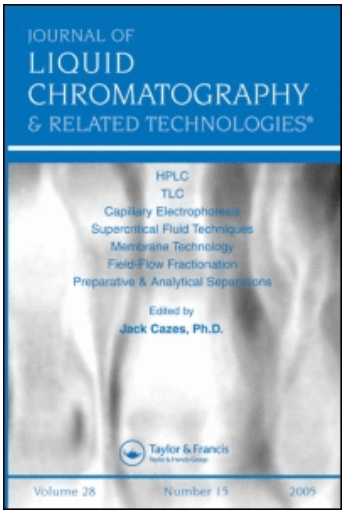

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A Bidentate Amino Stationary Phase for Hydrophilic Interaction Chromatography

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Abstract: A bidentate amino stationary phase (SG-EDA) for hydrophilic interaction chromatography (HILIC) was synthesized through Michael addition with methyl acrylate (MA) to aminopropyl silica and amidation to the resulting esters with ethylenediamine (EDA). FTIR and element analysis measurements were carried out to confirm the successful synthesis of the SG-EDA phase. The chromatographic characteristics of the SG-EDA phase were investigated by exploring the retention behaviors of different types of probes including naphthalene, acrylamide, berberine, and p-hydroxybenzoic acid. It revealed that the SG-EDA phase demonstrated a typical HILIC characteristic. To explore the retention mechanism of the SG-EDA phase, the retention behaviors of four organic acids were investigated by varying the pH of mobile phases, organic solvent content, and ionic strength. The results disclosed that hydrophilic interaction contributed to their retention at high acetonitrile content while ion exchange played a more important role. Finally, a comparison of separation for four organic acids was obtained with an aminopropyl silica phase (SG-NH_2) and SG-EDA phase.

Keywords: Aminopropyl stationary phase (SG-NH_2), Bidentate amino stationary phase (SG-EDA), Hydrophilic interaction, Hydrophilic interaction chromatography (HILIC), Ion exchange interaction, Organic acids

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INTRODUCTION

Liquid chromatography has been widely used for the separation of various compounds from non-polar compounds to polar compounds. However, until now the separation of highly polar compounds is still a difficult task. Normal phase liquid chromatography (NPLC) has proven to be a very useful separation technique for providing effective retention for polar molecules. However, generally NPLC is inapplicable for these analytes due to their poor solubility in organic solvents.[1]

Hydrophilic interaction chromatography (HILIC), which was first introduced by Alpert in 1990,[2] allows the use of aqueous mobile phases in normal phase mode, rendering itself an extremely potent method for analysis of polar solutes. The most important characteristic of HILIC lies in that the mobile phase contains massive polar organic solvent rather than water.[2] In such a case, the retention of analytes increases as their polarity increases while they decrease as the polarity of mobile phase increases.[2] Therefore, in contrast to reversed phase liquid chromatography (RPLC), polar compounds have stronger retention than non-polar compounds in HILIC mode.[3]

The mechanism of HILIC has not been definitely clarified due to the complex interactions such as ion exchange, hydrophobic interaction, etc. Several publications have explored HILIC retention mechanisms[4–7] and partition has been considered to be the most popular one. Nevertheless, ion exchange interaction has also been reported as a secondary retention mechanism[8–11] under some particular conditions. Despite the complexity of the mechanism, the technique is simple in practice and its general advantages can be summarized as follows: (1) it is particularly suitable for the separation of highly polar substances;[3] (2) it can enhance the sensitivity in mass spectrometry because of the high content of organic modifier in mobile phase;[12,13] (3) it can simplify sample preparation procedures;[1] (4) high flow rates are possible due to the low viscosity of typical mobile phases containing high organic content.[14] Therefore, HILIC has been applied in a broad range of applications such as carbohydrates, amino acids, peptides, proteins, oligonucleotides, natural product extracts, and small drug molecules and metabolites.[15]

In general, silica based packing materials are commonly used HILIC stationary phases. To date, a variety of silica based packings have been developed for HILIC, such as bare silica,[12,16] silica modified with polyols,[17] cyano,[18] amide,[2] or ion exchange groups.[18,19] Nevertheless, it is of great importance to explore some novel phases to expand the application of HILIC.

Here, a bidentate amino stationary phase (SG-EDA) was synthesized for this purpose. The HILIC retention mechanism was explored by investigating the retention behavior of several probes under different

conditions. Finally, the HILIC separation of organic acids on this stationary phase was discussed. For comparison, their separation on aminopropyl silica phase (SG-NH_2) was also carried out.

EXPERIMENTAL

Reagents and Materials

Spherical silica (5–7 μm) was home-made.[20] 3-Aminopropyltrimethoxysilane was purchased from Wuhan University Silicone New Material (Wuhan, China). Methanol, acetone, and acetonitrile were purchased from Shanghai General Chemical Reagent Factory (Shanghai, China). Benzoic acid, sorbic acid, acrylamide, p-hydroxybenzoic acid, o-aminobenzoic acid, methyl acrylate (MA), ethylenediamine (EDA), and ammonium formate were purchased from Sinopharm Chemical Reagent (Shanghai, China). Naphthalene, berberine, and other reagents were all obtained from various commercial sources and were analytical grade unless otherwise indicated. Double distilled water was used for all experiments.

Preparation of Stationary Phase

The synthesis of SG-NH_2 and SG-EDA were illustrated in Figure 1.[21] All reactions were carried out under nitrogen. First, 4.0 g silica was suspended in 50 mL of anhydrous toluene under stirring. To it, 2.06 g 3-aminopropyltrimethoxysilane was added drop by drop. The mixture was then kept at 110°C for 12 h under magnetic stirring to obtain

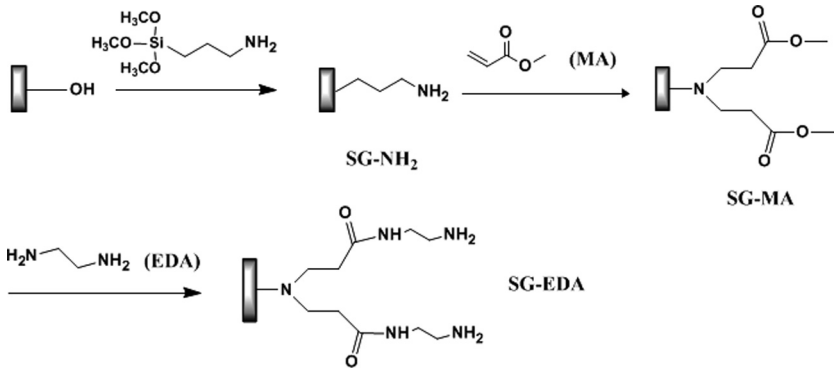

Figure 1. Preparation of SG-NH_2 and SG-EDA phase.

$SG\text{-}NH_2$. The obtained $SG\text{-}NH_2$ was washed with toluene, acetone, and methanol, respectively. To acquire SG-EDA, MA was covalently bonded with the amino groups on the $SG\text{-}NH_2$ through Michael addition. Thereafter, EDA was used for amidation of terminal groups in the same manner. The brief procedure was as follows: 1.5 mL MA, 2.0 g dried $SG\text{-}NH_2$, and 25 mL methanol were mixed and refluxed at 50°C under magnetic stirring for 12 h. The resulting solid was washed with methanol to remove the excess MA and then dried in vacuum at 60°C. Then EDA (2.0 mL) was used for amidation of terminal groups on the dried SG-MA at 50°C under magnetic stirring for 12 h. Finally, the resulting solid was washed with methanol and dried in vacuum at 60°C to obtain SG-EDA.

Instrumental and Chromatographic Procedures

The HPLC apparatus purchased from Shimadzu (Kyoto, Japan) was composed of two LC-20AD pumps, a SPD-20A ultraviolet detector, a Rheodyne 7725i injector with 20 μL sample loop, and a LGC-1025 M thermostat. The chromatographic data were acquired by Shimadzu LC Solution (Kyota, Japan). The FTIR instrument was Thermo Nicolet AVTAR-360 (Madison, USA). Element analysis was performed on an Elementar vario EL III Universal CHNOS Elemental Analyzer (Germany).

$SG\text{-}NH_2$ and SG-EDA were packed into a 150×4.6 mm (i.d.) stainless steel column with isopropanol as the slurry liquid and methanol as the eluent, respectively. A mixture solution of acetonitrile and ammonium formate buffer was used as the mobile phases. Prior to use, the mobile phases were filtered through a G-4 fritted glass funnel and degassed in an ultrasonic bath for about 5 min. The measured pH refers to the pH of prepared aqueous mobile phase. The flow rate was set at 1 mL/min. The wavelength used for detection was 254 nm. All measurements were carried out at 30°C and repeated at least twice.

RESULTS AND DISCUSSION

Characteristic of Stationary Phase

FTIR and element analysis measurements were carried out to confirm the successful preparation of the novel stationary phase (SG-EDA). The IR spectra (Figure 2) show that there is a strong peak in 1000–1200 cm^{-1}, corresponding to the stretching of Si-O groups. Two distinct peaks in the region of 1600−1700 cm^{-1} arose from C=O stretching. The peaks in 2800–2900 cm^{-1} and 3440 cm^{-1} are ascribed to the C-H stretching

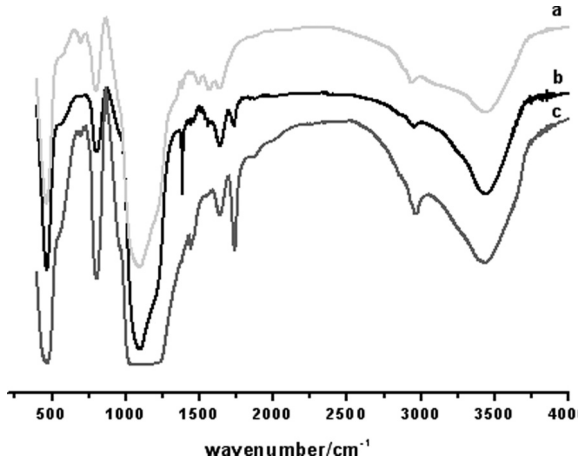

Figure 2. IR spectra of (a) SG-NH_2; (b) SG-EDA; (c) SG-MA.

and N-H stretching. Table 1 lists the element ingredients of the bonded silica. An obvious increase in nitrogen content from SG-NH_2 to SG-EDA indicated that the surface modification was successful.

Effect of ACN Content

In HILIC mode, water and high content acetonitrile were often employed as the mobile phase to ensure significant hydrophilic interaction. Generally, the hydrophilic retention increased with increasing acetonitrile content. In order to investigate the HILIC properties of the SG-EDA phase, naphthalene, p-hydroxybenzoic acid, acrylamide, and berberine were used as the test compounds. The acetonitrile content in the mobile phases was varied from 90 to 5% while the ammonium formate concentration was kept constant at 5 mM. Considering the limited solubility of ammonium formate in the mobile phase with high acetonitrile content, low salt concentration was used here.

Table 1. Elemental analysis of SG-NH_2, SG-MA and SG-EDA

	N(%)	C(%)	H(%)
SG-NH_2	4.6	4.3	1.0
SG-MA	5.2	9.1	0.8
SG-EDA	9.2	10.6	2.5

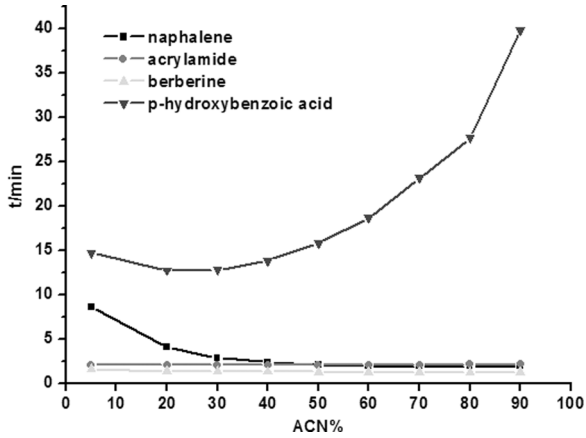

Figure 3. The influence of organic contents in the mobile phases on the retention behavior of analytes. Mobile phase, ACN/5 mM ammonium formate (pH 6.2); flow rate, 1 mL/min; injection volume, 20 μL; temperature, 30°C; detection, UV at 254 nm. Probes, naphthalene, acrylamide, berberine, and p-hydroxybenzoic acid.

The influence of acetonitrile content in the mobile phase on the retention of the four compounds was shown in Figure 3. It can be found that the retention time of naphthalene decreased as the acetonitrile content in the mobile phase increased from 5% to 40%, while it kept constant as the acetonitrile further increased from 60% to 90%. Retention of acrylamide and berberine almost kept constant as the acetonitrile increased from 5% to 90%. However, p-hydroxybenzoic acid revealed the opposite retention behavior. The retention time for p-hydroxybenzoic acid decreased slightly as the acetonitrile content increased from 5% to 20%, while it increased obviously as the acetonitrile content increased from 30% to 90%. These results demonstrated a typical HILIC retention mechanism at higher acetonitrile content.[22] However, the different trends of retention behavior implied that the separation mechanism might not be completely based on hydrophilic interaction. Some secondary interactions might be involved at low acetonitrile content such as hydrophobic interaction, etc.

Effect of pH

The pH of mobile phase is an important parameter in HILIC separation. It not only affected the hydrophilicity of stationary phase, but also affected the existent form of analytes, either charged or uncharged. In

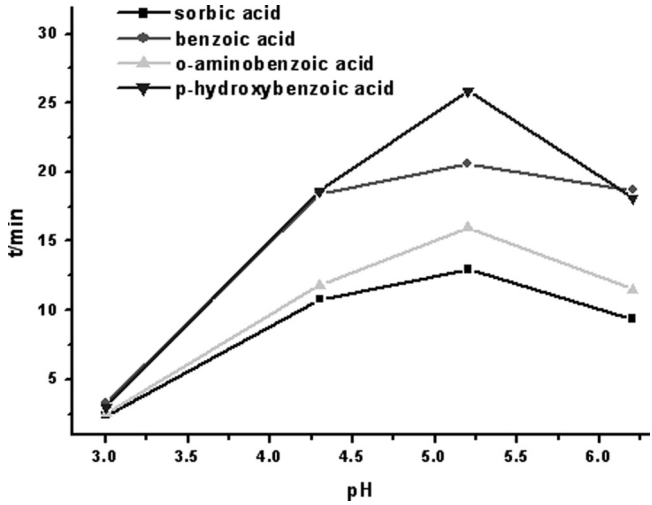

Figure 4. Effect of pH on the retention of organic acids. Mobile phase was composed of 70% ACN/30% ammonium formate (5 mM) with pH range from 3.0 to 6.2. Flow rate, 1 mL/min; injection volume, 20 μL; temperature, 30°C; detection, UV at 254 nm.

order to separate the studied organic acids, the pH of mobile phase was altered to explore the selectivity of SG-EDA. The effect of buffer pH ranging from 3.0 to 6.2 was evaluated, keeping the acetonitrile content constant at 70%. As can be seen from Figure 4, retention of organic acids increased as pH increased from 3.0 to 5.2, then decreased as pH increased from 5.2 to 6.2. A good separation of four organic acids could be obtained at pH 5.2. It was intelligible that as pH increased from 3.0 to 5.2, the analytes were gradually changed to a deprotonated form, resulting in the increase of the ion exchange interaction between the analytes and the protonated SG-EDA phase. Therefore, the retention of organic acids at pH 5.2 was found to be stronger than that at low pH range. Although, as the pH further increased to 6.2, the analytes existed in deprotonated form. Since the protonated SG-EDA phase lose charge between pH 5–9,[23] the ion exchange interaction was weakened due to the decreasing charge density of the stationary phase, thus the retention at pH 6.2 was found to be weaker than that at pH 5.2.

In order to gain more insight into the retention mechanism of organic acids, their retention was also investigated by varying acetonitrile content in mobile phase at pH 3.0. In this case, the organic acids were mostly in protonated form. As shown in Figure 5, the retention of organic acids revealed a typical HILIC characteristic as acetonitrile

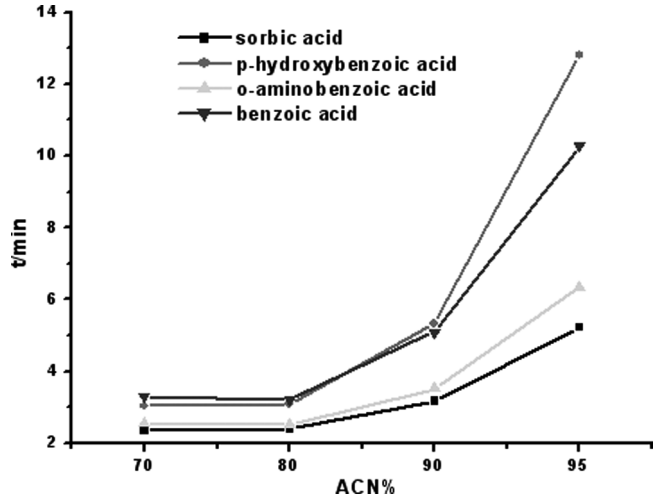

Figure 5. Effect of organic contents in mobile phase on the retention of organic acids. Mobile phase consisted of ACN and ammonium formate (5 mM, pH 3.0). Flow rate, 1 mL/min; injection volume, 20 μL; temperature, 30°C; detection, UV at 254 nm.

content was over 80%, suggesting the contribution of hydrophilicity interaction to the retention of organic acids besides ion exchange interaction. However, through comparison of the retention of organic acids at pH 3.0 and 5.2, it's obvious that ion exchange interaction played a more important role than hydrophilic interaction for the retention of organic acids.

Effect of Ionic Strength

Ionic strength also plays a very important role in HILIC.[4] The ionic strength of the mobile phase was examined at pH 5.2 with ammonium formate concentration varying from 5 mM to 100 mM. The retention behaviors of the organic acids were shown in Figure 6. It can be found that, as the ionic strength increased, the retention of organic acids decreased dramatically. As previously mentioned, the ion exchange interaction played an important role for the retention of the organic acids. Since higher salt concentrations increased the eluting strength of the mobile phase by weakening the ion exchange interaction, thus it lead to the decrease in retention. As a consequence, the retention behaviors of the organic acids decreased with increasing mobile phase ionic strength.

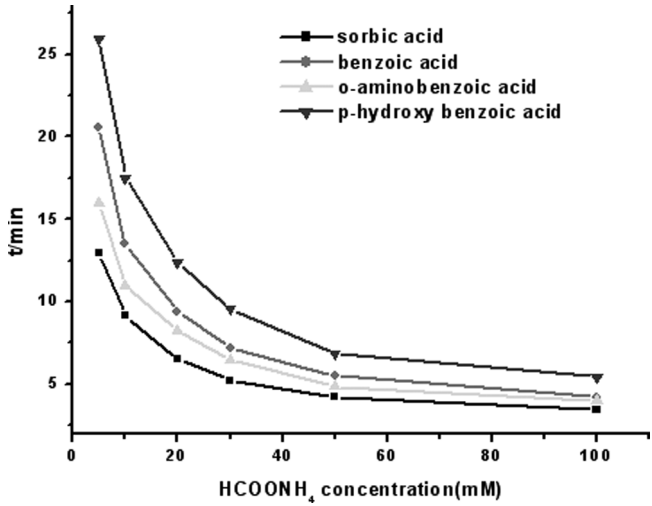

Figure 6. Effect of salt concentration in mobile phase on the retention of organic acids. Mobile phase consisted of 70% ACN and 30% ammonium formate (pH 5.2) with salt concentration ranging from 5 mM to 100 mM. Flow rate, 1 mL/min; injection volume, 20 μL; temperature, 30°C; detection, UV at 254 nm.

Comparison of Separation for Organic Acids

The separation of organic acids on the SG-EDA phase was achieved under the optimal condition with a mixture of 70% ACN and 30%

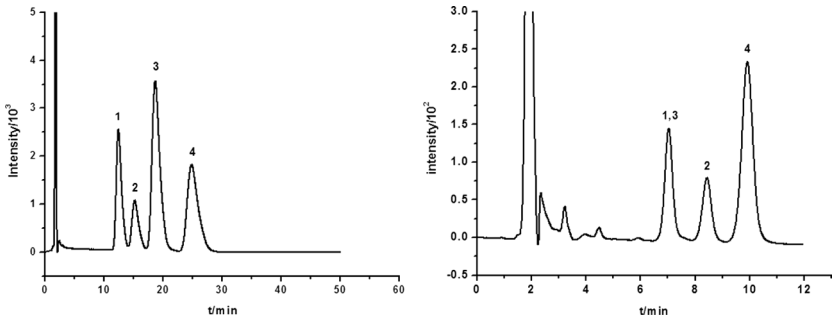

Figure 7. Separation chromatography of organic acids on SG-EDA phase (a) and SG-NH_2 phase (b). Mobile phase was composed of 70% ACN and 30% ammonium formate (5 mM, pH 5.2). Flow rate, 1 mL/min; injection volume, 20 μL; temperature, 30°C; detection, UV at 254 nm. (1) sorbic acid, (2) o-aminobenzoic acid, (3) benzoic acid, (4) p-hydroxybenzoic acid.

ammonium formate buffer solution (5 mM, pH 5.2) as the mobile phase. The chromatogram was shown in Figure 7a. In addition, their separation was also obtained on the SG-NH_2 phase, as shown in Figure 7b. It can be seen clearly that the SG-EDA phase showed stronger retention and better selectivity for organic acids.

CONCLUSIONS

A bidentate amino stationary phase has been successfully synthesized through Michael addition with methyl acrylate (MA) to aminopropyl silica and amidation to the resulting esters with ethylenediamine (EDA). Separation conditions for organic acids such as acetonitrile content, the pH of mobile phase, and ionic strength were carefully investigated. The retention behaviors of analytes indicated that hydrophilic interaction contributed to their retention to some degree at higher acetonitrile content. However, the ion exchange interaction played a more important role for their retention. In addition, the novel amino phase (SG-EDA) demonstrated stronger retention and better selectivity for organic acids compared to classic aminopropyl silica phase (SG-NH_2).

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