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Evaluation of reversed-phase liquid chromatographic columns for recovery and selectivity of selected carotenoids

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ABSTRACT

Sixty commercially available and five experimental liquid chromatography columns were evaluated for the separation and recovery of seven carotenoid compounds. Methanol- and acetonitrile-based solvents (either straight or modified with ethyl acetate or tetrahydrofuran) were compared to determine which solvent systems and which columns provided better selectivity and recovery. Methanol-based solvents typically provided higher recoveries than did acetonitrile-based solvents. Polymeric C_{18} phases generally provided better selectivity for the difficult separation of lutein and zeaxanthin than did monomeric C_{18} phases.

INTRODUCTION

Epidemiologists have observed a lower incidence of lung cancer in people who have an above-average intake of fruits and vegetables [1,2]. Studies also suggest that the intake of fruits and vegetables may reduce the risk of cancers of the mouth, pharynx, larynx, esophagus, stomach, colon, rectum, bladder, and cervix [2]. Because low serum levels of β-carotene are associated with the subsequent development of lung cancer, β-carotene may be the protective factor present in the fruits and vegetables [2]. However, the lowered incidence of cancer may be due to other carotenoids that are co-ingested with the β-carotene, and serum levels of these other carotenoids have not been adequately studied. Liquid chromatography (LC) has been used to separate and measure β-carotene in serum [3] with concurrent measurement of α-carotene [4–10] and sometimes lycopene [11–18]. In addtion to numerous *cis/trans* geometric isomers, human blood serum contains at least six structurally distinct carotenoids. To determine which carotenoid compound(s) may provide anti-cancer effects, it is important to separate and measure the major carotenoids in serum and in food. Consequently, a number of workers have reported the measurement of carotenoids other than lycopene, α-, and β-carotene in serum

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[19–31], in skin [32], in human milk [33] and in foods [34–48].

The majority of carotenoid separations reported in the literature involve the use of reversed-phase LC, generally on a C_{18} stationary phase, although a few normal-phase LC separations have been reported [21,24,35]. Most workers using reversed-phase LC have used one of the following solvent systems: an acetonitrile-based eluent, an acetonitrile-based eluent to which ammonium acetate has been added, or a methanol-based eluent.

Acetonitrile-based eluents are used most frequently. In 1979, Zakaria *et al.* [49] separated lycopene, α-carotene, and β-carotene in tomatoes on a C_{18} column using a mixture of chloroform and acetonitrile. In 1983, Nelis and De Leenheer [19] reported LC separations on C_{18} columns using acetonitrile or acetontrile and 8% methanol with various organic modifiers added [tetrahydrofuran (THF), diisopropyl ether, chloroform, dichloromethane, ethyl acetate]. Most workers have adapted one of these mixtures so that it provides acceptable results on their particular LC column [4,5,8,9,11–13,16–18,20,22,25,26,30,31,35,38,40,46,48]. Khachik and co-worker [37,41,42,47] typically add hexane to a mixture of methanol, acetonitrile, and methylene chloride. Other workers have used acetonitrile modified with THF and water [36], with water and 2-propanol [44,45], or in a 1:1 mixture with ethanol [27].

The second basic method was described by Peng [6], and employs a mixture of acetonitrile, THF, methanol, and ammonium acetate. This method has been adapted by Nierenberg *et al.* [3,14,29], Kalman *et al.* [10], and Culling-Berglund *et al.* [32].

Methanol-based separations cited include the use of straight methanol [15] or methanol that has been modified with THF or chloroform [35], with water and THF [7], with water and butanol [23], and with hexane [28].

Carotenoid separations reported in the literature employ a wide variety of C_{18} and other reversed-phase columns from different manufacturers. Several workers have compared separations on a small number of commercially available columns: Nelis and De Leenheer [19] have described the effects of five organic modifiers on retention and selectivity of carotenoids on two different C_{18} columns. Bushway [35] has compared the selectivity of eight columns (two normal-phase and six reversed-phase) using several different solvent systems. Lauren and McNaughton [50] have compared ten reversed-phase columns with respect to alfalfa carotenoids' elution order, retention time, peak height and shape, etc. using acetonitrile and ethyl acetate with or without 0.1% *n*-decanol.

Recovery of carotenoids from the LC column is an important factor in carotenoid analysis. Frequently, serum carotenoid concentrations are near the detection limit so maximum sensitivity is necessary for accurate carotenoid measurements. This can only be achieved if carotenoid recovery is essentially 100%. Similarly, since epidemiological studies continue for extended periods, reproducibility is extremely important to the outcome of studies. Therefore only LC columns offering consistent and maximum recovery can yield the most meaningful results. Finally, there is a need to correlate carotenoid analysis by LC to older spectrophotometric measurements. If LC columns do not provide complete and consistent recovery of carotenoids, then LC measurements cannot be correlated with spectrophotometric measurements of carotenoids. Only one limited study has been reported that compared serum carotenoids measured by LC to total carotenoid concentration determined statically in a spectrophotometer [20]. We have compared sixty-five reversed-phase LC columns (primarily C_{18}-modified silica) for selectivity and recovery of a mixture of seven carotenoids, six of which are present in the serum of most American populations. This is the first study to report absolute recovery of carotenoids from LC columns.

EXPERIMENTAL[a]

Test mixture

An ethanol solution containing approximately 2 to 10 mg/l each of lutein (Kemin Industries, Des Moines, IA, USA); zeaxanthin, β-cryptoxanthin (Atomergic Chemetals, Farmingdale, NY, USA); echinenone (Hoffmann-La Roche, Nutley, NJ,

[a] Certain commercial equipment, instruments, or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

USA); lycopene (extracted from tomato paste); and α- and β-carotene (Sigma, St. Louis, MO, USA) was prepared, and 2-ml aliquots of this solution were stored in amber glass vials at −20 °C. The respective concentrations of the compounds in the mixture were sufficiently different to provide some indication of identity based on chromatographic peak area. Prior to injection, 100 μl THF were added to a vial of the carotenoid mixture, and the mixture was placed in an ultrasonic bath for 10 min to ensure dissolution. This solution was then transferred to a vial and placed in an autosampler where it was held at a constant temperature of 15°C; the autosampler injected a 20-μl aliquot of the solution onto the column being tested.

Chromatographic conditions

A liquid chromatograph pumped the mobile phase at a rate of 1.5 ml/min. Solvents were premixed so as not to depend on reproducible mixing by the pump; the solvents were sparged with helium. The column being tested was held at 27°C by the LC column oven.

Columns

A list of the columns tested, serial number, manufacturer, and classification of the bonded C_{18} stationary phase (polymeric, monomeric, or intermediate [51]) is provided in Table I. Reversed-phase columns that were potentially useful for the separation of carotenoids were donated by LC column manufacturers. Columns prepared from different production lots from the same manufacturer were also requested in order to assess reproducibility. Several columns that were currently in use in our laboratory were tested as well. Most of the columns tested were C_{18}, 250 × 4.6 mm I.D., with 5-μm particles. All columns were made of stainless steel. All columns were alkyl-silane modified silica, except for the Biotage Unisphere Polybutadiene, which was modified alumina. The polymeric/monomeric/intermediate classification given in Table I refers to the type of stationary phase modification procedure, and is described in detail in the Results and Discussion section, as is the separation factor ($\alpha_{TBN-BaP}$ value) to which it is related.

Eluents based in methanol and acetonitrile were used in this study. Butylated hydroxytoluene-stabilized THF and ethyl acetate modifiers were added to the mobile phase for those columns that failed to elute all seven compounds with a capacity factor ($k' \leqslant 11$. Both THF [3,6,7,10,11,14,22,29,32,35, 36,43] and ethyl acetate [19,50] have been used for carotenoid separations and represent two different solvent selectivity groups, III and VI, respectively. (Chlorinated solvents such as methylene chloride and chloroform were not tested because of a previous report of carotenoid losses caused by the hydrochloric acid that may be a trace contaminant in these solvents [52].) Prior to performing a separation, each column was flushed with THF at 1.5 ml/min for 4 min to remove any compounds remaining on the column from a previous run. The column was then equilibrated at 1.5 ml/min for 15 min with a given eluent. In cases where 100% methanol or acetonitrile eluted the compounds from the column with a $k' < 7$, no attempt was made to increase retention by the addition of water because of the minimal solubility of carotenoids in water and the opportunity for on-column precipitation [19,23].

Recovery determination

The photodiode array detector (Model 990, Waters, Milford, MA, USA) used to monitor the LC effluent acquired absorbance spectra from 350 to 500 nm with 20 scans/s at 2-s intervals. Data were acquired for 25 min at 0.5 a.u.f.s. Spectral data were used for peak identification when necessary. Visible absorbance at 450 nm was recorded by the data system for the quantitative comparison discussed below.

For the recovery study, the column was replaced with a 195-cm length of PTFE tubing (0.8-mm I.D.) to provide "peak" dispersion similar to the LC column. Using ethanol as the mobile phase, a 20-μl injection of the test mixture was considered to have a peak area that represented 100% recovery. Five such replicate measurements were made at the beginning and at the end of each day's work. With a column in place, the individual areas of the integrated peaks were totalled, and the total area was normalized to the average area of the 10 replicate injections when no column was used. Because the test mixture contained small quantities (1 to 7%) of impurities that certain columns may have been capable of resolving, all peaks eluting from a column were measured, not just the seven main peaks.

The linearity of the detector response was verified

TABLE I

LIST OF COLUMNS USED

Columns are C_{18} except where noted.

	Supplier	Column name	Serial No.	$\alpha_{TBN/BaP}$[a]	Classification[b]
1	Beckman	Ultrasphere ODS	9UE1898	1.92	Monomeric
2	Beckman	Ultrasphere ODS	8UE2757	2.01	Monomeric
3	Beckman	Ultrasphere ODS DABS	7UE2306	2.00	Monomeric
4	Analytichem	Sepralyte C18	072851-18	1.82	Monomeric
5	Separations Group	Vydac 218TP	890130-16	0.83	Polymeric
6	Separations Group	Vydac 201TP	890130-23	0.78	Polymeric
7	Separations Group	Vydac 201TP	890131-26	0.80	Polymeric
8	Biotage	Unisphere-PBD[c]	2167	3.06	Monomeric
9	ES Industries	Gamma Bond C18	19589-57-17992	1.86	Monomeric
10	ES Industries	Chromegabond C22 (C_{22})	19589-4-58-17994	1.83	Monomeric
11	ES Industries	Chromegabond MC18	19589-4-57-17991	1.73	Monomeric
12	Supelco	LC-18	18744F	2.03	Monomeric
13	Supelco	LC-18	18745F	2.02	Monomeric
14	Supelco	LC318	110224	2.05	Monomeric
15	Supelco	LC318	110232	2.04	Monomeric
16	Supelco	LC-PAH	81695	0.70	Polymeric
17	Analytichem	Sepralyte C18	071912-14	1.92	Monomeric
18	Alltech	Adsorbosphere C18	08039GA	2.01	Monomeric
19	EM Science	LiChrospher 100 RP-18	86554563	1.45	Intermediate
20	Serva	Octadecyl	03118	1.84	Monomeric
21	Serva	Triacontyl (C_{30})	–[d]		
22	J&W	Accusphere ODS	9050825	2.07	Monomeric
23	J&W	Accusphere ODS	9072526	1.96	Monomeric
24	MacMod	Zorbax RX C8 (C_8)	AU2642	2.33	Monomeric
25	Perkin Elmer	Pecosphere C18	1119	2.00	Monomeric
26	YMC	A303	42511	1.97	Monomeric
27	YMC	AMP303	4259	2.01	Monomeric
28	YMC	AP303	4250	2.06	Monomeric
29	J.T. Baker	exper WP C18[e]	1294-43	0.57	Polymeric
30	J.T. Baker	exper WP C18[e]	1294-26	0.93	Polymeric
31	J.T. Baker	exper WP C18[e]	1294-29	0.73	Polymeric
32	J.T. Baker	exper WP C18[e]	1239-64C	1.42	Intermediate
33	J.T. Baker	exper WP C18[e]	1239-64D	0.22	Polymeric
34	ES industries	Chromegabond PFP[f]	19589-4-58-17993	0.88	Polymeric
35	ES Industries	Chromegabond BF-C18	21389-4-60-18079	1.04	Intermediate
36	J.T. Baker[g]	Bakerbond C18	A29113-01	1.25	Intermediate
37	J.T. Baker[g]	Bakerbond WP C18	B36097.25	0.51	Polymeric
38	J.T. Baker[g]	Bakerbond WP C18	none	0.54	Polymeric
39	J.T. Baker[g]	Bakerbond WP C18	B33125.16	1.09	Intermediate
40	Keystone Sci.	ODS Hypersil	03708	1.95	Monomeric
41	Shiseido	Capsell Pak C18	SG120	1.99	Monomeric
42	Nacalai Tesque	Cosmosil 5C18-P Waters	391-03	2.04	Monomeric
43	Nacalai Tesque	Cosmosil 5C18 Waters	390-47	1.85	Monomeric
44	Waters	Nova-Pak C18	T93242	1.97	Monomeric
45	YMC	ASP303-5	42541	2.05	Monomeric
46	Hewlett-Packard	ODS Hypersil	79926OD-584	1.98	Monomeric
47	Hewlett-Packard	LiChrospher 100 RP-18	79925OD-584	1.50	Intermediate
48	Brownlee	Spheri-5 ODS	102454	1.26	Intermediate
49	Bio-Rad	Hi-Pore RP318	890215-11 No. 82	0.59	Polymeric
50	Macherey-Nagel	Nucleosil 5 PAH	90702B	0.36	Polymeric
51	Rainin[g]	Microsorb C18	10681	1.78	Monomeric
52	Phase Separations	Spherisorb S5 PAH	23/123 00-1046	0.82	Polymeric
53	Phase Separations	Spherisorb S5 ODS	29/69 19-1372	1.68	Intermediate

TABLE I (*continued*)

Supplier	Column name	Serial No.	$\alpha_{TBN/BaP}$[a]	Classification[e]
54 Phase Separations	Spherisorb S5 ODS	30/35 06-10-F1	1.50	Intermediate
55 Carlo Erba	Erbasil 5 C18/L	517250502	1.76	Monomeric
56 Carlo Erba	Erbasil 5 C18/M	517250503	1.28	Intermediate
57 Carlo Erba[h]	Erbasil 5 C18/H	517250504	0.91	Polymeric
58 Brownlee	Spheri-5 RP-18	109083	1.92	Monomeric
59 MacMod	Zorbax RX C18	880967.902	1.50	Intermediate
60 Phenomenex	Ultracarb 5 ODS20	PP/4953C	1.95	Monomeric
61 Phenomenex	Ultracarb 5 ODS30	PP/4954C	2.01	Monomeric
62 J.T. Baker	Bakerbond WP C18	1314-13	0.89	Polymeric
63 J.T. Baker	Bakerbond WP C18	DS097	0.67	Polymeric
64 Brownlee	Spheri-5 ODS	102402	1.42	Intermediate
65 MacMod[g]	Zorbax ODS	F36560	1.80	Monomeric

[a] Relative retention of 1,2:3,4:5,6:7,8-tetrabenzonaphthalene (TBN) to benzo[a]pyrene (BaP); see Results and Discussion section or ref. 51.
[b] As determined by evaluation of SRM 869 (see refs. 51 and 53).
[c] PBD = polybutadiene.
[d] Returned to supplier before value could be determined.
[e] Experimental columns with varying surface coverage.
[f] PFP = pentafluorophenol.
[g] Used in laboratory prior to evaluation in this study.
[h] Not tested due to excessive backpressure (>200 bar).

by injecting varying amounts of the mixture of seven carotenoids. The detector response was linear from 50 to 300% of the amount injected for evaluation of the columns.

RESULTS AND DISCUSSION

Sixty-five reversed-phase LC columns (Table I) were evaluated to determine selectivity and recovery of selected carotenoids. Lutein, zeaxanthin, β-cryptoxanthin, echinenone, lycopene, and α- and β-carotene were selected for use in the test mixture, and are shown in Fig. 2. Excluding echinenone, which is present in the human populations that consume echinoderms (sea urchins, starfish), these compounds account for more than 90% of the carotenoids present in American serum [20]. Echinenone was included because it has intermediate polarity and has been used as an internal standard for carotenoid measurements [20].

Column classification

A system has been developed for the classification of C_{18} stationary phases based on their separation of a mixture of polycyclic aromatic hydrocarbons (PAHs) [51]. The application of this classification scheme has proven useful for selecting columns for separating PAH isomers and steroids, two classes of compounds with rigid molecular

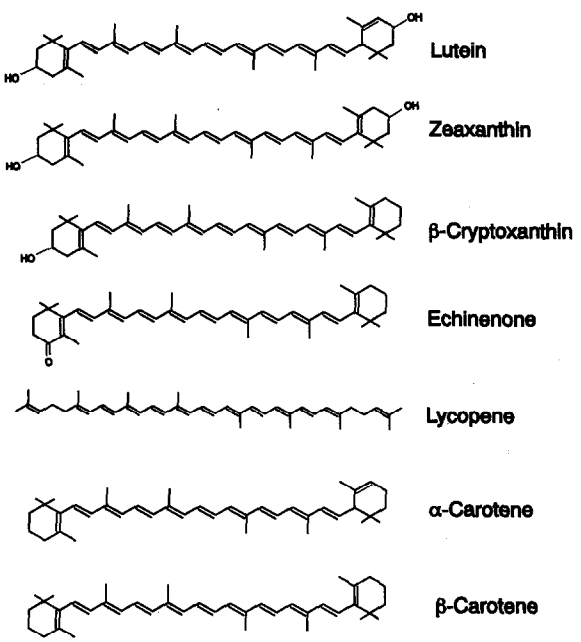

Fig. 1. Structures of carotenoids used in test mixture.

structures [51]. Because carotenoid isomers also have a rigid structure, it was thought that the classification scheme might provide insight into retention mechanisms for carotenoids, as well as provide assistance in column selection.

The C_{18} columns in this study were classified into three stationary phase types using Standard Reference Material (SRM) 869, Column Selectivity Test Mixture for Liquid Chromatography [51,53]. The SRM is a mixture of three PAHs: benzo[*a*]pyrene (BaP), 1,2:3,4:5,6:7,8-tetrabenzonaphthalene (TBN), and phenanthro [3,4-*c*]phenanthrene. Columns were classified by calculating the relative retention of TBN to BaP. The resulting $\alpha_{TBN-BaP}$ values were grouped as follows: those columns with an $\alpha_{TBN-BaP}$ less than 1 are classified as polymeric, those with an $\alpha_{TBN-BaP}$ between 1 and 1.7 are classified as intermediate, and those with an $\alpha_{TBN-BaP}$ greater than 1.7 are classified as monomeric. The $\alpha_{TBN-BaP}$ values for each column are provided in Table I. These values may not be directly comparable for the five columns whose stationary phases were not C_{18}. Nonetheless, 35 of the columns tested were classified as monomeric, 17 were polymeric and 12 were intermediate at 27°C.

This classification scheme is strongly related to the procedures used in the bonded-phase synthesis, *i.e.*, monomeric phases prepared by the reaction of silica with monofunctional silanes (usually in the absence of water) have properties that differ from polymeric phases prepared by reaction of silica with trifunctional silanes in the presence of water. (The third group is an arbitrary classification for which phase chemistry is less certain; this group has properties that are intermediate to the monomeric and polymeric classes.)

Selectivity

Optimum carotenoid selectivity of each column was difficult to assess, since a mobile phase was not tailored for each. Our design was limited to an isocratic, nonaqueous binary mobile phase separation, with the capacity factor, k', manipulated through the addition of THF or ethyl acetate such that $7 \leqslant k' \leqslant 11$. Under the defined conditions, lutein and zeaxanthin were the most difficult carotenoids to resolve due to their structural similarity and early elution.

No monomeric C_{18} column evaluated in this study was able to resolve lutein and zeaxanthin using methanol or methanol-based solvents. Using acetonitrile-based solvents, monomeric C_{18} columns were sometimes able to separate this pair partially. The polymeric C_{18} columns were usually able to separate lutein and zeaxanthin. Typical chromatograms for columns classified as monomeric, polymeric, and intermediate are shown in Figs. 2, 3 and 4, respectively; chromatographic conditions are described in those figures. In Table II, resolution (R) and α values for zeaxanthin/lutein and β-carotene/lycopene are shown for a representative one-third of the columns. The actual frequency and inability of a class of columns to separate lutein and zeaxanthin is represented, *i.e.*, if one in every three monomeric columns failed to separate lutein and zeaxanthin, then one of every three numbers shown for the monomeric columns in Table II is for a column that failed to separate this pair. Using monomeric and most intermediate columns, the elution of lycopene was followed by that of α- and β-caro-

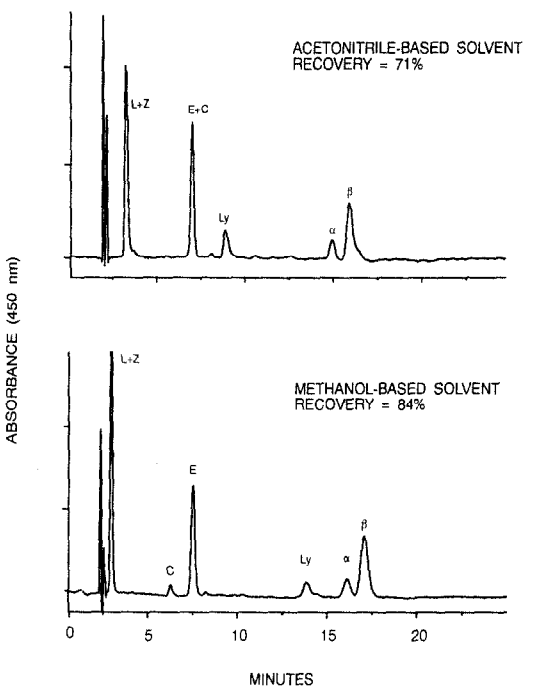

Fig. 2. Separation of test mixture on a monomeric C_{18} phase. Eluent is THF–methanol (10:90) or THF–acetonitrile (15:85) at a flow-rate of 1.5 ml/min. Legend: L = lutein, Z = zeaxanthin, C = β-cryptoxanthin, E = echinenone, Ly = lycopene, α = α-carotene, β = β-carotene.

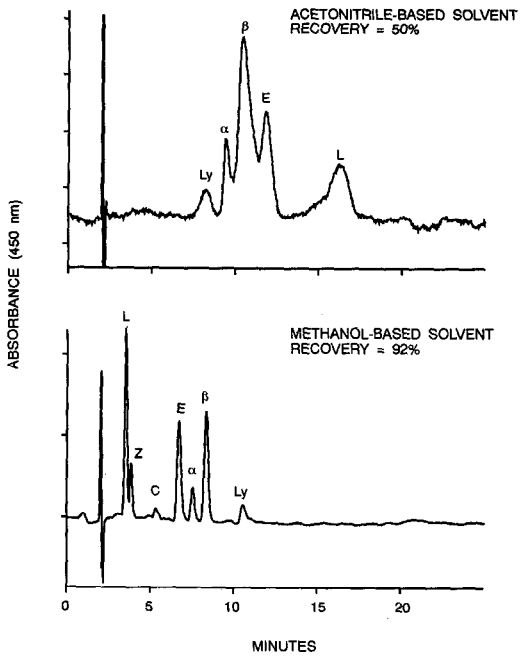

Fig. 3. Separation of test mixture on a polymeric C_{18} phase. Eluent is 100% methanol or 100% acetonitrile at a flow-rate of 1.5 ml/min. For legend, see Fig. 2.

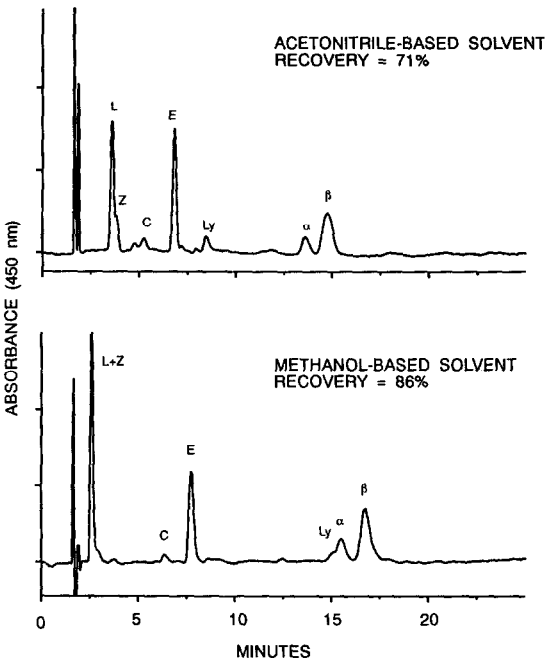

Fig. 4. Separation of test mixture on an intermediate C_{18} phase. Eluent is THF–methanol (7.5:92.5) or THF–acetonitrile (12.5:87.5) at a flow-rate of 1.5 ml/min. For legend, see Fig. 2.

TABLE II

RESOLUTION (R) AND SELECTIVITY (α) FOR ZEAXANTHIN/LUTEIN (Z/L) AND β-CAROTENE/LYCOPENE (B/Ly) ON SELECTED C_{18} COLUMNS USING METHANOL AND THF–METHANOL COMPARED TO THE TBN/BaP α VALUE ON THOSE COLUMNS

Column[a]	Character	$\alpha_{Z/L}$	$R_{Z/L}$	$\alpha_{B/Ly}$	$R_{B/Ly}$	$\alpha_{TBN/BaP}$
22	Monomeric	1	0	1.28	13	2.07
14	Monomeric	1	0	1.20	6.2	2.05
18	Monomeric	1	0	1.28	14	2.01
25	Monomeric	1	0	1.18	7.8	2.00
41	Monomeric	1	0	1.24	7.8	1.99
44	Monomeric	1	0	1.29	13	1.97
26	Monomeric	1	0	1.27	12	1.97
40	Monomeric	1	0	1.29	12	1.95
1	Monomeric	1	0	1.28	13	1.92
20	Monomeric	1	0	1.19	7.4	1.84
11	Monomeric	1	0	1.36	7.9	1.73
53	Intermediate	1	0	1.15	10	1.68
59	Intermediate	1	0	1.24	13	1.50
19	Intermediate	1	0	2.16	19	1.45
35	Intermediate	1.20	2.9	0.91	6.0	1.04
30	Polymeric	1.18	2.9	0.78	8.0	0.93
5	Polymeric	1.21	2.9	0.83	5.8	0.83
6	Polymeric	1.23	4.0	0.74	12	0.78
16	Polymeric	1.22	4.4	0.74	12	0.70
49	Polymeric	1.26	3.9	0.70	16	0.59
37	Polymeric	1.32	4.6	0.56	35	0.51

[a] See Table I for column descriptions.

tene. Using polymeric columns, the elution order often changed to α-carotene, β-carotene, lycopene, thus the α- values for the polymeric columns (and one intermediate column) in Table II are less than one. In general, if the column could resolve lutein and zeaxanthin, then lycopene eluted after α- and β-carotene. Quackenbush and Smallidge [54] and Bushway [35] have also observed that Vydac 201TP and 218TP columns, which are polymeric, reverse the order of elution of lycopene and β-carotene compared to other (monomeric) columns that they tested, although they did not recognize the monomeric or polymeric phase synthesis of the columns as the cause. Polymeric phases have also been reported to provide better separation of certain carotenoids and their *cis* isomers [35,54,55].

To investigate whether the selectivity for selected carotenoids correlates with the selectivities for the PAHs, $\alpha_{TBN/BaP}$ values for the columns are also shown in Table II. On the polymeric columns, $\alpha_{TBN/BaP}$ and $\alpha_{zeaxanthin/lutein}$ are correlated (correlation coefficient, $r = -0.94$), as are $\alpha_{TBN/BaP}$ and $\alpha_{\beta\text{-carotene/lycopene}}$ ($r = 0.86$). Despite the small sample size ($n = 6$ polymeric columns), there is more than 95% confidence that a correlation does exist, according to Pearson Product Moment correlation coefficient tables [56]. There is no correlation between the $\alpha_{TBN/BaP}$ and the $\alpha_{zeaxanthin/lutein}$ on the monomeric columns because all the $\alpha_{zeaxanthin/lutein}$ values are 1, as is the case for most of the intermediate columns' $\alpha_{zeaxanthin/lutein}$ values. There is also no correlation between the $\alpha_{TBN/BaP}$ and the $\alpha_{\beta\text{-carotene/lycopene}}$ on both the monomeric and intermediate columns. Thus, the $\alpha_{TBN/BaP}$ values are useful for predicting whether a column will be able to separate lutein and zeaxanthin inasmuch as it indicates whether the column is monomeric, polymeric, or intermediate, since mainly polymeric columns are capable of resolving this pair. However, it is not useful for predicting whether an intermediate column will be able to resolve lutein and zeaxanthin, nor can it predict how much separation of lycopene and β-carotene a monomeric or intermediate column is likely to provide.

For most columns it was necessary to add a mobile phase modifier to elute compounds with a $k' \leqslant 11$. The choice of modifier (THF or ethyl acetate) did not significantly affect selectivity for either lutein/zeaxanthin or lycopene/β-carotene, the two pairs that were examined. Columns that failed to resolve lutein and zeaxanthin using methanol-based solvents failed using each modifier. On a few columns, the use of one modifier in acetonitrile did provide higher α values than the other, but the difference was not great enough to significantly improve resolution. THF did not consistently provide higher α values than ethyl acetate or vice versa. For the separation of lycopene and β-carotene, selectivity differed slightly using acetonitrile-based mobile phases on some columns. On one or two columns using methanol-based solvents, the α values for lycopene and β-carotene may have been more significantly affected by the choice of modifier. Again, THF was not consistently better or worse than ethyl acetate at providing greater resolution.

Pore size of the column can affect the selectivity for carotenoids of sufficiently different size (*e.g.*, lycopene and β-carotene, zeaxanthin and β-carotene) but does not affect selectivities for carotenoids of very similar sizes (*e.g.*, α-carotene and β-carotene). In a comparison of four monomeric columns from the same manufacturer (column 45, 26, 27 and 28), the only difference being pore size of the silica substrate (*i.e.*, 60, 120, 200 and 300 Å, respectively), both $\alpha_{\beta\text{-carotene/lycopene}}$ and $\alpha_{\beta\text{-carotene/zeaxanthin}}$ decrease with increasing pore size. This also holds true for a comparison of two other sets of wide-pore columns and their narrow-pore counterparts (columns 12 and 13, 100 Å *vs.* columns 14 and 15, 300 Å; column 36, 120 Å *vs.* column 37, 300 Å). In addition, as pore size decreases, more modifier must be added in all three cases to elute all seven compounds with a $k' \leqslant 11$. This is as expected since the greater surface area of the base silica of the narrow-pore columns results in a higer carbon load. Although absolute retention of carotenoids increases with decreasing pore size (and increasing carbon loading), α values for similarly sized carotenoids do not change with column pore size. An example of this trend is the α values for β-carotene/α-carotene, which are the same for the sets of columns described above.

Recovery

Sample losses on the column are critical to the sensitivity and precision of an LC method and to quantitative analysis. Carotenoid recoveries ranging from 0 to almost 100% were observed. Recovery was dependent on the mobile phase, stationary phase type classification, and possibly the column bed support frit material.

Mobile phase. On almost all the columns tested, the use of methanol or methanol-based solvents provided a higher recovery of the carotenoids than did the use of acetonitrile of acetonitrile-based solvents, as shown by the mean recoveries given in Table III. When using acetonitrile-based mobile phases, the THF modifier resulted in a higher percent recovery on most columns than did the ethyl acetate. This difference in recovery was less noticeable when the methanol-based eluents were used. Addition of a modifier was not necessary for about half the columns when methanol was used and about one-third of the columns when acetonitrile was used. Typically, it took less THF than ethyl acetate to elute the compounds with $k' \leqslant 11$, and methanol required less modifier than acetonitrile, which is contrary to expectations based on solvent strength parameters, where acetonitrile is classified as a stronger solvent than methanol [57]. However, this observation is consistent with the findings of Nelis and De Leenheer, who reported that methanol acts as a stronger solvent than acetonitrile for the separation of carotenoids on C_{18} columns [19]. Since recoveries were lower and no improvements in selectivity were observed, ethyl acetate-modified solvents are not discussed further to simplify data analysis. Henceforth each column has just two sets of results: 100% methanol and 100% acetonitrile, or THF–methanol and THF–acetonitrile if it was necessary to use a modifier.

TABLE III

AVERAGE RECOVERY OF CAROTENOIDS ON ALL COLUMNS TESTED USING DIFFERENT MOBILE PHASES

THF or ethyl acetate are added to methanol and acetonitrile as modifiers such that $7 \leqslant k'_{max} \leqslant 11$.

Mobile phase	Recovery ± S.D. (%)
100% methanol	84 ± 8 (n = 29)[a]
Methanol–THF	86 ± 11 (n = 35)
Methanol–ethyl acetate	82 ± 12 (n = 35)
100% Acetonitrile	56 ± 19 (n = 21)
Acetonitrile–THF	68 ± 17 (n = 43)
Acetonitrile–ethyl acetate	47 ± 17 (n = 43)

[a] n = Number of columns.

Acetonitrile-based solvents are typically used for the carotenoid separations reported in the literature [3–12,14,17–20,22,25,26,29–38,40–48]. However, our studies show that recoveries using acetonitrile and acetonitrile-based eluents are generally lower than those obtained using methanol and methanol-based eluents. Nelis and De Leenheer [19] reported that the incorporation of methanol in acetonitrile-based solvents dramatically enhanced selectivity, which they speculated was due to hydrogen bonding. Most of the acetonitrile-based methods reported in the literature do involve the use of some methanol [3,6,7,9–11,14,17,19,20,22,26,29–35,37,38,40–44,46–48]. To determine whether the addition of methanol improved recovery as well as selectivity, a column that had provided 0% recovery when run with 100% acetonitrile and 77% recovery with 100% methanol was tested with a methanol–acetonitrile (10:90) mixture. Recovery with respect to the 100% acetonitrile run improved only slightly (up from 0 to 2%). A run using methanol–acetonitrile (20:80) as the eluent resulted in 4% recovery. Thus the addition of methanol did improve recovery marginally. The column tested performed poorly using 100% acetonitrile; it would be unfair to say that recovery on all columns using acetonitrile-based solvents would also show only a 2% increase in recovery with the addition of methanol.

The poor performance of certain columns appears to be real and not an artifact. When poorly performing columns were rechecked at a later date, performance had not changed. In addition, recovery was not affected when various sample sizes were injected (from 5 μl to 100 μl). Lauren and McNaughton [50] recommend the addition of 0.1% *n*-decanol to the mobile phase to improve performance by minimizing adsorption. We did not find *n*-decanol to be effective in improving the performance of columns with poor recoveries using acetonitrile-based solvents. However, we have observed that flushing the column with an ammonium acetate buffer improves recovery on these columns when an acetonitrile-based mobile phase is used. We have also found that the addition of ammonium acetate to the mobile phase improves recoveries.

To determine the reproducibility of recovery results, one column from each of the three classification groups was retested about four months after the initial test. Columns that had required 100%

methanol were selected to eliminate any effect caused by slight variations in the mobile phase composition. Columns were also selected that had provided recoveries greater than 80% in the first test. Relative standard deviations ranged from 2.5 to 5.6%. An insufficient number of runs were made on individual columns to allow statistical comparisons of methanol or methanol-based and acetonitrile or acetonitrile-based recoveries for each column; however, based on the reproducibility studies, 6% represents a liberal estimate of the relative standard deviation. Using this estimate of variability, columns with a 12% difference in recovery between methanol- and acetonitrile-based eluents would be significantly different. Columns are grouped according to their recoveries using methanol or THF–methanol and acetonitrile or THF–acetonitrile in Table IV.

Selective recovery. Experiments were performed to determine whether certain carotenoids contributed a greater share to recovery losses than did the other carotenoids. Individual carotenoids were injected into the system, both with and without a column, as described. Two monomeric-phase and three polymeric-phase columns were tested. All columns had previously required 100% methanol for elution of the carotenoids in less than 25 min. Four of the columns had required 100% acetonitrile; one required THF–acetonitrile (5:95). Two trends in recovery were observed. Losses of zeaxanthin and β-carotene, which each contain two β-rings (Fig. 1), were greater than losses of lutein and α-carotene, which each contain one β- and one ε-ring. Within the group of β,β-carotenoids used in the mixture (zeaxanthin, β-cryptoxanthin, echinenone, and β-carotene), recovery increased as polarity decreased: less zeaxanthin, a dihydroxy carotenoid, was recovered than β-cryptoxanthin, a monohydroxy carotenoid. Less β-cryptoxanthin was recovered than echinenone, a mono-keto carotenoid. And less echinenone was recovered than β-carotene, a hydrocarbon carotenoid. This trend also holds true for the two β,ε-carotenoids used in the mixture. The recovery of the dihydroxy carotenoid, lutein, was less than that of the hydrocarbon carotenoid, α-carotene.

Stationary phase. In these carotenoid studies, as a rule, polymeric phases required the least modifier (a mean value of 2% THF in methanol, 3% THF in acetonitrile) and monomeric phases required the most (a mean value of 6% THF in methanol, 9% THF in acetonitrile) to elute all seven compounds from the column within the k' range allowed ($7 \leqslant k' \leqslant 11$). Intermediate phases required an intermediate amount of modifier (4% THF in methanol, 6% THF in acetonitrile). Percent recovery across the three groups did not vary consistently. Recovery results for columns divided into the three classification groups are shown in Table V. Thirty percent of the columns gave >90% recovery using

TABLE IV

COLUMNS USED IN THIS STUDY GROUPED ACCORDING TO PERCENT RECOVERIES

Recovery (%)	Columns
Using methanol or THF–methanol:	
90–100	3 5 7 11 15 21 23 24 26 27 28 29 30 32 41 42 43 44 45 52 65
80–89	1 2 4 6 8 10 12 13 14 16 17 18 19 22 25 31 35 37 38 39 40 46 47 48 49 50 51 53 54 58 59 60 61
70–79	20 33 36 55 63 64
60–69	42 62
50–59	9
40 49	
30–39	56
Using acetonitrile or THF–acetonitrile:	
90–100	43 50 51
80–89	2 3 23 27 37 49 60 61
70–79	1 4 8 12 13 18 21 22 26 30 32 41 42 45 47 52 58 59
60–69	5 10 25 28 29 33 36 44 48 65
50–59	9 11 14 15 17 20 24 31 38 40 46
40–49	6 7 16 19 35 39 62 64
30–39	63
20–29	–
10–19	–
0–9	53 54 55 56

TABLE V

AVERAGE PERCENT RECOVERY FOR POLYMERIC, INTERMEDIATE, AND MONOMERIC COLUMNS TESTED

Eluent	Polymeric	Intermediate	Monomeric
MeOH[a]	83%	79%	88%
ACN[b]	62%	46%	67%

[a] MeOH = methanol and methanol-based eluents.
[b] ACN = acetonitrile and acetonitrile-based eluents.

methanol or THF–methanol as the solvent; of these, 70% were classified as monomeric and the remaining 30% were classified as polymeric.

In several cases, columns were available that contained the "same" packing material from different lots. Recovery results and k' values for selected compounds using THF–methanol for columns from different production lots are presented in Table VI. Mean recoveries for many of the columns appear to be different, but performance of Tukey's Multiple Comparison test [58] on these data shows that there is no difference between the mean recoveries at a 95% confidence level for almost all the column pairs.

Retention times of carotenoids using columns from different stationary phase lots varied substantially. (On one pair of columns, there was a 15% difference in β-carotene's retention time.) This variability can arise from two sources: the base silica from which the stationary phase is prepared and reproducibility of the bonding procedure. Most LC column manufacturers obtain silica from outside suppliers, and differences between column lots is strongly related to differences in the silica—particle size, pore size, surface area, silanol activity, and trace metal contamination. Thus, column lot differences can exist for both monomeric and polymeric phases. The only way to ensure reproducible columns is to purchase columns packed with the same production lot of stationary phase.

Column bed support frits. It has been suggested that losses occurring on column frit materials may be partially responsible for low recoveries of carotenoids [3,23]. Data from two sets of columns that were identical except for the presence of different frits are presented in Table VII. Although recoveries were slightly lower for stainless steel frits, no significant differences in recoveries between these sets of columns were detected. Later studies in which frits were placed in series (without a column in the system) showed no significant difference in recovery using stainless steel, titanium, or "biocompatible" frits.

In 1986, Nierenberg and Lester [3] observed dif-

TABLE VI

COMPARISON OF k' AND RECOVERY FOR COLUMNS FROM DIFFERENT LOTS

Column[a]	k'			Recovery (%)	n[b]
	Lutein	Echinenone	β-carotene		
4	0.30	2.91	8.38	87	3
17	0.20	2.75	8.08	82	3
12	0.26	2.60	7.07	92	3
13	0.20	2.66	7.25	89	3
14	0.27	2.21	5.86	88	3
15	0.30	2.31	6.07	90	5
16	0.73	1.83	2.80	86	5
66[c]	0.66	2.00	2.95	92	5
40	0.43	3.18	8.22	86	3
46	0.49	3.06	7.76	92	3
48	0.35	3.24	8.16	86	3
64	0.38	3.57	9.18	78	3
37	1.03	3.00	4.81	82	3
62	0.74	2.58	3.89	87[d]	4
63	0.59	2.60	3.82	73[d]	2

[a] See Table I for column descriptions.
[b] n = Number of runs.
[c] Supelco LC-PAH column tested after the completion of the other analyses.
[d] Statistical difference in recovery as determined by Tukey's Multiple Comparisons.

TABLE VII

COMPARISON OF k' AND RECOVERIES ON COLUMNS WITH DIFFERENT FRIT MATERIALS

Column[a]	Frit	k'			Recovery (%)	n
		Lutein	Echinenone	β-carotene		
6	SS[b]	0.75	2.46	3.30	86	5
7	Ti	0.75	2.53	3.42	88	5
1	SS	0.28	2.85	8.27	87	5
3	Ti	0.29	3.02	8.93	92	5
2	Hast	0.33	2.98	8.54	93	5

[a] See Table I for column description.
[b] SS = Stainless steel; Ti = titanium; Hast = Hastelloy.

ferences in recovery when they switched to a new column of the same brand from the same manufacturer, but with a different kind of frit, and they attributed these differences to the use of different frits (stainless steel *vs.* Hastelloy). They also observed this phenomenon when two columns were packed with the same lot of packing material, topped with either stainless steel or Hastelloy frits. During our studies, it was observed that a certain previously unused column did not provide the same recovery or retention times as its older, used, counterpart, but that the "new" column gradually attained the retention and recovery characteristics of the "old" column with repeated use. Columns that had been used in our laboratory prior to this study have been marked in Table I, in the event that results for these columns have been affected by previous use in the laboratory.

CONCLUSIONS

Several conclusions concerning the selectivity and recovery of carotenoids in reversed-phase LC can be drawn from this study: (1) In general, separations using acetonitrile or the frequently employed acetonitrile-based solvents resulted in lower recoveries than separations using methanol or methanol-based solvents. (2) Monomeric C_{18} columns generally provided high recoveries, but they were unable to resolve lutein and zeaxanthin using methanol or methanol-based eluents, which were the solvents yielding the highest recoveries. (3) Polymeric C_{18} columns were usually able to resolve lutein and zeaxanthin in methanol-based solvents, but on average had lower recoveries than monomeric C_{18} columns. (4) Using methanol-based solvents, the retention time of lycopene is greater than that of β-carotene on most polymeric columns. (5) Columns with the "same" stationary phases from different lots do not necessarily elute compounds with the same retention times. (6) When using a new column, it may be necessary to make several preliminary runs before the separation (*i.e.*, retention times, recovery, selectivity) becomes reproducible and comparable to that expected based on the performance of the corresponding "old" column. (7) The most appropriate system for the separation of carotenoids would probably include the use of a polymeric C_{18} column (to allow separation of lutein and zeaxanthin) and a methanol-based mobile phase (to obtain a high percent recovery).

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