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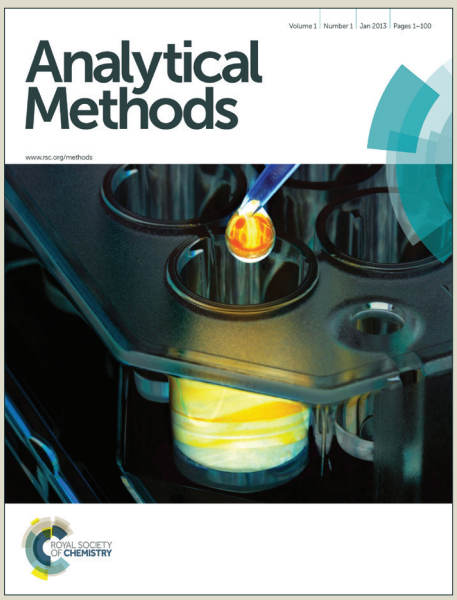

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Method development for the simultaneous analysis of *trans, trans*-muconic acid, 1, 2-dihydroxybenzene, *S*-phenylmercapturic acid and *S*-benzylmercapturic acid in human urine by liquid chromatography/tandem mass spectrometry

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This study reported a method to simultaneously determine *trans, trans*-muconic acid (*t,t*-MA), 1,2-dihydroxybenzene (or catechol, abbreviated as 1,2-DB), *S*-phenylmercapturic acid (*S*-PMA) and *S*-benzylmercapturic acid (*S*-BMA) in human urine. Samples were prepared through solid phase extraction and analyzed by ultra-high performance liquid chromatography/tandem mass spectrometer in negative electrospray ionization mode. The method was fully validated through the studies of precision, accuracy, matrix effects, detection limit, linear range, stability and real urine sample tests. Calibration curves of all target analytes showed favorable linearty within the wide concentration range of 0.2-4,000 μg/L. The detection limits in 10 times diluted pooled urine ranged from 0.08 to 7.8 μg/L. The method showed satisfactory accuracies and precisions. Except for the low spiked quality control (QC) level of 1,2-DB (73.1% recoveries), recoveries were in the range of 100 ±15% with a variation coefficient of less than

15%. Target analytes were stable in stock solutions and spiked urine samples under storage and test conditions. Twenty nine anonymous real urine samples from non-occupational donors were analyzed for all target analytes. Except for *S*-PMA, the other three compounds could be detected and quantified. Preliminary results showed that *S*-BMA was a sensitive and specific biomarker of toluene exposure and had significant correlation with *t,t*-MA ($r=0.631$, $p<0.01$). Moreover, *S*-BMA and *S*-PMA together would reflect the overall exposure to BT at low levels.

1. Introduction

Benzene and toluene (BT) have been widely used as the organic solvents and the source materials for industrial synthesis. They are also volatile components of gasoline and constitutes of tobacco smoke. Due to their extensive use and unintended release, they are ubiquitous air pollutants. Since benzene exposure was strongly associated with acute non-lymphocytic leukemia,[1,2] aplastic anemia[3] and chromosomal aberrations,[4] benzene has been classified as a human carcinogen by the International Agency for Research on Cancer (IARC).[5] Toluene, although less toxic than benzene, has significant toxic effects to the human central nervous system.[6] Therefore, it is particularly important to monitor BT together in human bodies at the same time to assess human exposure from various sources.

Benzene metabolizes in various pathways. Initially, the benzene epoxide is formed under the catalysis of cytochrome *P*450 (CYP), then transformed to different phenols, catechol (or 1,2-dihydroxybenzene, abbreviated as 1,2-DB), *trans, trans*-muconic acid (*t,t*-MA, also known as *trans, trans*-2,4 hexadienedioic) and *S*-phenylmercapturic acid (*S*-PMA). Eventually, they are excreted through urine.[5] *t,t*-MA is a common biomarker for benzene exposure. However, its urinary levels do not always correlate well with air concentration of benzene because it is also a metabolite of sorbic acid, a common food additive.[7] Recently, *S*-PMA is believed to be a sensitive and specific biomarker for evaluating benzene exposure at low levels.[8-10]

The primary route of toluene metabolism is hydroxylation to benzyl alcohol under the catalysis of CYP enzyme members. Most of toluene metabolites are excreted in the form of hippuric acid (HA), and about 1% metabolizes to form *S*-benzylmercapturic acid (*S*-BMA).[11] HA is also one of the main endogenous urinary metabolites and hence a less specific biomarker for toluene exposure.[11] Since *S*-BMA was identified as a metabolite of toluene, by Takahashi et al.,[12] it has been used as a more specific biomarker of low levels of toluene exposure than HA and o-cresol.[9,13-14]

Previous studies only measured one or two metabolites of benzene, such as *t,t*-MA or S-PMA[7-8] to assess benzene exposure by gas chromatography/mass spectrometry (GC/MS)[19] and liquid chromatography/tandem mass spectrometry (HPLC/MS/MS).[13-14,16-18] However, the exposure to BT often happened at the same time . In addition, metabolism pathways, metabolite types and levels of biomarkers also vary with external exposure levels and doses.[21-22] Hence it is crucial to develop a method using multiple biomarkers to assess the overall BT exposure. In this study, a new and fast ultra HPLC/MS/MS method to simultaneously quantify multiple urinary biomarkers (including *t,t*-MA, 1,2-DB, *S*-BMA and *S*-PMA) were developed with easy sample pretreatment procedure. Thirty three urine samples from anonymous donors were employed to validate the method.

2. Experimental

2.1 Chemicals and materials

t,t-MA (purity 98%), *S*-BMA (purity, no data), 1,2-DB (purity 99%) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). *S*-PMA (purity 98%) was purchased from Fluka (St. Louis, MO, USA). The chemical structures of *t,t*-MA, 1,2-DB, *S*-BMA and *S*-PMA are shown in Fig. 1. D_4-*t,t*-MA (D_4 99.7%), $^{13}C_1$-1,2-Dihydroxybenzene ($^{13}C_1$ 99%), D_5–*S*-BMA (D_5 99.1%) were obtained from C-D-N Isotope Inc. (Montréal, Quebec, Canada). D_5-*S*-PMA potassium (purity, no data) was purchased from Synthese Aptochem Inc. (Montréal, Quebec, Canada). Methanol (LC-MS Chromasolv®, ≥99.9%) was obtained from Fluka (St. Louis, MO, USA). Water (Chromosolv plus, HPLC grade) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Glacial acetic acid (HAC) was from Fisher Scientific (Houston, TX, USA). All other reagents were of analytical grade and used without further purification.

2.2 Standard preparation

A stock solution of *t,t*-MA was prepared in methanol and water (v/v, 4/1) with a concentration of 162 μg/mL. Other native and internal standards were prepared in methanol with concentrations ranging from 96 to 4,150 μg/mL. Calculated volumes of individual stock solutions were diluted to 10.0 mL to make native standard mixtures (NSM) with concentrations of 40 μg/mL for *t,t*-MA, 20 μg/mL for 1,2 -DB, 1μg/mL for *S*-BMA and 2 μg/mL for *S*-PMA. Similarly, an internal standard mixture (ISM) was prepared containing D_4-*t,t*-MA at 20 μg/mL, $^{13}C_1$-1,2-DB at 40 μg/mL, D_5-*S*-PMA at 0.4 μg/mL and D_5-*S*-BMA at 0.4 μg/mL. The concentration of D_5-*S*-PMA was derived from the concentration of D_5-*S*-PMA potassium. All standards were stored at -20°C until use. A pooled urine samples from 20 volunteer

donors was used to validate the analytical method. One hundred milliliter of urine was mixed with 900 mL of water, and ten calibration standard solutions were prepared using 2.0 mL of the diluted urine pool. The concentrations of native standards were in the range of 0.2-4,000 μg/L, while the concentrations of internal standards, D4-*t,t*-MA, $^{13}C_1$-1,2-BD, D_5-*S*-PMA and D_5-*S*-BMA, were kept at 1,000, 2,000, 20 and 20 μg/L, respectively, at each calibration level. HPLC grade water and urine samples without spiked analytes were used to examine whether the 0.1% HAC (v/v) buffer and the urine had matrix interference and/or background contamination. The method blank was measured by using the diluted urine pool spiked only with the internal standards. All of the calibration standards and blank samples were processed with the real samples in the same run batch.

2.3 Sample preparation

To prepare the quality control (QC) samples, 5.0 mL of high concentration (50-2,000 μg/L), 0.625 mL of medium concentration (6.25-250 μg/L) and 0.156 mL of low concentration (1.56-62.5 μg/L) NSM were added separately to three different volumetric flasks, each filled with 100 mL of the diluted urine pool. The QC samples were kept at -20°C until use and stability testing was performed to measure degradation over various time periods. Prior to solid phase extraction (SPE), the isotope labeled internal standard mixture solutions (100 μL) at concentrations of 20-2,000 μg/L were added to urine sample, native standards, method blank samples and QC samples. Twenty nine urine samples, donated from volunteers, were collected and stored at -20°C until sample preparation. Two milliliter of urine was taken from each sample for processing. Three mL of 0.1% acetic acid in water was then added to the urine samples to adjust the pH value to 4.5. The target analytes were extracted through SPE. The final SPE procedures were established as follows: the cartridges were conditioned with 5.0 mL of methanol, 5.0 mL of water and 10.0 mL of 0.1% HAC buffer, respectively. Then urine samples were passed through the cartridges, which were washed with 5.0 mL of 0.1% HAC buffer and 5.0 mL of distilled water. Finally, the cartridges were air-aspirated for 5-10 min and the target analytes were eluted with 4.0 mL of acetonitrile. The eluents were concentrated to 5-10 μL under a gentle current of nitrogen gas and reconstituted to 50 μL in methanol for the instrumental analysis.

2.4 Instrumental analysis

Each run batch includes freshly prepared calibration standards, blank samples and unknown urine samples. All samples were analyzed by an Agilent 6460 LC-MS Triple Quadrupole system (Santa Clara,

CA, USA), which was equipped with an Ultra HPLC 1290, G4220A Infinity Binary Pump, G1316C Infinity TCC, and a G4226A Infinity Sampler. A Zorbax Eclipse plus phenyl-hexyl (narrow bore RRHT, 600 bar, 4.6×100 mm, 1.8 μm, Agilent USA) column was employed in the LC system. The mobile phases were 0.1% HAC in water (v/v, solvent A) and methanol (solvent B). The flow rate was set at 0.4 mL/min and the column temperature was held at 40°C. The gradient elution program was set as follows: 0-5 min, 25% solvent B; 5-6 min, 25-35% solvent B; 6-10 min, 35% solvent B; 10-11 min 35-80% solvent B, 11-15 min 80-99% solvent B and holding at 99% solvent B for 3 min to elute all compounds. The LC column was then equilibrated for three more minutes. All target analytes could be eluted within 15 min, and the total analytical instrument time could be finished in 18 min. (Shown in Figure 2).Negative electrospray ionization (ESI) in multiple reaction monitoring (MRM) mode was used to record the signals of analytes and their isotope-labeled internal standards (ISs). The ion source and other MS/MS parameters were optimized by measuring a standard solution at 100 μg/L and applying the injector program or Automation Optimizer Software (Agilent, Santa Clara, CA, USA). To achieve the maximum sensitivity, the mass spectrometer parameters for the labeled ISs were also optimized. The nitrogen gas temperature was held at 300°C with a flow rate of 10.0 L/min. The nebulizer was set to 45 psi and the sheath gas temperature was also kept at 300°C with a flow rate of 11.0 L/min. The nozzle voltage was set at 2000 eV in negative ESI mode, while the capillary voltage was set at 2000 V. The mass spectrometer parameters for all analytes are listed in Table 1.

2.5 Method validation

Calibration standards were also prepared by the diluted urine pool. Calibration curve linearity, the detection limits (LODs), method precision, accuracy, matrix effects and stability were evaluated. The standard deviations of multi-calibration curve slopes were set as less than 20% to evaluate the instrumental method's accuracy and precision. The LODs of all analytes were calculated as three times the standard deviation (3×SD) of the replicate measurement (10 times) of the lowest calibration concentrations with the recoveries of 80-120%. The method precision was evaluated by calculating the relative standard deviations (RSDs) of the repetitive measurement of the QC materials at high, medium and low concentrations (50-2,000 μg/L, 6.25-250 μg/L and 1.56-62.5 μg/L, respectively). Over twenty runs were completed to evaluate the inter- and intra-days standard deviation. The relative recoveries and accuracies were calculated by dividing the measured concentrations of the target analytes by their actual spiked concentrations. Synthetic urine,[23] diluted urine pool, urine pool without dilution and unknown

urine samples were used to prepare calibration standards to quantify the three QC level samples for the assessment of the matrix effect. The measured amounts in these QC samples were compared with the spiked amounts and the acceptable recovery range was between 80%-120%. Several tests were carried out to evaluate the stability of BT metabolites in the stock solutions and in the urine after SPE. The Medium level of QC sample after SPE was analyzed 6 times under room temperature within 24 hours to evaluate the short-term stability. One batch of all three levels of QC samples after SPE were run once a week, for four weeks, to evaluate the analytes' stability in the stock solution. After 6 freeze/thaw cycles (-20°C to room temperature) over 6 days and SPE, all three levels of QC samples were found to be stable in urine. Finally, the sample storage (two months) stabilities were evaluated by comparing the amounts of analytes found with the amounts spiked in the urine; analytes were also found to be stable after this testing. Prior to daily instrumental analysis, the lowest calibration standard prepared in the diluted urine pool was analyzed. The LC/MS/MS response of each analyte in this solution was compared with the previous one to confirm acceptable LC resolution and MS sensitivity. If neither of deviations exceeded 15%, the instrumental performance (resolution and sensitivity) was considered acceptable for the analysis of a batch of samples. Two methanol blanks were run following the analysis of the highest levels of calibration standards and QC samples to examine and eliminate the potential carry-over. In order to fully confirm the practicality of this method, the 33 urine samples, were analyzed. All urine samples were collected anonymously in accordance with our institutional guidelines. For the real sample analysis, 15% of the samples in each run batch were analyzed in duplicate and the absolute relative percentage difference (RPD) was confined to a range of less than +20%.

2.6 Data statistical methods

All statistical analysis was performed by SPSS program, version 13. The significance level was $p<0.01$. Spearman's correlations (two tailed) were used to test the degree of the associations between variables as data were not normal distributed

3. Results and discussion

The method development and validation for the analysis of BT metabolites were completed over six months. Table 1-3 lists the validated parameters; including optimized MS/MS parameters, linearity, LODs, accuracy and precision data. The optimal MS/MS parameters included MRM transitions, fragmentor and CE (Table 1). The MRM transitions in this study (Table 1) are the same as those

previously reported.[9,15] The linearity range is wide enough and covered the reported concentration ranges in the real urine samples.[8,9,17] The analysis of the water blank and the urine blank indicated that there were no interferences or contaminations from the whole procedure. The methanol blank run after the highest calibration curve standards and QC samples were clean and no carry-overs were found.

3.1 Chromatographic separation and efficiency

Four HPLC columns were tested, including Zorbax Eclipse plus phenyl-hexyl column (narrow bore RRHT, 600 bar, 4.6×100 mm, 1.8 μm, Agilent, USA), Zorbax Eclipse XDB-C18 column (narrow bore RRHT, 600 bar, 2.1×30 mm, 1.8 μm, Agilent, USA), Chromolith performance RP-18e column (4.6x100 mm, 2 μm, Merck, Germany) and Zorbax RRHD Eclipse plus C18 column (2.1×100 mm, 1.8 μm, Agilent, USA). The Zorbax Eclipse plus phenyl-hexyl column was chosen as the separation column in this study because all target analytes could be eluted within 15 min and well separated, and the whole instrumental analysis could be finished within 18 min (Figure 1 and 2). Due to the fact that the four target analytes are polar and acidic compounds and most of them have benzene rings, Zorbax Eclipse plus phenyl-hexyl column offered better chromatographic separation and peak shapes when 0.1% acetic acid in water was used as a mobile phase, especially for *t,t*-MA. Alternatively, all the target analytes could be separated in 10 min when 35% methanol in water was used as an initial mobile phase at a flow rate of 0.5 mL/min. However, we found that the first eluted two analytes, *t,t*-MA and 1,2-DB, were affected by urine matrix. In order to reduce such interferences for these two analytes in real urine samples, several gradient programs were tested with varying ratios of the initial mobile phases. The results showed that the use of a low methanol (25%) content as an initial mobile phase and a lower flow rate (0.4 mL/min) could separate the more polar interference compounds from the two early eluting analytes, which allowed the early eluting analytes to be interference free and be quantified accurately.

3.2 Optimization of sample preparation

Liquid-liquid extraction (LLE) and SPE are commonly used to extract target analytes in urine. In contrast with LLE method, SPE method is more convenient and efficient, and has been applied to measure the urinary metabolites of BT in published studies.[14,16-20] A strong anion exchange (SAX) cartridge is often used to extract polar and ionic compounds due to its specificity.[22,24] In our study, two types of SPE cartridges, including the Bond Elut SAX polymer cartridge (100 mg, 3 mL, Agilent Technology, Santa Clara, CA, USA) and the Bond Elut C18 SPE cartridge (500 mg, 6 mL, Varian,

Santa Clara, CA, USA) were evaluated. The SPE clean-up procedure was established by comparing Bond Elut C18 cartridge (500 mg, 6 mL) and Bond Elut strong anion exchange (SAX) polymer cartridge (100 mg, 3 mL) by testing different conditioning and elution solvents. Five milliliter of methanol, and water were used to condition the cartridge. After the urine sample was loaded, it was then washed with 5.0 mL of 0.1% HAC buffer and 5.0 mL of water. In order to determine whether methanol or acetonitrile was the more efficient solvent to elute the target analytes, both cartridges containing the target analytes were eluted five times with 1.0 mL of methanol and 1.0 mL of acetonitrile. Five fractions were collected for each solvent for qualitative and quantitative analysis. Five milliliter of 0.1 mM ammonium buffer was used to condition the Bond Elut SAX cartridge. The Bond Elut C18 SPE cartridge was chosen due to its high recoveries, stability and low cost (data not shown). *t,t*-MA was found to elute together with the bulk urine during the sample loading step, which indicated that *t,t*-MA was not effectively retained on the C-18 SPE cartridge due to its strong polarity. In order to improve SPE efficiency, 10.0 mL of 0.1% HAC buffer was used to condition the cartridge before urine samples were loaded. In the washing step, we found that *t,t*-MA could elute with 10% methanol in water. Then 5.0 mL 0.1% HAC in water was used to wash the cartridge. In the elution step, it was found that 4.0 mL of acetonitrile could completely elute all of the target analytes, while 4.0 mL of methanol could not. Therefore, acetonitrile was chosen as the optimal elution solvent.

3.3 Linearity, LODs, accuracy and precision

Our results showed that the calibration curves in all run batches had good linearity and slope deviations (below 20%) with a weighted (1/x) linear regression (r^2 >0.990). Most compounds demonstrated a wider linearity range covering 3-4 orders of magnitude. The LODs were 7.8 µg/L for *t, t*-MA, 0.69 µg/L for 1, 2-DB, 0.10 µg/L for *S*-PMA and 0.08 µg/L for *S*-BMA (Table 2). LOD for *t,t*-MA in our study was comparable to the value reported by HPLC/UVD (ultra-violet detection) method.[20] LODs of the three other analytes in our study were two, six and even seventy times lower than those in the previously reported studies (Table 2).[8, 14, 16, 26-27] The imprecision or coefficient of variation (CV) for the method was within 15% and the accuracy was within 100±15% of the expected amounts. However, the accuracy for 1,2-DB was 73.1% in the lowest levels of QC samples, which was out of the range of 80%-120%. We observed that an interfering peak partially co-eluted with 1,2-DB in the urine sample and could cause a low result (Figure 2). The recoveries and accuracies of every other analyte ranged from 88.7% to 107%, which met the required recovery range of 80% to 120% (Table 3).[25]

3.4 Stabilities

The prepared samples remained stable after 24 hour post preparation at room temperature (low CVs shown in Table 4). The assessment of stability experiments showed good storage stabilities with the CVs lower than 10%, except for1,2-DB (Table 5). Six freeze/thaw cycle stabilities were tested and was acceptable based on low CVs (all<11.6%, Table 5). Long term freeze/thaw stabilities (-70ºC for over 2 months) on selected QC samples (250 µg/L for *t,t*-MA, 125 µg/L for 1,2-DB, 100 µg/L for *S*-PMA and 50 µg/L for *S*-BMA) were also acceptable based on low CVs with a range of 3.5% to 10.5% (Figure 3). It has been shown that *t,t*-MA in urine would degrade when kept under the extreme condition, such as in 1.0 mL of 9 M H_2SO_4 for 10 min, followed by a pH adjustment with addition of a 50% NaOH solution.[15] However, our experiment showed that all of the target compounds in solution remained stable in short and middle term, freeze/thaw cycle tests and under long term freeze conditions after SPE preparation. These results are consistent with those stability studies reported under similar conditions.[16]

3.5 Matrix effects

Matrix effects could cause suppression or enhancement of MS/MS signals and are a challenging factor in the mass spectrometric analysis of biological fluids. Many studies have examined matrix effects and found they varied with the analytes and analytical methods.[14-15, 28-29] Sabatini et al. used HPLC/MS/MS method for the determination of urinary *S*-PMA, *S*-BMA and *o-methylbenzyl* mercapturic and found no matrix effects during the quantification.[14] In our study, four different matrices were processed and the target analytes in the three QC levels were quantified (as described above and shown in Figure 4). Though there has been no consensus on how the matrix effects should be assessed or eliminated, the use of the isotope labeled internal standards could compensate for the matrix effects.[29] As no other commercial IS was available, 1,2-dihydroxybenzene-1-$^{13}C_1$ was used as the IS of 1,2-DB for quantification. The mass difference of two compounds is smaller than 3Amu. To check the interferences from IS, blank samples spiked with 1,2-DB at the upper limit of the calibration range but without IS was analyzed and no interferences was found. Thus, 1,2-dihydroxybenzene-1-$^{13}C_1$ was chosen as the IS to determine the urinary 1,2-DB.

As analyte-free matrices do not exist, to minimize the matrix effects, a 1/10 diluted urine pool was used as an alternative matrix to prepare calibration standards. Screening showed that the 1/10 diluted urine pool contained target analytes at the level much lower than the LODs. Another benefit of

using 1/10 diluted urine pool was that it compensated for matrix effects in real urine samples to the greatest degree. In contrast, synthetic urine free of target analytes couldn't compensate for the matrix effect for *t,t*-MA in the high levels of QC sample, indicated by an accuracy of larger than 120%. Additionally, *t,t*-MA and *S*-BMA in the low levels of QC sample could not be well quantified with calibration standards prepared in the pooled urine without dilution. Furthermore because the levels of endogenous analytes were comparable to those in calibration standards and to those in QC samples, *S*-BMA in the low levels of QC samples could also not be well quantified with calibration standards prepared in the real urine samples (Figure 4).

3.6 Application of method

The validated analytical method was applied to measure *t,t*-MA, 1,2-DB, *S*-PMA and *S*-BMA in the real urine samples (Figure 2). The samples are from anonymous donors. The results are shown in Table 6. QC samples were analyzed in the sample batches of real urine and the results met the established criteria. No contaminations were found in the water or urine blank and no carry-overs were observed in the methanol blank after analyzing the highest level of QC samples and calibration standard solutions. The RPDs of duplicate analysis were below 20%, except for 1,2-DB in one sample (sample had a concentration close to the quantitation detection limit). *S*-BMA and *t,t*-MA could be detected and quantified in most of the real urine samples (Table 6). In our tests, the detection frequency of S-BMA and *S*-PMA was close to 100%, which was higher than the value reported by Sabatini. [14] Besides of being stable, sensitive and specific, a good biomarker should be detected easily and measured reproducibly.[32] S-PMA meets all the requirement of being a good biomarker and was used to evaluate low levels of benzene exposure,[9, 16, 22] Hence, the determination of S-BMA and S-PMA together could comprehensively reflect the exposure levels to BT.

Interestingly, a strong correlation existed between *S*-BMA and *t,t*-MA ($r=0.631$, $p<0.01$) as well as *S*-BMA and the total concentration of both BT metabolites ($r=0.593$, $p<0.01$). As sample number in this study were limited and *t,t*-MA accounted for almost 80% of the total concentrations of the metabolites of BT, a large population study could be used to evaluate the relationships between individual metabolite both for non-occupational and occupational populations .

4. Conclusions

A new method was fully developed and validated to simultaneously determine urinary *t,t*-MA, 1,2-DB, *S*-PMA and *S*-BMA concentrations by UHPLC/MS/MS in negative ESI mode. All target analytes could be eluted and quantified within 18 min. Good precision and high accuracy were obtained by using the isotope internal standards. The LODs were 7.8 μg/L for *t,t*-MA, 0.69 for μg/L for 1,2-DB, 0.10 μg/L for *S*-PMA and 0.08 μg/L for *S*-BMA. Except for *t,t*-MA, the LODs of other three target compounds were much lower than those in previous reports. Matrix effects were noticed and it was found that using 10 times diluted urine pool to prepare calibration standards and the employing of isotope labeled standards could reduce the matrix effects effectively. Short, middle, and long-term storage stability studies after SPE and freeze/thaw cycles all showed that the analytes remained stable. The method was successfully employed in the simultaneous determination of four compounds to assess the overall human exposure to BT. Preliminary results showed that *S*-BMA was a sensitive biomarker of toluene and there were significant correlations between *S*-BMA and *t,t*-MA. Moreover, *S*-BMA and *S*-PMA together are effective and sensitive biomarkers to evaluate the exposure of general and/or occupational population to BT.

Conflict of interest:

The authors declare no conflicts of interests. All the urine samples for validation were obtained the anonymous donors in accordance with our institutional guidelines.

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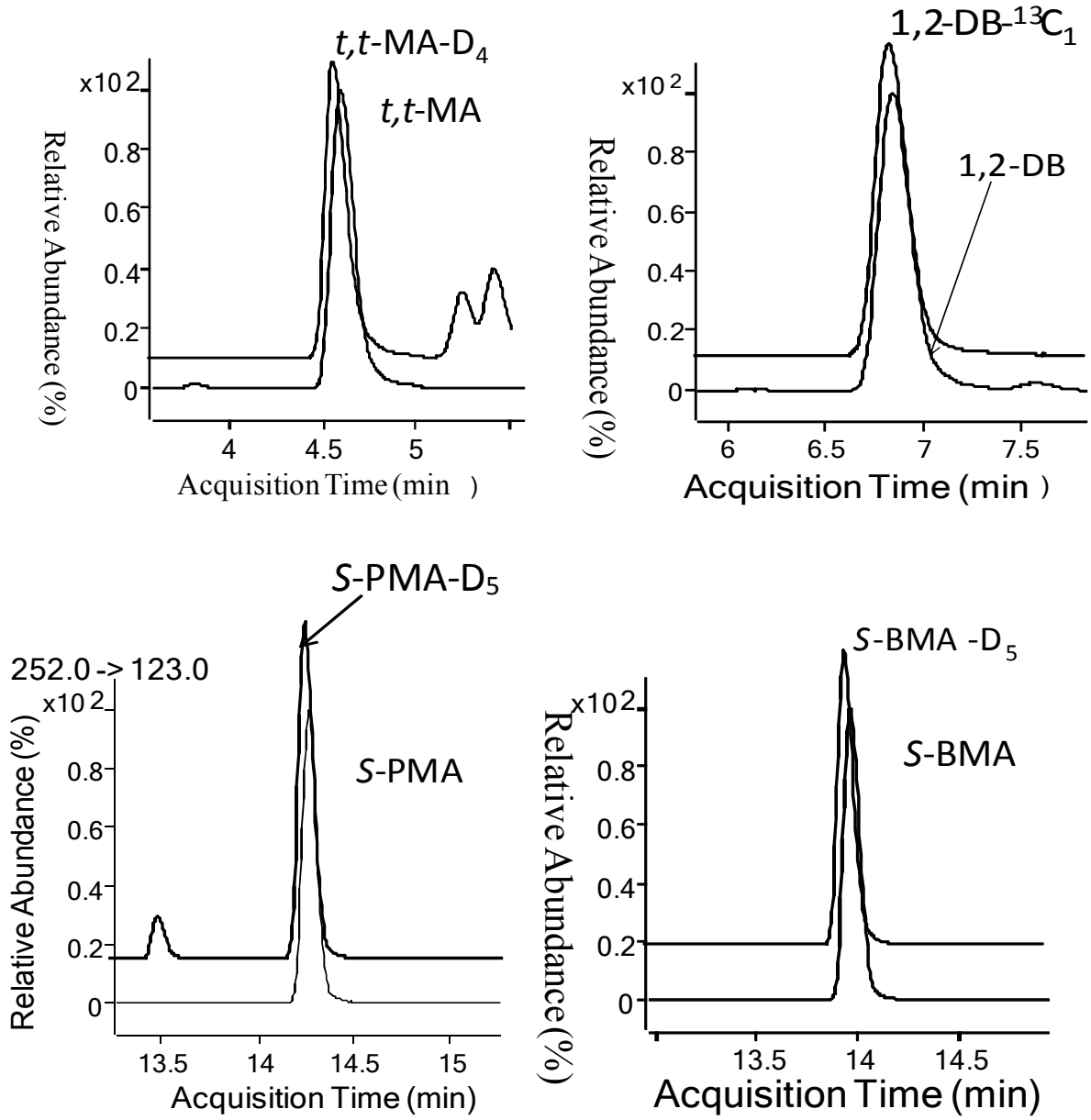


Fig. 1 The LC/MS/MS chromatograms of standards prepared in 1/10 diluted urine pool, the concentrations are 125 μg/L for *t, t*-MA, 62.5 μg/L for 1,2-DB, 3.13 μg/L for *S*-BMA and 6.25 μg/L for *S*-PMA, respectively.

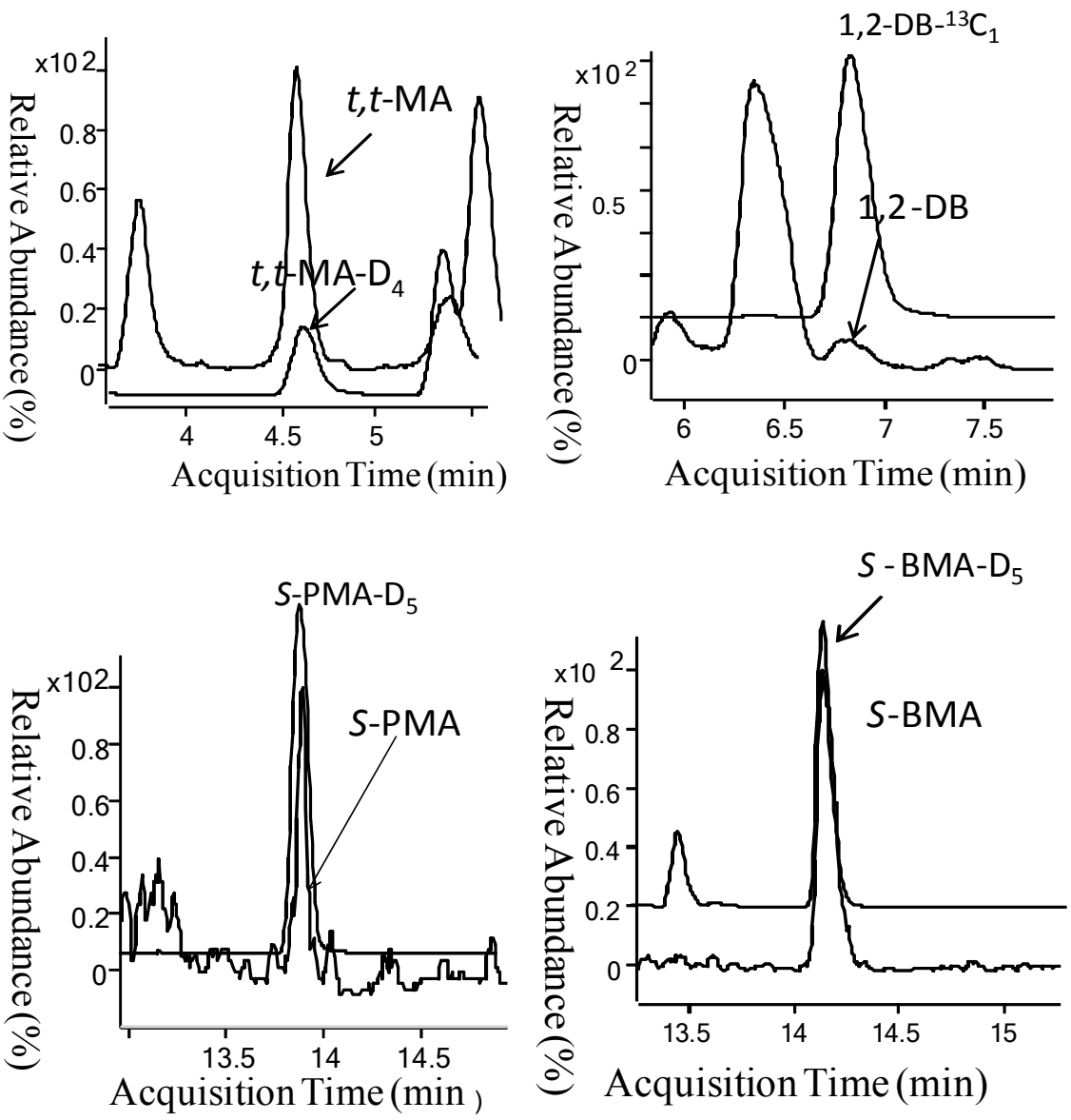


Fig. 2 The chromatograms of a real urine sample, the concentrations are 20.98 μg/L for *t, t*-MA, LOQ for 1, 2-DB, 0.67μg/L for *S*-BMA and 0.22 μg/L for *S*-PMA, respectively.

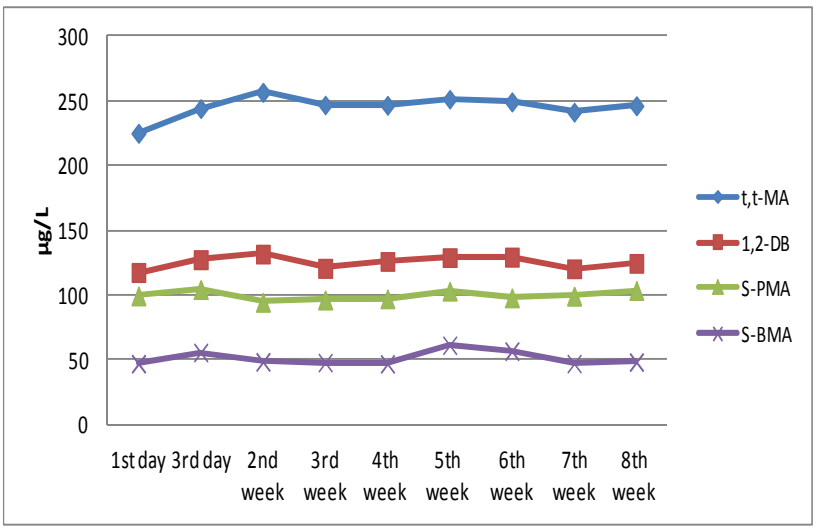


Fig. 3 The storage stability of target analytes at -20℃ for 8 weeks.

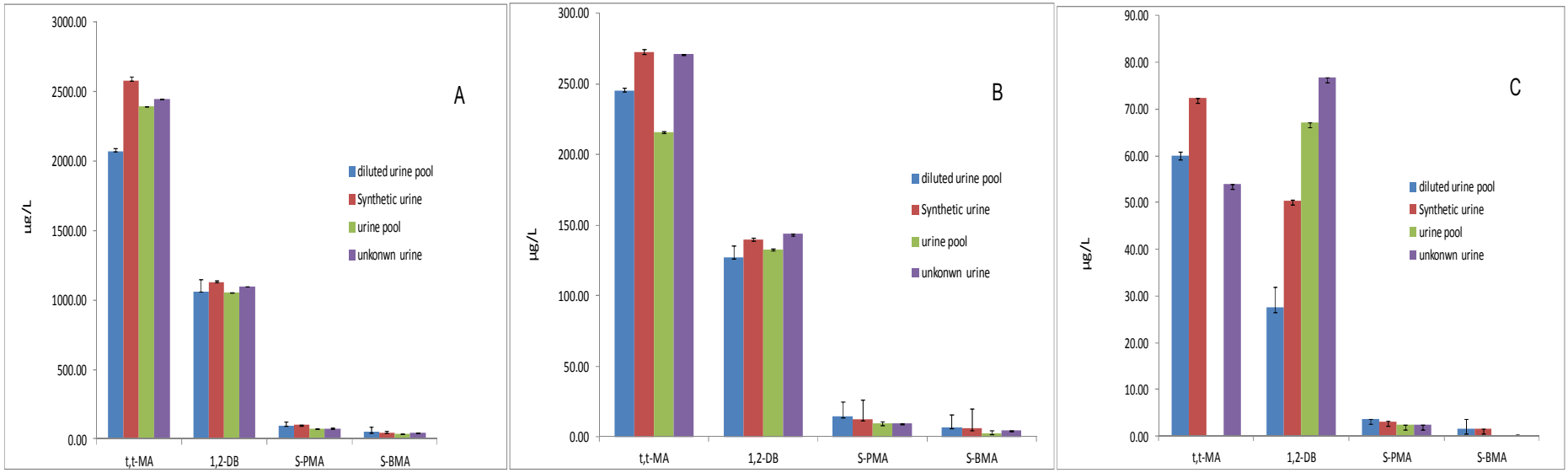


Fig 4 The mean concentrations of QC samples in three levels quantified by calibration curves prepared with 1/10 diluted urine pool, synthetic urine, urine pool and unknown urine, respectively (n=3 for each batch). A: QC high (50-2,000 μg/L); B: QC: medium (6.25-250 μg/L); C: QC low (1.56-62.5 μg/L)

Table 1 The parameters of tandem mass spectrometer, retention time, linear range and LODs in 1/10 diluted urine pool.

compound	*Abbreviation*	*MRM transitions*	*Fragmentor (V)*	*CE (V)*	*Linear range (μg/L)*	*LODs (μg/L)*	*Retention time (min)*
trans,trans-muconic acid	*t t*-MA	141-97	85	6	15.6-4000	7.8	4.60
trans,trans-muconic acid-D_4	*t,t*-MA-D_4	145-101	75	5			4.54
1,2-Dihydroxybenzene (catechol)	1, 2-DB	109-108	100	10	7.81-2000	0.69	6.86
1,2-Dihydroxybenzene-1-$^{13}C_1$	1, 2-DB-$^{13}C_1$	110-109	90	20			6.83
S-phenylmercapturic acid	*S*-PMA	238-109	90	8	0.20-200	0.10	14.00
S-phenylmercapturic acid-D_5 potassium	*S*-PMA-D_5	243-114	70	18			13.91
S-benylmercapturic acid	*S*-BMA	252-123	90	10	0.39-100	0.08	14.22
S-benylmercapturic acid-D_5	*S*-BMA-D_5	257-128	70	15			14.26

Table 2 Comparisons of LODs in this study with those reported in the literatures (unit:μg/L)

reference	*method*	*t, t*-MA	1,2-DB	*S*-PMA	*S*-BMA
In this study	*LC/MS/MS*	7.8	0.69	0.10	0.08
Marrubini et ***a****l, 2001*[26]	*HPLC-UV*	50			
Sabatini et al., 2008[14]	*LC/MS/MS*			0.30	0.60
Lin et al., 2006[16]	*LC/MS/MS*	1.27μg/g		0.042μg/g	
Pieri et al., 2003[8]	*LC/MS/MS*			5	
Lee et al., 2005[20]	*HPLC-UV*	5			
Takayasu et al., 2001[27]	*GC/MS*		50		
Ruppert et al., 1995[19]	*GC/MS*	10			
Schettgen et al, 2008[9]	*LC/MS/MS*			0.02	0.02
B'Hymer, 2011[31]	*LC/MS/MS*			0.2	0.2

Table 3 Method precision and accuracy (n=20) in 1/10 diluted urine pool spiked at various concentrations.

analyte	*Added amount (μg/L)*	*Expected amount (μg/L)*	*Measured mean (μg/L)*	*Accuracy (% of expected)*	*Precision CV (%)*
t, t-MA	0		8.274		
	2000	2008	2123	106	3.61
	250.0	258.3	245.0	94.9	2.61
	62.50	70.77	62.75	88.7	4.52
1,2-DB	0		4.247		
	1000	1004	1074	107	3.11
	125.0	129.2	127.0	98.3	3.39
	31.25	35.50	25.94	73.1	14.6
S-PMA	0		0.067		
	100.0	100.1	99.30	99.2	3.12
	12.50	12.57	14.17	113	6.39
	3.125	3.192	3.578	112	9.37
S-BMA	0		0.075		
	50.00	50.08	51.27	102	9.62
	6.250	6.325	6.904	114	9.15
	1.560	1.635	1.695	104	11.7

Table 4 The short-term stability results using QC urine samples during 24 hours at room temperature

Comp.	*Short-term stability*		
	Spiked Conc.	*mean±SD*	*CV*
t, t-MA	250	244±3.22	1.32
1,2-DB	125	117±1.12	0.96
S-PMA	12.5	12.6±0.11	0.85
S-BMA	6.25	6.51±0.12	1.79

Table 5 The stability results (stored in methanol after SPE) for one month; and 6 cycles of freeze-thaw stability of QC samples during 6 days

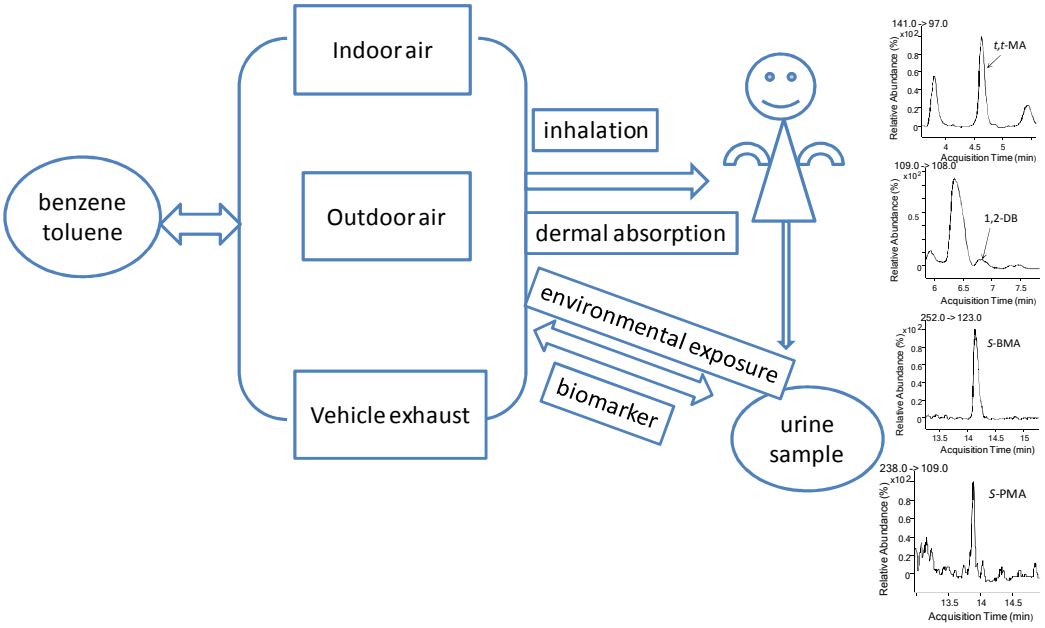
Comp.	*Stability of samples stored in solvent after SPE for one month*								
	Spiked Conc.	*Mean±SD*	*CV*	*Spiked Conc.*	*Mean±SD*	*CV*	*Spiked Conc.*	*Mean±SD*	*CV*
t, t-MA	2,000	2116±47.4	2.2	250	247.2±16.4	6.6	62.5	62.2±2.4	3.8
1, 2-DB	1,000	1047±29.5	2.8	125	130.1±12.1	9.3	31.2	33.4±8.7	25.9
S-PMA	100.0	95.9±6.3	6.6	12.5	12.2±1.1	8.9	3.1	2.9±0.2	6.7
S-BMA	50.0	49.9±2.4	4.8	6.2	6.0±0.2	2.6	1.6	1.5±0.1	7.9
Comp.	*Freeze-thaw stability of the analytes in QC samples for 6 cycles during 6 days*								
	Spiked Conc.	*Mean±SD*	*CV*	*Spiked Conc.*	*Mean±SD*	*CV*	*Spiked Conc.*	*Mean±SD*	*CV*
t, t-MA	2,000	2055±13.9	0.7	250	240±6.9	2.9	62.5	60.4±1.3	2.2
1, 2-DB	1,000	924±10.8	1.2	125	95.5±4.0	4.2	31.2	16.7±1.9	11.6
S-PMA	100.0	82.2±0.5	0.6	12.5	10.4±0.1	0.9	3.1	2.7±0.00	0.9
S-BMA	50.0	48.2±0.5	10.1	6.2	5.9±0.1	1.4	1.6	1.4±0.00	2.3

Table 6 The results of real urine samples (N=33, unit μg/L; As sample number is limited, only median concentrations were presented)

Compound	*Mean± SD*	*Median*	*Range*	*Numbers of N.D.*	*Numbers of B.L.Q.*
t, t-MA	74.4±85.9	67.8	N.D.-381	1	1
1,2-DB	192.5±182.6	130.7	N.D.-546	1	1
S-PMA	3.35±1.72	3.02	N.D.-1.84	2	1
S-BMA	15.0±15.7	8.66	B.L.Q.-17.3	0	1

N.D., no detection; B.L.Q., below quantitative limit

Graphical Abstract



This study reported a method to simultaneously determine *trans, trans*-muconic acid (*t,t*-MA), 1,2-dihydroxybenzene (or catechol, abbreviated as 1,2-DB), *S*-phenylmercapturic acid (*S*-PMA) and *S*-benzylmercapturic acid (*S*-BMA) in human urine. Samples were prepared through solid phase extraction and analyzed by ultra-high performance liquid chromatography/tandem mass spectrometer in negative electrospray ionization mode. The method was fully validated through the studies of precision, accuracy, matrix effects, detection limit, linear range, stability and real urine sample tests. Calibration curves of all target analytes showed favorable linearty within the wide concentration range of 0.2-4,000 μg/L. The detection limits in 10 times diluted pooled urine ranged from 0.08 to 7.8 μg/L. The method showed satisfactory accuracies and precisions. Except for the low spiked quality control (QC) level of 1,2-DB (73.1% recoveries), recoveries were in the range of 100 ±15% with a variation coefficient of less than 15%. Target analytes were stable in stock solutions and spiked urine samples under storage and test conditions. Twenty nine anonymous real urine samples from non-occupational donors were analyzed for all target analytes. Except for *S*-PMA, the other three compounds could be detected and quantified. Preliminary results showed that *S*-BMA was a sensitive and specific biomarker of

toluene exposure and had significant correlation with *t,t*-MA (r=0.631, p<0.01). Moreover, *S*-BMA and *S*-PMA together would reflect the overall exposure to BT at low levels.