Getting Off to a Good Start

The makings of a reliable method

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The Importance of Developing a Reliable Method*

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 that 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]

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)

Get Off to a Good Start

Method Development

- Goals
  - Good resolution: ≥2
  - Robust and reliable
- Key Considerations
  - Column
    - Particle size
    - Bonded phase
  - Conditions
    - Mobile phase
    - pH
    - Temperature
  - Sample
  - Instrument

Common Separation Goals and Method Performance Criteria

Good system suitability parameters

- Resolution: ≥2
- Peak shape: USP Tf close to 1 (<2)
- Inj. repeatability: areas, Tf, (RSD 0.1 - 0.25%)
- Absolute retention factors: 1< k<10
- Relative retention: α or k2/k1
- Signal-to-Noise Ratio: >10

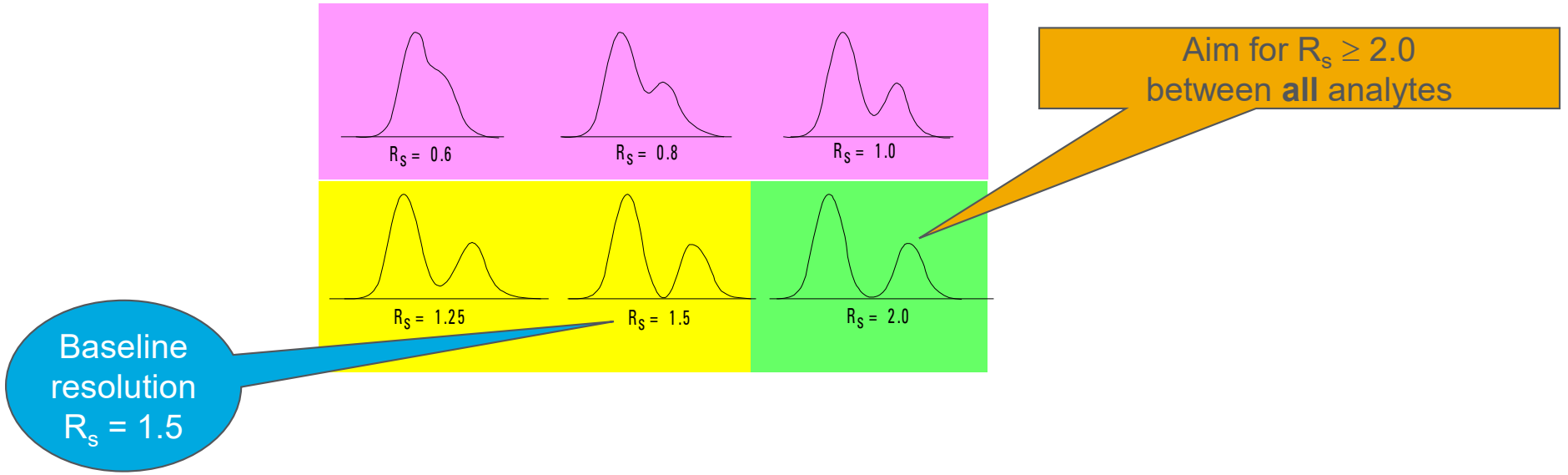
For system suitability criteria, *avoid:*
- Column efficiency (theoretical plates)
- Absolute retention time

May prevent ability to speed up method in the future!

Method performance criteria

- Accuracy
- Precision
  - Ruggedness
    - Multiple labs, analysts
    - Different instruments
    - Reagent lots, Columns
    - Different days
  - Robustness
    - Temperature
    - Mobile phase pH
    - Flow rate
- Analytical selectivity/specificity
- Linearity
- Range
- Quantitation limit (LOQ, 10x S/N)
- Detection limit (LOD, 3x S/N)

$R_s \geq 2.0$ is Important

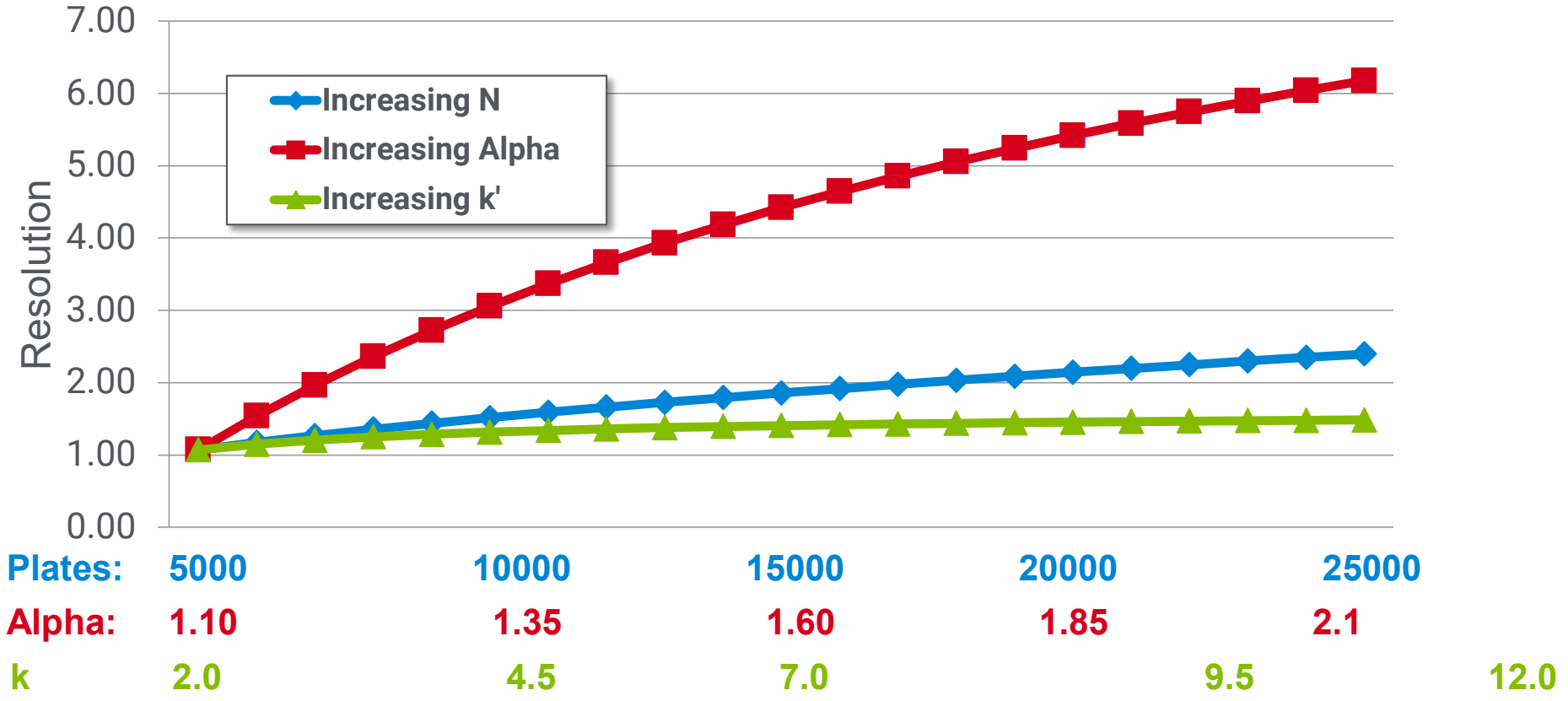


- Insufficient Rs, compromises accuracy, precision, robustness, and ruggedness
- Initial resolution can decrease due to changes in separation variables
- Build in robustness so ΔRs is small when separation variables are changed

Factors that Affect Resolution

$$R_s = \left(\frac{1}{4}\right) N^{0.5} \left(\frac{\alpha - 1}{\alpha}\right) \left(\frac{k}{1 + k}\right)$$

Resolution **Efficiency** **Selectivity** **Retention**



Selectivity impacts resolution the most

- Change bonded phase
- Change mobile phase

} **Typical analytical method development parameters**

Key Factors and Conditions to Consider

Column

- Bonded phase
- Lot

Conditions

- Mobile phase components
  - Aqueous
  - Organic
- pH
- Temperature

Sample

- Injection volume
- Solvent strength

Instrument

- Delay volume
- Extracolumn volume

Column Choice Considerations

Particle

- Size
- Totally porous or superficially porous

Bonded phase

- Affects selectivity (alpha)
- Different interactions for polar and non-polar compounds.
- Exploit other interactions with bonded phase (e.g., pi-pi)
- Changing the bonded phase can improve selectivity/resolution, reduce analysis time
- Having different bonded phases available on the same particle makes development easier

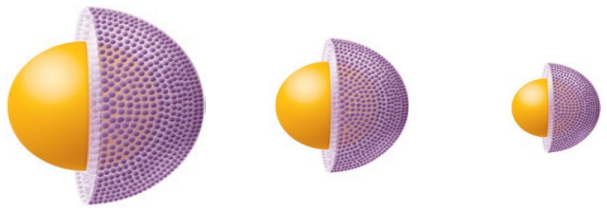
Lot

- Compare retention, selectivity, resolution, peak width and symmetry
- Method Validation kit

Agilent InfinityLab Poroshell 120 Portfolio

start here

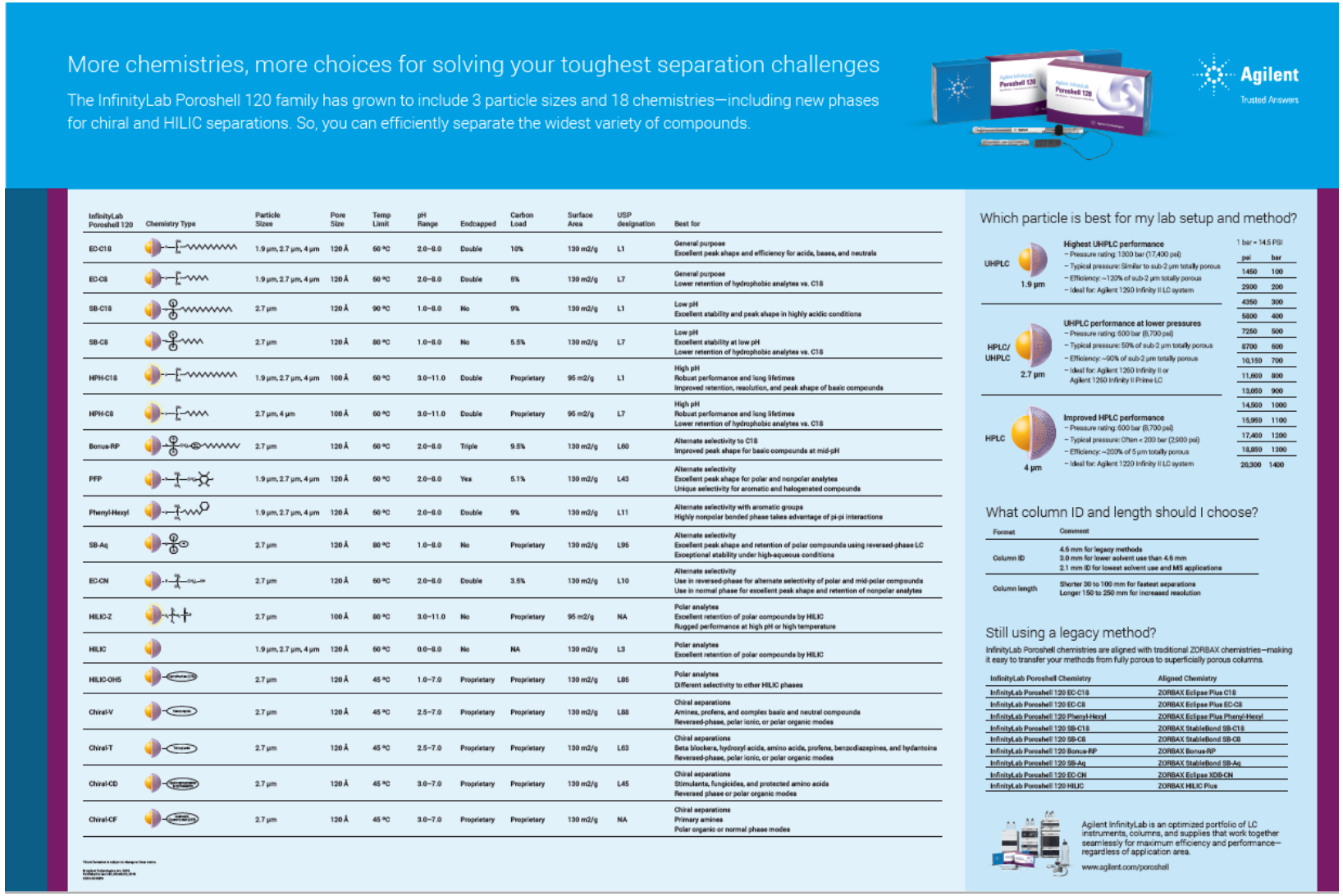
Best all around	Best for low pH mobile phases	Best for high pH mobile phases	Best for alternative selectivity	Best for polar Analytes	Best for Chiral
InfinityLab Poroshell **EC-C18** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **SB-C18** 2.7 µm	InfinityLab Poroshell **HPH-C18** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **Bonus-RP** 2.7 µm	InfinityLab Poroshell **HILIC** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **Chiral-V** 2.7 µm
InfinityLab Poroshell **EC-C8** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **SB-C8** 2.7 µm	InfinityLab Poroshell **HPH-C8** 2.7 µm, 4 µm	InfinityLab Poroshell **PFP** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **HILIC-Z** 2.7 µm	InfinityLab Poroshell **Chiral-T** 2.7 µm
			InfinityLab Poroshell **Phenyl-Hexyl** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell **HILIC-OH5** 2.7 µm	InfinityLab Poroshell **Chiral-CD** 2.7 µm
			InfinityLab Poroshell **SB-Aq** 2.7 µm		InfinityLab Poroshell **Chiral-CF** 2.7 µm
			InfinityLab Poroshell **EC-CN** 2.7 µm		



4µm **2.7µm** **1.9µm**

Reversed-phase chemistries

Select the Right Column Phase



More chemistries, more choices for solving your toughest separation challenges

The InfinityLab Poroshell 120 family has grown to include 3 particle sizes and 18 chemistries—including new phases for chiral and HILIC separations. So, you can efficiently separate the widest variety of compounds.

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InfinityLab Poroshell 120	Chemistry Type	Particle Sizes	Pore Size	Temp Limit	pH Range	Endcapped	Carbon Load	Surface Area	USP designation	Best for
EC-C18		1.9 µm, 2.7 µm, 4 µm	120 Å	60 °C	2.0–8.0	Double	10%	130 m2/g	L1	General purpose Excellent peak shape and efficiency for acids, bases, and neutrals
EC-C8		1.9 µm, 2.7 µm, 4 µm	120 Å	60 °C	2.0–8.0	Double	5%	130 m2/g	L7	General purpose Lower retention of hydrophobic analytes vs. C18
SB-C18		2.7 µm	120 Å	90 °C	1.0–8.0	No	9%	130 m2/g	L1	Low pH Excellent stability and peak shape in highly acidic conditions
SB-C8		2.7 µm	120 Å	80 °C	1.0–8.0	No	5.5%	130 m2/g	L7	Low pH Excellent stability at low pH Lower retention of hydrophobic analytes vs. C18
HPH-C18		1.9 µm, 2.7 µm, 4 µm	100 Å	60 °C	3.0–11.0	Double	Proprietary	95 m2/g	L1	High pH Robust performance and long lifetimes Improved retention, resolution, and peak shape of basic compounds
HPH-C8		2.7 µm, 4 µm	100 Å	60 °C	3.0–11.0	Double	Proprietary	95 m2/g	L7	High pH Robust performance and long lifetimes Lower retention of hydrophobic analytes vs. C18
Bonus-RP		2.7 µm	120 Å	60 °C	2.0–8.0	Triple	9.5%	130 m2/g	L60	Alternate selectivity to C18 Improved peak shape for basic compounds at mid-pH
PFP		1.9 µm, 2.7 µm, 4 µm	120 Å	60 °C	2.0–8.0	Yes	5.1%	130 m2/g	L43	Alternate selectivity Excellent peak shape for polar and nonpolar analytes Unique selectivity for aromatic and halogenated compounds
Phenyl-Hexyl		1.9 µm, 2.7 µm, 4 µm	120 Å	60 °C	2.0–8.0	Double	9%	130 m2/g	L11	Alternate selectivity with aromatic groups Highly nonpolar bonded phase takes advantage of pi-pi interactions
SB-Aq		2.7 µm	120 Å	80 °C	1.0–8.0	No	Proprietary	130 m2/g	L95	Alternate selectivity Excellent peak shape and retention of polar compounds using reversed-phase LC Exceptional stability under high-aqueous conditions
EC-CN		2.7 µm	120 Å	60 °C	2.0–8.0	Double	3.5%	130 m2/g	L10	Alternate selectivity Use in reversed-phase for alternate selectivity of polar and mid-polar compounds Use in normal phase for excellent peak shape and retention of nonpolar analytes
HILIC-Z		2.7 µm	100 Å	80 °C	3.0–11.0	No	Proprietary	95 m2/g	NA	Polar analytes Excellent retention of polar compounds by HILIC Rugged performance at high pH or high temperature
HILIC		1.9 µm, 2.7 µm, 4 µm	120 Å	60 °C	0.0–8.0	No	NA	130 m2/g	L3	Polar analytes Excellent retention of polar compounds by HILIC
HILIC-OH5		2.7 µm	120 Å	45 °C	1.0–7.0	Proprietary	Proprietary	130 m2/g	L85	Polar analytes Different selectivity to other HILIC phases
Chiral-V		2.7 µm	120 Å	45 °C	2.5–7.0	Proprietary	Proprietary	130 m2/g	L88	Chiral separations Amines, profens, and complex basic and neutral compounds Reversed-phase, polar ionic, or polar organic modes
Chiral-T		2.7 µm	120 Å	45 °C	2.5–7.0	Proprietary	Proprietary	130 m2/g	L63	Chiral separations Beta blockers, hydroxyl acids, amino acids, profens, benzodiazepines, and hydantoins Reversed-phase, polar ionic, or polar organic modes
Chiral-CD		2.7 µm	120 Å	45 °C	3.0–7.0	Proprietary	Proprietary	130 m2/g	L45	Chiral separations Stimulants, fungicides, and protected amino acids Reversed phase or polar organic modes
Chiral-CF		2.7 µm	120 Å	45 °C	3.0–7.0	Proprietary	Proprietary	130 m2/g	NA	Chiral separations Primary amines Polar organic or normal phase modes

Which particle is best for my lab setup and method?

UHPLC, 1.9 µm
Highest UHPLC performance
- Pressure rating: 1300 bar (17,400 psi)
- Typical pressure: Similar to sub-2 µm totally porous
- Efficiency: ~120% of sub-2 µm totally porous
- Ideal for: Agilent 1260 Infinity II LC system

HPLC/UHPLC, 2.7 µm
UHPLC performance at lower pressures
- Pressure rating: 600 bar (8,700 psi)
- Typical pressure: 50% of sub-2 µm totally porous
- Efficiency: ~90% of sub-2 µm totally porous
- Ideal for: Agilent 1260 Infinity II or Agilent 1260 Infinity II Prime LC

HPLC, 4 µm
Improved HPLC performance
- Pressure rating: 600 bar (8,700 psi)
- Typical pressure: Often < 200 bar (2,900 psi)
- Efficiency: ~200% of 5 µm totally porous
- Ideal for: Agilent 1220 Infinity II LC system

1 bar = 14.5 PSI

psi	bar
1450	100
2900	200
4350	300
5800	400
7250	500
8700	600
10,150	700
11,600	800
13,050	900
14,500	1000
15,950	1100
17,400	1200
18,850	1300
20,300	1400

What column ID and length should I choose?

Format	Comment
Column ID	4.6 mm for legacy methods 3.0 mm for lower solvent use than 4.6 mm 2.1 mm ID for lowest solvent use and MS applications
Column length	Shorter 30 to 100 mm for fastest separations Longer 150 to 250 mm for increased resolution

Still using a legacy method?

InfinityLab Poroshell chemistries are aligned with traditional ZORBAX chemistries—making it easy to transfer your methods from fully porous to superficially porous columns.

InfinityLab Poroshell Chemistry	Aligned Chemistry
InfinityLab Poroshell 120 EC-C18	ZORBAX Eclipse Plus C18
InfinityLab Poroshell 120 EC-C8	ZORBAX Eclipse Plus EC-C8
InfinityLab Poroshell 120 Phenyl-Hexyl	ZORBAX Eclipse Plus Phenyl-Hexyl
InfinityLab Poroshell 120 SB-C18	ZORBAX StableBond SB-C18
InfinityLab Poroshell 120 SB-C8	ZORBAX StableBond SB-C8
InfinityLab Poroshell 120 Bonus-RP	ZORBAX Bonus-RP
InfinityLab Poroshell 120 SB-Aq	ZORBAX StableBond SB-Aq
InfinityLab Poroshell 120 EC-CN	ZORBAX Eclipse XDB-CN
InfinityLab Poroshell 120 HILIC	ZORBAX HILIC Plus

Agilent InfinityLab is an optimized portfolio of LC instruments, columns, and supplies that work together seamlessly for maximum efficiency and performance—regardless of application area.

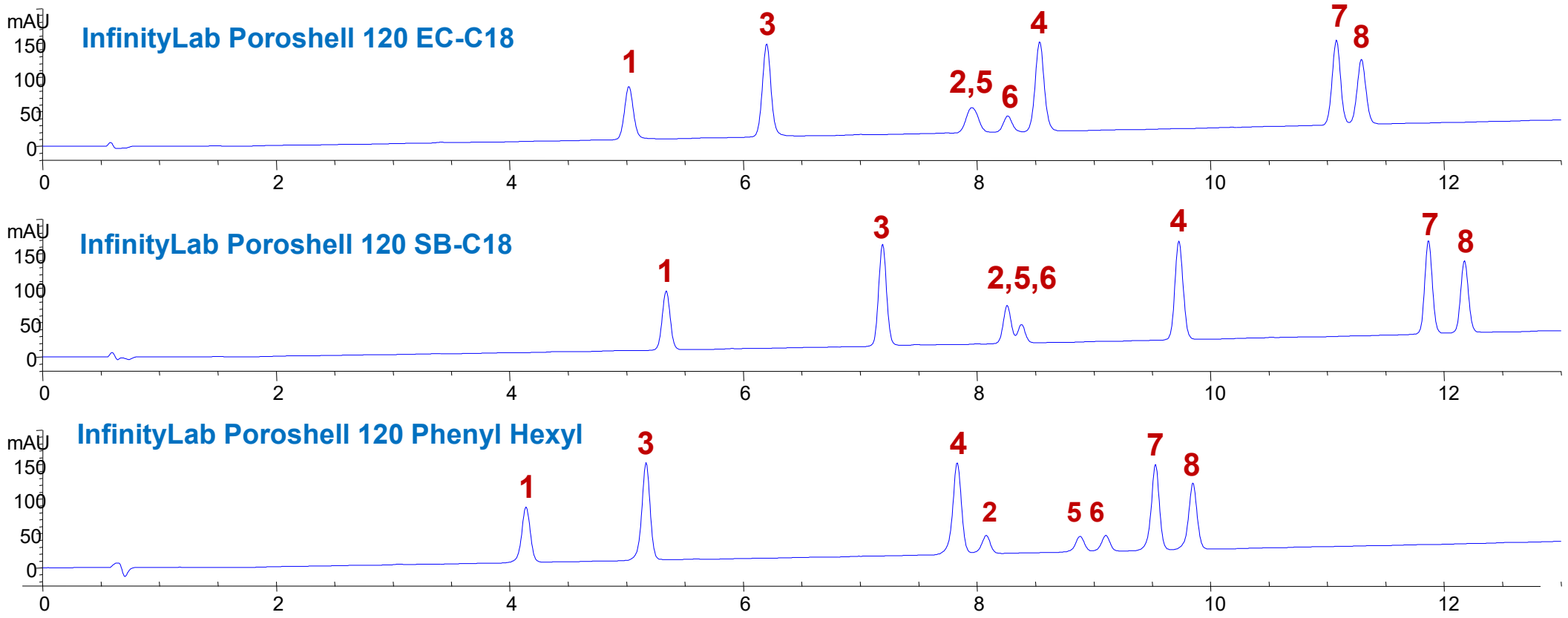
www.agilent.com/poroshell

Agilent column selection guidance poster!

Request yours today at:

csd.online_sales@agilent.com

Selectivity Differences Across InfinityLab Poroshell Bonded Phases



1. Hydrocortisone 2. B Estradiole, 3. Andostadiene 3. 17 dione, 4. Testosterone
5. Ethyestradione 6. Estrone 7. Norethindone acetate 8. Progestreone

Column: 2.1 x 100 mm
Mobile phase A: 0.1% Formic Acid in H2O
Mobile phase B: Methanol
Gradient: 40–80 %, 14 min
Flow: 0.4 mL/min,
Temp: 40 °C
Detection: DAD 260, 80 nm

Column – Lot-to-Lot

Best practice – Assess method performance in two or more labs, ideally over time

Lack of ruggedness is often attributable to

- Insufficient documentation
- Differing practices
- Reagents
- Instrumentation

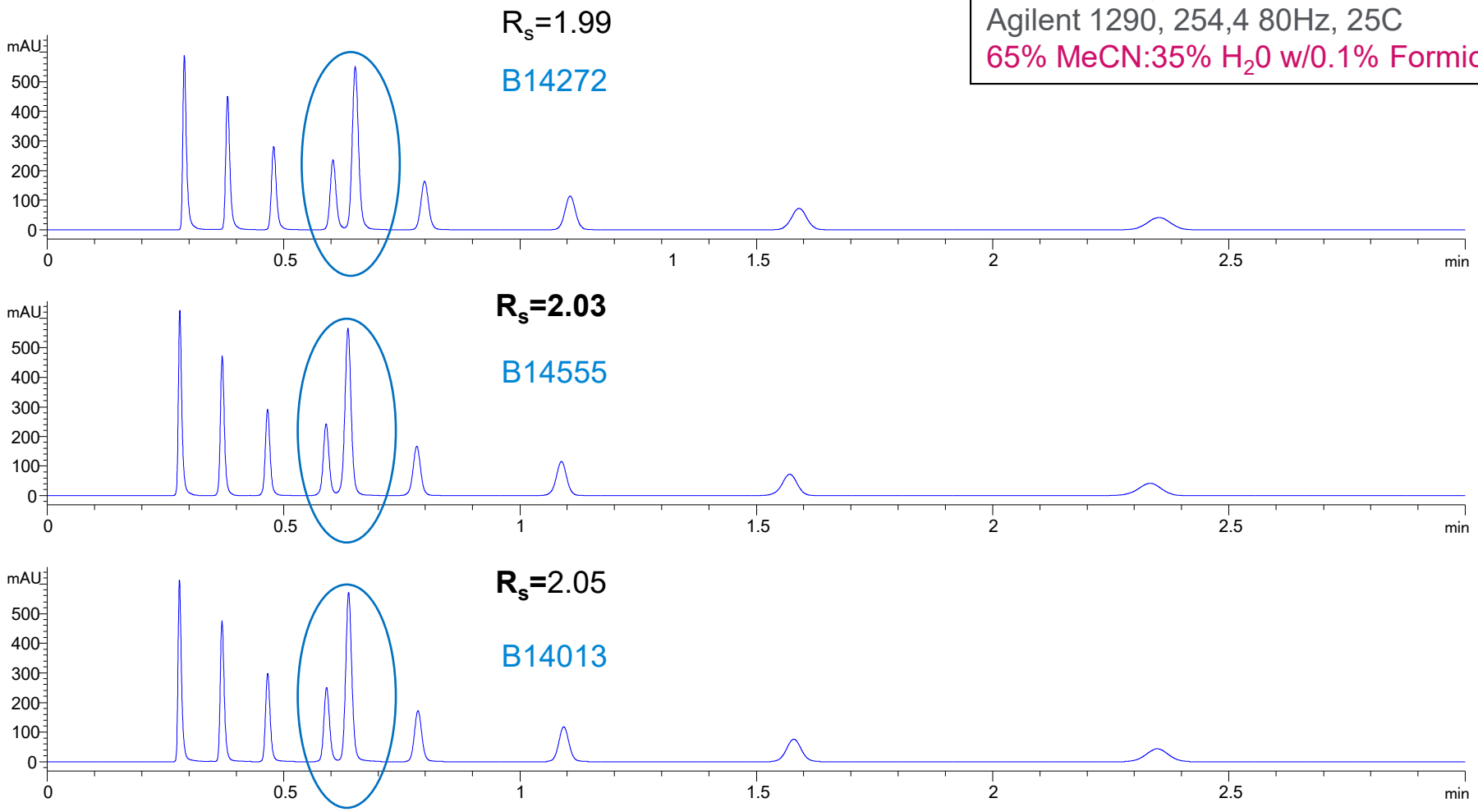
Ruggedness example: column lot

- Test 3 different column lots – Method Validation kit
- Compare RT for the 3 lots
- If ΔRT is too large…
  - Improve method
  - Consider a different column or manufacturer
  - Sequester “good” batch – **Should be avoided!**

Help Ensure Method Reproducibility

Test at least three lots

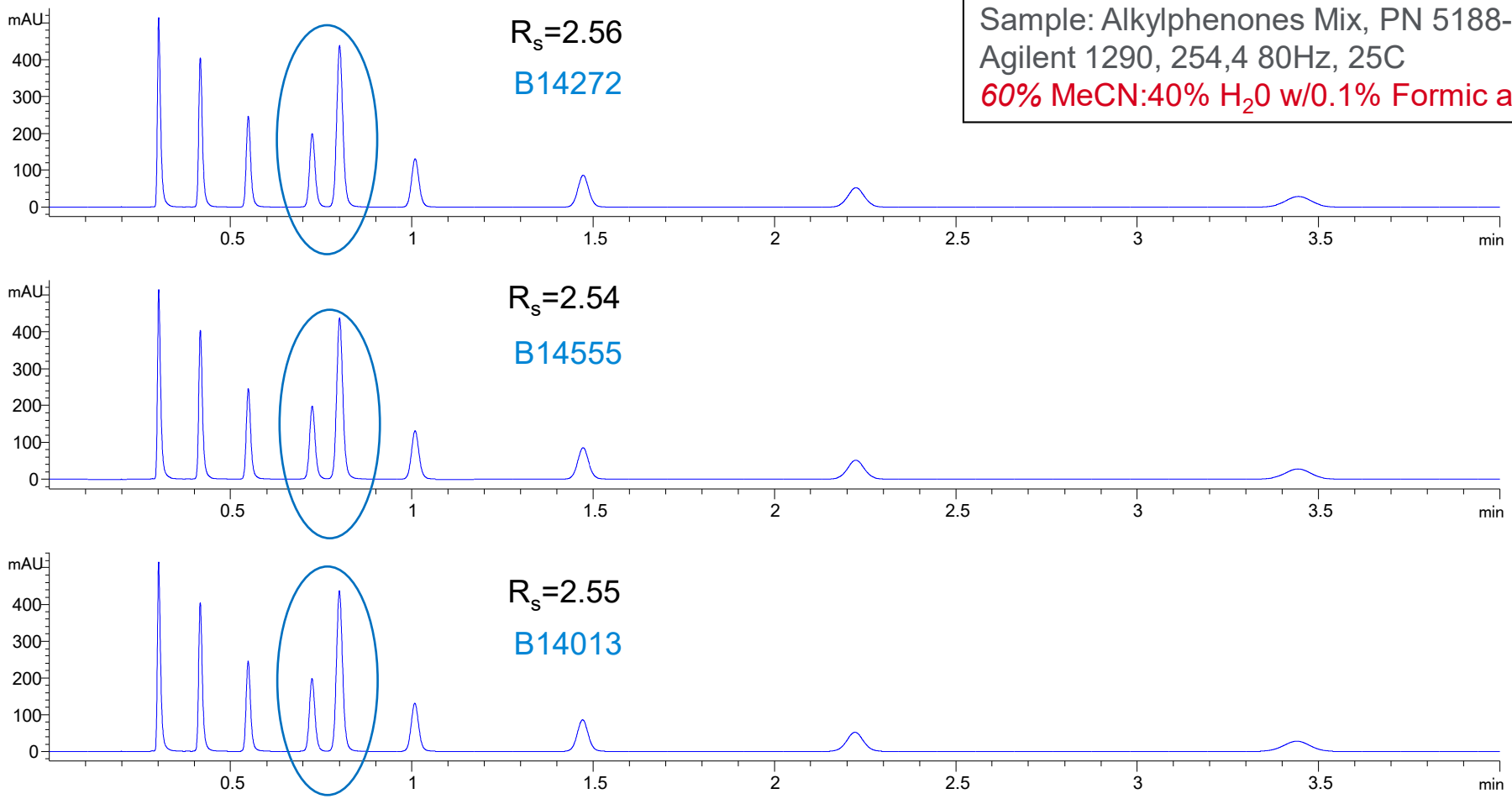
Poroshell 120 EC-C18 4.6 x 50mm 2.7um, PN 699975-902*
Sample: Alkylphenones Mix, PN 5188-6529, 2ul injection
Agilent 1290, 254,4 80Hz, 25C
65% MeCN:35% H_20 w/0.1% Formic Acid



*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

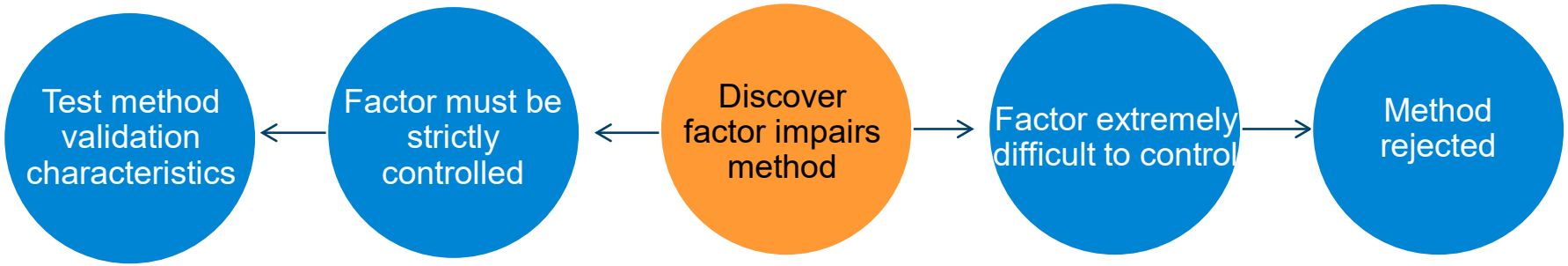
Help Insure a Reliable Method

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

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



Experimental Conditions That Affect Resolution

Column

Mobile phase components

- Aqueous
  - Buffers Control retention of ionizable compounds
    - Selection considerations
    - Concentration
- Organic

pH

Temperature

Sample

Instrument

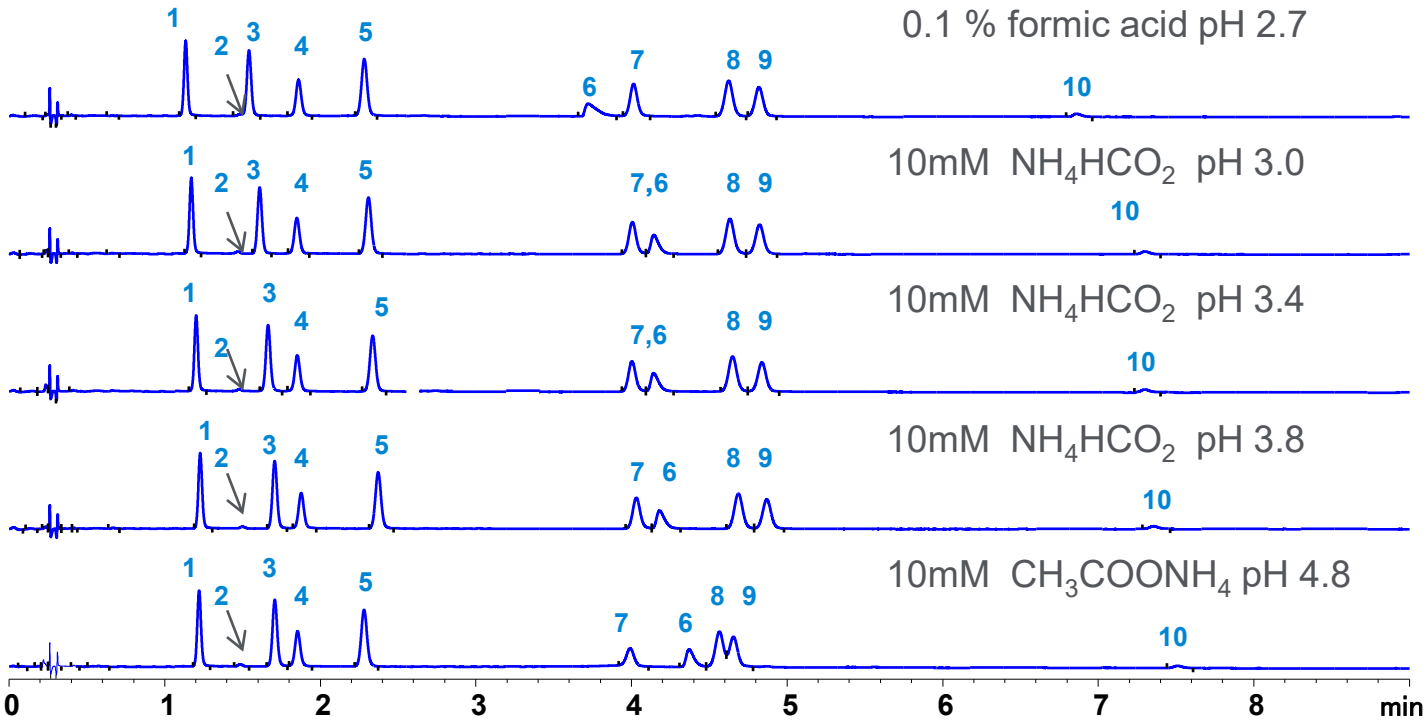
Your opportunity to improve robustness and ruggedness

Mobile Phase – Aqueous
Buffer

Buffer type

- Can affect R_s and column lifetime
- Detector choice
  - DAD
  - MS

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



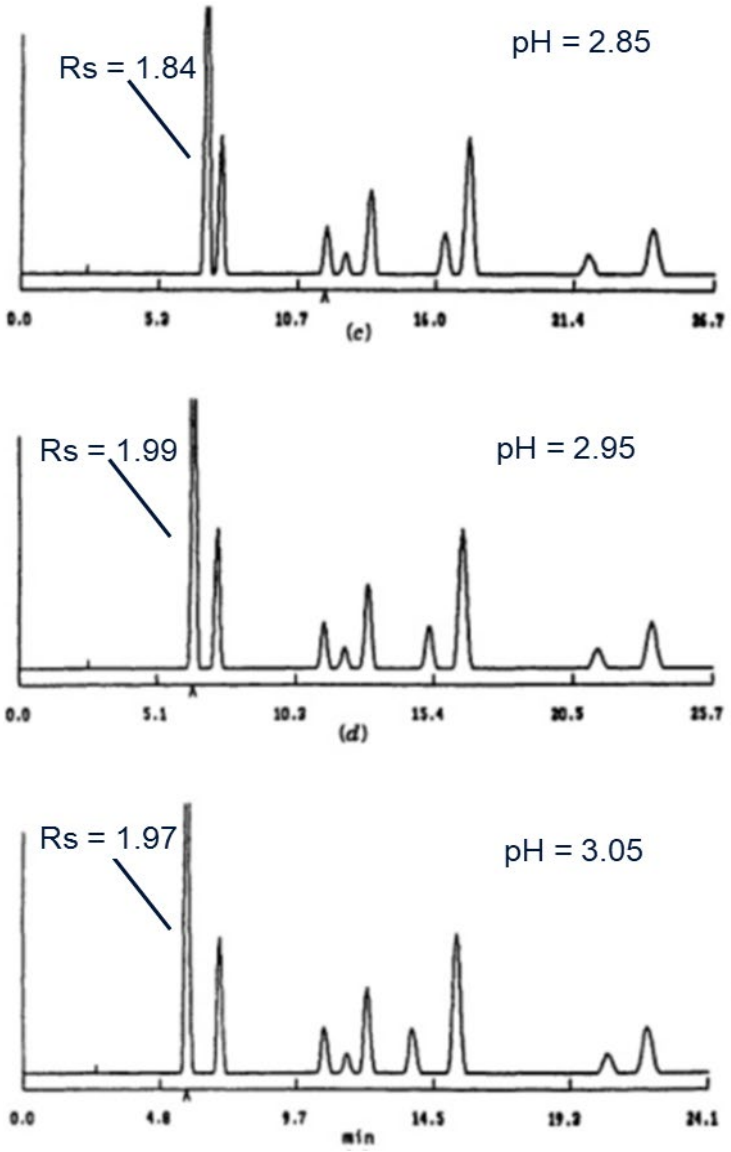
4.6 x 50 mm Poroshell 120 EC-C18; 205 Bar
10-40 %B (ACN)/12 min @ 2 mL/min, 0.5 µL injection 0.1 mg/mL each

pH and Resolution

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 effect.



DryLab simulations of substituted benzoic acid sample

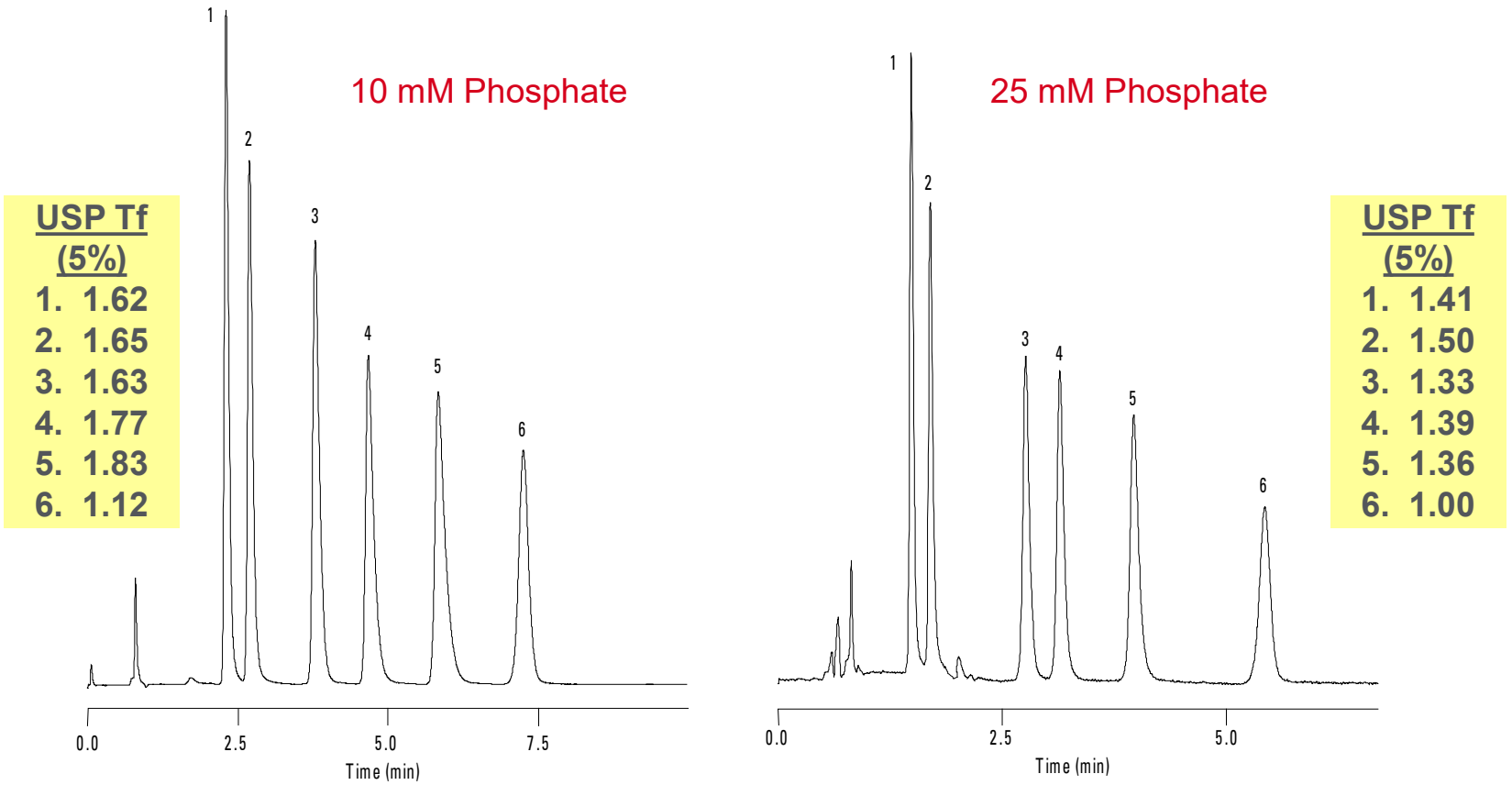
J.W. Dolan, D. C. Lommen, and L. R. Snyder, *J. Chromatogr.*, **535** (1990) 55, 75

Buffer Concentration

Retention, peak width, and peak shape

Concentration and ionic strength

- Start at 20 – 25 mM
- Avoid overshooting and readjusting pH
- Compare Rs 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

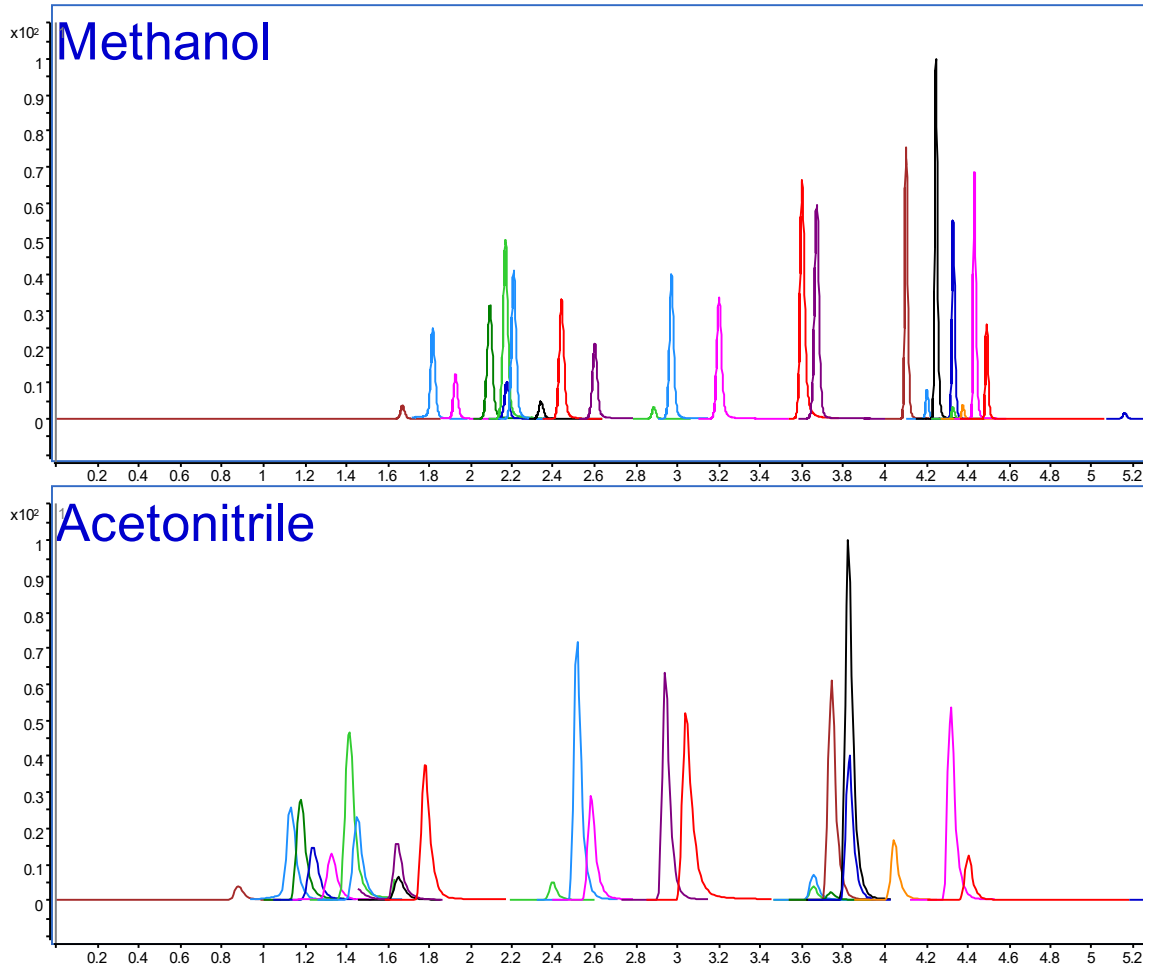
Mobile Phase – Explore Organic Options

Why?

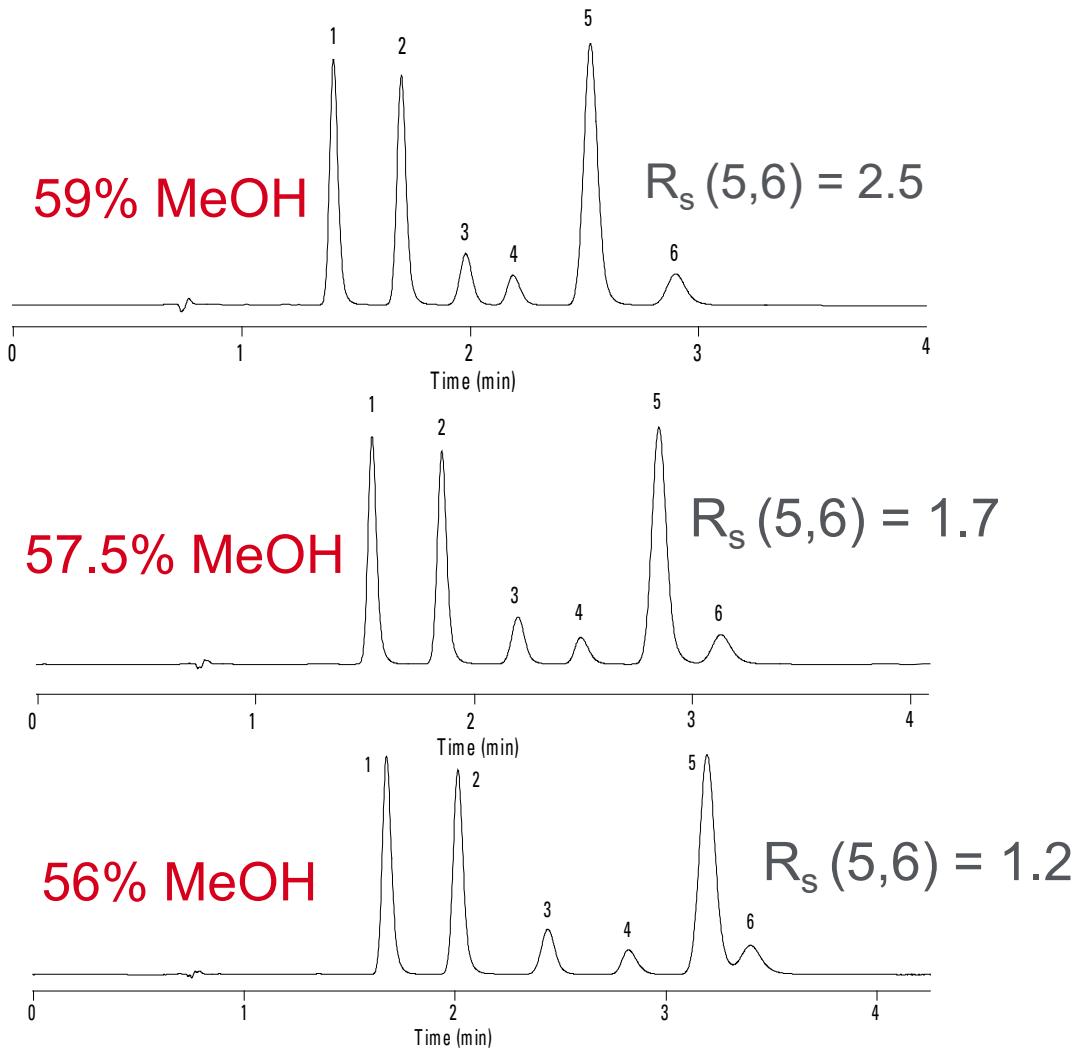
- It's easy – ACN and MeOH are readily available
- Works on any bonded phase – optimize separation no matter the column choice

MeOH – Higher pressure, generally better peak shape with bases, protic solvent

Acetonitrile – Aprotic, wider UV window, stronger than MeOH



% Organic



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

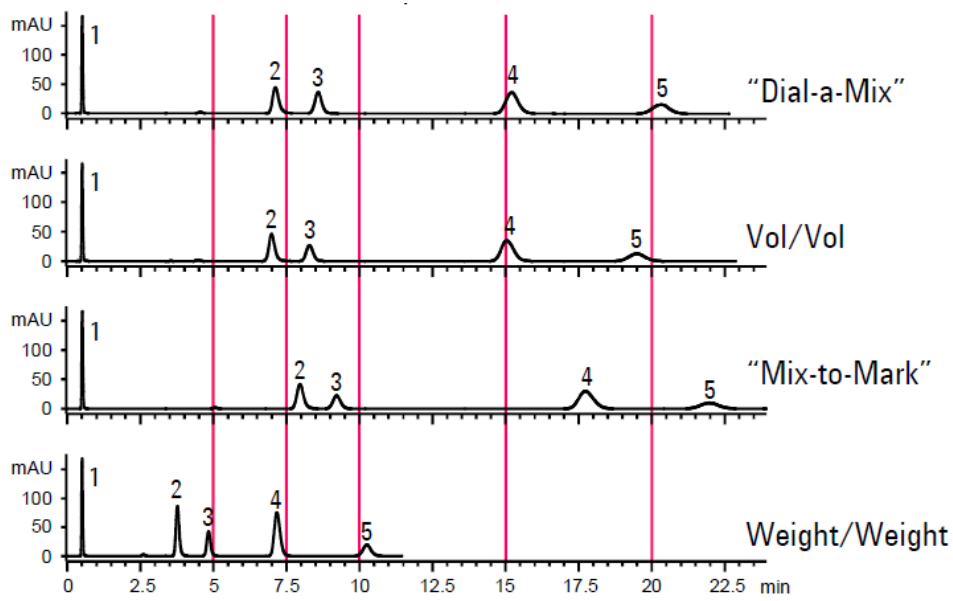
Detection: 275 nm

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

- How you make your mobile phase can affect %
- Verify resolution doesn't change around desired conditions (i.e. +/- 1-2% B)

Getting Off to a Good Start

Mobile phase preparation – be consistent and document



HPLC System:	Agilent 1100 with quaternary pump
Column:	ZORBAX Eclipse XDB-C8 Rapid-Resolution (3.5µm), 4.6 x 50 mm Agilent Part No. 935967-906
Mobile Phases:	Dial-a-Mix= A: water B: MeOH, pump 50% B Vol/Vol=250 mL water + 250 mL MeOH, pump 100% Mix-to-Mark = 250 mL MeOH, fill to 500 mL with water, pump 100% Premixed (w/w) = 200 g MeOH + 200 g water, pump 100%
Detection:	UV 254 nm
Flow:	1 mL/ min.
Temperature:	ambient

1. Uracil
2. Butylparaben
3. Napthalene
4. Dipropylphthalate
5. Acenaphthene

- Method used to prepare MP can significantly affect the elution pattern
- W/W is more accurate than V/V

Effect of Mobile Phase Preparation on Chromatography, Pub no. 5988-6476EN

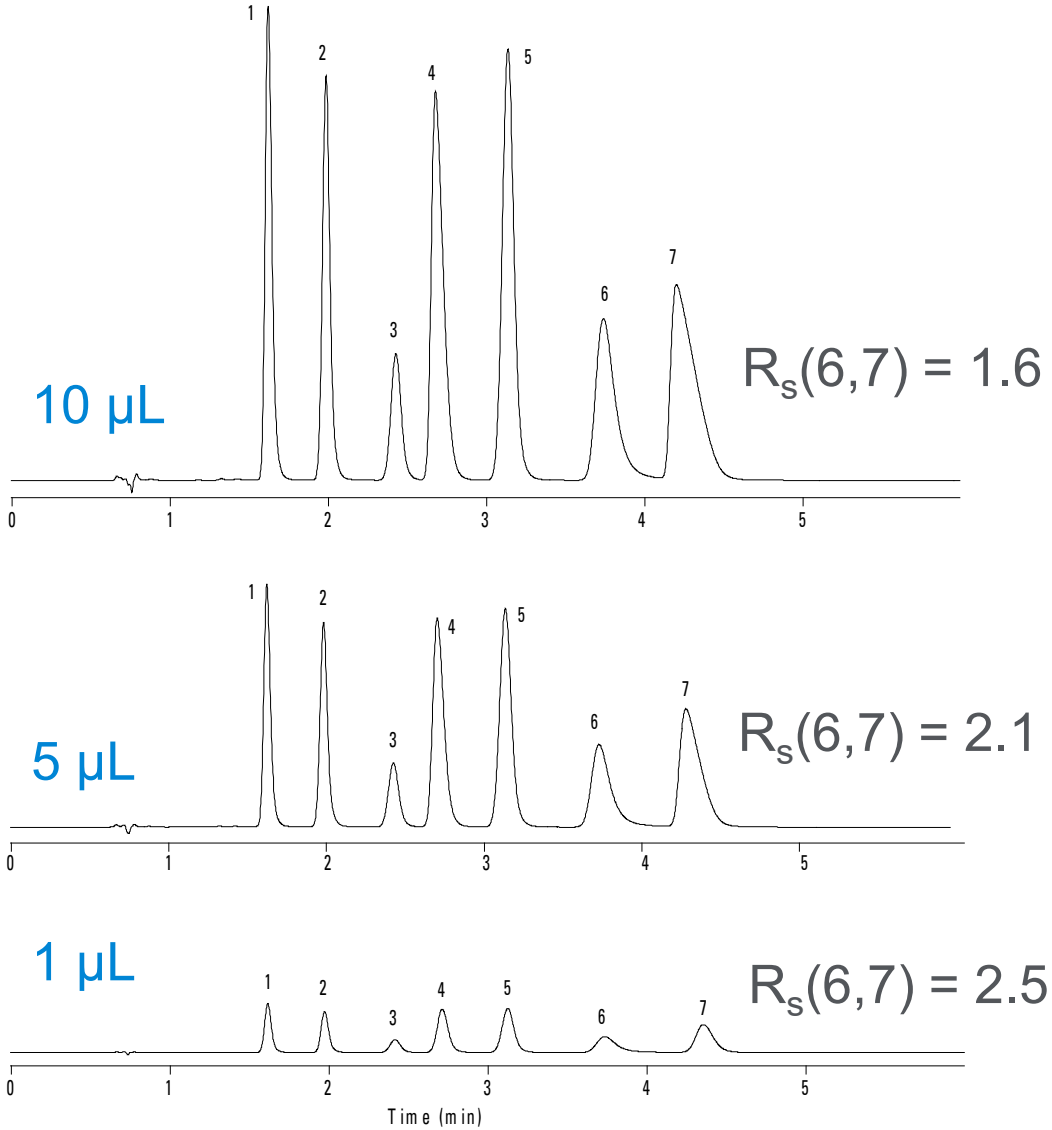
The Sample

Experimental Variables That Can 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

Injection Volume Robustness

Check Affect on Resolution and Peak Shape – Vary Volume

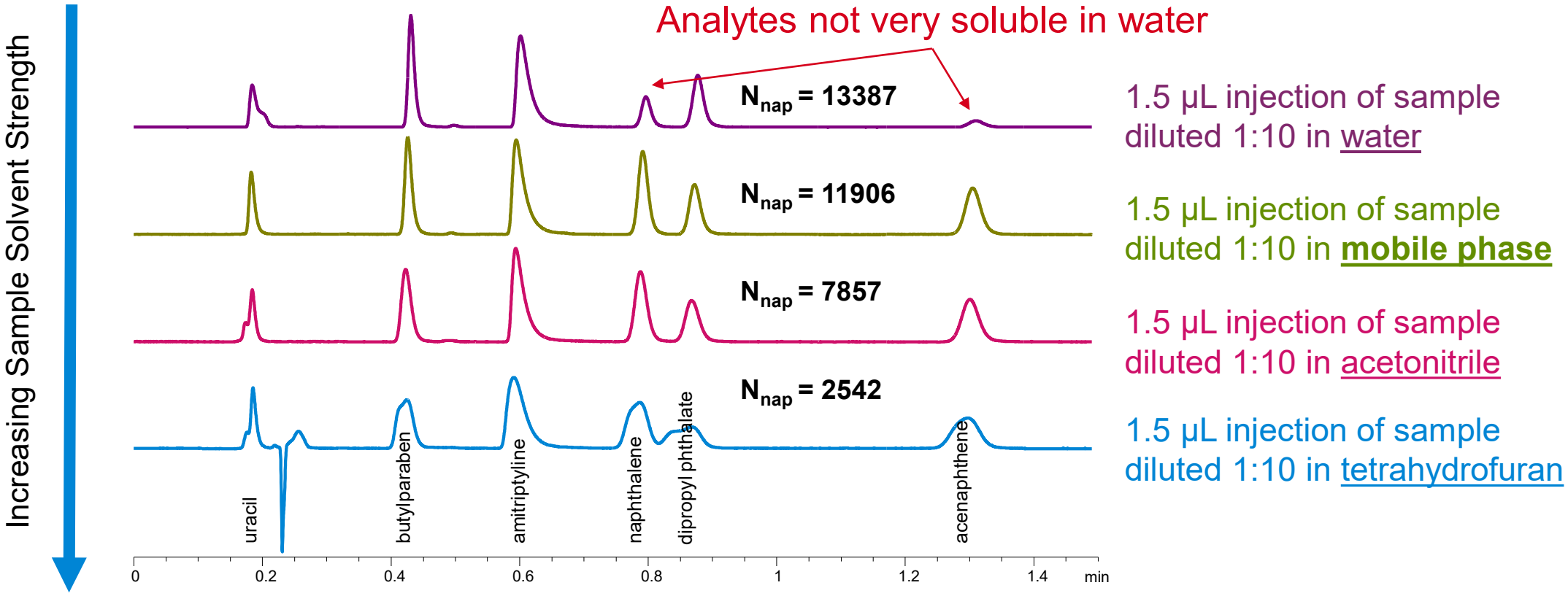


Column: ZORBAX Rapid Resolution Eclipse XDB-C8
4.6 x 75 mm, 3.5 µm

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

Reliability

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

- Is sample soluble in diluent?
- Sample solvents – equal or lesser strength than mobile phase

Instrument

Variability can affect resolution

- Column
- Mobile Phase
- Sample
- Instrument
  - Dwell volume
  - Extracolumn volume

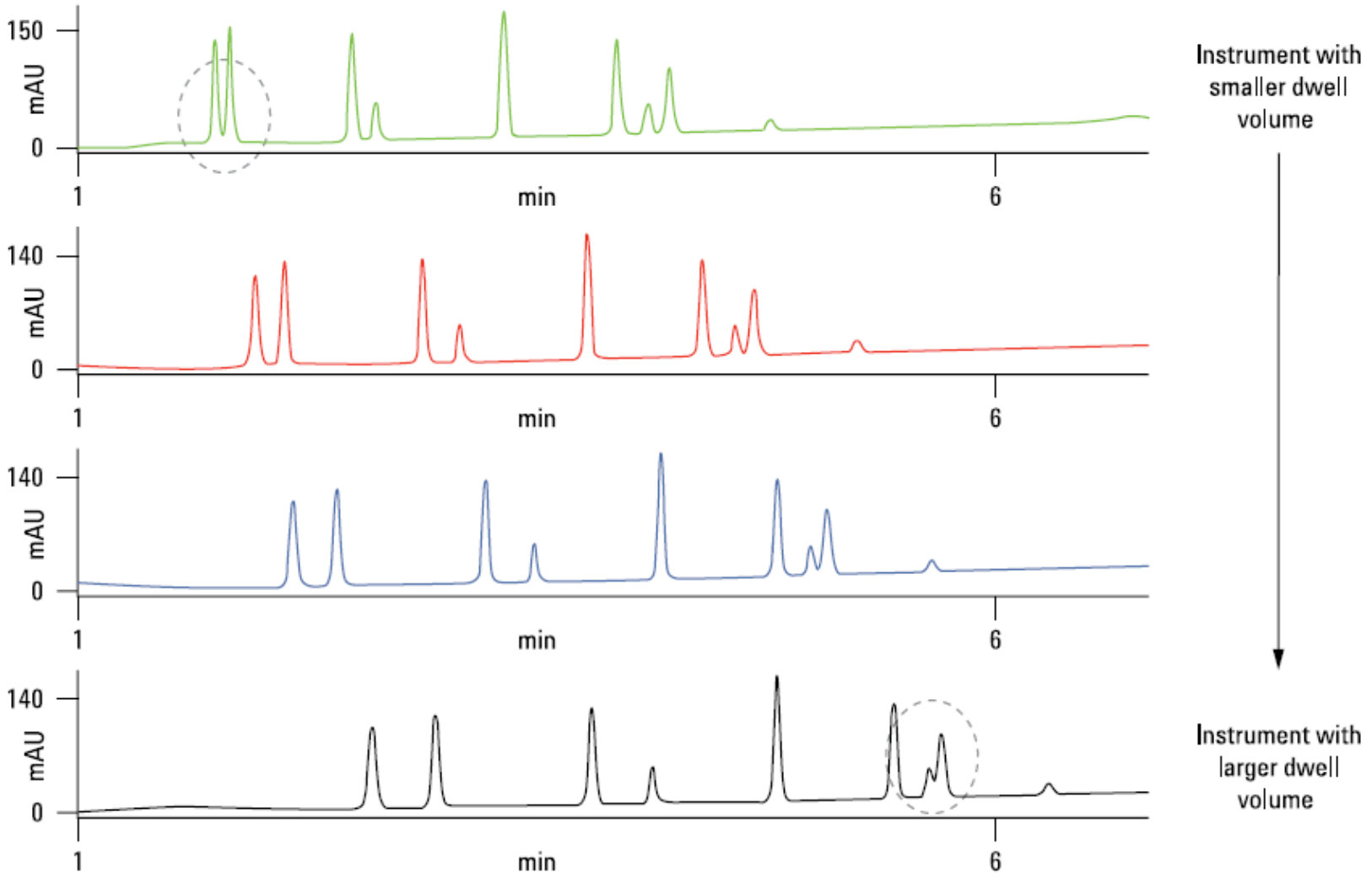
Gradient Delay Volume

Key parameter: Can cause major chromatography differences between systems

Why is delay/dwell volume important?

1. Different dwell volumes result in a RT time shift
   - Definition: The time (or volume) for mobile phase from point of mixing to reach the column head
2. Different dwell volume could affect resolution
   - Peaks spends different time under isocratic/gradient conditions
3. Dwell volume effects on gradient shape
   - Dispersion effects => the programmed gradient becomes deteriorated
4. Same “delay” volume – chromatograms could look different on different systems
5. Big impact for narrow bore applications, especially combined with fast gradient

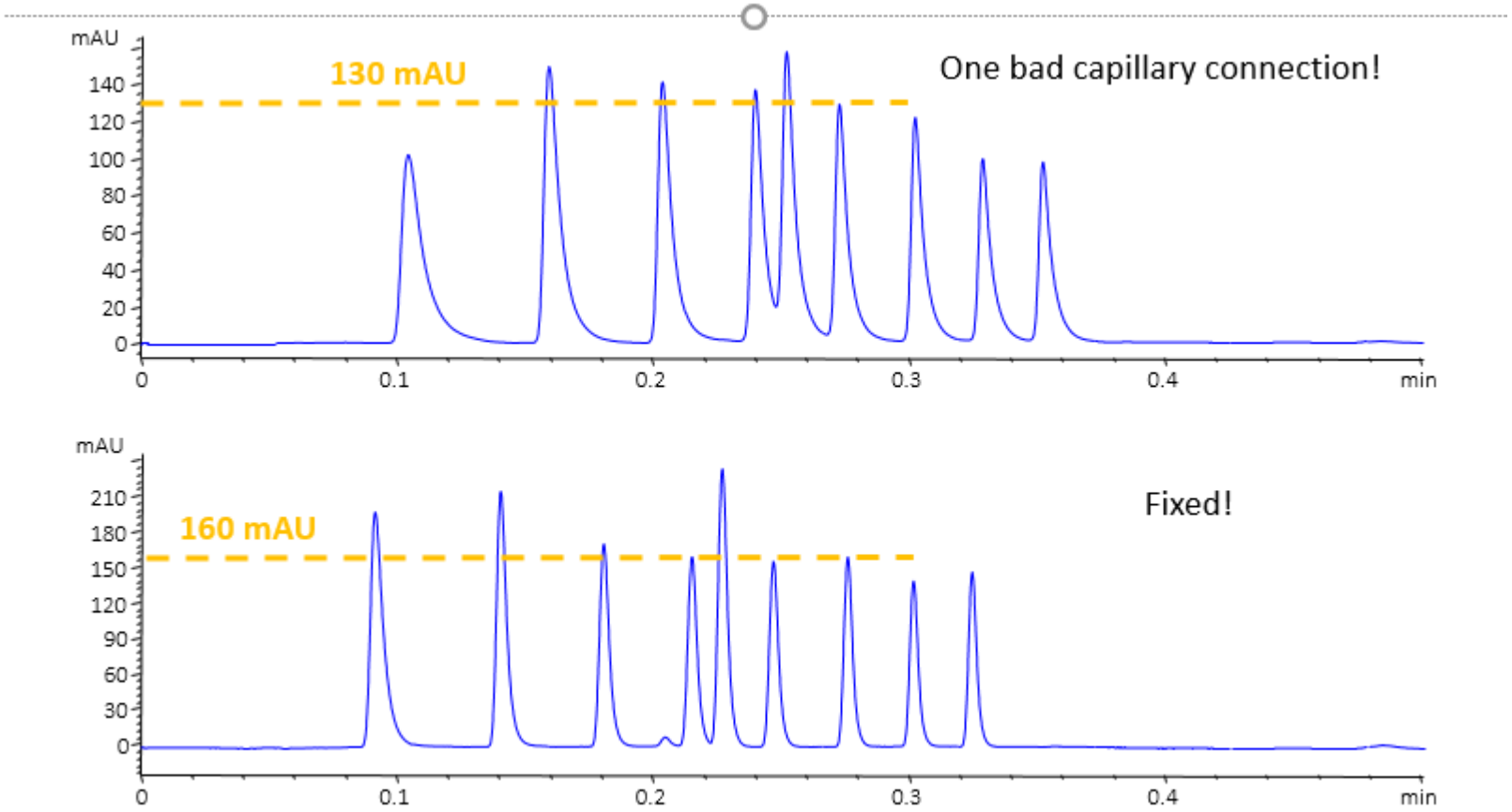
Chromatographic Test Result; Different Delay Volumes



Dispersion
Extracolumn volume

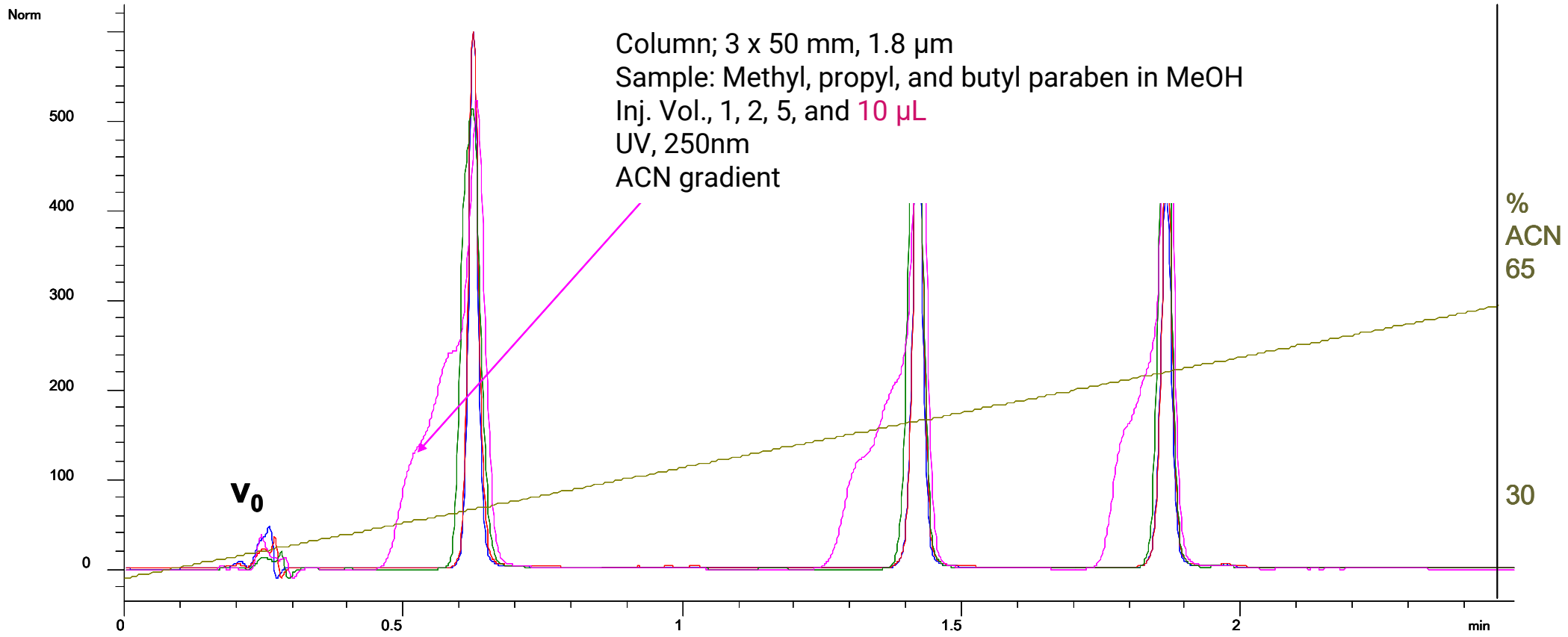
- ECV – System volume between point of injection and detector outlet
- ECV major contributors
  - Capillaries, length, and id
  - Heat-exchangers
  - Connectors and fittings
  - Flow cell
- Large ECV causes sample dispersion and band broadening of analytes
  - Result: Decreased resolution and less sensitivity
- Take special care with capillary connectors or when mounting columns into a system.
- Remember – Diluent strength and injection volume contribution
- Small id columns, <2.1 mm

Dispersion: Peak Broadening Extracolumn Volume



Column: StableBond SB-C18, 4.6 x 30 mm, 3.5 µm Mobile Phase: 85% H_2O with 0.1% TFA : 15% ACN Flow Rate: 1.0 mL/min
Temperature: 35°C Sample: 1. Phenylalanine 2. 5-benzyl-3,6-dioxo-2-piperazine acetic acid 3. Asp-phe 4. Aspartame

Tips for Minimizing Dispersion in LC Systems



Keep injection volumes small, especially when the diluent is significantly stronger than the mobile phase (isocratic) or gradient initial condition

Getting Off To a Good Start

Reliability is key

- Many variables to consider; some more apparent than others
- A reliable method minimizes “headaches” and repeat work
- Well-conceived and documented lab practices
  - Equilibration
  - Mobile phase
    - What’s in it and how do you make it?
- Column selection; particle size, bonded phase, etc.
  - Method validation kit
- Sample considerations
- Instrument role

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

Option 5 for Standards

gc-column-support@Agilent.com

lc-column-support@agilent.com

spp-support@agilent.com

spectro-supplies-support@agilent.com

chem-standards-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

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

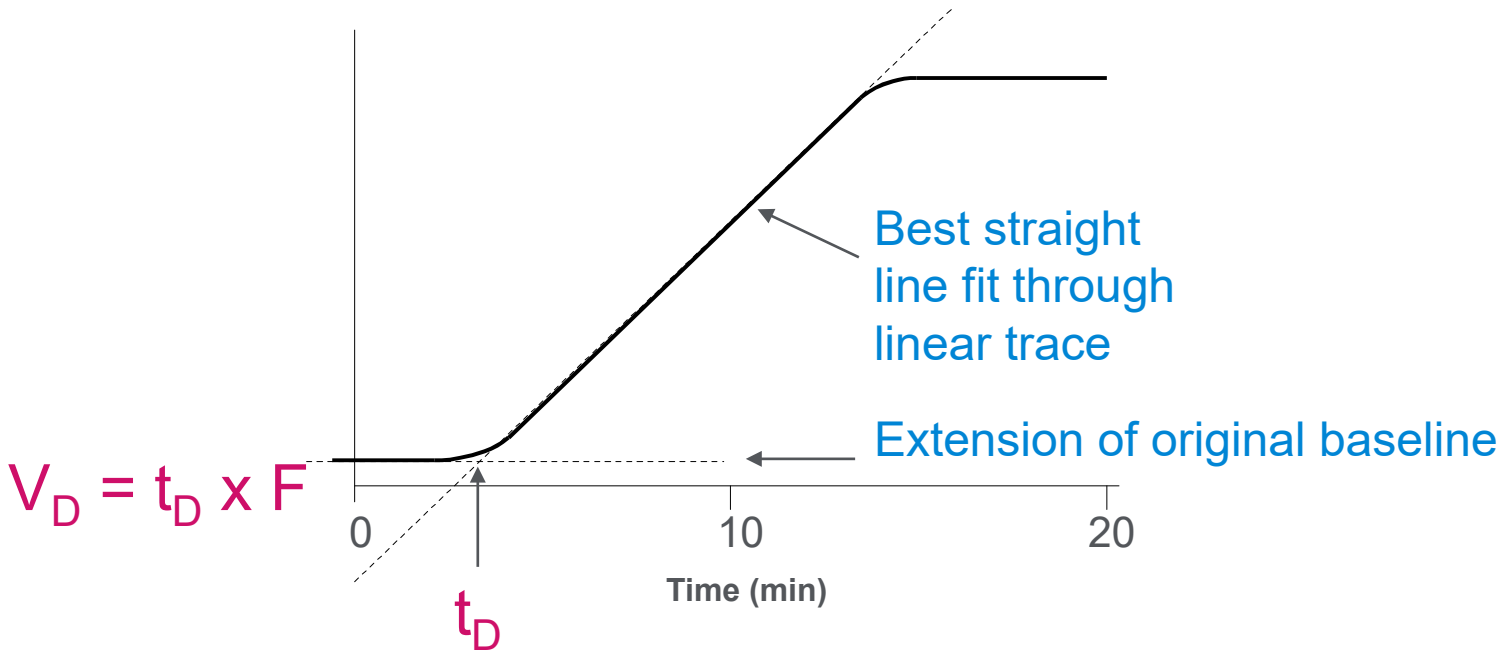
Monitor at 265 nm

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 (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.