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Classification of special octadecyl-bonded phases by the carotenoid test

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Abstract

Amongst the numerous base-deactivated ODS phases obtained by increasing the bonding density or/and by efficient endcapping treatments, some particular stationary phases have been developped, to limit the additional interactions of basic compounds with residual silanols, to work at extreme pH or with rich water mobile phases. Horizontal polymeric phases, sterically protected ones, hybrid silicas, propylene bridge, are particularly used for this purpose. Octadecyl chains with embedded polar groups and hydrophilic endcapping are also used in this goal. The properties of these phases were studied with a simple test consisting in the injection of carotenoid pigments in Subcritical Fluid Chromatography. The molecules used and the nature of the mobile phase allow the determination of hydrophobicity, polar site accessibility and type or/and bonding density of the stationary phases. Whatever the type of the phases, the particular stationary phases do not show any remarkable property, in comparison to other base-deactivated C18-bonded phases. On the other hand, embedded and polar-endcapped phases display a specific behaviour in regard of hydrophilic interactions, which are highlighted by the absence of water in the subcritical fluid. Additional properties of these phases are described, such as steric recognition and retention performances. As expected, polar-embedded phases are less retentive than classical ODS ones, but are sometimes able to provide greater steric recognition. On the other hand, the polar-endcapped phases display greater hydrophobicity than polar-embedded ones. From a simple classification diagram based on chromatographic properties, differences can be noticed between the polar-embedded groups (amide, carbamate, ether, sulfonamide) and between embedded and endcapped phases. Surprising behaviours are also noticed for some on the tested phases.

Keywords: Stationary phases; ODS; Carotenoid test; Subcritical fluid chromatography; Embedded polar group; Hydrophilic endcapping

1. Introduction

Among the different properties expected from octadecyl-bonded silicas, the absence of interactions with basic solutes is one of the most difficult to achieve. On the basis of 8 μmol/m^2 silanol groups on a fully hydroxylated silica surface, the maximum coverage of bonded ligands appears to be 4–4.5 μmol/m^2 [1]. The residual, or non-bonded, silanol groups on the silica surface are responsible for secondary interaction mechanisms in reversed phase liquid chromatography (RPLC), which modify both the retention and the peak asymmetry of polar analytes. This peak tailing can be explained in terms of kinetics of mass transfer, because the exchange rate between the compounds and silanol sites is slower than the one between the compounds and the alkyl chains. The contribution of silanols to retention is also smaller. An extensive review on silica structure, activity and species of silanols was done by Nawrocki [2]. The silanophilic activity includes hydrogen bonding and ionic interactions, the latter depending on the pH of the mobile phase. Ionic interactions are especially high at pH 7 due to ionisation of both acidic silanols and basic solutes such as amines.

Numerous attempts were done to improve the chromatographic behaviour of stationary phases in regard of these additional interactions with basic solutes. Obviously, the endcapping treatment by silylating agents such as trimethyl- or hexamethylchlorosilane is the first step in this direction, allowing the reduction of the residual silanol number. The use of polymer coated or polymer encapsulated silica favours the improvement in the peak symmetry [3–6], when vertical polymerization [7,8] was introduced to increase the phase density of the stationary phase to avoid solute/silica interactions (Fig. 1). High purity silica and rehydroxylation during the synthesis process also limit the peak tailing by decreasing the amount of metal impurities at the sil-

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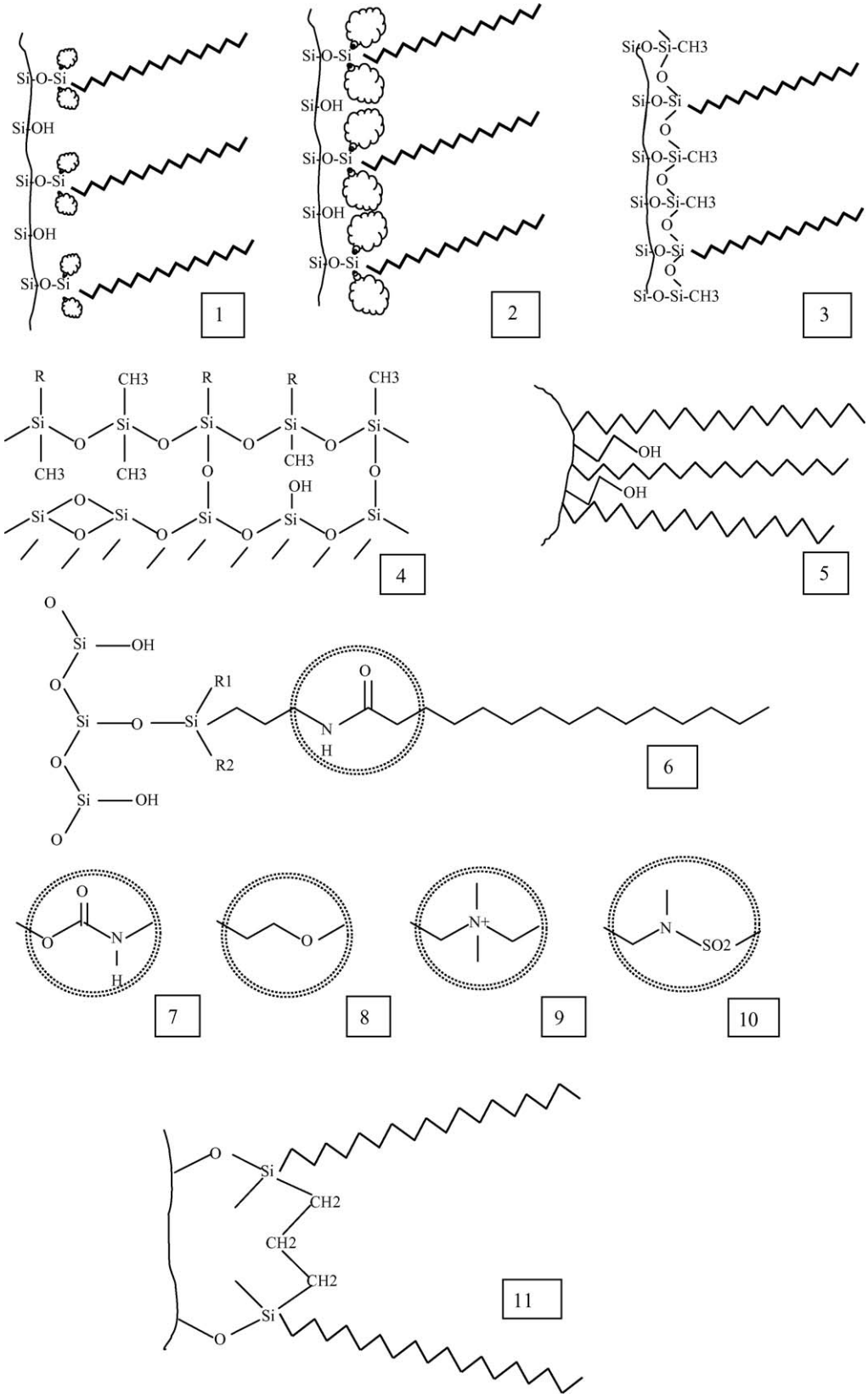

Fig. 1. Chemical structure of tested stationary phases. (1) Monofunctional phases (columns # 27, 42, 51, 63, 69, 79, 89, 100); (2) monofunctional sterically protected (#17); (3) horizontal polymerization (# 68); (4) coated polymer (#5, 53, 58); (5) hydrophilic endcapping (#133, 139, 141, 146, 148); (6) amide embedded phase (# 131,132,137,138,142,143,144,145,151); (7) carbamate-embedded phase (# 149); (8) ether-embedded (# 152); (9) quaternary ammonium-embedded non-endcapped (# 134) and endcapped (#135); (10) sulfonamide-embedded (#136); (11) bidentate-bonded phase (# 101).

ica surface, and the number of isolated silanols. These bonded silicas are often called "base deactivated" [9].

Other phases have been developped to avoid silanophilic interactions by steric protection with iso-propyl groups on the silinazing agent [8], by horizontal polymerization silanization [10,11], by the use of hybrid organic/inorganic silica in which OH groups were replaced by methyl ones [12], and by bidentate C18/C18 phases with a propylene bridge [13] (Fig. 1).

More recently, polar-embedded and polar-endcapped phases were successfully used for polar and basic compounds analyses in reversed phase liquid chromatography (Fig. 1).

Polar-embedded phases were obtained by the insertion of a polar functional group within the alkyl chain. These polar groups, are amide [14], carbamate [15,16], or ether, ammonium, and urea. Located at the bottom of the alkyl-bonded chains, these groups are supposed to increase the water concentration near the silica surface. The immobilized water layer acts as a shield with a high dielectric constant, which reduces the strength of the ionic interaction between the protonated bases and dissociated silanol groups [16]. However, a direct interaction between the embedded group and the residual silanols does not seem to occur, because of the weak dependance in the tailing factor of Amitriptiline with the surface concentration of embedded groups [16].

Different tests were used to study the silanol activity. They have been rewieved recently by Claessens et al. [17]. These studies underline great differences depending on the test conditions (pH of mobile phases, use of buffered or non-buffered eluents, test compounds used). Recently, polar-embedded and polar-endcapped phases were evaluated [18]. The polar-embedded phases displayed a lower hydrogen bonding capacity than conventional or polar-endcapped phases, and were shown to be less hydrophobic than conventional columns with ligands of identical chain length [18–20]. Moreover, the use of the butyl paraben and dipropyl phthalate separation factor allowed the discrimination of carbamate and amide groups [20–22]. The polar-embedded groups favour the retention of the phenolic compound (butylparaben).

Another separation factor, between amitiriptyline, a basic compound, and acenaphtene, is used to measure the silanophilic activity of packings [21,22]. This separation factor decreases as the surface coverage of the packing increases [23], showing that the relative influence of the residual silanols on the retention of basic compounds decreases. As expected, the embedded polar phases display a low relative retention for amitriptyline, but no clear differences appear between the two main types of polar groups (carbamate and amide).

These results shows that the embedded polar phases do not have the same behaviour in regards to the retention due to hydrogen bond because it favors the retention of butylparaben (hydrogen bond donor) and reduces the retention of amiltriptyline (hydrogen bond acceptor).

The polar-embedded groups modify the retention of basic compounds, because it is involved in interactions with the water of the hydro-organic mobile phase, which displays a high hydrogen bond donor character. The bound of water retained by the embedded group hinders the further hydrogen bonds of basic compounds.

Our purpose in this paper is to study numerous phases devoted to the analysis of basic compounds with the "carotenoid test" previously developped [24]. We will then investigate not only silanophilic interaction and hydrophobicity but also shape recognition. Classical stationary phases will be included in the set of columns to better compare the chromatographic behaviour of the particular "special base" phases studied.

2. Experimental

Experimental conditions of the carotenoid test are described elsewhere [24]. The experimental conditions selected for the test are: mobile phase methanol–carbon dioxide (15:85 (v/v)), 25 °C, flow rate 3 ml/min, and outlet pressure 15 MPa. UV–vis detection was carried out at 440 nm. The retention factors of all *trans* β-carotene (major compound of the isomer peaks), 13-*cis*-β-carotene (more intense *cis*-peak isomer), and zeaxanthine, as well as 13 *cis*/all *trans* β-carotene and β-carotene/zeaxanthine

Table 1
Specification of special base and conventional columns

Supplier	Column	Number	Surface area ($m^2\ g^{-1}$)	Carbon content (%)	Linkage type	Endcapping
DUPONT	Zorbax Eclipse XDB	63	180	10.3	DiMethylC18	Y
DUPONT	Zorbax Extend	101	185	12.1	Bidentate C18	ns
DUPONT	Zorbax RX-C18	89	180	12	DiMethylC18	N
DUPONT	Zorbax SB C18	17	180	10	DiButhylC18	N
EKA NOBEL	Kromasil C18	100	350	21.4	Monofunctional	Y
ES INDUSTRIE	Gammabond C18	5			Coated polymer	ns
INTERCHIM	Uptisphere NEC	27	320	16	Monofunctional	N
INTERCHIM	Uptisphere ODB	51	320	17	Monofunctional	Y
MACHEREY	Nucleosil 50 C18	69	450	14	Monofunctional	N
NAGEL	Nucleosil 5 C18 AB	103	350	25	Polyfunctional	Y
MERCK	Chromolith C18	79	300	17		Y
SHISEIDO	Capcell pak C18	58			Coated polymer	ns
SMT	SMT C18	68			Horizontal polyfunctional	ns
WATERS Ltd.	Delta-Pak C18	53	300		Coated polymer	ns
WATERS Ltd.	XTerra MS C18	42	175	15.5	Monofunctional	Y

The column numbers are the same as in ref. [23].

Table 2
Specification of embedded and endcapped stationary phases

Supplier	Column	No.	Surface area ($m^2\ g^{-1}$)	Carbon content (%)	Type of bonding group
ALLTECH	Platinum C18 EPS	130	200	5	
	Alltima HP C18 Amide	131	200	12	Amide
	Prevail amide C18	132	350		Amide
	Prevail C18	133	350	15	Polar-endcapped
CLUZEAU	Stability BS-C23 ne	134	250		Ammonium-C18
	Stability BS-C23 e	135	250		Ammonium-C18
DIONEX	Acclaim Polar Advantage	136	300	17	Sulfonamide C16
DUPONT	Zorbax Bonus RP	137	180	9.5	Amide-C14
INTERCHIM	PLAP	138			Amide
MACHEREY	Nautilus C18	139	350	16	Polar-endcapped
NAGEL	Nucleosil C8 Protect	140	350	11	Polar-embedded
	Nucleodur C18 Pyramid	155	340	14	Polar-endcapped
PHENOMENEX	Synergy Hydro-RP	141	474	19	Polar-endcapped
SUPELCO	Suplex pKb	142			Amide
	ABZ	143	170	12	Amide
	ABZ+	144	170	12	Amide
	Discovery RP Amide C16	145	200	11	Amide-C16
THERMO	Aquasil C18	146	310	12	Polar-endcapped
ELECTRON	HyPurity C8 Advance	147	200	10	Polar-embedded
	HyPurity Aquastar	148	200	10	Polar-endcapped
WATERS Ltd.	XTerra RP 18	149	175	15	Carbamate-C18
	Symmetry Shield RP 18	150	340	17.6	Carbamate-C18
	Atlantis dC18	120	330	12	Unknown
VARIAN	Polaris amide C18	151			Amide
	Polaris ether C18	152			Ether
	Polaris C18 A	153			Unknown
	Polaris C 18 B	154			Unknown
YMC	YMC Pack ODS AQ	23	300	16	Polar-endcapped

The column numbers begin at 130, to take into account the column number of ref. [23].

selectivity, are calculated and used to characterize ODS phases.

Conventional and special base C18 are listed in Table 1, embedded and endcapped polar phases are listed in Table 2. Structures of the tested phases are presented in Fig. 1.

3. Results and discussions

3.1. Base deactivated C18 phases

The chromatogram of the carotenoid pigments in SubFC depends on the stationary phase properties. The absence of water in the mobile phase and the great efficiency of the separation allow the achievement of fine separations, which provide information on stationary phase properties that could not be obtained in HPLC. However, these results can support the choice of a column in HPLC, as the polar site accessibility, the phase hydrophobicity and the ability of the stationary phase to separate compounds on the basis of their special conformation is essentially related to the stationary phase nature.

Fig. 2 displays some classical separations obtained from three types of phases. On monofunctional columns (Fig. 2A and B) the zeaxanthin is eluted before the all *trans* β-carotene, because low interactions occur between the hydroxyl groups of zeaxanthin and the residual silanols of the stationary phase. Moreover, for monofunctional phases, the separation factor between the 13-*cis* and all *trans* isomers of β-carotene depends on the bonding density [24]. Fig. 2A is obtained with a low bonding density phase (TSK ODS 80 TM) when Fig. 2B is obtained with a high bonding density phase (Nucleodur 100 C18 ec).

On polyfunctional phases, such as Vydac 201 TP 54, zeaxanthin is eluted after the all *trans* β-carotene, showing the higher interactions between the hydroxyl groups of zeaxanthin and the polar sites on the stationary phase (Fig. 2C). As described in the previous paper, the greater separation factor between the 13-*cis*/all *trans* isomers is due to the polymerization of the stationary phase, which improves the shape recognition of linear and bent compounds [24]. For these kind of phases, the polymerisation is due to the bonding of one (or more) additional ODS chain on the first chain grafted on the silica. These phases are obtained using trifunctional silans in the presence of water. The ability of these vertical polyfonctional phases to distinguish planar and non planar PAH was detailed extensively by Sander and Wise [7].

Fig. 3 shows the location of numerous stationary phases on the diagram β-carotene/zeaxanthin separation factor, related to the accessibility of polar sites on the stationary surface versus

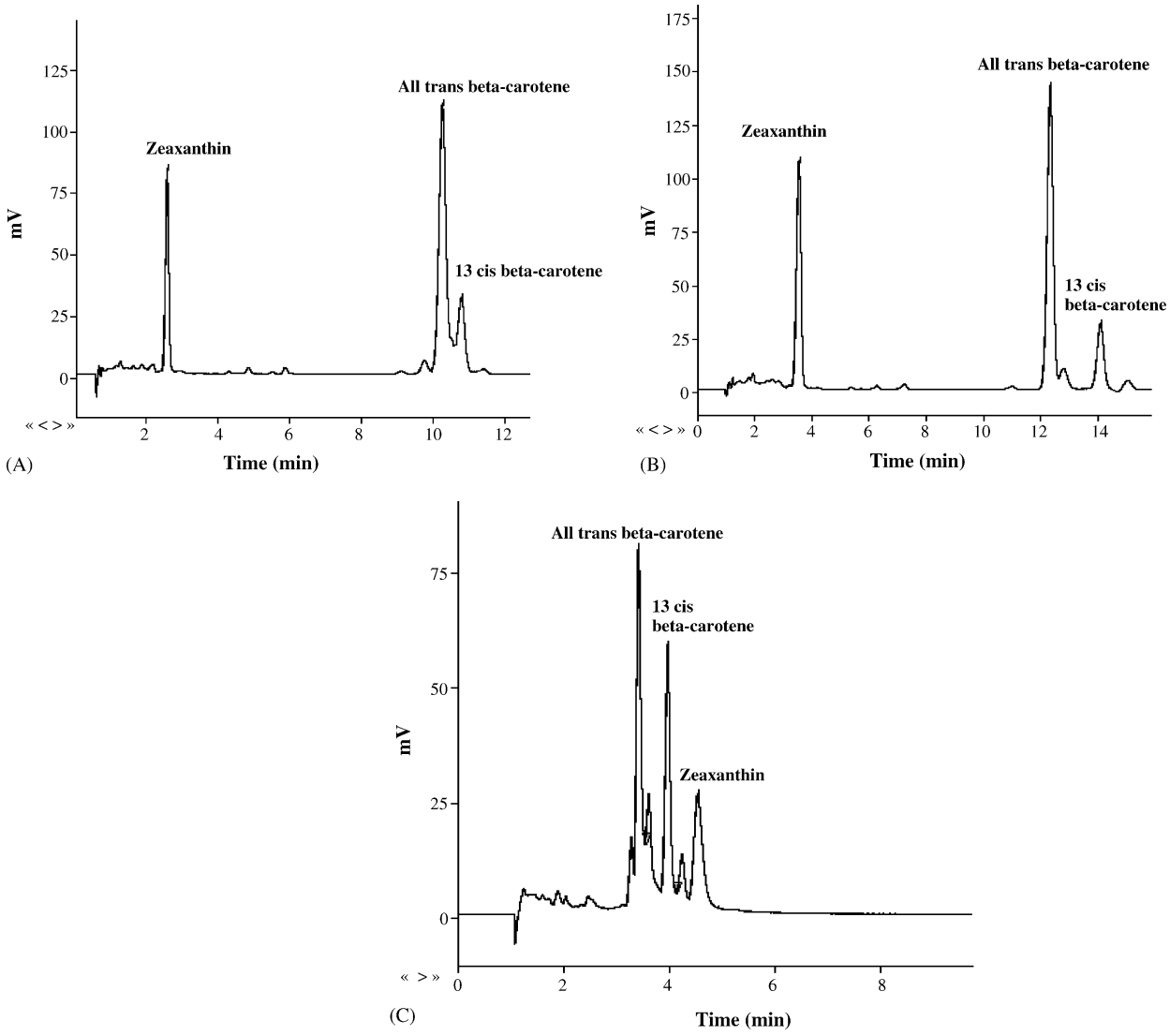

Fig. 2. Chromatograms of carotenoid pigments with different stationary phases. (A) Low bonding density monofunctional phase (α_{13} *cis*/all *trans* β-carotene = 1.05; α β-carotene/zeaxanthin = 4.9) (column: TSK ODS 80 TM); (B) high bonding density monofunctional phase (α 13 *cis*/all *trans* β-carotene = 1.16; α β-carotene/zeaxanthin = 4.46) (column: Nucleodur 100 C18 ec)); (C) polyfunctional phase (α 13 *cis*/all *trans* β-carotene = 1.25; α β-carotene/zeaxanthin = 0.65) (column: Vydac 201 TP 54).

13-*cis*/all *trans* β-carotene separation factor, related to the functionality (monofunctional or polyfunctional) of the stationary phase or to the bonding density for monomeric supports. Separation lines are plotted on this figure to indicate different areas, following the column properties, which will be discussed below.

Some of these stationary phases are used as references of classical ODS phases: Zorbax RX (#89), Kromasil (#100), Uptisphere ODB (#51), Uptisphere NEC (#27), Nucleosil 50 nec (#69) and Zorbax Eclipse XDB (#63). All these phases are monofunctional, and are located in six different parts of the classification diagram, varying in the bonding density (*x*-axis) or in the silanophilic interactions (*y*-axis).

Among the studied special base phases, three silicas studied are known to be coated by a polymer, which is bonded by C18 chains: Gammabond C18 (#5), Capcell pak (#58) and Delta Pak (#53). One of these phases, Gammabond C18, has the highest protection against silanophilic interactions.

This phase also displays a retention inversion of 13-*cis*/all *trans* β-carotene isomers, in comparison to the other phases ($\alpha_{cis/trans} < 1$). The retention order observed for Gammabond C18, first the bent isomer and secondly the linear one, is similar to the retention order of TbN/BaP reported by Sander and Wise [7]. It seems due to a stationary phase possessing slots in which compounds, such as carotenoid pigments or PAHs can be included.

The two other polymer-coated silica phases display a steric recognition identical to the one of monomeric-bonded phases with low bonding density ($1 < \alpha_{cis/trans} < 1.1$). No slots seem to be present on the stationary phase surface, and the interactions between the isomers and the stationary phases probably act only through the ODS chains bonded on the coated polymer. On the other hand, these two phases are both well covered to avoid interactions with polar compounds, as the all *trans* β-carotene/zeaxanthin separation factor is higher or equal to 10.

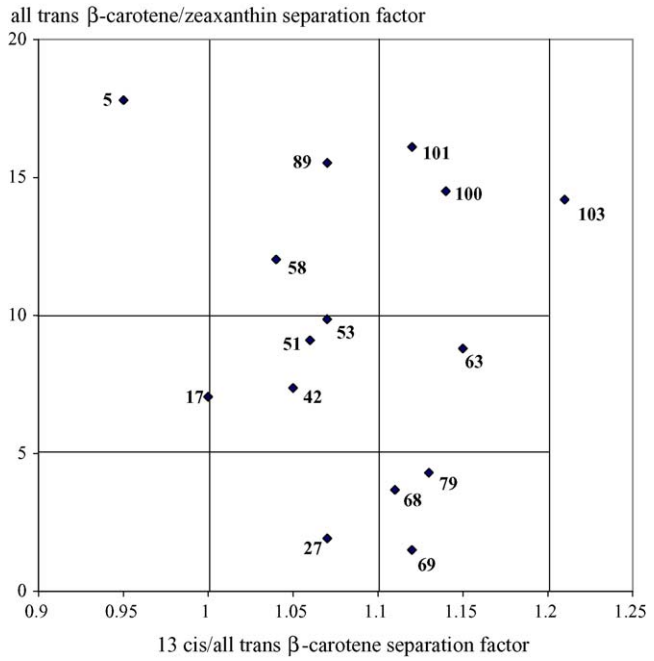

Fig. 3. Classification diagram β-carotene/zeaxanthin separation factor vs. 13 *cis*/all *trans* β-carotene separation factor for stationary phases referenced in Table 1.

The hybrid silica XTerra MS (#42) has a monomeric behaviour ($1 < \alpha_{cis/trans} < 1.1$) despite the use of a trifunctional silanizing agent. The presence of methyl groups into the hydrid silica decreases the silanol groups available on the silica surface, which is classically equal to 8 μmol/m^2 for the inorganic silica. Despite the hybrid nature of silica, the diagram shows that the interactions between the stationary phase and the polar probe are a little higher than on the two previous polymer coated phases having a monomeric behaviour ($5 < \alpha_{\beta\text{-carotene/zeaxanthin}} < 10$).

Based on the high purity of the Zorbax RX-sil silica, Zorbax Extend (#101) is a bidentate C18/C18 propylene stationary phase. Devoted first to increase the stability over high pH, it also reduces the accessibility to polar compounds, as underlined by the high value of the β-carotene/zeaxanthin separation factor. The shape recognition corresponds to a monofunctional phase with a high bonding density, such as Kromasil or Zorbax Eclipse XDB.

As it can be concluded from the *cis*/*trans* separation factor equal to 1, Zorbax SB-C18 (#17) is a purely monomeric phase. The low bonding density of Zorbax SB-C18 (2 μmol/m^2) can be explained by the steric hindrance of isopropyl side groups reducing the bonded chain density. The efficiency of this steric hindrance for reducing the interactions between polar compounds and non-endcapped residual silanols is of the same range that on silica XTerra MS hybrid silica (#42) because the values of the β-carotene/zeaxanthin separation factor are close for these two stationary phases.

The SMT silica (C18/C1) (#68), obtained by horizontal polymerization silanization method, seems not to avoid the silanophilic interactions, judging by the low value of the β-carotene/zeaxanthin separation factor, close to the one of the Chromolith C18 phase (#79).

Such behaviour of polar compounds on these "self assembled monolayer" SMT phases was also reported in HPLC [10]. On the other hand, the separation of *cis*/*trans* β-carotene isomers is around 1.1, related to a chromatographic behavior of a monomeric phase with a medium bonded density. This result is close of the one described in HPLC, by using the Sander and Wise test [11].

The Nucleosil 100 AB (#103) appears to be a polyfunctional octadecyl-bonded phase, obtained from a trichlorosilane silanizing agent in the presence of water, because the *cis*/*trans* β-carotene separation factor is higher than 1.2. It appears that the silanophilic interactions are particularly reduced on this phase, probably due to a very efficient endcapping treatment. This behaviour made this phase different in comparison to other polyfunctional C18 ones, such as Vydac [24].

On the other hand, many of these columns have close properties with conventional ODS ones [24]. Capcell Pak C18 looks like YMC Pack pro C18 or Excelsphere ODS 2 (#57 and 59 in ref. [24]), Deltapak resembles Luna C18 and Uptisphere ODB (#52 and 51 in ref. [24]), Xterra MS C18 resembles Inertsil ODS 3 and HAIsil C18 (#43 and 42 in ref. [24]), SMT C18 resembles Colosphere C18, Normasphere ODS 2 and Supersphere 100 RP 18 (# 67, 70 and 71 in ref. [24]), Zorbax Extend resembles Restek Ultra, Kromasil C18 and Omnisphere (# 99, 100 and 102 in ref. [24]), and Zorbax SB resembles Targa C18 and YMC Pack ODS-AQ (#18 and 19 in ref. [24]).

In conclusion, except the SMT phase, all these special phases display a medium (Zorbax SB, XTerra MS, Delta pak) or a low (Gammabond, Capsell pak, Zorbax Extend, and Nucleosil AB) accessibility to residual silanols. However, none of the particular treatments applied to obtain deactivated silicas produces specific retention behaviour allowing the discrimination of these phases by the carotenoid subcritical test.

Consequently, on the basis of the results obtained from the carotenoid test, it is not possible to distinguish these columns from more conventional ones.

3.2. Embedded and polar-endcapped stationary phases

Fig. 4 shows the classification of shielded or endcapped columns. The low β-carotene/zeaxanthin separation factor underlines the high interactions taking place between the polar groups of the stationary phase and the hydroxyl groups of zeaxanthin.

These embedded or polar-endcapped phases, devoted to the analysis of basic compounds, are located on the classification diagram in a very different place in comparison to polymer encapsulated or special base deactivated ones. This point is really different for tests done either in HPLC or in SubFC, because the polar interactions are suppressed with hydro-organic liquid, when they are highlighted by the subcritical conditions, because of the absence of water. The use of sub- or supercritical fluids enables this additional discrimination because the absence of water in the mobile phase, allows silanophilic interactions, which are avoided with hydro-organic mobile phases in HPLC, to occur. Consequently, one can suggest that the use of shielded phases in non-aqueous reversed phase liquid

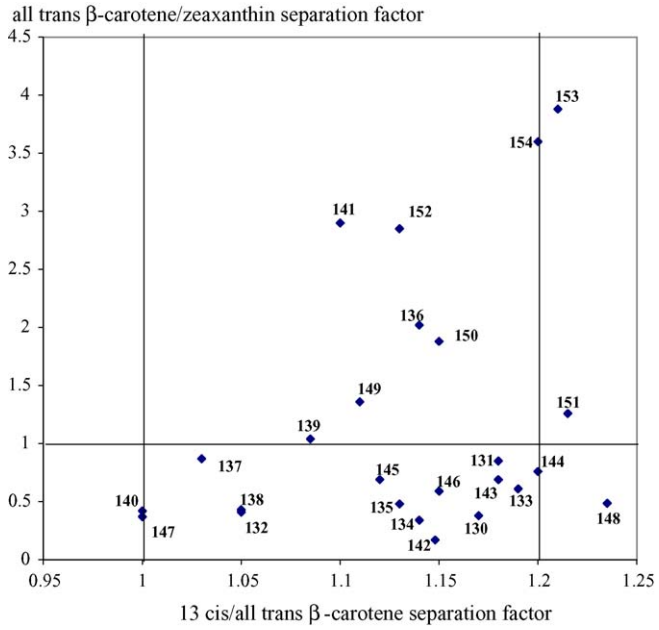

Fig. 4. Classification diagram β-carotene/zeaxanthin separation factor vs. 13 *cis*/all *trans* β-carotene separation factor for endcapped and embedded stationary phases referenced in Table 2.

chromatography (NARP-LC) would produce unusual separations.

The β-carotene/zeaxanthin separation factor values ranges from 0.17 (Suplex pKb) (#142) to 3.88 (Polaris C18 A) (#153). A value inferior to 1 underlines than the zeaxanthin is more retained than β-carotene. The values obtained for Polaris C18 A (#153) and Polaris C18 B (#154) seem too high for amide embedded phases. This is in good accordance with recent other works underlining that Polaris C18 A contains a limited degree of amide functionality [25].

On the other hand, this separation factor is higher for the ether embedded phase Polaris Ether (#152), than for the carbamate or amide groups, because of the lower polarity of an ether group. Despite one additional oxygen atom in the carbamate group, the XTerra RP 18 (#149) ($\alpha = 1.36$) and Symmetry Shield RP 18 (#150) ($\alpha = 1.88$) supports seem to have a lower ability to interact with polar compounds than the amide ones.

Recently, Engelhardt et al. [20] showed a retention inversion of butylparaben and dipropylphalate between carbamate and amide embedded phases. In the same manner, the retention order of β-carotene and zeaxanthine is reversed between amide and carbamate, except for Polaris amide C18 (#151).

In both cases, the compounds having a hydroxyl group (butylparaben and zeaxanthin) are more retained than the non-hydroxylated compounds (dipropylphalate or β-carotene) on the amide embedded phase, showing that the hydrogen-bond acceptor potential of the amide group is higher.

Amongst the amide functionalized supports, the ABZ (#143) and ABZ+ (#144) display very close properties. A decrease in the step number of the synthesis procedure was supposed between these two silicas [26,27]. The two-step procedure used for the ABZ leads to residual amino groups on the silica surface. However, the values of β-carotene/zexanthin separation factor do not show the residual amino groups. Moreover, both the elemental composition of these two phases [26] and the retention behaviour with regard to pyridinecarboxylic acids [27] are identical. Another study underlines the same ability of these two phases to interact through the amide functional group [28].

Both mono and trifunctional aminopropylsilanes can be used in the amide packing, but the 3-aminopropyltriethoxysilane used for ABZ and ABZ+ leads to a polyfunctional phase [26,27]. This stationary phase nature is assessed by their 13-*cis*/all *trans* β-carotene isomer separation factor, almost as high as the one of wide pore polyfunctional C18, which underlines their ability for shape recognition. The Prevail C18 (#133) and the Alltima HP C18 amide (#131) also have such ability.

The quaternary ammonium of the Stability BS C23 e (#134) and Stability BS C23 ne (#135) shielded phases is involved in polar interaction with as amine or carbamate groups do. It shows that electrostatic interactions can occur between permanent charges and zeaxanthin in SFC.

The endcapping treatment of this cationic phase, from BS C23 ne to BS C23 e, reduces the polar interactions but does not reverse the retention order of β-carotene and zeaxanthin, which are consequently mainly due to strong interactions between zeaxanthin and the quaternary ammonium.

As expected, the two C8 shielded phases (Hypurity Advance (#147) and Nucleosil Protect (#140)) strongly interact with polar compounds, and display no *cis*/*trans* selectivity due to the small alkyl chain length.

The five hydrophilic endcapped phases, Aquasil (#146), Prevail C18 (#133), Synergy Hydro-RP (#141), Hypurity Aquastar (#148) and Nautilus C18 (#139), display low β-carotene/zeaxanthin separation factors, that show the high interactions of endcapped polar groups with zeaxanthin.

However, the higher value obtained for Synergy Hydro-RP could underline a weaker hydrophilic endcapping treatment than on other polar-endcapped phases. Other works indicated that PCA analyses, done on polar phases, showed surprising changes in the localisation of Synergy Hydro-RP on the PC1–PC2 score plot [29].

From these studies, few differences appear between the different classes of studied columns. Hovewer, when plotting the phase hydrophobicity (retention factor of all *trans* β-carotene), versus the silanol groups accessibility (β-carotene/zeaxanthin separation factor), these columns can be classified into different groups (Fig. 5).

One group contains Prevail C18 (#133), Nautilus C18 (#139), Synergy Hydro-RP (#141), Aquasil C18 (#146) and Acclaim Polar Advantage (#136), because of the greater hydrophobicity of these polar-endcapped phases and of the sulfonamide one, in comparison to the hydrophobicity of polar-endcapped ones. Such behaviours were reported elsewhere [18,28,29].

All amide phases, are located in the same group, having both a low hydrophobicity and α β-carotene/zeaxanthin separation factor inferior to 1. The two carbamates, Xterra MS (#139) and Symmetry Shield (#140), can be distinguished from the amide groups, because of their higher β-carotene/zeaxanthin selectivity.

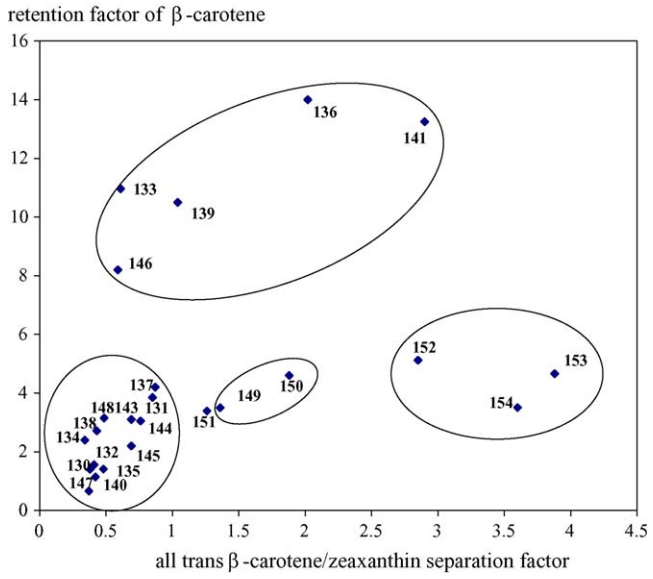

Fig. 5. Classification diagram β-carotene retention factor vs. β-carotene/zeaxanthin separation factor for endcapped and embedded stationary phases referenced in Table 2.

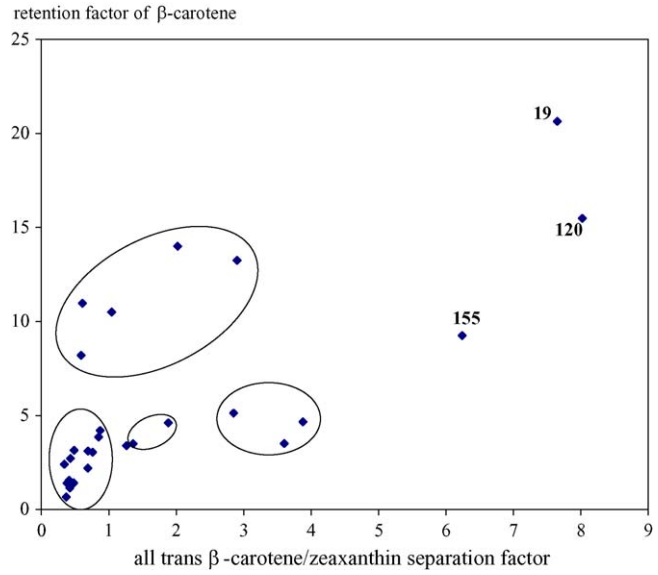

Fig. 6. Extention of the classification diagram presented in Fig. 5.

In an other study, the steric selectivity, based on triphenylene/*o*-terphenyl separation factor seems be able to distinguish polar-embedded and polar-endcapped phases[29].

In our case, Fig. 4 shows that no discrimination of these two-phase types was possible by using the steric selectivity of the carotenoid test. We reported in a previous paper that triphenylene/o-terphenyl separation factor provides poor discrimination in comparison to the TbN/BaP [7] or to the *cis*/*trans* carotenoid tests [24].

The Polaris Ether (#152) appears clearly different from polar-endcapped and polar-embedded phases, with a reduced ability to interact with polar compounds.

However, as discussed previously, one amide phase, Polaris Amide (#151), could be included into the carbamate phases group, when the Polaris A (#153) and Polaris B (#154) are located near the Polaris Ether (#152), and seem to be almost identical.

As the nature of the Polaris phases is not well known, no serious hypothesis can be done to explain their unusual chromatographic behaviour.

Other columns do not display the expected chromatographic behavior in regards to the supposed chromatographic properties: Atlantis dC18 (#120 in ref. [24]), YMC Pack ODS AQ (#19 in ref. [24]), Hypurity Aquastar (#148) and Nucleodur C18 Pyramid (#155). These phases are devoted to pure water mobile phase, suggesting that they possess polar groups in the bonded phase, either a polar endcapping or a proprietary endcapping.

On Fig. 5, the Hypurity Aquastar (#148) is located in the polar amide-embedded group, because of a weak hydrophobicity.

Surprisingly, Nucleodur C18 Pyramid, Atlantis dC18 and YMC Pack ODS AQ do not present a high retention of zeaxanthin in comparison to the retention of β-carotene (Fig. 6) (the β-carotene/zeaxanthin separation factor is higher than 5 for these three stationary phases). In this case, the hydrophilic endcapping does not seem to favour the hydrogen bonds with zeaxanthin, as it does for the other phases which are endcapped with hydrophilic groups. Moreover, the last two phases have the greatest hydrophobicity amongst the polar-endcapped phases tested in this study, showing high dispersive interactions between solutes and the alkyl chains.

It was reported elsewhere for YMC Pack ODS AQ, that the retention behaviour of polar compounds varies following the mobile phase nature (hydro-organic or purely aqueous).

Moreover, PCA analyses performed on numerous phases did not classify YMC Pack ODS AQ with other polar-endcapped phases such as Aquasil or Nautilus [29].

On the other hand, by using benzylalcohol in addition to phenol, which was inappropriate for the study of polar-embedded and polar-encapped phases, the Atlantis dC18 was included in the group of classical C18 phases [30]. With this Tanaka modified test [30], the Hypersil Aquastar also displays a different behaviour from other polar-endcapped phases due to a higher silanol activity, that could be related to the abnormal location of this phase by our carotenoid test (Fig. 5).

4. Conclusion

In conclusion, by using two classification diagrams, the carotenoid test allows to provide accurate information on the properties of the studied stationary phases.

The conclusions obtained on the phase nature in Subcritical Fluid Chromatography are often identical to the ones obtained from HPLC studies, showing the ability of this SubFC test to provide suitable conclusions on the use of these phases in HPLC.

These classifications are achieved with only one chromatographic analysis, and without the use of chemometric methods. On the basis of hydrophobicity and hydrogen bond interactions, it distinguishes polar-endcapped, amide, carbamate and ether embedded ones. Based on steric selectivity, most of the studied

embedded phases are classified as monofunctional phases with a high bonding density. Because these phases display a lower retention than classical ODS phases, the real bonding density is probably lower, and can not explain the high *cis*/*trans* selectivity observed. Consequently, one can suggest that the polar group included in the chain increases the chain rigidity that favors the *cis*/*trans* discrimination. Moreover, this steric selectivity measured by the carotenoid pigment test is not related to the type of polar group (embedded or endcapped).

Despite this satisfactory classification, two difficulties are noticed. First, these polar phases are located on the first classification diagram in the same area that conventional C18 which have a high accessibility to residual silanol groups (low values of β-carotene/zeaxanthine separation factor), which are non-endcapped phases, or phases made with type A silica.

This result is not surprising when considering the high capacity of both phase types to interact with polar compounds, but this close behaviour could prevent from clearly assessing the nature of these supports by using the carotenoid test.

Secondly, the location on the classification diagram by the carotenoid test of a sulfonamide-embedded phase in the group of hydrophilic endcapping ones, and the classification of the quaternary ammonium phases with the amide ones.

A further study using linear solvation energy relationships (LSER) will be developed to provide more accurate phase characterization.

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