Note

Determination of Green Leaf Carotenoids by HPLC[†]

Shigeaki TAKAGI

Department of Agricultural Chemistry,
Faculty of Agriculture,
Okayama University,
Okayama 700, Japan

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Lutein, one of the major carotenoids in green leaves, associates upon dispersion in water to form a large helical aggregate which induces strong circular dichroism (CD) in the visible region.^{1~6)}

Quantitative determination for each carotenoid of green leaves is essential to pursue the function and the role of lutein molecule in thylakoid membranes.

A number of papers^{7~14)} on liquid chromatography for analysis of leaf carotenoids have been published. These methods, however, involve no quantitative concept. The author presents a new technique for a high performance liquid chromatographic (HPLC) system permitting simultaneous determination of major leaf carotenoids with simplicity and reproducibility using silica gel columns.

Authentic carotenoids were obtained as follows; β -carotene (β,β -carotene) was purchased from Merck, and lutein (β,ϵ -carotene 3,3'-diol),¹⁵⁾ violaxanthin (5,6,5',6'-diepoxy-5,6,5',6'-tetrahydro- β,β -carotene-3,3'-diol), neoxanthin (5',6'-epoxy-6,7-didehydro-5,6,5',6'-tetrahydro- β,β -carotene-3,5,3'-triol),¹⁶⁾ and crocetin (8,8'-diapocarote-8,8'-dioic acid) were isolated from spinach leaves by preparative column chromatography. Carotenoid extraction from leaves was carried out substantially according to the ordinary method.^{17,18)} Less than 10 g of fresh green leaves were homogenized with 150 ml acetone and kept at room temperature for 1 hr in the dark. The homogenate was filtered through filter paper by suction. Extraction was repeated three times with the same volume of acetone. The extracts were combined and evaporated under reduced pressure, and the residue was reextracted by a mixture of diethyl ether and petroleum ether (1:1). The extract was dried over anhydrous sodium sulfate, concentrated by evaporation, and filled up to 20 ml with acetone. Chlorophylls were determined with the acetone solution according to the method of Wintermans *et al.*,¹⁹⁾ if necessary. Five ml of the acetone solution was saponified with 25 ml of 1N ethanolic solution of potassium hydroxide at 20°C for 8 hr in the dark. After addition of an equal volume of distilled water, unsaponified substances were extracted with a mixture of diethyl

ether and petroleum ether (1:1) three times, followed by washing of the extract with small volume of aqueous saturated solution of sodium chloride three times. The extract was dried over anhydrous sodium sulfate, concentrated by evaporation, and filled up to 5 ml with solvent A, the initial solvent for HPLC. Irrespective of the saponification time (7~24 hr), the determined values of the individual carotenoids were almost constant, so saponification at 20°C for 8 hr was used throughout the experiments.

HPLC was run on a Hitachi 638 instrument with a stainless steel column of 0.3 cm $\times$ 30 cm packed with Hitachi gel #3040. Carotenoids in the effluent were monitored at 450 nm with the UV detector, a Hitachi model 200 spectrophotometer, which was equipped with a micro flow cell of 10 mm light path. Two solvents were used for HPLC: 1) solvent A, a mixture of equal volumes of *n*-hexane and benzene and 2) solvent B, acetone. Carotenoids were eluted with solvent A containing an increasing concentration of solvent B in a linear gradient reaching 100% after 60 min. Elution speed was kept constant at 0.6 ml per min. The sample volume injected changed from 5 to 20 μ l.

A typical chromatogram of HPLC of carotenoids in green leaves is shown in Fig. 1. Usually seven peaks of the major components were observed. Sometimes minor ones appeared. Peaks 1, 3, and 6 were identified as carotenes (mainly β -carotene), lutein, and neoxanthin respectively by comparing their absorption spectra to those of the authentic carotenoids. Peak 4 was deduced to be antheraxanthin (5,6-epoxy-5,6-dihydro- β,β -carotene-3,3'-diol) from the retention time, absorption maximum, and positive hydrochloric acid test for 5,6-epoxide.²⁰⁾ Peak 5 was

FIG. 1. Typical HPLC Chromatogram of Leaf Carotenoids in Summer (*Perilla*).

For chromatographic conditions see the text.

[†] Biochemical Studies on Carotenoid XV. For Part XIV, see ref. 6.

TABLE I. COMPOSITION OF LEAF CAROTENOIDS IN SUMMER AND IN WINTER

Plants	Carotene (%)	Lutein (%)	Antheraxanthin (%)	Violaxanthin (%)	Neoxanthin (%)	Crocin (%)	Total carot. ($\mu\text{g}/10\text{ g}$ fresh weight)
(In summer)							
Carrot (<i>Daucus carota</i> var. <i>sativus</i>)	45.5	28.2	8.5	9.1	8.7	0	743.5
Maize (<i>Zea mays</i> L.)	50.0	37.0	6.3	4.2	2.5	0	1389.0
Soya (<i>Glycine max</i> MERRILL)	50.6	33.6	6.3	3.3	5.4	0.8	1376.0
Perilla (<i>Perilla frutescens</i> <i>viridis</i> MAKINO)	41.4	26.0	8.8	13.7	6.0	4.0	2233.0
Cherry (<i>Prunus yedoensis</i> MATSUM.)	36.1	35.3	5.7	16.6	6.3	0	2142.4
Maple (<i>Acer palmatum</i> THUNB.)	36.4	40.7	5.6	11.1	6.3	0	3158.4
Azalea (<i>Rhododendron indicum</i> SWEET)	30.1	33.9	8.4	13.6	9.1	0	1436.0
Spindle tree (<i>Euonymus japonica</i> THUNB.)	28.9	34.2	8.0	15.6	13.2	0	630.8
(In winter)							
Radish (<i>Raphanus sativus</i> L. var. <i>acanthiformis</i> MAKINO)	28.4	47.7	8.0	8.6	7.3	0	1058.0
Lettuce (<i>Lactuca scariola</i> L. var. <i>sativa</i> BISCH)	28.5	44.5	8.8	9.9	8.3	0	1019.5
Cabbage (<i>Brassica oleracea</i> L. var. <i>capitata</i> L.)	17.9	36.3	15.3	14.2	13.5	0	1731.3
Leaf mustard (<i>Brassica juncea</i> CZEM. et COSS.)	20.3	33.0	18.9	15.2	6.2	0	2175.3
Azalea (<i>Rhododendron indicum</i> SWEET)	22.1	42.1	11.9	11.2	5.2	0	2022.0
Spindle tree (<i>Euonymus japonica</i> THUNB.)	34.5	42.1	9.9	7.5	6.1	0	789.5

identified as violaxanthin by the retention time, absorption maximum, and positive hydrochloric acid test. Peak 7 was presumed to be crocetin,²¹⁾ peak 2 contained no carotenoid.

When β -carotene solutions of known concentrations in solvent A were subjected to HPLC, a linear relationship was observed between the quantities of β -carotene and the peak areas in those chromatograms up to 200 ng of β -carotene. Therefore, an equation, $C = 124 \times S/f$, can be set up, in which 'C', 'S' and 'f' were starting carotenoid quantity (ng), chromatographic peak area (cm^2) calculated by the half width method, and a correction factor, respectively. The correction factors were determined by ratios of $E_{1\text{cm}}^{1\%}$ of each carotenoid at 450 nm in solvent A to that of β -carotene ($E_{1\text{cm}}^{1\%} = 2590$) at the same wavelength, and values determined were 1.0, 0.87, 0.86, 0.86, 0.50, and 1.67 for β -carotene, lutein, antheraxanthin, violaxanthin, neoxanthin, and crocetin, respectively. The constant in the equation, 124, may change with chromatographic conditions.

The recovery of β -carotene was over 97%. The errors inherent in the chromatographic analysis were within a few per cent, which mainly depended on the errors of injection volume. A detection limit in this analytical system was about 2 ng for β -carotene. If one expects to obtain reproducible results, however, an injection quantity above 10 ng for each carotenoid is necessary. Standard deviations throughout the analyses (three samples) were 4.3, 12.1, 11.2, 6.3, and 15.4% for β -carotene, lutein, antheraxanthin, violaxanthin, and neoxanthin, respectively.

Carotenoids in leaves of some species were quantitatively analyzed by this method (Table I). In annual plants, β -carotene was predominant in summer, while lutein was predominant in winter. In leaves of almost all the species of three tested more lutein was found than β -carotene, but lutein contents usually increase in quantitative ratio to β -carotene in winter. The variation between the amounts of β -carotene and lutein is considered to be caused by differences of both temperature and light

intensity.

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