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Decreased Levels of Tobacco-Specific *N*-Nitrosamines in Moist Snuff on the Swedish Market

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Moist snuff, or snus, on the Swedish market in 2001 and 2002 was analyzed for tobacco-specific *N*-nitrosamines (TSNAs) using a recently developed LC-MS/MS method. All samples of moist snuff analyzed were found to contain detectable levels of *N*′-nitrosonornicotine (NNN), *N*′-nitrosoanatabine (NAT), *N*′-nitrosoanabasine (NAB), and 4-(*N*-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). In the survey in 2001, all samples except for one were produced by Swedish Match ($n = 14$), which is the dominating manufacturer on the Swedish snuff market. In the survey in 2002, samples from both Swedish Match ($n = 7$) and seven smaller manufacturers ($n = 20$) were analyzed. Total TSNA levels of between 0.15 and 3.0 μg/g wet weight were found. In the survey in 2001 and 2002, the mean level of the total TSNA content in moist snuff was 1.1 μg/g ($n = 14$) and 1.0 μg/g ($n = 27$), respectively. The result of the survey shows that the level of TSNAs in moist snuff on the Swedish market has been greatly reduced since the middle of the 1980s. Clearly, efforts have been made by the manufacturers to reduce the level of TSNAs in snuff.

KEYWORDS: Tobacco; moist snuff; snus; *N*-nitrosamines; TSNA

INTRODUCTION

In Sweden the use of oral moist snuff (snus) has increased considerably during the past three decades and sales in 2002 totaled 187 million snuff boxes, equivalent to about 6900 tonnes. Swedish Match produces more than 99% of all moist snuff on the Swedish market. However, during the past decade several small manufacturers have started to produce snuff, in most cases less than 100 tonnes per year. Snuff dipping is still increasing among both men and women. It has been estimated that ∼1 000 000 Swedes take snuff, including 190 000 women. Approximately 14% of the adult population in Sweden, 16 years of age and older, are snuff dippers. During the period 1996−2002, the number of women taking snuff in Sweden has increased by 137%. One reason for the increased use of snuff is that the users regard it 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 oral snuff (moist snuff) in the EU member countries—with Sweden as the sole exception. However, there is a market for dry snuff, used nasally, in the EU member countries Austria, France, Germany, and Italy. Nevertheless, recent studies have confirmed that incidences of oral cancer among Swedish men and women who take snuff are far below those of snuff users in the rest of the world (*2*, *3*). However, the results of the Swedish studies are compatible with a slightly increased risk, particularly for cancer in certain locations, such as the oral cavity (*4*).

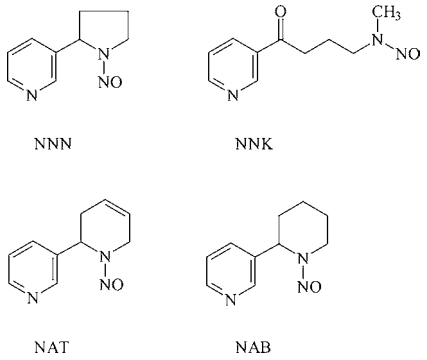

Figure 1. Chemical structures of the analyzed TSNAs: NNN = *N*′-nitrosonornicotine, NAT = *N*′-nitrosoanatabine, NAB = *N*′-nitrosoanabasine, and NNK = 4-(*N*-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).

Tobacco-specific *N*-nitrosamines (TSNAs), see **Figure 1**, are the most abundant carcinogens identified in tobacco and tobacco smoke, and are formed during the aging, curing, and fermentation of tobacco (*5*−*7*). NNK and NNN are the strongest animal carcinogens among the TSNAs, NAB is a weak carcinogen, and NAT was inactive in the rat study (*6*). Snuff and chewing tobacco have been reported to contain high concentrations, up to about 100 μg/g dry weight, of TSNAs (*8*, *9*). But during the period 1980−1992, a gradual decrease of up to 90% in two of the TSNAs, NNN and NNK, has occurred in the two most popular U.S. snuff brands (*10*, *11*). The TSNA content in Swedish moist snuff (snus) has also been considerably decreased—

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from a much lower level than the U.S. snuff—during the period 1983−1992, from 7.3 to 4.4 μg/g, based on the wet weight of the snuff, with a moisture content of about 50% (*11*, *12*). A study on snuff purchased in 1989/ 1990 shows TSNA levels in three samples of Swedish snuff ranging from 9.3 to 11.2 μg/g dry weight. (*13*). A recent study shows that U.S. brands of oral snuff still have rather high concentrations of TSNAs: the total levels of TSNAs ranged from 16.6 to 127.9 μg/g dry weight (*14*). In the study, one Swedish moist snuff sample (Ettan) was analyzed, and it had a far lower TSNA content, 2.8 μg/g dry weight. Recently, we found the same low levels of TSNAs in Swedish snuff (*15*). Previously, TSNAs have also been detected in the saliva of snuff users (*8*, *16*−*18*).

We have previously reported on the levels of TSNAs and volatile *N*-nitrosamines in snuff and chewing tobacco on the Swedish market between 1981 and 1983 (*19*, *20*) as well as on the levels of TSNAs in snuff and chewing tobacco in 1986 and 1992 (*12*). In the present study, the focus is on moist nonfermented oral snuff, the type of snuff commonly used in Sweden.

The purpose of the present study was to follow up the previous surveys by determining the TSNA levels in moist snuff on the Swedish market in 2001 and 2002. This was of particular interest as nowadays, in addition to Swedish Match, a number of small manufacturers are active in the Swedish market. A new EU Directive demands control of snuff as well as other tobacco products (*21*). On account of this, we have recently developed a rapid LC-MS/MS method for the analysis of TSNAs in snuff (*15*, *22*), which has been used in the present study.

MATERIALS AND METHODS

Materials. The snuff samples were purchased in 2001 and 2002 from shops in Uppsala and Enköping in central Sweden, and were also ordered on the Internet. Some of the small manufacturers also sent in snuff samples. The following Swedish manufacturers are included in the two surveys: Appeltofft's, Bengt Sändh:s Snusfabrique, Gellivare Snusfabrik HB, Gustavus Holding AB, KungsSnus HB (Åfors), Prisens Snus, SnusAB, and Swedish Match. All samples were analyzed within 8 weeks of purchase after storage in a refrigerator at 5 °C. Some of the manufacturers have labeled their snuff boxes with a "best before date". All samples were analyzed before this date.

Nitrosamine standards were purchased from Midwest Research Institute (Kansas City, MO); 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, pesticide grade (Lab-Scan), and methanol, gradient grade (Merck) were used for extraction and sample preparation, respectively. Sodium sulfate, analytical grade (Merck) was used for sample preparation. Formic acid 98−100% (Merck), 25% ammonium solution (Riedel-de-Haën, Hannover, Germany) and Milli-Q water were used for the preparation of mobile phase B, 10 mM ammonium formate, pH 4.0, in water/methanol (80 + 20, v/v). After the addition of 0.31 mL formic acid, ammonia was dropped into 800 mL Milli-Q water, to give a pH of about 4.0, and finally 200 mL methanol was added.

Methods. Ten milliliters ethyl acetate and 2.0 g sodium sulfate were added to 5.0 g snuff in a wide test tube capped with a Teflon-lined cap. The contents of the tubes were mixed by shaking before insertion in an ultrasonic bath for 10 min, and the sample was resuspended by shaking. Then, a 1.5 mL extract was evaporated to dryness under a stream of nitrogen, redissolved in 1.5 mL pure methanol and filtered through a 0.45 μm Teflon filter. Before analysis on LC-MS/MS, the sample was diluted five times with methanol to a concentration of 0.1 g snuff/mL.

Analyses were carried out using a liquid chromatograph (Waters Alliance 2690, Milford, MA) interfaced with a Quattro LC triple quadrupole mass spectrometer equipped with a standard pneumatically assisted electrospray (ES) ion source, operated in positive mode (Micromass, Manchester, U.K.). Experimental conditions were as follows: nebulizing gas at a flow of about 70 L/h, 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 optimum cone voltage was 17 V for all TSNAs, but the collision energy varied between 10 and 35 eV. For each compound, the following ions were detected: NNN, precursor ion $m/z = 178$, fragment ions $m/z = 105$, 120, and 148; NNK, precursor ion $m/z = 209$, fragment ions $m/z = 106$, 122, and 134; NAT, precursor ion $m/z = 191$, fragment ions $m/z = 79$, 106, and 160; NAB, precursor ion $m/z = 193$, fragment ions $m/z = 106$, 133, and 162.

For detection and quantification, 5.0 μL portions were analyzed against external standards by injection into a Genesis C18 column (100 × 3 mm, 4 μm, Jones Chromatography Ltd., Mid Glamorgan, U.K.) with a 1-cm long guard column packed with the same material. The mobile phase was filtered through a 0.45-μm-membrane filter (HVLP, Millipore, Ireland). Separation was performed using a gradient between methanol (mobile phase A) and 10 mM 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.

The accuracy of the method has been reported earlier (*22*). The mean recovery of the four TSNAs varied between 78 and 89%, and the relative standard deviation ranged from 5 to 11%. The limit of detection of the method was 0.01 μg/g for NNN, NNK, and NAT, and 0.005 μg/g for NAB.

All values are based on wet weight, the moisture content in moist snuff (snus) is about 55% when dried overnight at 70 °C. Freeze-drying of the snuff gave the same result.

Table 1. Tobacco-Specific Nitrosamines in Moist Snuff (Snus) on the Swedish Market in 2001[a]

country of origin	manufacturer and brand no.[c]		tobacco-specific *N*-nitrosamine content[b] (μg/g)				
			NNN	NNK	NAT	NAB	total
Sweden	A[d]	1	0.49	0.22	0.34	0.03	1.1
		2	0.49	0.22	0.34	0.03	1.1
		3	0.57	0.24	0.36	0.03	1.2
		4	0.41	0.20	0.32	0.03	0.96
		5	0.41	0.19	0.27	0.02	0.89
		6	0.36	0.16	0.27	0.03	0.82
		7	0.50	0.18	0.33	0.03	1.0
		8	0.50	0.21	0.32	0.04	1.1
		9	0.44	0.23	0.31	0.04	1.0
		10	0.50	0.13	0.28	0.03	0.94
		11	0.47	0.21	0.38	0.03	1,1
		12	0.76	0.32	0.49	0.04	1.6
		13	0.76	0.32	0.50	0.04	1.6
USA	I	14	0.71	0.24	0.33	0.03	1.3

[a] All values are based on wet weight, the moisture content in moist snuff (snus) is about 55%. [b] All values are from single determinations. [c] All samples have been purchased from local shops. [d] A is Swedish Match, which accounts for nearly 100% of the Swedish snuff market.

RESULTS AND DISCUSSION

The recently developed LC-MS/MS method has been used for the analysis of TSNAs in oral moist snuff (snus) on the Swedish market (*15*, *22*). In the survey in 2001, all samples except one, which was a snuff from the United States, were produced by Swedish Match, which accounts for nearly 100% of the Swedish snuff market. **Table 1** summarizes the results from the analysis of 14 samples of moist snuff. All samples represented different brands. All values in **Table 1** are from single determinations. The levels of TSNAs are based on the

Table 2. Tobacco-Specific Nitrosamines in Moist Snuff (Snus) of Swedish Origin on the Swedish Market in 2002[a]

manufacturer and brand no.[c]		source of sample[d]	tobacco-specific *N*-nitrosamine content[b] (μg/g)				
			NNN	NNK	NAT	NAB	total
A[e]	1	P	0.48	0.16	0.27	0.02	0.93
	2	P	0.56	0.18	0.31	0.03	1.1
	3	P	0.49	0.19	0.33	0.02	1.0
	4	P	0.46	0.18	0.26	0.02	0.92
	5	P	0.50	0.16	0.31	0.02	0.99
	6	P	0.48	0.15	0.24	0.02	0.89
	11	P	0.42	0.13	0.22	0.02	0.79
B	15	S	0.82	0.35	0.41	0.05	1,6
	16	S	0.98	0.53	1.40	0.13	3.0
C	17	O	0.52[3]	0.20[3]	0.29[3]	0.02[3]	1.0[3]
	18	O	0.53	0.18	0.31	0.02	1.0
D	19	S	0.53	0.09	0.20	0.03	0.85
	20	S	0.46	0.09	0.21	0.03	0.79
E	21	S	0.63[1]	0.26[1]	0.43[1]	0.05[1]	1.4[1]
	21	P	0.61	0.22	0.29	0.03	1.2
	22	S	0.65	0.20	0.35	0.03	1.2
	23	P	0.63[1]	0.36[1]	0.47[1]	0.05[1]	1.5[1]
	23	S	0.73	0.30	0.39	0.05	1.5
	24	S	0.59	0.39	0.48	0.05	1.5
F	25	S[f]	0.59[3]	0.45[3]	0.41[3]	0.05[3]	1.6[3]
G	26	S	0.73[3]	0.18[3]	0.32[3]	0.02[3]	1.3[3]
H	27	P	0.16[1]	0.03[1]	0.09[1]	0.01[1]	0.29[1]
	28	P	0.15[1]	0.04[1]	0.09[1]	0.01[1]	0.29[1]
	28	S	0.13	0.04	0.08	0.01	0.27
	29	P	0.23[1]	0.04[1]	0.13[1]	0.01[1]	0.41[1]
	29	S	0.28	0.08	0.22	0.02	0.60
	30	S	0.07	0.02	0.05	0.01	0.15

[a] All values are based on wet weight. [b] All values are the mean of two determinations, except for those marked "1" (single determination) or "3" (mean of three determinations). [c] The analyzed brands (1–6 and 11) of manufacturer A are the same brands as those analyzed in **Table 1**. [d] Samples marked "P" have been purchased from shops, those marked "O" have been ordered on the Internet, and those marked "S" have been sent in by the manufacturers. [e] A is Swedish Match, and B–H are small manufacturers. [f] This snuff was made in the laboratory by mixing all the ingredients.

wet weight of the snuff. The moisture content in moist snuff is about 55%. All the moist snuff samples contained detectable levels of all TSNAs, ranging from 0.82 to 1.3 μg/g wet weight. The mean level of the total TSNA content in snuff was 1.1 μg/g. As can be seen in **Table 1**, the range of levels of TSNAs in snuff is very small. NNN, NAT, and NNK were found at somewhat higher concentrations than NAB. The level of TSNAs in one moist snuff sample from the United States was in the same order, 1.3 μg/g, as in the Swedish moist snuff.

In the survey in 2002, moist snuff from Swedish Match, as well as a majority of the small manufacturers was tested. Many of the small manufacturers mainly sell their snuff in local shops. However, it is possible to purchase snuff on the Internet from some of them. Besides Swedish Match, seven small manufacturers were included in the survey in 2002. The samples were purchased from shops, sent in by the manufacturers, as well as ordered on the Internet. In one case, we had to make the snuff ourselves in the laboratory by mixing all the ingredients according to the manufacturer's instructions. **Table 2** summarizes the results from the analysis of 27 samples of moist snuff. The samples represented 23 different brands from eight manufacturers in Sweden. Most of the values in **Table 2** are the mean of two determinations. As in **Table 1**, the levels of TSNAs are on wet weight, with a moisture content of about 55%. All the samples contained detectable levels of all TSNAs, ranging from 0.15 to 3.0 μg/g wet weight. The mean of the total TSNA content in moist snuff was 1.0 μg/g. As noted previously, the range of levels of TSNAs in snuff was small, and NAB was found in somewhat lower concentrations than the other TSNAs.

Table 3. Levels of Tobacco-Specific *N*-Nitrosamines in Snuff and Chewing Tobacco on the Swedish Market in 1983–2002[a]

sample	country of origin	year	no. of brands[b]	tobacco-specific *N*-nitrosamine content (μg/g)					
				NNN	NNK	NAT	NAB	total	ref
moist snuff	Sweden	1983	16 (32)	3.8	0.80	2.5	0.17	7.3	*20*
		1986	18 (34)	4.3	0.75	2.9	–[c]	8.0	*12*
		1992	20 (20)	1.9	0.64	1.9	–	4.4	*12*
		2001	10 (10)	0.53	0.22	0.35	0.03	1.1	
		2002	23 (27)	0.49	0.19	0.32	0.03	1.0	
	USA	1983	1 (2)	2.0	0.30	0.70	0.05	3.1	*20*
		1986	3 (6)	23	13	29	–	65	*12*
		1992	1 (1)	2.1	0.12	1.3	–	3.5	*12*
		2001	1 (1)	0.71	0.24	0.33	0.03	1.3	
	Norway	1983	1 (2)	21	3.3	13	1.7	39	*20*
dry snuff	Germany	1986	2 (4)	0.68	0.10	0.31	–	1.1	*12*
	U.K.	1986	1 (2)	1.8	0.26	0.82	–	2.9	*12*
chewing tobacco	Sweden	1983	2 (4)	0.92	0.01	1.1	ND[d]	2.0	*20*
		1986	1 (2)	1.7	0.46	1.4	–	3.6	*12*
		1992	3 (3)	0.70	0.09	2.1	–	2.9	*12*
	USA	1983	3 (6)	1.1	0.11	0.94	0.02	2.2	*20*
		1986	2 (3)	0.25	0.08	0.15	–	0.48	*12*
	Denmark	1983	4 (8)	0.78	0.04	1.2	0.03	2.1	*20*
		1986	4 (8)	0.08	0.01	0.18	–	0.27	*12*
		1992	4 (4)	1.6	1.9	2.9	–	6.4	*12*

[a] All values are based on wet weight. [b] Number in parentheses is the number of samples analyzed. [c] NAB content is included in the figure for NAT. [d] Not detected.

The mean of the total TSNA content in the snuff from the small manufacturers (B−H), 1.1 μg/g, was nearly the same as the mean of the total TSNA content in the snuff from Swedish Match (A), 0.95 μg/g. However, the range of total TSNAs was larger, from 0.15 to 3.0 μg/g. The snuff from one of the small manufacturers (H) contained lower levels of TSNAs, from 0.15 to 0.60 μg/g (mean 0.34 μg/g), than the rest of the manufacturers, including Swedish Match. Samples that were sent in by the manufacturers, marked S in **Table 2**, showed the same content of TSNAs as those purchased from shops or on the Internet.

During the past two decades, the TSNA levels in moist snuff on the Swedish market have shown a noticeable decrease of about 85%, as can be seen in **Table 3**. Swedish Match produced all Swedish moist snuff and chewing tobacco samples from 1983, 1986, as well as 1992, in **Table 3**. No samples of chewing tobacco and dry snuff have been analyzed in the survey in 2001 and 2002. The TSNA levels in moist snuff of Swedish origin on the Swedish market are substantially lower than in the moist snuff sold in the United States in 2001 (*14*). There is one important difference in the way that Swedish and American moist snuff is manufactured, which may influence the TSNA content. Swedish moist snuff is heat-treated, giving rise to a sterile snuff product, while most American moist snuff is fermented, which may result in higher concentrations of, for instance, nitrite and nitrosamines, especially TSNAs.

In conclusion, the results of the present study show that, during the past two decades, the levels of TSNAs in moist snuff produced by Swedish Match, the leading snuff manufacturer in Sweden, have decreased noticeably. However, all new small manufacturers have also achieved the same low levels of TSNAs in their products. Thus, the efforts by the manufacturers to reduce the TSNA levels in moist snuff on the Swedish market have been successful.

SAFETY

All TSNAs are carcinogenic in animal studies. Thus, they have to be handled with great care.

ABBREVIATIONS USED

TSNAs, tobacco-specific *N*-nitrosamines; LC, liquid chromatography; MS, mass spectroscopy; NNN, *N*′-nitrosonornicotine; NAT, *N*′-nitrosoanatabine; NAB, *N*′-nitrosoanabasine; NNK, 4-(*N*-methylnitrosamino)-1-(3-pyridyl)-1-butanone; IARC, International Agency for Research on Cancer; EU, European Union; ES, electrospray.

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