Extraction of Nicotine and Metabolites from Urine, Serum, Plasma and Whole Blood Using ISOLUTE® SLE+ Prior to LC-MS/MS Analysis

This application note describes a Supported Liquid Extraction (SLE) protocol for the extraction of nicotine, cotinine, 3-OH-cotinine, nornicotine, norcotinine and anabasine from urine, serum, plasma and whole blood using ISOLUTE® SLE+ plates with LC-MS/MS detection.

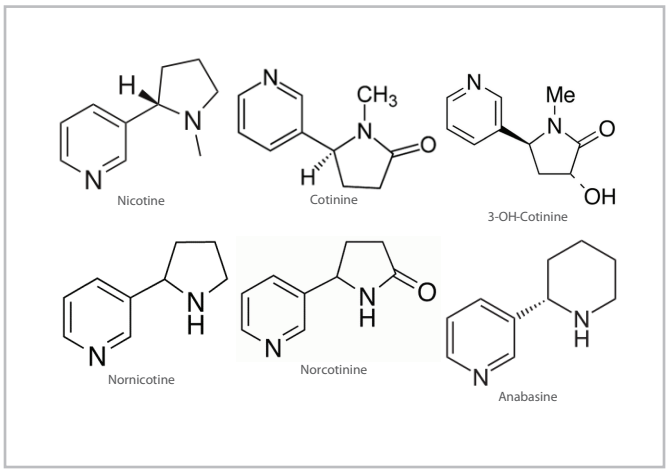

Figure 1. Structures of nicotine and metabolites

Introduction

The method described in this application note achieves high recoveries of nicotine and a number of common metabolites in a range of biological matrices. The method is sensitive enough to be able to discriminate between non-smokers and those regularly exposed to a passive smoking environment but also rugged enough (with appropriate dilution) to measure levels in heavy smokers.

A single treatment applies to all matrices tested including those that are blood based (plasma, serum and whole blood) and urine.

ISOLUTE® SLE+ products provide clean, rapid, robust, efficient , high throughput and automatable extraction solutions for a wide range of analytes.

Analytes

Nicotine, Cotinine, 3-OH-Cotinine, Nornicotine, Norcotinine, Anabasine

Sample Preparation Procedure

Column configuration ISOLUTE SLE+ 200 supported liquid extraction plate, part number 820-0200-P01

Sample pre-treatment:

1. Dilution: 120 µL matrix was combined with 10 µL of 2 µg/mL internal standard solution and 230 µL 0.25% ammonia solution.
2. This creates a volume of 360 µL from which a sample volume of 150 µL (corresponding to 50 µL of biological fluid) was taken.

Supported Liquid Extraction

Sample Loading: Load pre-treated sample (150 µL) onto each well. Apply a pulse of vacuum (VacMaster-96 Sample Processing Manifold, 121-9600) or positive pressure (Pressure+ 96 Positive Pressure Manifold, PPM-96) to initiate flow. Allow the sample to absorb for 5 minutes.

Analyte Elution: Elute with dichloromethane : isopropanol (95 : 5, v/v, 1 mL) and allow to flow under gravity into a 2 mL deep well collection plate (121-5203) containing 100 µL methanolic 200 mM HCl in each well. Apply vacuum or positive pressure to elute any remaining extraction solvent.

Post Elution: Dry the eluate in a stream of air or nitrogen using a SPE Dry (40 °C, 20 to 40 L min^{-1}) or TurboVap 96 (15 bar at 40 °C for 1 hr). Reconstitute in methanol:water (10:90, v/v, 200 µL)

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HPLC Conditions*

Instrument: Waters Acquity UPLC (Waters)

Column Waters 1.7µm 100 x 2mm BEH C18 column with an appropriate on-line filter

Mobile Phase: A: 0.1% ammonia solution
B: 0.1% ammonia solution in methanol

Flow rate: 0.3 mL min^{-1}

Injection: 5 µL

Gradient: Initial 10 % B
linear ramp to 65 % B in 3.5 min
linear ramp to 100 % B in 0.01 min, hold 1.49 min
linear ramp to 10% B in 0.01 min, hold 1.49 min
total run time 6.5 min

Column temperature 40 °C

Sample temperature: 20 °C

Table 1: Typical retention times for nicotine and metabolites using the LC-MS/MS method described

Compound	Retention Time (min)
3 OH Cotinine	1.94
Norcotinine	2.17
Cotinine	2.42
Nornicotine	3.23
Anabasine	3.77
D4 Nicotine	3.88
Nicotine	3.91

MS Conditions

Ions were selected in order to achieve maximum sensitivity using multiple reaction monitoring.

Instrument: Waters Quattro Premier XE

Ionization mode: ES+

Desolvation temperature: 450 °C

Source temperature: 120 °C

Table 2. Positive Ion Mode - MRM Parameters

MRM transition	RT	Compound ID	Cone, V	CE, V	MS Period
193.1 > 79.9	1.94	3-OH cotinine	34	25	
163.1 > 79.9	2.17	Norcotinine	36	20	1
177.1 > 80.0	2.42	Cotinine	37	22	
149.1 > 79.9	3.23	Nornicotine	26	11	2
163 > 91.1	3.77	Anabasine	34	18	
167 > 133.9	3.88	D4 nicotine	28	16	3
163 > 132	3.91	Nicotine	26	18	
Dwell = 0.1 sec (all analytes), Inter channel delay 0.005 sec					

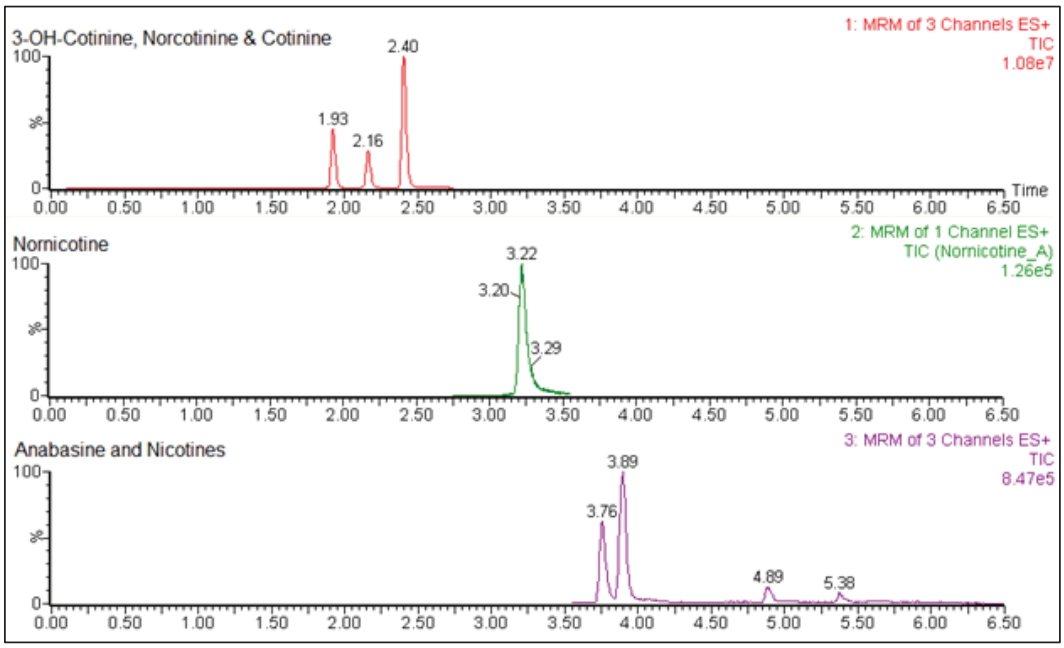

Figure 1. Total ion chromatograms in positive ion mode using ISOLUTE® SLE+ procedure (sample: 50 µL whole blood, analytes: 125 ng/mL).

Results

Figure 2 below shows typical linearity data achieved using this method. Tables 3-6 show analyte recovery and %RSD for nicotine and metabolites in urine, serum, plasma and whole blood.

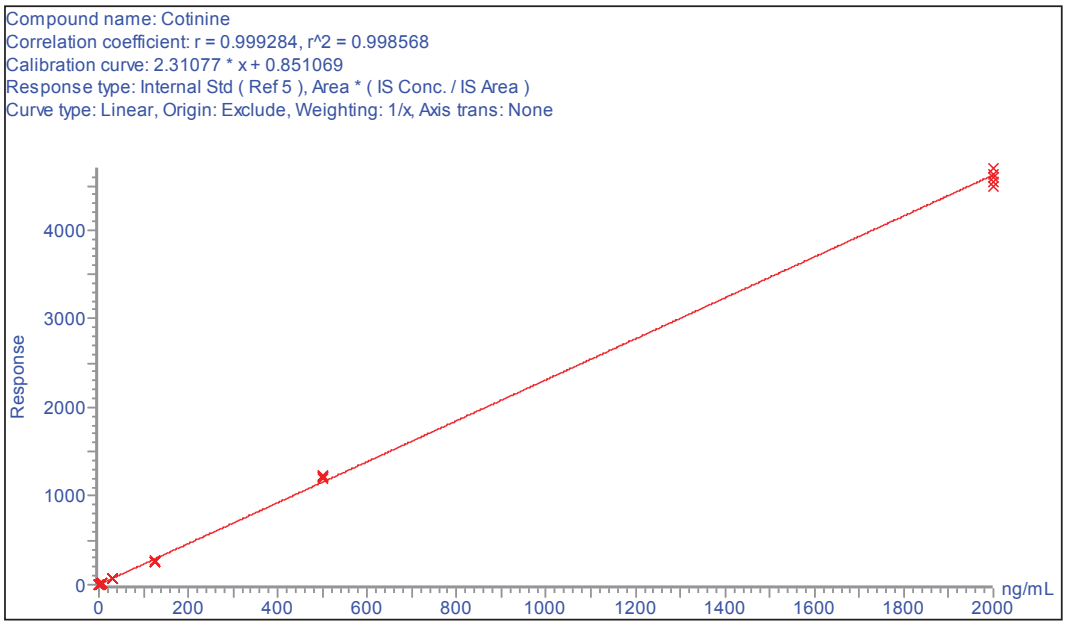

Figure 2. Typical calibration curve for cotinine, expressed on a linear scale

Table 3. Analyte performance and recovery data for nicotine and metabolites from urine

Analyte in Urine	r	% RSD	Recovery %
3 OH Cotinine	0.999	3.2	94.7
Norcotinine	0.999	2.8	87.9
Cotinine	0.999	3.4	100.5
Nornicotine	0.994	7.6	88.6
Anabasine	0.996	2.2	102.6
Nicotine	1.000	6.5	97.1

Recovery and RSD calculations based on extractions of blank matrix spiked at 10 ng/mL, r calculations based on a 0.25 to 2000 ng/mL calibration range with the 500 and 2000 ng/mL standards diluted 1 in 10 to avoid saturation in the instrument.

Table 4. Analyte performance and recovery data for nicotine and metabolites from serum

Analyte in Urine	r	% RSD	Recovery %
3 OH Cotinine	0.998	2.0	93.0
Norcotinine	0.997	1.6	98.4
Cotinine	0.999	1.9	98.2
Nornicotine	0.997	2.9	83.5
Anabasine	0.978	1.5	97.9
Nicotine	1.000	3.0	95.4

Recovery and RSD calculations based on extractions of blank matrix spiked at 10 ng/mL, r calculations based on a 0.25 to 2000ng/mL calibration range with the 500 and 2000 ng/mL standards diluted 1 in 10 to avoid saturation in the instrument.

Table 5. Analyte performance and recovery data for nicotine and metabolites from plasma

Analyte in Urine	r	% RSD	Recovery %
3 OH Cotinine	0.993	1.2	92.9
Norcotinine	0.994	1.9	98.9
Cotinine	0.997	4.4	98.9
Nornicotine	0.988	3.8	79.8
Anabasine	0.988	2.3	99.0
Nicotine	0.999	4.3	95.7

Recovery and RSD calculations based on extractions of blank matrix spiked at 10 ng/mL, r calculations based on a 0.25 to 2000ng/mL calibration range with the 500 and 2000 ng/mL standards diluted 1 in 10 to avoid saturation in the instrument.

Table 6. Analyte performance and recovery data for nicotine and metabolites from whole blood

Analyte in Urine	r	% RSD	Recovery %
3 OH Cotinine	0.955	2.5	77.7
Norcotinine	0.980	8.9	83.2
Cotinine	0.907	1.7	96.9
Nornicotine	0.977	5.6	97.9
Anabasine	0.981	5.4	107.0
Nicotine	0.996	3.2	97.9

Recovery and RSD calculations based on extractions of blank matrix spiked at 10 ng/mL, r calculations based on a 0.25 to 2000ng/mL calibration range with the 500 and 2000 ng/mL standards diluted 1 in 10 to avoid saturation in the instrument.

Relatively poor r values for whole blood are due to the fact that blank matrix was not available for r determinations at the time, substantial responses for metabolites, particularly cotinine and 3 OH cotinine from the available control matrix meant that small spikes could not be as accurately measured as they could in a blank matrix resulting in an artificially poor correlation coefficient calculation.

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Additional Notes

» Analytes were purchased or prepared at a concentration of 1 mg/mL in methanol except the internal standard (D4-nicotine) which was only available at 100 µg/mL.The 1 mg mL analytes were combined and diluted to a 50 µg/mL solution in methanol which was stored at approximately -20 °C when not in use.

» The D4-nicotine was diluted to a concentration of 2 µg/mL in 50/50 methanol water on a daily basis (1.2 mL required per 96 wells).

» Calibration lines in the procedure detailed here were constructed by preparing 0.6 mL of a calibration standard at 2000 ng/mL (576 µL of blank matrix and 24 µL of 50 µg mL sub stock). After mixing, 150 µL was transferred to another well of a 96 well block and diluted by the addition of 450 µL blank matrix. This 1 in 4 dilution procedure was performed a total of 6 times until a concentration of 0.488 ng/mL had been reached.

» The method detailed in this application note has an ultra-wide dynamic range because of the large differences in concentrations (particularly with cotinine) between heavy smokers and non-smokers. For best results it is recommended that operators set their own quantification limits depending on whether they wish to measure the differences between non and passive smokers or whether they need to quantify levels from heavier smokers. For samples expected to contain higher concentrations (e.g. greater than 500 ng/mL) it is recommended that these samples are diluted further on reconstitution to avoid instrument saturation, and/or that a non-linear calibration line is fitted.

» For calibration line construction an analyte free substitute matrix could not be found that applied to all matrices and so batches acquired from non-smokers were used instead. In the case of blood based matrices these may be able to be purchased as nicotine free or a screen performed on available stocks to make sure that they're from a non-smoker origin.

» Although the same experimental procedure was used for all matrices different types of samples should not be combined in the same run unless each has its own calibration line.

» The urine assay was performed on urine adjusted to pH4 and pH8 both sets giving acceptable recovery data (the normal pH range of urine is pH 4 to 8).

» There was no indication of a drop of column performance across the work however as a precautionary measure the column and filter were back-flushed with the aqueous and organic mobile phases separately for approximately 30 minutes each every 250 samples.

» The internal standard concentration was aimed significantly lower than would normally be expected (8.3% instead of 50% of the top concentration) as relatively little was available at the time of method development. Due to the large signal to noise ratios across the range quantification was still acceptable. Good precision was also returned without using an internal standard however the plots of most components required quadratic plotting for the closest fitting lines.

» It is important that the collection plate is completely dry prior to reconstitution, presence of any remaining hydrochloric acid could significantly affect chromatography and signal responses.

» Although not precisely measured there was evidence of some medium term instability of nicotine and nornicotine. It is recommended that the stock solutions and the 50 µg/mL nicotine suite sub-stock solution were stored at approximately-20°C in methanol. All other analyte or internal standard solutions were prepared daily.

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Ordering Information

Part Number	Description	Quantity
820-0200-P01	ISOLUTE SLE+ 200 Supported Liquid Extraction Plate	1
121-9600	VacMaster-96 Sample Processing Manifold	1
PPM-96	Biotage PRESSURE+ 96 Positive Pressure Manifold 96 Well.	1
121-5203	Collection plate, 2 mL	50
SD-9600-DHS	SPE Dry Sample Concentrator	1
C103264	TurboVap 96	1

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