DILUTE-AND-SHOOT METHOD FOR DETERMINATION OF TOBACCO-SPECIFIC NITROSAMINES (TSNAs) IN TOBACCO AND E-CIGARETTE PRODUCTS BY UPLC-MS/MS

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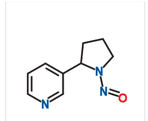
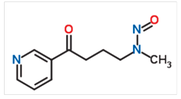
INTRODUCTION

TSNAs are a group of carcinogenic compounds found in tobacco and tobacco smoke. They have also been reported in emerging e-cigarette products that use nicotine extracted from tobacco. TSNAs need to be accurately measured and reported in tobacco products to regulatory agencies including NNN and NNK [1]. This study reports a rapid, sensitive and robust UPLC-MS/MS method for determination of TSNAs in both conventional tobacco and e-cigarette products.

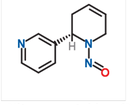
Industry standard HPLC-MS/MS methods for analysis of TSNAs in conventional tobacco products are available from CORESTA [2]. However, no standardized methods have been developed for TSNAs determination in e-cigarettes. In this study, a UPLC-MS/MS method for determination of TSNAs in both conventional tobacco and e-cigarette products was developed based on the CORESTA standard (CRM-72) with enhanced sensitivity, extended linear dynamic range, simplified sample workflow (dilute and shoot approach) and improved analysis throughput.



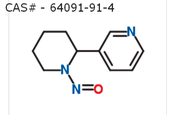
N-Nitrosonornicotine (NNN)
Molecular Formula - $C_9H_{11}N_3O$
Molar Mass - 177.21 Da
CAS# - 16543-55-8



4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)
Molecular Formula - $C_{10}H_{13}N_3O_2$
Molar mass - 207.23 Da
CAS# - 64091-91-4



N-Nitrosoanatabine (NAT)
Molecular Formula - $C_{10}H_{11}N_3O$
Molar Mass - 189.21 Da
CAS# - 71267-22-6



N-Nitrosoanabasine (NAB)
Molecular Formula - $C_{10}H_{13}N_3O$
Molar Mass - 191.23 Da
CAS# - 37620-20-5

Figure 1. Structure and chemical properties of TSNAs.

METHODS

UPLC CONDITIONS

LC system:	Waters ACQUITY UPLC H-Class
Column:	BEH C18 2.1 X 50 mm, 1.7 µm
Column temp.:	45 °C
Injection volume:	5 µL
Flow rate:	0.45 mL/min
Mobile phase A:	10 mM ammonium acetate in water
Mobile phase B:	0.1% acetic acid in methanol (v/v)
Weak needle wash:	50/50 water/methanol (v/v)
Strong needle wash:	10/90 methanol/water (v/v)
Seal wash:	90/10 water/methanol (v/v)
Analysis time:	7 min

Time (min)	Flow Rate (mL/min)	%A	%B	Curve
Initial	0.45	99	1	6
3.00	0.45	10	90	6
4.00	0.45	10	90	6
4.01	0.45	1	99	6
5.00	0.45	99	1	6
7.00	0.45	99	1	6

Table 1. UPLC gradient for TSNA analysis

MS CONDITIONS

MS system:	Xevo™ TQD
Ionization mode:	ESI+
Capillary voltage:	2.5 kV
Desolvation temp.:	550 °C
Desolvation gas flow:	1000 L/Hr
Source temp.:	150 °C

Compound	Retention Time (min)	Precursor Ion (m/z)	Product Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
NNN	2.10	178.06	148.02	24	10
		178.06	105.01	24	16
NNN-D4	2.10	182.10	152.10	24	10
NNK	2.22	208.07	122.01	28	12
		208.07	79.03	28	32
NNK-D4	2.22	212.15	126.07	28	12
NAT	2.41	190.06	160.08	18	10
		190.06	79.01	18	26
NAB	2.48	192.07	162.10	28	12
		192.07	133.07	28	20

Table 2. Optimized MS/MS conditions for TSNAs and labeled internal Standards using IntelliStart on the Xevo TQD.

TOBACCO SAMPLES

CORESTA reference products (CRP) for smokeless tobacco were provided by North Carolina State University (Raleigh, NC) including snus (CRP-1), moist snuff (CRP-2), dry snuff (CRP-3) and loose-leaf chewing tobacco (CRP-4). Commercial cigarette tobacco and e-liquids were purchased from a local retail store.

RESULTS AND DISCUSSIONS

METHOD OPTIMIZATION USING RADAR (SIMULTANEOUS FULL SCAN MS and MS/MS)

To optimize chromatographic separation for TSNAs and to improve the data quality, RADAR acquisition mode was utilized to characterize the tobacco matrix. RADAR enables simultaneous acquisition of full scan MS and MRM data, a unique capability that can both simplify and accelerate development of robust methods.

Significant matrix interference for NNN in presence of tobacco matrix as it co-elutes with Nicotine

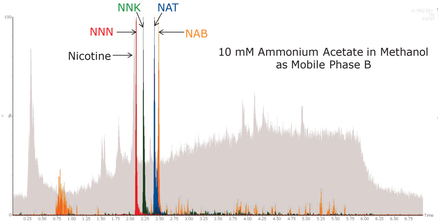


Figure 2A. Full scan TIC (in grey) and MRM chromatogram of TSNAs (in color) using 10mM ammonium acetate in methanol as mobile phase B.

Reduced matrix interference for TSNAs in presence of tobacco matrix

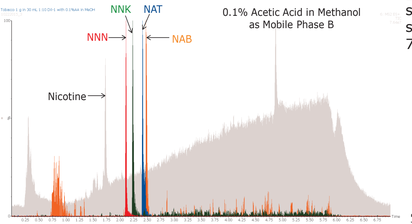


Figure 2B. Full scan TIC (in grey) and MRM chromatogram of TSNAs (in color) using 0.1% Acetic Acid in methanol as mobile phase B.

ENHANCED LINEARITY AND SENSITIVITY

The TSNAs showed excellent linearity with R^2 values greater than 0.999 (Figure 3) for the extended calibration range of 0.25 to 128 ng/mL for NNN, NNK, NAT and 0.0625 to 32 ng/mL for NAB. The wide calibration range enables determination of TSNAs in different tobacco products using a single sample preparation procedure.

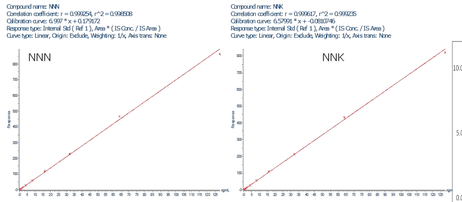


Figure 3. Calibration curves of TSNAs solvent standards

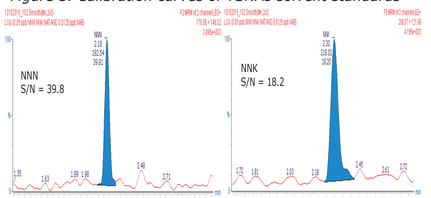


Figure 4. Signal to noise ratio of diluted TSNA solvent standard (0.05 ng/mL) analyzed using Xevo TQD.

TSNAs Analysis in Reference Tobacco Products

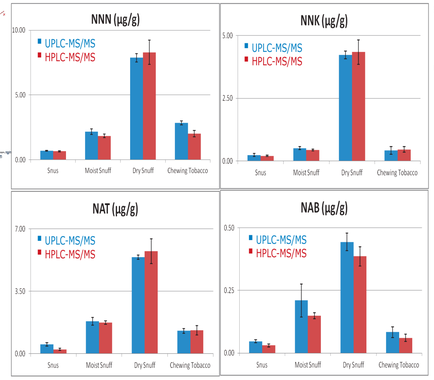


Figure 6. Comparison of TSNA yields using the UPLC-MS/MS method and the HPLC-MS/MS methods used in the CORESTA inter-lab study [3].

METHOD ACCURACY AND PRECISION

In the absence of a blank tobacco matrix, TSNA recoveries were determined by standard additions method. Tobacco sample extracts with incurred nitrosamines were fortified with 50 ng/g of NNN, NNK, NAT and 12.5 ng/g of NAB in triplicates. Both tobacco and fortified tobacco extracts were analyzed and quantified against solvent calibration curves. The error bars represent standard deviation (%RSD) that ranged from 0.1% to 7.2%.

TSNAs recovery ranged from 105-119% in different tobacco matrices

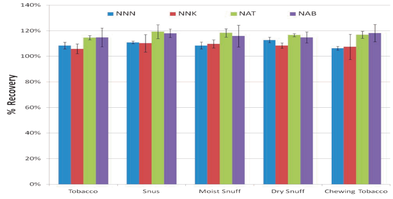


Figure 5. Percent recovery of TSNAs from different

Trace Level of TSNAs Detected in Selected Commercial E-liquids below LOQ

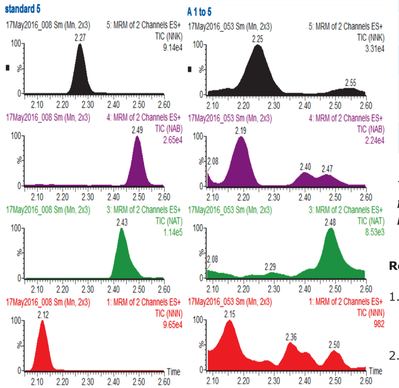


Figure 7. Chromatograms from analysis of TSNA

CONCLUSIONS

- **A rapid, sensitive and robust UPLC-MS/MS method has been developed for determination of TSNAs in tobacco and emerging e-cigarette products.**
- **The dilute and shoot approach for TSNA analysis eliminates the need for sample cleanup and minimizes instrument maintenance and downtime.**
- **UPLC-MS/MS method provides higher sensitivity, selectivity and reduced analysis time (7 min) for determination of TSNAs compared to industry standard CRM-72.**
- **RADAR helps understand sample complexity and leads to faster and robust method development.**
- **Use of lower sample injection volume reduces matrix effect, eliminates the need for SPE cleanup step without compromising data quality.**

Method Parameter	HPLC-MS/MS Standardized Method	UPLC-MS/MS Acquity Xevo TQD Method	Benefits
Calibration Range	2-80 ng/mL for NNN, NNK, NAT	0.25-128 ng/mL for NNN, NNK, NAT	Extended calibration range
Sample Cleanup	SPE Recommended	10-fold Dilution	Simplified workflow
Injection Volume	10 µL	5 µL	Reduced matrix load
Analytical Column	Waters X-terra MS C18, 2.1 x 50mm, 2.5 µm	Waters Acquity UPLC BEH C18, 2.1 x 50mm, 1.7 µm	Higher chromatographic resolution
Analysis Time	10 min	7 min	Reduced analysis time

Table 3. The key benefits of UPLC-MS/MS method for TSNA analysis are compared to HPLC-MS/MS based on CORESTA method.

References

1. FDA Guidance for the Industry, March 2012, Reporting Harmful and Potentially Harmful Constituents in Tobacco Products and Tobacco Smoke Under Section 904(a)(3) of the Federal Food, Drug, and Cosmetic Act".
2. CORESTA Recommended Method No 72 (July 2013): Determination of Tobacco-Specific Nitrosamines in Smokeless Tobacco Products by LC-MS/MS.
3. CORESTA STS Technical Report: 2014 Smokeless Reference Tobacco Product Analysis.

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