General Principles of HPLC Method Development

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Thailand
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Part 1. General Chromatographic Theory

Part 2. The Stationary Phase: An Overview of HPLC Media

Part 3. Role of the Mobile Phase in Selectivity
The Liquid Chromatographic Process

The Beginning of Liquid Chromatography

**Column Chromatography:**

- Empty Column
- Adsorbent particles added
- Sample is loaded onto the top of the column
- Solvent is added to the top of the column
- Separation occurs as the bands move down the column

Mikhail Tsvet, 1872-1919
Basis of Chromatographic Separation

Separation of compounds by HPLC depends on the interaction of analyte molecules with the **stationary phase** and the **mobile phase**.

The Liquid Chromatographic Process

Analytes

Mobile Phase → Stationary Phase
In any separation, almost never get a pure, single mode of separation. In RP, performance will be dictated by mixture of:

1. **Hydrophobic interactions**
2. **Polar interactions**
3. **Ionic interactions**

**Method Development** = modulating stationary phase and mobile phase conditions to optimize these interactions and achieve a specific separation goal.

**Mechanisms of Interaction In RP Chromatography**

**Tapentadol**

**Hydrophobic Interactions**

- Weak, transient interactions between a non-polar stationary phase and the molecules
- Hydrophobic & van Der Waals interactions
- Retention will be predicted by **Log P** values
**Polar Interactions**

- Interactions between polar functions groups of analyte and residual silanols or polar groups on media
- Hydrogen bonding, dipole-dipole interactions
- Relatively weak and transient

**Ion-exchange Interactions**

- Interactions between ionizable functional groups on analyte and counter-charged moiety on stationary phase
- Ion-exchange
- Strong, slow interactions
Chromatographic Measurements

<table>
<thead>
<tr>
<th>Ret Time [min]</th>
<th>k'</th>
<th>Area [NAT***]</th>
<th>Height [nA]</th>
<th>Symm</th>
<th>Width [min]</th>
<th>Selectivity</th>
<th>Activity</th>
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</thead>
<tbody>
<tr>
<td>2.938</td>
<td>0.71</td>
<td>116.4684</td>
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<tr>
<td>11.333</td>
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<td>179.8535</td>
<td>10.9418</td>
<td>0.87</td>
<td>0.1917</td>
<td>19344</td>
<td>2.22</td>
</tr>
</tbody>
</table>
Void Volume

Analytes which do not interact with the adsorbent elute from the column in a volume equal to the **void volume** in the column. The void volume of a column is the amount of mobile phase in the column between the adsorbent particles and in the pores of the porous adsorbent particles.

Mobile phase occupies the space between the particles or the interstitial volume.

Mobile phase fills the pores of the porous adsorbent particles.

A compound which does not interact with the adsorbent at all elutes at what is termed the **void volume** or the **solvent front**. The time that it takes for non-retained components to elute is the void time or \( t_0 \).
The retention factor of the eluting compound is its elution volume (time) relative to the elution volume (time) of an unretained compound. The k’ value for a given analyte will be determined by its relative affinity for the stationary phase and mobile phase.

\[ \text{Retention factor (k')} = \frac{t_R - t_0}{t_0} \]

For any given analyte, the k’ value will be most readily modified by changing the % of strong mobile phase (e.g. methanol or ACN).

Example: The Separation of Steroids:

Column used: Phenomenex Luna C18(2) 150 x 4.6 mm
Retention Factor ($k'$):
Effect of Changing % ACN

Peak Asymmetry ($Asym$)

The asymmetry value ($Asym$) for a peak is a measure of the divergence of the peak from a perfect Gaussian peak shape. Peaks with asymmetry values $>1.0$ are said to be “tailing”, those with asymmetry values $<1.0$ are said to be “fronting”.

Asymmetrical peaks can be attributed to a number of factors:

1. Strong secondary interactions (e.g. ionic interactions between basic compounds and residual silanols)
2. Sample overloading
3. Sample solvent incompatibility
4. Poor packing

\[ Asymmetry = \frac{b}{a} \]
Peak Tailing due to Secondary Interactions

Classical peak tailing in reversed-phase methods is most commonly caused by strong ionic interactions between basic analytes and residual silanols on the surface of the silica.

Example: The Separation of Tricyclic Antidepressants:

Column used: Kinetex 2.6µ XB-C18 50 x 2.1 mm
Brand H 2.7µ C18 50 x 2.1mm

Amitriptyline (pKa 9.4)  
Nortriptyline (pKa 9.7)  
Protriptyline (pKa 8.2)
Peak Tailing due to Secondary Interactions

Mobile phase: A = 0.1% Formic acid in water, B = 100% Methanol, Gradient: 15 to 60% B in 5 min, hold for 1 min
Flow rate: 400 µL/min
1. Amoxapine
2. Imipramine
3. Desipramine
4. Protriptyline*
5. Amitriptyline
6. Nortriptyline*
7. Clomipramine
8. Neflomipramine

Peak Tailing due to Sample Overloading

Strongly basic analytes are very sensitive to sample loading, and will display peak tailing effects as a function of increasing loading (µg on column):

- 2.5 µg on column: USP Tailing = 1.17, pKa 9.7
- 5 µg on column: USP Tailing = 1.34
- 12 µg on column: USP Tailing = 1.87
**Peak Fronting due to Sample Overloading**

Neutral and acidic compounds will typically show **peak fronting** when the column is overloaded.

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**Peak Fronting due to Sample Solvent Effects**

**Peak fronting** can also be due to **sample solvent effects**:

1. Sample insolubility
2. Sample solvent is too strong:

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**Sample in 50% Methanol**

- Morphine
- Hydromorphone
- Norhydrocodone

**Sample in 100% Methanol**

- Breakthrough!
Selectivity ($\alpha$)

Selectivity is a measure of the difference in the interactions of two compounds with the stationary phase. Selectivity is a function of both the stationary phase and the mobile phase.

$$\alpha = \frac{k_2}{k_1}$$

The choice of stationary phase will often have a dramatic effect on the selectivity of analytes.
Selectivity ($\alpha$)

But mobile phase is also a very powerful tool in modulating selectivity.

Column: Gemini 5 µm C6-Phenyl
150 x 4.6mm
Mobile phase: 20mM KH2PO4, pH 2.5; % organic as noted
Flow rate: 1.0 mL/min
Detection: UV at 220nm

1. Saccharin
2. p-Hydroxybenzoic Acid
3. Sorbic Acid
4. Dehydroacetic Acid
5. Methylparaben

35% Methanol

20% Acetonitrile

Column Efficiency ($N$)

The amount of band (peak) broadening or dispersion that occurs in the column is measured by calculating the column efficiency ($N$) expressed as the number of theoretical plates in the column:

$$N = 5.54 \left( \frac{\text{Retention time}}{W_{\text{half height}}} \right)^2$$

- Columns that cause a lot of peak broadening have few theoretical plates.
- Columns that produce very narrow peaks (little peak broadening) have a large number of theoretical plates.
Column Efficiency (N) is a Function of Particle Size

Efficiency of a well-packed column will be a function of several factors, one of which will be particle size. As particle size decreases, efficiency will increase. In addition, back-pressure also increases as particle size decreases.

<table>
<thead>
<tr>
<th>Particle Size</th>
<th>Core-Shell</th>
<th>sub-2 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µm</td>
<td>50,000 P/m</td>
<td>300,000 P/m</td>
</tr>
<tr>
<td>5 µm</td>
<td>100,000 P/m</td>
<td>300,000 P/m</td>
</tr>
<tr>
<td>3 µm</td>
<td>150,000 P/m</td>
<td></td>
</tr>
</tbody>
</table>

Efficiency

Back-Pressure

The Core-Shell Advantage
Fully Porous Particles

Columns packed with core-shell particles will deliver significantly higher efficiency (N) than columns packed with fully-porous particles of the same diameter.*

* Gritti et al., Journal of Chromatography A, 1217 (2010) 1589
Columns packed with core-shell particles will deliver significantly higher efficiency ($N$) than columns packed with fully-porous particles of the same diameter.*

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The van Deemter Equation

The three principle factors that cause band broadening and a decrease in column efficiency are described by the van Deemter equation:

\[ H = A \cdot d_p + B/\mu + C \cdot d_e^2 \cdot \mu \]

Simplified version:

\[ H = A \cdot d_p + B/\mu + C \cdot d_e^2 \cdot \mu \]

- Particle size
- Linear velocity (flow rate)
- Mass Transfer

Mobile phase viscosity
The Core-Shell Advantage

**A-term**

\[ H = A \cdot d_p + \frac{B}{u} + C \cdot d_e^2 \cdot u \]

Eddy Diffusion
Multi-path Effect

**B-term**

\[ H = A \cdot d_p + \frac{B}{u} + C \cdot d_e^2 \cdot u \]

Longitudinal diffusion
The Core-Shell Advantage

**Fully Porous C-term**

\[ H = A \cdot d_p + B/\mu + C \cdot d_e^2 \cdot \mu \]

Mass Transfer (Kinetics)

Dispersion due to resistance to mass transfer

The Core-Shell Advantage

**Core-Shell C-term**

\[ H = A \cdot d_p + B/\mu + C \cdot d_e^2 \cdot \mu \]

Mass Transfer (Kinetics)

Dispersion due to resistance to mass transfer
The Core-Shell Advantage

\[ H = A \cdot d_p + B/\mu + C \cdot d_e^2 \cdot \mu \]

**h vs. v Comparison**

<table>
<thead>
<tr>
<th>Column Length</th>
<th>Plates</th>
<th>Elution Time</th>
<th>Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>5cm</td>
<td>5,000</td>
<td>8 min</td>
<td>50 Bar</td>
</tr>
<tr>
<td>10cm</td>
<td>10,000</td>
<td>16 min</td>
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<tr>
<td>15cm</td>
<td>15,000</td>
<td>24 min</td>
<td>150 Bar</td>
</tr>
<tr>
<td>25cm</td>
<td>25,000</td>
<td>40 min</td>
<td>250 Bar</td>
</tr>
</tbody>
</table>

### Balancing Column Length and Particle Size

<table>
<thead>
<tr>
<th>Column Length (mm)</th>
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<th>Efficiency dp 3µm</th>
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<tr>
<td>250</td>
<td>25,000</td>
<td>37,500</td>
</tr>
<tr>
<td>150</td>
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<td>5,000</td>
<td>7,500</td>
<td>15,000</td>
<td>80</td>
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</table>

Use shorter columns packed with smaller particles to reduce analysis time while maintaining/improving efficiency!!
Quick Review

The chromatographic measurements that we have discussed so far will all play a significant role in method development.

1. **Retention factor (k')** – retention of analyte relative to void t,
   - Controlled by modulating % strong mobile phase

2. **Peak asymmetry** – peak shape (fronting, tailing, symmetrical)
   - Result of secondary interactions (e.g. Ionic in RP mode)
   - Sensitive to sample loading & sample solvent effects

3. **Selectivity (α)** – difference in the k’ of two analytes
   - Will be determined by mobile phase composition and nature of stationary phase

4. **Efficiency (N)** – function of peak width and retention
   - Determined by particle size, column length, flow rate
   - *Column packing will affect efficiency (vendor)*
Any Questions?

Resolution: The Goal of Chromatography
The goal of liquid chromatography is to resolve the individual components of a sample from each other so that they may be identified and/or quantitated.

Resolution is a measure of how well two peaks are separated from each other. It is calculated as the difference in retention time of two eluting peaks divided by the average width of the two peaks at the baseline.

\[ R \ (\text{resolution}) = \frac{RT_B - RT_A}{0.5 \ (\text{width}_A + \text{width}_B)} \]
(1) Retention time for the two peaks will be a function of retention factor \( k' \).

(2) The selectivity \( \alpha \) will also affect the retention time values for the two peaks.

(3) Peak width will be a function of column efficiency \( N \) and asymmetry \( \text{Asym} \).

**Resolution: The Goal of Liquid Chromatography**

The equation below allows us to calculate the relative importance of each of these three factors in overall resolution:

\[
R_s = \frac{\sqrt{N}}{4} \left( \frac{\alpha - 1}{\alpha} \right) \left( \frac{k}{k+1} \right)
\]

It is important to note that you (the analyst) have control over each of those factors through your choice of HPLC column and running conditions:

1. **Efficiency** \( N \) → Particle size/morphology and column length
2. **Selectivity** \( \alpha \) → Stationary phase and mobile phase
3. **Retention factor** \( k' \) → Stationary phase and mobile phase
Resolution: The Relative Effectiveness of $k'$, $\alpha$, and $N$

Most important determinant of resolution!!

Constant increase in resolution

Ineffective after $k' \sim 10$

The Impact of Efficiency on Resolution

Modulating column efficiency is a very effective way to optimize resolution. There is a strong, linear correlation between $N$ and $R_s$, but it is not a 1:1 ratio. Column efficiency is a flexible tool because we can easily modify it via changes in particle size and column length.

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<tr>
<td>50</td>
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<td>7,500</td>
<td>15,000</td>
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</tbody>
</table>

More Pressure
Longer Run Time

More Back-Pressure

More efficiency/resolution
The Impact of Efficiency on Resolution

Doubling column efficiency increases $R_s$ by a factor of 1.4x

5 µm
80,000 P/m

3 µm
150,000 P/m
Optimizing Efficiency for Maximum Resolution

1. For method development, start with an intermediate column length, packed with the **smallest particle size** that system pressure limitations will allow.
   • Conventional HPLC → 3µm 150x4.6mm or core-shell 100x4.6mm
   • UPLC → sub-2µm or core-shell particle
   • Work at **optimal flow rate** for that particle size

2. **Fine-tune** for maximum productivity:
   • Excessive resolution → shorter column, increase flow rate
   • Insufficient resolution → longer column; modify flow rate to compensate for pressure

The Impact of Retention Factor on Resolution

Retention factor is the **most important, yet limited, factor** in determining resolution. It is crucial to have a reasonable k’ value because analytes must be retained in order to separate them. The drawback is that at high k’ values, passive diffusion causes extensive band broadening and loss of performance.
Optimizing **Retention Factor** for Maximum Resolution

1. Adjust k’ value to be between 2 and 10
   - In RP, adjust % of organic (acetonitrile or methanol)
   - Altering nature of stationary phase/media can modulate k’ as well

2. At k’ < 2, have sub-optimal resolution
   - May also have interference from solvent, non-retained components

3. At k’ values > 10, band broadening due to diffusion limits resolution gain
   - In RP, complex mixtures of polar and non-polar components will require gradient for optimal performance/run time balance
   - Polar stationary phases can the “total elution window” of complex mixtures in isocratic mode

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The Impact of **Selectivity** on Resolution

Small changes in selectivity can have a dramatic effect on retention. This is one of the reasons why the same stationary phases from different manufacturers can sometimes give very different results, and also why changes to mobile phase composition can alter the results so strongly.

\[ R_s = \frac{\sqrt{N}}{4} \left( \frac{\alpha - 1}{\alpha} \right) \left( \frac{k}{k+1} \right) \]

- Selectivity = 1.05
- Selectivity = 1.1
- Selectivity = 1.2
Method Development Exercise 1: Optimization to Reduce Analysis Time and Increase Productivity

Column: C8 250 x 4.6mm 5µm
Mobile phase: 70 / 30 0.1M Ammonium acetate / THF
Flow rate: 1.0 mL/min
Components: 1-6 = Impurities A - G
7. Mupirocin

<table>
<thead>
<tr>
<th>Mupirocin Impurity Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column:</strong> C8 250 x 4.6mm 5µm</td>
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</tbody>
</table>

Mupirocin
Mupirocin: Original Method

Column: Luna 5µ C8(2) 250 x 4.6mm
Mobile phase: 70/30 0.1M Ammonium acetate pH 5.7/THF
Flow rate: 1.0 mL/min

Step 1. Adjust k’ for better Resolution

Step 1. Reduce % organic to increase k’:
- Increases Rs
- Increases run time
Step 2. Optimize Efficiency and Length

<table>
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<td>50</td>
<td>5,000</td>
<td>7,500</td>
<td>15,000</td>
<td>80</td>
</tr>
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</table>

Step 2. Switch to 150x4.6mm 3 µm media:
- Reduces analysis time
- Maintains efficiency

Step 3. Optimize the Flow Rate

Step 3. Increase flow rate to 1.5 mL/min:
- Optimizes efficiency for 3 µm
Mupirocin: Intermediate Method

Column: **Luna 3µ C8(2) 150 x 4.6mm**
Mobile phase: **80/20 0.1M Ammonium acetate pH 5.7/THF**
Flow rate: **1.5 mL/min**

- $R_s$ increased from 0.63 to 1.6
- Run time increased from 16 to 20 minutes

**Step 4. Switch to Core-Shell Media**

<table>
<thead>
<tr>
<th>Column Length (mm)</th>
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</tbody>
</table>

- Step 4. Switch to 100x4.6mm Core-Shell media:
  - Reduce analysis time
  - Increase efficiency
Mupirocin: Final Optimized Method

**Column:** Kinetex 2.6µ C8 100 x 4.6mm

**Mobile phase:** 80/20 0.1M Ammonium acetate pH 5.7:THF

**Flow rate:** 1.5 mL/min (2mL/min if pressure allows)

- *R*$_s$ increased from 1.6 to 2.3
- Run time decreased from 20 to 8 minutes
Any Questions?

Part 1. General Chromatographic Theory

Part 2. The Stationary Phase:  
An Overview of HPLC Media

Part 3. Role of the Mobile Phase in Selectivity
Fully Porous Silica

**Advantages:**
- Ability to derivatize with numerous bonded phases
- High mechanical strength
- Excellent efficiency
- Highly amenable to modulation of material characteristics (pore size, surface area, etc.)

**Disadvantages:**
- Dissolution of silica at pH > ~7.5 (may extend with bonded phase)
- Hydrolysis of bonded phase at pH <1.5
Organosilica Hybrid Particle

Conventional Silica Particle  Organosilica Hybrid Particle

Dissolution at pH > 7.5  Stable to pH ~12

Advantages:
- Extended pH range from 1-12
- Performance and strength of conventional silica particle
- Unique selectivity

Disadvantages:
- Fewer stationary phases available compared to conventional silica (e.g. cyano, amino)
Core-Shell Particle

- 0.35 µm Porous Shell
- 2.6 µm Core-Shell Particle
- 1.9 µm Solid Core

Advantages:
- 3x the efficiency of 5 µm fully-porous media & 2x the efficiency of 3 µm media
- Pressures compatible with conventional HPLC systems*

Disadvantages:
- Pressure is still higher than 3 µm media
- More sensitive to system extracolumn volumes
- More sensitive to overload in some cases
We use the methylene selectivity test to determine the ability of stationary phase to separate molecules based upon differences in their hydrophobic character. In general, very hydrophobic bonded phases (e.g. C18) will display higher levels of methylene selectivity than less hydrophobic phases.
**Methylene Selectivity**

![Graph showing methylene selectivity](graph.png)

**C18 > C8 > C5 ≥ Phenyl > CN > Amino**

**Columns:**
- 5µm **C18** 150x4.6mm
- 5µm **C8** 150x4.6mm
- 5µm **Phenyl** 150x4.6mm

**Mobile phase:** 65:35 Acetonitrile:Water

**Flow rate:** 1 mL/min

**Components:** Two steroids:
1. Testosterone
2. Methyltestosterone
**Phenyl Selectivity**

**Columns:**  
- C18  
- Phenyl

**Dimensions:** 150 x 4.6 mm

**Mobile phase:** 75:25 Methanol:water

**Flow rate:** 1 mL/min

**Components:**  
1. Estrone  
2. Estradiol

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**Graphs:**

- **C18 Phenyl**
  - Estradiol
  - Estrone

**Chart:**

- **C18 Phenyl Selectivity**
  - Components 1 and 2

---

**Graphs:**

- **Phenyl**
  - Estradiol
  - Estrone

**Chart:**

- **Phenyl Phenyl Selectivity**
  - Components 1 and 2
Aqueous Stability of Embedded Phases

Nucleic Acid Bases:

**Luna C18(2)**

**Polar-Endcapped C18**

Phase Collapse!

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Aqueous Stability of Embedded Phases

**LC/MS/MS Analysis of ETG & ETS in Urine:**

- Polar-Endcapped 2.5 µm C18 100x3.0mm
  - 10mM Ammonium formate
  - 1. Ethyl glucuronide
  - 2. Ethyl sulfate

ETG

ETS

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Part 1. General Chromatographic Theory

Part 2. Overview of HPLC Media

Part 3. Role of the Mobile Phase in Selectivity

Solvents for RP Chromatography

Mobile phase selection is much more challenging than stationary phase selection because the options are limitless. However, in practical method development, we can dramatically narrow down the options to focus on those conditions which will give us the highest likelihood of success.

Typical RP Solvents:

Weak Solvent: Water/Buffer

Strong Solvent: Acetonitrile (64)
Methanol (34)
Composite mixtures (1)
THF (1)

Frequency of use
The elution strength of a given solvent is determined by its hydrophobicity (e.g. heptane would be stronger than hexane because it is more hydrophobic). The selectivity of a solvent, however, is determined by its polar characteristics (e.g. heptane and hexane would have the same solvent selectivity).

**Methanol** is a strong proton donor and a strong proton acceptor in hydrogen bonding.

\[
\text{H}_3\text{C} \quad \text{OH}
\]

**Acetonitrile** has a dipole moment but is only a very weak proton acceptor in hydrogen bonding.

\[
\text{N} \quad \text{CH}_3
\]

**Tetrahydrofuran** is able to accept a proton in hydrogen bonding but cannot donate a proton.

\[
\text{O}
\]

**Solvent Selectivity**

The optimum separation of 4 steroids in different solvents:

- **27.5% acetonitrile**

- **50% methanol**

- **25% tetrahydrofuran**
1. Start at high % acetonitrile and work backwards until $k'$ is 2-10 (if possible)

- 80% ACN: $k' = 0$
- 40% ACN: $k' \approx 0.8$
- 25% ACN: $k' \approx 6$
- 21% ACN: $k' \approx 11$

2. Repeat with alternative solvent:

- 40% THF
- 30% THF
- 25% THF
Buffer Selection for RP-HPLC

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH&lt;sub&gt;b&lt;/sub&gt;</th>
<th>Buffer Range (pH)</th>
<th>MS Compatible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trifluoracetic Acid</td>
<td>&lt; 2</td>
<td>&lt; 2.5</td>
<td>1</td>
</tr>
<tr>
<td>Phosphoric Acid (pK&lt;sub&gt;a&lt;/sub&gt;)</td>
<td>2.1</td>
<td>1.1 - 3.1</td>
<td></td>
</tr>
<tr>
<td>Citric Acid (pK&lt;sub&gt;a&lt;/sub&gt;)</td>
<td>3.1</td>
<td>2.1 - 4.1</td>
<td></td>
</tr>
<tr>
<td>Formic Acid</td>
<td>3.8</td>
<td>2.8 - 4.8</td>
<td>1</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>4.8</td>
<td>3.8 - 5.8</td>
<td>1</td>
</tr>
<tr>
<td>Carbonate (pK&lt;sub&gt;a&lt;/sub&gt;)</td>
<td>6.4</td>
<td>5.4 - 7.4</td>
<td>1</td>
</tr>
<tr>
<td>Phosphate (pK&lt;sub&gt;a&lt;/sub&gt;)</td>
<td>7.2</td>
<td>6.2 - 8.2</td>
<td>1</td>
</tr>
<tr>
<td>Citrate (pK&lt;sub&gt;a&lt;/sub&gt;)</td>
<td>5.4</td>
<td>4.8 - 6.0</td>
<td>1</td>
</tr>
<tr>
<td>Triethanolamine</td>
<td>7.8</td>
<td>6.8 - 8.8</td>
<td>1</td>
</tr>
</tbody>
</table>

**Effect of pH on Base Silica**

Any silica-based RP material will have some residual silanols left after bonding and end-capping. These Si-OH groups can be deprotonated at values above pH ~3.5. The deprotonated silanols are more likely to engage in ion-exchange with basic analytes, leading to peak tailing.

- **pH <3.5**
  - Silanols protonated
  - Less ion-exchange
  - Less peak tailing

- **pH >3.5**
  - Silanols deprotonated
  - Increased ion-exchange
  - Increased peak tailing
The primary mechanism of retention in RP chromatography is hydrophobic interaction. Ionizing compounds will cause them to behave as more polar species, and reduce their hydrophobic interaction with the stationary phase, leading to decreased retention.

The ionization state of a molecule will be determined by the pH of the mobile phase. Therefore, mobile phase pH will dictate retention behavior of analytes with ionizable functional groups.
Effect of pH on Analyte Ionization

Alkaline

Acidic

Alkyl Stationary Phase

Aqueous Mobile Phase

Effect of pH on Analyte Ionization

Alkaline

Acidic

Alkyl Stationary Phase

Aqueous Mobile Phase
Effect of pH on Analyte Retention

Amitriptyline (pKa 9.4) = (B)ase
Toluene = (N)eutral
Naproxen (pKa 4.5) = (A)cid

Effect of pH on Analyte Retention

The gradient slope is analogous to solvent strength in isocratic elution.

**Isocratic Solvent Strength:**
- Increasing the solvent strength reduces analysis time but also reduces resolution.
- Decreasing the solvent strength increases resolution at the cost of increased analysis time.
- Solvent strength sometimes affects selectivity

**Gradient Slope:**
- Increasing the gradient slope reduces analysis time but also reduces resolution.
- Decreasing the gradient slope increases the resolution at the cost of increased analysis time.
- Gradient slope sometimes affects selectivity

The goal of gradient elution is to optimize resolution while minimizing analysis time.
The use of temperature in HPLC method development presents a challenge because it can have unpredictable effects on selectivity.

The use of elevated temperatures will:
1. Reduce mobile phase viscosity and back-pressure. This can allow you to operate at higher flow rates, or to use longer columns/smaller particle sizes.
2. Reduce elution time.
3. Improve method reproducibility (as opposed to operating at room temperature).

However, it is impossible to determine if the use of elevated temperatures will help or hinder a specific separation. For complex separations, improvements in one portion of the chromatogram are almost always accompanied by decreases in another part of the same chromatogram.

End of Section II

Any Questions?
End of the HPLC Method Development Portion