Resolution:

Too Much, Too Little or Just Right

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LC Columns and Consumables Technical Support
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Agilent CrossLab
From Insight to Outcome

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Topics for Discussion:

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Resolution & the Resolution Equation

- Parameters defining resolution
- Importance of these parameters

Getting the Desired Resolution

- Know your requirements
- Changing individual parameters to effect resolution

Mobile Phase & Gradient

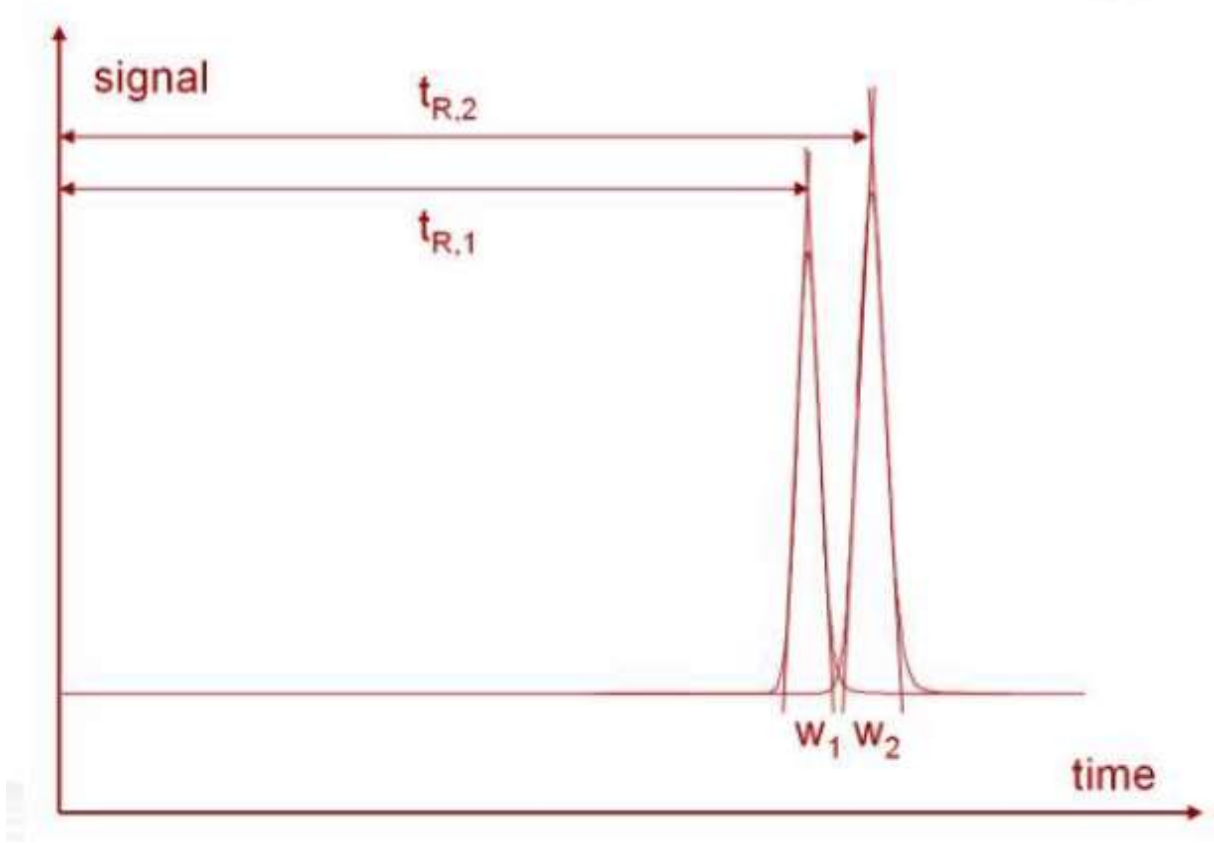
- **C**onsiderations for mobile phase, i.e. pH
  Choice of organic
- Gradient elution and the resolution relationship

Role of the Instrument

- Delay volume
- Dispersion
- Column Temperature
- Detector Flow Cell Volume
- Data Collection Rate and Importance

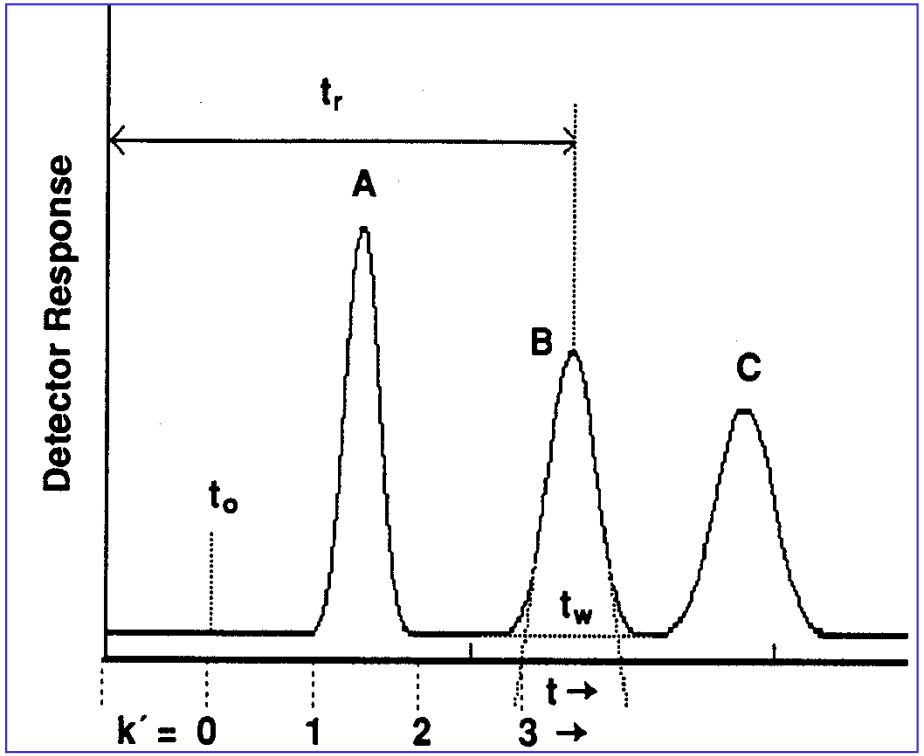
Resolution Definition

Resolution is a measure of the ability to separate two components



Basic Chromatography Parameters

Equations Describing Factors Controlling R_S



Retention Factor

$$k = \frac{(t_R - t_0)}{t_0}$$

Selectivity or Separation Factor

$$\alpha = k_2/k_1$$

Theoretical Plates-Efficiency

$$N = 16(t_R / t_W)^2$$

$$R_s = \frac{t_{R-2} - t_{R-1}}{(w_2 + w_1)/2} = \frac{\Delta t_R}{\bar{w}}$$

Resolution Equation

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The Fundamental Resolution Equation

$$R_S = \frac{\sqrt{N}}{4}\,\frac{(\alpha-1)}{\alpha}\,\frac{k}{(k+1)} = \frac{\Delta t_R}{\overline{w}}$$

Resolution …
Determined by 3 Key Parameters –
Efficiency, Selectivity and Retention

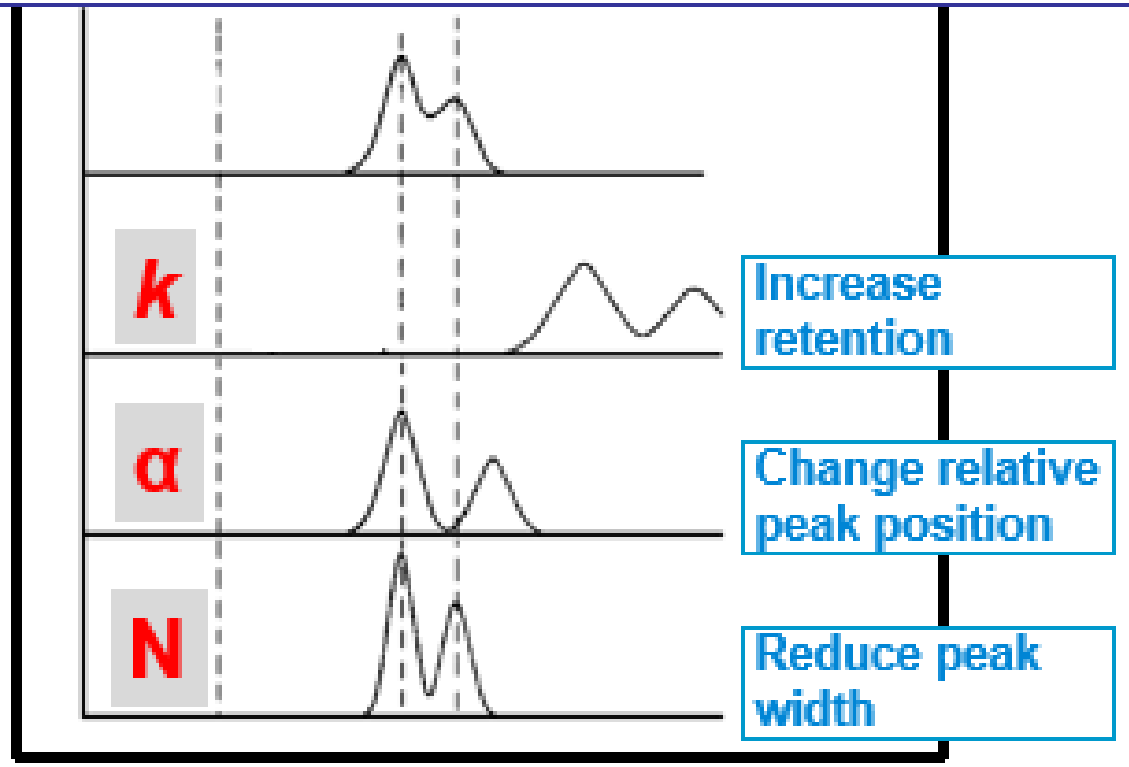
N = Column Efficiency – Column length and particle size

α = Selectivity – Mobile phase and stationary phase

***k* = Retention Factor** – Mobile phase strength

Factors that Improve Resolution

$$R_S = \frac{\sqrt{N}}{4}\,\frac{(\alpha-1)}{\alpha}\,\frac{k}{(k+1)} = \frac{\Delta t_R}{\overline{w}}$$



Resolution (R_s) & Parameters Affecting 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

Retention Factor (*k*):

Describes how well an analyte is retained by the stationary phase.

This is expressed as a ratio of column volumes.

This can be adjusted by making changes to the organic strength of the mobile phase

Resolution (R_s) & Parameters Affecting 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

Column Efficiency as Theoretical Plates (N)

As the number of plates increase, peaks become thinner and sharper, which improves resolution.

Plates are often described by their height (H), or Height Equivalent to the Theoretical Plate (HETP)

Number of plates and plate height are inversely proportional, i.e. H = L/N

Resolution (R_s) & Parameters Affecting Resolution

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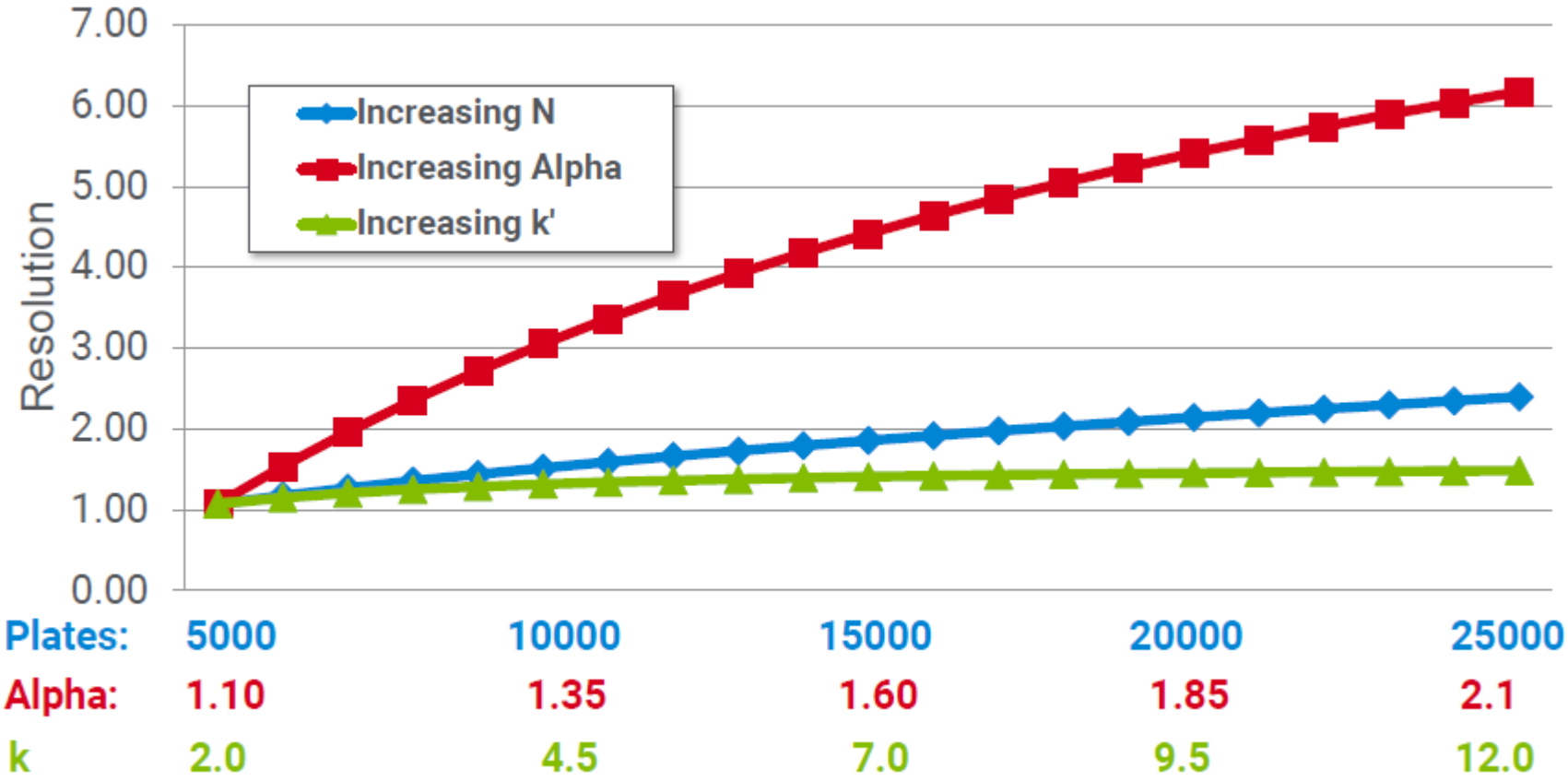
$$R_s = \left(\frac{1}{4}\right) N^{0.5} \left(\frac{\alpha - 1}{\alpha}\right) \left(\frac{k}{1 + k}\right)$$

Resolution Efficiency Selectivity Retention

Selectivity or Separation Factor (α)

- This is the ratio of retention factors for two adjacent peaks.
- Larger α values indicate better separation.
- Selectivity can be adjusted by changes to either the mobile phase or the stationary phase.

Factors That Effect Resolution

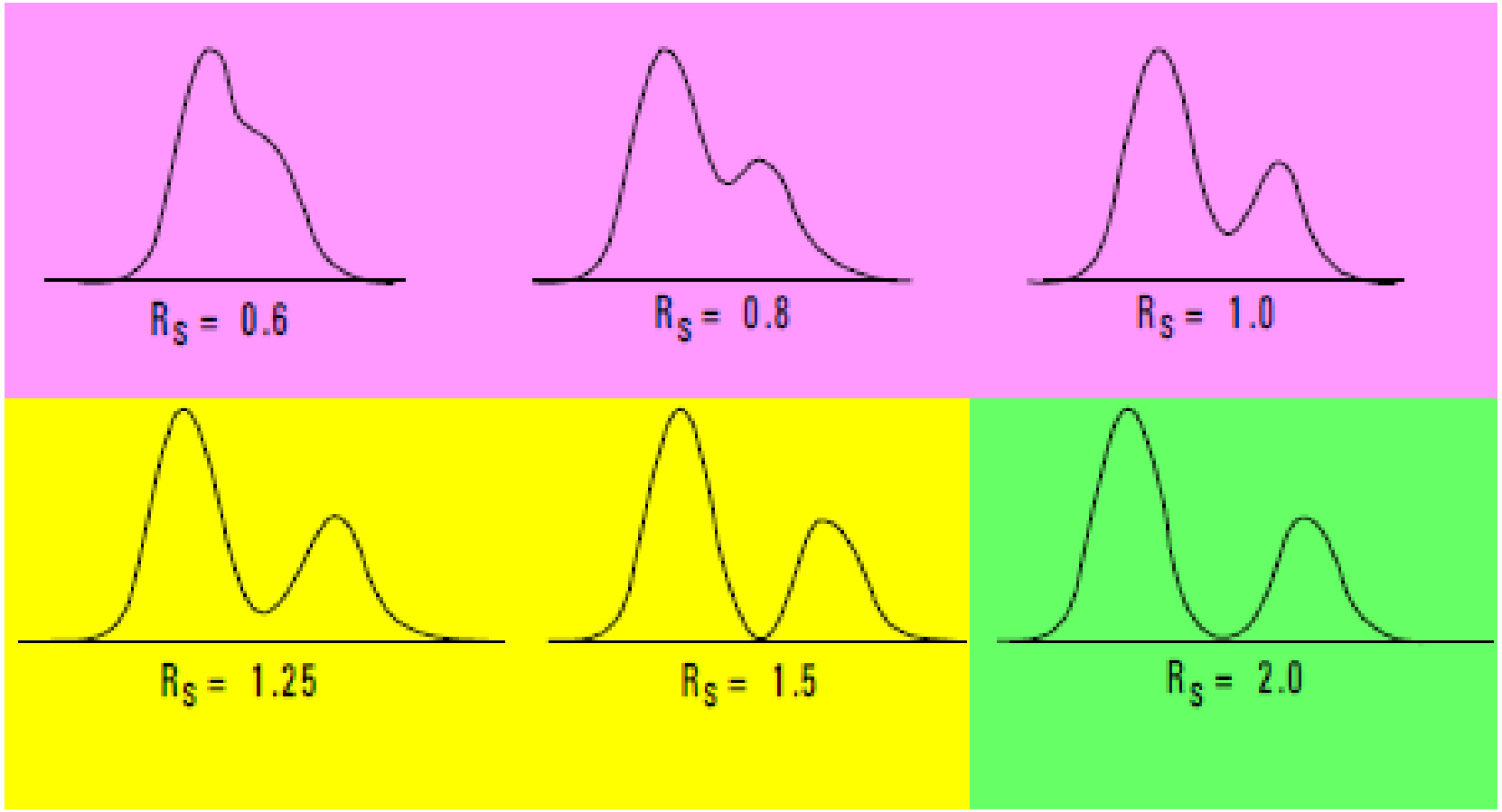


Selectivity impacts resolution the most

- Change bonded phase
- Change mobile phase

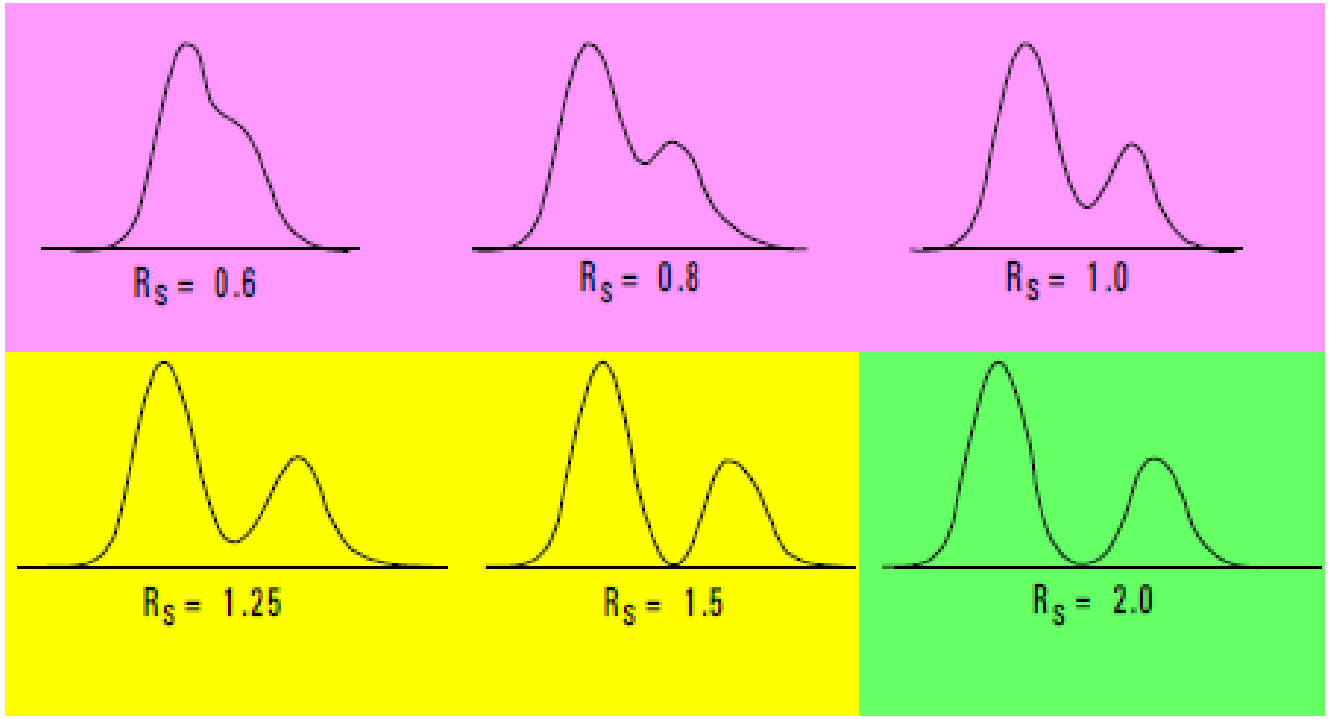
} **Typical Analytical Method Development Parameters**

Getting the Desired Resolution:
Resolution is a Key Goal in Chromatographic Separations



BUT how much is necessary?

How Much Resolution is Necessary?



Aim for $R_s \geq 2.0$ between all analytes

Baseline Resolution $R_s = 1.5$

- Insufficient R_s 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

Increase Resolution with no run time increase

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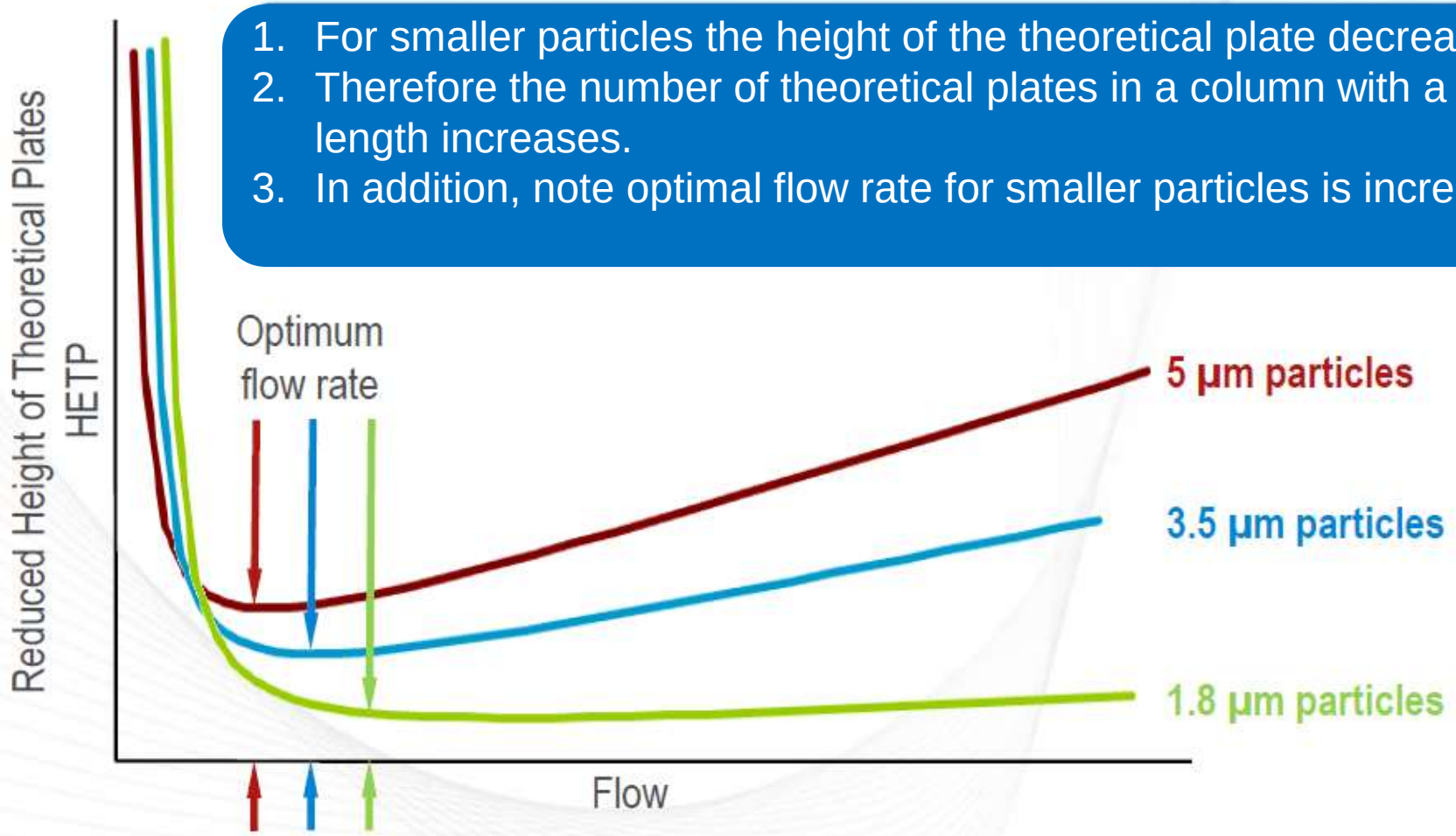
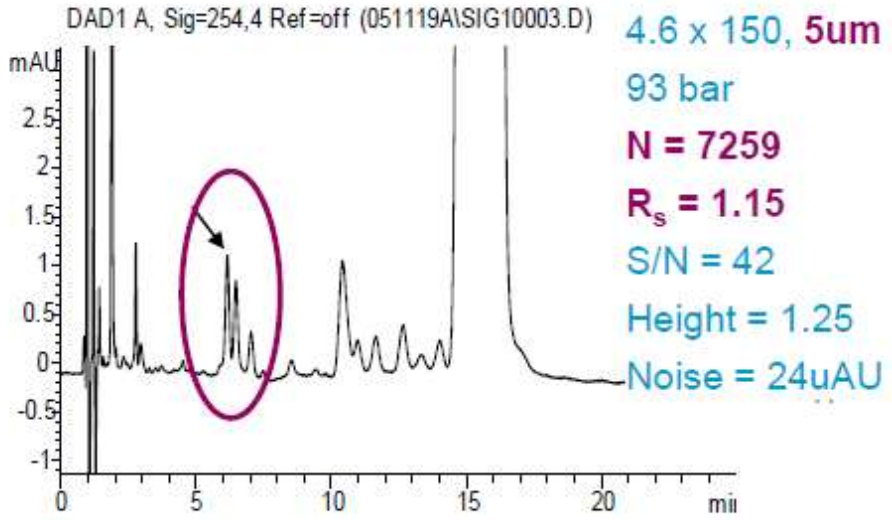
Column with higher number of theoretical plates required

1. For smaller particles the height of the theoretical plate decreases.
2. Therefore the number of theoretical plates in a column with a given length increases.
3. In addition, note optimal flow rate for smaller particles is increased.

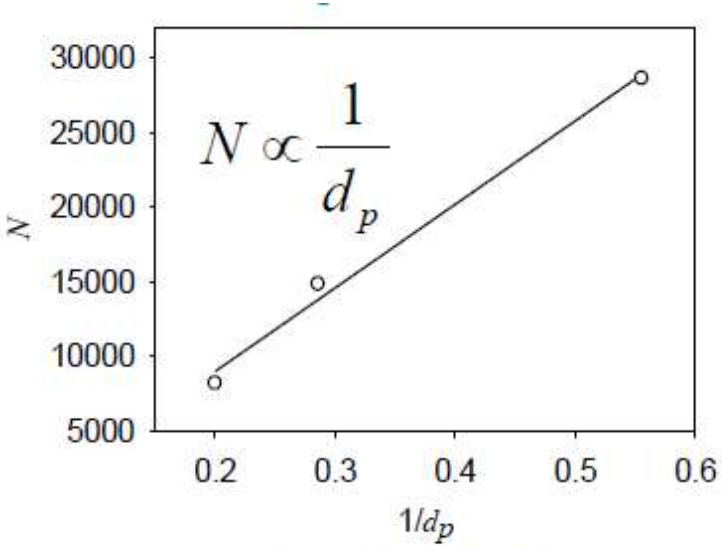


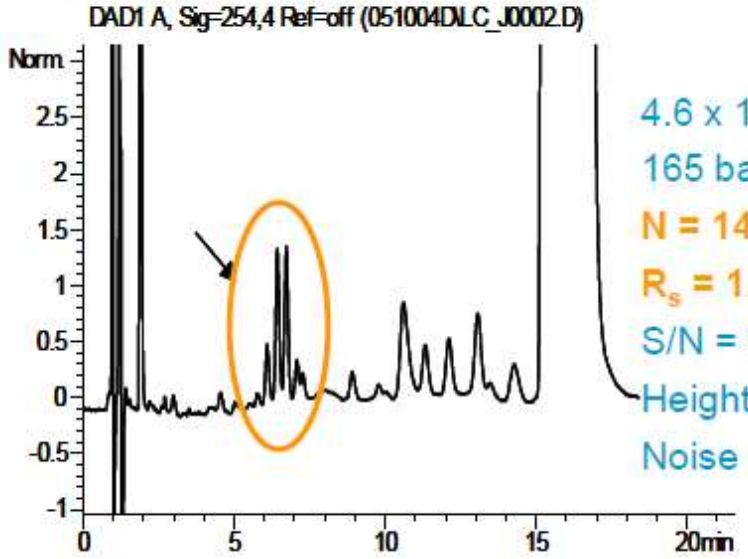
Reduce Particle Size & Maintain column length

Increased ‘N” in an isocratic separation – improved resolution

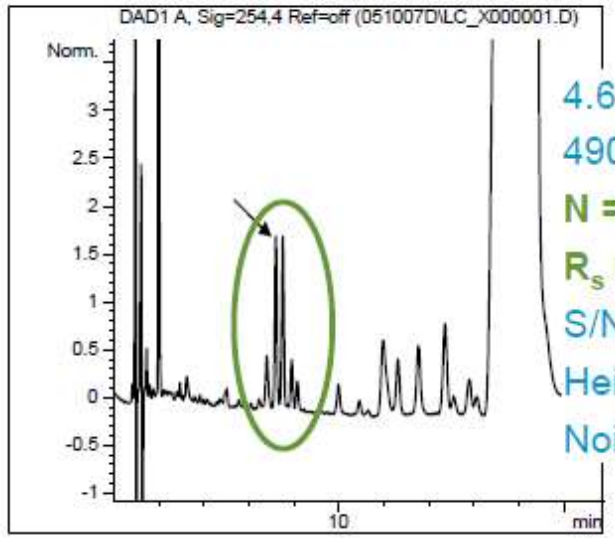


4.6 x 150, **5um**
93 bar
N = 7259
R_s = 1.15
S/N = 42
Height = 1.25
Noise = 24uAU





4.6 x 150, **3.5um**
165 bar
N = 14862
R_s = 1.37
S/N = 50
Height = 1.34
Noise = 20uAU



4.6 x 150, **1.8um**
490 bar
N = 28669
R_s = 1.80 (+57%)
S/N = 44
Height = 1.80
Noise = 30uAU

Maintaining Resolution

Reduce column length AND Particle size

Column Length (mm)	Column Efficiency N(5 µm)	Column Efficiency N(3.5 µm)	Column Efficiency N(1.8 µm)	Analysis Time*
150	12,500	21,000	35,000	-
100	8,500	14,000	23,250	-33%
75	6000	10,500	17,500	-50%
50	4,200	7,000	12,000	-67%
30	N.A.	4,200	6,500	-80%
15	N.A.	2,100	2,500	-90%

Efficiency (N) ↑ / Pressure ↓

Analysis Time ↓

Peak Volume ↓

Solvent Usage ↓

- **Shorter columns with small particles provide the efficiency of longer columns with larger particles**

Selectivity and Column Choice

Evaluate Different Bonded Phases

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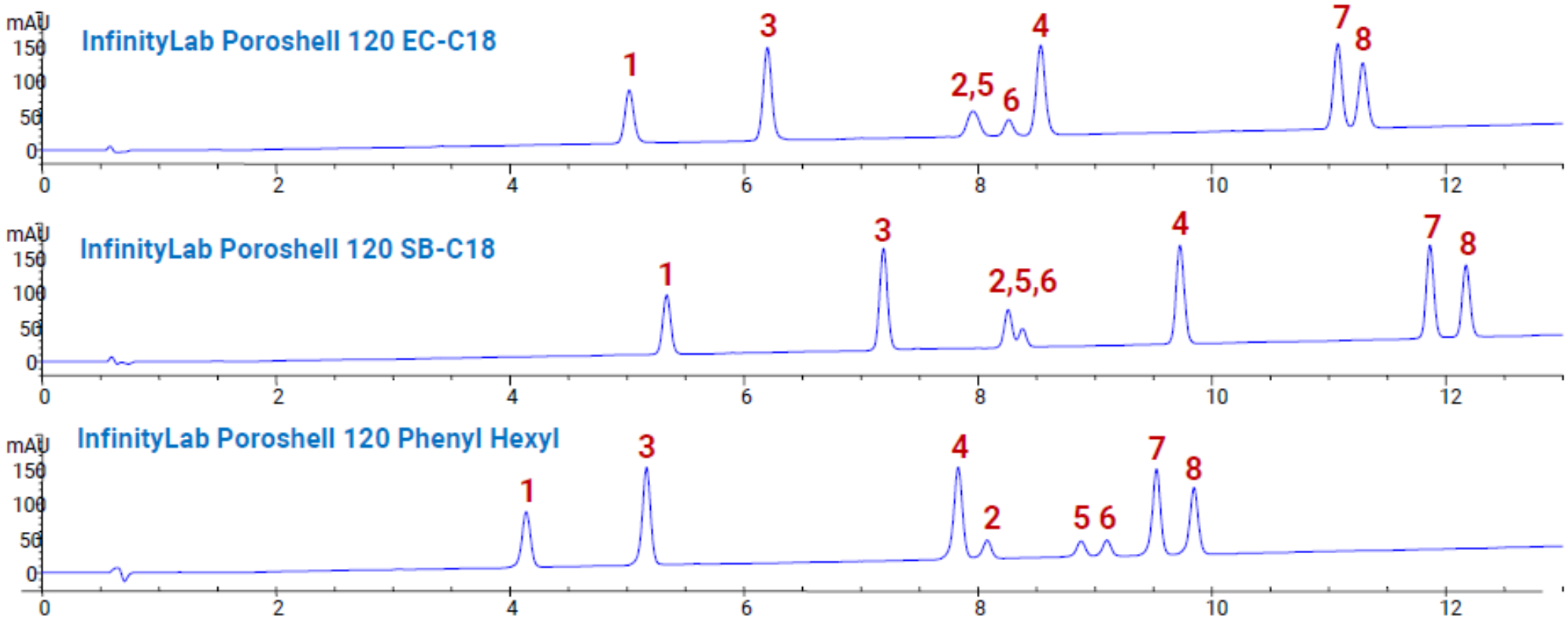
- Bonded phase affects selectivity (alpha)
- Different interactions for polar and non-polar compounds.
- Exploit other interactions with bonded phase (e.g., pi-pi)
- Changing the bonded phase can improve selectivity/resolution, reduce analysis time
- Having different bonded phases available on the same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

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

Differences in Selectivity

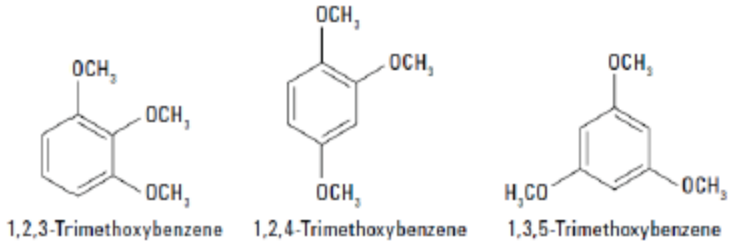
Agilent InfinityLab Poroshell Bonded Phases



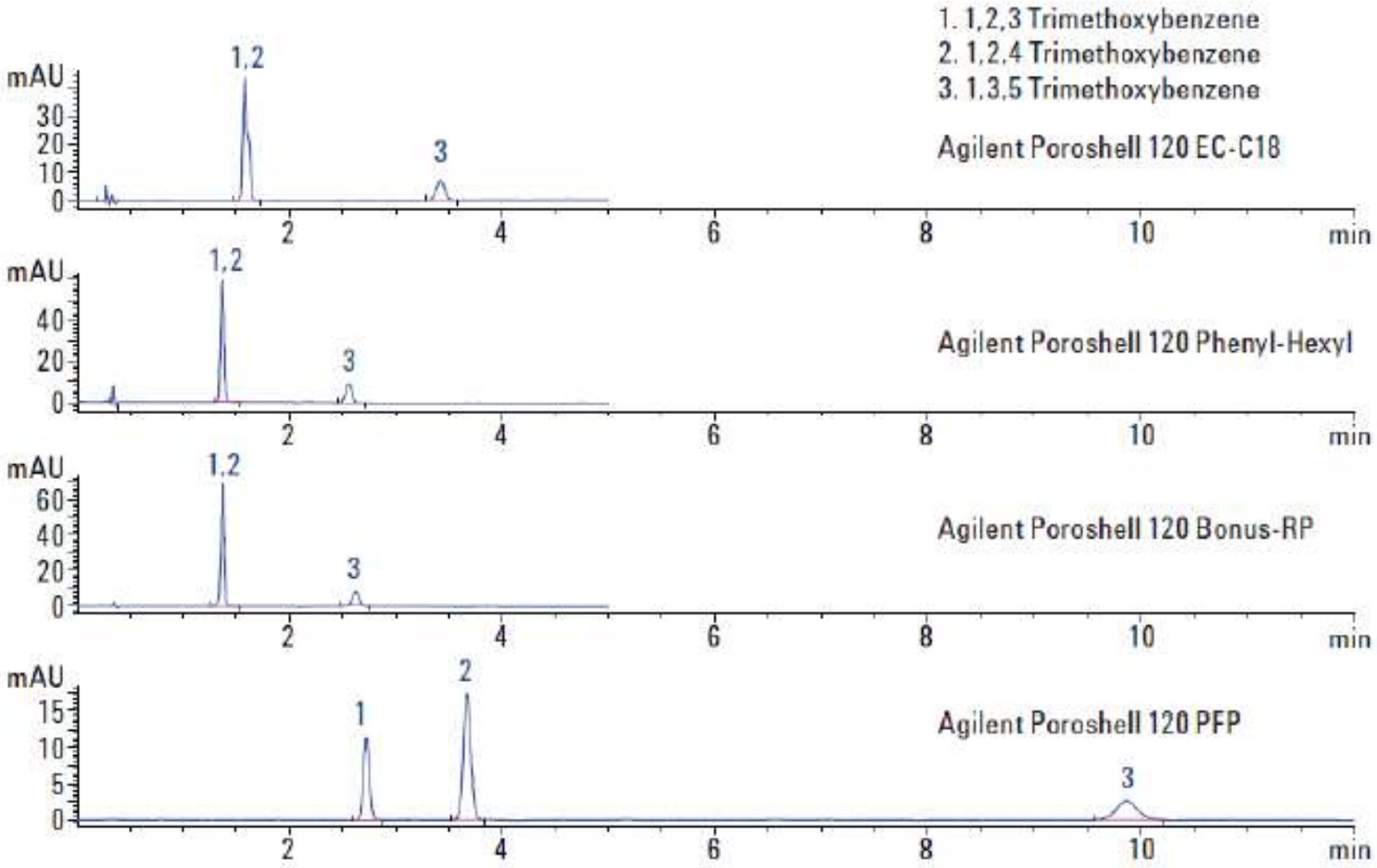
1. Hydrocortisone 2. B Estradiole, 3. Andostadiene 3. 17 dione, 4. Testosterone
5. Ethyestradione 6. Estrone 7. Norethindone acetate 8. Progestreone

40-80 % 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



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



Agilent InfinityLab Poroshell 120 columns 4.5 x 40 mm, 2.7µm
70/30 – MeOH/H2O, 1.5mL/min, 40C, 254nm

Polar Embedded Phase for Alternate Selectivity:

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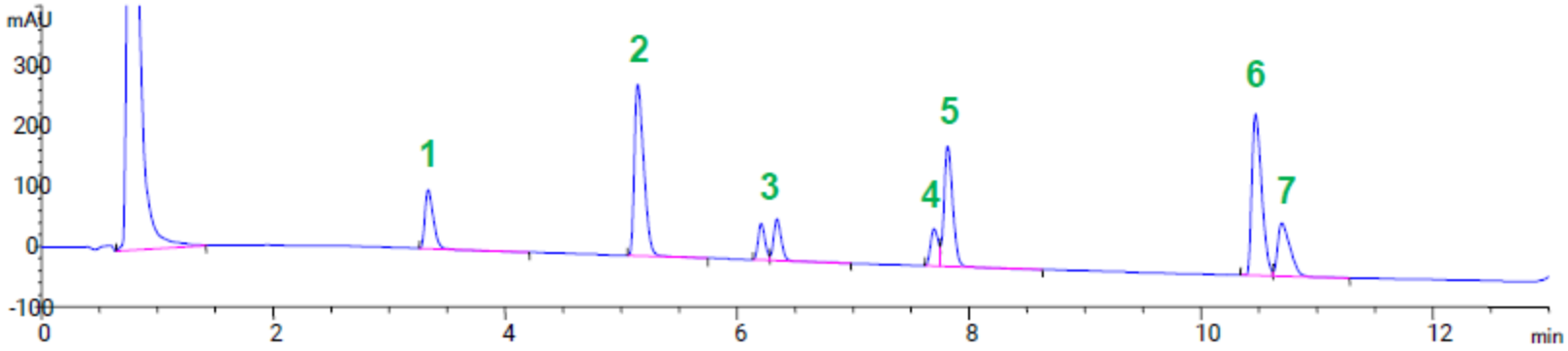
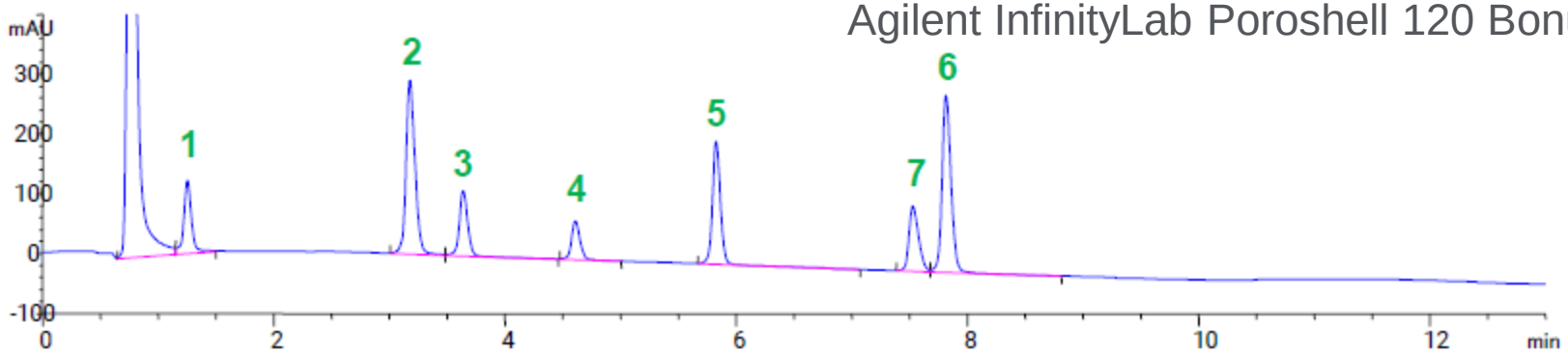
Agilent InfinityLab Poroshell 120 EC-C18



Beta Blockers

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

Agilent InfinityLab Poroshell 120 Bonus RP



Polar embedded group provides unique selectivity

10-70 % 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

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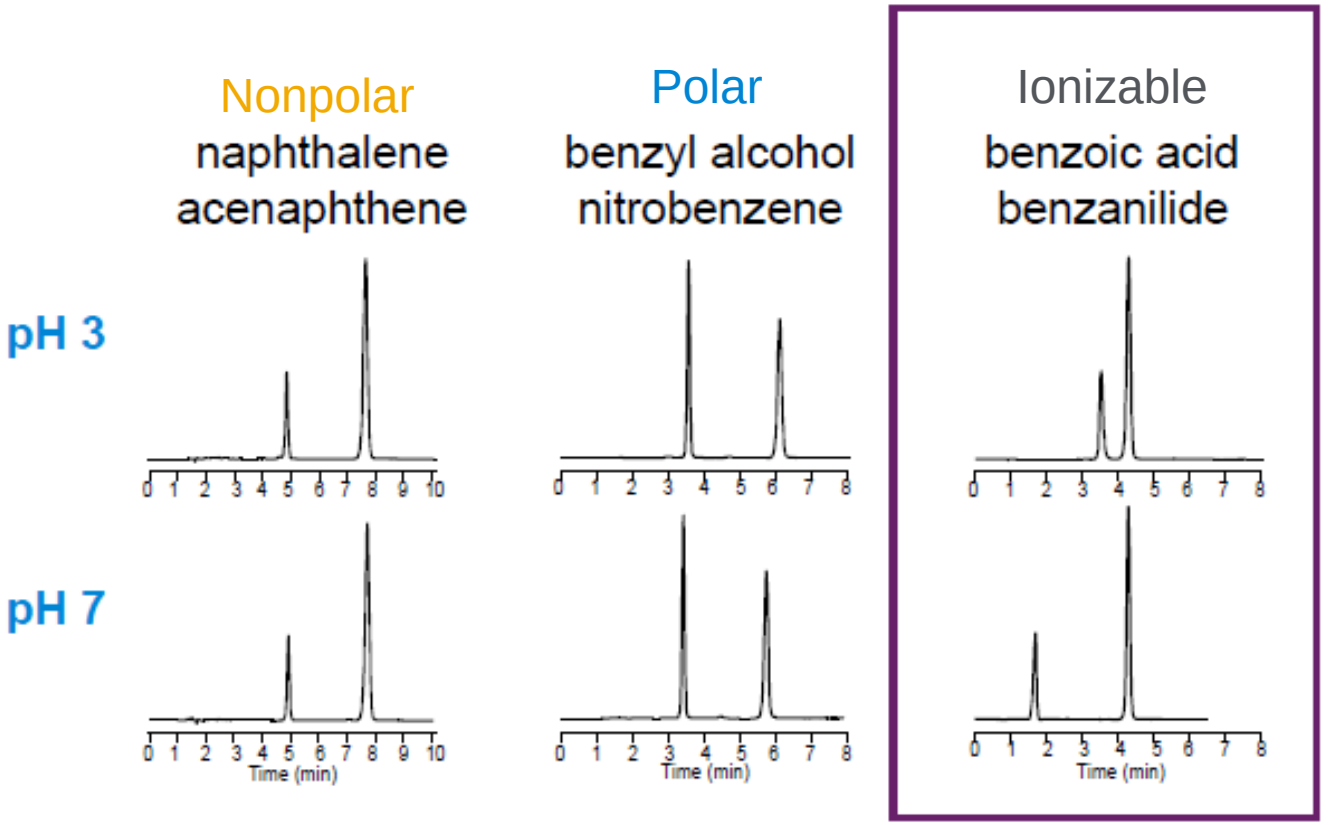
Best all around	Best for low pH mobile phases	Best for high pH mobile phases	Best for alternative selectivity	Best for polar Analytes	Best for Chiral
InfinityLab Poroshell 120 **EC-C18** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell 120 **SB-C18** 2.7 µm	InfinityLab Poroshell **HPH-C18** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell 120 **Bonus-RP** 2.7 µm	InfinityLab Poroshell 120 **HILIC** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell 120 **Chiral-V** 2.7 µm
InfinityLab Poroshell 120 **EC-C8** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell 120 **SB-C8** 2.7 µm	InfinityLab Poroshell **HPH-C8** 2.7 µm, 4 µm	InfinityLab Poroshell 120 **PFP** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell 120 **HILIC-Z** 2.7 µm	InfinityLab Poroshell 120 **Chiral-T** 2.7 µm
			InfinityLab Poroshell 120 **Phenyl-Hexyl** 1.9 µm, 2.7 µm, 4 µm	InfinityLab Poroshell 120 **HILIC-OH5** 2.7 µm	InfinityLab Poroshell 120 **Chiral-CD** 2.7 µm
			InfinityLab Poroshell 120 **SB-Aq** 2.7 µm		InfinityLab Poroshell 120 **Chiral-CF** 2.7 µm
			InfinityLab Poroshell 120 **EC-CN** 2.7 µm		

Choice of 18 chemistries

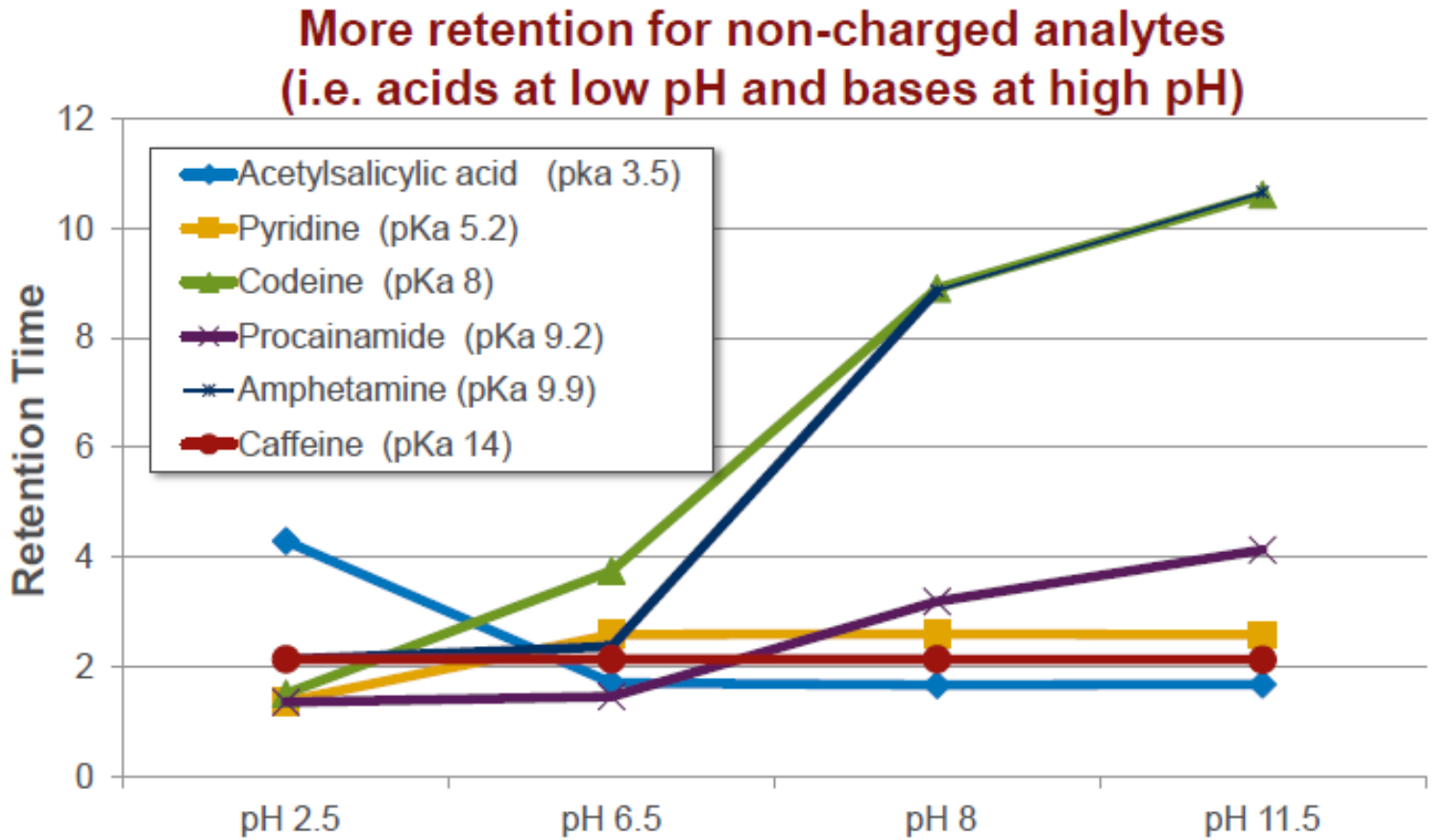
When does pH Affect Selectivity and Resolution?

Example of Compound Type Comparison



If an ionizable compound, acids and bases can change retention and selectivity with changes in pH

Change in Retention with pH for Ionizable Compounds Is Compound Dependent



Agilent InfinityLab Poroshell Column
HPH C18, 2.7um

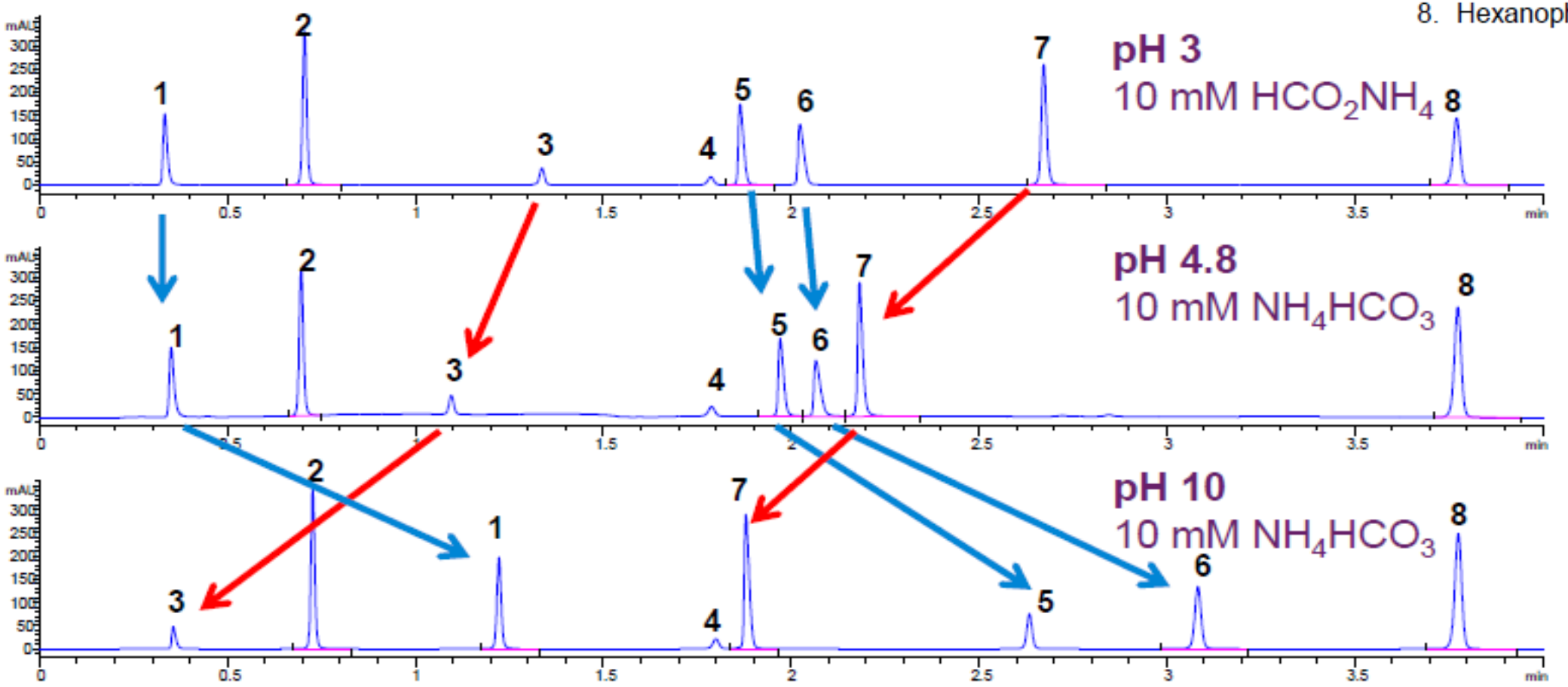
Mobile Phase: 45% Methanol, 55% 20 mM Phosphate Buffer

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 nm

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



Change in Retention w/pH for Ionizable Compounds is Key to Method Development

- Non-charged analytes have better retention (i.e. acids at low pH and bases at high pH)
- Silanols on silica ionize at mid-pH, increasing retention of basic analytes (i.e possible ion-exchange interactions)
- Choose mobile phase pH to optimize retention and selectivity during method development
- Ensure that your column is compatible with and stable in the mobile phase pH you select

Mobile Phase & Gradients
Evaluate Organic Modifiers

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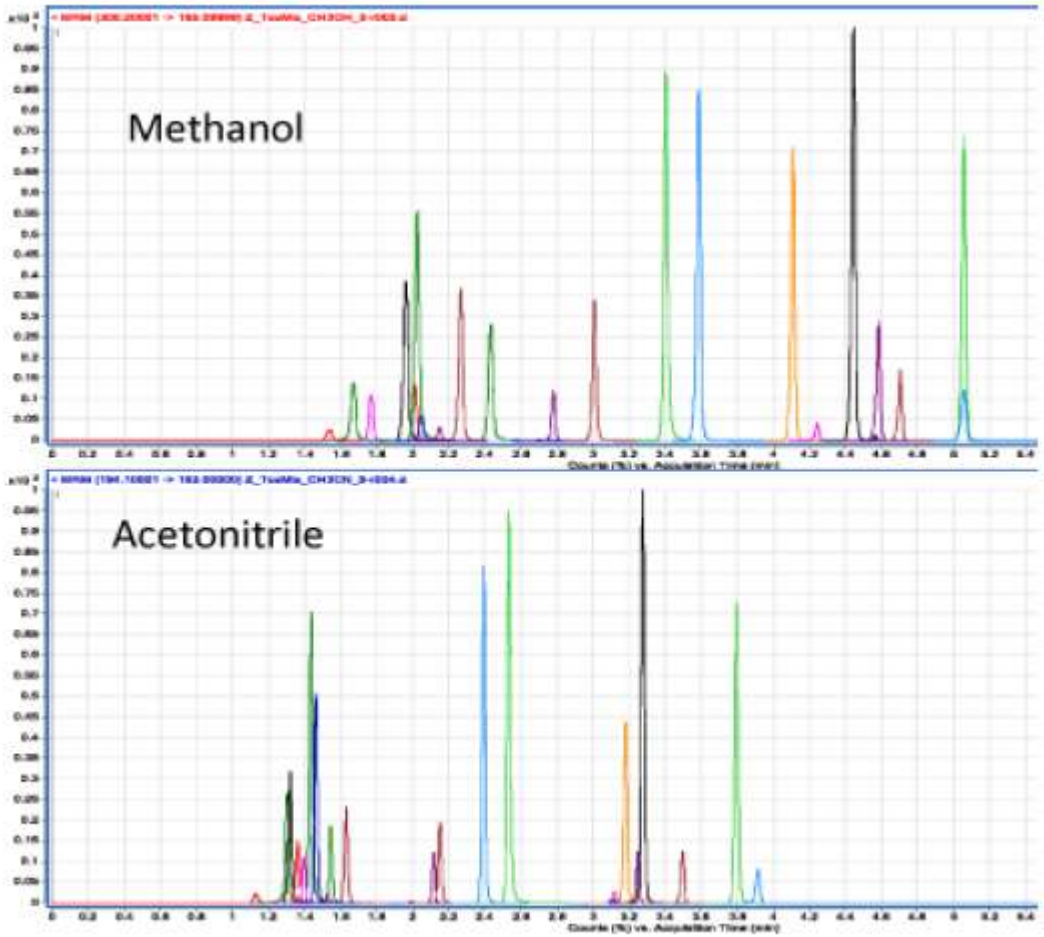
MeOH – higher pressure, generally better peak shape with bases, protic solvent

Acetonitrile – lower pressure, wider UV window, stronger than MeOH

WHY?

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



"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.

Further Optimization of Organic to Improve Resolution

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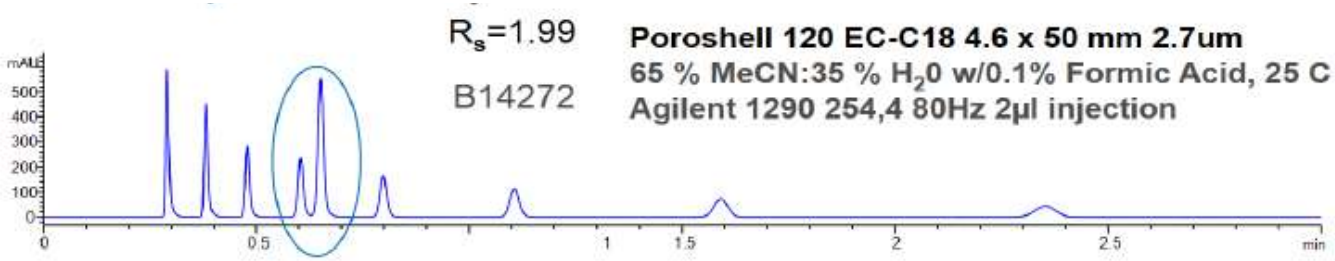
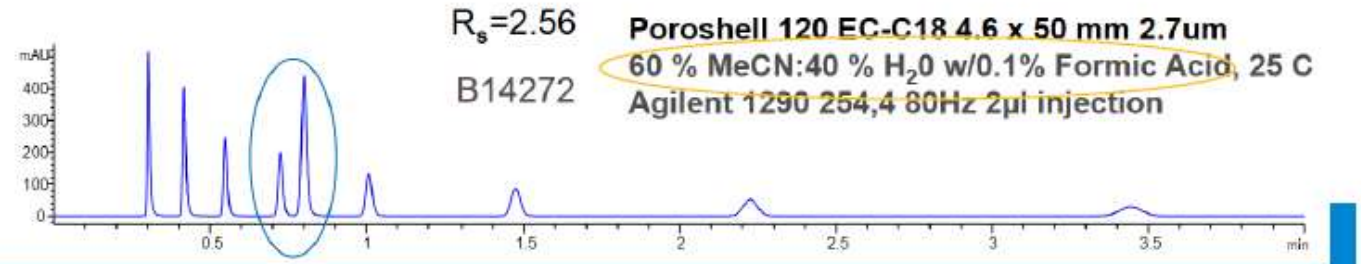
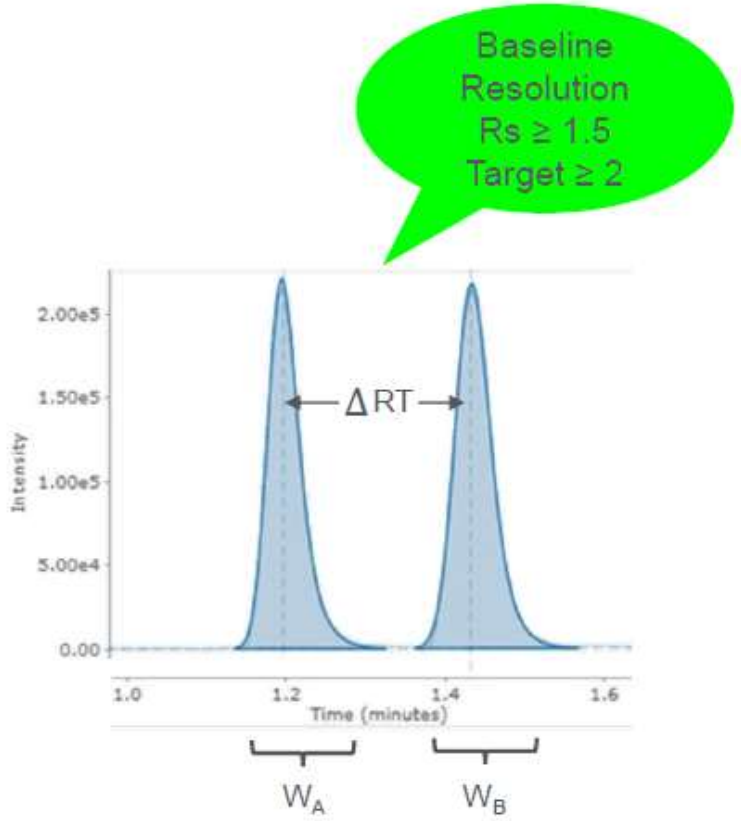
Method tested for reproducibility for resolution



Slight Change to Method can give more reproducible results





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

Resolution Relationship for Gradient Elution

$$R \approx \frac{\sqrt{N}}{4} \alpha \; k^*$$

k* - represents the fact that k changes constantly during a gradient

Gradient Retention

$$k^* = \frac{t_g F}{S\,(\Delta\%B)\,V_m}$$

- $\Delta\%B$ = difference between initial and final % B values
- S = constant (≈ 4 for 100 - 500 Da)
- F = flow rate (ml/min.)
- t_g = gradient time (min.)
- V_m = column void volume (ml)

This Relationship says that to Keep Relative Peak Position in the Chromatogram unchanged..

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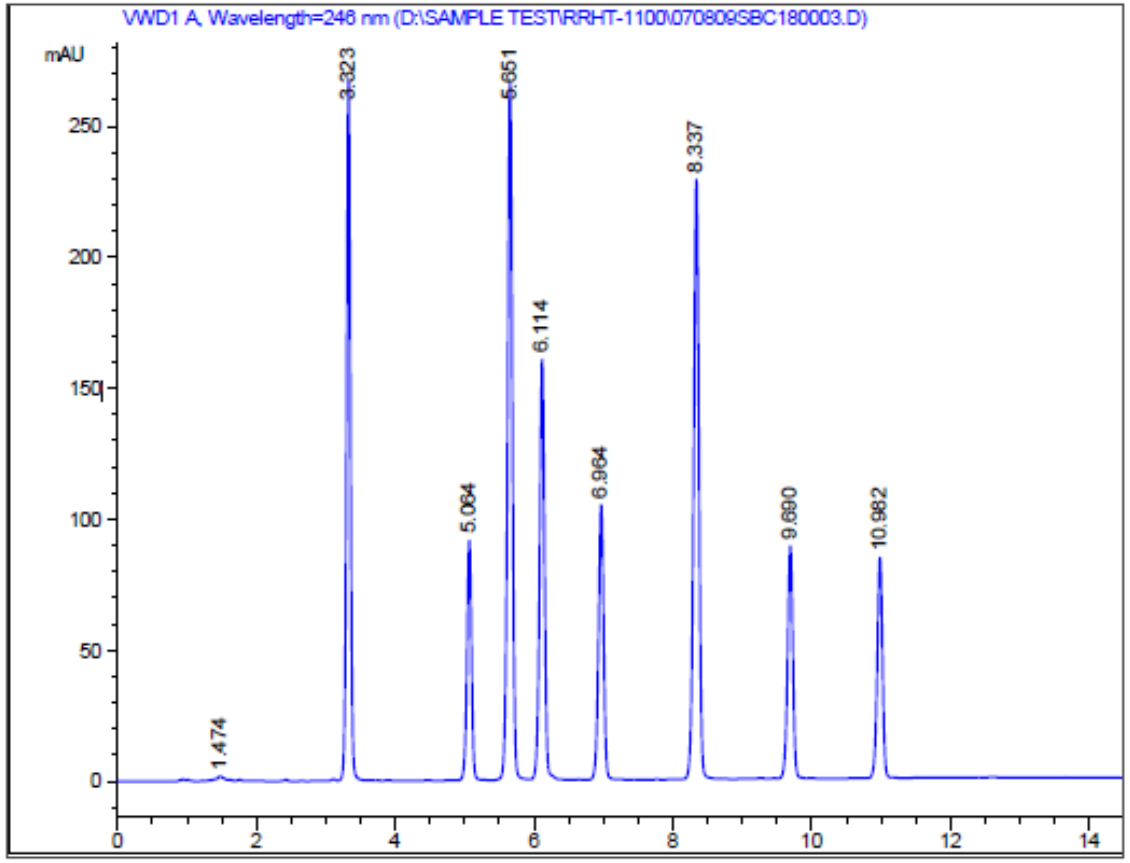
Any Decrease in	Can be Offset by a Proportional
• Column length	• Decrease in t_G or F • Increase in $\Delta\Phi$
• Column volume (i.d.)	• Decrease in t_G or **F** • Increase in $\Delta\Phi$
• $\Delta\Phi$ (same column)	• Decrease in t_G or F

$$k^* = \frac{t_G \bullet F}{S \bullet \Delta\Phi \bullet Vm}$$

Conventional Column – 4.6 x 150mm, 5µm SB-C18

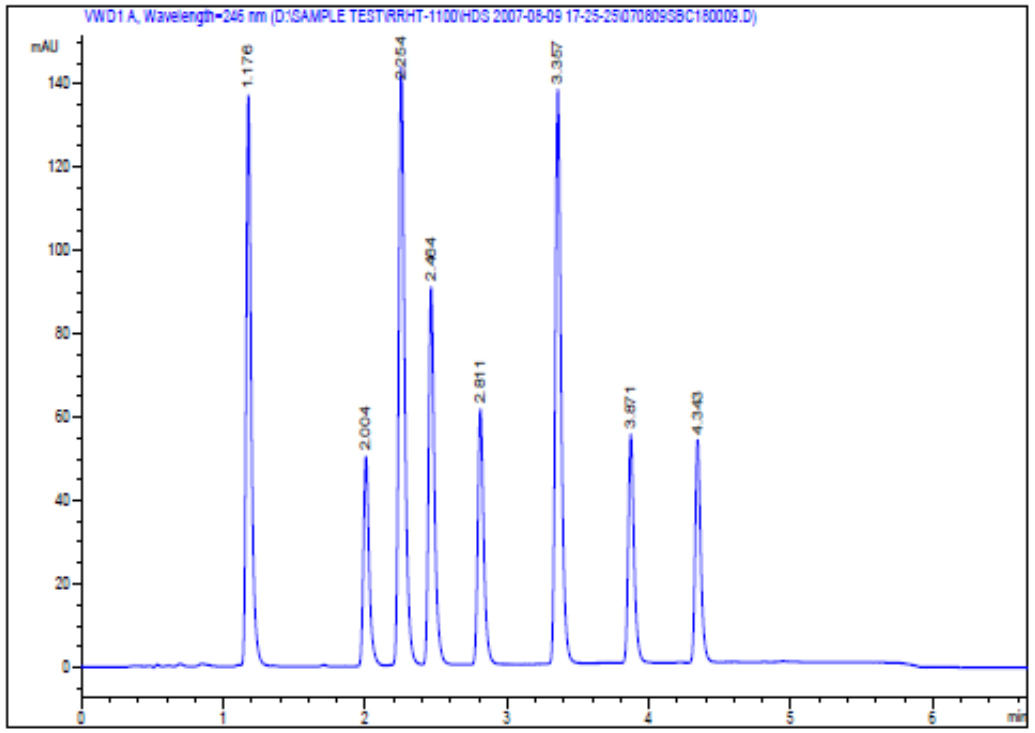


Flow Rate	1.0 ml/min
Injection Volume	15uL
Temperature	30° C
Wavelength	246nm
Sample rate	2.5 Hz

Time (min)	% Acetonitrile
0	50
10	90
13.5	90
13.6	50
15	50

Maintaining Peak Position & Resolution

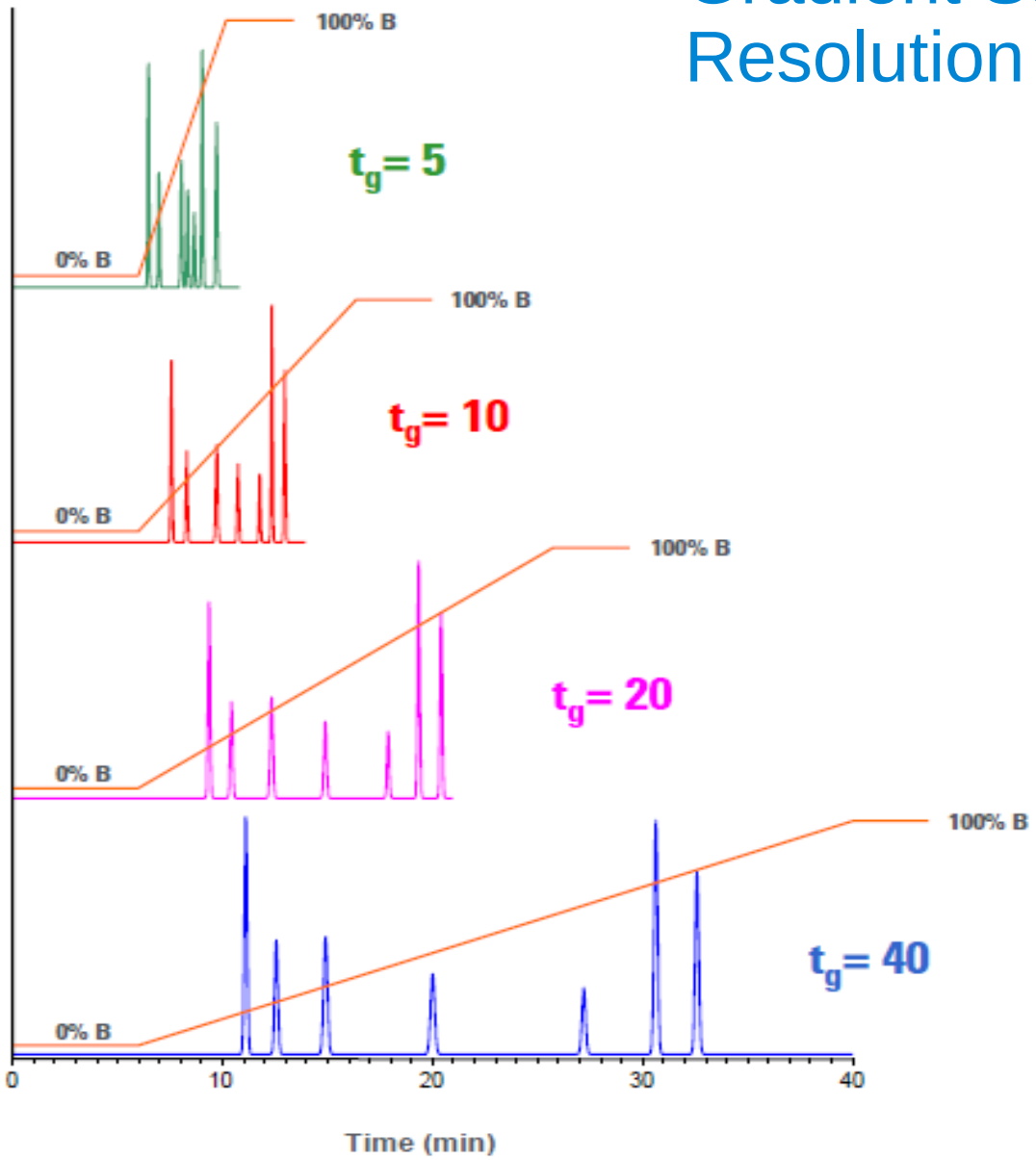
Have shortened column & Gradient time – Need to do so by the SAME factor
1/3 column length – 1/3 Gradient time
ex: RRHT column – 4.6 x 50 mm, 1.8µm, SB-C18



Flow Rate	1.0 ml/min
Injection Volume	5uL
Temperature	30° C
Wavelength	246nm
Sample rate	*13.74 Hz*

Time (min)	% Acetonitrile
0	50
3.33	90
4.5	90
4.53	50
5	50

Gradient Steepness Effects Resolution & Retention (k*)



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

1/k* = gradient steepness = b

DF = 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)

Gradient Steepness and Gradient Shape

1. Gradient shape

- Linear gradients are preferred
- Nonlinear, segmented, and step gradients can be used but can be harder to transfer

2. Gradient Steepness

- Change can affect resolution
- Compare resolution at desired gradient time and at tg +/- 10-20%
- Small changes likely due to instrument performance differences
- Need to compensate for any dwell/delay volume differences first

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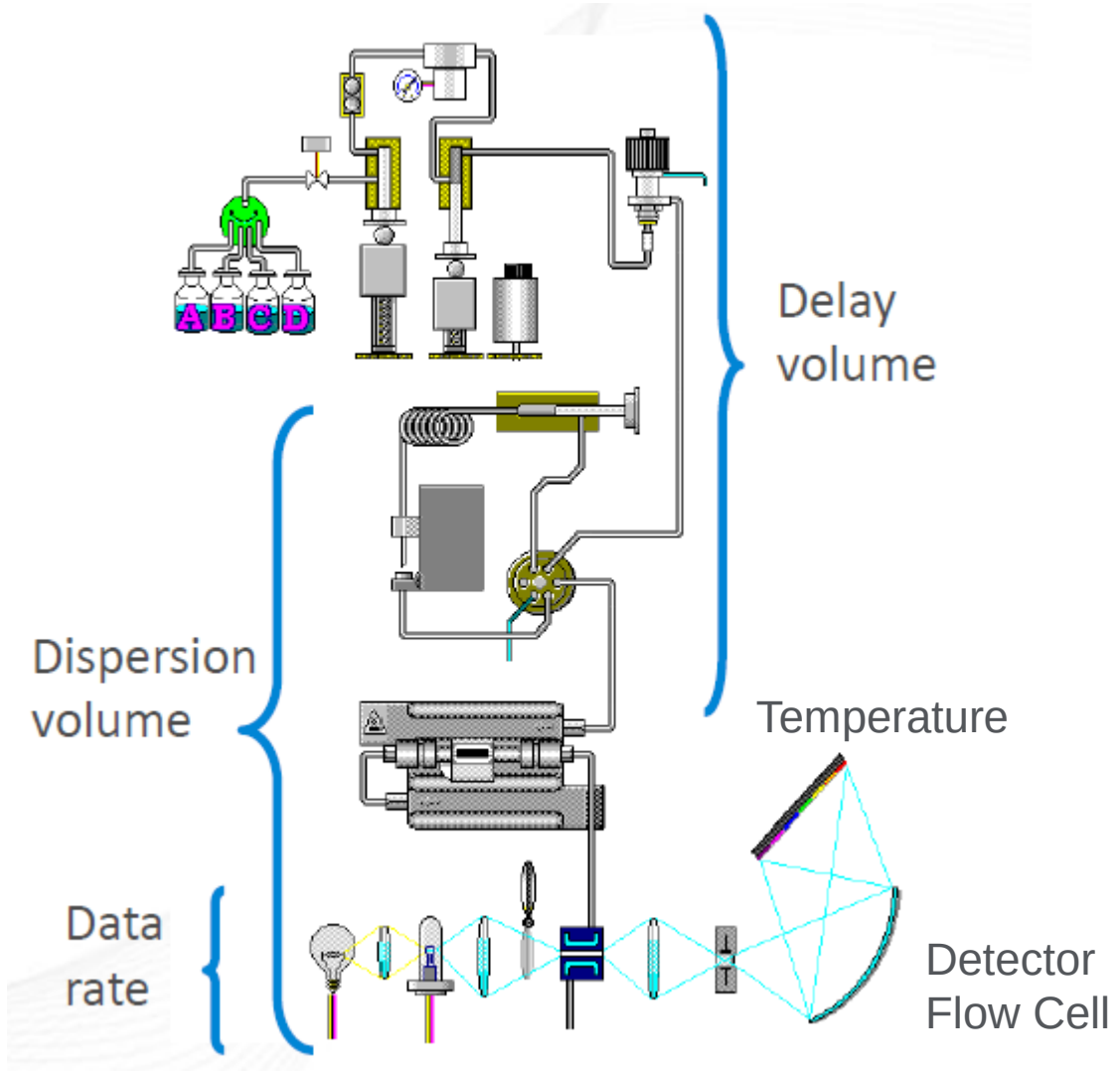
Instrument: Areas of Instrument that Impact Resolution

Volume Considerations for LC systems

1. Delay volume
2. Dispersion volume/Extra Column Volume
3. Temperature
4. Detector Flow cell volume
5. Data collection rate

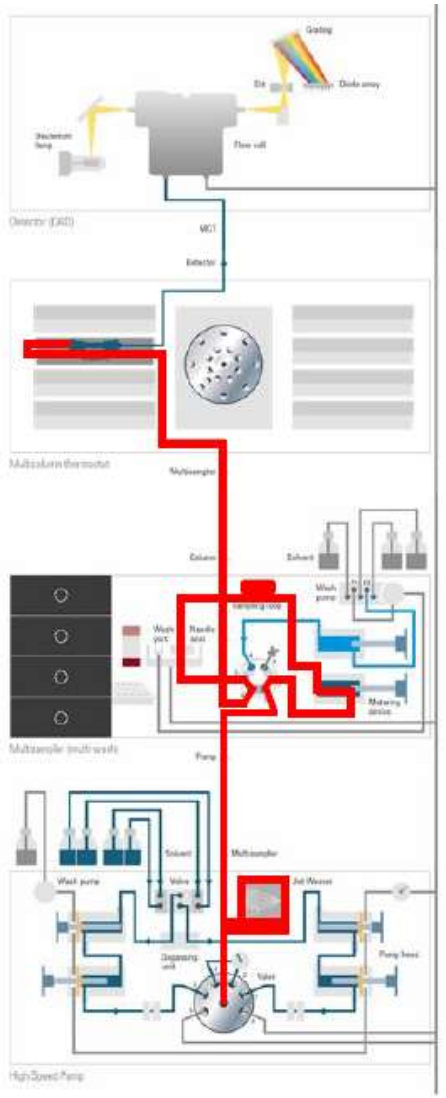


Gradient Delay Volume
System Design – Agilent 1290 Infinity II LC System

Affects our results:

- An isocratic hold step at the beginning of every gradient
- Sharpness of the gradient
- Required equilibration time and therefore total cycle time

Early eluting peaks are more affected than later eluting peaks



Gradient Delay Volume
Agilent 1290 vs 1200 systems

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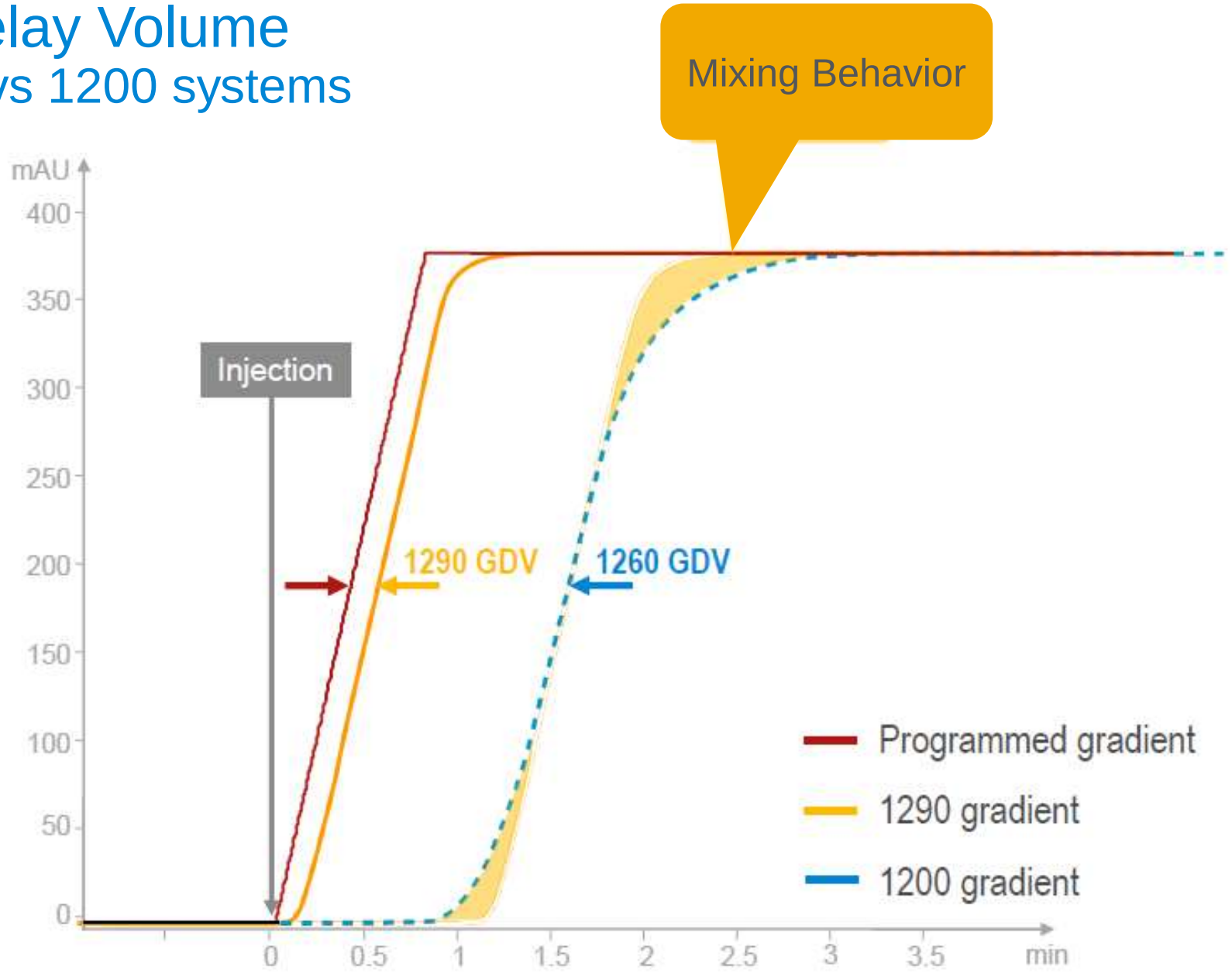

Instrument Delay Volume Differences
Can Cause Changes in Resolution & Retention

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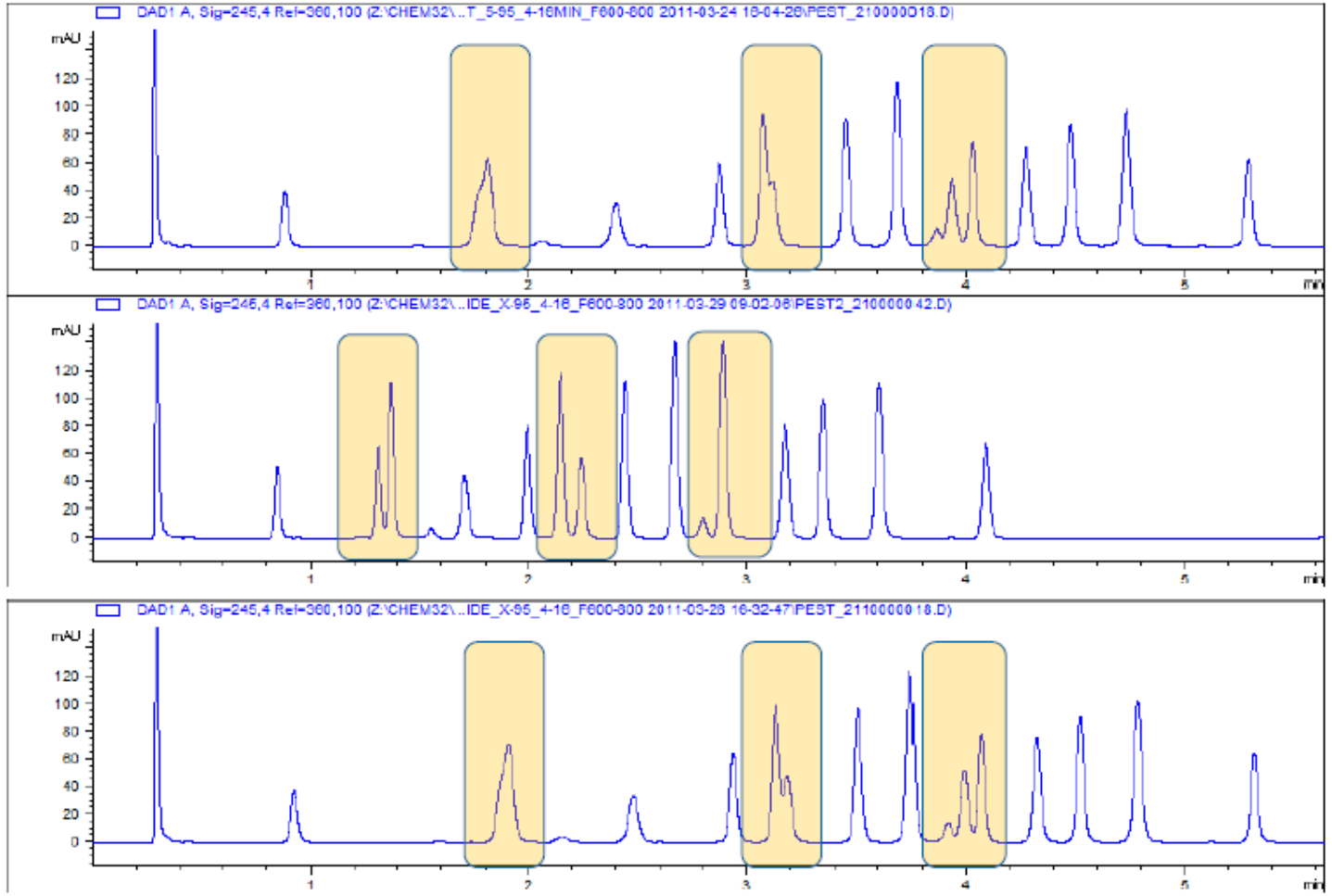
1100 Quaternary 400 bar

1290 Infinity II 1300 bar

1290 Infinity II 1300 bar with ISET



2.1 x 100 mm Agilent ZORBAX Eclipse Plus, 1.8 µm, flow = 0.8 mL/min

Instrument Considerations
Dispersion & how it can effect resolution

What is dispersion?

- Original sample concentration being diluted as it is carried through the system plumbing (extra-column volume)

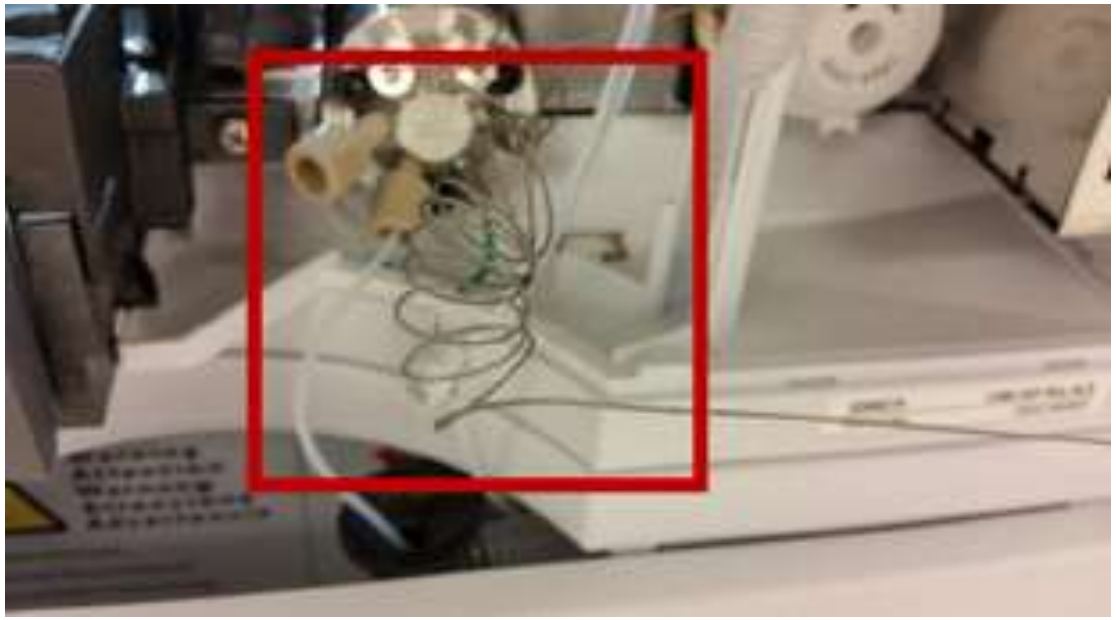
What increases dispersion?

- Connecting tubing that is too long
- Connecting tubing that is too large in diameter
- Connections that have gaps and form small mixing chambers

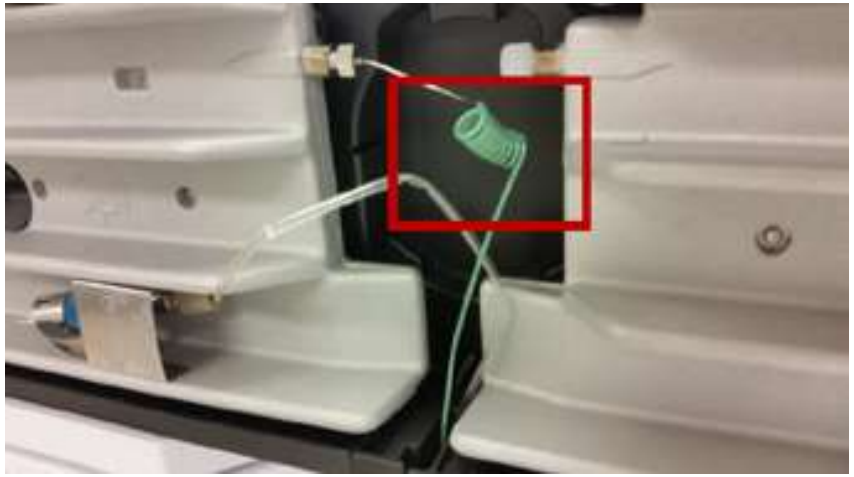
Dispersion & ECV : where on LC might it be?

ECV is the volume in the LC system outside of the column

@ Detector

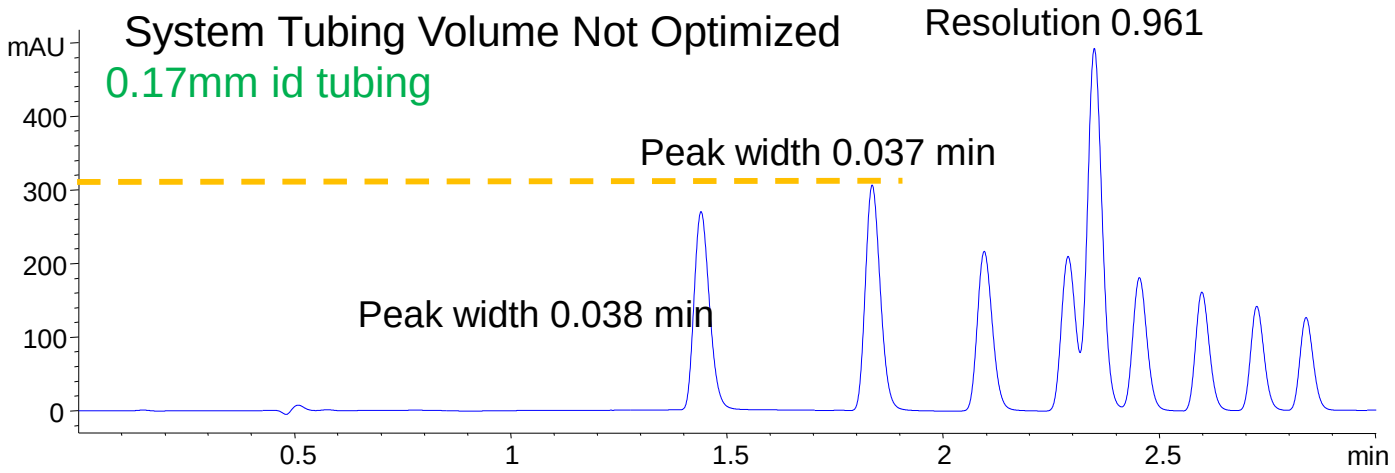


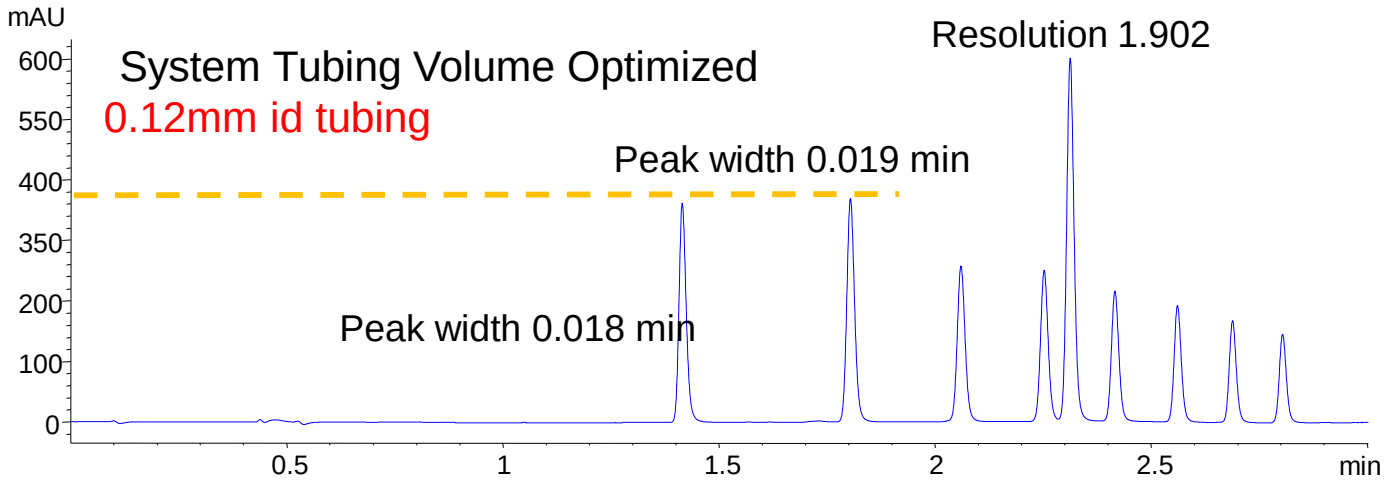
@ Autosampler



@ Column Compartment

Optimizing Connecting Tubing Volume For UHPLC Columns



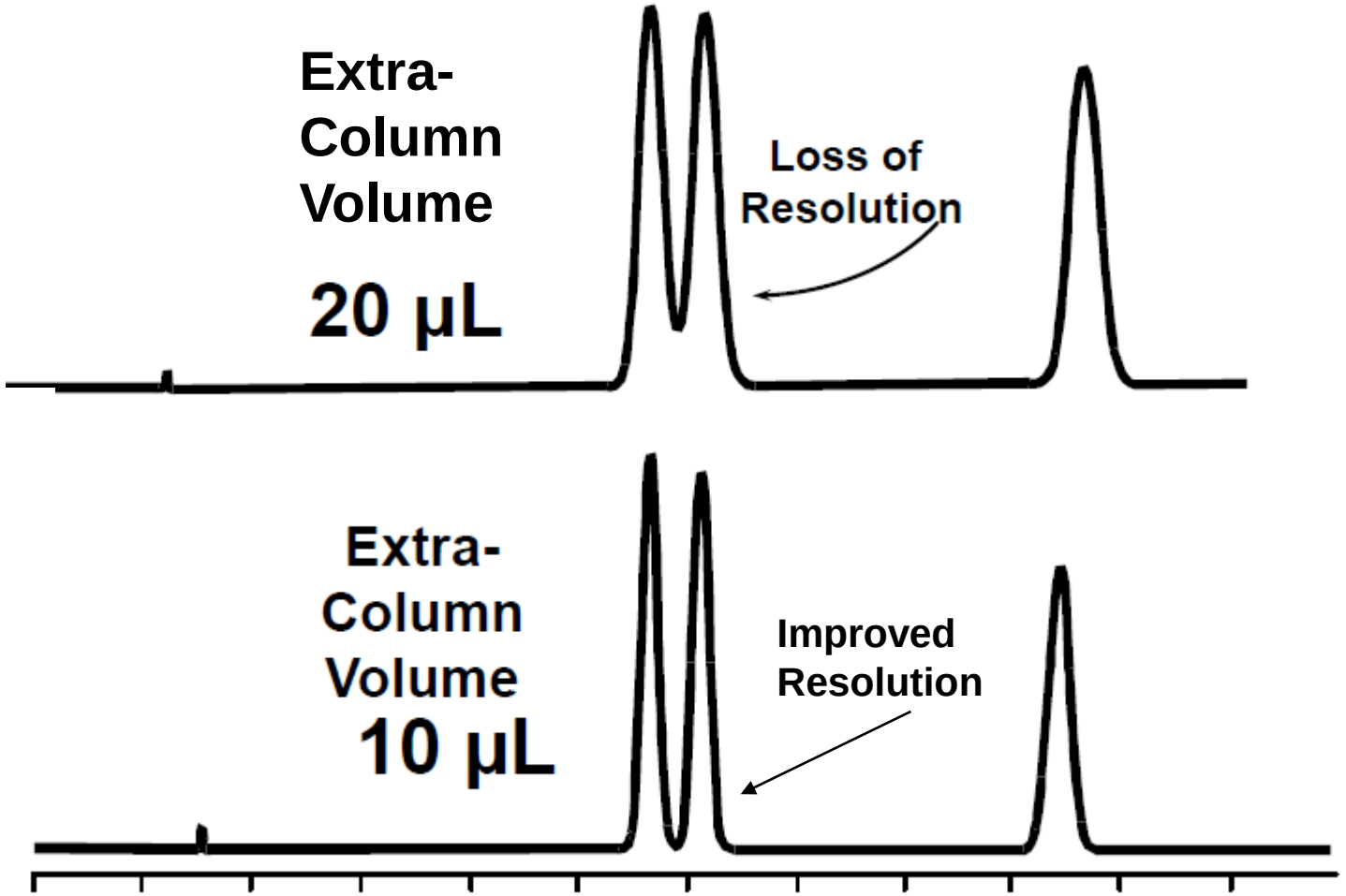


Length	10mm	50mm	100mm	150mm
Tubing ID	Volume	Volume	Volume	Volume
0.17mm (green)	0.227 uL	1.1uL	2.27 uL	3.3 uL
0.12mm (red)	0.113 uL	0.55uL	1.13 uL	1.65 uL

Resolution & Peak Shape
Effect of Extra Column Volume

2.1 x 150 mm columns
Flow rate: 0.2mL/min

Length	10mm	50mm	100mm	150mm
Tubing ID	Volume	Volume	Volume	Volume
0.17mm (green)	0.227 uL	1.1uL	2.27 uL	3.3 uL
0.12mm (red)	0.113 uL	0.55uL	1.13 uL	1.65 uL



Extra Column Volume – Resolution & Peak Shape Lost

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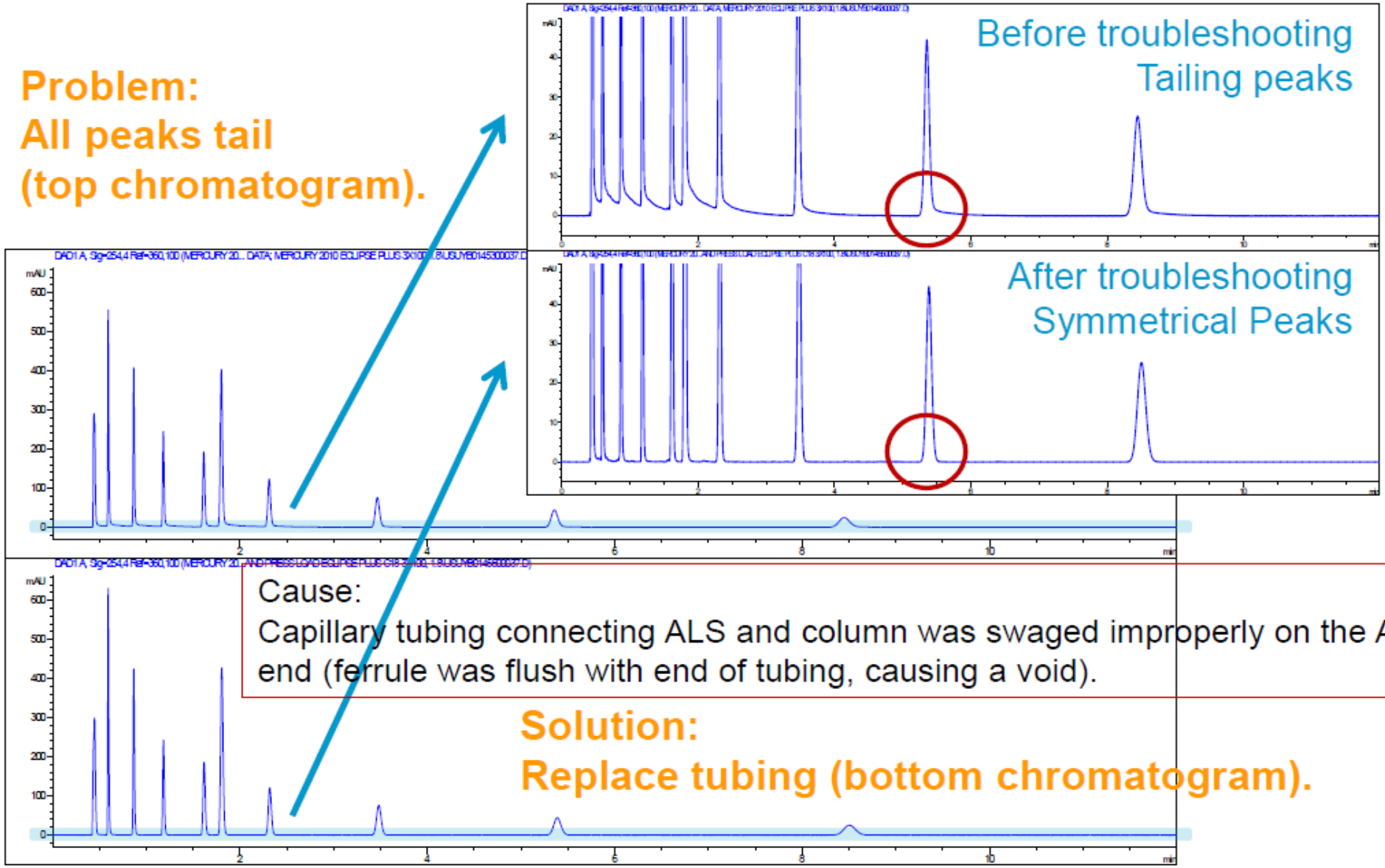
Problem:
All peaks tail
(top chromatogram).



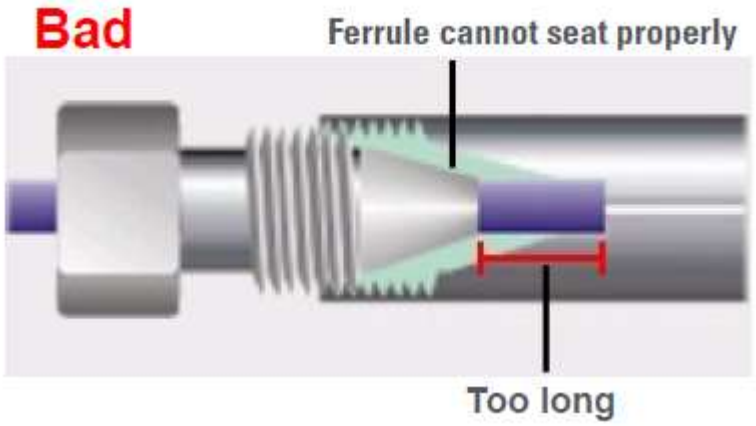
← Resolution lost

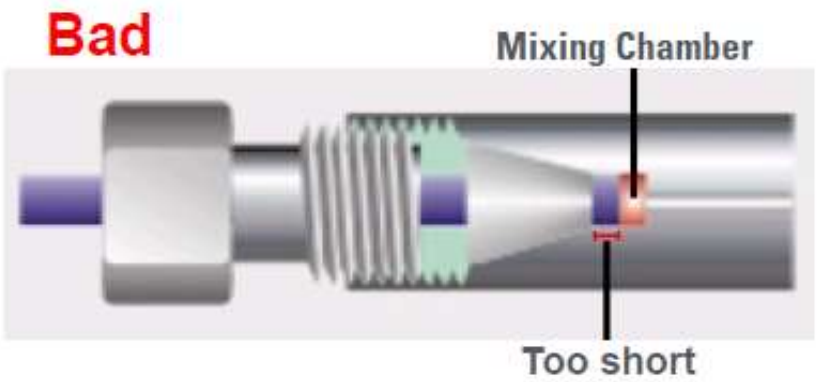
← Resolution improved

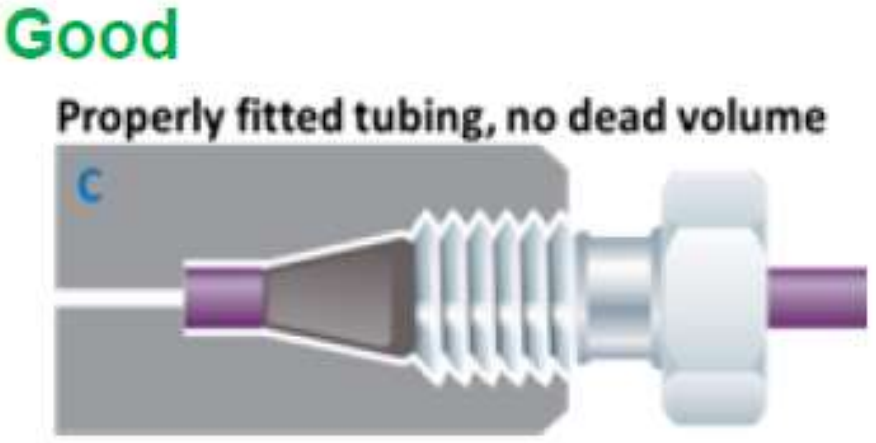
Cause:
Capillary tubing connecting ALS and column was swaged improperly on the ALS end (ferrule was flush with end of tubing, causing a void).

Solution:
Replace tubing (bottom chromatogram).

Making Correct Connections



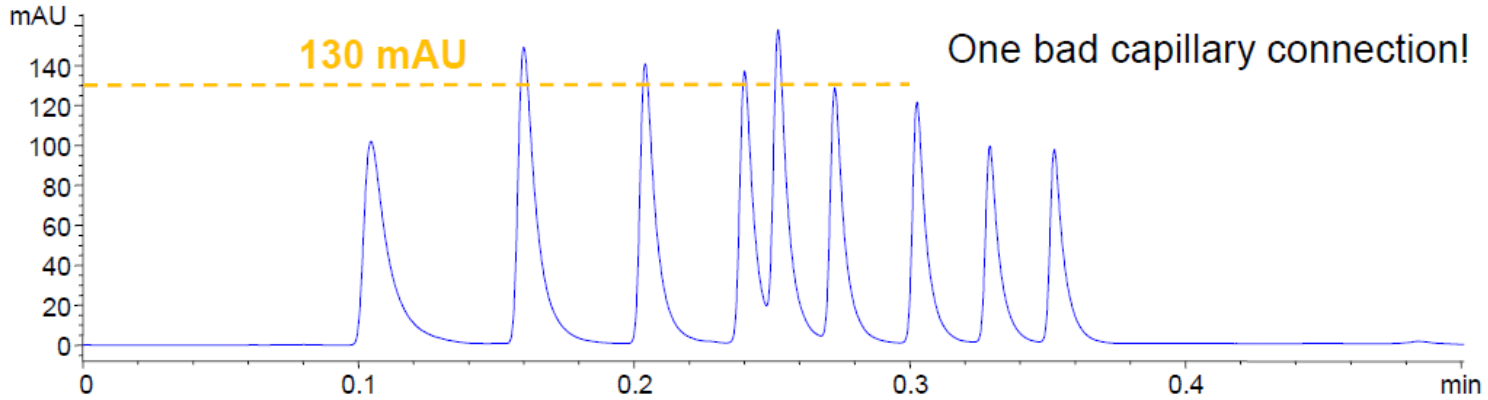


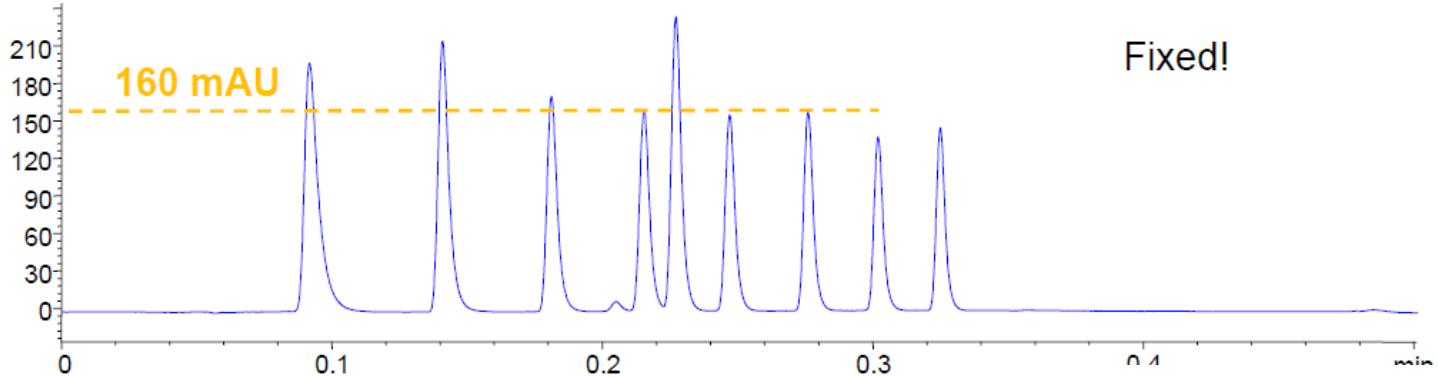


Poor Fitting Connections

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

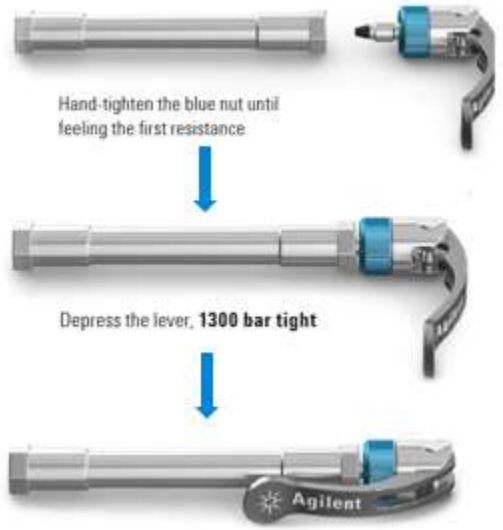
Importance of Correct Connections





Quick Turn

Quick Connect



Correct connection every time

Compatible to 1300 bar

Agilent Technical Note: Agilent InfinityLab UHPLC Fittings

Pub No 5991-5525EN

Evaluating Temperature

Column Temperature
Adequate Temperature Control is Essential

Provides more rapid mass transfer:

- Improves Efficiency – enhances resolution
- Decreases analysis time – faster separations with no loss in resolution

Decreases Mobile Phase Viscosity

- Lowers backpressure – allows for higher flow rates, faster separations, greater efficiency and use of sub 2-micron columns

Can change selectivity – optimize resolution

Temperature Can Optimize Resolution and Selectivity

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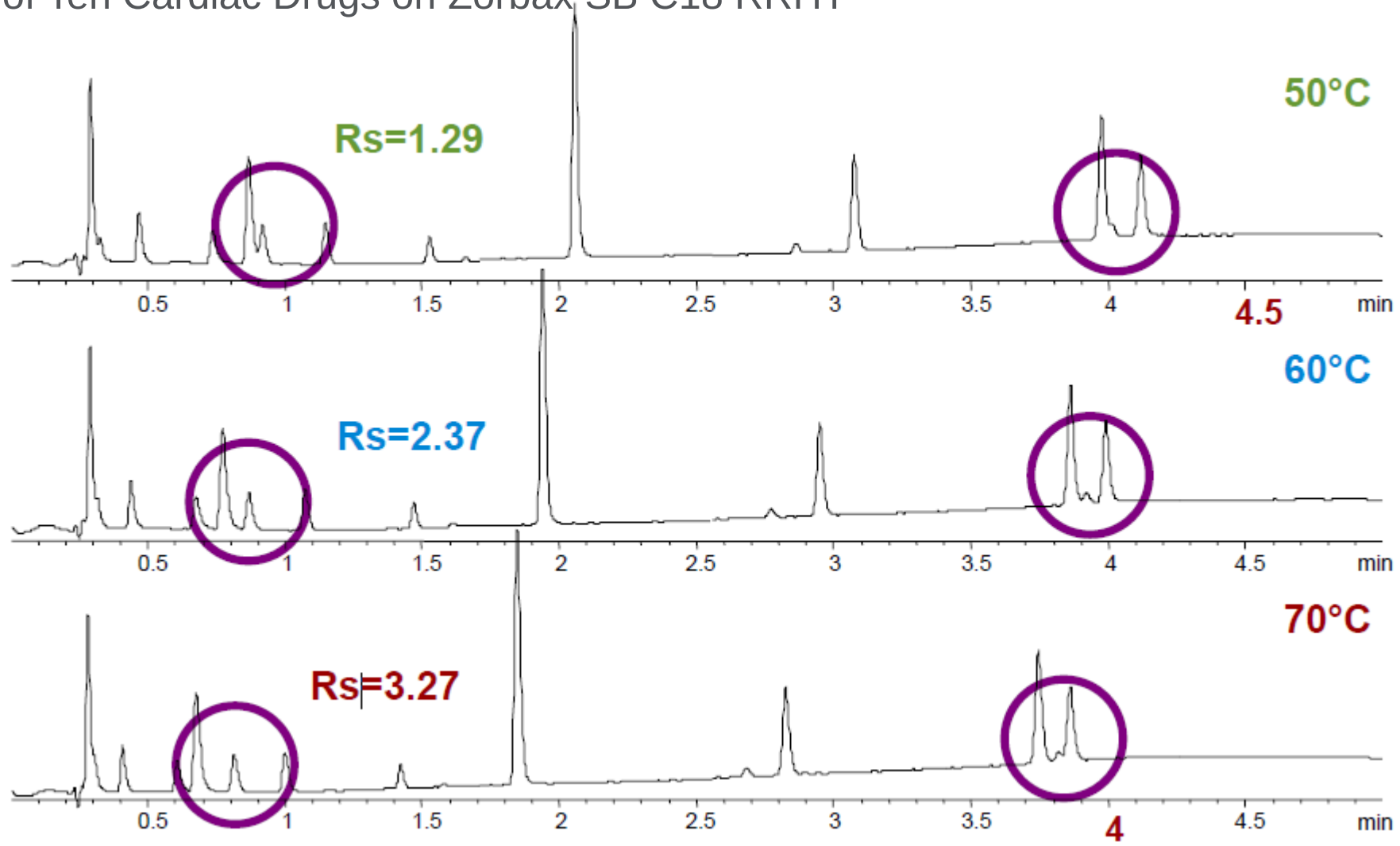
Gradient of Ten Cardiac Drugs on Zorbax SB C18 RRHT



Temperature Can Also be used to Optimize a HILIC Separation

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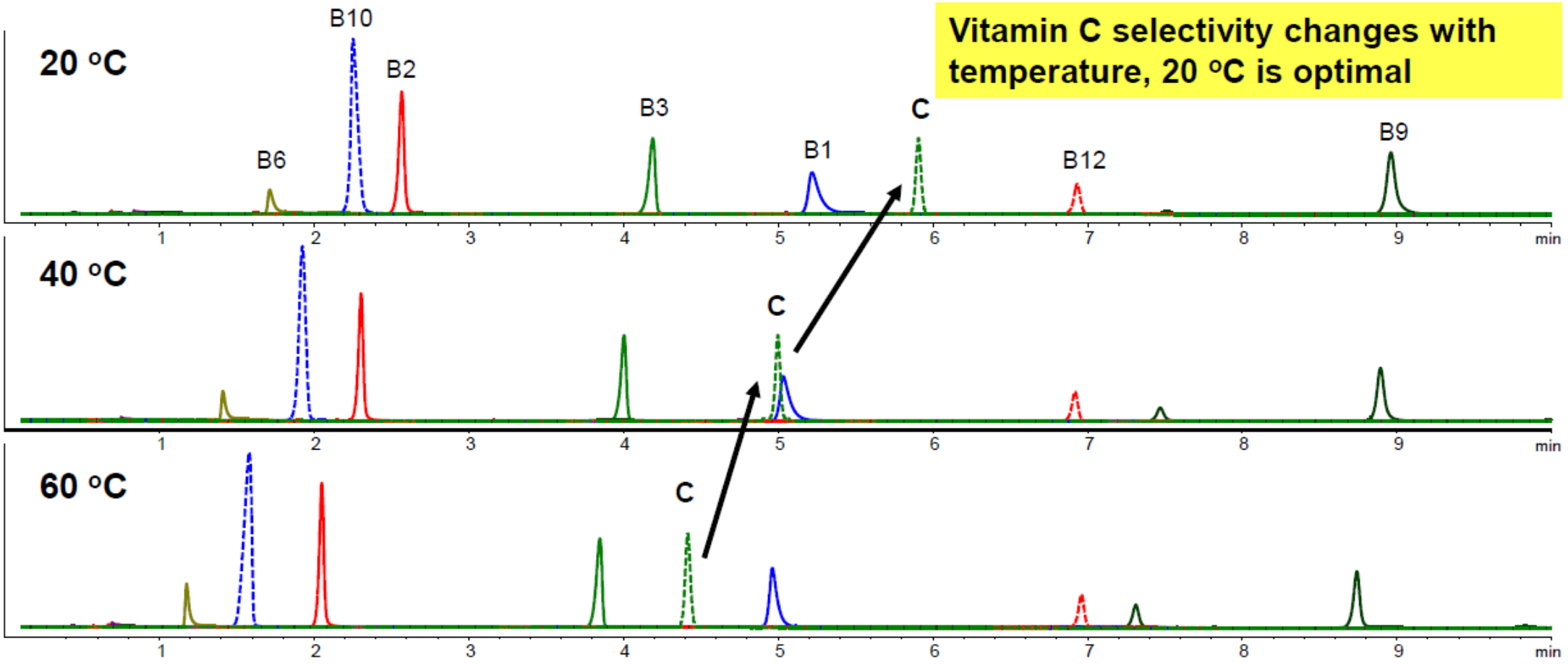
Water Soluble Vitamins on Agilent InfinityLab Poroshell 120 HILIC-OH5



Agilent InfinityLab Poroshell 120 HILIC-OH5 2.1 x 100 mm, 2.7 µm; A: 100 mM Ammonium Acetate (no pH adjustment) in H_2O, B: CH_3CN, 0.5 mL/min, 95-60%B in 10 min, 3 min re-equilibration, 1 µL injection of individual vitamin standards (0.1-0.4 mg/mL each), 20/40/60 °C, 260 nm, 80 Hz

Resolution & Importance of Flow Cell Volume

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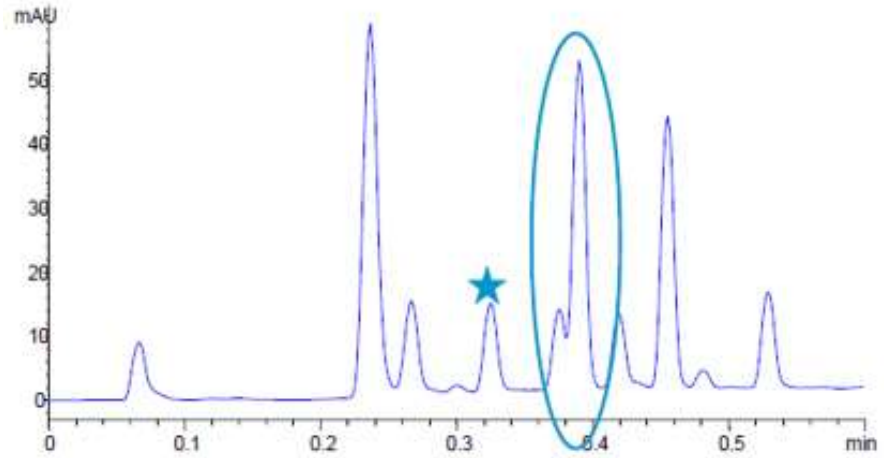
Differences in Detector Flow Cell Volume Can Affect N and R_s

Scenario: Agilent ZORBAX Rapid Resolution Column: 75 mm, 3.5 µm; Flow Rate: 1mL/min; k = 3

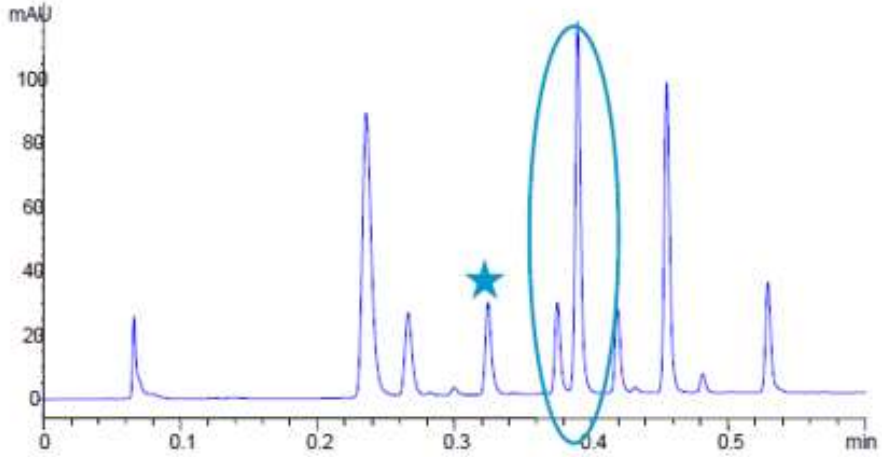
Flow Cell Volume	Band Broadening* (4.6 mm)	Band Broadening* (2.1 mm**)
1.7 µL	0.3%	6%
8 µL	6%	138%
14 µL	19%	423%

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

System Data Collection Rate
Optimize for peak Rs



★ *Peak width = 0.021min at 10Hz*



★ *Peak width = 0.017min at 80Hz*

Maintaining Resolution at High Analysis Speed

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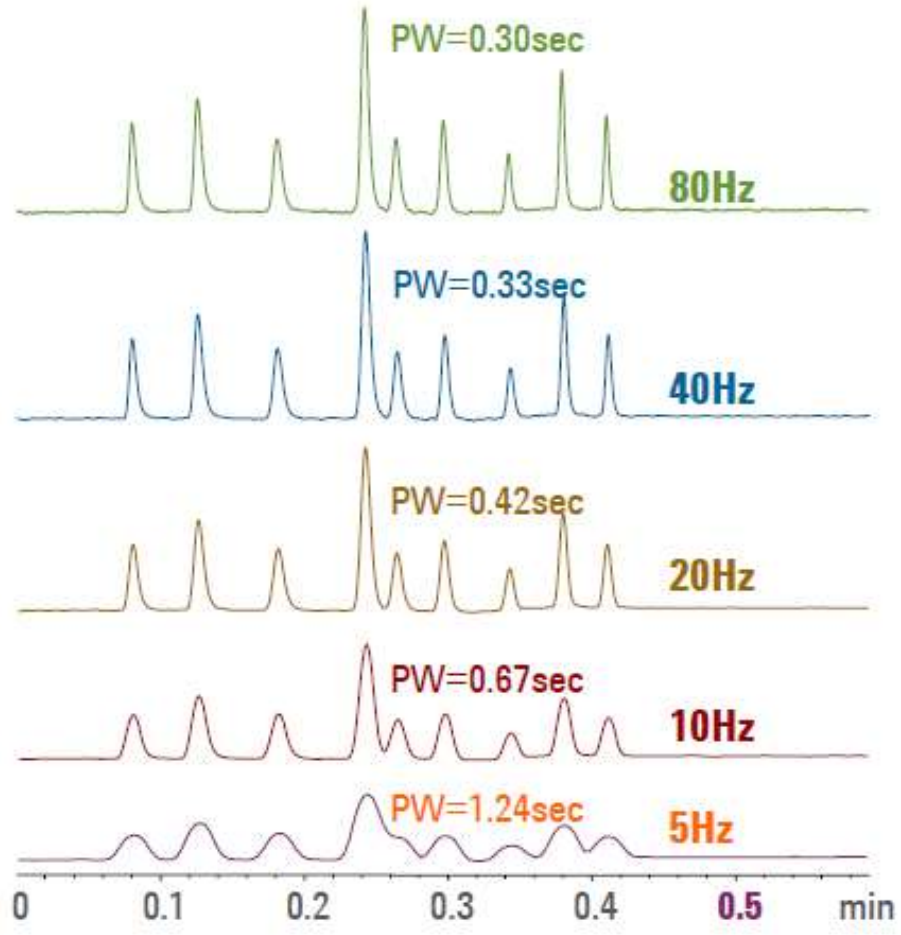
Importance of data collection rate for narrow peaks



80Hz versus 10Hz (20Hz) Data Rate

- Peak Width: – 55% (– 30%)
- Resolution: + 90% (+ 30%)
- Peak Capacity: + 120% (+ 40%)
- App. Column Eff.: + 260% (+ 70%)

Data Rate	Peak Width	Resolution	Peak Capacity
80 Hz	0.300	2.25	60
40 Hz	0.329	2.05	55
20 Hz	0.416	1.71	45
10 Hz	0.666	1.17	29
5 Hz	1.236	0.67	16

Sample: Phenones Test Mix
Column: Zorbax SB-C18, 4.6x30, 1.8um
Gradient:: 50-100%ACN in 0.3min
Flow Rate: 5ml/min

In Summary:

Resolution Equation

- Become familiar with the Resolution equation and its parameters Efficiency, retention, and selectivity. Selectivity is the main driver of resolution.

Required Resolution

- Baseline resolution is achieved at 1.5 but need to strive to achieve resolution of > 2.0.

Consider Alternates for Improving Resolution

- Explore alternate selectivity by choosing and evaluating different bonded phases.
- Also look to selectivity effects of both mobile phase organics and pH.

Gradient time & Steepness

- Optimal gradient is one where we have sufficient (i.e. >2.0) resolution at the shortest runtime.

Role of the Instrument

- From System delay volume, ECV & Dispersion, to correct fittings, temperature, flow cell & data collection rate, the LC system too needs to be optimized to ensure that acceptable resolution is achieved and maintained.

THANK YOU FOR ATTENDING

ANY QUESTIONS??

Contact Agilent Chemistries & 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

*available 8am – 5pm EST – PST in US and Canada

- gc-column-support@agilent.com
- lc-column-support@agilent.com
- spp-support@agilent.com
- spectro-supplies-support@agilent.com