Variation in Sterols, Alkaloids, and Polyphenols of Two *Nicotiana* Varieties under Different Nitrogen Fertilization and Drying Processes

Claus Grunwald, Lowell P. Bush,* and Carol J. Keller

Coker 139 and Burley 21 were cultured under high and low levels of nitrogen fertilization and sampled throughout the growing season. Analyses of polyphenols, alkaloids, and sterols were performed on primed fresh leaves, air-dried leaves, and oven-dried leaves. Coker 139 had higher quantities of rutin and chlorogenic acid than Burley 21, and in both varieties the polyphenols decreased over the growing season. Total alkaloid content increased uniformly during the growing season until the plants were topped, and then a greater rate of accumulation occurred. In Burley 21, nornicotine and anabasine increased most rapidly during the second half of the growing season, but in Coker 139 they increased uniformly throughout the growing season. Coker 139 and Burley 21 contained about equal quantities of sterols at time of transplanting. In both tobacco varieties, sterol content increased over the 84-day growing season. At transplanting, Coker 139 contained slightly higher quantities of stigmasterol than β-sitosterol, while Burley 21 contained about equal quantities of these two sterols. No difference in polyphenol or sterol content was observed between nitrogen treatments within tobacco variety. Different drying conditions lowered the polyphenol and sterol content in both varieties, but had very little influence on total alkaloid content.

Parups (1967) recognized that phenols influence color and quality of tobacco, and Shmuk (1927) proposed that polyphenols may be an important factor in the quality of tobacco smoke. Harlan and Moseley (1955) suggested that alkaloids and possibly other chemical components in tobacco leaf also may be essential to the quality of tobacco smoke. Unlike the alkaloids and polyphenols, the sterols have not been linked with tobacco quality or smoke flavor. But, like the alkaloids and polyphenols, the sterols have been implicated as possible precursors to compounds in tobacco smoke which may be carcinogenic (Public Health Service, 1964). Bischoff (1963) reported that at least three oxidative products of cholesterol are presently known to have tumorigenic activity. Because of the potential importance of these chemical constituents, any factors affecting their content in tobacco leaf are of interest. Genotype, environmental conditions, culturing practices, and curing methods may influence the chemical composition of leaf tobacco, and in 152 randomly selected tobacco samples the alkaloid content varied from 0.17 to 4.93%, the phenol content (including total reducing substances, except sugar) varied from 0.52 to 2.61%, and the sterol content varied from 0.1 to 0.3% (Tso, 1969).

Vickery *et al.* (1935) reported that nicotine synthesis began very early in the development of shade tobacco and accumulated rapidly in leaves throughout the growing season; however, they did not analyze any of the secondary alkaloids. Franklin *et al.* (1964) and Atkinson *et al.* (1969) reported an increase in total alkaloids in cured tobacco leaf as nitrogen fertilization rate increased, but they also reported a decrease in alkaloid content as water availability increased.

The most recent review of sterols in tobacco has been presented by Stedman (1968). Generally, four major sterols have been found in tobacco: stigmasterol; β-sitosterol; campesterol; and cholesterol. Stedman and Rusaniwskyj (1959) reported that immature and mature Maryland tobacco contained similar quantities of sterols; however, Davis *et al.* (1970) reported that the pattern of sterol distribution in the tobacco plant was a linear increase from the base to the apex of the plant and from the base to the tip of the leaf.

Penn and Weybrew (1958) reported that the content of the principal polyphenols increased with plant maturity. A similar observation was reported by Walker and Lee (1968). Penn and Weybrew (1958) reported that levels of chlorogenic acid varied only slightly with curing; however, Nagasawa (1958) and Walker and Lee (1968) found an appreciable increase in chlorogenic acid with curing. Conversely, Keller and Kasperbauer (1969) found that with storage there were moderate losses in chlorogenic acid content. Andersen *et al.* (1970) recently reported that polyphenol content of flue-cured tobacco decreased with increased nitrogen fertilization; however, Tso *et al.* (1967) showed that the sums of chlorogenic acid, rutin, scopolin, and scopoletin increased with increased nitrogen fertilization.

Much remains to be learned about the relationship between plant age, culturing practice, and curing method as to the levels of sterols, phenols, and alkaloids in tobacco. The objectives of this research were: (1) to determine the effect of nitrogen fertilization and plant maturity on levels of polyphenols, sterols, and alkaloids in two types of tobacco; and (2) to study the relative merits of various methods of

Department of Agronomy, University of Kentucky, Lexington, Ky. 40506

tissue processing as related to polyphenol, sterol, and alkaloid content.

RESEARCH METHODOLOGY

Experimental Procedure. Varieties, Burley 21 (an air-cured type) and Coker 139 (a flue-cured type), of *Nicotiana tabacum* L. were cultured in the field in 1968 at Spindletop Farm, Lexington, Ky., with high and low rates of ammonium nitrate fertilization. Nine-hundred plants were grown in each of four adjacent blocks, each 0.05 ha in area, at the following nitrogen levels: Burley 21, 112 kg N/ha and 448 kg N/ha; Coker 139, 56 kg N/ha and 224 kg N/ha. Plants were topped at 74 days after transplanting (50–75% of plants were in full bloom) and any axillary bud growth was removed by hand. The plants were sampled at the time of transplanting from the seed bed to the field plots, and at four subsequent 21 day intervals. At all sampling dates, all the leaves including midribs were primed from the appropriate experimental plants. For each harvest date tissue was analyzed fresh as well as after air- and oven-drying. Primed leaves to be air-dried for 14 days were strung and hung in an air-conditioned building with an approximate temperature of 22° C and relative humidity of 50%. Leaves to be oven-dried were placed in a forced-draft oven at 70° C until constant weight was reached—24 to 36 hr.

One-hundred-and-twenty plants of each variety were harvested on the initial sampling date (time of transplanting) and divided equally (40 plants) for fresh, air-dried, and oven-dried treatments. On the following two sampling dates (21 and 42 days) 20 plants of each variety and each nitrogen treatment were taken for each of the three sample treatments. To reduce the amount of tissue to be analyzed at 63 and 84 days, three composite plants were made from the leaves of 20 plants for the air- and oven-drying treatments, and two composite plants were made for the fresh treatments. The composite samples were then weighed and processed. Fresh weights of plants from each cultural treatment are shown in Table I. Depressed growth of both varieties at the high nitrogen fertilization rates was observed during the first part of the growing season. This is not an uncommon observation and is probably caused by a lowered soil pH due to nitrification of the ammonium nitrogen and a subsequent alteration of micronutrient availability (Atkinson *et al.*, 1969; Reneau *et al.*, 1968).

Processing of the Samples. Fresh Tissue. Slurries were made with fresh tissue by homogenization with 2 to 4 volumes of absolute methanol in a 4 l. Waring Blendor. Portions of these slurries were used for determination of dry weights and analysis of polyphenols, sterols, and alkaloids. Dry weight determinations were made by drying three 50 ml volumes of slurry to constant weight in an oven at 70° C. From this, the dry weight/unit volume of slurry was calculated.

Dried Tissue. Leaves which were either air- or oven-dried were ground to pass a 40-mesh screen in a Wiley mill and mixed by tumbling. Before chemical analysis, the ground samples were brought to 6% moisture by equilibration at laboratory conditions.

Alkaloid Analysis. Individual alkaloids from oven- and air-dried treatments were determined as described previously (Keller *et al.*, 1969). Fifty ml of the methanol slurry (fresh samples) was made basic by the addition of 150 ml 1 *N* NaOH and mechanically shaken for 30 min. The mixture was filtered and the filtrate extracted with four 10 ml portions of chloroform. The combined chloroform extracts were taken to dryness, the residue solubilized in 1.5 ml chloroform, and a measured amount streaked on thin-layer (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 chromatograms. Individual alkaloids were quantified by eluting the alkaloids in 0.1 *N* HCl and measuring the absorbance at 259 nm with a Beckman DU spectrophotometer. The anabasine fraction also contained anatabine and was quantified with an anabasine standard.

Table I. Fresh Weight of Leaves From Burley 21 and Coker 139 Plants Cultured under Two Nitrogen Regimes

Tobacco	Nitrogen Rate kg/ha	Sample Time, Days 0	21	42	63	84
		g fresh weight of leaf per plant				
Burley 21	112	15	76	662	1178	2070
	448	15	53	602	1220	2150
Coker 139	56	14	36	364	992	1680
	224	14	24	356	905	1650

Polyphenol Analysis. A measured portion of the methanol slurry (30 ml) or dried tissue (1 g) was exhaustively extracted in boiling absolute methanol and concentrated *in vacuo* to a volume of 5 ml.

Chlorogenic acid and rutin were determined by two-dimensional descending chromatography as described previously (Keller *et al.*, 1969). 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. The zone of rutin was eluted in a solution of 1% aluminum chloride in methanol and the absorbance was measured at 432 nm with a Beckman DU spectrophotometer. The chlorogenic acid zone was eluted in 30% methanol and assayed by the addition of Arnow's reagent and measuring the absorbance at 510 nm (Arnow, 1937).

Sterol Analysis. For fresh tissue analysis, 100 ml of the methanol slurry, about 3 g dry weight of tissue, were extracted with 150 ml of acetone in a Soxhlet apparatus for 24 hr. For dried sample analysis 5 g were extracted with 250 ml of acetone for 24 hr. The acetone extracts were used for subsequent sterol analysis, as described by Stedman and Rusaniwskyj (1959).

Individual sterols were separated by gas chromatography as free sterols on a 1.80-m column, 6 mm i.d., packed with 5% OV-101 on Anakrom ABS 80- to 90-mesh (Grunwald, 1969). The column temperature was 250° C and the flash heater temperature was kept 50° C above that of the column. The flame ionization detector temperature was 275° C. Helium was the carrier gas at a flow rate of 100 ml/min. The quantification was carried out as described previously (Grunwald, 1970a).

RESULTS AND DISCUSSION

Alkaloids. There was a steady rate of nicotine accumulation between setting and 63 days in all treatments except the 63 day fresh samples (Figure 1). Nicotine, in Burley 21, accumulated most rapidly after topping while in Coker 139 nicotine content increased more uniformly over the growing season and the effect of topping was less pronounced. The rate of nicotine accumulation in the first 21 days was less in Coker 139 than in Burley 21, and throughout the season nicotine was lower in Coker 139 than in Burley 21. No difference in nicotine content was observed in Coker 139 between low and high nitrogen treatments, whereas in Burley 21 the high

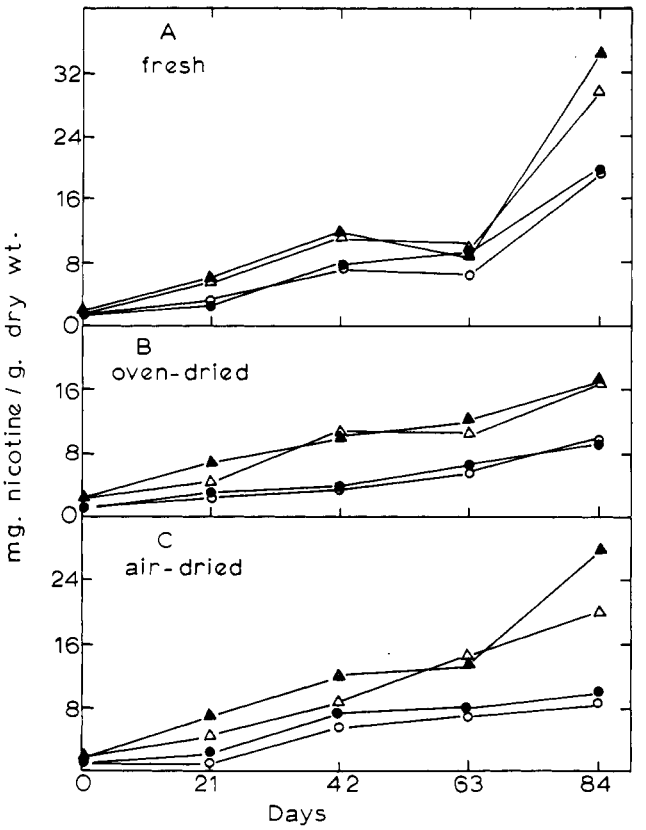

Figure 1. Nicotine content as influenced by nitrogen fertilization, age and drying process. △—△ Burley 21 at 112 kg N/ha; ▲—▲ Burley 21 at 448 kg N/ha; ○—○ Coker 139 at 56 kg N/ha; and ●—● Coker 139 at 244 kg N/ha

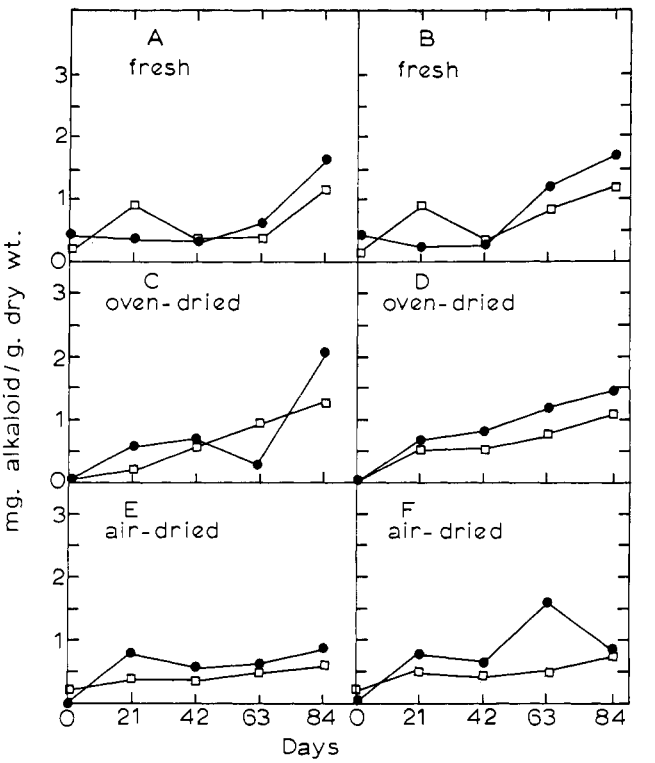

Figure 3. Nornicotine and anabasine in Coker 139 as influenced by nitrogen fertilization, age and drying process. Figure 3A, C, and E 56 kg N/ha; and Figure 3B, D, and F 244 kg N/ha; ●—● nornicotine and □—□ anabasine

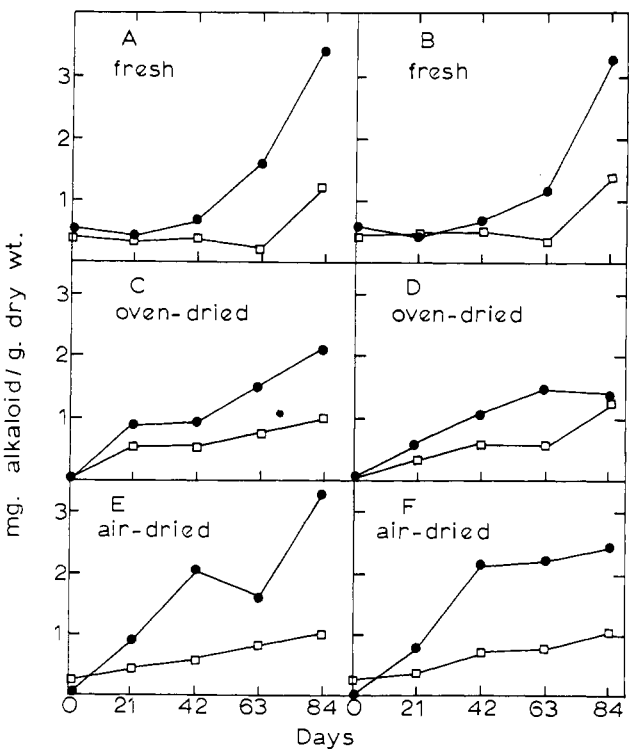

Figure 2. Nornicotine and anabasine in Burley 21 as influenced by nitrogen fertilization, age and drying process. Figure 2A, C, and E 112 kg N/ha. Figure 2B, D, and F 448 kg N/ha; ●—● nornicotine; and □—□ anabasine

nitrogen treatments had a greater nicotine content than the low nitrogen treatments.

In both nitrogen treatments of the fresh Burley 21 samples, anabasine was low until after topping (Figure 2). Nornicotine in the fresh samples accumulated at a slow rate from 0 to 42 days, and accumulated rapidly between 42 to 84 days. However, in the air-dried samples, anabasine increased at a steady rate throughout the season, whereas nornicotine increased more rapidly between 0 to 42 days than between 42 to 84 days. The accumulation of nornicotine was equal to or greater than the anabasine content in all samples of Burley 21 from 21 to 84 days.

A small increase of nornicotine and anabasine was observed in all treatments for Coker 139 throughout the growing season (Figure 3). In contrast to Burley 21 very little difference in accumulation was observed between nornicotine and anabasine, and both were lower in Coker 139 than in Burley 21.

Total alkaloids, calculated by summation of nicotine, nornicotine, and anabasine–anatabine, increased at a steady rate from 0 to 63 days, but after topping, between 63 and 84 days, a greater rate of accumulation occurred. This pattern of increased total alkaloid content during the growing season is in agreement to the earlier work of Mothes (1928) and Vickery *et al.* (1935). Contrasting to reports of Atkinson *et al.* (1969) and Breland *et al.* (1967) no differences were observed between high and low nitrogen application in Coker 139, but in Burley 21 at 84 days plants from the high nitrogen treatment contained 14% more alkaloids than plants from the low nitrogen treatment. Only minor differences were observed among drying treatments until the 84 day sample, when the fresh samples were much higher in alkaloid content than either the air- or oven-dried samples. The percentage of nornicotine and anabasine of the total

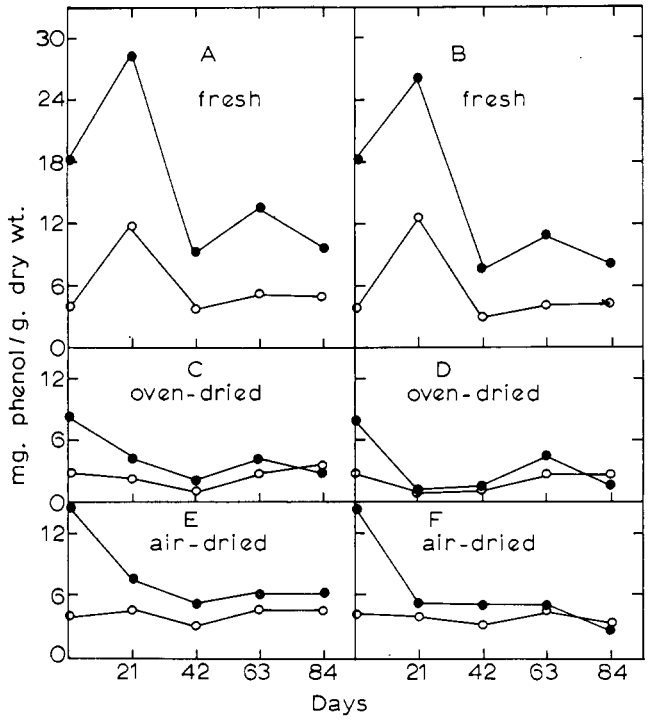

Figure 4. Chlorogenic acid and rutin in Coker 139 as influenced by nitrogen fertilization, age and drying process. Figure 4A, C, and E 56 kg N/ha and Figure 4B, D, and F 244 kg N/ha; O—O rutin and ●—● chlorogenic acid

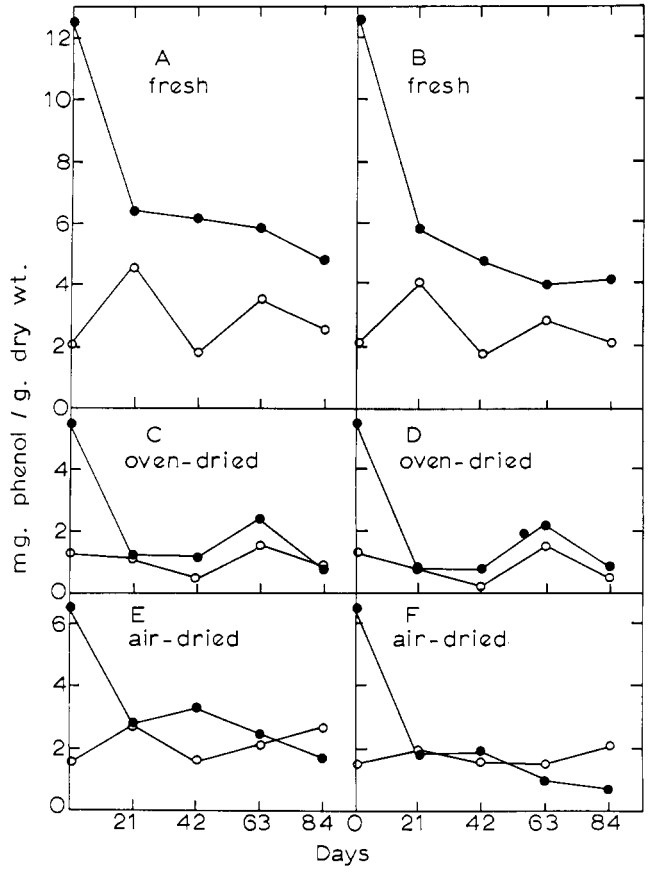

Figure 5. Chlorogenic acid and rutin in Burley 21 as influenced by nitrogen fertilization, age and drying process. Figure 5A, C, and E 112 kg N/ha and Figure 5B, D, and F 448 kg N/ha; O—O rutin and ●—● chlorogenic acid

alkaloid content was constant throughout the growing season with the exception of the 21 day samples of Coker 139. There was no apparent influence of the nitrogen treatments on this percentage, nor was a difference observed between Burley 21 and Coker 139 at maturity. Sims *et al.* (1970) found this percentage to increase with higher nitrogen levels, and Terrill (1965) reported the opposite effect. Our results are intermediate. However, water availability and tobacco variety are apparently involved in the observation because our 21 day samples were taken after a period of extreme moisture stress. It was only at the 21 day samples that we observed any difference in the percentage, and then it was mainly in Coker 139.

Polyphenols. Results of the determination of chlorogenic acid and rutin content, the principal polyphenols of tobacco, for all sample dates and treatments are shown in Figure 4 for Coker 139 and in Figure 5 for Burley 21. A sharp increase in both polyphenols was observed in the fresh samples of Coker 139 between the 0 and 21 day sampling periods. This was possibly related to the severe moisture stress on these plants during this period. The young seedlings made very little growth during this period (Table I) and may have accumulated polyphenols. A similar observation has been reported by Loche and Chouteau (1967). The Burley 21 seedlings appeared to be affected very little by this dry period, although an increase in rutin was observed. The greater increase in polyphenols in Coker 139 compared to Burley 21 during this period was similar to observed changes in the percentage of nornicotine and anabasine of the total alkaloid content.

Levels of chlorogenic acid and rutin were somewhat lower at the final harvest date than would be expected from other studies (Sheen and Calvert, 1969; Akaike and Yamada, 1966) in which there were general trends of increased amounts of polyphenols with increasing plant maturity. With maturity there was a trend for lower polyphenol production at higher nitrogen level. Andersen *et al.* (1970) and Loche and Chouteau (1967) reported a similar but larger effect between levels of nitrogen fertilization and polyphenol content in tobacco leaves. It is apparent from our results that the levels of chlorogenic acid and rutin in fresh and air-dried samples are higher in Coker 139 (Figure 4) than in Burley 21 (Figure 5). Sheen and Calvert (1969) found that dark tobacco had higher polyphenol levels than burley tobacco when the two types were cultured according to conventional burley tobacco practices. Our results provide further evidence that the differences in polyphenol content in the two tobacco types are not attributable to the low rates of nitrogen fertilization commonly employed in the culture of flue-cured tobacco types.

No differences in the recovery of the two polyphenols between tissue which had been air-dried or oven-dried could be shown. Both types of drying decreased the amounts of rutin and chlorogenic acid compared to the fresh tissue samples. This observation was surprising since it was expected that the more rapid cessation of enzyme activity under the high temperature in the oven would give better preservation of polyphenols.

Sterols. Burley 21 and Coker 139 at time of transplanting contained 1.5 and 1.6 mg total sterols per g dry weight, respectively. The fresh tissue of both tobacco varieties under both nitrogen treatments increased slightly in sterol content over the 84-day growing season. No published information is available concerning the influence of age and nitrogen fertilization on sterol content in tobacco. However, Eisenberger and Menke (1966) sampled *Antirrhinum majus, Spinacia*

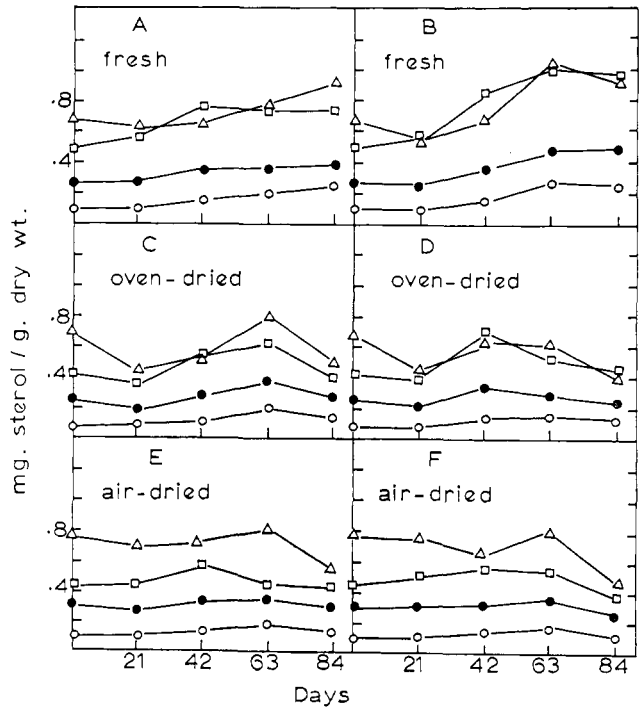

Figure 6. Individual sterols in Coker 139 as influenced by nitrogen fertilization, age and drying process. Figure 6A, C, and E 56 kg N/ha and Figure 6B, D, and F 244 kg N/ha. △—△ stigmasterol; □—□ β-sitosterol; ●—● campesterol; and ○—○ cholesterol

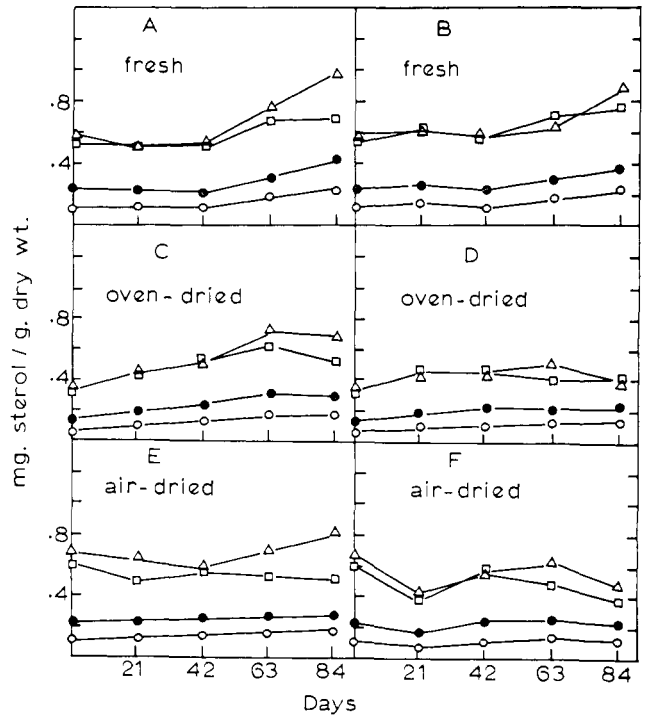

Figure 7. Individual sterols in Burley 21 as influenced by nitrogen fertilization, age and drying process. Figure 7A, C, and E 112 kg N/ha and Figure 7B, D, and F 448 kg N/ha. △—△ stigmasterol; □—□ β-sitosterol; ●—● campesterol; and ○—○ cholesterol

oleracea and *Allium porrum* leaves at different stages of development and found only very slight fluctuations in total sterol content, even if they compared their results over a 3-year period.

In fresh tissue the individual sterol analysis revealed that cholesterol and campesterol increased at a rather constant rate over the growing season in both varieties (Figures 6 and 7). At transplanting Coker 139 contained slightly higher quantities of stigmasterol than β-sitosterol (Figure 6), while Burley 21 at this point contained about equal quantities of these two sterols (Figure 7). In Coker 139 the ratio of stigmasterol to β-sitosterol fluctuated during the growing season, while the content of these two components increased during most of the growing season. In the fresh Burley 21 samples stigmasterol and β-sitosterol did not markedly increase during the first 42 days, but once stigmasterol started to increase it continued until the end of the experiment, while β-sitosterol leveled off after 63 days (Figures 7A, B).

Stigmasterol and β-sitosterol are C_{29} sterols and differ only in one double bond at C_{22} (trans). Using [2-^{14}C] mevalonate, Bennett *et al.* (1963) suggested that in *Dioscorea spiculiflora* stigmasterol may be formed by dehydrogenation of β-sitosterol. This suggestion finds support in the observations by Rowe (1965). He reported the absence of stigmasterol in pine bark, in which β-sitosterol predominates, and interpreted this as a failure of the genus *Pinus* to acquire the specific dehydrogenase. Davis (1970) observed that the ratio of stigmasterol to β-sitosterol within the tobacco plant is not constant, and other experiments suggest (Grunwald, 1970b) that light quality may have an effect on the stigmasterol to β-sitosterol ratio in tobacco. However Waters and Johnson (1965), administering ^{14}C labeled β-sitosterol and stigmasterol to excised soybean (*Glycine max*) leaves, did not find any relationship between those sterols.

Different drying conditions generally decreased the sterol content in both varieties under all nitrogen treatments. Sample processing had a very pronounced effect on the 84-day sample; however, the decrease was not constant for all sterols (Figures 6 and 7). Generally, no difference was found in the recovery of sterols between tissues which had been air-dried and oven-dried. Incomplete extraction of the sterols from dried tobacco samples cannot be ruled out; however, the unequal loss of individual sterols suggests that enzyme action may be responsible for the lower sterol values in the dried tobacco samples.

ACKNOWLEDGMENT

The authors are obliged to John Sims for assistance in the culturing of the tobacco plants. We gratefully acknowledge the technical assistance of William Scrivner, Donald Webb, and Pat Matheny.

LITERATURE CITED

Akaike, S., Yamada, S., *Hatano Tob. Exp. Sta. Spec. Bull.* **57,** 27 (1966).

Andersen, R. A., Chaplin, J. J., Currin, R. E., Ford, Z. T., *Agron. J.* **62,** 415 (1970).

Arnow, L. E., *J. Biol. Chem.* **118,** 531 (1937).

Atkinson, W. O., Ragland, J. L., Sims, J. L., Bloomfield, B. J., *Tobacco Sci.* **13,** 123 (1969).

Bennett, R. D., Heftmann, E., Preston, W. H., Hann, J. R., *Arch. Biochim. Biophys.* **103,** 74 (1963).

Bischoff, F., *Progr. Exp. Tumor Res.* **3,** 412 (1963).

Breland, H. L., Pritchett, W. L., Lundy, H. W., *Soil Crop Sci. Soc. Fla. Proc.* **27,** 235 (1967).

Davis, D. L., Department of Agronomy, University of Kentucky, Lexington, Ky., private communication, 1970.

Davis, D. L., Legg, P. D., Collins, G. B., *Crop Sci.* **10,** 545 (1970).

Eisenberg, W., Menke, W., *Z. Naturforsch. B.* **21,** 857 (1966).

Franklin, R. E., Teater, R. W., Curry, R. B., Schwab, G. O., *Agron. J.* **56,** 361 (1964).

Grunwald, C., *Anal. Biochem.* **34,** 16 (1970a).

Grunwald, C., Department of Agronomy, University of Kentucky, Lexington, Ky., unpublished data, 1970b.

Grunwald, C., *J. Chromatogr.* **44,** 173 (1969).
Harlan, W. R., Moseley, J. M., *Tobacco—Encycl. Chem. Tech.* **14,** 248, The Interscience Encycl. Inc., N.Y., 1955.
Keller, C. J., Bush, L. P., Grunwald, C., J. AGR. FOOD CHEM. **17,** 331 (1969).
Keller, C. J., Kasperbauer, M. J., J. AGR. FOOD CHEM. **17,** 327 (1969).
Loche, J., Chouteau, J., *Bull. Soc. Fr. Physiol. Veg.* **13,** 327 (1967).
Mothes, K., *Planta* **5,** 563 (1928).
Nagasawa, M., *Agr. Chem. Soc. Japan B* **22,** 31 (1958).
Parups, E. V., *Lighter* **37,** 11 (1967).
Penn, D. T., Weybrew, J. A., *Tobacco Sci.* **2,** 68 (1958).
Public Health Service Publications No. 1103, 47–65 U.S. Department of Health, Education and Welfare 1964.
Reneau, R. B., Ragland, J. L., Atkinson, W. O., *Tobacco Sci.* **12,** 50 (1968).
Rowe, J. W., *Phytochemistry* **4,** 1 (1965).
Sheen, S. J., Calvert, J., *Tobacco Sci.* **13,** 10 (1969).
Shmuk, A. A., *Tr. Inst. Tabak.* (Works of the Tobacco Cultivation Institute) *Bull. No. 33* (1927).
Sims, J. L., Bush, L. P., Atkinson, W. O., J. AGR. FOOD CHEM. **18,** 381 (1970).
Stedman, R. L., *Chem. Rev.* **68,** 153 (1968).
Stedman, R. L., Rusaniwskyj, W., *Tobacco Sci.* **3,** 44 (1959).
Tso, T. C., *Tobacco* **168,** 69 (1969).
Tso, T. C., Sorokin, T. P., Engelhaupt, M. E., Andersen, R. A., Bortner, C. E., Chaplin, J. F., Miles, J. D., Nichols, B. C., Shaw, L., Street, O. E., *Tobacco Sci.* **11,** 133 (1967).
Terrill, T. R., *Diss. Abstr.* **26,** 605 (1965).
Vickery, H. B., Parker, G. W., Leavenworth, C. S., Wakeman, A. J., *Conn. Agr. Exp. Sta. Bull.* 374 (1935).
Walker, E. K., Lee, T. T., *Can. J. Plant Sci.* **48,** 381 (1968).
Waters, J. A., Johnson, D. F., *Arch. Biochim. Biophys.* **112,** 387 (1965).

Received for review July 8, 1970. Accepted December 18, 1970. Investigation (No. 70-3-110) is in connection with a project of the Kentucky Experiment Station and is published with the approval of the director. Work supported by Crops Research Division, Agricultural Research Service, U.S. Department of Agriculture Contracts Nos. (12-14-100-8945-34) and (12-14-100-9523-34).