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A Rapid and Robust Sample Preparation Method for Quantitation of Nicotine from Oral Fluid

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Overview

- Single cartridge solid phase extraction of nicotine, anabasine, and cotinine from oral fluid
- Resolution of nicotine and anabasine isomers
- Linear regression values (R) of greater than 0.997

Introduction

Nicotine is the active ingredient in tobacco and vape products that is highly addictive. Nicotine, its metabolite cotinine, and anabasine, a tobacco alkaloid, are often used to detect tobacco exposure. For tests like this, oral fluid collection has emerged as an alternative to other biological matrices. The reason for its popularity is due to its low chance of adulteration. In addition, oral fluid collection is easy and non-invasive. As a test matrix, oral fluid shows great promise for detection of recent drug use, its disposition, and detection times.[1] However, it is not free of challenges. The additives and preservative buffer (critical for preservation of the chemical integrity of the oral fluid sample) present in the collection devices, must be removed for the proper upkeep of the mass spec detector.

In this technical note, we present a solid phase extraction (SPE) method for simultaneous detection and quantitation of nicotine, cotinine, and anabasine from oral fluid. We employ Intercept i2®, a commercially available oral fluid collection device for sample collection and transport. A polymeric, strong cation-exchange sorbent, Strata®-X-C was utilized for solid phase extraction (SPE). pH stable Kinetex® 2.6 µm EVO C18 column was used for the analysis purposes to obtain the best selectivity between the two isomeric compounds nicotine and anabasine under an ESI mode in LC/MS/MS analysis.

Materials and Methods

Analytical reference standards were purchased from Cerilliant® Corporation (Round Rock, TX). Negative calibrator oral fluid and Intercept i2 collection device were obtained from Orasure Technologies (Bethlehem, PA). All other chemicals, were obtained from the Sigma-Aldrich Company (St. Louis, MO). Ultrapure DI water was obtained from Sartorius® arium® comfort II, courtesy of Sartorius Corporation (Bohemia, NY)

Experimental Conditions

Calibrators for the 7-point linearity curve were prepared by serial dilution of the negative oral fluid control. The curve spans a total of seven concentration. level, covering a wide range. The QC samples for extraction were prepared at three concentration (low, medium and high) level.

Sample Collection and Pretreatment

Oral fluid specimens, calibrators, and QC samples were collected on the cellulose pad (on a plastic stick from Intercept i2 devices) until the indicator window turns blue. The saturated pad on the stick was then placed into the transport tube containing the buffer solution and left overnight to represent transit time. The plastic nipple from the transport tube was removed and cellulose tab was placed in a centrifuge tube, which was centrifuged at 600 g for 15 mins. The supernatant was collected for sample preparation.

SPE Method

Step	Basic analyte extraction
96-Well Plate:	Strata-X-C, 30 mg
Part No.:	8E-S029-TGB
Condition:	1 mL Methanol
Equilibrate:	1 mL DI Water
Load:	Combine 0.5 mL of pretreated sample with 1 mL 1% Formic acid, add 40 µL working internal standard solution (0.5 µg/mL). Mix/vortex 5-10 sec and load on Strata-X-C.
Weak Wash:	1 mL DI Water
Strong Wash:	1 mL Acetone/Water (50:50)
Dry:	3-4 minutes at maximum vacuum (15" Hg or higher)
Elute:	2 x 500 µL Ethyl acetate / Isopropanol / Ammonium hydroxide (7:2:1)
Dry down:	Evaporate to dryness under gentle stream N_2 at 45-50 °C
Reconstitute:	With 200 µL initial mobile phase

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LC/MS/MS Conditions

Column: Kinetex® 2.6 µm EVO 2.6 µm C18
Dimensions: 100 x 3.0 mm
Part No.: 00D-4725-Y0
Recommended Guard: SecurityGuard™ ULTRA EVO C18 Cartridges: AJ0-9297
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
Injection Volume: 5 µL
Detection: SCIEX Triple Quad™ 4500
Detection Mode: ESI+
Sample: 1. Cotinine
2. Cotinine-D3
3. Anabasine
4. Anabasine-D4
5. Nicotine
6. Nicotine-D4

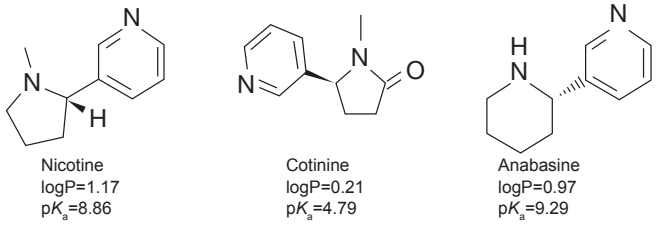

Figure 1.
Structure, pK_a and logP Value of Nicotine, Cotinine, and Anabasine

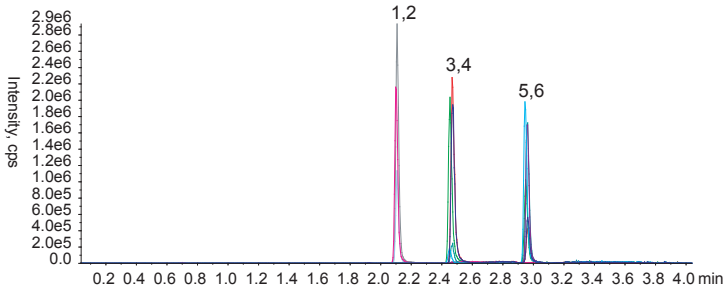

Figure 2.
Representative chromatogram of cotinine, anabasine, and nicotine from oral fluid extracted samples.

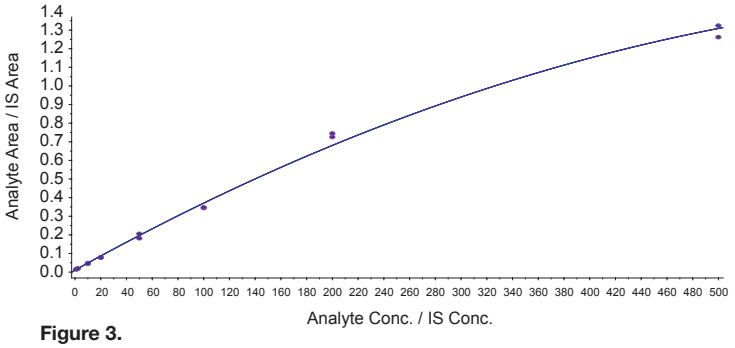

Figure 3.
Linearity curve for nicotine (1 to 500 ng/mL) from oral fluid extracted samples. R=0.9978.

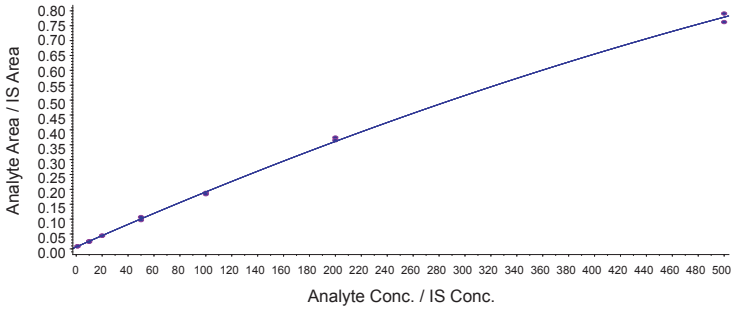

Figure 4.
Linearity curve for cotinine (1 to 500 ng/mL) from oral fluid extracted samples. R=0.9996.

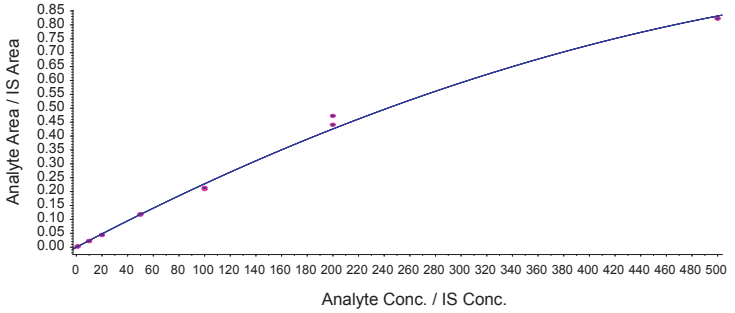

Figure 5.
Linearity curve for anabasine (1 to 500 ng/mL) from oral fluid extracted samples. R=0.9983.

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Table 1.
Precision and accuracy data for 3 different levels of QC.

a) Nicotine

Conc. (ng/mL)	Sample Name	Replicate	% CV	Accuracy
4	QC-Low	4	8.4	97.6
40	QC-Med	4	3.9	92.9
150	QC-High	4	5.1	94.0

b) Cotinine

Conc. (ng/mL)	Sample Name	Replicate	% CV	Accuracy
4	QC-Low	4	10.8	84.1
40	QC-Med	4	4.1	97.7
150	QC-High	4	4.6	95.2

c) Anabasine

Conc. (ng/mL)	Sample Name	Replicate	% CV	Accuracy
4	QC-Low	4	3.2	95.3
40	QC-Med	4	3.7	94.1
150	QC-High	4	2.3	98.0

Results and Discussion

Kinetex® EVO C18 column was chosen because it is robust and provided the best selectivity. The isomeric species nicotine and anabasine were well resolved, as shown in **Figure 2**.

For SPE, a strong cation-exchanger sorbent, Strata®-X-C, was used to allow the use a 50% acetone wash. This strong organic wash effectively removed the excipients from the sample and transport buffer.[2] An elution solvent composing of ethyl acetate, isopropanol, and ammonium hydroxide eluted the analytes selectively, leaving any residual impurities behind.

Calibration curves were constructed from spiked saliva samples ranging from 1 to 500 ng/mL, using seven points for all three analytes. The linearity curves with a quadratic fit and 1/x weighting factor showed the correlation coefficient value (R) for all analytes more than 0.997 (**Figures 3-5**) over a wide dynamic range.

Three levels of QC samples (low, medium and high) obtained a precision and accuracy ranging from 2-10% and 84-98% respectively, for 4 replicate extraction at each concentration level (**Table 1**).

Conclusion

In this technical note we demonstrated an effective sample preparation technique for quantitation of nicotine, cotinine, and anabasine from oral fluid. This is a reliable and reproducible assay with good separation of isomeric analytes and demonstrates linear regression values (R) more than 0.997 for all analytes that reflects the robustness of the assay over a wide dynamic range.

References

1. N. Robson, A. J. Bond and K. Wolff; “Salivary nicotine and cotinine concentrations in unstimulated and stimulated saliva”; African Journal of Pharmacy and Pharmacology, Vol. 4(2), 061-065, 2010.

2. S. Sadjadi, S. Huq, L. Snow; “An Investigation into Removing the Excipients from Selected Oral Fluids Collection Devices by SPE and LC/MS Detection”; Mass Spec Application for Clinical Laboratory Conference, 2016.

Kinetex® EVO C18 Ordering Information

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

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

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 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

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

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

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Strata®-X-C Ordering Information

Format	Sorbent Mass	Part Number	Unit
Tube			
	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)
Giga™ Tube			
	500 mg	8B-S029-HDG	12 mL (20/box)
	1 g	8B-S029-JDG	12 mL (20/box)
	1 g	8B-S029-JEG	20 mL (20/box)
	2 g	8B-S029-KEG	20 mL (20/box)
	5 g	8B-S029-LFF	60 mL (16/box)
96-Well Plate			
	10 mg	8E-S029-AGB	2 Plates/Box
	30 mg	8E-S029-TGB	2 Plates/Box
	60 mg	8E-S029-UGB	2 Plates/Box
96-Well Microelution Plate			
	2 mg	8M-S029-4GA	ea

** Tab-less tubes available. Contact Phenomenex for details.

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