The Secrets of Good Peak Shape in HPLC

Choosing Columns and Conditions for the Best Peak Shape

What is Good Peak Shape and Why is it Important ?

- Good peak shape can be defined as a symmetrical or gaussian peak and poor peak shape can include both peak fronting and tailing.
- Good peak shape can be defined by….
- Tailing factor of 1.0
- High efficiency
- Narrow peak width
- Good peak shape is important for….
- Improved resolution (Rs)
- More accurate quantitation
- Longer usable column lifetime (based on system suitability criteria)

How is Peak Shape Measured?

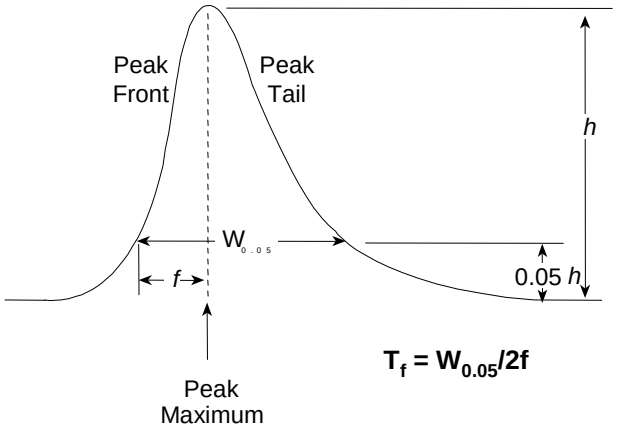
- **Measures**
- **USP Tailing Factor – at 5% of peak height***
- **Asymmetry – at 10% of peak height**

- **Indicators**
- **Efficiency – plates***
- **Peak Width – peak width at ½ height***

* Available in ChemStation reports

How is Peak Shape Measured?

USP Tailing Factor

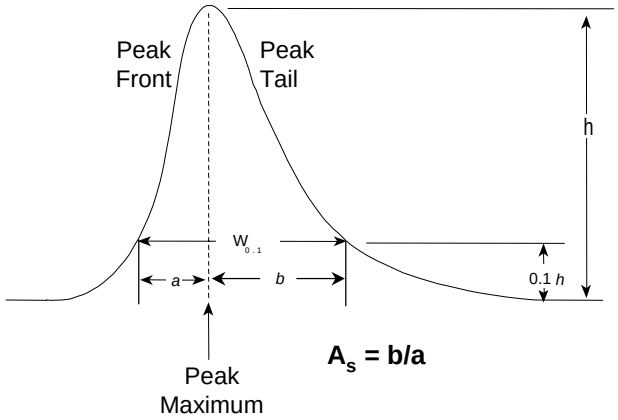


$T_f = W_{0.05}/2f$

Efficiency

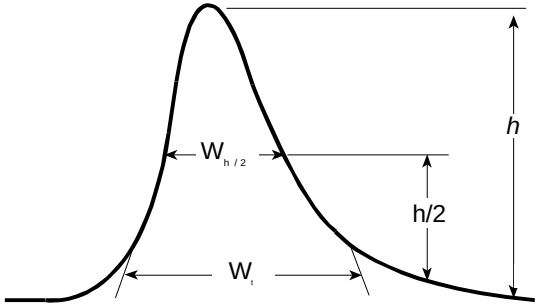
$$N = 5.54 \left(\frac{t_R}{W_{h/2}} \right)^2$$

Asymmetry



$A_s = b/a$

Peak Width at ½ Height



Factors Affecting Peak Shape

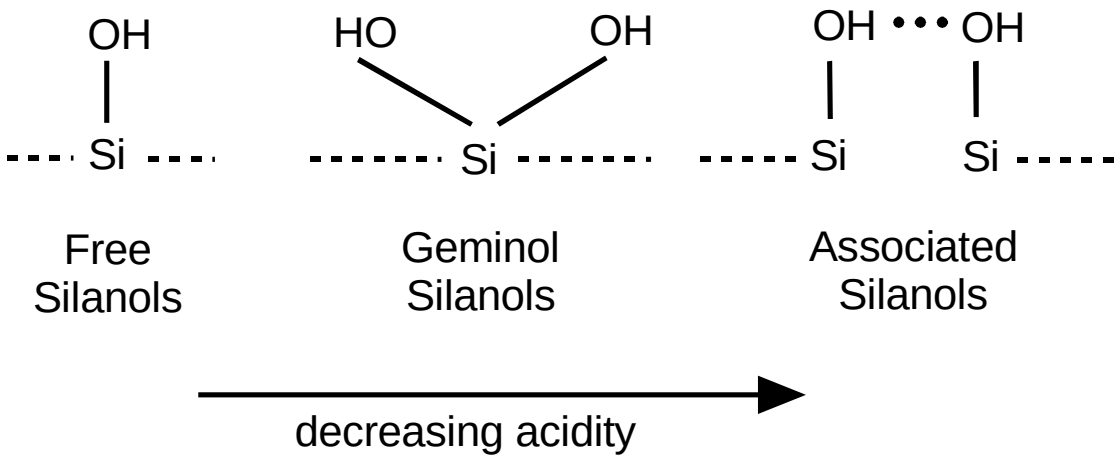
- Column packing factors
  - Silica type
  - Bonded phase and endcapping
- Mobile phase factors
- Sample factors

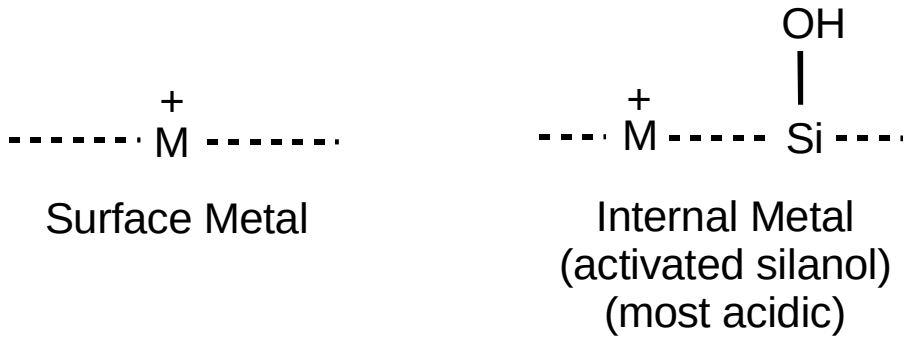
Column Factors Affecting Peak Shape

- **Silica type/acidity**
  - **Fully hydroxylated silica and silica metal content**
  - **Silica silanol ionization***
  - **Hydrogen bonding with silica silanols***
- **Column bonding**
  - **Endcapping**
  - **Type of bonded phase**
- **Packing Pore Size/Structure**

*Controlled by mobile phase

Silica Type - Fully Hydroxylated and Metal Free Silica Reduces Acidity



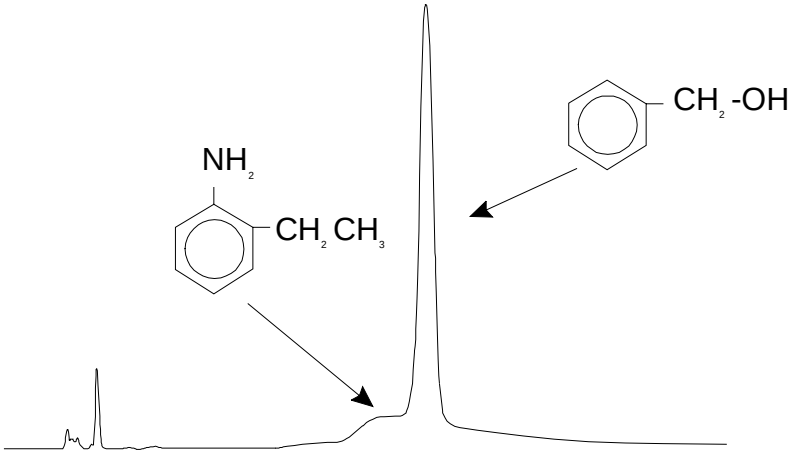


High Purity, Low Acidity Silica Improves Peak Shape

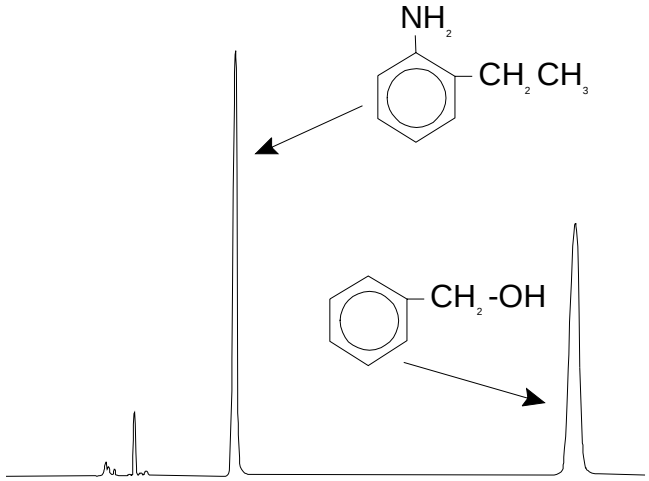
Mobile Phase: 5% 2-Propanol in Heptane

Flow Rate: 2.0 mL/min. Temperature: 35°C

Standard Silica



High Purity, Low Acidity ZORBAX Rx-SIL

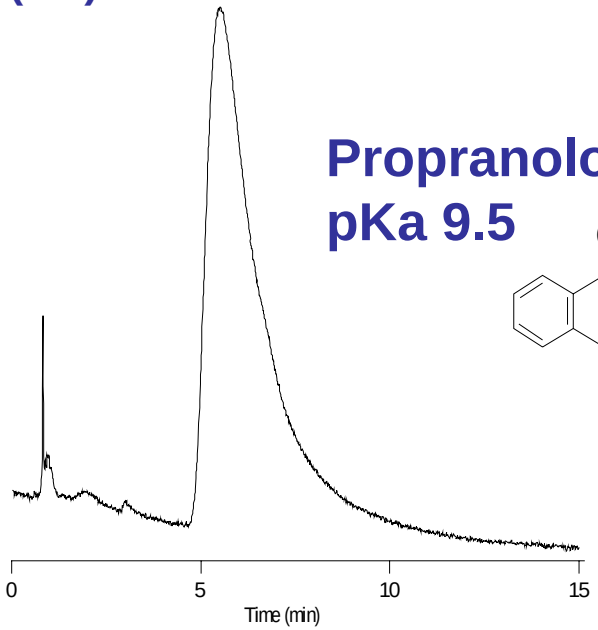


- **Improve peak shape for basic compounds with high purity, fully hydroxylated silica such as Rx-SIL**

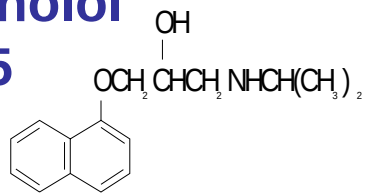
ZORBAX StableBond with Rx-SIL Improves Peak Shape

Mobile Phase: 75% 50 mM KH_2PO_4, pH 4.4 : 25% ACN Flow Rate: 1.5 mL/min

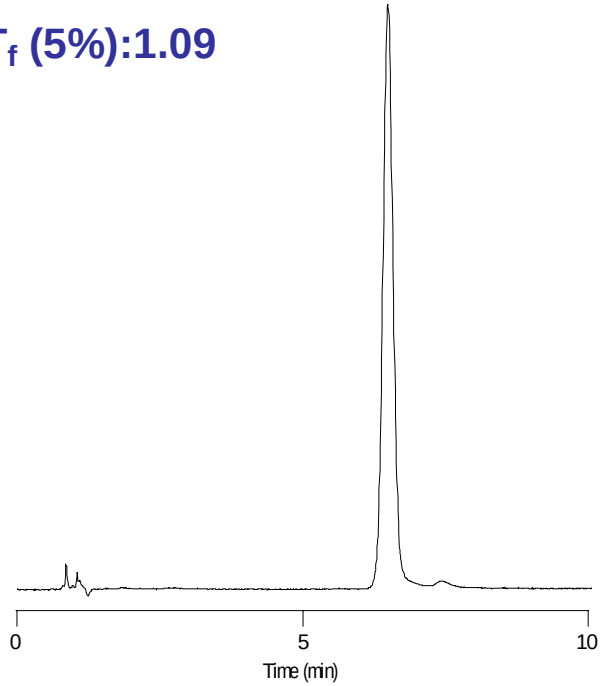
Silica Type – More Acidic
Column: ODS, 4.6 x 250 mm, 5 µm
Plates: 92
USP T_f (5%): 2.90



Propranolol
pKa 9.5



Silica Type – High Purity, Rx-Sil
Column: SB-C18, 4.6 x 150 mm, 5 µm
Plates: 6371
USP T_f (5%):1.09



- The high purity Rx-SIL improves the peak shape dramatically on a C18 column

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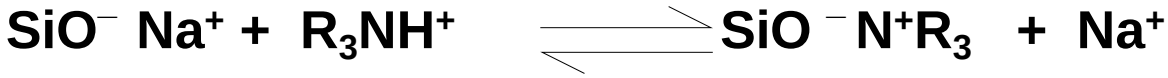
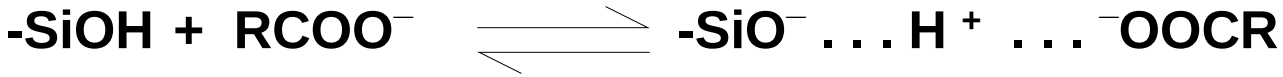
Potential Ion Exchange and Hydrogen Bonding Secondary Interactions

Ion-exchange



1. Ionized silanols (SiO^-) will ion-exchange with protonated bases (R_3NH^+) which can cause tailing and method variability. This occurs most often at mid pH where silanols are ionized.

Hydrogen Bonding



2. Unprotonated acids can compete for H^+ with protonated silanols. This can occur at low pH.

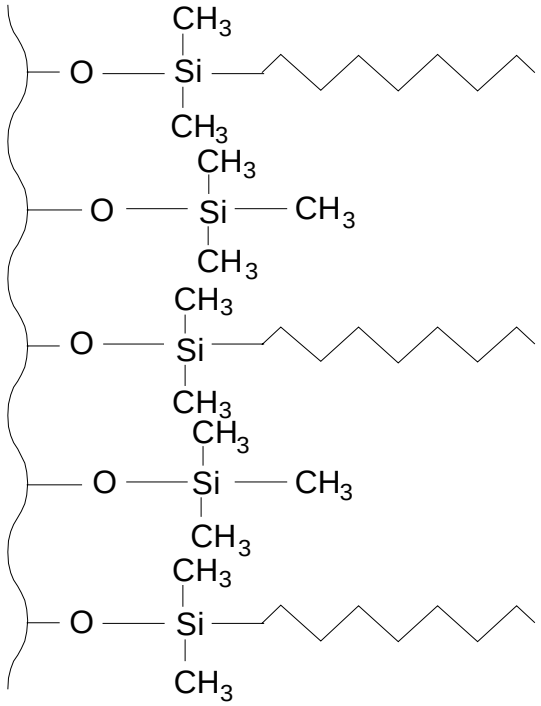
Some mobile phase additives can be added to the mobile phase to reduce these interactions and this will be discussed in the mobile phase section.

Bonded Phase Types for Better Peak Shape

- Bonded phases with endcapping
  - **Eclipse XDB columns are double endcapped**
  - **This minimizes the number of unreacted silanols and potential peak tailing interactions**
- Bonded phases with embedded polar groups
  - **Bonus-RP provides unique silanol shielding reducing peak tailing for basic compounds**
- Bonded phases for high pH
  - **Extend-C18 is stable at high pH**
  - **Basic compounds can not ion-exchange with the silica thereby reducing peak tailing**
- Bonded phases for high aqueous
  - **SB-Aq is more “wettable” improving analyte interactions and peak shape**

Eclipse XDB is Double Endcapped for Best Peak Shape

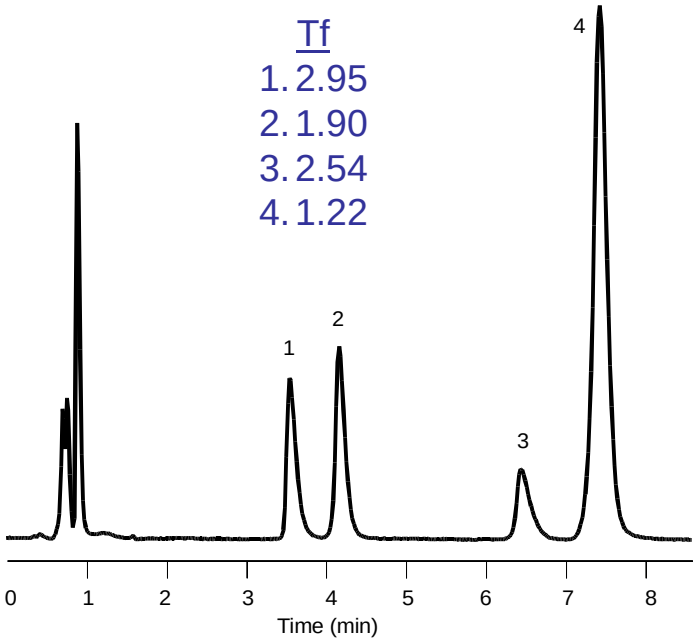
- Improved peak shape for basic compounds – especially at intermediate pH
- Double endcapped – with two different reagents for more complete silanol coverage
- Good for all sample types – acid, base, neutral
- Wide useable pH range – pH 2-9



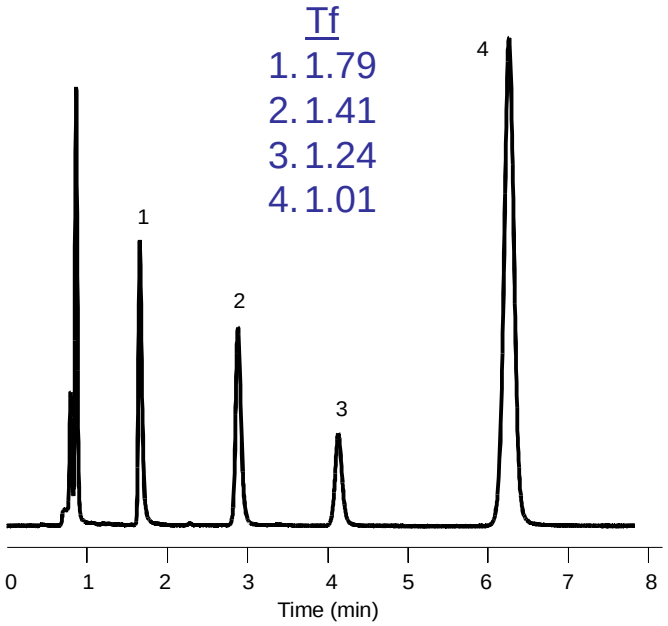
Double Endcapping Improves Peak Shape – pH 7

Columns: 4.6 x150 mm, 5 µm Mobile Phase: 60% ACN : 40% 10 mM phosphate buffer pH 7.0 Flow Rate: 1.5 mL/min.
Temperature: 40°C Sample: 1. Nortriptyline pKa 9.7 2. Doxepin pKa 9.0 3. Amitriptyline pKa 9.4 4. Trimipramine

Single Endcapped Competitive WS C18



Double Endcapped Eclipse XDB-C8



- Each column is made from high purity silica
- Fewer silanol interactions on the double endcapped column reduce tailing and retention

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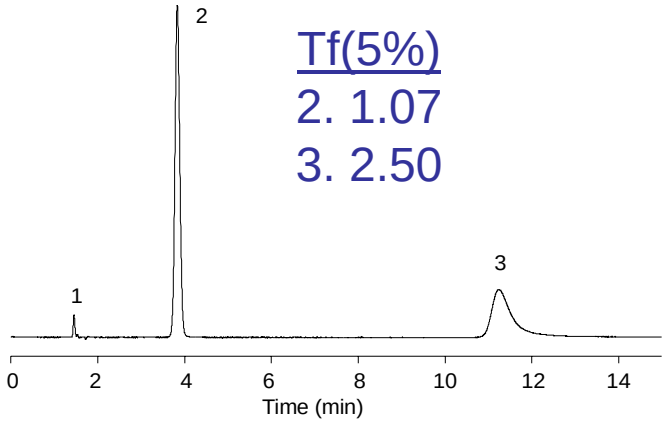
Double Endcapping Provides Superior Peak Shape
Eclipse XDB-C18 vs. Competitive C18

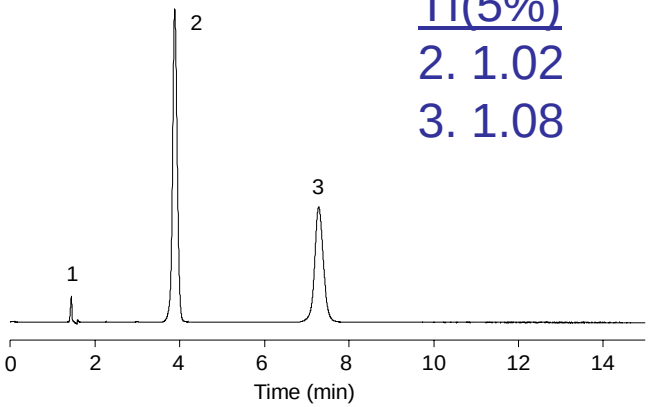
Columns: 4.6 x 150 mm, 5 µm Mobile Phase: 50% 25 mM Na_2HPO_4, pH 7.12: 50% MeOH
Detection: UV 254 nm Flow Rate: 1.0 mL/min Temperature: 25°C Sample: 1. Uracil 2. Amlodipine 3. Benazepril

Competitive L C18



Tf(5%)
2. 1.07
3. 2.50

Eclipse XDB-C18

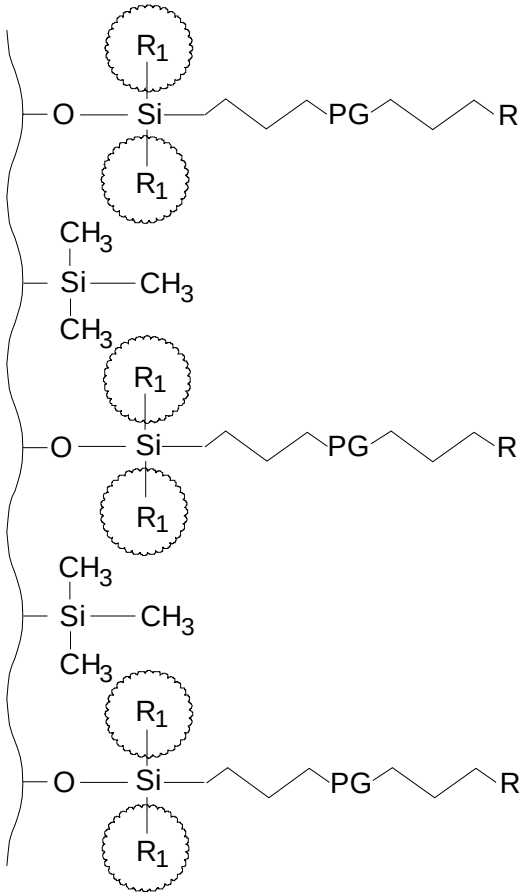


Tf(5%)
2. 1.02
3. 1.08

- Eclipse XDB shows superior peak shape for the basic compound, benazepril.

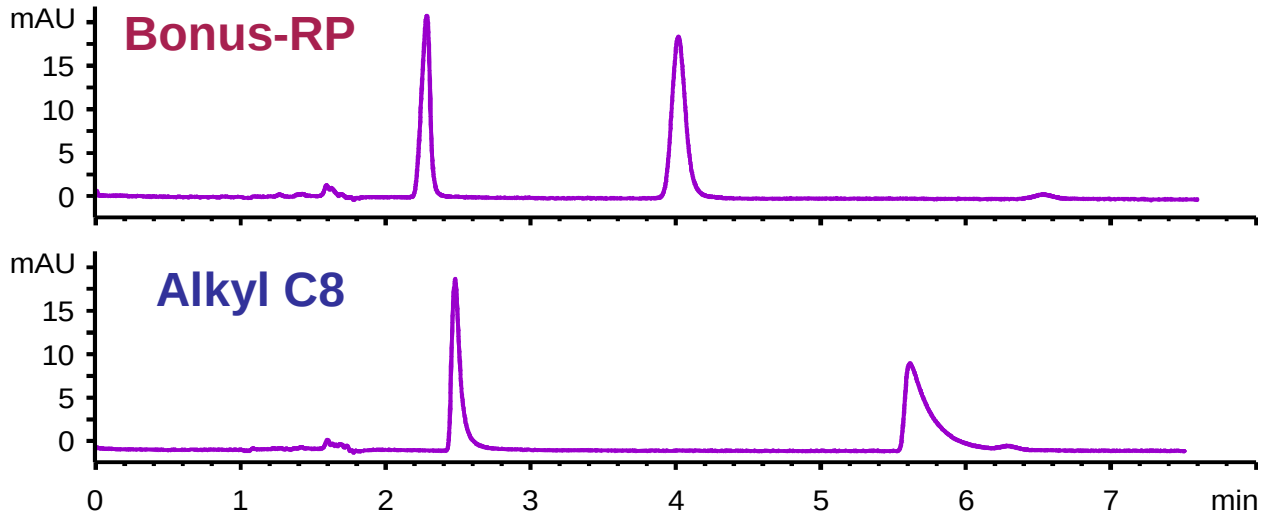
Bonus-RP with Embedded Polar Groups

- Good peak shape for basic compounds
- Polar alkyl-amide bonded phase
- Unique selectivity
- Enhanced low pH stability with sterically protecting bonding
- Triple endcapped



Polar Alkyl Amide Bonded Phase Provides Better Peak Shape than Traditional Alkyl Phase

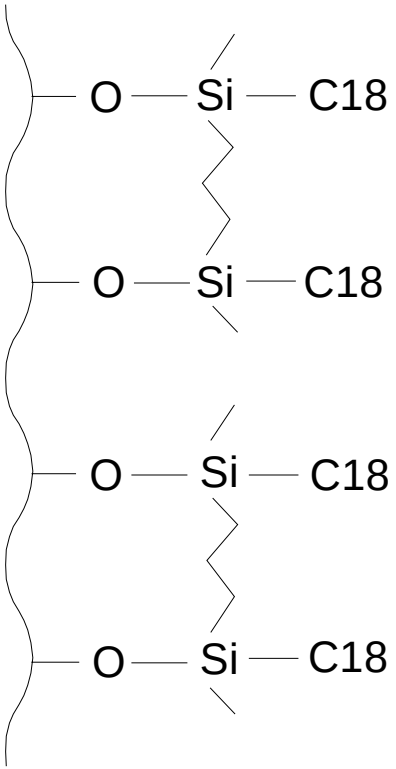
Columns: 4.6 x 150 mm Mobile phase: 25 mM K_2HPO_4, pH 7.2 / (MeOH: ACN, 50:50), 45/5 Flow rate: 1 mL/min.
Detection: UV 254 nm Injection vol: 5 µL Sample: Anorectics ("Fen-phen") 1. Phentermine pKa10.1 2. Fenfluramine pKa 9.1 3. Impurity



- Good peak shape of highly basic compounds is readily achieved on Bonus-RP.

ZORBAX Extend-C18 for High pH

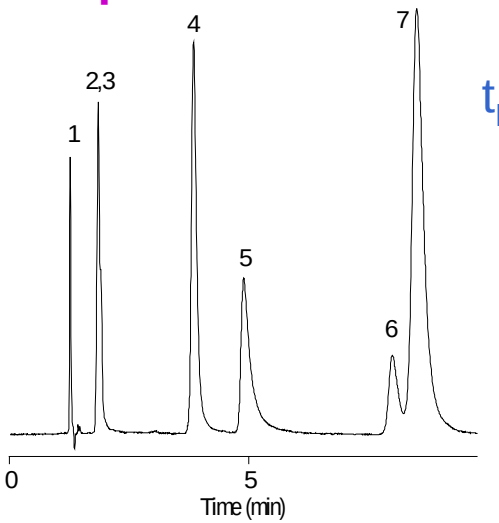
- **Superior high pH stability – up to pH 11.5 with silica particles**
- **Excellent reproducibility**
- **Patented bidentate, C18 bonding**
- **Double endcapping**



Extend-C18 Improves Retention and Peak Shape of Basic Compounds at High pH

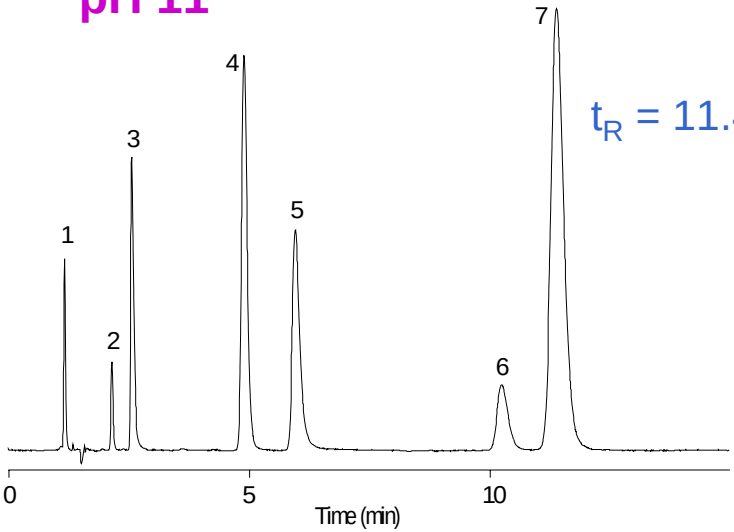
Column: ZORBAX Extend-C18, 4.6 x 150 mm, 5 µm Mobile Phase: 30% buffer: 70% MeOH pH 7 buffer=20 mM Na_2HPO_4 pH 11buffer = 20 mM TEA Flow Rate: 1.0 mL/min Temperature: RT Detection: UV 254 nm Sample: 1. Maleate 2. Scopolamine pKa 7.6 3. Pseudoephedrine pKa 9.8 4. Doxylamine pKa 9.2 5. Chlorpheniramine pKa 9.1 6. Triprolidine pKa 6.5 7. Diphenhydramine pKa 9.0

Extend-C18 pH 7



t_R = 8.5

Extend-C18 pH 11



t_R = 11.4

- **The retention of this sample of basic compounds increases at high pH on Extend-C18.**

ZORBAX SB-Aq for Good Retention and Peak Shape in High Aqueous Mobile Phases

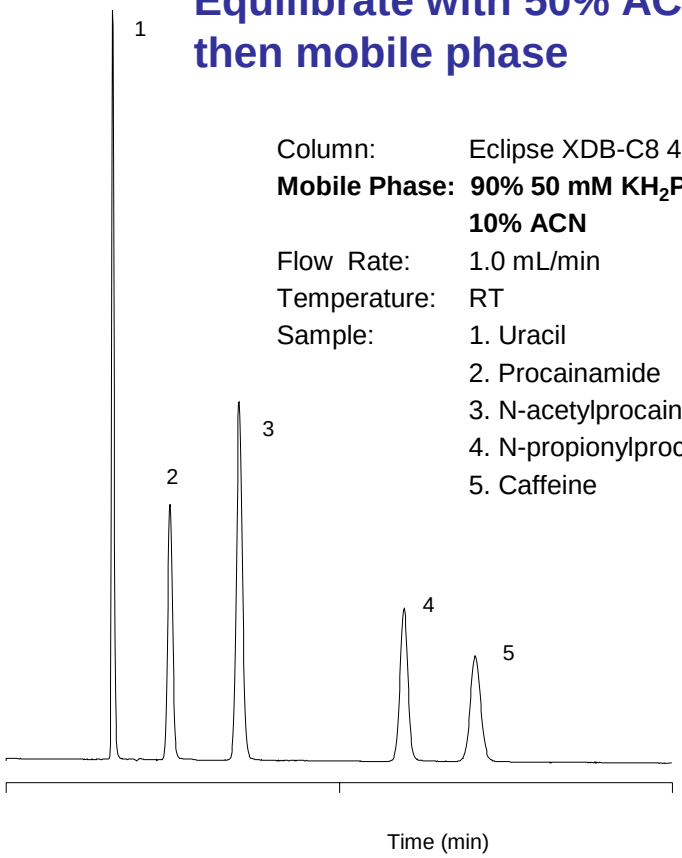
- Bonded phases that are more “wettable” in high aqueous mobile phases improve chromatography – both peak shape and retention reproducibility.
- A “wettable” bonded phase is one that does not fold over or collapse in a high aqueous mobile phase.
- These types of bonded phases can have polar endcapping or polar groups in the bonded phase, or other modifications to increase polarity.
- The SB-Aq column is an alkyl chain with more polar character that a typical C18 column.

Inconsistent Retention in High Aqueous Mobile Phase with Typical C18/C8 Columns

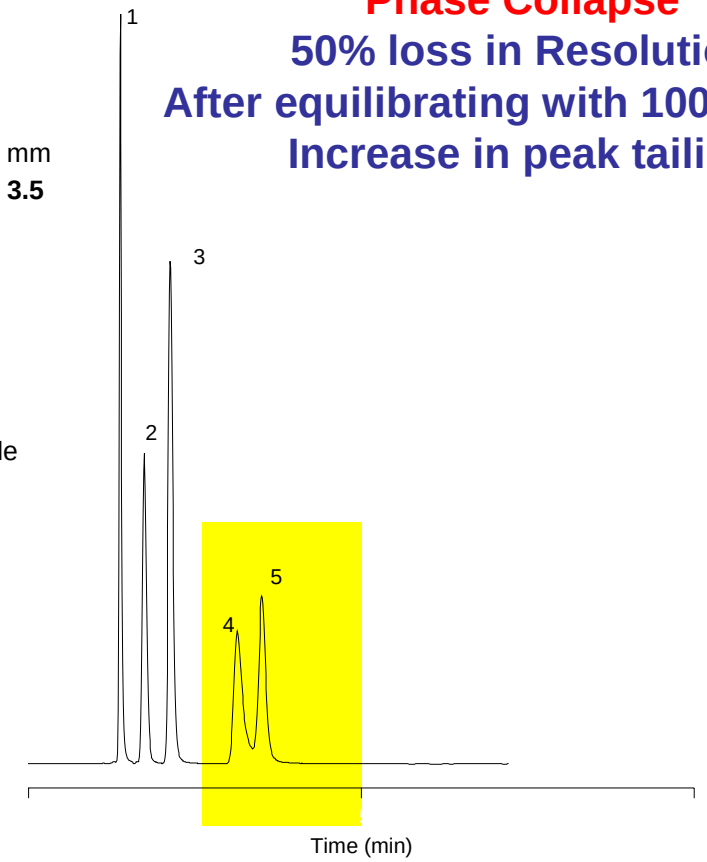
Example: Procainamides on Hydrophobic-C8 Column

Equilibrate with 50% ACN, then mobile phase

Column:	Eclipse XDB-C8 4.6 x 150 mm
Mobile Phase:	**90% 50 mM KH_2PO_4, pH 3.5**
	10% ACN
Flow Rate:	1.0 mL/min
Temperature:	RT
Sample:	1. Uracil
	2. Procainamide
	3. N-acetylprocainamide
	4. N-propionylprocainamide
	5. Caffeine



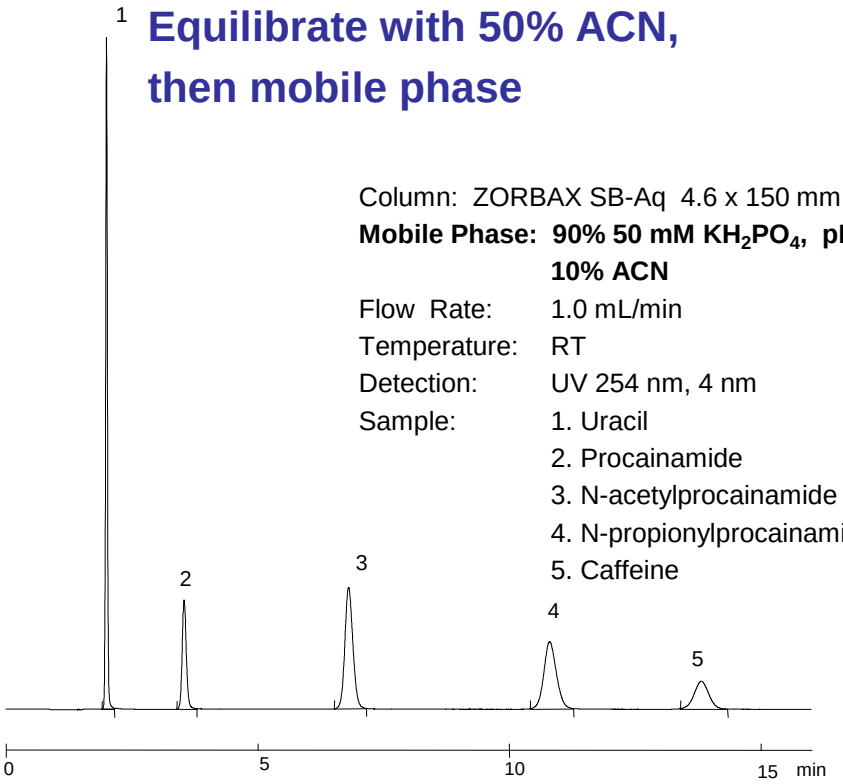
Phase Collapse
50% loss in Resolution
After equilibrating with 100% buffer
Increase in peak tailing



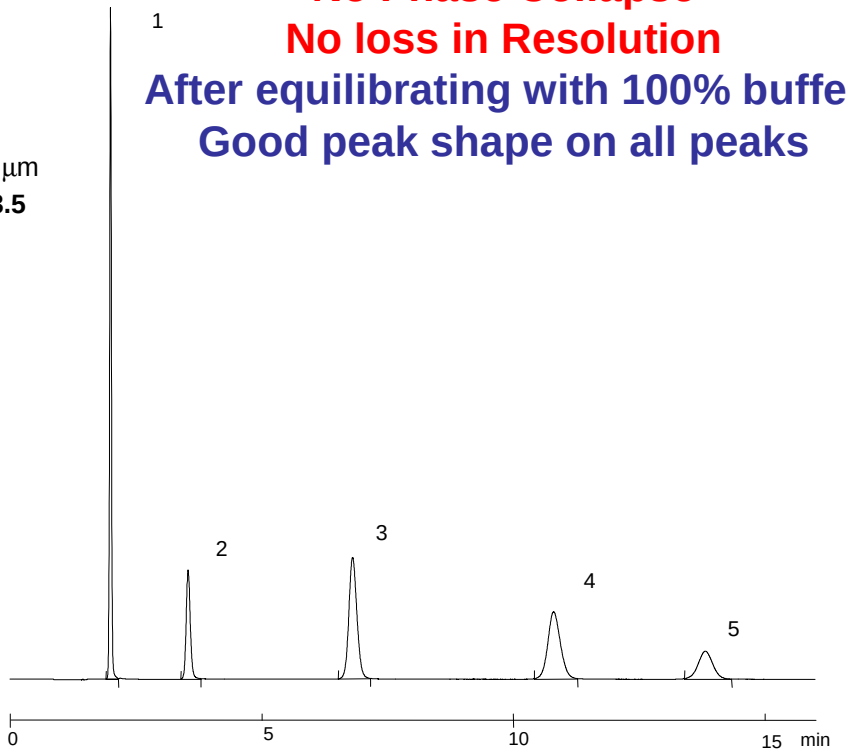
Consistent Retention of Procainamides on SB-Aq with Good Peak Shape

Equilibrate with 50% ACN, then mobile phase

Column: ZORBAX SB-Aq 4.6 x 150 mm, 5 µm
Mobile Phase: 90% 50 mM KH_2PO_4, pH 3.5
10% ACN
Flow Rate: 1.0 mL/min
Temperature: RT
Detection: UV 254 nm, 4 nm
Sample:
1. Uracil
2. Procainamide
3. N-acetylprocainamide
4. N-propionylprocainamide
5. Caffeine

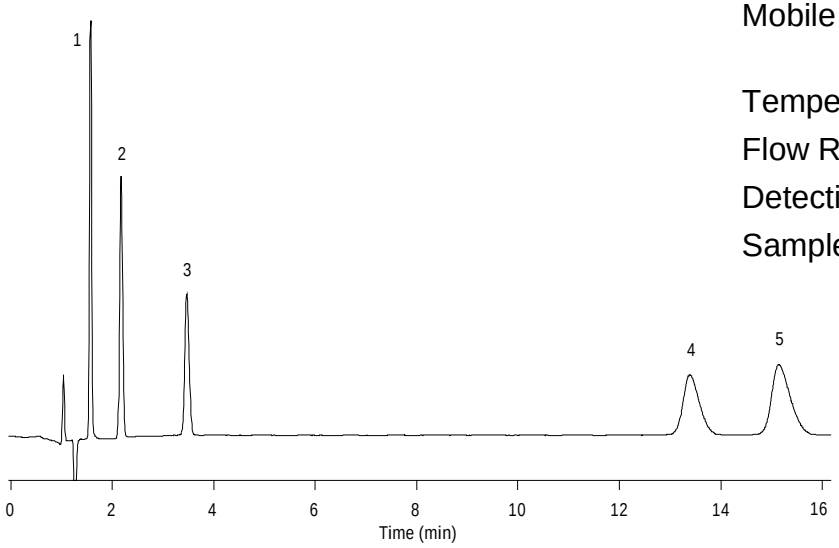


No Phase Collapse
No loss in Resolution
After equilibrating with 100% buffer
Good peak shape on all peaks



- **The SB-Aq column provides both good and consistent retention of the procainamides.**

ZORBAX SB-Aq Provides Good Retention and Peaks Shape of Polar Pharmaceutical Compounds

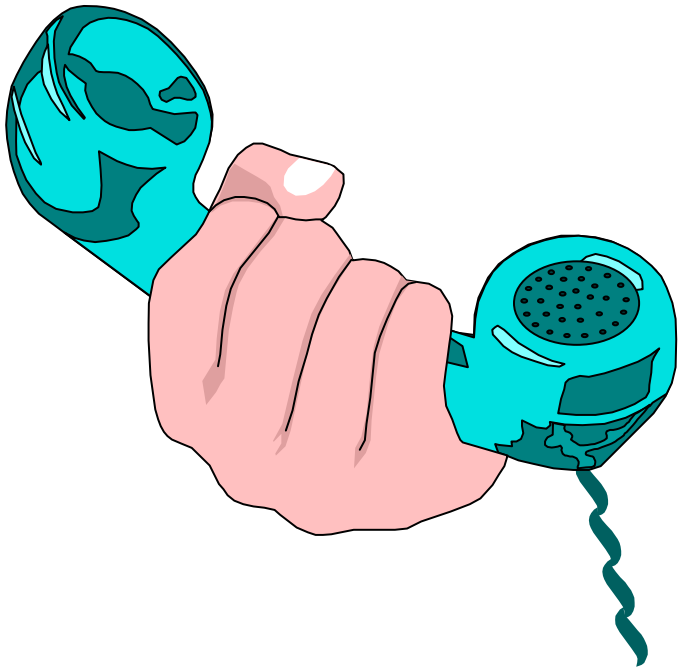


Columns:	**Zorbax SB-Aq, 4.6 x 150 mm, 5 µm**
Mobile Phase:	90% 0.2% TFA 10% ACN
Temperature:	25°C
Flow Rate:	1.5 mL/min.
Detection:	UV 254 nm
Sample:	1. Maleate 2. Phenylephrine 3. Phenylpropanolamine 4. Pyrilamine 5. Chlorpheniramine

- These small polar compounds are difficult to retain on most columns.
- The SB-Aq provides excellent retention with a 90% aqueous mobile phase.

Break Number 1

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

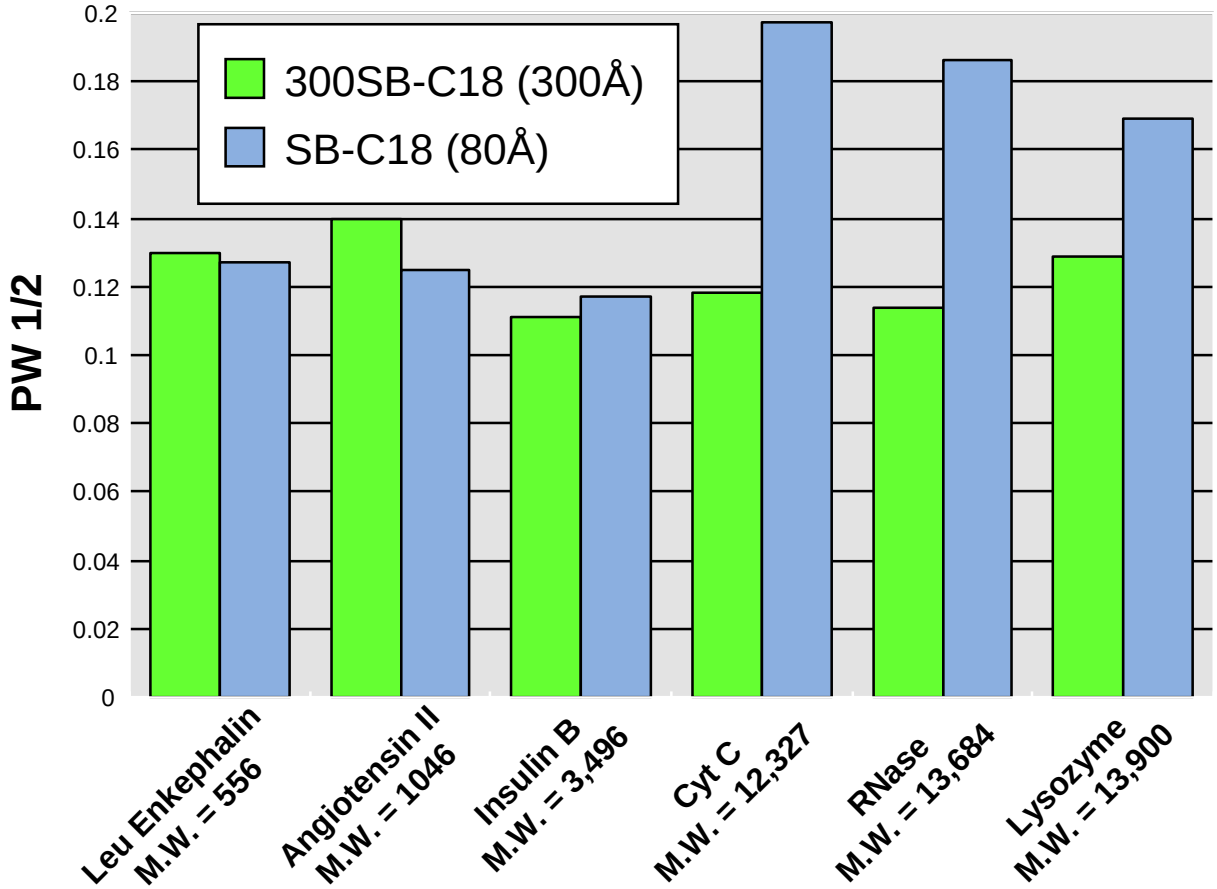


Large Molecules Need Large Pore Sizes for Good Peak Shape

- **Wide-pore 300Å totally porous columns can be selected for efficient peaks when separating proteins and peptides.**
- **Select wide-pore columns for lower molecular weight molecules with large hydrodynamic volumes.**
- **Select Poroshell columns for more rapid mass transfer and improved efficiency of large peptides and proteins at higher flow rates.**

Choose 300Å Columns for Good Peak Shape with Peptides and Proteins

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



- Proper pore size selection results in sharper peaks for large molecules

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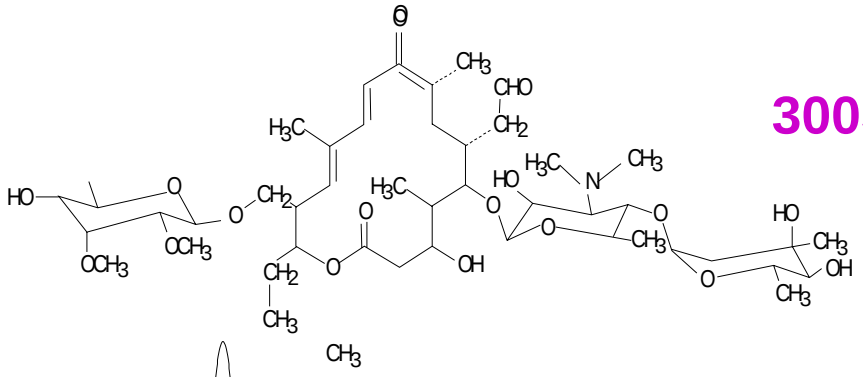
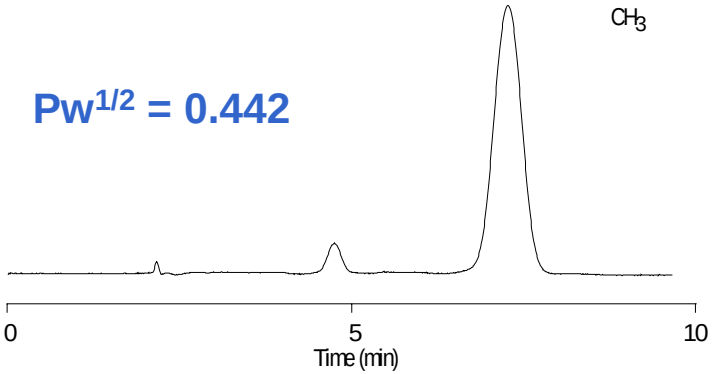
Improved Peak Shape for Large Molecules in Solution with 300Å Columns

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)



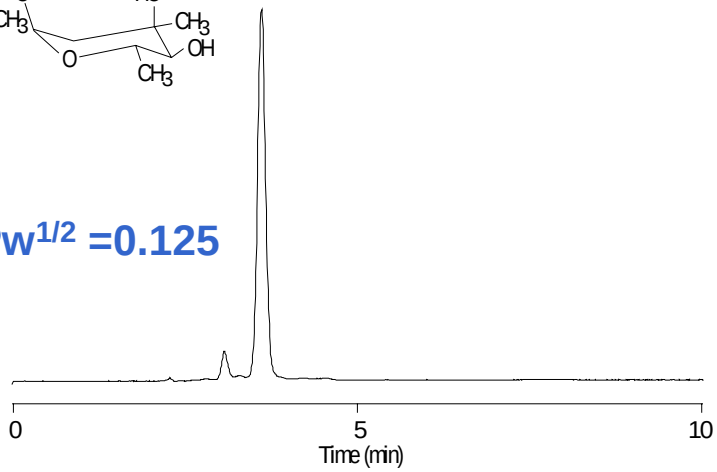
SB-C3 (80Å)

$Pw^{1/2}$ = 0.442



300SB-C3 (300Å)

$Pw^{1/2}$ =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.

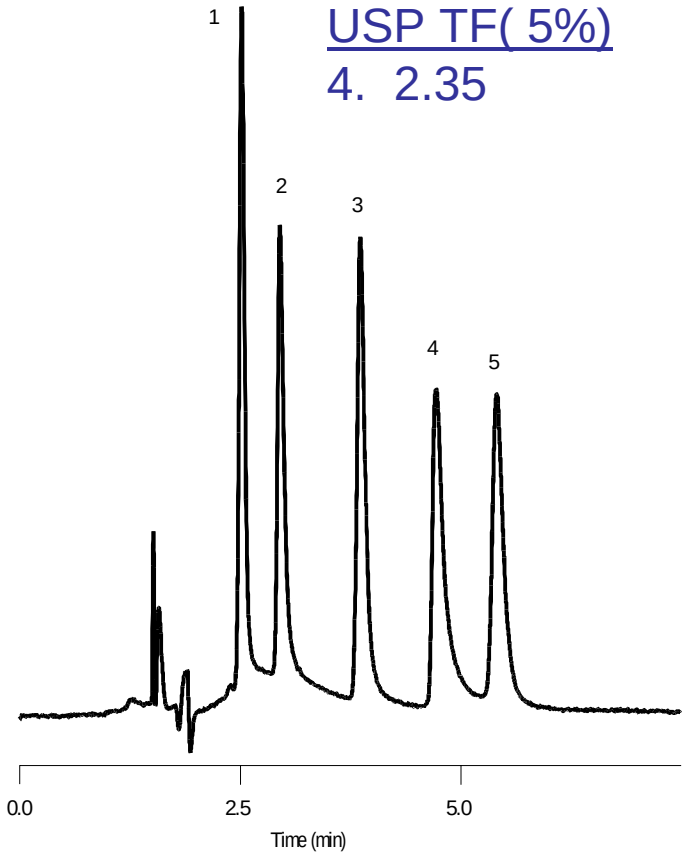
Mobile Phase Factors for Improved Peak Shape

- pH
- Buffers
- Organic modifiers
- Additional mobile phase modifiers (TEA, TFA)

Good Peak Shape at Low pH

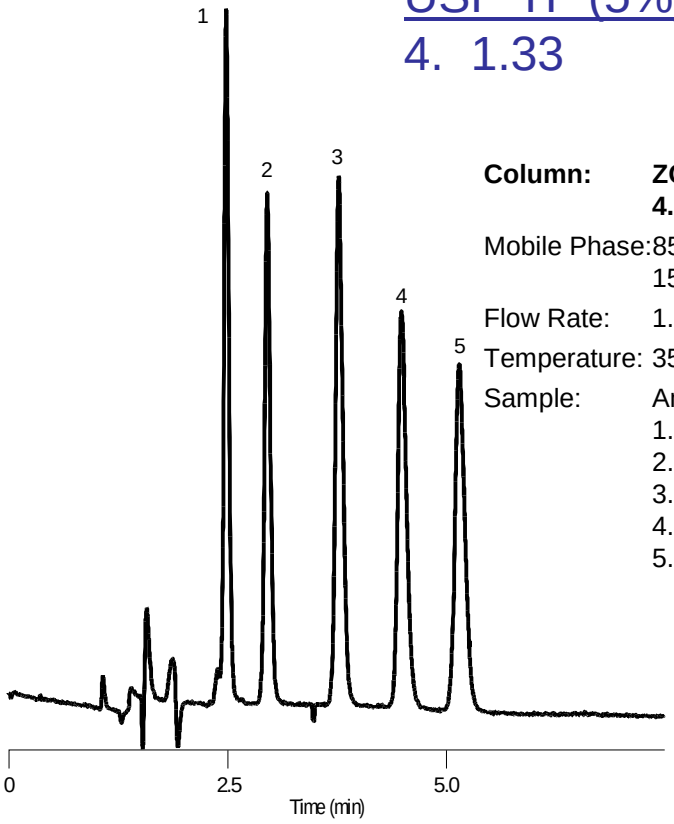
pH 7.0

USP TF(5%)
4. 2.35



pH 3.0

USP TF (5%)
4. 1.33



Column: **ZORBAX Eclipse XDB-C8 4.6 x 150 mm, 5 µm**

Mobile Phase: 85% 25 mM Na_2HPO_4
15% ACN

Flow Rate: 1.0 mL/min.

Temperature: 35°C

Sample: Amphetamines

1. Phenylpropanolamine pKa 9.4
2. Ephedrine pKa 9.6
3. Amphetamine pKa 9.9
4. Methamphetamine pKa 10.1
5. Phentermine pKa 10.1

- These basic compounds have good peak shape when the pH is lowered and the silica silanols are protonated.

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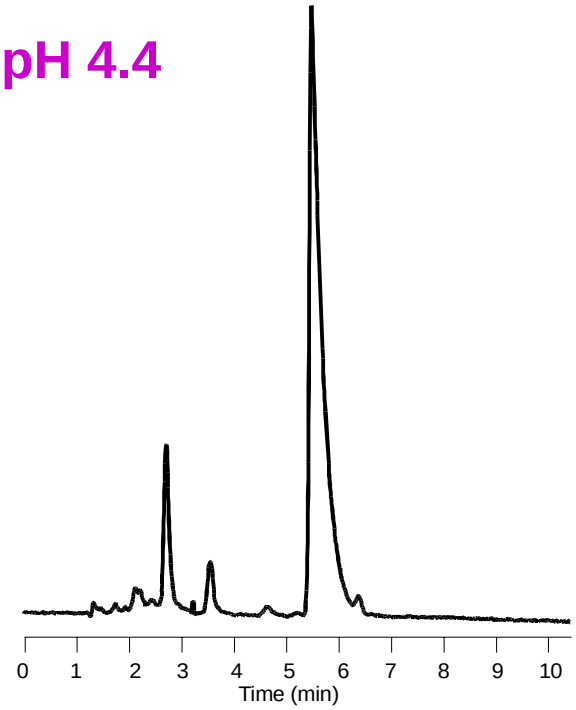
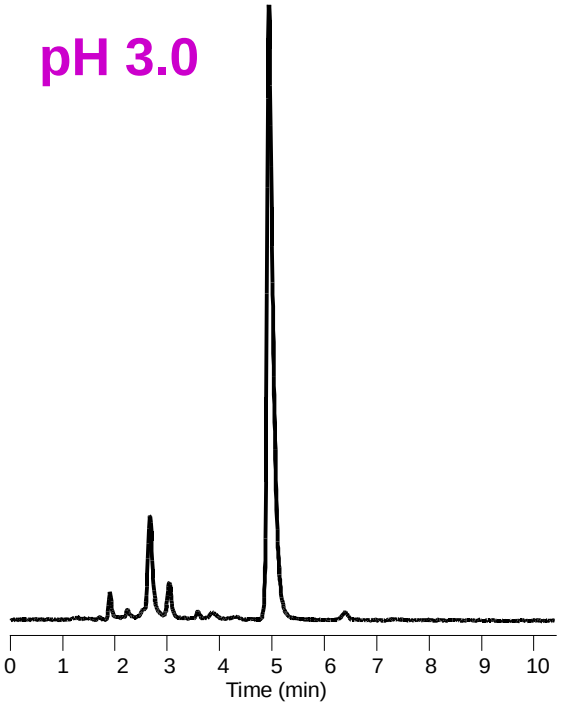
Effect of pH on Peak Shape at or Near the Sample pK_a

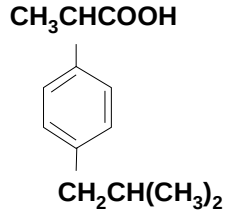
Column: ZORBAX SB-C8 4.6 x 150 mm, 5 µm Mobile Phase: 40% 5 mM KH_2PO_4: 60% ACN
Flow Rate: 1.0 mL/min. Temperature: RT

pH 4.4



pH 3.0





Ibuprofen
pK_a = 4.4

- Inconsistent and tailing peaks may occur when operating close to an analyte pKa and should be avoided.

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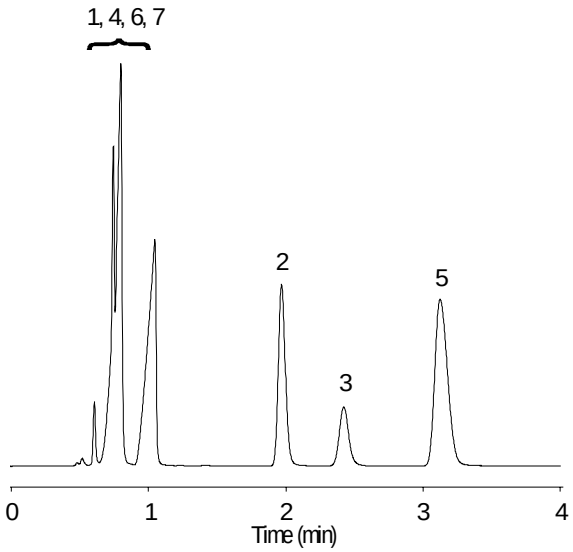
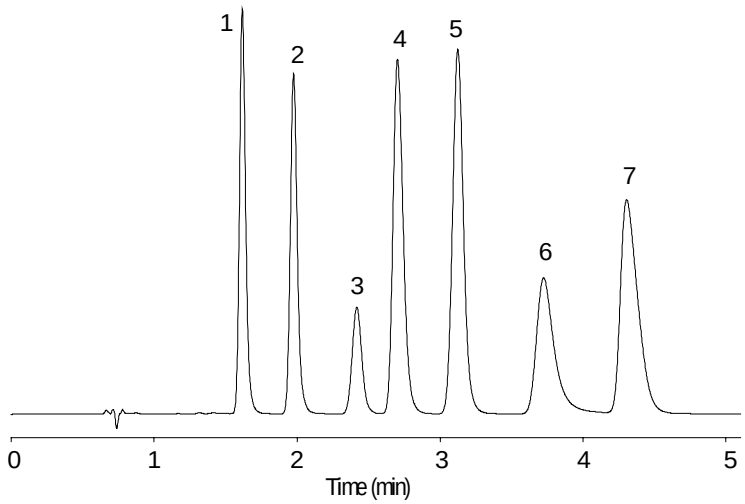
Use Buffered Mobile Phases for Best Peak Shape and Retention

Column: ZORBAX Rapid Resolution Eclipse XDB-C8, 4.6 x 75 mm, 3.5 µm Mobile Phase: 44% A : 56% methanol
Flow Rate: 1.0 mL/min Temperature: 25°C Detection: UV 250 nm
Sample: 1. ketoprofen 2. ethyl paraben 3. hydrocortisone pKa 5.1 4. Fenoprofen pKa 4.5 5. propyl paraben 6. Propranolol pKa 9.5 7. Ibuprofen pKa 4.4

A = pH 7.0 water



A = pH 7.0, 25 mM phosphate buffer

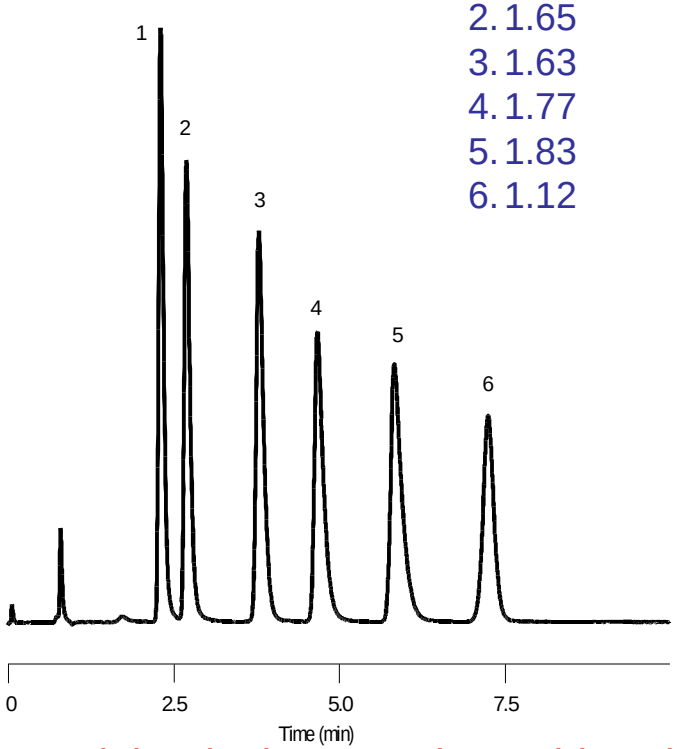


- Buffered mobile phases enhance retention, resolution, and peak shape.

Increasing Buffer Concentration Decreases Tf

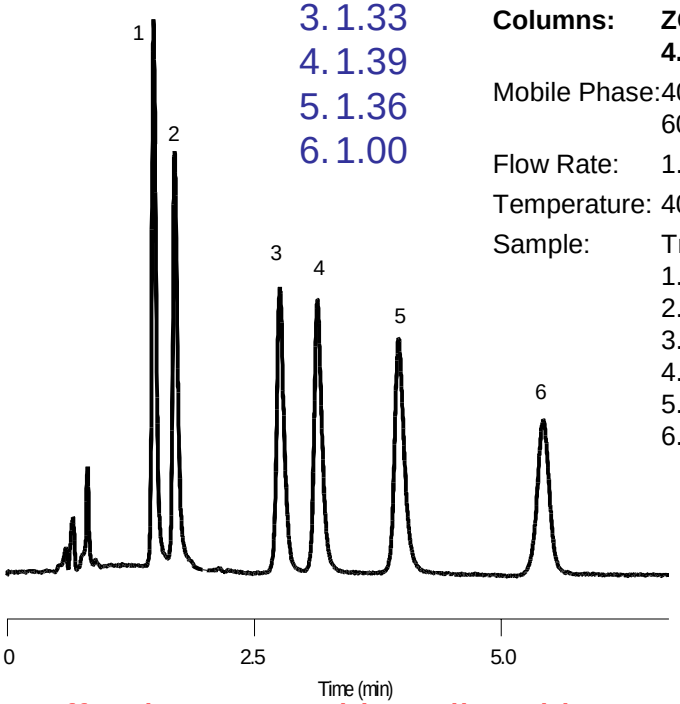
10 mM Phosphate
pH 7.0

	USP Tf
1.	1.62
2.	1.65
3.	1.63
4.	1.77
5.	1.83
6.	1.12



25 mM Phosphate
pH 7.0

	USP Tf
1.	1.41
2.	1.50
3.	1.33
4.	1.39
5.	1.36
6.	1.00



Columns: **ZORBAX Eclipse XDB-C8 4.6 x 150 mm, 5 µm**
Mobile Phase: 40% phoshpate buffer 60% ACN
Flow Rate: 1.5 mL/min.
Temperature: 40°C
Sample: Tricyclic Antidepressants
1. Desipramine
2. Nortriptyline
3. Doxepin
4. Imipramine
5. Amitriptyline
6. Trimipramine

- Higher ionic strength at mid pH is very effective at masking silanol interactions and reducing peak tailing.

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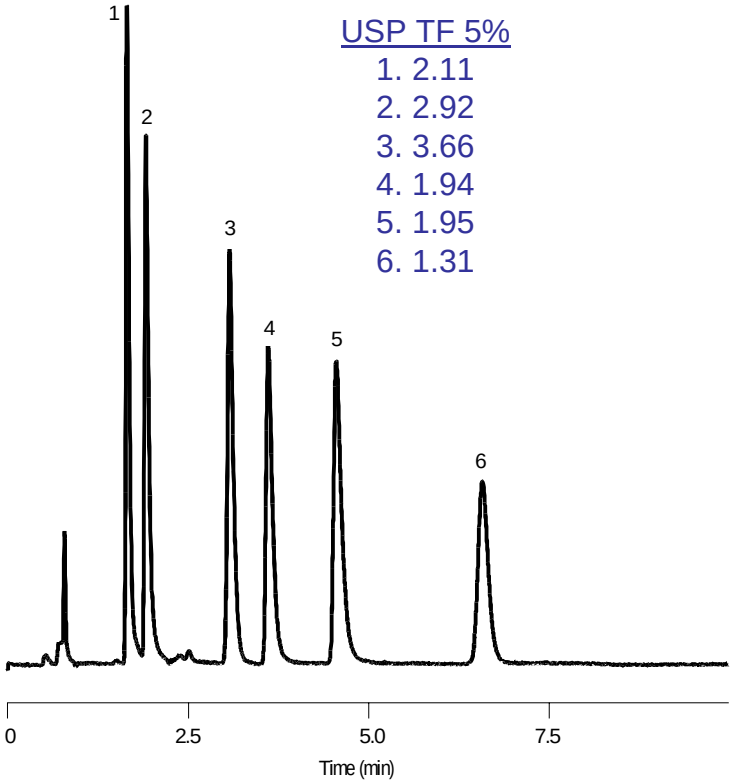
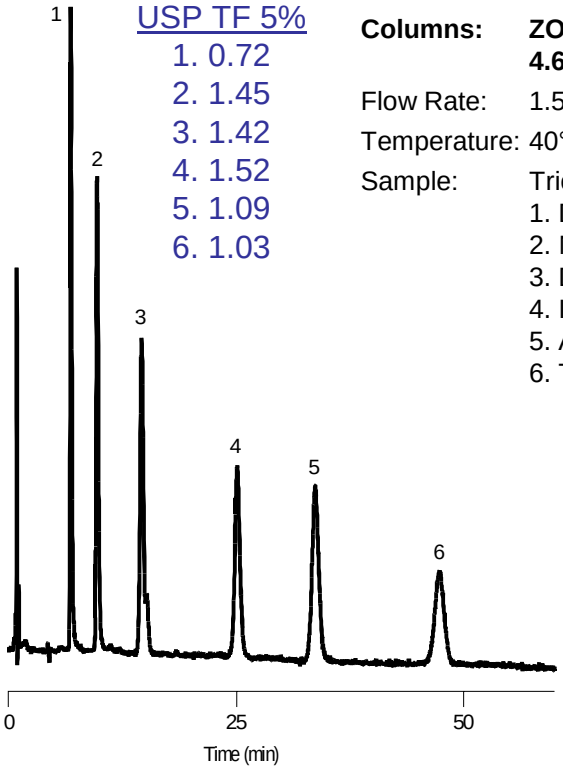
Mobile Phase Organic Modifier
Acetonitrile vs. Methanol

40% 25 mM Na_2HPO_4 pH 7.0
60% ACN



USP TF 5%
1. 2.11
2. 2.92
3. 3.66
4. 1.94
5. 1.95
6. 1.31

40% 25 mM Na_2HPO_4 pH 7.0
60% MeOH



USP TF 5%
1. 0.72
2. 1.45
3. 1.42
4. 1.52
5. 1.09
6. 1.03

Columns: **ZORBAX Eclipse XDB-C8 4.6 x 150 mm, 5 µm**
Flow Rate: 1.5 mL/min.
Temperature: 40°C
Sample: Tricyclic Antidepressants
1. Desipramine
2. Nortriptyline
3. Doxepin
4. Imipramine
5. Amitriptyline
6. Trimipramine

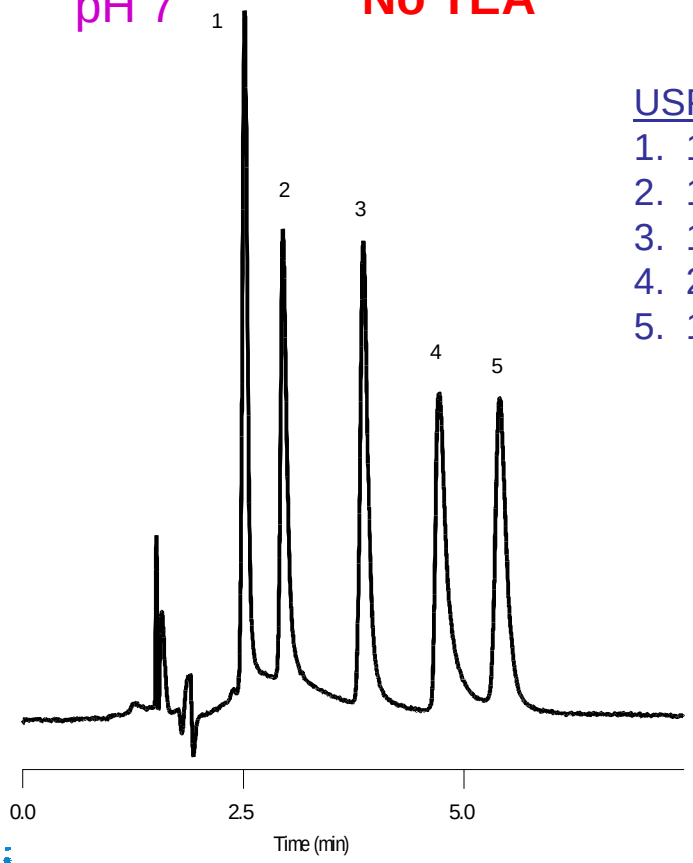
•Changing the organic modifier may improve peak shape due to secondary interactions.

Mobile Phase Modifiers – Effect of TEA on Peak Shape of Basic Compounds

Column: ZORBAX Eclipse XDB-C8, 4.6 x 150 mm, 5 µm Mobile Phase: 85% 25 mM Na_2HPO_4 : 15% ACN
Flow Rate: 1.0 mL/min. Temperature:35°C Sample: Amphetamines 1. Phenylpropanolamine 2. Ephedrine 3. Amphetamine 4. Methamphetamine 5. Phenteramine

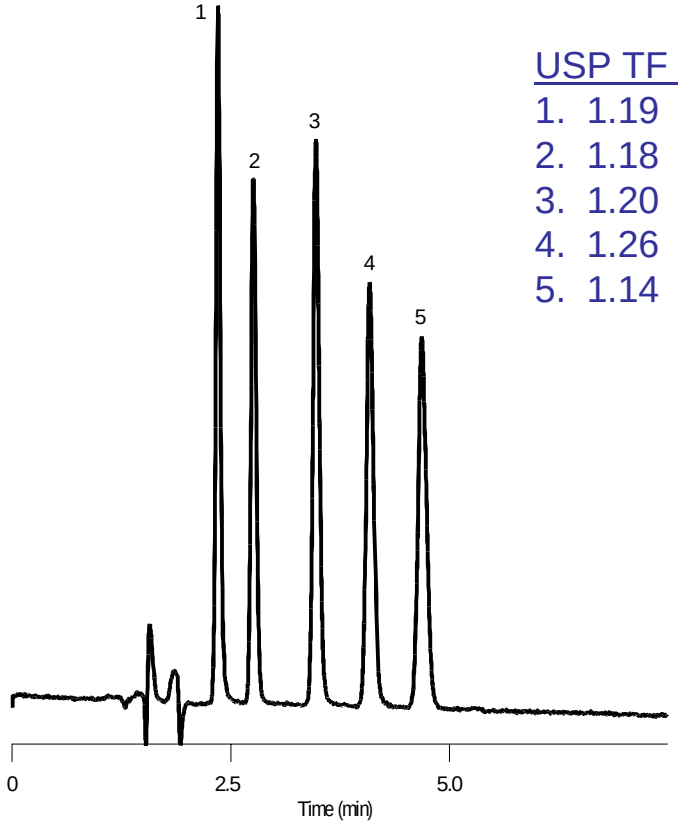
pH 7

No TEA



USP TF 5%
1. 1.29
2. 1.33
3. 1.63
4. 2.35
5. 1.57

10 mM TEA



USP TF 5%
1. 1.19
2. 1.18
3. 1.20
4. 1.26
5. 1.14

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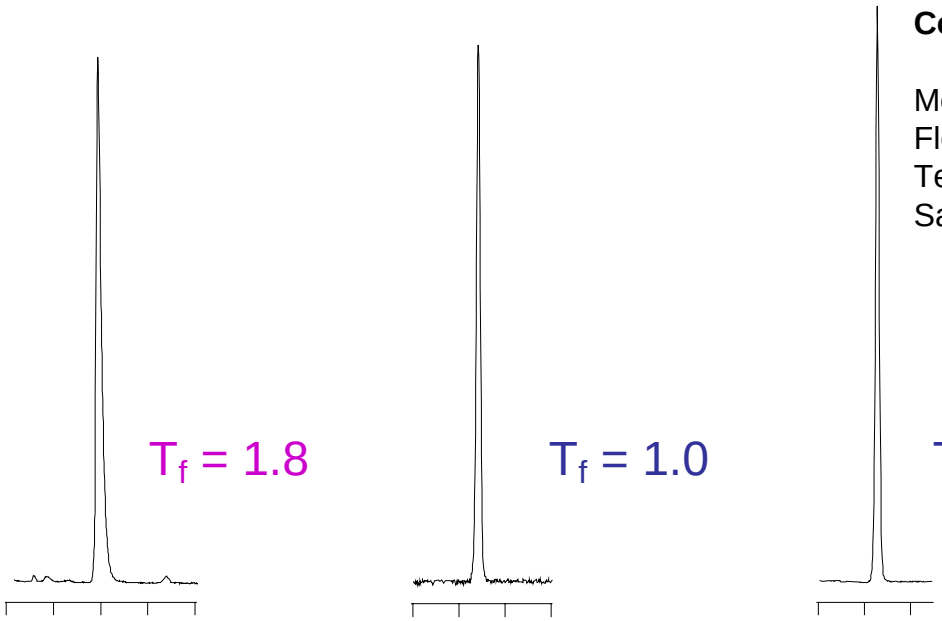
Mobile Phase Modifiers – Effects of Competing Acids on the Peak Shape of Acidic Compounds

A:

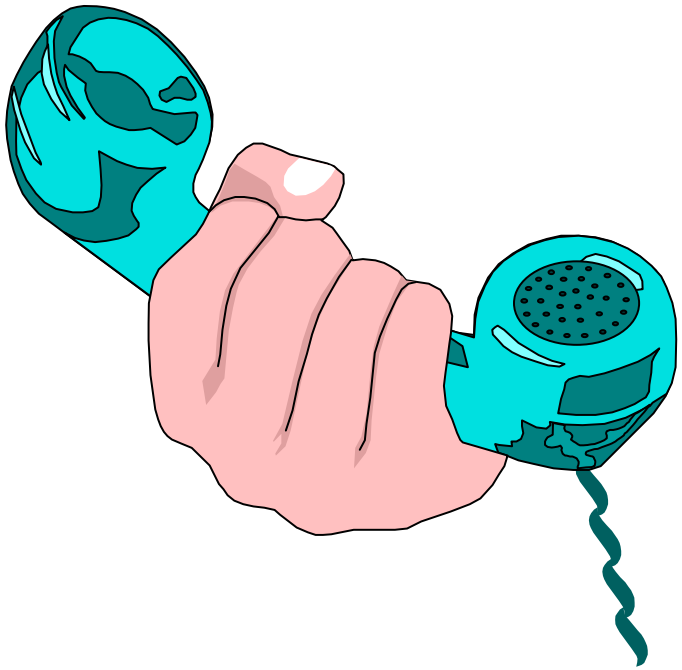
pH 3 5 mM NaH_2PO_4	pH 3 5 mM NaH_2PO_4 1% acetic acid	pH 2.5 0.1% TFA



Column:	**ZORBAX StableBond SB-C18 4.6 x 150 mm, 5 µm**
Mobile Phase:	40% A: 60% ACN
Flow Rate:	1.0 mL/min.
Temperature:	Room Temperature
Sample:	Ibuprofen pKa 4.4

- Both acetic acid and TFA (trifluoroacetic acid) act as competing acids.
- TFA can be used at a lower concentration and is the preferred choice.

Break



Sample and Additional Considerations for Good Peak Shape

- Sample overload (mass/volume)
- Sample solvent/injection solvent
- Metal complexation

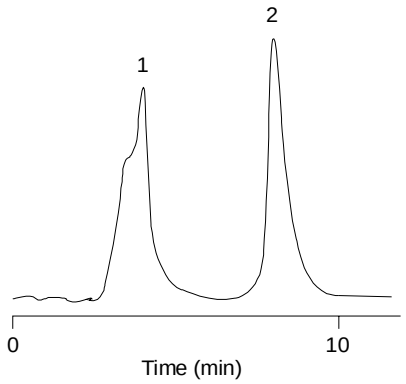
Sample Overload May Cause Peak Fronting and Tailing

- Peak fronting from sample mass overload – more sample than can effectively partition results in some sample preceding the rest of the peak
- Peak tailing from overload of silanols with basic compounds may be seen as peak tailing
- Reduce sample load to eliminate – 100 mAU for compounds with average absorptivity

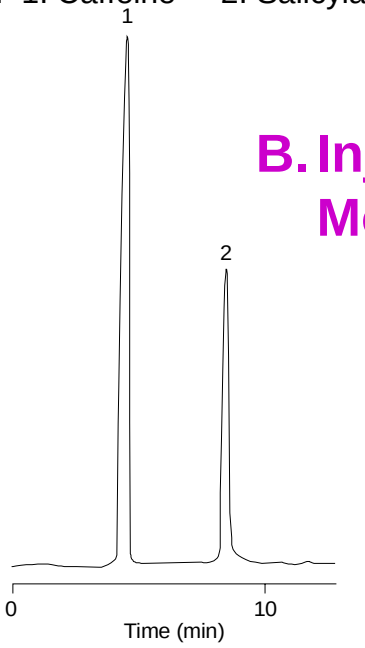
Strong Injection Solvent May Cause Poor Peak Shape

Column: ZORBAX StableBond SB-C8, 4.6 x 150 mm, 5 µm Mobile Phase: 82% H_2O : 18% ACN
Injection Volume: 30 µL Sample: 1. Caffeine 2. Salicylamide

A. Injection Solvent 100% Acetonitrile



B. Injection Solvent Mobile Phase



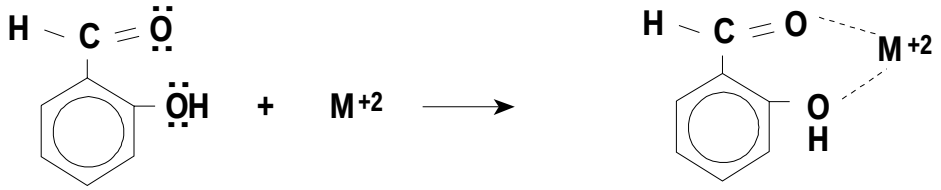
- Injecting in a solvent stronger than the mobile phase can cause peak shape problems, such as peak splitting or broadening.

Metal Complexation May Cause Poor Peak Shape

- Analytes that may complex with metals may show poor peak shape
- Both tailing and fronting may result from metal complexation
- Metals are present in LC systems from solvents, tubing, and stainless steel frits
- High purity silica eliminates silica as a source of metals

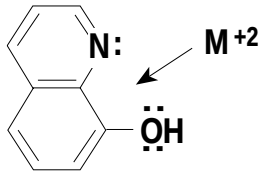
Metal Sensitive Compounds Can Chelate

Hint: Look for Lone Pair of Electrons on :O: or Ṅ
Which Can Form 5 or 6 Membered Ring with Metal

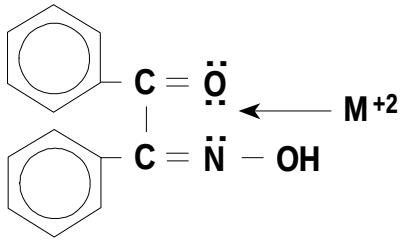


Salicylaldehyde

6-membered ring complex



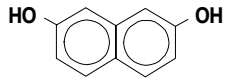
8-hydroxyquinoline 5-membered ring complex

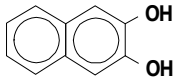


α-benzoinoxomine 5-membered ring complex

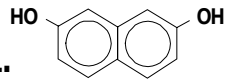
Acid Wash Can Improve Peak Shape

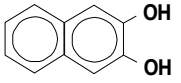
Before Acid Wash

1. 

2. 

After Acid Wash 50 – 100 mLs 1% H_3PO_4

1. 

2. 

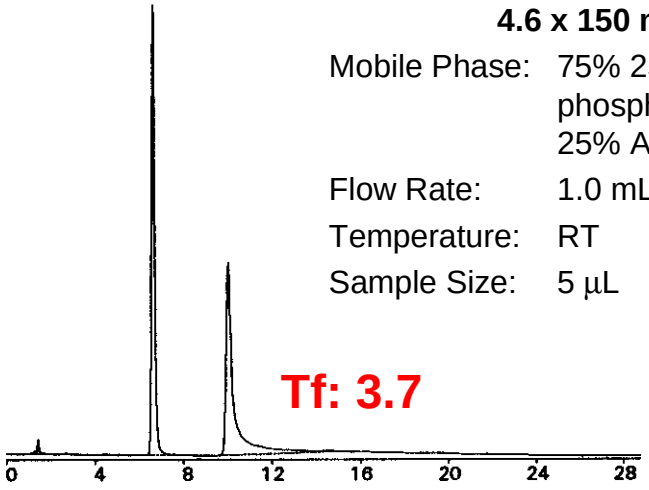
Columns: ZORBAX SB-Phenyl 4.6 x 150 mm

Mobile Phase: 75% 25 mM ammonium phosphate buffer
25% ACN

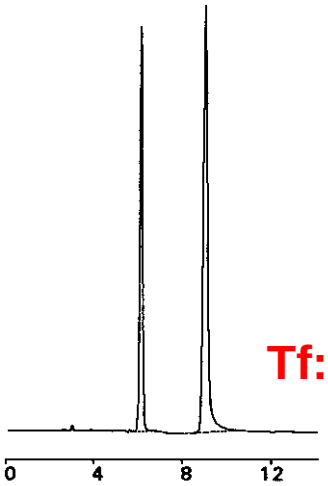
Flow Rate: 1.0 mL/min.

Temperature: RT

Sample Size: 5 µL



Tf: 3.7



Tf: 1.2

- A 1% H_3PO_4 solution is used on SB columns, 0.5 % can be used on endcapped columns.

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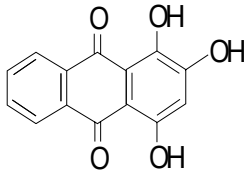
Purpurin

Column: Zorbax SB-C8, 4.6 x 250 mm, 5 µm Mobile Phase: 20% 0.02% TFA in water: 80% MeOH
Flow Rate: 1 mL/min Detection: UV 254 nm Temperature: 24°C

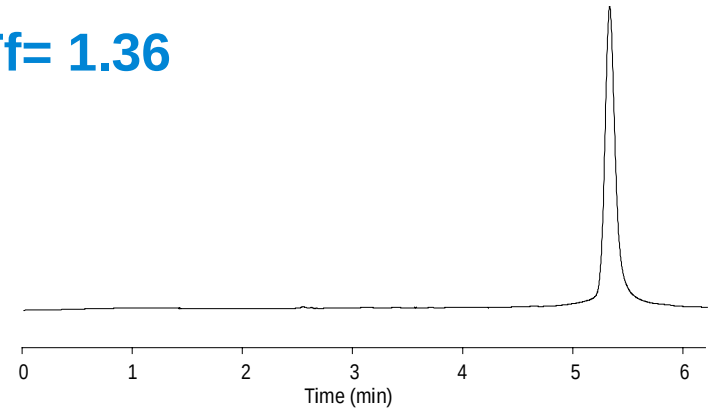
A. Before Acid Wash

B. After Acid Wash
1% phosphoric acid, 30 min.

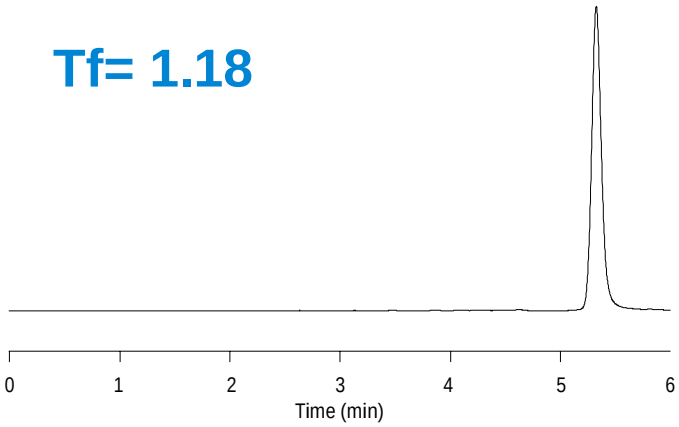
Purpurin



Tf= 1.36



Tf= 1.18



- Both fronting and tailing are evident on purpurin before the acid wash

Guidelines for Improved Peak Shape

- Select columns based on high purity fully hydroxylated silica – Zorbax Rx-Sil based columns, such as StableBond, Eclipse XDB, Bonus-RP and Extend
- Select double endcapped columns for mid pH or difficult basic compounds, such as Eclipse XDB
- Select special bonded phases (Bonus-RP, Extend-C18) for better peak shape at mid and high pH
- Select wide-pore columns for high molecular weight analytes
- Use buffered low pH mobile phases to reduce secondary interactions
- Use 25 – 50 mM buffered mobile phases at every pH
- Use additional additives only when needed
- Check sample solvents

HPLC Column Technical Support

800-227-9770 (phone: US & Canada)*
302-993-5304 (phone)*
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