



Simultaneous Determination of Nicotine and Metabolites by LC-MS Using an Acclaim Mixed-Mode HILIC-1 Column

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Environmental Tobacco Smoke (ETS), commonly called second-hand smoke, is generated from sidestream smoke and mainstream smoke exhaled by smokers. The relation between ETS and numerous disease states and mortality is well documented (1). Nicotine has been used as a marker for ETS exposure assessment for decades. Its major metabolites, cotinine and trans-3'-hydroxycotinine (HOC), have been used as biomarkers to study individual ETS exposure and tobacco related diseases.

Methods to quantify nicotine and its metabolites using reversed-phase HPLC suffer from significant peak tailing for nicotine that hampers quantification at low concentrations. A recent study showed much improved peak shape and a wide, controllable retention range for nicotine by hydrophilic interaction chromatography (HILIC) on a bare silica column (2). However, bare silica does not offer retention for cotinine and HOC, which are less hydrophilic than nicotine.

A fast and sensitive method has been developed for simultaneous determination of nicotine, cotinine, and HOC by LC-MS using an Acclaim® Mixed-Mode HILIC-1 column and mass

spectrometric detection. The hydrophilic and reversed-phase retentions of the HILIC-1 column offer considerable retention and resolution for cotinine and HOC without sacrificing peak shape, retention, and resolution for nicotine.

Experimental

LC-MS determination was performed on a Dionex Summit® HPLC system and an MSQ™ Plus single quadrupole mass spectrometer with an electrospray ionization source.

HPLC Conditions

Column: Acclaim Mixed-Mode HILIC-1, 5 μ m, 2.1 \times 150 mm

Mobile Phase: 85% acetonitrile / 6% 50 mM ammonium formate buffer (pH 2.7) / 9% H₂O (v/v/v)

Flow Rate: 0.25 mL/min

Injection Volume: 5 μ L

Column Temperature: 20 °C

Table I: SIM Scan Functions and Scan Events

Analyte	Adduct	SIM (m/z)	Time Range (min)	Dwell Time (s)	Cone Voltage (V)
Nicotine	[M+H] ⁺	163.2	2.0–4.0	0.5	80
Nicotine- ² H ₄	[M+H] ⁺	167.2	2.0–4.0	0.5	80
Cotinine	[M+H] ⁺	177.1	0.0–2.0	0.1	80
Cotinine- ² H ₄	[M+H] ⁺	181.1	0.0–2.0	0.1	80
HOC	[M+CH ₃ CN+H] ⁺	234.1	0.0–2.0	0.1	50
HOC- ² H ₃	[M+CH ₃ CN+H] ⁺	237.1	0.0–2.0	0.1	50

Table II: Precision, Accuracy and Method Detection Limits (MDL)

Analyte	Injected Amount (ng/mL)	Observed Amount* (ng/mL)	Standard Deviation	%RSD	Accuracy** %	MDL (pg)	S/N (Injected Amt.)
Nicotine	199.9	200.3	0.446	0.22	100.2	5.85	25.9 (25.0 pg)
Cotinine	100.8	99.25	1.76	1.78	98.46	23.2	26.8 (10.0 pg)
HOC	150.0	150.6	2.25	1.49	100.4	29.5	18.7 (25.0 pg)

*Observed amount was the calculated average of 15 replicate injections.

**Accuracy was calculated by (observed amount)/(injected amount) \times 100.

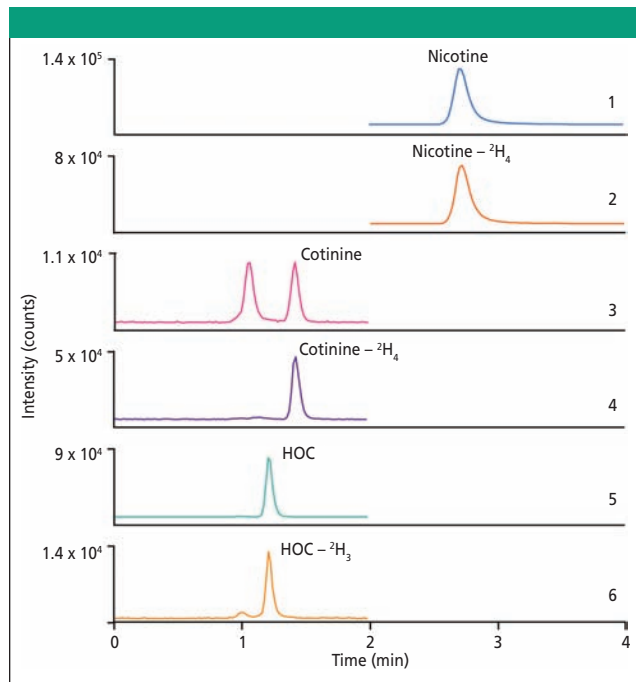


Figure 1: SIM chromatograms of nicotine and metabolites by LC-MS. Traces: 1) Nicotine, 163.2 m/z; 2) Nicotine-²H₄, 167.2 m/z; 3) Cotinine, 177.1 m/z; 4) Cotinine-²H₄, 181.1 m/z; 5) HOC, 234.1 m/z 6) HOC-²H₃, 237.1 m/z.

MS Conditions

Operating Mode: Positive selected ion monitoring (SIM)
 Probe Temperature: 500 °C
 Needle Voltage: 4 kV
 Nebulizer Gas: Nitrogen at 80 psi
 SIM Settings: See Table I for details.

Results

Nicotine, cotinine, and HOC were resolved within four minutes in a fast chromatographic run. SIM chromatograms are shown in Figure 1. Calibration curves generated from replicate injections ($n = 4$) are shown in Figure 2.

Precision, accuracy, and method detection limits (MDL) were evaluated by replicate injections ($n = 15$) of medium level calibration standards (nicotine at 199.9 ng/mL, cotinine at 100.8 ng/mL and HOC at 150 ng/mL). Results are shown in Table II.

MDLs were calculated based on the equation: $MDL = t_{99\%} \times S_{(n=15)}$ where t is Student's t statistic at 99% confidence intervals ($t_{99\%, n=15} = 2.624$) and S is the standard deviation. The signal to noise ratio (S/N) of each analyte at lowest calibration level is also shown in Table II.

Conclusion

A fast and sensitive LC-MS method for simultaneous determination of nicotine, cotinine, and HOC was demonstrated using an Acclaim Mixed-Mode HILIC-1 column and mass spectrometry with stable isotope-labeled internal standards. Excellent linearity was achieved for each analyte from < 10 ng/mL to 2000 ng/mL with a correlation coefficient (r^2) > 0.999. Excellent precision and accuracy were achieved with %RSD < 2% and accuracy bias < 2%.

Table III: Concentrations of Nicotine, Cotinine, and HOC Used for Calibration

Standard	Nicotine ^a (ng/mL)	Cotinine ^b (ng/mL)	HOC ^a (ng/mL)
Level 1	2.003	—	0.00
Level 2	5.006	5.020	5.00
Level 3	10.04	24.67	10.0
Level 4	50.79	100.8	50.0
Level 5	199.9	255.5	150
Level 6	509.0	507.9	500
Level 7	1002	994.3	1000
Level 8	2001	2016	2000

^a $n=5$ injections

^b $n=4$ injections

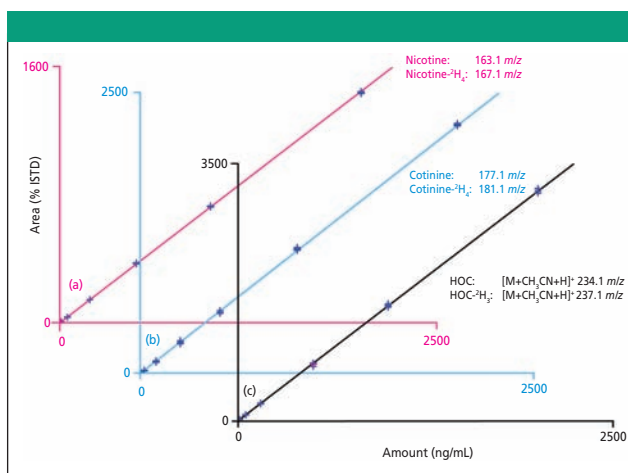


Figure 2: Calibration curves of A) nicotine, B) cotinine, and C) HOC using isotope-labeled internal standards. Calibration curves were generated using replicate injections of the calibration standards at different levels (Table III). Internal standard concentrations were 150 ng/mL for nicotine and HOC, 100 ng/mL for cotinine. All standards were prepared in pure acetonitrile.

References

- (1) *The Health Consequences of Involuntary Exposure to Tobacco Smoke: A Report of the Surgeon General*; U.S. Dept. of Health and Human Services, Centers for Disease Control and Prevention, Coordinating Center for Health Promotion, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health, U.S. Government Printing Office: Washington, DC, 2006.
- (2) Wang, L. Chemical Analysis of Environmental Nicotine and Nicotine Metabolites by Liquid Chromatography Tandem Mass Spectrometry. Masters Thesis, San Diego State University, San Diego, CA, 2006.

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