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Rapid Determination of Four Tobacco-Specific Nitrosamines in Mainstream Cigarette Smoke by UPLC-TOF-MS

SAI-JING ZHENG[1], JUN YANG[1,2,*], BAI-ZHAN LIU[1], WEN-JING ZHU[2], LI YAO[2], WEN-BO GU[1] and RONG QIAN[1]

[1]Key Laboratory of Cigarette Smoke Research of China National Tobacco Corporation, Shanghai 200082, P.R. China
[2]Department of Chemistry, University of Science and Technology of China, Hefei 230026, P.R. China

*Corresponding author: Tel/Fax: +86 551 3492065; E-mail: yjun8202@ustc.edu.cn

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A rapid method for determining four major tobacco-specific nitrosamines (TSNAs) in mainstream cigarette smoke based on ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry (UPLC-TOF-MS) has been developed. Cigarette smoke particulate, collected on a Cambridge filter pad, was extracted by using ammonium acetate buffer as solvent, purified by solid phase extraction (SPE) and analyzed by UPLC-TOF-MS with positive electrospray ionization (ESI^+). The method has shown excellent sensitivity, repeatability and accuracy.

Key Words: UPLC-TOF-MS, Tobacco-specific nitrosamines, Mainstream cigarette smoke, Solid phase extraction.

INTRODUCTION

Tobacco specific nitrosamines (TSNAs) have been identified in both tobacco and cigarette smoke and widely admitted as one of the main groups leading to cancer and some related preventable disease[1]. Four of the most commonly analyzed TSNA compounds are N'-nitrosonornicotine (NNN), 4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N'-nitrosoanatabine (NAT), N'-nitrosoanabasine (NAB) (Fig. 1). Because of health implications and the difficulties in the determination of TSNAs, there is a longstanding interest in the accurate analysis of TSNAs in tobacco products.

Previously, the occurrence of TSNAs in tobacco had usually been analyzed by gas chromatography (GC) with nitrogen-phosphorous detector (NPD) and high performance liquid chromatography (HPLC) with UV detector[2,3]. Thereafter, the introduction of thermal energy analyzer (TEA), a chemiluminescence detector, offered a means of identifying and quantifying TSNAs[4]. Subsequently, GC coupled with mass spectrometry (GC-MS)[5], capillary electrophoresis-electrospray ionization-mass spectrometry (CE-MS)[6], HPLC coupled tandem mass spectrometry (HPLC-MS-MS)[7-9] had also been applied to determine TSNAs with high efficiency and low detection limits. However, there were several disadvantages in those methods above, such as GC-NPD, HPLC-UV had a high detection limit which was not appropriate to detect trace TSNAs in cigarette smoke. TEAs are nitro-specific and offer satisfactory sensitivity for the determination of all the common nitrosamine compounds in the cigarette smoke. However, it cannot distinguish the coeluting nitroso compounds. GC-MS had a rather complicated sample preparation procedure while CE-MS was fitting for biological samples. Recently developed HPLC-MS-MS methods are sensitive and selective for determination of TSNAs. However, these methods were influenced markedly by matrix effect[8].

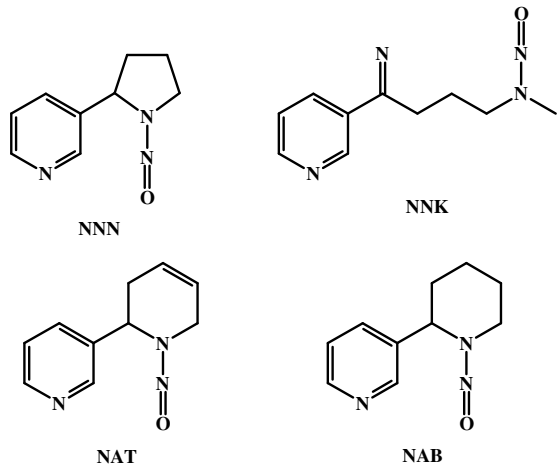

Fig. 1. Chemical structures of NNN, NNK, NAT, NAB

UPLC-TOF-MS is distinguished by its high-resolution, full scan data, which allows the testing of any compounds by extracting any desired exact mass chromatogram[9]. In this paper, a rapid screening and quantification method based on

UPLC-TOF-MS was developed to evaluate the trace TSNAs in mainstream cigarette smoke. TSNAs in the mainstream smoke samples were extracted by buffer solution, purified by mixed mode cation-exchange column, separated by UPLC, profiled by UPLC-TOF-MS with ESI^+ mode and quantified by using isotope dilution technique. These steps were used together in UPLC-TOF-MS analysis to eliminate the matrix effect and ensure an exact ultimate quantification.

EXPERIMENTAL

The TSNA analytical standards NNN, NNK, NAT, NAB and isotopically labeled analogues, NNN-d4, NNK-d4, NAT-d4, NAB-d4 were purchased from Toronto Research Chemicals (Toronto, Canada), dissolved in methanol as stock solutions and stored at -18 ºC. HPLC-grade acetonitrile and methanol were obtained from Burdick & Jackson (Muskegon, USA). Ammonia was purchased from Sigma-Aldrich (St. Louis, USA). Commercial solid phase column such as C_{18}, B-Al_2O_3, PCX columns (60 mg/3 mL) were purchased from Agela (Beijing, China). Other analytical-grade reagents mentioned were all obtained from Guoyao Group (Shanghai, China). Pure water (18.2 Ω) was obtained from Milli-Q water system (Millipore, USA).

The separation of four TSNAs was performed on ultra-performance liquid chromatography (UPLC) system (Acquity UPLC system; Waters, Milford, USA) and a column oven equipped with a reversed-phase Agilent UPLC XDB-C_{18} analytical column of 50 mm × 4.6 mm with 1.8 µm particle size and maintained at 50 ºC. UPLC mobile phase A water/ammonia (1,000:1, v/v) and phase B (methanol) lines were, respectively equipped with aqueous and organic solvent on-line filters. An iso-gradient elution was performed with 32 % B during running. Injection volume was 10 µL. The flow rate was 400 µL/min. The total running time was 6 min.

The UPLC system was combined with a time-of-flight mass spectrometer Waters-Micromass LCT Premier TOF (Waters, Milford, USA) equipped with an electrospray interface and lock spray operating in the positive ion V-mode. The parameters were as follows: capillary voltage, 2,500 V; cone voltage, 30 V; desolvation gas flow, 500 L/h; cone gas flow, 30 L/h; desolvation temperature, 350 ºC; source temperature, 120 ºC. The instrument was set to acquire over the mass range m/z 100-400 with acquisition time of 200 ms. Mass accuracy was maintained by employing a concentration of 200 pg/µL leucine-enkefaline ^{12}C $[M + H]^+$ ion (m/z 556.277) as lock mass. The instrument was calibrated by using 0.5 mM sodium formate solution.

Sample pre-treatment and extraction: All cigarette samples were purchased from Chinese market except Kentucky 2R4F and 1R5F reference cigarette was presented by Technical Centre of Shanghai Tobacco Corporation, P.R. China. Before smoking, cigarettes were all conditioned at 22 ºC and 60 % humidity for at least 24 h and then smoked using a Borgwaldt RM200 smoking machine with 20-port (Borgwaldt, Germany). Each cigarette was smoked at 1.0 puff/min, 2.0 s puff duration and 35 mL puff volume, trapped with a diameter of 92 mm Cambridge filter pad and stored in flask at -18 ºC for use.

The Cambridge filter pads with total particulate matters of 20 cigarettes and 50 ng of four isotopically labeled TSNA analogues were mixed with 40 mL 100 mmol/L ammonium acetate buffer solution, vibrated on a vibrational machine (GFL corp., Germany) at 140 rpm for 1 h. The pH of extraction solution was adjusted to 1.5 and then applied to a PCX-SPE column with the volume of 5.0 mL. The column was pre-conditioned with 3.0 mL methanol and 3.0 mL water, washed with 3.0 mL methanol, eluted with methanol/ammonia (95:5, v/v), then collected the eluting solution. All the solution passed through the cartridge at a flow rate of 1 mL/min. Before injecting into the equipment, a 0.22 µm filtration membrane was applied.

UPLC-TOF-MS analysis: Sample analysis was carried out using UPLC-TOF-MS in the full scan mode. ESI was operated in the positive ion mode. Prior to sample analysis, the MS conditions for each target analyte were optimized by using flow infusion with a syringe pump. The specific $[M + H]^+$ ions were acquired from the spectra and the used extraction mass window width was 0.10 Da in present study.

RESULTS AND DISCUSSION

Optimization of the SPE procedures: In this paper, a series of commercially available SPE columns: basic aluminum cartridge, C_{18} cartridge and PCX cartridge were evaluated to obtain optimal separation and recovery. In order to obtain optimal separation, different columns were preconditioned with varied reagents which referred to literatures. It was clearly indicated that C_{18} and B-Al_2O_3 columns had a rather high loading breakthrough, while PCX column was the one with fairly low loading penetration and small washing loss. The possible reason for this was due to the new material of mixed-mode cation-exchange PCX cartridges. Benefitted from the mixed-mode retention mechanism of PCX material, the protonated TSNAs were more easily to be retained than just polar or non-polar substances.

Considering both removal of unwanted components and retaining as much of the TSNAs as possible on PCX cartridge during SPE process, we carried out a series of preliminary experiments. The final SPE conditions, such as the pH value of loading solution, the composition of washing and eluent solution, were optimized by evaluating the recovery of TSNAs. In present study, pH at 1.5 was selected as the optimum loading condition on PCX column methanol/water (100:0, v/v) and methanol/ammonia (95:5, v/v) were ultimately chosen as the best washing and eluent solution.

UPLC column comparison: Different chromatographic columns such as Agilent Zorbax C_{18} (150 mm × 4.6 mm i.d. 5.0 µm), Agilent XDB-C_{18} (50 mm × 4.6 mm i.d. 1.8 µm) and Waters Acquity BEH-C_{18} (50 mm × 2.1 mm i.d. 1.7 µm) were selected to obtain the best resolution. It was visibly depicted that Agilent Zorbax C_{18} and Waters Acquity BEH-C_{18} had fairly worse resolution with the time difference of four TSNAs less than 0.10 min, while Agilent XDB-C_{18} possessed more than 0.5 min difference. A probable reason was that super-pressured chromatographic columns packed with smaller padding granularity resulted into the better resolution than conventional ones[10].

Width of the extraction mass window: An appropriate width of the extraction mass window should be selected to separate target compounds and avoid obtaining incorrect results. Reducing the width from 1-0.01 Da to optimize the best extracted ion chromatogram, it was demonstrated that the

extraction mass window set at 0.1 Da could separate four TSNAs and gain fairly good shape of extracted ion chromatograms (Fig. 2). There was too much false signal in the extraction mass window at 1 Da, while at 0.01 Da the signal of NNN was not presented. Therefore, the width of 0.1 Da was chosen to acquire data from total ion chromatogram of TSNAs for reliable screening results.

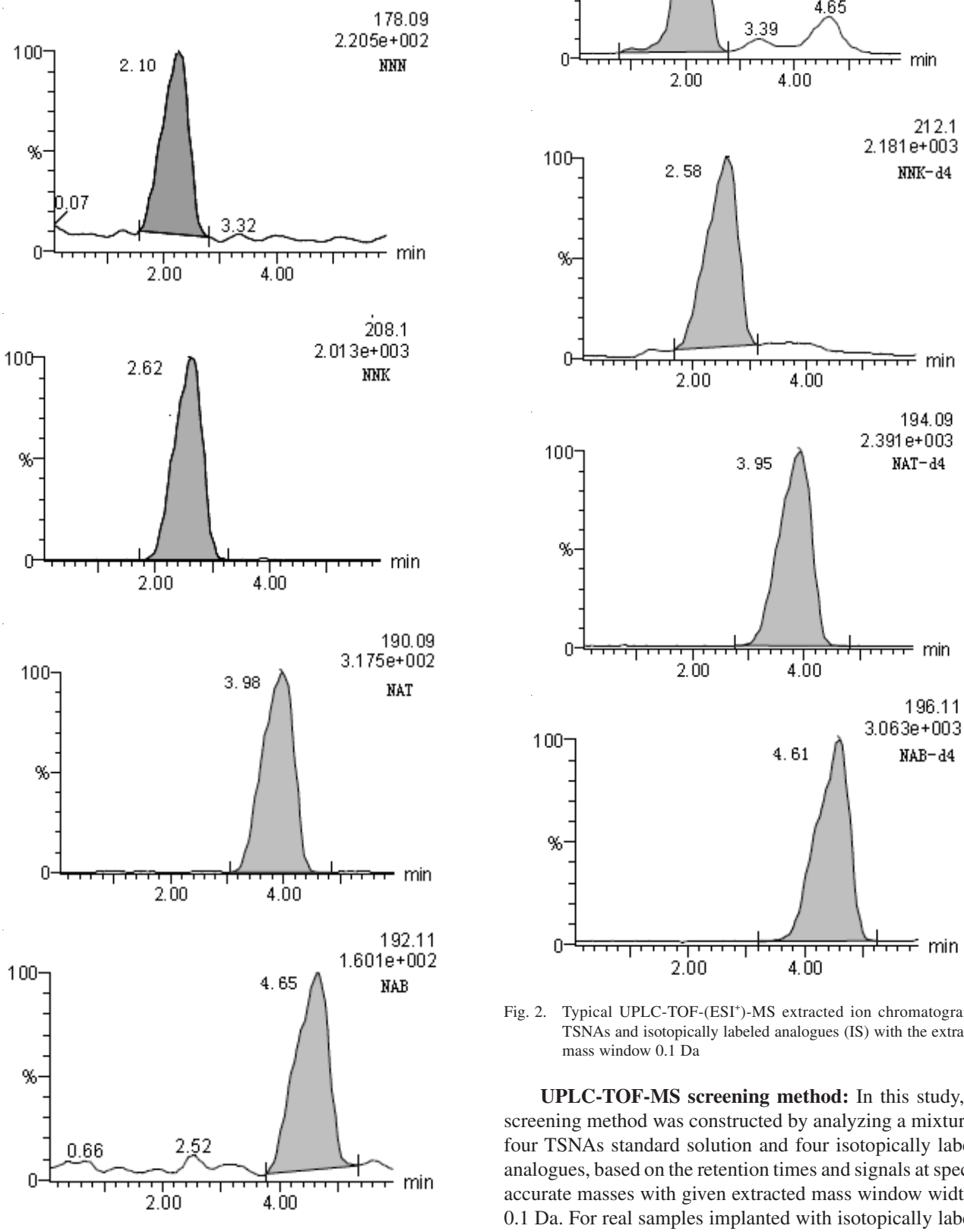

Fig. 2. Typical UPLC-TOF-(ESI$^+$)-MS extracted ion chromatogram of TSNAs and isotopically labeled analogues (IS) with the extraction mass window 0.1 Da

UPLC-TOF-MS screening method: In this study, the screening method was constructed by analyzing a mixture of four TSNAs standard solution and four isotopically labeled analogues, based on the retention times and signals at specific accurate masses with given extracted mass window width of 0.1 Da. For real samples implanted with isotopically labeled

analogues, the full scan chromatogram was obtained and a list of main ions of the eight target objects was extracted at the same extracted mass window width as the standard solution, furthermore, accurate mass and retention time tolerance between real samples and standard solution should also be acceptable.

Analytical method validation: The calibration curves were prepared in the range of 0-100 ng/mL for TSNAs with addition of 50 ng individual internal standard. Under the optimal conditions, excellent linearity was obtained by plotting the peak area ratios of the analytes relative to the internal standards with concentration. The correlation coefficients (R) were above 0.99. Limits of quantification (LOQ) were evaluated using signal/noise ratios of 10 in blank sample. All data were listed in Table-1.

TABLE-1
TYPICAL CALIBRATION PARAMETERS AND LOQ OF TSNAS

Compounds	R	Slope	Intercept	LOQ (ng/mL)
NNN	0.9958	10.02	-0.1349	1.2
NNK	0.9944	40.83	3.283	1.4
NAT	0.9908	18.79	6.341	1.0
NAB	0.9935	0.9436	-4.958	1.8

To ensure the reproducibility of the newly developed UPLC-TOF-MS method, validation tests of both precision and repeatability were performed. Considering the abundance levels and retention time distribution in the extracted ion chromatogram, the extracted ions of m/z 178.09 (NNN), 208.10 (NNK), 190.09 (NAT), 192.11 (NAB), 182.09 (NNN-d4), 212.10 (NNK-d4), 194.09 (NAT-d4), 196.11 (NAB-d4), were selected in mainstream smoke samples for validation. Five replicated samples were carried out on UPLC-TOF-MS using the same preparation protocol, a stable retention time of the ion peaks was observed. The variations of retention time RSD were less than 0.2 %, the RSD values of NNN, NNK, NAT, NAB peak areas ratios were 6.3, 5.5, 3.8 and 9.7 %, respectively. It showed that this novel method was acceptable in complicated samples.

The accuracy of this method was accessed by analyzing TSNAs in mainstream smoke of 2003 Kentucky 2R4F reference cigarette. The comparison values for TSNAs in 2R4F reference cigarettes determined by different methods were shown in Table-2. There was a good agreement among the different methods reported in literatures. The fortified recoveries (N = 5) for NNN, NNK, NAT and NAB in trapped Cambridge filter pads were 80.4, 77.3, 84.0 and 111.8 %, respectively.

TABLE-2
COMPARISIONC VALUES OF TSNAS IN 2R4F REFERENCE CIGARETTES

Compounds	UPLC-TOF-MS (ng/cig)	GC-TEA[(a)] (ng/cig)	LC-MS-MS[(b)] (ng/cig)	LC-MS-MS[(c)] (ng/cig)
NNN	121	147	142	138
NNK	111	125	121	114
NAT	114	136	125	123
NAB	16.8	11.8	17.3	15.2

(a) Determined by GC-TEA. (b) Reported by Lee *et al.*[11]
(c) Obtained by MRM mode in our previous study.

Application on real sample analysis: Finally, the method was employed to screen the TSNAs in mainstream smoke samples in order to validate its application on the real samples. It showed that the method can be applied to screen and quantify the TSNAs in mainstream smoke of flue-cured and blended type cigarettes.

Conclusion

This paper described a new screening and quantification UPLC-TOF-MS method of TSNAs in mainstream cigarette smoke. The method has been shown to produce excellent recoveries as well as reproducibility. Measurements TSNAs in reference cigarettes provided results comparable to those reported in literatures. Hence, UPLC-TOF-MS could be used as an alternative approach to investigate TSNAs in mainstream smoke.

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