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Characterization of major carotenoids in water convolvulus (*Ipomoea aquatica*) by open-column, thin-layer and high-performance liquid chromatography

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ABSTRACT

The major carotenoids present in water convolvulus (*Ipomoea aquatica*) were characterized by open-column chromatography, thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC). A 1:1 mixture of activated magnesium oxide and diatomaceous earth was used as the major adsorbent to separate carotenes, monohydroxy, dihydroxy and polyoxy pigments by open-column chromatography. Carotenes and cryptoxanthin were eluted with hexane–acetone at 96:4 and 90:10, respectively. Lutein, violaxanthin and neoxanthin were eluted with hexane–acetone–methanol at 85:15:0.2, 85:15:0.2 and 85:15:1.5, respectively. The elution sequence of lutein and violaxanthin was dependent on the amount of methanol present. A lutein band containing lutein and lutein epoxide was further separated by TLC. An HPLC isocratic solvent system of acetonitrile–methanol–ethyl acetate (75:15:10) was found to be appropriate for determining the reproducibility of retention time with respect to separated bands obtained by open-column chromatography. Each band was identified by comparing the absorption spectra and retention time with reference standards. The major carotenoids present in water convolvulus were β-carotene, lutein, lutein epoxide, violaxanthin and neoxanthin. The amount of each major carotenoid was also determined.

INTRODUCTION

Carotenoids are important biological compounds that are widely distributed in green plants. Carotenoids have been found effective in preventing photosensitization [1,2] and the formation of skin tumours in mice [3,4], and in increasing immune response in rats [5]. Water convolvulus (*Ipomoea aquatica*) is a popular green vegetable grown in Taiwan and China. It has been well documented [6] that increased consumption of water convolvulus is protective against high blood pressure and nosebleeds. As water convolvulus is also a rich source of carotenoids, it has received considerable attention regarding its role in human nutrition and health. However, no information is available as to the major carotenoids present in water convolvulus.

The current AOAC method (1984) [7] uses silica gel as the major adsorbent to separate carotenes, monohydroxy pigments (*e.g.*, zeinoxanthin and cryptoxanthin), dihydroxy pigments (*e.g.*, lutein and zeaxanthin) and polyoxy pigments (*e.g.*, violaxanthin and neoxanthin) by open-column chromatography. It has been reported [8] that losses or degradation of carotenoids could occur on silica columns. Hence it is

necessary to use magnesium oxide instead of silica gel as the major adsorbent to separate the major carotenoids in water convolvulus. The AOAC method also uses a binary solvent system of hexane–acetone in different proportions to separate polar xanthophylls. However, this solvent system failed to resolve polyoxy pigments such as violaxanthin and neoxanthin. Moreover, it has been established [9–11] that with ternary solvent mixtures it is possible to increase sample resolution by optimizing the mobile phase selectivity while maintaining a constant solvent strength. Therefore, by adding methanol as a modifier to the binary solvent system it is possible to separate some major xanthophylls such as lutein, violaxanthin and neoxanthin.

The objectives of this study were to use open-column chromatography to separate major carotenoids in water convolvulus, to use thin-layer chromatography (TLC) to separate pigments that could not be resolved by open-column chromatography and to use high-performance liquid chromatography (HPLC) to determine the reproducibility of retention time with respect to separated bands for the confirmation of major carotenoids in water convolvulus.

EXPERIMENTAL

Instrumentation

The HPLC instrument consisted of a Hewlett-Packard (Palo Alto, CA, U.S.A.) 1084B liquid chromatograph with a variable-wavelength UV–VIS detector and a Phenomenex (Torrance, CA, U.S.A.) stainless-steel column (25 cm × 4.6 mm I.D.) with an octadecyl (C_{18}) packing of 5 μm particle size. A sensitivity of 0.0064 a.u.f.s was used, and a Hewlett-Packard 79850B integrator was used to record retention times and chromatograms. Spectrophotometric determinations were made with a Hitachi (Tokyo, Japan) Model 220S double-beam spectrophotometer. A solvent system of acetonitrile–methanol–ethyl acetate (75:15:10) pumped at flow-rates of 1.0 and 2.0 ml/min was used.

Marerials

Water convolvulus and yellow corn were purchased from a local supermarket. *trans*-β-Carotene and lutein (75% purity) were purchased from Sigma (St. Louis, MO, U.S.A.). Zeinoxanthin, cryptoxanthin and zeaxanthin standards were prepared from yellow corn according to Quackenbush *et al.* [12]. Lutein epoxide was prepared from water convolvulus by TLC using hexane–acetone–methanol (90:29:1). Violaxanthin and neoxanthin standards were prepared from Bermuda grass according to Chen and Bailey [13].

All solvents were purchased from Merck (Taipei, Taiwan). Acetonitrile, methanol and ethyl acetate were of HPLC grade and hexane, acetone, ethanol and toluene were of ACS grade. HPLC-grade solvents were filtered through a 0.2-μm membrane filter and degassed under vacuum prior to use.

The silica gel, diatomaceous earth and MgO adsorbents were obtained from Merck. The silica gel TLC plates were made with a Camag spreader and activated at 110°C for 2 h before spotting samples.

Purification of lutein standard

Lutein standard purchased from Sigma was found not to be pure by open-

column chromatography, so a further purification step was necessary. A 1-mg amount of lutein dissolved in 1 ml of acetone was pipetted onto a column (30 cm × 12.5 mm I.D.) containing a mixture of activated MgO and diatomaceous earth (1:1). Three bands were observed on the column when employing hexane–acetone–methanol (89:10:1) [12]. One band with the deepest yellow colour was collected with the same solvent system and used as a reference standard of lutein.

Sample preparation and extraction procedure

A 1-kg amount of water convolvulus was freeze-dried to minimize oxidative loss and ground into a fine material, 10 g of which were extracted with 75 ml of hexane–acetone–methanol–toluene (10:7:6:7) in a 250-ml volumetric flask. The solution was saponified by adding 10 ml of 40% methanolic potassium hydroxide solution under nitrogen overnight. Hexane (75 ml) was added to the flask and the mixture was diluted to volume with 10% sodium sulphate solution. After shaking vigorously for 1 min and standing in the dark for 1 h, 5 ml of the upper phase were pipetted onto a column (30 cm × 12.5 mm I.D.) containing a mixture of activated MgO and diatomaceous earth (1:1) for open-column chromatography. All the sample preparations were conducted in the dark and samples were kept under nitrogen whenever possible during the procedure.

Separation and identification of major carotenoids by open-column chromatography and HPLC

Hexane–acetone (96:4 and 90:10) was used to elute the carotene band and monohydroxy pigment band, respectively. As monohydroxy pigments such as cryptoxanthin were not found in water convolvulus, a cryptoxanthin standard prepared from yellow corn was added to the crude extract and pipetted onto the column to determine the ability of this binary solvent system to elute monohydroxy pigments. After carotene and monohydroxy pigment bands had been eluted, dihydroxy pigments such as lutein and polyoxy pigments such as violaxanthin were eluted with hexane–acetone–methanol (85:15:0.2). The other polyoxy pigment, neoxanthin, was the last to be eluted with the same ternary solvent system in the proportions 85:15:1.5. Another ternary solvent system, hexane–acetone–methanol (85:15:1), was also used to compare the elution sequence of lutein and violaxanthin.

Each band eluted from the column was evaporated to dryness and dissolved in an appropriate solvent to determine the absorption spectra. The eluate from each band was also injected into an HPLC system to determine retention times. Reproducibilities of retention time with respect to the separated bands and standards were determined by making a series of five injections. An isocratic solvent system of acetonitrile–methanol–ethyl acetate (75:15:10) pumped at a flow-rate of 1 ml/min for the first 7.5 min and 2 ml/min thereafter. Each band eluted by open-column chromatography was monitored at 450 nm with a sensitivity at 0.0064 a.u.f.s.

The major carotenoids in water convolvulus were identified by comparing the absorption spectra and retention times of the separated bands with those of reference standards. A hypsochromic shift on acidification with hydrochloric acid was used to identify 5,6-epoxy carotenoids such as violaxanthin and neoxanthin.

Separation of carotenoid standards by HPLC

A mixture of neoxanthin, violaxanthin, lutein epoxide, lutein, zeaxanthin, zeinoxanthin, cryptoxanthin and β-carotene standards was dissolved in ethyl acetate and filtered through a 0.2-μm membrane filter before injection into the HPLC system. The HPLC conditions were the same as described above.

Separation of major carotenoids by TLC

A lutein band containing lutein and lutein epoxide eluted by open-column chromatography was further separated on silica gel TLC plates. Some carotenoid standards such as neoxanthin, violaxanthin and lutein epoxide were also separated and prepared from water convolvulus and Bermuda grass with the same procedure.

Development of the TLC plates was carried out in glass tanks lined with filter-paper and equilibrated for 30 min with 150 ml of methanol–acetone–hexane (1:29:90) prior to development. A 10-μl volume of extract was applied to the TLC plate with a syringe. The chromatograms were developed over a distance of 15 cm for *ca.* 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 measure the absorption spectra with a Hitachi 220S double-beam spectrophotometer. An epoxide test [14] was conducted to identify the presence of neoxanthin, violaxanthin and lutein epoxide. The amount of each major carotenoid was determined using Beer's law and the molar absorptivities [14]. The quantitative data were calculated based on averages of duplicate analyses.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatogram of carotenoid standards obtained by employing acetonitrile–methanol–ethyl acetate (75:15:10). This solvent system was chosen based on a similiar system developed by Nelis and De Leenheer [15], who used acetonitrile–methanol–dichloromethane (70:10:20) to separate nine carotenoids within 30 min. Although the nine carotenoids were adequately resolved, the separation time was too long. They also reported that with an isocratic non-aqueous solvent system it was possible to minimize the risk of solute precipitation on the column, increase the sample capacity and chromatographic efficiency and prolong the column lifetime. The total analysis time with this method could be reduced to 18 min by increasing the flow-rate from 1 to 2 ml/min after 7.5 min during a chromatographic run. The reproducibility of the retention times was determined by making a series of five injections of each standard and a mixture of standards. The relative standard deviation (R.S.D.) was less than 3% for all standards except β-carotene (3.38%). This result was expected because of increased sample diffusion at the end of separation.

Table I shows the identification data for major carotenoids in water convolvulus by open-column chromatography and TLC. β-Carotene was first eluted with hexane–acetone (96:4), followed by cryptoxanthin with the same solvent system at 90:10. However, this binary solvent system failed to resolve dihydroxy and polyoxy pigments. Thus, by adding methanol as a modifier to the binary solvent system it was possible to resolve lutein, violaxanthin and neoxanthin. Lutein and violaxanthin were thus eluted with hexane–acetone–methanol (85:15:0.2). Surprisingly, violaxanthin was eluted before lutein. Neoxanthin was the last eluted with the same ternary solvent
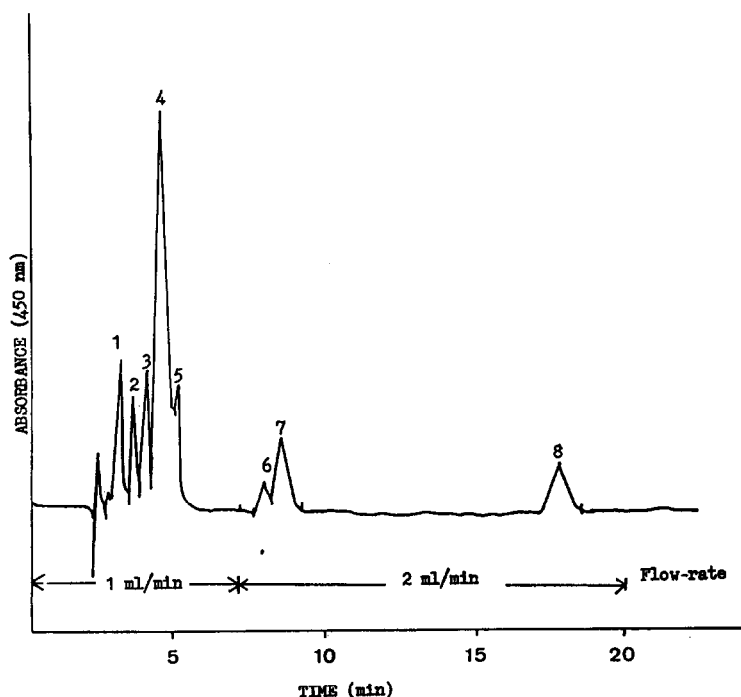
Fig. 1. HPLC of carotenoid standards by employing an isocratic solvent system of acetonitrile–methanol–ethyl acetate (75:15:10). Peaks: 1 = neoxanthin; 2 = violaxanthin; 3 = lutein epoxide; 4 = lutein; 5 = zeaxanthin; 6 = zeinoxanthin; 7 = cryptoxanthin; 8 = β-carotene.

TABLE I

DATA FOR IDENTIFICATION OF PIGMENTS IN WATER CONVOLVULUS BY OPEN-COLUMN CHROMATOGRAPHY AND TLC

Pigment	Visible spectra[a]			Epoxide test	
	λ_{max} found (nm)	Solvent	λ_{max} reported (nm) [14]	Hypsochromic shift	Colour
β-Carotene	429, 451, 477	Ethanol	427, 449, 475	—	—
	436, 462, 488	Choroform	435, 461, 485	—	—
Cryptoxanthin[b]	(427), 448, 471	Ethanol	(428), 449, 473	—	—
	(436), 458, 483	Chloroform	(435), 459, 485	—	—
Lutein[c]	421, 445, 474	Ethanol	422, 445, 474	—	—
	435, 461, 490	Chloroform	435, 458, 485	—	—
Lutein epoxide[c]	417, 440, 471	Ethanol	418, 442, 470	400, 422, 452	Green-blue
	426, 453, 485	Benzene	427, 454, 484	409, 436, 466	Green-blue
Violaxanthin[d]	419, 442, 471	Ethanol	417, 440, 469	381, 403, 431	Blue
	428, 453, 482	Chloroform	426, 449, 478	389, 411, 440	Blue
Neoxanthin[d]	416, 438, 466	Ethanol	415, 438, 467	397, 421, 450	Green-blue
	422, 446, 475	Chloroform	423, 448, 476	416, 430, 457	Green-blue

[a] Values in parentheses represent shoulders on spectral absorption curves.

[b] Cryptoxanthin was not originally present in water convolvulus. It was prepared from yellow corn and added to the crude extract during column chromatography.

[c] Lutein and lutein epoxide which eluted as one band on the column were further separated by TLC to determine absorption spectra.

[d] Both neoxanthin and violaxanthin were separated by open-column chromatography and TLC to determine absorption spectra.
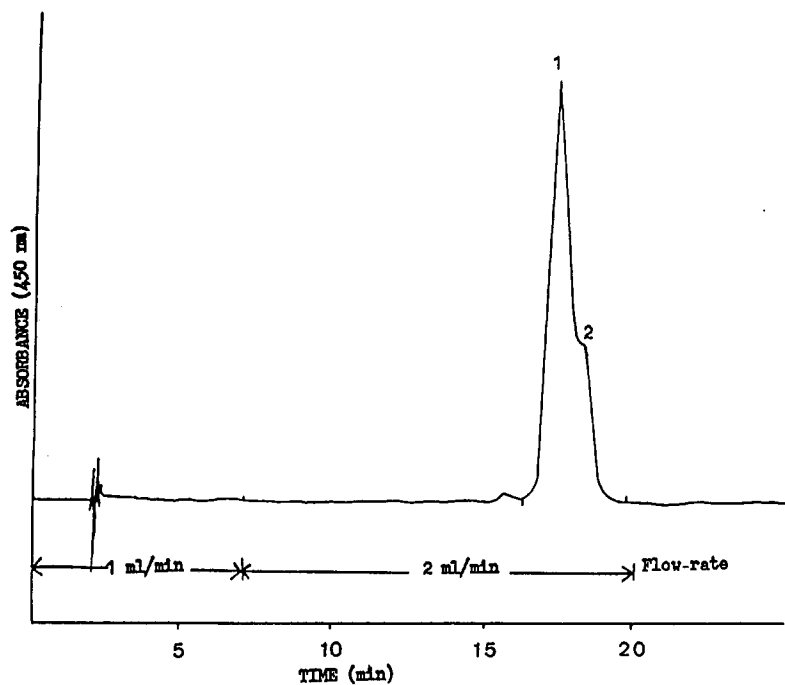
Fig. 2. Chromatogram of a carotene band eluted with hexane–acetone (96:4). Peaks: 1 = β-carotene; 2 = β-carotene isomer.
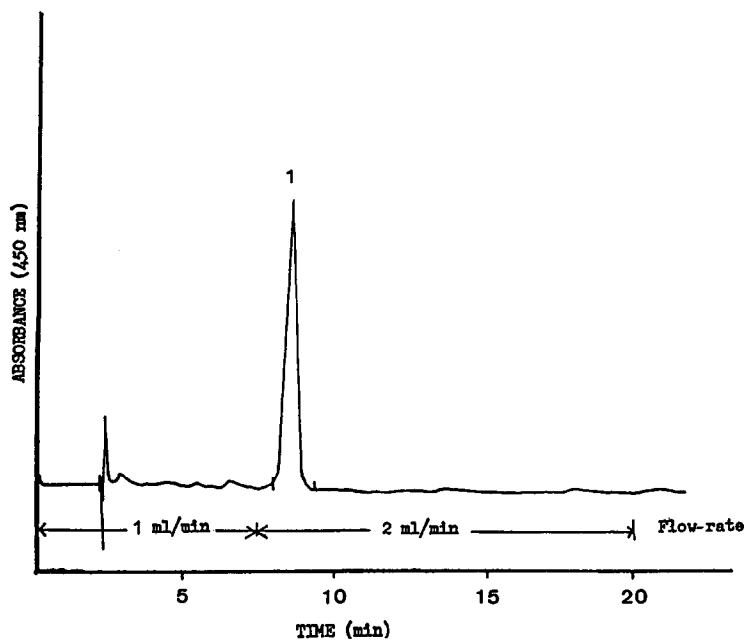

Fig. 3. Chromatogram of a cryptoxanthin band eluted with hexane–acetone (90:10). Peak 1 = cryptoxanthin.

system at 85:15:1.5. In contrast, by using the same ternary solvent system at 85:15:1 it was found that lutein was eluted followed by violaxanthin. This difference in the elution sequence of lutein and violaxanthin should be due to the amount of methanol present in the mobile phase.

Each carotenoid band eluted from the column was injected into the HPLC system to determine retention times. The low R.S.D. (less than 3% with five injections) demonstrates the high reproducibility of this method. Thus the various carotenoids in water convolvulus could be characterized by comparing the absorption spectra and retention times of the separated bands with those of reference standards.

Fig. 2 shows the chromatogram of a carotene band eluted with hexane–acetone (96:4). This band consisted mainly of β-carotene. However, there was a shoulder present, which might be due to the presence of small amount of cis-β-carotene.

Fig. 3 shows the chromatogram of a cryptoxanthin band eluted with hexane–acetone (90:10). Although cryptoxanthin was not present in water convolvulus, this result implied that this binary solvent system could adequately elute monohydroxy pigments.

Fig. 4 shows the chromatogram of a violaxanthin band eluted with hexane–acetone–methanol (85:15:0.2).

Fig. 5 shows the chromatogram of a lutein band eluted with hexane–acetone–methanol (85:15:0.2). The lutein band was not pure, as shown by the presence of two major peaks and one minor peak. This band was therefore further separated on silica
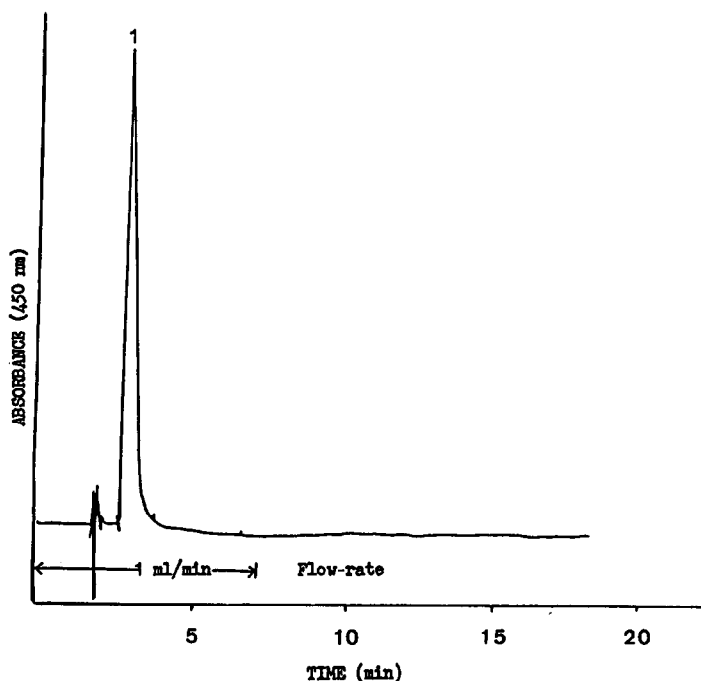

Fig. 4. Chromatogram of a violaxanthin band eluted with hexane–acetone–methanol (85:15:0.2). Peak 1 = violaxanthin.
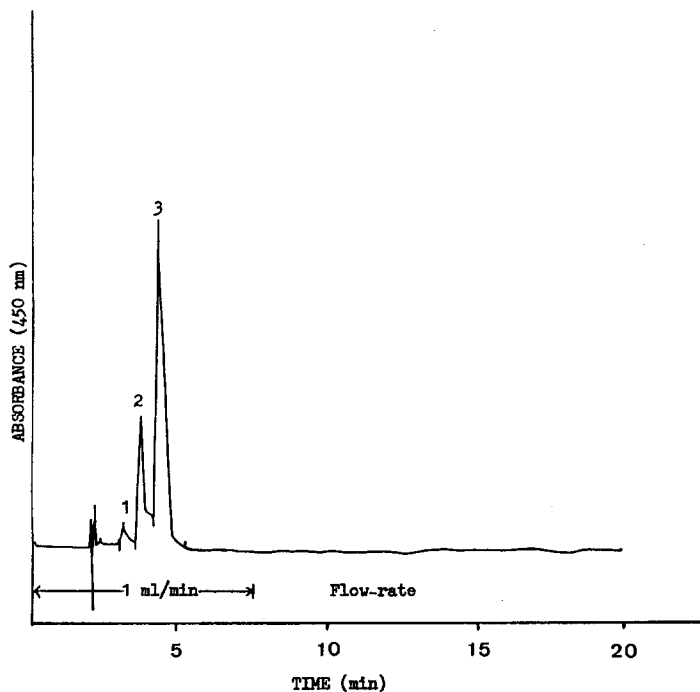
Fig. 5. Chromatogram of a lutein band eluted with hexane–acetone–methanol (85:15:0.2). Peaks: 1 = violaxanthin; 2 = lutein epoxide; 3 = lutein.
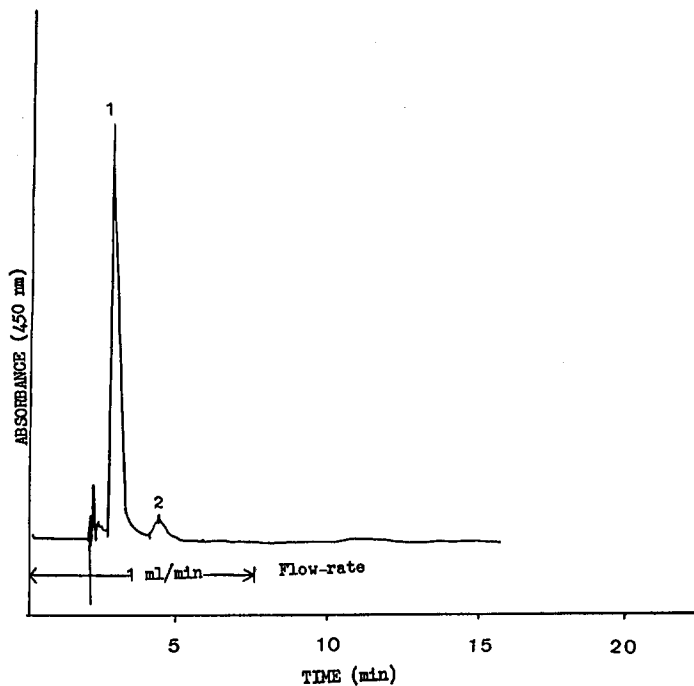

Fig. 6. Chromatogram of a neoxanthin band eluted with hexane–acetone–methanol (85:15:1.5). Peaks: 1 = neoxanthin; 2 = lutein.

gel TLC plates. Two bands were formed and were identified as lutein and lutein epoxide. Lutein epoxide was also confirmed by a hypsochromic shift on acidification with hydrochloric acid. The minor peak should be due to the presence of a violaxanthin residue, according to the retention time.

Fig. 6 shows the chromatogram of a neoxanthin band eluted with hexane–acetone–methanol (85:15:1.5). A minor peak is present, which should be due to the presence of a lutein residue, according to the retention time.

The amounts of neoxanthin, violaxanthin, lutein epoxide, lutein and β-carotene in water convolvulus were 216, 188, 28, 706 and 302 $\mu g/g$, respectively.

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REFERENCES

1 S. M. Anderson and N. I. Krinsky, *Photochem. Photobiol.*, 18 (1973) 403.
2 M. M. Mathews-Roth, *Nature (London)*, 203 (1964) 1092.
3 M. M. Mathews-Roth, *Oncology*, 39 (1982) 33.
4 M. M. Mathews-Roth, *Pure Appl. Chem.*, 57 (1985) 717.
5 A. Bendich, *J. Nutr.*, 119 (1989) 112.
6 H. M. Li and K. C. Liu, *A Survey of the Medicinal Plants in Taiwan*, National Research Institute of Chinese Medicine, Taiwan, 1973, p. 150.
7 Association of Official Analytical Chemists, *Official Methods of Analysis of the Association of Official Analytical Chemists*, AOAC, Washington, DC, 14th ed., 1984.
8 H. H. Strain, J. Sherma and M. Grandolfo, *Anal. Chem.*, 38 (1967) 926.
9 P. J. Schoenmakers, H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, 218 (1981) 259.
10 S. P. Bakalyar, R. McIlwrick and E. Roggendorf, *J. Chromatogr.*, 142 (1977) 353.
11 E. Roggendorf and R. Spatz, *J. Chromatogr.*, 204 (1981) 263.
12 F. W. Quackenbush, J. G. Firch, W. J. Rabourn, M. McQuistan, E. M. Petzold and T. E. Karl, *J. Agric. Food Chem.*, 9 (1961) 132.
13 B. H. Chen and C. A. Bailey, *J. Chromatogr.*, 393 (1987) 297.
14 B. H. Davies, in T. W. Goodwin (Editor), *Chemistry and Biochemistry of Plant Pigments*, Vol. 2, Academic Press, New York, 2nd ed., 1976, pp. 38–165.
15 H. J. C. F. Nelis and A. P. de Leenheer, *Anal. Chem.*, 55 (1983) 270.