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Determination of four tobacco-specific nitrosamines in mainstream cigarette smoke by gas chromatography/ion trap mass spectrometry

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A specific and sensitive method was developed and validated to quantitatively analyze four tobacco-specific nitrosamines (TSNAs) in the particulate phase of mainstream cigarette smoke. Cigarette smoke particulate matter was collected according to ISO 4387. The particulate matter was extracted with acetic ether, cleaned up with a Supelclean ENVI-Carb silod-phase extraction (SPE) cartridge, concentrated under the protection of nitrogen and analyzed by gas chromatography (GC)/ion trap mass spectrometry (MS) with a very-low-flow-loss column (VF-17 MS) in MS^n mode using N-nitrosopentyl-3-picolylamine (NNPA) as an internal standard. TSNAs were identified by chromatographic retention time, matching the spectra of the standards and the samples and the consistency of the ratio of the abundance of the ions detected in the standards and the samples. Limits of detection of each TSNA varied from 0.01 to 0.06 ng/cig. A linear calibration range for this method is large enough to measure TSNA levels in cigarette smoke. The recovery of each TSNA was from 91.5 to 102.7%. The method achieved excellent reproducibility (RSD: 1.8–4.8% for intra-assay, 3.4–7.1% for inter-assay). It also shows no evidence of artifact formation. This method is very suitable for the routine detection of TSNAs in mainstream cigarette smoke. Copyright © 2007 John Wiley & Sons, Ltd.

Tobacco-specific nitrosamines (TSNAs) are well-known carcinogenic components in tobacco and tobacco smoke.[1–3] The most commonly determined TSNAs are *N*′-nitrosonornicotine (NNN), *N*′-nitrosoanatabine (NAT), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosoanabasine (NAB).[4] Because of health implications and the difficulties in the determination of TSNAs, there is long-standing interest in the accurate analysis of TSNAs.

As early as 1960s, Boyland *et al*.[5] attempted to analyze NAB and NNN. However, until the introduction of the thermal energy analyzer (TEA) detector,[6] there was no method that could determine all of the common TSNAs (NNN, NAT, NAB, NNK) in the smoke of most commercial cigarettes,[7–10] although there were many efforts utilizing gas chromatography (GC)[11] and high-performance liquid chromatography (HPLC).[12] Gas chromatography/quadrupole mass spectrometry (GC/MS) was employed to determine TSNAs in cigarette tobacco,[13] but the method sensitivity was not high enough for the determination of TSNAs in cigarette smoke, because the levels of TSNAs in cigarette smoke are much lower than those in cigarette tobacco.

TEAs are nitro-specific and offer satisfisfactory sensitivity for the determination of all the common TSNAs in the smoke of most commercial cigarettes, so some standard methods and recommended methods for the determination of TSNAs were set up using the GC/TEA method.[14,15] However, coeluting nitroso compounds cannot be differentiated with a TEA. Moreover, the low level of TSNAs in the smoke of some Virginia-type cigarettes and cigarettes with reduced levels of TSNAs greatly challenges the sensitivity of the technique, especially for NAB and NNK. In addition, the difficulties in routine maintenance of TEA detectors also limited their wider application.

Recently developed HPLC/tandem mass spectrometry (MS/MS) methods are sensitive and selective for the determination of TSNAs in tobacco smoke.[16,17] However, these methods have seldom been used for routine analysis of TSNAs because of the extremely high cost for the test not only for the use of HPLC/MS/MS, but also for the use of isotope-labeled internal standards to reduce the negative effect of the signal suppression of the ion current by other constituents in the smoke matrix.

The use of GC/ion trap MS in MS^n mode provides higher throughput with analyte-specific detection based on both retention time and structurally specific analyte fragmentation information. We have reported the development of a new extraction with acetic ether and a new cleanup procedure with a Supelclean ENVI-Carb solid-phase extraction (SPE) tube followed by GC/MS^n (ion trap) analysis. Four

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TSNAs from cigarette smoke can be analyzed with a sensitive and selective GC/MS^n (ion trap) technique that provides excellent reproducibility and accuracy using *N*-nitrosopentyl-3-picolylamine (NNPA) as an internal standard. This approach has several additional advantages in terms of lower cost and easier maintenance over previous HPLC/MS/MS and GC/TEA methods.

EXPERIMENTAL

Chemicals

The TSNA analytical standards, NNN, NNK, NAT, NAB and internal standard, NNPA, were purchased from Toronto Research Chemicals (Ontario, Canada). Acetic ether (chromatographic grade) was obtained from Tianjin Fuchen Chemicals (Tianjin, China).

Smoke collection

Cigarettes and Cambridge filter pads (CFPs, Whatman, UK) used for this study were conditioned in a 1M1000 RHD controlled humidity chamber (Claison, Australia) at $22 \pm 1°C$ and a humidity of $60 \pm 2\%$ for at least 48 h. The CFPs were put into the cigarette holders with one pad each. The rough surface of the CFP was facing the entrance of smoke. The cigarette holder was closed and examined with a leak tester (KC Automation, Richmond, VA, USA) to ensure no leaking. The puff volume for each port was adjusted to 35.0 ± 0.3 mL with a puff volume tester (KC Automation). Cigarette smoking was carried out with SM 450 linear smoking machine (Cerulean, UK) according to ISO 4387 (2000 E) at $22 \pm 2°C$ and a humidity of $60 \pm 5\%$. The smoke particulate matter from five cigarettes was collected in one CFP.

Standard and sample preparation

Standards and internal standard were dissolved in acetic ether at 4 mg/mL, diluted to intermediate stock solutions of 10, 1 and 0.1 μg/mL and stored at −20°C. Intermediate stock solutions were used to prepare calibration standard solutions.

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 particulate matter from the inner surface of the holder. Next, two of the folded CFPs were transferred to a 250 mL Erlenmeyer flask and 40 μL of the internal standard solution (acetic ether solution of NNPA of 4000 ng/mL) and 100 mL of acetic ether were added. The flask was shaken on a horizontal shaker (Wode Instrumentation, Beijing, China) for 30 min. Then the solution was filtered through a glass suction filter into a 500 mL round-bottomed flask. The Erlenmeyer flask was washed with approximately 15 mL of acetic ether three times. The washing solution was added to the filter extract. The solution was concentrated under the protection of nitrogen to 2 mL at 78°C with a RE-52C rotary evaporator (Tianrong Biochemical Instruments, Shanghai, China). The concentrated solution was loaded on to a Supelclean ENVI-Carb SPE tube (500 mg/6 mL, Supelco, USA) that had been conditioned with 2 mL of acetic ether. The round-bottomed flask was washed with 2 mL of acetic ether three times and the washing solution was added to the SPE tube. Then the SPE tube was eluted with 5 mL of acetic ether twice and the eluate was all collected in a 100 mL Erlenmeyer flask. The solution was concentrated under the protection of nitrogen to 1.0 mL at 78°C with a RE-52C rotary evaporator. A 1.5 μL aliquot of this concentrated solution was injected onto a GC 3900/Saturn 2100T GC/MS (ion trap) system (Varian, CA, USA).

Sample analysis

The GC 3900/Saturn 2100T GC/MS (ion trap) system, including an autosampler, was controlled using a Varian GC/MS work station version 5.52 software (Varian). Separation of TSNAs from the cleaned up and concentrated smoke extract was performed using a Varian VF-17 MS very-low-flow-loss column (30 m × 0.25 mm × 0.25 μm). Helium was used as the carrier gas at a flow rate of 1.0 μL/min and the injector (splitless) temperature was 230°C. The temperature program of the column oven started at 50°C and was ramped at 15°C/min to 200°C, held for 15 min, then ramped at 40°C/min to 280°C at 40°C/min and held for 10 min. The conditions used for the ion trap mass spectrometer were as follows: Ionization mode, EI (70 ev); target, 5000; prescan ionization time, 1500 μs; scan time, 0.66 s; scan mode, MS/MS and MS^n; filament, 50 μA; multiplier, +200 V; RF dump value, 650; ion trap temperature, 200°C; transfer line temperature, 220°C; manifold temperature, 45°C. For increased sensitivity, the 26 min run time was divided into eight scan segments: from 0–12.50 min, turning off; 12.50–14.80 min, scanning for NNPA, 14.80–15.80 min, turning off; 15.80–16.86 min, scanning for NNN; 16.86–17.47 min, scanning for NAT; 17.47–19.00 min, scanning for NAB; 19.00–22.20 min, turning off; 22.20–26.00 min, scanning for NNK. In addition to

Table 1. Common MS parameters and ion preparation method (IPM) for the method

MS		IPM	
Solvent delay	12.5 min	Ejection amplitude	20 V
Emission current	50μA	Isolation window	3.0 *m/z*
Mass defect	0 mmu/100 u	Low-edge offset	6 steps
Count threshold	1	High-edge offset	2 steps
Background mass	60 *m/z*	High-edge amplitude	30.0 V
Target total ion current	5000	Isolation time	5 ms
Ion preparation technique	MS/MS and MS^n	Excitation time	20 ms
		Waveform	Resonant
		Modulation range	2 steps
		Number of frequencies	1
		Modulation rate	3000 μs/step

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common MS parameters (Table 1), each segment included an ion preparation method (IPM) which defines MS/MS and MS^n parameters and *m/z* scan range (Table 2).

Data analysis

All chromatographic data were processed using Varian GC/MS work station version 5.52 software. Each ion of interest in the chromatogram was automatically selected and integrated. The peak integrations were inspected for errors and re-integrated if necessary. Six standards were prepared in the range of 11–6600 ng/mL for NNN, 5–4000 ng/mL for NAT, 10–6000 ng/mL for NAB and 66–6600 ng/mL for NNK with the internal standard added at a concentration of 5000 ng/mL. 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 was carried out using Statistical Analysis System (SAS) software (SAS Institute, Cary, NC, USA).

Artifact formation

A separate smoking run was conducted to determine the extent, if any, of artifact formation of the four TSNAs during sample collection and preparation. Ten CFPs 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 at least 48 h at 22 ± 1°C and a humidity of 60 ± 2% before use; the five control sample CFPs were also conditioned for at least 48 h at 22 ± 1°C and a humidity of 60 ± 2% before use. Ten smoke samples were collected on two treated and five untreated CFPs during the same collection run. Samples were prepared and analyzed as described in the Standard and Sample Preparation, Sample Analysis and Data Analysis sections.

RESULTS AND DISCUSSION

Sample preparation

Some solvents, such as methanol, acetone, methylene chloride and acetic ether, were initially evaluated for extraction of TSNAs. It was found that all the solvents listed above were good for extraction and all gave acceptable recoveries of the TSNAs from the CPF with 30 min, but showed different sensitivity, especially in the experiment that detected cigarette sample with low TSNA delivery. The highest sensitivity and selectivity were obtained by using acetic ether; moreover, acetic ether is non-toxic, so acetic ether was finally selected as the optimum solvent for the extraction of TSNAs.

Different volumes of acetic ether were tried for the extraction of TSNAs. In general, acceptable recoveries were obtained for extraction of 5000 ng of each of the four TSNAs with 40 mL of acetic ether. Considering that some cigarettes might produce more TSNAs, 100 mL was finally used for the extraction.

To optimize the extraction of TSNAs from the CFPs, a series of 2R4F reference cigarettes (University of Kentucky, USA) were smoked and the CFPs were extracted with acetic ether and were shaken on a horizontal shaker for 5, 10, 20, 30 and 60 min, respectively. The time-dependent study showed that increased extraction time provided improved recovery for the four TSNAs, while very similar recoveries were obtained for the extraction times of 20, 30 and 60 min. Therefore, 30 min was finally selected for the extraction.

To further clean up smoke extracts, solid-phase extraction was performed following the liquid extraction. A basic aluminum (200–300 mesh, pore diameter 5.8 nm, 10 g; Aldrich, St. Louis, MO USA) cartridge (i.d. 1.5 cm, length 30 cm), a silica gel (40–63 μm, pore diameter 6.0 nm, 10 g; Silicycle, Quebec, Canada)/basic aluminum (200–300 mesh, pore diameter 5.8 nm, 10 g, Aldrich) cartridge (i.d. 1.5 cm, length 30 cm) and Supelclean ENVI-Carb (500 mg/6 mL) SPE cartridges were evaluated to obtain optimal recovery and sensitivity. It was found that Supelclean ENVI-Carb (500 mg/6 mL) SPE cartridges had the best overall recovery and sensitivity for all the four TSNAs. The selection of washing solvent in SPE considered both removal of unwanted compounds and retaining as much of the analytes as possible on the SPE cartridge. Preliminary experiments showed that the optimal conditions were as follows: washing the SPE cartridge with 5 mL of acetic ether three times. The recovery experiments[18] showed that about 99% of the TSNAs were washed out with 2×5 mL of acetic ether.

Sample analysis

To achieve maximum sensitivity and optimize peak shape, we evaluated different types of GC columns for the separation of TSNAs, including CP-sil8 CB Low Bleed/MS (Varian), VF-5MS (Varian) and VF-17MS (Varian) columns. After careful evaluation of these columns, we successfully optimized the TSNA peaks while maintaining good separation using a Varian VF-17MS very-low-flow-loss column ($30\,m \times 0.25\,mm \times 0.25\,\mu m$).

Table 2. Ion preparation method (IPM) for each segment of the MS/MS and MS^n method

Compound	Retention time (min)	Ion range (m/z)	Precursor ion (m/z)	Quantification ion (m/z)	Isolation window (m/z)	Excitation storage level (m/z)	Excitation voltage (V)
NNPA (ISTD)	13.8	117–119	177	118	3	77.9	0.48
			160		3	70.4	0.48
NNN	16.3	102–104	147	103	3	64.6	0.51
			130		3	57.1	0.54
NAT	17.2	102–104	159	103	3	69.9	0.45
			157		3	69.0	0.54
			130		3	57.1	0.56
NAB	17.7	132–134	161	133	3	70.8	0.48
NNK	22.9	145–147	177	146	3	77.9	0.39

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Data acquisition took place by utilizing both MS/MS and MS^n mode. Selection of MS^2, MS^3 or MS^4 transition was by considering both the selectivity and sensitivity of each analyte. Table 3 shows the major ions acquired in MS/MS and MS^n mode in the ion trap mass spectrometer. For NAB and NNK, MS^2 transitions were selected as they gave several diagnostic ions in sufficient abundance for the required sensitivity and for further identification. Implementation of MS^3 transitions for these analytes, to achieve even higher selectivity, proved unfruitful as their MS^3 ions were in lower abundance than the MS^2 ions. For a similar reason, MS^3 transitions were selected for NNPA and NNN. MS^4 transitions were suitable for NAT. Figure 1 shows, as an example, the MS, MS^2, MS^3, and MS^4 spectra of the NAT standard.

To further identify TSNAs in the complex matrix, we investigated the chromatographic retention time, the match of the spectra with the standards and the samples and the consistency of the ratio of the abundance of the ions detected in the standards and the samples. The analytes were further identified with all the investigation results, high consistency of the chromatographic retention time, the ratio of the abundance of the ions detected in the standards and the samples and the high match of the spectra of the standards and the samples. Figure 2 shows the total ion current (TIC) traces comparing a mixed standard sample, a 2R4F cigarette sample and a 2R4F cigarette sample with added mixed standard sample. The area of the peak of each TSNA in the 2R4F cigarette sample was increased with the addition of the TSNA standard sample.

Method performance

The performance of the method was evaluated by its sensitivity, accuracy and precision. The limit of detection (LOD) represents the sensitivity of the procedure. The 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 LODs were calculated at 3 S_0. The estimated LODs for NNN, NAT, NAB and NNK were 0.01, 0.01, 0.04 and 0.06 ng/cig or 0.1, 0.1, 0.4 and 0.6 ng/mL, respectively. The estimated LODs for this method are much lower than that for the GC/TEA method (LODs for NNN, NAT, NAB and NNK were 0.70, 0.24, 0.79 and 0.42 ng/cig or 4.67, 1.60, 5.29 and 2.82 ng/mL, respectively)[20] and are similar to those found using the LC/MS/MS method (LODs for NNN, NAT, NAB and NNK were from 0.02 to 0.1 ng/cig).[17]

Since there is currently no smoke matrix available that lacks TSNAs, an estimation of method recovery was performed using blank matrix spikes. In this recovery experiment, a blank CFP was spiked, in quadruplicate, with a known amount of each TSNA at four separate concentrations. The concentrations of blank matrix spikes were chosen to cover almost the full range of the concentrations of calibration standards. 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 (Table 4). The results were excellent and ranged from 90.5 to 102.7%. The recovery for this method is better than that for the GC/TEA method (87.3–98.9%)[20] and is similar to that for the LC/MS/MS method (96.0–102.1%).[17]

The accuracy of the method was established by spiking known amounts of the TSNAs on CFPs containing smoke samples from 1R5F cigarettes. TSNAs were added at three concentrations: about half the amount, about the same amount and about double the amount of the TSNAs in 1R5F cigarette smoke. The accuracy was calculated as the mean of the experimentally determined concentration from replicate analysis divided by the nominal concentration (Table 5). Percent spike recovery for the method was excellent with values from 95.5 to 109.7%, which is very similar to that for the LC/MS/MS method (92.1–104.8%).[17]

The precision of the method was evaluated as relative standard deviation (RSD) both for intra- and inter-assay measurements by analyzing five replicates of both 1R5F (University of Kentucky, USA) and 2R4F cigarette smoke extracts on three separate days. The precision for the intra-assay samples was determined by analyzing the sample five times on three separate days ($n = 15$). The precision for the inter-assay samples was determined by analyzing 15 1R5F and 2R4F samples, five of which were collected on three separate days. Method precision was excellent with RSDs for intra- and inter-assay measurements ranging from 1.78 to 4.77% and from 3.43 to 7.12%, respectively (Table 6). The precision for this method is better than that for the GC/TEA method (RSD for blended type cigarettes: 4.73–5.93%, RSD for Virginia-type cigarettes: 5.09–19.63%),[20] and is similar to that for the LC/MS/MS method (RSD for intra-assay: 2.7–4.7%, RSD for inter-assay: 5.1–8.5%).[17]

Artifact formation

Artifact formation of TSNAs during sample collection has been investigated by several research groups.[16,21–23] It has

Table 3. Major ions acquired in MS/MS and MS^n mode in the ion trap mass spectrometer

Compound	Precursor ion (*m/z*)	Product ions (relative abundance)
NNPA	177	94(45),118(60),134(25),148(35),160(100)
	160	118(100),131(25),132(35),143(35)
NNN	147	105(35),120(25),130(100),132(40),145(80)
	130	77(20),103(100),128(45)
NAT	159	157(100),158(20)
	157	130(100)
	130	77(40),103(100),128(55)
NAB	161	106(20),132(25),133(100),144(70)
NNK	177	146(100),149(20),159(20)

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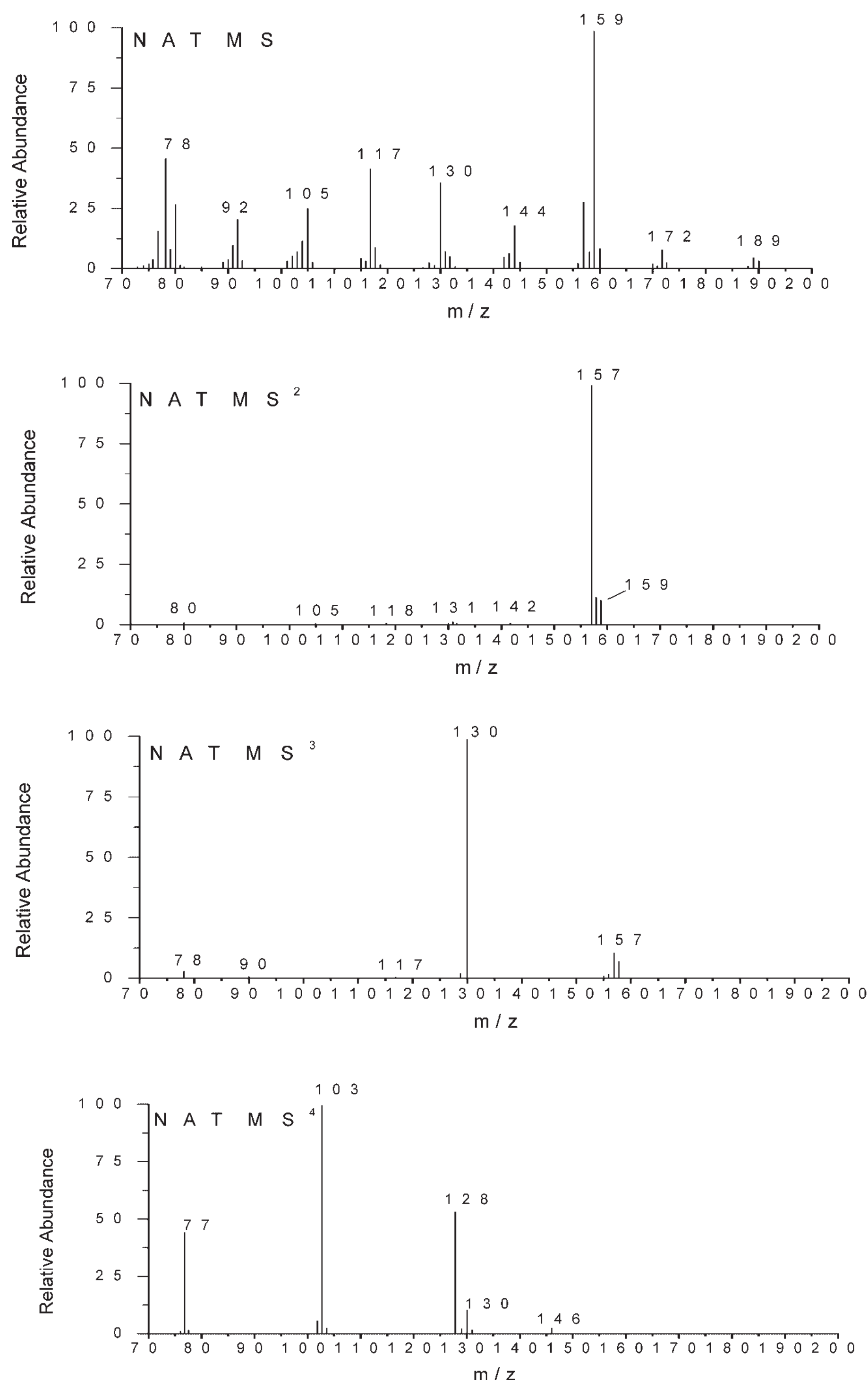

Figure 1. MS, MS^2, MS^3, and MS^4 spectra of NAT standard.

been reported that the formation of artifactual nitrosamines can be reduced with the addition of ascorbic acid to the sample collection system.[21] With our assay, we did not detect a difference in the four TSNAs with or without pretreatment of the CFP with ascorbic acid. To investigate this observation, we conducted an experiment in which 1R5F cigarette smoke samples were collected on treated and untreated CFPs during the same collection event ($n = 10$) as described in the Experimental section. The results (Table 7) show no statistical difference between samples collected on treated and untreated CFPs.

Quantitation of analytes

The analytes were quantified by means of calibration curves from known concentrations of a mixture of analyte standards. Six calibration levels (described in the Experimental section) were used with five replicate tests made at each concentration. The calibration curves were found to be linear over the entire range for all the four TSNAs with values for the coefficient of determination, R^2, >0.999.

The reference cigarettes 2R4F used for method validation are also used in our laboratory as method control samples during routine analysis of samples. Method control data

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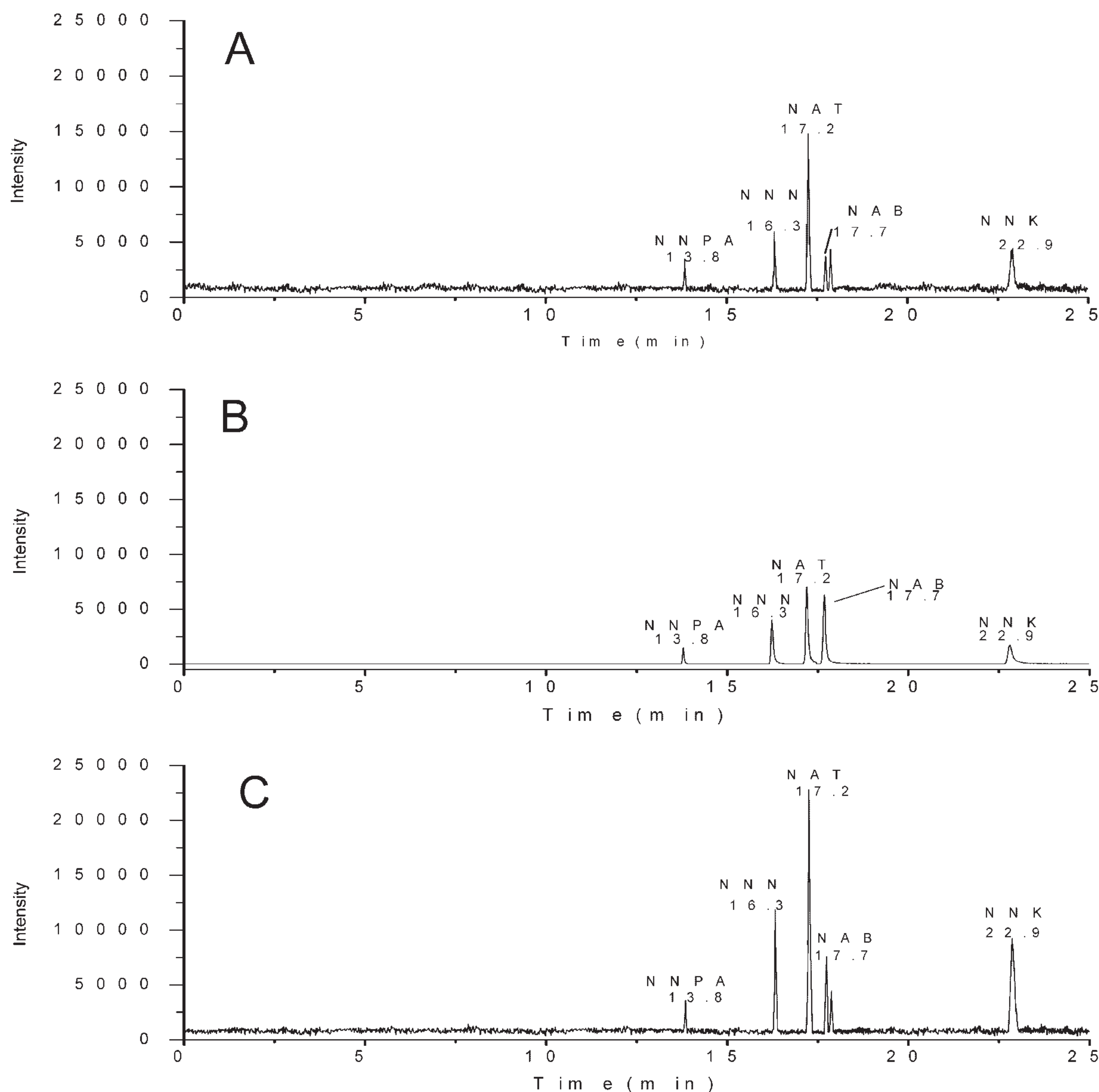

Figure 2. Total ion current (TIC) traces obtained by injection of 1.0 μL of 2R4F cigarette smoke extract (A), mixed TSNA standard sample (B) and 2R4F cigarette smoke extract added with mixed TSNA standard sample (C).

Table 4. Recovery of each TSNA from a blank CFP at four concentrations

Analyte	Spiked (ng/mL)	Calculated (ng/mL)	Recovery (%)
NNN	11	10.24	93.1
	110	105.26	95.7
	1100	1129.35	102.7
	6600	6204.12	94.0
NAT	10	9.55	95.5
	50	48.02	96.0
	500	498.56	99.7
	4000	3922.33	98.1
NAB	10	9.05	90.5
	100	91.52	91.5
	1000	964.23	96.4
	6000	5583.21	93.1
NNK	66	67.56	102.4
	132	130.25	98.7
	1320	1344.21	101.8
	6600	6204.74	94.0

Table 5. Method accuracy for laboratory-fortified matrix spikes in smoke extracts at three spike concentrations

Analyte	Spiked (ng/mL)	Recovered (ng/mL)	Accuracy (%)
NNN	65.35	68.25	104.4
	85.42	88.12	103.2
	126.56	125.12	98.9
NAT	61.43	63.45	103.3
	82.15	84.23	102.5
	123.45	128.12	103.8
NAB	11.43	10.91	95.5
	17.02	16.75	98.4
	25.47	27.95	109.7
NNK	46.18	47.25	102.3
	65.23	64.12	98.3
	110.37	106.34	96.3

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Table 6. Method precision for 2R4F smoke extract

Analyte	Sample	Intra-assay[a] (% RSD)	Inter-assay[b] (% RSD)
NNN	1R5F	2.35	4.56
	2R4F	1.78	3.43
NAT	1R5F	2.12	3.85
	2R4F	2.88	4.85
NAB	1R5F	4.77	7.12
	2R4F	4.76	6.78
NNK	1R5F	3.12	5.06
	2R4F	2.89	4.12

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

Table 7. Investigation of artifact formation of each TSNA during sample collection and preparation from a 1R5F cigarette

	NNN (ng/cig)	NAT (ng/cig)	NAB (ng/cig)	NNK (ng/cig)
Treated CFP				
Average	46.77	41.28	6.53	26.77
SD (n = 10)	1.20	0.91	0.71	1.30
Control				
Average	47.04	40.32	6.85	27.16
SD (n = 10)	1.18	1.08	0.70	1.08
Pr > F	0.209	0.129	0.179	0.230

Table 8. Comparison values for TSNA in the 2R4F reference cigarettes

Method	NNN (ng/cig)	NAT (ng/cig)	NAB (ng/cig)	NNK (ng/cig)
Various[24]	133	119	16	116
LC/MS/MS (n > 50)[17]	155 ± 17	122 ± 8	15.3 ± 2	134 ± 10
GC/ion trap MS^n (n = 50)	159.22 ± 2.63	115.48 ± 3.85	12.94 ± 1.29	135.18 ± 4.66

have been collected over a period of 10 months in our laboratory (n = 50). With this large amount of data, it was 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. Our data are in good agreement with other reported values.

CONCLUSIONS

A selective, quantitative, sensitive and reliable method has been developed for the determination of TSNAs in mainstream cigarette smoke using GC/ion trap MS^n. The resolving power of an ion trap mass spectrometer has the inherent advantage over the current, widely used TEA methods for TSNA quantification in terms of selectivity and sensitivity. The use of a very-low-flow-loss column (VF-17 MS) and acetic ether as extraction solvent ensured higher selectivity and sensitivity. In addition to unambiguous identification of four TSNAs, we achieved excellent reproducibility and accuracy without the use of expensive isotope-labeled analogues as internal standard. Importantly, acetic ether is non-toxic, while the solvents used in TEA methods[14,15,20] and HPLC/MS/MS[16,17] methods are all toxic to some extent.

We found no evidence for the formation of artifactual TSNAs with the use of our analytical procedure. While artifact formation has been reported in the literature, we believe the improvements made in our sample preparation procedures may explain the lack of artifact formation in our results.

Because of the limit of the sensitivity for the TEA method, NNK and NAB in the mainstream cigarette smoke of some Chinese Virginia-type cigarettes could not be detected.[20] Recently, some new cigarette brands with claims of reduced TSNA levels have been introduced. For example, Nanometer 3 mg ZNH, manufactured by Beijing Cigarette Factory, claims a 53.9% reduction of NNN, 53.0% reduction of NAT, 55.6% reduction of NAB and 52.9% reduction of NNK compared with the control cigarette when smoked using the ISO machine smoking protocol.[25] The TSNA levels in these and future modified tobacco products require stringent examination to assess these and other product claims. To facilitate such studies it is necessary to develop and maintain sensitive and reliable methods to measure toxic and carcinogenic agents in tobacco smoke.

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