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Note

Simultaneous separation and identification of carotenoids and chlorophylls in turf bermudagrass by high-performance liquid chromatography

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Both carotenoids and chlorophylls are important biological compounds that are present in green tissues of higher plants. It has been well established[1,2] that the primary function of carotenoids in plant tissue is to prevent photosensitization by endogenous photosensitizers such as chlorophylls. Due to the different polar characteristics of carotenoids and chlorophylls, the separation of these pigments has been difficult. Therefore, a simple, rapid method for the simultaneous separation of carotenoids and chlorophylls is important in order to learn more about the role of these pigments in photosynthesis.

The separation of carotenoids and chlorophylls by high-performance liquid chromatography (HPLC) has been well documented[3–9]. However, these methods are lengthy and time-consuming. Some methods[3,4] even require 75 min or longer for complete elution of these pigments. In a previous study[10] we reported the separation of major carotenoids in turf bermudagrass. By using a 15-cm, reversed-phase column we have since been able to resolve 19 peaks within 10 min, of which 10 pigments were identified. These pigments included xanthophylls, chlorophylls and β-carotene.

EXPERIMENTAL

Instrumentation

The HPLC instrument consisted of an IBM (Danbury, CT, U.S.A.) LC 9533 ternary gradient liquid chromatography with an IBM UV–VIS fixed-wavelength detector employing a 440 nm filter and a Water Nova-Pak C_{18} stainless-steel column (15 cm × 3.9 mm I.D.). A Sargent-Welch recorder (Skokie, IL, U.S.A.) was used for recording the chromatographs at 0.05 a.u.f.s. Spectrophotometric determinations were made with a Beckman (Irvine, CA, U.S.A.) DU-6 spectrophotometer. Water–acetonitrile–chloroform (2:83:15)[10], pumped at a flow-rate of 1.0 ml/min, was used as the solvent system.

Materials

Trans-β-carotene, chlorophyll *a* and chlorophyll *b* standards were purchased from Sigma (St. Louis, MO, U.S.A.). Neoxanthin and violaxanthin standards were prepared from saponified turf bermudagrass extract by thin-layer chromatograpy

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(TLC) as described by Chen and Bailey[10]. Lutein and zeaxanthin standards were prepared from corn by open-column chromatography using a method[11] similar to that described by Quackenbush *et al.*[12]. Lutein monoester was prepared from unsaponified turf bermudagrass extract by TLC. Pheophytins *a* and *b* were prepared by adding a few drops of 0.01 *N* ethanolic HCl to chlorophylls *a* and *b*, respectively[9]. The silica gel G TLC plates were purchased from Whatman (Clifton, NJ, U.S.A.). All solvents were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). Acetonitrile, water and chloroform were HPLC grade. Acetone, hexane, toluene, ethanol, methanol, ethyl acetate, light petroleum (b.p. 37.9–55.4°C) and benzene were ACS grade. HPLC-grade solvents were filtered through a 0.2-μm membrane filter and degassed under vacuum prior to use.

Turf bermudagrass was fresh-cut from Maxim Productions in South Central Texas. The grass was stored in a dark, ventilated room to dry for 1 week before grinding into fine materials with a hammer mill fitted with a 3.2 mm screen.

Preparation of lutein monoester from unsaponified turf bermudagrass extract

A solvent system of methanol–ethyl acetate–benzene (5:20:75)[13] was used to separate the major carotenoids, lutein monoester and chlorophylls in concentrated turf bermudagrass extracts on silica gel G TLC plates. Individual carotenoids, lutein monoester and chlorophylls were characterized by determining their R_F values and absorption spectra. For co-chromatographic test, β-carotene, chlorophyll *a*, chlorophyll *b*, pheophytin *a*, pheophytin *b*, lutein, violaxanthin and neoxanthin were used as reference standards.

Extraction and HPLC analysis of unsaponified turf bermudagrass extract

A 1-g amount of ground turf bermudagrass was extracted with 30 ml hexane–acetone–ethanol–toluene (10:7:6:7) in a 100-ml volumetric flask under nitrogen overnight. A 30-ml volume of hexane was added to each flask which was then diluted to volume with 10% sodium sulfate and mixed. After 1 h, 10 ml of the upper phase were evaporated to dryness and dissolved in 10 ml chloroform and filtered through a 0.2-μm membrane filter. A 10-μl volume of extract was injected onto the HPLC column and developed with water–acetonitrile–chloroform (2:83:15) at a flow-rate of 1.0 ml/min. Samples were monitored at 4.40 nm with a sensitivity of 0.05 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 various carotenoids and chlorophylls in turf bermudagrass was verified by comparison of retention time with standards and co-chromatography with added standards.

RESULTS AND DISCUSSION

Fig. 1 presents the chromatogram of carotenoid and chlorophyll standards. Although the water–acetonitrile–chloroform (2:83:15)[10] solvent system provides a clear separation of these standards, there were minor peaks present. These were probably lutein and zeaxanthin isomers originally present in corn extract.

Fig. 2 presents the chromatogram of unsaponified turf bermudagrass extract. The major peaks were identified by comparison of retention time with standards and

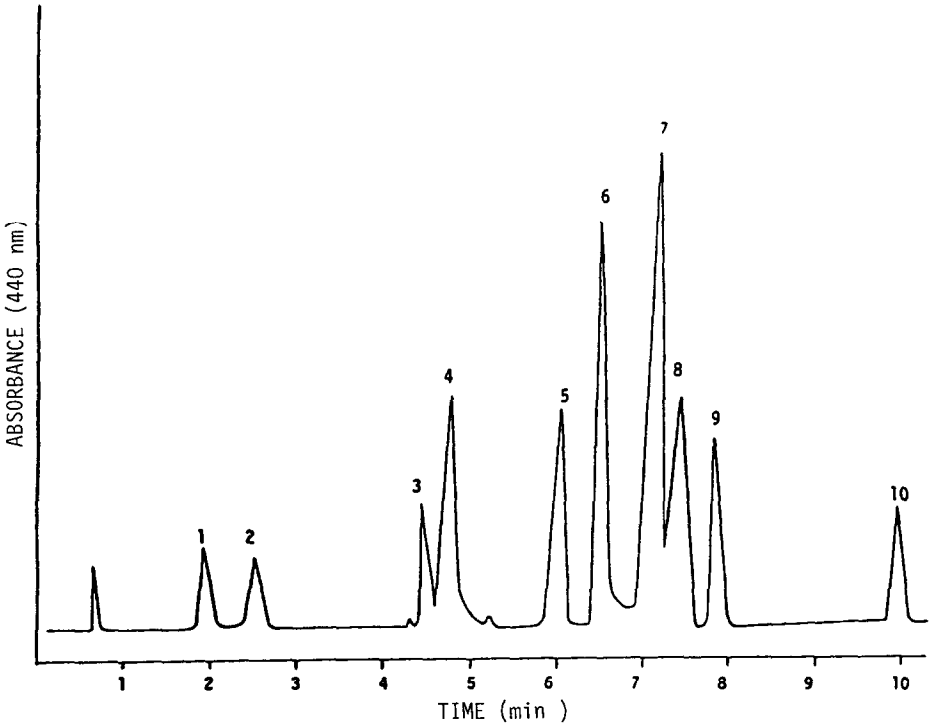

Fig. 1. A chromatogram of carotenoid and chlorophyll standards by employing an isocratic solvent system of water–acetonitrile–chloroform (2:83:15). Peaks: 1 = neoxanthin; 2 = violaxanthin; 3 = zeaxanthin; 4 = lutein; 5 = lutein monoester; 6 = chlorophyll *b*; 7 = chlorophyll *a*; 8 = pheophytin *b*; 9 = pheophytin *a*; 10 = *β*-carotene.

co-chromatography with added standards. Some minor peaks were not identified but may be due to the artifacts formed during storage of turf bermudagrass[10]. Peak 5 was identified as lutein monoester based on visible spectra and R_F value. It has been reported that xanthophylls normally occur unesterified in the chloroplast but during senescence, when chloroplasts disintegrate, the xanthophylls released into the cytoplasm are esterified[14]. Therefore, most xanthophylls in ripe or aged plants occur naturally acylated with fatty acids[15–19]. Lutein and lutein monoester have identical visible spectra, indicating that no color change occurs as a result of esterification. The presence of only small amounts of lutein monoester indicated that most xanthophylls in green turf bermudagrass remain unesterified.

Table I shows the identification data of various pigments from unsaponified turf bermudagrass extract by TLC. Compared to carotenoids, chlorophyll *a* and chlorophyll *b* were present in large amounts of turf bermudagrass extract as shown by the presence of two large peaks on the HPLC chromatogram (Fig. 2). Pheophytins *a* and *b*, the most common derivatives of chlorophylls *a* and *b*, were also present in turf bermudagrass. The absorption spectra of each pigment were compared with those reported in the literature[3–9]. A hypsochromic shift upon acidification with HCl was used to identify 5,6-epoxycarotenoids such as neoxanthin and violaxanthin[13].

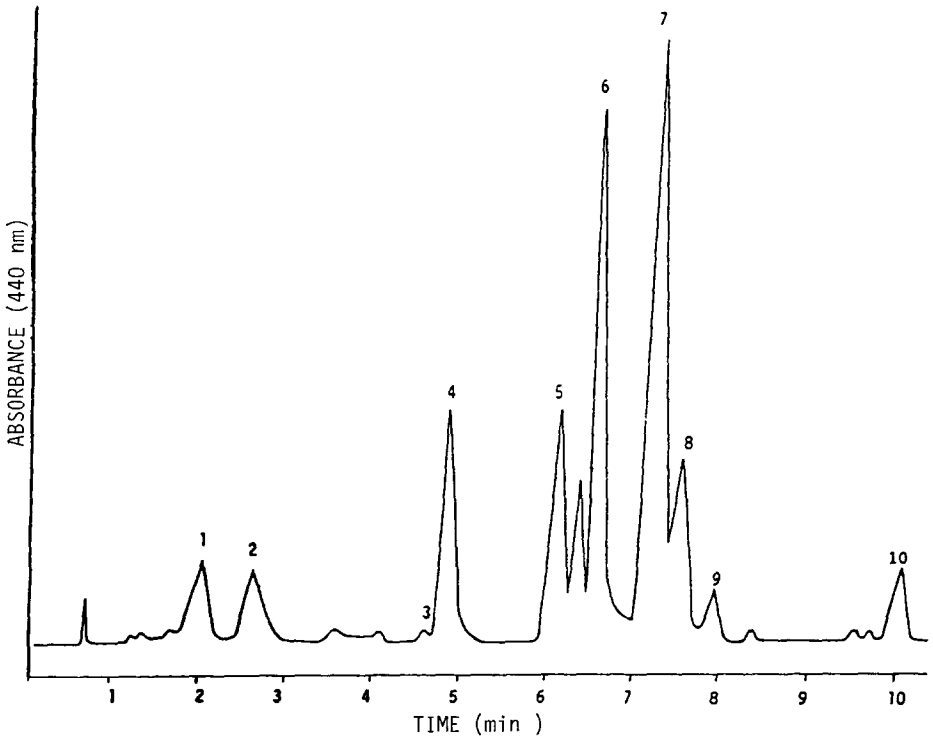

Fig. 2. A chromatogram of unsaponified turf bermudagrass extract. Peaks: 1 = neoxanthin; 2 = violaxanthin; 3 = zeaxanthin; 4 = lutein; 5 = lutein monoester; 6 = chlorophyll *b*; 7 = chlorophyll *a*; 8 = pheophytin *b*; 9 = pheophytin *a*; 10 = β-carotene.

TABLE I
TLC IDENTIFICATION OF VARIOUS PIGMENTS FROM UNSAPONIFIED TURF BERMUDAGRASS EXTRACTS

Pigment	*Visible spectra*		R_F	*Epoxide test*	
	Maxima found (nm)	*Solvent*		*Hypsochromic shift (nm)*	*Color*
β-Carotene	434, 460, 483	Chloroform	0.95	–	–
Chlorophyll *a*	660, 432	Acetone	0.74	–	–
Chlorophyll *b*	645, 454	Acetone	0.65	–	–
Lutein monester	423, 444, 475	Ethanol	0.61	–	–
Lutein	423, 445, 473	Ethanol	0.52	–	–
Violaxanthin	417, 440, 468	Ethanol	0.35	378, 400, 427,	Blue
Neoxanthin	416, 437, 465	Ethanol	0.14	400, 420, 448	Blue
Pheophytin *a**	670, 410	Acetone	0.78	–	–
Pheophytin *b*	658, 430	Acetone	0.75	–	–

* Pheophytin *a* and *b* were prepared from chlorophyll *a* and *b* standards, respectively. They were purified by TLC to determine visible spectra and R_F value.

Reproducibility and precision were checked by making a series of 10 injections of lutein, chlorophyll *a* and β-carotene standards with an IBM Instrument LC/9505 auto sampler. Coefficients of variation in regard to peak area were 2.0, 2.0 and 8.3%, respectively. Coefficients of variation with respect to retention time were 0.4, 0.4 and 0.5%, respectively.

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