

Robust and Fast Analysis with an RSLC PA2 Column of Four Tobacco Specific Nitrosamines in Cigarettes by LC-MS/MS

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INTRODUCTION

Tobacco Specific Nitrosamines (TSNA) are a group of carcinogens found only in tobacco products. They are formed from nicotine and related alkaloids during the production and processing of tobacco and tobacco products.¹ Due to their carcinogenic properties, efforts have been made to reduce TSNA levels in tobacco products. The desired goal of this investigation is to develop a sensitive, high-throughput method to monitor TSNA levels in tobacco and tobacco products. This study focuses on N'-nitrosornicotine (NNN), N'-nitrosoanatabine (NAT), N-nitrosoanabasine (NAB), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).

Conventional methods for TSNA analysis are based on gas chromatography with thermo energy analyzer (GC-TEA)^{2,3}, or high performance liquid chromatography (HPLC) with various detection techniques such as UV and mass spectrometry (MS).⁴⁻⁶

In this study, a robust and fast LC-MS/MS method was developed to analyze the four TSNAs in tobacco cigarettes after a simple sample preparation of ammonium acetate liquid extraction and filtration. The chromatographic separation of the four TSNAs was achieved within 3.5 minutes and these analytes were detected with great sensitivity and selectivity by tandem mass spectrometry. This method has been evaluated with respect to linearity, detection limits, precision, and accuracy. The ruggedness of the methodology was proven with more than 1000 replicate injections.

EXPERIMENTAL

Instrumentation

HPLC

P680 dual ternary pump
ASI-100 autosampler
TCC-100 thermostatted column compartment
UVD340U detector

Mass Spectrometer

TSQ Quantum Access™ triple quadrupole mass spectrometer with heated electrospray ionization (H-ESI) interface

Software

Xcalibur® 2.0
Dionex DCMS^{Link}™ for Xcalibur (version 2.0)*

* DCMS^{Link} is a Chromeleon®-based software module providing the interface for controlling a wide range of Dionex chromatography instruments from different mass spectrometer software platforms.

Chromatographic Conditions

Analytical Column: Acclaim® RSLC PA2 (5.0 × 2.1 mm, 2.2 μm)
Column Temperature: 60 °C
Injection Volume: 10 μL
Mobile Phase: 10% CH₃CN in buffer (1 mM NH₄OAc, pH adjusted to pH 8.0 by NH₄OH)
Flow Rate: 0.50 mL/min
Detection: UV at 230 nm
TSQ Quantum Access SRM

Mass Spectrometric Conditions

Needle Voltage: 2000 V
Vaporizer Temp.: 350 °C
Sheath Gas: 60 (arbitrary unit)
Auxiliary Gas: 50 (arbitrary unit)
Ion Sweep Gas: 2 (arbitrary unit)
Collision Gas Pressure: 1.5 mTorr
Capillary Temp.: 350 °C

| SRM Transitions: | Compound | m/z | Relative Abundance |
|--------------------|----------|---------------|--------------------|
| NNN | | 178.1 → 148.1 | 11 V |
| | | 178.1 → 120.1 | 18 V |
| NNK | | 208.1 → 122.1 | 11 V |
| | | 208.1 → 79.2 | 38 V |
| NNK-d ₄ | | 212.1 → 126.1 | 11 V |
| | | 212.1 → 83.2 | 38 V |
| NAT | | 190.1 → 160.1 | 10 V |
| | | 190.1 → 79.2 | 5 V |
| NAB | | 192.1 → 106.2 | 10 V |
| | | 192.1 → 133.1 | 24 V |

The 1st SRM transition of each compound is used for quantification and the 2nd SRM transition is used for confirmation.

Sample Preparation

Tobacco cuts from 5 brands of cigarettes were weighed to 0.25 grams into 20 mL glass vials, and extracted with 10 mL of 100 mM ammonium acetate solution. Thirty minutes of constant agitation was applied by placing vials on a swirl table. The extracts were filtered through 0.25 µm membrane syringe filters. A 1.0 mL aliquot of each extract was then spiked with 10 µL internal standard (IStd, NNK-d₄) in preparation for LC-MS/MS analysis.

RESULTS AND DISCUSSION

Chromatography

As shown in Figure 1, the four TSNA are retained and completely resolved within 3.5 min, with the minimum of the retention factors (K'_{NNN}) greater than 4 and minimum resolution (R_{sNAT}) greater than 2.0. These demonstrated retention factors ensure analytes of interest are chromatographically separated from early eluted compounds that can suppress ionization. The total chromatographic resolution minimizes the possibility of ionization suppression and eliminates cross contamination of SRM transitions between main analytes.

Mass Spectrometry

Tandem mass spectrometry provides specific and sensitive detection. The SRM chromatograms in Figure 1 show that quantification can be performed with a high level of confidence even at low concentration (10 ng/mL, 0.1 ng injected amount). By using specific SRM detection, the sample preparation was simplified and the total process time significantly reduced.

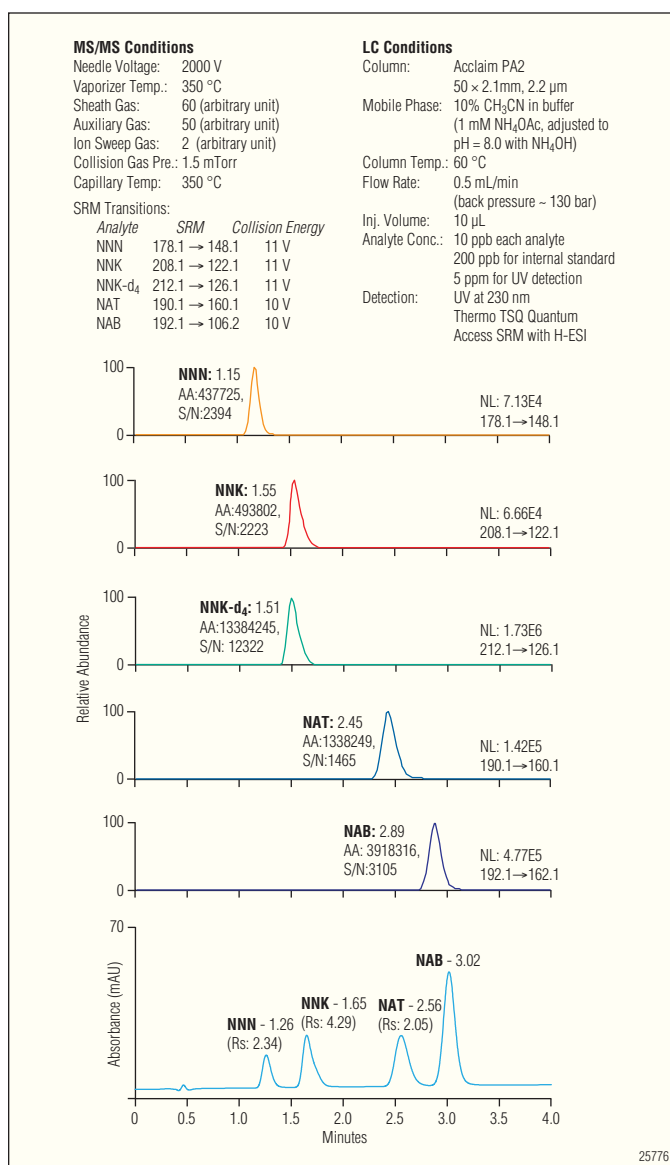


Figure 1. SRM and UV chromatograms of TSNA on RSLC PA2.

Method Performance

Internal standard quantification and calibration were performed using isotope labeled NNK- d_4 (IStd). Calibration standards were prepared at 8 levels from 2 ng/mL to 1000 ng/mL. Triplicate injections were performed to generate calibration curves, and a coefficient of determination (R^2) greater than 0.99 was achieved for each analyte. $1/X$ weighting provided best quantification for samples with lower concentration. Excellent linearity was achieved for NNK with $R^2 = 0.9998$, suggesting that the use of isotope-labeled analogs as internal standards could provide better quantification accuracy and precision. The calibration curve is shown in Figure 2.

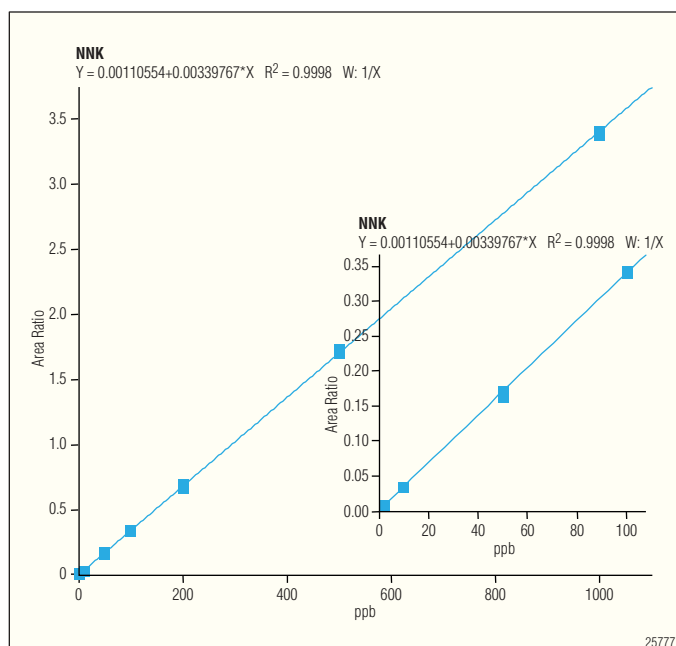


Figure 2. Calibration curve of NNK using NNK- d_4 as internal standard.

Method detection limits (MDLs) were statistically calculated using the equation $MDL = S \times t_{(99\%, n=7)}$ where S is the standard deviation, t is student's t at 99% confidence interval. Seven replicate injections of a standard solution at 2 ng/mL were performed, and used for MDL calculation. The MDL for TSNA ranges from 0.22 ng/mL (NAB) to 0.38 ng/mL (NNK).

Precision and accuracy were evaluated at 10 ng/mL and 100 ng/mL. Seven replicate injections of calibration standards at both levels were performed, and quantification was calculated based on the calibration curve. The results in Table 1 show that the quantification of NNK was very accurate and precise (< 4% difference, < 4.5% RSD) at both low (10 ng/mL) and medium levels (100 ng/mL). However, significant inaccuracy was observed for NAB and NAT at the low level, showing 54.2% and 36.6% quantification differences respectively. This observation suggests better quantification accuracy may be achieved using an isotope labeled internal standard for low-level TSNA analysis, for example, analyzing TSNA in biological matrices.

| Table 1. Precision and Accuracy | | | | |
|---------------------------------|--------------|------|--------------|------|
| | 10 ng/mL | | 100 ng/mL | |
| | Accuracy (%) | %RSD | Accuracy (%) | %RSD |
| NAB | 154.2 | 2.74 | 103.7 | 1.49 |
| NAT | 136.6 | 4.69 | 100.7 | 1.77 |
| NNK | 103.9 | 4.38 | 100.9 | 1.88 |
| NNN | 95.3 | 3.56 | 94.4 | 1.56 |

Method Ruggedness

Since the analytical column was running under the very harsh conditions of high temperature and high pH mobile phase, method ruggedness was evaluated by repeated injections of standard solutions and tobacco extracts. As shown in Figure 3, chromatographic retention and resolution were well maintained after more than 1000 injections.*

*The author suggests using an in-line filter to prevent column clogging.

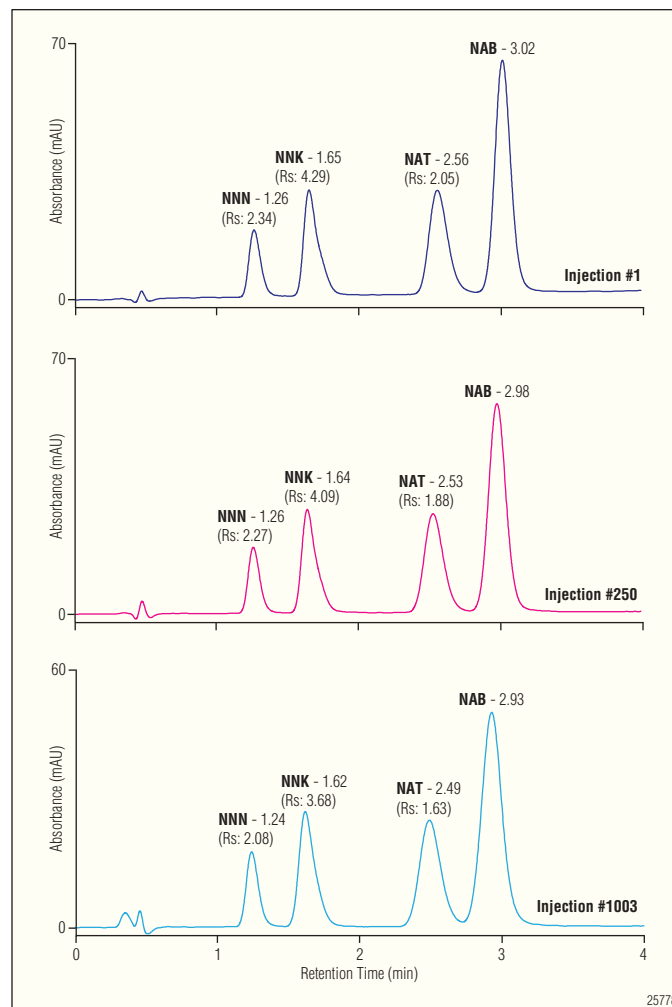


Figure 3. Ruggedness of the Acclaim RSLC PA2 column. The column was cleaned with 50/50 CH_3CN/H_2O before injection #250. A new inlet frit was installed before injection #1003.

Determination of TSNA in Tobacco Cigarettes

Five brands of tobacco cigarettes were purchased from a local convenience store. Tobacco cuts from each brand of cigarettes were prepared ($n = 3$) following the procedure described previously. The results are shown in Table 2. TSNA contents are significantly different between brands A and B and brands C, D, and E. The differences in TSNA content could be related to differences in the tobacco blended in each brand, such as type, origin, age of the tobacco, curing method, and storage conditions. The comparison of brands A and B, which are the regular and light varieties of a premium US brand of cigarettes, shows no difference for TSNA contents except for a change in the amount of NAB. See Figure 4 for SRM chromatograms of TSNA from a brand A cigarette.

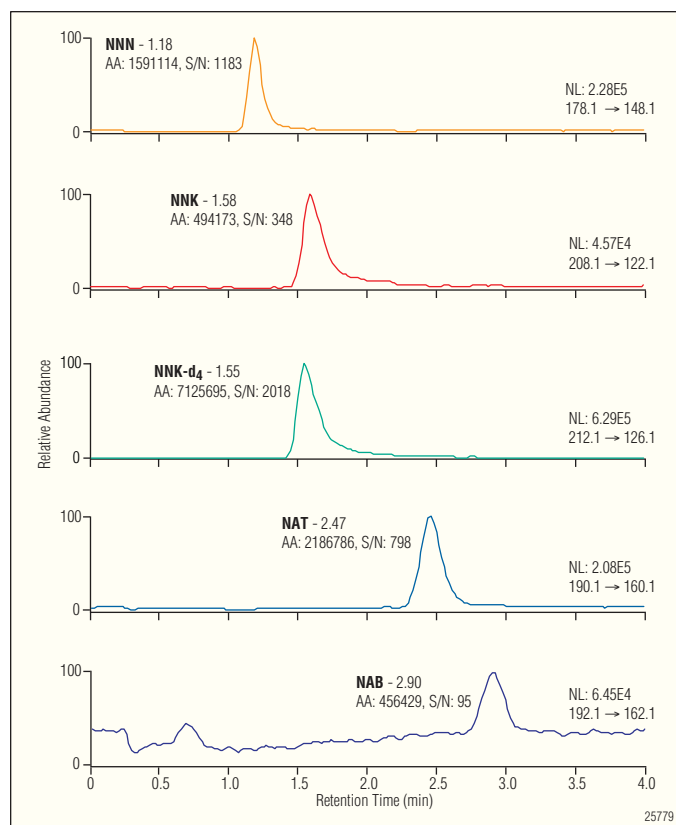


Figure 4. SRM chromatograms of TSNA from brand A cigarette.

| | NAB | NAT | NNK | NNN | Note |
|---------|------|--------|-------|--------|-----------------------|
| Brand A | 5.3 | 1145.1 | 676.5 | 2221.1 | US brand, regular |
| Brand B | 42.1 | 1218.1 | 696.0 | 2472.5 | US brand, lights |
| Brand C | 40.1 | 160.8 | 225.2 | 213.0 | US brand, all natural |
| Brand D | 21.2 | 103.9 | 70.2 | 155.4 | International brand |
| Brand E | 32.7 | 78.2 | 87.8 | 114.3 | Asian brand |

*Amounts shown in Table 2 are ng/g tobacco cuts.

CONCLUSION

A rugged and ultrafast method for TSNA analysis was developed using HPLC-MS/MS on an Acclaim RSLC PA2 column. Four TSNA were chromatographically retained and resolved within 3.5 min. Tandem mass spectrometry with the use of an isotope-labeled internal standard ensured a high level of selectivity and sensitivity. The ruggedness of the method was proven by more than 1000 injections of standards and tobacco extracts. The applicability of the method for the determination of TSNA in tobacco cuts from five brands of cigarettes was demonstrated.

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