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Development of a Quantitative Method for the Analysis of Tobacco-Specific Nitrosamines in Mainstream Cigarette Smoke Using Isotope Dilution Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry

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An improved method has been developed for the determination of the four major tobacco-specific nitrosamines (TSNA) in mainstream cigarette smoke. The new method offers decreased sample preparation and analysis time as compared to traditional methodologies. This method uses isotope dilution liquid chromatography coupled to a tandem mass spectrometer with electrospray ionization and is significantly more sensitive than traditional methods. It also shows no evidence of artifactual formation of TSNA. Sample concentrations were determined for four TSNA in mainstream smoke using two isotopically labeled TSNA analogues as internal standards. Mainstream smoke was collected on an industry standard 44-mm Cambridge filter pad, extracted with an aqueous buffer solution, and analyzed without further sample cleanup. This method has been validated through intra- and interlaboratory studies and has shown excellent recoveries, sensitivity, and repeatability. The limits of detection of each TSNA varied from 0.01 to 0.1 ng/mL, and the linear calibration range of the instrument in sample matrix spanned 0.5−200 ng/mL, which allowed for the determination of the TSNA levels in cigarettes with a wide range of deliveries. Data are also reported from two commercially available industry reference cigarettes and show excellent agreement and reproducibility over a six-month time period (n > 50).

Tobacco-specific nitrosamines (TSNA) are known components of tobacco products and tobacco smoke.[1−3] Four of the most commonly determined TSNA are *N*′-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N*′-nitrosoanatabine (NAT), and *N*′-nitrosoanabasine (NAB).[4] The determination of TSNA in cigarette smoke is of particular interest in tobacco chemistry as these compounds have been shown to be potent carcinogens in animal studies.[5]

Due to their carcinogenic potential, there is a trend in the tobacco industry to develop products with reduced levels of TSNA.[6] While there have been published methods for the determination of TSNA utilizing gas chromatography (GC),[7] high-performance liquid chromatography (HPLC),[8] gas chromatography/mass spectrometry (GC/MS),[9] and HPLC coupled to a thermal energy analyzer (TEA) detector,[10] the most widely used method [6] has been GC coupled with TEA detection.[11−13]

TEAs offer reasonable sensitivity and are nitroso-specific, which aids in selectivity. However, coeluting nitroso compounds cannot be differentiated with a TEA.[14] In addition, tobacco products with reduced levels of TSNA can challenge the sensitivity of the technique, especially for NAB and NNK.[6]

While the GC−TEA combination is commonly used for the determination of TSNA in mainstream cigarette smoke, the technique has several noteworthy disadvantages. Specifically, extensive sample cleanup involving liquid−liquid extractions and solid-phase extractions are necessary so as not to foul the GC

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(1) Hoffmann, D.; Adams, J. D.; Brunnemann, K. D.; Rivenson, A.; Hect, S. S. *Int. Agency Res. Cancer (IARC)* **1982**, 309−318 (Scientific Publications 41),.

(2) Hoffmann, D.; Brunnemann, K. D.; Webb, K. S. *IARC* **1983**, 69−83 (Scientific Publications 45).

(3) Adams, J. D.; O'Mara-Adams, K. J.; Hoffmann, D. *Carcinogenesis* **1987**, *8*, 729−731.

(4) Brunnemann, K. D.; Genoble, L.; Hoffmann, D. *Carcinogenesis* **1987**, *8*, 465−469.

(5) Hecht, S. S.; Hoffmann, D. *Cancer Surv.* **1989**, *8*, 273−294.

(6) Risner, C. H.; Reece, J. B.; Morgan, W. T. *The Determination of* Tobacco-Specific Nitrosamines In Tobacco; A Collaborative Investigation Of Current Methodology. 55th Tobacco Science Research Conference, Greensboro, NC, September 9, 2001.

(7) Hoffmann, D.; Hecht, S. S.; Ornaf, R. M.; Wynder, E. L. *Science* **1974**, *186*, 265−267.

(8) Hecht, S. S.; Ornaf, R. M.; Hoffmann, D. *Anal. Chem.* **1975**, *47*, 2046−2048.

(9) Munson, J. W.; Abdine, H. *Anal. Lett.* **1977**, *10*, 777−786.

(10) Hoffmann, D.; Adams, J. D.; Brunnemann, K. D.; Hecht, S. S. *Cancer Res.* **1979**, *39*, 2505−2509.

(11) Adams, J. D.; Brunnemann, K. D.; Hoffmann, D. *J. Chromatogr.* **1983**, *256*, 347−351.

(12) Begutter, H.; Klus, H.; Ultsch, I. *J. Chromatogr.* **1985**, *321*, 475−479.

(13) Djordjevic, M. V.; Brunnemann, K. D.; Hoffmann; D. *Carcinogenesis* **1989**, *10*, 1725−1731.

(14) Song, S.; Ashley, D. L., *Anal. Chem.* **1999**, *71*, 1303−1308.

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injection port and column. Furthermore, due to the limited sensitivity of the GC−TEA (LODs ~50−80 pg/injection),[15] the samples must be concentrated before injection.

A recent publication by Wu et al. described the determination of five TSNA by LC−MS/MS.[16] This LC−MS/MS method offers several advantages over the GC−TEA method. It has greater specificity, significantly lower limits of detection, and a greater linear dynamic range. However, the method described requires a liquid−liquid extraction, SPE sample cleanup, and sample concentration, which will undoubtedly limit sample throughput. In addition, the method has a low procedural recovery (75%) for NAT, and a correction factor must be applied to estimate the true concentration.

We have developed and validated a LC−MS/MS method that takes full advantage of the specificity and sensitivity of tandem mass spectrometry where sample preparation involves extraction of the Cambridge filter pad (CFP) on an orbital shaker followed by sample filtration. Furthermore, there is no evidence of TSNA artifact formation during the sample cleanup and concentration steps, as has been reported with other methods.[15]

EXPERIMENTAL SECTION

Caution. The work described involves the handing of hazardous agents and was therefore conducted in accordance with NIH guidelines for the Laboratory Use of Chemical Carcinogens.[17]

Reagents. Solvents included HPLC grade methanol and acetonitrile. Ammonium acetate was purchased from Aldrich (St. Louis, MO), and acetic acid was purchased from Mallinckrodt (St. Louis, MO). NNN, NNK, NAT, and NAB were purchased from Midwest Research Institute (Kansas City, MO). The deuterated standards, NNN-d_4, and NNK-d_4 were purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). The Kentucky Reference 1R4F and 2R4F research cigarettes were purchased from the University of Kentucky (Lexington, KY).

Apparatus. A 20-port linear smoking machine (Tri-City Machine Works, Chester, VA) was used as the smoking engine for puffing the cigarette. A leak tester (Catalog No. 80206390, KC Automation, Richmond, VA) was used to check the system for leaks prior to sample collection. A puff volume tester (Catalog No. 70241000, KC Automation) was used daily to set the desired puff volume. The MS trapping system consisted of a filter cassette holder that contained a 44-mm CFP (part 80202851, Borgwaldt Tecknik, Hamburg, Germany).

Two different LC−MS/MS systems were used for this experiment work: (system 1 Shimadzu HPLC system consisting of a LC-10Advp twin pumping unit, SCL-10Avp system controller, LC-10Advp degasser, and CT0-10Avp column oven (Kyoto, Japan). A CTC Analytics HTC PAL autosampler was used for sample introduction (Carrboro, NC). The HPLC was interfaced to a Micromass Quattro Ultima triple quadrupole mass spectrometer (Milford, MA). Separation was achieved using a Waters Xterra C_{18} (2.1 × 50 mm i.d. 2.5 μm) column (Milford, MA). (system 2) Agilent 1100 HPLC system consisting of a 1100 capillary LC pump, 1100 Series microvacuum degasser, and a heated column compartment. A CTC Analytics HTC PAL autosampler was used for sample introduction (Carrboro, NC). The HPLC was interfaced to a Micromass Micro triple quadrupole mass spectrometer. Separation was achieved using a Waters Xterra C_{18} (3.0 × 50 mm i.d. 3.5 μm) column.

Table 1. Typical MS Operating Conditions

capillary voltage	3.5 kV
cone voltage	35 V
desolvation temperature	450 °C
source temperature	130 °C
desolvation gas	~700 L/h
cone gas	~150 L/h
collision gas	~2.5 mTorr
collision energy	10 V

Table 2. Parent/Daughter Ions

analyte	precursor ion (m/z)	product ion (m/z)
NAB	192	162
NAT	190	160
NNK	208	122
NNN	178	148
NNK-d_4	212	126
NNN-d_4	182	152

Smoke Collection. The cigarettes used for this study were conditioned and smoked following the Federal Trade Commission (FTC) smoking protocol.[18] The FTC smoking protocol specifies that cigarettes be puffed with a 35 ± 0.2 mL puff volume with one puff every 60 s and a 2-s puff duration. Five cigarettes were smoked for each sample. As specified by the FTC protocol, the CFP and test cigarettes were conditioned for a minimum of 24 h at 23.9 ± 1 °C and a humidity of 60 ± 2% before use. All samples were smoked to a butt mark of 3 mm past the tipping paper overwrap.

Sample Preparation. The mainstream smoke samples were prepared by first removing the CFP from the pad holder assembly, folding the pad with the clean surface of the pad facing outward, and wiping any residual smoke condensate from the inner surface of the holder. Next, 100 μL of the internal standard solution (NNN-d_4, NNK-d_4) was added to the CFP, which was then transferred to a 50-mL amber flask. Subsequently, 10 mL of the extraction solution (100 mM ammonium acetate) was added to the flask containing the CFP, and the flask was agitated on a wrist action or an orbital shaker for 30 min. Finally, the sample was filtered into an autosampler vial using a syringe filter.

Sample Analysis. Sample analysis was carried out using LC−MS/MS. The analytical run time for each sample was 7 min at a flow rate of 0.2 mL/min for the 2.1 mm × 50 mm column and 0.5 mL/min for the 3.0 mm × 50 mm column. HPLC separation was achieved by employing a gradient using 0.1% acetic acid in water and methanol as mobile phases. The column was held at a temperature of 65 °C. Typical MS parameters are shown in Table 1. Precursor and product ions are shown in Table 2.

Standard stock solutions were prepared by weighing the appropriate commercially available analyte and dissolving with acetonitrile. Calibration standards were prepared in the range of

(15) Caldwell, W. S.; Conner, J. W. *J. Assoc. Off. Anal. Chem.* **1990**, *73*, 783−789.
(16) Wu, W.; Ashley, D. L.; Watson, C. H. *Anal. Chem.* **2003**, *75*, 4827−4832.
(17) *NIH Guidelines for the Laboratory Use of Chemical Carcinogens*; NIH Publication 81-2385, 1981.
(18) *Fed. Regist.* **1967**, *32*, 174, 11178.

2−200 ng/mL for all TSNA except for NAB (0.5−50 ng/mL) by dilution of the standard stocks with a mixture of 8% acetonitrile in extraction solution.

Artifact Formation. A separate smoking run was conducted to determine the extent, if any, of artifact formation of the four TSNA during sample collection. Five CFP were treated with 40 mg of ascorbic acid dissolved in 2 mL of methanol.[16] The treated CFPs were allowed to dry and then conditioned for 24 h at 23.9 ± 1 °C and a humidity of 60 ± 2% before use; the five control sample CFPs were also conditioned for 24 h at 23.9 ± 1 °C and a humidity of 60 ± 2% before use. Ten smoke samples were collected on five treated and five untreated CFPs during the same sample collection run. Samples were extracted as described in the Experimental Section.

Data Analysis. All chromatographic data were processed using the Masslynx 3.5 or 4.0 software package with Quanlynx. All chromatographic peaks were reviewed, and any integration corrections were made manually, when necessary. Calibration curves were prepared using a linear regression with $1/X$ weighting. All standard and sample concentrations were determined using internal standard areas versus analyte areas. The statistical analysis of the data generated in the artifact formation experiment was performed using Statistical Analysis System (SAS) software (SAS Institute, Cary, NC). The ANOVA function was used to compare the data sets.

RESULTS AND DISCUSSION

Method Development. Several different solutions were initially evaluated for extraction of the smoke samples. Buffer solutions with and without methanol were evaluated for their TSNA extraction efficiency. In general, we found that acidic aqueous solutions or solutions containing methanol adequately extracted the TSNA from the CFP within 30 min. Ammonium acetate, formic acid, and acetic acid all gave acceptable recoveries of the TSNA from the CFP. However, samples extracted with formic acid-, acetic acid-, or methanol-containing solutions all either exhibited poor chromatography for NNN or suppressed the ionization of the TSNA. Ammonium acetate had an additional benefit in that it selectively extracted the TSNA from the CFP from the bulk of the smoke sample. This resulted in a relatively clean sample that allowed for analysis of 500−1000 samples before the MS required cleaning. During the development of the separation of TSNA from the smoke matrix, an elevated temperature was found to give improved peak shape for NNN. The Waters Xterra C_{18} column was chosen based on its ability to operate at 65 °C for extended periods of time and its ability to produce an acceptable separation of the TSNA. The calibration range of the instrument was found to be linear up to 800 ng/mL with standard solutions; however, the sample matrix was found to cause progressively worse suppression of ionization at concentrations exceeding 12.5 mg of total particulate (TMP)/mL of extraction solution. In the presence of the smoke sample matrix, the upper limit on the linear range was found to be ∼200 ng/mL for NNN, NNK, and NAT. For samples outside of this range, the authors recommend increasing the volume of the extraction solvent to 20 mL.

Method Validation. This method was validated at two separate laboratories. Laboratory A validated the method using the Micromass Ultima with a 2.1 × 50 mm HPLC column and the 1R4F reference cigarette. Laboratory B validated the method using the Micromass Micro with a 3.0 × 50 mm HPLC column and the 2R4F reference cigarette. Each laboratory used the same sample collection and extraction procedures.

Figure 1 shows the chromatography obtained from a mainstream smoke sample obtained from the 2R4F cigarette. These data were collected by laboratory A using the Micromass Ultima with a 2.1 × 50 mm HPLC column. Excellent peak shape was achieved for all the compounds with minimal interference from the smoke matrix. Calibration curves were linear over the quantitation range with R^2 values of >0.998. Similar chromatography and calibration results were also collected by laboratory B.

Since there is currently no smoke matrix available that lacks TSNA, an estimation of method recovery was performed using blank matrix spikes. For this recovery experiment, a blank CFP was spiked, in triplicate, with a known amount of each TSNA at three separate concentrations. The concentrations of blank matrix spikes were chosen to cover the expected values in the 1R4F and 2R4F cigarettes. (NNN is present in the 1R4F cigarette samples at ∼45 ng/mL and accordingly, the blank matrix spikes were prepared at about 21, 32, and 62 ng/mL. These CFPs were extracted, prepared, and analyzed as described above. The recovery was determined by calculating the mean of the experimentally determined amount and dividing by the nominal amount. Data for these experiments are presented in Table 3. Results were excellent and ranged between 97 and 102% recovery.

Method accuracy was evaluated by completing laboratory-fortified matrix spikes in triplicate, with both the 1R4F and 2R4F smoke extracts. This experiment was performed by taking two aliquots of the same prepared smoke sample and then spiking one of the aliquots with a known concentration of each TSNA. The concentrations of fortified matrix spikes were chosen to approximately double the TSNA concentrations in the smoke samples and to prepare fortified matrix spikes at the high limit of the calibration range. (Both the spiked and unspiked samples were analyzed to determine the recovery of the spike. These data are displayed in Table 4. Percent spike recovery was excellent with all values within 100 ± 10%. These results indicate a lack of any significant matrix effect for the determination of the selected TSNA.

Precision was evaluated both for intra- and interassay measurements by analyzing five replicates of a 1R4F and a single 2R4F smoke extract on three separate days. The precision for the intraassay samples was determined by analyzing the same sample five times on three separate days ($n = 15$). The precision for the interassay samples was determined by the analysis of 15 1R4F samples, 5 of which were collected on three separate days. Data for the 2R4F and 1R4F cigarettes were collected in separate laboratories and are shown in Table 5.

Limits of detection (LODs) were estimated for each compound from the slope of the linear calibration curve and the standard deviation at zero concentration S_0.[19] The LOD was calculated at $3S_0$. The LODs are shown in Table 6. The LOD on a per cigarette basis was calculated using the number of cigarettes collected per sample ($n = 5$), the final volume (10 mL), and any detectable response in sample blanks. In all cases, the LODs were significantly below the levels determined in the test samples.

(19) *Fed. Regist.* **1997**, *62*, 96, 27463−27467.

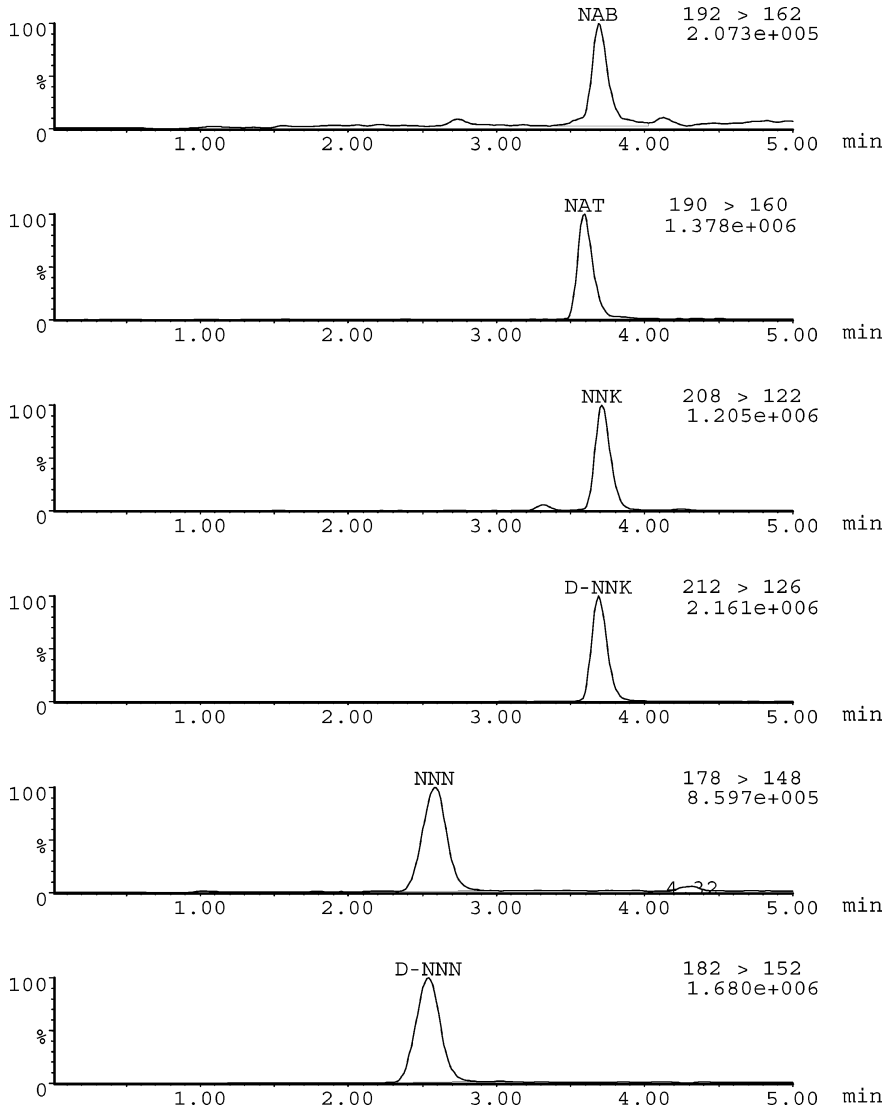

Figure 1. Typical multiple reaction monitoring chromatograms of TSNA in the 2R4F reference cigarette.

Table 3. Recovery of Each TSNA from a Blank CFP at Three Concentrations

analyte	spiked (ng/mL)	calculated (ng/mL)	recovery (%)
NNN	20.78	20.47	98.5
	31.17	31.73	101.8
	62.34	60.62	97.2
NNK	20.76	20.35	98.0
	31.14	30.16	96.8
	62.28	60.90	97.8
NAB	5.42	5.53	102.1
	8.12	8.22	101.2
	16.25	16.38	100.9
NAT	20.88	21.18	101.4
	31.32	30.42	97.1
	62.64	60.13	96.0

Table 4. Method Accuracy for Laboratory-Fortified Matrix Spikes in Smoke Extracts at Two Spike Concentrations

analyte	spiked (ng/mL)	recovered (ng/mL)	accuracy (%)
NNN	41.56	43.56	104.8
	140.0	131.0	93.6
NNK	41.52	41.02	98.8
	140.0	129.0	92.1
NAB	10.83	10.52	97.2
	35.00	33.00	94.3
NAT	41.76	39.53	94.7
	140.0	132.0	94.3

Artifact Formation. Artifact formation of TSNA during sample collection has been investigated by several groups.[15,16,20,21] It has been reported that the formation of artifactual nitrosamines can be reduced with the addition of ascorbic acid to the sample collection system.[15,21,22] For these studies, the extent of the artifactual TSNA formation was determined by measuring the percentage decrease in TSNA concentration with the addition of ascorbic acid versus an untreated sample. Using a series of impingers followed by an untreated CFP, Fisher and Spiegel-

(20) Brunnemann, K. D.; Hoffmann, D. *Rec. Adv. Tob. Sci.* **1991**, *17*, 71−112.
(21) Fisher, S.; Spiegelhalder, B. *Beitr. Tabakforsch.* **1989**, *14*, 145−153.
(22) Mirvisch, S. S.; Wallcave, L.; Eagen, M.; Shubik, P. *Science* **1972**, *177*, 65−67.
(23) Chen, P. X.; Moldoveanu, S. C. *Beitr. Tabakforsch. Int.* **2003**, *20* (70), 448−458.

Table 5. Method Precision for the 1R4F and 2R4F Smoke Extracts

analyte	sample	intraassay[a] (% CV)	interassay[b] (% CV)
NNN	1R4F	4.7	6.7
	2R4F	2.9	8.5
NNK	1R4F	3.4	5.8
	2R4F	2.5	5.8
NAB	1R4F	3.4	7.3
	2R4F	2.7	5.1
NAT	1R4F	2.9	6.9
	2R4F	3.3	5.9

[a] Replicate analysis of same sample, five times a day for 3 days (n = 15). [b] Replicate analysis of separate samples, five times per day for 3 days (n = 15).

Table 6. Instrument and Sample LOD of Each TSNA

analyte (ng/mL)	LOD[a] (ng/cigt)	LOD[a,c] (ng/mL)	LOD[b] (ng/cigt)	LOD[b,c]
NNN	0.05	0.1	0.1	0.2
NNK	0.03	0.06	0.03	0.06
NAB	0.02	0.04	0.04	0.08
NAT	0.02	0.04	0.04	0.08

[a] Micromass Ultima with 2.1 × 50 mm i.d. 2.5 μM column. [b] Micromass Micro with 3.0 × 50 mm i.d. 3.5 μM column. [c] Calculated using five cigarettes and 10 mL final volume.

halder[21] found that artifact formation of NNN could be reduced by over 100% if an ascorbic acid buffer solution was used in the impingers instead of cyclohexane. They also reported that when fresh mainstream smoke was collected directly on untreated CFPs they did not detect any artifact formation. However, a similar impinger trapping system was used by Caldwell and Conner,[15] and they reported decreases of 83, 38, 27, and 19% for NAB, NAT, NNK, and NNN, respectively, occurred if the CFP was pretreated with ascorbic acid. Wu et al.[16] reported a combined TSNA decrease of 25−35% when samples were collected on ascorbic acid-treated CFP compared to samples collected on untreated CFPs.

With our assay, we did not detect a difference in the four selected TSNA with or without pretreatment of the CFP with ascorbic acid. To investigate this observation, we conducted an experiment in which samples were collected (n = 5) on treated and untreated CFPs during the same collection event. This experiment was conducted by treating CFPs with a solution of ascorbic acid dissolved in methanol and then allowing the CFPs to dry before equilibration. Samples collected on treated and untreated pads were prepared as described in the Experimental Section. The results are presented in Table 7 and show no statistical difference between samples collected on treated or untreated CFPs.

Historical Data. The two reference cigarettes (Kentucky 1R4F and 2R4F) used for method validation are also used in our laboratories as method control samples during the routine analysis of test samples. Cigarettes are man-made products derived from natural materials and, therefore, have inherent variability. Method control data have been collected over a period of 24 months in laboratory A (n > 100) and 12 months in laboratory B (n > 50). With this large amount of data, it may be possible to estimate the true TSNA content for each of the reference cigarettes. The historical method control data are presented in Table 8 along with data from other published sources. The literature values for the TSNA concentrations in the 1R4F reference cigarette are widely varied for NNN and NNK and do not allow for direct comparison with our data, but our values for NAT and NAB are in good agreement with other reported values.

Table 7. Investigation Artifact Formation of Each TSNA during Sample Collection from a 2R4F Cigarette

		concentration (ng/cigt)			
		NNK	NNN	NAT	NAB
treated CFP	average	133.7	124.6	113.8	17.0
	SD (n = 5)	1.6	5.3	4.1	0.2
control	average	133.6	127.2	120.0	17.9
	SD (n = 5)	4.1	7.4	6.6	2.0
	Pr > F	0.97	0.54	0.11	0.37

Table 8. Comparison Values for TSNA in the 1R4F and 2R4F Reference Cigarettes

TSNA Values in for this LC−MS/MS Method (n > 50)

		sample (ng/cigt)			
		NNN	NNK	NAT	NAB
1R4F[a]	average	89.5	107	109	17.4
	SD	8.3	12	11	1.9
2R4F[b]	average	155	134	122	15.3
	SD	17	10	8	2

Literature Values for TSNA in Mainstream Smoke

	method	sample (ng/cigt) NNN	NNK	NAT	NAB
1R4F	various[c]	107	91	122	19
	LC−MS/MS[d]	91	54	96	12
	GC-TEA[e]	90	81	111	12
2R4F	various	133	116	119	16

[a] Micromass Ultima with 2.1 mm × 50 mm HPLC column. [b] Micromass Micro with 3.0 mm × 50 mm HPLC column. [c] Reference 23. [d] Reference 16. [e] Reference 15.

CONCLUSION

We have developed a rapid, quantitative, and reliable method for the determination of TSNA in mainstream cigarette smoke. This method takes advantage of the resolving power and selectivity of tandem mass spectrometry, which makes it possible to utilize a quick and simple sample preparation procedure. The method has been shown to produce excellent recoveries for the four major TSNA in solvent and matrix spikes as well as excellent reproducibility.

While there are several methods for the determination of TSNA in mainstream smoke cited in the literature, all of these methods involve extensive sample preparation steps that severely limit throughput. For these procedures, the sample is typically collected on a CFP and then either extracted with an aqueous solution followed by a liquid−liquid extraction into an organic solvent or direct extraction of the CFP with an organic solvent. Due to a lack of sensitivity, the organic solvent is usually concentrated prior to solid-phase extraction.

We found no evidence for the formation of artifact TSNA with the use of our analytical procedure. While artifact formation has

been reported in the literature, we believe that the improvements made in our sample collection and preparation procedures may explain the lack of TSNA artifact formation in our results.

The two laboratories that took part in this study have successfully used this method for four (laboratory A) and two years (laboratory B), respectively. The two Kentucky reference cigarettes are routinely run in each laboratory as method control samples. The data presented in Table 8 represent the analysis of >50 individual samples collected over at least a 12-month period. These data demonstrate the ruggedness of this test method and, due to the size of the population, can also be used to estimate the range of TSNA concentrations in the 1R4F and 2R4F reference cigarettes.

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