A Validated Analytical Method for Environmental Nicotine Exposure by Hydrophilic Interaction Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT

Environmental Tobacco Smoke (ETS) 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 in air, surface, and dust samples has been used as a marker for ETS exposure assessment for decades. Methods to quantify nicotine using reversed-phase HPLC methods suffer from significant peak tailing that negatively effects quantification at low concentrations.

We present for the first time a validated method for nicotine analysis from ambient air, surface and dust samples. Each sample type was extracted by solid phase extraction (SPE) and quantified by Hydrophilic Interaction Liquid Chromatography-Tandem Mass Spectrometry (HILIC-MS/MS) with electrospray ionization (ESI). Linearity ($R^2 > 0.999$) was achieved over a range of 50 pg to 20 ng injected on-column, using isotope dilution mass spectrometry. Peak shape was considerably improved over previous HPLC methods (peak tailing factor of 1.13), and excellent precision and accuracy were achieved and validated.

A low limit of quantification was set at 10 ng in consideration of the ubiquitous nature of nicotine as an environmental contaminant that affects sample collection and processing. The lowest nicotine levels detected in samples (including blank samples) that were delivered to our laboratory for analysis were 3 ng for wipes, 2.7 ng for air badges and 16 ng for 50 mg dust samples.

The ruggedness of the method has been verified by the analysis of hundreds of environmental nicotine samples obtained from dosimeters, surface wipes, and dust. Method precision and accuracy will be discussed for each sample type.

A mixed-mode column featuring reversed-phase retention and ion exchange mechanisms was applied for simultaneous determination of nicotine and nicotine metabolites: cotinine and trans-3'-hydroxycotinine. Results of the method validation will be presented upon completion.

EXPERIMENTAL

HPLC System

- Surveyor HPLC System consisting of a Surveyor autosampler and HPLC pump from Thermo Fisher Scientific, Inc.

Mass Spectrometer

- TSQ Quantum™ triple quadrupole mass spectrometer from Thermo Fisher Scientific, Inc. coupled to HPLC by ESI interface

Software

- Xcalibur™ 1.4 for Thermo Surveyor HPLC and TSQ Quantum systems

Analytical Column

- Thermo Hypersil-Keystone Silica (50 × 2.1 mm, 3 µm)

CHROMATOGRAPHIC CONDITIONS

- Flow rate: Isocratic 80/20 v/v = acetonitrile/50 mM pH 4 ammonium formate buffer (v/v) at 0.3 mL/min;
- Column temperature: Ambient, not controlled
- Injection volume: 10 µL

MASS SPECTROMETRIC CONDITIONS

The MS operating conditions were optimized by infusing nicotine standard (100 ng/mL) coeluted with the same mobile phase described above.

- Ion source: Positive ESI
- Spray voltage: 2000 V (Spray discharge current ~ 5.3 µA)
- Sheath gas pressure: 30 arbitrary units
- Auxiliary gas pressure: 5 arbitrary units
- Capillary temperature: 250 °C
- Source CID energy: 18 V
- Q2 collision cell: Argon at 1.5 mTorr
- Scan event: Multiple Reaction Monitoring (MRM), details listed in table 1.

Table 1: MRM Ion Transmissions for Nicotine and ITSDs			
Analyte	**Parent Ion (*m/z*)**	**Product Ion (*m/z*)**	**Collision Energy (volts)**
Nicotine	163.1	130.1	16
	163.1	117.1	24
Nicotine – d_3	166.1	130.1	16
	166.1	117.1	24
Nicotine – d_4	167.1	134.1	16
	167.1	121.1	24

INSTRUMENT FOR NICOTINE AND ITS METABOLITES BY MIXED-MODE HILIC CHROMATOGRAPHY

HPLC System

- Dionex Summit® HPLC System consisting of a P680 dual ternary pump, ASI-100 autosampler, TCC-100 column oven, and a UVD-340U UV/Vis detector

Mass Spectrometer

- Dionex MSQ™ Plus single quadrupole mass spectrometer coupled to HPLC by ESI interface

Software

- Chromeleon® 6.8 Chromatography Management Software for Dionex Summit HPLC system and Xcalibur 2.0 for MSQ Plus systems

Analytical Column

- Dionex Acclaim® Mixed Mode HILIC-1 column (150 × 2.1 mm, 5 µm)

CHROMATOGRAPHIC CONDITIONS

- Flow rate: Isocratic 85/9/6 v/v = acetonitrile (CH_3CN)/H_2O/ pH 2.7 ammonium formate buffer with 3 mM total ionic strength at 0.25 mL/min;
- Column temperature: 20 °C
- Injection volume: 5 µL

MASS SPECTROMETRIC CONDITIONS

- Operating Mode: Positive selected ion monitoring (SIM)

Nicotine:	$[M+H]^+$ +163 m/z
Cotinine:	$[M+H]^+$ +177 m/z
Trans-3'-hydroxycotinine:	$[M+H]^+$ +193 m/z

- Cone voltage: 70 V for all scans
- Dwell time: 0.5 seconds for all SIM channels
- Probe temperature: 500 °C
- Needle voltage: 4 kV

SAMPLE PREPARATION

Details for sample preparation are discussed in detail in another publication.[2] SPE assembly (shown in Figure 1) was attached to a 24 channel SPE vacuum manifold and conditioned with 3 mL methanol followed by 3 mL 0.1 M sodium hydroxide. Air filters, dust samples and surface wipes were removed from original container, spiked with ITSD (nicotine-pyridinal-d_4, i.e. nicotine-d_4) and soaked in formic acid solution. The pH of the extraction solution was then adjusted using sodium hydroxide, and loaded onto the SPE column. The SPE column was washed by 5% acetonitrile (v/v), and nicotine was eluted by 0.4 mL acetonitrile followed by 0.1 mL pH 4 buffer.

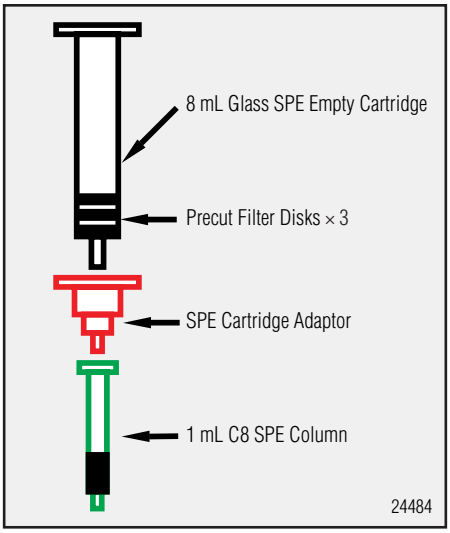

Figure 1. SPE assembly for nicotine sample preparation.

RESULT AND DISCUSSION

METHOD DEVELOPMENT

HILIC separation on a bare silica column can be affected by mobile phase composition, buffer pH, and ionic strength.[3] The effect on chromatography of mobile phase composition was investigated in terms of organic solvent percentage, buffer pH, and buffer concentration. Dependence of mass spectrometric response on mobile phase composition and sample injection solution was also explored.

MOBILE PHASE COMPOSITION AND BUFFER PH

Increased retention time of nicotine was observed as acetonitrile percent increased, as shown in Figure 2. The mobile phase containing pH 3 buffer showed the greatest effect on the retention of nicotine, with a capacity factor range from 4.94 to 17.4. This observation can be explained by the different forms nicotine presents; at lower pH, nicotine (pKa = 4.23) will shift to ionic form, which has a stronger affinity to the silica surface, and the retention of nicotine has greater dependence on mobile phase strength change.

When comparing natural nicotine (upper trace) and nicotine-d_3 (lower trace) in Figure 2, a noticeable difference of intersections was observed.[2]

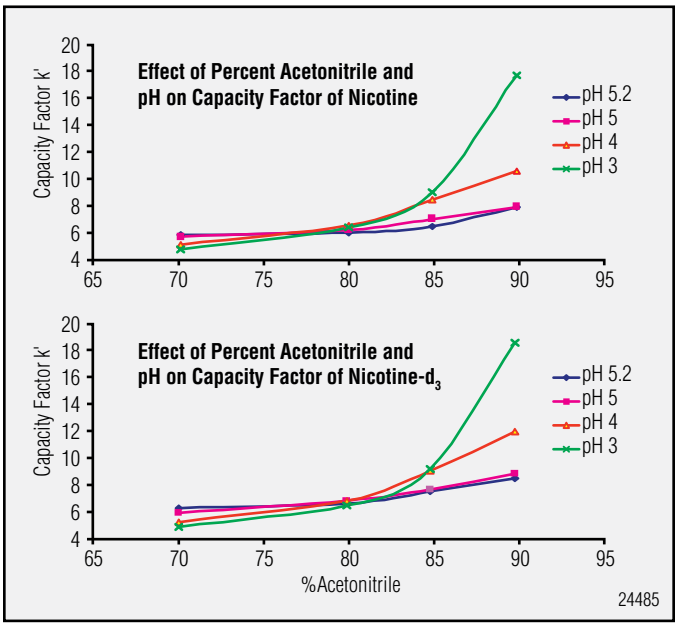

Figure 2. Effect of percent acetonitrile and buffer pH on capacity factor. Total ionic strength was kept at 4 mM for whole mobile phase. Percent acetonitrile ranged from 70 to 90. Aqueous buffer was made by dissolving appropriate amount of ammonium formate in D.I. water and adjusting pH by formic acid. Flow rate was 0.3 mL/min.

IONIC STRENGTH

Ionic strength is one of the most important factors effecting the retention of nicotine on a silica column in HILIC mode (See Figure 3). This characteristic offers a high degree of flexibility for controlling the retention of nicotine.

MASS SPECTROMETRIC RESPONSE

No significant change in MS response was observed while using different mobile phase combinations, although a clear trend was observed that a mobile phase with higher acetonitrile percentage showed higher MS response.

Injection solution was observed to be one critical factor effecting the MS response. Dissolving the sample in the mobile phase resulted in two orders of magnitude higher intensity than that dissolved in pure acetonitrile as shown in Figure 4.

The final conditions were achieved as a result of balancing the issues of sample throughput, resolution, MS response, and minimum ion suppression. The MRM chromatogram of nicotine under optimized condition is shown in Figure 5.

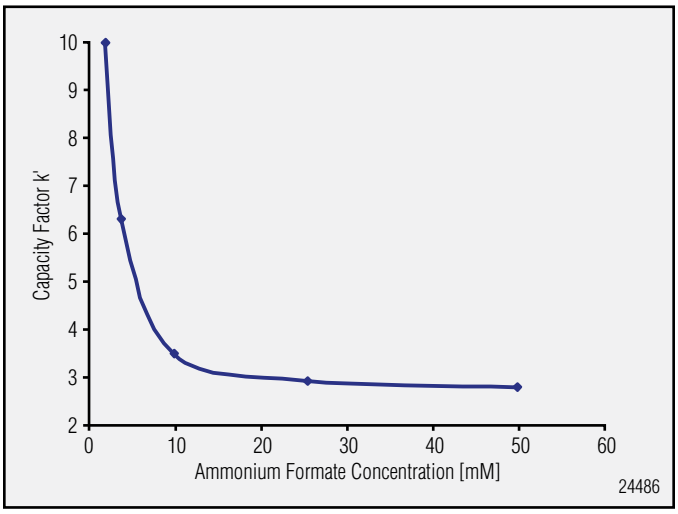

Figure 3. Effect of ionic strength on nicotine capacity factor. Mobile phase was 80% acetonitrile in aqueous buffer with various concentrations of ammonium formate (from 10 mM to 250 mM) buffered at pH 4. Flow rate was 0.3 mL/min.

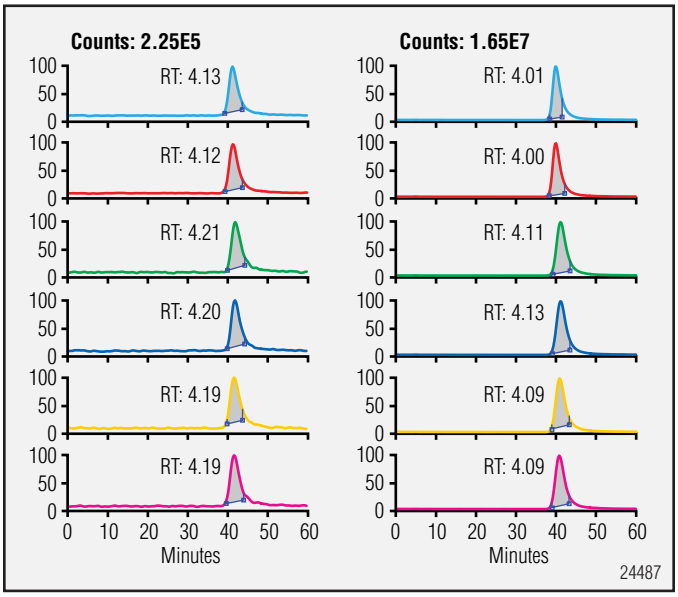

Figure 4. Effect of injection solution on mass spectrometric response. Left Panel: Sample reconstituted in pure acetonitrile. Right Panel: Sample reconstituted in mobile phase, 80/20 v/v = acetonitrile/pH 4 50 mM ammonium formate buffer.

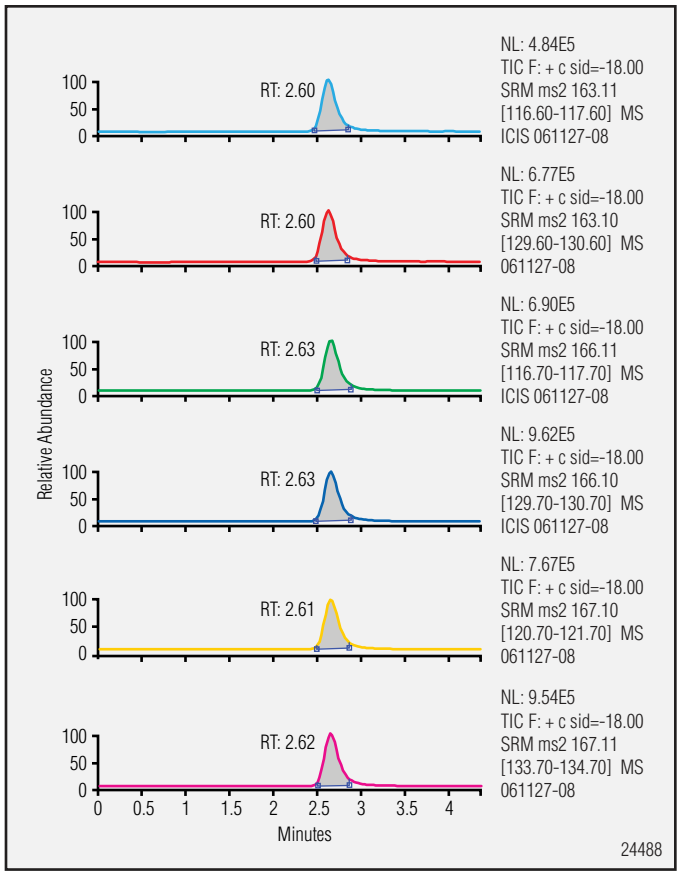

Figure 5. MRM chromatogram under optimized condition. An unknown sample spiked with nicotine-d_3 and nicotine-d_4 . Mobile phase: 80/20 v/v = acetonitrile/ pH 4 50 mM ammonium formate buffer; Injection volume: 10 µL; Mass Spectrometer conditions are listed in experimental section.

METHOD VALIDATION

LINEARITY AND LOWER LIMIT OF QUANTIFICATION

Because nicotine is a ubiquitous contaminant, the lower limit of quantification (LLOQ) was determined by environmental background instead of instrument detection capability. The lowest nicotine levels detected in samples (including blank samples) obtained for analysis and delivered to our laboratory were 3 ng for wipes, 2.7 ng for air badges, and 16 ng for 50 mg dust samples. LLOQ was set at 10 ng, which represents 200 pg nicotine injected on-column. Calibration standards ranged from 5.0 ng/mL to 2000 ng/mL. (50 pg to 20 ng on-column injection) Linearity was achieved throughout the range with a correlation coefficient (R^2) > 0.999. The calibration curves are shown in Figure 6, which were generated from four repeated injections of the seven calibration standards.

RECOVERY

Recovery was evaluated for overall sample preparation procedure. Nicotine-d_3 was used as an analog instead of nicotine due to the ubiquity of nicotine in environment. Nicotine-d_4 was spiked to each sample as ITSD after the nicotine analog eluted from SPE column, the result is shown in Table 2.

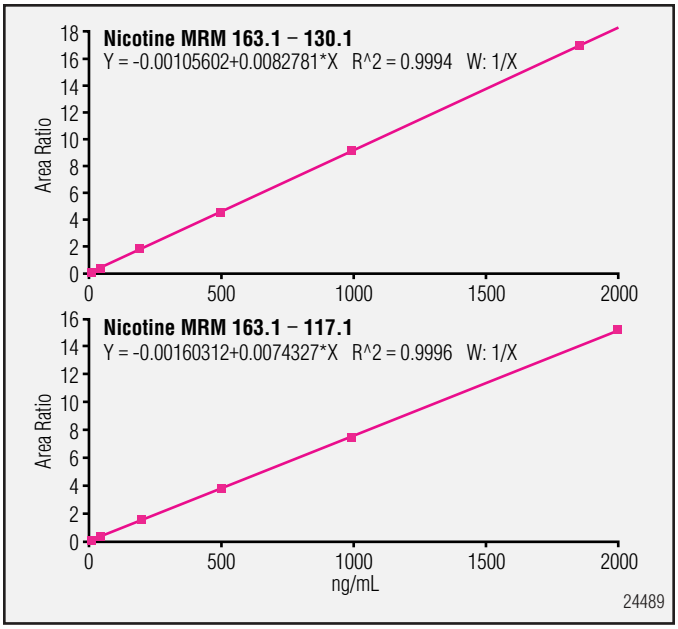

Figure 6. Calibration curves of nicotine by using isotope labeled nicotine-d_4 as ITSD generated from 5 runs of calibration standards ranging from 5 to 2000 ng/mL.

Consistent recoveries were observed except for air badge samples at medium level and wipe samples at low level. Higher recovery for dust sample was observed, possibly due to improved sample extraction.

PRECISION AND ACCURACY

Sample preparation precision and accuracy were determined by repeating the preparation of different samples with nicotine-d_3 spiked at low, medium, and high levels (n≥3) and quantified by nicotine-d_4 as ITSD. Results are shown in Table 3 and 4. Accuracy was not evaluated for the dust samples due to the limited sample size. Greater deviation of precision for sample A and sample B was observed when compared to sample C. The disagreement may be attributed to sample homogeneity which can be affected by particle size, shape, etc.

Instrument precision and accuracy evaluation was performed by repeating injections of calibration standards, and result is shown in Table 5.

STABILITY DURING STORAGE

Nicotine loss during storage was observed in this study, and a comparison of short-term and long-term storage stability under different conditions indicates that samples should be stored at -15°C (or lowest available temperature) and need to be processed within the shortest possible time.[2]

Table 2. Recovery of Nicotine							
Level (n≥4)	Spiked Amount (ng)	Air Badges Samples		Wipe Samples		Dust Samples	
		Observed (ng)	Recovery	Observed (ng)	Recovery	Observed (ng)	Recovery
Low	30	13.3 ± 2.7	44.2%	11.4 ± 0.9	38.1%	24.9 ± 4.7	75.2%
Medium	272	85.2 ± 4.3	32.2%	158 ± 5.6	59.8%	220 ± 18.1	81.1%
High	1088	451 ± 22.1	42.6%	651 ± 25.3	61.6%	812 ± 57.6	74.7%

Table 3. Precision and Accuracy of Sample Preparations							
Level (n≥3)	Spiked Amount (ng)	Air Badges Samples		Wipes			
		Observed (ng)	Accuracy	Precision (RSD)	Observed (ng)	Accuracy	Precision (RSD)
Low	30	29.3 ± 0.6	97.6%	2.06	29.7 ± 1.0	99.1%	3.20
Medium	247	208 ± 14.2	84.3%	6.85	231.3 ± 4.5	93.8%	1.97
High	986	920 ± 15.4	93.3%	1.68	893.7 ± 25.6	90.6%	2.86

Table 4. Method Precision and Accuracy for Dust Samples							
Sample	Specified Amount	Sample Preparation		Instrumental			
		Observed (ng/mg)	Accuracy	Precision (RSD)	Observed (ng/mg)	Accuracy	Precision (RSD)
Low	---	7.54 ± 0.50	----	6.62	----	----	N/A
Medium	---	12.4 ± 1.45	----	11.7	11.3 ± 1.1	----	10.2
High	---	1.77 ± 0.03	----	1.58	1.76 ± 0.04	----	2.54

Table 5. Instrumental Precision and Accuracy							
Level	Specified Amount (pg)	Intra-Day (n=5)		Inter-Day (n=9)			
		Observed (ng)	Accuracy	Precision (%RSD)	Observed (ng)	Accuracy	Precision (%RSD)
Low	25.00	5.12 ± 0.28	102%	5.44	5.57 ± 0.31	111%	5.53
Medium	1000	197 ± 4.73	98.9%	2.39	194 ± 5.85	99.8%	3.01
High	10000	1988 ± 30.0	99.3%	1.49	2008 ± 15.1	100%	0.75

POTENTIAL FOR NICOTINE BIOANALYSIS IN URINE

Urine samples from subjects exposed or suspicious of being exposed to ETS were spiked with nicotine ITSD, prepared by cotinine sample preparation procedure[2] and analyzed by this method. Excellent method performance was observed for quantification of nicotine in urine samples. Retention and peak shape are well maintained, with no interference observed near the nicotine peak.

FURTHER STUDY

We have developed a rugged, high-throughput method for simultaneous quantification of nicotine and its two major metabolites: cotinine and trans-3'-hydroxycotinine (HOC). Reversed-phase chromatography offers considerable retention for cotinine and HOC, but suffers from significant peak tailing as well as excessive retention time for nicotine. Conventional HILIC chromatography provides good retention for nicotine but elutes cotinine and HOC near the void, this may lead to interference by early eluting impurities; in addition, ion suppression may be observed from the injection matrix.

A Mixed-Mode HILIC-1 column was evaluated to take advantage of the mixed retention mechanisms (reverse phase and HILIC retention). Improved retention and resolution were achieved on this column, as shown in figure 7. Further study will be undertaken to validate and verify its application for biological sample quantification. Results will be released upon completion.

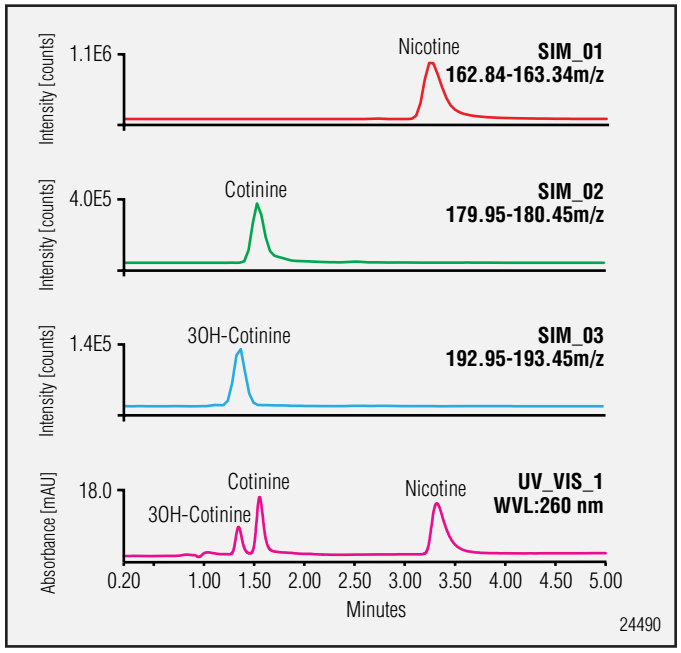

Figure 7. UV and SIM chromatograms of nicotine and its metabolites. Mobile phase: 85% CH_3CN/ 9% H_2O/ 6% 50 mM pH 2.7 Ammonium formate buffer; Flow rate: 0.5 mL/min; Injection volumn: 5 µL; Nicotine: 10 ng/µL, SIM=163 m/z*; Cotinine: 9.8 ng/µL, SIM =177* m/z*; HOC: 6.7 ng/µL, SIM=193* m/z*; Cone voltage: 70V, Dwell time: 0.5 second, Probe temperature: 500 °C; Needle voltage: 4 kV.*

CONCLUSION

- A comprehensive method for quantitative determination of environmental nicotine in different media by HILIC-MS/MS was developed and validated.
- Significant peak shape improvement was achieved.
- Excellent linearity was achieved from 50 pg to 20 ng on-column injection.
- Short run time and considerable capacity factor ensure rugged high sample throughput.
- Method has been verified by analyzing hundreds of real samples.
- A fast method for simultaneous determination of nicotine and its major metabolites: cotinine and trans-3'-hydroxycotinine has been developed.

REFERENCE

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3. Guo, Y.; Gaiki, S. *Journal of Chromatography A* **2005**, 1074, 71-80.

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