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Preparation, chromatographic evaluation and comparison between linear peptide- and cyclopeptide-bonded stationary phases

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ABSTRACT

In this paper, linear and cyclic tetrapeptide bonded stationary phases, were synthesized by the solid-phase synthesis method. Each amino acid coupling step was monitored by the ninhydrin test using UV/visible spectrophotometry. The proposed materials were characterized by UV spectroscopy, Fourier transform infrared spectroscopy, elemental analysis and thermogravimetric analysis, which proved the successful immobilization of tetrapeptide on the silica support. The cyclopeptide stationary phase displayed mixed-mode behavior and had the size selectivity for polycyclic aromatic hydrocarbon such as anthracene, while the linear peptide stationary phase only exhibited typical hydrophilic interaction chromatography (HILIC) characteristics and was more hydrophilic under the same mobile phase condition. Retention behaviors of polar compounds on the two stationary phases were studied through varying column temperature, the water content, pH and ionic strength in mobile phase. Applications in the separation of the mixture of nucleosides, sulfa compounds, organic acids and isomers of substituted benzoic acid compounds were demonstrated.

1. Introduction

Hydrophilic interaction chromatography (HILIC), as a complement to reversed phase liquid chromatography (RPLC) and variation of normal phase liquid chromatography (NPLC), has shown great potential and is becoming more and more a powerful technique in chromatographic analysis of various kinds of polar and hydrophilic compounds [1–6]. In HILIC, a hydrophilic stationary phase and an aqueous-organic solvent mobile phase with high organic-solvent (usually acetonitrile (ACN)) content (60–95%) are employed. The retention process in the HILIC mechanism is based on mobile phases having an increased content of the organic modifier [7,8]. Reviews detailing various advances in HILIC technology have been fairly numerous in recent years and can be categorized as those detailing general advances in HILIC as a whole and those which focus on specific aspects of HILIC technology, such as new stationary phases and advances in detection methods in general [9–12]. So far, HILIC has been applied in a wide variety of scientific fields including food stuffs, biological liquids, environmental samples, etc. [13–20].

There is a significant range of commercial HILIC columns currently available, mainly including bare-silica, amino-silica, amide-silica, sulfoalkylbetaine bonded silica, cyano- and diol-silica, carbohydrate-modified silica, triazol-bonded silica, and polymer-based monolithic columns [6,9–12,21]. Recently, many new stationary phases for HILIC have been developed. Shen et al. [22] prepared a novel zwitterionic stationary phase based on the "thiol-ene" click reaction between cysteine and vinyl silica, which exhibited great potential in the separation of oligosaccharides, peptides and basic compounds, as well as in the enrichment of glycopeptides. Guo et al. [23] developed bonded mono-, di- and oligosaccharides as separation materials for HILIC. Jiang et al. [24] prepared a porous poly(MPC-co-EDMA) monolithic column by thermal co-polymerization of MPC and EDMA, had been successfully used as a stationary phase in micro-HILIC.

Linear peptides, which offer a wide range of side chain chemistries, are suitable organic components in separation materials for possible applications such as chiral separation, the fractionation of DNA, purification, fractionation and isolation of peptides, proteins, etc. [25–29]. Although linear peptide-bonded stationary phases have been reported [22,30–34], there is no report on the cyclopeptide-bonded stationary phase. Cyclopeptide, which is a cyclocompound composed of amino acids, displays a broad range of biological effects. Due to its cyclic structure, cyclopeptide-based stationary phase might have size selectivity for relevant cyclocompounds.

In order to investigate the differences between the linear peptide and cyclopeptide bonded stationary phases, two stationary phases of bonded cyclopeptide (*c*-pep) and linear peptide (*l*-pep) with the same amino acid sequence (-alanine-alanine-alanine-glutamic acid,

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-Ala-Ala-Ala-Glu-) were prepared. After peptide molecules were immobilized on silica surface, the resulting materials possessed multiple amido bonds, which conferred good hydrophilicity on the new phases. The retention behavior of various compounds on the two stationary phases were studied and compared. It is found that the two stationary phases displayed different retention selectivities for aromatic and some hydrophilic compounds. The *l*-pep stationary phase seemed more hydrophilic than *c*-pep under the same mobile phase conditions.

2. Experimental

2.1. Reagents and materials

Spherical silica (5 μm particle size; 10 nm pore size; 320 m^2/g surface area) was purchased from Fuji Silysia Chemical (Aichi, Japan). Fmoc-Ala-OH and *N*-hydroxysuccinimide (NHS) were obtained from Aladdin reagent database Inc. (Shanghai, China). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimidehydrochloride (EDC) and Fmoc-Glu(O*t*Bu)-OH were purchased from GL Biochem Ltd. (Shanghai, China). Aminopropyltrimethoxysilane (APS) was from Alfa Aesar (Karlsruhe, Germany). Ninhydrin and piperidine were from Beijing Aoboxing Biochem Co., Ltd. (Beijing, China) and Shanghai Runjie Chemical Reagent Co., Ltd. (Shanghai, China), respectively.

Thymine, cytosine, adenine, inosine and adenosine used as test probes in the HILIC mode were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Melamine was obtained from J&K Chemical Ltd. (Beijing, China). Compounds including phenols, anilines and salicylic acid used as test probes in water-rich mobile phase were purchased from Tianjin Guangfu Chemical Reagent Co. (Tianjin, China). Sulfamethoxypyridazine (SMP), sulfamethoxazole (SMZ), sulfamonomethoxine (SMM) and sulfaquinoxaline (SQ) were obtained from Alfa Aesar (Karlsruhe, Germany). Sulfadimidine (SM_2) and sulfafurazole (SIZ) and substituted benzoic acid compounds were purchased from Aladdin reagent database Inc. (Shanghai, China). Acetonitrile (ACN) 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. Activation and immobilization of APS on silica surface

The silica (2.5 g) was suspended in 3 mol/L HCl (40 mL), and refluxed for 8 h. The obtained product was filtered and intensively washed with ultrapure water to neutral, and then dried under vacuum at 120 °C overnight.

The activated silica (2.0 g) was suspended in 30 mL anhydrous toluene, and APS (1.5 mL) was added with a stirring. The reaction mixture was heated under reflux with a N_2 atmosphere at 95–100 °C for 12 h. Then the obtained APS-bonded silica (APS-Sil) was filtered and intensively washed with dichloromethane, acetone and methanol respectively, and then dried under vacuum at 60 °C overnight.

2.3. Synthesis of tetrapeptide stationary phases

2.3.1. Preparation of Glu(OtBu)-bonded silica (Glu(OtBu)-Sil)

Firstly, Fmoc-Glu(O*t*Bu)-OH (3.0 g) was dissolved in 20 mL anhydrous DMF and adjusted pH to 4.5 by acetic acid. Secondly, the carboxyl groups of Fmoc-Glu(O*t*Bu)-OH were activated by adding 0.8 g of NHS and 1.3 g of EDC in solution in an ice bath. After 20 min, APS-Sil (2.0 g) was dispersed into the solution. The resulting mixture was stirred in an ice bath for 8 h and at room temperature for 8 h. Then, the obtained mixture was filtered and intensively washed with DMF and ethanol and then dried under vacuum at 50 °C overnight. Finally, the protective group Fmoc was removed. Typical deprotection procedure was as follows [35]: 20% piperidine in DMF (20 mL), 15 min; 20 mL of 20% piperidine in DMF, 30 min; washings (1.5 min each): 2 × 20 mL of DMF, 3 × 20 mL of

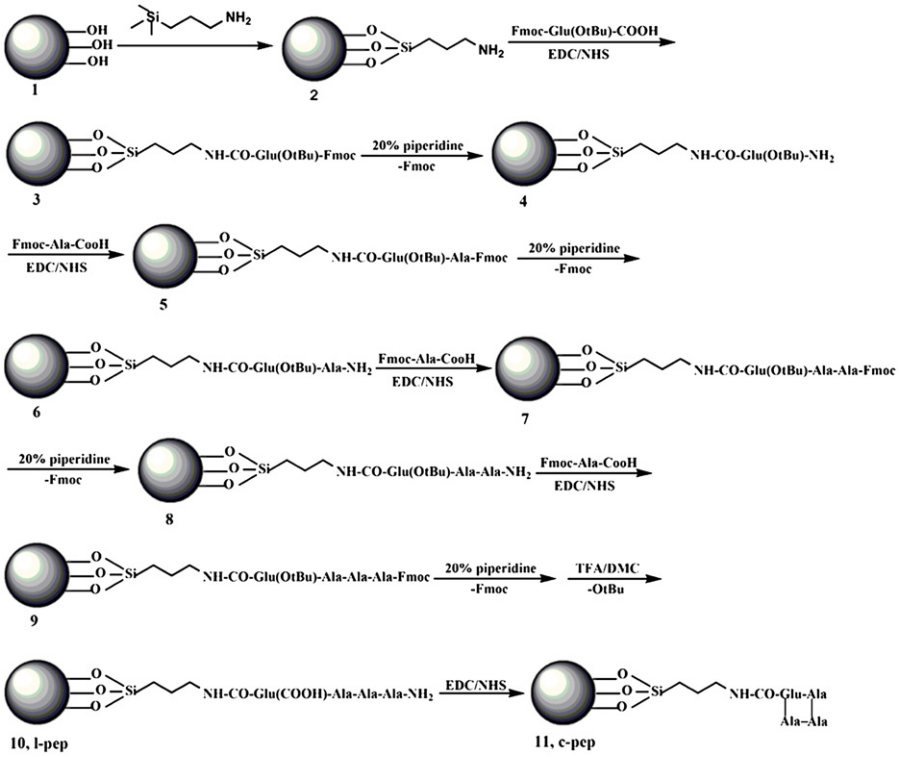

Fig. 1. Synthesis route of two new peptides materials.

DCM, 2 × 20 mL of DMF and 2 × 20 mL of ethanol. The obtained mixture was filtered and dried under vacuum at 50 °C overnight.

2.3.2. Preparation of Ala-Ala-Ala-Glu-Sil

Based on the above steps, three Ala can be bonded to Glu(O*t*Bu)-Sil in turn. Then, the protective groups O*t*Bu group for Ala-Ala-Ala-Glu(O*t*Bu)-Sil was removed by treatment with 55% (v/v) TFA/DCM (15 mL) for 2.5 h at room temperature [35]. The obtained mixture was neutralized by $NaHCO_3$, filtered, washed with water and ethanol and then dried under vacuum at 50 °C overnight. The product was named as *l*-pep.

2.3.3. Preparation of cyclo[-Ala-Ala-Ala-Glu-]Sil

After the synthesis of the linear peptide was accomplished, deprotection was achieved through repeating the deprotection experiments three times. Then, the cyclization reaction was allowed to proceed on solid-phase with EDC/NHS activation in DMF. Firstly, Ala-Ala-Ala-Glu-Sil was suspended in anhydrous DMF and adjusted pH to 4.5 by acetic acid. Then, NHS and EDC were added in an ice bath and stirred the mixture for 8 h in an ice bath and at room temperature for another 8 h. Finally, the obtained mixture was filtered, washed with DMF and ethanol and then dried under vacuum at 50 °C overnight. The product was named as *c*-pep.

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

2.4. The quantification of amino group

To insure the successful synthesis of *l*-pep and *c*-pep, the amino groups after each amino acid coupling step were measured. Amino group can react with ninhydrin, which produce a blue-violet compound [36]. In this work, the dual-wavelength UV method was used to measure the NH_2 group on the materials. Because the sample was the suspension, it was necessary to eliminate the interference from scattered light. The interference could be thought equal within $\lambda_{max} \pm 50$ nm. The product of ninhydrin and $-NH_2$ group generated UV absorption at specific wavelengths, particularly at 315 nm, 410 nm, and 585 ± 5 nm. Maximum absorption wavelength was 585 nm (λ_{max}). 555 nm ($\lambda_{max\text{-}30}$) was chosen as the second wavelengths to eliminate the interference from scattered light.

A series of amino silica solution with different NH_2 concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.5 μmol/mL) were prepared in ethanol, which had chromogenic reaction with ninhydrin. The difference of ultraviolet absorption value ΔA at 585 nm and 555 nm ($\Delta A = \lambda_{max} - \lambda_{max\text{-}30}$) was used to set the standard curve versus the concentration of amino groups. The standard curve equation was obtained: $\Delta A = 0.1790C - 0.0091$ ($R^2 = 0.9972$). Amount of the relative materials was weighed for the measurement of ΔA within the linear scope.

2.5. Instruments and chromatographic evaluation

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. Absorbance spectra were carried out using a Puxi UV-1810 visible spectrophotometer (Beijing, China). 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). Thermogravimetric (TGA) curves were carried out by a PET series Thermal Analyzer from Perkin-Elmer Instruments with a heating rate of 10 °C/min under nitrogen.

l-pep and *c*-pep were slurry-packed into a 150 mm × 4.6 mm I.D. stainless steel column with methanol as the slurry medium and packing solvent at 6000 psi pressure. C18 column for comparison (250 mm × 4.6 mm, 5 μm, 10 nm pore size) was from Hanbon Science and Technology Co., Ltd., China. Both materials of *l*-pep and *c*-pep were prepared twice and packed into columns twice too. The same kind of columns showed very similar retention behavior for the same analyte. One of the each kind was chosen for the total experiments in the manuscript.

The chromatographic evaluations were carried out at room temperature (15 ± 2 °C). A set of test probes (thymine, cytosine, adenine, inosine adenosine, melamine and salicylic acid) in the HILIC mode with a concentration of 20 μg/mL was prepared in ACN/water (4/1, v/v). The flow rate was 0.8 mL/min in ACN-rich mobile phase on two columns. A set of test compounds (SD, SMM, hydroquinone, phenol, 4-nitrophenol, adenine and salicylic acid) for water-rich mobile phase with a concentration of 20 μg/mL was prepared in methanol/water (1/4, v/v). The flow rate was 0.5 mL/min in water-rich mobile phase on *c*-pep column. All the evaluation experiments were repeated at least two times and the average retention data were used.

3. Results and discussions

3.1. Preparation and characterization of tetrapeptide bonded stationary phases

The ninhydrin test was performed to insure the successful amino acid coupling reaction and deprotection of amino groups (Table 1). The reaction would be continued until the amount of free amino groups kept stable or could not be quantified. The last cycle reaction was also evaluated by ninhydrin test.

FT-IR, elemental analysis, and TGA were used to evaluate the two materials and the relative data are shown in the support document. All of them can prove the successful immobilization of tetrapeptide on the silica support. Because of the little difference between the two materials, their characterization results are nearly the same.

3.2. Effect of organic solvent content in mobile phase on retention

The level of organic solvent content in mobile phase is the foremost factor on retention and HILIC separation requires much higher ACN content (typically $>70\%$) to ensure significant hydrophilic interaction. Thus, the retention behaviors of five nucleosides on the two columns were studied with a range of 70–95% ACN in mobile phases. The typical HILIC behaviors of test compounds are shown in Fig. 2(a and b). With the increase of ACN content, retention and separation selectivity of five polar

Table 1
The concentration of amino groups after each coupling reaction.

Materials	ΔA	Amino content (mmol/g)[a]
3	0.01	0.12
4	0.12	1.00
5	0.01	0.10
6	0.09	0.77
7	0.00	0.00
8	0.05	0.42
9	0.00	0.00
10	0.03	0.29
11	0.00	0.00

[a] Calculated from the standard curve.

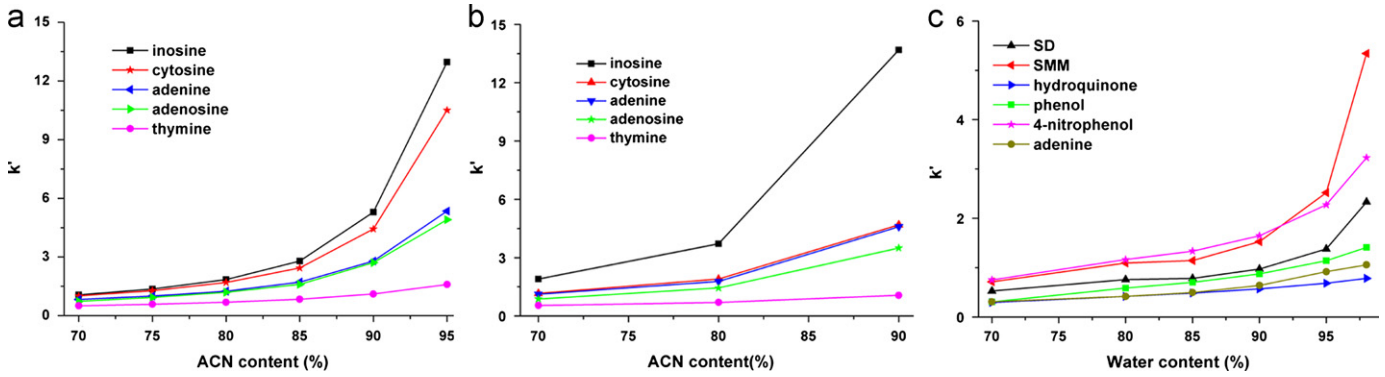

Fig. 2. Effect of ACN content on retention in ACN/water mobile phase. (a) *c*-pep column, UV 248 nm. (b) *l*-pep column, UV 248 nm. (c) *c*-pep column, UV 270 nm.

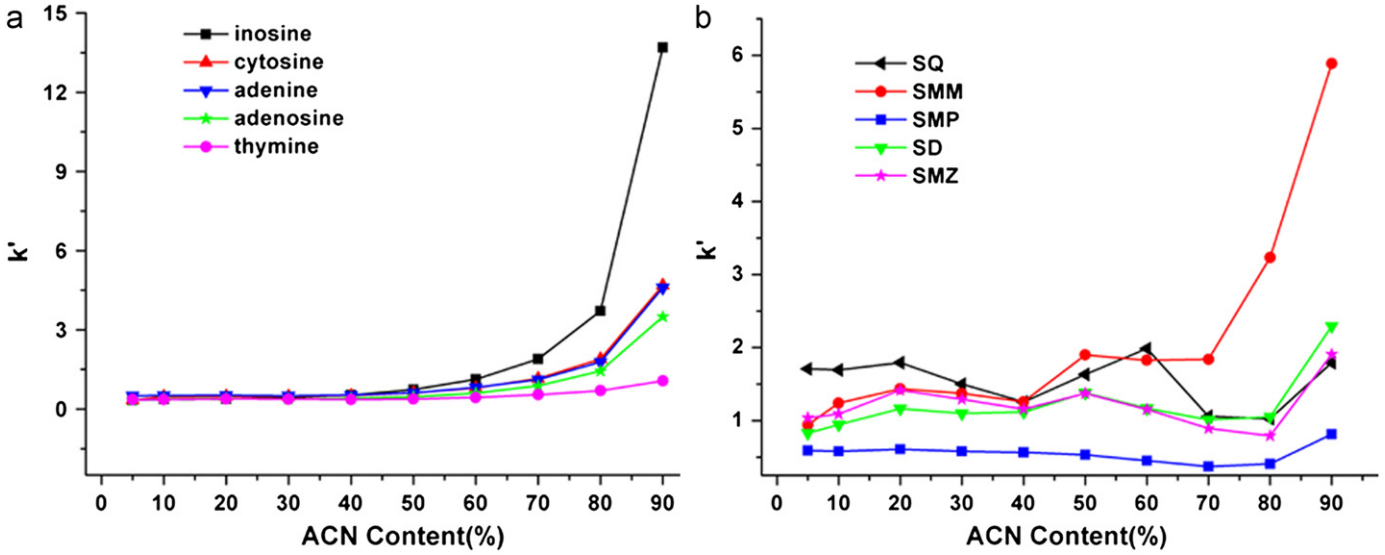

Fig. 3. Effect of ACN content on retention in ACN/water mobile phase. (a) *l*-pep column, UV 248 nm. (b) *l*-pep column, UV 270 nm.

probes increased gradually. It reflected the hydrophilic nature of the two stationary phases. Compared to the *c*-pep stationary phase, the retention on the *l*-pep stationary phase changed more obvious.

In water-rich mobile phase, the effect of water content on the retention was also studied on the two columns. Six test probes had been investigated on *c*-pep column by varying the percentage of water from 70% to 98% in eluent (Fig. 2c). In contrast, the retention increased with increasing water content in water-rich mobile phase. That is to say, reverse phase retention mechanism was exhibited on *c*-pep stationary phase, and the *c*-pep column displayed mixed retention modes just like some other HILIC stationary phases did [37–39]. The same compounds were used to evaluate the *l*-pep stationary phase and no retentions on them. More sulfa compounds were further used to study the retention behavior on the *l*-pep column and the same results were obtained. It is to say, reverse phase retention mechanism was not observed on the *l*-pep column [40].

In order to fully study the retention mechanism of the *l*-pep column, the retention behaviors of five nucleosides and five sulfonamides were studied in the range of 5–90% ACN in mobile phase (Fig. 3). When ACN concentrations were lower than 50%, all the compounds had little retention. When ACN concentration were in scope of 50–90%, all the compounds showed increased retention with increasing ACN concentration, especially when acetonitrile content was greater than 70%. The results were in accordance with the former experimental data. That is to say, *l*-pep stationary phase only exhibited typical HILIC characteristics.

3.3. 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 LC [41]. Fig. 4(a and b) shows the van't Hoff plots with column temperature varying from 25 to 60 °C for selected solutes on *c*-pep column. In ACN-rich and water-rich mobile phases, all the solutes decrease in retention with increasing 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'. In HILIC, linear van't Hoff plot was obtained, but in water-rich mobile phase, nonlinear van't Hoff plot was observed. Dong and Huang [42] attributed nonlinear van't Hoff plot to the mixed retention mechanism where electrostatic and adsorptive forces were responsible for the retention of solutes.

The same experiments were done on *l*-pep column in ACN-rich mobile phase. As can be seen in Fig. 4(c), the k' values of these five probes were basically unchanged in the temperature range surveyed, this might be due to the fast mass transfer on the *l*-pep stationary phase, which was affected little by temperature.

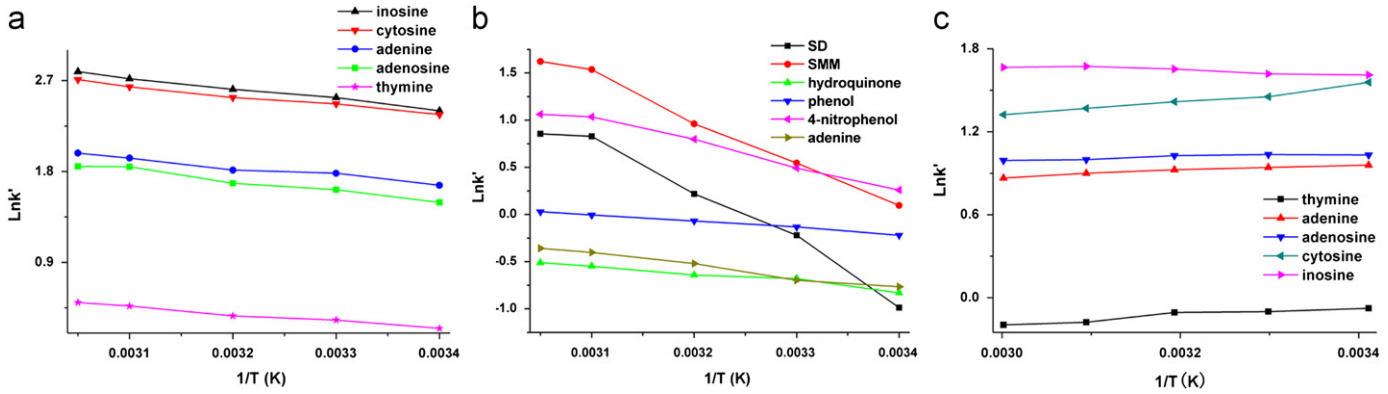

Fig. 4. Effect of column temperature on retention. (a) ACN/water (95/5, v/v), *c*-pep column. (b) ACN/water (5/95, v/v), *c*-pep column. (c) ACN/water (90/10, v/v), *l*-pep column.

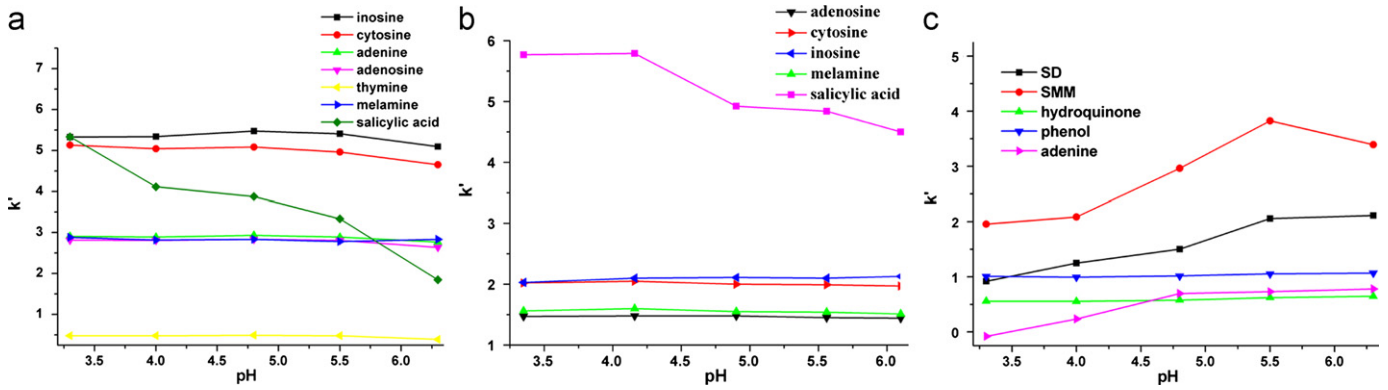

Fig. 5. Effect of mobile phase pH on retention. (a) ACN/water (90/10, v/v) containing 15 mM ammonium formate, *c*-pep column, UV 248 nm. (b) ACN/water (80/20, v/v) containing 15 mM ammonium formate, *l*-pep column, UV 248 nm. (c) ACN/water (5/95, v/v) containing 50 mM ammonium formate, *c*-pep column, UV 270 nm.

3.4. The effect of buffer pH on retention

Buffer pH can affect the retention and selectivity of solutes in LC. In HILIC, the effect of buffer pH on retention time was examined with 15 mM ammonium formate at pH 3.3, 4.0, 4.8, 5.5 and 6.3 (Fig. 5a). For five nucleosides and melamine, the retention time only fluctuated slightly on *c*-pep stationary phase in the pH range studied. The retention time of salicylic acid (pK_a∼2.8) increased significantly from 6.3 to 3.3. Moreover, it was been observed that salicylic acid eluted as a split peak from 4.8 to 3.3, possibly due to some secondary interactions with the functional groups. Therefore, it indicated a complex retention mechanism and further studies are still needed for HILIC. The similar experiments were done on *l*-pep column, and the same results were received, as can be seen in Fig. 5(b).

Fig. 5(c) shows the retention factor of the test probes in water-rich mobile phase on *c*-pep column made of 50 mM formate buffers of various pH values. The pK_a values of phenol and hydroquinone are greater than 9, and their acidic dissociation at pH below 7 is strongly suppressed. So their retention time remained essentially unchanged. For adenine with a pK_a∼4.2, the retention time had no change in the buffer pH from 6.3 to 4.8, but decreased sharply when the buffer pH decreased from 4.8 to 3.3. The possible reason was that adenine acquired more positive charge at pH below 4.2, generating electrostatic repulsion with the new phase, thus leading to a decrease in retention. The retention of SD (pK_a∼6.4) and SMM (pK_a∼6.5) decreased with pH values. Both SD and SMM acquired more positive charge when pH was decreased, generating electrostatic repulsion. Therefore, the ionization state and charge density of both the stationary phase and analytes contributed to the retention of the compounds.

3.5. The effect of ionic strength on retention [43–45]

Ionic strength of mobile phase can also have influence on the retention of polar compounds in HILIC. In this study, the effect of salt concentration was investigated on the two columns. In HILIC, the effect of ammonium formate concentration in the range of 5–15 mM (higher ionic strength was tried too, it was found that all the k' for the test probes decreased to near zero. In addition, the higher ionic strength was disadvantage for the columns in HILIC mode) on the retention of the model compounds at pH 6.3 is presented in Fig. 6(a and b). Both the stationary phases showed the same results. The retention of other substances (nucleosides and melamine) remained essentially unchanged except salicylic acid, whose retention time decreased with the increasing of salt concentration. The ion-exchange interaction could explain the retention decreasing on the new phases. The acid molecules have weak ion-exchange interaction with the amido groups on the new phases with low salt concentration. Higher salt concentration may have weakened any ion-exchange interaction, leading to the weaker retention of salicylic acid.

In water-rich mobile phase, the retention plot obtained from varying the concentrations of ammonium formate for the studied compounds on the *c*-pep phase is shown in Fig. 6(c). The retention factors for all the solutes decreased or were unchanged with

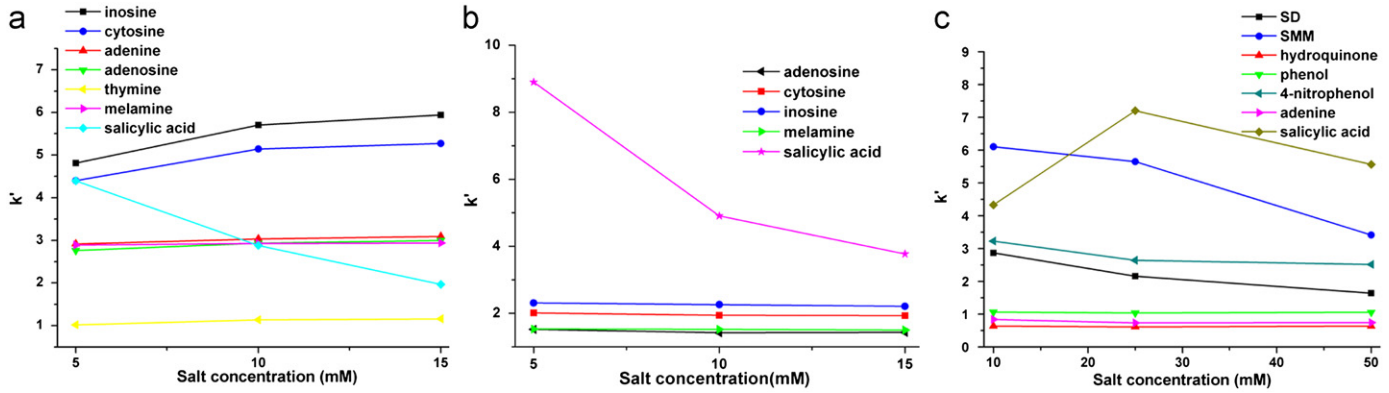

Fig. 6. Effect of salt concentration on retention. (a) *c*-pep, ACN/water (90/10, v/v), UV 248 nm. (b) *l*-pep, ACN/water (80/20, v/v), UV 248 nm. (c) *c*-pep, ACN/water (5/95, v/v), UV 270 nm.

increasing the salt concentration. It could be due to the decrease in hydrophobic interaction.

3.6. Hydrophilic comparison between c-pep and l-pep stationary phases

In order to investigate the hydrophilic of the two stationary phases, the hydrophilic and hydrophobic molecules were selected. As can been seen in Fig. 7, the order of the retention strength of five nucleosides was the same on the two columns, and the k' was smaller on *c*-pep column. However, the retention factors of phenol, hydrochinone, 4-aminophenol, 4-nitrophenol were increased on the *c*-pep column. The result showed the interaction of polar compounds with *c*-pep stationary phase was weaker than that with linear peptide stationary phase and the interaction of weak and non-polar compounds was stronger instead. Therefore, *l*-pep stationary phase was more hydrophilic than *c*-pep stationary phase, due to the existence of $-NH_2$ and –COOH groups on the surface of linear peptide stationary phase.

3.7. Comparison with C18 stationary phase

We compared the two new peptide stationary phases with C18 through investigating the effect on the retention time of different aromatic ring compounds. Firstly, the retention factor of naphthalene was kept the same by adjusting the mobile phase on *c*-pep and C18 columns, as shown in Table 2. The retention time of benzene was longer on C18 column than on other two columns due to stronger hydrophobicity of C18. Moreover, the retention of benzene was weaker on *l*-pep column than on *c*-pep column due to stronger hydrophilicity of *l*-pep. Compared with C18 column, the retention time of anthracene was increased more than two times on *c*-pep column and decreased more than three times on *l*-pep column. Cyclopeptides have ring structures with 14 carbon atoms per molecular on the *c*-pep stationary phase. Similarly, anthracene, which is polycyclic aromatic hydrocarbon, also includes 14 carbon atoms. Anthracene molecular just matched with space size of cyclopetide, thus leading to a significant increase in retention. It clearly indicated that the *c*-pep stationary phase had been prepared successfully and had size selectivity. Secondly, retention increased with decreasing polarity of the solutes, which indicated hydrophobic interaction in water-rich mobile phase on the *c*-pep stationary phase. Thirdly, the elution order of phenol and aniline, naphthol and naphthylamine could be reversed on the peptide-bonded columns compared with C18 column. The result showed the interaction of phenolic compounds with the peptide-bonded stationary phases was stronger than that with C18 column due to the existence of amide groups

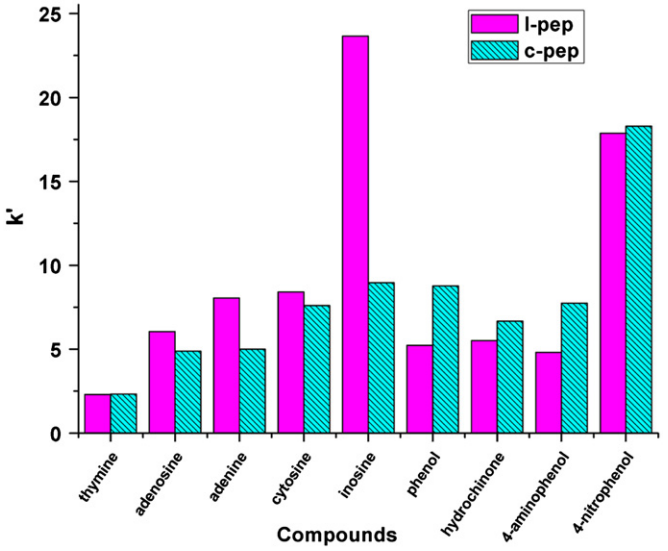

Fig. 7. Retention factor of five nucleosides on *c*-pep and *l*-pep columns. Mobile phase: ACN/water (90/10, v/v); UV 248 nm.

on surface of peptide-bonded silica. For all the test compounds, it could be found that their retentions are much lower on *l*-pep column, which is difficult to retain the hydrophobic analytes.

3.8. Application

In order to study the separation ability and selectivity of the new stationary phases, the separation of some polar small molecules was investigated.

3.8.1. The separation of the nucleosides

Nucleosides (nucleosides, bases, and its analogs) are the basic building blocks of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). A lot of diseases are connected with the abnormal of this kind of substances and their metabolites. Therefore, rapid and effective detection of a variety of nucleoside compounds has become an important issue in the field of natural pharmaceutical chemistry, pharmaceutical analysis, and disease diagnosis. It was found that five nucleosides can be successfully separated on the two peptide-bonded stationary phases (Fig. 8).

Table 2
Retention factor k' of the model compounds on tetrapeptide stationary phases[a] and C18[b] stationary phase.

Stationary phase	Phenol	Aniline	Benzene	Naphthol	Naphthylamine	Naphthalene	Anthracene
C18	5.93 ± 0.01	6.10 ± 0.01	8.20 ± 0.01	6.23 ± 0.01	7.16 ± 0.01	11.12 ± 0.01	20.17 ± 0.11
l-pep	3.86 ± 0.02	3.49 ± 0.01	3.67 ± 0.04	4.49 ± 0.01	4.03 ± 0.01	4.12 ± 0.00	5.47 ± 0.01
c-pep	5.11 ± 0.01	4.94 ± 0.01	5.49 ± 0.02	7.92 ± 0.01	7.13 ± 0.01	11.13 ± 0.01	48.63 ± 0.16

[a] Mobile phase: ACN/water (20/80, v/v).
[b] Mobile phase: ACN/water (80/20, v/v); UV detection: 254 nm. UV detection: 254 nm. $n=2$.

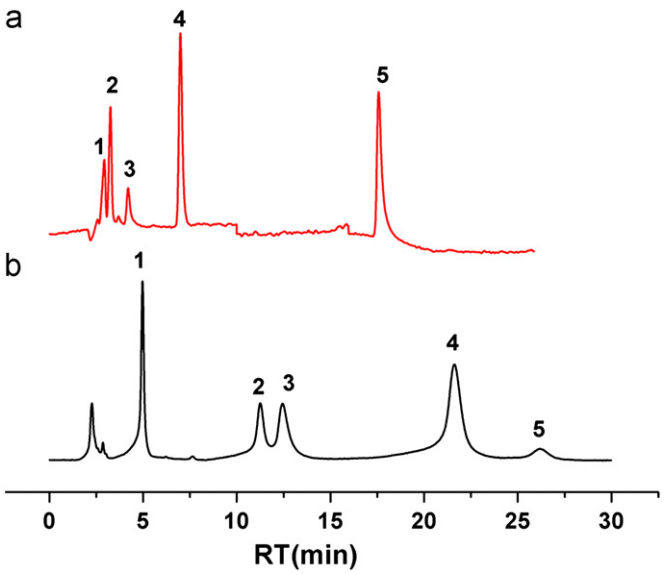

Fig. 8. Chromatograms of five nucleosides on two columns. a. *l*-pep, mobile phase: A: ACN; B: 0.5% trifluoroacetic acid aqueous solution. Gradient eluent: 0–2.5 min, 98% A; 2.5–10 min, 98–48% A. UV detection: 0–5 min, 248 nm; 5–8 min, 280 nm; 8 min–15 min, 248 nm. Analytes: (1) cytosine, (2) adenine, (3) adenosine, (4) thymine, (5) inosine. b: *c*-pep: mobile phase: ACN/water (95/5, v/v); UV detection: 248 nm. Analytes: (1) thymine, (2) adenosine, (3) adenine, (4) cytosine, and (5) inosine.

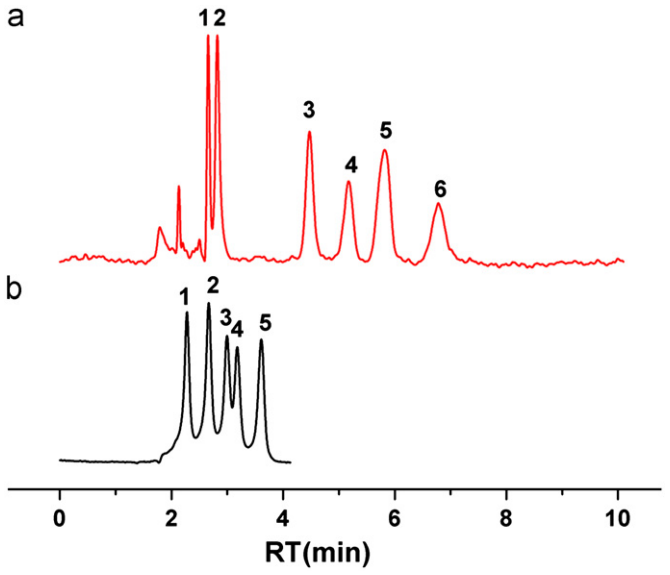

Fig. 9. Chromatograms of sulfa compounds on two columns. a: *l*-pep: mobile phase: ACN/water (85/15, v/v); UV detection: 270 nm. Analytes: (1) SM_2, (2) SMP, (3) SMZ, (4) SQ, (5) SIZ, (6)SMM. b: *c*-pep: Mobile phase: ACN/water (35/65, v/v); UV detection: 270 nm. Analytes: (1) SMP, (2) SD, (3) SMZ, (4) SMM, and (5) SQ.

3.8.2. The separation of sulfa compounds

Sulfonamide is a kind of antibiotic widely used in disease prevention and control. However, antibiotics have become a new kind of important environmental pollutant, and pose a serious threat to the ecological environment and human health because of their excessive use. The selectivity of the sulfonamides mixture was investigated on the two stationary phases (Fig. 9).

3.8.3. The separation of substituted benzoic acids

Substituted benzoic acid compounds are widely used as intermediates of medicine, dyes, fragrances and pesticides, many of which have physiological regulation and disease resistance, such as salicylic acid. The six substituted benzoic acids were successful separated on the *l*-pep stationary phase with high column efficiency, as shown in Fig. 10.

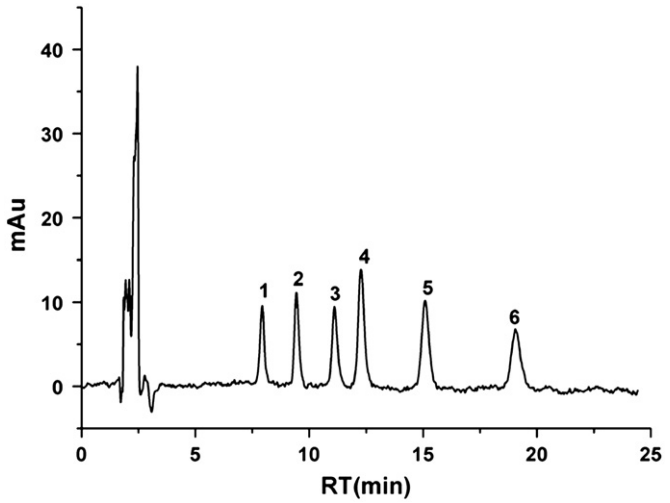

Fig. 10. Separation of six organic acids on *l*-pep column. Mobile phase: ACN/water (containing 10 mM ammonium formate) (73/27, v/v). UV 260 nm. Analytes: (1) salicylic acid, (2) *m*-nitrobenzoic acid, (3) *o*-aminobenzoic acid, (4) *o*-nitrobenzoic acid, (5) *p*-hydroxybenzonic acid, and (6) *m*-aminobenzoic acid.

3.8.4. The separation of substituted benzoic acid isomers

Isomers are the compounds which have the same formulas and different structures. The separation of isomers is difficult because of similar structures and polarities. The selectivity of the amino, nitro, and hydroxy-substituted benzoic acid isomers was investigated on the new *l*-pep stationary phase. A good separation was obtained (Fig. 11).

The tetrapeptide bonded stationary phases had been used more than 3 months (running over 800 h) in our laboratory and have no obvious change of column efficiency, which meant the high column stability obtained.

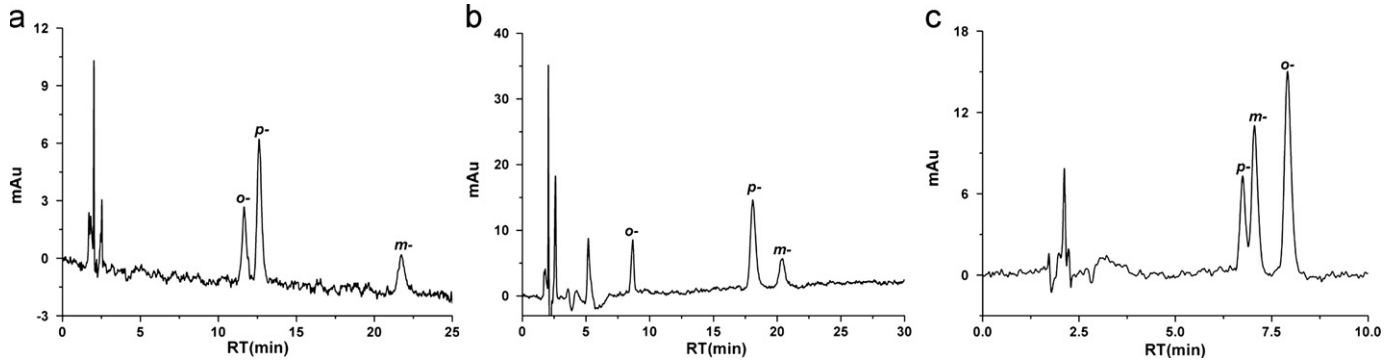

Fig. 11. Separation of substituted benzoic acid isomers on *l*-pep column. Mobile phase: A: ACN, B: 10 mM ammonium formate aqueous solution. Flow rate: 1.0 mL/min. UV 260 nm. (a) Aminobenzoic acid, A:B=78:22 (v/v). (b) Hydroxybenzoic acid, A:B=82:18 (v/v). (c) Nitrobenzoic acid, A:B=35:65 (v/v).

4. Conclusions

Two novel peptide-bonded stationary phases were designed, synthesized and compared. The representative polar compounds were employed to evaluate their chromatographic properties. Mixed-mode behavior was displayed on the *c*-pep stationary phase. However, the *l*-pep stationary phase only exhibited typical HILIC characteristics. Compared with *l*-pep stationary phase, *c*-pep stationary phase had size selectivity for polycyclic aromatic hydrocarbon and less hydrophilic property, which showed shorter retention for strong polar compounds and stronger retention for weak and non-polar compounds.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.02.005.

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