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Comparative Analysis of Tobacco-Specific Nitrosamines and Total *N*-Nitroso Compounds in Moldovan Cigarette Tobacco

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While previous studies have evaluated levels of tobacco-specific nitrosamines (TSNA) and total *N*-nitroso compounds (NOC) in tobacco, there are no reports in the literature on TSNA and total NOC in the same tobacco products. We compared levels of TSNA, total NOC, and NOC precursors (NOCP) in tobacco of cigarettes purchased in Moldova and in some tobacco types commonly used for the manufacturing of Moldovan cigarettes. Cigarette tobaccos included those from non-Moldovan, traditional Moldovan, and blended Moldovan cigarettes. The results demonstrate that tobacco of non-Moldovan cigarettes contains higher TSNA and NOC levels (mean, 16 and 63 nmol/g tobacco, $n = 6$) than that of Moldovan cigarettes (mean, 5 and 23 nmol/g tobacco, $n = 25$). TSNA and NOC levels were also generally higher in tobacco of blended than in traditional Moldovan cigarettes. NOCP levels in Moldovan and non-Moldovan cigarette tobacco were similar as follows: 29000 ± 30000 and 33000 ± 28000 nmol/g tobacco (mean ± SD). Total NOC were strongly correlated with total TSNA levels ($r = 0.66$; $P < 0.0001$). These findings demonstrate that current technologies involved in the manufacture of some blended cigarettes create conditions that favor *N*-nitrosation of alkaloids and other tobacco constituents.

KEYWORDS: Tobacco-specific nitrosamines; total *N*-nitroso compounds; Moldovan tobacco

INTRODUCTION

There are more than 1.2 billion smokers worldwide and more than 4 million of them die annually from smoking-related disease (*1*). Tobacco products cause up to 30% of all cancer deaths in developed countries (*2*, *3*). Cigarette smoking is causally related to the cancers of the lung, oral cavity, larynx, pharynx, nasal cavity, esophagus, liver, pancreas, kidney, urinary bladder, cervix, and myeloid leukemia (*4*, *5*).

Tobacco-specific nitrosamines (TSNA) comprise a class of the most important and abundant strongly carcinogenic agents in unburned tobacco (*6*, *7*). They are formed during the curing, processing, fermentation, and combustion of tobacco (*8*−*10*). 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN) are the most important of the TSNA because of their abundance and strong carcinogenicity (*11*−*13*). NNK induces lung tumors in rodents independent of the route of administration (*12*). NNN induces tumors of the esophagus and nasal cavity in rats, the lung in mice, the respiratory tract in hamsters, and the nasal cavity in mink (*11*, *12*). A mixture of NNK and NNN produced oral cavity tumors in rats (*14*). According to the International Agency for Research on Cancer, NNK and NNN are carcinogenic to humans (*15*). TSNA levels in tobacco vary according to blend, processing, and storage and are important factors in evaluating the carcinogenic potential of cigarette tobacco.

In addition to TSNAs, other *N*-nitroso compounds (NOC) are also present in cigarette tobacco. These include volatile nitrosamines (VNA) and *N*-nitrosamino acids (NAAC) (*16*−*21*), which also result from the nitrosation of natural tobacco constituents during tobacco curing and fermentation. The levels of VNAs are usually very low (*16*, *17*) as compared to those of TSNA and NAAC (*17*−*20*). 3-(Methylnitrosamino)propionic acid (MNPA) and *N*-nitrosoproline (NPRO) are the most abundant nitrosamino acids in cigarette and smokeless tobacco, with MNPA amounting to 70 μg/g dry weight of the tobacco of a U.S. moist snuff brand (*19*). Brunnemann et al. (*22*) demonstrated that NPRO was significantly correlated with NNN and the sum of TSNAs and on this basis proposed NPRO as an indicator of N-nitrosation of amines during tobacco processing.

The level of total NOC in cigarette tobacco was reported by Haorah et al. (*23*), who developed a method for the analysis of

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total NOC and NOC precursors (NOCP) in foods, chewing and cigarette tobacco, and tobacco smoke particulates. No previous studies have compared the levels of total NOC with those of TSNA in cigarette tobacco. Such a comparison would help to characterize *N*-nitrosation potential and add to our understanding of how some cigarette manufacturing methods create favorable conditions for more complete *N*-nitrosation of alkaloids and other tobacco constituents.

In a previous study (*24*), we showed that TSNA levels in tobacco of some Moldovan cigarettes are quite low as compared to those in American cigarettes. This is in agreement with a recent study conducted in 31 other countries, which showed that American cigarettes had significantly higher tobacco TSNA levels than did all locally popular non-U.S. brands (*25*). However, TSNA levels in Moldovan cigarette tobacco varied widely and in some Moldovan brands were similar to those in American cigarettes (*24*).

In the present study, we compared levels of TSNA with those of total NOC and NOCP in the tobacco of cigarettes purchased in Moldova. There are three major categories of cigarettes marketed in Moldova: (i) non-Moldovan cigarettes produced in the United States and Western Europe and imported to Moldova, henceforth, referred to as non-Moldovan cigarettes; (ii) traditional local cigarettes made from sun-cured tobaccos, referred to as traditional cigarettes; and (iii) locally produced blended cigarettes made from blends of Virginia, Burley, Oriental, and reconstituted tobaccos, referred to as blended cigarettes. The cigarette tobaccos analyzed here included tobaccos from these three categories. We also analyzed some types of tobacco that are used in the manufacture of traditional and blended cigarettes.

MATERIALS AND METHODS

Caution. NNN, NNK, and NAB are carcinogenic and mutagenic and should be handled with extreme care, using appropriate protective clothing and ventilation at all times. Total NOC may also include carcinogens.

Cigarettes and Tobacco. Cigarettes were purchased on the open market in Chisinau in 2001 and 2002. The samples of tobacco commonly used for Moldovan cigarette manufacturing were provided by the tobacco factory "Tutun-CTC" (Chisinau, Moldova). For 24 h before analysis, the tobacco was conditioned in a chamber at ambient temperature and a relative humidity of 60%.

Chemicals. Reference TSNA were synthesized (*26*), except that NAT was purchased from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada). 5-Methyl-*N*′-nitrosonornicotine (5-MeNNN) was synthesized (*27*). 5-(Methylnitrosamino)-1-(3-pyridyl)-1-pentanone (C5-NNK) was prepared by the same method as NNK (*26*), except that 1-methyl-2-piperidone (Aldrich Chemical Co., Milwaukee, WI) was used as the starting material. [CD_3]Nicotine was obtained from Sigma (St. Louis, MO).

Analyses of TSNA. This was performed by a slight modification of the method previously used by Stepanov et al. (*24*). In brief, 200 mg of humidity-conditioned tobacco and 10 mL of citrate-phosphate buffer containing ascorbic acid were added to 30 mL Nalgene centrifuge tubes (Nalge Nunc International, Rochester, NY). C5-NNK and 5-MeNNN (200 ng each) were added to each sample as internal standards. The mixtures were homogenized using a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY) and sonicated for 1 h. The buffer extracts were separated from the particles of tobacco and filtered into 50 mL glass centrifuge tubes (Kimble, Vineland, NJ), and the pH was adjusted to 5. Each sample was extracted three times with 10 mL of EtOAc. The extracts were combined, dried with ∼10 g of Na_2SO_4 for 30 min, and evaporated with a model SVT200H Speedvac concentrator (Savant Instruments, Farmingdale, NY). The dry residues were dissolved in 0.5 mL of CH_2Cl_2 and purified by solid phase extraction using Sep-Pak Silica cartridges (Waters Corp., Milford, MA). TSNA were eluted from the cartridges with 10 mL of EtOAc, collected in 15 mL centrifuge tubes, concentrated to dryness (SpeedVac), transferred into 200 μL GC vials (Waters Corp.) with 150 μL of CH_3-OH, dried, and redissolved in 100 μL of acetonitrile. Of the sample, 3 μL was analyzed by gas chromatography (GC) with thermal energy analysis (TEA) using a model 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) interfaced with a 450 °C oven to liberate NO from nitrosamines and a model 610 Thermal Energy Analyzer (Orion Research, Beverly, MA) to determine the NO. The gas chromatograph was fitted with a DB-1301 capillary column (30 m × 0.32 mm × 0.25 μm) [6% (cyanopropylphenyl)methylpolysiloxane; J&W Scientific, Folsom, CA] and a 2 m × 0.53 mm deactivated fused silica precolumn. The splitless injection temperature was 225 °C. The oven temperature program was as follows: 80 °C for 2 min, 12 °C/min to 160 °C, 15 min at 160 °C, 12 °C/min to 210 °C, and 8 min at 210 °C.

Total NOC and NOCP. To determine total NOC, 1 g samples of tobacco were suspended overnight in 15 mL of H_2O, vortexed, and centrifuged. The supernatants were filtered. To 400 μL of filtrate was added 50 μL of 2 N HCl and 50 μL of SA reagent (a freshly prepared saturated solution of sulfamic acid in H_2O) to destroy excess nitrite. The mixture was left for 15 min, and NOC in 50 μL samples were determined as before (*23*) using the HBr mode. In brief, samples were injected into a 500 mL round bottom flask containing a mixture of EtOAc, HOAc, HCl, and HBr, which was heated under reflux at reduced pressure at 28 °C. Under these conditions, NOC are denitrosated to give NO, which is swept in a stream of argon through seven washbottles containing reagents to remove H_2O and acids and then into a Thermal Energy Analyzer. To determine NOCP, a mixture of 200 μL of supernatant, 110 μL of 2 N $NaNO_2$, 110 μL of 2 N HCl, and 1.58 mL of H_2O (total 2 mL) was incubated for 1 h at 37 °C. Then, 300 μL of SA reagent was added, the mixtures were left for 15 min at room temperature, the sample was diluted 100 times in H_2O containing 1% SA reagent, and 100 μL aliquots were analyzed for NOC. The measured NOC represents the sum of NOC and NOCP.

Nicotine. Nicotine was analyzed as previously described (*24*). Humidity-conditioned tobacco (50 mg) and 20 mL of MeOH containing 50 mg of KOH were added to 30 mL Nalgene centrifuge tubes. The samples were homogenized with a Polytron and then sonicated for 3 h. The MeOH extracts were separated by high speed centrifugation. Then, 200 mL of the extracts was transferred into a silanized 4 mL vial and 50 ng of [CD_3]nicotine internal standard was added. The samples were transferred to GC-microinsert vials and analyzed by GC-mass spectrometry (MS)-selected ion monitoring (SIM) using a model 6890 GC equipped with an autosampler and interfaced with a model 5973 mass selective detector (Agilent Technologies, Palo Alto, CA) as previously described (*24*).

Nitrate and Nitrite. Humidity-conditioned tobacco (100 mg) and 10 mL of reagent grade water (Milli-Q, Millipore Corp.) were added to a 50 mL glass screw-top centrifuge tube (Kimble) prewashed with H_2O. Two H_2O negative controls and control solutions containing 16.22 ppm N as nitrate and 4.06 ppm N as nitrite were included in the sample set. Tobacco was homogenized with a Polytron, and the tubes were sonicated for 30 min. The suspensions were centrifuged, and the aqueous extract was applied to a C-18 SPE cartridge (Waters Corp.) conditioned with 2 mL of MeOH. The first 5 mL of eluant was discarded. The next 2 mL of eluant was collected in a prewashed plastic tube and stored at −20 °C until analysis. The samples were diluted 10-fold before analysis by ion chromatography using a Dionex ICS-2000 Ion Chromatograph. Conditions were as follows: an AS14 anion exchange column and guard column; carbonate/bicarbonate eluant; 50 mL sample loop; 1.0 mL/min flow. These analyses were performed at the University of Minnesota Geochemical Analysis Facility.

Statistical Analyses. These were performed using SigmaPlot 2001 software (version 7.101, SPSS Inc., Chicago, IL).

RESULTS

Table 1 lists the levels of total TSNA, total NOC, and total NOCP in the tobacco of cigarettes purchased in Moldova. Each of these values is the mean of two analyses, the results of which generally differed by <10%.

Table 1. TSNA, NOC, and NOCP in Tobacco of Cigarettes Purchased in Moldova

cigarette brand	total TSNA[a] (nmol/g)	total NOC (nmol/g)	total NOCP (nmol/g)
Non-Moldovan Cigarettes			
Monte Carlo[b]	12[c]	44	7500
Prima lux (lights)[b]	9[c]	28	11000
Lucky Strike [b]	23[c]	84	5500
LM (lights)	18	98	50000
LM (full flavor)	21	86	73000
West	13	40	47000
mean ± SD for non-Moldovan cigarettes	16 ± 5.5	63 ± 29	33000 ± 28000
Moldovan Cigarettes			
traditional cigarettes (made from sun-cured tobaccos)			
Astra[b]	1.4[c]	13	10000
Plai[b]	1.3[c]	23	9300
Risk[b]	2.1[c]	12	9900
Plugarul[d]	1.4[c]	8	8500
	1.3	25	73000
Prima[b]	3.3[c]	33	5700
Luceafar[b]	3.5[c]	10	14000
Foisor[b]	5.9[c]	12	14000
Cosmos[b]	2.1[c]	7	16000
Zimbru[b]	5.8[c]	18	15000
Ancora de Aur[b]	3.1[c]	17	16000
Doina (cl.4)[d]	3.0[c]	7	13000
	2.4	46	53000
Leana	1.6	20	71000
Flueras	2.9	25	78000
mean ± SD for traditional cigarettes	2.7 ± 1.5	18 ± 11	27000 ± 27000
blended cigarettes			
Flueras extra[b]	8.3[c]	25	12000
Chisinau[b]	8.5[c]	35	10000
Tandem[b]	17[c]	46	7500
Temp[d]	12[c]	33	17000
	15	51	91000
MT[b]	4.6[c]	21	6400
Doina lux (classic)[b]	4.3[c]	17	10000
Doina lux (premium)[d]	4.8[c]	17	5900
	5.7	18	86000
Doina lux (lights)	3.6	37	75000
mean ± SD for blended cigarettes	8.3 ± 4.7	30 ± 12	32000 ± 36000
mean ± SD for all Moldovan cigarettes	5.0 ± 4.2	23 ± 13	29000 ± 30000

[a] The sum of NNN, NAT, NAB, and NNK. [b] Cigarettes were purchased in 2001, stored in a cold room at 4 °C, and analyzed in 2002. [c] Calculated from previously published data (*24*). [d] The top and the bottom rows refer to tobacco purchased in 2001 and 2002, respectively.

Non-Moldovan cigarettes contained high total TSNA and NOC levels: mean, 16 and 63 nmol/g, respectively ($n = 6$). Mean total TSNA and NOC levels in all Moldovan brands were 5 and 23 nmol/g ($n = 25$). However, the levels of these analytes in Moldovan cigarette tobacco varied widely, being generally lower in traditional than in blended cigarettes. Mean total NOCP levels were similar in non-Moldovan and Moldovan cigarette tobacco: 33000 and 29000 nmol/g, respectively.

The differences in blends and manufacturing processes present an additional challenge in comparing TSNA and total NOC contents in cigarette tobacco. Therefore, we analyzed TSNA and NOC in tobacco commonly used for the manufacturing of traditional and blended Moldovan cigarettes. The sun-cured tobaccos listed in **Table 2** are used in the manufacture of traditional cigarettes, which contained medium to low TSNA and NOC levels. Burley 320 and Virginia 401 tobaccos are used in the production of Moldovan blended cigarettes. A typical GC trace of TSNAs in Burley 320 tobacco is shown in **Figure 1**.

Table 2. Total TSNA, NOC, and NOCP in Tobacco Commonly Used for Manufacturing of Moldovan Cigarettes

	nmol/g			mg/g		μg/g
tobacco type	total TSNA	total NOC	total NOCP	nicotine	nitrate	nitrite
sun-cured tobaccos used in the manufacture of traditional Moldovan cigarettes						
Moldovenesc 272						
cured	0.68	1.6	126000	8.5	2.4	2.8
further processed[a]	1.2	36	49000	7.7	2.1	ND[b]
Moldovenesc 456						
cured	1.1	1.2	66000	12	7.6	4.4
further processed	1.7	31	34000	11	7.0	1.8
Jubiliar 8						
cured	3.2	5.7	15000	12	8.9	4.2
further processed	4.8	92	6300	11	8.9	1.4
Doina 211						
cured	1.3	6.4	47000	16	4.2	6.1
further processed	2.8	90	16000	15	4.5	3.3
tobaccos used in the manufacture of Moldovan blended cigarettes						
Burley 320						
cured[c]	1.4	2.0	16000	8.9	5.6	8.2
further processed	2.0	37	8900	8.1	4.8	1.2
Virginia 401						
cured[d]	7.2	9.4	139000	20	17	46
further processed	9.6	21	36000	18	16	4.2

[a] Further processing: fermentation for sun-cured tobaccos; redrying for Burley and Virginia. [b] ND, not detected. [c] Air-cured. [d] Flue-cured.

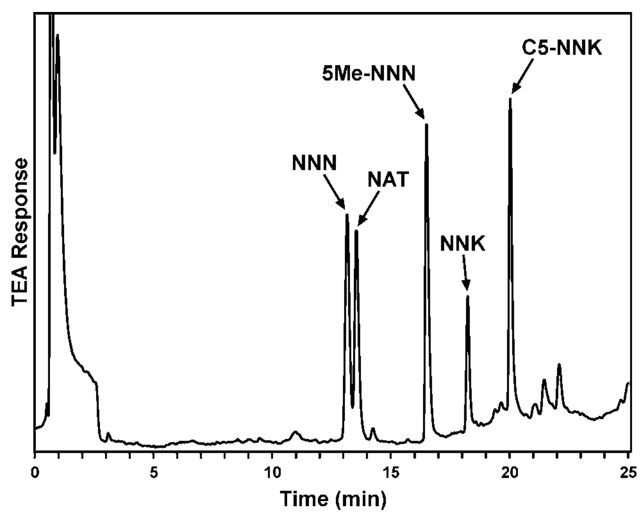

Figure 1. Chromatogram obtained upon GC-TEA analysis of Burley 320 tobacco.

Mean total TSNA and total NOC levels in sun-cured tobaccos were 1.6 and 3.7 nmol/g, respectively. After fermentation, these values increased to 2.6 and 62 nmol/g tobacco. In contrast, total NOCP decreased after fermentation, with a mean total NOCP of 64000 nmol/g cured tobacco and 26000 nmol/g fermented tobacco. Virginia 401 tobacco contained the highest levels of total TSNA, NOC, and NOCP among the cured tobaccos with mean values of 7.2, 9.4, and 139000 nmol/g, respectively (**Table 2**). Burley 320 contained moderate levels of all three analytes. Cured tobaccos usually contained higher levels of nicotine, nitrate, and nitrite than the same tobaccos, which had undergone further processing by fermentation or redrying (**Table 2**). The highest levels of almost all analytes were observed in Virginia 401 tobacco.

There was a strong correlation between total NOC and total TSNA levels ($r = 0.66$; $P < 0.0001$) (**Figure 2**) in the tobacco samples analyzed.

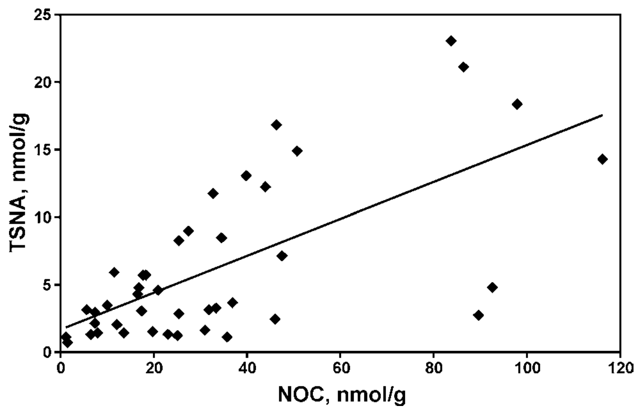

Figure 2. Correlation between total NOC and total TSNA in all tobacco samples analyzed in this study, $N = 42$.

DISCUSSION

In this study, we compared total TSNA, NOC, and NOCP levels in tobacco of cigarettes purchased in Moldova and in some types of tobacco grown in that Eastern European country. Cigarette tobaccos analyzed here included those from non-Moldovan, Moldovan traditional, and Moldovan blended cigarettes. The results of this study demonstrate that tobacco of non-Moldovan cigarettes contains higher TSNA and NOC levels than that of Moldovan cigarettes. The levels of TSNA and NOC were also generally higher in tobacco of Moldovan blended cigarettes when compared to traditional cigarettes. Total NOCP varied widely, the mean total NOCP levels being similar in non-Moldovan and Moldovan cigarette tobacco. Sun-cured tobaccos, used for the manufacturing of Moldovan traditional cigarettes, contained relatively low TSNA and NOC levels, comparable to those in the corresponding category of cigarettes. Virginia 401 tobacco, comprising up to 60% of Moldovan blended cigarettes, contained the highest levels of TSNA and NOC among all tobacco types analyzed. There was a strong correlation between total NOC and total TSNA levels in the analyzed tobacco samples.

Of particular interest are the relatively high TSNA and NOC levels in tobacco of some blended Moldovan cigarettes as compared to traditional cigarettes. Moldovan blended cigarettes are made from blends of Virginia, Burley, Oriental, and reconstituted tobaccos. In this study, cured Virginia tobacco contained the highest levels of TSNA, NOC, and NOCP, as well as of nicotine, nitrite, and nitrate (**Table 2**). The amount of precipitation, nitrogen content in the soil, fertilizers used, and specifics of curing practices in Moldova are probably responsible for the unusual levels of nicotine in tobacco types analyzed here. The same factors are probably responsible for high nitrite levels in Virginia tobacco, which may have led to high TSNA and NOC levels found in this tobacco type. Burley tobacco, which is the second major constituent of Moldovan blended cigarettes, contained relatively low levels of TSNA and NOC, similar to the levels in sun-cured tobaccos. Because the levels of tobacco constituents analyzed here could greatly fluctuate from crop to crop, further research is needed in order to understand why TSNA levels in some Moldovan blended cigarettes (Flueras, Chisinau, Tandem, Temp) are relatively high as compared to other blended and traditional Moldovan brands (**Table 1**). It should be mentioned that because of the lower nicotine and tar content as compared to traditional cigarettes, blended cigarettes produced in Moldova are thought by customers and manufacturers to be "less toxic". This category of cigarette is becoming more popular because of their better taste as compared to traditional cigarettes. They are also usually less expensive than imported American and other non-Moldovan blended cigarettes.

In accord with a previous observation by Haorah et al. (*23*), we did not find any correlation between NOC and NOCP. This supports the view that variations in the levels of *N*-nitrosatable compounds in tobacco are not responsible for the variation in total NOC levels, which are more likely to be due to varying levels of nitrite or other nitrosating agents. The TSNA levels in tobacco also depend on the amount of nitrate present during curing and fermentation (*30*, *31*), presumably because nitrate is reduced to nitrite by bacterial action. On this basis, we suggest that the nitrite level determines the levels of both total TSNA and total NOC and that this explains the correlation between TSNA and NOC (**Figure 2**). The correlation between total NOC and total TSNA observed here is similar to the findings that NPRO in tobacco correlates with the sum of TSNA (*22*).

NPRO along with MNPA are the main non-TSNA contributors to the total amount of NOC in cigarette tobacco (*17*–*20*). Thus, from the data reported by Tricker and Preussmann (*17*), one may calculate that the sum of four NAAC in cigarette tobacco—MNPA, NPRO, *N*-nitrososarcosine, and *N*-nitrosopipecolic acid—averaged approximately 33 nmol/cigarette in 20 cigarette brands, the sum of NPRO and MNPA contributing to 92% of this amount. Similar results were observed in U.S. moist snuff (*32*), where the sum of five NAAC averaged approximately 60 nmol/g dry weight, 95% of this amount being represented by the sum of NMPA and NPRO. *N*-Nitroso-1-deoxy-1-*N*-glycosyl amino acids may also contribute to the total NOC (*23*, *33*, *34*). The contribution of VNAs to the total amount of NOC in cigarette tobacco is usually low (*16*, *17*). These data suggest that the difference between levels of total NOC and TSNA in the cigarette tobaccos analyzed here is comprised mainly of NAAC. Mainstream smoke condensate of a regular cigarette does not contain any measurable amounts of NAAC, and the transfer rate of NAAC into the mainstream smoke was shown to be less than 1% (*18*). However, thermal decarboxylation of NAAC during smoking yields corresponding carcinogenic VNA (*17*). Some NOCP may also be important, yielding VNA through pyrolytic nitrosation during tobacco combustion (*17*). NOCP-derived NOC in snuff were directly mutagenic in the Ames test (*35*).

In summary, our results for the first time compare TSNA and total NOC levels in tobacco, demonstrating that total NOC levels are low in those cigarette brands that contain low TSNA levels. These findings support the concept that current technologies involved in the manufacture of some blended cigarettes create conditions that favor N-nitrosation of alkaloids and other tobacco constituents.

ABBREVIATIONS USED

C5-NNK, 5-(methylnitrosamino)-1-(3-pyridyl)-1-pentanone; GC, gas chromatography; 5-MeNNN, 5-methyl-*N*′-nitrosonornicotine; MNPA, 3-(methynitrosamino)propionic acid; MS-SIM, mass spectrometry with selected ion monitoring; NAAC, *N*-nitrosamino acids; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; NOC, *N*-nitroso compounds; NOCP, *N*-nitroso compound precursors; NPRO, *N*-nitrosoproline; SA reagent, saturated solution of sulfamic acid in water; TEA, thermal energy analysis; TSNA, tobacco-specific nitrosamines; VNA, volatile nitrosamines.

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