Conquer Method Variability

Evaluate Variables During Method Development

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Common Separation Goals and Method Performance Criteria

Good System Suitability Parameters

- Resolution: ≥2
- Peak shape: USP T_f close to 1 (<2)
- Inj. Repeatability: areas, T_f, (RSD 0.1 - 0.25%)
- Absolute retention factors: 1< k<10
- Relative Retention: α or k_2/k_1
- Signal-to-Noise Ratio: >10

AVOID THESE for System Suitability Criteria:

Column efficiency (theoretical plates)
Absolute retention time

These may prevent the ability to speed up your method in the future!

Method Performance Criteria

- Accuracy
- Precision
  - Ruggedness
  - Robustness
- Analytical Selectivity/Specificity
- Linearity
- Range
- Quantitation Limit (LOQ, 10x S/N)
- Detection Limit (LOD, 3x S/N)

Terms and Definitions

Robustness

"is a measure of [an analytical procedure] to remain unaffected by small, but deliberate variations in the method parameters"[2]

- Prerequisite for a rugged method
- Sensitivity of R to small changes that may occur from day to day
  - Temperature
  - Mobile phase pH
  - Flow rates
  - Extraction solvent composition

Ruggedness

"reproducibility of results when a method is performed as written under actual use conditions"[1]

- Separation ruggedness: long-term reproducibility of R
  - Multiple labs
  - Analysts
  - Different instruments
  - Reagent lots, Columns
  - Different days

1. According to The United States Pharmacopeia (USP)
2. International Conference on Harmonization (ICH) Guideline: Validation of Analytical Procedures: Text and Methodology, Q2 (R1), Nov. 2005.

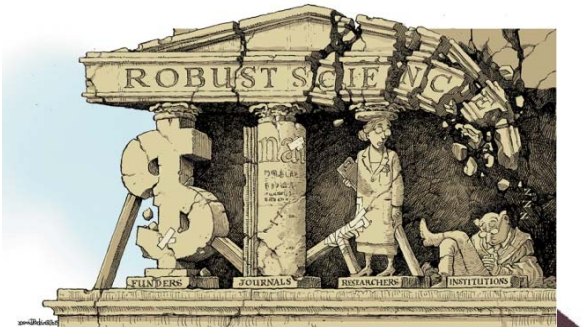
Why Incorporate Ruggedness and Robustness?

Scenario: You're trying to reproduce an experiment in a journal article but have been unsuccessful. You contact one of the authors, Marco the Magnificent. You explain your dilemma and ask him to help you understand why it's not working.

His response: *"OF COURSE YOU CAN'T REPLICATE MY EXPERIMENT. THERE'S A SECRET INCANTATION YOU HAVE TO CHANT, AND I'M NOT TELLING IT TO ANYONE."*

Unexpected Variables

- Differences in calibrating equipment
- Different instruments
- Different individuals with varying levels of experience
- Different lots of reagents/columns



Studies estimate that only around 40% of published findings can be replicated reliably.[1]

Cartoon Reference: Begley, CG, Buchan AM, Dirnagl. Robust research: Institutions must do their part for reproducibility. *Nature* ***525,*** 25-27 (03 September 2015)

1. Baker M, Penny D. Is there a reproducibility crisis? A Nature survey lifts the lid on how researchers view the 'crisis' rocking science and what they think will help. *Nature* ***533,*** 452-454 (26 May 2016)

It's Science Not Magic
Ruggedness

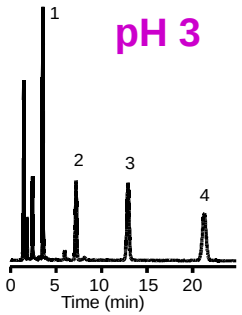
Assess method performance in two or more different labs—ideally over time

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

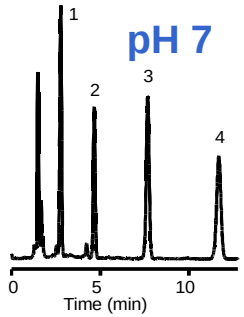
Ruggedness Example: Column Lot

1. Test 3 different column lots
2. Compare RT for the 3 lots
3. If ΔRT is too large…
   i. Sequester "good" batch – **Should be avoided!**
   ii. Improve method
   iii. Consider a different column or manufacturer

Lot-to-Lot Reproducibility Improves Method Ruggedness



RSD	Retention, k´				Selectivity, a			
	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



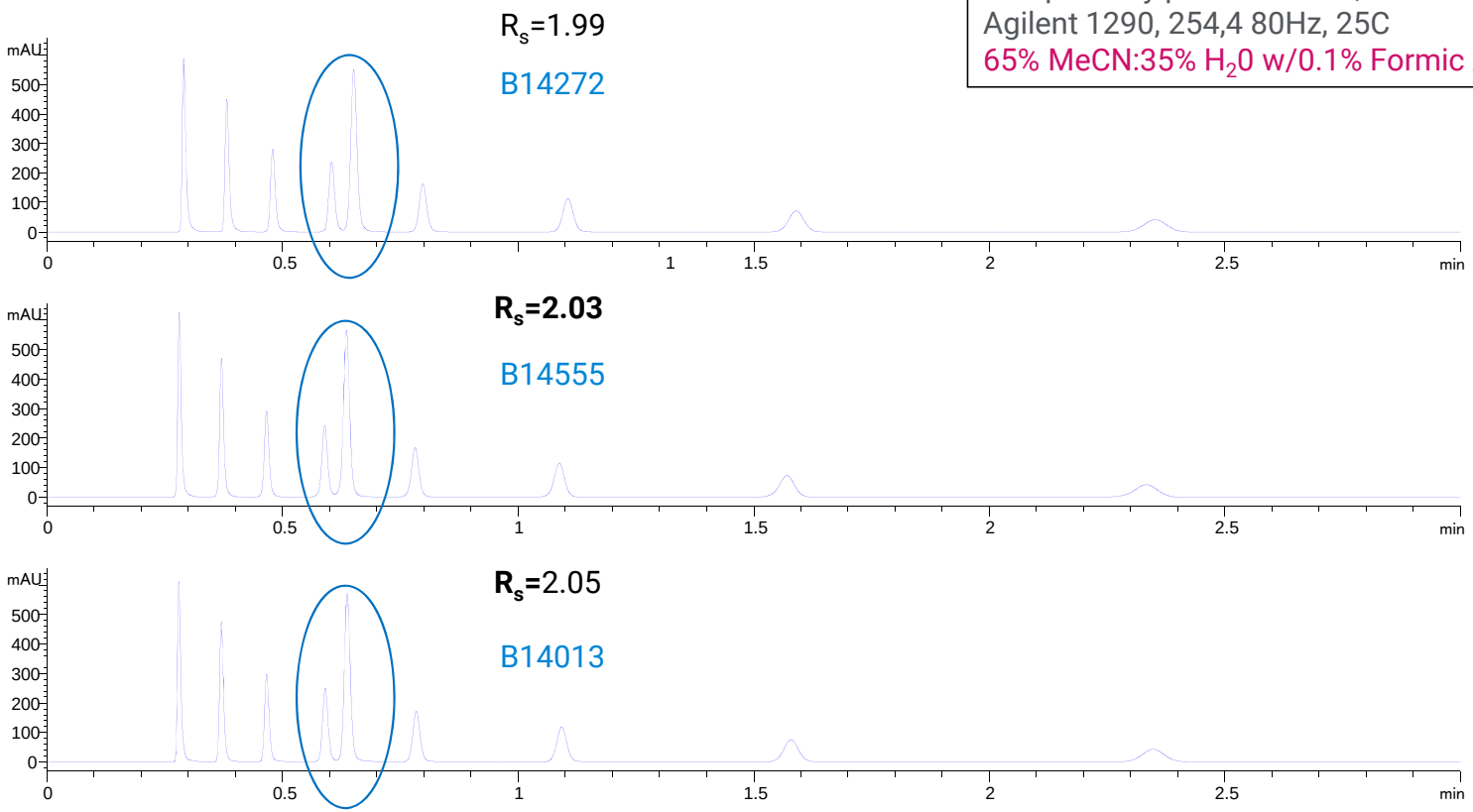
RSD	Retention, k´				Selectivity, a			
	Lot 1	Lot 2	Lot 3	% RSD	Lot 1	Lot 2	Lot 3	%
1. Cefotaxime	0.80	0.88	0.88	5.4	-	-	-	-
2. Cefoxitin	1.96	2.14	2.15	5.1	2.45	2.43	2.44	0.41
3. Cefamandole	3.83	4.15	4.16	4.6	1.96	1.94	1.94	0.59
4. Cephalothin	6.26	6.79	6.84	4.8	1.64	1.64	1.64	0.00

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

- *Agilent Method Validation Kits*

Help Ensure Method Reproducibility
Test at Least 3 Lots

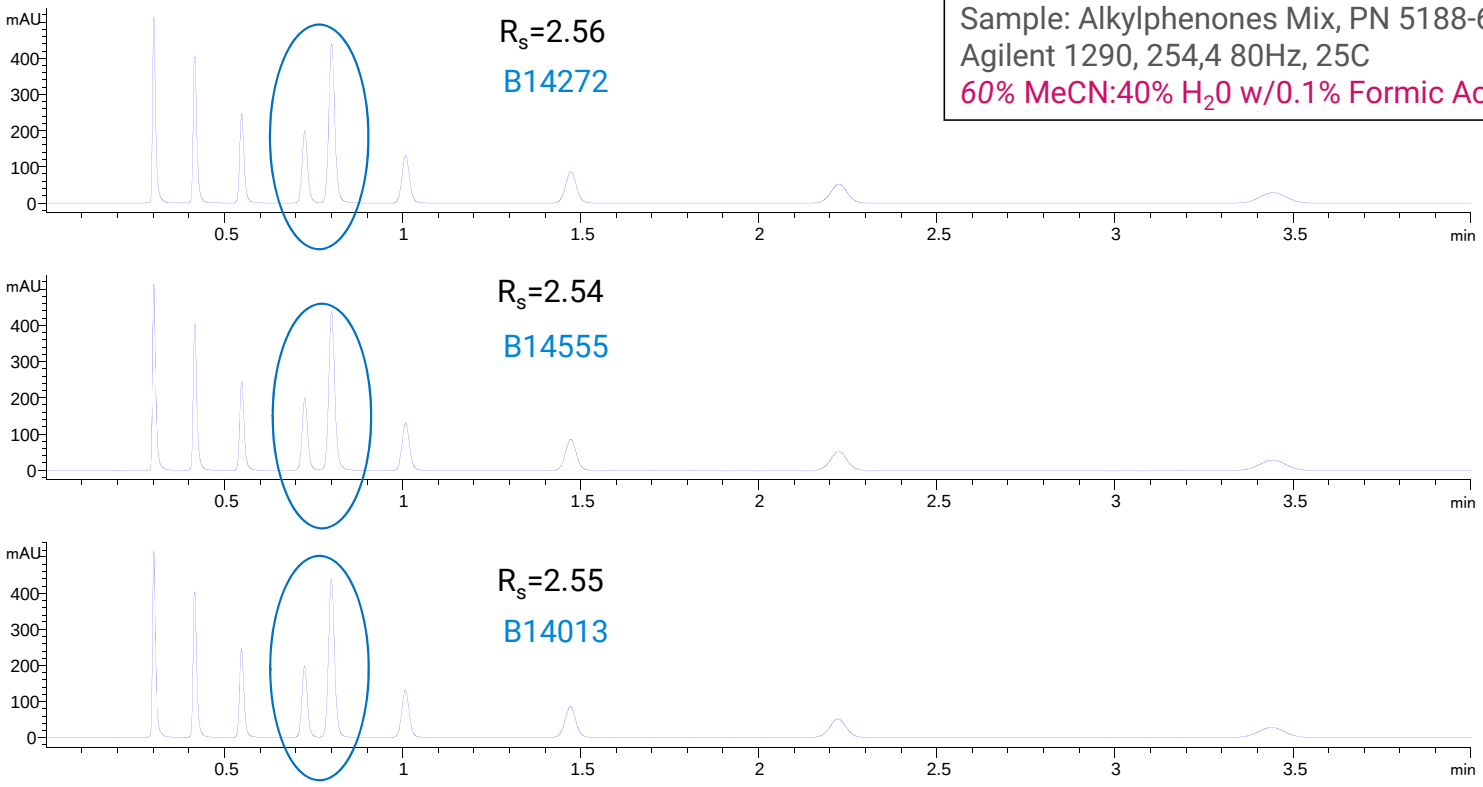
Poroshell 120 EC-C18 4.6 x 50mm 2.7um, PN 699975-902*
Sample: Alkylphenones Mix, PN 5188-6529, 2ul injection
Agilent 1290, 254,4 80Hz, 25C
65% MeCN:35% H_20 w/0.1% Formic Acid



*Poroshell 120 EC-C18 4.6 x 50mm 2.7um Validation Kit, PN 699975-902K

Slight Change to Method
More Reliable Results

Poroshell 120 EC-C18 4.6 x 50mm 2.7um, PN 699975-902*
Sample: Alkylphenones Mix, PN 5188-6529, 2ul injection
Agilent 1290, 254,4 80Hz, 25C
60% MeCN:40% H_20 w/0.1% Formic Acid



*Poroshell 120 EC-C18 4.6 x 50mm 2.7um Validation Kit, PN 699975-902K

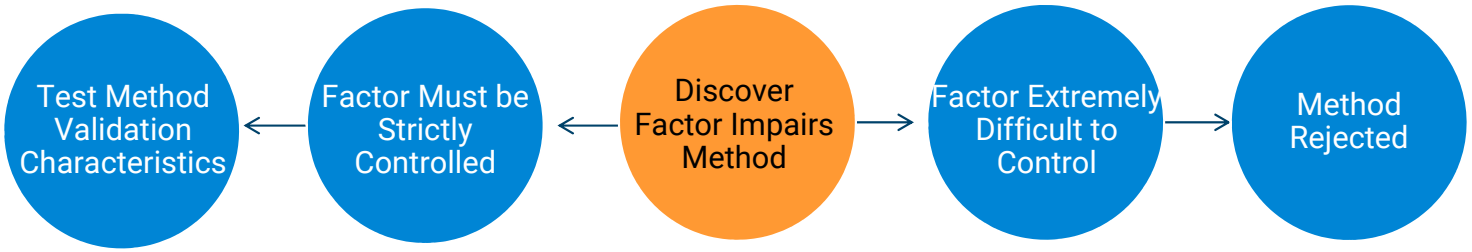
Determining Robustness

Systematically vary separation parameters and measure effects on R_s

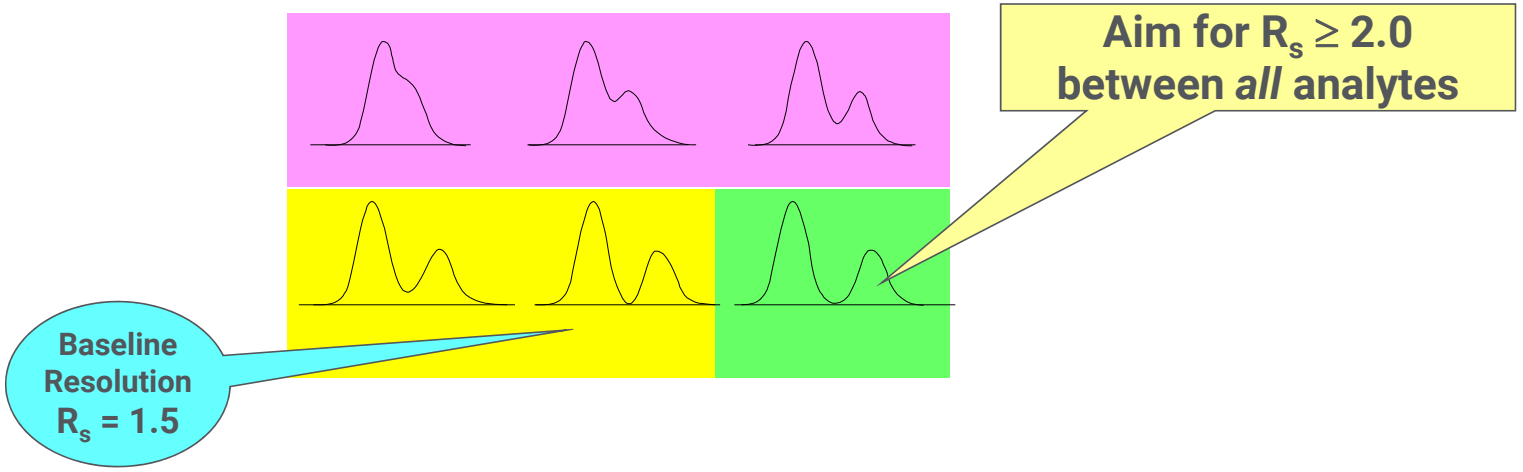
- Incorporate parameter ranges into written method to allow flexibility
  - Include precautionary statement if needed
- Helps minimize or avoid many ruggedness problems - but not all

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



How Much Resolution is Necessary?



- Insufficient R_s, compromises accuracy, precision, robustness, and ruggedness
- Initial resolution can decrease due to changes in separation variables
- Build in robustness so ΔR_s is small when separation variables are changed

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

Experimental Variables That Impact Resolution

Column

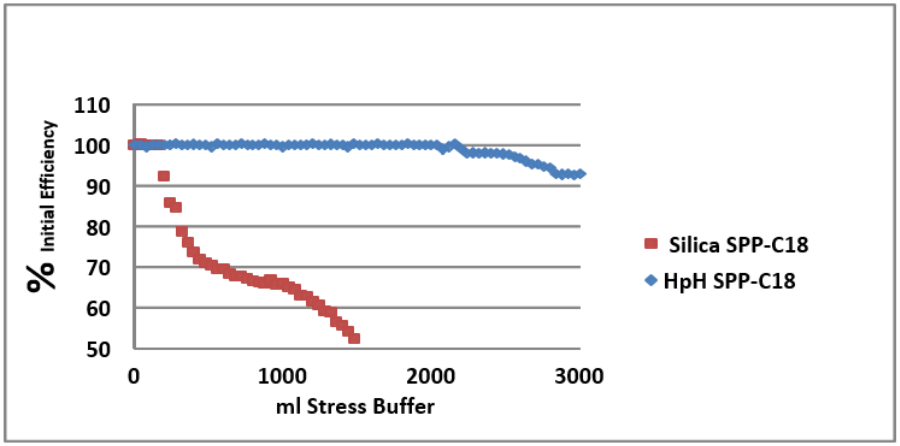
- Select high-quality column manufacturer
- Select column with long lifetime at desired pH
- Assess lot-to-lot reproducibility
  - Compare retention, selectivity, resolution, peak width and symmetry.
  - Method Validation Kit

Mobile Phase

Sample

Instrument

Gradient Separations



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

Mobile Phase: Aqueous Component
Experimental Variables That Impact Resolution

Column

Mobile Phase

Your **opportunity to improve robustness and ruggedness**

- Aqueous component
  - Importance of buffers
  - Selection considerations
  - pH
  - Concentration
- Organic component

Sample

Instrument

Gradient separations

Buffered Mobile Phase - Importance
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

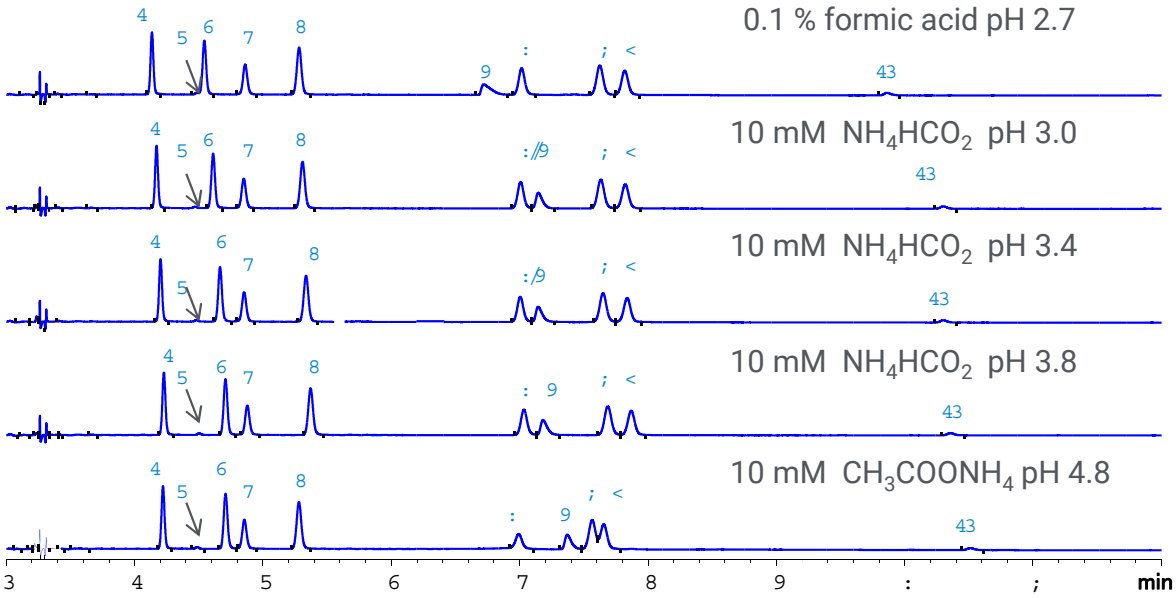
Allow efficient use of pH as separation variable during method development

Buffer Comparison

Buffer Type

- Can affect R_s and column lifetime
- MS or other detector

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



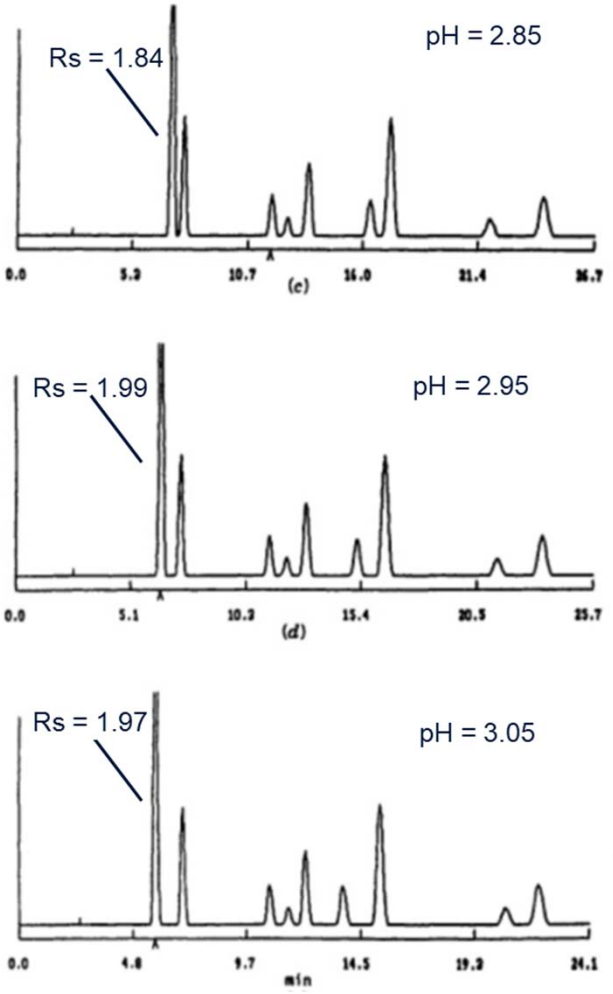
10-40 %B/12 min @ 2 mL/min 0.5 ul injection 0.1 mg/ml each
4.6 x 50 mm Poroshell 120 EC-C18; 205 Bar

pH and Resolution

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

Remember – Even small variations can have an affect.



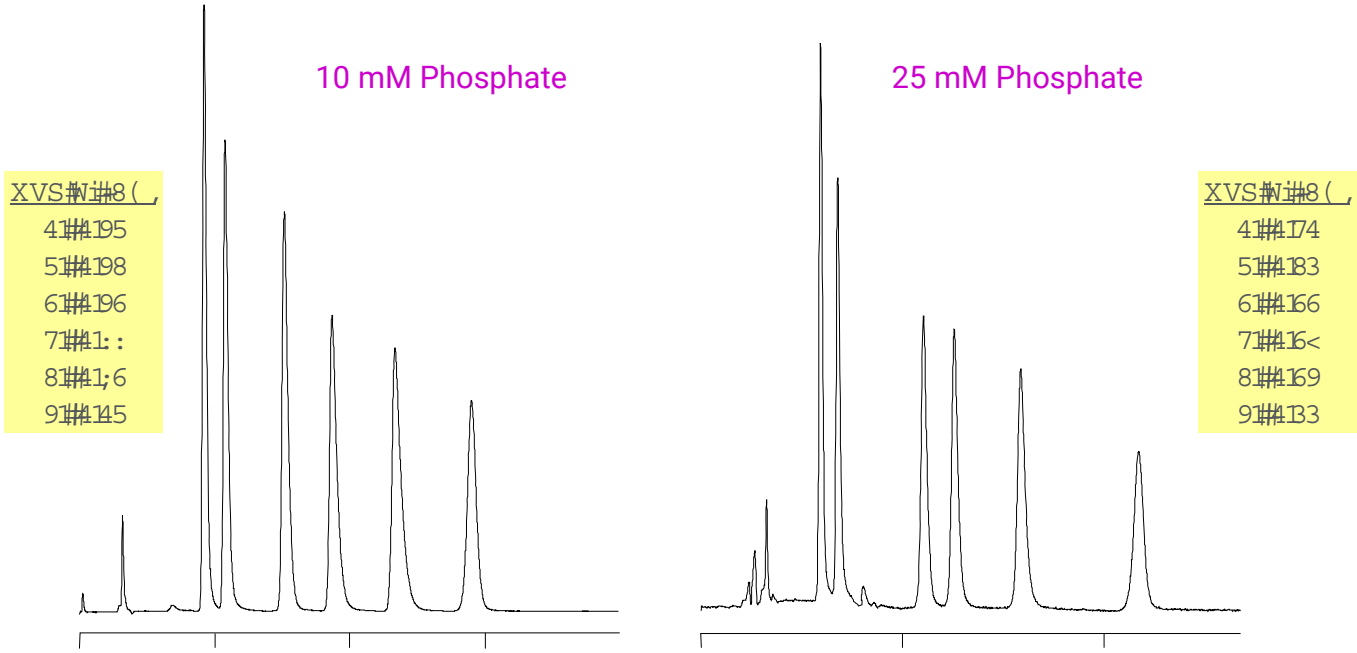
*DryLab simulations of substituted benzoic acid sample

1. J.W. Dolan, D. C. Lommen, and L. R. Snyder, *J. Chromatogr.*, **535** (1990) 55, 75.

Changes in Buffer Concentration
Retention, Peak Width and Peak Shape

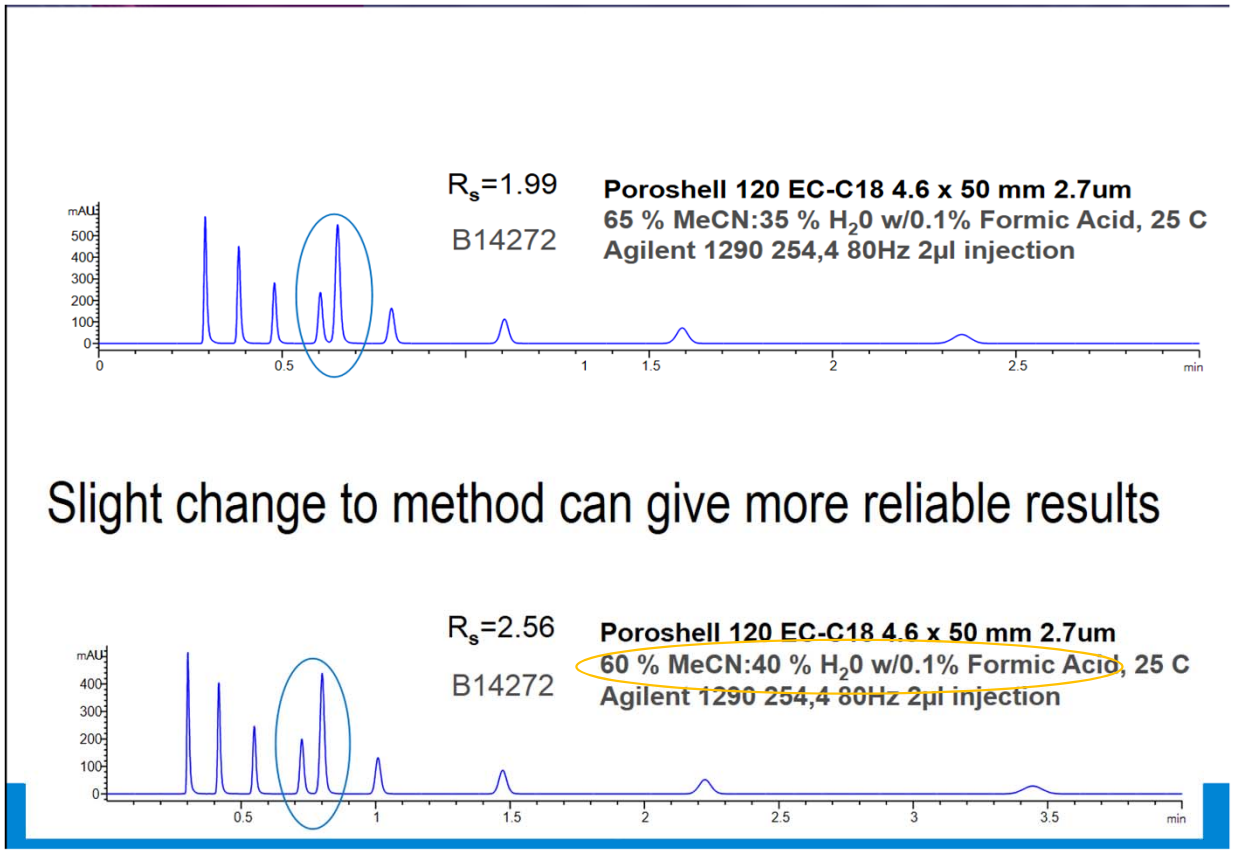
Buffer Concentration & Ionic Strength

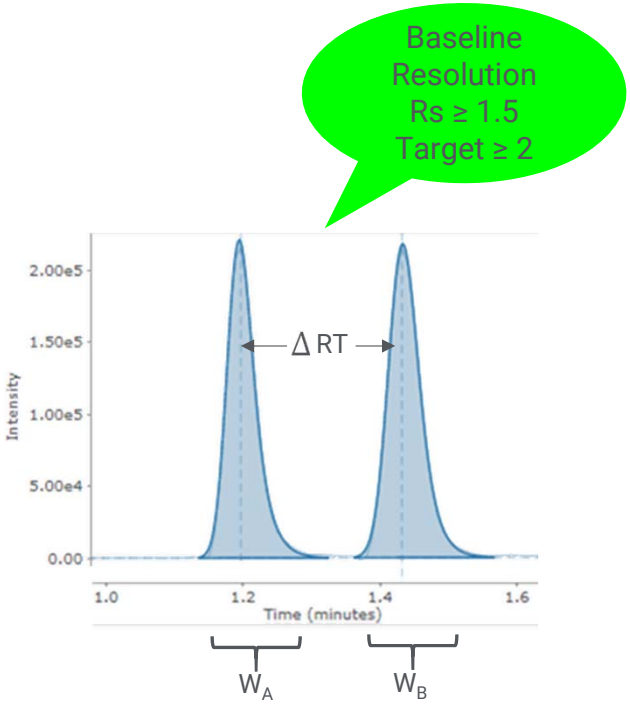
- Start at 20 – 25mM
- Avoid overshooting/ readjustment when pHing
- Compare R_s at desired concentration ± 5–10mM



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

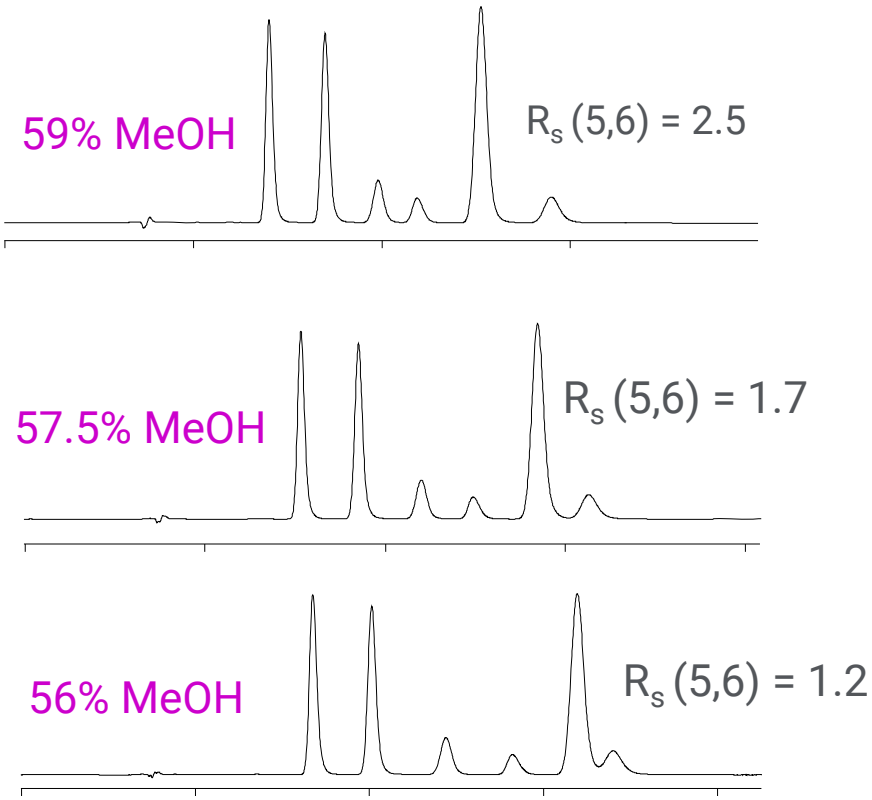
% Organic Modifier





1. W. Long, Best LC Practices for Efficient LC Operations; Par 3: Making LC method better (Webinar Series), Agilent Technologies, September 19, 2017.

Small Changes Can Affect Resolution



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

Mobile Phases:
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 resolution does not change around desired conditions (i.e. +/- 1-2%B)

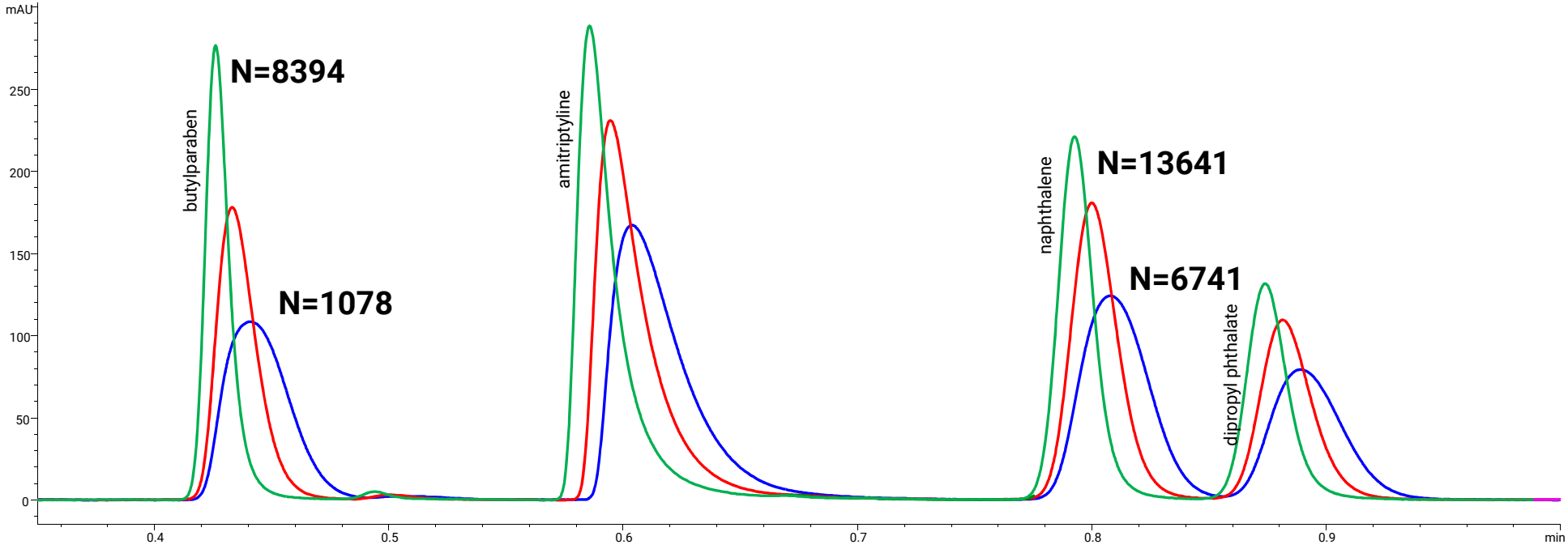
The Sample
Experimental Variables That Impact Resolution

- Column
- Mobile phase
- Sample
  - Injection volume ruggedness
    - Issue – V_{inj} is increased to improve (S/N) ratio
    - Issue – Decrease column size
    - Solution – Compare resolution, peak shape and repeatability at 20% and 200-500% V_{inj}
      - Use minimum V_{inj} for required repeatability and limit of detection
  - Sample solvent strength
    - Match starting mobile phase conditions (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
- Instrument
- Gradient separations

Sample Injection Volumes Can Affect Peak Shape and Resolution

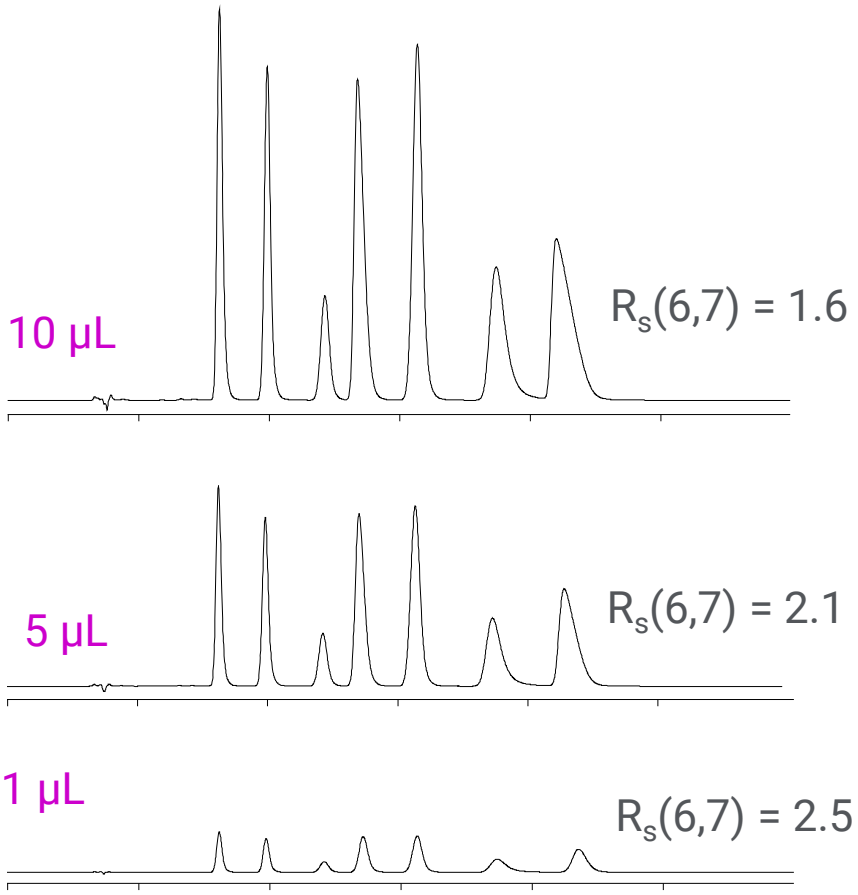
16 µL injection
8 µL injection
0.5 µL injection

- Injection volumes contribute to overall system volume
- Keep injection volumes to a minimum, while retaining solubility



Note: Sample concentrations adjusted to ensure same sample load on column regardless of injection volume.

Test For Injection Volume Robustness

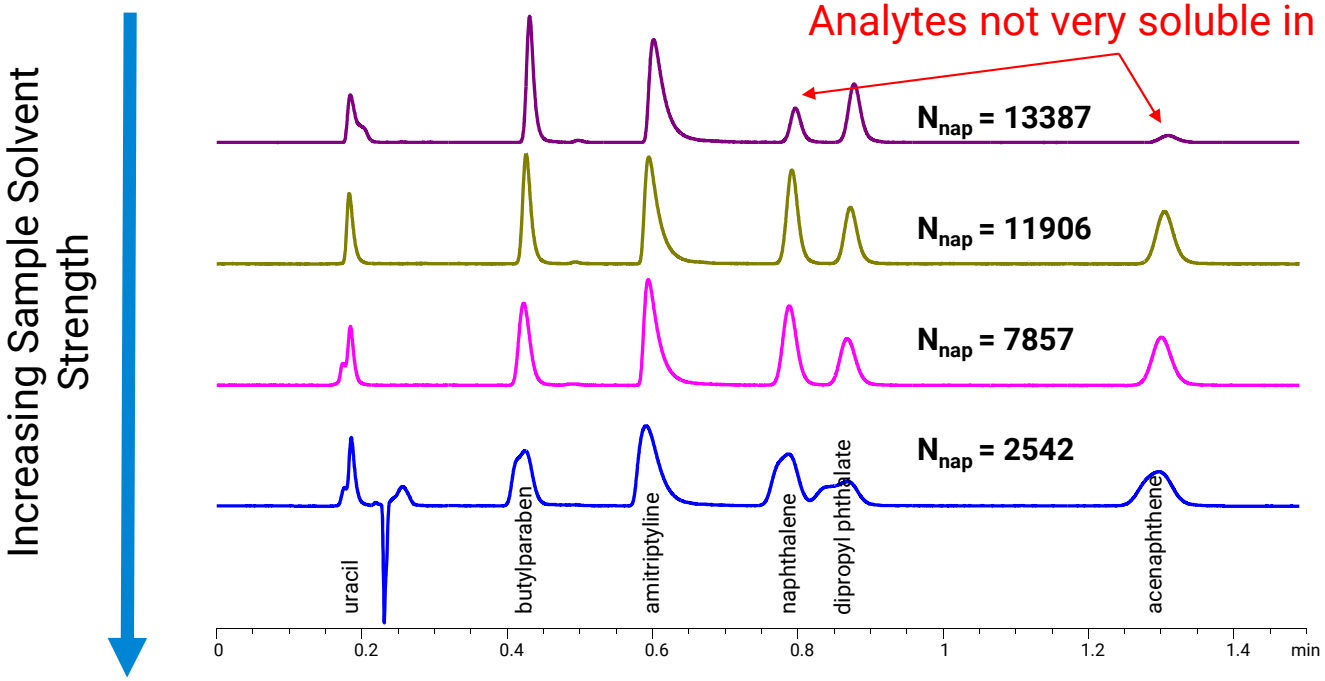


Column: ZORBAX Rapid Resolution Eclipse XDB-C8 4.6 x 75 mm, 3.5 µm

Mobile Phase: 44% 25 mM phosphate, pH 7, 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

Sample Considerations - Diluents and Solubility



1.5 µL injection of sample diluted 1:10 in **water**

1.5 µL injection of sample diluted 1:10 in **mobile phase**

1.5 µL injection of sample diluted 1:10 in **acetonitrile**

1.5 µL injection of sample diluted 1:10 in **tetrahydrofuran**

Sample solvents should be of equal or lesser strength than the mobile phase, otherwise poor peak shape can occur, resulting in poor efficiency

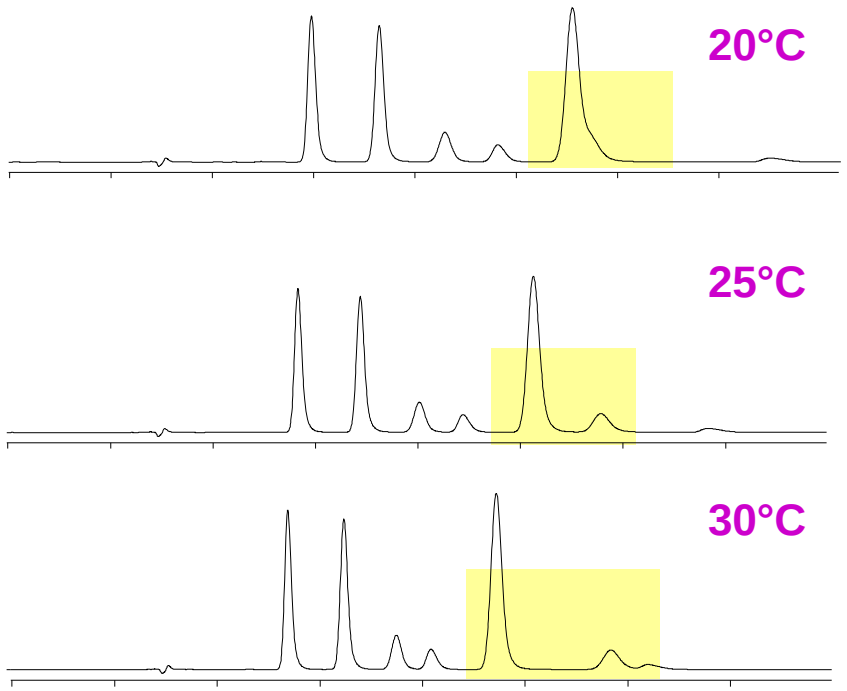
Instrument
Experimental Variables That Impact Resolution

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

Adequate Temperature Control
Small Temperature Changes Can Cause Significant Changes in R_s

Temperature

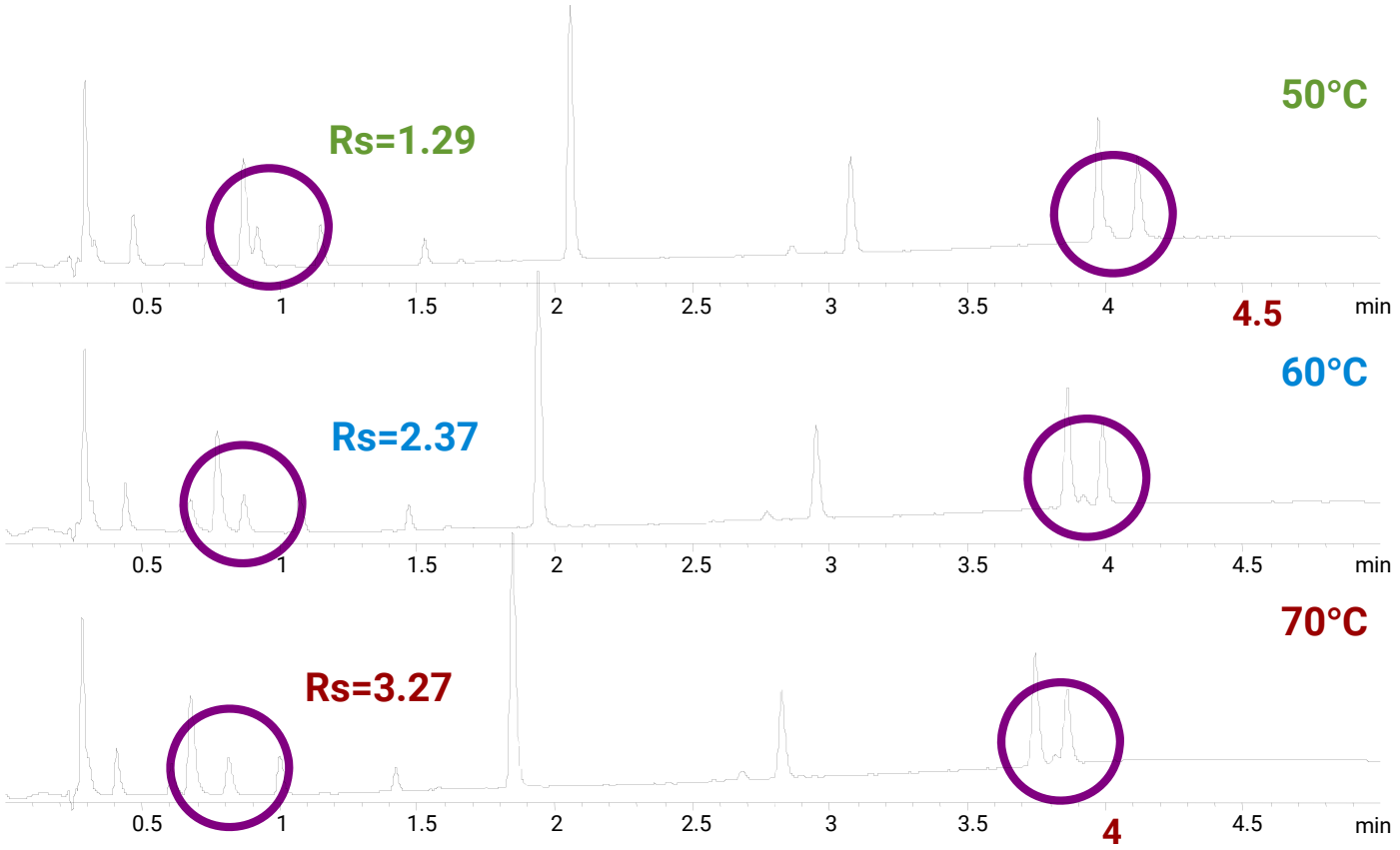
- Lab temps can vary ≥±5°C or more
- Column temp changes affect R_s and repeatability
- Useful changing selectivity, retention & efficiency
- Important parameter to control during MD & validation
- Compare R_s, peak width, & peak shape at desired temperature ±5°C



Column: ZORBAX Rapid Resolution Eclipse XDB-C8,4.6 x 75 mm, 3.5 µm
Mobile Phase: 72%A:28%B, A: 5/95 MeOH/pH 7 buffer, 25mM, 10mM TEA, B: 80/20 MeOH/pH 7 buffer, 25mM, 10mM TEA
Flow Rate: 1.0 mL/min.; Temperature: See Figure;
Injection: 5 µL; Sample:1. ketoprofen, 2. ethyl paraben, 3. hydrocortisone, 4. fenoprofen, 5. propyl paraben, 6. propranolol

Temperature Can Optimize Resolution and Selectivity

Gradient of Ten Cardiac Drugs on SB-C18 RRHT



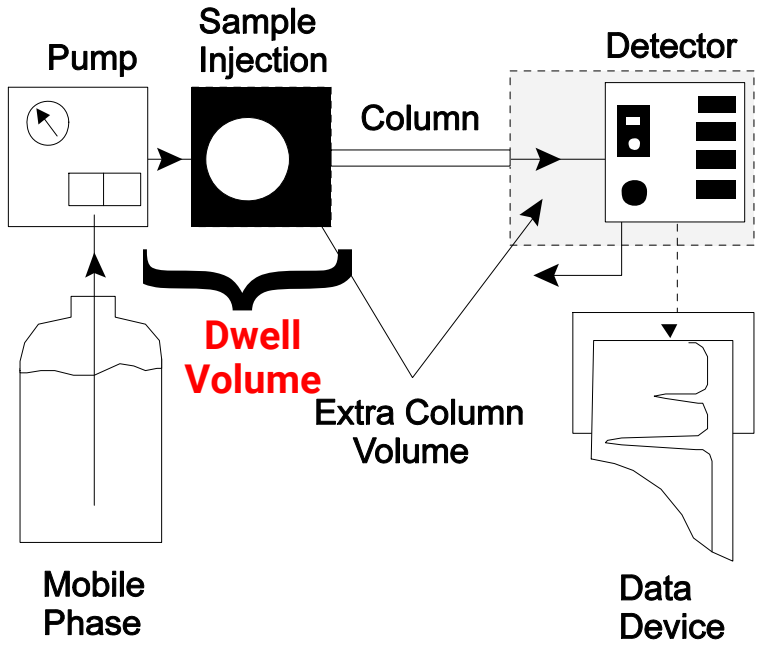
Gradient Separations
Experimental Variables That Impact Resolution

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

Gradient Separations
Effect of Dwell Volume on Ruggedness

Dwell volume = Volume from formation of gradient to column; Behaves as isocratic hold at gradient beginning

- Measure instrument dwell volume
- Assess effect of dwell volume on R_s during MD
  - 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

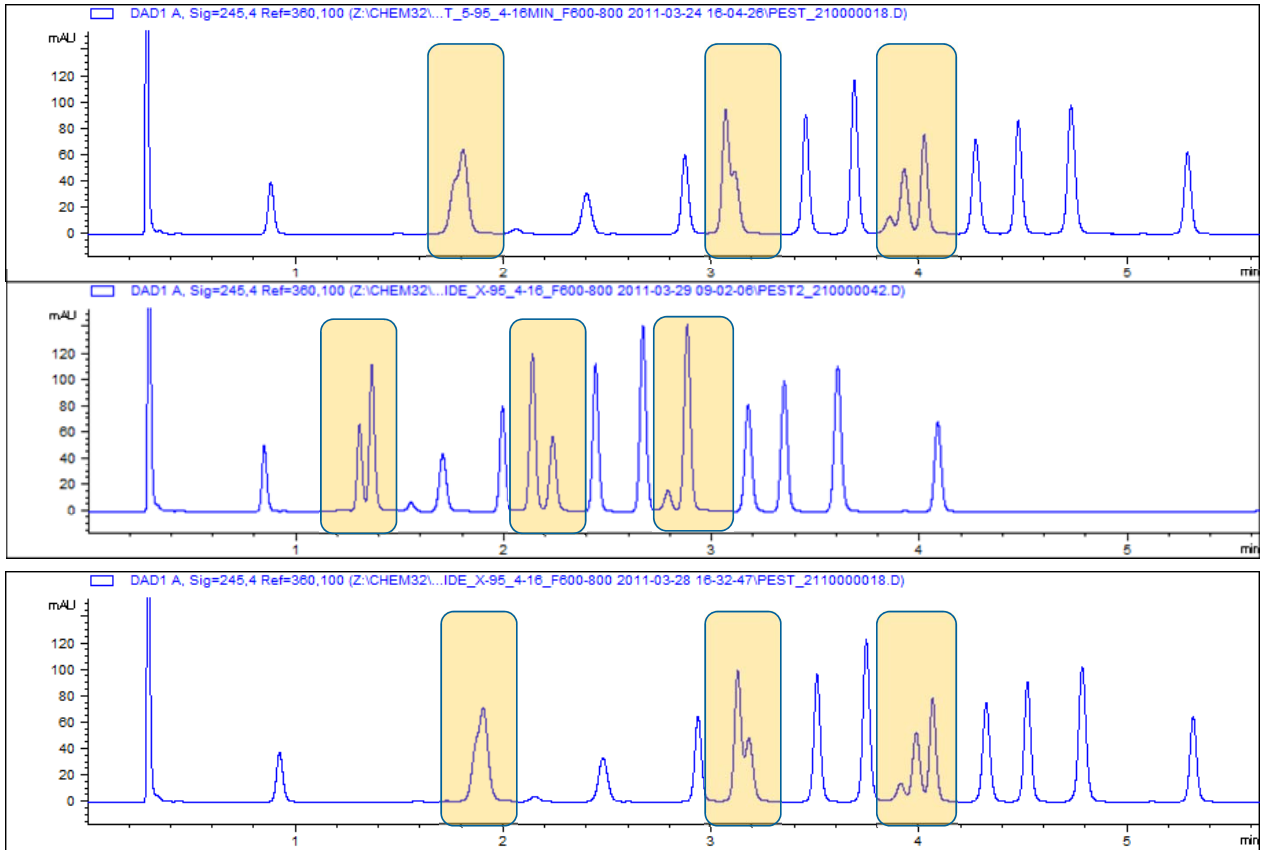


Instrument Dwell Volume Differences Can Cause Changes in Retention and Resolution

1100 Quaternary 400 bar

1290 Infinity II 1300 bar

1290 Infinity II 1300 bar with ISET

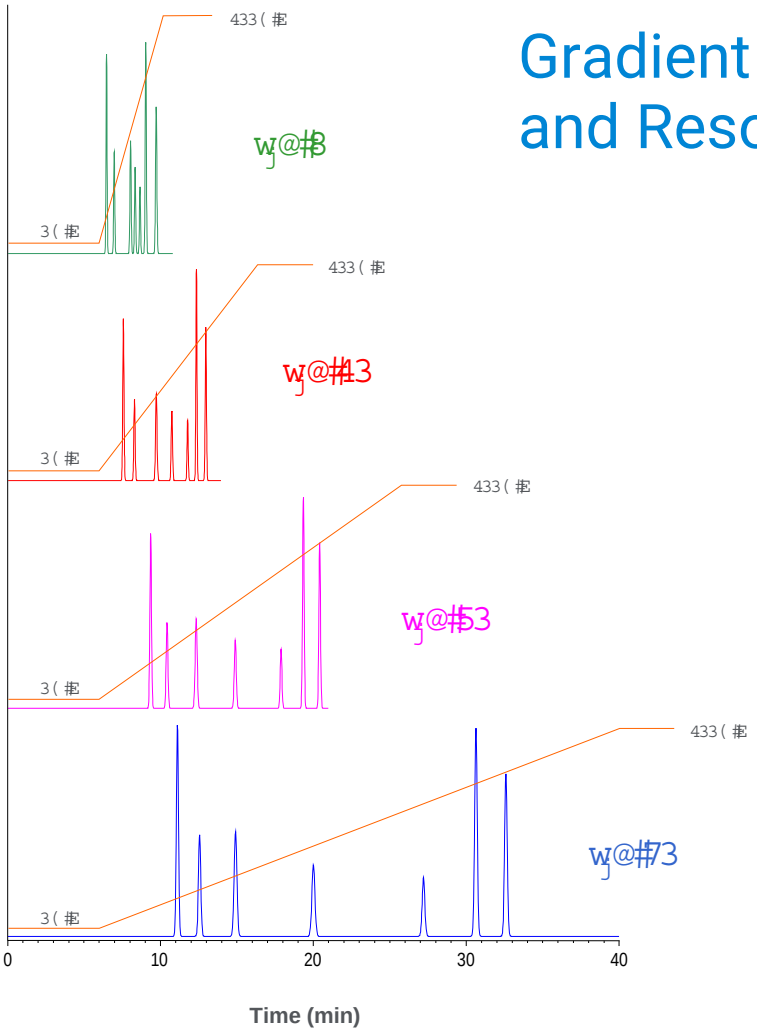


2.1x100 mm Zorbax Eclipse Plus, 1.8 µm column; Flow = 0.8 mL/min

Gradient Steepness and Gradient Shape

- Gradient steepness
  - Change can affect resolution
  - Small changes likely due to instrument performance differences
  - 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



Gradient Steepness Affects Retention (k*) and Resolution

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

1/k* = gradient steepness = b

ΔΦ = 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

Summary

When a method is not rugged and/or robust

- Method can fail unexpectedly, halting production
- "Method creep"
- Risk of redeveloping method after validation
- Compromise quality

HPLC separation robustness & ruggedness review

- Many variables to consider; some more apparent than others
- Careful consideration during MD can minimize "headaches" and repeat work
- Well-conceived and documented lab 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

Contact Agilent Chemistries and Supplies Technical Support

1-800-227-9770 Option 3, Option 3:

Option 1 for GC/GCMS Columns and Supplies

Option 2 for LC/LCMS Columns and Supplies

Option 3 for Sample Preparation, Filtration and QuEChERS

Option 4 for Spectroscopy Supplies

gc-column-support@Agilent.com

lc-column-support@agilent.com

spp-support@agilent.com

spectro-supplies-support@agilent.com

Resources for Support

- Agilent University http://www.agilent.com/crosslab/university
- Tech support http://www.agilent.com/chem/techsupport
- Resource page http://www.agilent.com/chem/agilentresources
  - Quick Reference Guides
  - Catalogs, Column User guides
  - Online Selection Tools, How-to Videos
- InfinityLab Supplies Catalog (5991-8031EN)
- Your local FSE and Specialists
- Youtube – Agilent Channel
- Agilent Service Contracts

Appendix

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

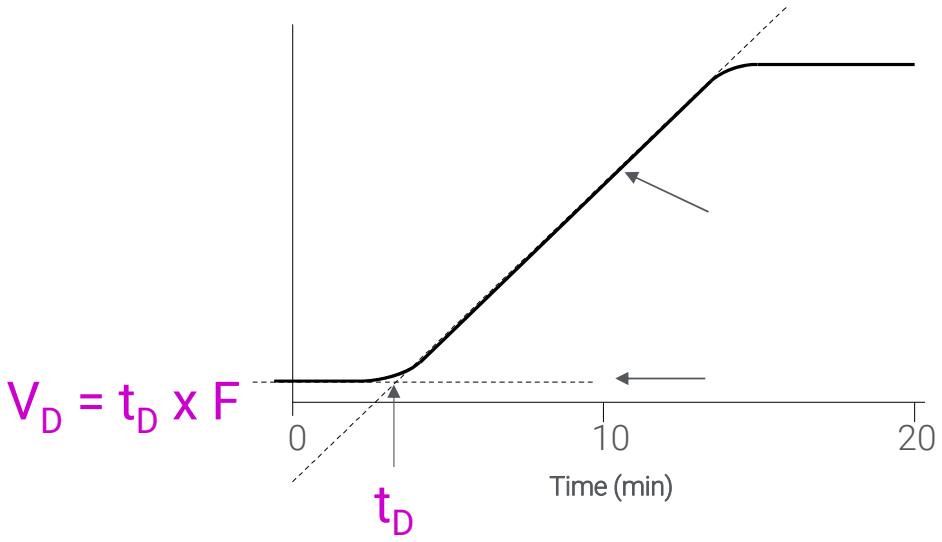
Monitor at 265 nm

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.

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.