Simplified Method Development

Low, Mid and High pH Recommendations

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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

Retention time (min):
- pH 2.5
- pH 6.5
- pH 8
- pH 11.5

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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**
- **ZORBAX Eclipse XDB-C18 or -C8**
  - pH 2.5 (2-3) 20 - 50 mM buffer,
  - T = 30°C (ambient – 60°C)
  - Adjust %ACN for 0.5 < k < 20

**STEP 1a**
- Bonus-RP or Add TEA

**STEP 1b**
- Poorly retained compounds
  - acids
  - Mid pH

**STEP 1c**
- Ion-Pairing of Basic Compounds
  - bases

**STEP 2**
- Band spacing problems
- Change % organic

**STEP 2a**
- Change Temperature

**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 Eclipse XDB-Phenyl, Bonus-RP**
- Try **ZORBAX SB-Phenyl, SB-CN, SB-C3**
- Restart at STEP 1

Band spacing problems
# Recommended Starting Conditions for RP-HPLC

## Method Development Approach

<table>
<thead>
<tr>
<th>Separation Variable</th>
<th>Preferred Initial Choice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column</strong></td>
<td></td>
</tr>
<tr>
<td>Primary Stationary Phase</td>
<td>Eclipse XDB-C18 or Eclipse XDB-C8</td>
</tr>
<tr>
<td>Secondary 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 or 5 µm</td>
</tr>
<tr>
<td>Pore Size</td>
<td>80Å: M.W. ≤ 4000, 300Å: M.W. ≥ 4000</td>
</tr>
</tbody>
</table>

| **Mobile Phase**     |                          |
| Solvents A-B         | Water-acetonitrile |
| % B solvent         | Variable |
| Buffer              | 25 mM NaH₂PO₄, pH ≤ 3 |
|                     | or 0.1% TFA or Formic acid |
| Additives i.e. amines and ion-pair reagents | TEA, Hexane sulfonate as needed |
| Flow Rate           | 1-2 mL/min |
| Temperature         | 30 - 35°C |
Choose Eclipse XDB First at Low pH

Wide useable pH range – pH 2- 9 – can use for low and mid pH without changing columns

Fully-hydroxylated ultra-pure silica improves peak shape

Double endcapping for good peak shape of basic compounds at low and mid pH

Three different bonded-phases for selectivity options
Method Development – Eclipse XDB-C18 at Low pH - Separation of Flavones

**Conditions:**
- **Column:** 4.6x75mm, 3.5µm
- **Temp:** Ambient
- **Eluent:** 30% ACN, 70% 25mM NaH₂PO₄ pH 2.5
- **Flow:** 0.75 mL/ min
- **Inj. Vol.:** 4 µL
- **Sample:** 1) Luteolin
  2) Apigenin
Excellent Reproducibility of ZORBAX Eclipse XDB at Low pH

**Conditions:**
- **Column:** Eclipse XDB-C8 4.6x150mm, 5µm
- **Temp:** 35°C
- **Eluent:** 15% ACN 85% 25mM Phosphate
- **Flow:** 1.0 mL/min

**Sample:**
1) Cefotaxime
2) Cefoxitin
3) Cefamandole
4) Cephalothin

**3 Lots % RSD**

<table>
<thead>
<tr>
<th>k'</th>
<th>α</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>3.1</td>
</tr>
<tr>
<td>2)</td>
<td>2.6</td>
</tr>
<tr>
<td>3)</td>
<td>2.8</td>
</tr>
<tr>
<td>4)</td>
<td>2.5</td>
</tr>
</tbody>
</table>

**pH 3**

**pH 7**
StableBond – Secondary Choice at Low pH

Superior column lifetime at very low pH – down to pH 1 – due to patented sterically protecting bonding technology

Fully-hydroxylated ultra-pure silica improves peak shape

Six different 80Å bonded-phases – SB-C18, SB-C8, SB-CN, SB-Phenyl, SB-C3, SB-Aq – provide optimum selectivity with exceptional lifetime

Four different 300Å bonded-phases for selectivity options with protein and peptide separations
Selectivity Options at Low pH
StableBond vs. Eclipse

Conditions:
- Column: 4.6x75mm, 3.5µm
- Temp: Ambient
- Eluent: 25% ACN, 75% 25mM NaH₂PO₄ pH 2.5
- Flow: 0.75 mL/min
- Inj. Vol.: 20 µL
- Sample: 1) Caffeic Acid
2) Luteolin
3) Naringenin
4) Apigenin
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.
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.

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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.
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 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 6a
- Change Temperature
- Band spacing problems

Step 6
- Band spacing problems
- Change % organic

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

Step 5b
- Poorly retained acidic compounds

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

| Solvents A-B            | Water-acetonitrile       |
| % B solvent             | Variable                  |
| Buffer                  | 25 mM Na₂HPO₄, pH =7      |
|                        | Acetate/ acetic acid      |
| Additives i.e. amines and ion-pair reagents | TEA, tetrabutylammonium as needed |
| Flow Rate               | 1-2 mL/min                |
| Temperature             | 30 - 35°C                 |
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
Low pH Provides Insufficient Selectivity for Some Samples

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

- Poor Selectivity with Eclipse at Low pH
- Selectivity Improves Somewhat with StableBond
Improved Selectivity and Resolution with Eclipse XDB-C18 at Mid-pH

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
- A little TEA also improves peak shape peak #4 (di-amine)
Optimize Separations with Eclipse XDB

Selectivity Options – Analysis of Sunscreens

Columns: 4.6 x 150 mm 3.5 µm  
Mobile Phase: 15% H2O: 85% MeOH  
Flow Rate: 1.0 mL/min  
Temperature: 30°C  
Detection: UV 310 nm  
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 µL
Sample: Cephalosporins
1. Cephalexin
2. Cephaclor
3. Cephuroxime
4. Cephoxitin
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
  - $T = 25^\circ C$ (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

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# Recommended Conditions for High pH Method Development

<table>
<thead>
<tr>
<th>Separation Variable</th>
<th>Preferred Initial Choice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column</strong></td>
<td></td>
</tr>
<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 5 µm</td>
</tr>
<tr>
<td>Pore Size</td>
<td>80Å: M.W. ≤ 4000, 300Å: M.W. ≥ 4000</td>
</tr>
</tbody>
</table>

| Mobile Phase        |                          |
| Solvents A-B        | Water-methanol           |
| % B solvent         | Variable                 |
| Buffer              | 20 mM TEA pH =11         |
| ammonium hydroxide, pH 10 |
| Flow Rate           | 1-2 mL/ min              |
| Temperature         | RT - 30°C                |
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

Extend-C18
Bidentate Structure

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High pH Increases Retention of Antihistamines


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
Match Method pH and Column Choice

Choose the Best Bonded-Phase for Each pH Range

**StableBond, pH 1-6**
Use at Low pH
1. Uses bulky silanes
2. Non-endcapped

**Eclipse XDB, pH 2-9**
**First choice at Low and Mid pH**
1. eXtra Densely Bonded dimethylalkylsilanes
2. proprietary double-endcapping

**Bonus-RP, pH 2-8**
Use at Low and Mid pH
1. polar alkyl phase
2. triple endcapped
3. uses bulky silanes

**Extend-C18, pH 2-11.5**
Use at High pH
1. unique bidentate structure
2. double endcapped

Silica Support

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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.

<table>
<thead>
<tr>
<th>pH Level</th>
<th>Bonded Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low pH</td>
<td>Eclipse XDB then StableBond</td>
</tr>
<tr>
<td>Mid pH</td>
<td>Eclipse XDB or Bonus-RP</td>
</tr>
<tr>
<td>High pH</td>
<td>Extend-C18</td>
</tr>
</tbody>
</table>
## 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>Pyrrolidine</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>
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
Reduce Column “Secondary Interactions” at Low pH

Column: Eclipse XDB-C8, 4.6 x 150 mm, 5µm
Mobile Phase: 85% 25 mM Na₂HPO₄ : 15% ACN, Flow Rate: 1.0 mL/min, Temperature: 35°C

Reducing the mobile phase pH reduces interactions with silanols that cause peak tailing.

• Reducing the mobile phase pH reduces interactions with silanols that cause peak tailing.

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SB-CN Optimizes Retention and Resolution

**Phytoestrogens and Isoflavones**

**Columns:** 4.6 x 75 mm, 3.5 µm  
**Mobile Phase:** 30% ACN: 70% NaH₂PO₄, 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.
Why Choose 300Å Columns?

Molecules must enter pores to interact with bonded-phase. Molecules must freely enter and exit pores to maximize efficiency.
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 nm, 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 (80 Å)  
300SB-C3 (300 Å)

$P_{w1/2} = 0.442$

$P_{w1/2} = 0.125$

- The size of a molecule in solution determines which pore size column is best.
- The narrower peak width indicates unrestricted access to the pores.

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Method Development Scheme

Start at Low pH

**STEP 1**
- **STEP 1a**
  - Bonus-RP or Add TEA
  - Tailing peaks

- **STEP 1b**
  - Poorly retained compounds
  - Band spacing problems
  - CHANGE % organic
  - Band spacing problems
  - Poorly retained compounds

**STEP 2**
- **STEP 2a**
  - Change Temperature
  - Band spacing problems

**STEP 3**
- **STEP 3a**
  - Change organic modifier (MeOH or THF)
  - Adjust %ACN for 0.5 < k < 20
  - Restart at STEP 2

**STEP 4**
- **STEP 4a**
  - Try ZORBAX Eclipse XDB-Phenyl, Bonus-RP
  - Try ZORBAX SB-Phenyl, SB-CN, SB-C3
  - Restart at STEP 1

Ion-Pairing of Basic Compounds

**Middle pH**