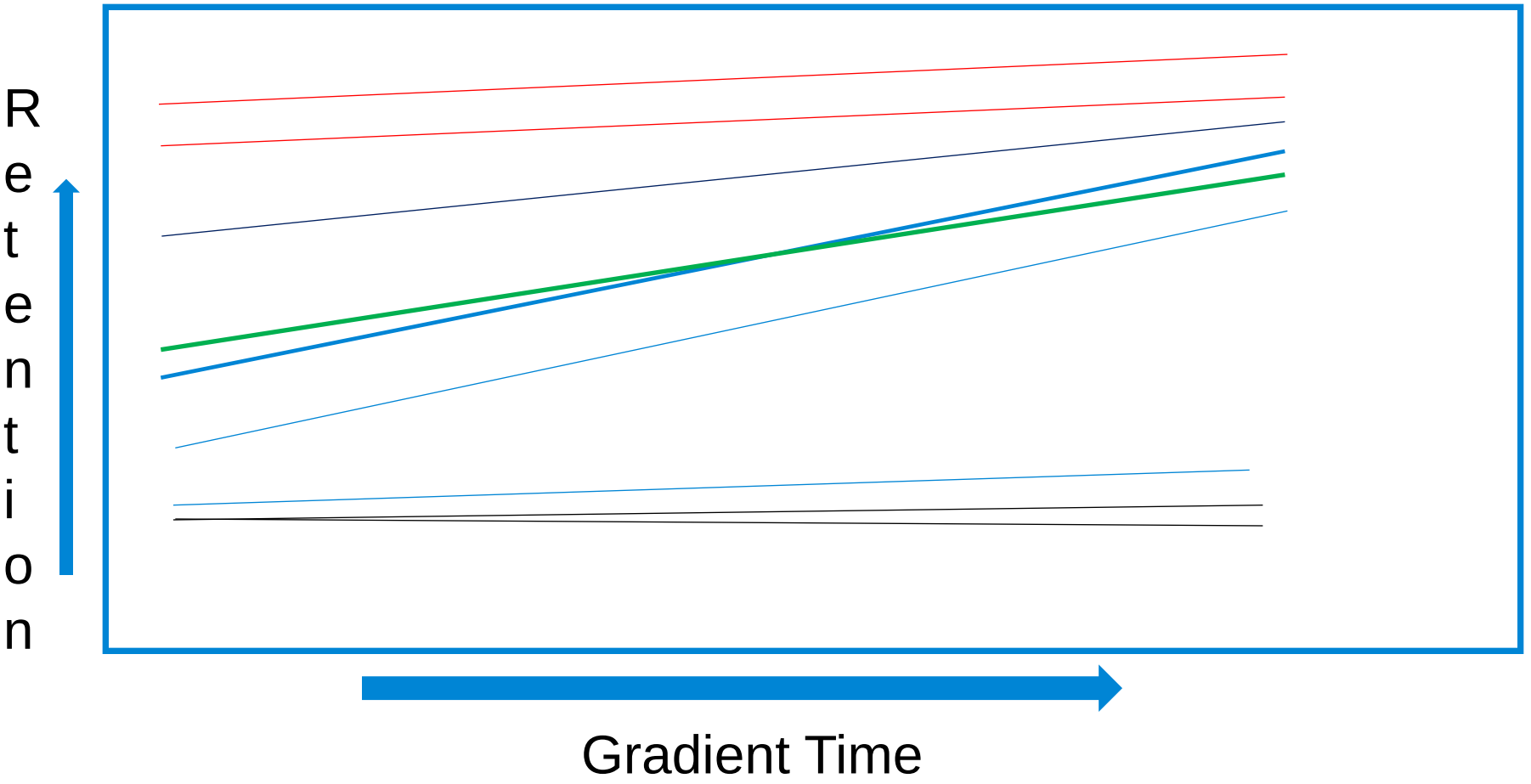
Eliminating Unwanted Variability in LC Methods: More Uptime, More Sample Throughput

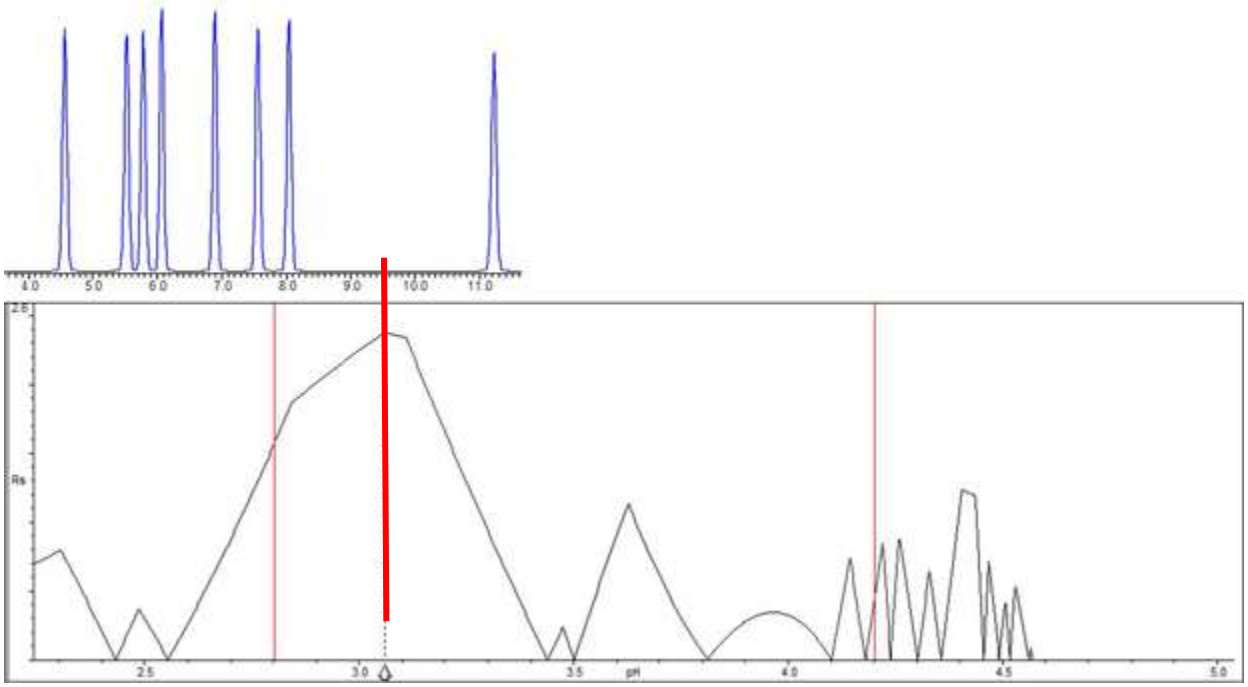
HPLC Separation
Robustness and Ruggedness

Different Compounds Interact Differently with the Column and Mobile Phase

Changes in Gradient Conditions do not Alter Retention for All Peaks the Same Way



Is Your Separation on a “Resolution Cliff”?



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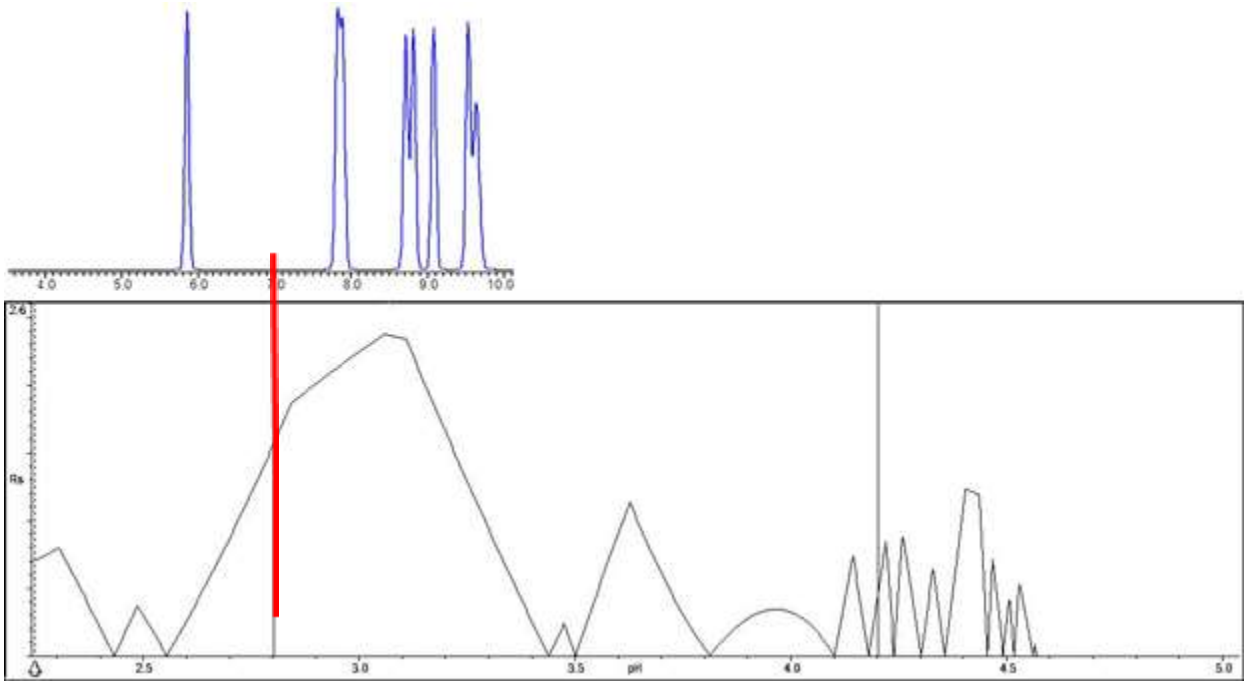


pH change of only 0.4 units with major change in resolution

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.

Robustness Example: % Organic Modifier

Vary % organic modifier ±1–2%.

Evaluate changes to R_s.

If ΔR_s is too large at either %B, modify method.

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.

Ruggedness Example:
Column Lot

1. Test 3 different column lots.
2. Compare R_s for the 3 lots.
3. If ΔR_s is too large, modify method.

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).

The Column

Experimental Variables That Impact Resolution

Column

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

Mobile Phase

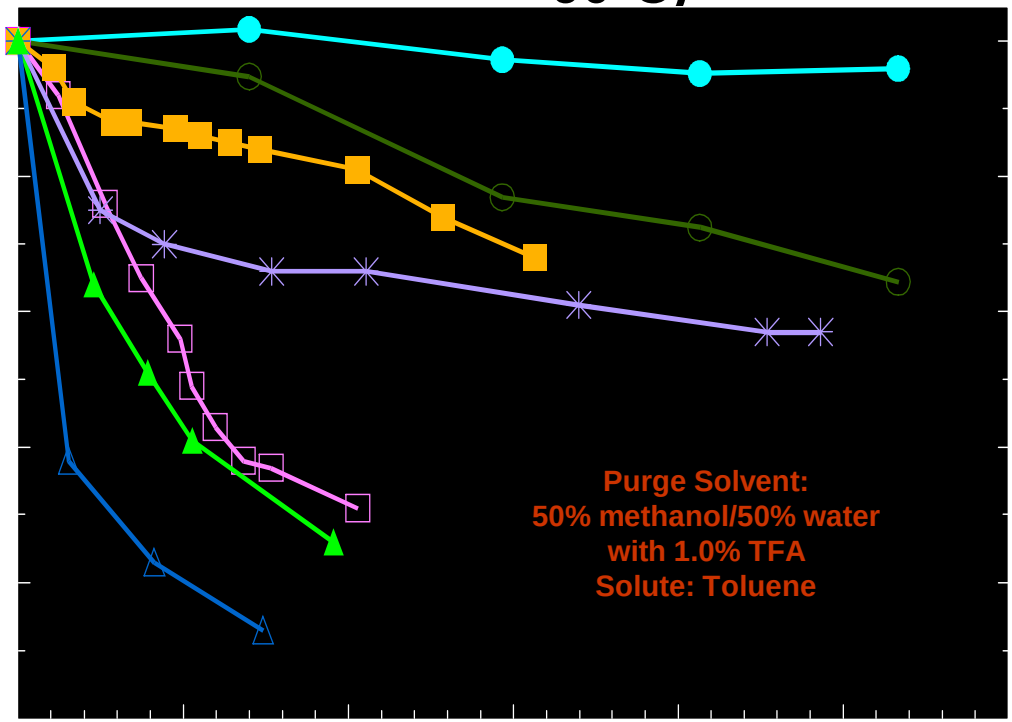
Sample

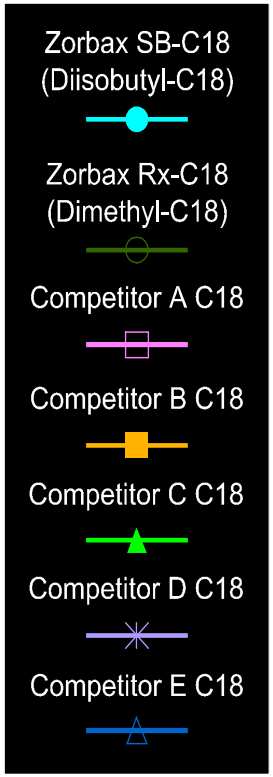
Instrument

Gradient Separations

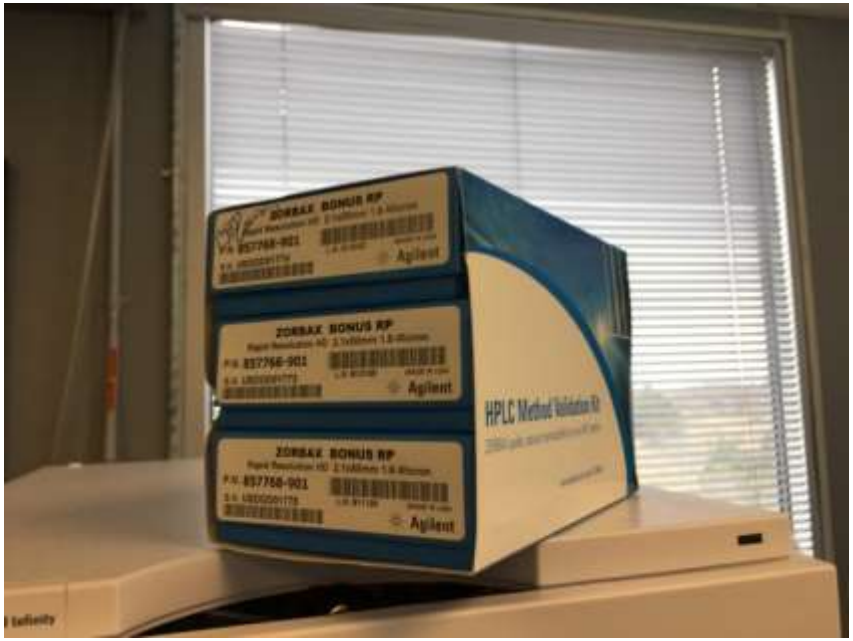
Select a Column With Long Lifetime ZORBAX StableBond C18:

Exceptional Stability At Low pH and High Temperatures (pH 0.8, 90°C)





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

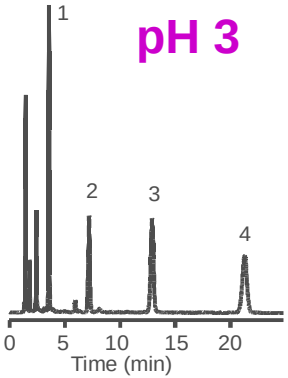


Meet “Texas TOF”

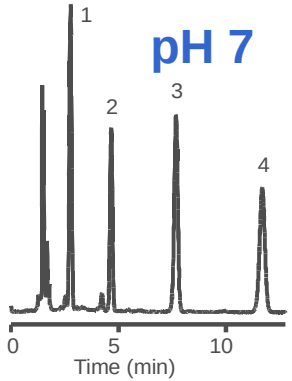
Tex knows the wisdom of multiple lot testing, but also making sure he has the correct conditions!

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



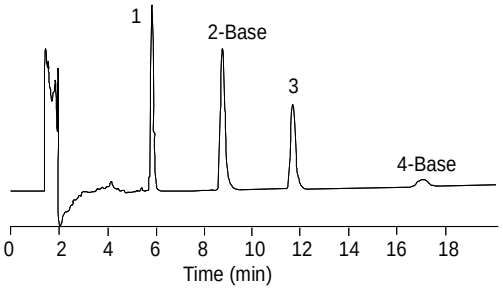
RSD	Retention, k´				Selectivity, α			
	Lot 1	Lot 2	Lot 3	% RSD	Lot 1	Lot 2	Lot 3	%
1. Cefotaxime	0.80	0.88	0.88	5.4	-	-	-	-
2. Cefoxitin	1.96	2.14	2.15	5.1	2.45	2.43	2.44	0.41
3. Cefamandole	3.83	4.15	4.16	4.6	1.96	1.94	1.94	0.59
4. Cephalothin	6.26	6.79	6.84	4.8	1.64	1.64	1.64	0.00

Column: ZORBAX Eclipse XDB-C8, 4.6 x 150 mm, 5 µm
Flow Rate: 1.0 mL/min

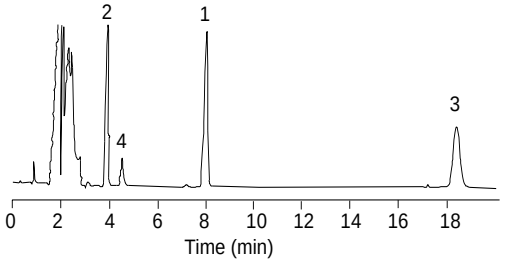
Mobile Phase: 85% 25 mM phosphate : 15% ACN
Temperature: 35°C

Lot-to-Lot Selectivity Change Related to pH Choice

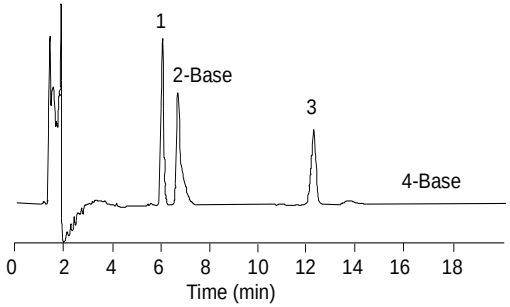
pH 4.5 - Lot 1



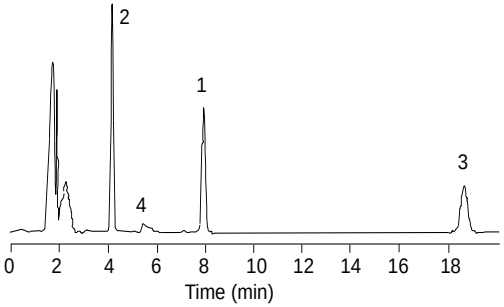
pH 3.0 - Lot 1



pH 4.5 - Lot 2



pH 3.0 - Lot 2



- pH 4.5 shows selectivity change from lot-to-lot for basic compounds
- pH 3.0 shows no selectivity change from lot-to-lot
- Indication of poorly controlled ionization

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' validation kits and special orders

- Call Agilent Technical Support, (800) 227-9770.

Mobile Phase: Aqueous Component

Experimental Variables That Impact Resolution

Your opportunity to improve robustness and ruggedness

Column

Mobile Phase

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

Sample

Instrument

Gradient Separations

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.

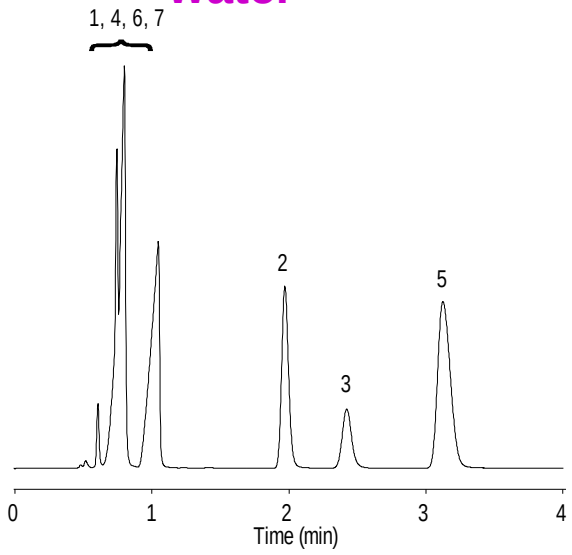
Allow efficient use of pH as separation variable during method development.

$\therefore$ Separation pH must be set accurately and reproducibly.

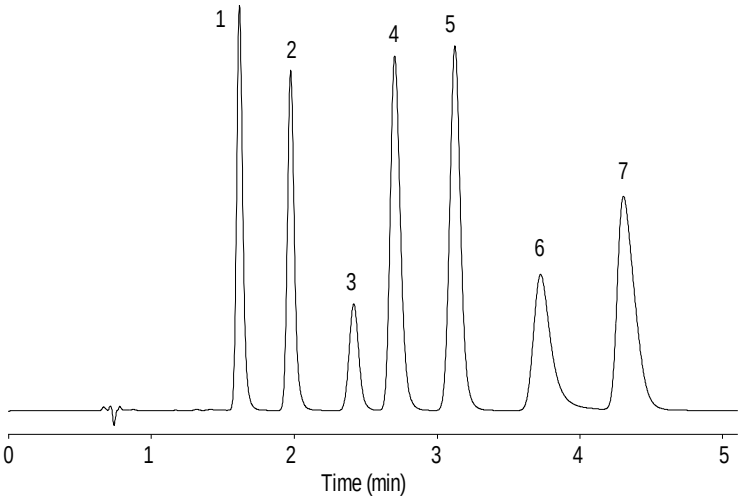
Why Use Buffered Mobile Phases?

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 and column lifetime.

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.

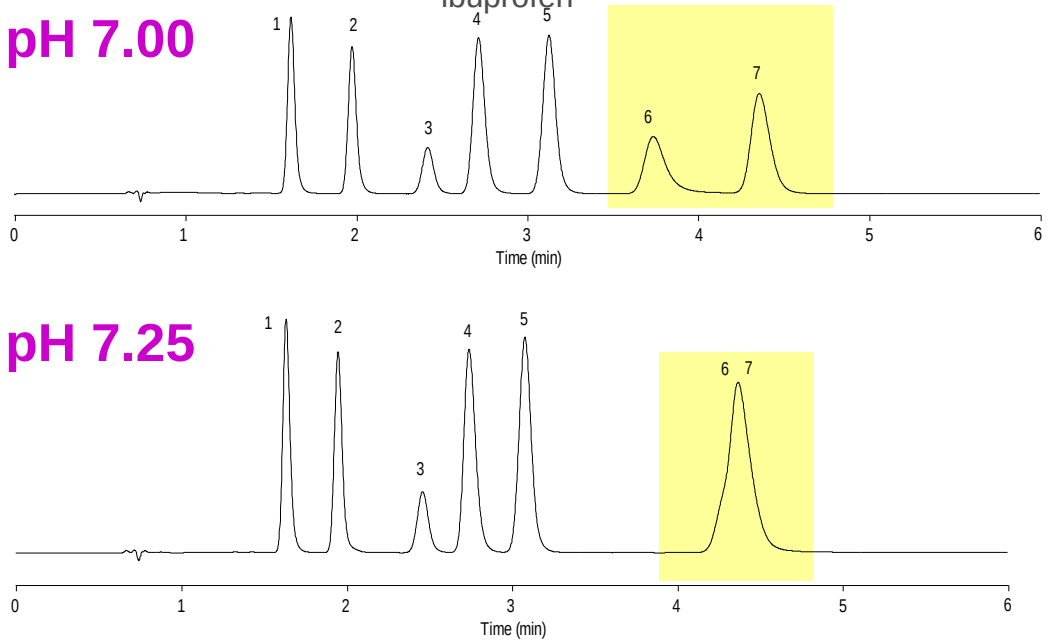
Test for pH Robustness

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

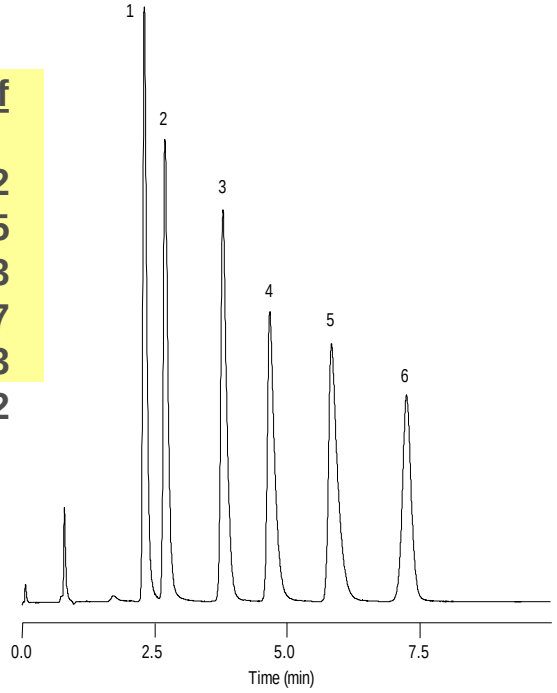
Column: ZORBAX Eclipse XDB-C8, 4.6 x 150 mm, 5 µm

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

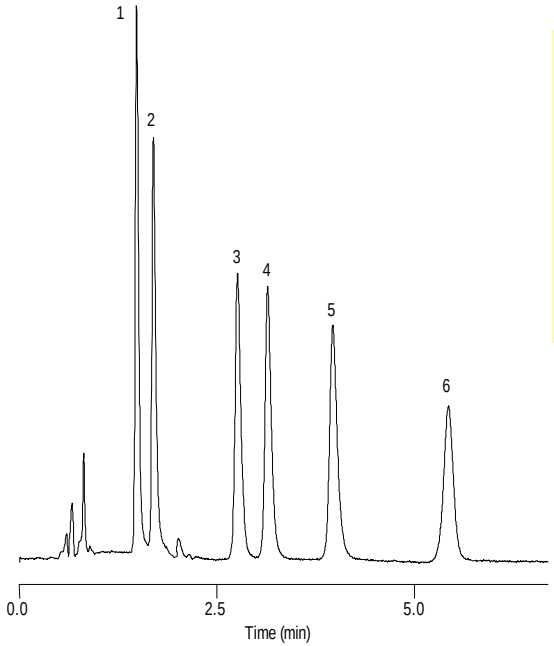
Sample: Tricyclic Antidepressants, 1. Desipramine 2. Nortriptyline 3. Doxepin 4. Imipramine 5. Amitriptyline 6. Trimipramine

10 mM Phosphate

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



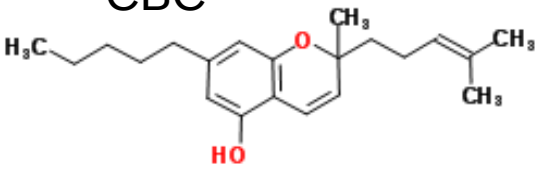
25 mM Phosphate



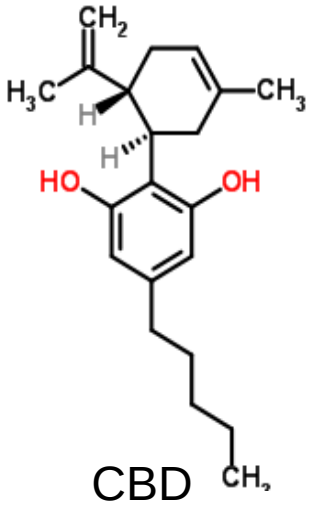
USP Tf (5%)
1. 1.41
2. 1.50
3. 1.33
4. 1.39
5. 1.36
6. 1.00

Cannabinoids of Interest

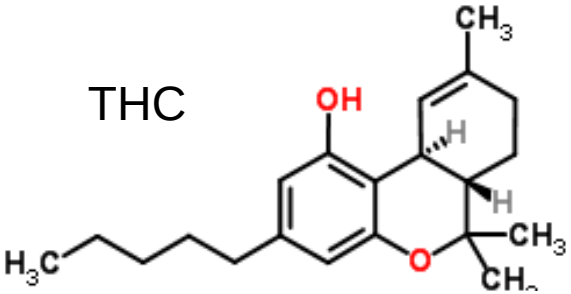
CBC

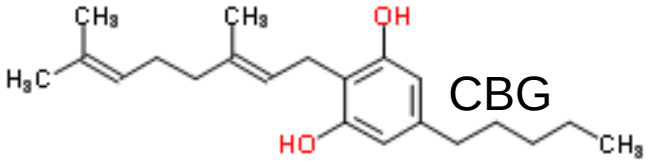


CBD



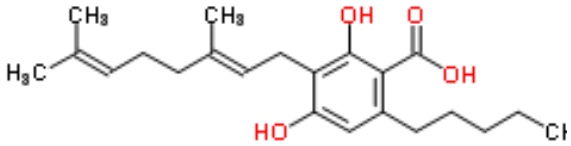
THC

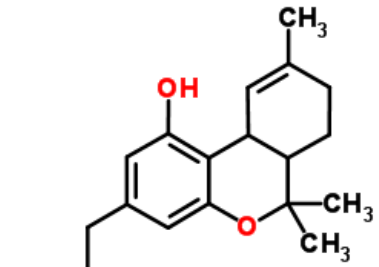




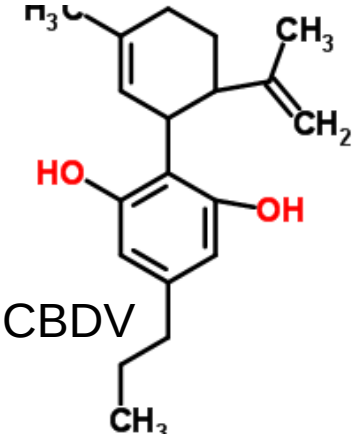
CBG

CBGA

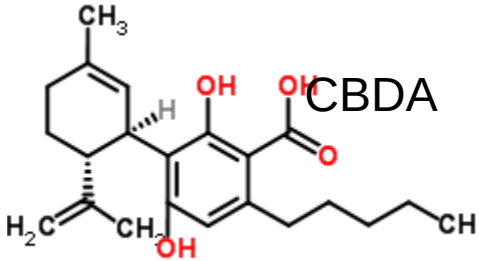




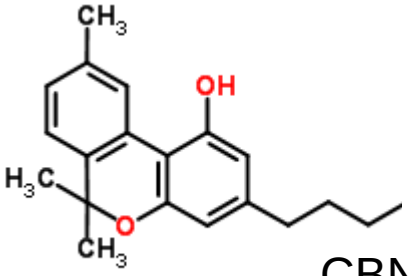
THCV



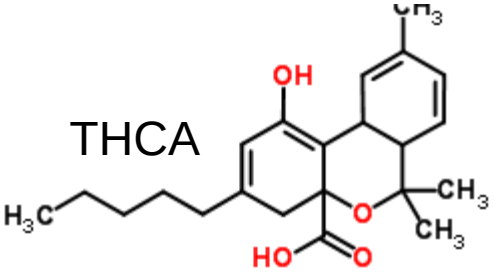
CBDV



CBDA

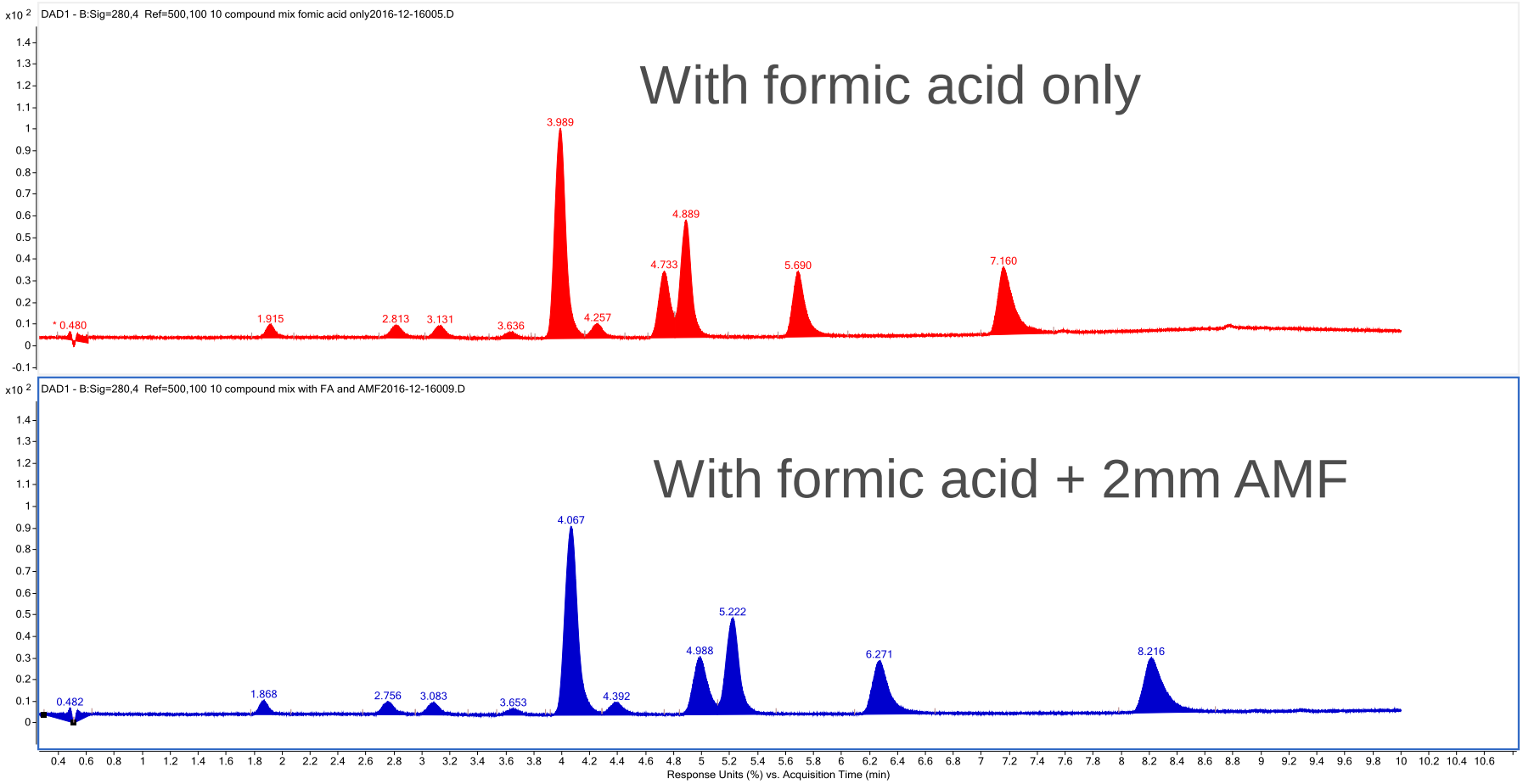


CBN



THCA

Peak Shape and Resolution Improvement with Buffer Added



Experimental Variables That Impact Resolution

Column

Mobile Phase

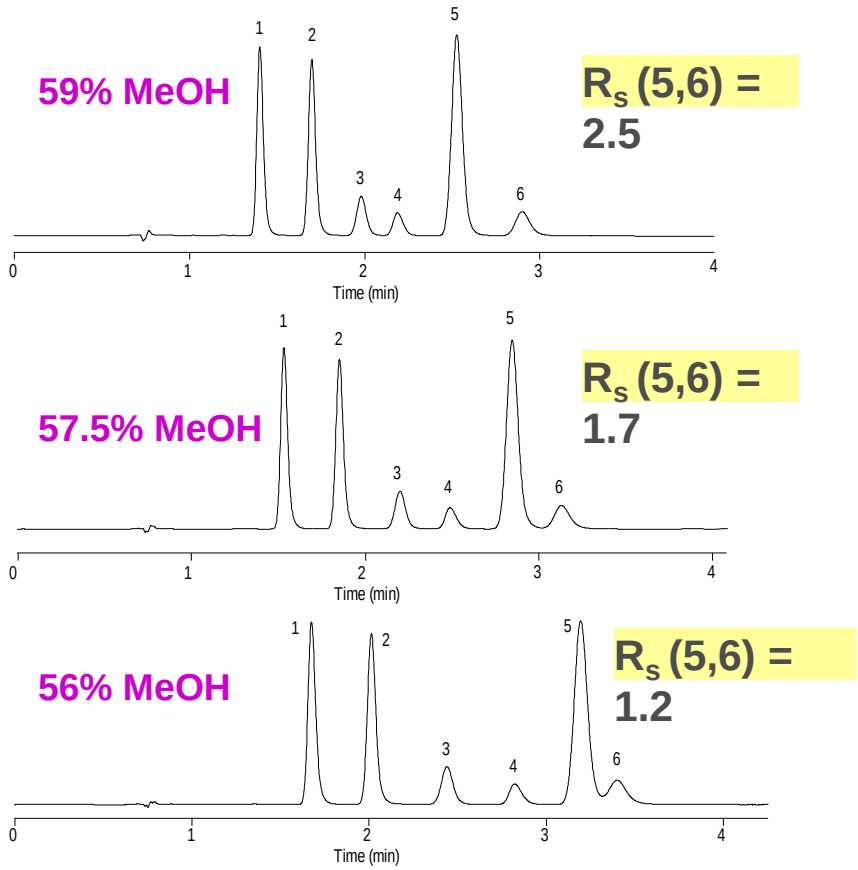
- Aqueous Component
- **Organic Component**
  - **% Organic Modifier**

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

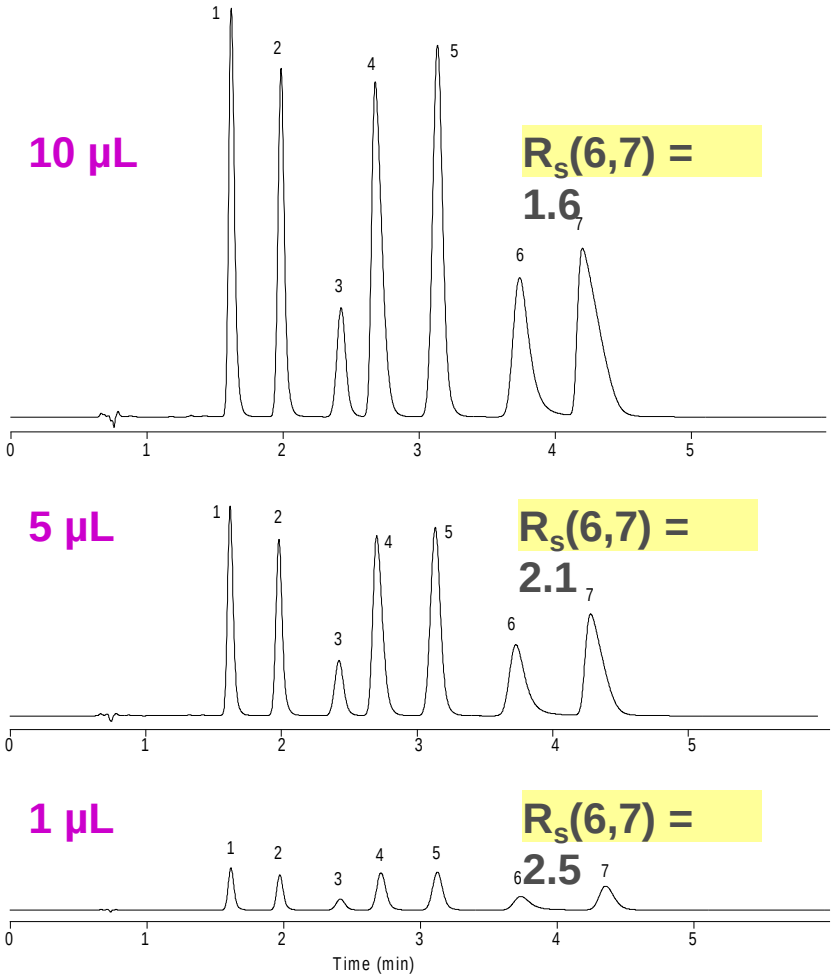
Injection Volume

- 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

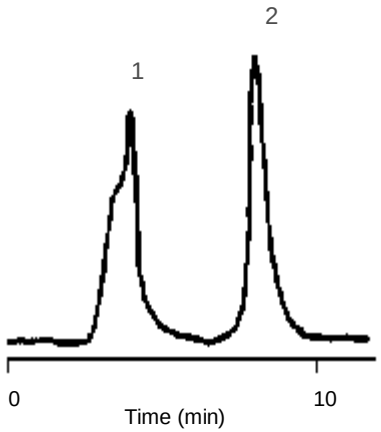
- Varying injection volume can sometimes reveal lack of robustness for resolution and peak shape.

Strong Sample Solvent Can Compromise Peak Shape

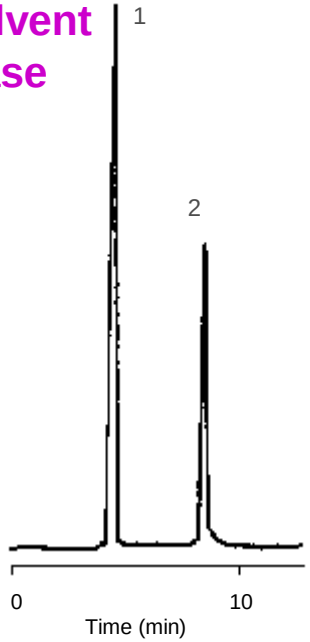
Column: ZORBAX SB-C8, 4.6 x 150 mm, 5 µm Mobile Phase: 82% H_2O:18% ACN Injection Volume: 30 µL

Sample: 1. Caffeine 2. Salicylamide

A. Sample Solvent 100% Acetonitrile



B. Sample Solvent Mobile Phase



Instrument

Experimental Variables That Impact Resolution

Column

Mobile Phase

Sample

Instrument

- **Column temperature**

Gradient Separations

Column Temperature

Adequate Temperature Control is Essential

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

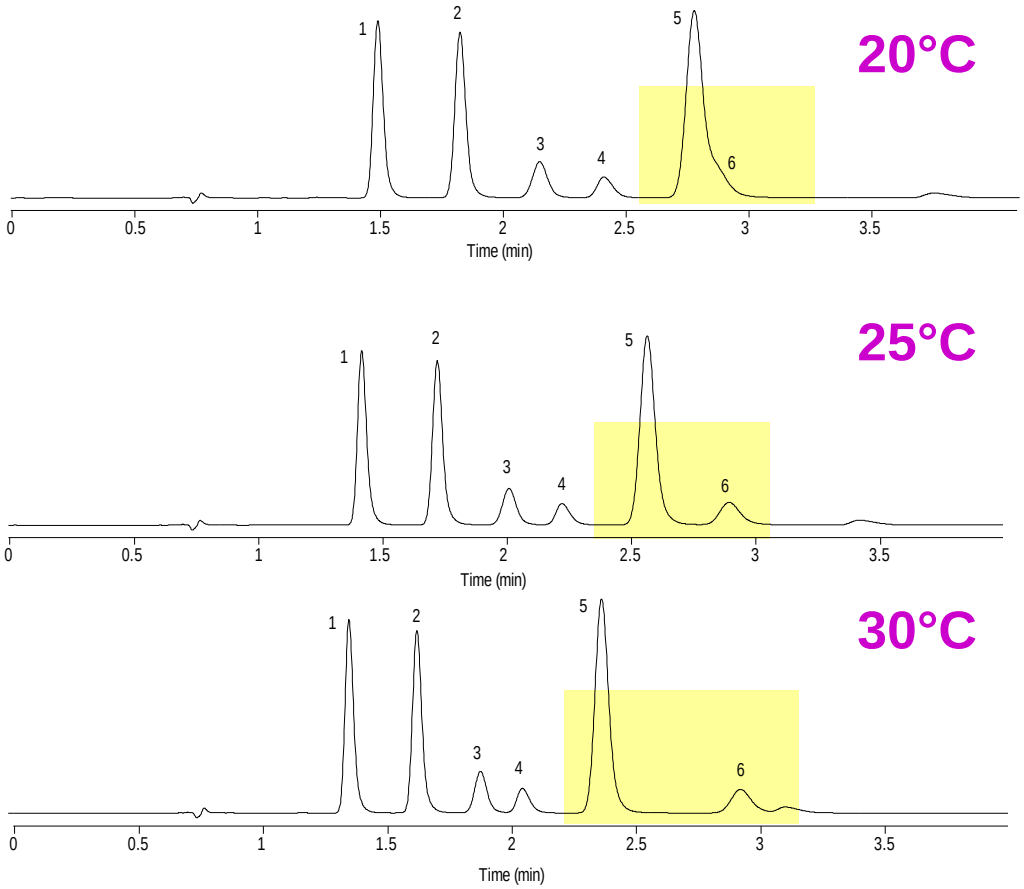
Column temperature changes affect resolution and repeatability.

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.

Gradient Separations

Experimental Variables That Impact Resolution

Column

Mobile Phase

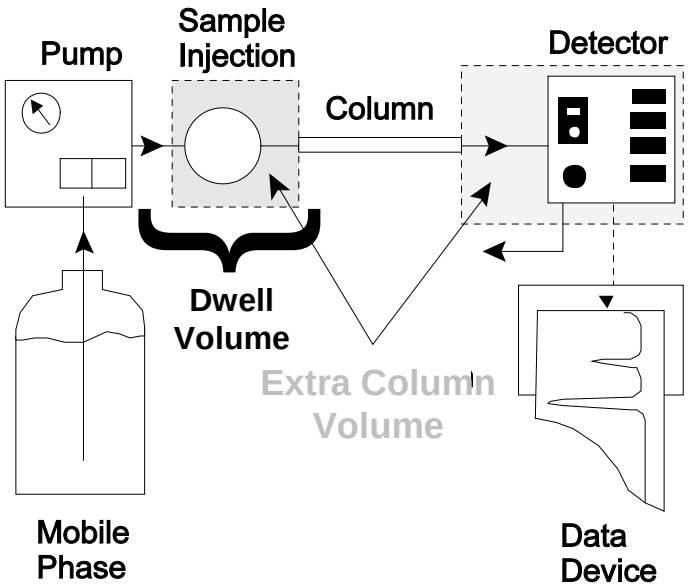
Sample

Instrument

Gradient Separations

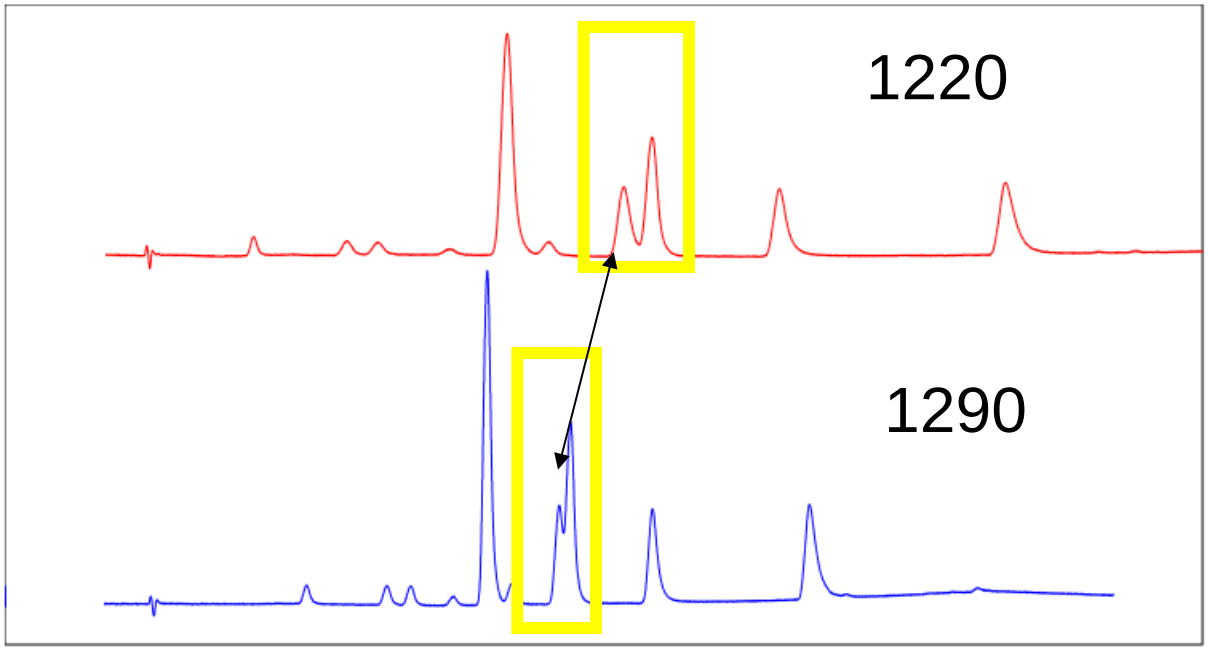
- **Dwell volume**
- **Gradient steepness**

Dwell Volume



Dwell Volume = volume from formation of gradient to top of column

Dwell Volume Differences Can Change Resolution



Column: **ZORBAX Bonus RP**

Mobile Phase: Gradient, 74 – 98.0 %B in 6.25 min.
A: H2O + 0.1% FA + 2.2 mM AMF

B: methanol
pH 2.50

Flow Rate: 1.0 mL/min

Temperature: 50°C

Injection: 1 µL

Detection: 280 nm

Sample: Cannabiniods mixture

Assess Dwell Volume Effect on Resolution

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.

Summary

HPLC Separation Robustness and Ruggedness

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

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

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

Choosing the right column from Agilent for your application is an excellent first step in developing a robust and rugged method.

If You Need Assistance with HPLC/UHPLC Methods:

Contact

John Palmer at john_palmer3@agilent.com

Or

LC Column Support at 1-800-227-9770 and follow the voice prompts

Appendix

Why Select Agilent Technologies as Your Column Manufacturer?

With ZORBAX HPLC columns, Agilent Technologies offers:

Long-standing commitment to quality and innovation

Total control over silica manufacture, bonding, and column packing

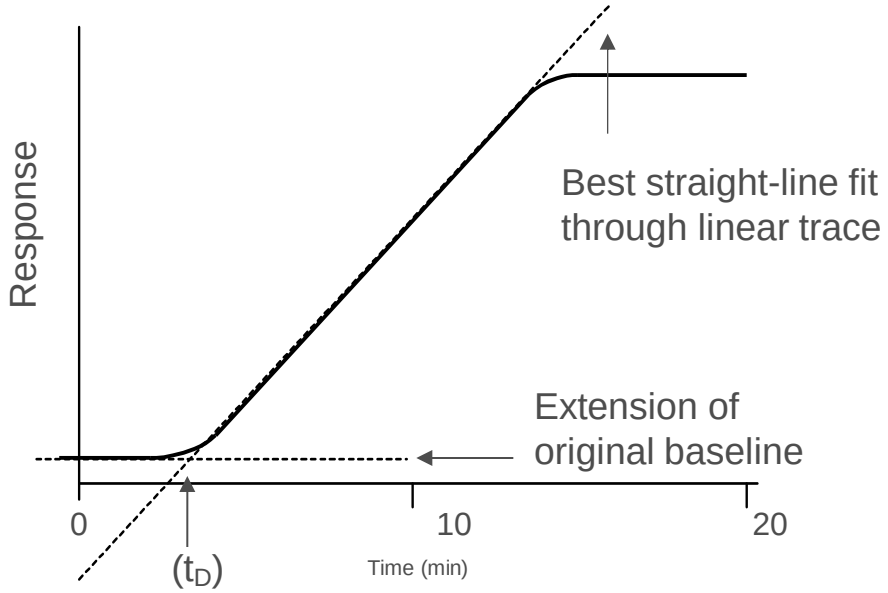
Reproducible separations (lot-to-lot, among different geometries)

Superior column lifetime (pH 1–12)

Alternative selectivities (C18, C8, Phenyl, CN, C3, etc.)’

Variety of geometries (i.d., length, particle size)

Measuring Dwell Volume



Intersection indentifies
dwell time (t_D)

$V_D = t_D \times F$

V_D = Dwell Volume

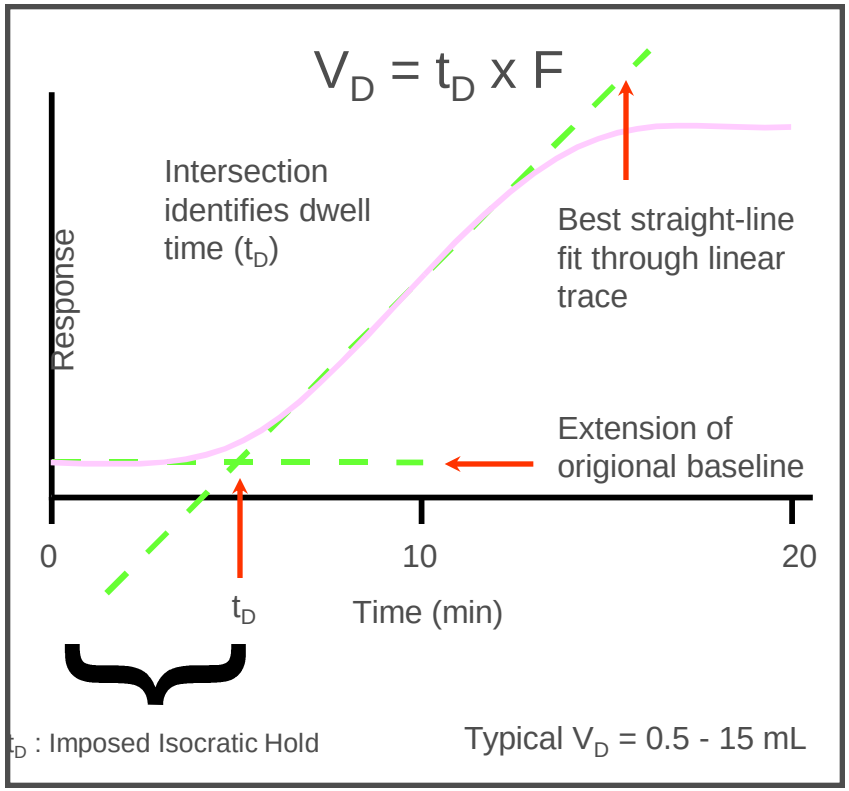
001815S1.PPT

Measuring Dwell Volume

Dwell Volume Impact

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

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

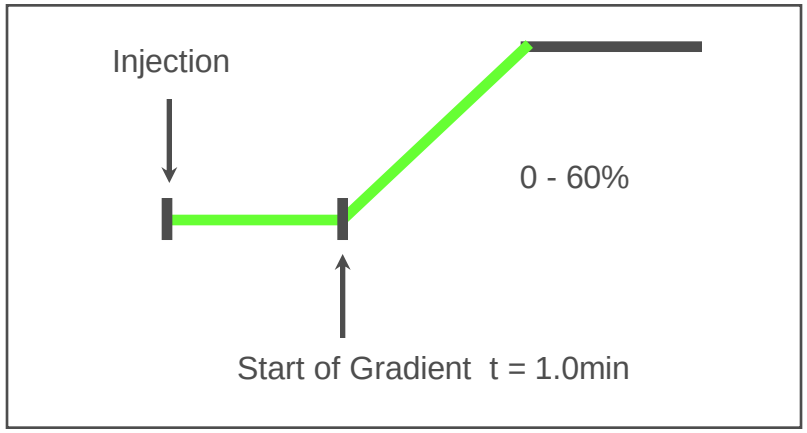


High Pressure Mixing: V_D = mixing chamber + connecting tubing + injector

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



Correcting for Dwell Volume

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

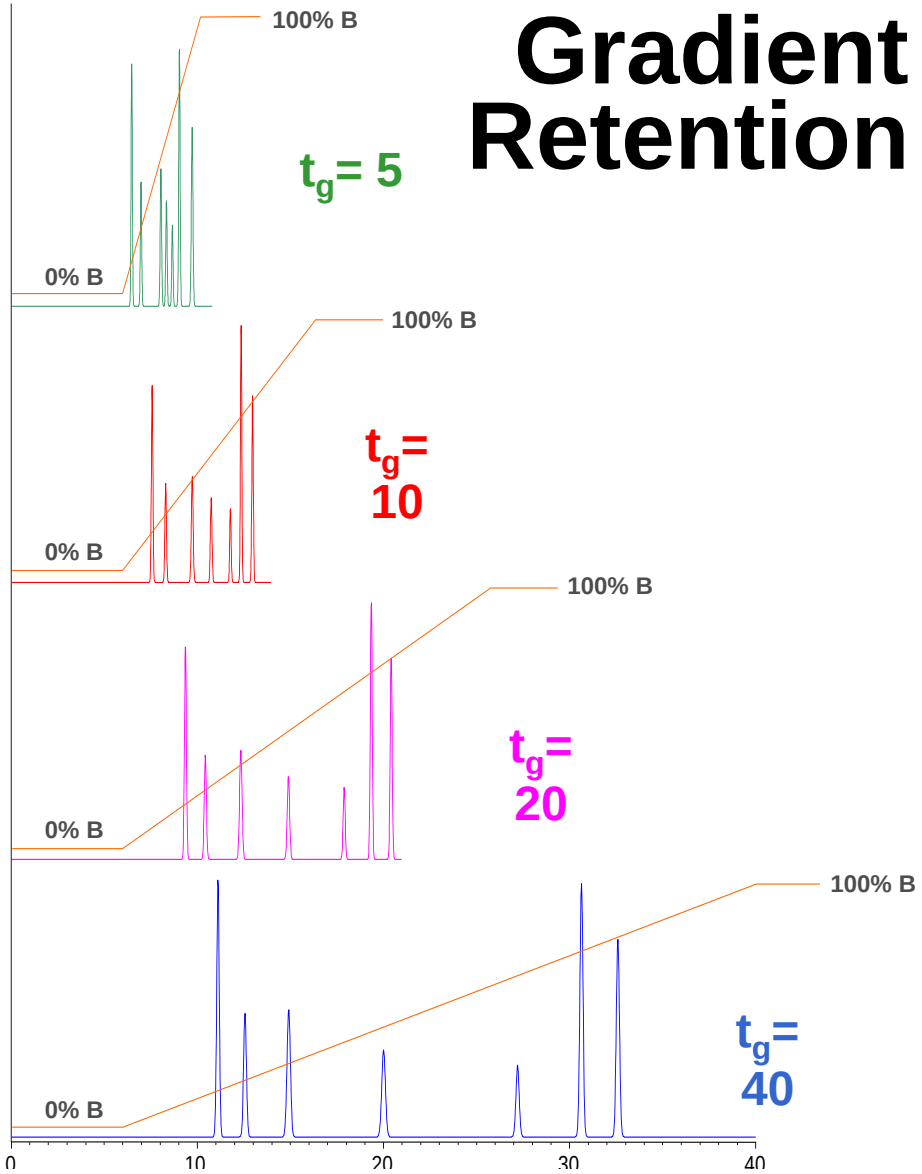
Compensate for longer V_{D1} by adding an isocratic hold to V_{D2}, such that Hold + $V_{D2} = V_{D1}$

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

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

(very difficult to accomplish in practice)

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

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.

Separation Criteria and Goals

Separation Criterion	Goal	Comment
Selectivity/Specificity	Peak elution order and relative RTs (α) same on different column lots.	Use ZORBAX products.
Resolution	Target R_s of 2.0 for least resolved peak pair.	Baseline separation of equal size peaks requires R_s = 1.5.
Column Lifetime	Adequate relative to overall analysis costs	Select the right column based on desired pH range. Follow care and use instructions.
Chromatographic Repeatability	Retention times: 0.05-0.25% RSD Peak area, height, width, symmetry: 0.05-0.5% RSD	Requires autosampler, symmetrical peaks, optimized integration parameters, etc.
Robustness of selectivity and chromatographic performance	Effects of key separation variables have been assessed and operating ranges specified.	Build in robustness during method development to avoid surprises during validation.

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

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.

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).

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).

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

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

Shutdown State

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.