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SEPARATION OF CAROTENOIDS IN TURF BERMUDA GRASSES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple, rapid high-performance liquid chromatographic method has been developed to separate and quantify the major carotenoids present in turf Bermuda grasses (*Cynodon dactylon*) without isomerization and oxidation. A reversed-phase isocratic solvent system of water–acetonitrile–chloroform (2:83:15) provided a clear separation of neoxanthin, violaxanthin, lutein and β-carotene. Separation occurred within 10 min with detection at 470 nm and a sensitivity at 0.01 a.u.f.s. *β-apo*-8′-Carotenal was used as an internal standard to quantify all the compounds. This method was found to be very reproducible with coefficients of variation less than 3% in 5 sample analyses. The amount of neoxanthin, violaxanthin, lutein and β-carotene in turf Bermuda grasses was 106, 127, 535 and 253 μg/g, respectively.

INTRODUCTION

Carotenoids are important biological compounds that are present in all green tissues of higher plants. Of the various higher plants, coastal Bermuda grass has been a potentially economic source of xanthophylls in pigmentation of egg yolk[1,2]. However, no information is available as to the various carotenoids present in turf Bermuda grasses (*Cynodon dactylon*).

The separation of carotenoids has been previously achieved by column chromatography[3,4] and thin-layer chromatography[5,6]. Both methods are lengthy and are susceptible to oxidative loss. Also, the separation and quantitation of the carotenoids are difficult. Recently, many high-performance liquid chromatographic (HPLC) techniques have been developed to separate carotenoids because of their fast resolution[7–13]. However, these authors used fresh fruits and vegetables from local supermarkets. It is possible that many artifacts can be formed during storage. Since rapid oxidation can occur in killed leaves[14], it is necessary to use fresh-cut and freeze-dried sample to avoid isomerization and oxidation. The purpose of this paper was to develop a HPLC procedure to separate and quantitate the major carotenoids in fresh-cut turf Bermuda grasses without isomerization and oxidation.

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MATERIALS AND METHODS

Instrumentation

The HPLC instrument consisted of a IBM LC/9533 Ternary Gradient liquid chromatograph with an IBM UV–VIS fixed wavelength detector emploring a 470 nm filter and an IBM stainless-steel column (25 cm × 4.5 mm I.D.) packed with octadecyl, C_{18} of 5 μm particle size (IBM Instrument, Danbury, CT, U.S.A.). A 0.01 a.u.f.s. was used. A Sargent-Welch recorder (Sargent-Welch Scientific, Skokie, IL, U.S.A.) was used for recording the chromatographs. Spectrophotometric determinations were made with a Beckman DU-6 Spectrophotometer (Beckman Instruments, Irvine, CA, U.S.A.). A solvent system of water–acetonitrile–chloroform (2:83:15) pumped at a flow-rae of 2.0 ml/min was used.

Materials

trans-β-Carotene was obtained from Sigma (St. Louis, MO, U.S.A.). Lutein, violaxanthin and neoxanthin standards were prepared from Bermuda grasses by thin-layer chromatography (TLC). The silica gel-G TLC plates were purchased from Whatman (Clifton, NJ, U.S.A.). Internal standard β-*apo*-8′-carotenal (20% suspension in vegetable oil) was purchased from Hoffman-LaRoche (Nutley, NJ, U.S.A.). All solvents were purchased from Fisher Scientific. Acetonitrile, water and chloroform were HPLC grade. Acetone, hexane, toluene, ethanol, methanol, ethyl acetate, benzene and light petroleum (b.p. 37.9–55.4°C) were ACS grade. HPLC-grade solvents were filtered through a 0.2-μm membrane filter and degassed under vacuum prior to use. Spinach was purchased from local supermarkets. Turf Bermuda grasses were fresh-cut from the Texas A&M University grass farm.

Standard solutions

Solutions of β-carotene were prepared in chloroform. β-*apo*-8′-Carotenal were prepared in light petroleum. Lutein, violaxanthin and neoxanthin were prepared in ethanol. Concentrations of the standard solutions were determined by the following formula

$$\text{Concentration} = E/E^{1\%}_{1\text{cm}} \cdot 100$$

where E = extinction at a given wavelength (β-carotene 465 nm, lutein 445 nm, neoxanthin 439 nm, violaxanthin 443 nm, β-*apo*-8′-carotenal 457 nm); $E^{1\%}_{1\text{cm}}$ = extinction coefficient of 1% solution measured in cell with 1 cm light path (β-carotene 2396, lutein 2550, neoxanthin 2243, violaxanthin 2550, β-*apo*-8′-carotenal 2640)[15].

Preparation and purification of internal standard

A 0.1229 g mixture of β-*apo*-8′-carotenal and vegetable oil was extracted with 30 ml hexane–acetone–ethanol–toluene (10:7:6:7) in a 100-ml volumetric flask. Fat was hydrolyzed by 2 ml 40% methanolic potassium hydroxide under nitrogen overnight. Thirty ml hexane was pipetted into a flask and diluted to volume with 10% sodium sulfate. One ml of upper phase was pipetted onto a column containing mixture of diatomaceous earth (Fisher Scientific) and adsorptive magnesia (Fisher Scientific) at 1:1 ratio. The β-*apo*-8′-carotenal was eluted with 30 ml methanol–acetone–hexane (1:1:8) at a concentration of 16.38 μg/ml.

Preparation of standard curve

Standard curves were prepared by the addition of 0.0819 mg internal standard (*β-apo*-8′-carotenal) and various concentrations of carotenoid standards to the grass sample. Results were calculated using the ratio of the peak area of the carotenoid compound over the peak area of the internal standard. Linearity and recovery were determined. Recovery studies were performed by adding 3 concentrations of *β-apo*-8′-carotenal to 1 g of Bermuda grasses. Reproducibility was conducted by using 5 samples of Bermuda grasses.

Sample preparation and extraction procedure

Bermuda grasses were fresh-cut from the turf grass farm at Texas A&M University. Immediately following cutting, the Bermuda grasses were freeze-dried and ground into fine materials. One gram of Bermuda grass was extracted with 30 ml hexane–acetone–ethanol–toluene (10:7:6:7) in a 100-ml volumetric flask. The solution was saponified by adding 2 ml 40% methanolic potassium hydroxide under nitrogen overnight. Thirty ml hexane was added to the flask and diluted to volume with 10% sodium sulfate. Ten ml of upper phase was evaporated to dryness and dissolved in 10 ml chloroform. Ten ml extract was then filtered through a 0.2-μm membrane filter. Ten μl of extract was injected onto the column and developed with water–acetonitrile–chloroform (2:83:15) with a solvent flow-rate of 2 ml/min. Samples were monitored at 470 nm with a sensitivity at 0.01 a.u.f.s. All the sample preparations were conducted under diffused light and samples were kept under nitrogen whenever possible during the procedure. Identification of the major carotenoids in Bermuda grasses was verified by comparison of retention time with standards and co-chromatography with added standards.

Separation and identification of carotenoid standards from Bermuda grasses by TLC

A solvent system of methanol–ethyl acetate–benzene (5:20:75)[15] was used to separate the major carotenoids in concentrated Bermuda grass extracts on silica gel-TLC plates. Separated pigment bands were marked and scraped into a small sintered-glass funnel attached to a 25-ml side-arm filtration flask. Pigments were eluted with acetone under slight suction. The acetone was evaporated under a stream of nitrogen and the pigments were dissolved in an appropriate solvent to measure the absorbances and spectra with a Beckman DU-6 spectrophotometer.

Individual carotenoids were characterized by comparing their R_F values and absorption spectra with values reported in the literature[15]. For co-chromatographic tests, β-carotene, lutein, violaxanthin and neoxanthin from spinach extract were used as reference standards. An epoxide test[15] was conducted to check if there is any epoxide formation during the separation of these compounds.

RESULTS AND DISCUSSION

Two typical chromatograms for carotenoid standards and carotenoids from Bermuda grass extract are shown in Figs. 1 and 2, respectively. Zakaria *et al.*[7] used acetonitrile–chloroform (92:8) to separate α- and β-carotene in tomatoes. However, this solvent system failed to resolve the xanthophylls in our column. An aqueous system, water–acetonitrile–chloroform (2:83:15), was thus developed to separate the

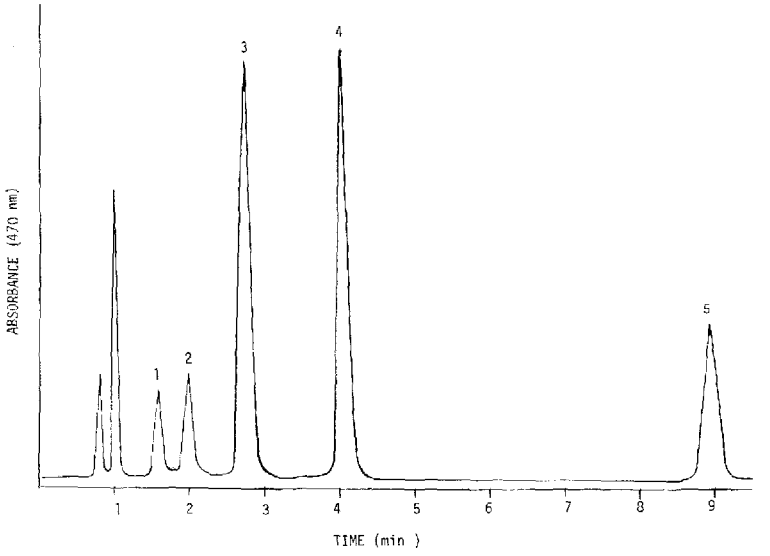

Fig. 1. A chromatogram of carotenoid standards from Bermuda grass extract. Peaks: 1 = neoxanthin; 2 = violaxanthin; 3 = lutein; 4 = *β-apo*-8′-carotenal; 5 = *β*-carotene.

xanthophyll and *β*-carotene in 10 min. Neoxanthin, violaxanthin, lutein and *β*-carotene were clearly separated to allow the quantitation of each compound. The compounds were identified by comparison of their retention times with those of the standards and co-chromatography with added standards. *β-apo*-8′-Carotenal was found to be a suitable internal standard because of its complete resolution in the column.

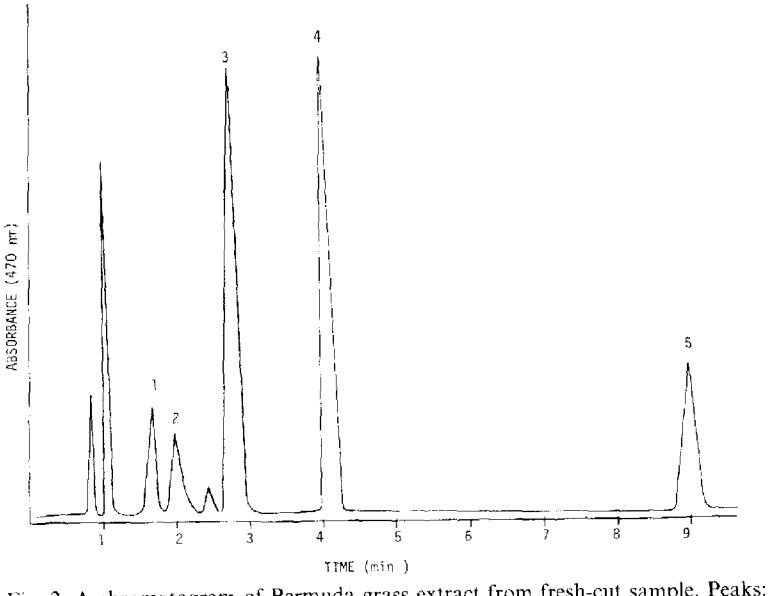

Fig. 2. A chromatogram of Bermuda grass extract from fresh-cut sample. Peaks: 1 = neoxanthin; 2 = violaxanthin; 3 = lutein; 4 = *β-apo*-8′-carotenal; 5 = *β*-carotene.

Also, it did not interfere with the separation of other compounds. When the internal standard was not added to the samples, a corresponding peak was not seen. One minor peak was not identified but may be due to the presence of another xanthophyll such as antheraxanthin.

Table I shows the identification data for the carotenoids of turf Bermuda grasses separated by TLC. A solvent system[15] of methanol–benzene–ethyl acetate (5:20:75) provided a clear separation of these compounds. An epoxide test showed that there was no epoxide formation during the separation of these compounds. Separated compounds were injected onto the column to check if there was any isomer formation. Fig. 1 shows the peak purity of carotenoid standards prepared from TLC. (We are unsure about the identity of the first two unnumbered peaks. They are present even when we inject 100% acetonitrile or 100% chloroform into the chromatography. We assume them to be an artifact of the injector used on our system.) Many minor peaks are shown in Fig. 3 when the Bermuda grasses were stored at room temperature under daylight for 2 weeks. Although these minor peaks were not identified, they are probably artifacts formed through isomerization and oxidation. The identification of carotenoids was further confirmed by co-chromatography of Bermuda grass extract with spinach extracts on silica gel TLC plates.

Repeated injections of standards and samples were used to demonstrate the reproducibility of peak areas and retention times. The chromatographic peaks of neoxanthin, violaxanthin, lutein and β-carotene were quantitated using the standard curve data shown in Table II. Table III shows the mean ± standard deviation (S.D.) concentrations of five samples. The coefficient of variation (C.V.) was less than 3% in all cases.

In the recovery study, 3 concentrations of *β-apo*-8′-carotenal were added to the grass sample. A mean recovery of 98.6% was obtained after the extraction. The

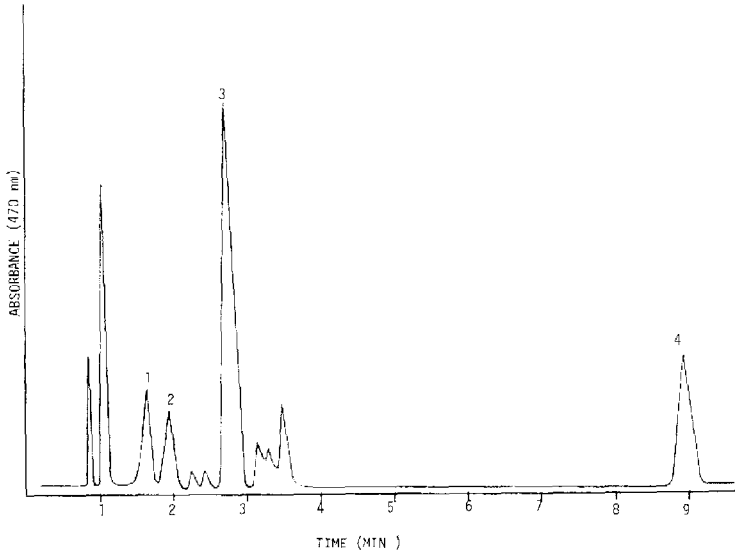

Fig. 3. A chromatogram of Bermuda grass extract from the sample stored under daylight at room temperature for two weeks. Peaks: 1 = neoxanthin; 2 = violaxanthin; 3 = lutein; 4 = β-carotene.

TABLE I

IDENTIFICATION DATA FOR PIGMENTS OF TURF BERMUDA GRASSES BY TLC

Pigment	*Visible spectra*			R_F *found*	R_F *reported*[15]	*Epoxide test*		*Color*
	Max. found	*Solvent*	*Max. reported*[15]			*Hyposochromic shift*	*Color*	
β-Carotene	434, 460, 483	Chloroform	435, 461, 485	0.96	–	–	–	Orange yellow
Lutein	423, 445, 473	Ethanol	422, 445, 474	0.56	0.57	–	–	Light orange
Violaxanthin	417, 440, 468	Ethanol	417, 440, 469	0.37	0.35	378, 400, 427	Blue	Yellow
Neoxanthin	416, 437, 465	Ethanol	415, 438, 467	0.16	0.15	400, 420, 448	Blue	Yellow

TABLE II

STANDARD CURVE DATA FOR NEOXANTHIN, VIOLAXANTHIN, LUTEIN AND β-CAROTENE

Pigment	*Slope*	*y-intercept*	r^2	*Linear range* (ng)*
β-Carotene	0.013	−0.027	0.9960	10–100
Lutein	0.013	−0.005	0.9960	20–200
Violaxanthin	0.011	−0.019	0.9266	5–30
Neoxanthin	0.011	−0.003	0.9711	5–30

* Quantity injected to obtain standard curve.

TABLE III

QUANTITATION OF β-CAROTENE, LUTEIN, VIOLAXANTHIN AND NEOXANTHIN IN TURF BERMUDA GRASSES (IN μg/g)

Sample No.	*β-Carotene*	*Lutein*	*Violaxanthin*	*Neoxanthin*	*Unknown fraction*
1	252.4	535.0	126.3	105.1	42.6
2	249.7	530.3	124.8	104.2	41.6
3	250.9	531.5	125.1	104.7	43.0
4	254.4	537.8	127.7	108.4	44.6
5	255.7	538.6	128.9	109.5	42.8
Mean	252.6	534.6	126.6	106.4	42.9
S.D.	2.5	3.7	1.7	2.4	1.1
C.V. (%)	1.0	0.7	1.3	2.3	2.6

recovery values of neoxanthin, violaxanthin, lutein and β-carotene were not calculated, but were assumed to be of the same order.

In summary, a rapid HPLC reversed-phase method was developed for the separation of carotenoids in turf Bermuda grasses. Separation occurred in less than 10 min by employing an isocratic system water–acetonitrile–chloroform (2:83:15) and a 5-μm particle column. β-*apo*-8′-Carotenal was found to be a suitable internal standard to quantify all the compounds. Repeated injections of standards and samples were used to demonstrate the reproducibility of peak areas and retention times. Isomerization and oxidation were minimized by using fresh-cut and freeze-dried samples and by performing the sample extraction under nitrogen atmosphere. Also, the pigment decomposition and formation of artifacts during the separation were minimized because the total elution time is so short.

REFERENCES

1 B. D. Barnett and C. L. Morgan, *Poult. Sci.*, 38 (1959) 1188.
2 H. O. Wheeler and D. E. Turk, *Tech. Bul.. S.C. Agric. Exp. Stan.*, 1009 (1963) 1.
3 D. B. Rodriguez, L. C. Raymundo, T. C. Lee, K. L. Simpson and C. O. Chichester, *Ann. Biol.*, 40 (1976) 615.
4 S. E. Gebhardt, E. R. Elkins and J. Humphrey, *J. Agric. Food. Chem.*, 25 (1977) 629.
5 A. K. Baloch, K. A. Buckle and R. A. Edwards, *J. Chromatogr.*, 139 (1977) 149.

6 K. A. Buckle and F. M. M. Rhaman, *J. Chromatogr.*, 171 (1979) 385.
7 M. Zakaria, K. Simpson, P. R. Brown and A. Krstulovic, *J. Chromatogr.*, 176 (1979) 109.
8 R. J. Bushway and A. M. Wilson, *Can. Inst. Food Sci. Technol. J.*, 15 (1982) 165.
9 Y. P. C. Hsieh and M. Karel, *J. Chromatogr.*, 259 (1983) 515.
10 C. R. Broich, L. E. Gerber and J. W. Erdman, Jr., *Lipids*, 18 (1983) 253.
11 K. W. Miller, N. A. Lorr and C. S. Yang, *Anal. Biochem.*, 138 (1984) 340.
12 J. L. Bureau and R. J. Bushway, *J. Food Sci.*, 51 (1986) 128.
13 F. Khachik, G. R. Beecher and N. F. Whittaker, *J. Agric. Food Chem.*, 34 (1986) 603.
14 H. H. Strain, *Leaf Xanthophylls*, Carnegie Institution of Washington, Washington, DC, 1938.
15 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.