Journal of Chromatography, 645 (1993) 29–39
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 232

Use of carotenoids in the characterization of octadecylsilane bonded columns and mechanism of retention of carotenoids on monomeric and polymeric stationary phases

E. Lesellier* and A. Tchapla

LETIAM, IUT d'Orsay, Plateau du Moulon, B.P. 127, 91403 Orsay Cedex (France)

A.M. Krstulovic

Synthelabo Recherche (LERS), Recherche Analytique et Controle Pharmaceutique, 23–25 Avenue Morane Saulnier, 92360 Meudon-la-Forêt (France)

(First received January 8th, 1993; revised manuscript received March 19th, 1993)

ABSTRACT

Different analyses were performed with 29 commercial octadecylsilane bonded columns in HPLC and subcritical fluid chromatography (SbFC) in order to evaluate the characteristics of stationary phases. Two types of test molecules were used, carotenoids and polycyclic aromatic hydrocarbons. The use of the *cis–trans* isomers of β-carotene allowed the characterization of the stationary phase (monomeric *versus* polymeric) and of the carbon content. The separation of luteine and zeaxanthine depends on the nature of the stationary phase and, in addition, allows the evaluation of the extent of accessibility of residual silanol groups. These results, and those of a study on the effect of temperature, allow a better understanding of the separation mechanisms in the retention of planar and non-planar compounds, and emphasize the similarity between HPLC and SbFC.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (HPLC) now occupies an important place amongst separation methods. Several factors contribute to this situation, in particular the ease of manipulation and the reproducibility of analyses. In addition, the better knowledge of retention mechanisms and the understanding of the influence of the solvent enable one to obtain increasingly reliable separation predictions.

Along with these developments, new synthesis methods and overlapping of silanol groups have also contributed to the ability to perform certain difficult separations and have improved the transfer of a separation between supports.

Many different parameters govern the choice of a chromatographic support. The diversity of silicas employed [1] (spherical or non-spherical, pore diameter from 60 to 300 Å) and pretreatment (acidic or basic, thermal, cladding with zirconia), bonding treatment (reactivity: chloro,

* Corresponding author.

0021-9673/93/$06.00

alkoxy), end-capping (trimethylsilyl) or steric protection (diisopropyl) lead to the appearance of columns on the market which, starting with the carbon chain C_{18}, have very different performances. It is therefore necessary, for a successful separation, to have a better knowledge of the characteristics of the adsorbent employed.

In addition to the classical techniques for the characterization of stationary phases (spectroscopy, microscopy or thermal analysis), which necessitate specialized equipment and/or skills, numerous tests allow for a better definition of the C_{18} support used and also allow the eventual replacement of one material by another [1,2].

The most often used tests include those employing a homologous series or the coupling of the benzene–anthracene pair for determining the solvophobic power of the chains, the phenyl series for the identification of structure, the selectivity of the pairs N,N-diethyltoluamide–anthracene [3] and caffeine–theophylline for the determination of residual silanols, the measurement of the asymmetry of peaks of N,N-dimethylaniline and the NIST 869 test for polycyclic aromatic hydrocarbons (PAHs) for determining the monomeric or polymeric nature of the phase [4].

Previous studies either in non-aqueous reversed-phase (NARP) liquid chromatography [5] or in subcritical fluid chromatography (SbFC) [6] have shown that the separation of the *cis–trans* isomers of carotenes is also dependent on the nature of the stationary phase. This separation cannot generally be achieved on a monomeric support at ambient temperature, in contrast to polymeric supports. Nevertheless, it is apparent that the treatment of columns that are employed specifically for the study of basic products, such as Ultrabase UB 225 bonded with monofunctional silanes, enabled this separation to take place [7]. Further, a different investigation showed the distinctive behaviour of non-oxygenated α- and β-carotenes and the dependence of their retention on the oxygenated groups of the stationary phase [8].

On the basis of this information, we were able to compare different commercial columns in SbFC employing a mixture of four carotenoids and some of their isomers. In addition, we compared these results with those obtained by the NIST 869 test.

EXPERIMENTAL

Chemicals

HPLC-grade methanol was purchased from Carlo Erba (Milan, Italy) and acetonitrile from Merck (Darmstadt, Germany).

Luteine, zeaxanthine and *cis*-β-carotene were kindly provided by Hoffman La Roche (Basle, Switzerland); all-*trans*-α- and β-carotene were purchased from Sigma (St. Louis, MO, USA).

Carbon dioxide (N45 grade, containing <7 ppm of water) was purchased from Alphagaz (Bois d'Arcy, France).

Apparatus

Subcritical fluid chromatography was performed using equipment manufactured by Jasco (Tokyo, Japan). The two pumps (Model 880-PU) were connected to a SEDERE pulse damper (Touzart et Matignon, Vitry sur Seine, France). The head of the pump used for carbon dioxide was cooled to −2°C by a cryostat (F 10 c; Julabo, Seelbach, Germany). The pulse damper was connected to an injection valve fitted with a 20-μl loop (Model 7125; Rheodyne, Cotati, CA, USA). The column was thermostated in a controlled oven (Crocosil; Cluzeau, Ste. Foy-la-Grande, France), regulated at 25°C by a cryostat (D 8 GH; Haake, Karlsruhe, Germany).

Detection was performed with a UV–Vis detector (Hewlett-Packard Model 1050) with a high-pressure-resistant cell. The eluent was discharged via an automatic back-pressure regulator (Model 880-81). Chromatograms were recorded at 450 nm, using an electronic integrator (CR 6A; Shimadzu, Kyoto, Japan). The flow-rate of the mobile phase was 3.0 ml/min, the output pressure of the fluid was 15 MPa, the temperature was 25°C and the composition of the mobile phase was carbon dioxide–acetonitrile–methanol (65:33.25:1.75, v/v/v).

Liquid chromatography of PAHs was performed using a quaternary pump (PU4100; Unicam, Cambridge, UK), an injection valve

(Rheodyne Model 7125) and a diode-array detector (PU 4121), connected to a PC compatible computer using the PU 6003 software (Unicam). The flow-rate of the mobile phase [water–acetonitrile (15:85)] was 2.0 ml/min. The analyses were carried out at ambient temperature.

The chromatographic columns used in this study were the following: 5-μm Hypersil ODS (150 × 4.6 mm I.D.) (Shandon, Sewickley, PA, USA); 10-μm Partisil ODS3 (250 × 4.6 mm I.D.) (Whatman, Clifton, NJ, US); 5-μm Pecosphere HS 5 (150 × 4.6 mm I.D.) (Perkin-Elmer, Norwalk, CT, USA); 5-μm Ultrasphere DABS (250 × 4.6 mm I.D.) (Beckman, San Ramon, CA, USA); 5-μm Adsorbosphere HS (250 × 4.6 mm I.D.) (Alltech, Deerfield, IL, USA); 5-μm Adsorbosphere UHS (250 × 4.6 mm I.D.) (Alltech); 5-μm Zorbax ODS (250 × 4.6 nm) (DuPont, Wilmington, DE, USA); 3-μm Ultracarb 3 C 18 (150 × 4.6 mm I.D.) (Phenomenex, Torrance, CA, USA); 5-μm Ultrabase UB 225 (250 × 4.6 mm I.D.) (SFCC Shandon, Eragny, France); 5-μm LiChrospher 100 RP 18 e (250 × 4 mm I.D.) (Merck); 5-μm LiChrospher 100 RP 18 (250 × 4 mm I.D.) (Merck); 5-μm Superspher 100 RP 18 e (250 × 4 mm I.D.) (Merck); 5-μm Superspher 100 RP 18 (250 × 4 mm I.D.) (Merck); 5-μm Hypersil BDS (250 × 4.6 mm I.D.) (Shandon); 5-μm Kromasil C_{18} (150 × 4.6 mm I.D.) (Eka Nobel, Surte, Sweden); 5-μm Nucleosil C_{18} (150 × 4.6 mm I.D.) (Macherey–Nagel, Düren, Germany); 5-μm Zorbax Rx (150 × 4.6 nm I.D.) (DuPont); 5-μm Bakerbond C_{18} wide pore (100 × 4.6 mm I.D.) (Baker, Phillipsburg, NJ, USA); 5-μm Supelcosil LC-PAH (150 × 4.6 mm I.D.) (Supelco, Bellefonte, PA, USA); 5-μm Vydac 201 TP (150 × 4.6 nm I.D.) (Separations Group, Hesperia, CA, USA); 5-μm PAH HC/ODS (250 × 4.6 nm I.D.) (Perkin-Elmer); 5-μm Vydac 218 TP (250 × 4.6 mm I.D.) (Separations Group); 5-μm Suplex pKb (150 × 4.6 mm I.D.) (Supelco); 5-μm Pecosphere 5-CR (150 × 4.6 mm I.D.) (Perkin-Elmer); 5-μm Inertsil IN 5 ODS2-15F (150 × 4.6 mm I.D.) (Gasukuro, Tokyo, Japan); 5-μm Spheri-5 ODS (250 × 4.6 mm I.D.) (Brownlee Labs., Santa Clara, CA, USA); 5-μm Vydac 201 HS (150 × 4.6 mm I.D.) (Separations Group); 5-μm RP Select B (250 × 4.6 mm I.D.) (Merck); and 5-μm Spherisorb ODS 2 (150 × 4.6 nm I.D.) (Phase Separations, Queensferry, UK).

The void volume was determined as described elsewhere [6].

RESULTS AND DISCUSSION

Comparison of the NIST results in LC and in SbFC

As has been shown by Sander and Wise [4], the selectivity between two PAHs such as benzo[*a*]pyrene (BaP) (planar compound) and tetrabenzonaphthalene (TBN) (non-planar compound) depends on the monomeric or polymeric nature of the stationary phase. However, this test is valid only for very precise mobile phase conditions. Therefore, we wanted to assess the validity of the test for the chromatographic conditions used in this work. Fig. 1 shows the selectivity obtained in HPLC as a function of that obtained in SbFC. A correlation coefficient of 0.975 was obtained, which indicates the good correlation between the results obtained by the two methods. A decrease in the selectivity values of *ca.* 0.2 can, however, be observed in SbFC (Table I). In SbFC, polymeric columns give a selectivity value of ≤ 0.8 (instead of a value of 1.0 for HPLC), whereas monomeric columns give values close to or above 1.5 (instead of a value of 1.7 for HPLC).

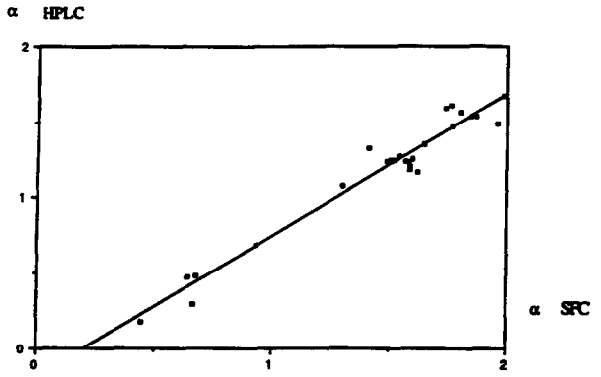

Fig. 1. TBN–BaP selectivity in HPLC *vs.* SbFC on different commercial columns. The equation of the linear regression is $y = -0.1839 + 0.9309x$. The correlation coefficient is 0.975.

TABLE I

EXPERIMENTAL VALUES AND CHARACTERISTICS OF DIFFERENT COMMERCIAL COLUMNS

The linkage functionality and the carbon content are given when data were available from the manufacturers.

Column	No.	k' (α-carotene)	Selectivity			Linkage functionality[a]	Carbon content (%)
			cis–trans-β-Carotene	TBN–BaP	α-Carotene–zeaxanthine		
Hypersil ODS	1	5.8	1	1.485	6.27	M	8
Partisil ODS 3	2	6.2	1	1.53	0.99	M	10
Pecosphere HS	3	9	1	1.608	2.28	M	8
Ultrasphere DABS	4	11.5	1.04	1.495	5.31		
Adsorbosphere HS	5	16	1.048	1.58	2.69	M	20
Adsorbosphere UHS	6	38	1.052	1.62	2.97	M	30
Zorbax ODS	7	14.5	1.068	1.466	1.046	M	20
Ultracarb 3 ODS	8	8.5	1.082	1.53	1.12	M	22
Ultrabase UB 225	9	13.5	1.84	1.185	4.428	M	19
LiChrospher 100 RP 18 e	10	9.1	1.09	1.238	2.53		22
Hypersil BDS	11	6.6	1.122	1.212	5.83	M	11
Kromasil C_{18}	12	10.1	1.092	1.153	4.9	M	19
Nucleosil C_{18}	13	7.2	1.104	1.354	0.625		
Zorbax RX	14	7.7	1.097	1.078	3.82		
Bakerbond C_{18} wide pore	15	1.5	1.153	0.183	0.218	P	
Supelcosil LC-PAH	16	2.2	1.181	0.30	0.205	P	8
Vydac 201 TP	17	2.5	1.208	0.476	0.184	P	8
PAH HC/ODS	18	2.4	1.194	0.487	0.155	P	8.5
Vydac 218 TP	19	1.8	1.213	0.69	0.271	P	8
Suplex pKb	20	2.4	1.148	0.815	0.17		
Pecosphere 5-CR	21	10.5	1.07	1.35	4.27	M	12
Superspher 100 RP 18 e	22	12.1	N.m.[b]	1.277	4		
Inertsil IN 5OD2-15F	23	7.1	N.m.	1.255	3.7		
Spheri-5 ODS	24	11.4	N.m.	1.238	1.836	P	
Vydac 201 HS	25	6.1	N.m.	1.561	0.541	M	13.5
RP Select B	26	1.9	N.m.	1.331	0.426		
Superspher 100 RP 18	27	11.1	N.m.	1.235	1.148		
LiChrospher 100 RP 18	28	10.4	N.m.	1.148	1.035		
Spherisorb ODS 2	29	5.8	N.m.	1.243	0.883		12

[a] M = monofunctional, P = polyfunctional.
[b] N.m. = Not measurable.

A similar change in selectivity to those between HPLC and SbFC was observed in HPLC as a function of the organic solvent in either binary aqueous–organic mixtures or in pure organic solvents. A decrease in the water content of the mobile phase leads to a decrease in the BaP–TBN selectivity. If one were to base the proposed model of Sander and Wise [9] on the presence of slots in the stationary phase which permit the insertion of planner compound, BaP, one could explain this phenomenon.

Indeed, some workers have shown a change in the state of the alkyl chains, collapsed on the silica in the presence of water and more extend-

ed in the mobile phase in the presence of organic solvents [10,11]. The probable unfolding of organic solvents increases either the number or the size of the slots and increases the possibility of penetration of BaP into the stationary phase.

This phenomenon is observed regardless of the type of silane used (mono- or polyfunctional), which implies that there are two types of sites that BaP can penetrate: those created by the network in the bulk of the polymeric phases, which would correspond to a macrostructure of those phases, and the surface sites arising from the state of the grafted chains, either at the surface of the polymeric phases or present in the monomeric phases.

As the changes in the values in this test are identical for NARP chromatography and SbFC, it would appear that the mixture of CO_2 and organic modifier used in SbFC has properties close to those of a non-aqueous mobile phase in NARP.

A study of the variation of the TBN–BaP selectivity in SbFC showed that the addition of modifier results in a decrease in selectivity, which is characteristic of a polymeric-type phase (Fig. 2). These modifiers seem to induce an elongation of the alkyl chains in the mobile phase. It is probable that pure CO_2 solvates the alkyl chains only weakly. The state of the stationary phase is probably comparable to that observed in liquid chromatography with water-rich mobile phases (collapsed conformation of the alkyl chains).

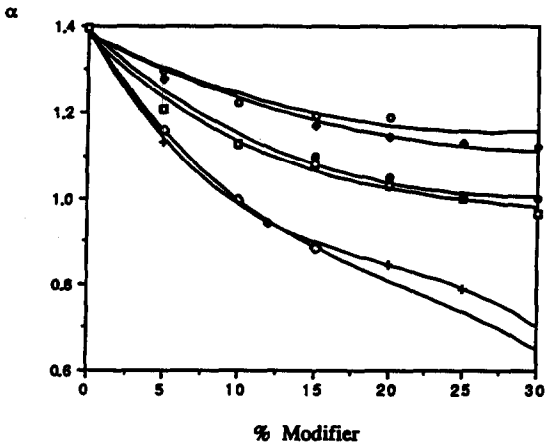

Fig. 2. Selectivity of TBN–BaP in SbFC as a function of the percentage of modifier. Flow-rate, 3.0 ml/min; temperature, 25°C; column, Ultrabase UB 225. ○ = Acetonitrile; ◆ = methanol; ● = acetone; □ = ethanol; + = methylene chloride; ◇ = THF.

The extent of the effect of organic modifiers varies depending on the type of modifier used, as shown in a previous study [7]. Modifiers can be classified into three categories on the basis of their dielectric constants: (i) acetonitrile and methanol (>30), (ii) acetone and ethanol (20–30) and (iii) tetrahydrofuran and methylene chloride (<10). The larger the dielectric constant, the smaller is the tendency of the TBN–BaP selectivity towards a polymeric-type phase. This underlines increased solvatation and elongation of the chains, as in HPLC with aqueous–organic mixtures [12–21].

Comparison of the selectivity of BaP–TBN and the selectivity of the cis–trans isomers of β-carotene

It must initially be emphasized that it is not possible to measure the value of selectivity between 9- or 13-*cis*- and *trans*-β-carotene, whose separation is difficult with certain columns owing either to the low k' values with some columns or to broad peaks with the particular mobile phase employed. The latter phenomenon has already been observed when using acetonitrile at the modifier. It was also not possible to inject the component separately as we did not have samples of the 9- and 13-*cis* isomers.

We chose to group together the results obtained according to their similarities, because for certain monomeric columns the use of linear regression does not reflect the reality when the selectivity of these isomers is equal to 1, whereas the value of the NIST test for the same columns varies between 1.48 and 1.61 (Fig. 3).

An identical value for the first selectivity and a varying value for the second can only strongly degrade the correlation coefficient, whereas the information given by the two selectivities is in this instance identical. In other words, the phase is monomeric with a low bondage density.

There is a tendency for the selectivity to increase between the *cis–trans* isomers of β-carotene when the value of the NIST test decreases. The correlation coefficient is 0.87,

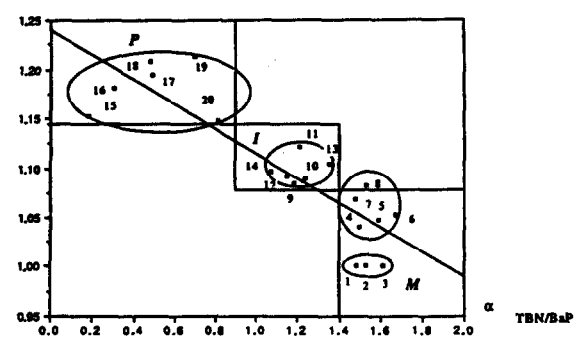

Fig. 3. Selectivity of 9- or 13-*cis*- and *trans* isomers of β-carotene as a function of the selectivity of TBN–BaP in SbFC on different commercial columns. P = polyfunctional; I = intermediate; M = monofunctional. Numbers are column numbers as given in Table I.

which, according to previous statements, shows some correlation between the two criteria. According to Sander and Wise [4], it is possible to separate columns into three categories, monomeric, intermediate and polymeric. This distribution in SbFC corresponds to selectivity values of >1.45 for the first group, 0.9–1.45 for the second group and <0.9 for the last group.

Among the monomeric columns, Hypersil ODS (column No. 1, Table I), Pecosphere HS (No. 3) and Partisil 5 ODS3 (No. 2) give a selectivity between the *cis–trans* isomers of β-carotene of 1. However, five columns give a selectivity >1 (*ca.* 1.05) for the *cis–trans* isomers of β-carotene but behave as monomeric according to the tests with PAH Zorbax ODS (No. 7), Adsorbosphere HS (No. 5), Adsorbosphere UHS (No. 6), Ultrasphere DABS (No. 4) and Ultracarb (No. 8). These five columns give capacity factors greater than all the other columns, which underlines their high carbon content, and above all takes account of the specific surface area and a carbon load that must be greater than those for the three aforementioned columns. It should be noted that Adsorbosphere UHS gives k' values that are three times greater than those of Ultrabase UB 225 used as a reference, and whose retentivity is already amongst the most important.

As expected, all the polymeric columns, Vydac 201 TP (column No. 16), Vydac 218 TP (No. 18), Supelcosil LC-PAH (No. 15), PAH HC/ODS (No. 17), Suplex pKb (No. 19) and Bakerbond C_{18} wide pore (No. 14) tested are found in the group of columns that have a polymeric stationary phase.

These structures of these columns allow one to separate the *cis–trans* isomers for which the selectivity is between 1.15 and 1.21. It must be noted, however, that despite this elevated value, these columns are less adapted to separations of carotenes under these analytical conditions. This is due to their poor retention, which is indicative of a relatively low carbon content (Table I).

The group of intermediate columns includes Ultrabase UB 225 (column No. 9), Nucleosil C_{18} (No. 13), Hypersil BDS (No. 11), Zorbax Rx (No. 14), Kromasil C_{18} (No. 12) and LiChrospher 100 RP 18 e (No. 10). They give intermediate value for PAHs, which, according to Sander and Wise [4], correspond to a monomeric phase with a high bonding density or a polymeric phase with a low bonding density. One effectively finds among these columns those for which the extent of bonding is important, such as Ultrabase UB 225. It is also interesting that several of these columns (Ultrabase UB 225, Zorbax Rx, Kromasil C_{18} and Hypersil BDS) are stated by the manufacturers as having been specially designed for use with basic products, and often present a high coverage density (Ultrabase UB 225 and Kromasil C_{18} 3.2 μmol/m^2, Hypersil BDS 3.6 μmol/m^2).

If one looks into the development of the selectivity of the PAHs between initially available supports and those designed for basic products, one realises that the coverage of the silanol groups after bonding (LiChrospher 100 RP 18 e and Superspher 100 RP 18) entails increased selectivity, which shows the development of the support towards a more monomeric natural phase. It could be that the groups used to react with the silanols decrease the depth between the grafts where the BaP becomes trapped, or that the BaP can no longer interact with the silanols. This type of interaction between the silanols and the PAHs has already been reported [22].

Conversely, comparison between thermally treated silicas and silicas that are untreated before bonding (Hypersil, Zorbax and Pecosphere) shows that the selectivity of the PAHs

decreases for treated supports, which gives the columns a more pronounced polymeric character. Even so, it is not really the structure of the phase that is modified but rather the carbon load which leads to an identical development of PAH selectivity, as was shown by Sentell and Dorsey [23].

In addition, it can be emphasized that the three supports designed for basic products (Hypersil BDS and Pecosphere 5 CR) are end-capped or sterically protected (Zorbax Rx). This observation indicates that a detailed understanding of the parameters influencing the separations studied is difficult. An additional parameter that could be of importance is the inorganic impurity content of the silicas used (sodium, aluminium, iron) (Ultrabase UB 225 and Kromasil C_{18}) whose presence can affect the chromatographic properties [1].

It appears nevertheless that the columns treated for the analysis of basic products allow the separation of the *cis–trans* isomer of β-carotene and have an adequate carbon content for this separation and also for the separation of mixtures containing other pigments.

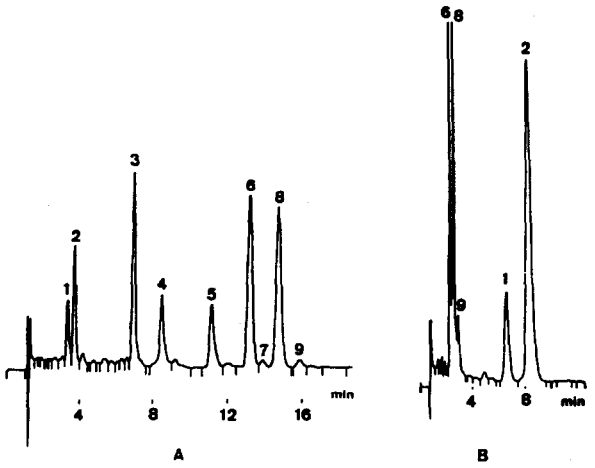

Fig. 4. Chromatograms of a mixture of carotenoids on two different types of stationary phases. Flow-rate, 3.0 ml/min; temperature, 25°C; mobile phase, acetonitrile–methanol–carbon dioxide (33.25:1.75:65, v/v/v). (A) Ultrabase UB 225; (B) Supelcosil LC-PAH. Peaks: 1 = luteine; 2 = zeaxanthine; 3 = β-cryptoxanthine; 4 = lycopene; 5 = all-*trans*-γ-carotene; 6 = all-*trans*-α-carotene; 7 = *cis*-α-carotene; 8 = all-*trans*-β-carotene; 9 = 9-*cis*-β-carotene.

Study of α-carotene–zeaxanthin selectivity

We have previously observed in SbFC that the retention of xanthophylls (luteine and zeaxanthine) greatly depends on the presence of an alcohol as a modifier [8]. A minimum amount of alcohol is necessary for the Ultrabase UB 225 column to ensure rapid elution of its components. Therefore, we deduced that a small amount of alcohol is indispensable for the recovery of the residual silanol groups that interact and strongly retain luteine and zeaxanthine. For this column the selectivity between *trans*-α-carotene and zeaxanthine, with 35% modifier [acetonitrile–methanol (95:5)], is 4.43 (Fig. 4A).

The same analysis carried out on a polymeric column (Supelcosil LC-PAH) gives a totally different result in that the xanthophylls are much more strongly retained than the carotenes, the selectivity of α-carotene–zeaxanthine being 0.205 (Fig. 4B). This result seems surprising as the use of a polymeric stationary phase for avoiding interactions of products with residual silanols is a recognized practice [24,25]. The addition of butylamine to the mobile phase at a level of 0.25% lowered the capacity factor of luteine from 8.28 to 5.69 and decreased the asymmetry factor of luteine from 2.03 to 1.3. It seems that the xanthophylls interact with certain oxygen atoms carried by the siloxane groups that are more accessible at the stationary polymeric phase/mobile phase interface, rather

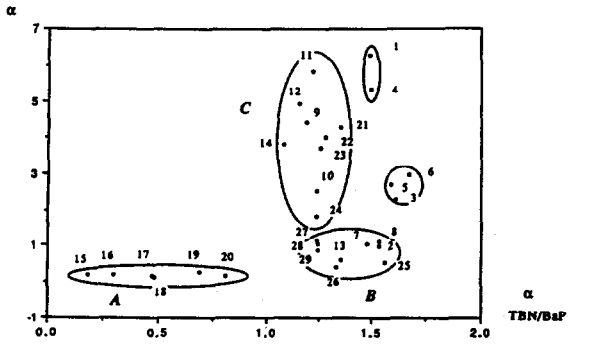

Fig. 5. Selectivity α-carotene–zeaxanthine as a function of the selectivity of TBN–BaP in SbFC for different commercial columns. Numbers are column numbers as given in Table I.

than with the residual silanol groups which are difficult to access with this type of bonding. This behaviour has also been noted in HPLC [25]. We therefore compared this selectivity as a function of that of PAHs (Fig. 5) and established a plan regrouping columns with similar behaviour. Comparison with partial results obtained for the mixture of residual silanols by a different method [3] shows an identical development between the two sources of results, which seems to confirm the hypothesis of the retention mechanism of xanthophylls on polymeric stationary phases.

The first group (A) (15–20) includes polymeric columns that give an inverted retention between the xanthophylls and the carotenes.

Also with these columns the *cis–trans* selectivity of β-carotene is highest. The combination of these two selectivities thus allows the discrimination of this type of support.

The second group (B) (2, 7, 8, 13, 25–29) contains columns that are classified as monomeric or as intermediate according to the NIST 869 test. Included here are the classical silica supports (Nucleosil, Spherisorb ODS 2, LiChrospher 100 RP 18).

The selectivity between α-carotene and zeaxanthine is close to 1, which often lead to co-elution of the carotene and the xanthophylls on these columns. It is possible, however, to suppress this co-elution by altering the amount of alcohol in the mobile phase since the retention of the xanthophylls depends more on the alcohol content than does that of the carotenes. The value of this selectivity shows that the residual silanols are accessible to the xanthophylls on these columns.

The third group (C) (9–12, 14, 21–24) also gives an intermediate PAH selectivity even though most of the supports are monomeric. The elevated value of the α-carotene–zeaxanthine selectivity shows that few of the silanol groups are available to the xanthophylls, which explains their low retention. This result is not surprising since these columns (except the Spheri-5 ODS) are treated for the analysis of basic products and therefore the number of residual silanol present is decreased. It appears that these columns are also those which give a selectivity for the *cis–trans* isomers of β-carotene of close to 1.1 with a sufficient retention to obtain, under these conditions (Fig. 6), a complete separation of the compounds studied.

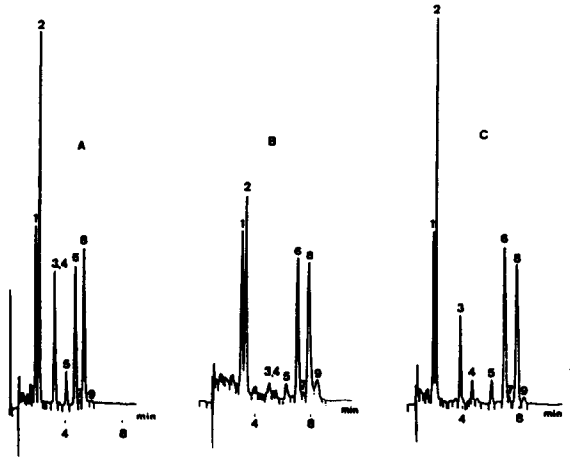

Fig. 6. Chromatograms of a mixture of carotenes on different chromatographic supports used for the analysis of basic products. Analytical conditions as in Fig. 4. (A) LiChrospher 100 RP 18; (B) Pecosphere 5-CR; (C) Zorbax Rx. Peaks: 1 = luteine; 2 = zeaxanthine; 3 = β-crypoxanthine; 4 = lycopene; 5 = all-*trans*-γ-carotene; 6 = all-*trans*-α-carotene; 7 = *cis*-α-carotene; 8 = all-*trans*-β-carotene; 9 = *cis*-β-carotene.

On studying the information obtained regarding these supports, it appears that certain pretreatment of the silica before bonding increases the number of reactive silanols, which leads to a high bonding density phase with homogeneous bonding. These columns therefore have comparable performances and can be used for the separation of complex mixtures of carotenoid pigments.

Two other groups can be distinguished according to this classification, composed of columns classified as monomeric by the NIST 869 test. Amongst these five columns, three give a selectivity close to 3 for the α-carotene–zeaxanthine coupling: Pecosphere HS (No. 3), Adsorbosphere UHS (No. 6), whereas the other two, Hypersil ODS (No. 1) and Ultrasphere DABS (No. 4), have a very high selectivity for the compounds studied.

These results with regard to the Hypersil ODS and the Pecosphere HS columns are surprising, in that these columns are the opposite of the three other columns which are either treated for

basic products (Ultrasphere DAB) or which give a high recovery as attested by the very high capacity factors (Adsorbosphere).

Mechanism of separation of the cis–trans isomers of carotenes

From the results obtained it is possible to propose a separation mechanism for the *cis–trans* isomers of carotenes. As polymeric phases exhibit superior selectivity for this separation, the separation mechanism depends on the shape selectivity, often observed for rigid or planar compounds. The conformation or the order of the bonded chains also plays an important role in this separation, as shown by the results obtained with highly bonded silica or in the presence of different solvents. However, several pieces of information indicate that the separation mechanisms of PAHs and *cis–trans* isomers of carotenes are probably different.

First, for polymeric columns, there does not appear to be a correlation between measured selectivity values within this group, which could indicate that the separation mechanisms between PAHs and carotenes are not identical. The slot model [9] suggests that the planar compounds are retained more than the non-planar compounds, which is the opposite to what is observed for the carotenes, where the linear *trans* isomer is retained less than the bent *cis* isomer, both in SbFC and in NARP HPLC.

Nevertheless, the selectivity of the carotene isomers is highest on a polymeric phase. It is possible that polymeric phases have an irregular thickness depending on the extent of local polymerization. This irregular thickness can lead to the presence of slots, which explains the higher retention of the BaP compared with the TBN and assumes that the surface of this phase has alternating troughs and bumps.

In this instance, owing to the cluttered nature of the interior of the polymeric network together with the tangling of the alkyl chains, it is possible that the carotenes, which are large, rigid molecules, do not penetrate the stationary phase but remain stuck to the surface or only penetrate superficially.

On this uneven surface, the bent form of the *cis* isomers would favour interactions in contrast to the *trans* compounds, for which none of the surface would be in contact with the adsorbent.

In the same way, high-bonded-density or/and homogeneously bonded monomeric phases (Ultrabase UB 225, Hypersil BDS, Kromasil C_{18}) could give a surface state similar to that described previously, but whose topology would be less pronounced, explaining the lower values of the selectivity for these phases. The value indicative of the coverage density of bonded groups for these supports is in the region of 3.4 μmol/m^2. The high density would favour rigidity of grafts [26] which could not move apart sufficiently to let the molecules penetrate the interior of the phase.

Dill [27], in his model of retention, emphasized a phenomenon of entropic exclusion (linked with the disruption of a spatial molecular arrangement) of the solute by stationary phases with high coverage density.

The separation mechanism of the *cis–trans* isomers results from an external contact with a stationary phase that covers the silica in the same way but whose surface is more or less regular.

Effect of temperature on the structure of the stationary phase

We investigated the effect of temperature on a polymeric column (Vydac 201 TP) and on a highly bonded monomeric column (Ultrabase UB 225) and on a monomeric column (Zorbax ODS). There have been several reports of the influence of temperature on the separation of *cis–trans* isomers [5,28] and the unique behaviour of polymeric phases has also been emphasized with the use of homologous series [29] or other test compounds [30,31].

A decrease in temperature leads to a decrease in TBN–BaP selectivity for the three columns studied, as has already been reported by Sander and Wise [32]. On the other hand, Fig. 7 shows that if one observes a phase transition for the monomeric column, Zorbax ODS, and for the Ultrabase column, the curve of log k' *vs.* $1/T$ for BaP is not linear for the polymeric column, Vydac 201 TP, in the temperature range 5–45°C, which shows the existence of a phase transition for this polymeric column. The variation of log k' for β-carotene shows a change in the slope for

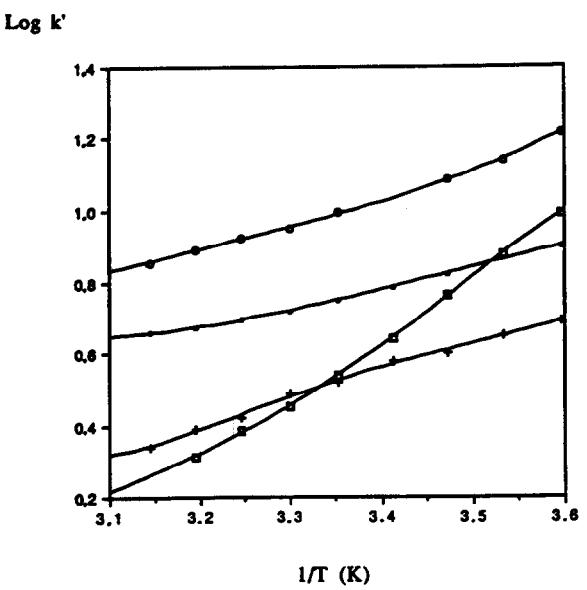

Fig. 7. Variation of the k' as a function of the analytical temperature for different types of linkage. O = BaP on Ultrabase UB 225 (15% of acetonitrile as modifier in CO_2); + = BaP on Vydac 201 TP (10% of acetonitrile as modifier in CO_2); ● = BaP on Zorbax ODS (15% of acetonitrile as modifier in CO_2); □ = all-*trans*-β-carotene on Vydac 201 TP (10% of acetonitrile as modifier in CO_2).

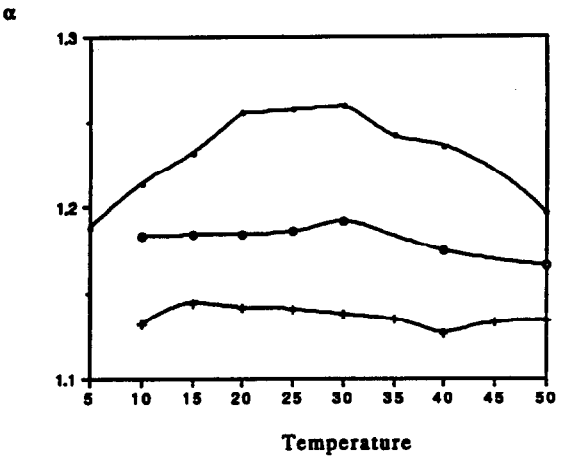

Fig. 8. Selectivity of fifteen all-*trans*–*cis* isomers of β-carotene *vs.* analytical temperature for two types of linkage. ● = Vydac 201 TP (10% of acetonitrile as modifier in CO_2); O = Ultrabase UB 225 (15% of acetonitrile as modifier in CO_2); + = Ultrabase UB 225 (30% of acetonitrile as modifier in CO_2).

this column, which seems to confirm this hypothesis. In HPLC, such behaviour has been reported by Jinno *et al.* [33] for a polymeric column using a similar sample, coronene. The same has been observed with smaller test molecules [29–31].

The different results with respect to that of Sander and Wise can be explained by the size of the range studied and the subtlety of the phenomenon. These results further underline the similarity in behaviour between the stationary phases in HPLC and SbFC.

If we now turn to the influence of temperature on the separation of the *cis*–*trans* isomers of β-carotene, we find that it has little effect for a high-bonded-density phase, whatever the percentage of acetonitrile in the carbon dioxide (Fig. 8). One could propose that the state of the surface is therefore not greatly affected by a change in temperature, either because the high coverage density leads to an increased rigidity of the grafts, or because the variation in temperature induces various opposing phenomena.

It is, in fact, known that with respect to the bonds there are two types of effects associated with a decrease in temperature: a lengthening of grafts (prevails in *trans* forms of the bonded chains) and an increased rigidity of the chains. These two changes conflict according to the penetrating power of the carotenes into the bulk of the stationary phase.

An increase in selectivity of 50 at 25°C followed by a decrease of 25 at 5°C is seen for the polymeric phase. Even then, one can suggest that there are numerous phenomena occurring. If we consider that the structure of the polymeric stationary phase is made up of a network of variable thickness and of octadecyl chain extremities which form an external layer, it could be that between 50 and 25°C the rigidity of the network increases, as do the local differences in the thickness and topology recognition. In the same way, the swelling of the polymeric phases under the influence of a solvent, like a sponge, has been described by Lochmüller and Kersey [34].

However, between 25 and 5°C, the free grafts take up a *trans* configuration which initiates their stretching and favours penetration of compounds which would in turn decrease the *cis*–*trans* selectivity.

On the other hand, these two phenomena (between 5 and 25°C and between 25 and 50°C) favour in the same way the insertion of BaP and are in accordance with the continuous increase in the TBN–BaP selectivity observed with a decrease in temperature.

Hence the use of carotenes as test molecules appears to allow the visualization of phenomena unobserved by the use of the selectivity between the PAHs, which further emphasizes the difference in behaviour between these compounds.

CONCLUSIONS

The retention mechanism of carotenes depends on different factors according to the type of compounds studied. The separation of the *cis–trans* isomer of carotenes is probably linked to the state of the surface of the stationary phase whereas that of the xanthophylls seems to be governed more by the presence of accessible oxygen groups for the support than that of carotenes. By combining these two criteria of selectivity it seems that it is possible to characterize the nature of the support used.

Polymeric columns give a selectivity between the *cis–trans* isomers of β-carotene which is greater than 1.15 and a selectivity between luteine and zeaxanthine which is always greater than 1.2. Monomeric columns with a high carbon content, used in particular for the analysis of basic products, give a value between 1.07 and 1.15 for the first selectivity and a value greater than 2.5 for the second. These columns seem particularly useful for the analysis of carotenoids by SbFC.

ACKNOWLEDGEMENTS

The authors gratefully thank Mrs. Nathalie Huchon and Miss Alexandra Jones for the preparation of the manuscript.

REFERENCES

1 K.K. Unger, in K.K. Unger (Editor), *Packings and Stationary Phases in Chromatographic Techniques* (*Chromatographic Science Series*, Vol. 47), Marcel Dekker, New York, 1990, pp. 1–2.
2 A. Goldberg, *Anal. Chem.*, 54 (1982) 342.
3 M.J. Walters, *J. Assoc. Off. Anal. Chem.*, 70 (1987) 465.
4 L.C. Sander and S.A. Wise, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 11 (1988) 383.
5 E. Lesellier, C. Marty, C. Berset, A. Tchapla, *J. High Resolut. Chromatogr.*, 12 (1989) 447.
6 E. Lesellier, A. Tchapla, M.R. Pechard, C.R. Lee and A.M. Krstulovic, *J. Chromatogr.*, 557 (1991) 59.
7 E. Lesellier, A.M. Krstulovic and A. Tchapla, *Chromatographia*, 36 (1993) 275.
8 E. Lesellier, A.M. Krstulovic and A. Tchapla, *J. Chromatogr.*, 641 (1993) 137.
9 L.C. Sander and S.A. Wise, *LC · GC*, 6 (1990) 24.
10 C.H. Lochmüller, C.H. Hunnicutt and M.L. Mullaney, *J. Phys. Chem.*, 89 (1985) 5770.
11 D.E. Martire and R.E. Boehm, *J. Phys. Chem.*, 87 (1983) 1045.
12 L.C. Sander, J.B. Callis and L.R. Field, *Anal. Chem.*, 55 (1983) 1068.
13 L.C. Sander, C.J. Glinka and S.A. Wise, *Anal. Chem.*, 62 (1990) 1099.
14 J. Stahlberg and M. Almgren, *Anal. Chem.*, 57 (1985) 817.
15 A.M. Stalcup, D.E. Martire and S. Wise, *J. Chromatogr.*, 442 (1988) 1.
16 J.W. Carr and J.M. Harris, *Anal. Chem.*, 58 (1986) 526.
17 K. Tani and Y. Susuki, *Chromatographia*, 31 (1991) 347.
18 L.A. Cole and J.G. Dorsey, *Anal. Chem.*, 62 (1990) 16.
19 P.B. Wright, E. Lamb, J.G. Dorsey and R.G. Kooser, *Anal. Chem.*, 64 (1992) 785.
20 P. Shah and L.B. Rogers, *J. Chromatogr.*, 388 (1987) 411.
21 S. Heron and A. Tchapla, *J. Chromatogr.*, 556 (1991) 219.
22 A.Y. Fadeev, G.V. Lisichkin, V.K. Runov and S.M. Staroverov, *J. Chromatogr.*, 558 (1991) 31.
23 K.B. Sentell and J.G. Dorsey, *J. Chromatogr.*, 461 (1989) 193.
24 D. Chan Leach, M.A. Stadalius, J.S. Berus and L.R. Snyder, *LC · GC Int.*, 5 (1988) 22.
25 K.S. Epler, L.C. Sander, R.G. Ziegler, S.A. Wise and N.E. Craft, *J. Chromatogr.*, 595 (1992) 89.
26 K.B. Sentell and J.G. Dorsey, *Anal. Chem.*, 61 (1989) 930.
27 K.A. Dill, *J. Phys. Chem.*, 91 (1987) 1980.
28 L.S. Sander and N.E. Craft, *Anal. Chem.*, 62 (1990) 1545.
29 S. Heron and A. Tchapla, *Chromatographia*, 36 (1993) 11.
30 L.A. Cole and J.G. Dorsey, *Anal. Chem.*, 64 (1992) 1317.
31 L.A. Cole, J.G. Dorsey and K.A. Dill, *Anal. Chem.*, 64 (1992) 1324.
32 L.C. Sander and A. Wise, *Anal. Chem.*, 61 (1989) 1789.
33 K. Jinno, T. Nagoshi, N. Tanaka, M. Okamoto, J.C. Fetzer and W.R. Biggs, *J. Chromatogr.*, 436 (1988) 1.
34 C.H. Lochmüller and M.T. Kersey, *Anal. Chem.*, 60 (1988) 1910.