APPLICATIONS

Ann Montross
Technical Specialist
Ann enjoys cooking/baking and roasting her own coffee beans.

A Sensitive Extraction Method for Nicotine and Metabolites from Urine using Simplified Liquid Extraction (SLE)

Xianrong (Jenny) Wei, Ann Montross, and Sean Orlowicz
Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501 USA

This method has been validated and shown acceptable accuracy and precision using a small volume of human urine in the analysis of nicotine and its metabolites (anabasine, nornicotine, cotinine and 3-hydroxycotinine). The modified method employed uses a Novum™ SLE MAX 96-well plate with pH adjusted organic elution solvent, and a Kinetex® EVO C18 column with high pH mobile phase to increase analyte recoveries and sensitivity using LC/MS/MS.

Introduction

Analysis of nicotine in urine has become more widespread with the increased use of tobacco-less products, such as nicotine lozenges, vaporizers, and gums. Analyzing nicotine metabolites (anabasine, nornicotine, cotinine and 3-hydroxycotinine) allows researchers to distinguish between an active smoker and someone using tobacco-less products. The presence of tobacco metabolites, anabasine and nornicotine, in urine is an indication of an active smoker.

The goals of this study were to simplify the extraction process using a Simplified Liquid Extraction (SLE) method, increase recoveries, and demonstrate the assay accuracy, precision and linearity for all compounds under (GLP) Good Laboratory Pratice guidance. The endogenous level of nicotine and its metabolites were monitored during the method development for the determination of LLOQ quantification level purpose. The method uses higher pH mobile phase at pH 8.2 to ensure the separations of all compounds, and therefore the Kinetex EVO C18 column was chosen for its widely stable pH range and chromatography.

Experimental Conditions

Reagents and Chemicals

All solvents and reagents were HPLC or analytical grade. HPLC Grade methanol was purchased from Honeywell Burdick & Jackson® (Muskegon, MI), Milli-Q® water was used for sample preparation. HPLC Grade water was purchased from Honeywell Burdick & Jackson and used to prepare the LC mobile phase.

Standards

Reference standards were purchased from Cerilliant® Corporation.

Equipment and Materials

Agilent® 1260 pumps and autosampler were used along with a SCIEX QTRAP® 4500, positive polarity, ESI for detection.

Sample Pretreatment

200 µL of 2 % NH_4OH in water and 50 µL of mixed deuterated internal standards (200 ng/mL each in water) were added to 100 µL of human urine (spiked with analytes and/or blank) and mixed.

SLE Conditions

Load: Load pretreated sample onto the Novum MAX 96-Well Plate (Phenomenex Part No.: 8E-S138-5GA) and apply 5" Hg of vacuum to initiate loading. Stop vacuum and wait 5 minutes.

Elute: Dispense 600 µL of 1 % Formic acid in DCM/IPA (95:5). Repeat two more times. Collect eluents in a collection plate (2 mL).

Dry Down: Evaporate the final extract to complete dryness under a slow stream of N_2 at 45 °C.

Reconstitute: Add 300 µL of 20 mM Ammonium Bicarbonate, pH 8.2/Methanol (90:10) and vortex.

LC/MS/MS Conditions:

Column: Kinetex® 2.6 µm EVO C18
Dimensions: 100 x 3.0 mm
Part No.: 00D-4725-Y0
Mobile Phase: A: 20 mM Ammonium Bicarbonate, pH 8.2
B: Methanol

Gradient:

Time (min)	B (%)
0	10
3	90
5	90
5.01	10
6.	10

Flow Rate: 0.75 mL/min
Temperature: Ambient
Detection: SCIEX QTRAP 4500 Turbo™
Sample:
1. 3-Hydroxycotinine
2. Nornicotine
3. Cotinine
4. Anabasine
5. Nicotine

ESI Ionization Source Parameters:

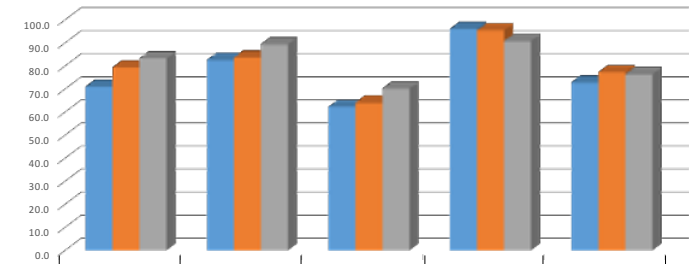
Curtain Gas (CUR): 20
Collision Gas (CAD): 7
Temperature (TEM): 600
Gas 1 (GS1): 50
Gas 2 (GS2): 50
IS: 4500
Entrance Potential (EP): 10
Collision Potential (CXP): 12

Table 1. Mass Transitions

Compounds	Q1 Mass (amu)	Q3 Mass (amu)	Dwell Time (amu)	DP	CE
Anabasine 1*	163	80	25	80	27
Anabasine 2	163	120.1	25	80	22
Anabasine D4 1*	167	84	25	80	27
Anabasine D4 2	167	124	25	80	22
Cotinine 1	177	80	25	80	29
Cotinine 2*	177	98	25	70	28
Cotinine D3 1*	180	80	25	80	28
Cotinine D3 2	180	101	25	80	28
3-Hydroxycotinine 1*	193	80	25	60	34
3-Hydroxycotinine 2	193	134	25	60	25
3-Hydorxycotinine D3 1*	196	80	25	60	34
3-Hydroxycotinine D3 2	196	134	25	60	25
Nicotine 1	163	130	25	60	26
Nicotine 2*	163	132	25	60	20
Nicotine D4 1*	167	134	25	60	26
Nicotine D4 2	167	136	25	60	20
Nornicotine 1	149	80	25	50	25
Nornicotine 2*	149	130	25	50	21
Nornicotine D4 1	153	84	25	55	29
Nornicotine D4 2*	153	134	25	55	18
Anabasine 1*	163	80	25	80	27

*Quantitation mass

Table 2. Novum Recovery



	Anabasine	Nicotine	Nornicotine	Cotinine	3-OH-Cotinine
QCL	71.1	82.8	62.4	96.2	73.0
QCM	79.5	83.9	64.3	95.6	77.5
QCH	83.5	89.6	70.4	91.0	76.7

Figure 1. Representative chromatograms of QCM at 200 ng/mL in human urine

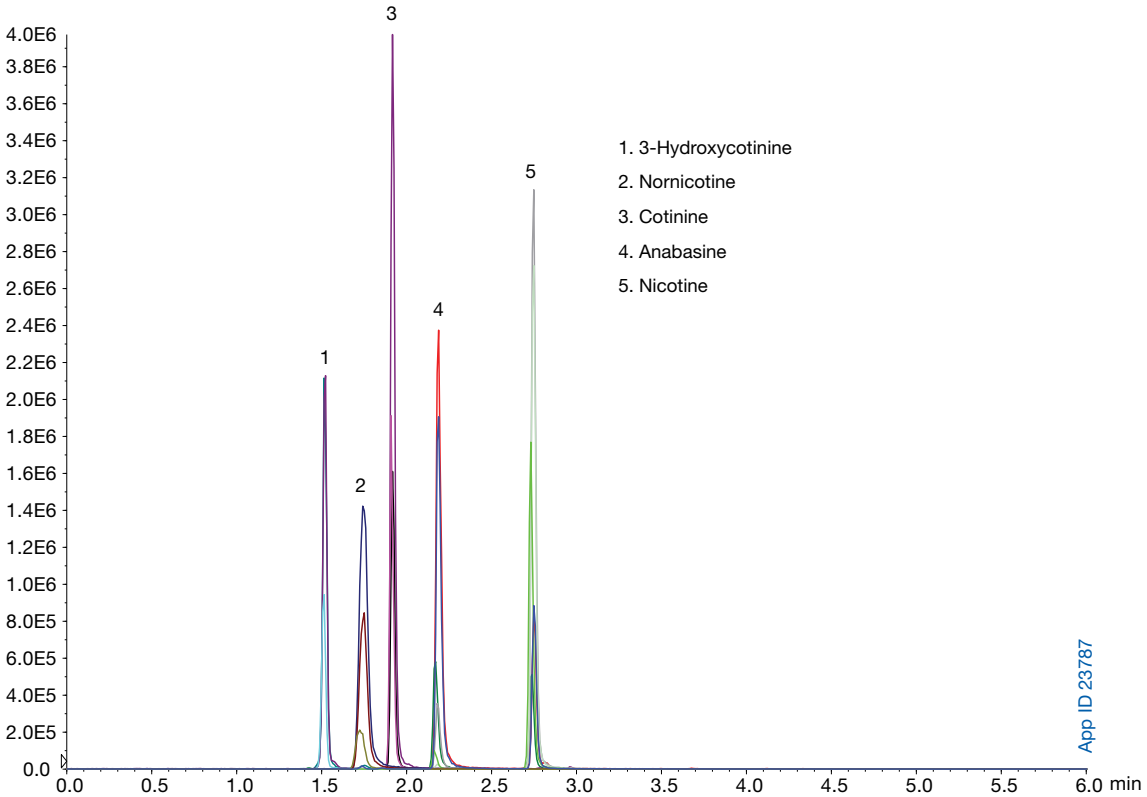


Table 3. Accuracy and Precision

	Anabasine	Nicotine	Nornicotine	Cotinine	3-OH-Cotinine
Sample ID	**Conc. Found (ng/mL) - LLOQ**				
Nominal Conc. (ng/mL)	**1**	**5**	**1**	**1**	**1**
LLOQ_1	0.946	5.14	1.15	1.05	0.979
LLOQ_2	0.950	4.45	0.994	0.900	0.900
LLOQ_3	0.993	4.27	1.04	0.941	0.987
LLOQ_4	0.986	6.56	0.942	0.971	0.988
LLOQ_5	0.962	5.29	1.02	1.08	1.18
LLOQ_6	1.09	5.32	1.15	1.04	1.10
Mean Conc. Found (ng/mL)	**0.988**	**5.172**	**1.05**	**0.997**	**1.02**
STDV	**0.054**	**0.812**	**0.085**	**0.070**	**0.10**
CV%	**5.42**	**15.7**	**8.06**	**7.06**	**9.80**
Accuracy (%)	**98.8**	**103**	**105**	**99.7**	**102**
n=	6	6	6	6	6
Sample ID	**Conc. Found (ng/mL) - QCL**				
Nominal Conc. (ng/mL)	**5**	**15**	**5**	**5**	**5**
QCL_1	5.71	17.0	5.12	5.49	5.53
QCL_2	5.20	17.2	4.90	4.82	5.06
QCL_3	4.70	15.8	4.91	5.32	5.75
QCL_4	5.43	17.1	5.27	5.38	4.90
QCL_5	5.47	15.4	5.08	5.56	4.57
QCL_6	5.50	16.8	5.22	5.57	5.04
Mean Conc. Found (ng/mL)	**5.34**	**16.55**	**5.08**	**5.36**	**5.14**
STDV	**0.351**	**0.758**	**0.154**	**0.281**	**0.430**
CV%	**6.58**	**4.58**	**3.03**	**5.24**	**8.36**
Accuracy (%)	**107**	**110**	**102**	**107**	**103**
n=	6	6	6	6	6
Sample ID	**Conc. Found (ng/mL) - QCM**				
Nominal Conc. (ng/mL)	**200**	**200**	**200**	**200**	**200**
QCM_1	213	210	213	197	222
QCM_2	221	211	202	198	194
QCM_3	225	203	207	222	215
QCM_4	212	214	206	224	213
QCM_5	214	224	222	209	217
QCM_6	234	222	204	231	218
Mean Conc. Found (ng/mL)	**220**	**214**	**209**	**214**	**213**
STDV	**8.61**	**7.87**	**7.38**	**14.29**	**9.87**
CV%	**3.92**	**3.68**	**3.53**	**6.69**	**4.63**
Accuracy (%)	**110**	**107**	**105**	**107**	**107**
n=	6	6	6	6	6
Sample ID	**Conc. Found (ng/mL) - QCH**				
Nominal Conc. (ng/mL)	**400**	**400**	**400**	**400**	**400**
QCH_1	413	414	409	395	393
QCH_2	370	396	411	367	399
QCH_3	402	397	385	378	387
QCH_4	421	438	390	395	413
QCH_5	381	392	407	417	396
QCH_6	378	377	410	371	391
Mean Conc. Found (ng/mL)	**394**	**402**	**402**	**387**	**397**
STDV	**20.8**	**21.1**	**11.4**	**18.8**	**9.1**
CV%	**5.27**	**5.24**	**2.84**	**4.85**	**2.29**
Accuracy (%)	**98.5**	**101**	**101**	**96.8**	**99.1**
n=	6	6	6	6	6

Figure 2. Representative chromatograms of blank human urine

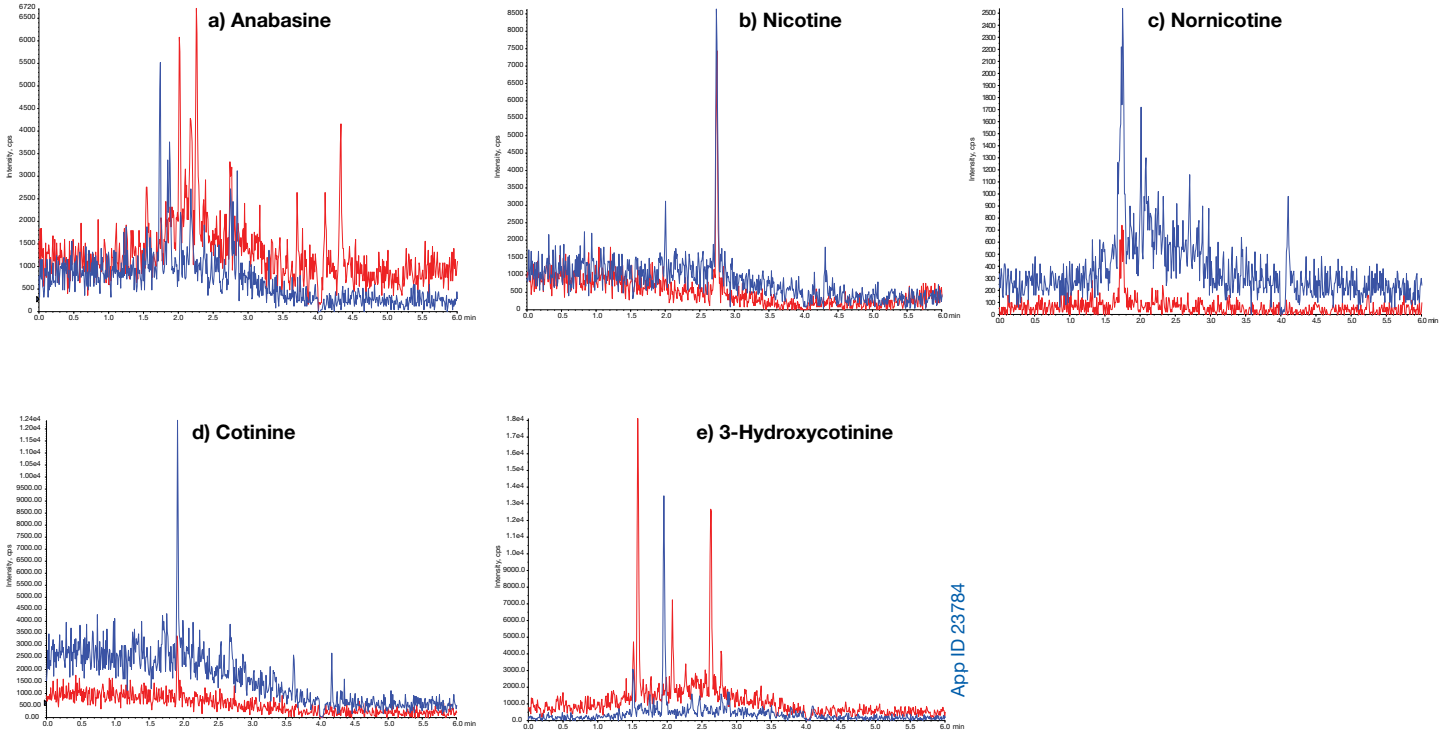


Figure 3. Representative chromatogram of LLOQ at 1.0 ng/mL (5.0 ng/mL for Nicotine) in human urine

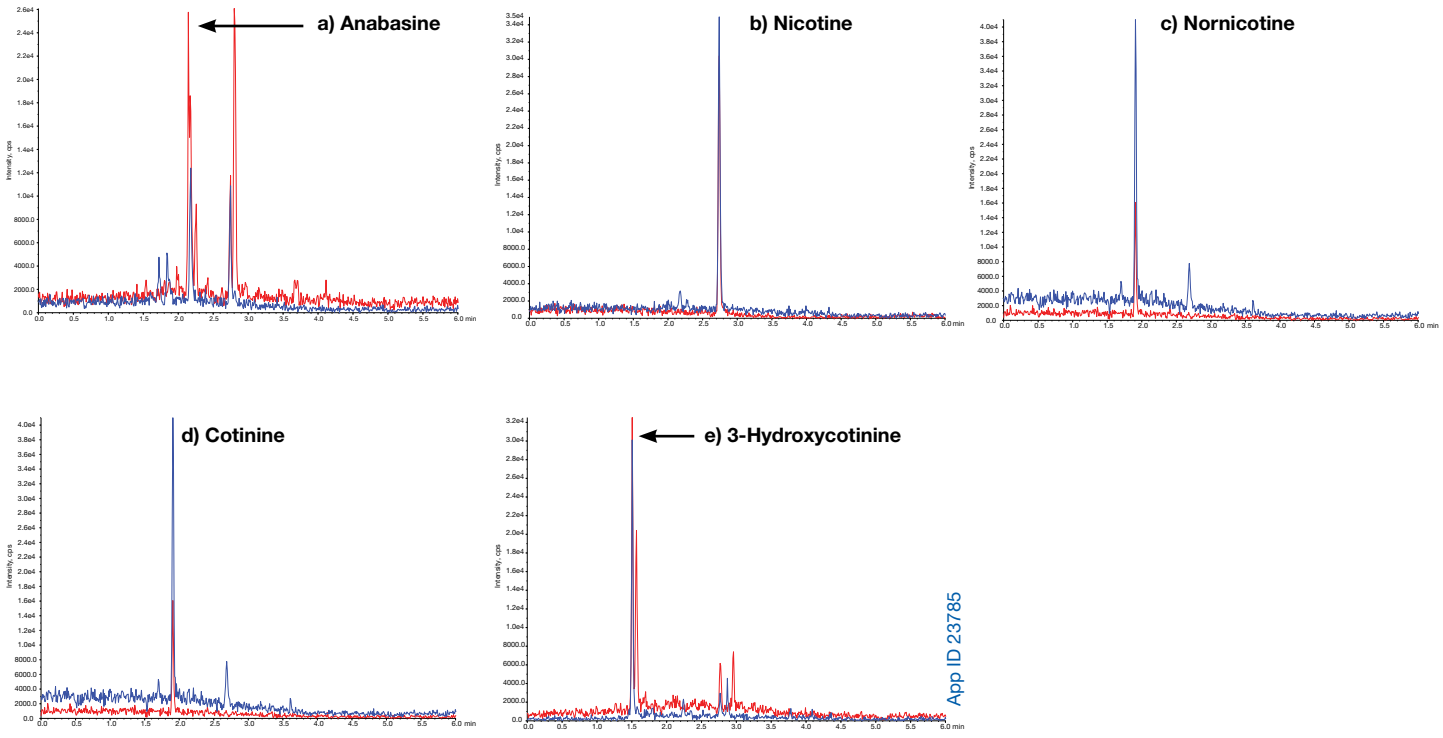


Figure 4. Representative chromatogram of ULOQ at 500 ng/mL in human urine

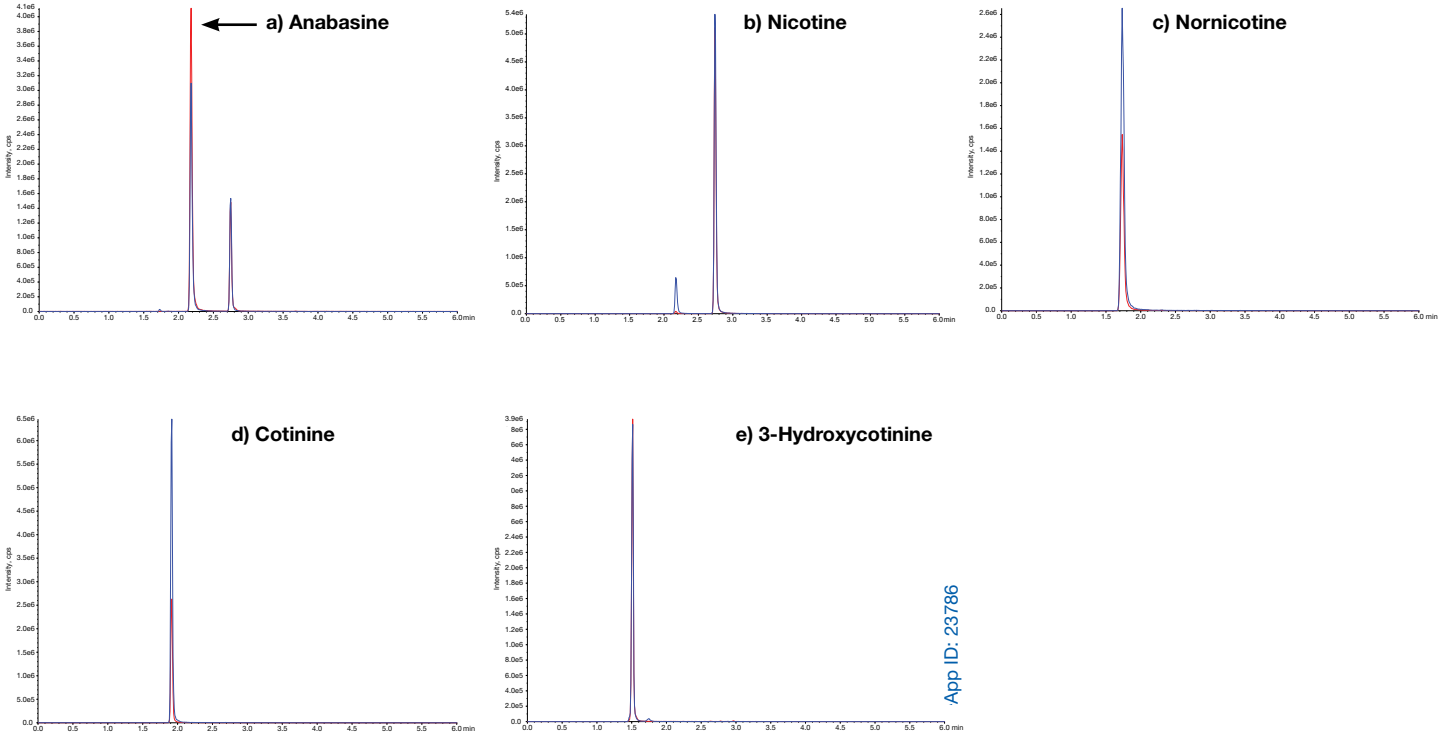


Figure 5. Representative calibration curves in human urine (n=2)

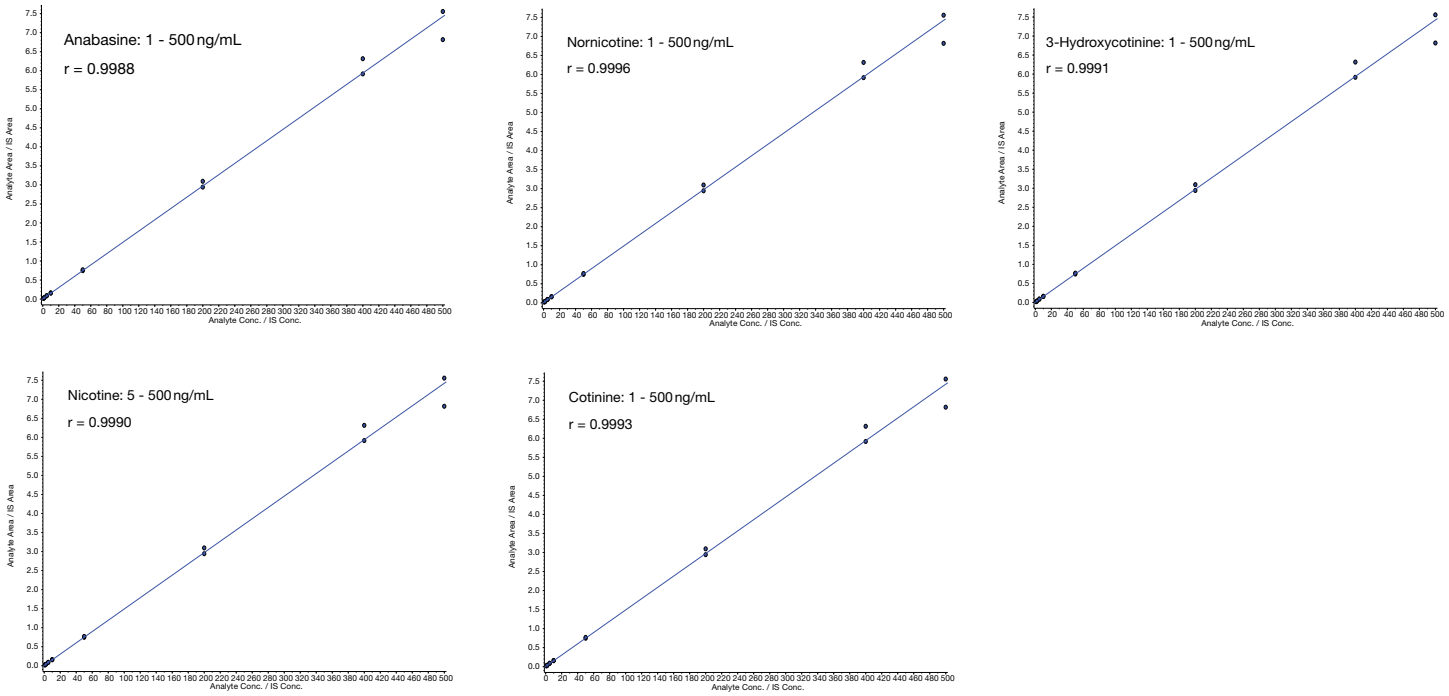
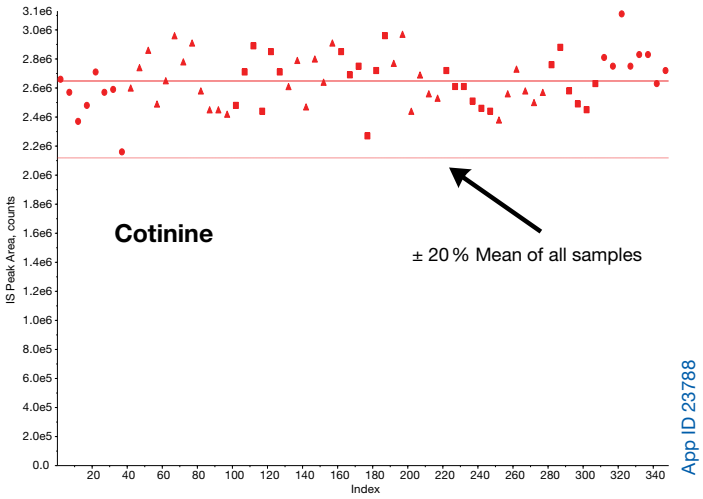


Figure 6. Representative Internal Standard Plots



Results and Discussion

The assay of nicotine and metabolites (anabasine, nornicotine, cotinine and 3-hydroxycotinine) was validated for linearity, accuracy and precision using five levels of calibration standards for all compounds. This method was able to obtain the LLOQ at 1 ng/mL (5 ng/mL for Nicotine) using SCIEX QTRAP® 4500. Also with the modifications to the SLE method with improved recovery of analytes, the need of sample volume was reduced to 100 µL making this method ideal for any laboratory environment.

There were several modifications made to the SLE extraction that produced the higher recoveries over the entire calibration range. The addition of 2 % ammonium hydroxide neutralized the analytes before the sample loading, and the 1 % formic acid added to the elution step were the two major modifications that increased recoveries as seen in **Table 2**. The mass transitions of the analytes were listed in **Table 1**, respectively. The optimal mobile phase required 20 mM ammonium bicarbonate with a pH of 8.2 to ensure separation and peak shape of analysis. A representative total ion chromatogram at medium QC level (200 ng/mL) is shown in **Figure 1**. A representative chromatogram of a blank, Lowest Limit of Quantitation (LLOQ), and Upper Limit of Quantitation (ULOQ) are seen in **Figures 2**, **3**, and **4** respectively.

The results of the accuracy and precision from the LLOQ, Quality Control Low (QCL), Quality Control Medium (QCM), and Quality Control High (QCH) meet the GLP acceptance criteria (**Table 3**). The assay accuracy at above four QC levels over all compounds was between 96.8 and 110 %. The Coefficient of Variation (% CV) for the LLOQ ranged between 5.42 % and 15.7 %; the QCL analytes had a % CV that ranged between 3.03 % and 8.36 %; the QCM analytes had a % CV that ranged between 3.53 % and 6.69 %; the QCH analytes had a % CV that ranged between 2.29 % and 5.27 %, respectively. The linearity of each calibration curve had the following R2 values from 0.9988 to 0.9996 for all analytes. The dynamic curve ranges are at 1-500 ng/mL for all compounds except Nicotine at 5-500 ng/mL due to the endogenous level of nicotine in human body (**Figure 2** and **5**).

The internal standard responses were also evaluated. The assay showed the consistent response of internal standard, which was within ±20 % of mean of standards and QCs in the run for all compounds (**Figure 6**).

Conclusion

The simplified quantitation method of analysis of nicotine, anabasine, nornicotine, cotinine and 3-hydroxycotinine in human urine shows acceptable, accurate, and precise results over the wide calibration range. The modified SLE method was optimized to provide higher recovery across all compounds. The Kinetex® EVO C18 column coupled with high pH mobile phase showed better peak shape, resolution and sensitivity in the analysis of nicotine and its metabolites in human urine. The lower sample volume and optimized simplified liquid extraction method can be easily transfer into various laboratory settings. The assay is also automation friendly which allows for high throughput laboratories to utilize this method.

Ordering Information

Kinetex EVO C18 Core-Shell LC Columns

5 µm Minibore Columns (mm)				SecurityGuard™ ULTRA Cartridges‡
30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
00A-4633-AN	00B-4633-AN	00D-4633-AN	00F-4633-AN	AJ0-9298 for 2.1 mm ID

2.6 µm Minibore Columns (mm)				SecurityGuard™ ULTRA Cartridges‡
30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
00A-4725-AN	00B-4725-AN	00D-4725-AN	00F-4725-AN	AJ0-9298 for 2.1 mm ID

5 µm MidBore™ Columns (mm)			SecurityGuard ULTRA Cartridges‡
50 x 3.0	100 x 3.0	150 x 3.0	3/pk
00B-4633-Y0	00D-4633-Y0	00F-4633-Y0	AJ0-9297 for 3.0 mm ID

2.6 µm MidBore™ Columns (mm)			SecurityGuard ULTRA Cartridges‡
50 x 3.0	100 x 3.0	150 x 3.0	3/pk
00B-4725-Y0	00D-4725-Y0	00F-4725-Y0	AJ0-9297 for 3.0 mm ID

5 µm Analytical Columns (mm)				SecurityGuard ULTRA Cartridges‡
50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	3/pk
00B-4633-E0	00D-4633-E0	00F-4633-E0	00G-4633-E0	AJ0-9296 for 4.6 mm ID

2.6 µm Analytical Columns (mm)			SecurityGuard ULTRA Cartridges‡
50 x 4.6	100 x 4.6	150 x 4.6	3/pk
00B-4725-E0	00D-4725-E0	00F-4725-E0	AJ0-9296 for 4.6 mm ID

1.7 µm Minibore Columns (mm)			SecurityGuard ULTRA Cartridges‡
50 x 2.1	100 x 2.1	150 x 2.1	3/pk
00B-4726-AN	00D-4726-AN	00F-4726-AN	AJ0-9298 for 2.1 mm ID

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

Novum™ Simplified Liquid Extraction (SLE) 96-Well Plates

Part No.	Description	Unit
8E-S138-FGA	Novum SLE MINI 96-Well Plate	1/pk
8E-S138-5GA	Novum SLE MAX 96-Well Plate	1/pk

Novum SLE Tubes

Part No.	Description	Unit
8B-S138-FAK	Novum SLE 1 cc tubes	100/pk
8B-S138-5BJ	Novum SLE 3 cc tubes	50/pk
8B-S138-JCH	Novum SLE 6 cc tubes	30/pk
8B-S138-KDG	Novum SLE 12 cc tubes	20/pk

Presston™ 100 Positive Pressure Manifold

Part No.	Description
AH0-9334	Presston 100 Positive Pressure Manifold, 96-Well Plate
AH0-9342	Presston 100 Positive Pressure Manifold, 1 mL Tube Complete Assembly
AH0-9347	Presston 100 Positive Pressure Manifold, 3 mL Tube Complete Assembly
AH0-9343	Presston 100 Positive Pressure Manifold, 6 mL Tube Complete Assembly

The Presston 100 96-Well Positive Pressure Manifold can also process 1, 3, and 6 mL tubes using the following adapter kits

Presston 100 Tube Adapter Kits (for AH0-9334)

Part No.	Description
AH0-9344	1 mL Tube Adapter Kit
AH0-9345	3 mL Tube Adapter Kit
AH0-9346	6 mL Tube Adapter Kit

WARRANTY Phenomenex warrants that for a period of 12 months following delivery, the Presston 100 Positive Pressure Manifold you have purchased will perform in accordance with the published specifications and will be free from defects in materials or workmanship. In the event that the Presston 100 Positive Pressure Manifold does not meet this warranty, Phenomenex will repair or replace defective parts. Please visit www.phenomenex.com/Presston for complete warranty information.

Ordering Information
Vacuum Manifolds

Part No.	Description	Unit
12–Position Vacuum Manifold for Tubes*		
AH0-6023	12-Position Vacuum Manifold Set, complete assembly	ea
24–Position Vacuum Manifold for Tubes*		
AH0-6024	24-Position Vacuum Manifold Set, complete assembly	ea
96-Well Plate Manifold		
AH0-8950	96-Well Plate Manifold, Universal with vacuum gauge	ea

* Manifolds include: Vacuum-tight glass chamber, vacuum gauge assembly, polypropylene lid withzgasket, male and female luers and yellow end plugs, stopcock valves, collection rack assemblies, polypropylene needles, lid support legs. Waste container included with 12-position manifold.

96-Well Plate Accessories

Part No.	Description	Unit
Collection Plates (deep well, polypropylene)		
AH0-7192	96-Well Collection Plate, 350 µL/well	50/pk
AH0-7193	96-Well Collection Plate, 1 mL/well	50/pk
AH0-7194	96-Well Collection Plate, 2 mL/well	50/pk
AH0-8635	96-Well Collection Plate, 2 mL/well Square/Round-Conical	50/pk
AH0-8636	96-Well Collection Plate, 2 mL/well Round/Round, 8 mm	50/pk
AH0-7279	96-Well Collection Plate, 1 mL/well Round, 7 mm	50/pk
Sealing Mats		
AH0-8597	Sealing Mats, Pierceable, 96-Square Well, Silicone	50/pk
AH0-8598	Sealing Mats, Pre-Slit, 96-Square Well, Silicone	50/pk
AH0-8631	Sealing Mats, Pierceable, 96-Round Well 7 mm, Silicone	50/pk
AH0-8632	Sealing Mats, Pre-Slit, 96-Round Well 7 mm, Silicone	50/pk
AH0-8633	Sealing Mats, Pierceable, 96-Round Well 8 mm, Silicone	50/pk
AH0-8634	Sealing Mats, Pre-Slit, 96-Round Well 8 mm, Silicone	50/pk
AH0-7362	Sealing Tape Pad	10/pk
Vacuum Manifold		
AH0-8950	96-Well Plate Manifold, Universal with Vacuum Gauge	ea

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APPLICATIONS

Australia
t: +61 (0)2-9428-6444
f: +61 (0)2-9428-6445
auinfo@phenomenex.com

Austria
t: +43 (0)1-319-1301
f: +43 (0)1-319-1300
anfrage@phenomenex.com

Belgium
t: +32 (0)2 503 4015 (French)
t: +32 (0)2 511 8666 (Dutch)
f: +31 (0)30-2383749
beinfo@phenomenex.com

Canada
t: +1 (800) 543-3681
f: +1 (310) 328-7768
info@phenomenex.com

China
t: +86 (0)20 2282-6668
f: +86 (0)20 2809-8130
chinainfo@phenomenex.com

Denmark
t: +45 4824 8048
f: +45 4810 6265
nordicinfo@phenomenex.com

Finland
t: +358 (0)9 4789 0063
f: +45 4810 6265
nordicinfo@phenomenex.com

France
t: +33 (0)1 30 09 21 10
f: +33 (0)1 30 09 21 11
franceinfo@phenomenex.com

Germany
t: +49 (0)6021-58830-0
f: +49 (0)6021-58830-11
anfrage@phenomenex.com

India
t: +91 (0)40-3012 2400
f: +91 (0)40-3012 2411
indiainfo@phenomenex.com

Ireland
t: +353 (0)1 247 5405
f: +44 1625-501796
eireinfo@phenomenex.com

Italy
t: +39 051 6327511
f: +39 051 6327555
italiainfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

Mexico
t: 01-800-844-5226
f: 001-310-328-7768
tecnicomx@phenomenex.com

The Netherlands
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

New Zealand
t: +64 (0)9-4780951
f: +64 (0)9-4780952
nzinfo@phenomenex.com

Norway
t: +47 810 02 005
f: +45 4810 6265
nordicinfo@phenomenex.com

Puerto Rico
t: +1 (800) 541-HPLC
f: +1 (310) 328-7768
info@phenomenex.com

Spain
t: +34 91-413-8613
f: +34 91-413-2290
espinfo@phenomenex.com

Sweden
t: +46 (0)8 611 6950
f: +45 4810 6265
nordicinfo@phenomenex.com

United Kingdom
t: +44 (0)1625-501367
f: +44 (0)1625-501796
ukinfo@phenomenex.com

USA
t: +1 (310) 212-0555
f: +1 (310) 328-7768
info@phenomenex.com

All other countries
Corporate Office USA
t: +1 (310) 212-0555
f: +1 (310) 328-7768
info@phenomenex.com

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