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# SEPARATION OF CAROTENES ON CYCLODEXTRIN-BONDED PHASES

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## SUMMARY

The separation of carotenoids and retinoids on a β-cyclodextrin-bonded stationary phase with conventional mobile phases is reported. Compounds studied include β-carotene (all-*trans*), 15,15′-*cis*-β-carotene, 7,8,7′,8′-dihydro-β-carotene, α-carotene, lycopene, lutein, zeaxanthin, retinal, retinol, retinol palmitate and retinol acetate. The best resolution of carotenes was obtained with low concentrations ($\leqslant 1\%$) of polar solvents (*e.g.*, 2-propanol or ethyl acetate) in hexane or cyclohexane. Xanthophylls required much higher concentrations of polar solvents. The best solvent for the resolution of lutein and zeaxanthin was found to be dichloromethane. The resolution of *cis*/*trans*-isomers and the tentative identification of other isomers present in newly synthesized carotenoid standards is also reported. All-*trans*-isomers were found to be eluted before *cis*-isomers.

## INTRODUCTION

Retinoids, carotenes and their more polar analogues, the xanthophylls, are ubiquitous in plant and animal tissues. Although the extensive conjugation makes visualization and detection of these compounds fairly straightforward, it also contributes to the instability of these compounds to acid, heat and light[1]. Much of the early work involving carotenes was concerned with the isolation of carotenes from biological matrices and with determining the total carotenoid concentration[2]. In many of these studies thin-layer or paper chromatography was employed as a means of isolating the carotenoids from other materials present in the matrix[3]. One of the difficulties encountered in the application of these methods to the analysis of carotenes was their decomposition or isomerization. Therefore it has been desirable to find an analytical technique that is rapid, highly selective, and requiring minimal sample manipulation to limit the exposure of the sample to factors that contribute to decomposition or isomerization.

High-performance liquid chromatography (HPLC) is rapidly becoming the method of choice for the analysis of carotenoids[4–6]. Although silica gel has proven effective for the separation of some *cis*/*trans*-isomers, resolution of structural isomers

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such as α- and β-carotene was not accomplished[7]. Adsorption chromatography has been used successfully to separate *cis/trans*-retinal isomers[8], but silica gel has been suspected of initiating carotenoid decomposition and/or isomerization[9]. Reversed-phase partition chromatography with hydro-organic mobile phases has limited applicability due to the relatively low solubility of carotenoids in aqueous mobile phases and low retention of hydroxycarotenoids, which often are present in carotenoid samples, on non-polar stationary phases. Non-aqueous reversed-phase chromatography was found useful for the isocratic resolution of individual carotenoids but *cis/trans*-isomeric pairs were not included in that study[4]. Separation of *cis/trans*-isomers of β-carotene[10] and canthaxanthin [11] has been achieved on calcium hydroxide columns. Cyclodextrins (CD) have been used extensively to separate a wide variety of optical and geometric isomers[12–16]. Although the most common applications of CD stationary phases involve hydro-organic mobile phases, CD can also be used in the normal-phase mode[17].

The recent interest in the role of carotenoids in cancer prevention[18,19] necessitates the synthesis and characterization of standards. Several carotenes were synthesized and analyzed chromatographically to determine isomeric purity. The purpose of the work presented here was to evaluate the unique selectivity characteristics of cyclodextrin-bonded phases in the normal-phase mode, as applied to natural and synthetic carotenoid samples.

## EXPERIMENTAL

The chromatographic measurements were made on a LC-6A liquid chromatograph, interfaced with a C-R2AX Chromatopac data system and a SCL-6A system controller (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.). Detection was accomplished using a Shimadzu Model SPD-6A variable-wavelength UV detector (Columbia, MD, U.S.A.).

The chromatographic column was made of 250 × 4.6 mm I.D. stainless steel and packed with 5-μm Cyclobond I (β-CD) (Advanced Separations Technology, Whippany, NJ, U.S.A.).

α-Carotene, β-carotene (all-*trans*), lycopene, retinal, retinol, retinol palmitate and retinol acetate were obtained from Sigma (St. Louis, MO, U.S.A.); the xanthophylls (lutein and zeaxanthin) from Atomergic Chemetals Corp. (Farmingdale, NY, U.S.A.); and the solvents (all HPLC grade) from Fisher Scientific (St. Louis, MO, U.S.A.).

15,15′-Didehydro-β-carotene was synthesized according to the method of Surmatis and Ofner[20]. The mixture of isomers obtained by this procedure was then thermally isomerized to all-*trans*-15,15′-didehydro-β-carotene. 15,15′-*cis*-β-Carotene was prepared by catalytic hydrogenation of all-*trans*-15,15′-didehydro-β-carotene. 11-*cis* and all-*trans*-15,15′-dihydro-β-carotene were synthesized by alkylation of β-ionylideneethylphenylsulfone[21,22] with 1,8-dichloro-2,7-dimethyl-2,6-octadiene[23], followed by elimination of benzenesulfinic acid with potassium isopropoxide[24]. 15-*cis* and all-*trans*-7,8,7′,8′-tetrahydro-β-carotene were obtained by $TiCl_3/LiAlH_4$ coupling[25] of all-*trans*-7,8-dihydroretinal[26].

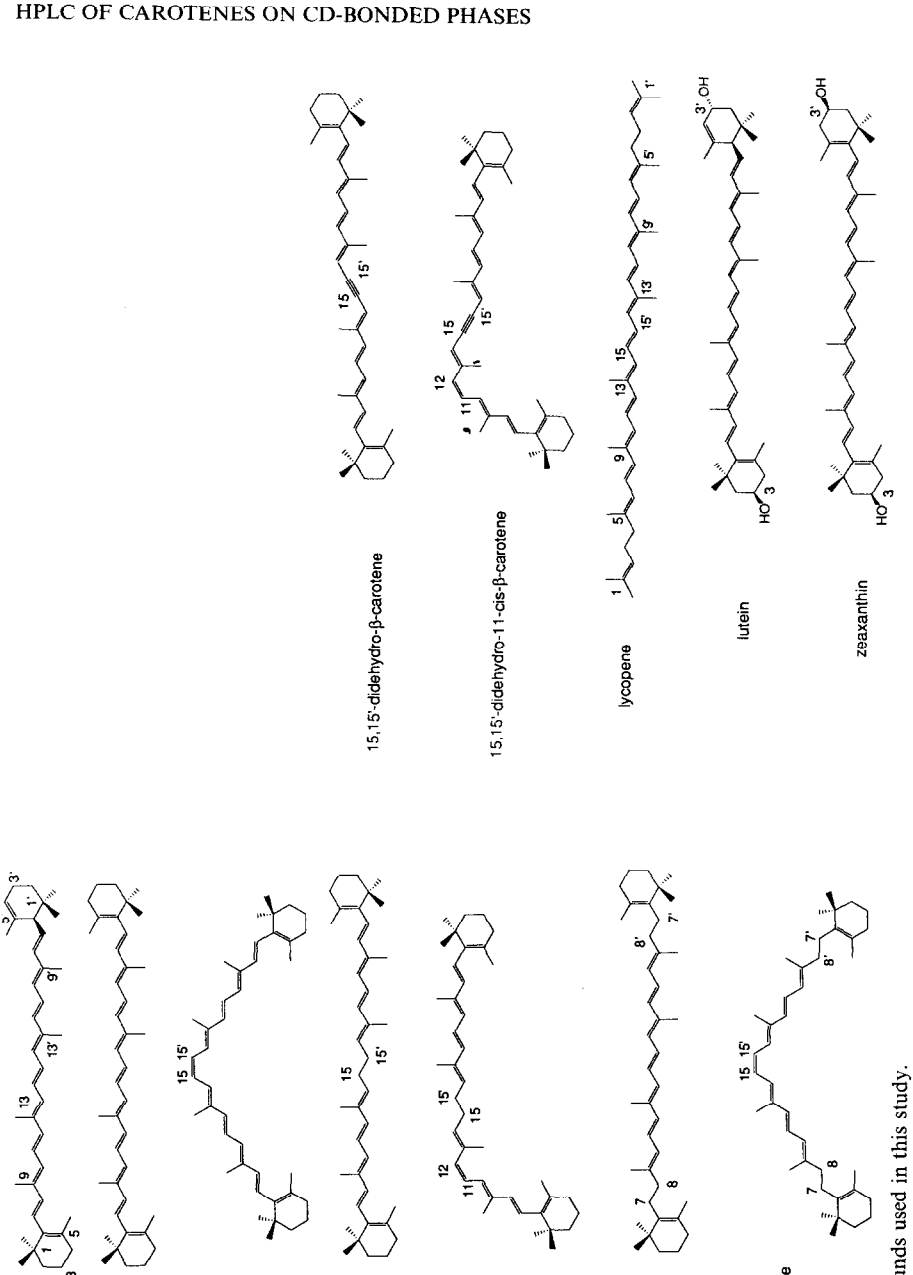


Fig. 1. Structures of compounds used in this study.

TABLE I

RETENTION TIMES ($t_R$), CAPACITY FACTORS ($k'$), AND CHROMATOGRAPHIC CONDITIONS FOR THE ELUTION OF CAROTENOIDS AND RETINOIDS FROM β-CYCLODEXTRIN-BONDED PHASES

Details given in Experimental.

| *Compounds* | $t_R$ | $k'$ | *Mobile phase* | *Detector (UV, nm)* |
|---|---|---|---|---|
| α-Carotene | 5.31 | 1.62 | Dichloromethane–hexane (1:99) | 350 |
| | 11.60 | 4.87 | Acetone–hexane (1:99) | 350 |
| β-Carotene | 5.41 | 1.67 | Dichloromethane–hexane (1:99) | 325 |
| | 5.88 | 1.31 | 2-Propanol–cyclohexane (0.5:99.5) | 350 |
| | 2.87 | 0.49 | Ethyl acetate–cyclohexane (1:99) | 350 |
| | 11.47 | 4.81 | Acetone–hexane (1:99) | 350 |
| | 4.04 | 0.98 | Chloroform–hexane (1:99) | 325 |
| 15,15′-*cis*-β-Carotene | 6.28 | 2.10 | Dichloromethane–hexane (1:99) | 325 |
| | 8.08 | 2.18 | 2-Propanol–cyclohexane (0.5:99.5) | 350 |
| | 3.31 | 0.72 | Ethyl acetate–cyclohexane (1:99) | 350 |
| | 12.29 | 5.22 | Acetone–hexane (1:99) | 350 |
| | 4.63 | 1.27 | Chloroform–hexane (1:99) | 350 |
| 15,15′-Dihydro-β-carotene | 12.49 | 5.11 | Dichloromethane–hexane (1:99) | 325 |
| 11-*cis*-15,15′-Dihydro-β-carotene | 13.43 | 5.58 | Dichloromethane–hexane (1:99) | 325 |
| | 22.75 | 10.11 | Ethyl acetate–hexane (1:99) | 325 |
| 7,8,7′,8′-Tetrahydro-β-carotene | 4.39 | 1.16 | Dichloromethane–hexane (1:99) | 400 |
| | 6.49 | 2.10 | Ethyl acetate–hexane (1:99) | 325 |
| | 3.61 | 0.77 | Chloroform–hexane (1:99) | 400 |
| 15-*cis*-7,8,7′,8′-Tetrahydro-β-carotene | 4.53 | 1.23 | Dichloromethane–hexane (1:99) | 400 |
| | 6.80 | 2.25 | Ethyl acetate–hexane (1:99) | 325 |
| | 3.72 | 0.82 | Chloroform–hexane (1:99) | 400 |

| | | | | |
|---|---|---|---|---|
| 15,15′-Didehydro-β-carotene | 3.30 | 0.64 | Ethyl acetate–hexane (1:99) | 450 |
| | 5.44 | 1.71 | Dichloromethane–hexane (1:99) | 330 |
| Retinol palmitate | 4.71 | 1.46 | Ethyl acetate–hexane (1:99) | 350 |
| | 6.71 | 2.55 | Dichloromethane–cyclohexane (5:95) | 350 |
| | 4.82 | 1.47 | Acetone–hexane (1:99) | 350 |
| Retinyl acetate | 17.53 | 8.26 | Dichloromethane–cyclohexane (5:95) | 350 |
| | 13.62 | 5.98 | Acetone–hexane (1:99) | 350 |
| Lycopene | 5.07 | 1.64 | Ethyl acetate–cyclohexane (1:99) | 350 |
| | 4.57 | 1.42 | Dichloromethane–cyclohexane (5:95) | 350 |
| | 7.11 | 2.64 | Acetone–hexane (1:99) | 350 |
| Retinal | 2.25 | 0.21 | Ethanol–hexane (5:95) | 280 |
| | 3.21 | 0.75 | Ethyl acetate–cyclohexane (12:88) | 350 |
| | 17.41 | 8.66 | Dichloromethane–cyclohexane (15:85) | 350 |
| | 5.52 | 1.88 | Acetone–hexane (4:96) | 350 |
| Retinol | 8.16 | 3.43 | Ethyl acetate–cyclohexane 12:88) | 350 |
| | 3.41 | 0.83 | Ethanol–hexane (5:95) | 280 |
| | 40.39 | 21.4 | Dichloromethane–cyclohexane (15:85) | 350 |
| | 22.22 | 10.6 | Acetone–hexane (4:96) | 350 |
| Lutein | 6.54 | 2.79 | Ethyl acetate–hexane (42:58) | 280 |
| | 5.74 | 1.67 | Chloroform–hexane (80:20) | 280 |
| | 11.03 | 5.21 | Dichloromethane | 280 |
| | 3.83 | 1.17 | Ethanol–hexane (10:90) | 280 |
| | 5.28 | 2.02 | Ethanol–$CH_2Cl_2$ (1:99) | 280 |
| Zeaxanthin | 6.50 | 2.77 | Ethyl acetate–hexane (42:58) | 280 |
| | 7.27 | 2.38 | Chloroform–hexane (80:20) | 280 |
| | 20.28 | 10.42 | Dichloromethane | 280 |
| | 5.12 | 1.89 | Ethanol–hexane (10:90) | 280 |
| | 6.50 | 2.72 | Ethanol–$CH_2Cl_2$ (1:99) | 280 |

## RESULTS AND DISCUSSION

The structures of the carotenoids used in this study are presented in Fig. 1. Various mobile phases were tested to optimize carotenoid separations. In general, the best separations of carotenes and retinoids were observed with fairly low concentrations ($\leqslant 1\%$) of a polar solvent in hexane or cyclohexane. In order to elute the more polar xanthophylls, it was necessary to employ higher concentrations of more polar solvents (*e.g.*, chloroform or ethanol). The retention times and capacity factors for several carotenoids and retinoids with various mobile phases are presented in Table I and sample chromatograms are shown in Figs. 2 and 3. As expected, the non-polar solutes are eluted first and the more polar solutes are retained the longest. For instance, retinyl palmitate and retinyl acetate are eluted earlier than retinol because they are less polar. The selectivity of the cyclodextrin column resembles that of the silica gel columns in that the cyclodextrin column is unable to resolve $\alpha$- and $\beta$-carotene but does resolve their xanthophyll analogues, lutein and zeaxanthin.

Separations of compounds on CD-bonded phases when using hydro-organic mobile phases are thought to be the result of preferential formation of inclusion complexes of the more retained solute within the CD cavity[27]. When using non-aqueous mobile phases, the CD cavities are most likely occupied by non-polar solvent molecules. The solutes in this study are probably too large to reside entirely within such a cyclodextrin cavity. Retention and selectivity are, therefore, most probably the result of solute interaction with the hydroxyl groups that line the CD cavity. This would account for the similarity in elution order of the solutes between the CD-bonded phase and silica gel. A similar conclusion regarding the role of the CD hydroxyls on retention and selectivity was drawn in a recent report of the separation of mono- and polysaccharides on CD phases[28]. In addition, the bulky CD groups limit the accessibility of solute molecules to silica surface silanols, which are more acidic than the CD hydroxyls and may contribute to isomerization[9]. Additional studies on this potentially useful aspect of CD-bonded phase are in progress.

The retention times and capacity factors for the carotenoid standards under various chromatographic conditions on the $\beta$-CD column are listed in Table I. These include 15,15′-dehydro-, 7,8,7′,8′-tetrahydro- and 15,15′-didehydro-$\beta$-carotene. Proton NMR of the synthetic carotenoid samples revealed the presence of *cis*-isomers along with the predominant all-*trans*-isomers. Based on the NMR, UV, and chromatographic data, the principal contaminants in the standards are tentatively identified and also listed in Table I. The chomatographic factors which aided in the determination of peak identity were elution order (all-*trans*-isomers eluted before *cis*-isomers) and the change in relative peak height with changing wavelength. The retention of 15,15′-dihydro-$\beta$-carotene seems anomalously long when compared to the retention of the other $\beta$-carotenes. It may be that the flexibility about the molecular center of symmetry afford enhanced solute bonded ligand interactions than are possible with the other, more rigid $\beta$-carotenes. Resolution and selectivity for geometric and structural isomeric pairs are presented in Table II. Note that *trans*-isomers are linear, while the presence of a *cis*-double bond within the polyene backbone confers a more bent configuration to the molecule. This spatial orientation of the *cis*-isomers may allow a more efficient interaction with the bulky CD moiety than is possible for the linear all-*trans*-isomers[29].

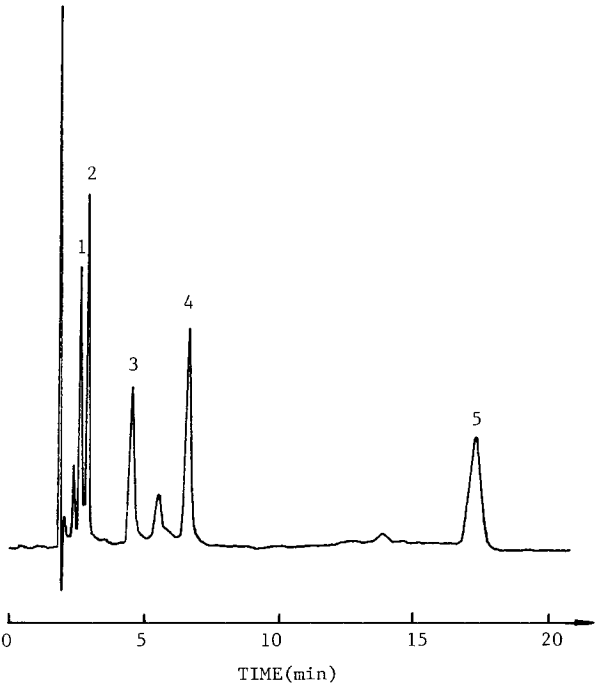


Fig. 2. Chromatographic separation of β-carotene (1), 15,15′-*cis*-β-carotene (2), lycopene (3), retinyl palmitate (4) and retinyl acetate (5) on a β-CD column with dichloromethane–cyclohexane (5:95) as mobile phase at 1.5 ml/mn. (Details given in Experimental and tables.)

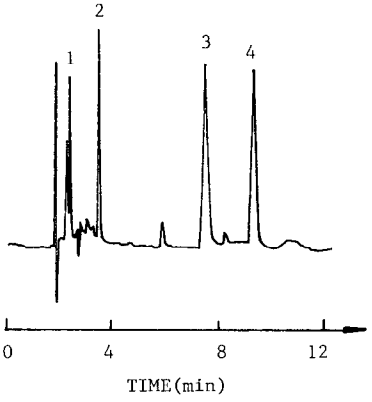


Fig. 3. Chromatographic separation of retinal (1), retinol (2), lutein (3), and zeaxanthin (4) on a β-CD column with ethanol–hexane (5:95) at 1.5 ml/min as mobile phase. (Details given in Experimental and tables.)

TABLE II

SEPARATION OF *CIS*- AND *TRANS*-ISOMERS AND OF α- AND β-ISOMERS

Details given in Experimental.

| *Compounds* | $\alpha^a$ | $R_s^b$ | *Mobile phase* |
|---|---|---|---|
| β-Carotene (all-*trans*-)/15,15′-*cis*-β-carotene | 1.26 | 1.74 | Dichloromethane–hexane (1:99) |
| | 1.66 | 2.36 | 2-Propanol–cyclohexane (05:99.5) |
| | 1.47 | 1.93 | Ethyl acetate–cyclohexane (1:99) |
| | 1.09 | 0.9 | Acetone–hexane (1:99) |
| | 1.30 | 1.66 | Chloroform–hexane (1:99) |
| Lutein (α-)/Zeaxanthin (β-) | 1.43 | 4.5 | Chloroform–hexane (80:20) |
| | 2.00 | 10.8 | Dichloromethane |
| | 1.62 | 4.5 | Ethanol–hexane (10:90) |
| | 1.35 | 2.3 | Ethanol–dichloromethane (1:99) |
| 15,15′-Dihydro-β-carotene | | | |
| (All *trans*- and 11-*cis*) | 1.09 | 1.24 | Dichloromethane–hexane (1:99) |
| | 1.07 | 0.8 | Ethyl acetate–hexane (1:99) |
| 7,8,7′,8′-Tetrahydro-β-carotene | | | |
| (All *trans* and 15-*cis*) | 1.07 | 0.7 | Ethyl acetate–hexane (1:99) |
| | 1.07 | 0.7 | Chloroform–hexane (1:99) |
| | 1.06 | 0.7 | Dichloromethane–hexane (1:99) |
| 15,15′-Didehydro- and 11-*cis*-15,15′-didehydro-β-carotene | 1.11 | 1.25 | Dichloromethane hexane (1:99) |

[a] $k_1/k_2'$.

[b] Resolution.

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