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Analysis of tobacco-specific N-nitrosamines in snuff by ethyl acetate extraction and liquid chromatography–tandem mass spectrometry

Christer Jansson*, Alexandre Paccou, Bengt-Göran Österdahl
Swedish National Food Administration, Research and Development Department, Box 622, SE-751 26 Uppsala, Sweden

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Abstract

A rapid, selective and sensitive method for routine analysis of the four tobacco-specific N-nitrosamines, *N′*-nitrosonornicotine, *N′*-nitrosoanatabine, *N′*-nitrosoanabasine and 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone in snuff has been developed. The nitrosamines were isolated by ethyl acetate extraction and analysed by LC–MS–MS. Except for evaporation and filtration, no additional clean-up steps are needed in the proposed method. The detection limits for standard in solvent are between 0.0005 and 0.001 μg/ml (0.005 and 0.01 μg/g).

Keywords: Tobacco; Nitrosamines

1. Introduction

In Sweden, the use of moist snuff has increased considerably during the last three decades and total sales in 2001 were 175 million snuff boxes, equivalent to about 6500×10^3 kg. About one million Swedes take snuff. One reason for the increased use of snuff is that it is regarded by the users as being safer than tobacco smoking. However, the International Agency for Research on Cancer (IARC) has stated that ‘‘there is sufficient evidence that oral use of snuffs of the types commonly used in North America and Western Europe is carcinogenic to humans’’ [1]. The European Union (EU) has a sales ban on snuff in the EU member countries—with Sweden as the sole exception. Tobacco-specific N-nitrosamines (TSNAs) are the most abundant carcinogens identified in tobacco and tobacco smoke, and are formed during the ageing, curing and fermentation of tobacco [2].

Previously, the occurrence of TSNAs in tobacco and snuff has usually been analysed by liquid extraction, purification of the extract and quantification by high-performance liquid chromatography (HPLC) or gas–liquid chromatography (GLC) interfaced with the highly specific and sensitive thermal energy analysis (TEA) [3–6]. Extraction has been performed using, for example, buffer solution [3–5], dichloromethane [6], and supercritical carbon dioxide [7–9]. The TEA detector exhibits extreme sensitivity and high specificity for N-nitrosamines, but it can only be used for this purpose, and thus its use is not widespread. Recently, the analysis of TSNAs in

*Corresponding author. Fax: +46-18-105-848.
E-mail address: chja@slv.se (C. Jansson).

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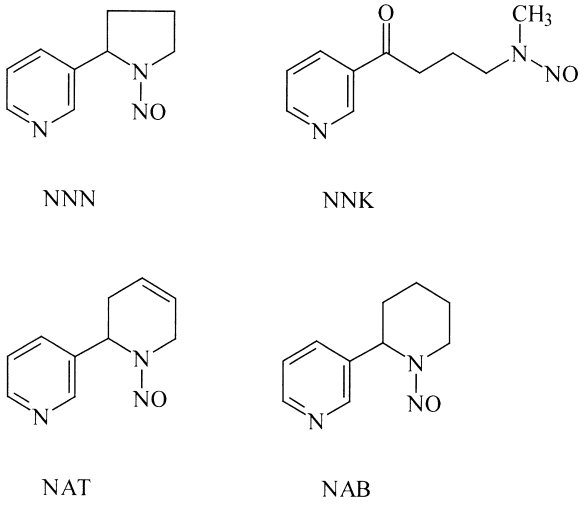

Fig. 1. Chemical structures of the TSNAs studied.

tobacco has been performed by gas chromatography–mass spectrometry (GC–MS) using single ion monitoring [9], as well as HPLC–particle beam-TEA, HPLC–electrospray interface (ESI)-MS and GC–electron impact (EI)-MS [10]. A new EU directive demands control of snuff as well as of other tobacco products [11]. Thus it would be desirable to have a rapid and specific method for analysis of TSNAs in snuff which does not rely on the use of TEA.

The purpose of the present study was to develop a rapid LC–MS–MS method for the analysis of four TSNAs in snuff, *N′*-nitrosonornicotine (NNN), *N′*-nitrosoanatabine (NAT), *N′*-nitrosoanabasine (NAB) and 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Fig. 1).

2. Experimental

2.1. Materials

Nitrosamine standards were purchased from Midwest Research Institute (Kansas City, MO, USA). The purity was >95%. Stock solutions were prepared in dichloromethane, pesticide-grade (Lab-Scan, Dublin, Ireland). Working solutions were prepared daily in methanol, gradient grade (Merck, Darmstadt, Germany). All standard solutions were kept in the dark at 2–10 °C and were found to be stable for at least 2 months.

Ethyl acetate and dichloromethane, pesticide grade (Lab-Scan), and methanol, gradient grade (Merck), were used for extraction and sample preparation. Formic acid 98–100% (Merck), 25% ammonia solution (Riedel-de Haën, Hannover, Germany) and Milli-Q water were used for preparation of mobile phase B, 10 m*M* ammonium formate, pH 4.0, in water–methanol (80:20, v/v). After the addition of 0.31 ml formic acid, ammonia was added dropwise to 800 ml Milli-Q water, to give a pH of about 4.0, and finally 200 ml methanol were added.

Mobile phases were also prepared to test HPLC conditions (Section 2.3) and buffer was prepared to test extraction efficiency (Section 2.4.3). Ammonium acetate (10 m*M*), pH 4.8 in water–methanol (80:20, v/v) was prepared by adding ammonia dropwise into 0.46 ml acetic acid in 800 ml Milli-Q water, to give a pH of 4.8, and finally 200 ml methanol were added. Ammonium formate (10 m*M*), pH 3.5, in water–methanol (80:20, v/v) was prepared by adding ammonia to 0.31 ml formic acid in 800 ml Milli-Q water to give a pH of 3.5, and finally 200 ml methanol were added. Phosphate–citrate buffer (100 m*M*) pH 3.9 was prepared by dissolving 1.9 g Na_2HPO_4 in 500 ml Milli-Q water. The pH was adjusted by adding 100 m*M* citric acid in Milli-Q water (10.5 g citric acid/500 ml). In the original method the pH was adjusted to pH 4.2 for use in the analysis of TSNA in tobacco filler. However, since Swedish snuff contains sodium or potassium carbonate, the pH in the final solution of buffer and moist snuff was 4.2 when the pH of the buffer was 3.9.

For comparison of the extraction methods, polymeric solid-phase extraction (SPE) columns Porapac RDX (500 mg with 6 ml reservoir) were purchased from Waters (Milford, MA, USA) and were used in the buffer extraction method. Kieselguhr (Extrelut, Merck), used in the dichloromethane extraction method, was dried overnight at 200 °C prior to use and stored at the same temperature.

2.2. Liquid chromatography–mass spectrometry

A Waters Alliance 2690 system with a quaternary gradient pump and vacuum degassing was used for

liquid chromatography. Separations were carried out using a Genesis C_{18} column, 100 mm×3 mm I.D., 4 μm particle size (Jones Chromatography, Mid Glamorgan, UK) with a 1-cm guard column with the same packing material. The mobile phase was filtered through a 0.45 μm PTFE membrane filter (HVLP, Millipore, Ireland). Separation was performed using a gradient between methanol (mobile phase A) and 10 m*M* ammonium formate, pH 4, in water–methanol (80:20, v/v) (mobile phase B). The gradient was: $t=0$ min, 0% A and 100% B; $t=5$ min, 30% A and 70% B; $t=10$ min, 30% A and 70% B; $t=12$ min, 0% A and 100% B, next injection after 15 min. The flow-rate was 0.3 ml/min, and the injection volume was 5 μl.

The nitrogen used for desolvation and as nebulising gas for the LC–MS was produced in situ by a nitrogen generator (Aquilo NG 11, Aquilo Gas Separation, Etten-Leur, The Netherlands) fed by compressed air at 7 bar. LC–MS was carried out using a Micromass Quattro LC (Manchester, UK) triple quadrupole mass spectrometer equipped with a standard pneumatically assisted electrospray ion source, operated in the positive ion mode. The experimental conditions were as follows: the nebulising gas had a flow-rate of about 80 l/h, the desolvation gas was heated to 400 °C at a flow-rate of 600–700 l/h, the capillary voltage was set at 4.0 kV, and the source block temperature was 120 °C. The resolution in both the first and the second quadrupoles was set to 15.0 (unit resolution) and the optimum cone voltage (CV) was 17 V for all TSNAs. The collision cell was filled with argon at a pressure of 2–3 mbar, and the collision energy was optimised for each fragment and the optimum was found to vary between 10 and 35 eV. The collision energy for each monitored fragment ion and the retention time for each TSNA are shown in Table 1. Detection was performed by switching between the different collision energies with a dwell time of 0.1 s and an inter-scan delay of 0.03 s.

2.3. Testing of HPLC condition

In order to optimise the separation, the HPLC conditions were also tested using two other buffer systems at two different pH values, as mobile phase B. Both 10 m*M* ammonium acetate, pH 4.8, in water–methanol (80:20, v/v) and 10 m*M* ammonium formate, pH 3.5, in water–methanol (80:20, v/v) were tested. The gradient was the same as described in Section 2.2.

2.4. Optimisation of extraction method

The extraction efficiency was studied by extraction of four moist snuff samples using three different extraction methods. The dichloromethane extraction method is a modification of the method earlier used for extraction of TSNAs at the National Food

Table 1
Retention times, fragment ions and collision energies for the analysed TSNAs. The cone voltage was 17 V for all compounds

TSNA	M_r[a]	t_R (min)	Precursor ion (m/z)	Fragment ion (m/z)	Collision energy (eV)
NNN	177.1	5.5+6.2	178	148	10
				120	25
				105	25
NAT	189.1	9.5	190	160	10
				106	20
				79	30
NAB	191.1	9.8	192	162	10
				133	25
				106	35
NNK	207.1	8.7	208	134	27
				122	15
				106	30

[a] M_r, monoisotopic molecular mass.

Administration [6]. Extraction using ethyl acetate is an adaptation of the multi-residue method for analysis of pesticides in fruit and vegetables used in the Swedish pesticide monitoring programme for many years [12]. The buffer extraction method has been developed by Lucke et al. for tobacco filler [13]. All extracts from the three extraction methods were transferred into methanol before analysis on LC–MS–MS.

2.4.1. Dichloromethane extraction

A 3.0 g amount of moist snuff was suspended in 25.0 ml dichloromethane in a beaker covered with plastic film. After being allowed to stand for 30 min at room temperature, the mixture was placed on a dried Extrelut column (15 cm×2 cm I.D.). The column was eluted with dichloromethane (4×25 ml) after 10 to 15 min. The eluate was evaporated in a water-bath at 55 °C. When about 0.5 ml remained, 1 ml methanol was added and the evaporation was continued until the boiling stopped. The extract was transferred to a volumetric flask, diluted to 3.0 ml with methanol and filtered through a 0.45 μm PTFE filter. Before analysis on LC–MS–MS, the sample was diluted 10 times with methanol to a final concentration of 0.1 g/ml.

2.4.2. Ethyl acetate extraction

A 10.0 ml volume of ethyl acetate and 2.0 g sodium sulphate were added to 5.0 g of moist snuff in a wide test tube (25 mm O.D.) capped with a PTFE-lined cap. The contents of the tube were mixed by shaking before insertion in an ultrasonic bath for 10 min, and then the sample was re-suspended by shaking. Then, after filtration through a 0.45 μm PTFE filter, 1.5 ml extract was evaporated to dryness under a stream of nitrogen, re-dissolved in 1.5 ml pure methanol and once again filtered through a 0.45 μm PTFE filter. The sample was diluted five times with methanol to a concentration of 0.1 g/ml before analysis on LC–MS–MS.

2.4.3. Buffer extraction

A 0.5 g amount of moist snuff was suspended in 10.0 ml 100 m*M* phosphate–citrate buffer pH 3.9 and set aside for 16 h at room temperature. The pH of the mixture was now 4.2. After filtration through a 0.45 μm nylon filter, 5.0 ml extract were transferred to an SPE column (Porapac RDX) preconditioned with 5 ml methanol and 5 ml buffer pH 3.9. The column was rinsed with 3 ml water, 5 ml 0.1% trifluoroacetic acid (TFA) in water, 3 ml water and finally with 4 ml 20% methanol in water, before drying with vacuum for 1 min. Then the TSNAs were eluted with 7 ml pure methanol. The extract was concentrated to about 100 μl under vacuum at 37 °C, transferred to a volumetric flask and diluted to 1.0 ml with methanol. The sample was diluted with methanol to a concentration of 0.1 g/ml before analysis on LC–MS–MS.

2.5. Recovery studies

Recovery tests were done at two spiking levels with three different snuff samples. The lowest level for each TSNA was about the same level as was expected to be found in the snuff, and the highest level was three times higher. At the lowest level, four recovery tests were done on each snuff sample, and two at the highest levels. After addition of the standards dissolved in methanol, the samples were kept at room temperature for 15 to 30 min before extraction. The recovery study was only performed using ethyl acetate extraction, since it had already been shown to be the most effective extraction method.

2.6. Test of matrix effect

One of the main problems when using LC–MS(–MS) detection is signal suppression, or sometimes enhancement, caused by co-eluting matrix components. To avoid these matrix effects, a thorough clean-up of the samples, or use of matrix matched standards is often required [14,15].

Matrix effect, expressed as the signal from the TSNAs in matrix compared to the signal in solvent, was determined for all snuff samples by standard addition at two levels to the snuff extract. The first level was addition of roughly the same concentration as was expected to be found in the snuff, and the second level was twice the first level. Both levels were within the linear range. The slope of the calibration curve of these standard additions was compared to the slope of the calibration curve made up from standards in pure methanol and analysed in

the same run. The matrix effect was calculated as the deviation between the slope in matrix and the slope in solvent.

Evaluation of the true nitrosamine concentration in the samples was done in the normal way when using standard additions, by extrapolation to where the calibration curve intersects the *x*-axis. By this method the concentrations are automatically corrected for matrix effect.

2.7. Quantification

Quantification was always done by using matrix matched standards. Since we did not have access to blank snuff, without TSNA, quantification was done by using standard additions at two levels, in the same way as described in Section 2.6. The linearity, R^2, of calibration curves prepared in this way was in the range 0.98–1.00. Calculations were done by both peak area and peak height, with no deviation in linearity, and the difference in calculated concentration was always less than 5%. Peak height was chosen as the preferred method for the calculations.

Analysis was normally carried out the same day as the samples were extracted, although the stability of the TSNAs was very good.

Stability of TSNAs in extract from snuff was examined by analysing extracts stored in ethyl acetate or in methanol. Three different extracts of TSNAs from snuff were stored at +4 °C for 1, 2 and 4 months before they were analysed again. The extracts stored in methanol, 0.1 g/ml, were injected on LC–MS directly as they were. The extracts stored in ethyl acetate, 1.0 g/ml, were evaporated to dryness using nitrogen and re-dissolved in methanol as described in Section 2.4.2.

Quantification of the methanol extracts was done by using the same standard additions, which were prepared when the samples were analysed the first time, 1, 2 or 4 months ago. Quantification of the samples stored in ethyl acetate was done by newly prepared standard additions.

For all four TSNAs, the stability was found to be very good in both methanol and in ethyl acetate. In extracts stored in methanol for 4 months, the calculated concentration of total TSNA was 90% compared to when they were newly prepared. The concentration of total TSNA in extracts stored in ethyl acetate was 102% when analysed 4 months later.

3. Results and discussion

3.1. Fragmentation

Each TSNA produced many different fragment ions. Three fragment ions from each TSNA were chosen for quantification and confirmation purposes. The ions chosen were the ones with highest m/z and at the same time with high intensity (Table 1). Attempts to deduce the identity of the ions have been made both theoretically and by sequential fragmentation. The sequential fragmentations were done by choosing a fragment as precursor ion, and possible fragments from this ion were determined. The first fragmentation step in all spectra is due to the loss of a NO·, resulting in an odd-electron fragment ion. This ion fragments further at higher collision voltages. Tentative interpretations of the TSNA spectra are given in Fig. 2.

3.2. Optimising of the mobile phase

HPLC performance regarding retention time, response and peak shape was tested at three different pHs using three buffer systems. The best response for each TSNA was achieved at pH 3.5 and 4.0, while the response at pH 4.8 was only about one fourth compared to the response at pH 3.5 and 4.0 (Fig. 3). At pH 3.5 and 4.0, NNN was divided up into two peaks and NAT was eluted as one single peak. However, at pH 4.8 the opposite was true: NNN elutes as one single peak and NAT was split up into two narrow peaks. The fragmentation pattern of both NNN peaks was exactly the same, which means that both peaks, most likely, came from the same compound. The split up of the signal for NNN into two peaks was probably due to the basicity of NNN and the formation of two structural isomers after the protonation of NNN.

3.3. Comparison of extraction methods

Extraction of snuff using dichloromethane has been applied at the National Food Administration for

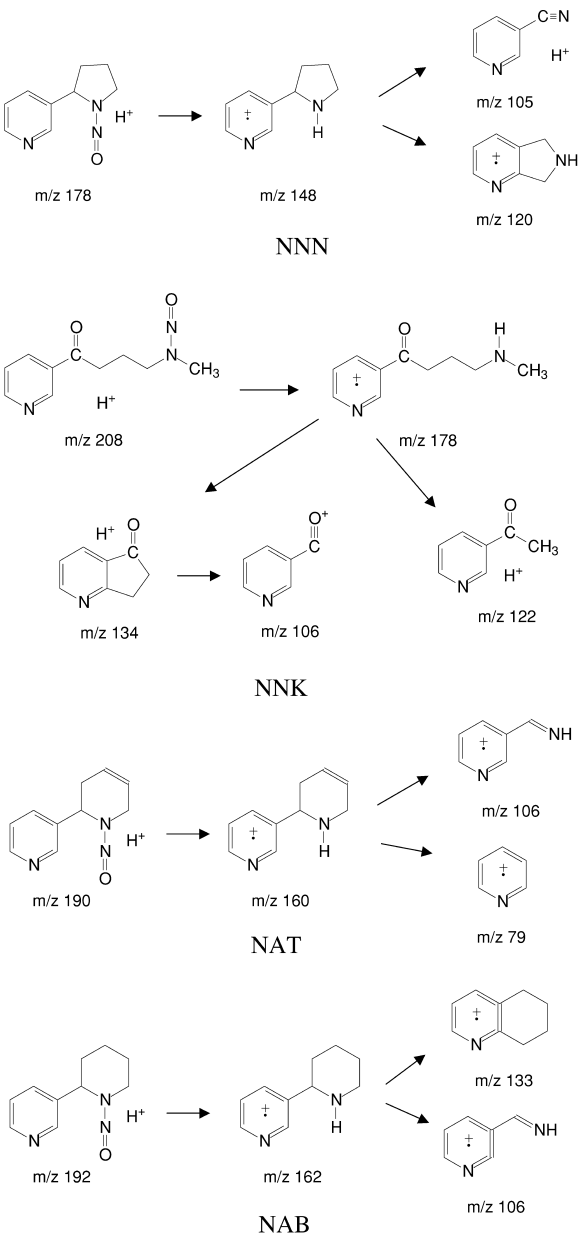

Fig. 2. Proposed fragmentation schemes for TSNA ions used in the method. Interpretations have been done in co-operation with Niessen [17].

many years [6]. The method we have used in this study is an adaptation of the former method. In order to use LC–MS–MS instead of GC–TEA for the analysis of TSNAs, the samples were transferred into methanol before quantification. The dichloromethane extraction was compared to extraction with ethyl acetate [12] and extraction with a 100 m*M* phosphate–citrate buffer at pH 3.9 [13]. Four different moist snuff samples were extracted using all three methods. A comparison of the results is shown in Table 2. The results show that ethyl acetate extracts the four TSNAs more effectively than dichloromethane and 100 m*M* phosphate–citrate buffer pH 3.9. Dichloromethane extraction gave an extraction efficiency of 84 to 93% for the four TSNAs compared to ethyl acetate extraction, and the buffer extraction at pH 3.9 gave an even lower extraction efficiency, 74 to 86%, also compared to ethyl acetate extraction. Furthermore, the ethyl acetate extraction method is the least time-consuming method of the three methods tested. One person can extract and prepare at least 10 samples per day.

3.4. Recovery, matrix effect and confirmation

The recovery of TSNAs spiked to moist snuff samples before extraction with ethyl acetate was tested with three different snuff samples. The mean recovery of the four TSNAs varied between 78 and 83% at the high level and between 84 and 89% at the low level. The relative standard deviation ranged from 5 to 11% and the limit of detection (LOD) was estimated to be between 0.005 and 0.01 μg/g (Table 3). The LOD was derived from injection of standards in solvent, and defined as the concentration giving a signal-to-noise ratio of at least 10:1, peak-to-peak, for the best ion from each compound.

All matrix effects were found to be quite similar in extracts from the three extraction methods. The signal was suppressed in the presence of matrix by 10 to 25%, with no difference between the four TSNAs. Although the signal was suppressed in these tests, when analysing the same samples with the same method on other occasions, the signal was found to be enhanced by up to 50% instead. The same enhancement was found for all fragment ions from the compound. The difference is probably due to the condition of the LC–MS instrument.

Three fragment ions per TSNA have been tested for confirmation. For positive confirmation the retention time, and the ratio between at least two fragments for each compound in the snuff, must be in accordance with what is found when injecting the

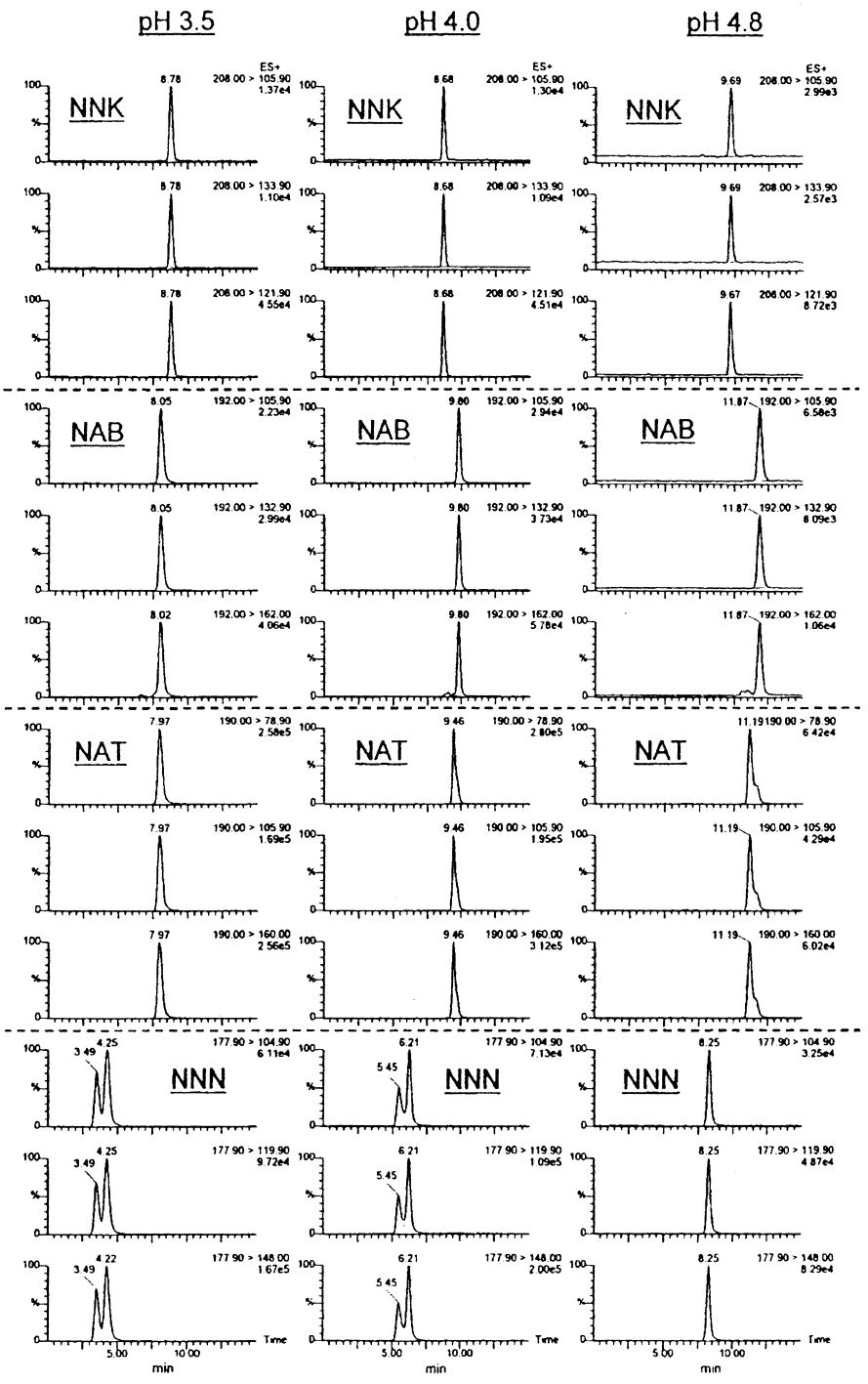

Fig. 3. Retention time and peak symmetry of the TSNAs at pH 3.5, 4.0, and 4.8 of the mobile phase. Three fragment ions from each compound. From the top NNK, NAB, NAT and NNN, all at 0.1 μg/ml methanol.

Table 2
Amount of TSNA extracted from four different Swedish moist snuff samples, using three different extraction solvents

TSNA	Concentration (μg/g)[a]		
	Ethyl acetate	Dichloromethane	Buffer pH 3.9
NNN	0.45±0.07	0.42±0.07	0.35±0.04
NNK	0.20±0.03	0.18±0.05	0.17±0.04
NAT	0.31±0.04	0.26±0.05	0.23±0.04
NAB	0.03±0.003	0.02±0.005	0.02±0.003

[a] Mean result and standard deviation from extractions of four different moist snuff samples.

corresponding standard. For all TSNAs, at least two fragments could be used (Table 1). However, sometimes one of the fragment ions for NNN and NNK had to be rejected because of matrix peaks (Fig. 4). In general, calculations using peak height was less affected by matrix peaks than calculations using peak area. The difference in ratio between the two or three ions in standard and in sample, was less than 10% at the low level and less than 5% at the high level of addition, for all compounds.

4. Conclusion

On the basis of the presented results, the described method using ethyl acetate extraction and quantification by LC–MS–MS is a rapid, selective and sensitive method for the analysis of TSNAs in snuff. Thus, the method could be suitable for routine analysis of TSNAs in moist snuff.

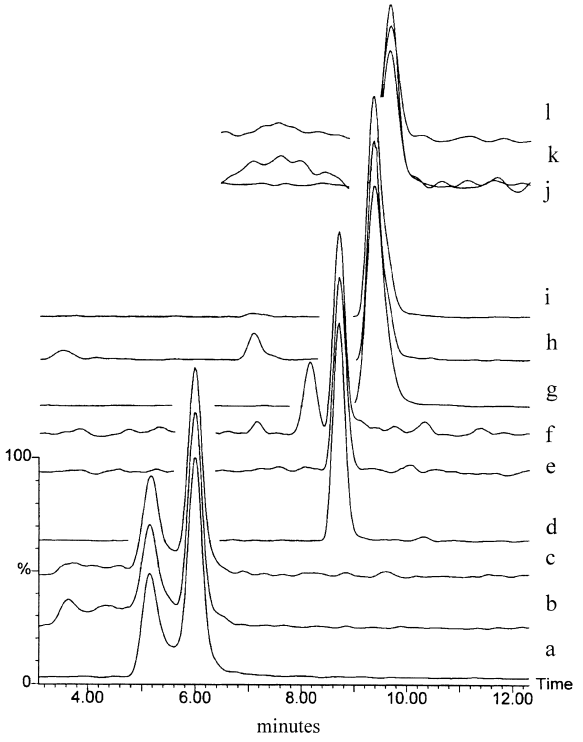

Fig. 4. Chromatogram of TSNAs in a Swedish snuff sample, at a concentration of 0.1 g snuff/ml. The peaks come in the following order: NNN ($m/z=178>105$ (a), 120 (b) and 148 (c)) which has a retention time of 5.5 and 6.2 min, NNK ($m/z=208>106$ (d), 122 (e) and 134 (f), t_R 8.7 min), NAT ($m/z=190>79$ (g), 106 (h), and 160 (i), t_R 9.5 min) and NAB ($m/z=192>106$ (j), 133 (k) and 162 (l), t_R 9.8 min). NNN gives a double peak in both standard and snuff, probably due to isomers. Concentrations are 0.05 μg/ml for NNN, 0.025 μg/ml for NNK, 0.04 μg/ml for NAT and 0.004 μg/ml for NAB.

Table 3
Recovery of spiked TSNA from moist snuff using ethyl acetate extraction

TSNA	Initial level (μg/g)	Amount added (μg/g)	*n*	Recovery[a] (%)	RSD[b] (%)	LOD[c] (μg/g)
NNN	0.4–0.7	0.50	12	84	9	0.01
		1.50	6	79	5	
NNK	0.2–0.3	0.25	12	85	10	0.01
		0.75	6	78	10	
NAT	0.3–0.4	0.40	12	89	6	0.01
		1.20	6	83	6	
NAB	0.02–0.03	0.04	12	85	11	0.005
		0.12	6	81	9	

[a] Recovery of added TSNA. Mean value of three fragment ions per compound, as in Table 1.
[b] RSD, relative standard deviation.
[c] LOD, limit of detection determined from injections of standard in solvent.

Although no clean-up at all is performed in the proposed method, only a very low matrix influence could be observed, which can be handled by using matrix-matched standards.

The developed method has recently been applied to study the levels of TSNAs in snuff on the Swedish market in 2001 and 2002 [16].

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