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Received for review March 16, 1978. Accepted July 12, 1978. Reference to a company or product name does not imply approval or recommendation by the USDA.

High-Speed, Low-Pressure Liquid Chromatography of Chloroplast Pigments from Tobacco Mutants

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A chromatographic method was developed for the separation and quantification of chloroplast pigments from tobacco (*Nicotiana tabacum* L.) leaves. The apparatus consisted of a low-pressure pumping system, a sealed silica gel column, and a recording spectrophotometer. Pigments were eluted with a stepped gradient solvent mixture containing heptane, diethyl ether, and acetone. The rapidity and accuracy of the technique provided a convenient means for individually measuring and collecting each of the chlorophyll and carotenoid components in leaf extracts from selected yellow mutant and green tobacco genotypes. The pattern of pheophytin a distribution in leaves harvested from different levels on the stalk showed no correlation with chlorophyll a content. It is suggested that pheophytin a is not necessarily an extraction artifact but could have a physiological role in photosynthesis.

Published methods for column chromatography of chlorophylls and carotenoids are tedious and time-consuming (Strain et al., 1971). They are difficult to replicate and require collection of fractions for quantification. As a consequence, paper and thin-layer methods are commonly used for rapid comparative analyses and column methods are used only for larger scale separations of individual pigments (Holden, 1965; Sěstâk, 1971). Weybrew (1957) reported that plastid pigments extracted from tobacco leaf would not separate on a conventional starch column, presumably because of the "poisoning" effect of nicotine. Whitfield and Rowan (1974) employed thin-layer chromatography to follow the decline of chlorophylls and carotenoids during senescence of tobacco leaves. Their work showed that the rate of chlorophyll loss equalled the rate of carotenoid loss in lower leaves, whereas chlorophyll disappeared faster in upper leaves. Some chlorophyll mutants have low levels of chlorophyll and appear yellowish throughout development; according to Benedict (1972) mutants of this type are widely distributed among crop plants. The existence of yellow and variegated mutants has stimulated research with regard to their mode of inheritance (Burk and Menser, 1964), chloroplast structure (Schmid and Gaffron, 1969; Shumway and Kleinhofs, 1973), and photosynthetic behavior (Highkin et al., 1969; Schmid, 1971). However, except for chlorophyll a/b ratios, which are generally higher in the mutants, little information is available on the range of total pigment levels in green and yellow genotypes. These differences could be important because, for example, the *Su/su* tobacco mutant examined by Homann and Schmid (1967) demonstrated high Hill rates (O_2 evolution) and superior photosynthetic capabilities. Boardman (1971) considered the C3 yellow mutants analogous to C4 type plants in terms of their photosynthetic efficiency.

In a previous paper we attempted to correlate growth vigor with the chloroplast properties of a normal green and a pale-yellow (*Py*) mutant of tobacco (De Jong and Woodlief, 1974). We have since added two genotypes to the study and have now examined the concentrations of chloroplast pigments in all four tobaccos. During preliminary efforts to develop a satisfactory methodology, we became frustrated with published methods of column chromatography. No fundamental improvement in the method of Perkins and Roberts (1962) has appeared in the literature and their technique was totally unsuited for rapid surveying of chlorophylls and carotenoids in comparative samples. After experimenting with a number of different solid phase absorbents and eluting solvents, we succeeded in assembling a reliable and reusable high-speed, low-pressure chromatographic system that totally separated all major leaf pigments during a run of 20-min duration. The eluate stream was monitored by passing it through a recording spectrophotometer. Fractionation of chloroplast pigments graphically displayed on the chromatogram was directly quantified from peak heights. In addition to conducting a comparative survey of pigment levels in leaves from four diverse tobacco genotypes harvested from three different portions of the stalk, we also examined the occurrence of pheophytin in the leaf extracts.

MATERIALS AND METHODS

Plant Types and Growth Conditions. Tobacco plants (*Nicotiana tabacum* L.) of four genotypes were grown in controlled-environment chambers (Sherer-Gillett CEL

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Chart I

step	composition	concn	amount, mL
1. washout	acetone	100%	200
2. reequilibrate	(a) H:E:A	(60/20/20)	60
	(b) H:E:A	(60/20/15)	95
3. load sample	heptane solution		1
4. develop	(a) H:E:A	(60/20/15)	95
	(b) H:E:A	(60/20/20)	95
	(c) H:E:A	(60/20/60)	95
	(d) H:E:A	(60/20/120)	60
	(e) acetone	100%	90

37-14). Nutrient-enriched water was supplied individually to each plant by means of an automatic watering system (De Jong and Woodlief, 1974). Growth conditions included day/night temperatures of 25/20 °C and an 8-h photoperiod with 18000-lux light intensity. The genotypes utilized were a commercial cultivar, NC-95; a high nicotine breeding line, SC-58; and two chlorophyll mutants, a Pale Yellow hybrid (NC-95 × T.I. 1372) and a Yellow Green hybrid (SC-58 × Consolation yg/yg). All genotypes have been previously characterized (Nolla, 1934; Chaplin, 1969; Gwynn et al., 1970).

Extraction. Routinely, 2.5 g (fresh weight) of deveined leaf lamina was combined with 50 mL of 100% acetone containing 20 mg of $MgCO_3$. The leaf tissue was vigorously pulverized with a Virtis No. 60 homogenizer for 1 min. The homogenate was filtered by passing through Miracloth and rinsing with acetone until all color was removed from the debris. The extract was adjusted to 100 mL, and suspended solids, primarily starch, were removed by centrifuging at 2500*g* for 5 min to clarify the green pigment solution. Extraction operations were carried out in a cold room at 5 °C under a dim green safelight.

Concentration. The preparation was concentrated by evaporating to near dryness over a hot water bath. When the smell of acetone disappeared, the remaining light-brown aqueous fluid was decanted to remove water-soluble impurities. The pigmented green film adhering to the surface of the evaporating dish was rinsed with 5–10 mL of deionized water and allowed to air-dry for a few minutes. After redissolving in 5 mL of heptane, the preparation was stored in a darkened cold room until analyzed. The pigment solution was stable under these conditions for several days.

Chromatographic Analyses. The chromatographic assembly consisted essentially of an EM No. 60-B prepacked silica gel column (trade name Lobar) available from E. Merck Laboratories, Inc., and a Fluid Metering Inc. proportionating pump (Model RPD-D) connected together with appropriate solvent-resistant tubing. A 250-mL separatory funnel was used as a reservior on the intake side and the eluate was monitored at 425 nm on the outlet side with a recording Gilford 240 Spectrophotometer equipped with a 10-mm IEC zonal flow cell. A Gilson Escargot volumetric collector was utilized to collect 5-mL fractions. The flow rate was adjusted to 20 mL/min with the vernier knob on the FMI pump. At this rate, a pressure of about 50 psi was developed. Approximately 30 min was required for each run, including wash-out, reequilibration, and sample application. A 1-mL aliquot of pigment concentrate was applied to the column by means of a sample injection loop. The chromatograph was developed with a three-component solvent mixture of heptane, diethyl ether, and acetone (HEA), changed at unequal intervals in a five-step gradient. The sequence shown in Chart I was used for the complete cycle. The proportion of diethyl ether was held constant at all stages in order to keep heptane and acetone freely miscible. Although the 425-nm monitor setting was not the optimal wavelength for any particular pigment, it represented a good compromise among the different Soret absorbance patterns.

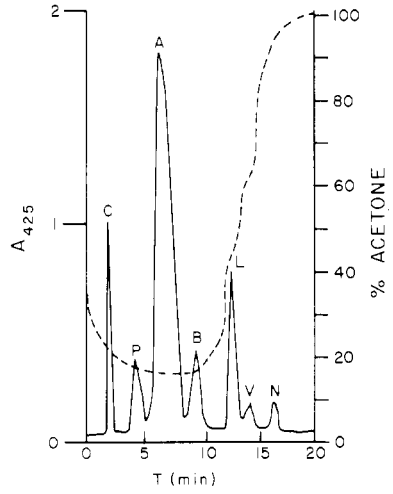

Figure 1. Representative chromatogram of chloroplast pigments extracted from tobacco leaves and eluted through a silica gel column with a stepped gradient consisting of heptane–diethyl ether–acetone. The dashed line trace shows the change of acetone concentration as determined by monitoring eluant stream at A_{320} without sample. Sample from pale yellow tobacco: (C) β–carotene, (P) pheophytin, (A) chlorophyll a, (B) chlorophyll b, (L) lutein, (V) violaxanthin, (N) neoxanthin.

Identification and Calibration. The collected 5-mL fractions were pooled for each separated component and diluted to 50 or 100 mL depending upon the concentration. These solutions were scanned spectrophotometrically and the absorbance spectra of the pigments used for identification. The recorder tracing was calibrated and the peaks quantified on a standardized chromatogram by correlating the known concentration as derived from values in the literature with each eluted peak. Equations were developed to calculate pigment concentrations on the basis of leaf dry weight and leaf area with appropriate correction factors.

Fluorescence Measurements. The fluorescence yields of chlorophyll a and pheophytin a were measured with a Turner Model 111 Filter Fluorometer equipped with a blue bulb and a photomultiplier sensitive in the red region of the spectrum. Filters recommended by the manufacturer were used for the measurement of pheophytin a or chlorophyll a fluorescence singly or in mixtures. A narrow pass (NP-425 nm) primary filter was placed on the excitation side with a sharp cut-off secondary filter (SC-687 nm) on the emission side. In addition, for some of the experiments, an accessory SC-415 nm filter was inserted on the excitation side to exclude wavelengths in the blue region of the spectrum so as to prevent excitation of pheophytin fluorescence. Pure solutions of chlorophyll a and pheophytin were diluted to approximately the same concentrations with acetone, and their fluorescence yields singly and in mixed combinations were measured.

RESULTS

Pigment Separation and Identification. Seven colored fractions were separated and recovered from all tobacco leaf samples. Their order of passage through the column agreed with their known polarities and elution solvent strength (Strain et al., 1971). The first pigment to elute from the column was β-carotene, followed in order by pheophytin a, chlorophyll a, chlorophyll b, lutein,

Table I. Values Used for Identifying and Quantifying Chloroplast Pigments in Tobacco Leaves

	absorption peak for identification and calibration, nm	correction factor for 425-nm reading in μg/OD	coeff. of variation
β-carotene	453	47.57	0.045
pheophytin	409	338.52	0.056
chlorophyll a	662	307.70	0.091
chlorophyll b	644	809.72	0.066
lutein	446	93.86	0.062
violaxanthin	442	115.33	0.576
neoxanthin	438	175.80	0.341

violaxanthin, and neoxanthin (Figure 1). Each compound was identified by collecting the fraction, scanning the visible wavelength with a spectrophotometer, and comparing the recorded spectra with published values (Davies, 1965; Holden, 1965). The technique of increasing solvent strength by sequential increments, rather than gradually, was effective in achieving complete separation of the major pigments with no indication of baseline drift and with minimal gap between each component. Isocratic elution with any combination of solvents we tested was not satisfactory. Changing the solvent system in five successive steps was particularly important for getting a clean separation between chlorophylls a and b and for resolving the lutein and violaxanthin peaks.

During our early efforts to develop a suitable chromatographic system, we noticed three bands between the β-carotene and chlorophyll a bands, all of which were spectrally identical and subsequently indentified as pheophytin a. The problem of triple pheophytin peaks disappeared when heptane was substituted for chloroform (Davies, 1965); however, we found that the amount of pheophytin relative to chlorophyll a could be further reduced by adding $MgCO_3$ to the homogenization media. Nevertheless, increasing $MgCO_3$ beyond 10 mg/g of leaf tissue never completely eliminated pheophytin. Instead, the amount of pheophytin always varied with the particular tobacco genotype and stage of leaf maturity. Under these conditions pheophytin levels showed no correlation with chlorophyll content.

Chromatogram Calibration. Due to the stable baseline and peak symmetry, calculation of pigment content by measurement of peak heights proved to be sufficiently accurate for our purposes. According to Baumann (1972), peak height is often more reliable than integrating the area of the peak if an electronic integrator is not available. After the basic elution sequence was established, all operations were standardized and each pigment collected manually and in toto as it exited the column. Depending upon the amount of pigment present, each fraction was diluted to 50 or 100 mL with eluting solvent. The optical densities of the pigment solutions were measured at two wavelengths; viz. at 425 nm and at the specific wavelength characteristically used to quantify a particular pigment (eg., at 453 nm for β-carotene). Published extinction coefficients were used to calculate the concentration of each pigment fraction and the relationship of peak height at 425 nm to pigment concentration was established. Dry weight/fresh weight ratios were obtained for every leaf sample to be extracted by weighing, drying, and reweighing representative leaf disks. A mathematical expression was developed from these data for calibrating the peak height readings for all samples analyzed. The equation relating peak heights at 425 nm to pigment concentration was in the following form: $C = (A \times f \times S)/(r \times L \times E \times 1000)$ where C = mg/g of dry weight or cm^2, A = absorbancy at 425 nm in OD units, f = correction factor in μg/OD for each pigment, S = sample size in milliliters after concentration, r = dry weight/fresh weight ratio or area in cm^2/fresh weight ratio, L = starting leaf tissue weight in g, and E = heptane extract in milliliters applied to column.

Specific absorbance maxima used for identification of each pigment as well as the correction factors and coefficients of variation are given in Table I. The 425-nm absorption band was linear for each pigment over a wide range of concentration. β-Carotene was used as the internal standard because it was stable and eluted quickly as a sharp symmetrical peak. The relatively high variability associated with violaxanthin was probably due to its low concentration and its inherent instability, i.e., desepoxidation to form zeaxanthin (Yamamoto and Takeguchi, 1972) which probably comigrated with lutein in our system.

Comparative Survey. For comparison of the four genotypes, leaves were harvested from the fourth, eighth, and twelvth nodes of plants after floral maturity but before the onset of senescence. This sampling arrangement provided a good representative selection of tobacco leaves in lower, middle, and upper stalk positions. Stalk position is known to have an effect on many chemical constituents in cured tobacco (Tso, 1972). Pigment concentrations

Table II. Chlorophyll and Carotenoid Content[a] in Tobacco Leaves as a Function of Variety and Leaf Position on the Stalk [Leaf Samples Taken from Fourth, Eighth, and Twelfth Nodes at Full Plant Maturity (2 Weeks after Removal of Flower Heads)]

	β-carotene	pheophytin	chl a	chl b	lutein	violaxanthin	neoxanthin
lower							
NC-95	0.345	1.030	3.381	1.459	0.705	0.091	0.170
PY	0.152	0.229	1.410	0.586	0.332	0.028	0.057
SC-58	0.238	0.347	2.735	1.102	0.526	0.079	0.121
YG	0.277	0.343	2.937	1.224	0.530	0.081	0.121
middle							
NC-95	0.315	0.219	3.968	1.291	0.619	0.154	0.169
PY	0.262	0.152	3.065	0.895	0.586	0.133	0.130
SC-58	0.247	0.137	3.152	1.046	0.588	0.183	0.133
YG	0.285	0.195	3.053	0.910	0.536	0.082	0.097
upper							
NC-95	0.519	0.424	6.442	1.730	1.049	0.236	0.280
PY	0.315	0.160	3.692	0.990	0.620	0.205	0.143
SC-58	0.394	0.221	5.020	1.419	0.862	0.233	0.234
YG	0.303	0.196	3.215	0.798	0.510	0.130	0.118

[a] Milligram pigment/gram of dry weight.

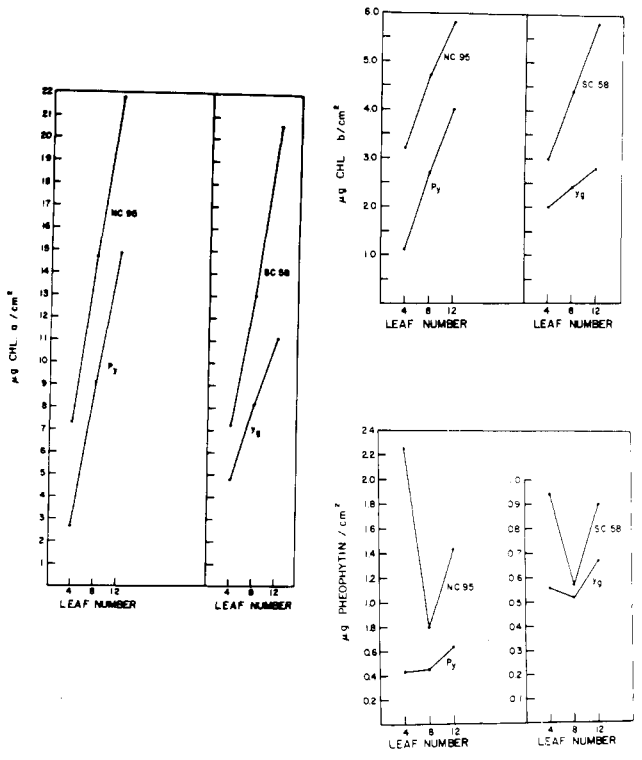

Figure 2. Chlorophyll concentration in tobacco leaves as a function of tobacco genotype and leaf position on the stalk. Numbers 4, 8, and 12 correspond to samples from low, middle, and upper leaves, respectively. Differences in individual pigment levels calculated from chromatograms on basis of leaf area.

expressed on a dry weight basis are given in Table II. The lower the position on the stalk, the less pigment was detected in most instances. However, some interesting exceptions were noted. Pheophytin, for example, was lowest in the middle leaves but significantly highest in lowermost leaves from the two green genotypes. On a dry weight basis, chlorophyll b in *Yg* tobacco increased whereas chlorophyll b in *Py* tobacco decreased with leaf age.

Plotted on a leaf area basis, concentrations of the two chlorophylls and β-carotene showed a linear relationship with leaf age regardless of genotype (Figures 2 and 3). Slight deviations from linearity were observed for lutein and the two lesser xanthophylls (Figure 3). The maturity gradient in chlorophyll a and b content was less steep for *Yg* tobacco, indicating greater uniformity in physiological age of the leaves. By far the most remarkable pigment pattern was that of pheophytin (Figure 2). The lack of correlation between chlorophyll a and pheophytin levels implied that pheophytin content in the extracts was not a result of chlorophyll degradation during sample preparation. Although pheophytin could conceivably result from chlorophyll breakdown during senescence, one would then expect pheophytin content in middle leaves to be intermediate between that for upper and lower leaves. Because of these puzzling anomalies, the behavior of pheophytin in tobacco leaf extracts was examined in more detail.

Occurrence of Pheophytin. The appearance of three pheophytin peaks under certain conditions of extraction indicated that the acid-catalyzed conversion of chlorophyll a to pheophytin a occurred by more than a single step. The data suggested that intermediates are formed which differ with respect to either the amount of magnesium bound or the manner in which the magnesium is held—perhaps the nature of the ligand complex. This interpretation is also supported by evidence that magnesium is more loosely bound to chlorophyll in *Py* preparations since the degree of conversion of chlorophyll a to pheophytin a was almost total in unprotected *Py* extracts compared to identically handled NC-95 extracts where the degree of conversion was considerably less. When pheophytin a fractions from the two plants were collected and scanned spectrally, the most obvious features in the *Py* spectrum were: (1) the existence of three fairly strong absorbance bands in the green region of the spectrum, viz., at 508, 535, and 610 nm and (2) the red peak is shifted upward from 662 nm to 676 nm (Figure 4). At first it appeared that a high pheophytin content was characteristic of the *Py* genotype, but later work showed that excessive pheophytin formation could be prevented by adhering to a rigorous extracting protocol. Nevertheless, pheophytin was never entirely absent. Pheophytin recovered under our standard operating conditions exhibited a Soret peak at 410 nm but the 662 nm peak was identical with the red absorbance band of chlorophyll a.

Artificial Generation of Pheophytin. To investigate the nature of chlorophyll conversion to pheophytin, we acidified purified chlorophyll a solutions with oxalic acid to displace magnesium. Figure 5 shows the shift in absorbance maxima that occurs at progressively greater concentrations of oxalic acid. An acid excess of at least 1000 times the concentration of chlorophyll a was required to achieve total conversion. The principal spectral changes were that the red band at 662 shifted upward to 676 nm and the Soret maximum at 435 nm shifted downward to 410 nm. Absorbancy in the red region was reduced 40% in accompaniment with additional peaks appearing in the green region. These experiments indicated artifically generated and naturally occurring pheophytin could reliably be distinguished by the position and shape of the red band. The peak appears rounded at 662 nm if naturally occurring but sharp-pointed at 676 nm with a shoulder at 662 nm if artifically generated.

Fluorescence Yields of Chlorophyll a and Pheophytin a in Varying Mixtures. If pheophytin occurs naturally (that is in vivo), it might either be a nonfunctional degradation product or alternatively have functional significance. We measured fluorescence yields of mixtures of chlorophyll a and pheophytin a to find out whether or not they reacted photochemically. As shown in Figure 6, the fluorescence yield of chlorophyll a is normally greater than that of pheophytin when each is measured separately. This becomes especially noticeable when a sharp cutoff filter is used in the optical pathway to screen out some of the Soret absorbancy of pheophytin. Mixtures of chlorophyll a and pheophytin excited with the SC-415 nm cutoff filter yielded fluorescence values that were less than expected from combining the yields of the two pigments analyzed separately. Moreover, the yields of mixtures excited without the filter were reduced to an even greater extent particularly at the higher pheophytin concentrations (lower end of the curve). These results indicated mutual quenching of chlorophyll and pheophytin fluorescence and demonstrated that the two pigments interact photochemically. The evidence further suggests that pheophytin absorbs light at wavelengths inaccessible to chlorophyll a and transfers the absorbed energy to chlorophyll a.

DISCUSSION

The chromatographic apparatus and technology described in this paper are a substantial advancement over methods based upon the Perkins-Roberts procedure. In spite of some obvious shortcomings, the latter procedure is the standard column method for leaf pigment separation described in current methodology texts (Strain et al., 1971;

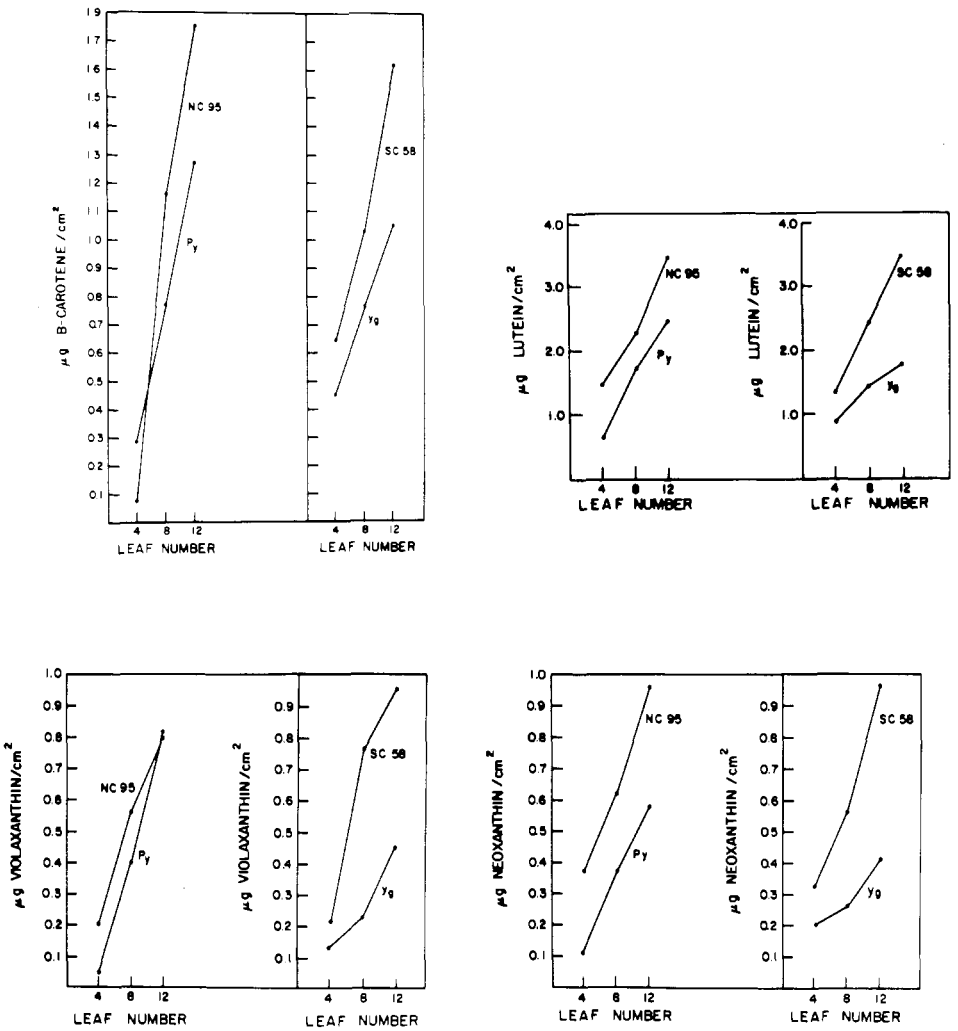

Figure 3. Carotenoid concentration in tobacco leaves as a function of tobacco genotype and leaf position on the stalk. Numbers 4, 8, and 12 correspond to samples from low, middle, and upper leaves, respectively. Differences in individual pigment levels calculated from chromatograms on basis of leaf area.

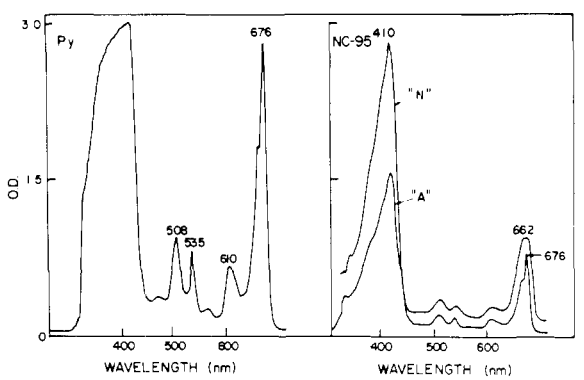

Figure 4. Spectral scans of purified pheophytin isolated chromatographically from Pale Yellow and NC-95 leaves following extraction without $MgCO_3$ and redissolving in chloroform. The spectrum of NC-95 pheophytin extracted in the presence of $MgCO_3$ (labeled "N") is also shown for comparison. See text for details. "A" = artificial; "N" = natural.

Sěsták, 1971). It has been our experience that even updated modifications of that method are quite unsatisfactory for tobacco samples. Recently, Iriyama et al. (1974) reported an innovation in chlorophyll isolation that prescribed selective aggregation of chlorophyll a and b with dioxane to separate these pigments from the carotenoids. Fractionation of the chlorophyll components was then accomplished by the laborious gravity flow method of Perkins and Roberts (1962), which utilizes powdered sugar with 3% starch as solid support. The chromatogram in their method is developed with petroleum ether containing 0.5% isopropyl alcohol. The high-speed–low-pressure liquid chromatographic method presented here totally resolves the seven major pigments in a single run of 20 min. The colum was readily regenerated by washing with acetone and reequilibrating with the starting solvent mixture. By calibrating the chromatographic record, each component was quantified—a decided advantage in conducting a rapid survey of pigment levels in numerous samples.

The two yellow mutants, *Py* and *Yg*, exhibited distinctly different chlorophyll profiles. In the upper leaf position, chlorophyll was highest in the *Py* sample, but in the lower leaf position chlorophyll was highest in the *Yg* sample. The shapes of the maturity gradients in NC-95 and *Py* were identical, although at each leaf node *Py* samples contained about 40% less chlorophyll. The shallow maturity gradient in *Yg* is clearly related to the virescent character (lagging chlorophyll synthesis) of the genotype. The chlorophyll b and lutein profiles were similar for the *Yg* type but not the other three types. The existence of extremely viable yellow mutants like *Py* and *Yg* tobaccos suggests that green types are either very inefficient in light capture or contain excessive amounts of chlorophyll for accidental or unknown reasons. As early as 1918, Willstatter and Stohl concluded that there was no correlation

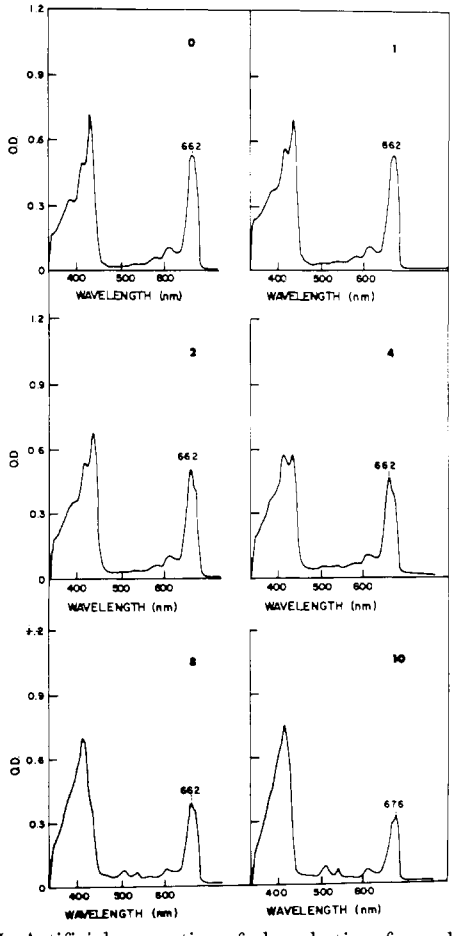

Figure 5. Artificial generation of pheophytin a from chlorophyll a with oxalic acid. Chlorophyll concentration was at 5 μM and oxalic acid was added at 1, 2, 4, 8, and 10 mM. Note progressive shifting of red band from 662 to 676 nm.

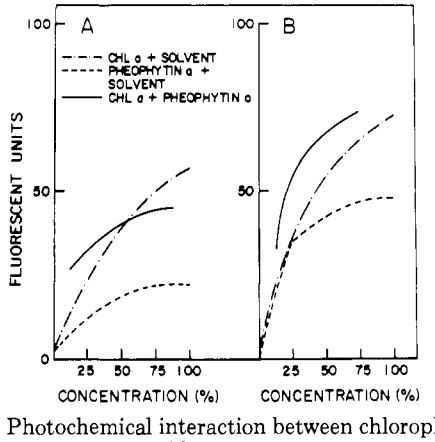

Figure 6. Photochemical interaction between chlorophyll a and pheophytin a as determined by fluorescence measurements: (A) with Sharp-cutoff filter (415 nm) interposed on excitation side to exclude major contribution from pheophytin; (B) without SC filter. Chlorophyll a and pheophytin a solutions of approximately equal concentration (5 μM = 100%) were diluted back with solvent to obtain yield curves. In chlorophyll–pheophytin mixtures, the percent values refer to content of chlorophyll a.

between photosynthetic rates in yellow varieties and the amount of chlorophyll in these plants (Wild, 1969). Even with green genotypes, pathologically chlorotic leaves as well as naturally senescent leaves often exhibit superior photosynthesis (Zaitseva, 1970; De Jong, 1974). Schmid and Gaffron (1969) postulated that photosynthetic efficiency (on a chlorophyll basis) is related to photosynthetic unit size; i.e., the smaller the unit the more light is needed to saturate it. Recent experiments in our laboratory (unpublished results) indicated that, while this explanation might hold true for the *Yg* genotype, it does not apply to *Py* plants. It would appear that the lower pigment content in certain chlorophyll deficient plants is offset by other factors relating to chloroplast structure and function.

One unanticipated outcome of the present work concerns the matter of pheophytin occurrence. The crucial issue is whether or not it is an artifact of extraction. Strain and Katz (1969) indicated that under carefully controlled conditions they were unable to detect its presence. However, we have shown: (1) that pheophytin content depends upon the stage of leaf development and (2) that pheophytin generated by oxalic acid catalysis is spectrally distinguishable from "natural" pheophytin. The facile conversion of chlorophyll a to pheophytin in unprotected *Py* extracts deserves further consideration. Sap expressed from *Py* leaves has a pH of about 6.3, only two-tenths units lower than the leaf sap of NC-95 (pH 6.5), hardly enough difference to have such a profound effect. One clue might be found in the work of Shlyk et al. (1969) who reported that chloroplast material most active in carrying out chlorophyll biosynthesis of chlorophyll is also apt to be the site of greatest pheophytinization. Alternatively, the fragile structure of *Py* chloroplasts (see De Jong and Woodlief, 1974) might allow the chlorophyll to be more susceptible to loss of magnesium. Even so, the suppression of pheophytin formation in *Py* by $MgCO_3$ addition during homogenization argues strongly for the effectiveness of this treatment. In our view, pheophytin formation may not always or necessarily result from detrimental extraction conditions. Confirmation for this position comes from the work of Hedin et al. (1976) who found pheophytin in leaf extracts prepared under weakly alkaline conditions. From this they concluded that pheophytin was already present in the leaf before disintegration. Quinlan (1968) investigated the red shift from 662 to 672 nm associated with the formation of colloidal chlorophyll a. According to Katz (1972), adjacent chlorophyll molecules couple by linkage of magnesium and the C-9 carbonyl. As the oligomer weight increased, the 677-nm band intensity increased and the 663-nm band correspondingly decreased. Therefore, the loss of Mg^{2+} from the pheophytin molecule accounts for the red shift that occurs during the conversion of chlorophyll a to pheophytin.

The natural existence of forms of chlorophyll other than a and b has been proposed by Michel-Wolwertz and Sironval (1965). It is also implicit in the data of Leicknam et al. (1975), who detected four chlorophyll a species with differing electron-accepting reactivities and differing states of bonding with magnesium. Because of these differences the degree of proximity of magnesium to the tetrapyrollic plane can be variable. It has been reported that the spillover of excitons between the two photosystems is regulated by cations such as Mg^{2+} (Gross et al. 1975). Although this effect has been variously interpreted, it appears that magnesium levels have a role in controlling the conformational relationships between photosystems I and II as well as determining the relative proportions of chlorophyll and pheophytin. According to Jones (1973), claims for the detection of special forms of chlorophyll must be viewed with caution, but the pheophytin spectra presented in his paper unmistakably show the red shift from 662 to 676 nm.

Several recent reports have suggested that pheophytin has a biological function. In this connection, Netzel et al. (1973) found that excitation of bacteriopheophytin with green light at 535 nm promoted photobleaching of bac-

teriochlorophyll monitored at 865 nm in reaction center preparations. More recent work has provided evidence that bacteriopheophytin links electron transfer between the plastocyanin and iron quinone components of the redox chain (Thornber et al., 1978). While studying redox reactions in higher plants with subchloroplast preparations treated with deoxycholate, Van Gorkom (1975) observed a band shift from 536 to 555 nm, in addition to the red band shift upward from 672 to 685 nm, which he attributed to oxidation of a bound or aggregated form of pheophytin a. The absence of a magnesium atom from the center of chlorophyll derivatives renders the molecule more resistant to photooxidation (Evstigneev, 1969). Similarly, oxygen quenches the fluorescence of pheophytin more effectively than that of chlorophyll (Krasnovsky, 1969). While investigating the action spectra of photosynthesis, Inada (1976) detected lower photoactivity in the ultraviolet and blue whenever there was higher absorbance in the green band. He reported that if A_{560} was higher than 0.60 OD only one peak rather than two was seen in the blue region of the spectrum. One interesting possibility is that pheophytin functions in higher plants as an accessory pigment. Its higher concentration in leaves in the lower portion of tobacco plants (Table II) suggests an analogy with the accessory pigments of marine algae. The water soluble phycobilins absorb substantial quantities of light in the "green window" for algae growing in the ocean at depths where light penetration is poor (Gantt, 1975). The phycobilins comprise the major portion of photosystem II in these marine organisms. Evidence that pheophytin increases in response to shading was obtained as we were finishing these experiments. While conducting a study of the effect of light intensity on pigment concentration in NC-95 plants growing in controlled environment chambers, we found that the leaf content of pheophytin from plants grown under 130 μeinsteins m^{-2} s^{-1} of white light was double that in comparable leaves from plants grown under 195 μeinsteins m^{-2} s^{-1}. There were no corresponding differences in chlorophyll a levels. If pheophytin extends the light-harvesting capabilities of partially shaded leaves into the green part of the spectrum, it might also explain the observation that only about 10% of the incident green radiation eventually penetrates into the lowermost portion of the leaf canopy (Saeki, 1975), even though green light is poorly absorbed by either chlorophyll a or b. If pheophytin has a role in photosynthesis, one would expect it to be particularly important for plants that are capable of existing in shaded habitats. To our knowledge this possibility has never been systematically studied. One of the problems has been lack of a rapid system of pigment analyses and the assumption that pheophytin is invariably a degradation product. Even the high-pressure liquid chromatography method recently described by Eskins et al. (1977) required an elution time of 4 h to develop the chromatogram compared to 20 min for the method reported here. The fractionation system discussed in this paper should be useful for rapid comparative analyses as well as for preparative-scale separations of the chloroplast pigments.

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Received for review April 11, 1978. Accepted June 26, 1978. Paper No. 5374 of the Journal Series of the North Carolina Agricultural Experiment Station, Raleigh, N.C. Cooperative investigations of the U.S. Department of Agriculture and North Carolina Agricultural Experiment Station. Mention of a trademark or proprietary product 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 that may also be suitable.

Compounds New to Essential Orange Oil from Fruit Treated with Abscission Chemicals

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The compositions of volatile constituents in essential oils from control and chemically treated Hamlin, Pineapple, and Valencia oranges were compared. The chemicals were abscission agents used to loosen fruit for mechanical harvesting. Six phenolic ethers—eugenol, methyleugenol, *cis*-methylisoeugenol, *trans*-methylisoeugenol, elemicin, and isoelemicin—were isolated and identified as citrus constituents for the first time, but only from the chemically treated fruit. Threshold levels in orange juice were determined for four of these ethers. The concentrations of all six compounds in the essential oil and processed juice from oranges were estimated.

The importance of mechanically harvesting citrus fruit has been accentuated in the last 10 years by over a 70% increase in cost of product production, a 25% increase in citrus available for processing, and the difficulty in retaining a stable work force. All promising mechanical harvesting systems include the application of abscission-inducing chemicals to loosen the fruit prior to harvesting.

Cooper et al. (1969) were first to report the use of cycloheximide (Acti Aid) as an effective abscission agent for citrus. However, cycloheximide was not suitable for late season (Valencia) oranges because it induced excessive droppage of the young fruit that were due to ripen the next season (Cooper and Henry, 1973). This problem was overcome by the use of two newer abscission agents: 5-chloro-3-methyl-4-nitro-1*H*-pyrozol (Release) reported by Wilson (1973) and glyoxal diamine (Pik-Off) reported by Wilcox et al. (1974). All three agents damage the peel, and this causes the fruit to release ethylene which promotes abscission. These chemicals have been shown to affect the chemical composition of cold-pressed orange oil and, thus, the flavor quality of processed juice and essential oil (Moshonas et al., 1976; Moshonas and Shaw, 1977). The change in chemical composition of the chemically treated oil could decrease the value of resulting citrus products if flavor changes are pronounced and detrimental. There is, therefore, a clear need to determine and evaluate the compositional changes brought about by the use of abscission-inducing agents. To date there has not been a systematic analysis to isolate and identify specific compounds that may be responsible for the compositional and flavor changes reported in previous studies.

We undertook to analyze essential oils from oranges treated with abscission agents, and now report six compounds not previously reported as citrus oil constituents.

EXPERIMENTAL SECTION

Sample Preparation. Each pair of samples, which included untreated control oranges and oranges treated with one of the listed abscission agents, was harvested from adjacent trees on similar rootstock. The trees had been sprayed, fertilized, and irrigated the same way. Samples were prepared both from oranges that had just reached legal maturity and from well-matured fruit of early- (Hamlin), mid- (Pineapple) and late-season (Valencia) oranges. Oranges were thoroughly washed and then processed with a commercial 5-cup FMC In-Line Extractor. The extracted crude oil emulsions were put through 0.20-in. screen openings and 30- and 60-mesh shaker screens for removal of residual solid materials. Each emulsion was then placed into a holding tank for 4 h, after which most of the water layer was removed. The oil rich emulsion was then separated in a continuous-type centrifuge operated at a relative centrifugal force of about 22 000*g*, which yielded the cold-pressed orange peel oil. The oils were stored at 0 °C until they were analyzed.

Separation Procedure. Each control and experimental orange oil sample (300 mL) was placed in a rotary evaporator and distilled at 36 °C at a pressure of 1–2 mm until most of the terpene hydrocarbons (99% limonene) were removed. A 4-g sample of the 13.3 g of residue was separated into three fractions on a 1 in. × 15 in., 9 °C water-jacketed column containing 100–200 mesh Florisil deactivated with 6% water (Lund and Coleman, 1977).

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