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응용 자료

Dilute and Shoot Method for the Determination of Tobacco-Specific Nitrosamines (TSNAs) in Smokeless Tobacco Products by UPLC-MS/MS

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Abstract

A rapid, sensitive and robust UPLC-MS/MS method has been developed for the determination of TSNAs in tobacco and smokeless tobacco products. The UPLC-MS/MS method further improves the performance of industry standard CRM-72 by utilizing a UPLC column to improve chromatographic resolution and reduce analysis time.

Benefits

- Sensitive and robust method for analysis of TSNAs in smokeless tobacco products including snus, moist snuff, dry snuff, chewing tobacco, and raw tobacco.
- Rapid separation of TSNAs within 7 minutes using an ACQUITY UPLC BEH C_{18} Column.
- Wide linear dynamic range for TSNAs (NNN, NNK, NAT–0.25 to 128 ng/mL and NAB–0.0625 to 32 ng/mL).
- Faster method development using RADAR Technology to overcome sample matrix effects.
- Simplified workflow using sample dilution and smaller injection volume to eliminate the need for SPE cleanup.

Introduction

Tobacco specific nitrosamines (TSNAs) are a group of carcinogenic compounds found in tobacco and tobacco smoke. Four different TSNAs (Figure 1) are monitored in tobacco and smoke emissions: N-nitrosonornicotine (NNN), 4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosoanatabine (NAT), and N-nitrosoanabasine (NAB).[1] These harmful constituents are formed from nicotine and related alkaloids by a nitrosation reaction that occurs during the curing and processing of tobacco.

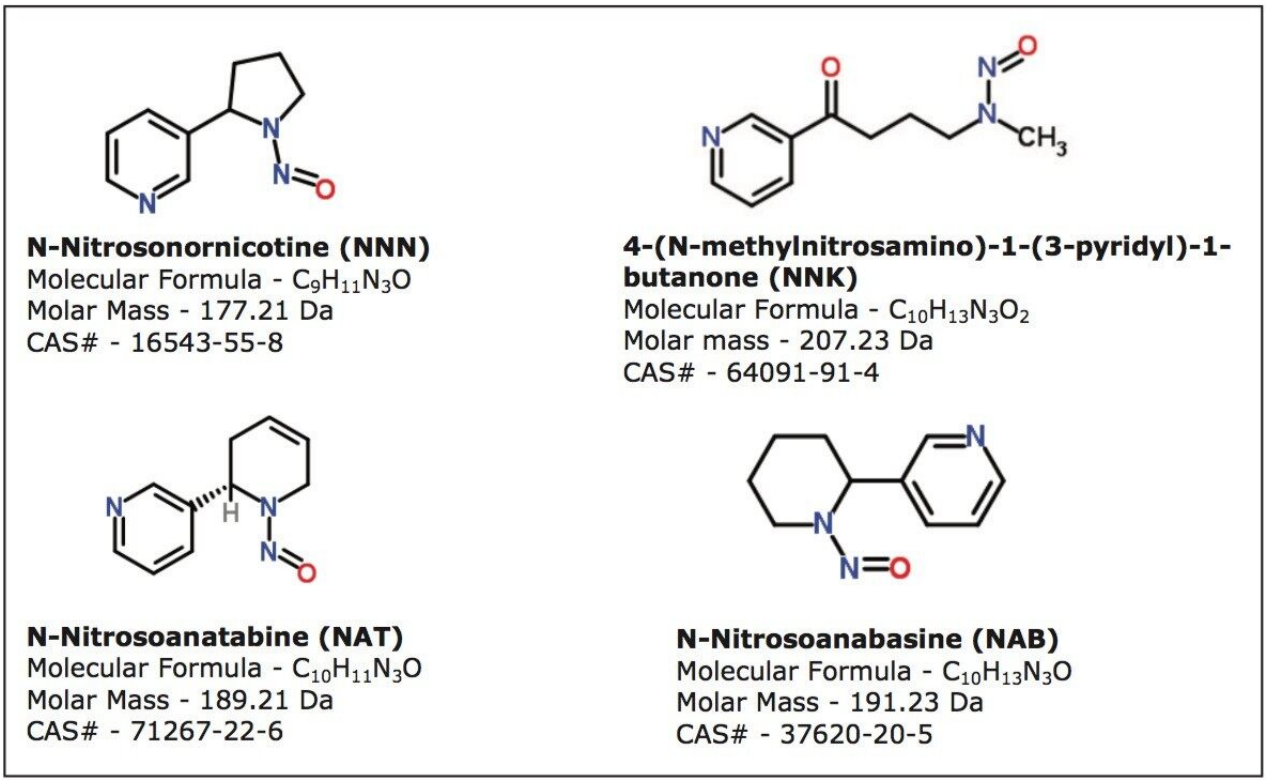

Figure 1. Structure and chemical properties of TSNAs.

Global tobacco product regulations require tobacco companies to disclose the contents of tobacco products, smoke emissions, and changes to their products. Several priority toxicants including TSNAs have been identified in tobacco and smoke emissions that need to be accurately measured and reported to regulatory bodies. The development of robust and reliable analytical methods for quantitative measurement of priority tobacco and smoke constituents is critical to global tobacco harm reduction initiatives.

Standardized methods for the analysis of TSNAs in tobacco products exist from CORESTA and ISO TC/126, developed through inter-laboratory collaborative studies.[2–4]

Experimental

UPLC conditions

UPLC system:	ACQUITY UPLC H-Class

Column:	ACQUITY UPLC BEH C_{18} 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

MRM transitions (Table 2) for TSNAs and labeled internal standards (NNN-D4 and NNK-D4), were optimized using IntelliStart and monitored in the analysis of calibration standards and sample extracts. The first and second MRM transitions of target analytes were used for quantification and confirmation, respectively. The data was acquired and processed using MassLynx Software with TargetLynx Application Manager.

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 MRM conditions for TSNAs and labeled internal standards using IntelliStart on the Xevo TQD.

TSNAs can be measured using gas chromatography with a thermal energy analyzer (GC-TEA), or liquid chromatography coupled to tandem quadrupole mass spectrometry (LC-MS/MS). LC-MS/MS methods for TSNA analysis provide significant advantages over GC-TEA as they offer high sensitivity, high selectivity and high sample throughput (simplified workflow).

In this application note, we describe a UPLC-MS/MS method for TSNA analysis in tobacco products that has

been developed based on the CORESTA standard (CRM-72), and provides further improvements in sensitivity, linear dynamic range, sample workflow, and overall analysis throughput. This method is applicable to the determination of TSNAs in tobacco and smokeless tobacco products including snus, moist snuff, dry snuff, and chewing tobacco.

Standards

TSNA reference standards (1 mg/mL) were purchased from Cerilliant (Round Rock Texas). Isotopically labeled internal standards (NNN-D4 and NNK-D4) were purchased from the Toronto Research Chemicals (Toronto, Canada). An intermediate stock solution consisting of 10 µg/mL of NNN, NNK, NAT, and 2.5 µg/mL of NAB, was prepared in acetonitrile and used to prepare calibration standards in the range of 0.25 to 128 ng/mL for NNN, NNK, NAT, and 0.0625 to 32 ng/mL for NAB. The calibration standards and internal standard spiking solution were prepared in 100 mM ammonium acetate in water. NNN-D4 was used as the internal standard for NNN quantification, while NNK-D4 was used as the internal standard for quantification of NNK, NAT, and NAB. For accurate quantification of TSNAs in different tobacco matrices, the use of all four labeled internal standards is recommended.

Tobacco samples

CORESTA Reference Products (CRP) for smokeless tobacco were provided by Nort Carolina State University (Raleigh, NC) including snus (CRP-1), moist snuff (CRP-2), dry snuff (CRP-3), and loose-leaf chewing tobacco (CRP-4). Cigarette tobacco was purchased from a local retail store.

Sample preparation

Recommendations on sampling and grinding tobacco products can be found in CRM-72.[3] The tobacco sample was weighed (1±0.05 gram) and extracted using 30 mL of 100 mM ammonium acetate in water. The amount of labeled internal standards was kept constant in the sample extracts and calibration standards to facilitate accurate quantification. The samples were shaken for 30 minutes on a hand motion shaker (Model EL680.Q, Eberbach Corporation, Ann Arbor, MI) at 350 rpm. Sample extracts were filtered using a 0.45-µm PTFE syringe filter and diluted 10-fold with 100-mM ammonium acetate in water prior to UPLC-MS/MS analysis.

Sample analysis

Method optimization studies were conducted to evaluate chromatographic separation and matrix effects using RADAR acquisition mode which enables simultaneous full-scan (MS) and MRM acquisitions. The method was also evaluated with respect to linearity, sensitivity, accuracy and precision. For linearity,

calibration curves were created using solvent standards in the range of 0.25 to 128 ng/mL for NNN, NNK, NAT, and 0.0625 to 32 ng/mL for NAB. The method sensitivity was evaluated using a diluted 0.05 ng/mL TSNA standard. Accuracy of the method was evaluated by determining recoveries for TSNAs from five different tobacco matrices, spiked (n=3) at 50 ng/g for NNN, NNK, NAT, and 12.5 ng/g for NAB.

Results and Discussion

Method optimization – RADAR (full scan MS and MRM)

To optimize the chromatographic separation for TSNAs and to improve the data quality, RADAR acquisition mode was utilized on Waters Xevo TQD 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. Two different mobile phase B solvents were evaluated: 10 mM ammonium acetate in methanol and 0.1% acetic acid in methanol. While both mobile phases provided improved chromatographic resolution for TSNA separation compared to the CORESTA standard CRM-72,[3] significant matrix suppression was observed for NNN using 10 mM ammonium acetate in methanol. The full scan data from RADAR revealed that the NNN peak co-eluted with the highintensity nicotine peak (Figure 2A). The use of 0.1% acetic acid in methanol as the mobile phase B improved separation between nicotine and TSNAs while minimizing matrix suppression for the NNN peak (Figure 2B).

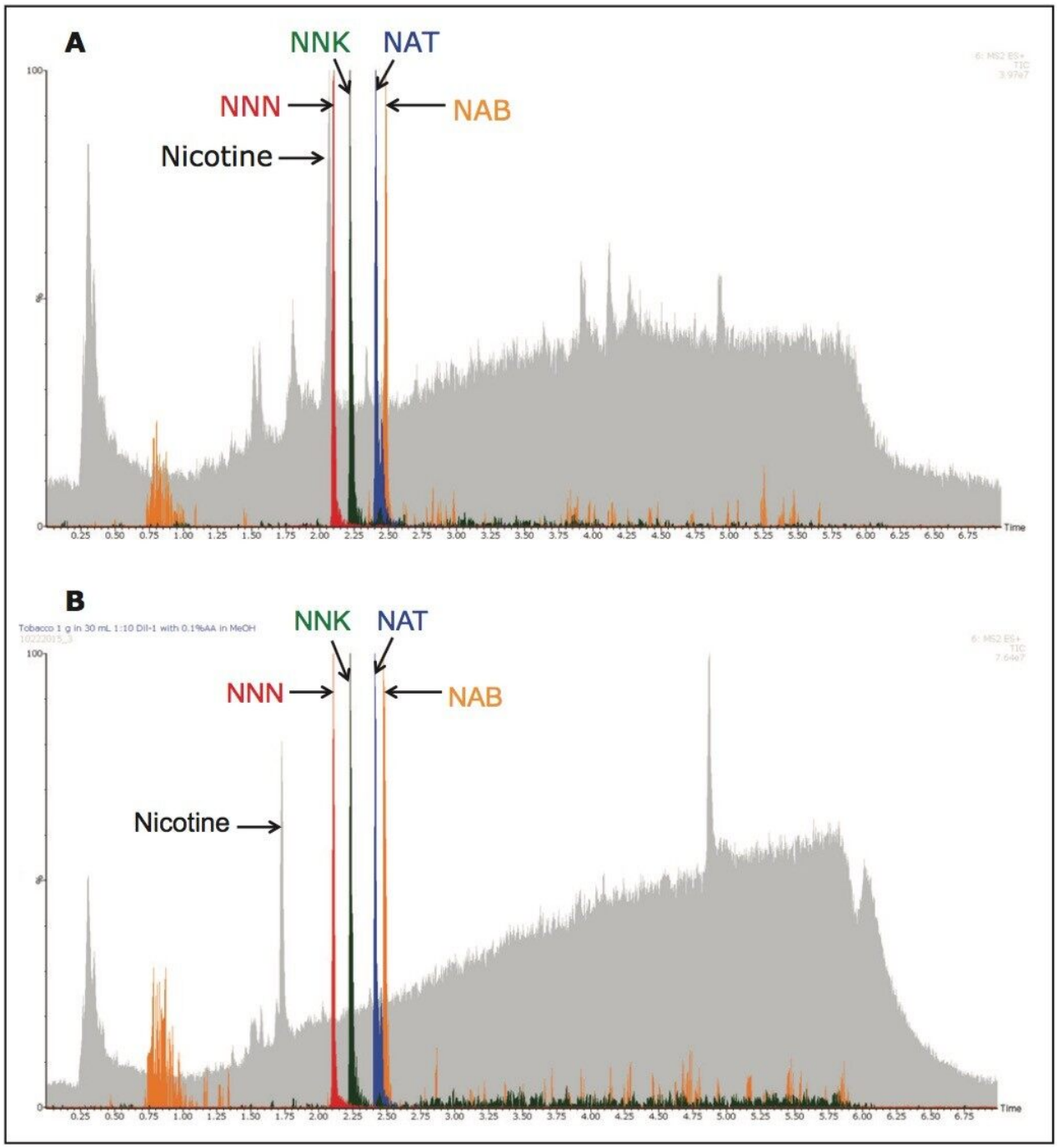

Figure 2A. Full scan TIC (in grey) and MRM chromatogram of TSNAs (in color) using 10 mM ammonium acetate in methanol as mobile phase B. Figure 2B. Full scan TIC (in grey) and MRM chromatograms of TSNAs (in color) using 0.1% acetic acid in methanol as mobile phase B.

The tobacco extracts (1 gram in 30 mL of 100 mM ammonium acetate in water) were analyzed directly and also with a 10-fold dilution to evaluate the method sensitivity for detection of TSNAs. This comparison

showed that there was adequate instrument sensitivity for the detection of TSNAs using 10-fold diluted tobacco extracts (data not shown). The advantage of the dilution step is that matrix effects are reduced and instrument maintenance and downtime is minimized. Alternatively, the CORESTA method[3] provides a sample cleanup procedure using Waters Oasis HLB SPE Cartridges to reduce matrix effects observed in TSNA analysis.

Linearity

The TSNAs showed excellent linearity with R^2 values >0.999 (Figure 3) for the 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 evaluated in this study exceeds the calibration range used in CRM-72 (0.5 to 100 ng/mL for NNN, NNK, NAT, and 0.125 to 25 ng/mL for NAB) and enables determination of TSNAs in different tobacco products using a single sample preparation procedure.

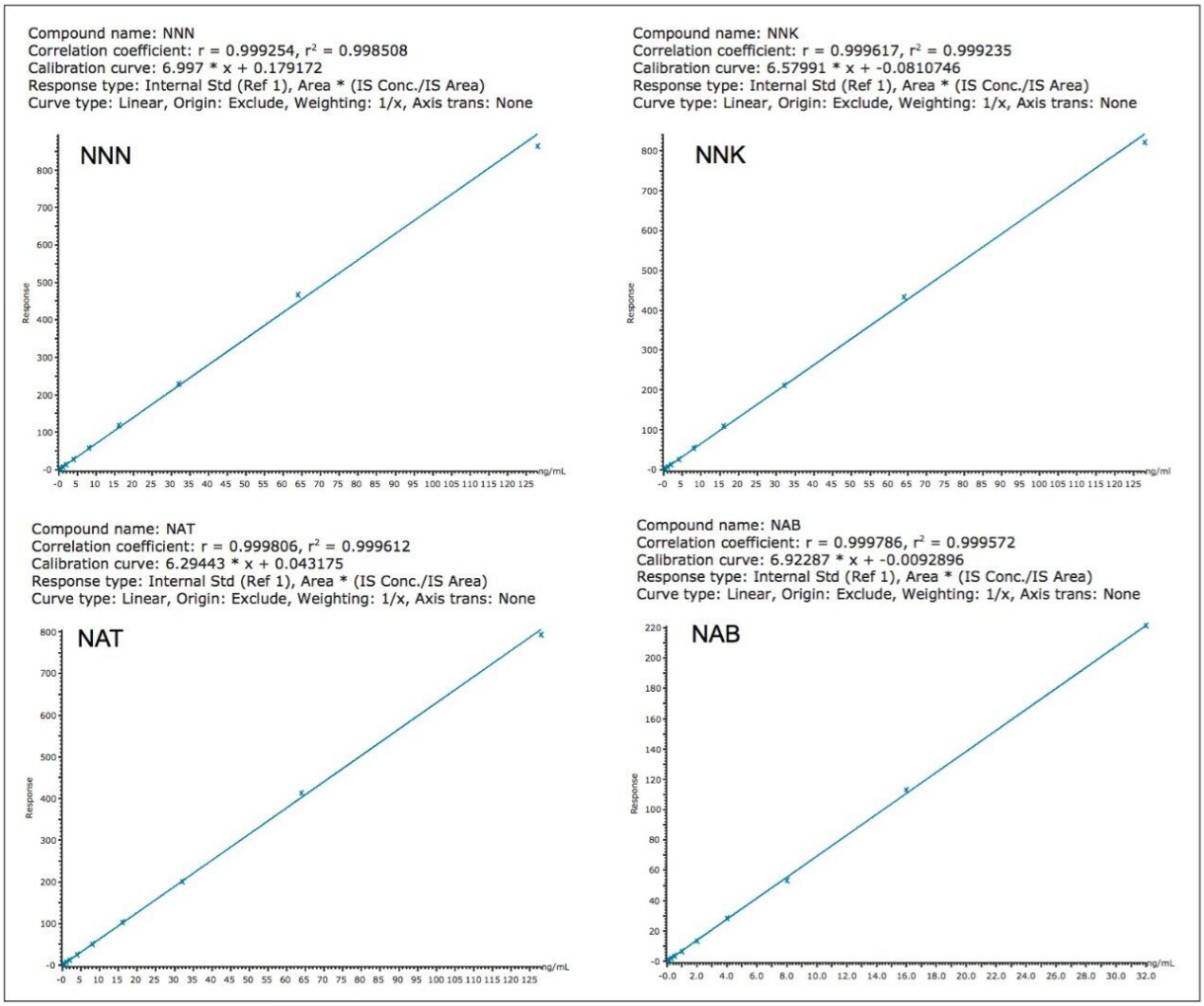

Figure 3. Calibration curves of TSNAs solvent standards.

Sensitivity

Method sensitivity was evaluated by determining analyte response in diluted TSNAs solvent standards, below the lowest calibration standard of 0.25 ng/mL for NNN, NNK, NAT, and 0.0625 ng/mL for NAB. As shown in Figure 4, the signal-to-noise (S/N) ratio calculated by peak-to-peak noise approach for a 0.05 ng/mL TSNA standard was >10 using a Xevo TQD System. The ability to detect and quantify TSNAs at such low levels allows users to inject diluted sample extracts to minimize ion suppression from tobacco matrix and eliminate the need for time-consuming SPE cleanup procedures.

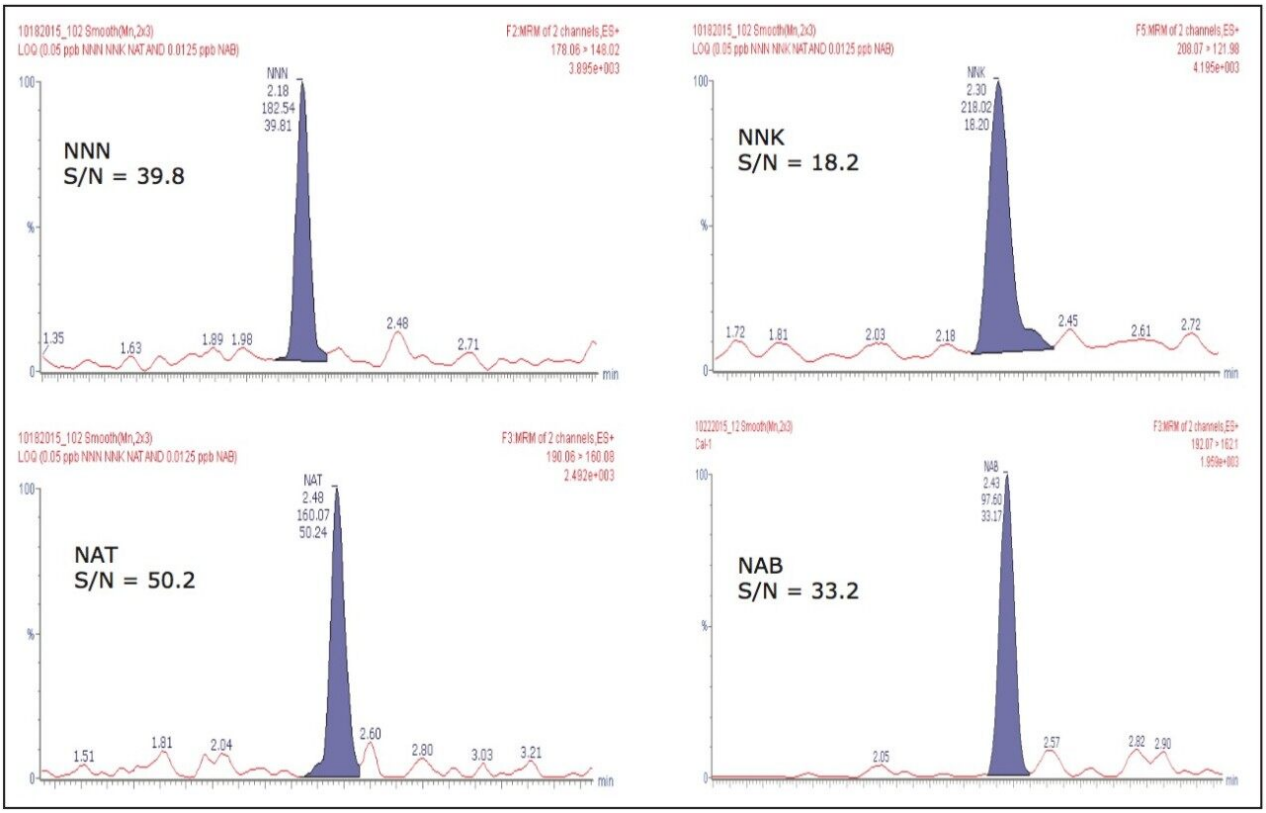

Figure 4. Signal-to-noise (S/N) ratio of diluted TSNA solvent standard (0.05 ng/mL) analyzed using the Xevo TQD.

Recovery

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 calculated recoveries for TSNAs after subtracting the incurred levels from tobacco matrices are shown in Figure 5. The recoveries for TSNAs were acceptable and ranged from 105% to 119% in the different tobacco matrices evaluated. The error bars represent percent relative standard deviation (%RSD) that ranged from 0.1% to 7.2%. NNN and NNK showed relatively lower mean recoveries of 109% and 108% from different tobacco matrices using respective isotopically labeled internal standards (NNN-D4 and NNK-D4). The mean recoveries for NAT and NAB were relatively higher (117% and 116%) as NNK D4, a structural analogue, was used as the internal standard for their quantification. For more accurate quantification in tobacco matrices, the use of all four labeled internal standards is recommended.

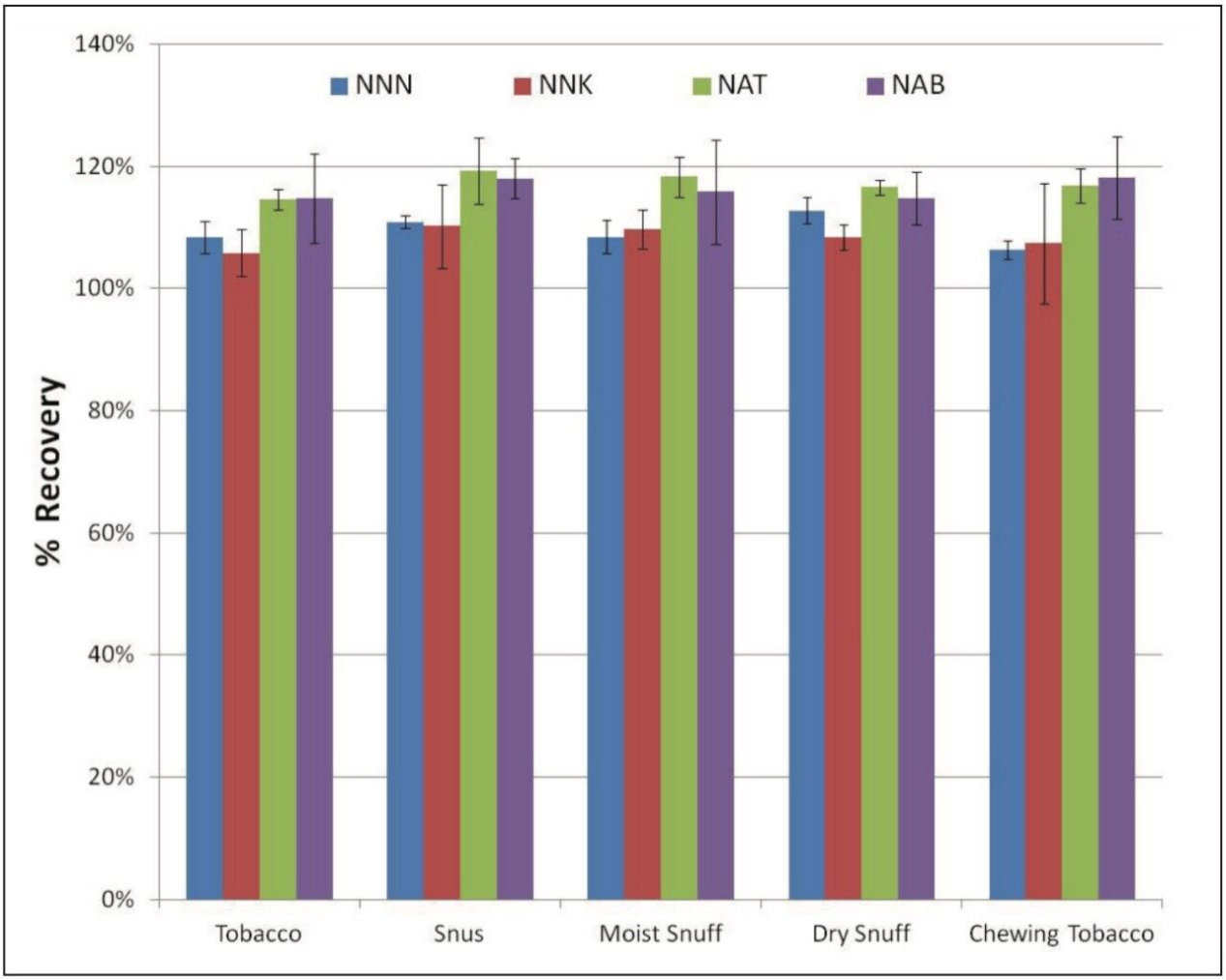

Figure 5. %Recovery of TSNAs from different tobacco product matrices.

Tobacco analysis

The raw tobacco and smokeless reference tobacco products were analyzed in triplicate following the optimized UPLC-MS/MS method. The results from the UPLC-MS/MS method were compared to results from HPLC-MS/MS methods used in the CORESTA inter-lab study (n=3 replicates, 11 labs) in which TSNA data was generated following CRM-72 (Figure 6). The TSNA yields from the UPLC-MS/MS method are within the range of CORESTA inter-lab study results. The error bars represent ± one standard deviation. As observed from the individual and total TSNA yields, the TSNA content varies significantly between the different tobacco products. The differences in TSNA yields among the various tobacco products are attributed to differences in tobacco blend, curing and processing methods, and storage conditions.

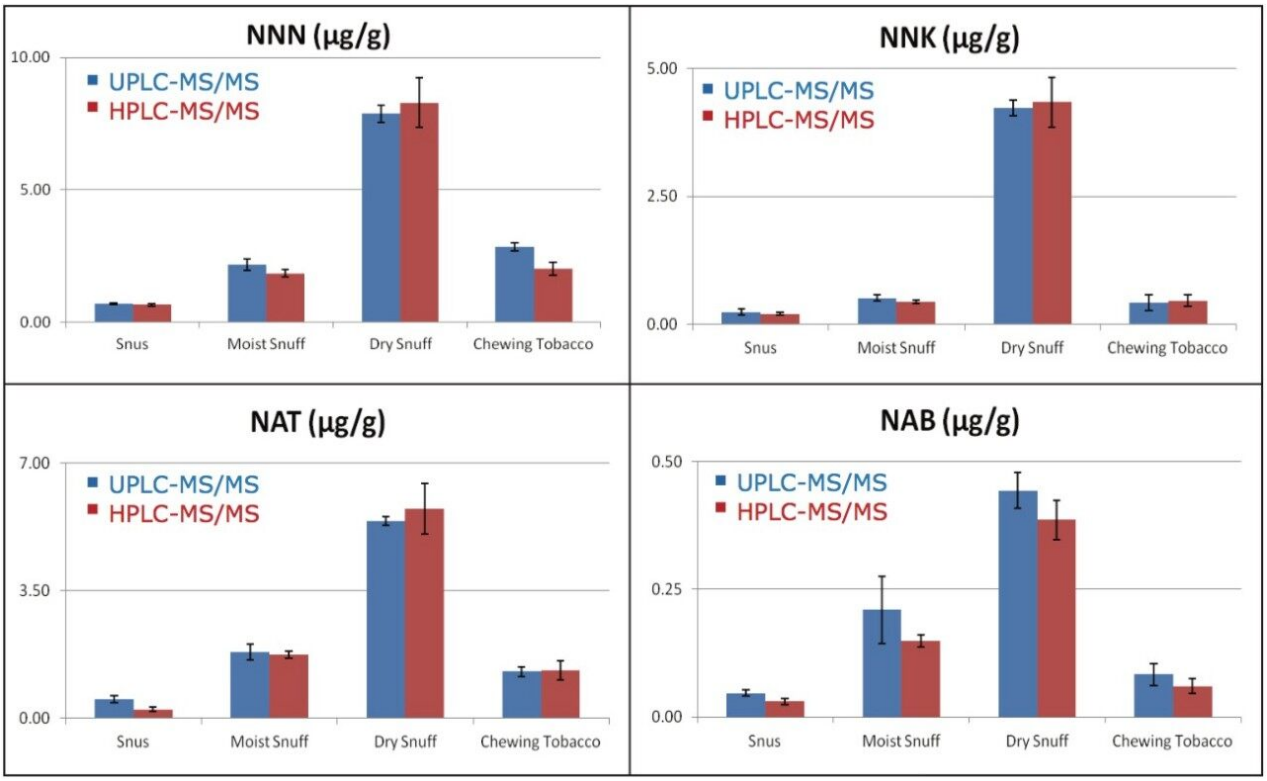

Figure 6. Comparison of TSNA yields from analysis of smokeless tobacco products using the UPLC-MS/MS method and the HPLC-MS/MS methods used in the CORESTA inter-lab study.[5]

Conclusion

A rapid, sensitive and robust UPLC-MS/MS method has been developed for the determination of TSNAs in tobacco and smokeless tobacco products. The UPLC-MS/MS method further improves the performance of industry standard CRM-72 by utilizing a UPLC column to improve chromatographic resolution and reduce analysis time. The key benefits of UPLC-MS/MS method for TSNA analysis are compared to HPLC-MS/MS based on CORESTA method in Table 3.

Method parameter	HPLC-MS/MS standardized method[3]	UPLC-MS/MS method	Key benefits
Calibration range	2–80 ng/mL for NNN, NNK, NAT and 0.125 to 25 ng/mL for NAB	0.25-128 ng/mL for NNN, NNK, NAT and 0.0625 to 32 ng/mL for NAB	Extended calibration range
Sample cleanup	SPE recommended	10-fold dilution	Simplified workflow
Injection volume	10 µL	5 µL	Reduced matrix load
Analytical column	XTERRA® MS C_{18}, 2.1 x 50 mm, 2.5 µm	ACQUITY UPLC BEH C_{18}, 2.1 x 50 mm, 1.7 µm	Higher chromatographic resolution
Analysis time	10 min	7 min	Reduced analysis time

Table 3. Key benefits of UPLC-MS/MS method for TSNA analysis compared to HPLC-MS/MS method.

The dilute and shoot approach for TSNA analysis eliminates the need for sample cleanup and minimizes instrument maintenance and downtime.

- This method is suitable for the determination of TSNAs in various tobacco products including snus, moist snuff, dry snuff, chewing tobacco, and raw tobacco.
- The UPLC-MS/MS method provides higher sensitivity, selectivity, and reduced analysis time (7 min) for the determination of TSNAs compared to CRM-72.
- RADAR helps understand sample complexity and leads to faster and robust method development.
- Dilution of tobacco extracts and use of a lower sample injection volume reduces matrix effects, and eliminates the need for SPE cleanup step without compromising data quality.

References

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3. *CORESTA Recommended Method No 72*. Determination of Tobacco-Specific Nitrosamines in Smokeless Tobacco Products by LC-MS/MS. July 2013.
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2008.

5. *CORESTA STS Technical Report:* Smokeless Reference Tobacco Product Analysis. 2014.

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