Changes in Content of Sterols, Alkaloids, and Phenols in Flue-Cured Tobacco during Conditions Favoring Infestation by Molds

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Sterols, alkaloids, and phenols were determined in two samples of flue-cured tobacco (*Nicotiana tabacum* L.) which differed only in that one sample was heavily infested with the molds *Aspergillus niger*, *Aspergillus flavus*, and *Penicillium*. Total sterol and total alkaloid contents were higher in the moldy tobacco sample; total phenol content was higher in the nonmoldy sample. Differences in amounts of nicotine, chlorogenic acid, rutin, scopolin, caffeic acid, and minor sterol constituents indicated that cured tobacco subjected to conditions favoring mold development may undergo alteration in a number of its chemical constituents.

A problem sometimes encountered during post harvest processing of tobacco (*Nicotiana tabacum* L.) is infestation of leaves in curing barns by molds when the environment is moist and warm. Young and Jeffrey (1943) noted that when tobacco is air-cured under conditions of high relative humidity, leaves mold and their nitrogen composition is significantly different from lamina cured under conditions of lower relative humidity. Since there is evidence that molds such as *Aspergillus niger* and *Aspergillus flavus* which commonly infest tobacco are capable of metabolizing or synthesizing sterols (Barton and Bruun, 1951; Vanghelovici and Serban, 1941), alkaloids (Bates, 1938; Erlich, 1917), and phenols (Hay *et al.*, 1961; Simpson *et al.*, 1960; Westlake *et al.*, 1959), it was the objective of this preliminary study to determine whether qualitative or quantitative differences in these three types of compounds occurred in flue-cured tobacco exposed to conditions favorable for mold infestation.

MATERIALS AND METHODS

Preparation of Tissue Samples. Field-grown *Nicotiana tabacum* L. var. NC2236 was harvested at maturity, flue-cured, and then divided randomly leaf by leaf into two equal lots of about 1 kg. each. The resulting composite samples were subsequently handled in an identical manner, except that while one sample was maintained under normal storage conditions unfavorable to mold development, the other was brought into high order and kept in a warm glasshouse. To hasten development of the mold, this sample was inoculated with scraps of moldy leaf obtained from curing barn trash. After 3 weeks the sample was removed from the glasshouse and allowed to dry to a keeping condition (moisture content less than or equal to 11%) under atmospheric conditions.

Both samples were ground to a homogeneous powder prior to chemical analysis. Moisture contents of the two samples at the time of analysis were approximately 6%.

Cultures of Microorganisms. Cultures of the two samples were made on three media: Czapek, potato dextrose agar, and water agar. Plates were read 3 weeks after inoculation.

Determination of Alkaloids. Total alkaloid content was determined by the steam distillation method of Griffith (1957).

Individual alkaloids were determined by a modification of the method of Cundiff and Markunas (1955). Dried ground tobacco (250 mg.) and approximately 100 mg. of $Ba(OH)_2$ were mixed in a 25-ml. Erlenmeyer flask. Saturated $Ba(OH)_2$ (1.5 ml.) was added and the tobacco thoroughly wetted. Following the addition of 10 ml. of a benzene-chloroform solution (9 to 1, v./v.), the contents of the flask were mechanically shaken for 30 minutes. Two hundred milligrams of Celite were then added and dispersed by shaking the flask. The organic phase was filtered through Whatman No. 1 filter paper. A measured amount of this filtrate was streaked on TLC plates of silica gel G. A mixture of benzene-dioxane-ethanol-NH_4OH (100:80:10:10, v./v.) was used to develop the plate. Individual alkaloids were quantitated by eluting spots in 0.1*N* HCl and measuring the absorbance at 259 mμ on a Beckman DBG spectrophotometer.

Determination of Sterols. Total sterol content was determined by a modification of the method of Stedman and Rusaniswkyi (1959). Five-gram samples of moisture-free tobacco were extracted in 250 ml. of acetone for 24 hours in a Soxhlet apparatus. The extracts were taken to dryness and the residues taken up with 25 ml. of 95% ethanol containing 0.15 ml. of sulfuric acid. The mixtures were refluxed for 15 hours. Fifteen milliliters of 10% KOH in 95% ethanol were then added and the mixtures were refluxed for 30 minutes. This procedure yielded total sterols, since both glycosides and esters of sterols had been hydrolyzed.

The sterols were extracted by adding 50 ml. of *n*-hexane and enough water to obtain two layers. The *n*-hexane layer was removed and the aqueous layer washed three times with *n*-hexane; each time sufficient water was added to obtain two layers. The *n*-hexane fractions were combined and extracted three times with 90% methanol. The methanol fraction was back-extracted twice with *n*-hexane and the *n*-hexane fractions were combined and taken to dryness. The resultant

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residue was dissolved in 20 ml. of boiling absolute ethanol and transferred to plastic centrifuge tubes. Five milliliters of hot 2% digitonin in 80% ethanol were added and the solution was boiled continuously; 5 ml. of water were added and the mixture was allowed to cool at room temperature overnight. The tubes were centrifuged at 15,000 × G for 30 minutes and washed three times with 30 ml. of 80% ethanol and three times with 30 ml. of diethyl ether. The centrifuge tubes were dried at 70° C. for 12 hours and weighed. The sterol weight was 25.3% of the weight of the digitonide precipitate.

Individual sterols contained in the digitonide precipitate were separated by gas chromatography (complete method will be published elsewhere). The digitonide precipitate was broken with 1.0 ml. of pyridine containing a known amount of cholestane as the internal standard. The pyridine mixture was heated to 70° C. for 1 hour and left at room temperature for 12 hours. The digitonin was removed by precipitation with 30 ml. of diethyl ether, and centrifugation at 10,000 × G for 30 minutes. The ether layer was recovered and taken to dryness. The residue was taken up in ethyl acetate and injected into a gas chromatograph (F&M Model 402) using a flame ionization detector. The sterols cholesterol, campesterol, stigmasterol, and β-sitosterol were separated as free sterols on a 1.80-meter column, 6-mm. i.d., packed with 5% OV-101 on Anakrom ABS 80- to 90-mesh. The column temperature was 250° C. and the flash heater temperature was kept 50° C. above that of the column. The flame detector temperature was 275° C. Helium was the carrier gas at a flow rate of 100 ml. per minute at 50 p.s.i. of pressure. The quantitation was carried out by measuring the peak areas of the sterols and the internal standard. Corrections were made for the differences in relative weight response.

Determination Phenols. Total phenol content was estimated by the method of Andersen and Todd (1968) based on the bonding of these compounds to polyvinylpyrrolidone. Results were expressed in chlorogenic acid equivalents.

Individual phenols were separated by paper chromatography. One gram of dried tobacco was exhaustively extracted in absolute methanol and concentrated to a volume of 5 ml. A measured portion of the methanol concentrate was streaked in a 1-inch band on Whatman No. 1 paper and developed in two dimensions by descending chromatography. First- and second-dimension solvent systems employed were butanol–acetic acid–water (6:1:2, v./v.) and 2-propanol–water–formic acid (5:95:0.1, v./v.), respectively. Zones containing chlorogenic acid (the 3-*o*-caffeoylquinic acid isomer), rutin, scopolin, scopoletin, and caffeic acid were previously determined by comparison of R_f values and spectral curves with standard compounds recovered from the same chromatography system. These zones were cut out of the chromatograms. Rutin was assayed by eluting its zone in a solution of 1% aluminum chloride in methanol and the absorbance was measured at 432 mμ. The remaining compounds were eluted in 30% methanol. Chlorogenic acid was then assayed by addition of Arnow's reagent (Arnow, 1937) and the absorbance of the solution was measured at 510 mμ. Absorbance of the caffeic acid solution was measured at 310 mμ and that of scopolin and scopoletin at 345 mμ.

Amounts of chlorogenic acid, rutin, scopoletin, and caffeic acid were determined by means of internal standard curves prepared by recovery of known amounts of both compounds from the paper chromatography system described above. Amounts of scopolin were approximated from the scopoletin standard curve and are expressed in units of scopoletin.

Assay of Polyphenol Oxidase. Five hundred milligrams of each tobacco sample were gently stirred for 15 minutes in 8 ml. of phosphate buffer, 0.025*M*, pH 7.2. To the supernatant solution recovered following centrifugation were added 3 volumes of saturated ammonium sulfate. The protein pellet recovered after centrifugation was dissolved in 2 ml. of water (5 mg. of protein per ml.) and portions of this enzyme preparation were assayed according to the method of Constantinides and Bedford (1967) using dihydroxyphenylalanine as substrate.

Presentation of Data. For analyses of total and individual sterols, alkaloids, and phenols, a minimum of two extracts was prepared for each tobacco sample. Values reported in the tables are the means and standard deviations obtained from two or more extracts of each sample. Differences between the two samples were statistically analyzed by a *t* test of significance.

Table I. Microflora in Inoculated and Uninoculated Tobacco Leaves

	Inoculated Sample		Uninoculated Sample	
Media	**Total plates**	**No. plates showing microflora**	**Total plates**	**No. plates showing microflora**
Potato dextrose agar	24	11–*A. niger* 3–*A. flavus* 2–*Penicillium spp.* 19–yeast and bacteria	20	1–*A. niger* 12–yeast and bacteria
Czapek	20	11–*A. niger* 6–*A. flavus* 8–*Penicillium spp.* 13–yeast and bacteria	22	0–fungus 15–yeast and bacteria
Water	8	4–*A. niger* 4–yeast and bacteria	12	0–fungus 5–yeast and bacteria

RESULTS

Results obtained from cultures of the inoculated and uninoculated tobacco sample on various media are expressed in Table I. Plates from inoculated tobacco were heavily infested with *A. niger*, *A. flavus*, and *Penicillium*. Only one plate of a total of 54 runs for the uninoculated tobacco showed fungal growth. Comparable amounts of yeast and bacteria were found in the two samples. The results of the quantitative chemical analyses are shown in Tables II, III, and IV. Moldy tobacco contained greater amounts of total sterols and total alkaloids, but less total phenols than the sound sample. The principal individual sterols, alkaloids, and phenols found in tobacco were also determined. Although minor constituents were not quantitatively determined, chromatograms were inspected for possible qualitative differences between the two samples. No qualitative differences in contents of alkaloids and phenols were noted between chromatograms from moldy and nonmoldy tobacco, but some qualitative differences were observed among minor sterol constituents. On the chromatogram obtained from the moldy sample, one small peak appeared between cholesterol and campesterol and two other peaks appeared past stigmasterol. These minor constituents, not observed in the nonmoldy sample, were not identified.

Table II. Total[a] and Individual[b] Sterols in Nonmoldy and Moldy Tobacco

	Mg. Sterol/G. Tobacco		
	Moldy	Nonmoldy	Moldy − nonmoldy
Total	2.0162 ± 0.278[c]	1.6294 ± 0.266	+0.3868
Cholesterol	0.1113 ± 0.009	0.1110 ± 0.008	+0.0003
Campesterol	0.2278 ± 0.025	0.2417 ± 0.001	−0.0139
Stigmasterol	0.4550 ± 0.036	0.4593 ± 0.009	−0.0043
β-Sitosterol	0.4103 ± 0.052	0.4368 ± 0.004	−0.0265

[a] Total sterol content determined gravimetrically.
[b] Individual sterols determined by GLC using cholestane addition procedure.
[c] Value differs significantly from that in nonmoldy tobacco at 5% level.

Table III. Total[a] and Individual[b] Alkaloids in Moldy and Nonmoldy Tobacco

	Mg. Alkaloid/G. Tobacco		
	Moldy	Nonmoldy	Moldy − nonmoldy
Total	32.4 ± 0.4[c]	30.4 ± 0.9	+2.0
Anabasine	1.1 ± 0.1	1.2 ± 0.1	−0.1
Nicotine	25.3 ± 2.6[c]	22.0 ± 0.4	+3.3
Nornicotine	4.4 ± 0.2	4.1 ± 0.1	+0.3

[a] Total alkaloids determined by steam distillation.
[b] Individual alkaloids determined by thin-layer chromatography technique.
[c] Value differs significantly from that in nonmoldy tobacco at 5% level.

Table IV. Total[a] and Individual[b] Phenols in Moldy and Nonmoldy Tobacco

	Mg. Phenol/G. Tobacco		
	Moldy	Nonmoldy	Moldy − nonmoldy
Total	58.5 ± 0.2[c]	64.5 ± 0.3	−6.0
Chlorogenic acid	18.00 ± 0.46[c]	22.50 ± 0.72	−4.50
Rutin	6.43 ± 0.29[c]	7.19 ± 0.28	−0.76
Scopolin	0.60 ± 0.14[c]	1.23 ± 0.24	−0.63
Scopoletin	0.50 ± 0.00	0.55 ± 0.22	−0.05
Caffeic acid	0.87 ± 0.01[c]	0.14 ± 0.00	+0.73

[a] Total phenol determined by polyvinylpyrrolidone adsorption technique.
[b] Individual phenols separated by paper chromatography.
[c] Value differs significantly from that in nonmoldy tobacco at 5% level.

The slight increase in total sterols in moldy tobacco does not seem to be due to an increase in any of the four individual sterols determined. Cholesterol, β-sitosterol, campesterol, and stigmasterol were found in approximately the same amounts in moldy and nonmoldy tobacco (Table II). Since additional sterol components were detected in the moldy tobacco sample, it is suggested that minor sterol constituents account for the difference in total sterol content as determined by the digitonin precipitation procedure.

The increase in amount of total alkaloids in the moldy tobacco (Table III) reflects a similar increase in the amount of nicotine in this sample. Levels of nornicotine and anabasine were approximately the same in both samples. None of the three molds infesting the inoculated sample is known to produce nicotine, and it is unlikely that the increase in this alkaloid is due to biosynthetic activity of the fungi. A possible explanation is that the observed increase in total alkaloids, particularly nicotine, results from a release of bound alkaloids such as those found in poppy seeds (Fairbairn and El-Masry, 1968) and hemlock (Fairbairn and Ali, 1968). Tobacco may contain similar bound alkaloids, since total and individual alkaloid content increases when tobacco is extracted under acidic conditions (Weeks and Bush, 1968).

The contents of chlorogenic acid and rutin, the principal soluble phenolic constituents of tobacco leaves (Stedman, 1968), as well as of total phenols, were lower in the moldy sample (Table IV). Although a number of minor phenolic constituents are known to occur in tobacco (Stedman, 1968), only three of these—the coumarins scopolin and its aglycone scopoletin, and caffeic acid—were assayed (Table IV). An increase of 620% in amount of caffeic acid, one of the products of hydrolysis of chlorogenic acid, was found in the moldy sample. The large increase seems to be due to the breakdown of chlorogenic acid in this sample. In contrast, no increase in amount of scopoletin was observed to account for the 49% decrease of its glycoside scopolin in the moldy sample. Similarly, although the content of rutin was lower in the moldy tobacco, its aglycone quercetin was not detected in this sample.

The changes in content of alkaloids, sterols, and phenols which resulted when flue-cured tobacco was exposed to conditions favorable for mold infestation may have resulted from one or more of the following factors:

A loss or gain in dry weight in the moldy sample.
Reactions of endogenous enzymes in the cured tobacco tissue.
Chemical reactions promoted by the environment during the inoculation period.
The presence and activities of the molds.

Since certain constituents increased, others decreased, some did not change, and new components appeared, a change in dry weight could not have accounted for all of the observed differences in the two samples.

Furthermore, it is improbable that the activities of endogenous enzymes of tobacco could account for these differences. Since the samples had been exposed to temperatures of 77° C. for 24 hours during flue-curing, their endogenous enzymes may be presumed to have been inactivated prior to the inoculation period of the moldy sample. Evidence favoring this hypothesis was obtained by assay of polyphenol oxidase, an enzyme which acts on both rutin and chlorogenic acid. No activity was detected in either sample, indicating that the decrease in levels of chlorogenic acid and rutin in the moldy sample was not due to this enzyme.

While it is not possible to exclude chemical reactions occurring during the inoculation period as the cause of some of the differences in the two samples, some of the changes seem more likely to be a result of the activity of the molds. The additional minor sterol constituents which appeared in the moldy sample were possibly biosynthetic products of the molds, since ergosterol and related compounds which are produced by *A. niger* (Barton and Bruun, 1951) would also be included in the digitonin precipitate and could account for the observed increase in total sterols. Decreases in amounts of total and individual phenols may also have been due to the activity of fungal enzymes. *A. flavus* and *A. niger* are known to contain enzymes which degrade rutin to water-soluble products (Westlake *et al.*, 1959). Similar fungal enzymes may account for the losses in other phenolic constituents in the moldy sample.

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