Maureen Joseph, Ph.D.
HPLC Column Support
October 3, 2000

Method Development by the Numbers
Low, Mid and High pH Recommendations

9:00 a.m. EST
Telephone Number: 816-650-0621
Chair Person: Tim Spaeder

Agilent Technologies
Innovating the HP Way
Method Development by the Numbers
Low, Mid and High pH Recommendations
pH – An Important Parameter for Method Development

• Retention of ionizable compounds is strongly affected by pH

• Ionizable compounds (acids and bases) may be analytes or matrix compounds

• Accurate pH control improves method reproducibility

• The pH range from 1 – 12 provides maximum method development flexibility
Change in Retention with pH for Ionizable Compounds is Compound Dependent

- **Acetylsalicylic acid** pKa 3.5
- **Pyridine** pKa 5.2
- **Codeine** pKa 8.0
- **Procainamide** pKa 9.2
- **Amphetamine** pKa 9.9
- **Caffeine** pKa 14

Mobile Phase:
- 45% MeOH: 55% 20 mM Phosphate Buffer

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Change in Retention with pH for Ionizable Compounds is Key to Method Development

- Non-charged analytes have better retention (i.e. acids at low pH and bases at high pH)
- Silanols on silica ionize at mid-pH, increasing retention of basic analytes (i.e. possible ion-exchange interactions)
- Choose mobile phase pH and column type to optimize retention and selectivity during method development
Recommended Method Development Goals

- Adequate resolution of all peaks, $Rs \geq 2.0$
- Retention of first peak preferred to be at least $k=1$
- Analysis time below 30 minutes, 20 minutes preferred
- Robust and rugged methods
- Use buffered mobile phases and try low pH first
Why Develop Methods at Low pH?

- Acids are protonated for maximum retention
- Silica silanols are protonated thereby minimizing ion-exchange interactions with basic compounds
  - Good peak shape
  - Long term reproducibility
- Excellent mobile phase choices
Method Development Scheme
Start at Low pH

STEP 1
- Add 20 mM TEA
- Tailing peaks

STEP 2
- Change Temperature
- Band spacing problems

STEP 3
- Band spacing problems
- Change organic modifier (MeOH or THF)
- Adjust % organic for 0.5 < k < 20
- Restart at STEP 2

STEP 4
- Band spacing problems
- Try ZORBAX SB-CN, SB-C3 or SB-Phenyl
- Restart at STEP 1

Poorly retained basic compounds

Ion-Pairing of Basic Compounds

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## Recommended Starting Conditions for RP-HPLC Method Development Approach

### Separation Variable

<table>
<thead>
<tr>
<th>Column</th>
<th>Preferred Initial Choice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary Phase</td>
<td>SB-C18 or SB-C8</td>
</tr>
<tr>
<td>Dimensions</td>
<td>4.6 x 75 mm or 4.6 x 150 mm</td>
</tr>
<tr>
<td>Particle Size</td>
<td>3.5 µm 5 µm</td>
</tr>
<tr>
<td>Pore Size</td>
<td>80Å: M.W. ≤ 4000, 300Å: M.W. ≥ 4000</td>
</tr>
</tbody>
</table>

### Mobile Phase

<table>
<thead>
<tr>
<th>Solvents A-B</th>
<th>Water-acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>% B solvent</td>
<td>Variable</td>
</tr>
<tr>
<td>Buffer</td>
<td>25 mM NaH$_2$PO$_4$, pH ≤ 3 or 0.1% TFA or Formic acid</td>
</tr>
<tr>
<td>Additives i.e. amines and ion-pair reagents</td>
<td>TEA, Hexane sulfonate as needed</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1-2 mL/min</td>
</tr>
<tr>
<td>Temperature</td>
<td>30 - 35°C</td>
</tr>
</tbody>
</table>
Choose StableBond at Low pH

- Superior column lifetime due to patented sterically protecting bonding technology
- Fully-hydroxylated ultra-pure silica improves peak shape
- Five different 80Å bonded-phases – SB-C18, SB-C8, SB-CN, SB-Phenyl, SB-C3 – provide optimum selectivity with exceptional lifetime
- Four different 300Å bonded-phases for selectivity options with protein and peptide separations
Method Development – SB-C18 at Low pH
Separation of Steroids

- Method development scheme recommends starting with SB-C18 at low pH, which provides an excellent separation of these steroids and impurities.

Column: ZORBAX Rapid Resolution SB-C18, 4.6 x 75 mm, 3.5 µm
Mobile Phase: 50% ACN
50% 20 mM NaH₂PO₄, pH 2.8
Flow Rate: 1.0 mL/min
Temperature: RT
Detection: UV 254 nm
Sample:
1. Estradiol
2. Ethynylestradiol
3. Dienestrol
4. Norethindrone
Method Development – SB-C18 at Low pH
Separation of Plant Extract

Flavones, Flavanones, and Phenolic Esters

Column: ZORBAX Rapid Resolution SB-C18
4.6 x 75 mm, 3.5 µm
Mobile Phase: 22% ACN
78% NaH₂PO₄, pH 2.5
Flow Rate: 1.0 mL / min
Temperature: RT
Detection: UV 254 nm
Sample:
1. Caffeic acid
2. Impurity
3. Luteolin
4. Naringenin
5. Apigenin

• To obtain k=1 for caffeic acid requires 22 minute analysis time
Method Development – Change Organic Modifier
Separation of Plant Extract

Flavones, Flavanones, and Phenolic Esters

- Methanol as the organic modifier changes selectivity and increases the analysis time.

Column: ZORBAX Rapid Resolution SB-C18
4.6 x 75 mm, 3.5 µm

Mobile Phase: 40% MeOH
60% NaH₂PO₄, pH 2.5

Flow Rate: 1.0 mL/min
Temperature: RT
Detection: UV 254 nm
Sample: 1. Caffeic acid
2. Impurity
3. Naringenin
4. Luteolin
5. Apigenin
Method Development – Change Bonded-Phase Separation of Plant Extract on SB-CN
Flavones, Flavanones, Phenolic Esters

Column: ZORBAX Rapid Resolution SB-CN, 4.6 x 75 mm, 3.5 µm
Mobile Phase: ACN: NaH₂PO₄, pH 2.5
Flow Rate: 1.0 mL/min
Temperature: RT
Detection: UV 254 nm

22% ACN: 78% Buffer

25% ACN: 75% Buffer

• SB-CN with stronger mobile phase reduces analysis time by 50% and maintains retention of k=1 on 1st peak.
SB-CN Optimizes Retention and Resolution
Phytoestrogens and Isoflavones

**Columns:** 4.6 x 75 mm, 3.5 µm  
**Mobile Phase:** 30% ACN: 70% NaH$_2$PO$_4$, pH 2.5  
**Flow Rate:** 1.0 mL/min  
**Temperature:** 35°C  

- SB-CN reduces analysis time by 50% and increases retention of early eluting peaks.
- Method development procedure followed to get to this point.
StableBond 300SB Columns Ideal for Separations of High MW Analytes

- 300Å pore size necessary for good peak shape and high efficiency separation of proteins and polypeptides.
- Exceptional stability with low pH “TFA containing” mobile phases.
- Improve peak shape for lower molecular weight analytes with large hydrodynamic volume.
- Four different bonded-phases allow bonded-phase optimization of all high MW samples.
Why Choose 300Å Columns?

- Molecules must enter pores to interact with bonded-phase.
- Molecules must freely enter and exit pores to maximize efficiency.
Comparison of Bonded-Phase Options – Affect on Selectivity and Retention of Polypeptides

Columns: ZORBAX 300SB, 4.6 x 150 mm, 5 μm
Mobile Phase: Gradient, 0 - 26% B in 30 min.
A = 0.1% TFA in Water
B = 0.1% TFA in Acetonitrile
Temperature: 40°C
Sample: 2 μg of each peptide
Flow Rate: 1.0 mL/min
Detection: UV 210nm
Why Choose 300Å Columns?

Effect of Pore Size and Molecular Size on Peak Width, Gradient Separations

- Proper pore size selection results in sharper peaks for large molecules
Improved Peak Shape for Large Molecules in Solution

Columns: 4.6 x 150 mm, 5 µm  Mobile Phase: 60% MeOH: 40% 0.1% TFA  Flow Rate: 0.75 mL/min
Temperature: RT  Detection: UV 282 nm  Sample: Tylosin (MW 916)

SB-C3 (80Å)  300SB-C3 (300Å)

\[ P_{w1/2} = 0.442 \]  \[ P_{w1/2} = 0.125 \]

- The size of a molecule in solution determines which pore size column is best.
- The narrower peak width indicates unrestricted access to the pores.
Why Develop RP-HPLC Methods at Mid-pH?

• Compounds of interest are unstable at low pH
• Improved solubility of analytes at mid pH
• Increase retention of basic compounds
• May have better selectivity in the pH range 3 – 8
Method Development Scheme
Mid pH Range

STEP 5
- ZORBAX Eclipse XDB-C18 or C8
- pH 7 (6-9) 20 - 50 mM buffer,
- T = 30°C (ambient – 60°C)
- Adjust %ACN for 0.5 < k < 20

STEP 5a
- Add 20 mM TEA
- Tailing peaks

STEP 5b
- Poorly retained acidic compounds

STEP 6
- Band spacing problems
- Change % organic

STEP 6a
- Change Temperature
- Band spacing problems

STEP 7
- Band spacing problems
- Change organic modifier (MeOH or THF)
- Adjust % organic for 0.5 < k <20
- Restart at STEP 6

STEP 8
- Band spacing problems
- Try ZORBAX Eclipse XDB-Phenyl or Bonus-RP
- Restart at STEP 5

Ion-Pairing of Acidic Compounds
# Recommended Conditions for Mid-pH Method Development

## Separation Variable

<table>
<thead>
<tr>
<th>Column</th>
<th>Preferred Initial Choice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary Phase</td>
<td>Eclipse XDB-C18</td>
</tr>
<tr>
<td>Dimensions</td>
<td>4.6 x 75 mm or 4.6 x 150 mm</td>
</tr>
<tr>
<td>Particle Size</td>
<td>3.5 µm 5 µm</td>
</tr>
</tbody>
</table>

## Mobile Phase

<table>
<thead>
<tr>
<th>Solvents A-B</th>
<th>Water-acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>% B solvent</td>
<td>Variable</td>
</tr>
<tr>
<td>Buffer</td>
<td>25 mM Na₂HPO₄, pH =7</td>
</tr>
<tr>
<td></td>
<td>Acetate/acetic acid</td>
</tr>
<tr>
<td>Additives i.e. amines and ion-pair reagents</td>
<td>TEA, tetrabutylammonium as needed</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1-2 mL/min</td>
</tr>
<tr>
<td>Temperature</td>
<td>30 - 35°C</td>
</tr>
</tbody>
</table>
Choose Eclipse XDB for Mid-pH

- Long lifetime at mid-pH with dense bonding and double endcapping
- Strong silica for long lifetime
- Double endcapping provides excellent peak shape
- Three different bonded-phases (C18, C8, Phenyl) for selectivity optimization
Good Resolution with Eclipse XDB-C18 at Mid-pH

SB-C18, 4.6 x 75 mm, 3.5 µm

Eclipse XDB-C18, 4.6 x 75 mm, 3.5 µm

Mobile Phase: 20% Methanol: 80% 20 mM phosphate buffer  
Flow Rate: 1.0 mL/min  
Temperature: RT  
Detection: UV 254 nm  

• Better selectivity and improved retention occur at pH 7.
Eclipse XDB Selectivity Options
Maximize Retention at pH 7

Columns: 4.6 x 150 mm, 5 µm  Mobile Phase: 10% ACN: 90% Na₂HPO₄, pH 7  Flow Rate: 1.5 mL/min  Temperature: 35°C
Detection: UV 254 nm  Sample: 1. Procainamide  2. n-Acetylprocainamide  3. n-Propionylprocainamide

Eclipse XDB-C18  Eclipse XDB-C8  Eclipse XDB-Phenyl

- Eclipse XDB-Phenyl provides improved retention of these procainamides.
Bonus-RP Provides Alternate Selectivity at Mid-pH

- Polar alkyl-amide bonded-phase for unique selectivity
- Improves peak shape of basic compounds
- Triple-endcapped for good lifetime at mid-pH
- Enhanced low-pH stability (sterically protecting bonding) for alternate selectivity at low pH
- Compatible with 100% aqueous mobile phases
Bonus-RP Provides Alternate Selectivity at Mid-pH

Mobile Phase: 75% mM NaCitrate, pH 6
             25% MeOH
Flow Rate:   1.0 mL/min
Temperature: Ambient
Detection:   UV 254 nm
Injection Vol: 3 mL
Sample:     Cephalosporins
            1. Cephalexin
            2. Cephaclor
            3. Cephuroxime
            4. Cephoxitin

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Why Develop RP-HPLC Methods at High pH?

• Compounds of interest not soluble at lower pH
• Compounds of interest not stable at lower pH
• Increase retention of basic compounds by analyzing them in non-charged form
• Improve selectivity
Method Development at High pH

**STEP 9**
- ZORBAX Extend-C18
- pH 10.5 (9-12) 5 mM ammonia, or TEA, or 10 – 50 mM organic or borate buffers
- T = 25°C (ambient – 40°C)
- Adjust MeOH for 0.5 < k < 20

**STEP 10a**
- Vary temperature within recommended range for bonded phase

**STEP 10**
- Band spacing problems
- Change organic modifier (ACN or THF)
- Adjust for 0.5 < k<20
- Try different HPLC mode

Band spacing problems
**Recommended Conditions for High pH Method Development**

### Separation Variable

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<tr>
<th>Column</th>
<th>Preferred Initial Choice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary Phase</td>
<td>Extend-C18</td>
</tr>
<tr>
<td>Dimensions</td>
<td>4.6 x 75 mm or 4.6 x 150 mm</td>
</tr>
<tr>
<td>Particle Size</td>
<td>3.5 µm or 5 µm</td>
</tr>
<tr>
<td>Pore Size</td>
<td>80Å: M.W. ≤ 4000, 300Å: M.W. ≥ 4000</td>
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### Mobile Phase

<table>
<thead>
<tr>
<th>Solvents A-B</th>
<th>Water-methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>% B solvent</td>
<td>Variable</td>
</tr>
<tr>
<td>Buffer</td>
<td>20 mM TEA pH =11</td>
</tr>
<tr>
<td></td>
<td>ammonium hydroxide, pH 10</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1-2 mL/min</td>
</tr>
<tr>
<td>Temperature</td>
<td>RT - 30°C</td>
</tr>
</tbody>
</table>
Silica–Based HPLC Columns are Now a High pH Choice

- New technologies to protect silica from dissolution provide good lifetimes at high pH
- Superior efficiency of silica-based columns provides high resolution
- Robust methods can be established using the same parameters as used at low pH
Choose Extend-C18 for High pH

- Patented bidentate C18-C18 bonding for superior high pH stability – up to pH 11.5
- Improved performance over polymeric columns
- Excellent peak shape with double endcapping
- LC/MS at high pH (ammonium hydroxide) with high efficiency
Good Lifetime of Extend-C18 at High pH

Columns: 4.6 x 150 mm, 5 µm
Purge: 50% ACN / 50% 0.02 M K₂HPO₄, pH 11
Flow Rate: 1.5 mL / min
Temperature: 25°C
Detection: Silicate concentration by silicomolybdate color reaction
High pH Increases Retention of Antihistamines

- Column: ZORBAX Extend-C18, 4.6 x 150 mm, 5 µm
- Mobile Phase: See Below
- Flow Rate: 1.0 mL/min
- Temperature: RT
- Detection: UV 254 nm


pH 7
30% 20 mM Na₂HPO₄
70% MeOH

pH 11
30% 20 mM TEA
70% MeOH

\[ t_R = 8.5 \]

\[ t_R = 11.4 \]

- The retention of this sample of basic compounds increases at high pH.
Extend-C18 Provides High Efficiency and Good Peak Shape

Mobile Phase: 65% 20 mM TEA, pH 11: 35% MeOH  
Temperature: RT  
Detection: UV 254 nm  

Polymeric-Based Column  
4.0 x 250 mm, 5 µm  
Flow Rate: 0.5 mL/min

Extend-C18  
4.6 x 250 mm, 5 µm  
Flow Rate: 1.0 mL/min

- In comparison to polymeric columns, the Extend-C18 has superior efficiency and peak shape
Summary

• This method development scheme follows an approach of trying different pH’s for ionizable compounds with an optimum bonded-phase for both small molecules and large biomolecules.

- Low pH StableBond
- Mid pH Eclipse XDB or Bonus-RP
- High pH Extend-C18
## Appendix

### Recommended Buffer Choices for High pH

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pKa</th>
<th>Effective pH range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrroldidine</td>
<td>11.3</td>
<td>10.3 – 12.3</td>
</tr>
<tr>
<td>Triethylamine (TEA)</td>
<td>10.7</td>
<td>9.7 – 11.7</td>
</tr>
<tr>
<td>1-methyl-piperidine</td>
<td>10.3</td>
<td>9.3 – 11.3</td>
</tr>
<tr>
<td>glycine</td>
<td>9.8</td>
<td>8.8 – 10.8</td>
</tr>
<tr>
<td>TRIS</td>
<td>8.1</td>
<td>7.1 – 9.1</td>
</tr>
<tr>
<td>Borate</td>
<td>9.2</td>
<td>8.2 – 10.2</td>
</tr>
<tr>
<td>Ammonia</td>
<td>9.2</td>
<td>8.2 – 10.2</td>
</tr>
<tr>
<td>Diethylamine</td>
<td>10.5</td>
<td>9.5 – 11.5</td>
</tr>
</tbody>
</table>