

N-Nitrosamines in Chewing Tobacco: An International Comparison

Klaus D. Brunnemann,* Lise Genoble, and Dietrich Hoffmann

Samples of commercial smokeless tobacco products from six countries were analyzed for nitrate, nicotine, and volatile, nonvolatile, and tobacco-specific *N*-nitrosamines. Volatile nitrosamines in these tobaccos were found to be at relatively low concentration (<100 ppb), while the nonvolatile nitrosodiethanolamine and nitrosoproline and the tobacco-specific *N*-nitrosamines exceeded 1000 ppb in all except the Russian nass samples. In the 11 moist snuff samples, the tobacco-specific *N*-nitrosamines ranged from 5900-240 000 ppb, exceeding by at least 2 orders of magnitude the levels of carcinogenic *N*-nitrosamines in other consumer products. This and the fact that in the U.S.A. alone more than 8 million snuff dippers are at an increased risk for oral cancer emphasize the need for product modifications with the goal to significantly reduce the levels of carcinogenic nitrosamines in snuff.

Chewing tobacco has found increasing acceptance as a substitute for cigarette smoking, primarily in the U.S.A. and in Scandinavian countries (IARC, 1985). In the U.S.A., one distinguishes between four kinds of chewing tobacco. These are plug tobacco, loose-leaf (or scrap) tobacco, twist (or roll) tobacco, and snuff tobacco (dry and moist). Each of these varieties is produced by mixing several types of tobacco and additives, as well as by using different manufacturing processes; their composition is often determined by the availability of tobacco types and by regional customs and preferences. Plug tobacco generally contains leaves of Burley and Virginia as well as cigar tobacco. It is sweetened with honey, sugar, molasses, syrup, and/or licorice, then pressed into flattened blocks, and wrapped in natural leaf. Loose-leaf or scrap tobacco is made of fermented cigar leaf tobacco; some brands are lightly sweetened, and others carry large amounts of sugars, syrups, licorice, and other flavoring materials. It is packaged as a batch of loose pieces or cut strips. Twist or roll tobacco is made of cured leaf, treated with a tarlike extract from tobacco leaves, and twisted into strands that are allowed to dry. Snuff consists of powdered dark, air-cured, and fire-cured tobaccos that may have been sweetened with sugars, molasses, and/or syrup. Some snuff brands are treated with wintergreen oil, licorice, and other flavors.

In India where betel quid chewing is more popular, the per capita consumption of chewing tobacco and snuff (often mixed with safran, menthol, and aromatic spices) is less pronounced. Most chewing tobacco is used as an ingredient of betel quid, which also contains areca nut and fine lime, and is wrapped in areca leaf. People in some of the Asian republics of the Soviet Union, especially in Uzbek, Tadzhik, and the Turkmenian Republics chew nass quid. The incidence rates for oral cancer in men and women in these republics are higher than that in the other areas of the USSR. Nass use has been incriminated as an etiologic factor for the increased oral cancer rates. Nass or nassvay is usually made with local tobaccos (sometimes only partially cured), ash, cotton oil or sesame oil, and lime (Paches and Milievskaya, 1980). In some European countries such as Germany the custom of sniffing tobacco in the nostrils is more popular than chewing of tobacco.

In Oct 1984, the International Agency for Research on Cancer concluded "There is sufficient evidence that oral

use of snuffs of the types commonly used in North America and in western Europe is carcinogenic to humans. There is limited evidence that chewing tobacco of the types commonly used in those areas is carcinogenic" (IARC, 1985). There is also, at this time, limited evidence that the use of nass practiced in the USSR is carcinogenic to humans (Zaridze et al., 1985). Currently, the only well-established groups of carcinogens in chewing tobaccos are the volatile, nonvolatile, and tobacco-specific *N*-nitrosamines. It is likely, though, that processed tobacco also contains traces of carcinogenic polynuclear aromatic hydrocarbons and polonium-210 (Hoffmann and Hecht, 1985; IARC, 1985). In addition to the dependence on concentrations of the alkaloids and nitrate in tobacco, the yields of the nitrosamines are greatly influenced by the processes involved in the manufacture of the smokeless tobaccos. These processes most likely affect the reduction of nitrate to *N*-nitrosating species (Anderson et al., 1982; Brunnemann et al., 1983). The highest yields of nitrosamines occur in fire-cured tobacco and/or highly fermented snuffs (Brunnemann et al., 1983; Hoffmann and Hecht, 1985).

It was the goal of this study to compare some chewing tobaccos from different countries on the basis of their nitrosamine yield, their nitrate and nicotine content, and their pH levels. The pH of tobacco is important in that it influences formation of nicotine-derived nitrosamines (Hecht et al., 1978).

EXPERIMENTAL SECTION

Materials. The smokeless tobacco products were purchased in 1984 and 1985 on the open markets in the various countries. However, nass tobaccos were collected in farmers' markets in Samarkand Oblast of Uzbek, SSR, by D. G. Zaridze of the International Agency for Research on Cancer, Lyon, France, and sent to the analytical laboratory in plastic bags. Upon arrival, the tobacco products were refrigerated ($\approx 4^\circ\text{C}$). Samples were analyzed immediately after opening the tightly closed containers or the plastic bags containing the nass samples.

Chemicals. The standard mixture of volatile *N*-nitrosamines (VNA) was purchased from Thermo Electron Corp., Waltham, MA, and the tobacco-specific *N*-nitrosamines (TSNA) were synthesized according to earlier published methods (Hoffmann et al., 1979). Nitrosoproline (NPRO) was synthesized from proline according to the method of Lijinsky et al. (1970).

Nitrosodiethanolamine (NDELA) was synthesized by nitrosation of diethanolamine. [^{14}C]Nitrosodimethylamine and [^{14}C]nitrosonor nicotine were purchased from New

Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, New York 10595.

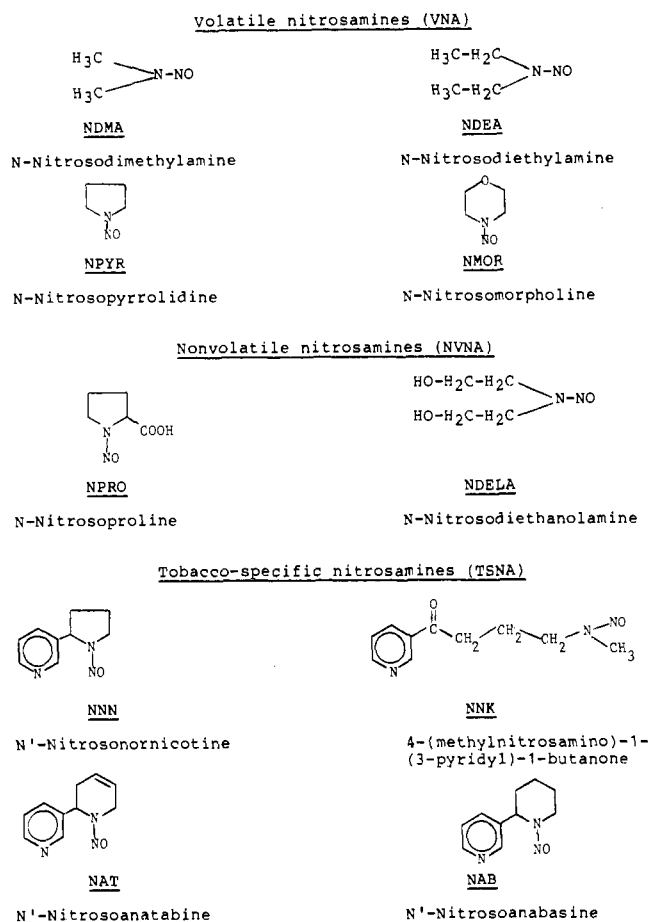


Figure 1. N-Nitrosamines in tobacco.

England Nuclear, Boston, MA. [^{14}C]NDELA was obtained by nitrosation of [$2\text{-}^{14}\text{C}$]diethanolamine hydrochloride (Dhom Products, North Hollywood, CA) (Brunnemann and Hoffmann, 1981), and [^{14}C]NPRO was synthesized from [$U\text{-}^{14}\text{C}$]-L-proline (ICN, Irvine, CA) (Brunnemann et al., 1983). Figure 1 presents the formulas of the N-nitrosamines determined in smokeless tobacco.

Apparatus. A Model 543 thermal energy analyzer (TEA) from the Thermo Electron Corp. (Waltham, MA) was interfaced directly with a Model 5890 gas chromatograph (Hewlett-Packard, Paramus, NJ) by using modifications described previously (Brunnemann and Hoffmann, 1981).

Analytical Procedures. For the analysis of VNA, TSNA, NDELA, and NPRO we employed earlier published methods all of which include precautions to avoid the artifactual formation of N-nitrosamines during extraction and analysis (Brunnemann et al., 1977; Hoffmann et al., 1979; Brunnemann and Hoffmann, 1981; Adams et al., 1983; Brunnemann et al., 1983). For the determination of moisture in tobacco, a modified Dean-Stark procedure was used (von Bethmann et al., 1961). The pH of the chewing tobaccos was measured after adding 10 mL of distilled water to 1 g of tobacco, followed by shaking. Nitrate in tobacco was determined by the specific ion electrode method (Jacin, 1970). Nicotine was analyzed by gas chromatography after enrichment by steam distillation (Schmeltz et al., 1976).

RESULTS AND DISCUSSION

Table I lists the data for the moisture content of tobacco, the pH of the tobacco suspensions, and nitrate, nicotine, and volatile, nonvolatile, and tobacco-specific N-nitrosamine contents in the smokeless tobacco products from six countries. The analytical data for the nitrosamines are

the actual concentrations of these compounds in the samples since all precautions were met to prevent their artifactual formation during extraction and analysis (Hoffmann et al., 1979).

Since the nass samples from the USSR contain lime, their pH is remarkably high (pH 11.0–11.8) in comparison to the other samples. Disregarding the buffering properties of saliva, at pH 11 or more, nicotine is completely unprotonated and its rate of absorption is thus accelerated (Brunnemann and Hoffmann, 1974; Armitage and Turner, 1970). When such nass samples are placed into the oral cavity, nicotine will reach the central nervous system very quickly.

The Indian and German samples have low pH values (4.4–6.4). At that pH, nicotine is mostly monoprotonated and less active. The domestic and the Swedish tobacco yielded values mostly around pH 8 at which approximately half of the nicotine is monoprotonated and half is unprotonated. It appears that by selectively "producing" a given pH value in chewing tobacco one may influence the absorption and thereby the extent of pharmacological activity of nicotine. Furthermore, at alkaline pH the possible endogenous formation of N-nitrosamines will be inhibited (Hecht et al., 1978; Hoffmann and Hecht, 1985).

The values for the volatile nitrosamines are fluctuating and do not reveal a specific trend. This had to be expected because the VNA formed during aging, curing, and/or fermenting of the tobaccos are at least partially lost during processing because of their volatility. The presence of N-nitrosomorpholine in some of the tobaccos indicates contamination with morpholine either due to the additives and/or because of diffusion from packing materials (Brunnemann et al., 1982). The levels of VNA are in agreement with those reported for Swedish chewing tobacco (Österdahl and Slorach, 1983).

The relatively high values for the carcinogenic N-nitrosodiethanolamine in some smokeless tobaccos (>200 ppb) are explained, at least partially, by the presence of residual diethanolamine due to tobacco treatment with the sucker growth inhibitor maleic hydrazide–diethanolamine (MH-30; Brunnemann and Hoffmann, 1981). In Oct 1981, MH-30 was banned for tobacco cultivation in the U.S.A. (Environmental Protection Agency, 1981). It is expected that smokeless tobaccos will not be entirely free of residues from MH-30, and thus of NDELA, until the later years of this decade. As observed for one moist snuff brand and one loose-leaf chewing tobacco brand, a gradual decrease of NDELA has already occurred for smokeless tobaccos in the U.S.A., indicating that recent commercial products contain tobaccos that were not treated with MH-30 (Table II). In the case of tobacco products with low NDELA values, we speculate that these traces originate, at least partially, from inadvertent contamination of the tobaccos with diethanolamine and/or triethanolamine during processing and packaging. These synthetic chemicals are occurring ubiquitously in our environment (IARC, 1978; Elder, 1983).

The occurrence of little or no nitrosoproline in the yellow-green nass samples from the USSR (Table I, P–S) is probably related to the fact that the curing process for these tobaccos is relatively brief (Zaridze et al., 1985). In fact, it appears that the intensive treatment during fermentation of the moist tobaccos favors nitrosation reactions as indicated by the NPRO yields (Table I, A–F and I–M), even though the concentrations of NPRO found in moist snuff samples were not correlated with the nitrate content of the tobaccos. The NPRO data indicate that the nitrosamine formation is markedly influenced by the

Table I. Analysis of Smokeless Tobacco Products^a

samples	type of product	% H ₂ O cont	pH	NO ₃ ⁻ , %	nicotine, %	VNA, ppb				NVNA, ppb				TSNA, ppb			
						NDMA	NDEA	NPYR	NMOR	NDELA	NPRO	NNN	NAT	NAB	NNK	total	
U.S.A.	A	52.4	8.88	2.68	3.64	46.5	ND	93.8	19.5	890	14000	9040	32500	2050	680	44270	
	B	43.8	5.99	1.58	3.05	26.5	ND	15.4	9.8	5910	7820	5790	3500	180	80	9550	
	C	19.7	6.10	1.34	0.73	ND	ND	12.4	4.2	160	7620	3120	1340	190	750	5400	
	D	50.2	8.14	3.65	2.50	46.9	ND	41.7	ND	880	7830	7060	6480	500	1190	15230	
	E	39.9	7.54	2.95	3.22	85.1	ND	291	29.4	4260	30500	28000	33300	1660	3260	66220	
	F	31.4	7.41	5.39	1.69	24.3	ND	86	30.0	1400	4930	8580	8440	350	1400	18770	
	G	16.4	6.37	4.45	0.75	4.12	ND	ND	ND	600	463	670	2300	140	380	3490	
Canada	H	13.9	5.28	0.59	1.71	ND	ND	ND	ND	110	100	2090	1580	100	240	4010	
	I	52.3	8.23	1.56	3.33	72.8	ND	321	21.9	2720	16600	79100	152000	4000	5800	240100	
	J	49.6	7.50	4.68	3.10	23.0	ND	337	32.8	1180	8800	50400	170000	4800	3200	228400	
Sweden	K	53.3	8.68	2.62	3.31	ND	ND	22.1	ND	290	8210	3050	1840	130	870	5890	
	L	50.3	7.30	2.22	3.31	ND	ND	12.2	ND	300	3120	3660	2200	130	950	6940	
	M	52.4	8.52	2.13	3.05	ND	ND	20.2	9.1	230	4680	4140	2180	150	1030	7500	
FRG	N	12.1	5.01	1.41	1.36	ND	ND	ND	ND	50	700	2130	500	50	40	2720	
	O	10.1	5.05	1.17	1.34	ND	ND	ND	ND	50	550	1420	330	30	30	1810	
	P	7.25	11.8	0.74	3.02	ND	ND	8.82	ND	40	ND	520	300	30	110	960	
	Q	8.63	11.0	0.58	2.61	ND	ND	1.80	ND	40	ND	140	32	8	20	200	
	R	10.1	11.1	1.06	1.31	ND	ND	1.74	ND	40	70	120	32	8	30	190	
	S	6.37	11.0	0.82	3.07	ND	ND	4.33	ND	40	180	520	170	10	130	830	
India	T	11.0	5.34	0.65	3.70	69.6	ND	1.55	ND	40	210	570	390	30	180	1170	
	U	13.0	6.42	0.66	2.05	ND	ND	4.48	ND	40	190	540	450	50	130	1170	
	V	12.3	5.31	0.70	3.87	ND	ND	1.73	ND	30	290	470	370	30	130	1000	
	W	11.5	4.36	0.80	2.77	0.56	ND	2.70	ND	110	410	850	300	70	230	1450	

^a Abbreviations for N-nitrosamines: see Figure 1. Abbreviations for type of product: MS, moist snuff; LL, loose-leaf or scrap tobacco; PL, plug tobacco; N, nass; IT, Indian chewing tobacco used in betel quid. ND, not detected (in the case of VNA <0.2 ppb, NVNA <1 ppb, TSNA <5 ppb). All values given for nitrate, nicotine, and nitrosamines are based on dry weight of tobacco.

Table II. N-Nitrosodiethanolamine in Two Brands of U.S. Smokeless Tobaccos (ppb), 1981-1985

sample	Feb 1981	Feb 1983	% cge ^a	Nov 1983	% cge ^a	Aug 1984	% cge ^a	Feb 1985	% cge ^a
moist snuff	6840	3035	-56	982	-86	4260	-38	2900	-58
loose-leaf chewing tobacco	224	151	-33	168	-25	165	-26	83	-63

^aRelative to Feb 1981.

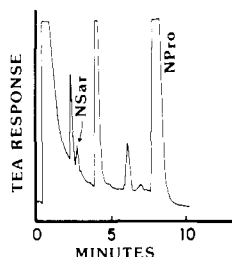


Figure 2. GC-TEA trace of the methyl esters of NPRO and NSAR in Swedish sample M.

processing of tobacco. We also detected N-nitrososarcosine (NSAR) in one sample (20 ppb, Swedish M, Figure 2).

The highest levels of tobacco-specific N-nitrosamines, including the highly carcinogenic NNN and NNK (Hoffmann and Hecht, 1985; IARC, 1985), were found in moist snuff. As in the case of NPRO, we did not detect a correlation between nitrate and/or nicotine content and the concentration of individual TSNA or total TSNA in the moist snuff samples. Again, the absence of such correlations is thought to relate to the differences in the processes leading to moist snuff, a fact well documented in an earlier study by Anderson et al. (1982). However, with one exception smokeless tobaccos low in nitrate (≤ 1.17) were also low in total TSNA (≤ 1810 ppb).

The analytical data of this study (Table I) underline the urgent need for quantitative reduction of the carcinogenic nicotine-derived nitrosamines in snuff. So far we have found only one moist snuff brand that had relatively low TNSA levels (1290 ppb; Brunnemann et al., 1982). This observation indicated that at least one process is available that effects partial inhibition of nitrosamine formation during the manufacturing of snuff. In addition, we have shown that TSNA are formed during "aging" of snuff on the open air and that wrapping of individual snuff portions in small aluminum envelopes prevents the increase of TSNA by "aging" (Hoffmann and Adams, 1981).

The occurrence in snuff products of the highly carcinogenic nitrosomorpholine (IARC, 1978; Preussmann and Stewart, 1984; Lijinsky, 1985) can be avoided as is indicated by the data for two Swedish samples (Table I, K and L). Moist snuff is used for snuff dipping, the practice of extracting juices from a pinch of fine-cut chewing tobacco, placed between the cheek and the gum. The concentration in moist snuff of NNN (3-79 ppm) and total TSNA (5-240 ppm) exceeds by at least 2 orders of magnitude the occurrence of carcinogenic N-nitrosamines in any other consumer product (IARC, 1978, 1985; National Research Council, 1981). Furthermore, TSNA have been detected in the saliva of snuff dippers (Hoffmann and Adams, 1981). The general popularity of snuff dipping, especially among young people, has increased substantially. Currently, there are an estimated 8 million snuff consumers in the U.S.A.

Epidemiological studies indicate that snuff dippers are at high risk for oral cancer (IARC, 1985). Furthermore, snuff contains high concentrations of the potent, nicotine-derived carcinogenic nitrosamines. Thus, the increased popularity of snuff dipping becomes a public health concern.

ACKNOWLEDGMENT

We thank Dr. David Zaridze, IARC, Lyon, France, for providing the nass samples, Dr. S. V. Bhide, Tata Memorial Centre, Bombay, India, for sending us the Indian tobaccos, and Dr. H. F. Stich, British Columbia Cancer Research Centre, Vancouver, Canada, for the Canadian samples.

Registry No. NDELA, 1116-54-7; NPRO, 7519-36-0; NDMA, 62-75-9; NDEA, 55-18-5; NPYR, 930-55-2; NMOR, 59-89-2; NNN, 16543-55-8; NNK, 64091-91-4; NAT, 71267-22-6; NAB, 1133-64-8; nicotine, 54-11-5; nitrate, 14797-55-8.

LITERATURE CITED

- Adams, J. D.; Brunnemann, K. D.; Hoffmann, D. *J. Chromatogr.* 1983, 256, 347.
- Anderson, R. A.; et al. *J. Agric. Food Chem.* 1982, 30, 663.
- Armitage, A. K.; Turner, D. M. *Nature (London)* 1970, 226, 1231.
- Brunnemann, K. D.; Hoffmann, D. *Food Cosmet. Toxicol.* 1974, 12, 115.
- Brunnemann, K. D.; Hoffmann, D. *Carcinogenesis (London)* 1981, 2, 1123.
- Brunnemann, K. D.; Scott, J. C.; Hoffmann, D. *Carcinogenesis (London)* 1982, 3, 693.
- Brunnemann, K. D.; Scott, J. C.; Hoffmann, D. *J. Agric. Food Chem.* 1983, 31, 905.
- Brunnemann, K. D.; Yu, L.; Hoffmann, D. *Cancer Res.* 1977, 37, 3218.
- Elder, R. L. *J. Am. Coll. Toxicol.* 1983, 2, 183.
- Environmental Protection Agency *Fed. Reg.* 1981, 46, (179), 45999.
- Hecht, S. S.; et al. *J. Org. Chem.* 1978, 43, 72.
- Hoffmann, D.; Adams, J. D.; Brunnemann, K. D.; Hecht, S. S. *Cancer Res.* 1979, 39, 2505.
- Hoffmann, D.; Adams, J. D. *Cancer Res.* 1981, 41, 4305.
- Hoffmann, D.; Hecht, S. S. *Cancer Res.* 1985, 45, 935.
- International Agency for Research on Cancer *IARC Monogr.* 1978, 17, 365.
- International Agency for Research on Cancer *IARC Monogr.*, in press.
- Jacin, H. *Tobacco Sci.* 1970, 14, 28.
- Lijinsky, W. Proceedings on the Organic and Biological Chemistry of Carcinogenic and Carcinostatic Agents Containing Nitrogen-Nitrogen Bonds, Harpers Ferry, WV, 1985.
- Lijinsky, W.; Keefer, L.; Loo, J. *Tetrahedron Lett.* 1970, 26, 5137.
- National Research Council In "The Health Effects of Nitrate, Nitrite and N-Nitroso Compounds"; National Academy Press: Washington, DC, 1981; Part 1, Chapter 7.
- Österdahl, B.-G.; Slorach, S. A. *Food Chem. Toxicol.* 1983, 21, 759.
- Paches, A. I.; Milievskaia, I. L. *NIH Publ.* 1980, No. 802044, 177.
- Preussmann, R.; Stewart, B. L. *Am. Chem. Monogr.* 1984, 182, 643.
- Schmeltz, I.; Brunnemann, K. D.; Hoffmann, D.; Cornell, A. *Beitr. Tabakforsch.* 1976, 8, 367.
- von Bethmann, M.; Lipp, G.; Van Nooy, H. *Beitr. Tabakforsch.* 1961, 1, 19.
- Zaridze, D. G.; et al. *Int. J. Cancer* 1985, 36, 153.

Received for review May 13, 1985. Accepted June 19, 1985. This is study No. LXXXIV of the series "Chemical Studies on Tobacco Smoke". It was presented in part at the 38th Tobacco Chemists' Research Conference, Atlanta, GA, Nov 5-8, 1984. The study was supported by Research Grant PO1-CA-29580 from the National Cancer Institute.