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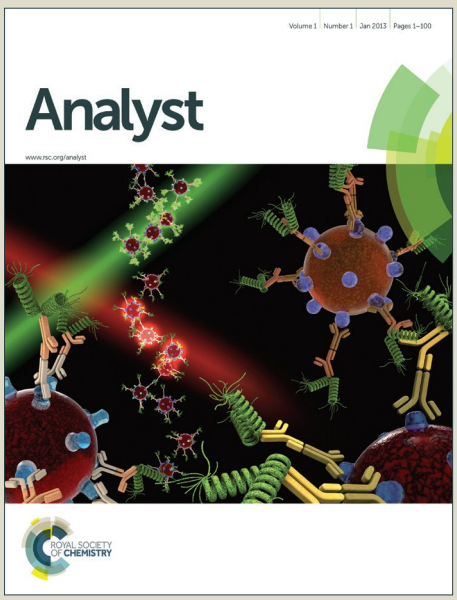

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Evaluation of separation properties of a modified strong cation exchange material named MEX and its application in 2D-MEX×C18 system to separate peptides from scorpion venom

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Abstract

Peptides from scorpion venom represent one of the most promising sources for drug discovery in some specific disease. Current challenges in their separation include high complexity, high homologies and huge range of peptides. In this paper, a modified strong cation exchange material named MEX was firstly introduced in a two-dimensional separation of peptides from complex scorpion venom. The silica-based MEX column was bonded with two functional groups of benzenesulfonic acid and cyano-propyl. To better understand its separation mechanisms, seven standard peptides with different properties were employed in evaluation study. Results showed that two interactions were involved in the MEX column: electrostatic interactions based on benzenesulfonic acid groups dominated the separation of peptides; weak hydrophobic interactions introduced by cyano-propyl groups increased the column's selectivity for peptides with the same charges. This characteristic allowed the MEX column to overcome some drawbacks of traditional strong cation exchange (SCX) columns. Furthermore, the study showed a great effect of acetonitrile (ACN) contents, sodium perchlorate ($NaClO_4$) concentrations and buffer pH in the mobile phase on peptides' retention and separation selectivity on the MEX column. Subsequently, the MEX column was combined with a C18 column to establish an off-line 2D-MEX×C18 system to separate peptides from scorpion *Buthus martensi* Karsch (*Bm*K) venom. Due to complementary

separation mechanisms in each dimension, high orthogonality of 47.62% was achieved. Moreover, good loading capacity, excellent stability and repeatability were exhibited by the MEX column, which were beneficial for its use in future preparation experiment. Therefore, the MEX column could be an alternative to traditional SCX columns for the separation of peptides from scorpion venom.

Key words: peptides, modified SCX column, 2D-LC, scorpion venom, separation

1. Introduction

In recent year, peptides has been an increasing pharmaceuticals in drug market, especially in the treatment of cancer, diabetes and cardiovascular diseases[1, 2]. As peptides from the animal venom have been approved with highly specific bioactivities[3-5], now they have become significant promising sources for peptide drug development, and scorpion venom peptide involving in this paper is the most representative one[6, 7]. Over the past years, many bioactive peptides have been purified and characterized from scorpion venom. However, they just occupied 1% among all peptides from 1500 scorpion species in the world[6]. High complexity[8], high homologies[9], low content[9] and huge range of peptides[7] (13-70 residues with no or three or four disulfide bridges) in scorpion venom have brought enormous challenges in separation.

Reverse phase liquid chromatography (RPLC) is the widely used method for its excellent resolving power and separation efficiency. However, it has been recognized that a single reverse phase column is insufficient to adequately resolve above mentioned separation problems for scorpion venom[10, 11]. Combination of two orthogonal separation procedures is necessary to increase the peak capacity and the power of separation system. The technique of two dimensional liquid chromatography (2D-LC) coupled with tandem mass spectrometry introduced by Yates et al.[12] has become the mainstream for peptide separation. Recently, Xu et al.[13, 14] employed a 2D-LC system to successfully purify 18 homologous short-chain peptides and 11 long-chain peptides from scorpion venom. Mostly, RPLC was chosen as the second dimensional method since it can provide effective desalting and good compatibility with MS detection to achieve the online analysis[15]. In a 2D-LC system, the separation mode of the first dimensional directly influences the separation capacity. Several chromatographic modes coupled with RPLC have been investigated by Gilar et al.[16], including RPLC, strong

cation exchange chromatography (SCX) and hydrophilic interaction liquid chromatography (HILIC) systems. For example, the RPLC×RPLC system that used at different pH was constructed. Since peptides' properties such as charge state and hydrophobicity could be altered by the changing pH, different separation selectivity in each dimension was obtained to provide good orthogonality[17]. However, similar separation mechanisms were still existed in both dimensional columns. For highly homologous peptides in scorpion venom, the resolving power may be not enough[18]. The SCX×RPLC system is another common strategy for peptide separation based on electrostatic interaction and hydrophobicity[19]. Different separation mechanisms between the two dimensions allow them exhibit good orthogonality for peptide separation. But for the SCX mode, a problem that the majorities of peptides with similar charges tend to elute within a narrow window was still remained, which resulted in relatively low separation efficiency[15, 17]. The HILIC×RPLC system had a highest degree of orthogonality among all systems. For HILIC column packed with bare silica sorbent in Gilar's experiment was involved two interactions of hydrophilic and ionic exchange(due to the charged silanols at pH 4.5), leading to significant improvement of separation selectivity for peptides[16]. In fact, most HILIC materials are involved ionic exchange interactions in separation, especially for those silica-based HILIC materials. However, HILIC materials are always designed to be more hydrophilic than ionic characters[20], and this may be -unbeneficial for the separation of hydrophobic peptides. For instance, HILIC material Click maltose used by Xu et al.[14] was just suitable for the separation of more hydrophilic short-chain peptides in scorpion venom rather than long-chain peptides. In addition, the utility of HILIC also could be hindered by its poor solubility of hydrophilic peptides in the mobile phase with high organic content. Therefore, some new stationary phases are required to further enhance the resolving power of 2D-LC system.

For peptide that has both charged and hydrophilic/hydrophobic characteristics, separation method involved in both ionic exchange and hydrophilic/hydrophobic interactions is superior to traditional RPLC, HILIC and SCX methods. In this case, stationary phases are always designed to be simultaneously boned with two or more functional groups. For example, new reverse phase/weak cation exchange stationary phase (named C18WCX) composed of mixed n-octadecyl and 3-carboxypropyl groups was synthesized by Cai et al.[18]. The orthogonality of

off-line 2D-C18WCX×RPLC system was higher than that of conventional 2D-RPLC×RPLC approach. In this study, we introduced a modified strong cation exchange column (named MEX) as the potential alternative to tradition SCX column in 2D-LC separation of peptides from scorpion venom. The MEX were synthesized by bonding two functional groups of benzenesulfonic acid and cyano-propyl to the surface of silica[21]. The cyano-propyl groups were introduced to reduce the density of sulfonic groups to decrease cation exchange capacity of the stationary phase, which led to moderate retention of peptides. In addition, the introduction of cyano-propyl groups was expected to improve the separation selectivity of the MEX column to overcome the drawbacks of traditional SCX columns in separation of peptides with the similar charges. In order to better understand the retention mechanism of peptide on the MEX column, we firstly evaluated the separation mechanisms of the MEX column by employing seven standard peptides with different properties in this paper. Moreover, the effects of key experimental factors including organic modifier concentrations, $NaClO_4$ concentrations and buffer pH in the mobile phase were further investigated. Subsequently, the MEX column were combined with a C18 column to develop a 2D-LC system for the separation of scorpion venom peptides from *Buthus martensi* Karsch (*Bm*K), a traditional Chinese medicine[22]. The orthogonality of the off-line 2D-MEX×C18 system also was investigated. According to the results of all studies, this modified strong cation exchange column MEX could be an alternative to existing SCX columns for separation of peptide.

2. Experimental

2.1 Apparatus and reagents

The high performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA) consisted of a Waters 2695 HPLC pump and a waters 2996 photodiode array detector. Data acquisition and processing were conducted using Waters Empower 3 software. The mass spectrometry (MS) experiments were performed on ultra performance liquid chromatography system (Agilent 1290, USA) couple to a quadrupole time-of-flight (Q-TOF) mass spectrometer (Agilent 6450, USA). The pH was measured by a FiveEasy Plus pH meter (Mettler Toledo, Model FE20, Swiss). The electrode system was calibrated using usual aqueous standard reference buffers (sodium chloride buffer at pH 4.0 and 7.0, respectively) before using.

Acetonitrile (ACN) and methanol of HPLC grade was from J&K (Beijing, China). Sodium perchlorate ($NaClO_4$) was obtained from Lingfeng chemical reagent Co., Ltd (Shanghai, China). Triuoroacetic acid (TFA), *ortho*-phosphoric acid (PA) and triethylamine (TEA) were obtained from Sigma-Aldrich (St. Louis, Mo, USA). Water was purified by a Milli-Q water purification system (Billerica, MA, USA).

2.2 Sample and Preparation

Seven standard peptides including FPRPGGGGNGDFEEIPEEYL, SLIGKV-amide, DRVYIHP, YSFKDMQLGR, Pyr-LYENKPRRPYIL, Acetyl-FGGF and Acetyl-FGGF-amide, were purchased from GL Biochem Co., Ltd (Shanghai, China). Their characteristic information listed in table 1 was calculated by using Peptide Property Calculator software (http://www.biosyn.com/peptidepropertycalculator/peptidepropertycalculator.aspx). The standard peptide mixture solution was prepared by dissolving them in water and the concentration of each peptide was at 1 mg/mL.

The scorpion venom from *Buthus martensi* Karsch (*Bm*K) was obtained from Luoyang, Henan province of China. Crude *Bm*K venom was firstly pretreated by using the solid phase extraction (SPE) procedure to enrich peptides and remove the unknown components. Crude venom was prepared as 50 mg/mL solution with water, and centrifuged with 10000 r/d for 10 min. The upper solution was then loaded to the SPE cartridge which packed with C18 materials (60 μm, 100 Å). The SPE cartridge was activated and conditioned with methanol, water with 0.1% TFA separately for three times. After loading the sample, the cartridge was firstly washed with water with 0.1% TFA, then 60% ACN with 0.1% TFA was used to elute the venom peptides, the fractions was collected and lyophilized. Finally, the lyophilized scorpion venom sample was dissolved in water at 200 mg/mL which was ready to use for next HPLC analysis.

2.3 Chromatographic conditions

2.3.1 Chromatographic evaluation of MEX column with standard peptide

MEX column (250 mm×4.6 mm i.d., 10 μm) was home-made[21]. Its dead time was 2.83 min by measuring with toluene. In this experiment, quaternary pump of HPLC was utilized by four eluents, including A: water, B: ACN, C: 1 mol/L $NaClO_4$ solution and D: 100 mM triethylamine phosphate (TEAP) aqueous buffer. 100 mM TEAP buffers at pH 2.5, 4.0, 6.3

and 8.0 were prepared as follows: 1.36 mL *ortho*-phosphoric acid was added to 200 mL water and triethylamine was added until the appropriate pH was attained. Chromatographic conditions: the flow rate and column temperature were 1.0 mL/min and 30°C, respectively, the signal was monitored at 214 nm and the injection volume of peptide mixture was 5 μL. Evaluation experiments included following three sections: (1) Organic modifier effect: B was employed for 20%, 25%, 30%, 35%, 40% and 45% ACN, respectively; C was in a linear gradient of 5% to 35% $NaClO_4$ (50 to 350 mM) over a 35 min time period, D was fixed at 10% TEAP (10 mM, pH 2.5). Here a comparison experiment was conducted on a Unitary C18 column (250 mm×4.6 mm i.d., 5 μm), this column was purchased from Acchrom Co., Ltd (Beijing, China). The mobile phase was same as that used for the MEX column but the ACN content was fixed at 25%. (2) Salt effect: in the condition of pH 2.5, C was utilized for 15%, 20%, 25%, 30%, 35% $NaClO_4$ whose concentrations were 150 mM, 200 mM, 250 mM, 300 mM and 350 mM, respectively, pump B was fixed at 25% ACN and D continuously was 10% TEAP (10 mM, pH 2.5); while in the condition of pH 6.3, C was utilized for 10%, 15%, 20%, 25% and 30% $NaClO_4$ whose concentrations were 100 mM, 150 mM, 200 mM, 250 mM and 300 mM, respectively, pump B was fixed at 25% ACN and D was fixed at 10% TEAP (10 mM, pH 6.3). (3) Buffer pH effect: D was continuously employed at 10% TEAP (10 mM), but at different pH of 2.5, 4.0, 6.3 and 8.0; C was in a linear gradient of 5% to 35% $NaClO_4$ (50 to 350 mM) over a 35 min time period, B was fixed at 25% ACN. For all evaluation experiments, A was water gradient changing to keep the whole mobile phase at 100%.

2.3.2 An off-line 2D-MEX× C18 system coupled with tandem mass spectrometry for separating and analyzing scorpion venom peptide

Scorpion venom peptide was separated on the MEX column (150 mm×4.6 mm i.d., 5 μm, homemade), the injection volumes were 2 μL and 50 μL, respectively. The mobile phase was A: water, B: ACN, C: l mol/L $NaClO_4$ and D: 100 mM TEAP, pH 6.3. The HPLC condition of the MEX column was optimized as: 0-5 min (5% to 30% B, 5% C), 5-30 min (30% B, 5% to 25% C), 30-40 min (30% to 50% B; 25% C). Pump D constantly delivered 10% TEAP (10 mM) and A was a water gradient changing to keep the whole mobile phase at 100%. Fractions were collected manually at 2 min intervals. In total 20 fractions were obtained and lyophilized to dry. Each fraction was dissolved with water and further re-analysised on an XCharge C18

column (150 mm×4.6 mm i.d., 5 μm, Acchrom). Mobile phase A and B were water and ACN, respectively, in which all contained 0.1% TFA (pH 1.8). An optimal elution condition was adopted as 0-10 min (5% to 20% B), 10-25 min (20% to 30% B), 25-35 min (30% to 40% B). The external conditions for the MEX and C18 columns were same: flow rate was 1.0 mL/min, column temperature was 30°C, and UV signal response monitored at two wavelengths of 214 nm and 280 nm. The second dimensional XCharge C18 column output was coupled to an electrospray ionization source, the split ratio for the mobile phase between MS and waste is 1/4. The mass spectrometer was operated in the positive ion mode. Ion source parameters were as follows: the capillary voltage was set to 3.0 kV, the cone voltage was set to 40 V. The nebulization gas was set to 800 L/h at a temperature of 350°C, the cone gas was set to 50 L/h, and the source temperature was set to 120°C. The MS survey was from m/z 200 to 2000. The acquisition time was 40 min. MS data were processed using a Mass Hunter Workstation software (Agilent, Version B.03.01).

2.4 Data analysis

The data calculations and plotting were performed on a personal computer using Microsoft excel 2010 and Origin 8.0. Retention times of peptides on the MEX column were recorded manually and their retention factors (k) was calculated according to Eq.(1),

$$k = (t_R - t_0)/t_0 \quad (1)$$

where t_0 and t_R represented dead time of the MEX column and the retention times of standard peptides, respectively. Orthogonality was evaluated according to a reported method[16, 23, 24]. Firstly, all peptides were identified by MS information, and repeated data in different fraction were deleted. Then all the retention data of peptides in the second dimension were normalized according to Eq.(2),

$$t_R^{i(normal)} = \frac{t_R^i - t_R^{min}}{t_R^{max} - t_R^{min}} \quad (2)$$

in which t_R^{max} and t_R^{min} represented the retention times for the peaks showing the greatest and least retention among all the second dimension runs, respectively. The retention times were converted to normalized $t_R^{i(normal)}$ values that range from 0 to 1. Finally, the 2D plots were constructed, in which the number of fractions was plotted on the *x-axis*, and the normalized retention times of the second dimension were plotted on the *y-axis*. Peptides were

distributed in fractions 1 to 20, so a two-dimensional separation space was divided into 20×20 bins, and the normalized retention data were plotted into this 2D separation space. The orthogonality O% was calculated according to Eq.(3),

$$O\% = \frac{\sum bins - \sqrt{P_{max}}}{0.63 P_{max}} \times 100\% \quad (3)$$

in which $\sum bins$ was the number of bins containing data points in the 2D plot. P_{max} was the sum of all bins, which represented the total peak capacity in this evaluation system.

3. Results and discussion

3.1 The property analysis of seven standard peptides

The MEX is a modified strong cation exchange stationary phase simultaneously boned with benzenesulfonic acid and cyano-propyl groups. Different from traditional SCX columns, the cyano-propyl groups are introduced to reduce the density of sulfonic groups to decrease cation exchange capacity. As a result, on the MEX column, some other interactions (like hydrophobic interactions) may be involved in its separation. Here seven standard peptides were employed to evaluate the separation mechanisms of the MEX column and their detail information was listed in table 1. According to their properties, these peptides can be classified as basic peptides (2 to 5, net charge>0), acidic peptides (1 and 6, net charge<0) and neutral peptide (7, net charge=0). When mobile phase's pH changed, peptides 1 to 6 were charged in different degrees, and their retentions would be affected by different electrostatic interactions with ionic groups of the MEX column, leading to their different retention behaviors. Thus according to the retention behaviors of standard peptides, electrostatic interactions contributed by benzenesulfonic acid groups could be deduced. Besides, the hydrophobicity of peptides 1 to 7 at pH 2.0 was also listed in table 1, and the hydrophobic property of the MEX column could be evaluated through peptides' retention behaviors when changing the ACN content in the mobile phase.

3.2 The effect of organic modifier on peptides' retention under the MEX column

To evaluate the effect of organic modifier on peptides' retention under the MEX column, ACN with different concentrations of 20%, 25%, 30%, 35%, 40% and 45% were employed. The $NaClO_4$ concentration was in gradient elution of 5% to 35% (50 mM to 350 mM) over 35 min and TEAP buffer fixed at 10 mM, pH 2.5. As showed in Fig.1, retention times of all

seven peptides decreased with the increase of ACN concentration, which was in accordance with the typical retention characteristics of RPLC. For RPLC mode, relationship between the logarithm of retention factor, $\ln k$, and the composition of strong modifier, C_B, can be expressed in Eq.(4)[25],

$$\ln k = \mathrm{a} + \mathrm{c}C_B \quad (4)$$

where C_B is the content of ACN in this study. Results of multiple regression analysis between $\ln k$ and C_B were presented in table 2, Relatively low correlation coefficients were showed by standard peptides 1 to 5. But high correlation coefficients of peptides 6 and 7 were indicated the presence of hydrophobicity on the MEX column. According to previous reports[26, 27], the retention model expressed in Eq.(5),

$$\ln k = \mathrm{a} + \mathrm{b}\ln C_B + \mathrm{c}C_B \quad (5)$$

was also utilized for the quantitative description of retention behaviors of peptides in this experiment. The omitted $\mathrm{b}\ln C_B$ related to absorption interaction was added into the model of RPLC mode. It was found correlation coefficients of peptides 6 and 7 were still high and little changed between these two models. As peptides 6 and 7 were all neural in the acid condition, their retentions were just affected by the hydrophobic property of the MEX column. Conversely, for charged peptides 1 to 5 in acid condition, their correlation coefficients were improved closely to 0.999 by Eq.(5).. This indicated that there was the other interaction on the MEX column to affect the retentions of peptides besides the hydrophobic interaction.

Obviously, the other interaction was electrostatic interaction contributed by benzenesulfonic acid groups.. Buffer pH 2.5 was used to maximize the basic character of peptides, where basic residues in peptide were completely protonated to be positively charged, while acid residues were protonated to be neutral. Thus the net positive charges of peptide 1 to 7 were 2, 2, 3, 3, 3, 0 and 0, respectively (table 1, including free α-Amino). As showed in Fig.1, peptides' elution orders on the MEX column were generally corresponding to the increasing of net positive charges (peptides 6 and 7 < peptides 1 and 2 < peptides 3, 4 and 5), although ACN content in the mobile phase was changed from 20% to 45%. Peptides with more positive charges showed longer retention because of their stronger electrostatic attractions with the negatively charged MEX column. This result indicated that the electrostatic interactions dominated the separation mechanism of the MEX column, which

was similar to a traditional SCX column. However, differently, the increase of ACN content was found to affect the separation selectivity of peptides with the same charges on the MEX column.. To be observed in Fig.1, the reversed retention order of peptides 1 and 2 (all with two net positive charges) happened when ACN content increased from 20% to 30%. And when ACN content reached to 35%, the resolution among peptides 3, 4 and 5 (all with three net positive charges) was becoming poor. Hydrophobicity of peptide 2 was higher than that of peptide 1, and hydrophobicity of peptide 4 was higher than those of peptides 3 and 5 (table 1). For retentions of those peptides with the same charges, higher hydrophobic ones (such as peptides 2 and 4) tended to retain more strongly along with the increase of ACN concentration. These results also demonstrated the presence of hydrophobic interaction on the MEX column, which was caused by the cyano-propyl groups.

Comparatively, seven standard peptides were further separated on a C18 column. The ACN content in mobile phase was fixed at 25%, and other conditions in mobile phase was same as that used for the MEX column. As seen from Fig.2, peptides 2, 3, 4 and 6 showed completely different retention behaviors from those on the MEX column, and peptides 1 and 5 were not eluted under this condition. This result indicated a potential orthogonality existing between the MEX and C18 columns. It should be noted that structure of peptide 6 was similar to the peptide 7 except the free C-terminus, and it was a neutral-liked peptide in acidic condition. On the MEX column they were hardly retained, whereas they were well separated on the C18 column. Compared with C18 column, the hydrophobic property of MEX column were too weak to retain small neutral peptides.

The above results revealed that electrostatic and weak hydrophobic interactions were involved in peptide separation on the MEX column. Separation property of the MEX column was mainly based on the electrostatic interactions just like that of the traditional SCX column. But for peptides with the same charges, their separation selectivities were further affected by the weak hydrophobic property of the column. Through optimization of ACN content in the mobile phase, peptides with the same charges could be separated on the MEX column according to their different hydrophobic properties. This characteristic further allowed the MEX column to overcome some drawbacks of the traditional SCX columns. Suitable ACN content was significant for peptide separation on the MEX column. According to Fig.1, 30%

ACN was the optimal organic modifier content in the mobile phase when considering suitable retention and good resolution of these standard peptides.

3.3 The effect of salt concentration on peptides' retention under the MEX column

In this study, to confirm that the electrostatic interaction mechanism contributed by benzenesulfonic acid groups dominated peptide separation on the MEX column, varying $NaClO_4$ proportions of 15%, 20%, 25%, 30% and 35% in mobile phase were utilized, whose concentrations were 150 mM, 200 mM, 250 mM, 300 mM and 350 mM, respectively. The content of ACN was kept at 25% and the TEAP buffer was kept at 10 mM, pH 2.5. Neutral salt $NaClO_4$ was employed in this paper due to its good solubility in high content of organic modifier[28]. The effect of $NaClO_4$ concentration on the retentions of peptides was shown in Fig. 3. Peptides 6 and 7 were neutral in acidic condition, so they were not in consideration. Retentions of other five peptides all decreased with increasing $NaClO_4$ concentration, which was because electrostatic attractions between positive peptides and negative MEX column were suppressed. Retention model expressed as Eq.(6)[29, 30],

$$\ln k = a + b \ln C_S \quad (6)$$

was employed to quantitatively descript the retention factors of peptides in ionic exchange chromatography, where C_S representted the content of $NaClO_4$ in the mobile phase. The results of multiple regression analysis between $\ln k$ and $\ln C_S$ was listed in table 3, and good linear relationships ($R^2 \geq 0.999$) were exhibited for all charged peptides. As the slopes (b) were related to the charge numbers of peptides, peptide with highest absolute b value indicated itself carried most charged numbers, such as peptide 5 with most positive numbers who had a strongest retention on the MEX column. In this experiment, retention times of peptide 1 and 2 (both with one positive) were overlapped, which was probably caused by the low separation efficiency with isocratic $NaClO_4$ condition.

The salt effect on peptides retention was further evaluated under the neutral condition of pH 6.3. Because peptides weakly retained at pH 6.3, the $NaClO_4$ proportions in mobile phase were adjusted to 10%, 15%, 20%, 25% and 30%, whose concentrations were 100 mM, 150 mM, 200 mM, 250 mM and 300 mM, respectively. Neutral peptide 7 was not in consideration here. As seen in Fig. 4, retentions of four basic peptides 2 to 5 declined along with the increase of $NaClO_4$ concentration while retentions of two acid peptides 1 and 6 increased. For

acidic peptides, electrostatic repulsions between dissociated carboxyl groups and negatively charged MEX column were suppressed by the increasing $NaClO_4$ content, leading to peptides' longer elution. The plots of $\ln k$ versus $\ln C_S$ still performed good linear relationships, but correlation coefficients in this case were lower than those at pH 2.5, especially for acid peptides. It was probably caused by errors of retention times. Due to poor retentions of peptides at pH 6.3, baseline separation for peptides did not achieve especially at high $NaClO_4$ concentration, resulting in the difficulty in reading retention times. It was to be noted the absolute values of slopes of basic peptides 3 to 5 in neutral condition were lower than those in acidic condition. This was because net positive charges of peptides were declined when pH changing to pH 6.3. Only the absolute values of slope of peptide 2 was slightly changed in two conditions, since peptide 2 only contained one protonated amino acid residue Lys (pKa 10.4)[15], which was hardly affected when pH was changed from 2.5 to 6.3.

In conclusion, the results in this section confirmed the dominated effect of electrostatic interactions on the peptides' separation on the MEX column. With the increase of $NaClO_4$ concentration, electrostatic attractions between basic peptides and negatively charged MEX column were suppressed to make the retention decrease. In contrast, the electrostatic repulsions between carboxyl groups of acidic peptides and negatively charged MEX column were weakened and resulted in their increased retention. Along with the increase of $NaClO_4$ concentrations, there was no change in retention orders of standard peptides. This indicated the $NaClO_4$ concentrations had no influence on separation selectivity of the MEX column to peptides.

3.4 The effect of buffer pH on peptides' retention under the MEX column

Since the sulfonic acid groups on the MEX column are strongly acidic and minimally change within the pH ranges of 1 to 7, thus pH in the mobile phase mainly influences the ionization of peptides. In Fig.5, the plots of retention factors of seven peptides versus different pH values of 2.5, 4.0, 6.3 and 8.0 were shown. At pH 2.5, the strongest retentions of peptides were obtained due to their highest positive capacities in this condition, but wide peak shapes were followed (not shown). With the pH rising, peptides' retentions were largely decreased and peak shapes became sharp (not shown). In specially, peptide 2 exhibited a distinctive retention curve. As described above, it just contained one basic residue but no an acidic group,

the effect of increased pH on its retention was limited in the ranges of 2.5 to 6.3. When pH reached to 8.0, the single Lys residue was deprotonated to be neutral, leading to its greatly decreased retention. For other peptides, like 1 to 6, all contained acidic residues. With increasing pH, electrostatic repulsions were increased due to the gradually dissociated carboxyls in acidic residues, leading to their strong decline of retentions. Neutral Peptide 7 was not affected by the changed pH values due to the lack of ionic residues. As can be seen, separation selectivity of peptides on the MEX column could be affected by the pH in mobile phase. At pH 2.5, peptides 1 and 2, 6 and 7 were not separated, but they obtained good separation when pH at 4.0 or 6.3. Therefore, a suitable buffer pH for the MEX column was necessary to obtain good resolution and moderate separation of peptides with good peak shapes, in this study, pH 6.3 was thought as the optimal conditions.

3.5 An off-line 2D-MEX× C18 system coupled with tandem mass spectrometry for separating and analyzing scorpion venom peptide

After the fundamental studies, the MEX column was employed in separation of peptides from scorpion venom. As discussed above, organic modifier concentration, salt concentration and buffer pH in the mobile phase were demonstrated to have important effects on peptides' retention and selectivity. After optimization, ACN content and pH were 30% and 6.3, and $NaClO_4$ was in a liner gradient of 50 mM to 250 mM within 25 min, TEAP buffer was fixed at 10 mM. As seen from Fig.6A, good performance was obtained for the separation of scorpion venom samples on the MEX column. It was notable that the MEX column (150 mm×4.6 mm i.d., 5 μm) could be subjected to 10 mg samples without losing separation efficiency (Fig.8A). When injection volumes (2 μL to 50 μL) increased, the peak shapes always kept well. And high repeatability of the chromatograms was obtained among twenty injections (50 μL). It demonstrated that the MEX column possessed good loading capacity, excellent stability and repeatability, which was beneficial for its use in future preparation experiment. In fact, this characteristic of the MEX column may be attributed to the silica-based supports, Zhen et.al.[31] has reported that silica-based SCX packing had high mechanical strength and excellent stability.

The separation of the sample was also performed on an XChargeC18 column (Fig. 6B). The results of complementary separations between two columns were observed. These two

columns were employed to established off-line 2D-LC system for separating peptides from the scorpion venom. Considering the salt used in mobile phase not compatible with MS, the MEX column was used in the first dimension. During the elution times of 40 min, 20 fractions were manually collected from the MEX column at 2 min intervals. Each fraction was re-separated on the XCharge C18 column directly to an ESI mass spectrometer with 0.1% TFA (pH 1.8) in the mobile phase. The separation chromatograms were showed in Fig.7, and baseline separations were observed in many fractions. Taking Fractions 6 and 8 as examples (Fig. 8A), for Fraction 6, two main peaks were separated in first dimension, whereby eight peaks were baseline-separated in second dimension (Fig. 8B). Similarly, one main peak with several small peaks in Fraction 8 in first dimension was separated into four peaks on the XCharge C18 column (Fig. 8C). All of these results potentially exhibited a high orthogonality between the MEX and C18 columns. Further ESI-MS analysis of peaks in Fig.7 revealed them all toxin peptides, and repeated data in near fractions were deleted. Based on the retention time of these peptides, a normalized 2D plot was constructed to quantitatively evaluate orthogonality. As shown in Fig.9, the plot was divided into 20×20 bins, and unique peptides occupied 140 bins. The orthogonality was calculated as 47.62%. Possible factors contributing to this high orthogonal result were explained as follows. In the first dimension, peptides separated on the negatively charged MEX column at pH 6.3 were mainly based on electrostatic interactions, where basic peptides were attracted while acidic peptides were repulsed. Meanwhile, the weak hydrophobic interactions enhanced column's selectivity for peptides with the similar charges. In the second dimension, peptides were separated on the XCharge C18 column, whose separation mechanism of hydrophobicity was complementary to that of the MEX column. Besides, under the acid condition of pH 1.8, weak anionic exchange interactions were involved in the XCharge C18 column[32], which showed different electrostatic interactions with the MEX column. Therefore, good orthogonality was realized by the 2D-MEX×RP system. Overall, the MEX column could be a powerful alternative to existing SCX column in 2D separation of peptide from complex scorpion venom.

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4. Conclusion

In the current study, a modified cation exchange column named MEX bonded with benzenesulfonic acid and cyano-propyl groups was firstly introduced by us to separate complex scorpion venom peptide. MEX stationary phase was bond with two functional groups of benzenesulfonic acid and cyano-propyl, in which the cyano-propyl groups were introduced to reduce the density of sulfonic groups to decrease cation exchange capacity of the stationary phase. To better understand the separation mechanisms of the MEX column, seven standard peptides with different properties were employed for the evaluation study. Results showed two interactions were involved in peptides' separation on the MEX column: electrostatic interactions based on benzenesulfonic acid groups were approved to dominate separation mechanism of the MEX column and weak hydrophobic interactions caused by the cyano-propyl groups were evolved to improve the separation selectivity for peptides withthe same chargs. This characteristic would allow the MEX column to overcome the drawbacks of the traditional SCX column which the similar charged peptides were eluted within a narrow window. Furthermore, the evaluated studies also demonstrated the great effects of the organic modifier contents, $NaClO_4$ concentrations and buffer pH in the mobile phase on retention and separation selectivity of peptides. Therefore, for good separation performance of the MEX column, the condition of the mobile phase was optimized as: ACN content and pH were 30% and 6.3, and $NaClO_4$ was in a liner gradient of 50 mM to 250 mM within 25 min, TEAP buffer was fixed at 10 mM. Subsequently, the MEX column was combined with a C18 column to establish an off-line 2D-MEX×C18 system for separating scorpion venom peptides from *Buthus martensi* Karsch (*Bm*K). The orthogonality of this system was calculated as 47.62%. The high orthogonality was attributed the complementary separation mechanisms of columns and the different pH conditions used in each dimension. Meanwhile, in the separation process, good loading capacity, excellent stability and repeatability were exhibited by the MEX column. These were beneficial for its use in future preparation experiment. Overall, the MEX column could be an alternative to existing SCX column in 2D separation of scorpion venom peptides.

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Figure list

Fig.1 Chromatograms of seven standard peptides separated on the MEX column (250 mm×4.6 mm i.d., 10 μm) under the different ACN concentration respectively: (A) 20%; (B) 25%; (C) 30%; (D) 35%; (E) 40%; (F) 45%. Seven standard peptides were referred to table 1. Conditions: 5%-35% $NaClO_4$ (50 mM to 350 mM) over 35 min; 10 mM TEAP, pH 2.5; flow rate: 1.0 mL/min; column temperature: 30°C; wavelength: 214 nm.

Fig.2 Chromatograms of seven standard peptides separated on the MEX column (A) and C18 column (B), peptides 1 and 5 were not eluted from C18 column within 35 min. Conditions: 25% ACN; 5%-35% $NaClO_4$ (50 mM to 350 mM) over 35 min; 10% TEAP (10 mM, pH 2.5); flow rate: 1.0 mL/min; column temperature: 30°C; wavelength: 214 nm.

Fig.3 Plots between logarithms of the retention factor ($\ln k$) versus $NaClO_4$ concentration ($\ln C_S$) on the MEX column (250 mm×4.6 mm i.d., 10 μm) at pH 2.5. $NaClO_4$ concentrations were 15%, 20%, 25%, 30% and 35%, respectively (150 mM, 200 mM, 250 mM, 300 mM and 350 mM). Conditions: 25% ACN; 10 mM TEAP; flow rate: 1.0 mL/min; column temperature: 30°C; wavelength: 214 nm.

Fig.4 Plots of logarithms of the retention factor ($\ln k$) versus $NaClO_4$ concentration ($\ln C_S$) on the MEX column (250 mm×4.6 mm i.d., 10 μm) at pH 6.3. $NaClO_4$ concentrations were 10%, 15%, 20%, 25%, 30%, respectively (100 mM, 150 mM, 200 mM, 250 mM and 300 mM). At low $NaClO_4$ content of 10% and 15%, retention of acid peptide 1 was so weak on the MEX column that its retention time was less than the void time. Their retention factors could not be calculated in this case. Conditions: 25% ACN; 10 mM TEAP; flow rate: 1.0 mL/min; column

temperature: 30°C; wavelength: 214 nm.

Fig.5 Plots of peptides' retention factors (*k*) on the MEX column (250 mm×4.6 mm i.d., 10 μm) against eluent pH (2.5, 4.0, 6.3 and 8.0). Conditions: 25% ACN; 5%-35% $NaClO_4$ (50 mM to 350 mM) over 35 min; 10 mM TEAP; flow rate: 1.0 mL/min; column temperature: 30°C; wavelength: 214 nm.

Fig.6 Chromatograms of scorpion venom sample separated on the MEX column (A) and XChargeC18 column (B). Condition for the MEX column (150 mm×4.6 mm i.d., 5 μm): 0-5-30-40 min, 5%-30%-30%-50% ACN, 5%-5%-25%-25% $NaClO_4$ and continuously 10 mM TEAP, pH 6.3; condition for the C18 column (150 mm×4.6 mm i.d., 5 μm): 0-10-25-35-40 min, 5–20-30-40-90% ACN (containing 0.1% TFA). Flow rate: 1.0 mL/min; column temperature: 30°C; wavelength: 214 nm; injection volume: 2 μL (200 mg/mL).

Fig.7 3D Chromatograms of 2D-MEX×C18 system, *x-axis* was the number of fractions, *y-axis* was retention times of peptides in the second dimension, *z-axis* was absorbance responses.

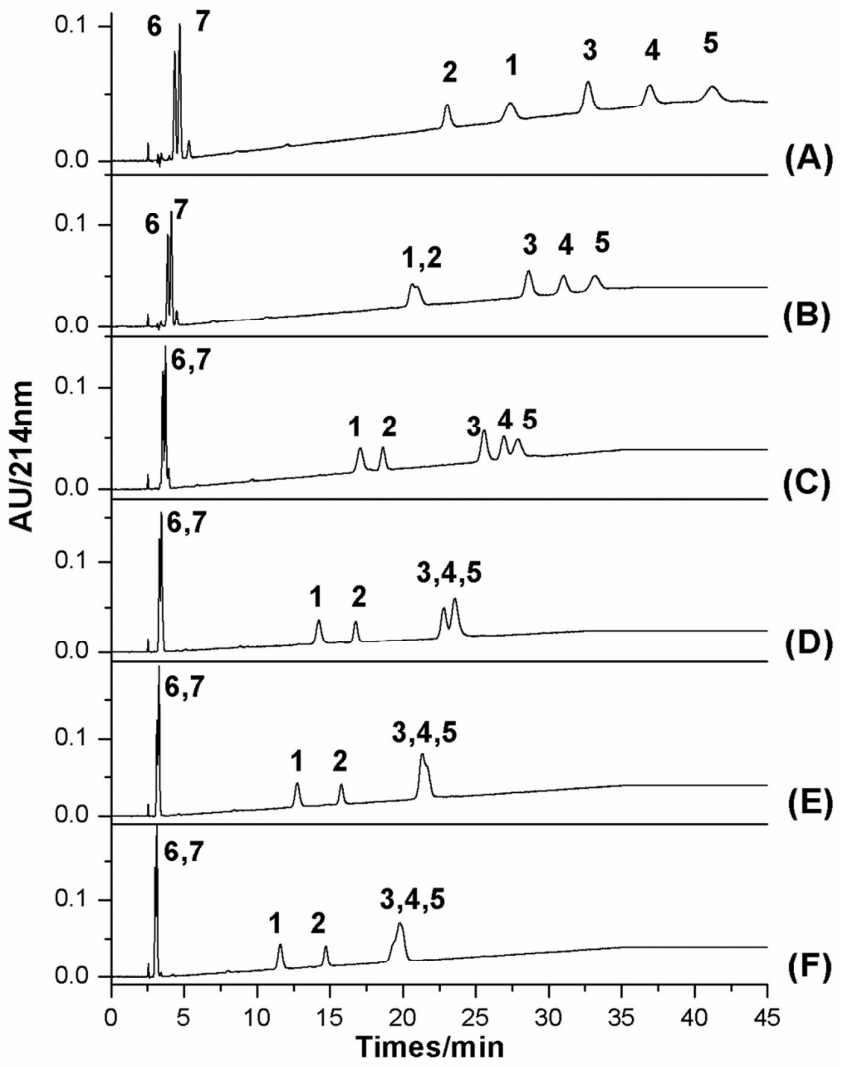
Fig.8 Chromatograms of scorpion venom sample separated on the MEX column in first dimension, and Fractions 6 and 8 showed by the arrows (A). Fraction 6 (B) and Fraction 8 (C) were re-separated on a C18 column in second dimension. Condition for the MEX column: 0-5-10-35-45 min, 5%-5%-30%-30%-50% ACN, 5%-5%-5%-25%-25% $NaClO_4$ and continuously 10 mM TEAP, pH 6.3; flow rate: 1.0 mL/min; column temperature: 30°C; injection volume: 50 μL (200 mg/mL); wavelength: 280 nm. Condition for the C18 column: 0-10-25-35-40 min, 5–20-30-40-90% ACN (containing 0.1% TFA); flow rate: 1.0 mL/min; column temperature: 30°C; injection volume: 20 μL (1 mg/mL); wavelength: 214 nm. The $NaClO_4$ salt was eluted within 10 min.

Fig.9 Normalized retention time plots for 2D-MEX (first dimension)×C18 (second dimension) in separation of scorpion venom peptides.

Table 1. Amino acid sequence, calculated mass weight, isoelectric point (p*I*), hydrophobicity at pH 2.0 and net charges of seven standard peptides.

Table 2. The results of regression coefficients of retention models $\ln k = \mathrm{a} + \mathrm{c}C_B$ and $\ln k = \mathrm{a} + \mathrm{b}\ln C_B + \mathrm{c}C_B$ for seven peptides separated on the MEX column.

Table 3. The results of regression coefficients of retention model $\ln k = a + b\ln C_S$ on the MEX column at pH 2.5 and 6.3, respectively.



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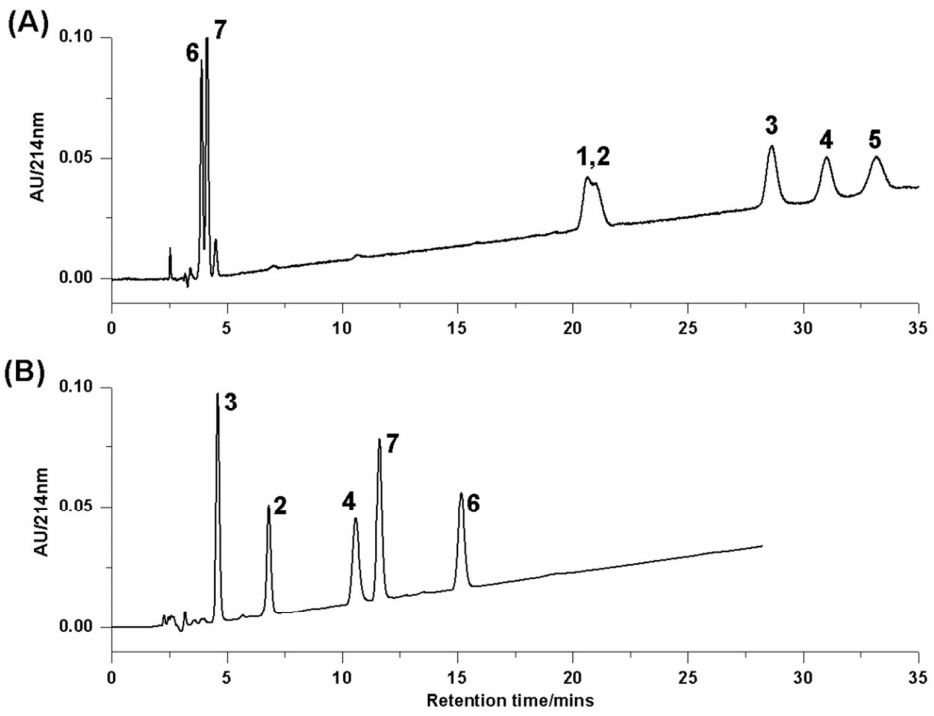

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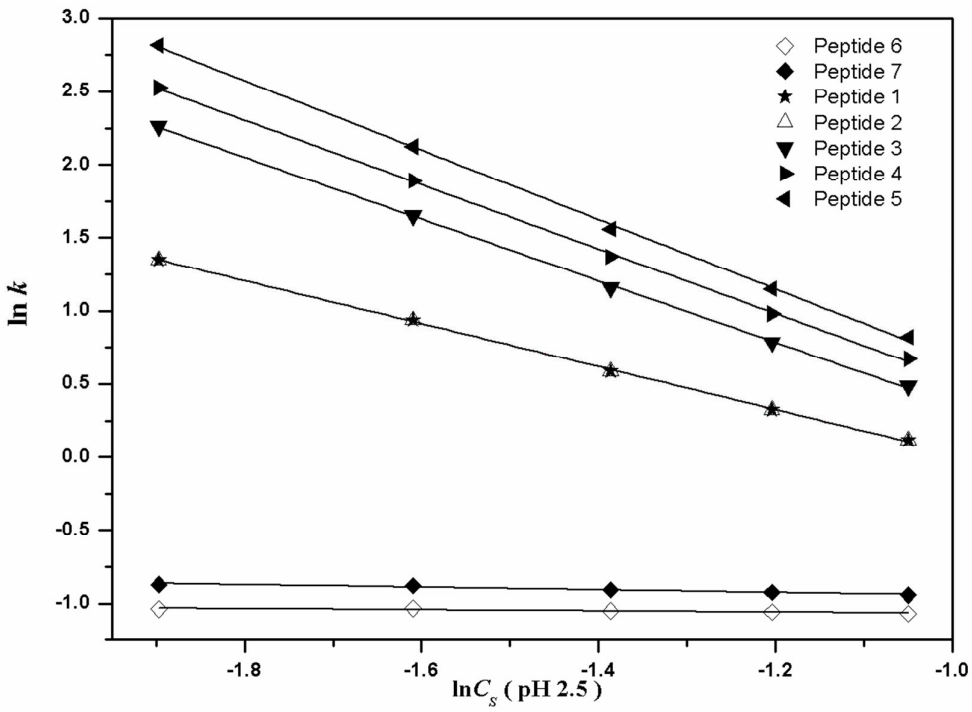

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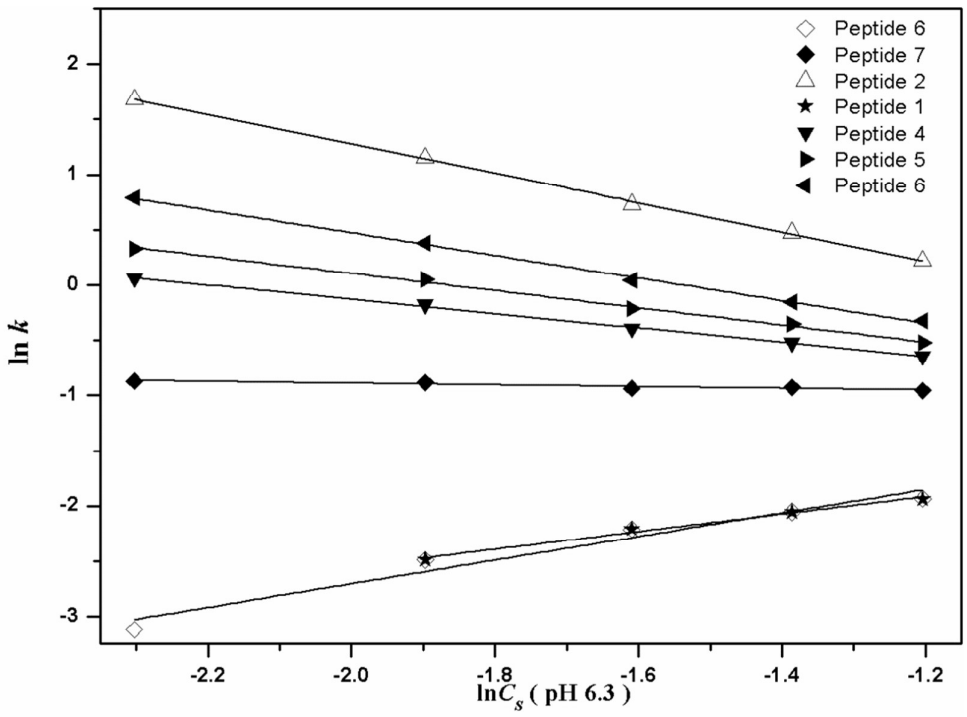

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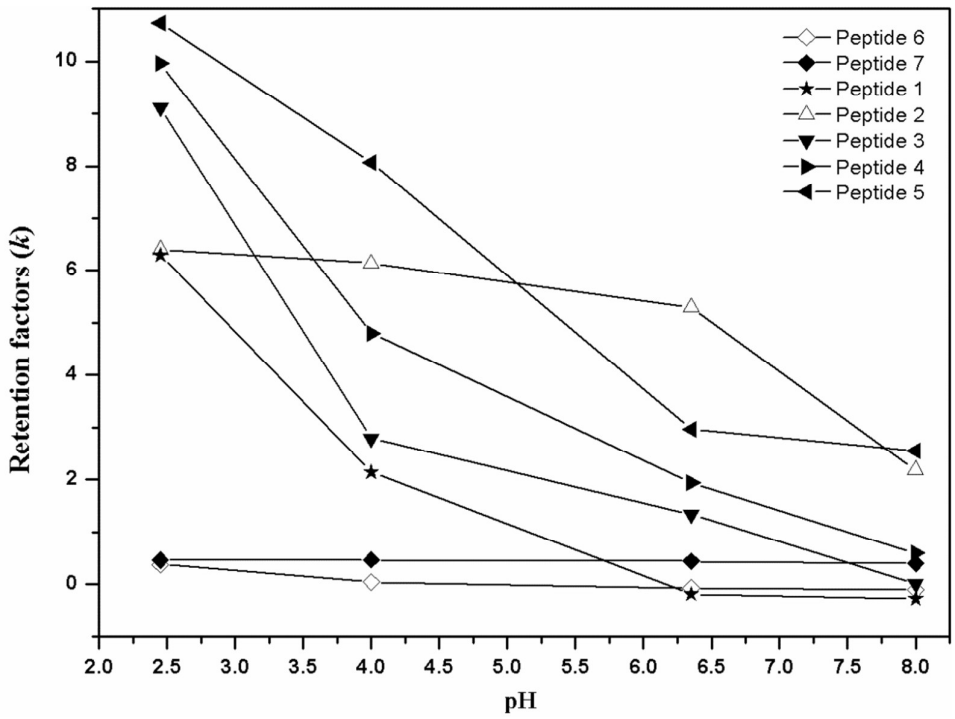

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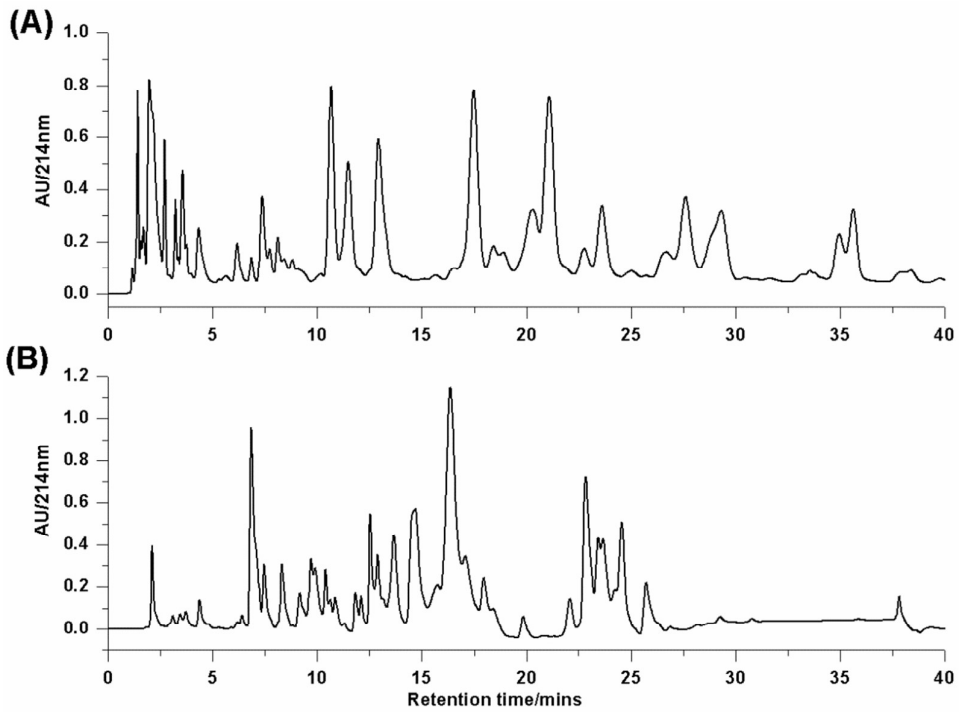

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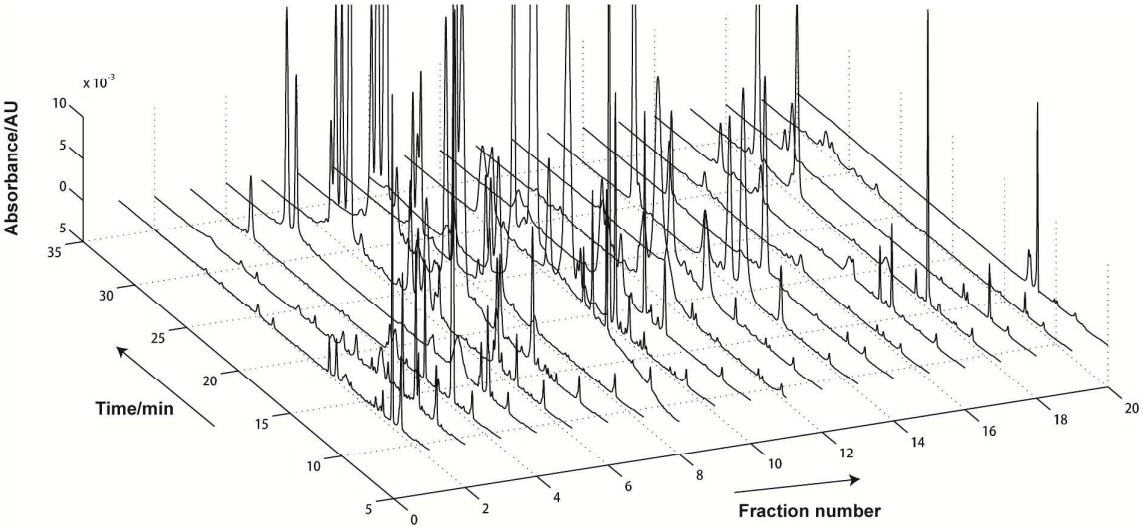

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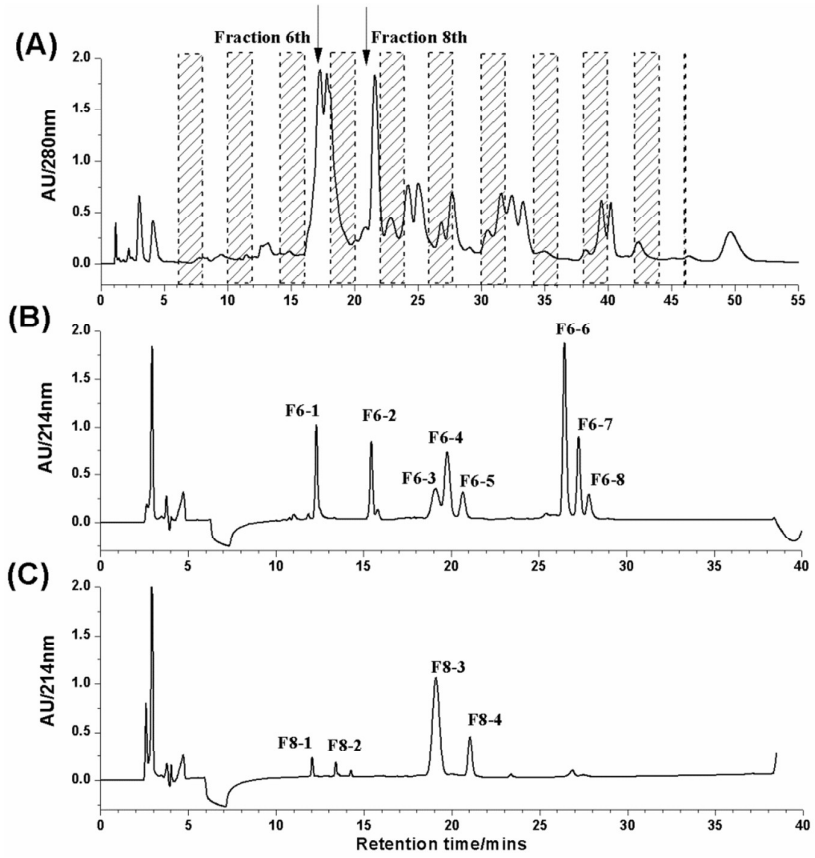

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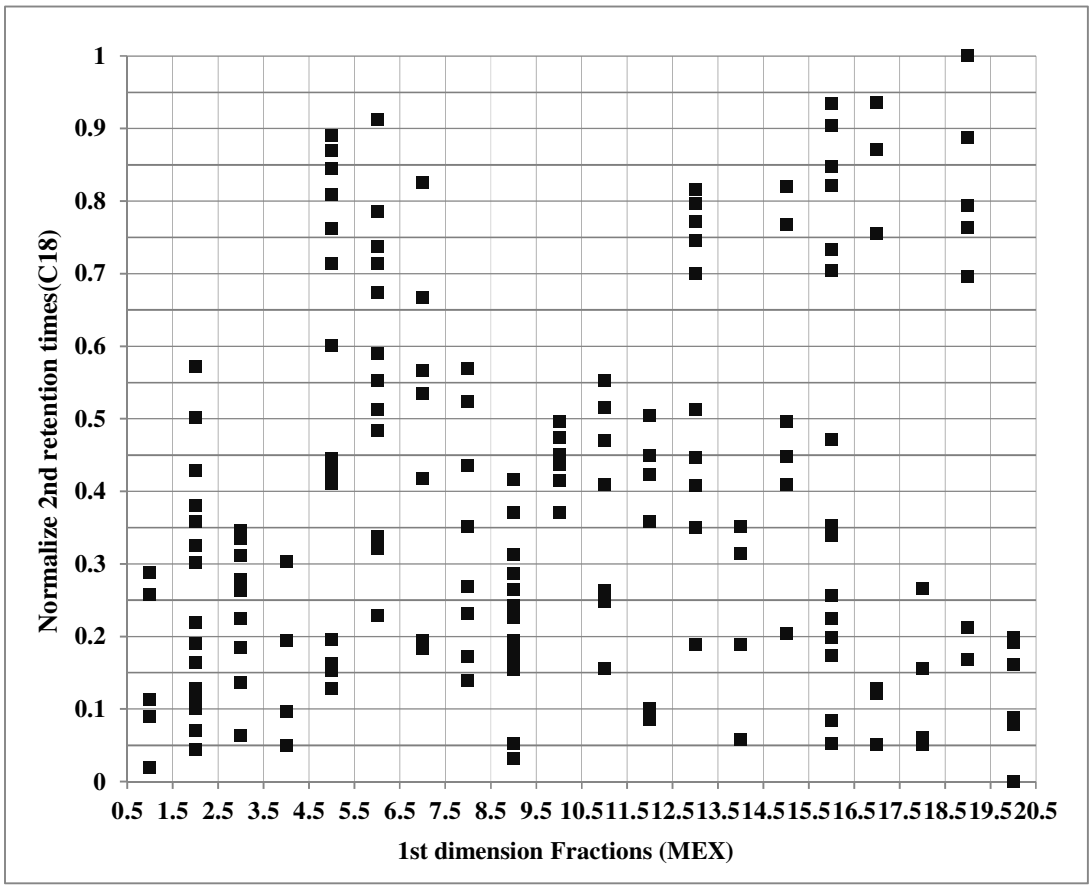

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Table 1. Amino acid sequence, calculated mass weight, isoelectric point (p*I*), hydrophobicity at pH 2.0 and net charges of seven standard peptides.

Peptide code	Sequence[a]	Mass weight	p*I*	Hydrophobicity at pH 2.0[b]	Net charge[c]
1	FP**R**PGGGGNG*D*F*EE*IP*EE*YL	2180.42	3.35	12	-4
2	SLIG**K**V-amide	614.83	10.28	39	1
3	*D***R**VYI**H**P	899.05	7.95	14	1
4	YSF**K***D*MQLG**R**	1244.48	9.79	21	1
5	Pyr-LY*E*N**K**P**RR**PYIL	1673.01	9.85	15	2
6	Acetyl-FGGF	468.53	6.09	46	-1
7	Acetyl-FGGF-amide	467.55	7.00	46	0

[a] Bold represents basic amino acid residue and Italic represents acid basic amino acid residue in the sequence, free α-Amino and α-Carboxyl are not written.

[b] Hydrophobicity value at pH 2.0 was calculated by referring to http://www.biosyn.com, it was hydrophobicity value of individual amino acid at pH 2.0 / total number of amino acid in the peptide, high value indicated higher hydrophobicity.

[c] Each acidic amino acid residue is assigned a value of -1, basic amino acid residue is assigned a value of 1 and neutral amino acid residue is assigned a value of 0.

Table 2. The results of regression coefficients of retention models $\ln k = \mathrm{a} + \mathrm{c}C_B$ and $\ln k = \mathrm{a} + \mathrm{b}\ln C_B + \mathrm{c}C_B$ for seven standard peptides separated on the MEX column.

Peptide code	$\ln k = \mathrm{a} + \mathrm{c}C_B$			$\ln k = \mathrm{a} + \mathrm{b}\ln C_B + \mathrm{c}C_B$			
	a	c	R^2	a	b	c	R^2
1	2.8869	-4.0629	0.9642	-1.1006	-1.8090	1.7456	0.9987
2	2.3846	-2.1608	0.9861	1.3504	-0.4691	-0.6544	0.9927
3	2.7828	-2.2741	0.9844	1.3739	-0.6391	-0.2218	0.9980
4	2.9921	-2.7339	0.9757	0.8116	-0.9892	0.4422	0.9986
5	3.2245	-3.3587	0.9736	0.4403	-1.2631	0.6969	0.9982
6	1.0490	-8.1182	0.9979	2.7321	0.7635	-10.5698	0.9993
7	0.9933	-7.0642	0.9995	0.4798	-0.2329	-6.3162	0.9996

Table 3. The results of regression coefficients of retention model $\ln k = a + b \ln C_S$ on the MEX column under pH 2.5 and 6.3，respectively.

Peptide code	$\ln k = a + b \ln C_S$ (pH 2.5)			$\ln k = a + b \ln C_S$ (pH 6.3)		
	a	b	R^2	a	b	R^2
1	-1.4353	-1.4683	0.9994	-1.3861	0.7951	0.9855
2	-1.4353	-1.4683	0.9994	-0.9605	-1.3325	0.9989
3	-1.7442	-2.1082	0.9993	-1.4241	-0.6476	0.9976
4	-1.6606	-2.2035	0.9993	-1.4507	-0.7782	0.9961
5	-1.6967	-2.3733	0.9990	-1.5756	-1.0246	0.9976
6	-1.1063	-0.0395	0.7381	-0.5700	1.0667	0.9515
7	-1.0322	-0.0889	0.9267	-1.0442	-0.0774	0.8191