Scaling up to Preparative Chromatography

Dr. Shulamit Levin

home page: www.forumsci.co.il/HPLC

Strategy for Preparative Separation

Selection of the appropriate mode of chromatography

Optimization of the Separation
(Stationary phase, Mobile Phase, Temperature, Additives)

Optimization of the throughput
(Sample amount: Column Overloading)
Adsorption Isotherm & Competition
Scaling up to Preparative Chromatography

Seven Basic Considerations in Choosing HPLC Operating Parameters

1) Solubility - Hexane, Chloroform, Methanol, Water (buffer pH), other?
2) Molecular Weight - Would GPC be useful in either the analysis or sample prep?
3) Functional Groups - Any ionizable groups? Acidic, Basic, or Neutral?
4) Sample Matrix - What amounts are expected in matrix for either analytical or preparative isolation?
5) Levels in Matrix - What amounts are expected in matrix for either analytical or preparative isolation?
6) Detectability - Any chromophores or fluorophores? Consider Redox or derivatization.
   Together with point #5, an appropriate detector is chosen.
7) How Do Species Differ - An important clue to manipulate selectivity in the separation, especially if compounds are similar in their structure.

High Performance Liquid Chromatography Modes

PRINCIPLE OF SEPARATION:

ADSORPTION
NORMAL PHASE

PARTITION
REVERSED PHASE

SOLUTES:
LIPOPHYLIC:
OILS, FATS, LIPIDS

MOBILE PHASE:
ORGANIC SOLVENTS: n-HEXANE, HEPTANE, CHLOROFORM, ALCOHOLS

MOST OF THE BIOMEDICAL SUBSTANCES
AQUEOUS MIXTURES WITH METHANOL, ACETONITRILE AND ADDITIVES (BUFFERS, ION-PAIRS)

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High Performance Liquid Chromatography Modes

PRINCIPLE OF SEPARATION:
- ION-EXCHANGE
- SIZE-EXCLUSION
- BIO-AFFINITY
- CHIRALITY

SOLUTES:
- INORGANIC IONS, ACIDS, BASES
- POLYMERS, PROTEINS, NUCLEIC ACIDS
- PROTEINS & ENZYMES
- ENANTIOMERS

MOBILE PHASE:
- AQUEOUS BUFFERS, IONIC SOLUTIONS
- AQUEOUS BUFFERS OR ORGANIC SOLVENTS
- AQUEOUS BUFFERS AND SPECIAL ADDITIVES
- AQUEOUS OR ORGANIC SOLVENTS

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PERFORMANCE CRITERIA BY ONE PEAK

RETENTION FACTOR or CAPACITY RATIO

\[ k' = \frac{t_R - t_0}{t_0} \quad k' = \frac{C_s}{C_m} \]

ASYMMETRY FACTOR

\[ A_f = B \left(10\% h\right) \]

TAILING FACTOR

\[ T_f = \frac{A + B}{2A} \left(5\% h\right) \]

NUMBER OF THEORETICAL PLATES

\[ N = 16 \left( \frac{t_R}{W} \right)^2 \]

PERFORMANCE BY TWO PEAKS

SELECTIVITY FACTOR

\[ \alpha = \frac{k'(2)}{k'(1)} \]

EXPERIMENTAL RESOLUTION

\[ R_s = \frac{t_R(2) - t_R(1)}{1/2 \left( w_1 + w_2 \right)} \]

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Gradient enables wide scope of chemical entities

General Introduction to Preparative Chromatography

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HPLC System-Prep

Solvent (Mobile Phase) → HPLC Column (Packing Material/Stationary Phase) → Injector AutoSampler → Detector → Fraction Collector (Purified Analyte(s)) → Data

Modern Setup of Preparative Chromatography: Mass and UV directed Separations

Gradient Pump → Autosampler → Column → Splitter → Purified Fractions → Makeup Pump → MS

Trigger of Fraction Collector by Detector on MS

Waste

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Purification of 0.1 µmole of 5'HEX labeled 25mer

Non-labeled failure sequences

Target Product

Labeled failure sequences

UV 260nm

UV 539nm

Minutes


Various Dimensions of Preparative Columns

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Problem Definition: Purifications

- What quantity of material needs to be isolated?
- Is the material a major or minor component?
- Do you need to maintain biological activity?
- What degree of purity (or specific activity) is required?
- How will purity or activity be verified?

If analyzed sample is to be collected; the above questions must be answered

Preparative Chromatography Terminology:
Different from Analytical Chromatography

- Sample Solubility
- Load - Overload
- Throughput
- Purity
- Recovery/Yield from Column
- Recovery from Fractions
- Cost of Purification

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Understanding the Chromatographic Process is Essential for Rational Scaling-up

Elution through the Column

\[ k' = \frac{t_R - t_0}{t_0} \]

Distribution:

\[ K = \frac{C_s}{C_m} \]

Chromatogram

Understanding the Chromatographic Process is Essential for Rational Scaling-up

RETENTION FACTOR = Capacity Factor

We know the measurement of \( k' \) from the analytical work:

Retention Factor:

\[ k' = \frac{t_R - t_0}{t_0} \]

We use its thermodynamic expression to rationally scale up the separation:

CAPACITY RATIO

\[ k' = \frac{C'_s}{C'_m} = \frac{C_s V_s}{C_m V_m} = \phi K \]

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**Adsorption Isotherms: The Key to Rational Scale-Up**

- **Langmuir type Adsorption Isotherm**
  - Linear
  - Non-Linear
  - Practical working limit in the analytical mode

\[
C_s = \frac{k'}{C_m} \phi
\]

**Linear**

\[
C_s = \frac{a}{1 + b} C_m
\]

**Non-Linear**

**Mechanism of Tailing**

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Mechanism of Diffuse Front

\[ C_s = aC^\alpha \]

\[ C_s = \frac{k'}{\phi} C_m \]

Example of the Lab Scale Purification of Single Stranded RNA

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Mass Overload

- Analytical load of 6 µg yields efficient peak shape
- Preparative load of 25 mg generates mass overload peak shape
- Note that the back of the peaks of the analytical and prep loads are at the same retention

Volume Overload

- Column Volume: 0.8 mL (800 µL)
- Wider peaks first observed at low retention
- Peak position shifts to higher retention in proportion to the injection volume
- Start of peak remains in the same position unless injected in a weaker solvent

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Scaling Comparison
Overlaid

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Optimization of the throughput
(Sample amount: Column Overloading)
Adsorption Isotherm & Competition

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From Small Scale To Purification

Parameters of Scaling Up and Optimization of the Overloaded Chromatography

- Mass Load
- Injection Volume
- Flow Rate
- Gradient/Run-Time Duration
- Column
  - Dimensions
  - Particle Size
  - Chemistry
- Chemistry of the Mobile phase

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**Scaling**
**Mass Load and Injection Volume**

- **Mass Load**
  - Proportional to column volume
  - Limited by solubility in mobile phase
- **Injection volume**
  - Approximately proportional to column volume
  - Approximately proportional to both length and cross-sectional area
  - Most strongly dependent on sample solvent

**Scale Up Equations - Mass**

\[
M_{\text{prep}} = M_{\text{anal}} \times \frac{V_{\text{prep}}}{V_{\text{anal}}}
\]

- \( M_{\text{prep}} = \text{mass injected on prep column} \)
- \( M_{\text{anal}} = \text{mass injected on analytical column} \)
- \( V_{\text{prep}} = \text{volume of prep column} \)
- \( V_{\text{anal}} = \text{volume of analytical column} \)

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**Scaling Equations**

**Mass**

\[ M_2 = M_1 \times \frac{L_2}{L_1} \times \frac{d_2^2}{d_1^2} \]

where:

- \( M_1 \) is Mass on Column 1 (Analytical)
- \( M_2 \) is Mass on Column 2 (Prep)
- \( L_1 \) is Length of Column 1
- \( L_2 \) is Length of Column 2
- \( d_1 \) is Diameter of Column 1
- \( d_2 \) is Diameter of Column 2

**Mass Scalability - XTerra® Prep MS C18**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Load (mg)</th>
<th>Column Details</th>
<th>Flow Rate (mL/min)</th>
<th>Mobile Phase</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mg</td>
<td>3 mg</td>
<td>XTerra MS C18 5 µm, 4.6x50 mm</td>
<td>1.0</td>
<td>A: Water with 0.1% Formic Acid, B: ACN with 0.1% Formic Acid</td>
<td>4.0</td>
</tr>
<tr>
<td>51 mg</td>
<td>51 mg</td>
<td>XTerra Prep MS C18 5 µm, 19x50 mm</td>
<td>3.0</td>
<td>A: Water with 0.1% Formic Acid, B: ACN with 0.1% Formic Acid</td>
<td>4.0</td>
</tr>
<tr>
<td>128 mg</td>
<td>128 mg</td>
<td>XTerra Prep MS C18 5 µm, 30x50 mm</td>
<td>7.0</td>
<td>A: Water with 0.1% Formic Acid, B: ACN with 0.1% Formic Acid</td>
<td>4.0</td>
</tr>
</tbody>
</table>

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Mass Scalability - XTerra® Prep MS C₁₈

<table>
<thead>
<tr>
<th>R (cm)</th>
<th>R²</th>
<th>L (cm)</th>
<th>V (mL)</th>
<th>Scaling Factor</th>
<th>Flow</th>
<th>Mass Load</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.230</td>
<td>0.053</td>
<td>5</td>
<td>0.83</td>
<td>1.8</td>
<td>1.8</td>
<td>3</td>
</tr>
<tr>
<td>0.950</td>
<td>0.903</td>
<td>5</td>
<td>14.18</td>
<td>17</td>
<td>30.7</td>
<td>51</td>
</tr>
<tr>
<td>1.5</td>
<td>2.250</td>
<td>5</td>
<td>35.34</td>
<td>43</td>
<td>76.6</td>
<td>128</td>
</tr>
</tbody>
</table>

Buspirone Impurities II

Structure of Buspirone

Column: Symmetry Prep C₁₈, 7μm (3.9 x 150) mm
Mobile Phase: 28% acetonitrile / 72%
0.18% TETA-MeCOOH pH 7.0
Flow Rate: 1.0 mL/min
Detection: UV at 360 nm
Sample: 12 mg/mL of Buspirone
Injection: from 0.4 to 1.6 mL

4.8 mg

9.6 mg

19.2 mg

El Fallah

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Approximate Mass Loading Capacity

Many factors affect the mass capacity of preparative columns. The listed capacities represent an “average” estimate.

<table>
<thead>
<tr>
<th>Length (mm)</th>
<th>3.9</th>
<th>4.6</th>
<th>7.8</th>
<th>8</th>
<th>10</th>
<th>19</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>40</th>
<th>47</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>2</td>
<td>3</td>
<td>8</td>
<td>15</td>
<td>45</td>
<td>50</td>
<td>110</td>
<td>310</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>100</td>
<td>4</td>
<td>5</td>
<td>15</td>
<td>15</td>
<td>25</td>
<td>40</td>
<td>155</td>
<td>225</td>
<td>400</td>
<td>620</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>6</td>
<td>8</td>
<td>25</td>
<td>40</td>
<td>135</td>
<td>150</td>
<td>335</td>
<td>930</td>
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<tr>
<td>200</td>
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<td>30</td>
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<td>795</td>
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</tr>
<tr>
<td>250</td>
<td>10</td>
<td>13</td>
<td>40</td>
<td>60</td>
<td>225</td>
<td>250</td>
<td>560</td>
<td>1550</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>12</td>
<td>16</td>
<td>45</td>
<td>50</td>
<td>75</td>
<td>270</td>
<td>300</td>
<td>470</td>
<td>670</td>
<td>1195</td>
<td>1650</td>
<td>1860</td>
</tr>
<tr>
<td>Reasonable Flow Rate (ml/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.4</td>
<td>4.0</td>
<td>4.2</td>
<td>6.6</td>
<td>24</td>
<td>27</td>
<td>42</td>
<td>60</td>
<td>105</td>
<td>145</td>
<td>164</td>
</tr>
<tr>
<td>Reasonable Injection Volume (µl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>15</td>
<td>20</td>
<td>60</td>
<td>65</td>
<td>100</td>
<td>350</td>
<td>390</td>
<td>610</td>
<td>880</td>
<td>1565</td>
<td>2160</td>
<td>2450</td>
</tr>
</tbody>
</table>

Parameters of Scaling Up and Optimization of the Overloaded Chromatography

- Mass Load
- Injection Volume
- Flow Rate
- Gradient/Run-Time Duration
- Column
  - Dimensions
  - Particle Size
  - Chemistry
- Chemistry of the Mobile phase

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**Scale Up of Flow Rate**

* Same Linear Velocity to maintain N and Rs and Retention Time
  - Van Deemter

**Scaling Equations**

**Flow Rate**

\[ F_2 = F_1 \times \frac{d_2^2}{d_1^2} \]

where:

- \( F_1 \) is Flow Rate for Column 1 (Analytical)
- \( F_2 \) is Flow Rate Column 2 (Prep)
- \( d_1 \) is Diameter of Column 1
- \( d_2 \) is Diameter of Column 2

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Valerophenone Impurities

Structure of Valerophenone

El Fallah

Column:
a: Symmetry C18 5 µm (3.9x150) mm
b: Symmetry Prep C18 7 µm (7.8x300) mm
Mobile Phase: 60% acetonitrile / 40% water
Sample: 4 mg/mL Valerophenone solution
a: 100 µL injection
b: 800 µL injection
Detection: UV at 340 nm

Example: Calculations

R (cm)  R^2  L (cm)  V (mL)  Flow (mL/min)  Scaling Factor
0.195  0.038  15  1.79  0.7
0.390  0.152  30  14.34  8  5.6

Column Volume = π x R^2 x L

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Parameters of Scaling Up and Optimization of the Overloaded Chromatography

- Mass Load
- Injection Volume
- Flow Rate
- Gradient/Run-Time Duration
- Column
  - Dimensions
  - Particle Size
  - Chemistry
- Chemistry of the Mobile phase

Understanding the Concept of Column Volumes

_column volume_ = \( \pi \times R^2 \times L \)
Scaling up to Preparative Chromatography

Valerophenone Impurities

a

b

Structure of Valerophenone

Column: 5 µm, 3.9 x 150
Mobile Phase: 60% acetonitrile / 40% water
Sample: 4 mg/mL Valerophenone solution
Detection: UV at 340 nm

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Example: Calculations

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 μm, 3.9 x 150</td>
<td>7 μm, 7.8 x 300</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a: 0.70 mL/min</td>
<td>b: 5.60 mL/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<p>| | | | | | | |</p>
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<thead>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>R^2</td>
<td>L</td>
<td>V</td>
<td>Scaling Factor</td>
<td>Flow</td>
<td>Run-Time (Min)</td>
</tr>
<tr>
<td>cm</td>
<td>(cm)</td>
<td>(mL)</td>
<td></td>
<td></td>
<td>(mL/min)</td>
<td></td>
</tr>
<tr>
<td>0.195</td>
<td>0.038</td>
<td>15</td>
<td>1.79</td>
<td>0.7</td>
<td>15</td>
<td>5.9</td>
</tr>
<tr>
<td>0.390</td>
<td>0.152</td>
<td>30</td>
<td>14.34</td>
<td>8</td>
<td>15</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Column Volume to Gradient Volume Relationship

Gradient

Column Volume: (πr^2L)~5 mL

Gradient at Flow = 2 ml/min

Column Volume: (πr^2L)~125 mL

Gradient at Flow = 50 ml/min

Gradient Volume: Analytical

25 min x 2 ml/min = 50 ml

Gradient Volume: Preparative

25 min x 50 ml/min = 1250 ml

No. of Column Volumes:

Gradient Vol/Column Vol

50/5 = 10

No. of Column Volumes:

Gradient Vol/Column Vol

1250/125 = 10
Scaling up to Preparative Chromatography

Scale Up Equations - Gradient

\[ t_{\text{prep}} = t_{\text{anal}} \times \frac{V_{\text{prep}}}{V_{\text{anal}}} \times \frac{F_{\text{anal}}}{F_{\text{prep}}} \]

- \( t_{\text{prep}} \) = run time with prep column
- \( t_{\text{anal}} \) = run time with analytical column
- \( V_{\text{prep}} \) = volume of prep column
- \( V_{\text{anal}} \) = volume of analytical column
- \( F_{\text{prep}} \) = prep flow rate
- \( F_{\text{anal}} \) = analytical flow rate

Example: Scale-up Gradient

SunFire™ C₁₈
4.6x50 mm
Total Mass Load: 5 mg

Flow Rate: 1.4 mL/min
Gradient: Time Profile
<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>1.0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>6.0</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>7.0</td>
<td>30</td>
<td>70</td>
</tr>
</tbody>
</table>
Injection Volume: 20 µL

Compounds: Metoprolol (50 mg/mL), Oxprenolol (25 mg/mL), Propranolol (5 mg/mL)

SunFire™ C₁₈ 19x50mm
Total Mass Load: 85 mg

Flow Rate: 23.9 mL/min
Gradient: Time Profile
<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>1.8</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>6.8</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>7.8</td>
<td>30</td>
<td>70</td>
</tr>
</tbody>
</table>
Injection Volume: 340 µL

Compounds: Metoprolol (50 mg/mL), Oxprenolol (25 mg/mL), Propranolol (5 mg/mL)
Scaling up to Preparative Chromatography

**Scale-up Gradient**

**SunFire™ C18 4.6x50 mm**
Total Mass Load: 5 mg

Injection Volume: 20 µL

Flow Rate: 1.4 mL/min
Gradient: Time Profile
(min) %A %B
0.0 95 5
1.8 95 5
6.8 30 70
7.8 30 70

**SunFire™ C18 19x50 mm**
Total Mass Load: 85 mg

Injection Volume: 340 µL

Flow Rate: 23.9 mL/min
Gradient: Time Profile
(min) %A %B
0.00 95 5
1.8 95 5
6.8 30 70
7.8 30 70

**Compounds:** Metoprolol (50 mg/mL), Oxpranolol (25 mg/mL), Propranolol (5 mg/mL)

<table>
<thead>
<tr>
<th>R (cm)</th>
<th>R²</th>
<th>L (cm)</th>
<th>V (mL)</th>
<th>Scaling Factor</th>
<th>Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.230</td>
<td>0.053</td>
<td>5</td>
<td>0.83</td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td>0.950</td>
<td>0.903</td>
<td>5</td>
<td>14.18</td>
<td>17</td>
<td>23.9</td>
</tr>
</tbody>
</table>

(Addition of 0.8 min to compensate for instrument’s delay volume)

**Prep Calculator**
Scaling up to Preparative Chromatography

Parameters of Scaling Up and Optimization of the Overloaded Chromatography

- Mass Load
- Injection Volume
- Flow Rate
- Gradient/Run-Time Duration
- Column
  - Dimensions
  - Particle Size
  - Chemistry
- Chemistry of the Mobile phase

HPLC Columns

<table>
<thead>
<tr>
<th>Length</th>
<th>Internal Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>3cm – 50cm</td>
<td>1mm -- 50mm</td>
</tr>
<tr>
<td>30mm – 500mm</td>
<td></td>
</tr>
</tbody>
</table>
Scaling up to Preparative Chromatography

Scale Up – Column’s Dimensions

- Dimensions
  - Mass capacity is proportional to column volume
  - Elution volume is proportional to column volume
  - Shorter columns preferred for speed and pressure
  - Longer columns preferred for resolution

Effect of Column Length

- Efficiency increases with length
- Backpressure increases with length
- Run time increases with length
- Cost increases with length
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Effect of Column Length on Capacity

As can be seen in this example, there is a linear relationship between column length and loading capacity - 3X increase in column length generates a >3X increase in loading capacity.

Disadvantage of long columns:
- higher pressure for equal run time (9x at 3x increase in length)
- higher pressure at equal velocity and longer run time

Effect of Column Length

As column length decreases, resolution between the minor impurity and main peak decreases. Loading capacity will also decrease.
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XTerra® Prep Columns for Speed – 40% Reduction in Retention and Peak Volume

- Peak # 1 13 ml
- Peak # 2 9 ml
- Peak # 3 13 ml

Scaling to Smaller Columns Allows:

- Faster chromatography
- Peak volume reduction
- Less expensive column
- Depending on the application, how far is it possible to downsize?
- Plate-to-plate mapping, injecting from a 96 well plate and collecting fractions in another 96 well plate, analytical size columns are suitable for this work
## Load Considerations

- **Mass Capacity**
  - Proportional to column volume

- **Injection Volume**
  - Scale in proportion to the column volume
  - Usually larger for gradient separations
  - Cannot be considered separately from sample solvent

- **Sample Solvent**
  - Good solvent needed to load high sample concentrations
  - Good sample solvents degrade chromatography
  - Good chromatographic diluents give low sample concentrations

## Parameters of Scaling Up and Optimization of the Overloaded Chromatography

- Mass Load
- Injection Volume
- Flow Rate
- Gradient/Run-Time Duration
- Column
  - Dimensions
  - Particle Size
  - Chemistry
- Chemistry of the Mobile phase

---

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As particle size increases, resolution of the minor impurities decreases.

Well resolved components not affected by change in particle size.

As particle size increases, resolution between the minor impurity and the main peak decreases.

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Parameters of Scaling Up and Optimization of the Overloaded Chromatography

- Mass Load
- Injection Volume
- Flow Rate
- Gradient/Run-Time Duration
- Column
  - Dimensions
  - Particle Size
- Chemistry
- Chemistry of the Mobile phase

Prochlorperazine: Effect of Loading Capacity on the Separation of Impurities

Column:

- a: SymmetryPrep C18 7µm (4.6x150) mm
- b: Kromasil C18 7µm (4.6x150) mm

Mobile Phase: 75% methanol / 25% 20 mM phosphate buffer pH 7.0
Flow Rate: 1 mL/min
Detection: UV at 254 nm
Sample: 10 µL of 0.97 mg/mL solution

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Impact of Column Chemistry

**Conditions:**
- Columns: 4.6 x 150 mm, 5 µm
- Mobile Phase A: 0.1% HCOOH in H2O
- Mobile Phase B: 0.1% HCOOH in ACN
- Flow Rate: 1.4 mL/min
- Gradient: Time Profile
  - (min) %A %B
  - 0.0 70 30
  - 10.0 10 90
  - 15.0 10 90
- Injection Volume: 10.0 µL
- Sample Diluent: DMSO
- Temperature: 30 °C
- Detection: UV @ 254 nm
- Instrument: Alliance® 2695 with 2996 PDA

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### All C18 Columns are NOT the Same

<table>
<thead>
<tr>
<th>Column</th>
<th>Rs</th>
<th>W&lt;sub&gt;1/2&lt;/sub&gt;</th>
<th>Total Load</th>
</tr>
</thead>
<tbody>
<tr>
<td>SunFire™ C&lt;sub&gt;18&lt;/sub&gt;</td>
<td>2.12</td>
<td>0.24</td>
<td>0.42 mg</td>
</tr>
<tr>
<td>Luna (2) C&lt;sub&gt;18&lt;/sub&gt;</td>
<td>1.31</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Kromasil C&lt;sub&gt;18&lt;/sub&gt;</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Hypersil Gold</td>
<td>0.71</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>Zorbax C&lt;sub&gt;18&lt;/sub&gt;</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

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Parameters of Scaling Up and Optimization of the Overloaded Chromatography

- Mass Load
- Injection Volume
- Flow Rate
- Gradient/Run-Time Duration
- Column
  - Dimensions
  - Particle Size
  - Chemistry
- Chemistry of the Mobile phase

Chemistry of the Sample Solution: Maximizing Sample Load

- Mobile phase solvent choice
- Buffer the mobile phase
  - Control of chromatographic pH conditions
  - Reduces breakthrough of sample
  - Use volatile buffers
    - Reduces sample handling and energy required for evaporation
    - Minimizes contamination of purified fraction
    - Minimizes risk of target decomposition
    - Mass spectrometry compatible
- Mobile phase pH impacts load
  - Low pH maximizes acid load
  - High pH maximizes base load

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Sample Solvent Affects Resolution and Peak Shape

Chromatography run at pH 3.8

DMSO in high aqueous mobile phases generates a high viscosity pressure “spike” (observed post injection) resulting in poor resolution of the chromatographic peaks. All weaknesses in HPLC system will become evident.

Increasing Load in DMSO: Mass Overload

Column: XTerra® MS C₁₈, 4.6 x 50 mm, 5 µm. Mobile phase A: water + 50 mM formic acid; mobile phase B: acetonitrile + 50 mM formic acid. Flow rate: 1.8 mL/min. Gradient: 35-65% B in 5 min. UV: 280 nm.

- 5.4 mg Load
- 5.7 mg Load
- 7.5 mg Load
- 12.5 mg Load

When DMSO is used as the diluent, mass loading is 5.4 mg.

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Non-Ionic Loading: Acids

When acidic sample is loaded in its non-ionic state, mass loading is up to 12.5 mg on XTerra® MS C₁₈ column.

Dicloxacillin @ 250 mg/mL
pH = 2

DMSO
12.5 mg Load

DMSO + 50 mM FA
Formic acid was added to DMSO directly, then use this acidified DMSO to dissolve sample

2.3X higher loading

Non-Ionic Loading: Bases

When a basic sample is loaded in its non-ionic state, peak tailing improves dramatically.

Econazole @ 200 mg/mL
pH = 11

XTerra® MS C₈

DMSO
6 mg Load

DMSO + 50 mM NH₄OH

Shoulder appears
USP Tailing: 1.60

USP Tailing: 0.78

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Loading a Base under Acidic Mobile Phase Conditions

Column: XTerra® MS C18, 4.6 x 50 mm, 5 µm. Mobile phase A: water + 50 mM formic acid; mobile phase B: acetonitrile + 50 mM formic acid. Flow rate: 1.8 mL/min. Gradient: 5-95% B in 5 min. UV: 280 nm.

- DMSO
- DMSO + NH₄OH
- DMSO
- DMSO + NH₄OH

When NH₄OH is added to the DMSO, no breakthrough was observed. 1.8X high mass load by adding base to the diluent.

Choice of Strong Solvent (Acetonitrile vs. Methanol)

Column: XTerra® MS C18, 4.6 x 50 mm, 5 µm. Mobile phase A: water + 50 mM formic acid; mobile phase B: acetonitrile/methanol + 50 mM formic acid. Flow rate: 1.8 mL/min. Gradient: 35-65% B in 5 min. UV: 254 nm.

- Acetonitrile, pH 2
- Methanol, pH 2

Applying the same gradient with both organic solvents, sample elutes later with methanol than with acetonitrile. Changing the organic solvent may improve peak shape due to the additional interaction with the sample.

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Use of Buffers

Buffered mobile phases enhance retention and mass loading. There is a high risk of breakthrough and retention loss leading to recovery problems when buffers are left out!

Modifier Impact on Load

As sample mass / volume loading increases, strong pH shifts can occur, affecting chromatographic peak shapes.

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Mobile Phase pH Effect:
Loading of Bases

- XBridge™ C₁₈
- Adjusted gradient to keep the same k’ values.

**Bases**
1. Nordoxepin
2. Doxepin
3. Amitriptyline

**pH 2.3**
- 0.1 mg

**pH 7**
- 0.1 mg

**pH 10**
- 0.5 mg

High pH for basic analytes results in the best loadability, as well as the best retention and separation.*


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Mobile Phase pH Effect:
Loading of Acids

- XBridge™ C₁₈
- Adjusted gradient to keep the same k’ values.

**Acids**
1. Oxacillin
2. Cloxacillin
3. Dicloxacillin

**pH 2.3**
- 0.8 mg

**pH 7**
- 0.3 mg

**pH 10**
- 0.2 mg

Low pH for acidic analytes results in the highest loadability, as well as the best retention and separation.*


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Loadability with Varied pH

- pH 2: Flurbiprofen - 4.80 mg
- pH 11: Amitriptyline - 10.5 mg

Increased retention for targets in their non-ionic state.

Utilizing pH to Manipulate Target Retention

- pH is the most powerful tool to manipulate retention of ionizable compounds
- Elution Order (Standard Gradient Mode)
  - early eluting peaks can be isolated more quickly
  - later eluting peaks can be purified at higher loadings and elute in higher concentration organic solvent
- Basic compounds chromatographed in their unionized state (2pH units above pKa) are retained longer and exhibit excellent peak shape
- A popular high pH volatile buffer is 0.1% NH4OH

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Example: Basic Test Compounds

Diphenhydramine  Oxybutynin

Terfenadine

Peak shape and retention comparison: Basic Compounds at Low and High pH

XTerra® Prep MSC$_{18}$ 19 x 50 mm, 5 µm

Analytes: 1. Diphenhydramine (2.5 mg/mL) 3 mg Load
2. Terfenadine (0.15 mg/mL) 0.18 mg Load

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### Scale-up Strategy - Summary

1. Define the problem
   - Find the chromatographic mode.

2. Develop and optimize the separation
   - Increase selectivity > 1.5

3. Maximize throughput
   - Measure adsorption isotherm.

4. Increase sample mass and volume to the maximum
   - while meeting purity objectives.
   - Examine the interferences

5. Determine recovery
   - Examine residuals on the column

6. Scale up to desired column size to meet
   - throughput/load objectives.
   - Keep the flow rate and sample load ratio

7. Pool fractions of comparable purity and rerun if necessary.

8. Check fraction purity using analytical column.

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