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Investigation on the preparation and chromatographic behavior of a new *para*-tert-butylcalix[4]arene-1,2-crown-4 stationary phase for high performance liquid chromatography

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ABSTRACT

In the present work, a new *para*-tert-butylcalix[4]arene-1,2-crown-4 bonded silica stationary phase (CBS4-4) was synthesized, structurally characterized, and employed to separate polycyclic aromatic hydrocarbons (PAHs), phenols, aromatic amines, benzoic acid and its derivatives. The chromatographic behaviors of the prepared stationary phase were investigated and compared with ODS. The effects of methanol concentrations on the retention index show that CBS4-4 exhibits high selectivity for the above analytes. The separation mechanisms based on the different interactions between calixarene and the analytes were discussed. With the assistance of quantum chemistry calculation, the interaction Gibbs free energy change ΔG_{solv} (in the mobile phase) of *p*, *m* and *o*-phenylenediamine positional isomers and *para*-tert-butylcalix[4]arene-1,2-crown-4 were obtained. The ΔG_{solv} values were consistent with the retention behavior of *p*, *m* and *o*-phenylenediamine on the CBS4-4. According to the chromatographic data, it can be concluded that the selectivity of CBS4-4 for analytes is mainly ascribed to hydrophobic interaction, accompanied by other effects such as hydrogen bonding interaction, π–π and inclusion interaction. The CBS4-4 column has been successfully employed for the analysis of benzoic acid in Sprite drink.

1. Introduction

With the development of high performance liquid chromatography (HPLC), various new bonded stationary phases were continuously explored and applied. However, until now there exists no universally accepted chromatographic test to choose an appropriate packing material for a particular separation problem [1]. The search for more efficient stationary phases with the widest possible spectrum of applications has become a significant direction in the study of compound separation by HPLC. Recently, macrocyclic compounds (cyclodextrins, crown ethers and calixarenes), which are capable of forming inclusion complexes with guest molecules, are commonly used in modern chromatography. Many chromatographic scientists have paid the exceptional attention to calixarenes because their unique structures. Calixarenes, cavity-shaped cyclic molecules consisting of phenol units linked via methylene bridges, are known as a typical representative of the third-generation host after crown ethers and cyclodextrins [2]. The peculiar configurations lead to the formation of typical host–guest interaction between calixarenes and numerous compounds, and result in widely varied applications in ion-selective membranes and electrodes [3–9], electrophoresis [10–15] and chromatography [16–27].

In the field of chromatography, their ability to provide π–π interactions or π-electron transfer [28–30] and form inclusion complexes [30–33] enable them to be a valuable tool for HPLC stationary phases. To date, more and more applications of different calixarene bonded stationary phases have been reported for the analyses, such as water-soluble vitamins [34], sulphonamides [35,36], estradiol epimers [37], metal ions [38], amino acid esters [39,40], aromatic positional isomers [41–44], polycyclic aromatic hydrocarbons (PAHs) [44–47], nucleosides [48], and so on.

Very recently, we developed six LC calixarene-bonded stationary phases [44] for HPLC and *p*-tert-butylcalix[4]arene modified sol-gel column for open-tubular capillary electrochromatography [49], and investigated their separation performance and separation mechanism assisted with quantum chemistry calculation [44,50,51]. These previous reports have shown that calixarene bonded stationary phases are excellent in reversed-phase chro-

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matography and exhibiting promising application in HPLC. In the present work, we describe the preparation of a new *para*-tert-butylcalix[4]arene-1,2-crown-4 bonded silica stationary phase (CBS4-4) and with its chromatographic behavior investigated. The new developed stationary phase has been characterized by using elemental analysis, FT-IR and thermal gravimetric analysis. The chromatographic performance was investigated by using PAHs, phenols, aromatic amines, benzoic acid and its derivatives as probes in comparison with ODS. The influence of methanol concentrations and pH on the chromatographic behavior of the solutes was also investigated.

2. Experimental

2.1. Apparatus and materials

Chromatographic analyses were carried out by using a Agilent 1200 series system equipped with a 1200 model quaternary pump, a G1314A model Multiple Wavelength UV–vis detector, a G1316A model thermostated column compartment, a 1322A model vacuum degasser, and an Agilent Chemstation B.03.02 Patch data processor. The home-made calixarene column was filled using a packing machine (Kerui Tech. Co. Ltd., Dalian, China) under the pressure of 50 MPa. An Eclipse XDB-C18 column (Agilent, 150 mm × 4.6 mm i.d., 5 μm) was used as a comparison with the home-made calixarene column. Elemental analysis was performed with a Flash EA 1112 elemental analyzer. ^{1}H NMR spectrum was recorded with a Bruker 400 MHz spectrometer in $CDCl_3$. Mass spectrum was acquired by using a Agilent XCT Trap mass spectrometer equipped with a gas nebulizer probe, nitrogen was used as drying gas at a flow rate of 5.0 L/min, the nebulizer pressure was 15.0 psi, the capillary was typically held at 3500 V and the source temperature was maintained at 325 °C. IR spectra were recorded with a Bruker Vector 22 instrument. Thermal gravimetric analysis (TGA) was carried out with a Shimadzu DT-40 thermal analyzer, the analysis was performed from 40 °C to 650 °C at heating rate of 10 °C/min in argon atmosphere with a gas flow rate of 20 mL/min.

All analytes and solvents used in this study were of analytical grade and obtained from Beijing Chemical Plant (Beijing, China) unless specially mentioned. Silica gel (with particle size of 5 μm, pore size of 100 Å and specific surface area of 300 m^2/g) was provided by Lanzhou Institute of Chemical and Physics of Chinese Academy of Sciences (Lanzhou, China). γ-Glycidoxypropyltrimethoxysilane (KH-560) was purchased from Wuhan University Chemical Plant (Wuhan, China). A phosphate buffer (0.5%, w/w) (pH 3.5–7.5) was prepared by mixing KH_2PO_4 with ultra-high quality pure water, and filtered through a 0.45 μm filter before use. HPLC-grade methanol (MeOH) was purchased from the Luzhong Reagent Plant of Shanghai (Shanghai, China). Water was purified by using Milli-Q purification equipment.

2.2. Synthesis of para-tert-butylcalix[4]arene-1,2-crown-4

Scheme 1 shows the synthesis scheme of *para*-tert-butylcalix[4]arene-1,2-crown. *Para*-tert-butylcalix[n]arene was prepared in good yield according to the previous literature [52]. *para*-tert-butylcalix[4]arene-1,2-crown-4 was obtained by reacting of *para*-tert-butylcalix[4]arene and triethylene glycol ditosylate [53]. NaH (0.43 g, 18 mmol) was added to a suspension of compound **2** (1.00 g, 1.54 mmol) in anhydrous DMF (100 mL) under the protection of argon, the mixture was stirred at room temperature for 1 h to ensure the material completely transformed into phenol sodium salt. Then triethylene glycol ditosylate (0.35 g, 0.77 mmol) dissolved in DMF (10 mL) was added dropwise, after that the mixture continued to stir at 50–70 °C. Progress of the reaction was detected by TLC. When the starting triethylene glycol ditosylate had disappeared, methanol (10 mL) was added dropwise to remove the excess NaH. The resulting solution was evaporated to dryness and the residue taken up with aqueous HCl (10%, 100 mL), extracted with methylene chloride (100 mL) for three times. The organic layer was dried by anhydrous Na_2SO_4 and evaporated to give a yellow residue. The product was purified by column chromatography with EtOAc–Petroleum ether (1:3, v/v) as eluent to give 0.40 g (68%, yield) white solid. Mp: 111–114 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.89 (s, 2H, OH), 7.25 and 6.93 (2d, 4H, J = 1.9 Hz, ArH), 7.06 and 6.98 (2d, 4H, J = 1.7 Hz, ArH), 4.68 (d, 1H, J = 12.3 Hz, $ArCH_2Ar$ axial), 4.30 (d, 3H, J = 12.6 Hz, $ArCH_2Ar$ axial), 4.26–3.89 (m, 12H, CH_2–CH_2), 3.38–3.33 (m, 4H, $ArCH_2Ar$ equatorial), 1.20 and 1.13 (2s, 36H, $C(CH_3)_3$). From the ^{1}H NMR data, we can see that the chemical shift of phenolic hydroxyls is in low field, which is due to the existence of intra-molecular hydrogen bonds, indicating that the crown ether ring is connected on the two adjoining hydroxyls. The product can easily form molecular ion ($[M–H]^-$) in negative detection mode with the MS result of m/z = 761 (M^-).

2.3. Preparation of para-tert-butylcalix[4]arene-1,2-crown-4 bonded silica stationary phase (CBS4-4)

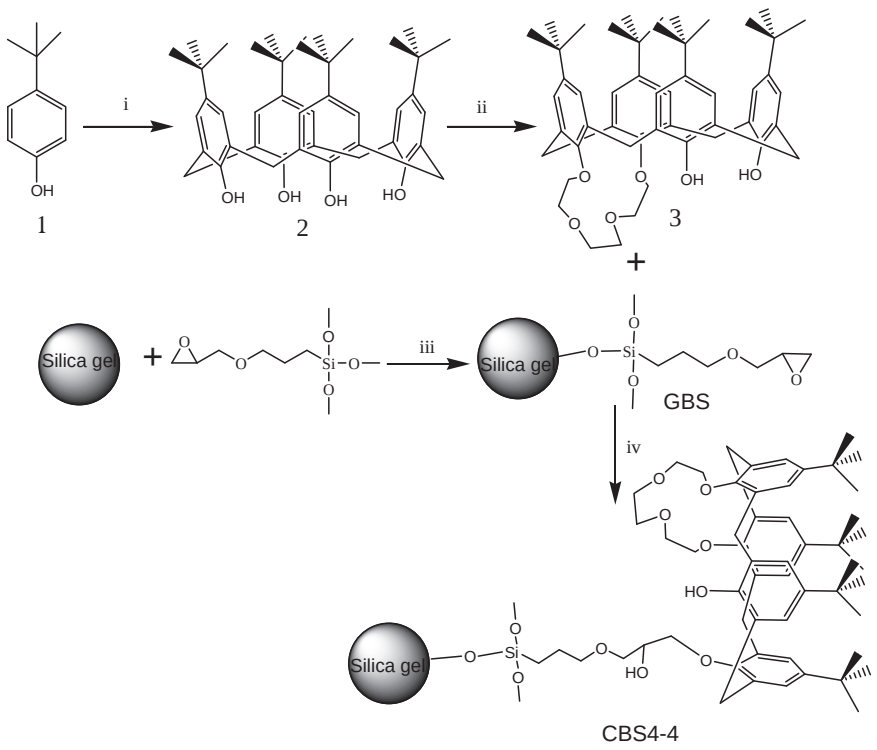
Scheme 1 shows the synthesis process of a new calix[4]arene-bonded silica gel stationary phase. Details of the bonding procedure are as follows. Active silica gel (5.0 g) was suspended in 50 mL dry toluene (freshly distilled), and then 6.0 mL KH-560 and 1.0 mL triethylamine (used as a catalyst) was added to this suspension. The mixture was stirred and heated to 80 °C under the protection of nitrogen atmosphere for 8 h. The solid was filtered by 1.5 μm filter, and washed in sequence with toluene and acetone, then dried at 80 °C under vacuum for 8 h. Finally, γ-glycidoxypropyl bonded silica gel (GBS, Scheme 1) was obtained and used as a precursor in the following reaction.

A mixture of 2.0 g *para*-tert-butylcalix[4]arene-1,2-crown-4, 0.6 g NaH, and 60 mL toluene (freshly distilled) were stirred at 80 °C for 30 min, then the supernatant liquid was transferred to a 100 mL three-neck flask, 3.0 g GBS was added, after that the mixture was refluxed with the catalyst for 48 h. The whole process was carried out under nitrogen atmosphere. After the reaction finished, the product was filtered and washed in sequence with toluene, acetone, methanol and distilled water. Subsequently, CBS4-4 was obtained, and was dried at 100 °C under vacuum for 8 h, then cooled to room temperature in a desiccator.

2.4. Characterization of para-tert-butylcalix[4]arene-1,2-crown-4 bonded silica stationary phase (CBS4-4)

As can be seen from Scheme 1, *para*-tert-butylcalix[4]arene-1,2-crown-4 (compound 3) was prepared by the reaction of *para*-tert-butylcalix[4]arene (compound 2) and triethylene glycol ditosylate in the presence of NaH, and with DMF as the solvent. Then the compound 3 was bonded onto γ-glycidoxypropyl bonded silica gel (GBS) by the breaking ring reaction method [44]. The characterization of the developed stationary phase was carried out by elemental analysis, IR and thermal gravimetric analysis.

The elemental analysis results of GBS and CBS4-4 are given in Table 1. The carbon content of CBS4-4 was obviously higher than that of GBS, which confirmed that the calixarene was successfully immobilized onto the silica gel. The bonded amount of *para*-tert-butylcalix[4]arene-1,2-crown-4 onto the silica gel was calculated by subtracting that of GBS. According to the carbon content of the bonded silica gel stationary phases, the resulting stationary phase contains 13.46% carbon corresponding 0.105 mmol *para*-



Scheme 1. Preparation of CBS4-4 (i) 30% HCHO, NaOH, diphenyl ether; (ii) triethylenglycol ditosylate, NaH, DMF, stir at 50–70 °C for 24 h; (iii) toluene, catalyst, N_2, 80 °C stirred for 24 h; (iv) toluene, catalyst, para-tert-butylcalix[4]arene-1,2-crown-4, N_2, reflux for 48 h.

Table 1
Elemental analysis results of the bonded phases.

Bonded phase	C%	H%	Bonded amount (mmol g^{-1})
GBS	7.13	1.21	0.743
BCS4-4	13.46	1.76	0.105

tert-butylcalix[4]arene-1,2-crown-4 of per gram silica gel. Also, the bonded stationary phase was characterized by infrared spectroscopy. From IR spectra, the characteristic absorption band of the benzene ring appears at 1635, 1556 and 1485 cm^{-1}, peaks at 2959 and 2867 cm^{-1} are assigned to C–H stretching frequency. The peak at 1398 cm^{-1} is assigned to the C–H bending frequency of the *tert*-butyl group. The peak at 1106 cm^{-1} corresponds to the groups of Si–O–Si and C–O–C. All IR spectra indicate that the organic ligands were bonded onto silica gel.

The thermal stability of GBS and CBS4-4 has been investigated by TG analysis. The results show that the temperatures of weight loss for GBS and CBS4-4 are both more than 300 °C. It indicates that the new CBS4-4 bonded phase possesses high thermal and chemical stability. Moreover, the weight losses in 25–650 °C are 8.75% and 16.52% for GBS and CBS4-4, respectively, which was in line with the results of elemental analysis.

The stability of the column was evaluated over 5 months of being used under different chromatographic conditions. The relative standard deviations (RSDs) of retention time of biphenyl were less than 2.0% ($n = 10$) during that time. The prepared column showed high chemical stability when water (phosphate buffer, pH from 3.5 to 7.5) and MeOH mixtures were used as mobile phases.

2.5. Chromatographic procedures

The bonded phase CBS4-4 was packed into 150 mm × 4.6 mm i.d. stainless steel column according to a slurry packing procedure by using methanol as the displacing agent (50 MPa, 2 h). The mobile phases used were methanol–water and methanol–phosphate buffers (0.5%, pH 3.5–7.5). The pH value of this buffer was adjusted with H_3PO_4 to 3.5 or with KOH to 7.5.

Analytes were dissolved in the mobile phase at the concentration in range of 5–100 μg/mL, and 20 μL of the solution was injected into the chromatographic column. The void time (t_0) for the calculation of the retention factor was determined by injecting 0.05 M sodium nitrate ($NaNO_3$) at UV detection 210 nm, with MeOH–H_2O (70/30, v/v) as mobile phase. All measurements were carried out at 30 °C and repeated three times.

3. Results and discussion

In order to exploit the chromatographic characteristics of the new bonded calixarene phase, solutes representing compounds with non-polar and polar characteristics and possessing basic, acidic and neutral properties were selected. The selected analytes are as follows: seven PAHs, phenols, aromatic amines, benzoic acid and its derivatives. Their retention factors (k') and the separation factors ($\alpha_{1,2}$) were calculated and listed in Tables 2 and 3. The influence of mobile parameters (such as methanol concentration, pH value), which may affect the retention and selectivity was investigated. And moreover, the chromatographic behaviors of the analytes on CBS4-4 were compared with these on ODS.

3.1. The separation of PAHs on CBS4-4

In this section, the separations for seven PAHs on CBS4-4 and ODS columns were carried out. The chromatogram (Fig. 1) and the retention factors k' (see Table 2) were obtained under the same chromatographic conditions on CBS4-4 and ODS, respectively. As can be seen from Fig. 1, the elution order of PAHs on CBS4-4 is the same as that on ODS, indicating that CBS4-4 and ODS have similar hydrophobic interactions with PAHs. The retention factors k' of aromatic hydrocarbons on CBS4-4 are less than that on ODS. This

Table 2
The retention factors (k') and separation factors (α) of PAHs on CBS4-4 and ODS (MeOH–water (70/30,v/v); flow-rate, 0.8 mL/min; detection wavelength, 258 nm).

Bonded phase		Benzene	Toluene	Biphenyl	Acenaphthene	Anthracene	Pyrene	Chrysene
CBS4-4	k'	2.62	3.05	6.24	7.19	10.28	15.13	21.58
	α	1.16	2.04	1.15	1.43	1.47	1.43	
ODS	k'	2.79	4.48	10.01	12.13	16.16	24.00	38.14
	α	1.60	2.23	1.21	1.33	1.49	1.59	

Table 3
The retention factors (k') and the separation factors (α) of some aromatic amines on BCS4-4 and ODS.

Bonded phase	Benzenediamines[a]	Anlines[b]	Nitroanilines[a]
BCS4-4	*p, m, o*	A, MA, DMA[c]	*m, p, o*
k'	1.37, 1.89, 2.55	2.82, 4.63, 7.19	13.64, 16.15, 19.11
α	1.38, 1.35	1.64, 1.55	1.18, 1.18
ODS	*p, m, o*	A, MA, DMA[c]	*p, m, o*
k'	1.18, 1.18, 2.04	2.15,5.39, 17.58	4.82, 6.49, 11.31
α	1.00, 1.76	2.51, 3.26	1.35, 1.75

[a] Mobile phase, MeOH/water (30:70,v/v).
[b] Mobile phase, MeOH/water (45:55, v/v).
[c] A, MA, DMA shorten for aniline, N-methyl aniline and N,N-dimethyl aniline, respectively.

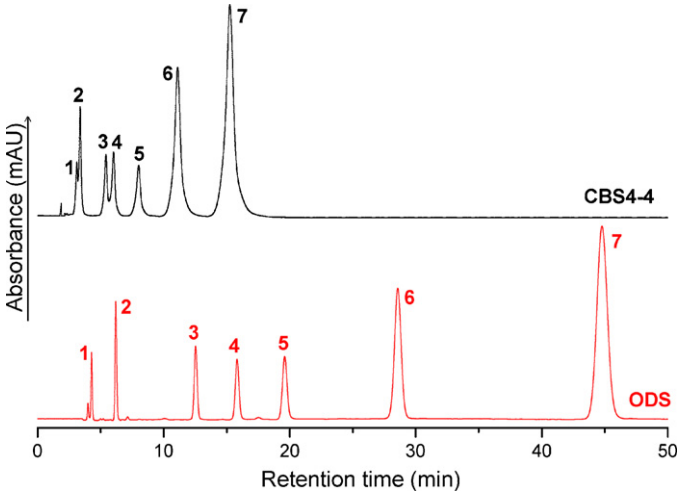


Fig. 1. The chromatograms of seven aromatic hydrocarbons on BCS4-4 and ODS. Mobile phase: MeOH–H_2O (70:30, v/v); flow rate 0.8 mL/min, detection UV at 280 nm, temperature 30 °C. Peaks: 1, benzene; 2, toluene; 3, biphenyl; 4, acenaphthene; 5, anthracene; 6, pyrene; and 7, chrysene.

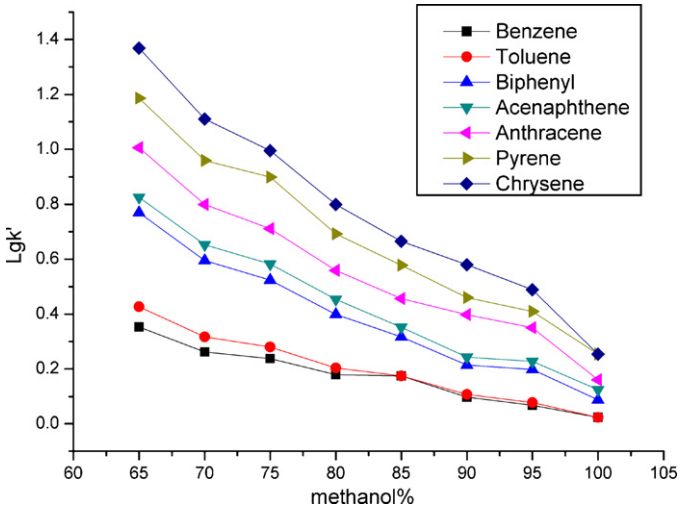


Fig. 2. Effect of the methanol content of mobile phases on the logarithmic retention factor of the PAHs on CBS4-4.

implies that hydrophobic interaction plays an important role in the separation, and the hydrophobic property of CBS4-4 is weaker than that of ODS.

Fig. 2 illustrates the plot of logarithmic retention factor of PAHs against the volume percentage of methanol in mobile phase. As methanol content of the mobile phase increasing, the retention factors k' values of the solutes are decreasing. In reversed-phase chromatography, increase of organic modifier content in the mobile phase leads to decrease in the retention time of analytes. This result indicates that the new stationary phase can behave as an excellent reversed-phase performance and the hydrophobic interaction is one of the factors playing a role in the separation of PAHs.

3.2. The separation of phenols on CBS4-4

Three phenolic compounds (phenol, 1,3-benzenediol and 1,3,5-trihydroxybenzene) were used as probes for further investigation of the chromatographic characteristic of the new calixarene stationary phase. From the chromatogram (Fig. 3), it can also be observed that both CBS4-4 and ODS exhibited high selectivity for phenolic compounds. With more hydroxy groups be attached to benzene ring, the analyte became a stronger polarity, and was first eluted

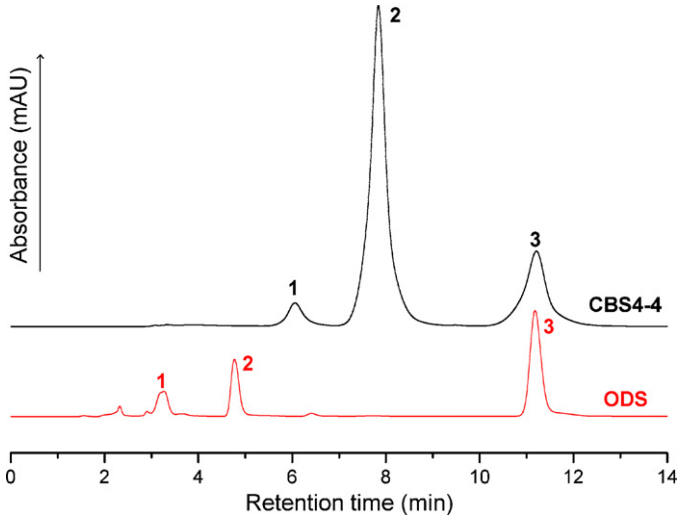


Fig. 3. The chromatogram of phenols on CBS4-4 and ODS. Mobile phases: MeOH/water (35:65, v/v), flow-rate, 0.5 mL/min; detection wavelength, 254 nm; temperature 30 °C. Peaks: 1. 1,3,5-trihydroxybenzene; 2, 1,3-benzenediol; 3, phenol.

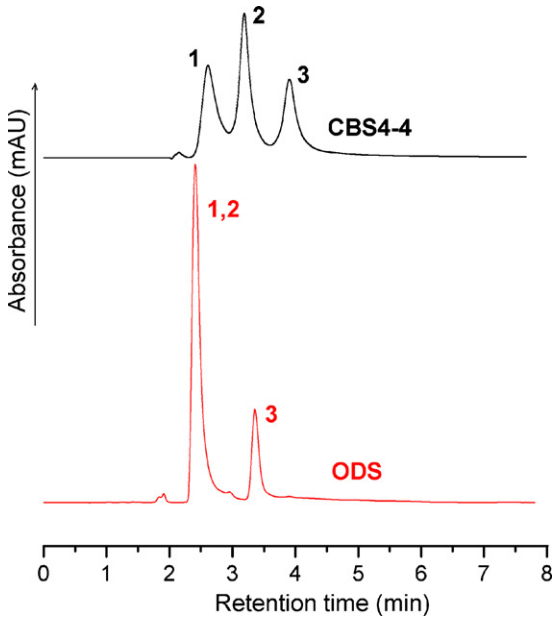


Fig. 4. The chromatogram of phenylenediamines on CBS4-4 and ODS. Mobile phases: MeOH/water (30:70, v/v), flow-rate, 0.8 mL/min; detection wavelength, 254 nm; temperature 30 °C. Peaks: 1. *p*-phenylenediamine; 2, *m*-phenylenediamine; 3, *o*-phenylenediamine.

out on both the CBS4-4 and ODS. The elution order is also the same on the both column, however, the analytes are more strongly retained on CBS4-4, specifically for 1,3,5-trihydroxybenzene and 1,3-benzenediol, which implies that the hydroxyl and the crown ether group play the additional roles on the separation mechanism for the phenols. These additional interactions are possibly π–π and hydrogen bonding interactions.

When we take the concentration of methanol into concern, it can be noted that the retention of the phenolic compounds decrease with the increasing methanol content as organic modifier in mobile phases. It was a further evidence of reversed-phase chromatographic separation mechanism of phenolic compounds on CBS4-4.

3.3. The separation of aromatic amines on CBS4-4

In order to exploit the chromatographic potential of the new calixarene stationary phase, the separation of aromatic amines was performed. Three groups of aromatic amines were separated on both CBS4-4 and ODS. For each group, the HPLC condition was optimized and they obtained better separation compared with their separation on ODS column. The chromatograms and the retention factors of aromatic amines on CBS4-4 and ODS were shown in Fig. 4 and Table 3.

3.3.1. The separation of phenylenediamines

As can be seen from Fig. 4, the analytes of *o*-phenylenediamine, *m*-phenylenediamine, *p*-phenylenediamine could achieve better separation on the BCS4-4 column, while *m*-phenylenediamine and *p*-phenylenediamine were eluted together on ODS column. However, the retention values of the three phenylenediamine positional isomers on BCS4-4 are larger than these on ODS. This case shows that there exist other retention mechanisms besides hydrophobic interaction in the separation of phenylenediamines on CBS4-4. The phenomenon can be explained as follows: on one hand, the group of $-NH_2$ can easily form hydrogen bond with CBS4-4. On the other hand, the π–π or inclusion interaction between the moiety of the calixarene and the analytes may improve the separation of phenylenediamines on BCS4-4.

In our previous work, we have developed a quantum chemistry calculation method to investigate the separation mechanism between calix[4]arene and benzenediols [50]. In this section, we try to use the same method to elucidate the interaction of phenylenediamines and *para*-tert-butylcalix[4]arene-1,2-crown-4. The calculations were performed using the Gaussian 03 series of programs by the basis set of DFT-B3LYP/STO-3G*. The geometries of the guest analytes, the host calixarene, and the host–guest complexes were first optimized, to ensure the structures obtained at the lowest energy. Then their stabilization energy change ΔE, entropy change ΔS, and Gibbs free energy change ΔG (Table 4) on formation of a so-called calixarene-analyte supramolecule (two molecules as close as possible) were calculated.

Fig. 5 illustrates that the optimized complex structures of supramoleculars are different, which means the interaction between guest analytes and the host calixarene may be also different from each other. For the supramoleculars, obviously there exists inclusion complexation, hydrogen bonding and π–π interaction. Besides, from the figure we can see that the two $-NH_2$ groups of *o*-phenylenediamine can both strongly attract the calixarene cavity, while for *p*- and *m*-phenylenediamine only one $-NH_2$ group take effects.

Table 4 indicates that the ΔG values of the supramoleculars are positive, and the ΔS values are negative, which imply that these so-called supramolecule are not stable. When in vacuum condition, the values of ΔS_{vac} and ΔG_{vac} increased in turn from *p*-phenylenediamine to *m*-phenylenediamine and to *o*-phenylenediamine, which indicates that the supramolecules become more and more instable, which is contrary to the elution order. However, if we take the solvent effects into consideration, the values of ΔS_{solv} and ΔG_{solv} were consistent with the elution order. This demonstrates that the hydrophilic mobile phases could change the supramolecular interaction between the calixarene and the analytes. The calculation results in consideration of solvent effects would be more identical with the practical elution results. The results can be useful in the investigation of separation mechanism

3.3.2. The separation of aniline, N-methyl aniline and N,N-dimethyl aniline

With the purpose of investigating the influence of $-NH_2$ in the separation on BCS4-4 column, aniline (A), N-methyl aniline (MA) and N,N-dimethyl aniline (DMA) were selected as probes. These aniline compounds contain the number of hydrogen atoms (which attached to nitrogen atoms) from 2 to 0. As was shown in Table 3, both the stationary phases exhibited good separation abilities for the above solute probes, and the probes were eluted in the same order on the two columns. This case shows that hydrophobic interaction is mainly responsible for the retention behavior of aromatic amines. However, the retention values were different. Aniline gave a little stronger retention on BCS4-4 columns than that on ODS. But for N-methyl aniline and N,N-dimethyl aniline, the retentions are weaker on BCS4-4 columns. This may be another powerful evidence for the existing of hydrogen bonding interaction between the $-NH_2$ and the BCS4-4 column. Aniline can easily form hydrogen bond with BCS4-4 column, while N-methyl aniline and N,N-dimethyl aniline do not have a group of $-NH_2$, so the retentions of N-methyl aniline and N,N-dimethyl aniline are weaker on BCS4-4 columns.

3.3.3. The separation of nitroanilines

For *o*, *m* and *p*-nitroaniline, it can be noted that the elution order of nitroaniline isomers on BCS4-4 column is $m < p < o$, which was consistent with the reported literatures [44]. As was shown in Table 3, the elution order of the analytes is different from that on the ODS columns. Moreover, nitroanilines gave comparatively stronger retention on CBS4-4 than that on ODS, which means that there

Table 4
Elution order, *k'* and calculated stabilization energy change ΔE, entropy change ΔS, and Gibbs free energy change ΔG of Phenylenediamine and *para*-tert-butylcalix[4]arene-1,2-crown-4.

Parmeter	Phenylenediamine-*para*-tert-butylcalix[4]arene-1,2-crown-4		
Elution	*p*-	*m*-	*o*-
k'	1.37	1.89	2.55
R	1.38	1.35	
ΔE_{vac} (kJ/mol)	−8.1487	−10.2312	−17.1483
ΔE_{solv} (kJ/mol)	16.0593	9.1695	2.1487
ΔS_{vac} (J mol^{-1} k^{-1})	−146.5880	−154.0456	−214.6444
ΔS_{solv} (J mol^{-1} k^{-1})	−204.6519	−169.3473	−158.5667
ΔG_{vac} (kJ/mol)	40.9170	41.4502	50.5548
ΔG_{solv} (kJ/mol)	97.4063	63.4720	63.2196

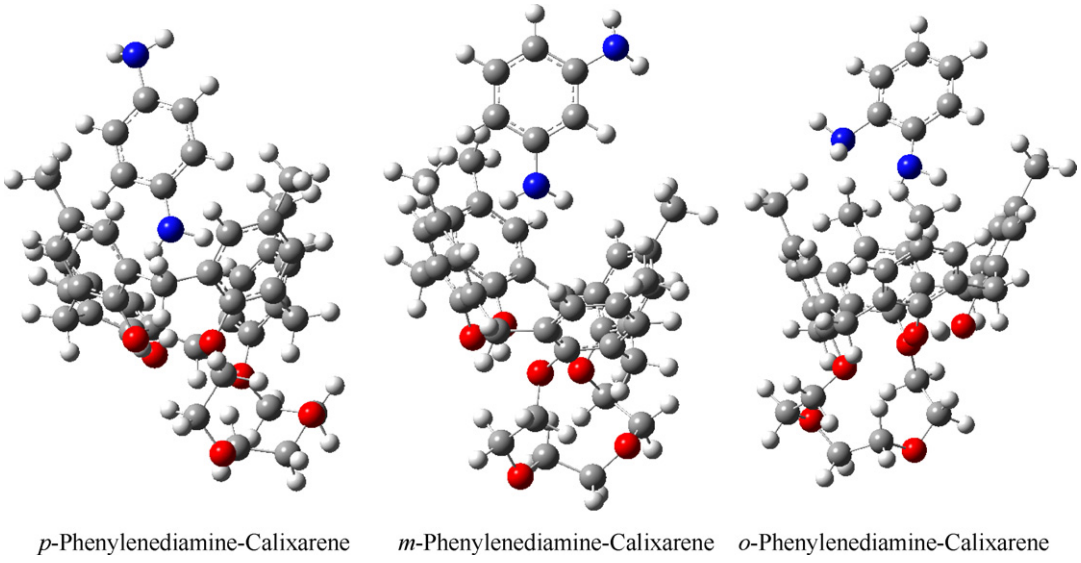


Fig. 5. Optimized complex structures of phenylenediamine and para-tert-butylcalix[4]arene-1,2-crown-4.

were the additional interactions with exception of the hydrophobic interaction between the analytes and CBS4-4. This may be associated with the guest–host inclusion interactions of the calixarene cavity with the analyte molecule. The group of $-NO_2$ is a strong electron-attracting group, which may strongly attract the electron-rich cavity of calixarene. In addition, there may exist π–π interaction, hydrogen bonding interactions between nitroanilines and BCS4-4.

From the discussion above, it is noteworthy that the aromatic amines which own polar groups such as $-NO_2$ and $-NH_2$, tend to be longer retained on the CBS4-4, and can obtain better separation and selectivity on calixarene-bonded phases by contrast.

3.4. The separation of benzoic acid and its derivatives on CBS4-4

In order to study the chromatographic property of CBS4-4 for acid solutes, in this section, the separation of five benzoic acid and its derivatives was carried out by using a buffered mobile phase at pH 4.5, in which all of the analytes exist in non-ionized forms. The effects of methanol concentrations and pH value on the retention were also investigated.

The chromatograms of benzoic acid and its derivatives on CBS4-4 and ODS were shown in Fig. 6. It can be seen from Fig. 6 that the elution order on CBS4-4 was different from that on ODS. This case shows that the CBS4-4 exhibited better separation abilities for benzoic acid and its derivatives than ODS under the given condition. This implies that there may exist other interactions besides hydrophobic interaction, which affected the results on the stationary phase. At the same time, we can see that benzoic acid and its derivatives gave comparatively stronger retention on calixarene columns than those on ODS, which further confirmed that there were the additional interactions with exception of the hydrophobic interaction between calixarene and the analytes. Moreover, the obvious difference in the selectivity for the analytes between BCS4-

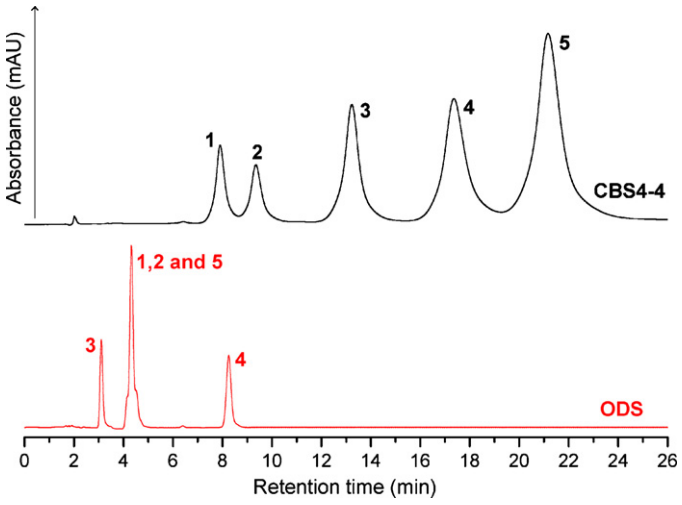


Fig. 6. The chromatogram of benzoic acid and its derivatives on CBS4-4 and on ODS. Mobile phases: MeOH/0.5% KH_2PO_4 (30:70, v/v), flow-rate, 0.8 mL/min; detection wavelength, 254 nm; temperature 30 °C. Peaks: 1, benzoic acid; 2, *m*-methoxybenzoic acid; 3, *p*-nitrobenzoic acid; 4, *p*-chlorobenzoic acid; 5, 3,5-dinitrobenzoic acid.

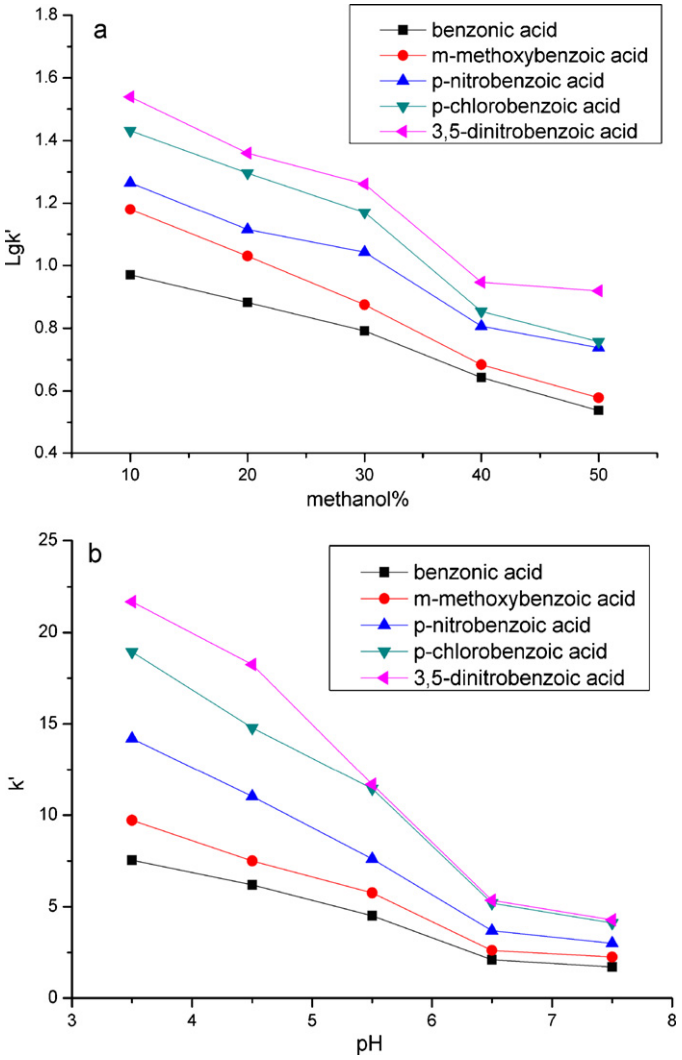


Fig. 7. (a) Effect of the methanol content of mobile phases on the logarithmic retention factor of the benzoic acid and its derivatives on BCS4-4. (b) Effect of the pH of mobile phases on the retention factors of benzoic acid and its derivatives on BCS4-4.

4 and ODS can also be observed under the same chromatographic conditions. For example, while benzoic acid, methoxybenzoic acid and 3,5-dinitrobenzoic acid were eluted together on ODS column, these analytes were eluted separately on BCS4-4 column. All these results can be explained as follows: on one hand, the carboxyl group in the benzoic acid and its derivatives can easily form hydrogen bond with the hydroxyl on the BCS4-4, which may importantly influence the retention time of the analytes. On the other hand, dipole–dipole interaction can occur between the polar groups (such as $-OCH_3$, $-NO_2$ and $-Cl$) of the analytes and the electron-rich cavity of the calixarene. At the same time there exists $\pi-\pi$ interaction between the analytes and the benzene skeleton of calixarene.

Fig. 7(a) illustrates the plot of logarithmic retention factor of benzoic acid and its derivatives against the volume percentage of methanol in mobile phase at constant pH (pH 4.5) and constant KH_2PO_4 buffer concentration (0.5%). As shown in Fig. 7(a), with the content of methanol increasing, the retention values of the solutes decreasing. It indicates that the BCS4-4 column can behave as a reversed-phase packing and the hydrophobic interaction is one of the factors in the separation of benzoic acid and its derivatives. However, the relationship line in Fig. 7(a) on CBS4-4 is not linear. It shows that there might exist other interactions.

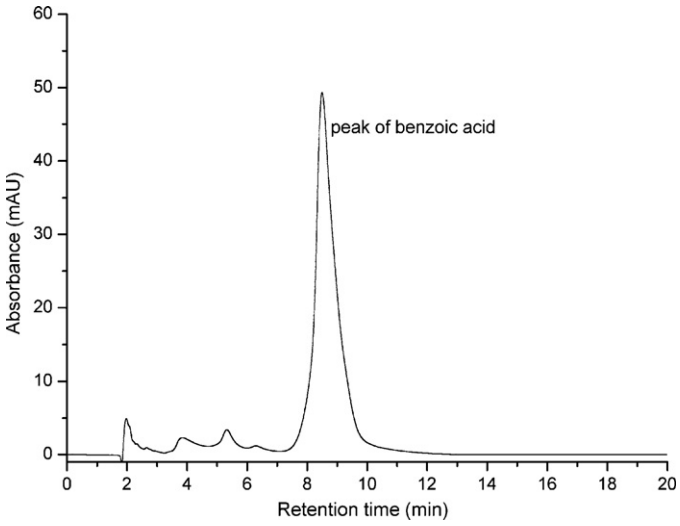


Fig. 8. Chromatogram of benzoic acid in Sprite drink on BCS4-4 column. Mobile phases: MeOH/0.5% KH_2PO_4 (25:75, v/v), flow-rate, 0.8 mL/min; detection wavelength, 230 nm, temperature 30 °C; sample: sprite drink, injection: 10 μL.

The influence of variation in pH of the mobile phase on retention of the selected analytes was studied in order to gain a better understanding of the retention mechanism on CBS4-4. The retention factors Fig. 7(b) for each analyte were measured at constant 30% methanol and 70% phosphate buffer at pH 3.5–7.5. As shown in Fig. 7(b), when the pH value of the mobile phases varies, the retention of the acidic compounds undergoes considerable changes. The changes of pH in mobile phase seriously influence the separation of acidic solutes. When the pH at 7.5, the benzoic acid and its derivatives lose a proton and their retentions are reduced several times in comparison to pH 3.5, which is consistent with a reversed-phase retention mechanism.

3.5. The determination of benzoic acid in the Sprite drink on BCS4-4 column

As we know, benzoic acid is commonly used as antiseptic in food and drinks. The quantity of which may serious affects our physical health, thus the fast and accurately determination of benzoic acid is necessary, and RP-HPLC method is typically applied.

The stock solution of benzoic acid (1.000 mg/mL) was prepared by dissolving 25.00 mg benzoic acid in MeOH to make 25 mL solution. The standard working solutions were prepared by diluting aliquots of the stock solution to obtain concentrations ranging from 1.000 to 100.0 μg/mL. The calibration graph was constructed by plotting the peak areas obtained at wavelength 230 nm versus the corresponding injected concentrations. The Sprite drink was treated according to the following method: 10 g sample (accurately weighed to 0.001 g) was transferred to a 25 mL volumetric flask. The solution of ammonia (1:1, v/v) was added to keep the pH value is neutral, and then diluted to volume with water. After that the solution was filtered by 0.45 μm filter membrane, the filtrate was ready for analysis.

The graph of the peak area (y) against concentration (x, μg/mL) proved linear in the range from1.000 to 100.0 μg/mL and the linearity equation was: $y = 51.260x + 20.804$ ($n = 6$, $R^2 = 0.9994$). The limit of detection (LOD) defined as the injected quantity giving S/N of 3 (in terms of peak area), was found to be 0.13 μg/mL. Inter-day precision was assessed by injecting the standard solution and the sample on each day for 5 days. The results show that there were high inter-day precisions (within 1.64%). The accuracy of the method was determined by recovery experiments. The analysis of benzoic acid in Sprite drink showed high accuracy with

a recovery of 98.80–101.88%. The chromatogram of benzoic acid in Sprite drink was shown in Fig. 8, from which we can see that benzoic acid was obtained better separation from the matrix in the Sprite drink. The content of benzoic acid in the Sprite drink is 117.10 μg/g.

4. Conclusion

A new *para*-tert-butylcalix[4]arene-1,2-crown-4 bonded stationary phase was prepared and characterized by elemental analysis, thermal analysis, FT-IR. The chromatographic behaviors of this new developed stationary phase were investigated by using PAHs, phenols, aromatic amines, benzoic acid and its derivatives, and the results were compared with those on ODS. The results show that the stationary phase exhibits high selectivity for these solutes and can behave as a reversed-phase retention mechanism with some hydrophobic interaction as compared with ODS. However, there are some other interactions such as π–π interaction, hydrogen bonding interaction and inclusion complexation, all of which manipulates the chromatographic behaviors of analytes on the BCS4-4. The BCS4-4 was used for the analysis of benzoic acid in Sprite drink. Furthermore, it showed the BCS4-4 stationary phase is a useful stationary phase for the determination of benzoic acid and the other analytes.

Acknowledgements

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