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Simultaneous analysis of 28 urinary VOC metabolites using ultra high performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC-ESI/MSMS)☆

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Abstract

Volatile organic compounds (VOCs) are ubiquitous in the environment, originating from many different natural and anthropogenic sources, including tobacco smoke. Long-term exposure to certain VOCs may increase the risk for cancer, birth defects, and neurocognitive impairment. Therefore, VOC exposure is an area of significant public health concern. Urinary VOC metabolites are useful biomarkers for assessing VOC exposure because of non-invasiveness of sampling and longer physiological half-lives of urinary metabolites compared with VOCs in blood and breath. We developed a method using reversed-phase ultra high performance liquid chromatography (UPLC) coupled with electrospray ionization tandem mass spectrometry (ESI/MSMS) to simultaneously quantify 28 urinary VOC metabolites as biomarkers of exposure. We describe a method that monitors metabolites of acrolein, acrylamide, acrylonitrile, benzene, 1-bromopropane, 1,3-butadiene, carbon-disulfide, crotonaldehyde, cyanide, *N,N*-dimethylformamide, ethylbenzene, ethylene oxide, propylene oxide, styrene, tetrachloroethylene, toluene, trichloroethylene, vinyl chloride and xylene. The method is accurate (mean accuracy for spiked matrix ranged from 84 to104%), sensitive (limit of detection ranged from 0.5 to 20 ng mL^{-1}) and precise (the relative standard deviations ranged from 2.5 to 11%). We applied this method to urine samples collected from 1203 non-smokers and 347 smokers and demonstrated that smokers have significantly elevated levels of tobacco-related biomarkers compared to non-smokers. We found significant ($p < 0.0001$) correlations between serum cotinine and most of the tobacco-related biomarkers measured. These findings confirm that this method can effectively quantify urinary VOC metabolites in a population exposed to volatile organics.

☆*Disclaimer*. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2012.04.009.

Keywords

VOC metabolites; Urinary biomarkers; Tobacco-related; UPLC-ESI/MSMS; Endogenous; Correlation

1. Introduction

Volatile organic compounds (VOCs) are ubiquitous in the environment, frequently detected at workplaces, in daily routines and in widely used consumer products. Inhalation is the predominant route of exposure due to their volatility, and many VOCs are also readily absorbed through skin [1]. Long-term exposure to certain VOCs may increase the risk for leukemia, bladder cancer, birth defects, and neurocognitive impairment [2–5]. In the United States tobacco smoke is the major non-occupational source of exposure to many harmful VOCs [6]. Tobacco smoke contains over 8000 chemicals, including carcinogenic and toxic VOCs [7]. An estimated 46 million people, or 20.6% of all adults (aged 18 years and older) in the United States, smoke cigarettes [8]. Cigarette smoking is the leading cause of preventable death in the United States, accounting for approximately 443,000 deaths, or 1 of every 5 deaths, in the United States each year. Additionally, second and third hand smoke affects many U.S. residents, including a disproportionate number of children and infants [9,10]. Regardless of exposure source, high levels of toxic VOCs are an area of significant public health concern.

Exposure to VOCs is often assessed by measuring VOCs in blood [11,12] or VOC metabolites in urine [13–15]. The main advantages of monitoring urinary VOC metabolites as biomarkers of exposure are the non-invasive sampling of urine, longer physiological half-lives of metabolites compared with parent compounds, and the specificity of most mercapturic acid metabolites. Mercapturic acids are *N*-acetyl-L-cysteine-*S*-conjugates that are formed in the human body via the glutathione pathway through metabolism of a range of endogenous and exogenous chemicals. VOCs and/or their metabolites can react with glutathione (GSH) to form mercapturic acids. These metabolites are then removed from the blood by the kidneys and excreted in urine. In the present study we monitor VOC metabolites as biomarkers of exposure to acrolein, acrylamide, acrylonitrile, benzene, 1-bromopropane, 1,3-butadiene, carbon disulfide, crotonaldehyde, cyanide, *N*-*N*-dimethylformamide, ethylbenzene, ethylene oxide, propylene oxide, vinyl chloride, styrene, tetrachloroethylene, toluene, trichloroethylene, and xylene (Table 1). Except for 1-bromopropane, tetrachloroethylene, and trichloroethylene all the other parent compounds are constituents of tobacco smoke [7]. 1-bromopropane, tetrachloroethylene, and trichloroethylene are widely used solvents that may be neurotoxic [16] or carcinogenic [17].

Although biological exposure limits have been established for occupationally exposed workers for some of the urinary VOC metabolites [18], there are no large scale data sets or reference levels for VOC metabolites available for the general U.S. population. To facilitate measuring many VOC metabolites in large epidemiological studies, it is important to develop a sensitive and high throughput biomonitoring method which can measure many VOC metabolites simultaneously in urine. Previously, liquid chromatography–mass

spectrometry (LC–MSMS) methods have been reported for the analysis of VOC metabolites [13,14,19–23] in human urine for small scale studies; however, those methods targeted only a small number of VOC metabolites. Therefore we developed and validated an UPLC-ESI/MSMS method that concurrently measures 28 VOC metabolites in urine.

2. Experimental

2.1. Materials and methods

The following solvents were used for the mobile phase (Solvent B, strong wash) and to prepare stock standards: Fisher optima grade acetonitrile, methanol and iso-propyl alcohol, and J.T. Baker HPLC grade water; Fisher HPLC grade dimethylsulfoxide. These chemicals were purchased from Fisher Scientific (Fairlawn, NJ). Ammonium acetate was purchased from Sigma–Aldrich (St. Louis, MO). Most of the analytes and the labeled internal standards were custom synthesized. The source of labeled and unlabeled analytes is listed in Table 1. The labeled ATCA was a gift from Dr. William Draper's Lab, California Department of Public Health, CA. The purity levels of analytes and the labeled analogs were ≥98%. The analytes and the labeled analogs were stored at temperatures specified in the certificate of analysis received from the vendors.

VOC metabolite stock solutions were individually prepared by weighing neat compounds (10–20 mg) using a calibrated analytical balance and dissolving each in 10 mL of solvent. The solvent used to prepare 2MHA, 3MHA, 4MHA, BPMA, MA unlabeled and labeled standards was methanol:water (1:1) mixture. Dimethylsulfoxide was used as the solvent to prepare MU unlabeled and labeled standards. Individual stocks of unlabeled and labeled 1,2DCVMA; 2,2DCVMA; 2,4DPMA; 2,5DPMA; 3,4DPMA; PHEMA; and TCVMA were prepared separately using methanol as the solvent. The remaining analytes and the corresponding analogs were prepped in HPLC-grade water. A working solution (100 mL) of analytes was then prepared from individual stocks, aliquoted (1 mL) into labeled 1.5 mL Dionex vials (Fisher Scientific, Fairlawn, NJ), and stored at −20 °C. Similarly, a working solution (25 mL) of internal standards was prepared from individual analog stocks, aliquoted (1 mL) and stored at 4 °C (stable for over 4 months). Aliquots of individual stocks of both labeled and unlabeled were stored at −20 °C.

2.2. Instrumentation

An ultra high performance liquid chromatography system (Waters Inc., Milford, MA) coupled with electro spray tandem mass spectrometry (Sciex API 5500 Triple Quad, Applied Biosystems, Foster City, CA) was employed to quantitatively measure urinary VOC metabolites. Chromatographic separation was achieved using an Acquity UPLC® HSS T3 1.8 μm × 2.1 mm × 150 mm (Waters Inc, Milford, MA) column with 15 mM ammonium acetate pH 6.8 (Solvent A) and acetonitrile (Solvent B) as mobile phases. The eluent from the column was ionized using an electrospray interface to generate and transmit negative ions into the mass spectrometer. Comparison of relative response factors (ratio of native analyte to stable isotope labeled internal standard) with known standard concentrations yielded individual analyte concentrations for unknowns. The Analyst software (version 1.5.1, Applied Biosystems, Foster City, CA) was used to operate both the UPLC and the

API5500. The separation conditions were optimized to obtain good resolution among VOC metabolites (Fig. 1). The final chromatographic parameters for the UPLC are presented in Table 2. Alternative mobile phases (0.05% acetic acid pH 3.5, 0.1% formic acid pH 2.5, and HPLC water pH 5) did not significantly improve overall performance (chromatography and ionization).

The mass spectrometer was operated under scheduled multiple reaction monitoring (SMRM) mode for negative ions, the ion source temperature was kept at 650 °C, and the electro spray ion voltage at −4000 V. The mass spectrometric parameters were optimized for each analyte and analog separately. Table 3 presents the MRM transitions used for quantification, confirmation and internal standard; hence 84 transitions were monitored for 28 analytes. The target scan time and the MRM window were optimized for SMRM monitoring.

2.3. Quantification

Analyst software (version 1.5.1, Applied Biosystems, Foster City, CA) was used for peak integration, calibration, and quantification. Relative response factors were calculated based on the ratio of relative peak area of the analyte quantification transition to that of the labeled internal standard transition.

2.4. Calibration

A set of 9 calibrators was analyzed with each set of unknown samples. A weighted, $1/x$ (where x is the standard concentration), least-square model was fit to the calibration except for TCVMA (quadratic fit). Calibration curves were linear, with $r^2 > 0.99$. Calibrators were prepared in 15 mM ammonium acetate buffer to avoid the background of some analytes found in urine. The limit of detection was defined as 3 times the standard deviation at zero concentration ($3S_0$) based on analysis of six sets of calibrators [24]. Each analytical batch consisted of a set of calibrators, two QC samples (low-concentration QC and high-concentration QC), a blank mobile phase, and 75 unknowns.

We validated this calibration model by confirming that calibrators prepared in ammonium acetate buffer had the same slope as calibrators prepared in urine ($p > 0.05$; Table 4). Similarly, bias plots (not shown) of this data indicated no systemic bias introduced by preparing calibrators in 15 mM ammonium acetate instead of urine. Therefore we recommended preparing calibrators in 15 mM ammonium acetate for this method.

2.5. Quality control (QC) samples

Two low-concentration QC (QCL) and two high-concentration QC (QCH) samples were included in each analytical batch. QC materials were prepared by spiking a known amount of mixed standards solution into 50 mL urine to achieve the target concentration (Table S1, online supplemental information). Two urine pools were prepared with levels within the linear range established, QC low and QC high. QC pools were aliquoted (150 μL) into 1.5 mL cryovials and stored at −20 °C. QC pools were subsequently characterized (mean and standard deviation) with 20 independent analyses of each QC pool, and results were analyzed using a multirule method to evaluate method precision for each batch of analyses

[25]. If QC results were not acceptably precise compared with the characterized means, then results for that analyte/batch were rejected.

2.6. Accuracy and proficiency testing

Four levels of proficiency testing (PT) materials were prepared by diluting original VOC metabolite stock solutions with water (HPLC-grade) (Table S2, online supplemental information). PT solutions were subsequently aliquoted into cryovials. These cryovials then were blind-coded by an independent QC officer and stored at −20 °C. PT samples were analyzed every 6 months or after major instrument maintenance. The assay passed PT if blind-analyzed amounts fell within 25% of the nominal values. The accuracy of the assay was established by analyzing PT samples blind to the analyst and also by spiking urine at three different levels of VOC metabolites.

2.7. Urine specimen collection, storage and preparation

Urine samples for VOC metabolites analysis were collected in sterile urinary cups. Immediately after collection, a 1.8-mL aliquot from each subject was transferred to a 2-mL cryovial and stored at −70 °C until assay. Individual consent of study participants and the human subjects (IRB) approval were obtained prior to this study. The anonymous subjects were multi-ethnic, male and female, and 12+ yr of age. Urine samples were assayed diluted 1:10 with buffer (50 μL urine + 25 μL working mixed internal standard + 425 μL 15 mM ammonium acetate pH 6.8). This dilution yielded minimal suppression of ionization while maintaining sensitivity. Nevertheless, we observed ion suppression for some analytes in <1% of urine specimens. Further dilution of the specimen overcame this problem, and produced accurate results.

2.8. Freeze–thaw and stability experiments

Spiked urine samples were frozen (−20 °C) and thawed 10 times to check the stability of analytes with number of freeze–thaw cycles. Also spiked samples were kept at different storage temperatures (22 °C, 4 °C and −20 °C) to check the stability of analytes under those storage conditions for up to one week.

2.9. Statistical analysis

For data analysis, we used SAS for Windows (ver. 9.3, SAS Institute Inc., Cary, NC). Descriptive analysis was done using SAS proc univariate. Spearman correlation (Proc Corr) was performed to determine the relationship among urinary VOC metabolites and between VOC metabolites and serum cotinine.

3. Results and discussion

We developed a high throughput method using UPLC/ESI-MSMS to simultaneously measure 28 VOC metabolites in urine samples. The higher chromatographic efficiency of UPLC allowed us to increase chromatographic resolution and throughput relative to previous methods [14,26]. As shown in Fig. 1, the method resolves crucial isomers and potential interferents. Specifically, we can now resolve the primary monohydroxylated human metabolite of 1,3-butadiene (MHBMA3) from other interferents and MHBMA

isomers. By coupling selective chromatography with selective mass spectrometry of 84 separate transitions, we achieved unambiguous quantification of the 28 target analytes. A typical set of extracted transition chromatograms for the analyte GAMA is shown in Fig. 2.

The method accurately measures the target analytes in urine. We evaluated method accuracy using spike recovery in the urine matrix and proficiency testing solutions in pure solvent. Urine matrix was spiked with three different levels and analyzed; the mean accuracy across all analytes ranged from 84 to 104% (Table 5), with an overall average of 98%. Proficiency testing solutions contained four different levels of analytes. Repeated analysis of blind coded PT solutions indicated excellence accuracy (102%) averaged across all PT levels for all analytes (online supplemental information Table S2).

We evaluated method precision by testing two sets of urine-based QC specimens with every batch of samples for a 7-month period. Based on 130 sets of measurements made within this time frame, the inter-day variability of QCs ranged from 2.5 to 11% for all the analytes (online SI Table S1). We observed slightly higher variability in the QCL results (average co-efficient of variation [CV] 6.9%) compared with QCH results (6.4% average CV), likely because analytical methods tend to be less precise with less analyte material to measure. Methodological precision was similar for mercapturate metabolites (6.6% average CV) compared with non-mercapturate metabolites (6.5% average CV), consistent with the precision of the method across a broad range of chemical structures. Because these QC pools were spiked human urine samples, we are confident that the method was similarly precise for human urine specimens analyzed with our method. Furthermore, analysis of these characterized QC pools also verifies assay accuracy for each batch of samples analyzed.

The method demonstrates better sensitivity compared to previous methods (Table 6), with the current limits of detection for urine specimens ranging from 0.5 to 20 ng mL^{-1}. This sensitivity is adequate enough for detecting background exposure from sources such as tobacco smoke or solvent use. Sample clean up as described by previous studies [13,15,26] could have further improved detection limits. However, because our goal was to balance sensitivity with a high throughput (75 samples/24-h) and less labor-intensive method capable of simultaneously quantifying multiple analytes, we chose a “dilute-and-shoot” approach instead of extensive sample preparation. As a result, the current method quantifies 28 different VOC metabolites at levels relevant to non-occupational exposure for each of 75 urine samples per batch.

We characterized the stability of analytes in urine stored at different temperatures and freeze–thaw cycles. All analytes were stable in urine samples stored at −20 °C for a week; similarly all analytes except for TTCA and PGA were stable at 4 °C for a week. We also tested freeze–thaw stability of analytes spiked into urine and found that all analytes were stable through 10 freeze–thaw cycles, except TTCA which decreased by about 20% ($p < 0.05$) after the fifth freeze–thaw cycle. Therefore, we recommend that samples be frozen immediately after collection and that freeze–thaw cycles be minimized (<5 times).

PMA is a selective urinary biomarker of benzene exposure. Paci et al. [22] and Sterz et al. [38] showed that strong acid pretreatment of urine is needed to convert pre-PMA

[N-acetyl-S-(1,2-dihydro-2-hydroxyphenyl)-L-cysteine] to PMA in order to measure total PMA. Samples pre-treated with concentrated sulfuric acid (pH −0.6) yielded more PMA compared with samples pre-treated with HCl (pH 0.5–1.0) and the origin of extra PMA is unknown [38]. The strong acid hydrolysis of pre-PMA reduces the variability in the results by reducing pH differences in urine samples and increasing the amount of measured PMA [22]. A small scale experiment carried out as part of this method development confirmed the observations of Paci et al. (data not shown). However, we omitted the strong hydrolysis step for this current multianalyte method because we found that the strong acid hydrolysis decreased the stability of several other VOC metabolites present in urine samples. Therefore, PMA values from our method represent only “free” PMA, and not the “total PMA” (the sum of PMA, pre-PMA and other possible PMA precursors) as measured by previous methods [22,38].

We applied this method to urine samples collected from 1203 non-smokers and 347 smokers, as categorized based on a serum cotinine cut point of 10 ng mL^{-1} [37,39], in order to evaluate the applicability of this method to determine higher levels of tobacco-related biomarkers (24 of the 28 analytes) in smokers compared with non-smokers. Not surprisingly, our results indicated that urine samples collected from smokers have significantly higher levels of most tobacco-related VOC biomarkers compared to urine collected from non-smokers (Table 6). Additionally, serum cotinine was significantly correlated with urinary VOC metabolites except for ATCA, BMA, and MU (Table 7). The lack of correlation between ATCA and cotinine may be due to multiple sources of exposure to cyanide other than tobacco smoke (e.g. cyanide from food and from amino acid catabolism) [40]. Similarly, BMA and MU are not specific biomarkers of exposure to tobacco smoke. BMA levels can be formed from multiple sources other than toluene; for example BMA is formed from benzyl alcohol, which is a widespread constituent of cosmetic products like shampoo, creams, after-shave lotions or fragrances [41]. The benzene metabolite *trans*, *trans*-muconic (MU) acid can also be formed from sorbic acid [34], which is a common food preservative.

MHBMA and DHBMA are metabolites of 1,3-butadiene exposure. DHBMA is formed by hydrolysis of butadiene monoepoxide (BMO) to butadiene-diol, which is then conjugated with glutathione (GSH) to form DHBMA [42]. MHBMA is formed by direct conjugation of BMO with GSH. Monkeys and humans produce primarily DHBMA because of their naturally high levels of epoxide hydrolase enzyme that catalyze hydrolysis. The metabolic ratio (DHBMA/(DHBMA + MHBMA) is a measure of the hydrolytic metabolism of BMO. Smokers are known to have less epoxide hydrolase activity compared with non-smokers [42]. Consistent with this literature, we found significantly lower metabolic ratios for smokers (0.92 ± 0.07) compared to non-smokers (0.98 ± 0.01). The median levels of MHBMA3 among smokers and non-smokers respectively were 26.6 and 4.53 ng mL^{-1}. The DHBMA levels were 385 and 273 ng mL^{-1} for smokers and non-smokers respectively.

By resolving the three MHBMA isomers via selective LC conditions, we were able to better evaluate butadiene metabolism compared with previous studies [13,14,26]. Among MHBMA isomers, MHBMA3 (N-acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine) was detected in majority of the samples (99%) tested (Fig. 3), while MHBMA1 was not. Analysis of fresh urine in this study indicated that the MHBMA3 measured was

not an artifact of storage or acidification as discussed in previous studies [43]. Selective chromatography also resolved 2HPMA (propylene oxide biomarker) from 3HPMA (acrolein biomarker), and also resolved trichloroethylene biomarkers 1,2DCVMA and 2,2DCVMA.

We monitored three sets of mercapturic acid metabolites that are biomarkers of the same parent VOCs: DHBMA and MHBMA metabolites of 1,3-butadiene; AAMA and GAMA metabolites of acrylamide; and CEMA and HPMA metabolites of acrolein. Because these pairs of metabolites are formed from the same parent compounds, it is highly probable that both analytes in each pair are correlated. As expected we found significant correlations (measured by Spearman correlation) between DHBMA and MHBMA3 ($r = 0.63$, $p < 0.0001$); AAMA and GAMA ($r = 0.75$, $p < 0.0001$); and CEMA and HPMA ($r = 0.79$, $p < 0.0001$) among smokers.

Some of the VOC metabolites are non-specific and can be formed through the metabolism of dietary sources; others are endogenous. AMCC may be formed from the dietary uptake of methyl isothiocyanate which is a component of wine and cruciferous vegetables such as cabbage, turnips and cress [44]. DHBMA may be formed from endogenous butadiene-diol [42]. Acrolein, which is a major cigarette-related lung cancer agent [45] can also be formed within the physiological metabolism of fatty acids in the human body [46]. As dietary and smoking habits, work exposure and endogenous factors contribute to biomonitoring results, these factors should be considered when interpreting VOC metabolite data.

4. Conclusions

We developed a selective, precise and sensitive method that uses UPLC/ESI-MSMS to measure 28 urinary biomarkers of exposure to 19 different VOC parent compounds. Importantly this method distinguishes smokers from non-smokers based on measured levels of metabolites of acrolein, acrylamide, acrylonitrile, benzene, 1,3-butadiene, carbon-disulfide, crotonaldehyde, *N*, *N*-dimethylformamide, ethylbenzene, propylene oxide, styrene, and xylenes. By improving exposure assessment for these hazardous VOCs, this method can serve as a valuable tool to improve studies related to VOC exposure and health effects such as cancer, birth defects, and neurocognitive impairment. This high throughput method will be applied to samples from the National Health and Nutrition Examination Survey (NHANES) to evaluate baseline urinary VOC metabolite levels and to determine VOC exposure among the U.S. population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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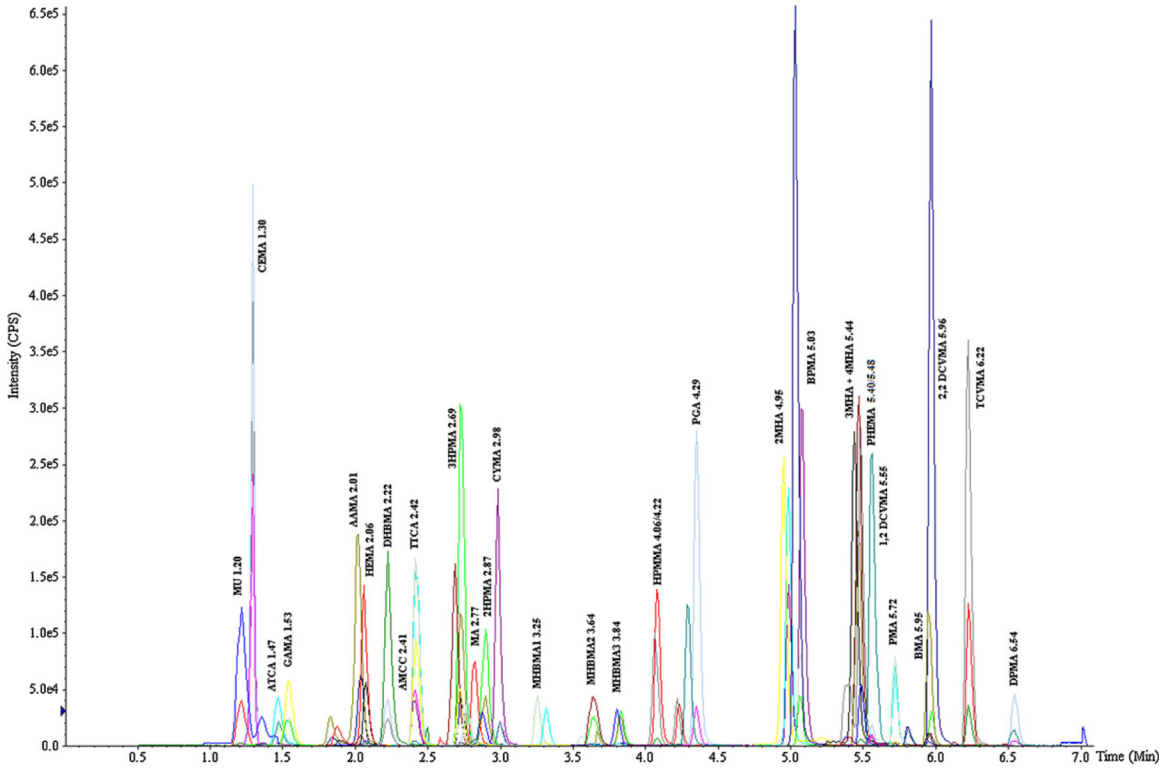

Fig. 1.

The extracted ion chromatogram of a high QC urine pool fortified with 28 VOC metabolites (analyte concentrations are given in Table S1 online supplemental information).

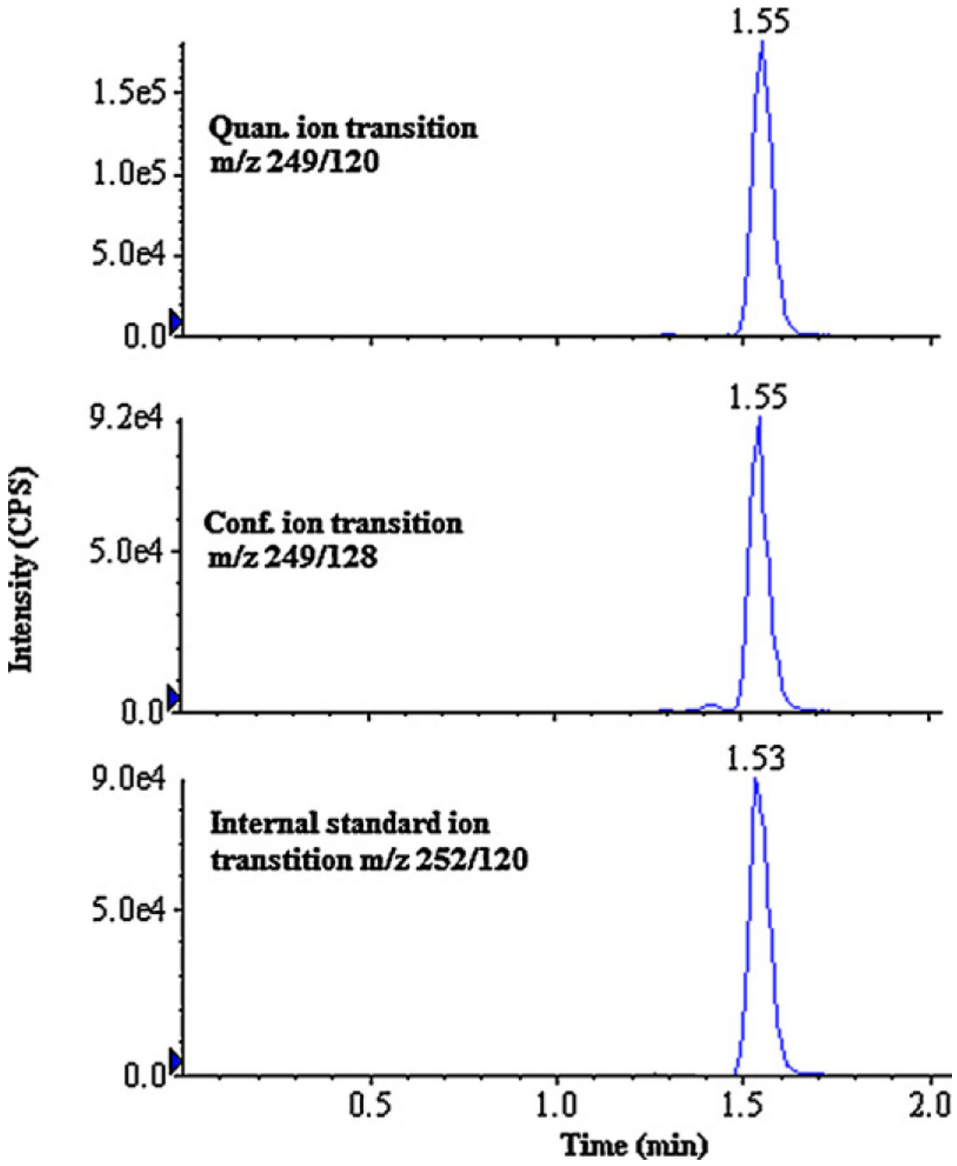

Fig. 2.
GAMA (236 ng mL^{-1}) in a spiked human urine sample.

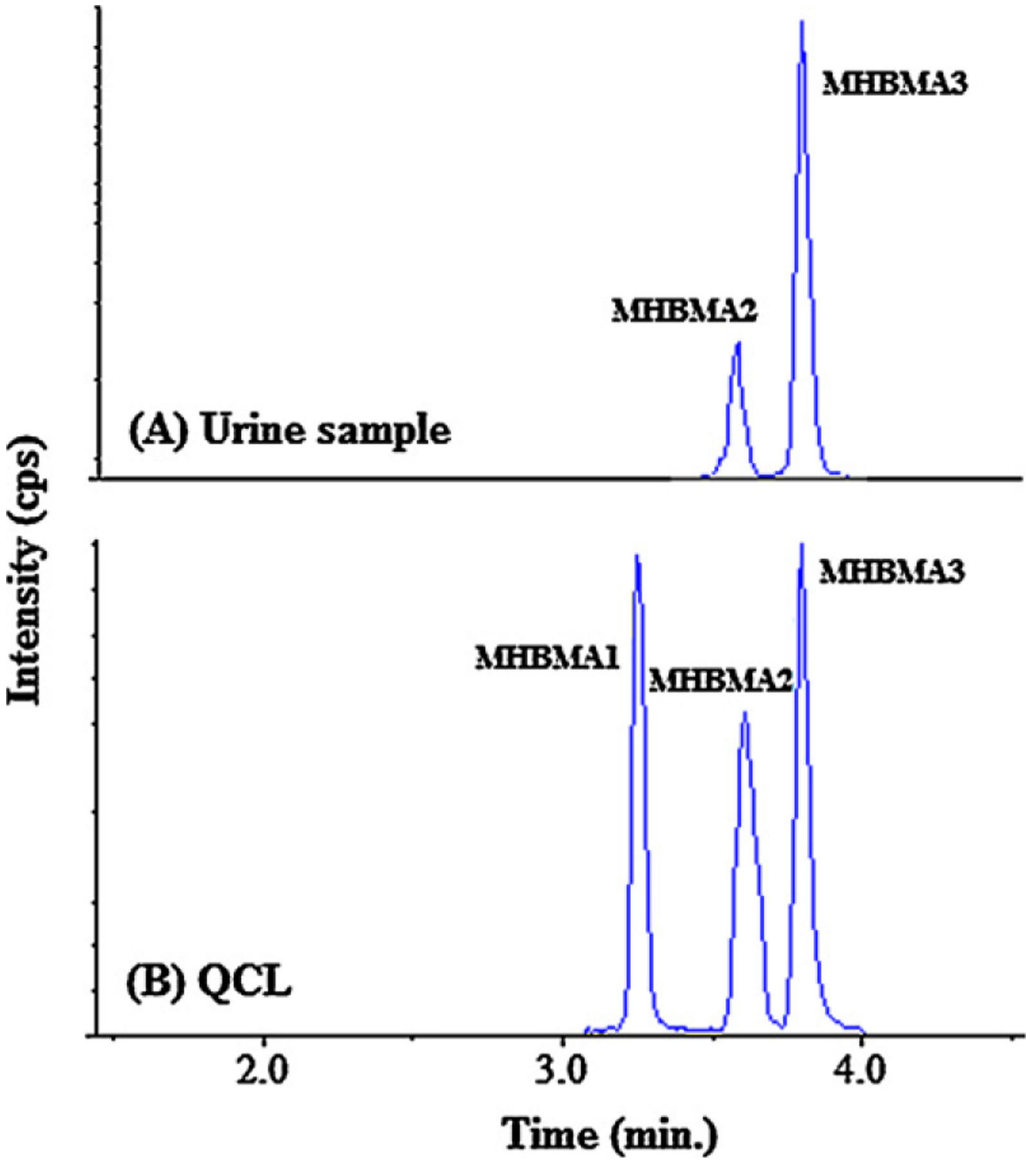

Fig. 3.

Urinary biomarkers of 1,3-butadiene exposure. (A) MHBMA2 (11 ng mL^{-1}) and MHBMA3 (98 ng mL^{-1}) in a human urine sample. (B) Three isomers in a QCL sample spiked with MHBMA1 (8.33 ng mL^{-1}). MHBMA2 (9.71 ng mL^{-1}) and MHBMA3 (23.0 ng mL^{-1}).

Table 1

VOC metabolites and the parent compounds.

Parent compound	VOC metabolite	Common name	Internal standard
Acrolein	*N*-Acetyl-S-(2-carboxyethyl)-L-cysteine[b]	CEMA	*N*-Acetyl-S-(2-carboxyethyl_$^{13}C_3$)-L-cysteine[b]
	N-Acetyl-S-(3-hydroxypropyl)-L-cysteine[b]	3HPMA	*N*-Acetyl-S-(3-hydroxypropyl_$^{2}H_6$)-L-cysteine[b]
Acrylamide	*N*-Acetyl-S-(2-carbamoylethyl)-L-cysteine	AAMA	*N*-Acetyl-S-(2-carbamoylethyl_$^{2}H_4$)-L-cysteine
	N-Acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine[g]	GAMA	*N*-Acetyl-$^{2}H_3$-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine[g]
Acrylonitrile	*N*-Acetyl-S-(2-cyanoethyl)-L-cysteine[g]	CYMA	*N*-acetyl-$^{2}H_3$-S-(2-cyanoethyl)-L-cysteine[g]
Acrylonitrile, vinyl chloride, ethylene oxide	*N*-Acetyl-S-(2-hydroxyethyl)-L-cysteine[b]	HEMA	*N*-Acetyl-S-(2-hydroxyethyl_$^{2}H_4$)-L-cysteine[b]
Benzene	*N*-Acetyl-S-(phenyl)-L-cysteine[b]	PMA	*N*-Acetyl-S-(phenyl_$^{13}C_6$)-L-cysteine[b]
	trans, *trans*-Muconic acid[f]	MU	*trans,trans*-Muconic-^{2}H4-acid[c]
1-Bromopropane	*N*-Acetyl-S-(*n*-propyl)-L-cysteine[g]	BPMA	*N*-Acetyl-S-(*n*-propyl_$^{2}H_7$)-L-cysteine[g]
1,3-Butadiene	*N*-Acetyl-S-(3,4-dihydroxybutyl)-L-cysteine[g]	DHBMA	*N*-Acetyl-S-(3,4-dihydroxybutyl_$^{13}C_4$)-L-cysteine[b]
	N-Acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine[b]	MHBMA1	*N*-Acetyl-S-(1-hydroxymethyl-2-propenyl_$^{2}H_6$)-L-cysteine[b]
	N-Acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine[e]	MHBMA2	*N*-Acetyl-S-(2-hydroxy-3-butenyl)-L-cysteine_$^{13}C_3$_^{15}N[e]
	N-Acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine[g]	MHBMA3	*N*-Acetyl-$^{2}H_3$-S-(4-hydroxy-2-buten-1-yl)-L-cysteine[g]
Carbon-disulfide	2-Thioxothiazolidine-4-carboxylic acid[f]	TTCA	2-Thioxothiazolidine-$^{13}C_3$-4-carboxylic acid[b]
Crotonaldehyde	*N*-Acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine[g]	HPMMA	*N*-Acetyl-$^{2}H_3$-S-(3-hydroxypropyl-1 methyl)-L-cysteine dicyclohexylammonium salt[g]
Cyanide	2-Aminothiazoline-4-carboxylic acid[d]	ATCA	2-Aminothiazoline-$^{2}H_3$-4-carboxylic acid
N,*N*-Dimethylformamide	*N*-Acetyl-S-(N-methylcarbamoyl)-L-cysteine[f]	AMCC	*N*-Acetyl-S-(*N*-methylcarbamoyl)-L-cysteine_$^{13}C_3$_^{15}N[e]
Ethylbenzene, styrene	Phenylglyoxylic acid[f]	PGA	Phenylglyoxylic-$^{2}H_5$-acid[c]
Propylene oxide	*N*-Acetyl-S-(2-hydroxypropyl)-L-cysteine[g]	2HPMA	*N*-Acetyl-S-(2-hydroxypropyl)-L-cysteine_$^{2}H_3$ dicyclohexylammonium salt[g]
Styrene	*N*-Acetyl-S-(1-phenyl-2-hydroxyethyl)-L-cysteine +	PHEMA	*N*-Acetyl-S-(1-phenyl-$^{13}C_6$-2-hydroxyethyl-L-cysteine +
	N-Acetyl-S-(2-phenyl-2-hydroxyethyl)-L-cysteine[g]		*N*-Acetyl-S-(2-phenyl-$^{13}C_6$-2-hydroxyethyl)-L-cysteine[g]
	Mandelic acid[f]	MA	Mandelic-2,3,4,5,6-$^{2}H_5$ acid[c]

Parent compound	VOC metabolite	Common name	Internal standard
Tetrachloroethylene	*N*-Acetyl-S-(trichlorovinyl)-L-cysteine[a]	TCVMA	*N*-Acetyl-S-(trichlorovinyl_$^{13}C_2$)-L-cysteine[a]
Toluene	*N*-Acetyl-S-(benzyl)-L-cysteine[a]	BMA	*N*-Acetyl-S-(benzyl_$^{13}C_6$)-L-cysteine[a]
Trichloroethylene	*N*-Acetyl-S-(1,2-dichlorovinyl)-L-cysteine[g]	1,2DCVMA	*N*-Acetyl-^{13}C-$^{2}H_3$-S-(1,2-dichlorovinyl)-L-cysteine[g]
	N-Acetyl-S-(2,2-dichlorovinyl)-L-cysteine[g]	2,2DCVMA	*N*-Acetyl-^{13}C-$^{2}H_3$-S-(2,2-dichlorovinyl)-L-cysteine[g]
Xylene	*N*-Acetyl-S-(2,4-dimethylphenyl)-L-cysteine[g] +	DPMA	*N*-Acetyl-$^{2}H_3$-S-(2,4-dimethylphenyl)-L-cysteine[g] +
	N-Acetyl-S-(2,5-dimethylphenyl)-L-cysteine[g] +		*N*-Acetyl-$^{2}H_3$-S-(2,5-dimethylphenyl)-L-cysteine[g] +
	N-Acetyl-S-(3,4-dimethylphenyl)-L-cysteine[g]		*N*-Acetyl-$^{2}H_3$-S-(3,4-dimethylphenyl)-L-cysteine[g]
	2-Methylhippuric acid[f]	2MHA	2-Methylhippuric-$^{2}H_7$-acid[c]
	3-Methylhippuric acid[f] + 4-Methylhippuric acid[f]	3MHA + 4MHA	3-Methylhippuric-$^{2}H_7$-acid[c] + 4-Methylhippuric-$^{2}H_7$-acid[c]

[a]Battelle Research Institute, Columbus, Ohio.

[b]Cambridge Isotopes Inc., Andover, MA.

[c]C/D/N Isotopes Inc., Quebec, Canada.

[d]Chem-Impex International Inc., Wood Dale, IL.

[e]Kalexsyn Inc., Kalamazoo, MI.

[f]Sigma Chemicals, St. Louis, MO.

[g]Toronto Research Chemicals, Toronto, Canada.

Table 2

Chromatographic parameters for the Acquity UPLC.

Parameter	Details
Column	Acquity UPLC® HSST3
	1.8 μm × 2.1 mm × 150 mm column
	Operating temp: 40 °C
Mobile phase	15 mM ammonium acetate pH 6.8 (Solvent A)
	Acetonitrile (Solvent B)
Weak wash	HPLC grade water
Strong wash	25% HPLC grade water,
	25% LCMS Optima grade methanol,
	25% LCMS Optima grade acetonitrile,
	25% LCMS Optima grade isopropyl alcohol
Gradient: Time, flow, Solvent A:Solvent B	Initial, 250 μL min^{-1}, 97%:3%
	2min, 250 μL min^{-1}, 95%:5%
	3 min, 300 μL min^{-1}, 90%:10%
	5 min, 300 μL min^{-1}, 70%:30%
	6.5 min, 300 μL min^{-1}, 60%:40%
	7 min, 300 μL min^{-1}, 85%:15%
	7.5 min, 300 μL min^{-1},90%:10%
	8 min, 300 μL min^{-1}, 97%:3%
	9 min, 300 μL min^{-1}, 97%:3%

Table 3

MRM transitions and collision energy voltage for VOC metabolites.

Analyte	RT (min)	Quantification ion transition	CE[a] (v)	Confirmation ion transition	CE (v)	Internal standard	Quantification ion transition	CE (v)
AAMA	2.01	233/104	−18	233/58	−50	AAMA_$^{2}H_4$	237/108	−22
AMCC	2.41	219/162	−10	220/163	−12	AMCC_^{15}N_$^{13}C_3$	223/166	−14
ATCA	1.47	145/67	−18	145/58	−14	ATCA_$^{2}H_3$	148/70	−18
BMA	5.95	252/123	−20	253/124	−22	BMA_$^{13}C_6$	258/84	−20
BPMA	5.03	204/75	−38	204/84	−14	BPMA_$^{2}H_7$	211/82	−20
CEMA	1.30	234/162	−16	234/105	−22	CEMA_$^{13}C_3$	237/162	−16
CYMA	2.98	215/86	−16	215/162	−12	CYMA_$^{2}H_3$	218/165	−12
1,2DCVMA	5.55	257/127	−10	257/128	−10	1,2DCVMA_^{13}C_$^{2}H_3$	261/127	−10
2,2DCVMA	5.96	257/127	−12	257/128	−14	2,2DCVMA_^{13}C_$^{2}H_3$	261/127	−14
DHBMA	2.22	250/121	−24	250/75	−40	DHBMA_$^{13}C_4$	254/125	−22
DPMA	6.54	266/137	−18	267/138	−14	DPMA_$^{2}H_3$	269/137	−16
GAMA	1.53	249/120	−22	249/128	−16	GAMA_$^{2}H_3$	252/120	−24
HEMA	2.06	206/77	−16	206/75	−34	HEMA_$^{2}H_4$	210/81	−22
2HPMA	2.87	220/91	−18	221/91	−58	2HPMA_^{2}H3	223/97	−20
3HPMA	2.69	220/91	−18	220/89	−32	3HPMA_$^{2}H_6$	226/97	−20
HPMMA	4.06/4.22[b]	234/105	−22	235/105	−20	HPMMA_$^{2}H_3$	237/105	−20
MA	2.77	151/107	−14	152/108	−14	MA_$^{2}H_5$	156/112	−14
2MHA	4.95	192/148	−18	192/91	−22	2-MHA_$^{2}H_7$	199/155	−18
3MHA + 4MHA	5.44	192/148	−18	192/91	−24	3-MHA_$^{2}H_7$ + 4-MHA_$^{2}H_7$	199/155	−18
MHBMA1	3.25	232/103	−16	233/103	−18	MHBMA1_$^{2}H_6$	238/109	−20
MHBMA2	3.64	232/103	−18	233/103	−18	MHBMA2_$^{13}C_3$_^{15}N	236/103	−18
MHBMA3	3.84	232/103	−18	233/103	−30	MHBMA3_^{2}H3	235/103	−20
MU	1.20	141/97	−12	141/53	−16	MU_$^{2}H_4$	145/100	−12
PGA	4.29	149/77	−18	149/105	−12	PGA_$^{2}H_5$	154/82	−16
PHEMA	5.40/5.48[c]	282/153	−22	282/123	−32	PHEMA_$^{13}C_6$	288/159	−18

Analyte	RT (min)	Quantification ion transition	CE[a] (v)	Confirmation ion transition	CE (v)	Internal standard	Quantification ion transition	CE (v)
PMA	5.72	238/109	−18	239/110	−14	PMA_$^{13}C_6$	244/115	−12
TCVMA	6.22	290/161	−10	290/35	−36	TCVMA_$^{13}C_2$	294/165	−10
TTCA	2.42	162/58	−14	162/33	−24	TTCA_$^{13}C_3$	165/58	−16

Note: Monitored same transitions for 1,2DCVMA and 2,2DCVMA; 2HPMA and 3HPMA; 2MHA and 3MHA + 4MHA; MHBMA1, MHBMA2 and MHBMA3 in scheduled MRM as the isomers eluted at different retention times.

[a]CE-collision energy.

[b]Isomers of each analyte monitored together.

[c]Isomers of each analyte monitored together.

Table 4

Validation of non-matrix based calibrators for quantifying analytes in human urine.

Analyte	Calibration range (ng mL^{-1})	Slope of the calibration curve		*p* [a]
		15 mM Ammonium acetate matrix	Urine matrix	
AAMA	0.11–109	0.9262	0.9242	0.81
AMCC	0.36–277	0.9626	0.9623	0.87
ATCA	0.90–357	1.0048	1.0047	0.97
BMA	0.04–44.0	1.0103	1.0111	0.86
BPMA	0.08–61.0	0.9737	0.9737	0.94
CEMA	0.60–240	0.9723	0.9725	0.90
CYMA	0.04–126	0.9993	0.9992	0.99
1,2DCVMA	0.90–378	0.9868	0.9866	0.99
2,2DCVMA	0.40–156	1.0239	1.0233	0.99
DHBMA	0.40–400	0.9530	0.9529	0.56
DPMA	0.02–9.00	1.0041	1.0040	0.99
GAMA	0.60–236	1.0110	1.0110	0.98
HEMA	0.04–24.0	1.1843	1.1831	0.99
2HPMA	0.30–1057	0.9640	0.9638	0.87
3HPMA	1.30–1296	1.0153	1.0149	0.94
HPMMA	0.30–973	0.9660	0.9662	0.47
MA	1.20–480	1.0022	0.9999	0.96
2MHA	0.30–275	0.9655	0.9646	0.99
3MHA + 4MHA	0.60–550	0.9906	0.9904	0.83
MHBMA1	0.04–18	0.9883	0.9881	0.96
MHBMA2	0.04–19	1.0131	1.0131	0.99
MHBMA3	0.06–44	1.2040	1.2050	0.97
MU	2.00–783	0.9902	0.9889	0.89
PGA	1.00–806	0.9929	0.9930	0.92
PHEMA	0.05–20	0.9837	0.9839	0.99
PMA	0.03–14	0.9915	0.9925	0.99
TTCA	0.03–268	0.9877	0.9873	0.99

Analyte	Calibration range (ng mL^{-1})	Slope of the calibration curve		p^a
		15 mM Ammonium acetate matrix	Urine matrix	
TCVMA	0.15–60	0.9412	0.9404	0.98

[a]Probability (Student T-test).

Table 5

Spike recovery for VOC metabolites.

Analyte	Spiked conc. in urine (ng mL^{-1})			Spike recovery (%) ±SD[a]		
	Level I	Level II	Level III	Level I	Level II	Level III
AAMA	21.7	109	217	98 ± 11	103 ± 6	104 ± 7
AMCC	27.7	139	277	100 ± 10	100 ± 9	99 ± 6
ATCA	66.9	335	669	94 ±8	97 ± 8	101 ± 10
BMA	3.96	19.8	40.0	96 ± 10	95 ± 13	99 ± 14
BPMA	30.7	154	307	91 ± 7	91 ± 5	102 ± 5
CEMA	60.0	300	600	98 ± 11	103 ± 11	95 ± 2
CYMA	3.16	15.8	32.0	99 ± 4	97 ± 3	99 ± 9
1,2DCVMA	63.0	315	630	96 ± 7	97 ± 6	95 ± 5
2,2DCVMA	32.4	162	324	93 ± 9	95 ± 7	99 ± 7
DHBMA	40.0	200	400	98 ± 6	106 ± 9	101 ± 4
DPMA	1.28	65.0	130	94 ± 3	97 ± 3	97 ± 2
GAMA	47.2	236	472	97 ± 12	103 ± 7	105 ± 5
HEMA	3.04	15.2	30.0	93 ± 13	96 ± 7	98 ± 5
2HPMA	66.1	165	661	84 ± 5	83 ± 2	86 ± 3
3HPMA	130	648	1296	96 ± 4	108 ± 4	107 ± 7
HPMMA	15.2	76.0	152	96 ± 13	96 ± 12	100 ± 8
MA	100	501	1001	95 ± 11	98 ± 8	94 ± 8
2MHA	25.0	126	251	98 ± 8	91 ± 2	88 ± 6
3MHA + 4MHA	101	503	1000	101 ± 4	101 ± 9	108 ± 7
MHBMA1	6.84	34.2	68.0	89 ± 11	94 ± 4	85 ± 2
MHBMA2	7.20	36.0	72.0	102 ± 5	105 ± 4	105 ± 4
MHBMA3	55.0	138	550	87 ± 1	86 ± 1	86 ± 3
MU	104	520	1039	102 ± 10	99 ± 9	99 ± 4
PGA	101	504	1007	104 ± 7	105 ± 4	105 ± 4
PHEMA	20.0	100	200	99 ± 7	101 ± 9	101 ± 6
PMA	2.76	13.8	28.0	97 ± 9	98 ± 6	100 ± 7
TTCA	40.2	201	402	98 ± 7	102 ± 3	99 ± 9

Analyte	Spiked conc. in urine (ng mL^{-1})			Spike recovery (%) ±SD[a]		
	Level I	Level II	Level III	Level I	Level II	Level III
TCVMA	15.1	75.5	151	93 ± 7	105 ± 4	97 ± 5

[a]Standard deviation.

Table 6

Smoker, non-smoker levels and limits of detection (LOD).

Analyte	Mean ± SD (ng mL^{-1})		LOD (ng mL^{-1})	LOD (ng mL^{-1}) [Literature]
	Smoker[a] (*N* = 347)	Nonsmoker *N* = 1203)		
AAMA	196 ± 180***	82 ± 128	2.20	2.5 [27]
AMCC	479 ± 410***	122 ± 135	5.50	5 [27]
ATCA	191 ± 340	167 ± 245	15.0	25 [28]
BMA	16 ± 29	15 ± 32	0.50	0.02 [15]
BPMA	21 ± 78	16 ± 29	1.20	10 [20]
CEMA	305 ± 294***	128 ± 119	8.00	200 [29]
CYMA	187 ± 181***	4.60 ± 35	0.50	1 [26]
1,2DCVMA	<LOD	< LOD	12.6	
2,2DCVMA	<LOD	< LOD	6.50	
DHBMA	440 ± 311***	331 ± 279	5.00	10 [26]
DPMA	<LOD	< LOD	0.50	0.1 [30]
GAMA	57 ± 57***	28 ± 36	9.40	10 [31]
HEMA	1.90 ± 3.70	0.66 ± 1.16	0.60	0.5 [27]
2HPMA	185 ± 235***	81 ± 118	1.30	5 [27]
3HPMA	1546 ± 1643***	406 ± 487	1.30	5 [27]
HPMMA	1992 ± 2009***	429 ± 478	2.00	28 [19]
MA	420 ± 357***	198 ± 226	12.0	100 [32]
2MHA	144 ± 265***	71 ± 277	5.00	5 [33]
3MHA + 4MHA	1020 ± 1379*	579 ± 3692	8.00	10 (3MHA), 6 (4MHA) [33]
MHBMA1	<LOD	<LOD	0.70	2 [26]
MHBMA2	1.80 ±2.10	<LOD	0.70	
MHBMA3	36 ± 34***	6.40 ± 10	0.60	
MU	473 ± 410***	358 ± 291	20.0	20 (LOQ) [34]
PGA	330 ± 425***	169 ± 224	12.0	45 [33]

Analyte	Mean ± SD (ng mL^{-1})		LOD (ng mL^{-1})	LOD (ng mL^{-1}) [Literature]
	Smoker[a] (*N* = 347)	Nonsmoker *N* = 1203)		
PHEMA	<LOD	<LOD	0.70	0.7 [35]
PMA	0.92 ± 2.11 ***	0.60 ± 0.40	0.30	0.1 [22]
TTCA	37.4 ± 170	27.3 ± 83	3.50	1 [36]
TCVMA	<LOD	<LOD	3.00	

SD: Standard deviation.

*** $p < 0.0001$,

** $p < 0.001$,

* $p \leq 0.05$; Student T-test.

[a] Defined as having serum cotinine ≥10 ng mL^{-1} [37].

Table 7

Relationship between serum cotinine levels with tobacco related urinary VOC metabolites.

Analyte	r [a]	p [b]
Mercapturic acids		
AAMA	0.25	<0.0001
AMCC	0.40	<0.0001
BMA	−0.02	0.73
CEMA	0.34	<0.0001
CYMA	0.46	<0.0001
DHBMA	0.12	0.03
GAMA	0.16	0.003
2HPMA	0.31	<0.0001
3HPMA	0.44	<0.0001
HPMMA	0.47	<0.0001
MHBMA3	0.42	<0.0001
PMA	0.24	<0.0001
Non-mercapturic acids		
2MHA	0.30	<0.0001
34MHA	0.35	<0.0001
ATCA	0.03	0.62
MA	0.29	<0.0001
MU	0.02	0.74
PGA	0.18	0.0006
TTCA	0.06	<0.0001

[a] Spearman correlation coefficient.

[b] Probability.