively, were used as collision and quench gas, at a flow of 1.5 mL min^{-1} and 2.25 mL min^{-1}, respectively. Temperatures of the transfer line, ionization source, and quadrupoles were set at 290 °C, 320 °C, and 150 °C, respectively. The solvent delay was set at 6.0 min. The dwell time of MRM transitions was in the range 40–100 ms. The resolution of the first (Q1) and third

(Q3) quadrupole was set at wide peak width. The details of MS–MS conditions are shown in Table 2.

Method validation and qualitative and quantitative analysis

Method detection limits (MDLs) were estimated as 2.625 times (student's $t_{(\alpha = 0.01(\text{one tail}),\ df = 15\text{-}1)}$ value) standard deviations of 15 replicate analyses of a urine extract, in which concentrations of the target analytes were adjusted by spiking standard solutions or diluting with hexane to a concentration where S/Ns of the peaks were in the range 3–5 (the corresponding concentration was in the range 0.9–12 pg mL^{-1}).

Reference urine samples and spiked reference samples were analyzed to determine method accuracy and precision, and were used as quality control (QC) samples in each batch.

Table 2 GC–MS–MS conditions and MDLs (pg mL^{-1}) for all analytes

RT channels (min)	Compound	Quantification ions (CE, eV)	Conformation ions (CE, eV)	Dwell time (ms)	C/Q (%)	RT (min)	MDL (pg g^{-1})
10–11.1	2-IPP	151→136 (17)	166→151 (11)	80	97.7	10.68	4.2
11.1–11.9	25-DCP	219→93 (13)	219→183 (9)	150	17.7	11.29	9.8
	$^{13}C_6$-25-DCP	225→93 (13)	225→189 (9)		18.1	11.28	–
	24-DCP	219→93 (13)	219→183 (5)		37.9	11.52	8.3
	$^{13}C_6$-24-DCP	225→93 (13)	225→189 (5)		37.3	11.52	–
11.9–12.7	CFP	236→205 (13)	179→149 (25)	50	146	12.12	4.4
	TCPY	254→93 (16)	256→95 (23)		28.6	12.57	6.7
	$^{13}C_5$-TCPY	259→93 (16)	261→95 (23)		33	12.56	–
12.7–13.5	246-TCP	253→217 (9)	253→159 (13)	20	40.4	13.08	7.5
	$^{13}C_6$-245-TCP	259→223 (9)	259→165 (13)		50.7	13.07	–
	Methyl paraben	224→209 (6)	224→177 (14)		20	13.13	3.4
	$^{2}D_4$-methyl paraben	228→213 (6)	213→95 (14)		34.4	13.10	–
	NTP	196→135 (19)	211→196 (6)		66	13.28	6.9
	^{2}D4-NTP	200→154 (19)	215→200 (6)		62	13.26	–
	245-TCP	253→93 (13)	268→253 (7)		20	13.40	6.0
	$^{13}C_6$-245-TCP	259→93 (13)	274→259 (7)		17.4	13.40	–
13.5–14.5	Ethyl paraben	223→151 (8)	238→195 (11)	35	62.4	13.98	3.7
	$^{2}D_4$-ethyl paraben	227→155 (8)	242→199 (11)		56	13.97	–
	OPP	242→227 (9)	211→165 (24)		25.6	14.42	3.9
	$^{13}C_6$-*ortho*-phenylphenol	248→233 (9)	217→171 (24)		54.3	14.42	–
14.5–15.5	tOP	278→207 (7)	207→73 (19)	50	3033.7	14.88	4.5
	Propyl paraben	252→210 (5)	252→195 (15)		97	15.12	4.6
	$^{2}D_4$-propyl paraben	256→214 (5)	256→199 (15)		100	15.09	–
15.5–16.9	Butyl paraben	266→195 (16)	210→195 (7)	70	1264	16.24	2.3
	$^{2}D_4$-butyl paraben	270→199 (16)	214→199 (7)		1076	16.21	–
16.9–19	PCP	323→93 (15)	323→288 (9)	35	19	17.37	2.5
	$^{13}C_6$-PCP	329→93 (15)	329→294 (9)		16	17.37	–
	4-*n*-NP	292→179 (10)	179→73 (13)		662	17.81	0.8
	$^{13}C_6$-4-*n*-NP	298→185 (10)	185→73 (13)		680	17.81	–
19–19.6	BP-3	285→242 (21)	285→241 (33)	70	45	19.49	0.7
	$^{13}C_6$-BP-3	291→248 (21)	291→247 (33)		42.1	19.48	–
19.6–20.35	Triclosan	200→185 (16)	360→345 (8)	35	20	19.90	4.9
	$^{13}C_{12}$-triclosan	206→191 (16)	372→357 (8)		11.2	19.90	–
	Benzyl paraben	300→193 (8)	193→73 (19)		1100.7	20.22	1.9
	$^{2}D_4$-benzyl paraben	198→74 (8)	289→91 (19)		104.3	20.20	–
20.35–40	BPA	357→191 (20)	372→357 (8)	70	36.8	20.62	5.1
	$^{13}C_{12}$-BPA	369→197 (20)	384→369 (8)		25.1	20.61	–

C/Q: percentage abundance ratios between confirmation ions and quantitation ions

Urine samples, which were screened with a lower level of phenols (<0.8 ng mL^{-1} for all analytes except methyl paraben (4 ng mL^{-1})) were combined and divided into two aliquots. One aliquot was diluted with water (1:1, *v*/*v*) as a reference sample for methyl paraben, because of its high concentration (4 ng mL^{-1}) in the combined sample. The other aliquot was used as a reference sample for the other 19 analytes. The two reference samples were spiked at two levels (low: 0.1 ng mL^{-1} and 5 ng mL^{-1}; high: 0.5 ng mL^{-1} and 10 ng mL^{-1}); the levels were based on the exposure levels of the general population [7, 17, 27, 28]. Each level was analyzed in five replicates, and validation was completed within five days.

The peaks were identified as target compounds by the following rules: (1) the retention time matched that of the standard compound within±5 %, (2) the response ratio of the quantification ion (Q) to confirmation ion (C) was within± 20 % compared with the standard compounds, and (3) the concentration of the corresponding analyte was above the MDL. The target analytes were quantified using the isotope-dilution method because their isotopic internal standards were available. 1-IPP, CFP and tOP were quantified by $^{13}C_6$-24-DCP, $^{13}C_6$-245-TCP, and D_4-propyl paraben, respectively, because their commercial isotopically labelled internal standards were unavailable.

Data analysis

Statistical analyses and plotting of graphs were performed using SigmaPlot 12.5 (Systat Software, Inc.).

Results and discussion

GC–MS–MS

Large-volume injection (LVI) and backflushing of GC

All relevant variables of LVI were investigated. The optimum initial temperatures of the LVI and GC column oven were 70 °C and 60 °C, respectively. The optimized vent flow and vent time were 20 mL min^{-1} and 0.2 min, respectively. Under the optimum conditions and with a 5 μL injection, no obvious loss was observed during injection for the more volatile analytes (for example 24-DCP and 25-DCP). Mean responses of analytes were 5±0.15 times higher than those in splitless injection mode (1 μL injection). The mid-column backflushing technique along with the guard column would substantially reduce contamination of the column [29]. No chromatographic separation abnormality was observed after analyzing 100 urine samples.

MS–MS transitions

The collision energy (CE) for each analyte was optimized by investigating values in the range 5–40 eV with an increment of 2 eV. The optimum MS–MS settings are summarized in Table 2. For most analytes, the MRM transitions with the highest abundance were used for quantitation and those with the second highest abundance for confirmation (Table 2). However, for four analytes (tOP, butyl paraben, benzyl paraben, and 4-NP) the MRM transitions with the second highest abundance generated much better peak shapes and more accurate quantitation results and were therefore used as quantitation MRM transitions. Analysis of 29 urine samples revealed that most MS–MS transitions selected (Table 2) were specific. One exception was $^{13}C_{12}$-propyl paraben, whose initial transitions (214 › 199 and 199 › 155) had severe interference in the test samples, and thus two alternative transitions (256 › 214 and 256 › 199) were used and the interference was avoided (Electronic Supplementary Material (ESM) Fig. S1). Similarly, the transition of $^{2}D_4$-methyl paraben was changed to 213 › 95 from the initial one of 213 › 89.

Sample preparation

Hydrolysis

HCl was used as a hydrolysis agent for acidic analytes in urine, e.g. pyrethroid insecticide metabolites [26], OPP [30], phenol, and chlorophenols (CPs) [31]. We hypothesized that HCl could be used as a hydrolysis agent for 20 phenolic metabolites in urine. Feasibility of HCl hydrolysis was investigated by comparing its hydrolysis performance with that of the enzymatic method. The combined urine sample was used for hydrolysis tests. The results revealed that the measured values for HCl hydrolysis were comparable to those obtained by the enzymatic method (Fig. 2). No significant difference (Z-statistic (based on positive ranks)=−1.489, p=0.145> 0.05) was observed using the Wilcoxon Signed Rank Test. For TCPY, methyl paraben, and ethyl paraben, HCl hydrolysis had higher efficiency than the enzymatic method (Fig. 2) because of its higher capacity to hydrolyze the coagulations with glycine [32, 33]. The degradation of analytes caused by HCl was not observed when comparing the responses of the isotopically labelled internal standards for both methods. We used HCl hydrolysis for further studies.

Liquid–liquid extraction (LLE) and SPE

Comparison of LLE and SPE LLE and SPE are common extraction methods for the determination of biomarkers in urine [34]. However, there have been no studies comparing the performance of LLE and SPE in extraction of phenols from urine samples. This study investigated the recoveries of

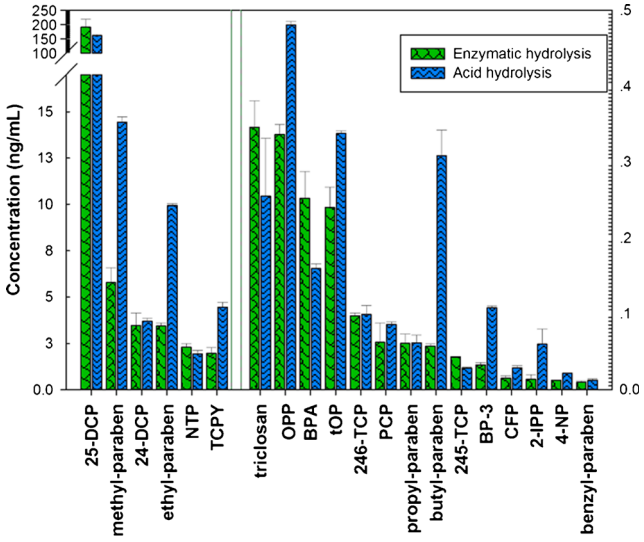

Fig. 2 Comparison of hydrolysis efficiency between enzymatic and HCl hydrolysis using a combined urine sample (n=3)

both methods. For LLE, absolute recoveries of all analytes were 65–121 % with RSDs of less than 11 %. For SPE, absolute recoveries were much lower for 2-IPP (9±13 %, n=2) and CFP (22±1 %, n=2). For other analytes, absolute recoveries were 60–129 % with RSDs of less than 19 %. Our results were in line with those in Ref. [8], revealing that SPE could not recover all target analytes and unsatisfactory recoveries would occur for some analytes [1]. The drying step for the SPE-cartridge method has been reported to be crucial [8], and affects the efficiency of both elution and derivatization. Furthermore, the cartridge drying is a poorly-controlled step in practical laboratory operation. Therefore, the LLE method was used in this study.

No degradation of analytes was observed when HCl was used in LLE at room temperature. Acidification could transform analytes from ionized forms to molecular forms and promote partition of the analytes from the aqueous phase to the organic phase. Therefore, HCl should be added before extraction in LLE for the enzymatic method (the effect of using HCl is described in ESM Fig. S2).

Solvent selection for LLE MTBE was initially used as an extraction solvent in this study, and the results revealed that more interferences were co-extracted. A nonpolar solvent (Hex) was thus mixed with MTBE in four ratios (1:1, 3:1, 5:1, and 7:1, *v*/*v*) to investigate LLE efficiency for a urine sample. The results revealed that the relative response of each analyte increased with the increasing proportion of MTBE, with the exception of 2-IPP (Fig. 3). The ratios 1:1 and 3:1 obtained much higher responses (Fig. 3). For the ratios 1:1 and 3:1, no statistical difference was observed in extraction efficiency for most analytes with the exceptions of TCPY, NTP, and PCP, which had 25 %, 11 %, and 28 % higher relative responses, respectively, in the 1:1 mixture (Fig. 3). Additionally, we found that the more-polar interferences were co-extracted from urine when using the solvent mixture with a ratio of 1:1. The mixture of Hex–MTBE in a ratio of 3:1 (*v*/*v*) was therefore used for LLE.

Sample cleanup

Identification of matrix interference After acidification, the organics in urine exist in protonated or acid forms and therefore can easily be co-extracted with the target analytes. These compounds might be present at concentrations several orders

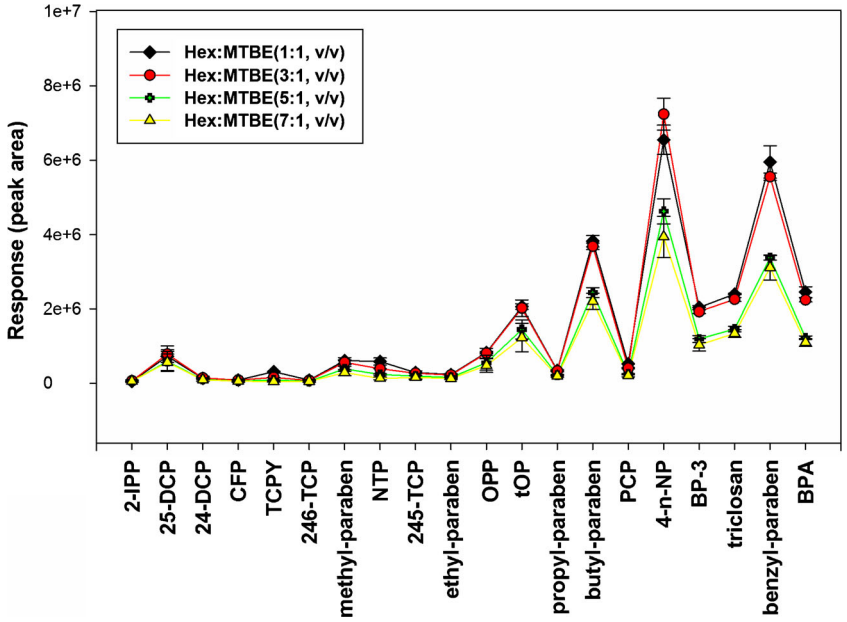

Fig. 3 Responses of each analyte using different ratios of hex and MTBE (1:1, 3:1, 5:1, and 7:1, *v*/*v*), relative to those using a ratio of 7:1 (*v*/*v*)

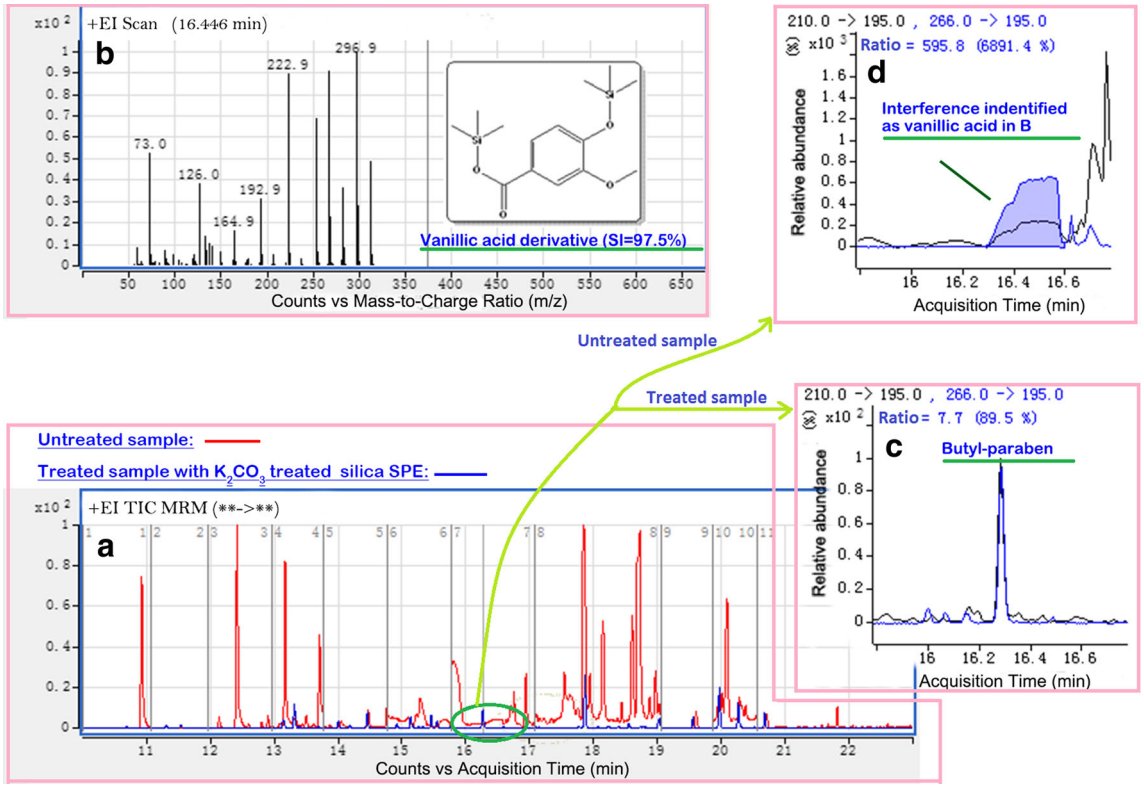

Fig. 4 Butyl paraben (**c**) and its interference ((**d**) and identified as vanillic acid in (**b**)) in a typical sample with (*blue peak* in (**a**)) and without (*red peak* in (**a**)) K_2CO_3-treated-silica-gel SPE

of magnitude higher than the target analytes, resulting in failures of identification and quantification of one or several target analytes. Among these organic-acid compounds, vanillic acid (metabolite of tyrosine and catecholamine in urine) was identified as a dominant interference (Figs. 4a,b). Vanillic acid has a similar chemical structure to butyl paraben. Co-elution of vanillic acid with butyl paraben in GC (Figs. 4a–c) made it impossible to identify and quantify butyl paraben (Fig. 4d). Therefore, elimination of the interference from vanillic acid was a critical sample-preparation step.

Selection and design of cleanup methods Vanillic acid is more polar and more soluble in water than the analytes. Adsorbents (for example PSA, MAX, and WAX) with amine

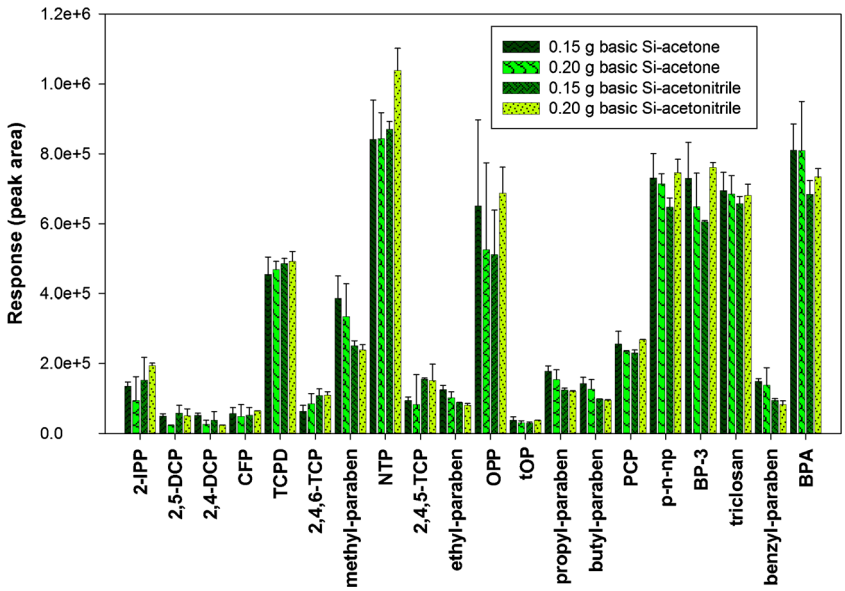

Fig. 5 Comparison of cleanup efficiency (expressed in peak responses) of each analyte in a urine sample spiked at 2 ng mL^{-1} using K_2CO_3-treated-silica-gel cartridges (0.15 and 0.2 g adsorbents; 3 mL acetone and acetonitrile)

functional groups and inorganic base (for example K_2CO_3) have a strong interaction with acidic functional groups of urinary content and were chosen for the sample cleanup. This study designed and tested a series of cleanup methods, including LLP by Hex–MTBE (3:1, *v*/*v*)–water solutions of KCl or K_2CO_3, DSPE with the adsorbents of PSA, MAX, WAX, or K_2CO_3 powder, and neutral-silica-gel SPE cartridges (ESM Table S1). The results (ESM Table S1 and Figs. S3–S6) revealed that neutral-silica-gel SPE and K_2CO_3 DSPE generated satisfactory recoveries for most analytes, and excellent results for the removal of vanillic acid (ESM Table S1). We combined the advantages of both methods and made K_2CO_3-treated-silica-gel SPE cartridges for the sample cleanup. When testing such new SPE cartridges (200 mg each), satisfactory recoveries (81–115 % with RSDs of less than 7 %) were obtained for all analytes, and the interference of vanillic acid was eliminated in a retention-time range of 16.3–16.7 min (Fig. 4c). GC–MS–MS had much lower background (Fig. 4a). A K_2CO_3-treated-silica-gel SPE cartridge was therefore used to treat urine samples.

Optimization of K_2CO_3-treated-silica-gel SPE The amount of silica gel (0.15 g and 0.2 g) and the use of 3 mL of two elution solvents (acetone and acetonitrile) were investigated, and no significant difference was observed in GC–MS–MS responses under four test conditions (Fig. 5). However, acetone was more easily evaporated for sample concentration, and was therefore selected as the elution solvent. An SPE cartridge filled with 0.15 g K_2CO_3-treated silica gel obtained slightly higher GC–MS–MS responses for 17 target analytes (accounting for 85 % of all analytes, Fig. 5). Therefore, a 0.15 g SPE cartridge and 3 mL acetone were used in this study.

Method validation

Linearity was established in the range 0.1–100 ng mL^{-1} for all target analytes with R^2>0.996. MDLs were in the range 1–11 pg mL^{-1} (Table 2). As a result of the effective cleanup, LVI, and the specificity of tandem MS, this method required only a 0.5 mL urine sample. As far as we are aware, 0.5 mL is lower

Table 3 Concentrations and detection rates for each analyte in a pilot study, and comparison with those reported by US CDC [28, 35]

Analytes	This study (0.3–4.1 years, *n*=11) (ng mL^{-1})			US CDC (6–11 years) (ng mL^{-1})		This study (adults, *n*=18) (ng mL^{-1})			US CDC (≥20 years) (ng mL^{-1})		Detection (%)
	Mean±SD	50th percentile	95th percentile	50th percentile	95th percentile	Mean±SD	50th percentile	95th percentile	50th percentile	95th percentile	
2-IPP[a]	0.01±0.02	0	0.04	<0.4	<0.4	0.19±0.25	0	0.59	<0.4	<0.4	21
25-DCP[c]	26.4±25.7	16.5	73.4	6.5	464	22.9±2.4	11.2	79.7	6.6	452	100
24-DCP[c]	2.46±2.13	2.02	5.8	0.9	11.4	0.69±0.46	0.57	1.56	0.8	13.2	100
CFP[b]	2.71±8.26	0.19	14	<0.4	<0.4	0.88±2.77	0.17	2.32	<0.4	<0.4	90
TCPY[a]	2.84±2.23	2.37	6.59	3.09	15.3	24.5±51.4	5.63	81.4	1.91	10.9	100
246-TCP[c]	0.64±0.52	0.59	1.46	<0.5	1.6	1.27±1.04	0.83	3.13	<0.5	1.1	100
Methyl paraben[c]	161±105	127	338	30.1	651	185±133	146	364	64	916	100
NTP[a]	5.44±2.92	5.16	9.49	0.79	4.1	3.63±2.9	2.59	10.3	<0.1	3.72	100
245-TCP[c]	0.05±0.08	0	0.19	<0.1	0.3	nd	nd	nd	<0.1	0.3	3
Ethyl paraben[c]	22.1±23.7	11.2	61.3	<1.0	35.6	137±120	88	336	1.1	77.4	100
OPP[c]	4.93±13.6	0.45	23.4	<0.1	0.6	2.76±5.82	0.59	10.1	<0.1	0.3	100
tOP[c]	0.4±0.2	0.41	0.64	<0.2	0.3	0.38±0.17	0.36	0.7	<0.2	0.3	93
Propyl paraben[c]	11.6±25.5	0.68	58.9	3.5	99.6	23.4±57.2	2.73	88.8	8.4	272	93
Butyl paraben[c]	6.36±16	0.46	30.7	<0.2	3.8	2.13±3.66	1.1	7.23	<0.2	24.6	86
PCP[b]	0.13±0.13	0.09	0.35	<0.5	5.67	0.18±0.22	0.1	0.59	<0.5	3.4	100
4-NP	0.01±0.02	0	0.04	NA	NA	0.04±0.01	0.04	0.05	NA	NA	69
BP-3[c]	3.74±8.01	0.38	15.7	17.2	1410	5.82±19.2	0.76	15.8	13.7	801	100
Triclosan[c]	7.9±8.93	2.54	22.4	9.8	296	2.63±4.07	1.09	7.94	12.3	504	100
Benzyl paraben	0.06±0.03	0.04	0.11	NA	NA	0.13±0.04	0.12	0.2	NA	NA	97
BPA[c]	0.33±0.37	0.18	1.05	2.4	13.4	0.76±0.6	0.61	1.87	1.8	10.7	93

nd: <MDL; NA: unavailable concentration

[a] Concentration reported by US CDC in 2001–2002

[b] Concentration reported by US CDC in 2003–2003

[c] Concentration reported by US CDC in 2007–2008

than the volumes used in all reported multi-analyte methods (1–4 mL) except US CDC's online SPE-LC–MS–MS method (0.1 mL). The method sensitivity (expressed as MDL or LOD) was higher than those of the reported methods [1, 3–7, 9, 27]. The MDLs were low enough for the measurement of the studied urinary phenols in the general population.

The results (ESM Table S2) revealed excellent accuracy, precision, and repeatability. The intraday (n=5) recoveries were 70–126 % with RSDs of 1–19 %, and interday (n= 5 days) relative recoveries were in the range 73–107 % with RSDs of 2–17 %. One exception was that 2-IPP had 62 % recovery on the 5th day; a possible reason is that its isotopically labelled internal standard was not available. Absolute recoveries were in the range 61–138 % with RSDs of less than 29 % for all isotopically labelled internal standards.

Application to urine samples

The developed method was used in a pilot study. The results are summarized in Table 3. More than 85 % of the target analytes were detected in all 29 samples, except for 2-IPP (21 %), 245-TCP (3 %), and 4-NP (69 %). No obvious overall concentration difference was observed between children and adults, although concentrations of some individual analytes differed (Table 3). To date, information regarding the exposure levels of these compounds has been limited in China. For children, the geometric-mean concentrations of this study (Shanghai) were 0.02, 3.28, and 0.22 ng mL^{-1} for 4-NP, TCS, and BPA, respectively, which were much lower than the levels reported in Guangzhou [27]. For adults, 50th-percentile concentrations were comparable with those in Nanjing for all analytes, but concentrations in the 95th percentile for PCP, BP-3, and triclosan [7] were much lower. Compared with the data from US CDC studies [28, 35], we found higher levels of CFP, NTP, ethyl and butyl paraben, and OPP, and lower levels of PCP, propyl paraben, BP-3, triclosan, and BPA (Table 4). The difference might be attributable to different lifestyles and exposure risks in the two countries [7].

Conclusion

This study developed a method for determination of multiple phenolic metabolites in human urine. HCl hydrolysis achieved the same efficiency as enzymatic hydrolysis, but with reduced hydrolysis time and cost. LLE obtained better recoveries than SPE. Vanillic acid in human urine was identified as the main interference for identification and quantification of butyl paraben. K_2CO_3-treated-silica-gel SPE could effectively remove vanillic acid and generate satisfactory recoveries for all analytes. The method also offers higher sensitivity as a result of the effective sample pretreatment, LVI, and the specific detection of tandem MS. Because isotopically labeled internal standards were used for most target analytes, satisfactory intra and interday accuracy, precision, reproducibility, and robustness were obtained. The method only required a 0.5 mL urine sample. As a result of the use of the guard GC column and mid-column backflushing, the instrument maintenance frequency was reduced. The method was used in a pilot study with more than 85 % detection for most analytes. Compared with reported methods, this method can simultaneously determine a broader range of phenolic biomarkers, and enables assessment of human exposure to more than 30 prominent xenobiotics that have endocrine activity. One analyst can treat more than 40 samples per day in our laboratory. However, use of automated technology for sample preparation is being considered.

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