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Separation of *trans/cis* α- and β-carotenes by supercritical fluid chromatography

II. Effect of the type of octadecyl-bonded stationary phase on retention and selectivity of carotenes

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ABSTRACT

Described in this paper are the effects of the type of bonding (monomeric *versus* arborescent-polymeric) of 22 octadecyl-bonded phases and of carbon loading on the separation of *cis* and *trans* isomers of α- and β-carotenes. Only arborescent-polymeric phases afforded the separation of *cis* and *trans* isomers, regardless of the degree of carbon loading. Incomplete separation of the α- and β-carotenes is obtained if the capacity factor for α-carotene is below 6. Also described is a method for estimating the void volume in supercritical fluid chromatography, derived from the two-solvent weight-difference method used in high-performance liquid chromatography. The porosity values are in good agreement with those reported in the literature.

INTRODUCTION

Commercial stationary phases of octadecyl-bonded silica have widely differing properties, and it is sometimes difficult to transpose a separation from one make of column to another. Among the factors that determine the performance of a column, such as the shape and size of the particles, the pore size, the specific surface area and the percentage surface coverage, the kind of function that is bonded to the support is particularly important.

Where the stationary phase is prepared with a monofunctional alkylsilane, there is one-to-one bonding between the reagent and the silanol groups, giving a "brush-type" structure [1]. Di- and trifunctional silanes can bond to more than one silanol group on the silica support to give essentially the same type of "brush-type"

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stationary phases as monofunctional silanes. However, they can also polymerize in the presence of traces of water [2]; under suitable conditions, a stationary phase can be prepared in which each alkylsilane that is bonded to the surface of the silica gives rise to an arborescent-polymeric structure that is not brush-like. Such stationary phases are frequently (and correctly) described in the literature as "polymeric", but as this can lead to confusion with column packings in which the support itself is polymeric we shall use the term "arborescent-polymeric" in this paper. Arborescent-polymeric stationary phases appear to be particularly suitable for separating closely related compounds that differ in the degree of planarity of their structures [3,4]. Although no systematic studies have been carried out, the best published reversed-phase separations of *cis*- and *trans*-carotenes have been obtained on this type of stationary phase, particularly with Vydac [5,6] and Spheri-5 [7] columns.

Several techniques have been applied to the study of the structure of bonded phases, but in most cases the results do not reflect the state of a phase in the presence of the mobile phase. More empirical studies have established the behaviour of different stationary phases in terms of capacity factors (k') and selectivities (α) [1,8–10]. As part of a study of the simultaneous separation of α and β *cis* and *trans* carotenes extracted from carrots [11], we have compared the results obtained on columns containing monofunctional and arborescent-polymeric C_{18} bonded phases, including some whose nature was not revealed by the manufacturer.

EXPERIMENTAL

Details of sample preparation and of the chromatographic system have been given in the preceding paper [11]. The octadecyl-bonded silica columns that were used are listed in Table I.

The void volume of a column was determined by first flushing it with pure carbon dioxide at 3 ml/min for 10 min. The column was then depressurized and left open to the atmosphere for another 10 min, by which time the mass was stable. It was weighed with the stoppers in place, then filled and equilibrated with methanol by pumping the solvent for 30 min, and finally stoppered and reweighed.

The extra-column void volume (0.206 μl) was estimated from the retention times for repeated injections of solvents (acetonitrile of methanol), the column being replaced by a union of negligible volume. The appropriate time was taken as the retention time of a positive peak that appeared shortly after the injections. Ten determinations of this time gave a relative standard deviation of 1.5%.

RESULTS AND DISCUSSION

The *cis* and *trans* α- and β-carotenes were separated by non-aqueous reversed-phase (NARP) high-performance liquid chromatography (HPLC), on a Spheri-5 ODS-5A column, which had an arborescent-polymeric bonded octadecyl stationary phase [7]. A column having a monomeric stationary phase (Spheri-5 ODS-5A) was not satisfactory. In the preceding paper [11], we showed that an improved separation can be obtained with the arborescent stationary phase by using mobile phases based on high-pressure carbon dioxide, the retention mechanism being the same. This technique is generally referred to as supercritical fluid chromatography (SFC), despite the

fact that in many cases, including the present one, the mobile phase is in the liquid state. However, since the chromatographically relevant physical properties (including compressibility) do not change abruptly at the critical temperature, it is usual to retain the term "SFC", even when the temperature is somewhat below the critical point. We have evaluated the performance of the other columns, using the SFC conditions (22°C, 150 bar, 15% methanol) that were found to be optimal for the Brownlee arborescent-polymeric column. Particular attention is paid to the estimation of k' for the analytes, because this parameter characterizes the stationary phase independently of factors such as the size and porosity of the column.

Determination of dead volumes in SFC

For an incompressible mobile phase, the capacity factor is given by the equation:

$$k' = (V_r - V_{0(\text{total})})/V_{0(\text{column})} \quad (1)$$

where V_r is the retention volume of an analyte and the V_0 terms represent the void volumes; the void volumes of the column and of the equipment must be known individually. Various methods have been proposed for estimating the column void volume: by injection of non-retained solvents, by extrapolation of retention volumes of solutes forming homologous series, and by weighing the column equilibrated with solvents of different densities [12]. The first two methods are the most sound from the theoretical point of view, because V_0 is determined under the conditions that will be used for chromatography. However, in the case of SFC, the density gradient along the column leads to complications that render precise definitions of V_0 and k' difficult to achieve in practice when (as in the present work) the mobile phase is used at temperatures and pressures not far removed from the critical values. Furthermore, since the temperature of the mobile phase is lower at the chilled pump head than at the top of the column, the average flow-rate (in ml/min) through the column is greater than that supplied by the pump.

Since our objective was to compare columns of fairly similar dimensions and particle size, and not to establish thermodynamic constants, we chose to neglect the errors due to compressibility. The void volume was taken as the volume of methanol (calculated from the mass) required to fill the columns that had previously been

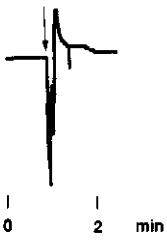

Fig. 1. Blank injection for determination of the void volume. Column: Nucleosil C_{18} (250 × 4.6 mm I.D.), 5 μm. Mobile phase: carbon dioxide–methanol (85:15, v/v). Temperature 22°C, pressure 15 MPa, flow-rate 3 ml/min, λ = 450 nm.

TABLE I

POROSITIES OF OCTADECYL-BONDED COLUMNS USED FOR SUPERCRITICAL FLUID CHROMATOGRAPHY, RETENTION PARAMETERS OF ALL-*trans* α-CAROTENE AND SELECTIVITIES AND RESOLUTIONS OF THE SEPARATION OF *cis* AND *trans* α- AND β-CAROTENES ON DIFFERENT C_{18} COLUMNS

M = monofunctional C_{18} groups; D = difunctional C_{18} groups; P = arborescent-polymeric C_{18} groups; – = exact nature unknown.

Column	*L* (mm)	V_0	Porosity ε (%)	Type of bonding	t_r α-carotene (min)	k' α-carotene	Separation of α- and β-carotenes	Selectivity of *trans/cis* β-carotene isomers	Resolution of *trans/cis* β-carotene isomers	Separation of α- and β-carotenes *trans/cis* isomers
Ultrabase UB 225	250	2.463	59	M	11.22	12.58	Yes	>1	>1.5	Yes
Spheri-5 ODS-5A	250	2.456	59	P (A)	11.40	12.85	Yes	>1	>1.5	Yes
LiChrospher 100 RP 18	250	2.150	68	D	8.35	10.57	Yes	>1	>1.5	Yes
LiChrospher 100 PR 18e	250	2.187	70	D	9.60	12.05	Yes	>1	>1.5	Yes
Nucleosil C_{18}	250	2.866	69	P (A)	7.38	6.66	Yes	>1	>1.5	Yes
ChromTech CT-Sil C_{18}	150	1.545	62	–	4.89	8.36	Yes	>1	>1.5	Yes
Superspher 100 RP_{18}	250	2.106	67	D	8.16	8.02	Yes	>1	>1.5	Yes
Spherisorb ODS-2	100	1.029	62	P (A)	3.75	9.73	Yes	>1	>1.5	Yes
Supelcosil LC-PAH	150	1.768	71	P (A)	2.05	2.37	Yes	>1	<1.5	No
Erbasil C18	150	1.656	66	P (A)	3.60	5.4	Yes	>1	<1.5	No
Suplex pKb-100	150	1.623	65	–	2.38	3.27	Yes	>1	>1.5	No
Ultracarb 5-ODS 20	150	1.789	72	–	10.32	16.2	Yes	>1	>1.5	No
Zorbax ODS	250	2.678	64	M	4.98	4.5	Yes	=1	<1.5	No
Ultrasphere ODS	250	2.520	61	M	2.48	1.86	No	=1	–	No
Vydac 201 HS	150	1.760	70	M	5.43	8.13	No	=1	–	No
Partisil 5 ODS-3	250	2.803	67	M	7.70	7.17	No	=1	–	No
μBondapak C_{18}	300	2.908	70		6.29	5.42	No	=1	–	No
Vydac 210 TP	150	1.680	67	P (A)	1.6	1.73	Yes	>1	<1.5	No
Perkin-Elmer HC-ODS/PAH	250	1.327	88	P (A)	1.25	2.05	Yes	>1	<1.5	No
Vydac 218 Tp	250	2.898	70	P (A)	3.28	2.32	Yes	>1	>1.5	No
Hypersil $15C_{18}$	150	2.635	63	M	7.01	6.9	No	=1	–	No
Synchropak SCD-100	150	1.874	75	M	1.68	1.59	Yes	>1	<1.5	No

flushed with supercritical carbon dioxide and equilibrated with air. Methanol was used because it is not absorbed to a significant extent by the stationary phase [13].

The value of V_0 for each of the columns is given in Table I, together with the porosity, calculated from V_0 and the total column volume. A representative HPLC trace is given in Fig. 1. As expected for C_{18} bonded-phase columns, the porosity ranged from 60 to 70%, except for the Perkin-Elmer analytical HC-ODS/PAH column, for which the value was anomalously high.

Influence of the type of stationary phase on the separation of carotenes

The effect of the stationary phase can be evaluated from overall variations in capacity factors or from variations in the extent of separation of different pairs or groups of compounds, such as:

(a) α- and β-carotenes (the isomers not being separated),
(b) *cis* and *trans* isomers of β-carotene, or
(c) *cis*/*trans* α- and β-carotenes.

Separation of α- from β-carotenes. All but five of the columns in Table I separated two classes of carotene (resultion, $R_s > 1.5$), under the conditions that had been optimized for the Spheri-5 ODS-5A column. Two of these five stationary phases are of the monofunctional type (Ultrasphere and Hypersil), one (μBondapak) is of an undetermined nature and the other two are arborescent-polymeric (Vydac 201TP and Synchropak SCD-100). The failure of the last two columns to provide a separation can be accounted for by the exceptionally low capacity factors (Table I), which are due to low percentages of carbon loading and, in the case of the Vydac 201 TP column, to a pore size of 30 nm. It is interesting that the end-capped version of this column, Vydac 218 TP, give higher capacity factors (for example 2.32 compared with 1.73 for α-carotene), together with an improved separation.

Four of the five columns that were unsatisfactory under the standard conditions gave adequate separations when the percentage of modifier in the mobile phase was reduced, confirming the suggestion that, as far as this particular separation is concerned, the major difference between the columns is in the degree of carbon loading. The only exception was the μBondapak column, which was already been reported to give anomalous results in a study of homologous series [9].

Separation of the cis and trans isomers of β-carotene. During a previous liquid chromatographic study, it was found that the separation of the *cis*- and *trans*-carotene isomers at ordinary temperatures (> 15°C) required a stationary phase of the arborescent-polymeric type. The same generalization applies to separations by SFC, since all the columns that failed to give this separation were of the monofunctional type: Hypersil 15C_{18}, Vydac 201 HS, Zorbax ODS, Ultrasphere ODS, μBondapak C_{18} and Partisil 5 ODS-3.

All of the columns with arborescent-polymeric stationary phases (including those that did not separate the α- and β-carotenes) gave selectivity factors (α) greater than 1 for the separation of the *cis* and *trans* isomers (Table I). However, the resolution (R_s) was not always satisfactory, especially when the capacity factor (measured for α-carotene) was less than 6. To summarize, this separation requires a arborescent-polymeric stationary phase and the percentage carbon loading must be sufficient to

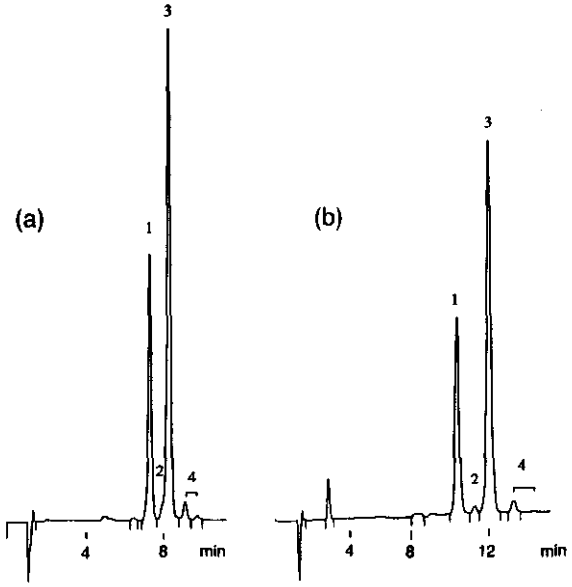

Fig. 2. Comparison of two chromatograms of a carrot extract, analysed on a Nucleosil C_{18} column (250 × 4.6 mm I.D.). Mobile phase: (a) carbon dioxide–methanol (85:15, v/v) at 15 MPa; (b) carbon dioxide–methanol (95:5, v/v) at 20 MPa. Temperature 22°C, flow-rate 3 ml/min, λ = 450 nm. Identification of peaks: 1, all-*trans* α-carotene, 2, *cis* isomers of α-carotene, 3, all-*trans* β-carotene, 4, *cis* isomers of β-carotene.

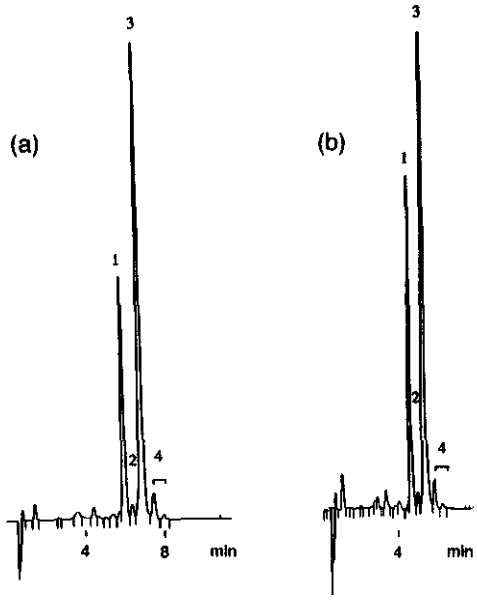

Fig. 3. Chromatograms of a carrot extract on (a) ChromTech CT-Sil C_{18} (150 × 4.6 mm I.D.), (b) Spherisorb ODS-2 (100 × 4.6 mm I.D.). Mobile phase: carbon dioxide–methanol (90:10, v/v). Temperature 22°C, pressure 15 MPa, flow-rate 3 ml/min, λ = 450 nm. Identification of peaks: see legend to Fig. 2.

TABLE II

EFFECT OF ADDITION OF ACETONITRILE TO THE MOBILE PHASE ON THE CAPACITY FACTOR (k') OF ALL-*trans* α-CAROTENE AND ON THE SEPARATION OF *cis* AND *trans* β-CAROTENES ON DIFFERENT C_{18} BONDED COLUMNS

Columns	k' of α-carotene with 15% organic modifier		Separation of β-carotene *trans*/*cis* isomers with 15% organic modifier [methanol–acetonitrile (1:1, v/v)]
	Methanol	Methanol–acetonitrile (1:1, v/v)	
Ultrabase UB 225	12.58	10.91	Yes
Spheri-5 ODS-5A	12.85	10.69	No
LiChrospher 100 RP 18	10.57	9.95	No
LiChrospher 100 RP 18e	12.05	9.4	No
Nucleosil C_{18}	6.66	6.15	Yes
ChromTech CT-Sil C_{18}	8.36	7.1	Yes
Superspher 100 RP-18	8.02	7.08	Yes
Spherisorb ODS-2	9.73	8.48	No

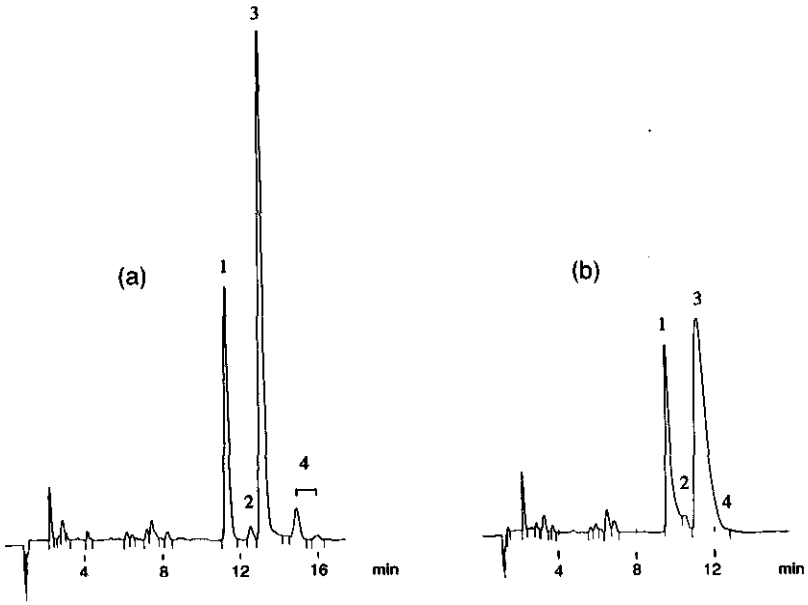

Fig. 4. Influence of the organic modifier on the chromatographic profile of a carrot extract analysed on an arborescent-polymeric column (Spheri-5 ODS-5A; 250 × 4.6 mm I.D.). Mobile phase: (a) carbon dioxide–methanol (85:15, v/v), (b) carbondioxide–methanol–acetonitrile (85:7.5:7.5, v/v). Temperature 22°C, pressure 15 MPa, flow-rate 3 ml/min, λ = 450 nm. Identification of peaks: see legend to Fig. 2. The early-eluting peaks are probably oxygen-containing degradation products.

give a capacity factor of at least 6 under the described conditions. The only column that deviated from this rule was Suplex pKb-100, which gave an adequate separation with a capacity factor of less than 6. However, the packing material of this column has been specially treated, in a manner that has not been divulged, to render it suitable for the chromatography of basic compounds.

Separation of the cis and trans isomers of α- and β-carotenes. The simultaneous separation of the *cis* and *trans* α- and β-carotenes was achieved on 8 of the 22 columns that were tested. Of these, six columns were of the arborescent-polymeric type: Spheri-5 ODS-5A, LiChrospher 100 RP 18, LiChrospher 100 RP 18e, Superspher 100 RP18 and Nucleosil C_{18}. In the four cases where capacity factors were less than 10 under the conditions that were optimal for Spheri 5 ODS-5A (ChromTech CT-Sil C_{18}, Superspher, Spherisorb ODS-2 and Nucleosil C_{18}), it was necessary to reduce the concentration of mobile phase modifier to 5 or 10% in order to obtain a clean separation of the *cis* isomers of α-carotene (Fig. 2). The consequent increase in retention times could be reduced by raising the pressure to 200 bar. The shortest analysis times (less than 8 min for a complete separation) were provided by ChromTech CT-Sil C_{18} and Spherisorb ODS-2 columns, which are the shortest of those tested (Fig. 3).

The quality of the separation obtained on the Ultrabase column is surprising for a monofunctional stationary phase (See Fig. 9 of preceding paper [11]). As with the Suplex pKb-100 column mentioned in the previous section, this column is specially deactivated for the analysis of basic compounds. Clearly, the proprietary treatment modifies the chromatographic properties for non-basic compounds.

Effects of the mobile phase modifier as a function of the stationary phase. The kinetics of mass transfer between the phases is influenced by the nature of the mobile phase modifier [14]. In the chromatography of phenolic compounds on unmodified silica, the addition of acetonitrile to the mobile phase leads to severe peak broadening, and in certain cases a loss of selectivity. We tested the effects of replacing half of the methanol in the mobile phase with acetonitrile (carbon dioxide–methanol–acetonitrile, 85:7.5:7.5, v/v/v), for the chromatography of carrot carotenes on the eight columns that gave a complete separation (Table II). In all cases, the capacity factors were reduced. With four of the columns, neither efficiency nor selectivity was

TABLE III

PERCENTAGE OF ACETONITRILE THAT PRODUCES A SIGNIFICANT LOSS IN EFFICIENCY ON DIFFERENT OCTADECYL-BONDED COLUMNS, IN RELATION TO THE CAPACITY FACTOR OF ALL-*trans* α-CAROTENE

Columns	k' of α-carotene with with 15% organic modifier [methanol–acetonitrile (1:1, v/v)]	Acetonitrile (%)
Spheri-5 ODS 5A	10.69	5
LiChrospher 100 RP 18	9.95	50
LiChrospher 100 RP 18e	9.4	70
Spherisorb ODS-2	8.48	95

reduced, and, as noted previously [11], a previously undetected peak was resolved when acetonitrile was used.

However, with the remaining four columns (Spheri-5 ODS-5A, LiChrospher 100 RP 18, LiChrospher 100 RP 18e and Spherisorb ODS-2) the peaks were broadened, so that the resolution between the *cis* and *trans* isomers was reduced (Fig. 4). These four columns, which are arborescent-polymeric, have the greatest carbon loadings of the columns of this type that were tested. It appears from the data in Table III that the deleterious effect of acetonitrile is directly correlated with the carbon loading (as estimated from the capacity factors). The peak broadening did not occur with the monofunctional Ultrabase column, despite its high carbon loading ($k' = 12.6$).

CONCLUSION

Both the type and the percentage carbon loading of the stationary phase influence the separation of the carotenes by SFC. Except for Ultrabase UB225, the stationary phases of the monofunctional type do not separate the *cis* and *trans* isomers. On arborescent-polymeric columns, the separation of α- and β-carotenes is incomplete if the capacity factor for α-carotene is below 6. Where the capacity factor is above 9.5 under the described conditions (high percentage carbon loading), the efficiency of the column decreases if part of the methanol in the mobile phase is replaced by acetonitrile; with less heavily loaded supports, the nature of the mobile phase modifier is relatively unimportant.

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