Simplified Method Development

Low, Mid and High pH Recommendations

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pH – An Important Parameter for Method Development

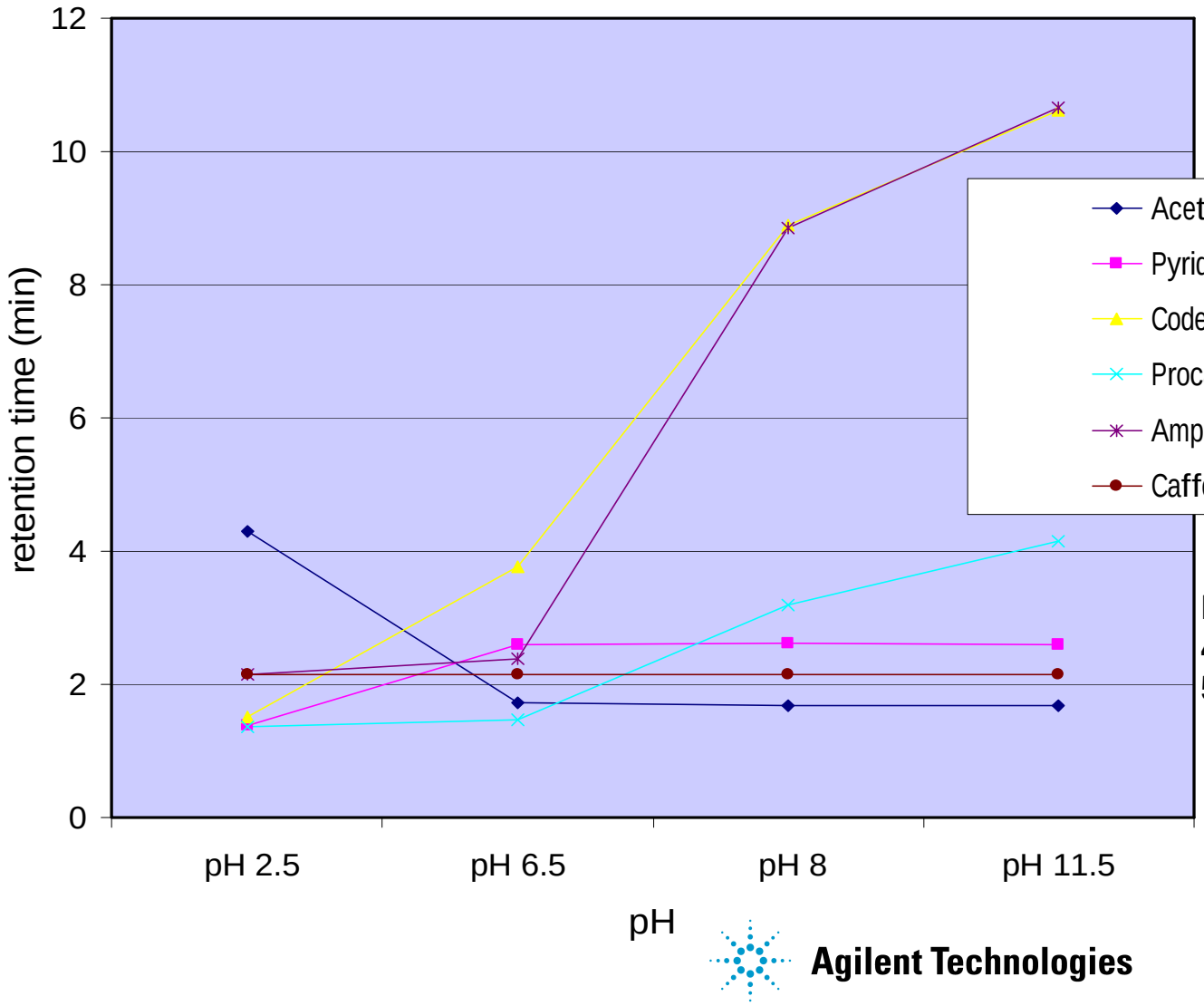
Retention of ionizable compounds is strongly affected by pH

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



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

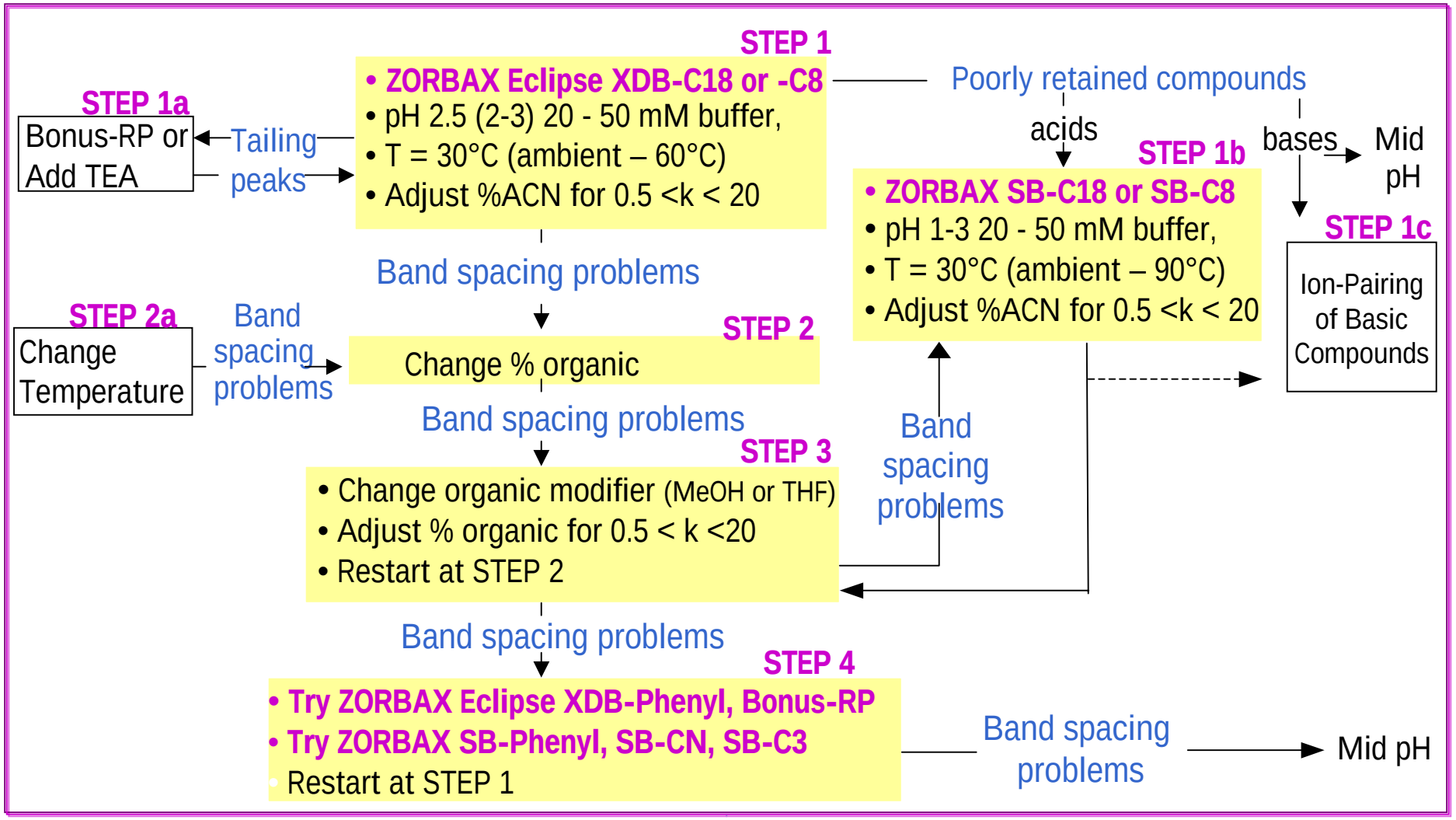
Silica silanols are protonated thereby minimizing ion-exchange interactions with basic compounds

- Good peak shape
- Long term reproducibility

Excellent mobile phase choices

Method Development Scheme

Start at Low pH



Recommended Starting Conditions for RP-HPLC Method Development Approach

Separation Variable	Preferred Initial Choice
Column	
Primary Stationary Phase	Eclipse XDB-C18 or Eclipse XDB-C8
Secondary Stationary Phase	SB-C18 or SB-C8
Dimensions	4.6 x 75 mm or 4.6 x 150 mm
Particle Size	3.5 µm 5 µm
Pore Size	80Å: M.W. ≤ 4000, 300Å: M.W. ≥ 4000
Mobile Phase	
Solvents A-B	Water-acetonitrile
% B solvent	Variable
Buffer	25 mM NaH_2PO_4, 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

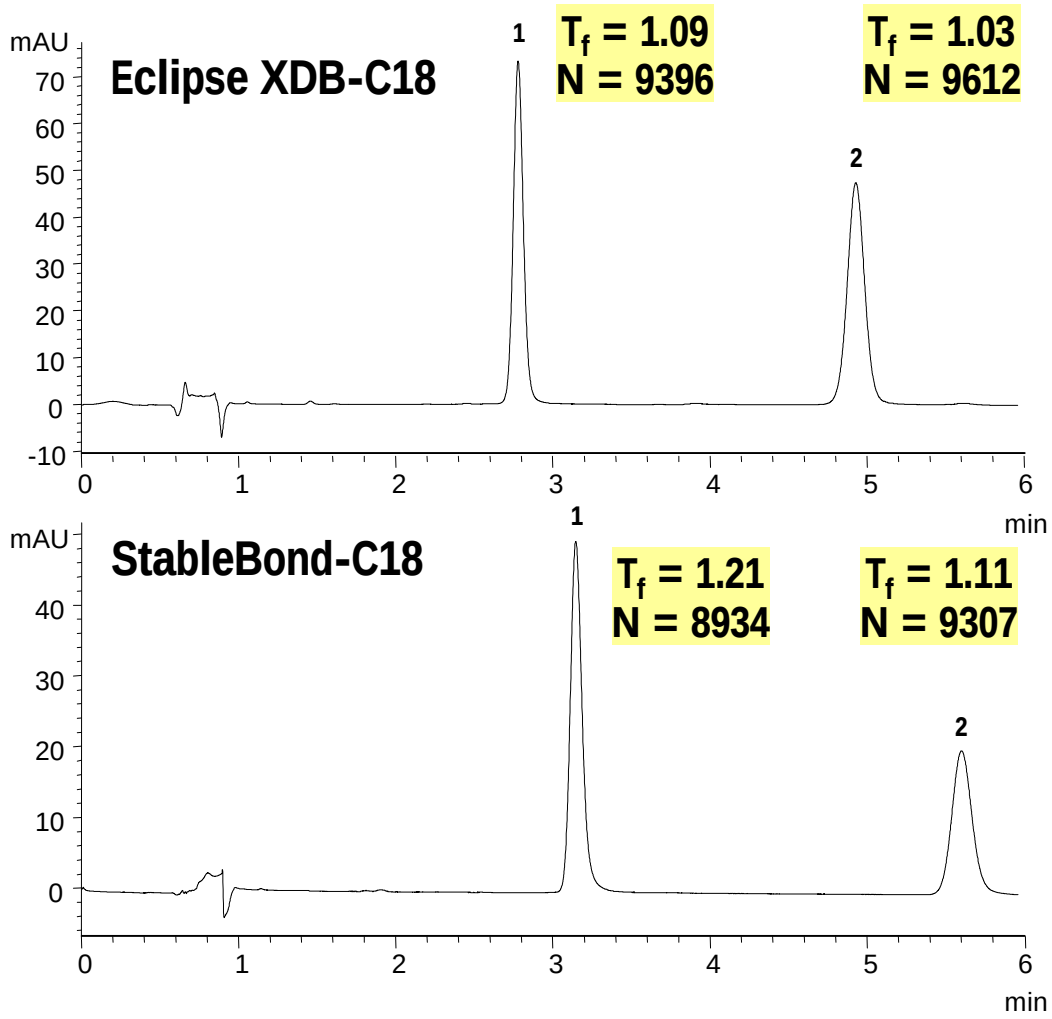
Wide useable pH range – pH 2- 9 – can use for low and mid pH without changing columns

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

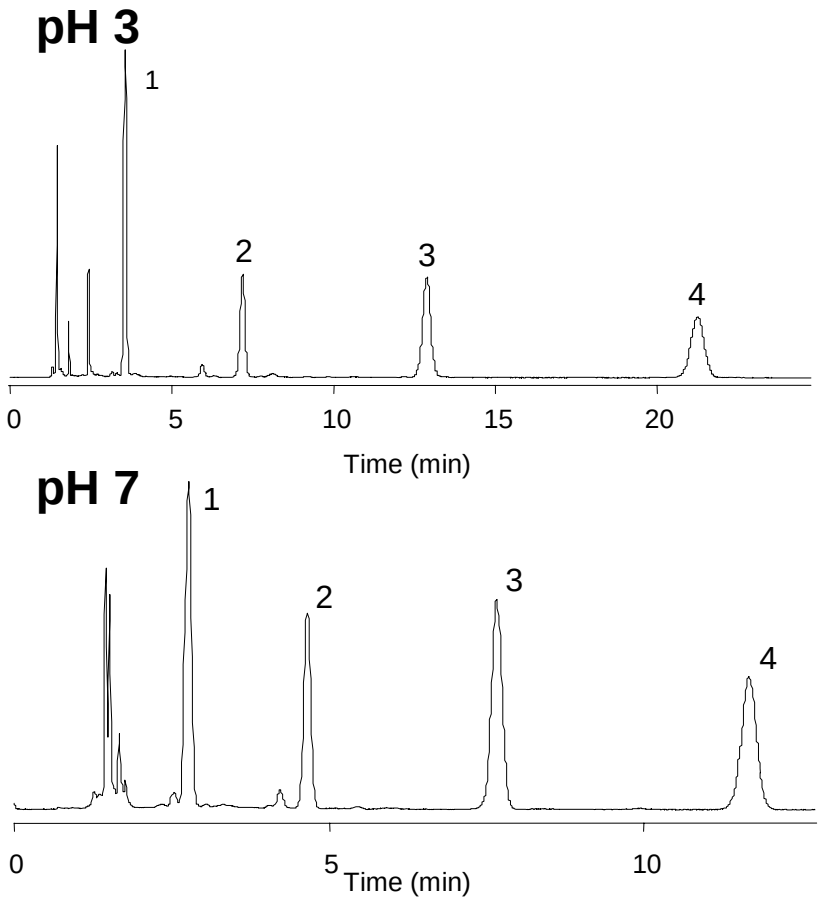
Method Development – Eclipse XDB-C18 at Low pH - Separation of Flavones



Conditions:

Column:	4.6x75mm, 3.5µm
Temp:	Ambient
Eluent:	30% ACN 70% 25mM NaH_2PO_4 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



3 Lots % RSD		
	k'	α
1)	3.1	-
2)	2.6	0.56
3)	2.8	0.29
4)	2.5	0.34

3 Lots % RSD		
	k'	α
1)	5.4	-
2)	5.1	0.41
3)	4.6	0.59
4)	4.8	0.00

Conditions:

Column: Eclipse XDB-C8
4.6x150mm, 5µm

Temp: 35ºC

Eluent: 15% ACN
85% 25mM Phosphate

Flow: 1.0 mL/min

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

StableBond –Secondary Choice at Low pH

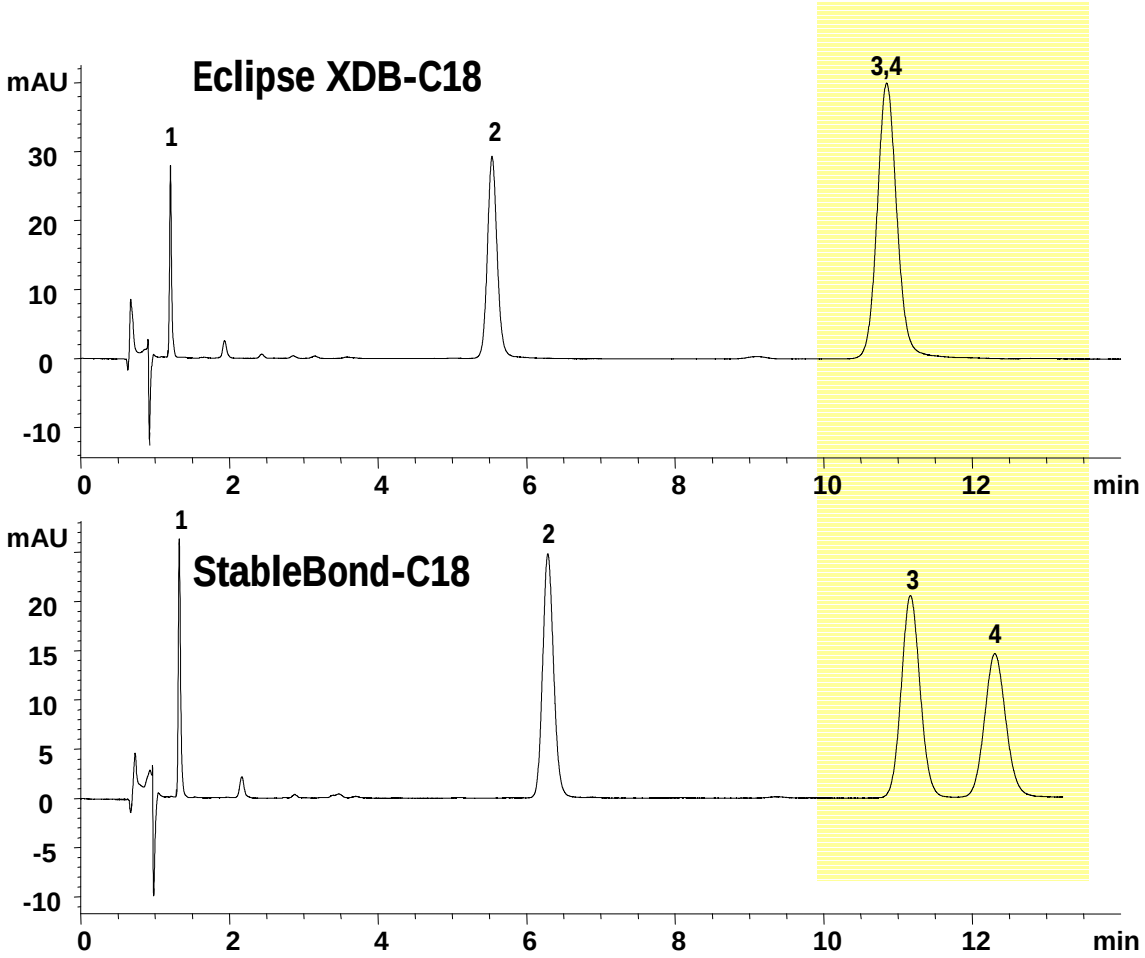
Superior column lifetime at very low pH – down to pH 1 – due to patented sterically protecting bonding technology

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 NaH_2PO_4
	pH 2.5
Flow:	**0.75 mL/min**
Inj. Vol.:	**20 µL**

Sample:
1) Caffeic Acid
2) Luteolin
3) Naringenin
4) Apigenin

DAD1 A, Sig=254,40 Ref=360,100 (021000\LY000018.D)
DAD1 A, Sig=254,40 Ref=360,100 (021000\LY000017.D)

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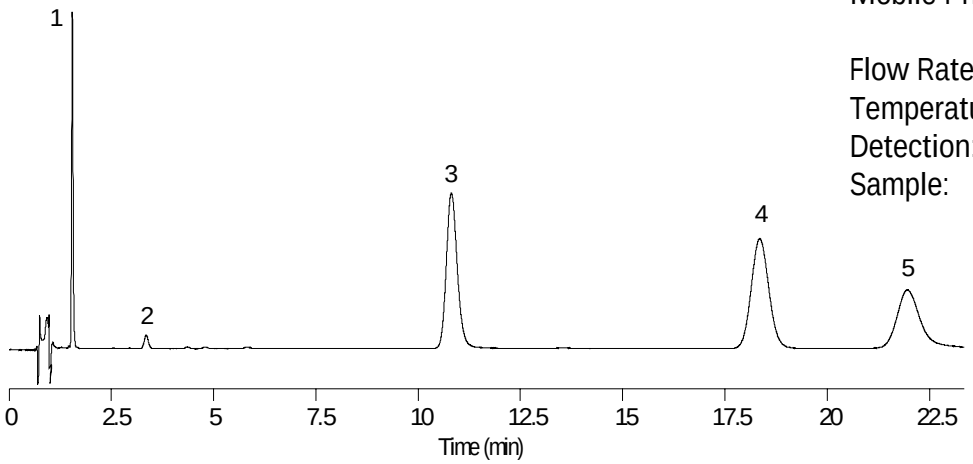
Method Development – SB-C18 at Low pH Separation of Plant Extract

Flavones, Flavanones, and Phenolic Esters

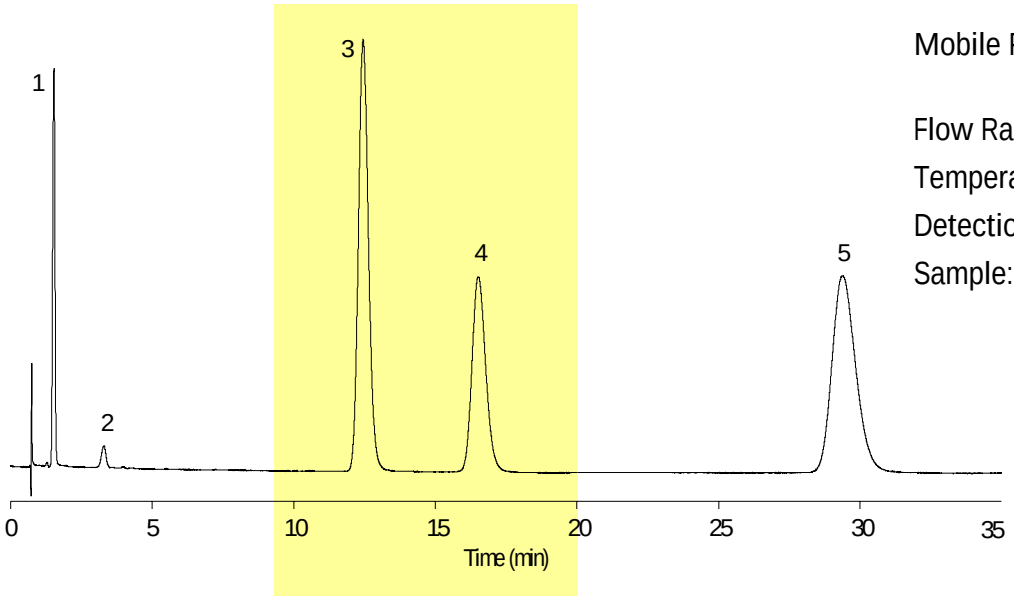


Column: **ZORBAX Rapid Resolution SB-C18 4.6 x 75 mm, 3.5 µm**
Mobile Phase: 22% ACN
78% NaH_2PO_4, pH 2.5
Flow Rate: 1.0 mL/min
Temperature: RT
Detection: UV 254 nm
Sample:
1. Caffeic acid
2. Impurity
3. Luteolin
4. Naringenin
5. Apigenin

- 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



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

Mobile Phase: 40% MeOH
60% NaH_2PO_4, 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

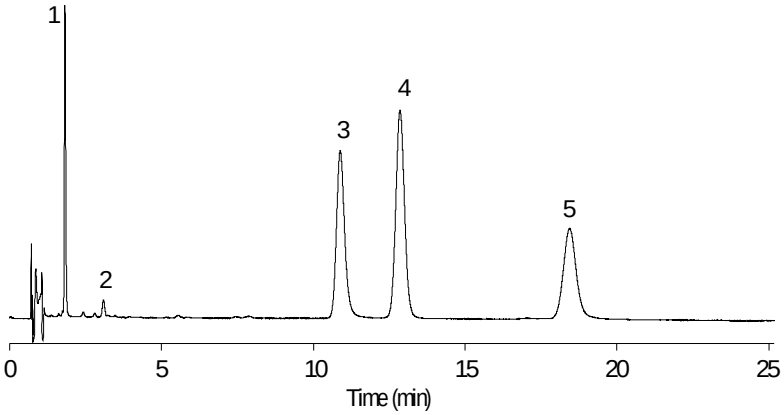
- Methanol as the organic modifier changes selectivity and increases the analysis time.

Method Development – Change Bonded-Phase Separation of Plant Extract on SB-CN

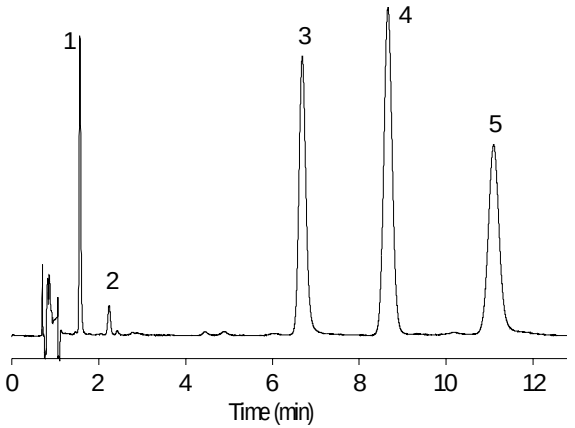
Flavones, Flavanones, Phenolic Esters

Column: ZORBAX Rapid Resolution SB-CN, 4.6 x 75 mm, 3.5 µm Mobile Phase: ACN: NaH_2PO_4, 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

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.

StableBond 300SB Columns Ideal for Separations of High MW Analytes

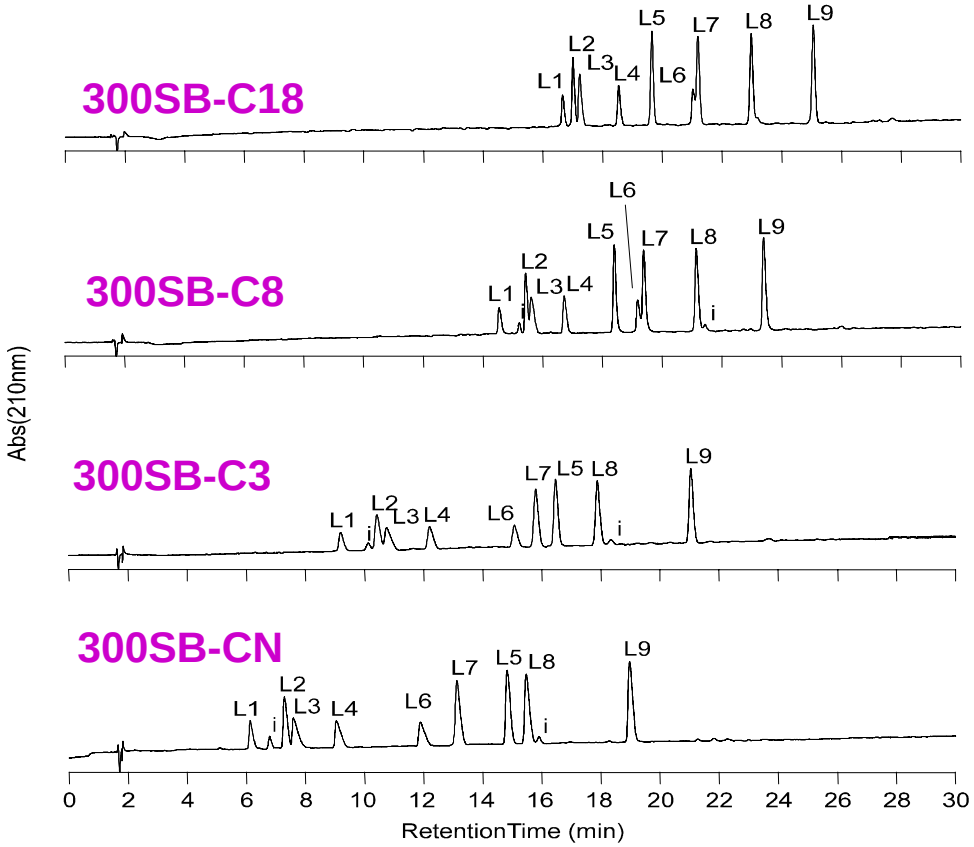
300Å pore size necessary for good peak shape and high efficiency separation of proteins and polypeptides.

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?

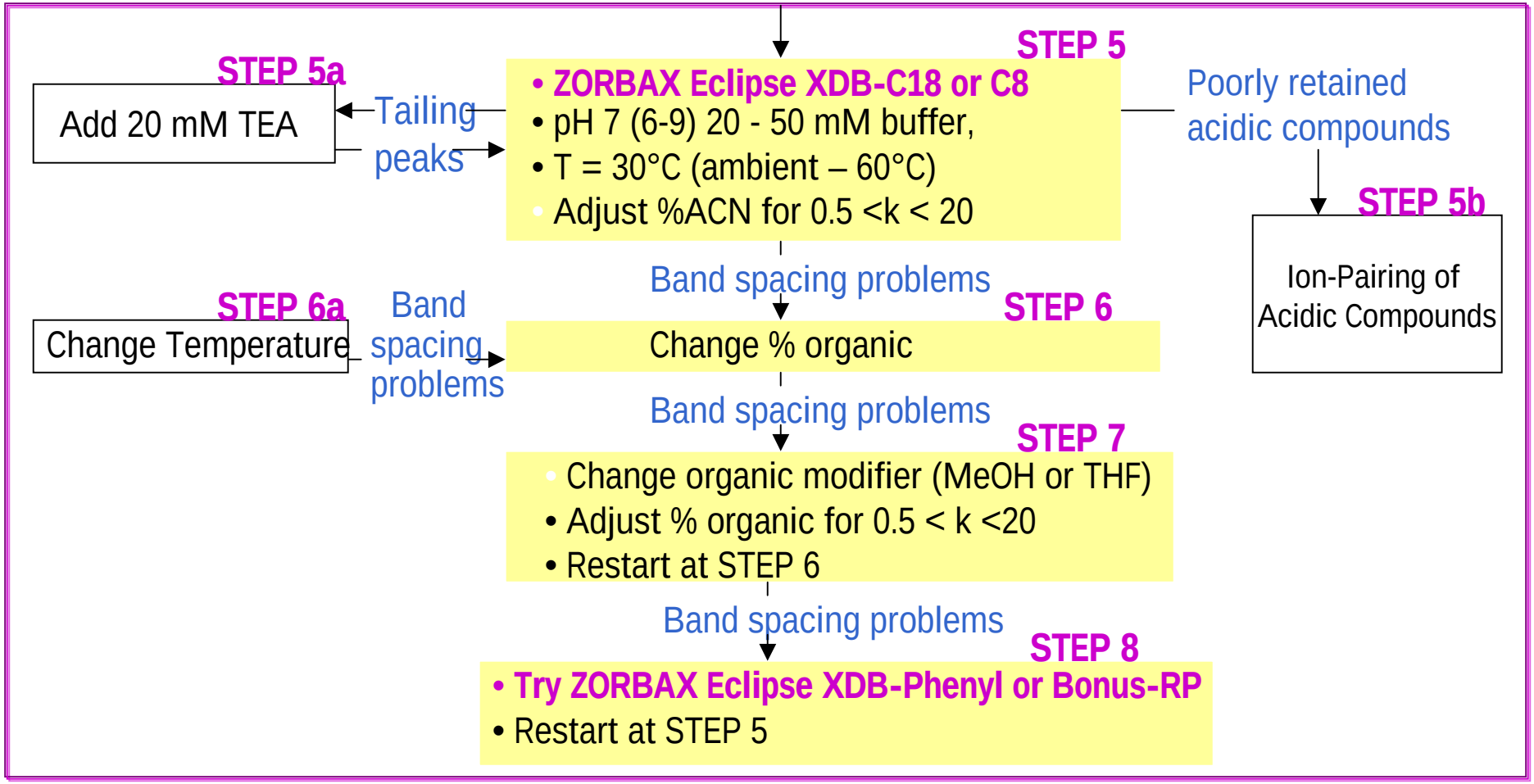
Compounds of interest are unstable at low pH

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



Recommended Conditions for Mid-pH Method Development

Separation Variable	Preferred Initial Choice
Column	
Stationary Phase	Eclipse XDB-C18
Dimensions	4.6 x 75 mm or 4.6 x 150 mm
Particle Size	3.5 µm 5 µm
Mobile Phase	
Solvents A-B	Water-acetonitrile
% B solvent	Variable
Buffer	25 mM Na_2HPO_4, 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

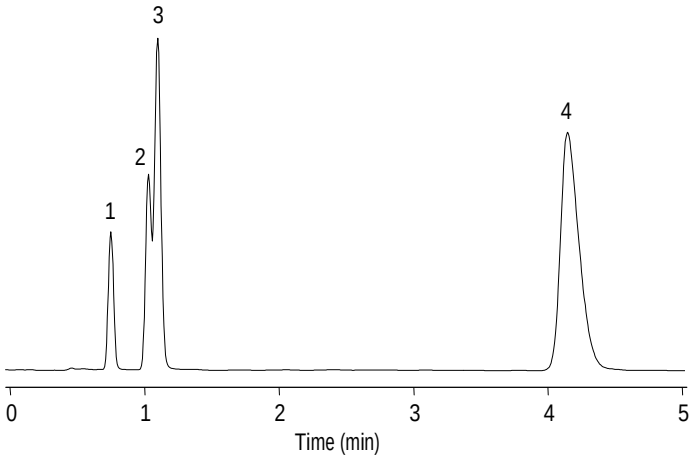
Double endcapping provides excellent peak shape

Three different bonded-phases (C18, C8, Phenyl) for selectivity optimization

Low pH Provides Insufficient Selectivity for Some Samples

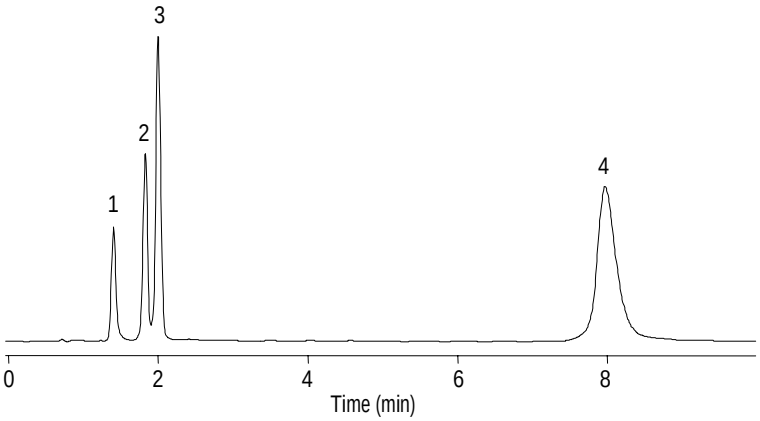
Eclipse XDB-C18, 4.6 x 75 mm, 3.5 µm

pH 3



SB-C18, 4.6 x 75 mm, 3.5 µm

pH 3



Mobile Phase: 20% Methanol: 80% 20 mM phosphate buffer Flow Rate: 1.0 mL/min Temperature: RT
Detection: UV 254 nm Sample: 1. Nizatidine 2. Famotidine 3. Cimetidine 4. Pirenzipine

- Poor Selectivity with Eclipse at Low pH
- Selectivity Improves Somewhat with StableBond

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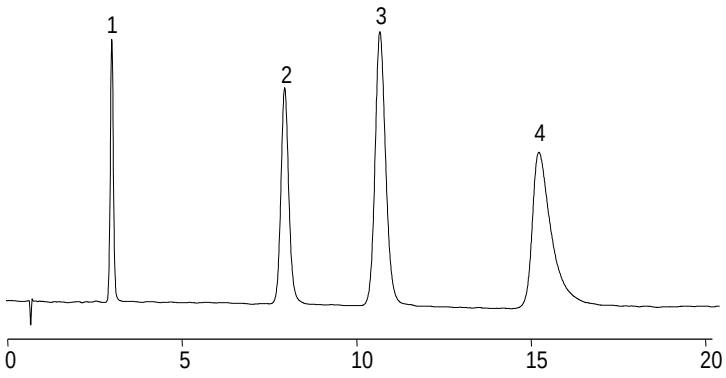
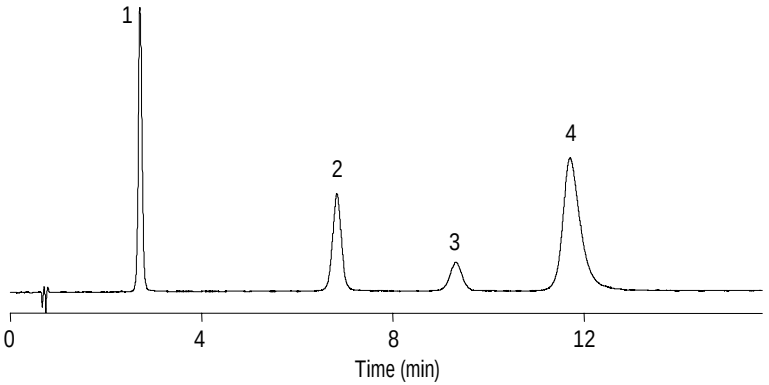
Improved Selectivity and Resolution with Eclipse XDB-C18 at Mid-pH

Eclipse XDB-C18, 4.6 x 75 mm, 3.5 μm

pH 7
No TEA



pH 7
10 mM TEA

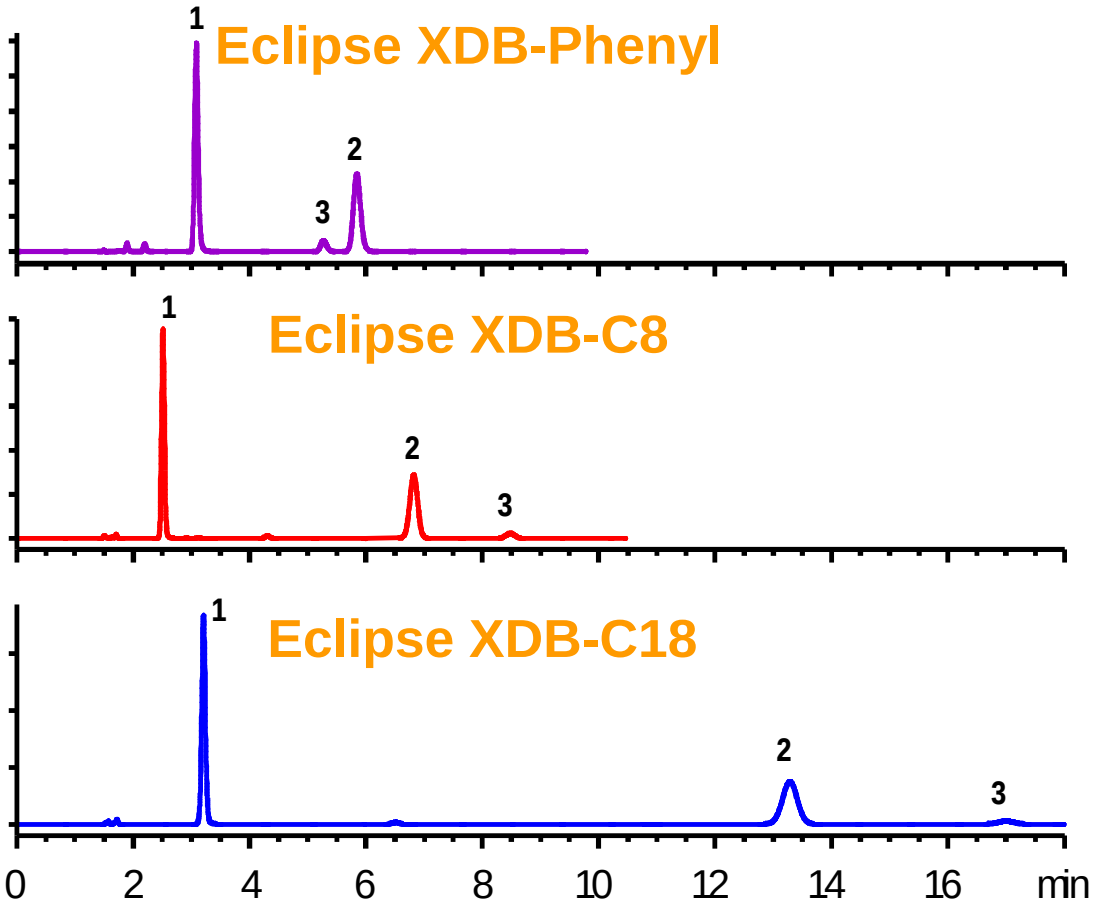


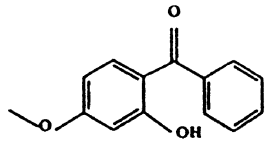
Mobile Phase: 20% Methanol: 80% 20 mM phosphate buffer Flow Rate: 1.0 mL/min Temperature: RT
Detection: UV 254 nm Sample: 1. Nizatidine 2. Famotidine 3. Cimetidine 4. Pirenzipine

- Better selectivity and improved retention occur at pH 7
- A little TEA also improves peak shape peak #4 (di-amine)

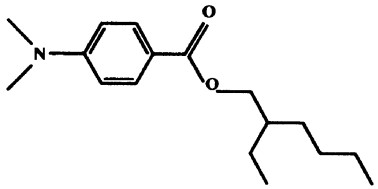
Optimize Separations with Eclipse XDB Selectivity Options – Analysis of Sunscreens

Columns: 4.6 x 150 mm, 3.5 µm Mobile Phase: 15% H_2O: 85% MeOH Flow Rate: 1.0 mL/min Temperature: 30°C
Detection: UV 310 nm Sample: 1. Oxybenzone 2. Padimate-O 3. Ethylhexylsalicylate

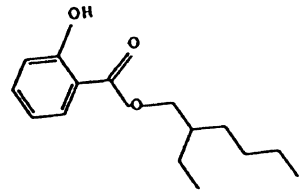




1. oxybenzone



2. padimate O



3. ethylhexyl salicylate

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Bonus-RP
Provides Alternate Selectivity at Mid-pH

Polar alkyl-amide bonded-phase for unique selectivity

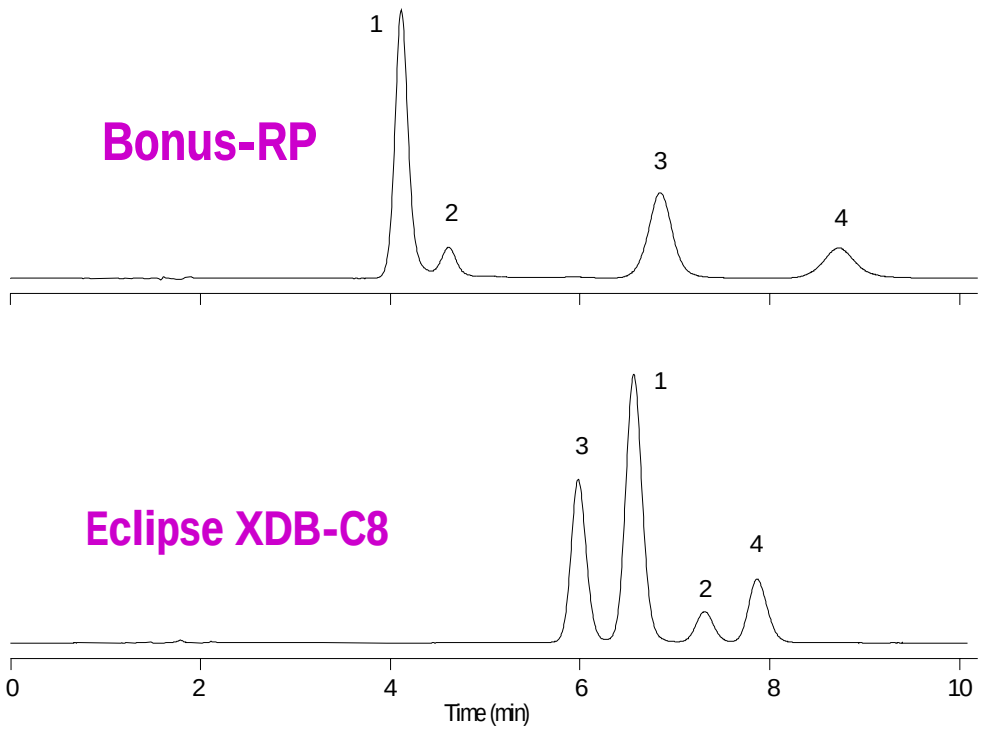
Improves peak shape of basic compounds

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?

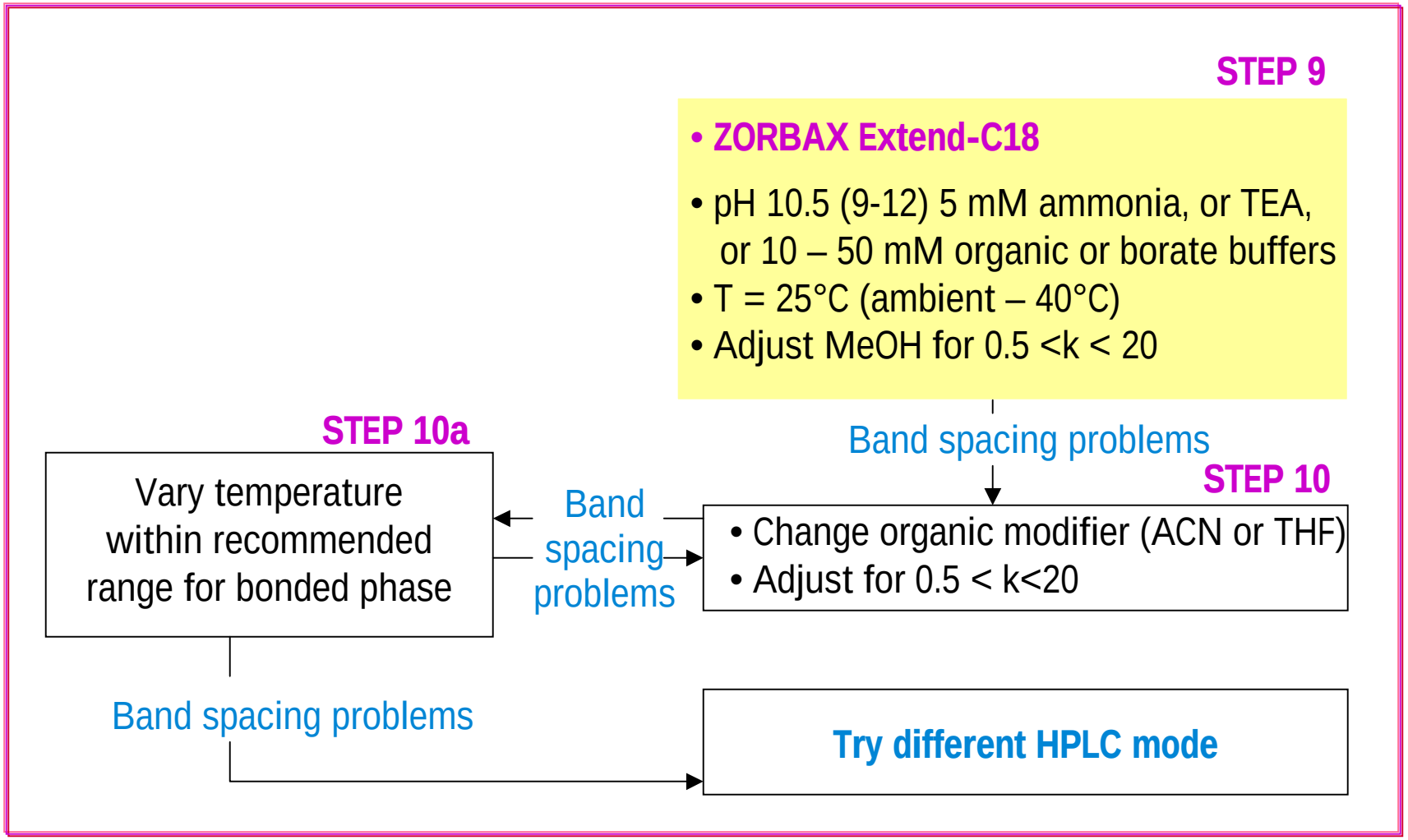
Compounds of interest not soluble at lower pH

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



Recommended Conditions for High pH Method Development

Separation Variable	Preferred Initial Choice
Column	
Stationary Phase	Extend-C18
Dimensions	4.6 x 75 mm or 4.6 x 150 mm
Particle Size	3.5 µm 5 µm
Pore Size	80Å: M.W. ≤ 4000, 300Å: M.W. ≥ 4000
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

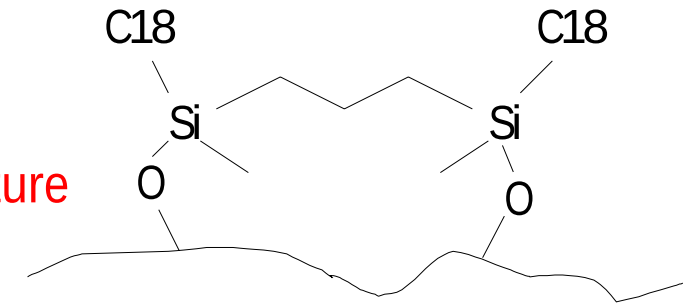
Patented bidentate C18-C18 bonding for superior high pH stability – up to pH 11.5

Improved performance over polymeric columns

Excellent peak shape with double endcapping

LC / MS at high pH (ammonium hydroxide) with high efficiency

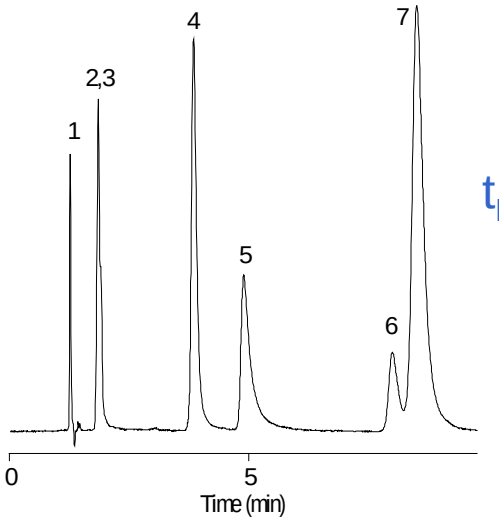
Extend-C18
Bidentate Structure



High pH Increases Retention of Antihistamines

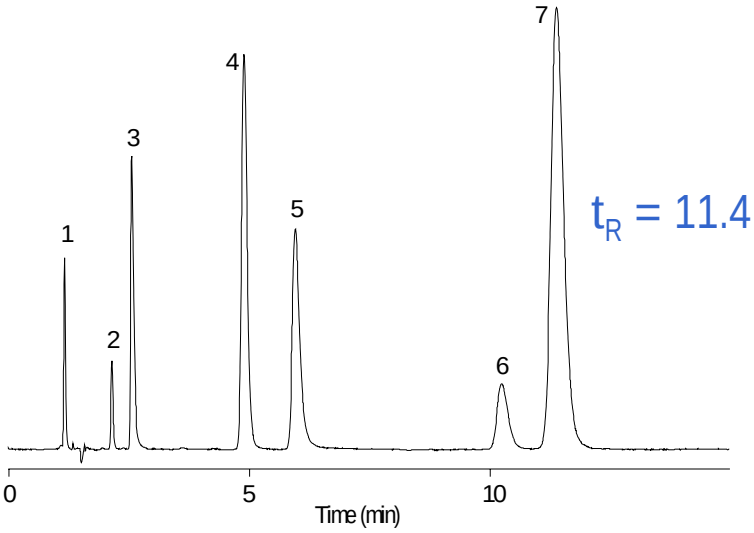
1. Maleate 2. Scopolamine 3. Pseudoephedrine 4. Doxylamine 5. Chlorpheniramine 6. Triprolidine 7. Diphenhydramine

pH 7
30% 20 mM Na_2HPO_4
70% MeOH



t_R = 8.5

pH 11
30% 20 mM TEA
70% MeOH



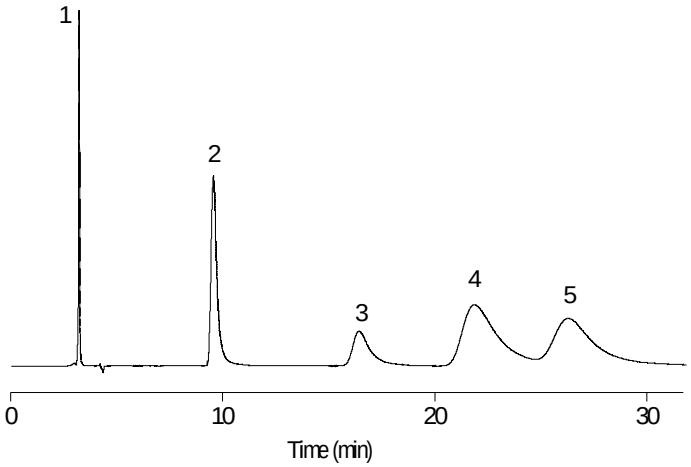
t_R = 11.4

The retention of this sample of basic compounds increases at high pH.

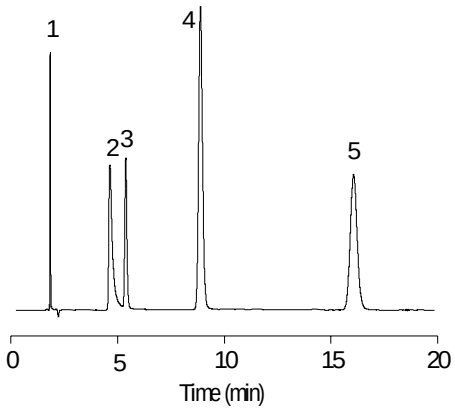
Extend-C18 Provides High Efficiency and Good Peak Shape

Mobile Phase: 65% 20 mM TEA, pH 11: 35% MeOH Temperature: RT Detection: UV 254 nm
Sample: 1. Pyridoxine 2. Pyridine 3. n-Methylbenzylamine 4. Procainamide 5. n-Acetylprocainamide

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



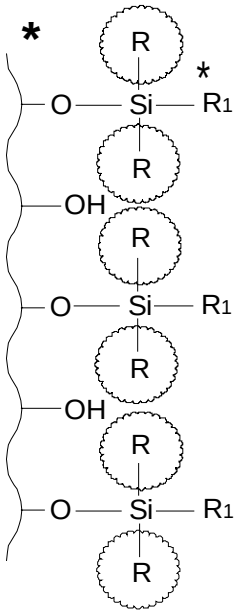
Extend-C18
4.6 x 250 mm, 5 µm
Flow Rate: 1.0 mL/min



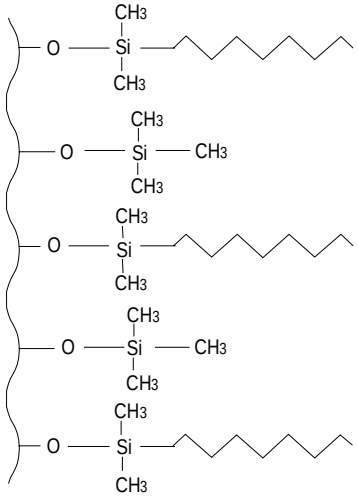
In comparison to polymeric columns, the Extend-C18 has superior efficiency and peak shape

Match Method pH and Column Choice
Choose the Best Bonded-Phase for Each pH Range

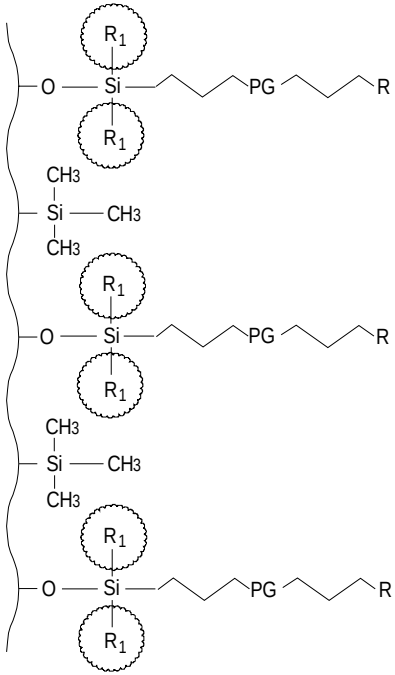
StableBond, pH 1-6
Use at Low pH
1. Uses bulky silanes
2. Non-endcapped



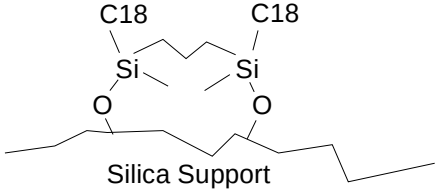
Eclipse XDB, pH 2-9
First choice at Low and Mid pH
1. eXtra Densely Bonded dimethylalkylsilanes
2. proprietary double-endcapping



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



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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.

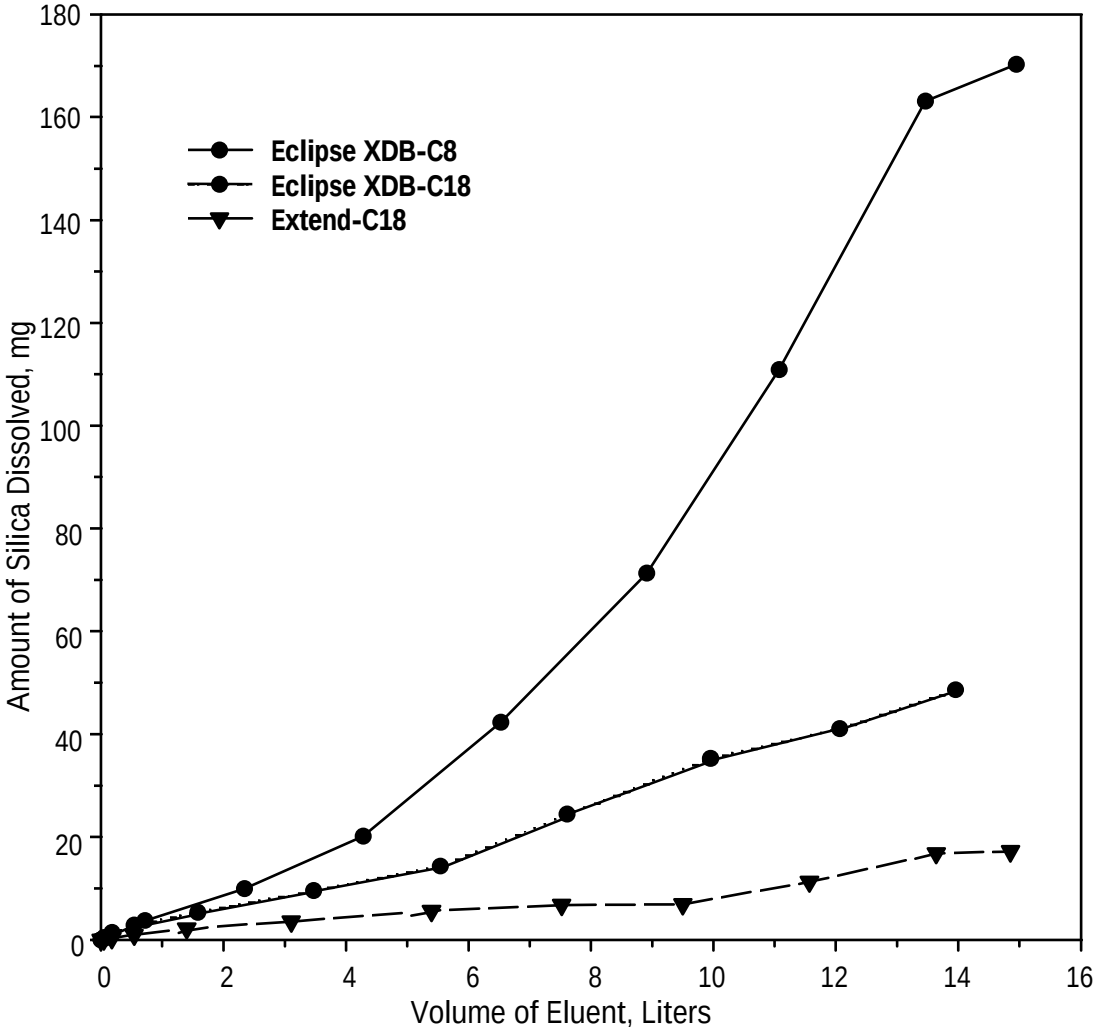
Low pH	**Eclipse XDB then StableBond**
Mid pH	**Eclipse XDB or Bonus-RP**
High pH	**Extend-C18**

Appendix

Recommended Buffer Choices for High pH

Buffer	pKa	Effective pH range
Pyrrolidine	11.3	10.3 – 12.3
Triethylamine (TEA)	10.7	9.7 – 11.7
1-methyl-piperidine	10.3	9.3 – 11.3
glycine	9.8	8.8 – 10.8
TRIS	8.1	7.1 – 9.1
Borate	9.2	8.2 – 10.2
Ammonia	9.2	8.2 – 10.2
Diethylamine	10.5	9.5 – 11.5

Good Lifetime of Extend-C18 at High pH



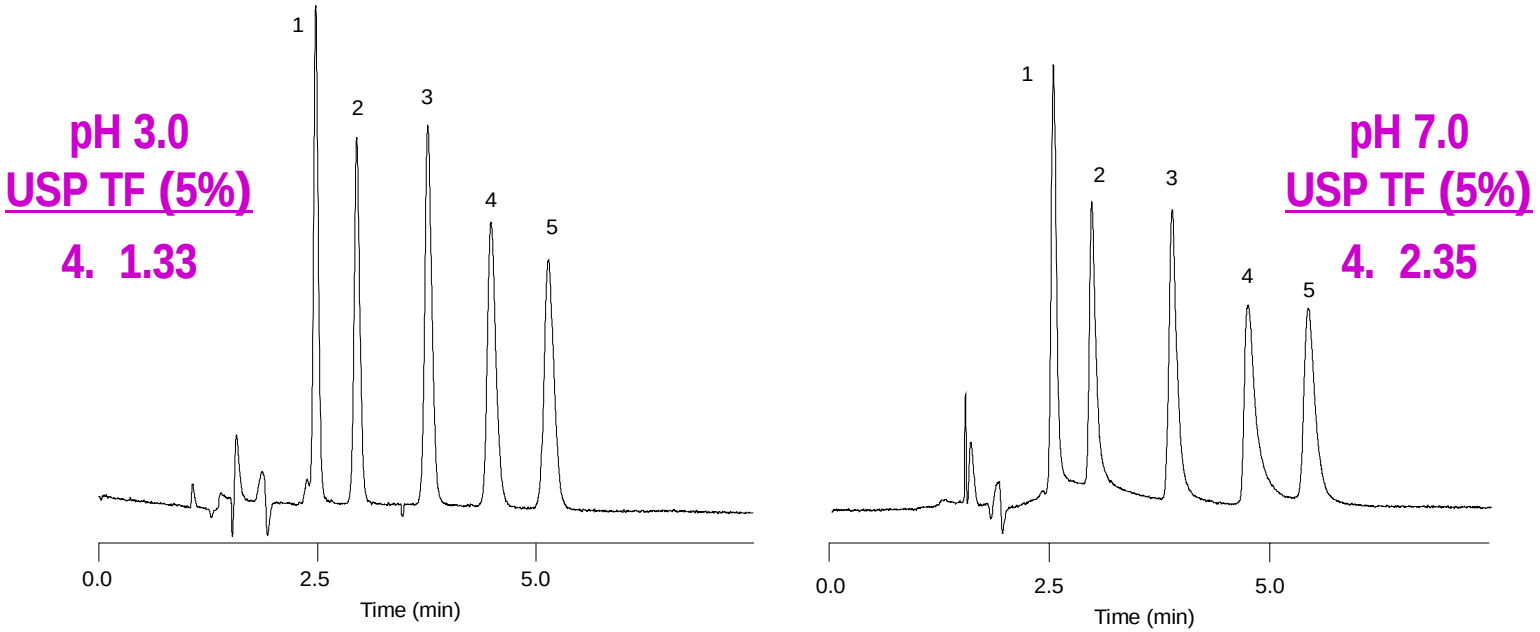
Columns:	4.6 x 150 mm, 5 µm
Purge:	50% ACN / 50% 0.02 M K_2HPO_4, pH 11
Flow Rate:	1.5 mL/min
Temperature:	25°C
Detection:	Silicate concentration by silicomolybdate color reaction

001369P1.PPT

Agilent Technologies

Reduce Column "Secondary Interactions" at Low pH

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

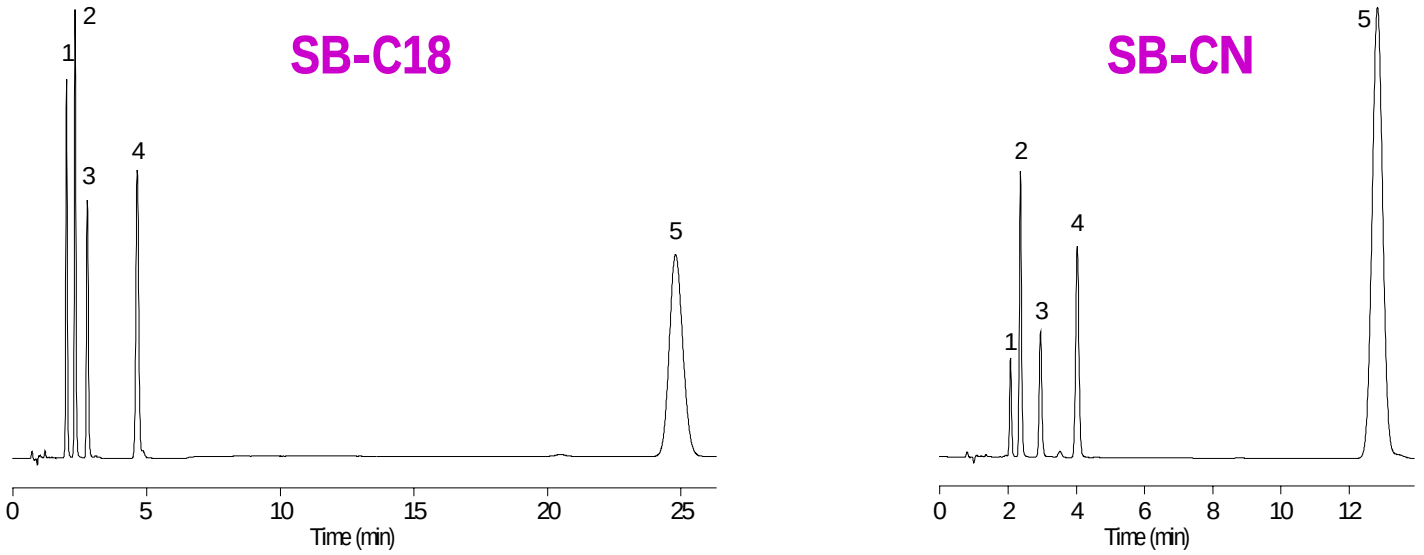


- Reducing the mobile phase pH reduces interactions with silanols that cause peak tailing.

SB-CN Optimizes Retention and Resolution

Phytoestrogens and Isoflavones

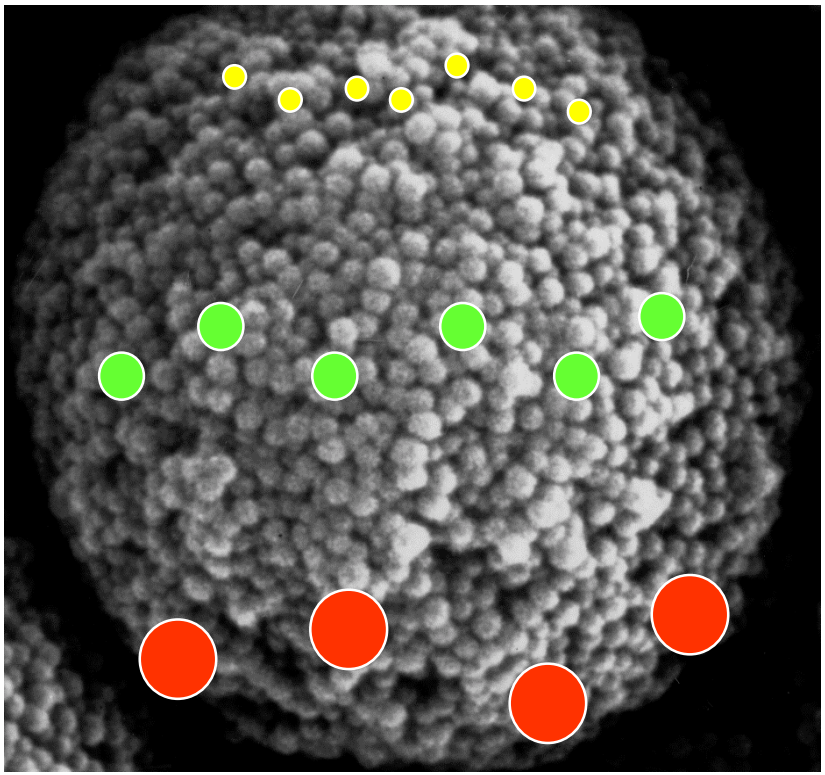
Columns: 4.6 x 75 mm, 3.5 µm Mobile Phase: 30% ACN: 70% NaH_2PO_4, pH 2.5 Flow Rate: 1.0 mL/min
Temperature: 35°C Sample: 1. Estriol 2. Daidzen 3. Quercetin 4. Genistein 5. Diethylstilbestrol



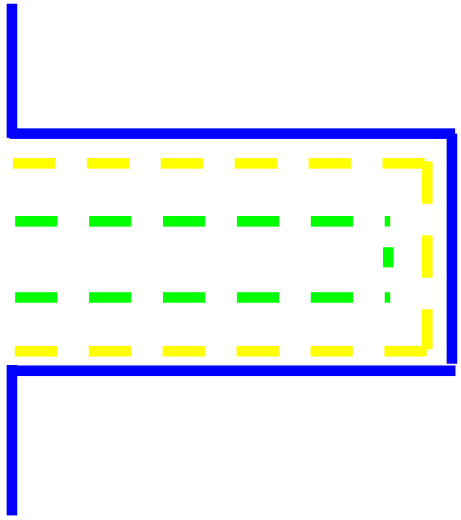
SB-CN reduces analysis time by 50% and increases retention of early eluting peaks.

Method development procedure followed to get to this point.

Why Choose 300Å Columns?



(A,B) Enter Pores

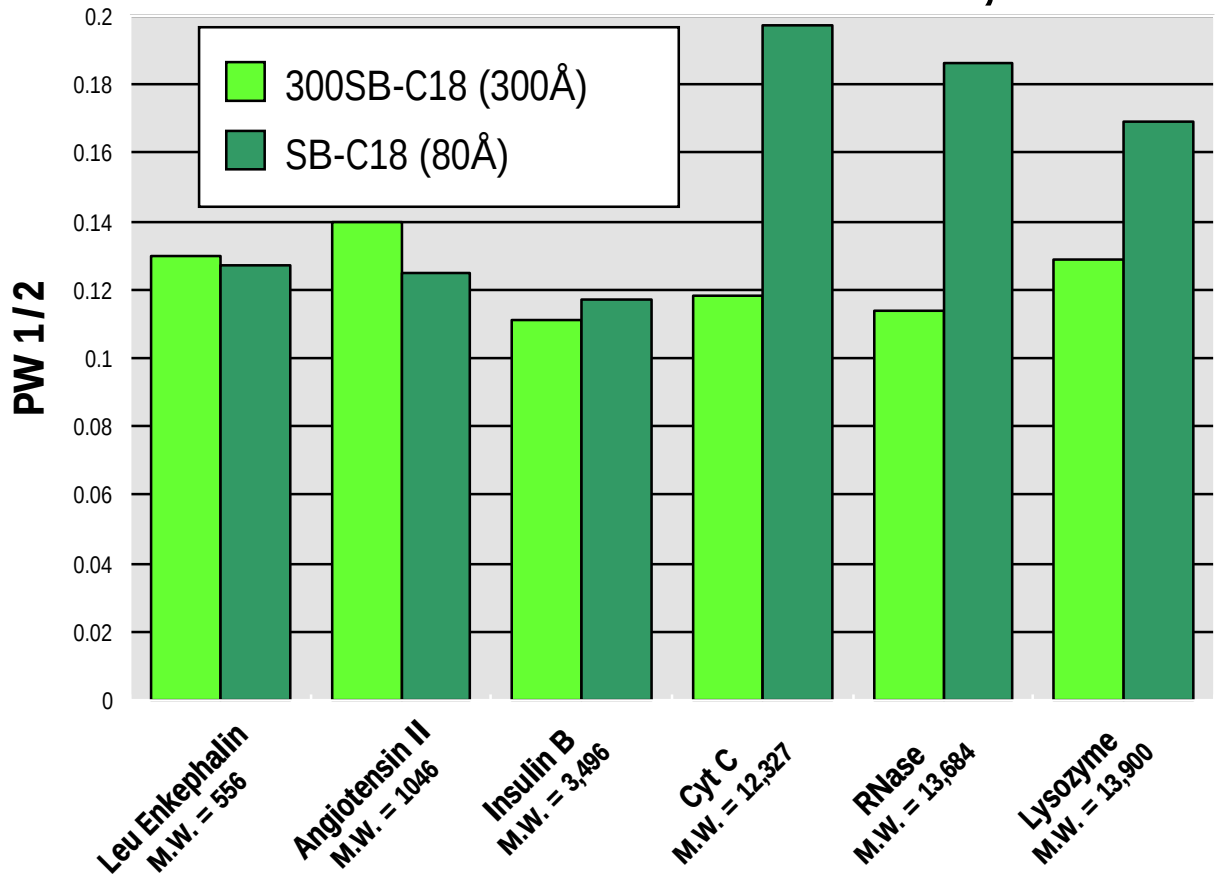


(C) Will be Excluded

Molecules must enter pores to interact with bonded-phase.
Molecules must freely enter and exit pores to maximize efficiency.

Why Choose 300Å Columns?

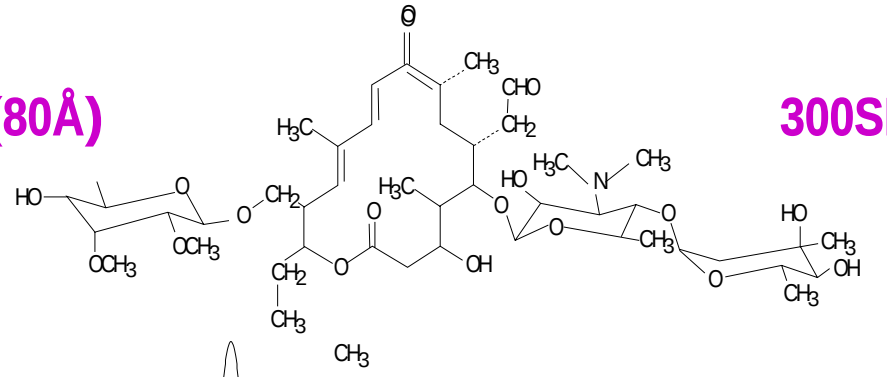
Effect of Pore Size and Molecular Size on Peak Width, Gradient Separations



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

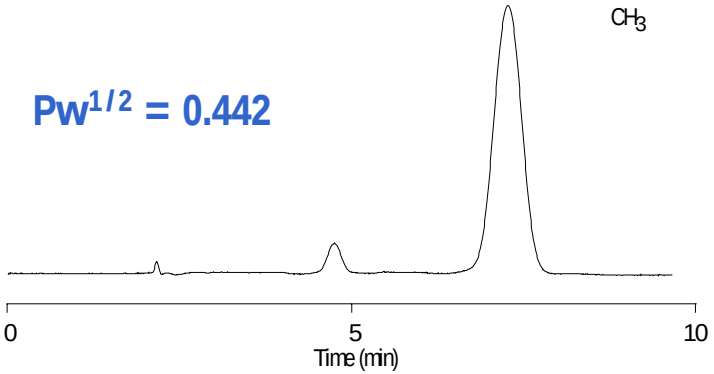
Improved Peak Shape for Large Molecules in Solution

Columns: 4.6 x 150 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)



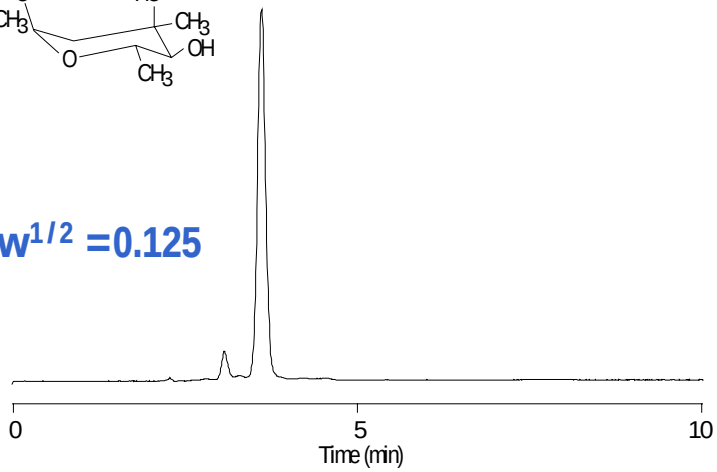
SB-C3 (80Å)

$Pw^{1/2}$ = 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.

Method Development Scheme
Start at Low pH