Automated Solid Phase Extraction and Polarity-Switching Tandem Mass Spectrometry Technique for High Throughput Analysis of Urine Biomarkers for 14 Tobacco-related Compounds

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ABSTRACT: Tobacco use is the leading preventable cause of premature disease and death in the United States. Approximately, 34 million U.S. adults currently smoke cigarettes. We developed a method for automated sample preparation and liquid chromatography-tandem mass spectrometry quantitation of 14 tobacco-related analytes: nicotine (NICF), cotinine (COTF), trans-3′-hydroxycotinine (HCTF), menthol glucuronide (MEG), anabasine (ANBF), anatabine (ANTF), isonicoteine (ISNT), myosmine (MYOS), beta-nicotyrine (BNTR), bupropion (BUPR), cytisine (CYTI), varenicline (VARE), arecaidine (ARD), and arecoline (ARL). The method includes automated solid-phase extraction using customized positive-pressure functions. The preparation scheme has the capacity to process a batch of 96 samples within 4 h with greater than 88% recovery for all analytes. The 14 analytes, separated within 4.15 min using reversed-phase liquid chromatography, were determined using a triple-quadrupole mass spectrometer with atmospheric-pressure chemical ionization and multiple reaction monitoring in negative and positive ionization modes. Wide quantitation ranges, within 1.2−72,000 ng/mL, were established especially for COTF, HCTF, MEG, and NICF to quantify the broad range of biomarker concentrations found in the U.S. population. The method accuracy is above 90% while the overall imprecision is below 7%. Finally, we tested urine samples from 90 smokers and observed detection rates of over 98% for six analytes with urinary HCTF and MEG concentrations ranging from 200−14,100 and 60−57,100 ng/mL, respectively. This high throughput analytical process can prepare and analyze a sample in 9 min and along with the 14-compound analyte panel can be useful for tobacco-exposure studies, in smoking-cessation programs, and for detecting changes in exposure related to tobacco products and their use.

INTRODUCTION

Tobacco use is the leading preventable cause of premature disease and death in the United States and overseas.1,2 The highly addictive primary tobacco-specific alkaloid, nicotine, can lead to tobacco-product dependence and chronic exposure to the carcinogens and toxic chemicals in tobacco and tobacco smoke. An individual’s exposure to tobacco emissions varies based on many factors, including the product type, use intensity, and duration of use.3−5 Improved quantitative methods for analyzing tobacco exposure are crucial for better characterizing health risks and for formulating more effective tobacco regulations associated with its use.1,6 A high-throughput method, with automated sample preparation capability, can also improve the process while reducing the sample analysis cost, enabling studies to assess more chemical exposures.7,8

The presence of nicotine and its major metabolites, cotinine and trans-3′-hydroxycotinine, in urine indicates exposure to tobacco products.7 Menthol is a major tobacco-product flavor enhancer that has been widely studied for its tobacco-use related public health effects,9−12 indicating the importance of having menthol-exposure measurements along with tobacco-exposure biomarkers. Menthol is metabolized and excreted in urine predominantly as menthol glucuronide (MEG), which is used as a biomarker for menthol exposure.13,14 The presence of...
the minor tobacco-specific alkaloids, anabasine (ANBF) and anatabine (ANTF), can be used to identify tobacco-derived nicotine, whether to monitor compliance to smoking-cessation programs that use nicotine-replacement therapy or to assess if the nicotine in an e-liquid was likely derived from tobacco or not.\textsuperscript{15−17} Bupropion (BUPR), cytisine (CYTI), and varenicline (VARE) are three widely used smoking cessation pharmaceuticals.\textsuperscript{18}

Including ANBF, ANTF, BUPR, CYTI, and VARE in our analyte panel will enable their use in studying smoking-cessation programs. The addition of minor tobacco-specific alkaloids such ANBF, ANTF, isonicoteine (ISNT), myosmine (MYOS), and beta-nicotyrine (BNTR) to our panel also provides information about the type or species of tobacco used and curing processes, as well as liquids in electronic cigarettes.\textsuperscript{19−21}

The use of tobacco with areca nut, the seed of the Areca catechu tree, is common in the Asian and Pacific regions and in those migrant communities.\textsuperscript{22} The health consequences associated with this mix include addiction and carcinogenic effects from the toxic chemicals.\textsuperscript{23,24} Adding arecoline (ARL), the most abundant areca-nut alkaloid, and its metabolite arecaidine (ARD) to our panel also provides information about the type or species of tobacco used and curing processes, as well as liquids in electronic cigarettes.\textsuperscript{25−27}

To quantify these 14 tobacco-related analytes, we developed a high throughput method for automated sample preparation and high-performance liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry (HPLC-APCI-MS/MS) technique with stable-isotope dilution for instrumental analysis. The novel aspects of our method are as follows: automation of entire sample preparation process; introduction of sample-specific step-pressure functions for automating SPE of 96 urine samples; efficient separation and detection of 14 compounds in 4.15 min with HPLC-APCI-MS/MS; establishment of broad quantitation ranges (1.2–72,000 ng/mL) for tobacco exposure biomarkers. The analytes covered in this method included, nicotine (NICF) and its major metabolites cotinine (COTF) and trans-3′-hydroxycotinine (HCTF) in their unconjugated forms, MEG, five minor tobacco-specific alkaloids, biomarkers of three smoking cessation pharmaceuticals, and two areca-nut biomarkers. Thus, our analytical method enables to study tobacco and menthol exposure, to monitor smoking-cessation programs, and to detect changes in exposure related to changes in tobacco products.

\section*{RESULTS AND DISCUSSION}

\textbf{Automated Sample Preparation.} The automated sample preparation process (Figure 1A), including aliquoting, SPE, drying, and reconstitution, was completed in 4 h for a batch of 96 samples. For aliquoting urine, we used capacitance-based automatic liquid-level sensing to ensure accurate sample delivery from containers with variable volumes, and to minimize user interference during the process. To accommodate varied turbidities,\textsuperscript{28} samples were thoroughly mixed with rapid aspiration-dispensation cycles to obtain representative sample fractions.\textsuperscript{25−27}

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with basic methanol. In this procedure, the formic-acid wash improved recovery for HCTF and ARD, instead of the manufacturer-recommended methanol wash.

Turbidity can vary widely in a batch of 96 urine samples, making it challenging and time-consuming to visually gauge the levels of liquid remaining in SPE wells before determining the pressure needed for samples to elute completely. Less-turbid samples require lower pressure and more residual time to interact adequately with the SPE material while the more turbid samples need moderate or high pressure to elute completely. To optimize the SPE process, we developed customized positive pressure functions with step increments (Figure 1B) based on eluent viscosities. Urine samples of different turbidities were used to assess and optimize the three different positive pressure functions. We started the pressure function for the initial urine elution at 0.5 psi and used step-increments up to 10 psi over a period of 9.3 min (Figure 1B). The pressure function for the subsequent formic-acid wash included additional 20 psi pressure segments, separated by a one-minute pause (Figure 1B), to allow for complete washing of the high-turbid samples. Efficient removal of the aqueous solvent at this stage promoted fast sample drying. For the final analyte elution, less-viscous alkaline methanol was used along with pressure function ranging from 0.25 psi to 3 psi over 10 min, followed by an isolated 10 psi pressure step (Figure 1B) to ensure complete elution. The optimized automated SPE process with three pressure functions, assessed with spiked urine samples, produced above 88% recovery for all the analytes (Table 1).

Table 1. SPE Recovery (n = 12), LODs, and Calibration Ranges for the Method With Reference Ranges for the 14 Analytes

| analyte | SPE recovery % (SD) a | LOD (ng/mL) | calibration range (ng/mL) | reference range (ng/mL) b |
|---------|-----------------------|-------------|---------------------------|---------------------------|
| ANBF    | 98 (4)                | 0.770       | 0.20−400                  | 0.1−1400                  |
| ANTF    | 91 (2)                | 0.263       | 0.10−198                  | 0.04−300                  |
| ARD     | 92 (3)                | 0.621       | 0.26−133                  | 1−300                     |
| ARL     | 100 (4)               | 3.16        | 0.95−486                  | 1−100                     |
| BNTR    | 95 (3)                | 3.60        | 0.93−478                  | not available             |
| BUPR    | 98 (3)                | 1.82        | 0.55−1,114                | 2−2000                    |
| COTF    | 95 (3)                | 1.18       | 1.18−9,603                | 4−5,000                   |
| CYTI    | 98 (3)                | 1.44        | 0.23−480                  | 0.03−1500                 |
| HCTF    | 90 (4)                | 2.39        | 1.95−15,901               | 37−24,500                 |
| ISNT    | 99 (5)                | 0.646       | 0.07−350                  | not available             |
| MEG     | 92 (4)                | 8.91       | 8.91−71,925               | 60−100,000                |
| MYOS    | 96 (3)                | 1.45        | 0.61−314                  | not available             |
| NICF    | 100 (1)               | 4.27        | 0.97−8,080                | 10−6,600                  |
| VARE    | 89 (5)                | 1.56        | 0.23−457                  | 2−1000                    |

a SD: standard deviation. b Nicotine and nicotine metabolites represent the total (conjugated + unconjugated) levels. c COTF and MEG LODs are set to their lowest standard concentrations, above the observed LODs (Table S5). d Preliminary assay findings and Benowitz et al.1,4

In LC–MS/MS analysis, matrix effects can be reduced by using stable-isotope labeled IS, APCI, SPE, or dilute urine matrix.29–32 In our method, diluted urine samples are subjected to SPE, and stable-isotope IS and an APCI source are used to quantify all 14 analytes (Table S1). Further, Chambers et al.33 highlighted significant reduction in matrix effects with the use of mixed-mode sorbent SPE, which we used for sample preparation. Consequently, we observed the slopes of the calibration curves of urine standards equivalent to those of water standards with the differences ranging from 0.1–5.0% (Table S8), for all the analytes. We also measured matrix effects and absolute recoveries at one calibrant point according to Matuszewski et al.30 (Figure S3). The observed matrix effects (Figure S3a) are low or insignificant (93–100%) for all the analytes. The absolute recoveries, ranging from 97–109%, are also comparable to Table 1 SPE recoveries as the matrix effects are minimal.

**Buffer Concentration and pH in Analyte Separation.** The 14 analytes were separated within 4.15 min (Figure 2). Another 1.85 min of cleaning and re-equilibrating time was added to maintain the consistency of chromatography when analyzing a batch of 96 samples. MEG, detected with the negative-ionization mode, was observed within the busy region (2.8–3.1 min) of the chromatogram with buffer concentrations at or below 15 mM. Improved MEG peak quality with retention times greater than 3.1 min was obtained with the use of 20 mM ammonium acetate buffer (pH 10 ± 0.15). Separation characteristics for NICF (pKₐ = 3.1 and 8.0), ANBF (pKₐ = 9.3), and ANTF (pKₐ = 8.8) are pH-dependent, and the importance of the run buffer pH for their retention variations has been shown.30 We thus assessed retention time variations for the 14 analytes using ammonium acetate buffers with pH ranging from 9.5 to 10.5 before selecting pH 10 (±0.15) buffer. ARD, ANBF, VARE, and MEG showed the highest (≤12 s) average retention time variations (Table S7) for the tested conditions while variations for the other 10 analytes were below 2.5 s.

**Calibration and Reference Ranges, LOD, Accuracy, and Precision.** To setup suitable calibration ranges, we considered our preliminary analysis and studied the previously published analyte levels (Table 1, reference range). The broad concentration ranges of COTF, HCTF, MEG, and NICF (Table 1) were covered using 15 calibration standards with the concentration of the second-highest standard set to 75% of the highest. The concentration ranges of ANBF, ANTF, and the three smoking-cessation pharmaceuticals were covered by 12 calibration standards, while that of the remaining five analytes were covered by 10 standards. The optimum collision-energy voltages (CE) of COTF, HCTF, MEG, and NICF provided LODs below the corresponding reference concentration ranges but saturated the detector at higher concentrations for these transitions. Hence, CEs of the corresponding quantitative and qualitative transitions were offset to enable determination of high concentrations; similar approaches have been previously used32,43 to mask transition-specific signal. The resulting LODs fit within reference ranges (Table 1) and were adequate to determine the exposure biomarkers of smokers. The obtained HCTF calibration range with CE offset is also adequate as our method is measuring the unconjugated biomarker levels of smoker urine; the reference range concentrations mostly represented total biomarker levels. Additionally, less-sensitive 13C-HCTF and 13C-COTF transitions were included to quantify their high concentrations. The MRM transitions and finalized MS voltage settings for the native analytes and their isotope-labeled internal standards are listed in Table S4; the method with these established parameters is used for the validation experiments and measurements. The steps we took to accommodate high-and low-concentration samples within one analytical run avoided time-consuming repetitive sample-preparation and
analysis. High-concentrated samples with carry-over issues for ANBF, BUPR, CYTI, NICF, and VARE were resolved using a thorough autosampler-cleaning program and strong rinsing solution mixture.

The slopes of the processed urine calibration standard curves were comparable to those of water, with differences ranging from 0.1 to 5.0% for all 14 analytes (Table S8); our method also uses isotope dilution mass spectrometry with isotopically labeled internal standards for all 14 analytes for quantitation. Consequently, we used calibration standards in water to further increase the method throughput as more samples could be accommodated during preparation.

The LODs for the 14 analytes were listed in Table 1; the CLSI recommended procedure was followed to determine LODs by characterizing the relationship between the standard deviation of the measurements and concentrations of the analyte at low concentrations.44,45 We used a blank and six low-concentrated urine pools (LP) for the LOD determinations (Table S5). The analyzed blank pools provided the mean concentration (mean_blank) and the standard deviation (SD_blank) for each analyte. Synthetic urine was used as an alternate matrix for MEG’s LOD determination due to the presence of MEG (235 ng/mL) in the blank-urine pool. Twenty-three batches of low-concentrated LPs were processed and analyzed on different days with each batch including at least three replicates from each LP. The mean concentration of each LP was plotted against the corresponding standard deviation for each analyte (Table S5) to obtain the linear regressions, where A and B are the gradient and the intercept (Table S5). The following equation, with the value 1.645 representing the 95th percentile from the measurement normal distribution for α = 0.05, was used to calculate the analyte LODs45

\[
\text{LOD} = \left( \frac{\text{mean_blank} + 1.645 \times (\text{SD_blank} + B)}{1 - 1.645 \times A} \right)
\]

Previously, Benowitz et al. observed 18% of urine samples from mentholated cigarette smokers below their established MEG LOQ of 1000 ng/mL.14 We obtained MEG LOD of 7.71 ng/mL (Table S5) and our preliminary analysis indicated 60 ng/mL MEG as the lower limit of the reference range (Table 1). The lower LOD of our method also helps determine MEG in non-mentholated smokers, which is attributed to the use of mentholated dental-hygiene products, gum, candy, and food.13,14,46

We performed the following LC–MS/MS method optimizations: selection of LC mobile phase for efficient separation of 14 compounds; establishment of analyte calibration ranges with suitable LODs and 13C transitions; assessment of calibration curves in matrix and non-matrix based standards; and minimization of carryover or high background issues for high concentrated samples. The two MRM transitions for each analyte were selected only after thorough screening of smoker urine samples to confirm no co-eluting interferents. The confirmation ion ratio for each analyte was also calculated and
The observed urinary concentration variations (Figure 3) were for the two minor tobacco alkaloids ANBF and ANTF. MEG, NICF and its two major metabolites COTF and HCTF, specimens were collected by Tennessee Blood Services for this study were cigarette smokers, and their urine processed urine samples when refrigerated at 4 to 10 freeze-thaw cycles (Table 3). The analytes were stable in 3 days at room temperature for up to three analytes in urine are stable at room temperature for up to three study planning and in ensuring the quality of assay results. The Stability of analytes in urine was confirmed (<7% deviation) up to 3 days at room temperature (Table 3). The analytes were stable in processed urine samples when refrigerated at 4 °C for 7 days. 

Analyte Stability. Although a 96-sample batch could be prepared and analyzed in 14 h, we conducted stability experiments over longer durations with consideration for the range of possible conditions during sample collection, shipping, storage, and preparation. The findings are vital in study planning and in ensuring the quality of assay results. The analytes in urine are stable at room temperature for up to three days and at or below −60 °C for 6 months (Table 3), while stability at −60 °C for longer durations is being monitored. Stability of analytes in urine was confirmed (<7% deviation) up to 10 freeze-thaw cycles (Table 3). The analytes were stable in processed urine samples when refrigerated at 4 °C for 7 days.

Analysis of Smoker Urine Samples. Participants targeted for this study were cigarette smokers, and their urine specimens were collected by Tennessee Blood Services (Memphis, TN) upon obtaining prior written consent. Urine samples from 90 smokers showed detection rates over 98% for MEG, NICF and its two major metabolites COTF and HCTF, and for the two minor tobacco alkaloids ANBF and ANTF. The observed urinary concentration variations (Figure 3) were included in the method for assessing interferences in unknown samples. The overall accuracy at a given concentration for an analyte was above 90% while the overall imprecision ranged from 1.2 to 6.9% (Table 2). From the LOD determinations, the overall precision of LP-4, LP-5, and LP-6 was calculated (Table S6) and the RSD % was below 20% when the concentration of the LP was above its LOD. Also, when the LP concentration was greater than three times the LOD, the corresponding RSD % was at or below 10%, except for VARE. We determined accuracy and precision using urine samples spiked with the analyte of interest at three different concentrations (Tables 2, S9, and S10), with three replicates at each concentration, within the reportable range; analyses were conducted on two different days with one batch per day.

### Table 2. Overall Precision and Accuracy of the Assay Determined by Analyzing Nine Replicates, With Three at Each Concentration, for Each Analyte on Two Different Days

| analyte | expected (ng/mL) | mean assayed<sup>a</sup> (ng/mL) | RSD %<sup>b</sup> | error %<sup>c</sup> | analyte | expected (ng/mL) | mean assayed<sup>a</sup> (ng/mL) | RSD %<sup>b</sup> | error %<sup>c</sup> |
|---------|-----------------|-------------------------------|-----------------|-----------------|---------|-----------------|-------------------------------|-----------------|-----------------|
| ANBF    | 10.2            | 10.1                          | 6.9             | −0.8            | CYTI    | 34.1            | 37.5                          | 2.6             | 9.9             |
|         | 19.8            | 18.6                          | 4.7             | −6.2            |        |                 |                               |                 |                 |
|         | 39.6            | 38.5                          | 6.6             | −2.8            |        |                 |                               |                 |                 |
| ANTF    | 5.55            | 5.43                          | 2.3             | −2.1            | HCTF    | 645             | 615                          | 2.7             | −4.7            |
|         | 11.2            | 11.5                          | 2.2             | 2.7             |        |                 |                               |                 |                 |
|         | 25.4            | 24.9                          | 2.7             | −1.9            |        |                 |                               |                 |                 |
| ARD     | 12.3            | 12.8                          | 3.7             | 4.0             | ISNT    | 1.95            | 2.08                          | 3.5             | 6.4             |
|         | 24.7            | 24.7                          | 2.8             | 0.2             |        |                 |                               |                 |                 |
|         | 49.4            | 49.9                          | 3.8             | 1.0             |        |                 |                               |                 |                 |
| ARL     | 30.1            | 29.4                          | 3.5             | −2.2            | MEG     | 7500            | 7420                          | 2.2             | −1.1            |
|         | 69.7            | 66.5                          | 4.3             | −4.5            |        |                 |                               |                 |                 |
|         | 198             | 186                           | 2.8             | −5.8            |        |                 |                               |                 |                 |
| BNTR    | 29.6            | 29.9                          | 2.7             | 1.0             | MYOS    | 17.5            | 19.2                          | 3.1             | 9.3             |
|         | 59.6            | 57.3                          | 2.4             | −4.0            |        |                 |                               |                 |                 |
|         | 119             | 116                           | 3.2             | −2.6            |        |                 |                               |                 |                 |
| BUPR    | 44.1            | 41.7                          | 3.2             | −5.4            | NICF    | 254             | 247                           | 1.5             | −2.8            |
|         | 104             | 97.3                          | 1.2             | −6.3            |        |                 |                               |                 |                 |
|         | 403             | 392                           | 4.1             | −2.8            |        |                 |                               |                 |                 |
| COTF    | 368             | 389                           | 3.1             | 5.8             | VARE    | 47.9            | 48.4                          | 5.2             | 0.9             |
|         | 668             | 719                           | 2.2             | 7.6             |        |                 |                               |                 |                 |
|         | 1280            | 1400                          | 3.6             | 9.4             |        |                 |                               |                 |                 |

<sup>a</sup>Mean of six assayed measurements obtained on 2 days at each expected concentration. <sup>b</sup>[(SD/Mean assayed concentration) × 100. <sup>c</sup>[mean assayed concentration − expected concentration)/expected concentration] × 100.

### Table 3. Stability of Analytes in Unprocessed and Processed Samples Estimated by Deviation (%) of Mean Concentration From the Initial Measurement (n = 6)

| analyte | deviation (%) of mean concentration from the initial measurement after | | | |
|---------|---------------------------------------------------------------|---|---|---|
|         | 3 days at room temp<sup>a</sup> | 6 months, ≤−60 °C<sup>b</sup> | 10 freeze-thaw cycles<sup>c</sup> | 7 days at 4 °C<sup>b</sup> |
| ANBF    | 2.9                                                        | −0.2                                      | 0.0                                      | 4.1                                      |
| ANTF    | 5.9                                                        | 0.5                                       | −0.5                                     | −0.2                                     |
| ARD     | 6.3                                                        | 1.5                                       | −4.7                                     | 2.1                                     |
| ARL     | 1.0                                                        | −1.9                                      | 1.8                                      | 4.3                                     |
| BNTR    | −5.2                                                       | 3.1                                       | −6.2                                     | 1.4                                     |
| BUPR    | −9.6                                                       | −4.1                                      | −4.9                                     | −1.3                                     |
| COTF    | 3.5                                                        | 3.4                                       | 0.4                                      | 0.1                                     |
| CYTI    | 3.0                                                        | −3.9                                      | 0.6                                      | 1.3                                     |
| HCTF    | 3.2                                                        | 0.5                                       | 3.0                                      | 2.3                                     |
| ISNT    | 2.7                                                        | −4.2                                      | 0.1                                      | 1.5                                     |
| MEG     | 3.1                                                        | 5.3                                       | 1.1                                      | 3.0                                     |
| MYOS    | 6.8                                                        | −1.9                                      | 4.7                                      | 2.3                                     |
| NICF    | 2.0                                                        | 7.3                                       | 0.3                                      | 1.0                                     |
| VARE    | 2.7                                                        | 3.2                                       | −0.6                                     | −4.9                                     |

<sup>a</sup>First three conditions with unprocessed samples. <sup>b</sup>Last condition with processed samples. <sup>c</sup>Freeze-thaw: freeze at or below −20 °C and thaw at room temperature.
demographics, the ability of our method to quantify multiple analytes within wide concentration ranges in a single analytical run improved the method efficiency while decreasing the analysis cost.

The observed average urinary ANBF concentration (10.4 ng/mL) was about half of ANTF (20.2 ng/mL), comparable with the previously observed ANBF/ANTF ratios. The urinary ANTF and ANBF concentrations are also far less than that of urinary NICF (Figure 3), following their levels in tobacco. Nicotine-containing medication in the form of gum and transdermal patches is used in smoking-cessation programs. ANBF and ANTF, which are not present in nicotine-containing medications, are measured in smoking cessation programs to validate compliance of participants receiving nicotine replacement therapy. Our method also measures smoking-cessation pharmaceuticals such as BUPR, CYT, and VARE, making it broadly applicable for monitoring compliance in such programs.

The method also enables monitoring of three other minor tobacco alkaloids, BNTR, ISNT, and MYOS in urine to detect corresponding changes in tobacco species or curing processes, or in the liquid contents of electronic cigarettes. IsNT and MYOS content in tobacco is 5 to 10 times less than that of ANBF, which is not present in nicotine-containing medications, are measured in smoking cessation programs to validate compliance of participants receiving nicotine replacement therapy. As expected, the three minor tobacco alkaloids were not detected in the analyzed urine samples from the smokers that showed less-diverse demographics and product use. However, detection of BNTR, ISNT, MYOS, and ARD and ARL in smoker urine could be related to the use of differently blended/processed tobacco or use of areca nut.

## CONCLUSIONS

Our methods for automated sample preparation and stable-isotope labeled polarity switching LC−MS/MS are applicable to tobacco-exposure studies and smoking-cessation programs, and for detecting changes in exposure related to changes in tobacco products and their use. The 14-analyte panel includes three tobacco-exposure biomarkers (COTF, HCTF, and NICF), the metabolite of the major tobacco flavoring agent (MEG), five minor tobacco-specific alkaloids (ANBF, ANTF, BNTR, ISNT, MYOS), three smoking cessation pharmaceuticals (BUPR, CYT, VARE), and two areca nut exposure biomarkers (ARD and ARL). The method is high throughput, having the capability of preparing and analyzing a 96-sample batch within 14 h, with an average of 8.75 min per sample. The overall method accuracy is above 90% while the imprecision is below 7% for all analytes, with LODs ranging from 0.26–8.91 ng/mL. Our automated sample-preparation method provided greater than 88% recovery, included automated SPE sample cleanup with customized step-pressure functions for enhanced efficiency, resulting in the ability to process 96 samples in 4 h. The LC−MS/MS method is efficient, separating 14 analytes in 4.15 min while detecting in both positive and negative ionization modes. Wide quantitation ranges, within 1.2–72,000 ng/mL, were established especially for COTF, HCTF, MEG, and NICF to quantify the broad range of biomarker concentrations found in the U.S. population. Additionally, transition samples were used, carryover potentials were experimented and resolved, and analyte stabilities were studied, enabling efficient measurement of high- and low-concentration samples in a single analytical run to avoid time-consuming repetitive sample preparation and analysis. Finally, urine samples from 90 smokers were analyzed and over 98% detection rates were observed for ANBF, ANTF, COTF, HCTF, MEG, and NICF, with widely spread urinary HCTF and MEG concentrations despite limited variations of smokers in CPD and demographics.

## EXPERIMENTAL SECTION

Details on stock and standard solutions, chemicals, materials, and equipment used are in the Supporting Information section.

### Sample Preparation.

A Hamilton Microlab STARlet liquid handling system with an 8-channel pipette head, a robotic arm, and a Monitored Multi-Flow Positive Pressure Evaporative Extraction ([MPE] 2) module was used for automating sample preparation (Figure 1A). We developed and optimized custom VENUS software programs for automating the aliquot, SPE, and reconstitution steps with the deck layouts (Figures S1 and S2, Supporting Information); the robotic arm was used for moving laboratory ware across the deck while the 8-channel pipette head was used for transferring all solutions. The automated aliquoting program obtained the number of samples, their barcodes, and desired starting-well position, and assigned the plate-wells to the corresponding sample information to be incorporated into the LC−MS/MS data files. Thawed and rotary-mixed (20 min) samples went through 5 rapid aspiration-dispensation mixing cycles (150 μL/cycle) prior to transferring to obtain representative sample fractions. After transferring the sample (100 μL), the internal standard (IS) mixture (100 μL), and 4% formic acid (200 μL) into the 96-well plate, it was sealed and mixed for 30 min.

The automated SPE process was carried out on the Hamilton [MPE] 2 unit using the Oasis MCX 96-well SPE
plates. The SPE and collection plates were manured from their deck positions to the [MPE] using the pre-programmed robotic arm. We developed and optimized three positive-pressure functions (Figure 1B) for the initial urine elution, formic-acid wash, and final analyte elution. These optimized-pressure functions were included in the MPE-controlling software program for automating the SPE process. The SPE plate on [MPE] was conditioned with methanol (500 µL) and water (500 µL), prepared samples (400 µL) were introduced, and the first 9.3-min pressure function (Figure 1B) was applied to each well to expel the non-retaining substances from the SPE plate. The retained substances were washed with 2% formic acid (500 µL) by applying the second 12.3 min pressure function. The waste solvents from these steps are collected with the [MPE] unit’s built-in waste tray. The retained analytes from the SPE plate were eluted into a fresh 96-well plate with alkaline (4% NH4OH) methanol using the third 12 min pressure function (Figure 1B). Eluted samples, dried with heated nitrogen (40 °C, 12 psi, 30 min), were reconstituted with LC/MS water (400 µL) and mixed for 30 min prior to the LC–MS/MS analyses.

LC–MS/MS Method. We performed chromatographic separations on the Shimadzu modular LC system using a Kinetex EVO C18 column (100 mm × 2.1 mm, 2.6 µm) and a 0.2 µm pre-column filter held at 45 °C. The gradient solvent program (Table S3), with the total flow rate of 0.6 mL/min, used ammonium acetate buffer (20 mM, pH 10) and LC/MS-grade acetonitrile as mobile phases A and B on two Shimadzu LC-30AD pumps, respectively. We used a thorough autosampler rinsing program with a strong rinsing solution (2-propanol, acetonitrile, and water in 4:3:3 volume ratio with 0.3% ammonium hydroxide) to avoid or minimize carryover and high background issues. The optimized rinsing program integrated to the LC-method included an external and internal needle rinse, injection-port rinse, and measuring-line purge.

A Sciex triple-quadrupole 6500+ mass spectrometer with APCI was used for analyte determinations with multiple reaction monitoring (MRM) in positive and negative ionization modes at unit mass resolution. We monitored two 13C MMR precursor/product transitions for each analyte, one MMR transition for the corresponding isotope-labeled internal standard, and additional 13C transitions for native COTF and HCTF (Table S4). The optimized MS source parameters were as follows: temperature, 500 °C; nebulizer current, +3/-3 µA; ion source gas 1, 35 psi; and curtain gas, 35 psi. Analyst software (version 1.6.3) was used to control and acquire LC–MS/MS data. Web-based Indigo Ascent Automated Data Analysis and Review software was used for automated peak integration and quantitation. We used peak area ratios of analytes to the corresponding internal standards to construct the calibration curves with linear least-squares regression and 1/x weighting factor.

Urine Pools for Recovery, Accuracy, Precision, and Limit-Of-Detection Determinations. Anonymous human urine specimens, collected at CDC under Internal Review Board-approved protocol (ID 3994.0), were screened for the targeted analytes, and the blanks were used to prepare the blank urine pools. Spiked blank urine samples (n = 12) and a set of standards in water with equal analyte concentrations were used to assess analyte recovery in the automated SPE process. The spiked urine samples were processed with the automated SPE, and the IS mixture was added prior to the subsequent drying and reconstitution steps. The processed urine samples were analyzed using LC–MS/MS with the standards containing the same IS mixture; the peak areas were calculated, and the analyte recoveries were determined with the following equation

\[
\text{SPE recovery} = \frac{[(\text{analyte peak area of processed sample})]}{[(\text{analyte peak area of the standard})]} \times \frac{[(\text{IS peak area of corresponding processed sample})]}{[(\text{IS peak area of the standard})]} \]

Six low-concentration analyte pools were prepared for limit-of-detection (LOD) determinations by spiking analytes into screened nonsmoker urine. Three low-concentration MEG pools were prepared in synthetic urine.

To determine the accuracy and precision, we prepared urine samples with the analytes of interest at three expected concentrations. The expected concentrations of different analytes needed to represent narrow or wide analyte-calibration ranges depending on their reference ranges. Thus, urine samples with known concentrations, standard solutions, and blanks were mixed in different proportions to obtain the expected concentrations within the reportable range of each analyte (Tables S9 and S10). The replicates, with three at each concentration, were then processed and analyzed on two different days to obtain six assayed measurements at each spiked concentration; the corresponding mean-assayed and the spiked concentrations were used to calculate the precision and accuracy for each analyte.

Comparison of Water and Urine Calibration Standards. The following experiment was carried out to compare the matrix effects. We prepared 10 urine calibration standards, bracketing each analyte’s measurement range with four replicates at each concentration, by spiking analytes into non-smoker urine. Another set of 10 calibration standards with identical concentrations were prepared in water for analyses without processing. All standards were analyzed and the calibration curves for each analyte in each matrix was constructed (1/x weighting) to compare the slopes.

Thermal and Freeze-Thaw Stability Testing. We investigated analyte stability in unprocessed and processed samples using two spiked urine pools at two different concentrations for each analyte. Analyte stability at room temperature was assessed for 1, 3, and 7 days in unprocessed samples while long-term stability was assessed by storing them at or below −60 °C for up to 6 months. To assess freeze–thaw stability, unprocessed urine samples were subjected to 2, 3, 5, and 10 freeze–thaw cycles (at or below −20 °C). The stability of analytes in processed samples was tested by refrigerating at 4 °C for 1, 3, and 7 days.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs omega.1c02543.

Additional information as noted in text (Piyankarage et al. Supporting Information); chemicals, materials, equipment, stock and standard solutions; Hamilton deck layouts for the automated sample preparation; gradient solvent program used for chromatographic separations; MRM transitions with MS voltage settings; determination of LOD; RSD % and the ratio of low pool mean
concentration to the LOD; retention-time variations with buffer pH; comparison of calibration standards made in water and urine; stock solution and spike mixture preparations for precision and accuracy analysis; and matrix effects and absolute recoveries (PDF)

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Notes
The authors declare no competing financial interest.

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