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Introduction

Multi-dimensional chromatography-mass spectrometry (MD LC-MS) is a useful tool for the separation of complex peptide mixtures. Since the samples may contain hundreds of thousands of peptides of various abundance, length and hydrophobicity, high separation power is required. This paper presents a critical look at multi-dimensional chromatography and its challenges for peptide separation. We discuss the importance of column peak capacity (number of peaks separated within a given gradient time) and the selectivity of selected chromatographic modes on the success of MD LC-MS analysis.

Results

We investigated column peak capacity using Symmetry300™ (C₁₈, 300 Å) columns of differing length and particle size, while inner diameter was kept constant. The separation efficiency of columns under gradient conditions is defined by column peak capacity P , rather than by theoretical plates. Peak capacity is the number of peaks that can be theoretically separated within a gradient time (not the actual number of peaks separated). Peak capacity was calculated from experimentally measured peak widths of nine peptides (Figure 3) using equation 1.

$$P = 1 + \frac{t_g}{w} \quad (1)$$

where t_g is time of the gradient run and w is peak width measured at 13.4% peak height. The experimental data were compared with P predicted from equation 2,

$$P = 1 + \frac{\sqrt{N}}{4} \cdot \frac{B \cdot \Delta c}{B \cdot \Delta c \cdot (t_0/t_g) + 1} \quad (2)$$

where N is column efficiency, B is the slope of the function $\ln k$ versus solvent composition, Δc is the organic modifier range used in the gradient run (0-50% → $\Delta c=0.5$), t_0 is column void time, and t_g is gradient time. The normalized gradient slope, s (equation 3) was kept constant for all columns (Table 1). Since t_0 varies with column length, t_g was adjusted to keep s constant.

$$s = \Delta c \cdot t_0/t_g \quad (3)$$

Both theoretical prediction and experimental data suggest that the practical number of peaks separated on columns reaches an upper limit, despite using highly efficient columns and very shallow gradients. The practical peak capacity value is about several hundred for state of the art RP-HPLC columns (Figure 1). Doubling the column efficiency (length) improves the peak capacity by only 40 %, and proportionally increases either the separation time or the backpressure (Figure 1A). Similarly, extremely shallow gradients have a positive effect on the peak capacity, but analysis becomes unacceptably long (Figure 1B). For example, a peak capacity of ~1400 was achieved in a 17 hour run using a 600 x 4.6 mm, 3.5 μm RP-HPLC column operated within the pressure limits of conventional HPLC instruments (using a flow rate of 0.4 ml/min).

Figure 1: Peak capacity shown as a function of column efficiency and gradient time. Peak capacity increases with an increase in column efficiency (A). Doubling column length leads to only a 40% increase in peak capacity. Peak capacity increases with increasing gradient time (B). However, extensively increasing gradient time offers no substantial increase in peak capacity.

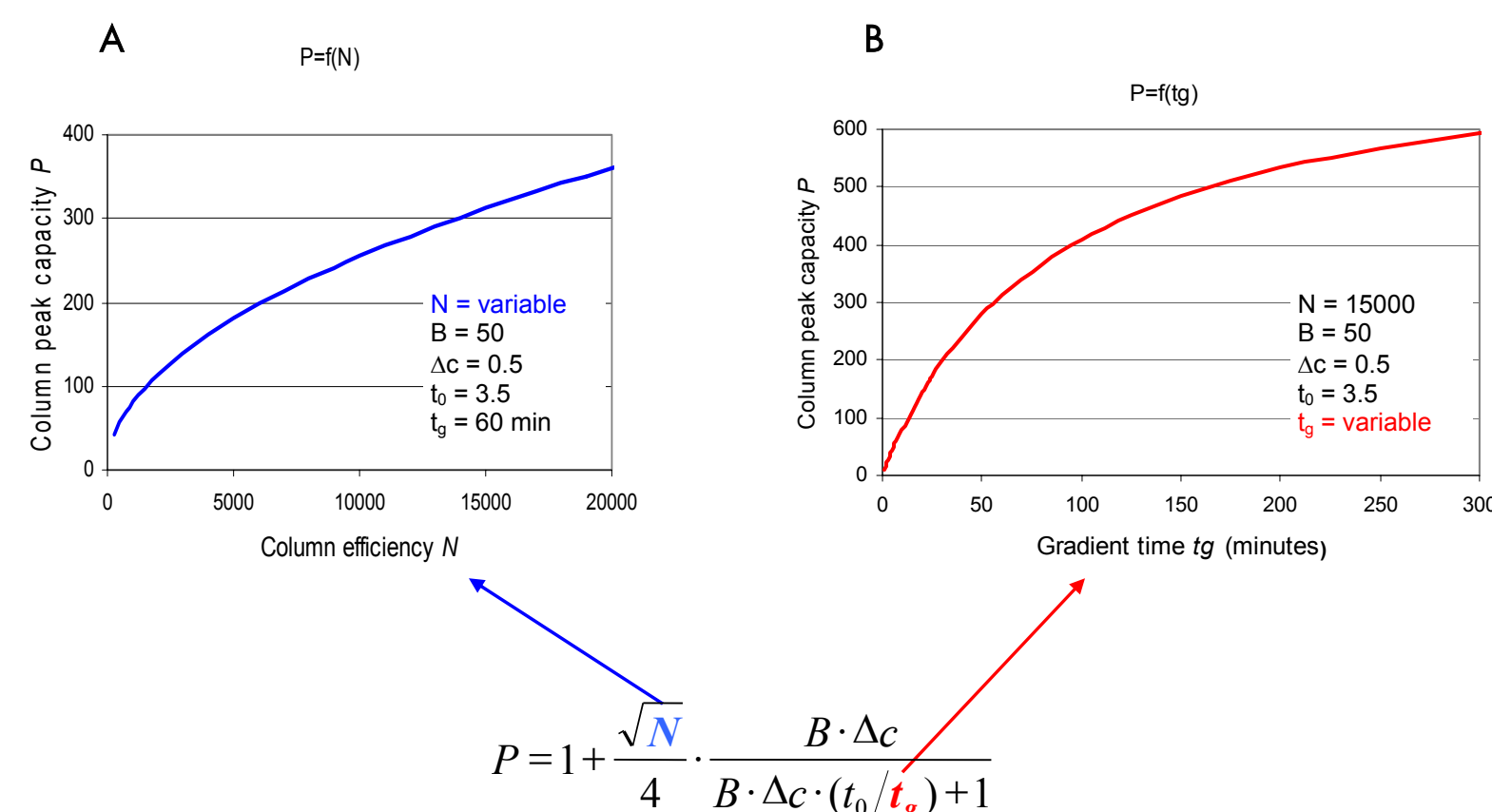


Figure 2: Calculated peak capacity (equation 2) as a function of both column efficiency and gradient time (B=50). This three-dimensional graph shows that the peak capacity plateaus at long gradient times and high column efficiencies.

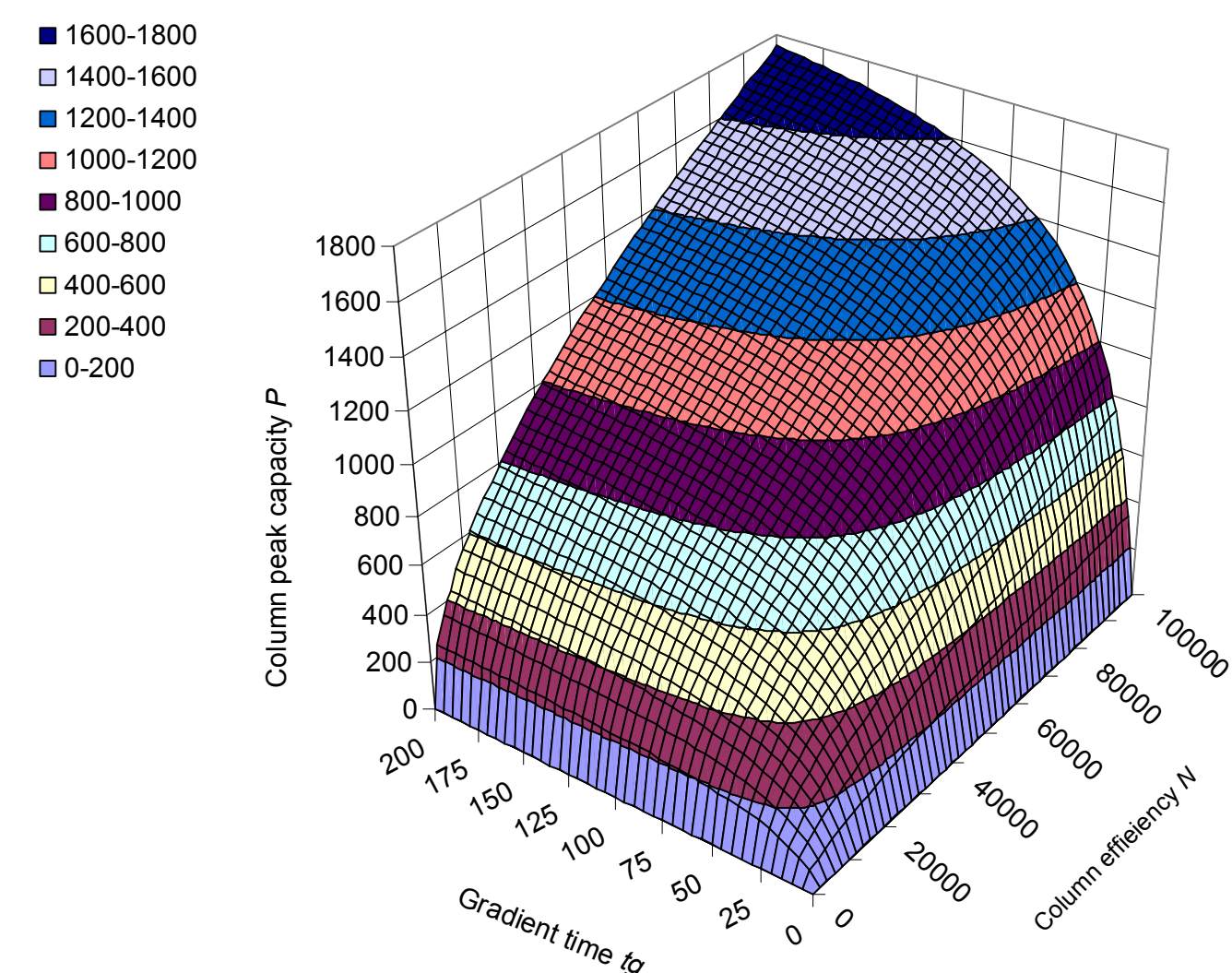


Table 1: Columns and gradients used in this study. All columns were packed with Symmetry300™ C₁₈ sorbent. The inner diameter was held constant while the particle size, column length, and gradient slope varied.

Length x ID (mm)	Particle Size d	Efficiency N ^a	Void Volume	t _g for slope s = 0.0123 ^b	t _g for slope s = 0.0246 ^b	t _g for slope s = 0.0492 ^b
50 x 4.6	5	2438	0.66	35.7	17.8	8.9
150 x 4.6	5	7313	1.86	100	50	25
150 x 4.6	3.5	12837	1.90	100	50	25
150 x 4.6	7	4179	1.80	100	50	25
300 x 4.6	5	14626	3.46	186	93.1	46.5

^a Efficiency calculated from Van Deemter Equation for peptides, D_m=2 x 10⁻¹⁰, and flow rate 0.75 ml/min
^b Normalized gradient slope $s = \Delta c \cdot t_0/t_g$

Figure 3: An example of peak capacity measurement for Symmetry300™ C₁₈, 5 μm, 4.6 x 150 mm column using three different gradient slopes. The peak capacity was calculated from the average measured peak widths of nine peptides for each gradient slope. As the gradient time increases, the peak capacity increases.

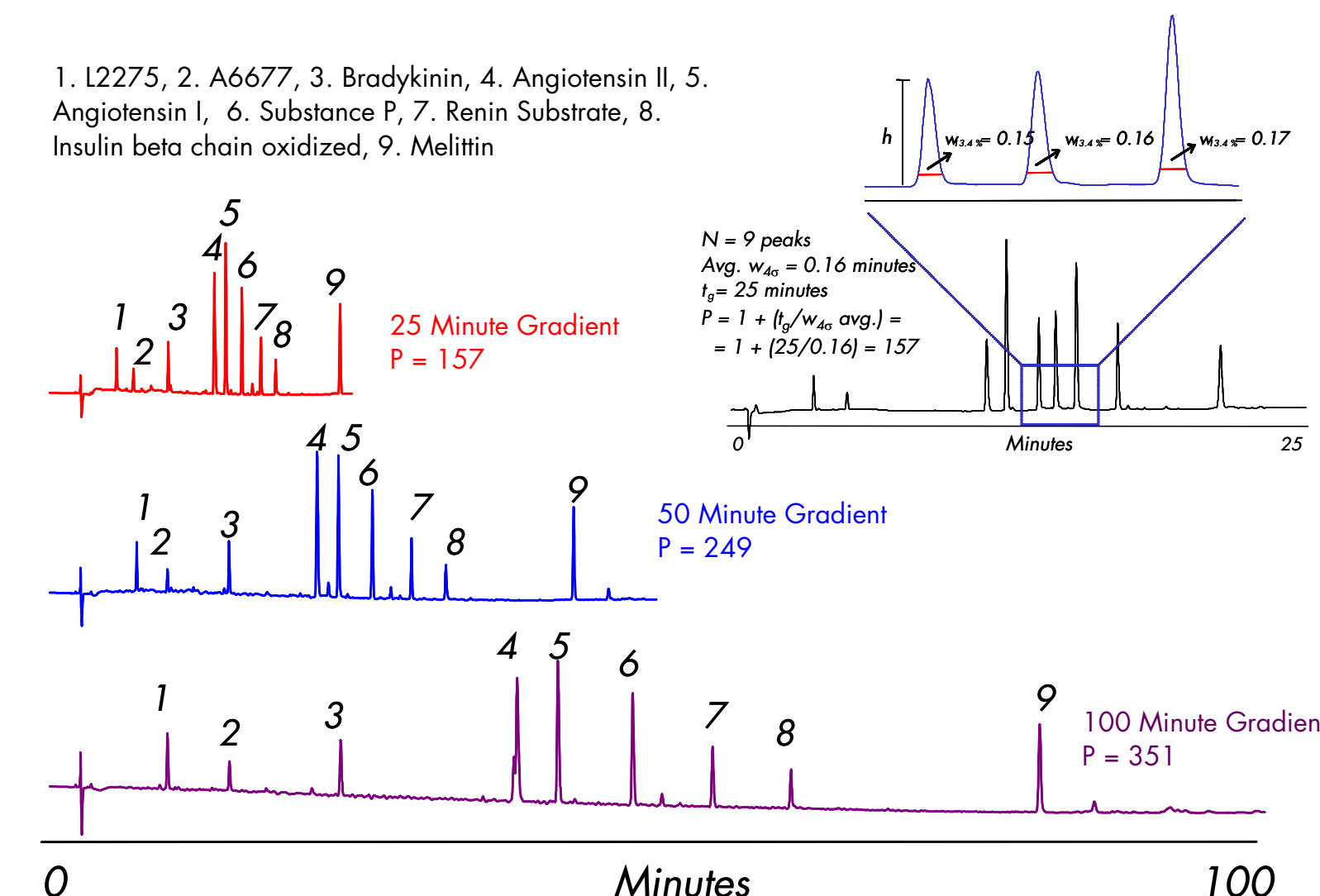
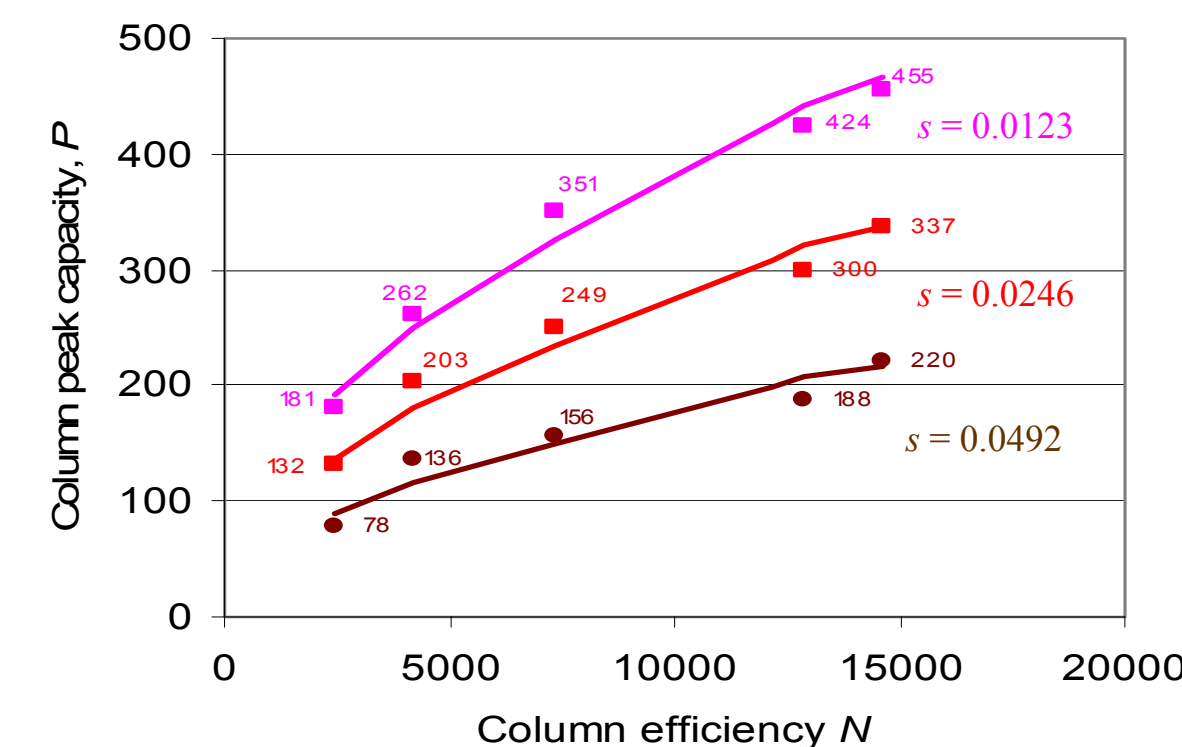


Figure 4: Comparison of experimentally measured and predicted peak capacity values (B=50, D_m=2 x 10⁻¹⁰) at three different normalized gradient slopes. Solid lines represent predicted P values and data points (numbers) represent experimentally measured P values.



Discussion

The currently used two-dimensional (2D) HPLC approach employs ion-exchange (IE) followed by reversed-phase (RP)-HPLC. However, the peak capacity of IE-HPLC is only 50-100. Even this moderate resolution power is underutilized when using the common step gradient approach, in which 10-20 fractions are recovered. Therefore, the peak capacity of 2D-IE-RP-HPLC is about 10-20 thousand peaks, assuming a peak capacity of ~1000 for RP. Some researchers proposed adding separation dimensions to 2D-LC-MS. However, the success of MD-LC-MS separation requires the orthogonal separation selectivity in each dimension. Available techniques such as size exclusion chromatography (SEC), hydrophobic interaction chromatography (HIC), and hydrophilic interaction chromatography (HILIC) show selectivity principally similar to those in RP-HPLC. Therefore, the MD-LC-MS separation of more than ten thousand peaks is difficult to achieve on a routine basis, even when taking into consideration the additional resolving power of MS.

Experimental

HPLC System: Alliance® 2796 Bioseparations Module (Waters) with a 996 PDA detector
Mobile Phases: A: 0.1% TFA in water, B: 0.08% TFA in acetonitrile
Column Dimensions: Symmetry300™ C₁₈ (See Table 1)
Gradient Conditions: 0-50% B; gradient slope was varied
Other Conditions: Column temperature: 40 °C; Flow rate: 0.75 ml/min; Injection volume: 20 μl (total peptide load: 11 μg); Peak capacity: determined at 4σ (13.4% peak height)

Conclusions

- State of the art RP-HPLC columns have a practical peak capacity of several hundred.
- Increasing column efficiency (longer columns, smaller particles) improves P , but leads to an increase in backpressure and/or analysis time, t_g .
- Gains in peak capacity plateau at very shallow gradients using highly efficient columns.
- Peak capacity of IE-HPLC columns is substantially lower than that of RP-HPLC.
- Current 2D-HPLC separations result in a peak capacity of < 10,000.
- Successful MD-LC-MS separation requires orthogonal selectivity in each separation dimension. This is difficult to achieve with currently available HPLC separation modes.