High-Throughput Separations for QA/QC, LC/MS, LC/MS/MS, Combinatorial Chemistry and Rapid Method Development
Ultra-Fast Chromatography

Powerful Tool

Less labor cost
Less cost per column
Less solvent usage / disposal
Ultra-Fast Chromatography

Acceptance

Rugged 3.5 µm Particles
Better HPLC’s Perceived
QA/QC, LC/MS, CombiChem
Outline

Theoretical

Selectivity (Bonded Phase and Mobile Phase)
Temperature
Column Configuration (Length and Particle Size)
Flow Rate

Practical Examples
Why Rapid-Resolution Chromatography?

Increases Productivity
Gaining Acceptance
Easily Achieved with Few Tradeoffs

Equal Resolution

4.6 x 250mm  
5 µm

4.6 x 150mm  
3.5 µm
Why Rapid-Resolution Chromatography?

Increases Productivity
Gaining Acceptance
Easily Achieved with Few Tradeoffs
Strategy for Ultra-Fast Separations

Drive Resolution Up Then Reduce Run Time

Rs factors that DON’T increase run time!

Rs factors that DECREASE run time!
How Resolution Varies with $k'$, $N$, and $\alpha$
Strategy for Ultra-Fast Separations
Analyzing the Fundamental Resolution Equation

\[ R_s = \frac{\sqrt{N}}{4} \]

**Plates**
- Particle Size
- Temperature
- Column Length

**Selectivity**
- Bonded Phase
- Mobile Phase
- Temperature
- pH

**Retention**
- Mobile-Phase Concentration
Outline

**Theoretical**

Selectivity (Bonded Phase and Mobile Phase)
Temperature
Column Configuration (Length and Particle Size)
Flow Rate

**Practical Examples**
Bulky diisobutyl (with C18) or diisopropyl (with C8, C3, CN, phenyl) side-chain groups are used to stabilize both long and short-chain monofunctional ligands.

300Å / 80Å  SB-CN

300Å / 80Å  SB-C3

80Å  SB-Phenyl

300Å / 80Å  SB-C8

300Å / 80Å  SB-C18
ZORBAX StableBond Optimizes Resolution with Five Bonded-Phases

- Each different bonded-phase changes selectivity and resolution of these compounds.

- Columns: 4.6 x 250 mm, 5 µm
- Mobile Phase: 0 - 100% B in 18.8 min
  - A: 50 mM NaH₂PO₄, pH 2.5 in 95% H₂O / 5% ACN
  - B: 50 mM NaH₂PO₄, pH 2.5 in 47% H₂O / 53% ACN
- Flow Rate: 1.5 mL/min
- Temperature: 26°C
- UV Detection: 254 nm
- Sample: 1. Procaine
  2. Lidocaine
  3. d-Cinchonine
  4. Butacaine
  5. Tetracaine

StableBond SB-C18
StableBond SB-C8
StableBond SB-Phenyl
StableBond SB-C3
StableBond SB-CN

Agilent Technologies
ZORBAX Eclipse XDB Offers Alternative Selectivity to ZORBAX StableBond

Columns: 4.6 x 75 mm, 3.5 µm  
Mobile Phase: 80% 25 mM NaH₂PO₄, pH 3.0 : 20% MeOH  
Flow Rate: 1.0 mL/min  
Temperature: 35°C  
Detection: UV 254 nm  

**Eclipse XDB-C8**  
**SB-C8**

- The selectivity differences between these two bonding technologies provide increased opportunities for maximizing sample resolution.
Comparison of Angiotensins Separation with TFA and NH4OH

Acidic Conditions
A- 0.1% TFA in water  
B- 0.085% TFA in 80%AcN

Basic Conditions
A- 10 mM NH₄OH in water  
B- 10 mM NH₄OH in 80%AcN

Zorbax Extend C18  
(2.1 x 150 mm)

HP 1100  
MSD: Pos. Ion ESI  
Vf 70V, Vcap 4.5 Kv  
N₂=35psi, 12L/min.  
325°C  
Gradient: 15-50%B / 15 min.  
0.2 mL/min  
Temp: 35°C  
Sample: 2.5 µL  
(50 pmol each)
Break Number 1

For Questions and Answers
Press *1 on Your Phone to Ask a Question
Outline

Theoretical

Selectivity (Bonded Phase and Mobile Phase)
Temperature
Column Configuration (Length and Particle Size)
Flow Rate

Practical Examples
New Selectivities Using Stable, Short-Chain Bonded Phases at High Temperature, Low pH

Resolution Improvement

35°C

8 9 10

300 SB-CN

60°C

8 9 10

300 SB-C3

8. Myoglobin
9. Calmodulin
10. Carbonic Anhydrase
Comparison of Gradient Performance at Different Temperatures

Zorbax SB-C8 (4.6x50mm)
A=90% H₂O : 10% MeOH containing 25 MM sodium phosphate buffer
B=10% H₂O : 90% MeOH containing 25 MM sodium phosphate buffer
[0-35%B in 5 min]
Detect.: 210 nm
Ion-Pairing Chromatography of B-Vitamins at Elevated Temperature for High-Throughput Analyses

**Isocratic Ion-Pairing**

Zorbax SB-C18 (4.6 x 75 mm) mixture containing 7.4 mM hexane sulfonate and 0.07% phosphoric acid

Absorbance

- **20°C**
- **60°C**
- **90°C**
Ultra-Fast Analysis of Parabens

Parabens: Preservatives used in food and personal care products against microbes
Outline

Theoretical

Selectivity (Bonded Phase and Mobile Phase)
Temperature
Column Configuration (Length and Particle Size)
Flow Rate

Practical Examples
## Increasing Analysis Speed in HPLC while Maintaining Resolution

<table>
<thead>
<tr>
<th>Dimension</th>
<th>5µm</th>
<th>3.5µm</th>
<th>5µm</th>
<th>3.5µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 x 4.6 mm</td>
<td>30 min.</td>
<td>150 x 4.6 mm</td>
<td>18 min.</td>
<td>150 x 4.6 mm</td>
</tr>
<tr>
<td>Solvent Waste, mL</td>
<td>30 mL</td>
<td>40% reduction</td>
<td>18 mL</td>
<td>40% reduction</td>
</tr>
<tr>
<td>N</td>
<td>20,000</td>
<td>20,000</td>
<td>12,000</td>
<td>10,000</td>
</tr>
<tr>
<td>Resolution α N^{1/2}</td>
<td>Unchanged</td>
<td>9% Difference</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**
- Increasing analysis speed in HPLC while maintaining resolution.
- Resolution changes due to the reduction in analysis time and solvent waste.
Rapid Resolution of Proteins / Peptides

- **ZORBAX 300SB-C8**
  - 4.6 x 250 mm, 5 µm
  - 40 min.

- **Rapid Resolution**
  - 4.6 x 150 mm, 3.5 µm
  - 24 min.

- **Rapid Resolution**
  - 4.6 x 50 mm, 3.5 µm
  - 9 min.

1. Met-enkephalin
2. Leu-enkephalin
3. Angiotensin II
4. Neurotensin
5. RNase
6. Insulin (Bov)
7. Lysozyme
8. Calmodulin
9. Myoglobin
10. Carbonic Anhydrase
Reduce Column Length to Reduce Run Time
Rapid Separation of Analgesics

SB-C18
4.6 x 15 mm

All 7 components resolved in less than 1 min

Conditions: LC: Hewlett-Packard HP1100
Columns: Zorbax SB-C18, 3.5 µm
Mobile Phase: 1 mM octane sulfonic acid, Na salt, pH 2.5 : ACN (80:20)
UV: 275 nm; Flow: 2.0 mL / min.; 70°C
Inj Vol: 1 µL

Sample:
1. Acetaminophen (4-acetamidophenol)
2. Caffeine
3. 2-Acetamidophenol
4. Acetanilide
5. Aspirin (acetsalicylic acid)
6. Salicylic acid
7. Phenacetin (acetophenetidin)
Improved Column Lifetime Using Ultra-Pure Silica Sol (Rx-SIL)

For 100Å PORE SIZE:
- STRUCTURE: UNIFORM SUB PARTICLES
- POROSITY (%): 50
- SURFACE AREA (M²/G): 150
- STRENGTH: HIGH
- HIGH pH RESISTANCE: GOOD
- PURITY: HIGH
- PORE SIZE DISTRIBUTION: NARROW

“SPONGE-LIKE,” POLYMERIC NETWORK
- STRENGTH: WEAK
- HIGH pH RESISTANCE: POOR
- PURITY: LOW - HIGH
- PORE SIZE DISTRIBUTION: BROAD

Silica-based packings made using agglutination of sol gel particles exhibit increased column lifetime
Outline

Theoretical

Selectivity (Bonded Phase and Mobile Phase)
Temperature
Column Configuration (Length and Particle Size)
Flow Rate

Practical Examples
Columns Packed with Smaller Particles Retain Efficiency at High Flow Rate

FLOW

N

3 micron

5 micron

10 micron
Gaining Full Potential in HPLC by Increasing Flow Rate

Pressure

- 90 bar
- 133 bar
- 181 bar

Flow Rate

- 2 mL/min
- 3 mL/min
- 4 mL/min

Rs_{2,1} = 2.2
Rs_{7,6} = 2.2

Rs_{2,1} = 2.1
Rs_{7,6} = 2.2

30 sec

ZORBAX SB-C18, 3.5 µm, 4.6 x 30 mm
Improving Resolution Using $k^*$

Resolution Relationship for Gradient Elution

$$R \approx \frac{\sqrt{N}}{4} \alpha^* k^*$$

$$k^* = \frac{t_G F}{S \Delta \Phi V_m} \sim \frac{V_G}{V_m}$$

$\Delta \Phi = \text{change in volume fraction of organic}$
$S = \text{constant}$
$F = \text{flow rate}$
$t_G = \text{gradient time (min.)}$
$V_m = \text{column void volume}$

$\text{b} = 1 / k^* = \text{gradient steepness}$

Thus, all of these increase $k^*$:

1. Longer gradient time $t_G$
2. Shorter column $V_m$
3. Higher flow rate $F$
4. Shorter organic range $\Delta \Phi$
Adjusting Gradient Parameters for High Throughput

0-35% B in 5 min, 1mL / min

0-35% B in 2 min, 1mL / min

0-35% B in 5 min, 2mL / min

Zorbax SB-C8 (4.6 x 50mm)
A=90% H₂O : 10% MeOH containing 25 mM Na phosphate buffer
B=10% H₂O : 90% MeOH containing 25 mM Na phosphate buffer
[0-35%B in 5 min]
Temp.: 90°C
Detect.: 210 nm

1. B6
2. B3
3. Pantothenic Acid
4. Folic Acid
5. B2
6. B12
Break Number 2

For Questions and Answers
Press *1 on Your Phone to Ask a Question
Outline

Theoretical
Selectivity (Bonded Phase and Mobile Phase)
Temperature
Column Configuration (Length and Particle Size)
Flow Rate

Practical Examples
LC for Combinatorial Chemistry

• 400 - 10,000 compounds/month

• Desire consensus method capable of separating a group of molecules of varying properties - “non-ideal” method development

• May require follow-up methods capable of high resolution separation

• Goal is rapid resolution in 1-4 minutes

• Preparative separations usually 15 - 50 mg
Optimized Column Configuration and Operating Parameters for High-Throughput Analyses*

A: 10/90, MeOH / H₂O + 0.2% H₃PO₄
B: 90/10, MeOH / H₂O + 0.2% H₃PO₄
20 mM each compound, 5µL inj.

Vendor A-C18
4.6 x 30 mm
Flow: 4 mL / min.
Gradient: 0-100% B / 2 min.
Detect.: 220 nm

Eclipse
XDB-C18
4.6 x 30 mm
Flow: 4 mL / min.
Gradient: 0-100% B / 2 min.
Detect.: 220 nm

Eclipse
XDB-C18
4.6 x 30 mm
Flow: 4 mL / min.
Gradient: 0-100% B / 1 min.
Detect.: 268 nm

<1 min

* Note improved baseline for HP 1100 DAD
Heterogeneity of Anthocyanins

<table>
<thead>
<tr>
<th>Anthocyanidin</th>
<th>R</th>
<th>R'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanidin</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Delphinidin</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>Malvidin</td>
<td>OCH₃</td>
<td>OCH₃</td>
</tr>
<tr>
<td>Pelargonidin</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Peonidin</td>
<td>OCH₃</td>
<td>H</td>
</tr>
<tr>
<td>Petunidin</td>
<td>H</td>
<td>OCH₃</td>
</tr>
</tbody>
</table>
Computer-Predicted vs. Observed Separations of a Complex Anthocyanin Sample

Zorbax SB-C18
4.6 x 250 mm
26°C, F=1.5

A = 5% HCOOH
B = 100% MeOH

<table>
<thead>
<tr>
<th>Time</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14%</td>
</tr>
<tr>
<td>10.2</td>
<td>17%</td>
</tr>
<tr>
<td>35.3</td>
<td>23%</td>
</tr>
<tr>
<td>64.6</td>
<td>47%</td>
</tr>
<tr>
<td>64.9</td>
<td>47%</td>
</tr>
<tr>
<td>65.0</td>
<td>14%</td>
</tr>
</tbody>
</table>

Final predicted vs. observed anthocyanin separation with <60 min. run time and separation of overlapped peaks.
Comparison of Column Configuration in Rapid LC/MS Analysis of Anthocyanins

Base-Peak Chromatograms

Relative Abundance

- 4.6 x 150mm
- 4.6 x 50mm

Multisegment Gradients of A: 5% formic acid, B: 1000% MeOH
EICs (extracted ion chromatograms) Based on Anthocyanin Fragments

Myrtillus extract

Malvidin m/z 331
Peonidin m/z 301
Petunidin m/z 317
Cyanidin m/z 287
Delphinidin m/z 303
BPC

The MS detector allows component analysis without the same high resolution necessary for UV detection

Agilent Technologies
Anthocyanins: Improved Separation Using New Mobile Phase

**A** = 5% HCOOH
**B** = 100% MeOH

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</thead>
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<tr>
<td>0</td>
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</tr>
<tr>
<td>15.4</td>
<td>17%</td>
</tr>
<tr>
<td>52.9</td>
<td>23%</td>
</tr>
<tr>
<td>96.9</td>
<td>47%</td>
</tr>
<tr>
<td>97.4</td>
<td>47%</td>
</tr>
<tr>
<td>97.5</td>
<td>14%</td>
</tr>
</tbody>
</table>

**A** = 3% H₃PO₄
**B** = 100% MeOH

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>23%</td>
</tr>
<tr>
<td>35</td>
<td>26%</td>
</tr>
<tr>
<td>97</td>
<td>60%</td>
</tr>
</tbody>
</table>
**Anthocyanins: Improved Separation With Shorter Columns**

\[
\begin{align*}
A &= 3\% \text{ H}_3\text{PO}_4 \\
B &= 100\% \text{ MeOH}
\end{align*}
\]

Excellent resolution obtained by change of mobile phase, allows use of shorter columns with smaller particle-size, for higher throughput.

---

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Percent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>23%</td>
</tr>
<tr>
<td>35</td>
<td>26%</td>
</tr>
<tr>
<td>97</td>
<td>60%</td>
</tr>
</tbody>
</table>

4.6 x 250mm, 5µm

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Percent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>23%</td>
</tr>
<tr>
<td>21</td>
<td>26%</td>
</tr>
<tr>
<td>58.2</td>
<td>60%</td>
</tr>
</tbody>
</table>

4.6 x 150mm, 3.5µm

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Percent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>23%</td>
</tr>
<tr>
<td>10.5</td>
<td>26%</td>
</tr>
<tr>
<td>29.1</td>
<td>60%</td>
</tr>
</tbody>
</table>

4.6 x 75mm, 3.5µm

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Agilent Technologies
Anthocyanins: Ultra-Fast Separation at High Flow Rates
Zorbax SB-C18, 3.5 µm Cartridge Columns

Abs (525 nm)

4.6 x 30 mm
F = 1 mL/min
12 min

4 mL/min
7.5 min

4.6 x 15 mm
F = 1 mL/min
6.5 min

4 mL/min
4.5 min

0 - 30 %B in 15 min (30mm)
A= 1% TFA, B= ACN, 1%TFA

0 - 30 %B in 7.5 min (15mm)
A= 1% TFA, B= ACN, 1%TFA
Ultra-Fast LC-MS Analysis of Analgesics (20 sec)

Zorbax XDB-C8 (2.1 x 15 mm)

- **Response Time**: < 0.01 min
- **Column**: 2.1 x 15 mm, 3.5 µm
- **Flow**: 0.834 mL/min
- **Temp**: 70 °C
- **Mobile Phase**: 18% ACN +1% TFA

**DAD 275 nm**

- **Cycle Rate**: 4.76 cycle/sec
- **Fragmentor**: 30V
- **Capillary**: 3500V
- **Gas Flow**: 13 L/min
- **Nebulizer Temp**: 350 °C

**MS - BPC**

**ES**

**Positive Mode**
Ultra-Fast LC-MS Analysis of Analgesics (20 sec)
Zorbax XDB-C8 (2.1 x 15 mm)

Response Time
< 0.01 min

Column
2.1 x 15 mm
3.5 µm

Flow
0.834 mL/min

Temp
70 °C

Mobile Phase
18% ACN
+1% TFA

DAD 275 nm

MS - BPC

Acetaminophen 152 EIC
2-Acetaminophenol

Caffeine 195 EIC

Acetanilide 136 EIC

Phenacitin 180 EIC

Cycle Rate
4.76 cycle/sec

Fragmentor
30V

Capillary
3500V

Gas Flow
13 L/min

Nebulizer Temp
350 °C
Summary

• Save time and money by choosing the shortest possible column with smallest particle size to achieve the desired separation
  • 150- vs. 15-mm length
  • 3.5µm vs. 5µm particles
• Adjust chromatographic parameters to obtain optimal separation
  • bonded phase functionality
  • mobile-phase pH
  • temperature
  • flow rate
  • highly selective detection (e.g., RI, Fluorescence, MS)
Break Number 3

For Questions and Answers
Press *1 on Your Phone to Ask a Question
Wrap-up E-Seminar Questions