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Analysis of Tobacco-Specific Nitrosamines in Moldovan Cigarette Tobacco

IRINA STEPANOV,[†] STEVEN G. CARMELLA,[‡] STEPHEN S. HECHT,*[,‡] AND GHEORGHE DUCA[†]

Department of Industrial and Ecological Chemistry, Moldova State University, MD-2009 Chisinau, Moldova, and University of Minnesota Cancer Center, Mayo Mail Code 806, 420 Delaware Street Southeast, Minneapolis, Minnesota 55455

Tobacco-specific nitrosamines (TSNA) are among the most important and abundant strongly carcinogenic agents in unburned tobacco. It has been established that 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induces lung tumors in rodents independent of the route of administration. *N*′-Nitrosonornicotine (NNN) causes tumors of the esophagus and nasal cavity in rats, lung in mice, and respiratory tract in hamsters. Although the manufacturing of cigarettes is an important domain of Moldovan industry, there are no reports in the literature on TSNA analysis in Moldovan tobacco. The main purpose of the present study was an initial evaluation of TSNA levels in Moldovan cigarette tobacco. Eighteen brands of Moldovan cigarettes, representing 78% of all brands produced in Moldova, were analyzed. Four TSNA—NNN, NNK, *N*′-nitrosoanatabine (NAT), and *N*′-nitrosoanabasine (NAB)—were analyzed by gas chromatography with nitrosamine selective detection (GC-TEA). Levels of TSNA in most Moldovan cigarettes were substantially lower than in American brands. Mean levels of NNN in three commercial American brands were 3.32 ± 0.88 (SD) μg/g as compared to 0.579 ± 0.548 μg/g, range 0.093−2.09 μg/g ($N = 18$), in the cigarettes produced in Moldova. For NNK and NAT, mean levels in the American brands were 1.57 ± 0.178 and 1.99 ± 0.579 μg/g, respectively, while the corresponding values for Moldovan cigarettes were 0.193 ± 0.089, range 0.104−0.484 μg/g, and 0.160 ± 0.114 μg/g, range 0.055−0.481 μg/g. The highest levels of NNN—1.10−2.09 μg/g—were observed in "American type" cigarettes manufactured from high-quality tobacco. The results of this study should be useful in heightening the awareness of the dangers of smoking in Moldova and can be envisioned as the initial step in the control of tobacco-related cancer in this republic.

KEYWORDS: Tobacco-specific nitrosamines; Moldovan tobacco

INTRODUCTION

Cigarette smoking causes 30% of all cancer deaths in developed countries and is the largest single preventable risk factor for premature death (*1*). Lung cancer is the most common cause of cancer death both in the U.S. and Moldova (*2*, *3*). In the U.S., about 87% of lung cancer deaths can be directly attributed to cigarette smoking (*4*). Tobacco control interventions are necessary in the Republic of Moldova, where about 70% of men and 20% of women 20−40 years old are smokers (*5*). Moldova occupies a relatively small territory between the Prut and Nistru rivers, between Romania and the Ukraine. The country covers 33 700 km^2. It became an independent nation in 1991 when the USSR disbanded. The Republic of Moldova is also one of the smallest states in Europe by its population—about 4.5 million, two-thirds of whom are Moldovans/Romanians with the remainder being Ukrainians and Russians.

Tobacco-specific nitrosamines (TSNA) are among the most important and abundant strongly carcinogenic agents in unburned tobacco (*6*, *7*). Two TSNA, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN), are of considerable interest because they are strongly carcinogenic (*8*). NNK induces lung tumors in rodents independent of the route of administration. It also causes tumors of the nasal mucosa, liver, and pancreas. NNN causes tumors of the esophagus and nasal cavity in rats, lung in mice, and respiratory tract in hamsters. A mixture of NNK and NNN produced oral cavity tumors in rats. *N*′-nitrosoanabasine (NAB) is a weak esophageal carcinogen in rats while *N*′-nitrosoanatabine is apparently noncarcinogenic (*8*). The structures of NNK, NNN, NAT, and NAB are shown in **Figure 1**. The levels of TSNA in tobacco vary according to blend, processing, and storage and are important factors in evaluating the carcinogenic potential of cigarette tobacco (*9*).

Although the manufacturing of cigarettes is an important domain of Moldovan industry, there are no reports in the literature on TSNA analysis of Moldovan tobacco. This contrasts

* To whom correspondence should be addressed. Tel.: 612-624-7604. Fax: 612-626-5135. E-mail: hecht002@umn.edu.
[†] Moldova State University.
[‡] University of Minnesota Cancer Center.

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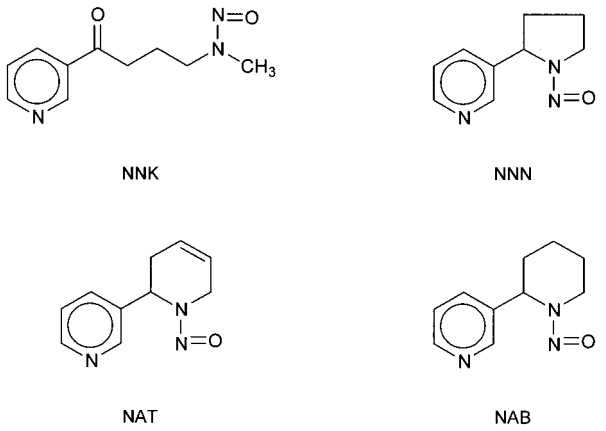

Figure 1. Structures of NNK, NNN, NAT, and NAB.

to extensive data on TSNA available for products sold in other parts of Europe (*10*). Therefore, we carried out the present study to provide an initial evaluation of TSNA levels in Moldovan cigarette tobacco. We envision this as a first step toward tobacco-related cancer control in Moldova.

MATERIALS AND METHODS

Cigarettes. Moldovan cigarettes were purchased on the open market in Chisinau, Moldova in 2001. The 18 brands of Moldovan cigarettes represent 78% of all brands produced in Moldova. All categories—from the most inexpensive cigarettes to fine blends—were included in this work. For 24 h prior to analysis, the cigarette tobacco was conditioned in a chamber at a relative humidity of 60%. University of Kentucky research cigarette 1R3 and American commercial cigarettes Marlboro, Camel, and Lucky Strike (purchased in Minneapolis) were analyzed for comparison.

Apparatus. Gas chromatography with nitrosamine selective detection (GC-TEA) was carried out with a model 5890 gas chromatograph (Hewlett Packard, Palo Alto, CA) interfaced with a model 610 Thermal Energy Analyzer (Orion Research, Beverly, MA). The GC was equipped 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 flow rate was 2.6 mL/min He, and the splitless injection temperature was 225 °C. The oven temperature program was as follows: 80 °C for 2 min, then 12 °C/min to 160 °C, then 15 min at 160 °C, then 12 °C/min to 210 °C, then 13 min at 210 °C.

Nicotine was 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). The GC was equipped with an HP-5 fused silica capillary column (30 m × 0.5 mm, 0.25 μm film thickness). The splitless injection port temperature was 250 °C. The oven temperature was 70 °C for 0.5 min, then increased to 180 °C at 10 °C/min, held for 1 min, then increased to 275 °C at 50 °C/min, then held for 5 min, and returned to initial conditions. The carrier gas was He, and the flow rate was 1 mL/min.

Ion chromatography for nitrate and nitrite analysis in tobacco was carried out on a Dionex-500 Ion Chromatograph with a W/CD 20 detector and GP40 gradient pump. Conditions were as follows: 2 AS4A anion exchange columns; carbonate/bicarbonate eluent; 50 mL sample loop; flow rate, 2.1 mL/min. These analyses were performed at the University of Minnesota Geochemical Analysis Facility.

Reagents. Reference TSNA, except for NAT, was synthesized as described (*11*). NAT was purchased from Toronto Research Chemicals, Inc., Toronto, Ontario, Canada. 5-Methyl-*N*′-nitrosonornicotine (5-MeNNN) was synthesized as described (*12*). 5-(Methylnitrosamino)-1-(3-pyridyl)-1-pentanone (C5-NNK) was prepared in the same manner as NNK, except that 1-methyl-2-piperidone (Aldrich Chemical Co., Milwaukee, WI) was used as starting material. It was characterized by ^{1}H nuclear magnetic resonance (NMR) and MS. [CD_3]Nicotine was obtained from Sigma Chemical Co., St. Louis, MO.

Analyses of TSNA, Nicotine, and Nitrate in Cigarette Tobacco. The TSNA analysis of cigarette tobacco was performed by a slight modification of a method previously described by Carmella et al. (*12*). One gram of humidity-conditioned tobacco and 25 mL of citrate−phosphate buffer (pH 4.5) containing ascorbic acid were added to a 30 mL NALGENE centrifuge tube. Fifty nanograms each of C5-NNK (internal standard for NNK) and 5-MeNNN (internal standard for NNN, NAT, and NAB) were added. The samples were homogenized using a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY) and sonicated for 1.5 h. The buffer extracts were separated from fine particles of tobacco by high-speed centrifugation (15 000 rpm, 10 min). The buffer extracts were filtered into 50 mL glass screw-top centrifuge tubes (Kimble, Vineland, NJ), and the pH was adjusted to 5 by adding a few drops of 10 N NaOH. Each sample was extracted three times with 12 mL of EtOAc, and the extracts were combined. Emulsions encountered in this step were separated by centrifugation. The combined extracts were dried with ~10 g Na_2SO_4 for 30 min and concentrated to dryness with a model SVT200H Speedvac concentrator (Thermo Savant, Farmingdale, NY). Residues were dissolved in 0.5 mL of methylene chloride and further purified by solid phase extraction using Sep-Pak Plus silica cartridges (Waters Corp., Milford, MA), preequilibrated with methylene chloride. TSNA were eluted with methylene chloride/ethyl acetate:50/50 and ethyl acetate. The extracts were concentrated to dryness (Speedvac). Residues were transferred into microvials with 3 × 60 μL methanol, dried, and redissolved in 50 μL of acetonitrile. Two microliters of the prepared sample was injected on the GC-TEA.

For nicotine analysis, 50 mg of tobacco and 20 mL of methanol containing 50 mg of potassium hydroxide were added to 30 mL NALGENE centrifuge tubes. The samples were homogenized (Polytron) and then sonicated for 3 h. The methanol extracts were separated from the fine particles of tobacco by high-speed centrifugation. Then, 200 μL of the methanol extract was transferred into a silanized 4 mL vial, 40 μL of the internal standard mixture ([CD_3]nicotine) was added, and the samples were frozen at −20 °C until analysis. The prepared tobacco samples were analyzed by GC-MS-SIM, after transferring to a GC microinsert vial.

For nitrate and nitrite analysis, 100 mg of humidity-conditioned tobacco 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 water. Two water negative controls were included in the sample set. Tobacco was broken up into a fine suspension with the Polytron tissue homogenizer. The tubes were sonicated for 30 min and shaken briefly, and the suspension was centrifuged. The aqueous tobacco extract was applied to a C-18 SPE cartridge (Waters Corp, Milford, MA) conditioned with 2 mL of methanol. The first 5 mL of eluant was discarded. The next 2.5 mL of eluant was collected in a prewashed plastic tube and stored at −20 °C until analysis. Nitrate and nitrite in the samples were analyzed by ion chromatography.

RESULTS

Figure 2 presents a typical GC-TEA trace of TSNA in Moldovan cigarette tobacco. The analytes and internal standards were well-resolved; peak shape and signal-to-noise ratios were excellent. **Table 1** summarizes nicotine, nitrate, and TSNA levels of Moldovan and American cigarettes. Each value is the mean of two analyses, which agreed on average within less than 7% of each other for NNN, NNK, and NAT. Recoveries of internal standards averaged 57.1%.

Levels of TSNA in most Moldovan cigarettes were substantially lower than in the American commercial brands. The mean level of NNN in the three commercial American brands was 3.32 ± 0.88 (SD) μg/g as compared to 0.579 ± 0.548 μg/g, range 0.093−2.09 μg/g (N = 18), in the cigarettes produced in Moldova. For NNK and NAT, mean levels in the American brands were 1.57 ± 0.178 and 1.99 ± 0.579 μg/g, respectively, while the corresponding values for Moldovan cigarettes were

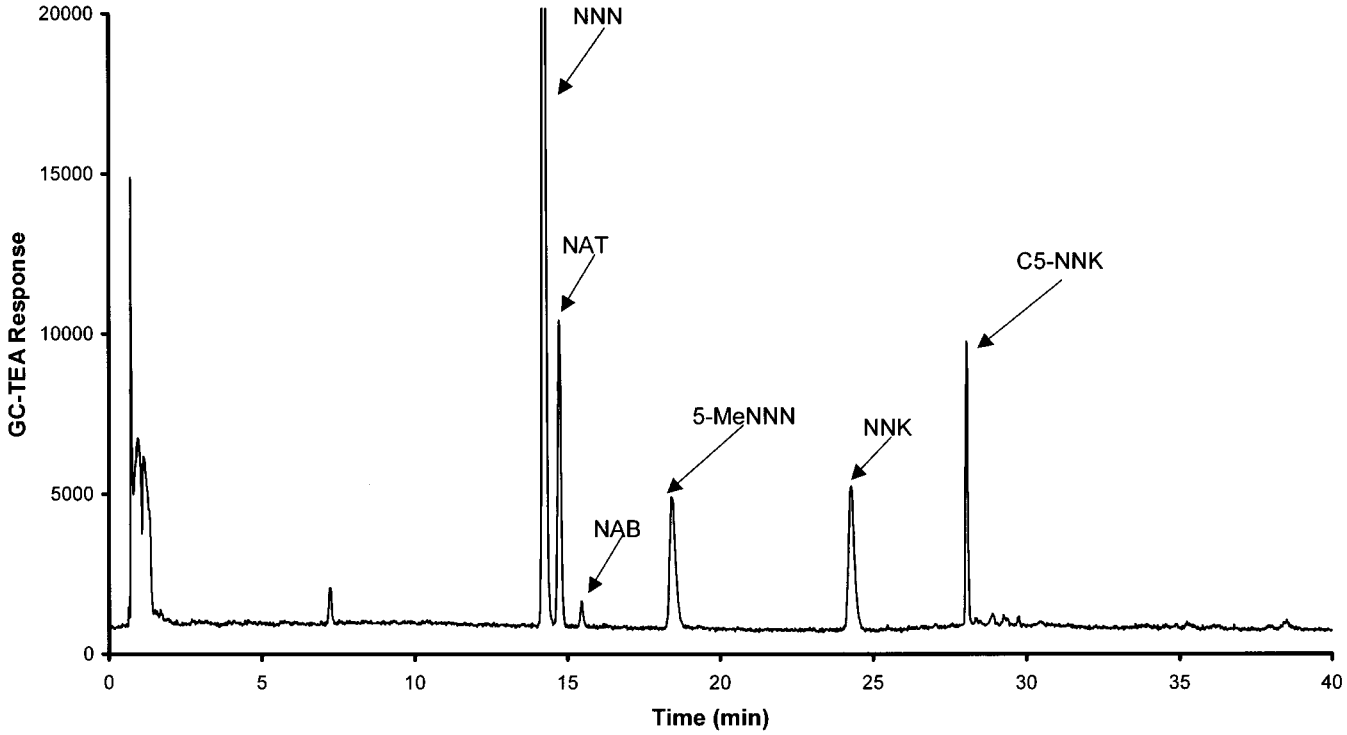

Figure 2. Chromatogram obtained upon GC-TEA analysis of tobacco from a Moldovan cigarette, Tandem.

Table 1. Nicotine, Nitrate, and TSNA in Cigarette Tobacco (Per Gram Wet Weight)[a]

cigarettes	filter (f) or nonfilter (nf)	nicotine (mg)	nitrate (mg)	TSNA[b] (μg)			
				NNN	NNK	NAT	NAB
U.S. Cigarettes							
1R3[c]	nf	12.7	16.1	1.65	0.805	0.842	0.040
Camel	nf	19.5	10.3	3.09	1.41	1.61	0.110
Marlboro	f	18.7	7.46	4.30	1.76	2.66	0.140
Lucky Strike	f	17.6	7.69	2.59	1.56	1.72	0.100
Cigarettes Produced in Moldova							
Astra	nf	16.8	7.33	0.098	0.114	0.060	ND[e]
Plai	nf	17.9	16.3	0.094	0.104	0.055	ND
Risc	nf	19.6	2.67	0.150	0.153	0.092	0.001
Plugarul	f	15.6	2.76	0.093	0.105	0.075	ND
Prima	nf	14.0	5.49	0.284	0.185	0.146	0.003
MT	f	11.2	5.46	0.535	0.163	0.144	ND
Luceafar	f	12.7	3.02	0.303	0.237	0.108	0.014
Foisor	f	12.7	5.30	0.629	0.304	0.156	0.018
Cosmos	f	11.2	2.59	0.173	0.119	0.099	0.014
Zimbru	nf	11.1	7.16	0.709	0.148	0.171	0.026
Ancora de aur	f	13.1	5.54	0.247	0.184	0.133	0.016
Flueras (extra)	f	9.65	5.21	1.10	0.194	0.192	0.024
Chisinau	f	9.60	9.58	1.17	0.200	0.157	0.015
Tandem	f	15.3	9.32	2.09	0.485	0.481	0.021
Temp	f	12.3	3.14	1.44	0.251	0.426	0.019
Doina	nf	14.0	9.85	0.27	0.176	0.100	0.013
Doina Classic	f	13.0	4.46	0.465	0.179	0.140	0.015
Doina Premium	f	11.8	6.46	0.562	0.168	0.152	0.011
Foreign Cigarettes Commercialized in Moldova							
Monte Carlo	f	13.5	11.2	1.15	0.377	0.705	0.040
Prima lux	f	15.1	7.56	0.865	0.376	0.415	0.025
Lucky Strike[d]	f	14.5	9.17	2.01	0.942	1.29	0.075

[a] Tobacco was conditioned at 60% relative humidity for at least 24 h before analysis. [b] All samples were analyzed in duplicate; values are the means. [c] University of Kentucky Tobacco and Health Research Institute research cigarette containing blended tobaccos used in U.S. commercial products. [d] European. [e] ND, not detected.

0.193 ± 0.089, range $0.104-0.484$ μg/g, and 0.160 ± 0.114 μg/g, range $0.055-0.481$ μg/g.

The tobacco of the best-selling Moldovan brands Astra, Prima, Temp, Chisinau, Doina, and Doina Premium had varying levels of TSNA and nitrate. The highest levels of NNN—1.10—2.09 μg/g—were observed in "American type" cigarettes manufactured from high-quality tobacco: Tandem, Chisinau, Temp, and Flueras extra. Levels of NNK and NAT were also generally higher in these brands than in the other Moldovan cigarette tobaccos. The highest concentrations of all TSNA were recorded for the brand Tandem. The concentrations of NNN and other TSNA were generally relatively low in the more inexpensive

brands: Astra, Plai, Risc, and Plugarul. The levels of TSNA in the most popular foreign cigarettes commercialized in Moldova were higher than in Moldovan cigarettes and lower than in the American commercial brands.

A positive correlation was observed between the levels of NNN and other TSNA ($P < 0.0001$), but there was no correlation between levels of nicotine or nitrate and TSNA. Nitrite levels were below the detection limit of our assay (0.01 mg/g wet weight tobacco).

DISCUSSION

In this study, we analyzed TSNA, nicotine, and nitrate in 18 brands of Moldovan cigarettes purchased in June 2001 on the open market in Moldova. These brands accounted for 78% of the Moldovan market in 2000. Some of them have dominated the Moldovan market for more than 20 years with at least 40% of the sales each year. A survey in 1999 showed that users aged 20−40 years preferred Temp and Chisinau. These brands contain relatively high levels of TSNA, comparable to the levels in the American brands. On the other hand, the most inexpensive nonfilter cigarettes such as Astra, Risc, and Prima are very popular among users aged 40−60 years. Though these brands are fairly high in nicotine (from 14.0 to 19.6 mg/g), levels of TSNA are relatively low. The brand Tandem, which of the Moldovan cigarettes had the highest TSNA levels, is not very popular, accounting for only 5% of the market.

It is well-established that significant amounts of TSNA form during the curing and processing of tobacco (*9*, *13*, *14*). Tobacco type as well as curing practices can have significant effects on the levels of these carcinogens in tobacco. Our results suggest that these are important factors in determining the levels of TSNA in Moldovan cigarette tobacco.

The principal traits of cigarette production in the tobacco factory "Tutun-CTC' in Chisinau, Moldova consist of two manufacturing lines. The ordinary line is designated for processing of low-quality tobacco, which is used for inexpensive cigarettes. Classes III−IV tobacco from Moldova, Crimea (American and Diubeca types), and Apkhasia (Samsun type) are used in this line. These tobaccos are fermented at 60−70 °C and 17% relative humidity. The optimal time interval for fermentation is 2−3 months. During this time, different biochemical and chemical processes in the tobacco contribute to its specific aroma and flavor. However, in Moldova, for economic reasons, the fermentation process continues for only 8−10 days. On the basis of the fact that *N*-nitrosamine formation takes place mainly during curing and fermentation, we propose that the short duration of fermentation accounts for the low levels of TSNA in tobacco of some Moldovan cigarettes.

The second line is designed for preparation of American type cigarettes, such as Temp, Tandem, Chisinau, and Flueras extra. Burley, Virginia, and Oriental tobacco brands are used in this manufacturing line. This line is based on modern equipment and includes treatment with additional aromatizing agents, which takes place at increased temperatures. Thus, another step in tobacco processing is included, and possibly, this step along with the higher quality tobacco determines the higher levels of TSNA in the American type cigarettes.

It should be noted that the majority of young smokers use foreign cigarettes commercialized in Moldova. Among these are Lucky Strike, Prima lux, and Monte Carlo. These cigarettes are produced in Europe under license with American corporations. The comparative analysis has shown that the levels of TSNA in these brands are close to those in American cigarettes.

Levels of NNN and NNK in Moldovan cigarette tobacco were lower than in American tobacco and appear to be in the lower range of those reported for cigarette tobaccos from several other European countries (*10*, *15*). TSNA in cigarette tobacco have a significant impact on the amounts in smoke (*16*, *17*). Therefore, Moldovan smokers will be exposed to these carcinogens.

We found no correlation between nitrate or nicotine levels and amounts of TSNA. Nevertheless, nitrate levels in Moldovan cigarette tobacco were frequently lower than in American blends; this may contribute to the lower TSNA levels. Previous studies reported correlations between either nitrate or nitrite and TSNA levels in tobacco. No relationship of TSNA concentrations to alkaloid levels in tobacco has been observed (*9*, *18*, *19*).

In summary, this study presents the first data on TSNA concentrations in the tobacco of cigarettes available in Moldova. Although many brands have lower levels of these carcinogens than those found in American cigarette tobacco, possibly due to abbreviated curing and processing practices, some of the products with higher levels are widely used in particular groups of smokers. The information presented here should be useful in heightening the awareness of the dangers of smoking in Moldova and can be envisioned as the initial step in the control of tobacco-related cancer in this republic.

ABBREVIATIONS USED

5-MeNNN, 5-methyl-*N*′-nitrosonornicotine; C5-NNK, 5-(methylnitrosamino)-1-(3-pyridyl)-1-pentanone; GC-TEA, gas chromatography with nitrosamine selective detection; NAB, *N*′-nitrosoanabasine; NAT, *N*′-nitrosoanatabine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitrosonornicotine; TSNA, tobacco-specific nitrosamines.

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