group was dried for analysis after 2 days of refrigerated storage in the green state. Almost 58% of the starch originally present was lost. This difference could not be accounted for by the oven-drying process since such a difference was not seen for samples harvested July 14, 1981, and dried immediately. This points up the need for rapid processing of green tobacco to obtain reliable results. The most practical approach appears to be *immediate* freeze-drying after sampling green tobacco to get it into a form which is stable and can be analyzed reproducibly.

It is also interesting to observe that the starch content of Coker 319 *field-grown* tobacco (Table II) harvested at the early "button" stage is about 5 times that of Coker 411 *greenhouse-grown* tobacco (Table I) harvested in the early flowering stage (~15% vs. ~3%). Similar low values were found for a Coker 319 *greenhouse-grown* tobacco that had a starch content of 3%. This appears to be another manifestation of the evidence that field-grown plants are more lush and full bodied than plants grown under the conditions maintained in our greenhouse. The magnitude of the difference is such that it cannot be due to varietal variation between the two Coker varieties.

No recovery studies were made of starch added to tobacco because it was felt that topically applied starch would wash off during the ethanol extraction.

CONCLUSIONS AND RECOMMENDATIONS

An enzymatic method for the determination of starch in tobacco in various forms has been developed. With slight modifications it has been applied to other starches. While it is a somewhat laborious method requiring solvent extraction, starch solubilization, and two enzymatic treatments before the manual colorimetric readout, it is felt to be an absolute representation of the starch content of materials rather than a relative value. It has been applied successfully to samples which could not be analyzed by an automated starch–iodine method.

Future improvements in speed and possibly in precision can be made by automation of the glucose oxidase readout using a Technicon AutoAnalyzer II system or a Yellow Springs Instrument Co. Model 27 industrial analyzer.

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N-Nitrosoproline, an Indicator for N-Nitrosation of Amines in Processed Tobacco

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N-Nitrosoproline (NPRO) was isolated from processed tobacco; a method for its quantitative assessment was developed, based on the enrichment of NPRO by solvent distributions, derivatization to its methyl ester, and gas chromatography with a thermal energy analyzer (detection limit 0.5 ng/g of tobacco). Cigarette and cigar tobaccos contained 0.33–2.3 ppm of NPRO; chewing tobacco and snuff had levels of 0.45–21.8 ppm. On the basis of the analyses of 14 tobacco products, the formation of the noncarcinogenic NPRO is significantly correlated with the formation of *N'*-nitrosonornicotine and the sum of the carcinogenic tobacco-specific *N*-nitrosamines (TSNA). Fine-cut snuff is relatively rich in NPRO as well as in TSNA. The latter are the only known carcinogens in snuff tobaccos where they occur in relatively high concentrations. It is suggested that efforts be undertaken to inhibit *N*-nitrosamine formation, especially during the preparation of fine-cut snuff. NPRO was not detected in cigarette smoke (<1 ng/cigarette).

Tobacco and tobacco smoke contain three types of *N*-nitrosamines, namely, the volatile nitrosamines, *N*-nitrosodiethanolamine, formed from the residue of an agricultural chemical, and the tobacco-specific *N*-nitrosamines (Hoffmann et al., 1981; Brunnemann et al., 1982). These agents are not present in freshly cut green leaves but are formed during tobacco processing (Hecht et al., 1978; Adams et al., 1983). All of the *N*-nitrosamines so far identified in tobacco products are known carcinogens (U.S. Surgeon General, 1982).

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Recently, *N*-nitrosoproline (NPRO) has become an important tool in nitrosamine research because it is considered nonmutagenic and noncarcinogenic (International Agency for Research on Cancer, 1978). NPRO is formed in man by endogenous N-nitrosation of proline and is excreted in the urine in unchanged form (Ohshima and Bartsch, 1981). Upon administration of dietary proline supplements, we found higher amounts of NPRO in the urine of some cigarette smokers than in urine of nonsmokers (Hoffmann et al., 1983); thus, we questioned whether tobacco products contain NPRO. It was the goal of this study to explore the possible presence of NPRO in tobacco and tobacco smoke and to investigate whether this compound could serve as an indicator for the formation of TSNA during tobacco processing and smoking. The advantage of measuring N-nitrosation potential by determining the noncarcinogenic *N*-nitrosoproline lies in avoiding the carcinogenic tobacco-specific *N*-nitrosamines for which reference compounds are not always readily available.

EXPERIMENTAL SECTION

Materials. All commercial tobacco products were purchased on the open market in Westchester County, NY, during 1982. Green Burley 21 tobacco plants were made available by Dr. T. C. Tso, U.S. Department of Agriculture, Beltsville, MD. The leaves were harvested when the plants had reached maturity.

Chemicals. NPRO was synthesized from proline according to Lijinsky et al. (1970). Its purity was ascertained by gas chromatography (GC) and mass spectrometry (MS). The standard mixture of volatile *N*-nitrosamines was purchased from Thermo Electron Corp., Waltham, MA; the tobacco-specific *N*-nitrosamines (TSNA) were synthesized according to earlier published methods (Hoffmann et al., 1979). L-Proline-*U*-^{14}C (sp act. 250 μCi/μM) was obtained from ICN, Irvine, CA, and 14% BF_3 in methanol from Pierce Chemical Co., Rockford, IL.

Apparatus. A Model 543 thermal energy analyzer (TEA) from the Thermo Electron Corp., Waltham, MA, was interfaced directly with a Model 700 gas chromatograph (Hewlett-Packard) by using modifications previously described (Brunnemann and Hoffmann, 1981). The mass spectral analysis was done with a Hewlett-Packard Model 5982 GC–MS instrument. Cigarettes were smoked with a 20-port automatic smoker with a rotating head (H. Borgwaldt, Hamburg, FRG). Every second port of the smoking machine was connected to a nitrogen source which replaced the air with nitrogen in order to prevent the possible artifactual formation of nitrosamines in the headspace of the device and the trap (Hoffmann et al., 1976).

Determination of NPRO in Tobacco. Five grams of tobacco was extracted for 2 h with 100 mL of water containing 6 mL of AS solution (20% ammonium sulfamate in 3.6 N sulfuric acid) and NPRO-*U*-^{14}C (96 500 dpm/27 ng) as an internal standard. Subsequently, the mixture was filtered through Celite 545. After the addition of 20 g of NaCl, the filtrate was partitioned with 4 × 150 mL of ethyl acetate. The combined organic layers were dried (Na_2SO_4) and reduced to 0.1 mL in a rotary evaporator. One milliliter of 14% BF_3 in MeOH was added to the residue and the mixture was heated at 60 °C for 1 h. After the mixture was cooled to ambient temperature, 1 mL of dichloromethane and 4 mL of water were added. The organic phase was removed and placed into a 1-mL serum vial together with 100 mg of Na_2SO_4. An aliquot was used to determine the recovery rate by scintillation counting, and a second aliquot was analyzed by GC–TEA on a 6 ft × 1/4 in. o.d. (2 mm i.d.) glass column packed with 10% Carbowax 20 M on Chromosorb W (oven temperature 150 °C).

For the mass spectral identification of NPRO, 100 g of snuff tobacco was extracted in 2000 mL of water and the extract was worked up as described under Determination of NPRO in Tobacco and further enriched by column chromatography on 60 g of silica gel with a 1:1 mixture of methanol–ethyl acetate. The radioactive fraction was purified by preparative TLC. After extraction of the NPRO band, the final fraction was further concentrated and methylated prior to GC–MS identification. We used a 25 m × 0.24 mm (i.d.) fused silica capillary column (WCOT) coated with chemically bonded methyl silicone (film thickness 0.25 μm). The GC program was 4 min at 50 °C and then 2 °C/min to 250 °C. Under these conditions, the methyl ester of NPRO had a retention time of 11.3 min.

Determination of Proline in Tobacco as *N*-Nitrosoproline (NPRO). One gram of processed tobacco was stirred for 2 h with 50 mL of water containing 1 g of $NaNO_2$, 2 mL of 1 N acetic acid, and 1 mL of proline-*U*-^{14}C (84 000 dpm/19 ng). The determination of proline as NPRO was then carried out with the analytical procedure described above. In the case of green tobacco we homogenized ≃25 g of leaves, immediately after their removal from the stalk, in 150 mL of diluted AS solution (see Determination of NPRO in Tobacco) in a blender. The analysis was completed in the same manner as in the case of processed tobacco.

Analysis of NPRO and *N*-Nitrosopyrrolidine in Tobacco Smoke. For the untreated control, 100 cigarettes were smoked with a 20-unit automatic smoker (see Apparatus) and the mainstream smoke was led through two gas wash bottles containing citrate buffer and 20 mM ascorbic acid (Hoffmann et al., 1979) with NPRO-*U*-^{14}C serving as the internal standard (96 500 dpm/27 ng). After addition of 80 g of NaCl, the buffer solution was extracted with ethyl acetate and the latter was dried (Na_2SO_4). After concentration to 2 mL, the extract was further enriched by column chromatography (60 g of silica gel, mesh 40–140, Baker Chemicals) and then analyzed for NPRO (see Determination of NPRO in Tobacco) and *N*-nitrosopyrrolidine (Brunnemann et al., 1977). Only 10 cigarettes per analysis were smoked in the case of applications of 5 mg of either proline or NPRO in 50 μL of water by the microsyringe technique (Hoffmann et al., 1977).

Other Analytical Methods. For the analysis of VNA and TSNA in tobacco we employed earlier published methods (Brunnemann et al., 1977; Hoffmann et al, 1979). For the determination of moisture in tobacco, a modified Dean-Stark procedure was used (von Bethmann et al., 1961). Nitrate in tobacco was determined by the specific ion electrode method (Jacin, 1970).

Statistical Evaluations. Linear regression models were calculated on an IBM 4341 Model II mainframe by using a statistics analysis system (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Analysis of Tobacco for NPRO. A schematic representation of the NPRO analysis in tobacco is shown in Figure 1. It involves extraction, solvent partition, and subsequent methylation as well as GC–TEA analysis. Figure 2 shows the GC–TEA trace of a concentrate from a cigarette tobacco extract depicting the methyl ester of NPRO (retention time 5.5 min). In one case NPRO was enriched from 100 g of snuff tobacco following the analytical scheme (Figure 1) with an additional column chromatographic step and preparative TLC; NPRO was

Table I. Analytical Data for Commercial Tobacco Products[a]

tobacco product[b]	NO_3, %	nicotine, %	NNN, ppm	% nitrosation × 10^{-2} [c]	total TSNA, ppm	PRO, ppm	NPRO, ppm	% nitrosation[d]
U.S., F, 85 (A)	0.81	1.82	2.64	1.33	3.73	1620	1.50	0.075
U.S., F, 85 (B)	1.23	1.45	2.17	1.37	4.95	1070	1.60	0.12
U.S., NF, 85	0.70	2.05	1.83	0.81	3.61	990	0.88	0.071
U.S., NF, 70	1.08	1.81	1.96	0.99	4.09	860	1.20	0.11
U.S., F, 85, menthol	1.14	2.04	1.94	0.87	4.09	800	1.45	0.14
U.S., F, 85, light	0.89	1.66	4.44	2.44	8.90	960	2.30	0.19
U.S., F, 85, ultra light	0.74	1.72	3.20	1.70	6.44	890	1.58	0.14
French, NF, 70	0.93	1.25	1.80	1.32	2.70	980	1.55	0.13
French, F, 70	0.98	1.20	0.64	0.48	0.98	565	1.43	0.20
Kentucky 1R1, 85	0.53	2.30	0.68	0.27	1.08	1280	0.33	0.021
cigar	1.98	1.10	2.94	2.43	4.78	100	1.13	0.92
fine-cut snuff	3.48	2.42	20.5	7.71	33.3	480	21.8	3.66
snuff (pouches)	2.73	1.91	6.63	3.17	15.1	400	3.48	0.70
loose leaf	2.20	0.92	1.16	1.16	1.98	160	0.45	0.22

[a] Based on dry tobacco weight. Abbreviations: NNN = N'-nitrosonornicotine; TSNA = tobacco-specific N-nitrosamines; PRO = proline; NPRO = N-nitrosoproline. [b] Numbers indicate length in millimeters of cigarettes; F = filter; NF = nonfilter. [c] Percent N-nitrosation of nicotine to NNN. [d] Percent N-nitrosation of PRO to NPRO.

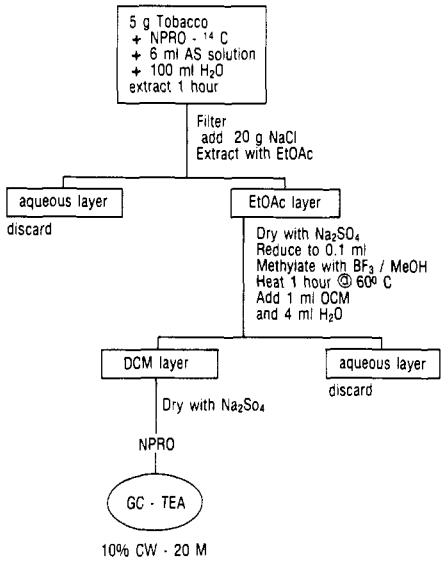

Figure 1. Schematic for the analysis of NPRO.

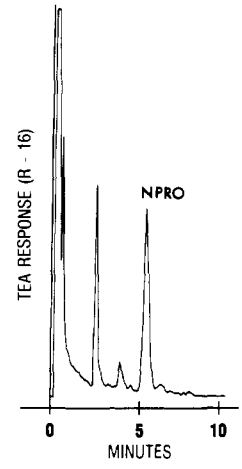

Figure 2. GC–TEA trace of cigarette tobacco extract (U.S., F, 85 mm, Ultra Light) showing the presence of NPRO.

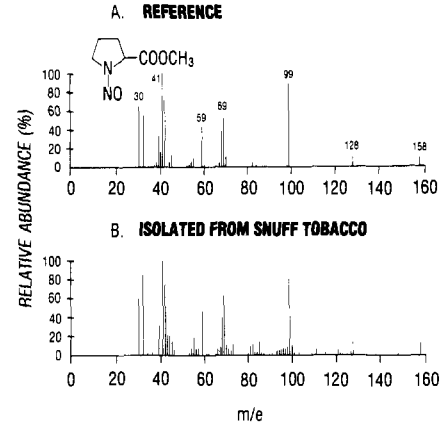

Figure 3. Mass spectra of reference and isolate from snuff tobacco showing NPRO as the methyl ester.

then identified as the methyl ester by capillary GC–MS (Figure 3).

The identity of NPRO isolated from each tobacco sample was assured by established confirmatory methods (Krull et at., 1979). When both the standard solution of NPRO and the concentrates from the tobacco extracts were treated with HBr, the NPRO peak in the GC–TEA disappeared completely. For a more positive confirmation we oxidized NPRO to the corresponding nitramine [C_4H_7-(COOH)N-NO → C_4H_7-(COOH)N-NO_2] using the method of Emmons (1957) modified to include acetic anhydride and 50% hydrogen peroxide in place of trifluoroacetic acid anhydride and 90% H_2O_2 (Sen et al., 1982). (This treatment had to precede the methylation in order to avoid hydrolysis of the methyl ester of NPRO prior to GC–TEA analysis.) A NPRO standard solution treated with BF_3–MeOH showed that approximately half of the NPRO was oxidized to the corresponding nitramine as detected by GC–TEA at a TEA pyrolyzer temperature of 800 °C. The retention time of the methylated nitramine was exactly twice that of the methyl ester of NPRO.

On the basis of five analyses of the Kentucky reference cigarette, the relative standard deviation for NPRO and PRO was ±8%. According to the loss of the ^{14}C-labeled internal standard, 65–75% of NPRO and proline were enriched in the concentrate from which NPRO was determined as the methyl ester. When we started with 5 g of processed tobacco, the detection limit was about 0.2 ng/g.

When using 25 g of green leaves (nitrate content 0.2%) which was analyzed immediately after removal from the stalk of a Burley 21 tobacco plant, we were unable to detect measurable amounts of NPRO (<5 ppb). This result supports our earlier observation (Hecht et al., 1978; Adams et al., 1983) that N-nitrosamines are primarily formed during tobacco processing.

Table II. Volatile N-Nitrosamines in Commercial Tobacco Products (ppb)[a]

tobacco product[b]	NDMA	NDEA	NDPA	NDBA	NPIP	NPYR	NMOR	total VNA
U.S., F, 85 (A)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
U.S., F, 85 (B)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
U.S., F, 85	280	47	n.d.	n.d.	15.3	4.9	3.7	350
U.S., NF, 70	250	n.d.	n.d.	1.5	5.5	n.d.	4.1	260
U.S., F, 85, menthol	6.0	n.d.	n.d.	n.d.	n.d.	3.6	n.d.	10
U.S., F, 85, light	6.7	2.0	2.3	5.0	7.0	9.9	10	43
U.S., F, 85, ultra light	4.4	n.d.	n.d.	2.8	n.d.	5.9	n.d.	13
French, NF, 70	42	7080	n.d.	n.d.	n.d.	7.5	1.1	7120
French, F, 70	58	7870	n.d.	n.d.	11	n.d.	n.d.	7940
Kentucky 1R1, 85	12.7	2.7	2.7	4.4	4.0	n.d.	7.6	34
cigar	n.d.	3.2	11.8	0.9	22	20	6.4	64
fine-cut snuff	74	n.d.	n.d.	n.d.	14	115	44	247
snuff (pouches)	15	n.d.	2.7	n.d.	13	140	36	207
loose leaf	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	

[a] Based on dry tobacco weight. Abbreviations: NDMA = nitrosodimethylamine; NDEA = nitrosodiethylamine; NDPA = nitrosodipropylamine; NDBA = nitrosodibutylamine; NPIP = nitrosopiperidine; NPYR = nitrosopyrrolidine; NMOR = nitrosomorpholine; VNA = volatile nitrosamines; n.d. = not detected (<0.5 ppb). [b] Numbers indicate length in millimeters of cigarettes; F = filter; NF = nonfilter.

Table III. Statistical Evaluations (Commercial Tobacco Products)[a]

no. in model	independent variable	dependent variable	r^2
1	NO_3	NNN	0.592
1	nicotine	NNN	0.238
1	NO_3	NPRO	0.522
1	PRO	NPRO	0.052
1	NPRO	NNN	0.961
1	NPRO	TSNA	0.899
2	NPRO, NO_3	NNN	0.966
2	NPRO, NO_3	TSNA	0.918
2	NPRO, PRO	NNN	0.962
2	NPRO, PRO	TSNA	0.903
3	NPRO, PRO, NO_3	NNN	0.966
3	NPRO, PRO, NO_3	TSNA	0.918

[a] Abbreviations: see footnote a of Table I.

Table I lists the analytical data from 13 commercial tobacco products and from the Kentucky reference cigarette 1R1 for nitrate, nicotine, and N'-nitrosonornicotine, the percentage of nicotine which is N-nitrosated to N'-nitrosonornicotine, total TSNA [N'-nitrosonornicotine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, N'-nitrosonanatabine, and N'-nitrosoanabasine], proline, and NPRO, and the percentage of N-nitrosated proline. The results for up to seven volatile N-nitrosamines in the tobacco of the same 14 tobacco products are summarized in Table II.

It was the goal of this study to explore whether the noncarcinogenic N-nitrosoproline could serve as an indicator for the formation of the tobacco-specific N-nitrosamines of which 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone is a strong animal carcinogen (U.S. Surgeon General, 1982). As summarized in Table III, the data from these 14 tobacco products support the concept that NPRO can serve as an indicator for the formation of N'-nitrosonornicotine (r^2 = 0.961) and of TSNA (r^2 = 0.899). These statistically significant correlations are not much further enhanced by adding nitrate as a second variable (r^2 = 0.966 and 0.918) or proline (r^2 = 0.962 and 0.903) or both variables (r^2 = 0.966 and 0.918).

Fine-Cut Snuff. In agreement with earlier findings (U.S. Surgeon General, 1982; Brunnemann et al., 1982), the highest amounts of N'-nitrosonornicotine and other tobacco-specific N-nitrosamines are found in fine-cut snuff (Table I). During the processing of tobacco to this product, including fire curing, in this sample about 0.07% of nicotine have been N-nitrosated to carcinogenic N'-nitrosonornicotine. As to be expected, the nitrosation of proline to NPRO is also proportionately high (3.7%), demonstrating further that the processing to fine-cut snuff enhances the potential for the formation of N-nitrosamines. This observation cannot be explained by the relative high nitrate content of the snuff tobacco (≃3.5%) alone. The values for volatile N-nitrosamines are not especially high, with the possible exception of N-nitrosopyrrolidine (0.12 ppm) and N-nitrosomorpholine (0.04 ppm). These two compounds are the least volatile ones of the group of volatile N-nitrosamines. Since the amounts of carcinogenic nitrosamines are about 2 orders of magnitude higher in fine-cut snuff than in any other nonoccupational materials in man's environment, this observation strengthens our working hypothesis that the nitrosamines, especially the tobacco-specific N-nitrosamines, contribute significantly to the increased risk of snuff dippers for cancer of the oral cavity (Winn et al., 1981; Hoffmann and Adams, 1981). While it may be difficult to document the role of TSNA in oral cancer risk of snuff dippers, it is a fact that they are the only known carcinogens in snuff. Therefore, the reduction of the carinogenic nitrosamines deriving from the tobacco alkaloids should certainly be an important goal for the modification of snuff products.

Cigarette Smoke Analysis. Finally, we explored whether cigarette smoke contains NPRO and whether proline or NPRO in tobacco gives rise to NPRO in cigarette smoke. One hundred cigarettes were smoked under standard conditions with a 20-port automatic smoker (see Experimental Section) and the mainstream smoke was trapped in two gas wash bottles containing a buffer solution with ascorbic acid (pH 4.5; Hoffmann et al., 1979). The actual analytical procedure beginning with repeated ethyl acetate extractions was identical with the procedure used for the tobacco analysis as outlined in Figure 1. Despite repeated analyses we were unable to detect NPRO in tobacco smoke. When 5 mg of proline or 5 mg of NPRO was added to the 62-mm cigarette column with a microsyringe and when the mainstream smoke of 100 cigarettes spiked with proline or NPRO was analyzed, we found that PRO and especially NPRO serve as precursor for N-nitrosopyrrolidine in the smoke (Table IV). Upon spiking of the cigarettes with 5 mg of NPRO we found 7140 ng of NPRO/cigarette in the mainstream smoke corresponding to a transfer rate of 0.14%. Since this test cigarette contained 0.88 ppm (680 ng) of NPRO in the 62-mm tobacco column smoked, a 0.14% transfer rate would account for 0.9 ng of the nitroso compound in the smoke/cigarette. Since this calculated amount is close to the detection limit,

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

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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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