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Characterization of 1,3-alternate calix[4]arene-silica bonded stationary phases and their comparison to selected commercial columns by using principal component analysis

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ABSTRACT

Twelve calix[4]arene stationary phases in *1,3-alternate* conformation, synthesized in the authors' laboratory, were characterized in terms of their surface coverage, hydrophobic selectivity, aromatic selectivity, shape selectivity, hydrogen bonding capacity and ion-exchange capacity. The set of tests commonly used for evaluation of commercially available stationary phases was applied to assess fundamental chromatographic properties of the calixarene phases. The new calixarene phases were compared to each other, to Caltrex and LiChrosorb C-18 columns. Principal component analysis has been used to provide comparison between *1,3-alternate* calix[4]arene phases and commercially available phenyl, fluorophenyl and fluoroalkyl columns.

1. Introduction

The continuous efforts to synthesize the "universal and unfailing" HPLC stationary phase with excellent chemical stability, improved selectivity and efficiency have increased significantly in recent years. Among many different types of HPLC phases, working in different separation modes, the reversed phases play presently a dominant role [1] and a number of RP columns differing in ligand types and the way these are bonded to the solid support is commercially available. Apart from classical reversed phase columns, calixarene stationary phases have lately attracted the attention of many researchers and the potential of this class of macrocyclic compounds for HPLC applications has been shown [2,3]. The modifications of calixarene molecules influencing their chromatographic properties include: conformations in which they are blocked, the type of functional groups and substituents present at their upper and lower rims, the calixarene ring-size, and the type of spacer fixing the macrocycles to the solid support. The calixarene supramolecular host molecules in a cone and *1,3-alternate* conformation, bonded to the silica gel, form inclusion complexes with the analytes and posses unique chromatographic properties [4–12]. In this paper we characterize twelve novel calix[4]arene stationary phases in *1,3-alternate* conformation in terms of surface coverage, hydrophobic and shape selectivity as well as hydrogen bonding and ion-exchange capacity. The Tanaka characterization protocol, which is a well-established, still favored by many academic groups approach, was used to characterize fundamental chromatographic descriptors of calixarene phases [13–17]. Based on this protocol it is possible to compare the new type of calixarene columns to the great number of other RP-phases, characterized by other research groups. Although this test was originally developed for *n*-alkyl based RP columns, it may also be applicable to other phase types [18,19]. In order to assess the aromatic selectivity of these phases, we have additionally used the approach proposed by Horak and Lindner [18]. They have suggested that the retention ratio of the Tanaka test solutes (*n*-pentylbenzene/*o*-terphenyl) may provide an indication of the capacity of the phase to undergo aromatic interactions with aromatic analytes. The calixarene phases were compared to each other, to Caltrex columns possessing calixarene molecules in cone conformations and to LiChrosorb C-18 phase. Additionally, principal component analysis has been used to explore the differences and similarities between *1,3-alternate* calix[4]arene phases and selected commercially available RP columns. From among great number of RP stationary phases, we choose phenyl, fluorophenyl as well as fluoroalkyl columns with chemical structures resembling the building blocks of the calixarene phases.

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2. Materials and methods

2.1. Chemicals and reagents

All buffer chemicals and solvents used as mobile phases were of HPLC grade and were purchased from Merck (Darmstadt, Germany). HPLC water was obtained by passing distilled water through a Milli-Q system. Pentylbenzene (PB), butylbenzene (BB), triphenylene (T), *o*-terphenyl (O), caffeine (C), phenol (P), benzylamine (B) were purchased from Lancaster (Eastgate, UK). Reversed phase test mixture (LA 87221) was obtained from Supelco (Deisenhofen, Germany). Silica gels: LiChrosorb C-18 and LiChrosorb Si–OH (particle size 5 μm, pore size 100 Å, specific surface area 300–400 $m^2\ g^{-1}$) were obtained from Merck (Darmstadt, Germany). Spherical silica gel Nucleosil Si–OH (particle size 5 μm, pore size 100 Å, surface area 350 $m^2\ g^{-1}$) was obtained from Macherey-Nagel (Düren, Germany).

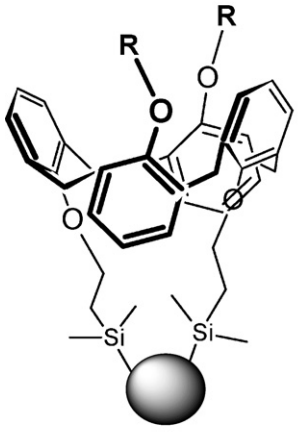
2.2. Chromatography

1200 Series Quaternary LC System (Agilent Technology Inc.) equipped with a quaternary pump, autosampler, thermostated column compartment and diode-array detector was used. Analytes were dissolved in the mobile phase at the concentration in range of 0.25–0.5 mg ml^{-1} and 5 μl of the solution were injected onto the chromatographic column. The retention time of potassium nitrate was used as void time marker for the calculation of the capacity factors. In all cases, the column temperature was set at 40 °C and the flow rate of the mobile phase was 1.0 ml min^{-1}. Diode-array detector was operated at 254 nm in single wavelength mode.

Six chromatographic column parameters were experimentally determined based on the modified protocol of Tanaka and coworkers [13–17]. The test proposed by Horak and Lindner [18] was performed for determination of aromatic selectivity.

The different chromatographic parameters used in the characterization procedures are described below:

Retention factor of *n*-pentylbenzene (k_{PB}), mobile phase: MeOH/H_2O (8:2, v/v);
hydrophobicity or hydrophobic selectivity (α_{CH_2}), retention factor ratio of *n*-pentylbenzene (PB) and *n*-butylbenzene (BB), individually injected, mobile phase: MeOH/H_2O (8:2, v/v);
aromatic selectivity (π-acidity) ($\alpha_{PB/O}$), retention factor ratio of *n*-pentylbenzene (PB) and *o*-terphenyl (O), individually injected, mobile phase: MeOH/H_2O (8:2, v/v);
shape selectivity ($\alpha_{T/O}$), retention factor ratio of triphenylene (T) and *o*-terphenyl (O), individually injected, mobile phase: MeOH/H_2O (8:2, v/v);



Column	R substituents
CalixPr	$-CH_2CH_2CH_3$
CalixHex	$-CH_2(CH_2)_4CH_3$
CalixDdc	$-CH_2(CH_2)_{10}CH_3$
CalixBz	$-CH_2$–C$_6$H$_5$
CalixBz-Cl	$-CH_2$–C$_6$H$_4$–Cl
CalixBz-NO_2	$-CH_2$–C$_6$H$_4$–NO_2
CalixBzF_5	$-CH_2$–C$_6$F$_5$
CalixBn	C$_6$H$_5$–C(=O)–
CalixBph	C$_6$H$_5$–C$_6$H$_4$–C(=O)–
CalixBn-OMe	CH_3O–C$_6$H$_4$–C(=O)–
CalixNph	C$_{10}$H$_7$–C(=O)–
CalixAmide	C$_6$H$_5$–CH(CH$_3$)–NH–C(=O)–

Fig. 1. Chemical structures of the investigated silica based *1,3-alternate* disubstituted calix[4]arene stationary phases.

hydrogen bonding capacity ($\alpha_{C/P}$), retention factor ratio of caffeine (C) and phenol (P), individually injected, mobile phase: MeOH/H_2O (3:7, v/v);
total ion-exchange capacity ($\alpha_{B/P}$ pH 7.6), retention factor ratio of benzylamine hydrochloride (B) and phenol (P), individually injected, mobile phase: 20 mM KH_2PO_4 at pH 7.6 in MeOH/H_2O (3:7, v/v);
acidic cation-exchange capacity ($\alpha_{B/P}$ pH 2.7), retention factor ratio of benzylamine hydrochloride (B) and phenol (P), individually injected, mobile phase: 20 mM KH_2PO_4 at pH 2.7 in MeOH/H_2O (3:7, v/v).

2.3. Chromatographic columns

All the investigated 25,27-disubstituted-26,28-bis-[3-propyloxy]-calix[4]arene-bonded silica gel stationary phases (Fig. 1), blocked in *1,3-alternate* conformation, were synthesized in our laboratory [4–12]. The ligands were immobilized on LiChrosorb silica gel, with the exception of CalixBzF5 and CalixBzNO_2 phases, which were bonded to Nucleosil silica gel. The carbon content and coverage densities of the investigated packing materials are summarized in Table 1. The modified silica gels were slurry packed into stainless steel columns (150 mm × 4.6 mm i.d.) and the columns efficiencies were determined with a test mixture containing dimethyl phthalate, diethyl phthalate, biphenyl and *o*-terphenyl, using methanol–water (70:30, v/v) as the mobile phase at flow rate of 1.0 ml min^{-1}.

2.4. Calculation

Principal Component Analysis (PCA) and chemometric computations were performed using STATISTICA software (StatSoft, Inc., Tulsa, USA).

Table 1
The investigated columns details and characterization.

Column code	Description	Column chemistry	Endcapping	Carbon content (%)	Coverage (μmol/g)	Silica gel/reference
Cluster 1 (see Fig. 3)						
5F	Perfluorpropyl ESI	Perfluorpropyl				[14]
6F	Fluofix (ec)	Branched perfluorohexyl	Y			Base deactivated/[16]
7F	Flouphase RP	Perfluorohexyl	Y			Base deactivated/[16]
8F	Fluofix (nec)	Branched perfluorohexyl	N			Base deactivated/[16]
9F	FluoroSep RP Octyl	Perfluorooctyl				Base deactivated/[16]
8Ph	Hypersil phenyl	Monomeric	N	5.0	408	[17]
14Ph	Prodigy phenyl 3	Polymeric phenyl ethyl	N	10.0	1845	[17]
Cluster 2 (see Fig. 3)						
1F	Monochrom MS	Perfluorophenyl				Low acidic/[16]
2F	Fluophase PFP	Perfluorophenyl	Y			Base deactivated/[16]
3F	Curosil PFP	Perfluorophenyl				[16]
4F	Discovery F5 HS	Perfluorophenyl				[16]
10F	Pursuit PFP	Perfluorophenyl				[17]
16Ph	Synergi Polar RP	Phenoxyethyl	Polar	11.0	1496	[17]
20Ph	Zorbax SB phenyl	Di-isopropyl side chain, phenyl	N	5.5		[17]
CalixBz		Modified 1,3-alternate calix[4]arene	Y	11.9	220	LiChrosorb Si-100/[6]
CalixBzCl		Modified 1,3-alternate calix[4]arene	Y	17.1	351	LiChrosorb Si-100/[10]
Calix$BnNO_2$		Modified 1,3-alternate calix[4]arene	Y	17.3	392	Nucleosil Si-100/[9]
CalixBzF5		Modified 1,3-alternate calix[4]arene	Y	12.2	260	Nucleosil Si-100/[11]
CalixNph		Modified 1,3-alternate calix[4]arene	Y	12.0	210	LiChrosorb Si-100/[12]
Cluster 3 (see Fig. 3)						
1Ph	ACE Phenyl	Monofunctional bonding	Y	9.5		[17]
2Ph	Ascentis phenyl	Phenylbutyl, polymeric	Y	19.0	1935	[17]
3Ph	BDS Hypersil phenyl		Y	5.0	425	[17]
4Ph	BetaBasic phenyl		Y	7.0	448	[17]
5Ph	Betasil phenyl hexyl		Y	11.0	648	[17]
6Ph	Fortis phenyl	Diphenyl	Y	13.0		[17]
7Ph	Gemini phenyl hexyl	Phenyl hexyl	Y	14.0		[17]
9Ph	Inertsil phenyl		Y	10.0	886	[17]
10Ph	Kromasil phenyl		Y	14.0		[17]
11Ph	Luna phenyl hexyl	Phenyl hexyl	Y	17.5	1600	[17]
12Ph	Novapak phenyl		Y	5.0	281	[17]
13Ph	Nucleodur Sphinx	C-18/Phenyl		15.0		[17]
15Ph	Pursuit diphenyl	Diphenyl	Y	11.0		[17]
17Ph	XBridge phenyl	Trifuctionalized phenyl hexyl	Proprietary	15.0	555	[17]
18Ph	XTerra phenyl	Difunctionalized 2-phenylpropyl		12.0	402	[17]
19Ph	Zorbax Eclipse XDB-phenyl	Monomeric	Doubly	7.2		[17]
CalixPr		Modified 1,3-alternate calix[4]arene	Y	14.5	303	LiChrosorb Si-100/[4]
CalixHex		Modified 1,3-alternate calix[4]arene	Y	17.1	391	LiChrosorb Si-100/[12]
CalixDdc		Modified 1,3-alternate calix[4]arene	Y	18.2	336	LiChrosorb Si-100/[12]
CalixBn		Modified 1,3-alternate calix[4]arene	Y	19.9	345	LiChrosorb Si-100/[5]
CalixBph		Modified 1,3-alternate calix[4]arene	Y	16.3	302	LiChrosorb Si-100/[12]
CalixBnOMe		Modified 1,3-alternate calix[4]arene	Y	8.1	151	LiChrosorb Si-100/[7]
CalixAmide		Modified 1,3-alternate calix[4]arene	Y	16.3	275	LiChrosorb Si-100/[8]
Columns not included in cluster analysis						
Caltrex AI		Cone calix[4]arene	Y	14.0	266	Kromasil/[25]
Caltrex AII		Cone calix[6]arene	Y	8.8	86	Kromasil/[25]
Caltrex AIII		Cone calix[8]arene	Y	15.3	149	Kromasil/[25]
Caltrex BI		Cone *p-tert*-butyl calix[4]arene	Y	14.3	195	Kromasil/[25]
Caltrex BII		Cone *p-tert*-butyl calix[6]arene	Y	8.1	53	Kromasil/[25]
Caltrex BIII		Cone *p-tert*-butyl calix[8]arene	Y	15.3	109	Kromasil/[25]
LiCh C-18	LiChrosorb C-18	Octadecyl		20.2	1222	LiChrosorb Si-100

3. Results and discussion

There have been numerous tests to characterize LC phases by chromatographic, spectroscopic, thermodynamic and physicochemical properties [20–24]. However, until now none of these evaluation methods have been widely accepted as a universal one. Limited studies [25–27] have been performed to characterize hydrophobic and steric properties of calixarene (Caltrex) and resorcinarene phases by means of empirically based test mixtures. In the present study, six chromatographic parameters of the prepared column were experimentally determined based on the Tanaka protocol [13–17]. In order to assess the aromatic selectivity of these phases, we have additionally used the approach suggested by Lindner (π-acidity of the phase) [18]. The results of the column characterization are placed in Table 2. The data concerning chromatographic characteristics and three Tanaka parameters (hydrophobic retention capacity, hydrophobic selectivity and steric selectivity) of Caltrex phases were taken from [25] (numerical data of these parameters are not available; hence they were approximated from graphical schemes).

The stationary phases evaluated by us contain supramolecular selectors consisting of two building blocks. One of them is the calix[4]arene moiety blocked in a *1,3-alternate* conformation which creates ordered hydrophobic channels [28] capable to form inclusion complexes with analytes. The second fragment comprises of different types of aromatic and aliphatic groups positioned at the upper rim of the calixarene scaffold. In order to improve the systematic data, the structures of these stationary phases were specially designed to determine how the presence of these two components placed in close proximity influenced the column properties, i.e., retention power, elution order of analytes and retention mechanism. The aromatic groups constitute of single phenyl rings (benzyl, benzoyl), phenyl rings additionally derivatized in para-position by electron-withdrawing (*p*-methoxybenzoyl, N-phenylethyl-carbonylmethyl) or electron-releasing substituents (*p*-chlorobenzyl, *p*-nitrobenzyl, pentafluorobenzyl), and fused phenyl rings of different planarities (naphthoyl, biphenyl-4-carbonyl). The aliphatic substituents are *n*-alkyl chains of different lengths (*n*-propyl, *n*-hexyl and *n*-dodecyl). These two units of the stationary phase possess various chemical properties. Therefore, numerous interactions may exist between analytes and the moieties of the bonded calixarenes, including hydrophobic interactions, inclusion complex formation together with interactions outside the calixarene channels, as well as π–π and π-electron transfer interaction between aromatic rings.

Table 2
The Tanaka and Lindner chromatographic test parameters of *1,3-alternate* calix[4]arene, phenyl and perfluorinated columns.

Column code	k_{PB}	$\alpha_{PB/O}$	α_{CH_2}	$\alpha_{T/O}$	$\alpha_{C/P}$	$\alpha_{B/P\,7.6}$	$\alpha_{B/P\,2.7}$
CalixPr	3.21	0.65	1.34	1.64	1.20	0.46	0.13
CalixHex	4.89	0.73	1.51	1.76	0.84	0.40	0.13
CalixDdc	3.96	0.63	1.35	1.76	0.84	0.40	0.12
CalixBz	2.65	0.64	1.21	1.56	1.56	0.34	0.24
CalixBzCl	3.22	0.50	1.37	2.34	1.55	0.45	0.31
CalixBnNO$_2$	8.15	0.55	1.38	3.32	1.47	0.35	0.14
CalixBzF5	1.54	0.81	1.27	4.13	1.88	0.94	0.10
CalixBn	2.91	0.55	1.37	1.62	0.89	0.74	0.08
CalixBph	2.22	0.56	1.32	1.72	1.29	0.39	0.09
CalixBnOMe	1.30	0.58	1.31	1.78	1.21	0.62	0.12
CalixNph	1.31	0.67	1.27	1.58	1.72	0.59	0.17
CalixAmide	1.82	0.58	1.31	1.88	0.74	0.18	0.01
LiCh C-18	7.51	1.14	1.42	1.66	0.81	1.18	0.22
1F	1.69	n/a	1.27	2.53	0.83	0.66	0.15
2F	1.60	n/a	1.23	2.50	0.63	0.70	0.30
3F	1.77	n/a	1.27	2.49	0.71	0.85	0.10
4F	1.70	n/a	1.26	2.55	0.68	0.85	0.34
5F	0.16	n/a	1.15	1.00	1.15	1.47	0.39
6F	0.57	n/a	1.24	0.58	0.81	2.06	0.38
7F	0.98	n/a	1.21	0.62	0.73	2.12	0.60
8F	0.48	n/a	1.20	0.67	1.37	3.90	0.26
9F	1.44	n/a	1.23	0.63	0.75	4.12	0.52
10F	1.03	1.06	1.28	2.46	0.73	0.40	0.12
1Ph	1.20	0.76	1.26	1.00	1.14	0.46	0.14
2Ph	2.32	0.61	1.31	1.00	0.96	0.44	0.14
3Ph	0.52	0.67	1.24	1.00	0.85	0.56	0.25
4Ph	0.39	0.56	1.21	0.92	1.00	0.64	0.19
5Ph	1.78	0.87	1.32	0.75	0.42	0.39	0.12
6Ph	1.22	0.42	1.27	0.87	0.88	0.46	0.14
7Ph	2.79	0.78	1.36	1.04	0.60	0.30	0.05
8Ph	0.47	0.60	1.25	0.92	2.04	2.00	0.85
9Ph	1.03	0.69	1.26	1.00	1.04	0.57	0.09
10Ph	2.02	0.69	1.30	1.00	0.96	0.48	0.13
11Ph	2.82	0.68	1.33	1.10	0.91	0.33	0.11
12Ph	0.98	0.86	1.29	1.00	1.56	0.89	0.25
13Ph	3.40	0.73	1.38	1.00	0.64	0.42	0.08
14Ph	0.87	0.57	1.22	1.00	1.00	2.52	0.16
15Ph	0.52	0.60	1.21	0.89	0.77	0.44	0.23
16Ph	1.18	0.60	1.22	1.35	2.53	1.00	0.14
17Ph	1.45	0.74	1.31	1.00	0.86	0.41	0.19
18Ph	1.26	0.75	1.28	0.88	0.62	0.35	0.11
19Ph	1.35	0.63	1.30	1.00	1.22	0.63	0.13
20Ph	1.09	0.73	1.30	1.18	3.69	1.08	0.13

n/a, not available.

3.1. Hydrophobic retention capacity (k_{PB})

The pentylbenzene was used as the test solute in order to determine the hydrophobicity of the calix[4]arene stationary phases. Its retention factor reflects the surface coverage of the gel and ligand density. Hydrophobic interactions between aliphatic chains are mainly responsible for retention of this alkylbenzene in case of classical *n*-alkyl stationary phases. The calixarene phases exhibit differences in k_{PB} values depending on the type of substituents present at the upper rim of the macrocycle. Generally, *n*-alkyl substituted calix[4]arene phases posses higher values of k_{PB} than their aromatic analogues, and these values are comparable to results obtained for Caltrex B column with *tert*-butyl substituents. However, the pentylbenzene retention capacity factor does not increase in the order of increasing chain length from hexyl- to dodecyl group, and variation in k_{PB} values are not always in correlation with coverage densities of packing materials. Although it is difficult to compare surface density of calixarene and alkyl chain stationary phases, it is worth to notice that LiChrosorb C-18 phase possessing high ligand density (1222 μmol g^{-1}) in comparison to the rest of the investigated columns has hydrophobicity parameter lower than CalixBzNO$_2$ phase with coverage density of 392 μmol g^{-1}. This may be due to dominant role of π–π interactions in the retention mechanism on the calixarene phase.

3.2. Hydrophobic selectivity (α_{CH_2})

Retention factor ratio of pentylbenzene and butylbenzene, known as hydrophobic selectivity, is a measure of ability of the phase to separate alkylbenzene solutes differentiated by one methylene group depending on the hydrophobic potency of the stationary phase and surface coverage. In most cases, the hydrophobic selectivity should correspond to the hydrophobic retention capacity of a given column. However, regardless of the retention power, parameter α_{CH_2} better describes the capability of a packing material to distinguish solutes of similar hydrophobicity. For that reason, not every phase with high hydrophobicity values simultaneously possess comparable high hydrophobic selectivity. As can be seen in Table 2, CalixBz phase has the lowest hydrophobic selectivity of all the calixarene phases, probably due to the low surface coverage (151 μmol g^{-1}). On the other hand, CalixHex and CalixBzNO$_2$ phases of almost equal ligand density differ in hydrophobic selectivities (α_{CH_2} = 1.383 for CalixBzNO$_2$ and

$\alpha_{CH_2} = 1.509$ for CalixHex). In spite of greater retentivity of octadecyl phase with relatively high coverage density, its hydrophobic selectivity is moderate ($\alpha_{CH_2} = 1.421$). Apparently, the calixarene phases have better separation power for alkylbenzene homologues as it was observed by Jira and co-workers [25] for Caltrex phases.

3.3. Aromatic selectivity ($\alpha_{PB/O}$)

Retention factor ratio of *n*-pentylbenzene and *o*-terphenyl describes the aromatic selectivity of stationary phase which is an indication of the capacity of the phase to undergo aromatic interactions with aromatic solutes, as has been previously suggested by Lindner [18,19] and later by Euerby et al. [17]. Making comparison of the results displayed in Table 2, it is evident that only the C-18 phase gives a selectivity factor of $\alpha_{PB/O} > 1$(*o*-terphenyl eluted before pentylbenzene). In contrast, *o*-terphenyl with higher number of aromatic rings than pentylbenzene is retained longer on all the calixarene phases, hence $\alpha_{PB/O} < 1$. The slight variation in aromatic selectivity values within the calixarene phases can arise from differences in chemical character of the substituents present at their upper rims, the steric effect of the analytes, or may result from differences in coverage density of these stationary phases. The aromatic selectivity parameter of CalixBzCl, CalixBzNO_2 and CalixBzF5 columns increases with electron deficiency in their benzyl substituents possessing electron-withdrawing groups (Cl < NO_2 < F). Taking into account the relatively low hydrophobicity of these calixarene phases, it indicates high aromatic interaction with increasing predominance for π–π interaction.

3.4. Shape selectivity ($\alpha_{T/O}$)

Retention factor ratio of triphenylene and *o*-terphenyl is a descriptor influenced by the spacing of the ligands and functionality of the silylating reagent. Both analytes are of nearly the same size (length-to-width ratio). However, they differ in spatial conformation since triphenylene is planar while *o*-terphenyl is twisted out of plane. Values of $\alpha_{T/O} > 3$ represent phases with enhanced shape selectivity whereas values $\alpha_{T/O} < 2$ suggest that a phase has low shape-recognition characteristics [29]. Most of the *1,3-alternate* calix[4]arene phases show similar values of $\alpha_{T/O}$ parameter in range of 1.56–1.77. These results are comparable to data obtained by Euerby et al. [14,17] for a great number of commercially available RP-phases, however they are higher than obtained by Jira for Caltrex A and Caltrex B phases [25]. CalixBzCl, CalixBzNO_2 and CalixBzF5 columns have exceptionally high steric selectivities increasing in order CalixBzCl < CalixBzNO_2 < CalixBzF5 with maximum $\alpha_{T/O} = 4.13$ characteristic for polymeric RP-phases [30,31]. The calixarene molecules seems to be more rigid than octadecyl chains possessing a lower degree of order, therefore application of the "slot model" proposed by Sander and Wise [32] may be questionably useful. Similar conclusions concerning Caltrex phases have been assumed by Jira and co-workers [25]. We rather speculate that shape selectivity of these calixarene columns can be attributed to interactions between the aromatic π-electrons of the test compounds and the Lewis acid sites of *p*-substituted calixarene ligands.

3.5. Hydrogen bonding capacity ($\alpha_{C/P}$)

Retention factor ratio of caffeine and phenol measures the number of available silanol groups and the efficiency of endcapping process. It also illustrates the strength of the hydrogen bonding interactions occurring between surface silanol groups and caffeine. Caffeine will have a stronger retention on stationary phases with high silanol activity, and may even change the elution order with phenol, which results in $\alpha_{C/P}$ values below 1. The immobilization of all the calixarene ligands onto silica gel has been performed by the same hydrosilylation reaction and all the phases have been endcapped by using hexamethyldisilazane. Nevertheless, the values of $\alpha_{C/P}$ differs significantly for these stationary phases and do not correspond to the degree of their surface coverage. For example, CalixNph and CalixBnOMe phases exhibit comparable values of retention factor ratio of caffeine and phenol (1.21 and 1.29), whereas the coverage density of CalixBnOMe column is two times lower than that of CalixNph phase (see Table 1). On the other hand, CalixBzF5 packing possesses highest hydrogen bonding capacity, although its degree of surface coverage places it in the middle of the synthesized calixarene phases. Phenol is retained stronger than caffeine ($\alpha_{C/P} \sim 0.8$) on CalixHex, CalixDdc, CalixBn, CalixAmide and LiChrosorb C-18 columns, while the elution order is opposite on the rest of the calixarene phases. The results demonstrate that the retention factor ratio of caffeine and phenol does not provide a complete description of the hydrogen bonding nature of calixarene stationary phases, probably due to other intermolecular interactions, e.g. π–π interactions and inclusion complex formation.

3.6. Ion-exchange capacities ($\alpha_{B/P}$ at pH 7.6 and pH 2.7)

The retention factor ratio of benzylamine and phenol at pH 7.6 estimates the total silanol activity of stationary phase, while analogous value obtained at pH 2.7 is a measure of the acidic activity of the silanol groups. Phenol (pK_a 10.0) is not ionized at both pH levels of mobile phase and it should have constant retention times. On the other hand, benzylamine exists in ionized form (pK_a 9.4) under both pH conditions, thus at the two pH levels of mobile phase it interacts with different types of silanols which undergo deprotonation to the extent strongly dependent on their pK_a value (from 1 to 10). It is postulated [33,34] that at pH 2.7 a small but highly acidic silanol population less than 1% is dissociated and therefore can interact with benzylamine via ion-exchange interaction. At pH 7.6 nearly 50% of silanol groups are fully dissociated producing stronger retention of benzylamine and higher value of $\alpha_{B/P}$. All the stationary phases in our tests show larger $\alpha_{B/P}$ values at pH 7.6. This strong tendency is especially evident for CalixBzF5, CalixBn, CalixNph, CalixBph, and CalixBnOMe phases. This behavior is justifiable for CalixNph and CalixBnOMe phases, which have the lowest surface coverage of the gel, however for the rest of the phases these results are unexpectedly high. Again, we assume that not only ion-exchange, but also other specific effects, e.g. host-guest interactions could be responsible for the strong retention of benzylamine on calixarene columns. The apparent silanol activity of all the calixarene phases was lower than that of LiChrosorb C-18 which may be due to the steric hindrance caused by bulky ligands.

3.7. Chemometrics

Chemometric approach was applied to the column evaluation due to lack of sufficient theory and ambiguous interpretation of the chromatographic test results. The data were normalized to give all variables the same importance by subtracting the variable average form each variable and dividing the difference by standard deviation. The number of principal components was chosen on the basis of magnitude of the eigenvalues which is a measure of the amount of information conveyed by each principal component. In the instance of calixarene column data set we applied the scree test and the first three PCAs with eigenvalues above unity were kept. Comparison to selected commercially available columns was done using the same number of PCAs although in this case the decision of retaining the third of them with eigenvalue of 0.89 was somewhat ambiguous. The clustering of the columns was done using *k*-mean algorithm with number of clusters equal to the number of principal components retained.

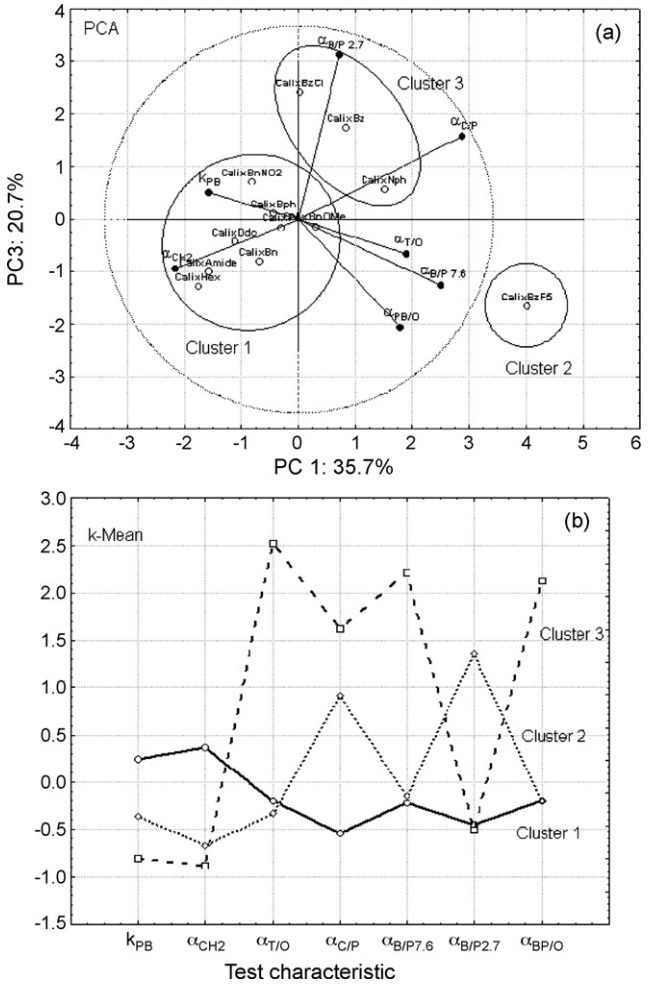


Fig. 2. PC1–PC3 plots of *1,3-alternate* calix[4]arene columns for the Tanaka and aromatic selectivity probes: (a) score plot and (b) *k*-mean cluster analysis.

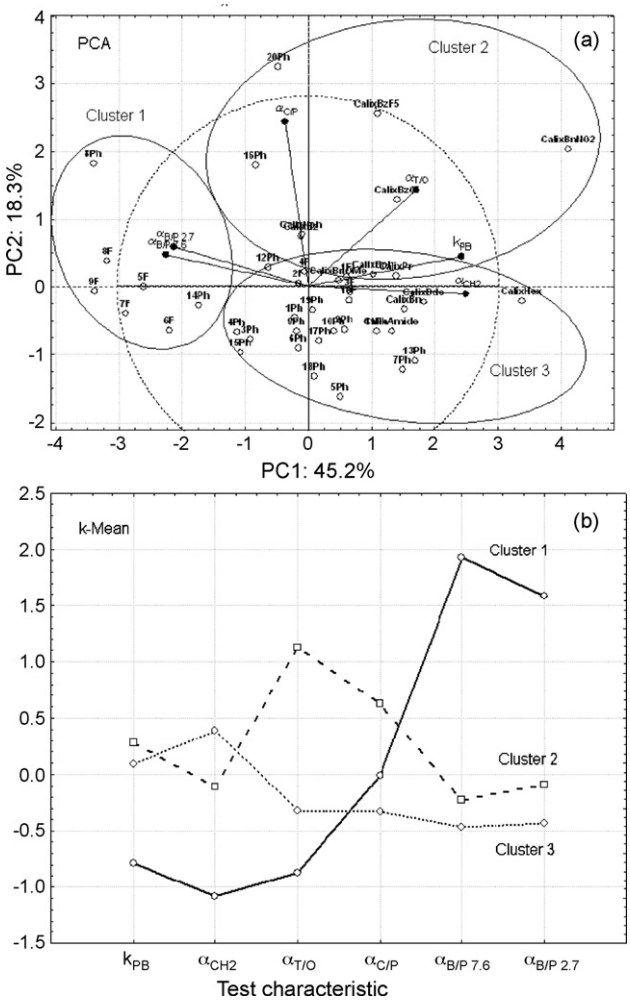


Fig. 3. PC1–PC2 plot of *1,3-alternate* calix[4]arene, phenyl and perfluorinated columns for the Tanaka probes: (a) score plot and (b) *k*-mean cluster analysis.

3.8. Calixarene column data set

At the first approach, the data of the seven chromatographic tests described in Sections 3.1–3.6 were used to compare the twelve calixarene phases. Caltrex phases were not included into the set, because Jira and co-workers [25] provided data only for k_{PB}, α_{CH_2} and $\alpha_{T/O}$ which would significantly reduce the dimension of the test space. Principal component analysis indicates that the seven tests are correlated to some extent, with maximum correlation factor of hydrophobic retention capacity (k_{PB}) to hydrophobic selectivity (α_{CH_2}) approaching $r \sim 0.6$. This can be rationalized from chromatographic point of view since the probe compounds undergo not only the sole interaction process the test is aimed at but also complex chromatographic process described in Sections 3.1 and 3.2. The first three principal components explain about 80% of variability observed in the calixarene stationary phases set. The stationary phases may be divided into three clusters shown in Fig. 2 together with vector loadings. The PC1–PC3 score plot explaining over 56% of the chromatographic variability is displayed instead of PC1–PC2 plot (~58% of variability) because this projection better visualized differences and similarities between tested calixarene columns. *k*-Mean cluster analysis points out that the calixarene stationary phases belonging to cluster 1 have better hydrophobic selectivity and higher hydrophobic retention capacity than the rest of the columns. The stationary phase with pentafluorobenzene moiety is exclusive in shape selectivity, hydrogen bonding capacity, total silanol activity and aromatic selectivity (cluster 2). The third cluster consists of CalixBzCl, CalixBz and CalixNph stationary phases with distinct mean value of silanol groups acidic activity.

3.9. Comparison to selected commercially available columns

The twelve calixarene stationary phases were compared to the set of commercially available columns with chemical structures resembling the building blocks of the calixarene phases. The data for stationary phases bearing phenyl, fluoroalkyl and fluorophenyl moieties selected from papers published by Euerby et al. [14,16,17] include six parameters of Tanaka tests. Therefore, aromatic selectivity of the calixarene phases was excluded from the test results and the evaluation was carried out in six-dimension space. The space dimension was reduced to three principal components describing about 75% of the total variability. Hydrophobic retention capacity was found again to be significantly correlated to hydrophobic selectivity ($r \sim 0.75$), and total silanol activity was correlated to acidic activity of the silanol groups ($r \sim 0.59$). The evaluated columns may be divided into three clusters (Fig. 3, for column description see Tables 1 and 2). The five fluoroalkyl and two phenyl stationary phases belonging to cluster 1 have high silanol group activity. Another five fluorophenyl and two phenyl columns together with CalixBz, CalixBzCl, CalixBnNO$_2$, CalixBzF5 and CalixNph stationary phases have elevated shape selectivity in comparison to the rest of the assessed columns (cluster 2). CalixPr, CalixHex, CalixDdc, CalixBn, CalixBph, CalixBnOMe, and CalixAm-

ide columns were found to be similar to the functionalized phenyl or mixed C-18/phenyl stationary phases (cluster 3 in Fig. 3). The results are in good agreement with column classification published by Euerby et al., although the set of data for PCA was different in each case. The cluster 1 corresponds to group containing fluoroalkyl bonded phases and the cluster 2 matches the group of fluorophenyl phases found in the investigation of fluorinated stationary phases [16]. The third cluster corresponds to heterogeneous group of phenyl phases described in [17], although in this investigation the new probe (nitroaromatic solutes) of π-acidity was included into primary component analysis along with Tanaka test results.

The principal components loading and the tests characteristics correlation observed for the two data sets together with Euclidian distances of the tested stationary phases are included in Supporting Data.

4. Conclusion

The examined twelve calixarene stationary phases comprise two building blocks with considerably different physico-chemical properties. The first is a calix[4]arene moiety with a hydrophobic cavity capable of forming inclusion complexes with guest molecules. The second block consists of various aliphatic or aromatic substituents positioned at the upper rim of the calixarene scaffold. Due to the fact, that these two components are placed in direct vicinity and possess different chemical properties, various interactions between analytes and the calixarene moieties may be expected.

The present paper describes chromatographic characterization of the *1,3-alternate* calix[4]arene columns by using Tanaka test protocol and approach proposed by Lindner. The phases have been evaluated in terms of their surface coverage, hydrophobic selectivity, shape selectivity, hydrogen bonding capacity, ion-exchange capacity at pH 2.7 and 7.6 as well as aromatic selectivity. The chromatographic parameters obtained for the calixarene columns have been compared to Caltrex phases and additionally to LiChrosorb C-18 phase used as reference RP column.

Calixarene phases generally exhibited lower hydrophobic retention capacity than LiChrosorb C-18 phase, however CalixBzNO$_2$ column was the exception. The k_{PB} value of this phase was highest in spite of its low coverage density in comparison to the octadecyl column. This may be due to π–π and charge-transfer interactions playing an important role in retention of pentylbenzene on CalixBzNO$_2$ phase beside hydrophobic interactions.

In aromatic selectivity test, it was found that only the C-18 phase gives the selectivity factor above 1, whereas on all the calixarene phases *o*-terphenyl was retained longer than pentylbenzene, producing $\alpha_{PB/O}$ values below 1. The aromatic selectivity parameters of CalixBzCl, CalixBzNO$_2$ and CalixBzF5 columns increased in order of increasing electron deficiency in the phenyl rings derivatized by electron-withdrawing groups (Cl < NO$_2$ < F$_5$). It can be explained by predomination of charge-transfer over π–π interactions in retention mechanism.

All the tested columns exhibited comparable values of methylene selectivity, however the calixarene phases had better separation properties for alkylbenzenes than can be expected from their hydrophobic retention capacity. The results imply that different sites of the calixarene phases contribute to the selectivities toward these solutes. Most of the *1,3-alternate* calix[4]arene phases showed similar values of shape selectivity ($\alpha_{T/O} \sim 1.7$), comparable to LiChrosorb C-18 column, but higher than those obtained on Caltrex phases. Enhanced shape selectivity ($\alpha_{T/O} > 3$), characteristic for polymeric-type RP-phases, was observed on CalixBzNO$_2$ and CalixBzF5 columns. In this case, shape selectivity must be attributed to interactions between the aromatic π-electrons of the tested compounds and the Lewis acid sites of *p*-substituted calixarene ligands.

The calixarene phases showed significant differences in hydrogen bonding capacities ($\alpha_{C/P}$) which did not correspond to the degree of their surface coverage. Moreover, some calixarene columns retained phenol stronger than caffeine, whereas on the other phases the elution order was opposite, without any connections to their ligands densities, the type of silica gel or the character of substituents attached to the upper rim of calixarene molecules. All these facts suggest that this test is not suitable for determination of the number of available silanol groups on calixarene phases. Probably other intermolecular interactions (e.g.: π–π, inclusion complex formation) may occur between phenol, caffeine and calixarene ligands.

CalixBzF5, CalixBn, CalixBph, and CalixBnOMe columns exhibited highest total silanol activity among calixarene phases. The high value of $\alpha_{B/P}$ parameter for the two last phases could be explained by their relatively low coverage densities. However, the results for CalixBzF5 and CalixBn columns were extraordinary. Again, it is most likely that the enhanced retention of benzylamine on these phases may be attributed to π–π interactions of benzylamine (non-ionized at pH 7.6) and the aromatic-rich stationary phases. CalixBzCl and CalixBz packing material exhibit in contrast the highest acidic activity of the silanol groups at pH 2.7.

Principal component analysis identified some relationships between seven parameters studied on calixarene phases. These phases can be divided into three sub-groups. The cluster 1 grouped columns which were characterized by better hydrophobic selectivity and higher hydrophobic retention capacity. All *n*-alkyl substituted calixarene columns and five phases possessing aryl substituents belong to this group. The cluster 2, located in the lower right quadrant in Fig. 2, is created by the single CalixBzF5 column. This phase exhibited enhanced shape selectivity, hydrogen bonding capacity, total silanol activity as well as aromatic selectivity. Based on the chemical structure of CalixBzF5, it can be concluded that fluorine atoms present at the phenyl substituents are responsible for its specific character. CalixNph, CalixBz and CalixBzCl columns, which formed the third cluster, displayed high silanol activity. It could be explained by relatively low surface coverage of CalixNph and CalixBz phases, however the reason of enhanced acidic activity of the silanol groups for CalixBzCl column was not clear.

The comparison of calixarene columns to selected commercially available phenyl and perfluorinated stationary phases (PC1–PC2 score plot in Fig. 3) showed that the forty-two evaluated stationary phases can be categorized into three clusters containing columns, which possessed different dominant chromatographic properties. The smallest cluster 1 assembles all fluoroalkyl columns with enhanced total ion and acidic ion-exchange capacities. Perfluorophenyl columns as well as fife calixarene phases with aryl substituents including electron-withdrawing groups (fluorine, chlorine, and nitro group) belong to the cluster 2. These phases are distinguished by high retentivity and shape selectivity in comparison to the rest of the columns. The last cluster 3 consists of phenyl phases, all *n*-alkyl substituted calixarene columns and four packing materials coated by aryl substituted calixarene ligands. The high methylene selectivity seems to be dominant for these phases.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2009.11.052.

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