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A novel congo red (CR) derivatized silica stationary phase was prepared and packed into a fused silica capillary tube for nano-flow HPLC. A variety of analytes including poly-aromatic hydrocarbons, parabens, acids, sulfonamides, bases, and nucleosides were successfully separated using the CR. In comparison to commercial ODS columns，this new stationary phase has a different separation mechanism (hydrophobically-assisted ion-exchange), which was evident in the separation of benzoic acid derivatives and sulfonamides. The successful application of CR-bonded silica stationary phase in the HILIC and PALC modes demonstrates the effectiveness of this potential chromatographic material in nano flow HPLC.

1. Introduction

With the advancement of analytical techniques and increasing importance of chromatographic analysis, single mode chromatography such as the classical reverse phase liquid chromatography (RPLC), cannot always meet the needs of contemporary separation science. This is partly because RPLC is limited in its adaptability and thus the range of analytes is also necessarily limited. Thus, the mixed-mode chromatographic approach has begun to emerge as a more useful chromatographic method due to a need for stronger retention of both polar and non-polar molecules. Multiple mechanisms of interaction[1, 2] permit the use of one stationary phase for a much wider range of applications as compared to the reversed-phase or ion-exchange approaches. A major benefit of mixed-mode chromatography is that the same column can be used either in a single mode (such as ion exchange[3-7], or reversed-phase[8-13]), or in a combination of modes[14-17]. Therefore, an increasing variety of mixed-mode or multi-mode stationary phases have recently begun to be explored.[18-22] For example, Lin et al. prepared a glutathione–silica hybrid monolith based on “thiol-ene” click chemistry for mixed-mode capillary liquid chromatography.[23] Li et al. prepared a poly-L-lysine stationary phase for hydrophilic interaction/reversed-phase mixed-mode chromatography.[24]

Sulfonic-azobenzene[25-27], a type of stationary phase, was discovered and creatively used in mixed-mode chromatography. The rigidly functionalized azobenzene and the ionized sulfonic acid groups allowed several potential mechanisms of interactions between analytes and chromatography materials. Besides hydrophobic interactions, the intermolecular π-π interactions and ion-exchange provide a wider range of application. The azobenzene dye methyl orange (MO), exhibits excellent chromatographic performance as a stationary phase. Preparation of the MO stationary phase simply uses bonded silica[28] or chemical modification of counter anions in ionic liquid polymer phase[29], or co-immobilization with alkylimidazolium[30, 31]. The MO stationary phase demonstrates outstanding separation characteristics for poly-aromatic hydrocarbons (PAHs), steroids, and other similar molecules as compared with the more typical C-18 (octadecylsilane) stationary phase. Another sulfonic-azobenzene dye which is closely analogous to MO, congo red (CR), is commonly used as an acid-base indicator and in the medical diagnosis of amyloidosis[32-36]. With CR, the two azo groups are protonated in the pH range of 3.0 - 5.2 causing a colour change of the dye from blue to red at pH 3.0 - 5.2. Aimed at this condition, CR is found to exist as a zwitterion. Besides, CR is also used as an amyloid-specific dye. It is known to form supramolecular structures in solution via pi-pi-stacking[37]. Dissociated sulfonic groups, protonation of azo groups and pi-pi-stacking behaviour must have a strong impact on the chromatographic properties of CR. Since the potential of CR as a chromatographic stationary phase has not previously been explored, we have prepared and analyzed this material for use in mixed-mode chromatographic separations.

As described herein, CR modified silica (Sil-CR) was prepared through the bonding of CR to glycidoxypropyl-modified silica. The modified silica was then packed into a capillary for nano-flow HPLC. This column provided different chromatographic modes for analyzing various compounds such as parabens, bases, and nucleosides by

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HPLC, hydrophilic interaction liquid chromatography (HILIC), and per aqueous liquid chromatography (PALC) respectively. Furthermore, this material was also used for the separation of benzoic acid derivatives and sulfonamides in a combination of chromatographic modes (reverse phase and ion-exchange).

2 Experimental

2.1 Materials and reagents

Congo red was obtained from Yuanhang Reagent Plant (Shanghai, China). 3-Glycidoxypropyltrimethoxysilane and sodium silicate were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Porous silica particles (diameter 5 μm, pore size 12 nm, specific surface area 300 m^2 /g were obtained from YMC (Kyoto, Japan). Fused silica capillaries were purchased from Yongnian Optic Fiber Plant (Hebei, China). Benzene, toluene and priority pollutant polyaromatic hydrocarbons (PAHs) were obtained from Chemical Reagent Plant (Shanghai, China). Uracil, methylparaben, ethylparaben, propylparaben, and butylparaben were obtained from Aladdin Reagent Company (Shanghai, China). Adenine, cytosine, thymine, various nucleoside bases such as *2*'-deoxycytidine-*5*'-monophosphate (dCMP), disodium uridine-*5*'-monophosphate (UMP), tryptamine, tyramine, dopamine and norepinephrine bitartrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). *4*-hydroxybenzoic acid, protocatechuic acid, m-toluic acid, benzoic acid, *2, 4*-dihydroxybenzoic acid and *2*-hydroxybenzoic acid were purchased from TCI (Tokyo, Japan). Sulfaguanidine, sulfadiazine, sulfamethoxazole, sulfadimethoxine, and sulfachinoxalin were purchased from Dr. Ehrenstorfer (Augsburg, German),

2.2 Preparation of Sil-CR

3-Glycidoxypropylsilica (Sil-GPS) was prepared by the reaction of activated silica gel and 3-glycidoxypropyltrimethoxysilane (GPS) mixed in toluene-water as described previously[38]. Briefly, a specific amount of silica was heated under reflux for 30 min with 10% wt hydrochloric acid, rinsed with deionized water, and dried under vacuum at 180 °C for 4 h to remove all adsorbed water from the surface. The activated silica (2 g) thus obtained was suspended in dry toluene (20 mL) in a three-necked round-bottomed flask equipped with a reflux condenser and then 2 mL 3-glycidoxypropyltrimethoxysilane and 440 μL of water were added. The mixture was then heated under reflux conditions, under nitrogen, for 24 h. The slurry was then filtered through a glass frit and washed successively with toluene, methanol, and finally acetone. 3-glycidoxypropylsilica was dried overnight under a vacuum at room temperature overnight.

The dried Sil-GPS (0.2 g) was added to a 50 mL three-necked round-bottomed flask. Then 3 equivalents of CR were separately dissolved in 25 mL of ethanol and the insoluble matter was removed by centrifugation. The CR solution was then added to the above flask and the mixture was heated under reflux conditions with a nitrogen protection for 24 h. The CR-modified silica (Sil-CR) particles were filtered and washed successively with ethanol, methanol, methanol-water (1:1, v/v), methanol and acetone. Finally Sil-CR was dried under vacuum at 60 °C overnight.

2.3 Nano-flow HPLC Sil-CR column packing

Untreated fused silica capillary columns (1 m long × 100 μm i.d.) were sequentially rinsed with 1.0 M NaOH for 0.5 h, ultra-pure water for 0.5 h, 0.1 M HCl for 0.5 h, and ultra-pure water again until the outflow was neutral. Finally, the capillary was washed with acetone and dried with a nitrogen purge for 2 h at room temperature. A piece of capillary was cut and injected with a sodium silicate solution from one side. A very narrow porous outlet-frit was sintered by gentle heating to form a temporary frit (**step 1**). The other end of the capillary was connected to an empty stainless steel jar containing a slurry of the packing material. The slurry was prepared by measuring out 0.15 g of Sil-CR, then adding methanol-water (8:2) to prepare a suspension. The Sil-CR-packed column was prepared under 20 MPa using an HPLC pump and methanol-water (8:2, v/v) as the packing solvent. The capillary was immersed into an ultrasonic bath to remove potential air-pockets and ensure close packing of the particles (**step 2, 3**). A retaining frit or an injection-side frit was sintered in the same manner as described for the outlet-frit. The frit was formed from the packing material itself. The end frit was prepared at an appropriate position (**step 4**). All operations were conducted under high pressure. Subsequently, the capillary was removed from the packer, the outside portion of the retaining frit was removed, and excess packing material (from the retaining frit to the end frit) was washed out in the reverse direction (**step 5**). Finally, a ~1-2 mm detection window was made by removing the poly-imide coating next to the frit (**step 6**).

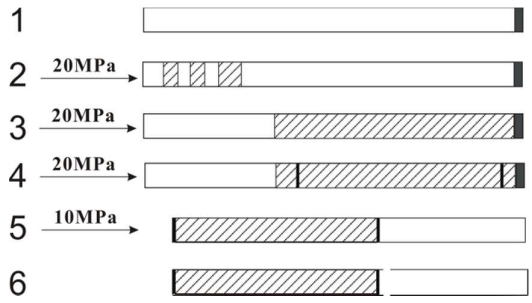


Fig. 1 Schematic overview of the Sil-CR packing procedure in six steps: (1) fabrication of a temporary frit, (2,3) filling the capillary with stationary phase, (4) fabrication of the permanent frits, (5) removal of the excess of stationary phase particles and (6) fabrication of a detection window.

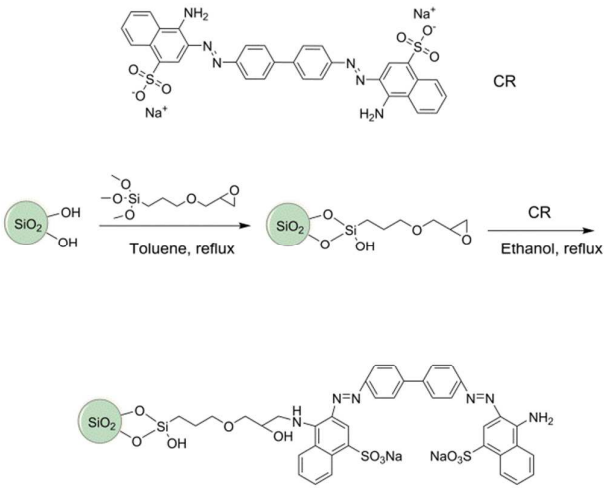
2.4 Equipment and chromatographic conditions

Sil-CR was packed into a fused silica capillary column (50 cm long ×100 μm i.d., 22 cm effective length) using methanol/water (8:2, v/v) as the packing solvent, as described above. A commercial ODS (octadecylsilane, C-18) capillary column (Unimicro C18, particle diameter 3 μm, 45 cm long ×100 μm i.d., 20 cm effective length) used as the reference was obtained from Unimicro Technologies (Shanghai, China). HPLC-grade methanol and acetonitrile were used as the mobile phase solvents. Deionized water (18.25 MΩ) was prepared using a Milli-Q system from Millipore (Bedford, MA, USA). DRIFT spectra were performed on Spectrum 100 with a smart diffuse reflection accessory (Perkin Elmer, USA) in the range of 4000–400 cm^{-1}. Elemental analysis for the adsorbents was performed by using a SERIES II 2400 (PerkinElmer) elemental analyzer. The analytes were directly dissolved in methanol or water. All chromatographic experiments were performed on a TriSep-2100 pressurized capillary electrochromatography (pCEC) instrument (this instrument can also be utilized as capillary liquid chromatography system, Unimicro Technologies, Pleasanton, CA, USA). Samples were injected through an injection valve with an internal 1 μL sample loop. A four-port splitter was set between the injection valve and the packed column to split the flow into a desirable and stable flow rate. Since the splitting ratio was set at 400:1, the actual injection volume was about 2.5 nL.

3 Results and Discussion

3.1 Synthesis and characterization

The silica surface-modification procedures are simple and facile as outlined in **Scheme 1**. In brief, the synthesis includes the preparation of 3-glycidoxypropylsilica (Sil–GPS) followed by the bonding of CR on 3-glycidoxypropylsilica.



Scheme 1 Preparation of Sil-GPS and Sil-CR.

Infrared spectroscopy is a highly useful instrumental approach for the precise identification of molecular structure, and is also valuable to identify and verify chemical modifications. Some clear differences were observed between the spectra of Sil-GPS and Sil-CR as shown in **Fig. 2**. In the spectrum for the Sil-CR surface (**Fig. 2b**), characteristic infrared (IR) signals were observed at 1503 cm^{-1}, 1586 cm^{-1} and 1612 cm^{-1}. These IR bands can be attributed to stretching vibrations of the phenyl bond of sulfonic azobenzene, which confirms the anchoring of the CR molecule onto the silica surface.

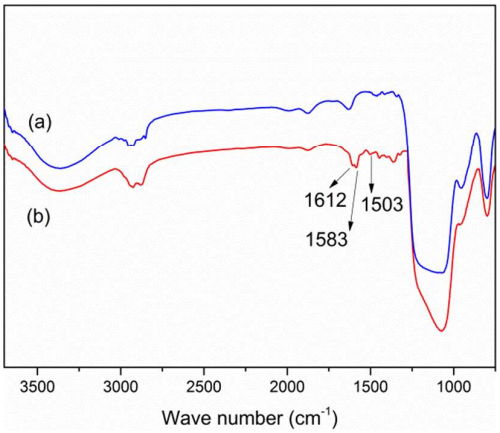


Fig. 2 Diffuse reflectance infrared Fourier transform (DRIFT) spectra of (a) Sil-GPS, and b) Sil-CR.

The results of elemental analysis further confirmed the immobilization of CR on activated silica gel. The elemental content (%) of Sil-GPS and Sil-CR was C 8.22, H 1.43 and C 14.51, H 1.82, N 1.38, respectively. The degree of surface coverage for Sil-GPS and Sil-CR was calculated from the following equations [Eqs. (1) and (2)][28,29]:

$$\text{Sil-GPS } [\mu\text{mol m}^{-2}] = \frac{\%C}{72 \times (1 - \%C - \%H) \times S} = 4.21 \quad (1)$$

$$\text{Sil-CR } [\mu\text{mol m}^{-2}] = \frac{\%N}{84 \times (1 - \%C - \%H - \%N) \times S} = 0.67 \quad (2)$$

In the above equations, which %C, %H, and %N represent the percentages of carbon, hydrogen, and nitrogen, respectively, and S is the surface area. The number 72 represents the total molecular weight of six C in GPS molecules and 84 represent the total molecular weight of six N in CR molecules. The amount of 3-glycidoxypropyl and CR molecules attached to the silica surface can thus be calculated as 4.21 μmolm^{-2} for Sil-GPS and 0.67 μmolm^{-2} for Sil-CR.

The column efficiency of the prepared nano-flow Sil-CR column was measured by using uracil, 4-hydroxybenzoic acid, benzoic acid, benzene, toluene and ethylbenzene as test compounds. Plate height curves (**Fig. S1**) shown that the optimum flow rate of 0.03 mL/min (linear velocity 0.33 mm/s), corresponded to the minimum height equivalent of theoretical plate. Under the optimal conditions (**Fig.3**), over 72000 plates/m of theoretical plates was obtained. As shown in **Table S1**, when benzene was used as a test compound, 90440 plates/m were obtained. The stability of this column was tested by the repetitive experiments of run-to-run (n = 6) and day-to-day (n = 3), and the repeatability was satisfactory with RSD values less than 3.1% for column efficiency and less than 2.7% for retention factor in the HPLC mode. In contrast to other reports where a high concentration of organic solvents is used as the mobile phase for a column efficiency test[39, 40], this work utilized a low concentration of acetonitrile (Acetonitrile/Acetate buffer (20:80)) as the mobile phase. The presence of the sulfonic acid and other polar groups in this column increases the polarity of the stationary phase, thus lowering the retention of non-polar compounds.

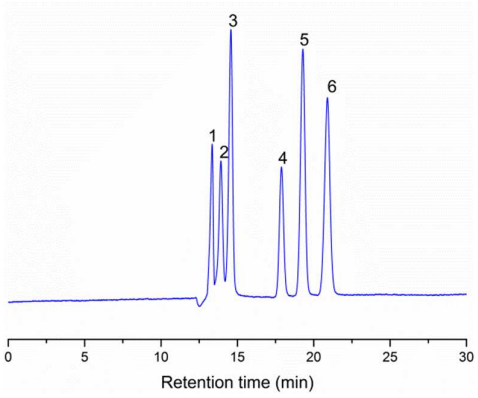


Fig. 3 Sil-CR column efficiency test on optimum flow rate: 1) uracil, 2) 4-hydroxybenzoic acid, 3) benzoic acid, 4) benzene, 5) toluene and 6) ethylbenzene with 12.5mM sodium acetate (pH = 4.2) buffer/acetonitrile (80:20, v/v). Column: 50 cm ×100 μm, 22 cm effective length; Pump flow: 0.03 mL/min, λ = 254 nm

3.2 Chromatographic performance

3.2.1 Sil-CR used as a stationary phase in RPLC mode

In RPLC, the properties of the stationary phase determine retention and selectivity. An ODS (octadecylsilane, C-18) stationary phase is the most commonly used column packing material due to its efficient separation characteristics and reproducible properties. In this work, we have successfully used the new Sil-CR stationary phase in the application of reverse phase chromatography. This stationary phase shows good retention and selectivity for PAHs (**Fig. 4a**). Benzene and five PAHs tested all showed baseline separation. Furthermore, this column can be used in the pure reversed-phase mode for separation of neutral hydrophobic compounds as shown in **Fig. 4b**. Methylparaben, ethylparaben, propylparaben and butylparaben are baseline-separated using methanol/water (20:80, v/v) as the mobile phase. With increasing length of the alkyl chain on the analytes, the hydrophobic properties of the compound are increased, resulting in stronger retention.

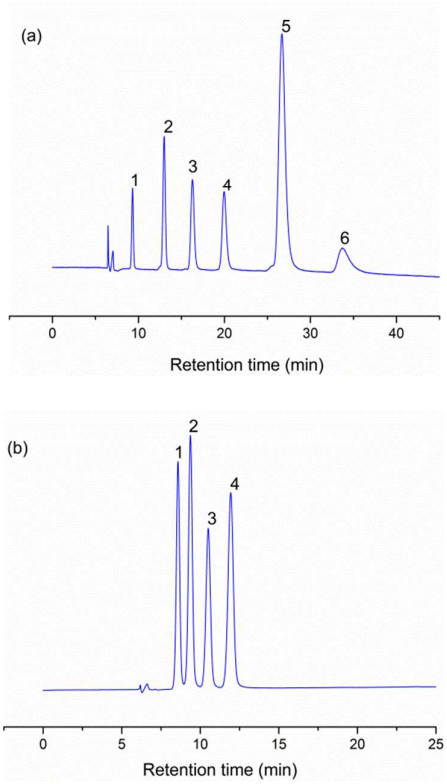


Fig. 4 Separation of neutral hydrophobic compounds (a) Benzene and PAHs: 1) benzene, 2) naphthalene, 3) fluorine, 4) anthracene, 5) pyrene, 6) chrysene and with methanol/water (60:40, v/v); (b) Parabens: 1) methylparaben, 2) ethylparaben, 3) propylparaben and 4) butylparaben with methanol/water (20:80, v/v); Column: 50 cm ×100 μm, 22 cm effective length; Pump flow: 0.06 mL/min, λ = 254 nm.

The Sil-CR column described herein can also be used to separate polar compounds. As shown in **Fig. 5** (Structures are given in **Fig. s2**), a comparable separation was performed by the ODS and Sil-CR columns on a series of benzoic acid derivatives, which included *4*-hydroxybenzoic acid, protocatechuic acid, *m*-toluic acid, benzoic acid, *2, 4*-dihydroxybenzoic acid and *2*-hydroxybenzoic acid was performed by the ODS and Sil-CR columns. Two different points can be observed clearly. The first one is that the retentions of *2, 4*-dihydroxybenzoic acid and *2*-hydroxybenzoic acid are even stronger than that m-toluic acid and benzoic acid in Sil-CR. The second one is that the retention order of *4*-hydroxybenzoic acid, protocatechuic acid, m-toluic acid and benzoic acid are different among the ODS and Sil-CR columns. It is known that the chemical polarity order from strong to weak for these analytes is protocatechuic acid, *2, 4*-dihydroxybenzoic acid, *4*-hydroxybenzoic acid, *2*-hydroxybenzoic acid, benzoic acid and *m*-toluic acid. It is no doubt that the order of retention is consistent with the order of polarity for these compounds on the ODS column as shown in Fig 5b**.** The order of pK_a from large to small is *4*-hydroxybenzoic acid, protocatechuic acid, *m*-toluic acid, benzoic acid, *2, 4*-dihydroxybenzoic acid and *2*-hydroxybenzoic acid, and their degrees of deprotonation increase gradually, which also corresponds well to the peak order (1-2-3-4-5-6) in **Fig. 5a**. The pKa values of these six analytes are listed in **Table S3**. So the ion-exchange chromatography might be the dominant separation mechanism on separation of benzoic acid derivatives. In addition, because of the small differences in the pKa values between *4*-hydroxybenzoic acid and protocatechuic acid, *m-t*oluic acid and benzoic acid, *2, 4*-dihydroxybenzoic acid and *2*-hydroxybenzoic acid respectively, their retention times are obviously very close, as seen from peak 1 and 2, 3 and 4, 5 and 6 in Fig. 5a. For this separation, a HAc-NaAc buffer solution at pH 5.18 is used. This buffer not only provides acidic surroundings, but also, the presence of sodium ions can accelerate separation of the analytes.

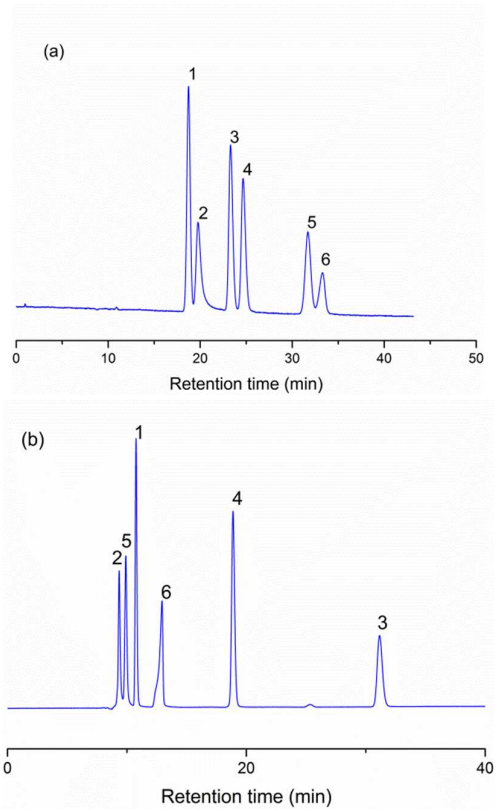


Fig. 5 Separation of six benzoic acid compounds: 1) *4*-hydroxybenzoic acid, 2) protocatechuic acid, 3) *m*-toluic acid, 4) benzoic acid, 5) *2, 4*-dihydroxybenzoic acid and 6) *2*-hydroxybenzoic acid with (a) Sil-CR with 12.5 $mmolL^{-1}$ sodium acetate buffer/acetonitrile (60:40, v/v, pH 5.18) Column: 50 cm ×100 μm, 22 cm effective length; Pump flow: 0.03 mL/min, λ = 254 nm. (b) ODS column with 12.5 $mmolL^{-1}$ sodium acetate buffer/methanol (70:30, v/v, pH 4.80). Column: 45 cm ×100 μm, 20 cm effective length; Pump flow: 0.05 mL/min, λ = 254 nm

Separation of a group of sulfa drugs whose structures are shown in **Fig. S3**, is also a good illustration of the effectiveness of the CR modified column for separation of similar analytes (**Fig. 6a**, **6b**, **6c**). In this work, five sulfonamides were separated by using HAc-NaAc /methanol/acetonitrile as the mobile phase. However, when utilizing water instead of the HAc-NaAc buffer, excessive retention was observed. Even after 150 minutes, the separation was incomplete. Furthermore, sulfamethoxazole and sulfadimethoxine (peak 3 and 4) had the same retention times (**Fig. 6c**). The differential properties of the solvent mixture vs. water also affected the separation of sulfonamides (**Fig. 6a**, **6b**). When HAc-NaAc was added in the buffer solution, the retention time was significantly decreased. Also, the retention time of the analytes was decreased with increasing of buffer salt concentration. Another result was that the resolution of sulfamethoxazole and sulfadimethoxine (peak 3 and 4) was obviously increased. This can be attributed to the restrained

ionization of sulfonic acid at a relatively low pH. It is also possible that variations in ionic strength of solvents could accelerate or alter the elution time. In comparison, this phenomenon was not observed with ODS column (**Fig. 6d** and **6e**). The peaks 4 and 5 were broad and tailing strongly in the absence of acidic surroundings, which indicates that the retention of analytes was hardly affected on ODS by the acidic medium and the ionic strength of solvents.

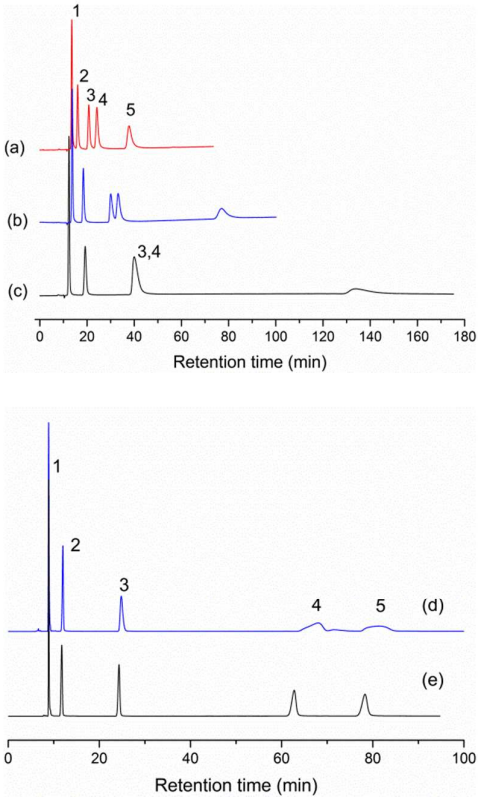


Fig. 6 Separation five sulfonamides: 1) sulfaguanidine, 2) sulfadiazine, 3) sulfamethoxazole, 4) sulfadimethoxine, 5) sulfachinoxalin with (a) methanol/acetonitrile/4 mM HAc-NaAc (20:5:75, v/v/v; pH = 5.4), (b) methanol/acetonitrile/1 mM HAc-NaAc (20:5:75, v/v/v; pH = 5.8) and (c) methanol/acetonitrile/water (20:5:75, v/v/v; pH = 6.8) with Sil-CR. Column: 50 cm ×100 μm, 22 cm effective length; Pump flow: 0.03 mL/min, λ = 254 nm; (d) methanol/water(30:70, v/v; pH = 6.6) and (e) methanol/5 mM HAc-NaAc (30:70, v/v; pH = 4.8) with ODS. Column: 45 cm ×100 μm, 20cm effective length; Pump flow: 0.05 mL/min, λ = 254 nm.

3.2.2 Sil-CR used as a stationary phase in HILIC mode

This sulfonic-azobenzene stationary phase was not only used in RPLC, as described above, but also used in HILIC to separate nucleoside bases including thymine, adenine, and cytosine. Due to the sulfonic and other polar moieties of this stationary phase, it is suitable for application in HILIC mode. The chromatographic separation was performed in a highly organic mobile phase. From the results (**Fig. 7**), we can see thymine, adenine and cytosine are separated in this nano-flow HPLC column with different proportions of 20 mmol L^{-1} ammonium acetate /acetonitrile as the eluent. The retention of adenine and cytosine increased with an increasing concentration of acetonitrile. It was not until the acetonitrile concentration was increased to 90%, that the retention of cytosine was higher than that of adenine. In this case, adenine and cytosine can be baseline separated (**Fig. 7c**). Upon continued increase of the acetonitrile concentration in mobile phase, adenine and cytosine demonstrated stronger retentions and separation.

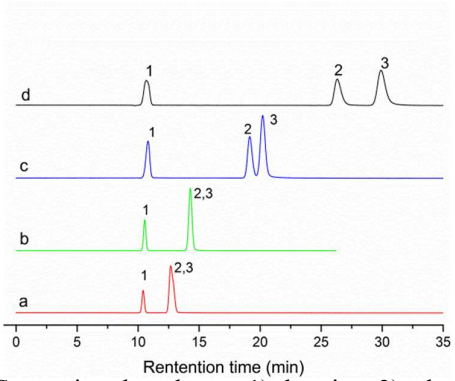


Fig. 7 Separation three bases: 1) thymine, 2) adenine, 3) cytosine with (a)20 mmolL^{-1} ammonium acetate/acetonitrile (30:70, v/v)and (b) 20 mmolL^{-1} ammonium acetate/acetonitrile (20:80, v/v) and (c) 20 mmolL^{-1} ammonium acetate/acetonitrile (10:90, v/v) and (d) 20 mmolL^{-1} ammonium acetate/acetonitrile (5:95, v/v). Column: 50 cm ×100 μm, 22 cm effective length; Pump flow: 0.04 mL/min, λ = 254 nm.

In another example, separation of four types of biogenic amines (BA) was evaluated with 20 mmol L^{-1} ammonium acetate/acetonitrile as the mobile phase. As shown in Fig. 8, both retention and separation increased concomitantly with an increased proportion of acetonitrile in the mobile phase. The chemical polarity order for the biogenic amines, from strong to weak, for the biogenic amines is: norepinephrine bitartrate, dopamine, tyramine and tryptamine. However, the order of retention for these compounds is just the reverse. This is a classic example of HILIC separation of polar compounds which clearly illustrates the utility of the sulfonic-azobenzene stationary phase in HILIC mode.

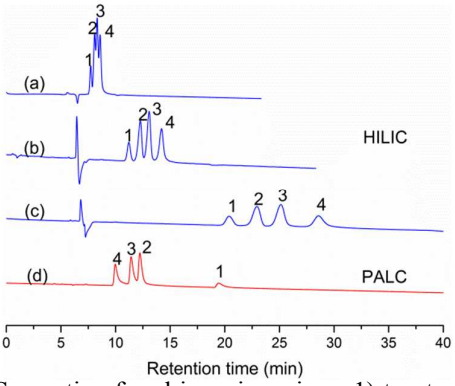


Fig. 8 Separation four biogenic amines: 1) tryptamine, 2) tyramine, 3) dopamine and 4)norepinephrine bitartrate with (a)20 mmolL^{-1} ammonium acetate/acetonitrile/HAc (30:70:0.1, v/v/v); (b) 20 mmolL^{-1} ammonium acetate/acetonitrile/HAc (20:80:0.1, v/v/v); (c) 20 mmolL^{-1} ammonium acetate/acetonitrile/HAc (15:85:0.1, v/v/v) and (d)20 mmolL^{-1} ammonium acetate. Column: 50 cm ×100 μm, 22 cm effective length; Pump flow: 0.05 mL/min, λ = 230 nm

3.2.3 Sil-CR used as a stationary phase in PALC mode

PALC[41, 42] is an alternative to hydrophilic interaction chromatography, and is complementary to reversed-phase chromatography. Due to both economic and environmental reasons, PALC is usually used for the separation of polar and hydrophilic substances. However, our Sil-CR stationary phase can also be used in PALC mode to separate polar and hydrophilic substances, such as four types of biogenic amines as shown in Fig. 8d. Here, 20 mmolL^{-1} ammonium acetate was used as the mobile phase. The retention orders of the samples were different in the two modes (PALC and HILIC). Another example was the separation of five polar and hydrophilic substances including dCMP, UMP, adenine, thymine and cytosine. All of these compounds were well separated by the Sil-CR column with 20 mmolL^{-1} ammonium acetate, as shown in **Fig. 9**. By comparison with **Fig. 7**, it can be seen that the results for adenine, thymine, and cytosine differed between the two chromatographic methods. It appears that two different retention mechanisms dominate for PALC with zero acetonitrile vs. HILIC with very high acetonitrile concentrations. These observations may be useful for the further exploration of this stationary phase in PALC chromatography.

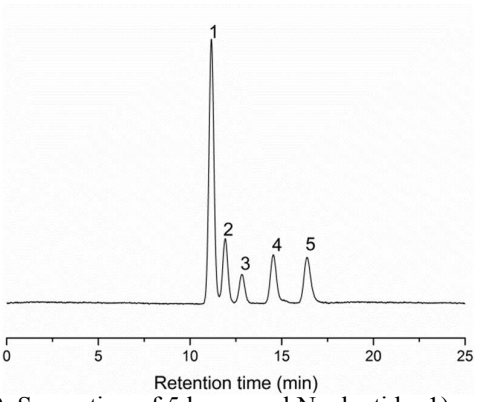


Fig. 9. Separation of 5 bases and Nucleotide: 1) cytosine, 2) adenine, 3) thymine, 4) dCMP and 5) UMP with 20 mmolL^{-1} ammonium acetate. Column: 50 cm ×100 μm, 22 cm effective length; Pump flow: 0.04 mL/min, λ = 254 nm

4. Conclusion

In this work, CR was bonded on silica and packed in fused silica capillary for nano-flow HPLC. The material was used in mixed-mode chromatography, including both hydrophobic and hydrophilic chromatography, for several analytes using RPLC, HILIC and PALC. The effective separation of benzoic acid derivatives and sulfonamides shows that the CR bonded-silica exhibited a mixed-mode retention behavior including hydrophobic and ion-exchange interactions, which was different from the single mode displayed by ODS columns. The multiple modes of interaction make this column more powerful with versatility, reproducibility and selectivity for the separation of a wide range of compounds. Owing to the existence of multi-functionalized groups, it is foreseeable that this material can not only be used as a versatile chromatographic stationary phase, but also as a solid extraction adsorbent in other chemical fields.

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