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Development of a V-shape bis(tetraoxacalix[2]arene[2]triazine) stationary phase for High performance liquid chromatography

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ABSTRACT

A new stationary phase for high-performance liquid chromatography was prepared by covalently bonding a V-shape cage heteroatom-bridged calixarene onto silica gel using 3-aminopropyltriethoxysilane as coupling reagent. The structure of the new material was characterized by infrared spectroscopy, elemental analysis and thermogravimetric analysis. Linear solvation energy relationship method was successfully employed to evaluate the new phase with a set of 34 solutes. The retention characteristic of the new phase shows significant similarities with ODS, as well as distinctive features. Multiple mechanisms including hydrophobic, hydrogen bonding, π–π and n–π interaction are involved. The chromatographic behavior of the phase was illustrated by using alkylbenzenes, aromatics positional isomers and flavonoids as probes. Moreover, inorganic anions were individually separated in anion-exchange mode by using the same column. Thus, multi-interaction mechanisms and mixed-mode separation of the new phase can very likely guarantee its excellent chromatographic performance for the analysis of complex samples. The column has been successfully employed for the analysis of clenbuterol in animal urine, and it is demonstrated to be suitable and a competitive alternative analytical method for the determination of clenbuterol.

1. Introduction

Traditionally, the separation function of the stationary phase is mainly based on a single mechanism. For example, the main function mechanism of alkyl silica-based stationary phase and ion-exchange packing phase are hydrophobic interaction and ion-exchange interaction. However, complex samples of pharmacology, environment or biological tissues are often mixtures with different properties and thus difficult to be separated. Developing stationary phases with mixed-mode separation mechanisms has been shown to be a flexible and versatile method to tackle these challenges [1–5].

In recent years, the macrocyclic hosts stationary phase has attracted considerable attention in liquid chromatography due to its varieties of separation mechanisms, including hydrophobic, host-guest inclusion complexation, hydrogen bonding, π–π or π–electron transfer interaction [6–19]. The outstanding solute recognition ability of macrocyclic stationary phases makes them superior to classic alkyl bonded stationary phases. Therefore, the exploitation of new functional macrocyclic host molecules and the utilization of them as selectors in chromatography separation have become one of the hotspots in both supramolecular chemistry and chromatographic science.

Along with the advances in the field of calixarenes, heteroatom-bridged calixaromatics has been emerging as a type of novel macrocyclic molecules and they exhibit unique, versatile structures and molecular recognition properties [20]. This new class of macrocyclic molecules is usually made up of benzene rings and triazine rings linked by an oxygen or nitrogen atom. The construction of a new calixarene by replacing its phenol units with benzene rings or triazine rings cannot only tune the cavity, but also improve its recognizing efficiency and selectivity toward various guest species including cations, anions and neutral molecules. The presence of oxygen atoms instead of methylene bridges on oxa-calixarene gives rise to multiple interactions notably toward guest molecules. Oxygen, unlike methylene, can adopt either sp^2 or sp^3 electronic configuration, providing different conjugation systems with adjacent aromatic rings [21]. Moreover, the benzene rings and triazine rings, the cavity and the conjugated system composed of aromatic rings with oxygen

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atoms in oxa-calixarene may serve to improve the selectivity of chromatographic separation. Therefore, it is reasonable to anticipate that the bonding of oxa-calixarene onto silica gel may bring certain changes to the retention mechanisms over traditional reversed- and normal-stationary phase. Though many oxa-calixarenes have been synthesized, very limited effort has been made into their applications to chromatography. Our previous work described the synthesis and characterization of a calix[2]arene[2]triazine-bonded stationary phase (OCATS) for HPLC [18], which was highly selective towards various compounds, such as PAHs and positional aromatic isomers.

In this paper, we reported the preparation of a V-shape cage heteroatom-bridged calixarene and its stationary phase, namely bis(tetraoxacalix[2]arene[2]triazine) stationary phase (BTOSP). Its retention property was elucidated by linear solvation energy relationship (*LSER*) study with a set of 34 solutes, and furthermore, the chromatographic behavior by using alkylbenzenes, aromatics positional isomers and flavonoids as probes was illustrated. Moreover, the separation of inorganic anions was achieved under the ion exchange mode. The column has been successfully employed for the analysis of clenbuterol in animal urine.

2. Experimental

2.1. Apparatus and materials

HPLC was performed by using an Agilent 1260 series system equipped with a 1200 model quaternary pump, a 1260 Infinity DAD detector and a G1316A model thermostatic column compartment. Elemental analysis was performed on a Flash EA 1112 elemental analyzer (Thermo Electron Corporation). Thermal gravimetric analysis (TGA) was carried out using a Shimadzu DT-40 thermal analyzer, and the analysis was performed from 40 °C to 600 °C at a heating rate of 10 °C per minute in nitrogen atmosphere with a gas flow rate of 20 mL min^{-1}.

Silica gel (particle size of 5 μm, pore size of 100 Å and specific surface area of 300 m^2 g^{-1}) was provided by Lanzhou Institute of Chemical and Physics of CAS (Lanzhou, China). 3-Aminopropyltrimethoxysilane was purchased from Aladdin Industrial Corporation (Shanghai, China). Methanol and acetonitrile of HPLC grade were purchased from Dikma (Beijing, China). All other chemicals and solvents used in this study were of analytical grade unless specially mentioned. Water used was obtained from Millipore water purification system.

2.2. Preparation of BTOSP stationary phase

2.2.1. Synthesis of 3-aminopropyl bonded silica gel

Activated silica gel (5.0 g) and anhydrous toluene (80 mL) were added to a flask equipped with a gas inlet valve and a reflux condenser. After the addition of 3-aminopropyltrimethoxysilane (10 mL) and triethylamine (0.15 mL), the mixture was magnetically stirred and refluxed at 115 °C under nitrogen atmosphere for 48 h. Then, the mixture was cooled to room temperature and filtered. The residue was washed in sequence with toluene, acetone and distilled water. Finally, the product of 3-aminopropyl bonded silica gel (APS) was dried under vacuum at 100 °C for 8 h and used as a precursor in the following reaction.

2.2.2. Synthesis of bis(tetraoxacalix[2]arene[2]triazine)

Bis(tetraoxacalix[2]arene[2]triazine) was prepared by the following literature procedures with slight modifications [22]. The solution of phloroglucinol (1.26 g, 10 mmol) and DIPEA (4.83 g, 37.6 mmol) in THF (80 mL) was added dropwise over a period of 1 h to an ice-cooled and well-stirred solution of cyanuric chloride (9.30 g, 45.0 mmol) in THF (100 mL). The resulting mixture was stirred for another 2.5 h at 0 °C. After filtration and concentration, the crude product was chromatographed on a silica gel column (100–200 mesh) with a mixture of petroleum ether and ethyl acetate, yielding pure trimer compound (yield:60%) as a white solid, mp 241–243 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ=7.16 (s, 3 H).

Both solutions of phloroglucinol (0.5 mmol, in 50 mL acetone) and the trimer compound synthesized above (0.5 mmol, in 50 mL acetone) were added dropwise at the same time and the same rate to a solution of DIPEA (3.6 mmol, in 60 mL acetone) at room temperature. The addition of the two reactants last over a period of 12 h, and was stirred at room temperature for another 60 h. After concentration, the residue was purified on a silica gel column (100–200 mesh) with a mixture of petroleum ether and acetone. Pure cage molecule bis(tetraoxacalix[2]arene[2]triazine) was obtained as a white solid (yield: 30%). ^{1}H NMR ($CDCl_3$, 400 MHz): d=6.69 ppm (s, 6 H).

2.2.3. Synthesis of BTOSP

Fig. 1 shows the synthesis process of the new oxa-calixarene stationary phase (BTOSP). Details of the bonding procedure are as follows: a mixture of bis(tetraoxacalix[2]arene[2]triazine) (2.0 g), APS (4.0 g) and THF (100 mL) was stirred at reflux for 24 h under argon atmosphere. After completion of the reaction, the product

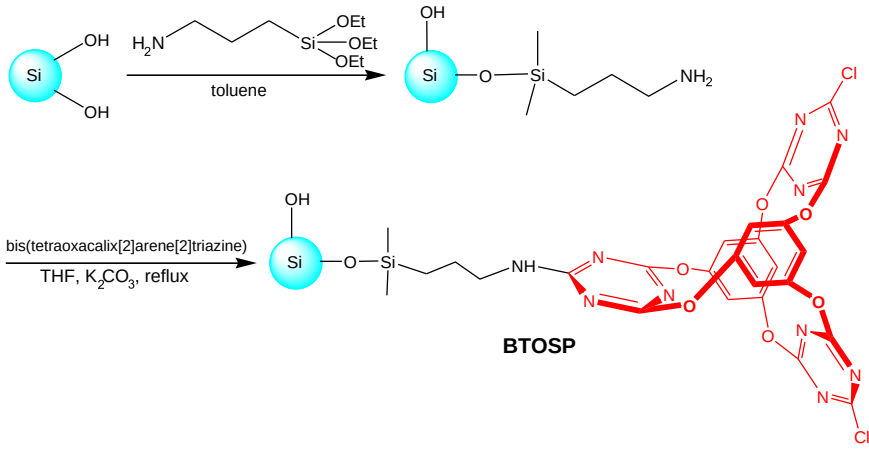

Fig. 1. Preparation scheme of bis(tetraoxacalix[2]arene[2]triazine)-bonded silica gel stationary phase (BTOSP).

was filtered and washed in sequence with DMF, acetone, methanol and distilled water. Subsequently, the oxa-calixarene bonded material (BTOSP) was dried under vacuum at 100 °C for 8 h before packing and characterization.

The prepared BTOSP was packed into a steel tube column (150 mm × 4.6 mm i.d.) by using a packing machine (Kerui Tech. Co. Ltd., Dalian, China) according to a slurry packing procedure by using methanol as the displacing agent (50 MPa, 30 min).

3. Results and discussion

3.1. Characterization of BTOSP

A comparison of APS and BTOSP IR spectra showed that new absorptions appeared at 1603, 1540, 1389, 1361 and 1109 cm^{-1}, which correspond to the groups of benzene rings (C–H) and Si–O–Si. The C–O stretching frequency of the ether-bridge almost overlaps with the Si–O–Si appearing for a broad bond at 1109 cm^{-1}. These differences suggest that the cage molecular bis(tetraoxacalix[2]arene [2]triazine) was successfully immobilized onto APS.

Quantitative determination of the packing material was achieved by elemental analysis. Elemental analysis results showed that the content of C, H and N in APS was 4.91%, 1.22% and 1.39%, respectively; the C, H and N in BTOSP was 12.38%, 1.42% and 5.19%, respectively. The bonding amount of BTOSP was about 296 μmol g^{-1} based on the change of carbon content. Both the IR spectra and the elemental analysis results indicate successful preparation of BTOSP.

TG analysis curve showed the weight loss temperature of BTOSP was higher than 330 °C, indicating the packing material possessed better thermal stability and chemical stability. The total weight loss was 15.53% for BTOSP in the temperature range of 40–650 °C, which was attributed to the breakage of oxacalixarene group bonded on the silica-gel surface together with the condensation of remaining silanol groups.

3.2. LSER analysis

The chromatographic property may be greatly influenced by the intermolecular interactions among stationary phase, analytes and mobile phase, so it is beneficial and necessary to evaluate the interactions by a qualitative and quantitative approach. LSER method has been regarded as an effective means to investigate the properties of HPLC stationary phase [7,8,23–25]. To gain a deeper insight into the chromatographic behaviors of BTOSP, LSER model [26,27] was introduced to elucidate the separation mechanisms of the new phase.

Table 1 shows the 34 solutes that carefully selected from Ref. [28], which covers the non-polar and polar ones (basic and acidic). Unsuitable selection of the solutes may increase the uncertainty of LSER coefficient, or cause more serious multiple coordinated variation. The issue of cross-correlations between R_2, π^{H2}, $\Sigma\alpha^{H2}$, $\Sigma\beta^{H2}$ and Vx was examined to verify the limitation of LSER induced by potential covariance of solute descriptors. Table 2 shows the cross-correlations matrix of the solute descriptors. No occurrence of significant correlation between each pair was observed. Nevertheless, there is minute correlation between R_2 and π^{H2}, which may be because both the descriptors reflect the solute polarizability and it is sensitive to the presence of π–electron [29,30]. In addition, R_2 seems to be somewhat correlated to Vx. A possible explanation could be the volume of the solute that is typically associated with the number of double bonds [29]. In general, the low correlation coefficients (less than 0.6) confirm the suitability of chosen set of solutes for employment with the LSER study.

The LSER regression coefficients of BTOSP and the referred ODS stationary phase were characterized under similar system conditions and are listed in Table 3. Evaluation of the LSER model was performed by comparing the experimentally-determined log$k$ values (log$k_{(exp)}$) with the calculated values (log$k_{(calc)}$). Fig. 2 shows the results in 65% methanol as a representative example. Good linear fitting between log$k_{(exp)}$ and log$k_{(calc)}$ was observed (correlation coefficient > 0.94), confirming the competence of selected solutes in specifying the interactions between solutes and the stationary phases under reverse-phase condition.

In the LSER equation, the positive or negative sign of the coefficient indicates the strength of interaction between mobile phase and stationary phase. The negative s, a and b coefficients reveal higher affinity of methanol–water for the solute concerning the dipolarity/polarizability and hydrogen bonding interactions; on the other hand, positive coefficients stand for stronger molecular interactions in the stationary phase than in mobile phase.

The v coefficient is influenced by cavity formation and dispersion interaction. Table 3 shows that the v coefficient of BTOSP is positive and slightly smaller than that of ODS, suggesting that BTOSP is more hydrophobic than mobile phase and less hydrophobic than ODS. In addition, the v values rise up with the decrease of methanol content in the mobile phase, emphasizing

Table 1
Solutes and solute descriptors.

No.	Analytes	R_2	π^{H2}	$\Sigma\alpha^{H2}$	$\Sigma\beta^{H2}$	Vx
1	Benzaldehyde	0.820	1.00	0.00	0.39	0.8730
2	Nitrobenzene	0.871	1.11	0.00	0.28	0.8906
3	Naphthalene	1.340	0.92	0.00	0.20	1.0854
4	Biphenyl	1.360	0.99	0.00	0.26	1.3420
5	Aniline	0.955	0.96	0.26	0.41	0.8162
6	Benzene	1.224	1.33	0.00	0.58	1.4808
7	Toluene	0.601	0.52	0.00	0.14	0.8573
8	4-Nitrotoluene	0.870	1.11	0.00	0.28	1.0315
9	Anisole	0.708	0.75	0.00	0.29	0.9160
10	Bromobenzene	0.882	0.73	0.00	0.09	0.8914
11	Chlorobenzene	0.718	0.65	0.00	0.07	0.8388
12	2-Nitroaniline	1.180	1.37	0.30	0.36	0.9904
13	3-Nitroaniline	1.200	1.71	0.40	0.35	0.9904
14	4-Nitroaniline	1.220	1.91	0.42	0.38	0.9904
15	Diphenylamine	1.585	0.88	0.10	0.57	1.4240
16	Pyridine	0.631	0.84	0.00	0.52	0.6753
17	2-Chlorophenol	0.853	0.88	0.32	0.31	0.8975
18	2,4-Dimethylphenol	0.843	0.80	0.53	0.39	1.0569
19	Ethylbenzene	0.613	0.50	0.00	0.15	0.9982
20	*n*-Propylbenzene	0.604	0.50	0.00	0.15	1.1391
21	*n*-Butylbenzene	0.600	0.51	0.00	0.15	1.2800
22	Iodobenzene	1.188	0.82	0.00	0.12	0.9750
23	2-Nitrotoluene	0.866	1.11	0.00	0.27	1.0320
24	Acetophenone	0.818	1.01	0.00	0.48	1.0139
25	N-Methylaniline	0.948	0.90	0.17	0.43	0.9571
26	N,N-Dimethylaniline	0.957	0.84	0.00	0.42	1.0980
27	2-Cloroaniline	1.033	0.92	0.25	0.31	0.9390
28	4-Cresol	0.820	0.87	0.57	0.32	0.9160
29	4-Methylpyridine	0.598	0.75	0.00	0.58	0.8160
30	Benzoic Alcohol	0.803	0.87	0.33	0.56	0.9160
31	Cyclohexanone	0.403	0.86	0.00	0.56	0.8610
32	Cyclopentanone	0.373	0.86	0.00	0.52	0.7202
33	4-Xylene	0.613	0.52	0.00	0.16	0.9980
34	Phenol	0.805	0.89	0.60	0.30	0.7750

Table 2
Variance–covariance matrix of solute descriptors used in LSER equation.

	R_2	π^{H2}	$\Sigma\alpha^{H2}$	$\Sigma\beta^{H2}$	Vx
R_2	1	0.558	0.207	0.062	0.581
π^{H2}		1	0.375	0.356	0.113
$\Sigma\alpha^{H2}$			1	0.159	−0.161
$\Sigma\beta^{H2}$				1	0.004
Vx					1

Table 3
System coefficients of BTOSP and C18.

Column	Eluent, MeOH–water (%,v/v)	*c*	*r*	*s*	*a*	*b*	*v*	*R*	*n*	*SE*	*F*
BTOSP	55:45	−0.31	0.39	−0.13	−0.39	−0.79	0.54	0.958	34	0.054	113
	65:35	−0.26	0.35	−0.16	−0.42	−0.68	0.50	0.948	34	0.053	100
	75:25	−0.21	0.28	−0.09	−0.30	−0.48	0.31	0.952	34	0.045	118
Nucleosil-C18[a]	55:45	0.11	0.22	−0.48	−0.43	−1.48	1.55	0.994	35	0.04	523
	65:35	0.13	0.20	−0.34	−0.37	−1.13	1.10	0.992	35	0.05	351
	75:25	0.09	0.15	−0.28	−0.29	−0.77	0.76	0.991	32	0.04	299

R: overall correlation coefficient, *n*: number of solutes, *SE*: standard error in the estimate, *F*: statistic.
[a] Values obtained from Ref. [29].

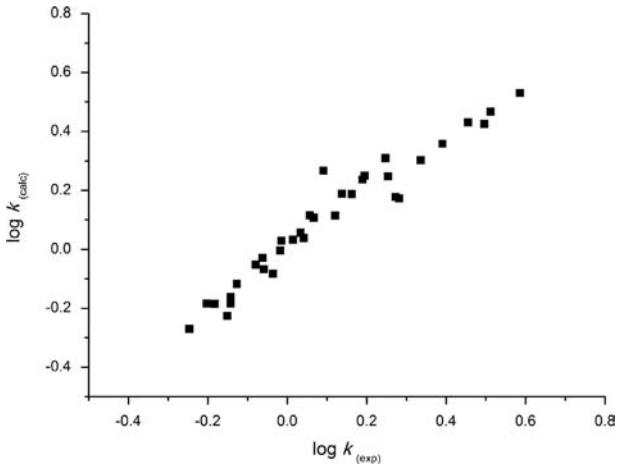

Fig. 2. Plot depicting log $k_{(calc)}$ versus log $k_{(exp)}$ with 65/35 (v/v) methanol/water.

the hydrophobic feature of the BTOSP phase. With the absence of long alkyl chain, the hydrophobicity of BTOSP may be attributed to the hydrophobic cavity and benzene rings.

The *r* coefficient reflects the difference between mobile phase and stationary phase in interacting with the solute through π- and *n*- electron pairs [30]. As shown in Table 3, the *r* coefficients of BTOSP are positive in all mobile phase compositions, implying BTOSP has more electron-involved interaction with the solute than does the mobile phase. With the increase of methanol content in the mobile phase, the *r* values of BTOSP decrease, and the trend is consistent with that of ODS, indicating the electron-involved interactions of BTOSP become weaker with the decrease of water in mobile phase. Compared with ODS, the *r* coefficients of BTOSP are larger, which indicates the electron-involved interactions between the solutes and BTOSP are more significant. However, comparisons between the values of *r* and *v* coefficients show that *v* is a much more important factor that impacts the retention of solute than *r*.

The *s* coefficient is a measure of dipolar interaction discrepancy of stationary phase and mobile phase with the solute. The *s* coefficients of BTOSP are small and negative, indicating the difference of dipolarity/polarizability between the mobile and stationary phases is quite small, and the dipolar solutes only slightly prefer the mobile over the stationary phase, which may be due to the substantial sorption of strongly polar molecules (i.e. methanol and water) into the stationary phase. Moreover, the *s* coefficient value (close to zero, tend to be more positive) ascends with the increasing methanol content in the mobile phase, implying that the increase of methanol content weakens the difference in dipolarity/polarizability between the mobile and stationary phases. Similar to tetraazamacrocycle-bonded stationary phase reported [8], the smaller negative *s* value indicates the higher dipolarity/polarizability of BTOSP over ODS, which can be explicated by the chemical properties of the oxygen bridged groups, aza-groups and the residual silanol on BTOSP.

The *b* and *a* coefficients represent the difference between mobile and stationary phases in HBD acidity and HBA basicity, respectively. As can be seen from Table 3, both BTOSP and ODS possess negative *b* coefficients. However, the smaller *b* coefficient of BTOSP indicates it has a much stronger HBD ability than ODS in the mobile phases investigated. The stronger acidity of BTOSP may arise from the stronger hydrogen bond donor groups (ether and amino groups), sorbed mobile-phase components and accessible silanol groups on the support surface. Since water is a very strong HBD acid, the value of *b* coefficients on both BTOSP and ODS are ascending with the increase of water content in the mobile phase. With more water molecules sorbed into stationary phase in the high aqueous mobile phase, the hydrogen bonding interaction between solutes and sorbed water molecules as well as between solutes and the residual silanol groups will be stronger too, so the HBD acidity will be enhanced. The *a* coefficients of BTOSP are close to those of ODS in all mobile phase conditions, indicating the basicity of them are similar.

From the above LSER results, it can be concluded that BTOSP possesses obvious reversed-phase chromatography properties, and the separation process is a synergistic effect including hydrogen bonding, dipole–dipole, π–π and *n*–π interactions.

3.3. Chromatographic behavior of BTOSP

3.3.1. Separation of alkylbenzenes

As a method for evaluating the hydrophobicity of new packing, separation of alkylbenzene homologous (or methylene selectivity) has been frequently used to characterize the property of RP-HPLC stationary phase. In this section, the chromatographic retention behaviors of *n*-alkylbenzenes, including benzene, toluene, ethylbenzene, propylbenzene and butylbenzene were investigated on BTOSP. Fig. 3 shows the typical chromatogram on BTOSP. As can be seen from Fig. 3, baseline separations of the five *n*-alkylbenzenes were achieved on the BTOSP, and the analytes were eluted out gradually with the increase of the alkyl chain of *n*-alkylbenzenes. This suggests that BTOSP has good methylene selectivity.

The log*k* of each analyte was plotted versus concentration of methanol content in mobile phase. As shown in Fig. 4, the value of log*k* ascended with the increase of water content in the mobile phase. This indicates the new material exhibits an excellent reversed-phase property, which is similar to conventional ODS.

3.3.2. Separation of positional isomers of disubstituted aromatics

In our previous work, the calixarene bonded stationary phases usually behaved better separation selectivity toward position isomer due to their diversity of separation mechanisms. In this section, the separations for some disubstituted aromatic positional

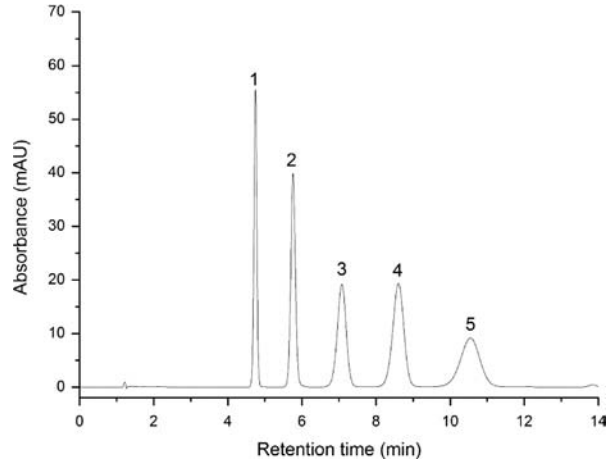

Fig. 3. Chromatogram of alkylbenzenes on BTOSP. Mobile phase: methanol–water (45/55, v/v); flow rates: 1.0 mL min^{-1}; detection wavelength, 254 nm. Peaks: 1, benzene; 2, toluene; 3, ethylbenzene; 4, *n*-propylbenzene; 5, *n*-butylbenzene.

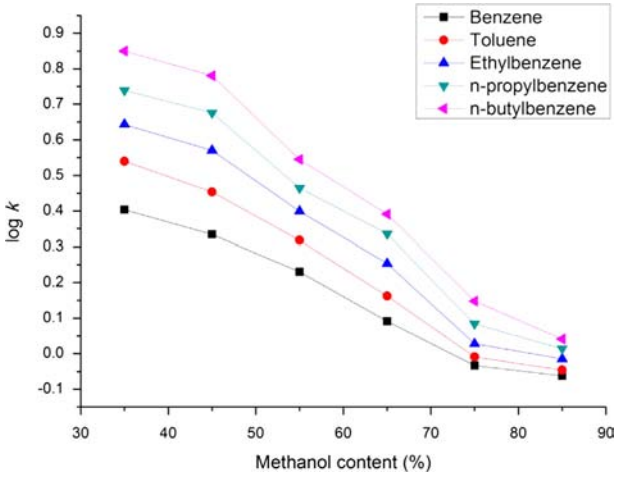

Fig. 4. Effect of methanol contents on log*k* of *n*-alkylbenzenes.

isomers with different polar and nonpolar groups were individually investigated on BTOSP, and compared with OCATS and C4BS (calix[4]arene-bonded stationary phase) [6]. The elution order, retention factors (k), peak width (W), theoretical plates (N) and resolution (R) values of these isomers were calculated and listed in Table 4. It showed that all of the isomers could obtain baseline separation on BTOSP except nitrotoluenes, moreover, the elution orders of the isomers were totally different among the four phases, indicating different separation mechanisms that were involved in the separation process.

For all the positional isomers selected (except nitrophenol), the ortho-isomers with an intramolecular hydrogen bonding were finally eluted out on BTOSP and ODS. Generally, ortho-isomers have stronger hydrophobicity than meta- and para-isomers, so the hydrophobic interaction between them and the stationary phase are stronger. Therefore, they were eluted out the latest on BTOSP and ODS. This phenomenon indicates that hydrophobic interaction played an important role in the separation of the isomers.

As can be seen from Table 4, the retentions of benzenediol, benzenediamine, aminophenol, nitrophenol and nitroaniline on BTOSP are stronger than those on C4BS and ODS. Obviously, this behavior is mainly related to the oxygen-bridge and the nitrogen atoms on triazines. The explanation should be that the additional interactions between BTOSP and the isomers enhanced the retentions. As the macrocyclic molecule that bonded on silica gel possesses multiple oxygen and nitrogens atoms, hydrogen bonding interaction between the hydrogen-donor (OH, NH_2) of the isomers and the heteroatoms existed. In addition, the strong conjugation system formed by the oxo-bridged atoms being conjugated with their adjacent aromatic rings makes the bis (tetraoxacalix[2]arene[2]triazine) have stronger delocalization π–electrons than conventional calixarenes. Thus, the synergistic effects of hydrogen bonding and π–π interaction enhance the retention of the solutes on BTOSP.

The interactions of π–π and π–electron transfer are the common features of conventional calixarene stationary phases [6,9,14–17]. As for BTOSP, stronger π–π and π–electron transfer interaction is expected due to the strong conjugation system in the oxa-calixarene compound. Table 4 shows that the retentions of the solutes containing NO_2 substituent at the phenyl ring, such as nitrophenol and nitroaniline were stronger than others, which can be due to the effect of π–π and π–electron transfer interaction. However, nitrotoluenes were weakly retained on BTOSP compared with the ODS, which was probably because the presence of non-polar methyl group increased their hydrophobicity.

Compared with OCATS, the solutes were weakly retained on BTOSP. This behavior is partly because of the lower bonded amount of BTOSP. On the other hand, the cage structure of bis (tetraoxacalix[2]arene[2]triazine) weakens the complexation ability toward solutes. For example, *para*-benzenediol retained stronger than *meta*-benzenediol on OCATS, which probably because the linear shape of the *para*-benzenediol facilitates its penetration into the cavities of the calix[2]arene[2]triazine. Inversely, *para*-benzenediol was first eluted out on BTOSP, which was because the cage structure of bis(tetraoxacalix[2]arene[2]triazine) makes the inclusion interaction weaker.

3.3.3. *Separation of flavonoids*

In this section, five flavonoids (Fig. 5) with –OH and phenyl conjugate groups were selected as probes and their separations on BTOSP and ODS were carried out. The chromatograms of their complete separation on BTOSP and ODS are exhibited in Fig. 6. The elution order of the five flavonoids on BTOSP is genistein < tangeretin < kaempferol < chrysin < quercetin, and did not change in all studied methanol compositions. The elution order is of great difference with that on ODS, which indicates that there exists additional interactions besides hydrophobic interaction.

As can be seen from Figs. 5 and 6, all the solutes with –OH group (genistein, kaempferol, chrysin, quercetin) give comparatively stronger retention on BTOSP than on ODS, which can be ascribed to the stronger hydrogen bonding interaction between –OH group and BTOSP. It is interesting that with the increase of hydroxyl groups on genistein, kaempferol and quercetin, the retention of them became longer and longer on BTOSP. For the analyte of quercetin that owns the most –OH groups, it was retained the most strongly on BTOSP, while it was eluted first on ODS due to its strong polarity. Moreover, obvious differences in the selectivity for the analytes between BTOSP and ODS can also be observed under the same chromatographic condition. For example, while tangeretin was secondly eluted out on BTOSP, it was retained the most strongly on ODS. One possible reason is that BTOSP interacts with solutes simultaneously through hydrophobic, dipole–dipole and hydrogen bonding interactions, while the retention of solutes on ODS is mainly governed by hydrophobic interaction.

Accordingly, it can be inferred that the separation of the five flavonoids on BTOSP is due to not only hydrophobic interaction, but also the contribution of hydrogen bonding interaction and other interaction.

Table 4
The elution order, *k*, *W*, *N* and *R* values of disubstituted beneze positional isomers on various columns.

Analytes	BTOSP					OCATS[b]					C4BS[a]			ODS				
	Elution Order	*k*	*W*	*N*/ 10^4	*R*	*Elution Order*	*k*	*W*	*N*/ 10^4	*R*	*Elution Order*	*k*	*R*	*Elution Order*	*k*	*W*	*N*/ 10^4	*R*
Benzenediol	*p*-	1.56	0.1398	1.09	-	*m*-	2.09	0.1662	0.99	-	*m*-	0.89	-	*p*-	0.52	0.0568	1.39	-
	m-	1.86	0.1586	1.10	3.42	*p*-	2.53	0.1922	1.00	3.68	*p*-	1.32	3.13	*m*-	0.66	0.0625	1.41	3.05
	o-	2.30	0.1838	1.14	4.37	*o*-	3.04	0.2206	1.01	3.71	*o*-	1.53	3.21	*o*-	0.93	0.0741	1.42	5.14
Aminophenol	*m*-	1.03	0.1058	1.08	-	*p*-	1.98	0.1628	0.95	-	*p*-	-	-	*p*-	0.40	0.0535	1.29	-
	p-	1.17	0.1131	1.12	2.17	*m*-	2.27	0.1786	0.97	2.55	*m*-	-	-	*m*-	0.77	0.0701	1.30	7.78
	o-	2.61	0.2018	1.16	15.5	*o*-	4.27	0.2932	1.02	12.72	*o*-	-	-	*o*-	1.56	0.1051	1.32	11.7
Nitrophenol	*m*-	6.87	0.4906	1.07	-	*m*-	15.70	0.9887	0.98	-	*m*-	3.24	-	*p*-	2.41	0.1451	1.30	-
	o-	7.92	0.5563	1.08	3.71	*o*-	19.60	1.1950	1.03	5.36	*o*-	3.79	0.73	*m*-	2.87	0.1652	1.31	3.85
	p-	10.71	0.7332	1.10	7.36	*p*-	27.10	1.5970	1.08	8.06	*p*-	3.79	0.00	*o*-	3.91	0.2105	1.33	7.20
Nitroaniline	*m*-	5.56	0.4132	1.02	-	*m*-	13.00	0.8407	0.95	-	*m*-	4.26	-	*p*-	0.86	0.0775	1.19	-
	p-	6.39	0.4658	1.04	3.21	*p*-	15.60	0.9878	0.98	4.27	*p*-	6.18	1.18	*m*-	1.31	0.0980	1.22	6.67
	o-	7.47	0.5312	1.07	3.68	*o*-	19.40	1.2070	0.99	5.19	*o*-	7.26	1.23	*o*-	2.65	0.1605	1.23	13.48
Phenylenediamine	*p*-	1.18	0.1206	0.99	-	*p*-	1.69	0.1435	0.97	-	*p*-	0.14	-	*p*-	0.43	0.0585	1.13	-
	m-	1.70	0.1523	1.04	6.48	*m*-	2.18	0.1722	0.98	4.66	*m*-	0.28	1.73	*m*-	0.54	0.0625	1.18	2.36
	o-	2.87	0.2268	1.07	10.49	*o*-	2.93	0.2171	0.99	5.78	*o*-	0.46	0.94	*o*-	0.99	0.0835	1.20	8.01
Nitrotoluene	*o*-	4.83	0.3568	1.07	-	*o*-	9.36	0.6175	0.95	-	*o*-	-	-	*o*-	10.30	0.5135	1.26	-
	m-	5.01	0.3678	1.07	0.84	*m*-	10.00	0.6534	0.95	1.51	*m*-	-	-	*p*-	10.90	0.5382	1.27	1.48
	p-	5.13	0.3762	1.07	0.55	*p*-	11.20	0.7232	0.97	2.62	*p*-	-	-	*m*-	12.30	0.5935	1.31	3.22

Mobile phase: methanol–water (40/60, v/v), flow rates:1.0 mL min^{-1}, detection wavelength:254 nm.
*k, W, N and R were shorten for retention factor, peak width, theoretical plates and resolution, respectively.

[a] Data taken from Ref. [6].
[b] Data taken from Ref. [18].

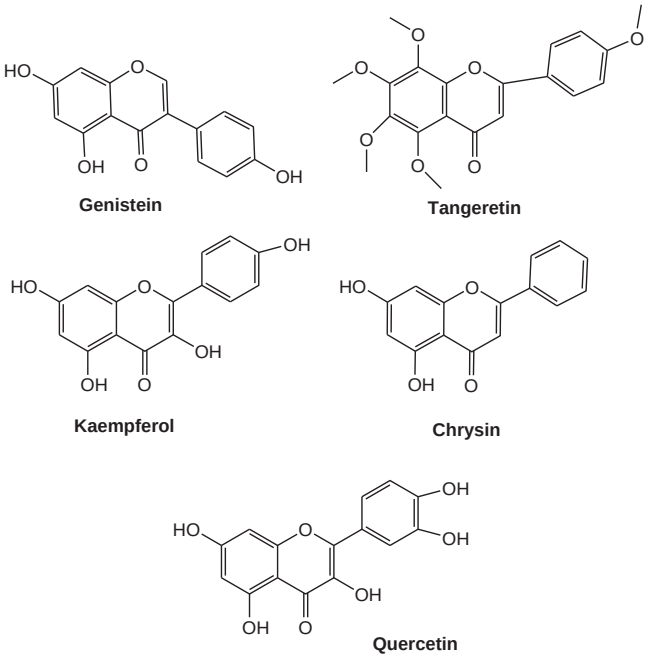

Fig. 5. The structures of flavonoids.

3.3.4. *Separation of anions in anion-exchange mode*

As reported in the Ref. [22], for the V-shape cage heteroatom-bridged calixaromatics, the electron-deficient triazine ring incorporated into a conformational rigid host can form various anion–π complexes. Multiple anion–π interactions along with other non-covalent bond interactions, such as hydrogen bonding, halogen bonding, and lone-pair-electrons–π interactions, directed the formation of different molecular assemblies in the solid state [22]. In this case, we envisioned that the newly bonded phase had distinctive separation selectivity for anions. To our delight, most inorganic anions in the mixture could be effectively separated with 25 mmol L^{-1} KCl aqueous solution as mobile phase.

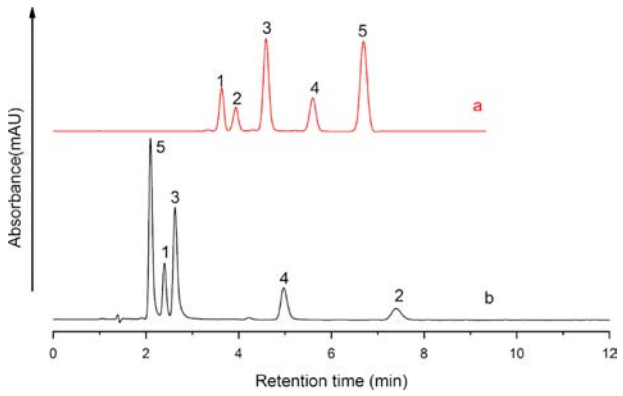

Fig. 6. Chromatogram of flavonoids on BTOSP(a) and ODS (b). Mobile phase: methanol–water (70/30, v/v); flow rates: 1.0 mL min^{-1}; detection wavelength, 254 nm. Peaks: 1, genistein; 2, tangeretin; 3, kaempferol; 4, chrysin; 5, quercetin.

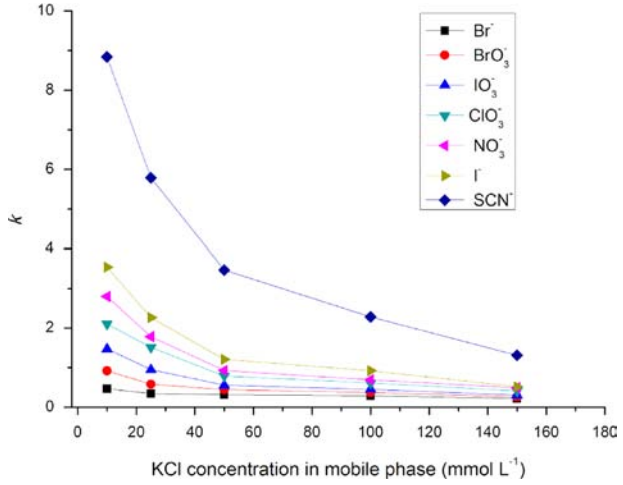

Fig. 7. Effect of KCl concentration on retention *k* of inorganic anions.

In this section, BTOSP was applied as an anion-exchange stationary phase with KCl solution as the mobile phase. Seven inorganic anions including Br^-, BrO_3^-, IO_3^-, ClO_3^-, NO_3^-, I^- and SCN^- were selected as probes to investigate the anion-exchange characteristic of BTOSP. The effect of the KCl concentration varied from 10 to 150 mmol L^{-1} (pH 6.8) was examined. As can be seen from Fig. 7, the retentions of inorganic anions decrease with the increase of KCl concentration of mobile phase, indicating the concentration of mobile phase could regulate the anion-exchange strength of BTOSP to obtain appropriate retention and better separation.

As shown in Fig. 8, the separation of seven inorganic anions could be achieved on BTOSP, which indicates the packing exhibits typical anion-exchange property. Compared with the oxa-bridged calix[2]arene[2]triazine-bonded stationary phase (OCATS) reported previously [17], significant differences were observed in Fig. 8. The newly developed stationary phase exhibited better separation for the anions, indicating the V-shape structure oxa-calixarene has distinctive recognition and complexation performance toward anions. The formation of various anion$-\pi$ complexes between the V-shape cage host and the diversity of anions resulted in the distinct separation results with those on OCATS. In addition, most of the anions obtained longer retention times than on OCATS, and the elution order was not entirely consistent with that on OCATS phase. What is more, iodide and bromate were coeluted on OCATS, while they were completely separated on BTOSP. All the above observations imply that the interaction between V-shape cage heteroatom-bridged calixaromatics and anions was more complicated, and multi-interaction may be involved in the retention behavior of anions on BTOSP.

3.4. Column efficiency, repeatability and column stability

In order to better understand the column performance of BTOSP, comparative studies were conducted on both BTOSP and ODS by using a series of solutes with different polarities, including phenol, aniline, benzene and nitrobenzene. The results of the column characterization are placed in Table 5, both the columns exhibited good separation abilities for the above solute probes. However, the elution order of the solutes was inconsistent on the two columns, indicating different mechanisms were taking affects in the separation process. In addition to this, BTOSP usually has a slightly larger peak width and slightly lower column efficiency, which is probably due to the multiple-function interactions between solutes and stationary phase enlarge the peak width.

The repeatability and column stability have also been measured. The column has alternately been eluted with ACN-water, MeOH – water and MeOH-0.01 mol/L potassium chloride solution (pH 4.5) for 2 months (20 injections for each mobile phase per day). The column efficiency reduction was less than 3%, and the relative standard deviations (RSDs) of the retention times in the repeated chromatographic runs ($n=10$) were in the range of 0.08–0.40% for benzene, 0.07–0.25% for aniline, 0.09–0.32% for NO_3^-. The effect of pH on column life was investigated, it showed that the efficiency reduction was less than 2% after it has been eluted with

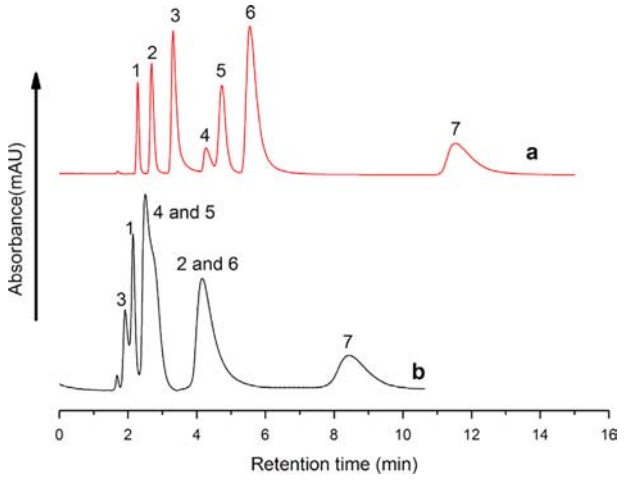

Fig. 8. Separation of anions on BTOSP (a) and OCATS (b). Mobile phase: 25 mmol L^{-1} KCl aqueous solution, flow rates: 1.0 mL min^{-1}, detection wavelength, 210 nm. Peaks: 1, bromide; 2, bromate; 3, iodate; 4, chlorate; 5, nitrate; 6, iodide; 7, thiocyanate.

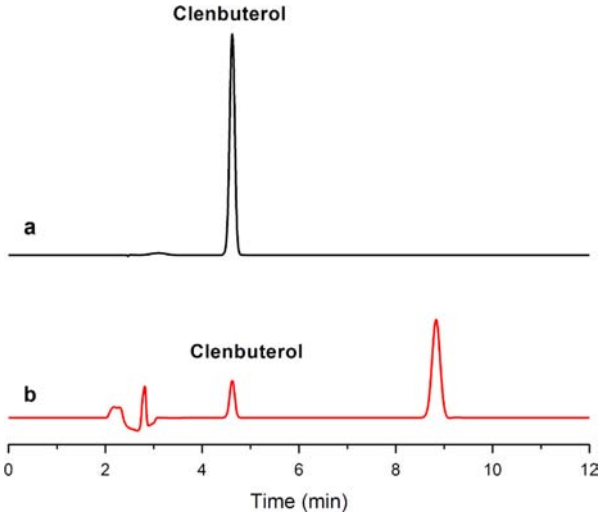

Fig. 9. Chromatograms of standard clenbuterol (a) and animal urine sample (b) on BTOSP column. Mobile phase, 0.2% formic acid solution /acetonitrile (80:20, v/v), pH 5.0; flow rate, 1.0 mL min^{-1}; detection wavelength, 243 nm.

Table 5
Comparative study of BTOSP and ODS using solutes of different polarities.

BTOSP						ODS					
Elution order	*k*	*α*	*R*	*W*	*N*/10^4	*Elution order*	*k*	*α*	*R*	*W*	*N*/10^4
Phenol	1.36			0.1287	1.06	Aniline	2.05			0.1265	1.34
		1.24	4.05					1.29	5.77		
Aniline	1.69			0.1482	1.09	Phenol	2.65			0.1522	1.37
		1.33	5.77					1.90	15.59		
Benzene	2.25			0.1815	1.13	Nitrobenzene	5.03			0.2547	1.40
		1.41	7.60					1.10	2.52		
Nitrobenzene	3.18			0.2346	1.19	Benzene	5.54			0.2736	1.44

Mobile phase, MeOH–H_2O (45:55, v/v); flow rate: 1.0 mL min^{-1};
*k, α, R, W and N were shorten for retention factor, separation factor, resolution, peak width and theoretical plates, respectively.

the mobile phase in the pH range of 3–8 for half a month. All these results indicate that BTOSP is stable and repeatable.

3.5. The determination of clenbuterol in animal urine on BTOSP column

Clenbuterol is usually misused as nutrient repartitioning agent in livestock by diverting nutrients from fat deposition in animals to the production of muscle tissues [31]. However, the misuse may severely endanger human health. Thus, clenbuterol is banned for growth promotion in animal production in China. In order to protect consumers, specific and sensitive methods for the identification and quantification of clenbuterol in biological sample are required. Clenbuterol is retained weakly on ODS column due to its strong polarity, while BTOSP has multiple-function mechanisms towards strong polarity solutes, which may improve the separation selectivity. Thus, in this paper, we try to develop a rapid analysis method for clenbuterol by using the newly developed column.

The urine sample was treated with ammonium acetate and ß-glucuronidase-aryl sulfatase to dissociate the target analyte from the sample matrix. The mixed solution was centrifuged and the supernate was applied to a Waters Oasis MCX cartridge column clean up. The resulting solution was filtered through 0.22 μm filter and the filtrate was ready for analysis.

The optimized chromatogram of clenbuterol on BTOSP column was shown in Fig. 9, from which we can see that clenbuterol was obtained better separation from the matrix. The linearity was satisfactory in the range of 0.05–10.0 $\mu g\,mL^{-1}$, with a regression coefficient of 0.9995. Under the optimal conditions, the limit of detection (LOD) defined as the injected quantity giving S/N of 3 (in terms of peak area), was found to be 0.025 $\mu g\,mL^{-1}$. The limit of quantitation (LOQ) defined as the injected quantity giving S/N of 10 (in terms of peak area), was found to be 0.074 $\mu g\,mL^{-1}$. Inter-day precision was assessed by injecting the standard solution of different concentrations (0.1 $\mu g\,mL^{-1}$, 1.0 $\mu g\,mL^{-1}$, 10 $\mu g\,mL^{-1}$) each day for 5 days, the RSD% of retention times was within 0.036 and the RSD% of peak areas was within 2.32. Intra-day precisions were assessed by injecting the standard solution at the three concentrations five times during a day, and the intra-day RSD% of retention time was within 0.032 and the RSD% of peak area was within 2.11. The analysis of clenbuterol in animal urine showed high accuracy when the spiked concentrations were 0.1 and 0.5 $\mu g\,mL^{-1}$, and the recovery was in the range of 94.3–107.6%.

4. Conclusion

A new stationary phase modified by V-shape cage heteroatom-bridged calixaromatics was successfully prepared and characterized. The retention property of this new material in RP-HPLC mode was investigated by LSER study. Different series of analytes including *n*-alkylbenzene, positional isomers of disubstituted aromatics, flavonoids and inorganic anions were successfully separated on this novel stationary phase, respectively. The formation of anion–π complexes between the V-shape cage host and the diversity of anions improved the separation effect of inorganic anions on BTOSP. The multiple-function mechanisms including hydrophobic, π–π, hydrogen bonding and anion-exchange interactions were demonstrated to be involved in the chromatographic separation. With such multi-interaction mechanism, the new stationary phase has promising prospect for separation of complex samples. A new method for the analysis of clenbuterol in animal urine was set up by using the BTOSP column, and it has been proven suitable for routine determination of clenbuterol in animal urine.

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References

[1] T.L. Chester, Anal. Chem. 85 (2013) 579.
[2] M. Zhang, X. Liang, S. Jiang, H. Qiu, TrAC Trend Anal. Chem. 53 (2014) 60.
[3] J. Li, Y. Li, T. Chen, L. Xu, X. Liu, X. Zhang, H. Zhang, Talanta 109 (2013) 152.
[4] S. Bocian, S. Studzińska, B. Buszewski, Talanta 127 (2014) 133.
[5] H. Aral, T. Aral, B. Ziyadanoğulları, R. Ziyadanoğulları, Talanta 116 (2013) 155.
[6] C. Ding, K. Qu, Y. Li, K. Hu, H. Liu, B. Ye, Y. Wu, S. Zhang, J. Chromatogr. A 1170 (2007) 73.
[7] L. He, M. Zhang, L. Liu, X. Jiang, P. Mao, L. Qu, J. Chromatogr. A 1270 (2012) 186.
[8] L. He, M. Zhang, W. Zhao, J. Liu, X. Jiang, S. Zhang, L. Qu, Talanta 89 (2012) 433.
[9] M. Śliwka-Kaszyńska, G. Gorczyca, M. Ślebioda, J. Chromatogr. A 1217 (2010) 329.
[10] J. Zhang, Y. Du, Q. Zhang, Y. Lei, Talanta 119 (2014) 193.
[11] T. Sokoließ, U. Menyes, U. Roth, T. Jira, J. Chromatogr. A 898 (2010) 35.
[12] S. Erdemir, M. Yilmaz, Talanta 82 (2010) 1240.
[13] S.K. Thamarai Chelvi, E.L. Yong, Y.H. Gong, J. Chromatogr. A 1203 (2008) 54.
[14] L.S. Li, S.L. Da, Y.Q. Feng, M. Liu, Talanta 64 (2004) 373.
[15] C. Schneider, T. Jira, J. Chromatogr. A 1216 (2009) 6285.
[16] K. Hu, W. Zhao, F. Wen, J. Liu, X. Zhao, Z. Xu, B. Niu, B Ye, Y. Wu, S. Zhang, Talanta 85 (2011) 317.
[17] W. Zhao, W. Wang, H. Chang, S. Cui, K. Hu, L. He, K. Lu, J. Liu, Y. Wu, J. Qian, S. Zhang., J. Chromatogr. A 1251 (2012) 74.
[18] W. Zhao, K. Hu, C. Wang, S. Liang, B. Niu, L. He, K. Lu, B. Ye, S. Zhang, J. Chromatogr. A 1223 (2012) 72.
[19] K. Hu, Y. Zhang, K. Chen, J. Liu, W. Zhao, W. Zhu, Z. Song, B. Ye, S. Zhang, J. Sep. Sci. 36 (2013) 445.
[20] M.X. Wang, Chem. Commun.. (2008) 4541.
[21] M.X. Wang, Acc. Chem. Res. 45 (2012) 182.
[22] D. Wang, Q. Wang, Y. Han, Y. Wang, Z Huang, M. Wang, Chem. Eur. J. 16 (2010) 13053.
[23] P.B. Ogden, J.W. Coym, J. Chromatogr. A 1218 (2011) 2936.
[24] P.R. Fields, Y. Sun, A.M. Stalcup, J. Chromatogr. A 1218 (2011) 467.
[25] M. Zhang, J. Chen, T. Gu, H. Qiu, S. Jiang, Talanta 126 (2014) 177.
[26] M.J. Kamlet, R.W. Taft, J. Am. Chem. Soc. 98 (1976) 377.
[27] M.J. Kamlet, R.W. Taft, J. Am. Chem. Soc. 98 (1976) 2886.
[28] L.C. Tan, P.W. Carr, M.H. Abraham, J. Chromatogr. A 752 (1996) 1.
[29] M.H. Abraham, M. Roses, C.F. Poole, S.K. Poole, J. Phys. Org. Chem. 10 (1997) 358.
[30] M.H. Abraham, Chem. Soc. Rev. 22 (1993) 73.
[31] E Shishani, S.C. Chai, S. Jamokha, G. Aznar, M.K. Hoffman, Anal. Chim. Acta 483 (2003) 137.