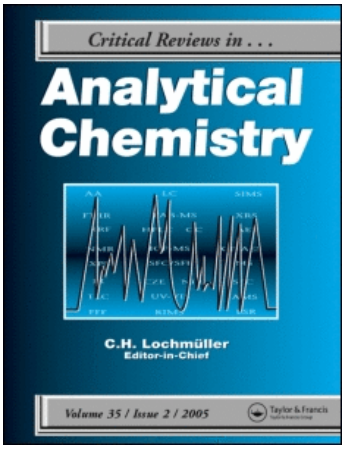

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Calixarenes as Stationary Phases in High Performance Liquid Chromatography

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Calixarenes are a class of host molecules with three-dimensional cavity capable of accepting guest molecules. The interest of calixarenes in analytical and separation chemistry has increased in recent years because of their ability to form reversible complexes with both neutral and charged molecules. Calixarenes have been utilized in gas chromatography, solid-phase extraction, capillary electrophoresis and overall in high performance liquid chromatography. This short review is focused on recent advances in synthesis and characterization of calixarene, calixresorcinarene and calixpyrrole stationary phases, chemically bonded or dynamically adsorbed onto silica gel or used as mobile phase additives, and its application to separation of organic and inorganic solutes by high performance liquid chromatography.

Keywords calixarene, calixresorcinarene, calixpyrrole, stationary phase, separation, analytes, high performance liquid chromatography

INTRODUCTION

Since identification and rationalization of calixarenes and the structurally related calixresorcinarenes (resorcinarenes) and calixpyrroles syntheses, they have been of interest in almost all fields of chemistry. Starting from pioneering work of Gutsche, the chemistry of these metacyclophanes has been extensively discussed in several books concerning synthesis (1, 2) structural features and host-guest interactions (3–5). Many review articles deal with more specific applications of calixarenes, e.g., in separation chemistry (6–8). In the subject of application of these macromolecules in chromatography, relatively recent literature has been available. There are several reasons for the current widespread interest in calixarenes in chromatography. One of them is the remarkably simple way of synthesis and modification of the parent compounds. In addition, lower and upper rim functionalization of these macrocycles resulted in massive expansion in the range of derivatives available. The properties of calixarenes are also strongly influenced by its conformation, which is fixed after introduction of bulky substituents at the phenolic oxygen atoms.

To summarize, calixarenes represent compounds with great potential for chemical alternation through variation of the ring-size and by insertion of functional groups at the upper and lower rim of the macrocycle. The interest in calixarenes as stationary phases in chromatography resulted from unique opportunities to influence the specificity and selectivity of the macrocycles. However, in contrast to crown ethers and cyclodextrin, the host-guest interactions of calixarenes with solutes are not determined solely by their hydrophobic cavities, but are also influenced by additional functional groups attached at their rims. It can contribute to potential variation in these interactions. The great potential of this class of macrocyclic compounds has been shown for several applications e.g., in gas chromatography (9–16), capillary electrophoresis (17–30), solid phase microextraction (31–38) and overall high-performance liquid chromatography.

Until now, calixarenes have been chemically bonded or dynamically adsorbed onto silica gel or used as mobile phase additives in reversed-phase liquid chromatography.

The present review dealing with recent advances in synthesis and characterization and HPLC application of calixarene, calixresorcinarene and calixpyrrole stationary phases. The paper is organized according to the structures and cavity size of calixarene derivatives used as selectors in HPLC and type of anchor molecule immobilizing macromolecules to the solid support.

CALIXARENE AND CALIXRESORCINARENE STATIONARY PHASES CHEMICALLY BONDED ONTO SILICA GEL

Calixarene in *Cone* Conformation

Calix[n]arenes (n = 4,5,6,8) with tert-Butyl Substituents at the Upper Rim. Historically first p-*tert*-butyl calix[4]arene HPLC stationary phase **1a** (Figure 1) was synthesized by Friebe

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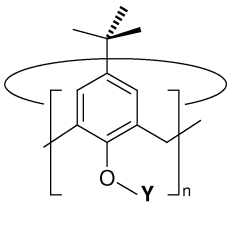


1

1	n	Y
a	4	spacer—†
b-e	4,5,6,8	CH_2C(=O)–spacer—†
f-h	4,6,8	$(CH_2)_3$—††
i-k	4,6,8	$CH_2CH(OH)CH_2O(CH_2)_3Si$(I)O—
l	8	$(CH_2)_3Si$(I)O—
m	8	$P(OC_2H_5)_2$ (OH)
n-s	8-12	H

†Spacer not specified by the authors
††Caltrex B phases

FIG. 1. Structures of stationary phase type 1.

and co-workers (39) in the reaction of parent calixarene modified in the narrow rim and irregular silica gel via short, not specified by the authors, hydrophilic spacer. The structure and purity of novel phase were checked by infrared, elemental analyses and electron microscopy. The linkage to the matrix showed high chemical stability with respect to many solvents used over 6-month period of time. p-*tert*-Butyl calix[4]arene stationary phase was successfully applied to the HPLC separation of position isomers of nitroaniline, nucleosides (cytidine, uridine, guanosine, adenosine) and *cis*/*trans* isomers of proline containing dipeptides (N = 18,000/m). The results indicated that the calixarene silica gel behaves predominantly as a reversed-phase material. It is assumed that charge transfer, π-donor-π-acceptor and electrostatic interactions between the substituted aromatic ring of the calix[4]arene and the analyte molecules are responsible for discrimination process, though. Carboxylic acid derivatives of p-*tert*-butyl calix[n]arenes (n = 4, 5, 6, 8) **1b-e**, similarly attached to the silica gel, were tested in separation of regioisomers of nitroaniline and diphenols, uracil derivatives, estradiol stereoisomers and *cis*/*trans* isomers of proline containing dipeptides (40, 41). It was demonstrated that regioisomers of nitroaniline were best resolved on calix[4]- and [6]arene stationary phases (elution order: *meta*<*para*<*ortho*), whereas the best results for diphenols isomers were obtained on calix[8]arene (*para*<*meta*< *ortho*). Separation of uracil derivatives (5-hydroxymethyluracil, uracil, 5-methyluracil, 6-methyluracil, 3-methyluracil) achieved optimal parameters at pH 3 of the buffered mobile phase, and increasing cavity size of calixarene only marginally improved resolution.

Chromatographic behavior of p-*tert*-butyl calix[n]arenes (n = 4,5,6,8) stationary phases checked in separation of estradiol stereoisomers (17α- and 17β-estradiol), showed their capable selectivity. With an increasing ring-size of calixarenes, a significant increase in retention time was observed in connection with slightly higher resolution factors of the epimers. Experiments with isomers of proline containing dipeptides show for instance, that Ala-Pro was only separated by the use of the calix[6]arene column, whereas resolution of dipeptides with bulky, hydrophobic side chain (Phe-Pro, Ile-Pro) increased on the calix[8]arene. The composition of mobile phase (cation in the buffer formula, acetonitrile concentration) showed a pronounced influence on the resolution of the dipeptides conformers.

Patented materials known as Caltrex B **1f-h** contain propylether spacer at the lower rim of p-*tert*-butyl calix[n]arenes (n = 4, 6, 8), covalently linked to the silica gel (42). Several analytes were tested to demonstrate the potential of these calixarene-bonded stationary phases: aromatic positional isomers (43, 44), phenol derivatives, alkylated and unsubstituted aromatics, benzoic acid esters, PAHs, barbituric acid derivatives, xanthine (45–47) and dibenz[*b*, *e*]oxepin derivatives, thioxanthene *cis*/*trans* isomers (48) and steroids (49–50).

Caltrex B phases exhibited high selectivity toward analytes of a very similar structure, e.g., aromatic positional isomers. Separation of a substituted phenols mixture (phenol, guaiacol, p-hydroxybenzoic acid methyl ester, p-hydroxybenzoic acid ethyl ester, β-naphtol, α-naphtol, thymol, p-hydroxybenzoic acid propyl ester) showed a strong dependence on the ring-size of the calixarene. It was postulated that this effect is possibly related to differences in the strength of the inclusion of the analytes in calixarene cavities based on the size of analytes. In contrast, separations of alkylated and unsubstituted aromatics (3,5-dinitrobenzoic acid, 2,6-dinitrotoluene, acetophenone, benzene, toluene, ethylbenzene, naphthalene, anthracene) were not complete which was explained by the presence of distinct substituents-dependency at the upper rim of the calixarenes for p-*tert*-butyl groups. The same hypothesis was supported for separation of benzoic acid methyl, ethyl, propyl, butyl and benzyl esters separated on Caltrex B columns.

The separation of selected PAHs (naphthalene, acenaphtalene, fluorine, phenanthrene, anthracene, fluoranthene, chrysene, perylene, benzo[*a*]pyrene) provided an example for differences between calixarenes cavities size. Better separations were observed on phases containing larger calixarenes than on those with calixarenes of a smaller ring-size. Detected favorable inclusion of large PAHs into calix[8]arenes relative to calix[4]arenes, which might contribute to a better selectivity of those hosts. The best chromatographic separations of barbituric acid derivatives (barbital, primidone, crotylbarbital, phenobarbital, hexobarbital, pentobarbital, phenytoine) were attained on p-*tert*-butyl calix[4]arene using water/1,4-dioxane (7:3, v/v) as eluents. The advantages of this chromatographic system result from the partial adsorption of dioxane onto stationary phase, enabling formation of hydrogen bonds between NH-acid solutes and dioxane molecules.

Caltrex B phases showed very different behavior in separating xanthine derivatives (xanthine, diprophylline, theobromine, theophyline, etophyline, proxyphyline, caffeine) and was strongly influenced by the kind of organic modifier of the mobile phase used. p-*tert*-Butyl calix[4]arene completely

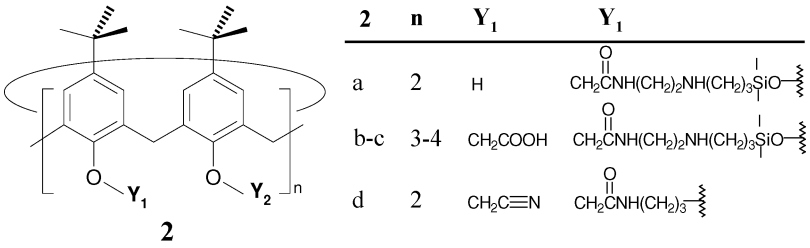


FIG. 2. Structures of stationary phase type 2.

separated diprophylline and theobromine whereas on p-*tert*-butyl calix[8]arene phase this separation was not achieved.

The selectivity of Caltrex B phases for *cis/trans* geometric isomers of doxepin and thioxanthene derivatives (flupentixol, clopenthixol, chlorothixene) was investigated as a function of the ring-size of the calixarene phases. In all cases *cis*- isomers of thioxanthenes eluted before *trans* due to stronger interaction between the steric less hindered halogen substituted aromatic of the *trans* isomers with the host molecules. Columns with the largest ring-size of the calixarenes were not able to separate both geometric isomers completely. In contrast to the thioxanthenes, *trans* isomers of dibenz[*b*, *e*]oxepin eluted before *cis*, moreover Caltrex B phases showed similar selectivity independent from the calixarene cavity size. Separations of a mixture of four steroids (norethisterone, chlormadinon acetate, norethisterone acetate, and testosterone propionate) were also complete on all investigated columns, regardless of their ring-size. It was concluded that the main retention mechanism is due to hydrophobic interactions because all separated steroids were separated in accordance to their lipophilicity.

Several p-*tert*-butyl calix[n]arene (n = 4,6,8) stationary phases which were fixed to the silica gel via the narrow rim by different spacer groups were synthesized by the research group from Wuhan University. Their research program focused on determination of the influence of the spacer linkage on stability and selectivity synthesized calixarene stationary phases. Experiments concerning the effect of calixarene ring-size on selectivity were also performed.

p-*tert*-Butyl calix[4]arenes **2a** (Figure 2) were linked to the silica gel support via γ-(ethylenediamino)-propyltriethoxysilane (51, 52), whereas phase **1i** via 3-glycidoxypropyltriethoxysilane (53). The structures of bonded phases were characterized by ^{29}Si and ^{13}C solid-state NMR, FTIR and elemental analysis. Chromatographic performance of the phases were performed by using PAHs, nucleosides, nucleobases, aromatic positional isomers, N-substituted anilines, aromatic amines and sulfonamides as solutes. All the mentioned analytes were well resolved. The elution order for PAHs and nucleosides was similar to the ODS phase which was explained by predominant reversed-phase character of the examined calixarene phases. Higher selectivity toward alkylbenzenes on p-*tert*-butyl calix[4]arene end-capped phase **2a** (52) was observed than on ODS as a result of host-guest interaction between the phase and solutes. In separation of sulfonamides the steric and hydrogen bonding interaction was detected.

p-*tert*-Butyl calix[6]arenes **1j**,**2b** (54–56) and p-*tert*-butyl calix[6]-1,4-benzocrown-4-arene **3** (57) (Figure 3) were immobilized onto silica gel by using the same above-mentioned coupling reagents. Better surface coverage of calixarene molecules on silica was achieved for γ-(ethylenodiamino)-propyl spacer group. Chromatographic efficiency of phase **1j** was evaluated by using nitrophenol, aminophenol and nitroaniline positional isomers, PAHs and nucleosides as analytes. The selectivity for selected solutes was acceptable, however resolution of aminophenol and nitrophenol isomers was not complete because of poor surface coverage of that phase (0.06 mmol/g). Screening evaluation of calixarene phase **2b** linked to the solid support via γ-(ethylenediamino)-propyltriethoxysilane, higher efficiency and selectivity toward selected nucleosides, nucleobases, sulfonamides and quinolones were observed in comparison to the **1j** phase.

While making comparison between p-*tert*-butyl calix[4]arene **2a**, **1i** and p-*tert*-butyl calix[6]arene **2b** phases, the same elution order was observed for examined nucleobases (cytosine<uracil<hypoxanthine<guanine<6-mercaptopurinr <caffeine) and sulfonamides (sulfadiazine<sulfadimidine< sulfamethoxazole <sulfaacetamide<trimethoprim). The influence of mobile phase variables (methanol content, ionic strength, ion-pairing agent and pH) on retention of the above-mentioned compounds was also evaluated. For these new HPLC phases, RP-analogous retention behavior was detected, however inclusion complexation and charge interactions were taken into consideration. The chromatographic performance of p-*tert*-butyl calix[6]-1,4-benzocrown-4-arene **3** was evaluated in separation of PAHs, N-substituted anilines and several aromatic

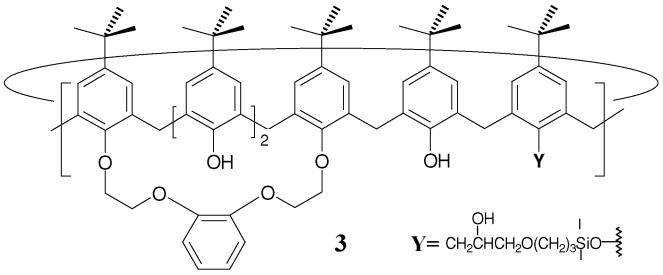


FIG. 3. Structures of stationary phase type 3.

positional isomers. Lower selectivity toward PAHs with the same elution order was observed on **3** phase in comparison to **2b** phase which was explained by ether-bridge substituents stiffen conformation. In contrast, the baseline separation of positional isomers and N-substituted anilines were achieved on calix[6]-1,4-benzocrown phase with the greater retention than those on p-*tert*-butyl calix[6]arene **2b** phase. The results were explained by additional hydrogen bond interaction between hydrogen-donor substituents of the analytes and the oxygen atoms of the ether bridge existed on phase **3**.

The researchers' great attention was paid to synthesis and evaluation of p-*tert*-butyl calix[8]arene stationary phases. Immobilization based on treatment of appropriate calixarene derivatives with γ-(ethylenediamino)-propyltriethoxysilane **2c** (58), 3-glycidoxypropyl-triethoxysilane **1k** (59-63) and chloropropyltriethoxysilane **1l** (64, 65) as coupling reagents. The coverage densities of calixarenes on silica gel were comparable for these calixarene phases, however bonded amount of phase **1l** was the highest (0.092 mmol/g). The columns (**2c** and **1k**) efficiency was first determined by separations of PAHs mixture (58, 59). The baseline resolution was obtained on both phases with the same elution order of the analytes (benzene< toluene<xylene<naphthalene<α-methylnaphtalene<biphenyl <fluorine<anthracene<*ortho*-terphenyl) which also corresponded to that achieved on p-*tert*-butyl calix[4]arene stationary phases **1i** under the same chromatographic conditions. Moreover, retention of PAHs on phases **2c** and **1k** was greater than that on **1i** according to the greater ability of calix[8]arene in the formation of inclusion complexes. HPLC stationary phase **1k** was also used for studies with aromatic positional isomers (59), aromatic carboxylic acids (60), nucleosides and nucleobases (61), water soluble vitamins (62), N-substituted anilines, phenols and *azo*-PAHs (63). Chromatographic performance of phase **1l** was evaluated in separation of steroid hormone (64, 65). All the investigated analytes were well resolved on the above-mentioned phase.

Stronger retention and better selectivity were observed in separation of aromatic carboxylic acids, nucleosides and nucleobases and water soluble vitamins in comparison to the ODS phase under the same chromatographic conditions. In contrast, weaker retention relative to ODS was detected for *azo*-PAHs, phenols and N-substituted anilines. The retention behavior of steroid hormone medicines on **1l** phase was additionally compared to β-cyclodextrin stationary phase, on which the optimal resolution of these solutes was obtained. The influence of mobile phase composition (methanol content, pH and ionic strength) on retention behavior of the examined analytes was studied. According to the chromatographic data it was concluded that calixarene stationary phases behave as a reversed-phase material, but other retention mechanisms (hydrogen bonding interaction, π-π interaction, π electron transfer interaction and inclusion complexation) occur in the separation process.

The chromatographic separation of selected nucleobases (adenine, adenosine, cytosine) and aromatic compounds (phenol, benzene, toluene) were investigated on p-*tert*-butyl-25,27-bis(cyanomethoxy)-26-(chloroformyl)-28-hydroxy calix[4]-arene bonded aminopropyl silica gel stationary phase **2d** (66). The effective separation ability of the **2d** was observed for target compounds. Again, hydrophobic interactions were appointed as a dominant in separation process, however, interactions between the nitrile groups, substituted at lower rim of calixarene, and polar analytes were also considered.

Calix[n]arenes (n = 4,5,6,8) with Other Substituents at the Upper Rim. p-Propyl calix[4]arenes ethylacetate **4a** and p-propyl calix[6]arenes ethylacetate **4b** (Figure 4) were obtained through immobilization of triethoxysilane derivatives of p-allyl calix[n]arene (n = 4,6) in the presence of hexachloroplatinic acid (H_2PtCl_6) as a catalyst. These phases fixed to the silica gel at the upper rim were employed for the separation of alkali and alkaline earth metal chlorides with conductivity detection (67). Selectivity for sodium ions over potassium and cesium ions was observed on calix[4]arene stationary phase **4a** with methanol/water mobile phase (7:3, v/v). The retention order being sodium> potassium>caesium>lithium ions was detected. In contrast, little selectivity was observed on the calix[6]arene hexaethyl ester stationary phase **4b** and only increases in retention and no improvement in selectivity was observed with increasing organic modifier concentrations.

Silica-bonded tetrameric calix[4]arene ester stationary phase **4a** has been also used in the chromatographic separation of amino acid esters. Under reversed-phase conditions, baseline resolution of standard test mixture of benzamide, benzophenone and biphenyl was achieved using methanol/water mobile phase. Retention of the amino acid ester hydrochlorides (L-aspartyl-L-phenylalanine methyl ester, β-alanine ethyl ester and D-tryptophan methyl ester) appeared to be related to the hydrophobicity of the ester studied (68). p-di-Propylsulfide calix[4]arene tetraacetyldiethylamide **4c** and p-di-propylsulfide calix[4]arene tetrahydroxamate **4d** were prepared by the treatment of the p-allyl calix[4]arene derivatives with mercaptopropyltriethoxysilane in the presence of cumene hydroperoxide and subsequent reaction with silica gel (69).

The chromatographic performance of tetradiethylamide phase **4c** was investigated using alkali and alkaline earth metal ions (71). Selective retention of sodium ions over other alkali ions and of calcium over magnesium ions was found using water as the mobile phase and conductivity detection. A series of amino acid ester hydrochlorides was shown to be retained according to their hydrophobicity on tetraester phase **4a** with aqueous mobile phases containing lithium perchlorate and acetonitrile. Calix[4]arene tetrahydroxamate phase **4d** enabled the selective pre-column concentration of lead traces in water samples collected from a river polluted by industrial effluent discharge (71). Specifically, a silica-bonded calix[4]arene, functionalized at the lower rim with L-(-)-ephedrine **4e**, was prepared from triethoxysilyl derivatives of appropriate derivatives of calixarene. Using reversed-phase conditions, such functionalised

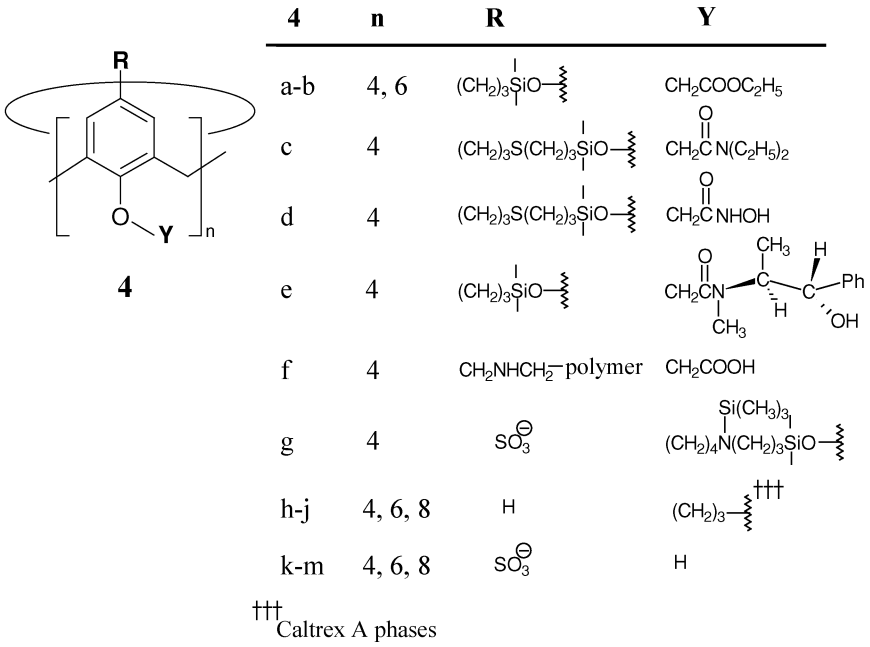


FIG. 4. Structures of stationary phase type 4.

chiral phase was capable of resolving the enantiomers of *R*(-) and *S*(+)-1-phenyl-2,2,2-trifluoroethanol in less than 45 seconds at 4.0 ml/min flow rate (72).

Novel type of calix[4]arene carboxylate resin **4f** immobilized with polyallylamine was prepared to investigate the adsorption behavior for lead and zinc ions (73). It was found that the resin possesses significantly higher separation efficiency for lead away from zinc. The lead concentration could be enriched almost 200-fold. It was confirmed to highly selectively adsorb trace amount of lead ions over large excess amount of zinc ions.

p-Sulfonate calix[6]arene stationary phase **4g** fixed on silica gel via 3-aminopropyldiethoxysilane was used in the reversed-phase separation of mono-substituted phenol regioisomers (cresol, methoxyphenol, nitrophenol, aminophenol and chlorophenol) and some other positional isomers (nitroaniline, nitrotoluene, 1-bromo-2-nitrobenzene, 1-bromo-4-nitrobenzene, 1,3-dichlorobenzene and 1,4-dichlorobenzene) (74). Phenol isomers with polar and hydrogen bonding groups could be well separated while isomers with non-polar groups were only partially resolved by highly aqueous, methanol mobile phase.

Another group of patented columns material Caltrex AI-AIII (**4h**, **4i**, **4j**), possessing hydrogen atoms at *para* positions of calix[n]arene (n = 4,6,8) molecules, were used for separation of the all earlier mentioned analytes (see paragraph 1) under the same chromatographic conditions as for Caltrex B phases. The results showed that Caltrex A phases possess considerable selectivity for chromatographic separations. Comparative study between phases Caltrex A and Caltrex B (45, 48–50) showed in many cases formation of stronger inclusion complexes for Caltrex B with solutes (e.g., PAHs, barbituric acid derivatives, thioxanthene derivatives, structurally similar steroids), which might be explained by a stabilizing effect of the p-*tert*-butyl substituents present in the upper rim of Caltrex B phases or by additional interactions of these substituents with solutes. On the other hand, better separations of alkylated and unsubstituted aromatics, benzoic acid esters and dibenz[*b*, *e*]oxepin derivatives with phases containing bond calixarenes without such substituents were observed, indicating that it is not possible to predict chromatographic selectivity for a given mixture. Caltrex A phase **4j** was also tested in separation of selected tricyclic neuroleptics (75) and retinoids (76). Here, formation of inclusion complexes with the analytes appeared to benefit the separation process in comparison to the other commercially available RP-phases. The effect of different chromatographic conditions (buffer system, pH-value, type and content of organic modifier) and effect of structural differences of the analytes were also studied.

The diastereomeric calixarene stationary phases **5a** and **5b** (Figure 5) were synthesized by inter-linking 9-amino-9-deoxyquinine, 9-amino-9-deoxyepiquinine and calix[4]arene platform via an urea functional units (78) and subsequent reaction with mercaptopropyl silica gel. The chiral recognition of N-protected cyclic and acyclic amino acids was achieved on these phases in buffered water/organic modifier mobile phases. The quinine containing phase **5a** showed higher enantioselectivity for open-chained amino acids bearing π-acidic protecting groups. The epiquinine type phase **5b** exhibited broad chiral recognition capacity for open-chained and cyclic amino acids.

Calixarenes in 1,3-*Alternate* Conformations

1,3-*alternate* calix[4]arenecrown-6 **6a-b** and calix[4]arenecrown-5 **6c** (Figure 6) were obtained by covalently linking

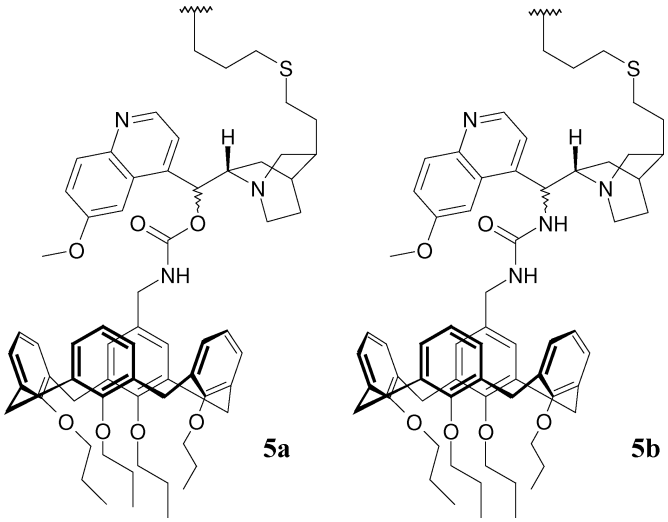


FIG. 5. Structures of stationary phase type 5.

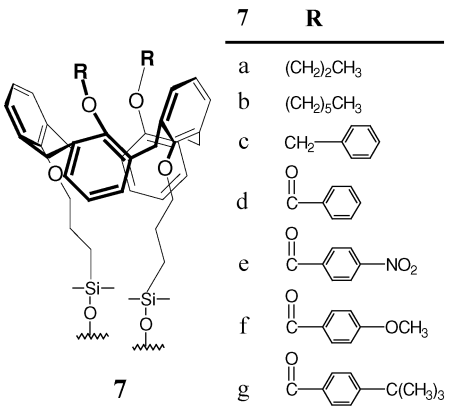


FIG. 7. Structures of stationary phase type 7.

suitably derivatized calix[4]arene receptors onto silica gel via p-allyl or p-1-octene residues in the reaction with triethoxysilane (78, 79). The degree of coverage of the stationary phases was estimated by elemental analysis and X-ray photoelectron spectroscopy (XPS) data. The novel packing material exhibited high selectivity toward alkali metal ions. Complete separations of cesium ions from potassium and sodium ions were achieved on **6a** and **6c** phases by using water/methanol mixture (8:2, v/v) as a mobile phase. No separation of cesium from potassium ions was observed on calix[4]arenecrown-6 **6b** phase under the same chromatographic conditions.

A series of 25,27-disubstituted calix[4]arene stationary phases **7a-g** (Figure 7) blocked in 1,3-*alternate* conformation were prepared by immobilization of appropriate calix[4]arene allyl-derivatives in hydrosililation process with high yields (coverage density ~350 μmol/g). Chromatographic performance of the above-mentioned phases were tested in separation of various analytes with neutral, acidic and base character, including compounds biologically active one. The baseline resolutions of: aromatic positional isomers (80), phenol derivatives (81), PAHs (82), purine and pyrimidine bases, non-steroidal anti-inflammatory drugs (NSAIDs) and water-soluble vitamins (83–85) were demonstrated. In many cases separations of the studied analytes were better on novel calixarene phases in comparison to the ODS one under the same chromatographic conditions.

Moreover, calixarene phases exhibited stronger retention power toward ionized solutes than ODS. Relations between structural elements of additional substituents introduced to 27,27 positions in the upper rim of calix[4]arene molecule (aliphatic chains **7a-b** and aromatic rings with electron-releasing or electron-withdrawing groups **7c-g**) and the retention behavior on the stationary phases were found. Better resolution factors of aromatic positional isomers were obtained on calix[4]arene phases possessing benzene rings with nitro- and methoxy groups placed in *para* positions than phases possessing aliphatic substituents or aromatic rings without substituents. The influence of chromatographic conditions (pH of the mobile phase, column temperature and organic modifier addition) on retention and selectivity was also evaluated. Again, the results indicated that the 1,3-*alternate* calix[4]arene stationary phases first of all behaved like reversed-phase packing, however, inclusion complex formation, hydrogen bonding and $\pi t \pi$ interactions seem to be involve in separation process.

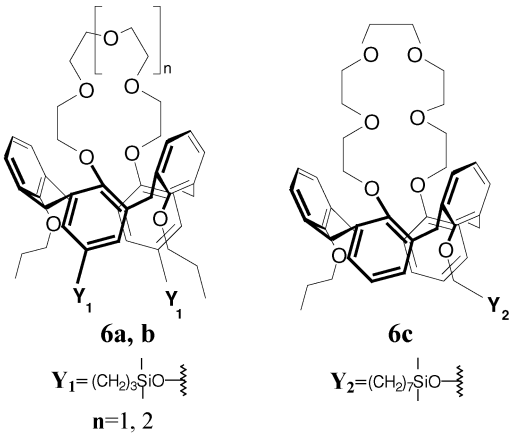


FIG. 6. Structures of stationary phase type 6.

Calixpyrroles and Calixresorcinarene Stationary Phases

Two novel solid supports containing amido calix[4]pyrrole groups were synthesized by coupling calix[4]pyrrole monoacids with aminopropyl silica gel **8a-b** (86) (Figure 8). Several anions (e.g. chloride, dihydrogen phosphate, hydrogensulfate, and fluoride) as their tertbutylammonium salts were well separated on both columns. Phases **8a** and **8b** were found to be also capable of separating nucleotides, oligonucleotides, Cbz-N-protected amino acids and polyfluorobiphenyls.

Calix[4]resorcinarene stationary phase **9a** (Figure 9) immobilized on silica gel via a propyl spacer were used for separations of *cis*/*trans* isomers of three thioxanthene (flupentixol,

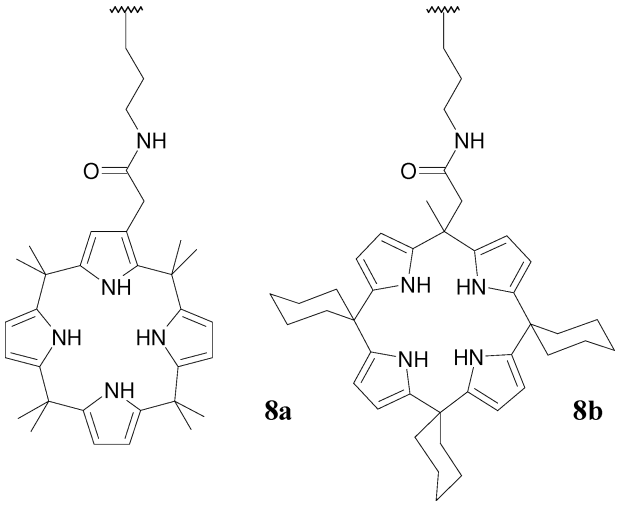


FIG. 8. Structures of stationary phase type 8.

clopenthixol, chlorprothixene) and one benz[*b*,*e*]oxepin derivative (48, 49). The influence of different organic modifiers on the separation of the isomers was described. In most cases, methanol containing mobile phase was advantageous for separations. The selectivity of resorcinarene stationary phase was compared with Caltrex phases (**1f**, **g**, **h** and **4h**, **i**, **j**) and ODS one. Specific interactions between resorcinarene phase and isomers possess polar side chains with hydroxyl group (flupentixol and clopenthixol) were found. In contrast, lipophilic isomers of chlorprothixene and doxepin were most discriminated on calixarene phases possess hydrophobic cavities.

Another type of calix[4]resorcinarene stationary phase called by the authors "polar headed" RP-type **9b** was prepared by attaching a carbamate functionalized calix[4]resorcinarene selector to the solid support via alkenyl chains containing a terminal double bond by free radical-inducted reaction on mercaptopropyl-functionalised silica gel (87). A comprehensive characterization of the resulting phase was carried out by solid state NMR, IR and elemental analysis. The chromatographic behavior of the novel resorcinarene phase **9b** was demonstrated in separation of a test mixture containing four compounds possessing hydrophilic or hydrophobic properties (uracil, phenol, naphthalene and anthracene). The chromatographic data was compared with four commercially available reversed-phases. Calix[4]resorcinarene stationary phase exhibited different retention characteristics that was governed by a multitude of interaction mechanisms.

Calixresorcinarene phases containing L-phenylalanine ethyl ester **9c**, *S*-1-(2-naphthyl)-ethylamine **9d** and *R*-2-amino-1-butanol **9e** were found to be enantioselective toward compounds of pharmaceutical interest (88) and amino acid derivatives (89).

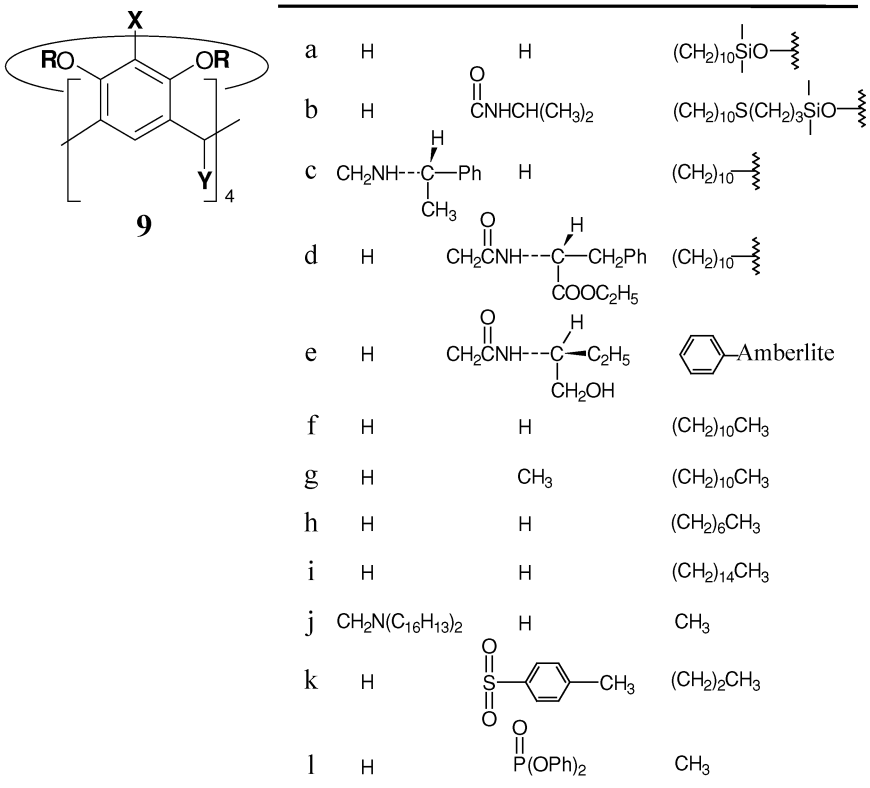


FIG. 9. Structures of stationary phase type 9.

The baseline separation of mianserin was obtained on **9c-d** under reversed-phase conditions. In contrast, the separations of chlorthalidone and ketoprofen enentiomers were achieved by using hexane-containing eluents. Chiral discriminations of phenylglycine and tryptophan sodium salts were achieved on **9e** by using methanol/water (1:9, v/v) as mobile phase.

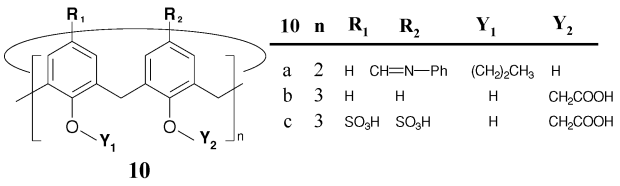
CALIXARENES AND CALIXRESORCINARENES DYNAMICALLY LOADED ONTO SILICA GEL

Lipophilic C-tetra-n-undecyl calix[4]resorcinarene **9f** and its octamethylether **9g** were dynamically loaded onto ODS phases and used in HPLC. The modified silica material showed high stability and reproducibility over several months of intensive use. The separation of selected regioisomeric phenol derivatives (nitrophenols, cresols, chlorophenols, dimethylphenols) (90) and pyrimidine bases (cytosine, uracil, thymine) (91, 92) were obtained under reversed-phase conditions. Much better separations of substituted phenols positional isomers were noted for **9f** phase in comparison to non-modified RP-18 phase and to the column coated with **9g**, due to formation of hydrogen bonding interaction between calix[4]resorcinarene selector **9f** containing free hydroxyl groups and the analytes. The baseline separation of three pyrimidine bases was achieved on modified material **9f**, however, a strong dependence of the elution behavior from the pH and mobile phase compositions was observed. The retention factors for the bases studied on the abovementioned column were higher than on non-modified RP-phase, in terms of pronounced interaction of the analytes with the calix[4]resorcinarene immobilized on the silica gel surface. An ODS phase modified with selector **9f** was also used for the separation of cyclic and linear alcohols, diols and sugars under reversed-phase conditions (93). Most of the alcohols were well resolved, however, their retention times decreased due to the increased polarity of the modified phase used. Only for 1,2-, 1,3- and 1,4-cyclohexandiol the retention times slightly increased in comparison to those measured on untreated RP-phase, which was explained by host-guest interaction through hydrogen bonds.

Calix[n]arene sulfonates (n = 4,6,8) **4k**, **l**, **m** were used as dynamic coatings to modify ion exchange resin (IC anion SW) and served as stationary phases for the resolution of fullerenes C_{60} and C_{70} (94). The baseline separations were achieved on all examined columns by using toluene/hexane (4:6, v/v) as the mobile phase, respectively. It was found that the cavity size of calixarene selectors did not influence on retention and separation factors of C_{60} and C_{70}.

CALIXARENES AND CALIXRESORCINARENES AS MOBILE PHASE ADDITIVES

Several calixarenes and calixresorcinarenes, functionalized at their upper or lower rim, were found to be efficient as mobile phase additives in reversed-phase chromatography. Modifications first of all based on introduction to these cavity compounds of polar substituents to improve their solubility in water. Park et al. first tested calix[6]arene hexasulfonates **4l** as mobile phase additive for separation of some monosubstituted phenol regioisomers (methoxyphenol, aminophenol, nitrophenol) in RP-LC system (95). Retention and separation factors of analytes were measured using methanol/water and methanol/acetonitrile mobile phases in different proportions containing calix[6]arene **4l** at the concentration of 1 mM. The results were compared with those obtained using mobile phase without additive. In most cases the reduction of retention times but increased selectivity of the phenol isomers were observed compared with a calixarene-free system. This was explained by enhanced solubility of the analytes in the mobile phase due to host-guest interactions and due to strong absorbance of calix[6]arene hexasulfonates in the UV region.

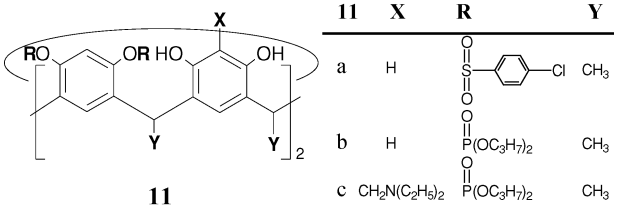


10	n	R_1	R_2	Y_1	Y_2
a	2	H	CH=N—Ph	$(CH_2)_2CH_3$	H
b	3	H	H	H	CH_2COOH
c	3	SO_3H	SO_3H	H	CH_2COOH

FIG. 10. Structures of stationary phases type 10.

Investigations of the chromatographic properties of **4l** and calix[4]arene tetrasulfonates **4k** (96) as mobile phase additives were also examined by another research group. The observed reduction in the HPLC capacity factors of nitrophenols, in the presence of the sodium salts of **4k**-**l** here was explained due to pH effects and not due to complexation between the nitrophenols and the calixarene additives, as it had been postulated earlier (95). Reversed-phase liquid chromatography was also applied to the study of the host-guest complexation of **4k** with amino acids as guests in the mobile phase (97). It was established that formation of the inclusion complexes results in changes in the retention times of the amino acids. The variations in stability constants determined were explained in terms of the various interactions (ion-pairing, hydrophobic, aromatic-aromatic and electrostatic interactions) that occurred between a given amino acids and calixarene selector **4k**.

An octaphosphorylated p-*tert*-butyl calix[8]arene **1m** additive was used under reversed-phase conditions for the separation of mono-, di- and trisubstituted benzene derivatives (98). It was shown that addition of calixarene **1m** in the concentrations from 0.1 to 0.4 mmol to the mobile phase led to decreasing



11	X	R	Y
a	H		CH_3
b	H		CH_3
c	$CH_2N(C_2H_5)_2$		CH_3

FIG. 11. Structures of stationary phase type 11.

TABLE 1
Assembly of analyte separated on HPLC calixarene phases

Analytes	Type of calixarene phase (Ref.)
Aromatic positional isomers	**1b-e** (40, 41); **1f-h**, **4h-j** (43, 44); **1i-1k** (53, 54, 59); **3** (57); **4g** (74); **4k-l** (95, 96); **7a-d** (80, 83, 81, 84); **9f-g** (90, 92); **9l** Add. (102).
Alkylated and unsubstituted aromatics	**1f-h**, **4h-j** (45); **1i** (53); **1m** Add. (98); **1n-s** Add. (100); **2d** (66); **9h-k** Add. (103); **9l** Add. (102); **10a** Add. (99); **11a-c** Add. (103).
Halobenzenes	**2a** (52).
Phenol derivatives	**1f-h**, **4h-j** (45); **1k** (63); **4g** (74); **9l** Add. (102)
Aromatic amines and substituted anilines	**2a** (52); **1i** (53); **1k** (63); **3** (57).
PAHs	**1f-h**, **4h-j** (45); **1i** (53); **1j** (54); **1k** (59, 60, 63); **2a** (51, 52); **2c** (58); **3** (57); **7e** (82).
azo-PAHs	**3** (57); **1k** (63).
Fullerenes C_{60}, C_{70}	**4k-m** (94).
Benzoic acid derivatives	**1k** (60); **9l** Add. (102).
Benzoic acid esters	**1f-h**, **4h-j** (45).
Sulphonamides	**1i** (53); **2b** (56).
Quinolones	**2b** (56).
Xanthines	**1f-h**, **4h-j** (45).
Barbituric acid derivatives	**1f-h**, **4h-j** (45).
Thioxanthene isomers	**1f-h**, **4h-j**, **9a** (48, 49).
Dibenz[*b*, *e*]oxepins	**1f-h**, **4h-j**, **9a** (48).
Steroids	**1f-h**, **4h-j** (49, 50); **9a** (48, 49); **1l** (64, 65).
Tricyclic neuroleptics	**4j** (75).
NSAIDs	**7d** (84).
Water soluble vitamins	**1k** (62); **7d** (84).
Estradiol stereoisomers	**1b**, **1d-e** (40).
Retinoids	**4j** (76).
Uracil derivatives	**1b**, **1d-e** (40).
Nucleobases	**1k** (61); **2a** (51); **2b** (55); **2d** (66); **7d** (84); **7f-g** (85); **9f**, **g** (91, 92).
Nucleosides	**1a** (39); **1j** (54); **1k** (61); **2a** (51); **2b** (55); **8a-b** (86).
Amino acids	**4k** Add. (97); **10b-c** Add. (101).
Amino acid esters	**4a** (68); **4c-d** (70).
Proline containing dipeptides	**1a** (39); **1b-e** (41).
Carbamate N-protected racemic amino acids	**5a-b** (77); **8a-b** (86).
Racemic Phe, Trp, PheGly	**9e** (89).
Racemic 1-phenyl- 2,2,2-trifluoroethanol	**4e** (72).
Racemic drugs	**9c-d** (88).
Inorganic anions	**8a-b** (86).
Na^+, K^+, Cs^+	**4a-b** (67); **4c-d** (70); **6a-c** (78, 79).
Pb^{2+}, Zn^{2+}	**4d** (71); **4f** (73).

Add. - calixarene derivatives used as mobile phase additives under reversed-phase conditions.

sorption of aromatic solutes on the sorbent's surface due to formation of host-guest inclusion complexes. Stability constants of these complexes were determined from relationship between the solute capacity factors and the calixarene concentration in the mobile phase. Similar calculations for RP-HPLC determination of stability constants of: 5,17-*bis*(N-tolyliminomethyl)-25,27-dipropoxy calix[4]arene **10a** (Figure 10) and p-*tert*-butyl calix[8-12]arenes **1n-s** complexes with a number of benzene derivative (99, 100); p-H-37-(2-carboxy-methyloxy) calix[6]arene **10b** and p-sulfonato-37-(2-carboxy-methyloxy) calix[6]arene **10c** complexes with selected amino acids were proposed by the same authors (101).

The variations in stability constant values were explained in terms of the different interactions, which may occur between analytes and calixarene mobile phase additives. Also tetra-alkyl calix[4]resorcinarenes **9h-i** and their derivatives functionalized at upper rim of the macrocycle by N,N-dialkylaminomethyl **9j**, arylsulfonyl **9k**, **11a**, dipropoxyphosphoryl **11b-c** (Figure 11) and dibenzyloxyphosphoryl **9l** groups were used as mobile phase additives to study of host-guest complexation. As analytes,

various aromatic compounds (alkylbenzenes, halobenzenes, aldehydes, substituted phenol derivatives and carboxylic acids) were tested (102, 103). In all cases, the formation of the complexes with calixresorcinarenes changed the retention of the investigated aromatic molecules on the sorbent's surface and led to an improvement of the separation of compounds possessing similar properties. The association constants of the complexes depended on the size, nature, position and quantity of substituents on the benzene ring of the guest molecules.

SUMMARY

Several functionalized calixarenes, calixresorcinarenes and calixpyrroles have been utilized as selectors in liquid chromatography. The modifications of these macrocycles comprised: conformation in which calixarenes molecule are blocked, the type of functional groups and substituents present at their upper and lower rim, the calixarenes ring-size and finally the type of spacer fixing macrocycles to the stationary phases. The choice of appropriate derivative of the macrocycles depended upon the type of separation technique adopted.

Calixarenes permanently attached to solid support attracted the great attention of researchers. Most of these stationary phases possessed calixarenes in cone conformation functionalized at the upper rim in *para* positions with *tert*-butyl substituents. However, some examples of functionalized calixarenes in *1,3-alternate* conformations were also tested. Calixarenes fixed via lower rim served for the separation of uncharged analytes. Immobilization by the upper rim allowed for effective separation of both charged and uncharged solutes. Multitude of interactions occurring between solutes and calixarene phases makes it difficult to discern a general rule for separation mechanism. According to the chromatographic data obtained, it was often concluded that calixarene stationary phases behaved as reversed-phase material, however, other retention mechanisms (hydrogen bonding interaction, π–π interaction, π electron transfer interaction) has been postulated. Investigations of the selectivity of calixarene phases as a function of their ring-size also did not give a clear answer concerning the inclusion complexation mechanism. In some cases, regardless of the calixarene ring-size, the retention of solutes examined did not change. Calixarenes substituted with chiral residues were also successfully used as selectors for the separations of optical active compounds. Again, it is questionable if the three-dimensional cavity structure of calixarenes is decisive for enantioselectivity or if it simply acts as a support frame.

Modified calixresorcinarenes and calixarenes dynamically adsorbed onto RP phase or onto ion exchange resin were also successfully applied in chromatographic separations. In this case adsorption leads to an increased polarity of the stationary phase and thus decreased retention factors of the analytes, however the separation factors were generally higher in comparison to non-modified RP phases. The same observations were also made when water-soluble calixarenes or calixresorcinarenes were introduced as mobile phase additives. It was established that formation of the inclusion complexes results in changes in the retention times of the analytes and the stability constants of these complexes were determined based on relationship between the solute capacity factors and the calixarene concentration in the mobile phase.

The interest in calixarenes as stationary phases in high performance liquid chromatography has been dynamic and fruitful developed for 13 years. All of the investigations quoted here clearly confirm that these cavity compounds are promising starting point for the synthesis of HPLC stationary phases. Several examples showed high selectivity and efficiency of these phases toward many charged and uncharged, organic and inorganic solutes, including biologically active compounds (Table 1). Moreover, in many cases the chromatographic data obtained were better in comparison to the conventional RP-stationary phases.

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