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Two-dimensional liquid chromatography separation of peptides using reversed-phase/weak cation-exchange mixed-mode column in first dimension

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ABSTRACT

Selection of a first-dimensional separation method is very important in proteomic studies performed with two-dimensional liquid chromatography (2D-LC), since reversed-phase chromatography (RPLC) is usually chosen as the second-dimensional separation method in most studies. In this paper, we assess the potential use of mixed-mode reversed-phase/weak cation-exchange (RP/WCX) chromatography in 2D-LC proteomic studies. First, a new RP/WCX mixed-mode stationary phase (named C18WCX) was synthesized based on the polar-copolymerized approach, and a C18WCX column with separation efficiency comparative to conventional C18 columns was developed. This new mixed-mode column primarily provides hydrophobic interactions under acidic condition, but can offer hydrophobic and cation-exchange interactions under neutral and weak basic conditions. An off-line 2D-RP/WCX–RPLC system was established using the C18WCX column with a mobile phase of pH 6.5 in the first dimension and a C18 column with a mobile phase of pH 3.0 in the second dimension. The orthogonality of this two-dimensional system, evaluated through the separation of 123 tryptic peptides, was shown to be higher than that of the conventional 2D-RP–RPLC approach. Applying this 2D-LC method to rat brain samples, we identified 1031 proteins and 4397 unique peptides. In addition, this new two-dimensional method improved the identification of basic peptides. Therefore, we propose that this novel 2D-RP/WCX–RPLC system can be used as an alternative approach for the two-dimensional separation of peptides.

1. Introduction

At present, LC coupled with tandem mass spectrometry is one of the most commonly used techniques in shotgun or bottom-up proteomics [1,2]. Current challenges in proteomic studies include sample complexity and the huge dynamic range of the peptides [3]. Despite great advancements in column and instrument technologies [4–6], the separation performance of one-dimensional LC is insufficient for reducing the complexity of peptide mixtures for comprehensive analysis. However, Yates et al. introduced the multidimensional liquid chromatography (MDLC) technique for proteomic analysis, and naming it multidimensional protein identification technology (MudPIT) [7,8]. This technique has been shown to greatly improve the comprehensiveness in shotgun proteome analysis [1,2].

In most shotgun proteomic studies, RPLC is usually chosen for the last dimension of separation before MS analysis due to its high separation efficiency and the compatibility of its mobile phase with MS detection. Selection of the first-dimensional separation mode directly influences the separation power of a 2D-LC system, and the most commonly used first-dimensional LC technique in 2D-LC proteome analysis is strong cation-exchange chromatography (SCX) [1,2,9–15]. SCX can provide good orthogonality to RPLC, since the retention mechanism of peptides on SCX is very different from RPLC. SCX uses electrostatic interactions to retain peptides, while the retention of peptides in RPLC is mainly based on hydrophobic interactions [8]. However, the main drawbacks of SCX are relatively low separation efficiency and poor resolution; the majority of peptides cluster in a relatively narrow elution window since most of them contain charges of +2 or +3 [16]. Furthermore, the need to desalt of fractions from the SCX column is time-consuming and causes sample loss. In order to overcoming these shortcomings of the SCX-RP approach, size exclusion chromatography (SEC) [17,18] and affinity chromatography (AC) [19] have also been combined with RPLC for shotgun proteome analysis. However, poor separation power limits their extensive application. Recently, HILIC has been suggested as a good alternative candidate

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for the first dimension in a two-dimensional hyphenated system, since it shows comparative separation power and good orthogonality to RPLC [20]. The 2D-HILIC–RPLC approach has been used to separate various peptides and modify peptides from cellular lysates and tissues [21–23]. However, the utility of HILIC may be hindered by its ambiguous separation mechanism and poor solubility of peptides in solvents with high organic content. In recent years, 2D-RP–RPLC system with different pH values in the two dimensions has become another attractive option for shotgun proteomics [16,20,24], especially since RPLC provides significantly greater peak capacity than other LC techniques. It is well-known that the physicochemical properties such as hydrophobicity, hydrophilicity and charge state of peptides can be altered by changing pH value; thus, selectivity for the RPLC separation of peptides could also be changed so that considerable orthogonality is achieved in this two-dimensional system. However, the orthogonality of this 2D-LC system is not optimal due to the similarity of retention mechanism of RP stationary phases used in both LC dimensions. In order to improve the orthogonality of the 2D-RP–RPLC separation, Song et al. [25] recently described a new fractionation system for the first dimension.

Another potential way to improve orthogonality in the 2D-RP–RPLC system could be the use of a column with similar separation performance but different selectivity compared to RPLC in the first dimension. For peptides, which have both charged and hydrophobic characteristics, LC separations involving mixed-mode ion-exchange and reversed phase columns could be superior to conventional RP columns, because the selectivity of the mixed-mode columns would be improved by the addition of ion-exchange interactions. The chromatographic technique, which employs a mixed-mode column and allowing more than two interactions in one separation, is known as mixed-mode chromatography (MMC) [26]. It has been widely used in the solid phase extraction since the end of the twentieth century [27,28] and has gradually been applied to the HPLC separations in recent years [29]. The most representative MMC technique is the mixed-mode HILIC/IEC technique, which has shown excellent performance in the comprehensive analysis of peptides and phosphopeptides in recent years. Recently, MMC techniques which embed ionic groups into the RPLC columns have also been developed and widely applied to the separation of basic and acidic compounds [30–32]. Although there have been studies about the retention mechanisms of peptides on a mixed-mode RP/anion-exchange column [33] and the use of mixed-mode RP/anion-exchange columns for the purification of target peptides [34], there have been few studies about the use of ion-exchange and RP mixed-mode columns in proteomics, especially in 2D-LC proteomic studies.

Stationary phase is important for successful LC or 2D-LC separation. Over the past few years, we have developed many new stationary phases which show remarkable performance in 2D-LC separation [35–38] and post-translational modification proteomics [39,40]. Recently, a "polar-copolymerized" approach has been developed in our lab for preparation of RP stationary phases with various embedded polar or ion groups [41]. In this work, we developed a new silica-based stationary phase composed of mixed n-octadecyl and 3-carboxypropyl groups based on the "polar-copolymerized" approach. In this stationary phase, the octadecyl ligands provide hydrophobicity while copolymerized 3-carboxypropyl groups provide cation-exchange sites. A mixed-mode reversed-phase/weak cation-exchange column named C18WCX was packed with this stationary phase, and the separation selectivity of peptides on this column was evaluated. Then, we optimized the most suitable separation conditions for conducting an off-line 2D-RP/WCX–RPLC system. The optimized two-dimensional system was used to characterize tryptic peptides from the soluble protein fractions of rat brain samples.

2. Experimental procedures

2.1. 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 (Aichi, Japan). Octyltrichlorosilane and 3-cyanopropyltrichlorosilane were obtained from ABCR (Karlsruhe, Germany). The peptide standards, hemoglobin, α-casein, bovine serum albumin (BSA), phosphorylase b, trypsin, Tris, Triton-100, guanidine hydrochloride, sodium orthovanadate (Na_3VO_4), and sodium fluoride (NaF) were purchased from Sigma (St. Louis, MO, USA). Urea, ammonium bicarbonate, DTT, iodoacetamide (IAA), EDTA and PMSF were all purchased from BioRad (Hercules, CA, USA). Protease inhibitor cocktail tablets (Complete Mini) were purchased from Roche Diagnostics (Mannhaim, Germany). Formic acid (FA) and ammonium formate (NH_4FA) were obtained from ACROS Organics (New Jersey, USA). HPLC grade acetonitrile (ACN) was purchased from Fisher (Fair Lawn, NJ, USA). Water was purified by a Milli-Q water purification system (Billerica, MA, USA). All other chemicals were of analytical grade and used without purification.

2.2. Synthesis of the stationary phases and column packing

The polar copolymerized C18WCX stationary phase was synthesized according to a previous report [41–43]. In brief, 15 g of humidified silica was placed in a flask under a blanket of nitrogen, along with a stirring bar and 45 mL of anhydrous toluene. A solution of 6 mL octyltrichlorosilane, 4.7 mL 3-cyanopropyltrichlorosilane (1:2 molar ratio of the two silanes) and 30 mL of toluene was added to the pretreated silica gel. The reaction was allowed to continue for 24 h. The silanized silica was filtered and washed with 20 mL each of dichloromethane, methanol, water and methanol in sequence successively. Then, the silanized silica was added to 120 mL of 30% sulfuric acid/methanol solution (3/1, v/v) and was stirred at 80 °C for 10 h to obtain carboxyl groups through the hydrolysis of cyan. The suspension mixture was cooled to room temperature and filtered, and the solid product was washed with water and methanol successively. The product was dried at 80 °C overnight to obtain the resulting C18WCX stationary phase. The C18WCX phase was slurry-packed into a stainless steel column with 20 mL of toluene/acetone (1/1, v/v) as the slurry solvent and acetone as the propulsion solvent under a pressure of 40 MPa. Chromatographic evaluation of the C18WCX column was performed on an Alliance HPLC system consisting of a Waters 2695 HPLC pump and a Waters 2996 DAD (Waters, Milford, MA, USA).

2.3. Sample preparation

The peptide standard mixture was prepared by mixing together equal volumes of solutions of six oligopeptides, including angiotensin III, Phe-Gly-Gly-Phe, L-Leucyl-glycyl-glycine, Gly-Gly-His, Glu-Glu, Lys-Gly. The six peptides were dissolved in 0.2% FA at 1 mg mL^{-1}. Tryptic protein digests and soluble protein fraction of rat brain were prepared by reported methods [24,44] (see SI-1 and SI-2).

2.4. LC–MS/MS

One-dimensional LC–MS experiments were performed on an ACQUITY UPLC system (Waters, Milford, MA, USA) which was coupled with a Q-TOF Premier system (Waters MS Technologies, Manchester, UK). The standard peptide mixture and protein digests were subjected to the C18WCX column (2.1 mm × 150 mm, 5 μm, 100 Å, packed in house), which was packed in house, and the XTerra MS C18 column (2.1 mm × 150 mm, 5 μm, 143 Å), which

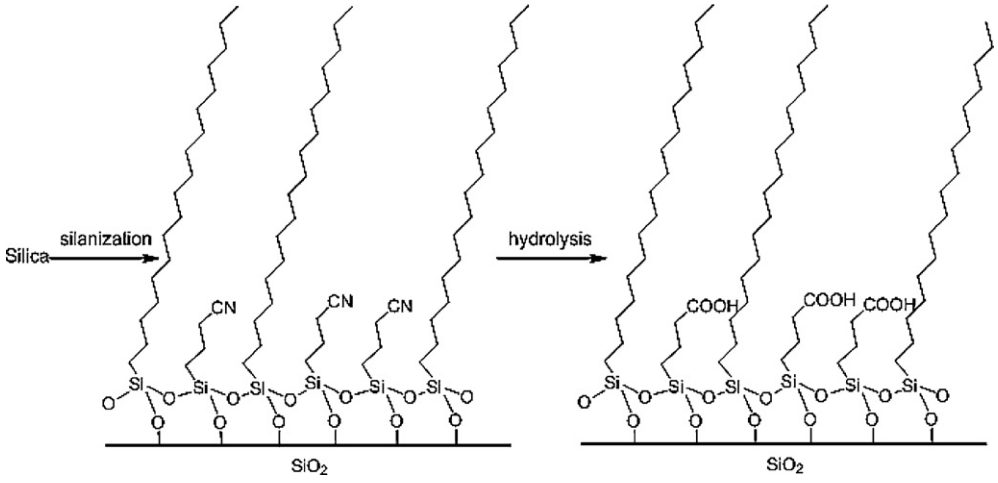

Fig. 1. Surface chemistry of C18WCX.

was purchased from Waters corporation (Milford, MA, USA). The mobile phase consisted of A (20 mM NH_4FA buffer, pH3.0, 4.5, 6.5 and 8.0) and B (ACN). The pH 2.6 mobile phase consisted of A (0.2%FA in water) and B (0.16%FA in ACN). Gradient elution on the C18WCX column for all pH conditions was set as follows: 2%B–50%B in 55 min and then 50%B–90%B in 5 min. Gradient elution on the XTerra column for all pH conditions was set as follows: 5%B–40%B in 60 min and then 40%B–90%B in 2 min. The flow rate was 0.2 mL min^{-1} and the column temperature was maintained at 25 °C for all of the analysis. Column output was coupled to an electrospray ionization source. The mass spectrometer was operated in the positive ion mode in V optics mode. The capillary voltage was set to 35 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 100 °C. The MCP detector voltage was set to 1700 V. Data-dependent analysis was employed. The MS survey was from *m*/*z* 200 to 2000, with a scan time of 0.6 s/scan. The intensity threshold for switching from the MS scan to MS/MS was set at 20 ion counts. The top three ions of greatest intensity were selected for MS/MS study. MS/MS spectra were recorded in the range of 100–3000 Da, with a scan time of 0.9 s/scan. The optimal collision energy was determined by a charge state recognition algorithm. The acquisition time was 60 min.

2.5. 2D-LC–MS/MS

2.5.1. First-dimensional separation

First-dimensional C18WCX separation of tryptic peptides from rat brain samples was performed on an Alliance HPLC system. The mobile phase consisted of A (water), B (ACN), C (200 mM NH_4FA in water, pH 6.5) and D (2% FA in water). 100 μg of the digested proteins of rat brain was loaded onto the C18WCX column. The gradient profile was set as follows: 2%B–50%B, 10%C and 0%D in 0–55 min; 50%B, 10%C and 0%D–10%D in 55–58 min; 50%B–80%B, 10%C and 10%D in 58–60 min. The flow rate was 0.2 mL min^{-1}. Fractions were collected every 1 min.

2.5.2. Nano-LC–MS/MS analysis

The second-dimensional separation was performed on a nanoACQUITY UPLC system (Waters, Milford, MA, USA) coupled to a Q-TOF Premier mass spectrometer. The lyophilized fractions from the first dimension were resuspended in 40 μL of 0.1% FA solution, and 5 μL of the sample were then loaded onto a symmetry C18 trap column (180 μm × 20 mm, 5 μm, Waters, Milford, MA, USA) at a flow rate of 3 μL min^{-1} using 0.1% FA in water for 10 min. Separation of the sample was then performed on a BEHC18 capillary column (75 μm × 250 mm, 1.7 μm, Waters, Milford, MA, USA). The mobile phase consisted of A (0.1% FA in water) and B (0.1% FA in ACN). Gradient elution was set as follows: 2%B–35%B in 0–55 min; 35%B–90%B in 55–60 min; 90%B–90%B in 60–65 min; 90%B–2%B in 65–66 min; 2%B–2%B in 66–80 min. The flow rate of separation was 300 nL min^{-1}. The peptides were eluted into a nanoelectrospray ionization Q-TOF mass spectrometer. The nanoACQUITY UPLC and the Q-TOF MS were controlled by MassLynx 4.1 software. Data were collected by the data directed analysis (DDA) MS method in positive mode. The MS survey was from *m*/*z* 200 to 2000. The top three ions of greatest intensity were selected for MS/MS study. MS/MS spectra were recorded in the range of 50–2000 Da. The collision voltage for MS/MS was selected based on peptide mass and charge. Acquisition time was 0–80 min, while capillary voltage was 3.5 kV.

2.6. Data analysis

The LC–MS/MS and nanoLC–MS/MS data were processed by Micromass ProteinLynx 2.2.5. Databank searching was carried out on a Mascot version 2.3.0 (Matrix Science) platform. The resulting .pkl files of standard proteins and digested rat brain proteins were searched against, respectively, Uniprot-Swiss-Prot database with taxonomy: other mammalian and IPI-Rat database. Monoisotopic masses were used for the search. Searches were performed for fully tryptic peptides containing up to 2 missed cleavages, and the variable modifications, N-terminal acetylation, methionine oxidation, and phosphorylation (S, T, Y), were included in the search parameters. Peptide mass tolerance was set at 1 Da, and MS/MS tolerance was set at 0.8 Da. The minimum peptide score was set at 20 and a minimum mascot protein score of 40 was used for confident identification. Peptides were identified with a Mascot Score over the 95% confidence limit. The MS data of standard proteins were processed with MassLynx software (Waters), and retention times of peptides were recoded manually according to their mass and entered into Microsoft Excel. Retention data normalization was carried out in Excel.

3. Results and discussion

3.1. Preparation and characterization of C18WCX stationary phase

The surface chemistry of the new polar-copolymerized stationary phase C18WCX is shown in Fig. 1. Mixed C18 ligands for RP

Table 1
Amino acid sequence, calculated hydrophobicity, isoelectric point (PI) and charges of standard peptides.

Peptide code	Sequence	Mass	p*I*[a]	GRAVY[b]	Net charge[a]			
					pH 3.0	pH 4.5	pH 6.5	pH 8.0
1	Glu-Glu	276.2	3.1	−3.00	0.07	−1.27	−1.99	−2.01
2	Lys-Gly	203.2	10.1	−1.50	+1.18	+1.01	+1.00	+0.98
3	Gly-Gly-His	269.3	7.8	0.20	+1.17	+0.98	+0.24	−0.01
4	Leu-Gly-Gly	245.3	6.0	0.67	+0.18	+0.01	0	−0.02
5	Phe-Gly-Gly-Phe	426.5	6.0	1.20	+0.18	+0.01	0	−0.02
6	Arg-Val-Tyr-Ile-His-Pro-Phe	931.1	9.8	0.129	+2.18	+1.98	+1.24	+0.98

[a] The embl-heidelberg.de: EMBL WWW Gateway to Isoelectric Point. Service was employed for calculating the isoelectric points (p*I* values) as well as the net-charge values of peptides at different pH values.
[b] GRAVY was estimated on http://www.expasy.org with ProtParam. It was tenable when the amino-acid residues in the peptides were in un-ionized form.

and carboxyl groups for weak cation exchange are copolymerized on the silica surface. Organic elemental analysis (EA) results indicated that the carbon content of C18WCX stationary phase is 12.60% while the nitrogen content is 0.43%. An infrared (IR) spectrum (not shown) was used to confirm the identity of the mixed ligands on the silica surface. The peaks at 2965 cm^{-1} and 2850 cm^{-1} indicate the presence of C18 ligands and the strong adsorption at 1750 cm^{-1} confirms the presence of carboxyl groups on the silica surface.

A mixture of toluene and ethyl benzene was used to evaluate the basic RP retention characteristics of the C18WCX stationary phase. An XTerra MS C18 column, which primarily separates compounds based on their hydrophobic characteristics, was used for comparison. The chromatographic results are shown in Fig. S1 (S means supplementary information). For ethyl benzene on the C18WCX, the theoretical plate number is 55,800 plates m^{-1} and the USP tailing was 0.93. These results are comparable to those obtained from the XTerra MS C18 column, and are much higher than those obtained from mixed-mode stationary phases used in previous studies [33,45]. These results demonstrated that the C18WCX stationary phase is effective under reversed phase mode. However, the retention of the two solutes on the C18WCX column was weaker than their retention on the C18 column. This is because the polar groups on the C18WCX column make its hydrophobicity weaker than that of the C18 column.

A mixture of six peptides with different hydrophilicities and p*I* values (Table 1) and a tryptic digestion of hemoglobin (16 identified peptides, the chemical character of peptides were shown in Table S1) have been employed to evaluate the ion-exchange retention characteristics of the C18WCX stationary phase. The chromatograms of the mixture of six peptides and hemoglobin digests are shown in Fig. 2 and Fig. S1, respectively. The additional information of retention times of the 22 peptides was provided in Table S2. A comparison of normalized retention times on the C18WCX and XTerra MS C18 columns is shown in Fig. 3.

As shown in Fig. 3, high correlation (R^2 = 0.9806) of the two columns was observed under acidic condition, indicating that the retention mechanism and separation selectivity of the two columns are very similar. However, the similarity of the two columns decreased from R^2 = 0.9806 to R^2 = 0.8005 under neutral pH condition. These results indicate that additional retention interactions other than hydrophobic interactions are present when separation on the C18WCX column is performed under neutral pH condition.

In order to further investigate these additional retention interactions, the retention times of individual peptides were compared with respect to peptide charge. Taking peptides 5 and 6 in Fig. 2 as an example, the retention time of the neutral peptide 5 decreased on the C18WCX column (Fig. 2B and D) when changing the pH from 3.0 to 6.5, while the retention time of the basic peptide 6 on the C18WCX column clearly increased with pH (Fig. 2B and D). The decrease of retention time of neutral peptide 5 was also observed on the XTerra MS C18 column (Fig. 2E and F) when changing the pH from 2.6 to 6.5. However, the change in retention time of peptide 6 on the XTerra MS C18 column resulting from pH change was very small (Fig. 2E and F), indicating that pH does not strongly influence the hydrophobicity of peptide 6. Therefore, we concluded that the greatly enhanced retention of peptide 6 on the C18WCX column is likely caused by the cation-exchange interactions between deprotonated carboxyl groups on the stationary phase and the positive charges on the basic peptide (mainly on the basic residues K). This phenomenon of increased retention time was observed for most of the basic peptides in the hemoglobin digests (Fig. S2). Fig. S2 shows that most basic peptides in the hemoglobin digests cluster to the latter region of the retention window while most acidic peptides cluster earlier under neutral (pH 6.5) and basic (pH 8.0) conditions. However, this was not the case on the XTerra MS C18 column. All results demonstrate that the C18WCX stationary phase could offer both hydrophobic interactions and cation-exchange interactions.

Additionally, the retention time of basic peptide 6 increased step by step from 20.1 min to 26.6 min, and then to 45.9 min when pH was increased from 3.0 to 4.5, and then to 6.5 (Fig. 2B–D), while the positive charge of the peptide (in Table 1) was decreased with the pH increased. This showed that increased cation-exchange interactions could be induced by the stationary phase, and the cation-exchange interactions of the C18WCX column could be controlled by adjusting the pH value of the mobile phase. This is because the number of deprotonated carboxyl groups changes with pH value, and this trait makes it possible for the C18WCX stationary phase to provide adjustable selectivity for the separation of ionic compounds. The retention time of peptide 6 decreased when the pH was changed from 6.5 to 8.0 (Fig. 2A and B); this may have been because there was little increase in depronated carboxyl groups on the stationary phase, but the positive charges of the peptide decreased.

3.2. Orthogonality evaluation

An ideal 2D-LC system is always highly orthogonal and combined by two LC modes with high separation performance and very different separation selectivities. As shown above, the mixed-mode C18WCX column possesses different selectivity and comparative separation performance from RP columns under neutral and weak basic conditions. Thus, it would be expectation to establish a 2D-RP/WCX–RPLC system with higher orthogonality than the conventional 2D-RP–RPLC system, and the separation efficiencies of both dimensions in our new RP/WCX–RP system were as high as in the RP–RP system. To further investigate the performance of the mixed-mode C18WCX column and identify the most suitable conditions for conducting separation on a 2D-RP/WCX–RPLC system with high orthogonality, tryptic digestions of α-casein, BSA and phosphorylase b were separated under different pH conditions. The primary amino acid sequence of the detected peptides from the three proteins was shown in Table S3. Good separations of α-casein, BSA and phosphorylase b digests on the C18WCX column under neutral pH condition (pH 6.5) are shown in Figs. S3 and S4.

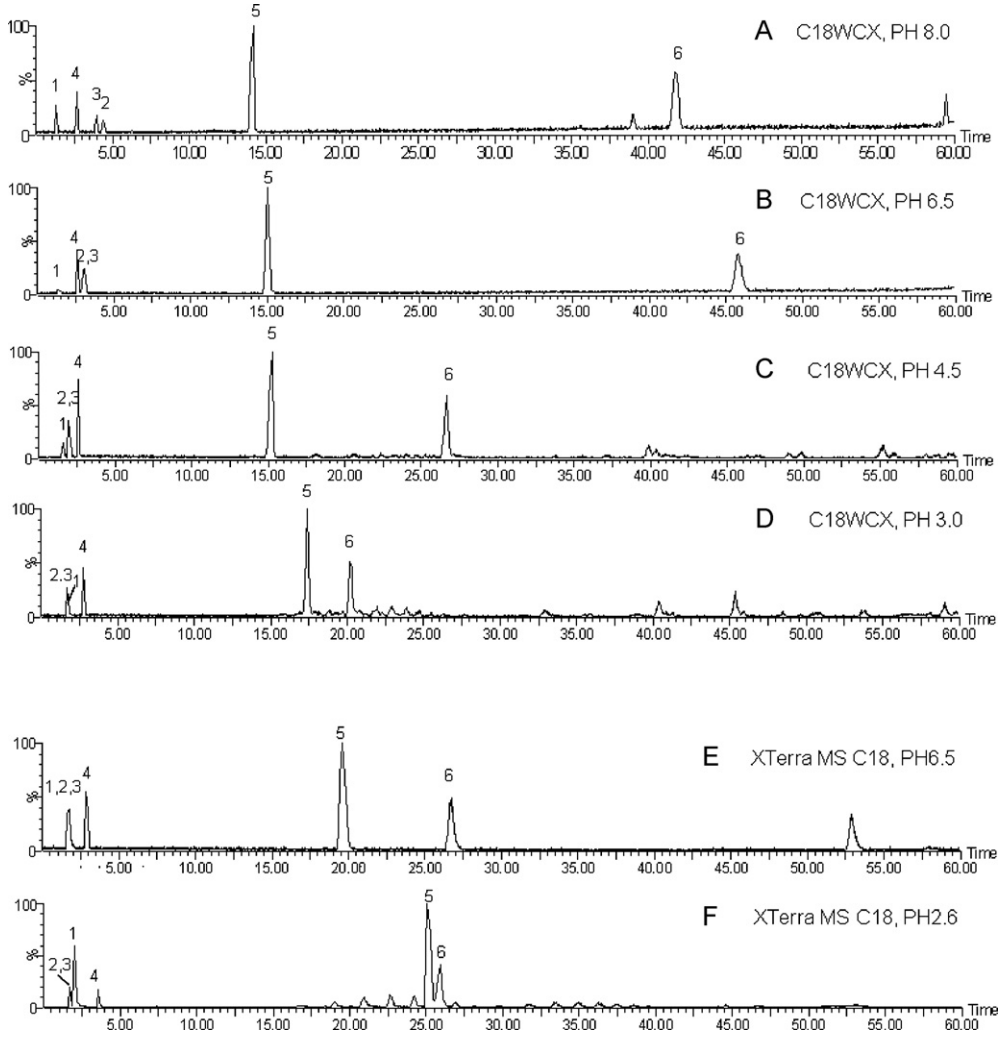

Fig. 2. Separation of standard peptide mixture with C18WCX column (A–D) and XTerra MS C18 column (E and F). Buffer pH was as indicated. 1: Glu-Glu; 2: Lys-Gly; 3: Gly-Gly-His; 4: Leu-Gly-Gly; 5: Phe-Gly-Gly-Phe; and 6: Arg-Val-Tyr-Ile-His-Pro-Phe.

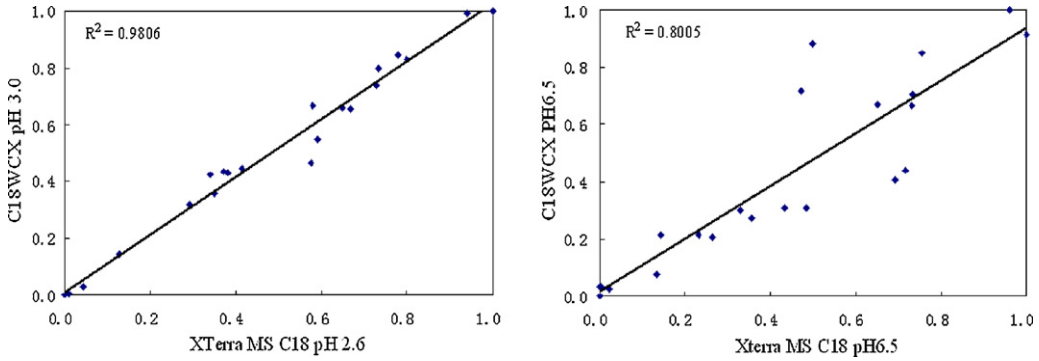

Fig. 3. Normalized retention times of 22 peptides detected in peptide mixture (see Table 1) and hemoglobin digests (see Table S1) on the C18WCX and XTerra MS C18 column at acid and neutral pH conditions.

The orthogonality was visualized as a dotplot, with the normalized retention times of the peptides marked on the two LC dimensions making up the axes [16,46]. The retention times of the detected peptides are listed in Table S4. Normalized two-dimensional plots were constructed for an identical set of 123 peptides ranging in length from 2 to 42 amino acids and PI from 3.1 to 11.1. The XTerra MS C18 column, operated under conditions suitable for a LC–MS experiment (pH 2.6), was used to generate the basic set of retention time data. The results of orthogonality evaluation were shown in Fig. 4. As shown in Fig. 4C and D, although considerable separation orthogonality can be achieved with RP–RP systems, the correlation of the two dimensions was obvious and a substantial amount of two-dimensional space is not used when separation is performed on RP–RP systems. However, a more random distribution of peptides and greater occupation of two-dimensional space are achieved when the mixed-mode C18WCX stationary phase with a neutral or weak basic mobile phase is used in the first dimension (Fig. 4A and B). In Fig. 4, the

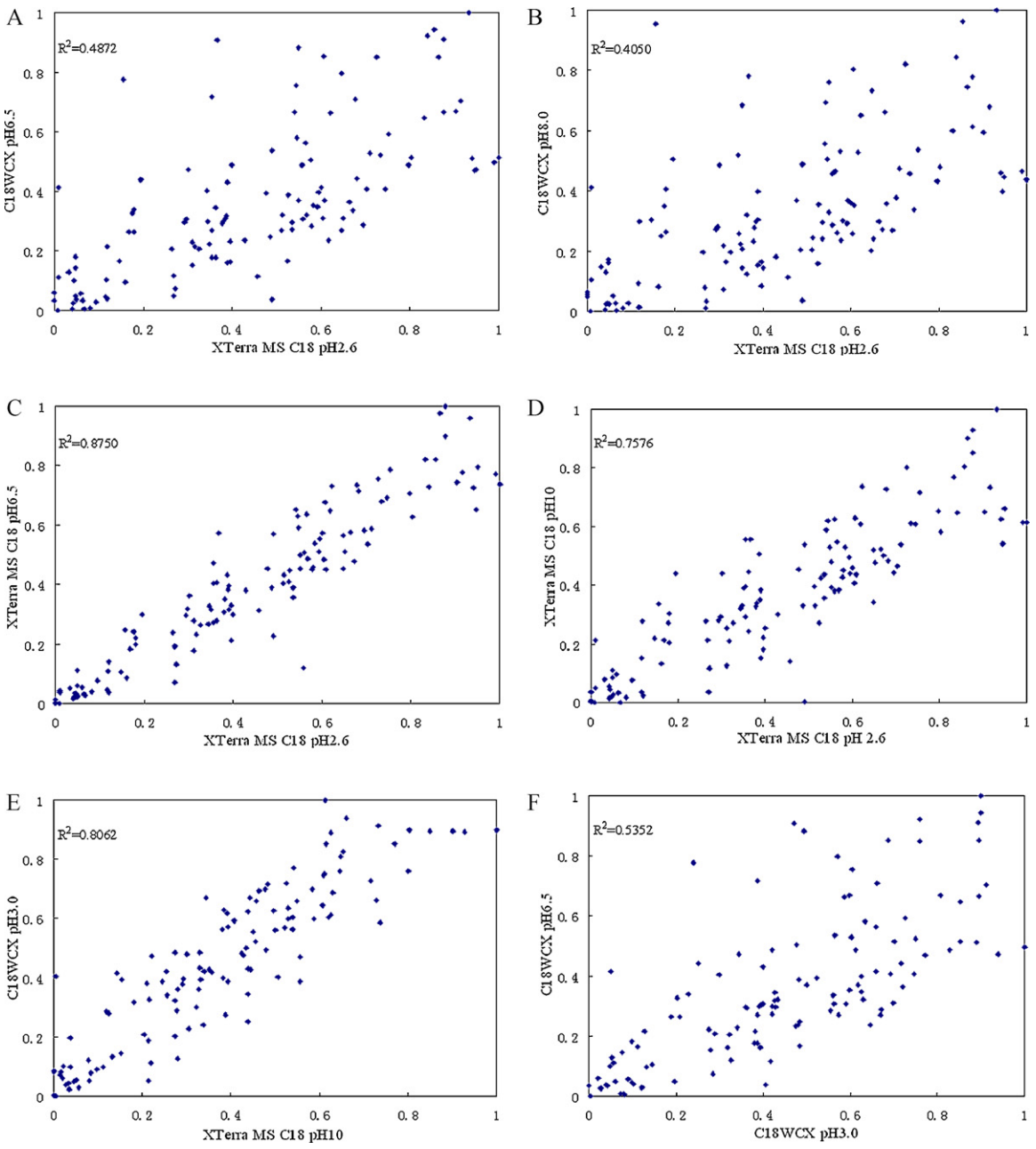

Fig. 4. Normalized retention time plots of 123 peptides for RP/WCX–RPLC and RP–RPLC systems. The 123 peptides including 6 standard peptides (see Table 1), 16 peptides in hemoglobin digest (see Table S1), 21 peptides in α-casein digest (see Table S3), 27 peptides in BSA digest (see Table S3) and 53 peptides in phosphorylase b digest (see Table S3).

lower correlation value is, the higher the orthogonality of the two separation dimensions will be. Three main points could be drawn from Fig. 4: (1) combination of RP/WCX and RP (Fig. 4A and B) provides for greater orthogonality than RP–RP system (Fig. 4C and D); (2) RP/WCX–RP system with low pH in the RP/WCX separation dimension (Fig. 4E) provides similar results as RP–RP system (Fig. 4C and D), this observation is consistent with Fig. 3A; (3) C18WCX at pH 3 combined with C18WCX at pH 6.5 provides good orthogonality and has promising potential for 2D-LC of peptides (Fig. 4F).

The degree of orthogonality of the RP/WCX–RP system slightly increased when the pH of the first dimension shifted from 6.5 to 8.0 (Fig. 4A and B). However, a closer look at the retention times of peptides in Table S4 reveals that over 70% of the peptides show a decrease in retention time on the C18WCX column in response to a pH shift from 6.5 to 8.0. This indicates that the peptides are better retained and distributed in the separation window of the first dimension at pH 6.5 than pH 8.0. Therefore, we concluded that a pH of 6.5 is more suitable for the first dimension of a 2D-RP/WCX–RPLC peptide separation system.

3.3. 2D-LC–MS/MS analysis of tryptic digestion of soluble protein fraction of rat brain

As suggested above, the mixed-mode C18WCX column could be combined with RP columns to form a two-dimensional separation system with high orthogonality. However, the practical application of this two-dimensional mode for the separation of complex proteome samples requires further validation. For this purpose, we developed an off-line 2D-RP/WCX–RPLC system that uses the

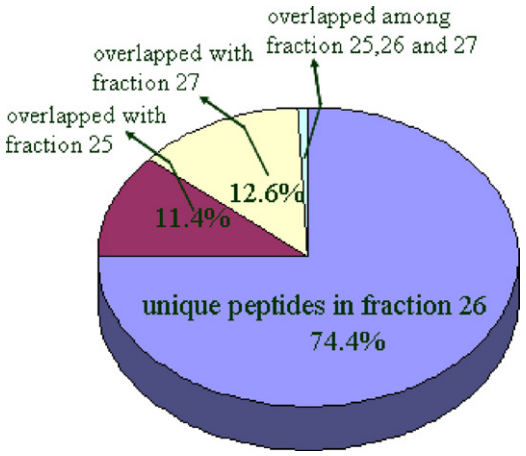

Fig. 5. Evaluation of peak broadening in the first-dimensional separation. The peptide sample was prepared from the soluble protein fraction of rat brain after enzymatic digestion. The sample was subjected to C18WCX column for first-dimensional separation, and the separated fractions were analyzed by RPLC–MS/MS. The relative number of peptides identified in only fraction 26 and the relative number of peptides overlapped between fractions 25 and 26 or fractions 26 and 27 are showed in different colors.

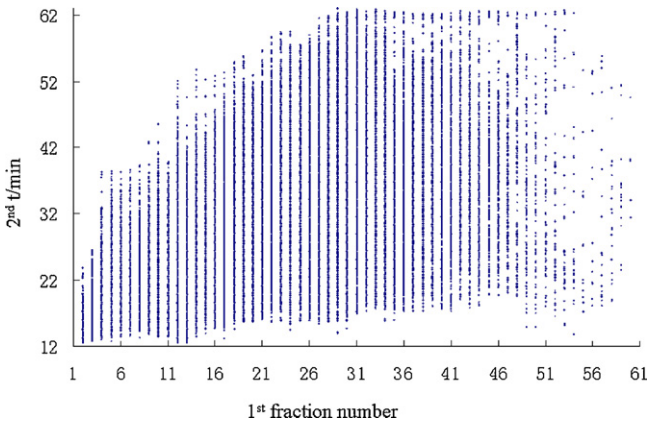

Fig. 6. Elution windows of the 59 fractions on the nano-RPLC column. The proteome sample was prepared from the soluble protein fraction of rat brain after enzymatic digestion. The sample was subjected to C18WCX column for first-dimensional separation, and the separated fractions were analyzed by RPLC–MS/MS.

C18WCX column (pH 6.5) for the first dimension, with the second dimension consisting of a 55-min gradient analysis conducted with nano-RPLC–MS/MS (pH 3.0). In order to assess the practicality of this 2D-RP/WCX–RPLC system, it was used to separate proteome samples from rat brain samples. It should be noted that, during the last 5 min of the 60-min first-dimensional separation, the pH of the mobile phase was decreased from 6.5 to 3.0 by adding a 3-min pH gradient. The addition of this pH gradient within the separation system allowed for the ion-exchange interactions of the column to be switched off, and ensured the elution of peptides abundant in positive charges. It was not necessary to increase the concentration of salt, which was necessary in the mixed mode RP/SCX separations [47]. Fractions were collected every minute and were subjected to a nano-scale ultra-performance LC system for high efficiency RPLC–MS/MS analysis.

The chromatogram of 100 μg rat brain tryptic digestion on the first dimensional C18WCX column is provided in Fig. S5. Although the complexity of the sample made it impossible to obtain a baseline separation in the first dimension, narrow peaks with good peak shape could be observed in the chromatograms. The narrow peak width associated with the first separation dimension was confirmed by the low overlap of peptides among the fractions. Fig. 5 shows the overlap of peptides before and after fraction 26. More than 74.4% of the peptides were unique to fraction 26, while only about 11.4% of the peptides overlapped with fraction 25, and 12.6% of the peptides overlapped with fraction 27. Similar results were obtained in the other fractions. In a study by Nakamura et al. [24] only 47.8% of the peaks were unique to a 2 min fraction when a PolyRP column was applied in the first dimension, and less than 30% of the peaks were unique to the same fraction when a SCX column was used. Nakamura et al. also showed that peak broadening in the first dimension is unfavorable to the identification of peptides; narrower peaks could result in the identification of a greater number of peptides.

The orthogonality of the off-line 2D-RP/WCX–RPLC system in the practical analysis was assessed by examining the retention window of the 59 fractions on the second dimension (Fig. 6). The separation window of the fractions on the nanoLC column ranged approximately from 12.5 min to 63.0 min. The retention time of detected ions in the second dimension were plotted to their fraction number in the first dimension. The elution windows of most of the 1 min fractions from the first dimension increased to more than 25 min in the second dimension during a 55-min nanoLC–MS/MS analysis; a 49-min elution window was even observed in the sep-

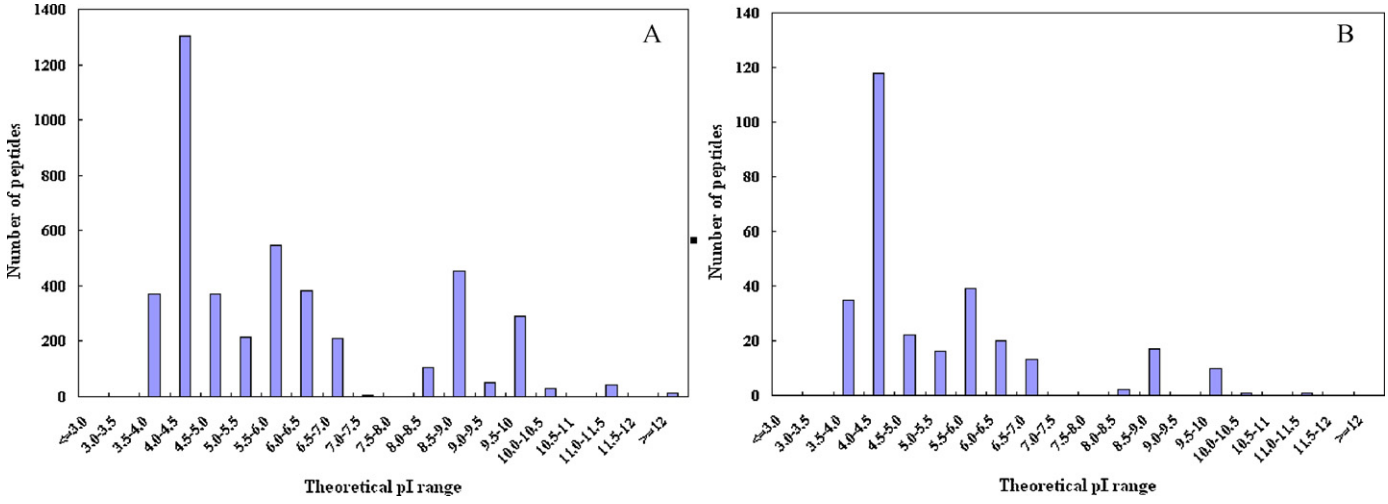

Fig. 7. Distribution of peptides according to their p*I*: (A) 2D-LC separation, and (B) 1D-LC separation. The proteome sample was prepared from the soluble protein fraction of rat brain after enzymatic digestion.

aration of fraction 29 on the second dimension. The distribution of the components in the two-dimensional plot demonstrates good orthogonality of the two-dimensional system. The dense plots in the figure also highlight sample complexity.

The use of this off-line 2D-RP/WCX–RPLC system allowed the identification of 1031 proteins with 4397 unique peptides from two replicates. Subjecting the same sample solely to nano-RPLC-Q/TOF analysis, only 84 proteins and 292 peptides could be identified from two replicates. Proteins were identified based on two unique peptides having ion scores > 20. The false positive discovery rate for protein identification was 1.9%. On peptide level, the degree of overlap between the two 2D-LC replicates was 52.7%, and the degree of overlap between the two 1D-LC replicates was 66.4%. Such a low degree of overlap was mainly related to the limited sequencing speed of mass spectrometer and the overwhelmingly complex analytes in the sample. Fig. 7 shows the distribution of theoretical p*I* values of the identified peptides and proteins from the 2D-LC and 1D-LC experiments. Clearly, 2D-LC analysis identified a greater number of peptides and proteins than 1D-LC analysis in every p*I* value range. The difference was even greater with basic peptides. For instance, 427 unique peptides with a p*I* value over 9.0 were identified in the 2D-LC analysis, and the relative proportion of these peptides in the 2D-LC analysis (9.7%) was higher than in the 1D-LC analysis (4.1%). This result might indicate that the two-dimensional method developed in this study improves the identification of basic peptides. This is likely because basic and hydrophobic peptides are retained better on the C18WCX column, and the orthogonality of the two-dimensional separation of these peptides is greater than that for other peptides.

4. Conclusions

In this study, we present an application of mixed-mode RP/WCX chromatography in 2D-LC proteome analysis. A mixed-mode RP/WCX stationary phase was synthesized and packed into a C18WCX column for peptide separation. This C18WCX column showed adjustable selectivity under different pH conditions. Under acidic conditions, the column mainly provided hydrophobic interactions and showed selectivity similar to that of the common RP column. Under neutral and weak basic conditions, it offered multiple retention mechanisms, including RP and cation-exchange mechanisms, and differed in selectivity from the RP column. So, the change in peptide separation selectivity induced by change in pH was much more noticeable on the mixed-mode C18WCX column than on the XTerra MS C18 column. Taking this into account, we developed an off-line 2D-RP/WCX–RPLC–MS system using the C18WCX column with a mobile phase of pH 6.5 in the first dimension and an Xterra MS C18 column with a mobile phase of pH 3.0 in the second dimension. The introduction of the mixed-mode C18WCX column in the first separation dimension allowed for high orthogonality and high performance two-dimensional separation at lower pH levels, which were shown to be favorable to peptide separation. This off-line hyphenation of a mixed-mode C18WCX column with a RP column displayed great separation power in the analysis of rat brain proteome samples, showing that it can be used in practical applications. Moreover, improved identification of basic and highly hydrophobic peptides was observed. Overall, this study introduces a novel two-dimensional separation approach for proteomics that provides both high orthogonality and performance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.06.042.

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