

Clinical, Forensic & Toxicology Applications

# Rapid and Accurate LC-MS/MS Analysis of Nicotine and Related Compounds in Urine Using Raptor™ Biphenyl LC Columns and MS-Friendly Mobile Phases

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## Abstract

A rapid, accurate, and reproducible method was developed for high-throughput testing of nicotine, cotinine, *trans*-3'-hydroxycotinine, nornicotine, norcotinine, and anabasine in urine. Data show that a fast and highly efficient analysis of these basic compounds can be achieved with the Raptor™ Biphenyl column using standard low-pH, reversed-phase LC-MS mobile phases that are compatible with a variety of LC-MS instrumentation.

## Introduction

Nicotine is the major tobacco alkaloid that underlies addiction in tobacco users. Anabasine and anatabine are the most abundant minor alkaloids in tobacco [1]. Anabasine is frequently used as a unique marker for recent tobacco use as it can only be detected in the urine of tobacco users and is not present in the urine of those who use nicotine replacement therapies (e.g., nicotine patches). In humans, more than 70% of nicotine is transformed to cotinine, which is subsequently converted to *trans*-3'-hydroxycotinine, the main nicotine metabolite detected in urine [2]. Nornicotine and norcotinine are minor metabolites (0.5–2%) produced by the demethylation of nicotine and cotinine, respectively. The urinary measurement of nicotine metabolites has several applications, including public tobacco exposure monitoring, nicotine replacement therapy evaluation, drug therapy assessment, forensic toxicology analysis, and life or health insurance application. In addition, nicotine metabolites can be used as the biomarkers for pharmacogenomics evaluation and disease profiling [3].

A variety of chromatographic methods have been developed for nicotine metabolite analysis. However, most methods use high-pH chromatography with relatively high concentrations of additives to increase retention, improve peak shape, and reduce peak tailing. The intent of this application was to develop a method for the analysis of nicotine-related compounds in urine using solutions that are “friendly” to LC-MS/MS systems. A Raptor™ Biphenyl column was chosen as the analytical column because it provides good retention and peak shape for the target analytes when used with standard low-pH, reversed-phase mobile phases. The clinical applicability of this method was demonstrated by the accurate and reproducible analysis of fortified analytes in urine.

## Experimental

### Instrument and Analytical Conditions

The instrument and analytical conditions are listed in Table I. The analyte MRMs are shown in Table II.

**Table I:** Analytical Conditions for Waters Xevo™ TQ-S with Acquity UPLC®

Analytical Column	Raptor Biphenyl 5 µm 100 mm x 2.1 mm (cat.# 9309512)	
Guard Column	Raptor Biphenyl 5 µm 5 µm, 5 mm x 2.1 mm (cat.# 930950252) EXP® direct connect holder (cat.# 25808)	
Injection Volume	5 µL	
Mobile Phase A	0.1% Formic acid, 5 mM ammonium formate in water	
Mobile Phase B	0.1% Formic acid in methanol	
Gradient	Time (min)	%B
	0.0	10
	1.0	10
	2.0	30
	3.0	70
	3.01	10
	5.0	10
Flow Rate	0.4 mL/min	
Column Temp.	30 °C	
Ion Mode	Positive ESI	
Capillary Voltage	1.0 kV	
Gas Flow	1,000 (L/Hr) desolvation	
	150 (L/Hr) cone	
	7.0 (bar) nebulizer	
Desolvation Temp.	450 °C	

**Table II:** Analyte MS/MS Transitions

Analyte	Precursor Ion	Product Ion Quantifier	Product Ion Qualifier
Nornicotine	149.10	80.05	105.89
Nornicotine-D4	153.17	84.03	-
Norcotinine	163.09	80.05	135.10
Norcotinine- <sup>13</sup> C <sub>3</sub>	166.22	80.05	-
Nicotine	163.15	132.10	117.07
Nicotine-D4	167.16	136.64	-
Cotinine	177.12	98.07	146.09
Cotinine-D3	180.20	101.07	-
trans-3'-Hydroxycotinine	193.12	80.05	134.12
trans-3'-Hydroxycotinine-D3	196.19	80.05	-
Anabasine	163.13	91.63	120.12
Anabasine-D4	167.20	96.19	-

### Sample/Calibration Standard Preparation

Blank urine from an unexposed non-tobacco user was fortified to prepare calibration standards and fortified QC samples. A 5,000 ng/mL standard mix was prepared in urine and diluted with urine to make calibration standards at 2, 5, 10, 25, 50, 100, 250, 500, 1,000, 2,500, and 5,000 ng/mL (only cotinine and *trans*-3'-hydroxycotinine were tested at 2,500 and 5,000 ng/mL levels). Three levels of QC samples (7.5, 75, and 750 ng/mL for nicotine, nornicotine, norcotinine, and anabasine; 75, 750, and 10,000 ng/mL for cotinine and *trans*-3'-hydroxycotinine) were prepared in urine for accuracy and precision testing. The 10,000 ng/mL QC sample for cotinine and *trans*-3'-hydroxycotinine was diluted 5-fold in water before the sample preparation procedure. Analyses were performed on three different days.

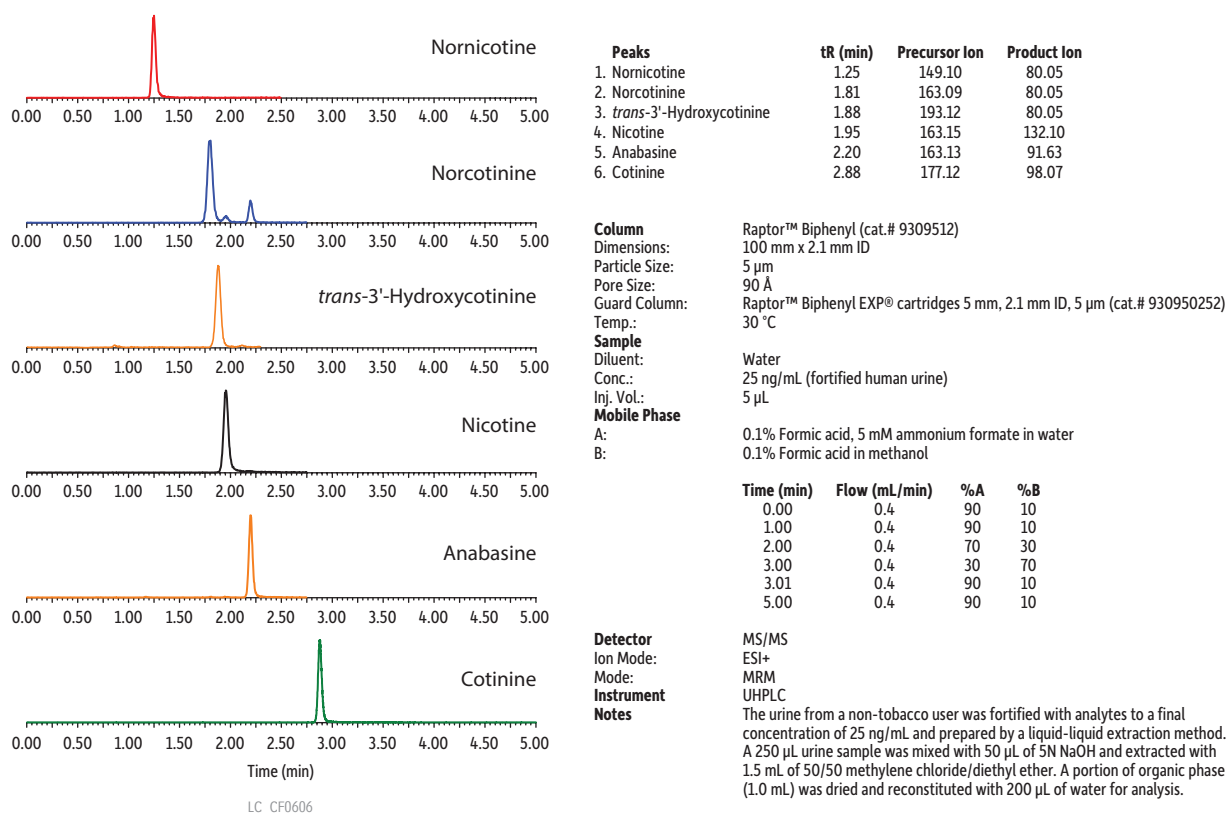
All fortified standards and QC samples were processed according to the following liquid-liquid extraction (LLE) procedure.

1. Mix a 250  $\mu$ L aliquot of urine with 40  $\mu$ L of internal standard solution (250 ng/mL in methanol) and 50  $\mu$ L of 5 N sodium hydroxide in a 4 mL glass vial.
2. Extract by adding 1.5 mL of 50:50 methylene chloride:diethyl ether and stirring for 1.5 minutes.
3. Centrifuge at 4,000 rpm for 5 minutes, then transfer 1 mL of the organic phase to a 1.5 mL HPLC vial and mix with 10  $\mu$ L of 0.25 N hydrochloric acid.
4. Evaporate to dryness at 35  $^{\circ}$ C under a gentle stream of nitrogen.
5. Reconstitute the dried extract with 200  $\mu$ L of water.

## Results and Discussion

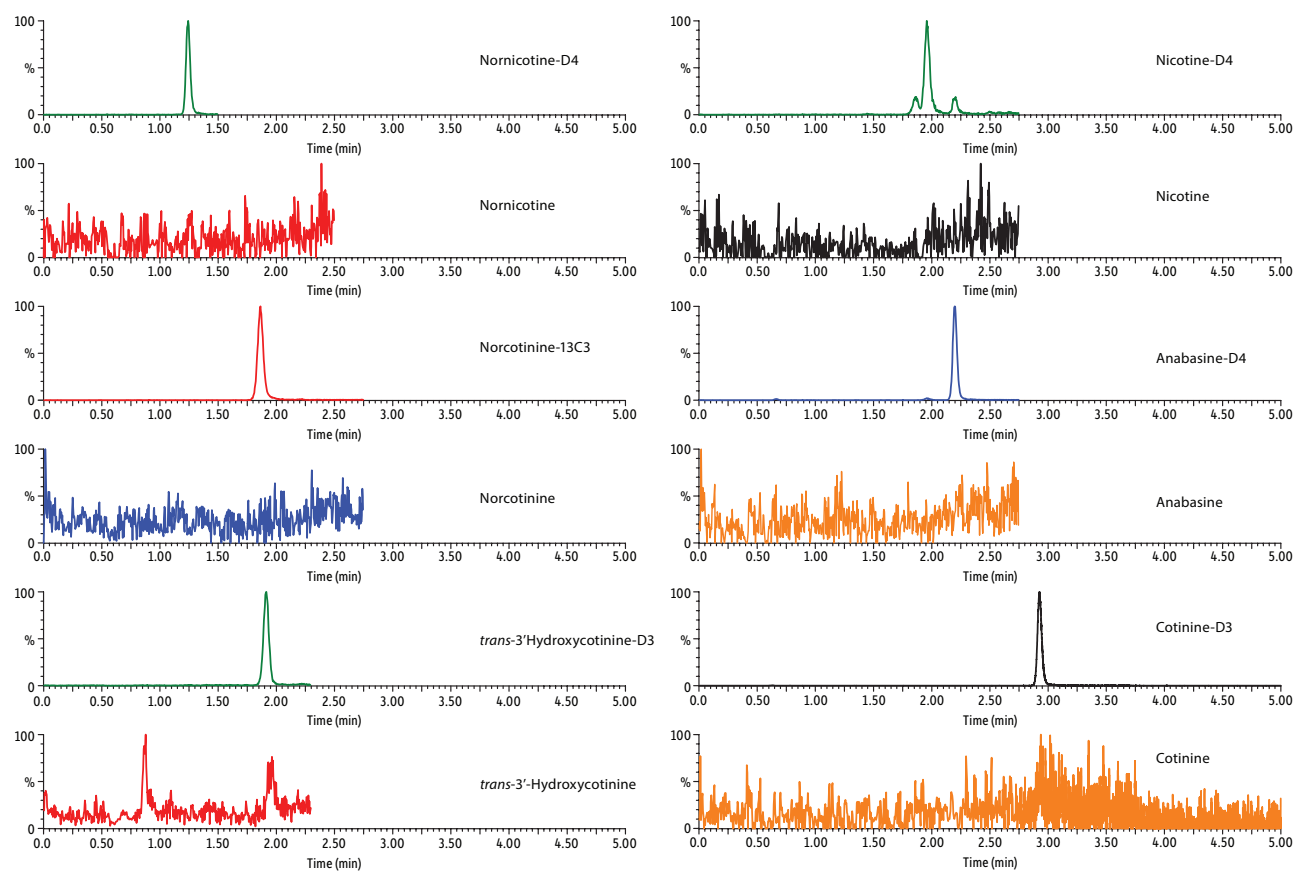
Since nicotine, norcotinine, and anabasine all share the same mass transitions, it is necessary to chromatographically separate these three compounds for accurate quantitation. As shown in Figure 1, baseline resolution was obtained for nicotine, norcotinine, and anabasine with the Raptor™ Biphenyl column. Simultaneous analysis of all six analytes was performed with a fast 3-minute gradient and a 5-minute total analysis time for each injection. Note that good peak shape was obtained for all analytes with the use of standard, low-pH, reversed-phase mobile phases. The Raptor™ Biphenyl column allowed an MS-friendly mobile phase to be used without any evidence of the peak tailing that is often observed in the analysis of these basic compounds, unless high-pH mobile phases with higher concentrations of additives are used.

**Figure 1:** Good chromatographic separations and peak shapes for nicotine-related compounds were achieved using the Raptor™ Biphenyl column and a simple, low pH, MS-friendly mobile phase.



Analysis of blank urine samples spiked with internal standards and subjected to the extraction procedure confirmed there were no significant matrix interferences for the target analytes (Figure 2). Analysis of the calibration standards established the linear ranges for each compound as 2-1,000 ng/mL (nicotine, nornicotine, norcotinine, and anabasine); 5-5,000 ng/mL (cotinine); and 10-5,000 ng/mL (*trans*-3'-hydroxycotinine). To achieve detection across these wide concentration ranges, a detuning of the MS detector was necessary for all analytes in order to avoid saturation of the MS signal at the highest concentration. It was determined that a 1/x weighted quadratic regression gave the best and most consistent fit for all analytes, except anabasine. For anabasine, a 1/x<sup>2</sup> weighted linear regression provided the best fit. As shown in Figure 3, r<sup>2</sup> was ≥ 0.995 for all analytes, and the percent deviation was within 15% of the nominal concentration (≤ 20% for the lowest concentrated standard). Based on the signal-to-noise value for the 10 ng/mL standard, the LLOQ is estimated to be 0.4 ng/mL for all analytes.

**Figure 2:** Chromatographic analysis of blank urine fortified with internal standards confirmed that no significant matrix interferences were present in the samples.



LC\_CF0628

**Column** Raptor™ Biphenyl (cat.# 9309512)  
**Dimensions:** 100 mm x 2.1 mm ID  
**Particle Size:** 5 µm  
**Pore Size:** 90 Å  
**Guard Column:** Raptor™ Biphenyl EXP® guard cartridge 5 mm, 2.1 mm ID, 5 µm (cat.# 930950252)  
**Temp.:** 30 °C  
**Sample**  
**Diluent:** Water  
**Conc.:** Blank human urine with internal standards  
**Inj. Vol.:** 5 µL  
**Mobile Phase**  
**A:** 0.1% Formic acid, 5 mM ammonium formate in water  
**B:** 0.1% Formic acid in methanol

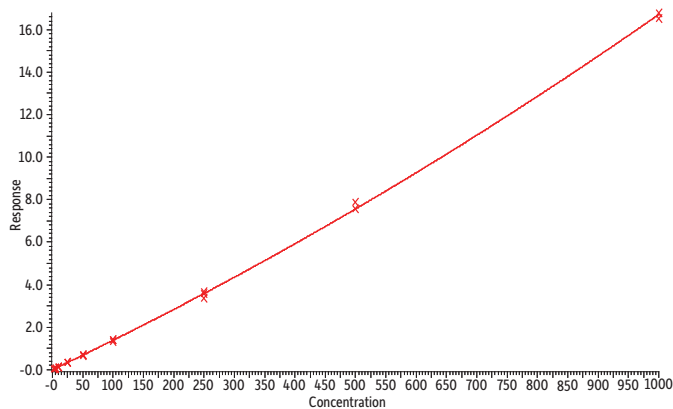
**Detector** MS/MS  
**Ion Mode:** ESI+  
**Mode:** MRM  
**Instrument** UHPLC  
**Notes**

MS/MS  
 ESI+  
 MRM  
 UHPLC  
 Samples were processed with a liquid-liquid extraction (LLE) procedure. An aliquot of 250 µL urine was mixed with 40 µL of internal standard solution (250 ng/mL in methanol) and 50 µL of 5N sodium hydroxide in a 4 mL glass vial. Extraction was performed by adding 1.5 mL of 50:50 methylene chloride:diethyl ether and stirring for 1.5 minutes. After centrifugation at 4,000 rpm for 5 minutes, 1 mL of the organic phase was transferred to a 1.5 mL HPLC vial and mixed with 10 µL of 0.25 N hydrochloric acid before evaporating to dryness at 35 °C under a gentle stream of nitrogen. The dried extract was reconstituted with 200 µL of water.

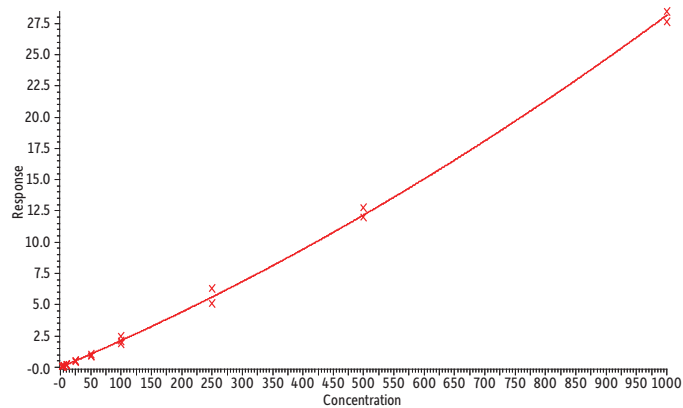
Time (min)	Flow (mL/min)	%A	%B
0.00	0.4	90	10
1	0.4	90	10
2	0.4	70	30
3	0.4	30	70
3.01	0.4	90	10
5	0.4	90	10

**Figure 3:** Good linear responses were obtained for all analytes using weighted regression.

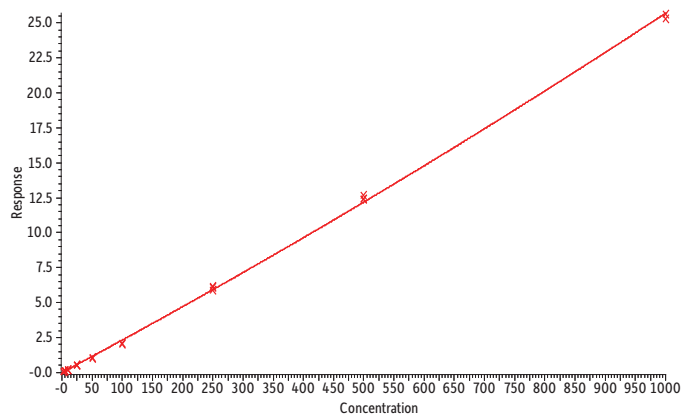
**A. Nor nicotine,  $r^2 = 0.999$**



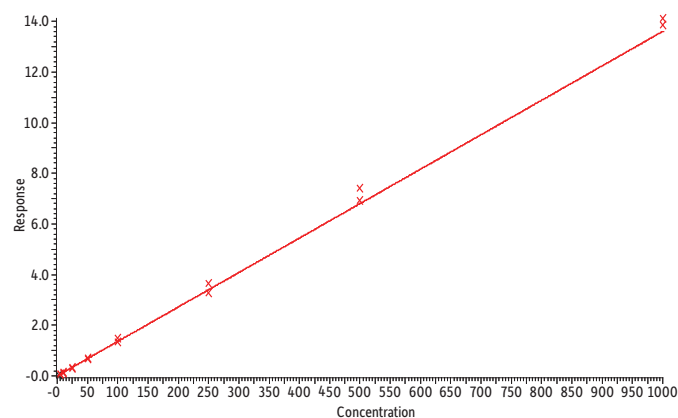
**D. Nicotine,  $r^2 = 0.997$**



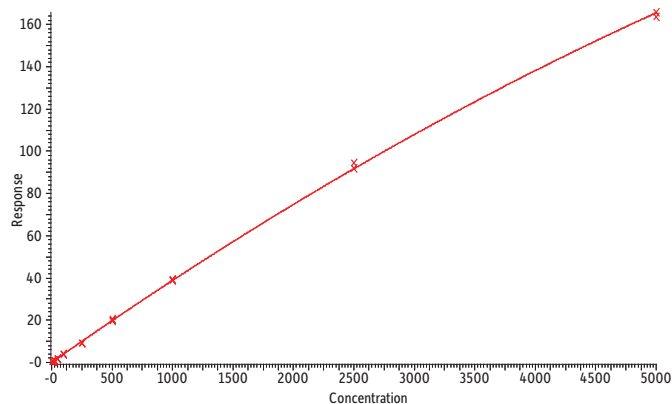
**B. Norcotinine,  $r^2 = 0.999$**



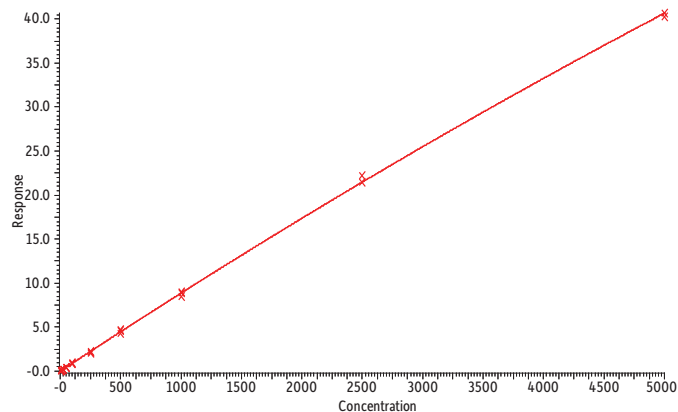
**E. Anabesine,  $r^2 = 0.995$**



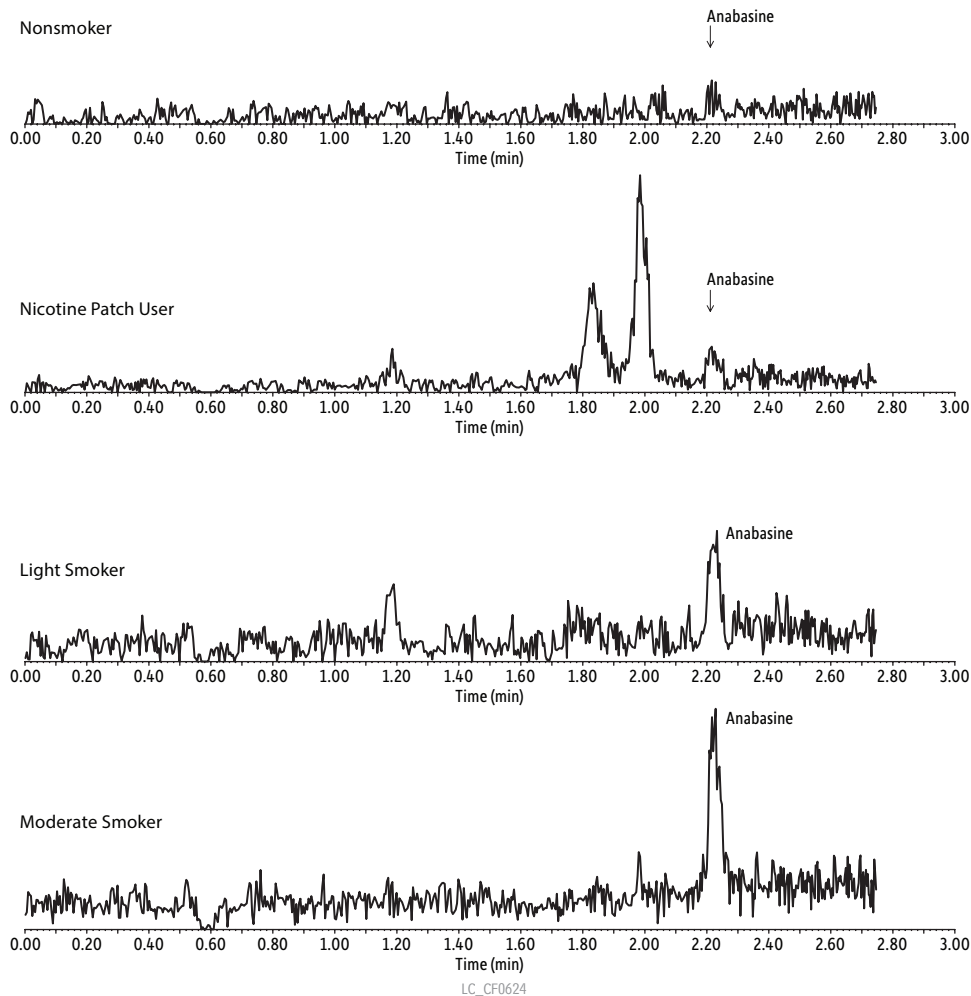
**C. *trans*-3'-Hydroxycotinine,  $r^2 = 0.999$**



**F. Cotinine,  $r^2 = 0.999$**



**Figure 4:** Specific detection of anabasine made it possible to distinguish different types of tobacco/nicotine product use.



LC\_CF0624

**Column** Raptor™ Biphenyl (cat.# 9309512)  
**Dimensions:** 100 mm x 2.1 mm ID  
**Particle Size:** 5 µm  
**Pore Size:** 90 Å  
**Guard Column:** Raptor™ Biphenyl EXP® guard cartridge 5 mm, 2.1 mm ID, 5 µm (cat.# 930950252)  
**Temp.:** 30 °C  
**Sample**  
**Diluent:** Water  
**Conc.:** Unknown concentration in human urine sample  
**Inj. Vol.:** 5 µL  
**Mobile Phase**  
**A:** 0.1% Formic acid, 5 mM ammonium formate in water  
**B:** 0.1% Formic acid in methanol

**Detector** MS/MS  
**Ion Source:** Waters Zspray™ ESI  
**Ion Mode:** ESI+  
**Instrument** UHPLC  
**Notes**

Samples were processed with a liquid-liquid extraction (LLE) procedure. An aliquot of 250 µL urine was mixed with 40 µL of internal standard solution (250 ng/mL in methanol) and 50 µL of 5N sodium hydroxide in a 4 mL glass vial. Extraction was performed by adding 1.5 mL of 50:50 methylene chloride:diethyl ether and stirring for 1.5 minutes. After centrifugation at 4,000 rpm for 5 minutes, 1 mL of the organic phase was transferred to a 1.5 mL HPLC vial and mixed with 10 µL of 0.25 N hydrochloric acid before evaporating to dryness at 35 °C under a gentle stream of nitrogen. The dried extract was reconstituted with 200 µL of water.

Time (min)	Flow (mL/min)	%A	%B
0.00	0.4	90	10
1	0.4	90	10
2	0.4	70	30
3	0.4	30	70
3.01	0.4	90	10
5	0.4	90	10

Method accuracy was demonstrated using percent recovery and these values fell well within 10% of the nominal concentration for all QC levels (93.8–104.9%). Good method precision was also obtained, as indicated by %RSD ranges of 0.6–8.2% and 1.4–9.9% for intraday and interday results respectively. The interday accuracy and precision data are summarized in Table III.

The minor tobacco alkaloid, anabasine, can only be detected in the urine of current tobacco users; it is not present in the urine of non-smokers or nicotine patch/gum users. Using the method established here, specific and sensitive urinary analysis of anabasine was used to distinguish different types of tobacco users. As shown in Figure 4, different levels of anabasine were detected from a light smoker, a moderate smoker, and a non-smoking nicotine patch user.

**Table III:** Results from fortified samples demonstrate the method was both accurate and precise for all compounds at all QC levels.

Analyte	QC-1 (7.5 ng/mL)			QC-2 (75 ng/mL)			QC-3 (750 ng/mL)			QC-4 (10,000 ng/mL)		
	Avg. Conc. (ng/mL)	Avg. Accuracy	%RSD	Avg. Conc. (ng/mL)	Avg. Accuracy	%RSD	Avg. Conc. (ng/mL)	Avg. Accuracy	%RSD	Avg. Conc. (ng/mL)	Avg. Accuracy	%RSD
Nicotine	7.520	100.3	4.2	71.52	95.4	8.1	779.0	103.9	6.6	-	-	-
Nornicotine	7.568	100.9	7.6	75.63	100.8	6.6	754.9	100.7	3.8	-	-	-
Norcotinine	7.546	100.6	5.3	75.08	100.1	9.9	786.5	104.9	4.0	-	-	-
Anabasine	7.181	95.8	3.4	73.53	98.0	4.9	778.1	103.7	3.8	-	-	-
Cotinine	-	-	-	73.82	98.4	6.3	774.6	103.3	4.7	10,076	100.8	2.3
<i>trans</i> -3'-Hydroxycotinine	-	-	-	72.31	96.4	2.7	730.0	97.3	1.4	9,379	93.8	4.6

## Conclusions

While many current methods for the analysis of nicotine-related compounds require the use of high-pH mobile phases with additives that may damage the analytical system, the method established here using the Raptor™ Biphenyl column produced good results using a simple, MS-friendly mobile phase. The method provided excellent performance for the simultaneous analysis of nicotine, two major metabolites (cotinine and *trans*-3'-hydroxycotinine), two minor metabolites (nornicotine and norcotinine), and a minor tobacco alkaloid, anabasine, in human urine. Accurate and reproducible analysis was achieved in less than 5 minutes of chromatographic analysis time, making the column and method well suited to low-cost, high-throughput analysis of nicotine-related compounds.

## References

- [1] P. Jacob, L. Yu, A.T. Shulgin, N.L. Benowitz, Minor tobacco alkaloids as biomarkers for tobacco use: comparison of users of cigarettes, smokeless tobacco, cigars, and pipes, *Am J Public Health*, 89 (5) (1999) 731–736.
- [2] N.L. Benowitz, J. Hukkanen, P. Jacob, Nicotine chemistry, metabolism, kinetics and biomarkers, *Handb Exp Pharmacol*, 192 (2009) 29–60.
- [3] K.S. Derby, K.C.C. Caberto, S.G. Carmella, A.A. Franke, S.S. Hecht, Nicotine metabolism in three ethnic/racial groups with different risks of lung cancer, *Cancer Epidemiol Biomarker Prev*, 17 (2008) 3,526–3,535.