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Imidazoline type stationary phase for hydrophilic interaction chromatography and reversed-phase liquid chromatography

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ABSTRACT

An imidazoline was prepared by solvent-free microwave-assisted organic synthesis and immobilized on porous silica particles by polymerization. The resulting material was composed of both hydrophobic alkyl ester chains and hydrophilic imidazoline rings, which gave it both hydrophilic interaction and reversed-phase characteristics. The titration curve suggests that the new material has buffering capacity and acquires increasing positive charge over the pH range 9–4, and is "zwitterionic" in the upper part of this pH range. Through investigating the effect of column temperature, the water content, pH and ion strength of mobile phase on the retention time of polar compounds in highly organic eluents, it was found that the new material could be used as a hydrophilic interaction liquid chromatography (HILIC) stationary phase which involved a complex retention process consisting of partitioning, surface adsorption and electrostatic interactions. In addition, the retention behavior of aromatic compounds in different mobile phase conditions was also studied, which showed the new material mainly exhibited a partitioning mechanism in the reversed-phase liquid chromatography (RPLC) mode. The separation of six water-soluble vitamins and five aromatic compounds were achieved by using the new material in the HILIC and RPLC modes, respectively.

1. Introduction

The separation of polar compounds and highly hydrophilic compounds is often challenging due to lack of retention on conventional reversed-phase columns [1–6]. Although normal phase liquid chromatography (NPLC) can be used, its eluents are non-polar and polar analytes usually have a low solubility in these eluents. This greatly limits its application [2,4,7–10]. HILIC has been used since 1975 for the analysis of sugar and oligosaccharides [11,12] and the abbreviation "HILIC" was suggested by Alpert in 1990 [8]. Similar to NPLC, polar compounds are more strongly retained on HILIC columns, but the non-aqueous mobile phase in NPLC is replaced by an aqueous–organic mixture with water as a strongly eluting solvent. This feature not only helps to eliminate the problem associated with low aqueous solubility, but also makes HILIC highly compatible with mass spectrometry (MS) and improves the MS sensitivity [2–4,13–15]. Therefore, researchers have paid attention to HILIC to study the separation of polar compounds in a wide variety of scientific fields and literature on HILIC has been increasing dramatically [1,2,4–7,13,16–21,30–34].

Separation materials for HILIC are usually polar, whether coated or not, mainly including bare-silica, amide-silica, amino-silica, sulfoalkylbetaine bonded silica, carbohydrate-modified silica, cyano- and diol-silica, triazol-bonded silica, and polymer-based monolithic columns [2,4,13,29]. Recently, mixed-mode separation materials have been reported [19,22–29]. They are operated in different chromatography modes based on different retention mechanisms. However, mixed-mode separation materials for HILIC are uncommon. Guo et al. [22] prepared a HILIC/RPLC mixed-mode "Click β-cyclodextrin" stationary phase. Liu and Pohl [24] developed a new stationary phase that combined both hydrophilic interaction and reversed-phase characteristics. The new phase consisted of hydrophobic alkyl chain and hydrophilic diol functionality. Ma et al. [19] successfully synthesized novel pH-responsive polymer-grafted silica whose hydrophilicity/hydrophobicity changes with pH. Lin et al. [28] prepared a hydrophilic/cation-exchange monolithic column which was dynamically modified with a cationic surfactant. The mixed-mode separation materials demonstrate flexibility and increase analyte coverage in some samples.

In this study, we have prepared a new imidazoline type stationary phase. Zwitterionic imidazoline molecules were immobilized by polymerization on silica surfaces, which effectively avoided residual silanols' irreversible adsorption of basic compounds when operating in RPLC mode [35,36]. The resulting material possesses

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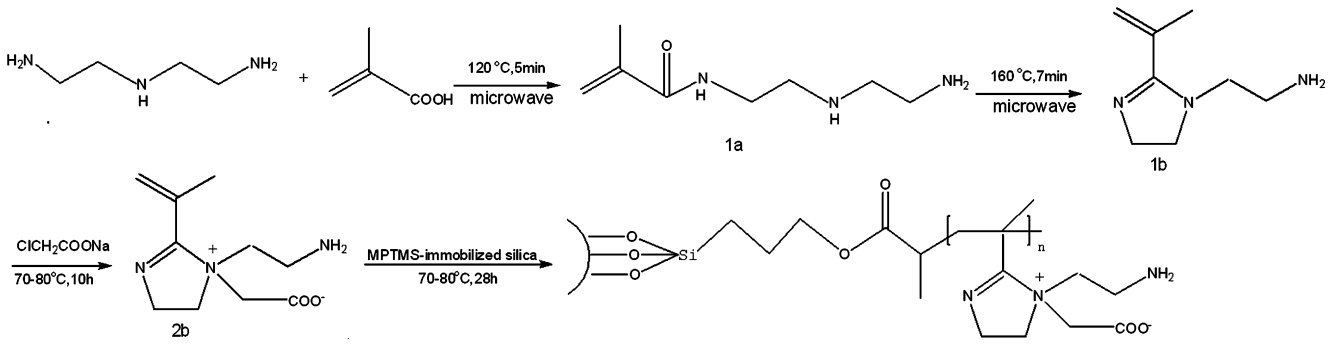


Fig. 1. The routes for synthesis of the new separation material.

both alkyl ester chains and imidazoline rings, which confers both HILIC and RPLC characteristics on the new phase. This feature has great potential in 2D-LC separation, in which just one column could be used in two different HPLC modes. Lastly, applications in the separation of water-soluble vitamins in HILIC mode and aromatic compounds in RPLC mode were demonstrated.

2. Experimental

2.1. Reagents and materials

Spherical silica (7 μm particle size; 10 nm pore size; 400 $m^2\ g^{-1}$ surface area) was purchased from Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences (Lanzhou, China). 3-(Methacryloyloxy)propyltrimethoxysilane (MPTMS) and sodium chloroacetate were obtained from Alfa Aesar (Karlsruhe, Germany). Diethylenetriamine (DETA) and p-dihydroxybenzene were purchased from Shanghai Chemical Reagents (Shanghai, China). Methacrylic acid (MAA), azobis(isobutyronitrile) (AIBN) and calcium oxide were obtained from the Tianjin Chemicals Corporation (Tianjin, China).

Vitamin B_1 (VB_1), vitamin B_2 (VB_2), vitamin B_6 (VB_6), vitamin C (VC), folic acid, nicotinic acid (VB_3), uracil, clenbuterol and salbutamol used as test probes in the HILIC mode were purchased from Sigma (St. Louis, MO, USA). Melamine was obtained from J&K Chemical Ltd. (Beijing, China). Phenylamine, acetophenone, benzene, toluene and dimethylbenzene used as test probes in the RPLC mode were purchased from Tianjin Guangfu Chemical Reagent Co. (Tianjin, China). Acetonitrile of HPLC grade was from Dima Technology (Richmond Hill, ONT, Canada). All other reagents were of analytical-reagent grade (Tianjin Chemicals, China) and purified water from a Milli-Q system was used throughout the experiments.

2.2. Synthesis of imidazoline

20 mL DETA, 15 mL MAA, 0.003 g p-dihydroxybenzene and 3.6 g calcium oxide were added to a three-necked flask equipped with a stirrer and a temperature probe and were carefully mixed. After 30 min stirring, the mixture was irradiated by microwave at 160 °C for 7 min. Then the reaction mixture was allowed to cool to room temperature. Ethanol was added to the obtained mixtures and heated until boiling and filtered off while hot. The filtrate was concentrated under vacuum to dryness, yielding a yellowish brown semisolid product.

2.3. Preparation of quaternary imidazoline

2.0 g imidazoline was dissolved in 25 mL ethanol, and 1.5 g sodium chloroacetate was added to the solution. The reaction mixture was refluxed with constant stirring for 10 h, then was filtered and the filtrate was concentrated under vacuum to dryness, yielding the corresponding quaternary imidazoline.

2.4. Immobilization of MPTMS on silica surfaces

The silica (2.0 g) was suspended in 30 mL anhydrous toluene, and 2 mL MPTMS was added with stirring. The reaction mixture was heated under reflux and a N_2 atmosphere at 95–100 °C for 20 h. Then the obtained MPTMS-bonded silica was filtered and intensively washed with dichloromethane, acetone and methanol, respectively, and then dried under vacuum at 60 °C overnight.

2.5. Polymerization

The MPTMS-bonded silica (1.0 g) and quaternary imidazoline (1.0 g) were added to 30 mL ethanol, and the polymerization was performed with the initiator AIBN (1.0 wt.% of monomer) under a N_2 atmosphere at 70–80 °C for 28 h. The final product was filtered, intensively washed with dichloromethane, acetone and methanol, respectively, and then dried under vacuum at 60 °C overnight.

The routes for synthesis of the new separation material are shown in Fig. 1.

2.6. Titration of the new stationary phase [37,38]

Reagents were prepared with purified water from which carbon dioxide had been eliminated. Twenty seven milligrams of the new stationary phase to be titrated was placed in a beaker and suspended with stirring in 5 mL of 1.0 M sodium chloride. The suspension was adjusted pH to 2.0 and titrated with 0.01 M sodium hydroxide. The pH was then measured with a Leici pH Meter PHSJ-3F (Shanghai, China).

2.7. Instruments and chromatographic evaluation

The microwave oven used for this research was made by Sineo Microwave Chemical Technology Co. Ltd. (Shanghai, China). 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. 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).

The new separation material was slurry-packed into a 150 mm × 4.6 mm I.D. stainless steel column with methanol as slurry medium and a tetrachloromethane-methanol mixture was used as the packing solvent at 6000 psi pressure. The chromatographic evaluations were carried out at room temperature (18 ± 2°C). The flow rate was 1.0 mL/min. A set of test probes (melamine, VB_1, VB_2, VB_6, VC, folic acid, VB_3, uracil, clenbuterol and salbutamol) in

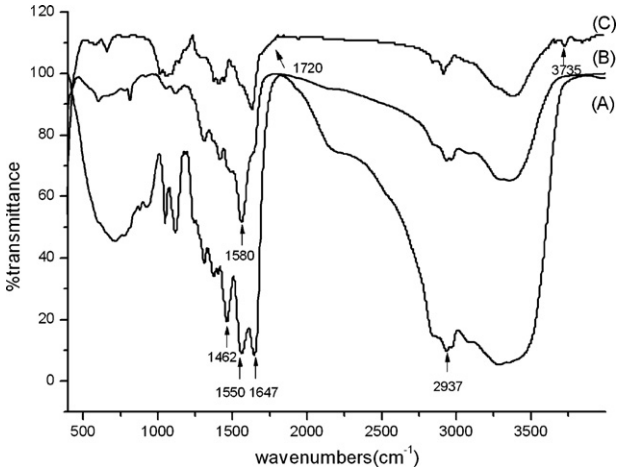


Fig. 2. FT-IR spectra of the amide1a (A), imidazoline 1b (B) and quaternary imidazoline 2b (C) in Fig. 1.

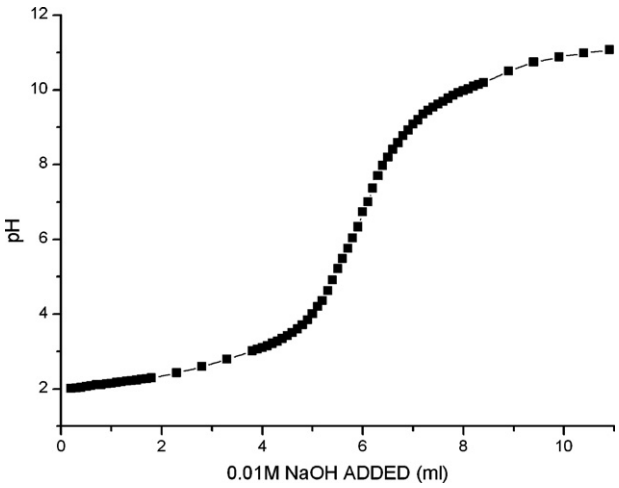


Fig. 3. Titration of the new material.

the HILIC mode with a concentration of 20 μg/mL was prepared in water/acetonitrile (1/1, v/v). A set of five aromatic standards (phenylamine, acetophenone, benzene, toluene and dimethylbenzene) for the RPLC mode with a concentration of 10 μg/mL was prepared in methanol/water (1/1, v/v). The dead time was 1.5 min, which was determined by injecting 5 μL methanol with water/acetonitrile (1/1, v/v) as mobile phase [17]. Each measurement was replicated three times.

3. Results and discussion

3.1. Preparation and characterization of imidazoline stationary phase

During the last decade, compared to conventional heating, as a way of minimizing safety problems microwave-assisted organic synthesis has received a great deal of attention due to shorter reaction time, minimization of reaction by-products, increased yields, and in many cases solvent-free conditions [39–42].

We synthesized imidazoline in solvent-free conditions using CaO as support in a microwave, according to the method reported in the literature [39–42]. Reaction was monitored by FT-IR. When the reaction mixture was irradiated with microwaves 5.0 min at 120 °C, only traces of the amide (1a) were detected. As can be seen from Fig. 2(A), peaks at 1647 and 1550 cm^{-1} were the absorption peaks of the amide. Other peaks were 3300–3280 (ν_{N-H}), 2937 (ν_{C-H}), 1462 (ν_{C-N}) cm^{-1}, respectively. Similarly, the reaction was also carried out under microwave irradiation 7.0 min at 160 °C. The amide peaks at 1647 and 1550 cm^{-1} completely disappeared, and a strong peak appeared at 1580 cm^{-1} that belonged to the absorption of C=N of the imidazoline ring (Fig. 2(B)). A FT-IR spectrogram of the quaternary imidazoline (2b) (Fig. 2(C)) showed that peaks from C=O and O–H stretching at 1720 and 3735 cm^{-1} appeared. The structures of the synthesized imidazoline (1b) and quaternary imidazoline had been identified.

After immobilization of MPTMS on silica surfaces, its FT-IR spectrogram was also measured. Absorption bands at 1730 and 1640 cm^{-1} were attributed to C=O and C=C stretching. The weak band at 3736 cm^{-1} belonged to residual silanols stretching. The result indicated that MPTMS had been immobilized on silica surfaces. The stronger absorption peak at 1640 cm^{-1} in the FT-IR spectrogram of the resulting new material was evidence of the imidazoline ring. The weak band at 3736 cm^{-1} had completely disappeared, indicating that residual silanols had been covered.

The results of elemental analysis of immobilization of MPTMS (N: 0; C: 9.52%; H: 1.55%) and quaternary imidazoline (N: 1.98%; C: 15.70%; H: 3.00%) on silica surfaces clearly indicated that the new phase had been prepared successfully.

Ionizable groups on the new stationary phase in suspension can be titrated and the results are seen in Fig. 3. The new stationary phase exhibited a broad range of ionization, buffering between pH 4 and 9. The carboxylic acid ligands in the new phase lose their (−) charge and the material acquires more net positive charge with decreasing pH. The new material is "zwitterionic" only in the higher part of this pH range when the carboxyl- groups are fully ionized.

3.2. Retention properties in the HILIC mode

3.2.1. The effect of water content on retention

Mobile phase strength in the eluent is probably the factor that has the largest effect on retention of polar compounds, because hydrophilic interaction is enhanced by decreasing the polarity of the eluent. Six polar compounds, VB_1, VB_2, VB_6, VB_3, folic acid, and melamine that were difficult to retain and separate by RPLC, were selected as test probes to investigate the HILIC properties of the new phase. A range of 5–30% water in mobile phases was studied while keeping ammonium formate concentration constant at 10 mM with pH 3.0. The pH of the aqueous phase was adjusted with formic acid. The logarithmic capacity factors (k') of six test probes were plotted against the volume fraction of water in the eluent as shown in Fig. 4(A). The retention times were observed to be proportional to the water content in the eluent, characteristics typical of HILIC. Hence, retention at high levels of organic solvents reflected the hydrophilic nature of the new phase.

Alpert [8] 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. According to McCalley and Neue [43], about 4–13% of the pore volume of a silica phase was occupied by a water-rich layer when using acetonitrile–water containing 95–70% (v/v) acetonitrile. But the final retention mechanism is most probably a complex process of partitioning and electrostatic interactions or hydrogen bonding to the stationary phase [44,45]. The relationship that is established for partitioning can be described in Eq. (1).

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

where k'_w is the capacity factor for the weaker eluent component only as mobile phase, φ is the volume fraction (concentration) of

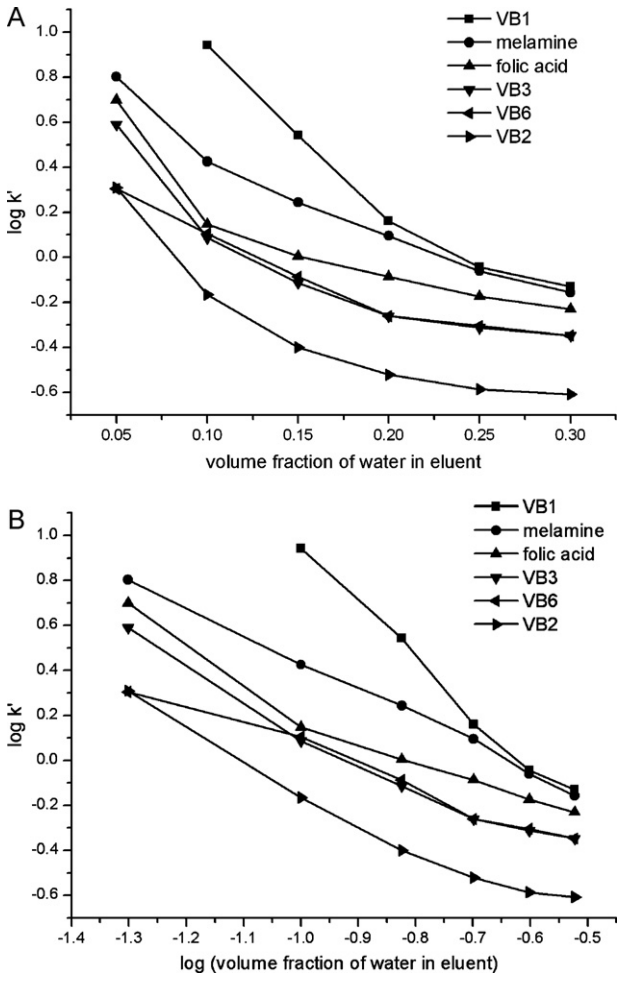


Fig. 4. (A) Plot of log k′ versus the water volume fraction in eluent. (B) Plot of log k′ versus logarithm of the water volume fraction in eluent. Mobile phase contained 10 mM ammonium formate, pH 3.0, UV detection: 260 nm.

the stronger member of a binary mobile phase mixture, and S is the slope of $\log k'$ versus φ when fitted to a linear regression model.

If retention is based on surface adsorption, the relationship can be described in Eq. (2).

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

where k' 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 [45–47].

In order to obtain more insight into the mechanism of the new stationary phase, we made an attempt to plot $\log k'$ versus the linear and logarithmic function of the volume fraction of water in eluent according to Eqs. (1) and (2) to assess whether the mechanism actually was due to partitioning or adsorption (Fig. 4). The linear relationship for melamine was only found in the log–log plot ($R^2 = 0.9976$). As shown in Fig. 4 (A) and (B), between 5 and 20% water, the adsorption model seemed to fit better except for VB_6, but between 20 and 30% water the partition model seemed to fit better. At pH 3.0, VB_6 has a net (+) charge and would presumably be repelled electrostatically by the stationary phase, which would have two net (+) charges per residue at that pH. So the nature of analytes might affect the types of retention mechanisms in HILIC. Discontinuous retention patterns were observed on the new phase and it was difficult to draw a conclusion about it manifesting a unique retention mechanism. It might be a complex retention process composed of partitioning of solutes between a surface water layer and the bulk mobile phase, surface adsorption and electrostatic interactions with the imidazoline molecule in HILIC.

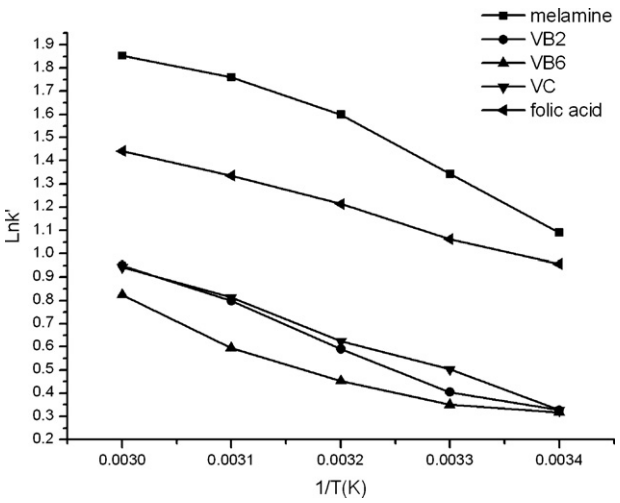


Fig. 5. Effect of column temperature on retention. Mobile phase: acetonitrile/water (95/5, v/v) containing 10 mM ammonium formate; UV detection: 260 nm.

3.2.2. The effect of column temperature on retention

Column temperature is also an important factor that has great influence on the retention of polar compounds in HILIC. The relationship between capacity factor (k') and column temperature (T) in RPLC is described by the van't Hoff equation [48]:

$$\ln k' = \frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln$$

where k' is the retention factor for the solute, ΔH° is the standard partial molar enthalpy of transfer, ΔS° is the standard partial molar entropy of transfer, R is the gas constant, T is the absolute temperature, and φ is the phase ratio, respectively. In this study, the retention data for the model compounds were used to construct van't Hoff plots with column temperature varying from 20 to 60 °C. The van't Hoff plots (Fig. 5) showed retention decreased for all test probes with an increase in column temperature. Since the k' value is strongly dependent on the column temperature, an increase in column temperature could lead to a decrease in the value of k' by changing the partition coefficient of the analyte between the mobile phase and the immobilized aqueous layer.

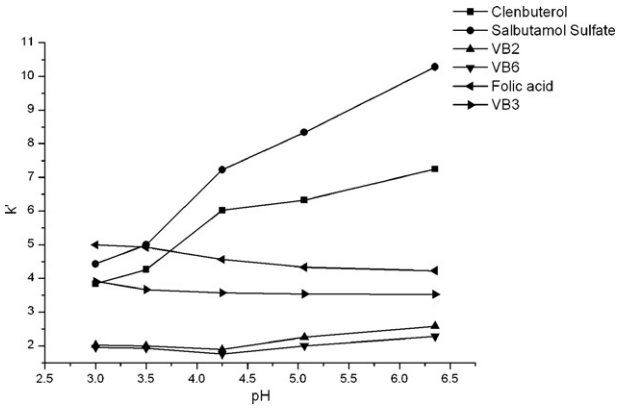


Fig. 6. Effect of pH on retention. Mobile phase: acetonitrile/water (95/5, v/v) containing 10 mM ammonium formate; UV detection: 260 nm.

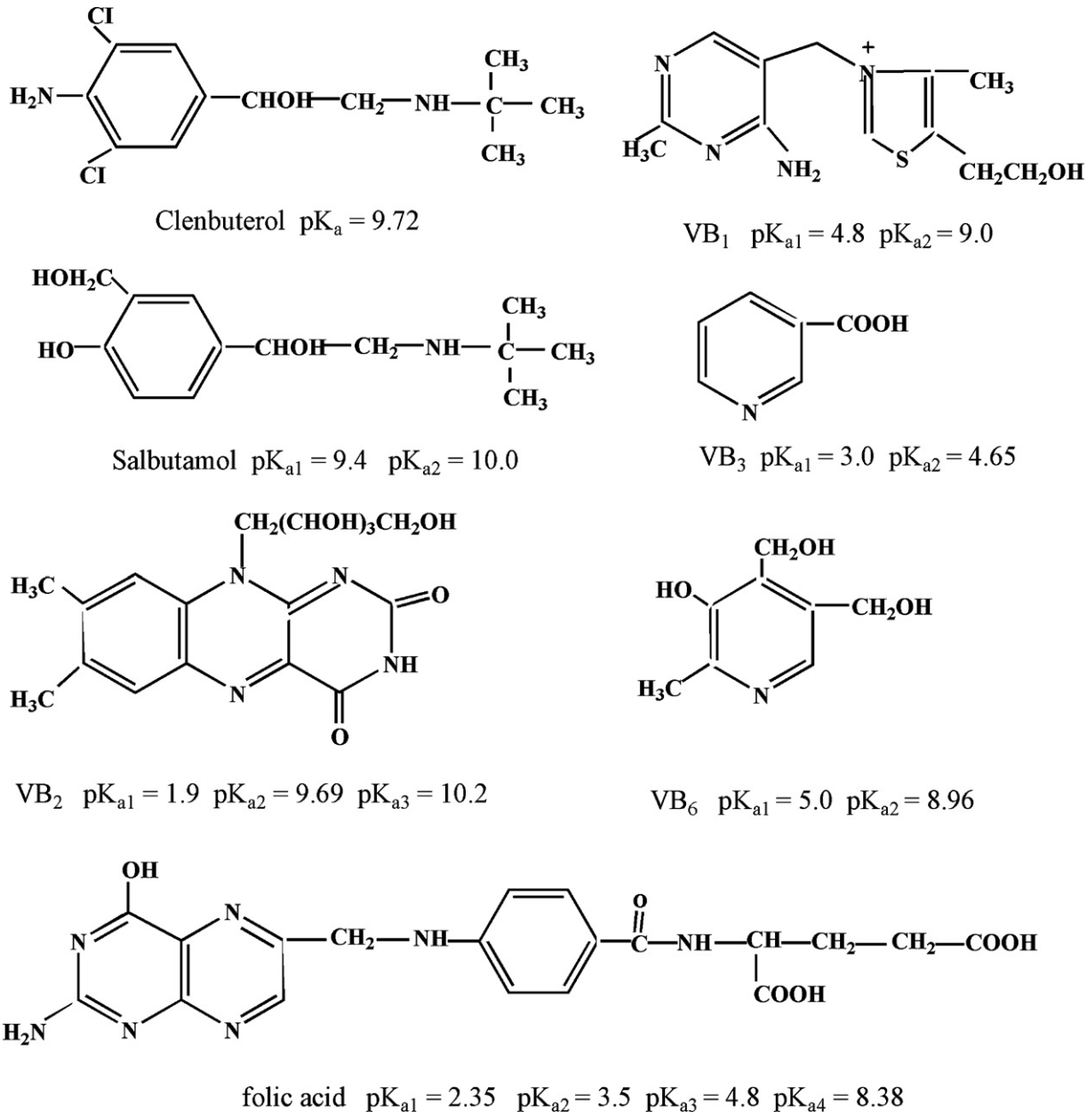


Fig. 7. Structures and p*K*a values of seven polar compounds.

3.2.3. The effect of buffer pH on retention

Mobile phase pH also plays an important role by influencing solute ionization in HILIC. Ammonium formate buffer was selected to investigate the effect of mobile phase pH by adjusting the pH of ammonium formate aqueous solutions to 6.3, 5.1, 4.3, 3.5 and 3.0 before mixing with ACN while keeping the concentration of ammonium formate constant at 10 mM (Fig. 6). Structures and p*K*a values of seven compounds can be seen in Fig. 7 [49–53]. The retention times for clenbuterol and salbutamol significantly decreased when buffer pH was decreased. The p*K*a values of clenbuterol and salbutamol are greater than 9. They will have a full (+) charge below pH 8, so their ionization state will not change over the pH range studied. The change in their retention time is due to a change in the charge state and density of the stationary phase. Within that pH range, the carboxylic acid ligands in the new phase will lose their (−) charge and the (+) charge density on the new phase will increase when the buffer pH is decreased from 6.3 to 3.0, thus leading to increased electrostatic repulsion between the protonated clenbuterol or salbutamol molecules and the positively charged ligands on the derivatized silica surfaces. The retention of VB_6 decreased with pH too. The possible reason was that both VB_6 and the stationary phase acquired more positive charge when pH was decreased, generating electrostatic repulsion. For VB_2 with pK_{a1} ~ 1.9, its ionization did not change significantly in the pH range studied, so the retention time remained essentially unchanged. As for VB_3, it started with (−) charge and became zwitterionic with decreasing pH, pretty much paralleling the change in net charge on the stationary phase. Therefore there was little change in its retention too. For folic acid with pK_a ~ 3.5 and 4.8, it acquired more (−) charge and the stationary phase acquired more (+) charge as the pH was decreased, generating electrostatic interaction, thus leading to an increase in retention.

3.2.4. The effect of ionic strength of mobile phase on retention

Ionic strength of mobile phase can also have influence on the retention of polar compounds in HILIC [54]. In this study, we

Table 1
Retention time[b] of the model compounds at different ammonium formate concentrations in the mobile phase[a].

Concentration (mM)	Uracil	Salicylic acid	VB_3	VB_2	VB_6
5	2.13 ± 0.011	2.15 ± 0.013	3.18 ± 0.009	3.20 ± 0.021	3.11 ± 0.026
10	2.13 ± 0.020	2.59 ± 0.018	3.20 ± 0.017	3.21 ± 0.015	3.25 ± 0.033
15	2.55 ± 0.015	2.77 ± 0.022	3.36 ± 0.025	3.32 ± 0.031	3.33 ± 0.022

[a] Mobile phase: acetonitrile/ammonium formate solution (90/10, v/v); pH 6.3; UV detection: 260 nm.
[b] Retention time (min) ± standard deviation (n = 3).

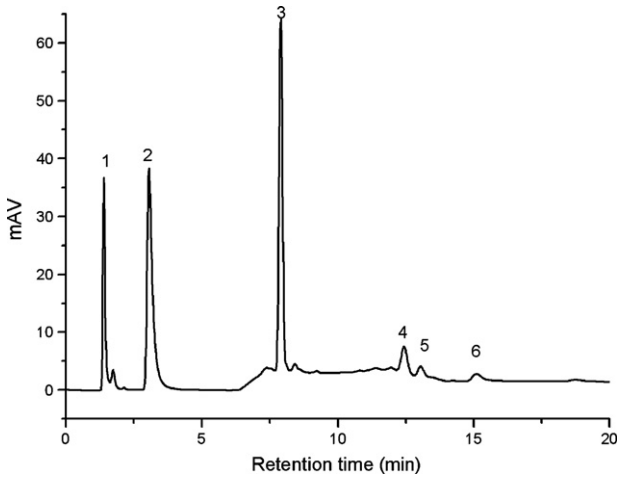


Fig. 8. Separation of vitamins on imidazoline column. Conditions: mobile phase A, acetonitrile; mobile phase B, 10 mM ammonium formate aqueous solution; gradient: 0–5 min 20% → 10% B, 5–15 min 10% → 5% B, 15–20 min 5% B; UV detection: 260 nm. Solutes: (1) VC, (2) VB_6, (3) VB_2, (4) folic acid, (5) VB_3, (6) VB_1; UV detection: 260 nm.

selected five compounds (uracil, salicylic acid, VB_3, VB_2 and VB_6) as test probes to investigate the effect of ionic strength on retention time at pH 6.3 with ammonium formate concentration at 5 mM, 10 mM and 15 mM, respectively (Table 1). The retention time increased 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 6.3 and with low salt concentration. Higher salt concentrations may have weakened any electrostatic repulsion, leading to the stronger retention of the polar solutes [3,15,55,56].

3.2.5. Separation of water-soluble vitamins

The separation of some water-soluble vitamins was achieved in the HILIC mode with a mixture of acetonitrile – 10 mM ammonium formate buffer solution as mobile phase at pH 6.3. Their separation chromatogram is shown in Fig. 8. The new phase shows good separation selectivity for vitamins.

3.3. Chromatographic behaviors in the RPLC mode

We plotted log k′ versus the linear and logarithmic function of the volume fraction of water in the eluent according to Eqs. (1) and (2) for investigating the mechanism in the RPLC mode. Structures

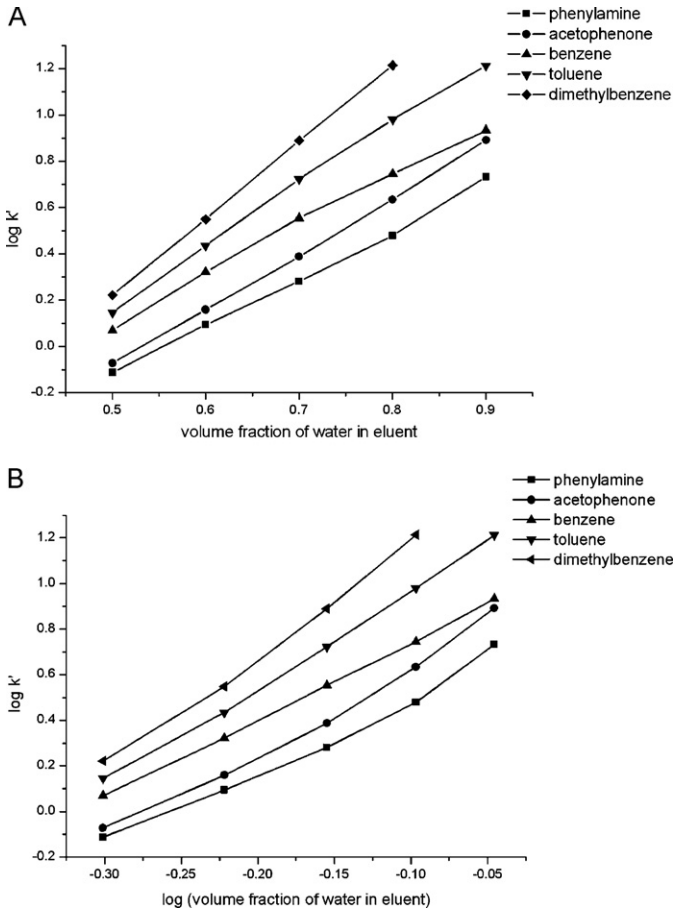
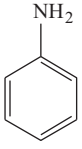
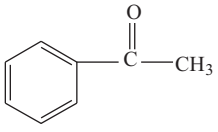
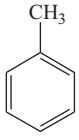
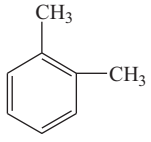


Fig. 9. (A) Plot of log k' versus the water volume fraction in eluent. (B) Plot of log k' versus logarithm of the water volume fraction in eluent. UV detection: 254 nm.

of five aromatic compound standards are summarized in Table 2. As can be seen from Fig. 9(A), between 50 and 90% water, the partition model fit better for phenylamine, acetophenone, benzene, toluene and dimethylbenzene (R^2 = 0.9968, 0.9993, 0.9955, 0.9978 and 0.9999), respectively. Moreover, the slope increased with decreasing polarity of the solutes (polarity order: dimethylbenzene < toluene < benzene< acetophenone < phenylamine), which indicated stronger hydrophobic interaction as the number of available polar sites decreased in the solutes. The fit was not consistent with an adsorptive process in Fig. 9(B). Accordingly,

Table 2
Structures of five aromatic compound standards.

Compound	Phenylamine	Acetophenone	Benzene	Toluene	Dimethylbenzene
Structure					

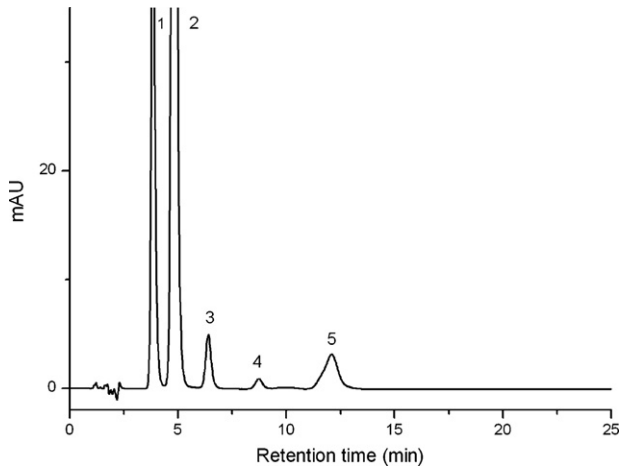


Fig. 10. Separation of aromatic compounds on imidazoline column. Mobile phase: ACN/10 mM ammonium formate aqueous solution (30/70, v/v); UV detection: 254 nm. Analytes: (1) phenylamine, (2) acetophenone, (3) benzene, (4) toluene, (5) dimethylbenzene.

retention in the RPLC mode was probably based on a partitioning mechanism.

The separation of the same aromatic compounds was achieved in the RPLC mode. Their separation is shown in Fig. 10. The new phase exhibited good separation of these aromatic compounds. The orthogonality of HILIC and RPLC was also evident with the new phase. As shown in Fig. 11, the elution order of benzene (a hydrophobic non-polar molecule) and melamine (a highly polar molecule) could be reversed in HILIC and RPLC. Due to the nonpolarity of benzene, it eluted in the dead time in the HILIC mode. This result suggests that the new phase could be used for both RPLC and HILIC applications.

Compared with previously reported phases with the same properties, the results here are consistent, for example, the retention behaviors of toluene in the mode of RPLC and hydrophilic compounds in HILIC mode. Ion-exchange is weaker here than that in the literature [22]. The differences between this new phase and existing ones lie in different hydrophilic and hydrophobic moieties with HILIC/RP properties, which led to the different applications. The "Click β-cyclodextrin" stationary phase prepared by Guo et al. [22] has not only hydrophilic/hydrophobic properties but also chiral separation capacity. The utility of this new material in various practical applications will be studied in the future.

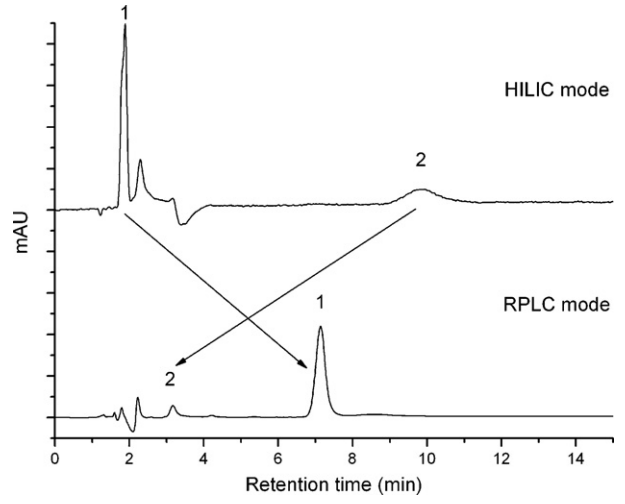


Fig. 11. Dual operation modes:RPLC and HILIC. Mobile phase: acetonitrile/water, 95/5 and 30/70 (v/v) for HILIC and RPLC modes, respectively; UV detection: 236 nm. Peaks: (1) benzene and (2) melamine.

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