Quantitation of Urinary Volatile Nitrosamines from Exposure to Tobacco Smoke*

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A sensitive and selective method was developed and validated to detect six volatile nitrosamines [N-nitrosodimethylamine, N-nitrosomethylamphetamine,  N-nitrosodiphenylamine, N-nitrosodiphenylmethane, N-nitrosodiphenylamine, N-nitrosopyrrolidine and N-nitrosomorpholine] in human urine. This method uses a liquid–liquid extraction cartridge followed by analysis with gas chromatography–tandem mass spectrometry (GC–MS-MS) and quantification based on isotopic dilution. This is the first GC–MS-MS method reported for measuring volatile nitrosamines in human urine. This method reduces the sample volume required in other methods from 5–25 to 2 mL. The limits of detection (2.62, 1.99, 2.73, 0.65, 0.25, 3.66 pg/mL, respectively) were better than existing methods, largely because of improved positive chemical ionization achieved by using ammonia gas and reducing background noise. Using nitrogen as the collision gas allowed the confirmation transition in the low mass region to be monitored. The analysis of human urine using this validated method is accurate (relative bias of 0–19%) and precise (relative standard deviation of 0.2–18% over two months of analyses). The validated method was applied to 100 urine samples and the levels of all six volatile nitrosamines were reported for the first time in urine specimens collected from smokers and nonsmokers, with smoking status determined by urinary cotinine measurement. Among 100 smokers and nonsmokers, the levels of three analytes (N-nitrosodimethylamine, N-nitrosomethylamphetamine and N-nitrosodiphenylamine) were significantly higher in smokers than nonsmokers (p < 0.05).

Introduction

Cigarette smoking is a significant risk factor for human cancer, cardiovascular disease and respiratory illness (1). Volatile nitrosamines (VNAs) are teratogens and carcinogens (2, 3) found in tobacco smoke at high levels (4, 5). They also have relatively high cancer risk indexes (6). People are potentially exposed to VNAs from contaminated water (average mean 2 mg nitrate/L), food (0.2–17 μg/kg), certain occupational uses (0.15–0.31 μmol/day) and tobacco products (0.1–27 ng/cigarette in mainstream, 7–415 ng/cigarette in sidestream) (5, 7–19). Smoking or using smokeless tobacco products may cause higher exposure to VNAs compared with most other sources (4, 20–22). Exposure to secondhand smoke may also lead to exposure to VNAs because the levels of these compounds are significantly higher in sidestream smoke than in mainstream smoke (5). In their work with four different US commercial cigarettes, Adams et al. reported that the levels of N-nitrosodimethylamine (NDMA), N-nitrosopiperidine (NPIP) and N-nitrosopyrrolidine (NPYR) are 4.1–31.1, 5.9–14.2 and 10.2–64.5 ng/cigarette, respectively, in mainstream smoke. Higher levels of VNAs are found in sidestream smoke [NDMA (597–735 ng/cigarette), NPIP (4.8–19.8 ng/cigarette) and NPYR (117–234 ng/cigarette)] (4). Brunemann et al. also found similar levels for NDMA and NPYR in mainstream and sidestream smoke generated by Swiss and German cigarettes (5). Other studies have found similar or higher levels of VNAs in tobacco smoke or environmental tobacco smoke (23, 24). According to Hoffmann et al., VNAs are likely formed during the pyrolysis of proteins and amino acids (25). Given the toxicity of these compounds, monitoring these compounds in smokers and nonsmokers is prudent. Other methods have measured some VNAs in urine as biomarkers of exposure from food (7, 13, 14, 26, 27) or drinking water (8–10, 18, 28). These reported methods utilized gas chromatographic analysis followed by mass spectrometry, thermal energy analysis or nitrogen chemiluminescence detection. They also required relatively high sample volumes of 5–25 mL of urine. High sample volume is often not feasible for large epidemiological or population studies such as the National Health and Nutrition Examination Survey (NHANES) because of the limited availability of specimens. Therefore, a method was needed that not only required minimal sample volume, but was also sensitive enough to measure these VNAs in urine, collected as part of large population studies. A highly sensitive method is crucial for measuring VNA exposure attributable to secondhand smoke exposure. In this article, a sensitive and selective method is presented for measuring six volatile nitrosamines [NDMA, N-nitrosomethylamphetamine (NMEA), N-nitrosodiphenylamine (NDEA), NPIP, NPYR and N-nitrosomorpholine (NMOR)] in human urine (Figure 1). The reported method utilizes gas chromatography–tandem mass spectrometry (GC–MS-MS) with positive chemical ionization (PCI). PCI with ammonia significantly improved the limit of detection (LOD) more than PCI with methane or electron impact (EI) (0.09-0.4 ng/mL). This gain in sensitivity allowed the sample volume to be reduced from 5–25 to 2 mL. The validated method was applied to 100 urine samples from smokers and nonsmokers. For the first time, the levels of six VNAs in human urine were reported as biomarkers for tobacco exposure in smokers and non-smokers. It is also the first GC–MS-MS method applied to the measuring of VNAs in human urine.

Method and Materials

Materials

Native analytes were purchased from Supelco (Belleville, PA) as a mixed standard of 2,000 μg/mL in dichloromethane (DCM). 2H-labeled analytes were purchased from Cambridge Isotope

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Laboratory (Andover, MA) and Toronto Research Chemical (Toronto, Canada). All solvents were from Honeywell Burdick & Jackson (VWR, Suwanee, GA). ChemElut cartridges (3 mL, pH 9.0) were obtained from Agilent (New Castle, DE). Siliconized test tubes (13 × 125 mm) were purchased from Lab Depot (Dawsonville, GA). All GC liners (double taper, dimple, single taper helix, double taper helix and focus) were purchased from Agilent.

Samples and quality control
Nonsmoker urine samples were collected in-house with Institutional Review Board (IRB) approval. Fifty spot urine samples from nonsmokers were collected anonymously via donating a sample in a restroom during a working day. Nonsmoker donors indicated on the collection cup whether they consumed any caffeinated beverage. Among 50 nonsmokers, 30 participants checked caffeine consumption and 20 checked no caffeine consumption. Fifty urine samples from smokers were purchased from Tennessee Blood Service (Nashville, TN) with no identifiable information. Because the smoker urine samples were acquired from commercial sources, the analysis of these samples did not meet the definition of human subjects as specified in 45 CFR 46.102 (f). Quality control (QC) materials were prepared by pooling two liters of non-smoker urine and spiking the pooled urine with mixed VNA standards. The final concentrations were 20 and 200 pg/mL for low and high QC, respectively. Blank QC material was prepared from pooled nonsmoker urine that contained no measurable levels of the monitored VNA compounds. The precision of the assay was characterized based on 20 separate analyses of QC pools. QC results were subsequently used to verify methodological precision for each analytical run, according to modified Westgard QC rules (29). If QC sample results did not meet acceptance criteria for an analyte, all results for that analyte in that batch were rejected. All QC and blank samples were stored at −70°C.

Sample preparation
Each two milliliters of urine samples were spiked with 100 pg of internal standard (ISTD). If the samples were frozen, they were thawed at room temperature before spiking with the ISTD. The sample was vortexed and loaded onto ChemElut cartridges, allowed to stand for 10 min and gravitationally eluted with 5 × 2 mL of DCM into siliconized test tubes. The eluent was then dried to approximately 200 µL of residual volume in a Savant vacuum evaporator and transferred to a 300 µL amber vial with insert. Twenty-five microliters of acetonitrile (ACN) was added as a keeper, and all of the remaining DCM was evaporated using a Savant vacuum evaporator (ThermoFisher Savant SPD2010 Speedvac Concentrator). Drying times were examined initially, and the optimal time was used on a designated Savant to maintain consistent evaporation.

Instrumental analysis
An Agilent 7000B GC–MS-MS was used for analyses. The GC was an Agilent 7890 equipped with a multimode inlet (MMI). The injection volume was 5 µL. The GC column was an Agilent DB-WaxERT (15 m × 0.32 mm, 0.25 µm). Two columns, collected by a metal union, were used for analysis with constant flow for column one and constant pressure for column two. A 2-column setup allowed for more efficient back-flush after each GC run. Programmed temperature vaporization (PTV), pulsed splitless and solvent vent injection modes were examined, and all final analyses were conducted with solvent vent injection. Solvent vent injection allowed for significant solvent removal in a large volume injection before transferring the analyte onto a GC analytical column. In solvent vent mode, the inlet was kept at a temperature lower than the boiling point of the solvent. Initially, the inlet was in split mode, and the evaporating solvent was removed through the out-venting valve. After an optimal hold time for solvent evaporation, the inlet was heated and switched to splitless mode, and analytes were transferred to the analytical column along with any remaining solvent. Finally, the inlet was switched to purging to remove any remaining material. The GC oven initial temperature was 35°C and held for 1 min. The temperature gradient was 20°C/min, and the final temperature was 240°C. The chemical ionization (CI) gas was ammonia (blue grade) and the collision gas was nitrogen (ultra-high purity). Quantitative analyses were conducted using multiple reaction monitoring (MRM) mode. All other operation parameters are listed in the Supplemental tables.

Analyte identification was confirmed by both retention time and the ratio of primary quantification and confirmation ions. A full set of 10 calibrators and one blank was analyzed with each set of samples to generate calibration curves for that analytical run. A linear 1/x fit was used to create calibration curves ($r^2 ≥ 0.99$) that typically spanned 3–4 orders of magnitude. The LOD was calculated according to the guideline for determination of limits of detection by the Clinical and Laboratory Standard Institute (30). Armbruster and Pry also reported a similar
approach in calculating the LOD and limit of quantification (LOQ) (31).

**Proficiency testing**

Proficiency testing (PT) was conducted by analyzing a series of five blind, VNA-spiked urine samples every six months. PT samples were blind-coded by an external QC officer. If blind-analyzed concentrations fell within 25% of known values for an analyte, the PT was passed.

**Method Validation**

Method validation was conducted as described in the following sections.

**Recovery**

Recovery was calculated by comparing the average ISTD counts from 10 standard solutions to the average of internal standard counts from 20 samples, each conducted in duplicate.

**Precision**

Intermediate precision was obtained from at least two pools at different levels from multiple runs over a period of at least one week, with \( N > 5 \) for each pool. The coefficient of variation (CV) was calculated for each level and accepted only if it was less than or equal to 10%.

**Short-term precision**

At least six repetitive injections of a low, a medium and a high standard were made, and the standard deviation (SD) and CV were calculated.

**Accuracy**

If precision was acceptable, assays were performed with blank urines spiked with analyte at low, medium and high range levels. Each spiked pool was analyzed at least three times, and the bias in the results was calculated as: 

\[
\text{Bias} \, (\%) = \frac{\text{Observed} - \text{Expected}}{\text{Expected}} \times 100\%.
\]

**Blanks**

A series of blanks was analyzed through the entire procedure and any interference was determined.

**Linearity**

Data from the accuracy evaluation were used to ensure the linearity of the results over the concentration range.

**Carryover**

Runs in which high and low samples were intermixed with low samples and/or blanks were conducted immediately following the high samples. This arrangement was maintained during both cleanup and MS analysis, and carryover was assessed under these conditions.

**Stability**

(i) Two well-mixed pools were prepared and assayed at least in duplicate. Over a period of about a week, this pool was frozen, re-thawed and reanalyzed. The effect of freeze-thaw cycles on the results was evaluated. (ii) Residual frozen aliquots from a series of analyses was re-assayed over a period of days, and the storage stability of these processed samples was determined.

**LOD and LOQ**

An initial LOD was estimated from a series of standard injections \( (N = 6) \); the final LOD was calculated according to the guideline for the determination of LODs by the Clinical and Laboratory Standard Institute \( (30) \). Four urine pools at 0.05, 0.1, 0.5 and 5 \( \mu \)g/\( \mu \)L of all six analytes and a urine blank were used. The LODs were obtained from 39 independent runs and by two different analysts.

**Robustness**

A control pool was analyzed for ruggedness by varying: (i) wait time after sample loading on a ChemElut cartridge at 5, 10 (standard condition) and 15 min; (ii) volume of DCM used for analyte elution at 4 \( \times 2 \) mL, 5 \( \times 2 \) mL (standard condition) and 6 \( \times 2 \) mL; (iii) source temperature at 200, 250 (standard condition) and 300°C; (iv) chemical ionization gas flow at 20%, 25% (standard condition) and 30%; and (v) volume injection at 3, 4 and 5 \( \mu \)L (standard condition).

**QA and QC**

Final characterization was conducted (at least 20 replicates over at least 10 days) for the QC pools to be used in the studies. The QC chart was obtained using an SAS program.

**Results**

Compared to direct extraction with DCM \( (8, 10, 14, 18, 32–35) \), a simple liquid–liquid (L–L) extraction using ChemElut cartridges and DCM increased analyte recovery by 50%. The addition of 10% methanol or ethanol in DCM to improve the extraction of polar compounds did not improve analyte recovery. Because DCM is volatile (boiling point, 40°C), the ACN keeper solvent reduced sample loss up to 90% in cases when all of the DCM evaporated to dryness. In addition to ACN, MeOH and toluene were evaluated as keeper solvents; all retained 70–90% of the target analytes. ACN was chosen because it was most compatible with the solvent vent-injection mode with starting temperature at 35°C. Subsequent GC fully resolved all six VNAs with a run time of 12 min (Figure 2).

Five inlet liners were tested: regular double taper, dimple, single taper helix, double taper helix and focus. The single taper helix produced the highest sensitivity and best peak shapes. Compared to pulsed splitless injection mode with a double taper liner, the LOD was improved approximately twofold when
solvent vent injection mode was applied with a single taper helix liner. Optimal collision energy (CE) for each MS-MS transition is listed in Table I, and other optimal operational parameters are provided in the Supplemental section. Together with solvent vent injection mode and a single taper helix liner, positive CI with ammonia improved the sensitivity from 10-fold to 50-fold compared to EI or CI with methane, and significantly improved the LOD (Table II).

Other parameters that greatly affected the sensitivity were source temperatures and CI gas flow. A CI gas flow of 25% (approximately 1.2 mL/min) and source temperature of 250°C was the best combination for optimizing sensitivity and reproducibility for all analytes.

Method validation

The recovery averaged 70–90% for all six measured VNAs. Short-term precision was estimated by the repetitive analysis of three levels of standards with concentrations of 0.5, 5.0 and 50.0 pg/mL. A single run with five replicates each resulted in relative standard deviations (RSDs) of 6.7, 4.6 and 1.7%, respectively, for the three concentrations. Inter-day precision was estimated over a period of two weeks with daily analyses of the three pools of low, medium and high levels. Two independent runs were analyzed each day, and the observed day-to-day precision for analytes except NMEA was less than 5% for all pools (details provided in Supplemental tables). The day-to-day precision for NMEA was 16.98, 18.11 and 17.21% for low, medium and high pools, respectively. The accuracy was examined by using fortified urine samples. From nine independent runs, the percent biases from the expected values were +0.6–18% (details provided in Supplemental tables). Linear responses were confirmed for all analytes across a broad range of ten analyte concentrations from 0.05 to 200 pg/mL, relevant to urinary levels of VNAs ($r^2 = 0.99, N = 6$) (Table II). The linear range of the analytical method extended from the LOD to the highest calibrator. A weighting factor of 1/x was used for all analytes. To verify matrix equivalency, 10 standard solutions were prepared in ACN (non-matrix). Another 10 standard solutions were prepared in urine (matrix) and sample preparation was conducted, as described previously. No significant differences were observed between the calibration curve prepared in ACN and that prepared in urine.

**Table I**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MRM Transitions for Quantitation, Confirmation, Optimal CE and Dwell Time*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDMA</td>
<td>92 → 75</td>
</tr>
<tr>
<td>NMEA</td>
<td>106 → 89</td>
</tr>
<tr>
<td>NDEA</td>
<td>120 → 103</td>
</tr>
<tr>
<td>NPIP</td>
<td>132 → 115</td>
</tr>
<tr>
<td>NPYR</td>
<td>118 → 101</td>
</tr>
<tr>
<td>NMOR</td>
<td>134 → 117</td>
</tr>
<tr>
<td>NDMA-2H6</td>
<td>98 → 81</td>
</tr>
<tr>
<td>NDEA-2H10</td>
<td>130 → 113</td>
</tr>
<tr>
<td>NPIP-2H10</td>
<td>142 → 125</td>
</tr>
<tr>
<td>NPYR-2H8</td>
<td>126 → 109</td>
</tr>
<tr>
<td>NMOR-2H8</td>
<td>142 → 125</td>
</tr>
</tbody>
</table>

*Note: All precursor ions are [M + 18]+ adducts. DT: dwell time.

**Table II**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>LOD (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDMA</td>
<td>2.62</td>
</tr>
<tr>
<td>NMEA</td>
<td>1.99</td>
</tr>
<tr>
<td>NDEA</td>
<td>2.73</td>
</tr>
<tr>
<td>NPIP</td>
<td>0.65</td>
</tr>
<tr>
<td>NPYR</td>
<td>0.25</td>
</tr>
<tr>
<td>NMOR</td>
<td>3.66</td>
</tr>
</tbody>
</table>

*Note: Linear range for all six analytes was 0.05–200.0 ng/mL.
Results for the other five analytes were similar and the difference in slopes ($N = 3$) was $< 5\%$ for all six analytes. To examine potential carryover, 20 runs were conducted in which low samples or blanks followed immediately after high samples. This arrangement was maintained during both cleanup and MS analysis. No carryover was observed under these conditions. Normal and accelerated stability studies of the analytes in a urine matrix were conducted. Repeated analysis of processed samples, either frozen or stored at room temperature, indicated that the analytes were stable at $-20\degree C$ for at least four months, but became unstable after one day at room temperature (sample loss of 97–100\% for all six analytes). Urine samples are routinely stored in the dark at approximately $-70\degree C$, and analyte levels should not be affected by long-term storage with these conditions. Additional experiments indicate that four analytes (NDEA, NPIP, NPYR and NMOR) were stable after at least seven thaw–refreeze cycles (sample loss of $5–10\%$), however, NDMA and NMEA lost approximately 30 and 60\%, respectively. Finally, three different pools were analyzed for ruggedness by varying: (i) wait time after sample loading on ChemElut cartridges; (ii) volume of DCM used for analyte elution; (iii) source temperature; (iv) CI gas flow; and (v) volume of injection. Except for injection volume, values were chosen that were higher and lower than the final standard condition. A higher volume of 6 $\mu$L was not tested because the volume would exceed the capacity of the liner, which would contaminate the gas line and cause an inaccurate volume to be injected. The observed variation had no significant effect on the calculated values of the analytes. Detailed results are provided in the Supplemental section. To ensure method performance, QC pools were analyzed to assess long-term stability and present assay sensitivity (Figure 4). The results acquired during a three-month period showed no significant deviations or trends, which was consistent with the stability of both the analyte and the method during that time.

The validated method was applied to 100 urine samples collected from smokers and nonsmokers. Geometric means and geometric standard errors for measurements of 50 smokers and 50 nonsmokers are listed in Table III. Urine from smokers contained significantly higher levels of NDMA (62.85 versus 49.22 pg/mL), NMEA (14.10 versus 10.87 pg/mL) and NPIP (22.47 versus 17.45 pg/mL) than urine from nonsmokers ($p$-value $< 0.05$).

**Discussion**

Most methods reported in the current literature have used direct L–L extraction with DCM, followed by direct injection of the extract. These methods require a relatively large sample volume (e.g., 5–25 mL). A high specimen volume is not feasible for large epidemiology studies; thus, other alternatives were examined. Compared to EPA Method 8270 or other methods (8, 10, 14, 16, 18, 36), a simple L–L with ChemElut cartridges eliminated emulsification, tedious and hazardous steps associated with traditional approaches, reduced background interference and significantly improved sample cleanup, with 70–90\% recovery for all six analytes. Injecting high eluent volumes without concentration was employed in other studies (8, 10, 14, 18, 32–35), but did not result in sufficient sensitivity for this study. Therefore, a keeper solvent was used, combined with centrifuge vacuum evaporation to improve sensitivity without significant sample loss. Although toluene retained 70–90\% of analytes as a keeper solvent, it resulted in a poor chromatographic peak shape, which was possibly due to its relatively high boiling point and tendency to be retained in the GC system. Both methanol and ACN are effective keepers. ACN was chosen as the keeper in the final method because it did not exceed the GC liner capacity at 5 $\mu$L injection volume. Using a combination of ChemElut/DCM and ACN as a keeper solvent, better LODs were achieved while using sample volumes of 2 mL instead of 5–25 mL, as reported in other methods (8, 10, 14, 16, 18, 36).
Pulsed PTV splitless injection mode resulted in poor chromatographic peak shapes, especially for late eluting peaks, most likely due to excess solvent in the analytical column. Compared with other injection modes, the solvent vent injection mode was the most sensitive and yielded the most reproducible results. The solvent vent allowed for the removal of excess solvent without significant loss of analytes, while dramatically improving analyte peak shape. GC liners affect sensitivity, and double taper liners are the most commonly used. In this experiment, a single taper helix liner was the most sensitive; the helix structure allowed for extra surface area that retained analytes while avoiding the material activity or interference of the inserted wool traditionally observed in focus liners. The performance of a single taper was more sensitive than a double taper helix liner, most likely due to less obstruction during the solvent venting period.

CI offered significant improvement in sensitivity; it is a softer ionization mechanism than EI (8). The ammonia adducted ion \([M + 18]^+\) peaks were the most abundant. Taking advantage of the adduct abundance, \([M + 18]^+\) to \(M^+\) transition was used as the quantitation transition for all analytes. This also allowed the monitoring of confirmation ions in a relative higher mass region for four of six analytes. For the other two analytes, NDMA and NPYR, confirmation ions of 43 and 55 Da could be monitored.

![Figure 4. QC characterization of NDMA; a total of 21 independent runs in one month.](image-url)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Statistics</th>
<th>NDMA</th>
<th>NMEA</th>
<th>NDEA</th>
<th>NPIP</th>
<th>NPYR</th>
<th>NMDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsmokers</td>
<td>Mean</td>
<td>49.22</td>
<td>10.87</td>
<td>34.64</td>
<td>17.45</td>
<td>19.70</td>
<td>190.66</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>1.06</td>
<td>1.22</td>
<td>1.08</td>
<td>1.13</td>
<td>1.16</td>
<td>1.05</td>
</tr>
<tr>
<td>Smokers</td>
<td>Mean</td>
<td>62.58</td>
<td>14.10</td>
<td>37.37</td>
<td>22.47</td>
<td>24.19</td>
<td>173.53</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>1.07</td>
<td>1.28</td>
<td>1.11</td>
<td>1.16</td>
<td>1.18</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>54.45–72.53</td>
<td>8.49–23.41</td>
<td>30.23–46.20</td>
<td>16.54–30.52</td>
<td>17.25–33.91</td>
<td>155.55–193.58</td>
</tr>
</tbody>
</table>

*Note: p-value < 0.05 for NDMA, NMEA and NPIP.*
with reduced noise levels because nitrogen was used as the collision. VNAs are small molecules, and because ammonia has a closer energy transfer gap than methane, sensitivity was dramatically improved. The LOD was greatly improved with the use of ammonia gas as the CI reagent gas. Compared to EI or PCI with methane, the sensitivity was significantly improved for all six analytes, ranging from at least 10-fold for NDMA and NMOR to 50-fold for the other four analytes. Improved LODs allowed the trace level analysis of VNAs in nonsmokers in addition to the reduction in the required sample volume. Other parameters that greatly affected the sensitivity were source temperatures and CI gas flow. Source temperatures were tested from 200 to 300 °C, with 250 °C producing the best sensitivity and reproducibility. Although the sensitivity was slightly increased at 300 °C, the reproducibility was significantly reduced. Ammonia gas was more reactive with the amine functional group at high temperature, resulting in less reliable analyte ionization. With CI gas flow ranging from 20% to 40%, equivalent to 1.0–1.5 mL/min, 30% provided the highest intensity of the [M + 18]+, but the results were not reproducible at high analyte concentration (above 500 pg on-column). It is likely that the kinetics of CI gas and analyte ions had approached an unstable level in the source. A CI gas flow of 25% provided the highest sensitivity and reproducible results for all analytes at all levels; therefore, it was chosen as the optimal flow rate.

The validated method was applied to 100 urine samples collected from smokers and nonsmokers. The geometric means for urinary levels of the six analytes are listed in Table III. No correlation was observed between NMOR or NPYR and caffeine intake, as reported among nonsmokers by Levallois et al. (18). Among 50 nonsmokers, 30 participants drank caffineinated beverages (tea, coffee or soda); 20 participants did not. Regarding caffeine intake, no significant difference was observed in NMOR or NPYR levels. The effect of alcohol use with tobacco product consumption on the urinary excretion of VNA was not investigated (37). Based on smoking status among this group of 100 smokers and nonsmokers, significant differences were found in three compounds: NDMA, NMEA and NPIP. Studies of VNAs in humans exposed to contaminated ground water or foods have been conducted (12, 15–18, 32–35, 38, 39); however, this study is the first to characterize these compounds in smokers and nonsmokers. Studies of VNA exposure from tobacco smoke are important, given the relatively high cancer risk index (6) of VNAs in tobacco smoke. Brunnenmann et al. tested samples of 22 different commercial cigarettes from Germany and Switzerland and found NDMA (0.1–27 ng/cigarette) and NMEA (2–13 ng/cigarette) in mainstream smoke (5). Similarly, Adams et al. examined samples of four U.S. commercial cigarettes and found that NDMA, NPIP and NPYR presented at 4.1–31.1, 5.8–14.2 and 10–264.5 ng/cigarette, respectively, also in mainstream smoke (4). Several studies have found concentrations of these VNAs at 10–167 times higher in sidestream smoke (4, 5, 23, 24). Considering the levels of NDMA, NMEA and NPIP detected in tobacco smoke and human urine, these VNAs may be important potential biomarkers for comparing the exposure of smokers who use different tobacco products.

In conclusion, this is the first time a GC–MS–MS method is presented for measuring VNAs in human urine. It is also the first time these six VNAs were investigated and measured in human urine, as related to smoking status. The levels of all six measured VNAs in human urine were quite stable. The concentrations of NDMA, NMEA and NPIP were shown to be significantly higher in smokers than in nonsmokers. Using tobacco products causes exposure to harmful VNAs; therefore, monitoring these biomarkers in smokers may provide useful information on exposure changes that may be the result of modified tobacco products.

Acknowledgments
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