Analytical Studies on Tobacco-Specific N-Nitrosamines in Tobacco and Tobacco Smoke

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Key Words: gas chromatography, thermal energy analyzer (TEA), capillary GC/TEA, snuff, cigarette smoke, N'–nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N'-nitrosoanatabine (NAT), N'-nitrosoanabasine (NAB), nitrosoamino acids.

I. INTRODUCTION

In 1962, Druckrey and Preussmann first suggested that the tobacco alkaloids nornicotine and nicotine may be precursors which could form the then suspected carcinogen *N'*-nitrosonornicotine (NNN) during smoking.[1] Shortly thereafter, Roe et al. pointed out the possible role of N'-nitrosoanabasine as a carcinogen in tobacco smoke.[2] It was then Boyland et al. who, in 1964, determined that NNN was in fact a lung carcinogen in mice;[3] later that year, Boyland et al. also reported that N'-nitrosoanabasine gives rise to benign as well as malignant tumors of the rat esophagus.[4]

II. ANALYTICAL DEVELOPMENTS

In the same communication, Boyland described his first attempt to detect nitrosamines in tobacco smoke, yet no suitable method was available.[4] At the same time, Preussmann et al. developed an analytical method using thin layer chromatography,[5] while Neurath et al. devised a relatively specific test for the analysis of nitrosamines in tobacco smoke.[6] In 1973, Klus and Kuhn described a semiquantitative method, reporting 40 ng NNN per cigarette in the smoke of nornicotine-rich cigarettes.[7] Shortly thereafter, we described a gas chromatographic method for the quantitative analysis of NNN and found 1.9 to 6.6 ppm in smoking tobacco and 3.4 to 88.6 ppm in chewing tobacco,[8] while 137 ng of NNN were detected in the mainstream smoke of an American blend cigarette.[9] In the following years, several analytical studies on tobacco-specific N-nitrosamines (TSNA) were published, using thin layer chromatography,[10,11] high performance liquid chromatography (HPLC),[12] gas chromatography (GC),[13] gas chromatography/mass spectroscopy (GC/MS),[14] and radioimmunoassay (RIA).[15] During that time, another TSNA, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was detected in chewing tobacco and snuff ranging from 0.6 to 2.4 ppm.[15]

A breakthrough in the nitrosamine analysis came with the introduction of the thermal energy analyzer (TEA), a chemiluminescence detector which is very sensitive and rather specific for nitrosamines.[17] Using this detector in connection with HPLC, TSNA could be analyzed after minimal sample enrichment; it was with this method, that a third TSNA, N'-nitrosoanatabine (NAT), was identified.[18] NAT was found in tobacco at levels ranging from 0.4 (bright cigarette tobacco) to 44 ppm (fine-cut chewing tobacco), while in mainstream and sidestream smoke the level of NAT ranged between 0.3 and 4.6 and between 0.2 and 1.5 μg/cigarette, respectively. The possible presence of NAT in cigarette smoke was first suggested by Klus and Kuhn.[11] Since the HPLC-TEA interface is rather tedious to use on a routine basis, we modified the procedure by directly interfacing the TEA pyrolysis tube into the oven of a gas chromatograph.[19] This improvement led to the identification of a fourth TSNA, N'-nitrosoanabasine (NAB).[20]

Our analytical studies using GC-TEA have led to the identification of a total of seven TSNA in tobacco and tobacco smoke. In addition to NNN, NAT, NAB, and NNK, we also identified 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanal (NNAL), 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (iso-NNAL), and, most recently, 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid (iso-NNAC).[21,22] Of these, NNN and NNK are strong carcinogens in mice, rats, and hamsters where they induce benign and malignant tumors of the lung, nasal cavity, mouth, esophagus, and/or pancreas. NNAL elicits carcinoma of the lung and pancreas in rats and NAB causes esophageal tumors in rats and lung tumors in mice. Iso-NNAL has so far not been bioassayed and NAT and iso-NNAC are inactive as carcinogens.[23]

Figure 1 shows how the TSNA are formed from the tobacco alkaloids.

III. ANALYTICAL METHODS

As discussed earlier, GC-TEA is the method of choice for the analysis of TSNA. It is important to prevent artifactual formation of nitrosamines during the analytical work-up. This can be achieved by using nitrosation inhibitors such as ammonium sulfamate or ascorbic acid.[22,24] In order to compensate for losses during the analysis, we have employed either ^{14}C-labeled NNN or N-nitrosoguvacoline as internal standard.[21,22] Figure 2 shows a GC-TEA trace of TSNA in snuff tobacco while Figure 3 depicts such a trace from cigarette smoke. Both chromatograms were obtained using a 12 ft × ¼" (2 mm I.D.) glass column packed with 10% UCW-982 on Gas Chrom Q,

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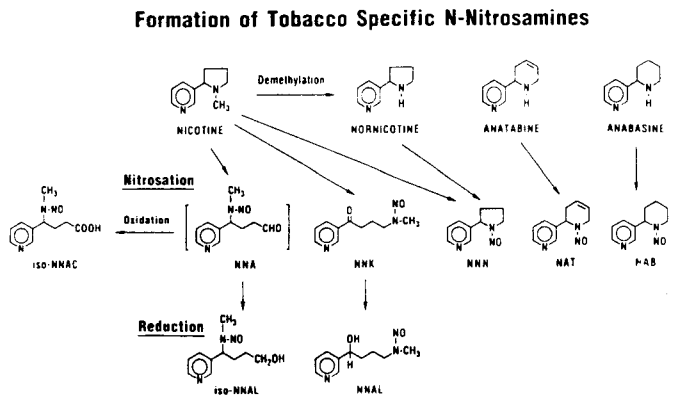

FIGURE 1. Formation of tobacco-specific N-nitrosamines.

resulting in a satisfactory separation. For the separation of the major TSNA, we have later chosen a GC packing consisting of 3% XE-60 on Gas Chrom Q.[21,22] Figure 4 shows the chromatographic separation of six TSNA. Although packed GC columns provide adequate separation and are easy to use for most applications, capillary columns offer better resolution and are essential for applications such as GC-mass spectral identification. Figure 5 represents a capillary GC trace of a standard mixture consisting of volatile nitrosamines (VNA) and TSNA, while Figure 6 shows a capillary GC-TEA trace of a snuff tobacco extract.

We recently refined our procedure for the analysis of nitrosamines in tobacco;[22] this allows the determination of nitrosamino acids in addition to the TSNA. The procedure consists of fractionation of the tobacco extract at different pH levels. The pH 2 fraction contains nitrosamino acids, such as nitrososarcosine, 3-(methylnitrosamino)propionic acid, 4-(methylnitrosamino)butyric acid, and nitrosoproline; the pH 4 fraction contains the nicotine-derived nitrosamino acid iso-NNAC and the pH 9 fraction contains the other TSNA, namely, NNN, NAT, NAB, and NNK. Figure 7 illustrates a schematic of the fractionation and Figure 8 shows a representative GC-TEA trace of the different pH fractions. Recently, Spiegelhalder et al. published a modified method for the analysis of TSNA in both tobacco and tobacco smoke.[25,26] This method employs the use of either *N*-nitrosopentylpicolylamine or *N*-nitrosodibenzylamine as internal standard. For the gas chromatographic separation, a column packed with 10% OV-17 on Chromosorb WHP was used; however, it did not separate NAT from NAB.

IV. RESULTS AND DISCUSSION

Table 1 lists the levels of TSNA in various snuff tobaccos and one type of chewing tobacco (loose leaf). As can be seen, the TSNA concentrations are the lowest in chewing tobacco.

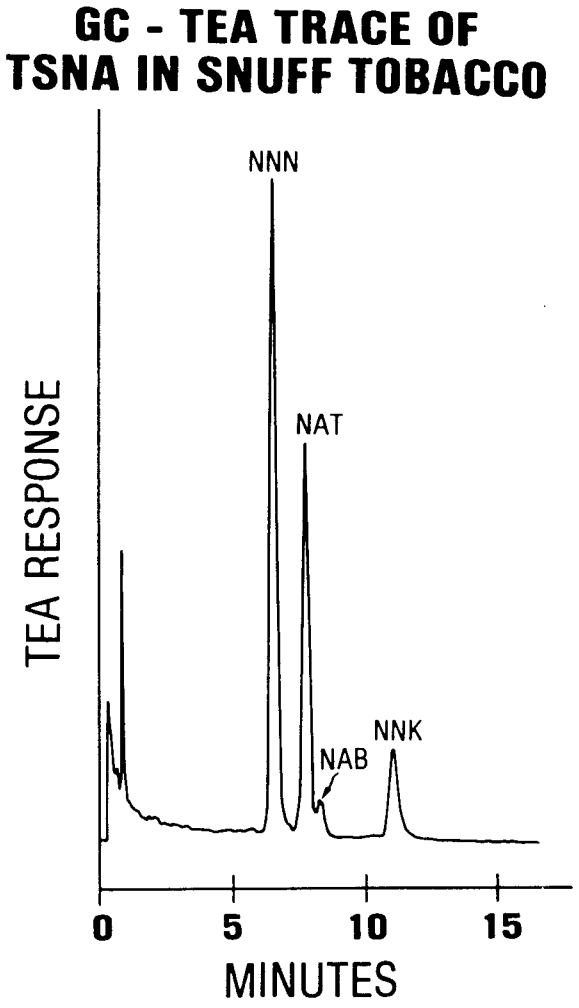

FIGURE 2. GC-TEA trace of tobacco-specific N-nitrosamines in snuff tobacco.

In moist and dry snuff, they range from a total of 8.4 to 166.3 μg/g (based on dry weight). These high levels of carcinogenic nitrosamines exceed those found in other consumer products by several orders of magnitude.[27] Remarkably, a recently introduced moist snuff product (U.S. moist snuff D) which bears the same brand name as the chewing tobacco in Table 1 (where the lowest nitrosamine levels were found) contained the highest TSNA levels of any snuff analyzed, even though the levels of nicotine, nornicotine, and other *Nicotiana* alkaloids were comparable to those of the other U.S. snuff brands. This finding suggests that the processing of this new snuff brand resulted in increased formation of N-nitrosamines, leading to a higher carcinogenic potential of this new snuff brand. On the other hand, TSNA levels in the two moist snuff brands A and B which account for about 90% of the current U.S. market have shown a decreasing trend in the concentrations of TSNA since

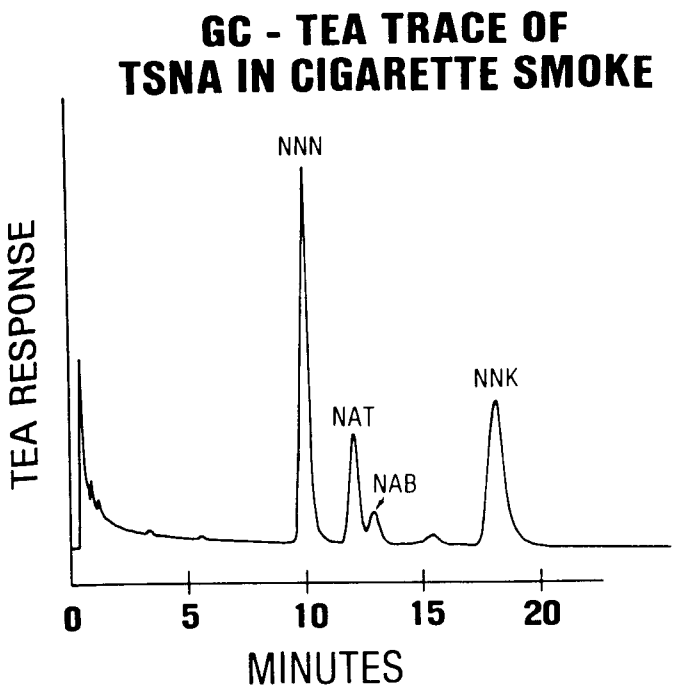

FIGURE 3. GC-TEA trace of tobacco-specific N-nitrosamines in cigarette smoke.

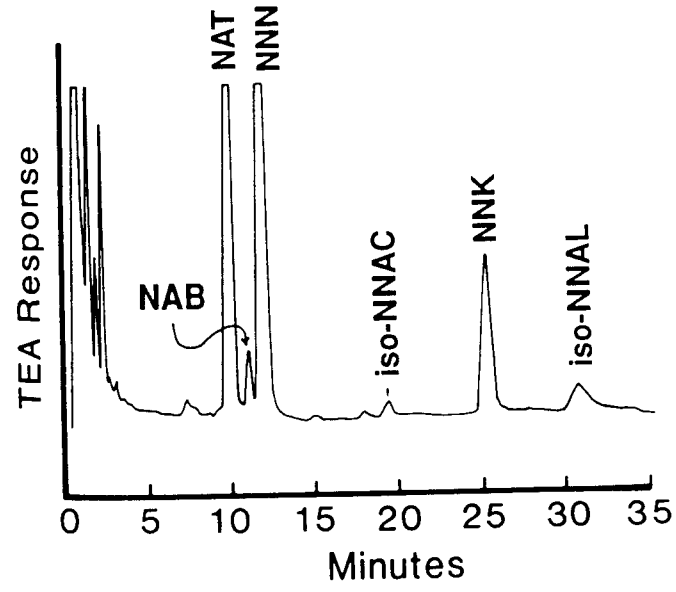

FIGURE 4. GC-TEA separation of six major tobacco-specific N-nitrosamines in snuff using XE-60 GC packing.

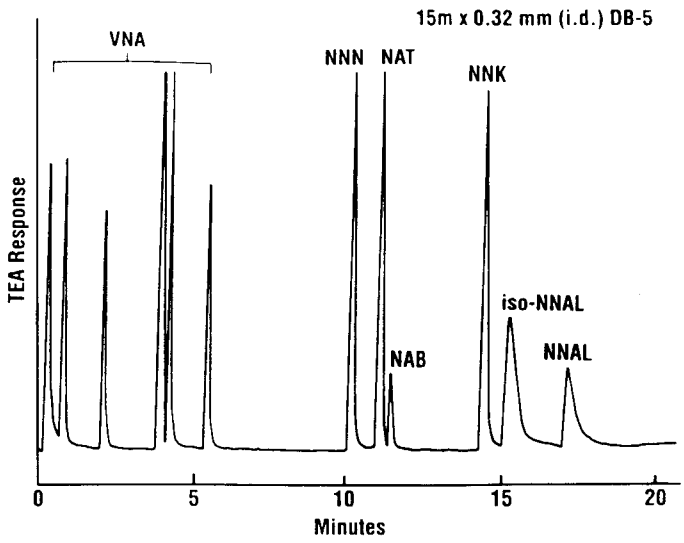

FIGURE 5. Capillary gas chromatogram of a nitrosamine reference mixture.

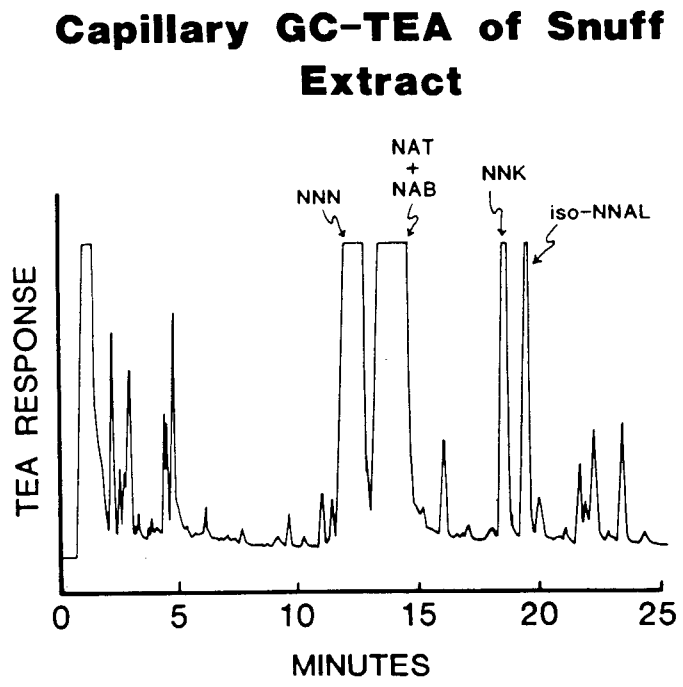

FIGURE 6. Capillary gas chromatogram of snuff extract.

1980.[28] The new U.S. moist snuff brand C had relatively low levels of TSNA and are in line with those of the Swedish moist snuff brand A and B.

Table 2 lists the levels of TSNA in the mainstream smoke of several brands and types of cigarettes, including the Kentucky 1R4F reference cigarette as well as the German reference

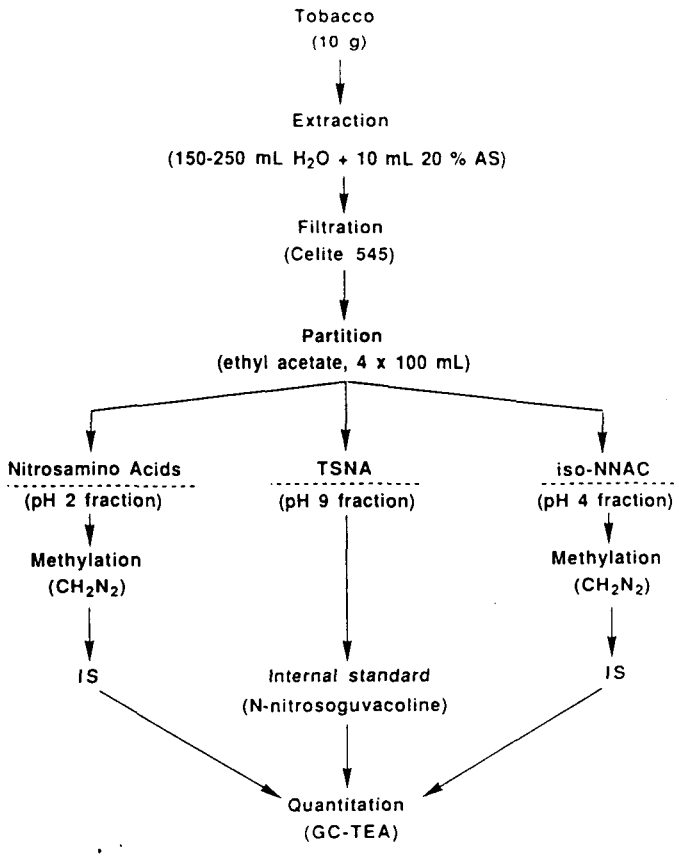

FIGURE 7. Fractionation scheme for the analysis of different nitrosamines in tobacco.

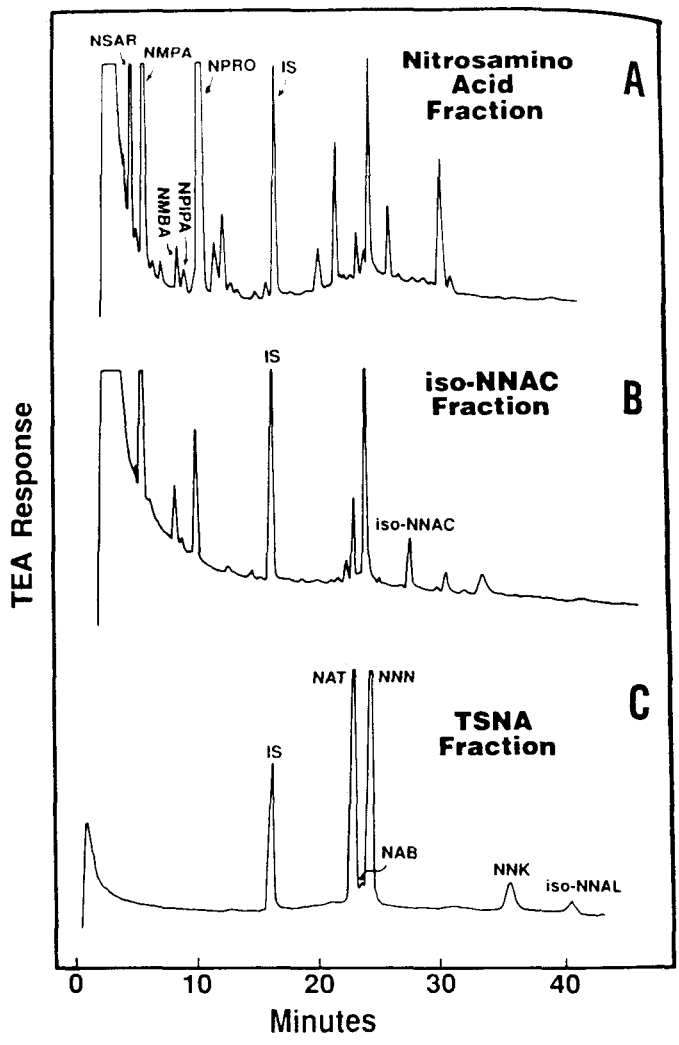

FIGURE 8. GC-TEA traces of the different nitrosamine fractions (IS = internal standard, N-nitrosoguvacoline).

cigarette C20. Altogether, the levels of total TSNA range from 113 to 788 ng/cigarette. A recently introduced U.S. cigarette treated by supercritical fluid extraction to remove most of the nicotine yielded TSNA concentrations in the mainstream smoke similar to those of an ultra low-yield cigarette. The highest levels of TSNA were observed in the smoke of a French non-filter cigarette. This is due to the tobacco fermentation which is performed with most French cigarette tobaccos. The levels of NNN and NNK in the smoke of other European and U.S. cigarettes, published recently by Fischer et al., are comparable with our data (Table 2).[29] In another communication, Fischer et al. suggest that all TSNA in mainstream smoke are transferred from preformed TSNA in the tobacco rather than pyrosynthesized during the combustion process,[30] as had been surmised on the basis of studies on the transfer rate of NNN-^{14}C into cigarette smoke.[31] The data in Table 2 suggest that means are available to reduce the carcinogenic TSNA in cigarette smoke. Industry should be encouraged to work toward such a goal.

V. SUMMARY

Chemical-analytical studies have led to the identification of approximately 3000 compounds in tobacco and 4000 in tobacco smoke. These include carcinogens in processed tobacco as well as tumor initiators, tumor promoters, cocarcinogens, and organ-specific carcinogens in tobacco smoke. The latter group includes N-nitrosamines, in particular those that derive from nicotine and other tobacco alkaloids, the TSNA. *In vitro* nitrosation of nicotine yields NNN, NNA, and NNK. Nitrosation of other tobacco alkaloids leads to the formation of NAT, and NAB. Our analytical studies using GC-TEA have led to the identification of seven TSNA in tobacco and tobacco smoke. In addition to NNN, NAT, NAB, and NNK, we also identified

Table 1
TSNA in Snuff and Chewing Tobacco (μg/g dry weight)

Product type	NNN	NAT + NAB	NNK	iso-NNAC	Total TSNA
U.S. moist snuff A	10.4	9.8	2.2	0.1	22.5
U.S. moist snuff B	9.6	7.9	3.1	0.2	20.8
New U.S. moist snuff C	4.1	3.0	1.2	0.1	8.4
New U.S. moist snuff D*	57.1	91.5	7.2	10.5	166.3
Sweden moist snuff A	5.7	3.5	2.1	0.1	11.4
Sweden moist snuff B	5.3	2.9	1.4	0.1	9.7
Sweden moist snuff C	5.2	2.6	1.4	0.1	9.3
U.S. dry snuff	10.6	13.1	0.9	0.1	24.7
U.S. chewing tobacco*	1.5	0.7	0.1	0.01	2.3

* Same brand name.

Table 2
TSNA in Cigarette Mainstream Smoke (ng/cigarette)

Cigarette type	NNN	NAT	NAB	NNK	Total TSNA
KY 1R4F	85	158	10	30	283
US, NF	162	156	12	49	379
US, F	162	167	12	75	416
US, F, Lights	188	144	n.d.	47	379
US, F, Ultra lights	46	58	n.d.	8.7	113
US, F, low nicotine[a]	34	50	9	36	129
French, NF	527	177	15	69	788
German C20, F	82	83	5.6	20	191
Russian, F	389	196	n.d.	37	622

Note: NF = nonfilter; F = filter; n.d. = not determined.

[a] Nicotine = 0.032 mg/cigarette. (The bulk of nicotine has been removed from this cigarette tobacco by means of supercritical fluid extraction.)

NNAL, iso-NNAL, and, most recently, iso-NNAC. Their levels range from 0.01 to 92 ppm in tobacco and from 6 to 530 ng/cigarette in tobacco smoke. The high levels observed in snuff are primarily due to fermentation and aging. Technological methods exist today to reduce the levels of TSNA in both tobacco and cigarette smoke.

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