HPLC Method Development: from Beginner to Expert Part 2

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Agilent
Trusted Answers

HPLC Method Development

From beginner to expert part 1

- Review of key terminology
- Discussed method goals
- Column selection
- Mobile phase selection
- Flow rates and injection volumes
- Scouting gradient

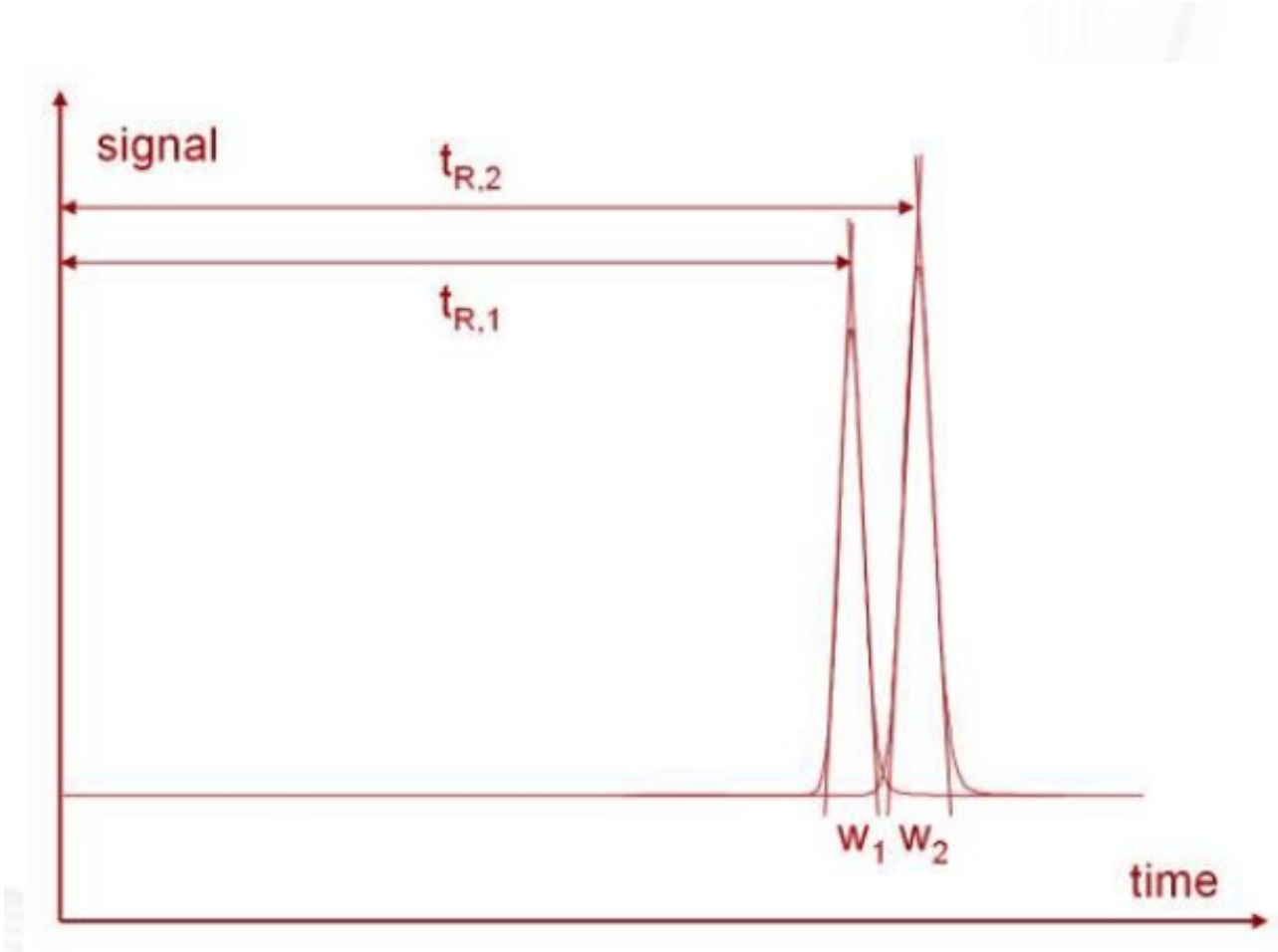
HPLC Method Development

From beginner to expert part 2

- Review of resolution and resolution equation
- Choosing isocratic versus gradient
- Scouting gradient and gradient optimization
- Method transfer: considerations for columns
- Method transfer: considerations for HPLC systems

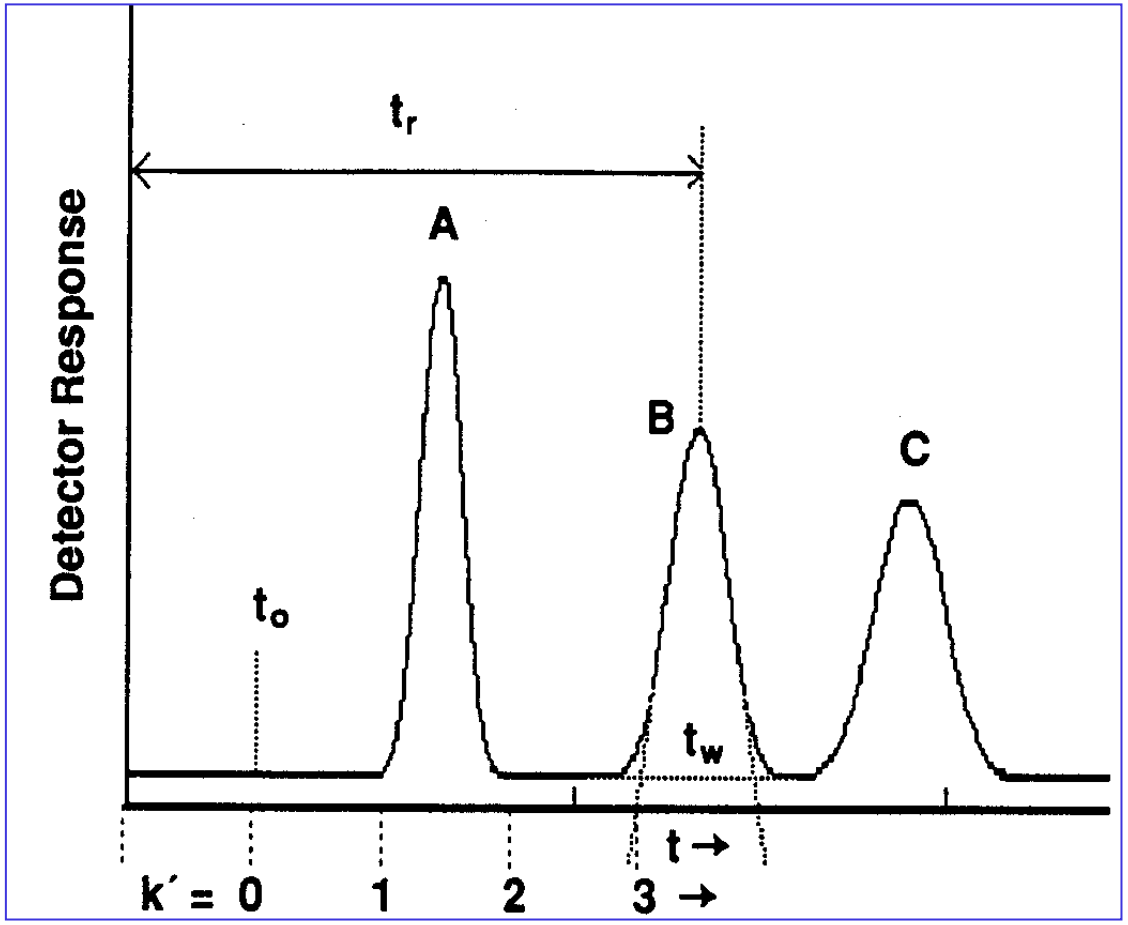
Resolution Definition

Resolution is a measure of the ability to separate two peaks of interest



Fundamental 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

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

Key Terminology

Resolution: Influencing factors

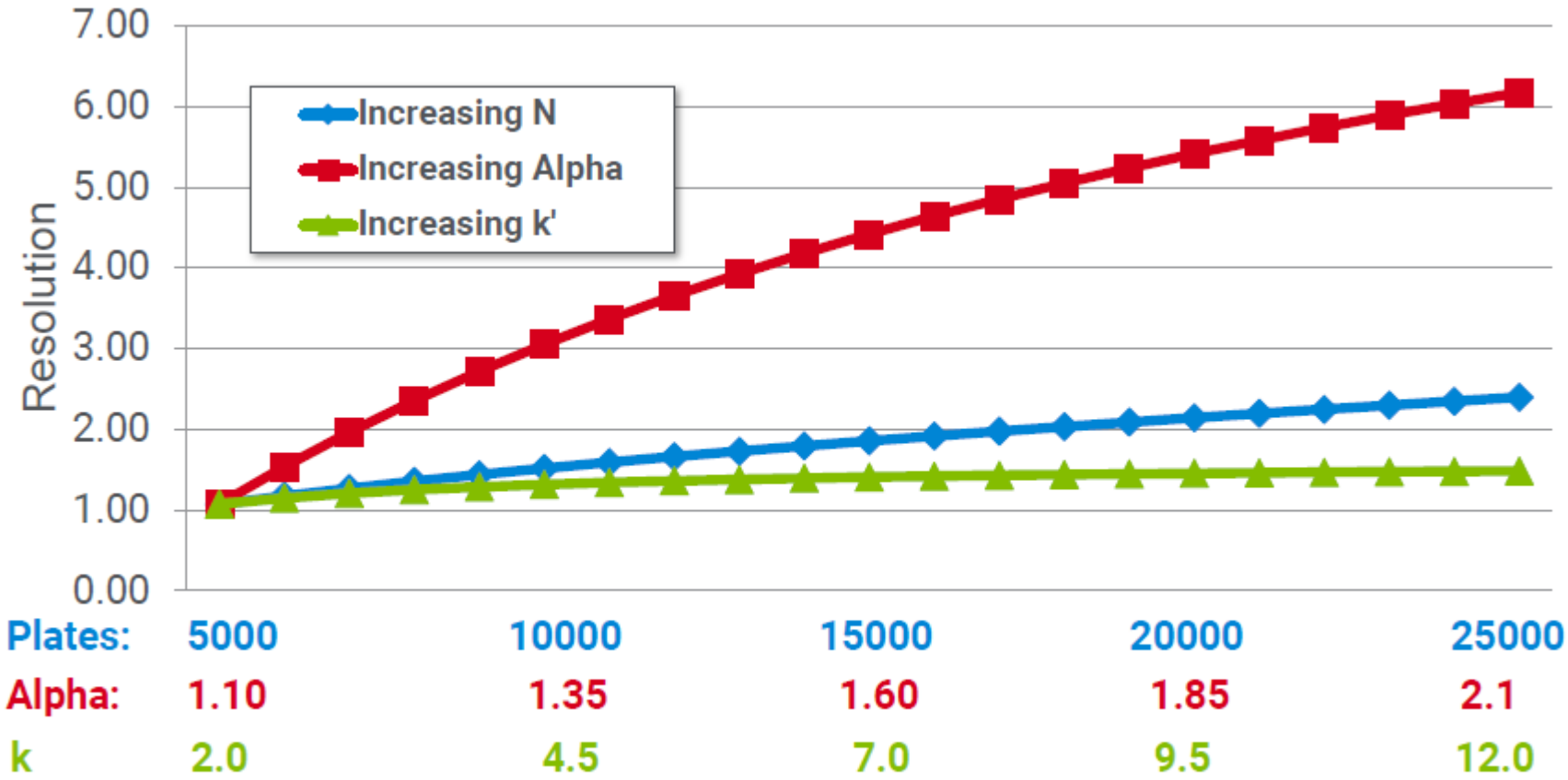
$$R_s = \frac{1}{4}\sqrt{N} \cdot \left(\frac{\alpha - 1}{\alpha}\right) \cdot \left(\frac{k}{1+k}\right)$$

Efficiency **Selectivity** **Retention**

Improve resolution by improving any of these parameters:

- **Efficiency** describes the separation power of the column
- **Retention** has only a significant influence at small k values
- **Selectivity** has the highest influence on the resolution; small changes in selectivity can lead to big changes in resolution

Factors That Effect Resolution



Selectivity impacts resolution the most

- Change bonded phase
- Change mobile phase

} **Typical Analytical Method Development Parameters**

-- Plates are the easiest to increase

Method Development Part 2

Considerations for isocratic versus gradient

What Is an Isocratic HPLC Method

- 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

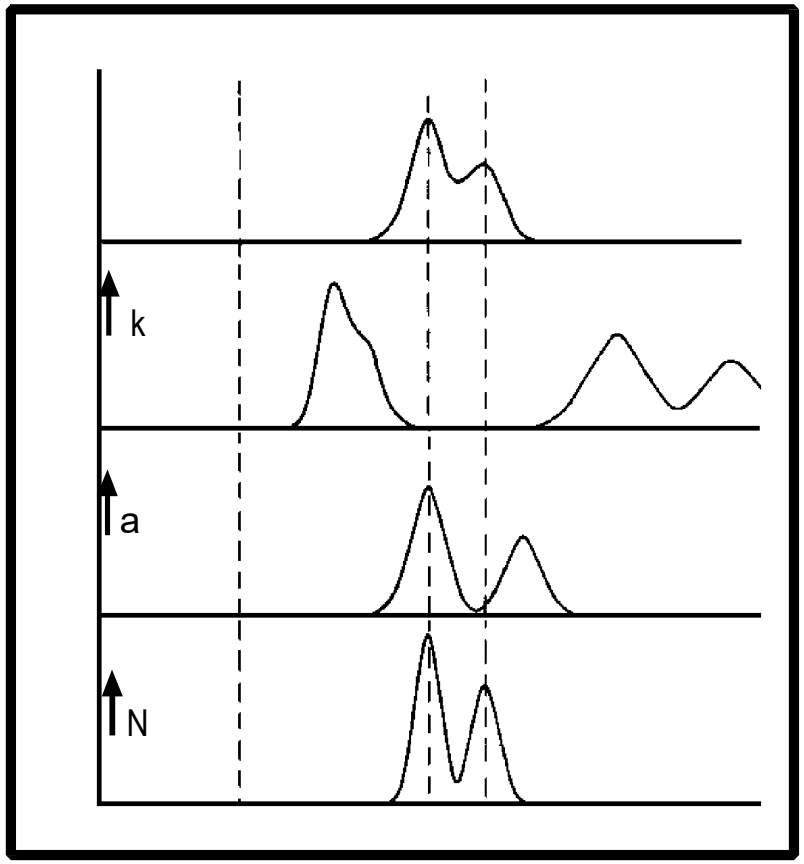
[1]L.R. Synder, J.J. Kirkland, J.W. Dolan. *Introduction to Modern Liquid Chromatography*, -3rd Ed., John Wiley & Sons, 2010

Factors that Maximize Isocratic Resolution Between Peaks

Increasing retention

Changing relative peak position

Increasing retention time faster than peak width



Decreasing %organic

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

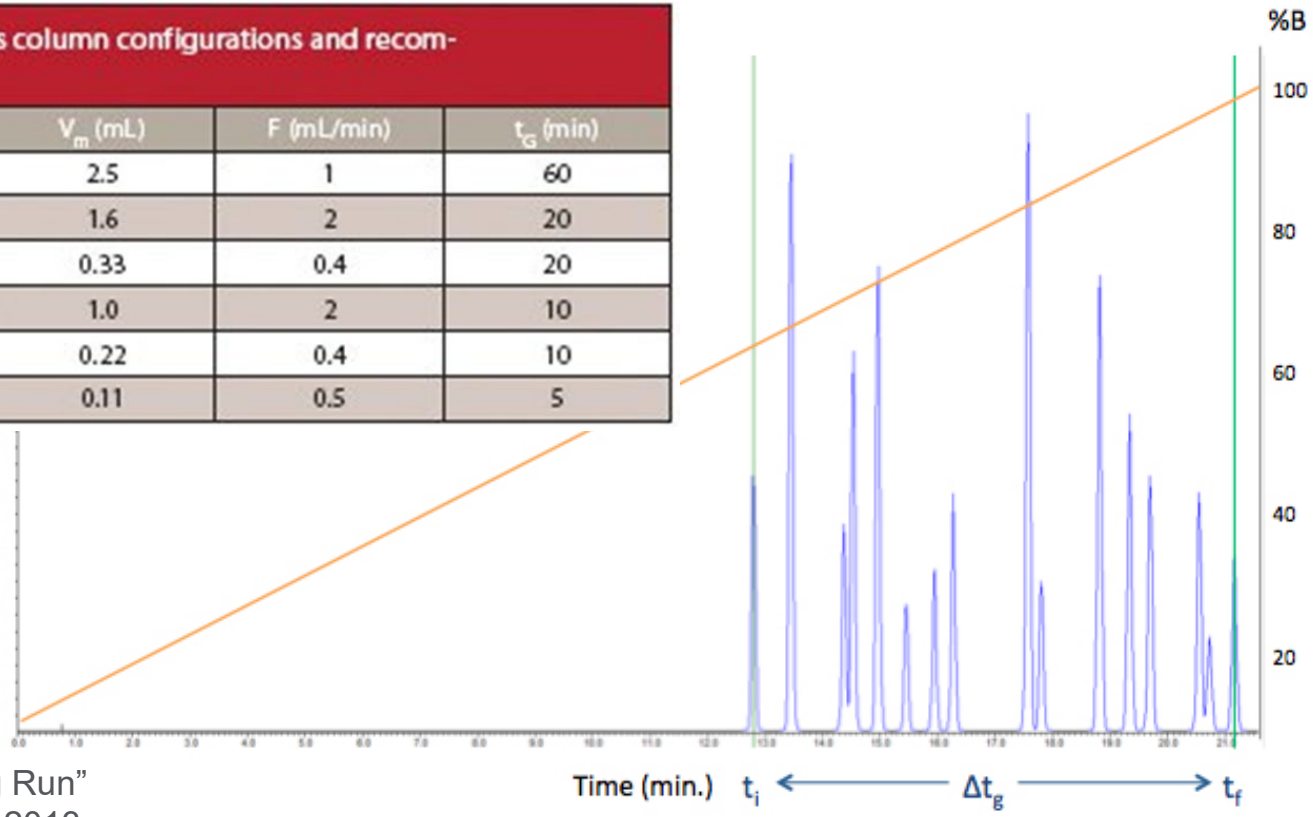
- Changing column dimensions or flowrate
- Decreasing particle size

Starting Point Scouting Gradient

- A good starting point for work is a scouting gradient.
- The conditions recommended by John Dolan are 5 to 95-100% acetonitrile, low pH, and they are dependent on the column length.
- Where 100 mm columns are chosen, using a 10 minute gradient is suggested.
- This example shows a 150 mm column.

Table I: Column volumes for various column configurations and recommended scouting gradient times

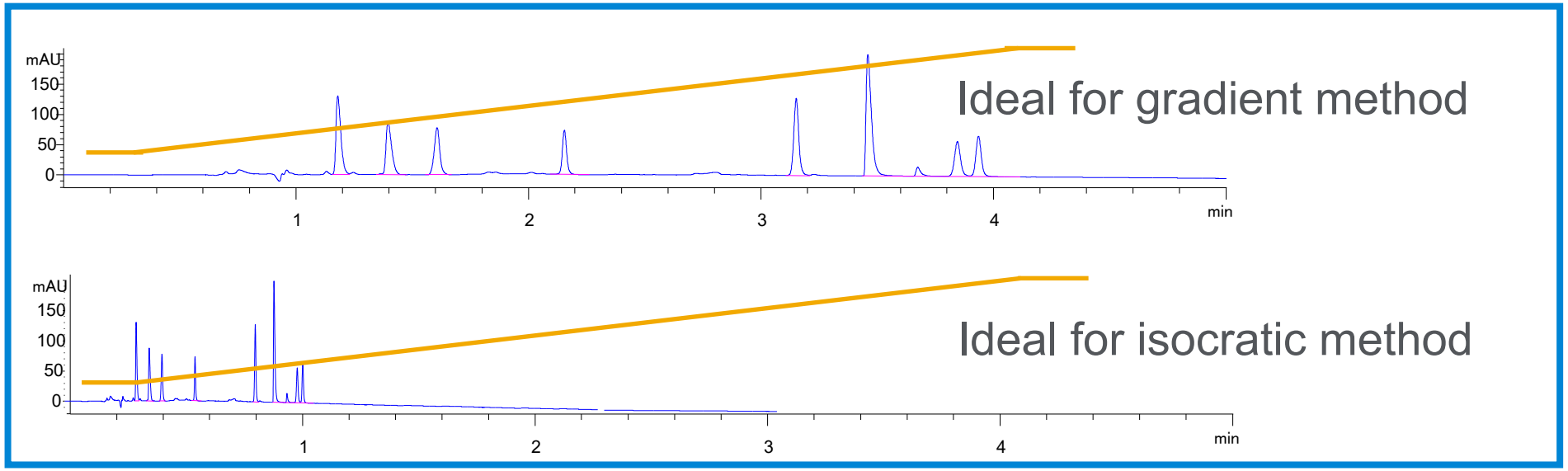
L (mm)	d_c (mm)	V_m (mL)	F (mL/min)	t_G (min)
250	4.6	2.5	1	60
150	4.6	1.6	2	20
150	2.1	0.33	0.4	20
100	4.6	1.0	2	10
100	2.1	0.22	0.4	10
50	2.1	0.11	0.5	5



"Making the Most of a Gradient Scouting Run"
LCGC North America Vol. 31,Number 1, 2013.

Gradients Are Critical Tools for Faster Methods

- Run a scouting method at 5% to 95% organic (reversed phase)
- Quick evaluation: how much of the gradient is occupied
  - $\frac{\Delta t_G}{t_G} \leq 25\%$ isocratic is recommended
  - $\frac{\Delta t_G}{t_G} \geq 40\%$ gradient is recommended



Estimating Isocratic Conditions from Our Scouting Gradient Results

This is 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:

$$\%B = 9.0\left(t_{avg} - t_{void}\right) - 2^{1}$$

Where:

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

t_{void} is the void time of the column

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

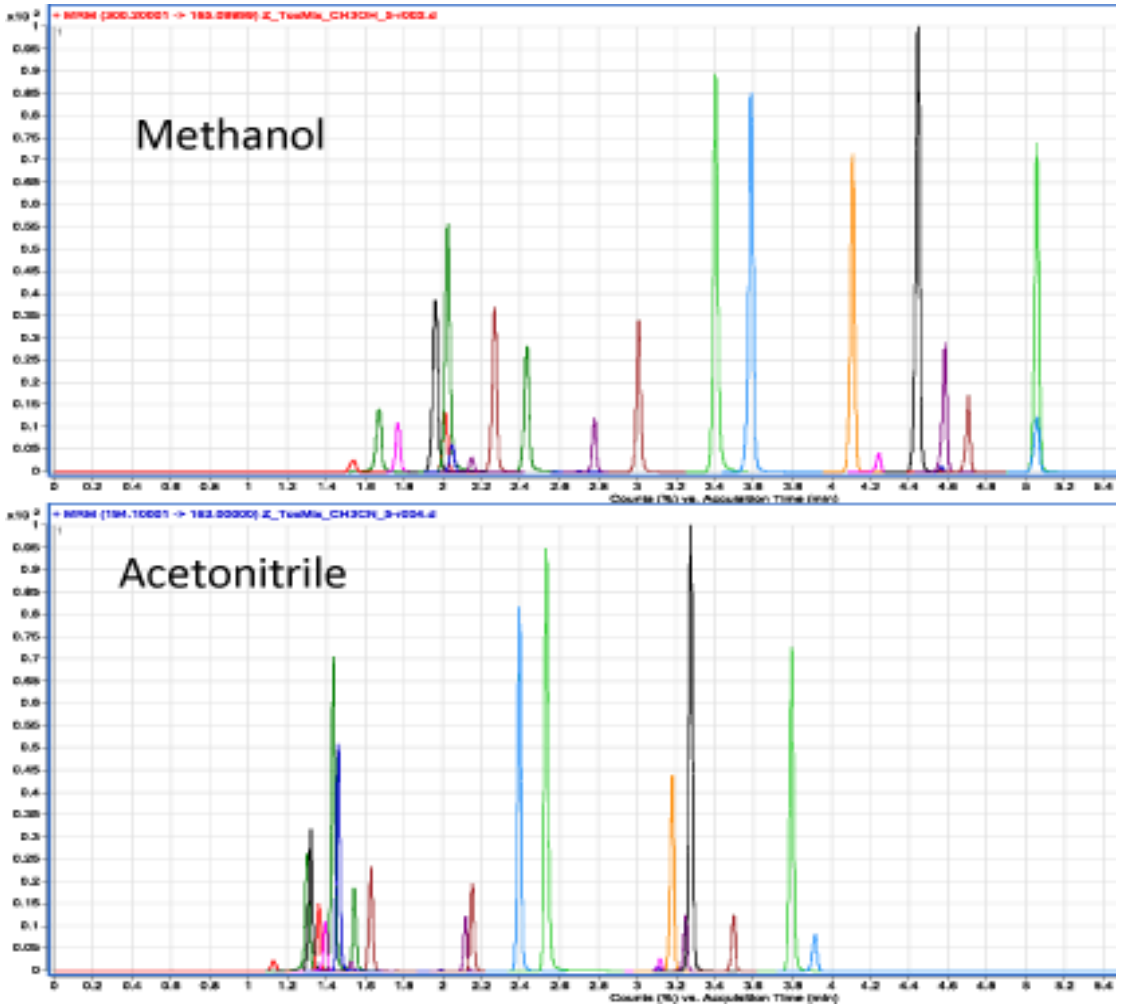
Exploring Organic Modifiers

Why?

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

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

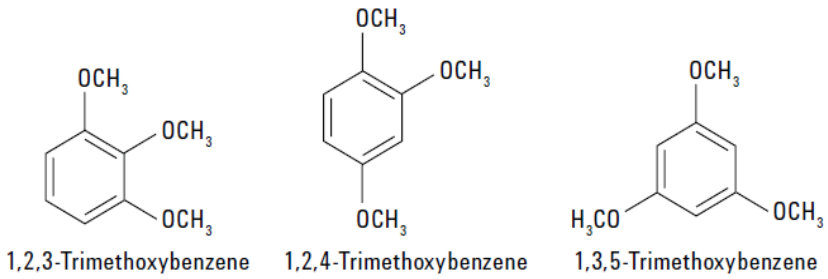
Acetonitrile – Aprotic, wider UV window, stronger than MeOH



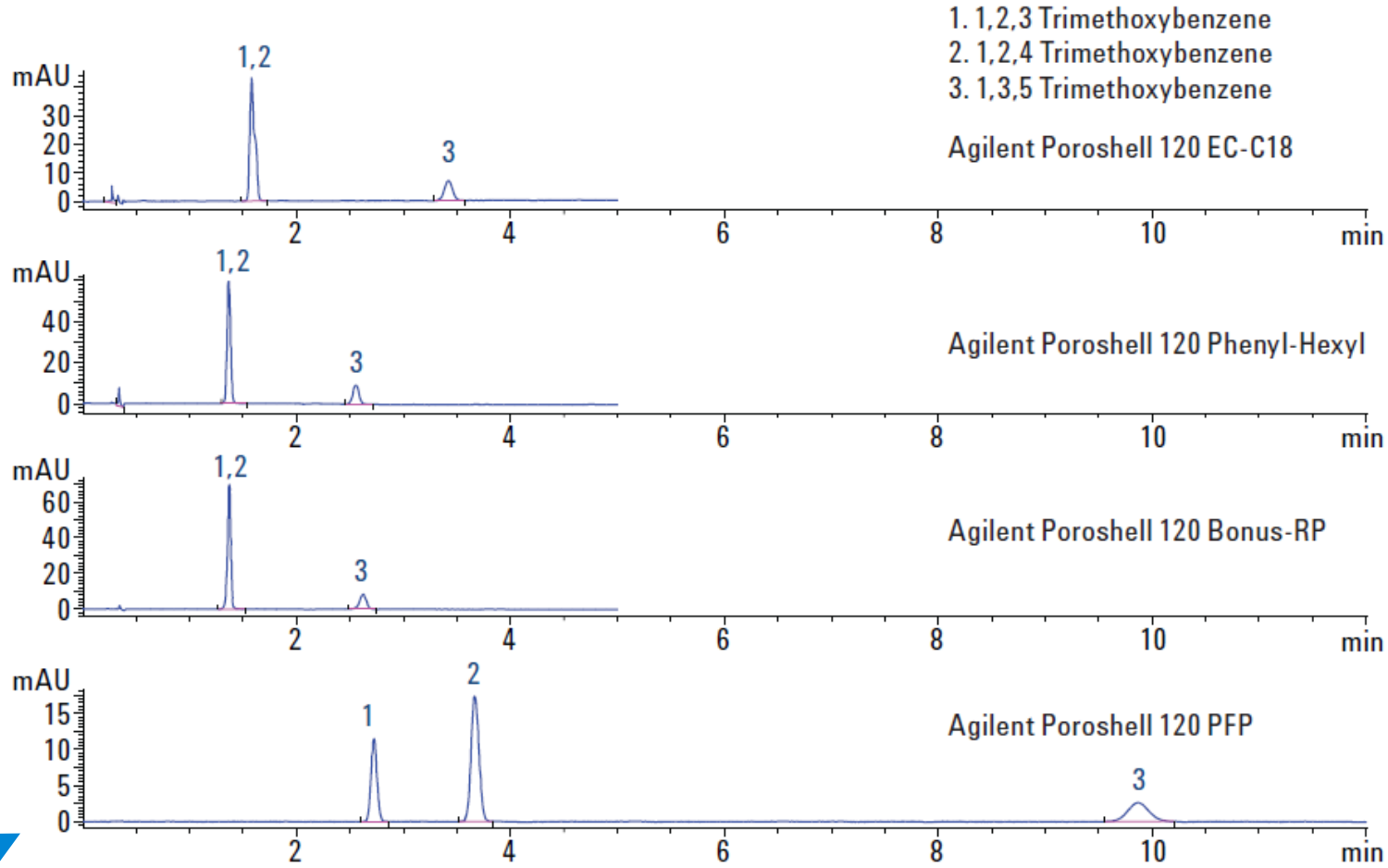
"Fast Analysis of Illicit Drug Residues on Currency using Agilent Poroshell 120", Anne E. Mack, James R. Evans and William J. Long, September 2010, 5990-6345EN.

Importance of Alternate Selectivity Chemistries

Isocratic separations and selectivity for samples



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



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

Importance of Evaluating Different Bonded Phases

- Bonded phase affects selectivity (alpha)
- Different interactions for polar and nonpolar compounds.
- Exploit other interactions with bonded phase (for example, pi-pi)
- Changing the bonded phase can improve selectivity/resolution
- May 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

The Poroshell 120 Family

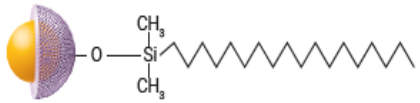
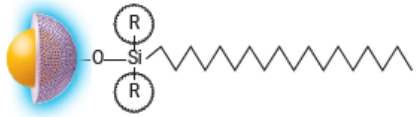
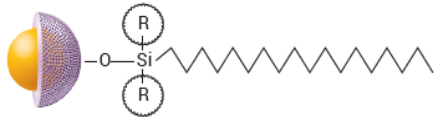
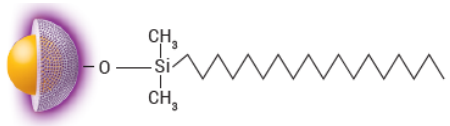
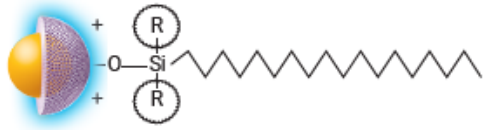
InfinityLab Poroshell 120 offers a broad portfolio to suit your needs

Best All Around	Best for Low pH Mobile Phases	Best for High pH Mobile Phases	Best for Alternative Selectivity	Best for More Polar Analytes	HILIC for polar analytes	Chiral
EC-C18 1.9 µm, 2.7 µm, 4 µm	**SB-C18** 1.9 µm, 2.7 µm, 4 µm	**HPH-C18** 1.9 µm, 2.7 µm, 4 µm	**Bonus-RP*** 2.7 µm	**Aq-C18*** 2.7 µm New!	**HILIC** 1.9µm, 2.7 µm, 4 µm	**Chiral-V** 2.7 µm
EC-C8 1.9 µm, 2.7 µm, 4 µm	**SB-C8** 2.7 µm	**HPH-C8** 2.7 µm, 4 µm	**PFP*** 1.9 µm, 2.7 µm, 4 µm	**SB-Aq*** 1.9 µm, 2.7 µm, 4 µm	**HILIC-Z** 1.9 µm, 2.7 µm, 4 µm	**Chiral-T** 2.7 µm
Phenyl-Hexyl* 1.9 µm, 2.7 µm, 4 µm		**CS-C18** 2.7 µm New!		**EC-CN*** 2.7 µm	**HILIC-OH5** 2.7 µm	**Chiral-CD** 2.7 µm
						Chiral-CF 2.7 µm

* Can be operated at 100%aqueous mobile phase conditions

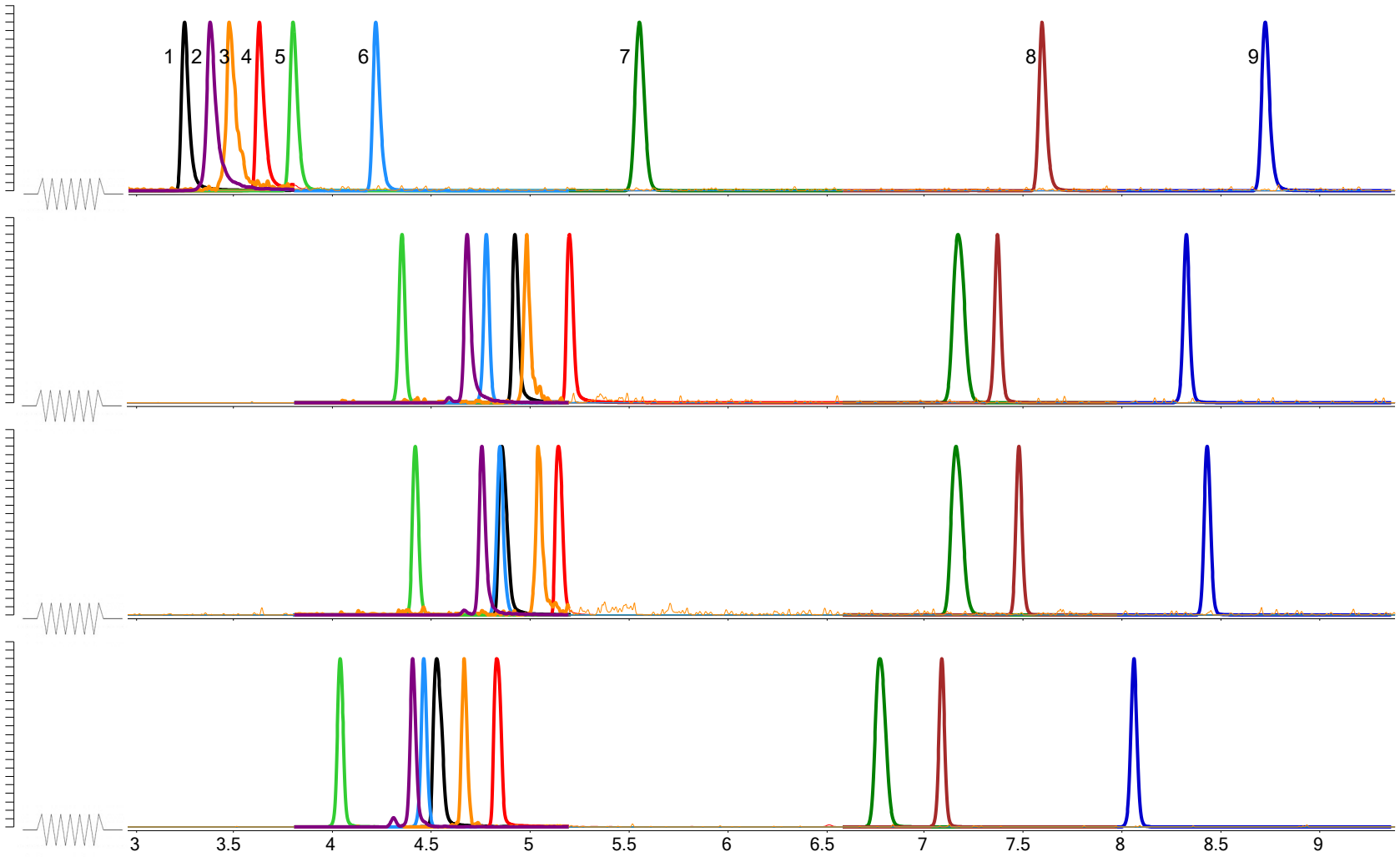
5991-9013EN_InfinityLab_Poroshell120_poster

Choosing between C18s

InfinityLab Poroshell 120	Chemistry	Pore Size	Endcapped	Carbon Load	Surface Area	Best For
EC-C18 1.9 µm, 2.7 µm, 4 µm		120 Å	Yes	10%	130 m2/g	General Purpose Excellent peak shape and efficiency for acids, bases, neutrals
Aq-C18 * 2.7 µm		120 Å	Yes	Proprietary	130 m2/g	Enhanced retention for challenging polar compounds 100% aqueous mobile phase compatibility and low pH stability
SB-C18 1.9 µm, 2.7 µm, 4 µm		120 Å	No	9%	130 m2/g	Low pH Excellent stability and peak shape in highly acidic conditions
HPH-C18 1.9 µm, 2.7 µm, 4 µm		100 Å	Yes	Proprietary	95 m2/g	High pH capable Robust performance and long lifetimes
CS-C18 2.7 µm		100 Å	Yes	Proprietary	95 m2/g	Alternate selectivity Improved peak shape and sample capacity for basic compounds with low ionic strength mobile phases High pH capable

* - can be operated at 100% aqueous mobile phase conditions

Alternative Selectivity with InfinityLab Poroshell 120 CS-C18



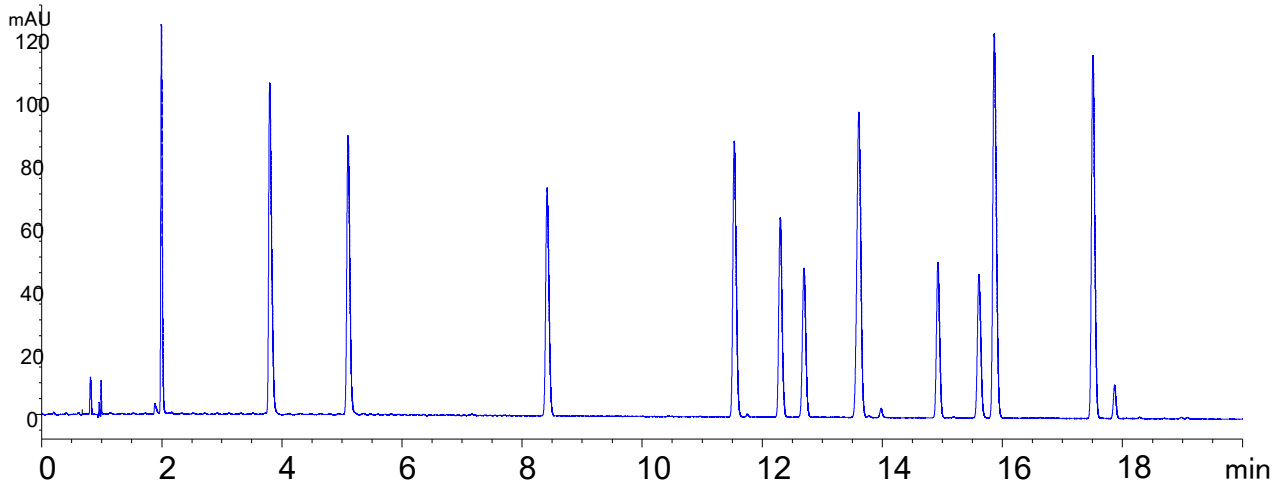
1	Ciprofloxacin	-----
2	Oxytetracycline	-----
3	Tetracycline	-----
4	Enrofloxacin	-----
5	Sulfamerazine	-----
6	Sulfamethazine	-----
7	Erythromycin	-----
8	Penicillin-G	-----
9	Oxacillin	-----

Method parameters:
A: 0.1% formic acid in water
B: Acetonitrile
0.4 mL/min, 0-95% B in 15 min
0.05 µL injection
Sample: 0.1 mg/mL in water
Column: 30 °C, 2.1 x 100 mm, 2.7 µm
Detection: LC/MS, ESI+, dMRM

Agilent application note 5994-2358EN

What Is Gradient Elution?

A separation that occurs by continuously increasing the solvent strength of the mobile phase over a period of time



1. Hydroquinone
2. Resourcinol
3. Catechol
4. Phenol
5. 4-Nitrophenol
6. p-cresol
7. o-cresol
8. 2-Nitrophenol
9. 3,4 di methyl phenol
10. 2,3 di methyl phenol
11. 2,5 di methyl phenol
12. 1-napthol

Column: Poroshell 120 EC-C18, 4.6 x 150 mm, 2.7 µm

Mobile phase: **Solvent A**: water with 0.1% formic acid, **solvent B**: acetonitrile

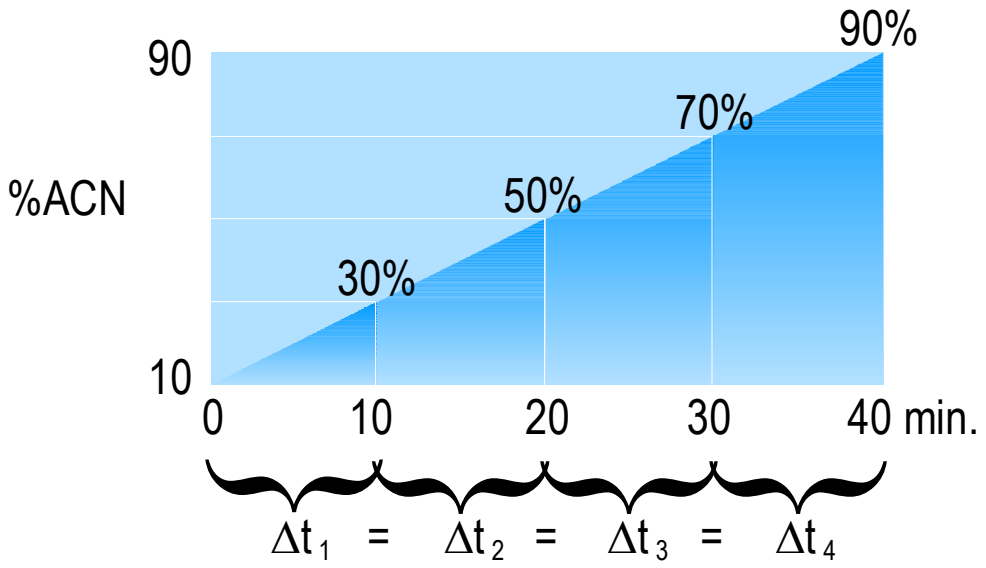
Gradient: 0–3 min 5% B, 5 to 60% B over 22 minutes

Agilent 1200 SL controlled temperature at 25 °C, 2 µL flow cell

Gradient Elution for Reversed-Phase HPLC

Increasing the solvent strength = Increasing the %organic in the mobile phase

Linear solvent strength gradient = % per min is a constant



$\Delta\phi = 80\%$

$t_G = 40$ min.

$\frac{\Delta\phi}{t_G} = 2\%/\text{min.}$

For every 20% change in ACN, △t is 10 min

Three Major Reasons to Choose Gradient Elution

1. Faster separation of samples having components that vary in polarity

2. To separate mixtures having a large number of components

3. Consistent, better peak shape throughout the run

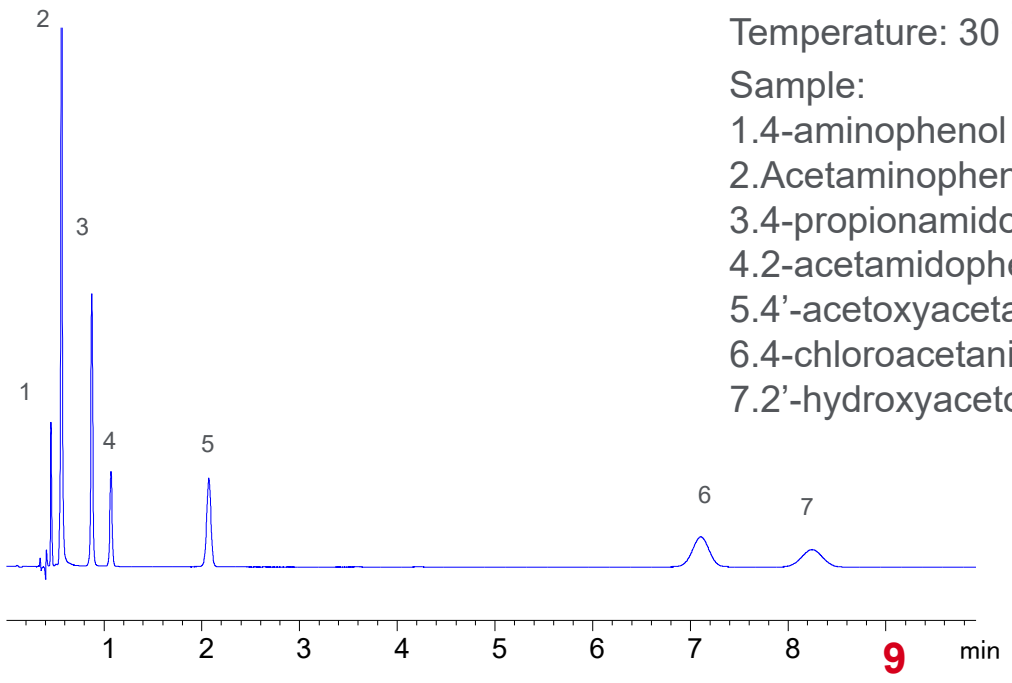
Gradient Separation is Faster Than Isocratic

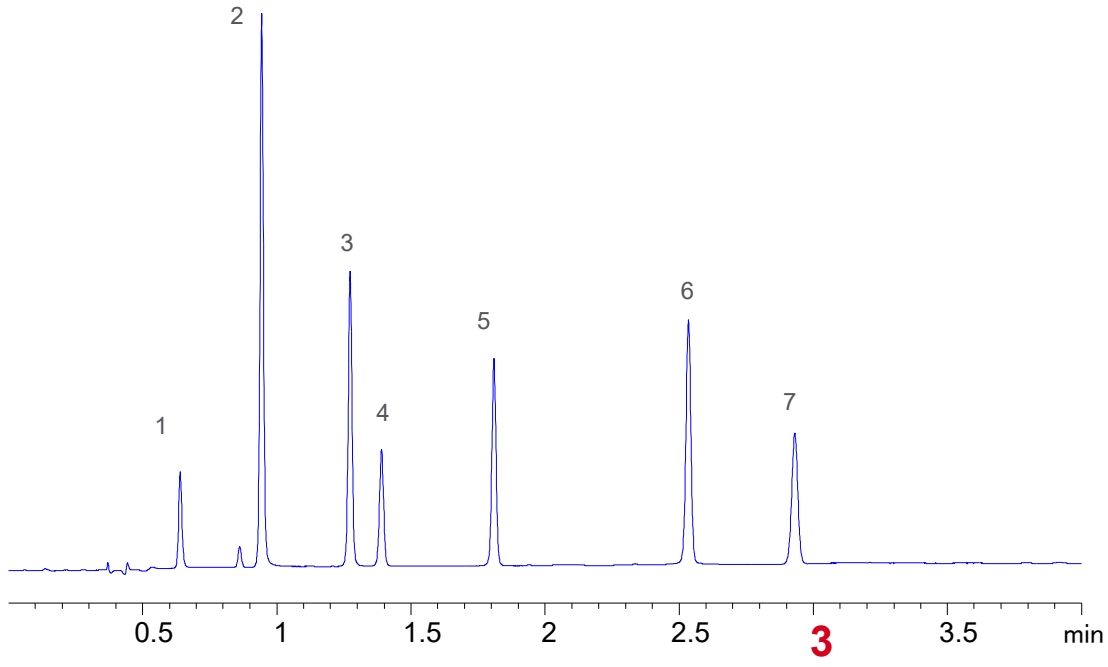
Separation of acetaminophen impurities on Poroshell 120 EC-C18

Isocratic elution:
85% aqueous/15% ACN

Column: Poroshell 120 EC-C18, 4.6 x 50 mm, 2.7 µm
p/n 699975-902
Mobile phase: A: 10 mM ammonium acetate, pH 6.8 B: Acetonitrile
Flow rate: 1.5 mL/min
Temperature: 30 °C
Sample:
1.4-aminophenol
2.Acetaminophen
3.4-propionamidophenol
4.2-acetamidophenol
5.4'-acetoxyacetanilide
6.4-chloroacetanilide
7.2'-hydroxyacetophenone

Gradient elution:
5 to 50% ACN in 5 min





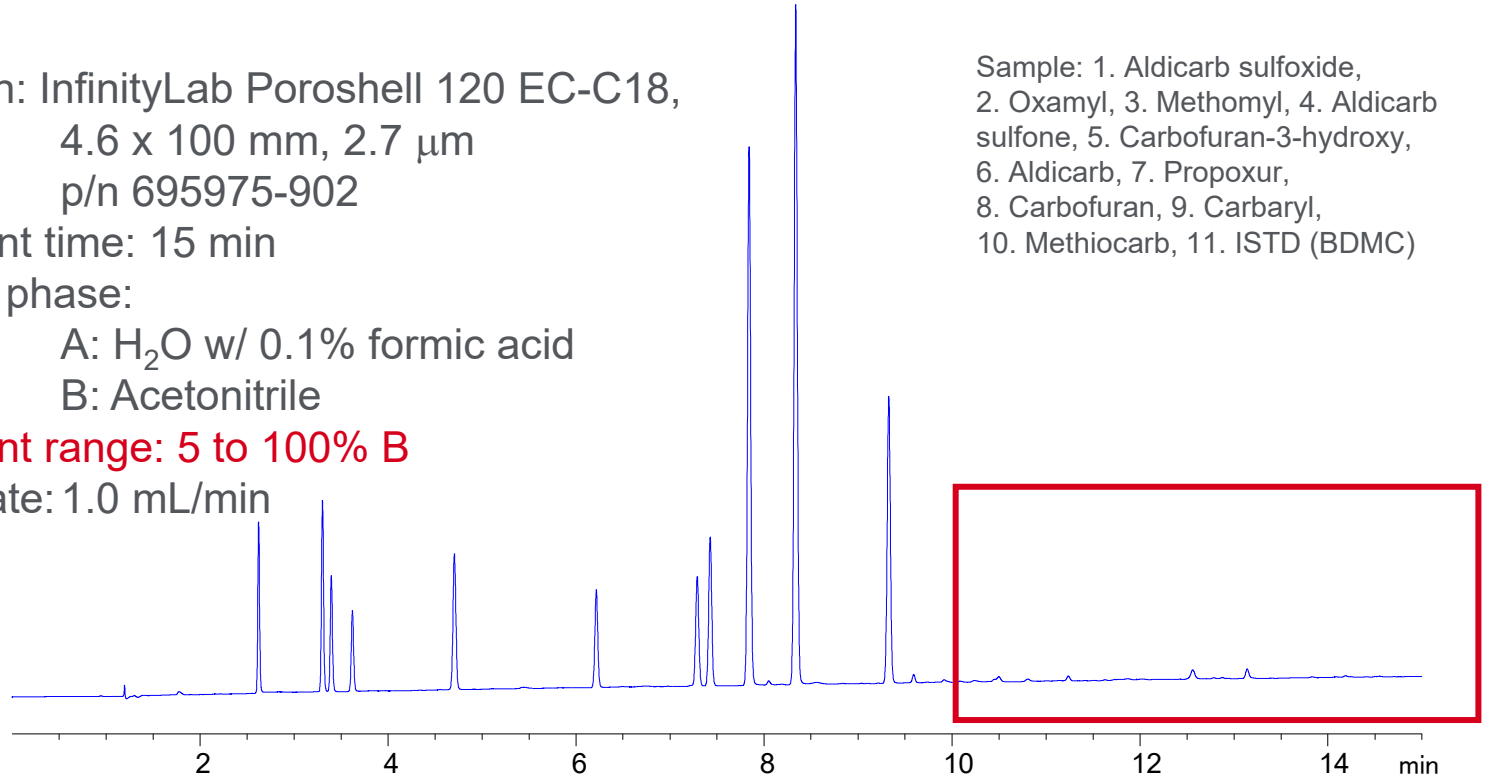
Perform Scouting Gradient

Choose shorter, efficient column

Column: InfinityLab Poroshell 120 EC-C18,
4.6 x 100 mm, 2.7 µm
p/n 695975-902
Gradient time: 15 min
Mobile phase:
A: H_2O w/ 0.1% formic acid
B: Acetonitrile
Gradient range: 5 to 100% B
Flow rate: 1.0 mL/min

Linear gradient
Change %B = 95%
Rate of change: 6.3%/min

Sample: 1. Aldicarb sulfoxide,
2. Oxamyl, 3. Methomyl, 4. Aldicarb
sulfone, 5. Carbofuran-3-hydroxy,
6. Aldicarb, 7. Propoxur,
8. Carbofuran, 9. Carbaryl,
10. Methiocarb, 11. ISTD (BDMC)



The scouting shows that there is wasted time in this chromatogram and resolution of all components can be achieved. Optimization is possible!

Gradient Optimization

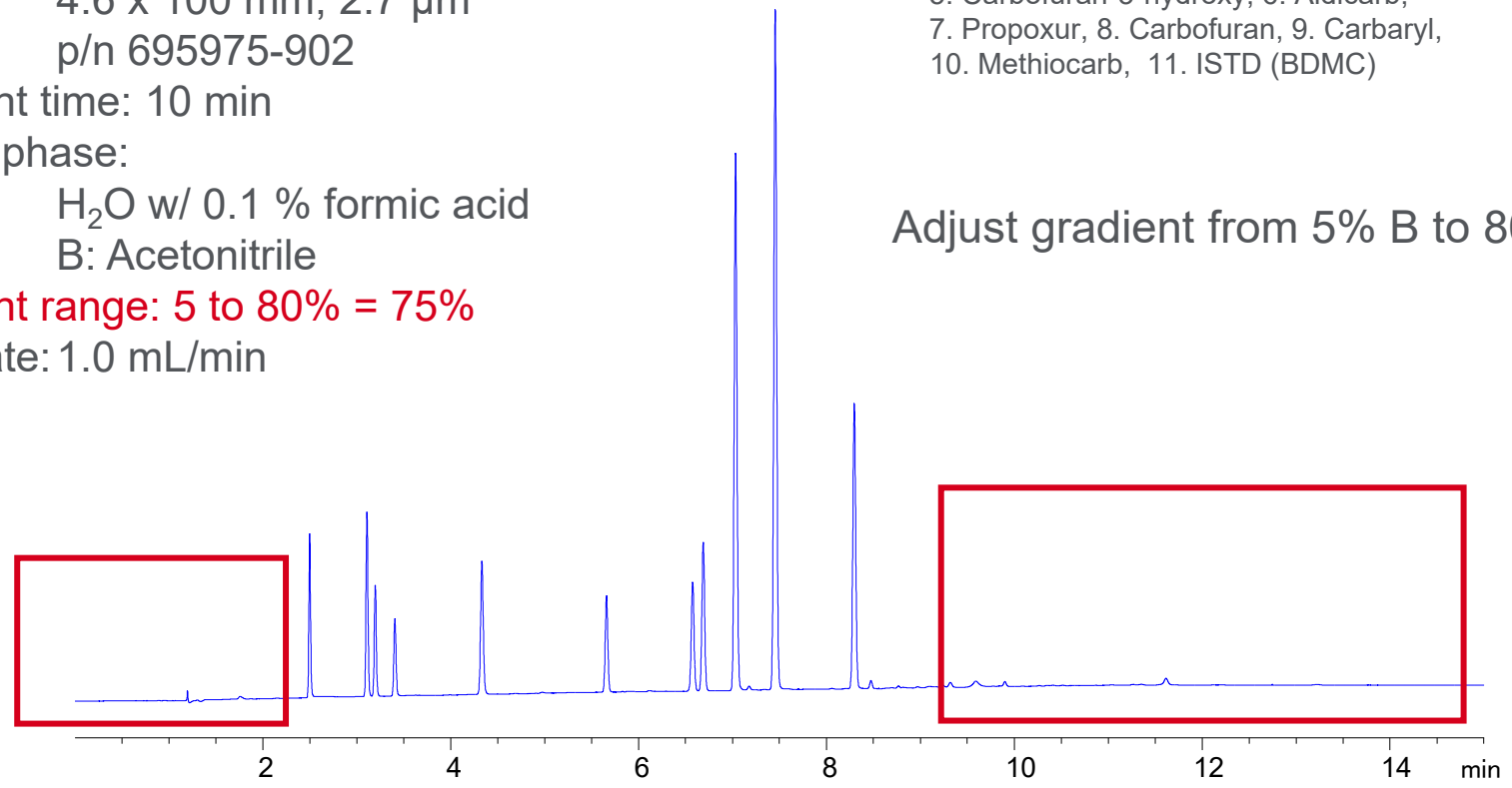
Reduce gradient range to minimize time

Column: InfinityLab Poroshell 120 EC-C18,
4.6 x 100 mm, 2.7 µm
p/n 695975-902
Gradient time: 10 min
Mobile phase:
H_2O w/ 0.1 % formic acid
B: Acetonitrile
Gradient range: 5 to 80% = 75%
Flow rate: 1.0 mL/min

Linear gradient
Change %B = 75%
Rate of change: 7.5%/min

Sample: 1. Aldicarb sulfoxide, 2. Oxamyl,
3. Methomyl, 4. Aldicarb sulfone,
5. Carbofuran-3-hydroxy, 6. Aldicarb,
7. Propoxur, 8. Carbofuran, 9. Carbaryl,
10. Methiocarb, 11. ISTD (BDMC)

Adjust gradient from 5% B to 80% in 10 minutes



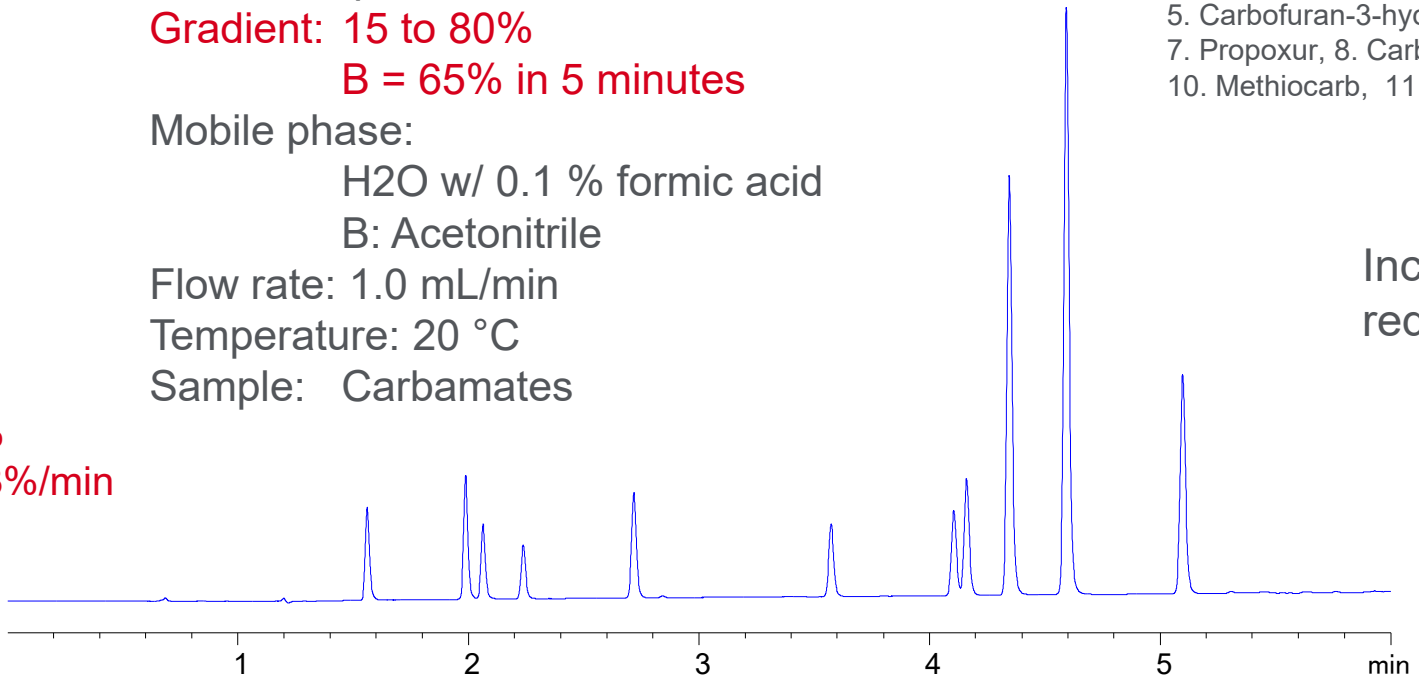
There is still excess time

Further Optimization

Finalize your results

Column: InfinityLab Poroshell 120 EC-C18,
4.6 x 100 mm, 2.7 µm
p/n 695975-902
Gradient: 15 to 80%
B = 65% in 5 minutes
Mobile phase:
H2O w/ 0.1 % formic acid
B: Acetonitrile
Flow rate: 1.0 mL/min
Temperature: 20 °C
Sample: Carbamates

Sample: 1. Aldicarb sulfoxide, 2. Oxamyl,
3. Methomyl, 4. Aldicarb sulfone,
5. Carbofuran-3-hydroxy, 6. Aldicarb,
7. Propoxur, 8. Carbofuran, 9. Carbaryl,
10. Methiocarb, 11. ISTD (BDMC)



Linear gradient
Change %B = 65%
Rate of change: 13%/min

Increase starting % organic and reduce time

Saved 50% of the time with further method optimization.
Used Poroshell 120 2.7 µm for high efficiency and resolution.

Scouting Gradient Works for Any Sample

Acetaminophen example

Column: Poroshell 120 EC-C18, 4.6 x 50 mm 2.7 µm
p/n 699975-902

Mobile phase: A: 10 mM ammonium acetate, pH 6.8
B: Acetonitrile

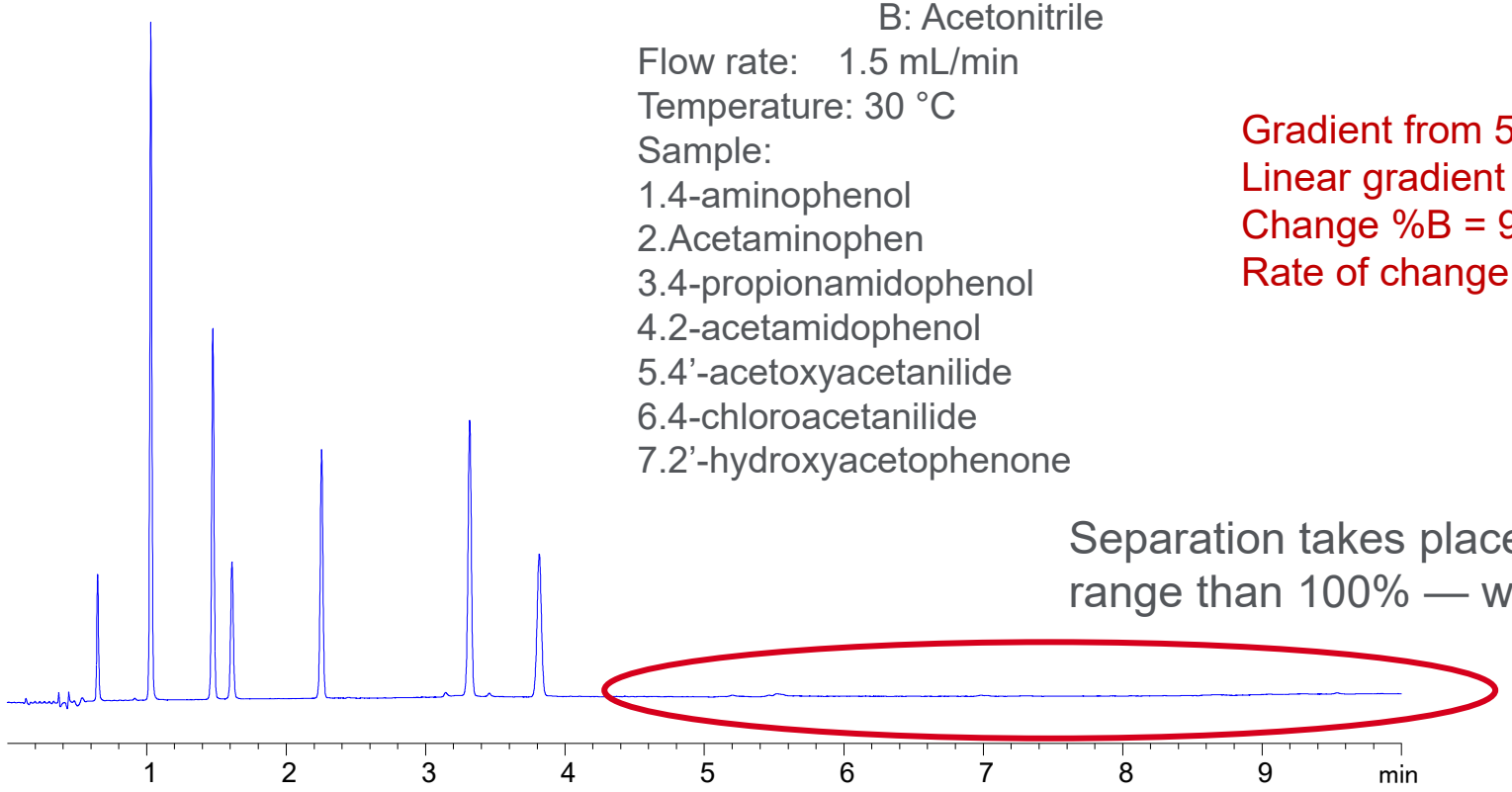
Flow rate: 1.5 mL/min

Temperature: 30 °C

Sample:

1.4-aminophenol
2.Acetaminophen
3.4-propionamidophenol
4.2-acetamidophenol
5.4'-acetoxyacetanilide
6.4-chloroacetanilide
7.2'-hydroxyacetophenone

Gradient from 5% to 100% in 10 minutes
Linear gradient
Change %B = 95%
Rate of change: 9.5%/min



Separation takes place over shorter organic range than 100% — wasted time

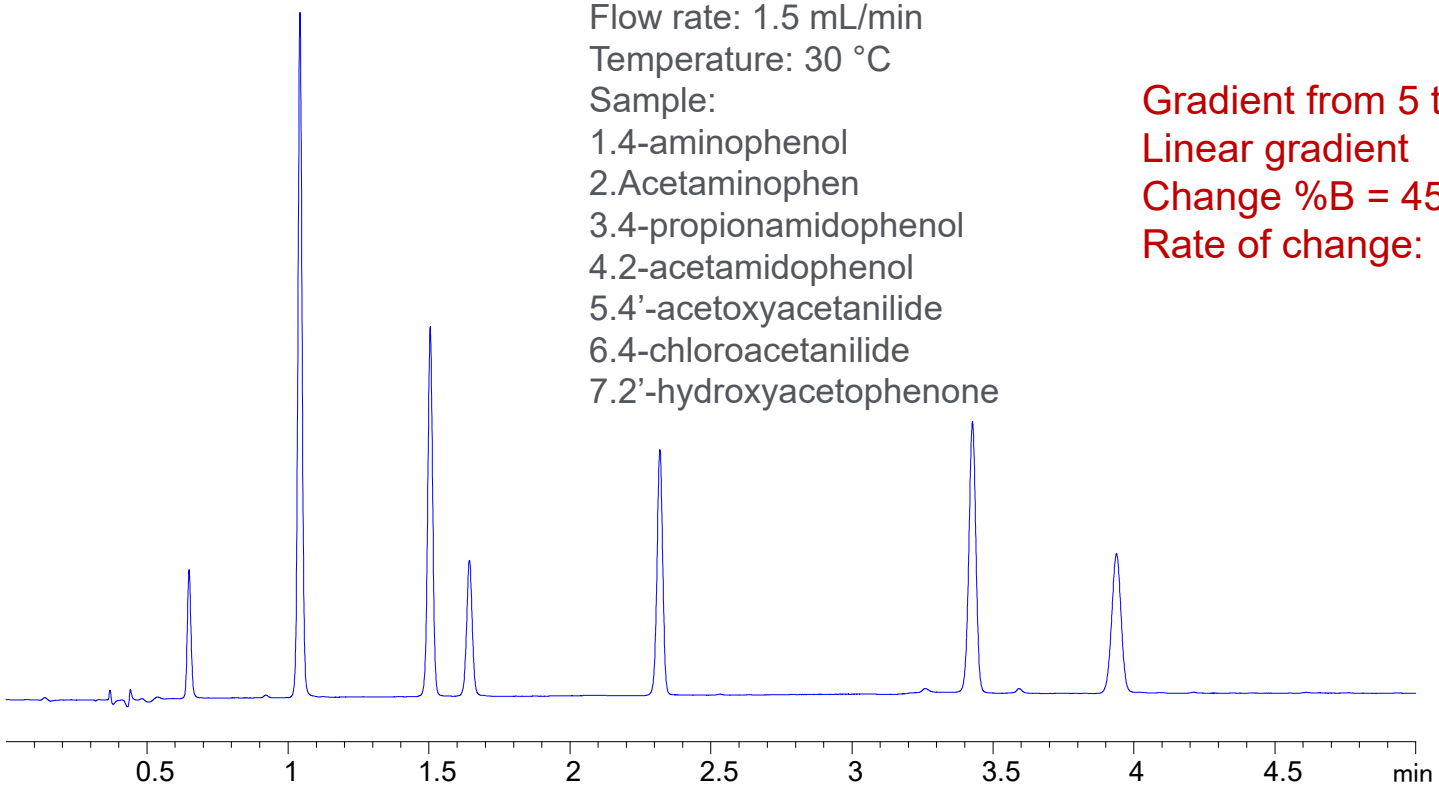
Optimizing Gradient

Adjust gradient to 5 to 50% in 5 minutes to reduce wasted time

Column: Poroshell 120 EC-C18, 4.6 x 50 mm, 2.7 µm
p/n 699975-902

Mobile phase: A: 10 mM ammonium acetate, pH 6.8
B: Acetonitrile
Flow rate: 1.5 mL/min
Temperature: 30 °C
Sample:
1.4-aminophenol
2.Acetaminophen
3.4-propionamidophenol
4.2-acetamidophenol
5.4'-acetoxyacetanilide
6.4-chloroacetanilide
7.2'-hydroxyacetophenone

Gradient from 5 to 50% in 5 minutes
Linear gradient
Change %B = 45%
Rate of change: 4.7%/min



Excellent resolution and distribution of peaks in the gradient – within 5 minutes

With Further Optimization

Reduce time and increase flow rate

Column: Poroshell 120 EC-C18, 4.6 x 50 mm, 2.7 µm
p/n 699975-902

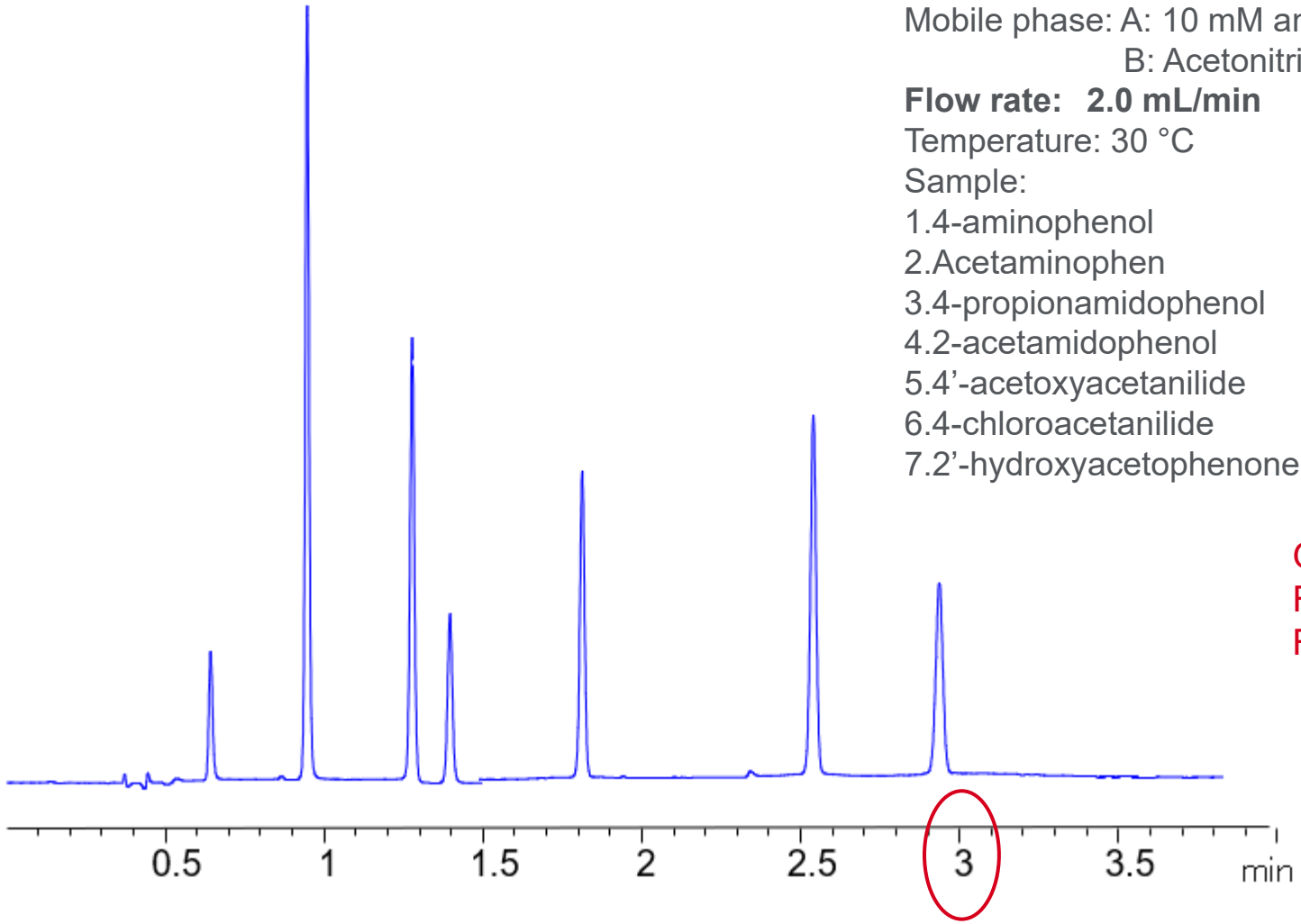
Mobile phase: A: 10 mM ammonium acetate, pH 6.8
B: Acetonitrile

Flow rate: 2.0 mL/min

Temperature: 30 °C

Sample:

1.4-aminophenol
2.Acetaminophen
3.4-propionamidophenol
4.2-acetamidophenol
5.4'-acetoxyacetanilide
6.4-chloroacetanilide
7.2'-hydroxyacetophenone



Gradient from 5 to 50% in 3 min
Flow rate : 2 mL/min
Rate of change: 22.5%/min

Method Development Part 2

Method transfer: considerations for columns

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

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

Δ%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)

Maintaining k*

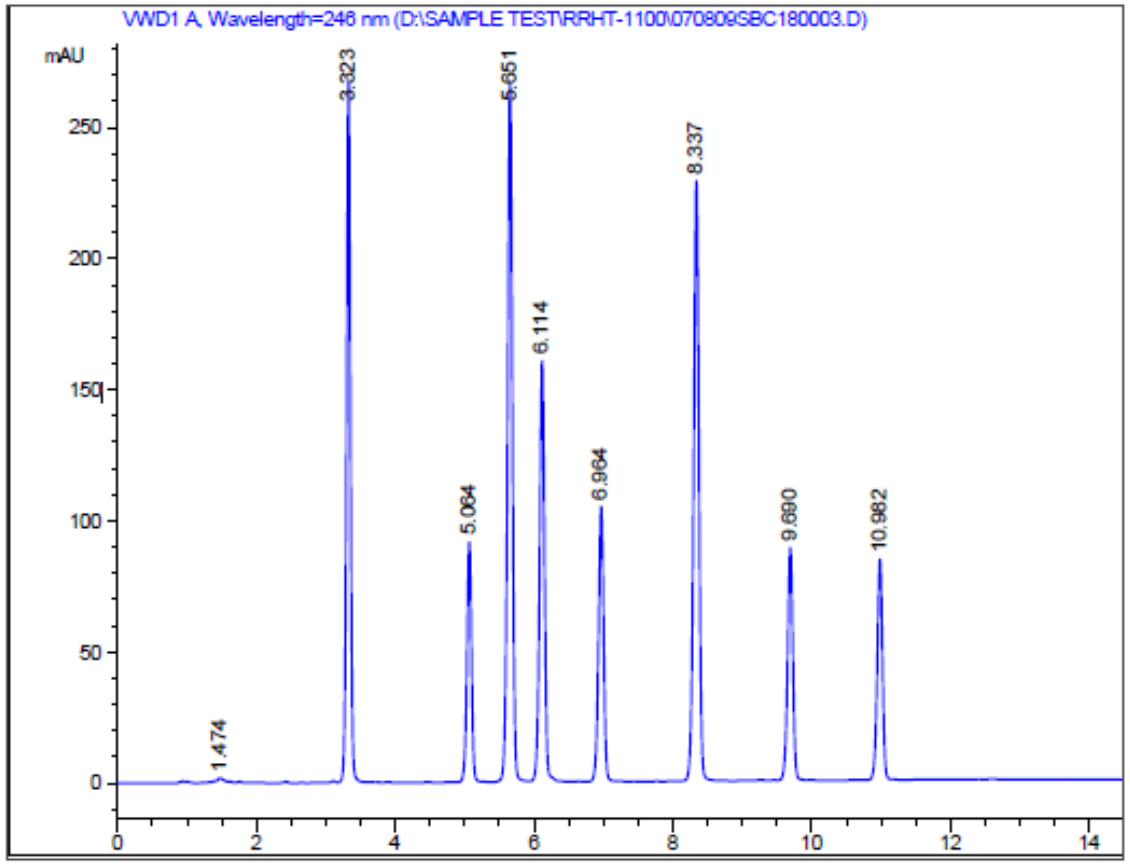
To keep relative peak position unchanged while changing analysis parameters

Any decrease in	Can be offset by a proportional
• Column length	• Decrease in t_G or F • Increase in Δ%B
• Column volume (id)	• Decrease in t_G or F • Increase in Δ%B
• Δ%B (same column)	• Decrease in t_G or F

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

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

p/n 883975-902



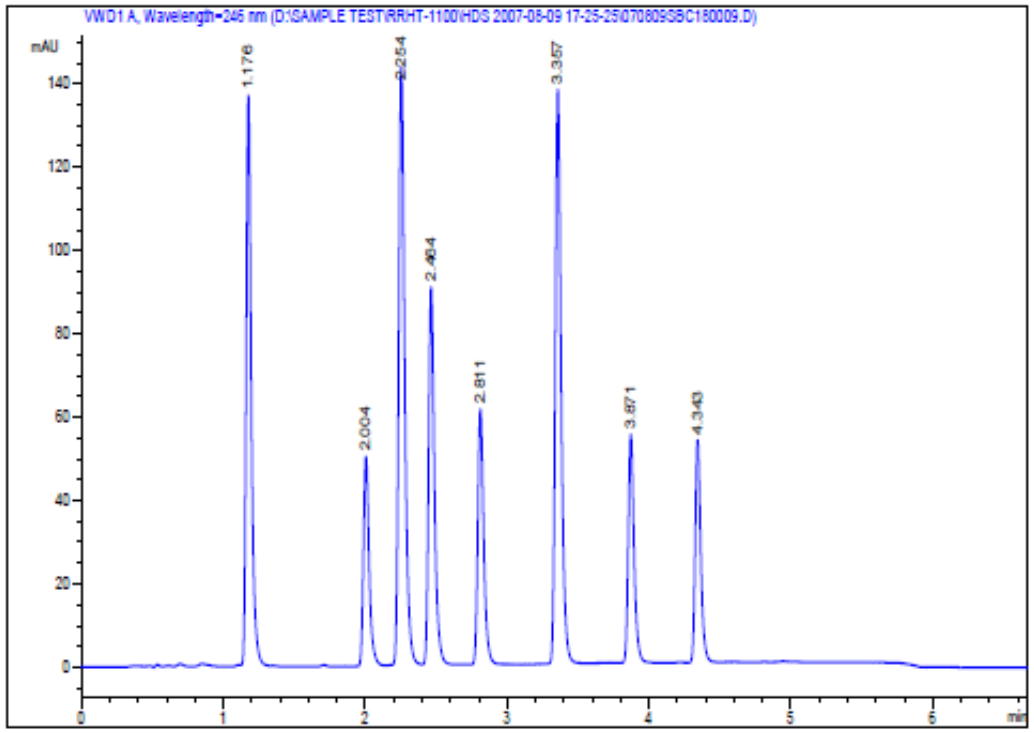
Flow rate	1.0 ml/min
Injection volume	15 µL
Temperature	30° C
Wavelength	246nm
Sample rate	2.5Hz

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

Agilent Zorbax Family of Columns 5994-2212EN

Maintaining Peak Position and Resolution

Have shortened column and gradient time – need to do so by the **same** factor
1/3 column length – 1/3 gradient time
Example: RRHT column – 4.6 x 50 mm, 1.8 µm, SB-C18 p/n 846975-902



Flow rate	1.0 ml/min
Injection volume	15 µL
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

Agilent Zorbax Family of Columns 5994-2212EN

Gradient Steepness and Gradient Shape

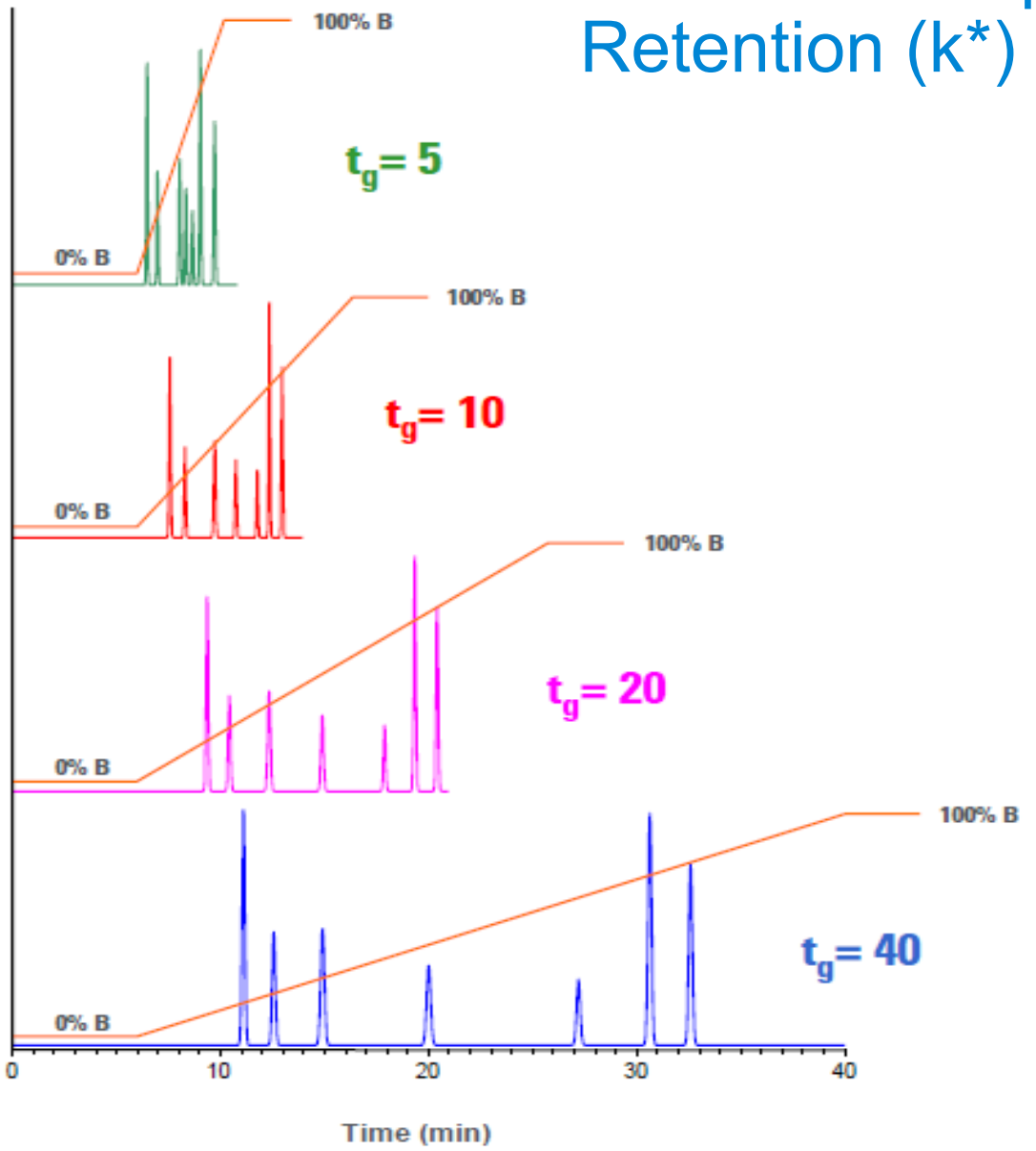
1. Gradient shape

- 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 the desired gradient time and at tg +/- 10-20%
- Small changes are likely, due to instrument performance differences
- Need to compensate for any dwell/delay volume differences first

Gradient Steepness Effects Resolution and 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)

Adapting Gradient Methods to Different Column Dimensions

To adjust gradient methods to different column dimensions, keep gradient steepness (b) the same.

$$1/k^* \propto \text{Gradient steepness} = b = \frac{S \bullet \Delta\Phi \bullet Vm}{t_G \bullet F}$$

S = constant
$\Delta\Phi$ = change in %organic during the gradient run
Vm = void volume of column
F = flow rate
t_G = gradient time
k* = k of solute at mid point of column

If “b” is kept constant from run-to-run, peaks will elute in the same relative pattern.

Adjusting a Gradient from a 4.6 x 150 mm Column to a 2.1 x 100 mm Column

4.6 x 150 mm	2.1 x 100 mm
ΔΦ = 40 (20 to 60%)	ΔΦ = 40 (20 to 60%)
Vm = 1.5 mL	Vm = 0.2 mL
F = 1.0 mL/min	F = 0.2 mL/min
t_G = 15 min	t_G = ? (10 min)

$$b = \frac{\Delta\Phi_1 \cdot V_{m_1}}{F_1 \cdot t_{G_1}} = \frac{\Delta\Phi_2 \cdot V_{m_2}}{F_2 \cdot t_{G_2}}$$

$$t_{G_2} = t_{G_1} \cdot \frac{\Delta\Phi_2}{\Delta\Phi_1} \cdot \frac{V_{m_2}}{V_{m_1}} \cdot \frac{F_1}{F_2}$$

$$t_{G_2} = 15 \cdot \frac{40}{40} \cdot \frac{0.2}{1.5} \cdot \frac{1}{0.2} = 10$$

Adjusting a Gradient from a 4.6 x 150 mm Column to a 2.1 x 100 mm Column for Constant %B

$$t_{G_2} = t_{G_1} \cdot \frac{F_1}{F_2} \cdot \left(\frac{d_2}{d_1}\right)^2 \frac{L_2}{L_1} \cdot \frac{\Delta\Phi_2}{\Delta\Phi_1}$$

for constant %B

$$t_{G_2} = t_{G_1} \cdot \frac{F_1}{F_2} \cdot \left(\frac{d_2}{d_1}\right)^2 \frac{L_2}{L_1}$$

4.6 x 150 m

F = 1.0 mL

2.1 x 100 mm

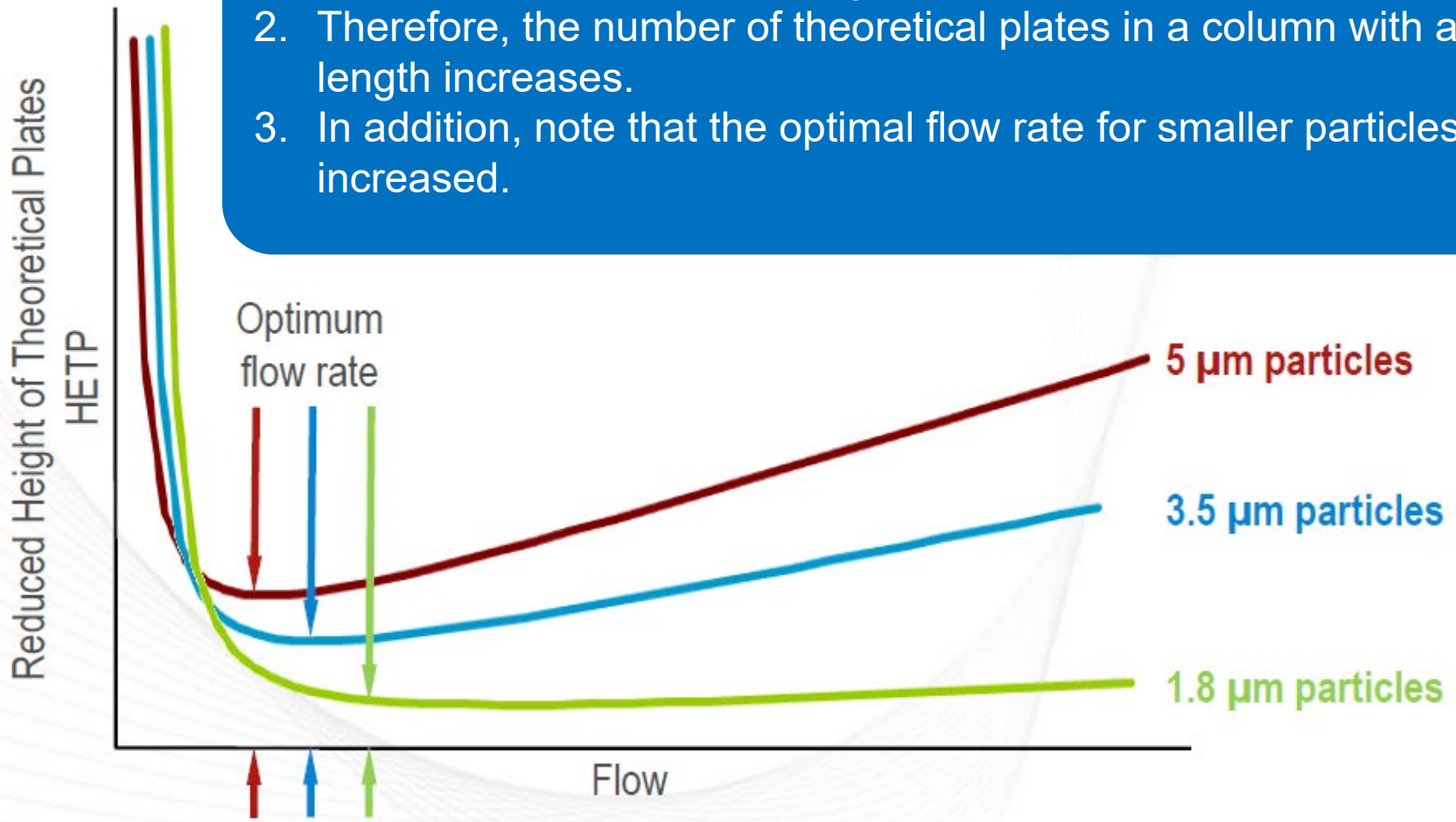
F = 0.2 mL

	%A	%B	t_{old}	t_{new}
	95	5	0	0
	95	5	1	0.7
	35	65	13	9.0
	35	65	14	9.7
	0	100	14.5	10.0
	0	100	16.5	11.5
	95	5	17	11.8
	95	5	20	13.9

Increase Resolution With No Run Time Increase

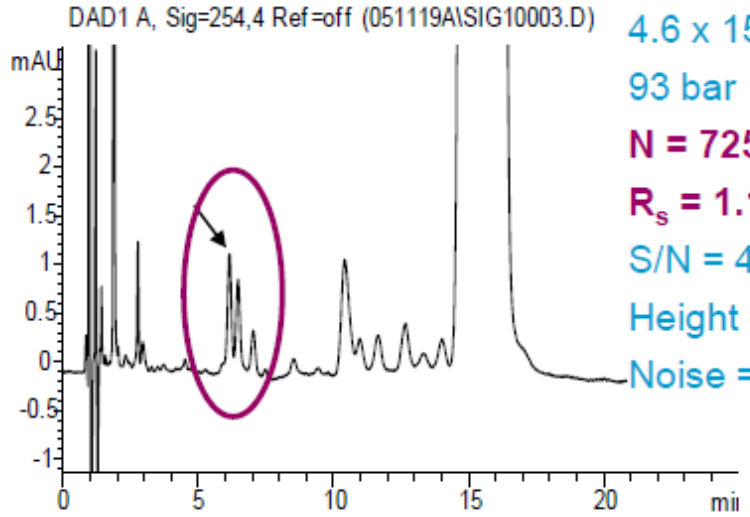
Use a column with a higher number of theoretical plates (N)

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 that the optimal flow rate for smaller particles is increased.

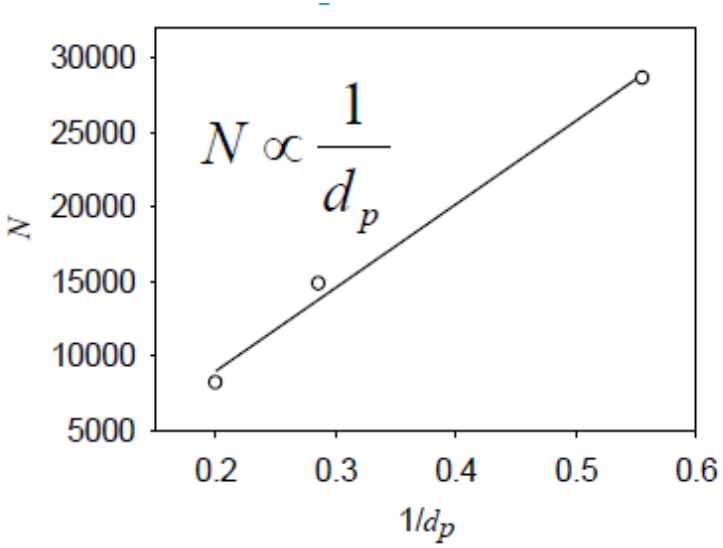


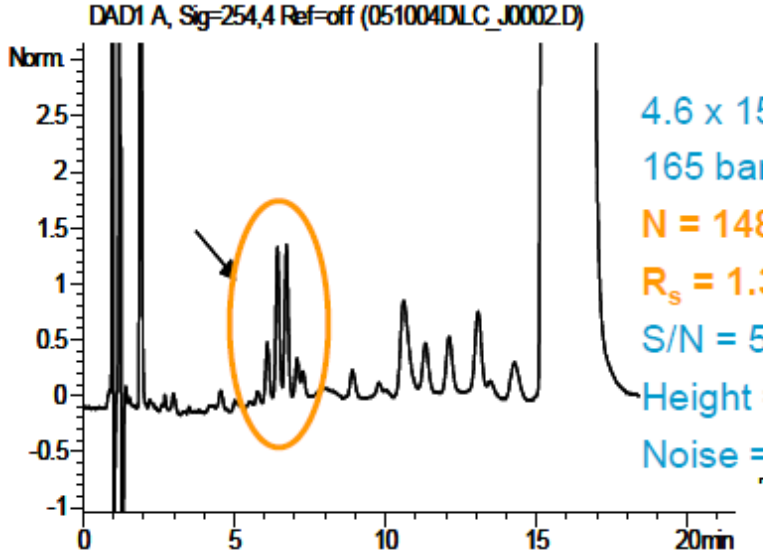
Reduce Particle Size and Maintain Column Length

Increased ‘N” in an isocratic separation – improved resolution

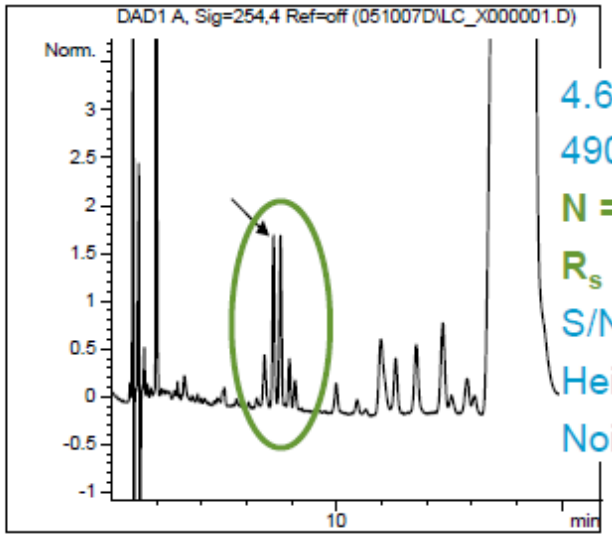


4.6 x 150, **5um**
93 bar
N = 7259
R_s = 1.15
S/N = 42
Height = 1.25
Noise = 24uAU





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 similar efficiency to longer columns with larger particles.

Method Development Part 2

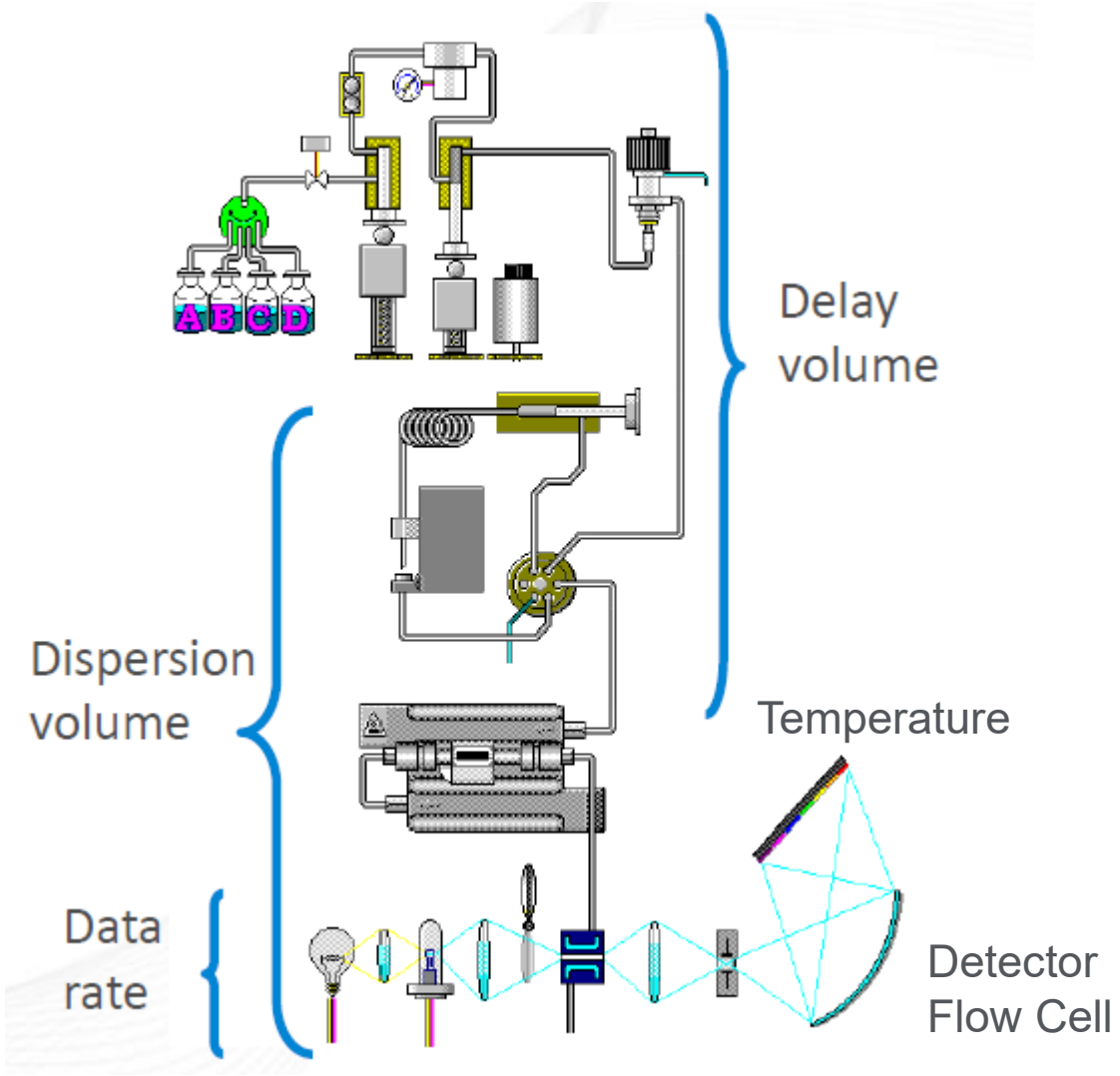
Method transfer: considerations for HPLC systems

Agilent

Transfer of Method to Different Instrumentation

Considerations for LC systems

1. Delay volume
2. Dispersion volume/extracolumn volume
3. Temperature
4. Detector flow cell volume
5. Data collection rate



Delay Volume/Dwell Volume

Why is it important in LC?

- What is it?
- How is it calculated?
- Impact on method transfer

What is it?

Delay volume/dwell volume/gradient delay volume

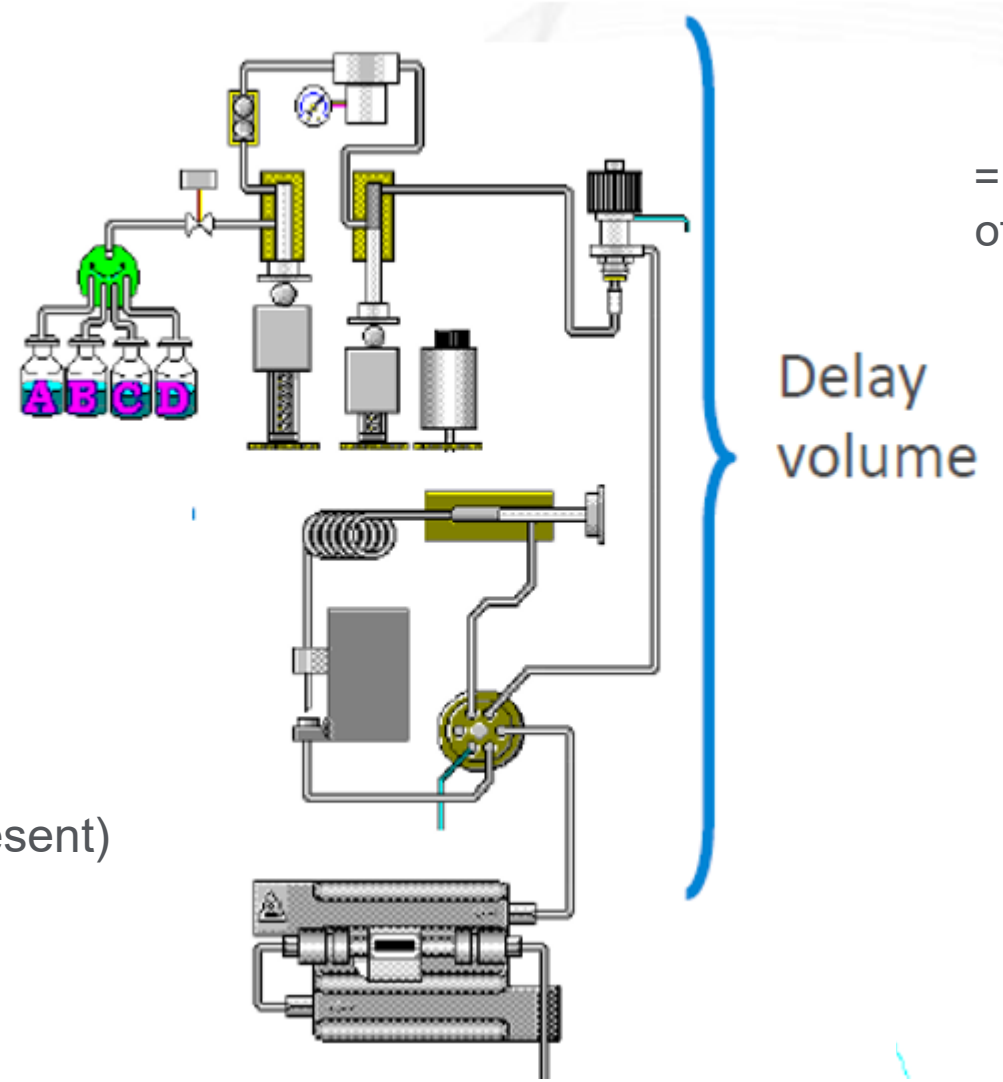
= the system volume from the point of solvent mixing to the head of the column

Delay Volume

Volume Considerations for LC systems

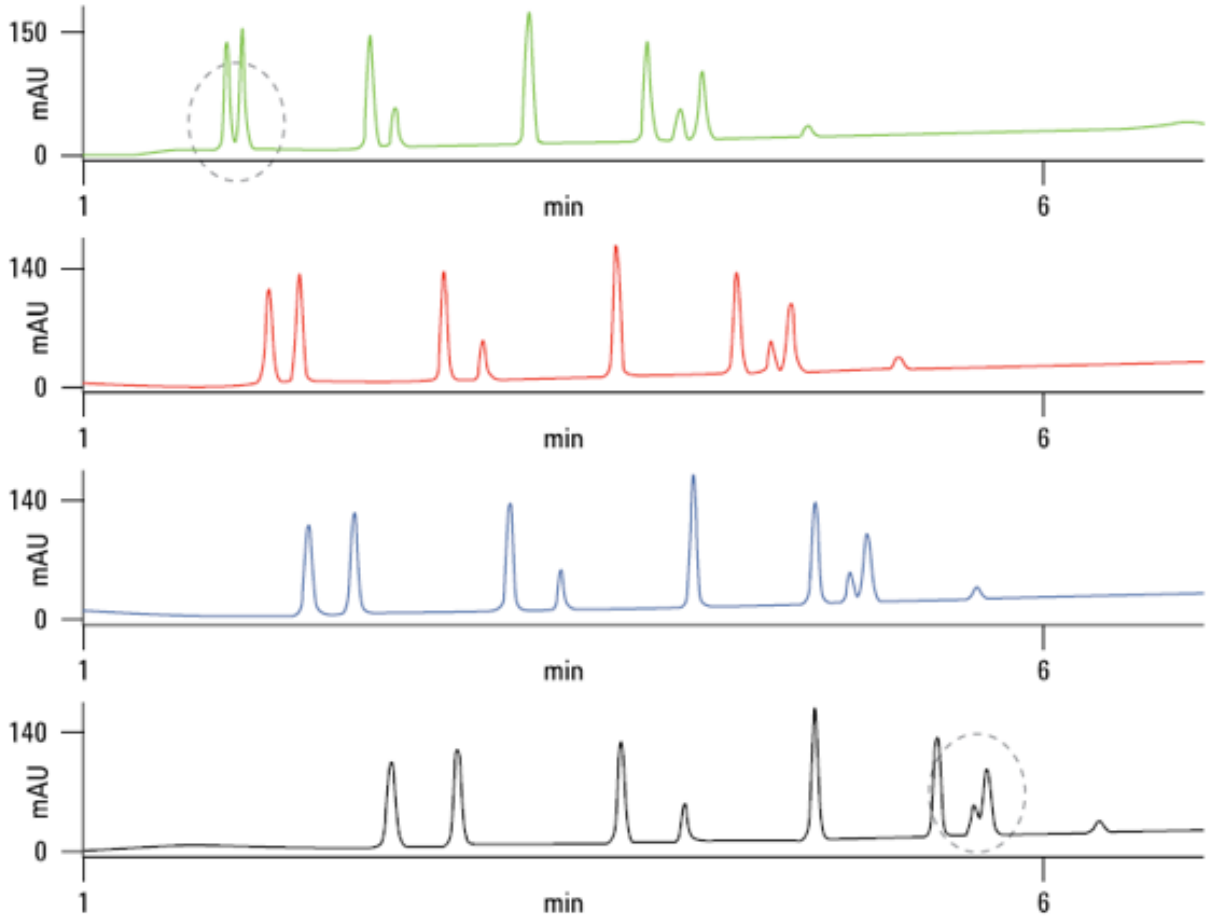
Components of system that influence the delay volume

- Solvent mixer and dampener
- Connecting capillaries
- Hydraulic volume of the sampler
- Volume of heat exchangers (if present)



= the system volume from the point of solvent mixing to the head of the column

Chromatographic Test Results with Different Delay Volumes



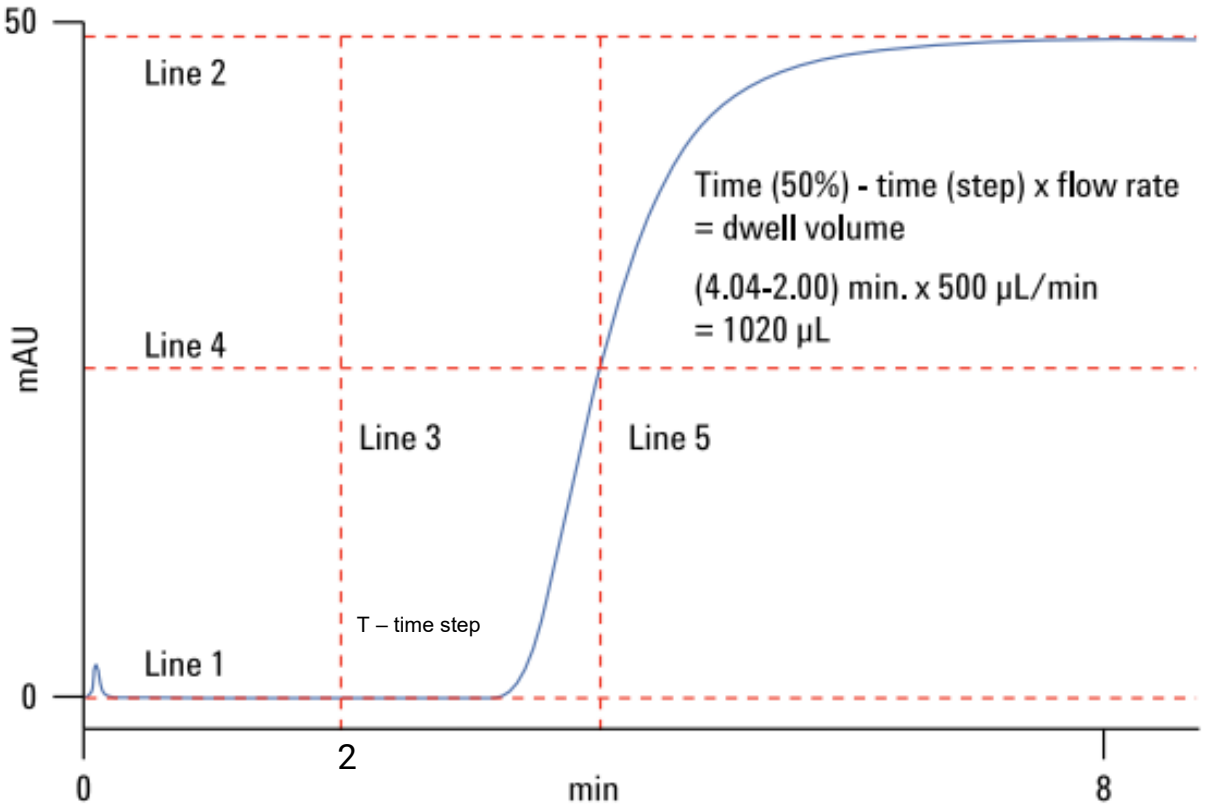
Instrument with smaller dwell volume

Instrument with larger dwell volume

Determining the Delay Volume of Your System

- Look it up in the LC manual or follow the procedure below
- Replace the column with a 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
- Run gradient profile 0 to 100% B for 10 min at 0.5 mL/min
- Record, then print out your gradient trace
- Calculate using the formula and record the value

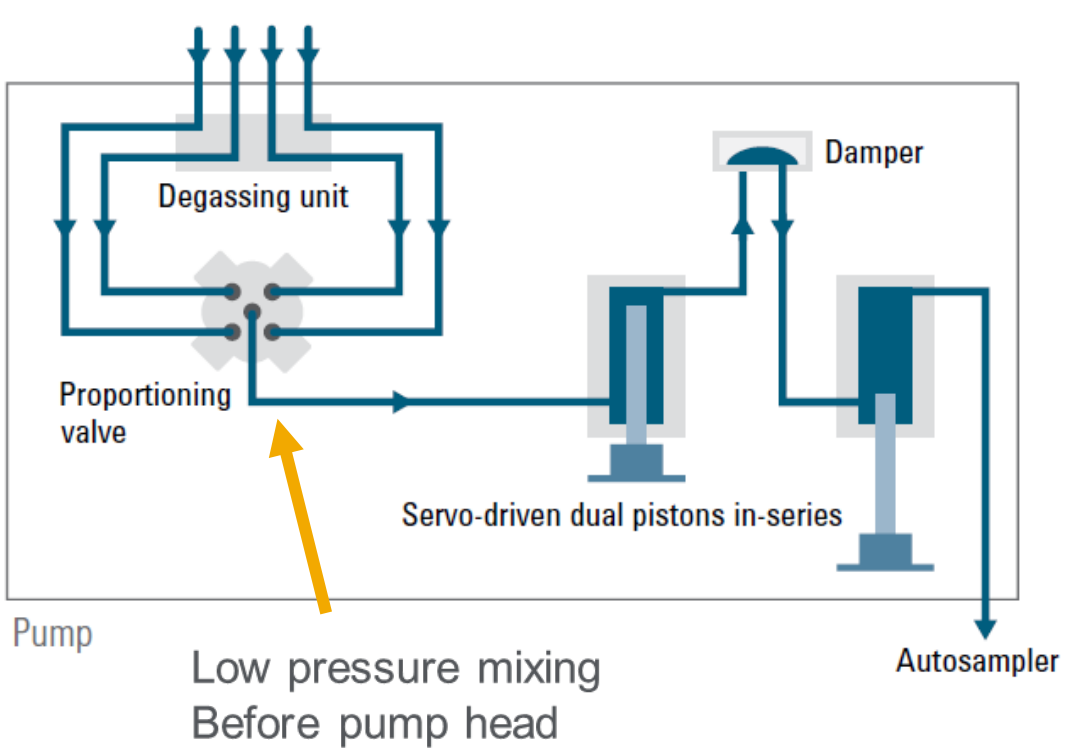
Calculating for Delay Volume



[Importance of Delay Volume in LC - Videos - Webinar Notifications - Agilent Community]

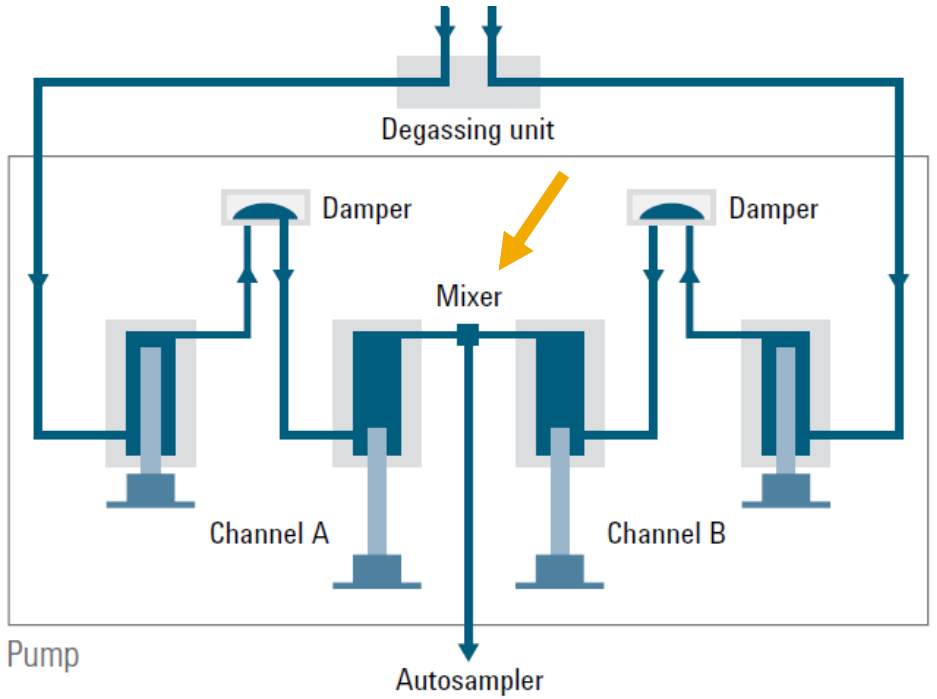
Comparison of Gradient Delay Volume (Dwell Volume)

Quaternary pump example



Low pressure mixing
Before pump head

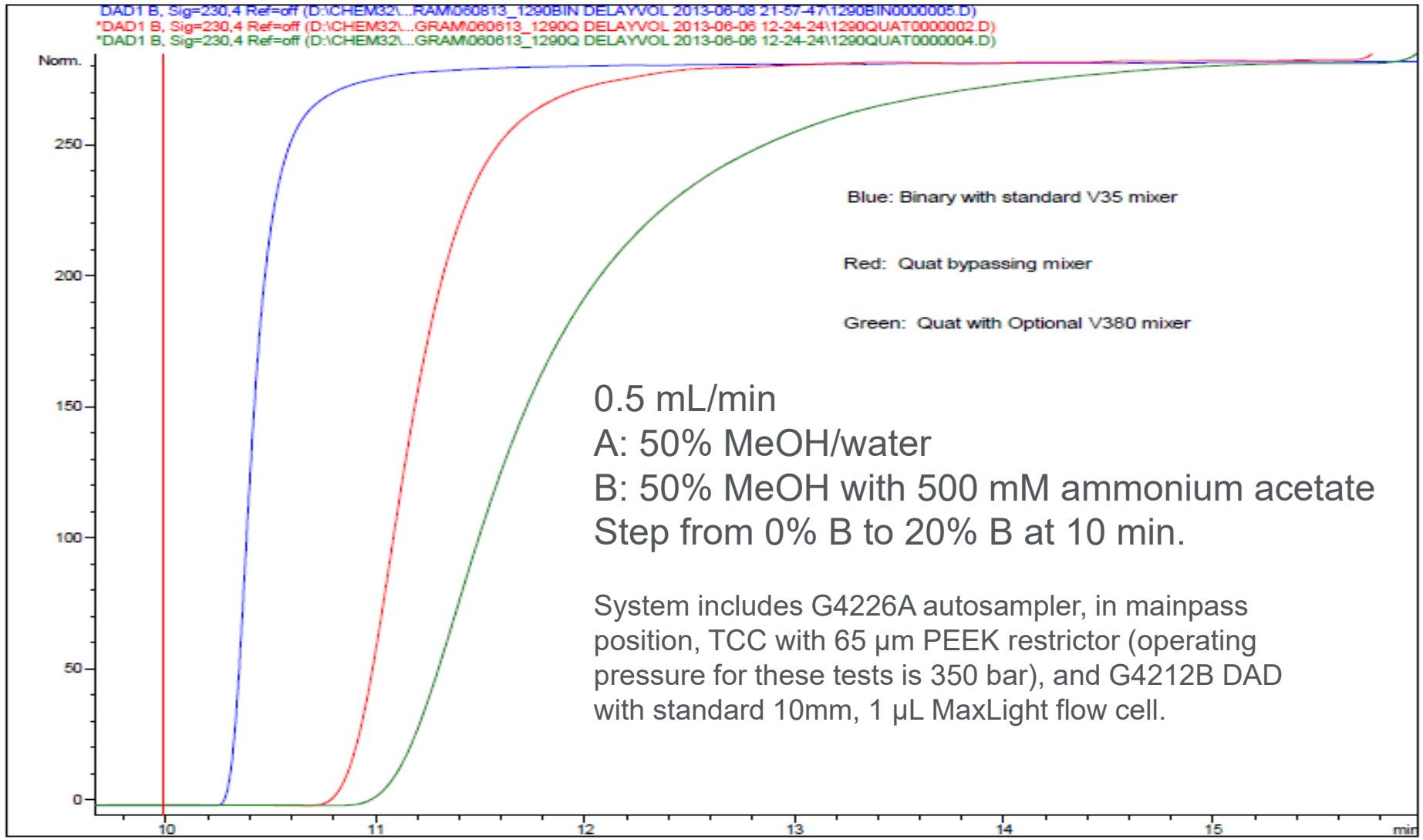
Binary pump example



High pressure mixing
After pump head

A helpful reference is the LC Handbook: [LC-Handbook-Complete-2.pdf (Agilent]

Delay Volume Profiles



Further Instrument Considerations

Dispersion and ECV differences between systems

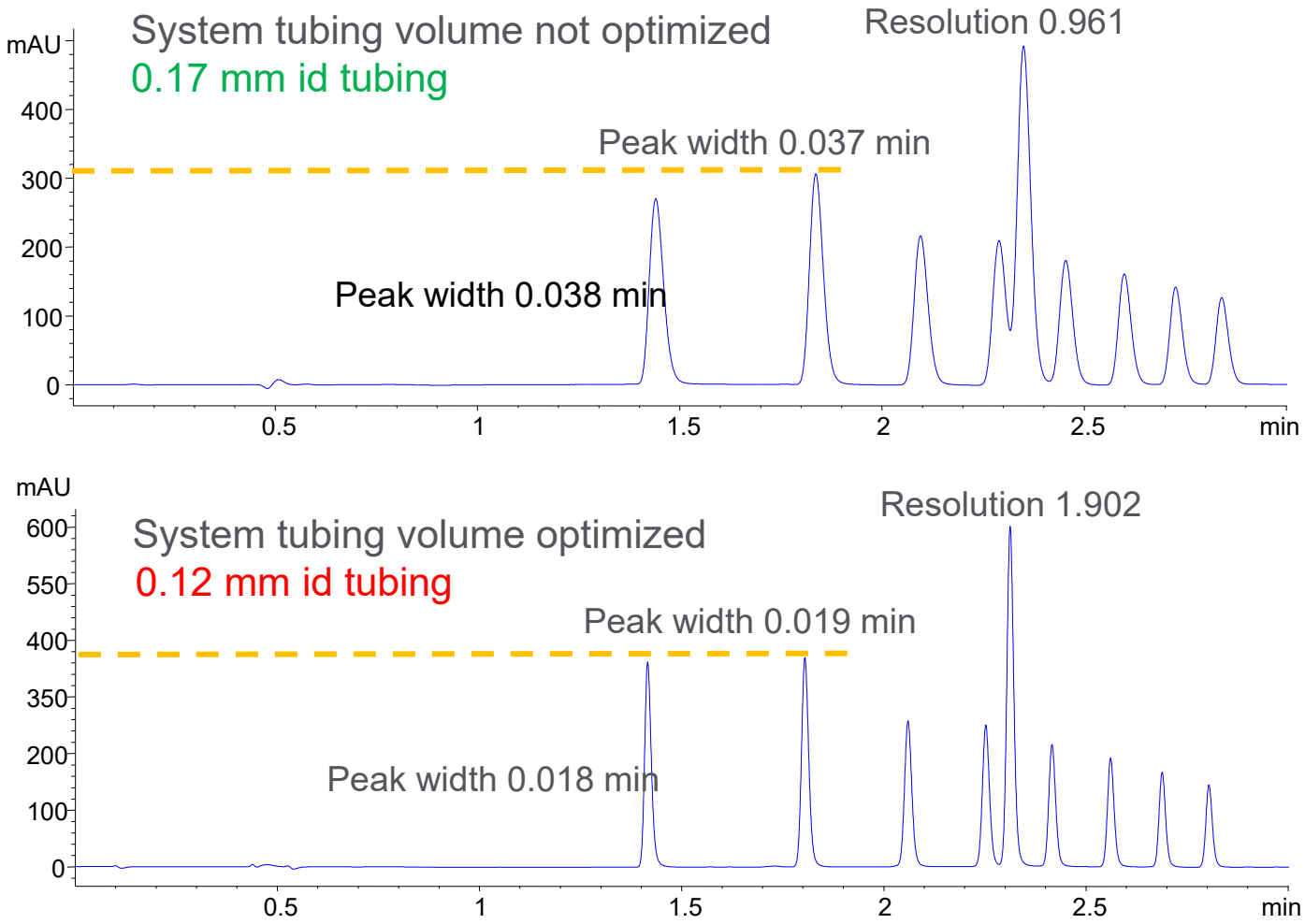
What is dispersion?

- Dispersion is the original sample concentration being diluted as it is carried through the system plumbing (extracolumn volume)

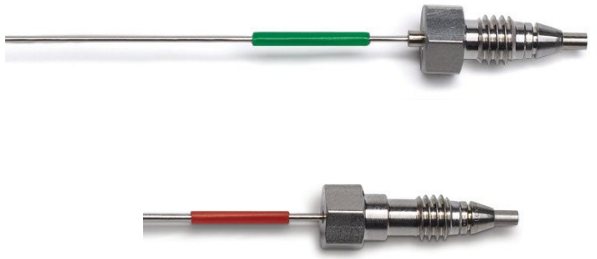
What increases dispersion in the system?

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

Optimizing Connecting Tubing Volume For UHPLC Columns

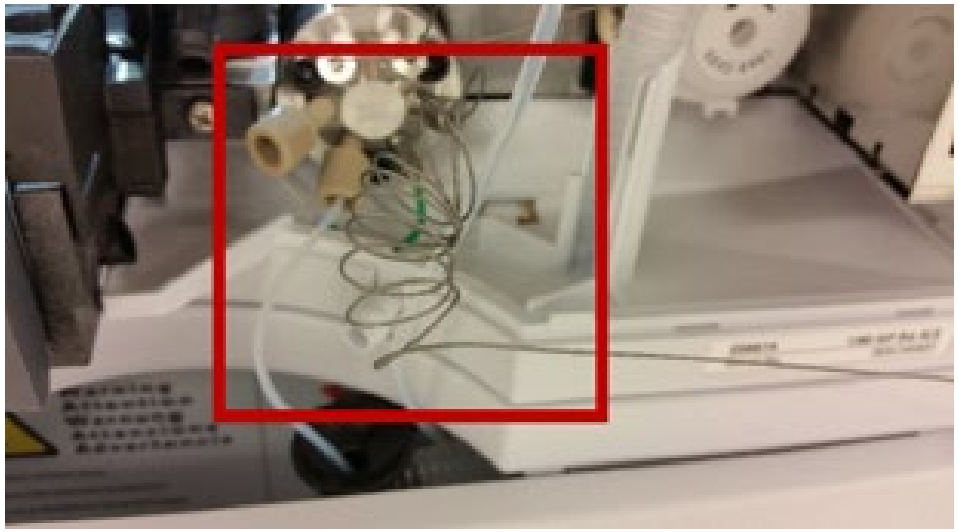


Length	10 mm	50 mm	100 mm	150 mm
Tubing id	Volume	Volume	Volume	Volume
0.17 mm (green)	0.227 µL	1.1 µL	2.27 µL	3.3 µL
0.12 mm (red)	0.113 µL	0.55 µL	1.13 µL	1.65 µL



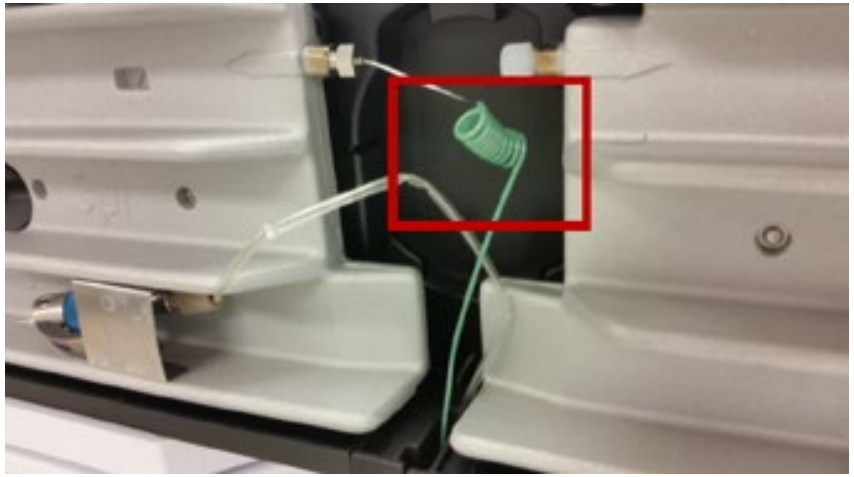
Dispersion and ECV
Where is it found?

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



At the autosampler

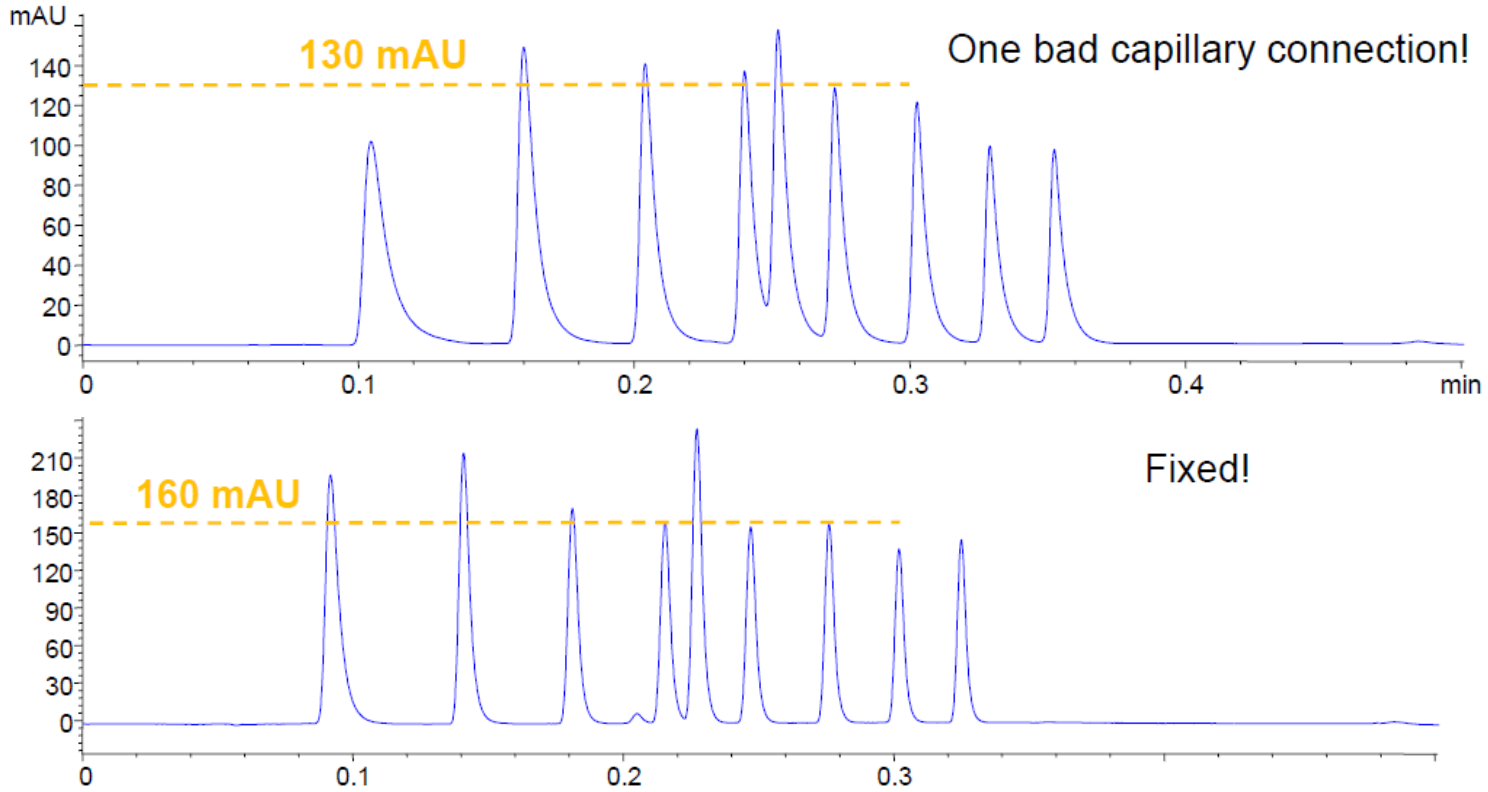
At the detector



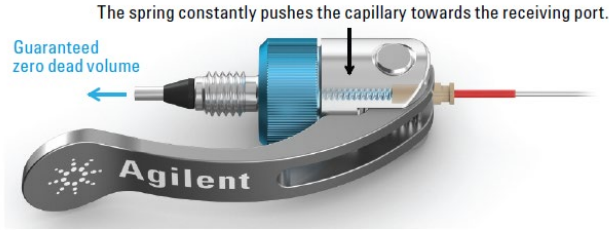
At the column compartment

Between Instruments

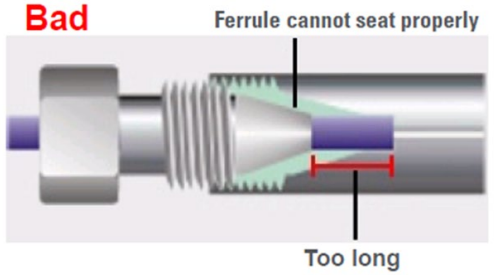
Importance of correct connections

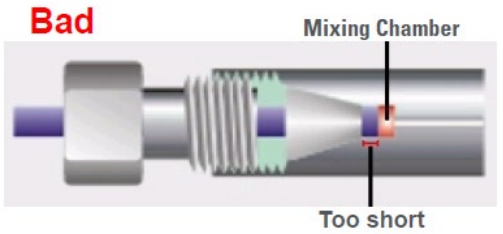


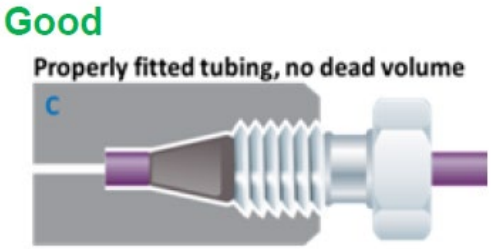
Quick Turn



Quick Connect







Provide for:

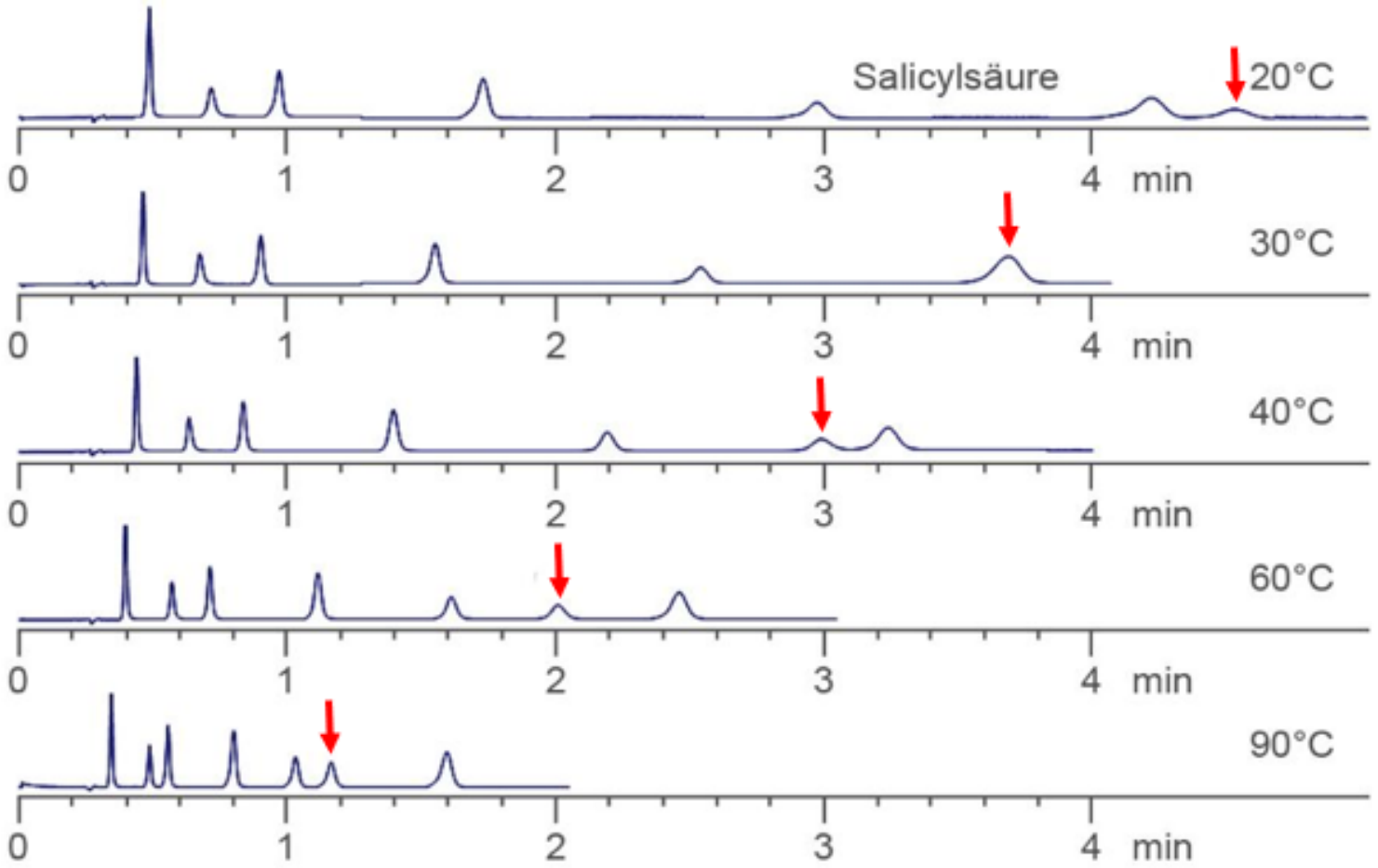
- Correct connection every time
- Compatiblity to 1300 bar

Agilent technical note: Agilent InfinityLab UHPLC Fittings
Publication number: 5991-5525EN

Between Instruments
Temperature Considerations

Same sample analyzed with same column, mobile phase and gradient, but run at different temperatures.

Noticeable differences can be seen for both retention times and peak shape



Between Instruments

Flow Cell volume

Differences in detector flow cell volume can affect resolution (R_s) and plate count (N)

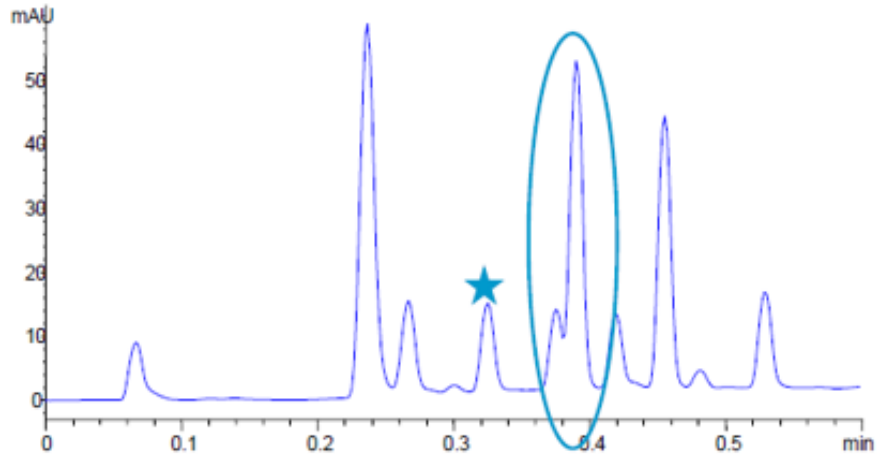
Scenario: Agilent ZORBAX Rapid Resolution column: 75 mm, 3.5 µm; flow rate: 1 mL/min; k = 3

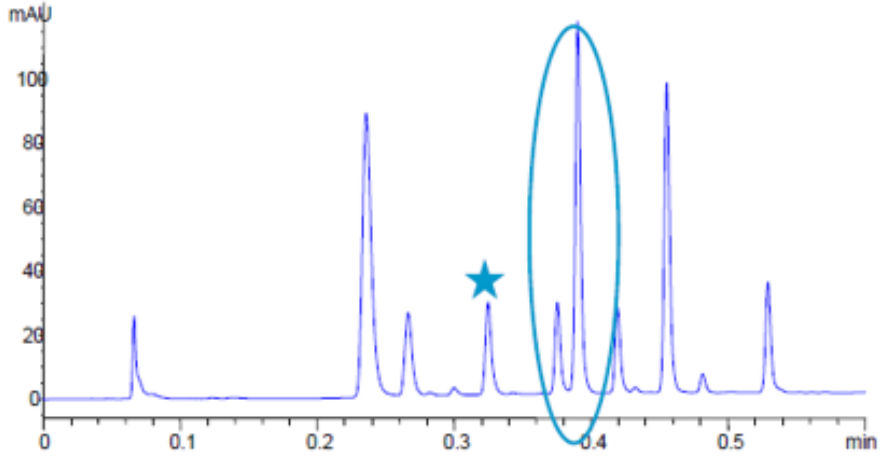
Flow Cell Volume	Band Broadening* (4.6 mm)	Band Broadening* (2.1 mm**)
1.7 µL	0.3%	6%
8 µL	6%	138%
14 µL	19%	423%

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

Between Instruments

System data collection rate





Be sure data collection rate is set properly between instruments

★ *Peak width = 0.021min at 10Hz*

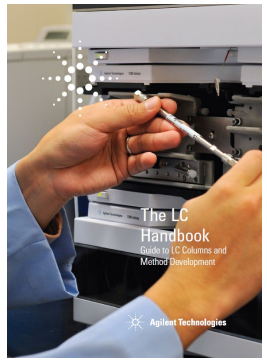
★ *Peak width = 0.017min at 80Hz*

Reminder Points for Method Development

- Outline method goals
- Column selection
- Mobile phase selection
- Flow rates and Injection volumes
- Scouting gradient
- Choosing isocratic versus gradient and optimizing those conditions
- Remember important considerations for method transfer between columns and instrumentation
  - Changes for column particle size, id, length
  - Delay/dwell volume differences
  - ECV/dispersion
  - Temperature/Flow Cells/Data rates

LC Columns and Supplies Resources

- LC Handbook: LC-Handbook-Complete-2.pdf (Agilent
- InfinityLab Poroshell Columns catalog: InfinityLab Poroshell 5991-8750EN
- InfinityLab Poroshell Poster: InfinityLab_Poroshell120 poster 5991-9013EN
- Zorbax Column Family Poster: Agilent Zorbax Family of columns poster
- InfinityLab Supplies catalog: InfinityLab LC Supplies (agilent.com)
- Resource page Agilent HPLC Resources
  - Quick reference guides, product catalogs
  - Online selection tools, “How-to” videos
  - Column user guides - Agilent HPLC Column User Guides
- Tech support: http://www.agilent.com/chem/techsupport
- App finder: Application Finder | Agilent
- LC troubleshooting poster: LC Troubleshooting Guide (Agilent.com)
- Agilent Community: Agilent Community
- Consumables Community: Agilent Collection of Columns, Supplies, and Standards Resources - Consumables - Agilent Community
- Your local product specialists
- Webinars, upcoming and recorded: LC & LC/MS Column Webinars | Agilent



Contact Agilent Chemistries and Supplies Technical Support

Available in the U.S. and Canada, 8-5 all time zones

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

Option 5 for chemical standards

Option 6 for Prozyme products

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