HPLC Separation Robustness and Ruggedness

Assessing the Effects of Experimental Variables During Method Development

Rita Steed
Application Engineer
Agilent Technologies, Inc.
November 19, 2015

Agilent Technologies

Method Development Goals

- **Performance**
  - —**Robust**
  - —**Rugged**
  - —Specific/selective
  - —Accurate
  - —Precise
  - —Excellent linearity
  - —Broad range
  - —Low LOD and LOQ
- **Cost**
  - —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"*
    - Is indicative of its reliability during normal usage
  - —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 under the variation in conditions normally expected from lab to lab and analyst to analyst"
  - —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.

Why Develop Robust and Rugged Methods?

- Provide greater day-to-day reliability
- Minimizes rework
  - —Saves time and resources ($$$)
- Likelihood of successful method transfer improved
- Robustness and ruggedness are regulatory requirements for the pharmaceutical industry (ICH, FDA, USP)

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
    1) Validation Kits
2. Compare R_s for the 3 lots
3. If ΔR_s is too large, modify method

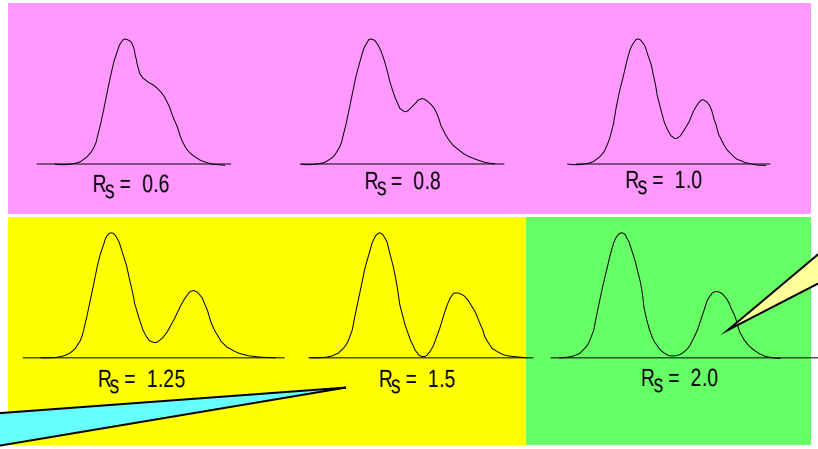
How is Method Robustness Determined

- Systematically vary separation parameters and measure effects on R_s
  - —Incorporate parameter ranges into written method to allow flexibility
    - Include precautionary statement if needed
  - —Helps minimize or avoid many ruggedness problems - but not all

Robustness Example: % Organic Modifier

1. Vary % organic modifier ±1–2%.
2. Evaluate changes to R_s.
3. If ΔR_s is too large at either %B, modify method.

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

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

***ruggedness variable**

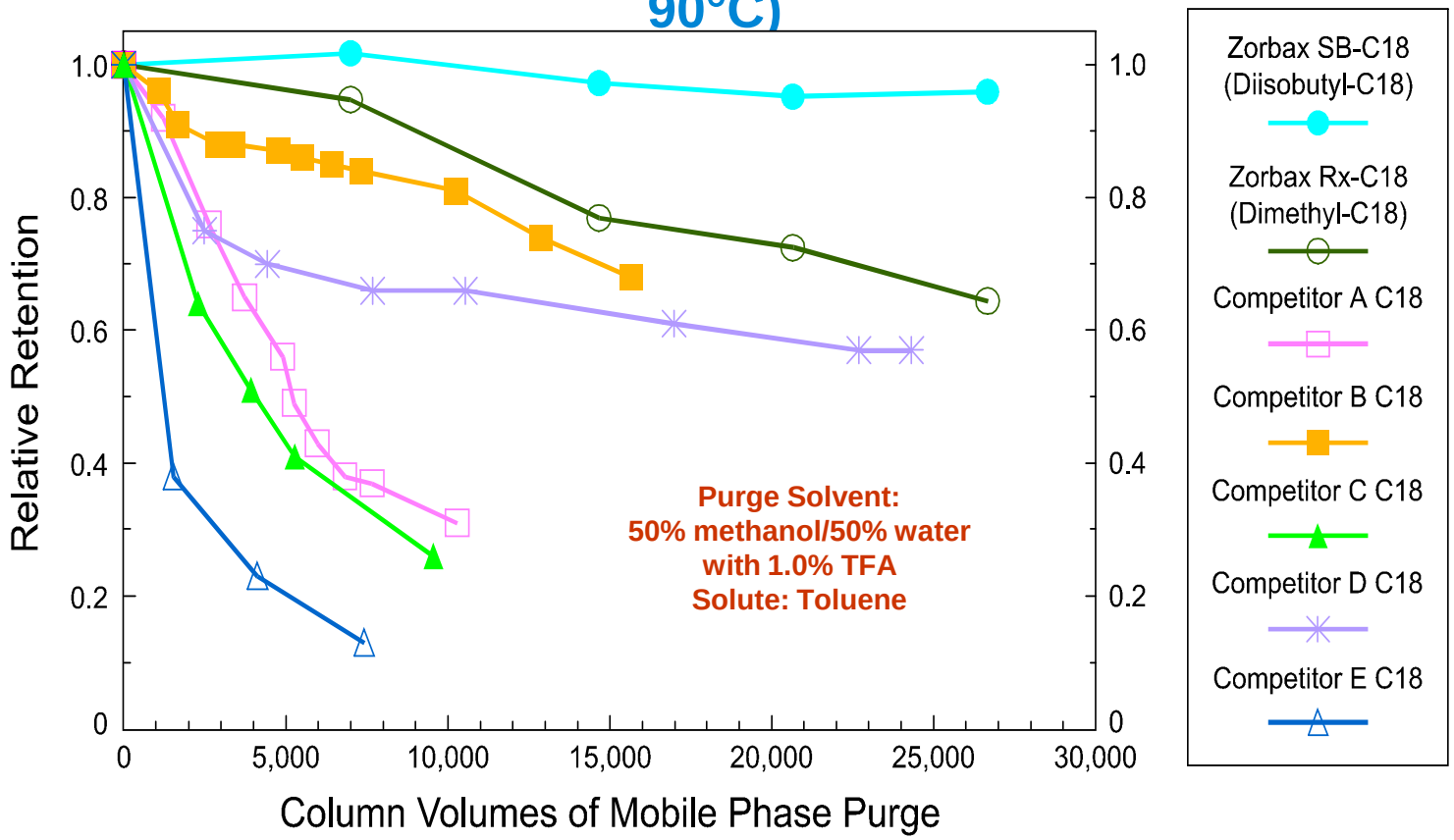
The Column

Experimental Variables that Impact R_s

- **Column**
  - **High-quality column manufacturer**
  - **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:

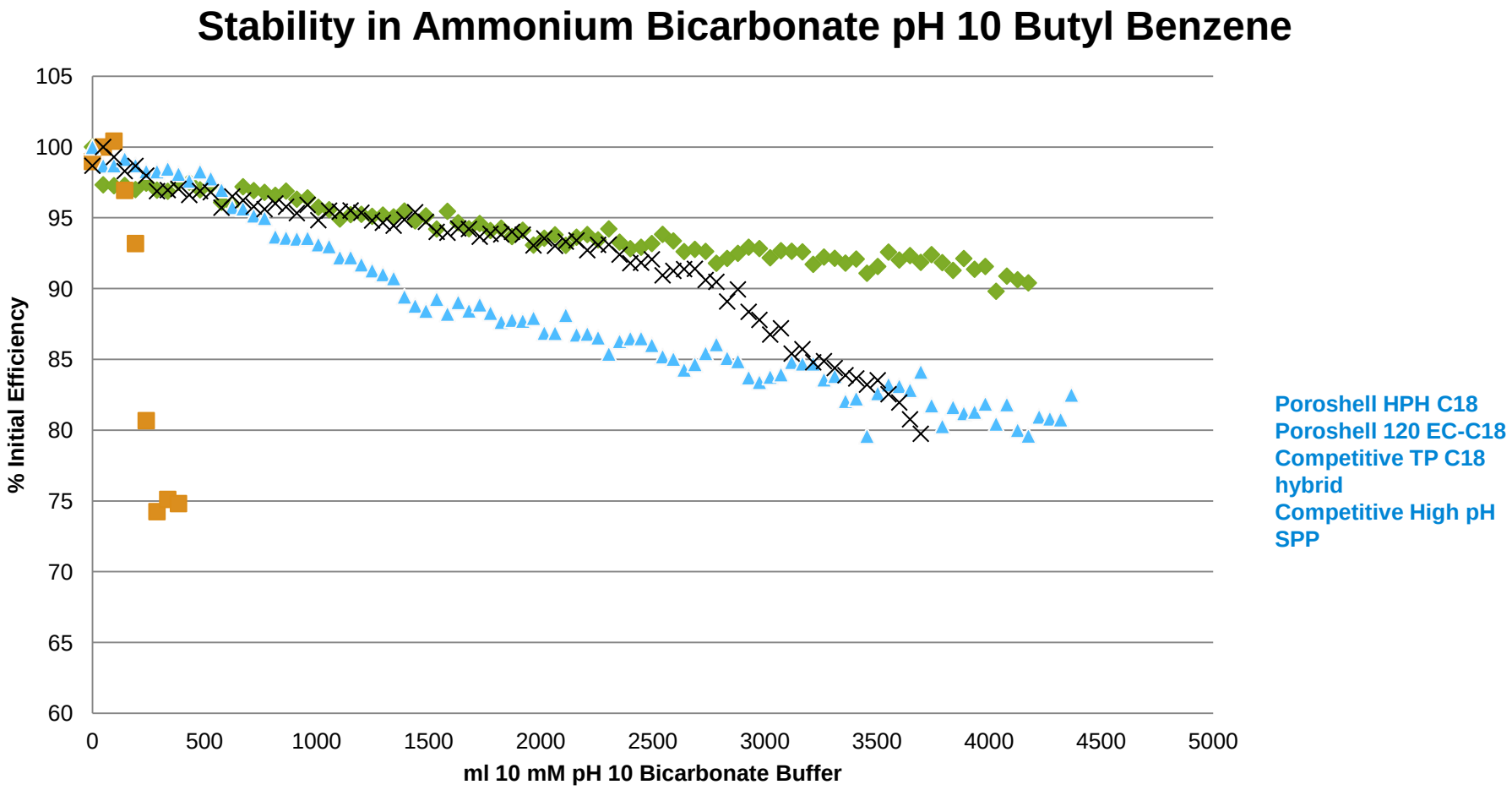
Exceptional Stability At Low pH and High Temperatures (pH 0.8, 90°C)



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

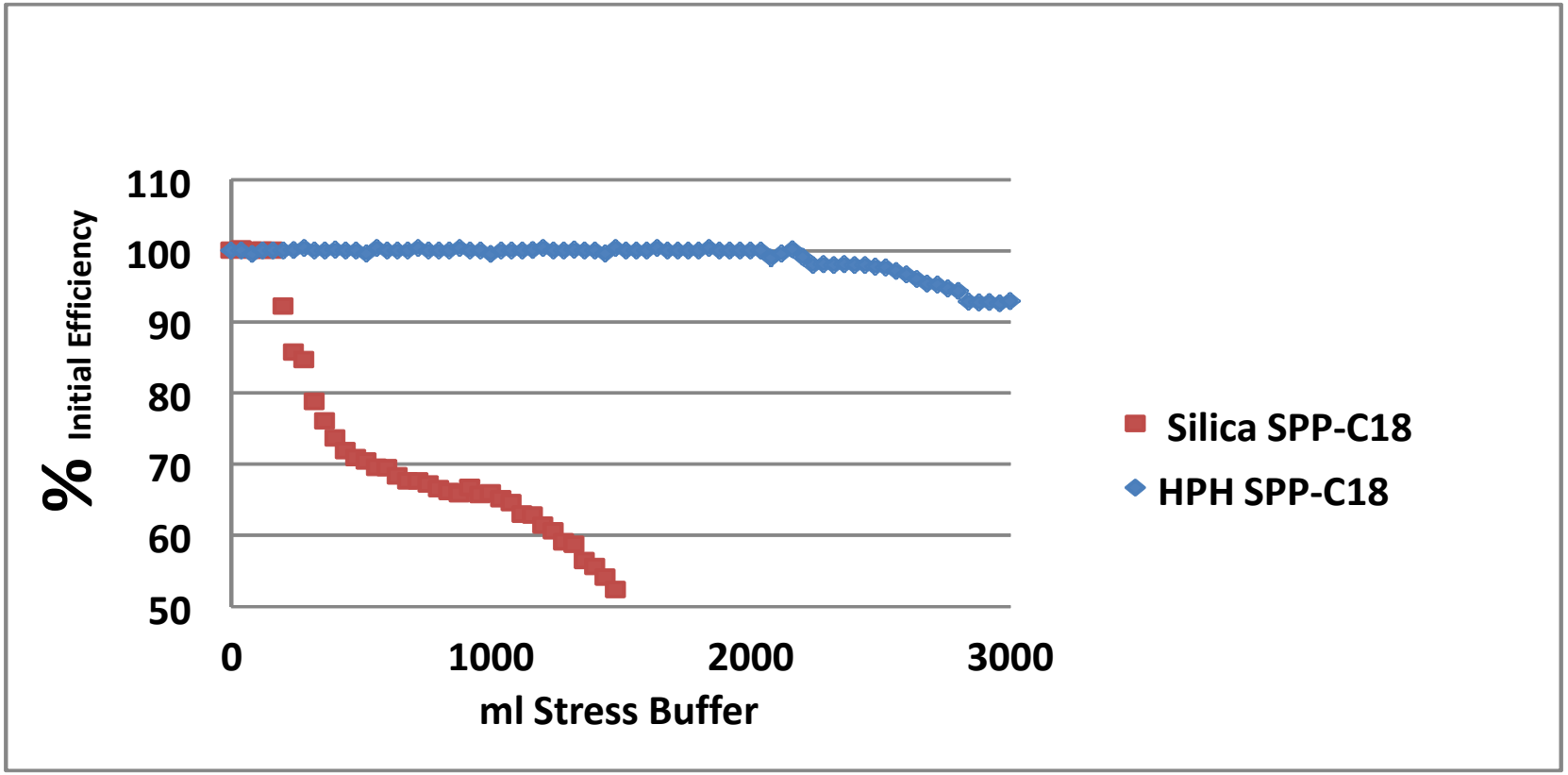
Agilent Technologies

HpH Lifetime in Bicarbonate Buffer at 50 C Excellent



Stress Test - Isocratic pH10, 50C, 10mM Ammonium Bicarb

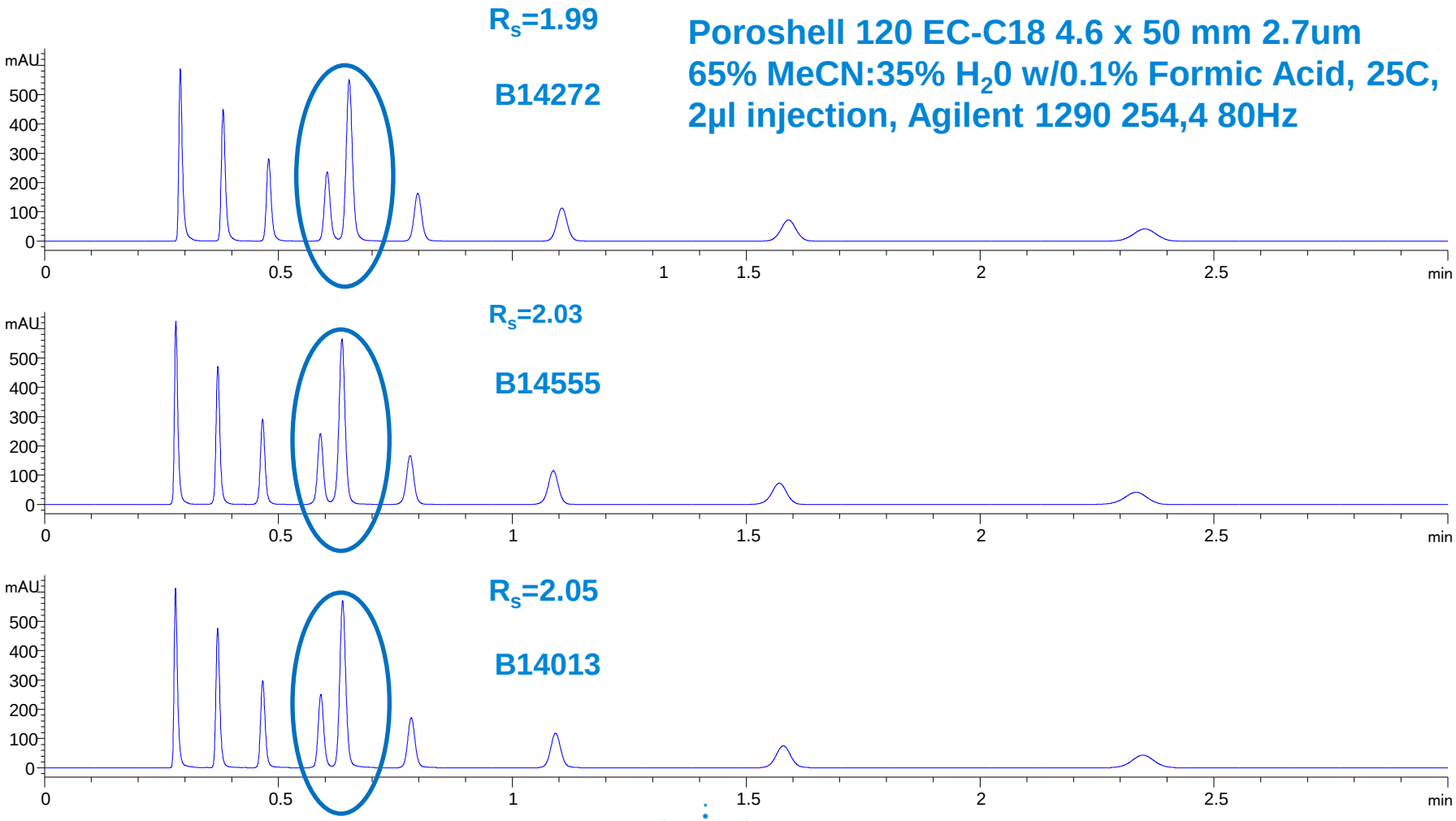
Poroshell HPH C18 lasts longer in phosphate buffer



Lifetime of SPP columns in phosphate buffer, pH 8, at elevated temperature. Mobile phase: Premixed 60% 30 mM sodium phosphate buffer at pH 8 and 40% acetonitrile; Flow rate 0.4 mL/min; UV absorbance 254 nm; 65 °C; Columns: 2.1 x 50 mm, 2.7 µm; Analyte: Naphthalene.

Agilent Technologies

Test at Least 3 Lots
Helps Ensure Method Reproducibility

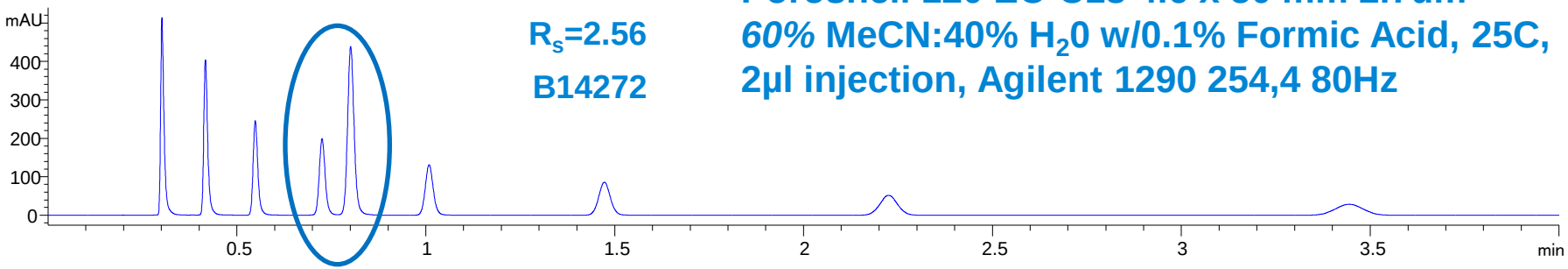


Agilent Technologies

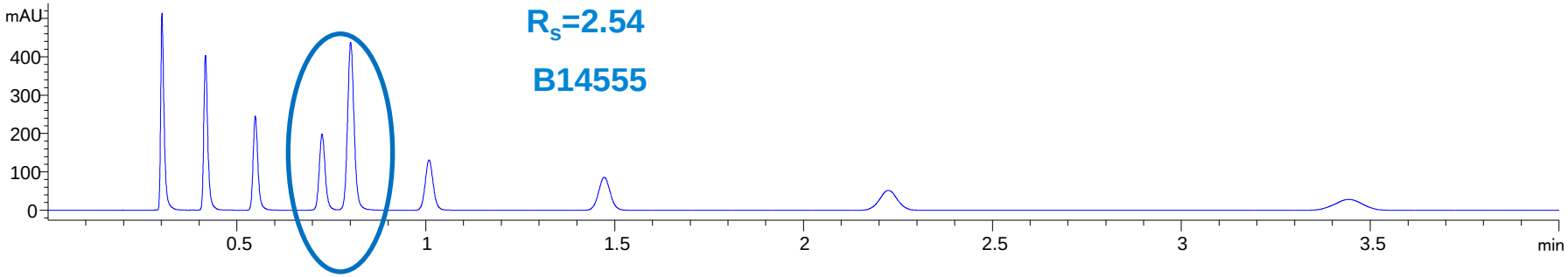
Alkyl Phenones Mix

Slight Change to Method
More Reliable Results

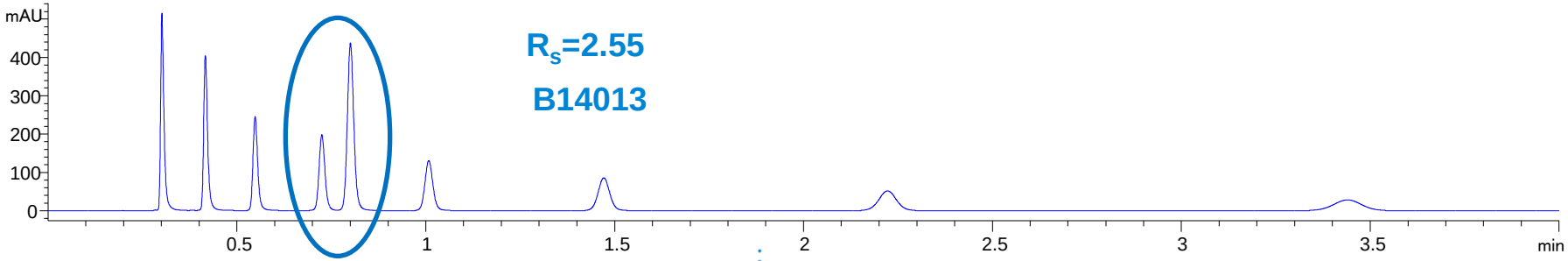
Poroshell 120 EC-C18 4.6 x 50 mm 2.7um
60% **MeCN:40% H_20 w/0.1% Formic Acid, 25C,**
2µl injection, Agilent 1290 254,4 80Hz



R_s=2.56
B14272



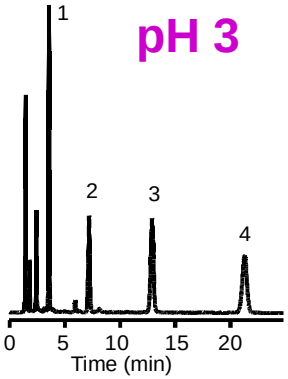
R_s=2.54
B14555



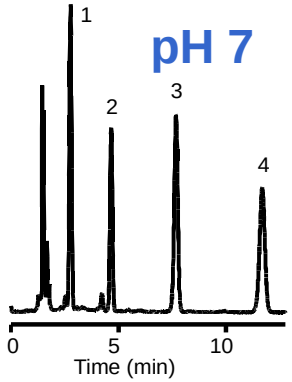
R_s=2.55
B14013

Agilent Technologies

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
Mobile Phase: 85% 25mM phosphate:15% ACN; Flow Rate:1.0 mL/min; Temperature: 35°C

Key Contributor to Method Ruggedness

How Do You Assess Lot-to-Lot Reproducibility?

- Test 3 different column lots and evaluate separation performance.
  - —Compare retention, selectivity, resolution, peak width and symmetry.
- Validation kits – For assistance
  - —Call Agilent LC columns Technical Support, (800) 227-9770, options 3, 3, and 2 or
  - —Email lc-column-support@agilent.com

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

Your **opportunity to improve robustness and ruggedness**

Buffered Mobile Phases

Control Retention of Ionizable Analytes

BUFFERS:

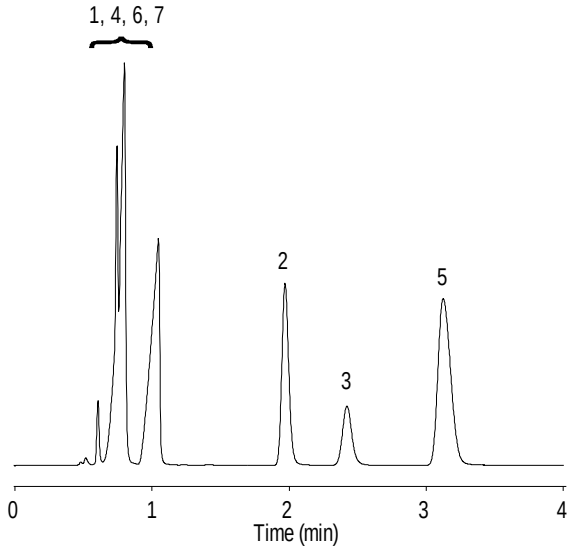
- Provide effective means for varying and controlling pH
- Improve retention, peak width and symmetry (especially for pH $\leq$ 3)
- Minimize or eliminate column-to-column differences
- Eliminate differences in water pH
- Allow efficient use of pH as separation variable during method development

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

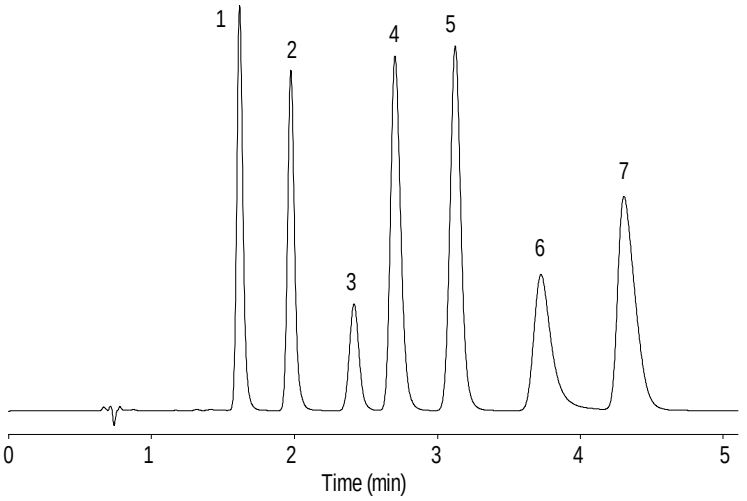
Why Use Buffered Mobile Phases?

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, 25mM phosphate buffer



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

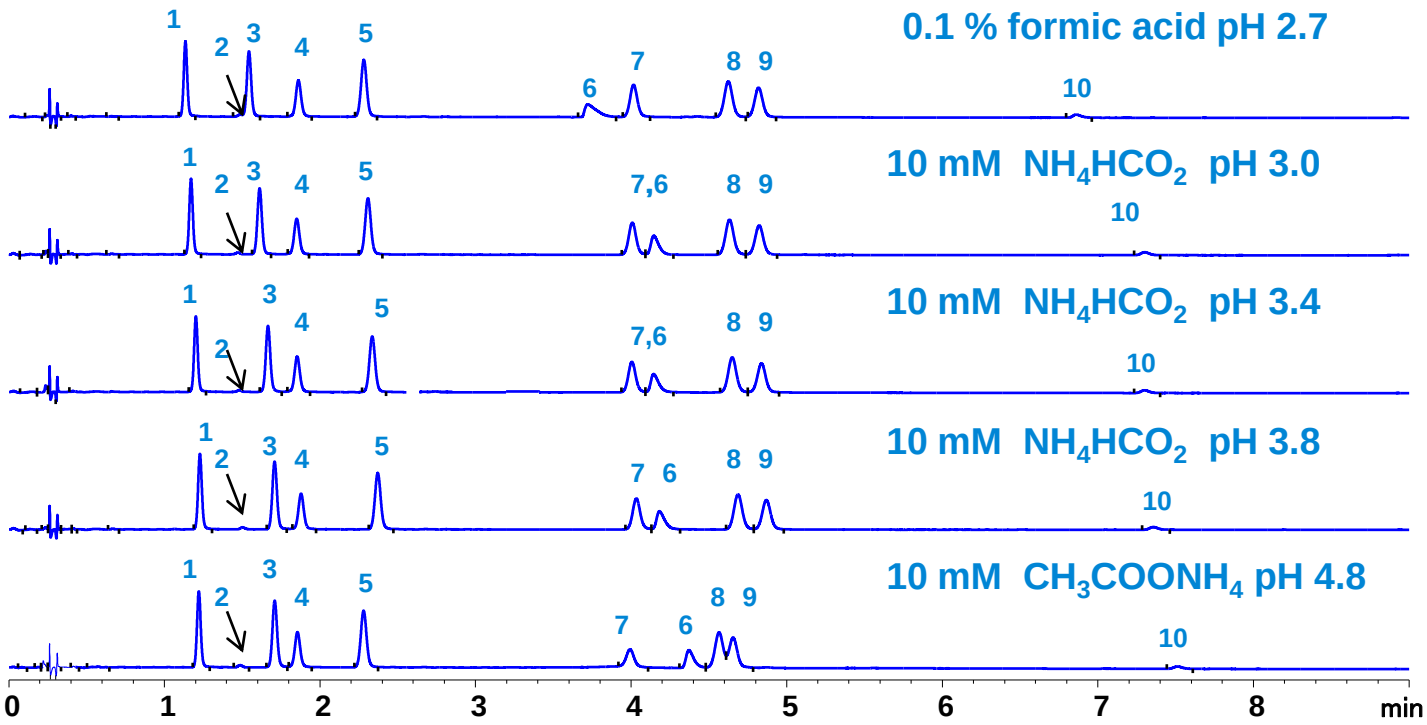
Buffer Selection Considerations

- Buffer Type
  - Inorganic vs. organic buffers
    - Choice can affect resolution and column lifetime
- Buffer pH
  - Select 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 – 25mM
  - Prepare buffer according to accepted procedures
  - Avoid overshoot and readjustment when setting pH
  - Compare resolution at desired buffer concentration ± 5–10mM

Buffer Comparison

Vary Mobile Phase Additive in Acetonitrile

1. SMR
2. TPC
3. SDD
4. FZD
5. SMMX
6. PYM
7. OXA
8. SDMX
9. SQZ
10. DFZ

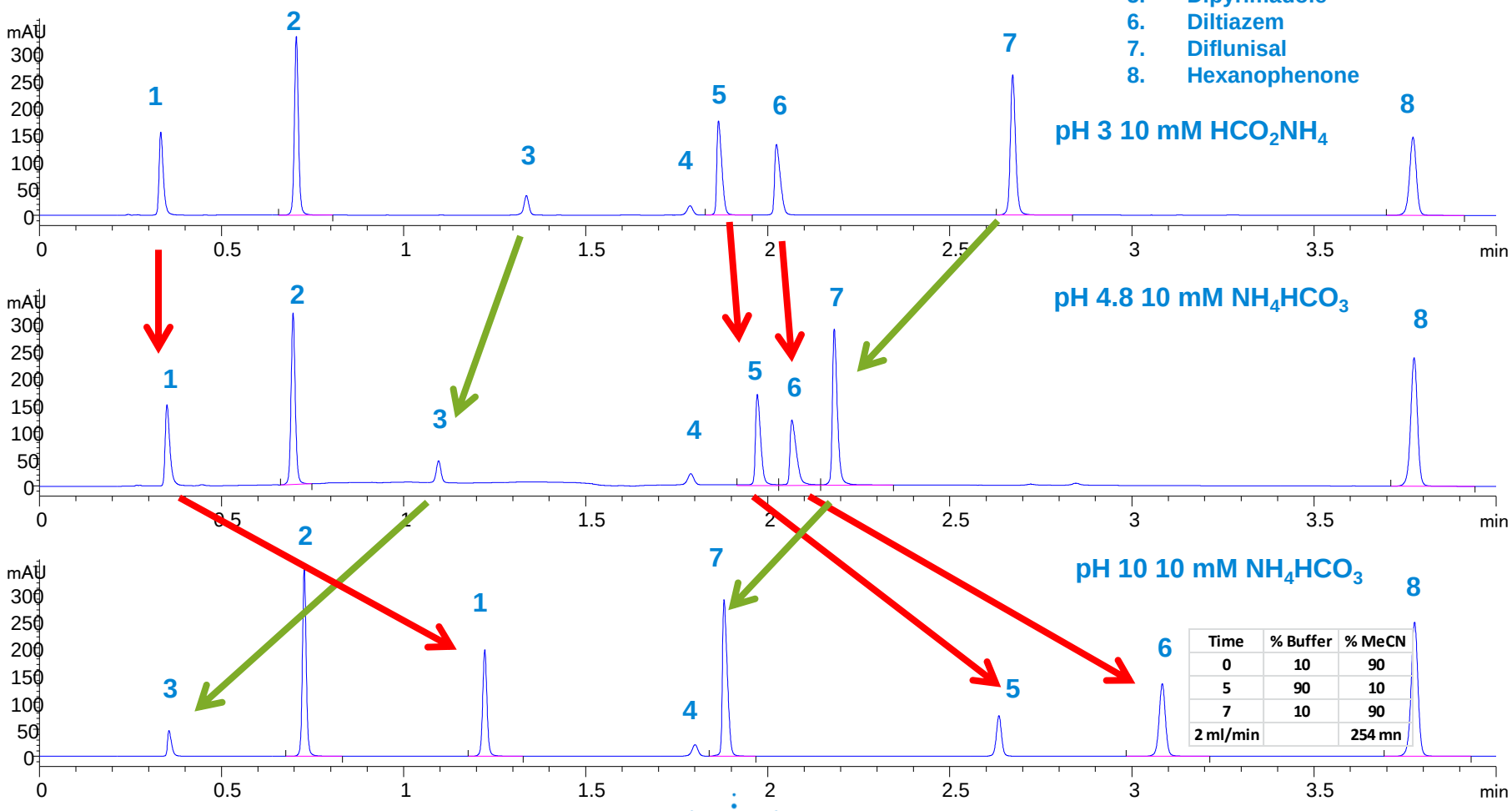


10-40 %B/12 min @ 2 mL/min 0.5 ul injection 0.1 mg/ml each
4.6 x 50 mm Poroshell 120 ECC18; 205 Bar

Agilent Technologies

Selectivity can be controlled by changing pH

1. Procainamide
2. Caffeine
3. Acetyl Salicylic Acid
4. Hexanophenone Deg.
5. Dipyrimadole
6. Diltiazem
7. Diflunisal
8. Hexanophenone



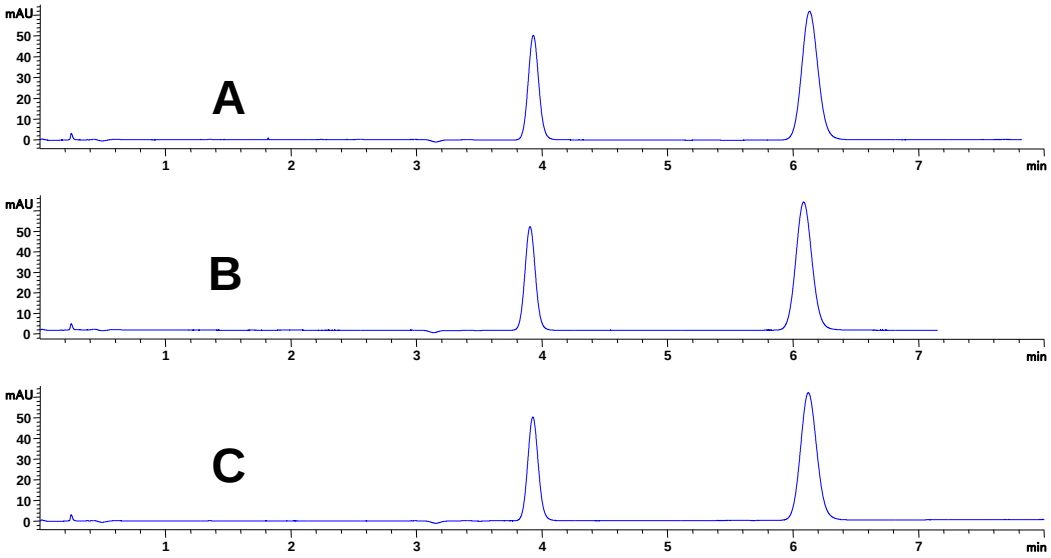
Time	% Buffer	% MeCN
0	10	90
5	90	10
7	10	90
2 ml/min		254 mn

Poroshell HPH C18 4.6x50 mm, 2.7µm

Agilent Technologies

Robustness- pH

Column: XDB-C8, 4.6 x 50 mm, 1.8 µm **Mobile Phase: 68% 0.01M H3PO4 : 32% ACN** **Flow Rate: 2.0 mL/min**
Temperature: 25C **Sample: 1. Benzophenone 1 mg/mL 2. Ibuprofen 1.2 mg/mL** **Injection Volume: 1.7 µL**



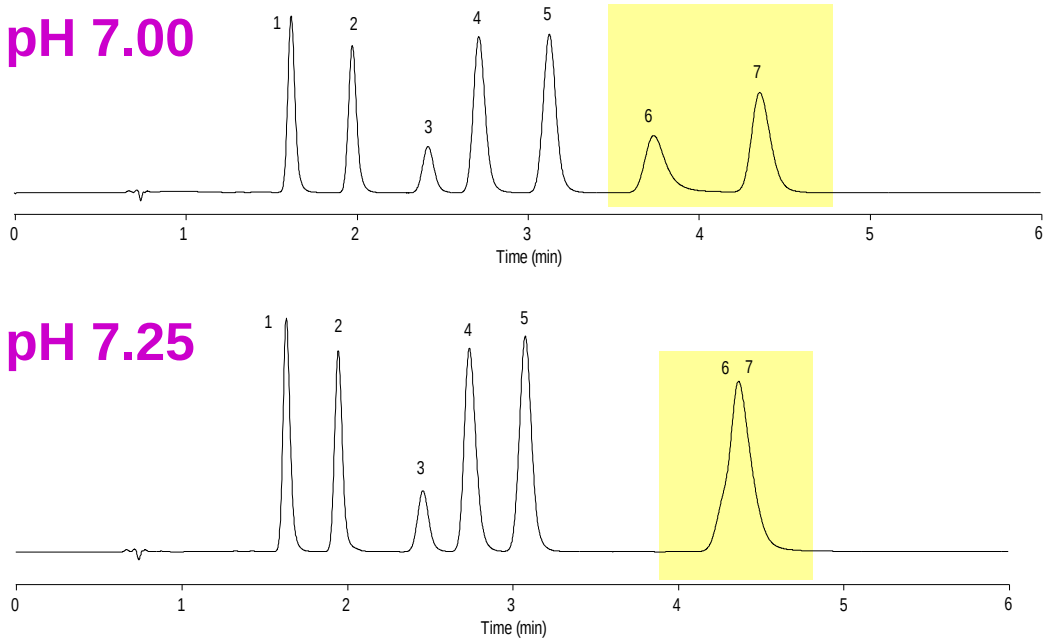
	mL acid	α	T_f
A	0.4	1.60	1.09
B	0.7	1.60	1.08
C	0.8	1.60	1.09

- Tested 3 pHs-mobile phase variation
- Monitor for changes in selectivity (α) and peak shape (T_f) of ibuprofen

Agilent Technologies

Test for pH Robustness

Column: ZORBAX Rapid Resolution Eclipse XDB-C8, 4.6 x 75 mm, 3.5 µm
Mobile Phase: 44% 25mM phosphate, pH 7 : 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



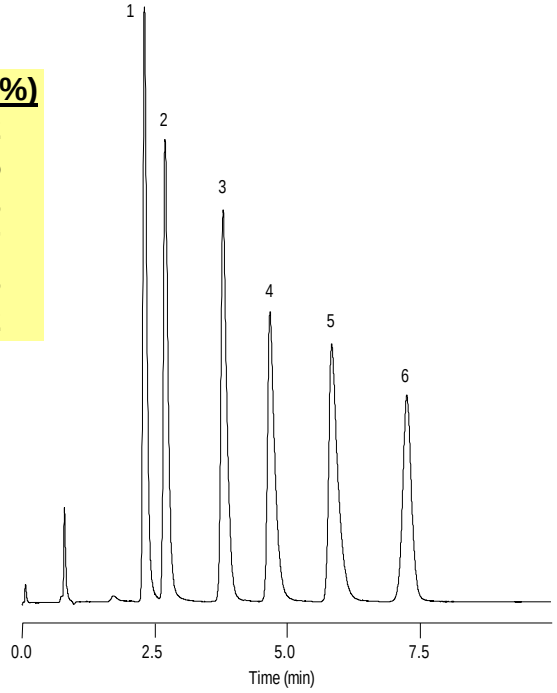
- The resolution of ionizable compounds can change markedly with pH changes—even as small as 0.05–0.25 pH units.

Buffer Concentration Changes
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

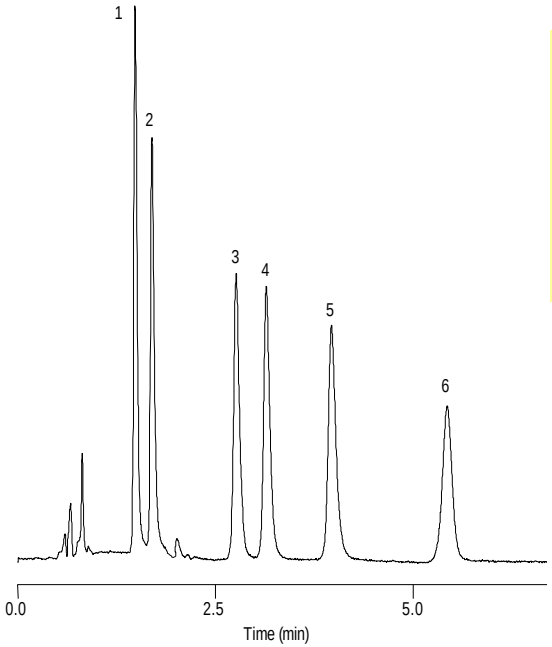
10 mM Phosphate

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

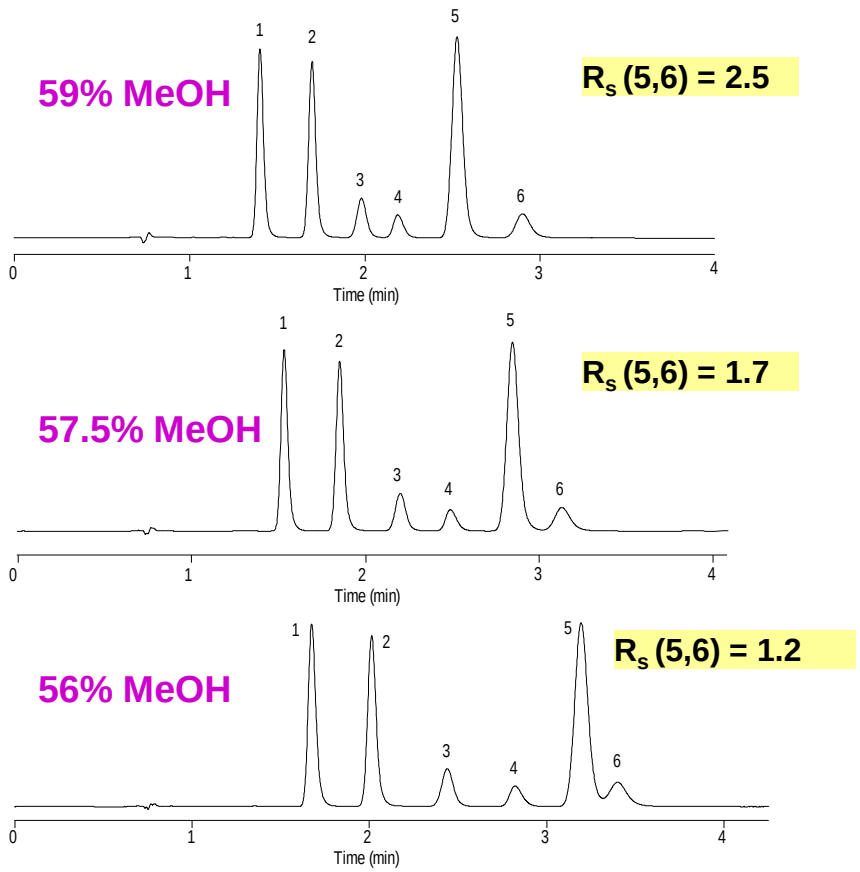


Agilent Technologies

Mobile Phase: Organic Component
Experimental Variables That Impact Resolution

- Column
- **Mobile Phase**
  - Aqueous Component
  - **Organic Component**
    - **% Organic Modifier**
- Sample
- Instrument
- Gradient Separations

Small Change in % Organic Modifier Can Change Resolution



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

Mobile Phase::A: 25 mM phosphate, pH 7,(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%).

Agilent Technologies

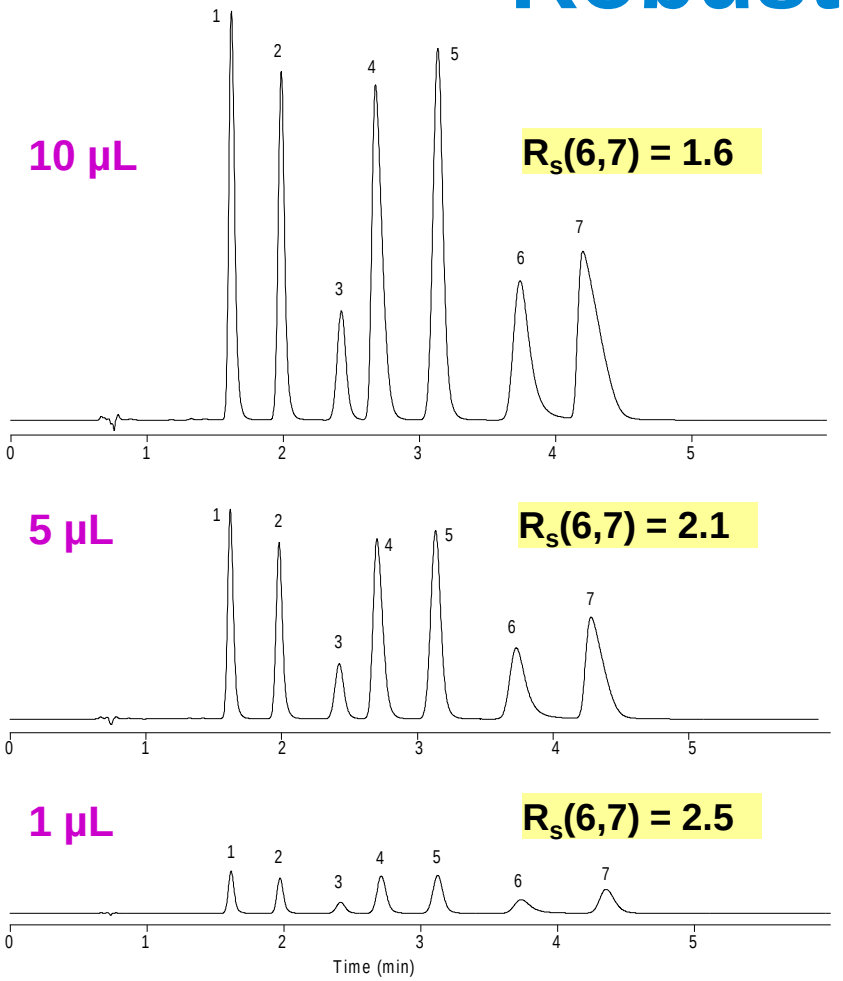
The Sample
Experimental Variables That Impact Resolution

- 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

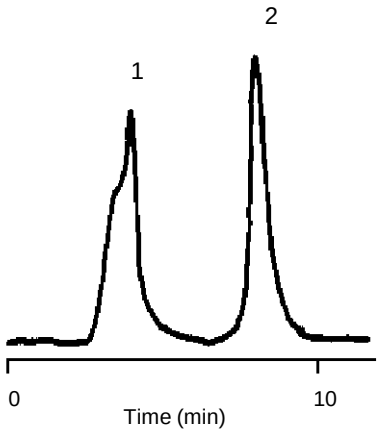
- Varying injection volume can sometimes reveal lack of robustness for resolution and peak shape.

Strong Sample Solvent Can Compromise Peak Shape

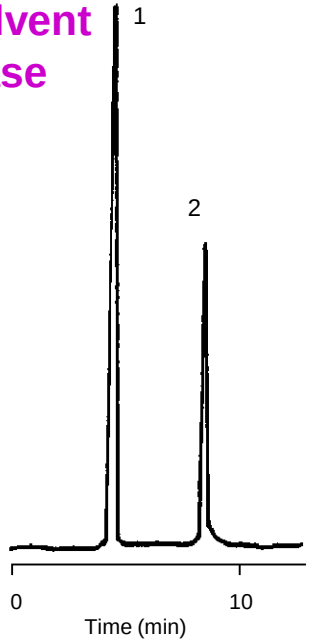
Column: ZORBAX SB-C8, 4.6 x 150 mm, 5 µm Mobile Phase: 82% H_2O:18% ACN Injection Volume: 30 µL

Sample: 1. Caffeine 2. Salicylamide

A. Sample Solvent 100% Acetonitrile



B. Sample Solvent Mobile Phase



Agilent Technologies

Instrument
Experimental Variables That Impact Resolution

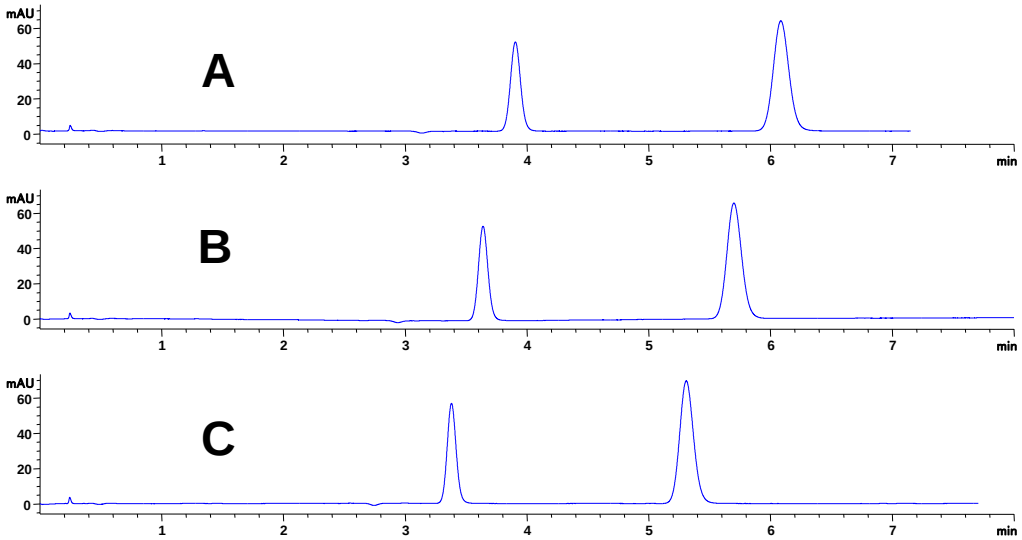
- Column
- Mobile Phase
- Sample
- **Instrument**
  - **Column temperature**
  - **Detector flow cell volume**
- Gradient Separations

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.

Robustness- Temperature

Column: XDB-C8, 4.6 x 50 mm, 1.8 µm **Mobile Phase: 68% 0.01M H3PO4 : 32% ACN** **Flow Rate: 2.0 mL/min**
Temperature: see below **Sample: 1. Benzophenone mg/mL 2. Ibuprofen .005 mg/mL** **Injection Volume: 1.7 µL**

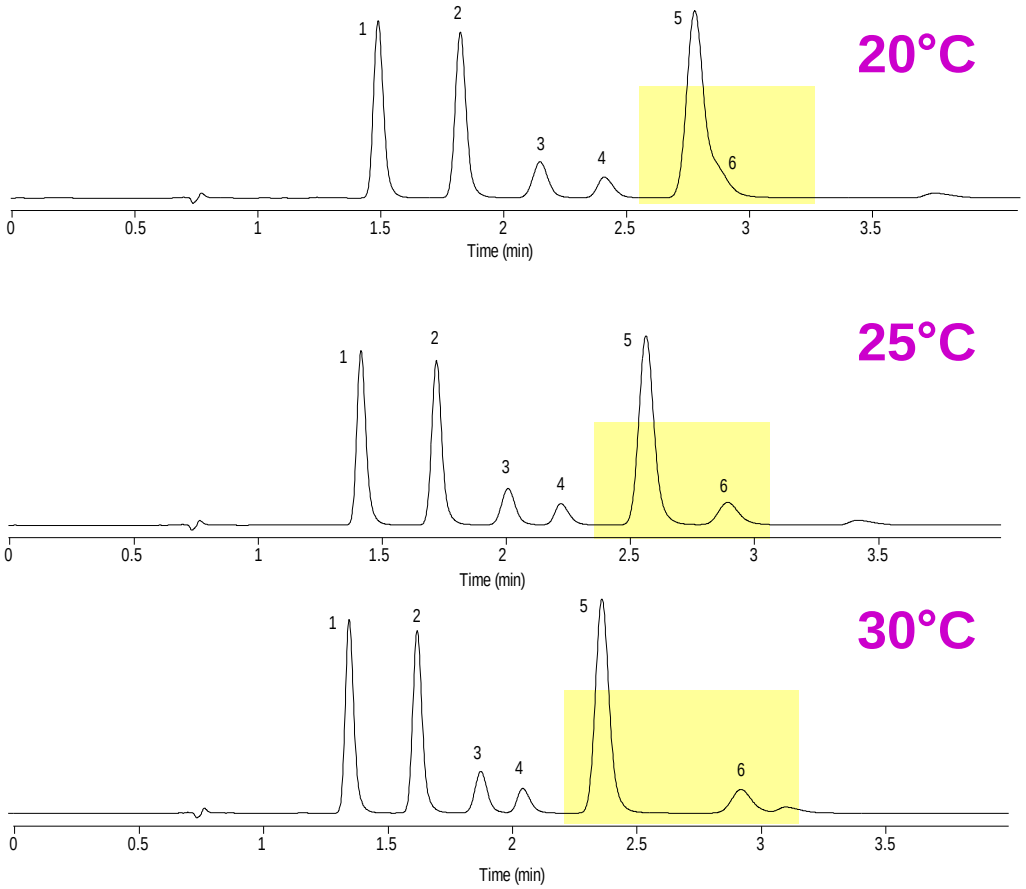


	°C	α	T_f
A	25	1.60	1.08
B	30	1.61	1.09
C	35	1.62	1.10

- Tested 3 temperatures – Room Temperature, 30°C and 35°C
- Monitor for changes in selectivity (α) and peak shape (T_f) of ibuprofen

Agilent Technologies

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

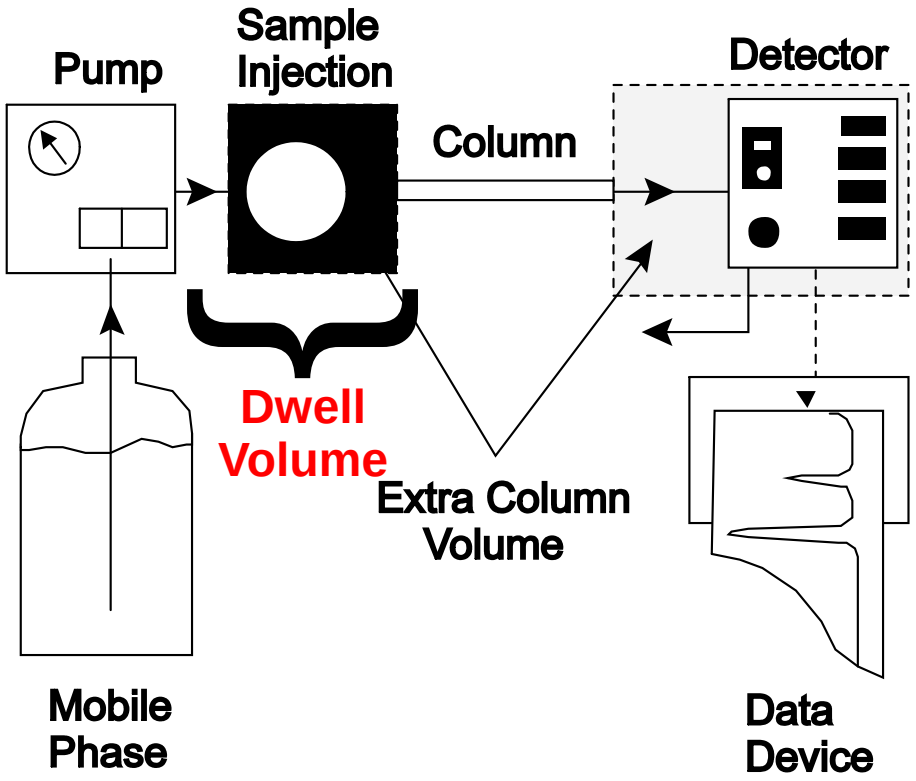
Flow Cell Volume	Band Broadening* (4.6 mm)	Band Broadening* (2.1 mm**)
1.7 µL	0.3%	6%
8 µL	6%	138%
14 µL	19%	423%

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

Gradient Separations
Experimental Variables That Impact Resolution

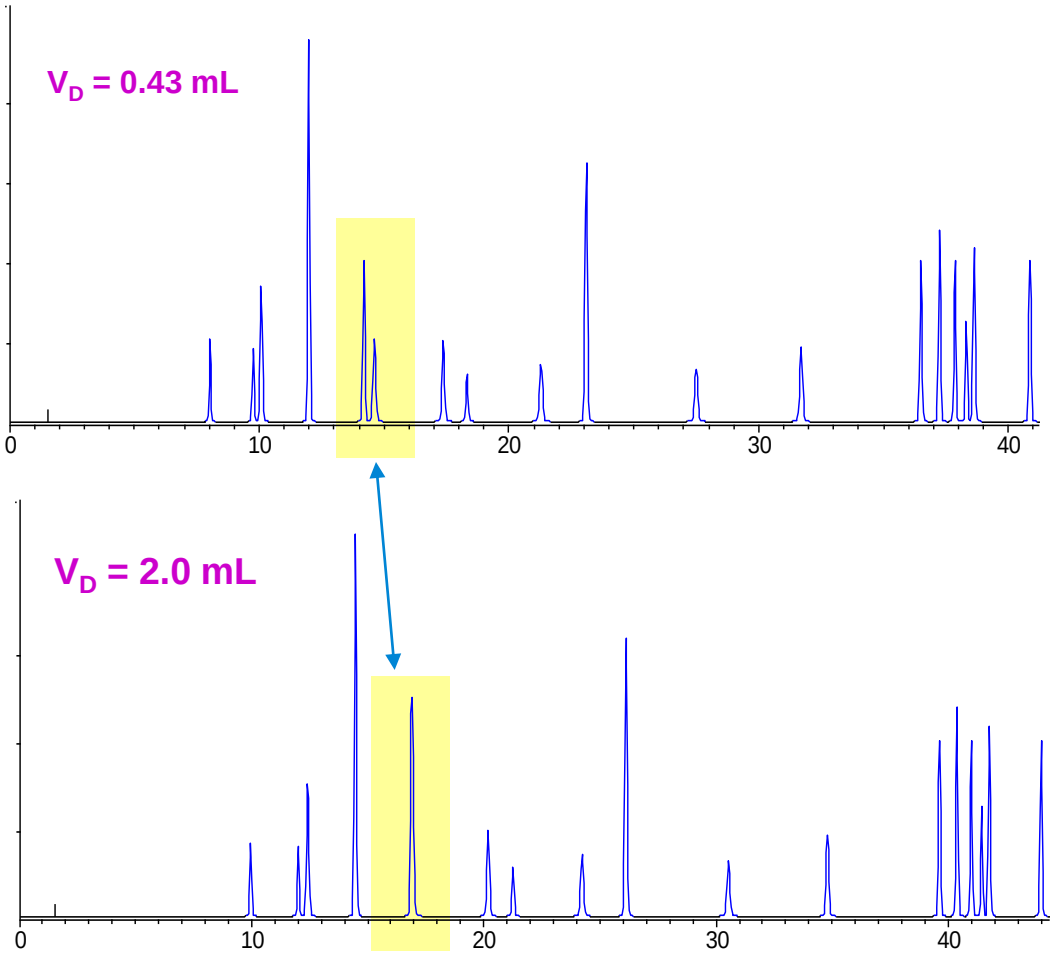
- Column
- Mobile Phase
- Sample
- Instrument
- **Gradient Separations**
  - **Dwell volume**
  - **Gradient steepness**

Gradient Separations
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

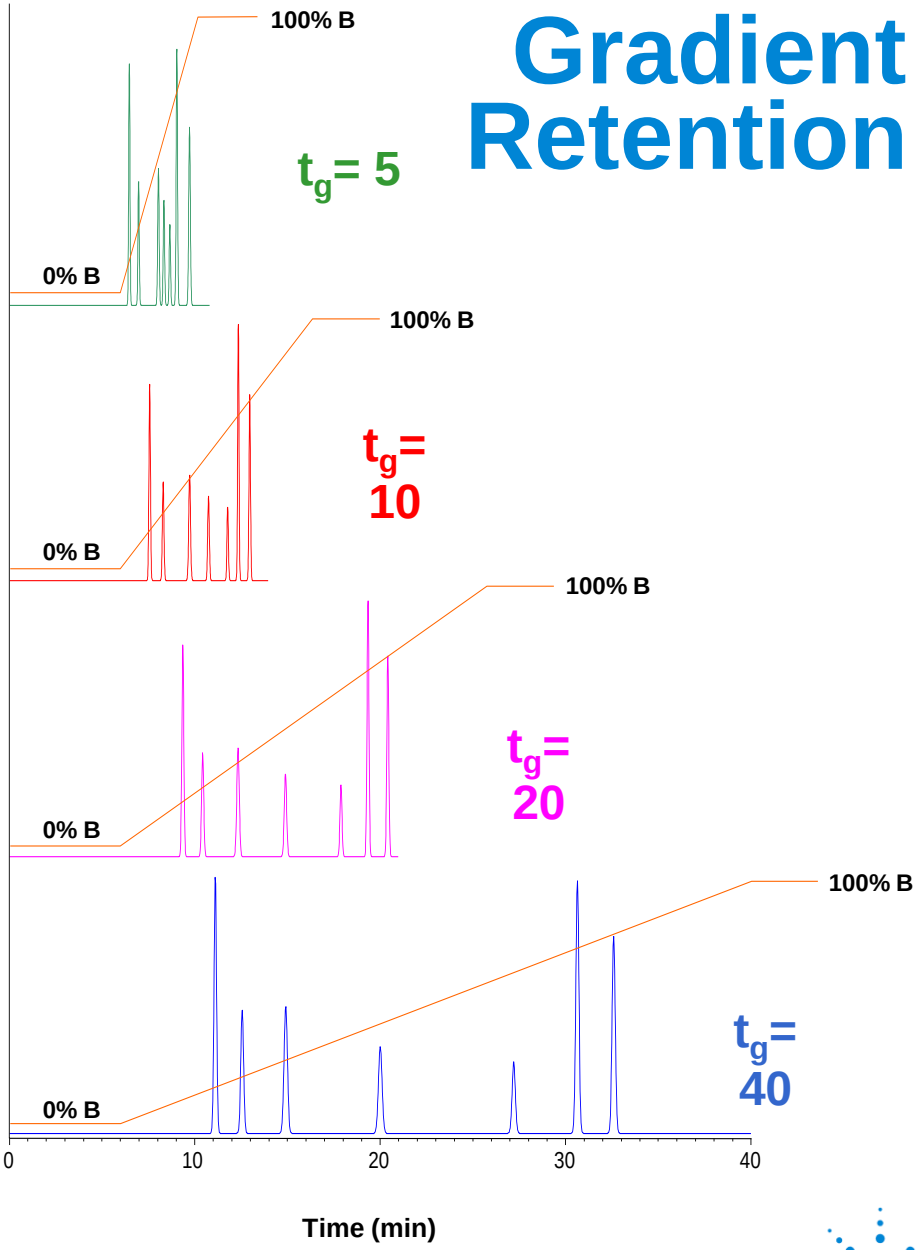
Lower trace is simulated chromatogram for larger V_D

Agilent Technologies

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
- 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

Agilent Technologies

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
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

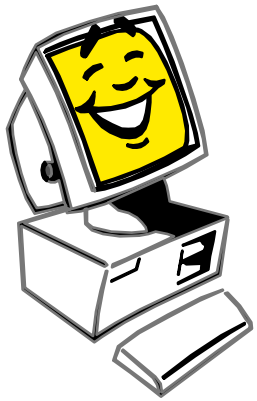
Agilent Technical Support

- **800-227-9770 (Toll Free US & Canada)**

  - **For LC columns**
  - ***Select option 3, then option 3, option 2***
  - ***For GC Columns***

Select option 3, then option 3, option 1

  - **916-608-1964 (fax)**



- **www.agilent.com/chem**

Agilent Technologies

Exclusive Offer for Attending Agilent Seminars

Receive a 25% discount off any Agilent J&W GC Columns, Agilent LC Columns, Sample Prep products, and your most often used GC and LC supplies*

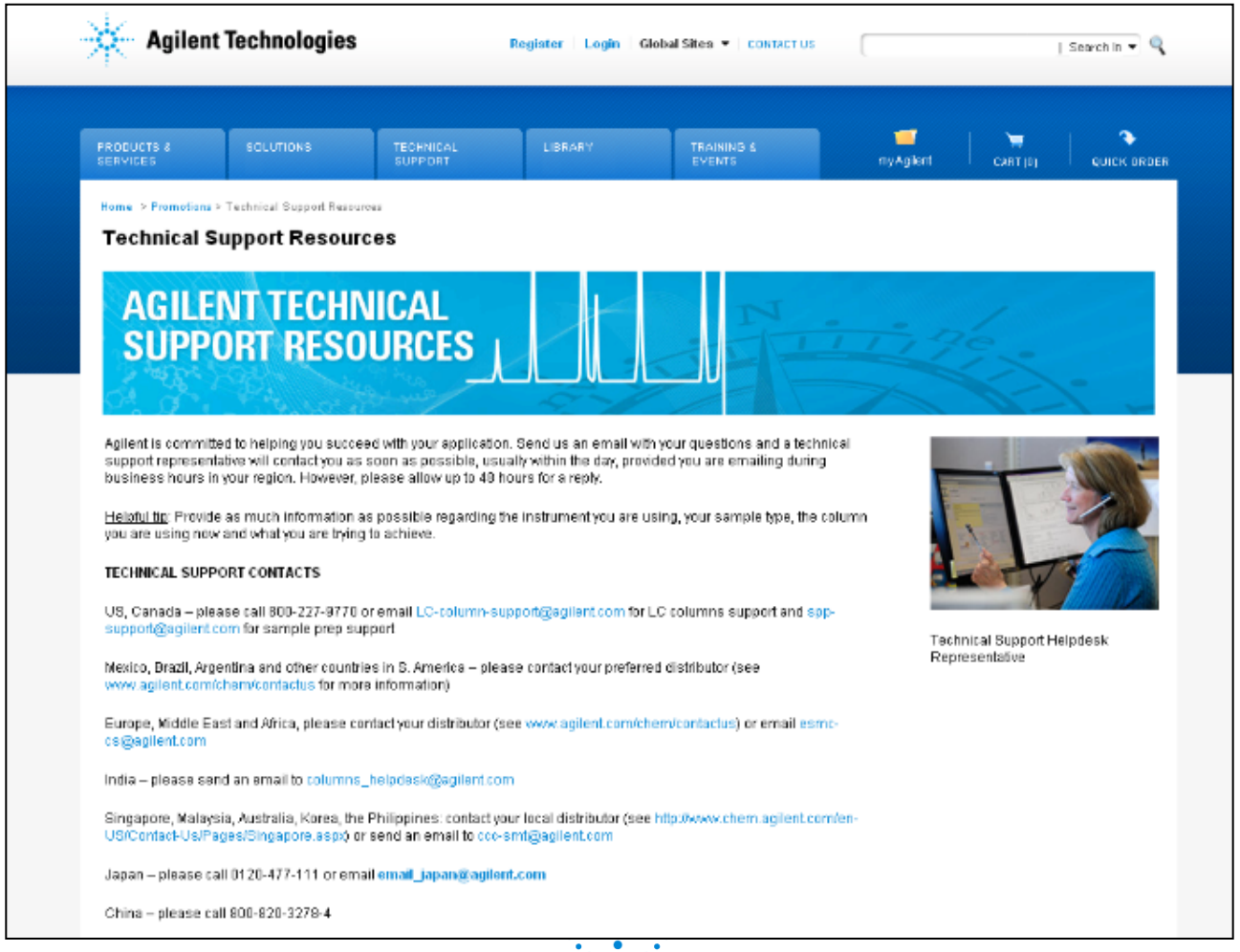
- **Discount applies on future purchase of up to $3,000 (USD)**
- **Offer good 30 days from the Agilent eSeminar**
- **Use promotion code 8.066**

***Some restrictions apply on supplies**

Agilent Technologies

Agilent is Here to Help

- Visit www.agilent.com/chem/cstechsupport



Agilent Technologies

Upcoming LC and LC/MS eSeminars

HPLC - There's a Lot to Learn –
Tips for Avoiding Pitfalls

January 19, 2016 – 11:00am ET

Gradient, Reversed Phase HPLC –
The Why, What, When and How

February 23, 2016 – 11:00am ET

Visit our website to register for these live seminars

www.agilent.com/chem/LCsem

Agilent Technologies

The Agilent LC Column and Sample Prep Navigator can help you find the optimal products for your separation. Find your LC solution!

We are happy to announce a new online tool to help easily find Agilent LC Columns or Sample Prep products for any application. Look for the tool at www.agilent.com/chem/navigator

This tool enables users to find an Agilent column and sample prep product recommendation in four easy ways:

1. By entering a competitive column or sample prep method (for a comparable Agilent cross-reference)
2. By entering some information about their analysis (i.e., instrument type, sample type, pKa of sample, hydrophobicity of sample, etc.)
3. By compound look-up
4. By USP code.

Agilent Technologies

Don’t Forget to Follow Agilent

Stay up-to-date with the latest news from Agilent…join the conversation today!

- twitter.com/AgilentChem (@AgilentChem)
- twitter.com/AgilentLife (@AgilentLife)
- youtube.com/AgilentChem
- youtube.com/AgilentLife
- linkedin.com/company/Agilent-Technologies
- facebook.com/Agilent.Tech

Agilent Technologies

Appendix

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

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

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.

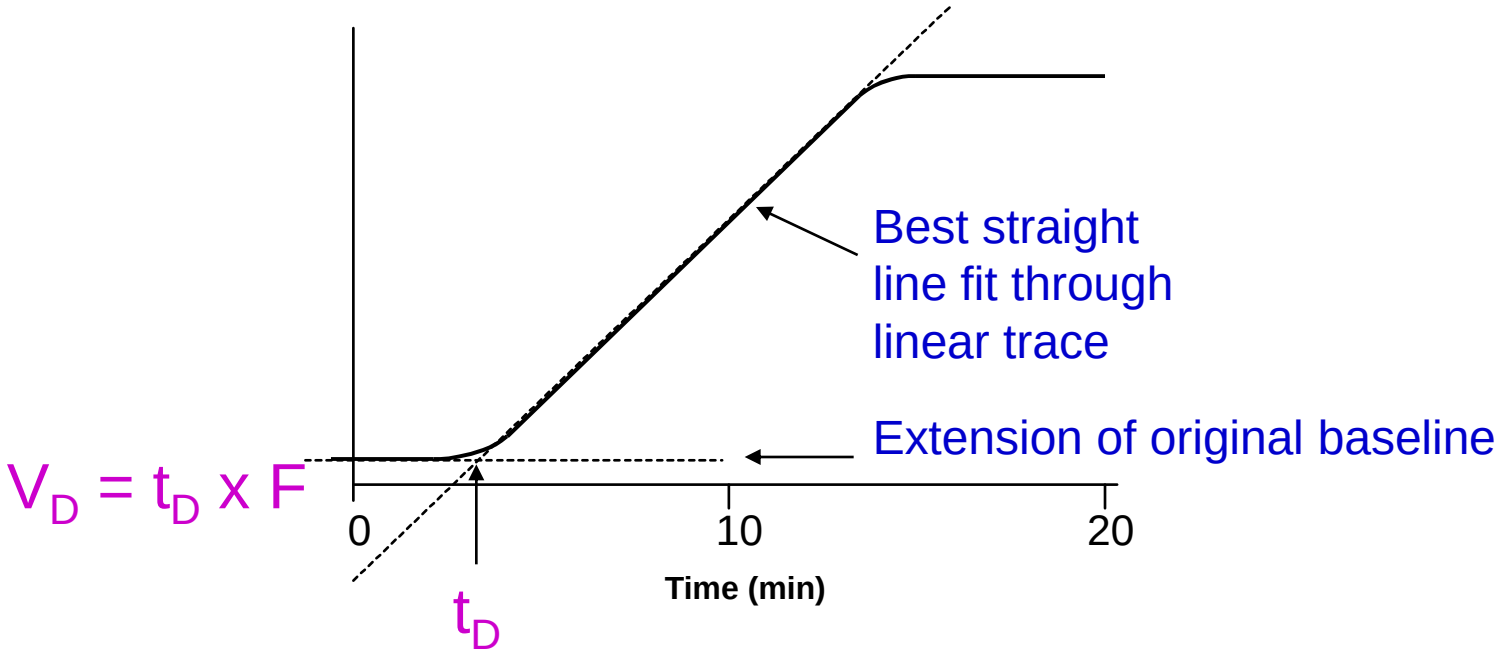
Instrument flushing

- 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.

Determining the Dwell Volume of Your System

- Replace column with short piece of HPLC stainless steel tubing
- Prepare mobile phase components
  A. Water -V-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 (V_D)



- Intersection of the two lines identifies dwell time (t_D)
- Dwell volume is equal to product of the flow rate and the dwell time.