HPLC Separation
Robustness and Ruggedness

Stopping Problems Before They Start

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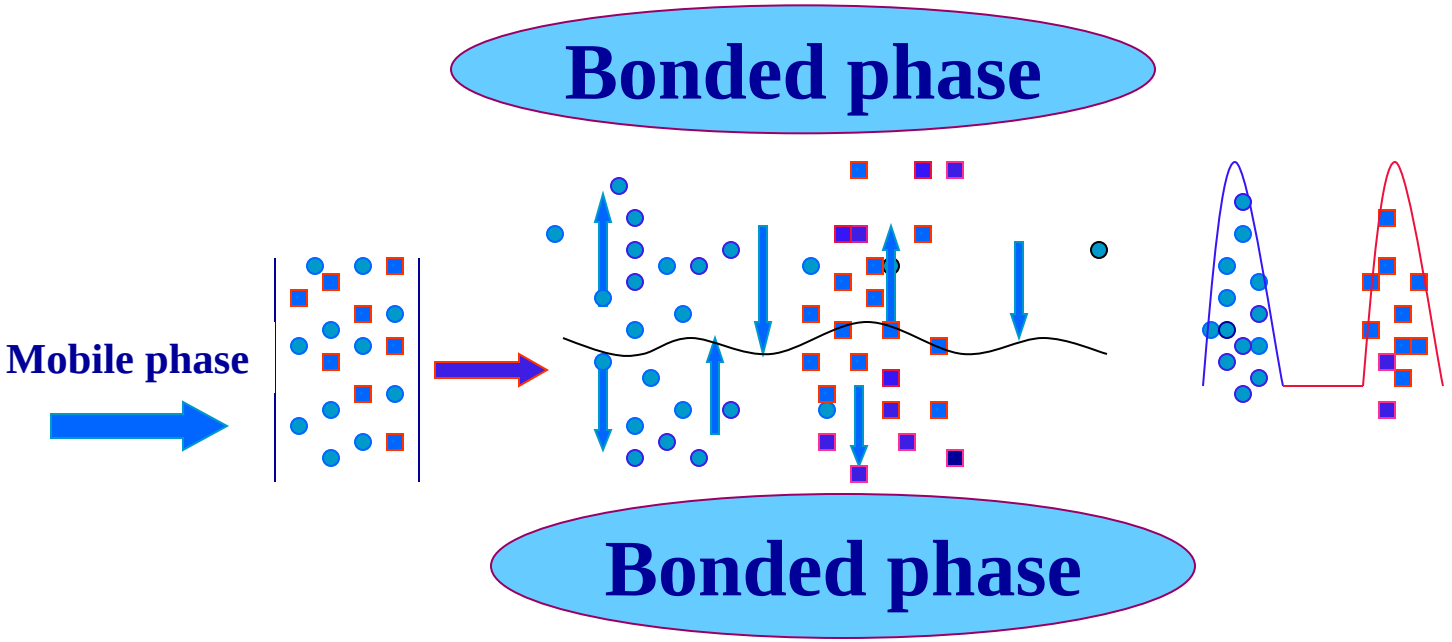
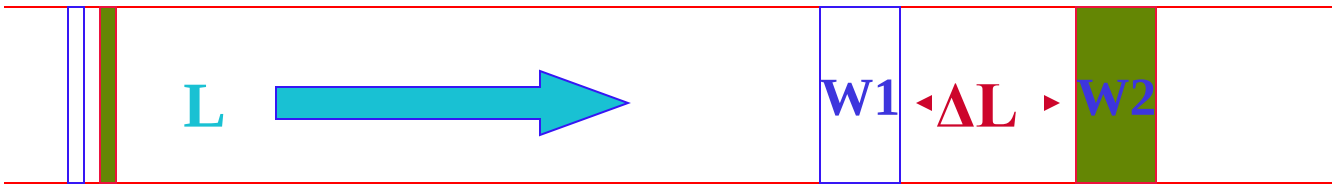
The Separation Process

- Differential partitioning of the components into the stationary and mobile phases.
- Separation controlled by chemical interaction of mobile phase/sample/bonded phase

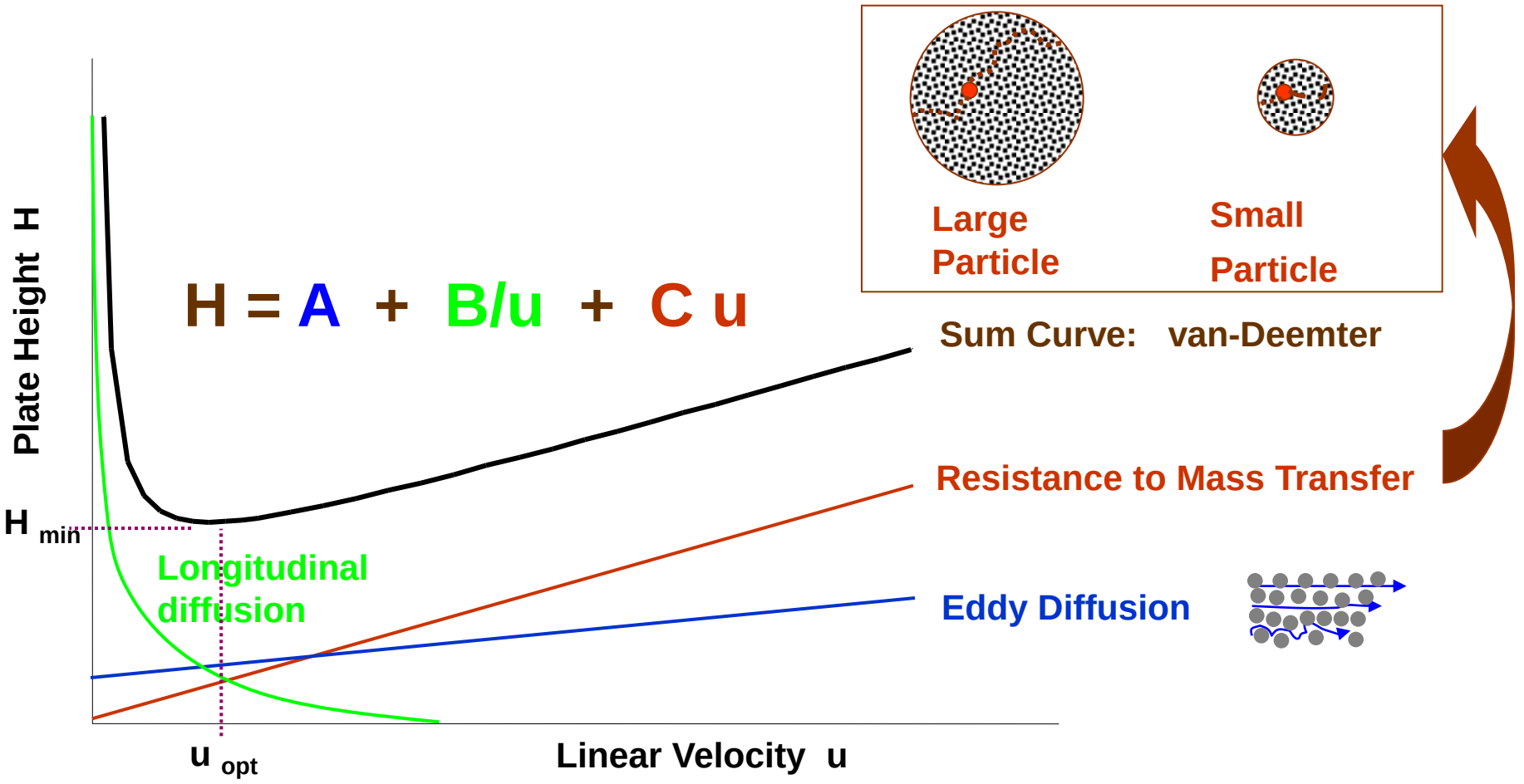


Separation Mechanism

- As Bands Travel the Length of the Column the Respective Distance (ΔL) Increases
- As Bands Move Through the Column (while in the Bonded Phase and Mobile Phase) Dispersion Causes the Band Width to Increase
- Choosing a High Efficiency Short Column Minimizes On-Column Dispersion and Improves Resolution



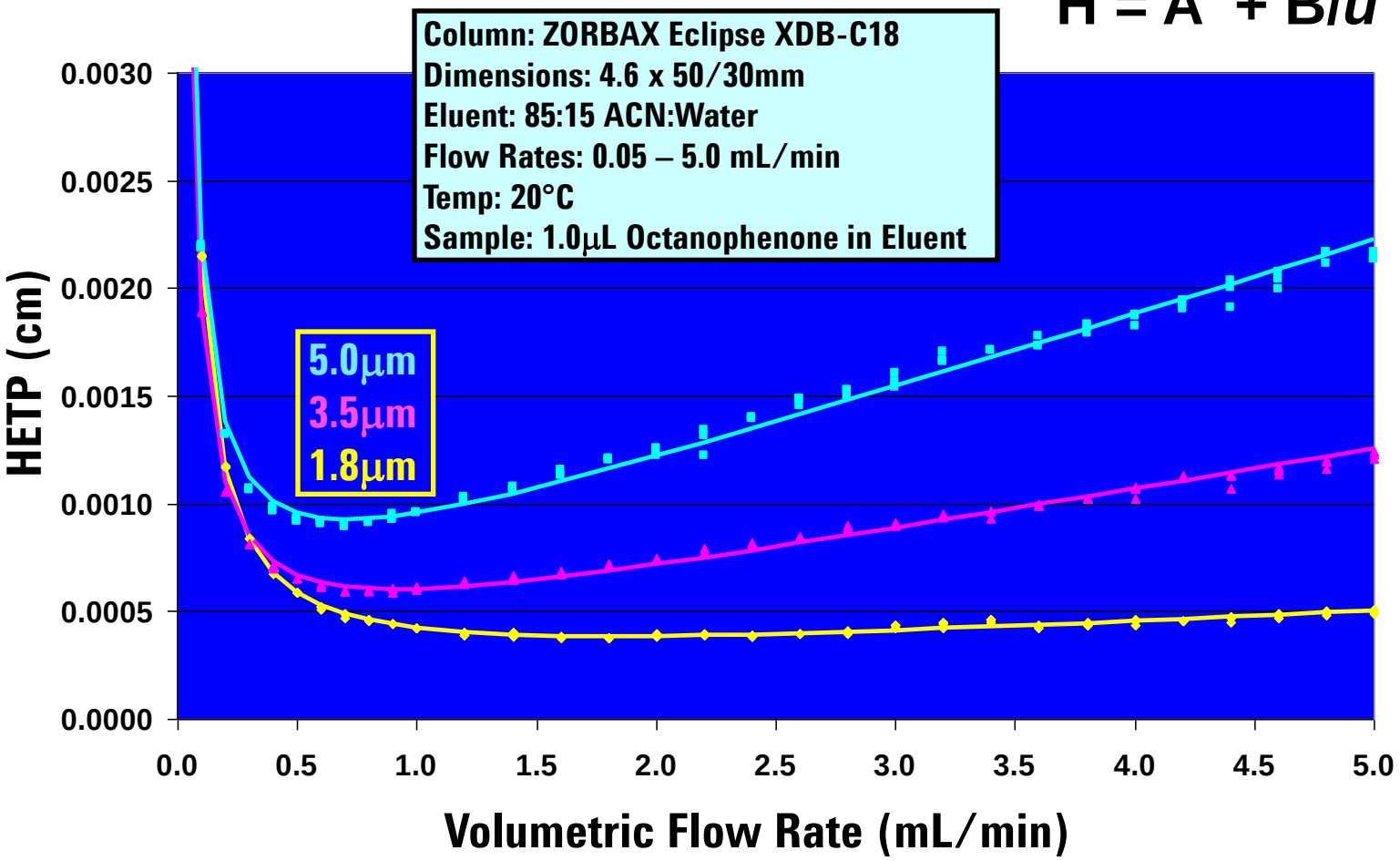
Smaller Particles Minimize Dispersion Effects



The smaller the plate height, the higher the plate number and the greater the chromatographic resolution

Smaller Particles More Efficient

$$H = A + B/u + Cu$$



Smaller particle sizes yield flatter curves, minima shift to higher flow rates

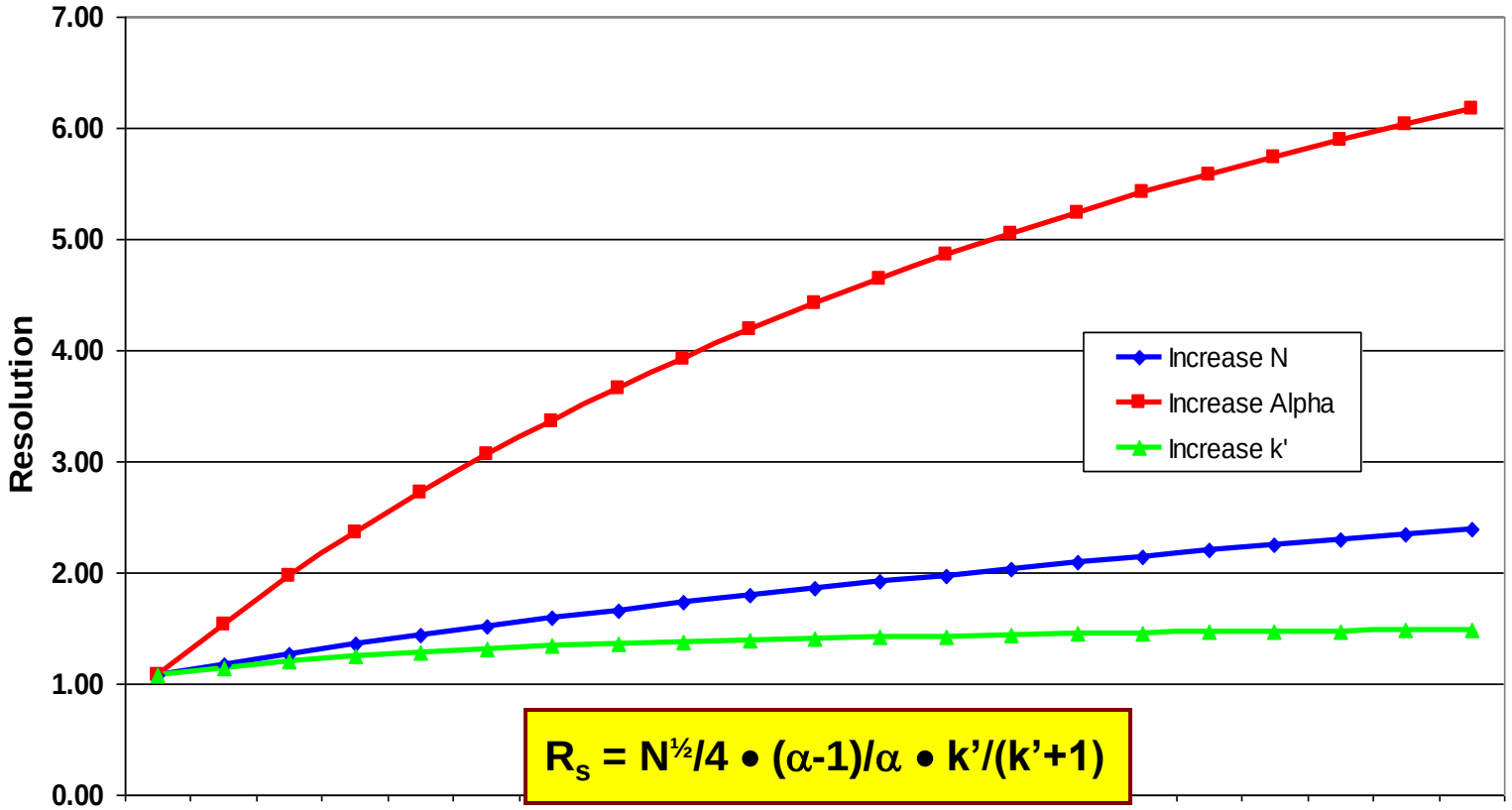
Combination of Factors Control Resolution

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{(\alpha-1)}{\alpha} \cdot \frac{k'}{k'+1}$$

Theoretical Plates ***Selectivity*** ***Retention***

Selectivity Impacts Resolution and Robustness

Change bonded phase or Change mobile phase – Change Chemistry



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 Goals

Performance

- **Robust**
- **Rugged**
- Specific/selective
- Accurate
- Precise
- Excellent linearity
- Broad range
- Low LOD and LOQ

Resource Use

— Minimal analysis time, solvent consumption, waste

— Adequate column lifetime

— Easy to use and minimal training required

Robustness and Ruggedness
Definitions

Robustness

- “a measure of [an analytical procedure’s] capacity to remain unaffected by small, but deliberate variations in method parameters”*
- prerequisite for a rugged method
- separation robustness: sensitivity of resolution to small, intentional changes in separation variables that may occur from day to day

Ruggedness

- “reproducibility of results when a method is performed as written under actual use conditions”*
- separation ruggedness: long-term reproducibility of resolution

*According to USP (United States Pharmacopoeia)

Method Validation Criteria

Where Do Robustness and Ruggedness Fit In?

ICH Validation Criteria

Specificity
Linearity
Range
Accuracy
Precision
Detection Limit
Quantitation Limit
Robustness
System Suitability Testing

Precision

- Repeatability
  - Single Lab: one day, analyst, instrument
- Intermediate Precision
  - Single Lab: multiple days, analysts, instruments
- Reproducibility **"Ruggedness"**
  - Multiple labs, days, analysts , instruments, etc.

How is Method Robustness Determined?

Systematically vary separation parameters and measure effects on R_s.

Incorporate parameter ranges into written method to allow flexibility.

Helps minimize or avoid many ruggedness problems, but not all.

How is Method Ruggedness Determined?

Assess method performance in two or more different labs—ideally over time.

Lack of ruggedness is often attributable to insufficient documentation, or differing practices, reagents, apparatus, and instrumentation.

Why Develop Robust and Rugged Methods?

They provide greater day-to-day reliability.

Rework is minimized—saving time and resources ($$$).

Likelihood of successful method transfer is improved.

Robustness and ruggedness are regulatory requirements for the pharmaceutical industry (ICH, FDA, USP).

Robustness and Ruggedness
Experimental Variables That Impact Resolution

Column
- column lot*

Mobile Phase
- buffer pH
- buffer concentration
- ionic strength
- % organic modifier

Sample
- injection volume
- solvent strength

Instrument
- column temperature
- detector flow cell volume*

Gradient
- dwell volume*
- gradient steepness

The Column

Column

- **Select high-quality column manufacturer.**
- **Select column with long lifetime at desired pH.**
- **Assess lot-to-lot reproducibility.**

Mobile Phase

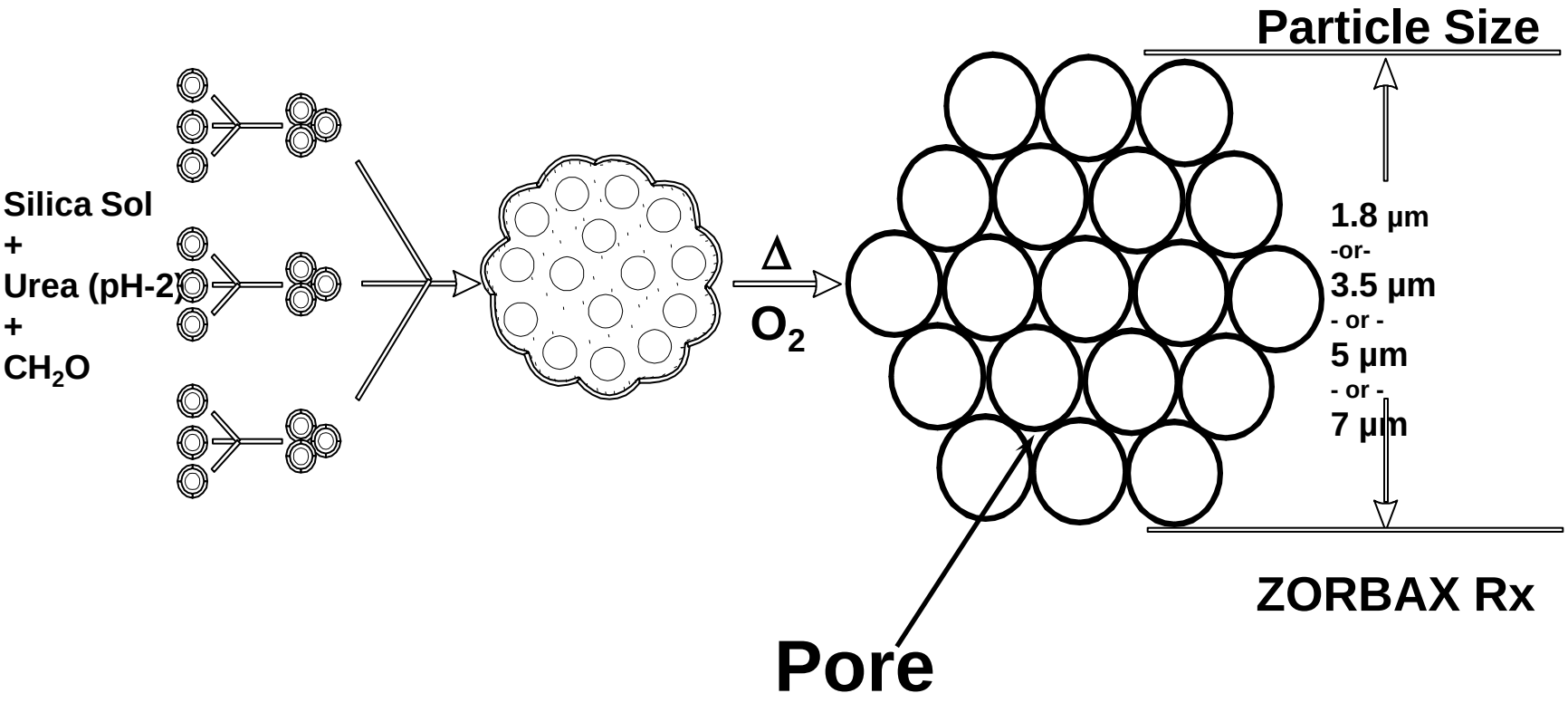
Sample

Instrument

Gradient Separations

ZORBAX® Manufacturing Process

Sol Aggregation



Uniform Silica Surface Key to Uniform Bonding

- **Silica Particle Production Usually Yields Heterogeneous Surface Chemistry**
  - Underlying -Si-O-Si- Chemical Structure
  - Free Silanols
  - Geminal Silanols
  - Not ideal for Uniform Chemical Bonding

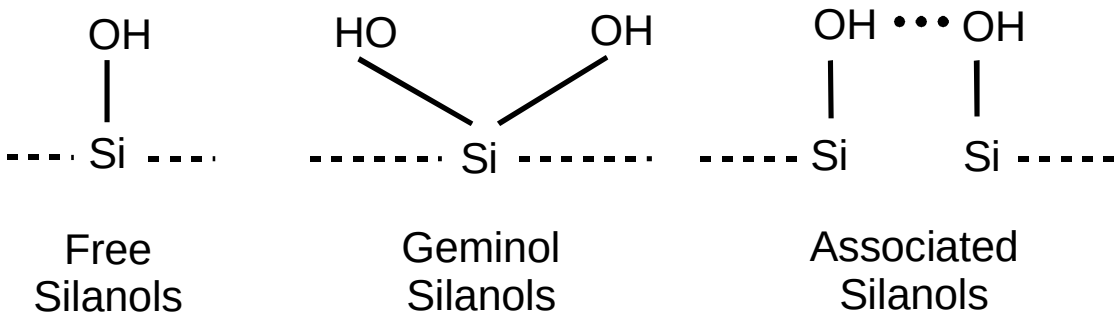
- **Re-Hydrolysis Increases Silanol Surface Population**

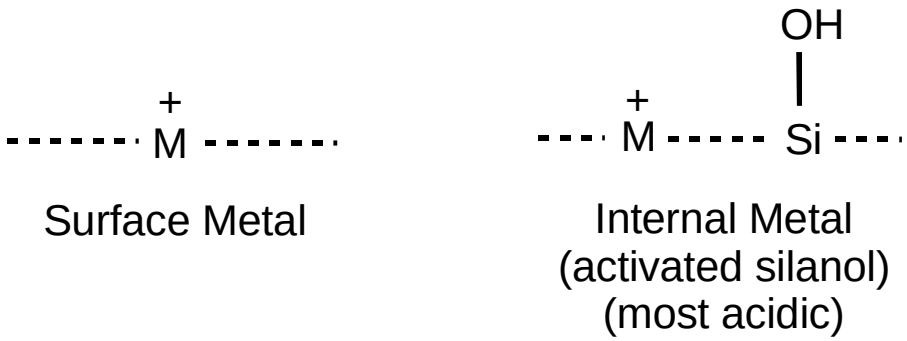
- **Theoretical Silanol Surface of ZORBAX PSM Particles**
  - 400µmole/M^2 for 80Angstrom Pore Particle
  - ~180M^2/gram (~1.5gram in 4.6x150mm Column dimension)

- **Thorough Re-Hydrolysis Produces Uniform Silanol Surface**
  - Maximum Silanol coverage
  - Associated Silanols with lower acidity

Silica Particle Surface Chemistry

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

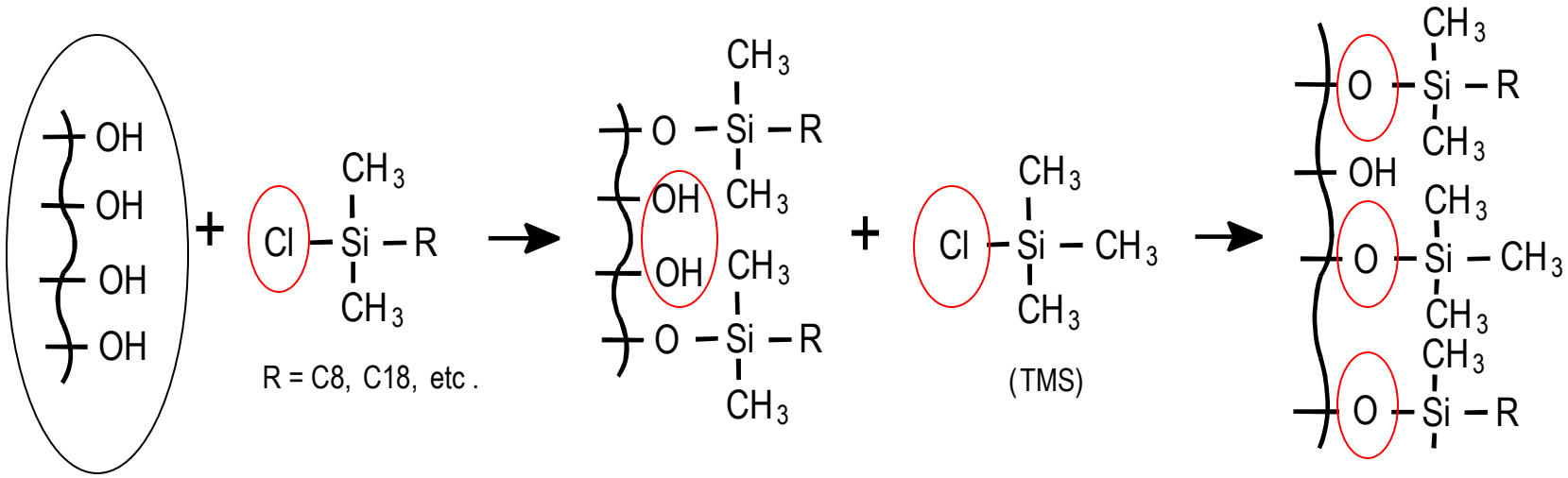




Caused by Using Impure Raw Material

Traditional Stationary Phase Silane Reaction

Dense Bonded Phase with Endcapping Reaction



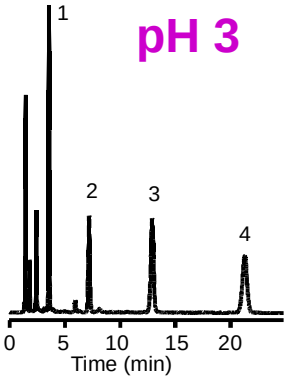
- **Dimethyl silanes**
- **Endcapped with TMS**

Selected ZORBAX Quality Testing Points

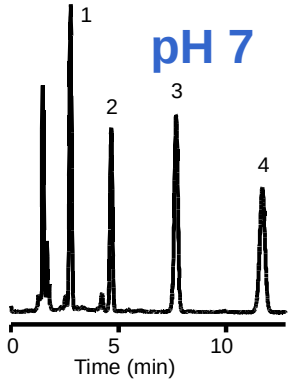
- Trace Metal Content
- Particle Size
- Particle Size Distribution
- Silanol Activity
- Bonded Phase Coverage
- Tailing Factor
- Non-polar Compound Retention
- Packing Efficiency (N)

ZORBAX HPLC Columns

Lot-to-Lot Reproducibility Improves Method Ruggedness



RSD	Retention, k´				Selectivity, α			
	Lot 1	Lot 2	Lot 3	% RSD	Lot 1	Lot 2	Lot 3	%
1. Cefotaxime	1.51	1.48	1.42	3.1	-	-	-	-
2. Cefoxitin	4.08	4.02	3.88	2.6	2.70	2.72	2.73	0.56
3. Cefamandole	8.17	8.04	7.74	2.8	2.00	2.00	1.99	0.29
4. Cephalothin	14.02	13.92	13.39	2.5	1.72	1.72	1.73	0.34



RSD	Retention, k´				Selectivity, α			
	Lot 1	Lot 2	Lot 3	% RSD	Lot 1	Lot 2	Lot 3	%
1. Cefotaxime	0.80	0.88	0.88	5.4	-	-	-	-
2. Cefoxitin	1.96	2.14	2.15	5.1	2.45	2.43	2.44	0.41
3. Cefamandole	3.83	4.15	4.16	4.6	1.96	1.94	1.94	0.59
4. Cephalothin	6.26	6.79	6.84	4.8	1.64	1.64	1.64	0.00

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

How Do You Assess Lot-to-Lot Reproducibility?
Key Contributor to Method Ruggedness

Test 3 different column lots and evaluate separation performance.

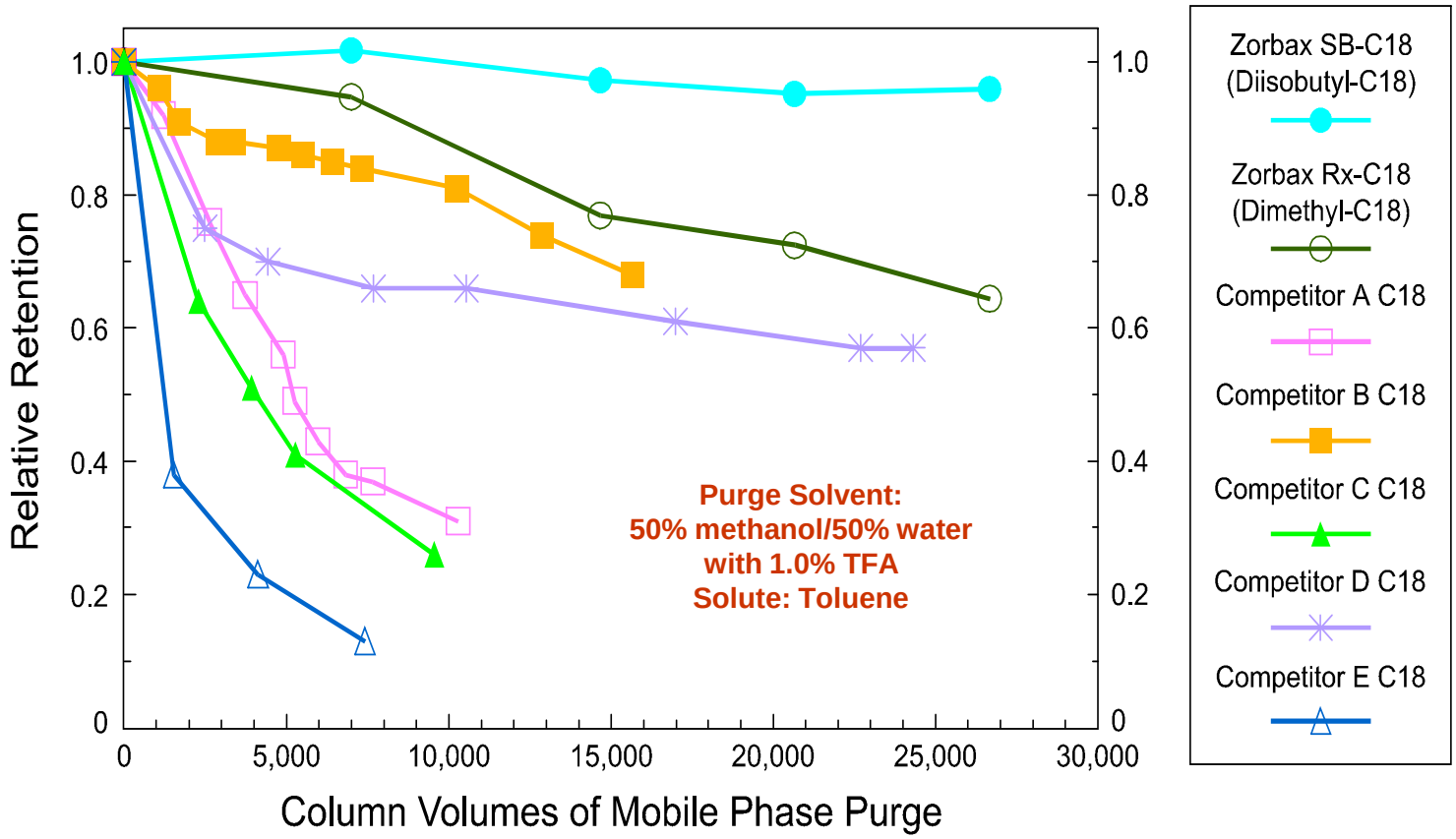
- Compare retention, selectivity, resolution, peak width and symmetry.

Agilent Technologies ZORBAX validation kits and special orders

- Contact local Agilent LC Column Product Specialist
- Call Agilent Technical Support, (800) 227-9770.

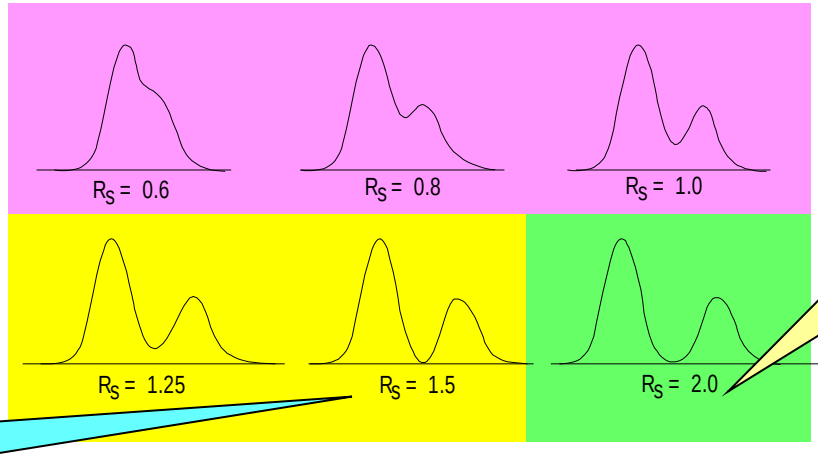
Select a Column With Long Lifetime

Low pH and High Temperature (pH 0.8, 90°C) Stress Test



Kirkland, J.J. and J.W. Henderson, Journal of Chromatographic Science, 32 (1994) 473-480.

Method Development
How Much Resolution is Necessary?



Aim for $R_s \geq 2.0$ between all analytes

Baseline Resolution $R_s = 1.5$

If your method has insufficient R_S, you will compromise accuracy, precision, robustness and ruggedness.

Initial resolution can decrease due to changes in separation variables.

Build in robustness so that ΔR_s is small when separation variables are changed.

Mobile Phase: Aqueous Component

Experimental Variables That Impact Resolution

Column

Mobile Phase

- **Aqueous Component**
  - **Importance of Buffers**
  - **Considerations for Buffer Selection**
  - **Buffer pH**
  - **Buffer Concentration**
- Organic Component

Sample

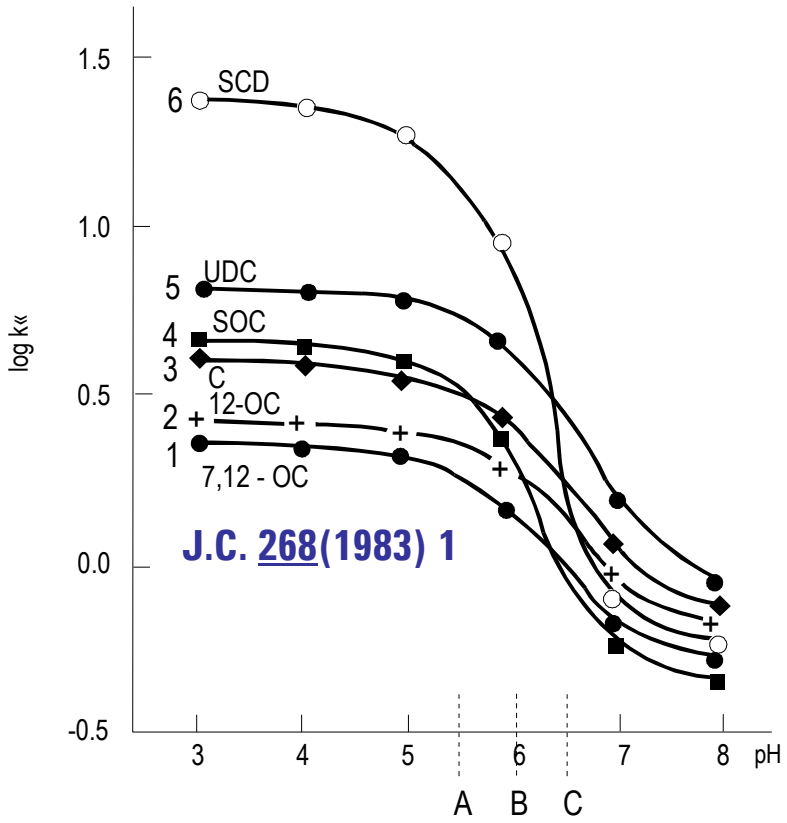
Instrument

Gradient Separations

<u>Your</u> opportunity to improve robustness and ruggedness

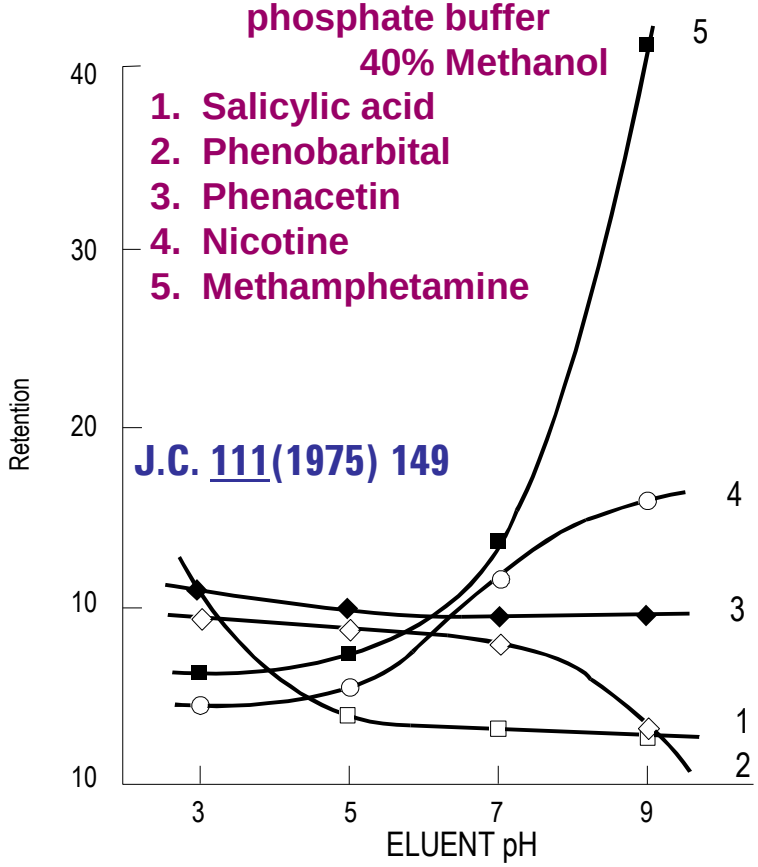
pH vs. Selectivity for Acids and Bases

Column: Nucleosil-C18
Mobile Phase: 45% ACN/55% phosphate buffer
Sample: Bile Acids



Column: µBondapak-C18
Mobile Phase: 60% 25 mM phosphate buffer
40% Methanol

1. **Salicylic acid**
2. **Phenobarbital**
3. **Phenacetin**
4. **Nicotine**
5. **Methamphetamine**



- **Retention and selectivity can change dramatically when pH is changed.**

Buffered Mobile Phases are Important for Controlling the Retention of Ionizable Analytes

BUFFERS:

Provide effective means for varying and controlling pH

Improve retention, peak width and symmetry (especially for pH $\leq$ 3)

Minimize or eliminate column-to-column differences

Eliminate differences in water pH

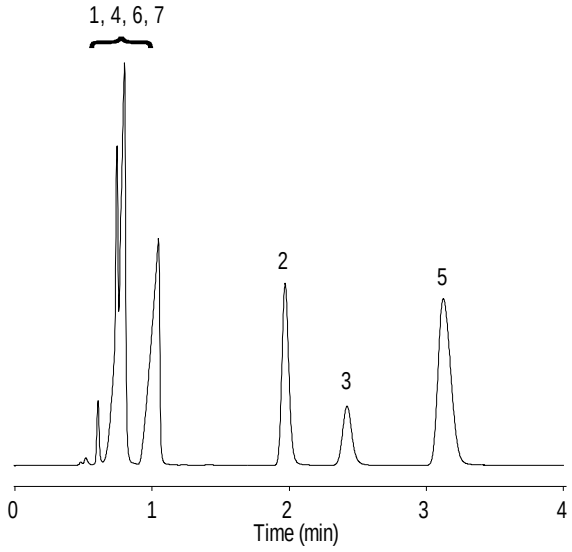
Allow efficient use of pH as separation variable during method development

Separation pH must be set accurately and reproducibly

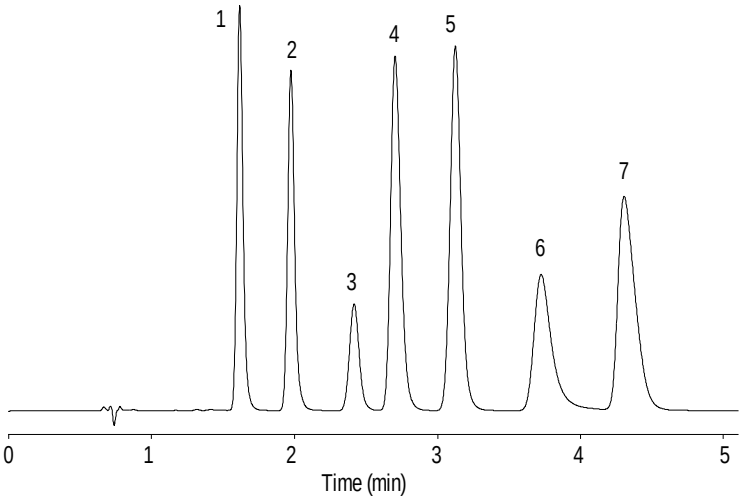
Why Use Buffered Mobile Phases?

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 4. fenoprofen 5. propyl paraben 6. propranolol 7. ibuprofen

A = pH 7.0 water



A = pH 7.0, 25 mM phosphate buffer



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

Considerations For Buffer Selection

Buffer Type

- Inorganic vs. organic buffers—choice can affect resolution, column lifetime and MS compatibility

Buffer pH

- Select buffer based on desired pH and optimum buffer pH range.
- Measure pH of buffer solution before mixing with organic modifier.
- Compare resolution at desired pH ± 0.1–0.2 pH units.

Buffer Concentration and Ionic Strength

- Start at 20 – 25 mM.
- Prepare buffer according to accepted procedures.
- Avoid overshoot and readjustment when setting pH.
- Compare resolution at desired buffer concentration ± 5–10 mM.

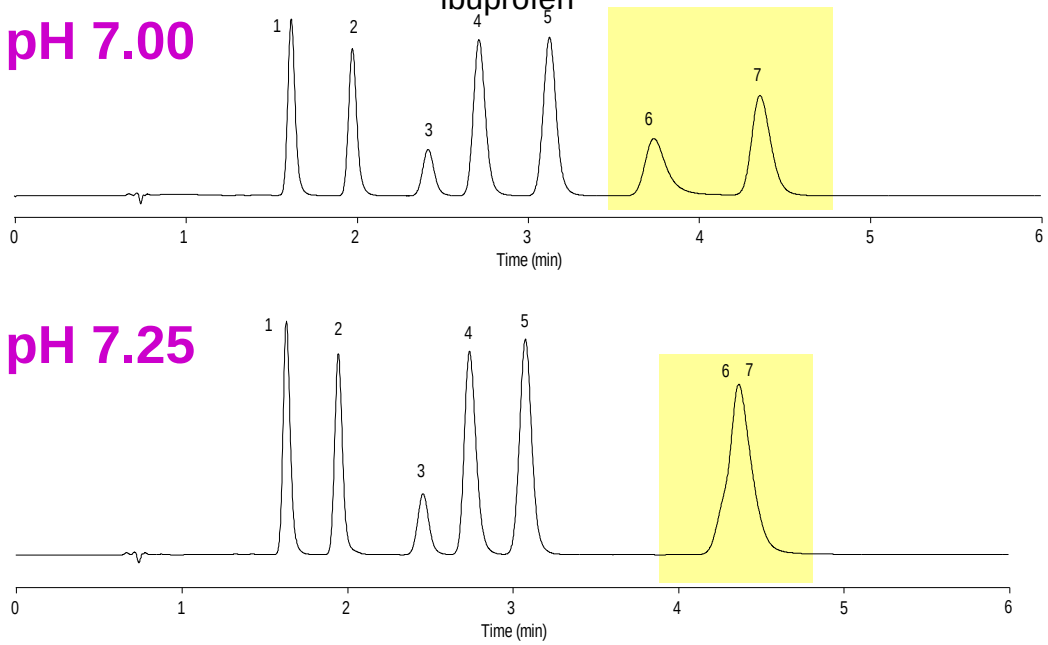
Test for pH Robustness

Column: ZORBAX Rapid Resolution Eclipse XDB-C8, 4.6 x 75 mm, 3.5 µm

Mobile Phase: 44% 25 mM phosphate, pH 7.00 : 56% methanol Flow Rate: 1.0 mL/min Temperature: 25°C

Detection: UV 250 nm

Sample: 1. ketoprofen 2. ethyl paraben 3. Hydrocortisone 4. fenoprofen 5. propyl paraben 6. Propranolol 7. ibuprofen



- The resolution of ionizable compounds can change markedly with pH changes—even as small as 0.05–0.25 pH units.

Changes in Buffer Concentration Can Affect Retention, Peak Width and Peak Shape

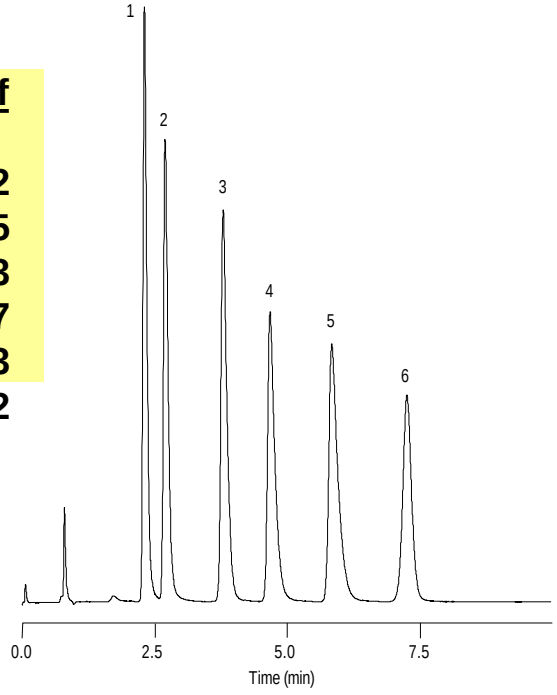
Column: ZORBAX Eclipse XDB-C8, 4.6 x 150 mm, 5 µm

Mobile Phase: 40% phosphate buffer (pH 7.0) : 60% ACN Flow Rate: 1.5 mL/min Temperature: 40°C

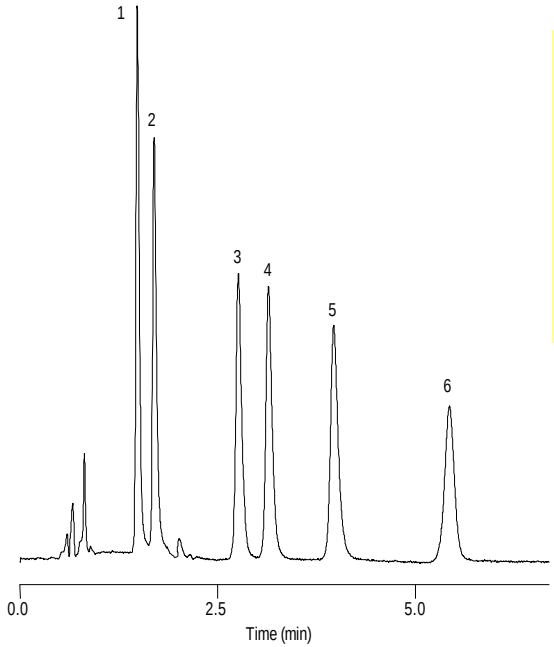
Sample: Tricyclic Antidepressants, 1. Desipramine 2. Nortriptyline 3. Doxepin 4. Imipramine 5. Amitriptyline 6. Trimipramine

10 mM Phosphate

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



25 mM Phosphate



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

Mobile Phase: Organic Component

Column

Mobile Phase

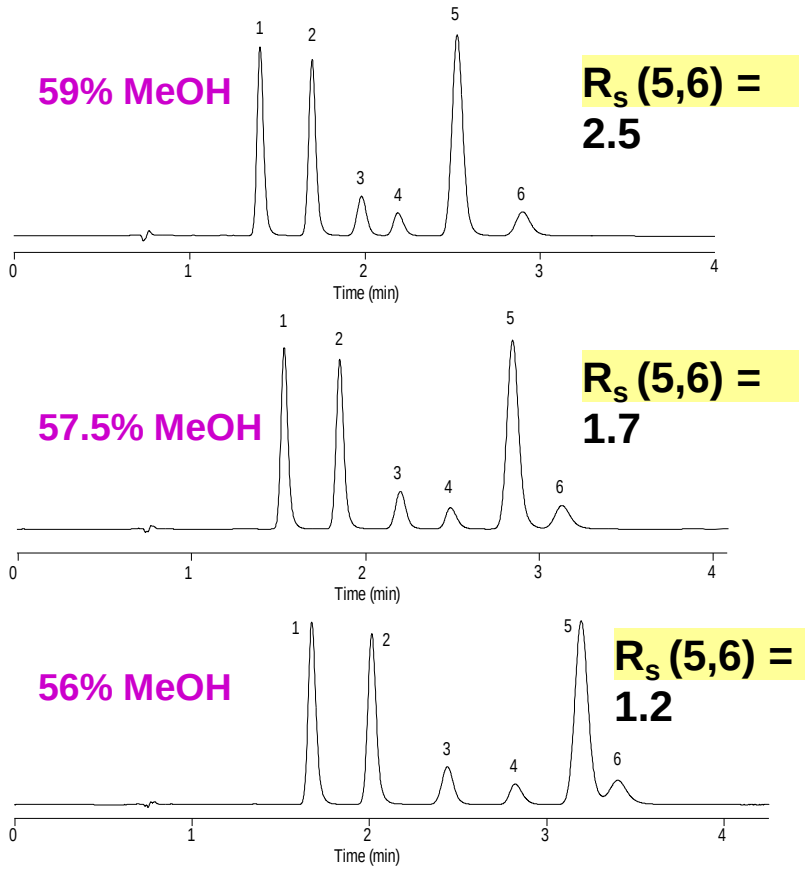
- Aqueous Component
- **Organic Component**
  - **% Organic Modifier**

Sample

Instrument

Gradient Separations

Even a Small Change in % Organic Modifier Can Change Resolution



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

4.6 x 75 mm, 3.5 µm

Mobile Phase: A: 25 mM phosphate, pH 7.00 (10 mM TEA)

B: methanol (10 mM TEA)

Flow Rate: 1.0 mL/min

Temperature: 25°C controlled

Injection: 5 µL

Detection: 275 nm

Sample: 1. ketoprofen 2. ethyl paraben 3. hydrocortisone

4. fenoprofen 5. propyl paraben 6. propranolol

- Verify that resolution doesn't change significantly around desired conditions (for example, %B ± 1–2%).

The Sample
Experimental Variables That Impact Resolution

Column

Mobile Phase

Sample

- **Injection volume**
- **Sample solvent strength**

Instrument

Gradient Separations

Injection Volume and Sample Solvent Strength

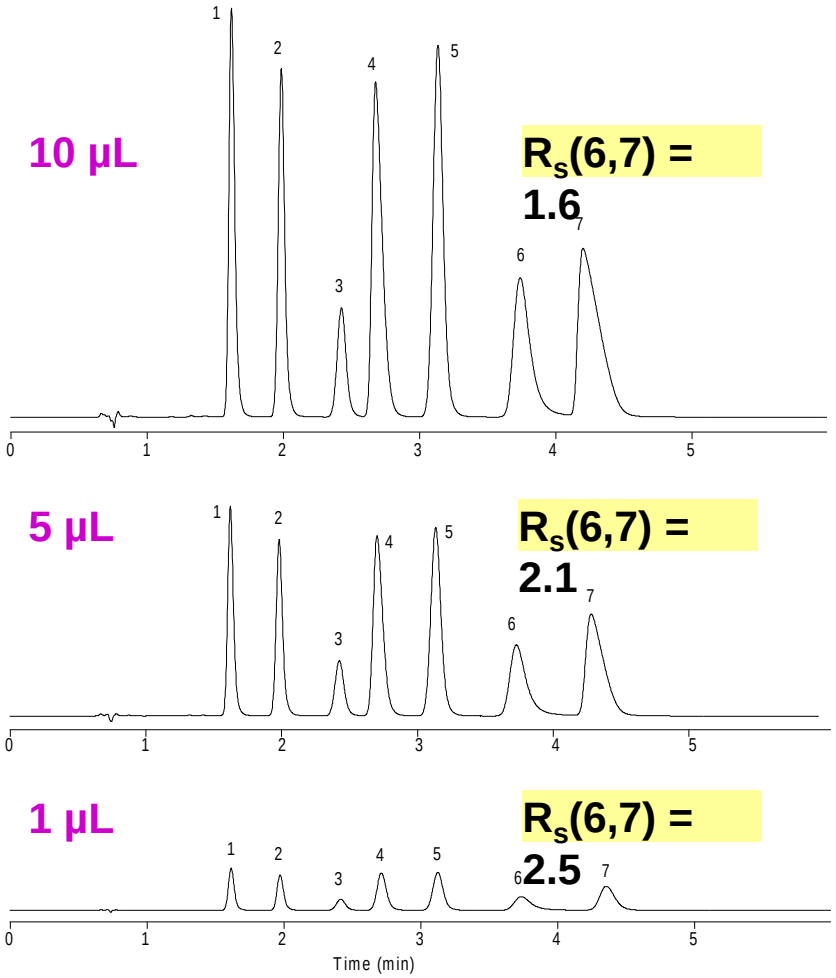
Injection Volume

- Lack of ruggedness typically seen
  - when V_{inj} is increased to improve signal-to-noise (S/N) ratio, or,
  - when column size is decreased.
- Use minimum V_{inj} for required repeatability and limit of detection.
- Compare resolution, peak shape and repeatability at 0.2X, 1X and 2–5X V_{inj}.

Sample Solvent Strength

- Match % organic modifier in mobile phase (or weaker).
- If stronger sample solvent needed (solubility, stability), keep V_{inj} to minimum.
- Compare resolution, peak shape and width at desired solvent strength ±50% relative.

Test For Injection Volume Robustness



Column: **ZORBAX Rapid Resolution Eclipse XDB-C8**
4.6 x 75 mm, 3.5 µm

Mobile Phase: 44% 25 mM phosphate, pH 7.00
56% methanol

Flow Rate: 1.0 mL/min

Temperature: 25°C

Detection: UV 250 nm

Sample:
1. ketoprofen
2. ethyl paraben
3. hydrocortisone
4. fenoprofen
5. propyl paraben
6. propranolol
7. ibuprofen

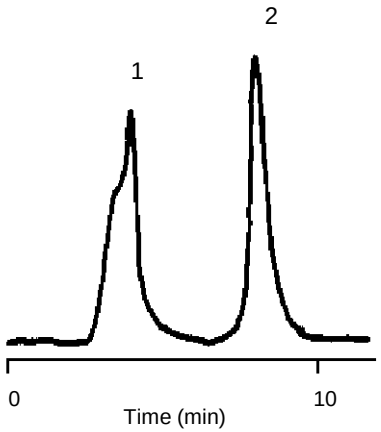
- Varying injection volume can sometimes reveal lack of robustness for resolution and peak shape.

Strong Sample Solvent Can Compromise Peak Shape

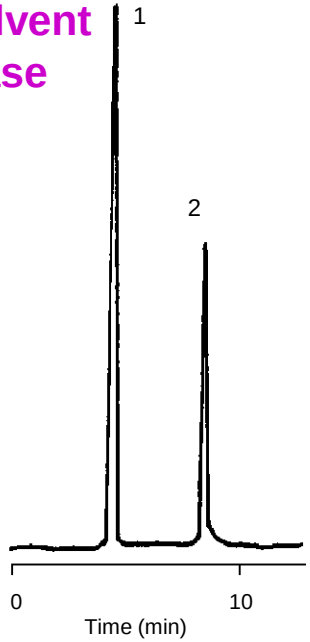
Column: ZORBAX SB-C8, 4.6 x 150 mm, 5 µm Mobile Phase: 82% H_2O:18% ACN Injection Volume: 30 µL

Sample: 1. Caffeine 2. Salicylamide

A. Sample Solvent 100% Acetonitrile



B. Sample Solvent Mobile Phase



Instrument Parameters

Column

Mobile Phase

Sample

Instrument

- **Column temperature**
- **Detector flow cell volume**

Gradient Separations

Column Temperature
Adequate Temperature Control is Essential

Laboratory temperatures can vary by ±5°C or more.

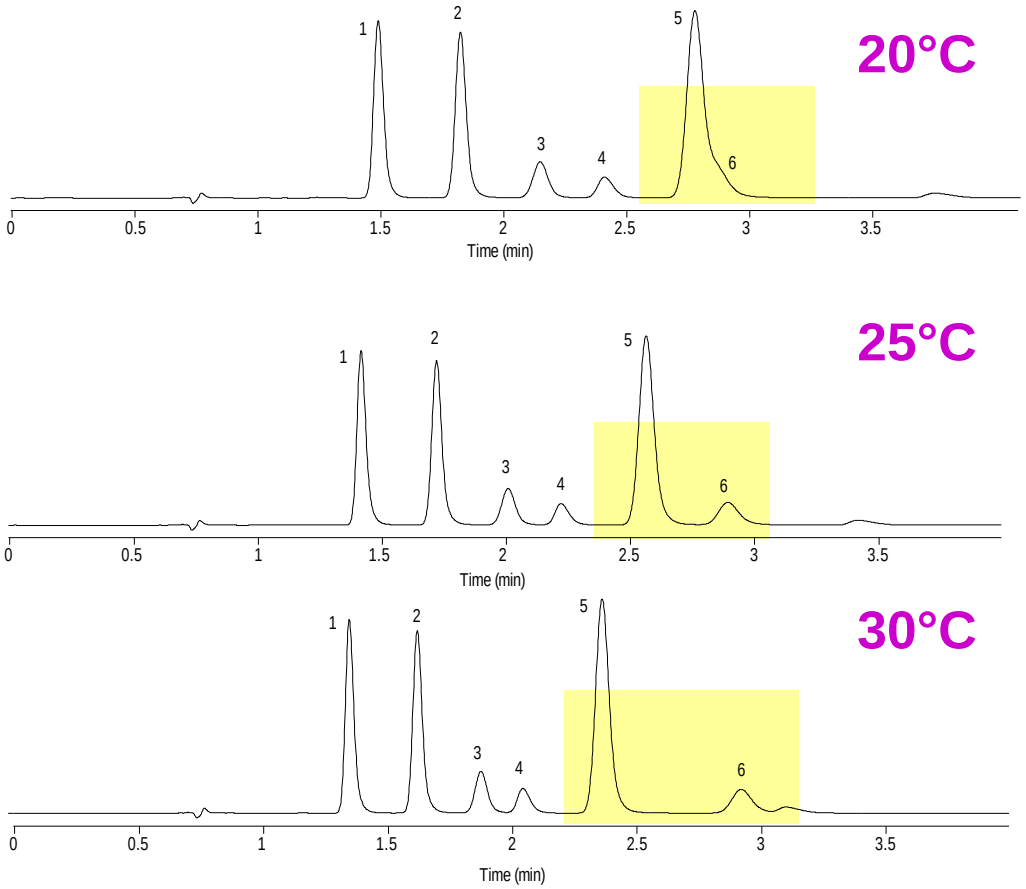
Column temperature changes affect resolution and repeatability.

Useful tool for changing selectivity, retention and efficiency when developing separations.

Important parameter to control during method development and validation.

Compare resolution, peak width and peak shape at desired temperature ±5°C.

Small Temperature Changes Can Cause Dramatic Changes in Resolution



Column: **ZORBAX Rapid Resolution Eclipse XDB-C8 4.6 x 75 mm, 3.5 µm**

Mobile Phase: Isocratic, 28%B : 72%A

A: 5/95 methanol/pH 7.00 buffer 25 mM, 10 mM TEA

B: 80/20 methanol/pH 7.00 buffer 25 mM, 10 mM TEA

Flow Rate: 1.0 mL/min.

Temperature: See Figure

Injection: 5 µL

Detection: 275 nm

Sample: 1. ketoprofen 2. ethyl paraben 3. hydrocortisone 4. fenoprofen 5. propyl paraben 6. propranolol

- Column temperature control will produce the most consistent results.

Differences in Detector Flow Cell Volume Can Affect N and R_s

Scenario: ZORBAX Rapid Resolution Column: 75 mm, 3.5-µm; Flow Rate: 1 mL/min; k = 3

Flow Cell Volume	Band Broadening* (4.6 mm)	Band Broadening* (2.1 mm**)
1.7 µL	0.3%	6%
8 µL	6%	138%
14 µL	19%	423%

*Versus 8571 theoretical plates (HPLC Calculations Assistant, Version 2.1, Savant Audiovisuals)
**Flow Rate, 0.2 mL/min

Gradient Separations

Column

Mobile Phase

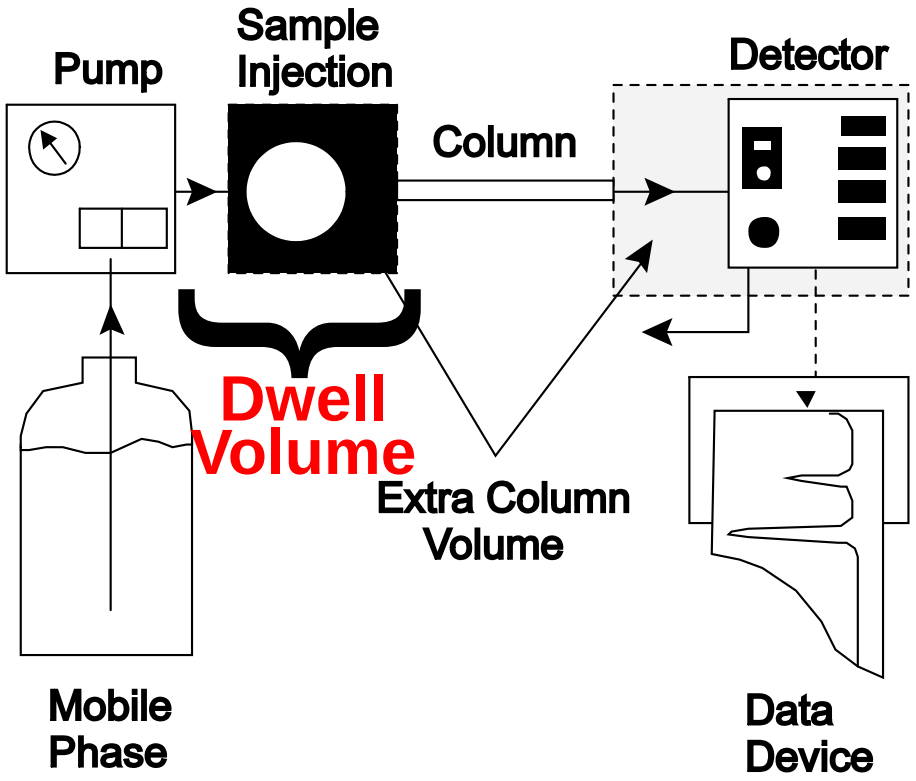
Sample

Instrument

Gradient Separations

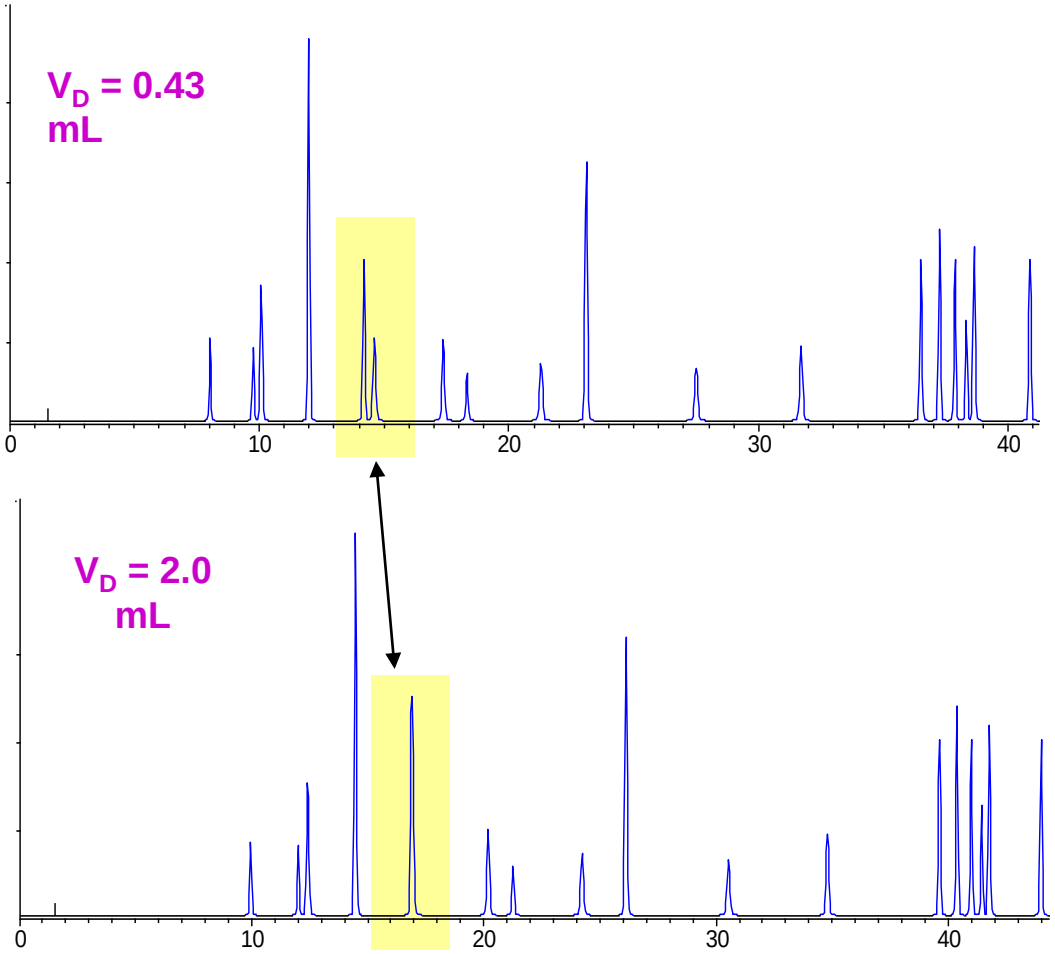
- **Dwell volume**
- **Gradient steepness**

What is Dwell Volume?



- Dwell volume = volume from formation of gradient to the column
- Behaves as isocratic hold at the beginning of gradient.

Minor Dwell Volume Differences Can Change Resolution



Column: **ZORBAX Rapid Resolution Eclipse XDB-C8 4.6 x 75 mm, 3.5 µm**

Mobile Phase: Gradient, 0 - 100 %B in 52.5 min.
A: 5/95 methanol/ 25 mM phosphate pH 2.50
B: 80/20 methanol/25 mM phosphate pH 2.50

Flow Rate: 0.5 mL/min

Temperature: 25°C

Injection: 5 µL

Detection: 250 nm

Sample: Mixture of antibiotics and antidepressants

Upper trace simulates actual run data entered into DryLab® 3.0 software

Lower trace is simulated chromatogram for larger V_D

Effect of Dwell Volume on Ruggedness
Gradient Separations

Measure instrument dwell volume. (See Appendix.)

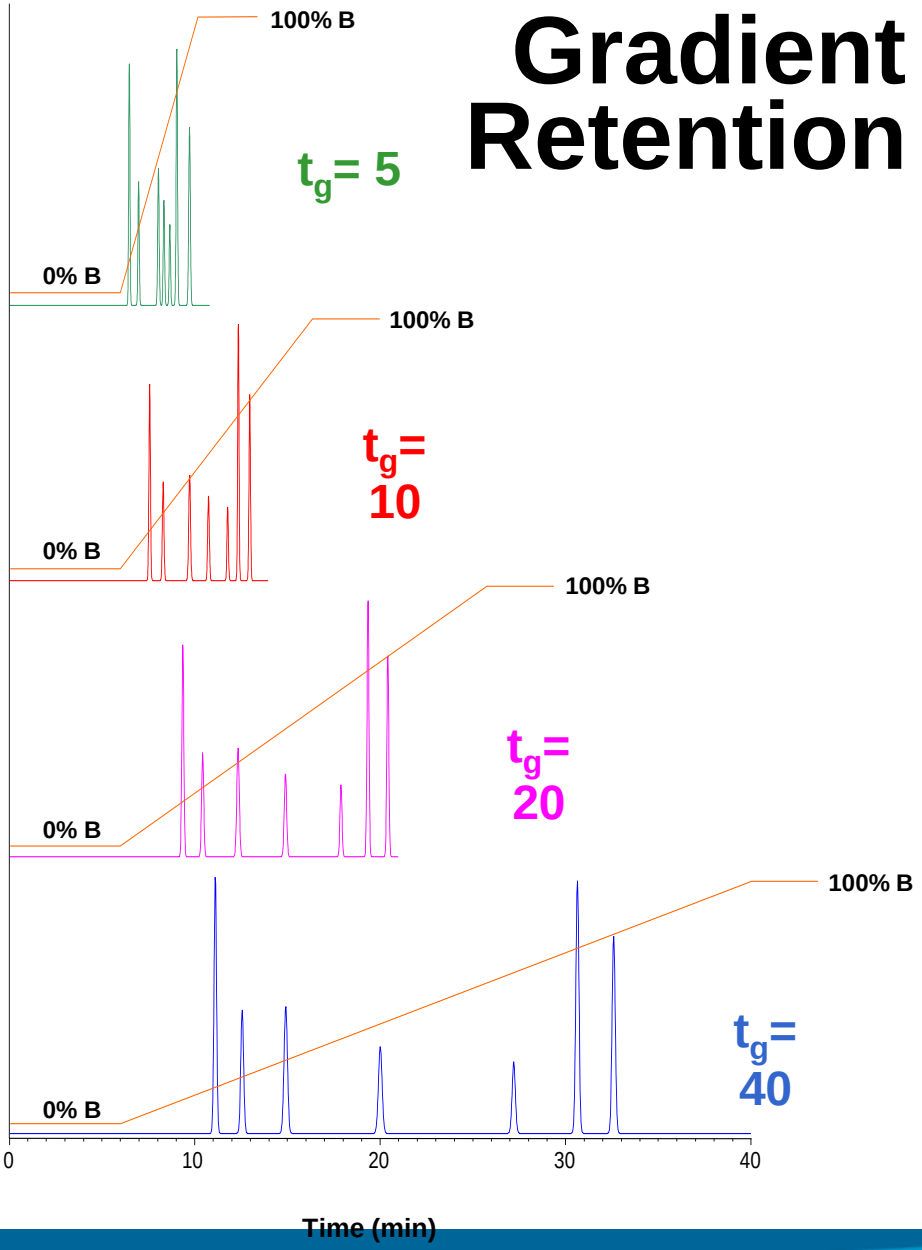
Assess effect of dwell volume on R_s during method development.

- To simulate larger V_D, use initial isocratic hold before gradient start.
- To simulate smaller V_D, use injection delay.
- Model dwell volume changes using computer simulation software.
- Compare gradient performance and resolution on different instruments.

Specify dwell volume in written method.

- Allows other users to compensate for instrument differences.

Gradient Steepness Affects Retention (k*) and Resolution



$$k^* = \frac{t_g F}{S \Delta\Phi V_m}$$

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

Fast Re-equilibration with Rapid Resolution and Rapid Resolution HT Columns

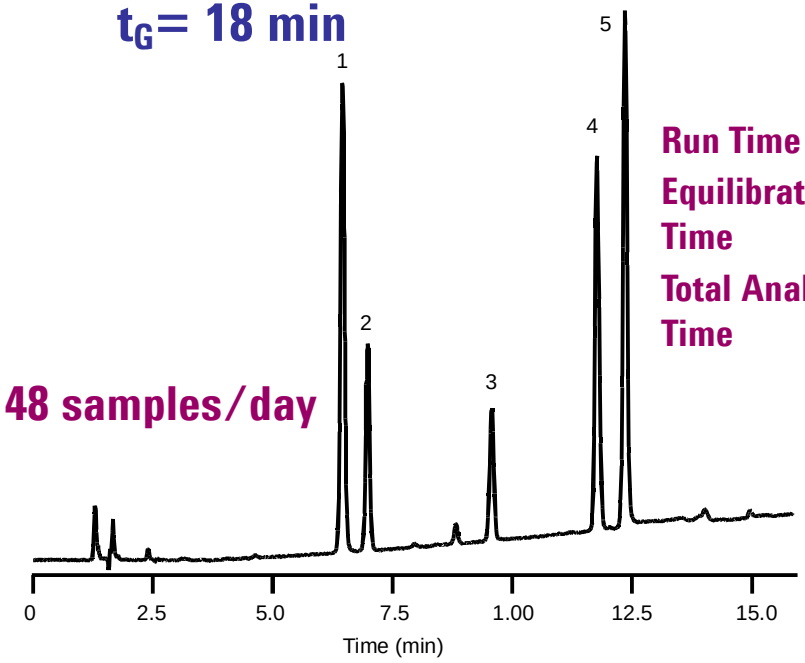
Column Dimension (mm)	Internal Volume (Vm)	Equilibration Time at 1.0 mL/min (Vm x 10 x F)	
4.6 x 50	0.5 mL	5 min	
4.6 x 30	0.3 mL	3 min	
4.6 x 15	0.15 mL	1.5 min	
4.6 x 150	1.54 mL	15 min	
		at 0.2 mL/min	at 1.0 mL/min
2.1 x 50	0.10 mL	5 min	60 sec
2.1 x 30	0.06 mL	3 min	36 sec
2.1 x 15	0.03 mL	1.5 min	18 sec

Gradient Analysis Time = Run Time + Equilibration Time using single step return

Short Columns Reduce Total Gradient Analysis Time

Gradient Separation of Cardiac Drugs

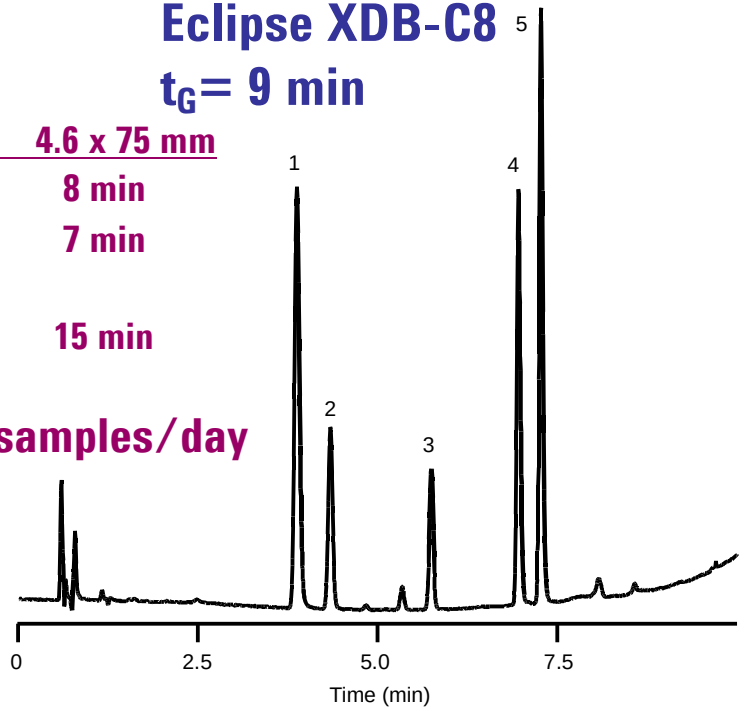
A. 4.6 x 150 mm, 5 µm
Eclipse XDB-C8
t_G = 18 min



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

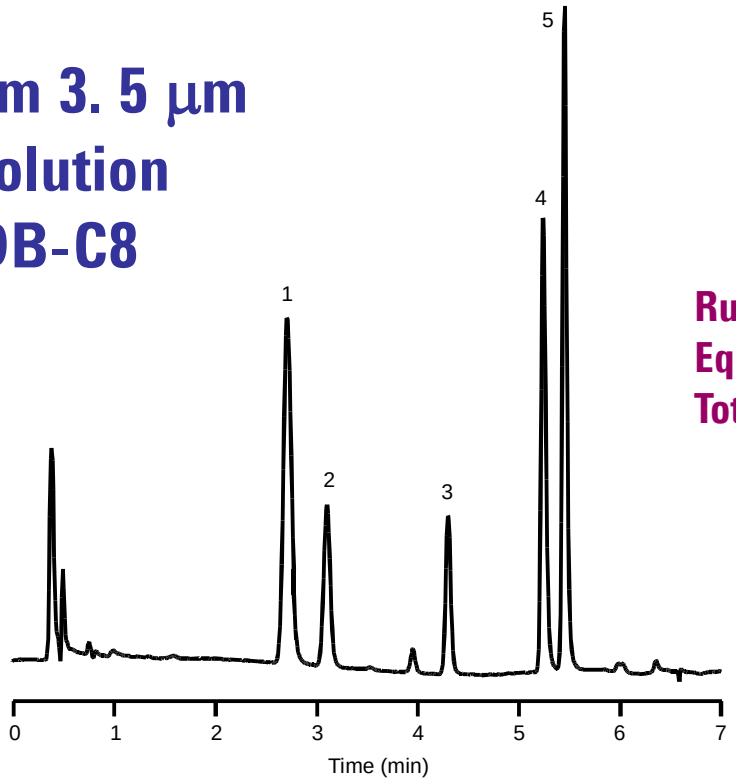


96 samples/day

Very Short Columns Reduce Analysis Time

Gradient Separation of Cardiac Drugs

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



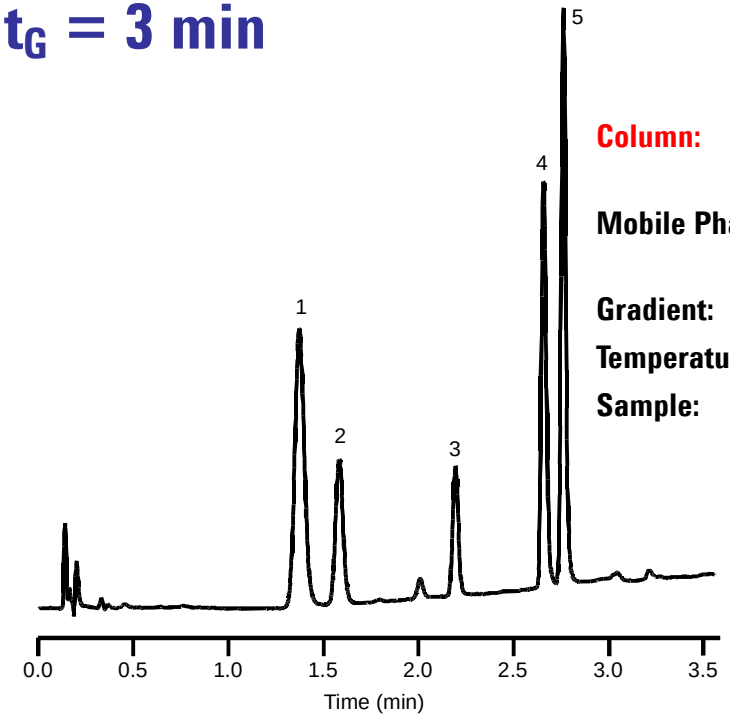
Run Time	6 min
Equilibration Time	5 min
Total Analysis Time	11 min

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

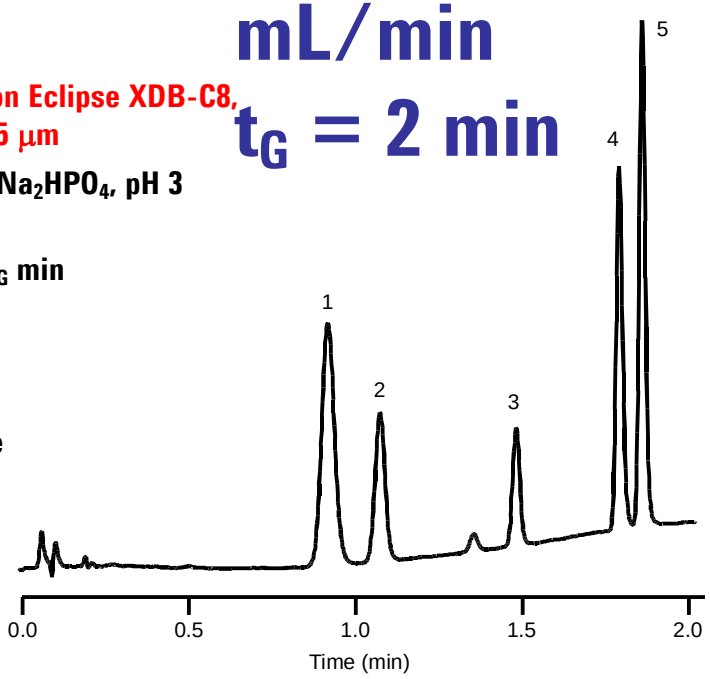
Mobile Phase: A: 55% 25 mM Na_2HPO_4, pH 3
B: 45% MeOH

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

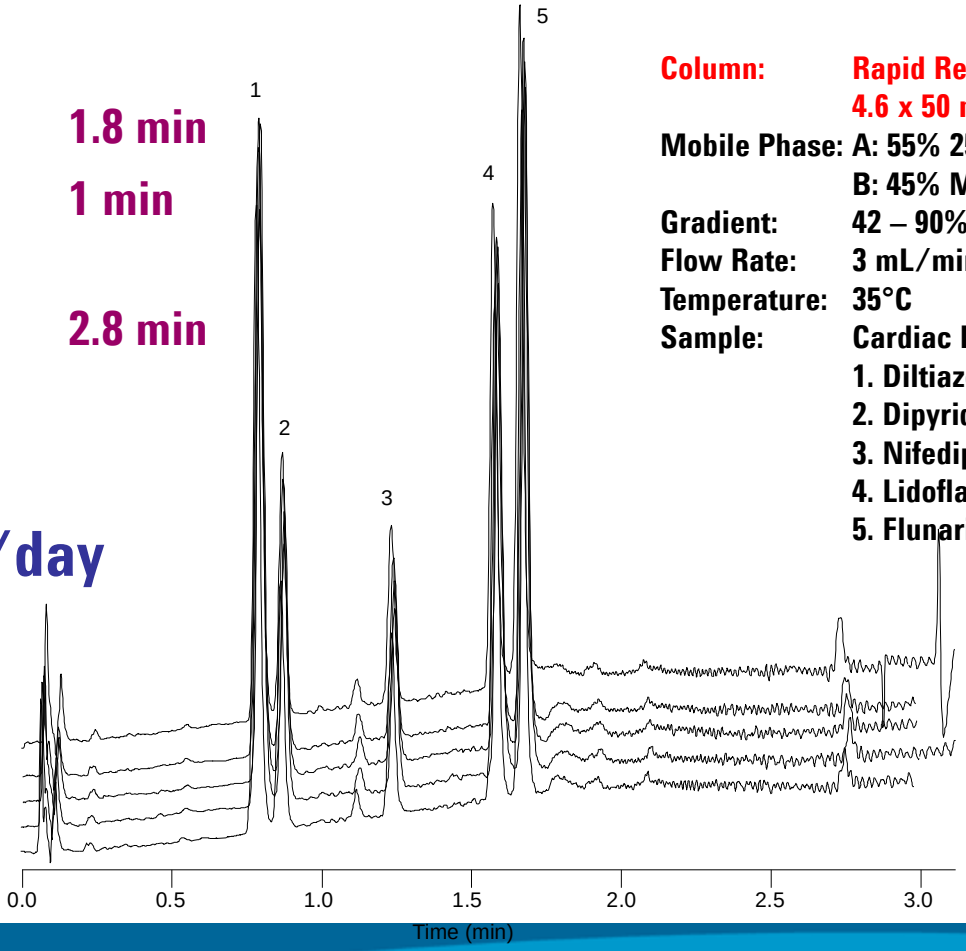


Fast Gradient Analysis of Heart Drugs

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

Gradient Steepness and Gradient Shape
Gradient Separations

Gradient steepness

- Change in gradient steepness, “b”
  - changes retention
  - may change resolution
- Small changes in “b” typically due to instrument performance differences (t_g, F, $\Delta\Phi$).
- Compensate for any dwell volume differences first.
- Compare resolution at desired gradient time and at t_g ±10–20%.

Gradient shape

- Linear gradients are preferred.
- Non-linear, segmented and step gradients harder to transfer.

Summary

Many variables to consider; some are more apparent than others.

Careful consideration during method development can minimize “headaches” and repeat work.

Well-conceived documented laboratory practices are important to successful development of rugged methods.

Appendix

Important Buffer Systems

Buffer Selection

Buffer	pK_a	pH Range	UV Cutoff (A > 0.5)
Trifluoroacetic acid	<<2 (0.5)	1.5-2.5	210 nm (0.1%)
KH_2PO_4/phosphoric acid	2.12	1.1-3.1	<200 nm (0.1%)
tri-K-Citrate/hydrochloric acid 1	3.06	2.1-4.1	230 nm (10 mM)
Potassium formate/formic acid	3.8	2.8-4.8	210 nm (10 mM)
tri-K-Citrate /hydrochloric acid 2	4.7	3.7-5.7	230 nm (10 mM)
Potassium acetate/acetic acid	4.8	3.8-5.8	210 nm (10 mM)
tri-K-Citrate /hydrochloric acid 3	5.4	4.4-6.4	230 nm (10 mM)
Ammonium formate	3.8 9.2	2.8-4.8 8.2-10.2	(50 mM)
Bis-tris propane•HCl/Bis-tris propane	6.8	5.8-7.8	215 nm (10 mM)
Ammonium acetate	4.8 9.2	3.8-5.8 8.2-10.2	(50 mM)
KH_2PO_4/K_2HPO_4	7.21	6.2-8.2	<200 nm (0.1%)
Tris•HCl/Tris	8.3	7.3-9.3	205 nm (10 mM)
Bis-tris propane•HCl/Bis-tris propane	9.0	8.0-10.0	225 nm (10 mM)
Ammonium hydroxide/ammonia	9.2	8.2-10.2	200 nm (10 mM)
Borate ($H_3BO_3/Na_2B_4O_7$•10 H_2O)	9.24	8.2-10.2	
Glycine•HCl/glycine	9.8	8.8-10.8	
1-methylpiperidine•HCl/1-methylpiperidine	10.1	9.1-11.1	215 nm (10 mM)
Diethylamine•HCl/diethylamine	10.5	9.5-11.5	
Triethylamine•HCl/triethylamine	11.0	10.0-12.0	<200 nm (10 mM)
Pyrollidine•HCl/pyrollidine	11.3	10.3-12.3	

Adapted from Practical HPLC Method Development, 2nd Edition, Snyder, L.R., Kirkland, J.J. and Glajch, J.L., page 299.

Separation Ruggedness
Buffer Preparation

1. Dissolve salt in organic-free water in 1- or 2-L beaker. Use appropriate volume to leave room for pH adjustment solution. Equilibrate solution to room temperature for maximum accuracy.

2. Calibrate pH meter. Use 2-level calibration and bracket desired pH. Use appropriate audit solution to monitor statistical control (for example, potassium hydrogen tartrate, saturated solution, pH = 3.56).

3. Adjust salt solution to desired pH. Minimize amount of time electrode spends in buffer solution (contamination). Avoid overshoot and readjustment (ionic strength differences can arise).

4. Transfer pH-adjusted buffer solution quantitatively to volumetric flask, dilute to volume, and mix.

5. Filter through 0.45 µm filter. Discard first 50 – 100 mL filtrate. Rinse solvent reservoir with small volume of filtrate and discard. Fill reservoir with remaining filtrate or prepare premix with organic modifier.
   - Agilent Solvent Filtration Kit, 250-mL reservoir, 1000-mL flask, p/n 3150-0577
   - Nylon filter membranes, 47 mm, 0.45 µm pore size, p/n 9301-0895 (not for proteins!)

Using Buffers Successfully
Initial Column and System Equilibration

In an appropriate vessel, test highest % organic/buffer ratio to verify that buffer will not precipitate. With stirring, add organic to buffer first, not vice versa.

Equilibrate column with, in order:

- 100% organic modifier (if brand new)
- mobile phase minus buffer
- buffered mobile phase containing highest % organic modifier (gradient high end)
- buffered mobile phase containing lowest % organic modifier (gradient low end).

Inject standard or sample several times until RTs stable, or for gradient methods, precede former with 1 or 2 blank gradients.

Using Buffers Successfully
Shutdown State and Instrument Flushing

Next day use—using same buffers

- Pump mobile phase very slowly (for example, 0.01 – 0.1mL/min).

When flushing column or for longer term column storage

- Flush with 20/80 organic/water, then 80/20 organic/water or 100% organic.

Instrument flushing

- Replace column with capillary tubing. Leave disconnected from detector.
- Flush pumps with water, then connect capillary tubing to detector.
- Inject water 2-3 times at maximum injection volume setting.
- Flush all pumps with 100% organic for long term storage.

Determining the Dwell Volume of Your System

Replace column with short piece of HPLC stainless steel tubing

Prepare mobile phase components

A. Water - UV-transparent

B. Water with 0.2% acetone - UV-absorbing

Monitor at 265 nm

Adjust attenuation so that both 100% A and 100% B are on scale

Run gradient profile 0 - 100% B/10 min at 1.0 ml/min

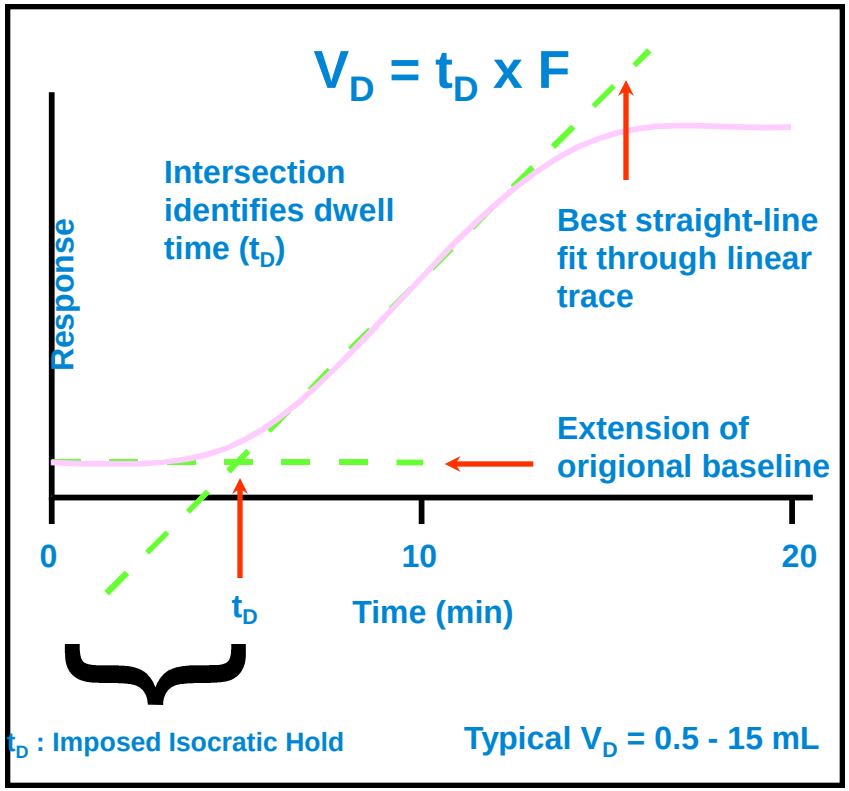
Record

Measuring Dwell Volume

If using gradient conditions - report dwell volume (V_D)
V_D varies from instrument to instrument

Dwell Volume Impact

A chromatogram generated on one instrument (V_{D1}) can have a very different profile if generated on another instrument (V_{D2})

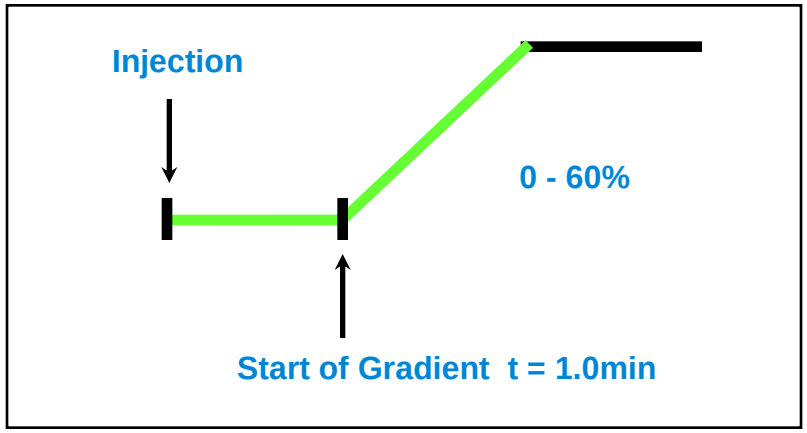


High Pressure Mixing: V_D = mixing chamber + connecting tubing + injector

Low Pressure Mixing: V_D = the above + pump heads + associated plumbing

Correcting for Dwell Volume

1. Measure the Dwell Volume of your HPLC System
   V_D = 1.0 mL
2. Draw Effective Gradient Profile at First Flow Rate
   Calculate the time delay (imposed isocratic hold) caused by dwell volume
   $V_D = t_D \bullet F$ 1.0 mL = $t_D \bullet$ 1.0 mL / min
   where F = 1.0 mL / min for 4.6 x 150 mm column
   V_D = 1.0 mL
   $t_D = F/V_D$ t_D = 1.0 mL / min / 1.0 mL
   t_D = 1.0 min



To Accommodate Different Column Sizes

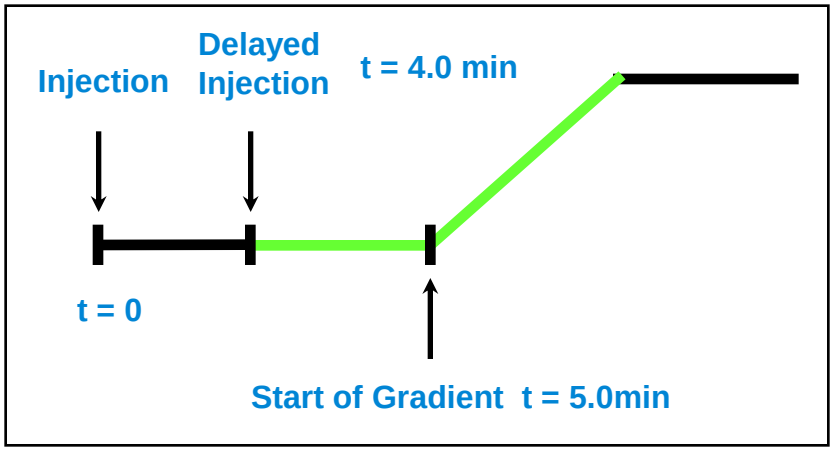
3. Draw Effective Gradient Profile at Second Flow Rate

$t_D = F / V_D$ $t_D = (0.2\ mL / min) / 1.0mL$

$t_D = 5.0$ min

where $F = 0.2$ mL / min for 2.1 x 150 mm column

$V_D = 1.0$ mL (same for HPLC system)



Delay injection on the 2.1 x 150 mm column by 4.0 min (5.0 min - 1.0 min) so that the gradient profile is the same on both columns

Correcting for Dwell Volume

If $V_{D1} > V_{D2}$

Compensate for longer V_{D1} by adding an isocratic hold to V_{D2}, such that Hold + V_{D2} = V_{D1}

If $V_{D1} < V_{D2}$

Delay injection, such that V_{D2} - delay = V_{D1}