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Development of a UPLC-ESI-MS/MS method to measure urinary metabolites of selected VOCs: benzene, cyanide, furfural, furfuryl alcohol, 5-hydroxymethylfurfural, and *N*-methyl-2-pyrrolidone

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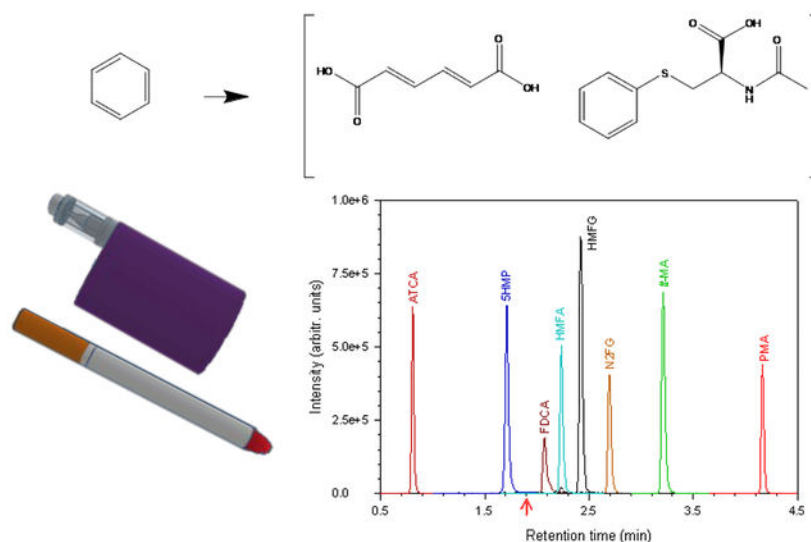
Abstract

We report on the development of an ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method for simultaneously measuring eight biomarkers of volatile organic compound (VOC) exposure, with potential application to e-cigarette aerosol biomonitoring. Phenylmercapturic acid (PMA) and *trans, trans*-muconic acid (*tt*-MA) are metabolites of benzene; 2-aminothiazoline-4-carboxylic acid (ATCA) is a metabolite of cyanide; *N*-2-furoylglycine (N2FG) is a metabolite of furfural and furfuryl alcohol; 5-hydroxymethylfuroic acid (HMFA), 5-hydroxymethyl-2-furoylglycine (HMFG), and 2,5-furandicarboxylic acid (FDCA) are metabolites of 5-hydroxymethylfurfural; and 5-hydroxy-*N*-methylpyrrolidone (5HMP) is a metabolite of *N*-methyl-2-pyrrolidone. A pentafluorophenyl-modified silica column was used for chromatographic separation. The overall run time for the method is about 6 min per sample injection. The method has low to sub-nanograms per milliliter sensitivity, linearity over 3 orders of magnitude, and precision and accuracy within 15%. The method was used to measure human urine samples. Results showed that people with known benzene exposure (daily cigarette smokers) had higher levels of *tt*-MA and PMA compared with non-smokers. The method is advantageous for high-throughput analysis of selected VOC metabolites in large-scale, population-based studies such as the National Health and Nutrition Examination Survey (NHANES). Quantifying these urinary biomarkers is important to public health efforts to understand human exposure to VOCs from various sources, including tobacco products and electronic nicotine delivery systems.

Graphical Abstract

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Keywords

Benzene; Furfural; Furfuryl alcohol; 5-Hydroxymethylfurfural; *N*-Methylpyrrolidone; Cyanide; VOC metabolites

1. Introduction

Volatile organic compounds (VOCs) are ubiquitous in the environment. Chronic exposure to certain VOCs increases the risks for cancer, Parkinson's disease, adverse reproductive outcomes, DNA mutations, respiratory irritation, cardiovascular toxicity, and developmental abnormalities [1–4]. Exposure to VOCs can come from occupational, residential, and environmental sources, including combusted tobacco products, e-cigarettes, and food contaminants [5–7]. Urinary VOC metabolites can be biomonitored to non-invasively assess parent VOC exposure. This work describes the development of a quantitative method for simultaneously measuring 8 metabolites of select parent VOCs: benzene, cyanide, furfural, furfuryl alcohol, 5-hydroxymethylfurfural (5HMF), and *N*-methyl-2-pyrrolidone (NMP).

Benzene is classified as a Group 1 carcinogen (known human carcinogen) by the International Agency for Research on Cancer [8]. Numerous studies have shown that benzene exposure can cause leukemia and other hematotoxic effects [7, 9, 10]. The most significant sources of benzene exposure to the general population are active and passive smoking and automobile use [9]. Occupational exposures to benzene from widespread industrial applications are prevalent, including use in the production of plastics, resins, synthetic fibers, dyes, detergents, drugs, and pesticides [11]. Additionally, benzene is present in crude oil and gasoline [7]. The metabolic pathway of benzene begins with catalytic oxidation aided by cytochrome P450 (CYP) 2E1. The benzene oxide intermediate then undergoes three different metabolic pathways leading to varying urinary excretion of the retained dose: 3.9% to *trans,trans*-muconic acid (*tt*-MA), 0.11% to *S*-phenyl mercapturic acid (PMA), and 40% to phenol and its conjugates [10–12]. PMA and *tt*-MA are the most commonly used urinary biomarkers of benzene exposure [10, 12].

Hydrogen cyanide and cyanide salts are well-known toxins. Exposure to cyanide can cause loss of consciousness, cardiac arrhythmias, and neurophysiological changes [13]. The acute neurotoxic effects of cyanide exposure include altered respiration, nausea, and vomiting. These result from cellular hypoxia, which can lead to convulsions, coma, and death [13, 14]. One of the major sources of exposure to cyanides is cigarette smoke [3]. Other sources include biomass burning, volcanic eruptions, industrial processing, and natural biogenic processes from plants, bacteria, and fungi [3, 15]. Rhodanese enzymatically converts 60–80% of the cyanide dose to thiocyanate. The other metabolic pathway involves cysteine, which converts approximately 15% of the dose to 2-aminothiazoline-4-carboxylic acid (ATCA) and its tautomer, 2-iminothiazolidine-4-carboxylic acid (ITCA). ATCA is frequently used as a stable cyanide exposure biomarker [16, 17].

Furfural and furfuryl alcohol are reported to cause eye and mucous membrane irritation, abdominal pain, diarrhea, headache, and vomiting [18–22]. They are widely used for various industrial applications. They are neo-formed contaminants (i.e. intermediate in the Maillard reaction) produced during thermal decomposition of carbohydrate-rich food [19, 23]. Potential sources of their exposure include resins, adhesives, fungicides, inks, coffee, tobacco smoke, and e-cigarette aerosol [21, 24]. Furfural and furfuryl alcohol undergo reversible reactions in the gut of exposed humans. These include oxidation of furfuryl alcohol to furfural and vice-versa, followed by rapid oxidation to furoic acid. They are then primarily excreted as glycine conjugates (i.e. *N*-2-furoylglycine [N2FG]), with other minor metabolites, including furoic acid [19, 20, 25, 26].

5-hydroxymethylfurfural (5HMF) causes eye, upper respiratory tract, and mucous membrane irritation [4, 27, 28]. It is also a potential carcinogen [4, 29]. 5HMF is a neo-formed contaminant produced along with furfural and furfuryl alcohol during heating or cooking of carbohydrate-containing food. Potential sources of exposure to 5HMF include cooked food, food flavoring agents, dried fruits, coffee, caramel products, and e-cigarette aerosol [4, 23, 28]. 5HMF is believed to follow the same metabolic pathway as furfural metabolism. In animal models, 78–85% of the administered dose gets converted into 5-hydroxymethyl-2-furanoic acid (HMFA), 5–8% into 5-hydroxymethyl-2-furoylglycine (HMFG), and 2–6% into 2,5-furandicarboxylic acid (FDCA) [4, 28].

N-Methyl-2-pyrrolidone (NMP) can cause severe skin, eye, and respiratory tract irritation [30]. It is primarily used as an organic solvent for various industrial applications [31, 32]. It is a constituent of paint stripper and graffiti remover and is used as a polymer-resist stripping solvent in microelectronics fabrication. NMP is used as a formulating agent in pigments, dyes, and agricultural products such as insecticide, fungicides, and herbicides. It is also a pyrolysis product in tobacco smoke, and an additive in some tobacco products [33, 34]. Exposure to NMP can occur through inhalation, ingestion, and skin contact during its manufacturing, handling, and application. The major urinary metabolite of NMP is 5-hydroxy-*N*-methyl-2-pyrrolidone (5HMP), with the largest fraction (44%) excreted in urine [35]. Other metabolites include *N*-methylsuccinimide and 2-hydroxy-*N*-methylsuccinimide [31, 35].

Previously, several standalone analytical methods were developed to measure urinary biomarkers of exposure to the aforementioned VOCs [36–38]. Most of the established assays focused on measuring the metabolites of these VOCs and other ubiquitous VOCs [3, 6, 14, 36, 39, 40]. In our laboratory, we currently measure 28 VOC metabolites of exposure to 20 parent VOCs using a validated analytical assay [6]. The method comprises metabolites of exposure to combusted tobacco VOCs, including acrolein, acrylamide, acrylonitrile, 1,3-butadiene, and crotonaldehyde, along with non-tobacco-related VOCs. However, rapid increases in e-cigarette use among young adults and the presence of neo-formed contaminants in vaping aerosol [5, 23, 41] have shifted the focus of the biological monitoring beyond combustible VOCs. Quantitating exposures to furfural, furfuryl alcohol, 5HMP, and other harmful and potentially harmful constituents from electronic nicotine delivery systems is of significant interest to public health research. Therefore, we developed an ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method for the simultaneous measurement of 8 urinary biomarkers of exposure to benzene, cyanide, furfural, furfuryl alcohol, 5HMF, and NMP to characterize human exposure to these select VOCs. The method expands on the ability of our laboratory and others for the rapid and high-throughput analysis of VOC metabolites associated with environmental and dietary exposure to the corresponding parent VOCs.

2. Experimental

2.1. Materials.

HPLC-grade J.T. Baker water, LCMS Optima-grade ammonium formate, LCMS Optima-grade formic acid, and LCMS Optima-grade methanol were purchased from Fisher Scientific (Suwanee, GA). Analytical standard-grade FDCA was purchased from Sigma-Aldrich (St. Louis, MO). Analytical standard-grade HMFA was purchased from Santa Cruz Biotechnology (Dallas, TX). Analytical standard-grade ATCA, 5HMP, HMFG, N2FG, *tt*-MA, and PMA were purchased from Toronto Research Chemicals (Ontario, Canada). ATCA- ^{13}C , $^{15}\text{N}_2$], HMFG- $^{13}\text{C}_2$, ^{15}N], 5HMP- $^2\text{H}_3$], FDCA- $^{13}\text{C}_6$], N2FG- $^2\text{H}_3$], and HMFA- $^{13}\text{C}_6$] were purchased from Toronto Research Chemicals (Ontario, Canada). *tt*-MA- $^{13}\text{C}_6$] was purchased from Sigma-Aldrich (St. Louis, MO). PMA- $^{13}\text{C}_6$] was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). Mass Spect Gold Urine was purchased from Golden West Diagnostics (Temecula, CA). ClinChek urine controls for occupational medicines, levels I/II (lyophilized), were purchased from IRIS Technologies International GmbH (Olathe, KS). A human urine pool was collected anonymously following a protocol approved by the CDC Institutional Review Board.

2.2. Calibration Solutions.

Individual master stocks of neat standards and internal standards were prepared in methanol. Master stocks were diluted in water to make working stocks. All calibration solutions were prepared by diluting the working stock solution in 5 mM ammonium formate buffer (pH 2.9). The buffer is prepared by mixing 50 mL of 200 mM ammonium formate solution, 3 mL of formic acid, and 1.947 L of water. A 200 mM ammonium formate solution is prepared by adding 3.15 g of neat ammonium formate to 250 mL HPLC grade water. All stock solutions were stored at $-70\text{ }^\circ\text{C}$ before use.

2.3. Sample Preparation.

A dilute-and-shoot method was selected to minimize sample preparation time. Urine samples were thawed, homogenized with a rugged rotator, and diluted 1:10 with 5 mM ammonium formate buffer before analysis.

2.4. UPLC-ESI-MS/MS Analysis.

The analytical run was performed using an Acquity I-Class UPLC system (Waters Corporation, Milford, MA) equipped with a Waters high-strength silica (HSS)-pentafluorophenyl (PFP) column (2.1×100 mm, $1.8 \mu\text{m}$) and a Waters HSS-PFP VanGuard pre-column (Waters Corporation, Milford, MA). The UPLC system was coupled to a 5500 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Sciex, Framingham, MA). Chemical separation was performed using a solvent gradient of 5 mM ammonium formate buffer at pH 2.9 (mobile phase *A*) and methanol (mobile phase *B*) (Table 1). Column and sample manager temperatures were set to 30°C and 25°C , respectively. The injection volume was $2 \mu\text{L}$ using full loop injection mode. The mass spectrometer was operated in positive ion ESI and negative ion ESI scheduled multiple reaction monitoring modes using the polarity-switching feature. For each injection, data were collected in positive ion mode for the first 1.9 min before switching to negative ion mode for the remainder of the analysis time. The optimized ion source parameters were as follows: ESI voltage, 3.5 kV (positive mode) and -4.5 kV (negative mode); collisionally activated dissociation (CAD) gas, 7 psi; curtain gas flow, 20 psi; nebulizing gas (GS1) flow, 55 psi; heating gas (GS2) flow, 65 psi; and heater temperature, 650°C . Fig. 1 shows the chemical structures of the urinary metabolites of interest.

2.5. Data Analysis.

All UPLC-MS/MS data were generated in Analyst 1.6.2 (Sciex, Framingham, MA) and processed in MultiQuant 3.0.3 (Sciex, Framingham, MA). Statistical analysis was performed with JMP 13 statistical software (SAS Institute, Cary, NC).

3. Results and Discussion

3.1. Method Development.

The compound-specific parameters (Table 2) for the triple quadrupole mass spectrometer were optimized by direct infusion of $0.1 \mu\text{g/mL}$ neat compound in methanol/water (50/50%, v/v) at a flow rate of $10 \mu\text{L/min}$. The chromatographic separation was performed using a PFP-modified HSS column with reversed-phase gradient elution (Table 1). We chose the HSS-PFP column for the unique selectivity it provides for our compounds of interest. It offers various physiochemical interactions in addition to hydrophobic interactions, such as π - π , electrostatic, shape selectivity, and hydrogen bonding [42]. The buffer pH (mobile phase *A*) was optimized at pH 2.9 to maintain consistent reversed-phase type separation. This pH is especially important for analytes such as *tt*-MA, a dicarboxylic acid, which is doubly negatively charged at neutral pH. With a $\text{p}K_{\text{a}1}$ and $\text{p}K_{\text{a}2}$ of 3.87 and 4.65, respectively [43], and a drastic polarity difference, this dicarboxylic acid elutes closer to the void volume at pHs higher than 5 when performing a reversed-phase separation. This buffer

pH was still optimal for all other analytes because their $\log P$ and $\log D$ values were comparable at pH 2.9 (Table 2). Converting these analytes to a neutral form did not affect their retention times, except diminished buffering capacity (e.g., below pH 2.7). For these reasons, pH 2.9 was optimal, and as such, analytes with the lower $\log D$ eluted first. As the percentage composition of methanol (mobile phase *B*) increased, the analytes with higher $\log D$ eluted sequentially, with a few exceptions (Fig. 2, Table 3). For example, N2FG and HMFG eluted later than expected, based on $\log D$, but both compounds contain an aliphatic amide. This suggests that amide interactions might be preferentially involved in secondary retention mechanisms. The metabolites ATCA and 5HMP contain heterocycles or amine moieties, but followed the expected $\log D$ retention relationship.

Methanol (mobile phase *B*) is an important contributor to the column selectivity. Acetonitrile disrupts π - π interactions between solvated analytes and the PFP-modified surface by way of its carbon-nitrogen triple bond. Methanol resolved analytes more effectively because it does not inhibit such interactions [44].

3.2. Quantitative Analysis.

Quantitative studies were performed using calibration curves with stable isotope-labelled internal standards. The concentration of calibration curves spanned more than 3 orders of magnitude with the coefficient of determination (r^2) exceeding 0.99 when fitted by linear regression on $1/x$ weighted data. Because the working stock calibrators were prepared in water, a matrix validation experiment was performed to investigate the use of non-urine-based calibrators. The slopes of two calibration curves, one made in water and one in pooled human urine, were compared for each analyte. The experiments were repeated 3 times. Table 4 shows the average percentage difference in the slopes for each analyte. The results were within 4% difference, which verified the use of non-urine-based calibrators for this assay. It is noteworthy that at least 10 times sample dilution was required to be able to use non-urine based calibrator.

Table 5 shows the method limits of detection (LODs) and the range of linearity in non-urine matrix. The LODs were calculated based as $3S_0$, where S_0 is the standard deviation at zero concentration from a standard deviation versus concentration plot [45]. The calculated LODs were in the low to sub-nanograms per milliliter range, with the lowest value (0.097 ng/mL) for PMA and the highest value (16.4 ng/mL) for FDCA. Because the injection volume was 2 μ L, the on-column injection detection limit was between 0.19 pg/mL for PMA and 33 pg/mL for FDCA. The carryover effect was studied using the highest calibrator. Following a highest calibrator, no detectable peak was observed for any target analytes on the next injection of a blank solvent.

Previous studies of humans who had no occupational benzene exposure have shown that the mean or median concentration of urinary *tt*-MA ranged from 30 to 300 μ g/g creatinine and that *tt*-MA was successfully quantified using methods with LOD 5 ng/mL [10, 46]. Similarly, the central tendency of urinary PMA concentrations in non-occupationally exposed people ranged from 0.3 to 8.9 μ g/g creatinine, and PMA was quantified using methods with LOD 0.2 ng/mL [10]. The LODs calculated in this method are lower than those reported values (Table 5). Our method has sufficient sensitivity for the proper

assessment of biomarkers of environmental and occupational exposures to benzene. For the remaining metabolites, the method LODs or the average baseline concentration of the non-exposed urine samples were higher than the LODs calculated in our method [3, 4, 27, 39].

Accuracy was checked through spiking on two different urine specimens: 1) Mass Spect Gold Urine containing low endogenous levels of analytes and 2) a urine pool with higher endogenous levels of analytes. Each specimen was spiked with standard mix to make 3 samples of varied concentration. Samples were run in triplicate and repeated for 2 days. The results show that accuracy through spiking and the relative standard deviation (RSD) were within 15% (Table 6). Accuracy relative to the reference materials was determined when applicable. The ClinChek control reference materials containing benzene metabolites *tt*-MA and PMA were repeated for 5 days. The results were within 10.1% difference with respect to the nominal value provided by the vendors (Table 7). No other target analytes were available in the ClinChek controls. The interday precision of the method also was evaluated in spiked urine pools at two different concentrations for 10 days over 4 weeks. The percentage RSD varied between 2.2% for ATCA and 12.5% for PMA.

3.3. Stability.

The sample stability of spiked urine pools at two different concentrations and prepared in triplicate were examined to determine optimal handling and storage conditions. Bench-top stability was assessed for the length of time needed to handle study samples at room temperature (i.e. 4 h). The calculated percent error for the bench-top stability ranged from -0.8% (ATCA) to 11.4% (HMFG), with reference to the control (i.e. $t = 0$ h). Similarly, processed sample stability was evaluated on prepared samples stored in the autosampler at room temperature for 24 h. The percentage error for the processed sample stability compared with controls prepared the same day was within 10%.

Freeze-thaw stability was assessed for 3 freeze-thaw cycles. Samples were stored at -70°C for at least 24 h between each cycle and then thawed for sample preparation. The percentage error ranged from -0.3% (5HMP) to 11.4% (HMFA).

3.4. Application to Human Specimens.

We measured urinary biomarkers of exposure to benzene in urine collected from 25 daily smokers and 25 non-smokers (Tennessee Blood Services). Cigarette smoke is known to contain benzene; smoking a pack of cigarettes leads to inhalation of low milligram quantities of benzene [47]. In this study, smokers and non-smokers were categorized based on the self-reported data provided by the vendor. The total detection frequencies for PMA and *tt*-MA were 70% and 100%, respectively. The calculated data below LOD were imputed as $\text{LOD}/(2)$ for the descriptive statistics [48]. The results were then logarithmically transformed for the Student's *t*-test at 95% confidence interval ($\alpha = 0.05$). Smokers had significantly higher urinary PMA and *tt*-MA compared with non-smokers. Table 8 summarizes the urinary geometric mean concentration and standard error, along with *p*-values for smokers and non-smokers samples. Our results are consistent with previously published reports of statistically higher urinary concentrations of PMA and *tt*-MA in smokers compared with non-smokers [10].

3.5. Sample Throughput.

This method is based on the dilute-and-shoot approach and requires minimal sample preparation. Including column equilibration and autosampler movement, the total sample analysis time per injection is 6 min. Therefore, the instrument time required to analyze 96 samples is 9.6 h. In addition, the sample preparation time per 96-well plate using an automated liquid handling system is about 1 h. In its current stage, the method can generate 768 analytical results in less than 12 h.

4. Conclusions

We developed a novel UPLC-ESI-MS/MS method to measure urinary metabolites of exposure to selected VOCs. This assay includes the assessment of urinary metabolites of known carcinogens, such as benzene, and other neo-formed toxicants of emerging interests. The method monitors parent compounds found in mainstream cigarette smoke (benzene, cyanide, furfuryl alcohol, NMP) and e-cigarette aerosol (furfural, 5HMP). Validation of the method's accuracy, precision, and ruggedness showed it can be used as a rapid, high-throughput assay for nationally representative studies. The method showed that people with known benzene exposure (daily cigarette smokers) had higher levels of PMA and *tt*-MA compared with non-smokers. The method offers short run times via a simple dilute-and-shoot approach, but maintains superior selectivity and lower limits of detection.

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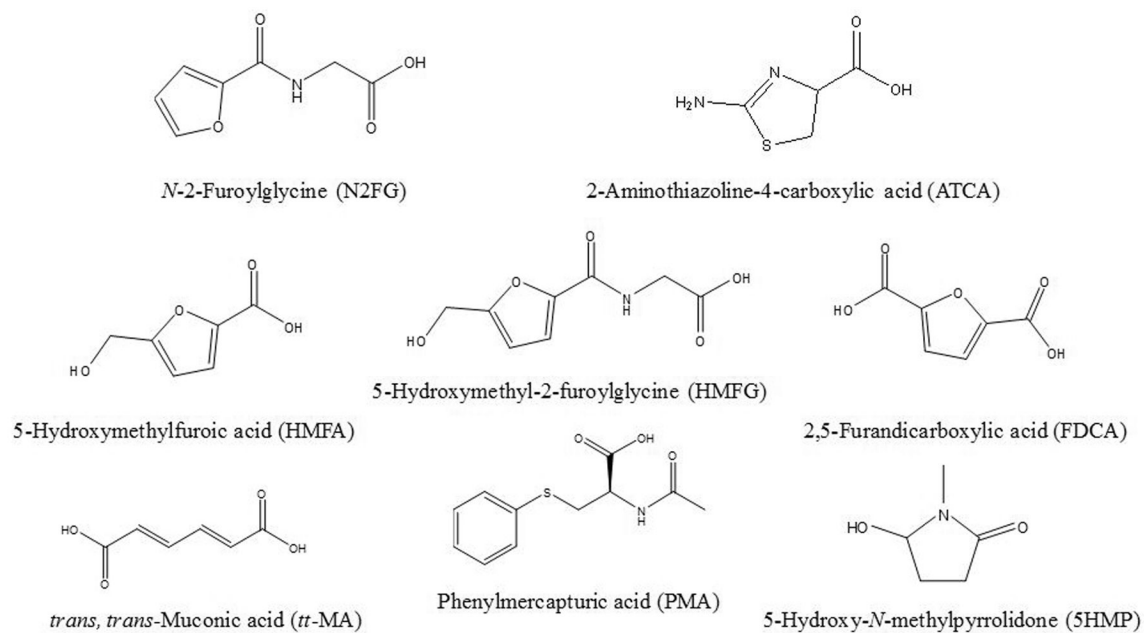


Fig. 1.
Chemical structures of urinary metabolites of interest.

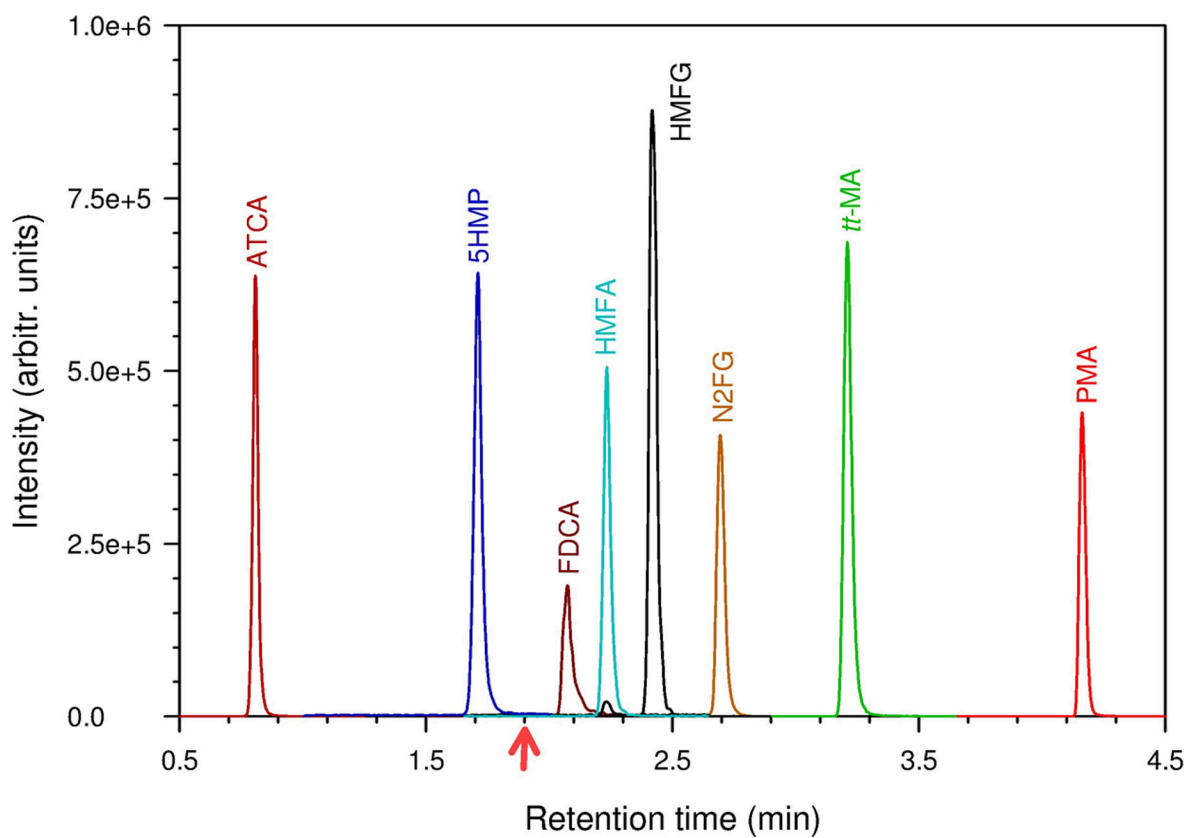


Fig. 2. Extracted-ion chromatograms of the analytes of interest. Data were collected in positive ion electrospray ionization (ESI) mode until 1.9 min and in negative-ion ESI thereafter. Arrow on the bottom represents the time at which the ESI polarity was switched to negative ion mode from positive ion mode.

Table 1.

Gradient elution table for chromatographic separation.

Time (min)	Flow Rate (mL/min)	% Mobile Phase B (v/v)	Curve [†]
Initial	0.4	2.5	Initial
0.6	0.4	2.5	6 (linear)
2.8	0.4	35	6 (linear)
4.5	0.4	80	5 (concave)
5.5	0.4	2.5	1 (step)

[†]For the gradient curve algorithm refer to www.waters.com

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Table 2.

Compound specific mass spectrometry parameters.

Analyte	Ion transition (m/z)		DP	EP	CE	CXP
	Quantitation	Confirmation				
ATCA	147.3→58.9		60		44	12
		147.3→73.9	60	10	38	12
ATCA- $^{13}\text{C}_6$	150.0→103.9		60		22	12
5HMP	116.1→85.2		50		16	10
		116.1→57.0	50	10	30	15
5HMP- $^2\text{H}_3$	119.1→85.2		55		18	12
FDCA	155.1→111.0		-30		-10	-9
		155.1→82.9	-30	-10	-16	-9
FDCA- $^{13}\text{C}_6$	161.1→116.0		-40		-10	-9
HMFA	141.1→97.1		-45		-6	-9
		141.1→69.0	-45	-10	-16	-9
HMFA- $^{13}\text{C}_6$	147.1→102.1		-45		-12	-9
HMFG	198.0→97.0		-45		-18	-8
		198.0→124.0	-45	-10	-17	-8
HMFG- $^{13}\text{C}_2,^{15}\text{N}$	201.0→97.0		-45		-18	-8
N2FG	168.0→66.9		-60		-11	-10
		168.0→124.1	-60	-10	-14	-10
N2FG- $^2\text{H}_3$	171.0→70.0		-60		-19	-10
<i>tt</i> -MA	141.1→97.1		-40		-10	-9
		141.1→53.2	-40	-10	-15	-3
<i>tt</i> -MA- $^{13}\text{C}_6$	147.0→102.0		-40		-10	-9
PMA	238.1→109.1		-50		-22	-9
		239.1→110.1	-50	-10	-21	-5
PMA- $^{13}\text{C}_6$	244.1→115.1		-50		-22	-9

DP = declustering potential, EP = entrance potential, CE = collision energy, CXP= cell exit potential.

Table 3.

Physicochemical properties of analytes of interest [43]. The analytes are listed based on their chromatography elution order (Fig. 2). $\log D$ is a logarithm of the ratio of chemical species in octanol to water at a specific pH. $\log P$ is equivalent to $\log D$ for neutral species.

Analytes	Strongest acidic pK_a	Strongest basic pK_a	$\log P$	$\log D$ at pH=2.9
ATCA	2.28	8.15	-1.75	-1.825
5HMP	13.49	-	-0.95	-0.949
FDCA	2.76	-	0.27	-0.171
HMFA	2.96	-	-0.16	-0.364
HMFG	3.40	-	-1.26	-1.382
N2FG	3.34	-	-0.41	-0.577
<i>tt</i> -MA	3.87	-	0.49	0.412
PMA	3.80	-2.00	1.05	1.002

Table 4.

Comparison of calibration curve slopes for different types of matrices.

Analyte	Slope (N = 3)		Percent Difference
	Water	Pooled Urine	
ATCA	0.0026	0.0027	3.4
5HMP	0.34	0.36	3.2
FDCA	0.017	0.017	0.10
HMFA	0.0040	0.0041	2.1
HMFG	0.0050	0.0049	2.1
N2FG	0.0036	0.0036	0.70
<i>tt</i> -MA	0.016	0.016	0.020
PMA	0.17	0.17	0.29

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Table 5.

Method LODs and linearity range.

Analytes	Method LOD (ng/mL)	Linearity Range (ng/mL)
ATCA	0.835	0.184 – 582
5HMP	0.274	0.0253 – 80.0
FDCA	16.4	9.99 – 3160
HMFA [†]	13.1	2.37 – 7500
HMFG	7.12	1.00 – 3160
N2FG [†]	6.71	3.95 – 12500
<i>tt</i> -MA	1.20	0.759 – 759
PMA	0.0970	0.00950 – 9.49

[†]HMFA and N2FG instrumental parameters (collision energy) were detuned to avoid detector saturation.

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Table 6.

Accuracy through spiking.

Analyte	Sample ID	Spiked Conc. (ng/mL)	Sample 1 (Mass Spect Gold Urine)			Spiked Conc. (ng/mL)	Sample 2 (Urine Pool)		
			Calculated Conc. (ng/mL)	Accuracy (% Error)	RSD (%)		Calculated Conc. (ng/mL)	Accuracy (% Error)	RSD (%)
ATCA	Blank	0	7.04	NA	4.7	0	101.25	NA	3.4
	Blank + Spike 1	29.1	34.7	-3.96	2.2	29.1	126	-2.99	2.2
	Blank + Spike 2	92.0	96.8	-2.26	2.7	92.0	195	0.66	2.4
	Blank + Spike 3	920	99.3	-0.738	2.6	920	997	-2.37	2.8
5HMP	Blank	0	3.02	NA	4.0	0	13.3	NA	4.5
	Blank + Spike 1	12.6	16.6	5.61	2.6	12.7	25.9	-0.184	2.9
	Blank + Spike 2	40.0	43.4	0.771	1.3	40.0	55.6	4.19	1.8
	Blank + Spike 3	400	403	-0.010	2.6	400	403	-2.48	2.0
FDCA	Blank	0	0	NA	0.0	0	858	NA	4.0
	Blank + Spike 1	158	166	4.99	4.8	158	1020	0.110	5.8
	Blank + Spike 2	500	472	-5.59	5.6	500	1410	3.95	3.6
	Blank + Spike 3	5000	5096	1.93	2.9	5000	5720	-2.44	4.2
HMFA	Blank	0	0	NA	0.0	0	1098	NA	8.8
	Blank + Spike 1	158	163	3.32	6.3	1580	2790	3.98	7.0
	Blank + Spike 2	500	513	2.49	9.1	5000	5960	-2.27	0.9
	Blank + Spike 3	5000	5480	9.58	14.7	10000	11330	2.09	7.2
HMFG	Blank	0	0.00	NA	0.0	0	148	NA	8.6
	Blank + Spike 1	158	166	5.24	3.9	1580	1690	-2.27	7.7
	Blank + Spike 2	500	547	9.44	5.2	5000	5170	0.41	6.1
	Blank + Spike 3	5000	4990	-0.166	6.2	10000	10040	-1.05	6.5
N2FG	Blank	0	0	NA	0.0	0	739	NA	6.4
	Blank + Spike 1	158	160	1.02	5.0	1580	2450	5.54	5.4
	Blank + Spike 2	500	528	5.56	1.4	5000	5960	3.78	8.2
	Blank + Spike 3	5000	5330	6.49	7.4	10000	10800	0.450	3.0
<i>tt</i> -MA	Blank	0	0	NA	0.0	0	13.3	NA	7.5

Analyte	Sample ID	Spiked Conc. (ng/mL)	Sample 1 (Mass Spect Gold Urine)			Spiked Conc. (ng/mL)	Sample 2 (Urine Pool)		
			Calculated Conc. (ng/mL)	Accuracy (% Error)	RSD (%)		Calculated Conc. (ng/mL)	Accuracy (% Error)	RSD (%)
PMA	Blank + Spike ₁	38.0	37.5	-1.06	4.0	38.0	46.3	-9.61	3.6
	Blank + Spike ₂	120	114	-5.25	2.8	120	129	-3.33	5.4
	Blank + Spike ₃	1200	1160	-3.13	1.1	1200	1150	-5.53	1.6
	Blank	0	0	NA	0.0	0	0	NA	0.0
	Blank + Spike ₁	0.632	0.576	-9.78	8.4	0.630	0.500	-13.9	11
	Blank + Spike ₂	2.00	1.94	-3.34	3.8	2.00	2.00	-2.19	7.2
	Blank + Spike ₃	20.0	19.6	-2.10	1.6	20.0	19.0	-5.092	0.8

Table 7.Accuracy with respect to ClinChek control reference materials for PMA and *tt*-MA.

Analyte	Nominal Concentration (ng/mL)	Level 1 (N = 10)			Level 2 (N = 10)			
		Calculated Concentration (ng/mL)	Accuracy (% Error)	RSD (%)	Nominal Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (% Error)	RSD (%)
PMA	4.83	5.25	8.8	3.7	44	48.4	10.1	2.7
<i>tt</i> -MA	976	906	-7.2	3.1	2990	2889	-3.4	2.6

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Table 8.

Urinary geometric mean (GM) concentration and standard error (SE) of benzene metabolites in 25 smoker and 25 non-smoker samples.

Analyte	GM [SE] ng/mL		<i>p</i> -value
	Smoker	Non-smoker	
PMA	1.23 [0.737]	0.298 [0.252]	0.0002
<i>tt</i>-MA	144 [92.7]	68.0 [16.0]	0.0171

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