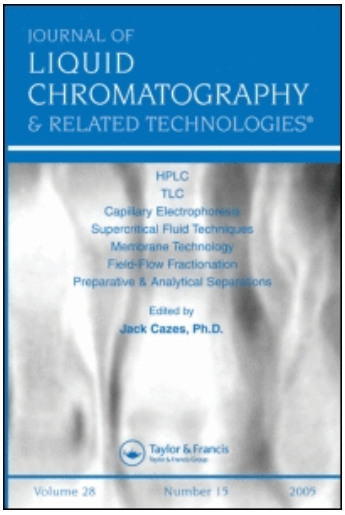


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# Comparison of Column Packings for Isocratic High-Performance Liquid Chromatographic Analysis of Carotenoids Using Non-Aqueous Reversed-Phase

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# COMPARISON OF COLUMN PACKINGS FOR ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF CAROTENOIDS USING NON-AQUEOUS REVERSED-PHASE

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ABSTRACT

Ten different columns are compared for the isocratic non-aqueous reversed-phase separation of carotenoids, using solvent mixtures of ethyl acetate-acetonitrile both with and without 0.1% n-decanol as modifier. Conditions were established for separation of a mixture of alfalfa carotenoids containing mainly neoxanthin, violaxanthin, lutein and β-carotene. The best material for use for rapid isocratic separation of all major components was a high carbon loading non-endcapped material with ODS functionality, although one endcapped C8 material gave similar results. The use of n-decanol as mobile phase modifier is imperative to rapidly condition new columns to give optimum peak shape and definition and system linearity.

## INTRODUCTION

Carotenoid compounds are of commercial value for both nutritional and pigmenting properties. Accordingly, their analysis in animal feeds and additives is important. In recent



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times, high-performance liquid chromatography (HPLC) has become increasingly accepted as the method of choice for pigment analysis (1), since it has the potential to provide full component description and quantitation in comparison with the non-specific nature of traditional methods based on spectrophotometric measurements of class fractions.

Both normal-phase and reversed-phase methods have been described which allow analysis of the range from polar to non-polar carotenoids to be analysed. However, either gradient analysis or long analysis times are required. More recently, isocratic non-aqueous reversed-phase systems have been described (2,3) capable of separating a range of carotenoids. Both employed the same reversed-phase packing material. The method we described (3) used n-decanol as a mobile phase modifier. It had improved chromatographic stability, allowed a greater range of compounds to be analysed and was also rapid and versatile. This paper describes the comparison of different column packing materials using the method, further examines the role of n-decanol as a mobile phase modifier, and shows separations of a mixture of common carotenoid compounds extracted from alfalfa-based feed concentrates.



## EXPERIMENTAL

### Materials and Reagents

Solvents used for HPLC were acetonitrile (Ajax HPLC grade), ethyl acetate (Mallinckrodt Analytical grade) and n-decanol (Eastman Kodak). Other solvents and reagents have been described elsewhere (3).

### Carotenoid Standards and Mixed Extract

The source of preparation of carotenoid standards are described elsewhere (3). A mixed extract of alfalfa carotenoids

was obtained by extracting alfalfa leaf protein concentrate (LPC) (12 g) with dichloromethane-acetone (50 ml total volume) (4). After saponification, aqueous washing and centrifugation, a portion of the yellow organic layer was removed and diluted with an equal volume of acetonitrile. This material was used as the concentrated mixed stock, and further dilutions were made with dichloromethane-acetonitrile (1:1). The concentration of the major carotenoids in this mixed stock solution were; β-carotene, 15 ppm; lutein, 92 ppm; epoxides, neoxanthin plus violaxanthin, 52 ppm. All standard and extract solutions contained butylated hydroxytoluene (BHT) antioxidant.



## Apparatus

Since the different column packings were each tested using mobile phases both with and without n-decanol mobile phase modifier, two isocratic pumps were used alternately on the same modular analytical system. These were a Waters M45 pump (no-modifier solvents) and a Metering Pumps Ltd. (UK) E2B chromatography pump (plus-modifier solvents) fitted with relief valve, pressure gauge and pulse dampener. Feed lines to both pumps contained stainless steel filters (2-μm). Injection was with a Valco fixed loop (10-μl) injector, and the analytical column was preceded by a 2-μm in-line filter (Rheodyne). The detector was a Tracor 970A set at 450 nm. Peak integration was with a Spectra Physics Minigrator.

## HPLC Columns

Both commercial and home-packed columns were used. These are listed in Table 1, along with some characteristics of the packing material used.

## HPLC Conditions

The HPLC mobile phases were isocratic mixtures of ethyl acetate-acetonitrile containing between 3-25% of ethyl acetate,

TABLE 1 Columns and Packing Materials Tested

| Column | Packing material | Manufacturer | Column size (mm) | Source[a] | % Loading[b] | Particle size (μm) | Endcapped? |
|---|---|---|---|---|---|---|---|
| 1 | Zorbax ODS | Dupont | 150 x 4.6 ID | H | High | 8 | No |
| 2 | Zorbax ODS | Dupont | 150 x 4.6 ID | H | High | 8 | No |
| 3 | Zorbax ODS | Dupont | 250 x 4.6 ID | C | High | 6 | No |
| 4 | Hypersil ODS | Shandon | 100 x 4.0 ID | H | High | 5 | Yes |
| 5 | Nova-Pak C18 | Waters | 150 x 3.9 ID | C | Low | 4 | Yes |
| 6 | Spherisorb ODS1 | Phase Sep | 250 x 4.6 ID | H | Low | 10 | No |
| 7 | Zorbax C8 | Dupont | 150 x 4.6 ID | H | High | 8 | No |
| 8 | CPSpher C8 | Chrompack | 250 x 4.6 ID | C | High | 8 | Yes |
| 9 | Zorbax silica | Dupont | 250 x 4.6 ID | C | n/a | 6 | n/a |
| 10 | PRP-1 | Hamilton | 150 x 4.1 ID | C | n/a | 10 | n/a |

a - H = homemade, C = commercially made. Column 1 had been well used for carotenoid analysis before these tests; all other columns were unused prior to testing.

b - Extent of reaction of silica base with reversed-phase reagent. For ODS materials, Low and High refer to materials with 7-8% and at least 11% carbon content respectively.

and either with or without n-decanol modifier. Percentages of the major components varied for different columns and are given in the text. For each mixture, a bulk was made then divided into two portions. One was used as is and the other was treated with 0.1% by volume of n-decanol . Each new column was tested first with a series of solvents containing no modifier. Injections of the mixed stock standard solution were made to observe the separation of the four major carotenoids present. Once suitable separation conditions were determined, a series of dilutions of the mixed stock were injected. The mobile phase was then changed to the plus-modifier solvent of equal ethyl acetate concentration, and the mixed stock and a series of dilutions again injected. At each solvent change, the system was equilibrated for 60 min before injecting the next sample.

The chromatograms were studied for compound elution order, absolute and relative retention times of carotenoids, peak height and peak shape and irregularities such as distorted baselines.



## RESULTS AND DISCUSSION

Since our work involved mainly alfalfa products, conditions which rapidly separated the major carotenoids occuring in alfalfa, neoxanthin, violaxanthin, lutein and β-carotene, were studied. Zorbax ODS, a material with high carbon loading but not endcapped, was found to have preferred characteristics. It showed good selectivity for the polar carotenoids without at the same time requiring a prolonged analysis time for concurrent analysis of the non-polar β-carotene. Results for the other materials will therefore be discussed relative to Zorbax ODS.

A typical chromatogram obtained with a well-used and n-decanol conditioned Zorbax ODS column is shown in Figure 1. With the mobile phase used, neoxanthin and violaxanthin, peaks 1 and 2, are only partly separated, but lutein and β-carotene

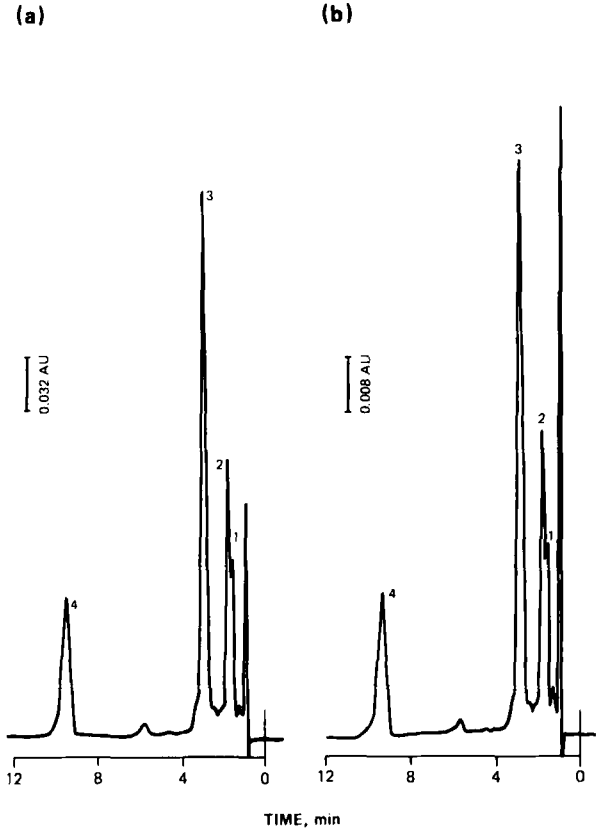


FIGURE 1 Separation of a mixture of alfalfa carotenoids on Zorbax ODS column 2. Mobile phase was ethyl acetate-acetonitrile (25+75) containing 0.1% (v/v) of n-decanol. Flow rate = 1.6 ml/min. Other conditions as in text. Samples were: a, concentrated stock solution; b, stock solution diluted 4 times. Peak identification for all figures; 1 = neoxanthin; 2 = violaxanthin; 3 = lutein; 4 = β-carotene.

are well separated from other major components. The small amount of zeaxanthin present in the extracts occurs as a small trailing shoulder on the lutein peak. Thus from Figure 1, the contents of the alfalfa extract may be quantified as class fractions of epoxide xanthophylls, lutein plus zeaxanthin, and β-carotene. The small peak between lutein and β-carotene has tentatively been ascribed to β-cryptoxanthin. Full separation of all these components was possible within 25 min with a mobile phase containing 12% ethyl acetate (3).

The column used for Figure 1 (column 2) had been used for four months and for several hundred samples when the illustrated analysis was performed. Peak shape and definition were still good, showing that the conditions used are conducive to long column life. The retention times were within 3% of those obtained when the column was first used and the response from the chromatographic system was linear over the full test range from the concentrated stock mixture to one diluted 32-times.



The three Zorbax ODS columns had similar, although not identical, capacity factors for the main alfalfa carotenoids (Table 2). Column 1 had greater retention for the polar carotenoids but less for β-carotene. Retention of the polar carotenoids was virtually identical on columns 2 and 3, while β-carotene was more strongly retained on column 2. The addition of 0.1% n-decanol to the eluent reduced the capacity factors for all compounds by a similar percentage in the range 6-16%.

Figures 2 to 8 show the separation of the alfalfa carotenoids achieved with each of the other columns. In general, the isocratic mobile phase composition shown for each column was chosen to allow a total analysis time similar to the 11 min shown in Figure 1 for Zorbax ODS. The capacity factors of the carotenoids for the different columns and conditions is also given in Table 2.

TABLE 2 Capicity Factors for Major Alfalfa Carotenoids on Ten Different Columns[a,b]

| Column | Packing | Mobile phase | | Capacity factor (k') | | |
|---|---|---|---|---|---|---|
| | | EtOAc (%) | n-decanol (+/-) | epoxides | lutein | β-carotene |
| 1 | Zorbax ODS | 25 | + | 1.75 | 3.21 | 9.44 |
| 2 | Zorbax ODS | 25 | - | 1.12 | 2.59 | 11.39 |
| | | | + | 1.00 | 2.18 | 10.06 |
| 3 | Zorbax ODS | 25 | - | 1.20 | 2.65 | 9.31 |
| | | | + | 1.01 | 2.22 | 8.79 |
| 4 | Hypersil ODS | 3 | - | 0.88 | 1.81 | 17.88 |
| | | | + | 0.78 | 1.56 | 15.22 |
| 5 | Nova-Pak C18 | 12 | - | 0.89 | 2.23 | 17.75 |
| | | | + | 0.85 | 2.15 | 17.58 |
| 6 | Spherisorb ODS1 | 12 | - | 2.39,1.44 | 1.44 | 0.25 |
| | | | + | 1.86,1.07 | 1.07 | 0.15 |
| 7 | Zorbax C8 | 6 | - | 0.89 | 1.34 | 4.11 |
| | | | + | 0.82 | 1.25 | 3.94 |
| 8 | CPSpher C8 | 6 | - | 0.71 | 1.38 | 5.92 |
| | | | + | 0.64 | 1.24 | 5.55 |
| 9 | Zorbax Silica | 3 | - | 1.39,0.76 | 0.56 | 0.17 |
| | | | + | 1.20,0.67 | 0.48 | 0.13 |
| 10 | PRP-1 | 3 | - | 0.93 | 2.06 | 20.73 |
| | | | + | 0.86 | 1.88 | 18.52 |

a - Capacity factors calculated from mean retention times obtained over the test period. Mean values are given for epoxides except for columns 6 and 9 which show values for neoxanthin then violaxanthin in order.

b - Mobile phases were mixtures of ethyl acetate-acetonitrile either with or without n-decanol (0.1% v/v) as indicated. Flow rate = 1.6 ml/min. Other instrumental conditions as in the text.

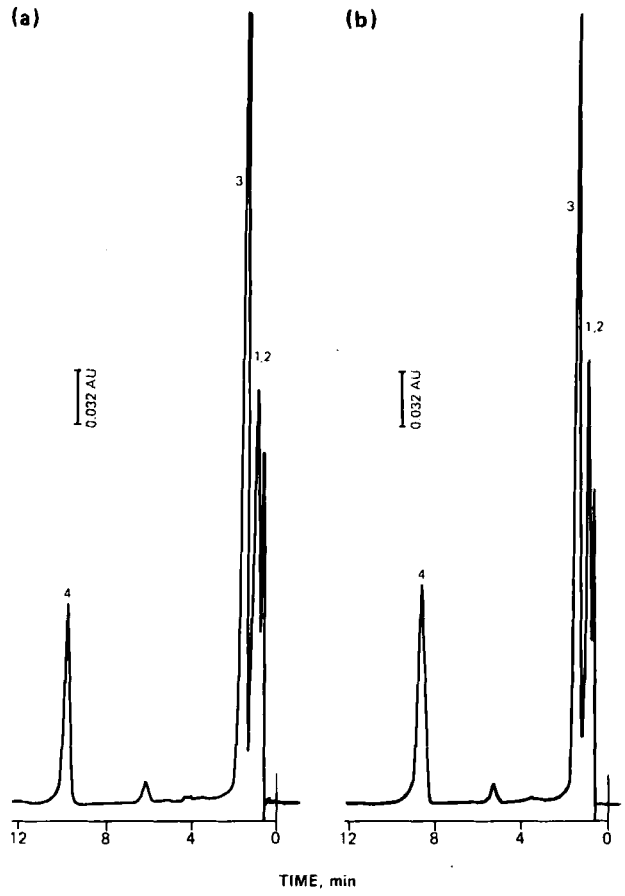


FIGURE 2 Separation of concentrated carotenoid mixture on Hypersil ODS column. Mobile phase conditions as in Table 2; a, no n-decanol; b, plus n-decanol.

Figures 2a and b show results with the 10 cm Hypersil ODS column. In contrast to the Zorbax ODS column, this high carbon loading endcapped material showed much less retention of the carotenoids, and a mobile phase composition of only 3% ethyl acetate was chosen for final testing. Even at this level, the polar carotenoids were not sufficiently retained to allow

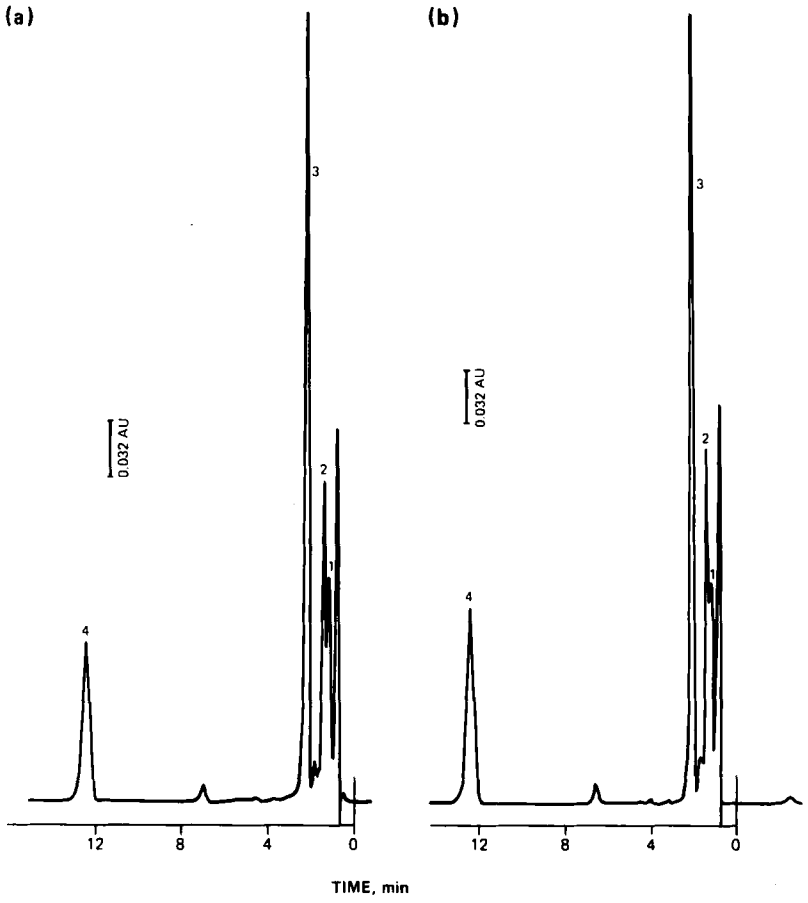


FIGURE 3 Separation of concentrated carotenoid mixture on Nova-Pak C18 column. Mobile phase conditions as in Table 2; a, no n-decanol; b, plus n-decanol.

accurate analysis. At the same time, any further drop in ethyl acetate content would increase analysis time for β-carotene to an unacceptable level. This material was therefore considered less suitable. Comparison of Figures 2a and b does show, however, that the presence of n-decanol had little effect on peak definition with this column.

Figures 3a and b show results for the Nova-Pak C18 column. This low carbon loading endcapped material had a retention intermediate to the Zorbax ODS and Hypersil ODS columns and a solvent containing 12% ethyl acetate was used. Although analysis of polar carotenoids would be possible on this column under these conditions, the separation of the polar compounds was not as good as with the Zorbax ODS column. The total analysis time to include β-carotene was also longer. Comparison of Figures 3a and b indicate that the addition of n-decanol to the mobile phase improves peak definition and response although retention times are only slightly affected. While separation and peak shape on the Nova-Pak C18 column was reasonable, this type of column could not be recommended for carotenoid analysis under these conditions. The column structure appears to be unstable to the solvent conditions. Two new Nova-Pak C18 columns were tested. On use, they were flushed first with acetonitrile and then with the carotenoid analysis solvents. Both exhibited low pressures with acetonitrile but developed back pressures in excess of 3000 psi within 4 h of use with ethyl acetate-acetonitrile solvents. One column became unusable. No obvious reason could be found for the high pressure which persisted even if the solvent was returned to 100% acetonitrile or methanol.

Figures 4a and b show results for the Spherisorb ODS1 column. This material, which has low carbon loading and is not endcapped, had a reversed elution order for carotenoids when compared to the three other ODS packing materials. The order is in fact identical to that shown on a silica column (Figures 5a and b), except that lutein and violaxanthin co-elute on the Spherisorb ODS1 column. The presence of n-decanol in the eluent had a major effect on peak shape, and doubled the response for each peak. The badly drifting baseline evident in the absence of n-decanol (Figure 4a) and presumably caused by gradual

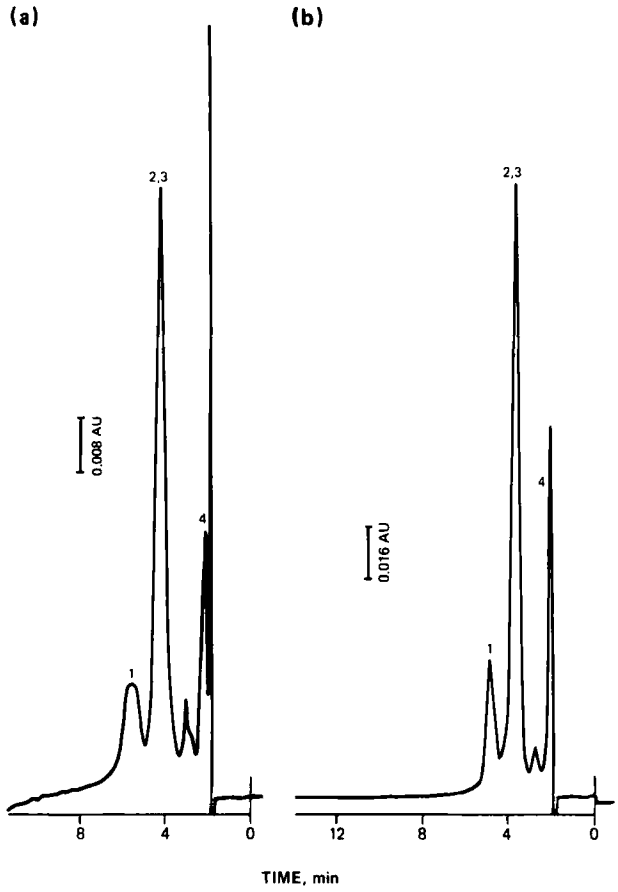


FIGURE 4 Separation of concentrated carotenoid mixture on Spherisorb ODS1 column. Mobile phase conditions as in Table 2; a, no n-decanol; b, plus n-decanol.

elution of a strongly retained portion of the analyte (3), was also largely removed.

Results for the two C8 packing materials are shown in Figures 6a and b and 7a and b. Both columns gave adequate separation of the three carotenoid classes, while the CPSpher column gave additional separation of the two epoxide

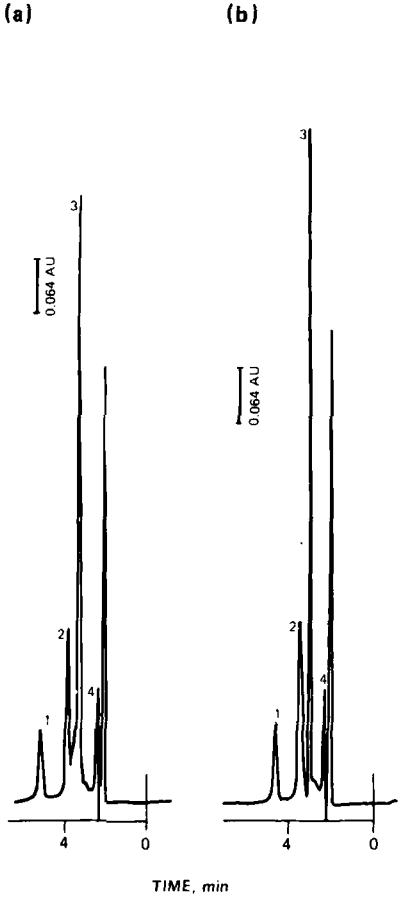


FIGURE 5 Separation of concentrated carotenoid mixture on Zorbax silica column. Mobile phase conditions as in Table 2; a, no n-decanol; b, plus n-decanol.

xanthophylls. The Zorbax C8 column appears less suitable than Zorbax ODS for carotenoid analysis since its capacity, peak shape and base-line were less than optimum. On the other hand, the CPSpher C8 column had relative capacity factors identical to those of an equivalent length Zorbax ODS column, therefore this type of column would be a suitable alternative to Zorbax ODS.

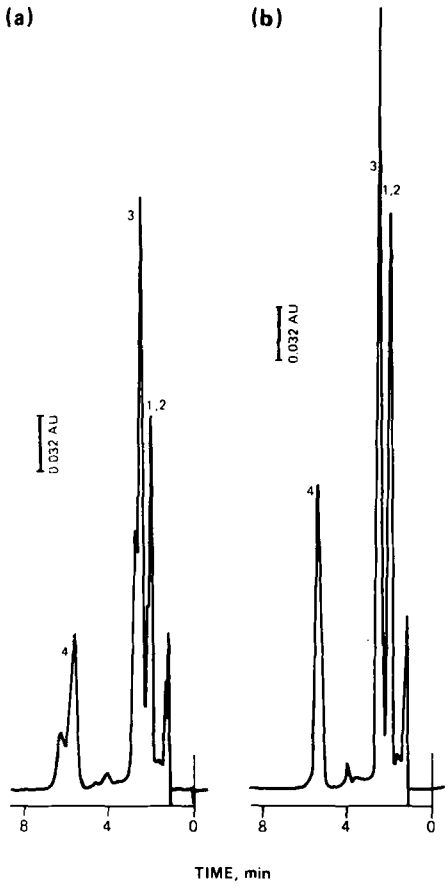


FIGURE 6 Separation of concentrated carotenoid mixture on Zorbax C8 column. Mobile phase conditions as in Table 2; a, no n-decanol; b, plus n-decanol.

The presence of n-decanol had a dramatic effect with both C8 columns. Without n-decanol, Zorbax C8 gave double peaks (Figure 6a). Comparison of Figures 7a and b show that n-decanol improved peak shape and definition and response on the CPSpher C8 column. A more dramatic effect to be discussed in the following section was on the response of low concentration samples.

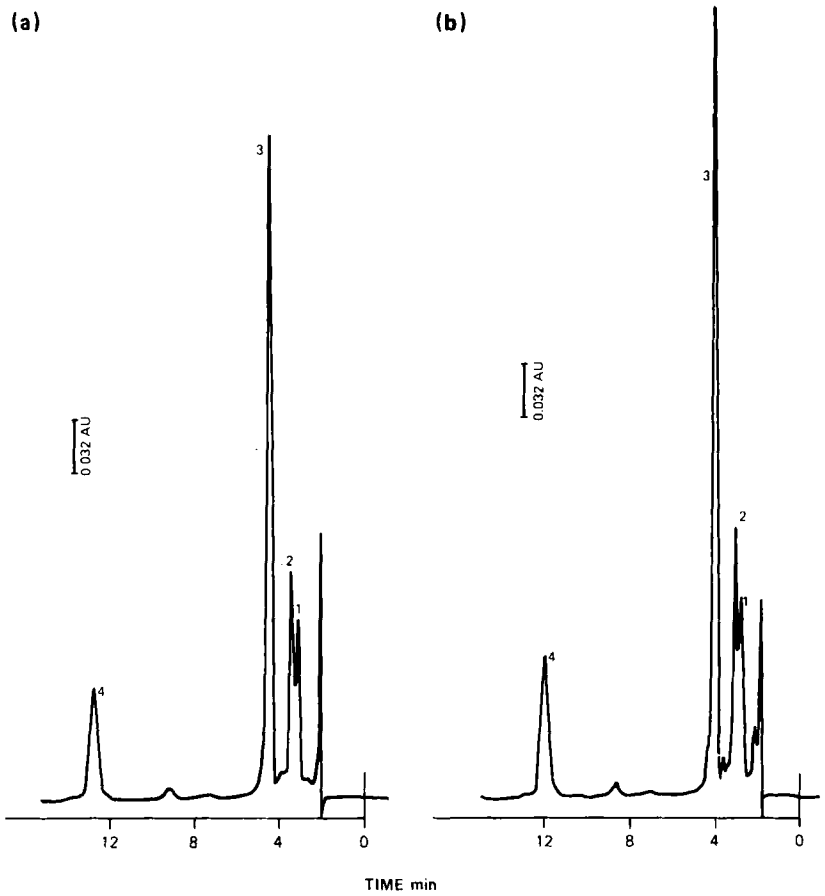


FIGURE 7 Separation of concentrated carotenoid mixture on CPSpher C8 column. Mobile phase conditions as in Table 2; a, no n-decanol; b, plus n-decanol.

Figure 8 shows the separation of carotenoids on the PRP-1 polymer column. The elution order was the same as obtained with the majority of other reversed-phase materials, but under the conditions used, the column is obviously unsuitable for carotenoid analysis. Addition of n-decanol had little effect if any.

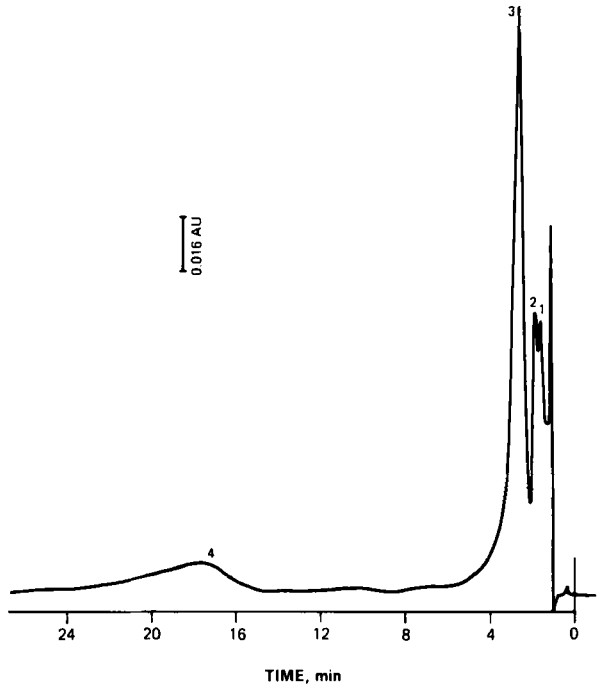


FIGURE 8 Separation of concentrated carotenoid mixture on PRP-1 column. Mobile phase conditions as in Table 2 for plus n-decanol.



## Linearity of Response with Concentration

As stated in the earlier publication (3) n-decanol had been added to the mobile phase used with Zorbax ODS columns to give chromatographic stability and to rapidly condition new columns. In the absence of n-decanol, the chromatographic system displayed nonlinearity at lower concentrations due to adsorbtive-type losses caused by activation of residual silanol groups. This aspect was further examined with the different columns tested in the present work, and results for the standard reversed-phase columns (except Spherisorb ODS1) are given in

Table 3. The results given are for β-carotene which is the component most affected when the symptom occurs.

The effect of n-decanol on the response curve of β-carotene on the two Zorbax ODS columns, 2 and 3, has been discussed more fully elsewhere (3). Briefly, the presence of n-decanol rapidly brings the response curve closer to linearity. In fact with column 2, full linearity was achieved within 2 h. The longer 25 cm column 3 had not reached linearity within the test time. This illustrates that different columns will vary in speed of conditioning. Although the initial improvement was the most dramatic and rapid, further gradual improvement to full linearity would be expected to occur with continued column use.

With the Hypersil ODS column 4, the response with concentration was linear either with or without n-decanol, and this also appeared to be the case with the Nova-Pak C18 column 5.

The Zorbax C8 column, 7, gave an apparently linear response either with or without n-decanol, although the double peaking which occured without n-decanol obviously indicated a non-homogeneous separation system existed. The values in Table 3 were taken off the major of the two β-carotene peaks, but the double peak shape for all components was consistent over the full concentration range tested.

The CPSpher C8 column showed the most dramatic change in response with addition of n-decanol. The column was grossly non-linear at low concentration in the absence of n-decanol, and became non-linear at high concentrations in the presence of n-decanol. However, observation of peak shapes and definition during the 3 h test period with solvent containing n-decanol suggested that these improved towards the end for the low concentration samples. This suggests that with further conditioning, this column would give a linear response.

TABLE 3

Effect of Packing Material and Mobile Phase Modifier on the Response Curves of β-Carotene for Different Reversed-Phase Columns[a]

| Column | n-Decanol added? | Apparent dilution for concentrated stock dilution of | | | | | |
|---|---|---|---|---|---|---|---|
| | | nil[b] | 2X | 4X | 8X | 16X | 32X |
| 2[c] | No | (17) | 2.4 | 6.3 | 16.5 | 36.7 | 66.0 |
| | Yes | (18) | 2.0 | 3.7 | 8.0 | 16.4 | 34.3 |
| 3[c] | No | (23) | 2.7 | 6.3 | 15.4 | 43.6 | 115.5 |
| | Yes | (25) | 2.2 | 4.4 | 9.7 | 22.4 | 54.7 |
| 4 | No | (35) | 1.9 | 3.7 | 7.6 | 17.1 | 29.2 |
| | Yes | (41) | 2.0 | 4.0 | 7.5 | 15.6 | 32.5 |
| 5 | No | (32) | 2.1 | 4.0 | 8.0 | | |
| | Yes | (33) | 1.9 | 4.2 | 7.2 | 17.0 | 34.1 |
| 7 | No | (30) | 2.0 | 3.9 | 7.8 | | |
| | Yes | (58) | 2.1 | 3.9 | 7.7 | 18.4 | 32.2 |
| 8 | No | (23) | 2.3 | 6.6 | 23.0 | 76.7 | 230.0 |
| | Yes | (24) | 1.8 | 3.3 | 6.3 | 12.2 | 23.6 |

a - Mobile phase conditions as for Table 2. Instrumental conditions as in the text. Detector sensitivity set at 0.32 a.u.f.s. for the most concentrated sample which contained 15 ppm β-carotene, then reduced to 0.08 a.u.f.s. for 4-32 times dilution samples. The concentrated sample was in dichloromethane-acetone-acetonitrile (4:1:5), and dilutions were made with dichloromethane-acetonitrile (1:1). Results are based on peak height. Peak area values agreed closely with these.

b - Actual response at 0.32 a.u.f.s. for nil dilution given in brackets.

c - Data for columns 2 and 3 have also been presented in reference 3.

The results obtained from these different columns suggest that packings such as Zorbax ODS with high carbon loading but no endcapping is preferred for non-aqueous reversed-phase separations of carotenoids. Involvement of exposed silanol groups aids the separation, but must be controlled by the addition of a mobile phase modifier such as n-decanol for optimum results. An obvious mechanism that would fit the results presented here would have retention of the non-polar carotenoids controlled primarily by carbon loading with the additional effect of exposed silanol groups selectively retaining polar carotenoids. This would account for the relatively poor selectivity of the endcapped ODS materials for the three polar carotenoids. Endcapped materials became more suitable as total carbon loading decreased, either by percent reaction, Nova-Pak C18, or carbon chain length, CPSpher C8. For non-endcapped materials, the high surface coverage by hydrocarbon found in the two Zorbax materials was essential. The ODS material gave improved peak shape over the C8 material. The results obtained with the Spherisorb ODS1 column illustrate that with low carbon non-endcapped materials, the exposed silanols can dominate the separation mechanism.

Several other properties of packing materials such as surface area, pore size, packing density, pH and trace impurities can influence retention, selectivity and efficiency of separation on both silica materials and the reversed-phase materials made from them (5). These factors have not been studied in this work, and some of them could be contributing to the unsatisfactory results observed in the absence of n-decanol.

The value of n-decanol as a mobile phase modifier to rapidly condition new columns and to stabilise the chromatographic system has once again been shown. The low percentage added generally affected all components to the same extent, and therefore allowed isocratic separation of the full range from

dihydroxy to hydrocarbon carotenoids to be achieved rapidly by correct choice of the main mobile phase constituents. This contrasts to solvent systems used in several recent publications (2,6,7) in which 10% or more of methanol are included in the mobile phase. In these cases, concurrent isocratic analysis of the epoxy-xanthophylls in the presence of lutein and β-carotene would not be possible. The deactivation of residual silanols caused by large amounts of methanol means that the retention of polar carotenoids is reduced to a much greater extent than that of non-polar carotenoids (2,3). The recent publication by Bieri et al. (6) used the solvent system of Nells and De Leenheer (2) with a Supelco LC18 column. This column, which has medium carbon loading and is endcapped, would be expected to give behaviour between the Hypersil ODS and Nova-Pak ODS columns with our solvent systems. The publication by Bushway (7) is complementary to our work, but can not be readily compared due to the high concentration of methanol used in the solvent systems. In that case, it can be seen that the two low carbon loading (8%) Vydac materials, one with and one without endcapping, gave a similar elution order.



## Conclusion

The isocratic non-aqueous reversed-phase separation of carotenoids with aprotic solvents is best achieved with packing materials of high carbon loading but without endcapping. The modifier n-decanol added at 0.1% to the mobile phase should be used to stabilize the system and give linearity of response and optimum peak shape and definition by minimizing absorptive losses. ODS packing materials are preferred but some C8 materials may be suitable.

## ACKNOWLEDGEMENTS

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