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New calixarene-bonded stationary phases in high-performance liquid chromatography: comparative studies on the retention behavior and on influences of the eluent

Torsten Sokoließ[a], Ulf Menyes[b], Ulrich Roth[b], Thomas Jira[a,*]

[a]*Institute of Pharmacy, Pharmaceutical/Medicinal Chemistry, Ernst-Moritz-Arndt-University of Greifswald, Friedrich-Ludwig-Jahn-Strasse 17, D-17487 Greifswald, Germany*

[b]*Synaptec GmbH, Brandteichstrasse 19, D-17489 Greifswald, Germany*

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Abstract

The chromatographic behavior of six calixarene-bonded stationary phases is reported. Varying analyte selectivities (i.e., for phenols, substituted aromatics, polycyclic aromatic hydrocarbons, barbituric acid derivatives, xanthines) exist as a function of the ring-size of the calix[*n*]arenes ($n=4$, 6, 8) and the substitution at the ''upper rim'' with *para-tert.*-butyl groups. Although eluents with unusually high proportions of water were used, a comparison with conventional reversed-phase (RP) columns shows a predominantly reversed-phase character with remarkable selectivities of these phases. The influences of several organic solvents on retention variations of solutes are compared for RP-C_{18}, phenyl and calixarene phases.

Keywords: Stationary phases, LC; Mobile phase composition; Methylene selectivity; Phenyl selectivity

1. Introduction

Calixarenes are macrocyclic molecules composed of phenol units linked by alkylidene groups. They belong to the class of [1*n*]cyclophanes [1,2]. It is the ability to form reversible complexes similar to cyclodextrins and crown ethers that has led to an increased interest in employing calixarenes for chromatographic separations. Some of the advantageous properties of this new class of ''supramolecules'' are the recognition of both metal cations and organic molecules, a facile large-scale preparation as well as the possibility of synthesizing optically-active isomers [3]. In recent years, the potential of this class of macrocycles has been shown for several applications in gas chromatography (GC) [4–7], high-performance liquid chromatography (HPLC) [8–18] and solid-phase extraction (SPE) [19]. Until now, calixarenes have been used as mobile phase additives in reversed-phase liquid chromatography (RPLC) as well as chemically bonded on silica gel. Calixarene-bonded stationary phases are preferable to the use of calixarene additives because the UV detection of analytes is not hindered by strong absorbance of calixarenes. Furthermore, poor solubility of most calixarenes precludes an application as additives in aqueous eluents. Nevertheless, separations with

*Corresponding author. Tel.: +49-3834-86-4850; fax: +49-3834-86-4843.

E-mail address: jira@pharmazie.uni-greifswald.de (T. Jira).

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water-soluble calixarene additives with refractive detection have been described [9,15].

There remain many questions concerning the separation mechanisms on calixarene-bonded stationary phases. Studies of the retention behavior of several nucleosides, free amino acids, proline-containing dipeptides and disubstituted aromatic analytes show a predominantly reversed-phase character of these new materials. However, we observed improved separations for phenols and barbituric acid derivatives on these columns compared to RP-C_{18}, phenyl and phenyl ether stationary phases [16]. Host–guest complexation, charge–transfer complexes and hydrophobic interactions have been discussed as possible factors for the discrimination of regio- and stereoisomers and for changes in elution order [10,13].

This paper describes several applications for the separations on calixarene-bonded stationary phases. Six different materials were investigated containing calix[4]-, calix[6]- and calix[8]arenes and its derivatives substituted with *p-tert.*-butyl-groups bonded on silica gel. By comparing these six stationary phases with a RP-C_{18}, a phenyl and a phenyl ether stationary phase, a new theory on the retention mechanisms and effects of several organic modifiers on retention behavior have been proposed.

2. Conditions

2.1. Chemicals

Naphthalene, acenaphthene, fluorene, 3,5-dinitrobenzoic acid, 2,6-dinitrotoluene, benzoic acid methyl, ethyl and propyl ester and xanthine were purchased from Merck (Darmstadt, Germany). Phenanthrene was obtained from Acros Organics (New Jersey, USA). Anthracene, ethylbenzene, α-naphthol, β-naphthol and etophylline were from Berlin-Chemie (Berlin, Germany). Fluoranthene, chrysene, perylene, benzo[*a*]pyrene, benzoic and *p*-hydroxybenzoic acid butyl ester and guaiacol were purchased from Sigma–Aldrich (Steinheim, Germany). Acetophenone, benzene and toluene were purchased from Riedel-de-Haën (Seelze, Germany). Benzoic acid benzyl ester and thymol were obtained from Laborchemie Apolda (Apolda, Germany). *p*-Hydroxybenzoic acid methyl, ethyl and propyl ester were from Chem.-Pharm. Werk Oranienburg (Oranienburg, Germany). Barbital, primidone, crotylbarbital, phenobarbital, hexobarbital, diprophylline, theobromine, proxyphylline and coffeine were purchased from AWD (Dresden, Germany). Pentobarbital, phenytoine and theophylline were purchased from Caesar u. Loretz (Hilden, Germany). All analytes used were of the best quality available.

Acetonitrile (ACN) and isopropanol (IPA) were of gradient HPLC grade and purchased from Scharlau (Barcelona, Spain). HPLC-grade methanol (MeOH) was purchased from J.T. Baker (Deventer, The Netherlands). 1,4-Dioxane (Dx) and tetrahydrofuran (THF) of extra pure quality were obtained from Merck and distilled before use. Water was obtained by bidistillation (pH 6.4–6.6).

2.2. Equipment

The separations were achieved with a HP1090 II Model equipped with a diode array detection (DAD) system (Hewlett-Packard, Weinheim, Germany).

2.3. Columns

Caltrex phases (calixarene phases) and the phenyl ether phase were obtained from Synaptec (Greifswald, Germany). Calixarene phases contain silica-bonded calixarenes of different ring-size and substitution immobilized via a propyl spacer on Polygosil (Polygosil Si 100, 10 μm, specific surface area/BET: 250 m^2/g, pore volume: 1 ml/g, manufacturer: Macherey-Nagel) by a patented procedure [DE 19 602 393, EP 0786661 A2 and Wo 97/27479]. [Caltrex AI – calix[4]arene (0.17 $\mu mol/m^2$), Caltrex AII – calix[6]arene (0.12 $\mu mol/m^2$), Caltrex AIII – calix[8]arene (0.20 $\mu mol/m^2$), Caltrex BI – *p-tert.*-butyl-calix[4]arene (0.33 $\mu mol/m^2$), Caltrex BII – *p-tert.*-butyl-calix[6]arene (0.19 $\mu mol/m^2$), Caltrex BIII – *p-tert.*-butyl-calix[8]arene (0.23 $\mu mol/m^2$)]. The phenyl ether phase was made by the same procedure (1.28 $\mu mol/m^2$).

Nucleosil-100 C_6H_5 (phenyl phase, 1.96 $\mu mol/m^2$) and Eurospher 100-C_{18} (RP-C_{18} phase, 2.69 $\mu mol/m^2$) were purchased from Knauer (Berlin, Germany).

All phases have mean pore diameters of 100 Å

and, except Caltrex AIII (200×4 mm I.D.), dimensions of 250×4 mm I.D. The particle diameters were different for the Caltrex (10 μm), phenyl ether (10 μm), phenyl (7 μm) and RP-C_{18} phase (5 μm).

2.4. Chromatography

Chromatographic experiments were performed with isocratic elution throughout. The binary mobile phases consisted of different proportions of ACN, MeOH, THF, Dx and IPA in water. The eluent was degassed prior to use. UV detection was used at different wavelengths given in the tables and figures. In all cases the column temperature was set at 40°C, the flow-rate was 1 ml/min and injection volumes were 10 μl. The hold-up times (t_0) were determined from injections of MeOH with UV detection at 220 nm in IPA–water (50:50, v/v) as the mobile phase.

3. Results and discussion

3.1. Suitability of calixarene-bonded stationary phases for separations of several model analytes

Several analytes were tested to demonstrate the potential of the calixarene-bonded stationary phases. Mobile phases with water and five different organic modifiers (MeOH, ACN, THF, Dx, IPA) were used in varying proportions. It was found that mixtures of nonpolar aromatic hydrocarbons [polycyclic aromatic hydrocarbons (PAHs), alkylated aromatics], mixtures of aromatics with polar substituents (nitroaromatics, benzoic acid esters, phenols, sulfonamides, xanthines) and mixtures of alicyclics (barbituric acid derivatives) could be separated, suggesting that an affinity for the stationary phase must exist with analytes of very different structures. Calixarenes form a cavity due to the cone conformation. A ring inversion can be prevented by substitution of all phenol units with a hydrophobic spacer and binding to silica gel. Hence, an inclusion of hydrophobic solutes can be assumed [9]. During such complex formations, various interactions may exist between aliphatic and aromatic groups of the analytes with aromatic moieties and *p-tert.*-butyl substituents of bonded calixarenes. Moreover, during the inclusion of solutes, interactions between polar structures of the analytes with the ether–oxygen of the calixarenes are conceivable because the macrocycles are bonded with ether groups to the silica gel. Thus, several solutes with hydrogen-donor properties (phenols, barbituric acid derivatives) could form hydrogen bonds with the calixarenes.

A much stronger retention of nonpolar analytes, such as aromatic hydrocarbons, relative to solutes containing polar structures was observed. Thus, it can be assumed that the retention mechanism of these stationary phases occurs mainly by varying degrees of hydrophobic interactions. Hence, there is a certain similarity with RPs, which has already been described for other solutes [10]. On the other hand, there must exist other effects between stationary phase and analytes that are responsible for the remarkable selectivities of these columns, as will be discussed below.

3.2. Comparison between calixarene-bonded stationary phases

Differences as well as similarities between calixarene phases as a function of ring-size of the calixarenes and the substitution at the ''upper rim'' with *tert.*-butyl groups were investigated. The following sections give information on the properties of these columns with various substance classes.

3.2.1. Separation of phenols

The separation of a mixture of eight phenols with all six calixarene phases showed a strong dependence on ring-size of the calixarenes, whereas the substitution with *p-tert.*-butyl groups at the ''upper rim'' influenced the separations to a lesser extent. Similar changes in selectivity with increasing ring-size of bonded calixarenes under the same chromatographic conditions were observed in systems with binary aqueous eluents containing ACN, Dx, IPA or MeOH. With MeOH as the organic modifier these effects were stronger than in the other systems. Relative retentions of the *p*-hydroxybenzoic acid esters decreased compared to adjacent phenols in the chromatogram (Fig. 1).

These effects are possibly related to differences in the strength of the inclusion of the analytes in calixarene cavities based on the size of analytes. The *p*-hydroxybenzoic acid ester contains a bulky func-

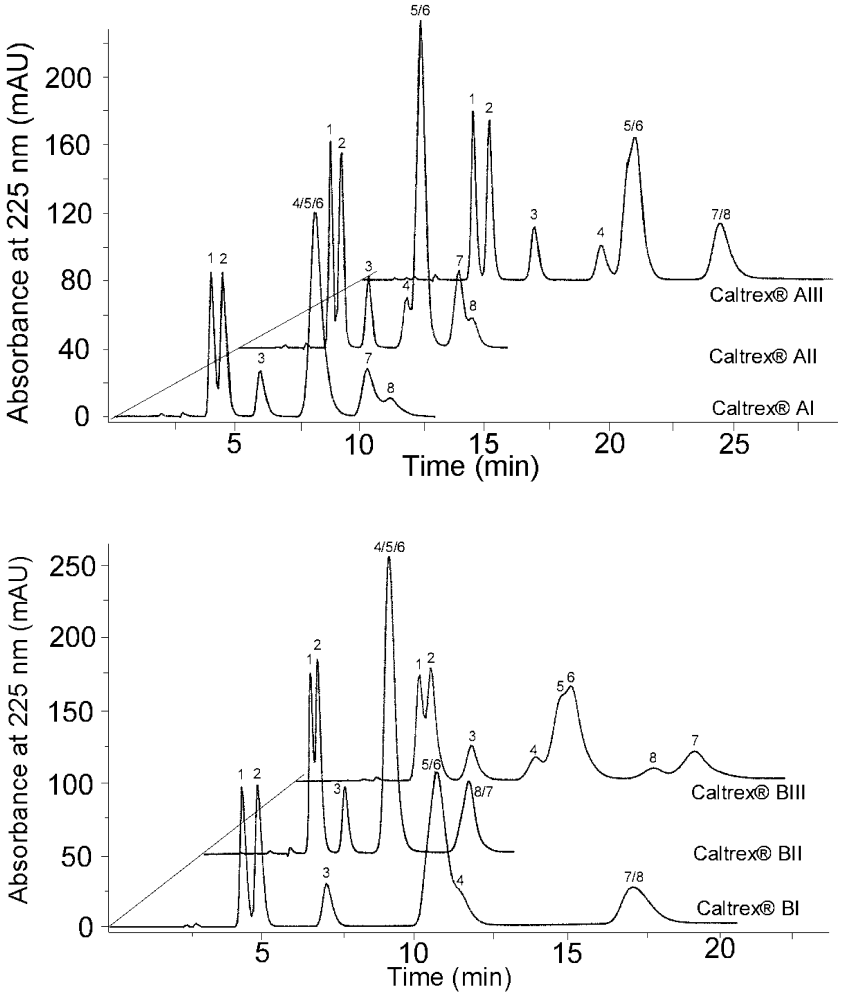

Fig. 1. Comparison of isocratic separations of phenols on calixarene phases. Analytes: 1=phenol, 2=guaiacol, 3=*p*-hydroxybenzoic acid methyl ester, 4=*p*-hydroxybenzoic acid ethyl ester, 5=β-naphthol, 6=α-naphthol, 7=thymol, 8=*p*-hydroxybenzoic acid propyl ester. Conditions: Caltrex AI: $\alpha_{4/5}=1.00$, $\alpha_{7/8}=1.12$; Caltrex AII: $\alpha_{4/5}=0.88$, $\alpha_{7/8}=1.08$; Caltrex AIII: $\alpha_{4/5}=0.83$, $\alpha_{7/8}=1.00$; Caltrex BI: $\alpha_{4/5}=1.06$, $\alpha_{7/8}=1.00$; Caltrex BII: $\alpha_{4/5}=1.00$, $\alpha_{7/8}=1.00$; Caltrex BIII: $\alpha_{4/5}=0.87$, $\alpha_{7/8}=0.86$; MeOH–water (30:70, v/v).

tional group with an ester functionality. Therefore, an inclusion of only the aromatic part into the cavity is likely to occur. At the same time, an interaction between the phenol group and the ether–oxygen of the silica-bonded calixarenes could contribute to a stabilization of the formed complex. In contrast to this, an inclusion of the whole analyte molecule in the larger cavities of calix[6]- and calix[8]arenes is also possible. Nevertheless, it is questionable whether these inclusion complexes are more stable than those with calix[4]arenes. A complex between toluene and *p-tert*.-butyl-calix[4]arene [20] has a higher stability than other complexes of larger calixarenes [21]. The reasons for these phenomenona are more likely to be due to better formation of π–π interactions in smaller calixarenes.

In contrast to *p*-hydroxybenzoic acid esters it is assumed that β-naphthol is too large to form good complexes with calix[4]arenes [22]. If this is true, better inclusions into larger calixarenes should be possible. Similar considerations are applicable for thymol, where an inclusion of this analyte into

smaller calixarenes is hindered by bulky alkyl groups. These effects could explain the changes in selectivity by changes in ring-size of the calixarenes.

A baseline separation of β-naphthol and α-naphthol could not be achieved on any phase with this chromatographic system (Fig. 1). However, good separations were obtained with mobile phases containing ethers (THF, Dx) in the aqueous eluent (e.g., on Caltrex AII with THF–water, 30:70, v/v: α= 1.29). This may be connected with a partial absorption of those solvent molecules into the stationary phase, possibly even in the calixarene cavities (see also Section 3.5.2). Such effects of absorption of organic modifiers have already been described in the literature for RP-C_{18} phases [23]. For this reason, additional sites for interactions of solutes with the stationary phase are possible, for example through hydrogen bonding.

3.2.2. Separation of alkylated and unsubstituted aromatics

There was a distinct substituent-dependency at the ''upper rim'' of the calixarenes for *p-tert*.-butyl groups on separations between alkylated and unsubstituted aromatic hydrocarbons. The calixarene-bonded stationary phases that do not have these substituents were able to separate ethylbenzene and naphthalene. In contrast to this, separations on Caltrex B phases were worse under the same chromatographic conditions. This effect was especially pronounced when Dx–water eluents were used (Fig. 2). Otherwise, the other organic modifiers (MeOH, THF, ACN, IPA) that were tested showed the same behavior. It may be that during inclusion of ethylbenzene into the hydrophobic cavities of the *p-tert*.-butylcalixarenes an interaction between both alkyl groups takes place. Naphthalene does not give such interactions.

If there is an inclusion into a calixarene without *p-tert*.-butyl substituents, no additional interactions between alkyl groups of ethylbenzene and the calixarenes can take place. Hence, the retention of ethylbenzene on these columns was not as strong as on Caltrex B columns. This could be the reason for the higher separation factors for the pair ethylbenzene/naphthalene on calixarene phases without *p-tert*.-butyl substituents.

3.2.3. Separation of benzoic acid esters

The hypothesis discussed in Section 3.2.2 can be supported by comparing the retention behavior of several benzoic acid esters on the six calixarene-bonded stationary phases. They differ mainly in their ability to separate benzoic acid butyl ester from benzoic acid benzyl ester. All separation factors for this pair of solutes gave higher values on Caltrex A columns compared with those on Caltrex B columns (Fig. 3). This may be due to a stronger retention of

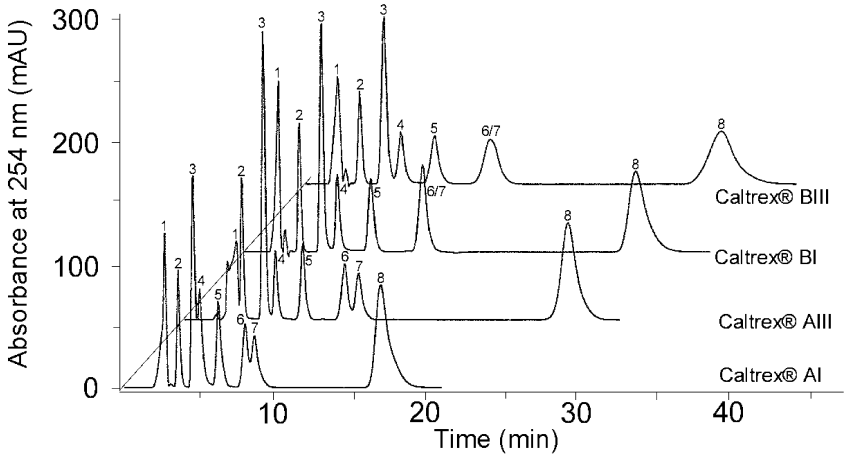

Fig. 2. Comparison of isocratic separations of a test mixture on Caltrex phases. Analytes: 1=3,5-dinitrobenzoic acid, 2=2,6-dinitrotoluene, 3=acetophenone, 4=benzene, 5=toluene, 6=ethylbenzene, 7=naphthalene, 8=anthracene. Conditions: Caltrex AI, AIII, BI, BIII; Dx–water (35:65, v/v).

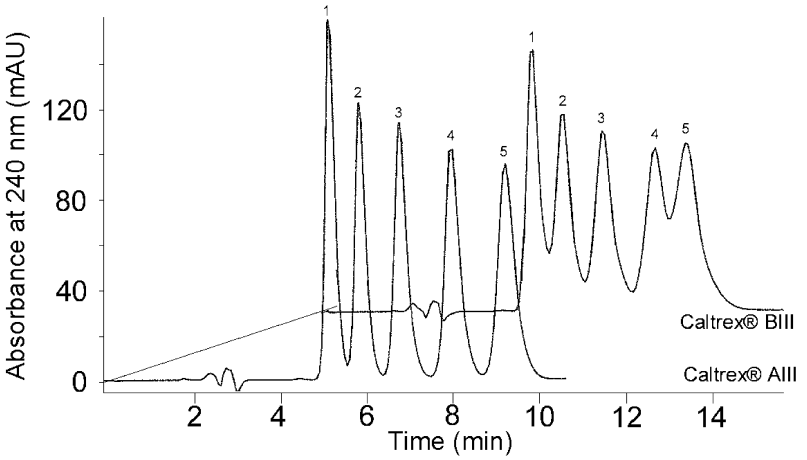

Fig. 3. Comparison of isocratic separations of benzoic acid esters on Caltrex AIII and Caltrex BIII. Analytes: 1=benzoic acid methyl ester, 2=benzoic acid ethyl ester, 3=benzoic acid propyl ester, 4=benzoic acid butyl ester, 5=benzoic acid benzyl ester. Conditions: Caltrex AIII, BIII; IPA–water (40:60, v/v).

the butyl ester relative to the benzyl ester on *p-tert.*-butyl-calixarene phases owing to additional interactions between alkyl chains of the alkyl ester and the stationary phase.

3.2.4. Separation of PAHs

The separation of nine PAHs provides an example for differences within the groups of Caltrex A and Caltrex B columns as well as between both groups. Better separations were observed on phases containing larger calixarenes than with calixarenes of smaller ring-size (Fig. 4). Favorable inclusion of large PAHs into calix[8]arenes relative to calix[4]arenes might contribute to a better selectivity of silica gels with calixarenes of large ring-size. Gutsche and Alam [22] described that association constants of a lot of PAHs with calix[6]- and calix[8]arenes are higher than those with smaller calixarenes. A deeper inclusion of large PAHs into the hydrophobic cavities of calix[8]- and *p-tert.*-

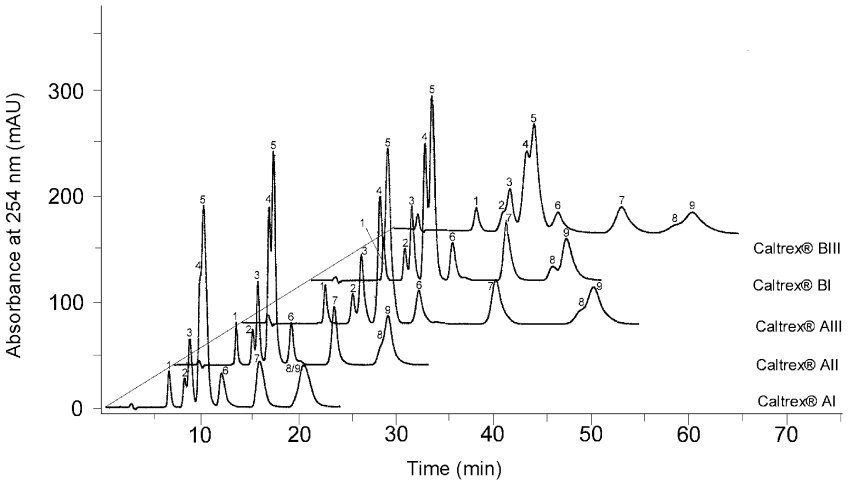

Fig. 4. Comparison of isocratic separations of PAHs on Caltrex phases. Analytes: 1=naphthalene, 2=acenaphthene, 3=fluorene, 4=phenanthrene, 5=anthracene, 6=fluoranthene, 7=chrysene, 8=perylene, 9=benzo[*a*]pyrene. Conditions: Caltrex AI–AIII, Caltrex BI, BIII; ACN–water (40:60, v/v).

butyl-calix[8]arenes is more likely due to the higher conformational flexibility of those hosts. That is different from a conformational rigid calix[4]arene. Our investigations show that there is no selectivity of Caltrex AI to separate perylene from benzo[*a*]pyrene whereas on Caltrex AIII a distinct shoulder was seen. Nevertheless, under the same chromatographic conditions no baseline separation was achieved for this pair of solutes on these columns.

Surprisingly, a better selectivity of Caltrex B phases for perylene and benzo[*a*]pyrene is found, where higher values for the relative retentions also of other pairs of solutes (acenaphthene/fluorene, phenanthrene/anthracene) on these columns are usually observed. Nevertheless, under these conditions no baseline separations for these analytes were achievable. It is known that *p-tert*.-butyl substituents at the ''upper rim'' of calixarenes stabilize inclusion complexes with other molecules. The isolation of such complexes is only successful if these groups exist, while calixarenes lacking such groups cannot form sufficiently stable complexes [24]. Thus, the better selectivity for PAHs on Caltrex B columns might be explained by a stabilizing effect of the *p-tert*.-butyl substituents.

Although there were better selectivities for PAHs on stationary phases with large calixarenes, resolution often became worse. This observation was especially distinct when comparing Caltrex BI and Caltrex BIII. These effects may be explained by a stronger inclusion of analytes in larger calixarenes and a slower partition process between mobile and stationary phases. That may also be the cause of the broader peak shape and the poor resolution between peaks. This tendency was not as strongly pronounced on Caltrex A columns. There is possibly a shorter partition process between solutes and bonded calixarenes without *p-tert*.-butyl substituents.

Under the same chromatographic conditions the retention of PAHs was quite different between calixarene phases. Increased retention times for phases containing calixarenes with large cavities compared to those with small ones were obtained in systems with ACN and MeOH in the aqueous eluent. This behavior is in part contrary to the surface coverage, e.g., the surface coverages of Caltrex BIII and Caltrex BII are lower than on Caltrex BI. This might be explained by better inclusion possibilities of large PAHs into the hydrophobic cavities of large calixarenes. Similar effects of longer retention times on stationary phases with bonded *p-tert*.-butyl-calix-[8]arenes relative to the bonded hexamer or tetramer have already been observed by Gebauer et al. [10] for the separation of regioisomer nitranilines with a MeOH–NaH_2PO_4 buffer as eluent.

It is interesting that we could not transfer these rankings between the columns for mobile phases with Dx and THF instead of ACN or MeOH. For most analytes retention times on Caltrex AII were shorter compared to those on Caltrex AI under these conditions which would correspond to the differences in surface coverage. If IPA was used as organic modifier, we observed just little differences in retention times for these solutes. In contrast to these effects, all retention times on Caltrex BIII were higher than on Caltrex BI with all five binary eluents described above, although differences between retention times became smaller in the same order ACN, MeOH, IPA, Dx and THF. Parts of organic modifiers are possibly extracted into the stationary phase and also included into cavities of the calixarenes. Such inclusion complexes are described for several organic solvents [21,25,26] and may also influence the inclusion of solutes in those cavities. In particular Dx and THF, which are larger molecules compared to ACN and MeOH, might hinder complex formation between analytes and calixarenes. If that is true, a shorter retention time would result, which was confirmed by our studies.

3.2.5. Separation of barbituric acid derivatives, primidone and phenytoine

Many similarities in the separations of related compounds with barbituric acid were observed within the groups of Caltrex A and Caltrex B columns but, we also observed distinct differences between the groups. Separations of hexobarbital and pentobarbital with a MeOH–water eluent were realizable on Caltrex B but not on Caltrex A phases (Fig. 5). Both analytes differ only in the aliphatic substituents. The selectivity of Caltrex B columns for these molecules might be attributed to additional interactions of these substituents with the *p-tert*.-butyl substituents of bonded calixarenes, which are not given on Caltrex A columns.

Furthermore, there existed problems in the sepa-

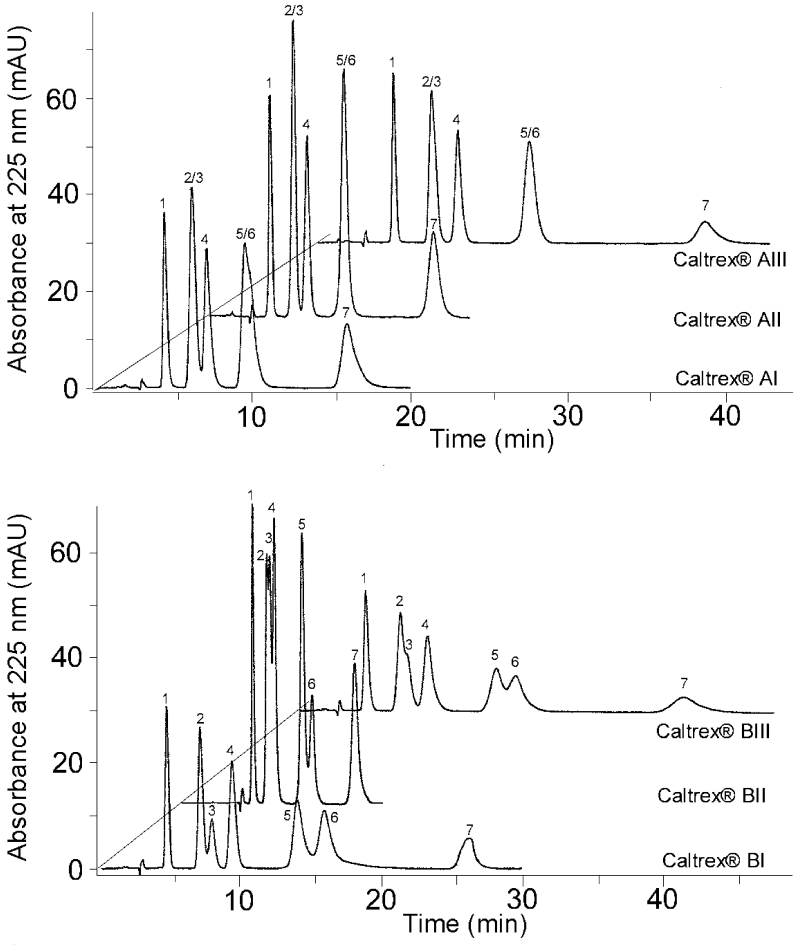

Fig. 5. Comparison of isocratic separations of the barbituric acid derivatives including related compounds (primidone, phenytoine) on calixarene phases. Analytes: 1=barbital, 2=primidone, 3=crotylbarbital, 4=phenobarbital, 5=hexobarbital, 6=pentobarbital, 7=phenytoine. Conditions: Caltrex AI–AIII, BI–BIII; MeOH–water (30:70, v/v).

ration of primidone from crotylbarbital on almost all six stationary phases under the above chromatographic conditions. In these cases, Caltrex B phases showed better selectivities than Caltrex A phases, although there were only shoulders on Caltrex BII and Caltrex BIII. Similar reasons discussed above may be responsible for this different behavior.

The best chromatographic separations for this substance mixture were attained with Dx–water eluents. The advantages of this chromatographic system could result from the partial absorption of Dx into the stationary phase, enabling hydrogen bonds between NH-acid solutes and Dx to be formed.

3.2.6. Separation of xanthine derivatives

Calixarene phases showed a very different behavior in separating xanthine derivatives. Often just a few columns distinguished between two solutes with only minor differences influenced by the kind of organic modifier in proportions of only 2.5% (v/v) in the aqueous eluent. Strong similarities between calixarene phases containing calixarenes of the same ring-size were obtained in systems with Dx (Fig. 6) or IPA in the eluents. Caltrex AI and Caltrex BI were able to separate diprophylline and theobromine whereas a separation was not achieved on any other column. Caltrex AIII and Caltrex BIII showed, in

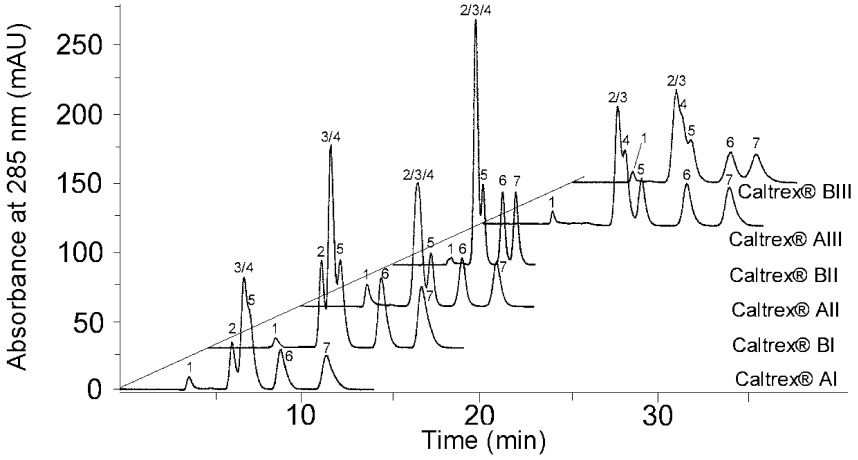

Fig. 6. Comparison of isocratic separations of the xanthine derivatives on Caltrex phases. Analytes: 1=xanthine, 2=diprophylline, 3=theobromine, 4=theophylline, 5=etophylline, 6=proxyphylline, 7=coffeine. Conditions: Caltrex AI–AIII, BI–BIII; Dx–water (2.5:97.5, v/v).

contrast to all other phases, shoulders in the separations between theobromine and theophylline.

In many cases selectivities of the columns were completely different if other organic modifiers like ACN, MeOH or Dx were used. Interestingly, often only one special calixarene phase was able to distinguish between two solutes in a given chromatographic system. For example, with THF–water and ACN–water eluents (2.5%, v/v) only the Caltrex AIII separated theobromine from diprophylline (α= 1.12 and α=1.11, respectively) whereas the other five columns showed no selectivity under these conditions (α=1.00).

3.3. Comparison between calixarene phases and conventional RPs by separation of PAHs

For comparison between calixarene phases with other RPs, a phenyl and a phenyl ether phase were chosen because of their formal chemical similarity and a RP-C_{18} phase was used because of their wide acceptance. For comparative purposes, the strength of the mobile phase was adjusted in such a way that almost the same analysis times were achieved on all columns. Under the given chromatographic conditions the calixarene phase was able to separate all analytes completely (Fig. 7). Otherwise, the resolutions on the RP-C_{18} phase and the phenyl phase were much better which could be due to smaller particle sizes for these columns (5 μm and 7 μm, respectively) in contrast to that for the calixarene column (10 μm). Better selectivities on all three phases were possible with eluents containing higher amounts of water. However, also analysis times were rising and resolution worsened as well.

The phenyl ether phase showed just as poor a selectivity for this mixture and was not suitable for the separation of the compounds because of strong tailing. This was especially the case with the large PAHs.

Another advantage of calixarene phases is the low consumption of organic solvents in the aqueous eluent relative to the phenyl and RP-C_{18} phases. In this example the proportion of MeOH is nearly twice as high on the RP-C_{18} column compared to the calixarene phase.

It is known that RP-C_{18} phases have stronger hydrophobic properties than phenyl phases [27]. However, the phenyl ether phase is more polar than the phenyl phase because the ether function varies the electronic distribution in the aromatic systems. These different properties of stationary phases polarity correlate with the strength of necessary eluent composition. Otherwise, also other factors influence retention that could contribute to this behavior (base silica, surface coverage). Hence, a comparison be-

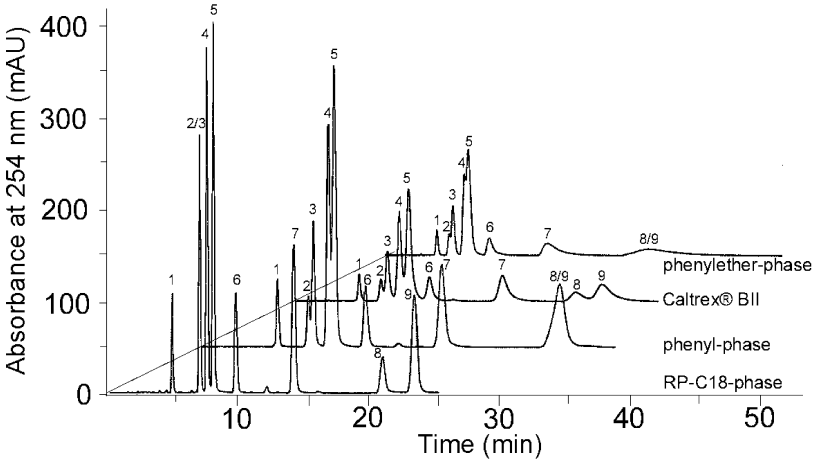

Fig. 7. Comparison between isocratic separations of the PAHs on different stationary phases. Analytes: 1=naphthalene, 2=acenaphthene, 3=fluorene, 4=phenanthrene, 5=anthracene, 6=fluoranthene, 7=chrysene, 8=perylene, 9=benzo[*a*]pyrene. Conditions: RP-C_{18} phase: MeOH–water (85:15, v/v); t_{Rmax}=23.28 min; phenyl phase: MeOH–water (60:40, v/v); t_{Rmax}=27.43 min; Caltrex BII: MeOH–water (50:50 v/v); t_{Rmax}=23.67 min; phenyl ether phase: MeOH–water (20:80, v/v); t_{Rmax}=20.24 min.

tween the polarities of these phases solely by comparing solvent strength is not possible.

We assume that calixarene phases possess a polarity more similar to phenyl ether phases than to phenyl phases because both are bonded on silica gel by ether functions (Fig. 8). Nevertheless, the proportion of organic solvent for Caltrex BII was higher relative to the phenyl ether column (Fig. 7). This behavior stands also contrary to the higher surface coverage of the phenyl ether phase compared to this calix[6]arene phase normalized for the phenyl-ether group content (see Section 2.3). It could indicate that in addition to hydrophobic interactions between solutes and stationary phase also an inclusion into the formed cavities of the calixarenes could contribute to this behavior. This possibility is not present

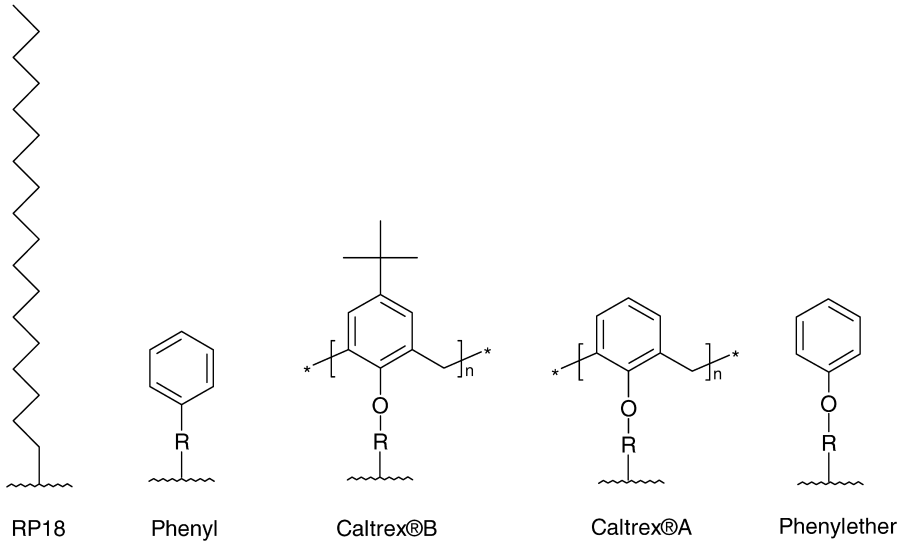

Fig. 8. Comparison of different functional groups chemically bonded on silica gel R-hydrophobic spacer.

on a phenyl ether phase because no comparable hosts are formed by bonded ligands. Hence, less retention results.

3.4. Methylene and phenyl selectivities

Methylene and phenyl selectivities characterize dispersive effects in chromatographic systems that are nonspecific interactions between structurally related compounds (homologous and phenylogous series) and bonded ligands [28]. The values show the ability of a stationary phase to distinguish between such analytes under the given chromatographic conditions, especially depending on the strength of the eluent. Selectivities a are expressed by:

$$\ln k' = \ln a \cdot n + b \quad (1)$$

where k' is the retention factor, n the number of phenyl or methylene groups and b a constant. Methylene selectivities were determined for all six Caltrex, the phenyl and the RP-C_{18} columns for the homologous series of benzoic acid esters ($n=1$–4), p-hydroxybenzoic acid esters ($n=1$–4) and for the series benzene/toluene/ethylbenzene. Furthermore, retention factors of benzene/naphthalene/anthracene were used to obtain phenyl selectivities for all phases. We were able to confirm the linear relationship between ln k' and n for a given eluent composition for all columns. With decreasing strength of the mobile phase, the slope ln a increases, indicating a better selectivity to separate solutes.

The RP-C_{18} column has the highest values for the methylene selectivity under the same chromatographic conditions with IPA in the aqueous eluent compared to all other columns (Table 1 and Fig. 9). Similar results for methylene and phenyl selectivities were obtained with other organic solvents. The values of ''a'' for the phenyl phase are significantly higher than those on the calixarene phases. These results correlate with different polarities of the stationary phases discussed in Section 3.3. Different polarities might also be responsible for the slight differences between Caltrex A and Caltrex B selectivities.

The RP-C_{18} phase with the greatest methylene selectivity for the given example also resulted in the longest analysis time (Table 1). Therefore, values of ''a'' obtained with mobile phases of different strength were comparable because the selectivities strongly depend on the chromatographic system chosen. We observed that there were linear dependences between ln a and the proportions of organic modifier (Fig. 10). Thus, this fundamental relationship can be applied not only for the phenyl and the RP-C_{18} phases but also for all calixarene phases. Likewise, it could be shown that equal selectivities on all columns were reached by varying the composition of the eluent. Methylene selectivities of ln $a=0.35$ are sufficient to attain baseline separations for these analytes on every column. The advantage of the calixarene phases is a diminished proportion of organic modifier compared to the other columns without loss of methylene selectivity. The same results were obtained for eluents containing ACN, MeOH, THF and Dx.

Otherwise, the RP-C_{18} phase has likely advantages in separating more polar analytes that differ by only one methylene group because of its higher methylene selectivity.

Table 1
Methylene selectivities a, correlations r and analysis times of different phases for the homologous series of benzoic acid esters with an eluent of IPA–water (45:55, v/v)

Phase	a	r	Analysis time (min)
Caltrex AI	1.21	0.999	5.40
Caltrex AII	1.21	0.999	5.22
Caltrex AIII	1.24	0.999	6.76
Caltrex BI	1.25	0.991	6.15
Caltrex BII	1.23	0.995	4.14
Caltrex BIII	1.25	0.999	6.16
phenyl	1.28	0.999	7.64
RP-C_{18}	1.45	0.999	12.38

3.5. Influence of several mobile phases on retention behavior

For our investigations concerning the influence of the eluent compositions on retention behavior, five frequently used organic modifiers were chosen (Table 2). The organic solvents belong to three different selectivity groups according to Snyder and Kirkland [27] through which we could make comparisons between the groups as well as within the groups.

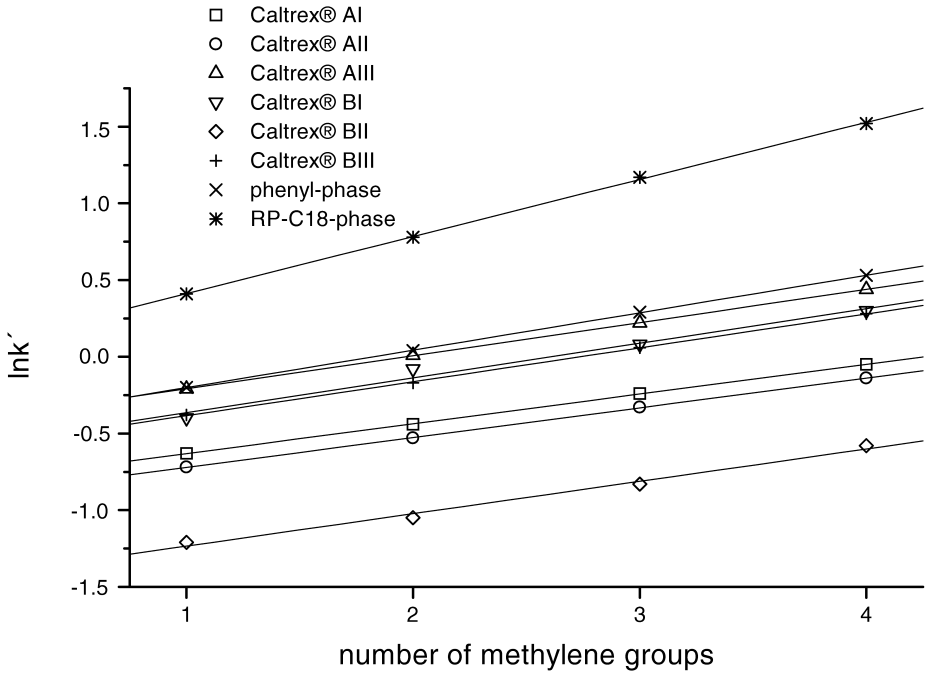

Fig. 9. Variations of ln k' values as a function of the number of methylene groups on different stationary phases for the homologous series of benzoic acid esters. Conditions: IPA–water (45:55, v/v).

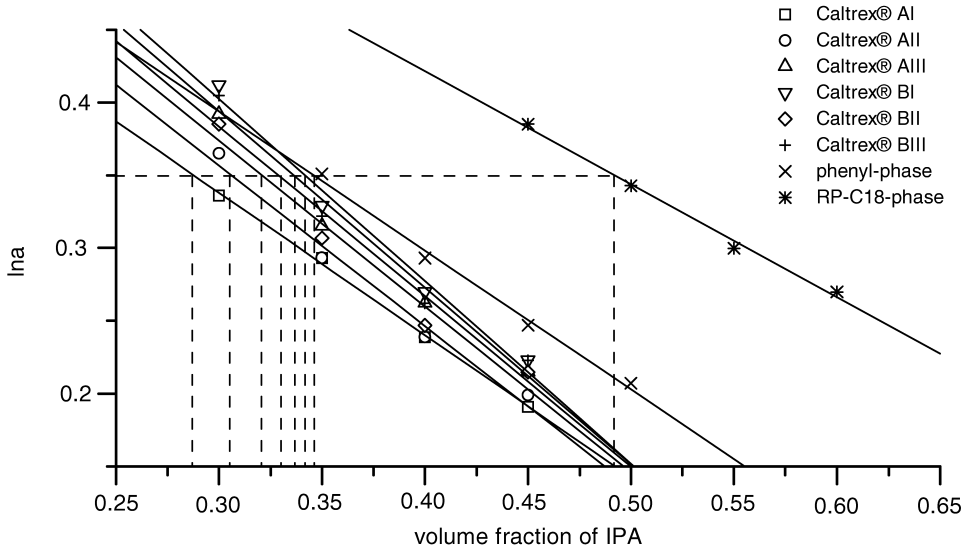

Fig. 10. Dependence of the logarithm of the methylene selectivity ln a from the composition of the mobile phase on different stationary phases for the homologous series of benzoic acid esters.

Table 2
Subdivision of chosen solvents according to Snyder and Kirkland [27]

Selectivity group	Solvent	P'^{a}	x_e^{a}	x_d^{a}	x_n^{a}	S_{RP}^{a}
II	MeOH	5.10	0.48	0.22	0.31	2.6
II	IPA	3.90	0.55	0.19	0.27	4.2
III	THF	4.00	0.38	0.20	0.42	4.5
VI	ACN	5.80	0.31	0.27	0.42	3.2
VI	Dx	4.80	0.36	0.24	0.40	3.5
VIII	Water	10.2	0.37	0.37	0.25	–

[a] P': Solvent polarity parameter; x_e, x_d, x_n: selectivity parameters (x_e: proton-acceptor properties, x_d: proton-donor properties, x_n: dipole properties); S_{RP}: solvent strength parameter for RPLC.

3.5.1. Linearity between ln k′ and φ

There are many mathematical models which attempt to describe the influence of solvent strength on retention factors of solutes [29]. Snyder et al. [30] used the linear relationship:

$$\ln k' = \ln k_W - S \cdot \varphi \quad (2)$$

for isocratic RPLC. Here k' is the retention factor, k_W is the value of k' in pure water as mobile phase, S is a constant for a given solute and φ is the volume fraction of the organic solvent in a given RPLC system. This relationship is approximately valid for a wide range of φ, whereas it is limited for basic or cationic solutes when $\varphi > 0.5$ because of interactions with accessible silanols. Altogether 28 analytes (aromatic hydrocarbons, phenols, benzoic acid esters and nitroaromatics given in Figs. 1–4 without 3,5-dinitrobenzoic acid) with four different volume fractions (between 20 and 65%, v/v) of the five organic solvents given in Section 3.1. were tested. In most cases the proportions of organic modifiers were varied around 5% (v/v). We confirmed the validity of Eq. (2) for all calixarene phases for the given range of organic solvent by linear regression analysis. S and ln k_W values were calculated for all compounds with correlations r higher than 0.99. If S and ln k_W are known or calculated by two or three runs, unknown retention factors of a solute can be approximated. Therefore, optimization procedures (e.g., window diagram method [31]) common in RPLC are also applicable for separations on calixarene phases.

All calculated values of S have a positive sign, meaning that the retention of the analytes decreases as the proportion of organic modifier in the aqueous mobile phase is increased. Thus, calixarene phases behave predominantly like RPs regarding to changes in the strength of the eluent. The fundamental difference to conventional RP columns exists in remarkable low proportions of organic solvents in the aqueous mobile phase.

3.5.2. ln k′–ln k′ correlations

The procedure of ln k'–ln k' correlation was used for our investigations on the retention behavior of aromatic analytes with calixarene, RP-C_{18} and phenyl phases. This method compares the retention parameters for several chromatographic systems, which can be represented in a plot of ln k_1' against ln k_2' where subscripts 1 and 2 refer to chromatographic solvent systems 1 and 2. The parameters A and B of the equation:

$$\ln k_1' = A + B \log k_2' \quad (3)$$

were determined by the least-square method. According to Dzido and Engelhardt [23], analytes were subdivided into nonpolar aromatic hydrocarbons, compounds with electron-donor properties and phenolic compounds (Table 3). The strength of the mobile phase was adjusted so that ln k' values had comparable quantities for all columns.

All calixarene phases showed comparable ln k'–ln k' plots. Thus, the results with Caltrex AI were chosen as representative for all the Caltrex columns.

3.5.2.1. Retention in THF, MeOH and ACN systems

Phenols were retained more strongly on all columns compared to the other analytes in systems with THF in the aqueous eluent than in systems with MeOH or ACN (Fig. 11). That is apparent from the regression line for phenols (dashed line), which is located above that for the electron-donor compounds (continuous line). This observation corresponds with

Table 3
Classification of chosen analytes for ln k'–ln k' correlations

1. Aromatic hydrocarbons	2. Compounds with an electron-donor group	3. Phenols
Benzene, toluene,	Acetophenone,	Phenol,
ethylbenzene,	benzoic acid methyl ester,	guaiacol,
naphthalene,	benzoic acid ethyl ester,	β-naphthol,
acenaphthene, fluorene,	benzoic acid propyl ester,	α-naphthol,
phenanthrene, anthracene,	benzoic acid butyl ester,	thymol
fluoranthene, chrysene,	benzoic acid benzyl ester	
perylene, benzo[*a*]pyrene		

results for RP-C_{18} columns [32]. The effect is explained by an extraction of planar THF molecules into partly ordered structures of the stationary phase with the formation of an ''interphase'' [23]. In this way, hydrogen bonds between absorbed modifier (THF) and phenols are formed, which contributes to the enhanced retention of those solutes. In contrast to this, an inclusion of the more polar MeOH molecules into the stationary phase is less likely. The same can be said for ACN, which bears a polar nitrile group. A stronger retention of phenols relative to electron-donor compounds was also obtained for the phenyl and the calixarene phase. Hence, formation of an ''interphase'' by extraction of THF could also be responsible for these effects.

When comparing THF and MeOH, there is a distinct distance between both regression lines of the phenyl phase (Fig. 11). Planar THF molecules are likely to be absorbed between planar phenyl groups bonded to silica gel and this might cause the very strong retention of phenols relative to systems with MeOH in the mobile phase. It is also possible that the ether–oxygen of the THF molecules may interact with accessible silanol groups, leading to a higher hydrophobicity of the stationary phase. Such effects are described for RP-C_4 columns [23]. Under such conditions, solutes with electron-donor properties can interact less with silanols and thus retention would be expected to decrease in systems with THF. In contrast, MeOH only forms weak complexes with silanols and stronger retention of electron-donor solutes compared to phenols results. The calixarene phase displays a smaller distance between both regression lines, suggesting that silanol groups are more strongly shielded through bonded calixarenes and play less of a role for the separation process compared to the phenyl phase.

If there was absorption of ACN on the stationary phase similar to that observed with THF, the weaker retention of phenols relative to electron-donors could be explained by the weaker proton acceptor properties of ACN ($x_e = 0.31$) relative to THF ($x_e = 0.38$) [23,27].

In systems with MeOH or ACN the larger aromatic hydrocarbons showed an enhanced retention compared to smaller ones relative to systems with THF. This might be connected with different polarities of the solvents. Thus, a partition of the large hydrophobic aromatics from the stationary phase into the THF mobile phase is more favorable relative to the weaker eluents MeOH and ACN.

The most distinct difference in the systems with MeOH and ACN are the locations of the regression lines (Fig. 11). Stronger retention of phenols on Caltrex AI and on the phenyl column in systems with ACN in contrast to the RP-C_{18} column might be due to the varying degrees of absorption into the stationary phase. The effects of accessible silanol groups (especially on the phenyl column) may possibly have greater influences on the retention behavior of electron-donor compounds. It is conceivable that there is more shielding of silanols by calixarenes than by the phenyl groups of phenyl phases, which would result in smaller distances between regression lines on Caltrex AI.

All aromatic hydrocarbons show a similar retention behavior in systems with ACN and MeOH in the aqueous eluent. This is understandable because

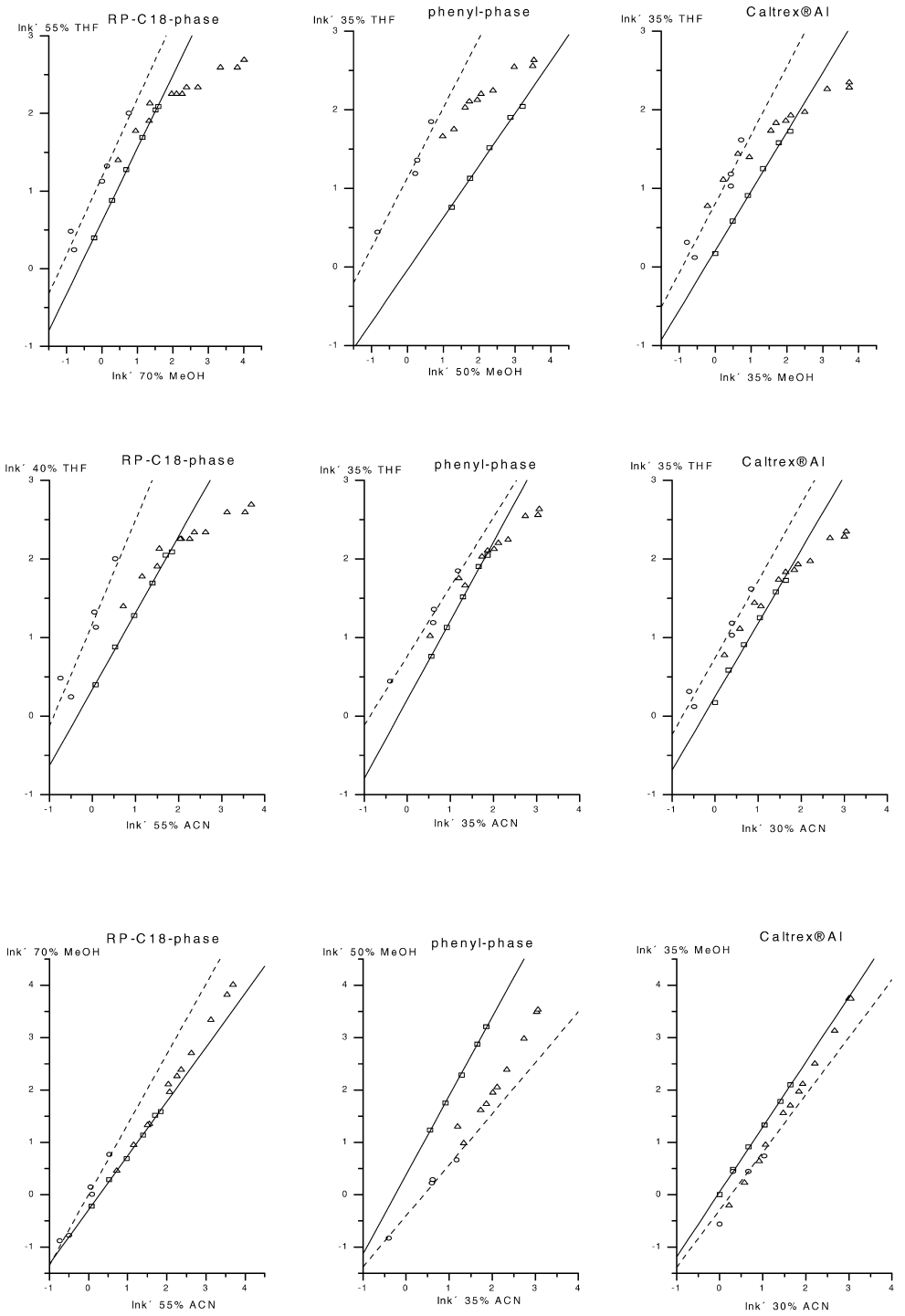

Fig. 11. Comparison of ln k' values of different stationary phases in systems with mobile phases containing THF–water, MeOH–water or ACN–water. △ Aromatic hydrocarbons; □ aromatic compounds with an electron-donor group; ○ phenols.

there is less difference in the polarities of MeOH ($S_{RP}=2.6$) and ACN ($S_{RP}=3.2$) relative to THF ($S_{RP}=4.5$).

3.5.2.2. Retention in ether systems

A stronger retention of phenols relative to compounds bearing electron-donor groups in systems with THF compared to systems with Dx on the RP-C_{18} phase was observed (Fig. 12). Dx has two oxygen atoms, so a more favorable interaction of phenols with Dx than with THF might be expected. On the other hand, it is known that there is hardly any absorption of Dx molecules into the stationary phase due to the nonplanar, rigid and chair or boat structure. In contrast, the planar THF molecules can lead to ordered structures and the extent of the absorption into the stationary phase is higher. Consequently, the retention of phenols in Dx systems is not stronger [23]. Differences between the three phases at that level are not recognizable, thus usual princip-

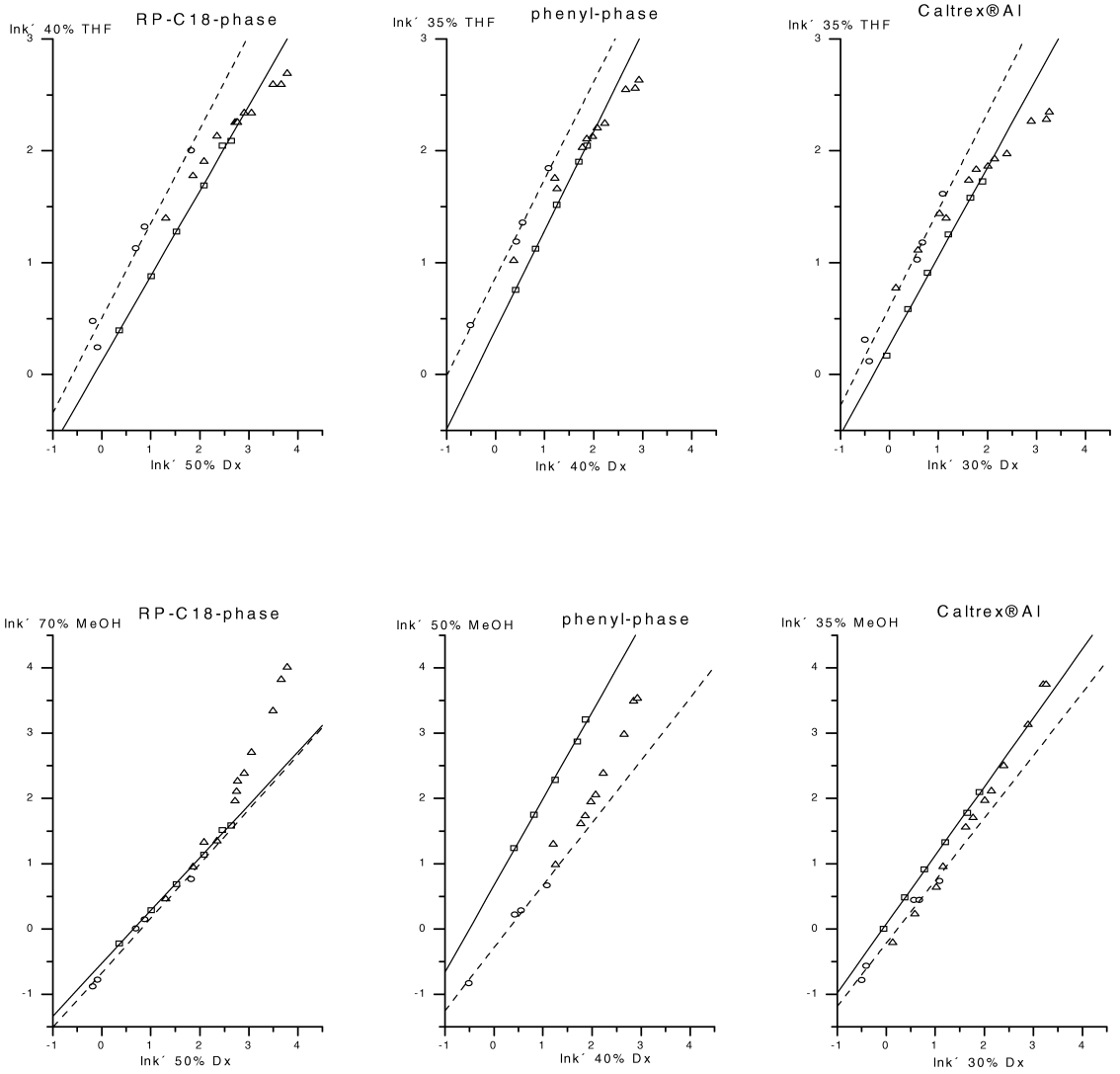

Fig. 12. Comparison of ln k' values of different stationary phases in systems with mobile phases containing Dx–water and THF–water or MeOH–water. △ Aromatic hydrocarbons; □ aromatic compounds with an electron-donor group; ○ phenols.

les of the RP-C_{18} column can be applied to the phenyl and the calixarene phase.

A stronger retention of large, aromatic hydrocarbons was seen in systems with Dx compared to THF. Otherwise, the retention of those solutes in MeOH systems is stronger than in Dx systems. All these effects can again be explained by differences in the polarities of organic modifiers.

The comparison of systems with MeOH and Dx (Fig. 12) is completely different for every column relative to the comparison of systems with MeOH and THF (Fig. 11). The similarities in retention behavior between systems with MeOH and Dx on the RP-C_{18} columns can be explained by just a small proportion of the two extracted solvents into the stationary phase [23]. None of the organic modifiers is able to form highly ordered structures with C_{18} chains, however, such an inclusion into the stationary phase of Caltrex AI and the phenyl phase appears to occur readily. Thus, there is an enhanced retention of phenols in these systems compared to MeOH systems. This is possibly connected with a better accessibility of silanol groups on the phases to interact with analytes, respectively with Dx. One ether–oxygen of Dx molecules might form a hydrogen bond with a silanol group and the second with an analyte molecule, for instance with a phenol like described for other RPs [33].

3.5.2.3. Retention in IPA and MeOH systems

Stronger retention of phenols on the RP-C_{18} phase in IPA systems compared with MeOH systems is explained by a slightly stronger extraction of IPA into the stationary phase because IPA is less polar than MeOH [23]. There are many similarities with Caltrex AI whereas the phenyl phase shows a different behavior (Fig. 13). These effects are difficult to explain. It is less likely that these results are caused by a much stronger absorption of IPA into the stationary phase because structural differences between MeOH and IPA are small and their chemical properties are not fundamentally different.

In summary, we could not confirm a stronger absorption of organic solvents into calixarene-bonded stationary phases compared to phenyl and RP-C_{18} phases. Otherwise, extractions of organic solvents appear responsible for the similarities seen between calixarene and other columns. Differences in retention behavior of analytes may be due to a varying accessibility of silanol groups of each column. Another reason could be different interactions of solutes with alkyl groups or aromatic systems that are bonded on silica gel. Nevertheless, it is remarkable that Caltrex phases occasionally show more similarity with a RP-C_{18} phase than with a phenyl phase, as in the case with the aqueous–alcoholic

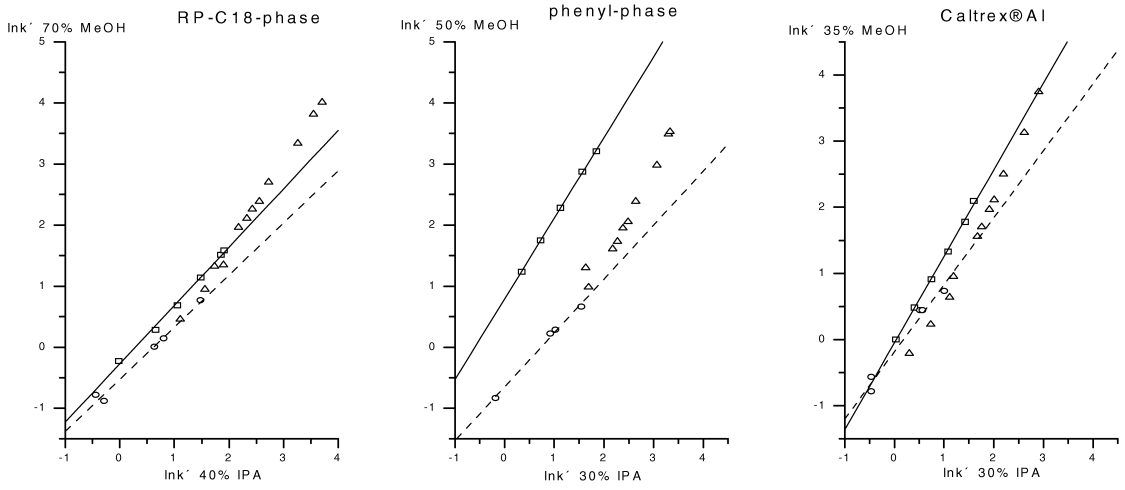

Fig. 13. Comparison of ln k' values of different stationary phases in systems with mobile phases containing MeOH–water and IPA–water. △ Aromatic hydrocarbons; □ aromatic compounds with an electron-donor group; ○ phenols.

eluents. Work is in progress to clarify these phenomenona.

4. Conclusions

The results show that silica-bonded calixarenes possess considerable selectivities for chromatographic separations. Formation of stronger inclusion complexes with solutes appear responsible for advantages of Caltrex B phases compared to Caltrex A phases because the Caltrex B calixarenes are substituted with *tert*.-butyl groups at the ''upper rim''. In other cases, better separations of analytes with phases containing bond calixarenes without such groups were observed, indicating that it is not possible to predict chromatographic selectivities for a given mixture.

Moreover, a dependence of the different ring-size of the calixarenes on the retention process of several analytes was demonstrated.

Compared to conventional RPs, these new calixarene phases possess certain advantages regarding high selectivities for PAHs separation and a low consumption of organic solvent. The main retention mechanisms seem to be hydrophobic interactions and formation of inclusion complexes. In addition to this, some effects might be explained by an extraction of organic solvents into the stationary phases.

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