Determination of Tobacco Carotenoids by Resonance Raman Spectroscopy

Gary Forrest* and Gunars Vilcins

Quantitative analysis of β-carotene and lutein in tobacco by resonance Raman spectroscopy reveals significant differences in Raman intensity enhancements for the two carotenoids. As a result the carotenoids must be separated from each other for quantitative analysis. Tobacco extracts and ground tobacco in a $NaNO_3$ matrix have been examined quantitatively using the matrix bands for normalization. The standard deviation of the method is less than 5%. Carotenoid levels in the nanomole/gram range have been measured for tobacco at all stages of processing, including green leaves, freshly cured leaves, aged tobaccos, and processed cigarettes. The greatest decrease in carotenoids is found during the curing process.

The analysis of leaf carotenoids by resonance Raman spectroscopy offers two unique potential advantages over conventional methods. First, the selective enhancement of carotenoid scattering via the resonant absorption of 488.0- or 514.5-nm laser radiation obviates the need to remove other pigments, such as chlorophyll, prior to the analysis. Secondly, the method is potentially capable of analyzing solid materials, eliminating the need for solvent extractions. The quantitative aspects of such carotenoid analysis are examined in detail in this paper. While carotenoids have been detected by resonance Raman spectroscopy in native materials previously, little consideration has been given to mixture of carotenoids containing major components other than β-carotene (Gill et al., 1970; Lutz, 1972; Salares et al., 1977; Rimai et al., 1973; Hoskins and Alexander, 1977). As will be shown here, despite similar extinction coefficients in the visible region of their spectra, β-carotene and lutein have markedly different resonance Raman scattering coefficients. This result has important implications for the measurement of carotenoids in tobacco leaves, in which the principal carotenoids are β-carotene and lutein (Wright et al., 1959).

EXPERIMENTAL SECTION

Resonance Raman spectra were recorded with a Jarrell-Ash 25-500 spectrometer equipped with a Spectra Physics Model 164 argon ion laser. A Hamamatsu R300 (S-4 response) photomultiplier tube was used for signal detection. Typical instrument settings were 10 cm^{-1} slit width, 1 cm^{-1}/s scan speed, 2 s time constant, 2×10^4 photon/s full-scale gain, and 5–30 mW laser power. Excitation with both 488.0- and 514.5-nm laser lines was examined. In general the 514.5-nm line was found to be more suitable for natural leaf materials, which are prone to weak fluorescence under 488.0-nm excitation. To avoid sample decomposition, solutions were rotated at 900 rpm with a sample holder (Figure 1) similar in design to that of Kiefer and Bernstein (1971). Typical sample volumes of 1×10^{-7}–1×10^{-6} mol L^{-1} solutions were 10–100 μL. Solids were examined quantitatively by pressing 5 mg of leaf material into a 200-mg $NaNO_3$ matrix using a standard 1.2-cm KBr die. For qualitative analysis the leaf material could be attached directly to the sample rotator with adhesive. Solid samples were rotated at 2000 rpm.

Visible spectra (Figure 2) were recorded with a Beckman Acta CV spectrometer. For quantitative analysis of ethanol solutions the molar absorptivities used were 1.40×10^5 L/(mol cm) at 453 nm and 1.37×10^5 L/(mol cm) at 445 nm for β-carotene and lutein, respectively (Goodwin, 1976).

Tobacco leaves were harvested and placed immediately on dry ice and subsequently lyophilized. All samples were equilibrated in a 60% relative humidity, 75 °F environment prior to analysis. Samples of 0.1–3.0 g were extracted three times for 2 min with 10 mL of acetone in a stainless steel Sorval blender. The acetone was removed by rotary evaporation at room temperature. Cured tobacco extracts were examined directly or separated on silica gel by thin-layer chromatography with a hexane/ether (3:7) solvent. Isolated β-carotene and lutein bands were eluted into 0.5 mL of acetone or ethanol and 0.1 mL sampled for the resonance Raman spectrum. Green tobacco extracts were saponified for 19 h with 6% KOH in methanol. This step greatly reduced fluorescence background problems encountered in resonance Raman spectra of chlorophyll. The carotenoids were then extracted with ether and separated by either thin-layer chromatography as described above or solvent partitioning between ether and 90% methanol–10% water. The standards were β-carotene, purchased from the Sigma Chemical Co. (St. Louis, MO), and lutein, which was isolated from Gold'n Bloom feed (S. B. Penick and Company, Lyndhurst, NJ) by saponification and extraction.

RESULTS AND DISCUSSION

Resonance Raman spectra of green Bright and Burley tobacco leaves reveal bands identical in frequency to those reported previously for β-carotene (Figure 3) (Gill et al., 1970). Further, the band frequencies observed for β-carotene and lutein are identical because of their similar polyene structures. Only those functional groups involved in the electronic excitation are observed to be enhanced in the resonance Raman spectrum. Thus the hydroxyl groups of lutein are insufficient to allow it to be distinguished from β-carotene.

Standard curves for β-carotene and lutein in ethanol and acetone were prepared by comparison of the normalized resonance Raman scattering intensity to the carotenoid concentration measured by visible absorption spectroscopy (Figures 4–7). Excitation with both 488.0- and 514.5-nm radiation was used. The 884 and 792 cm^{-1} bands of ethanol and acetone, respectively, were used to normalize the carotenoid resonance Raman bands due to C–C (1160 cm^{-1}) and C=C (1530 cm^{-1}) stretching motions. This technique eliminates variations in laser power and sample alignment and allows precise measurements of band intensities to be made. It was found that the sample volumes of 0.01–0.8 mL had little or no effect on the normalized band intensities.

The precision of quantitative analysis of carotenoids by resonance Raman spectroscopy was examined by six

Philip Morris Research Center, Richmond, Virginia 23261.

0021-8561/79/1427-0609$01.00/0

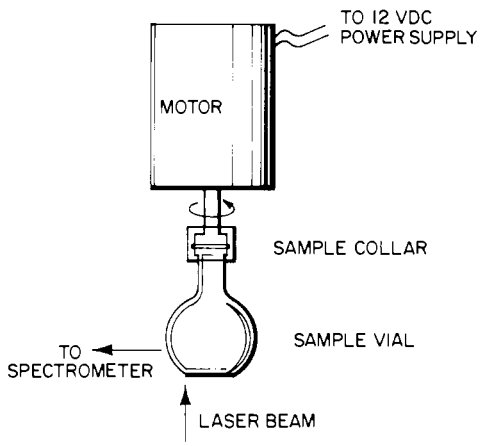

Figure 1. Sample rotator.

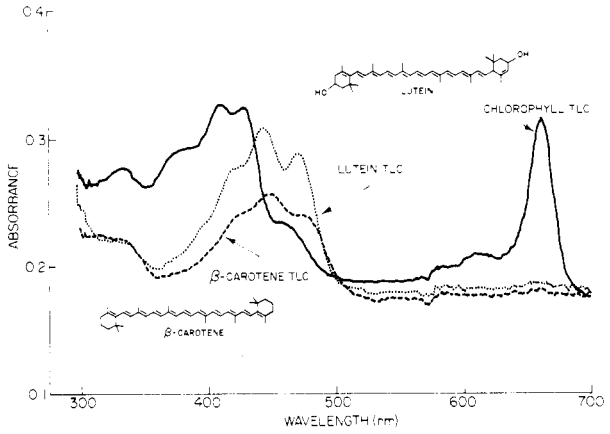

Figure 2. Visible spectra of pigments isolated from green bright tobacco.

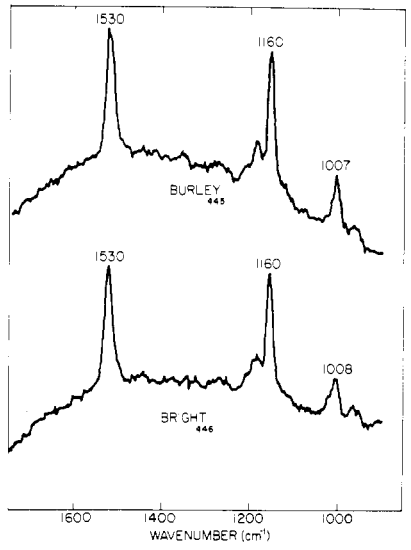

Figure 3. Resonance Raman spectra of green bright and burley leaves.

replicate analyses of samples of β-carotene in ethanol at a concentration of 8.2×10^{-6} mol L^{-1}. The band intensity ratios were $I_{1160}/I_{884} = 1.13 \pm 0.06$ and $I_{1530}/I_{792} = 1.08 \pm 0.07$. Analysis of three separate extracts of cased Bright tobacco separated by thin-layer chromatography gave values for β-carotene and lutein of 11.6 ± 0.4 and 22.3 ± 0.2 nmol/g of tobacco, respectively.

Analysis of solid leaf samples was facilitated by the incorporation of ground samples into a $NaNO_3$ matrix.

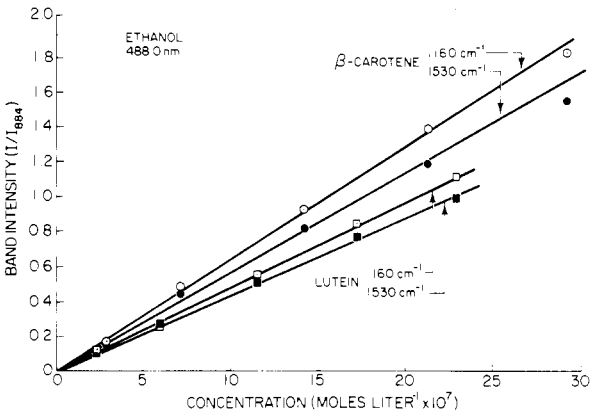

Figure 4. Standard curves in ethanol with 488.0-nm excitation.

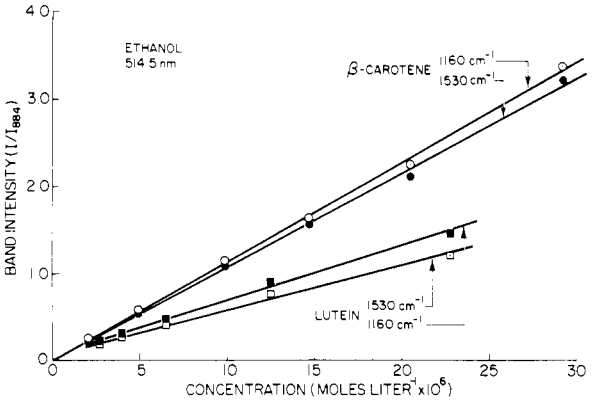

Figure 5. Standard curves in ethanol with 514.5-nm excitation.

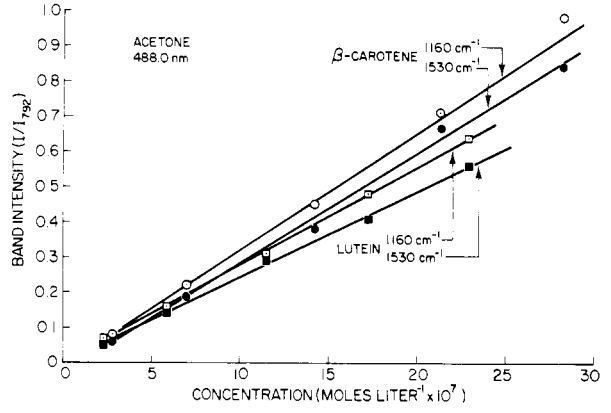

Figure 6. Standard curves in acetone with 488.0-nm excitation.

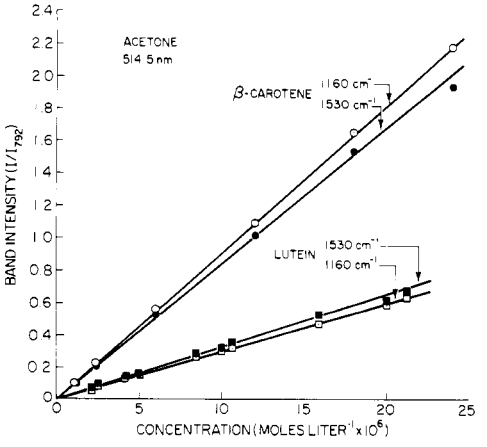

Figure 7. Standard curves in acetone with 514.5-nm excitation.

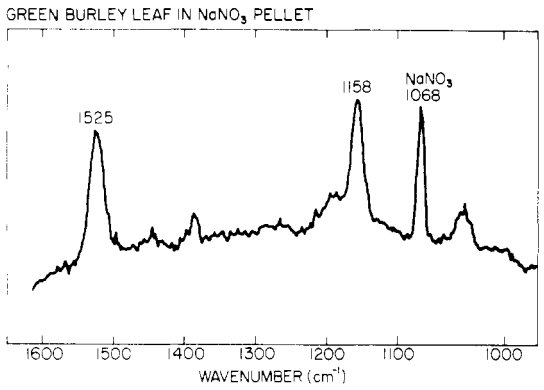

Figure 8. Resonance Raman spectrum of 5 mg of green burley leaf in 200 mg of $NaNO_3$.

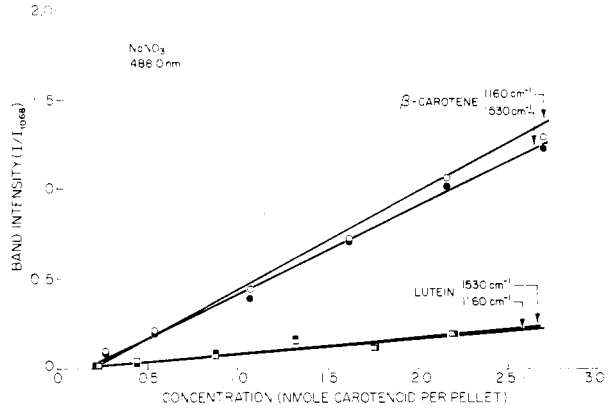

Figure 9. Standard curves in $NaNO_3$ with 488.0-nm excitation.

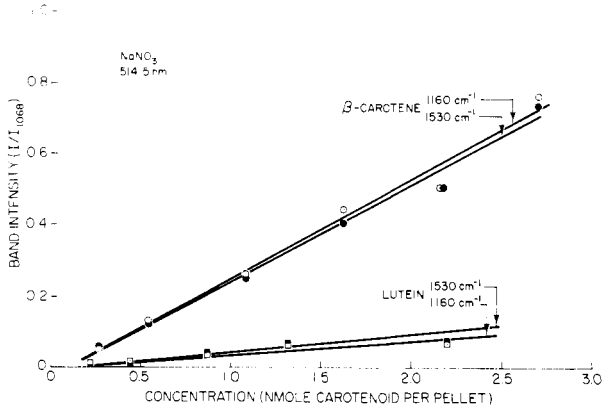

Figure 10. Standard curves in $NaNO_3$ with 514.5-nm excitation.

The 1068 cm^{-1} band of $NaNO_3$ could then be used for band normalization (Figure 8). Standard curves were prepared with excitation at both 488.0 and 514.5 nm (Figures 9, 10). Replicate analysis of 1×10^{-9} mol of β-carotene in 200 mg of $NaNO_3$ for six samples gave normalized band intensities of 0.47 ± 0.05 and 0.57 ± 0.06 for the 1160 and 1530 cm^{-1} bands, respectively.

Examination of the standard curves in Figures 4–7 and 9–10 reveals that, despite very similar visible absorption coefficients, the Raman intensity enhancements for β-carotene and lutein are quite different. When the excitation frequencies are compared to the visible absorption spectrum (Figure 2), it is clear that excitation is occurring on the edge of the visible absorption band. The consequence of this difference in resonance Raman extinction coefficients is that for quantitative analysis with a single exciting frequency the β-carotene and lutein must first be separated. Attempts to utilize measurements at two exciting frequencies have not been successful. This result means that while it is technically possible to record resonance Raman spectra of solid carotenoid containing samples, it is in general not possible to interpret these results in strict quantitative terms. As a consequence, thin-layer chromatography has been used in the following work to separate β-carotene and lutein prior to analysis.

Table I. Carotenoids in Green Bright Tobacco Leaves

stalk position	plant no.	nmol/g of tobacco β-carotene	nmol/g of tobacco lutein
top	1	330	733
	2	275	603
	3	290	635
middle	1	275	850
	2	225	538
	3	150	463
bottom	1	214	678
	2	213	545
	3	45	173

Table II. Carotenoids in Green and Freshly Cured Bright Tobacco

stalk position	plant no.	nmol/g of tobacco β-carotene green	β-carotene cured	lutein green	lutein cured
top	1	330	112	733	237
	2	275	223	603	263
	3	290	170	635	374
middle	1	275	130	850	319
	2	225	202	538	293
	3	150	118	463	330
bottom	1	214	83	678	261
	2	213	58	545	165
	3	45	38	173	125

Table III. Carotenoids in Aged, Cured Bright Tobaccos

year	nmol/g of tobacco β-carotene	nmol/g of tobacco lutein	β-carotene/lutein
1976	46.3	81.8	0.57
1975	45.0	91.5	0.49
1974	27.0	78.5	0.34
1974	37.3	73.8	0.50
1974	23.0	39.8	0.58
1974	33.0	60.3	0.55
1974	27.8	60.5	0.46
1973	11.5	26.0	0.44
1969	10.5	23.3	0.45

Table IV. Carotenoids in Cured Tobaccos

sample	nmol/g of tobacco β-carotene	nmol/g of tobacco lutein	β-carotene/lutein
bright	11.6	22.3	0.52
burley	20.5	51.0	0.44
oriental	7.3	12.0	0.60
burley stems	0.3	0.7	0.43

Carotenoids in green tobacco leaves vary as a function of stalk position and to a lesser extent plant location (Table I). Since all the leaves were harvested at the same time, variations exist in leaf maturity as well as in stalk position. The variation among plants for a given stalk position is greatest for the bottom leaves where senescence has already begun. For a particular plant this is apparent with the bottom leaves consistently containing less carotenoid. On curing (Table II), both β-carotene and lutein decrease significantly. However, the fresh cured leaf carotenoid levels are still significantly higher than those found for aged, cured Bright tobaccos (Table III). In fact, the older aged tobaccos show the lowest levels of carotenoid, with

Table V. Carotenoids in Cigarette Blends

	nmol/g of tobacco		β-carotene/ lutein
	β-carotene	lutein	
cigarette A	6.3	25.4	0.26
cigarette B	13.0	27.8	0.47
cigarette C	10.4	30.1	0.34

a trend toward a preferentially greater loss of β-carotene compared to lutein. While there is some variation in carotenoid levels among cured, aged tobaccos (Tables III and IV), the differences between freshly cured and aged tobaccos are greater. As might be expected, stems from cured leaves show the lowest carotenoid levels of all. Typically commercial cigarettes have carotenoid levels similar to those found in cured, aged tobaccos (Table V). These results suggest that the major decomposition of tobacco carotenoids occur during the curing process with continuous decomposition during aging. Such degradation of carotenoids may have important implications for flavor compounds formed from carotenoids by degradation (Fujimori et al., 1976).

LITERATURE CITED

Fujimori, T., Kasuga, R., Matsushita, H., Kaneko, H., Noguchi, M., *Agric. Biol. Chem.* **40**, 303 (1976).
Gill, D., Kilponen, R. G., Rimai, L., *Nature (London)* **227**, 743 (1970).
Goodwin, T. W., "Chemistry and Biochemistry of Plant Pigments", Vol. 2, Academic Press, New York, 1976, p 150.
Hoskins, L. C., Alexander, V., *Anal. Chem.* **49**, 695 (1977).
Kiefer, W., Bernstein, H. J., *Appl. Spectrosc.* **25**, 500 (1971).
Lutz, M. M., *C. R. Acad. Sci. Paris B*, 497 (1972).
Rimai, L., Heyde, M. E., Gill, D., *J. Am. Chem. Soc.* **95**, 4493 (1973).
Salares, V. R., Young, N. M., Bernstein, H. J., Carey, P. R., *Biochemistry* **16**, 4751 (1977).
Wright, H. E., Burton, W. W., Berry, R. C., *Arch. Biochem. Biophys.* **82**, 107 (1959).

Received for review November 7, 1978. Accepted January 11, 1979.

Preparation and Agronomic Evaluation of Long-Chain Crystalline Ammonium and Potassium Ammonium Polyphosphates

Richard C. Sheridan,* John F. McCullough, and Seward E. Allen

Long-chain crystalline ammonium polyphosphates and potassium ammonium polyphosphates were readily produced by thermal dehydration of orthophosphate or short-chain polyphosphates in a stream of gaseous ammonia. The products were high in total P but low in available phosphorus (citrate + water soluble); however, greenhouse tests showed that they are highly effective sources of nitrogen and phosphorus.

Crystalline ammonium polyphosphates with a chain length greater than 50 have a composition approaching that of ammonium metaphosphate $(NH_4PO_3)_x$. They contain up to 14% N and 73% P_2O_5 and are the most highly concentrated fertilizers obtainable in the ammonium polyphosphate system. Several crystalline modifications have been prepared by heating urea with monoammonium orthophosphate (Shen et al., 1969), and a mixed cation polyphosphate was obtained by the reaction of urea, ammonium orthophosphate, and potassium orthophosphate (Lyons and Vandersall, 1970). The urea serves as a condensing agent by combining chemically with the water released by condensation of the phosphate. Because the urea is consumed in the reaction, a more practical process for the production of these long-chain polyphosphates is desirable. Thermal dehydration of ammonium phosphates produces an amorphous mixture of polyphosphates from which most of the ammonia has been lost (Margulis et al., 1966; Knorre, 1900; Terem and Akalan, 1949). However, laboratory research at TVA showed that long-chain crystalline ammonium or potassium ammonium polyphosphate can be made by simple thermal dehydration of orthophosphates in a stream of gaseous ammonia (Sheridan and McCullough, 1975; McCullough and Sheridan, 1975). Similar processes have been reported in the recent literature (Shen et al., 1969; Vol'fkovich et al., 1972; Hecht et al., 1974a,b).

National Fertilizer Development Center, Tennessee Valley Authority, Muscle Shoals, Alabama 35660.

This paper summarizes the preparation and crop response to N and P of these experimental products in comparison with standard fertilizer sources.

MATERIALS AND METHODS

Chemical analyses of the polyphosphates that were tested as potential fertilizers are shown in Table I. The preparation of these materials is described below.

Preparation of Ammonium Polyphosphates. The long-chain crystalline ammonium polyphosphates have the general formula $(NH_4)_nH_2P_nO_{3n+1}$, where n is about 50 or greater. These compounds are stable, nonhygroscopic, and sparingly soluble in water. They were prepared in the laboratory by thermal condensation of ammonium ortho-, pyro-, tripoly-, tetrameta-, or oligophosphates in a stream of ammonia. The ammonia promotes the formation of long-chain ammonium polyphosphates by preventing excessive loss of ammonia from the reaction bed and by sweeping away the water released by condensation of the ortho- and short-chain phosphates. The products were made in batches and also in continuous-type operations.

In the batch tests, samples usually were heated at 225–300 °C for 4–8 h in a current of ammonia. For example, 20.0 g of crude monoammonium orthophosphate prepared by ammoniation of wet-process phosphoric acid was heated at 225 °C for 8 h in a slow stream of ammonia at atmospheric pressure. The crystalline product weighed 13.4 g, and X-ray analysis showed that it was a mixture of forms I, II, and V long-chain ammonium polyphosphate. Form I has the shortest average chain length of several polymorphic forms having essentially the same chemical

0021-8561/79/1427-0612$01.00/0