

OVERVIEW

- Several alternative off-line 2D-LC systems were evaluated
- A geometric method for 2D-LC separation orthogonality description was developed
- RP-RP (high/low pH) is a promising tool for 2D-LC proteome research
- RP-RP 2D-LC system provides the highest practical peak capacity

INTRODUCTION

2D-LC separation with tandem mass spectrometry (MS/MS) is a powerful tool for proteome research. 2D-LC is expected to provide for a greater peak capacity (theoretical number of resolved components) than 1D-LC; the peak capacity is typically calculated from equation 1.

$$P_{2D} = P_1 \times P_2 \quad (1)$$

However, a total 2D peak capacity can be calculated as multiplication of peak capacities in first and second dimensions only when the LC modes are ideally orthogonal. In this work we measured retention times of 196 tryptic peptides using 1D-LC-MS in order to construct the normalized peptide retention maps (Figure 2) and compared the orthogonality of 2D-LC systems. A geometric approach for orthogonality description was developed, employing a surface area of retention plots utilized for 2D separation. Those retention maps were normalized according to equation 2 and plotted in a normalized 2D separation space (Figure 2).

$$RT_{i(norm)} = \frac{RT_i - RT_{min}}{RT_{max} - RT_{min}} \quad (2)$$

RT_{min} and RT_{max} represent the retention times of first and last eluting peptides in each of 1D-LC data set. Normalized peptide $RT_{i(norm)}$ values vary between 0-1. The normalized 2D-separation space is divided into 14 x 14 bins representing total 2D peak capacity of 196. The principles of a geometric orthogonality are outlined in Figure 1. Each single bin containing a peptide is highlighted; each bin represent an area of an eluting peak. Sum of all bins represent a total area used for separation. In practice, a completely non-orthogonal system (Figure 1A) area coverage is 10%. Ideally orthogonal system (random distribution) coverage is ~63% (Figure 1B). The orthogonality is defined by equation 3, where $\sum bins$ is the number of bins containing a peptides in the 2D plot,

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

and P_{max} is total 2D peak capacity (sum of all bins). Practical peak capacity of 2D separation systems can be calculated from the knowledge of normalized area used for separation, and the peak capacities in both 1D-LC separation modes (equation 4). The $P_{1,2}$ in 1D was calculated from the average peak width w measured at 4 sigma and the useful gradient time t_g (equation 5).

$$N_p = P_1 \times P_2 \times \frac{\sum bins}{P_{max}} \quad (4)$$

$$P_{1,2} = 1 + t_g / w_{13.4\%} \quad (5)$$

The retention data of peptides were measured using 5 protein digests (MassPREP digestion standards, Waters) analyzed by LC-MS (example in Figure 3). The columns and conditions are described in Figure 3 and 4 captions. **Reversed-phase (RP), hydrophilic interaction chromatography (HILIC), size exclusion chromatography (SEC), and strong cation exchange (SCX) LC modes were evaluated.** Orthogonality of separation is due to the pH effect. The orthogonality and practical peak capacity of RP-RP and SCX-RP modes are comparable (Table 1). Figure 4 shows peptide analysis in RP-RP (high/low pH) mode; both columns were C18.

RESULTS AND DISCUSSION

Figure 1: Geometrical approach to orthogonality description. 10 x 10 separation space, total 2D peak capacity=100. 100 data points were plotted in 2D space. **(A)** example of completely non-orthogonal 2D separation; 10% area is used. **(B)** completely orthogonal system with randomized retention data plot; in average 63% area is used.

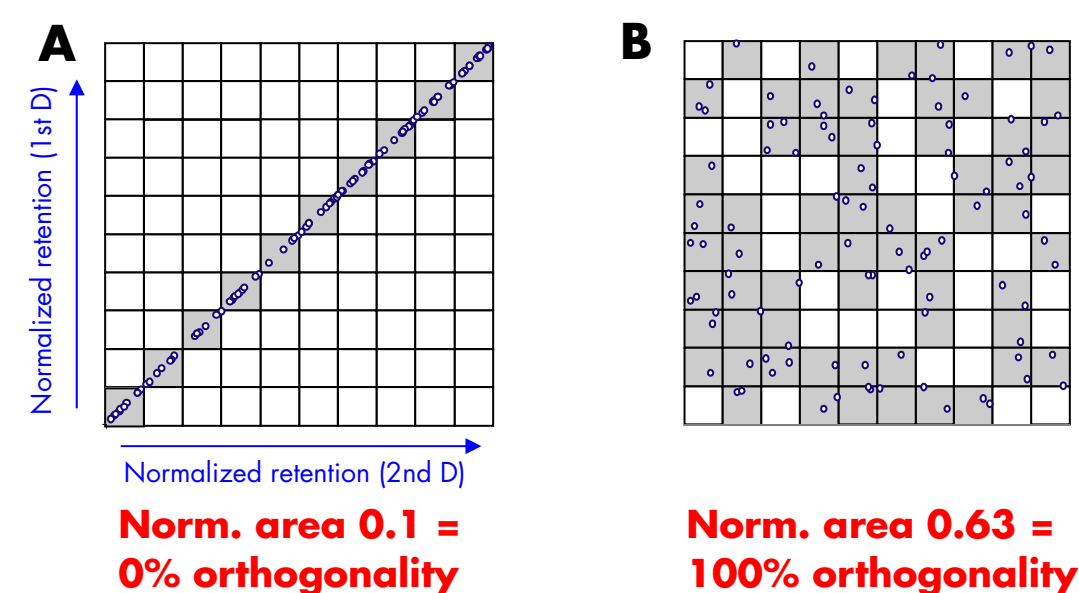
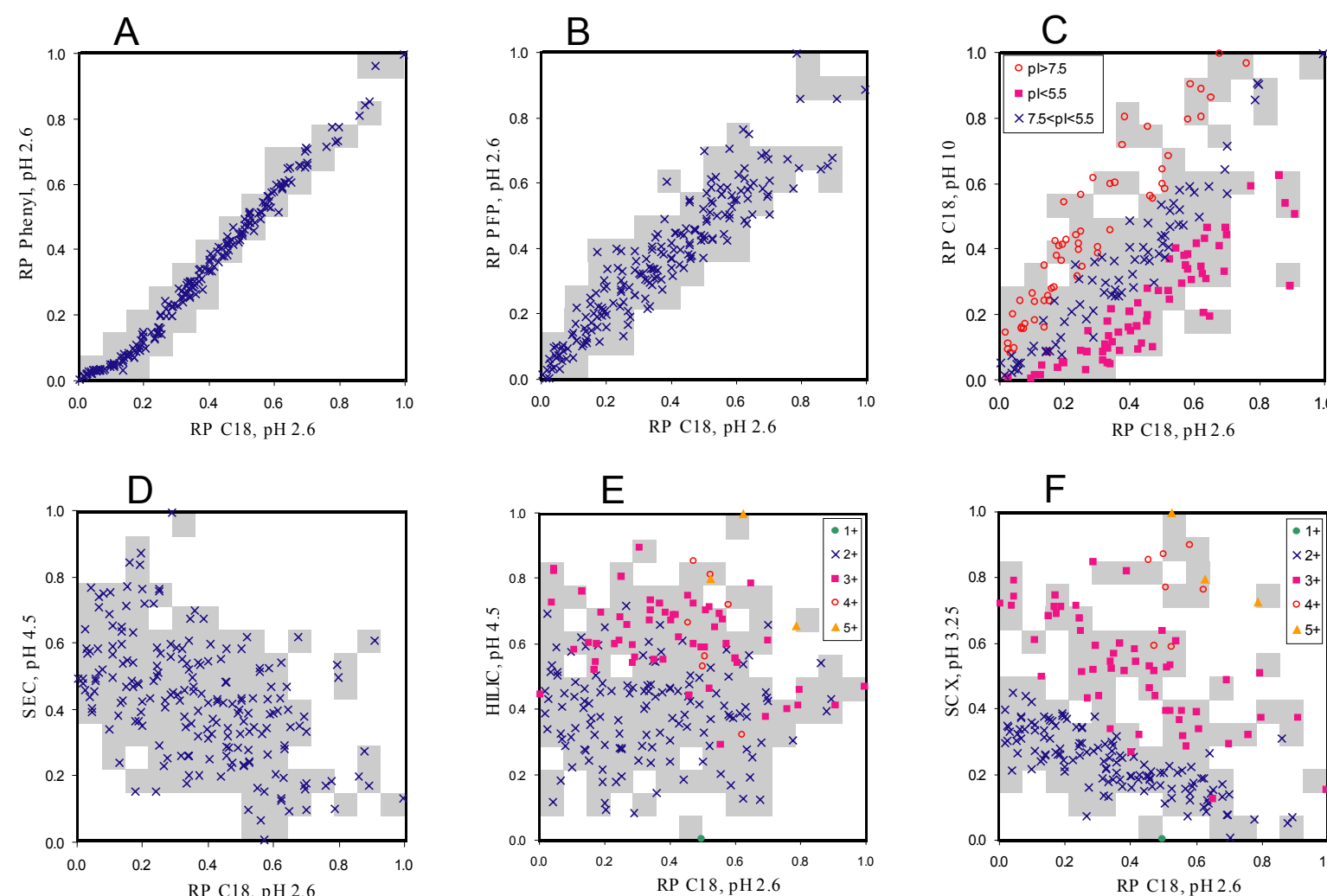


Figure 2: Peptide retention (196 tryptic peptides) was measured in 1D-LC-MS; 2D retention plots were then constructed in a 2D-LC normalized space: **(A)** Phenyl x C18, **(B)** Pentafluorophenyl x RP C18, **(C)** C18, pH 10 x C18, pH 2.6, **(D)** SEC x C18, **(E)** HILIC x C18, **(F)** SCX x C18. Examples of chromatograms see in Figure 3. The 2D-space was divided into 14 x 14 bins, total peak capacity is 196. Bins containing peptides are highlighted in grey. Greater surface coverage indicates a greater orthogonality (see Table 1).



LC conditions: All columns were 150 x 2.1 mm, except SEC (YMC diol, 60 Å, 750 x 4.6 mm). Flow rate was 0.2 mL/min, column temperature 40 °C, except for SCX (30 °C). Mobile phase A was 0.2% FA, pH 2.6 or appropriate ammonium formate buffer (pH is indicated in the figures); mobile phase B was acetonitrile. For RP systems **(A-C)** the gradient was 0.8% acetonitrile per min for 50 minutes. SEC column was used with a mobile phase containing 20% acetonitrile and 40 mM ammonium formate. HILIC gradient started from 90% acetonitrile, peptides were eluted with 0.8% water gradient per minute. SCX column (PolyLC Polysulfoaspartamide, 200 Å) was run with constant 25% acetonitrile content in mobile phase. Gradient was from 40 mM to 300 mM of ammonium formate, pH 3.25. SCX was compatible with a direct LC-MS detection.

Discussion of Figure 1: 2D retention plots

- Orthogonality of several RP modes tested is low. Varying ion-pairing buffer composition, or type of RP stationary phase (Fig.1A) does not generate a high degree of orthogonality. The best orthogonality was observed for PFP x C18 2D-LC system (Fig.1B).
- Because peptides are ionogenic compounds, the pH can be used to alter the selectivity. High degree of orthogonality was achieved when using C18 columns in both dimensions, but varying the pH of separation (Fig.1C). Acidic peptides (pI<5.5) are more effectively retained in RP mode at acidic pH, basic peptides (pI>7.5) at basic pH.
- SEC-RP system shows an interesting orthogonality (Fig.1D), in part due to a secondary interaction of peptides with SEC sorbent. Partial loss of hydrophobic peptides was observed.
- HILIC-RP system (Fig.1E) has the greatest surface coverage (orthogonality). Silica based Atlantis™ HILIC column was used without an observable loss of peptides. Selectivity of separation suggests that the retention mechanism includes a secondary interaction with silanols (cation-exchange mode).
- SCX chromatography with NaCl eluents is not compatible with MS. We have successfully used ammonium formate eluents with direct LC-MS detection (Fig.1F). Interestingly, a more complete peptide recovery was observed with volatile buffers than with NaCl eluent. Both systems show a similar selectivity; peptides are retained according to their charge. Additional separation within the charge groups is due to the charge density (shorter 2+ peptides are better retained than the long ones). Most abundant classes of tryptic peptides (2+ and 3+) cluster in small portion of 2D separation space, which lowers the overall orthogonality of SCX-RP system.

Figure 3: Examples of LC-MS analyses utilized to acquire peptide retention data for the retention plots (Figure 1). Separation of Phosphorylase b tryptic digest (~100 peptides) using various LC modes: **(A)** RP, Atlantis dC18, pH 2.6, **(B)** RP, Pentafluorophenyl, pH 2.6, **(C)** RP, XTerra MS C18, pH 10, **(D)** SEC, YMC diol 60 Å, pH 4.5, **(E)** HILIC, Atlantis HILIC, pH 4.5, and **(F)** SCX, PolySULFO A, pH 3.25.

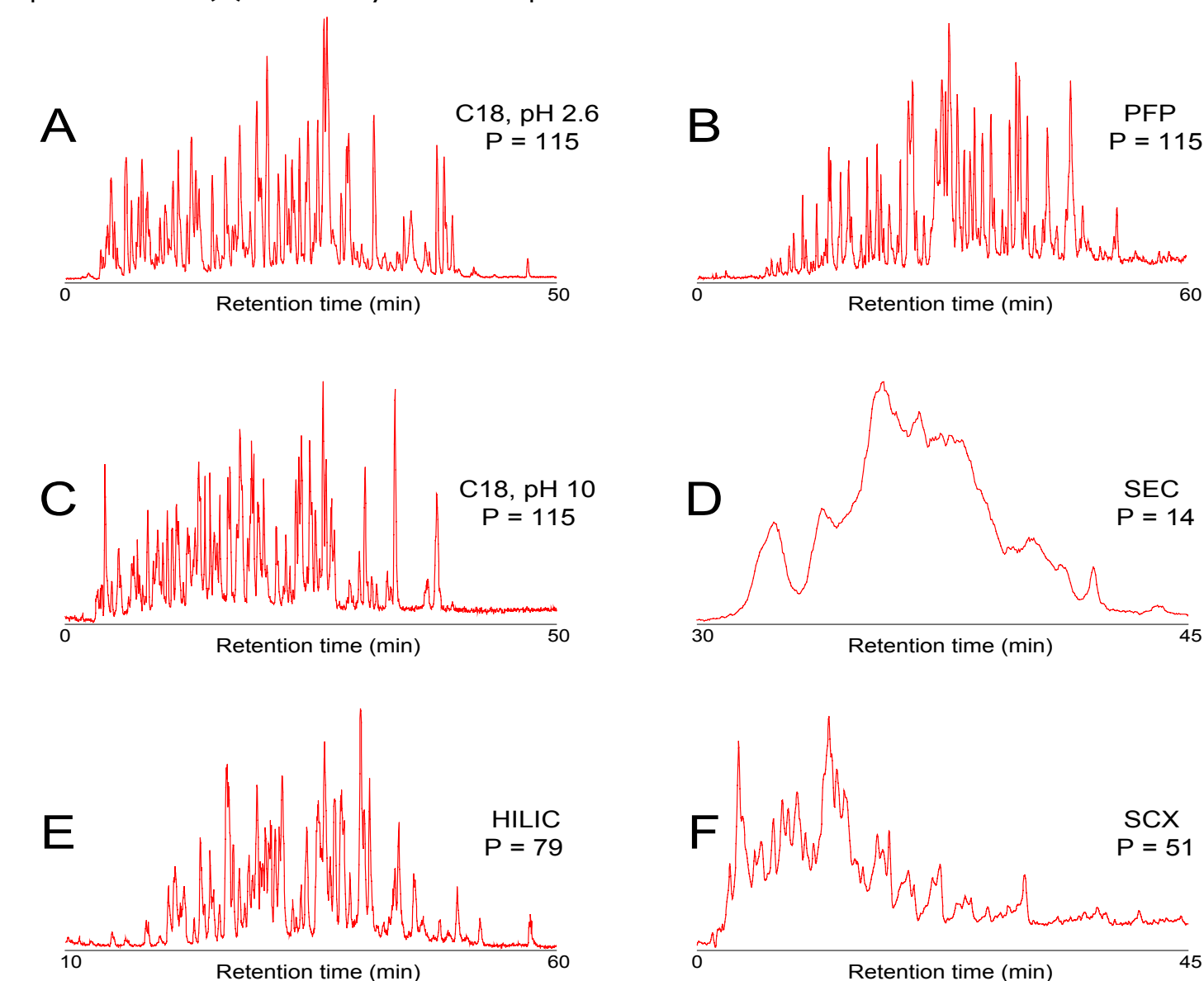


Table 1: Orthogonality and peak capacity of investigated 2D-LC systems (Figures 2 and 3).

	First LC dimension					
	Phenyl	PFP	C18 pH 10	SEC	HILIC	SCX
1D-LC peak capacity ^a	115	115	115	14	79	51
2D number of bins ^b	30	52	80	86	100	81
2D normalized area fraction ^c	0.15	0.27	0.41	0.44	0.51	0.41
2D-LC orthogonality % ^d	13	31	53	58	69	54
2D-LC theoretical peak capacity P_{2D} ^e	13225	13225	13225	1610	9085	5865
2D-LC practical peak capacity N_p ^f	1984	3571	5422	708	4633	2405
2D-LC practical peak capacity N_p ^g (10 fractions collected in 1 st D)	172	311	472	506	587	472

^a equation 5; ^b from Figure 2, 14 x 14 bins space (196 bins total); ^c see Figure 1; ^d equation 3; ^e equation 1; ^f equation 4; ^g only 10 fractions collected in first LC dimension (P1=10).

Figure 4: High/low pH RP-RP 2D-LCMS/MS separation in off-line mode. **(A)** - XTerra MS C18 separation of 5 protein digests (~200 peptides). **(B)** Collected fractions were partially evaporated and injected on 0.3 x 150 mm NanoEase Atlantis dC18 column (0.1% FA, 1% ACN/min.) Fraction numbers and collection intervals are shown in figures.

