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Intergroup cross-comparison for the evaluation of data-interchangeability from various chromatographic tests

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ABSTRACT

The recently dramatic increase in the available choices of reversed-phase columns could be an advantage of this mode of separation. However, due to the insufficiency of available information in terms of the exact functionality of these phases and the similarities and differences between these newly introduced and conventional reversed-phase columns, it is now somehow problematic to determine which could be the best column for a given analytical problem. There is no single column that will give us a good separation for all applications. As a result, there have been several attempts to develop testing strategies to characterize column chemistries. In this study three of the most widely used and acceptable approaches for the characterization of reversed-phase columns, which are Tanaka, United States Pharmacopeia (USP), and Snyder–Dolan, are systemically applied to investigate the chromatographic properties of calixarene- and resorcinarene-bonded stationary phases, polar-embedded and polar-endcapped stationary phases, phenyl and ether-linked phenyl with the presence of conventional alkyl-bonded phases (octyl- and octadecylsilane). Although all column classification systems aim to evaluate "more or less" the same characteristics, each system uses different test mixtures in different chromatographic conditions. It is therefore very important to evaluate the similarities and differences in the resulted "column parameters" and the possible interchangeability of them. The results of this comparative study show that the used parameters of Tanaka and of Snyder–Dolan have in many cases a good to very good correlation. The USP approach, which is based on single run, is related to Tanaka and Snyder–Dolan only in terms of hydrophobic characters, and no relation could establish in the other parameters. The hydrophobic-subtraction model could be extended to describe the ligand–solute interactions of calixarene- and resorcinarene-bonded stationary phases, which are belonging to reversed phase material. However they show, depending on the analytes, some additional interactions, since their steric, polar and ionic properties are different compared to those of conventional alkyl-bonded phases.

1. Introduction

High-performance liquid chromatography (HPLC) has become, and still is, the technique of choice for chemical analysis in biochemistry, toxicology, environmental and pharmaceutical analysis, polymer and food chemistry, and many other areas, probably due to the broad applicability to separate, analyze, and/or purify compounds from the low up to the very high molecular weight range in almost any sample matrix [1,2].

The most widely used mode in HPLC is by far reversed-phase chromatography (RPC) based on its versatility comparing to other HPLC modes such as normal-phase chromatography (NPC), hydrophilic interaction liquid chromatography (HILIC), which is becoming a well-accepted alternative to reversed-phase chromatography for polar analytes, ion-exchange chromatography (IEC) or size exclusion chromatography (SEC) [3,4].

A recent survey on HPLC columns shows clearly that C_{18} phases (octadecylsilane) were most popular followed by C_8 (octylsilane) [4]. However, there is a need, for new column chemistries. Many types of stationary phases are gaining popularity, such as the polar-embedded and the polar-endcapped stationary phases. The alkyl group could be substituted by other functional groups, which gives the so-called embedded-polar-group (EPG) phases; these phases are interesting due to their compatibility with high aqueous containing mobile phases, their reduced silanol interactions and unique selectivity. The polar-endcapped phases contain a polar functional group as an endcapping agent, which are, however, usually not defined by the manufacturer. These polar or hydrophilic endcapping chemicals allow the silica surface to be wetted with water and enable the full interaction with the longer alkyl chains, making the retention of polar analytes under highly aqueous conditions more reproducible [3,5–7].

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Phenyl bonded phases have been utilized in reversed-phase HPLC for many years [8]. These phenyl phases have been reported to exhibit π–π as well as steric recognition interactions [9]. The phase may also contain a heteroatom between the spacer and the phenyl ring, which could maximize retention and selectivity for polar and aromatic analytes. A popular example is an ether-linked phenyl phase (e.g. Synergi Polar RP), which also possess polar endcapping.

Macrocycle molecules bonded stationary phases based on supramolecular interaction have been receiving also much attention due to their special separation selectivity. The most useful property of calixarenes is their ability to function as molecular baskets which can engulf small neutral or ionic guests. Calix[n]arenes, which are macrocyclic compounds composed of phenol units linked by methylene bridges at positions ortho to the hydroxyl groups, have also been found to be useful in HPLC. However, calixarene bonded stationary phases are preferable comparing to the use of calixarene as additives to the mobile phase, as the interference of the strong UV absorption of the calixarenes in the mobile phase is undesirable. Furthermore, poor solubility of most calixarenes precludes an application as additives in aqueous eluents. Meyer and Jira reviewed and summarized the application possibilities and interactions of calixarenes as a stationary phase in liquid chromatography [10]. These novel LC stationary phases, which could have an enhanced selectivity for some molecules, are providing new tools to solve classical problems in pharmaceutical analysis [11–13].

After all of that, the continuously increasing number of commercially available HPLC stationary phases makes the selection of a suitable column for a specific analytical problem very difficult. On the other hand, the available chromatographic information supplied from manufacturers regarding their phases is not always useful, and comparisons between different manufacturers data are not always possible because of differences in testing conditions [3]. Consequently, the interest in the development of testing approaches for the characterization of the properties of chromatographic phases continues to be of interest, and that has prompted many researchers to try to develop a universal test method, which is capable to make proper column selection [14–34]. Column characterization methods are typically based on one or more test mixtures to identify the so called “column parameters”. These methods should be therefore able to identify the different interactions between the solute and the stationary phase. Despite the central role of hydrophobic interactions in RPC, other aspects such as electrostatic, hydrogen bonds and dipole–dipole interactions should be tested to have a complete overview.

Till now, there is unfortunately no universally accepted chromatographic test as a uniform approach for RPC phases. However, the chosen approaches are between the most widely used and acceptable methods, namely: Tanaka, United States Pharmacopeia (USP), which uses the Standard Reference Material (SRM) 870 mixture, and Snyder–Dolan.

A few studies in the literature tried to compare different characterization methods at the same time for the same group of stationary phases [1,35].

Claessens et al. [1] tested approximately 20 silica, alumina and polymer based C_8 and C_{18} columns using the following methods: Engelhardt [17,22], Tanaka [16], Galushko [18] and Walters [15]. This comparison showed clearly that both hydrophobicity and hydrophobic selectivity data of the tested phases were highly correlated. In contrast, silanol activity results are generally not in agreement and not interchangeable between the different characterization methods.

Sokoließ et al. [35] have compared the hydrophobic and steric properties of calixarene- and resorcinarene-bonded stationary phases using different approaches (Engelhardt [22], Walters [15], Tanaka [16], Olsen [19], Goldberg [14] and Neue [23]). This study, nevertheless, does not include any comparison of silanol activity data, which is critical and has a little correlation in other works [1].

Recently, Borges et al. [36] applied different classical chromatographic tests to stationary phases prepared by thermal immobilization on poly(methyloctylsiloxane) (PMOS) onto silica. The applied tests were: Engelhardt [22], Tanaka [16] and the USP (SRM 870) [26]. However, the only difference between the used stationary phases was the amount of PMOS immobilized onto the silica surface. These classical chromatographic tests showed high correlation between the retention factors of neutral and basic test solutes, except for poorly retained solutes such as aniline and phenol in the Engelhardt test; benzylamine in acidic mobile phase; phenol and caffeine in the Tanaka’s test. No cross-comparison to the data from the tests with each other was done. Moreover, the use of very similar stationary phases to compare chromatographic tests does not enable the evaluation of similarities and differences between the tests.

The aim of this paper is to spread the spectrum of the testing columns while comparing very different characterization methods, using calixarene- and resorcinarene-bonded stationary phases and some other relatively new RPC phases with the presence of conventional alkyl-bonded phases. It is also very important to evaluate the interchangeability of the resulted parameters from very different methods.

To our knowledge, this is the first systematic study, which includes an evaluation and comparison of Snyder–Dolan approach with other “classical” methods for the same group of columns. On the other hand, calixarene- and resorcinarene-bonded stationary phases will be characterized for the first time using the Snyder–Dolan approach.

2. Materials and methods

2.1. Reagents and chemicals

Thiourea, phenol, amitriptyline, triphenylene and *o*-terphenyl of analytical grade were purchased from Sigma–Aldrich (Steinheim, Germany). Toluene, ethylbenzene, *n*-butylbenzene, *n*-pentylbenzene, *trans*-chalcone and *n*-butylbenzoic acid were purchased from Alfa Aesar (Karlsruhe, Germany). Quinizarin (1,4-dihydroxyanthraquinone), berberine and anisole were obtained from Acros Organics (Geel–Belgium). Benzonitrile, benzylamine were from Merck (Darmstadt, Germany). *N*,*N*-diethylacetamide and caffeine from Merck Schuchardt OHG (Hohenbrunn, Germany). Acetophenone were purchased from Fluka (Buchs, Switzerland).

Phosphoric acid (85%), potassium hydroxide (85%) and potassium dihydrogen phosphate (99.5%) were obtained from Merck (Darmstadt, Germany).

Acetonitrile and methanol HiPerSolv CHROMANORM® for HPLC gradient grade were purchased from VWR International (Leuven, Belgium). Water was obtained by bi-distillation.

2.2. Equipment

Chromatography was performed on a Shimadzu Prominence equipped with on-line degassing unit (DGU-20A), two solvent delivery units (LC-20AD) with low pressure gradient unit, autosampler with cooling function (SIL-20AC), column oven (CTO-20A), photo-diode Array detector (SPD-M20A) and system controller (CBM-20A) (Shimadzu Europe, Duisburg, Germany). The dwell volume was 360 μl.

The pH-value of the solutions was adjusted with a Knick pH-meter (Berlin, Germany).

Table 1
List of the investigated stationary phases and their properties.

	No.	Abbr.	Stationary phase	Dimensions (mm)	Particle size (μm)	Pore size (Å)	Ligand type	Source	Fs (Krm)
Alkyl-bonded phases	1	Krm	Kromasil C_{18}	125 × 4.6	5	100	Octadecyl	VDS optilab	–
	2	LiCh	LiChrosorb RP_{18}	125 × 4.6	5	100	Octadecyl	VDS optilab	32.9
	3	Sph	Spherisorb ODS-2	125 × 4.6	5	80	Octadecyl	VDS optilab	79.9
	4	Lun	Luna	150 × 4.6	5	100	Octadecyl	Phenomenex	4.0
	5	Sym	Symmetry C_{18}	150 × 3	5	100	Octadecyl	Waters	9.8
	6	XT	XTerra MS C_{18}	150 × 3	5	125	Octadecyl	Waters	13.0
	7	XB	XBridge C_{18}	150 × 3	5	135	Octadecyl	Waters	8.7
	8	AQ	Prontosil C_{18}-AQ	125 × 4.6	5	120	Octadecyl (with polar end-capping)	Bischoff	9.7
	9	EPS	Prontosil C_{18}-ace-EPS	150 × 3	5	120	Octadecyl (with an embedded amide group)	Bischoff	54.5
	10	EC8	Eclipse XDB-C_8	150 × 4.6	5	80	Octyl	Agilent	7.6
Aromatic-RP columns	11	PhH	Luna Phenyl-Hexyl	150 × 4.6	5	100	Hexyl linked phenyl	Phenomenex	23.4
	12	Syn	Synergi Polar-RP	150 × 4.6	4	80	Ether-linked phenyl (with polar end-capping)	Phenomenex	27.4
	13	AI	Caltrex AI	125 × 4	5	100	Calix[4]aren	Syntrex	29.9
	14	AII	Caltrex AII	125 × 4	5	100	Calix[6]aren	Syntrex	33.8
	15	AIII	Caltrex AIII	125 × 4	5	100	Calix[8]aren	Syntrex	31.6
	16	Sci	Caltrex Science	125 × 4	5	100	Calix[4]aren and *p*-*tert*-butyl-calix[4]aren[a]	Syntrex	55.2
	17	BI	Caltrex BI	125 × 4	5	100	*p*-*tert*-butyl-calix[4]aren	Syntrex	20.9
	18	BII	Caltrex BII	125 × 4	5	100	*p*-*tert*-butyl-calix[6]aren	Syntrex	23.5
	19	BIII	Caltrex BIII	125 × 4	5	100	*p*-*tert*-butyl-calix[8]aren	Syntrex	25.9
	20	Res	Caltrex Resorcinaren	125 × 3	5	100	Resorcinarene	Syntrex	100.5

[a] In a 1:1 ratio.

2.3. Columns

The columns used in this study are shown in Table 1 and some of them are illustrated in Fig. 1.

For Caltrex® columns, the ligands were immobilized via hydrophobic spacers on endcapped silica (Kromasil Si 100, 5 μm, specific surface area/BET: 300 m^2/g, manufacturer: EKA Chemicals (Bohus, Sweden). Calixarenes, which are modified with olefin-containing groups with linkers at the oxygen group via ether function, were utilized. In case of resorcinarenes, the olefin function is the end group of the side chain of the bridged group between the resorcine units [37]. All calixarenes are products of Syntrex GbR and produced using modified procedures of Gutsche et al. [38–40].

2.4. Chromatographic conditions

The tests were performed according to the chromatographic conditions described in the testing protocols [16,28,32]. For all

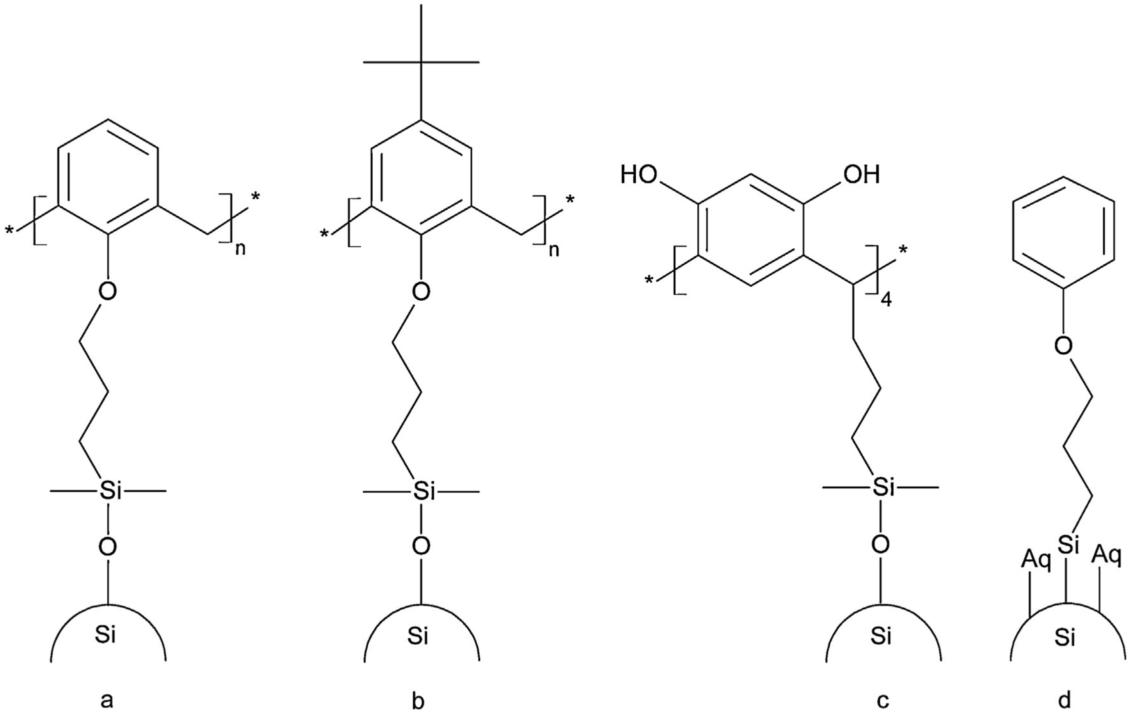

Fig. 1. Representative structures for stationary phases (a) Caltrex A (AI: *n* = 4; AII: *n* = 6; AIII: *n* = 8); (b) Caltrex B (BI: *n* = 4; BII: *n* = 6; BIII: *n* = 8); (c)Caltrex Resorcinaren (*n* = 4); and (d) Synergi Polar-RP.

tests the injection volume was 5 μl and detection was performed at 254 nm and at 210 nm for Snyder–Dolan. Flow rates depending on column internal diameter were applied. All pH-values were measured in the aqueous component of the mobile phase. Phosphoric acid and potassium hydroxide were used for pH adjustment.

Tanaka [16]: The mobile phase composition is shown in Table 2. Flow rates of 1 ml/min for the 4.6 mm and 4 mm ID, and of 0.6 ml/min for the 3 mm ID were used. Column temperature was set at 40 °C. Uracil was used as a dead time marker.

USP [26,32]: The mobile phase composition is shown in Table 2. Flow rates of 1 ml/min for the 4.6 mm and 4 mm ID, and of 0.6 ml/min for the 3 mm ID were used. Column temperature was set at 23 °C. Uracil was used as a dead time marker.

Snyder–Dolan (10-solute procedure) [28]: The mobile phase composition is shown in Table 2. Flow rates of 2 ml/min for the 4.6 mm and 4 mm ID, and of 1 ml/min for the 3 mm ID were used. Column temperature was set at 35 °C. Thiourea was used as a dead time (t_0) marker.

2.5. Calculations

Retention factors (k) were calculated using the following relationship:

$k = (t_R - t_0)/t_0$, where t_R is the retention time for the peak and t_0 is the retention time of the unretained component (in this study uracil or thiourea depending on the test method).

Tailing factors (TF) were calculated using the following relationship:

TF = (LW5% + RW5%)/(2 LW5%), where LW5% is the left peak width in 5% of the peak height, and RW5% is the right peak width in 5% of the peak height.

Column comparison function: The Fs value of a column relative to the reference column (Kromasil C_{18} in this study; see Table 1). Fs is the distance separating two columns in the plot (of values of $\mathbf{H}$, $\mathbf{S}^*$ and so on) in five-dimensional space. The Fs value is the basis for ranking the similarity or difference between two columns in the Snyder–Dolan approach [30].

$$Fs = \{[12.5(\mathbf{H}_2 - \mathbf{H}_1)]^2 + [100(\mathbf{S}_2^* - \mathbf{S}_1^*)]^2 + [30(\mathbf{A}_2 - \mathbf{A}_1)]^2 + [143(\mathbf{B}_2 - \mathbf{B}_1)]^2 + [83(\mathbf{C}_2 - \mathbf{C}_1)]^2\}^{1/2}$$

2.6. Software

LabSolutions chromatography software was used to control the modules and acquire data. DryLab® 2010, ColumnMatch® (Molnár-Institute, Berlin, Germany) was used in Snyder–Dolan approach to calculate the parameters.

3. Results and discussion

Most of characterization methods classify columns in terms of several fundamental chromatographic characteristics, which depend mainly on hydrophobic and/or silanol interactions. While the first influenced dominantly by the water content of the eluent, furthermore by the surface area of the silica, the amount of carbon bonded to the surface, and bonding density of the ligands on the stationary phase, play the number and the accessibility of residual silanol groups on the silica surface also an important role in hydrophilic and ion-exchange interactions [3]. In spite of the fact that all column classification systems aim to evaluate "more or less" the same characteristics, each system uses different test mixtures in different chromatographic conditions using different calculation procedures. All of that make the resulted column parameters using these systems deviate from one to another.

In general, the empirically based evaluation methods are based on the chromatographic information resulting from a limited number of selected test compounds, which are supposed to reflect a specific column property, e.g. silanol activity. On the other hand, the so-called model-based evaluation methods are based on a specific chromatographic model.

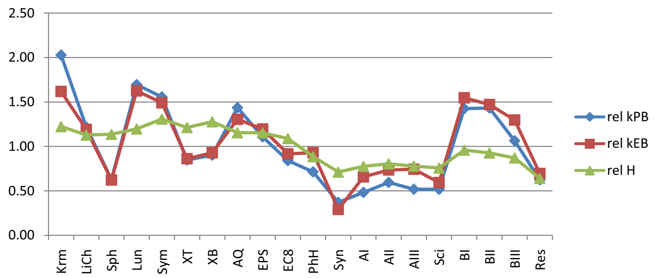

Fig. 2. Comparison of rel k_{PB}, rel k_{EB} and rel $\mathbf{H}$ from Tanaka, USP and Dolan/Snyder tests for all investigated stationary phases.

Two of the three selected methods (Tanaka and USP) are empirically based evaluation methods and both use methanol as an organic modifier in the mobile phase. Both methods are originally developed to characterize alkyl-bonded phases. However, they were used to evaluate different types of RP-phases, especially Tanaka's method. The last method (Snyder–Dolan) is a model-based evaluation method, which uses the hydrophobic-subtraction model and acetonitrile–water (50:50, v:v) as an organic modifier in the mobile phase. Solute retention factors k are given by the following model [30]:

$$\log k = \log k_{EB} + \underset{(i)}{\eta' \mathbf{H}} - \underset{(ii)}{\sigma' \mathbf{S}^*} + \underset{(iii)}{\beta' \mathbf{A}} + \underset{(iv)}{\alpha' \mathbf{B}} + \underset{(v)}{\kappa' \mathbf{C}}$$

Terms i–v describe five different solute–column interactions (see Table 4). Each of interactions i–v is based on a column parameter ($\mathbf{H}$, $\mathbf{S}^*$, $\mathbf{A}$, $\mathbf{B}$, or $\mathbf{C}$) and a solute parameter (η', σ', β', α', or κ'). The regression of the previous equation when applied to values of k for the test-solutes at pH 2.8 yields a value of $\mathbf{C}$ (2.8) for a given column. Values of the column cation-exchange capacity $\mathbf{C}$ at pH 7.0 are estimated using the retention k of a permanently ionized cationic solute (the quaternary-ammonium compound berberine), which is measured at pH 2.8 ($k_{2.8}$) and pH 7.0 ($k_{7.0}$). $\mathbf{C}$ (7.0) is then calculated:

$$\mathbf{C}(7.0) = \mathbf{C}(2.8) + \log(k_{7.0}/k_{2.8})$$

Snyder et al. [41] reviewed recently the hydrophobic-subtraction model of RP-column selectivity.

3.1. Hydrophobic interactions

Hydrophobic characters of stationary phases are represented using both hydrophobic capacity, i.e. retention factor k (of n-pentylbenzene "k_{PB}" and of ethylbenzene "k_{EB}"), and hydrophobic selectivity, i.e. retention factor ratio α (between n-pentylbenzene and n-butylbenzene "$\alpha_{PB/BB}$" and between ethylbenzene and toluene "$\alpha_{EB/To}$"), in Tanaka and USP methods, respectively. In Snyder–Dolan approach, $\mathbf{H}$ represents the hydrophobic characters of stationary phases.

3.1.1. Hydrophobic capacity

Fig. 2 illustrates the resulted values of k_{PB}, k_{EB} and $\mathbf{H}$. To facilitate the visual comparison between the methods, the relative values (which equal to the value divided by the mean of the same group)

Table 2
Summary of characterization tests used in the study.

	Parameter	Property of stationary phase[a]	Test mixtures	Mobile phase
Tanaka	Hydrophobicity (k_{PB})	Ligand density	*n*-Pentylbenzene (PB)	Methanol/water 80:20
	Hydrophobic selectivity ($\alpha_{PB/BB}=k_{PB}/k_{BB}$)	Surface coverage	*n*-Butylbenzene (BB) *n*-Pentylbenzene (PB)	Methanol/water 80:20
	Shape selectivity ($\alpha_{T/O}=k_T/k_O$)	Surface coverage Silane functionality	*o*-Terphenyl (O) Triphenylene (T)	Methanol/water 80:20
	Hydrogen-bonding capacity ($\alpha_{C/P}=k_C/k_P$)	Residual silanols Degree of end-capping	Caffeine (C) Phenol (P)	Methanol/water 30:70
	Cation-exchange capacity at pH 2.7 $\alpha_{B/P}=k_B/k_P$ (pH 2.7)	Acidic silanol activity	Phenol (P) Benzylamine (B)	Methanol/aqueous 20 mM KH_2PO_4 (pH 2.7) 30:70
	Cation-exchange capacity at pH 7.6 $\alpha_{B/P}=k_B/k_P$ (pH 7.6)	Total silanol activity	Phenol (P) Benzylamine (B)	Methanol/aqueous 20 mM KH_2PO_4 (pH 7.6) 30:70
USP	Hydrophobicity (k_{EB})	Hydrophobic retentivity	Ethylbenzene (EB)	Methanol/aqueous 50% 20 mM KH_2PO_4 and 50% 20 mM K_2HPO_4 (v/v) (pH 7.0) 80:20
	Hydrophobic selectivity ($\alpha_{EB/To}=k_{EB}/k_{To}$)	Methylene selectivity	Toluene (To) Ethylbenzene (EB)	
	Chelation (TF_Q)	Metal content (activity toward chelators)	Quinizarin (Q)	
	Silanol activity (k_{Am} and TF_{Am})	Total silanol activity (activity toward bases)	Amitriptyline (Am)	
Snyder–Dolan	H	Hydrophobicity	Mix #1	Acetonitrile/aqueous 60 mM KH_2PO_4 (pH 2.8) 50:50
	S^*	Steric resistance	Thiourea	
	A	Hydrogen-bond acidity Non-ionized silanols	Amitriptyline Benzonitrile	
	B	Hydrogen-bond basicity Sorbed water[b]	*n*-Butylbenzoic acid Trans-chalcone	
	C (2.8)	Cation-exchange activity at pH 2.8	Mix #2 *N*,*N*-Diethylacetamide Acetophenone Anisole Ethylbenzene Mix #3 berberine	
	C (7.0)	Cation-exchange activity at pH 7.0	Mix #3 berberine	Acetonitrile/aqueous 60 mM KH_2PO_4 (pH 7.0) 50:50

[a] As described in the original work.
[b] For some, but not all columns.

are presented. All correlation coefficient *r* in this study are calculated based on the original values.

It is clear that the curves obtained from k_{PB} and k_{EB} are parallel, and that suggests a good correlation ($r=0.962$). Fig. 2 indicates also that ***H***, which refers to hydrophobicity in Snyder–Dolan approach, have a weak relation to k_{PB} and k_{EB}. For example, Fig. 2 shows that the relative k_{PB}- and k_{EB}- values of Caltrex BI, BII and BIII are higher than those of Sph, XT, XB and EC8. However, the relative ***H*** values of Caltrex BI, BII and BIII, as of all aromatic-RP columns, are lower than those of alkyl-bonded phases.

Using methanol in the Tanaka and USP tests resulted, on the other hand, increasing values of k_{PB}, k_{EB} of the aromatic-RP columns. This phenomenon was clearer in the case of Caltrex B probably due to the presence of *p-tert*-butyl groups attached to the upper rim of Clatrex B, which could interact with the alkyl substituents of the solute, i.e. *n*-pentylbenzene and ethylbenzene.

The correlations between k_{PB}, k_{EB} and ***H*** for all phases, for alkyl-bonded phases and for aromatic-RP columns are shown in Table 3. k_{PB}- and k_{EB}- values are highly correlated in all cases ($r=0.962$, 0.976 and 0.971). However, the values of ***H*** have a good correlation only within the group of aromatic-RP columns ($r=0.813$ and 0.856) with k_{PB} and k_{EB}, respectively (Fig. 3). If we exclude the resorcinarene-bonded phase from this group the correlations will be even higher ($r=0.928$ and 0.959). In the case of alkyl-bonded phases there were poor correlation values for ***H***-values with k_{PB} and k_{EB}.

3.1.2. *Hydrophobic selectivity*

Gilroy et al. [42] calculated the corresponding correlation for 19 type-B alkyl–silica columns between ***H*** reported in their study and $\alpha_{PB/BB}$ for these columns calculated by Euerby and Petersson [43]. The result was: $\boldsymbol{H}=0.09+5.34\log\alpha_{PB/BB}$; $r^2=0.77$.

Fig. 4 shows the resulted values of relative $\alpha_{PB/BB}$, $\alpha_{EB/To}$ and ***H***. The results of $\alpha_{PB/BB}$ and $\alpha_{EB/To}$ suggest a similar hydrophobic profile of all columns, as they varies in the range of (1.26–1.49) and of (1.23–1.45), respectively. However, that does not reflect the real situation, since the selected phases have very different physical and chemical properties. The results of hydrophobic selectivity in our study are also in agreement with the results from Claessens et al. [1] and Cruz et al. [21]. However it is surprisingly that despite choosing alkyl-bonded and aromatic aromatic-RP columns, the results of hydrophobic selectivity were not very different. As a result, it is clear that the retention of a nonpolar solute (such as pentylbenzene or ethylbenzene) is a simple indicator of absolute retention.

Table 3
Comparison of the values of correlation coefficient (r) of k_{PB}, k_{EB} and ***H*** for all stationary phases, for alkyl-bonded phases and for aromatic-RP columns.

	k_{EB}			***H***		
	All	Alkyl	Aromatic	All	Alkyl	Aromatic
k_{PB}	0.962	0.976	0.971	0.658	0.364	0.813
k_{EB}				0.584	0.345	0.856

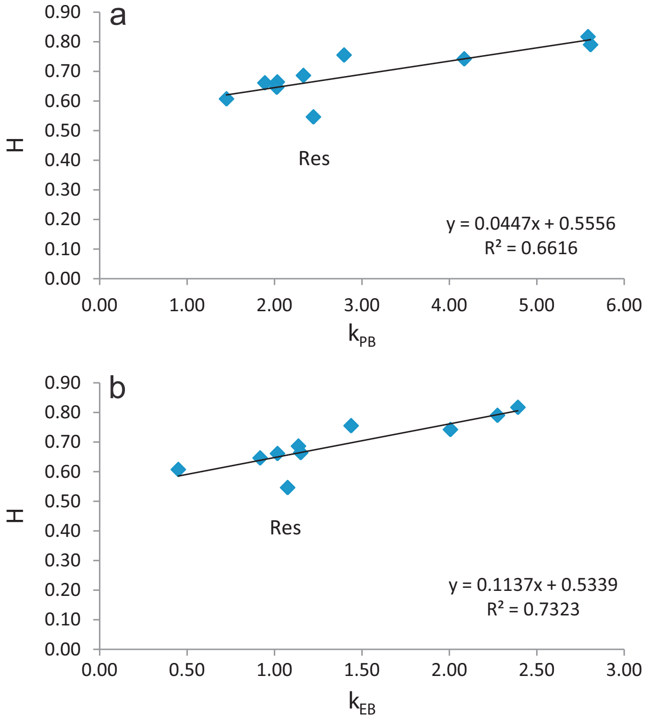

Fig. 3. Plot of (a) k_{PB} versus $\boldsymbol{H}$; (b) k_{EB} versus $\boldsymbol{H}$ for RP-aromatic columns showing the correlation between these parameters.

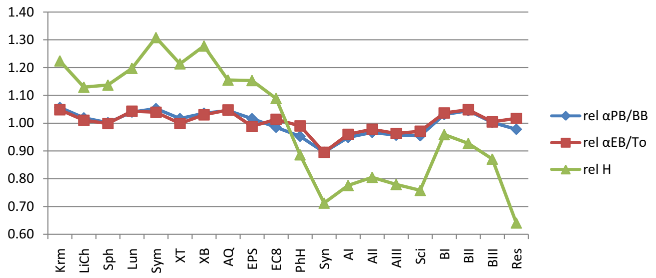

Fig. 4. Comparison of rel $\alpha_{PB/BB}$, rel $\alpha_{EB/To}$ and rel $\boldsymbol{H}$ from Tanaka, USP and Dolan/Snyder tests for all investigated stationary phases.

It seems that $\boldsymbol{H}$ reflects the hydrophobicity of the phases in a better way. Fig. 4 shows also clearly the effect of organic modifier switching from methanol in the Tanaka and USP tests to acetonitrile in Snyder–Dolan. $\boldsymbol{H}$-values are over the mean in all alkyl-bonded phases, since they do not support π-π interactions with aromatic solutes and the use of acetonitrile, therefore, do not reduce the retention on these phases. At the same time, using acetonitrile reduced the retention of aromatic solutes and consequently $\boldsymbol{H}$-values in aromatic-RP columns [44].

Table 4 shows the correlations between $\alpha_{PB/BB}$, $\alpha_{EB/To}$ and $\boldsymbol{H}$ for all phases, for alkyl-bonded phases and for aromatic-RP columns. As previously mentioned, $\alpha_{PB/BB}$- and $\alpha_{EB/To}$- values are highly correlated in the whole group (r = 0.925). Without the resorcinarene-bonded phase in the group of aromatic-RP columns, the correlation of $\boldsymbol{H}$ with $\alpha_{PB/BB}$ and $\alpha_{EB/To}$ will be much higher (r = 0.883 and 0.926), respectively, instead of (r = 0.678 and 0.566).

Table 4
Comparison of the values of correlation coefficient (r) of $\alpha_{PB/BB}$, $\alpha_{EB/To}$ and $\boldsymbol{H}$ for all stationary phases, for alkyl-bonded phases and for aromatic-RP columns.

	$\alpha_{EB/To}$			$\boldsymbol{H}$		
	All	Alkyl	Aromatic	All	Alkyl	Aromatic
$\alpha_{PB/BB}$	0.925	0.789	0.949	0.790	0.729	0.678
$\alpha_{EB/To}$				0.623	0.440	0.566

3.2. Steric interactions

Steric characters of stationary phases are described in Tanaka's approach using shape selectivity, i.e. retention factor ratio between triphenylene and *o*-terphenyl $\alpha_{T/O}$. This pair differs strongly in their molecular planarity, since triphenylene have much more planar structure in comparison to the "bulky" *o*-terphenyl, which is twisted out of plane. On the other hand, steric interaction $\boldsymbol{S^*}$ in Snyder–Dolan will represent the steric exclusion contribution to retention.

The comparison between shape selectivity and steric interaction is complicated, since both represent different aspects of a single phenomenon, namely the retention of hindered molecules in RPC. The differences are that shape selectivity is significant for more rigid solute molecules, polymeric stationary phases, and high-organic mobile phases (80–100% B); steric interaction is important for less rigid molecules, monomeric phases, and intermediate mobile phase compositions (e.g. 50% acetonitrile–buffer) [42,45]. Monomeric phases result from the reaction of monofunctional silanes with silanol groups at the silica surface. On the other hand, polymeric phases result from the reaction of di- or trifunctional silanes. The so-called "bulky" molecules face steric hindrance and are not able to penetrate into polymeric phases, since these phases have usually higher ligand concentrations. As a result, polymeric stationary phases should exhibit greater shape selectivity than monomeric phases [46]. On the other hand, steric interaction in Snyder–Dolan is based on the steric exclusion of larger solute molecules from the stationary phase.

The calculated correlation for a number type-B alkyl–silica columns by Gilroy et al. [42] between $\boldsymbol{S^*}$ in their study and $\alpha_{T/O}$ for these columns calculated by Euerby and Petersson [43] was r^2 = 0.40.

Fig. 5 illustrates the resulted relative values of shape selectivity ($\alpha_{T/O}$) and steric interaction $\boldsymbol{S^*}$ from Tanaka and Snyder–Dolan tests, respectively. Fig. 5 indicates clearly that the tested stationary phases could be divided into two groups according to the relationship between $\alpha_{T/O}$ and $\boldsymbol{S^*}$. While the relative values in the first half, i.e. alkyl-bonded phases, are not interchangeable and have low correlation (r = 0.395), the curves obtained from the relative values of $\alpha_{T/O}$ and $\boldsymbol{S^*}$ in the second half, i.e. aromatic-RP columns, are parallel and that suggests a good correlation, which is also much better when excluding the resorcinarene-bonded phase (r = 0.837 and 0.964). The data of all phases are poorly correlated (r = 0.291). This interesting founding about the difference between alkyl-bonded and aromatic-RP columns in term of the correlation of $\alpha_{T/O}$ and $\boldsymbol{S^*}$ values are better shown in Fig. 6.

In the case of stationary phases with larger values of $\boldsymbol{S^*}$ (Res, AI, AII, AIII and EPS), there is an increasing difficulty of a solute

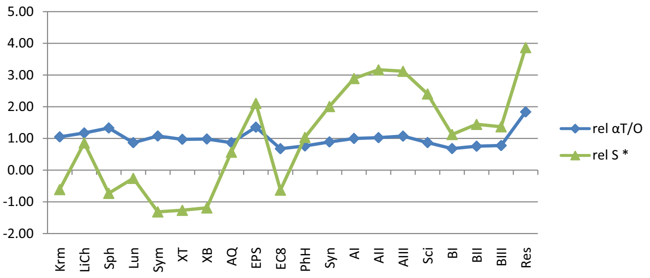

Fig. 5. Comparison of rel $\alpha_{T/O}$ and rel $\boldsymbol{S^*}$ from Tanaka and Dolan/Snyder tests for all investigated stationary phases.

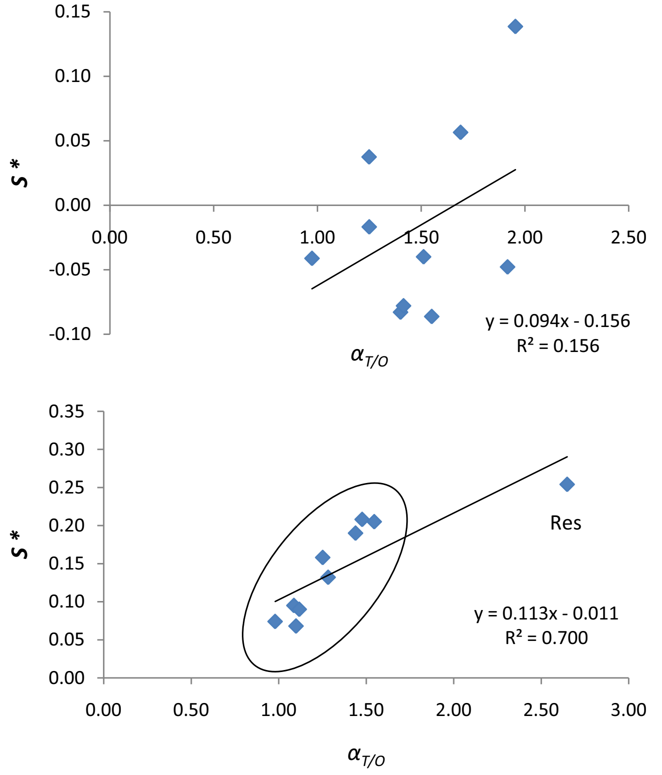

Fig. 6. Comparison of the correlation between $\alpha_{T/O}$ versus $\boldsymbol{S^*}$ for (a) alkyl-bonded phases and (b) RP-aromatic columns.

molecule, especially those with larger values of σ' which increases with molecular length, to enter the stationary phase, resulting in decrease in retention. These phases have also higher values of $\alpha_{T/O}$, which depends mainly on the ratio of length to width of a solute molecule.

Horak et al. [47,48] suggested the relation between the elution order of the pair (*n*-pentylbenzene and *o*-terphenyl) in Tanaka's test and the capacity of the stationary phase to show π–π interactions with aromatic solutes. They founded that $\alpha_{PB/O}$ decreases gradually in the order of increasing π–π activity, so that the value of $\alpha_{PB/O}$ could be used as an "indicator" for π–π activity. The results in our study support also this finding, as values of $\alpha_{PB/O}$ are >1 in the alkyl-bonded phases, i.e. the retention order was *o*-terphenyl, *n*-pentylbenzene). On the other hand, values of $\alpha_{PB/O} < 1$ are resulted in all aromatic-RP columns. Here, π–π interactions play an important role in the longer retention of *o*-terphenyl on aromatic-RP columns.

However, to make this relationship between the value of $\alpha_{PB/O}$ and the π–π activity of the stationary phase clearer, the value of $(1 - \alpha_{PB/O})$ is used, because it reflects directly the π–π activity of the stationary phase. Values of $(1 - \alpha_{PB/O})$ for all columns are shown in Fig. 7. In the group of aromatic-RP columns: the more the value of $(1 - \alpha_{PB/O})$, the stronger the π–π activity of the phase (according to the rule of Horak et al.).

3.3. Hydrogen-bonding interactions

Residual silanols are responsible for two important interactions: hydrogen-bonding and electrostatic interactions. Both of them are measured in the test methods depending on the test solute. While polar solutes are used to measure hydrogen-bonding capacity, electrostatic interactions (here, cation-exchange capacity) are measured using ionized bases.

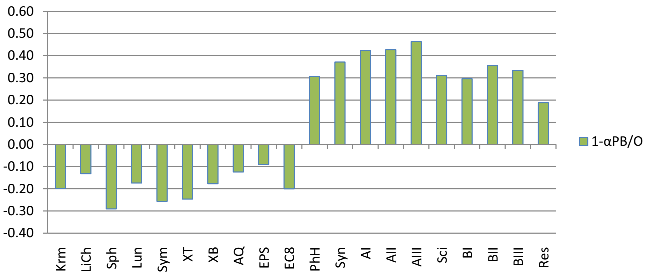

Fig. 7. Values of $(1 - \alpha_{PB/O})$ for investigated stationary phases showing the possible role of π–π interactions in each stationary phase (based on the rule of Horak et al.).

As seen in Table 2, hydrogen-bonding capacity of a stationary phase using Tanaka's test is measured by the retention factor ratio between caffeine and phenol $\alpha_{C/P} = k_C/k_P$, and that reflect two properties of this phase, which are the residual silanols and the degree of end-capping. The used mobile phase contains bi-distilled water and methanol only (unbuffered). In the Snyder–Dolan approach there are two types of hydrogen-bonding interactions, which are hydrogen-bond acidity ***A*** and hydrogen-bond basicity ***B***. Hydrogen-bond acidity ***A*** is based on non-ionized silanols, therefore columns with larger values of ***A*** provide increased retention of solutes that are hydrogen-bond acceptors. On the other hand, larger values of ***B*** are responsible for increasing retention of solutes that are hydrogen-bond donors. It is also important to state that USP approach does not include any parameter for hydrogen-bonding interactions.

The calculated correlation by Gilroy et al. [42] between the values of ***A*** for some type-B alkyl-silica columns from their study and $\alpha_{C/P}$ for the same columns calculated by Euerby and Petersson [43] was negligible $r^2 = 0.03$.

The resulted values of $\alpha_{C/P}$ and ***A*** are shown in Fig. 8. The mean of ***A*** in this case was negative (−0.17), which makes the relative values meaningless. Fig. 8 shows therefore the original values of $\alpha_{C/P}$ and ***A***. It is clear, that the curves of $\alpha_{C/P}$ and ***A*** are surprisingly quite parallel in the first half, i.e. in the group of alkyl-bonded phases, that suggest a good correlation between $\alpha_{C/P}$ and ***A*** within this group, which is shown in Fig. 9a. In the group of aromatic-RP columns, it seems that $\alpha_{C/P}$ from the Tanaka test is not related to ***A*** from the Snyder–Dolan approach, and there is a low correlation within this group between $\alpha_{C/P}$ and ***A*** (Fig. 9b). The correlations between $\alpha_{C/P}$, ***A*** and ***B*** for all phases, for alkyl-bonded phases and for aromatic-RP columns are shown in Table 5.

That could be related to the enormous structural difference between caffeine in the Tanaka test and *N,N*-diethylacetamide in the Snyder–Dolan test, which have the highest ß’ value in the 10-solute procedure. In the first case, i.e. alkyl-bonded phases, $\alpha_{C/P}$ and ***A*** are good correlated ($r = 0.930$), since the retention of caffeine and *N,N*-diethylacetamide is mainly based on hydrogen-bonding interactions with residual silanols. On the other hand, the long retention of caffeine in the case of aromatic-RP columns could not

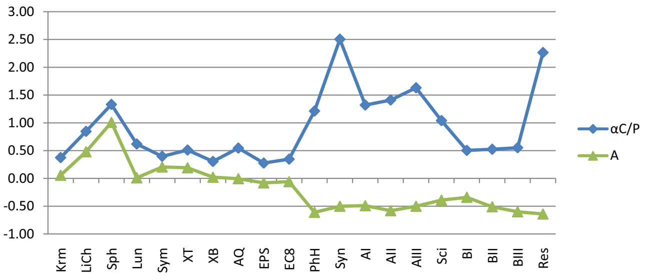

Fig. 8. Comparison of the original values of $\alpha_{C/P}$ and ***A*** from Tanaka and Dolan/Snyder tests for all investigated stationary phases.

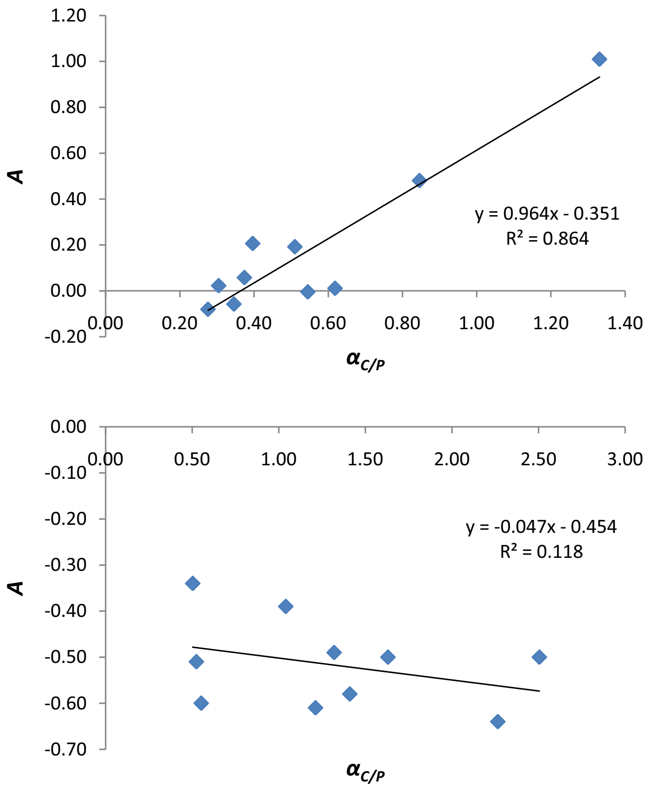

Fig. 9. Plot of $\alpha_{C/P}$ versus ***A*** for (a) alkyl-bonded phases; (b) RP-aromatic columns, showing the correlation between $\alpha_{C/P}$ versus ***A*** in each group of stationary phases.

be explained based only on hydrogen-bonding interactions with residual silanols, and it seems that strong π–π interactions are also the reason behind this longer retention. The small values of ***A*** for the same columns can be interpreted with the dependence of the retention of *N,N*-diethylacetamide on hydrogen-bonding interactions without any role of π–π interactions.

All of that make hydrogen-bond acidity ***A*** have the ability to reflect the interaction with residual silanols in the case of alkyl-bonded as well as in aromatic-RP columns. On the other hand, Tanaka test is originally developed to characterize alkyl-bonded phases, and is now widely used to characterize other RP-phases, which is possible in the case of hydrophobicity, hydrophobic selectivity, shape selectivity and cation-exchange capacity, but not to evaluate the hydrogen-bonding interactions with residual silanols in aromatic-RP columns.

3.4. Electrostatic interactions

The role of electrostatic interactions in RPC depends on the used column (value of cation-exchange capacity), on the pH of the mobile-phase and on the buffer concentration. While electrostatic interactions are evaluated in the Tanaka and Snyder–Dolan tests at two different pH values (2.7 and 7.6) and (2.8 and 7.0) respectively, the USP test uses a mobile phase of pH 7.0 only.

Table 5
Comparison of the values of correlation coefficient (r) of $\alpha_{C/P}$, ***A*** and ***B*** for all stationary phases, for alkyl-bonded phases and for aromatic-RP columns.

	A			***B***		
	All	Alkyl	Aromatic	All	Alkyl	Aromatic
$\alpha_{C/P}$	−0.356	0.930	−0.344	−0.138	−0.411	0.216
A				−0.163	−0.442	−0.149

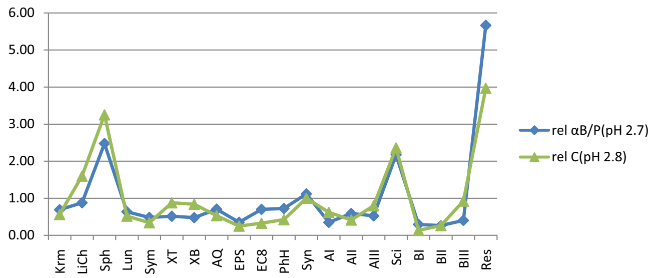

Fig. 10. Comparison of rel $\alpha_{B/P}$ (2.7) and rel ***C*** (2.8) from Tanaka and Dolan/Snyder tests for all investigated stationary phases.

In Tanaka's test the retention factor ratio between benzylamine and phenol $\alpha_{B/P}$ is measured at pH 7.2, as well as at pH 7.6. Benzylamine (pK_a 9.4) is protonated in both situations. On the other hand, residual silanols are to a certain extent dissociated at pH 7.6, but only the most acidic silanol groups (less than 1%) remain dissociated at pH 2.7, which are capable to establish electrostatic interactions [49,50]. Marchand et al. [51] provided also evidence for cation exchange (and presumably silanol ionization) at a pH as low as 3 for most columns. As a result the retention of benzylamine should decrease when the pH changes from 7.6 to 2.7 due to the large decrease in the number of dissociated silanols. The retention factor of phenol should be the same or only slightly affected by the variation of pH. Consequently, $\alpha_{B/P}$ (pH 2.7) evaluates the "acidic silanol activity", while $\alpha_{B/P}$ (pH 7.6) reflect the "total silanol activity" of a stationary phase.

The USP test uses the retention factor k_{Am} and the tailing factor TF_{Am} of amitriptyline (pK_a 9.4) at pH 7.0 to evaluate the "total silanol activity".

Values of cation-exchange activity in Snyder–Dolan test are given for pH 2.8 and 7.0, which are determined by the concentration and/or acidity of residual silanols. The retention of protonated bases increases on columns with large values of ***C***, which also lead to decreased retention of ionized acids.

Gilroy et al. [42] calculated the correlation for a number of type-B alkyl-silica columns between ***C*** (2.8) and ***C*** (7.0) reported in their study and $\alpha_{B/P}$ at pH 2.7 and 7.6, respectively, for these columns calculated by Euerby and Petersson [43]. The correlation between ***C*** (2.8) and $\alpha_{B/P}$ (2.7) was moderate ($r^2 = 0.70$). On the other hand, no correlation ($r^2 = 0.32$) resulted between ***C*** (7.0) and $\alpha_{B/P}$ (7.6).

Fig. 10 shows the resulting relative values of $\alpha_{B/P}$ (2.7) and ***C*** (2.8). From the curves a good correlation is to be expected. The values of $\alpha_{B/P}$ (2.7) and ***C*** (2.8) for all phases, for alkyl-bonded phases and for aromatic-RP columns were fairly good correlated in all cases ($r = 0.912$, 0.934 and 0.964), respectively.

Fig. 11 illustrates the relative values of $\alpha_{B/P}$ (7.6), TF_{Am} and ***C*** (7.0) from the Tanaka, USP and Snyder–Dolan tests, respectively. The curves of $\alpha_{B/P}$ (7.6) and ***C*** (7.0) are somehow parallel, but it is clear the values of TF_{Am} are not in agreement with them.

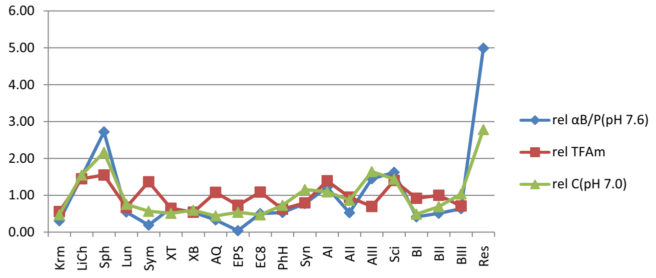

Fig. 11. Comparison of rel $\alpha_{B/P}$ (7.6), rel TF_{Am} and rel ***C*** (7.0) from Tanaka and Dolan/Snyder tests for all investigated stationary phases.

Table 6
Summary of correlation coefficient (r) of $\alpha_{B/P}$ (7.6), **C** (7.0), k_{Am}, TF_{Am}, k_Q and TF_Q for all stationary phases, for alkyl-bonded phases and for aromatic-RP columns.

	$C_{(pH\ 7.0)}$			k_{Am}			TF_{Am}			k_Q			TF_Q		
	All	Alkyl	Aromatic	All	Alkyl	Aromatic	All	Alkyl	Aromatic	All	Alkyl	Aromatic	All	Alkyl	Aromatic
$\alpha_{B/P(pH\ 7.6)}$	0.941	0.963	0.954	0.432	0.758	−0.414	0.576	0.618	0.505	0.524	−0.068	0.632	0.365	0.473	0.007
$C_{(pH\ 7.0)}$				0.400	0.767	−0.566	0.500	0.677	0.156	0.522	0.138	0.593	0.297	0.472	−0.159
k_{Am}							0.385	0.592	0.075	0.343	0.085	0.379	−0.160	−0.083	−0.135
TF_{Am}										−0.008	0.157	−0.114	0.267	0.324	0.075
k_Q													−0.070	0.261	−0.235

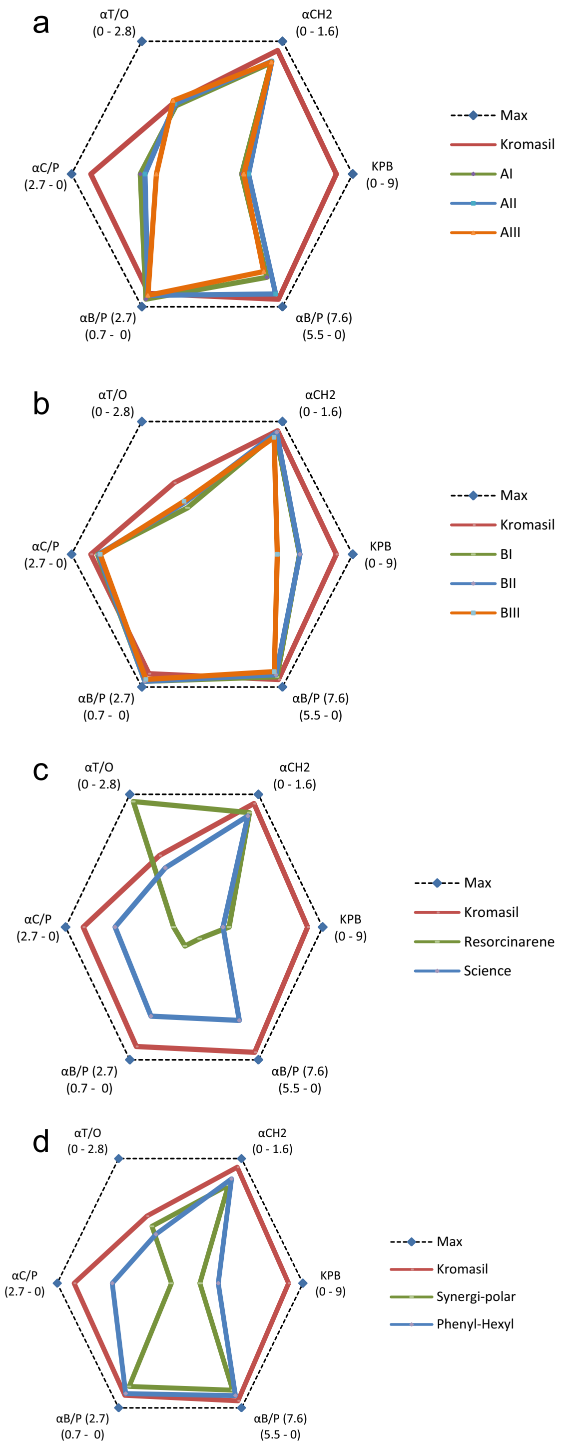

Fig. 12. Radar plots for Tanaka's parameter illustrating the similarities and differences for (a) Caltrex A; (b) Caltrex B; (c) Caltrex Science and Caltrex Resorcinarene; and (d) Luna Phenyl-Hexyl and Synergi Polar-RP; using Kromasil as a reference.

A high correlation resulted after calculating the values of r between $\alpha_{B/P}$ (7.6) and **C** (7.0) for all phases, for alkyl-bonded phases and for aromatic-RP columns (r = 0.941, 0.963 and 0.954), respectively. In the case of resorcinarene-bonded stationary phase amitriptyline could not be eluted from the column under the chromatographic conditions of the USP test.

Table 6 presents the correlation for the values of $\alpha_{B/P}$ (7.6), **C** (7.0), k_{Am} and TF_{Am} including also the retention factor k_Q and the tailing factor TF_Q of quinizarin (1,4-dihydroxyanthraquinone),

Table 7
Characterization parameters of calixarene- and resorcinarene-bonded stationary phases using Snyder/Dolan (10-solute procedure).

Name	***H***	***S****	***A***	***B***	***C*** (2.8)	***C*** (7.0)
Caltrex AI	0.661	0.190	−0.489	0.029	0.205	0.860
Caltrex AII	0.686	0.208	−0.578	0.049	0.137	0.683
Caltrex AIII	0.664	0.205	−0.502	0.022	0.264	1.292
Caltrex Science	0.646	0.158	−0.391	−0.013	0.781	1.132
Caltrex BI	0.817	0.074	−0.342	−0.061	0.044	0.384
Caltrex BII	0.790	0.095	−0.515	−0.025	0.087	0.544
Caltrex BIII	0.742	0.090	−0.597	−0.033	0.306	0.815
Caltrex Resorcinarene	0.546	0.254	−0.637	−0.031	1.314	2.189

which is used in USP test to evaluate the activity toward metal chelators. The presence of metals as impurities in the silica is also supposed to increase the acidity of residual silanols [50].

Data of the Tanaka test are usually normalized and presented through radar plots, allowing a simple visualization of the properties and represent multidimensional data simply in two dimensional diagrams. However, that can be used only to compare a limited number of columns on the same diagram. Radar plots allow therefore a rapid and simple assessing of column to find similarities with or differences from other columns. However, to make it easier, it is better to use different scales as seen in Fig. 12. On the other hand, the so called column comparison function (Fs) as a single parameter allows defining the difference between a reference column and other columns. Values of Fs using Kromasil C_{18} as a reference are summarized in Table 1.

The characterization of calixarene and resorcinarene phases using Snyder–Dolan test was performed for the first time (Table 7).

4. Conclusions

Making a comparative study between three widely used tests when applied to a group of different types or reversed-phase columns at the same time gives us a better overview of these approaches than making an evaluation of them based on the work of their developers. Very important is to consider that Tanaka (through the researches of Euerby and Visky) and the hydrophobic-subtraction model (Snyder–Dolan) are more particularly used for the study of widely different phases (alkyl phases, cyano, fluorinated and phenyl phases or even zirconia-based phases), where the USP approach are developed to characterize C_{18}-bonded phases.

Some interesting findings could be drawn from the results of this study:

(I) Hydrophobicity ***H*** in Snyder–Dolan approach has good correlation with k_{PB} and k_{EB} only within the group of aromatic-RP columns. Values of ***H*** are moderately correlated with the values of $\alpha_{PB/BB}$, $\alpha_{EB/To}$. On the other hand values of k_{PB} and k_{EB} are highly correlated in all cases. The same is applied for $\alpha_{PB/BB}$, $\alpha_{EB/To}$. The use of same criteria to evaluate the hydrophobic characters in Tanaka and USP tests is the reason in this case.

(II) A good correlation between steric interaction (***S****) and shape selectivity ($\alpha_{T/O}$) has been shown only in the group of aromatic-RP columns, which is also much better when excluding the resorcinarene-bonded phase ($r = 0.837$ and 0.964), respectively. In the group of alkyl-bonded phases the values of ***S**** and $\alpha_{T/O}$ are low correlated ($r = 0.395$). The data of all phases are also poorly correlated ($r = 0.291$).

(III) Surprisingly hydrogen-bonding capacity ($\alpha_{C/P}$) and hydrogen-bond acidity (***A***) are good related but only in the group of alkyl-bonded phases.

(IV) Silanol activity (cation-exchange capacity) at lower pH, i.e. $\alpha_{B/P}$ (2.7) and ***C*** (2.8) in Tanaka and Snyder–Dolan tests, respectively, is highly correlated in all cases. At high pH, values of $\alpha_{B/P}$ (7.6) and ***C*** (7.0) are also highly correlated. However, values of the retention factor k_{Am} and the tailing factor TF_{Am} of amitriptyline at pH 7.0, which are used in USP test to evaluate the "total silanol activity", showed moderate or low correlation with $\alpha_{B/P}$ (7.6) and ***C*** (7.0). This is probably due to the use of different interpretations of the silanol activity in USP test, which uses only amitriptyline as basic test solute.

Considering that all of these approaches have merit, for example the USP test evaluates also the activity toward metal chelators. Moreover, it is very simple, based on analyzing only five analytes in only one single run and it provides column performance characterization. Further works in this field are necessary to have a universal test for RPC-columns, taking into account that column characterization based on small molecular test substances, which are used in the most of theses tests except the hydrophobic-subtraction model (Snyder–Dolan) as it includes a number of complex molecules, do not necessarily provide the information that is needed for the proper selection of columns for the separation real samples, which are often more complex and contains larger molecules.

Supplementary materials

Figs. (2*, 4*, 5*, 10* and 11*), which are based on the original values of the test parameters, are given as supporting information.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.04.081.

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