Effects of Storage Conditions on Levels of Tobacco-Specific *N*-Nitrosamines and *N*-Nitrosamino Acids in U.S. Moist Snuff†

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Oral snuff is the only commercial tobacco product with increasing sales on the U.S. market. The carcinogenic activity of snuff and other smokeless tobacco products is largely attributed to the presence of *N*-nitrosamines, and especially to tobacco-specific *N*-nitrosamines (TSNA). In this study of the effects of aging and storage on the levels of TSNA, *N*-nitrosamino acids (NAA), and volatile *N*-nitrosamines (VNA) in commercial moist snuff, it was found that during storage at 4 °C none of these compounds increased significantly. However, at ambient room temperature, or at 37 °C, the levels of *N*-nitrosamines and nitrite of the snuff increased significantly after 4 weeks of storage; whereby levels of carcinogenic TSNA rose from 6.24 to 18.7 ppm, NAA from 3.13 to 16.3 ppm, and VNA from 0.02 to 0.2 ppm. The importance of this finding with respect to cancer risk through the use of oral tobacco products is discussed. This study also led to the identification and quantitative determination of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol in moist snuff.

INTRODUCTION

The consumption of oral snuff in the United States has steadily risen from 43.8 million pounds in 1982 to 56.0 million pounds in 1992 (U.S. Department of Agriculture, 1992). One major reason for this trend is the growing prevalence of snuff dipping among male adolescents (Orlandi and Boyd, 1989); constraint of smoking in public places may be another reason. Smokeless tobacco is known to contain at least 28 carcinogens, including the tobacco-specific nitrosamines (TSNA; Hoffmann et al., 1992). The latter are known carcinogens in animals and are regarded as at least partly responsible for the excess of oral cancer among snuff-dippers (Preston-Martin, 1991). During the past decade, the levels of *N*-nitrosamines in the leading snuff brands in the United States and Sweden have gradually declined (Djordjevic et al., 1993; Brunnemann and Hoffmann, 1991), even though a new snuff brand, introduced in the United States in 1990 and sold until 1992, contained the highest concentrations of TSNA (up to 177 ± 95 ppm) and *N*-nitrosamino acids (NAA; up to 85 ± 58 ppm) ever determined in a commercial tobacco product (Djordjevic et al., 1993). Andersen et al. (1989, 1991) have recently shown that nitrite and TSNA levels increased substantially in the University of Kentucky reference moist snuff when the product was stored at 24 and 32 °C, respectively, for 52 weeks, in sealed 1-L Mason jars or in the original tins that were exposed to ambient air. The increases of the highly carcinogenic 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N'*-nitrosonornicotine (NNN), were 14- and 33-fold, respectively. Higher moisture content had a greater effect than higher temperature on the increases of TSNA and nitrite levels in the snuff. NNK concentration in a commercial moist snuff exposed to air in a porous paper bag doubled in just 2 weeks (Hoffmann and Adams, 1981). When burley tobacco (approximately 10% in moist snuff) was exposed to adverse temperature and humidity conditions (32 °C, 90% RH), significant increases in NNN, NNK, and nitrite levels occurred within 7 days, but then the levels of these constituents declined (Burton et al., 1989). Treatment of tobacco with streptomycin or rifampicin did not inhibit the formation of nitrite or nitrosamines. When air-cured or fire-cured KY 171 dark tobaccos, which are also utilized for moist snuff formulations (approximately 25%), were stored at 24 and 32 °C for 52 weeks, no significant changes in TSNA levels were observed (Andersen et al., 1990). In view of these findings with reference snuff and raw tobaccos, and because of the rising consumption of moist snuff and the carcinogenicity of several TSNA and NAA, we deemed it to be important to undertake a systematic study of the effect of storage conditions on *N*-nitrosamine formation in a leading commercial U.S. snuff brand.

In this study we also identified and quantified the NNK-derived 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in moist snuff. Like NNK, NNAL is a strong inducer of tumors in laboratory animals (Hoffmann et al., 1993), and its presence in moist snuff adds to the overall carcinogenic potential of smokeless tobacco.

EXPERIMENTAL PROCEDURES

Materials. A leading commercial U.S. moist snuff brand was purchased from retailers in Westchester County, NY, in 1991 and 1992. The 1991 snuff was manufactured on June 27, 1991, purchased on July 2, and stored immediately in a cold room. The first analysis of this sample was done on July 9, 1991, i.e., within 2 weeks of the manufacture date. Another 1991 snuff sample (batch 2) was manufactured on August 15, purchased on August 20, and first analyzed on August 21. The 1992 snuff was manufactured on September 24, 1992, purchased on October 6, and first analyzed on October 8, 1992. The experimental design was as follows: (A) Snuff was stored in a cold room (4 °C). (B) Snuff was stored on the shelf at ambient temperature and humidity. (C) Snuff was kept in an incubator at 37 °C, at 85% relative humidity (RH), with an air flow of 20 mL/min. Both batches of the 1991 snuff were stored in individually sealed cardboard tins, whereas 1992 snuff was stored in plastic-wrapped 10-tin sleeves. Samples to be analyzed were taken at various

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Table I. Changes in the Chemical Composition of a U.S. Moist Snuff Brand during Storage: Cold Room (4 °C)[a]

			mg/g			TSNA, μg/g			NAA, μg/g		
storage duration, weeks	H_2O, %	pH	NO_2 N	NO_3 N	nicotine	NNN	NNK	NAT	MNPA	MNBA	NPRO
individual tins (1991)											
0	56.7	6.69	0.084	4.17	18.6	7.99	0.77	5.64	4.45	0.41	8.09
2	58.0	6.80	0.080	3.94	19.4	8.11	0.82	5.38	4.23	0.42	8.32
4	57.6	6.95	0.066	3.53	16.7	7.09	0.64	4.69	4.37	0.39	7.13
8	59.8	7.09	0.060	3.77	18.1	7.40	0.68	5.27	4.69	0.40	8.24
11	59.3	6.99	0.083	3.93	16.8	7.31	0.58	4.68	3.54	0.36	6.18
16	61.8	6.82	0.087	3.79	19.5	6.12	0.52	3.78	3.42	0.37	5.92
26	56.3	6.83	0.086	4.82	17.4	7.05	0.64	4.58	2.96	0.28	5.05
32	55.6	7.02	0.064	4.72	16.6	6.60	0.77	4.59	3.37	0.51	3.66
av	58.1	6.90	0.076	4.08	17.9	7.21	0.68	4.83	3.88	0.39	*b*
SD	1.9	0.13	0.011	0.43	1.1	0.62	0.10	0.55	0.59	0.06	
10-tin sleeves, wrapped in plastic (1992)											
0	48.5	6.96	0.058	5.68	16.7	5.64	0.60	3.40	2.84	0.29	4.50
8	49.9	7.12	0.088	5.47	16.3	5.85	0.54	3.27	2.96	0.24	4.86

[a] Values are based on dry weight. [b] Significant decreasing trend over time ($P < 0.001$).

time points, up to 32 weeks from the beginning of the experiment. At each time point a set of three to five individually sealed tins or one 10-tin sleeve was opened and the tobacco was frozen in liquid nitrogen, freeze-dried, and ground.

For the identification of NNAL, a newly introduced commercial snuff brand containing high levels of carcinogenic TSNA (65 ppm of NNN and 4.3 ppm of NNK; Djordjevic et al., 1993) was utilized. This brand had been purchased in Reno, NV, in 1991.

Reagents and Standards. All chemicals and solvents were analytical reagents of the highest purity from J. T. Baker Chemical Co., Phillipsburg, NJ, Fischer Scientific Co., Fair Lawn, NJ, Aldrich Chemical Co. Inc., Milwaukee, WI, and Alltech Associates, Inc., Deerfield, IL. Individual TSNA and NAA, which were utilized as standards for the analyses, were obtained as follows: NNN and NNK were synthesized according to the methods of Hu et al. (1974) and Hecht et al. (1977); NNAL was synthesized by reduction of NNK with sodium borohydride (Hecht et al., 1980); *N'*-nitrosoanatabine (NAT), *N*-nitrososarcosine (NSAR), and *N*-nitrosoproline (NPRO) were purchased from the NCI Chemical Carcinogen Reference Standard Repositories, Midwest Research Institute, Kansas City, MO. 3-(Methylnitrosamino)propionic acid (MNPA) and 4-(methylnitrosamino)butyric acid (MNBA) were synthesized according to the method of Gerjovich and Harrison (1966), and *N*-nitrosoguvacoline (NG) was prepared according to the procedure of Christensen and Krogsgaard-Larsen (1977). The purity of the reference compounds (≥99%) was verified by capillary GC with flame ionization detection and by NMR and MS. The reference mixture of seven volatile N-nitrosamines (VNA) and *N*-nitrosodipropylamine (NDPA) were purchased from Thermedics, Woburn, MA.

Apparatus. The instruments used in this study were previously described (Djordjevic et al., 1989, 1991; Brunnemann et al., 1982). Snuff was stored at "high temperature/high humidity" conditions (37 °C, 85% RH) in an incubator of the Thermolyne Compact CO_2 Series 5000 (Fisher Scientific, Springfield, NY). The ambient temperature was monitored by a Dickson self-contained 7-day temperature recorder (7.2–32.2 °C range; Dickson Co., Addison, IL).

GC–MS analysis of derivatized NNAL was carried out with a Hewlett-Packard GC Model 5890 interfaced with a Hewlett-Packard 5970 MSD in the electron impact mode. The analyses were performed by splitless injection (purge time 1 min) on a 0.25-mm × 30-m OV-225 (0.5-μm film thickness) fused silica capillary column purchased from On-Site Instruments, Columbus, OH. The injector was kept at 230 °C; the transfer line temperature was 250 °C. Helium was the carrier gas (head pressure 9 psi). The oven temperature was programmed as follows: 60 °C for 2 min raised at 30 °C/min to 140 °C; this was held for 3 min; heated at 4 °C/min to 210 °C and held for 120 min. Under these conditions, TMSi–NNAL eluted after 37.62 min.

Methods. The analytical methods for the determination of water, alkaloids, nitrite and nitrate-nitrogen, pH, tobacco-specific *N*-nitrosamines, and *N*-nitrosamino acids in snuff were previously reported (von Bethmann et al., 1961; Armstrong et al., 1967; Crutchfield and Burton, 1989; Djordjevic et al., 1989, 1991). Volatile *N*-nitrosamines (VNA) were analyzed by means of a procedure that was slightly modified from that described by Brunnemann et al. (1977, 1982): 10 g of snuff was extracted for 2 h under magnetic stirring with 100 mL of citrate buffer (pH 4.5) containing 20 mM ascorbic acid and 5 g/mL NDPA as an internal standard (dichloromethane used for the partitioning of the aqueous tobacco extract was free of VNA). The concentrations of individual VNA were calculated on the basis of their recoveries, which were determined in separate experiments in which 1 mL of a reference standard mixture was carried through the same analytical procedure as the tobacco samples. The recoveries of the seven VNA ranged from 46% [*N*-nitrosodimethylamine (NDMA)] to 80% [*N*-nitrosodibutylamine (NDBA)] with coefficients of variations ranging from 9.4% for NDBA to 35% for NDMA (average of four experiments). The detection limit of each VNA was 50 pg/injection. All samples were both extracted and analyzed by GC-TEA in duplicates.

NNAL and other TSNA were extracted from moist snuff by partitioning an aqueous extract of the tobacco at pH 9 with ethyl acetate (Djordjevic et al., 1989). Upon removal of ethyl acetate by evaporation in vacuum, the extract was derivatized with bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMC) at 70 °C for 15 min prior to analysis by GC-TEA, GC-FID, and GC–MSD.

RESULTS AND DISCUSSION

Table I lists analytical data for tobacco-specific *N*-nitrosamines, *N*-nitrosamino acids, and related parameters in a leading U.S. moist snuff brand that had been stored in a cold room at 4 °C. During 32 weeks of storage no significant change in the levels of any of the measured components was observed except for *N*-nitrosoproline (NPRO), which had decreased significantly ($P < 0.001$). The coefficients of variation ranged from 2% for pH to about 15% for nitrate and nitrite, NNK, MNPA, and MNBA. Moist snuff purchased in 1992 contained lesser amounts of both TSNA and NAA by comparison to the same type of snuff purchased in 1991, a trend which we reported earlier (Djordjevic et al., 1993).

The chemical composition of snuff changed significantly during storage at ambient room temperature (Table II). When individually sealed tins were kept at room temperature (range 13–27 °C), the moisture content of the snuff was reduced by more than 50% within 24 weeks, probably due to evaporation. Nicotine content varied somewhat during storage and pH decreased, while levels of nitrite, individual TSNA, and NAA in tobacco started to rise after about 4 weeks of storage. These changes were

Table II. Changes in the Chemical Composition of a U.S. Moist Snuff Brand during Storage: Laboratory Shelf (Ambient Conditions)[a]

			mg/g			TSNA, μg/g			NAA, μg/g		
storage duration, weeks	H_2O, %	pH	NO_2 N	NO_3 N	nicotine	NNN	NNK	NAT	MNPA	MNBA	NPRO
individual tins (1991)											
(batch 1)											
0	56.7	6.69	0.084	4.17	18.6	7.99	0.77	5.64	4.45	0.41	8.09
2	54.5	6.78	0.136	4.13	14.9	8.86	1.33	6.63	5.70	0.63	9.29
4	55.6	7.09	0.622	3.34	16.2	8.88	1.31	6.75	5.55	0.60	8.57
8	51.6	7.58	0.652	4.39	17.4	11.34	1.74	7.08	7.80	1.08	11.00
(batch 2)											
0	56.7	6.91	0.113	4.88	20.6	4.76	0.54	3.13	2.81	0.26	4.28
12	42.5	6.84	0.469	3.71	13.7	6.64	0.80	3.52	4.39	0.68	5.56
24	21.7	6.85	0.270	3.70	13.1	13.70	2.39	9.74	8.08	1.64	15.56
10-tin sleeves, wrapped in plastic (1992)											
0	48.5	6.96	0.058	5.68	16.7	5.64	0.60	3.40	2.84	0.29	4.50
2	53.1	7.00	0.122	5.74	19.4	5.48	0.54	3.24	2.66	0.25	4.08
4	56.0	7.01	0.369	5.39	17.8	5.50	0.85	3.85	3.42	0.47	5.08
8	57.6	7.31	1.009	5.54	20.2	8.02	1.31	5.12	4.26	0.68	8.22

[a] Values are based on dry weight.

Table III. Changes in the Chemical Composition of a U.S. Moist Snuff Brand during Storage: Incubator (37 °C, 85% RH, 20 mL/min Air Flow)[a]

			mg/g			TSNA, μg/g			NAA, μg/g		
storage duration, weeks	H_2O, %	pH	NO_2 N	NO_3 N	nicotine	NNN	NNK	NAT	MNPA	MNBA	NPRO
10-tin sleeves wrapped in plastic (1992)											
0	48.5	6.96	0.058	5.68	16.7	5.64	0.60	3.40	2.84	0.29	4.50
2	54.3	7.16	0.392	5.93	16.7	7.42	0.93	5.00	3.67	0.38	6.13
4	58.0	7.33	0.551	5.73	14.2	9.33	2.94	7.94	7.41	1.25	10.27
8	58.0	7.44	0.466	5.42	16.3	15.22	3.51	8.98	13.87	2.47	19.63

[a] Values are based on dry weight.

Table IV. Changes in the Chemical Composition of a U.S. Moist Snuff Brand during Storage: Volatile *N*-Nitrosamines[a]

		ng/g				
place	storage duration, weeks	NDMA	NDBA	NPIP	NPYR	NMOR
10-tin sleeves, wrapped in plastic (1992)						
cold room (4 °C)	0	7.4	ND[b]	ND	15.3	ND
	8	5.3	ND	8.2	17.6	2.8
lab shelf (13–23 °C)	8	21.3	41.0	8.6	70.4	19.7
incubator (37 °C)	8	38.6	66.1	8.9	71.4	17.6

[a] Values are based on dry weight. [b] ND, not detected.

even more pronounced after 24 weeks of storage (NNN and MNPA levels increased 2.9-fold; NNK and MNBA levels increased 4.4 and 6.3 times, respectively). This is the first study that reports on NAA levels in tobacco during storage.

When moist snuff was shelved in plastic-wrapped 10-tin sleeves for 8 weeks at room temperature (range 13–23 °C), trends of increasing concentrations of TSNA, NAA, and nitrite were also observed; surprisingly, in this case moisture appeared to accumulate in tobacco over time.

When moist snuff was stored in an incubator (37 °C, 85% RH, 20 mL/min air flow), moisture, pH, nitrite, TSNA, and NAA increased throughout the storage period (Table III). Changes in nicotine levels were not significant. During 8 weeks, the individual TSNA increased up to 4.7-fold (NNN and NAT rose 2.5-fold, NNK 4.7-fold). The greatest increase, both at ambient temperature and at 37 °C, occurred for NNK, one of the most potent carcinogens in smokeless tobacco products (Hoffmann et al., 1993).

During storage, not only the levels but also the composition of the VNA in snuff changed (Table IV). At the beginning of the study, snuff contained only NDMA and *N*-nitrosopyrrolidine (NPYR; 7.4 and 15.3 ppb, respectively). During 8 weeks of storage in a cold room, the levels of these two VNA did not change significantly. However, two additional VNA were detected, namely *N*-nitrosopiperidine (NPIP) and *N*-nitrosomorpholine (NMOR; 8.2 and 2.8 ppm, respectively). The occurrence of the latter was probably due to migration of morpholine from the waxed cardboard container into the snuff, where it was subsequently nitrosated. This phenomenon had been reported by us earlier (Brunnemann et al., 1982). After 8 weeks of storage, at ambient temperature or at 37 °C, the levels of all VNA had increased significantly. The concentrations of the highly carcinogenic NDMA and NPYR had at this point increased 5-fold. The carcinogens NDBA, NPIP, and NMOR, which were not originally detected in moist snuff, by now amounted to 66.1, 8.9, and 19.7 ppb, respectively. Although the levels of VNA in tobacco are 2–3 orders of magnitude lower than those of TSNA and NAA, the latter finding is important in view of the high carcinogenic potency of these compounds (Hoffmann et al., 1993; Preussmann and Stewart, 1984). In addition to known VNA, two unknown volatile compounds formed during storage were observed in the GC-TEA chromatogram (peaks 1 and 2, Figure 1). When the VNA extracts containing unknown peaks were exposed to UV radiation (365 nm; Krull et al., 1979) for 1 h, peak 1 disappeared while peak 2 remained in the GC-TEA chromatogram, suggesting that only peak 1 is a *N*-nitroso

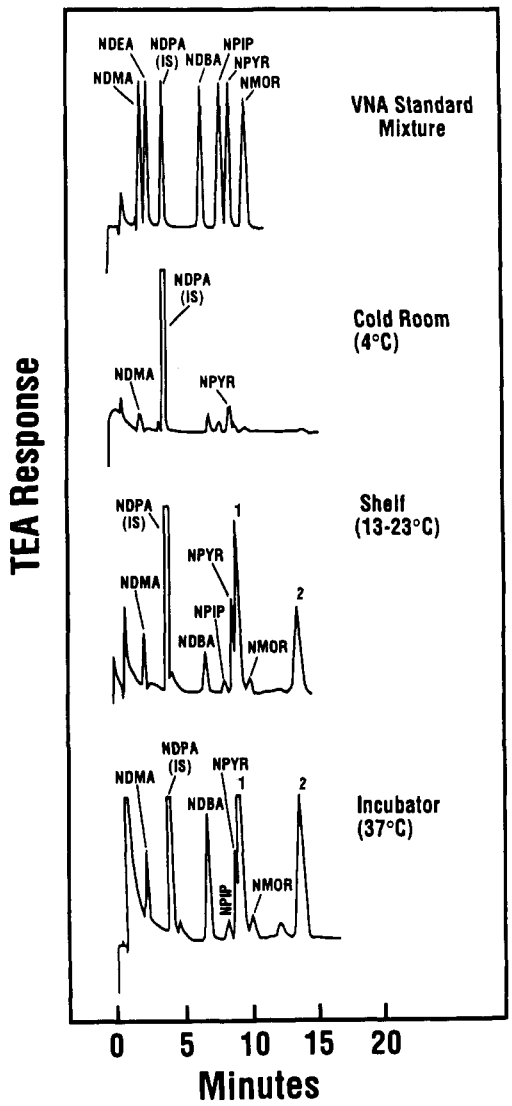

Figure 1. GC-TEA traces of volatile *N*-nitrosamines (VNA) in a commercial moist snuff brand stored at different temperatures for 8 weeks (peaks 1 and 2 are unknown compounds).

compound. The VNA extracts that contained peaks of unknowns were also treated with 10% hydrobromic acid in glacial acetic acid. Again, only peak 1 vanished while peak 2 remained in the chromatogram, though its intensity was somewhat reduced, suggesting that this unknown is possibly an organic nitro compound (N–NO_2, O–NO_2, or C–NO_2; Krull et al., 1979).

It is evident from this study that the levels of the highly carcinogenic tobacco-specific *N'*-nitrosamines and volatile *N*-nitrosamines in commercial moist snuff can rise significantly upon storage at room temperature over extended periods (≥4 weeks). Because it has been shown that morpholine from package material can diffuse into the snuff, where it forms NMOR (Brunnemann et al., 1982), additional studies on packaging materials and product quality are indicated. Certainly, the nature of packaging materials can influence product composition as was earlier shown in this study. In addition, there is sufficient evidence to conclude that factors other than storage conditions, such as blending of the tobacco and manufacturing technology, contribute to extremely high levels of TSNA and NAA. A primary example of this is seen in the chemical analysis of a commercial moist snuff that had been introduced on the U.S. market in 1990 (Djordjevic et al., 1993).

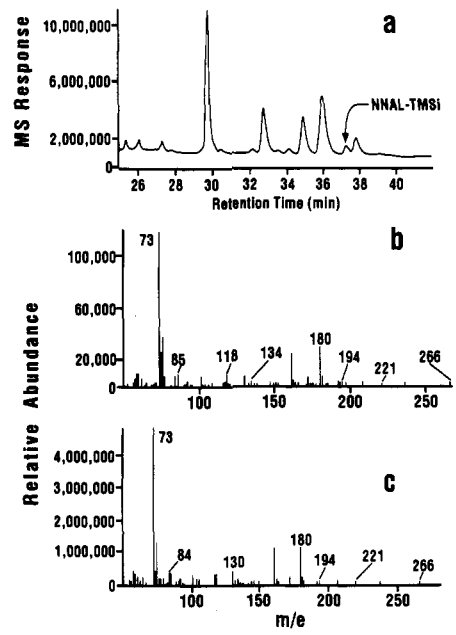

Figure 2. GC–MSD analysis (electron impact mode) of NNAL–TMSi: (a) total ionization chromatogram of pH 9 fraction from snuff extract; (b) mass spectrum of NNAL–TMSi from snuff; and (c) mass spectrum of reference NNAL–TMSi.

Earlier, NNAL had been only tentatively identified in moist snuff (Brunnemann et al., 1987). Since the level of NNAL in moist snuff was about twice that of NNK in a sample purchased in Nevada (9.6 *vs* 4.3 ppm), this sample afforded us the opportunity to positively identify the structure of this TSNA. Solvent partition of the aqueous tobacco extract at pH 9 and subsequent silylation with BSTFA plus 1% TMC yielded the compound which eluted at the same time as reference NNAL–TMSi when analyzed by GC-TEA, GC-FID, and GC–MSD. Also, the mass spectra of the reference compound and of NNAL–TMSi isolated from moist snuff were identical Figure 2.

The concentration of NNAL in a leading U.S. moist snuff brand did not increase significantly during 8 weeks of storage at ambient temperature, but it doubled during storage at 37 °C (from 0.29 to 0.65 ppm).

ABBREVIATIONS USED

MNBA, 4-(methylnitrosamino)butyric acid; MNPA, 3-(methylnitrosamino)propionic acid; NAA, *N*-nitrosamino acids; NAT, *N'*-nitrosoanatabine; NDBA, *N*-nitrosodibutylamine; NDMA, *N*-nitrosodimethylamine; NDPA, *N*-nitrosodipropylamine; NG, *N*-nitrosoguvacoline; NMOR, *N*-nitrosomorpholine; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N'*-nitrosonornicotine; NPIP, *N*-nitrosopiperidine; NPRO, *N*-nitrosoproline; NPYR, *N*-nitrosopyrrolidine; TSNA, tobacco-specific *N*-nitrosamines; VNA, volatile *N*-nitrosamines.

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