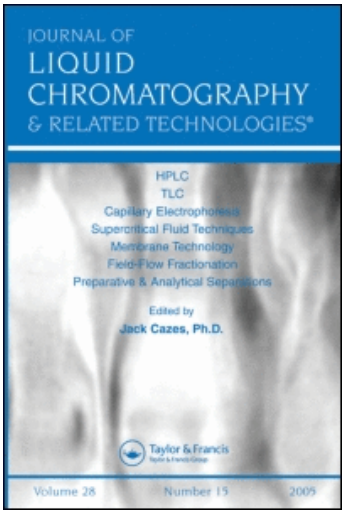

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Preparation and Evaluation of a New Calix[4]arene-Bonded Stationary Phase for HPLC

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ABSTRACT

A new *p-tert*-butyl-calix[4]arene-bonded silica gel stationary phase (C4BS) was prepared via breaking ring reaction of 3-glycidoxypropyltrimethoxysilane used as coupling reagent for high performance liquid chromatography (HPLC). The structure of the new stationary phase was characterized by Fourier transform infrared (FTIR) spectroscopy, elemental analysis, and thermal analysis. Its chromatographic property was evaluated by using neutral, acidic, and basic solutes as probes. Meanwhile, several bonded phases, such as a *p-tert*-butylphenyl-ether-bonded stationary phase (PEBS, the monomer-bonded phase), a *p-tert*-butyl-calix[8]arene-bonded phase (C8BS), and ODS were also used for the comparative study of C4BS, under the same chromatographic conditions. The results

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show that C4BS is an excellent reversed-phase packing with versatile chromatographic performance. The hydrogen bond, $\pi-\pi$ and synergistic interactions between the calix[4]arene ligand of C4BS and solutes can be observed, which result in that the chromatographic property of the new packing is different from both PEBS and ODS, especially, the latter. Additionally, the steric selectivity is also an important factor in the separation of sulfonamides. C4BS exhibits fast analysis for hydrocarbons and high selectivity for the sulfonamides. However, typical inclusion complex contribution on the new packing cannot be expected, which might be because of its small cavity (3 Å) relative to C8BS (11.7 Å).

Key Words: High performance liquid chromatography; Chromatographic evalution; Retention mechanism; *P-tert*-Butyl-calix[4]arene-bonded stationary phase.

INTRODUCTION

In the field of separation material, calixarenes used as additives or immobilization ligands have attracted much attention in the last few years. The versatile chromatographic characteristics are chiefly connected with their supramolecular interactions with many analytes.[1–3] The potential of this class of macrocycles has been shown for several applications, in gas chromatography (GC), high performance liquid chromatography (HPLC), and capillary electrophoresis.[4–9] However, the strong UV absorption and poor solubility of calixarenes preclude its application as additives in reversed-phase liquid chromatography (RPLC).[10,11] Therefore, it is necessary to develop the calixarene-bonded stationary phases.[12]

In 1993, Glennon and coworkers[13–15] prepared silica-bonded calix[4]arene tetraester and silica-bonded calix[4]arene tetradiethylamide stationary phases to separate metal ions and amino acid esters for the first time. Gebauer et al.[16–18] successfully separated disubstituted aromatics, nucleosides, uracil derivatives, estradiol epimers, and *cis*/*trans* isomers of proline-containing peptides on calix[-*n*]arene-bonded ($n = 4$, 5, 6, and 8) silica gel. Menyes et al. reported that a hexapropylether of *p-tert*-butyl-calix[6]arene was covalently linked to silica for the separation of polycyclic aromatic hyrocarbons (PAHs) and fullerenes, and showed higher selectivity and lower consumption of solvent than conventional RP-C_{18}.[19] In the past few years, our research group[20–22] prepared *p-tert*-butyl-calix[6]arene-bonded and *p-tert*-butyl-calix[*n*]arene-bonded ($n = 4$, 6, and 8) silica gel stationary phases using coupling reagents by one-pot method, and investigated the chromatographic separations of some positional isomers, PAHs, nucleosides, bases, and quinolones drugs. The results show that calixarene-bonded stationary phases are excellent reverse-phase packings with

inclusion capability. The calixarene-bonded phase exhibits the promising application for HPLC. However, in contrast to cyclodextrin-bonded phases, the studies on calixarene-bonded stationary phases are too little.

In this paper, we reported that a new *p-tert*-butyl-calix[4]arene-bonded silica gel stationary phase (C4BS) was prepared via breaking ring reaction of 3-glycidoxypropyltrimethoxysilane used as coupling reagent in the presence of NaH and phase-transfer catalyst for HPLC (scheme shown in Fig. 1). Meanwhile, its structure was characterized, and chromatographic property was evaluated via the comparative study of C4BS with PEBS (the monomer), ODS, and a *p-tert*-butyl-calix[8]arene-bonded silica gel stationary phase (C8BS) by using different compounds as probes. The retention mechanism was proposed.

EXPERIMENTAL

Apparatus

Elemental analysis was performed with a MOD-1106 elemental analyzer (Italy). A Model 710 instrument (Nicolet Analytical Instruments) was used for

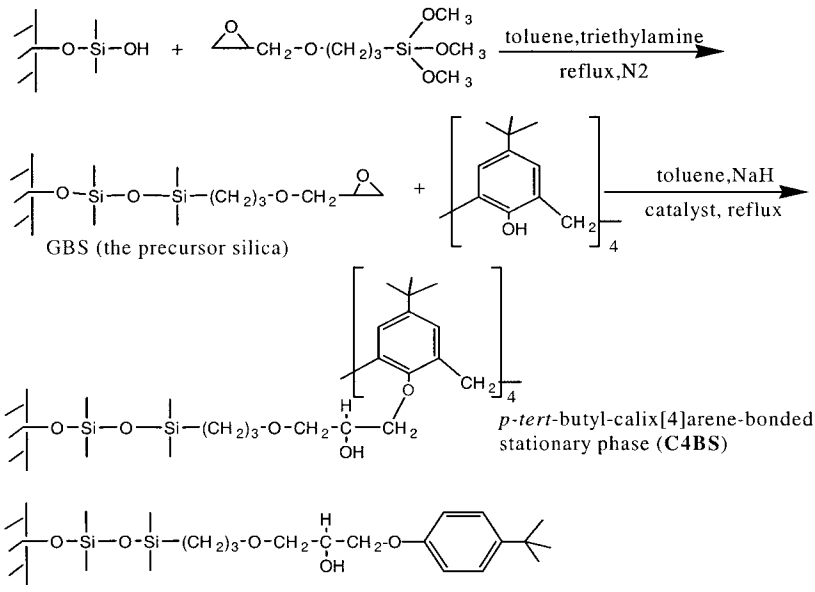

Figure 1. The preparation scheme of C4BS and PEBS.

Fourier transform infrared (FTIR) spectroanalysis. A Shimaduz DT-40 thermal analyzer was used for thermogravimetric analysis. The liquid chromatographic system was composed of a P200 II pump, a UV200 II variable wavelength UV-detector, attached Echrom 98 chromatographic data system (Dalian Elite company, Dalian, China), and a Rheodyne Model 7125 injector with 20-μL loop.

Reagents

Silica (Kromasil, particle size 5 μm, pore size 100 Å, the surface area 310 $m^2 g^{-1}$ and pore volume 0.9 mL g^{-1}), and C_{18} (Kromasil, particle size 5 μm, bonded amount 1.054 mmol g^{-1} silica) were purchased from Akzo Nobel (Sweden). 3-Glycidoxypropyltrimethoxysilane was purchased from Wuhan University Chemical Plant (Wuhan, China). *p-tert*-Butyl-calix[4]arene was synthesized according to a reported procedure.[23] A C8BS[24] with the same spacer and matrices of C4BS, was also prepared for the comparative study. Sulfadiazine (SD), sulfadimidine (SM_2), sulphamethoxazole (SMZ), sulfacetamide sodium (SA-Na), and trimethoprim (TMP) were obtained from the Northeastern Pharmaceutical Factory (Shengyang, China). *p-tert*-Butyl-phenol and other reagents were purchased from various commercial sources and were analytical grade, unless indicated. Water was doubly distilled water.

Preparation Procedures of C4BS and PEBS

The preparation scheme of the new stationary phase (C4BS) was shown in Fig. 1. The silica gel (10 g) was pretreated according to conventional methods, by hydrochloric acid to remove metal ion and maximize the number of silanol groups on the surface, then was heated at 160°C for at least 10 hr, and kept in a dessicator before use.

A mixture of 5.0 mL 3-glycidoxypropyltrimethoxysilane and 4.0 g activated silica gel in 50 mL dry toluene distilled fresh, was stirred and heated at reflux under streaming dry nitrogen gas, with 0.1 mL triethylamine as catalyst for 6 hr. The bonded silica gel was filtered, washed with toluene and acetone. 3-Glycidoxypropyl-bonded stationary phase (4.82 g) (the precursor silica) was obtained, which was to be used as precursor of following reaction.

A mixture of 1.2 g *p-tert*-butyl-calix[4]arene and 0.15 g NaH in 50 mL anhydrous toluene was heated, with stirring, under an inert atmosphere at 80°C for 30 min. Subsequently, 3.0 g of the precursor silica and 0.5 g phase-transfer catalyst were added to the suspension and heated immediately to

reflux for 24 hr. The bonded-material was filtered and washed in sequence with toluene, acetone, doubly distilled water, dimethylformamide, and acetone. Finally, the calix[4]arene-bonded stationary phase (C4BS) was dried at 120°C and weighed. The gain weight was 29.94% to bare silica gel in all.

The *p-tert*-butylphenyl-ether-bonded stationary phase (PEBS, the monomer-bonded phase) was synthesized by the above similar method, with some modification. The procedure was described briefly as follows: a mixture of 0.6 g *p-tert*-butyl-phenol, 0.05 g NaH, 2.5 g GBS, and 0.2 g catalyst in 30 mL anhydrous toluene, was refluxed with stirring, under an inert atmosphere for 24 hr. Subsequently, the mixture was treated by the same processes. The bonded phase (PEBS) was obtained. Its structure was also shown in Fig. 1 and characterized by FTIR and elemental analysis (Table 1).

Chromatographic Procedure

The bonded phases (C4BS, PEBS, C8BS, GBS, and ODS) were, respectively, packed into stainless-steel columns (150 mm × 4.6 mm i.d.) by using the balanced-density slurry technique. The mobile phases were methanol–H_2O or phosphate buffers, unless indicated. The flow rates of mobile phases were generally set at 0.8 mL min^{-1}. The samples were dissolved in methanol or mobile phases and kept in a refrigerator (in the dark). The wavelength of detection was at 254 nm or 270 nm. The concentration of samples was from 50 to 200 μg mL^{-1}. Typically, 5 μL of sample solutions were injected. The aqueous solution of a 0.05 M sodium nitrate was used as probe to determine void time. Each sample was chromatographed under ambient temperature (25 ± 2°C) at least twice, and the average retention times for solutes were reported.

Table 1. Results of elemental analysis of the bonded stationary phases.

Bonded phases	Element (%)		Bonded amounts (mmol g^{-1} silica)
	C	H	
C4BS	19.52	2.89	0.211
PEBS	12.85	2.13	0.371
C8BS	15.93	2.85	0.071
GBS (the spacer)	8.40	1.42	0.875
ODS (Kromasil C_{18})	19.0	3.16	1.054

Note: The same coupling reagent and matrices were used.

RESULTS AND DISCUSSION

Preparation of Bonded Phases

Column packing material is always a key factor in influencing the development of HPLC. Unfortunately, the most synthesizing methods of the calixarene-bonded phases were patents. To our knowledge, the coupling reagents play significant roles in the syntheses of bonded silica gel stationary phases. The addition reaction of *p*-allycalixarenes with triethoxysilane under catalysis with hexachloroplatinic acid was a usual method to prepare the calixarene-bonded phases.[13] In our laboratory, Xu et al.[20] prepared the *p-tert*-butyl-calix[6] arene-bonded phase via breaking ring reaction of 3-glycidoxypropyltrimethoxysilane as coupling reagent and perchloric acid as catalyst. The bonded amount of the stationary phase was less than 0.06 mmol g^{-1}, which could not exhibit ideal separation because of poor column efficiency for the solutes to be tested. The phenolic hydroxyl groups of the calix[6]arene were suppressed by $HClO_4$ as catalyst, leading to lower-bonded amount. The new approach used includes two steps, the precursor, 3-glycidoxypropyl-bonded stationary phase, was first synthesized, then the calix[4]arene-bonded phase was prepared by using phenolic sodium of calix[4]arene in the presence of phase-transfer catalyst, to cleave the epoxy group of GBS.

Characterization of the New Calix[4]arene-Bonded Phase

The results of elemental analysis were shown in Table 1. The bonded amount of C4BS was found to be 0.211 mmol g^{-1}, according to the carbon content.

The FTIR spectrum shows the disappearance of a strong absorption band at 3600–3700 cm^{-1}, which is characteristic of the residual Si–OH stretching frequency after subtraction of bare silica (Fig. 2). Peaks at 2960.46, 2883.64 cm^{-1} can be assigned to C—H stretching frequency. The characteristic absorption band of the benzene ring should appear at 1669.99, 1593.17, 1470.27 cm^{-1}, and the peak at 1388.34 cm^{-1} is attributed to C—H bending frequency of the butyl group. Peaks at 805.14, 744.75 cm^{-1} should be the C—H out-planar bending frequency of the benzene rings. The above spectra data indicate that the calix[4]arene ligand existed on the silica gel.

Additionally, the thermal analysis of C4BS shows that lost weight occurred in the range of temperature from 300°C to 600°C, which was over GBS (250–500°C). It indicates that the new packing is possessed of high heat and chemical stability, which is preferable to GBS (the spacer silica). Meanwhile, thermal analysis (from 30°C to 750°C, temperature rate

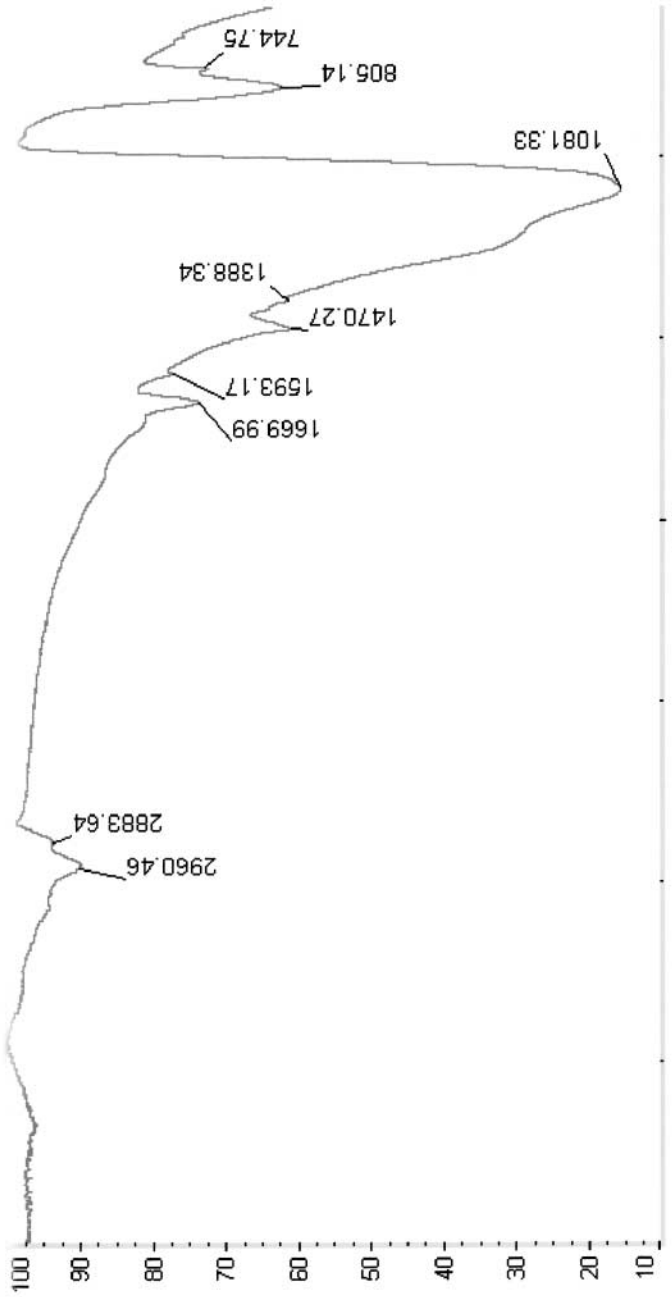

Figure 2. FTIR spectrum (KBr) of the new C4BS.

$10^{\circ}C\,min^{-1}$) shows that the C4BS loses weight by 30.94%, which is consistent with the weight gain of C4BS preparation. In the chromatographic characterization, we also found that the retention values of PAHs on C4BS were much higher than those on GBS. The results indicate that the calix[4]arene was successfully immobilized to the spacer silica gel.

Column Efficiency and Stability of C4BS

The column efficiency of the new calix[4]arene-bonded phase was determined by using methanol–water (70 : 30, v/v) as mobile phase and biphenyl as solute, at a flow rate of 0.8 mL min^{-1}. In this condition, the retention time of biphenyl is 4.85 min and the theoretical plate number was about 10,200 per meter. The column has alternately been eluted with methanol and methanol–0.02 M H_3PO_4 for a week. The retention time of biphenyl on C4BS was 4.85 ± 0.1 min and the column efficiency almost did not change. The results show that the new bonded phase is stable and repeatable in chromatographic procedures. The long-time stability of C4BS will be further investigated.

Separations of PAHs and Alkylbenzenes

Many empirical tests[25–27] were used to evaluate hydrophobicities of stationary phases for RP-C_{18}. Though there exists no universally accepted chromatographic test system for particular packing because of other special interactions coexisting, especially, the calixarene-bonded phases, the alkylbenzene homologues and PAHs were often used as probes to investigate hydrophobicity of the new packing.

In this paper, the above probes were employed for the hydrophobic evaluation of the new packing. Meanwhile, several bonded phases with the same spacer were employed for the comparative study of C4BS.

As can be seen in Fig. 3, the carbon number rule was observed in the separation of alkylbenzene homologues on C4BS. Further investigation also shows that the retention values of PAHs decreased largely, with increasing methanol content in mobile phases. The results indicate that the new stationary phase can behave as an excellent reversed-phase packing, and the hydrophobic interaction plays a significant role in the retention of the hydrocarbons on the new column. The slopes of the three curves were in the order of ODS $>$ C4BS $\approx$ PEBS, which are correlated to their hydrophobicities.

The rapid separation of nine PAHs, except for phenanthrene and its isomer on C4BS, can be achieved within 10 min. This ascribes to the

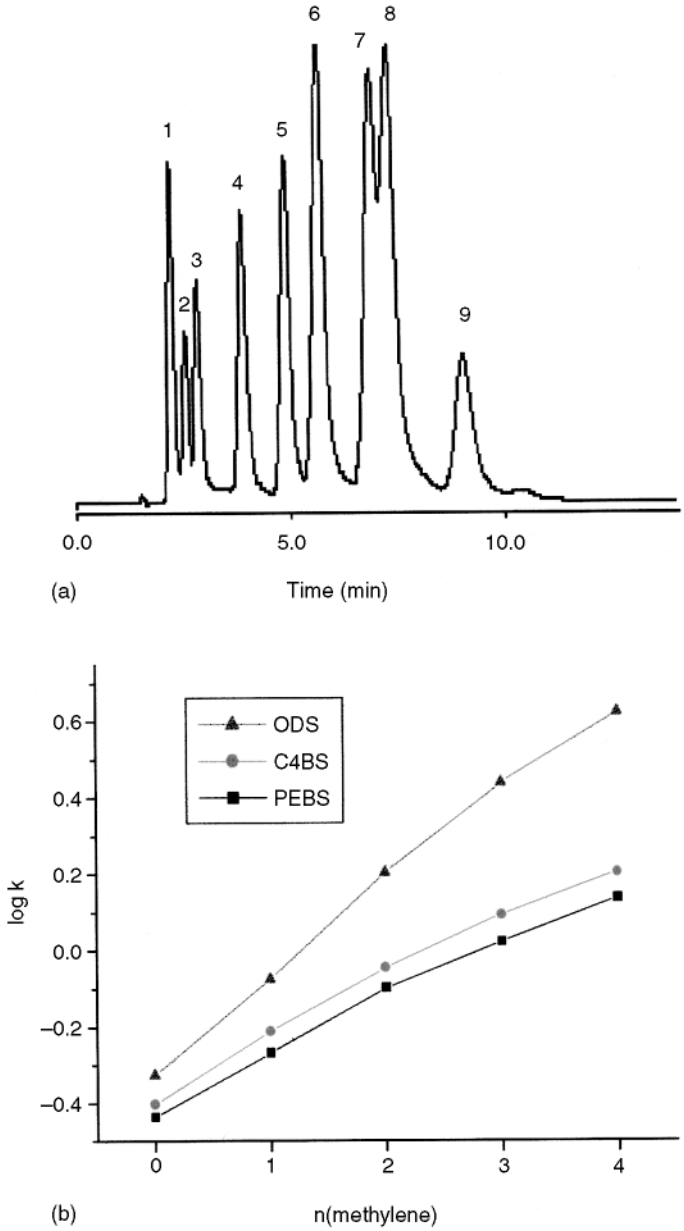

Figure 3. The chromatogram (a) of PAHs on C4BS and the plot (b) of the log k vs. the number (n) of the methylene groups in alkylbenzenes on three bonded phases.

natural reversed-phase property of C4BS with moderate hydrophobicity relative to ODS. As shown in Table 2, the retention of PAHs on C4BS was much less than that on C8BS, which is inconsistent with the higher bonded amount and carbon loading of C4BS (see Table 1). Obviously, the ring size of the calixarene ligands is also one of factors in the above separation. Other additional interactions, such as inclusion complex on large cyclic phase (C8BS) can be assumed. For example, better separation of phenanthrene and anthracene on C8BS can be achieved, instead of C4BS, which implies that inclusion complex tend to occur on the phases with large cavities. Table 2 also shows that the similar retention of PAHs can be found on C4BS and its oligomeric bonded phase (PEBS). It illustrates that the hydrophobicities mainly derived from the aromatic ring or moiety of ligands.

In order to further investigate the hydrophobicity of new stationary phase, the selectivities (α) of C4BS for methylene and phenyl groups were calculated by the method,[28] and the result was given in Table 3. As can be seen, the α (methylene) value of C4BS (1.4189) was similar to PEBS (1.3926), while the α (phenyl) value of the former (2.9142) was more than that of the latter (2.4393). The phenomenon can be explained as follows: on one hand, the selectivities for methylene groups was partially dependent on the flexible *p-tert*-butyl groups, thus, similar α (methylene) can be observed on the two columns. On the other hand, the selectivities for phenyl groups can be determined by the rigid moiety, in which the tetrapolymers moiety of C4BS is rigid relative to the rotatable single anchor of PEBS. In other words, the low flexibility of moiety facilitates the recognition of phenylogues via efficient hydrophobic and $\pi-\pi$ interactions with the analytes. It can also be noticed from

Table 2. The retention factors (k) of PAHs on several bonded stationary phases.

Solutes	C4BS	PEBS	C8BS
Benzene	0.430	0.523	1.356
Toluene	0.778	0.681	1.967
Xylene	0.968	0.859	2.622
Naphthalene	1.429	1.295	3.567
Biphenyl	2.079	1.710	5.267
Fluorene	2.571	2.343	7.567
Phenanthrene	3.587	2.996	9.344
Anthracene	3.865	3.115	10.083
Fluoranthene	4.698	4.242	13.778

Note: Mobile phases: methanol–water (70 : 30, v/v); flow rates: 0.8 mL min^{-1}.

Table 3. The selectivities (α) of several bonded phases for methylene and phenyl groups.

Bonded phases	Groups	Equations of linear regression	R	α
C4BS	Methylene	$\ln k = 0.3499n - 0.8623$	0.9946	1.4189
	Phenyl	$\ln k = 1.0696n - 1.8702$	0.9975	2.9142
PEBS	Methylene	$\ln k = 0.3312n - 0.9509$	0.9936	1.3926
	Phenyl	$\ln k = 0.8917n - 1.5342$	0.9999	2.4393
ODS	Methylene	$\ln k = 0.5561n - 0.7110$	0.9925	1.7439
	Phenyl	$\ln k = 0.9870n - 1.1451$	0.9995	2.5176

Note: $\ln k = \ln(\alpha)n + b$, where n is the number of methylene or phenyl groups, $\ln(\alpha)$ and b are constants. Mobile phases: methanol–water (70 : 30,v/v) for C4BS and PEBS (80 : 20) for ODS.

Table 3, that ODS has higher α values as compared with both C4BS and PEBS, which is obvious evidence of hydrophobic interaction in the separation of nonpolar hydrocarbons. Nevertheless, C4BS can provide rapid separation of PAHs and alkylbenzenes in comparison with conventional RP-C_{18}.

Separation of Aromatic Positional Isomers

A lot of experiments show that cyclodextrin-bonded stationary phase exhibits high selectivity for substituted benzenes, which is based on its host–guest inclusion characteristic. The relative retention value of isomers of nitroaniline on the cyclodextrin-bonded phase was taken as one of the evaluation index of its bonded amount.[29] Lee and Friebe et al.[16] also described the similar inclusion properties of the calixarene-bonded phases for nitroaniline, respectively. However, the available literatures of the calixarene-bonded phase were very limited. So, some position isomers with polar groups (OH, NH_2, and COOH) were used as probes so as to investigate the chromatographic contribution of other interactions besides hydrophobic interaction.

The first study is to investigate the chromatographic behavior of GBS (the spacer-bonded silica gel). The limited capability to separate the position isomers on GBS was observed. Moreover, the retention values of the analytes on this packing fluctuate in acidic mobile phases, which may result from the hydrolysis of the epoxy group partially. However, better resolution for the isomers on C4BS can be observed as compared with GBS, which illustrates that the separation was largely dependent on the calix[4]arene ligand.

After the investigation of the separation for hydrocarbons, it can be concluded that the hydrophobicities of C4BS and PEBS were much less than that of ODS. However, as shown in Table 4, most isomers exhibited stronger affinity to C4BS and PEBS, instead of ODS. Obviously, other interactions, besides hydrophobic interaction, existed in the separation of aromatic isomers. Based on the experimental data, some conclusions can be made as follows.

Hydrogen Bond Interaction

Most polar solutes with hydrogen-donor on C4BS exhibit stronger retention. Moreover, the elution orders of solutes on C4BS and ODS were also different. For example, methylphenol isomers on ODS can be eluted in the order of $o < m < p$, which are consistent with their dissociation constants (pK_a). However, the *ortho*-methylphenol on C4BS was finally eluted. Different elution orders for other isomers can also be found, such as methylanilines, naphthols, naphthylamines and so on, which implied different

Table 4. The elution order and retention factors (k') of the position isomers on several bonded phases.

Positional isomers	Retention factors (k')		
	C4BS	PEBS	ODS
Benzenediol[a]	0.540, 0.841, 1.016 (*pmo*)[b]	0.46, 0.65, 0.74 (*pmo*)	0.51, 0.74, 1.36 (*pmo*)
Methylphenol[c]	2.444, 2.492, 2.603 (*mpo*)	2.04, 2.08, 2.27 (*pmo*)	2.15, 3.50, 7.96 (*omp*)
Naphthol[d]	4.905, 6.213 ($\beta\alpha$)	3.38, 4.07 ($\beta\alpha$)	6.15, 7.28 ($\alpha\beta$)
Naphthylamine[d]	3.402, 3.4879 ($\alpha\beta$)	2.65, 3.09 ($\alpha\beta$)	2.91, 3.02 ($\beta\alpha$)
Methylaniline[c]	0.794, 1.397, 1.429 (*pom*)	1.56, 1.95, 2.37 (*omp*)	0.25, 1.18, 1.20 (*omp*)
Nitroaniline[c]	2.032, 3.651, 4.794 (*pmo*)	2.59, 2.79, 3.47 (*mpo*)	0.83, 3.90, 3.92 (*pmo*)
Nitrobenzoic acid[e]	2.017, 2.344 (*mp*)	0.80, 0.83 (*mp*)	1.02, 1.12 (*mp*)
Phthalic acid[e]	0.735, 0.947, 6.471 (*mpo*)	0.38, 0.41, 0.88 (*mpo*)	0.30, 0.48, 0.97 (*mpo*)

Note: Flow rates: 0.8 mL min^{-1}.
[a]Mobile phase: methanol–water (30 : 70, v/v).
[b]Elution order.
[c]Mobile phase: methanol–water (40 : 60, v/v).
[d]Mobile phase: methanol–water (50 : 50, v/v).
[e]Mobile phase: methanol–0.02 M H_3PO_4 (70 : 30, v/v).

mechanisms were employed on the two columns. The same situation occurred between PEBS and ODS. Hence, the hydrogen bonding interaction between the solutes and the ether-oxygen of C4BS or PEBS are conceivable.

π-Electron Transfer

The isomers of nitroaniline and nitrobenzoic acid on C4BS exhibit much stronger retention than those on ODS. These nitro compounds have also longer retention times relative to other analytes. Moreover, the separation of these isomers on C4BS is improved largely. The reason for these phenomena is more likely to be due to the π-electron transfer between the calix[4]arene and the nitro compounds for the electron-withdrawing effect of nitro groups. However, the same phenomenon cannot appear on PEBS, which might be the rotation of the monomer around the single ether-bond.

$\pi-\pi$ Interaction

Making a comparison with C4BS and ODS, it was found that $\pi-\pi$ interaction can contribute to the chromatographic processes. For example, after further investigation of the behavior of the phenols and anilines, we found that theses solutes exhibit stronger affinity to C4BS, instead of ODS, regardless of the acidity changes of mobile phases. The phenomenon reveals that there is the additional interaction between the parents of solutes (e.g., benzene ring, naphthalene ring) with the moiety of the calixarenes. In this case, $\pi-\pi$ interaction can be assumed.

Synergistic Effect

As can been noticed in Table 4, the elution order of naphthols and naphthylamines on C4BS were similar to PEBS, and different from ODS. Obviously, it ascribed to the synergistic effect of $\pi-\pi$ interactions and hydrogen bonding interaction (OH, NH_2), lacking of ODS. The elution order of naphthol isomers was β (pK_a 9.57) $<\alpha$(pK_a 9.30), while α (pK_b 10.08) $<\beta$ (pK_b 9.89) for naphthylamines on C4BS. This can be explained as follows: on one hand, α-naphthol with stronger acidity can ionize into more α-naphthoxylic anions, and β-naphthylamine has stronger alkaline compared to α-naphthylamine. Both α-naphthoxylic anions and β-naphthylamine is better electron-donors with high electron density. So, the stronger $\pi-\pi$ interaction with C4BS finally led to elution. On the other hand, both α-naphthoxylic anions and β-naphthylamine are also better hydrogen-acceptors. They facilitated the formations of hydrogen bonding with the residual phenolic hydroxyl groups of C4BS, which also contributes to the

retention of the isomers in different degrees. The two effects determine the elution order of the naphthols and the naphthylamines. Additionally, it can also be noticed in Table 4, that the k' values of naphthols and naphthylamines on C4BS are largely more than those on PEBS, which indicates the $\pi-\pi$ interaction is dependent on the size of π-electron system.

As can be observed in Table 4, C4BS exhibited strong retention and high selectivity for nitroaniline, which were obviously superior to both PEBS and ODS. The synergistic effect of π-electron transfer and hydrogen bond interactions should be responsible for the behavior of C4BS.

Obviously, the above additional interactions improve the selectivity of C4BS for the isomers in some degree. Even so, special selectivity of C4BS for the analytes cannot be observed in this case, which might be because of the small cavity (3 Å) and weaker hydrophobicity, as compared with C8BS (11.7 Å) and ODS, respectively.

Separation of *N*-Substituted Anilines

In order to further investigate the property of new stationary phase for these basic compounds, aniline, *N*-methylaniline, *N*, *N*-dimethylaniline, and diphenylamine were used as probes. The chromatograms and the separation factors of the N-substituted anilines were shown in Fig. 4 and Table 5, respectively.

That hydrophobic interaction is still predominant in the separation of the N-substituted anilines on C4BS and ODS. An advantage of C4BS is the fast analysis of the analytes using the mobile phases at pH 5.5 within 10 min. The five anilines exhibit weak retention on C4BS, which indicates the new phase has weaker ion-exchange capacities and is suitable for basic solutes.

According to the k' and $\alpha_{1,2}$ values of anilines shown in Table 5, hydrogen bonded interaction between the anilines and C4BS can be confirmed. For example, *N*,*N*′-dimethylaniline cannot form hydrogen bonds with the ligands of C4BS, because the ternary aniline contain no N—H group and other anilines do. Thus, the retention value of *N*, *N*′-dimethylaniline was less than the expectant value, which led to the small $\alpha_{1,2}$ value of *N*, *N*′-dimethylaniline against *N*-methylamine. As can be noticed in Table 5, the $\alpha_{1,2}$ values of the analytes on ODS changed slightly from 2.357 to 2.772. In contrast to this, obvious changes of the $\alpha_{1,2}$ can be found from 1.494 to 5.293 on C4BS, C8BS, and PEBS. This is because the separation of the above anilines on ODS is based on only hydrophobic interaction, which was different from other phases.

The k' and α values of the anilines on C4BS were much less than those on C8BS, especially, diphenylamine, which may result from the inclusion

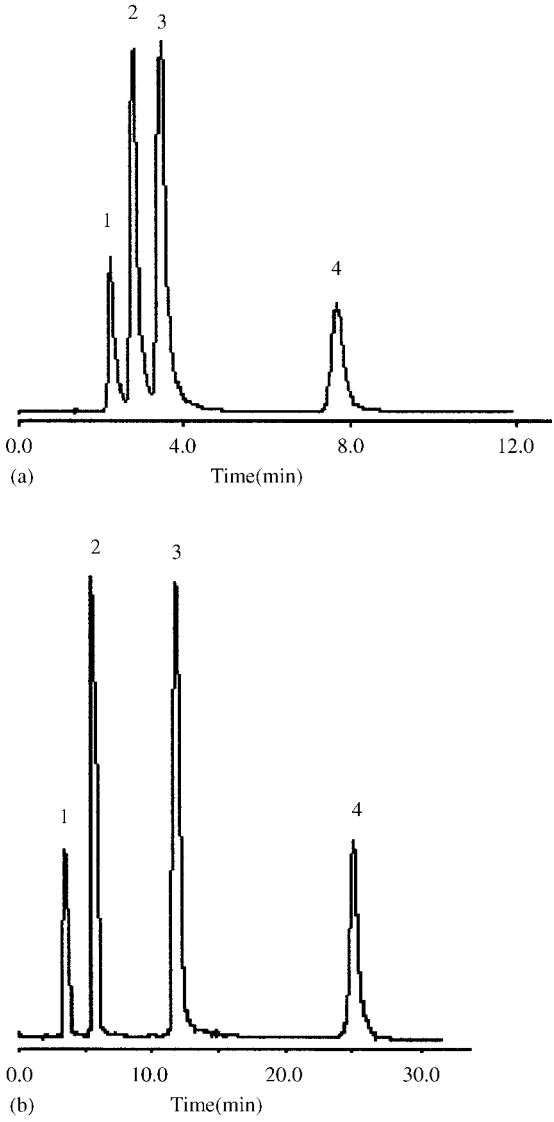

Figure 4. The chromatograms of the N-substituted anilines on C4BS (a) and on ODS (b). Mobile phases: methanol–0.02 M KH_2PO_4 (60 : 40, v/v, pH 5.5); flow rates: 0.8 mL min^{-1}; UV: 270 nm. Peaks: (1) aniline; (2) *N*-methylaniline; (3) *N*,*N*-dimethylaniline; (4) diphenylamine.

Table 5. The k and $\alpha_{1,2}$ values of the *N*-substituted anilines on several bonded phases.

Bonded phases		Aniline	*N*-Methylaniline		*N*,*N*′-Dimethylaniline		Diphenylamine
C4BS	k'	0.418	0.778		1.180		3.868
	$\alpha_{1,2}$		1.861	1.517		3.278	
PEBS	k'	0.465	0.763		1.140		3.144
	$\alpha_{1,2}$		1.641	1.494		2.758	
C8BS	k'	0.939	1.761		3.050		16.144
	$\alpha_{1,2}$		1.875	1.732		5.293	
ODS	k'	0.806	1.972		5.467		12.883
	$\alpha_{1,2}$		2.447	2.772		2.357	

Note: Separation factors ($\alpha_{1,2}$); mobile phases: methanol–0.02 M KH_2PO_4 (60 : 40, v/v, pH 5.5); flow rates: 0.8 mL min^{-1}; UV: 270 nm.

complex of diphenylamine in the large calix[8]arene ligand. The above results also illustrate the C4BS is a good hydrogen-acceptor and poor donor.

Separation of Sulfonamides

Sulfonamides (Fig. 5) are antibacterial agents widely used both in clinical application and in the prevention of diseases in food-producing animals. These compounds contain two aromatic rings linked via sulfonamido groups, and the planes of the rings are relatively rigid.[30] As expected, sulfonamides were also better probes to investigate the steric selectivity of stationary phases.

Figure 6 shows that better separation of sulfonamides on C4BS can easily be achieved under the same condition, instead of both PEBS and ODS. The retention value of SA-Na (pK_{a1} 1.8, pK_{a2} 5.4) was small on all the columns, because it was in ionic form in the mobile phase at pH 3.5. Better separation of the three solutes including SD (pK_a 6.52), SM_2 (pK_a 7.4), and SMZ (pK_a 6.0) on ODS can be obtained with high column efficiency, which were consistent with their hydrophobicities. The strong retention of SMZ might be related to its vertical stacking.[31] The results imply that hydrophobic interaction was predominant in the separation.

However, it is interesting that the behavior of TMP on the three columns was different. Stronger retention of TMP can be observed on ODS and PEBS, especially, the latter. Obviously, hydrophobic interaction, hydrogen bond, and $\pi-\pi$ interactions can clearly not explain this phenomenon. TMP can first be eluted on C4BS, C8BS, and C6BS.[22] Different steric selectivities of three

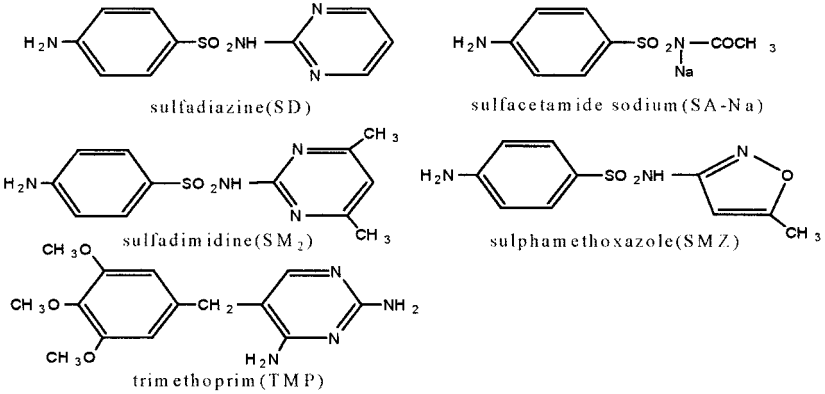

Figure 5. The chemical structures and dissociation constants of sulfonamides.

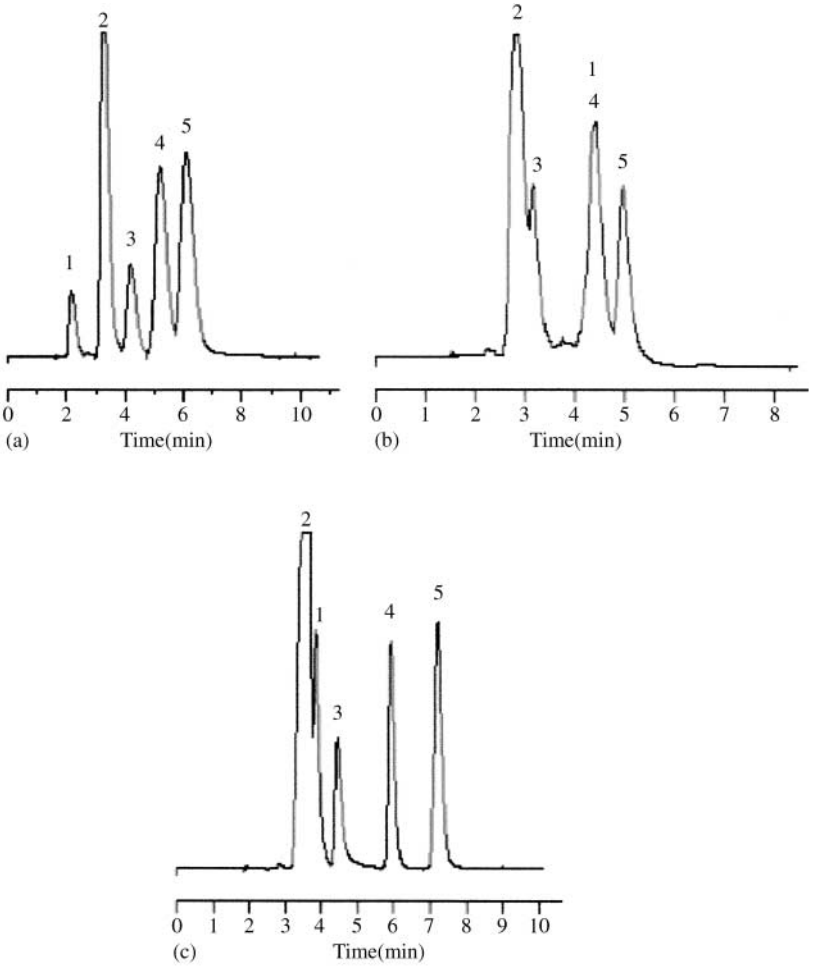

Figure 6. The chromatograms of sulfonamides on C4BS (a), PEBS (b), and ODS (c). Mobile phases: methanol–0.02 M KH_2PO_4 (50 : 50, v/v, pH 3.5); flow rates: 0.8 mL min^{-1}; UV: 254 nm. Peaks: (1) TMP; (2) SA-Na; (3) SD; (4) SM_2; (5) SMZ.

stationary phases should be responsible for the behavior. The two aromatic rings in sulfonamides were relatively planar via $\pi-\pi$ and $n-\pi$ conjugations of sulfamido groups. However, the aromatic rings linked by methylene groups does not conjugate, thus, the rings can rotate around the single bond. It weakens the hydrophobic and $\pi-\pi$ interactions between TMP and the rigid moiety of C4BS and results in weak retention of the analyte on C4BS.

In contrast to this, the flexible PEBS exhibits stronger infinity to TMP via hydrophobic and $\pi-\pi$ interactions.

CONCLUSIONS

A new C4BS phase was prepared via 3-glycidoxypropyltrimethoxysilane as coupling reagent in the presence of NaH and catalyst, by one-pot method. Its structure was characterized by FTIR, elemental analysis, and thermal analysis. The HPLC property of the new packing was evaluated by using various analytes as probes and several stationary phases as references. The chromatographic data indicates that C4BS has excellent reversed phase properties and can provide various sites for analytes, which endow versatile performance relative to PEBS and ODS. For example, stronger hydrogen bond, $\pi-\pi$ and steric interactions contribute to the fast analysis of C4BS for hydrocarbons and its high selectivity for sulfonamides. Nevertheless, the continuing efforts on retention mechanism and application of the new material are being carried out in our laboratory.

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REFERENCES

1. Shinkai, S.; Araki, K.; Manabe, O. NMR determination of association constants for calixarene complexes. Evidence for the formation of 1:2 complex with calix[8]arene. J. Am. Chem. Soc. **1988**, *110*, 7214–7215.
2. Nomura, E.; Taniguchi, H.; Kawaguchi, K.; Otsuji, Y. Catalytic ability of flexible *octapus*-type calix[6]arene in ester-forming reactions and its structural properties. J. Org. Chem. **1993**, *58*, 4709–4715.
3. Gutche, C.D.; Alam, I. Calixarenes. 23. The complexation and catalytic properties of water soluble calixarenes. Tetrahedron **1988**, *44*, 4689–4694.
4. Mnuk, P.; Feltl, L. Gas chromatographic study of the inclusion properties of calixarenes I. *p-tert*-Butylcalix[4]arene in a micropacked column. J. Chromatogr. A **1995**, *696*, 101–112.

5. Lin, L.; Wu, C.Y.; Yan, Z.Q.; Yan, X.Q.; Su, X.L.; Han, H.M. Calix[4]-arene derivatives as stationary phases for capillary gas chromatography. Chromatographia **1998**, *47*, 689–694.
6. Kalchenko, O.I.; Lipkowski, J.; Kalchenko, V.I.; Vysotzky, M.A.; Markovsky, L.N. Effect of *octakis* (diethoxyphosphoryloxy)-*tert*-butyl-calix[8]arene in mobile phase on the reversed-phase retention behavior of aromatic compounds: host–guest complex formation and stability constants determination. J. Chromatogr. Sci. **1998**, *36*, 269–273.
7. Arena, G.; Casnati, A.; Contino, A.; Mirone, L.; Sciotto, D.; Ungaro, R. Synthesis of new calixcrowns and their anchoring to silica gel for the selective separation of Cs^+ and K^+. J. Chem. Soc. Chem. Commun. **1996**, 2277–2278.
8. Shohat, D.; Grushka, E. Use of calixarenes to modify selectivities in capillary electrophoresis. Anal. Chem. **1994**, *66*, 747–750.
9. Zhao, T.; Hu, X.; Cheng, J.K.; Lu, X. Use of calix[4]arene to separate positional isomers in capillary electrophoresis. Anal. Chim. Acta **1998**, *358*, 263–268.
10. Millership, J.S.; Mckervey, M.A.; Russell, J.A. Complexation and chromatographic studies involving water soluble calixarenes and nitrophenols. Chromatographia **1998**, *48*, 402–406.
11. Kalchenko, O.I.; Perret, F.; Nicole, D.M.; Wcoleman, A. A comparative study of the determination of the stability constants of inclusion complexes of *p*-sulfonatocalix[4]arene with amino acids by RP-HPLC and ^{1}H NMR. J. Chem. Soc. Perkin Trans. II **2001**, 258–263.
12. Mibradt, R.; Bohmer, V. *Calixarenes 2001*; Kluwer Academic Publishers: Dordrecht, NL, 2001, 663–676.
13. Glennon, J.D.; O'Connor, K.; Srijaranai, S.; Manley, K. Enhanced chromatographic selectivity for Na^+ ions on a calixarene-bonded silica phase. Anal. Lett. **1993**, *26*, 153–162.
14. Glennon, J.D.; Horne, E.; O'Connor, K.; Kearney, G.A.; Harris, S.J.; McKewey, M.A. Chromatographic selectivity for amino acid esters and alkali metal ions on a silica bonded calix[4]arene. Anal. Proc. **1994**, *31*, 33–35.
15. Brindle, R.; Albert, K.; Harris, S.J.; Tröltzsch, C.; Horne, E.; Glennon, J.D. Silica-bonded calixarenes in chromatography I. Synthesis and characterization by solid-state NMR spectroscopy. J. Chromatogr. A **1996**, *731*, 41–46.
16. Friebe, S.; Gebauer, S.; Krauss, G.J. HPLC a calixarene bonded silica gels. I Characterization and applications of the *p-tert*-butyl-calix[4]arene bonded material. J. Chromatogr. Sci. **1995**, *33*, 281–284.
17. Gebauer, S.; Friebe, S.; Gübitz, G.; Krauss, G.J. High performance liquid chromatography on calixarene-bonded silica gels. II separations

of *regio-* and *stereo*isomers on *p-tert*-butyl-calix[*n*]arene phases. J. Chromatogr. Sci. **1998**, *36*, 383–387.
18. Gebauer, S.; Friebe, S.; Scherer, G.; Gubitz, G.; Krauss, G.J. High performance liquid chromatography on calixarene-bonded silica gels. III Separations of *cis/trans* isomers of proline-containing peptides. J. Chromatogr. Sci. **1998**, *36*, 388–394.
19. Menyes, U.; Roth, U.; Troeltzsch, C. European Patent 786,661 A2, 1997.
20. Xu, W.; Li, J.S.; Feng, Y.Q.; Da, S.L.; Chen, Y.Y.; Xiao, X.Z. Preparation and characterization of *p-tert*-butyl-calix[6]arene-bonded silica gel stationary phase for high-performance liquid chromatography. Chromatographia **1998**, *48*, 245–250.
21. Xiao, X.Z.; Feng, Y.Q.; Da, S.L.; Zhang, Y. Preparation and evaluation of *p-tert*-butyl-calix[8]-arene-bonded silica stationary phase for high performance liquid chromatography. Anal. Lett. **2000**, *33*, 3355–3372.
22. Xiao, Y.X.; Xiao, X.Z.; Feng, Y.Q.; Wang, Z.H.; Da, S.L. High-performance liquid chromatography of sulfonamides and quinolones on *p-tert*-butyl-calix[6]arene-bonded silica gel stationary phase. Talanta **2002**, *56*, 1141–1151.
23. Gutsche, C.D.; Dhawan, B.; No, K.H.; Muthukrishnan, R. Calixarenes.4. The synthesis, characterization, and properties of the calixarenes from *p-tert*-butylphenol. J. Am. Chem. Soc. **1981**, *103*, 3782–3792.
24. Li, L.S.; Li, M.; Feng, Y.Q.; Da, S.L. High performance liquid chromatography of aromatic carboxylic acids on *p-tert*-butyl-calix[8]arene-bonded silica gel stationary phase. Talanta **2004**, *62* (3), 643–648.
25. Kimata, K.; Iwaguchi, K.; Onishi, S.; Jinno, K.; Eksteen, R.; Hosoya, K.; Araki, M.; Tanaka, N. Chromatographic characterization of silica C_{18} packing materials. Correlation between a preparation method and retention behavior of stationary phase. J. Chromatogr. Sci. **1989**, *27*, 721–728.
26. Neue, U.D.; Alden, B.A.; Walter, T.H. Universal procedure for the assessment of the reproducibility and the classification of reversed-phase packing. J. Chromatogr. A **1999**, *849*, 101–116.
27. Sokoließ, T.; Schonherr, J.; Menyes, U.; Roth, U.; Jira, T. Characterization of calixarene- and resorcinarene-bonded stationary phases I. Hydrophobic interactions. J. Chromatogr. A **2003**, *1021*, 71–82.
28. Sokoließ, T.; Menyes, U.; Roth, U.; Jira, T. New calixarene-bonded stationary phases in high-performance liquid chromatography: comparative studies on the retention behavior and on influences of the eluent. J. Chromatogr. A **2000**, *898*, 35–52.
29. Armstrong, D.W.; Demond, W. Cyclodextrin bonded phase for the liquid chromatographic separation optical, geometrical, and structural isomers. J. Chromatogr. Sci. **1984**, *22*, 411–415.

30. Zhang, W.S.; Li, A.L. *Medicinal Chemistry*; Chinese Higher Education Press: Beijing, 2002, 540–545.
31. Brown, P.R.; Robb, C.S.; Geldart, S.E. Perspectives on analyses of nucleic acid constituents: the basis of genomics. J. Chromatogr. A **2002**, *965*, 163–173.

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