Table IV. Fate of PRO and NPRO during Smoking[e]

cigarette[a]	mainstream smoke NPRO ng/cig	% transfer	NPYR, ng/cig
control	n.d.[b]		5
+5 mg of PRO	trace[c]		49
+5 mg of NPRO	7140	0.14	2530[d]

[a] 85-mm nonfilter cigarette; 0.70% NO_3, 990 ppm of PRO, 0.88 ppm of NPRO. [b] n.d. = not detected (detection limit 0.5 ng/cigarette). [c] Approximately 1 ng/cigarette. [d] 0.073% by decarboxylation. [e] Abbreviations: see footnote a of Tables I and II.

one may conclude that if cigarette smoke contains any NPRO, it is less than 1.0 ng/cigarette.

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Registry No. NPRO, 7519-36-0; *N*-nitrosopyrrolidine, 930-55-2; *N'*-nitrosonornicotine, 16543-55-8.

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An Alternate Method for the Analysis of *N*-Nitrosonornicotine in Tobacco

William J. Chamberlain* and Richard F. Arrendale

A method is described for the analysis of *N*-nitrosonornicotine (NNN) in tobacco. This method has been applied to a number of flue-cured tobacco samples, differing in nicotine and nornicotine contents, in order to determine whether or not there was a direct correlation between nicotine and nornicotine levels and NNN level in tobacco leaf. There appears to be no correlation between the alkaloid content of tobacco leaf and the level of NNN.

The tobacco-specific nitrosamines are the only known cigarette smoke carcinogens that are also found in unburned tobacco. Initially, Hoffmann et al. reported the presence of *N*-nitrosonornicotine (NNN) in tobacco smoke and outlined a method for analysis, using gas chromatography–mass spectrometry (GC–MS) as the detection system (Hoffmann et al., 1973). Subsequently, a method (Hoffmann et al., 1974; Hecht et al., 1975) was developed based on high-speed liquid chromatography and a thermal energy analyzer (TEA) for GC detection and analysis. The TEA has been shown to be the simplest and best detector for *N*-nitrosamines that occur in both tobacco and tobacco smoke. However, many laboratories, including our own, are not equipped with the expensive TEA. We have, therefore, developed a method to separate NNN in tobacco by gas chromatography (GC) on fused silica glass capillary Superox-4 columns combined with detection by a nitrogen–phosphorus (N–P) thermionic detector.

The major problem in using a N–P detector for NNN analysis is the removal of other nitrogen-containing compounds, which could interfere, from the matrix. We have overcome this problem by alumina column chromatography of the crude extract to yield a refined NNN fraction, sufficiently pure for GC analysis. Details of the methodology are presented. In addition, the method was applied in a correlation study between NNN and nicotine–nornicotine levels of tobacco leaf.

It has been shown that NNN in smoke is formed from both nicotine and nornicotine (Hoffmann et al., 1977).

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Since the concentration of nicotine in conventional tobacco types is 20–100 times as great as that of nornicotine, nicotine is considered to be the more important precursor for NNN in both leaf and smoke. Accordingly, we have determined the NNN levels of tobaccos that varied in nornicotine levels and the ratios of nornicotine:nicotine in order to determine the effect of the concentrations of these two alkaloids on the concentration of NNN in the unburned tobacco. The results are presented and discussed.

EXPERIMENTAL SECTION

Flue-tobacco samples (2–5 g), ground to pass a 20-mesh sieve, were ultrasonically extracted by a 150-mL volume of aqueous citrate buffer [similar to reported buffer (Hoffman et al., 1979)]. The buffer solution was then extracted 3 times with 100-mL quantities of dichloromethane in a procedure similar to that reported by Hoffmann et al. (1979). The combined organic extracts were dried over anhydrous sodium sulfate, concentrated to volume of 5 mL, and chromatographed on 30 g of basic alumina (activity III), contained in a 200 mm × 20 mm glass column. The nitrosamine fraction was eluted with 250 mL of dichloromethane and then concentrated to dryness on a rotary evaporator. One milliliter of a methanol solution, containing 0.24 mg of 2,4′-dipyridyl internal standard, was added to the fraction prior to GC analysis.

The samples were analyzed with a Hewlett-Packard Model 5710A gas chromatograph, equipped with a thermionic N–P detector. The standard 5710A instrument was modified for glass capillary GC analyses, as previously described (Severson et al., 1980). NNN analyses were performed in the split mode (100:1) on a 25 m × 0.3 mm i.d. wall-coated open tubular column, coated with Superox-4 [a 4 000 000 M_r poly(ethylene glycol)]. The column was prepared according to Arrendale et al. (1983). The oven temperature was programmed from 150 to 250 °C at 2 °C/min, the He flow was 20 cm/s, and the injection port temperature was 280 °C. The thermionic N–P detector was operated under hydrogen and air flow conditions as recommended by the manufacturer. MS data were obtained with a Hewlett-Packard 5985 GC–MS system. The standard injection port of the HP-5540 gas chromatograph of the MS system was also modified to an all-glass capillary inlet system, as previously described (Severson et al., 1980). The GC conditions were as described above. The capillary column was connected by an open split interface (Arrendale et al., 1982) and 50 cm of 0.15 mm i.d. fused silica glass capillary tubing, which had been deactivated with Superox-4 (Arrendale et al., 1982). The MS conditions were as follows: interface temperature, 275 °C, scan range, 40–300 amu; scan ratio, 400 amu/s; electron multiplier voltage, 2400 V; source temperature, 200 °C.

NNN was prepared by adding a solution of 10 g of sodium nitrite in 20 mL of water, dropwise, to a stirred solution of 0.05 g of nornicotine in 100 mL of 3 N hydrochloric acid. This mixture was allowed to stand 18 h at room temperature and the pH was then adjusted to 10.0 with 3 N sodium hydroxide. The NNN was extracted with chloroform.

RESULTS AND DISCUSSION

The greatest difficulty in analyzing nitrosamines in tobacco is the required prior separation of these compounds from other interfering substances (Brunnemann and Hoffmann, 1978). This difficulty was reportedly overcome by the use of the specific thermal energy analyzer (Hoffmann et al., 1979). However, when we used a recommended extraction procedure (Brunnemann and Hoffmann, 1978) with the thermionic N–P detector, we observed an interfering GC peak, representing a compound

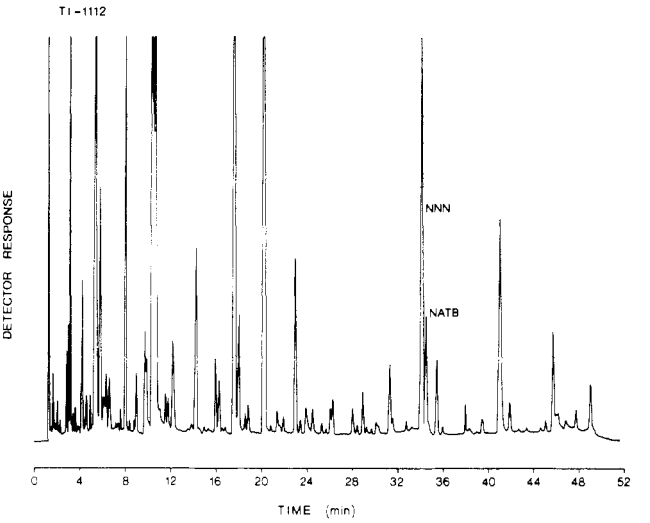

Figure 1. Gas chromatogram of the nitrosamine fraction of flue-cured tobacco TI-1112, separated on a fused silica glass column coated with Superox-4. (NATB = nitrosoanatabine; conditions: 25 m × 0.3 mm i.d. WCOT column; 150–250 °C, 2 °C/min; 20 cm/s helium, split injection mode.)

Table I. Nitrosonornicotine (NNN) Levels in Tobaccos of Varying Total Alkaloid (TA) Contents (ppm)

tobacco[a]	nicotine	nornicotine	TA	NNN
1	8 700	1200	10 300	21.4
2	21 500	900	23 300	97.7
3	28 900	900	31 100	53.0
4	35 700	1100	38 700	36.7
5	44 100	1800	47 100	47.7
6	44 200	1700	47 100	20.8
7	4 700	200	5 100	3.4
8	15 900	600	17 100	27.6
9	21 600	800	23 100	9.8
10	27 400	800	29 100	43.6
11	40 300	3400	44 900	130.0
12	50 400	3000	55 200	79.3

[a] Samples 1–6 are NC-95 tobacco crosses, while samples 7–12 derived from SC-58 bright tobacco.

with a molecular weight of 176. It could not be separated from NNN on the Superox GC column. However, by adjusting the activity of the alumina used in the column chromatographic step, to activity III, it was possible to elute the NNN in the first fraction, while the interfering compound eluted in the next fraction. Subsequent GC and GC–MS analyses indicated that there were no other N-containing compounds eluting with the same retention time as NNN in the tobacco extracts analyzed by this method. A representative gas chromatogram is shown in Figure 1.

The developed methodology was applied to a number of flue-cured tobacco samples, differing in nicotine and nornicotine contents, in order to determine whether or not there was a direct correlation between alkaloid levels and NNN levels in tobacco. Alkaloid levels in crosses of two tobacco varieties, known to contain varying alkaloid levels, were first determined (Table I) (Severson et al., 1981). NNN levels were determined by the reported procedure. Examination of the data indicated no obvious correlations of NNN levels with percentage of nicotine, nornicotine, total alkaloids, or with nornicotine:nicotine ratios. These tobaccos were all "normal" tobaccos in which nicotine constituted approximately 92% of the total alkaloids. In order to determine any possible effects of higher nornicotine levels on the formation of NNN, we examined a series of "converter" tobaccos, which appear to convert

Table II. Alkaloid and NNN Levels in High Nornicotine Tobacco Varieties (ppm)

tobacco	nicotine	nornicotine	NNN
TI-1112	4 800	31 400	1000
V8	13 300	3 700	70
V18	43 800	6 700	142
V48	25 600	600	169
V445	4 000	2 700	100
V446	13 900	10 800	ND[a]

[a] ND = none detected.

nicotine to nornicotine and thus have ratios of nornicotine:nicotine significantly larger. These results are given in Table II. Again, there appeared to be no correlation of nornicotine levels with NNN levels. In the case of TI-1112, where the nornicotine level was very high, the NNN level was also high. However, for variety V446, containing nornicotine at about 10 times a normal level, there was no NNN in the tobacco. Consequently, there appears to be no correlation between alkaloid contents of tobacco leaf and levels of NNN. We will be examining the smoke from these tobaccos to determine any effects of varying alkaloid levels on the formation of NNN in smoke.

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COMMUNICATIONS

Identification of Two New Volatile Amines in Wine

Two amines, 1-pyrroline and 4,5-dimethyl-1,3-dioxolane-2-propanamine, were identified in wine. This was accomplished by comparison of retention times and mass spectra of trifluoroacetamide (TFA) derivatives of the wine components with those of synthetic TFA derivatives.

Schreier (1979) reviewed previous amine work in wines. In an earlier paper Ough et al. (1981) reported the identification of and mass spectra for 10 amines in grapes and wines. Gas chromatographic analysis revealed a number of other unidentified amines in wine. This paper reports the structures of two such amines which were not previously characterized.

MATERIALS AND METHODS

Grapes and Wine. Pinot noir wine, the same made for a previous study (Ough et al., 1981; Daudt, 1980), and sherries, both baked and flor, were used for analysis by gas chromatography–mass spectrometry (GC–MS).

Separation and Derivatization of Amines in Wine. The procedure for isolation of amines and their derivatization with trifluoroacetic anhydride (TFAA) has been reported (Ough et al., 1981). In addition, the amines studied here were also obtained from the wine, made basic, by distillation (30 °C at 4 mmHg) under N_2 gas, and then derivatized (Daudt, 1980).

An alternative procedure, not involving distillation, gave the same dioxolaneamine TFA that had been produced by the procedure described above: The wine sample (750 mL) was acidified (pH 1.5) with HCl, concentrated (40 °C and 22 mmHg) in a rotary evaporator to 100 mL, made basic (pH 11), and extracted with 100 mL of 1-butanol. The solvent layer was washed with 1 M NaOH and then with two portions of 1 M HCl. The HCl solution was extracted twice with butanol and then concentrated at reduced pressure to a dry salt.

The salt was derivatized as before with 8 mL (TFAA) and washed with 30 mL of saturated aqueous $NaHCO_3$. Solid $NaHCO_3$ was added to make the solution basic and the mixture was extracted with 15 mL of ether. The ether was washed with saturated $NaHCO_3$ and then with water. The ether solution was dried, filtered, and concentrated under a stream of N_2 to ca. 0.1 mL of liquid which was analyzed immediately by GC–MS.

1-Pyrroline, 1, and Its Hydrochloride Salt, 1a. The procedure of Amoore (1979) was scaled up by a factor of 20 for the preparation of aqueous 1-pyrroline. The hydrochloride salt of 1 was prepared under conditions similar to the isolation of salts from wine (Ough et al., 1981) to yield 150 mg of a white solid.

Reaction Product, 1b, of 1a with TFAA. The salt **1a** was combined with 1 mL of TFAA. The reaction condi-

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