Simultaneous Determination of Tobacco Alkaloids, TSNA, and Solanesol in Consumer Products using UPLC-ESI-MSMS

John R. Shifflett, Leah Watson, Devin J. McNally, and Dawit Z. Bezabeh

United States Department of the Treasury, Alcohol and Tobacco Tax and Trade Bureau (TTB)

6000 Ammendale Road, Beltsville, MD 20705 USA

Introduction

The U.S. Alcohol and Tobacco Tax and Trade Bureau (TTB) is responsible for collecting Federal excise taxes on tobacco products. Tobacco products in the U.S. may fall into several taxable categories including cigars, cigarettes, snuff, chewing tobacco, pipe tobacco and roll-your-own. The existence of these taxable categories means that the TTB is also responsible for the determination of proper tax classification. Not only does proper classification determine the amount of tax owed, comprehensive classification procedures must also determine if a consumer product is subject to the tobacco excise tax. Since a product must contain tobacco to be subject to the excise tax, laboratory methods that test for the presence of tobacco can provide useful information to ascertain taxable status of a product.

To test for the presence unique chemical markers in tobacco, an analytical method was developed that permits the simultaneous determination of nicotine and related alkaloids, tobacco specific nitrosamines (TSNA), and solanesol. Nicotine is the predominant pyridine alkaloid found in *Nicotiana tabacum* and *Nicotiana rustica*, the primary cultivated tobaccos. In these plants it accounts for more than 95% of the total alkaloid content and is highly concentrated in the leaf material [1,2]. Nicotine can be found at levels as high as 8% by weight but is typically observed in the 1 to 3% range in cured *Nicotiana tabacum* [3]. Typically, almost all of the remaining alkaloid content in cultivated tobacco is divided between cotinine, nornicotine, anatabine, and anabasine. Mysomine and isonicoteine are usually observed at low levels and, occasionally, nornicotyrine and nicotyrine can be detected.

The second group of compounds listed above, tobacco specific nitrosamines (TSNA), are chemicals known to form from nicotine alkaloids as tobacco leaves are subjected to the curing conditions typically used by the tobacco industry. Specifically, N-nitrosoanatabine (NAT), N-nitrosoanabasine (NAB), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and N'-nitrosonornicotine (NNN) form from anatabine, anabasine, nicotine, and nornicotine, respectively [4]. The last compound listed, solanesol, is an all-trans nonaprenol that is found in the plant kingdom but is notable in *Nicotiana tabacum* due to its high concentration in the leaf. Solanesol concentration tends to increase in the leaf as curing progresses and it is generally found at higher levels in flue-cured when compared to air-cured tobacco [3]. Additionally, solanesol content in *Nicotiana rustica* has been found to be low relative to various types of cured *Nicotiana tabacum* leaves [5].

The method described here has been optimized for the determination of the aforementioned tobacco alkaloids, TSNA, and solanesol in methanolic extracts of tobacco using UPLC-ESI-MS/MS in positive ion mode. While there is precedent in the literature for simultaneous multi-component analysis of the tobacco alkaloids and TSNA using LC-ESI-MS/MS [6], co-elution occurs between multiple analytes, particularly anabasine and nicotine. This is problematic because anabasine and nicotine are isobaric and ESI-MS/MS experiments reveal many common fragment ions [7]. For these two alkaloids, baseline separation is required for unambiguous identification of each species. Additionally, the analytical method was designed to attenuate the instrument response of nicotine, which is overwhelming, to permit simultaneous analysis of all analytes.

Materials

***Standards*:** Purchased from Toronto Research Chemicals (North York, ON, CAN).

***Reference Tobacco Products*:** Kentucky Reference Cigarette 3R4F was obtained from The University of Kentucky (Center for Tobacco Reference Products, Kentucky Tobacco Research & Development Center). Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) Monitor No. 7 (CM7) was produced in June 2010 at the Imperial Tobacco Group factory in Berlin, Germany.

Sample Preparation

All laboratory glassware used in the extraction of samples was cleaned with concentrated (28 – 30%) ammonium hydroxide followed by rinsing with high purity (18 MΩ) water and LC-MS grade methanol.

***Extraction blanks*:** These were prepared in the same 125 mL Erlenmeyer flasks as extracts of suspect and reference tobaccos. 20 mL of LC/MS grade methanol was added to each flask. These flasks were agitated on a wrist action shaker for 1 hour. A sample of each extract was filtered using a 0.2 µm PTFE syringe filter. Each filtered blank extract was then diluted to 20% methanol with high purity water.

***Sample extracts*:** Approximately 0.5 grams of each suspect and reference sample was extracted with 20 mL of LC/MS grade methanol in the same 125 mL Erlenmeyer flasks that were used to prepare the blanks described above. Each flask had a unique identification number to match the results of the analysis for each sample and blank extract. The agitation, filtration, and dilution steps described for the extraction blanks were also used to process the suspect and reference samples.

To ensure that the results were within the range of the UPLC-ESI-MS/MS, the processed blank and suspect sample extracts described above were further diluted 10-fold prior to analysis using a 20% solution of LC/MS grade methanol in high purity water.

UPLC-ESI-MS/MS Instrument Conditions

Ultra Performance Liquid Chromatography (UPLC)

Analyses were performed using a Waters I-Class UPLC coupled to a Xevo TQ-S triple quadrupole mass spectrometer using electrospray ionization. The column was a Waters Acquity UPLC BEH C18 column, 1.7 µm particle size, 2.1 mm X 50 mm. The column temperature was 30 °C. Mobile phase A was 10 mM ammonium formate in water buffered at pH 9.5. Mobile phase B was methanol with 10 mM ammonium formate. The injection volume was 2 µL. A segmented gradient was used to elute the analytes at a flow rate of 0.4 mL/min. Following injection, initial solvent conditions (80% mobile phase A) were maintained for 0.4 minutes. This was followed by two sequential linear gradient segments for 2.1 minutes to 70% mobile phase A and 1.5 minutes to 100% mobile phase B. The gradient segments were followed by am isocratic segment at 100% mobile phase B for 4 minutes. The column was then re-equilibrated at the initial conditions for 3.5 minutes.

Waters Xevo TQ-S Tandem Mass Spectrometer

Source parameters: Positive ion mode, capillary voltage 2.5 kV, source temperature 150 °C, desolvation temperature 500 °C, desolvation gas flow 800 L/Hr, cone gas flow 150 L/Hr.

Parameters for Multiple Reaction Monitoring (MRM) optimized from infusion analysis of standards are shown below:

compound	r.t. (min)	r.t. window (min)	$[M+H]^+$ (m/z)	product ion (m/z)	cone voltage (V)	collision energy (eV)	product ion ratio	LOD (ng/L)
cotinine	1.01	0.90 - 1.35	177.1	98.1	4	20	1.0	80
				146.1		16	0.2	
nornicotine	1.66	1.40 - 2.00	149.1	80.1	30	18	1.0	370
				132.1		12	1.0	
anatabine	2.37	2.25 - 2.75	161.1	107.0	4	14	0.1	880
				144.1		14	1.0	
myosmine	2.43	2.35 - 2.85	147.0	104.9	4	22	1.0	400
				130.1		20	0.6	
anabasine	2.82	2.60 - 3.25	163.1	118.0	4	22	0.9	780
				146.1		16	1.0	
isonicoteine	2.95	2.85 - 3.35	157.0	78.1	30	26	0.8	70
				130.1		22	1.0	
nicotine	3.32	3.15 - 3.65	163.1	106.1	80	16	0.9	1780
				132.1		14	1.0	
nornicotyrine	3.37	3.30 - 3.65	145.1	91.1	55	26	0.7	240
				118.0		20	1.0	
nicotyrine	3.59	3.40 - 3.85	159.0	117.2	50	26	0.8	770
				144.1		22	1.0	
NNN	1.22	1.15 - 1.50	178.0	105.1	4	16	0.3	190
				148.2		10	1.0	
NNK	1.64	1.55 – 2.00	208.1	122.1	4	10	1.0	140
				148.2		12	0.2	
NAT	2.37	2.30 - 2.80	190.1	106.1	4	16	0.6	280
				160.2		10	1.0	
NAB	2.66	2.60 – 3.05	192.1	133.1	4	22	0.5	380
				162.2		10	1.0	
solanesol	6.84	6.35 - 7.55	613.7 *	69.2	45	55	0.2	3720
				81.2		55	1.0	

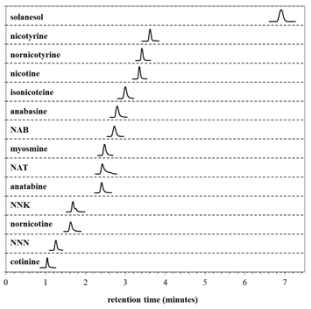
* $[M - H2O + H]^+$

Optimized Chromatography

Shown here are stacked total ion chromatograms (TIC) from an analysis of the 14 analytes described above. An important objective of this work was to avoid isobaric interferences by ensuring baseline separation of nicotine and anabasine. The stacked TIC's on the right result from the simultaneous analysis of the 14 standards from a single injection. The resolution (R_s) between nicotine and anabasine was greater than 5.

Another advantage of this method is the ability to study all analytes simultaneously by attenuating the peak intensity for nicotine. This was necessary because nicotine comprises > 95% of the total alkaloid content of cultivated tobaccos.

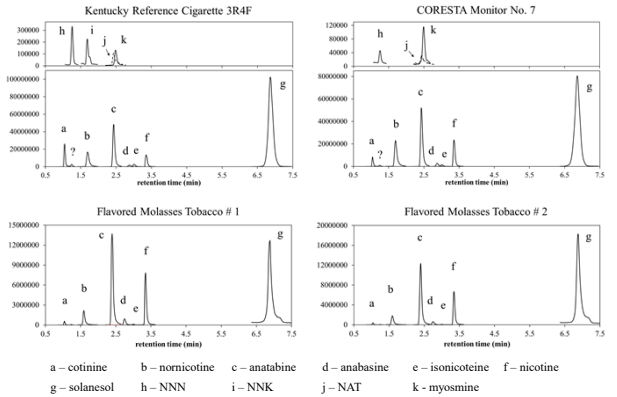
Solanesol is another abundant component of tobacco leaves. However, it did not ionize well under the conditions of the analysis and, as a result, did not require further manipulation to attenuate the peak intensity.



Results – Reference Cigarettes and Molasses Tobacco

A qualitative method has been developed for simultaneous determination 9 tobacco alkaloids, 4 TSNAs, and solanesol. This procedure has been used to detect and characterize the relative ratios of these 14 analytes in a group of tobacco products that includes reference cigarettes from the University of Kentucky and the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA). Future study is aimed at employing this method to determine the presence and relative ratios of these chemical markers in an expanded sampling of consumer products (both tobacco and non-tobacco) and cured tobacco leaves such as flue-cured, air-cured, oriental, and rustica.

The chromatograms shown below are overlays of total ion chromatograms taken from analyses of Kentucky and CORESTA reference cigarettes as well as two flavored molasses or "Hookah" water pipe tobaccos. An important feature of these plots is the peak intensity of nicotine (f) relative to the other analytes. Without attenuation of the nicotine response, it would be impossible to perform this analysis in a single run.



a – cotinine b – nornicotine c – anatabine d – anabasine e – isonicoteine f – nicotine
g – solanesol h – NNN i – NNK j – NAT k - myosmine

Acknowledgments

The authors would like to thank Dr. Abdul Mabud of the Alcohol & Tobacco Tax & Trade Bureau for his support of this research project.

References

1. Sisson VA, Severson RF (1990) Alkaloid composition of the Nicotiana species. Beitr. Tabakforsch. 14(6): 327-339
2. Saitoh F, Noma M, Kawashima N (1985) The alkaloid contents of sixty Nicotiana species. Phytochemistry 24(3): 477-480
3. Leffingwell, JC (1999) Basic chemical constituents of tobacco leaf and differences among tobacco types. In: Davis DL, Nielsen MT (eds) Tobacco – Production, Chemistry and Technology. Blackwell Science, Oxford, UK, pp 265-284.
4. Clayton PM, Cunningham A, van Heemst JDH (2010) Quantification of four tobacco-specific nitrosamines in cigarette filter tips using liquid chromatography-tandem mass spectrometry. Anal Methods 2: 1085-1094
5. Kotipalli KP, Rao NCV, Raj K (2008) Estimation of solanesol in tobacco and non-tobacco plants from the solanaceae family. J. Med Arom. Plant Sci. 30: 65-68
6. Li Y, Pang T, Shi J, Lu X, Deng J, Lin Q (2006) Simultaneous determination of alkaloids and their related tobacco-specific nitrosamines in tobacco leaves using LC-MS-MS. J Chromatogr Sci 53(10): 1730-1736
7. von Weymarn LB, Thomson NM, Donny EC, Hatsukami DK, Murphy SE (2016) Quantitation of the minor tobacco alkaloids nornicotine, anatabine, and anabasine in smokers' urine by high throughput liquid chromatography-mass spectrometry. Chem Res Toxicol 29(3): 390-397