Rapid Optimization of Speed and Resolution in HPLC Method Development

Exploiting Both Physics and Chemistry

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The Objective

Demonstrate a systematic approach to method development with a rapid path to success.

Method Development Goals

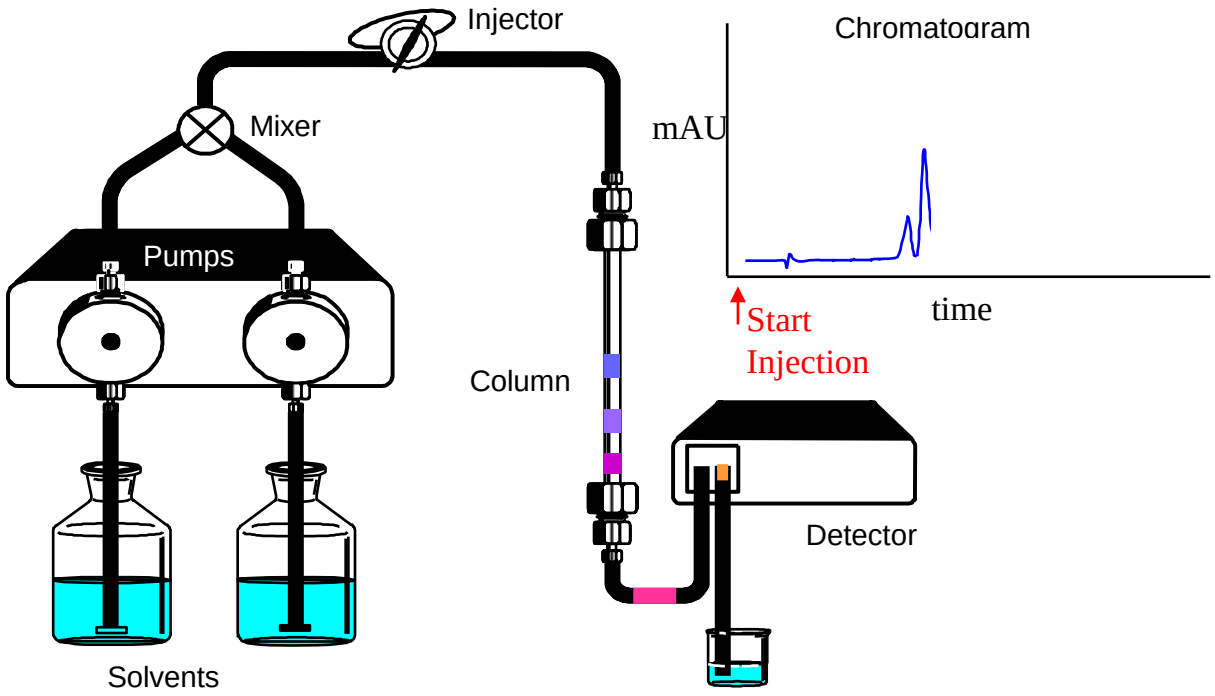
Adequate resolution of all peaks, Rs $\geq$ 2.0

Retention of first peak preferred to be at least k=1

Analysis time 2 - 10 minutes preferred

For Ionizable Compounds Use Buffered Mobile Phases

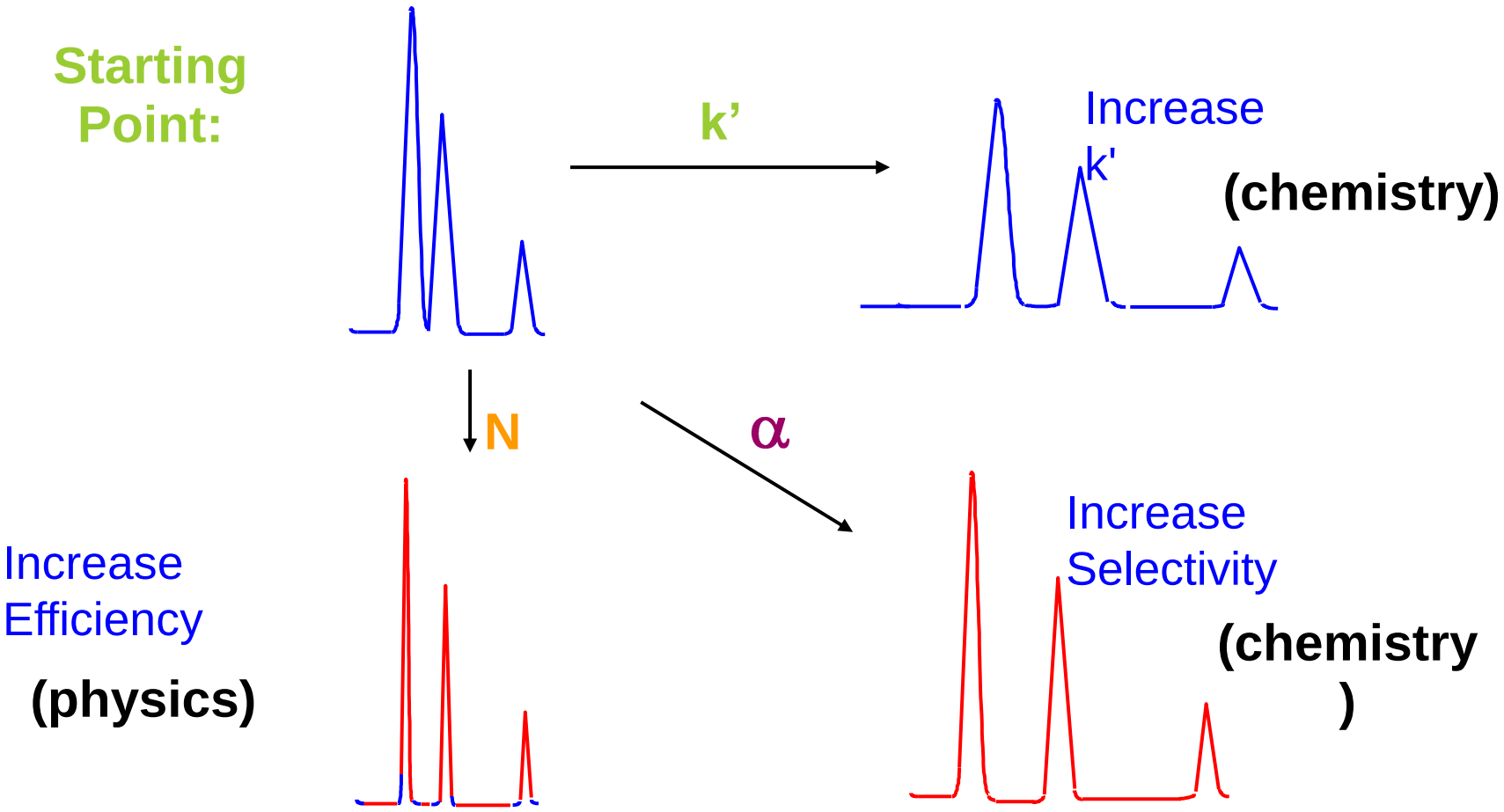
Separation in HPLC



"Separation is the art and science of maximizing separative transport relative to dispersive transport"

J. Calvin Giddings, "Unified Separation Science", John Wiley Publ, 1991, Chapt 1.

Increasing Resolution, R

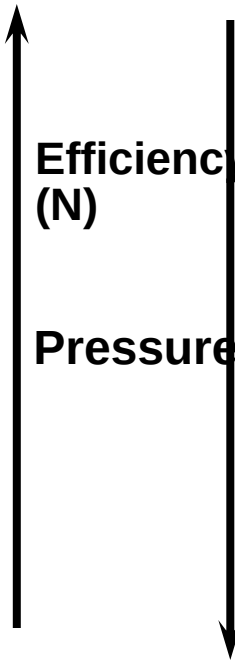


Increased Efficiency

It’s just physical!

Smaller Particle/Short Columns Provide Efficiency of Larger Particle/Longer Columns

Column Length (mm)	Resolving Power N(5 µm)	Resolving Power N(3.5 µm)	Resolving Power N(1.8 µm)	Analysis Time*
150	12,500	21,000	N.A.	-
100	8,500	14,000	N.A.	-33%
75	6000	10,500	N.A.	-50%
50	4,200	7,000	12,000	-67%
30	N.A.	4,200	6,500	-80%
15	N.A.	2,100	2,500	-90%



Analysis Time
Peak Volume
Solvent Usage

* Reduction in analysis time compared to 150 mm column

- **Rapid Resolution HT columns provide the efficiency of longer columns used for h**

N.A. = not available

Presentation Overview

- Particle Synthesis and Surface Chemistry
  - Process
  - Why pH Affects Surface Chemistry
  - Effects on Bonded Phase Reproducibility

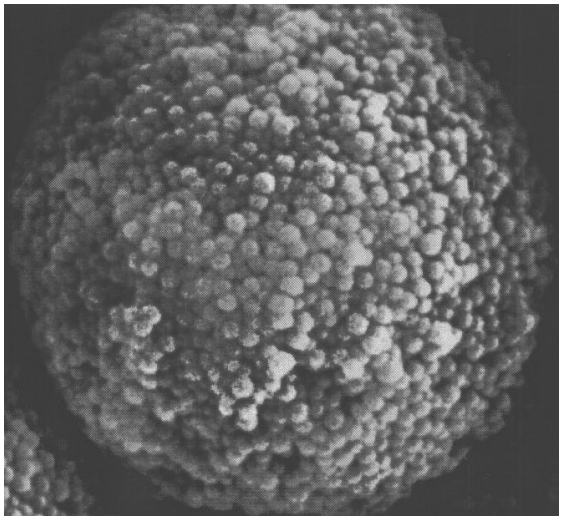
Bonded Phase Chemistry and Surface Structure
  - Surface Coverage
  - Sample interaction

Improving Sample Interaction
  - Altering Bonded Phase
  - Pore Size Isn't Just About Molecular Size
  - Does Carbon Load Matter?
  - Separation Exploiting Sample Shape or "Planarity"

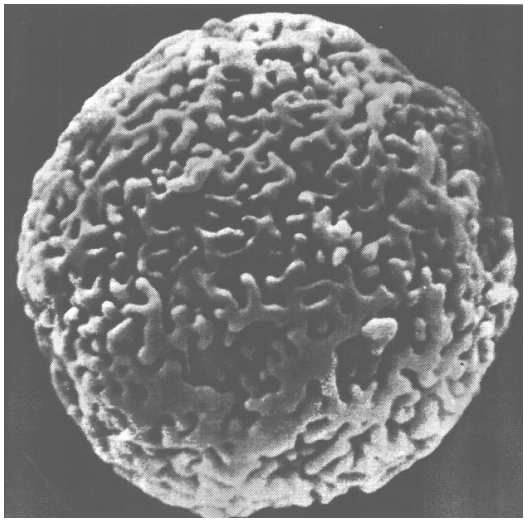
- Tailoring Column and Particle Dimensions to Suite Laboratory Needs
  - If you really Need It You Can Use
  - Faster Columns
  - Longer, High Plate Count Columns

Silica Particles Can Be Made by Different Methods

Silica Sol Aggregation

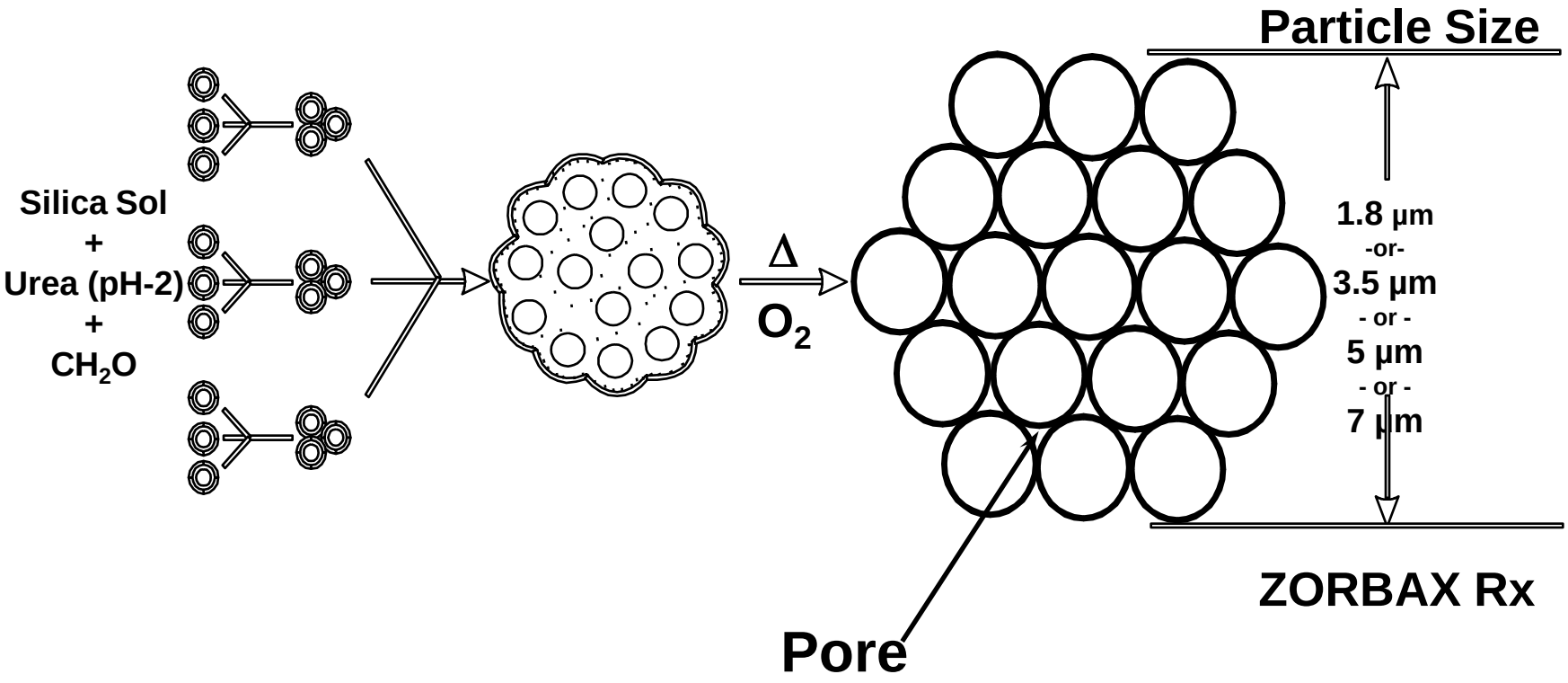


Xerogel



- **The small sol particles that come together to form a larger spherical particle are clearly visible in silica-sol particle.**
- **The silica sol particle will have thicker walls for a more rugged particle.**
- **The more "spongy" structure of particles made by the xerogel process that essentially involves precipitation from a silica solution orsuspension**

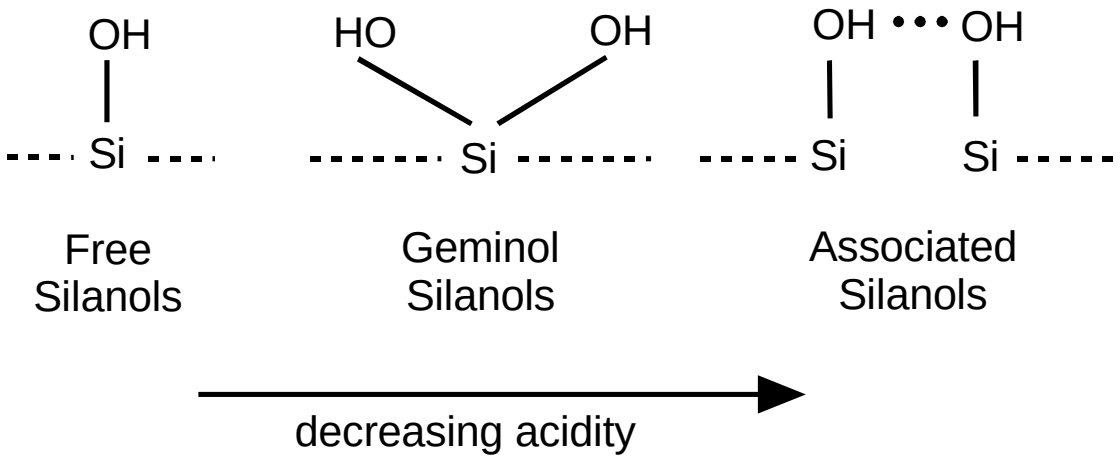
ZORBAX® Porous Silica Microsphere (PSM) Manufacturing Process- *Sol Aggregation*

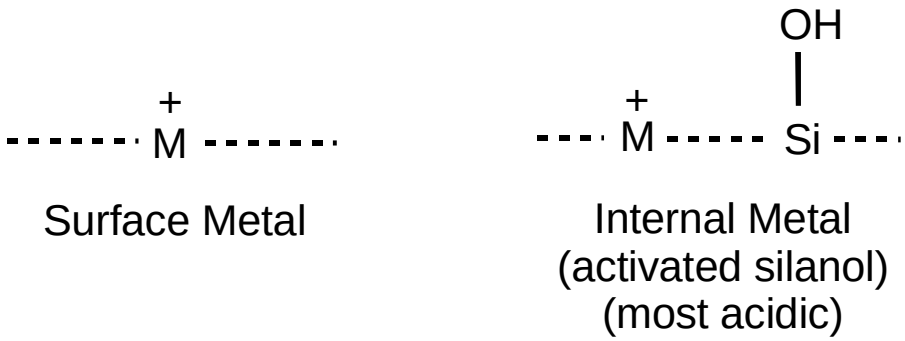


Agilent owns the patent for sol-gel HPLC packings

Silica Particle Surface Chemistry

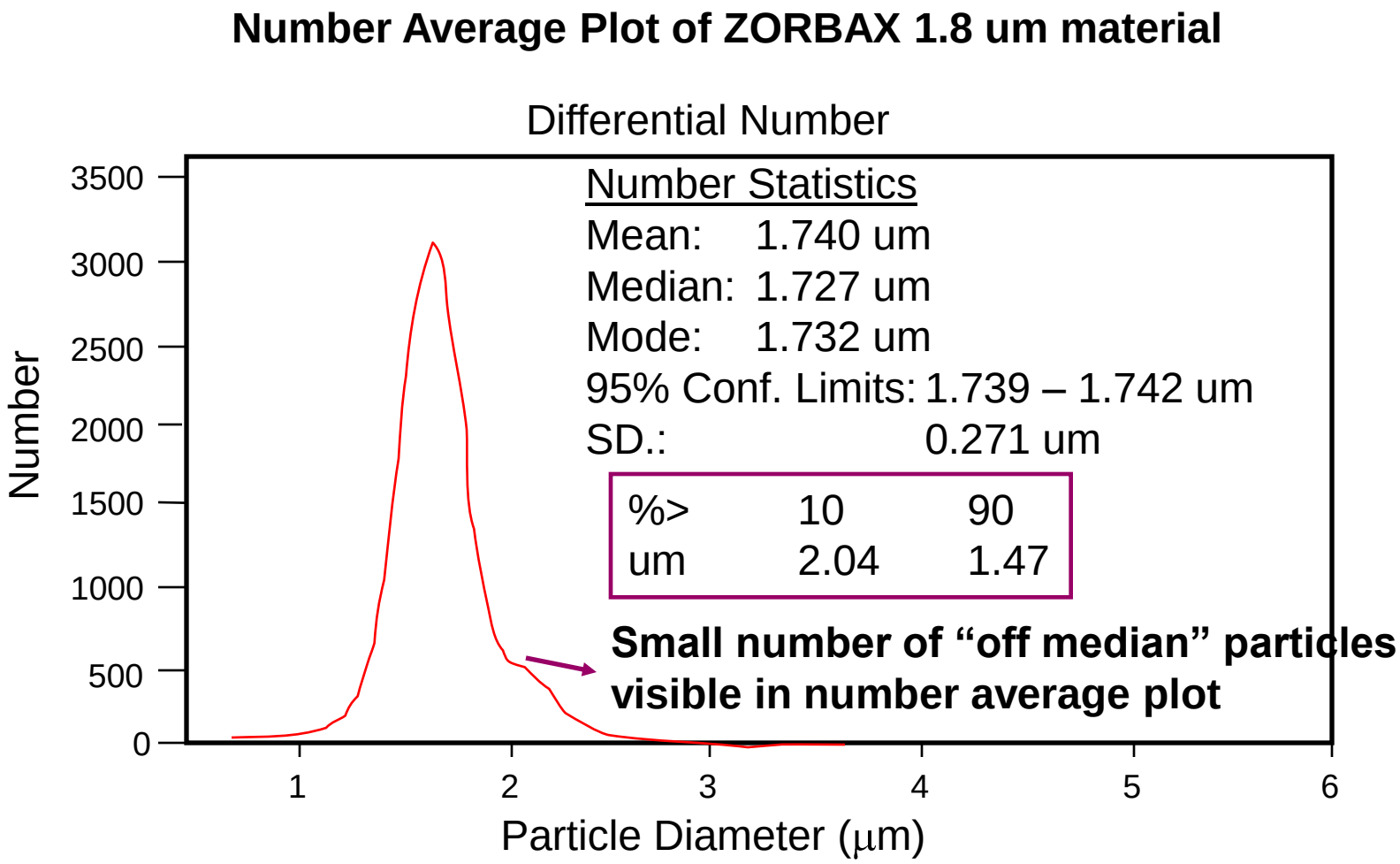
Non-Ideal Surface Re-Hydrolysis **Ideal Surface State**



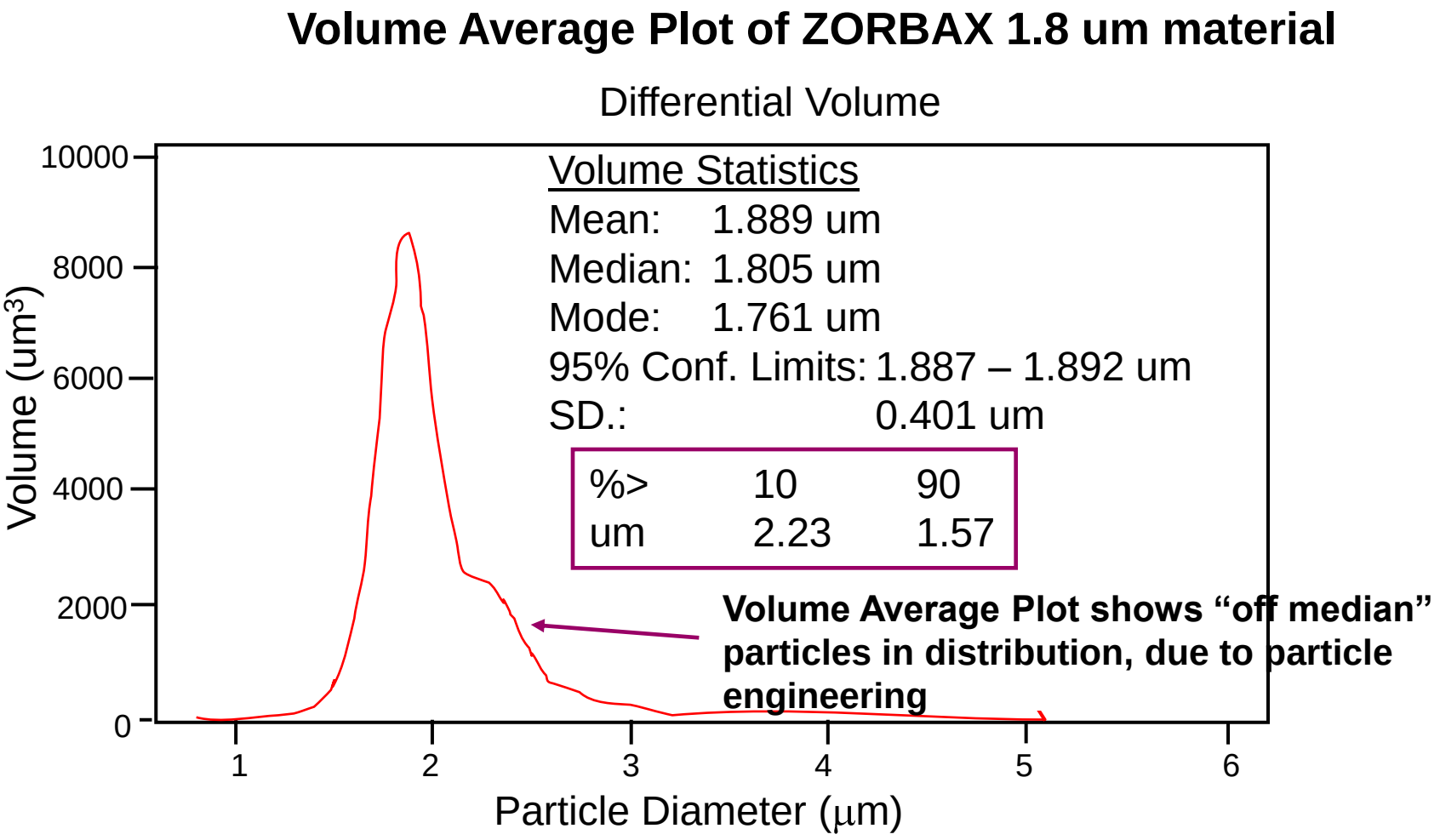


Caused by Using Impure Raw Material

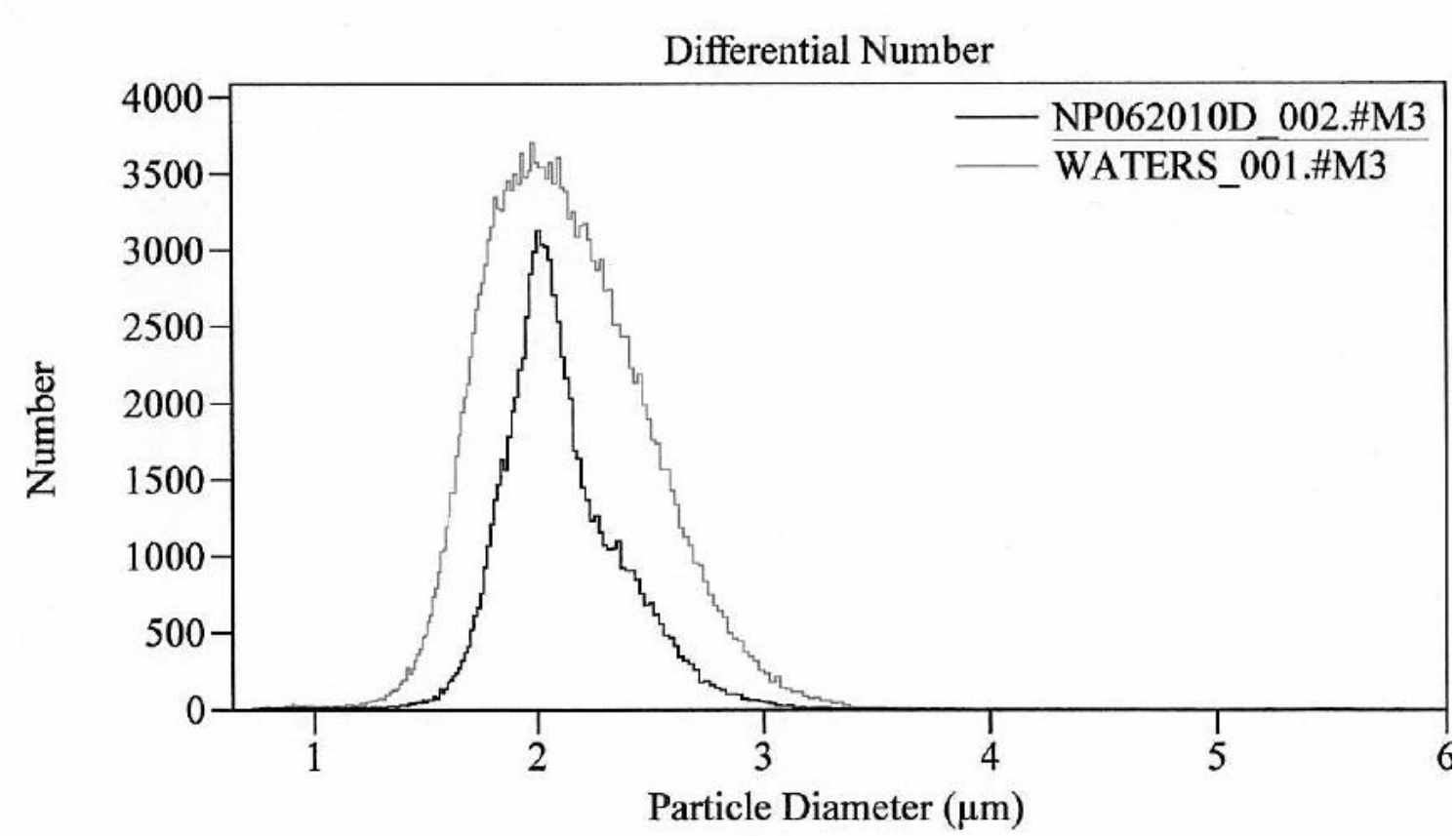
Actual Example of Particle Size Distribution on ZORBAX 1.8um Particles



Actual Example of Particle Size Distribution on ZORBAX 1.8um Particles

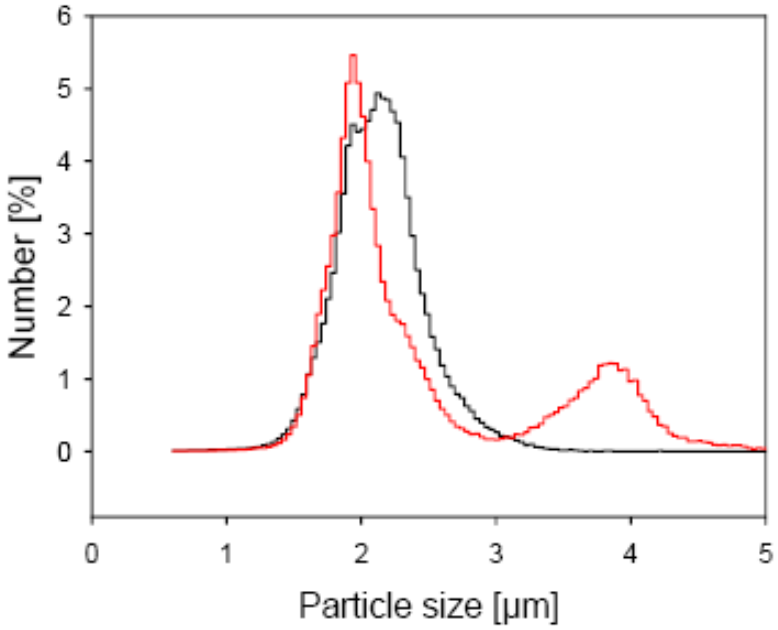


Particle Size Distribution – Agilent vs. Waters
Agilent has a tighter, narrow PSD



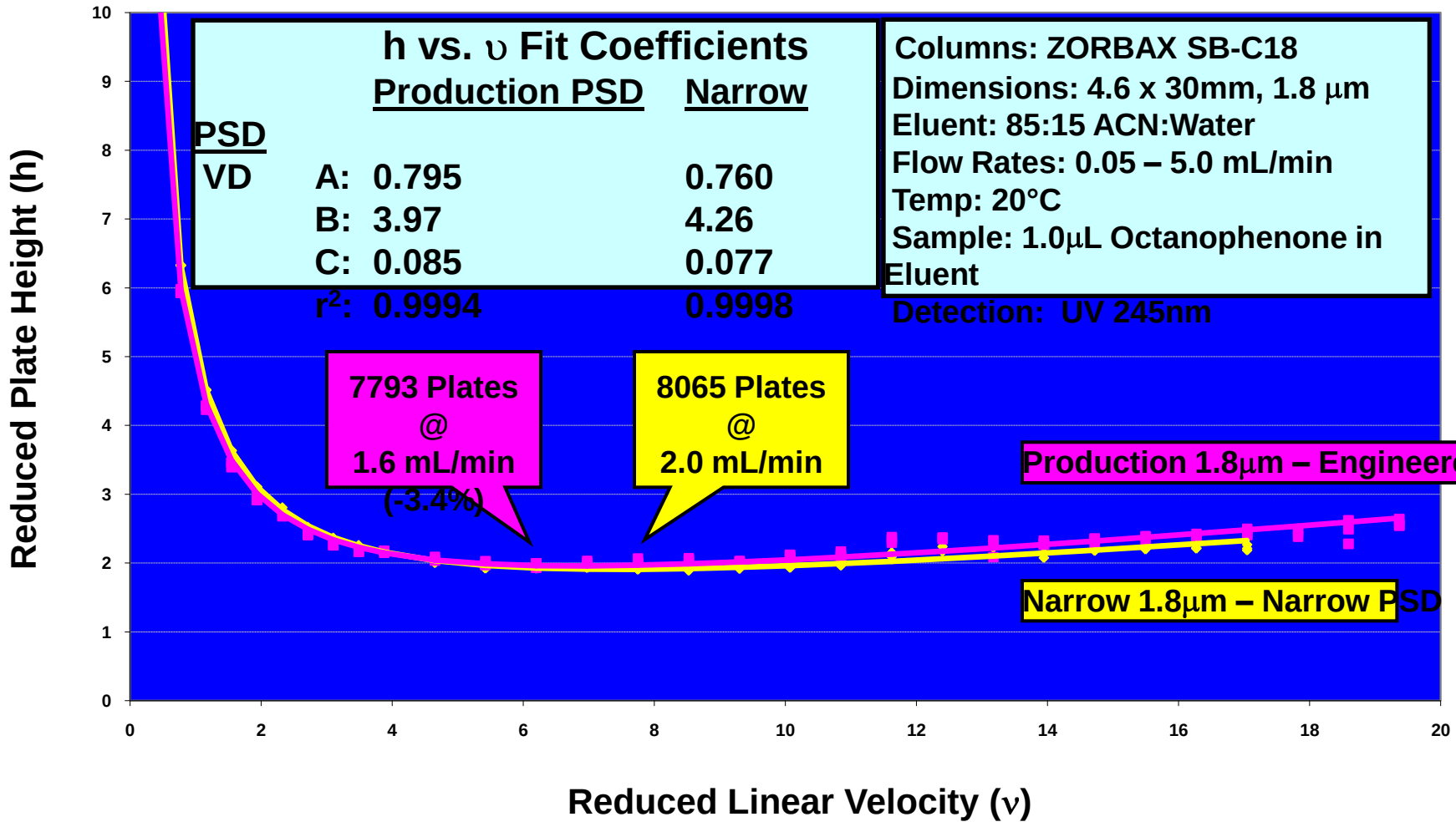
Packing from a new Waters Acquity 2.1 x 100mm, 1.7um column

What would a PSD look like with 3.5um blended in?



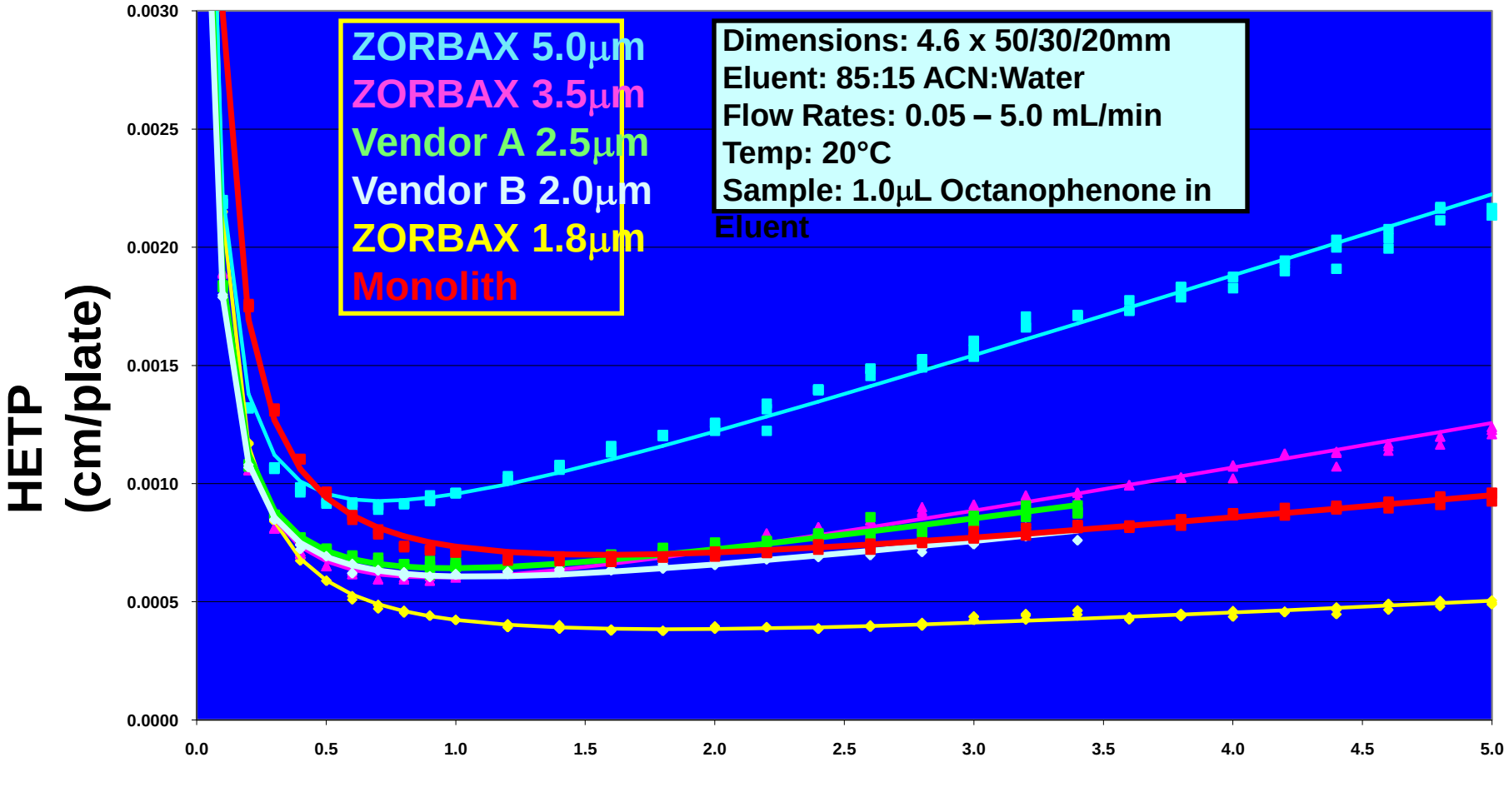
with 3.5um
without 3.5um

Van Deemter Curves for Narrow vs. Engineered Particle Size Distribution 1.8µm SB-C18

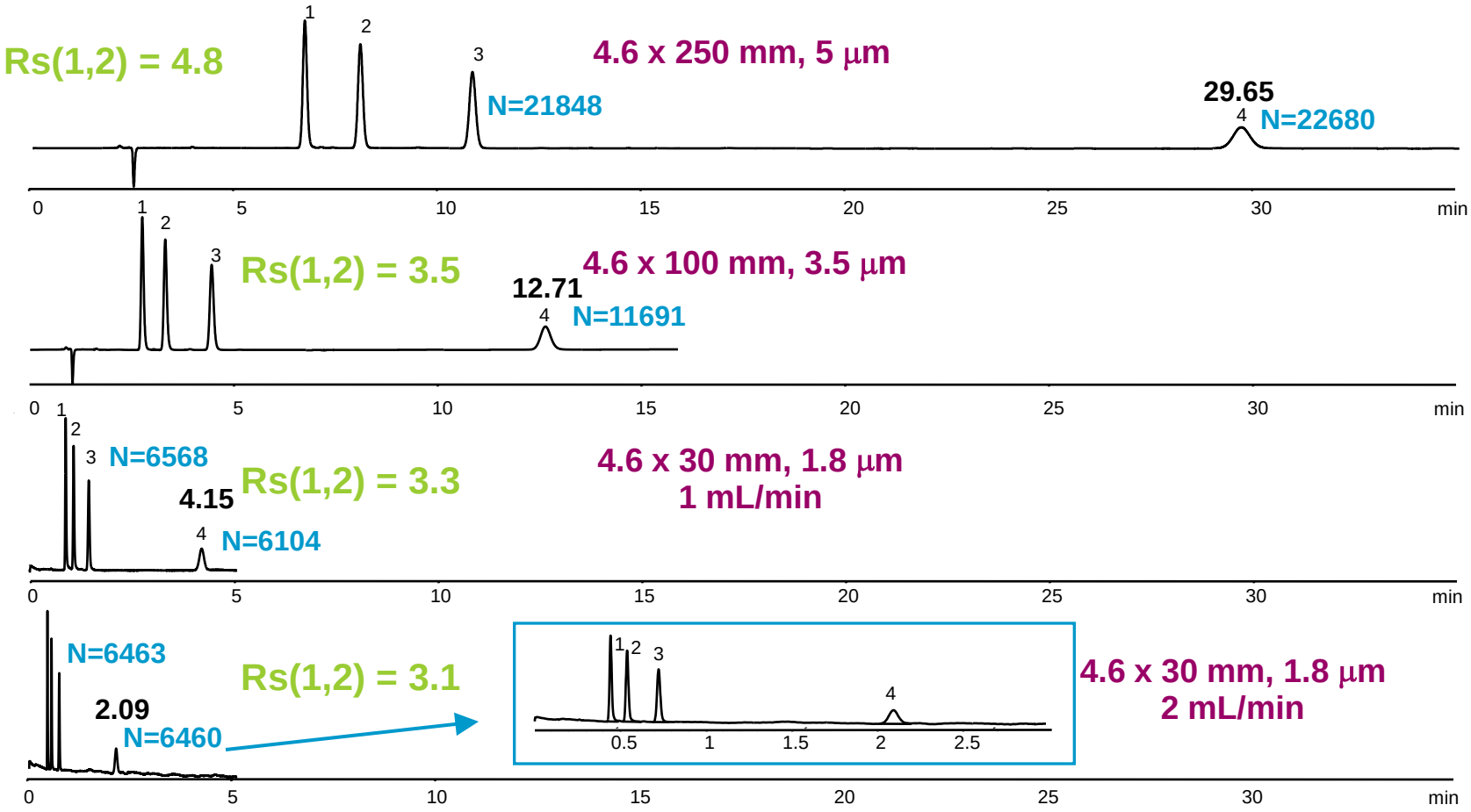


HETP vs. Volumetric Flow Rate

Small Particle Columns including Monolith



Reduce Analysis Time by up to 95% Using Sub-Two Micron Columns



olumns: ZORBAX SB-C18 Mobile Phase: 50% 20 mM NaH_2PO_4, pH 2.8: 50% ACN Flow Rate: 1 mL/min Temperature: RT
Detection: UV 230 nm Sample: 1. Estradiol 2. Ethynylestradiol 3. Dienestrol 4. Norethindrone

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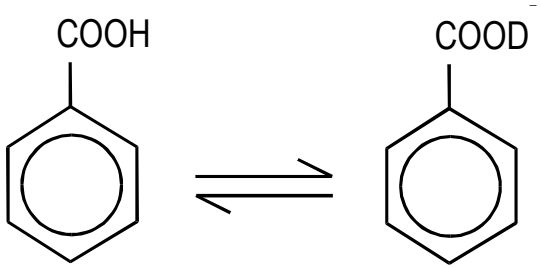
pH, pKa and Weak Acids

$$RCOOH \rightleftharpoons RCOO^- + H^+$$

$$K_a = \frac{[RCOO^-][H^+]}{[RCOOH]}$$



$$+ \quad H^+$$

$K_a = 6.4 \times 10^{-5}$

$pK_a = 4.2$

At pH 4.2 – the sample exists as benzoic acid and the benzoate ion in a ra
of 1:1. Peak shape can be poor
At pH 5.2 – 91% of the sample exists as the benzoate ion. RP retention de
At pH 3.2 – 91% of the sample exists as benzoic acid. RP retention increas

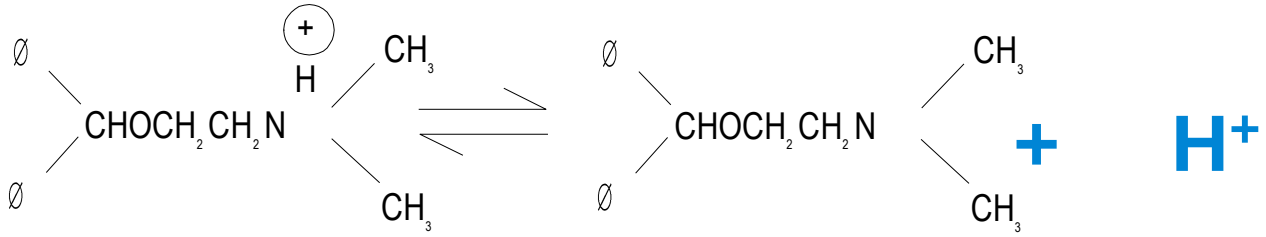
pH, pKa and Weak Bases

$$R_3NH^+ \rightleftharpoons R_3N + H^+$$

$$K_a = \frac{[R_3N][H^+]}{[R_3NH^+]}$$

$K_a = 1 \times 10^{-9}$

$pK_a = 9$



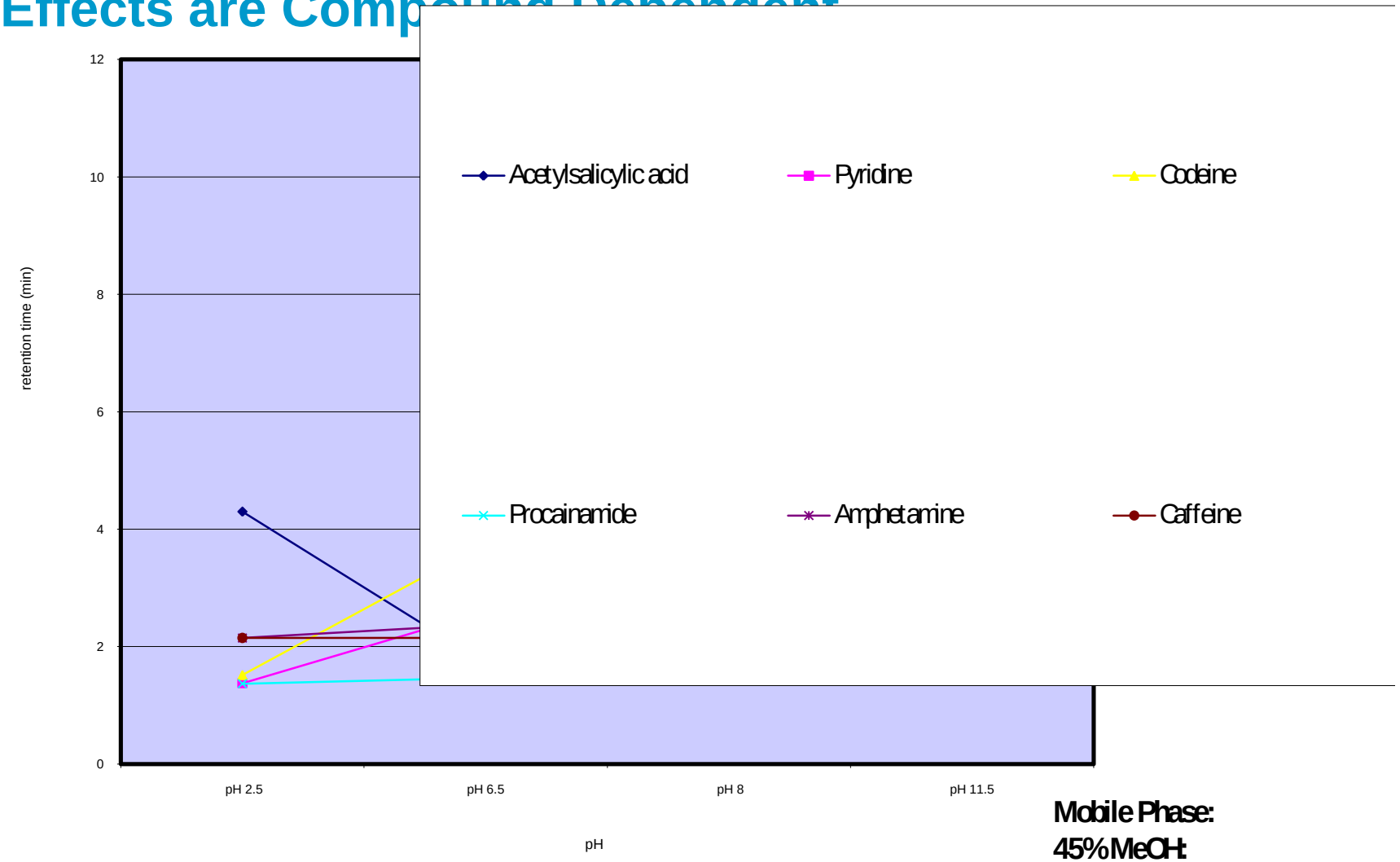
At pH 9 – the sample exists as protonated and unprotonated diphenhydramine in a ratio of 1:1. Peak shape can be poor.

At pH 10 – 91% of the sample exists as unprotonated diphenhydramine.

At pH 8 – 91% of the sample exists as protonated diphenhydramine.

Retention vs. pH for Ionizable Compounds

Effects are Compound Dependent



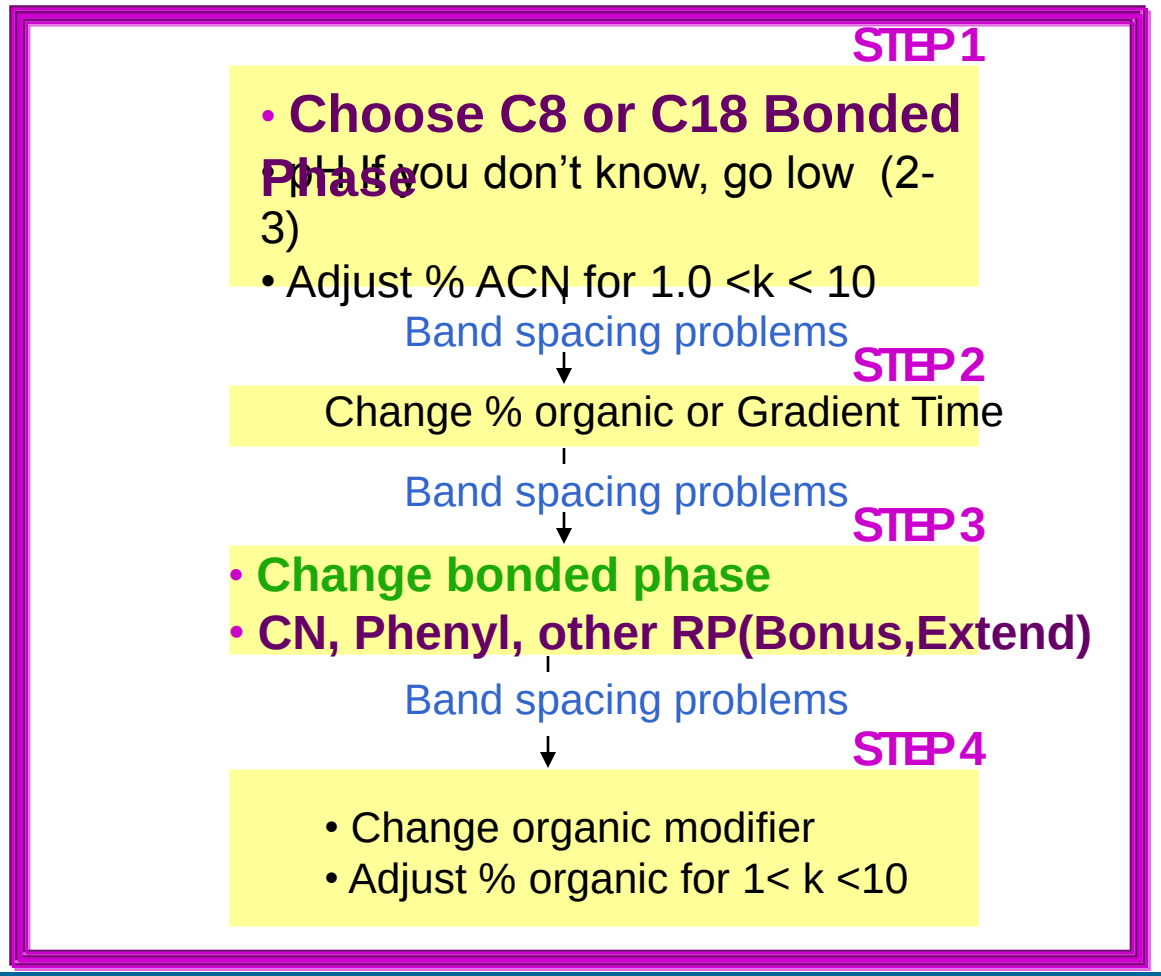
Mobile Phase:
45% MeOH:
55% 20 mM Phosphate Buffer

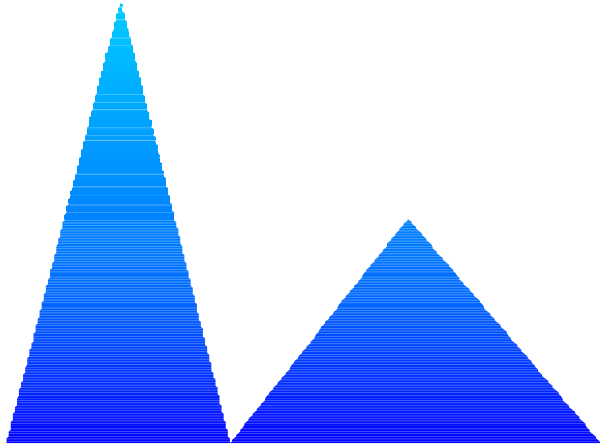
Common Buffers Used in HPLC
General Purpose Buffers

*Potassium dihydrogen phosphate (monobasic)	1.1 – 3.1
*Dipotassium hydrogen phosphate (dibasic)	6.2 – 8.2
Tripotassium phosphate (tribasic)	11.3 – 13.3
Sodium dihydrogen citrate	2.1 – 4.1
Disodium hydrogen citrate	3.7 – 5.7
Trisodium citrate	4.4 – 6.4
*Sodium acetate	3.8 – 5.8
TRIS [tris(hydroxymethyl)aminomethane]	8.0 – 10.0
Ammonium hydroxide	8.3 – 10.3

***most common**

Suggested Method Development Path

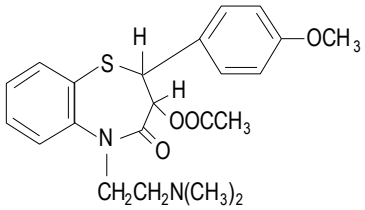




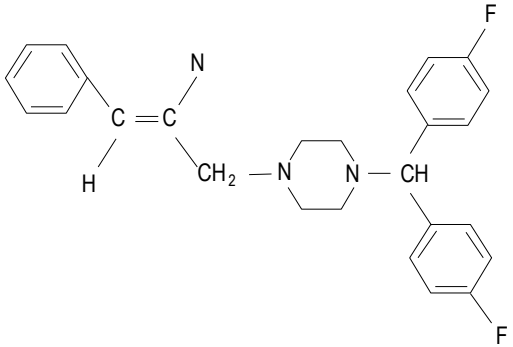
ISOCRATIC
ELUTION

Cardiac Drugs

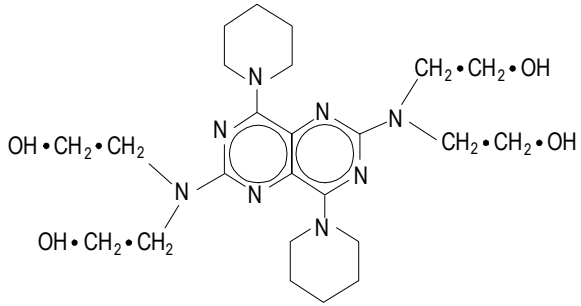
Diltiazem
pKa: unknown
calcium channel blocker



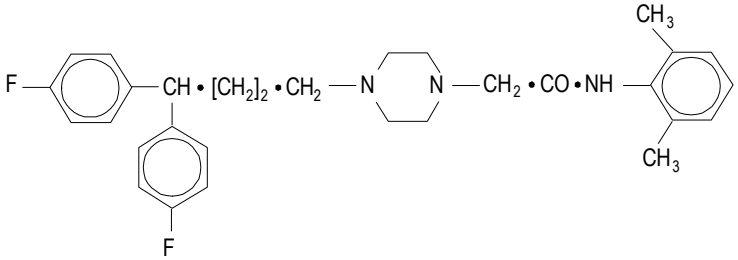
Flunarizine
pKa: unknown
calcium channel blocker



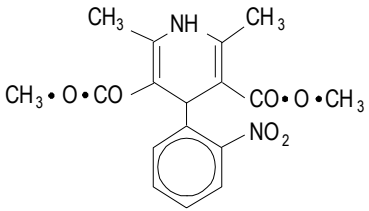
Dipyridamole
pKa: 6.4
anti-thrombotic/anti-anginal



Lidoflazine
pKa: unknown
calcium channel blocker

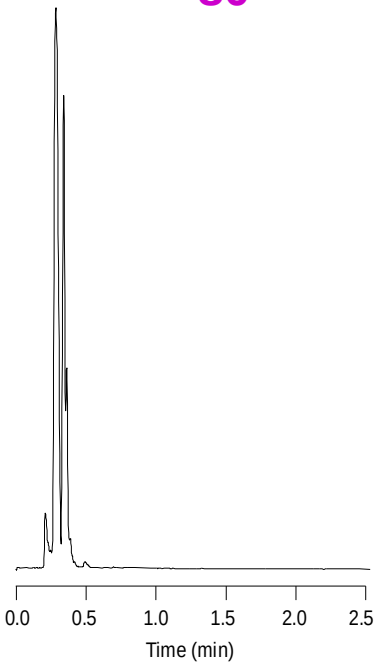


Nifedipine
pKa: unkbown
anti-anginal vasodilator

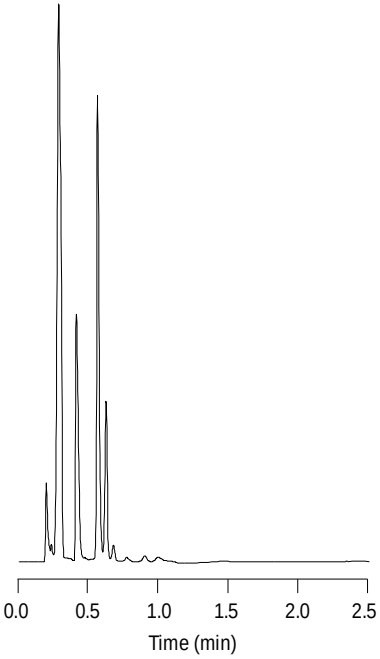


Fast Scouting Isocratic Runs
Cardiac Drugs with Acetonitrile

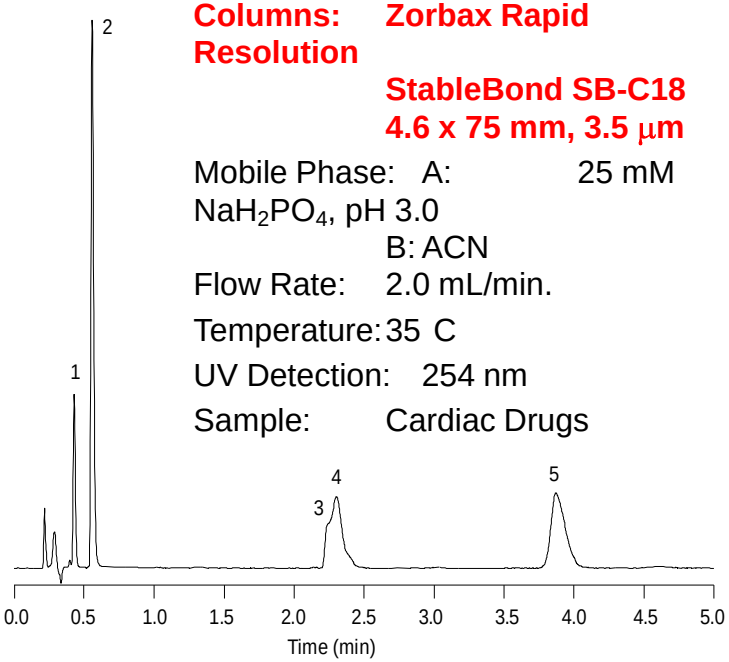
A : B **20 : 80**



40 : 60



60 : 40



Columns: **Zorbax Rapid Resolution**
StableBond SB-C18
4.6 x 75 mm, 3.5 µm

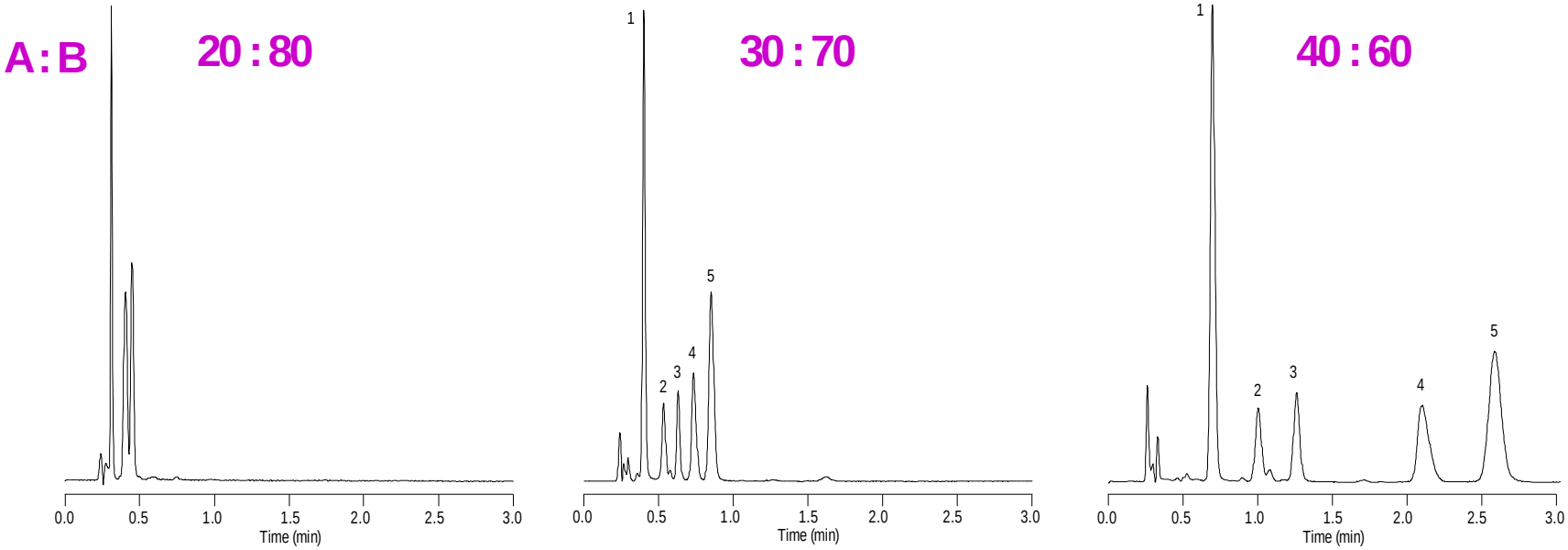
Mobile Phase: A: 25 mM NaH_2PO_4, pH 3.0
B: ACN
Flow Rate: 2.0 mL/min.
Temperature: 35 C
UV Detection: 254 nm
Sample: Cardiac Drugs

Fast Scouting Isocratic Runs
Cardiac Drugs with Methanol

Column: Zorbax Rapid Resolution SB-C18, 4.6 x 75 mm, 3.5 µm Mobile Phase: A: 25 mM NaH_2PO_4, pH 3.0 B: MeOH

Flow Rate: 2.0 mL/min Temperature: 35°C Detection: UV 254 nm

Sample: Cardiac Drugs 1. Diltiazem 2. Dipyridamole 3. Nifedipine 4. Lidoflazine 5. Flunarizine



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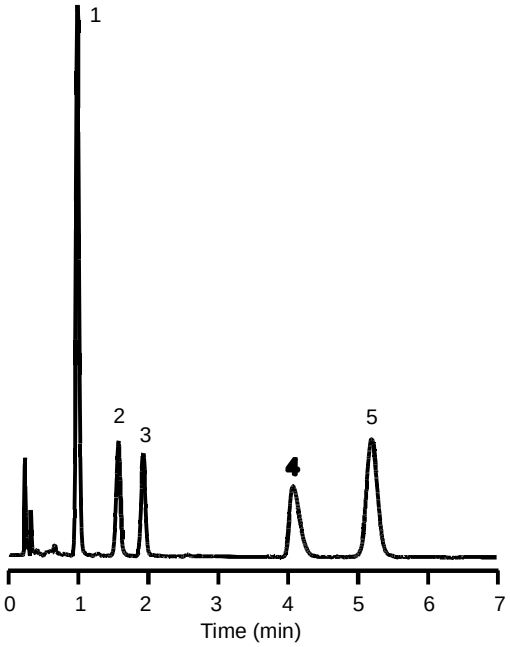
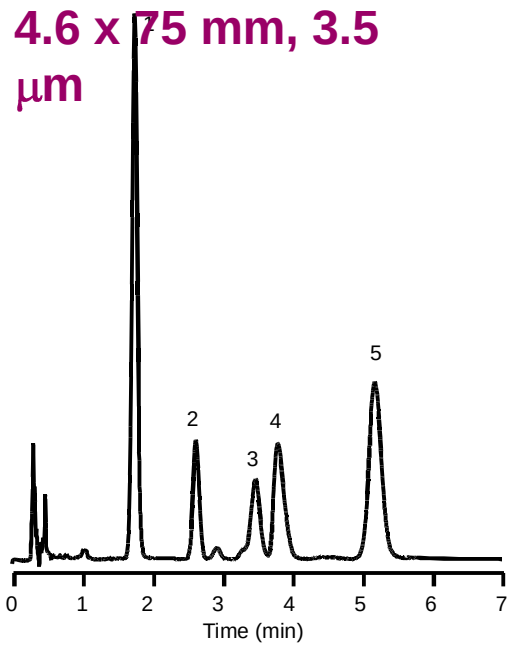
Bonded Phase Selectivity Differences

Mobile Phase: A: 25 mM NaH_2PO_4, pH 3.0 B: MeOH Flow Rate: 1.0 mL/min. Temperature: 35 C
UV Detection: 254 nm
Sample: Cardiac Drugs 1. Diltiazem 2. Dipyridamole 3. Nifedipine 4. **Lidoflazine** 5. Flunarizine

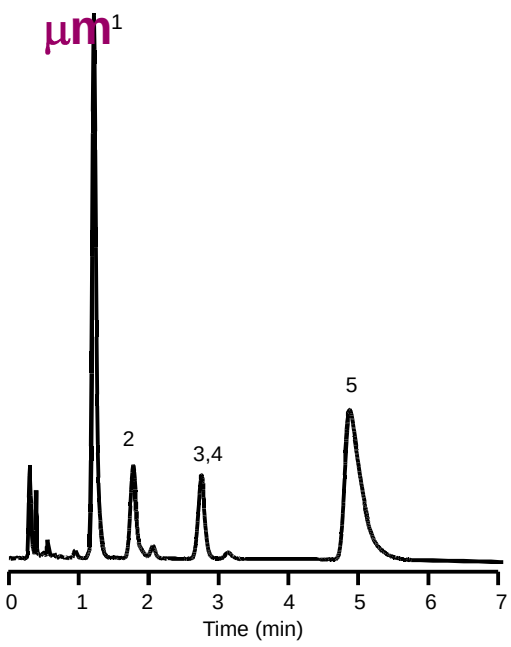
Rapid Resolution StableBond SB-C18 4.6 x 75 mm, 3.5 µm



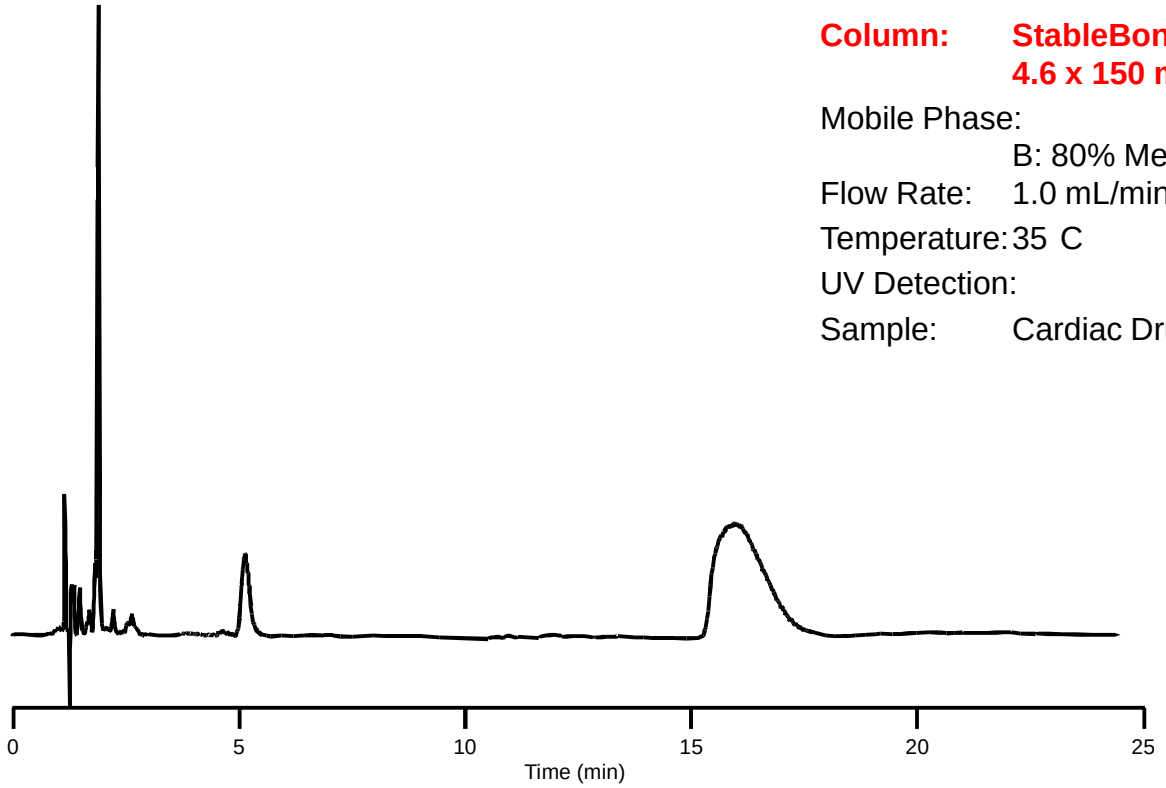
Rapid Resolution StableBond SB-Phenyl 4.6 x 75 mm, 3.5 µm



Rapid Resolution StableBond SB-CN 4.6 x 75 mm, 3.5 µm



I Don’t Have Time to Make Buffers…

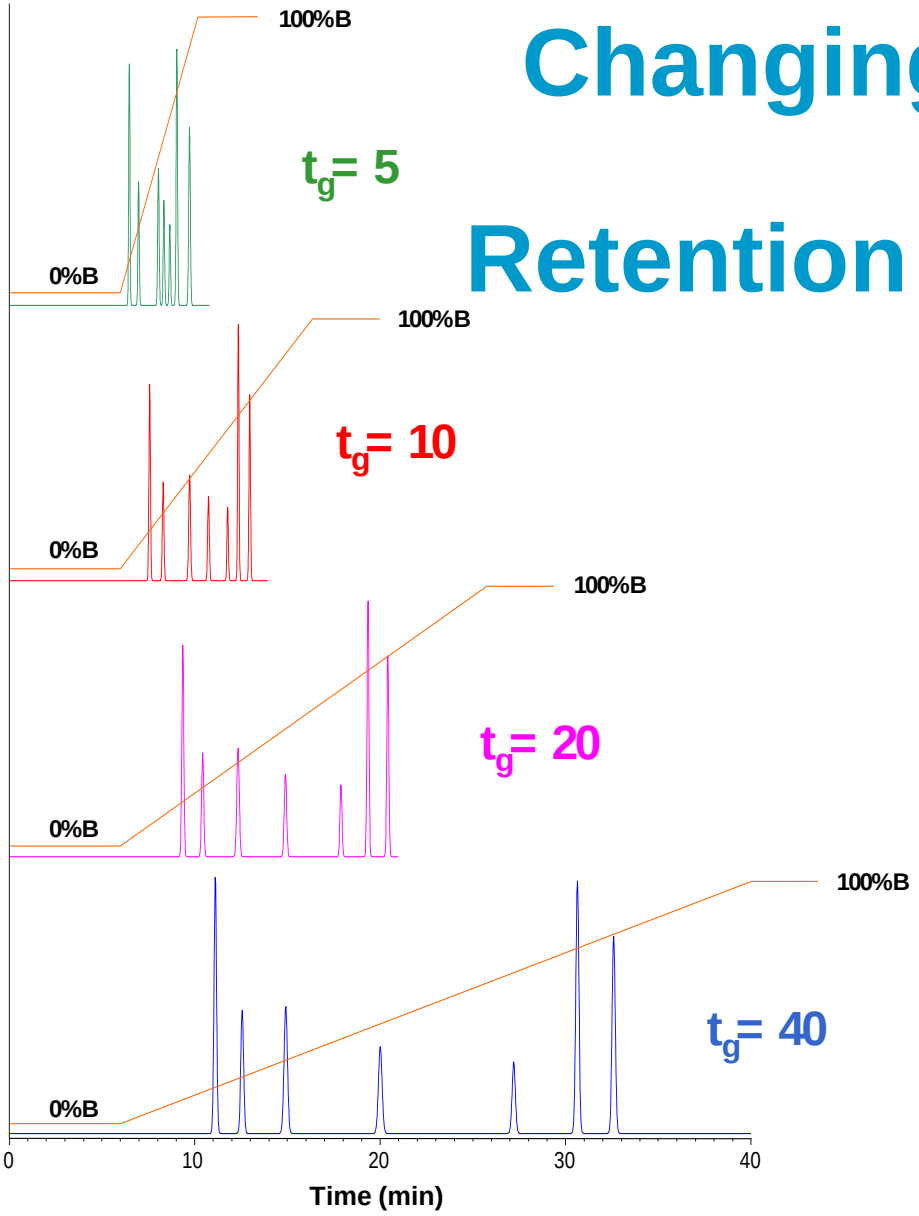


Column: **StableBond SB-C18**
4.6 x 150 mm, 5 µm

Mobile Phase: A: 20% H_2O
B: 80% MeOH
Flow Rate: 1.0 mL/min.
Temperature: 35 C
UV Detection: 254 nm
Sample: Cardiac Drugs

- **Buffers are critical to good retention and peak shape in many separati**

Changing Gradient Time to Affect Retention (k*) and Resolution



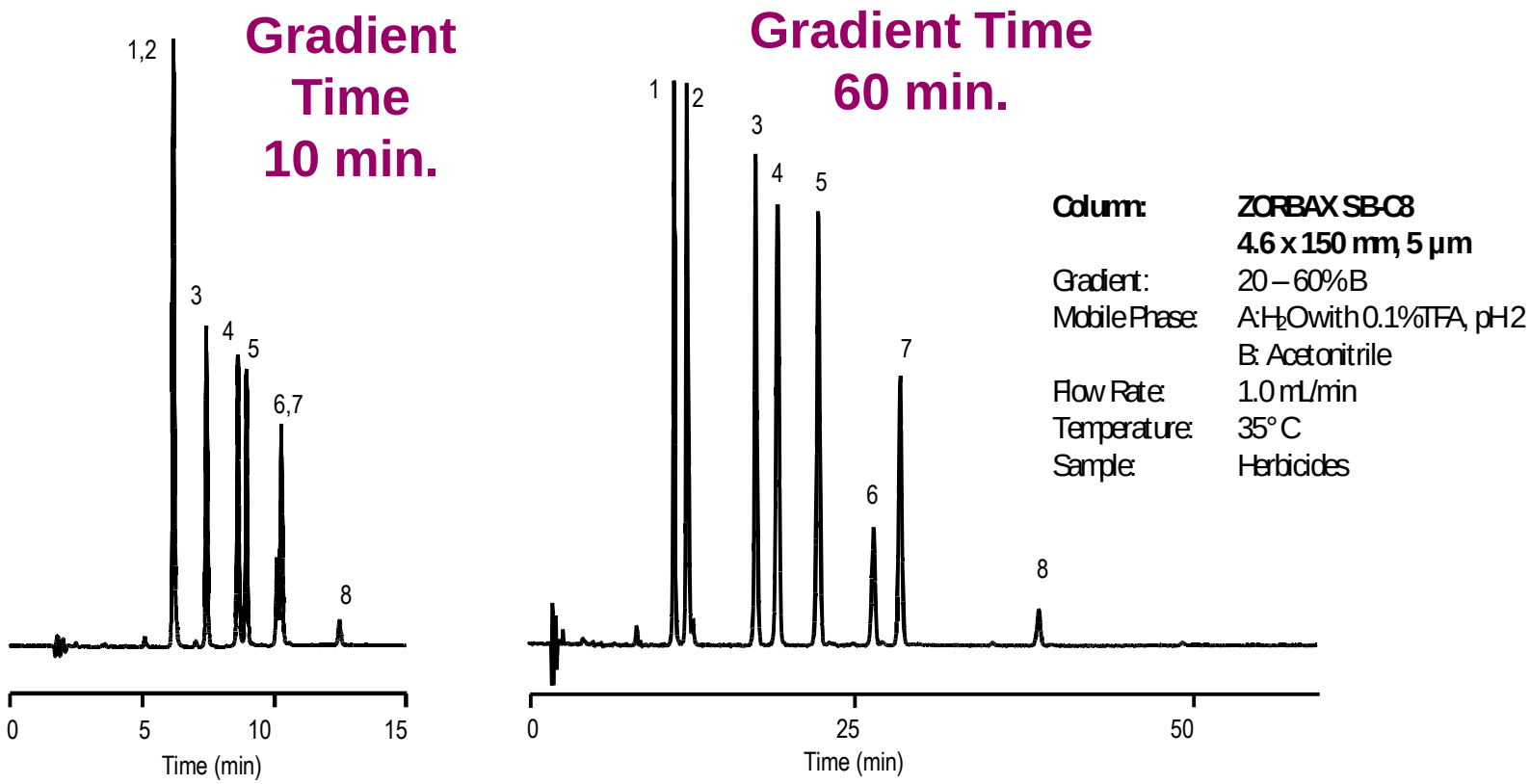
$$k^* = \frac{t_g F}{S \Delta\%B \; V_m}$$

$1/k^*$ = gradient steepness = b

$\Delta\Phi$ = change in volume fraction of B solvent
S = constant
F = flow rate (mL/min.)
t_g = gradient time (min.)
V_m = column void volume (mL)

- S ≈ 4–5 for small molecules
- 10 < S < 1000 for peptides and proteins

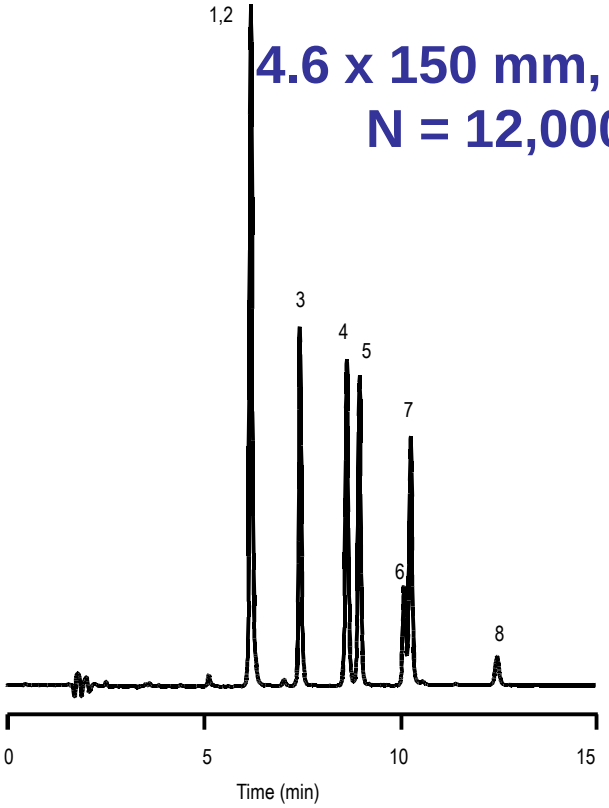
Use of a Longer Gradient Time Increases Gradient Retention



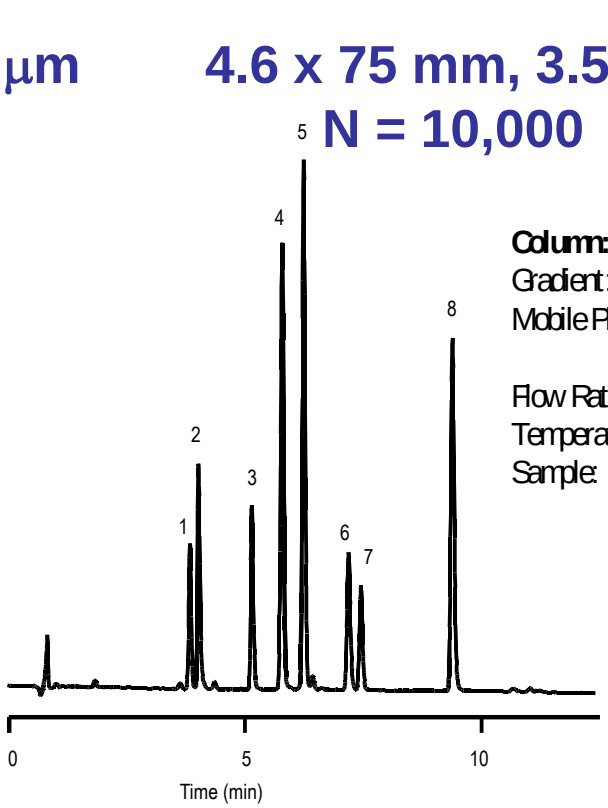
- **Increased gradient retention improves resolution of several peak pairs – 1,2 a**

A Shorter Column (smaller Vm)

Increases Gradient Retention, Increases Overall Resolution, Assumes Constant N



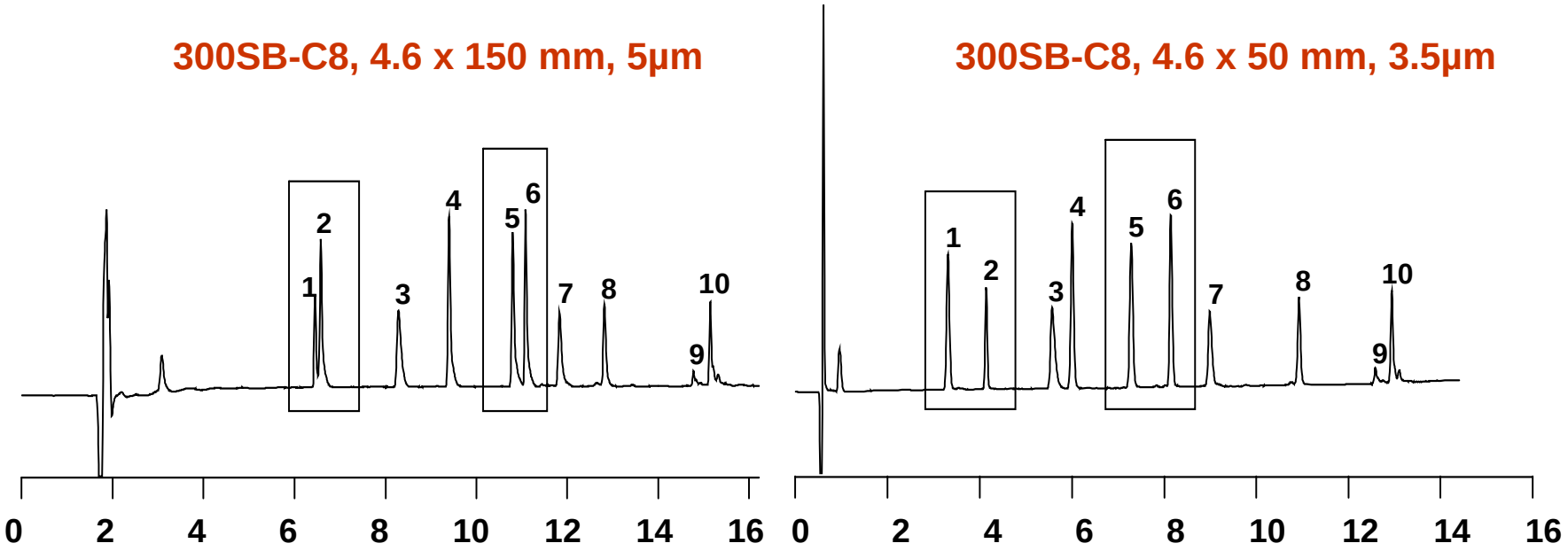
4.6 x 150 mm, 5 µm
N = 12,000



4.6 x 75 mm, 3.5 µm
N = 10,000

Column:	**ZORBAX SB-C8**
Gradient:	20 – 60% B
Mobile Phase:	A: H_2O with 0.1% TFA, pH 2
	B: Acetonitrile
Flow Rate:	1.0 mL/min
Temperature:	35° C
Sample:	Herbicides

Improving Resolution Using Short Column Length (Vm) and Particle Size (N)



Mobile Phase: A: 95% Water : 5 % ACN, 0.1% TFA
B: 5% Water : 95% ACN, 0.085% TFA
Gradient: 10-60% B in 30 min.

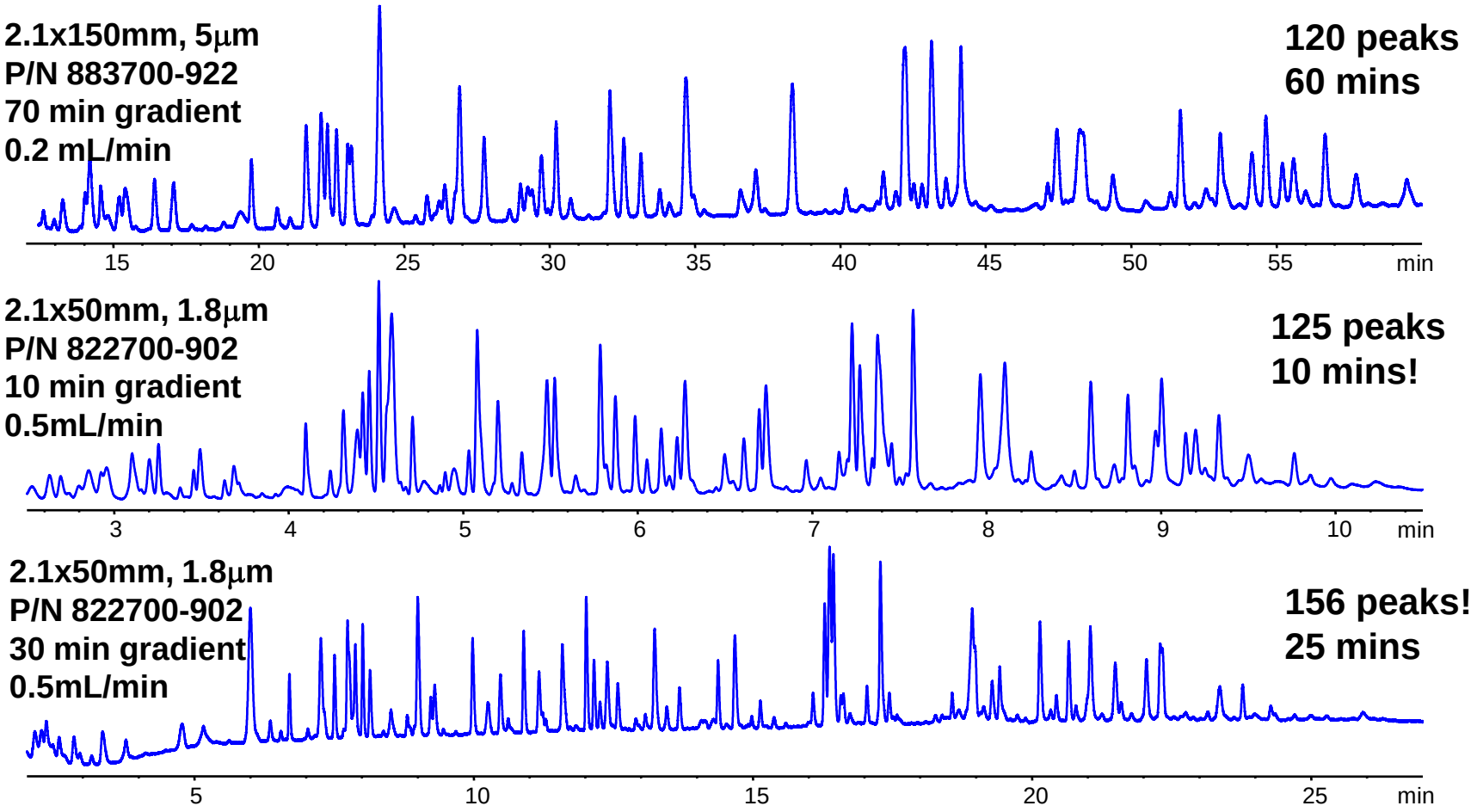
Flow Rate: 1.0 mL / min.

Temperature: Ambient

Sample:
1. Gly-Tyr
2. Val-Tyr-Val
3. [Gln^{11}] Amyloid-β-Protein Fragm 1-16
4. (TYR8) Bradykinin
5. Met-Enk
6. Leu-Enk
7. Angiotensin II
8. Kinetensin
9. RNase
10. Insulin (Eq.)

Gradient Resolution and Shorter Columns

Faster Analyses for Complex Samples: HSA Tryptic Digest



Conditions: Mobile Phase A: Water w/ 0.1% TFA, B: ACN w/0.1% TFA, Gradient 2%B to 50%B, Temp: 50°C
UV 214 nm

Maintaining k* - To Keep Relative Peak Position in a Chromatogram Unchanged

Any Decrease in Proportional	Can be Offset by a
Column length	Decrease in $\mathbf{t_G}$ or F Increase in Δ%B
Column volume (i.d.)	Decrease in t_G or **F** Increase in Δ%B
Δ%B (same colum)	Decrease in $\mathbf{t_G}$ or F

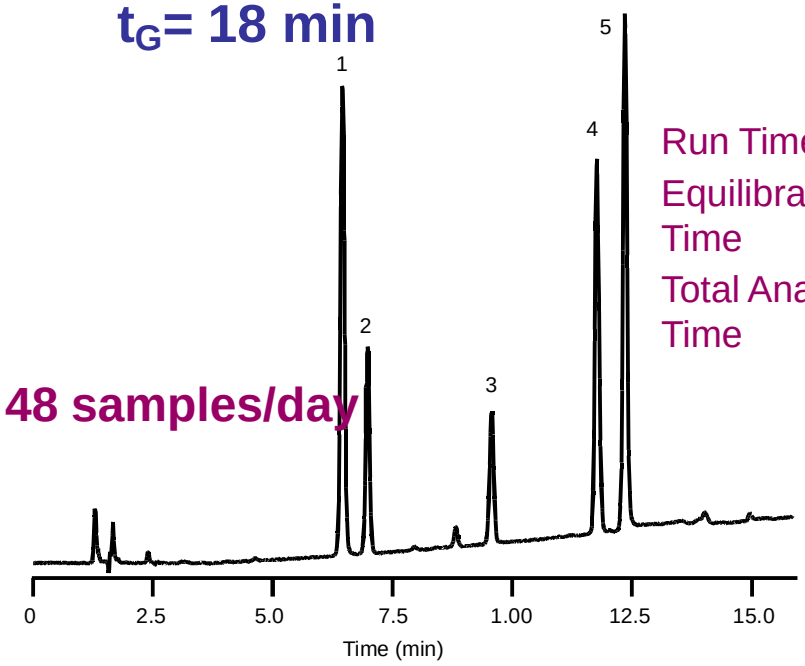
$$k^* \propto \frac{t_G \bullet F}{S \bullet \Delta\Phi \bullet Vm}$$

001809S1.PPT

Short Columns Reduce Total Gradient Analysis Time

Gradient Separation of Cardiac Drugs

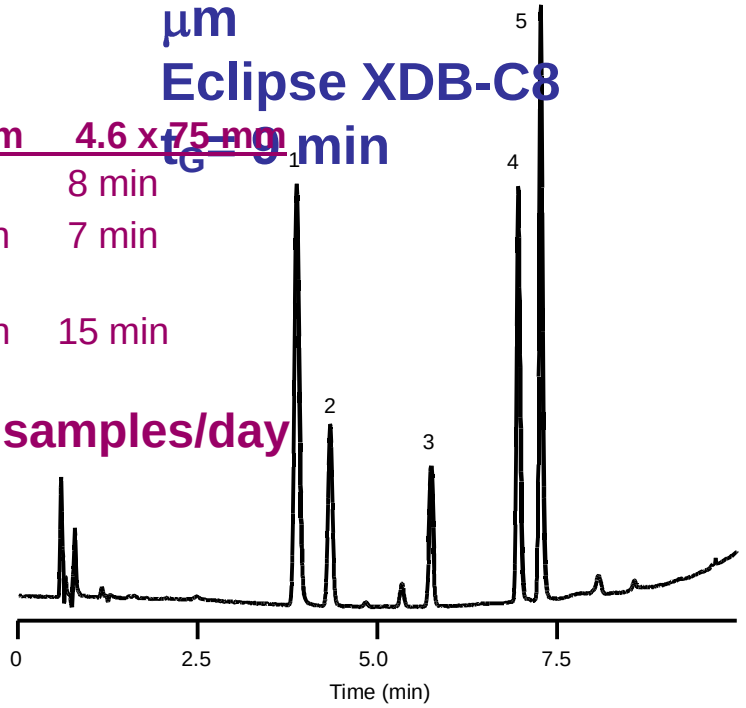
A. 4.6 x 150 mm, 5 µm Eclipse XDB-C8
t_G= 18 min



48 samples/day

	4.6 x 150 mm	4.6 x 75 mm
Run Time	13 min	8 min
Equilibration Time	15 min	7 min
Total Analysis Time	28 min	15 min

B. 4.6 x 75 mm, 3.5 µm Eclipse XDB-C8
t_G= 9 min

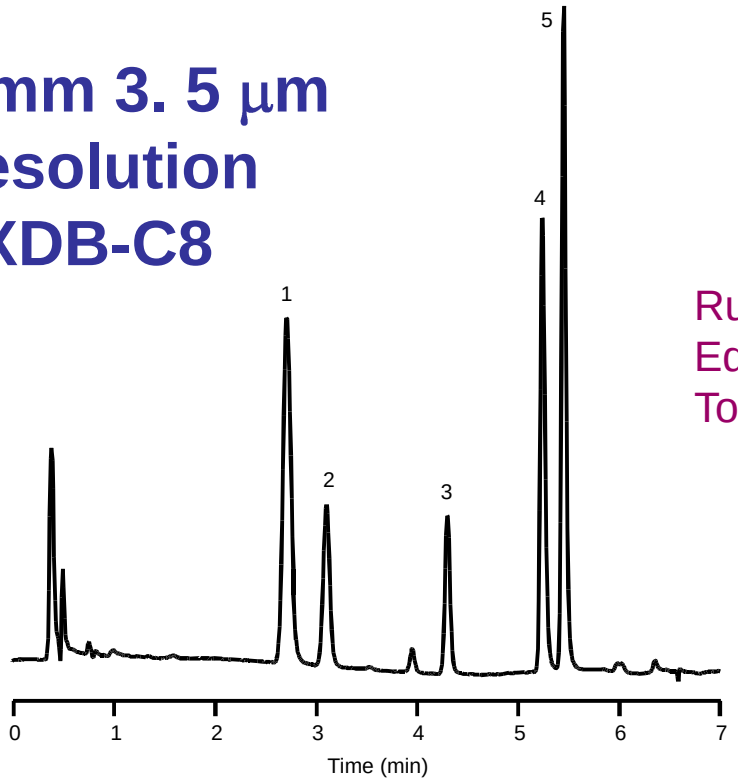


96 samples/day

Very Short Columns Reduce Analysis Time

Gradient Separation of Cardiac Drugs

4.6 x 50 mm 3. 5 μm
Rapid Resolution
Eclipse XDB-C8



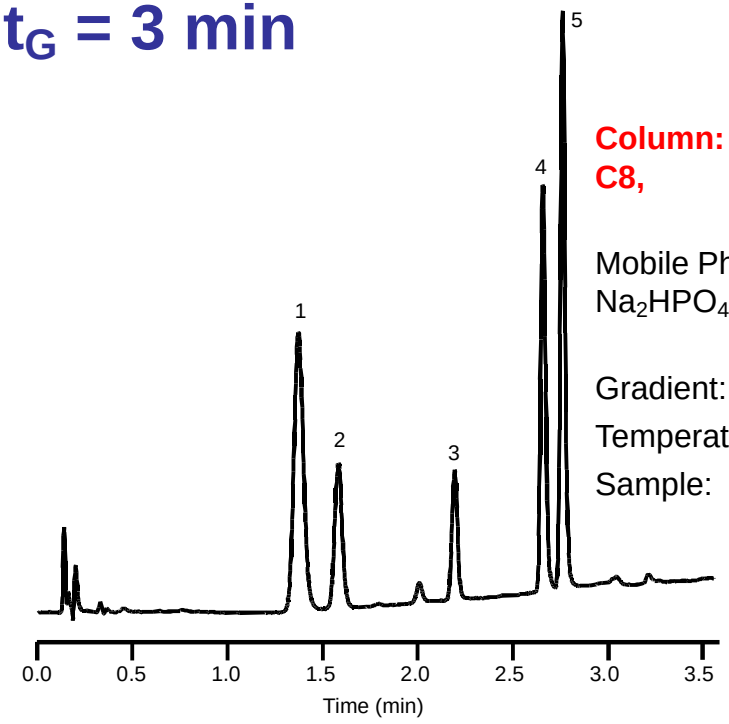
Run Time	6 min
Equilibration Time	5 min
Total Analysis Time	11 min

130 samples/day

Increasing Flow Rate Reduces Gradient Run Time Further

Cardiac Drugs – Gradient Time ∝ 1/F

F = 2.0 mL/min
t_G = 3 min



Column: **Rapid Resolution Eclipse XDB-C8,**
4.6 x 50 mm, 3.5 μm

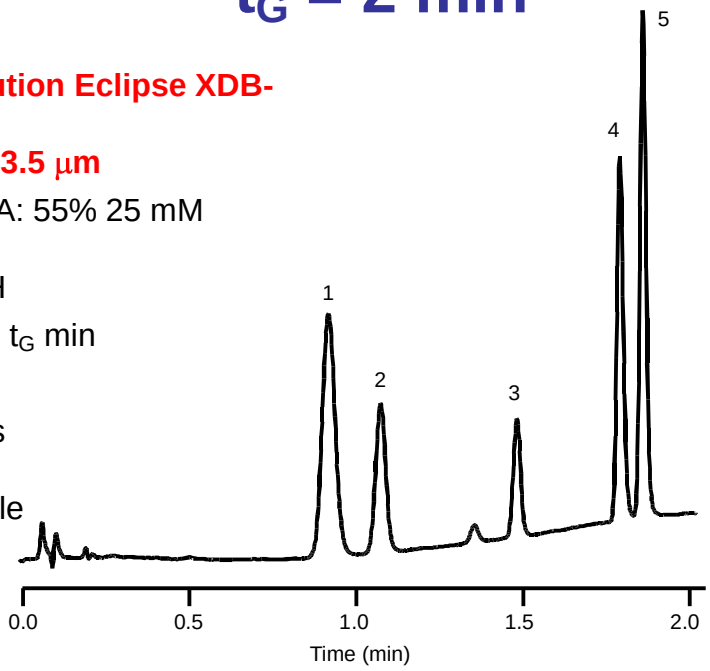
Mobile Phase: A: 55% 25 mM Na_2HPO_4, pH 3
B: 45% MeOH

Gradient: 45 – 90% B in t_G min

Temperature: 35°C

Sample: Cardiac Drugs
1. Diltiazem
2. Dipyridamole
3. Nifedipine
4. Lidoflazine
5. Flunarizine

F = 3.0 mL/min
t_G = 2 min



Fast Gradient Analysis of Heart Drugs

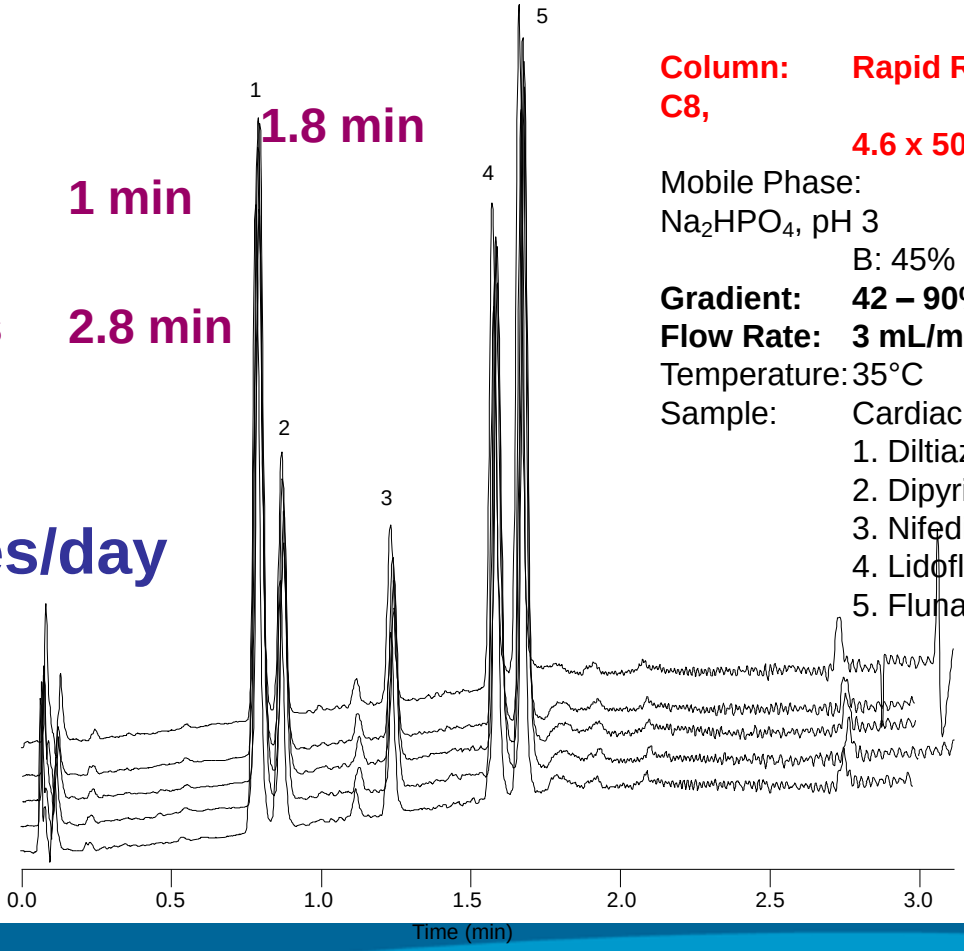
Optimized Gradient

Run Time **1.8 min**

Equilibration Time **1 min**

Total Analysis Time **2.8 min**

480 Samples/day



Column: **Rapid Resolution Eclipse XDB-C8,**

4.6 x 50 mm, 3.5 μm

Mobile Phase: A: 55% 25 mM Na_2HPO_4, pH 3

B: 45% MeOH

Gradient: **42 – 90% B in 3 min**

Flow Rate: **3 mL/min**

Temperature: 35°C

Sample: Cardiac Drugs

1. Diltiazem
2. Dipyridamole
3. Nifedipine
4. Lidoflazine
5. Flunarizine

Why Use Different Bonded-Phases in Method Development?

Develop the most effective method

Optimize selectivity

Optimize resolution

Minimize analysis time

Bonded Phase Choice Drives Resolution

Changing selectivity (α) influences resolution the most

Bonded phase is the column choice that controls selectivity

$$R_s = \square N \square \left[\frac{(\alpha - 1)}{\alpha}\right] \left(\frac{k}{1 + k}\right)$$

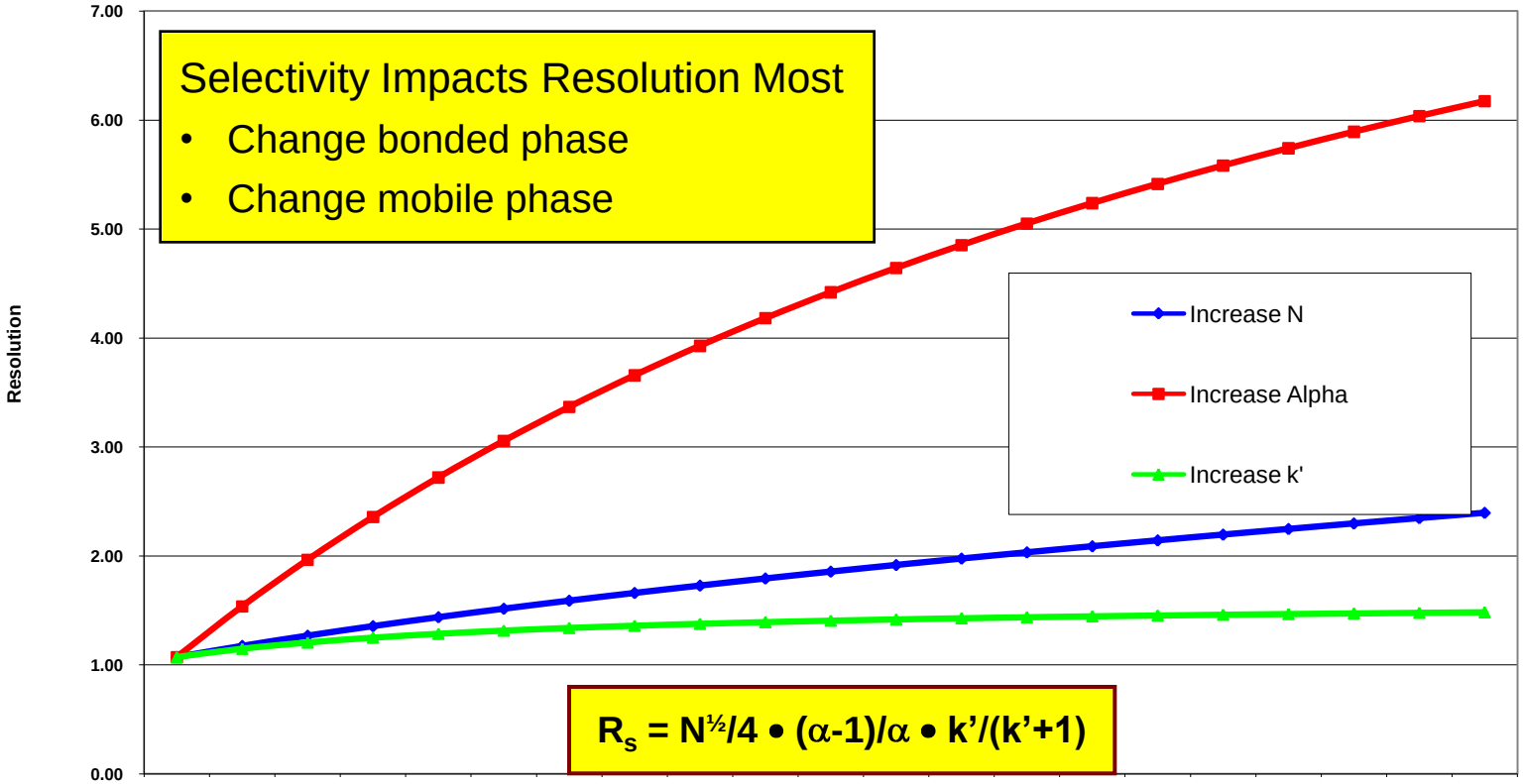
α= selectivity – increase by changing bonded phase and mobile

N = plates – increase by using longer column or reducing particl

k = retention – increase by changing bonded phase and mobile phase

does not improve Rs above k ≈10

Resolution as a Function of Selectivity, Column Efficiency, or Retention

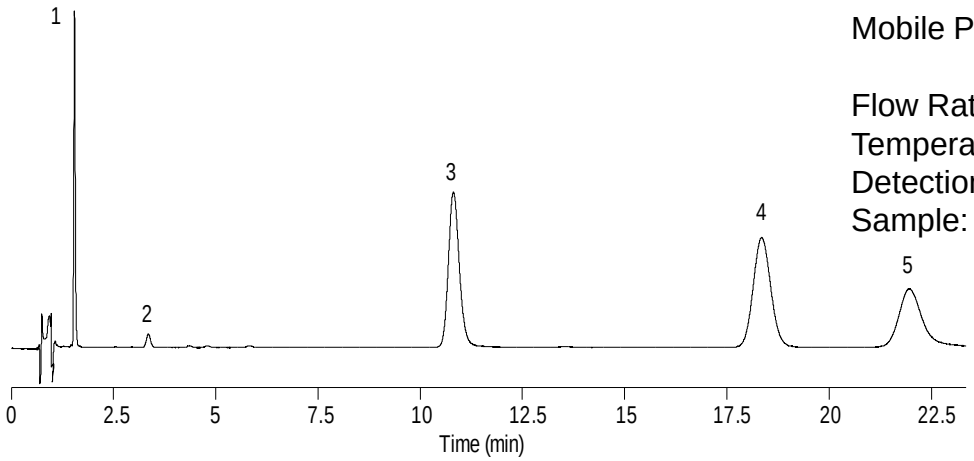


Plates:	5000	10000	15000	20000	25000
Alpha:	1.10	1.35	1.60	1.85	2.1
k':	2.0	4.5	7.0	9.5	12.0

Method Development – Start with C18/C8

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:

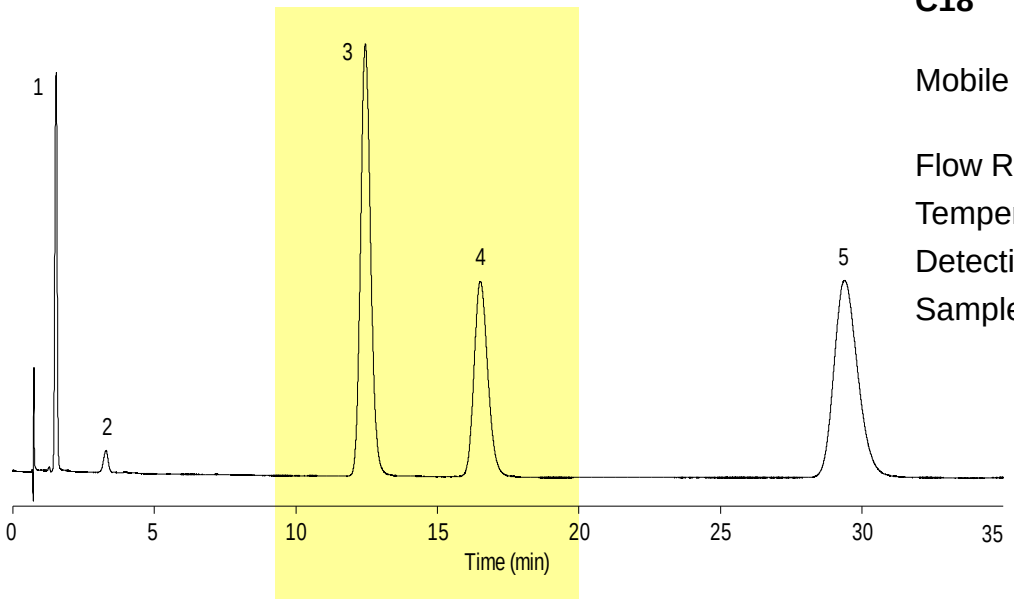
1. Caffeic acid
2. Impurity
3. Luteolin
4. Naringenin
5. Apigenin

- Analysis time too long!
- 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

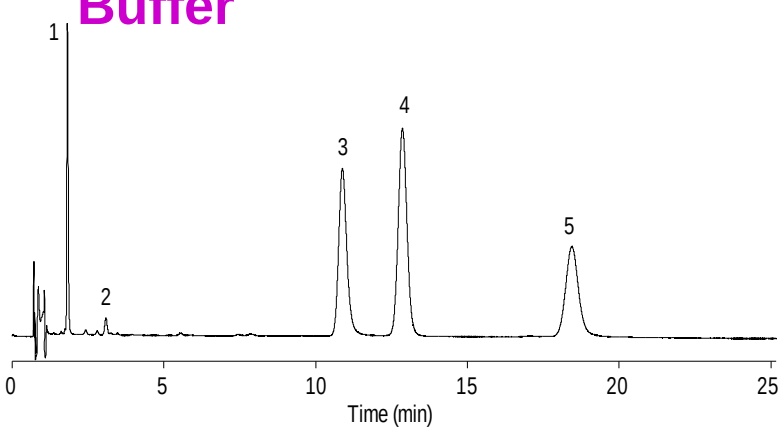
- Methanol as the organic modifier changes selectivity and increases the analys

Method Development – Change Bonded-Phase Separation of Plant Extract on Cyano Bonded Phase: Flavones, Flavanones, Phenolic Esters

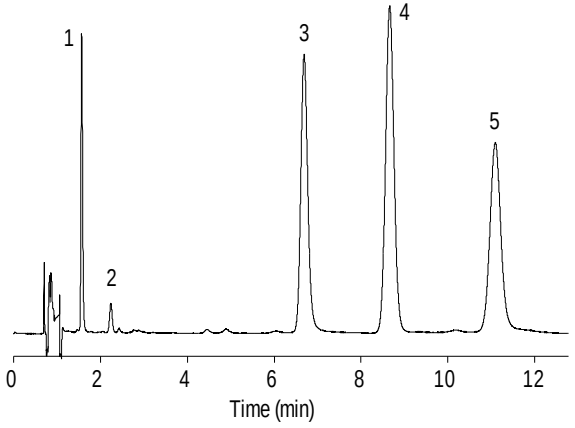
Column: ZORBAX Rapid Resolution SB-CN, 4.6 x 75 mm, 3.5 μm Mobile Phase: ACN: NaH_2PO_4, pH 2.5 Flow Rate: 1.0 mL/min

Temperature: RT Detection: UV 254 nm Sample: 1. Caffeic acid 2. Impurity 3. Luteolin 4. Naringenin 5. Apigenin

22% ACN: 78% Buffer

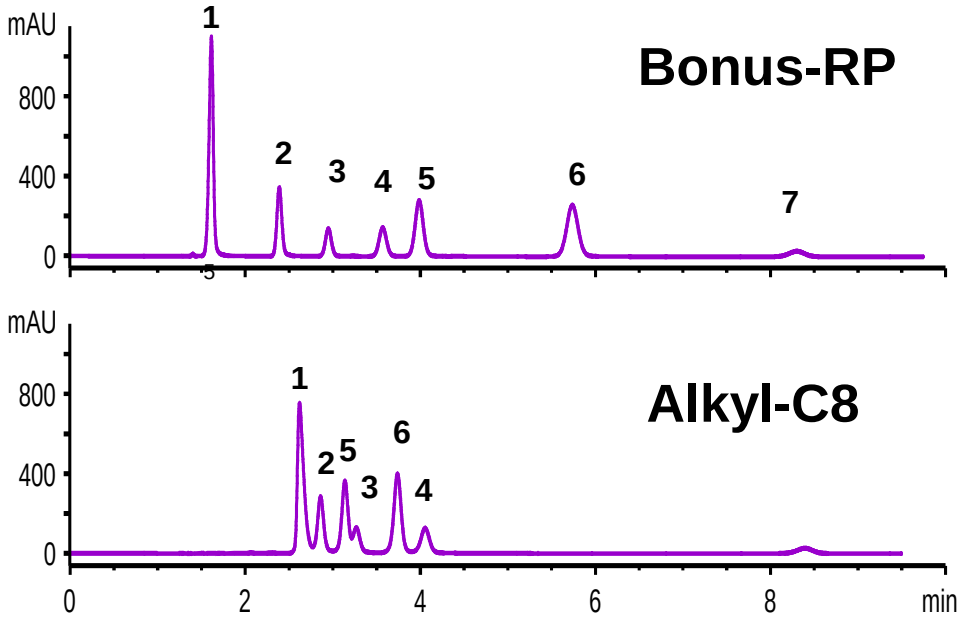


25% ACN: 75% Buffer



CN bonded phase with stronger mobile phase reduces analysis time by 50% and maintains retention of k=1 on 1st peak. Optimize and Test and Robustness!!!!

Bonus-RP Can Provide Alternate Selectivity to Alkyl Phases



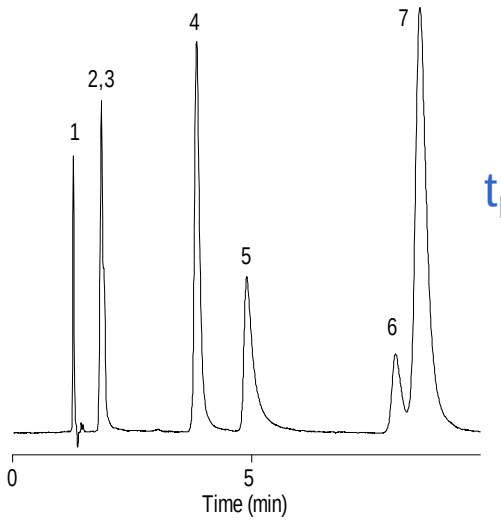
Columns:	4.6 x 150 mm	
Instrument:	HP 1100	
Mobile phase:	MeOH:0.1% TFA (70:30)*	
Flow rate:	1 mL/min.	
Temperature:	Ambient	
Inj.:	2 µL	
UV:	254 nm	
Sample:	1. Prometryne	5. Diuron
	2. Tebuthion	6. Propanil
	3. Atrazine	7. Dacthal
	4. Propazine	

*For low pH work, a TFA mobile phase is often preferred over phosphate, and is compatible with LC/MS.

High pH Increases Resolution of Antihistamines

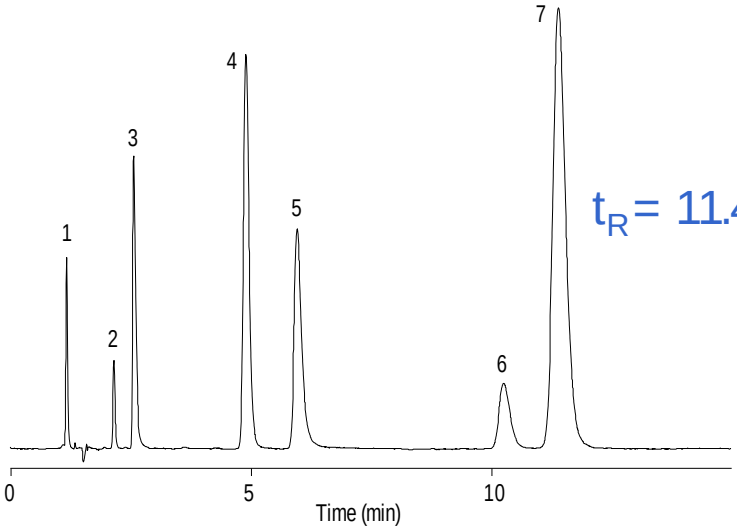
Extend-C18, 4.6 x 150 mm, 5 µm Mobile Phase: See Below Flow Rate: 1.0 mL/min Temperature: RT Detection
1. Maleate 2. Scopolamine 3. Pseudoephedrine 4. Doxylamine 5. Chlorpheniramine 6. Triprolidine 7. Diphenhydramine

pH 7
30% 20 mM Na_2HPO_4
70% MeOH



t_R = 8.5

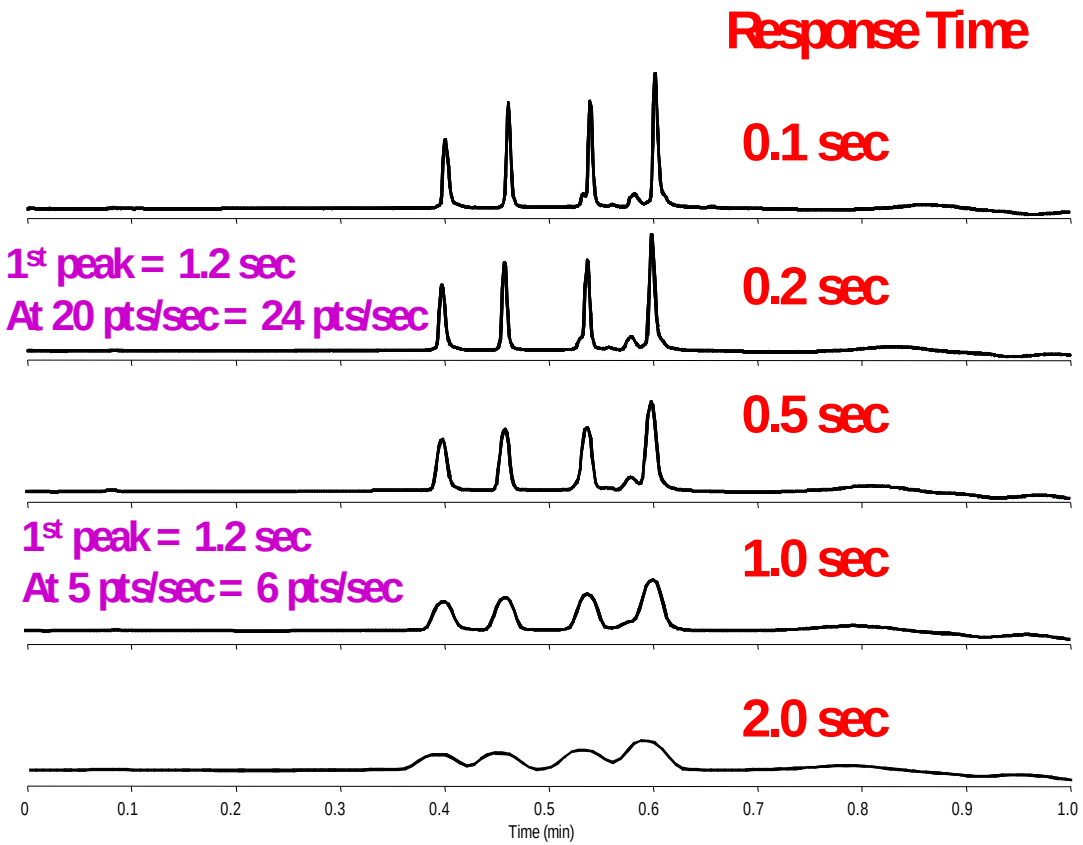
pH 11
30% 20 mM TEA
70% MeOH



t_R = 11.4

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

Effect of Detector Response Time on Fast Gradient Analyses



Agilent 1100 DAD
Agilent 1100 WPS with ADVR

Column:	**Poroshell 300SB-C18** 2.1 x 75 mm, 5 µm
Mobile Phase:	
A: 95% H_2O, 5% ACN with 0.1% TFA	
B: 5% H_2O, 5% ACN with 0.1% TFA	
Flow Rate:	2 mL/min
Temperature:	70° C
Detector:	UV 215 nm
Piston stroke:	20
Sample:	
1. Neurotensin	3. Lysozyme
2. RNaseA	4. Myoglobin

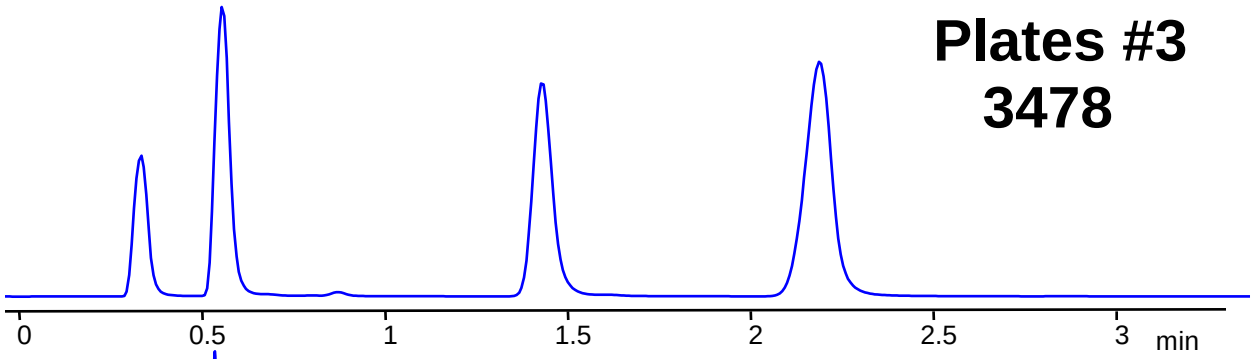
- You may have to adjust the response rate of your detector for rapid peak detecti

Optimization of Results with Rapid Resolution HT Columns - Data Acquisition Rate Comparison

Column: ZORBAX Rapid Resolution HT SB-C18 4.6 x 30 mm, 1.8 μm Mobile Phase: 60% Methanol: 40 Water Flow Rate: 1mL/min Temperature: RT Detection: UV 254 nm Sample: QC Test 1. Uracil 2. Phenol 3. 4-Cl-Nitrobenzene 4. Toluene

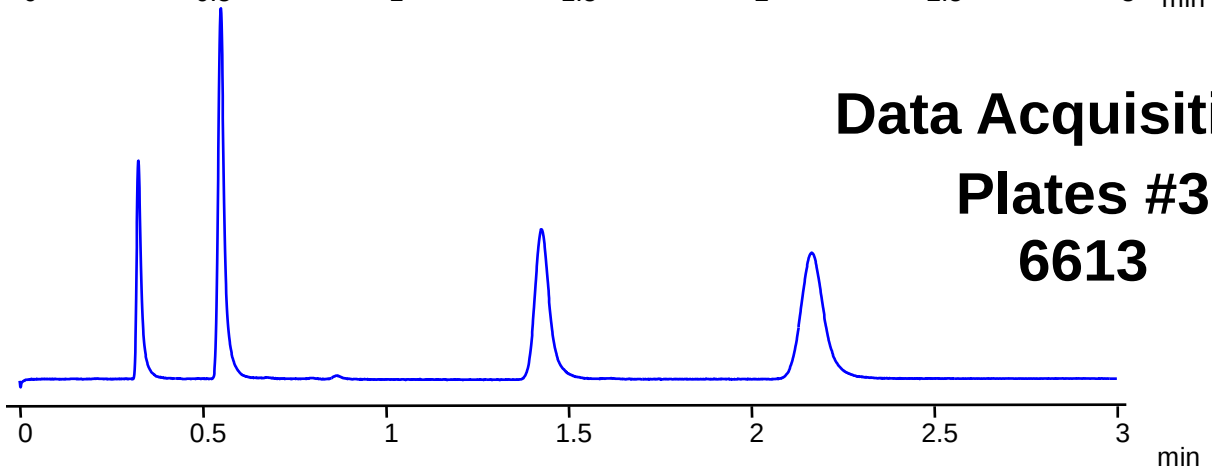
Data Acquisition Rate = 2 sec

Plates #3 3478 **Plates #4 4384**



Data Acquisition Rate = 0.1 sec

Plates #3 6613 **Plates #4 6138**



Conclusions

Get answers to key chemistry questions about your sample before you begin method development.

Use Small Particle columns (short columns with 3.5 u or 1.8u particles) to decrease method development time.

Evaluate methanol AND acetonitrile organic solvents for the best peak shape and selectivity.

Explore the use of Bonded Phase Selectivity to decrease analysis time and improve resolution. Consider alternate polarity phases to improve selectivity.

Use buffered mobile phases for ionizable compounds to get optimum performance on silica-based columns.

Choose proper type of column for varying pH conditions.

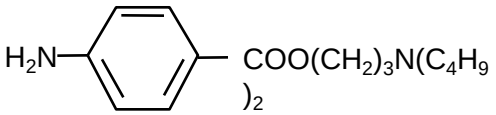
Appendix

Faster HPLC Methods Require Faster Data Acquisition

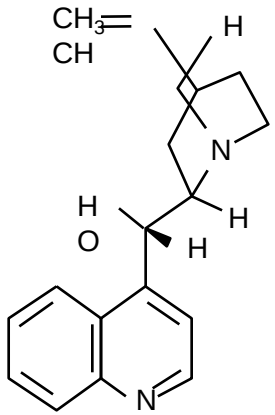
- Less Time Spent in the System Reduces Diffusion
- Reduced Diffusion Yields Narrower Peaks
- Narrower Peaks require Faster Rates of Data Acquisition

Basic Anesthetics

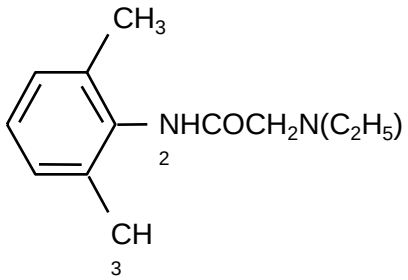
Butacaine



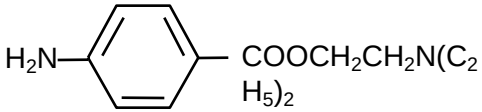
Cinchonine



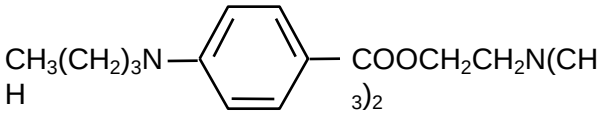
Lidocaine



Procaine



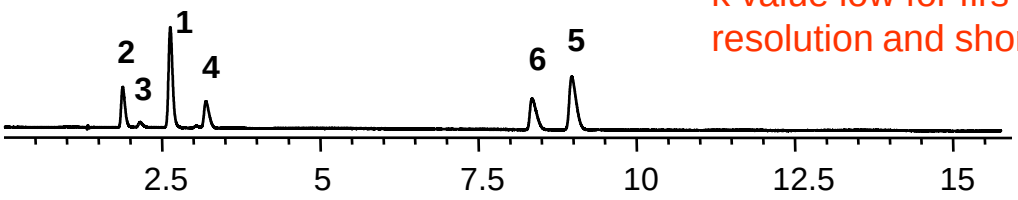
Tetracaine



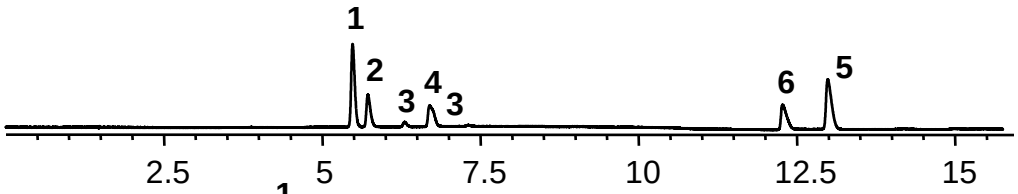
Basic Anesthetic Separation on Eclipse XDB Columns

k value low for firs peak, but excellent resolution and short analysis time

Eclipse XDB-CN

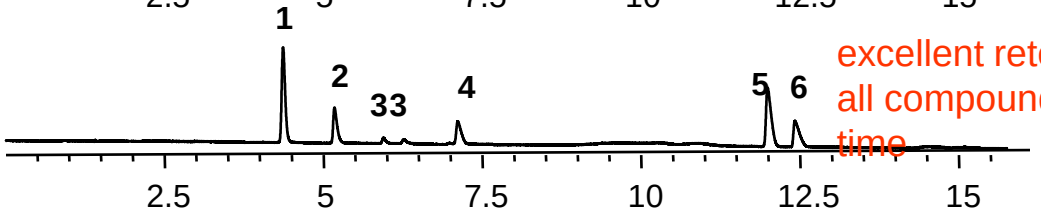


Eclipse XDB-Phenyl

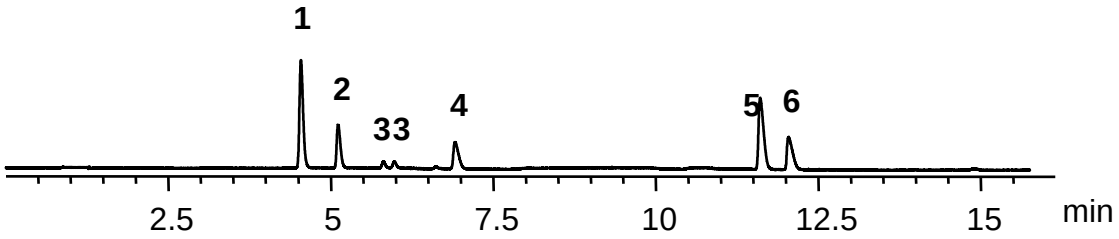


Eclipse XDB-C8

excellent retention and resolution of all compounds, with good analysis time



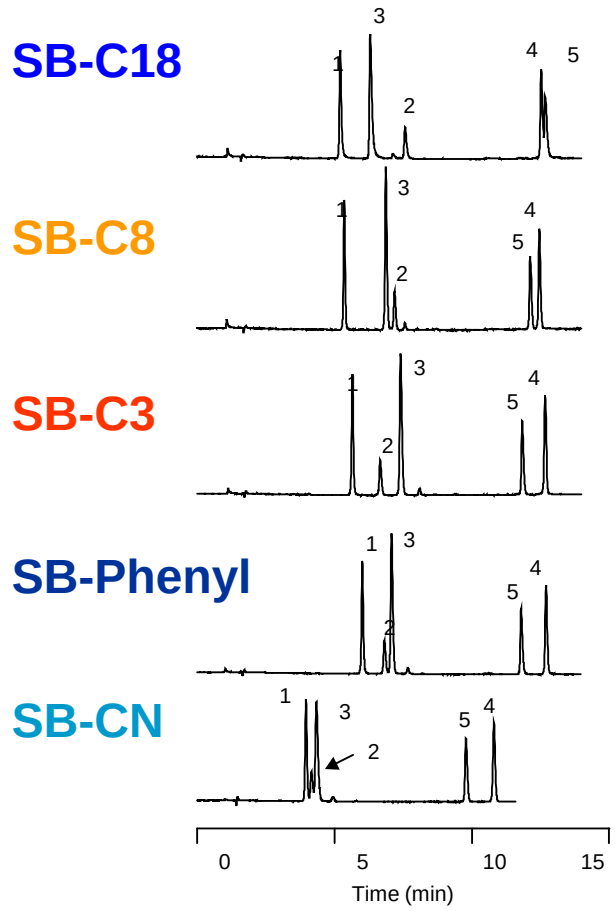
Eclipse XDB-C18



Dimensions: 4.6x150mm, 5μm Mobile Phase: 100% B in 18.8 min; A: 95:5 50mM NaH_2PO_4, pH 2.5:ACN; B: 47:53 50mM NaH_2PO_4, pH 2.5:ACN Flow Rate: 1.5 mL/min Injection: 5μL Temperature: 25 C Detector: UV, 254 nm

Sample: 1. Procaine (0.210μg/μl), 2. Cinchonine (0.224μg/μl), 3. Cinchonine impurity, 4. Lidocaine (0.232μg/μl), 5. Butacaine (0.214μg/μl), 6. Tetracaine (0.232μg/μl), 7. Mobile phase impurity

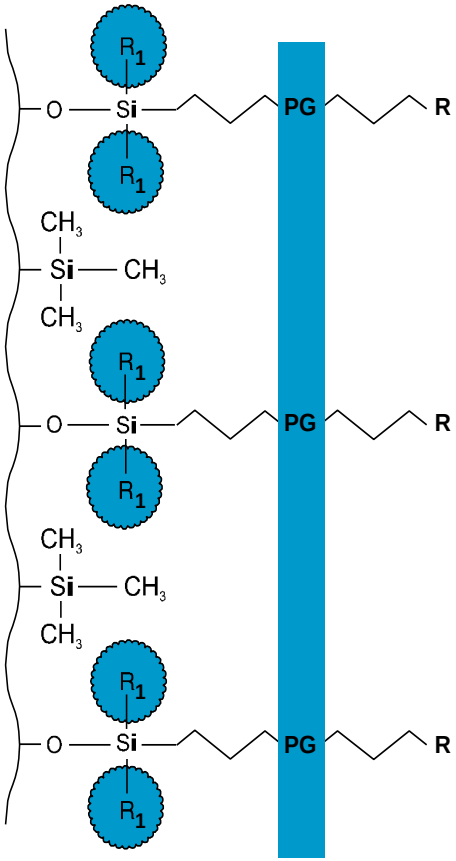
Separation of Basic Anesthetics at Low pH on StableBond Columns



Columns: 4.6 x 250 mm
Mobile Phase:
0 - 100% B in 18.8 min
A: 50 mM NaH_2PO_4, pH 2.5 in 95% H_2O/5% ACN
B: 50 mM NaH_2PO_4, pH 2.5 in 47% H_2O/53% ACN
Flow Rate: 1.5 mL/min
Temperature: 26°C
UV Detection: 254 nm
Sample: 10 µL
1. Procaine
2. Lidocaine
3. d-Cinchonine
4. Butacaine
5. Tetracaine

- **Closely related structures show changes in elution order as the polarity of the bonded phase changes.**

Zorbax Bonus-RP
Polar-linked alkyl phase



- Superb peak shape for basic compounds
- Long column life (pH 2-8)
- Unique selectivity
- Patented bonding technology
  - polar-linked alkyl phase for fast mass transfer giving good peak shape
  - bulky side groups give low pH stability
  - triple endcapped for mid-range pH stability and good peak shape

Choose Extend-C18 for High pH

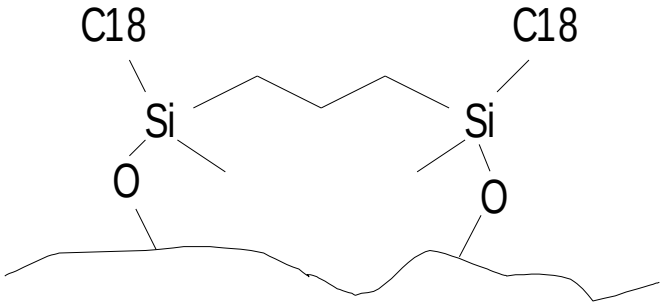
Patented bidentate C18-C18 bonding for superior high pH stability – up to pH 11.5

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



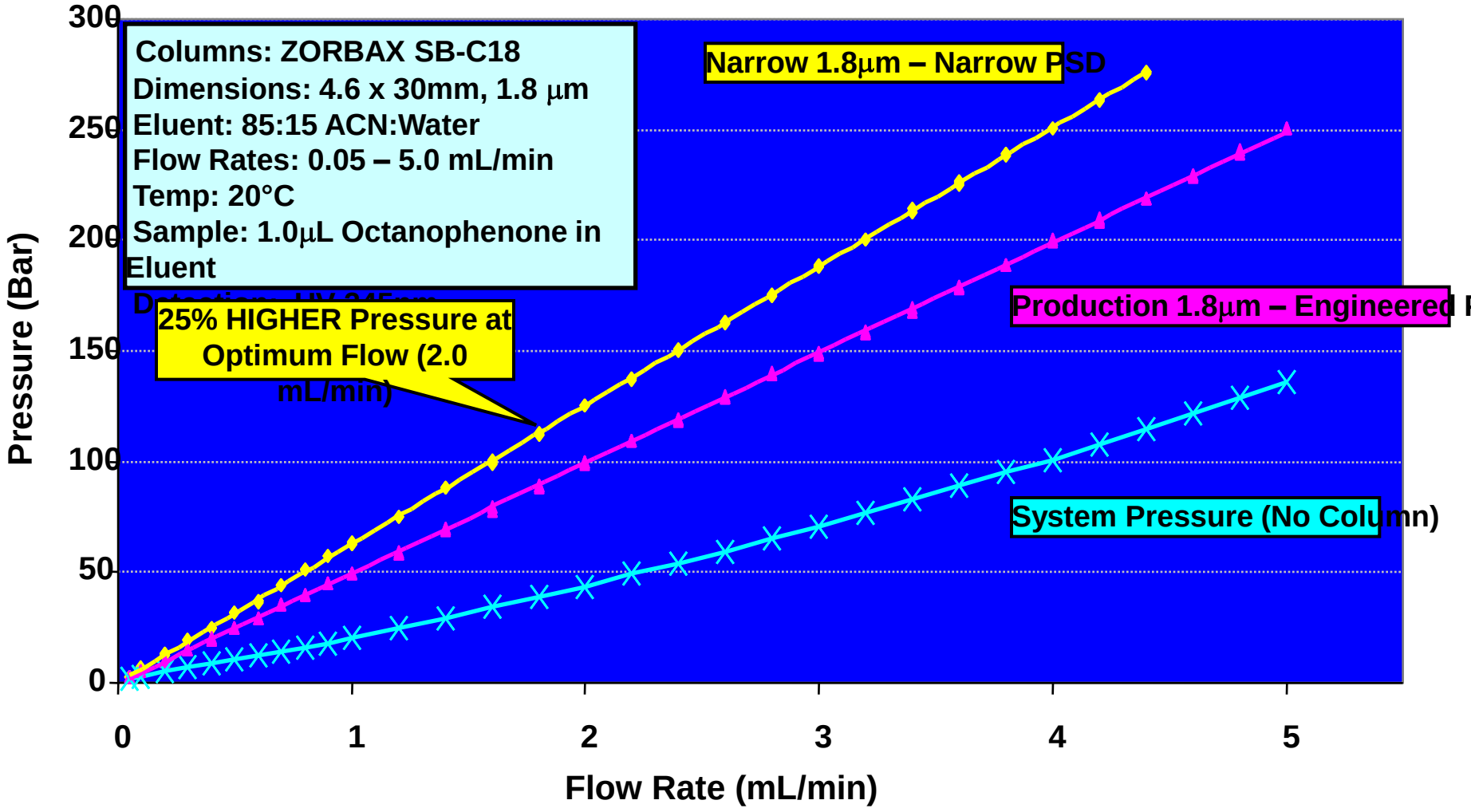
Zorbax RRHT (1.8 μm) Product Development Decisions Based on Performance Data

During product development, we investigated the effect of PSD on chromatographic performance and back pressure.

We decided to INTENTIONALLY alter the PSD because:

- Narrower PSD packings resulted in columns with MUCH higher back pressures.
- Engineered PSD packings resulted in LITTLE loss of column efficiency compared to the narrow PSD packings.
- Van Deemter plots for narrow vs. engineered PSD packings (1.8 μm) were nearly identical up to the optimum flow and then deviated only slightly at higher flows than optimum.

Back Pressure for Narrow vs. Engineered Particle Size Distribution 1.8µm SB-C18



Particle Size Distribution – ZORBAX RRHT

February 2007