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Two-dimensional separation of peptides using RP-RP-HPLC system with different pH in first and second separation dimensions

Two-dimensional high performance liquid chromatography is a useful tool for proteome analysis, providing a greater peak capacity than single-dimensional LC. The most popular 2D-HPLC approach used today for proteomic research combines strong cation exchange and reversed-phase HPLC. We have evaluated an alternative mode for 2D-HPLC of peptides, employing reversed-phase columns in both separation dimensions. The orthogonality of 2D separation was investigated for selected types of RP stationary phases, ion-pairing agents and mobile phase pH. The pH appears to have the most significant impact on the RP-LC separation selectivity; the greatest orthogonality was achieved for the system with C18 columns using pH 10 in the first and pH 2.6 in the second LC dimension. Separation was performed in off-line mode with partial fraction evaporation. The achievable peak capacity in RP-RP-HPLC and overall performance compares favorably to SCX-RP-HPLC and holds promise for proteomic analysis.

Key Words: Two-dimensional; Liquid chromatography; Peptides; Proteomic; Orthogonal

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1 Introduction

Despite the recent progress in ultrahigh-performance liquid chromatography [1, 2] and mass spectrometry (MS), the liquid chromatography (LC)-MS analysis of highly complex proteomic samples remains a challenging task [3–5]. Since the top-down LC-MS analysis of intact proteins is difficult [6–8], the proteomic analysis is usually performed on a peptide level after sample proteolysis with trypsin (or alternative enzymes). However, the tryptic cleavage generates multiple peptides *per* protein, proteomic samples typically consist of hundreds of thousands of peptides. To date, no separation method is capable of resolving so many components in a single analytical dimension prior to the MS analysis. Consequently, multiple peptides enter the mass spectrometer at any given time and overwhelm the MS/MS capability of the instrument. This results in a reduced number of peptide identifications, and greatly adds to the LC-MS/MS analysis (and database search) variability [3, 9, 10].

Off-line and on-line two-dimensional high-performance liquid chromatography (2D-HPLC) separations have been applied for analysis of proteomic samples, both having unique advantages and disadvantages [3, 11–13]. While the on-line approach minimizes the manipulation with the sample and its potential losses, it requires a sophisticated instrumental setup with additional pumps, switching valves, and trapping columns [14]. The requirements for the mobile phase compatibility for direct fraction transfer to the second separation dimension narrows the choice of useful LC separation modes. An off-line approach is simpler to implement, for example, *via* an appropriate fraction collector. The collected fractions can be further processed, concentrated and/or chemically modified prior to analysis in the second LC-MS dimension. Therefore, the off-line 2D-HPLC has less stringent requirements on mobile phase compatibility, and offers more freedom for timing of analysis. The implementation of both 2D-HPLC approaches is further complicated by the fact that analysis is often performed in capillary- or nano-LC format. Dedicated and well maintained instrumentation is absolutely essential to maintain data quality.

Current 2D-HPLC approaches for proteomic analysis usually combine a strong cation exchange (SCX) chromatography with reversed-phase (RP) HPLC [3, 9, 11]. Besides the differences in selectivity, the rationale for the choice is also a good compatibility of SCX mobile phases with the second separation dimension (RP). The peptides/salt fractions can be directly introduced on a RP-HPLC; while peptides are retained on sorbent, salts (required to elute peptides from SCX) are washed out unretained. High concentrations of salts are typically diverted to waste *via* an additional switching valve in order to avoid the contamination of the MS ionization source. The choice of mode in the second LC dimension is generally RP, due to its high

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efficiency and compatibility with on-line electrospray ionization (ESI) MS analysis (or other MS modes).

An interesting approach of dual-column has been proposed, combining SCX and RP sorbents in a single column housing [9]. The peptides injected on the SCX sorbent, at the head of column, are sequentially desorbed with the salt plug of desirable strength, concentrated on-column on the RP sorbent, and eluted with a subsequent RP gradient into an MS. The cycles of progressively stronger salt plugs followed by RP analysis are typically applied. This approach is well suitable for nano-scale HPLC and potentially simplifies the LC system setup. However, the column longevity and the carryover between fractions, as well as samples, could be a problem.

Although fractionation reduces the sample complexity, the LC-MS analysis of multiple fractions in the second dimension extends the duration of the experiment proportionally. In addition, the database search for multiple MS data is also time consuming. Despite the potential benefits of sample fractionation (greater number of proteins identified), the 2D-HPLC is only slowly gaining a wider acceptance for proteome analysis. Availability of robust commercial 2D-nanoLC systems, high quality columns (and trapping columns), well designed separation schemes, and user-friendly nano-ESI interfaces are expected to improve the acceptance of the 2D-LC-MS technique in proteomic laboratories.

In our recent study [15] we evaluated the potential of 2D-HPLC for complex peptide analysis. The goal was to estimate a theoretical number of components that can be separated with current state-of-the-art columns, and to assess the productivity of separation. The peak capacity [15, 16] (defined as a maximum theoretical number of peptides that can be separated in a given 2D-LC system with resolution R_s=1) was found to be approximately 15000 *per* eight hours of separation time. This estimate is based on the assumption that the peak capacitys of 2D-separations is a linear combination of peak capacities in both dimensions [17]. For example, when combining SCX-HPLC (typical peak capacity is 50 in 50-min analysis) with RP-HPLC (peak capacity may be 100 for 50-min analysis), the total 2-D peak capacity is 5000. In reality, this value also depends on the orthogonality of separation [18, 19]. To date little effort has been focused on investigation of orthogonality of various HPLC modes for peptides. Few available reports suggest that orthogonality of SCX and RP modes is not ideal [3, 14, 15]. It is known that separation in SCX is directed by the peptide charge. Since the tryptic peptides are mostly 2+ and 3+ charged, the peptides cluster in a narrow retention window. The majority of desirable peptides elute from the column early in the analysis, leaving a portion of separation space relatively devoid of peaks. Therefore, the peak capacity of the 2D-HPLC based on SCX-RP combination may be lower than generally expected.

In this work we describe the development of 2D-HPLC as an alternative to commonly used SCX-RP-HPLC. We have investigated several approaches to achieve a useful degree of orthogonality for peptide separation in RP-RP systems. Along with different types of RP sorbents, we evaluated the impact of ion-pairing agents, and pH. The performance of the RP-RP-HPLC approach was compared to SCX-RP-HPLC.

2 Experimental

2.1 Materials and reagents

Formic acid (FA), trifluoroacetic acid (TFA), ammonium hydroxide, ammonium bicarbonate, sodium chloride, dibasic sodium phosphate and phosphoric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA). A Milli-Q system (Millipore, Bedford, MA, USA) was used to prepare deionized water (18 MΩcm) for HPLC mobile phases. MassPREP™ protein digestion standards of Enolase, ADH, Phosphorylase b, Hemoglobin, BSA and MassPREP™ CHCA MALDI matrix were obtained from Waters (Milford, MA, USA).

2.2 HPLC instrumentation, columns and conditions

HPLC experiments were carried out using the following instruments: model 2795 Alliance® HPLC system with a 996 photodiode array detector equipped with micro UV cell (Waters, Milford, MA, USA). Single quadrupole Micromass ZQ 4000 MS instrument was used for LC-MS peptide analysis with narrow-bore (2.1 mm i.d.) LC columns. Micromass Q-Tof Micro and Q-Tof ULTIMA were used for capillary LC-MS and LC-MS/MS, respectively (Waters, Milford, MA, USA). CapLC (Waters, Milford, MA, USA) instrument was employed for capillary LC experiments. The 0.3 × 150 mm NanoEase BEH C18 column, 3.5 μm was obtained from Waters (Milford, MA, USA).

Reversed phase HPLC columns used in this study were obtained from Waters (Milford, MA, USA) or packed in-house with the indicated sorbent. The column dimensions used were 150 × 2.1 mm (L × i.d.), unless specified otherwise. Mobile phases were A: appropriate buffer in water, and B: appropriate buffer in acetonitrile. Gradients started from 0% ACN, the gradient slope was 0.8% ACN *per* minute. Ammonium formate buffer pH 10 was prepared by mixing ammonium hydroxide (25%) and water to final concentration 200 mM (5.4 g ammonium hydroxide and 425.3 g water). pH was adjusted by addition of 0.7 mL of formic acid (99%). Other HPLC conditions are given in the figure captions. The columns were operated at 40°C; the temperature was controlled by a built-in column heater.

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The SCX HPLC experiments were carried out using PolySULFOETHYL Aspartamide™ SCX columns purchased from PolyLC Inc. (Columbia, MD, USA). A 50 × 4.6 mm SCX column packed with 5 μm, 300 Å sorbent was used for initial experiments. The column was operated at 25°C; mobile phases were A: 20 mM NaH_2PO_4, pH 2.6 with 25% acetonitrile, and B: 20 mM NaH_2PO_4, pH 2.6 with 0.25 M NaCl. Mobile phases contained either 5 or 25% of acetonitrile. Gradient was from 0-60% B in 100 min (1.5 mM NaCl *per* min). In addition, 150 × 2.1 mm, 5 μm polySULFOETHYL A SCX columns were used for experiments, packed with either 300 Å or 200 Å pore size sorbent. The 150 × 2.1 mm columns were used with the following conditions: 30°C; mobile phases were A: water, B: 500 mM NaCl, C: 100 mM NaH_2PO_4 buffer, pH 2.6, D: 100% acetonitrile in water. The mobile phases C and D were delivered at constant percentage throughout the analysis to maintain the NaH_2PO_4 buffer at 10 mM strength and 5 or 25% of acetonitrile background. Peptides were eluted with NaCl gradient (10 mM NaCl *per* min) for 40 min.

Fractions from SCX were either desalted using SPE or partially evaporated to reduce the acetonitrile content and directly injected on LC-MS. The SPE desalting was as follows: Evaporated fractions were reconstituted in 0.2 ml of 0.1% TFA in water and loaded onto Oasis HLB mElution plate (previously conditioned with 0.5 mL of acetonitrile, and 0.5 mL of 0.1% aqueous solution of TFA). Sample was washed with additional 0.5 mL of 0.1% aqueous solution of TFA, and eluted with 10 mL of 60% acetonitrile in water. About 0.5 mL of eluted solution was mixed with CHCA MALDI matrix and analyzed by Micromass M@LDI R TOF instrument from Waters (Milford MA, USA). Peptides eluting in each fraction were identified by comparing the observed accurate mass with the theoretical mass of expected tryptic peptides.

3 Results and discussion

3.1 Off line SCX-RP HPLC

In our earlier work we briefly evaluated SCX-RP-HPLC performance for separation of peptides [15]. For this study we used a mix of five digested proteins mixed together with an additional nine standard peptides. This sample comprising ~250 peptides was separated in the SCX. Collected fractions were analyzed with MALDI MS and LC-MS in order to identify the peptide retention times. The data were plotted against the peptide retention obtained from RP-HPLC (Fig. 1).

As expected, the peptides are retained on the SCX column according to their charge. Since trypsin cleaves at the C terminus of basic amino acids (arginine and lysine), the most common tryptic peptides carry 2+ charge at the pH used for SCX separation (second charge is due to terminal amine). Only peptides containing histidine(s) and/or so-called missed cleavage peptides, incompletely cleaved by trypsin (additional arginine or lysine in the sequence), carry a charge greater than two.

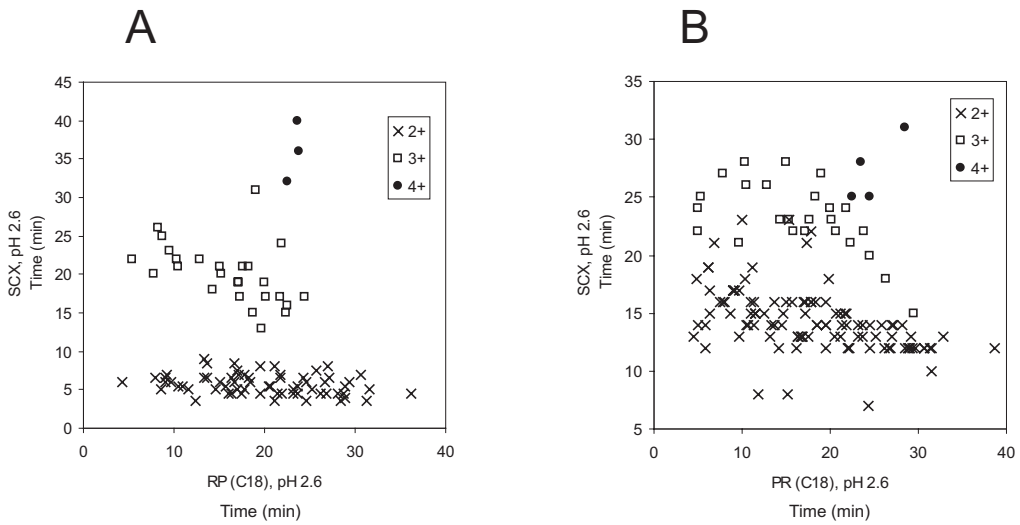

Figure 1. A 2D-selectivity plot for peptides separated in SCX and RP-HPLC. The RP retention times were measured for 150 × 2.1 mm BioSuite PA-A, C18, 3 μm column. Both RP mobile phases A and B contained 0.2% of FA; the gradient started at 0% acetonitrile, the slope was 0.8% *per* min. The retention times of peptides were plotted against the SCX for (A) 50 × 4.6 mm, 5 mm, 300 Å PolySULFOETHYL Aspartamide SCX column or (B) 150 × 2.1 mm, 5 μm, 200 Å PolySULFOETHYL Aspartamide SCX column. In case (A), the peptides were eluted with 1.5 mM NaCl/min gradient, flow rate was 1mL/min. The mobile phases contained 5% of acetonitrile and 20 mM NaH_2PO_4 buffer, pH 2.6. In case (B), the peptides were eluted with 10 mM NaCl/min gradient; flow rate 0.2 mL/min; mobile phases contained 25% of acetonitrile and 10 mM NaH_2PO_4 buffer, pH 2.6. Separation temperature was 30°C in both cases. Fraction collection interval was 0.5 min in the first 10 min, and 1 min for the rest of the LC run. Charges of the eluting peptides are indicated in the figure legend.

Figure 1A shows that the two-dimensional SCX-RP separation space is not completely covered by eluting peptides, which means that orthogonality of SCX and RP modes is not ideal (at least for SCX column packed with 300 Å sorbent). Contrary to other reports [3]; we did not observe a clear correlation between peptide hydrophobicity (calculated from reference [20]) and secondary retention on SCX column. Instead, the longer peptides (usually more hydrophobic) exhibited lower ion-exchange retentivity than the short ones.

The differences in separation selectivity observed for 200 Å and 300 Å SCX columns (Fig. 1) are in part due to their different length and conditions used for experiments. Since the overall retention of peptides was greater for the longer 200 Å SCX column, a sharper gradient was used to complete the elution within the same 45-min window. Therefore the differences in retention of 2+, 3+, and 4+ charged peptides are not as distinct as for the 300 Å column (Fig. 1A), and the apparent separation 2D-HPLC orthogonality is enhanced. The retention behavior of peptides was further investigated using a longer (150×2.1 mm, 5 mm) 300 Å PolySULFOETHYL Aspartamide column with the same gradient as in Fig. 1B (for conditions see experimental). Overall retention was similar, however, more distinct separation of 2+, 3+, and 4+ charged peptide clusters was still noticeable for the 300Å column (data not shown). Therefore, the SCX columns packed with 200 Å SCX sorbent may be more suitable for proteomic applications.

Careful analysis of SCX data revealed some loss of peptides regardless of the SCX experimental conditions; of the ~250 expected peptides only 96 were detected in the experiment represented by Fig. 1A. Addition of 25% of acetonitrile to the mobile phases improved the numbers of detected peptides only moderately (111 peptides). A possible explanation for peptide loss may be the sample handling prior to LC-MS (*e.g.* during the SPE desalting – see experimental). In order to eliminate this possibility, the experiment was repeated using a 2.1×150 mm column packed with 200 Å sorbent with 25% acetonitrile in the mobile phases (as suggested by manufacturer to reduce a non-specific peptide adsorption). The collected fractions were partially evaporated to reduce the organic content, and directly injected into LC-MS. Data for 161 observed peptides are shown in Fig. 1B. Despite the improvement, a number of peptides were still undetected. The loss of peptides correlates loosely with their length; few peptides longer than 15 amino acids were recovered from the SCX columns. The loss of 2+ and 3+ peptides appears to be equally severe.

3.2 Investigation of RP-RP 2D-HPLC orthogonality

Selectivity of peptide separation in RP-HPLC depends on various factors, namely the separation temperature, type of stationary phase, sorbent pore size (100 Å *versus* 300 Å), mobile phase additive, choice of organic modifier, and gradient slope. All of those factors have been utilized to alter the resolution of peptides [21–26], *e.g.* for peptide mapping applications. We have evaluated the impact of selected factors on the orthogonality of peptide separation in the RP-RP 2D-HPLC system. The experimental setup was as follows: Five protein digests (each comprising 20–100 peptides) were sequentially analyzed by LC-MS and the peptide retention was recorded. All five digests represent together approximately 250 peptides ranging from 4 to 45 amino acids, providing for 250 data points. The retention data were acquired for each single-dimensional LC system of interest (varying the mobile or stationary phase). Since all RP-LC modes were MS compatible, the identity of eluting peptides was confirmed by their MS signal (molecular weight). The data for different LC systems were then plotted into 2D retention graphs and cross-correlated. This experimental arrangement is simpler than the one described in the previous chapter (fraction collection from a first LC system and peptide reinjection in a second LC dimension). However, the results are equivalent and interchangeable.

Figure 2A illustrates the impact of an ion-pairing agent on the peptide separation selectivity. As seen from the plot and the correlation coefficient R^2, substitution of TFA for FA has only a subtle impact on separation. Selectivity changes are certainly sufficient to alter the resolution of critical peptide pairs, and could be useful for certain HPLC applications [24]; however, the overall separation orthogonality is insignificant. A more promising approach was to alter the type of stationary phase (Phenyl *versus* C18), rather than the ion-pairing agent. A greater degree of selectivity changes was achieved (Fig. 2B). Nevertheless, the resulting orthogonality is still low and likely not suitable for 2D-HPLC applications. Several other types of RP sorbents were evaluated with similar results (data not shown).

Since peptides are charged molecules comprised of ionizable basic and acidic functional groups, the change of mobile phase pH should have a pronounced effect on their retention behavior. The isoelectric constant (p*I*) of peptides varies from 3 to 12, based on the amino acid composition. The impact of pH on separation selectivity was first evaluated using an Enolase digest (approximately 35 peptides). The retention times acquired for the mobile phase with pH 7.9 (10 mM ammonium bicarbonate) were plotted against the data recorded previously for the mobile phase with FA, pH 2.6 (Fig. 3A).

The orthogonality of separation was noticeably greater than in previous experiments. To clarify that the change in orthogonality is indeed due to the pH difference, we carried out another experiment outlined in Fig. 3B. HPLC col-

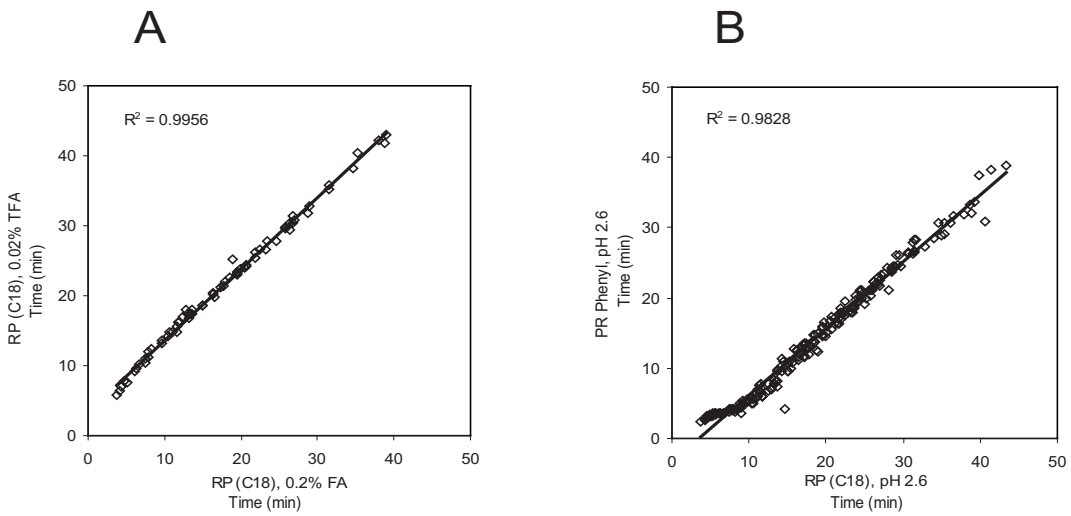

Figure 2. Impact of mobile phase additive (A), and stationary phase (B) on the peptide separation selectivity in RP-LC. 150 × 2.1 mm BioSuite C18, 3 μm PA-A column was used in experiment (A) with either 0.2% FA or 0.02% TFA as ion-pairing additive. BioSuite C18, 3 μm PA-A and XTerra Phenyl, 3.5 μm columns were used in (B) with 0.2% FA. The gradient for all experiments was from 0% ACN, slope was 0.8% ACN *per* min; temperature 40°C, flow rate 0.2 mL/min. All column dimensions were 150 × 2.1 mm.

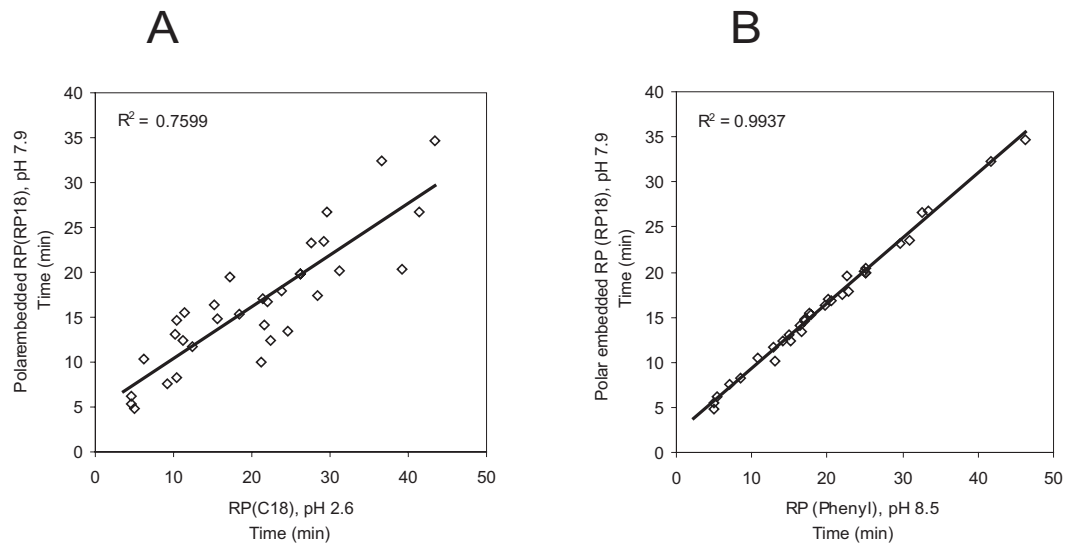

Figure 3. Impact of mobile phase pH on the peptide separation selectivity. BioSuite C18, 3 μm PA-A column, 0.2% FA, pH 2.6 *versus* SymmetryShield RP-18 (polar embedded RP-18), 10 mM ammonium bicarbonate, pH 7.9 (A). Panel (B) shows minimal selectivity changes using SymmetryShield RP-18 column with mobile phase pH 7.9 and XTerra Phenyl column with mobile phase pH 8.5, (10 mM ammonium formate). For other conditions see Fig. 2. Only Enolase digest (40 peptides) was used in this experiment.

umns were packed with polar embedded RP-18 and Phenyl sorbent; separation was carried out using similar pH (7.9 and 8.5, respectively). In contrast to Fig. 3A, no significant orthogonality was observed. It is clear that the pH is a more potent factor for achieving an orthogonal separation (of peptides) than different types of RP stationary phases.

The impact of pH was further investigated using a wider pH gap in both separation dimensions. The experimental data collected at pH 8.5 and 10 showed a significant degree of orthogonality when plotted against pH 2.6 retention data. Greater orthogonality was achieved for the wider pH difference between RP separation dimensions; therefore, further discussion focuses on the pH 2.6 *versus* pH 10 experiment. The separation at pH 10 was performed on hybrid-silica XTerra MS C18 stationary phase, known to be stable at elevated pH [27, 28]. The 20 mM ammonium formate buffer used for separation sup-

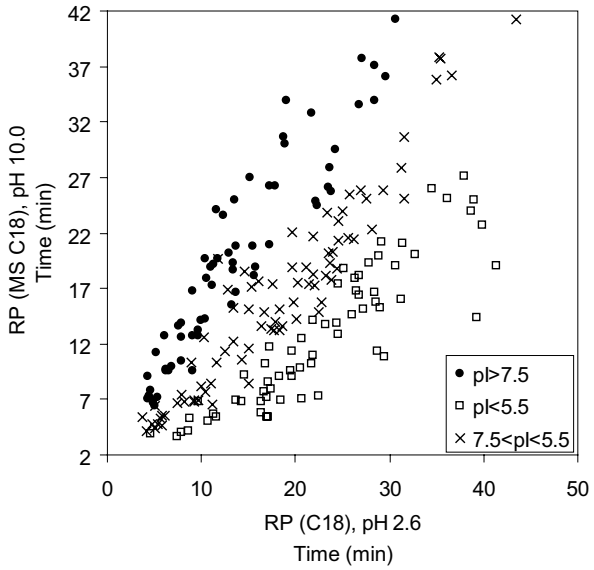

Figure 4. Achieving orthogonality of separation in RP-RP-HPLC – the impact of pH. BioSuite C18, 3 μm PA-A column, with 0.2% FA, pH 2.6 retention data were plotted against the XTerra MS C18 column used with the 20 mM ammonium formate buffer, pH 10. For other conditions see Fig. 2. p*I* ranges of peptides are indicated in the plot.

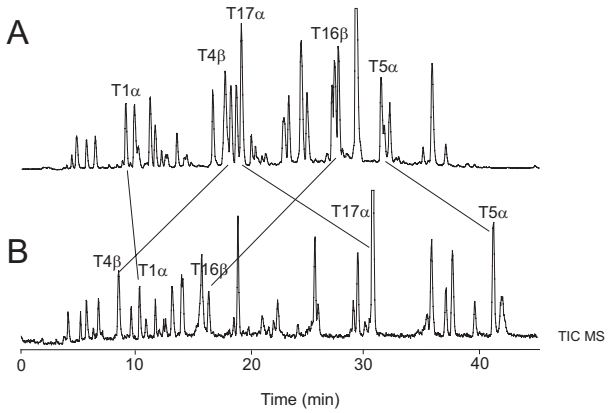

Figure 5. Analysis of bovine hemoglobin tryptic digest using BioSuite C18, 3 μm PA-A column at pH 2.6 (A) and XTerra MS C18 column, using pH 10 (B). Columns and conditions are the same as in Fig. 4. Retention shifts of selected basic peptides T17α, and T5α, "neutral" peptide T1α, and acidic peptides T16β, and T4β are indicated by lines. The assignment α and β denotes tryptic peptide origin (α- or β-hemoglobin).

pressed the MS signal to a degree. However, all peptide masses were clearly detectable. Retention data at pH 10 were plotted against data from BioSuite C18 PA-A column using 0.2% FA in the mobile phases (pH 2.6).

Figure 4 illustrates a relatively high spread of peptides over the 2-D separation space. The data were subdivided into three groups, according to the peptide p*I* values. A spatial separation emerging from the graph suggests that the main separation factor is indeed the p*I* (charge) of peptides. A class of acidic peptides (p*I* < 5.5) is more strongly retained at pH 2.6, when the carboxylic moieties are not ionized, compared to basic peptides (p*I* > 7.5) that are more strongly retained under pH 10 conditions (when they are, at least partially, discharged).

Some degree of orthogonality is observed also for the group of peptides within the 5.5–7.5 p*I* range. This observation deserves further comment. Peptides p*I* values represent the pH at which the molecule net charge is equal to zero. The p*I* is a sum of contributions of many ionizable amino acids, such as basic arginine (pK_a 12.5), lysine (pK_a 10.2), histidine (pK_a 6.45), the terminal amino group NH_2 (pK_a 7.6), acidic amino acids such as aspartic acid (pK_a 3.95), glutamic acid (pK_a 4.45), tyrosine (pK_a 9.8), and the terminal COOH group (pK_a 3.6) [29]. Even "neutral" peptides in p*I* range 5.5-7.5 must be affected by pH, since at least some of the ionizable groups will be differently charged/discharged under the separation conditions (pH 2.6 or 10).

The fact that the retention selectivity of peptides is affected by the pH of the mobile phase is expected. The variation of pH has been used earlier, for example for peptide mapping [26], and the pH is the classical factor influencing the RP-HPLC behavior of small (charged) molecules. However, the high degree of orthogonality achieved for peptides is rather surprising.

Figure 5 illustrates the retention shift of the selected peptides upon changing the mobile phase pH. Bovine hemoglobin digest (approximately 20 peptides) was analyzed by LC-MS at pH 2.6 (Fig. 5A) and pH 10 conditions (Fig. 5B). As shown in the chromatograms, the retention of acidic peptides T16β (p*I* 4.3) and T4β (p*I* 4.8) was reduced at pH 10 *versus* pH 2.6, while basic peptides T5α (p*I* 8.9) and T17α (p*I* 10.1) behaved in a directly opposite manner. Retention of a "neutral" T1α peptide (p*I* 6.0) was affected to a lesser degree.

3.3 Two-dimensional RP-RP-HPLC separation of peptides using an off-line mode.

Figures 4 and 5 strongly suggest that pH is a useful tool for achieving separation orthogonality. Further investigation was performed to find out whether the RP-RP-HPLC is a practical and efficient approach to 2D-HPLC separation of peptides. An off-line 2D-HPLC experiment was set up to confirm the degree of achievable orthogonality. A sample comprising 5 digested proteins mixed in equimolar ratio (10 pmole each) was injected in a first RP-HPLC dimension at pH 10, using a 150 × 1 mm column packed with a novel 3.5 mm C18 bridged-ethyl hybrid (BEH) silica sorbent, recently developed for RP-HPLC applications [28]. The BEH sorbent is highly stable at a broad range of

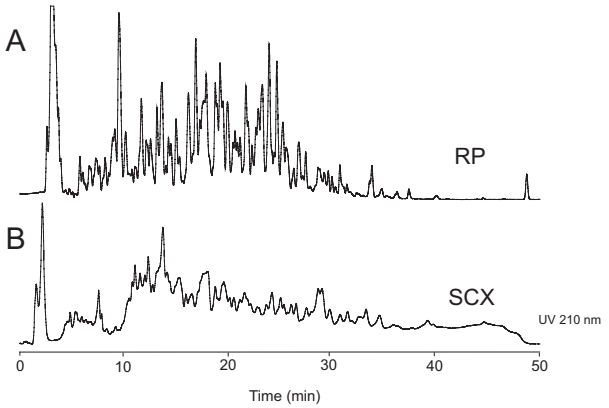

Figure 6. Fractionation of a 5 protein digest in RP-HPLC, pH 10 (A), and SCX-HPLC (B). Fraction collection interval was adjusted to approximately five peak widths at the baseline (*i.e.* 2.5 and 5 min fraction collection interval for RP and SCX, respectively). RP-HPLC column was 1×150 mm, bridged ethyl hybrid silica C18 sorbent, 3.5 μm; mobile phase A: water, B: ACN, and C: 200 mM ammonium formate. Gradient started at 90% A, 10% C and finished at 60 min at 23.3% A, 66.7% B and 10% C (1% ACN/minute), flow rate 50 μL/min. SCX-HPLC conditions in experiment (B) are the same as in Fig. 1B except for the gradient, which was 10 mM NaCl/min. LC-MS analyses of collected fractions are shown in Figs. 7 and 8.

pH, and provides an ideal material for separation at both pH 2.6 and 10.

The chromatogram in Fig. 6A shows a rich mix of partially resolved peptides. The average peak width is typically 0.5 min at the baseline; 2.5 min fractions (5 peak widths) were collected, and partially evaporated in order to reduce the acetonitrile and ammonium hydroxide content. The final volume of fractions was 10 μL; 1 μL was injected into a second LC-MS dimension using 150×0.3 mm, 3.5 μm BEH C18 capillary column. MS chromatograms are shown in Fig. 7.

The peptides eluting earlier in the first LC dimension tend to elute earlier in the second dimension as well. Similarly, the peptides collected in later fractions elute later in the second separation dimension (as suggested by the plot in Fig. 4). The orthogonality of separation appears to be good, especially when considering that the same type of C18 sorbent was used for the first and second dimension, thus the orthogonality is generated solely *via* the pH effect. Note that the gradient delay of the capillary LC system used is approximately 13 min; this specific time is not populated by the peaks. Few if any tryptic peptides elute beyond 43 min, making the useful separation window only 30 min wide. Figure 7 illustrates that in most collected fractions eluting peptides cover 50–70% of the useful LC-MS time. Adjustment of the gradient starting strength and span for early, medium and later collected fractions can potentially spread the peptides over the separation space more evenly.

The orthogonality of the RP-RP approach was compared to the SCX-RP-HPLC system. Figure 6B illustrates a SCX fractionation of a five protein digest; the fractions were collected every five min, which represents 3–5 peak widths. The 150×1 mm, 5 μm SCX, 200 Å, column performance did not match the performance of the 150×2.1 mm SCX column used for previous experiments. Therefore, we utilized the later column (2.1 mm i.d.) with 4.4 × greater peptide mass load of (in order to maintain the load proportional to RP-PR experiment with 1 mm i.d. column). Collected fractions were desalted by SPE and the final volume was reduced by evaporation to 44 μL. About 1 μL was analyzed

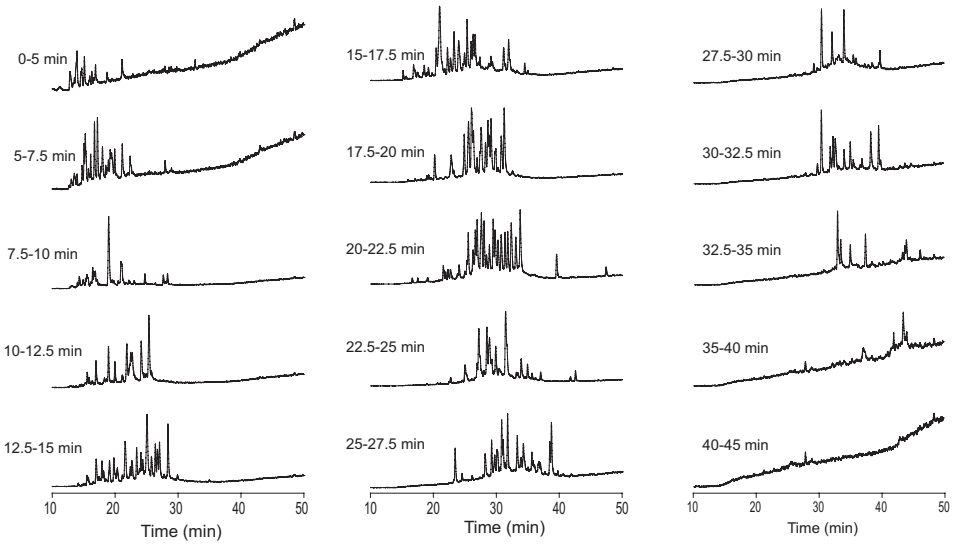

Figure 7. LC-MS analysis of RP fractions (see Fig. 6A). Fraction collection timing is indicated next to chromatograms. Analysis was performed on QTof micro MS instrument operated in MS mode. CapLC system was equipped with 150×0.3 mm bridged-ethyl hybrid silica MS C18 column, 3.5 μm; mobile phase A: 0.1% FA, B: 80% ACN in water, 0.1% FA, pH 2.7, separation temperature 40°C, flow rate 5 μL/min; gradient was from 0% to 80% B in 64 min (1% ACN *per* min).

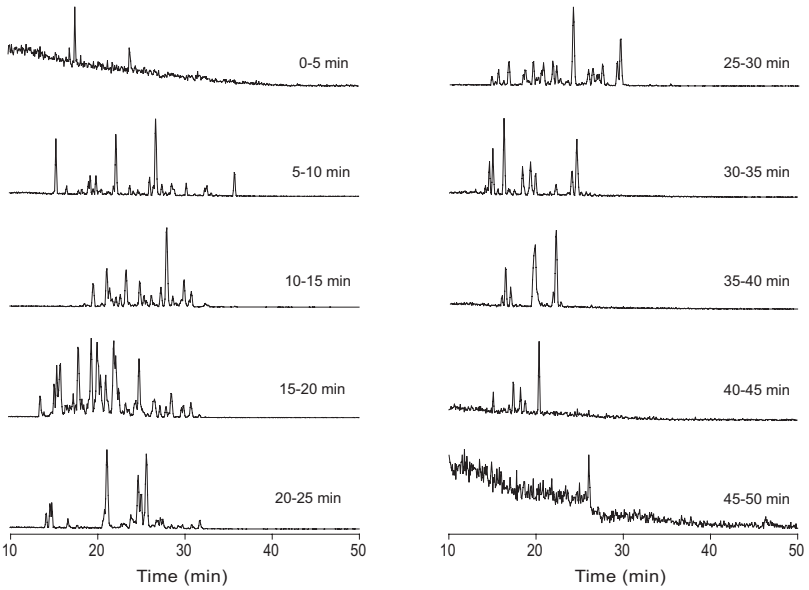

Figure 8. LC-MS analysis of SCX fractions (see Fig. 6B). Fractions collection timing is indicated next to chromatograms. Analysis was performed on QTof ULTIMA MS instrument operated in MS mode. CapLC system was equipped with NanoEase 150 × 0.3 mm Atlantis dC18 column, 3 μm; mobile phase A: 1% ACN in water, 0.1% FA, B: 80% ACN in water, 0.1% FA, pH 2.7, separation temperature 40°C, flow rate 5 μL/min; gradient was from 0% to 56% B in 45 min (approximately 1% ACN *per* min).

by LC-MS; the mass load and separation conditions were identical to a previous RP-RP experiment. Comparison of chromatograms shown in Figs. 7 and 8 reveals that the separation orthogonality is comparable for both 2D-HPLC systems. The retention behavior of peptides correlates well with the results presented in Fig. 1B (SCX-RP) and Fig. 4 (RP-RP).

3.4 Comparison of SCX-RP and RP-RP 2D-HPLC approaches

In an earlier publication we suggested that collecting multiple fractions from an efficient LC dimension in concert with fast (comprehensive) analysis in a second LC dimension is the most productive approach for 2D-HPLC. Figures 5 and 6 illustrate high peak capacity of the peptide separation in RP, at both high and low pH. It is not unusual to achieve peak capacity of 300 for state-of-the-art columns and shallow gradients. It is, therefore, possible to collect multiple fractions without severe fraction oversampling. On the contrary, SCX columns available on the market have practical peak capacity of 50 or less (at least for peptide separation). This is in part due to the fact that the separation in SCX is based on the charge of analytes, therefore the 2+, 3+, and 4+ peptides elute within a relatively narrow gradient strength window. In addition, even at relatively steep gradients peptides typically elute as 1–2 min broad peaks. If the useful gradient range (0–500 mM of NaCl for 200 Å PolySULFOETHYL Aspartamide sorbent) is covered in 50 min and the gradient slope generates peaks approximately 1 min wide, the peak capacity does not exceed 50. We have utilized shallower gradients, but the SCX peak capacity improved only marginally (contrary to RP-HPLC).

The difficulty of the frequent fractionation approach with fractions analyzed in comprehensive LC-MS lies in the limitations of current LC and MS instrumentation. A gradient delay of capillary and especially nano-LC systems unnecessarily extends the time of analysis and undermines the utility of the fast (several minutes long) gradients. Also, the current MS instruments using data-dependent analysis (DDA) may have difficulties in maintaining an effective switching from survey MS mode to MS/MS mode for peaks that are only several seconds wide (at the baseline). Although the development of MS instrumentation and data analysis systems is rapidly progressing [30], experts skilled in the art currently prefer to collect a limited number of fractions and utilize rather extensive gradients (2–4 hours) in the second LC dimension. Due to time constraints, only 10 fractions or less is typically analyzed in 2D-LC-MS. However, an efficient fractionation in the first LC dimension still represents a significant advantage in terms of reduced content overlap between fractions. For example, if one uses a collection window equal to a peak width, virtually all peptides are divided into two neighboring fractions. This causes an unnecessary sample dilution and reduction MS signal.

Table 1 suggests that even with wider collection windows one still observes a significant overlap between neighboring fractions (some components are always divided). When collecting five peak widths wide fractions, the overlap is still 33% (or 18%, assuming that only component overlap greater than 10% is detectable by MS). Therefore, the efficient separation in the first dimension has a considerable impact on 2D-HPLC performance even when a limited number of fractions is collected. The peak overlap was experimentally investigated for selected fractions in Fig. 7. The overlap measured as the percentage of peptides with the same mass in neighboring fractions was 7–22%, which is within the expected range (Table 1).

Table 1. Fraction components overlap dependence on the collection frequency. When collecting fractions equal to one peak width (4σ), virtually all components are divided into two fractions, and the collected fractions contain as many contaminants (from neighboring fractions) as expected components (100% overlap). When employing a wider fraction collection window, the percentage of overlap decreases. Last column indicates the overlap only for the contaminants present in greater than 10% of their original concentration (contaminants present in lower level are considered to be undetectable). Model assumes, Gaussian peaks regularly spaced throughout the separation time; all present at the equimolar level.

Fraction collection width	Overlapping components(all)	Overlapping components (<10% area)
(4σ)	%	%
1	100.0	61.5
2	66.7	38.1
3	50.0	27.6
4	40.0	21.6
5	33.3	17.8
6	28.6	15.1
7	25.0	13.1
8	22.2	11.6
9	20.0	10.4
10	18.2	9.4

The full advantage of the high peak capacity of the RP-RP-HPLC system will be best realized in conjunction with a rapid LC-MS analysis. The ability of 2D-HPLC to generate a large number of well defined fractions (384 or more) may also find its application in combination with high-throughput MALDI-MS analysis [13].

The loss of peptides in SCX mentioned above is a common problem, well known to the scientists skilled in the art [31]. A careful analysis of high pH RP fractionation did not reveal any losses, peak broadening or peak splitting. Some precipitation of peptides was observed when storing the sample at pH 10; therefore the sample was dissolved prior to analysis in neutral pH. It has to be mentioned that elevated pH and temperature may induce a deamidation of asparagines and glutamine and convert them into the corresponding aspartic and glutamic acids. Although we did not observe this, the issue deserves further investigation. From the recovery point of view, the RP mode clearly outperforms SCX fractionation.

Originally, SCX was used for 2D-HPLC mainly due to two factors: good orthogonality with RP, and good compatibility of mobile phases. In principle, it is possible to directly load the fractions into a second LC dimension in both on- or off-line mode. However, the secondary interaction of hydrophobic peptides with the SCX sorbents reduces the peptide recovery and makes an addition of organic solvent to mobile phases desirable.

The presence of organic solvent has detrimental effect on the retention of peptides on a trapping (or analytical) column; some hydrophilic peptides may be lost. A useful compromise is addition of only 5% ACN in SCX mobile phases; short peptides are not completely retained on RP sorbents, but it is believed that they do not represent a great information value for protein identification. In our experience, however, the loss of 7–10mer peptides can be also observed, especially when using short trapping columns and large volume loads of sample. Addition of 25% ACN in the SCX mobile phases (suggested by manufacturer) may further improve the recovery of peptides from the first LC dimension, but then it is no longer compatible with on-line 2D-HPLC approach. The reduction in organic content in the fractions is required, and dictates the use of off-line 2D-HPLC approach [3]. We have evaluated numerous silica and polymer-based SCX columns to identify the most suitable sorbents for peptide analysis with minimal secondary interaction (data not shown). The SCX columns used in this paper were selected for their good peak shape and peptide recovery even when using fully aqueous mobile phases. However, the selective loss of hydrophobic peptides is still noticeable.

RP-RP 2D-HPLC approach suffers a similar incompatibility of mobile phases as mentioned above. It is theoretically possible to use at-column dilution [32, 33] to reduce the organic content in fractions and feed them directly to the second separation dimension, but that is difficult to perform in capillary- or nano-LC scale. We have used the off-line approach with partial evaporation of collected fractions prior to their injection on the second LC dimension with little difficulty. No apparent loss of peptides was observed upon evaporation.

The database search and identification of proteins from peptide MS/MS data is a challenging task, in particular for highly complex samples. The peptides identifications are often based on incomplete MS/MS fragmentation spectra, polluted with noise from other (incidental) precursors; false negative and false positive rate of identification depends greatly on the choice of probability scores [9, 10, 34]. Any additional information about peptides derived from the separation can be used to eliminate low probability peptide assignments [35, 36]. The p*I* values (isoelectric focusing position) or the retention behavior of peptides in RP-LC has been successfully utilized for additional data filtering [10, 36]. SCX peptide retention provides some information about the peptide charge [3], but we are not aware of any report using this information as a data filter. This may be due to the low predictive information value of data – peptides cluster (according to their charges, see Fig. 1) only loosely into partially overlapping retention windows. How-

ever, the retention prediction of peptides in RP is rather accurate and informative [20, 34, 37–39]. Using RP in both LC dimension one can derive predictive models for both high pH and low pH retention, potentially enhancing the data filtering and the robustness of peptide identification.

4 Concluding remarks

A novel method for 2D-HPLC separation of proteomic samples has been developed, utilizing RP sorbents in both LC dimensions. Due to the ionic nature of peptides, it was possible to achieve a substantial separation orthogonality in the RP-RP system using pH 10 in the first and pH 2.6 in the second separation dimension, even for the columns packed with the identical C18 sorbent. The performance of RP-RP-HPLC compares well to current state-of-the-art SCX-RP-HPLC. Proposed novel 2D-HPLC method has several advantages: (i) A high peak capacity in the first separation dimension permits the collection of multiple fractions with minimal content overlap. Eluting peptides are not divided in several consecutive fractions, thus their signal is not diluted. (ii) No peptide losses were observed in the first RP-LC dimension. Better recovery of short as well as long peptides was obtained compared to SCX-LC. (iii) The mobile phases were salt free and compatible with MS detection. No desalting or salt diverting from MS source was required. Although the mobile phases used in RP-RP-HPLC have limited compatibility and on-line 2D-HPLC may be difficult to implement, an off-line approach with partial sample evaporation was performed without complications. A proposed 2D-HPLC method represents a promising tool for proteomic research, and could be used as an alternative to SCX-RP-HPLC approach.

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