SPE-LC-MS/MS Method for the Determination of Nicotine, Cotinine, and Trans-3-hydroxycotinine in Urine

J. Jones, Thermo Fisher Scientific, Runcorn, Cheshire, UK

Application Note 20709

Key Words
SPE, SOLA CX, Syncronis C18, nicotine, cotinine, trans-3-hydroxycotinine

Abstract
A liquid chromatography tandem mass spectrometry method for nicotine, cotinine, and trans-3-hydroxycotinine in urine has been developed. Using Thermo Scientific™ SOLA™ CX cartridges or plates, sample preparation is fast, giving excellent reproducibility and recovery levels for each compound. The analysis was carried out on a Thermo Scientific™ Syncronis™ C18 1.7 µm, 50 x 2.1 mm column for a fast separation with a cycle time of 3 minutes while maintaining excellent peak shape.

Introduction

Nicotine is a naturally occurring chemical compound that is found in the leaves of the Solanaceae. The contribution of nicotine in tobacco can be up to 3% of the total dry weight. Both cotinine and trans-3-hydroxycotinine are the major metabolites of nicotine. The extraction of nicotine, cotinine, and trans-3-hydroxycotinine from urine is demonstrated in this application.

SOLA SPE products introduce next-generation, innovative technological advancements, which give unparalleled performance characteristics compared to conventional SPE, phospholipid, and protein precipitation products.

These include:

- Higher levels of reproducibility
- Higher levels of extract cleanliness
- Reduced sample and solvent requirements
- Increased sensitivity

SOLA SPE plates or cartridges have significant advantages when analyzing compounds in complex matrices, particularly in high-throughput bioanalytical and clinical research laboratories where reduced failure rates, higher analysis speed, and lower solvent requirements are critical. SOLA products' superior performance gives higher confidence in analytical results and lowers cost without compromising ease-of-use or requiring complex method development.

One of the key goals for the chromatographer is to achieve a consistent, reproducible separation. The selection of a highly reproducible HPLC column is essential if this goal is to be attained. The Syncronis column range has been engineered to provide exceptional reproducibility due to its highly pure, high surface area silica, dense bonding, and double endcapping, which are all controlled and characterized through the use of rigorous testing.

Thermo SCIENTIFIC

Experimental Details

Consumables	Part Number
Fisher Scientific™ LC/MS grade water	W/011217
Fisher Scientific LC/MS grade methanol	M/4062/17
Fisher Scientific LC/MS grade acetonitrile	A/0638/17
Fisher Scientific Analytical grade formic acid	F/1900/PB08
Fisher Scientific Analytical grade ammonia	A/3295/PB05
Thermo Scientific™ National™ Mass Spec Target DP Certified 2 mL clear vial with ID patch, blue DP cap with bonded PTFE/silicone septum	MSCERT4000-34W

Sample Handling Equipment	Part Number
Thermo Scientific 96 well plate vacuum manifold	60103-351
Thermo Scientific™ UltraVap™ high speed sample concentrator	CLS-229070

Sample Pretreatment

A standard spiking solution of nicotine, cotinine, and trans-3-hydroxycotinine was prepared in water.
A working internal standard solution of cotinine-*d3* was prepared in methanol
Add 180 µL of blank urine to 1000 µL of 5 mM ammonium formate pH 2.5.
For standards and quality control (QC) samples, 10 µL of standard spiking solution was added; for blanks, 10 µL of water was added.
For standards and QCs 10 µL of working internal standard solution was added, and for blanks 10 µL of methanol was added.
All samples were vortexed for 30 seconds and then centrifuged for 5 minutes at 5000 rpm.

Sample Preparation		Part Number
Compounds:	Nicotine, cotinine, and trans-3-hydroxycotinine	
Internal standards:	Cotinine-*d3*	
Matrix:	Urine	
Plate type:	SOLA CX 10 mg/2 mL	60309-002
Conditioning stage:	Apply 500 µL of methanol then 500 µL of 5 mM ammonium formate pH 2.5 to the SPE cartridge at approximately 1 mL/min.	
Application stage:	Apply 1200 µL of sample at approximately 1 mL/min.	
Washing stage 1:	Apply 1000 µL of 5 mM ammonium formate pH 2.5 at approximately 1 mL/min.	
Washing stage 2:	Apply 1000 µL of 1% formic acid in methanol at approximately 1 mL/min	
Elution stage:	Dry down under nitrogen without heat and reconstitute in 200 µL water / methanol (70:30 v/v), then mix well.	

Separation Conditions				Part Number
Instrumentation:	Thermo Scientific™ Accela™ 600 LC pump			
Column:	Syncronis C18 1.7 µm, 50 x 2.1 mm			97102-052130
Guard column:	Syncronis C18 Holder			97105-012101 852-00
Mobile phase A:	Water + 0.1% ammonia			
Mobile phase B:	Methanol + 0.1% ammonia			
Gradient:	Time (min)	A	B	
	0	70	30	
	3.0	0	100	
	3.5	0	100	
	3.6	70	30	
	6.0	70	30	
Flow rate:	0.2 mL/min			
Column temperature:	30 °C			
Injection details:	10 µL			
Injection wash solvent 1:	Water			
Injection wash solvent 2:	Propan-2-ol / acetonitrile / acetone (45:45:10 v/v/v)			

Note: The pH of the mobile phase is outside of the recommended range for this column. We therefore strongly recommend the use of a guard column with frequent changing to maximize the lifetime of the analytical column.

MS Conditions	
Instrumentation:	Thermo Scientific™ TSQ Vantage™ MS
Ionization conditions:	HESI
Polarity:	Positive
Spray voltage (V):	3500
Vaporizer temperature (°C):	400
Sheath gas pressure (Arb):	50
Aux gas pressure (Arb):	30
Capillary temp (°C):	380
Collision pressure (mTorr):	1.5
Scan time (s):	0.02
Q1 (FWHM):	0.7
Q3 (FWHM):	0.7
Transition details:	Table 1

Compound	Nicotine	Cotinine	Trans-3-hydroxycotinine	Cotinine-*d3*
Parent (*m/z*)	163.1	177.1	193.1	180.1
Products (*m/z*)	130.1	80.1	80.1	80.1
Collision energy	19	22	28	22
S-lens	51	67	76	67

Table 1: Compound transition details

Data Processing	
Software:	Thermo Scientific™ LCQUAN™ version 2.6

Results

Analysis was carried out on a Syncronis C18 UHPLC column. The high surface area and high carbon load of the Syncronis column allowed for the elution and separation of nicotine, cotinine, and trans-3-hydroxy-cotinine to occur in less than 4 minutes.

Extracted standards of nicotine and cotinine from urine gave a linear calibration curve over the dynamic range 1–1000 ng/mL (Figures 1 and 2). Extracted standards of trans-3-hydroxycotinine from urine gave a linear calibration curve over the dynamic range 10–1000 ng/mL (Figure 3). Chromatograms for each compound at the lower limit of quantitation (LLOQ) are shown in Figures 4 to 6. QC samples were run in replicates of six at concentrations of 15, 250, and 600 ng/mL. Accuracy was assessed at each standard and QC level, and all data points were within ±15% or ±20% at the LLOQ. Table 2 shows the mean relative error (%) for QCL, QCM, and QCH (n=6). The precision at each of the QC levels for each compound is less than 9.0% RSD (Table 3).

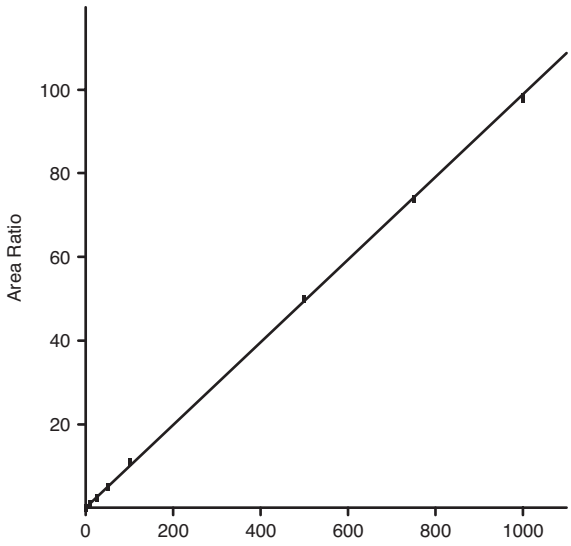

Figure 1: Nicotine linearity over the dynamic range 1–1000 ng/mL

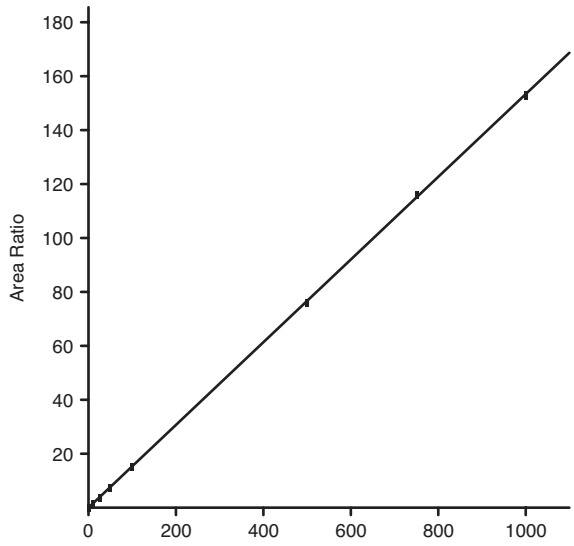

Figure 2: Cotinine linearity over the dynamic range 1–1000 ng/mL

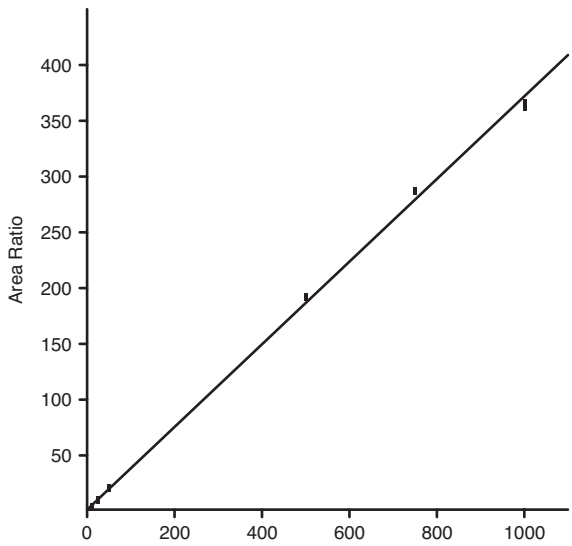

Figure 3: Trans-3-hydroxycotinine linearity over the dynamic range 10–1000 ng/mL

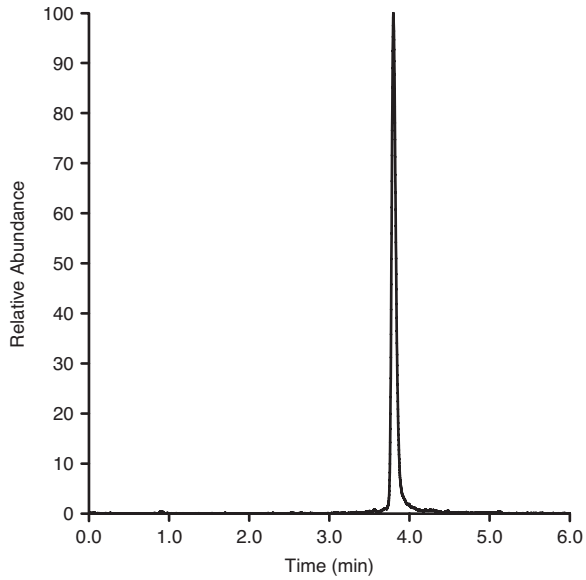

Figure 4: Representative chromatogram of nicotine SRM, extracted from urine at 1 ng/mL

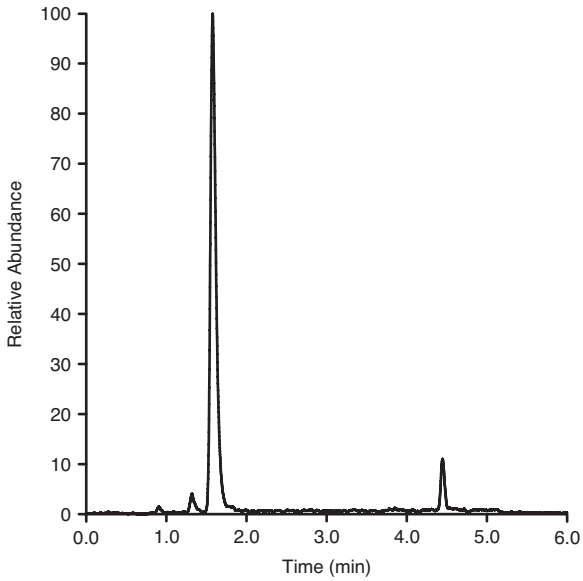

Figure 5: Representative chromatogram of cotinine SRM, extracted from urine at 1 ng/mL

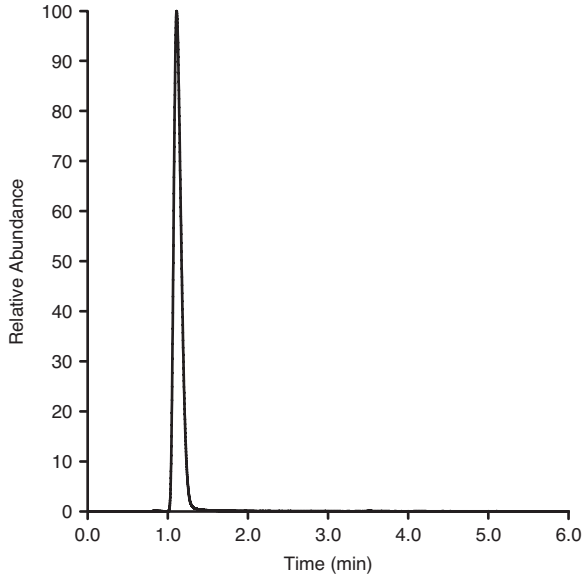

Figure 6: Representative chromatogram of trans-3-hydroxycotinine SRM, extracted from urine at 10 ng/mL

Overspikes were analyzed in triplicate at concentrations of 15, 250, and 600 ng/mL and used to calculate recovery. The overall percentage recovery level was calculated using the average recovery at the three QC levels and was greater than 79.3% for all compounds (Table 4).

Accuracy

Compound	Linearity Range (ng/mL)	Coefficient of Determination	Mean Relative Error (%) at QCL (n=6)	Mean Relative Error (%) at QCM (n=6)	Mean Relative Error (%) at QCH (n=6)
Nicotine	1–1000	0.9996	14.6	12.3	1.7
Cotinine	1–1000	1.0000	-10.0	-8.7	-10.4
Trans-3-hydroxycotinine	10–1000	0.9991	6.4	13.3	2.2

Table 2: Accuracy data for nicotine, cotinine, and trans-3-hydroxycotinine

Precision

Compound	QCL at 15 ng/mL (n=6)	QCM at 250 ng/mL (n=6)	QCH at 600 ng/mL (n=6)
Nicotine	7.8	5.0	6.6
Cotinine	9.0	4.4	0.7
Trans-3-hydroxycotinine	6.4	4.0	2.1

Table 3: Precision data for nicotine, cotinine, and trans-3-hydroxycotinine

Recovery

Compound	% Recovery at QCL	% Recovery at QCM	% Recovery at QCH	Average % Recovery
Nicotine	81.2	77.3	79.5	79.3
Cotinine	92.6	84.4	88.5	88.5
Trans-3-hydroxycotinine	87.0	81.0	88.0	85.3

Table 4: Recovery data for nicotine, cotinine, and trans-3-hydroxycotinine

Conclusion

- SOLA CX SPE plates and Syncronis C18 UHPLC columns allow for a simple extraction and rapid quantification of nicotine, cotinine, and trans-3-hydroxycotinine from urine using an internal standard.
- LLOQs ranged from 1 to 10 ng/mL for all compounds.
- Extraction recoveries for each compound were greater than 79.3%.
- The method showed excellent accuracy and precision with %RSD less than 9.0% for each compound.

USA and Canada +1 800 332 3331
France +33 (0)1 60 92 48 34
Germany +49 (0) 2423 9431 20 or 21
United Kingdom +44 (0)1928 534110
Japan +81 3 5826 1615

China +86 21 68654588 +86 10 84193588
+86 20 83145199 800 810 5118
India +91 22 6742 9494 +91 27 1766 2352
Australia 1 300 735 292 (free call domestic)
New Zealand 0800 933 966 (free call domestic)
All Other Enquiries +44 (0) 1928 534 050

Technical Support
North America +1 800 332 3331
Outside North America +44 (0) 1928 534 440

Thermo SCIENTIFIC
Part of Thermo Fisher Scientific

AN20709_E 02/13S