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Received for review January 14, 1985. Accepted April 29, 1985. This study was supported by Grant CA-29580 from the National Cancer Institute.

Changes in Chemical Composition of Tobacco Lamina during Senescence and Curing. 1. Plastid Pigments

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Chlorophyll a, chlorophyll b, neoxanthin (I), violaxanthin (II), lutein (III), and β-carotene (IV) were quantified in burley tobacco (*Nicotiana tabacum* L.) during senescence and air-curing. During senescence the decrease of pigment concentrations varied from 70% reduction of chlorophyll b to a 30% decrease of violaxanthin. During air-curing 99% of the chlorophylls were catabolized, whereas, approximately 70% of the lutein (III) and carotene were degraded. The majority of pigment degradation occurs during the first two weeks of curing. Curing environments also influenced the rates of degradation of the pigments. Presence of light during curing retarded destruction of the carotenoids during early stages, whereas at final cure there were few differences in the concentrations of the pigments for different treatments.

INTRODUCTION

In recent years there has been increased interest in changes in chemical composition during plant senescence. This is particularly true for tobacco since the final consumable product has gone through growth, senescence, and curing. Chemical changes that occur during curing have a dramatic impact on the cured product. A recent review (Long and Weybrew, 1981) summarized many of the major chemical changes which occur during senescence and curing of tobacco. Also, a review (Burton et al., 1983) reported on many of the chemical changes occurring during air-curing of burley tobacco. One major change during the cell death is the destruction of chrlorophyll a, chlorophyll b, and the four major carotenoids: neoxanthin (I), violaxanthin (II), lutein (III), and carotene (IV). The degradation of the chlorophylls is almost complete with only low levels detected in the cured lamina; whereas, there is approximately 80% destruction of the carotenoids (Forest and Vilicens, 1979). The degradation of the carotenoids can lead to the formation of many components which enhance the aroma of tobacco. Recent reviews (Enzell and Wahlberg, 1980; Demole and Dietrich, 1977) have indicated approximately 80 aroma constituents in tobacco can be derived from the oxidative degradation of the carotenoids. Because of the importance of carotenoid degradation to the formation of flavor components, a study was initiated to quantify the decrease of carotenoids in tobacco lamina during air-curing. This allowed for identifying the stage of curing when the majority of the carotenoids were being metabolized.

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EXPERIMENTAL SECTION

Plant Materials and Procedures. Burley tobacco (*Nicotiana tabacum* L. cv. Ky 14) plants were grown at the Kentucky Agricultural Experimental Farm near Lexington. Recommended fertilization and cultural practices were followed during the growing season (Atkinson et al., 1976). Plants designated for all curing environments were harvested on the same date. At harvest, plants were stalk cut and six stalks speared on each stick. One third of the tobacco was cured in a conventional curing barn at ambient conditions. Another third was placed in a controlled environmental chamber and cured at 24 °C and 70% RH in darkness. The remainder was also cured at the same conditions but under continuous illumination from cool white fluorescent lamps. The lamps were arranged for uniform illumination of the tobacco to be sampled with light intensity of 80 ft-c.

Four randomized replicate samples were taken at harvest and 1, 2, 3, 4, 7, 9, 11, 14, 16, 18, 21, 24, 27, 35, 42, and 90 days to determine the concentration changes that occurred during curing. Five leaves—excepting the top two—were taken from the upper one-third of each plant sampled. Midveins were removed from the leaf, the lamina weighed, and the leaf areas were determined with a leaf area meter. The lamina was freeze-dried and reweighed to determine moisture content. Samples were ground to pass a 40-mesh screen and stored at −40 °C until analyzed.

Chemical Analyses of Plant Pigments. The procedure for the analyses of chlorophyll a, chlorophyll b, neoxanthin, violaxanthin, lutein, and β-carotene was a modification of a procedure described by Eskins and Dutton (1979). A 250-mg sample of the dried tissue was placed in a 10 × 40 mm extraction thimble and extracted with acetone (20 mL) in a microsoxhlet apparatus for 1 h.

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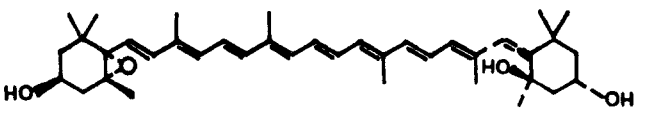
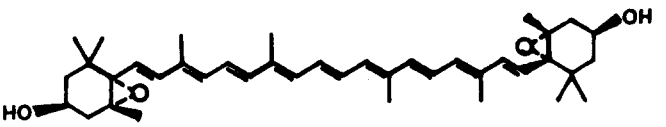
Chart I. Major Carotenoids in Tobacco



I



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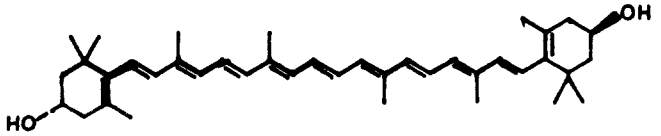
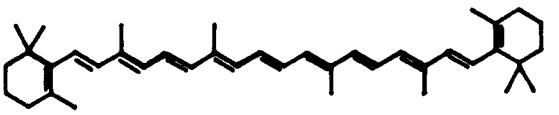


III



IV

The extract was filtered under vacuum through a Buchner medium-porosity filter and a Sep-Pak reverse-phase cartridge. The cartridge was rinsed with a small volume (3 mL) of acetone and the filtrate was transferred to a 25-mL actinic volumetric flask and diluted to volume with acetone. For HPLC analyses a 50-μL aliquot was injected on a C_{18} reverse-phase column with methanol–water (90:10 v/v) and ethyl acetate as the elution solvents. The conditions for elution were the same as those described by Eskins and Dutton (1979). Order of elution for the plastid pigments were neoxanthin, violaxanthin, lutein, chlorophyll b, chlorophyll a, and carotene. Quantification of the plant pigments were determined at 436 nM. Calibration curves for chlorophyll a and chlorophyll b were obtained from chlorophyll standards obtained from Sigma Chemical. Carotene calibration curves were obtained from carotene (Eastman Chemicals) and lutein; neoxanthin and violaxanthin curves were obtained by extrapolation of their extinction coefficients at 436 nM from the literature to the response of carotene standard at 436 nM.

Petroleum ether extractables (PEE), saturated hydrocarbons, and neophytadiene in PEE were quantified by using the following procedures. Tobacco (0.25 g) was placed in a microsoxhlet apparatus and extracted with 20 mL of petroleum ether (bp 30–60 °C) in a tared flask for 1 h. The petroleum ether was evaporated on a steam bath and the flask was placed in a drying oven at 95 °C for 1 h. After cooling, the flask was reweighed to obtain the residue weight and to calculate the percent petroleum ether extractables. For the analyses of neophytadiene and individual hydrocarbons, the extract was spiked with internal standard (eicosane, 1 mg/mL in hexane). The residue was redissolved in hexane (ca. 5 mL), passed through a silica gel cartridge (Sep-Pak), and hydrocarbons and neophytadiene were eluted completed with 15 mL of hexane. The hexane was evaporated to dryness on a steam bath and the residue was dissolved in 1. 5 mL of hexane and transferred to vial for gas chromatographic quantification. Neophytadiene and the saturated hydrocarbons were separated and quantified on a 1.83 m × 6.3 mm glass column packed with 3% Dexsil 300 on 90–100 Anakrom Q. After 5 min at 100 °C, the column oven temperature was increased at 3 °C/min to 280 °C and maintained at this temperature until the hydrocarbons had eluted.

Calcium content of the lamina was quantified with atomic absorption spectroscopy. A sample of tobacco lamina (0.2 g) was placed in a 50-mL Erlenmeyer flask, 10 mL of HNO_3–$HClO_4$ (9:1 v/v) was added, the flask was covered with a watch plate, and the sample was digested overnight at 80 °C. The flask was placed on a hot plate in a perchloric acid hood and the excess nitric and perchloric acid were removed by boiling to dryness. The residue was dissolved in 10 mL of N HCl, an aliquot was diluted 200:1, and the concentration of calcium was determined by AA in an acetylene–nitrous oxide flame.

Table I. Percent Moisture Content of Burley Tobacco Lamina during Air-Curing in Three Environments

days after harvest	conventional barn	controlled[a] environment	
		darkness	light
0	79.1	79.1	79.1
1	79.4	78.7	78.0
2	79.8	78.0	77.2
3	79.8	77.5	76.9
4	79.1	77.3	76.2
7	78.9	75.1	74.9
9	78.8	71.1	69.8
11	79.4	69.8	67.9
14	75.4	63.2	64.1
16	75.5	54.2	60.1
18	70.1	31.7	53.8
21	65.7	29.9	36.9
23	66.5	20.2	33.4
25	52.4	19.3	21.2
28	36.1	16.7	14.7
35	35.3	17.0	15.6
42	25.9	15.1	14.0
$LSD_{(0.05)}$	4.21	5.02	6.00
$LSD_{(0.01)}$	5.62	6.72	8.00

[a] 24 °C and 70% RH.

RESULTS AND DISCUSSION

Chemical changes during plant cell death are difficult to quantify. If all cells in an organism lost their viability within a short time, it would be possible to equate a chemical change with a particular physiological event. The study of whole leaf changes is more difficult, since the cells lose their viability over several weeks. Therefore, the quantification of specific leaf components are mean values of different physiological events during the death of tissue.

The moisture content of the lamina may be one of the better indicators of cell viability. Moisture content of burley lamina is presented in Table I. Upper leaves of tobacco cured at ambient conditions maintained a constant moisture for approximately 16 days before there was a

Table II. Pigment Concentration for Burley Tobacco from Time of Topping to Harvest[a]

weeks after topping	pigment, mg/g					
	neoxanthin (I)	violaxanthin (II)	lutein (III)	chlorophyll b	chlorophyll a	carotene (IV)
0	0.263	0.223	1.220	4.056	16.404	0.694
1	0.230	0.198	1.211	3.166	16.110	0.643
2	0.225	0.159	0.989	2.382	12.193	0.637
3	0.206	0.164	0.841	1.786	9.118	0.520
4	0.140	0.160	0.751	1.211	6.507	0.460
$LSD_{(0.05)}$	0.021	0.037	0.123	0.366	1.934	0.099

[a] Samples taken from top third of plant.

significant decrease in moisture content. Maintenance of water in the lamina during this period could be supplied by stalk and midveins, both of which contain a significant amount of water. This would allow the cells in the lamina to maintain their integrity. The moisture loss from days 18 to 25 was gradual for tobacco cured at ambient conditions. Even after the fifth week of curing the lamina contained approximately 35% moisture. Comparison of moisture content in tobacco lamina cured under a controlled environment indicates the influence of temperature on the loss of water. Samples cured at 24 °C and 70% RH, which has been reported to be the ideal curing condition for burley tobacco (Jeffrey, 1940), exhibited different responses to curing than the samples cured at ambient conditions (Table I). Tobacco cured under these controlled conditions exhibited significant water losses nine days earlier than the tobacco cured at ambient conditions. The 1982 mean temperature was 18 °C, which was 6 °C lower than the temperature in the curing chambers. This lower temperature could decrease the enzymatic or autolytic activity responsible for the catabolism of the cell organelles, thereby retarding the death of the cells and decreasing water loss from the leaf (Burton et al., 1984). Moisture loss from tobacco cured in the presence of light closely paralleled the tobacco cured in total darkness, except it was delayed slightly between days 16 and 23. This suggests that there is some indirect influence of light on water loss from the lamina.

In discussing the changes in pigment concentration during lamina cell death, it should be noted that visual senescence of tobacco begins before harvest. Significant decreases in concentration of lamina plastid pigments occur between topping and harvest (Table II) which is in agreement with an earlier study (Sheen et al., 1982). These decreases are not as large as those reported for the flue-cured tobacco (Court and Hendel, 1984). Except for neoxanthin (I) and chlorophyll b, the plastid pigment concentrations did not decrease significantly during the first week after topping. Two weeks after decapitation significant decreases of all individual pigments occurred, whereas violaxanthin concentration was reduced by only 30%. The decreases in concentrations of the four other pigments fall between the values for chlorophyll b and violaxanthin. These data show that there is a decrease of the plant pigments after decapitation, yet the rates of degradation of the individual pigments differ.

The curing environment had a significant influence on the rate of pigment disappearance. Chlorophyll content presented in Table III shows this influence. Tobacco cured at ambient conditions had less chlorophyll degradation in comparison to those samples cured at 24 °C. After the seventh day of ambient curing there was a statistically significant decrease in chlorophyll b concentration, whereas, for the tobacco cured at 24 °C, there was a significant decrease of chlorophyll b after the third day. The increased disappearance of chlorophyll b was a result of higher temperatures of the curing chamber in comparison to the tobacco cured at ambient temperatures.

Table III. Chlorophyll Content of Burley Tobacco Lamina during Curing

days after harvest	chlorophyll b, mg/g of dry matter			chlorophyll a, mg/g of dry matter		
		controlled environment[a]			controlled environment[a]	
	barn	dark	light	barn	dark	light
0	1.211	1.211	1.211	6.507	6.507	6.507
1	1.325	1.113	0.967	6.023	5.135	4.540
2	1.260	1.097	0.999	6.067	4.620	4.537
3	1.424	1.162	0.853	6.550	4.652	3.793
4	1.292	0.869	0.934	5.447	3.692	4.173
7	1.064	0.680	0.789	4.285	2.679	2.955
9	0.885	0.176	0.387	3.380	0.734	1.441
11	0.789	0.197	0.403	3.292	0.836	1.448
14	0.357	0.082	0.325	1.388	0.311	0.705
16	0.484	*b*	0.120	1.897	0.103	0.464
18	0.071	*b*	0.090	0.241	0.073	0.329
21	0.064	*b*	0.048	0.177	0.123	0.113
28	0.067	*b*	0.019	0.059	0.191	0.052
42	0.003	*b*	0.025	0.056	*b*	0.052
90	0.003	*b*	0.014	0.045	*b*	0.021
$LSD_{(0.05)}$	0.259	0.203	0.251	0.852	1.87	0.796

[a] 24 °C and 70% RH. [b] Not detected.

Curing tobacco in the presence of light also had an influence on the metabolism of the lamina pigments. There was a significant decrease in concentration of both chlorophylls during the first day of curing. Chlorophyll concentrations remained relatively constant during the next six days. During the subsequent two weeks the chlorophyll content decreased approximately 10-fold. For tobacco cured in the absence of light, chlorophyll b could not be detected 16 days after harvest. It was possible to detect chlorophyll a during the first four weeks after harvest but after this time it could not be detected. The disappearance of chlorophyll a is very similar to chlorophyll b. For the tobacco cured at ambient conditions chlorophyll a disappearance occurred at a decreased rate in comparison to the samples cured at a constant temperature and humidity. Again these differences were probably caused by the lower mean temperatures of the samples cured at ambient conditions.

The disappearance of the carotenoids in tobacco is of particular interest since many of their degradation products are known to be aroma consitutents (Enzell and Wahlberg, 1980). Neoxanthin (I) and violaxanthin (II), the two most polar carotenoids, had approximately the same concentration in lamina at harvest and approximately the same concentration after curing (Table IV). Except for neoxanthin from tobacco cured in the presence of light, there was a statistically significant decrease in the concentration of I and II the first day after harvest. The sample cured at ambient conditions showed a decreased rate of degradation in comparison to the tobacco cured under a controlled environment. This, again, reflects the lower mean temperature of curing (Burton et al., 1984).

Table IV. Carotenoid Content of Burley Tobacco Lamina during Air-Curing[a]

days after harvest	neoxanthin (I), μg/g			violaxanthin (II), μg/g			lutein (III), μg/g			carotene (IV), μg/g		
	A	B	C	A	B	C	A	B	C	A	B	C
0	140	140	140	160	160	160	752	752	752	460	460	460
1	97	100	129	98	87	127	700	635	498	458	408	480
2	105	83	127	95	77	119	681	603	524	468	381	456
3	118	105	119	112	79	117	703	669	457	487	391	449
4	117	78	130	101	74	116	672	526	506	446	359	446
7	109	79	120	88	62	97	637	479	480	403	314	414
9	109	57	68	77	29	41	581	325	358	383	228	233
11	113	65	64	72	32	40	548	371	405	380	248	238
14	86	67	67	47	21	36	388	329	382	264	206	231
16	87	37	43	42	15	23	386	290	284	295	201	177
18	61	25	49	29	*b*	25	279	255	295	205	179	171
21	53	32	38	23	*b*	21	288	284	227	233	201	167
28	38	*b*	27	19	*b*	12	272	275	183	185	156	151
42	28	*b*	19	21	*b*	14	274	275	230	159	167	179
90	12	*b*	13	11	*b*	12	220	247	179	115	152	126
$LSD_{(0.05)}$	14	14	20	14	5	20	82	63	91	56	50	69

[a] A, barn cured; B, chamber cured 24 °C, 70% RH; C, chamber cured 24 °C, 70% RH, incandescent light. [b] Not detected.

After 90 days there were no differences in the concentrations of I and II between tobacco cured at ambient conditions and in a controlled environment in the presence of light. At the end of curing there were no detectable quantities of either I or II when the tobacco was cured in darkness. This indicates some biosynthesis or retention of I and II occurred during the final stages of senescence in light.

Results for the concentration of lutein (III) and carotene (IV), the two major carotenoid pigments in tobacco, are presented in Table IV. Unlike the previously discussed chloroplastid pigments, III and IV are not as extensively metabolized as I and II. Depending on the curing regime, the decrease of lutein and carotene ranges from 58 to 76% and 67 to 75%, respectively. Therefore, degradation of lutein in burley tobacco is less than that reported for flue-cured tobacco (Court and Hendel, 1984). These differences could be due to differences in curing regimes or genetic characteristics of the tobacco cultivars.

There was no significant change in lutein or carotene concentration during the first week for ambient-cured tobacco. The most significant decrease in these pigments occurred during the second week after harvest. Between the third week and twelfth week there was no statistically significant change in lutein, whereas, there was a significant decrease of carotene concentration in the lamina. These results may, in part, reflect the relative stabilities of these two carotenoids to oxidation.

Tobacco cured in the controlled environments had a significant decrease of III and IV during the first 24 h. The concentration of lutein decreased to the greatest extent in the presence of light. This was opposite to the influence of light observed for carotene, neoxanthin, violaxanthin, chlorophyll a, and chlorophyll b. This indicates that light enhanced the degradation of lutein. During the next 9 days there was a further decrease in lutein concentration. For the tobacco cured in darkness the lutein concentration decreased dramatically after the third day. There is a steady decrease of its concentration until the second week of curing. This indicates, under controlled conditions, the degradation was occurring during the first two weeks after harvest. This was also true for the carotene levels in the lamina.

Carotene concentrations for the tobacco cured in the absence of light were different from those of the other two curing regimes, at least during the first week after harvest. The concentrations of IV in tobacco cured at ambient conditions and in the presence of light paralleled each other during the first 7 days. Carotene concentrations decreased less rapidly in tobacco cured at ambient conditions. At the last sampling date there was no statistical difference in the concentrations of carotene in samples obtained from the three different curing regimes. This shows curing regimes had no influence on the final concentrations of carotene even though there were differences in rates of carotene degradation during the earlier stages of curing.

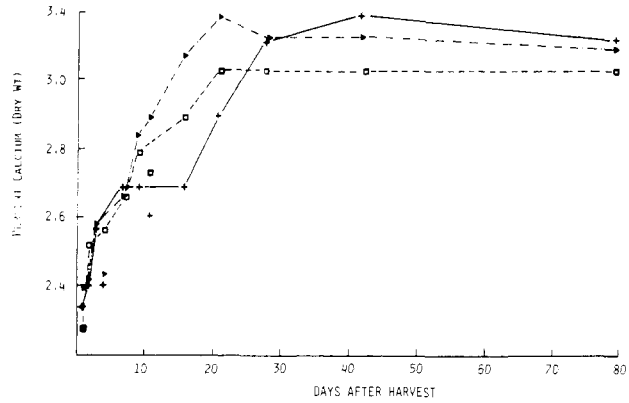


Figure 1. Changes in calcium content during air-curing of burley tobacco: (+) barn cured; (Δ) 24 °C/70% RH; (□) 24 °C/70% RH.

Another facet of curing is the total weight losses of the lamina due to respiration during the curing process. The change of weight due to respiration may give inflated values if concentration of plant constituents are reported on a per gram basis and they are not adjusted for respiration losses. Since there is no convenient means for direct measurement of these weight losses, an indirect method was employed. This involved determination of calcium concentration in the lamina. It is assumed that there is little translocation of calcium from the lamina, since it is in the insoluble form in the lamina, and would not be expected to translocate easily to the midrib or stalk of the plant. Increases in calcium content will reflect the dry matter decrease due to respiration.

Calcium content changes in the lamina during curing are presented in Figure 1. During the first week after harvest there was little difference between the respiration losses as determined by calcium levels of the tobacco cured under the three curing regimes. After this time there was a divergence of respiration losses among the curing treatments as indicated by the calcium concentration of the

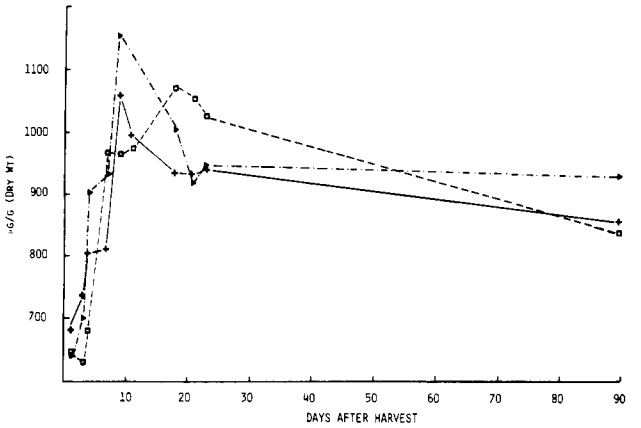


Figure 2. Neophytadiene changes during air-curing of burley tobacco: (+) Barn cured; (Δ) 24 °C/70% RH; (□) 24 °C/70% RH.

lamina. Respiration losses were retarded in samples cured at ambient conditions and at 24 °C in the presence of light. However, the samples cured at 24 °C in the absence of light maintained higher respiration losses. Respiration ceased by day 21. This is approximately the time of maximum moisture loss from the lamina (Table I). Respiration losses for tobacco cured at ambient conditions did not cease until approximately 4 weeks after harvest. This, again, reflects the influence of temperature on the curing process. It should be noted that even though respiration rates are different among the three curing regimes, there is no statistical difference between the final calcium concentration in cured lamina of the different curing environments.

Degradation of the plastid pigments in the lamina and weight loss due to metabolic respiration have been discussed in this paper with little emphasis placed on the metabolic products. One of the major degradation products found in tobacco is neophytadiene. This diterpene has been proposed to arise from the dehydration of phytol (Enzell et al., 1977; Amin, 1979). Degradation of the chlorophylls would lead to scission of the ester to yield phytol which would, in turn, undergo dehydration to form neophytadiene. Therefore, it is assumed neophytadiene is a metabolite of the chlorophylls.

Neophytadiene concentrations increase significantly during the first 10 days after harvest (Figure 2). Only 20% of this increase can be attributed to respiration losses. As neophytadiene increases during the first 10 days there is a large decrease in the chloorophyll concentrations of the lamina during the same time span. This observation suggests that chlorophylls can contribute to the neophytadiene concentration in the cured lamina. Curing environments had little influence on the formation of this diterpene. The maximum accumulation for neophytadiene was delayed for nine days in tobacco cured in light compared to the other two curing regimes. However, at the end of the experiment the concentration of neophytadiene from the tobacco exposed to light was lowest of the three curing regimes.

In summary, degradation of the lamina plastid pigments during senescence and air-curing of burley tobacco is a slow process for the lamina from the upper one-third of the plant. During senescence photosynthetic pigments decrease from 30 to 70%, depending on the individual pigments. During curing, for samples taken from the top third of the plant, 18 days were required for cessaton of plastid pigment degradation. Curing environment has been shown to have an influence on the rate of degradation of the plant pigments and indirectly measured respiration losses. The largest overall decreases of neoxanthin, volaxanthin, chlorophyll a, and chlorophyll b occurred when tobacco was cured in the dark. It is possible that temperature has an influence on the rate of degradation of these pigments during air-curing. Studies designed to provide more information on the influence of temperature on the degradation of tobacco pigments and to determine the changes of volatile tobacco constituents during curing are in progress.

Registry No. I, 14660-91-4; II, 126-29-4; III, 127-40-2; IV, 7235-40-7; chlorophyll b, 519-62-0; chlorophyll a, 479-61-8.

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Received for review January 24, 1985. Accepted May 13, 1985. This article is published with the approval of the Director of the Kentucky Agricultural Experiment Station as paper 84-3-244. The authors acknowledge financial support of the U.S. Department of Agriculture.