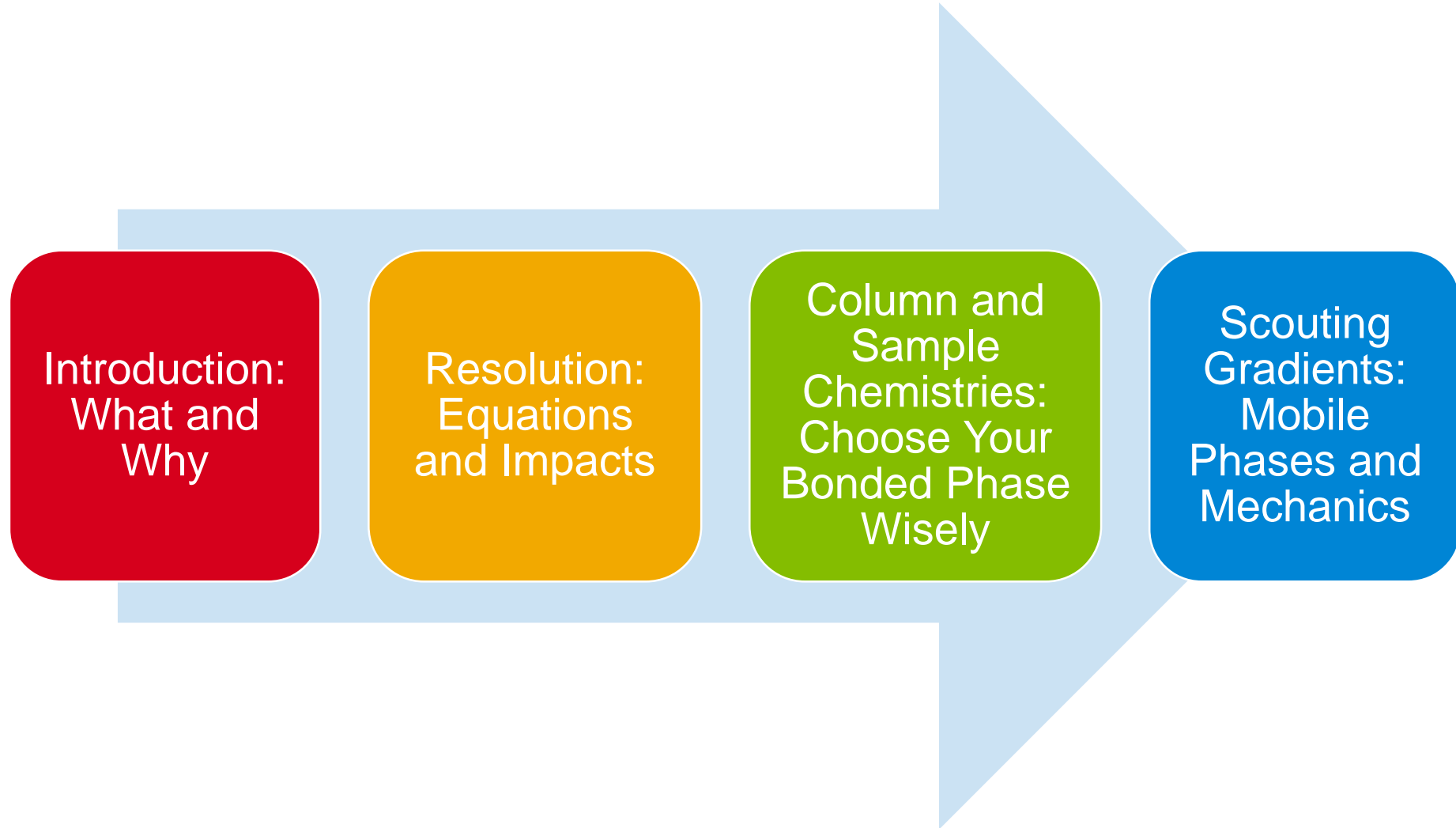


Getting Off to a Good Start

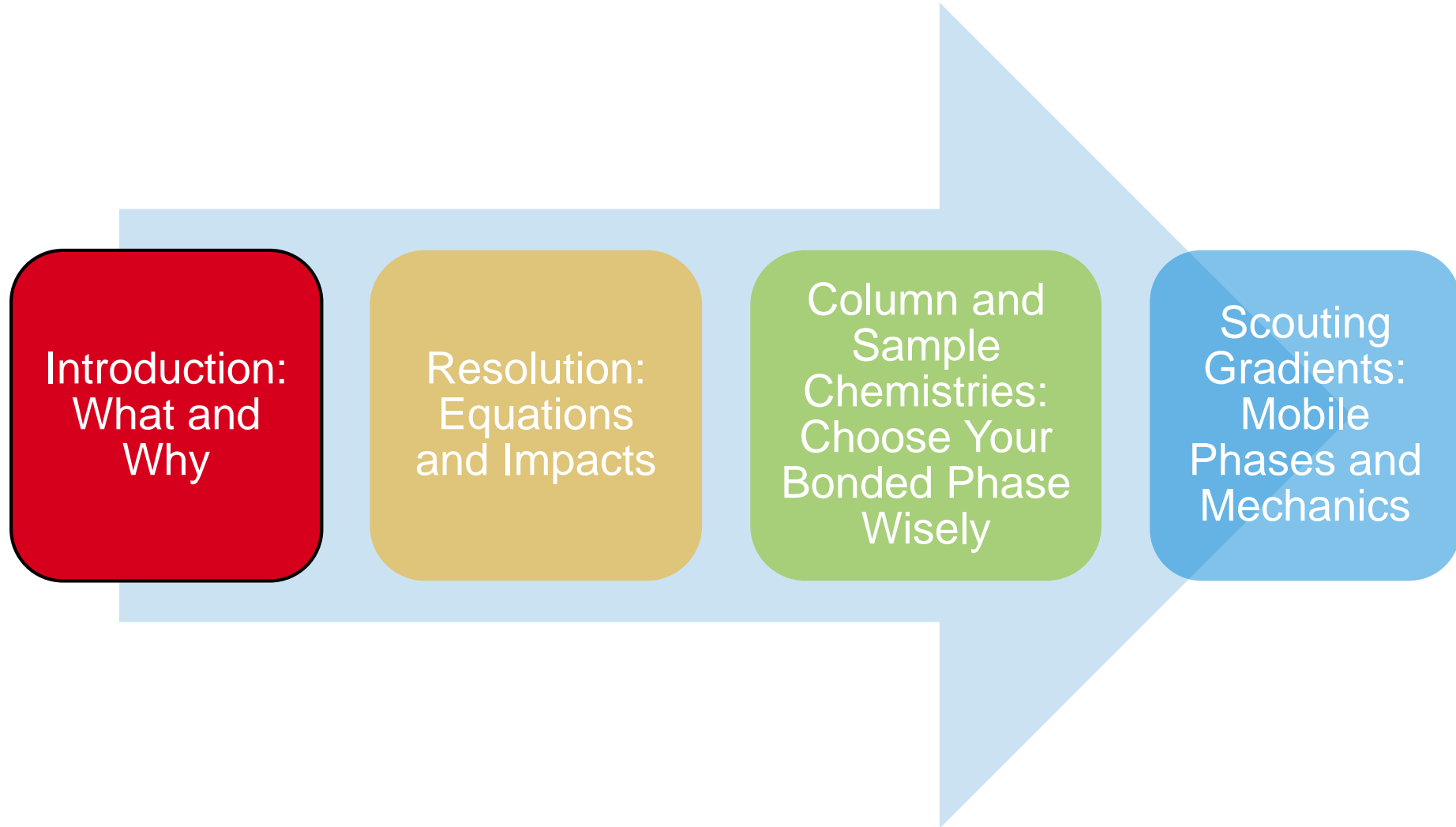
Isocratic method development

Paul Altiero
Applications Chemist, CSD
February 13, 2020

Isocratic Method Design and Development



Isocratic Method Design and Development



What is an Isocratic HPLC Method and Why Do I Want to Use One (or not)?

- An isocratic separation is: (one where) “the composition of the solvent remains constant throughout the separation”¹.
- Benefits of an Isocratic Method
 - Simple to adjust
 - No baseline drift
 - No Re-equilibration time
 - Not impacted by delay volume – easily transferrable
- Drawbacks
 - Time
 - Peak Shape
 - Column Cleaning

¹L.R. Snyder, J.J. Kirkland, J.W. Dolan. *Introduction to Modern Liquid Chromatography*, -3rd Ed., John Wiley & Sons, 2010

Getting Started

- Define the objective
 - What are the goals of the separation? Resolving multiple, critical pairs or just one component from matrix?
 - Is speed/throughput important?
 - What are the requirements around LoQ, accuracy, and precision?
 - How do we assess system/method performance?
- Gather sample information
 - List the analytes and their physical/chemical properties; for example, LogP, pKa, and solubility
 - Matrix and Sample Preparation
 - What is the sample in? (diluent)
 - What else is in the sample? (matrix)
 - List resources available
 - Instrumentation, capabilities, and limitations; for example, flowrates, pressure ratings, and types of detectors
 - What columns are available? Choices of bonded phase? New or used?
 - Literature references or subject matter experts

Getting Started (Continued)

- List Known Challenges – examples include:
 - Analytes with very similar structures/properties
 - Compatibility of analytes and detector – lack of chromophores, poor ionization
 - Additives/buffers – solubility of buffers, interference from additives, for example, TFA
- Plan out an approach
 - What sample preparation/cleanup do we need and why?
 - What columns/bonded phases do we want to try, and why?
 - Based upon pKas what pHs do we want to look at
 - What organic mobile phases do we want to use
 - What do we expect a scouting gradient to look like

Examples of Common Separation Goals and Method Performance Criteria

Good system suitability parameters

- Resolution: ≥ 2
- Peak shape: USP T_f close to 1 (< 2)
- Injection 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

Method performance criteria

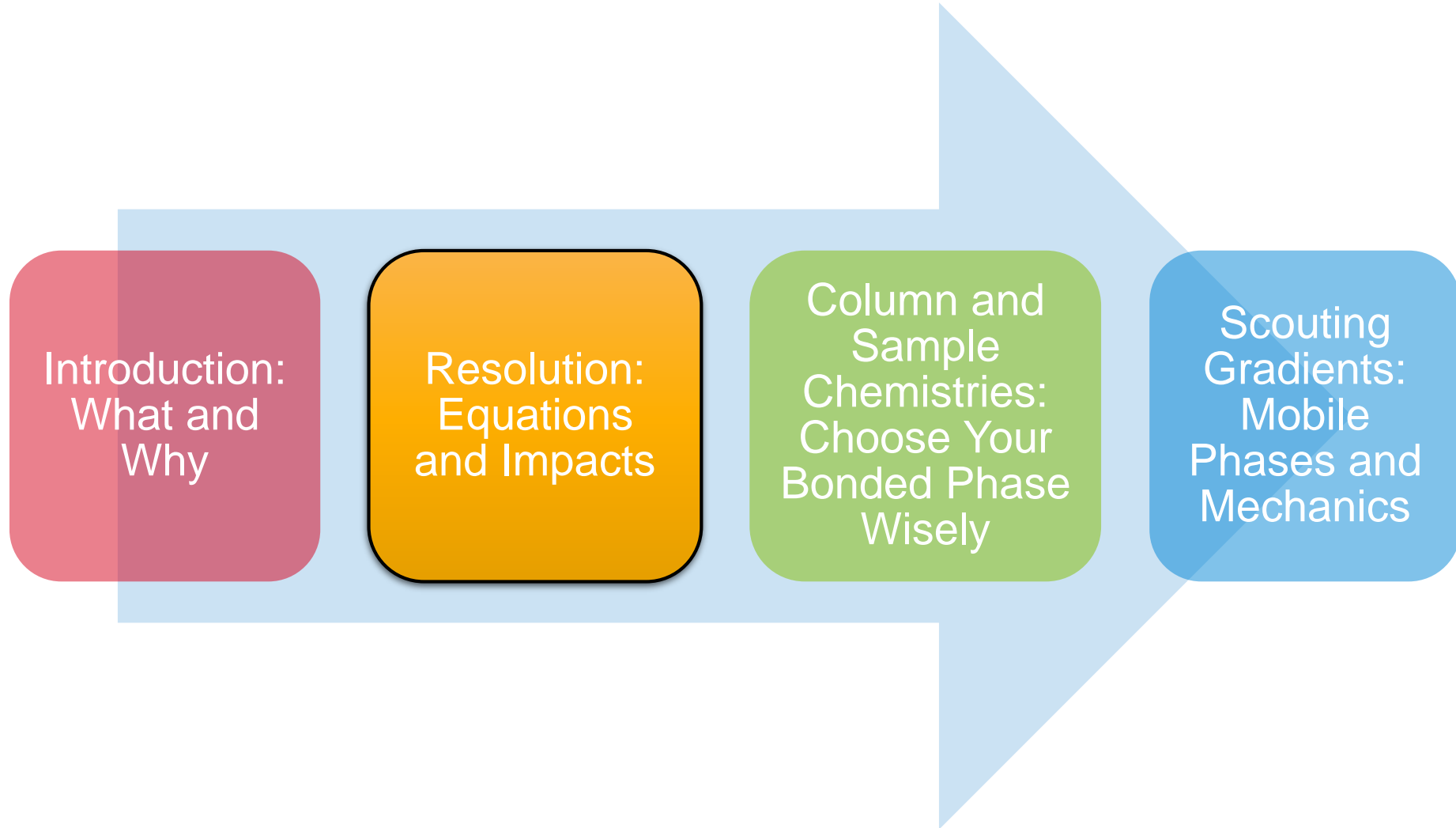
- Accuracy
- Precision
 - Ruggedness
 - Robustness
- Selectivity/specificity
- Linearity
- Range
- Quantitation limit (LOQ, 10x S/N)
- Detection limit (LOD, 3x S/N)

Avoid these for System Suitability Criteria:

*Column efficiency (theoretical plates)
and absolute retention time*

These inhibit the ability to speed up your method in the future

Isocratic Method Design and Development



Introduction:
What and
Why

Resolution:
Equations
and Impacts

Column and
Sample
Chemistries:
Choose Your
Bonded Phase
Wisely

Scouting
Gradients:
Mobile
Phases and
Mechanics

Fundamental Resolution Equation – Isocratic Separations

$$R_s = \frac{1}{4}(N)^{1/2} \left[\frac{(\alpha - 1)}{\alpha} \right] \left[\frac{k}{1 + k} \right]$$

α = Selectivity – increase by changing bonded phase and mobile phase

N = Plates – increase by using longer column or reducing particle size

k = Retention – increase by changing bonded phase and mobile phase

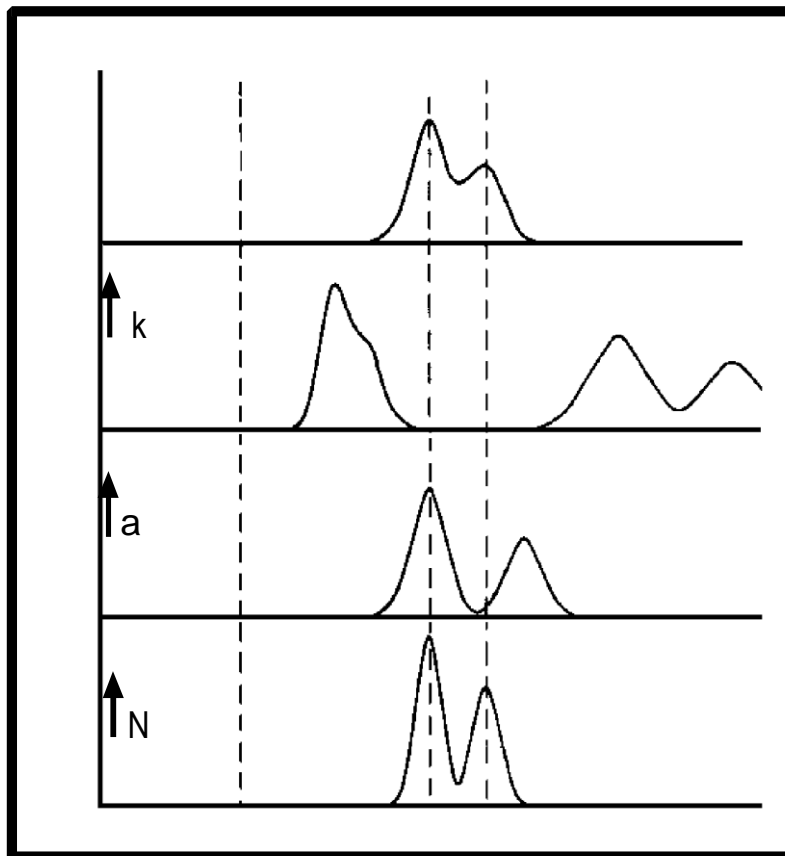
Does not improve R_s above $k \approx 10$

Factors that Maximize Isocratic Resolution Between Peaks

Increase retention

Change relative peak position

Increase retention time faster than peak width

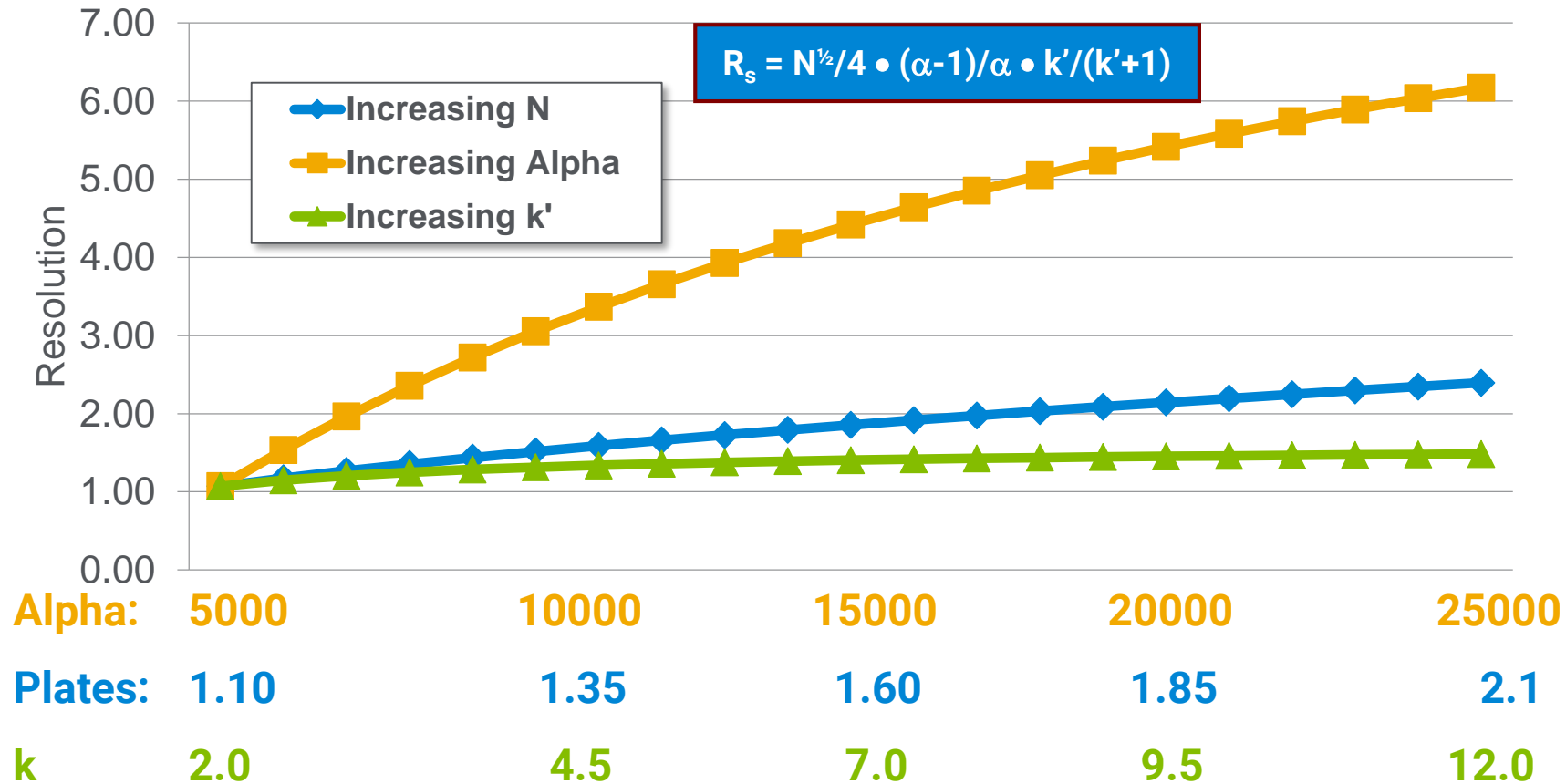


Decrease %organic

- Change the chemistry of the mobile or stationary phase
- Change %organic

- Change column dimensions or flowrate
- Decrease particle size

Selectivity Impacts Resolution the Most



Selectivity impacts resolution most

- Change bonded phase
 - Change mobile phase
 - Plates are easiest to increase
- } Typical method development parameters

The Van Deemter Equation for Band Broadening

$$H = A + \frac{B}{u} + Cu$$

Where A, B, and C represent a different set of constants for a particular solute, column, and set of experimental conditions¹.

The A term is *eddy diffusion* and is independent of linear velocity.

The B term is the *longitudinal diffusion* term and we can see that the contribution to total band broadening goes down as linear velocity increases.

The C term is the *mass transfer* term. The impact on total band broadening increases as linear velocity increases.

¹L.R. Snyder, J.J. Kirkland, J.W. Dolan. *Introduction to Modern Liquid Chromatography*, -3rd Ed., John Wiley & Sons, 2010

Optimal Flow Rates for Agilent InfinityLab Poroshell 120 Columns

Smaller particles have higher optimal flow rates

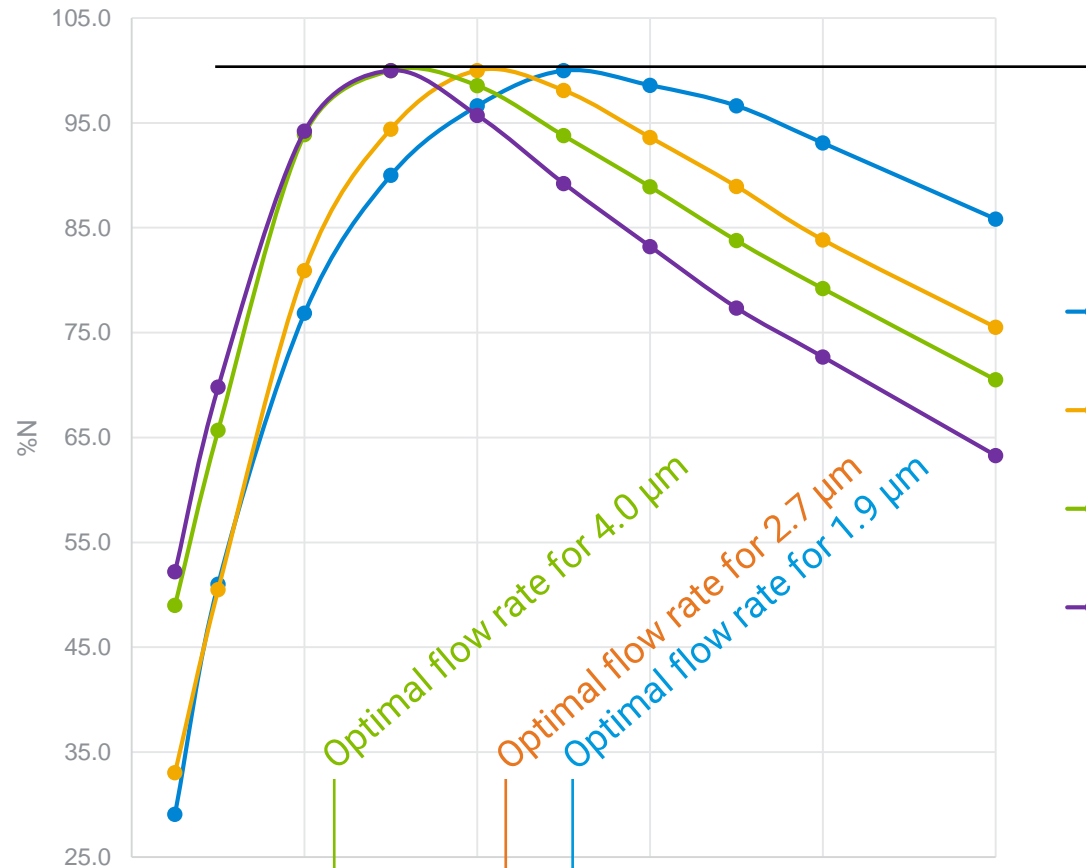
- Operating below the column's optimal flow rate negatively impacts efficiency (and resolution)
- Operating above the optimal flow rate also can negatively affect efficiency, but to a lesser extent.
- When comparing different column dimensions, flow rates should be geometrically scaled relative to column id to maintain a constant linear velocity.

$$F_2 = F_1 * (id_2 / id_1)^2$$

- F_1 and F_2 : Original and new flow rate
- id_1 and id_2 : Original new column id

2.1 mm id columns	0.2	0.4	0.6	0.8	1.0
3.0 mm id columns	0.4	0.8	1.2	1.6	2.0
4.6 mm id columns	1.0	2.0	3.0	4.0	

Equivalent flow rates (mL/min) for different column internal diameters



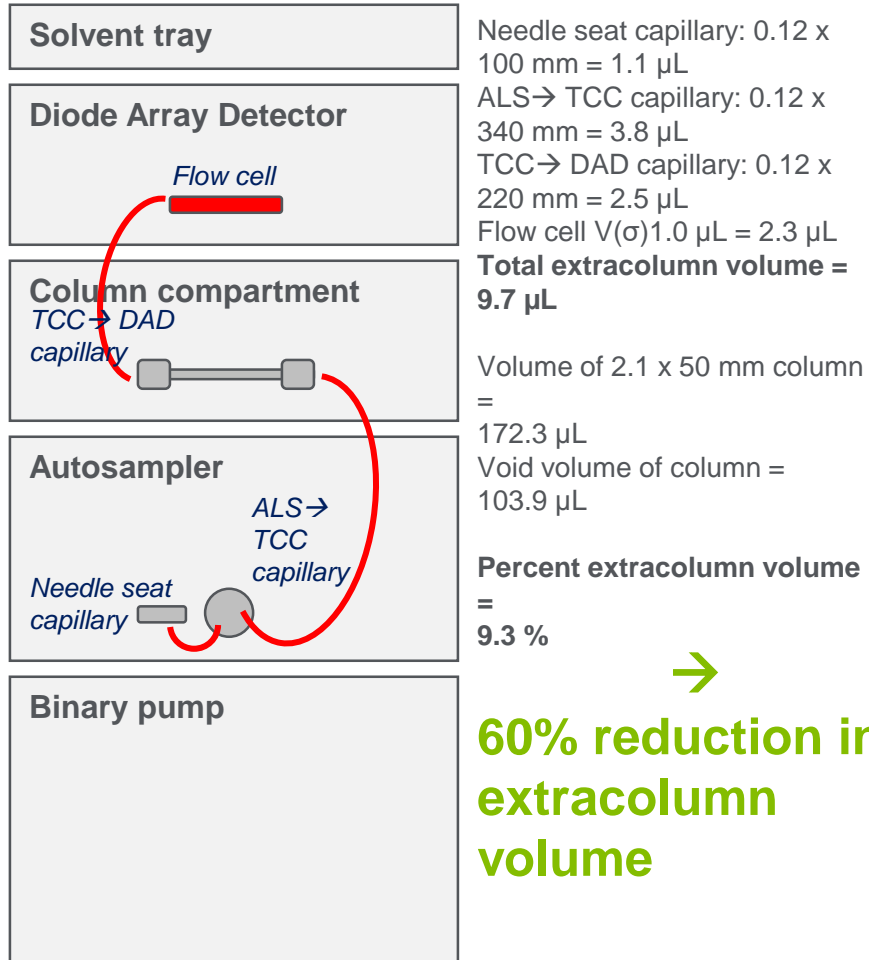
Maximum efficiency for each column

- 2.1 x 50 mm, 1.9 μm Poroshell 120 EC-C18
- 3.0 x 75 mm, 2.7 μm Poroshell 120 EC-C18
- 4.6 x 100 mm, 4.0 μm Poroshell 120 EC-C18
- 4.6 x 150 mm, 5.0 μm ZORBAX Eclipse Plus C18

A: Water
B: Acetonitrile
Flow rate: Variable
Elution: Isocratic 60% B
Sample: 0.5 μL of phenones (5188-6529) for 2.1 x 50 mm, geometrically scaled for each column dimension
Autosampler temperature: 5 °C
Column temperature: 30 °C
DAD: 254, 8 nm, Ref = Off, 80 Hz
Analyte: Octanophenone

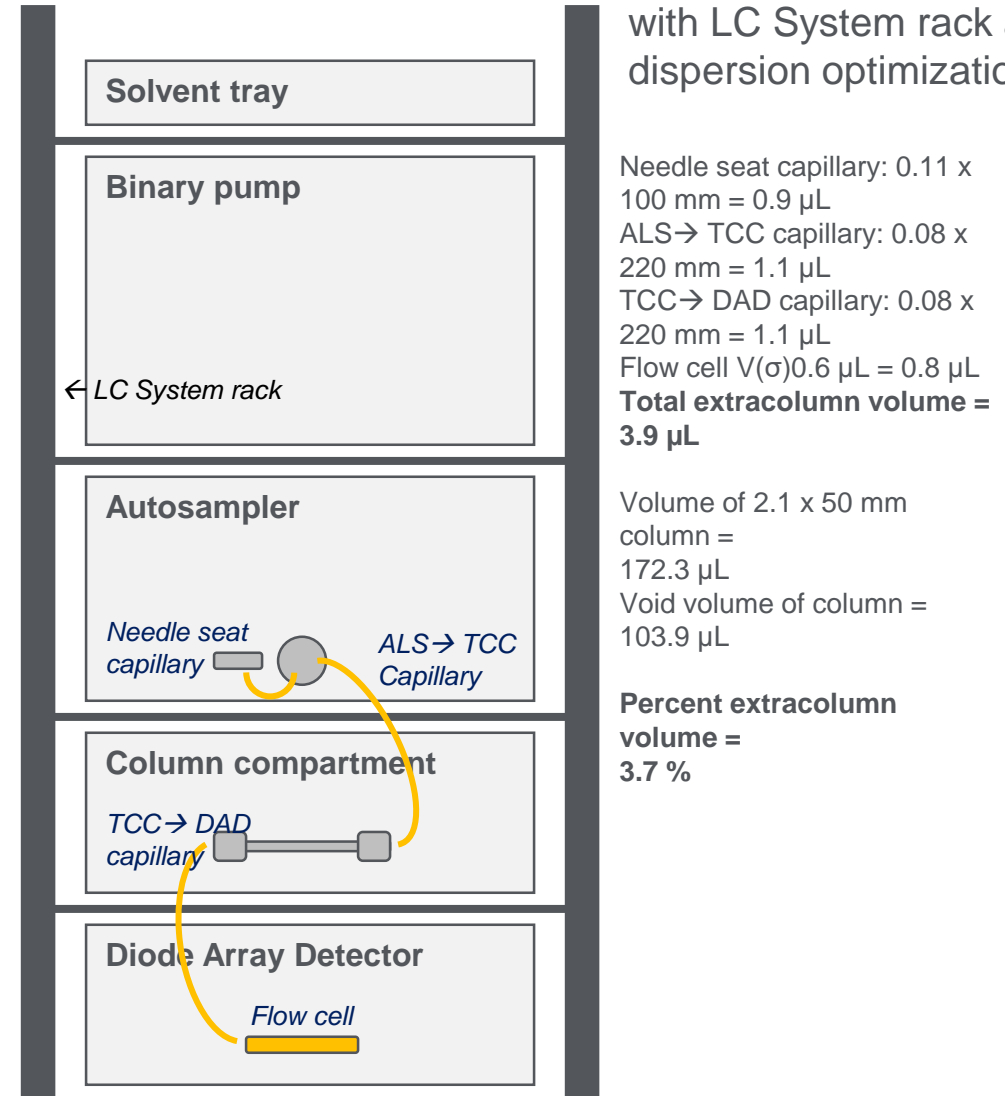
Improve Performance by Reducing LC System Volume

Agilent 1290 Infinity LC System:
Default stacking and capillary tubing configurations



\rightarrow
60% reduction in extracolumn volume

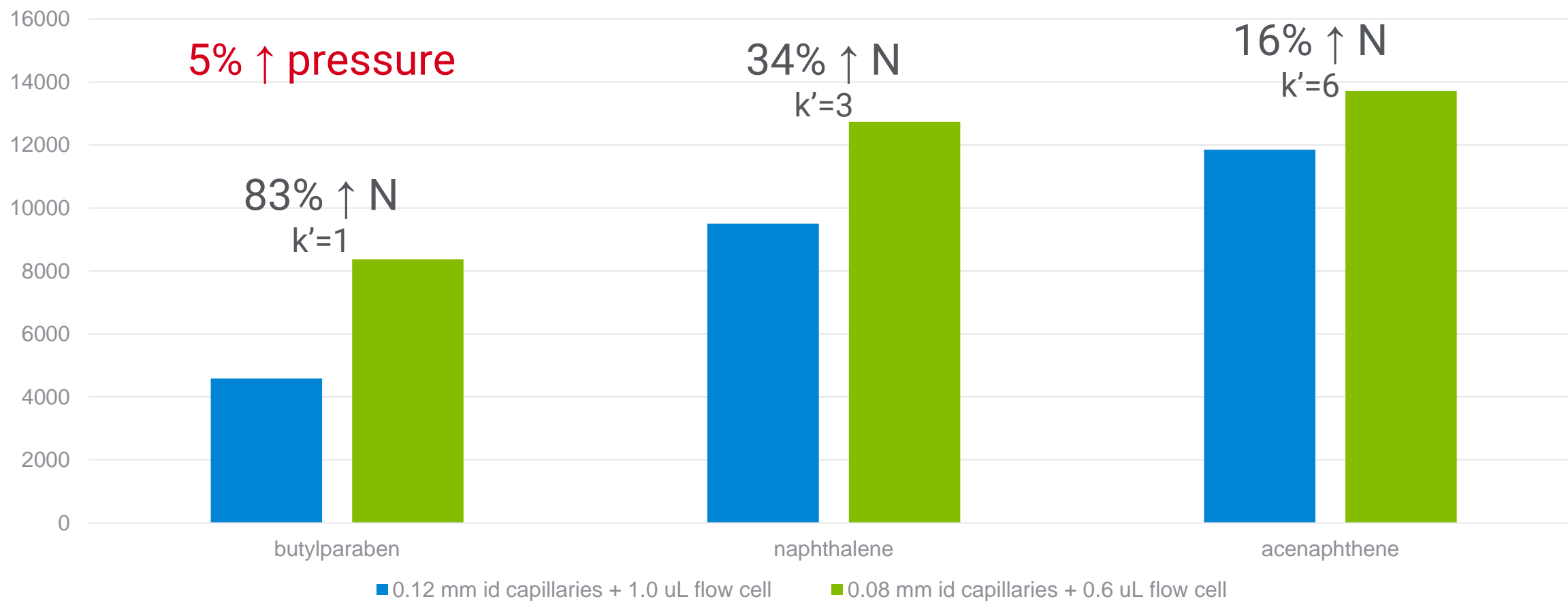
Agilent 1290 Infinity LC System:
with LC System rack and ultra-low dispersion optimizations



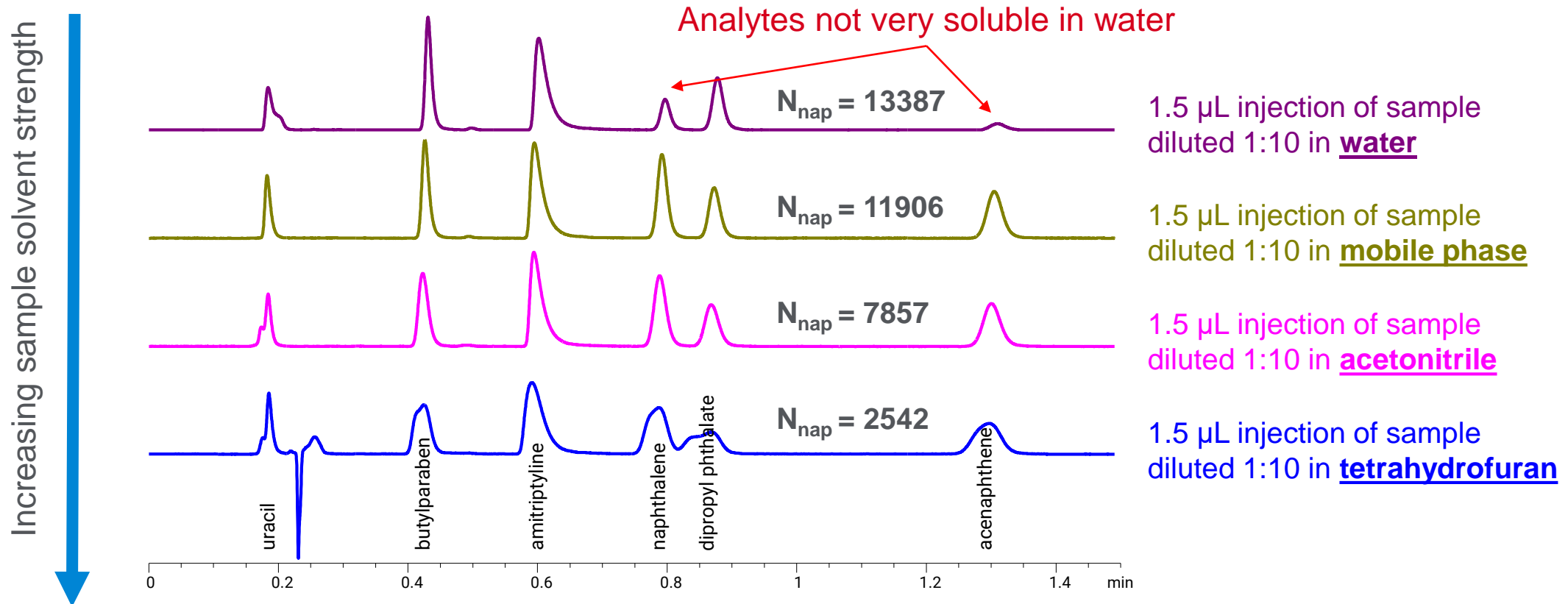
Improve Performance by Reducing LC System Volume

Use smaller internal diameter capillaries and a smaller volume detector flow cell

Effect of Capillary id + Flow Cell Volume on Efficiency



Sample Considerations – Mobile Phase Diluents and Solubility

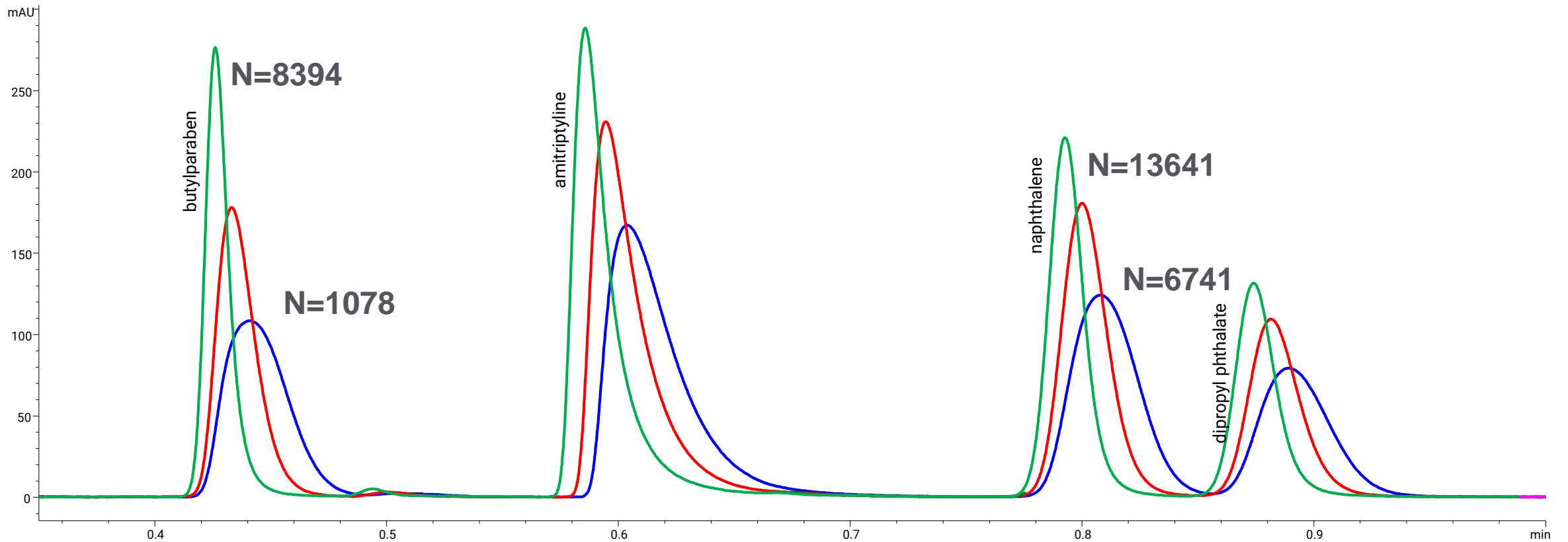


Sample solvents should be of equal or lesser strength than the mobile phase, otherwise poor peak shape can occur, resulting in poor efficiency.

Sample Injection Volumes Can Affect Peak Shape and Resolution

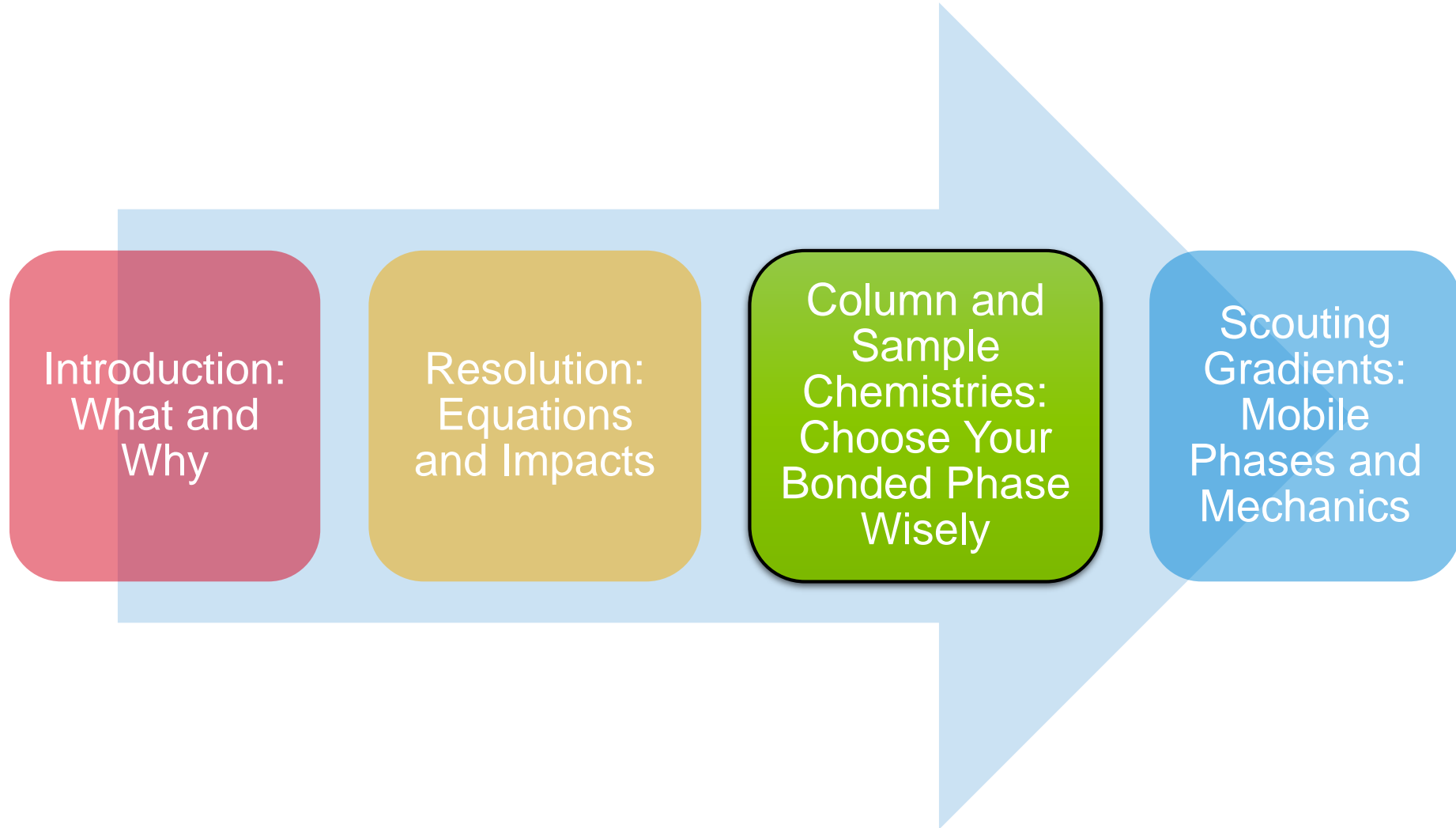
16 μL injection
8 μL injection
0.5 μL injection

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

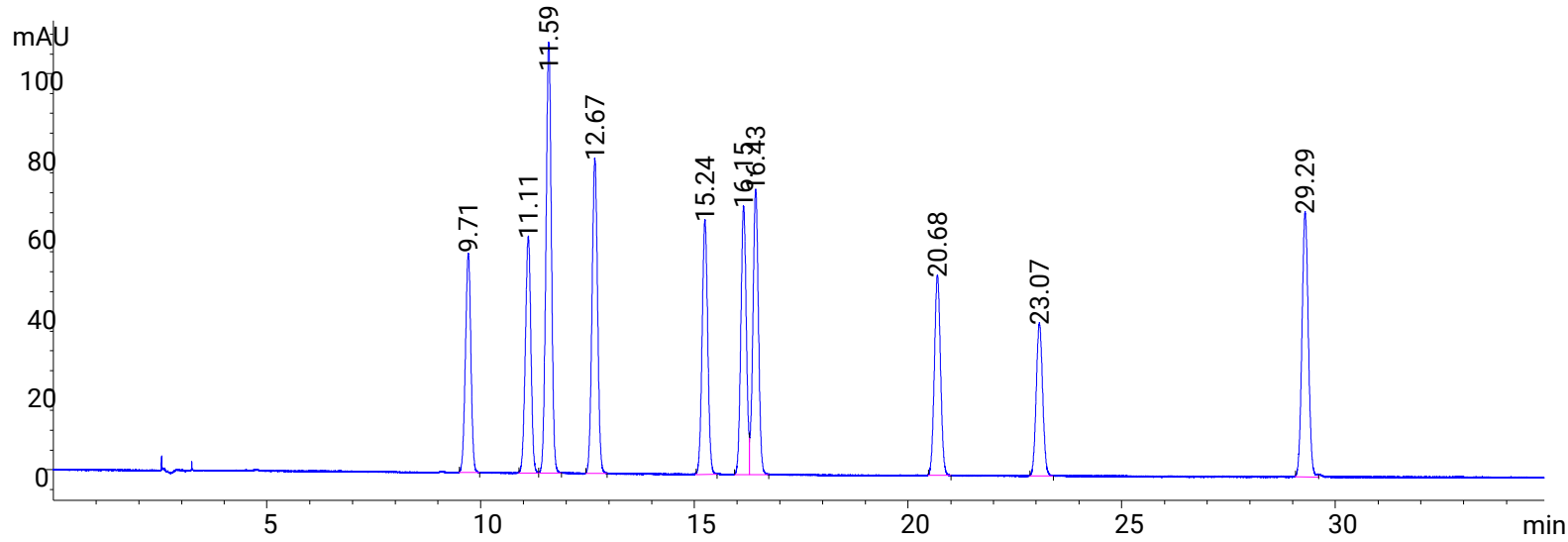


Sample concentrations are adjusted to ensure the same sample load on the column, regardless of injection volume.

Isocratic Method Design and Development



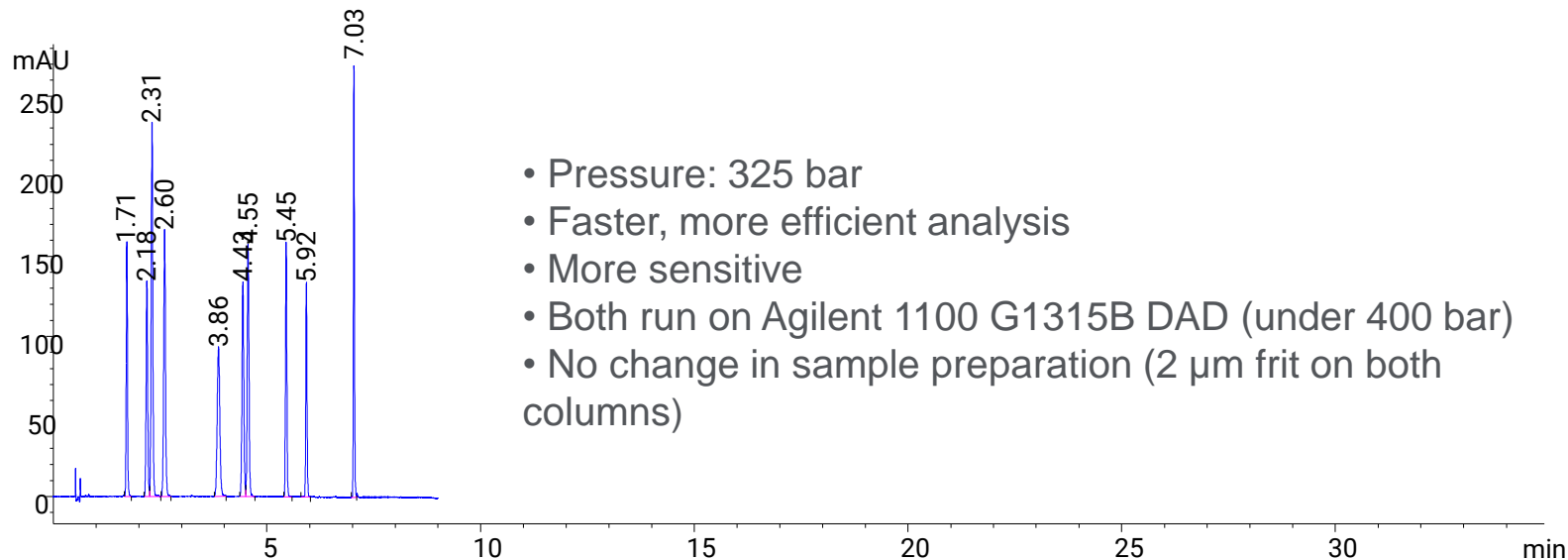
Column Choices – Which Particle Type to Choose?



Totally porous particle

ZORBAX Eclipse Plus C18
4.6 x 250 mm, 5 μ m

Run time: 35 min



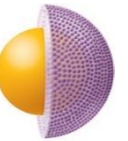
- Pressure: 325 bar
- Faster, more efficient analysis
- More sensitive
- Both run on Agilent 1100 G1315B DAD (under 400 bar)
- No change in sample preparation (2 μ m frit on both columns)

Superficially porous particle

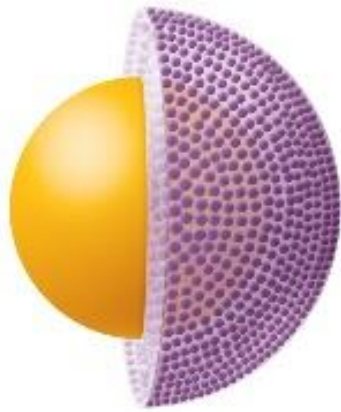
InfinityLab Poroshell 120 EC-C18
4.6 x 100 mm, 2.7 μ m

Run time: 9 min

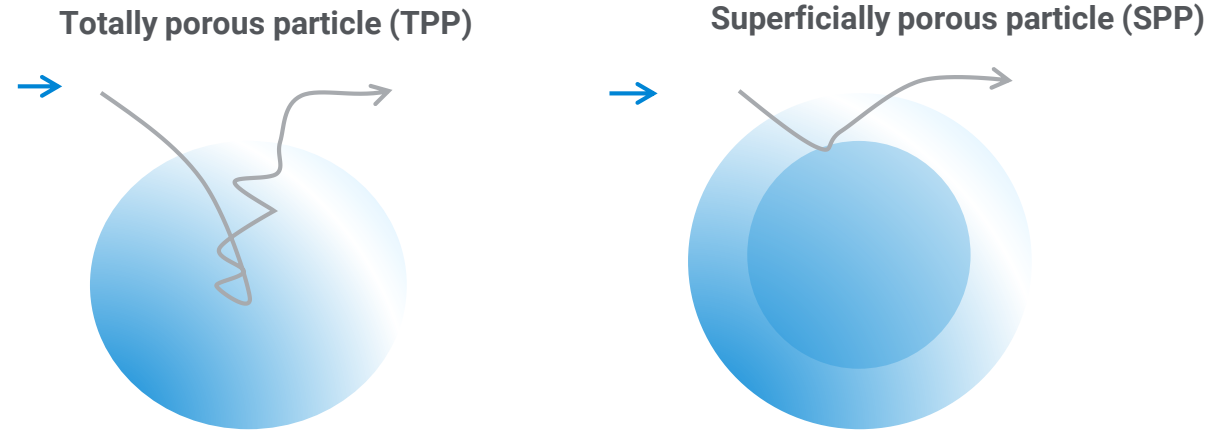
A: 0.1% formic acid in water, B: ACN
Gradient: 8-33% ACN in 30 or 8 min
1 or 2 mL/min, 25 °C, 254 nm
Agilent application note, 5990-5572EN



Poroshell Technology – What Makes it Better?



Poroshell is made of a solid core with a porous outer layer.



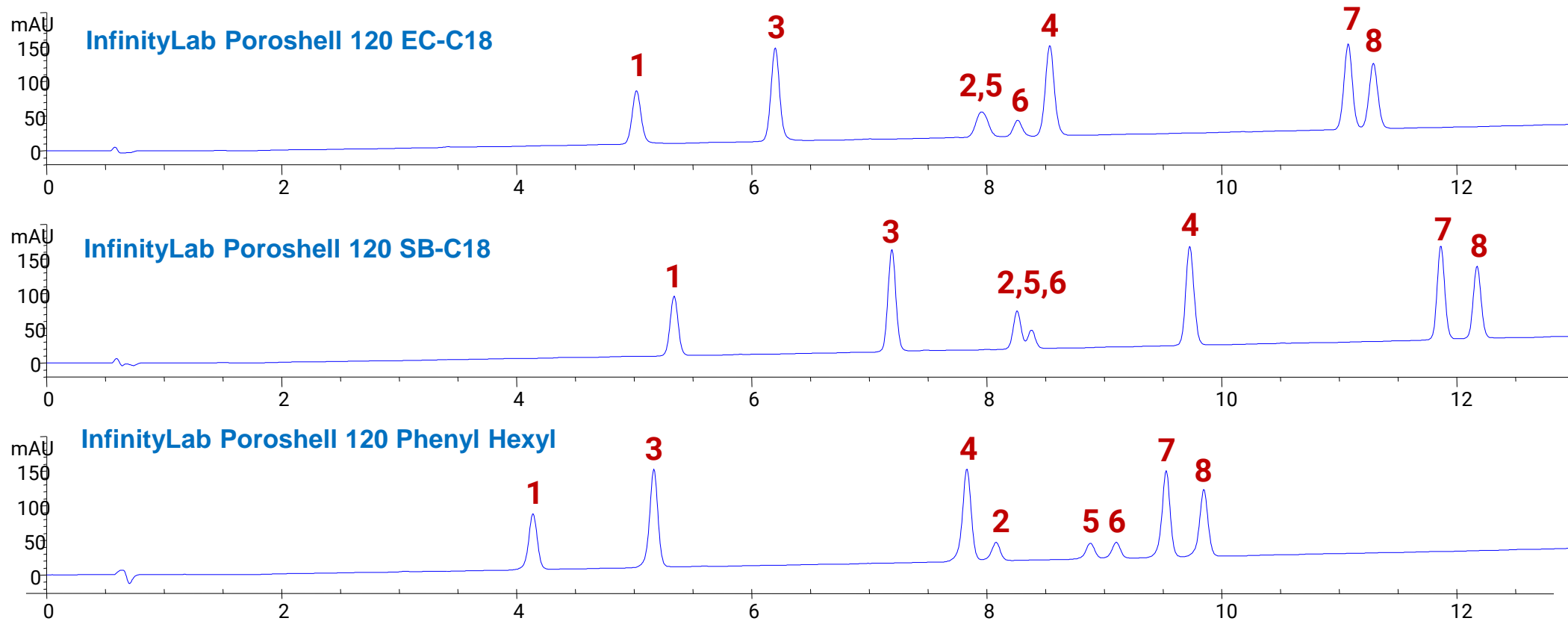
- Analytes travel through the particle more efficiently, improving peak shape and resulting in faster run times.
- High efficiency allows you to use a larger SPP (2.7 μm) for nearly equivalent performance to a smaller TPP column (sub-2 μm).
- Using a larger particle allows for lower backpressure than comparable TPP columns, and flexible use on HPLC or UHPLC systems.

Column Choice: Evaluate Different Bonded Phases

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

Evaluating different bonded phase chemistries early can save time in optimization and generate a more robust method.

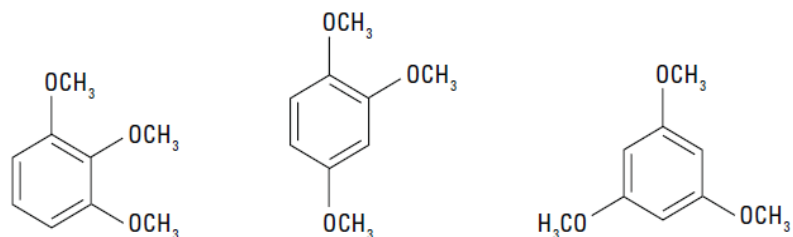
Selectivity Differences Across InfinityLab Poroshell Bonded Phases



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

40 to 80% methanol in 14 min, DAD 260, 80 nm 0.4 mL/min,
2.1 x 100 mm column, 40 C, 0.1% formic acid in water and
methanol, Agilent 1260 Method Development Solution

Importance of Alternate Selectivity Chemistries

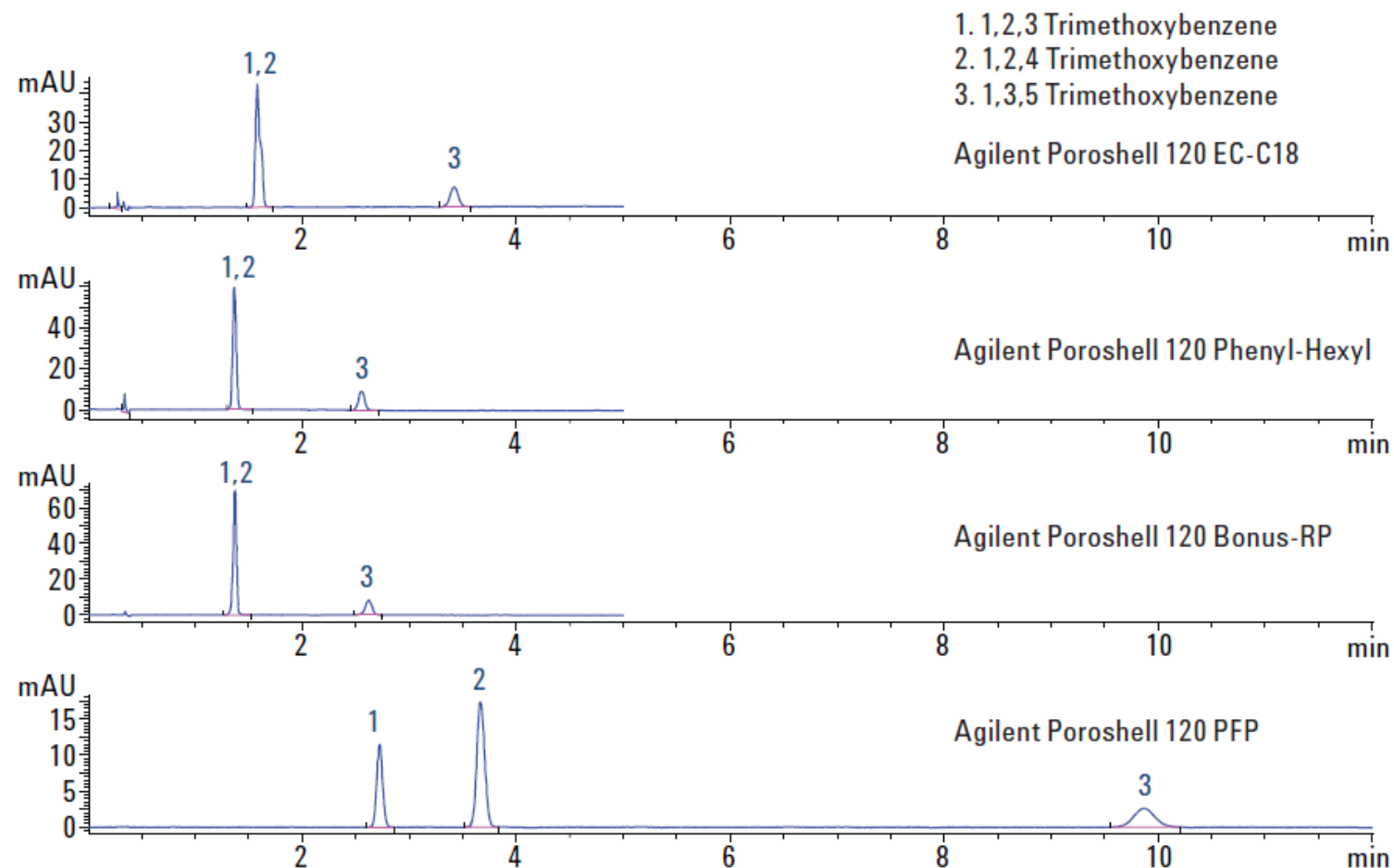


1,2,3-Trimethoxybenzene

1,2,4-Trimethoxybenzene

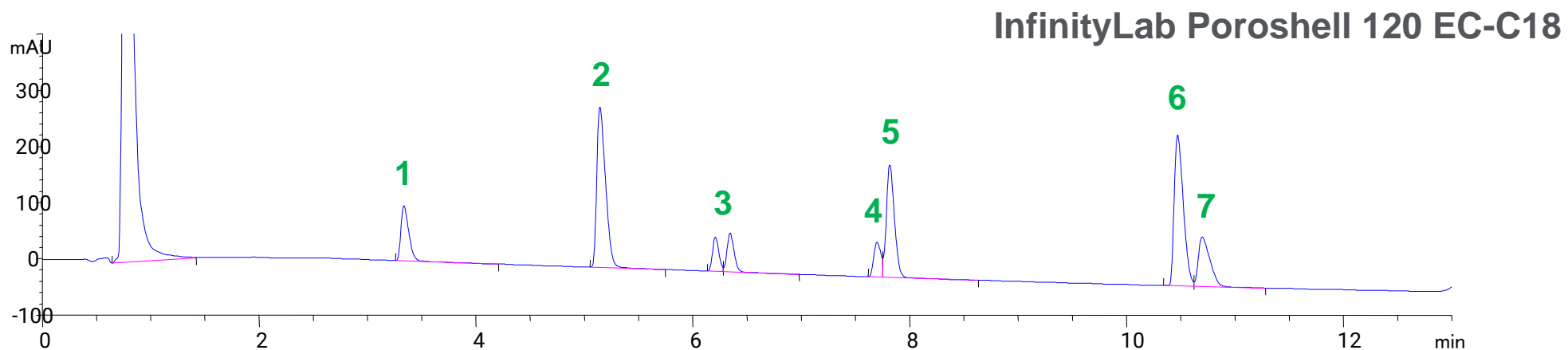
1,3,5-Trimethoxybenzene

- Three compounds
 - Same molecular weight
 - Only differ by positional location of the functionality



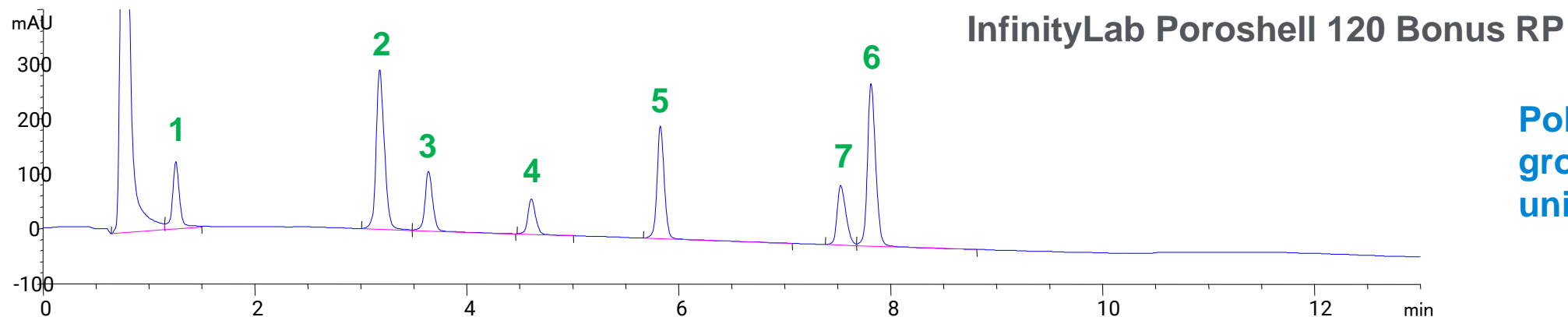
InfinityLab Poroshell 120 columns 4.6 x 50 mm, 2.7 μ m
70:30 – MeOH/H₂O, 1.5 mL/min, 40° C, 254 nm

Polar Embedded Phase for Alternate Selectivity



Beta blockers

1. Atenolol
2. Pindolol
3. Naldolol
4. Metoprolol
5. Acebutolol
6. Propranolol
7. Alprenolol

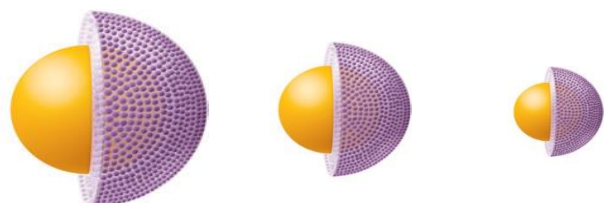


**Polar embedded
group provides
unique selectivity**

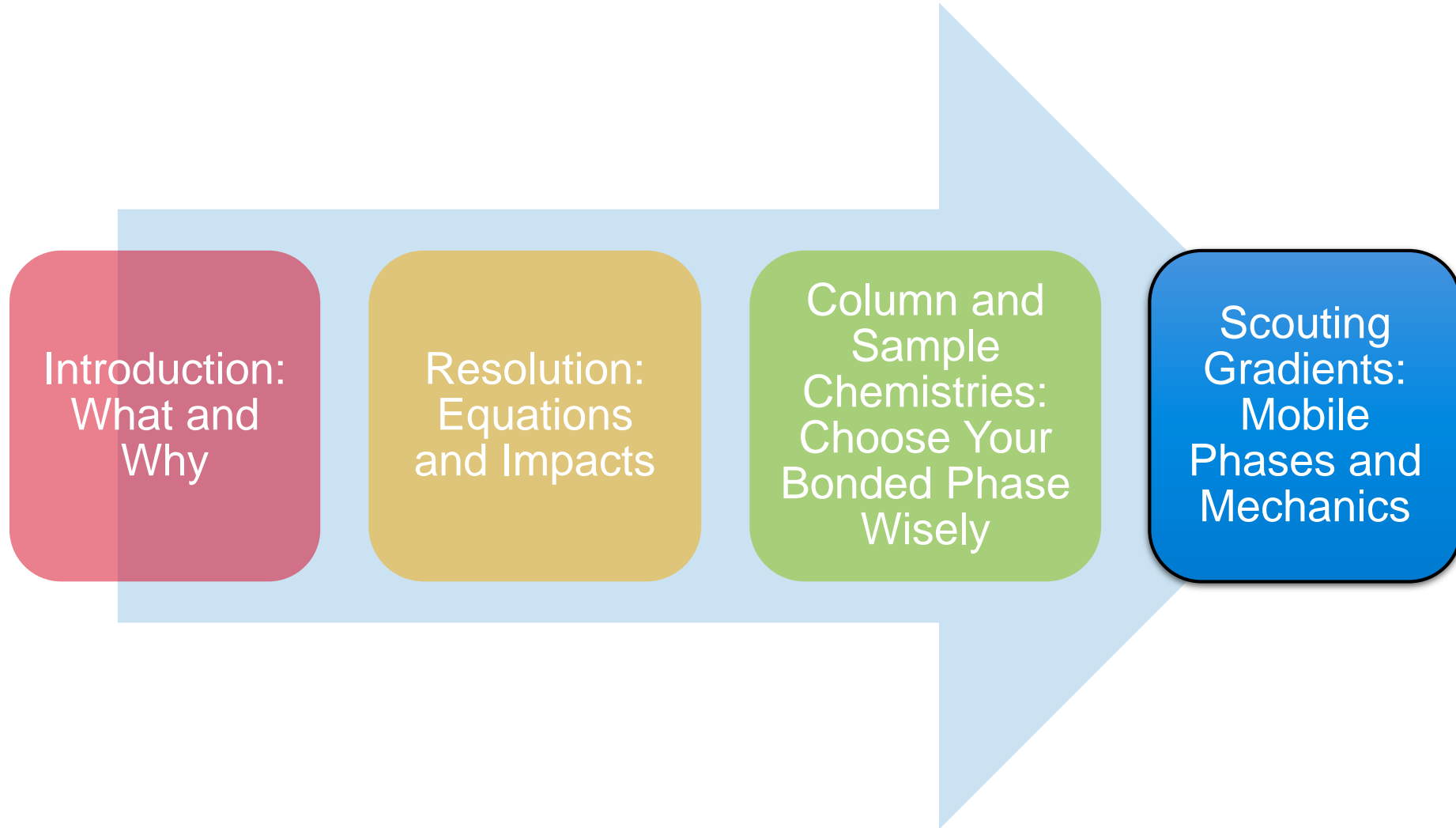
10 to 70% methanol/12 min, DAD 260 nm 0.35 mL/min, 2.1 x 100 mm 40 °C 10 mM pH 3.8 ammonium formate buffer and methanol

Agilent InfinityLab Poroshell 120 Portfolio

start here

Best All Round	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
 <p>4µm 2.7µm 1.9µm</p>			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		
Reversed-phase chemistries					

Isocratic Method Design and Development

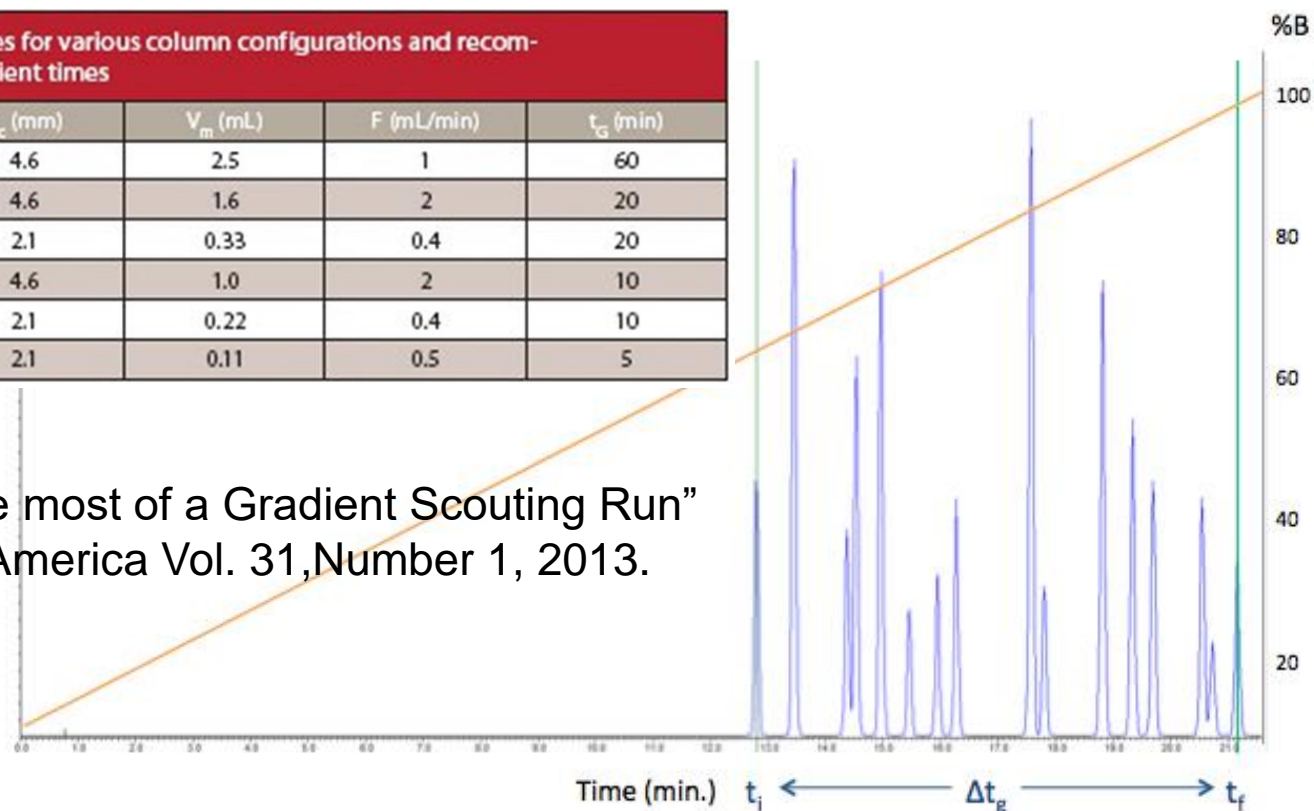


Starting Point Scouting Gradient

- A good starting point for work is a scouting gradient.
- The conditions recommended by John Dolan are 5–95% acetonitrile, low pH, and are dependent on the column length.
- Where 10 cm columns are chosen, use a 10 minute gradient.
- This example shows a 150 mm column.

Table I: Column volumes for various column configurations and recommended scouting gradient times				
L (mm)	d _c (mm)	V _m (mL)	F (mL/min)	t _G (min)
250	4.6	2.5	1	60
150	4.6	1.6	2	20
150	2.1	0.33	0.4	20
100	4.6	1.0	2	10
100	2.1	0.22	0.4	10
50	2.1	0.11	0.5	5

“Making the most of a Gradient Scouting Run”
LCGC North America Vol. 31, Number 1, 2013.



Estimating Isocratic Conditions from Our Scouting Gradient Results

For a scouting gradient on a 4.6 x 100 mm column, from the previous table. Where the run time is 10 minutes and the gradient goes from 5% to 95%. The starting percentage of organic can be estimated as:

$$\%B = 9.5(t_{avg} - t_{void}) - 2^1$$

Where:

t_{avg} is the average of the first and last retention times

t_{void} is the void time of the column

¹L.R. Snyder, J.J. Kirkland, J.W. Dolant *Introduction to Modern Liquid Chromatography*, -3rd Ed., John Wiley & Sons, 2010

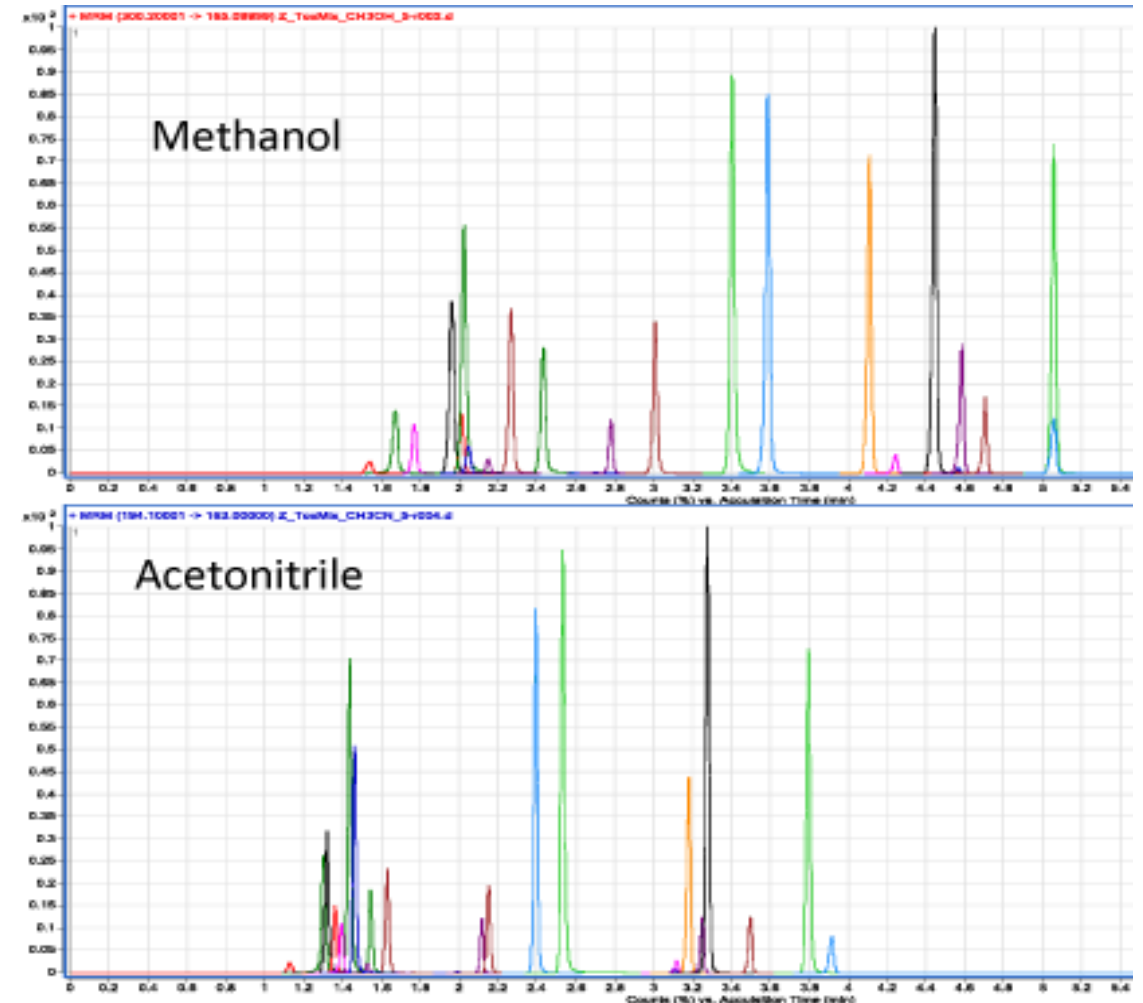
Exploring Organic Modifiers

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



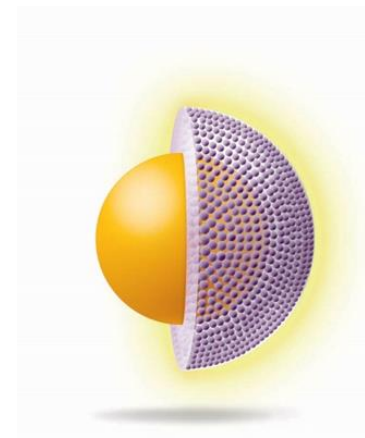
“Fast Analysis of Illicit Drug Residues on Currency using Agilent Poroshell 120”,
Anne E. Mack, James R. Evans and William J. Long, September 2010, 5990-6345EN.

pH – A Method Development Tool for Ionizable Compounds

- Ionizable compounds will be in a charged or uncharged state, based on pH.
- Choose a mobile phase pH that will help optimize retention and selectivity.
- Noncharged analytes have better retention
 - For example, acids at low pH and bases at mid or high pH
- Silanols on silica ionize at mid-pH, with possible ion-exchange interaction of basic analytes
- Ensure that your column is **compatible with and stable in the mobile phase pH you select.**

Agilent InfinityLab Poroshell HPH particles

Hybridized Poroshell 120 silica offers more rugged silica particle and enhanced stability up to pH 11



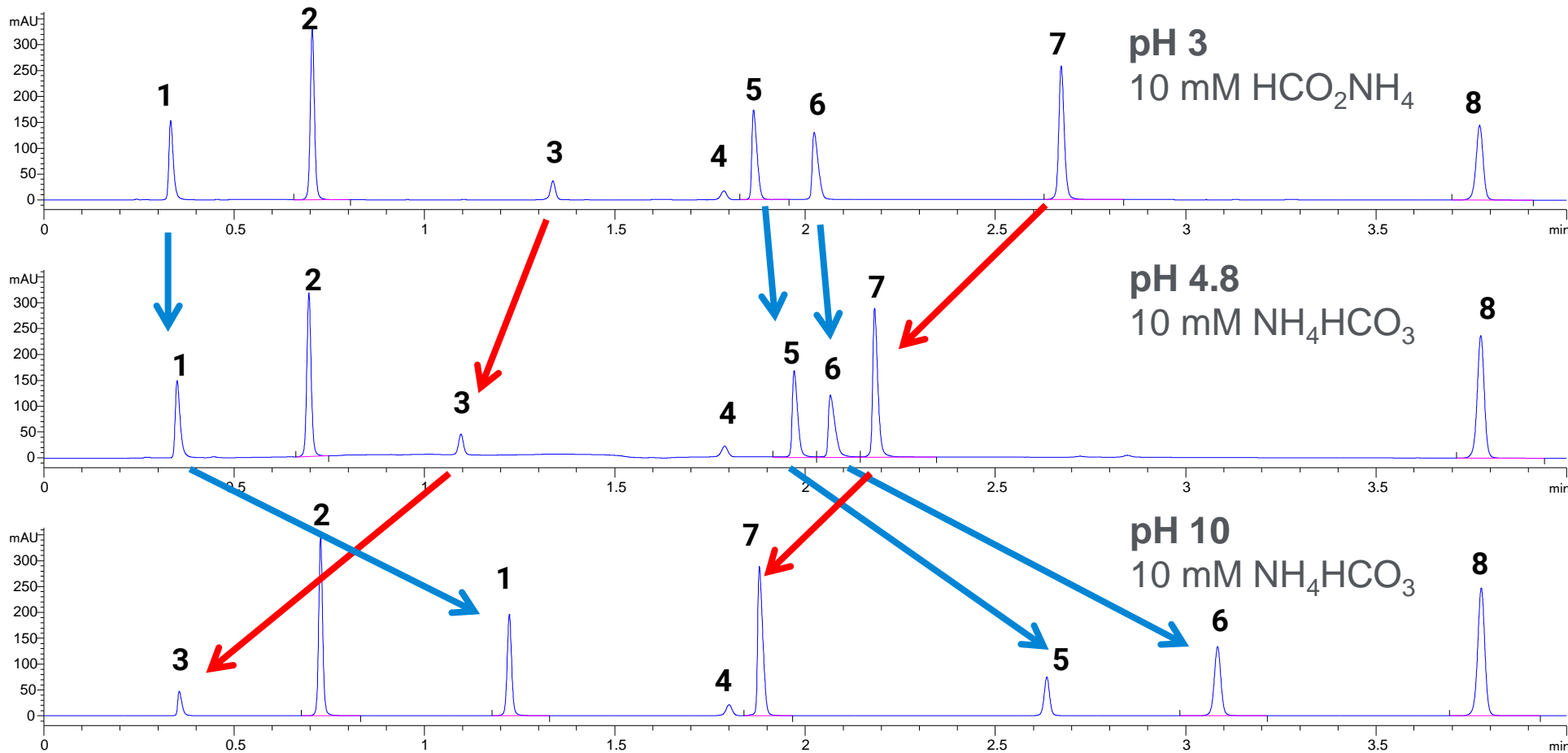
Best for High pH

InfinityLab Poroshell
HPH-C18
1.9 μm , 2.7 μm , 4 μm

InfinityLab Poroshell
HPH-C8
2.7 μm , 4 μm

Selectivity Can be Controlled by Changing pH

Agilent InfinityLab Poroshell HPH-C18 4.6 x 50 mm, 2.7 μm



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

 Acids
 Bases

Simplify Solvent Handling

- **Keep solvent concentrations constant** and solvent vapors out of the lab.
- Venting valve for mobile phase, time strip
- Safer handling, easier to grab
- Prevents tubing from twisting
- Can be used on **any** system

**InfinityLab
solvent bottle
and Stay Safe
cap**



Tips for Robust Methods

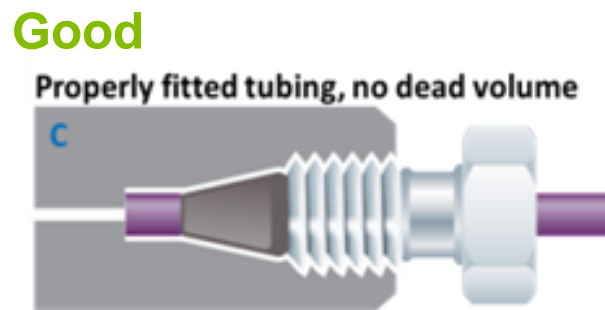
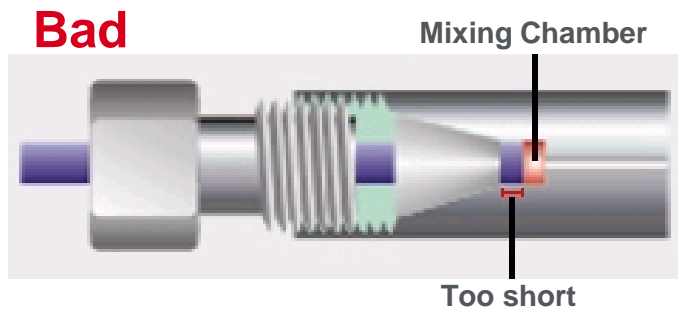
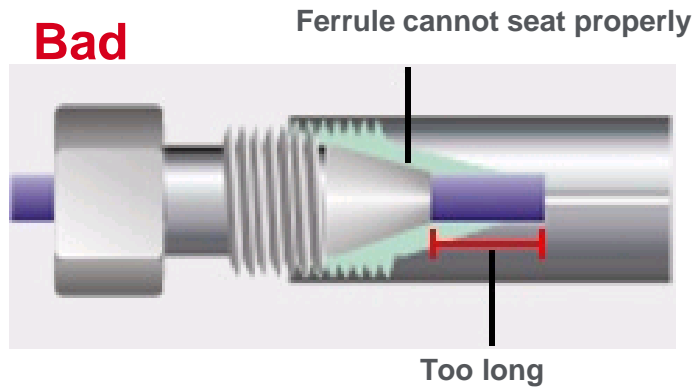
- Always start method development with a new column
- Select columns with robust properties at the pH of the method
- Choose a high-quality column with a long lifetime
- Consider batch-to-batch reproducibility
- Consider scalability of particle sizes and chemistries for downstream method transfer



Agilent employs end-to-end process control for quality LC columns

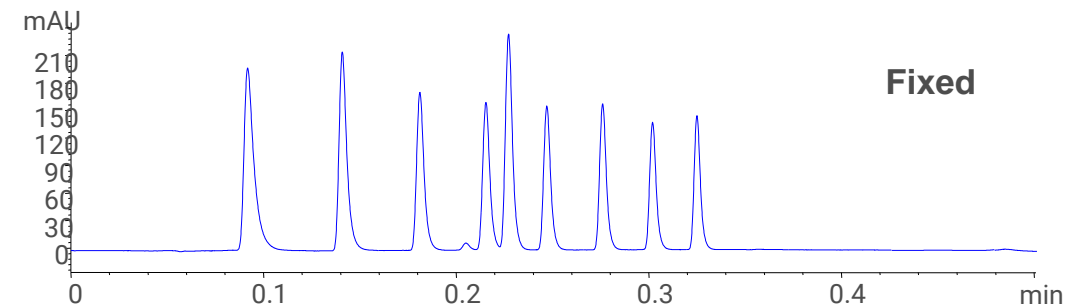
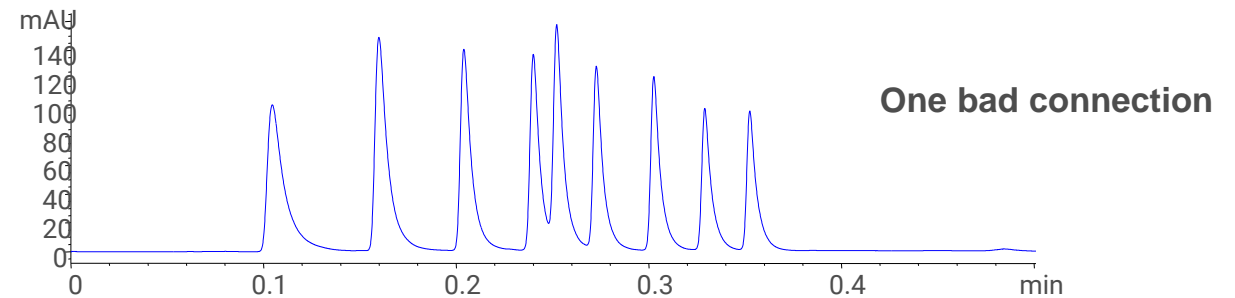
www.agilent.com/chem/qualitylc

Ensure Proper Column Connections



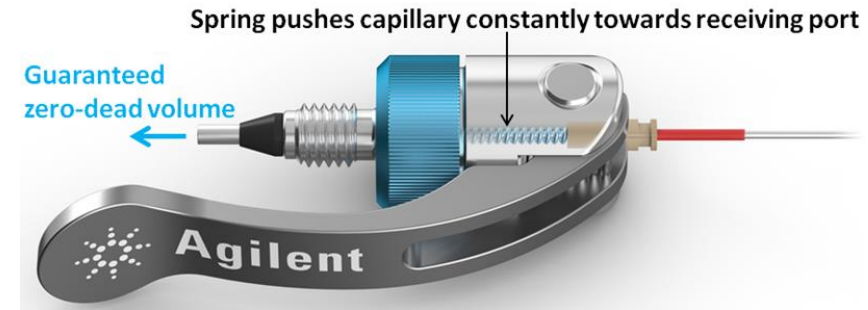
Poor fitting connections

- Will broaden or split peaks, or cause tailing
- Will typically affect all peaks, but especially early eluting peaks
- Can cause carryover



InfinityLab Quick Connect and Quick Turn Fittings

- Spring loaded design
- Easy, **no tools needed**
- Works for all column types
- Reusable
- Consistent ZDV connection



Quick Connect fitting

- Finger tight up to 1300 bar
- Hand tighten the nut, then depress the lever



Quick Turn fitting

- Finger tight up to 400 bar
- Up to 1300 bar with a wrench
- Compact design



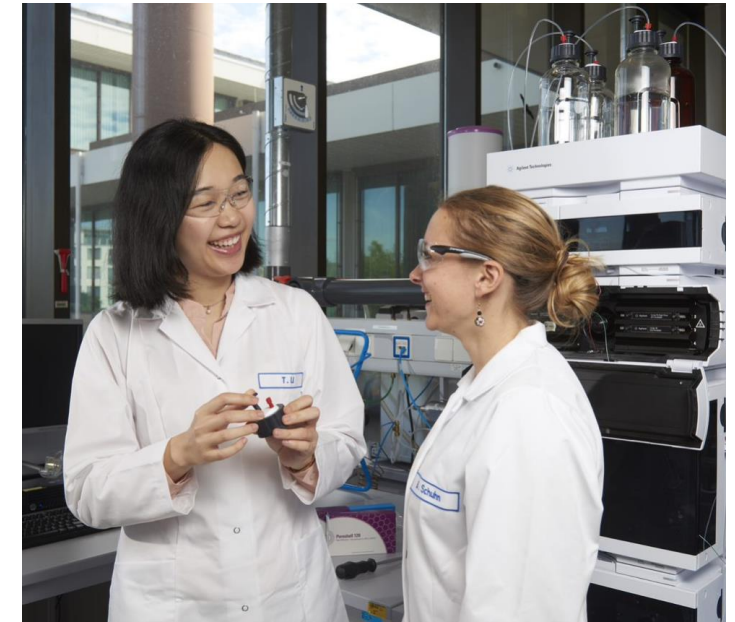
Resources for Support

- 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



Conclusions

- Resolution is a common method development goal
 - Selectivity is a main driver of resolution
- Column choices
 - Superficially porous particles speed up analysis
 - Explore alternate selectivity to increase resolution
- Method conditions
 - Consider the selectivity effects of mobile phases
 - Final tips
 - Adequately prepare samples
 - Be sure your system is optimized to maximize resolution



Contact Agilent Chemistries and Supplies Technical Support



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

Option 1 for GC and GC/MS columns and supplies

Option 2 for LC and LC/MS columns and supplies

Option 3 for sample preparation, filtration and QuEChERS

Option 4 for spectroscopy supplies

Available in the USA and Canada 8-5 all time zones



gc-column-support@Agilent.com

lc-column-support@agilent.com

spp-support@agilent.com

spectro-supplies-support@agilent.com