Tobacco Specific Nitrosamines and Nicotine Degradants: A Method for Combined Analysis in ENDS E-liquids and Aerosol by LC-MS/MS

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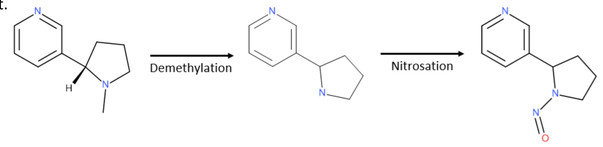
Introduction

Nicotine, glycerin, propylene glycol, and flavoring compounds comprise the bulk of ENDS products on the market today, and it is typical for these products to utilize US Pharmacopeia (USP) grade nicotine as their source. USP grade nicotine requires that single impurities are no more than 0.3% and total impurities to be no more than 0.8% of nicotine. However, once in the ENDS formulation or heated by a device, nicotine may generate nicotine related degradants that can add to the total and single impurities. Impurities that can be attributed to nicotine degradants per USP are nornicotine, myosmine, cotinine, anabasine, anatabine, nicotine-n-oxide, and β-nicotyrine. Other impurities of nicotine and its related compounds fall under a class of HPHCs called Tobacco Specific Nitrosamines (TSNAs) with the two most prominent TSNAs being NNN (n-nitrosonornicotine) and NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone).[1] These impurities and degradants include carcinogenic and respiratory irritants reportable to the FDA under Section 904(a)(3) of the Federal Food, Drug, and Cosmetic Act, and can vary depending on the tobacco source used to produce the USP grade nicotine. The levels of nicotine impurities and degradants present in the final product depend on several factors including purity of the nicotine, storage conditions, and age of the product.

Currently, published methods used for the quantitation of the TSNA and nicotine degradant compounds include either GC/MS or LC/MS as the instrumentation and separate the quantitation of TSNAs and nicotine degradants into multiple methodologies. We propose that utilizing LC-MS/MS instrumentation with the capabilities of MRM (Multiple Reaction Monitoring) analysis, quantitation of TSNA impurities and nicotine degradants can be combined and analyzed as one methodology. Having a combined analysis with the ability to obtain detection ranges equivalent to that of published methods adds to the extended benefit of increased throughput, waste reduction, reduced sample collection time, and direct comparison of related nicotine constituents all at the same time.

Methods

Nicotine degradants and TSNA compounds are intrinsically linked through their relation to nicotine and through their potential nitrosation reaction pathways. For example, nicotine can undergo demethylation to form nornicotine, a degradant product, which may then undergo nitrosation in the presence of nitrite to form NNN, a TSNA. Similar pathways exist for other TSNA compounds, and degradants associated with nicotine are also impurities derived from the tobacco plant.



In currently established methods, the quantitation of TSNA compounds or nicotine degradants utilize either GC/MS or LC/MS instrumentation, such as CORESTA Recommended Method No.72,[2] and separate the methodology of the two compound groups entirely. As proposed, we have utilized LC-MS/MS instrumentation with multiple reaction monitoring (MRM) to yield optimal selectivity and sensitivity for the combined analysis of TSNA and nicotine degradant compounds.

Leveraging key advantages of triple quadrupole (MS/MS) analysis with ultra high-pressure liquid chromatographic separation, parameters were found that maximized the reliable identification of analytes with little to no interference from co-eluting compound or matrix and allowed for accurate and reproducible quantitation. An Agilent Ultivo Triple Quadrupole mass spectrometer with a 1290 Infinity II UPLC fitted with a Waters Acquity BEH C18 2.1x100 mm. 1.8 µm column was used for method development and a hybrid approach of ICH and FDA guidance was used for the validation.

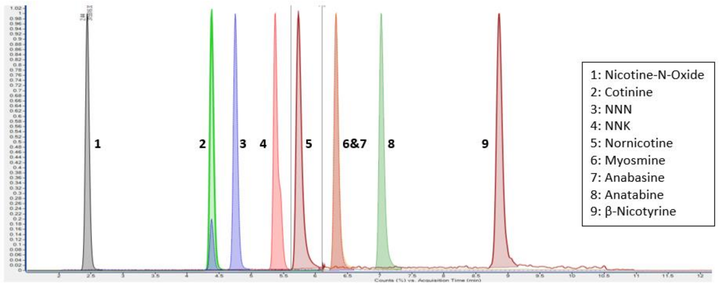
Time (min)	Flow (mL/min)	Mobile Phase A % (10 mM Ammonium Acetate pH10)	Mobile Phase B % (Methanol)
0.00	0.30	95	5
6.00	0.30	54	46
9.00	0.30	54	46
11.00	0.30	95	5
13.00	0.30	95	5

Parameter	Specification
Injection Volume	1 µL
Flow Rate (mL/min)	0.3 mL/min
Run Time	13 min
Autosampler Temp.	10°C
Column Temp.	25°C
Needle Wash/Seal Wash	50:50 ACN:DI H2O

Transitions	Window Start Time	Analyte	m/z Transitions	Frag. Voltage (V)	Collision Energy (V)
	1.5 min	Cotinine	177—80	50	25
		Cotinine-d3	180—80	50	25
		Nicotine-N-Oxide	179.2—130	50	25
		NNK	208.1—121.9	50	25
		NNK-d4	212.1—125.9	50	25
		NNN	178.1—119	50	25
		NNN-d4	182.1—123	50	25
		Nornicotine	149.1—80	50	25
		Nornicotine-d4	153.1—84	50	25
	6 min	Anabasine	163.1—90.9	50	35
		Anabasine-d4	167.1—96	50	35
		Anatabine	161.1—142	50	35
		β-Nicotyrine	159—143	50	35
		Myosmine	147—77.9	50	35
		Myosmine-d4	151—81.9	50	35

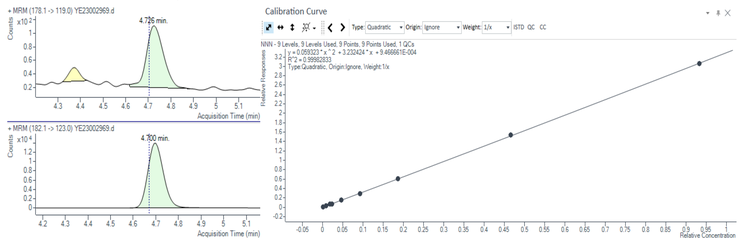
Results

A fit-for-purpose validation was performed in matrix for both e-liquid and aerosol analysis to show the performance of the combined method. Method results were obtained in the areas of accuracy, precision, detection limit, quantitation limit, linearity, and quantitation range.

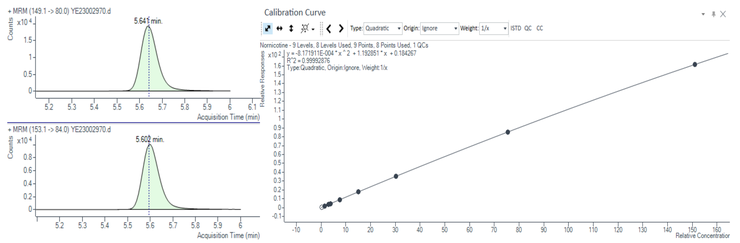


Combined chromatography of TSNA and nicotine degradant compounds. All compounds are resolved through UPLC separation except for Myosmine and Anabasine which are separated through MS/MS MRM detection.

NNN/NNN-d4 low standard peak and calibration curve



Nornicotine/Nornicotine-d4 low standard peak and calibration curve



For all compounds, it was found that for an appropriate analytical range for sample analysis a quadratic regression was the most suitable. With this calibration regression, the LOQ range was also assessed and found to show acceptable accuracy and %CV that is within 7% at the greatest for repeat injections.

Analyte	Type	R^2
Nicotine-N-Oxide	Quadratic	0.9963
Cotinine	Quadratic	0.9997
NNN	Quadratic	0.9998
NNK	Quadratic	0.9998
Nornicotine	Quadratic	0.9995
Myosmine	Quadratic	0.9990
Anatabine	Quadratic	0.9992
Anabasine	Quadratic	0.9991
β-Nicotyrine	Quadratic	0.9994

LOQ Analytes	Avg % Acc.	Avg S/N	% CV
Nornicotine	94%	3062	0.7%
Cotinine	94%	4638	0.7%
NNN	96%	172	2.3%
NNK	97%	278	2.5%
Nicotine-N-Oxide	83%	2995	1.7%
Myosmine	99%	775	2.2%
Anatabine	94%	886	2.0%
Anabasine	92%	386	3.3%
β-Nicotyrine	93%	108	6.5%

Accuracy determination for ENDS Aerosol Matrix

Aerosol Accuracy Tobacco	AVG Low Spike Amt (ng/mL)	AVG Low % Recovery	AVG Mid Spike Amt (ng/mL)	AVG Mid % Recovery	AVG High Spike Amt (ng/mL)	AVG High % Recovery
Nicotine-N-Oxide	83.7	115%	172.1	108%	197.2	105%
Cotinine	79.1	111%	167.4	105%	197.8	105%
NNN	21.0	106%	50.2	103%	59.1	101%
NNK	23.0	116%	54.6	112%	64.3	110%
Nornicotine	184.0	108%	272.4	105%	295.4	101%
Myosmine	105.5	101%	193.9	101%	225.1	103%
Anatabine	63.6	107%	152.5	104%	182.8	104%
Anabasine	62.0	104%	152.8	104%	184.2	105%
β-Nicotyrine	94.8	84%	176.1	89%	202.5	90%

Accuracy determination for E-liquid Matrix

E-liquid Accuracy Tobacco	AVG Low Spike Amt (ng/mL)	AVG Low % Recovery	AVG Mid Spike Amt (ng/mL)	AVG Mid % Recovery	AVG High Spike Amt (ng/mL)	AVG High % Recovery
Nicotine-N-Oxide	996.9	72%	1028.2	55%	813.6	103%
Cotinine	344.9	99%	421.9	101%	473.9	100%
NNN	21.3	106%	49.2	109%	118.2	107%
NNK	21.6	108%	50.6	112%	123.4	112%
Nornicotine	143.3	105%	221.0	104%	389.8	105%
Myosmine	301.5	101%	386.9	108%	462.3	103%
Anatabine	58.2	97%	136.4	101%	342.7	103%
Anabasine	57.1	95%	139.4	102%	340.2	102%
β-Nicotyrine	118.3	95%	194.4	98%	361.7	99%

Instrument Method Precision

Method Precision N=15	AVG % CV Across Precision
Nicotine-N-Oxide	2.9%
Cotinine	0.7%
NNN	1.4%
NNK	0.7%
Nornicotine	1.2%
Myosmine	2.2%
Anatabine	2.2%
Anabasine	2.3%
β-Nicotyrine	6.7%

Method Repeatability per matrix type

Analyte Repeatability	Tobacco %CV	Menthol %CV
Nicotine-N-Oxide	0.5%	2%
Cotinine	1%	0.4%
NNN	1%	0.5%
NNK	0.4%	0.3%
Nornicotine	1%	1%
Myosmine	1%	1%
Anatabine	1%	2%
Anabasine	1%	1%
β-Nicotyrine	3%	3%

As part of the combined method validation, accuracy and precision of the method were analyzed. Accuracy was shown to be within ±20% of the nominal matrix spiked value at three different spiking levels in both the aerosol and e-liquid matrix. The only deviation that was seen was for nicotine-n-oxide in the e-liquid matrix. In the samples analyzed, nicotine-n-oxide values were already in the upper portion of the calibration curve lending to a higher degree of variability between the matrix spiked sample possibly causing the low recovery for the low and mid spiking levels.

Method precision was observed to be within 10% CV across the e-liquid and aerosol matrix as repeat analysis of the same sample vial. An expanded precision was observed for aerosol when replicate devices were analyzed by ISO (55/3/30) and Intense (110/6/30) regimes. This was to be expected as the increase in the precision is directly correlated to the devices used for the analysis.

Method validation criteria as performed with a hybrid approach of ICH and FDA validation guidance overall showed results for acceptable accuracy, precision, LOQ, LOD, Linearity, and repeatability in the matrices of both e-liquid and trapped ENDS aerosol.

Conclusions

Utilizing LC-MS/MS instrumentation with the capabilities of MRM analysis, a combined method for the analysis of TSNAs and nicotine degradants in e-liquids and trapped ENDS aerosol was developed. The performance of this method was confirmed with both bulk e-liquid and collected aerosol utilizing a hybrid validation approach from both ICH and FDA validation guidance material. Electrospray Ionization (ESI) with a low initial fragmentation voltage of 50V generated a combined calibration curve of TSNA and nicotine degradant compounds with observed limit of quantitation (LOQ) values of 0.2 ng/mL and 30 ng/mL respectively, in line with current reportable limits. Overall, it is shown that through appropriate selection of analytical instrumentation and sample handling, a fit-for-purpose methodology for the analysis of TSNA and nicotine degradant compounds in both e-liquid and aerosol matrices is possible.

References:

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This study was funded by JUUL Labs, Inc.