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Preparation and chromatographic evaluation of a cysteine-bonded zwitterionic hydrophilic interaction liquid chromatography stationary phase

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ABSTRACT

A cysteine-bonded zwitterionic hydrophilic interaction chromatography (HILIC) stationary phase (Click TE-Cys) was prepared based on the "thiol-ene" click chemistry. The Click TE-Cys material was characterized by solid state ^{13}C cross polarization/magic-angle spinning (CP/MAS) NMR and elemental analysis. The dynamic evaluation for cytosine, cytidine and orotic acid was performed using Van Deemter plots. The plate height values were no more than 24 μm for the flow rate between 0.5 and 5.4 mm s^{-1} (0.3–3.5 mL min^{-1}), which proved the excellent separation efficiency of Click TE-Cys stationary phase. The influences of the content of water, concentration of salt and pH of the buffer solution on the retention of model compounds were investigated. The results demonstrated that the separation of polar analytes was dominated by the partitioning mechanism, while the contribution of electrostatic interaction was minor. The thermodynamic characteristic of Click TE-Cys stationary phase was also studied according to van't Hoff plot. An exothermic process for transferring analytes from the mobile phase to the stationary phase was observed and a linear relationship for $\ln k$ and $1/T$ was achieved, indicating no change of retention mechanism within the measured temperature range. Besides, the zwitterionic stationary phase exhibited good stability. Considering the high hydrophilicity of Click TE-Cys stationary phase, the application in the separation of protein tryptic digests was carried out using hydrophilic interaction chromatography–electrospray ionization mass spectrometry (HILIC–ESI-MS). More peaks were adequately resolved on the Click TE-Cys column comparing with that on the TSK Amide-80 column. In addition, the orthogonality between HILIC and RPLC system was investigated utilizing geometric approach. The XTerra MS C_{18} and Click TE-Cys column displayed great difference in separation selectivity, with the orthogonality reaching 88.0%. On the other hand, the orthogonality between Click TE-Cys and TSK Amide-80 system was 21.4%, i.e. the selectivity was similar but slightly different from each other. The successful separation of protein digests indicated the great potential of Click TE-Cys stationary phase in the separation of complex samples and applicability in two-dimensional liquid chromatography (2D-LC).

1. Introduction

As an alternative approach to reversed-phase liquid chromatography (RPLC) which is an indispensable and versatile technique for the separation of a wide range of analytes, hydrophilic interaction chromatography (HILIC) plays an important role in separating highly polar and hydrophilic compounds [1–10], such as oligosaccharides, peptides, nucleosides, polar pharmaceuticals, etc. Substantial separation materials have been designed specially for HILIC [8,11,12]. Nonetheless, the application of HILIC is not so popular as RPLC owing to the poor solubility of highly hydrophilic compounds in high organic content solvents and limited separation efficiency of HILIC columns. Thus, the development of HILIC stationary phases with improved hydrophilicity and separation efficiency is of great significance. Among those dedicated HILIC stationary phases, zwitterionic stationary phases [13–16], which contain both positive and negative charges at a stoichiometric ratio, have been found suitable for the separation of polar analytes. Although the surface charge of zwitterionic stationary phases was supposed to be neutral, both the sulfobetaine and phosphorylcholine types of zwitterionic stationary phases exhibited slightly negative surface charge. And the spatial arrangement of positive and negative charged groups was perpendicular to the surface [15,16].

The term "click chemistry" was first coined by Sharpless in 2001 [17]. And the typical Cu(I)-catalyzed alkyne-azide Huisgen reaction has been successfully applied in the synthesis of chromatographic stationary phases [18,19]. However, the leaving of heavy metal ions cannot be avoided using this click strategy. Based on the copper-free click chemistry, i.e. "thiol-ene" click chemistry [17,20,21], we first reported the design of a cysteine-bonded HILIC

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stationary phase [22] (designated Click TE-Cys, in which "Click TE" stands for "thiol-ene" click chemistry and "Cys" represents cysteine). The Click TE-Cys stationary phase belonged to zwitterionic stationary phases, with the surface charge switching slightly from positive to negative between pH 3 and 7. Unlike the typical zwitterionic stationary phases, the distribution of oppositely charged groups was paralleled to the surface of the silica gel. Besides, the Click TE-Cys stationary phase exhibited excellent hydrophilicity and great potential in the separation of polar compounds, as well as in the enrichment of glycopeptides [22]. In this study, the retention behaviors under HILIC mode were investigated in detail using a set of polar compounds to further characterize the chromatographic properties of Click TE-Cys stationary phase. Additionally, the dynamic evaluation was performed to illustrate the separation efficiency on Click TE-Cys column. And the thermodynamic characterization was conducted based on the van't Hoff plots. The results will provide basis for the understanding and application of the cysteine-bonded zwitterionic stationary phase and HILIC.

Although RPLC is important for separating and purifying peptide mixtures, the peak capacity and separation selectivity in the analysis of complex samples are limited in one-dimensional separation. Consequently, two-dimensional liquid chromatography (2D-LC) has been utilized to improve the separation [23–26]. The commonly used 2D-LC system for peptide separation is the combination of strong cation exchange (SCX) chromatography with RPLC. However, as the elution in SCX mode is driven by the difference in solute charge, the separation of peptides with the same charge is difficult. Besides, the separation is usually achieved using NaCl solution that is incompatible with MS detection [25,27]. Although HILIC × RPLC 2D-LC system is considered to be a promising method, the application in peptide separation is not so popular because of the limited column efficiency and hydrophilicity of HILIC stationary phases. Considering the advantages of Click TE-Cys stationary phase, the separation of protein tryptic digests using hydrophilic interaction chromatography–electrospray ionization mass spectrometry (HILIC–ESI-MS) was carried out and compared with the separation on the TSK Amide-80 column. Furthermore, the orthogonality between HILIC and RPLC system was studied in the separation of peptide mixtures.

2. Experimental

2.1. Apparatus

Chromatographic evaluation was performed on an Alliance HPLC system equipped with a Waters 2695 HPLC pump and a Waters 2996 diode array detection (DAD) system (Waters, Milford, MA, USA). Chromatograms were recorded on a personal computer with Empower workstation software. Elemental analysis was measured on a Vario EL III elemental analysis system (Elementar, Hanau, Germany). Solid state ^{13}C cross polarization/magic-angle spinning (CP/MAS) NMR characterization was performed on a Bruker AVIII 600 NMR Spectrometer (600 MHz, 14.1 T) (Karlsruhe, Germany) and the chemical shifts of ^{13}C were referenced to tetramethylsilane (TMS). Magic-angle spinning was performed at a spinning rate of 10 kHz with a 4-mm double resonance probe. The spectra was recorded at a ^{13}C frequency of 150.9 MHz with $\pi/2$ pulse length of 5 μs, a contact time of 3 ms and recycle delay of 4 s. Approximately 2050 scans were accumulated.

2.2. Chemicals and materials

Spherical silica (5 μm particle size, 10 nm pore size, 300 $m^2\,g^{-1}$ surface area) was purchased from Fuji Silysia Chemical (Kasugai, Japan). Trichlorovinylsilane was obtained from ABCR (Karlsruhe, Germany), α,α′-azodiisobutyronitrile (AIBN) was purchased from Shanghai Chemical Reagents (Shanghai, China) and cysteine was from Acros (Fair Lawn, NJ, USA). Acetonitrile (ACN) of HPLC grade was from Merck (Darmstadt, Germany). Water (H_2O) was purified by a Milli-Q water purification system (Billerica, MA, USA). Ammonium formate (NH_4FA) and formic acid (FA) were obtained from Acros (Fair Lawn, NJ, USA). Polar solutes uracil, uridine, cytosine, cytidine and orotic acid were from Acros. Melamine was from Tianjin Chemical Reagents (Tianjin, China). Salbutamol was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and hyoscyamine was obtained from TCI (Tokyo, Japan). All other reagents were analytical grade reagents and used without purification.

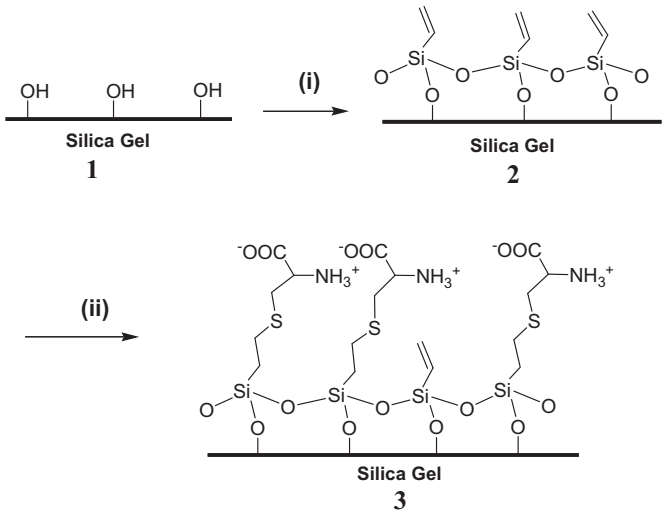

Fig. 1. The preparation of Click TE-Cys stationary phase. (i) trichlorovinylsilane, toluene, RT; (ii) cysteine, AIBN, H_2O/MeOH (2:1, v/v), 65 °C.

XTerra MS C_{18} (5 μm particle size, 150 mm × 2.1 mm I.D.) was purchased from Waters (Milford, MA, USA), TSK Amide-80 (5 μm particle size, 150 mm × 4.6 mm I.D.) was from Tosoh (Tokyo, Japan).

2.3. Preparation of cysteine-bonded stationary phase and column packing

The synthesis procedure of cysteine-bonded stationary phase was shown in Fig. 1. Silica gel was dried at 120 °C overnight. After that, humidification of the dried silica was performed by flowing 50% water humidified nitrogen through the silica until the weight increment was 5–6% of the silica. Then, 80 mL of anhydrous toluene was added into 30 g of humidified silica **1** in a flask under nitrogen atmosphere. And 7.8 mL of trichlorovinylsilane was gradually added into the solution with vigorous stirring. The reaction was kept at room temperature with stirring for 24 h. The silanized silica **2** was filtered, washed successively with anhydrous toluene, dichloromethane, methanol, water and methanol, and then dried at 80 °C overnight.

Under nitrogen atmosphere, 160 mg of AIBN was added to the solution of cysteine (3 g) in 90 mL of water–methanol (2:1, v/v). Then, 10 g of the silanized silica **2** was added into the stirred solution and the reaction was allowed to reflux for 12 h at 65 °C. Finally, the resulting material was filtered, washed with water and methanol in succession. The product was dried at 80 °C overnight to obtain the stationary phase **3** Click TE-Cys.

With 40 mL of methanol as slurry solvent and 80 mL of methanol as propulsion solvent under a pressure of 60 MPa, 2.5 g of the resulting Click TE-Cys material was slurry-packed into stainless steel column (150 mm × 4.6 mm I.D.).

2.4. Chromatographic conditions

For chromatographic evaluations, the flow rate was 1.0 mL min^{-1} and the column temperature was 30 °C unless otherwise specified. Mobile phases were prepared by mixing acetonitrile with stock solutions of ammonium formate. 100 mM NH_4FA was prepared as stock solutions and adjusted to the required pH by the addition of FA before mixing with ACN. The salt concentration in this paper represented the final concentration of ammonium formate in the mobile phase. The void time of Click TE-Cys column was measured using toluene as t_0 maker and the resulting t_0 was 1.41 min.

Four proteins (α-casein, phosphorylase b, hemoglobin, bovine serum albumin/BSA) tryptic digests were separately dissolved in ACN/H_2O (50:50, v/v). In the RPLC mode, eluent A was 0.1% FA in ACN, eluent B was 0.1% FA in water. The protein digests were separated by a linear gradient from 5% A to 45% A in 50 min, and then 45% A to 60% A in 10 min. And the flow rate was 0.2 mL min^{-1}. In the HILIC mode, eluent A was 10 mM NH_4FA in ACN/H_2O (80/20, v/v), and eluent B was 10 mM NH_4FA. The separation condition on the Click TE-Cys column was a linear gradient from 100% A to 60% A in 50 min, then 60% A to 10% A in 10 min. And a linear gradient from 100% A to 70% A in 50 min, then 70% A to 10% A in 10 min was performed on the TSK Amide-80 column. The flow rate was 0.5 mL min^{-1} for both types of HILIC columns. All the separation conditions were not optimized.

MS detection was performed in ESI$^+$ ion mode. The capillary voltage and cone voltage were set to 3.0 kV and 35 V. Source temperature and desolvation temperature were held at 120 °C and 350 °C respectively. Cone gas flow was 50 L/h and desolvation gas flow was 800 L/h. Each separation was conducted at least two times to validate the reproducibility of the system.

2.5. Data analysis

The data calculations and plotting were performed on a personal computer using Microsoft excel 2003 and Origin 8.0.

The orthogonality evaluation of the chosen two-dimensional liquid chromatography (2D-LC) modes for peptide separation was performed according to previous reports [25,28].

Retention times of peptides under RPLC and HILIC modes were recorded manually according to their mass and normalized according to Eq. (1). Where RT_{max} and RT_{min} represented the retention times of the most and least retained peptides in the data set, and the retention times RT_i were converted to normalized $RT_{i(norm)}$.

$$RT_{i(norm)} = \frac{RT_i - RT_{min}}{RT_{max} - RT_{min}} \quad (1)$$

Then the normalized retention times $RT_{i(norm)}$ were plotted into a two-dimensional separation space which was divided into 9×9 bins. Consequently, the orthogonality O which was defined by Eq. (2) was calculated for the chosen 2D-LC system.

$$O = \frac{\sum \text{bins} - P}{0.63P^2 - P} \quad (2)$$

$\sum$bins was the number bins in the 2D plot containing data points, and P represented one-dimensional peak capacity, i.e. 9 in the present evaluation.

3. Results and discussion

3.1. Preparation and characterization of Click TE-Cys material

Based on the horizontal polymerization [29,30] of trichlorovinylsilane on the humidified silica, the vinyl group was introduced on the silica support. Subsequently, the "thiol-ene" click reaction between vinyl silica and cysteine was carried out in the presence of AIBN to obtain the resulting material Click TE-Cys. The "thiol-ene" reaction, which was identified as a click reaction in 2007 [31], has been utilized in the preparation of chromatographic stationary phases [32–34]. Different from the classical Cu(I)-catalyzed alkyne-azide Huisgen click reaction [17], the copper-free "thiol-ene" click reaction can avoid the leaving of heavy metal ions in the stationary phase. Additionally, the selective reactivity between vinyl and thiol group is helpful to the preservation of the amino and carboxyl group in the cysteine, i.e. the zwitterionic characteristic of Click TE-Cys stationary phase.

The resulting Click TE-Cys material was characterized by solid state ^{13}C CP/MAS NMR. As displayed in Fig. 2, the resonance at 172.6 ppm was assigned to the carbon atom that belonged to the carbonyl group. The signals at 131.6 and 138.8 ppm were assigned to the carbon atoms of unreacted vinyl group. The peaks at 13.5 and 27.3 ppm were assigned to the carbon atoms of the reacted vinyl group. And the signals at 33.5 and 53.3 ppm were assigned to the carbon atoms on the cysteine. Thus, the solid state ^{13}C CP/MAS NMR spectra demonstrated the successful immobilization of cysteine on the silica support.

Besides, the vinyl silica and Click TE-Cys were characterized by element analysis. The results showed that the carbon content of vinyl silica and Click TE-Cys were 4.45% and 6.00%, while the nitrogen content of vinyl silica and Click TE-Cys were 0.00% and 0.85%, respectively. The surface coverage was calculated from the increase of carbon content based on the equation proposed by Kibbey and Meyerhoff previously [35]. The surface concentration of the vinyl group on the vinyl silica was 6.89 μmol m^{-2} and the surface concentration of cysteine on Click TE-Cys stationary phase was 1.52 μmol m^{-2}.

3.2. Separation efficiency of Click TE-Cys stationary phase

High separation efficiency is of great importance for the application of chromatographic stationary phases. As outlined in the comprehensive review by Ikegami et al. [12], separation efficiency on HILIC columns was generally lower than that on RPLC columns. However, the Click TE-Cys column exhibited high separation efficiency in the separation of polar compounds, with the column efficiency as high as 72,000, 75,000 and 82,000 N/m for cytosine, cytidine and orotic acid, respectively [22]. In order to further investigate the chromatographic performance of the Click TE-Cys stationary phase, the kinetic evaluation of Click TE-Cys stationary phase was carried out by plotting the plate height (H, μm) vs. linear velocity (u, mm s^{-1}, 1 mm s^{-1} corresponded to 0.65 mL min^{-1}).

As shown in Fig. 4, the stationary phase had minimum plate heights of 13–15 μm in the linear velocity between 0.62 and 1.85 mm s^{-1} for cytosine, cytidine and orotic acid. The optimal flow rate of Click TE-Cys stationary phase was higher than the typical zwitterionic stationary phases [12,36]. And the plate heights were no more than 24 μm within the tested flow rate 0.5–5.4 mm s^{-1} (0.3–3.5 mL min^{-1}), demonstrating the high separation efficiency of Click TE-Cys stationary phase. Besides, the data were fitted to the Van Deemter curve:

$$H = A + \frac{B}{u} + Cu \quad (3)$$

And the A, B and C (Table 1) values were comparable with the coefficients in previous reports [36,37].

3.3. Retention characteristics under HILIC mode

In order to understand the chromatographic properties of Click TE-Cys stationary phase, firstly, the influence of water content on the retention behavior was investigated. We proposed five polar

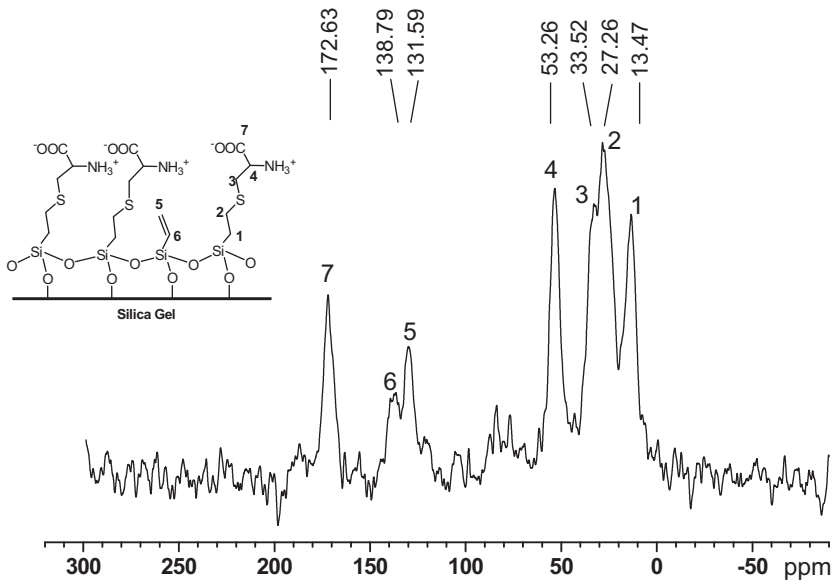

Fig. 2. Solid state ^{13}C CP/MAS NMR spectra of Click TE-Cys material.

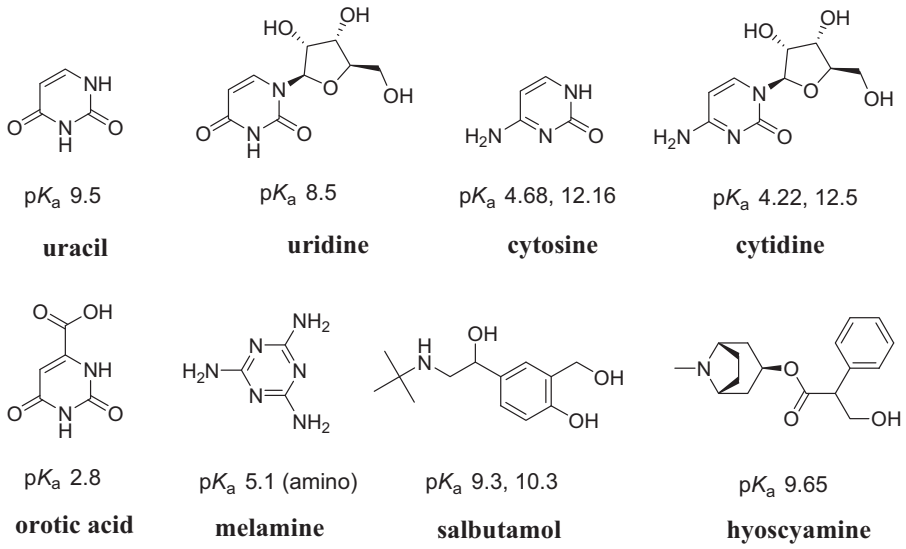

Fig. 3. Structures and p*K*as of tested probes.

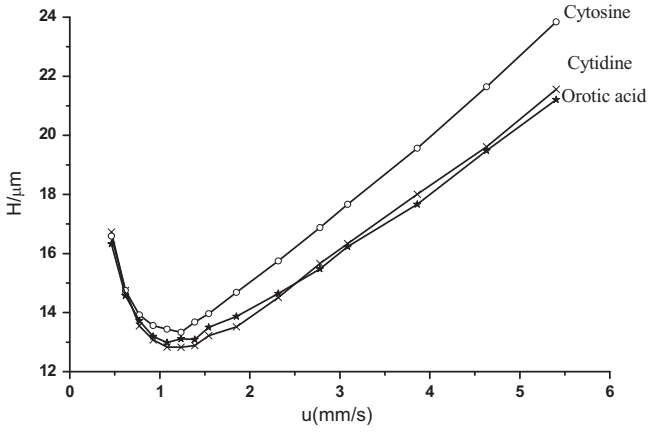

Fig. 4. Van Deemter plots on the Click TE-Cys column for cytosine, cytidine and orotic acid. *Conditions*: mobile phase, ACN/100 mM NH_4FA, pH 3.40 (85:15, v/v); corresponding flow rate range 0.3–3.5 mL min^{-1} (1 mm s^{-1} corresponded to 0.65 mL min^{-1}); column temperature, 30 °C; UV detection: 265 nm.

compounds as test mixture for HILIC, including uracil, uridine, cytosine, cytidine and orotic acid (Fig. 3). The volume percentage of water in the mobile phase was changed from 15% to 40% while the concentration and pH of ammonium formate buffer solution were kept at 15 mM and 3.30, respectively. The results (not shown) indicated that the retention factors decreased with the increase of water content, which was in accordance with the typical retention characteristics of HILIC [1,6,8]. According to previous reports [38–41], the retention model proposed by Lu et al. was suitable for the quantitative description of retention factors in HILIC mode. As

Table 1
The coefficients for data fitted to the Van Deemter equation.

Solutes	A^a (μm)	B^a ($\times 10^5$ cm^2/s)	C^a (ms)
Cytosine[b]	6.2	4.2	2.7
Cytidine[b]	6.8	3.8	3.1
Orotic acid[b]	7.1	3.7	2.5

[a] The coefficients *A*, *B* and *C* refer to the Van Deemter equation.
[b] The ACN content in the mobile phase is 85% in this experiment.

Table 2
The results of regression coefficients of Eq. (4) for five model compounds on Click TE-Cys column.

Solutes	*a*	*b*	*c*	R^2 [a]
Uracil	−1.54	−1.04	0.21	0.9996
Uridine	−2.21	−1.77	0.75	0.9998
Cytosine	−1.40	−1.60	0.70	0.9994
Cytidine	−3.14	−2.63	2.62	0.9997
Orotic acid	−3.09	−2.73	2.28	0.9991

[a] Square of correlation coefficient.

expressed in Eq. (4), k is the retention factor of the analyte and C_B is the content of water in the mobile phase. Thus, multiple regression analysis was performed and the results were presented in Table 2. All of the regression coefficients were above 0.999.

$$\ln\ k = a + b\ln C_B + cC_B \quad (4)$$

Besides, the effect of the concentration of buffer solution in the mobile phase was studied when the content of acetonitrile was kept at 85%. Ammonium formate with different concentrations (5–25 mM) was used because of its high solubility at higher concentration of organic modifier. And the pH of ammonium formate aqueous solution was kept at 3.35. The Click TE-Cys stationary phase was positively charged in this pH value. Therefore, the separation might involve both ionic interaction and hydrophilic interaction for ionic solutes. According to previous reports [1,6], high organic content in the mobile phase would drive more salt ions into the water-enriched layer with the increase of salt concentration, which might increase the hydrophilicity of the surface water-enriched layer. And the retention of polar compounds would be stronger. On the other hand, the retention factor of orotic acid would decrease as the electrostatic attraction between Click TE-Cys stationary phase and orotic acid decreased at higher salt concentration. However, the retention factors for all the model compounds increased with the increase of buffer concentration (Fig. 5). The results indicated that the separation of polar analytes was based predominantly on partitioning mechanism and the influence of ionic interaction was weak.

In addition, the mobile phase pH usually affects the selectivity and retention of analytes in HILIC, especially for ionic solutes. Consequently, the effect of mobile phase pH was investigated using six small compounds (structures as shown in Fig. 3). Buffer solutions of 100 mM ammonium formate with different pH values (pH 6.04, 4.78, 4.10 and 3.28) before mixing with ACN were employed. As shown in Fig. 6, the retention factors of the model compounds with

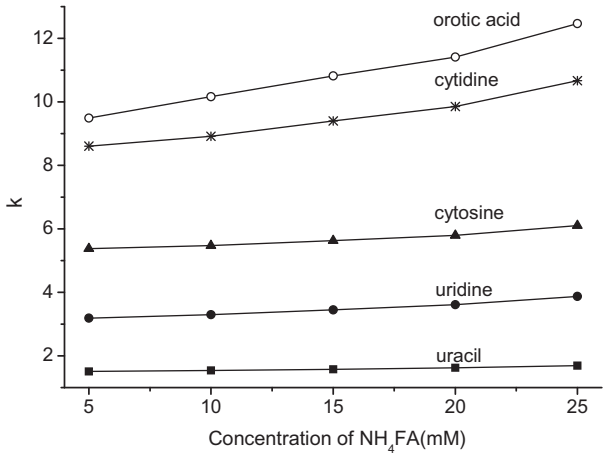

Fig. 5. The influence of buffer concentration on the retention of model compounds. *Conditions*: mobile phase, NH_4FA buffer (concentration as noted), pH 3.35, with 85% ACN; flow rate: 1.0 mL min^{-1}; column temperature, 30 °C; UV detection: 265 nm.

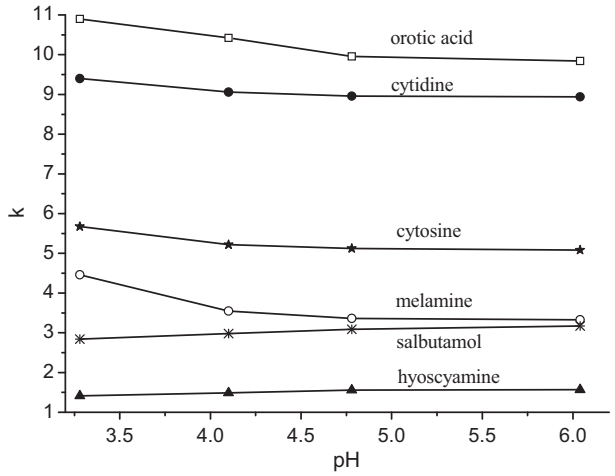

Fig. 6. The effect of different pH in the mobile phase on the retention of six small compounds. *Conditions*: mobile phase, 15 mM $HCOONH_4$ in ACN/water (85:15), pH as noted; flow rate, 1.0 mL min^{-1}; column temperature, 30 °C.

different p*K*a values were almost not changed within the pH range from 3 to 6. The retention of hyoscyamine (p*K*a 9.65) and salbutamol (p*K*a 9.3, 10.3) increased slightly when the pH was changed from 3 to 6, which was probably due to the increase of negative charge in the stationary phase while the charges of the solutes were almost unchanged, i.e. an increase in the electrostatic attraction between the two analytes and stationary phase. On the contrary, the retention times for orotic acid (p*K*a 2.8) decreased with pH, since the negative charge of the solute increased as well as the stationary phase. Thus, the electrostatic repulsion between orotic acid and stationary phase increased. For melamine (p*K*a 5.1, amino), cytosine (p*K*a 4.68, 12.16) and cytidine (p*K*a 4.22, 12.5), retention times reduced as the pH changed from 3 to 4 and remained almost unchanged between pH 4 and 6, which might be due to the decrease of positive charges in the analytes and the electrostatic attraction was reduced. More experiments would be needed to further illustrate the separation mechanism. In summary, the separation of ionic solutes on Click TE-Cys stationary phase involved both partitioning mechanism and ion-exchange interaction. Nonetheless, as the Click TE-Cys zwitterionic stationary phase contained oppositely charged groups at a stoichiometric ratio, the electrostatic interaction between the stationary phase and charged analytes was weak. Therefore, the partitioning interaction between mobile phase and water-enriched layer on the surface of Click TE-Cys stationary phase was the dominating retention mechanism in the separation of polar compounds.

3.4. Thermodynamic characterization of Click TE-Cys stationary phase

Column temperature which has significant effect on diffusion coefficient, mobile phase viscosity and analyte transferring enthalpy from mobile phase to stationary phase, is an important parameter that affects analyte retention in HILIC [6,42–44]. The influence of temperature on the retention of model compounds can be expressed by the van't Hoff equation.

$$\ln k_i = -\frac{\Delta H_i}{RT} + \frac{\Delta S_i}{R} + \ln \Phi \quad (5)$$

where k_i is the retention factor of the analyte, ΔH_i and ΔS_i are the molar enthalpy and molar entropy of transfer of the analyte in the chromatographic system, Φ is the phase ratio of the chromatographic column ($\Phi = V_S/V_M$) and R is gas constant.

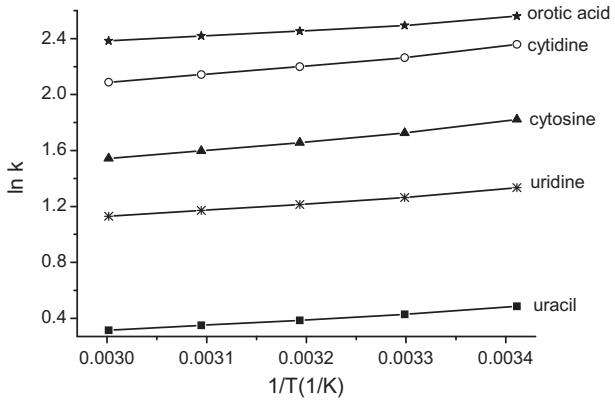

Fig. 7. The van't Hoff plots for five model compounds on Click TE-Cys column. *Conditions*: mobile phase, ACN/100 mM NH_4FA, pH 3.25 (85:15, v/v); flow rate, 1.0 mL min^{-1}; UV detection: 265 nm.

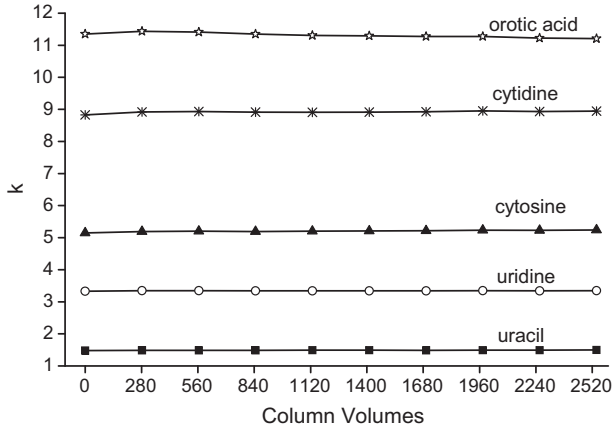

Fig. 8. The effect of column volumes of elution solution on the retention of polar compounds on Click TE-Cys column. *Conditions*: mobile phase, ACN/100 mM NH_4FA, pH 3.27 (85:15, v/v); flow rate, 1.0 mL min^{-1}; column temperature, 45 °C; UV detection: 265 nm.

The relationship between ln k and $1/T$ on Click TE-Cys column was explored in the temperature range of 20–60 °C. As shown in Fig. 7, the van't Hoff curves displayed positive slopes, i.e. the retention times for the five polar analytes decreased with elevated temperatures. The linear relationship for the model compounds with correlation coefficients of 0.982–0.993 indicated no variation of retention mechanism as the temperature changed. In addition, negative enthalpy values (Table 3) were observed for the model compounds, reflecting an exothermic process of transferring analytes from the mobile phase to the stationary phase. Hence, it was assumed that the contribution of electrostatic interaction to the overall retention was minor, i.e. the separation was based mainly on partition mechanism.

Table 3
The thermodynamic parameters for five model compounds on Click TE-Cys column.

Analytes	ΔH (kJ/mol)	ΔS^a (J/mol K)	Correlation coefficients
Uracil	−3.45	−5.54	0.9926
Uridine	−4.07	−0.63	0.9908
Cytosine	−5.59	−1.79	0.9903
Cytidine	−5.39	3.35	0.9895
Orotic acid	−3.5	11.48	0.9820

[a] ΔS was calculated from the plot intercept. And the phase ratio Φ was obtained according to the equation $\Phi = V_S/V_M$, where V_M was calculated from the dead time t_0 and the flow rate (assuming negligible extra-column volume) and V_S was calculated via the geometric internal volume of the column minus V_M.

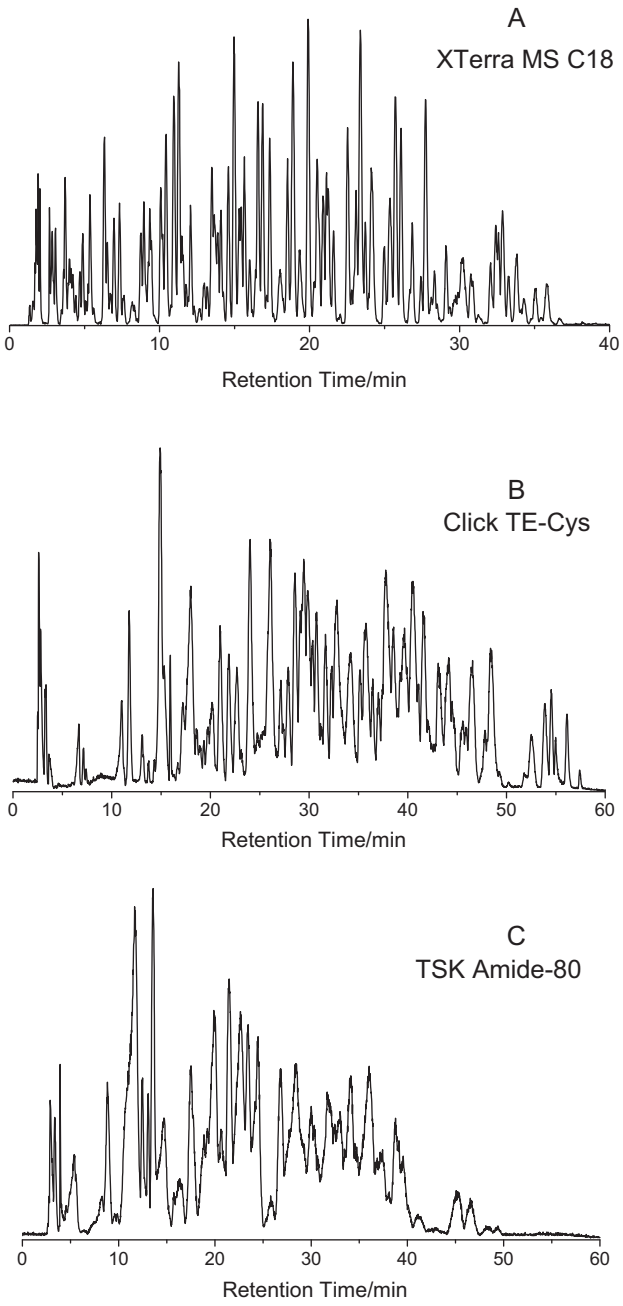

Fig. 9. LC–MS chromatograms of BSA tryptic digest. (A) XTerra MS C_{18} in RPLC mode; (B) Click TE-Cys in HILIC mode; (C) TSK Amide-80 in HILIC mode.

3.5. Chemical stability

The chemical stability of chromatographic stationary phase provides basis for its application and generalization. Herein, the proposed model compounds were chose to investigate the stability of the zwitterionic stationary phase. The Click TE-Cys column was eluted continuously with 20 mM ammonium formate (pH 3.30) for 72 h when the column temperature was maintained at 45 °C, and the separation was performed every 8 h. There was no obvious change of capacity factors for the polar compounds (Fig. 8). Additionally, intra-day and inter-day reproducibility of retention factors for model compounds were also studied and the RSDs were no more than 0.6% and 0.7% respectively, revealing the good stability of Click TE-Cys stationary phase. The horizontal polymerization of trichlorovinylsilane on silica formed a dense, two-dimensionally

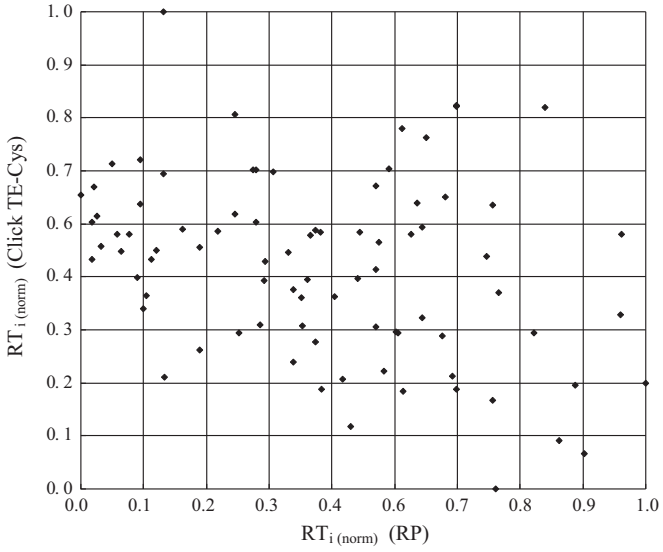

Fig. 10. Normalized peptides retention times plots for Click TE-Cys and XTerra MS C_{18} system.

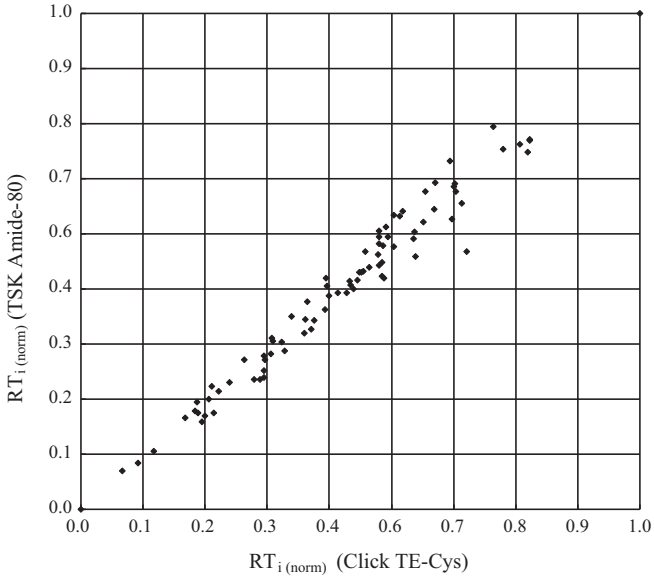

Fig. 11. Normalized peptides retention times plots for Click TE-Cys and TSK Amide-80 system.

cross-linked network with the surface coverage of 6.89 μmol m^{-2}, which enhanced the stability of vinyl silica. Besides, the thioether bond generated by the "thiol-ene" click reaction was of chemical inertness. As a result, the Click TE-Cys stationary phase exhibited good stability.

3.6. Application in the LC–MS analysis of peptide mixtures

Considering the excellent separation efficiency and hydrophilicity of Click TE-Cys stationary phase, the separation of four protein tryptic digests was carried out using HILIC–ESI-MS. Examples of chromatograms for the separation of BSA digest were displayed in Fig. 9. The separation of peptides on the Click TE-Cys column was better than that on the TSK Amide-80 column, with more peaks adequately resolved. This further proved the high separation efficiency of Click TE-Cys column. Nonetheless, the separation efficiency in HILIC mode was not as good as that in RPLC mode. Less and broadened peaks were observed on the Click TE-Cys column in comparison with that on the C_{18} column.

Owing to the limitation of peak capacity and separation selectivity in one-dimensional analysis, 2D-LC has been gradually applied to the separation of complex samples. The HILIC × RPLC system was supposed to be a promising 2D-LC system in peptide separation. Thus, the orthogonality was evaluated between the HILIC and RPLC system in the separation of peptides. XTerra MS C_{18} column was used to generate the basic set of retention time data, which represented a common second dimension of 2D-LC. Some peptides were eluted in the void volume in RPLC/HILIC, while some peptides had poor intensity in ESI-MS and were not detected. In consequence, only the most intense peptides common for all LC–MS experiments were chose for the comparison of orthogonality of the 2D-LC systems. The orthogonality between XTerra MS C_{18} and Click TE-Cys system was 88.0% (Fig. 10), while the orthogonality between XTerra MS C_{18} and TSK Amide-80 system was 78.5%. Peptide pairs LSQKFPK (RT_{C18} = 6.724 min, $RT_{Click\ TE\text{-}Cys}$ = 40.426 min) and YICDNQDTISSKLK (RT_{C18} = 6.724 min, $RT_{Click\ TE\text{-}Cys}$ = 55.019 min), VLYPNDNFFEGK (RT_{C18} = 21.387 min, $RT_{Click\ TE\text{-}Cys}$ = 27.452 min) and ARPEFTLPVHFYGR (RT_{C18} = 21.387 min, $RT_{Click\ TE\text{-}Cys}$ = 35.635 min), as well as VLVDLER (RT_{C18} = 15.308 min, $RT_{Click\ TE\text{-}Cys}$ = 25.296 min) and TNFDAFPDK (RT_{C18} = 15.308 min, $RT_{Click\ TE\text{-}Cys}$ = 35.322 min) which could not be separated on XTerra MS C_{18} column exhibited great difference in retention characteristics on Click TE-Cys column. On the contrary, peptide pairs VAAALTK ($RT_{Click\ TE\text{-}Cys}$ = 29.443 min, RT_{C18} = 5.728 min) and EIWGVEPSR ($RT_{Click\ TE\text{-}Cys}$ = 29.313 min, RT_{C18} = 16.414 min), DYYFALAHTVR ($RT_{Click\ TE\text{-}Cys}$ = 26.07 min, RT_{C18} = 20.023 min) and LVTDLTK ($RT_{Click\ TE\text{-}Cys}$ = 26.07 min, RT_{C18} = 10.943 min) that could hardly be separated on Click TE-Cys column were well separated on XTerra MS C_{18} column. On the other hand, the retention behaviors of peptides on Click TE-Cys column were similar but different slightly from that on TSK Amide-80 column and the orthogonality reached 21.4% (Fig. 11), which further demonstrated the typical HILIC retention properties for Click TE-Cys stationary phase. The successful separation of protein digests indicated the applicability of Click TE-Cys in 2D-LC separation and great potential in the separation of polar and complex samples.

4. Conclusion

The chromatographic retention behaviors and application of the cysteine-bonded zwitterionic HILIC stationary phase were investigated in detail. The dynamic evaluation demonstrated the excellent separation efficiency of Click TE-Cys stationary phase. The chromatographic retention behaviors of Click TE-Cys stationary phase revealed that the separation of polar compounds was based predominantly on partitioning mechanism. And the thermodynamic characterization reflected that no change of retention mechanism existed within the tested temperature range. Meanwhile, the zwitterionic stationary phase exhibited good stability. In addition, the separation of protein digests was performed on Click TE-Cys column using HILIC–ESI-MS. Compared to the separation on TSK Amide-80 column, more peaks were adequately resolved on Click TE-Cys column. The orthogonality of HILIC and RPLC system was also studied. The orthogonality between XTerra MS C_{18} and Click TE-Cys system was 88.0%, indicating the applicability of Click TE-Cys in 2D-LC separation and the great potential in the separation of polar and complex samples. Future work will be focused on biological sample analysis in our laboratory and the results will be reported subsequently.

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