

## A Sensitive, Specific, Accurate, and Fast LC/MS/MS Method for Measurement of Nicotine, Cotinine, 3-Hydroxycotinine, Nornicotine and Anabasine in Human Urine

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*A method is illustrated for simultaneous analysis of nicotine, two of its major metabolites, cotinine and 3-hydroxycotinine, as well as nornicotine and anabasine from human urine samples. The method described uses Strata™-X-C solid phase extraction (SPE) cartridges for sample clean-up and concentration, followed by fast (<6 min) LC/MS/MS analysis using a Gemini® NX-C18 column.*

### Introduction

Nicotine is the most abundant alkaloid present in all tobacco products along with being a major tobacco-specific component in both mainstream tobacco smoke and environmental tobacco smoke (ETS). Likewise it is also a potential and viable pharmacological ingredient found in most nicotine replacement therapeutic drugs (Tuomi T, 1-5). Determination of nicotine metabolism / pharmacokinetics provides a useful tool for estimating uptake of nicotine and tobacco-related toxicants, understanding the pharmacologic effects of nicotine and nicotine addiction, and for optimizing nicotine dependency treatment (Xu et al 2004).

In addition to nicotine and its metabolites, tobacco products also contain other alkaloids that can serve as unique markers of tobacco use. Two such examples are anabasine and nornicotine, which are present in tobacco products but not in nicotine replacement therapies. Detection of these alkaloids indicates current tobacco use, irrespective of whether the subject is using nicotine replacement therapy.

Development of highly specific and sensitive analytical methods for quantification of urinary nicotine and multiple nicotine metabolites would improve the estimation of nicotine exposure and dose for passively exposed nonsmokers (Davis RA). Our goal was to develop a sensitive, specific, accurate and fast analytical method to simultaneously quantify nicotine and metabolites in human urine using SPE for sample cleanup and concentration, and fast LC/MS/MS analysis using a Gemini NX-C18 column.

### Materials and Methods

All reagents and solvents were HPLC or analytical grade. HPLC grade methanol and acetonitrile was purchased from Honeywell, Burdick & Jackson (Muskegon, MI). Milli-Q Water was used for reagents preparation, SPE, sample preparation and to prepare the LC mobile phase. Anabasine, Cotinine, Nicotine, Nornicotine, and Ammonium Bicarbonate was purchased from J.T. Baker, Inc., (Phillipsburg, NJ). Ammonium hydroxide was purchased from Sigma-Aldrich. Trans-3'-Hydroxycotinine-d3 was purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). Nicotine-d4 was purchased from C/D/N Isotopes, (Quebec, Canada). Cotinine-d3 was purchased from Cerilliant, (Round Rock, TX). Nornicotine-d4 was purchased from C/D/N Isotopes, (Quebec, Canada).

An Agilent 1200 Series HPLC (Agilent® Technologies Inc., Santa Clara, CA USA) was interfaced with API 4000™ MS/MS with ESI TurbolonSpray® (AB SCIEX Foster City, USA) operated in positive ionization mode (ESI+).

### Sample Preparation

The individual deuterated internal standard (IS) stock solutions (1000 ng/mL) were prepared in acetonitrile and stored at 4 °C until use. A working solution containing all IS at a final concentration of 100 ng/mL was prepared in acetonitrile. 100 µL of the IS working solution was then added to 1 mL of diluted human urine specimen, yielding a final urine IS concentration of 100 ng/mL.

For calibrators, a stock solution of 1 mg/mL of each analyte was prepared in acetonitrile and stored at 4 °C until use. The working solution of 25 µg/mL was prepared by dilution with methanol. A standard calibration curve was generated by spiking different aliquots of the working stock solution into blank human urine specimen; yielding a nine-point calibration curve (1, 2.5, 5, 10, 25, 50, 100, 250, and 500 ng/mL).

Three human urine quality control (QC) samples were prepared in triplicates with a different lot of working standard solution to yield QC concentration of 4, 40 and 400 ng/mL, respectively. The samples were prepared for analysis using the SPE procedure.

The urine sample was prepared by diluting 0.5 mL urine samples with 0.5 mL of 20 mM ammonium acetate, pH 4, and adding 100 µL internal standards.

### Solid Phase Extraction (SPE)

**Cartridge:** Strata-X-C (60 mg/3 mL)  
**Part No.:** 8B-S029-UBJ  
**Condition:** 2 mL Methanol (1-2 mL/min)  
**Equilibrate:** 2 mL Ammonium acetate buffer  
**Load:** 0.5 mL Diluted urine sample  
**Wash 1:** 2 mL Ammonium acetate buffer  
**Wash 2:** 2 mL 30 % Methanol in water  
**Dry:** > 10<sup>3</sup> Hg for 5 min to remove residual water  
**Elute:** 2 x 2 mL 1.5 % Ammonium hydroxide in methanol  
**Dry down:** Nitrogen gas at 55 °C  
**Reconstitute:** 500 µL of Acetonitrile/20 mM Ammonium bicarbonate (10:90)

### LC/MS/MS

**Column:** Gemini 3 µm NX-C18  
**Dimensions:** 50 x 2.0 mm  
**Part No.:** 00B-4453-B0  
**Mobile Phase:** A: 20 mM Ammonium bicarbonate  
 B: Acetonitrile

Gradient	Time (min)	B (%)
	0	10
	3	75
	3.1	10
	5	10

**Flow Rate:** 0.5 mL/min  
**Temperature:** 25 °C  
**Injection:** 10 µL  
**Detection:** AB SCIEX API 4000™ MS/MS (ESI+)



**MS/MS Conditions**

**Ionization:** ESI  
**Polarity:** Positive  
**Scan Type:** MRM  
**Curtain Gas (CUR):** 50  
**Gas 1 (GS1):** 50  
**Gas 2 (GS2):** 50  
**IS:** 5500  
**Collision Gas (CAD):** 5  
**Interface Heater (Ihe):** On  
**Temperature (TEM):** 550 °C  
**Entrance Potential (EP):** 10

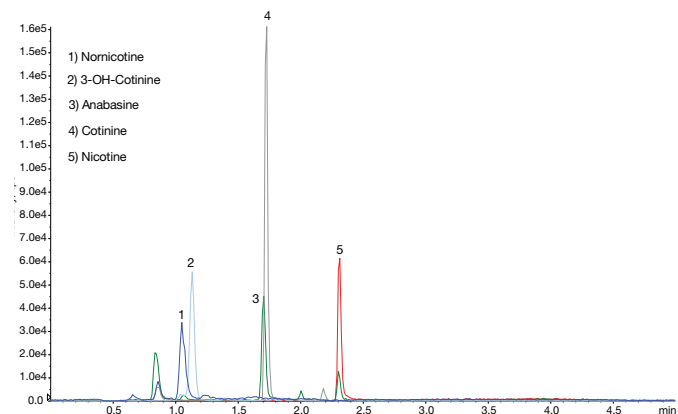
**Table 1.**  
Mass Dependant Parameters

Compounds	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (msec)	DP	CE	CXP
Nicotine	163.1	132.1	50	56	23	14
Nicotine-d4	167.1	136.0	50	56	21	14
Cotinine	177.1	80.1	50	70	31	8
Cotinine-d3	180.1	80.1	50	70	31	8
Normicotine	149.1	80.1	50	71	29	8
Normicotine-d4	153.1	84.1	50	70	29	8
3-OH-cotinine	193.1	80.1	50	70	31	8
3-OH-cotinine-d3	196.1	80.1 <td 50	66	35	8	
Anabasine	163.1	120.1	50	70	31	8

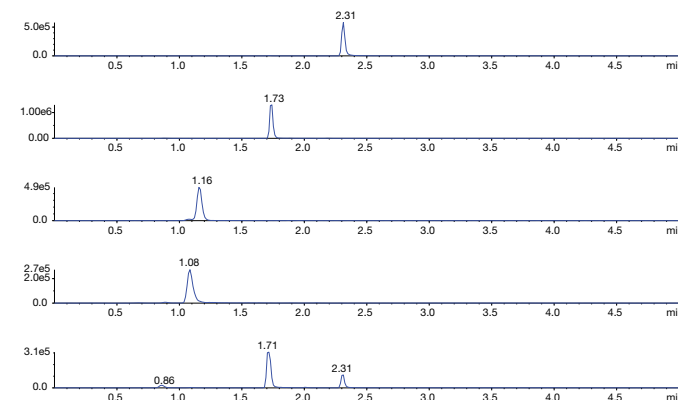
**Results and Discussion**

The use of the rugged pH stable Gemini<sup>®</sup> NX-C18 column allowed for fast elution of nicotine and its metabolites in less than 3 minutes (**Figure 1**). This fast separation allows for multiplexing techniques to handle the analysis of large numbers of samples. In ESI positive mode, nicotine and its metabolites were detected by monitoring the mass transitions and their deuterium-labeled internal standards listed in **Table 1**. The most abundant transition for each analyte was used for quantification. The second mass transition (not listed) served as a confirmation for each analyte. **Figure 2** shows the extracted ion chromatograms for nicotine, cotinine, 3-hydroxycotinine, normicotine and anabasine at concentrations of 10 ng/mL in extracted human urine. Note that anabasine and nicotine have the same parent mass, accounting for the 3rd peak in the anabasine XIC (**Figure 2**). Since the deuterium-labeled internal standard was not available for anabasine, cotinine-d3 served as its internal standard because it has the nearest retention time (**Figure 2**). The pH of the mobile phase was adjusted so that anabasine and cotinine elute as closely as possible. Anabasine can be separated at a slightly higher pH (pH 9) if the deuterated internal standard were to become available.

**Figure 1.**  
Nicotine, Cotinine, 3-OH-cotinine, Normicotine and Anabasine analysis (10 ng/mL urine extracted standard)



**Figure 2.**  
Extracted Ion Chromatograms for Nicotine, Cotinine, 3-OH-cotinine, Normicotine and Anabasine at a concentration of 10 ng/mL in human urine



App ID 22022

App ID 22035

### Results and Discussion (con't)

Standard calibration curves were generated over the concentration range of 1.0 ng/mL to 500 ng/mL by plotting the relative response (peak area of nicotine and its metabolites / peak area of internal standards) versus concentration. The standard calibration curves were linear over the calibration ranges with R<sup>2</sup> values of 0.9997 for nicotine, 0.9979 for cotinine, 0.9997 for nornicotine, 0.9973 for 3-OH-cotinine and 0.9995 for anabasine, separately (Figure 4).

Sensitivity of the method was evaluated by determining the lowest level concentration with a signal to noise of at least 10:1 for limit of quantification (LOQ). At the lowest level standard concentration (1.0 ng/mL) the signal-to-noise ratios were 46.1 for nicotine, 141.5 for cotinine, 16.1 for nornicotine, 50.7 for 3-OH-cotinine and 24.5 for anabasine, separately (Figure 3 and Table 2). Therefore, the LOQs were estimated to be < 1.0 ng/mL for nicotine, cotinine, nornicotine, 3-OH-cotinine and anabasine (Table 2). Furthermore, the current method achieved additional sensitivity by reducing specimen size from 1000 µL to 500 µL urine. If greater sensitivity is required or if the intended detector cannot meet the 1 ng/mL sensitivity, reducing the final elution volume by half is an easy approach. The upper limit of quantification was 500 ng/mL for all the analytes (Table 2).

**Table 2.**  
Statistical Data of Nicotine and Metabolites in Urine by LC/MS/MS

Analyte	LOQ	ULOQ	Y-intercept	R <sup>2</sup>	Intra Assay Precision % (N = 3)						S/N	RT		
					4 ng/mL		40 ng/mL		400 ng/mL				1 ng/mL	min
					Mean	% CV	Mean	% CV	Mean	% CV				
Nicotine	1	500	0.0495x +0.0503	0.9997	96.13	3.72	105.67	2023	102.00	0.80	46.10	2.31		
Cotinine	1	500	0.0386x +0.0372	0.9979	94.84	3.06	100.00	0.99	101.37	3.98	141.50	1.73		
3-OH-cotinine	1	500	0.0398x +0.044	0.9973	103.10	5.85	98.20	1.92	107.33	4.95	50.70	1.16		
Nornicotine	1	500	0.0285x +0.0233	0.9997	101.53	5.94	100.67	1.66	105.43	6.09	16.10	1.08		
Anabasine	1	500	0.0132x +0.0311	0.9995	97.03	2.77	107.00	2.64	95.53	4.67	24.50	1.71		

**LOQ** = Limit of quantification

**ULOQ** = Upper limit of quantification

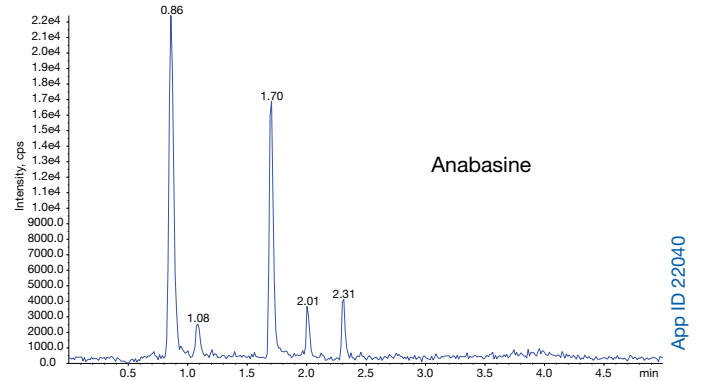
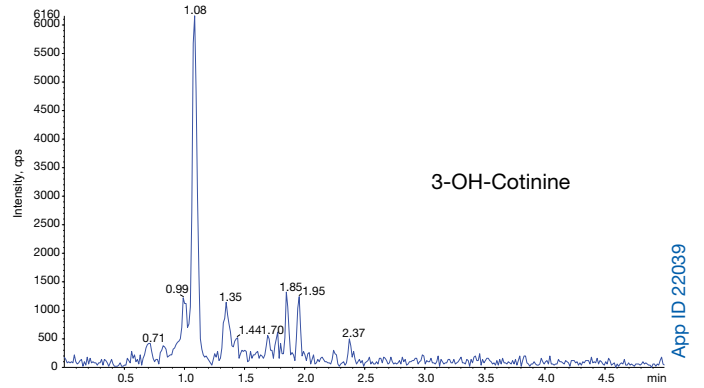
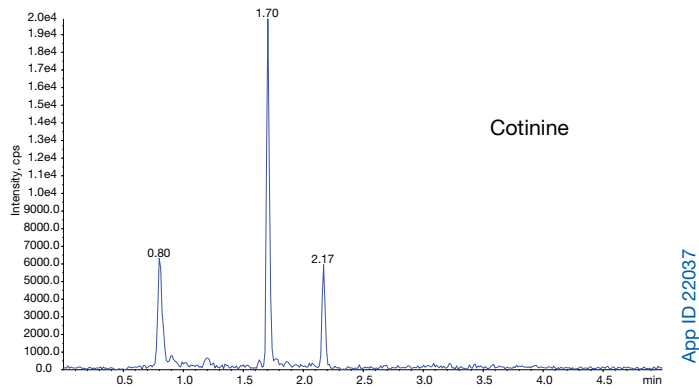
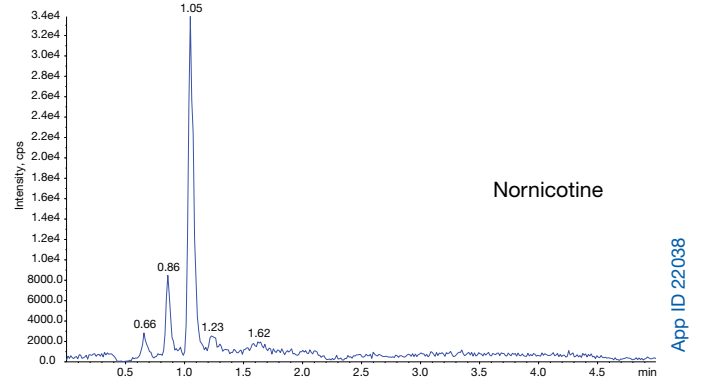
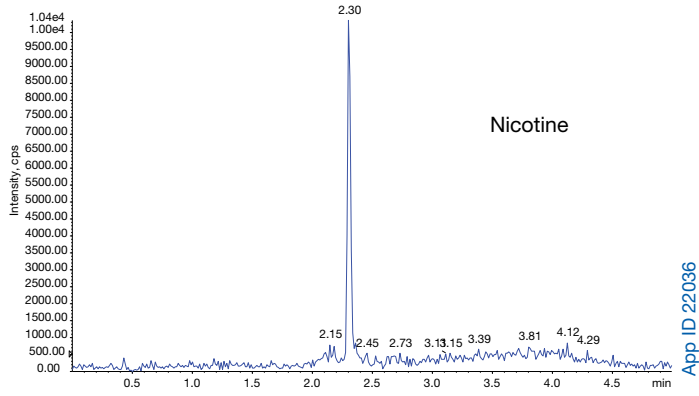
**CV** = Coefficient of Variation (Standard Deviation / Mean)

**S/N** = Signal / Noise

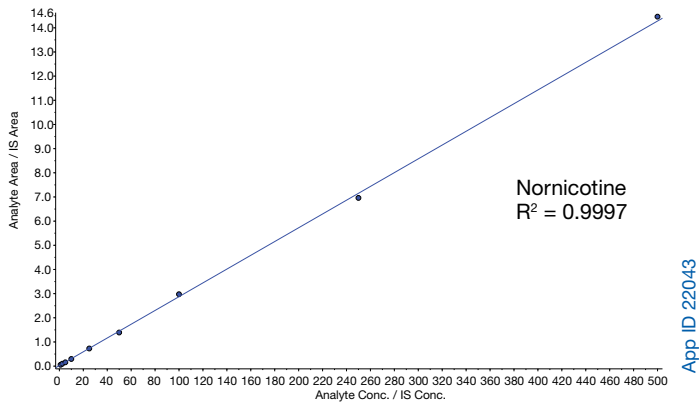
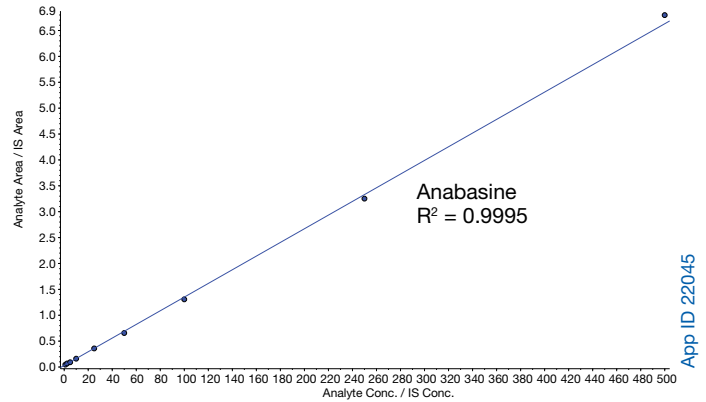
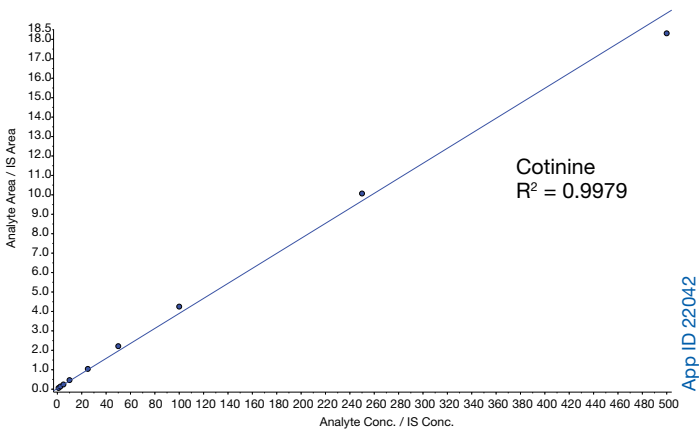
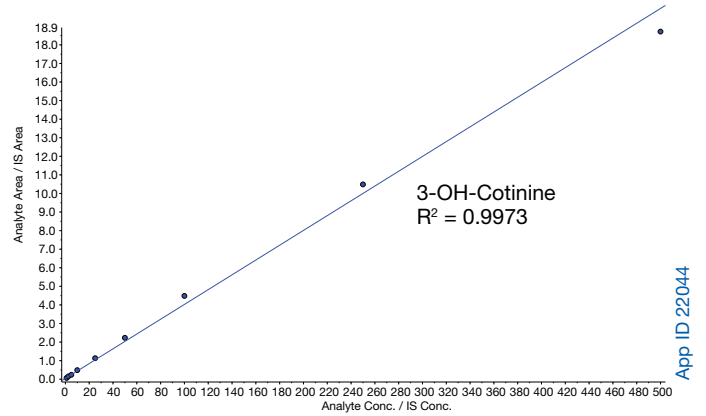
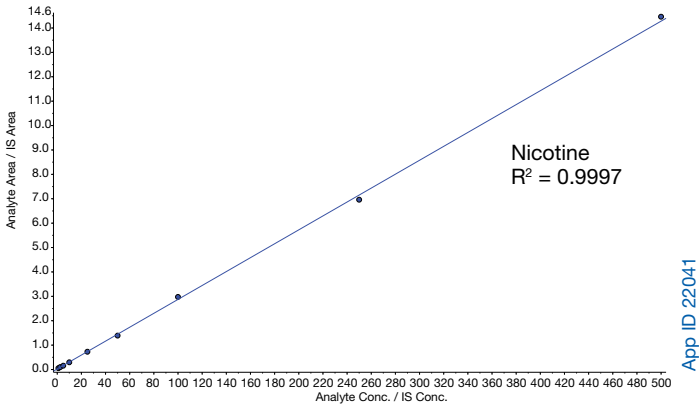
**RT** = Retention time



**Figure 3.**  
Nicotine and metabolites analysis (1 ng/mL extracted standard)



**Figure 4.**  
Standard curves from 1 ng/mL to 500 ng/mL for Nicotine, Cotinine, Nor-  
nicotine, 3-OH-cotinine and Anabasine



## Results and Discussion (con't)

Three levels of QC samples were prepared at 4, 40 and 400 ng/mL. These concentrations were selected to represent low, medium, and high concentration across the calibration range for each analyte. The three level QC samples were extracted in the same way as the actual sample described above and analyzed in triplicates to assess reproducibility. The mean expected recovery of the lowest level QC samples at 4 ng/mL was 96.13 % for nicotine, 94.80 % for cotinine, 103.10 % for 3-OH-cotinine, 101.53 % for nornicotine and 97.03 % for anabasine (Table 2).

The percentage Coefficient of Variation (%CV) for the intra assay precision were from 2.77 % to 5.85 % for levels of 4 ng/mL, 0.99 % to 2.64 % for levels of 40 ng/mL and 0.8 % to 6.09 % for levels of 400 ng/mL, respectively (Table 2). No endogenous signal was found in 3 nonsmoker urine specimens, demonstrating the selectivity of the method. There were no carryovers observed by injecting blank urine samples after the highest calibrator (500 ng/mL).

The chromatogram in Figure 5 illustrates a urine/matrix suppression study when using an ESI source and represents a post-column infusion of high concentration standards of nicotine, cotinine, 3-OH-cotinine, nornicotine and anabasine, while a low level urine extract is injected on column. The top trace contains the MRM transitions for all five analytes and the bottom trace is the four internal standards channel (nornicotine-d4, 3-OH-cotinine-d3, cotinine-d3 and nicotine-d4). As expected, this section of the chromatogram contains highly polar components of the extract and is virtually un-retained. Nicotine, its metabolites and their internal standards elute between 1 to 2.5 min, in the region of stability between 0.8 – 2.8 min. The region where the mobile phase is mostly organic, 2.9 – 4.5 min, produces the most dramatic signal increase. This is to be expected as the ionization source becomes more productive with a lower viscosity and lower boiling point solvent.

We optimized the SPE extraction procedure to allow for the best recovery of the urinary nicotine and its metabolites and to have the greatest potential for high-throughput sample preparation and automation. The Strata™-X-C sorbent represented the most durable and selective material.

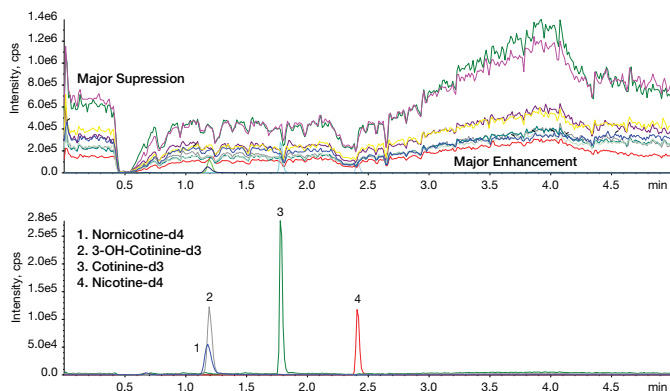
## Conclusion

A rapid analysis of urinary nicotine and its metabolites was developed. The application of the Gemini® NX-C18 column in this method results in a shorter chromatographic analysis time, providing a productivity benefit for clinical testing laboratories with a dramatic increase in efficiency while simultaneously reducing costs due to solvent consumption. Sample preparation using Strata-X-C SPE concentrates the nicotine and metabolites and removes potential sample matrix interferences, which coupled with high efficiency Gemini NX-C18 column provides for the low level detection and quantitation of nicotine and its metabolites in human urine.

## References

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**Figure 5.**  
Urine matrix effect on Nicotine, its metabolites and their IS's response using an ESI source



App ID 22046

## Ordering Information

### Gemini® HPLC Columns

3 µm Microbore, Minibore and Narrow Bore Columns (mm)								SecurityGuard™ Cartridges (mm)	
Phases	20 x 2.0	30 x 2.0	50 x 2.0	100 x 2.0	150 x 2.0	50 x 3.0	100 x 3.0	150 x 3.0	4 x 2.0*
NX-C18	00M-4453-B0	00A-4453-B0	00B-4453-B0	00D-4453-B0	00F-4453-B0	00B-4453-Y0	00D-4453-Y0	00F-4453-Y0	AJ0-8367

for ID: 2.0-3.0 mm

3 µm Analytical Columns (mm)					SecurityGuard Cartridges (mm)
Phases	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	4 x 3.0*
NX-C18	00B-4453-E0	00D-4453-E0	00F-4453-E0	00G-4453-E0	AJ0-8368

for ID: 3.2-8.0 mm

5 µm Minibore and Narrow Bore Columns (mm)								SecurityGuard Cartridges (mm)	
Phases	30 x 2.0	50 x 2.0	150 x 2.0	50 x 3.0	100 x 3.0	150 x 3.0	250 x 3.0	4 x 2.0*	
NX-C18	00A-4454-B0	00B-4454-B0	00F-4454-B0	00B-4454-Y0	00D-4454-Y0	00F-4454-Y0	00G-4454-Y0	AJ0-8368	

for ID: 2.0-3.0 mm

5 µm Analytical Columns (mm)					SecurityGuard Cartridges (mm)
Phases	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	4 x 3.0*
NX-C18	00B-4454-E0	00D-4454-E0	00F-4454-E0	00G-4454-E0	AJ0-8368



for ID: 3.2-8.0 mm



For Gemini Capillary or Preparative HPLC Columns, Guards, and Adapter, contact your Phenomenex technical consultant or local distributor.

## Ordering Information

### Strata™ -X-C

Format	Sorbent Mass	Part Number	Unit
<b>Tab-Less Tube</b>			
	30 mg	8L-S029-TAK	1 mL (100/box)
	60 mg	8L-S029-UBJ	3 mL (50/box)
<b>Tube</b>			
	30 mg	8B-S029-TAK**	1 mL (100/box)
	30 mg	8B-S029-TBJ	3 mL (50/box)
	60 mg	8B-S029-UBJ**	3 mL (50/box)
	100 mg	8B-S029-EBJ	3 mL (50/box)
	100 mg	8B-S029-ECH	6 mL (30/box)
	200 mg	8B-S029-FBJ	3 mL (50/box)
	200 mg	8B-S029-FCH	6 mL (30/box)
	500 mg	8B-S029-HBJ	3 mL (50/box)
	500 mg	8B-S029-HCH	6 mL (30/box)
<b>96-Well Plate</b>			
	10 mg	8E-S029-AGB	2 Plates/Box
	30 mg	8E-S029-TGB	2 Plates/Box
	60 mg	8E-S029-UGB	2 Plates/Box

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