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A simple subcritical chromatographic test for an extended ODS high performance liquid chromatography column classification

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Abstract

This paper describes a new test designed in subcritical fluid chromatography (SFC) to compare the commercial C18 stationary phase properties. This test provides, from a single analysis of carotenoid pigments, the absolute hydrophobicity, the silanol activity and the steric separation factor of the ODS stationary phases. Both the choice of the analytical conditions and the validation of the information obtained from the chromatographic measurements are detailed. Correlations of the carotenoid test results with results obtained from other tests (Tanaka, Engelhard, Sander and Wise) performed both in SFC and HPLC are discussed. Two separation factors, calculated from the retention of carotenoid pigments used as probe, allowed to draw a first classification diagram. Columns, which present identical chromatographic behaviors are located in the same area on this diagram. This location can be related to the stationary phase properties: endcapping treatments, bonding density, linkage functionality, specific area or silica pore diameter. From the first classification, eight groups of columns are distinguished. One group of polymer coated silica, three groups of polymeric octadecyl phases, depending on the pore size and the endcapping treatment, and four groups of monomeric stationary phases. An additional classification of the four monomeric groups allows the comparison of these stationary phases inside each group by using the total hydrophobicity. One hundred and twenty-nine columns were analysed by this simple and rapid test, which allows a comparison of columns with the aim of helping along their choice in HPLC.

Keywords: ODS stationary phases; Column classification; Hydrophobicity; Steric separation factor; Silanophilic interactions

1. Introduction

Since the introduction of modern liquid–liquid chromatography on packed columns by Kirkland [1], numerous separations have been achieved with ODS phases, owing to the easiness of use and to the well-known relationships between retention and analytical parameters (mobile phase composition, temperature).

However, even working with identical analytical conditions, the performances of stationary phases having the same chain length can vary greatly and transpositions of analytical conditions from one commercial support to another can produce very disappointing chromatograms.

In addition, minor changes in the process for preparing the silica or in the bonding conditions can decrease the reproducibility and the ruggedness of the chromatographic method [2–4]. Consequently, to reach a successful separation, it is necessary to have a better overall knowledge of the column used. Two types of methods are used to characterize stationary phases: static and dynamic [3,5].

The first ones are either non-destructive (Fourier transform infra-red spectroscopy, spectrofluorometry, mass spectrometry, microscopy, thermal analysis, thermal neutron diffusion, ^{29}Si and ^{13}C solid state nuclear magnetic resonance (NMR)) or destructive (elemental analysis, chemical degradation by hydrofluoric acid or alkaline reaction, followed by gas chromatographic analysis) [5].

The dynamic methods are based on measurement of chromatographic properties. Attempts made to establish recognised procedures involving standardized test solutes and conditions have been largely studied and reviewed [3–22]. Among the different tests, the properties mainly studied are: efficiency, hydrophobicity, steric separation factor also called shape recognition, H-bonding and ion-exchange ability. The determination of the whole properties on the basis of chromatographic

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measurements often requires the use of numerous analytical conditions (up to four mobile phase compositions). The results are sometimes difficult to use because the narrow range of their variations does not allow a clear discrimination of the studied phases and because of their apparent opposition depending on the chemical nature of the probes used [14–22].

Hydrophobicity can be measured either from absolute retention factors [12,23,24] or from the separation factor, called hydrophobic selectivity, measured from the retention of two compounds differing either by one methylene group: α_{CH_2} [12–14,17] or by one aromatic ring [12].

These tests are often performed with methanol/water mobile phases, using compounds containing at least one aromatic cycle, either alkylbenzenes or polycyclic aromatic hydrocarbons (PAHs) [12,25].

Relationships between methylene selectivity and carbon content have been reported. Linear increase in α_{CH_2} versus the carbon content is observed when using the same silica [13], but working with different silica, the hydrophobic separation factor is no longer a function of carbon content above 12% [14]. In this case, the α_{CH_2} value variations, which range from 1.45 to 1.55, can be greater between different C18 stationary phases than between RP8 and RP18 phases [14,17]. Moreover, this relationship does not depend on the functionality of the silylating agent (mono, di, or trifunctional) [13,14,17]. Besides, Sentell and Dorsey reported that from 2 to 4 μmol/m^2 of bonded chains, the methylene separation factor was unaffected by the chain order due to the increase in bonded density, as well with methanol/water as with acetonitrile/water mobile plases [25].

Furthermore, the surface area, which is involved in the hydrophobicity of the phase, is not taken into account by the methylene selectivity. Consequently, Engelhardt and Grüner recently stated that methylene selectivity did not follow the hydrophobic retention [26]. In line with this conclusion, Claessens reported that the hydrophobic selectivity (α_{CH_2}) is unable to clearly differenciate ODS stationary phases due to the small differences between numerous columns [18].

On the other hand, linear relationships are reported between the absolute retention factor of numerous compounds and the surface coverage, underlying the ability of the absolute retention factor to measure the change in carbon content between different bonded phases. This parameter depends both on the carbon content and on the specific area of the silica. The retention factors of the compounds studied vary in a larger range than methylene separation factor, for instance from 3.5 to 7.5 for amylbenzene [13] and from 2 to 6 for ethylbenzene [14].

These different points explain why the absolute retention factor is a better descriptor of column hydrophobicity than methylene selectivity for both endcapped (ec) and non-endcapped (nec) phases.

The most important interactions in RPLC are the dispersion interactions related to bonded alkyl chains. However, residual unbonded silanols are able to establish hydrogen bonding and ion-exchange interactions [6]. These additional interactions can modify the retention of polar compounds, and lead to tailing peaks, especially with basic compounds.

Depending on the pH of the partially aqueous mobile phase, the ionisation of silanols varies. Except particularly acidic sites, silanols are undissociated below pH 3 and anionic above pH 7. At neutral pH, silanols are able to create ionic interactions with protonated basic compounds (cations). It is generally accepted that isolated silanols (less than 1%) mainly are responsible for these unwanted interactions with polar solutes. Both the use of high purity silica and a full hydroxylation of silica (silica B) reduce the amount of these isolated silanols providing an improvement in the peak symmetry of basic compounds.

Because water strongly interacts with silanols, their H-bonding ability should be estimated in non-polar or non-aqueous solvents [6]. However, most of the probes used for the evaluation of these interactions are not retained in pure organic mobile phases. Both retention of neutral polar compounds (diethylphthalate [12], phenol or ethylbenzoate [14]) and relative retention (caffeine/phenol [13]) have been used to study the effect of hydrogen bonding due to the amount of residual silanols. This last test was largely used with rich water mobile phase compositions (typically MeOH–water; 30:70, v/v). Nevertheless, a good correlation between the caffeine/phenol separation factor and the hydrophobicity of phases having different bonding density was found when using the same silica (Develosil) [13], showing that the increase in bonding density reduces the H-bonding interaction. However, such relationship was not always observed with stationary phases provided by different sources [18], due to the variation of other parameters such as silica purity or silanol distribution and type.

The ion-exchange ability was measured in aqueous environment, by comparing the benzylamine/phenol separation factor at pH 2.7 and 7.6 [13], or by the measurement of the peak asymmetry of *p*-ethylaniline at pH 7 [14,15]. Contrary to the metal activity [22], ion-exchange interactions should be studied in buffered mobile phases [13,14,19].

These tests are performed with methanol-based mobile phases. Mc Calley showed by using different basic compounds that silanophilic effects were worse in acetonitrile than in methanol at neutral pH [19]. Peak asymmetry of basic compounds is reduced by a decrease of pH (from 7 to 3) [27] because silanols should be undissociated at this low pH.

However, this asymmetry factor often still remains higher than 1 at pH 3 due to remaining ion exchange sites (the 1% of more acidic silanols) on the silica surface.

Moreover, the results depend both on the size and the shape hindrance of the compounds [27,28], and are not clearly related to the pK_a of the tested solutes (ranging from 5.17 to 10.0) [28].

Recently, Claessens et al. [18] showed that there is little correlation between the Tanaka et al. [13] and the Engelhardt and Jungheim [14] tests, which are limited to the column generation type: A (containing metal impurities) or B (free of metal impurities and rehydroxylated). Moreover, opposite results are obtained when different compounds are used. Based on the Tanaka test, three columns (Kromasil C18; Inertsil ODS2; Symmetry C18) display close properties [17] whereas they are very different according to the Mc Calley study [19].

Two tests are mainly used to study the "steric selectivity" of the stationary phase. Sander and Wise [11,29–31]

demonstrated a relationship between the tetrabenzonaphtalene/benzo(a)pyrene (TBN/BaP) separation factor and the stationary phase organisation. Because of the planarity difference between these two polycyclic aromatic hydrocarbons, their retention order depends on the thickness and the bonding density of the stationary phase. Important thickness is reached when a chain polymerisation occurs during the bonding by using trichlorosilane in the presence of water, leading to polymeric stationary phases. Besides, the increase in the bonding density favors the order of the ODS chains, allowing a greater shape discrimination when the solutes penetrate into the stationary phase. For this last type of phase, called monomeric, the functionality of the chlorosilane used (mono, di or tri) is not the main factor determining the shape recognition.

Three classes of octadecyl stationary phases were discriminated with this test: polymeric, monomeric and intermediate, mainly including densely loaded monomeric phases and lightly polymerized ones. The slot model of solute insertion [29] allows to explain the retention order between the non-planar and rigid TBN and the planar BaP following the stationary phase nature.

Tanaka [13] developped another test with planar triphenylene (TRI) and non-planar *o*-terphenyl (TER). A satisfactory correlation can be observed between the two tests to discriminate polymeric C18 stationary phases from monomeric ones [18].

However, the TRI/TER test fails to distinguish between monomeric and intermediate supports and sometimes between C8 and C18 chain length [15], whereas according to the TBN/BaP test, octyl phases exhibited reduced shape separation factor [31].

Moreover, the high temperature used in the Tanaka test (40 °C) reduces the shape discrimination of the stationary phases [31]. Recently, Engelhardt et al. showed differences between the two tests with polymeric encapsulated and cholestane phases [32].

Our previous studies in subcritical fluid chromatography (SFC) have underlined the relationship between *cis*/*trans* β-carotene separations and the stationary phase nature [33,34]. We have also reported the great variations in retention of the xantophylls with the modifier content in comparison to the retention of the carotenes, due to the additional hydroxyl groups at the extremity of xanthophylls [35]. Based on these studies, preliminary experiments for checking the ability of carotenoid pigments to study the stationary phase properties were carried out on a set of twenty commercial supports [36,38].

In this paper, we describe the complete analytical conditions providing a simple and rapid test for characterization of reversed bonded phases: hydrophobicity, silanophilic interactions, shape recognition. Validation of conclusions is discussed based on the properties of classical stationary phases and by comparison with some tests used in HPLC.

The main objective of this study is to provide a classification column diagram allowing an easier comparison of the stationary phase properties. Thus, it will help the choice of ODS bonded silicas when changing the column type either to improve separation or to reduce the analysis duration but keeping the separation quality constant.

2. Experimental

Apparatus and chemicals are described elsewhere [35–38]. β-Carotene isomers were obtained by iodine isomerization [38,39]. Columns used are listed in Table 1. 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. These conditions were used in the part validation of evaluation.

The retention factors of *all trans* β-carotene (major compound of the isomer peaks), 13-*cis*- β-carotene (more intense cis-peak isomer), and zeaxanthin were determined. The, k 13-*cis*/k *all trans* β-carotene and k *all trans* β-carotene/k zeaxanthin are calculated and used to characterize ODS phases. These to separation factors were always calculated following the previous ratio, allowing to obtain values lower than 1 in the case of peak inversion.

3. Results and discussion

3.1. Test conditions

Our test mixture contains two pigments: zeaxanthin and *all trans* β-carotene (Fig. 1). In comparison to *all trans* β-carotene, zeaxanthin possesses two additional hydroxyl groups located at the cyclic extremities.

Obviously, these hydroxyl groups favour the interactions between zeaxanthin and the polar modifier of the mobile phase, but also between zeaxanthin and the polar sites on the stationary phase. However, working at constant mobile phase composition, the retention of zeaxanthin compared to that of β-carotene (relative retention) only depends on the silanol activity of the stationary phase studied.

The isomerization of *all trans* β-carotene due to the addition of iodine produces at least three mono-cis isomers, the main of which being the 13 mono-*cis*. Due to the numerous conjugated double bonds on the central chain of β-carotene (9), the compound is rigid and linear for the *all trans* conformation, or bent for the *cis* conformations. Because these compounds have similar hydrophobicity but different conformations, the separation factor between the *cis*/*trans* isomers depends on the steric or shape recognition.

Finally, the *all trans* β-carotene retention factor was selected to measure the stationary phase hydrophobicity. As discussed previously, for columns having the same bonded chain length, absolute retention depends both on the coverage density and on the specific area of the silica.

Methanol was preferred to acetonitrile as modifier for its ability to easily tune the relative retention of zeaxanthin, which carries two hydroxyl groups. Moreover, due to the better solvation of the stationary phase, leading to a more rigid an ordered chain-packing, methanol was also preferred to acetonitrile because the *cis*/*trans* separation factor, i.e. the shape recognition of the stationary phase was not depending on the methanol content from 5 to 50% [37,40–42]. Fig. 2 shows the variation of the carotenoïd retention factors versus methanol percentage in carbon dioxide. The increase in methanol dramatically decreases the zeaxan-

Table 1
List and properties of the columns used

Columns	Manufacturer	No.	Specific area ($m^2\,g^{-1}$)	Carbon content (%)	Coverage density ($mmol\,m^{-2}$)	Linkage type	Endcapping
Acclaim	DIONEX	115					
Adsorbosil	ALLTECH	28					
Adsorbosphere HS	ALLTECH	55	350	21	3.27	Monofunctional	Y
Adsorbosphere XL	ALLTECH	82	200	11		Monofunctional	Y
Alltima C18	ALLTECH	85	310	16.2			D
Alltima HP C18	ALLTECH	125	200	12			
Alltima HP C18 HL	ALLTECH	124	450	24			
Alphabond	ALLTECH	12	300	10		Monofunctional	Y
Apex C18	JONES	46	170				
Atlantis dC18	WATERS	120	330	12		Difonctional	Y
Baker C18 NP	BAKER	110	170	17.2			
Baker C18 WP	BAKER	105		7.3			
Betabasic	HYPERSIL	113	200	13			Y
Bondasorb	SFCC	25					
Brava BDS C18	ALLTECH	78	185	8.5			Y
C18 micro-bondapak	WATERS	13	330	10	1.1	Monofunctional	Y
Capcell pak C18	SHISEIDO	58				Coated polymer (CP)	
Chromegabond C22	ES Industries	30	350	22		Monofunctional	N
Chromolith C18	MERCK	79	300	17			Y
Clipeus C18	HIGGINS	47	350	18		Monofunctional	
Colosphere C18	COLOCHROM	67					
Cosmosil C18 AR II	NACALAI	122	300	17			
Cosmosil C18 MS II	NACALAI	121	300	16			
Cosmosil C18 PAQ	NACALAI	123	300	11			
Delta-Pak C18	WATERS	53	300			Coated polymer (CP)	
Develosil C18	DEVELOSIL	45	350	20	3.1		Y
Discovery C18	SUPELCO	91	200	12.5	3		
Discovery HS C18	SUPELCO	127	300	20	3.8		
Econosil	ALLTECH	29	450	15	1.74		Y
Econosphere	ALLTECH	9	200	10	2.41		Y
Exelsphere 120 C 18 H	COLOCHROM	21	300	15			Y
Exelsphere ODS 2 120	COLOCHROM	59	300	17			
Exsil ODS	SGE	75					
Gammabond C18	ES Industries	5				Coated polymer (CP)	
Gemini C18	PHENOMENEX	128	390				
Genesis C18	JONES	54	300		3.2		Y
HAIsil C18	HIGGINS	41	190	12		Monofunctional	Y
HAIsil HL C18	HIGGINS	98	300	18		Monofunctional	Y
Hydrosphere C18	YMC	4	340	12			
Hypersil 100 C18	TSP-SHANDON	49	300	16			
Hypersil BDS	TSP-SHANDON	90	170	11.1	3.6		Y
Hypersil Elite	TSP-SHANDON	96	250	15			Y
Hypersil Gold	TSP-SHANDON	126					
Hypersil Green-PAH	TSP-SHANDON	35	170	13.5			Y
Hypersil HyPurity	TSP-SHANDON	92	200	13		Monofunctional	Y
Hypersil ODS	TSP-SHANDON	48	170	9.5	2.8		Y
Hypersil PAH	TSP-SHANDON	32	170	13.5			Y
Inertsil ODS 2	GL SCIENCE	95	320	18		Monofunctional	Y
Inertsil ODS 3	GL SCIENCE	43	450	15			Y
Kromasil C18	EKA NOBEL	100	350	21.4	3.3	Monofunctional	Y
Lichrosorb RP 18	MERCK	10	300	18			N
Lichrospher 100 RP 18	MERCK	74	350	18			N
Lichrospher 100 RP 18 e	MERCK	88	350	21			Y
Lichrospher LC-PAH	MERCK	34	200	20			N
Luna C18(2)	PHENOMENEX	52	440	19	3		Y
NormasphereODS 2	COLOCHROM	70	450	21			Y
Nova-Pak C18	WATERS	84	120	7	2.7		Y
Nucleodur 100 C18 ec	Macherey-Nagel	117	340	17.5			
Nucleodur Gravity C18	Macherey-Nagel	118	340	18			
Nucleosil 100 C18	Macherey-Nagel	37	350	14		Monofunctional	Y
Nucleosil 100 C18 HD	Macherey-Nagel	97	350	20	3.6	Monofunctional	Y

Table 1 (*Continued*)

Columns	Manufacturer	No.	Specific area ($m^2\ g^{-1}$)	Carbon content (%)	Coverage density ($mmol\ m^{-2}$)	Linkage type	Endcapping
Nucleosil 100 C18 PAH	Macherey-Nagel	33	350				N
Nucleosil 300 C18	Macherey-Nagel	83	100	6.5			Y
Nucleosil 5 C18 AB	Macherey-Nagel	103	350	25		Polyfunctional	Y
Nucleosil 50 C18	Macherey-Nagel	69	450	14		Monofunctional	N
Nucleosil 50 C18 ec	Macherey-Nagel	73	450	14.5		Monofunctional	Y
Omnisphere	VARIAN	102	350	20	3.5		Y
Partisil ODS 1	WHATMANN	6	350	4.7	0.6		Y
Partisil ODS 2	WHATMANN	31	350	17.3	2.4		N
Partisil ODS 3	WHATMANN	8	350	10.7	1.4		Y
PE CR C18	PERKIN	40					
Platinum C18	ALLTECH	24	200	6		Monofunctional	Y
Prosphere C18 300 Å	ALLTECH	106	100	9		Polyfunctional	Y
Purospher 100 RP 18	MERCK	72	350	18			N
Purospher 100 RP 18 e	MERCK	86	350	21	3.2		Y
Purospher star RP18e	MERCK	114					Y
Pursuit C18	VARIAN	119					
RES-ELUT 5C18	VARIAN	11					
Resolve C18	WATERS	39	200	10	2.8		N
Restek Allure C18	RESTEK	61		27			
Restek Ultra C18	RESTEK	99		20			
Satisfaction RP 18 AB	CLUZEAU	62	320	17		Monofunctional	Y
Separon C18	TESSEK	26					N
Separon C18 ec	TESSEK	38					Y
SGE-250 GL4 P-C18	SGE	2				Coated polymer (CP)	
SMT C18	SMT	68					
Spheri-5 ODS	BROWNLEE	80	180	14			Y
Spherisorb ODSB	WATERS	66	220	12	2.72	Monofunctional	Y
Spherisorb ODS 1	WATERS	36	220	7	1.7	Monofunctional	N
Spherisorb ODS 2	WATERS	76	220	12	2.6		Y
Stability ODS 2	CLUZEAU	81	320	15		Monofunctional	N
Supelcosil LC-18	SUPELCO	44	170	11	3.1		
Supelcosil LC-18 DB	SUPELCO	56	170	11			Y
Supelcosil LC-18S	SUPELCO	50	170	11			
Supelcosil LC-18T	SUPELCO	93	170	12.3			
Supersphere 100 RP 18	MERCK	71	350	18	3.6		N
Supersphere 100 RP 18 e	MERCK	94	350	22	4.1		Y
Symmetry C18	WATERS	87	330	19.4	3.2		Y
Synchropak C18	EICHROM	16					
Synergy Fusion RP	PHENOMENEX	129	475				
Targa C18	HIGGINS	18	330	16		Monofunctional	
TSK ODS 80TS	TOSO-HASS	111		15			Y
TSK ODS 120T	TOSO-HASS	77	200	22			Y
TSK ODS 120A	TOSO-HASS	112		22			N
TSK ODS 80TM	TOSO-HASS	15		15			Y
Ultrasphere ODS	BECKMANN	60	200	12	3.5	Monofunctional	
Ultrasphere XL ODS	BECKMANN	65	250	12			Y
Unisphere C18	INTERCHIM	1					
Uptisphere HDO	INTERCHIM	20	320	18		Monofunctional	Y
Uptisphere HSC	INTERCHIM	64	310	20			Y
Uptisphere OBD nec	INTERCHIM	27	320	16		Monofunctional	N
Uptisphere ODB	INTERCHIM	51	320	17		Monofunctional	Y
Uptisphere TF	INTERCHIM	116	310				
Vydac 201 HS	GRACE Vydac	23	450	13.5	1.53	Monofunctional	Y
Vydac 201 TP 300 Å	GRACE Vydac	109	90	8		Polyfunctional	
Vydac 202 TP 300 Å	GRACE Vydac	104	90			Polyfunctional	
Vydac 218 MR 300 Å	GRACE Vydac	108	90				
Vydac 218 TP 300 Å	GRACE Vydac	107	90	8		Polyfunctional	
Vydac 238 TP 300 Å	GRACE Vydac	14	90			Monofunctional	
Wakosil C18 RS	SGE	22	350	17		Monofunctional	Y
XTerra MS C18	WATERS	42	175	15.5	2.2	Trifunctional	Y
YMC Pack ODS-AQ	YMC	19	300	14.6			
YMC Pack ProC18	YMC	57	340	17			Y
Zorbax 300 SB C18	DUPONT	3					

Table 1 (*Continued*)

Columns	Manufacturer	No.	Specific area ($m^2\ g^{-1}$)	Carbon content (%)	Coverage density ($mmol\ m^{-2}$)	Linkage type	Endcapping
Zorbax Eclipse XDB	DUPONT	63	180	10.3	3.5	DiMeC18	D
Zorbax Extend	DUPONT	101	185	12.1		Bidentate	
Zorbax ODS	DUPONT	7	330	20	3.5		Y
Zorbax RX-C18	DUPONT	89	180	12		DiMeC18	N
Zorbax SB C18	DUPONT	17	180	10		DiBuC18	N

The numbers, from 1 to 129, correspond to the tested columns located on Figs. 6–10.

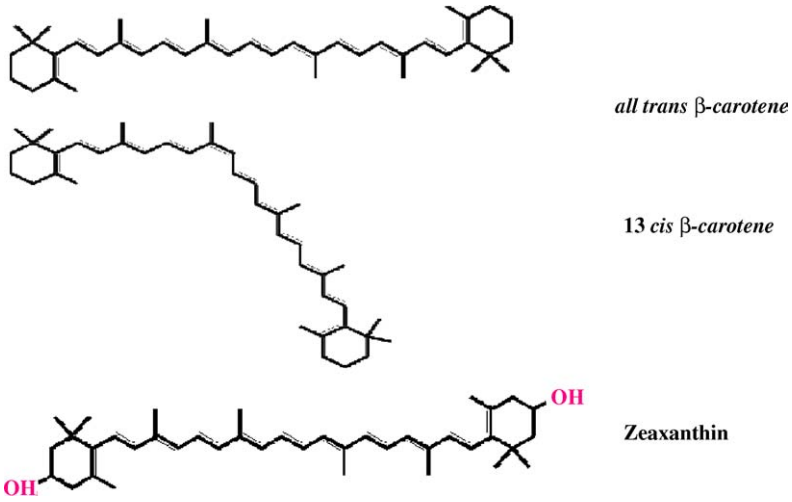

Fig. 1. Structures of the carotenoid pigments used for the chromatographic test.

thin retention factor, whereas the ones of the β-carotene isomers range in a more narrow area. The greater retention of the more polar compound (zeaxanthin) with low methanol shows strong interactions between polar sites of the stationary phase and zeaxanthin. Because of the strong regular decrease in the zeaxanthin retention when increasing the methanol content, a retention inversion between zeaxanthin and β-carotene isomers occurs between 15 and 25% of methanol in carbon dioxide with polymeric C18 stationary phases (Vydac 201 TP).

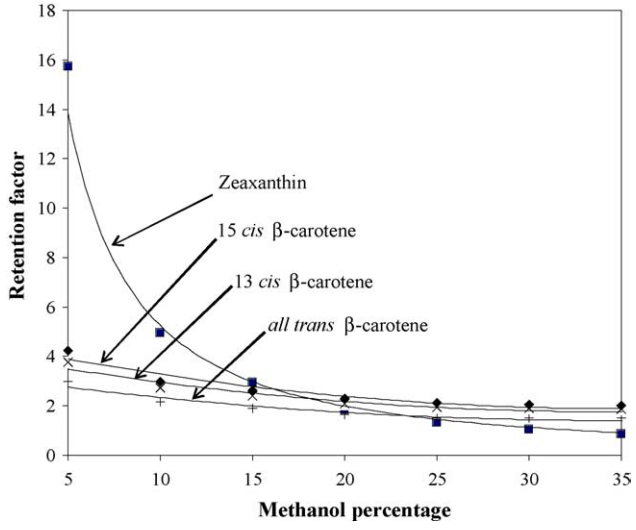

Fig. 2. Variation of carotenoid retention factor vs. methanol content in the carbon dioxide mobile phase stationary phase: Vydac 201 TP 54; outlet pressure: 15 MPa; $T = 25\,^{\circ}C$; flow rate: 3 ml/min.

A final content of 15% of methanol was selected due to the ability of this mobile phase to elute, on polymeric C18 stationary phases, all the isomers of β-carotene before zeaxanthin in a reduced analysis time.

Moreover, because the shape recognition is increased by decreasing the analytical temperature [42,43], the carotenoid test temperature was set at 25 °C, below the critical temperature. On the other hand, pressure was set up at 15 MPa to increase the mobile phase density. In these conditions, the density of the subcritical fluid is close to that of a liquid, and the density variations, due to changes in the flow resistance between the different columns, do not significantly modify the role of silanol groups [44], or the shape recognition [42].

3.2. Test validation

3.2.1. Silanol activity

Due to the inability of carbon dioxide to establish H-bonding interactions with the silica, supercritical fluid chromatography has been successfully employed in the investigation of the packing material activity [44,45]. For instance, correlations between the silanols group concentration of silica and the peak shape of phenol were reported in SFC [44]. On the other hand, using identical silica (Develosil) with different bonding density, Tanaka reported that the increase in the retention of butylbenzoate in normal phase liquid chromatography mode was related to the

Table 2
Comparison of *all trans* β-carotene/zeaxanthin separation factor (A) and hydroxyl group number per nm^2 on Partisil stationary phases (B)

Column	A	B
Partisil ODS 1	0.178	2.73
Partisil ODS 2	0.511	1.75
Partisil ODS 3	1.21	1.05

amount of silanol on the silica surface [13]. This result underlines that the silanol activity was also observed on bonded silica with non-aqueous mobile phases. Consequently, silanol activity could be studied with supercritical fluid.

Table 2 shows the *all trans* β-carotene/zeaxanthin separation factor and the silanols group concentration values for three Partisil supports, one of them being endcapped (ODS 3).

The increase in the silanol group number per nm^2 from ODS 3 (1.05) to ODS 1 (2.75) strongly modifies the *all trans* β-carotene/zeaxanthin separation factor because of the change in the elution order of the two compounds. The higher the silanol group concentration, the higher the zeaxanthin retention. On ODS 1 and ODS 2 zeaxanthin is eluted after *all trans* β-carotene, showing the strong interactions between the hydroxyl groups of zeaxanthin and residual silanols of these two phases. Such inversion of retention related to the silanols amount was also reported between caffeine and phenol in LC [13].

Besides, the *all trans* β-carotene/zeaxanthin separation factor of non-endcapped stationary phases was compared to that of endcapped ones (Fig. 3).

First, no decrease in the retention factor of the hydrophobic compound (*all trans* β-carotene) was observed between non-endcapped and endcapped phases, as it is sometimes observed when the endcapping treatment is carried out above 300 °C [46], due to hydrolysis of the bonding.

For two phases, Separon and Nucleosil, the endcapping treatment does not strongly increase the measured separation factor, showing the weak effectiveness of the treatment used on these phases. However, for the four other phases (Lichrospher, Superspher, Purospher, and Uptisphere), the endcapping treatment induces a dramatic improvement of the *all trans* β-carotene/zeaxanthin separation factor.

Except for Purospher, this improvement is not related to the increase in *all trans* β-carotene retention factor. Consequently, this enhancement is mainly caused by a decrease in retention of zeaxanthin. The large decrease in the concentration of residual silanols onto the endcapped phases reduces the H-bond interactions between zeaxanthin and silanols.

These two studies show that, as expected, the *all trans* β-carotene/zeaxanthin separation factor is able to measure the accessibility to silanol groups on the silica surface, and will be used as a silanophilic activity descriptor.

3.2.2. Steric selectivity

Two preliminary investigations were done, first by testing the correlation of the TBN/BaP separation factor obtained both in high performance liquid chromatograpy and in subcritical fluid chromatography, and secondly between the 13-*cis*/*all trans* β-carotene and the TBN/BaP separation factor in SubFC. We choose to compare the *cis*/*trans* β-carotene separation factor to TBN/BaP separation factor rather than comparing it to the triphenylene/*o*-terphenyl separation factor because of the greater discrimination reached by the Sander and Wise test.

Fig. 4 shows the good correlation of the TBN/BaP test between HPLC and SubFC. An identical classification of stationary phases in polymeric, intermediate and monomeric is obtained in SubFC. It is worthy to note a decrease of the separation factor values in SubFC. The TBN/BaP separation factor was then compared to the 13-*cis*/*all trans* β-carotene separation factor (Fig. 5). A satisfactory correlation was observed between the two separation factors.

Among about thirty columns, few differences were observed: the Nova-Pak C18 column being classified as a monomeric by the TBN/BaP test [29] and intermediate by the 13-*cis*/*all trans* β-carotene one, and the Partisil ODS 1 being classified as

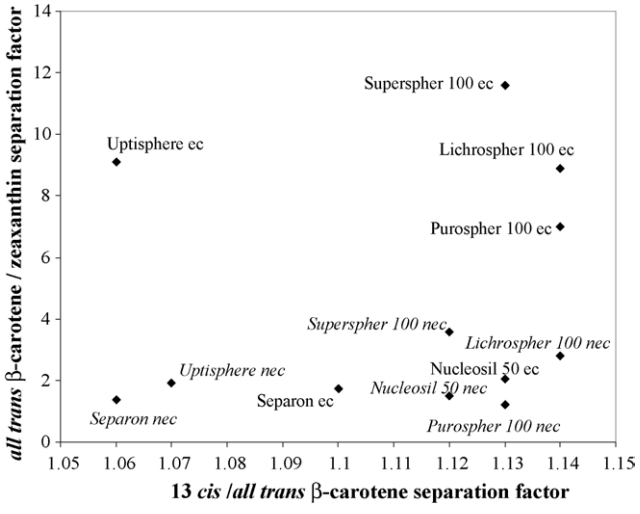

Fig. 3. Plot of the *all trans* β-carotene/zeaxanthin separation factor vs. the 13-*cis*/*all trans* β-carotene separation factor for endcapped (ec) and non-endcapped (nec) stationary phases.

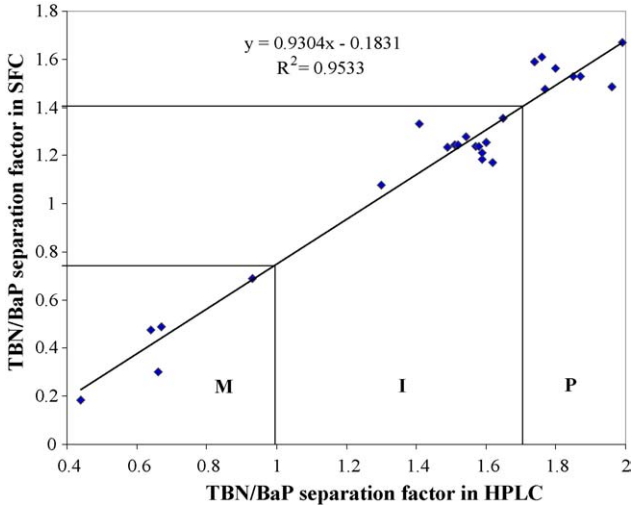

Fig. 4. Plot of the relationship between SubFC and HPLC retention factors of TbN/BaP (M: monomeric; I: Intermediate; P: polymeric).

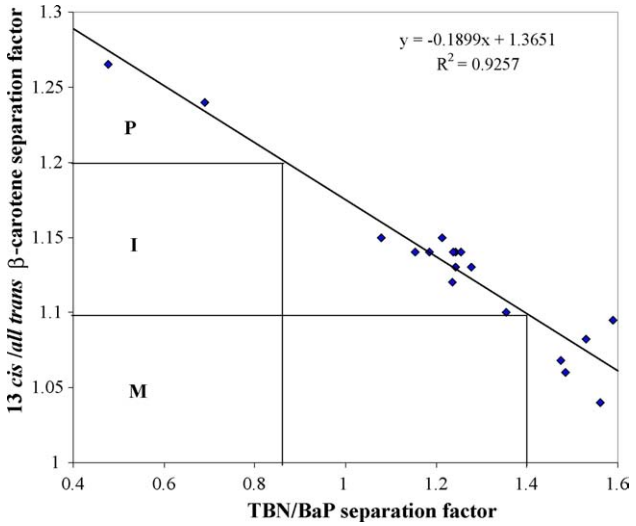

Fig. 5. Plot of the relationship between 13-*cis*/*all trans* β-carotene and TbN/BaP selectivities in SubFC (M: monomeric; I: Intermediate; P: polymeric).

intermediate by the TBN/BaP [29] test and monomeric by the 13-*cis*/*all trans* β-carotene one.

Since the classification of ODS stationary phase is almost identical by using these two tests, it proves that the 13-*cis*/*all trans* β-carotene separation factor is well suited for the evaluation of reversed phase shape recognition related to the bonding density and to the functionality of the stationary phases.

3.2.3. Hydrophobicity

To assess the choice of this hydrocarbonous pigment as a relevant hydrophobicity probe, a comparative classification of different stationary phases was carried out (Table 3), based either on the *all trans* β-carotene or on the amylbenzene (Tanaka test), or the ethylbenzene retention factor (Engelhardt test) [data in ref. 18].

Few inversions are observed in the three classifications showing that the results are very close and provide similar patterns of hydrophobic column classification. Moreover, the increase in the β-carotene retention factor is related to the increase in the carbon content of the three Partisil ODS phases: ODS 1 (4.7%; $k = 1.25$), ODS 3 (10.7%; $k = 6.5$) and ODS 2 17.3%; $k = 9.3$). A similar increase in retention has been reported with the PAHs included in the test mixture SRM 869 with these Partisil phases [30].

Table 3
Comparison of column hydrophobicity from different tests

Columns	A	B	C	D
Hypersil Hypurity	1	1	1	1
Hypersil ODS	2	2	2	2
Zorbax RX	3	3	4	4
Nucleosil 100-5 HD	4	4	3	3
Symmetry C18	5	6	5	5
Purosphere RP 18 e	6	7	8	6
Kromasil	7	8	7	7
Eclipse XDB	8	5	6	9
Alltima	9	9	9	8

A: Engelhardt test (ref. [18]); B: Tanaka test (ref. [18]); C: ref. [46]; D: our work.

3.3. Classification diagram

3.3.1. Description of the results

A diagram is plotted by combining the two separation factors, *all trans* β-carotene/zeaxanthin and 13-*cis*/*all trans* β-carotene, which allows a first classification of the tested columns (Fig. 6). The accessibility to polar sites is related to the *all trans* β-carotene/zeaxanthin separation factor, plotted on *Y*-axis. The higher this separation factor, the lower the interactions of zeaxanthin with polar sites.

On the *X*-axis, the 13-*cis*/*trans* β-carotene separation factor allows to classify four main types of apparent bonded phase organisation: polymer coated silica minor to 1, monomeric with low bonding density from 1 to 1.1; intermediate monomeric with high bonding density from 1.1 to 1.2 and polymeric above 1.2. The location of the stationary phases on this diagram can be related to their polar site accessibility and to their shape recognition. By combining these two selectivities, eight groups of columns can be distinguished from this diagram.

Six columns (#1, 2, 3, 4, 5 and 129) display a retention inversion between the 13-*cis* and *all trans* β-carotene. In fact, all the *cis* isomers elute before the *all trans* β-carotene that is in agreement with the slot model of Sander and Wise (the non-linear compounds do not penetrate into the slots of the stationary phase as easily as the linear compounds). Among these phases, at least two (#2, 5) are polymer-coated silica.

The monomeric columns have a 13-*cis*/*all trans* separation factor ranking from 1 to 1.19, and the *all trans* β-carotene/zeaxanthin separation factor from 0 to 20. Among all the monomeric columns, the Ultrasphere XL ODS (#65) displays the highest protection against silanophilic interactions and the Hypersil Hypurity (#92) provides the highest 13-*cis*/*all trans* β-carotene separation factor.

Old silica generations (Zorbax ODS (#7), Partisil ODS 1 (#6) have great silanophilic interactions, as reported elsewhere. Their 13-*cis*/*all trans* separation factor is equal to one, indicating monomeric stationary phases with low bonding density, allowing the accessibility of polar compounds to residual silanols. Other type A supports such as Lichrosorb RP 18 (#10), Partisil ODS 3 (#8) and μ-Bondapak (#13) are also monomeric with low bonded density, but seem a little bit better protected due either to the use of difunctional silylating agent (Lichrosorb C18) or to endcapping treatment (Partisil ODS 3). The lack of full rehydroxylation of this silica type could explain the weak protection against polar interactions.

Recent phases also display 13-*cis*/*all trans* separation factor equal to one, but with a lower accessibility to residual silanols: Wakosil C18 RS (#22); Targa C18 (#18), Zorbax Stablebond C18 (SB) (#17), and YMC-Pack ODS AQ (#19), Uptisphere HDO (#20), Exelsphere 120 C18 H (#21), Atlantis dC18 (#), Synchropak C18 (#16), Vydac 238 TP 300A°(#14), Gemini C18 (#129).

Moreover, the isomer separation observed with these phases is unusual, because of the 9 cis isomer is eluted before the all

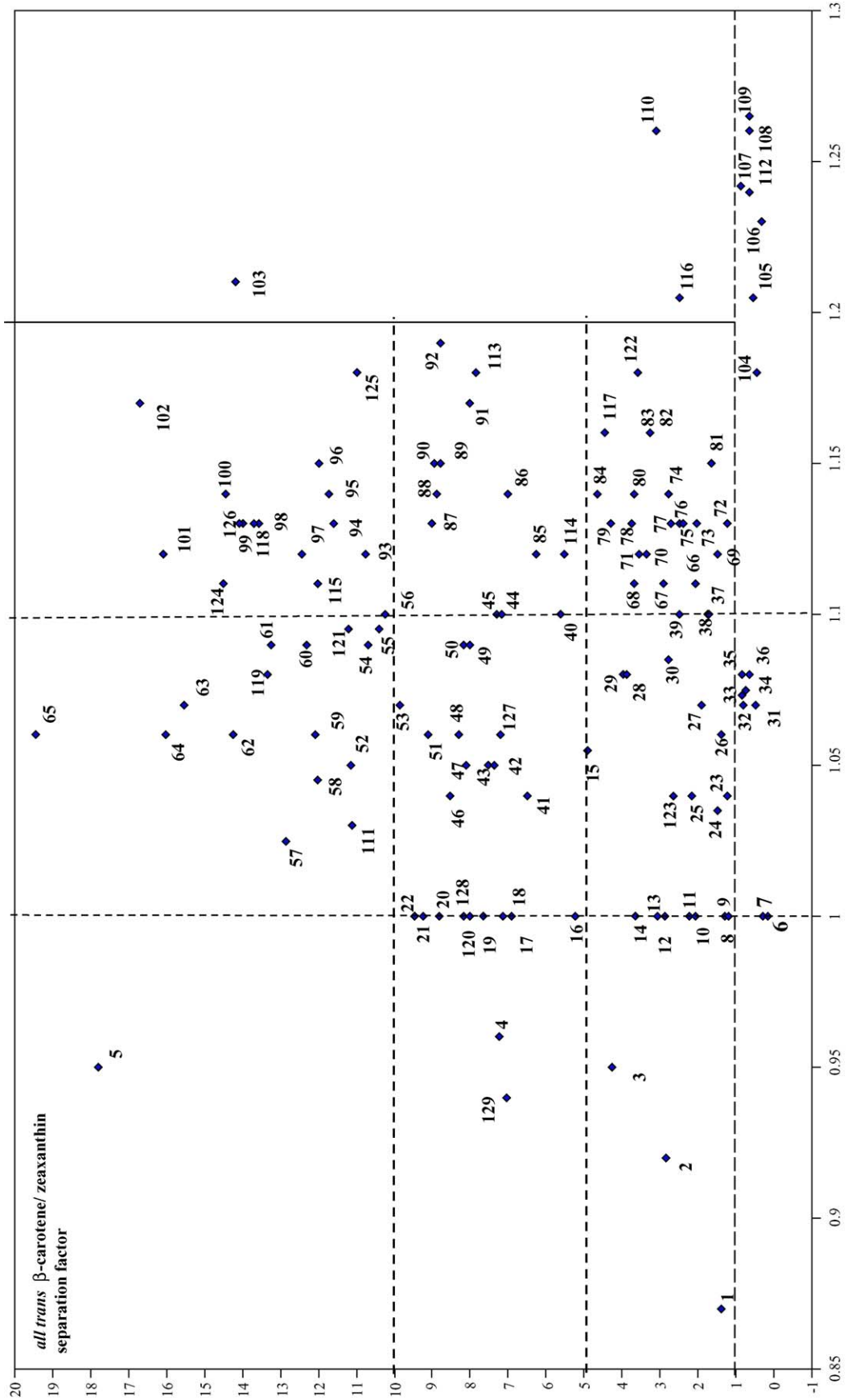

Fig. 6. Classification diagram of columns.

trans β-carotene, when 9-*cis* isomer elutes with the *all trans* β-carotene for all other monomeric phases. This particular separation could be provided by special stationary phase organisation, such as chain rigidity. One can remark that one of these phase (#17) is sterically protected by lateral isopropyl chain.

For other monomeric stationary phases, the increase in the *cis*/*trans* separation factor, ranging from 1.025 to 1.19, is related to an increase in the apparent bonding density, which favours the separation between the 13-*cis*/*all trans* β-carotene isomers, i.e. the shape recognition.

However, this increase in the bonding density can not be correlated to a decrease in the polar site accessibility, as would be expected from the Dorsey and Dill model with stationary phases having a surface coverage above 3 μmol/m^2 [47]. Besides, other parameters such as surface area and pore diameter are able to change the shape recognition, i.e. the apparent bonding density.

Some of the stationary phases which have a medium accessibility (from 1 to 5 for *all trans* β-carotene/zeaxanthin separation factor) are non-endcapped such as: Uptisphere ODB n-ec (#27), Nucleosil 50 C18 (#69), Separon C18 (#38), Resolve C18 (#39), Supersphere 100 RP18 (#71), Lichrospher 100 RP18 (#74), Purospher 100 RP18 (#72), explaining their ability to interact with polar compounds, whatever their bonding density. Others are endcapped, but have low carbon content (from 5 to 8%) such as: Platinum C18 (#24), Brava BDS (#78), Nova-Pak C18 (#84).

Among the phases displaying low accessibility to residual silanols (*all trans* β-carotene/zeaxanthin separation factor ranging from 5 to 10), the encapped versions of the previous ones can be found, such as: Uptisphere ODB (#51), Lichrospher (#88) and Purospher (#86).

Some of these stationary phases are considered as fully endcapped packing: Develosil C18 (#45), Symmetry C18 (#87), Hypersil BDS (#90) and Hypersil HyPurity (#92), but at least one of these packing is non-endcapped and based on high-purity silica : Zorbax RX C18 (#89).

Other monomeric columns show a very low accessibility to polar compounds (*all trans* β-carotene/zeaxanthin separation factor ranging from 10 to 20). Several have both a high surface coverage (above 3 μmol/m^2) and endcapping treatment.

Based on high purity silica, they are often called “base desactivated” or “special base” because they are especially devoted to the analysis of basic compounds.

The classical columns are: Kromasil C18 (#100), Zorbax Eclipse XDB (#63), Nucleosil 100 C18 HD (#97), Luna C18(2) (#52), Supelcosil LC-18 DB (#56), Hypersil Elite (#96), Inertsil ODS-2 (#95), Ultrasphere ODS (#60) and XL ODS (#65).

New supports have been developped corresponding to these criteria: Omnisphere C18 (#102), Restek Ultra C18 (#99), HAIsil HL C18 (#98), Satisfaction RP 18 AB (#62), Restek Allure C18 (#61), Exelsphere ODS 2 (#59), Genesis C18 (#54), YMC-Pack Pro C18 (#57), Nucleodur Gravity C18 (#118), Pursuit C18 (#119).

All the silica which present a 13-*cis*/*all trans* isomer separation factor higher than 1.2 are polymeric supports: Vydac 201 TP (#109); 218 TP (#107), 218 MR (#108), Prosphere C18 (#106), Baker C18 WP (#105), TSK OD S 120A (#112).

Moreover, the order of elution of zeaxanthin and β-carotene on these phases is opposite to that observed on the other stationary phases, zeaxanthin being more retained than β-carotene. This inversion of elution order shows the great accessibility to polar sites on these stationary phases, despite the coverage of the silica by the polymeric bonded phase.

Consequently, Vydac 202 TP (#104) can also be considered as a polymeric stationary phase even if the 13-*cis*/*all trans* separation factor is only equal to 1.19.

This kind of stationary phase is obtained by using trifunctional silylating reagents, in the presence of water traces, leading to the bonding of more than one octadecyl chain from one surface silanol, through condensation reaction [11]. The shape recognition of polymeric stationary phases has been extensively studied [11,29–31], and their ability to separate 13-*cis*/*all trans* isomers of β-carotene previously discussed. However, other stationary phases, described as polymeric ones by the TbN/BaP test [29], do not display such high 13-*cis*/*all trans* β-carotene separation factor: Hypersil Green PAH (#35); Lichrospher LC-PAH (#34); Nucleosil C18 PAH (#33); Spherisorb ODS1 (#36) Hypersil PAH (#32) and Partisil ODS 2 (#31). A number of these phases are especially devoted to the PAH separation as indicated by their name.

The 13-*cis*/*all trans* β-carotene separation factor difference between these two polymeric column types seems due to the pore diameter, equal to 300 Å for the first ones and around 100 Å for the second ones. Sander and Wise reported an increase in the shape separation factor related to the increase in the pore diameter for polymeric stationary phases, whereas little difference was observed for the monomeric ones [48].

Consequently, despite the identical functionality of the bonded phase, these two types of polymeric phases, according to the carotenoid test, do not have an identical behaviour regarding shape recognition.

Finally, three polymeric columns have a lower silanol activity: Uptishere TF (#116), Nucleosil 100 C18 AB (#103) and Baker C18 NP (#110). The reduced silanol activity can be due to chemically cross-linked C18 modification, or additional endcapping treatment.

3.3.2. Hydrophobicity of monomeric C18 columns

Due to their low carbon content, the polymeric C18 phases display a weak hydrophobicity, when the hydophobicity of monomeric phases strongly varies. To study these variations, monomeric columns were classified into four groups, in which columns have both close shape recognition and silanophilic interactions.

The columns in group 1 are monomeric with a low bonding density (13-*cis*/*all trans* β-carotene separation factor ranges from 1.0 to 1.1) and a medium accessibility to residual silanols (*all trans* β-carotene/zeaxanthin separation factor ranges from 1 to 5) (Fig. 7).

This medium accessibility to residual silanols was expected on Resolve C18 (#39), Uptisphere ODB nec (#27), Separon C18 (#26), Nucleosil 100 (#37) which are not endcapped. The low carbon content (6%), associated to a low specific surface area (200 m^2/g), of platinum C18 (#24) induces a low apparent bond-

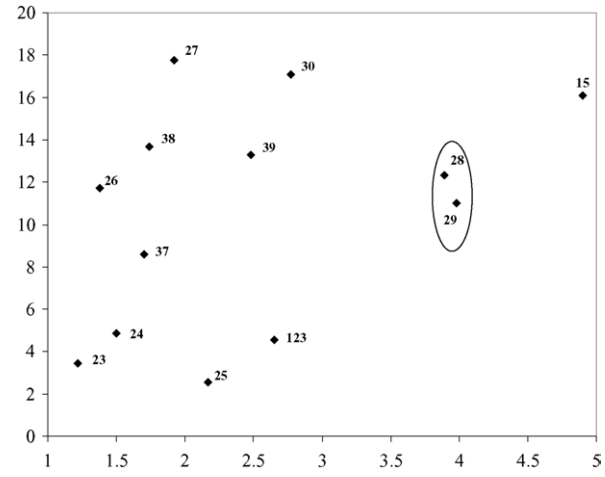

Fig. 7. Retention factor of *all trans* β-carotene vs. *all trans* β-carotene/zeaxanthin separation factor for columns of Group 1.

ing density which explains the polar site accessibility. Vydac 201 HS (#23) and Bondasorb C18 (#25) have the lowest hydrophobicity in this group. This seems to be surprising for Vydac 201 HS because its carbon content and its surface area are twice those of Platinum C18. However, the bonded phase coverage remains low (1.53 μmol/m^2).

Despite different bonding technology reported by the supplier, two columns in this group have identical chromatographic behaviour, Adsorbosil (#28) and Econosil (#29), with both the same specific surface (450 m^2/g) and the same carbon content (15%).

The columns in group 2 are monomeric with a high bonding density (13-*cis*/*all trans* β-carotene separation factor ranges from 1.1 to 1.2), with the same accessibility to residual silanol as columns of group 1 (*all trans* β-carotene/zeaxanthin separation factor ranges from 1 to 5) (Fig. 8).

The low hydrophobicity of some columns can be explained by a surface area around 200 m^2/g leading to a final carbon content ranging from 7 to 12%: Adsorbosphere XL (#82), Spherisorb ODS 2 (#76), Exsil ODS (#75), Brava BDS (#78), Nova-Pak C18 (#84), TSK 120 TM (#77).

The Exsil ODS (#75), the TSK ODS 120 (#77) are quite similar to Spherisorb columns, as well for the silanophilic interactions as for hydrophobicity.

Other columns have a higher hydrophobicity due both to great surface area, from 350 m^2/g (Lichrospher (#74), Superspher (#71)) to 450 m^2/g (Nucleosil 50 (#69), Normasphere ODS 2 (#70)) and higher carbon content, from 14 (Nucleosil 50) to 21% (Normasphere ODS2).

Numerous couples of columns have close properties: Adsorbosphere XL (#82) and Nucleosil 300-5 C18 (#83); Spheri-5 ODS (#80), Brava BDS (#78) and Cosmosil C18 AR II (#122); Colosphere 18 (#67) and Lichrosphere C18 (#74); Stability ODS 2 (#81) and Nucleosil 50 C18 (#69).

One monolithic silica is also included in these phases: Chromolith RP 18e (#79). Classically in HPLC, this silica rod column is compared, in terms of separation factor, to the Purospher 100 RP 18e (#86) [49].

If the shape recognition on these two silicas is very close, the results show that the accessibility of polar compounds to the monolithic silica surface is twice to that measured on the Purospher one.

The columns in group 3 are monomeric with a low bonding density (13-*cis*/*all trans* β-carotene separation factor ranges from 1.0 to 1.1) and a low accessibility to residual silanols (*all trans* β-carotene/zeaxanthin separation factor ranges from 5 to 20) (Fig. 9)

One of these columns has the highest hydrophobicity of all columns tested: Uptisphere HSC (#64). In comparison to the Uptisphere ODB ec (#51) having the same surface area, the apparent bonding density remains low (1.06) whereas the silanophilic interactions are reduced. Consequently, this great hydrophobicity seems rather due to a stronger endcapping treatment than to an increase in the bonding density of the ODS chains.

Two other columns also display a high hydrophobicity and a close chromatographic behavior: YMC-Pack Pro C18 (#57) and Restek Allure (#61).

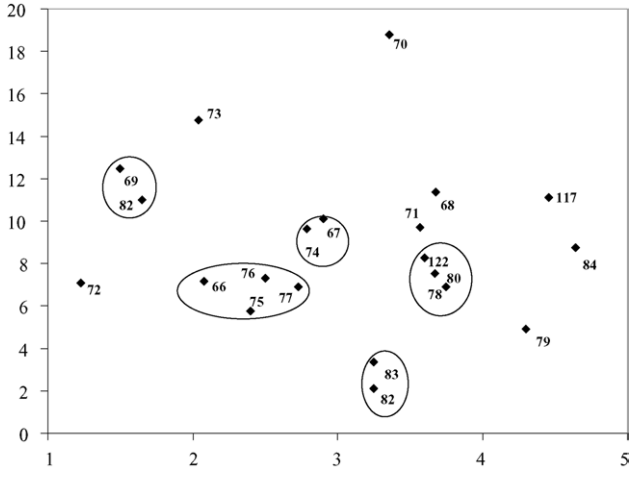

Fig. 8. Retention factor of *all trans* β-carotene vs. *all trans* β-carotene/zeaxanthin separation factor for columns of Group 2.

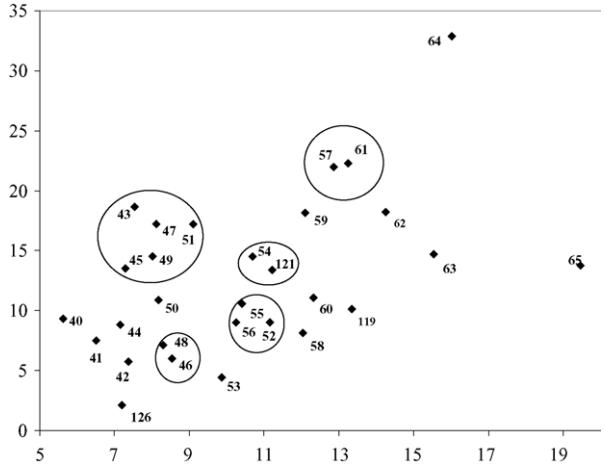

Fig. 9. Retention factor of all trans β-carotene vs. *all trans* β-carotene/zeaxanthin separation factor for columns of Group 3.

For Clipeus C18 (#47), Inertsil ODS 3 (#43), Hypersil 100 C18 (#49), Develosil C18 (#45) and Uptisphere ODB (#51) the high hydrophobicity seems rather due to a great surface area (from 350 to 450 m^2/g) than to a high bonding density on the silica surface. On the base of the studied performances, these columns could be interchangeable.

Hypersil ODS (#48) and Apex C18 (#46) have the same surface area (170 m^2/g), and identical chromatographic properties, when Genesis C18 (#54) and Cosmosil C18 MS (#121) have the same surface area and probably close carbon content.

However, it seems surprising that Supelcosil LC 18 DB (#56) having both a low surface area (170 m^2/g) and a low carbon content (11%) should be close to Luna C18(2) (#52) and Adsorbosphere HS (#55) having high carbon contents (around 20%) and greater surface areas.

Capcell Pak (#58) and Delta pak (#53) made by polymer encapsulation, present a low silanophilic interaction ability, with regard to their low hydrophobicity. The encapsulation of these columns seems to be efficient to avoid silanophilic interaction, but does not induce an inversion in the retention of *cis*/*trans* isomers as other polymer coated phases do (#2 and 5). The better shielding of silanols by polymeric coated stationary phases was also assessed by the study of the retention order between ethylbenzoate and toluene [14].

As reported previously, among all monomeric columns the lowest ability to interact with polar compounds is reached by Ultrasphere ODS XL (#65), despite a weak apparent bonding density (13 *cis*/*all trans* separation factor = 1.06).

The columns in group 4 are monomeric with a high bonding density (13-*cis*/*all trans* β-carotene separation factor ranges from 1.1 to 1.2) and a medium or low accessibility to residual silanols (*all trans* β-carotene/zeaxanthin separation factor ranges from 5 to 20) (Fig. 10)

Most of these phases are made from high-purity silica (type B), explaining why they display low silanophilic interactions. The silicas, having a surface area ranging from 170 to 200 m^2/g (Discovery C18 (#91), Hypersil HyPurity (#92), Zorbax RX (#89), Hypersil BDS (#90), Supelcosil LC 18T (#93)) display a lower hydrophibicity than those having a surface area greater than 300 or 350 m^2/g (Lichrospher RP 18e (#88), Superspher RP 18e (#94), Alltima C18 (#85), Symmetry C18 (#87), Omnisphere C18 (#102), and Kromasil C18 (#100)).

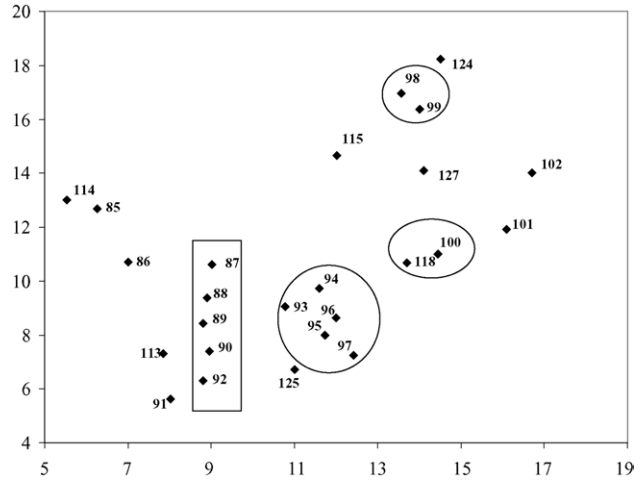

Fig. 10. Retention factor of *all trans* β-carotene vs. *all trans* β-carotene/zeaxanthin separation factor for columns of Group 4.

Nucleodur Gravity C18 (#118) looks like Kromasil C18 (#100) which is often chosen as a reference material.

Numerous columns have close chromatographic properties: Supelcosil LC-18 T (#93), Superspher 100 RP 18e (#94), Inertsil ODS 2 (#95), Hypersil Elite (#96), Nucleosil 100 C18 HD (#97) and Alltima HP C18 (#125), despite their different carbon content and specific area.

Nucleosil 100 C18 HD (#97) displays a low accessibility to polar sites in regards of its hydrophobicity. However, given its carbon content (20%), its hydrophobicity is low in comparison to the carbon content of Discovery C18 (#91) (12.5%), Hypersil HyPurity (#92) (13%), Hypersil BDS (#90) (11.1) or Betabasic C18 (#113) (13%), which have a close Hydrophobicity.

Moreover, despite this high carbon content, the bonding density of Nucleosil 100 C18 HD is lower (13-*cis*/*all trans* separation factor = 1.12) than the one of the previous phases. A great part of the carbon content of Nucleosil 100 C18 HD could be provided by the special base deactivated treatment, which strongly reduces the silanol accessibility, increasing neither hydrophobicity nor the bonding density.

Haisil HL C18 (#98), Restek Ultra C18 (#99) and Alltima HP C18 HL (#124) are the most hydrophobic supports in this group, when Omnisphere (#102) and Zorbax Extend (#101) have the lowest silanol accessibility.

A column can be replaced by another keeping one property constant. For instance, between Hypersil Hypurity (#92), Hypersil BDS (#90), Zorbax RX C18 (#89), Lichrospher 100 RP 18e (#88), Symmetry C18 (#87), the accessibility to polar sites is nearly the same, while the hydrophobicity increases from Hypersil Hypurity to Symmetry. In this range of retention factors (from 6 to 11), and following the Purnell equation, this increase favours the resolution.

On the other hand, by keeping the retention factor of apolar compounds quite constant, the use of Zorbax Extend (#101), or Kromasil (#100), or Nucleodur Gravity C18 (#118), or Purospher RP 18e (#86), or Symmetry C18 (#87) or Superspher 100 RP 18e (#94) will change the retention of polar solutes as the *all trans* β-carotene/zeaxanthin separation factor ranges from 7 to 16 showing the decrease in the polar compound retention.

Such methods can be used to choose or replace a column in any group of monomeric column.

Moreover, column of group 1 and 2 can be selected if the aim is to favour retention of polar compounds, when column of group 3 and 4 could be preferred to avoid silanophilic interactions of basic compounds. Besides, high steric recognition will be favored by columns of group 2 and 4, which display higher bonding density.

3.4. Comparison with other tests

Two points should be discussed when working on column classification:

(1) How relevant is the used test to measure hydrophobicity, silanol activity and steric separation factor?
(2) Do the calculation methods (PCA or ranking with *F*-value) performed provide better classification than simple and direct comparison (radar plots, classification diagrams).

Concerning the first question, the hydrophicity study based on the carotenoid test in SFC displays almost identical classification to the ones obtained from the retention of all classical compounds analysed in HPLC (amylbenzene).

About the silanol activity, the variations of the SFC separation factor between *all trans* β-carotene/zeaxanthin (ranging from 0.3 to 20) are larger than the one of caffeine/phenol, or benzylaniline/phenol at pH 7 or 2.6 often used in HPLC [49].

This important range observed in SFC favours a direct comparison of column properties, without the use of chemometric methods.

Nevertheless, we compared our results to those obtained in refs. [17,49–52], because of the large data set available from these references. Applied on more than fifty columns (data in refs. [49,51,52]), identical conclusions on silanol activity were drawn from *all trans* β-carotene/zeaxanthin separation factor and both caffeine/phenol or benzylamide/phenol selectivities at pH 7.6. However, no correlation appears between *all trans* β-carotene/zeaxanthin separation factor and benzylamide/phenol separation factor at pH 2.7.

Consequently, due to results gained from the comparison of endcapped and non-endcapped stationary phases, our test is rather related to hydrogen-bond ability rather than ionic interactions. Indeed, protonation of zeaxanthin might not occur in CO_2/MeOH subcritical phase, contrary to protonation of amines in HPLC at neutral pH. The ionic interactions can probably not be evaluated by the carotenoid test.

However, few differences appear in PCA classification when using separation factor at pH 7.6 or at pH 2.7. This shows the difficulty to clearly identify the part of the ionic and hydrogen bonding interactions even in HPLC.

For studying the steric selectivity, our previous comparison with the data of Sander and Wise [29] shows that the range of the 13-*cis*/*all trans* separation factor between 1 and 1.2 is sufficient to allow a accurate classification on the basis of stationary phase apparent functionality. No satisfactory correlation was found between our results and the triphenylene/o-terphenyl separation factor proposed by Tanaka and coworkers [13], mainly because no polymeric phases were included in the set we used for this comparison (Euerby data, ref. [49]), and also because the triphenylene/o-terphenyl separation factor does not describe differences for monomeric stationary phases [15,53].

The second question concerning the use of a calculation method is uneasy, but general trends can be drawn.

PCA analyses are required for the selection of the more relevant parameters from a large set of parameters [50–52]. However, the same authors concluded that the definition of column groups based on PCA plot was difficult [54]. Besides, whatever the nature of the descriptors retained, PCA analyses use at least three or four chromatographic measurements (retention factors, selectivities).

It allows to distinguish C18 endcapped/non-endcapped columns or the silica type (A or B), alkyl polar embedded ones, and C8 stationary phases (which obviously display a lower hydrophobicity than C18) [17,47–50,54,55]. In regard of the numerous classification trials performed to select the most pertinent descriptors of stationary phase properties, the resulting discrimination seems a little bit disappointing.

Visky et al. [50,55], by using four chromatographic parameters, obtained with three analytical conditions, classified columns in six groups: Ia, Ib, IIa, IIb, IIc, III. By comparison with our classification, we agree with some conclusions such as:

(1) The lower hydrophobicity of columns in group Ib compared to columns in group Ia.
(2) The greater silanol activity of columns in groups IIa and IIb.
(3) Columns having a *all trans* β-carotene/zeaxanthin separation factor higher than 5 are classified into groups Ia and Ib.

However, the classification obtained by PCA is not always very clear and does not allow a fine discrimination:

(1) Group 1a is supposed to contain only type B silica, but at least 4 type B silica are also found in group 1b.
(2) According to the carotenoid test, YMC Hydrosphere, Wakosil RS, Zorbax SB, Uptisphere HDO (group Ia) display special steric recognition that can not be estimated by the TER/TRI separation factor of the Tanaka test.
(3) On the other hand, no significant difference in the silanol activity appears from the carotenoid test between Hypersil ODS (group IIc), Supelcosil LC-18 (group IIb), Luna C18(2) and Uptisphere ODB (group Ia).

Moreover, columns not classified in the same PCA group do perform identical separation of acetylsalicylic acid and its impurities (Kromasil, ACE C18, Spherisorb ODS2), when columns classified in the same group do not provide the same separation performance eon this separation (ACE C18 and Hypersil Elite) [55].

The column ranking is an other way to classify the columns [54,56–58]. This method is based on the results obtained for a reference column. Then, a *F* (or *F**) factor is calculated from the differences of five test values between the reference column and a column i. The *F*-value of the reference column is equal to 0. The smaller the *F*-value, the closer the chromatographic behaviour of the column compared to the selected reference. Such columns can be exchanged because they provide identical separations.

However, it is difficult to determine the cut-off *F*-value, from which the columns are really different. On the basis of the hydrophobic subtraction model used for reversed phase columns, the *F*-values obtained for C8 and type B silica C18 do not allow a clear classification of these phases, when Hydrophobicity is really different between C8 and C18 chains [58]. Consequently, analyses performed on different samples display close separations for most of the studied phases despite their very varied *F*-values [57]. One hypothesis to explain this lack of dis-

crimination is related to the term used for the hydrophobicity evaluation (**H**). This term is obtained by a $\log k$–$\log k$ plot of chosen compounds on a reference column and the tested column. **H** is the slope of this plot. Consequently, in the same manner as the methylene separation factor, **H** does not take into account the phase ratio of the columns, i.e. the surface area or the bonding density differences between two C18 columns. Both criteria (α CH_2, **H**) are not satisfactory parameters to measure the hydrophobicity of a column, as their values vary in a narrow range between different C18 bonded phases.

On the other hand, the steric hindrance term (**S**) is different from the shape selectivity studied by Tanaka or Sander and Wise, and neither related to the first nor to the second known tests. The relevance of the **S** value is not warranted at this time.

The use of an additional criterion such as the chromatographic response function (CRF) is often required. This CRF-value, determined on a chosen separation, varies from 0 (no separation) to 1(baseline separation). A classification was done on 59 columns, by using the *F* parameter calculated from the Tanaka test experiments. Results show that columns having a *F*-value equal to 10.087 (Lichrospher RP 18), and classified at the rank 52, have a CRF-value equal to 1, meaning that despite its apparently very different properties, Lichrosphere RP 18 was able to perform the same separation as the reference column [56].

In our case, the use of classification diagrams, selecting the column family on two complementary separation factors (steric recognition and silanol accessibility) allows to select easily the columns having close separation properties.

The second diagram, displayed for monomeric stationary phases, can be used to choose the hydrophobicity. An increase of hydrophobicity can enhance the resolution thanks to the higher retention factors. A decrease of hydrophobicity favors the reduction of the analysis time, and of solvent consumption.

4. Conclusions

Results obtained on 129 columns allowing a rational classification of the stationary phases are obtained without the use of chemometric or ranking treatments. This simple subcritical test allows the measurement of the main column properties: hydrophobicity, silanol accessibility and shape recognition. Based on the three measurements done from one carotenoid pigments analysis, the tested columns can easily be compared by using two classification diagrams. These diagrams make easier the choice of the most appropriate stationary phase in HPLC, in regard to the compound structural differences.

General trends of the silica treatment effect clearly appear, supported by targeted investigations and the knowledge of numerous classical phases. However, as reported by numerous other works, it is impossible to explain all the differences between columns because of the diversity of silica treatments before and after the linkage of the C18 chains, and the combination of these treatments.

Moreover, the results show that different treatments can lead to close chromatographic behaviour, not only on the residual silanol separation factor, but also on Hydrophobicity and steric recognition.

Whatever the bonding chemistry, column having close chromatographic behaviours are located in the same area of these diagrams, and can be exchanged without great changes in mobile phase conditions. On another hand, the change of chromatographic properties requires the use of a column clearly located in another part of the classification diagram plotted from two selectivities.

The results obtained on special stationary phases such as embedded or hydrophilic endcapped ones will be discussed in a further paper.

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