Journal of Chromatography, 442 (1988) 412–419
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 504

Note

Normal-phase high-performance liquid chromatography of carotenes

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(Received March 7th, 1988)

High-performance liquid chromatography (HPLC) of carotenoids has become an invaluable analytical and preparative technique in recent years with considerable advantages of resolution and stability for sensitive compounds over older open-column and thin-layer procedures (for reviews see refs. 1–3). In most cases separation of the hydrocarbon carotenoids from the oxygenated xanthophylls (*e.g.* β-cryptoxanthin, lutein and zeaxanthin) is easily performed by either reversed- or normal-phase HPLC. Studies of individual carotenes in blood[4–6], plant materials and foodstuffs[7–10] have been concerned with the determination of the major provitamin A components β- and α-carotene, as well as lycopene, ζ-carotene or phytoene which may also be present in significant concentrations.

Reversed-phase systems, in particular use of non-aqueous solvents, for carotenoid analysis have achieved wide application, but separation of the carotenes is difficult and may require the use of gradients or complex ternary or even quaternary solvent mixtures. Broich *et al.*[5] used a methanol–acetonitrile–chloroform mixture to separate lycopene, α- and β-carotene from human serum. Beyer *et al.*[7] used water–methanol–tetrahydrofuran–acetonitrile to achieve resolution of five carotenes from *Narcissus pseudonarcissus* chromoplasts. Ruddat and Will[3] described the analysis of saponified extracts of the fungus *Ustilago violacea* using a shallow concave gradient of 2-propanol in aqueous acetonitrile. Separation of up to six carotenes was obtained, with lycopene eluting first, after 25 min, and phytoene last, after 40 min.

These examples illustrate some of the disadvantages of carotene analysis by reversed-phase HPLC: long run times and use of less volatile solvents which make solvent removal difficult in preparative work. Also variation between columns (especially due to different C_{18} loadings) requires careful consideration of column selection and evaluation. Other problems encountered in reversed-phase carotenoid HPLC have been discussed by Lauren *et al.*[11].

Normal-phase analyses of carotenes have been developed using a variety of stationary phases. Magnesium oxide was used to separate α-, β- and ζ-carotene from citrus juices[9]. Alumina has been more frequently used, for separations of α- and β-carotene in orange juice[10], several algal carotenes using a gradient of methyl-*tert.*-butyl ether in acetonitrile-modified hexane[12], and for the separation of stereoisomers of β-carotene[13]. Silica has been less useful for carotene analyses[8], giving either long retention times or poor resolution. Fiksdahl *et al.*[14], however, separated lycopene, γ- and β-carotene on Spherisorb 5-μm silica using 0.1% methanol in hexane. Sepa-

0021-9673/88/$03.50

ration of phytoene, β-carotene and lycopene has been reported on an amino-bonded silica column[2].

The influence of moisture content in adsorption chromatography has been known for some time[15], and separations of pentafluorobenzyl (PFB) esters of fatty acids[16], triglycerides[17], and stereoisomers of β-carotene[18] have been reported which depend on controlled levels of water in the eluting solvent. In the case of the fatty acid PFB esters and triglycerides retention is determined by the degree of unsaturation. In this paper we report the resolution of the carotenes of the biosynthetic sequence from phytoene to β-carotene using an isocratic mobile phase of defined water content on a silica column.

EXPERIMENTAL

Materials

Synthetic all-*trans*-β-carotene and all-*trans*-α-carotene were purchased from Sigma. Other carotenes were isolated from mutant strains of *Phycomyces blakesleanus*[19] and purified by standard procedures[20]. Identification of these pigments was established from their chromatographic properties and their UV–visible absorption spectra obtained both on-line from a diode array detector, and also using a Pye-Unicam SP8-2000 scanning spectrophotometer. The carotenes were dissolved in eluting solvent to 100–500 μg ml^{-1} and injected in 5–20 μl.

HPLC-grade hexane and acetonitrile were obtained from Mallinckrodt Australia (Sth. Oakleigh, Australia) and dried over Union Carbide molecular sieve 13X. Hexane was saturated with water by allowing to stand in contact with a small volume of deionized water. Half-water-saturated hexane was prepared by mixing equal volumes of dry and wet hexane before adding the required volume of dry acetonitrile (0.12%) and stirring to dissolve, then degassing and filtering before use.

HPLC

A Brownlee silica Spheri-5 analytical column (250 × 4.6 mm) was used in

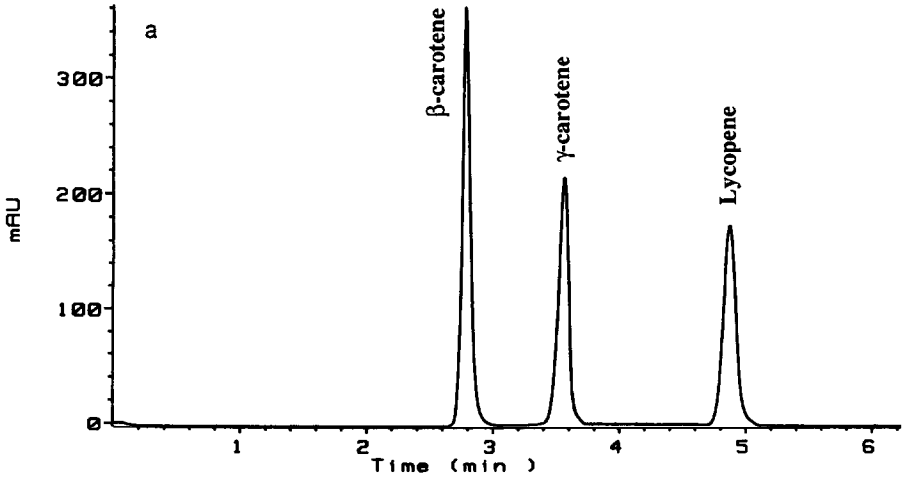

Fig. 1.

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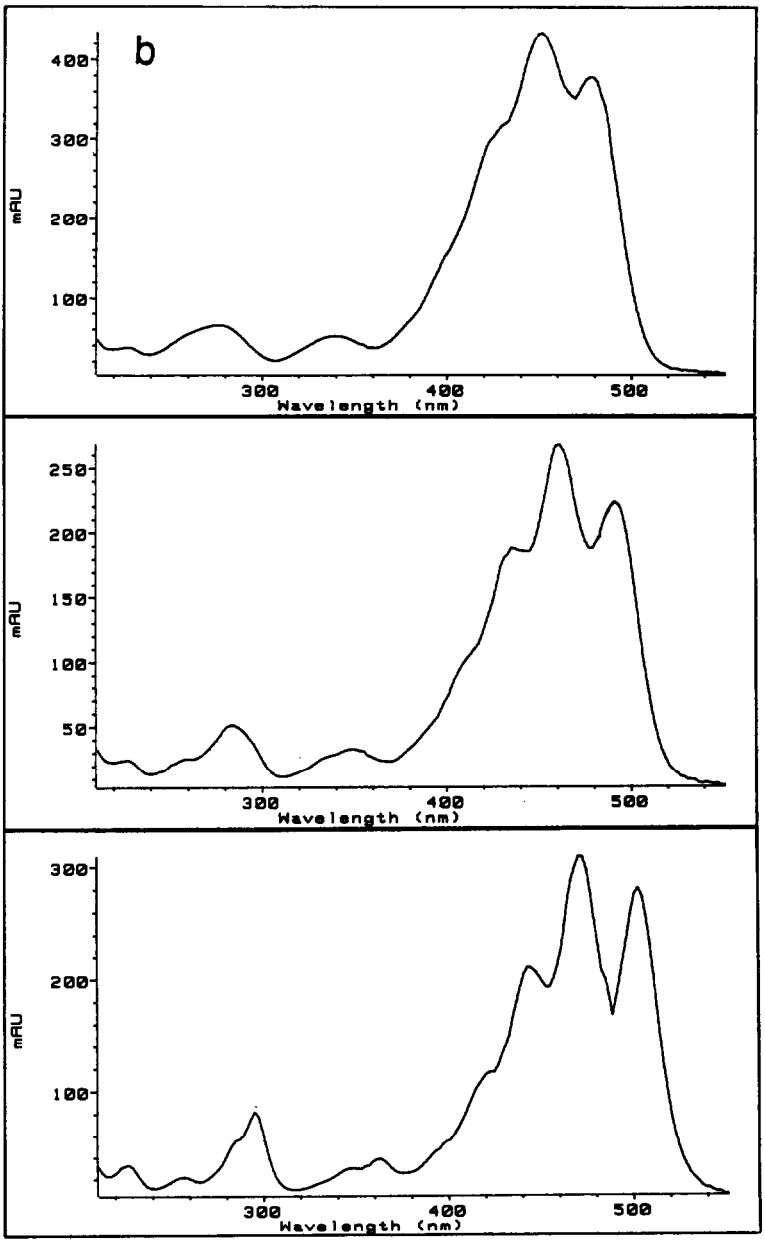

Fig. 1. (a) Chromatogram of β-carotene, γ-cerotene and lycopene. Signal recorded at 450 nm. Conditions as described in the text. (b) Spectra of β-carotene, γ-carotene and lycopene (from top to bottom) obtained on-line from diode array detector.

conjunction with an Upchurch Scientific silica guard column. The HPLC system comprised a Waters Model 510 pump and U6K injector. Solvent flow-rate was 2.0 ml min^{-1}. A Hewlett-Packard 1040A diode array detector and HP 79994A worksta-

tion with 20 Mb hard disk data storage was used to store chromatographic data at up to eight wavelengths, including spectra of the carotenes from 200 to 550 nm. Standard signals recorded were at 280, 370 and 450 nm with bandwidth 4 nm.

RESULTS

Chromatography of β- and γ-carotene and lycopene is shown in Fig. 1a, with the spectra obtained on-line from the diode array detector shown in Fig. 1b. Retention times are 2.78, 3.57 and 4.89 min, respectively, and clear baseline separation is achieved. α-Carotene (not shown) co-chromatographed with β-carotene.

Chromatography of phytoene, phytofluene, ζ-carotene, neurosporene and lycopene is shown in Fig. 2a, with the spectra shown in Fig. 2b. Retention times are 2.60, 2.88, 3.33, 4.06 and 5.10 min, respectively. Clear baseline separation is achieved for all of the carotenes tested except for β-carotene and phytofluene which partially co-elute.

Table I lists the observed absorption maxima with literature values for comparison.

The lower limit of detection of the diode array detector was found to be 20 ng for β-carotene (monitoring at 450 nm) and proportionately more for those carotenes with lower extinction coefficient. Recoveries were routinely better than 95% for β-carotene. A calibration curve for β-carotene using peak areas calculated by the HP workstation computer is shown in Fig. 3.

DISCUSSION

Normal-phase chromatography is particularly suited to the analysis of carotenes and other substances with minimal solubility in aqueous or polar solvents. Previous normal-phase systems, however, were incapable of resolving the various carotenes or required the use of solvent gradients or stationary phases such as mag-

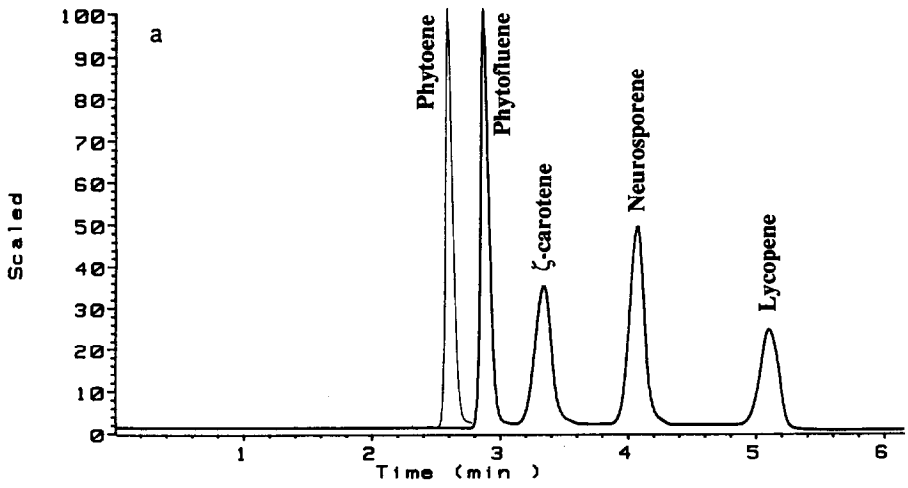

Fig. 2.

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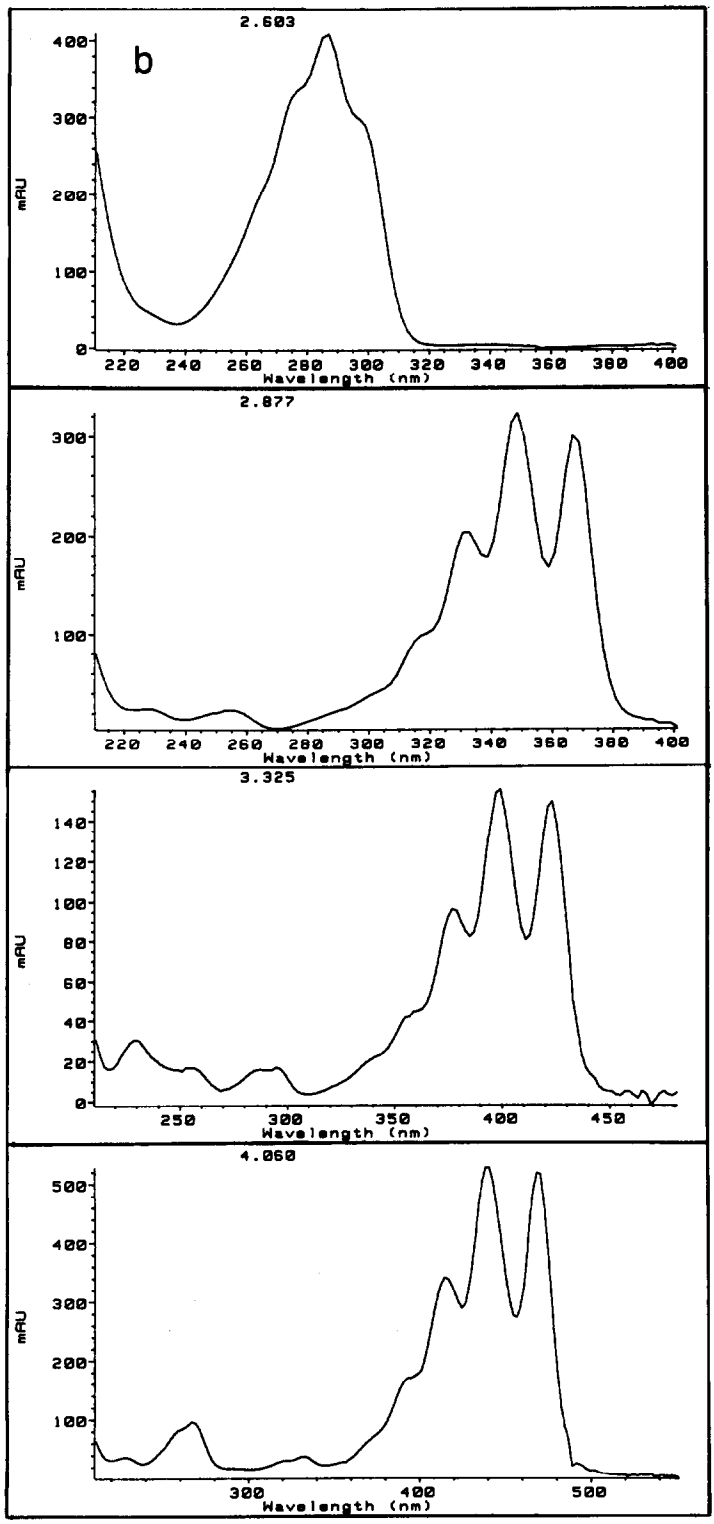

Fig. 2. (a) Chromatogram of acyclic carotenes. Signals recorded at 370 and 280 nm (shown only to 2.8 min; chromatograms normalized and superimposed). Conditions as described in the text. (b) Spectra of acyclic carotenes (identified by retention time) obtained on-line from diode array detector.

TABLE I

ABSORPTION MAXIMA OBTAINED ON-LINE WITH HP 1040A DIODE ARRAY DETECTOR (IN 0.12% ACETONITRILE IN 50% WATER-SATURATED HEXANE), COMPARED WITH PUBLISHED VALUES FROM SPECTRA OBTAINED IN HEXANE OR LIGHT PETROLEUM[21]

Compound	*Observed maxima (nm)*	*Published data (nm)*
Phytoene	278, 286, 297	276, 286, 298
Phytofluene	332, 349, 368	331, 347, 366
ζ-Carotene	378, 399, 423	380, 401, 425
Neurosporene	416, 440, 470	416, 440, 470
Lycopene	444, 472, 503	443, 472, 504
γ-Carotene	435, 461, 491	433, 459, 490
β-Carotene	428, 450, 478	427, 449, 477

nesium oxide[9], or alumina[12]. The HPLC method described here utilizes a silica column and was developed from earlier work using a moisture-controlled mobile phase for the separation of PFB esters of fatty acids and triglycerides[16,17], in which retention was determined primarily by the number of double bonds.

It has been applied now to the analysis of carotenes and is capable of resolving the seven pigments of the main biosynthetic sequence from phytoene to β-carotene. Retention times were essentially determined by the degree of unsaturation, ranging from the most saturated (phytoene, 9 double bonds) eluting first, to the most unsaturated (lycopene, 13 double bonds) eluting last. In addition, the presence of cyclic end groups decreases the retention times observed: β-carotene and γ-carotene have the same number of double bonds as the acyclic ζ-carotene and neurosporene (11 and 12 respectively), but elute about 0.5 min earlier. The positional isomers α-carotene and β-carotene were not separated.

A plot of log retention time *versus* number of double bonds (Fig. 4) shows a non-linear relationship, unlike the fatty acid PFB esters and triglycerides studied

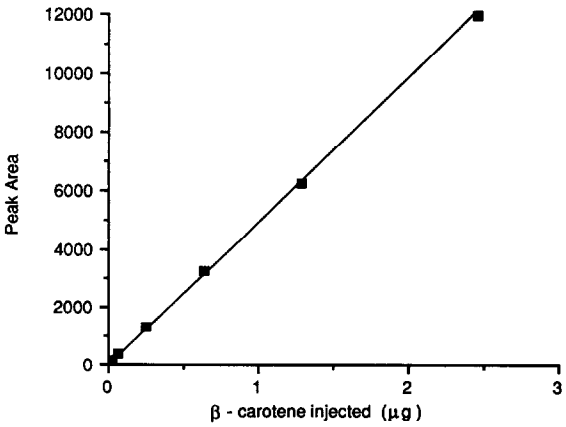

Fig. 3. Calibration curve for β-carotene. Signal at 450 nm integrated by HP workstation.

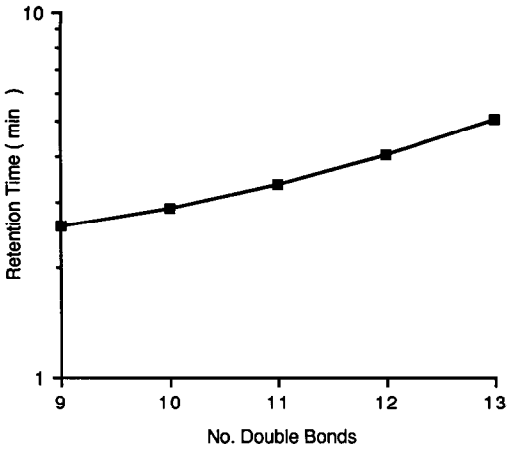

Fig. 4. Semi-log plot of retention time of acyclic carotenes *vs.* number of double bonds. HPLC conditions as described in text.

previously[16,17] in similar systems. It appears that for each additional double bond the increase in retention was more than expected for the addition of a single double bond. This may be correlated with the increased conjugation in the more unsaturated molecules, where the contribution to the observed retention is greater for the conjugated chain than for an equal number of isolated double bonds.

Despite a rather long column equilibration time for this solvent system the advantages over other HPLC methods of carotene analysis are manifest. The short run-times (5 min per injection) and isocratic operation combine to reduce dramatically the time required for analyses in comparison with, *e.g.*, the reversed-phase gradient system of Ruddat and Will[3]. Other problems with reversed-phase methods[11] are avoided; recoveries were quantitative and reproducible. In combination with the diode array detector and computerized multiple signal integration, this separation will allow rapid on-line spectral analysis, and simple quantitation of carotene pigments from a wide variety of animal, plant or microbial sources. In particular it would be useful in studies of the biosynthesis of β-carotene in which quantitation of the precursor pigments is required.

ACKNOWLEDGEMENTS

The authors wish to thank the Clive and Vera Ramaciotti Foundations for the HP workstation grant, and S.H.R. also wishes to thank Biotechnology Australia Pty. Ltd. for their generous support. This work was supported by the Australian Research Grants Committee.

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