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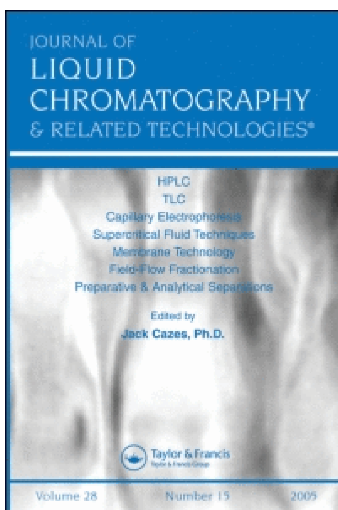

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JOURNAL OF LIQUID CHROMATOGRAPHY, 9(9), 1997-2012 (1986)

THE USE OF DECANOL FOR IMPROVING CHROMATOGRAPHIC STABILITY IN ISOCRATIC NON-AQUEOUS REVERSED-PHASE ANALYSIS OF CAROTENOIDS BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

A non-aqueous isocratic solvent system for reversed-phase analysis of a full range of carotenoids from polar to non-polar is described. The major advance in the method is the use of n-decanol added at 0.1% by volume as modifier to a mobile phase of ethyl acetate-acetonitrile. The presence of the modifier rapidly conditions new columns and avoids problems with trace solvent impurities, both of which would otherwise give unsatisfactory chromatographic behaviour. In the absence of n-decanol, activation of residual silanol groups in the reversed-phase packing can cause a drastic deterioration in column behaviour. The method is rapid, versatile and simple. Information on separation conditions, solvent effects, column conditioning and carotenoid response curves is given.

INTRODUCTION

Carotenoid compounds are commodities valuable for their nutritional and pigmenting properties. Because of this, their

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analysis in foodstuffs and animal feeds is important. Also of considerable recent interest is the analysis of carotenoid and photosynthetic pigments in aquatic ecological studies (1,2,3).

Analytical methods for these pigmenting compounds have been traditionally based on spectrophotometric measurements of class fractions. The lack of specificity of the values obtained by these methods makes them of questionable value unless the compositions of the fractions are also determined. Accordingly, high-performance liquid chromatography (HPLC) is becoming increasingly accepted as the method of choice for pigment analysis (4).

Elegant methods exist for the analysis of pigments in aquatic samples (2,3) when a wide range of components may be expected. However, these methods are more complex than required when analysis of only one structural group (eg. carotenoids) is desired. In addition, frequently in foodstuff analysis, the concentration of a few major components is required in preference to total sample characterisation.

The samples of primary interest to our work were alfalfa-based feed concentrates reported to contain mainly neoxanthin, violaxanthin, lutein, and β-carotene (5). The compounds cover the wide range of polar to non-polar carotenoids. We required a rapid analytical method suitable for routine quality control and product description, that is, one which retained the relative technical simplicity of standard methods employing column chromatography and spectrophotometric measurement, and yet had the added advantages of full component description and quantitation.

The various published methods for carotenoid separation of HPLC have been recently listed at length (6,7). General methods were few, rather, specific methods had been devised for specific applications. Methods used to cover the whole range of polar and non-polar carotenoids generally required gradient systems (6,8). Nells and DeLeenheer (6) reported non-aqueous reversed-phase (NARP) systems for separating a range of carotenoids including some of the common naturally occurring carotenoids, lutein, zeaxanthin, β-cryptoxanthin and β-carotene. One of the main

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strengths of their proposed method was that it was isocratic, and accordingly their method has gained wide acceptance (7,9).

In our early tests with this NARP system, several problems with reproductibility became evident, and some were further examined. It was also apparent that the system could be improved to allow the more polar compounds such as neoxanthin and violaxanthin to be analysed. In view of the wide acceptance of the NARP system, publication of our findings in detail is warranted. The work described in this paper improves on the work of Nells and De Leenheer (6) by the incorporation of a mobile phase modifier which stabilizes the chromatographic system. Reasons for the change and potential problems resulting without it are discussed.

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EXPERIMENTAL

Materials and Reagents

Solvents and reagents used were acetonitrile, hexane, dichloromethane and methanol (Ajax HPLC grade), acetone and glacial acetic acid (BDH Analar), ethyl acetate (BDH or Mallinckrodt Analytical grade) and n-decanol (Eastman Kodak). All were used as received. Water was glass distilled and then passed through a Millipore Milli-Q water purifier.

Carotenoid Standards

β-Carotene was purchased from Sigma Chemical Company. Stock solutions were made in hexane or ethanol containing butylated hydroxytoluene (BHT) antioxidant and accurate concentrations calculated using published extinction coefficients (10) following spectroscopic measurements at 453 nm of the stock and a series of dilutions. Zeaxanthin was donated by Hoffman La Roche. Stock solutions were made in ethanol containing BHT and concentrations determined at 450 nm as above. Lutein was recovered from a bulk alfalfa extract and purified by preparative HPLC. Stock solutions were made in ethanol containing BHT and concentrations determined

at 445 nm. Neoxanthin and violaxanthin were also recovered from a bulk extract of alfalfa separated and purified by HPLC. Stock solutions were prepared in ethanol as above and concentrations determined at 439 nm and 443 nm respectively. Direct probe mass spectra taken from aliquots of the stock solutions were consistent with the designated compounds. All stocks were stored in the dark at $0^{o}C$. HPLC standards were made by evaporation of an aliquot of the stocks under reduced pressure at $\leq 40^{o}C$ and in subdued light, and redissolving the residue in the appropriate quantity of a solvent compatible with the mobile phase. A mixed extract of alfalfa carotenoids was obtained by extracting alfalfa leaf protein concentrate with dichloromethane-acetone (11).

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Apparatus

Several modular liquid chromatographs were used. Solvent delivery was with a Spectra-Physics 740B gradient system, a Waters M45 pump, or a Metering Pumps Ltd (UK) E2B chromatography pump. All feed lines to the pumps contained a stainless steel filter (2-μm). Injection was by fixed loop valves (Valco or Rheodyne) or with a Micromeritics 725 autosampler. The analytical column was preceded by a 2-μm in-line filter (Rheodyne). Detection was at 450 nm with either a Tracor 970A or Perkin-Elmer LC75 variable wavelength detector. A Spectra Physics Minigrator was used for peak integration.

HPLC Columns

Both commercial and home-packed columns were used. The commercial column was 25 cm x 4.6 mm ID Zorbax ODS (Du Pont). Homemade columns slurry-packed with Zorbax ODS had the column dimensions of 15 cm x 4.6 mm ID and 30 cm x 3.9 mm ID. Analytical columns used for routine samples were directly preceded by a 6-cm guard column packed with CO:PELL ODS (Whatman).

Recommended HPLC Separation Conditions

Separation conditions varied depending on the analysis required and the column length used, but always employed isocratic

mobile phases of ethyl acetate-acetonitrile containing 0.1% by volume of n-decanol as modifier. Typical analytical conditions for a 15-cm column and a flow rate of 1.6 ml/min may be listed.

Repetitive β-carotene analysis was achieved in 4 minutes using 50% ethyl acetate. With 28% ethyl acetate, the carotenoids in alfalfa extracts were quantified within 10 minutes as β-carotene, non-epoxide xanthophylls (lutein and zeaxanthin) and epoxide xanthophylls (neoxanthin and violaxanthin). The use of 20% ethyl acetate allowed partial separation (not baseline) of neoxanthin and violaxanthin and of lutein and zeaxanthin plus β-carotene analysis within 15 minutes. Total separation of all these components was achieved with 12% ethyl acetate at 1 ml/min with an analysis time of 25 minutes. Separation of α-carotene and β-carotene was obtained with 28% or less of ethyl acetate. The synthetic pigments, canthaxanthin and apocarotenoic acid ethyl ester, were separated from each other and from lutein using 12% ethyl acetate.

Other separation conditions used during method development are listed in the text.

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RESULTS AND DISCUSSION

The advantages of high carbon loading packings for general carotenoid analysis may be inferred from earlier publications (2,6) and for this reason Zorbax ODS was chosen for the present work. This is the same material chosen by Nells and De Leenheer (6), but initial tests with a selection of the isocratic systems listed by them, including those containing methanol, proved unsatisfactory. We experienced poor or indefinite peak shape, especially for non-polar materials, highly variable retention times and failure of injected material to elute. An extract of carotenoids from alfalfa injected in methanol produced some early eluting peaks that occurred within an elevated baseline plateau that stretched beyond 20 min from injection time.

In an attempt to isolate the problem, some of the NARP systems already tested were further examined along with some including

hexane. Mobile phases including methanol were not used since it was obvious from capacity ratios that these would not be satisfactory for analysis of the more polar compounds, neoxanthin and violaxanthin. The results of these tests are shown in Figure 1. The dichloromethane-acetonitrile mixtures (Figure 1a,b,c) all gave unsatisfactory peak shape. Ethyl acetate-acetonitrile mixtures (Figure 1d,e,f) gave improved peak shape, although still not ideal. Comparison of these three chromatograms indicated that solvent compositions near 20:80 ethyl acetate-acetonitrile would give good retention of lutein (k' about 3) without unnecessarily long retention of β-carotene ($k' = 12$). Addition of hexane to the mobile phase was less suitable since as with methanol (6), it decreased the retention of lutein relative to β-carotene (compare Figure 1e and f).

Our choice of ethyl acetate in preference to dichloromethane and the elimination of methanol from the mobile phase are in contrast to the solvent apparently preferred by Nells and De Leenheer, namely 70:20:10 acetonitrile-dichloromethane-methanol (6). The following subsequent work we have done suggests that in both cases the final choice of the main mobile phase components may have been serendipitous and have resulted from the order in which different solvent mixtures were tested. Gradual conditioning of the column eventually produced better results. Nells and De Leenheer indicated in their concluding statement that conditioning slowly occurred with their system while one of the main purposes of n-decanol in our proposed method is to rapidly bring the system to a stable state.

Effect of n-Decanol Modifier

n-Decanol was chosen as modifier in an attempt to partly mimic the reversed-phase packing with the modifier. It was added to the ethyl acetate-acetonitrile solvent at 0.1% by volume. The quantity of modifier was kept low to avoid the reduction in retention of polar carotenoids caused by addition of large amounts of alcohol. Figure 2 shows the influence of the modifier. Figure

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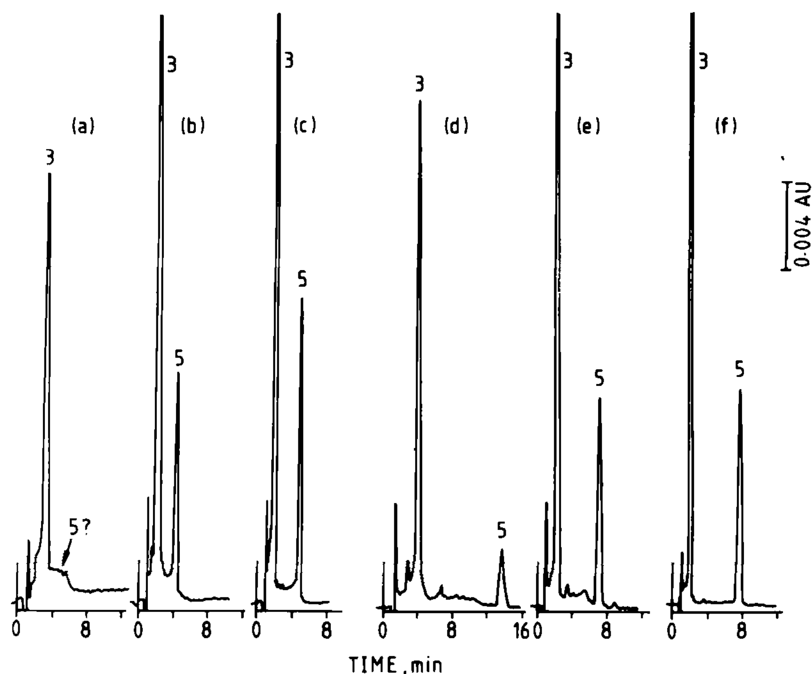

Figure 1. Preliminary tests of non-aqueous solvent systems for analysis of carotenoid mixtures on Zorbax ODS column (15 x 0.46 cm). Sample of lutein (4.47 μg/ml) and β-carotene (2.49 μg/ml) in dichloromethane-acetonitrile (30:70) (a,b,c) or ethyl acetate-acetonitrile (30:70) (d,e,f). Sample size = 20 μl. Solvent flow rate = 2 ml/min. Peak identification for all figures: 1 = neoxanthin; 2 = violaxanthin; 3 = lutein; 4 = zeaxanthin; 5 = β-carotene. Mobile phases were: a, dichloromethane-acetonitrile (20:80); b, dichloromethane-acetonitrile (30:70); c, dichloromethane-hexane-acetonitrile (20:5:75); d, ethyl acetate-acetonitrile (20:80); e, ethyl acetate-acetonitrile (30:70); f, ethyl acetate-hexane-acetonitrile (20:5:75).

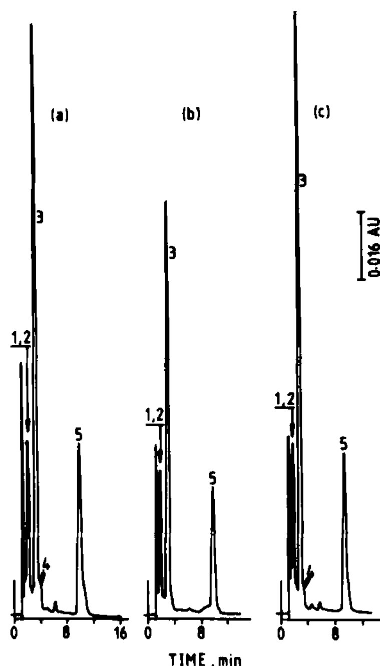

Figure 2. Influence of n-decanol modifier on column performance. Sample was an extract of carotenoids from alfalfa dissolved in dichloromethane-acetone-acetonitrile (4:1:5) containing BHT. Conditions as for Figure 1 except for flow rate = 1.6 ml/min and mobile phase which was ethyl acetate-acetonitrile (30:70) with or without 0.1% by volume of n-decanol. Columns were: a, a well-used column (15 x 0.46 cm) showing satisfactory behaviour and further conditioned with n-decanol; b, a new column (15 x 0.46 cm) using mobile phase without n-decanol passed for 30 min; c, column from b with mobile phase containing n-decanol passed for 30 min.

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2a shows a carotenoid sample analysed using recommended conditions on a column both well used and conditioned with n-decanol. Figure 2b shows the same sample on a freshly packed column with no modifier in the mobile phase. Note the reduced response for all peaks, diminuition of the minor peak, and the plateau before β-carotene. Figure 2c shows the same column using recommended conditions after conditioning for only 30 min with mobile phase

containing n-decanol. Note the improved result with enhanced response for all components and greater definition of minor peaks.

All columns have slight variations in behaviour. Thus when two apparently identical columns are interchanged, differences may be evident not only in the response of individual peaks, but also in their relative retention times. The length of time required to condition an individual column was easily assessed by occasional injection of a carotenoid mixture until further changes in peak height and peak shape were insignificant. Any further conditioning that takes place occurs so gradually that consistent results (standard deviation less than 0.65%) have been obtained from long automated runs lasting over 18 h or more.

Measurements taken from several new columns have shown that the small quantity of n-decanol modifier reduces the retention of both polar and non-polar carotenoids to a similar extent (k' reduced by 6-16%). This is in contrast to the use of larger quantities of methanol modifier which substantially reduce the retention of polar carotenoids (by 66%) with much less effect (7.4%) on non-polar carotenoids (6). This property of n-decanol allows, by choice of the main mobile phase constituent concentration, the full range from dihydroxy to hydrocarbon carotenoids to be analysed.

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Comparison of Old and New Columns

The effect of column conditioning first became apparent when the column used for all initial experiments was replaced with a freshly packed column. In the absence of n-decanol, the responses of all materials, but in particular β-carotene, were significantly reduced. This effect was consistent for a number of new commercial or homemade columns. Coincident with this reduced response was a loss of definition of minor peaks and occasional elevated plateau formation prior to β-carotene. With experience, a number of tell-tale signs became evident, and these are illustrated in Figure 3 and accentuated by shading. Figure 3b shows a generally elevated baseline which only gradually returns

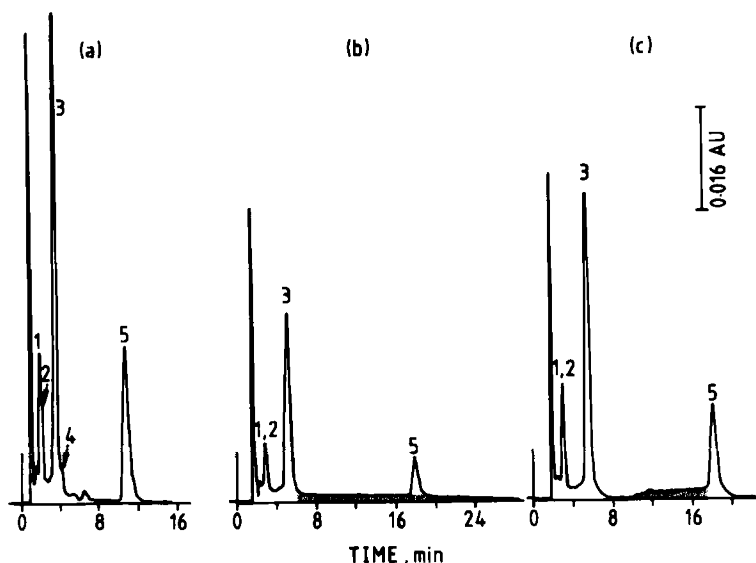

Figure 3. Typical symptoms observed when new columns packed with Zorbax ODS were used for carotenoid analysis without n-decanol in the mobile phase. Sample was as for Figure 2. Conditions as for Figure 2 except for mobile phase = ethyl acetate-acetonitrile (25:75). Coumns were: a, column from Figure 2a; b, a new column (30 x 0.39 cm) after 15 min use; c, as for b after 45 min.

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to zero; the minor peak between lutein and β-carotene is also lost. Figure 3c illustrates the plateau effect into β-carotene.

These effects indicated additional competing analyte-solid phase interactions in new columns compared to well-used ones which cause a portion of the analyte to be eluted in an ill-defined form. The effects were felt to originate from variable involvement of residual silanol sites in the packing material. In the absence of n-decanol, several weeks of use for carotenoid analysis were needed to condition new columns to a relatively stable state. Clearly the need for lengthy conditioning of new columns is unacceptable in a general method and this has been overcome by the addition of n-decanol which rapidly conditions new columns to a reproducible state.

Sample Response with Concentration

While carotenoid standard solutions injected directly into the detector cell produced a linear response within the limits of the detectors used, samples analysed through the chromatographic system displayed an unusual non-linearity at lower concentrations. As the concentration of the injected sample dropped, the chromatographic response measured by either peak height or peak area dropped to a relatively greater degree. Clearly part of the sample was being adsorbed or eluted as an ill-defined form as illustrated for new columns in Figure 3b and 3c. The extent of the effect varied from column to column but was minimized in the recommended solvents containing n-decanol and tended to diminish with column use. Results for β-carotene, which was most affected, are shown in Table 1. The effect is clearly in the chromatographic system rather than in the detector, and this was supported by the two different detectors showing the same effect.

In practical terms, the non-linear response curve means that a range of standards should be used in analysis and that all samples should be diluted to similar concentration range for accurate quantitation.

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Effect of Other Potential Modifiers

The effects illustrated suggest that the separation of carotenoids on Zorbax ODS, which contains residual silanol groups, results from the interaction of reversed-phase and straight-phase effects. Accordingly, analyses may be adversely affected by chance influences to either one. Involvement of the silanol groups is beneficial since it appears to enhance selectivity of the polar carotenoids (12), but in an uncontrolled system (e.g. without n-decanol) conflicting interactions could occur. For example, in the separation of amines by reversed-phase chromatography using amine modifiers (13), involvement of hydrophobic, ion exchange and hydrogen bonding interactions has been suggested. We believe the effects shown on new columns in the absence of n-decanol indicate the influence of conflicting adsorption mechanisms producing inferior results.

TABLE 1

Response Curves for β-Carotene on Three Zorbax ODS Columns[a]

Column[b]	n-decanol added?	Apparent dilution for stock dilution of					
		nil[c]	2X	4X	8X	16X	32X
A	Yes	(33)	2.1	4.3	8.0	16.9	33.0
B	No	(17)	2.4	6.3	16.5	36.7	66.0
	Yes	(18)	2.0	3.7	8.0	16.4	34.3
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

a - Different dilutions of a mixed carotenoid stock solution containing 15 ppm β-carotene in dichloromethane-acetone-acetonitrile (4:1:5) containing BHT were analysed. Dilutions were with dichloromethane-acetonitrile (1:1) containing BHT. Mobile phase was ethyl acetate-acetonitrile (25:75) with or without n-decanol (0.1% by volume) as indicated. Flow rate = 1.6 ml/min. Injection size = 10 μl. Detection was at 450 nm with sensitivity for the stock set at 0.32 a.u.f.s. to give from 16-32% deflection, then changed to 0.08 a.u.f.s. for 4-32X dilution samples.

b - Column identification: A = well-used 15 cm column; B = new homemade 15 cm column; C = new commercial 25 cm column.

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

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A number of modifier types have been used previously to stabilize or reduce activity of silica packings, and such additives could be useful to modify the influence of silanol groups in reversed-phase packings. Water or an alcohol may be used (14), and alcohols have been used as modifiers for silica gel analysis of retinol and related compounds (15). Our use of n-decanol is an example of this type. Aliphatic carboxylic acids (16) and various ionic compounds (17, 18) have been used but these may serve to either regenerate active sites (17) or to make them more accessible or to act as sources of electrostatic interaction (13, 18). In fact, β-carotene has been reported not to elute from

Zorbax ODS with an ion-pair gradient system (2). The alternate use of a carboxylic acid as modifier was examined, however.

When acetic acid was added at 0.5% to the mobile phase, an immediate effect was that the response for β-carotene was reduced by 20-25%. It was subsequently shown that even trace amounts of acetic acid can cause problems with chromatographic stability as has been reported in normal phase chromatography on silica (16). Free acetic acid is a potential impurity in ethyl acetate, and a major problem with chromatographic stability occurred when ethyl acetate containing 0.017% acetic acid was used in place of analytical grade ethyl acetate which contained from 0.0025-0.0045% of free acid. The effects, which were not immediate, are shown in Figure 4. Figure 4a shows separation of a carotenoid sample soon after the pump reservoir was filled with solvent made from ethyl acetate with the higher level of free acid. After 8 h of solvent flow, analysis of the same sample gave Figure 4b. This marked degeneration of the column only became obvious after about 4 hours of solvent flow, although in retrospect, slight degeneration began within the first 1-2 hours. The solvent was left in the column for about 64 hours, then solvent made from analytical grade ethyl acetate was pumped through. The first injection of the carotenoid sample applied at this time (Figure 4c) bore no resemblance to anything previously obtained, with all compounds very strongly retained. However after about 90 min of this solvent flow, the separation in Figure 4d was obtained, and after a further 1 hour, the separation was back to the original (Figure 4e). Over this period, the retention of lutein moved in stages from 611 sec to 451, 428, 257 and finally to 203 sec. The retention of β-carotene went from undefined to 1241, 945, 715, and finally to 576 sec.

The behaviour described in Figures 4b-d is consistent with the report that β-carotene could not be eluted from Zorbax ODS using an ion-pair gradient system (2). However, the summation of work described in this paper suggests that rather than not being eluted, the β-carotene would elute as an ill-defined plateau or general elevation of baseline over a protracted period.

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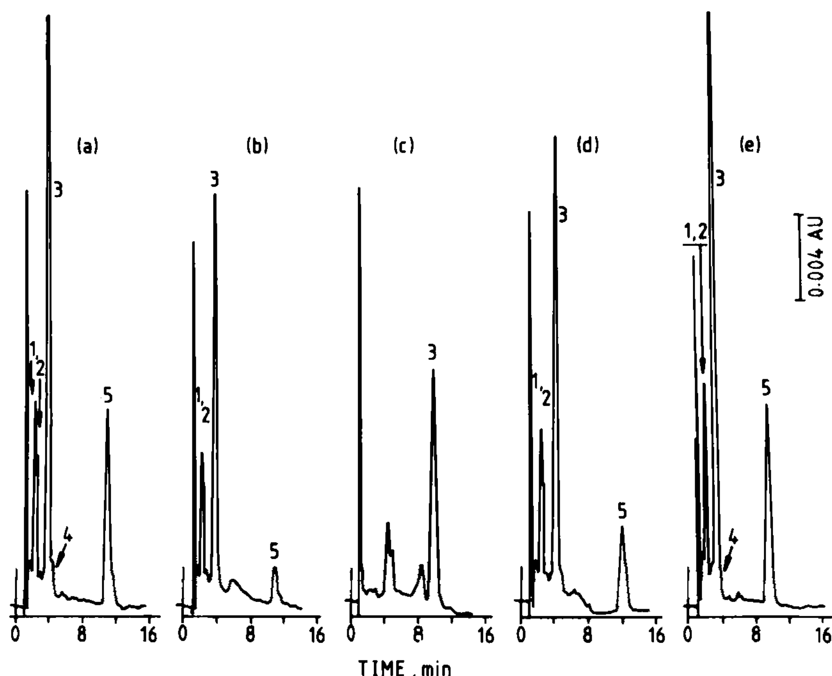

Figure 4. Effect of free acetic acid on the chromatographic behaviour of a well-conditioned Zorbax ODS column (15 x 0.46 cm) used for analysis of carotenoid mixtures. Sample was an extract of carotenoids from alfalfa dissolved in dichloromethane-acetone-acetonitrile (4:1:20) containing BHT. Conditions as for Figure 3 with mobile phase: a, ethyl acetate-acetonitrile (25:75) made from ethyl acetate containing 0.017% acetic acid and on column for 1 h; b, as for a after 8 h; c, same concentration made from ethyl acetate containing 0.0025% acetic acid and on column for 10 min; d, as for c after 1.5 h; e, as for c after 2.5 h.

Conclusion

Over the last year, the use of n-decanol modifier added at 0.1% by volume has proven to both avoid the detrimental effects of trace impurities in the mobile phase and to rapidly condition new columns. The method described is very versatile and well suited for general carotenoid analysis from extracts of alfalfa, alfalfa products and feedstocks. The system is readily modified for

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specific analytical purposes and has proven satisfactory for many hundreds of samples on a number of columns. It is usable on simple HPLC pumping systems. The non-aqueous solvent system allows high flow rates with low back-pressure and therefore allows rapid sample throughput, especially when combined with an automatic injection system. The method should find use in quality control as well as in research laboratories and should be applicable in any field where analysis of polar through to non-polar carotenoids is required.

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