APPLICATIONS

# 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, 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 levels, covering a wide range. The QC samples for extraction were prepared at three concentration (low, medium and high) levels.

### *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 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

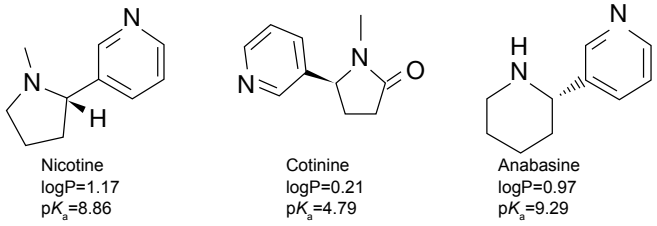


**Figure 1.**
Structure, p$K_a$ and logP Value of Nicotine, Cotinine, and Anabasine

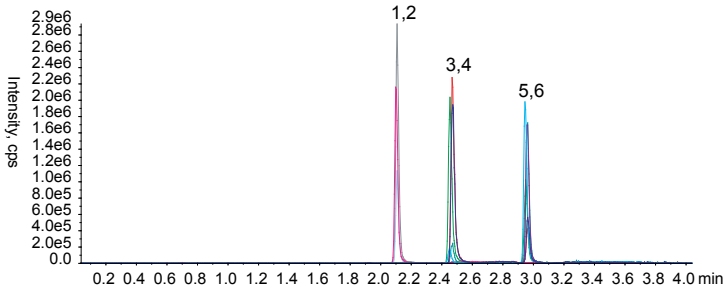


**Figure 2.**
Representative chromatogram of cotinine, anabasine, and nicotine from oral fluid extracted samples.

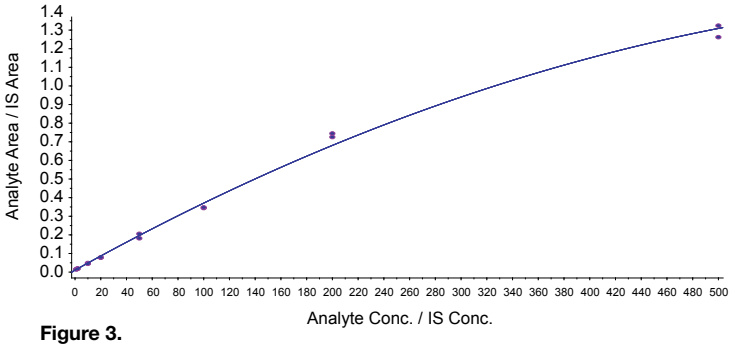


**Figure 3.**
Linearity curve for nicotine (1 to 500 ng/mL) from oral fluid extracted samples. R=0.9978.

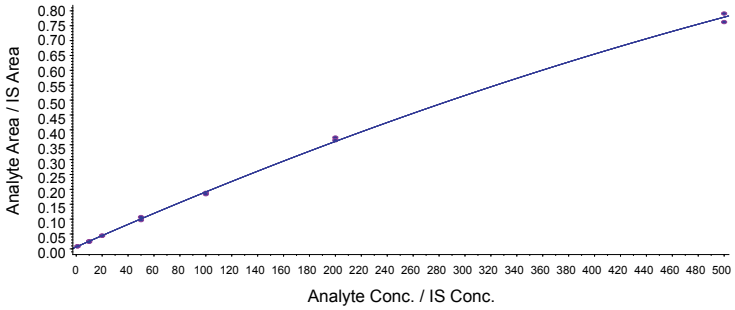


**Figure 4.**
Linearity curve for cotinine (1 to 500 ng/mL) from oral fluid extracted samples. R=0.9996.

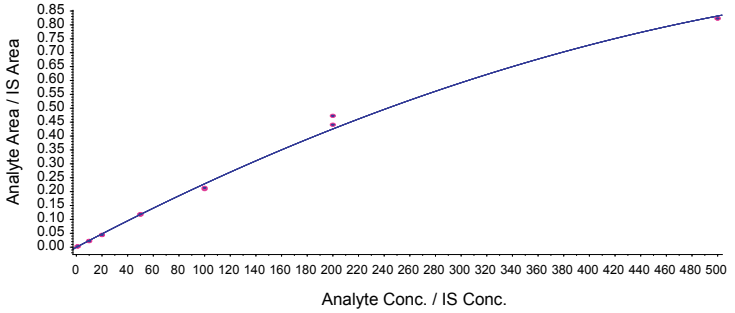


**Figure 5.**
Linearity curve for anabasine (1 to 500 ng/mL) from oral fluid extracted samples. R=0.9983.

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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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