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Resolution of lutein and zeaxanthin using a non-endcapped, lightly carbon-loaded C_{18} high-performance liquid chromatographic column

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ABSTRACT

A new rapid and reproducible high-performance liquid chromatographic method using Spherisorb ODS-1, a non-endcapped, lightly carbon-loaded column material, for the separation of higher-plant chloroplast pigments is described. The method resolves lutein and zeaxanthin, as well as all other major and most minor pigments at or near baseline by either of two solvent programs. Program I is faster and more sensitive than program II while the latter resolves pheophytin *a* and β,ε-carotene slightly better than program I. Both programs use an initial buffered aqueous mixture that appears critical for this application of ODS-1. The method is well suited for analysis of xanthophyll-cycle pigment changes.

INTRODUCTION

Baseline separation of plastid pigments from higher plants in a simple, reproducible, one-step method has yet to be reported. Difficulties arise from the wide-ranging polarities of the comprising pigments and the limited selectivity of the columns. The carotenes are non-polar whereas at the other extreme 9′-*cis*-neoxanthin is polar. Separation of structural isomers such as lutein from zeaxanthin and β,ε-carotene from β,β-carotene is usually incomplete in most reversed-phase high-performance liquid chromatographic (HPLC) procedures. Of several aqueous [1–9] and non-aqueous [10,11] reversed-phase HPLC methods reported for plant and algal pigments, only one method separates lutein from zeaxanthin at the baseline [12]. Rapid quantitative separation of lutein and zeaxanthin has become important for research on photoprotective processes in plants because of the apparent relationship between zeaxanthin and non-photochemical quenching of excess energy in the antennae chlorophylls of photosystem II [13]. Light induces changes in zeaxanthin levels via interconversions with violaxanthin and antheraxanthin in the xanthophyll cycle [14].

Thayer and Björkman [12] obtained baseline separation of lutein and zeaxanthin with a non-endcapped Zorbax-ODS column. Unfortunately this packing material is not presently being manufactured and therefore is not widely available. We sought an alternative solution and here report a new method using ODS-1, a non-endcapped and

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lightly carbon-loaded material. The method separates lutein and zeaxanthin and most other chloroplast pigments at or near baseline. Procedures for analyses of pigment extracts from whole leaves and isolated chloroplasts and the applicability of the method for xanthophyll-cycle studies are detailed.

EXPERIMENTAL

Instrumentation

The chromatographic system was a Beckman/Altex Model 334 gradient liquid chromatograph (Beckman Instruments, Berkeley, CA, U.S.A.) equipped with a Waters 990 photodiode array detector (Millipore, Milford, MA, U.S.A.). All solvents were HPLC grade and obtained from Fisher Scientific, Fair Lawn, NJ, U.S.A. Pigment standards were quantitated spectrophotometrically with a DW-2000 UV–VIS dual-wavelength dual-beam spectrophotometer (SLM Instruments, Urbana, IL, U.S.A.).

Spherisorb ODS-1 columns (5-μm particle size, 250 mm × 4.6 mm I.D.) were from Alltech, Deerfield, IL, U.S.A. ODS-1 is a non-endcapped, 6% carbon, spherical silica material manufactured by Phase Separations, Clwyd, U.K. The guard column was ODS-1 or Adsorbosphere C_{18} direct-connect cartridge, also from Alltech.

Liquid chromatography

The flow-rate for all separations was 2 ml min^{-1} and all sample injections were 20 μl. Two solvent programs were developed. Program I: solvent A-1 was ran isocratically from 0 to 4 min followed by a 2.5-min linear gradient to 100% solvent B. Program II: solvent A-1 was ran isocratically for 6 min followed by a 10-min linear gradient to 100% solvent C. Solvent A-2 replaced solvent A-1 in some experiments. Solvent mixtures were: A-1, acetonitrile–methanol–Tris · HCl buffer 0.1 *M* pH 8.0 (72:8:3); A-2, acetonitrile–methanol–Tris · HCl buffer 0.1 *M* pH 8.0 (75:12:4); B, methanol–hexane (4:1); C, methanol–ethyl acetate (68:32).

The columns were re-equilibrated between samples for a minimum of 10 min with solvent A-1 for both solvent programs. When changing solvent programs the columns were equilibrated with 60 ml of solvent B or C, and then with 30 ml of solvent A-1. This extensive re-equilibration was necessary when changing programs to remove residual effects of the prior solvents B or C. All runs were at room temperature.

Pigment identification and calibration

β,ε-Carotene and β,β-carotene were obtained from Sigma, St. Louis, MO, U.S.A. Violaxanthin, lutein and zeaxanthin were isolated according to Yamamoto *et al.* [15]. Antheraxanthin, 9′-*cis*-neoxanthin, lactucaxanthin and pheophytins *a* and *b* were identified by absorption spectra.

Chlorophylls *a* and *b* were quantitated according to Vernon [16]. Lutein, violaxanthin and zeaxanthin standards were in ethanol and β,ε-carotene and β,β-carotene were in hexane. Extinction coefficients ($E^{1\%}_{1cm}$) used for quantitation were: lutein and violaxanthin (2550), zeaxanthin (2540), β,ε-carotene (2725) and β,β-carotene (2590) [17]. Linearity of the peak-area (absorbance units × minutes) calibrations against pigment concentrations was $r^2 \geqslant 0.991$ for all pigment standards. The photodiode-detector wavelength for integration of peak areas was 440 nm.

Conversion factors for peak area to nmol per injection for program I, solvent A-1 were: violaxanthin (20.72); lutein (27.10); zeaxanthin (26.90); chlorophyll *a* (34.94); chlorophyll *b* (38.53); β,ε-carotene (18.51); β,β-carotene (18.94). Antheraxanthin was estimated with the conversion factor for lutein. Lactucaxanthin and 9′-*cis*-neoxanthin concentrations were estimated using the conversion factor for violaxanthin.

Preparation of isolated chloroplast and leaf-disk samples

Leaf disks and isolated chloroplasts with high and low levels of zeaxanthin were prepared to demonstrate the effectiveness of the method for quantitative analysis of the xanthophyll cycle. Chloroplasts were isolated from market lettuce (*Lactuca sativa* L. cv. Romaine) according to Yamamoto *et al.* [18]. Prior to isolation, the leaves were dark-adapted for 12 h to reduce the background level of zeaxanthin. Addition of 10 m*M* sodium ascorbate induced zeaxanthin formation in osmotically shocked chloroplasts suspended in 50 m*M* sodium citrate buffer at pH 5.0. Zeaxanthin formation was stopped after 10 min with 1.5 m*M* DTT. All reactions had a final volume of 3 ml and the chlorophyll concentration was 30 μg total chlorophyll per ml. Chloroplast suspensions were divided into two microcentrifuge tubes, centrifuged for 5 min and the resulting pellet extracted as described under pigment extractions.

Leaf disks (10 cm^2, approximately 0.25 g) were punched from fully developed leaves of shade-grown *Anthurium andraeanum* cv. Brown Tulip. Prior to removal of disks, the plant was dark-adapted for 12 to 15 h to reduce the background level of zeaxanthin. For light-induced zeaxanthin formation, a leaf disk was floated on water in a water-jacketed beaker and exposed to 2000 $\mu E\, m^{-2}\, s^{-1}$ white light for 20 min from a Model 640-HD lamp (Acme Light, Skokie, IL, U.S.A.). The light was filtered through 2.5 cm of refrigerated circulating water to remove heat. Leaf-disk temperature remained between 18 and 25°C.

Pigment extractions

Pigments were extracted at room temperature and under dim laboratory light. Chloroplast pellets (45 μg total chlorophyll) were suspended in 0.25 ml 100% acetone for 5 min at room temperature with occasional vortex mixing, centrifuged for 5 min in a microcentrifuge and the resulting supernatant saved. The acetone-dried pellets were re-extracted as above to ensure complete extraction of β,β-carotene and β,ε-carotene. The supernatants were pooled and then filtered through 0.2 μm nylon-66 microcentrifuge filters (Microfilterfuge; Rainin, Woburn, MA, U.S.A.). Leaf disks were ground in a tissue homogenizer with 25 mg $CaCO_3$ and 2.5 ml 100% acetone. The extract was divided into two microcentrifuge tubes and spun for 5 min. The supernatants were removed and the pellets extracted again with 1.25 ml 100% acetone each at room temperature for 5 min, with occasional vortex mixing before spinning again for 5 min. The supernatants were pooled and filtered as described for chloroplast extracts. The pigment extracts were either analyzed immediately or after 1 to 2 days storage at −20°C under argon. No pigment degradation was observed during this storage period.

RESULTS AND DISCUSSION

Solvent programs

Fig. 1 shows chromatograms of *Anthurium* extracts using solvent program I. The extracts were prepared from dark-adapted (A) and light-treated leaf disks (B). Fig. 1C shows the extract in (B) after acid treatment. The program resolved lutein, zeaxanthin and most of the other plastid pigments at baseline in about 13 min. A low level of zeaxanthin was detectable in the dark-adapted sample. Separation of β,β- and β,ε-carotene was incomplete although adequate for detection and estimation.

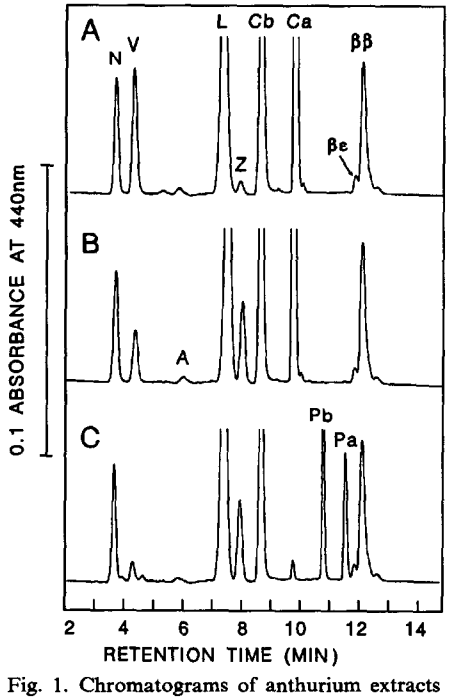

Fig. 1. Chromatograms of anthurium extracts on column 1 with program I, solvent A-1. (A) Before zeaxanthin formation; (B) after zeaxanthin formation; and (C) after acid treatment of the same extract used in B. Abbreviations: N = 9′-*cis*-Neoxanthin; V = violaxanthin; A = antheraxanthin; L = lutein; Z = zeaxanthin; Cb = chlorophyll *b*; Ca = chlorophyll *a*; $\beta\varepsilon$ = β,ε-carotene; $\beta\beta$ = β,β-carotene; Pb = pheophytin *b*; Pa = pheophytin *a*.

Pheophytins *a* and *b* were resolved but the former just barely from β,ε-carotene. Fig. 2 shows that the method also resolves lactucaxanthin in lettuce extracts. Zeaxanthin was undetectable in the dark-adapted lettuce (Fig. 2A). Lettuce also apparently lacks β,ε-carotene.

Increasing the hexane content in solvent B from methanol–hexane (4:1) to (3:1) improved the separation of the pheophytins and carotenes but also introduced a refractive-index change that interfered with the quantitation of the carotenes (data not shown). The proximity of pheophytin *a* to β,ε-carotene with program I is not critical for most applications since pheophytins are not usually detectable at 440 nm in

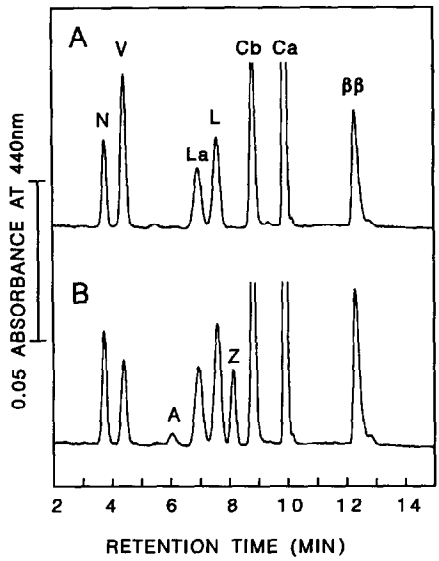

Fig. 2. Chromatograms of lettuce extracts on column 1 with solvent program I, solvent A-1. (A) Before zeaxanthin formation; (B) after zeaxanthin formation. La = lactucaxanthin; other abbreviations as in Fig. 1.

undegraded pigments extracts. When maximum separation of the carotenes and pheophytins is important, program II can be used. Fig. 3 shows that the separations with program II were comparable to program I except that the pheophytins eluted after the carotenes (Fig. 3C) and were more completely resolved from the carotenes. Program II, however, is appreciably longer than program I.

Separation of lutein from zeaxanthin is reportedly less enhanced with endcapped materials [7,12]. Indeed, endcapped column materials such as Lichrosorb RP-18 or ODS-2 did not separate zeaxanthin and lutein satisfactorily with this method (data not shown). Presumably interaction of these pigments with the exposed silanol sites of the non-endcapped ODS-1 material is important. The aqueous condition in solvent A-1 was a key to the successful application of ODS-1 inasmuch as zeaxanthin and chlorophyll *b* were otherwise unresolved. We speculate that water is required for sufficient interaction between the pigments and the lightly carbon-loaded ODS-1 material.

The Tris buffer in solvent A-1 neutralizes the acidity of the ODS-1 columns. Without Tris the chlorophylls and carotenoids degraded. Tris buffer also neutralizes acids inherently present in the acetonitrile solvent [1]. Although chloride ions are known to harm stainless steel we have seen no evidence of corrosion in our system. We flush the system with 20–30 ml of methanol after each days' runs to minimize corrosion and to eliminate residual hexane in the column material. This method demonstrates that non-endcapped column materials, which may have been previously avoided because of their tendency to isomerize and degrade pigments, can be used successfully for pigment separations with proper precautions.

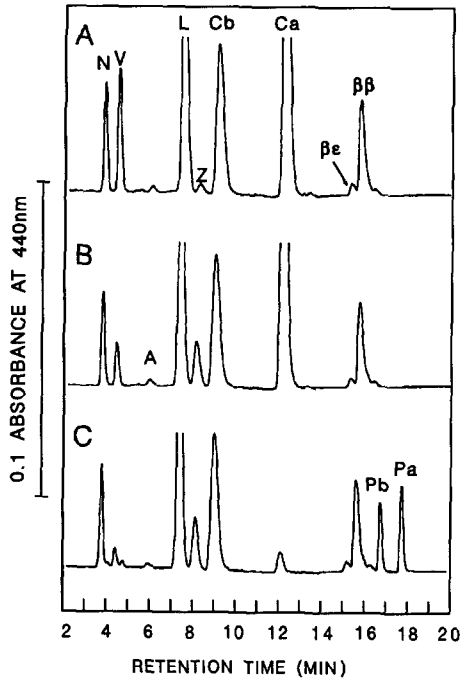

Fig. 3. Chromatograms of the same extracts as in Fig. 1 on column 1 with program II, solvent A-1. Abbreviations as in Fig. 2.

Column variability

Column variability is common in HPLC and may be even greater in non-endcapped materials. We tested three ODS-1 columns designated 1, 2 and 3. Whereas columns 1 and 2 gave similar separations, column 3 performed poorly. As shown in Fig. 4A, the resolution of zeaxanthin and chlorophyll *b* was poor and peak sensitivity

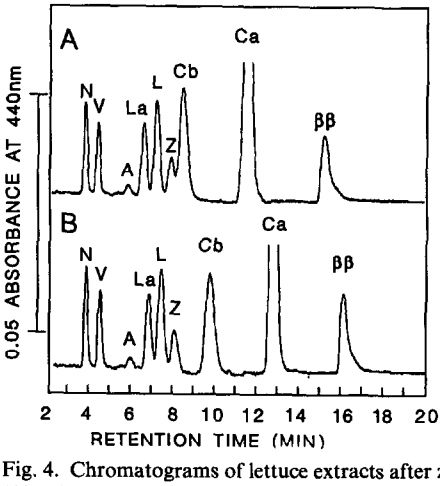

Fig. 4. Chromatograms of lettuce extracts after zeaxanthin formation on column 3 with program II using (A) solvent A-1 and (B) solvent A-2. Abbreviations as in Fig. 2.

was low on column 3 (solvent A-1, program II). We found that the poor resolution of column 3 was improved by further increasing the water and methanol content of the mobile phase. Fig. 4B shows that using the more aqueous A-2 mixture resolved lactucaxanthin, lutein, zeaxanthin and chlorophyll *b* adequately but still not as well as on columns 1 and 2. Apparently, increasing the polarity of the mobile phase by increasing the Tris · HCl buffer and methanol higher than in solvent A-1 caused chlorophyll *b* to interact sufficiently with the stationary phase to separate zeaxanthin. Solvent A-2 also worked satisfactorily with columns 1 and 2, but the resolution and sensitivity for lactucaxanthin, lutein and zeaxanthin was higher with solvent A-1. Thus, we regard solvent A-2 as a second-line solvent to be used only with columns that do not perform satisfactorily with solvent A-1.

The source of the observed column differences is not known. The supplier's test chromatograms for columns 1, 2 and 3 for the separation of ethyl benzene were 75 575, 125 479, and 101 429 plates m^{-1}, respectively. Thus, the reported efficiencies do not explain the performance difference between the columns. Column 3, however, separated N,N-diethyl-*m*-toluamide and toluene by only 0.56 min whereas columns 1 and 2 separated these compounds by 0.91 and 1.22 min, respectively. The separation of these test components appears to correlate with the poorer resolution of chlorophyll *b* and zeaxanthin on column 3 (Fig. 4A). The increased water-content requirement in the mobile phase suggests column 3 may have slightly less carbon loading than the other columns.

Retention time and sensitivity of solvent programs I and II

Table I compares the relative retention times for the major pigments of lettuce on column 1. Retention times varied by less than 0.05 min from the mean in five successive runs for either solvent program. The polar xanthophylls eluted earlier in program II than in program I, whereas the chlorophylls and β,β-carotene eluted later. The peak heights (sensitivity) of the major non-polar pigments were significantly higher in program I than program II, whereas the sensitivities for the polar xanthophylls were similar. For example, chlorophylls *b* and *a* were approximately two- and three-fold higher, respectively, for program I than for program II. Also, β,β-carotene peaks were over 50% higher in program I than in program II. In our studies the rapidity and increased sensitivity of program I outweighed the resolution problem of pheophytin *a* and β,ε-carotene. The detectable limit for individual xanthophyll pigments (V, A or Z) was about 5–7 pmol per 20-μl injection for program I. This is lower sensitivity than that reported by Thayer and Björkman [12] but was more than sufficient for studies involving intact leaf tissue and isolated chloroplasts. The sensitivity can be increased by slowing the flow-rate in both programs to 1 ml per min and proportionally adjusting the changeovers to solvents B or C.

Quantitative analyses of xanthophyll cycle changes with ODS-1

The following data demonstrate the usefulness of this method for quantitative analysis for the violaxanthin-cycle. Table II shows the relative pigment content in lettuce chloroplasts before and after dark ascorbate-induced zeaxanthin formation. Stimulation of violaxanthin de-epoxidation converts violaxanthin to zeaxanthin with virtual mol to mol stoichiometry [14]. The total relative concentrations of the violaxanthin cycle pigments (V + A + Z) were consistent within standard deviation for

TABLE I

RETENTION TIMES (MIN) FOR MAJOR LETTUCE CHLOROPLAST PIGMENTS FOR FIVE SUCCESSIVE SEPARATIONS WITH BOTH SOLVENT PROGRAMS I AND II

Separation	Retention time (min)						
	N	V	La	L	Cb	Ca	ββ
Solvent program I[a]							
1	3.81	4.47	7.00	7.66	8.89	9.96	12.34
2	3.81	4.46	7.02	7.68	8.89	9.96	12.36
3	3.82	4.47	7.03	7.68	8.90	9.99	12.37
4	3.85	4.53	7.11	7.76	8.93	10.00	12.38
5	3.87	4.52	7.11	7.76	8.95	10.00	12.38
Mean	3.83	4.49	7.05	7.71	8.91	9.98	12.37
S.D.	0.03	0.03	0.05	0.05	0.03	0.02	0.02
Solvent program II[b]							
1	3.74	4.40	6.78	7.48	9.13	12.33	15.96
2	3.75	4.41	6.79	7.47	9.11	12.28	15.88
3	3.75	4.41	6.80	7.49	9.11	12.28	15.84
4	3.77	4.43	6.80	7.48	9.17	12.34	15.85
5	3.81	4.47	6.86	7.55	9.24	12.40	15.88
Mean	3.76	4.42	6.81	7.49	9.15	12.33	15.88
S.D.	0.03	0.03	0.03	0.03	0.05	0.05	0.05

[a] Solvent system same as in Fig. 1.
[b] Solvent system same as in Fig. 3.

TABLE II

RELATIVE PIGMENT CONCENTRATION FOR LETTUCE CHLOROPLASTS BEFORE AND AFTER DARK, pH 5.0, ASCORBATE-INDUCED ZEAXANTHIN FORMATION

All values are relative to chlorophyll *a* (mmol mol^{-1} Ca), except Cb/Ca which is (mol/mol). All values are the mean of three individual experiments either before or after zeaxanthin formation. Solvent program I, solvent A-1, and column 1 were used for all runs. Abbreviations same as Fig. 2.

	Pigment concentration								
	N	V	A	Z	V+A+Z	La	L	Cb/Ca	ββ
Pre-zeaxanthin									
Mean	81.89	163.75	0.00	0.00	163.75	93.96	171.98	0.35	134.85
S.D.	0.54	2.11	0.00	0.00	2.11	1.44	0.88	0.01	0.83
C.V. (%)	0.66	1.29	0.00	0.00	1.29	1.53	0.51	2.66	0.62
Post-zeaxanthin									
Mean	81.91	70.64	15.32	77.92	163.88	93.35	173.34	0.36	134.71
S.D.	0.85	1.44	1.32	1.22	2.14	0.11	1.08	0.00	1.64
C.V. (%)	1.04	2.04	8.61	1.57	1.31	0.12	0.62	0.47	1.22

before and after zeaxanthin formation. The variance of the individual violaxanthin cycle components (V, A and Z) in the post-zeaxanthin runs reflected the variance of replicate treatments. All non-violaxanthin cycle pigments remained unchanged. The coefficient of variance for the other major pigments was less than 2.66% for both the before and after runs.

CONCLUDING REMARKS

ODS-1, a currently available high-performance liquid chromatographic column material, with the solvent programs described gives rapid and quantitative separation of all major and most minor chloroplast pigments. The method is well suited for studies on the xanthophyll cycle. Although the method has not been thoroughly tested for separation of more complex pigment compositions such as those found in phytoplankton, we have observed good separation of pigments in undegraded extracts of diatoms and several species of brown algae.

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NOTE ADDED IN PROOF

The authors of ref. 12 have informed us that non-endcapped Zorbax ODS recently became commercially available again.

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