Sarkanen, K.; Tillman, D. A., Eds. "Progress in Biomass Conversion"; Academic Press: New York, 1979; Vol. 1, pp 1–259.
Sarkanen, S.; Teller, D. C.; Abramowski, E.; McCarthy, J. L. *Macromolecules* **1982**, *15* (4), 1098–1104.
Sarkanen, S.; Teller, D. C.; Hall, J.; McCarthy, J. L. *Macromolecules* **1981**, *14*, 426–434.
Selke, S. M.; Hawley, M. C.; Hardt, H.; Lamport, D. T. A.; Smith, G.; Smith, J. *Ind. Eng. Chem. Prod. Res. Dev.* **1982**, *21* (1), 11–16.
SERI "Alcohol Fuels Program Technical Review"; Solar Energy Research Insitute: Golden, CO, Winter 1981; p 51.
St. Pierre, L. E.; Brown, G. R., Eds. "Future Sources of Oganic Raw Materials—CHEMRAWN I"; Pergamon Press: New York, 1980; pp 1–651.
Worthy, W. *Chem. Eng. News* **1981**, *59* (49), 35–42.

Received for review March 4, 1983. Accepted June 3, 1983. Financial support for this study was provided by the U.S. Department of Agriculture, Science and Education Administration, Arlington, VA, under Grant 59-2511-1-2-121-0.

# Partial Characterization of Condensate Derived from Volatiles-Sublimate of Homogenized Leaf-Cured Burley Tobacco during Storage

Roger A. Andersen

Approximately 100 mg dry weight of an orange-red water-soluble condensate was deposited as a viscous oil on the interior glass walls of the sealed storage chamber housing 1 kg of homogenized leaf-cured tobacco during a 20-weak storage period at 16% moisture and 30 °C. The condensate presumably emanated from the tobacco by volatilization and sublimation. Partial characterization of the substance indicated that it contained a mixture of organic compounds. One or more of these compounds was amphoteric. Elemental composition of the material was C 47.0%, H 5.5%, N 17.1%, and O 20.5%. Chemical, HPLC, GC, and GC–MS analyses showed that nicotinic acid (3.4%), nicotine (6.3%), and *N*-nitrosonornicotine (0.07–1.4%) were constituents accounting for about 10% of the dry weight. Total volatile bases as nitrogen equivalent were 6.6 mequiv/g.

An experimental homogenized leaf-cured (HLC) tobacco procedure for the rapid curing of burley tobacco is currently under investigation. The process involves homogenization of green leaves, incubation of the homogenate, and dehydration of the material (Tso et al., 1975; Yoder et al., 1976). During a recent investigation conducted to determine chemical changes that occur during prolonged storage ("aging") of HLC and conventionally air-cured burley tobacco under controlled environmental conditions, it was noted that quantities of an orange-red substance condensed on the upper walls of the storage chambers housing the HLC tobacco (Andersen et al., 1982). No measurable (weighable) amount of this kind of condensate was observed with the air-cured tobacco, however. Chemical changes in the HLC tobacco accompanied this loss in volatile or sublimed components with resultant decreases in concentrations of total alkaloids as nicotine, nitrate N, nitrite N, ammonia N, total volatile nitrogenous base N, and petroleum ether extractables in addition to a concomitant increase in *N*-nitrosonornicotine (NNN) levels. NNN was shown to have carcinogenic activity (Hecht et al., 1978). The purpose of this investigation is to characterize the condensed material that collected on storage chamber walls during the aging of HLC tobacco.

## MATERIALS AND METHODS

**Growth, Curing, and Storage (Aging) of Tobacco.** Burley tobacco (*Nicotiana tabacum* L. cv. Ky 14) plants were grown, harvested, homogenized leaf-cured, and prepared for sampling as previously described for tobacco that was not treated for the removal of protein (Andersen et al., 1982).

Agricultural Research Service, U.S. Department of Agriculture, and Department of Agronomy, University of Kentucky, Lexington, Kentucky 40546-0091.

A 1-kg sample of tobacco that had been stored temporarily at a 3–4% moisture content was moisturized in a high-humidity chamber to 16% moisture on a "wet weight" basis and then packed into an 8-L glass desiccator that served as the storage container. The container was placed in an unlighted controlled-environment chamber maintained at 30 ± 0.5 °C. The desiccator cover had a gas-inlet tube that was closed except for a brief opening to equilibrate air pressure followed by removal of the cover for single 1-min periods at weekly intervals to allow exchange of storage-container gases with ambient air. The short periods of gaseous exchange did not cause an appreciable change in the tobacco moisture content.

**Isolation of Condensate.** After 20 weeks of storage, three-fourths of the orange-red condensate that collected as a viscous oil on the upper walls and cover of the storage chamber (desiccator) was transferred to a 1-mL glass vial. The vial contents were dried over $P_2O_5$ for 96 h. The transferred dried material weighed 75 mg and portions of it were used for the analyses.

**Elemental and Chemical Analyses of Aged Tobacco and Condensate.** Elemental analyses for carbon, hydrogen, nitrogen, oxygen, chlorine, and sulfur were performed on an 18-mg sample of the condensate material by Gailbraith Laboratories, Inc., Knoxville, TN. A Beckman Acta III recording spectrophotometer was used to scan the absorbance of a 1 mg/mL aqueous solution of the condensate material from 400 to 190 nm.

Total volatile nitrogeneous bases as nitrogen equivalence (TVB-N) were determined on a 20-mg sample of the condensate material by titration with standard sulfamic acid after the following pretreatment sequence: initially, bases in the sample were steam distilled from an alkaline solution (pH 12.0) into dilute hydrochloric acid, the resultant solution that contained excess hydrochloric acid was concentrated and a Kjeldahl digestion of the residue with

sulfuric acid was carried out, the solution was again made basic, and the bases as ammonia were steam distilled (Milner and Zahner, 1960; Neurath et al., 1966).

Total alkaloids in tobacco were determined spectrophotometrically at 460 nm after reaction with cyanogen bromide and aniline (Harvey et al., 1969). Results were expressed as nicotine equivalent on a dry weight basis.

Total free amino acids in tobacco were estimated with the aid of a Technicon autoanalyzer (Piez and Morris, 1960) with modifications as previously described (Andersen et al., 1982). Quantitation was achieved by comparison with a standard mixture of amino acids (mean molecular weight of 120).

The values for concentrations of components in tobacco were corrected for changes in dry weights of samples by factors obtained from Ca contents of aged relative to zero-time control samples. Calcium was determined by atomic absorption as previously described (Andersen et al., 1982) and was assumed to undergo no loss during storage of the tobacco.

**High-Performance Liquid Chromatographic (HPLC) Analysis of the Condensate.** Components were chromatographed with an ALC/GPC 204 Waters Associates system that included two 6000A pumps, a 660 solvent flow programmer, a U6K injector, a 440-UV absorbance detector adapted for 254 nm, a 10-$\mu$m $\mu$Bondapak $C_{18}$ column (3.0 × 300 mm), and a 730 data module. Two paired-ion solvent systems were used: (1) methanol/water, 15/85, with 0.005 M tetrabutylammonium phosphate in phosphate buffer at pH 7.5 (PIC Reagent A, Waters Associates, Milford, MA) and (2) methanol/water, 15/85, with 0.005M pentane sulfonic acid and glacial acetic acid at pH 3.5 (PIC Reagent B, Waters Associates, Milford, MA).

*N*-Nitrosonornicotine (NNN) in the condensate was determined by two methods. In one, a GC procedure was used that is described in the foregoing two paragraphs. In the other method, an 18-mg sample was analyzed by the Analytical Service Laboratory of the Thermo Electron Corp., Waltham, MA. In their analysis a high-performance liquid chromatography (HPLC)–thermal energy analysis (TEA Model 502 analyzer) procedure was used with the following two columns and solvent systems: (1) a 10-$\mu$m 4.6 × 250 mm LiChrosorb $NH_2$ column and isooctane/dichloromethane/methanol, 80/16/4, and (2) a 10-$\mu$m 4.6 × 250 mm LiChrosorb Si60 column and isooctane/acetone, 93/7.

Nicotine, NNN, and nicotinic acid were identified by gas chromatography–mass spectrometry and quantitatively analyzed by a gas chromatography procedure as described in the next section.

**Gas Chromatographic (GC) Analysis and Gas Chromatography–Mass Spectrometry (GC–MS) of the Condensate.** Trimethylsilyl ($Me_3Si$) derivatives of condensate constituents were prepared by heating 150–300 $\mu$g of a condensate sample dissolved in 20 $\mu$L of acetonitrile and 30 $\mu$L of bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 75 °C in a sealed vial for 30 min. The $Me_3Si$ derivatives were chromatographed in a microprocessor-controlled Hewlett-Packard 5880A GC by using a splitless/split injection technique with a 30 m × 0.25 mm fused silica SE-54 column. The inlet temperature was 220 °C. In one analysis a flame ionization detector (FID) at 250 °C was used, and in a replicate analysis a nitrogen/phosphorus detector (NPD) at 280 °C was used. The inlet septum purge flow was 1.2 mL/min and the He carrier gas linear velocity through the column was 32 cm/s. A 0.5–1.0-$\mu$L aliquot was injected with an inlet septum purge inactivation time of 35 s and an initial column temperature of 100 °C. After 1 min, the oven was temperature-programmed at 4 °C/min to 200 °C and then maintained at 200 °C for 20 min. 2,4′-Dipyridyl was used as an internal standard, and it was added to the condensate prior to silylation. Quantitations of nicotinic acid, nicotine, and NNN were accomplished by use of the internal standardization method after calibration with authentic compounds and the internal standard.

The GC–MS analyses were performed in the electron impact mode at 70 eV with a computer-controlled Hewlett-Packard Model 5985A system. A 30 m × 0.31 mm fused silica SE-54 W.C.O.T. column was used at 300 °C with He as the carrier gas at a linear flow velocity of 30 cm/s; inlet and flame ionization detector (FID) temperatures were 300 °C. The preparation of $Me_3Si$ derivatives of the condensate sample and the use of 2,4′-dipyridyl as the internal standard were carried out in the same manner described for the GC analysis.

## RESULTS AND DISCUSSION

The elemental analysis, solubility, partitioning, and spectrophotometric results indicated that the condensate material contained organic compounds as components with some of the following characteristics: presence of nitrogen, oxygen, unsaturated carbon–carbon bonds, and polar functional groups. The solubility results were consistent with (but did not prove) the presence of a component with amphoteric properties. The results of elemental analysis of the condensate material as percentages of dry weight were C = 47.0, H = 5.5, N = 17.1, O = 20.5, Cl = 0.8, and S = 0.3. Thus, carbon, hydrogen, oxygen, and nitrogen accounted for 90% of the mass. Minor amounts of chlorine and sulfur were also present. The condensate material was completely soluble in water, 1 N HCl, and 1 N NaOH at 1 mg/mL. At this same concentration, it was partially soluble in anhydrous methanol, but it was relatively insoluble in diethyl ether, ethyl acetate, and chloroform. The pH of a 1 mg/mL aqueous solution of the condensate at 20 °C was 6.0. The material was not completely extractable from either 1 N HCl or 1 N NaOH by equal volumes of either diethyl ether or chloroform. An aqueous solution of the condensate material had strong wavelength absorbance maxima at 260 and 205 nm.

Evidence for the presence of nicotinic acid was subsequently obtained by paired-ion high-performance liquid chromatography and gas chromatography–mass spectrometry. The HPLC results are summarized in Table I. The retentions of the major peaks of the condensate material formed with the two solvent system reactive ions corresponded to those of authentic nicotinic acid paired ions. Although nicotinic acid appeared to be a major component, other components which absorbed light at 254 nm were present that yielded peaks in the paired-ion solvent system.

Nicotinic acid, nicotine, and *N*-nitrosonornicotine (NNN) were identified by GC and GC–MS procedures. Chromatograms of silylated condensate material obtained by GC utilizing FID and NPD are shown in Figure 1. The major peaks appear as overloaded because sufficient condensate was injected to demonstrate the presence of several trace constituents. Detector sensitivities obtained with NPD were 3, 7, and 27 times greater than with FID for $Me_3Si$-nicotinic acid, nicotine, and NNN, respectively. Probable identifications of compounds were made based on GC retention times, reactivities of condensate components with silylation reagents, and GC–MS results. The GC–MS results are summarized in Table II. Mass spectra of components in peaks obtained by GC of silylated extracts of condensed material were compared with those of

Table I. HPLC Analysis of Condensate Material Using Paired-Ion Chromatography

| solvent system with reactive ion | condensate material or reference compound | $k'$ (retention) on reverse-phase column[a] |
|---|---|---|
| methanol/water (15/85) with tetrabutylammonium phosphate at pH 7.5[b] | condensate | 1.13 (predominant peak), 1.26, 1.34, 1.70, 1.85, 2.11, 2.22, 2.44, 2.63 |
| | nicotinic acid | 1.15 |
| | picolinic acid | does not form paired ion |
| | nicotinamide | 1.72 |
| methanol/water (15/85) with pentanesulfonic acid at pH 3.5[c] | condensate | 1.18, 1.35 (predominant peak), 1.67, 1.80, 1.93, 2.27, 3.10 |
| | nicotinic acid | 1.37 |
| | picolinic acid | does not form paired ion |
| | nicotinamide | 1.91 |

[a] 10-μm 3.9 × 300 mm μBondapak $C_{18}$ column. [b] PIC A, Waters Associates. [c] PIC B, Waters Associates.

Table II. GC-MS Identification of Silylated Condensate Material Separated on a W.C.O.T. Fused Silica Capillary Column[a]

| proposed identity of GC peak | $t_R$[b] | electron impact MS data (probable fragment and rel intensity, %) |
|---|---|---|
| $Me_3Si$-nicotinic acid | 0.59 | *m/e* 195 (M, 11), 180 (M − $CH_3$, base ion), 136 (M − $CH_3CO_2$, 50), 106 (M − $OSiMe_3$, 53), 78 ($C_5H_4N$, 39) |
| nicotine | 0.69 | *m/e* 162 (M, 35), 161 (M − H, 31), 133 (M − H − $C_2H_5$, 57), 84 (M − $C_5H_{10}N$, base ion) |
| 2,4′-dipyridyl (internal standard) | 1.00 | *m/e* 156 (M, base ion), 155 (M − H, 49), 129 (M − CHN, 48), 78 (M − $C_5H_4N$, 18) |
| *N*-nitrosonornicotine | 1.29 | *m/e* 177 (M, base ion), 147 (M − NO, 28), 118 (M − $CH_2N_2OH$, 39), 105 (M − $C_2H_4N_2O$, 73), 78 (M − $C_4H_7N_2O$, 45) |

[a] Column dimensions were 30 m × 0.31 mm; column temperature 300 °C. [b] Relative retention time calculated by considering 2,4′-dipyridyl as 1.00.

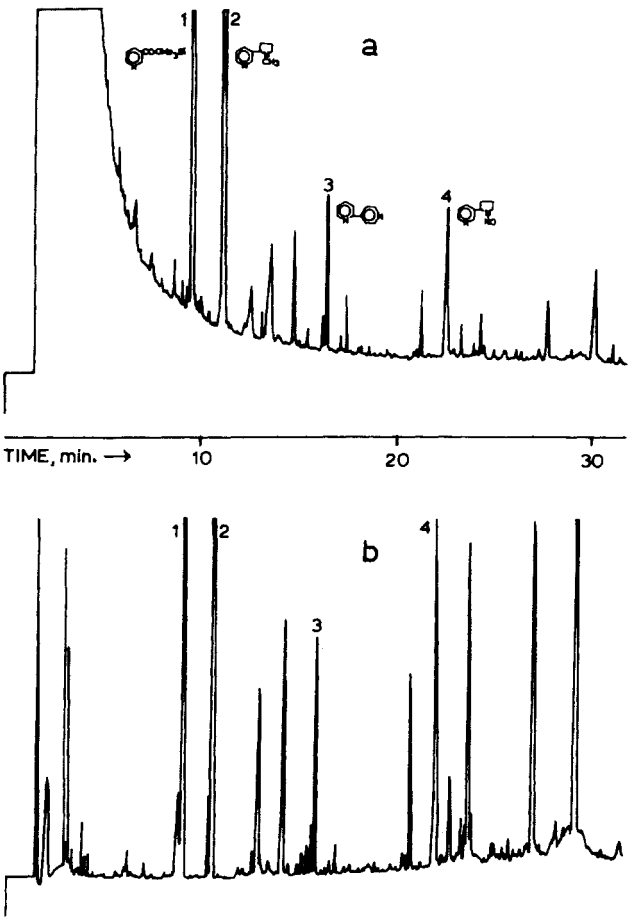


**Figure 1.** (a) GC-FID chromatogram of 3.8 μg of condensate material after silylation. Peaks 1, 2, 3, and 4 correspond to $Me_3Si$-nicotinic acid (130 ng calculated as nicotinic acid), nicotine (253 ng calculated), 2,4′-dipyridyl internal standard (57 ng), and *N*-nitrosonornicotine (47 ng calculated). (b) GC-NPD chromatogram of 1.9 μg of condensate material after silylation. Peaks 1, 2, 3, and 4 correspond to $Me_3Si$-nicotinic acid (63 ng calculated as nicotinic acid), nicotine (114 ng calculated), 2,4′-dipyridyl internal standard (54 ng), and *N*-nitrosonornicotine (29 ng calculated).

Table III. Comparison of Nitrogenous Component Concentrations in Condensate Material Determined by GC-FID and GC-NPD

| % of dry wt | | | | | |
|---|---|---|---|---|---|
| nicotinic acid | | nicotine | | *N*-nitrosonornicotine | |
| FID | NPD | FID | NPD | FID | NPD |
| 3.42 | 3.30 | 6.65 | 6.01 | 1.24 | 1.54 |

available reference compounds.

Quantitative GC analytical results obtained with the NPD and FID for nicotinic acid, nicotine, and NNN in the condensate are given in Table III. Nicotine content (6.33% average) was about twice that of nicotinic acid (3.36% average). NNN content (1.39% average) determined by GC was considerably higher than the content (0.07%) determined by HPLC–thermal energy analysis. The reason for the discrepancy between these results for NNN is unknown. No other nitrosamines were detected in the condensate material by HPLC–thermal energy analysis; there were several peaks obtained by GC (Figure 1) that were not identified, however. A previous study showed that NNN concentrations were much higher in homogenized leaf-cured burley tobacco than in conventionally air-cured burley tobacco and that most of the NNN formed during postharvest processing and aging of the HLC tobacco (Andersen et al., 1982).

As the volatiles–sublimate evolved and deposited on the storage chamber interior walls during storage, the nicotine content of the tobacco from which it originated decreased from 11.5 mg/g in the unaged sample to 1.3 mg/g in the 20-week-aged tobacco (Table IV). It seems probable that some or all of the nicotinic acid that we determined in the condensed material was formed in the tobacco as a breakdown product of nicotine and other tobacco alkaloids such as nornicotine, anabasine, and anatabine; the nicotinic acid subsequently sublimed. It is known that authentic nicotinic acid sublimes readily without decomposition. It was previously shown by Frankenburg and Gottscho (1952) that 15% of the nicotine in a cigar tobacco was converted to nicotinic acid during fermentation. The NNN content

Table IV. Chemical Composition of HLC Burley Tobacco Aged for 20 Weeks at 16% Moisture and 30 °C Compared to Unaged (Zero-Time) Controls[a]

| component | mg/g unaged | mg/g aged | % change |
|---|---|---|---|
| total alkaloids, nicotine equivalent | 11.5 | 1.30 | −88.7 |
| *N*-nitrosonornicotine | 0.36[b] | 0.74[c] | +106 |
| free amino acids | 23.8 | 11.3 | −52.5 |

[a] Each entry is the mean value of three to five laboratory analyses per sample and aged sample entries have been corrected for change in dry weight by a factor obtained from calcium analyses performed on aged and zero-time control samples. [b] NNN determined by HPLC-thermal energy analysis. [c] NNN analysis performed by GC-NPD (unpublished experiments). The method was based on procedural modifications described herein for NNN in condensate material and for nicotine and nornicotine in tobacco (Andersen et al., 1982).

of the tobacco increased 106% from 0.36 mg/g in the unaged sample to 0.74 mg/g in 20-week-aged tobacco (Table IV). It seeems probable that some of the NNN and nicotine in the tobacco volatilized or sublimed and deposited on the storage chamber wall along with nicotinic acid during storage. The evolution of carcinogenic NNN and its deposition as a condensate component from aging tobacco needs to be dealt with from the safety standpoint in any future industrial application of homogenized leaf curing or similar technology. There was a greater magnitude of chemical change in our homogenized leaf-cured tobacco than in conventionally air-cured tobaccos during aging (Andersen et al., 1982).

The concentration of total volatile nitrogenous bases (as nitrogen) in the condensate material was 6.60 μequiv/mg. This indicated that about 50% of the N content of the material was present as basic N. Thus, other basic nitrogen containing compounds such as ammonia and unidentified amines and alkaloids were probably present in the condensate. The high concentration of TVB-N in the condensate may have derived in part from the presence of ammonia released by deamination of free amino acids in the tobacco during the storage period. The basis of support for this possibility is that there was a 52% decrease in free amino acids during the 20-week-aging period; amino acid concentrations decreased from 198 μmol/g in the unaged tobacco to 94 μmol/g in the 20-week-aged tobacco (Table IV). Considerable amounts of ammonia are known to be released during air curing (Young and Jeffrey, 1943), and ammonia gradually evolves as a gas during the fermentation of cigar tobacco (Frankenburg and Gottscho, 1952). Also, there was a 22% decrease in α-amino N during the aging of flue-cured tobacco for 30 months (Tso, 1972).

ACKNOWLEDGMENT

The advice and assistance generously provided by M. Kasperbauer, H. Burton, T. Kemp, T. Vaughn, T. Sutton, and C. Hughes are gratefully acknowledged.

**Registry No.** *N*-Nitrosonornicotine, 16543-55-8; nicotine, 54-11-5; nicotinic acid, 59-67-6; nitrogen, 7727-37-9.

LITERATURE CITED

Andersen, R. A.; Kasperbauer, M. J.; Burton, H. R.; Hamilton, J. L.; Yoder, E. R. *J. Agric. Food Chem.* **1982**, *30*, 663.
Frankenburg, W. G.; Gottscho, A. M. *Ind. Eng. Chem.* **1952**, *44*, 301.
Harvey, W. R.; Stahr, H. M.; Smith, W. C. *Tob. Sci.* **1969**, *13*, 13.
Hecht, S. S.; Chen, C. B.; Hirota, N.; Ornaf, R. M.; Tso, T. C.; Hoffmann, D. *J. Natl. Cancer Inst. (U.S.)* **1978**, *60*, 819.
Milner, O. I.; Zahner, R. J. *Anal. Chem.* **1960**, *32*, 294.
Neurath, G.; Krull, A.; Pirmann, B.; Wandrey, K. *Proc. Int. Tob. Sci. Congr., 4th* **1966**, 774.
Piez, K. A.; Morris, L. *Anal. Biochem.* **1960**, *1*, 187.
Tso, T. C. "Physiology and Biochemistry of Tobacco Plants"; Dowden, Hutchinson and Ross, Inc.: Stroudsburg, PA, 1972; p 174.
Tso, T. C.; Lowe, R.; DeJong, D. W. *Beitr. Tabakforsch.* **1975**, *8*, 44.
Yoder, E. R.; Lowe, R. H.; Lam, J. J., Jr.; DeJong, D. W.; Tso, T. C. *Trans. ASAE* **1976**, *19*, 359.
Young, J. R.; Jeffrey, R. N. *Plant Physiol.* **1943**, *18*, 433.

Received for review November 1, 1982. Revised manuscript received March 24, 1983. Accepted May 20, 1983. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may be suitable. This investigation is in connection with a project at the Kentucky Agricultural Experiment Station, and the results are published as KAES Journal Article 82-3-243.