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The Impact of Column Peak Capacity on the Multi-dimensional Chromatography of Complex Peptide Mixtures

Martin Gilar

Life Sciences Chemistry, Waters Corporation

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Outline

- Highly complex peptide samples
- Column peak capacity definition
- 1D RP-HPLC - where are the limits?
- Peak capacity prediction
- Peak capacity in 2D and MD-HPLC
- Productivity of MD separation
- Future development

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What is the required peak capacity?

- Proteomic samples = peptides
- 10-50 thousand proteins
- 100-500 thousand peptides
- Current MD-HPLC systems: peak capacity > 10 000
  - orthogonal selectivity - 1D x 2D x …MD
  - SCX and RP-HPLC
- MS = additional separation dimension

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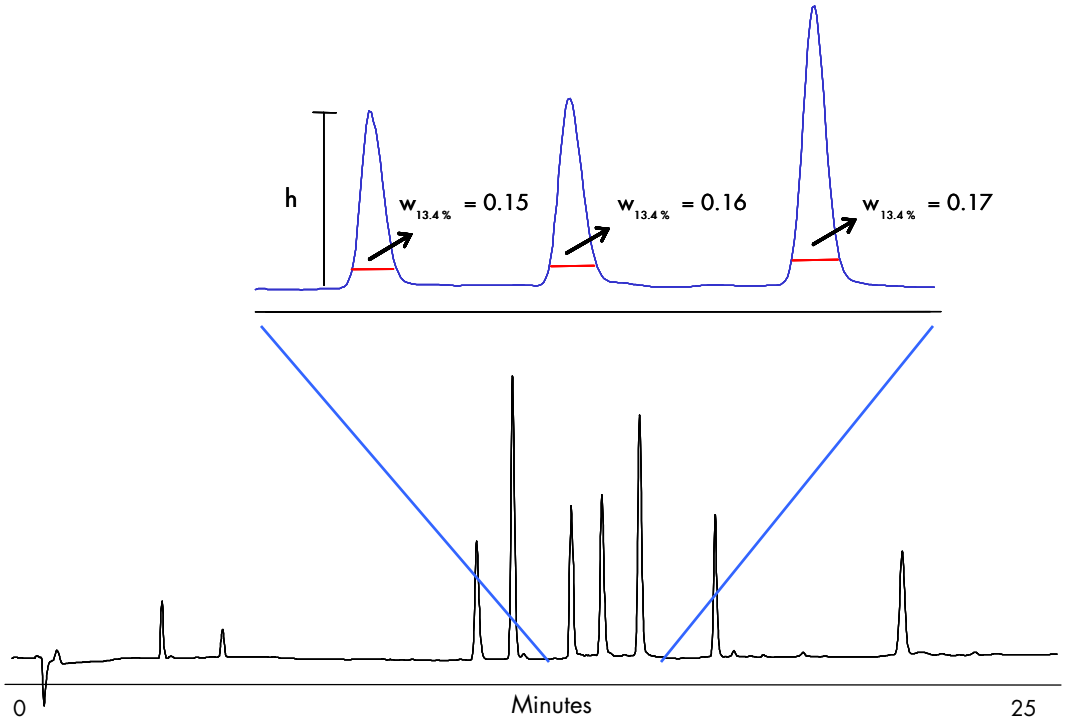
Column peak capacity

Column peak capacity: Maximum number of peaks that can be separated on a column within a given gradient time.

$$P = 1 + \frac{t_g}{w}$$



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1D HPLC peak capacity

Column type Lx ID (mm)	Particle size (mm)	Void volume (ml)	Gradient time (s=0.0123)	Gradient time (s=0.0246)	Gradient time (s=0.0492)
Symmetry300 C18 50 x 4.6	5	0.66	37.5	17.8	8.9
Symmetry300 C18 150 x 4.6	5	1.86	100	50	25
Symmetry300 C18 150 x 4.6	3.5	1.90	100	50	25
Symmetry300 C18 150 x 4.6	7	1.80	100	50	25
Symmetry300 C18 300 x 4.6	5	3.46	186	93.1	46.5
			Gradient time	Gradient time	Gradient time
PolySULFOETHYL aspartamide 50 x 4.6	5	0.7	20	40	80

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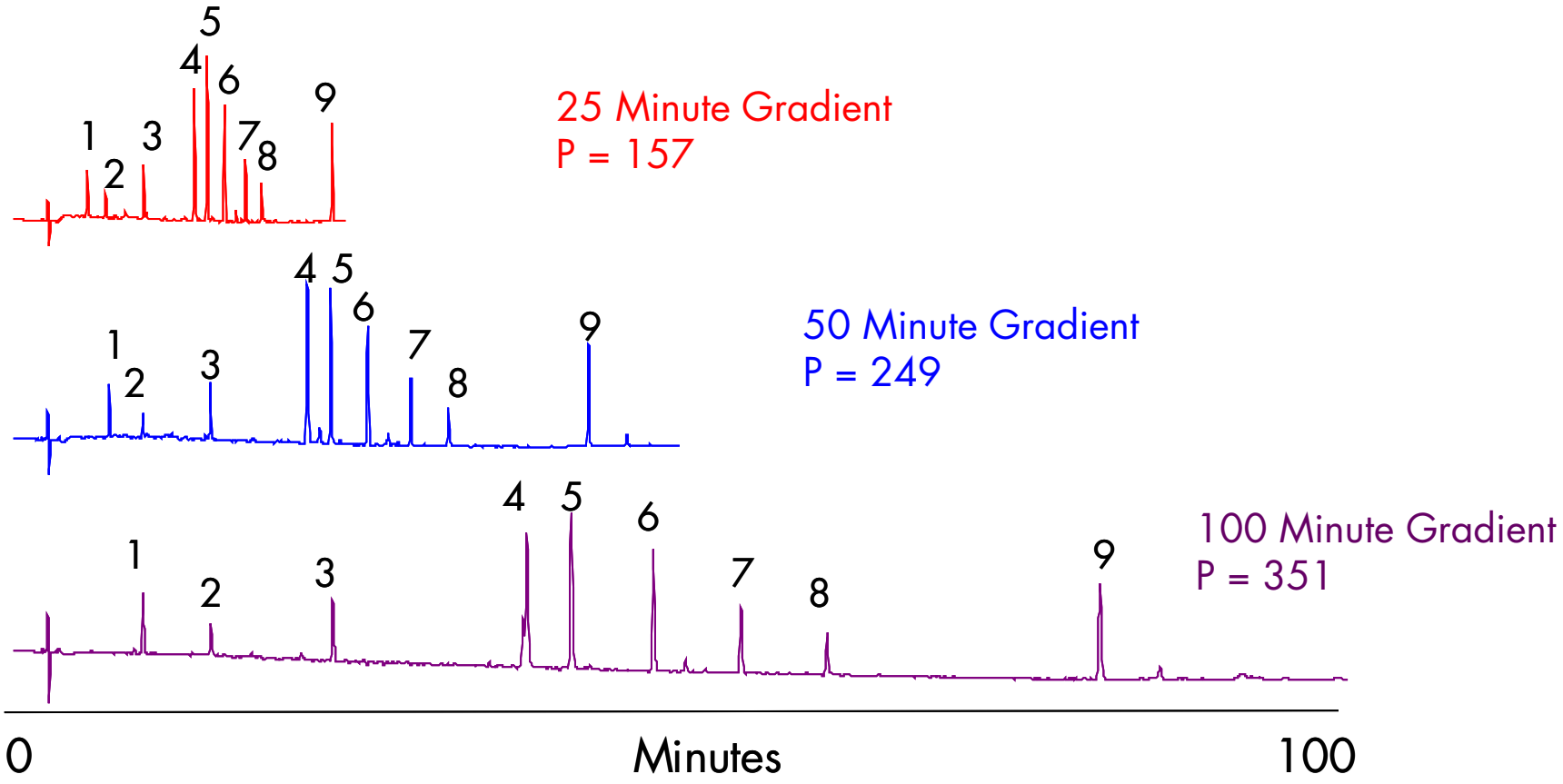
1D HPLC peak capacity

Symmetry300 C18, 150 x 4.6 mm, 5µm



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1D HPLC peak capacity

Strategies for increasing the column peak capacity

- Long gradient time (shallow gradients)
- Extend the column length + gradient time
- Use small particle size sorbent

$$s = \Delta c \cdot \frac{t_0}{t_g} = 0.5 \cdot \frac{1}{50} = 0.01$$

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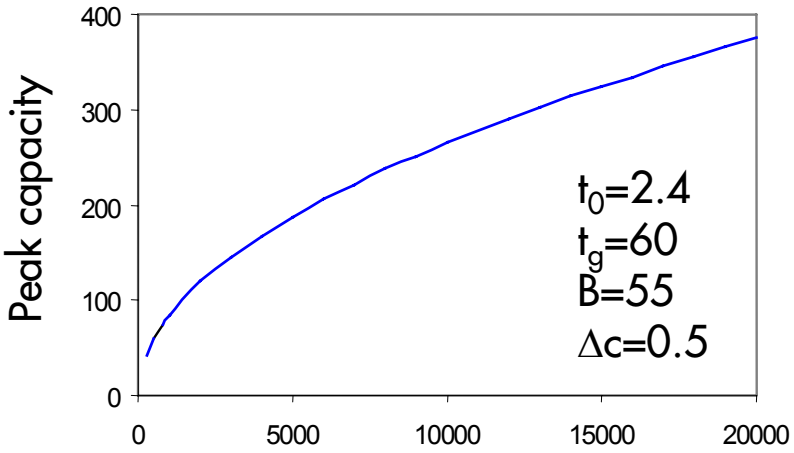
Peak capacity prediction

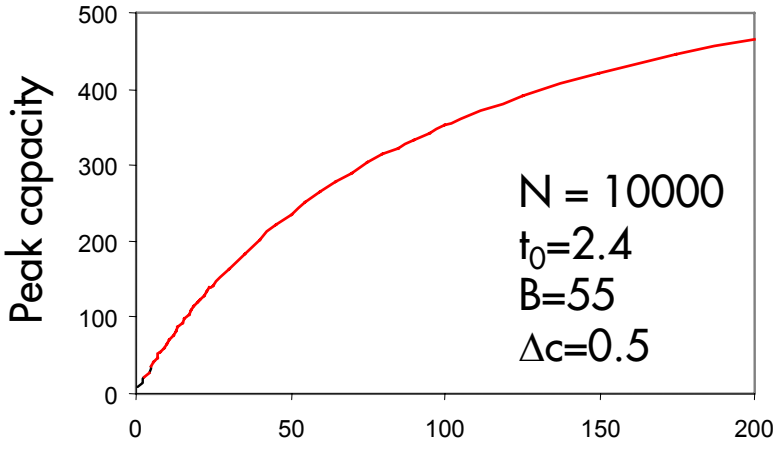
$$P = 1 + \frac{\sqrt{N}}{4} \cdot \frac{B \cdot \Delta c}{B \cdot \Delta c \cdot (t_0 / t_g) + 1}$$

N



Column efficiency N

tg



Gradient time (minutes)

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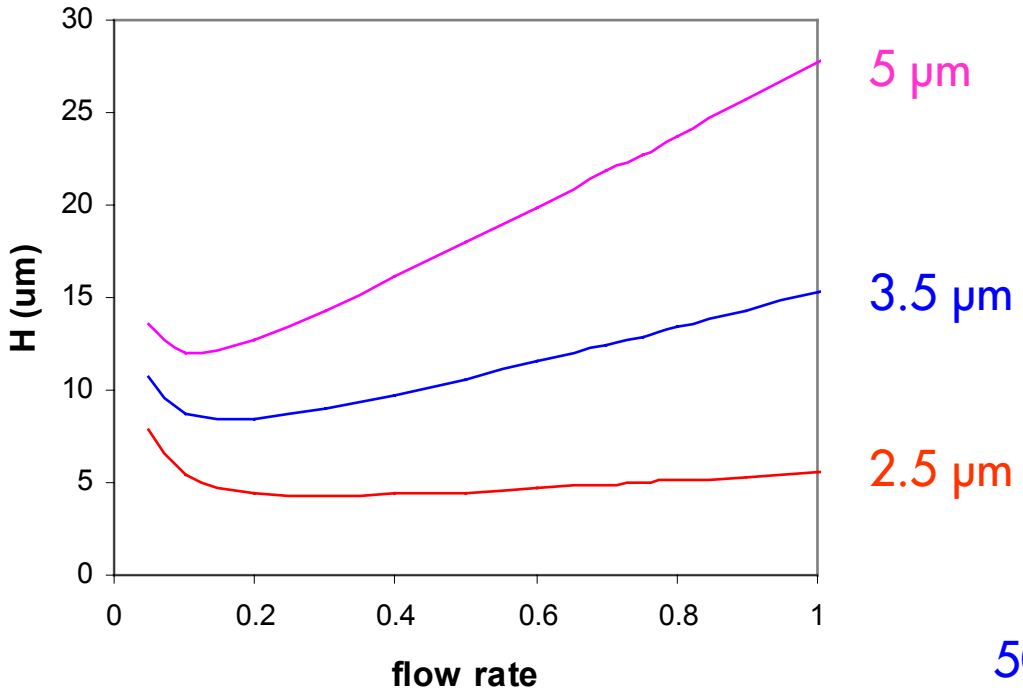
Peak capacity prediction

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

Column efficiency N, $D_m = 3 \times 10^{-10}$ m^2/s



50 x 4.6 mm I.D.

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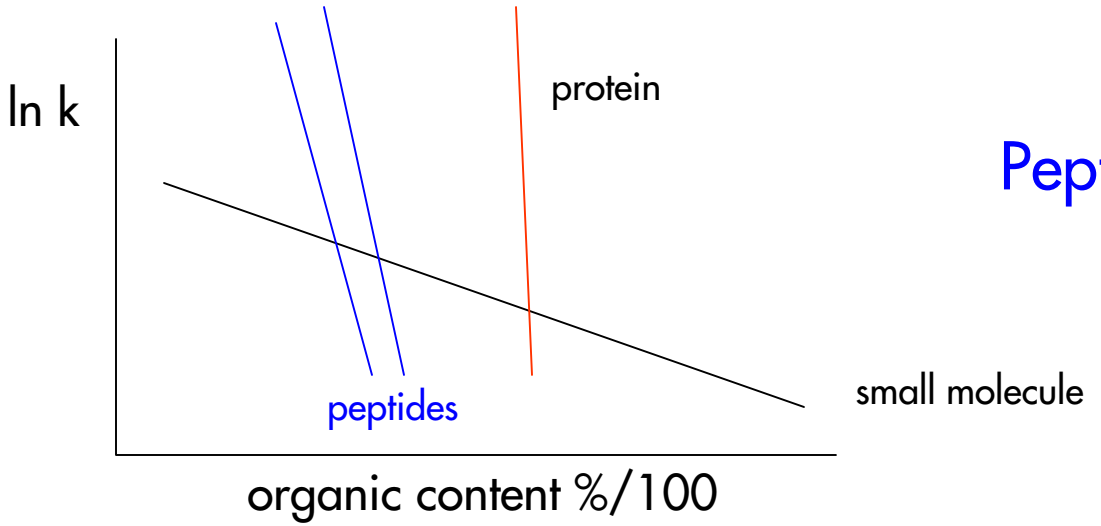
Peak capacity prediction

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



Peptides B~45-65

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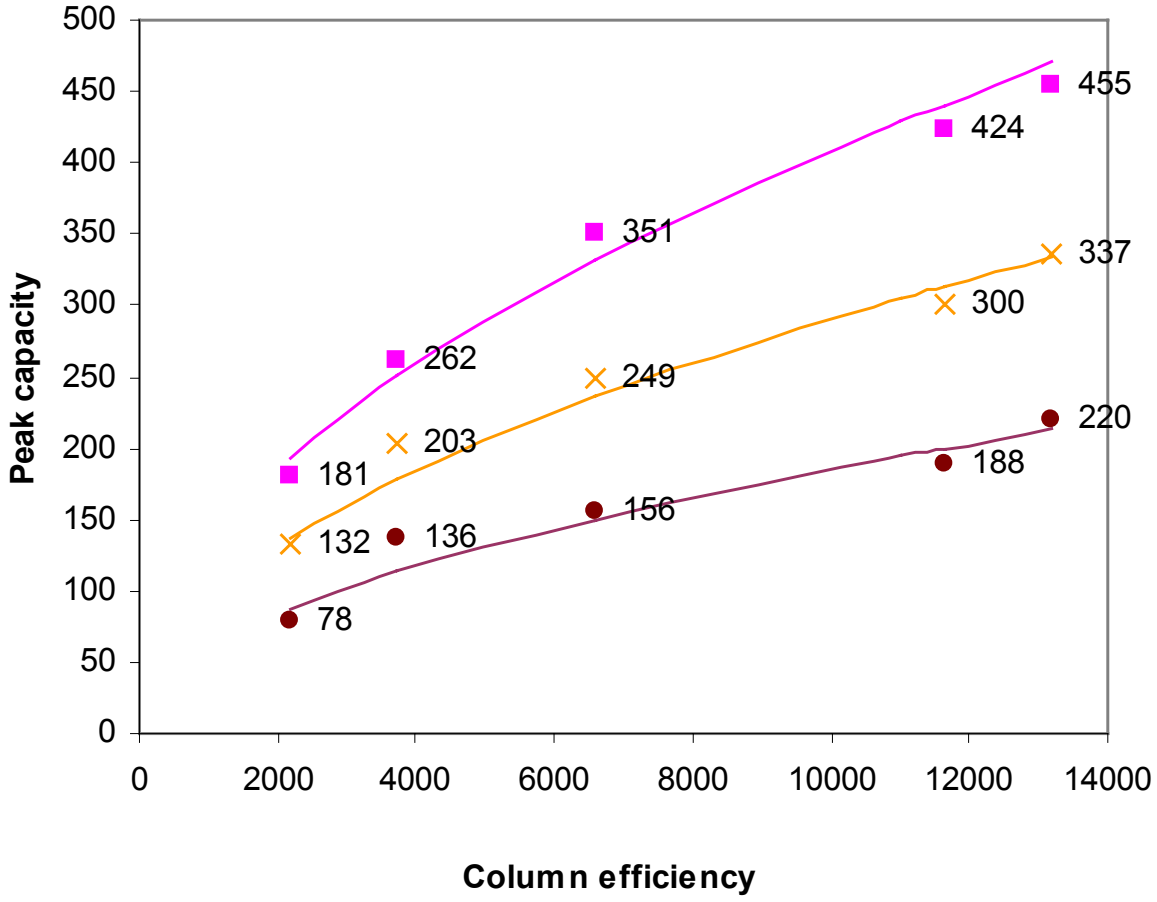
Predicted versus experimental peak capacity

Five columns, 3 gradient slopes



s = 0.0123

s = 0.0246

s = 0.0492

B=55

$D_m = 3 \times 10^{-10}$ m²/s

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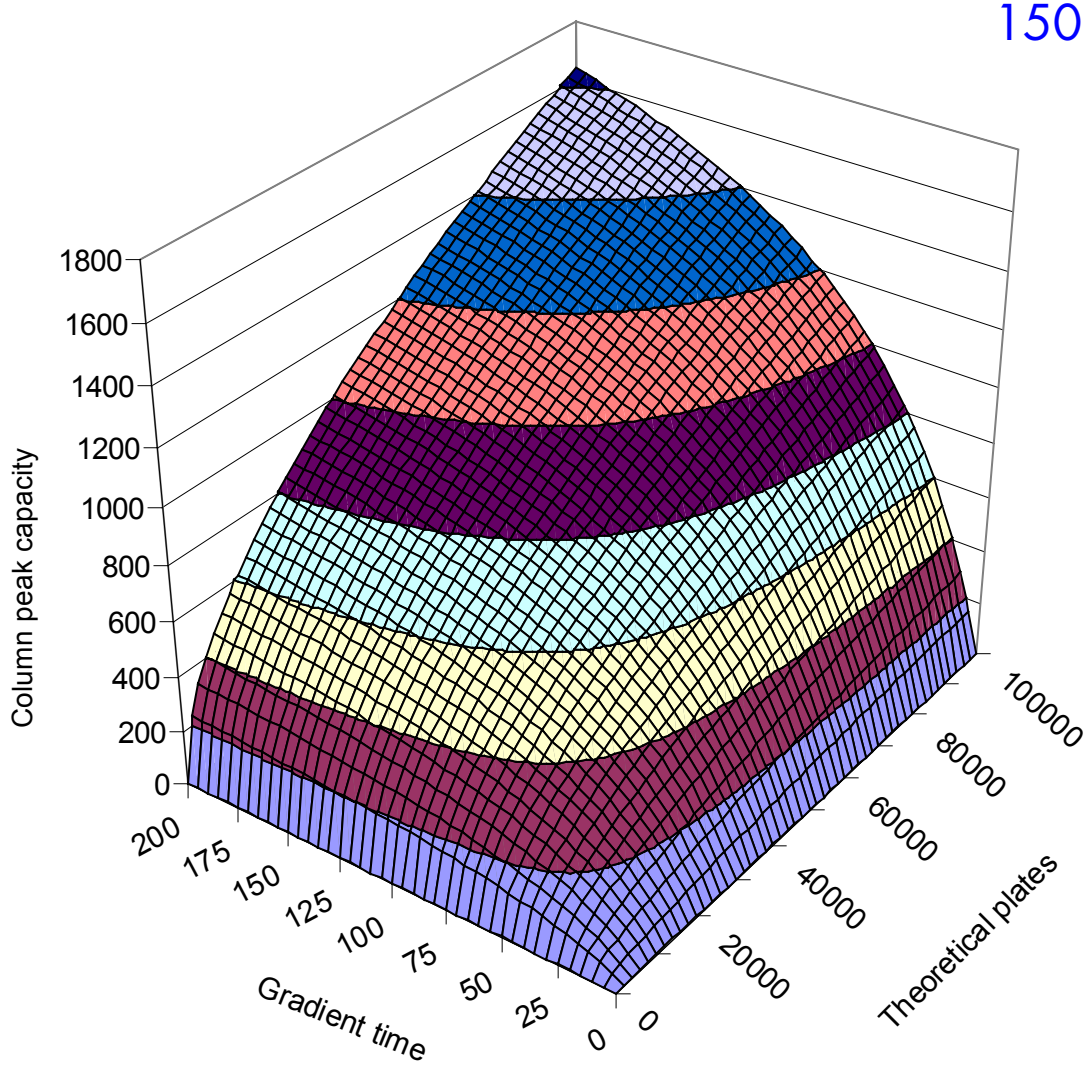
Peak capacity prediction

150 x 4.6 mm column, decreasing d_p



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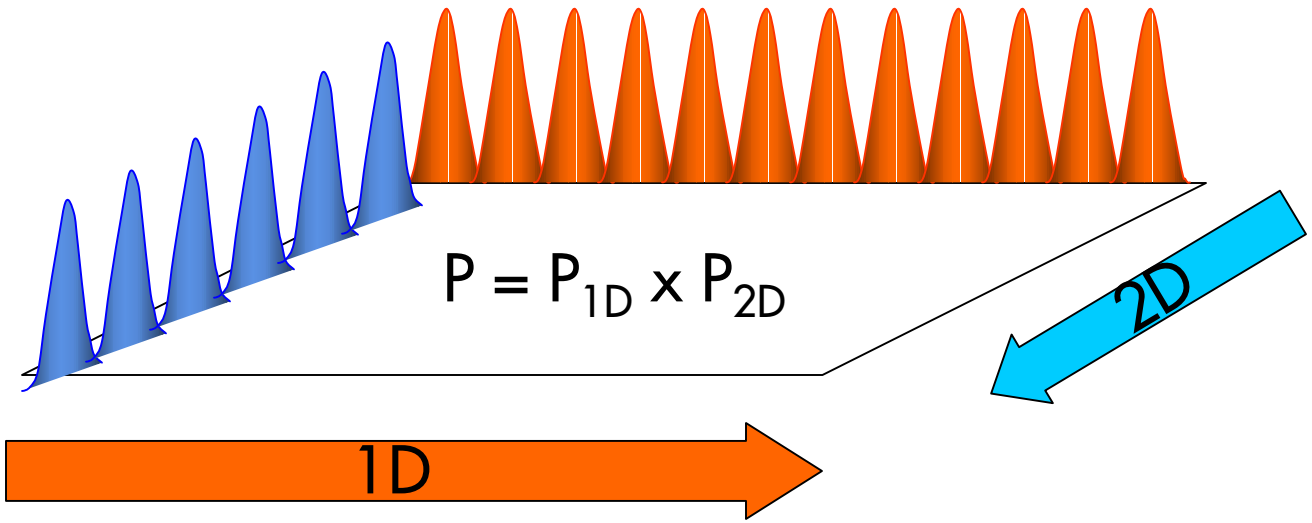
2D-HPLC peak capacity

- Orthogonal selectivity - P multiplication
- SCX + RP-HPLC



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SCX-HPLC peak capacity

- SCX peak capacity ~ 50 % of RP-HPLC values or less (polysulfoethyl aspartamide, PolyLC Inc.)
- SCX step gradient elution - peak capacity = no. of fractions

Column L x ID (SCX)	Gradient time (minutes)	Peak capacity
50 x 4.6	20	65
50 x 4.6	40	83
50 x 4.6	80	113

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2D-HPLC peak capacity

- SCX step gradient elution - extreme demand on 2D
- Fast 1D, very long 2D
- low peak/min productivity
- extensively long total analysis time
- complex sample enters MS
- limited number of identified peptides / dynamic range
- great demand on data interpretation (software)

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2D-HPLC peak capacity

1D fractions SCX	RP column length (mm)	2D grad time RP (minutes)	Peak capacity	Time (hours)
10	150	60	2700	10
10	250	120	3800	20
10	500	240	5300	40
40	150	60	10800	40

* 5 µm sorbent

Productivity?

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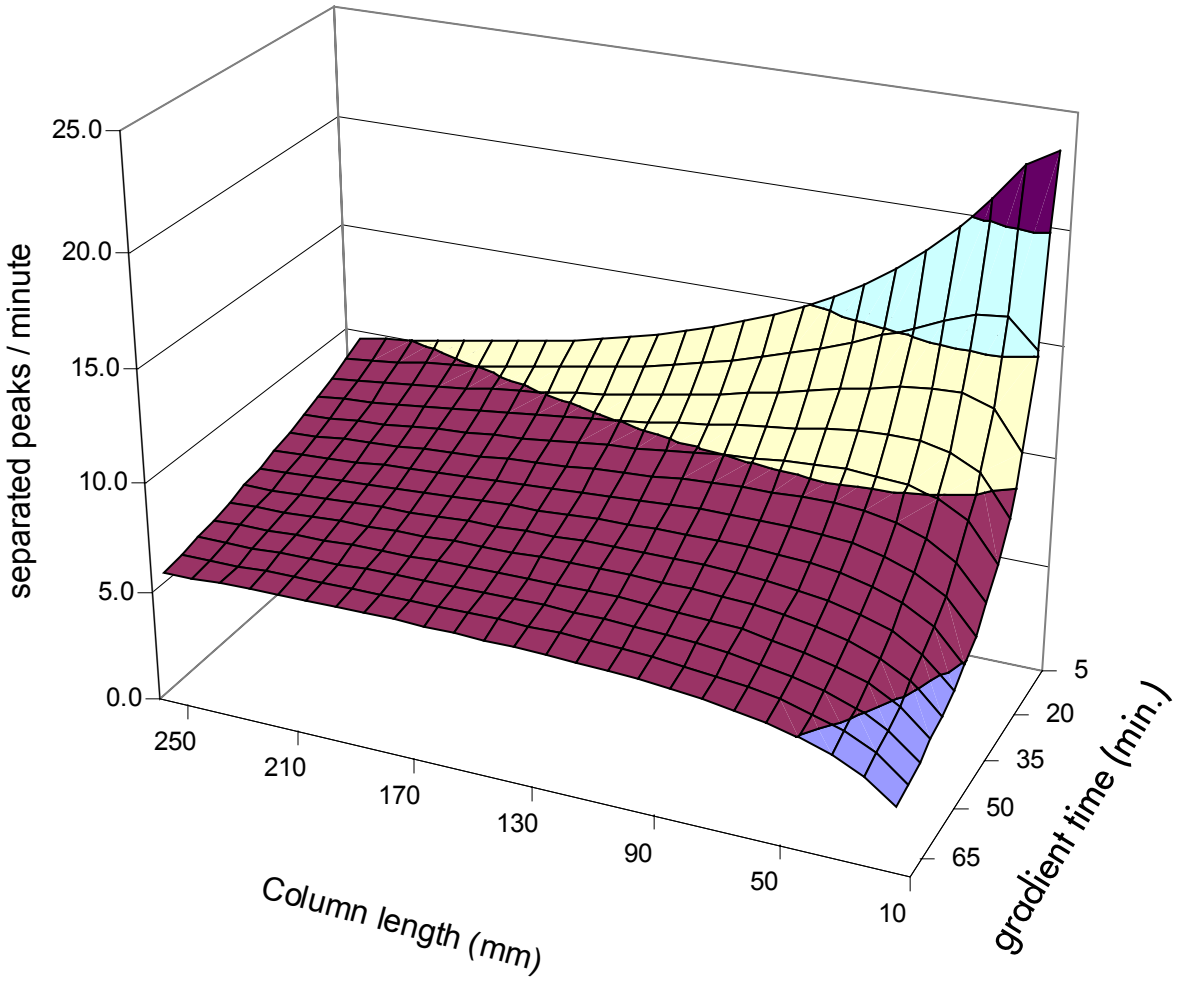
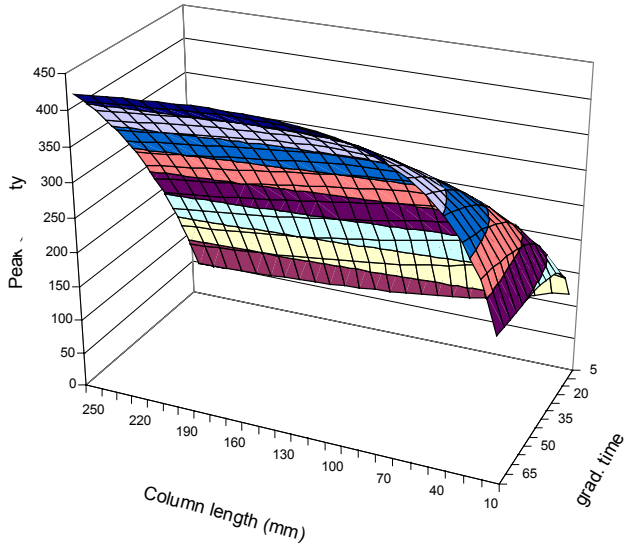
Productivity of separation



Productivity = peaks / min

Best for short, efficient columns using fast gradient



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2D-HPLC productivity

1D fractions SCX	RP column length (mm)	2D grad time RP (minutes)	RP-HPLC peak capacity	Total peak capacity	Time (hours)	Productivity Peaks/min.
20	250 (3.5 μm)	240	657	13000	80	2.7
40	150 (3.5 μm)	60	354	14000	40	5.9
80	50 (3.5 μm)	15	175	14000	20	11.6
80	20 (1.8 μm)	6	178	14000	8	29.6

Short + efficient columns = best productivity

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Validity of the model

- Uses assumption of porous sorbents
- Dm, and B = ? Changes with peptide MW.
- Model overestimates P for small d_p and fast gradients
- Extra-column peak broadening?
- Developed for 4.6 mm I.D. (lower P for nano-columns)
- Peak capacity ≠ no. of separated peaks
  - 100 components injected → 37 observed peaks (Giddings)
  - 1D & 2D will resolve ~ 37 % components (assuming that no. of components injected = peak capacity of the system)

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Conclusions

- RP-HPLC peak capacity ~ 200-400
- SCX-HPLC peak capacity ~50-100
- 2D-HPLC peak capacity
  - on-line ~5000-10,000
  - off-line ~10,000-40,000
- limited productivity of current setups
- long analysis time (second dimension)
- What is the expected sample complexity?
- What is the best pre-fractionation?

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Future outlook

- Protein pre-fractionation prior to 2D-HPLC (peptides)
- Abundant protein depletion (serum proteomics)
- ~ 1 µm particles
- ultra-HPLC
- monolithic columns
- New orthogonal modes of 2D-HPLC
- Progress in MS/MS and data acquisition and handling
- Fast 2D-HPLC systems ~10,000 peak capacity

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