*Journal of Chromatography*, 157 (1978) 111–117


CHROM. 10,995

# HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF CAROTENOIDS

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(Received March 6th, 1978)

## SUMMARY

The potential of high-pressure liquid chromatography (HPLC) for separation of carotenoids on silica has been studied with selected model carotenoids (carotenes, diols, *cis–trans* isomers and diastereoisomers) in comparison with conventional thin-layer chromatography and circular paper techniques. Fast and efficient separation on the analytical scale was obtained, including that of epimers not previously separated. The advantage of HPLC coupled with a UV–VIS scanning spectrophotometer for the analysis of *cis–trans* isomerization mixtures of carotenoids is pointed out.

## INTRODUCTION

Traditionally, carotenoids have been separated by column chromatography[1,2] and more recently, by thin-layer chromatography (TLC)[3]. Various types of paper chromatography have also been used[4,5], and gas chromatography (GC) has been applied to perhydrocarotenes[6].

Despite the application of high-pressure liquid chromatography (HPLC) to other types of compounds, few reports on HPLC of carotenoids have been published. Stewart and Wheaton[7] separated carotenes on magnesium oxide and xanthophylls on zinc carbonate; separation of double-bond isomers and *cis–trans* isomers was achieved, but retention times were long. More recently, diastereoisomeric furanoxides have been separated on magnesium oxide columns[8], and, in the apo-series, *cis–trans* isomers of retinal have been separated on Zorbax SIL[9]. Hajlbrahim *et al.*[10] have recently achieved good separation of components of algal extracts on Partisil (5-$\mu$m silica) columns. Recently, Stewart[21] employed HPLC on silica for separation of citrus carotenoids.

We have investigated the scope and limitations of analytical HPLC on silica for the separation of carotenes (Group 1), diols (Group 2), *cis–trans* isomers (Group 3) and diastereoisomers (Group 4) in comparison with common TLC and paper chromatographic systems.

## MATERIALS AND METHODS

### *Samples*

The samples used were authentic compounds from our carotenoid collection and were members of four groups, *viz.*:

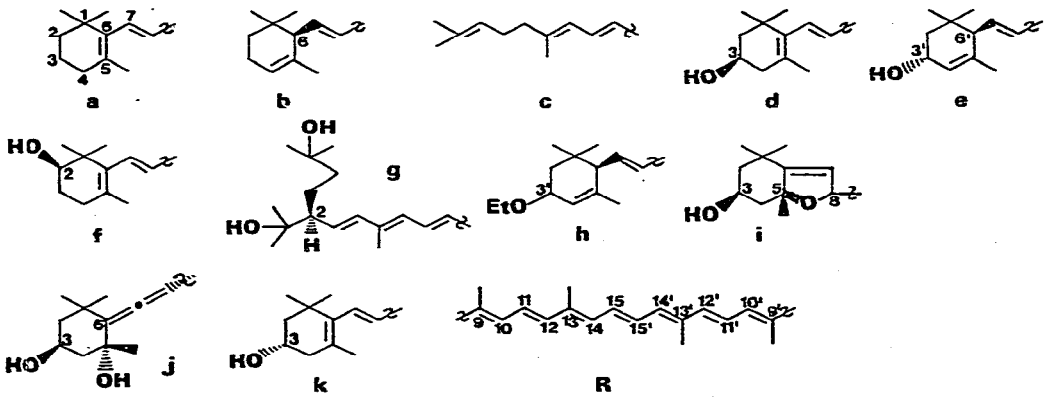
Group 1 (carotenes): 6′*R*-β,ε-carotene (1 in Scheme 1); β,β-carotene (2); β,ψ-carotene (3) and lycopene (4).

Group 2 (diols): 3*R*,3′*R*,6′*S*-lutein (5); 3*R*,3′*R*-zeaxanthin (6) and 2*R*,2′*R*-β,β-carotene-2,2′-diol (7).

Group 3 (*cis–trans* isomers): 3*R*,3′*R*,6′*S*-lutein (5) (stereoisomeric set) and 2*S*,2′*S*-bacterioruberin (8) (stereoisomeric set).

Group 4 (diastereoisomers): 3*R*,3′*R*,*S*,6′*S*-lutein 3′-ethyl ethers (9); 3*R*,5*R*, 8*R*,*S*,3′*R*,5′*R*,8′*R*,*S*-auroxanthins (10); 3*S*,5′*R*,6′*R*,3′*R*,5′*R*,8′*R*,*S*-neochromes (11) and 3*R*,3′*R*-zeaxanthin (6) plus 3*R*,3′*S*-zeaxanthin (12).

*Cis–trans* isomerization of double bonds in the polyene chain was effected by iodine-catalyzed isomerization in benzene in daylight[11]. The 3′-epimers of compound 9 were prepared by treatment of lutein (5) with acidified ethanol, occurring by $S_N1$-mechanism[12]. The 8,8′-diastereoisomers of auroxanthin (10) and the 8′-epimers of neochrome (11) were obtained by treatment of violaxanthin and neoxanthin, respectively, with acidified chloroform[13,14]. Epizeaxanthin (12) was obtained, by double-bond migration, from lutein (5) (see ref. 15).




| | | | | | |
|---|---|---|---|---|---|
| 1 | a–R–b | 5 | d–R–e | 9 | d–R–h |
| 2 | a–R–a | 6 | d–R–d | 10 | i–R–i |
| 3 | a–R–c | 7 | f–R–f | 11 | j–R–i |
| 4 | c–R–c | 8 | g–R–g | 12 | d–R–k |

### *Equipment*

A DuPont 830 liquid chromatograph equipped with gradient-elution acessories and thermostat-regulated oven was used in conjunction with a Varian Series 634 double-beam spectrometer as detector, which was set monochromatically at 400–490 nm, depending on the wavelength of max. absorption for the sample. The spectrometer was equipped with an 8-μl flow-through cell and, during separation, absorption spectra in the range 350–600 nm were recorded for the components of each HPLC peak.

## RESULTS AND DISCUSSION

### *Optimum conditions*

Columns of silica (various brands; particle size 5–10 $\mu$m), dry- or slurry-packed and commercial columns were tested. Optimum performance was obtained with a commercial Spherisorb (5-$\mu$m) column (4.6 × 250 mm) operated at 300 p.s.i. by gradient elution (0 to 40% of acetone in hexane containing 0.1% of methanol) at 1.25 ml/min (see ref. 16). For this column, the number of theoretical plates was 4350 (for toluene) and 6250 (for nitrobenzene). Resolution factors ($R_s$) for the carotenoids were calculated[17]; these varied from 0.86 (for 1 and 3) to 2.16 for the 8′-epimers of 11). Retention times (see Table I) ranged from 4–26 min.

### *Group 1 (carotenes)*

As can be seen from Table I, aliphatic (4) monocyclic (3) and bicyclic (1 and 2) carotenes were separated in hexane containing 0.1% of methanol; no separation of the double-bond positional isomers $\beta,\varepsilon$-carotene (1) and $\beta,\beta$-carotene (2) was achieved on the silica column. The HPLC separation compared well with that obtained by TLC on silica plates, but was inferior to that obtained on special plates[18].

### *Group 2 (diols)*

The 3,3′-diols lutein (5) and zeaxanthin (6) [corresponding to $\beta,\varepsilon$-carotene (1) and $\beta,\beta$-carotene (2)] were separated from eachother, and from the 2,2′-diol (7), with a mobile phase of up to 30% of acetone in hexane (see Table I). The separations compared favourably with those obtained by TLC and by circular development on silica-filled paper[5] (separations on the latter commercial paper are now inferior to those attained when the paper was first introduced[5]).

### *Group 3 (cis–trans isomers)*

The stereomutation mixture of lutein (5) gave three zones on TLC and circular paper chromatography and five peaks by HPLC (see Table I) (the designations neo-U, neo-V, etc., denote lower $R_F$ value, and neo-A, neo-B, etc., higher $R_F$ value, than the all-*trans*-isomer[11]).

Stereochemical assignment of the *cis*-isomers has recently been discussed[19]. Four sterically unhindered mono-*cis*-isomers of lutein (5) are possible, and our results indicate better chromatographic resolution by HPLC than was previously obtained (two major *cis*-isomers). However, isolation of the individual isomers for further characterization is required.

Bacterioruberin (8), after iodine-catalyzed stereomutation, yielded five peaks by HPLC (see Fig. 1). Identification of neo-U as di-*cis*, neo-A as 5-mono-*cis*, neo-B as 9-mono-*cis* and neo-C as 13-mono-*cis* is based on shifts in $\lambda_{max}$, and relative *cis*-peak intensities[11], excluding the formation of sterically hindered and central *cis*-bonds. The separation of the mono-*cis*-isomers is superior to that previously attained on silica-filled paper[20].

The possibility of scanning the electronic spectra during the HPLC development of such labile stereoisomers is a great advantage. In this context, HPLC of carotenoids is superior to conventional TLC or paper chromatography, both of which

TABLE I

SEPARATIONS OF CAROTENOIDS

| *Carotenoid* | *HPLC*[a] | | | *TLC ($R_C$)* | | *Circular paper ($R_E$)*[d] |
|---|---|---|---|---|---|---|
| | *$t_R$ (min)* | *Eluent*[b] | *Gradient rate (%/min)* | *Silica* | *Special plates*[c] | |
| *Group 1* | | | | | | |
| β,ε-Carotene (1) | 4.2 | 0 | 0 | 0.71[f] | 0.69[g] | 0.70[e] |
| β,β-Carotene (2) | 4.2 | | | 0.71 | 0.57 | 0.70 |
| β,ψ-Carotene (3) | 4.6 | | | 0.64 | 0.46 | 0.55 |
| Lycopene (4) | 5.0 | | | 0.57 | 0.03 | 0.35 |
| *Group 2* | | | | | | |
| 2,2′-Diol (7) | 8.0 | 0–30 | 10 | 0.67[k] | 0.52[k] | 0.85 |
| Lutein (5) | 9.5 | | | 0.61 | 0.48 | 0.77 |
| Zeaxanthin (6) | 9.7 | | | 0.61 | 0.29 | 0.63 |
| *Group 3* | | | | | | |
| Lutein (5) | | | | | | |
| Neo B | 12.4 | | | | | |
| Neo A | 13.1 | 0–40 | 3 | | | |
| All-*trans* | 14 | | | 0.70[l] | | 0.70[h] |
| Neo U | 14.8 | | | 0.62 | | 0.55 |
| Neo V | 15.4 | | | 0.52 | | 0.40 |
| Bacterioruberin (8) | | 20–60 | 3 | | | |
| Neo A | 14.1 | | | 0.61[l] | | 0.55[j] |
| All-*trans* | 14.6 | | | 0.41 | | 0.50 |
| Neo U | 15.1 | | | | | 0.40 |
| Neo V | 16.0 | | | | | |
| Neo W | 16.3 | | | | | |
| *Group 4* | | | | | | |
| Lutein 3′-ether (9) | | 0–30 | 1 | | | |
| Epimer 1 | 17.5 | | | 0.54[k] | | 0.57[g] |
| Epimer 2 | 17.9 | | | | | |
| Auroxanthin (10) | | 0–40 | 1 | | | |
| Epimer 1 | 25.5 | | | 0.20[k] | | 0.70[l] |
| Epimer 2 | 25.9 | | | | | |
| Neochrome (11) | | 0–40 | 3 | | | |
| Epimer 1 | 17.5 | | | 0.63[l] | | 0.80[j] |
| Epimer 2 | 17.8 | | | 0.60 | | 0.70 |
| Mono-*cis* | 18.4 | | | | | |
| Zeaxanthins (6 + 12) | 13.6 | 0–40 | 1 | | | 0.63[h] |

[a] Pressure, 300 p.s.i.; flow-rate, 1.4 ml/min.
[b] Percentage of acetone in hexane containing 0.1% of methanol.
[c] See ref. 18.
[d] S. & S. No. 287 (silica-filled) (see ref. 5).
[e] S. & S. No. 288 (alumina-filled); mobile phase, hexane.
[f] Mobile phase: acetone–hexane (2:98).
[g] Mobile phase: acetone–hexane (5:95).
[h] Mobile phase: acetone–hexane (10:90).
[i] Mobile phase: acetone–hexane (20:80).
[j] Mobile phase: acetone–hexane (30:70).
[k] Mobile phase: acetone–hexane (40:60).
[l] Mobile phase: acetone–hexane (60:40).

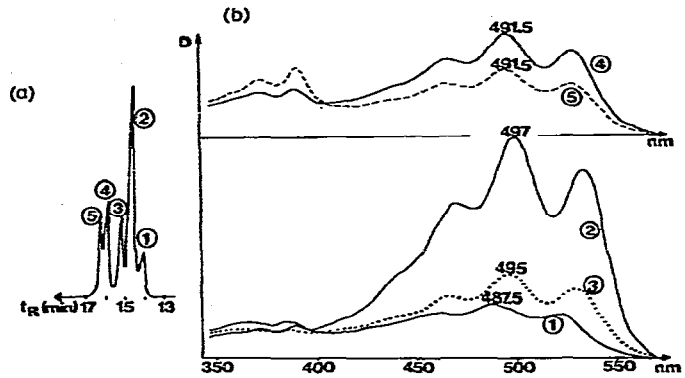



Fig. 1. HPLC separation of bacterioruberin (8) (stereoisomeric set) on Spherisorb (5 μm) at 300 p.s.i., with acetone–hexane 20–60% (gradient-rate, 3%/min) at 1.4 ml/min as mobile phase and the detector set at 490 nm. (a) Chromatogram; (b) electronic spectra of components. Peak and spectra numbers: 1 = neo-A, di-*cis*; 2 = all-*trans*; 3 = neo-U, 5-*cis*; 4 = neo-V, 9-*cis*; 5 = neo-W, 13-*cis*.

require elution and concentration procedures (with the inevitable partial reversible isomerization of the individual isomers) before spectra can be recorded.

*Group 4 (diastereoisomers)*

The two 3′-epimeric lutein ethyl ethers (9) have been separated for the first time by HPLC (see Fig. 2 and Table I). Of the expected three 8(8′)-diastereomers of auroxanthin (10), two were separated by HPLC and none by the other methods tested. Neochrome (11) was shown by HPLC to contain the two 8′-epimers and a mono-*cis*-isomer formed during acid treatment. The electronic spectra permitted reliable distinction between epimeric or *cis–trans* relationships during HPLC; separation into two zones only was obtained by TLC or circular development on paper. Finally, separation of natural zeaxanthin (6) and epizeaxanthin (12) was not achieved in any system.

The results indicate that separation of diastereoisomers in the HPLC system tested requires the presence of two chiral centres in the same end-group, with epimeric relationship for one of these centres. This could explain the separation of mono-ols (9, end-group h) and triols (11, end-group i) and failure to separate diols (6 and 12,

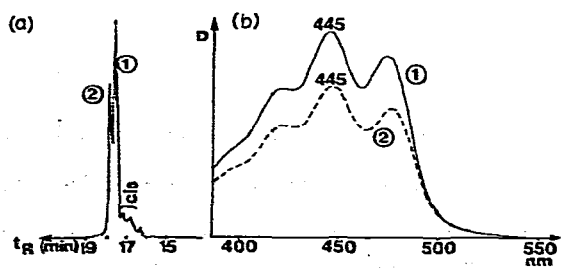



Fig. 2. HPLC separation of 3′-epimers of lutein 3′-ethyl ether (9), with acetone–hexane 0–40% (gradient-rate, 1%/min) at 1.4 ml/min as mobile phase and the detector set at 445 nm; other conditions as in Fig. 1. (a) Chromatogram; (b) electronic spectra of 3′-epimers.

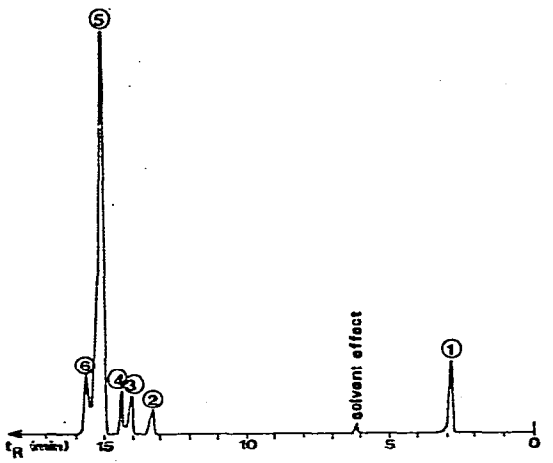



Fig. 3. HPLC separation of crude acetone extract of *Ochromonas* sp. Conditions as in Fig. 2, except that the gradient-rate was 3%/min and the detector was set at 450 nm. Peaks: 1 = β,β-carotene (2); 2 = zeaxanthin (6) plus chlorophyll; 3 and 4 = neochromes (11); 5 = fucoxanthin; 6 = neoxanthin.

end-groups d and k). However, the poor separation of the auroxanthin diastereoisomers (10, diol with two end-groups i) was unexpected.

*Application of the method*

Separation of a crude acetone extract of an *Ochromonas* sp. during 16 min (excluding the time for recording the spectra) is shown in Fig. 3, demonstrating separation of β,β-carotene (2), zeaxanthin (6), the neochromes (11), fucoxanthin and neoxanthin; in this instance, the detector was set at 450 nm. The peak areas do not reflect the exact concentrations of the individual carotenoids, owing to the different $\lambda_{max.}$ and extinction coefficients at 450 nm. The analytical advantages are obvious. For unknown mixtures, tentative identification based on electronic spectra and co-chromatography with authentic carotenoids can be made. The amount of an unknown mixture required is determined by the amount needed to record good quality electronic spectra for each component during the HPLC run (in this instance estimated to be *ca.* 0.2 μg of coloured carotenoid for adequate detection). However, for certain identification, mass spectra need to be obtained also.

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