Food Chemistry 45 (1992) 129–134
Analytical Methods Section

Determination of carotenoids and chlorophylls in water convolvulus (*Ipomoea aquatica*) by liquid chromatography

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(Received 25 July 1991; revised version received 4 November 1991; accepted 7 November 1991)

The carotenoids and chlorophylls present in water convolvulus (Ipomoea aquatica) were analyzed by high-performance liquid chromatography (HPLC) with photodiode-array detection and their identify confirmed by thin-layer chromatography (TLC). An HPLC quaternary solvent system of acetonitrile methanol chloroform hexane (75 : 12·5 : 7·5 : 7·5, v/v/v/v) resolved 14 peaks in 20 mih by using a 25 cm reversed-phase column, of which 12 pigments were identified. These pigments include carotenoids, chlorophylls and their isomers. The coefficient of variation for pigment concentrations was less than 12% in five sample analyses. A TLC quaternary solvent system of hexane acetone chloroform methanol in different proportions also resolved 10 pigments on silica gel layers. In addition to *cis*-lutein and *cis*-β-carotene, all major pigments were adequately resolved by TLC.

INTRODUCTION

Carotenoids and chlorophylls are important biological compounds that are widely present in green plants. It has been well documented (Mathews-Roth, 1981) that carotenes can transfer the light energy they absorb between 400 and 600 nm to the chlorophyll, thus providing it with additional energy for photosynthesis. In addition, the carotenoid pigments can function as protective agents against their own endogenous photosensitizer such as chlorophyll (Mathews-Roth, 1964; Anderson & Krinsky, 1973). Moreover, carotenoids have been found effective in preventing formation of skin tumors in mice (Mathews-Roth, 1982, 1985) and increasing immune response in rats (Bendich & Shapiro, 1986; Bendich, 1989). Water convolvulus (*Ipomoea aquatica*), a green vegetable grown in Taiwan and China, is well known for its protective effect against nosebleed and high blood pressure. Since water convolvulus is also a rich source of carotenoids and chlorophylls, it has received considerable attention regarding its role in human nutrition and photosynthesis. In a previous study the authors reported the separation of major carotenoids in water convolvulus by open-column chromatography (Chen *et al.*, 1991). However, this method failed to resolve carotenoid isomers. Also, the composition of chlorophylls and their isomers remains unknown.

The separation of carotenoids and chlorophylls has been mainly achieved by high-performance liquid chromatography (HPLC). Eskins *et al.* (1977) developed an HPLC method for separation of plant pigments by stepwise gradient system. Good resolutions of chlorophylls a and b, pheophytin, carotene, lutein, violaxanthin and neoxanthin were obtained in approximately 270 min. Braumann and Grimme (1979) utilized a reversed-phase column and a stepwise methanol water gradient for the separation of photosynthetic pigments by HPLC. A total of 15 pigments was resolved. These pigments include neoxanthin, neoxanthin isomer, violaxanthin, lutein, lutein epoxide, chlorophylls a, a' and b, pheophytin and β-carotene. The total elution time was 40 min. In a later study, Braumann and Grimme (1981) also described an HPLC method for separation of carotenoids and chlorophylls in spinach and algae by using two reversed-phase columns and a linear gradient of methanol/acetonitrile/water. Good resolutions of neoxanthin, *cis*-neoxanthin, violaxanthin, *cis*-violaxanthin, lutein, *cis*-lutein, α-carotene, *cis*-α-carotene, β-carotene and *cis*-β-carotene were completed in 32 min. Wright and Shearer (1984) developed an HPLC

Food Chemistry 0308-8146/92/$05.00

method for analysis of photosynthetic pigments in phytoplankton by using a reversed-phase column and a linear gradient from 90% acetonitrile to ethyl acetate. A total of 18 pigments was resolved in a single step in approximately 20 min. These pigments include carotenes, xanthophylls, chlorophylls and their degradation products. Khachik *et al.* (1986) used a C-18 column and a combination of isocratic and gradient solvent system for the separation of carotenoids and chlorophylls in green vegetables. A total of 20 pigments including carotenoids, chlorophylls and their geometrical isomers was resolved in 32 min. Siefermann-Harms (1988) separated carotene and xanthophyll isomers, and chlorophylls and pheophytins within 20 min by using a C-18 column and a combination of isocratic and gradient solvent system. Some positional isomers such as α-carotene and β-carotene, and lutein and zeaxanthin were adequately resolved. Bailey and Chen (1988) used a reversed-phase isocratic method to separate carotenoids and chlorophylls in turf Bermuda grass. Although the total elution time is only 10 min, some geometrical isomers are not detected in grass samples. Daood *et al.* (1989) reported a reversed-phase isocratic method for the separation of carotenoids and chlorophylls in vegetables. A total of 18 peaks was resolved in approximately 30 min, of which 15 pigments were identified. These pigments include carotenoids, chlorophylls and their isomers. Although these methods could adequately resolve both carotenoids and chlorophylls simultaneously, some are still lengthy and cannot resolve carotenoid and chlorophyll isomers in a single step. Moreover, most methods use gradient solvent system to resolve carotenoids, chlorophylls and their isomers, which may in turn affect the accuracy in quantifying each pigment because of baseline instability. Here a method which can resolve carotenoids, chlorophylls and their geometrical isomers in water convolvulus in approximately 20 min by using a 25-cm, reversed-phase column and an isocratic solvent system with photodiode-array detection was reported. Thin-layer chromatography (TLC) was also used to verify the identity of these pigments.

MATERIALS AND METHODS

Instrumentation

The HPLC instrument consisted of a SSI 222D pump (Scientific System Inc., State College, PA, USA) with a linear photodiode-array detector (Linear Instrument, Reno, NV, USA) and a Phenomenex stainless-steel column (25 cm × 4·6 mm i.d.) packed with Ultremex C-18 5 μm particle size (Torrance, CA, USA). The data were stored and processed with an Axxiom 727 Dual-Channel Chromatography Data System (Axxiom Chromatography Inc., Calabasas, CA, USA). A sensitivity of 0·01 AUFS was used. Spectrophotometric determinations were made with a Beckman DU-70 double-beam spectrophotometer (Irvine, CA, USA). A solvent system of acetonitrile methanol chloroform hexane (75 : 12·5 : 7·5 : 7·5, v/v/v/v) pumped at a flow rate of 1·0 ml min^{-1} was used.

Materials

Trans-β-carotene, lutein (75% purity), chlorophyll a (CHL a) and chlorophyll b (CHL b) standards were purchased from Sigma (St Louis, MO, USA). Neoxanthin and violaxanthin standards were prepared from saponified spinach extract by TLC (Chen & Bailey, 1987). Internal standard β-apo-8'-carotenal (20% suspension in vegetable oil) was a gift from Hoffman-La Roche (Basel, Switzerland). β-Apo-8'-carotenal was further purified on the column (30 cm × 12·5 mm i.d.) containing a 1 : 1 mixture of activated MgO and diatomaceous earth using a method described by Chen and Bailey (1987). All HPLC-grade solvents were purchased from Merck (Taiwan) Ltd (Taipei, Taiwan) and filtered through a 0·2-μm membrane filter under vacuum prior to use. The silica gel TLC plates (20 cm × 20 cm) were purchased from Merck. Lutein was further purified on the column (30 cm × 12·5 mm i.d.) containing a 1 : 1 mixture of activated MgO and diatomaceous earth using a method described by Chen *et al.* (1991). Water convolvulus was purchased fresh from a local supermarket.

Extraction and HPLC analysis of unsaponified water convolvulus extract

Samples of leaves of fresh water convolvulus (10 g) were mixed with $MgCO_3$ (0·2 g) and 30 ml extractant (hexane/acetone/absolute alcohol/toluene-10 : 7 : 6 : 7, v/v/v/v) and the mixture was blended for 3 min. The extract was vacuum-filtered through a Buchner funnel fitted with a Whatman filter paper. The residue was re-extracted with the same procedure to remove all the carotenoids and chlorophylls. After mixing, the crude extract was transferred to a separatory funnel. Hexane (30 ml) was added to the crude extract followed by 10% Na_2SO_4 (30 ml). This mixture was shaken for 3 min with the bottom layer being extracted once more with hexane (30 ml). The hexane fractions were combined and poured into a 100-ml volumetric flask and diluted to volume with hexane. Portions of the crude extract (2 ml) were further purified by passing through a silica cartridge (Sep-PAK, Water Assoc., Boston, MS, USA) and eluted with 5 ml hexane acetone methanol (70 : 20 : 10, v/v/v). The crude extract was evaporated under nitrogen and dissolved in chloroform (10 ml). Then the solution was filtered through a 0·2-μm membrane filter. Portions of the extract (10 μl)

were injected on to the HPLC chromatograph. A quaternary solvent system of acetonitrile/methanol/chloroform/hexane (75 : 12·5 : 7·5 : 7·5, v/v/v/v) pumped at a flow rate of 1 ml min^{-1} was used. The eluate was monitored at 440 nm with a sensitivity of 0·01 AUFS. The various carotenoids and chlorophylls were identified by comparing retention time of separated peaks with reference standards and co-chromatography with added standards. Also, the individual HPLC peaks of 10—15 runs were collected, pooled and solvents removed under vacuum. The carotenoids and chlorophylls were dissolved in an appropriate solvent to determine absorption spectra with a Beckman DU-70 double-beam spectrophotometer. In addition, scans of 190—365 and 366—800 nm for each peak were collected throughout the chromatographic runs with a Linear photodiode-array detector.

Separation and identification of carotenoids and chlorophylls by TLC

A solvent system of hexane/acetone/chloroform/methanol in proportions of 70 : 25 : 10: 5 (v/v/v/v) and 70 : 15 : 10 : 5 (v/v/v/v) was used to separate the major carotenoids and chlorophylls in concentrated water convolvulus extracts on silica gel TLC plates. The former was used to separate xanthophylls, β-carotene, pheophytin a, and CHL a' while the latter was used to separate CHL b, CHL b', CHL a, and CHL a'. Development of the TLC plates was carried out in glass tanks lined with filter paper and equilibrated for 30 min with 150 ml of hexane/acetone/chloroform/methanol at 75 : 25 : 10 : 5 (v/v/v/v) and 70 : 15 : 10 : 5 (v/v/v/v) prior to development. A portion of extract (10 μl) was applied to the TLC plate with a micropipette. The chromatograms were developed over a distance of 15 cm for *c.* 25 min in a dark room at ambient temperature. Separated bands were scraped into a small sintered-glass funnel attached to a 25-ml side-arm filtration flask. Pigments were eluted with an appropriate solvent to determine the absorption spectra with a Beckman DU-70 double-beam spectrophotometer. Individual carotenoid and chlorophyll were characterized by comparing their absorption spectra with reference values and co-chromatography with added standards. An epoxide test (Davies, 1976) was used to identify the presence of epoxy-containing carotenoids such as lutein epoxide, violaxanthin and neoxanthin. Each pigment obtained by TLC was also injected into an HPLC chromatograph so that the identity of each peak on the chromatogram could be confirmed.

Quantification of carotenoids and chlorophylls by HPLC

Internal standard β-apo-8'-carotenal (0·1 mg) was added to the sample for extraction and the concentration of each pigment was calculated using the response factor and ratio of the peak area of each pigment over the peak area of the internal standard. The relative detector response of lutein, CHL a, CHL b and β-carotene to β-apo-8'-carotenal at 440 nm were determined at four different concentrations. The response factors (slope of the curve) for lutein, CHL a, CHL b and β-carotene were 1·27, 1·38, 1·49 and 1·42, respectively. Neoxanthin, violaxanthin and lutein epoxide were calculated as lutein equivalents. *Cis*-β-carotene was calculated as β-carotene and CHL a' and CHL b' as CHL a and CHL b equivalents, respectively. The coefficient of variation for pigment concentrations was determined in five samples.

RESULTS AND DISCUSSION

Table 1 shows the identification data of carotenoids and chlorophylls in water convolvulus by TLC. A quarternary solvent system of hexane/acetone/chloroform/methanol (70 : 25 : 10 : 5, v/v/v/v) provided a clear separation of neoxanthin, violaxanthin, lutein epoxide, lutein, β-carotene, pheophytin a and CHL a'. However, several pigments such as CHL b, CHL b', CHL a and CHL a' were not adequately resolved by using this solvent system. Thus, a further separation step is necessary. The same quaternary solvent system of hexane/acetone/chloroform/methanol in proportion of 70 : 15 : 10 : 5 (v/v/v/v) was then employed to separate CHL b, CHL b', CHL a and CHL a'. An epoxide test showed that only three pigments contained epoxy groups. These epoxy-containing carotenoids were identified as lutein epoxide, violaxanthin and neoxanthin based on hypsochromic shifts of 17, 38 and 17 nm upon acidification with 0·1 N HCl, and color changes from yellow to green, blue, and green, respectively. In addition, the individual pigment was identified by comparing its absorption spectra with reference values reported in the literature and co-chromatography with added standards. Chlorophylls a' and b', the C-10 epimers of chlorophylls a and b, were resolved by using TLC. Since no commercial standards of chlorophylls a' and b' are available, they can be only tentatively identified. These two pigments were identified based on absorption spectra and retention behaviour after injection on to the HPLC chromatograph. Pheophytin a, the magnesium-free derivative of chlorophyll a, was also found on TLC plates. Since pheophytin a is not shown on the HPLC chromatogram (Fig. 1), it is probably an artifact formed during chromatography because of the slightly acidic nature of silica gel.

Figure 1 shows the chromatogram of carotenoids and chlorophylls in water convolvulus by HPLC. A quaternary solvent system of acetonitrile/methanol/chloroform/hexane (75 : 12·5 : 7·5 : 7·5, v/v/v/v) pumped at a flow rate of 1 ml min^{-1} provided a clear separation of carotenoids, chlorophylls and their isomers. This

Table 1. Identification data of carotenoids and chlorophylls in fresh water convolvulus by HPLC[a]

Pigment	Visible spectra			R_f found	Epoxide test	
	λ Max. found	Solvent	λ Max. reported[a]		Hypsochromic shift	Color
β-Carotene	(424),448,476	Hexane	(425),450,477	0·94	—	—
Pheophytin a	408,664	Acetone	409,664	0·79	—	—
Chlorophyll a'[b]	430,662	Acetone	431,662	0·61	—	—
Chlorophyll a	430,662	Acetone	431,662	0·58	—	—
Chlorophyll b'[b]	453,644	Acetone	453,645	0·52	—	—
Chlorophyll b	453,644	Acetone	453,645	0·49	—	—
Lutein	423,446,473	Ethanol	442,445,474	0·42	—	—
Lutein epoxide	419,440,469	Ethanol	420,442,471	0·30	402,423,451	Green
Violaxanthin	417,440,469	Ethanol	417,440,469	0·27	381,402,430	Blue
Neoxanthin	413,436,465	Ethanol	415,438,467	0·16	396,420,448	Green

[a] Reported values of absorption spectra are from two references by Davies (1976) and Wright *et al.* (1984).
[b] Tentatively identified.

solvent system was chosen based on a similar solvent system developed by Khachik *et al.* (1986), who used a combination of isocratic and gradient solvent mixtures. The authors used an isocratic mixture of methanol/acetonitrile/methylene/chloride hexane (15 : 75 : 5 : 5, v/v/v/v) at time 0 followed by a gradient beginning at 12 min and completed at 32 min. The final composition of the gradient solvent system was methanol acetonitrile methylene chloride hexane (15 : 40 : 22·5 : 22·5, v/v/v/v). Although a total of 20 peaks were separated in 32 min, some geometrical isomers such as *trans*-β-carotene and *cis*-β-carotene were not well resolved. Also, the solvent systems used by the authors are too complex and may cause baseline instability. By using the authors' method a total of 14 peaks were resolved in approximately 20 min. Each pigment was identified by comparing retention time of unknowns with standards and co-chromatography with added standards. Table 2 shows the absorption spectra of each pigment dissolved in various solvents. From those data, each peak on the HPLC chromatogram can be clearly identified by comparing its absorption spectrum with reference values reported in the literature (Davies, 1976). *Cis*-lutein and *cis*-β-carotene were identified by a hypsochromic shift of about 6 nm and a strong peak present in the UV region at about 340 nm. Since the presence of a strong peak in the UV region indicate the presence of central *cis* isomers of carotenoids, the loca-

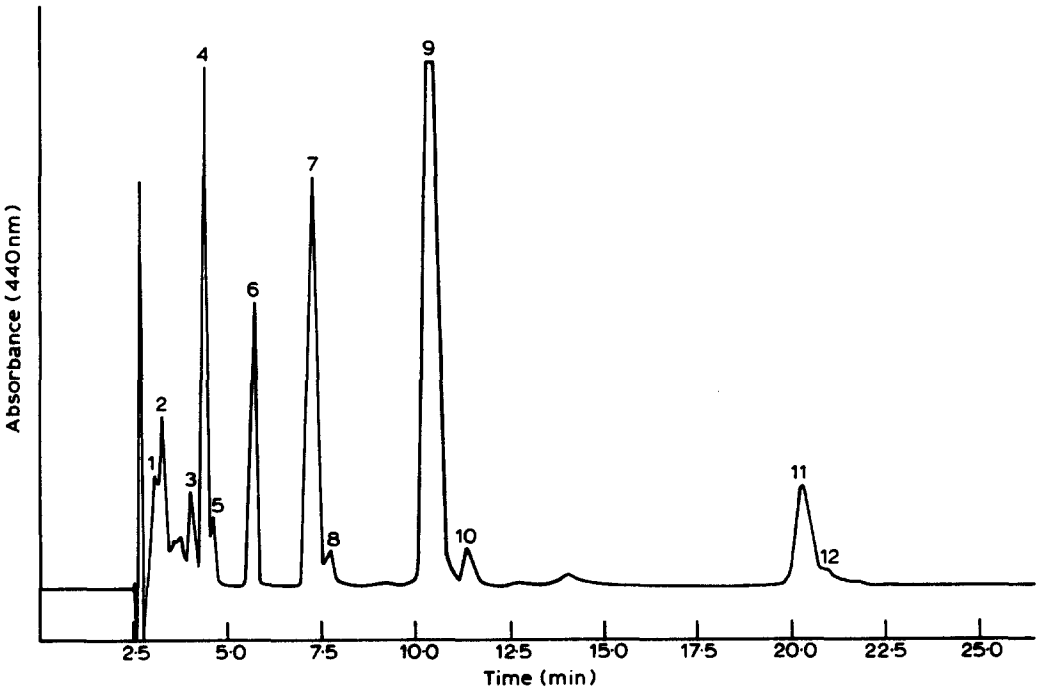

Fig. 1. A chromatogram of unsaponified extract prepared from fresh water convolvulus. Chromatographic conditions described in text. Peaks: 1, neoxanthin; 2, violaxanthin; 3, lutein epoxide; 4, lutein; 5, *cis*-lutein; 6, β-apo-8'-carotenal; 7, chlorophyll b; 8, chlorophyll b'; 9, chlorophyll a; 10, chlorophyll a'; 11, β-carotene; 12, *cis*-β-carotene.

Table 2. Identification data of carotenoids and chlorophylls in water convolvulus by TLC

Peak no.	Pigment	Visible spectra[b]			Epoxide test	
		λ Max. found	Solvent	λ Max. reported	Hypsochromic shift	Color
1	Neoxanthin	417,441,468	Eluant[c]	—	—	—
		413,436,465	Ethanol	415,438,467	396,420,448	green
2	Violaxanthin	421,445,473	Eluant	—	—	—
		417,440,469	Ethanol	417,440,469	381,402,430	blue
3	Lutein epoxide	421,443,473	Eluant	—	—	—
		419,440,469	Ethanol	420,442,471	402,423,451	green
4	Lutein	425,448,478	Eluant	—	—	—
		423,446,473	Ethanol	422,445,474	—	—
5	*Cis*-lutein	340,421,445,473	Eluant	—	—	—
		340,417,440,467	Ethanol	—	—	—
7	Chlorophyll b	459,648	Eluant	—	—	—
		453,644	Acetone	453,645	—	—
8	Chlorophyll b'[d]	458,648	Eluant	—	—	—
		453,644	Acetone	453,645	—	—
9	Chlorophyll a	428,663	Eluant	—	—	—
		430,662	Acetone	431,662	—	—
10	Chlorophyll a'[d]	430,663	Eluant	—	—	—
		430,663	Acetone	431,662	—	—
11	β-carotene	(430),456,485	Eluant	—	—	—
		(424),448,476	Hexane	(425),450,477	—	—
12	*Cis*-β-carotene	340,(422),451,479	Eluant	—	—	—
		340,(413),442,470	Hexane	—	—	—

[a] Reported values of absorption spectra are from two references by Davies (1976) and Wright *et al.* (1984).
[b] Values in parenthesis represent shoulder on spectra absorption curves.
[c] Eluant = acetonitrile/methanol/chloroform/hexane (75 : 12·5 : 7·5 : 7·5, v/v/v/v)
[d] Tentatively identified.

tion of *cis* double bonds of these carotenoids isomers may be designated as 13-, 13'- or 15-, 15'-*cis* (Khachik *et al.*, 1986). Chlorophylls a' and b' were identified based on absorption spectra and retention behaviour on HPLC chromatogram as reported by Schwartz *et al.* (1981) and Khachik *et al.* (1986). Fresh water convolvulus was found to contain chlorophylls a and b and their C-10 epimers, chlorophylls a' and b'. Khachick *et al.* (1986) also reported the presence of small amounts of chlorophylls a' and b' in several green vegetables. Pheophytins a and b, the most common derivatives of chlorophylls a and b, were not detected in fresh water convolvulus. This is in agreement with a report by Schwartz *et al.* (1981) who found that chlorophylls a and b were the only green pigments present in fresh spinach. Two minor peaks between violaxanthin and lutein epoxide were not identified but may be due to the presence of neoxanthin or violaxanthin isomer. Although lutein and *cis*-lutein, and β-carotene and *cis*-β-carotene, were not adequately resolved (Fig. 1), they can still be identified by comparison of absorption spectra with reference values reported in the literature (Davies, 1976; Schwartz & Patroni-Killam, 1985).

Table 3 shows the quantitative data of carotenoids and chlorophylls in water convolvulus. The coefficient of variation (CV) with respect to concentration of all

Table 3. Quantitative data of carotenoids and chlorophylls in water convolvulus

Pigment ($\mu g\ g^{-1}$)[a]	Sample no.					Mean	SD	CV (%)
	1	2	3	4	5			
Neoxanthin	55·9	53·6	51·6	42·8	48·5	50·5	5·1	10·0
Violaxanthin	58·5	62·3	60·5	65·8	52·6	59·9	4·9	8·1
Lutein epoxide	32·3	25·8	28·6	27·5	30·8	29·0	2·6	9·0
Lutein	85·6	74·6	79·2	68·5	82·3	78·0	6·7	8·6
Cis-lutein	12·2	10·9	11·2	12·5	10·4	11·4	0·9	7·9
Chlorophyll b	109·2	121·5	115·6	128·7	110·8	117·2	8·0	6·8
Chlorophyll b'	8·7	7·6	7·1	6·8	8·2	7·7	0·8	10·1
Chlorophyll a	337·2	352·8	358·6	342·3	368·5	351·9	12·5	3·6
Chlorophyll a'	13·1	12·5	11·8	12·2	10·8	12·1	0·9	7·1
β-Carotene	104·8	98·7	95·6	90·2	110·6	100·0	7·9	7·9
Cis-β-carotene	7·9	6·8	5·9	7·2	6·2	6·8	0·8	11·8

[a] Wet basis

pigments was less than 12% in five sample analyses. The result implied that some variation still existed among samples even when we used leaves for pigment analyses. The mean concentration of neoxanthin, violaxanthin, lutein epoxide, lutein, *cis*-lutein, chlorophyll b, chlorophyll b', chlorophyll a, chlorophyll a', β-carotene and *cis*-β-carotene was 50·5, 59·9, 29·0, 78·0, 11·4, 117·2, 7·7, 351·9, 12·1, 100·0 and 6·8 $\mu g\ g^{-1}$, respectively.

ACKNOWLEDGEMENTS

This study was supported by a grant (NSC-79-0409-B030-01) from the National Science Council, Taiwan.

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