Making the Leap

Small Molecule – Biologics

Rita Steed
LC Columns Application Engineer
June 26, 2018
Outline

➢ Small and/or biomolecules; Similarities/differences
➢ Column Choice
➢ Things to Consider
➢ Method Conditions
➢ Troubleshooting
Small and/or Biomolecule; Similarities/Differences

Define
Types of chromatography
• Similar
• Different
Sample types
• Simple
• Complex
Functional groups
• Polarity
Small v. Biomolecules

Small Molecules

**Size** – MW <1000 (although some may be somewhat larger)

**Structure** – Simple
  - Drugs, pesticides, chemicals…

**Manufacturing** – Chemical synthesis, predictable process

**Characterization** – straightforward

**Stability** – Stable

Biologics

**Size** – Mostly >1000 with some exceptions

**Structure** – Diverse; need to consider chemistry, biology, and biochemistry
  - MAbs, aggregates, charge variants, conjugates, peptides, amino acids, nucleic acids

**Manufacturing** – living cells; “dirty” (early) process with similar components; difficult to control

**Characterization** – complex molecular makeup; heterogeneity

**Stability** – Sensitive to external conditions; Temp, pH, Shearing, Folding, Glycoforms
Do you know what this is?

C$_{22}$H$_{30}$N$_6$O$_4$S  
MW=476.6 g/mol

Sildenafil
# Polarity of Functional Groups – Nonionic

Affect Solubility and Elution Order

<table>
<thead>
<tr>
<th>Functional Group</th>
<th>Polarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>-(\text{C} - \text{C})</td>
<td>Hydrocarbon (saturated)</td>
</tr>
<tr>
<td>-(\text{C} - \text{C})</td>
<td>Hydrocarbon (unsaturated)</td>
</tr>
<tr>
<td>-(\text{C} - \text{C} - \text{C} - \text{C})</td>
<td>Hydrocarbon (conjugated)</td>
</tr>
<tr>
<td>-(\text{C} - \text{C})</td>
<td>Aromatic Ring (benzene)</td>
</tr>
<tr>
<td>-(\text{C} - \text{Cl})</td>
<td>Chlorinated (halogenated)</td>
</tr>
<tr>
<td>-(\text{C} - \text{O} - \text{C})</td>
<td>Ether</td>
</tr>
<tr>
<td>-(\text{CO}_2\text{R})</td>
<td>Ester (R = organic chain)</td>
</tr>
<tr>
<td>-(\text{C} = \text{O})</td>
<td>Carbonyl (aldehyde, ketone)</td>
</tr>
<tr>
<td>-(\text{CONH}_2)</td>
<td>Amide</td>
</tr>
</tbody>
</table>
Polarity of Functional Groups

Affect Solubility and Elution Order

<table>
<thead>
<tr>
<th>Functional Group</th>
<th>Polarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic ring</td>
<td>polar, slightly ionic</td>
</tr>
<tr>
<td>Alcohol</td>
<td>polar, non-ionic</td>
</tr>
<tr>
<td>Carboxylic acid</td>
<td>polar, ionic, acidic</td>
</tr>
<tr>
<td>Acid Salt</td>
<td>polar, ionic, acidic</td>
</tr>
<tr>
<td>Amine (primary)</td>
<td>polar, ionic, basic</td>
</tr>
<tr>
<td>Amine Salt</td>
<td>polar, ionic, basic</td>
</tr>
</tbody>
</table>
Do you know what this is?

\[
\begin{align*}
C_{6470}H_{10012}N_{1726}O_{2013}S_{42} \\
MW &= 145421.50 \text{ g/mol}
\end{align*}
\]
HPLC Common Separation Mechanisms

**Small Molecules**
- Reversed Phase*
- Ion Exchange
- HILIC
- Normal Phase
- Chiral

**Biomolecules – Intact Protein**
- Reversed Phase*
- Ion Exchange
- Size Exclusion/Gel Filtration
- Affinity
- Hydrophobic Interaction (HIC)

*Of the many different HPLC separation mechanisms and column types, reverse phase is commonly used for both small molecules and biomolecules.
Choosing the Right Column …

Sorbent characteristics
– Particle size
– Plate count
– Back pressure
– Pore size
– Bonding chemistry
  • Small Molecule – C18, C8
  • Proteins – C4, C8

Column characteristics
– Internal diameter
– Length
– Material

Instrument compatibility
# Particle Size

Diameter  
Plate count  
Back pressure

<table>
<thead>
<tr>
<th>Diameter (µm)</th>
<th>Reduced Plate Height</th>
<th>Plate Count</th>
<th>Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.8</td>
<td>5.04</td>
<td>198000</td>
<td>380 bar</td>
</tr>
<tr>
<td>3</td>
<td>8.4</td>
<td>119000</td>
<td>140 bar</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>71000</td>
<td>50 bar</td>
</tr>
<tr>
<td>10</td>
<td>28</td>
<td>36000</td>
<td>12 bar</td>
</tr>
</tbody>
</table>

10µm  
5µm  
3µm  
1.8µm
# Agilent InfinityLab Poroshell 120 Portfolio Small Molecules & Peptides

<table>
<thead>
<tr>
<th>Best all around</th>
<th>Best for low pH mobile phases</th>
<th>Best for high pH mobile phases</th>
<th>Best for alternative selectivity</th>
<th>Best for polar Analytes</th>
<th>Best for Chiral</th>
</tr>
</thead>
<tbody>
<tr>
<td>InfinityLab Poroshell EC-C18 1.9 µm, 2.7 µm, 4 µm</td>
<td>InfinityLab Poroshell SB-C18 2.7 µm</td>
<td>InfinityLab Poroshell HPH-C18 1.9 µm, 2.7 µm, 4 µm</td>
<td>InfinityLab Poroshell Bonus-RP 2.7 µm</td>
<td>InfinityLab Poroshell HILIC 1.9 µm, 2.7 µm, 4 µm</td>
<td>InfinityLab Poroshell Chiral-V 2.7 µm</td>
</tr>
<tr>
<td>InfinityLab Poroshell EC-C8 1.9 µm, 2.7 µm, 4 µm</td>
<td>InfinityLab Poroshell SB-C8 2.7 µm</td>
<td>InfinityLab Poroshell HPH-C8 2.7 µm, 4 µm</td>
<td>InfinityLab Poroshell PFP 1.9 µm, 2.7 µm, 4 µm</td>
<td>InfinityLab Poroshell HILIC-Z 2.7 µm</td>
<td>InfinityLab Poroshell Chiral-T 2.7 µm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>InfinityLab Poroshell Phenyl-Hexyl 1.9 µm, 2.7 µm, 4 µm</td>
<td>InfinityLab Poroshell HILIC-OH5 2.7 µm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>InfinityLab Poroshell SB-Aq 2.7 µm</td>
<td>InfinityLab Poroshell Chiral-CD 2.7 µm</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>InfinityLab Poroshell EC-CN 2.7 µm</td>
<td>InfinityLab Poroshell Chiral-CF 2.7 µm</td>
</tr>
</tbody>
</table>

## Reversed Phase Chemistries

- 4µm
- 2.7µm
- 1.9µm
Columns for Intact Protein Analysis

No “One size fits all” Column …
Pore Size & Molecular Size Effect on Peak Width

Gradient Separations

![Bar chart showing PW 1/2 for different molecules across two pore sizes: 300SB-C18 (300Å) and SB-C18 (80Å).]
## Choose Column Configuration for Application

<table>
<thead>
<tr>
<th>Column Type</th>
<th>I.D. (mm)</th>
<th>Lengths (mm)</th>
<th>Particle Sizes (mm)</th>
<th>Flow Rate Ranges</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary</td>
<td>0.3, 0.5</td>
<td>35 – 250</td>
<td>≤5</td>
<td>1 – 10 mL/min</td>
<td>Max sensitivity LC/MS</td>
</tr>
<tr>
<td>MicroBore</td>
<td>1.0</td>
<td>30 – 150</td>
<td>≤5</td>
<td>30 – 60 mL/min</td>
<td>Higher sensitivity LC/MS</td>
</tr>
<tr>
<td>Narrow Bore</td>
<td>2.1</td>
<td>15 – 150</td>
<td>1.8 – 5</td>
<td>0.1 – 0.3 mL/min</td>
<td>High sensitivity LC/MS</td>
</tr>
<tr>
<td>Solvent Saver</td>
<td>3.0</td>
<td>100 – 250</td>
<td>1.8 – 5</td>
<td>0.3 – 1.0 mL/min</td>
<td>Analytical</td>
</tr>
<tr>
<td>Analytical</td>
<td>4.6</td>
<td>15 – 250</td>
<td>1.8 – 5</td>
<td>1 – 4 mL/min</td>
<td>Analytical</td>
</tr>
<tr>
<td>Semi-prep</td>
<td>9.4</td>
<td>50 – 250</td>
<td>≥5</td>
<td>4 – 10 mL/min</td>
<td>Small scale prep (mg)</td>
</tr>
<tr>
<td>Preparative</td>
<td>&gt;21.2</td>
<td>50 – 250</td>
<td>≥5</td>
<td>20 – 100 mL/min</td>
<td>Large scale prep</td>
</tr>
</tbody>
</table>
Things to Consider

Instrument van Deemter Recovery

Quick Connect fitting
Column and Instrument Materials

Nucleotide Phosphates on AdvanceBio MS Spent Media (HILIC stationary phase in PEEK lined SS hardware)

A: 10 mM Ammonium Formate pH 6.8 in water, B: acetonitrile + 10 mM Ammonium Formate pH 6.8, 95-30% B in 10 minutes, 0.25 mL/min, 0.2 μL injection (5 ng each on column), MS Source: ESI-, m/z 191.02, 346.06, 426.02, 505.99, 521.99, 742.067, 743.067, 259.03
Instrument Considerations

Bioinert system

- **100% Bio-inert**
  - Precious sample does not touch metal surfaces
  - pH range 1-13 (short-term 14)
  - 2 M salt, 8 M urea
  - No stainless steel in mobile phase flow path
  - New capillary technology

Phosphoric acid passivation

- Improve both peak shape and sensitivity
  - Run 90:10 acetonitrile:water with 0.5% (v/v) phosphoric acid overnight through Channel B, column, and MS nebulizer
Acid Wash Can Improve Peak Shape

Metal sensitive compounds can chelate

1% H$_3$PO$_4$ is used on SB columns, 0.5% on endcapped columns and system

Before Acid Wash

After 1% H$_3$PO$_4$ Acid Wash

**Columns:** ZORBAX SB-Phenyl
4.6 x 150 mm

Mobile Phase: 75% 25mM Am phosphate 25% ACN
Flow Rate: 1.0 mL/min.
Temperature: RT
Sample Size: 5 uL

Tf: 3.7

Tf: 1.2

**Hint:** Look for lone pair of electrons on “O” or “N” which can form 5 or 6 membered ring with metal
Factors Affecting Plate Count
van Deemter Equation

\[ h = A + \left( \frac{B}{u} \right) + (C \times u) \]

- **A** term, eddy diffusion
- **B** term, axial diffusion
- **C** term, Resistance to mass transfer
- **Sum**

\[ N = \frac{L}{H} \]

Column length

HETP

\[ h = \frac{L}{N} \]

A, B, and C are constants for a particular compound and set of experimental conditions as flow rate is varied.
Factors Affecting Plate Count

**A term**  Eddy diffusion (multiple path effect)

Increase in peak width due to self-diffusion of the analyte

\[ w_{\text{eddy}} \sim \lambda d_p \quad \lambda: \text{Quality of column packing} \]

**B term**  Molecular diffusion

**C term**  Mass transfer (within particle)

\[ w_C \sim d_p^2 \]
van Deemter Equation – C term
“Resistance to Mass Transfer”

\[ w_C \sim d_p^2 \]

Different diffusion paths
Porous particle
Stationary layer of mobile phase

Poroshell is made of a solid core with a porous outer layer. Reduces C term.
Van Deemter Equation
Measured for Different Particle Sizes

- Small particles = lower heights of theoretical plates \( \Rightarrow \) higher separation efficiency
- For smaller particles, separation efficiency suffers less when increasing the flow

➢ Point where minimum is reached, is the “optimal” flow rate at which maximal plate number is reached
van Deemter Equation
Curves for Different Analytes

- van Deemter equation for isocratic runs only
- Compound and instrument specific
- Even for sub-2-μm particles not horizontal
- Optimum flow rate depends on compound

Are proteins like small molecules?

Van Deemter Plots

Are proteins like small molecules? … NO!

Agilent RP-mAb Diphenyl - myoglobin

Linear Velocity, $u$ (mm/sec)

HETP ($\mu$m)

0.0 1.0 2.0 3.0 4.0 5.0 6.0

0.0 50.0 100.0 150.0 200.0 250.0
Slower Diffusion of Large Molecules Broadens Peaks at High Flow

So... decrease **diffusion time** for macromolecules!

Increase the Diffusion Rate

- Elevated operating temperature -- Works with StableBond
- Decreased solvent viscosity -- Helps but changes elution

Decrease the Diffusion Distance

- Small particles (<2-um) – Increased back pressure
- Limit diffusion distance into a particle!
Resolution
Factors that Affect It

Selectivity impacts resolution the most
- Change bonded phase
- Change mobile phase

Typical Analytical Method Development Parameters

Resolution
Efficiency
Selectivity
Retention

Resolution \( R_s \) = \( \left( \frac{1}{4} \right) N^{0.5} \left( \frac{\alpha - 1}{\alpha} \right) \left( \frac{k}{1 + k} \right) \)

- Increasing N
- Increasing Alpha
- Increasing \( k' \)

<table>
<thead>
<tr>
<th>Plates:</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>5000</td>
<td>1.10</td>
</tr>
<tr>
<td>10000</td>
<td>1.35</td>
</tr>
<tr>
<td>15000</td>
<td>1.60</td>
</tr>
<tr>
<td>20000</td>
<td>1.85</td>
</tr>
<tr>
<td>25000</td>
<td>2.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plates:</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>5000</td>
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</tr>
<tr>
<td>15000</td>
<td>7.0</td>
</tr>
<tr>
<td>20000</td>
<td>9.5</td>
</tr>
<tr>
<td>25000</td>
<td>12.0</td>
</tr>
</tbody>
</table>

Plates:
- 5000
- 10000
- 15000
- 20000
- 25000

Alpha:
- 1.10
- 1.35
- 1.60
- 1.85
- 2.1

\( k \):
- 2.0
- 4.5
- 7.0
- 9.5
- 12.0
Resolution Relationship for Gradient Elution

\[ R \approx \frac{\sqrt{N}}{4} \propto k^* \]

- represents the fact that \( k \) changes constantly during a gradient

\[ k^* = \frac{87 \cdot t_g \cdot F}{S \cdot (\Delta \% B) \cdot V_m} \]

- \( \Delta \% B \) = difference between initial and final \( \% B \) values
- \( S \) = Constant that changes with MW:
  - \( 4 \) for \( 100 - 500 \) Da; \( 10 < S < 1000 \) for peptides and proteins
- \( F \) = flow rate (mL/min)
- \( t_g \) = gradient time (min)
- \( V_m \) = column void volume (mL)
Larger Molecules v. Small Molecules
More Sensitive to Changes in % Organic

- Lysozyme is 15X more sensitive to changes in organic modifier than benzene
- 4X more sensitive than leucine enkephalin.
Method Conditions

Mobile phase
- Organic Modifier
- Additives

pH
Temperature
Sample
Exploring Organic Modifiers – Small Molecules

Why?

✓ It’s easy – ACN & MeOH are readily available
✓ Works on any bonded phase – optimize separation no matter the column choice

MeOH – Higher pressure, generally better peak shape with bases, Protic solvent

Acetonitrile – Aprotic, wider UV window, stronger than MeOH

Mobile Phase: Organic Modifier

<table>
<thead>
<tr>
<th>Columns</th>
<th>2.1x50mm</th>
</tr>
</thead>
</table>
| Mobile phase | A: water:IPA 98:2 + 0.1% TFA  
B: IPA:ACN:MPA 70:20:10 |
| Temperature | 80 °C |
| Flow rate | 0.5 mL/min |
| Gradient | 25 – 45% B in 10 min |

AdvanceBio RP-mAb Diphenyl

Polyphenyl column (other vendor)
TFA Concentration
Affect on Reversed-Phase Peptide Separation

Conditions: Column: Zorbax 300SB-C8, 4.6 x 150 mm, Mobile Phase: A= H₂O and TFA, B= ACN and TFA, Gradient: 0-30% B in 30 min., Flow: 1 mL/min., Temp.: 40°C, Detect.: UV-254 nm, Sample: 6 µL injection volume, Peptide Standards S1-S5, decapetides differing slightly in hydrophobicity

Retention Time, (min.)
6 8 10 12 14 16 18 20 22 24
0.05 % TFA
0.25 % TFA
1.0 % TFA

Peptide Standards S1-S5, decapetides differing slightly in hydrophobicity
Selectivity Can be Controlled by Changing pH

Poroshell HPH-C18 4.6 x 50 mm, 2.7 µm

1. Procainamide
2. Caffeine
3. Acetyl Salicylic Acid
4. Hexanophenone Deg.
5. Dipyrimadole
6. Diltiazem
7. Diflunisal
8. Hexanophenone
Increasing Temperature

- Reduces Analysis Time
- May Change Selectivity

![Graph showing the relationship between Log k and 1/T (°K) for Cytochrome C, Insulin (BOV), and RNase.](image-url)
Chromatography Optimization: Column Temperature (Herceptin)

Zorbax 300 diphenyl Column, 2.1 x 50 mm (or 100mm), 1.8µm, 0.5ml/min

RT
FWHM = 11s with significant tailing

80 °C
FWHM = 5.5s with some tailing
Using Temperature to Improve Resolution

35°C  \[ \text{Resolution Improvement} \]  60° C

300 SB-CN

8. Myoglobin
9. Calmodulin
10. Carbonic Anhydrase

300 SB-C3

8
9
10

000822P1.PPT
# Protein and Peptides

Selected Sample Solvents and Applications

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Application</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05-5% TFA in Water</td>
<td>General</td>
<td>Effective solubilization of many samples</td>
</tr>
<tr>
<td>6 M Guanidine, buffered at pH 6-8</td>
<td>General</td>
<td>Very good for many proteins and peptides</td>
</tr>
<tr>
<td>5-80% Acetic Acid or Formic Acid; 0.1-0.5M Perchloric Acid</td>
<td>Peptides</td>
<td>Frequently used to extract peptides from tissues, precipitating many proteins and cellular debris</td>
</tr>
<tr>
<td>6 M Urea/5% Acetic acid</td>
<td>Hydrophobic Peptides, Proteins</td>
<td>Useful for membrane proteins, fragments, aggregating systems</td>
</tr>
<tr>
<td>Water-Miscible Organic Solvents: Acetonitrile, Methanol, THF, Dioxane, DMSO; +/- TFA; +/- Water</td>
<td>Hydrophobic Peptides, Polypeptides</td>
<td>Limit injection volume to avoid problems; add water, as possible, to improve volume tolerance; acidify with TFA as required</td>
</tr>
</tbody>
</table>
Troubleshooting

Recovery
  • Molecule size
  • Metals
Detection
  • Chromophore
  • MS
Column cleaning
What Affects Recovery?

Chain length
Hydrophobicity / Aggregation
Mobile Phase
Solubility of sample
Size
Recovery of Polypeptides from ZORBAX 300SB Columns

Columns: 4.6 x 150 mm
Mobile Phase: 5 - 40% B in 20 min.
A: 0.1% TFA / Water
B: 0.1% TFA / ACN
Flow Rate: 1 mL / min.
Temperature: 60°C
Sample: 4 µg each protein
25 µL injection
Complications/Challenges

Aggregation
Degradation
Folding/conformational changes
Size
Poor fragmentation within collision cell (QQQ)
Poor solubility in organics
MP additives

PTMs; oxidation, isomerization, deamidation
• Little or no difference in mass

Multiple glycosylation sites
• Difficult to interpret/deconvolute

Things to do

Denature
• Chaotropes: Guanidine, Urea
• Reductants: DTT, BME, Ascorbic Acid
• Temperature

Digestion
Derivatize
Effect of Modifier on MS Response: 10 pmol BSA
Column Cleaning – Small Molecules

Flush with stronger solvents than your mobile phase Make sure detector is taken out of flow path

Reversed-Phase Solvent Choices in Order of Increasing Strength
Use at least 10 x $V_m$ of each solvent for analytical columns

1. Mobile phase without buffer salts (water/organic)
2. 100% Organic (MeOH or ACN)
3. Is pressure back in normal range?
4. If not, discard column or consider more drastic conditions:
   - 75% Acetonitrile:25% Isopropanol, then
5. 100% Isopropanol
6. 100% Methylene Chloride*
7. 100% Hexane*

* When using either Hexane or Methylene Chloride the column must be flushed with Isopropanol before returning to your reversed-phase mobile phase.
Column Cleaning Suggestions - Biomolecules

**Routine**

Each Run
- Run out gradient

Daily
- Stronger protocol

Periodic
- Back flush (if column permits)

Storage
- $\geq 50\%$ Organic

**Additional**

General: IPA ramp 0-100% and hold (1/2 flow rate)

Lipids and small hydrophobics: 100% MeCl

Synthetic Peptides: General followed by 100% MeCl

More drastic: Plug flow (repetitive injections) of 3M Guanidine in 50% IPA

Silica based RP columns only

Prior to using either Hexane or Methylene Chloride the column must be flushed with Isopropanol and again before returning to your reversed-phase mobile phase.
Summary

Chromatography of biomolecules is often different from small molecules
- Chemistry, Biology, Biochemistry
  - Temperature
  - pH
  - Shearing
  - Folding, glycoforms, heterogeneity

Structural diversity
- Proteins
- Nucleic Acids
- Conjugates, complexes

Instrumentation
Contact Agilent Chemistries and Supplies Technical Support

1-800-227-9770 Option 3, Option 3:

Option 1 for GC/GCMS Columns and Supplies
Option 2 for LC/LCMS Columns and Supplies
Option 3 for Sample Preparation, Filtration and QuEChERS
Option 4 for Spectroscopy Supplies

gc-column-support@Agilent.com
lc-column-support@agilent.com
spp-support@agilent.com
spectro-supplies-support@agilent.com
Resources for Support

  - Quick Reference Guides
  - Catalogs, Column User guides
  - Online Selection Tools, How-to Videos
- InfinityLab Supplies Catalog ([5991-8031EN](http://www.agilent.com/chem/chem/5991-8031EN))
- Your local FSE and Specialists
- Youtube – [Agilent Channel](http://www.youtube.com/agilentchannel)
- Agilent Service Contracts