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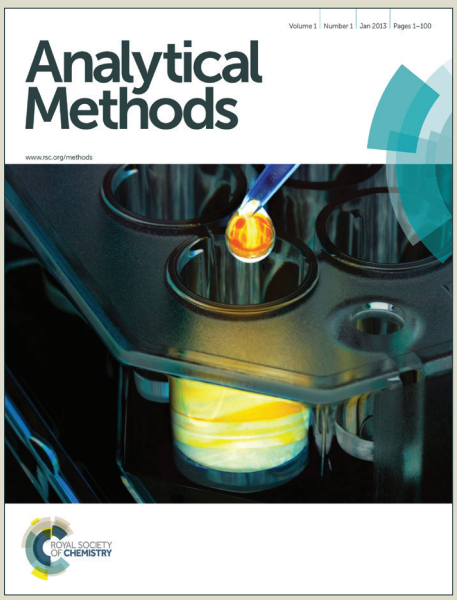

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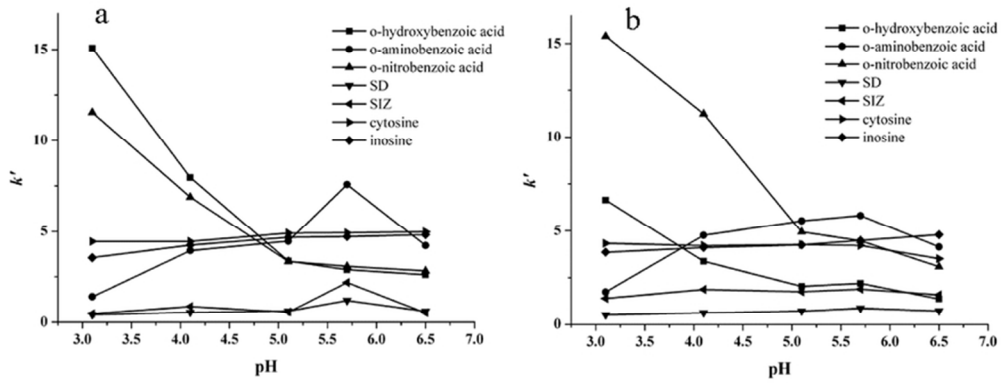

Effect of buffer pH on retention in HILIC mode
(a). Sil- (Cys)2 (b). Sil- Cys
Mobile phase: ACN/5 mM ammonium acetate (90/10, v/v)

74x32mm (300 x 300 DPI)

Preparation, chromatographic evaluation and comparison between cystine- and cysteine-bonded stationary phases

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Abstract

In this paper, cystine- and cysteine-bonded stationary phases were synthesized and characterized by UV spectroscopy, Fourier transform infrared spectroscopy and elemental analysis which proved the successful immobilization of cystine and cysteine on the silica support. The new stationary phases both displayed mixed-mode behavior, hydrophilic interaction chromatography (HILIC) mode and reversed-phase liquid chromatography (RPLC) mode. Retention behaviors of polar compounds on the two stationary phases were studied under different mobile phases with varying the water content, pH and ionic strength. The separation of sulfanilamide and substituted benzoic acid compounds were demonstrated on the new phases.

Keywords cystine bonded stationary phase; cysteine bonded stationary phase; hydrophilic interaction chromatography

Introduction

The first HPLC separations of polar compounds such as carbohydrates on polar stationary phases were published in the 1970s [1], using mobile phases containing water and a higher percentage of an organic solvent (typically acetonitrile). However, it was not until the early 1990s that new phases started emerging and Alpert [2] gave the practice a name by “hydrophilic interaction chromatography” (HILIC) to

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emphasize the presence of water in the mobile phase as the stronger eluting member, and the partition mechanism involved in the retention. In recent years it has obtained increasing popularity in separating polar solutes with HILIC as a complementary to reversed-phase liquid chromatography (RPLC)[3] and HILIC is accepted as a common separation mode [4,5], essentially with the separation of very polar compounds, such as glycopeptides [6-9] amino acids [2,10], oligonucleotides [11-13] and highly polar natural products.

The exact retention mechanism for HILIC is still open to considerable debate. The partitioning mechanism arises from the preferential adsorption of water on the polar stationary phase, which based on the differential distribution of the analyte molecules between the acetonitrile-rich mobile phase and a water-enriched layer at the surface of the hydrophilic stationary phase [2,4,14]. It has been shown that polar groups bonded on silica surface have a certain degree of solvation by water molecules. This would apparently support a partitioning model for retention [15]. Others reported that the separation in the HILIC mode is mainly governed by polar-polar interactions, such as hydrogen bonding, dipole-dipole and charge-dipole interactions, because of the strong dependence of the elution order on the number of polar functional groups involved [4,16-18]. In some cases, a combination of both partitioning and surface adsorption can take place, depending on the nature of the stationary phase, the properties of the solutes and the mobile phase composition [19].

The development of new stationary phases for HILIC has been the subject in recent years for the sake of increasing the diversity of HILIC stationary phases to enlarge the coverage to polar and hydrophilic compounds [3]. Thiol groups present on the silica surface also act as an intermediate silica species to provide a reactive site for the attachment of a chromatographic ligand on the surface via a range of reactions including free radical addition [20], disulfide formation and Michael addition [21,22]. For example, Shen et al synthesized a cysteine-bonded zwitterionic stationary phase [23]. In this work, we prepared both cystine- and cysteine-bonded stationary phases and the retention behavior of various compounds on the two stationary phases were studied and compared. The resulting stationary phases displayed excellent selectivity

and efficient retention for various polar solutes in HILIC and RP mode.

2. Experimental

2.1 Reagents and Materials

Spherical silica (7 μm particle size; 10 nm pore size; 400 $m^2 \cdot g^{-1}$ surface area) was purchased from Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences (Lanzhou, China). 3-(Aminopropyl)trimethoxysilane (APTMS) was purchased from Alfa Aesar (Tianjing, China). 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC • HCl) and Fmoc-Cys(Trt)-OH were obtained from GL Biochem Ltd. (Shanghai, China) N-hydroxysuccinimide (NHS), Triethylsilane (TES) and 5,5'-Dithio bis-(2-nitrobenzoic acid) (DTNB) were from aladdin reagent Ltd. Co (Shanghai, China). Dimethyl sulfoxide (DMSO), N, N-dimethyl formamide (DMF), trifluoroacetic acid (TFA) and dichloromethane (DCM) were purchased from Rionlo. Co., Ltd (Tianjin, China).

Sulfamethazine (SM2), sulfamethoxypyridazine (SMP), and sulfamethoxazole (SMZ), sulfadiazine (SD), and sulfafurazole (SIZ) were obtained from Alfa Aesar (Tianjin, China). Thymine, cytosine, adenine, inosine, adenosine and organic acids were all purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Acetonitrile (ACN) of HPLC grade was from Dima Technology (Richmond Hill, ONT, Canada) and purified water from a Milli-Q system was used throughout the experiments (Billerica, MA, USA). All other reagents were of analytical-reagent grade.

2.2 Preparation of new stationary phases

2.2.1 Immobilization of Fmoc-Cys(Trt)-OH on Silica-APTMS

APTMS-bonded silica (Sil-NH_2) was prepared from active silica according to the method [24].

Fmoc-(Cys)Trt-OH (4.5 g) was dissolved in 30 mL of anhydrous DCM and stirred at 0 °C, in which were added 1.5 g EDC •HCl and 0.9 g NHS. The mixture was

adjusted to pH 4.5-5 using acetic acid and kept for 20 min before Sil-APTMS was suspended in the solution. The reaction was lasted under stirring at 0 °C for 4 h and at 20 °C for another 20 h. The resulting product was washed with DMF and ethanol to remove the residual reactants. The obtained precipitate was dried under vacuum at 60 °C [25].

2.2.2 Synthesis of Sil-$(Cys)_2$ and Sil-Cys

Deprotection of thiol-protecting group and triphenylmethyl (Trt) were carried out according to the literature [26].

Sil-Fmoc-Cys(Trt)-OH was removed to a round bottomed flask, 27 mL DCM, 1.5 mL TES and 1.5 mL TFA were added and stirred for 30 min to deprotect the thiol-protect groups. After that, the material obtained (Sil-Fmoc-Cys) was washed by DCM and ethanol successively and dried under vacuum at 60 °C.

Sil-Fmoc-Cys was suspended in 20% DMSO (6 M guanidine hydrochloride) solution and stirred 48 h. The resulting material containing disulfide bond (Sil-Fmoc-$(Cys)_2$) was washed with water and ethanol, dried under vacuum at 60 °C.

In order to deprotect the amino-protection group, fluorenylmethoxy carbonyl (Fmoc), Sil-Fmoc-$(Cys)_2$ and Sil-Fmoc-Cys were suspended in 20% piperidine/DMF and stirred for 30 min, respectively. The final product Sil-$(Cys)_2$ and Sil-Cys were obtained after washing by DMF and ethanol successively and dried under vacuum at 60 °C.

The preparation of Sil-Cys and Sil-$(Cys)_2$ were shown in Fig. 1.

(Fig.1)

2.3 Free SH-groups identification

Free SH-groups were identified using Ellman's method [27]. Fig. 2 showed the reaction procedure of Sil-Fmoc-Cys and Ellman reagent. We prepared cysteine solutions with different concentration (1, 2, 3, 4, 5 μg/mL) in 0.1mmol/L phosphate buffer (pH 8.0) as standard solution to quantify free -SH group. In cysteine solution was added 150 μL of DTNB (4 mg/mL) and kept for 15 min before the absorbance of solutions were measured by UV-VIS spectrophotometer at 410 nm. The standard

curve could be obtained between cysteine concentration and absorbance as $A = 0.1156c - 0.0116$ with $R^2 = 0.9949$.

(Fig. 2)

The free SH-groups of Sil-Cys and Sil-$(Cys)_2$ were both measured by the same procedure as above.

2.4 Instruments and chromatographic conditions

FT-IR spectra were obtained on a Nicolet 20 NEXUS 670 FT-IR (Madison, USA) using KBr pellets. Elemental analysis was measured on a Vario EL elemental analysis system (Elementar, Germany). Absorbance spectra were carried out using a Puxi UV-1810 visible spectrophotometer (Beijing, China).

The materials of Sil-Cys and Sil-$(Cys)_2$ were slurry-packed into 150 × 4.6 mm I.D. stainless steel column and methanol was used as the packing solvent at 60 MPa pressure, respectively. The chromatographic system consisted of a Varian 210 high-performance liquid chromatographic pump (Palo Alto, CA, USA), a Varian 325 UV-Vis detector, and a Varian Star chromatographic workstation.

The chromatographic evaluations were carried out at 25° C. The test probes of 1mg/mL were prepared in ACN and diluted with water to 20 μg/mL for water-rich mobile phase and diluted with ACN for ACN-rich mobile phase. The flow rate was 1.0 mL/min. For water-rich mobile phase, ACN peak was used to calculate the dead time and for ACN-rich mobile phase, benzene was used to find the dead time. All the evaluation experiments were repeated at least twice and the average retention data were used.

3 Results and Discussion

3.1 Characterization of Sil-$(Cys)_2$ and Sil-Cys

3.1.1 FT-IR analysis

IR spectroscopy was used to identify the chemical modifications of the silica phase. As can be seen from Fig. 3, in the IR absorption spectrum for Sil-APTMS, broad Si-O-Si bands appeared at 1300-1000 cm^{-1}. The band at 1631 cm^{-1} was attributed to N-H bending vibrations of Sil-APTMS. In the IR spectrum for Sil-Fmoc-Cys(Trt)-OH, peaks at 1662 and 1550 cm^{-1} were the absorption peaks of the

amide. Peaks at 774 and 701 cm^{-1} belonged to the adsorption of benzene. In the IR absorption spectrum for Sil-$(Cys)_2$ and Sil-Cys, the peaks at 774 and 701 cm^{-1} disappeared which indicated protection groups had been removed. The spectrum of Sil-$(Cys)_2$ and Sil-Cys were almost same because their structures were very similar.

(Fig. 3)

3.1.2 Element analysis and Ellman reaction

The element analysis results for the silica materials were shown in Table 1. From the percentage of nitrogen (%N), the concentration of amino groups bonded to the bare silica was calculated as 2.33 μmol/m^2 for Sil-APTMS. From the percentage of sulfur (%S), the concentration of the bonding density of Fmoc-Cys(Trt)-OH was calculated as 0.52 μmol/m^2 for Sil-Fmoc-Cys(Trt)-OH. The calculation formulas of the surface coverage are as follows [28]:

(Table 1)

$$Sil-APTMS(\mu mol/m^2)=\frac{\%N}{14\times(100\%-\%C-\%N-\%H)\times S'}=2.33$$

$$Sil-Fmoc-Cys(Trt)-OH(\mu mol/m^2)=\frac{\%S}{32\times(100\%-\%C-\%N-\%H-\%S)\times S'}=0.52$$

Where *%C*, *%H*, *%N* and *%S* represent the percent of carbon, hydrogen, nitrogen and sulfur, respectively, as determined by elemental analysis shown in Table 1 , *S'* is the specific surface area of the silica support (m^2/g) (BET). When the thiol protection group trityl triphenylmethyl was removed, the thiol of Sil-Fmoc-Cys was identified by Ellman reaction. After the determination, thiol density on silica gel was 0.11 μmol/m^2. And the absorbance of Sil-$(Cys)_2$ had already below detection limit. Hence, we considered that all the thiol on the silica gel had oxidized to disulfide bond.

3.2 Chromatographic evaluation

The retention of a solute on stationary phase was affected by a variety of experimental factors such as water content, pH and salt concentration in mobile phase. Many kinds of substances were used to investigate the retention capacities of the new columns, including quinolones (cinoxacin, norfloxacin, oflxacin and enoxacin), amine compounds (aniline, p-nitroaniline, o-nitroaniline and p-chloroaniline), phenols

(phenol, p-chlorophenol and hydroquinone), vitamins (VB1, VB2, VB3 and VB6) and caffeine. However the above substances could not have enough retention on the new columns. In the following study, we investigated the influence of different chromatographic conditions on retention factors using a series of test probes which could retain on the new columns (listed in Fig. 4 with their structures and pKa values).

(Fig.4)

3.2.1 Retention properties in HILIC mode

(1) Effect of ACN content on retention

Hydrophilic interaction was enhanced by decreasing the polarity of the eluent. In order to investigate the HILIC properties of the new column, we chose a set of nucleosides as test probes. A range of 5-30% water containing 5 mmol/L of ammonium acetate in mobile phases was studied with pH 5.7. The retention factors (k') of test probes were plotted against the volume fraction of water in the eluent as shown in Fig. 5. The retention of all test compounds displayed a decreased trend as increasing the water content in mobile phase, which indicated a typical HILIC retention mechanism. The stationary phase of Sil-Cys had a bit of stronger retentions for the probes. The capacity factors of the probes were nearly same on Sil-Cys column as on the commercial NUCLEODUR® 100-5 HILIC column (150×4.6 mm, 5 μm), which meant the two kinds of stationary phases had the similar hydrophilic property (data were not shown).

(Fig.5)

We also investigated the possible retention mechanisms. Alpert suggested that the retention mechanism for HILIC was a partitioning between the bulk eluent and a water-rich layer, partially immobilized on the stationary phase [2]. But the final retention mechanism was most probably a complex process of partitioning and electrostatic interactions or hydrogen bonding to the stationary phase [29].The relationship that was established for partitioning in RPLC separations is

$$\log k' = \log k'_w - S\varphi \quad (1)$$

where k'_w was the capacity factor for the weaker eluent component (water) only as mobile phase, φ was the volume fraction (concentration) of the stronger member of a binary mobile phase mixture, and S was the slope of log k' versus φ when fitted to a linear regression model [30]. In this study, φ, the volume fraction of the stronger member of a binary mobile phase mixture, was water. The weaker eluent component was acetonitrile.

For conventional normal phase (NP) chromatographic systems, where retention was based on surface adsorption, the relationship between the retention and the mole fraction X_B of the stronger solvent B in the eluent should adhere to the following expression [30]:

$$\log k' = \log k'_B - \frac{A_S}{n_B} \log N_B \quad (2)$$

where k'_B is the solute retention factor with pure B as eluent, A_S and n_B are the cross-sectional areas occupied by the solute molecule on the surface and the B molecules, respectively, and N_B is the mole fraction of the stronger member B in the eluent. In this study, the eluent B was water, and k'_B is the solute retention factor with pure water as eluent.

The retention factor of every test solute vs. water volume fraction was used to perform linear regression analysis respectively based on Eqs. (1) and (2), and the corresponding correlation coefficients were listed in Table 2. The results showed that Eq. (2) provided better correlation coefficients for most of solutes, which seemingly indicated that the retention on the Sil-$(Cys)_2$ and Sil- Cys were more based on the adsorption mechanism rather than partitioning mechanism. From the structures of the Sil-$(Cys)_2$ and Sil- Cys functionality, it can be deduced that these two stationary phases had the capacity to interact with solutes by adsorptive forces.

(Table.2)

(2) Effect of salt concentration in mobile phase

Ionic strength of mobile phase can also have influence on the retention of polar

compounds in HILIC. Ammonium acetate with different concentrations (5, 10, 20, 50 and 100 mmol/L) in the mobile phase was employed to adjust ionic strength owing to its relatively high solubility at high organic levels. The polar compounds including inosine, cytosine, sulfadiazine, sulfisoxazole, o-hydroxybenzoic acid, o-aminobenzoic acid and o-nitrobenzoic acid were selected to study the influence of ionic strength in eluent with high ACN content. The data of retention factors under different ionic strength were shown in Fig. 6. For Sil-$(Cys)_2$, the retention factors of o-hydroxybenzoic acid and o-aminobenzoic acid decreased significantly with an increase in salt concentration. The change of o-nitrobenzoic acid was slight. The possible reason might be that high ionic strength weakened the ion-exchange interaction, leading weaker retention of these solutes. For inosine and cytosine, the retention factors of these two compounds increased slightly which might attribute to the increasing of the water enrichment layer at high salt concentration. The retention factors of SD and SIZ did not change significantly when salt concentration changed. For Sil-Cys, The retention factors decreased slightly for all test probes with an increase in salt concentration. The possible reason could be that the new material is repelling the acidic probes electrostatically at the pH of 5.7 and with low salt concentration. Higher salt concentrations may have weakened any electrostatic interacion, leading to the weaker retention of the polar solutes.

(Fig.6)

(3) Effect of buffer pH on retention

Mobile phase pH also played an important role by influencing solute ionization in HILIC. The mobile phase pH was adjusted to 6.5, 5.7, 5.0, 4.0 and 3.0 before mixing with ACN while keeping the concentration of ammonium acetate at 5 mM. Fig. 7 showed the retention of test solutes with different mobile phase pH. For Sil-$(Cys)_2$, as shown in Fig.7(a), the retention of o-hydroxybenzoic acid (pKa 2.98) and o-nitrobenzoic (pKa 2.2) acid decreased dramatically with the increasing of mobile phase pH. Both of compounds were dissociated gradually with the increase of pH and then kept the stable ionization state over the pH range studied. The change in their retention was due to the change of charge state and density of the stationary phase.

The (+) charge density on the new phase would increase when the buffer pH decreased from 6.5 to 3.0, thus leading electrostatic attraction strengthened between these two compounds and stationary phase. For o-aminobenzoic acid, its retention was increased first, and then decreased from pH 5.7 to 3.0. O-aminobenzoic acid was a kind of zwitterionic compound which pI value was in the range from 4 to 3. Under the low pH condition, o-aminobenzoic acid carried (+) charges and could had electrostatic repulsion with stationary phase. Adversely, under the high pH condition, it carried (-) charges and might had electrostatic attraction with stationary phase. For SD, SIZ, inosine and cytosine, there were no significant ionization changes in the pH range studied. And the effect of pH on retention of Sil-Cys was similarly to Sil-$(Cys)_2$, showed in Fig. 7(b).

(**Fig.7**)

3.2.2 Retention properties in RP mode

(1) Effect of water content on retention

In RP mode, five aromatic compounds were chosen as test probes to investigate the effect of water in eluent on retention. We plotted retention factor k' versus volume fraction of water in eluent as shown in Fig. 8. We found that the retention of test probes on the two new stationary phases increased when the water content increased from 50-90%, which exhibited typical behavior of RPLC.

(Fig.8)

(2) Effect of salt concentration in mobile phase

Fig.9 showed the effect of salt concentration on retention in mobile phase having high water content. The retention of inosine and cytosine did not change on both stationary phases. It was found that the retention of carboxylic compounds experienced the most dramatic decrease from salt concentration 5 to 100 mM. It could be explained that electrostatic attraction or ion-exchange between the stationary phase and these carboxylic compounds which carried (-) charges under the studied pH was weakened. The retention of SD and SIZ also decreased as salt concentration increased which might attribute to the increasing of solutes solubility.

(**Fig.9**)

(3) Effect of buffer pH on retention

The effect of buffer pH on retention with mobile phase having high water content was showed in Fig. 10. O-hydroxybenzoic acid and o-nitrobenzoic acid decreased remarkably when pH changed from 3.0 to 6.5 because the intramolecular hydrogen bonds of solutes became weaker which increased their solubilities in water. The retention of o-aminobenzoic acid and SIZ were both increased first and decreased later. The change to o-aminobenzoic acid was similar to the phenomenon in high ACN content. The change of SIZ (pKa 4.7) could be explained that the solutes had electrostatic attraction with the stationary phases under low pH conditions and electrostatic repulsion under high pH conditions. The retention of inosine and cytosine did not change conspicuously.

(**Fig.10**)

3.3 Retention properties in the mixed-mode

The retention of five sulfanilamide compounds and benzoic acid compounds depending on the water content in mobile phase exhibited "U-shape" curves with a minimum of retention for 70/30 water-ACN mixture on the two new columns (Fig. 11). Similar "U-shape" curves have been also observed with a great many of stationary phases and mobile phase conditions.[30, 31] U-shaped elution curves indicate mixed retention effects.

(**Fig.11**)

3.4 Application

3.4.1 Separation of sulfanilamide compounds in HILIC and RP mode

To demonstrate the separation performance and high selectivity of new stationary phases, sulfanilamide compounds, which are highly polar and were sucessfully separated in HILIC mode and RP mode respectively, as shown in Fig. 12. From the Fig.12, we could find that separation selectivity of sulfanilamide compounds on Sil-$(Cys)_2$ was better than that on Sil-Cys in HLIC mode and separation time of Sil-$(Cys)_2$ was longer. It might because that disulfide bond was more stable than thiol group. It was obvious that the separation time in HILIC was much shorter than in RP mode.In addition, the separation order of sulfanilamide compounds was not in accordance with their log P which indicated the interaction forces between the solutes

and the stationary phases should more than one. Sulfanilamide compounds' structures were shown in Fig.4.

(**Fig. 12**)

3.4.2 Separation of benzoic acid compounds

To demonstrate the special selectivity of the new phases, benzoic acid compounds were used for further evaluation. As shown in Fig. 13, both two new columns could separate benzoic acid mixtures successfully. And their separation time and resolution were essentially the same.

(**Fig.13**)

The stationary phases were prepared double times for the comparison and nearly offered same retention behaviors for the test compounds. Both the column had endured more than 800 injections and no column efficiency decreased. However, it should be cited that the thiol groups on the Sil-Cys stationary phase could react with some substances under a certain conditions, it should avoid to contact with the analytes with aldehyde groups.

4. Conclusion

In this work, we prepared a cysteine- and a cystine-bonded stationary phase, which could be used in two different chromatographic mode, HILIC mode and RP mode. In HILIC mode, the main factor for solutes to retain on the stationary phases was adsorption besides some other interaction forces, such as ion-exchange and electrostatic interaction. The resulting stationary phases displayed excellent selectivity and efficient retention for various polar solutes in HILIC and RP mode.

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Captions

Fig.1 Synthesis routes of Sil-$(Cys)_2$ and Sil-Cys

Fig. 2 Reaction procedure of Sil-Fmoc-Cys and Ellman reagent

Fig. 3 FT-IR spectra of the modified silica materials

Fig.4 The probes and their properties

Fig.5 Effect of ACN content in mobile phase on retention in HILIC mode

(a). Sil- $(Cys)_2$ (b). Sil- Cys

Mobile phase: ACN/5 mM ammonium acetate, pH=5.7, UV detection: 260 nm.

Fig.6 Effect of salt concentration on retention in HILIC mode

(a). Sil- $(Cys)_2$ (b). Sil- Cys

Mobile phase: ACN/ ammonium acetate (90/10, v/v), pH=5.7

Fig.7 Effect of buffer pH on retention in HILIC mode

(a). Sil- $(Cys)_2$ (b). Sil- Cys

Mobile phase: ACN/5 mM ammonium acetate (90/10, v/v)

Fig.8 Effect of water content in mobile phase on retention in RP mode

(a). Sil- $(Cys)_2$ (b). Sil- Cys

Mobile phase: ACN/5 mM ammonium acetate, pH=5.7, UV detection: 254 nm

Fig.9 Effect of salt concentration on retention in RP mode

(a). Sil- $(Cys)_2$ (b). Sil- Cys

Mobile phase: ACN/ ammonium acetate (30/70, v/v), pH=5.7

Fig.10 Effect of pH of mobile phase on retention in RP mode

(a). Sil- $(Cys)_2$ (b). Sil- Cys

Mobile phase: ACN/5 mM ammonium acetate (30/70, v/v)

Fig.11 "U-shape" curves in mixed-mode

(a)(b). Sil- $(Cys)_2$ (c)(d). Sil- Cys

Mobile phase: ACN/5 mM ammonium acetate, pH=5.7

Fig. 12 Chromatogram of sulfanilamide compounds in different modes

Analytes: 1. SM2; 2. SMP; 3. SD; 4. SMZ; 5.SIZ; UV detection: 270nm;

(a)(b).Mobile phase: ACN/5 mM ammonium acetate (10/90, v/v), pH=5.7;

(c)(d).Mobile phase: ACN/5 mM ammonium acetate (95/5, v/v), pH=5.7;

(a)(c). Sil- $(Cys)_2$, (b)(d). Sil- Cys

Fig.13 Chromatogram of benzoic acid compounds

(a). Sil- $(Cys)_2$ (b). Sil- Cys

Mobile phase: ACN/5 mM ammonium acetate (80/20, v/v), pH=5.7, UV detection: 254 nm.

Analytes: 1.o-hydroxybenzoic acid; 2.p-nitrobenzoic acid; 3.o-nitrobenzoic acid; 4.o-aminobenzoic acid; 5. m-hydroxybenzoic acid; 6.p-hydroxybenzoic acid; 7.m-aminobenzoic acid

Table 1. Elemental analysis of bonded silica gels

	N(%)	C(%)	H(%)	S(%)
Sil-APTMS	1.200	5.670	1.237	
Sil-Fmoc-Cys(Trt)-OH	1.990	14.290	2.062	0.538
Sil-Fmoc-Cys	1.666	9.795	1.482	
Sil-$(Cys)_2$	1.230	5.820	1.234	0.745
Sil-Cys	1.350	4.770	1.025	0.844

Table.2 Fitting results of nucleosides on Sil-$(Cys)_2$ and Sil-Cys

Analytes	R^2			
	Eq.(1)[a]	Eq.(2)[a]	Eq.(1)[b]	Eq.(2)[b]
cytosine	0.8860	0.9829	0.9022	0.9931
thymine	0.9580	0.9951	0.9334	0.9976
adenosine	0.8693	0.9816	0.9488	0.9966
inosine	0.9015	0.9895	0.8899	0.9921

a: Sil-$(Cys)_2$; b: Sil-Cys.

Captions

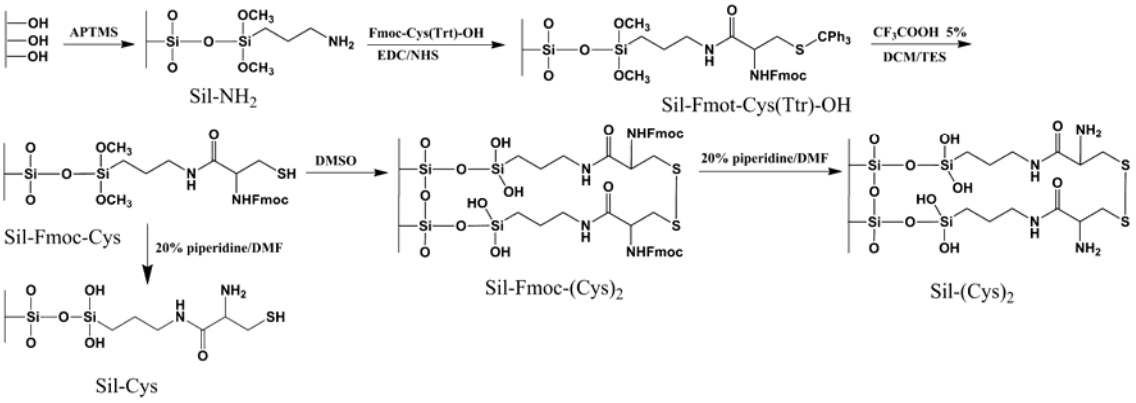

Fig.1 Synthesis routes of Sil-$(Cys)_2$ and Sil-Cys

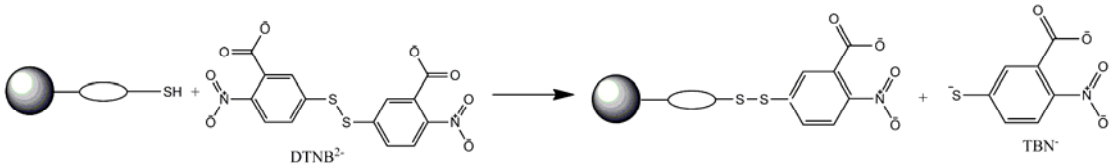

Fig. 2 Reaction procedure of Sil-Fmoc-Cys and Ellman reagent

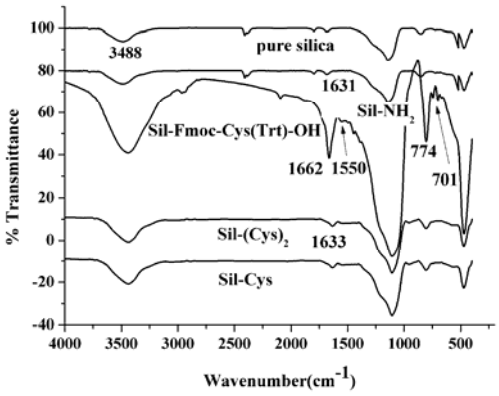

Fig. 3 FT-IR spectra of the modified silica materials

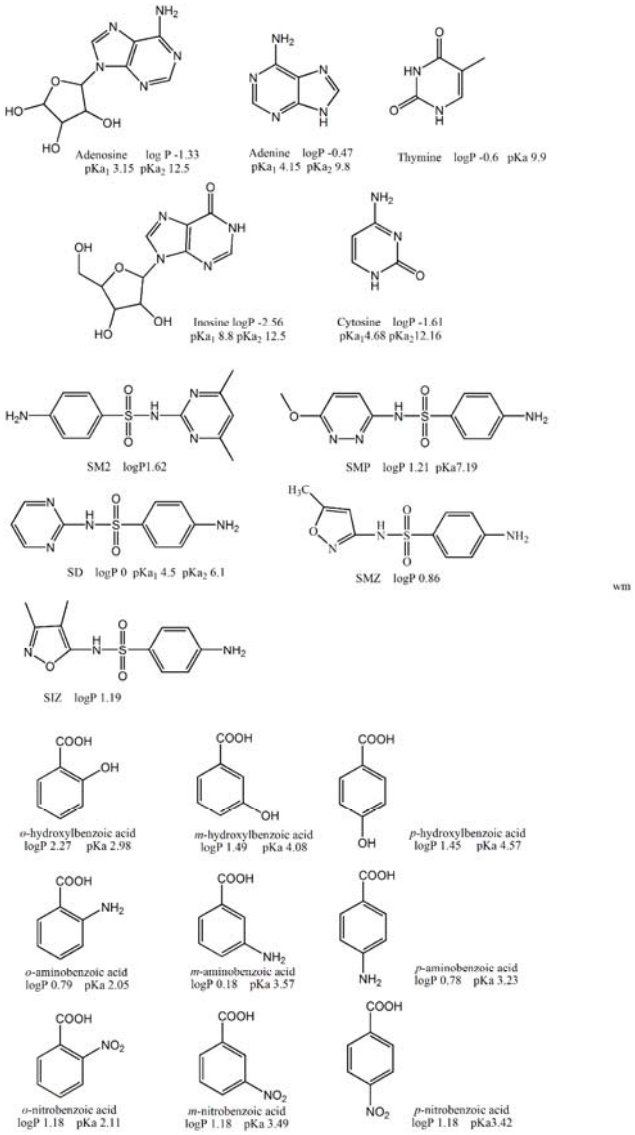

Fig.4 The probes and their properties

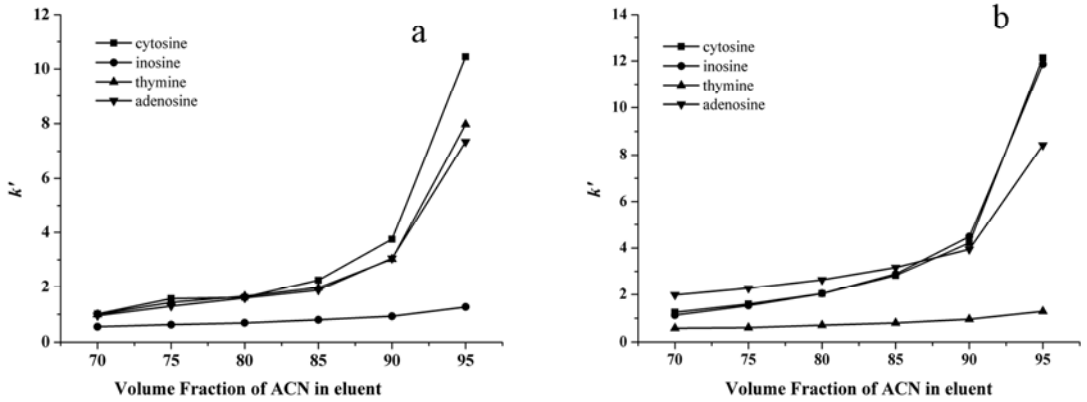

Fig.5 Effect of ACN content in mobile phase on retention in HILIC mode

(a). Sil- $(Cys)_2$ (b). Sil- Cys

Mobile phase: ACN/5 mM ammonium acetate, pH=5.7, UV detection: 260 nm.

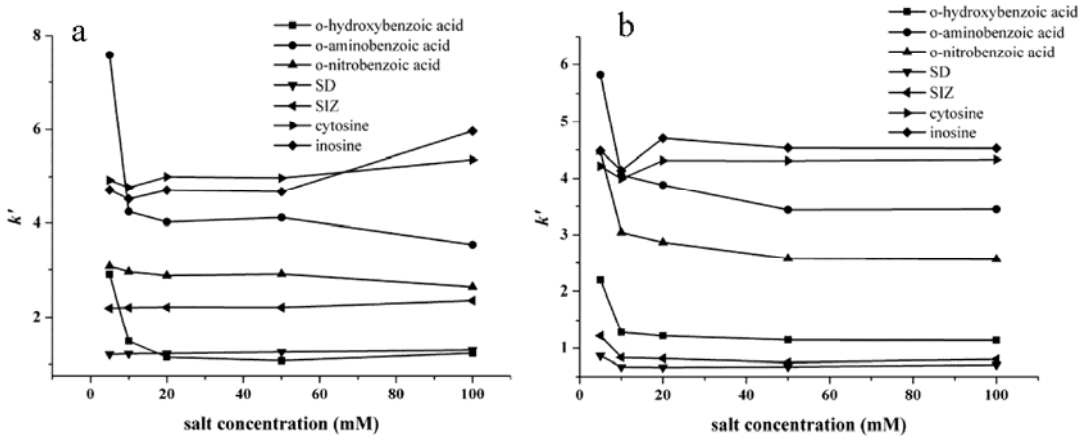

Fig.6 Effect of salt concentration on retention in HILIC mode

(a). Sil- $(Cys)_2$ (b). Sil- Cys

Mobile phase: ACN/ ammonium acetate (90/10, v/v), pH=5.7

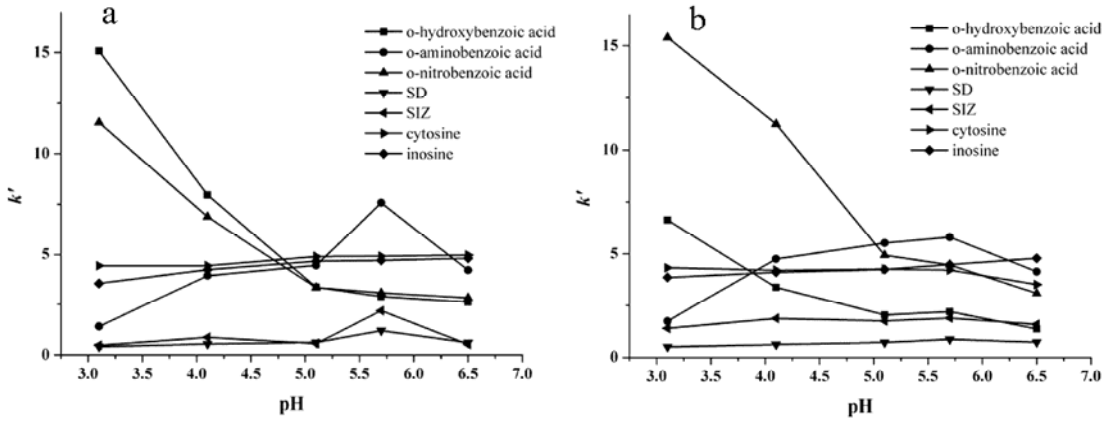

Fig.7 Effect of buffer pH on retention in HILIC mode

(a). Sil- $(Cys)_2$ (b). Sil- Cys

Mobile phase: ACN/5 mM ammonium acetate (90/10, v/v)

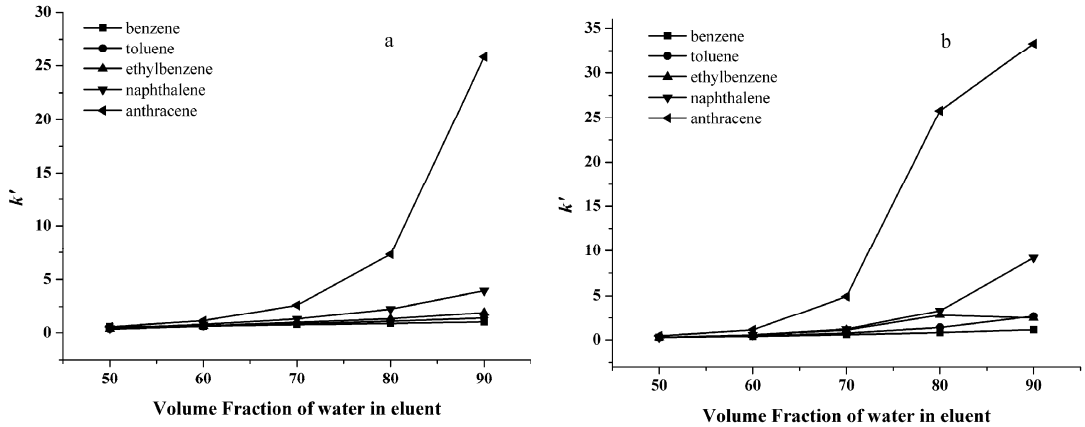

Fig.8 Effect of water content in mobile phase on retention in RP mode

(a). Sil- $(Cys)_2$ (b). Sil- Cys

Mobile phase: ACN/5 mM ammonium acetate, pH=5.7, UV detection: 254 nm

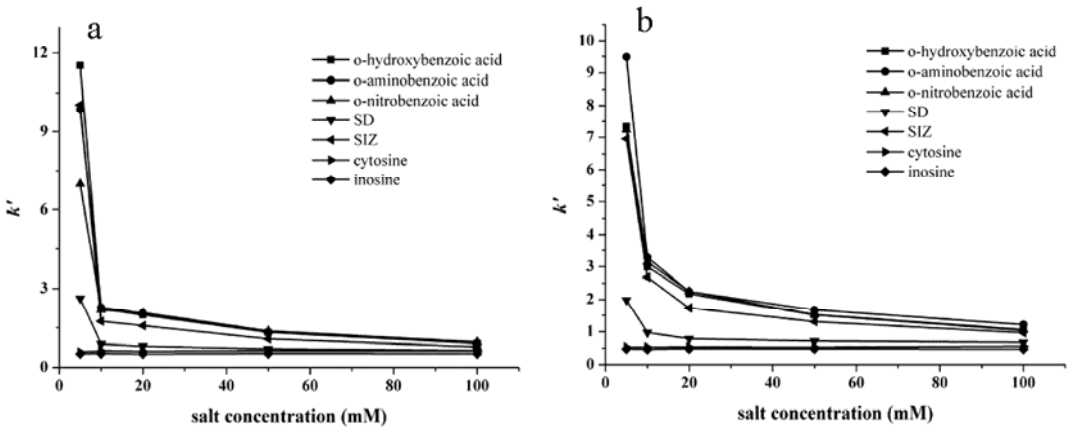

Fig.9 Effect of salt concentration on retention in RP mode

(a). Sil- $(Cys)_2$ (b). Sil- Cys

Mobile phase: ACN/ ammonium acetate (30/70, v/v), pH=5.7

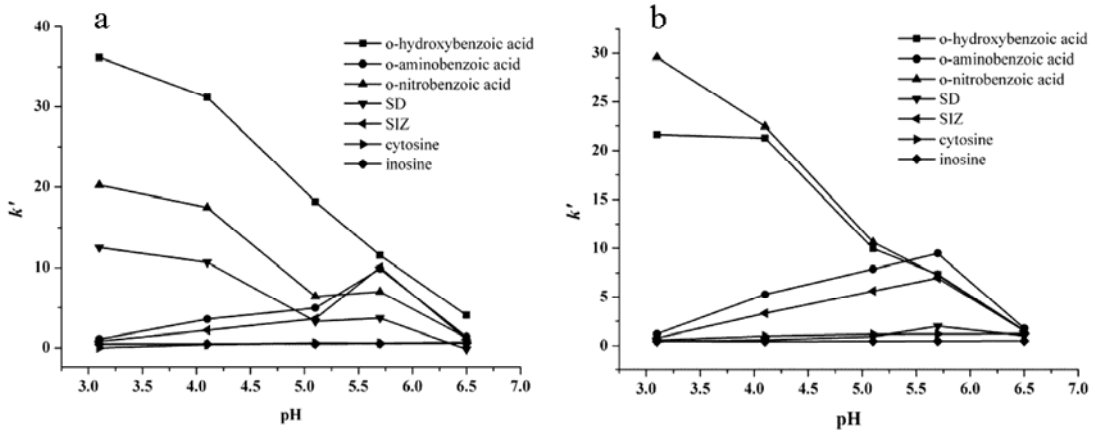

Fig.10 Effect of pH of mobile phase on retention in RP mode

(a). Sil- $(Cys)_2$ (b). Sil- Cys

Mobile phase: ACN/5 mM ammonium acetate (30/70, v/v)

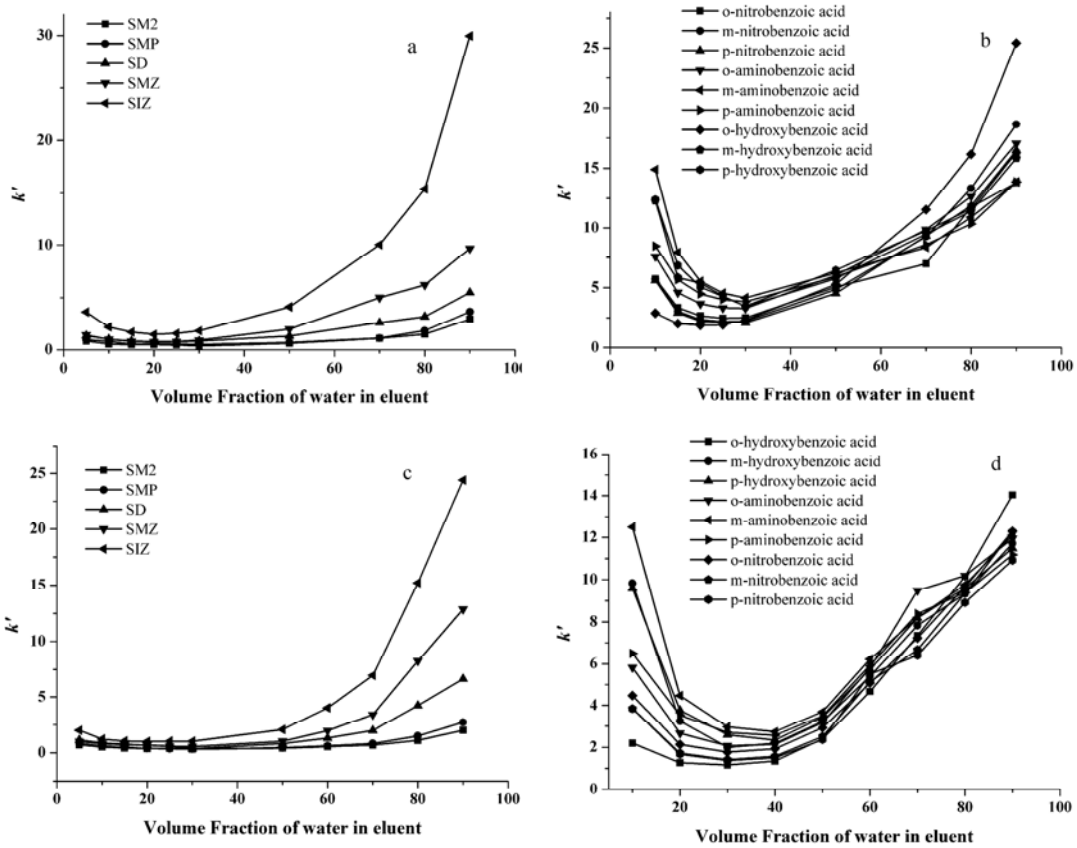

Fig.11 "U-shape" curves in mixed-mode

(a)(b). Sil- $(Cys)_2$ (c)(d). Sil- Cys

Mobile phase: ACN/5 mM ammonium acetate, pH=5.7

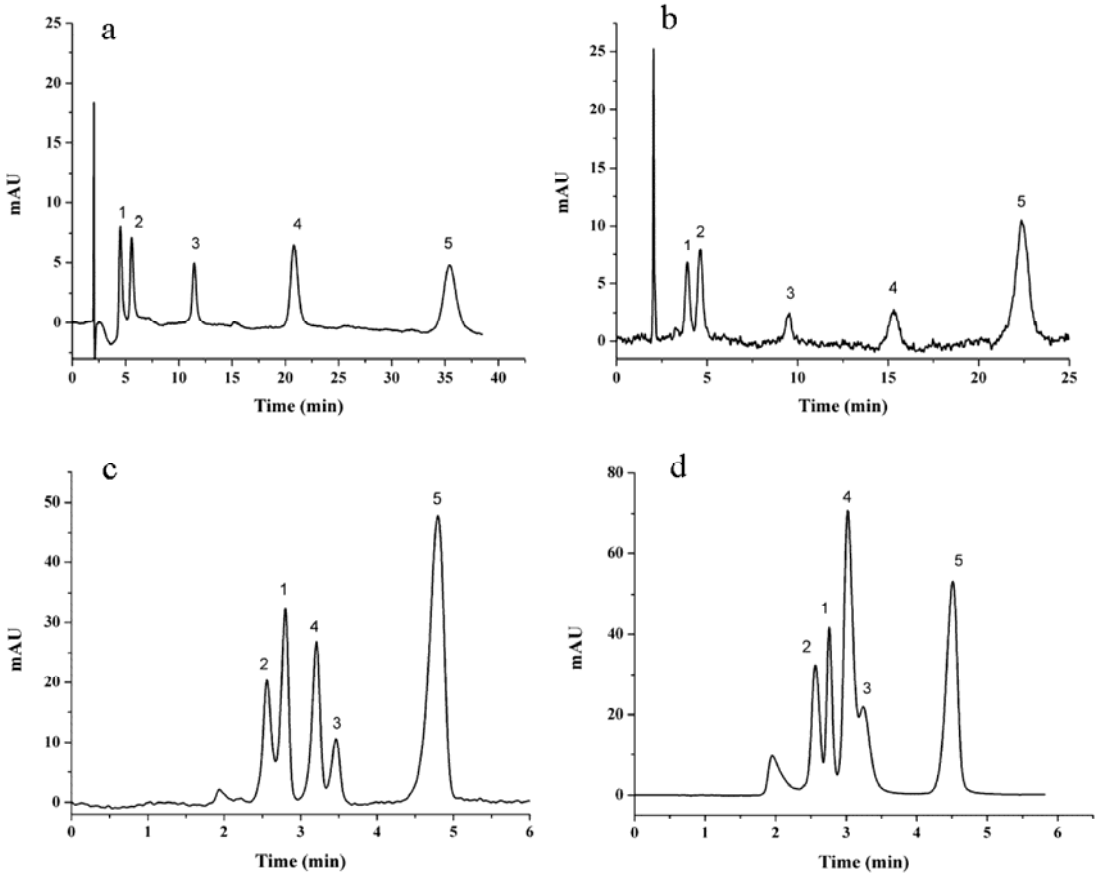

Fig. 12 Chromatogram of sulfanilamide compounds in different modes

Analytes: 1. SM2; 2. SMP; 3. SD; 4. SMZ; 5.SIZ; UV detection: 270nm;

(a)(b).Mobile phase: ACN/5 mM ammonium acetate (10/90, v/v), pH=5.7;

(c)(d).Mobile phase: ACN/5 mM ammonium acetate (95/5, v/v), pH=5.7;

(a)(c). Sil- $(Cys)_2$, (b)(d). Sil- Cys

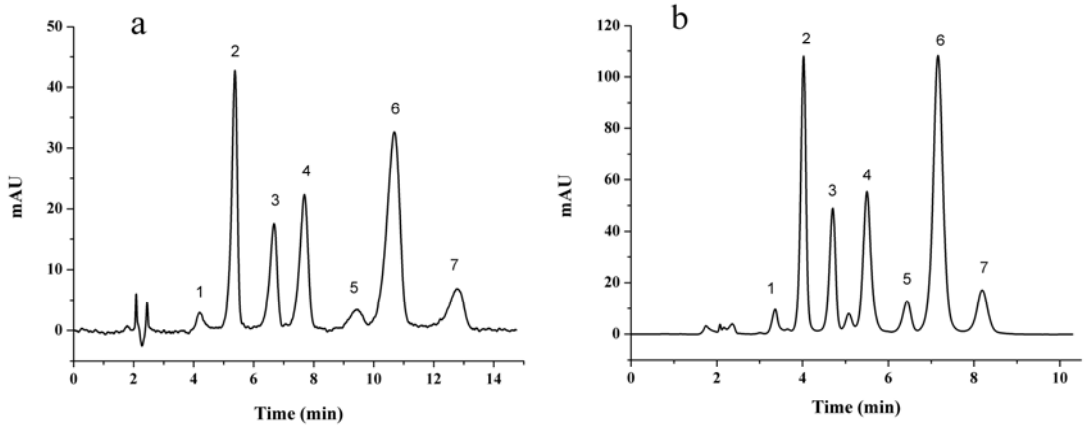

Fig.13 Chromatogram of benzoic acid compounds

(a). Sil- $(Cys)_2$ (b). Sil- Cys

Mobile phase: ACN/5 mM ammonium acetate (80/20, v/v), pH=5.7, UV detection: 254 nm.

Analytes: 1.o-hydroxybenzoic acid; 2.p-nitrobenzoic acid; 3.o-nitrobenzoic acid; 4.o-aminobenzoic acid; 5. m-hydroxybenzoic acid; 6.p-hydroxybenzoic acid; 7.m-aminobenzoic acid