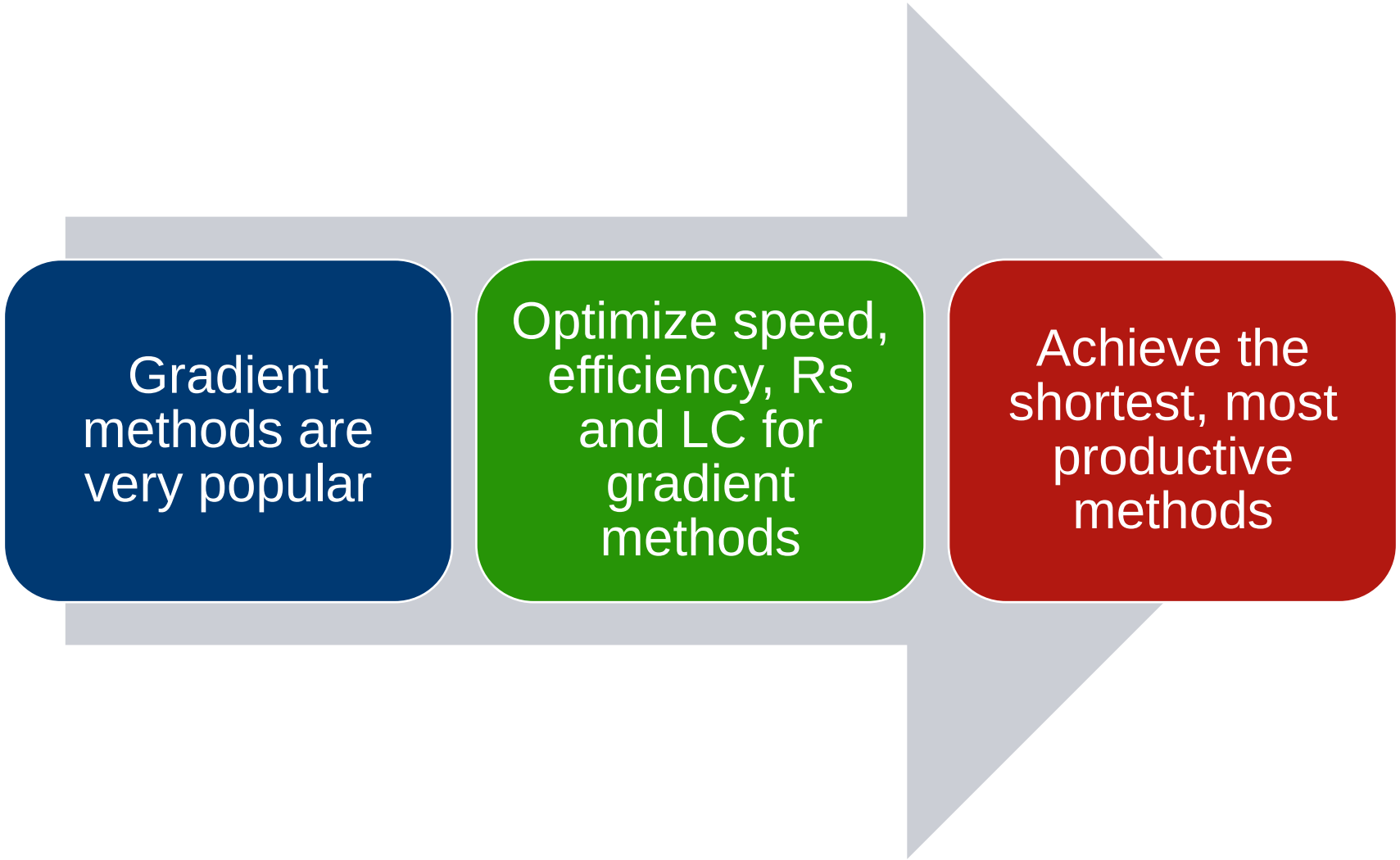
Good Habits for Successful Gradient Separations

Getting the most from your method

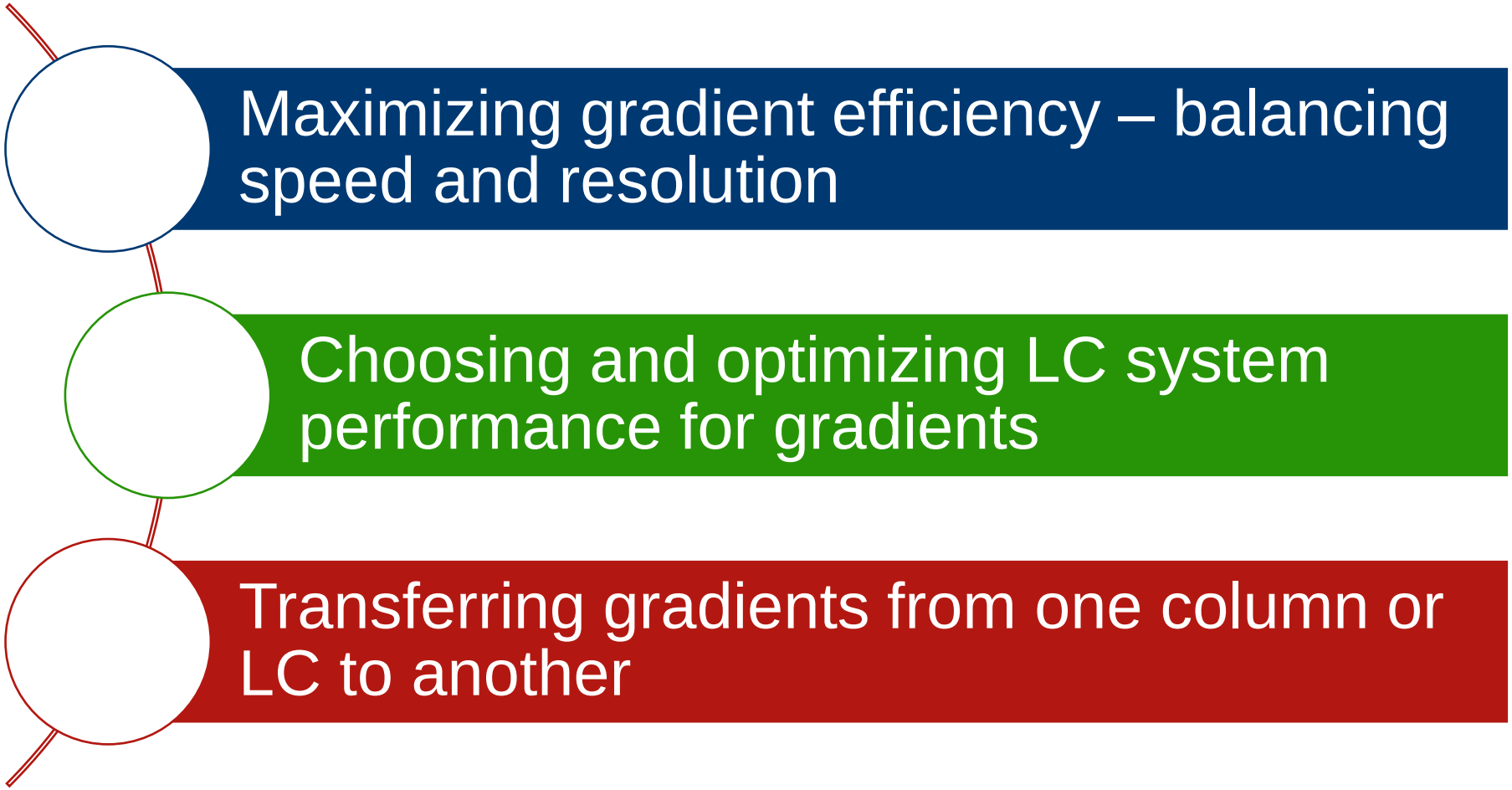
Good Habits for Successful Gradient Separations

Developing good gradient habits is the key to long term success. In this session we will start by discussing what it takes to maximize gradient efficiency by balancing gradient speed with adequate resolution needs. Since even the best gradient can be compromised we are going to look at optimizing LC system performance by minimizing un-needed physical volume, making full use of system functions for maximum efficiency and understanding the gradient delay volume effect on performance. Last, but not least we will demonstrate successfully transferring gradients from one instrument to another.

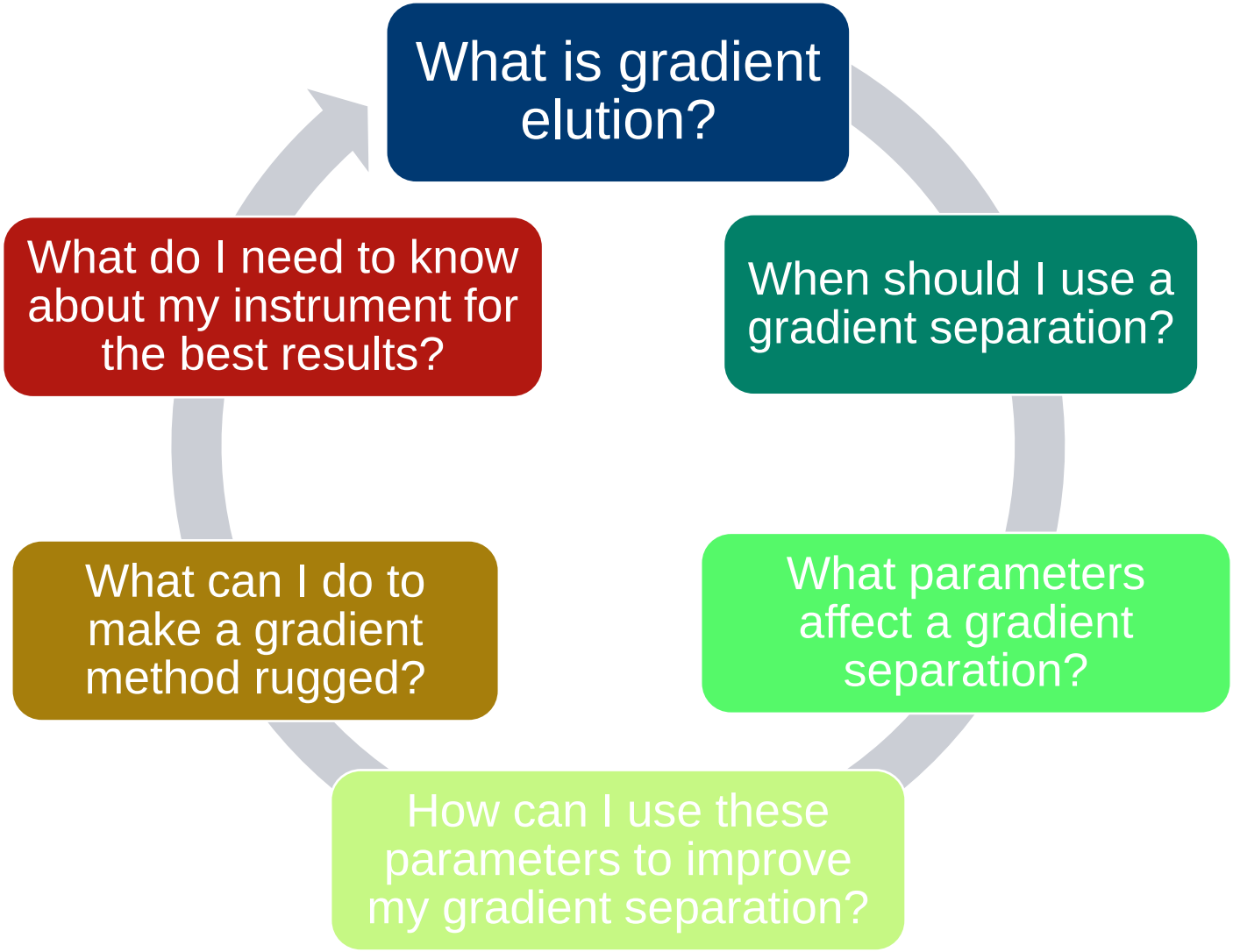
Good Habits for Successful Gradient Separations



Good Habits for Successful Gradient Separations

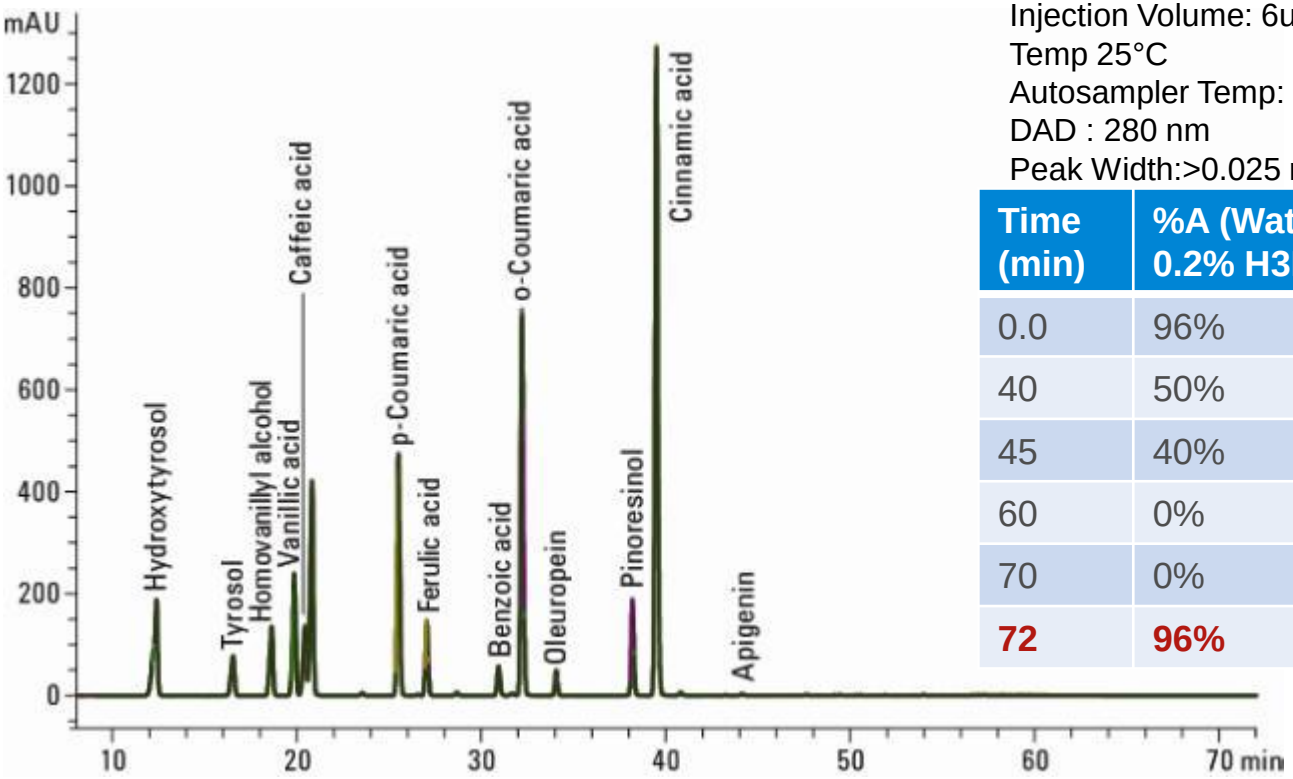


Gradient Elution – So Many Questions!



Gradient HPLC Separation of Phenolic Compounds in Olive Oil – Traditional Column

The old approach! Long column, long analysis!

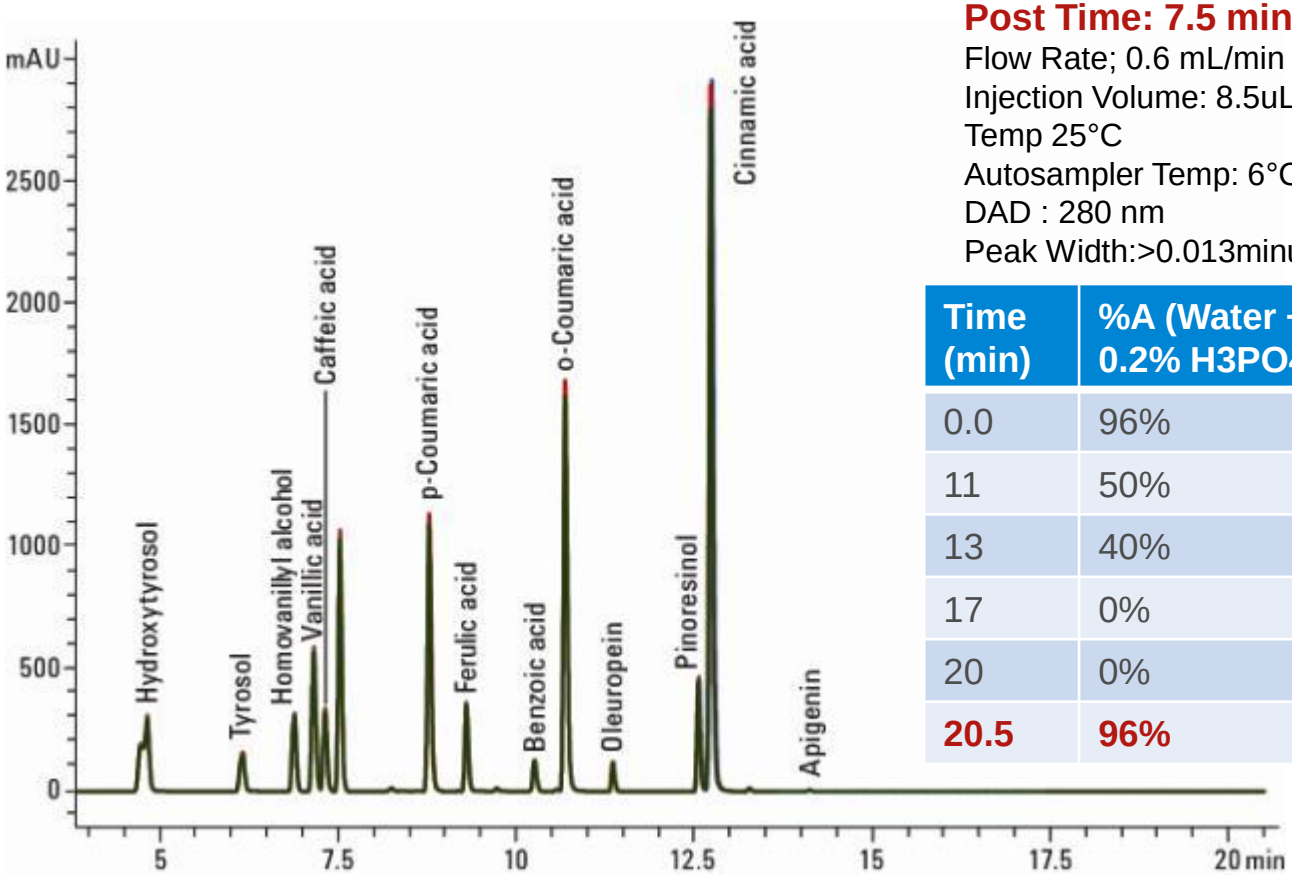


Column: Eclipse Plus C18, 4.6 x 250mm, 5um
Mobile Phase: A: water +0.2% H3PO4 B: Methanol C: Acetonitrile
Gradient: see below
Post Time: 20 min
Flow Rate; 1mL//min
Injection Volume: 6uL
Temp 25°C
Autosampler Temp: 6°C
DAD : 280 nm
Peak Width:>0.025 minutes (10Hz)

Time (min)	%A (Water + 0.2% H3PO4	%B (Methanol)	%C (ACN)
0.0	96%	2%	2%
40	50%	25%	25%
45	40%	30%	30%
60	0%	50%	50%
70	0%	50%	50%
72	**96%**	**2%**	**2%**

Gradient HPLC Separation of Phenolic Compounds in Olive Oil – UHPLC Column

Save 64 min per run and improve resolution!



Column: Eclipse Plus C18, 3.0 x 100mm, 1.8um
Mobile Phase: A: water +0.2% H3PO4 B: Methanol C: Acetonitrile
Gradient: see below
Post Time: 7.5 min
Flow Rate; 0.6 mL/min
Injection Volume: 8.5uL
Temp 25°C
Autosampler Temp: 6°C
DAD : 280 nm
Peak Width:>0.013minutes (20Hz)

Time (min)	%A (Water + 0.2% H3PO4	%B (Methanol)	%C (ACN)
0.0	96%	2%	2%
11	50%	25%	25%
13	40%	30%	30%
17	0%	50%	50%
20	0%	50%	50%
20.5	**96%**	**2%**	**2%**

What Habits do we Need to Get from Too Long to Short and Efficient Gradient Results

Selecting a more time efficient column – shorter, smaller particle size

- Reduce analysis time
- Reduce re-equilibration time, use appropriate post time

Optimize the gradient

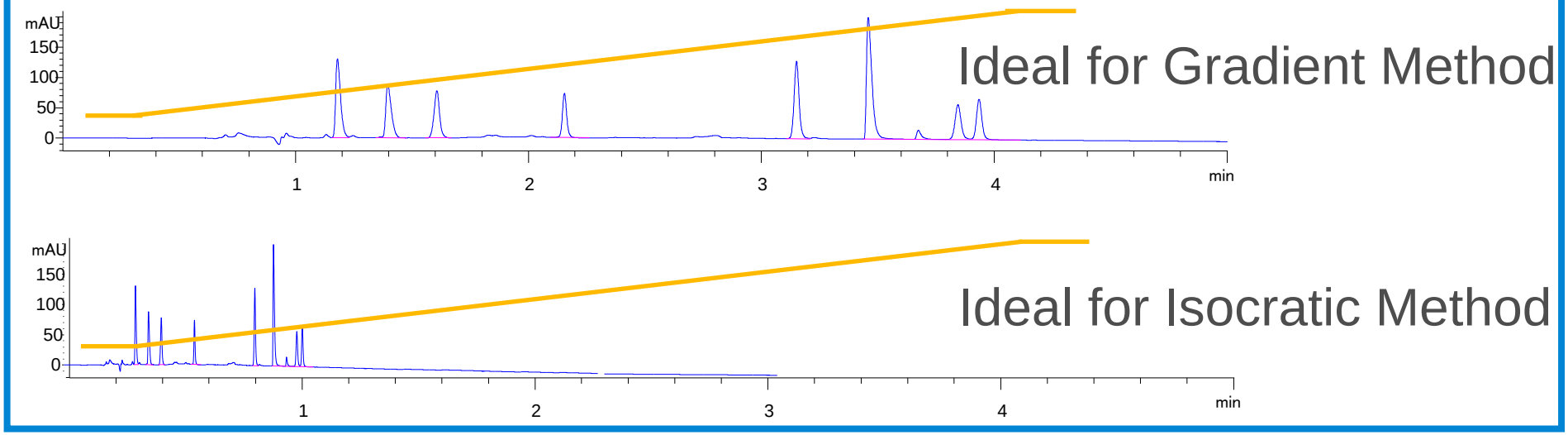
- Achieve maximum resolution in the minimum time
- Consider the complete gradient range

Optimize the LC

- Are LC parameters set correctly?
- Is it configured to deliver the rapid results you need?

Gradients are Critical Tools for Faster Methods

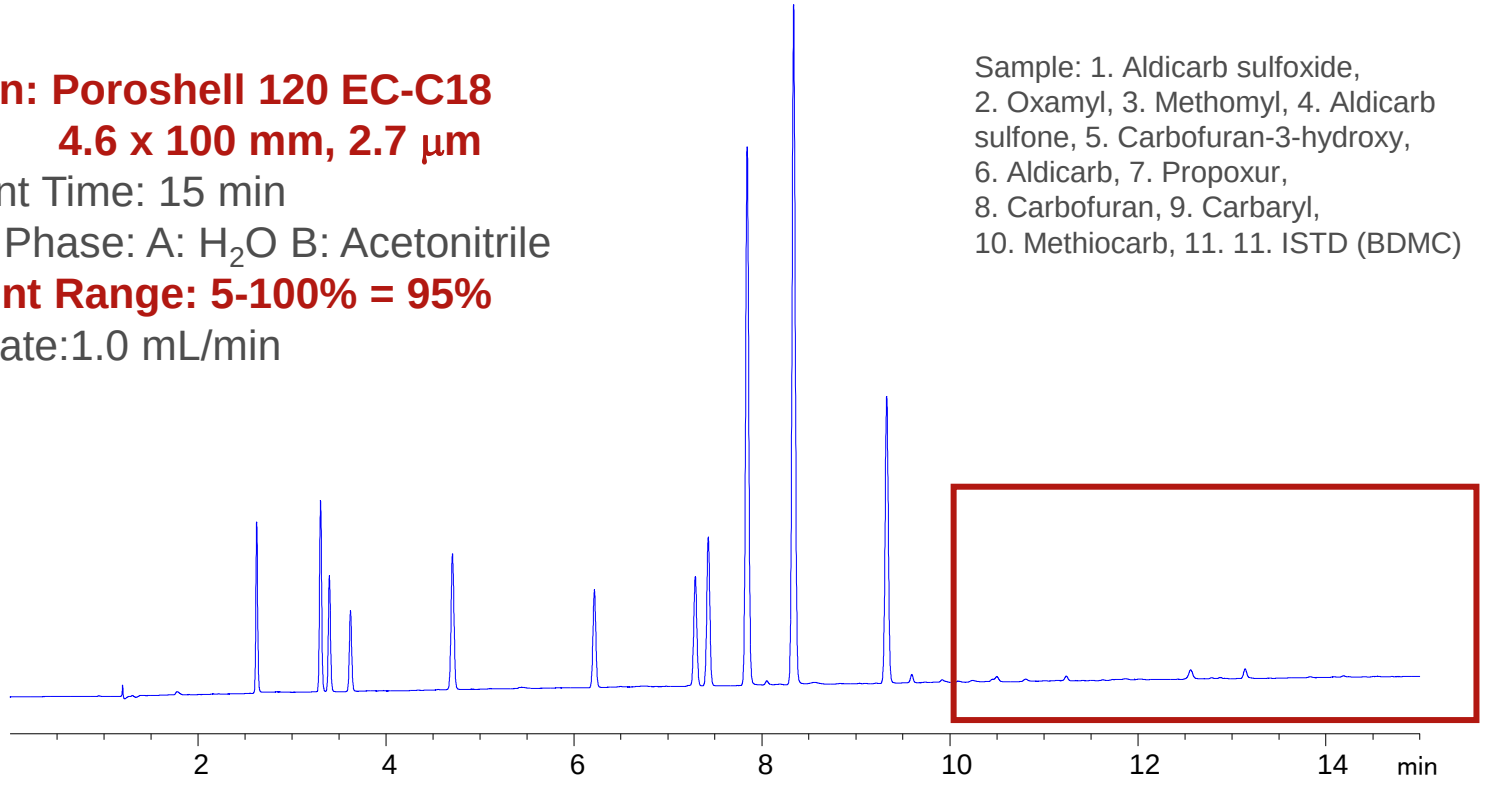
- Gradients provide faster method development
  - Run a scouting method 5% to 95% organic (reversed phase)
- Evaluate whether gradient or isocratic method is best
  - Quick evaluation: how much of the gradient is occupied



Step 1: Choose, Shorter Efficient Column and do Gradient Scouting from 5%B-100% in 15min

Column: Poroshell 120 EC-C18
4.6 x 100 mm, 2.7 μm
Gradient Time: 15 min
Mobile Phase: A: H_2O B: Acetonitrile
Gradient Range: 5-100% = 95%
Flow Rate:1.0 mL/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. 11. ISTD (BDMC)

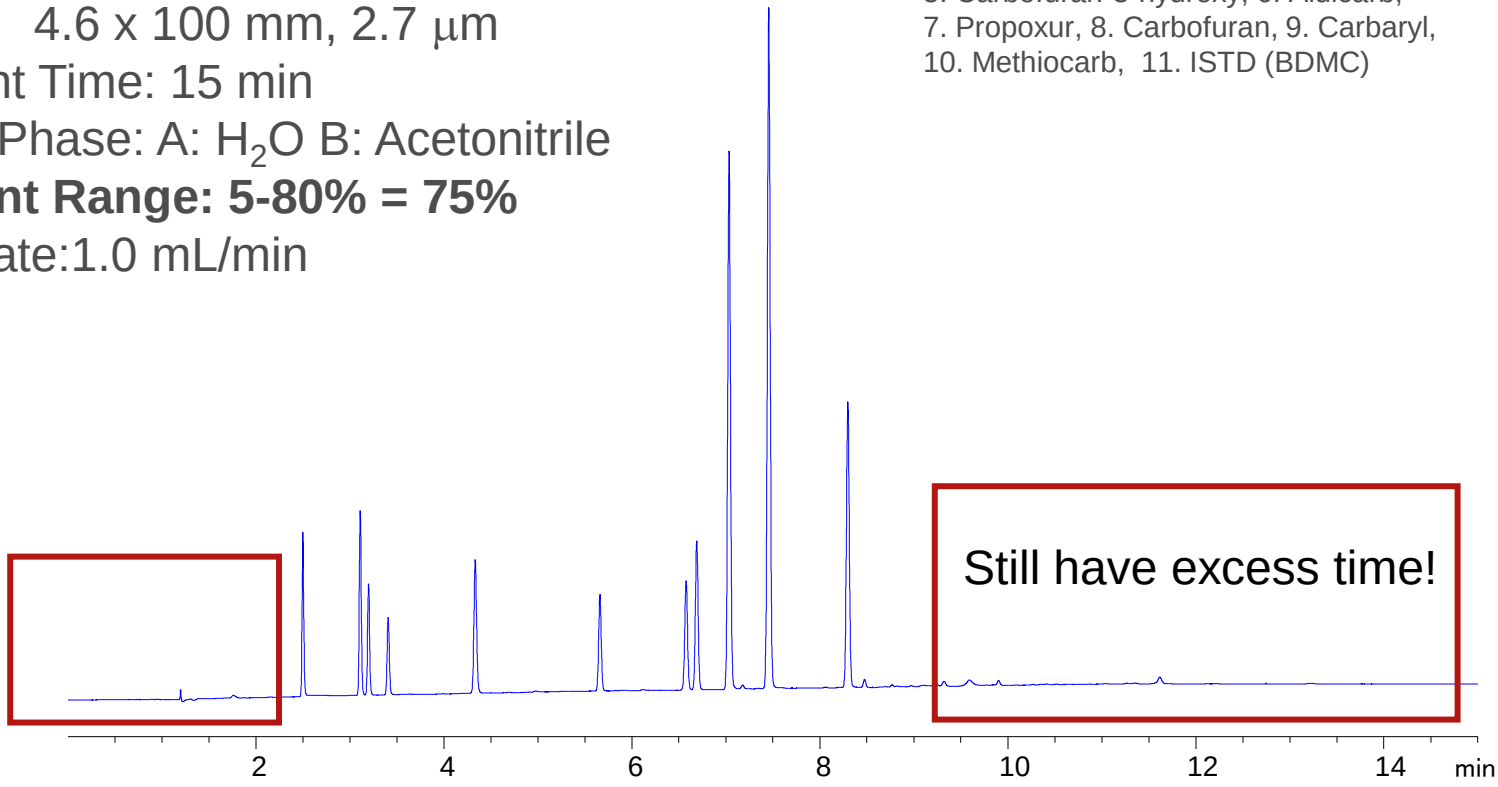


The scouting shows that there is wasted time in this chromatogram and resolution of all components can be achieved. Optimization possible!

Step 2: Reduce Gradient Range to Minimize Time
Adjust Gradient from 5%B-80% in 10min

Column: Poroshell 120 EC-C18
4.6 x 100 mm, 2.7 μm
Gradient Time: 15 min
Mobile Phase: A: H_2O B: Acetonitrile
Gradient Range: 5-80% = 75%
Flow Rate:1.0 mL/min

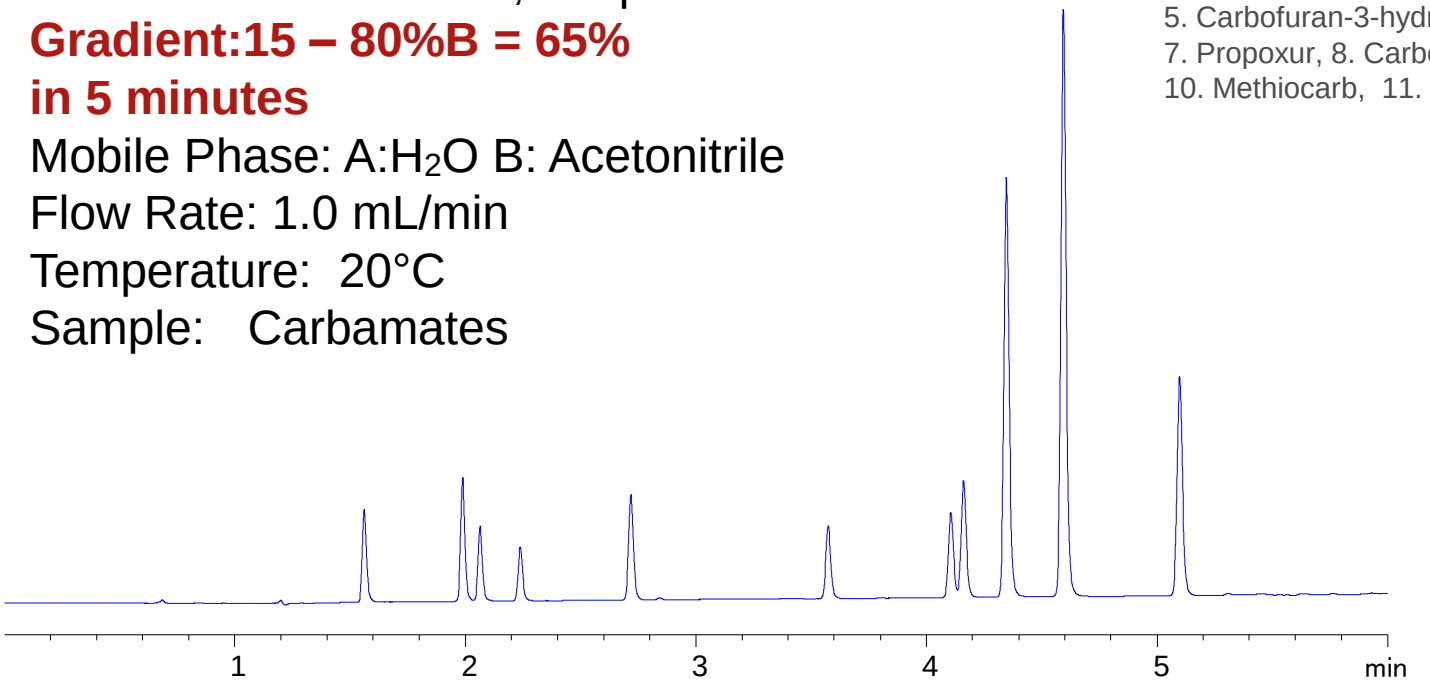
Sample: 1. Aldicarb sulfoxide, 2. Oxamyl,
3. Methomyl, 4. Aldicarb sulfone,
5. Carbofuran-3-hydroxy, 6. Aldicarb,
7. Propoxur, 8. Carbofuran, 9. Carbaryl,
10. Methiocarb, 11. ISTD (BDMC)



Step 3: Finalize Your Results - Increase Starting % Organic and Reduce time

Column: Poroshell 120 EC-C18
4.6 x 100 mm, 2.7 µm
Gradient:15 – 80%B = 65%
in 5 minutes
Mobile Phase: A:H_2O 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)



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

Shorter Columns are Good Choices -Save Time and May Not Compromise Resolution

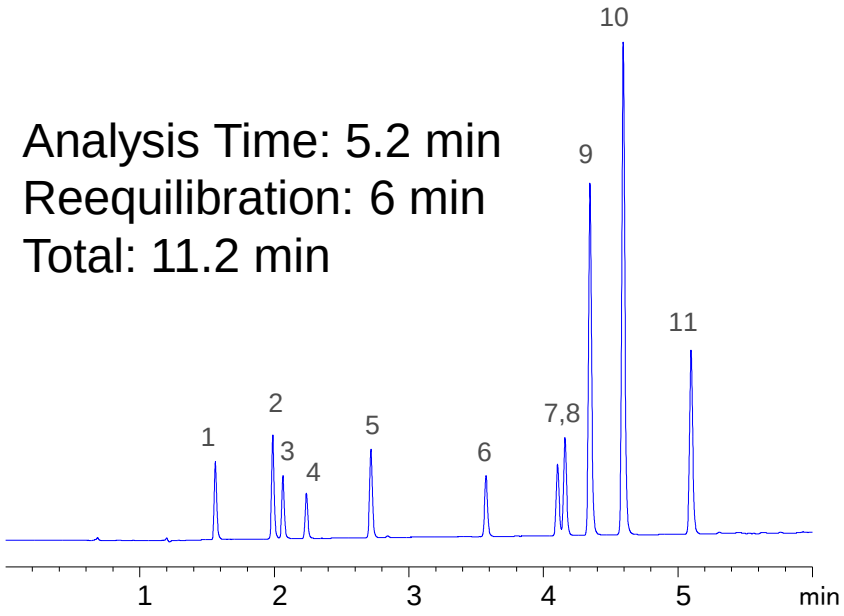
Because the gradient time was kept to 5 minutes, the Rs was maintained and the analysis time reduced (Vm increased).

Poroshell 120 EC-C18
4.6 x 100 mm, 2.7 μm
N = 22,000

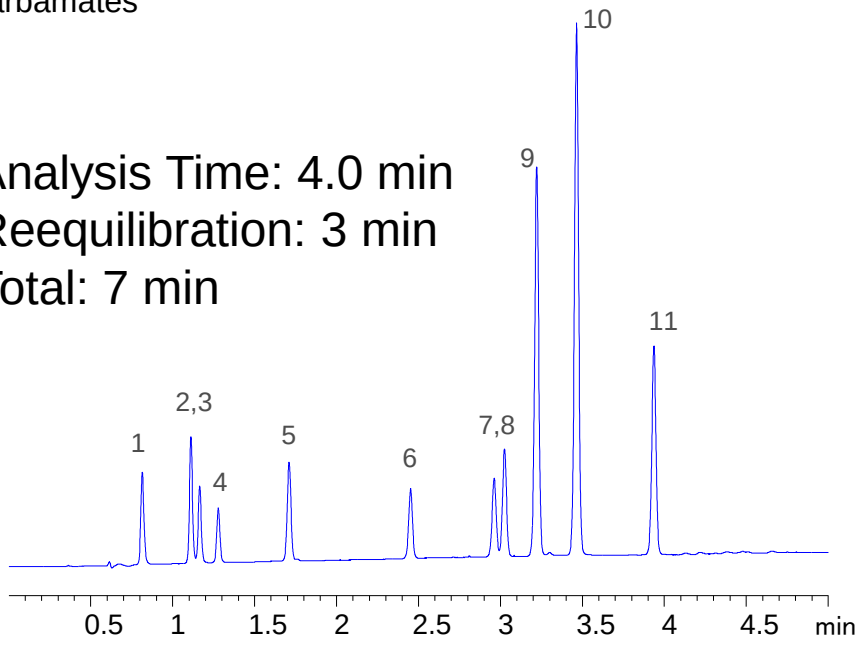
Gradient: 15 – 80%B in 5 min
Mobile Phase: A:H_2O
B: Acetonitrile
Flow Rate: 1.0 mL/min
Temperature: 20°C
Sample: Carbamates

Poroshell 120 EC-C18
4.6 x 50 mm, 2.7 μm
N = 11,000

Analysis Time: 5.2 min
Reequilibration: 6 min
Total: 11.2 min



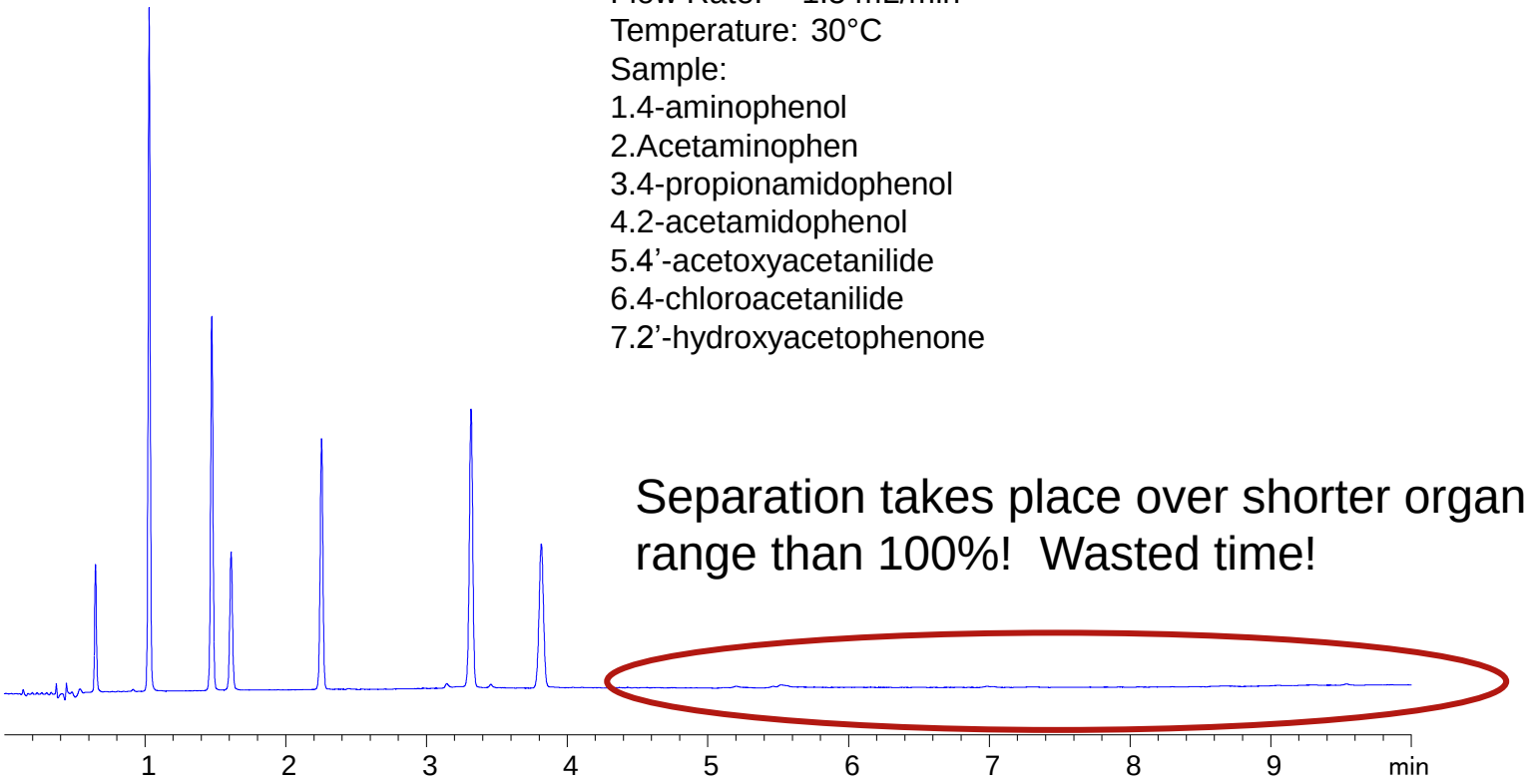
Analysis Time: 4.0 min
Reequilibration: 3 min
Total: 7 min



Gradient Scouting Works for any Sample – Gradient from 5%-100% in 10min for Acetaminophen

Column: Poroshell 120 EC-C18, 4.6x50mm 2.7um

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

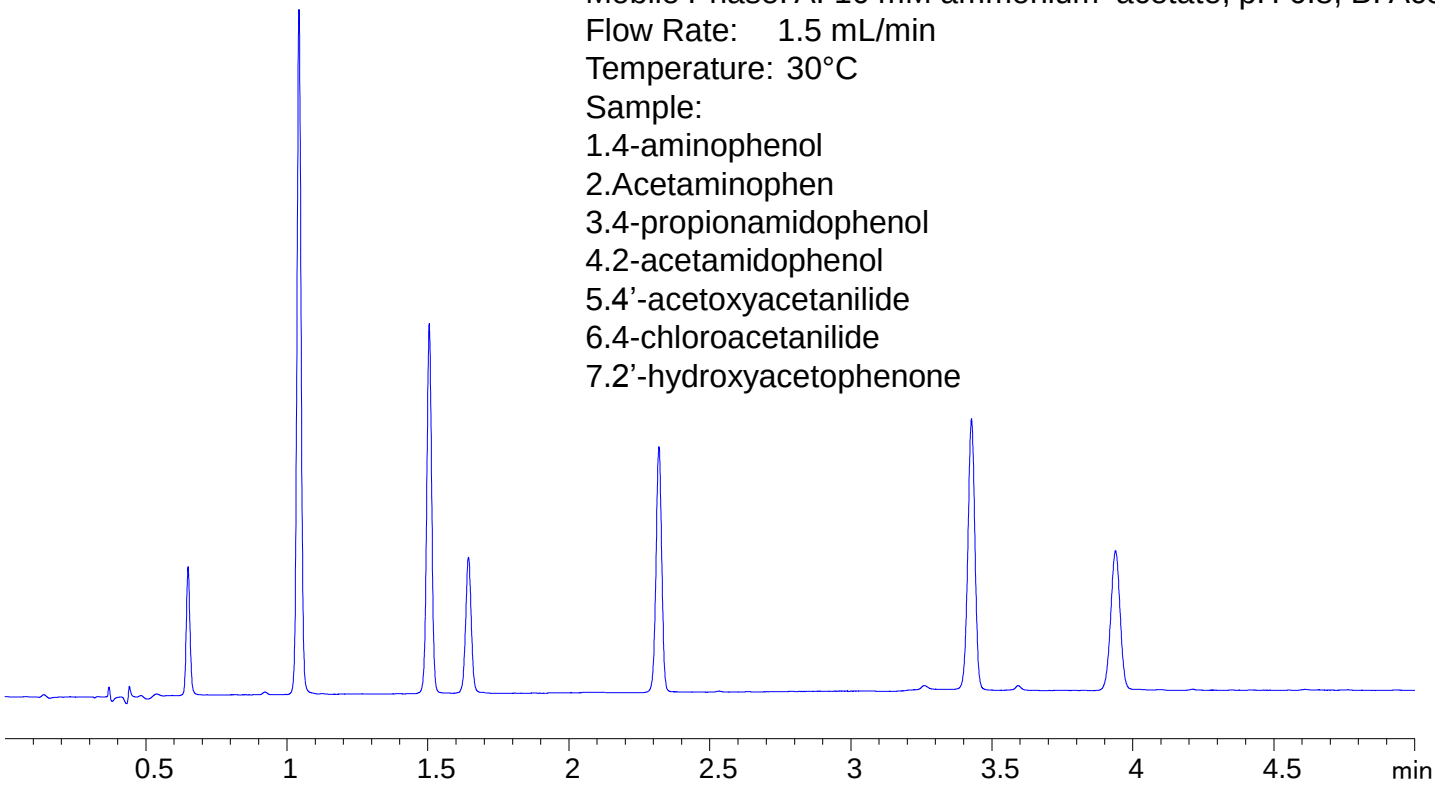


Separation takes place over shorter organic range than 100%! Wasted time!

Optimizing Gradient from 5%-50% in 5min to Reduce Wasted Time

Column: Poroshell 120 EC-C18, 4.6x50mm, 2.7um

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

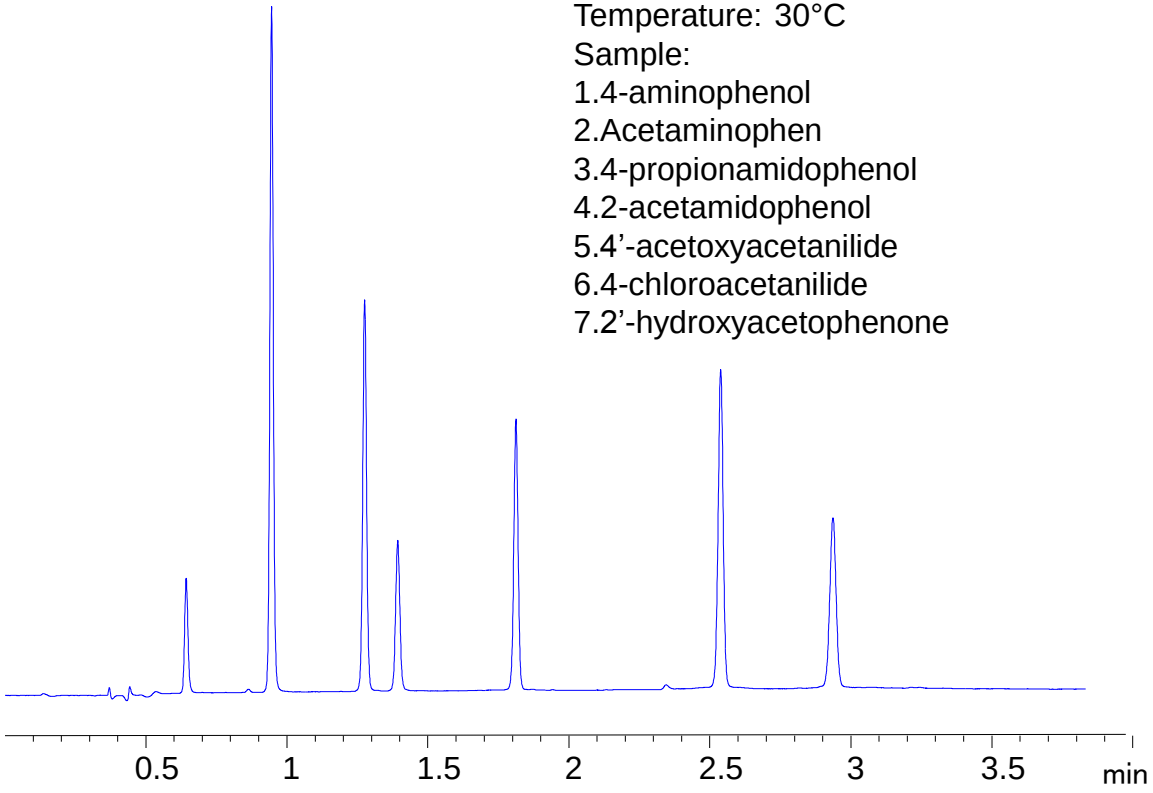


Excellent resolution and distribution of peaks in the gradient – within 5 minutes.

Final Optimization to Reduce Time - Gradient from 5%-50% in 3 min

Column: Poroshell 120 EC-C18, 4.6x50mm, 2.7um

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



Adapting Gradient Methods to Different Columns

1. Determining conditions based on “gradient equation”
2. Practical dwell/delay volume and other instrument considerations

1. 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.

1. 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
$\Delta\Phi$ = 40 (20% - 60%)	$\Delta\Phi$ = 40 (20% - 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)

$$\frac{\Delta\Phi \cdot Vm}{F \cdot t_G} = \frac{40 \cdot 1.5}{1.0 \cdot 15} = 4$$

Using b = 4

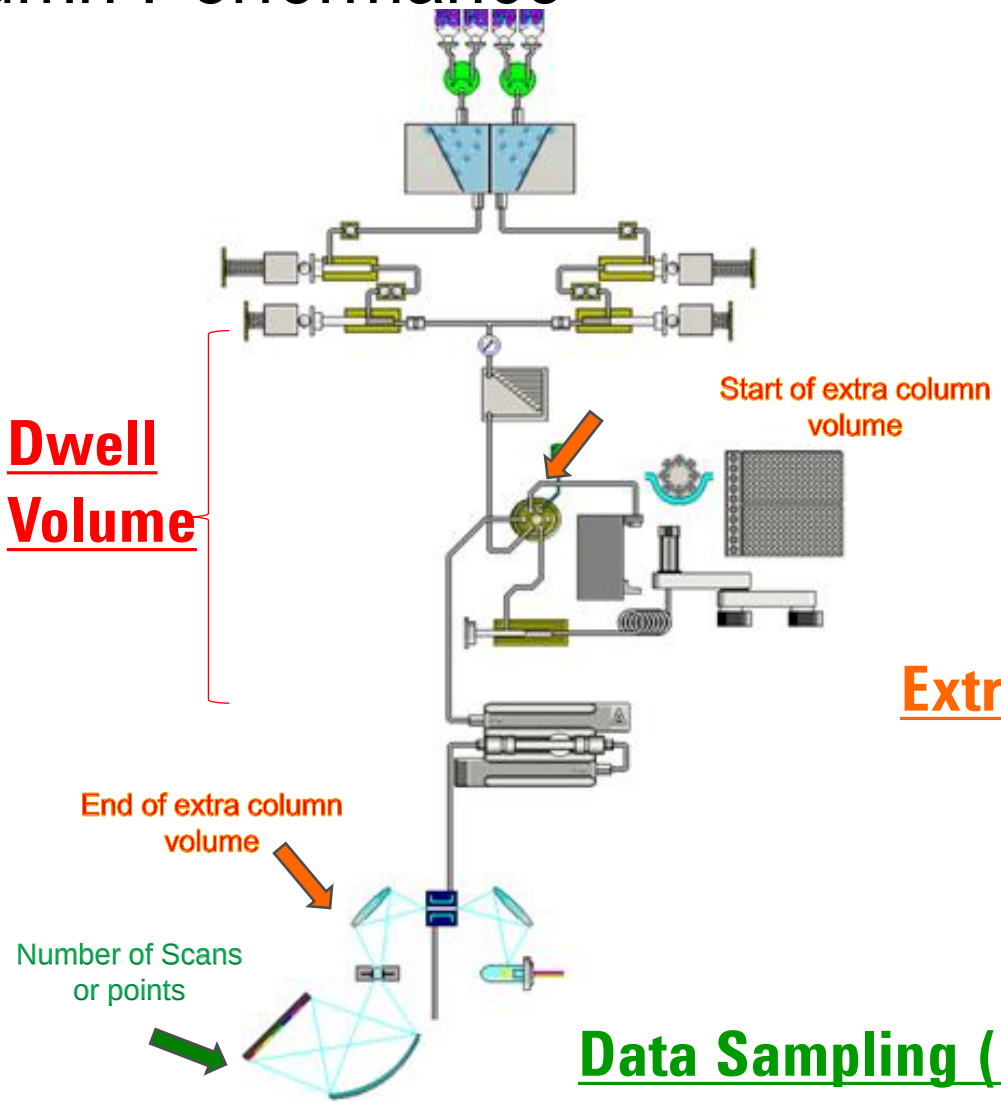
$$4 = \frac{40 \cdot 0.2}{0.2 \cdot t_G}$$

Using new dimensions

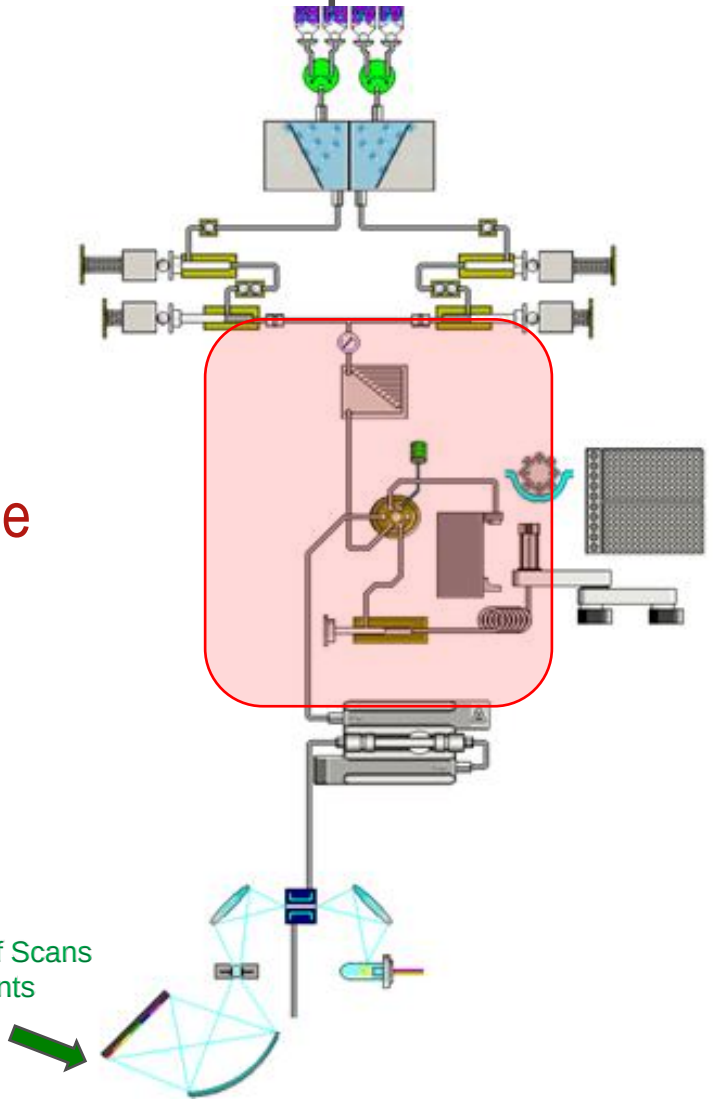
Solve for t_G

$$t_G = \frac{40 \cdot 0.2}{0.2 \cdot 4} = 10 \text{ min}$$

2. Gradient Separations - Instrument Impact on Column Performance



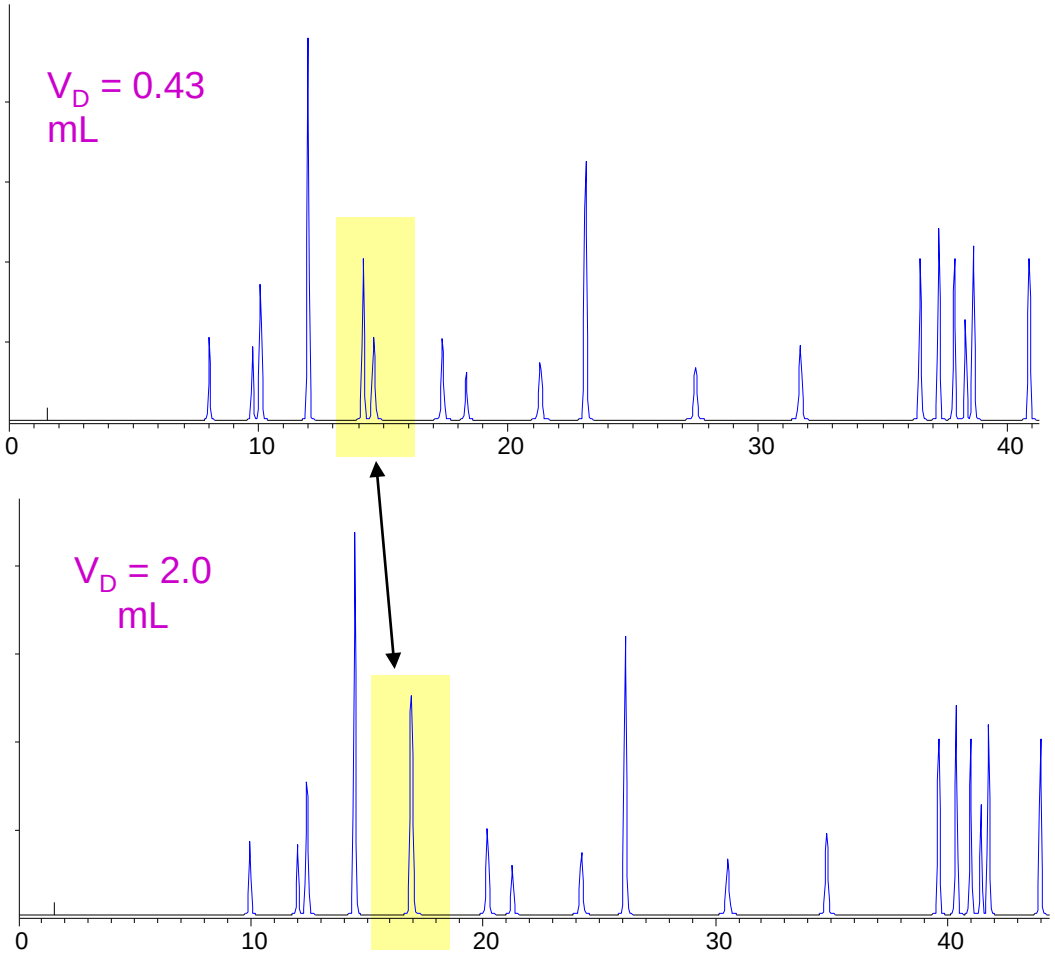
Instrument Impact on Column Performance



#1 Dwell Volume

- Dwell volume = volume from formation of gradient to the column

Minor Dwell Volume Differences Can Change Resolution



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

Mobile Phase: Gradient, 0 - 100 %B in 52.5 min.
A: 5/95 methanol/ 25 mM phosphate
pH 2.50
B: 80/20 methanol/25 mM phosphate
pH 2.50

Flow Rate: 0.5 mL/min

Temperature: 25°C

Injection: 5 µL

Detection: 250 nm

Sample: Mixture of antibiotics and antidepressants

Upper trace simulates actual run data entered into DryLab® 3.0 software

Lower trace is simulated chromatogram for larger V_D

Determining the Dwell Volume of Your System

Look it up in the LC manual or……

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

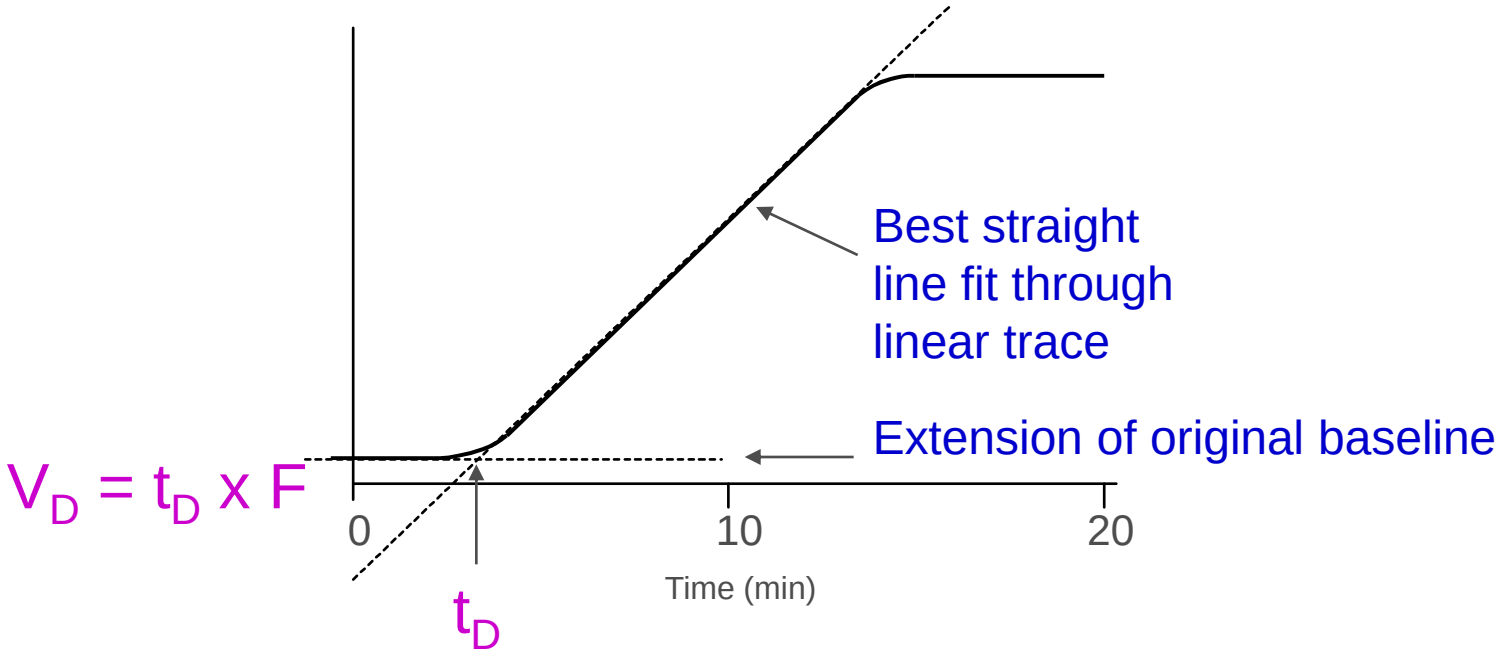
Adjust attenuation so that both 100% A and 100% B are on scale

Run gradient profile 0 - 100% B/10 min at 1.0 ml/min

Record

Expected Dwell Volume in UHPLC's – uL range!

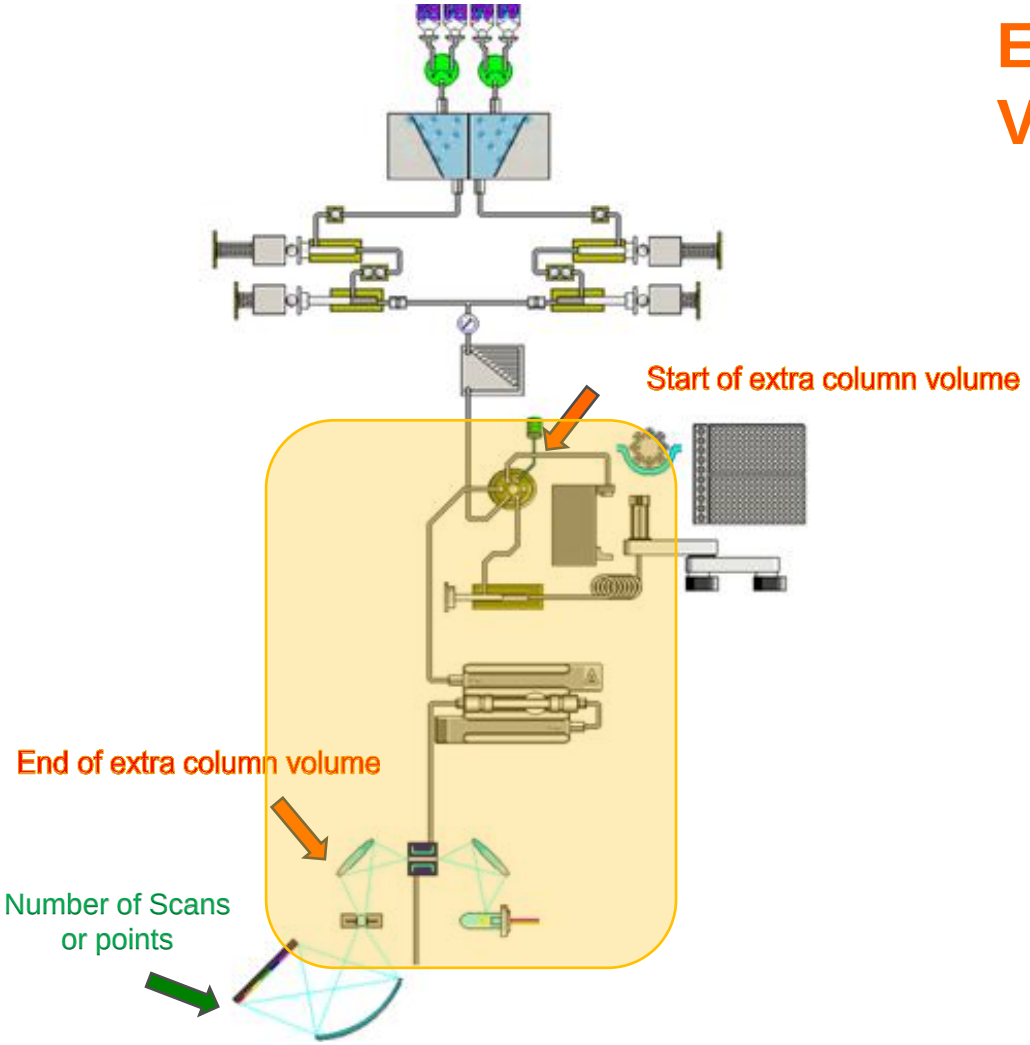
Measuring Dwell Volume (V_D)



- Intersection of the two lines identifies dwell time (t_D)
- Dwell volume is equal to product of the flow rate and the dwell time.

2. Gradient Separations- Instrument Impact on Column Performance

Extra-column Volume



How Does Instrument Design Effect Performance?

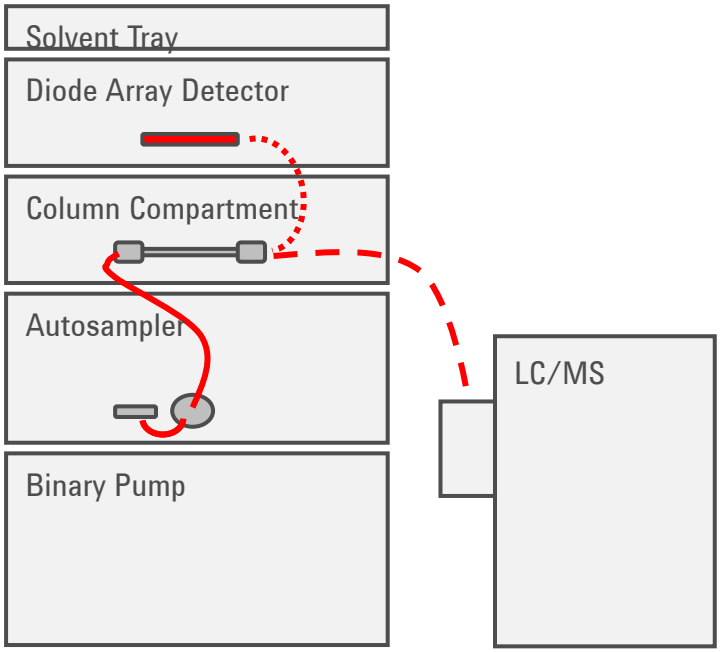
- The Flow Path the Sample “Sees” from injection to Detection Contributes to Dispersion and Peak Broadening
- Limiting excess volume will greatly reduce dispersion impact on performance
- UHPLCs (Agilent 1290 Infinity) are already optimized for Sub-2um , Poroshell and HPLC column performance
- Have options for ULD (ultra-low dispersion)
- What about other instruments?

1290 Infinity UHPLC

Comparison of 1290 Gradient Performance

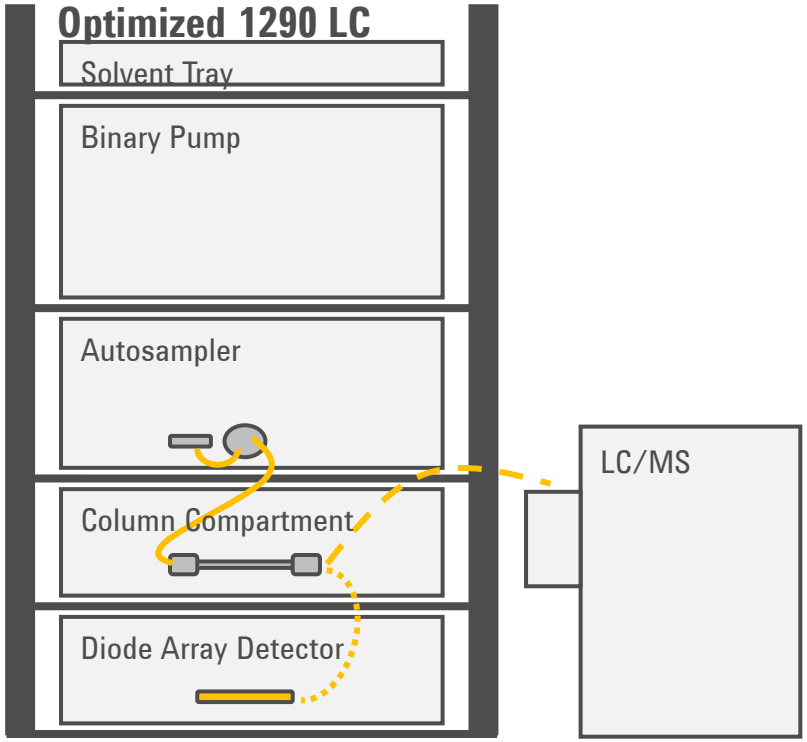
LC/UV systems extra column volume is reduced by 60% (from 9.7 to 3.9uL)
LC/MS system extra column volume is reduced by 64% (from 8.7 to 3.1 uL)

Default 1290 LC



Needle Seat Capillary: 0.12 x 100 mm = 1.1 µL
ALS→TCC Capillary: 0.12 x 340 mm = 3.8 µL
TCC→DAD Capillary: 0.12 x 220 mm = 2.5 µL
Flow Cell V(σ)1.0 µL = 2.3 µL
TCC→MS Capillary: 0.12 x 340 mm = 3.8 µL
2.1 x 50 mm Column = 172.3 µL
Void Volume of Column = 103.9 µL

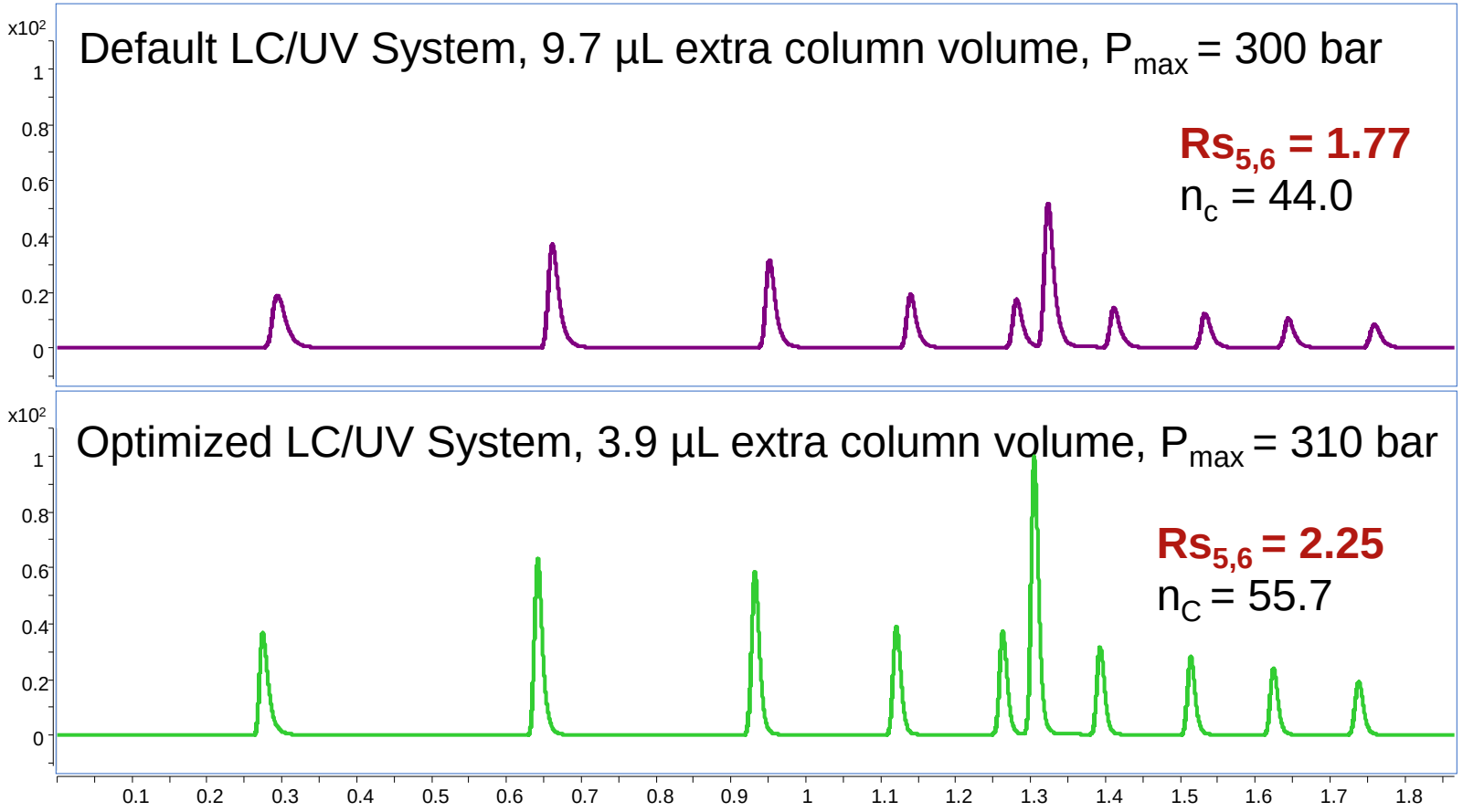
Optimized 1290 LC



Needle Seat Capillary: 0.11 x 100 mm = 0.9 µL
ALS→TCC Capillary: 0.08 x 220 mm = 1.1 µL
TCC→DAD Capillary: 0.08 x 220 mm = 1.1 µL
Flow Cell V(σ)0.6 µL = 0.8 µL
TCC→MS Capillary: 0.08 x 220 mm = 1.1 µL
2.1 x 50 mm Column = 172.3 µL
Void Volume of Column = 103.9 µL

Optimized LC Improves Gradient Resolution

Column: RRHD Eclipse Plus C18, 2.1 x 50mm, 1.8um Gradient: 25-95% CH_3CN in 1.2 min, Flow Rate: 0.4 mL/min LC: Agilent 1290 Infinity Sample: Alkylphenones



>20% improvement in gradient Rs and peak capacity with optimized LC

Tubing Volume

Tubing Length	10mm	50mm	100mm	150mm
Tubing i.d.	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

Dispersion in the Tubing

Dispersion Calculation

$$\sigma_t^2 = \pi^2 r^6 L u / 24 D_m$$

Dispersion in the tubing is proportional to the

- Length of tubing
- 6^{th} power of the tube radius

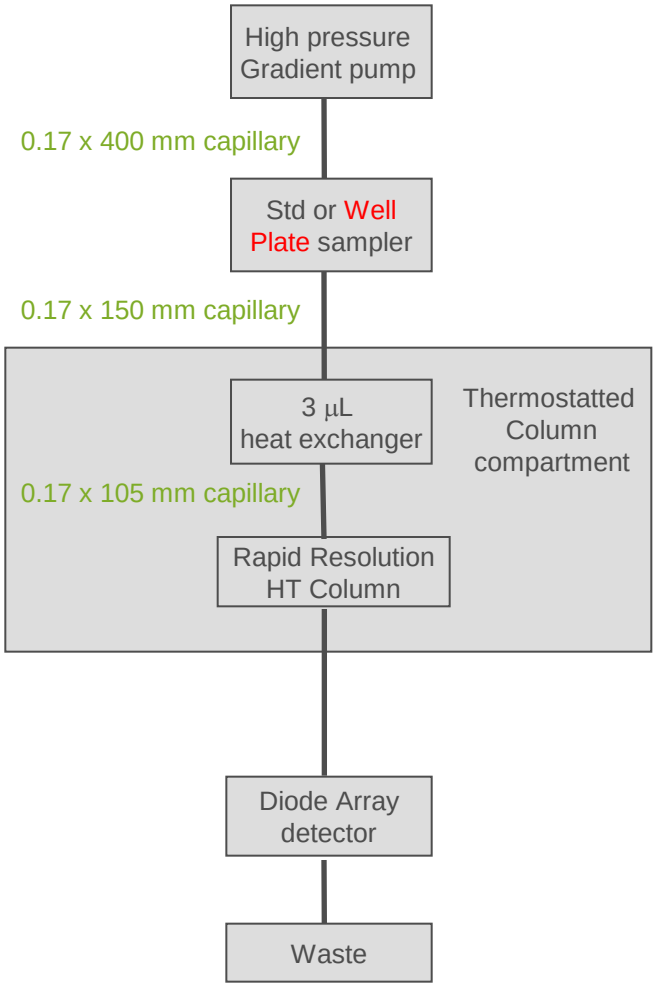
Shortest tubing lengths possible minimize dispersion

Small changes in tubing i.d. have major effects on peak width and efficiency

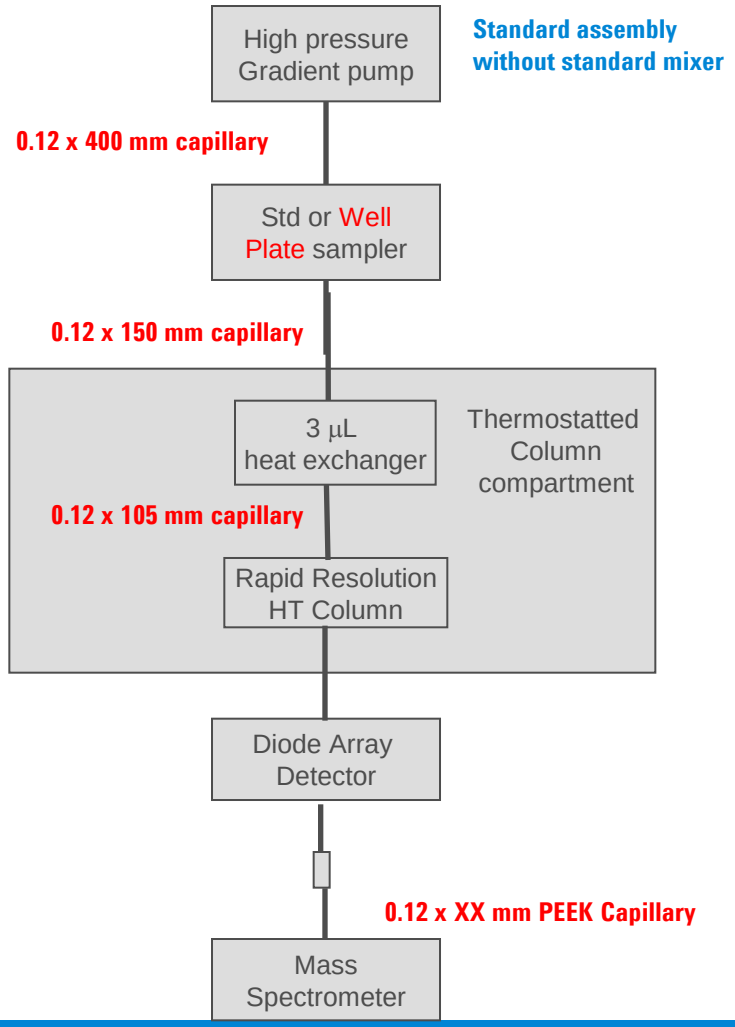
Conversion for Fast and Ultra-Fast HPLC

1200 through 1260 Series LC Systems

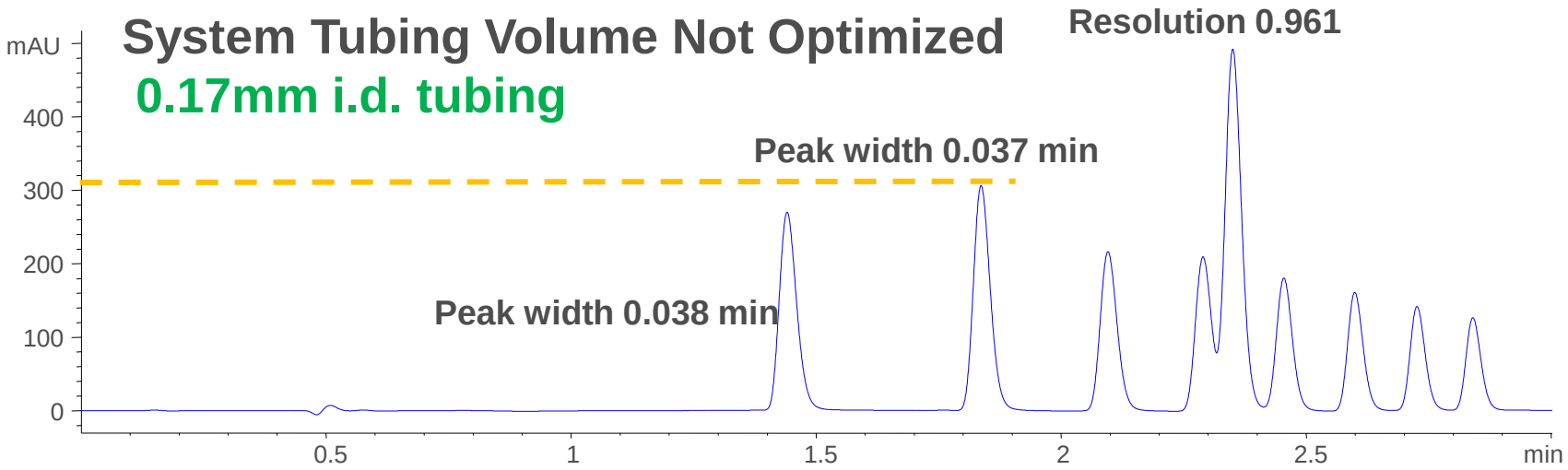
Traditional LC Columns

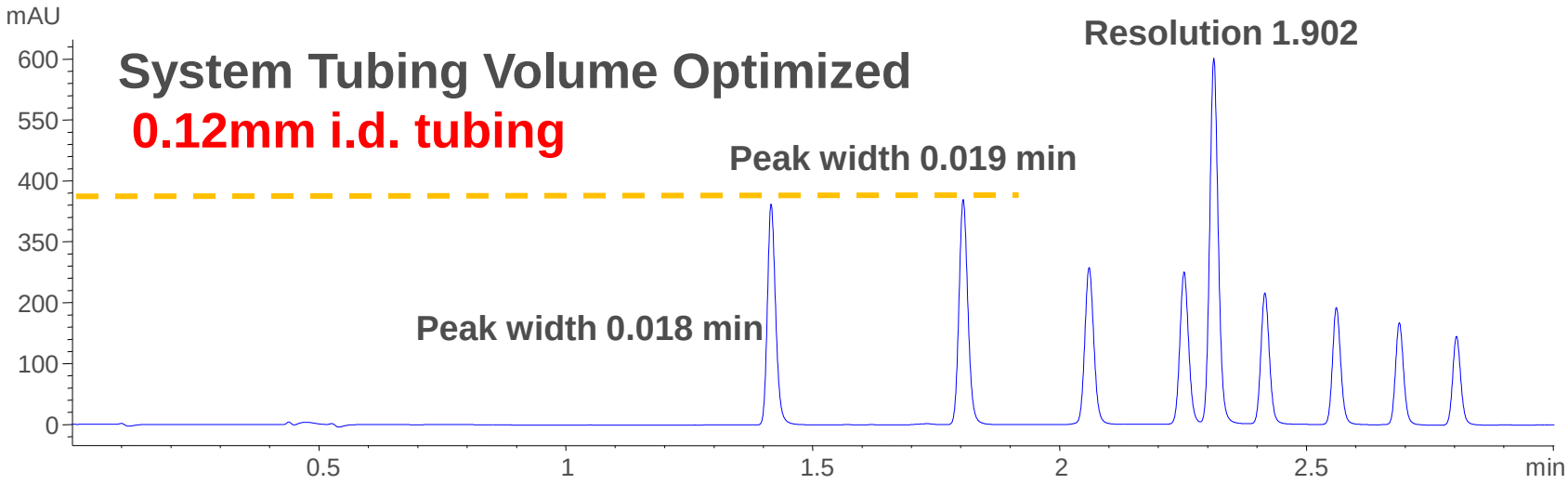


Fast LC/UHPLC Columns



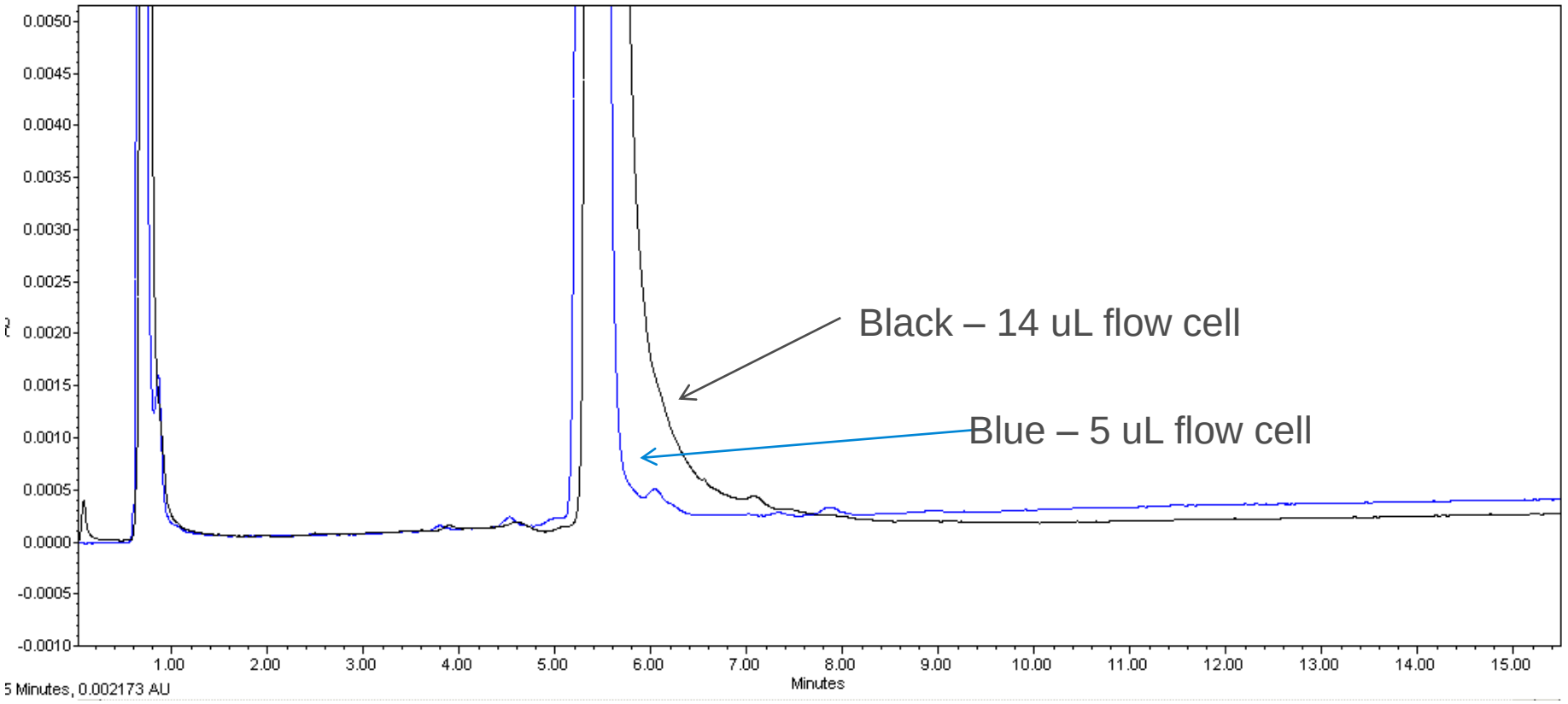
Optimizing Connecting Tubing Volume For UHPLC Columns



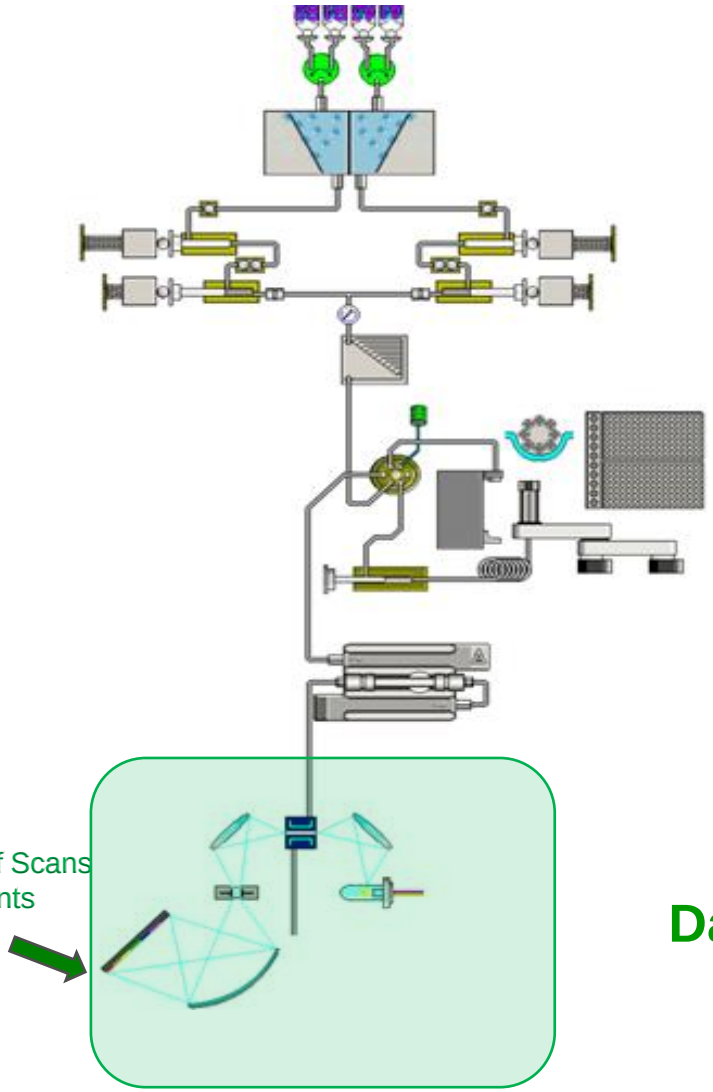


Smaller Column i.d. Requires a Lower Detector Cell Volume

3 x 100mm Column



2. Gradient Separations - Instrument Impact on Column Performance

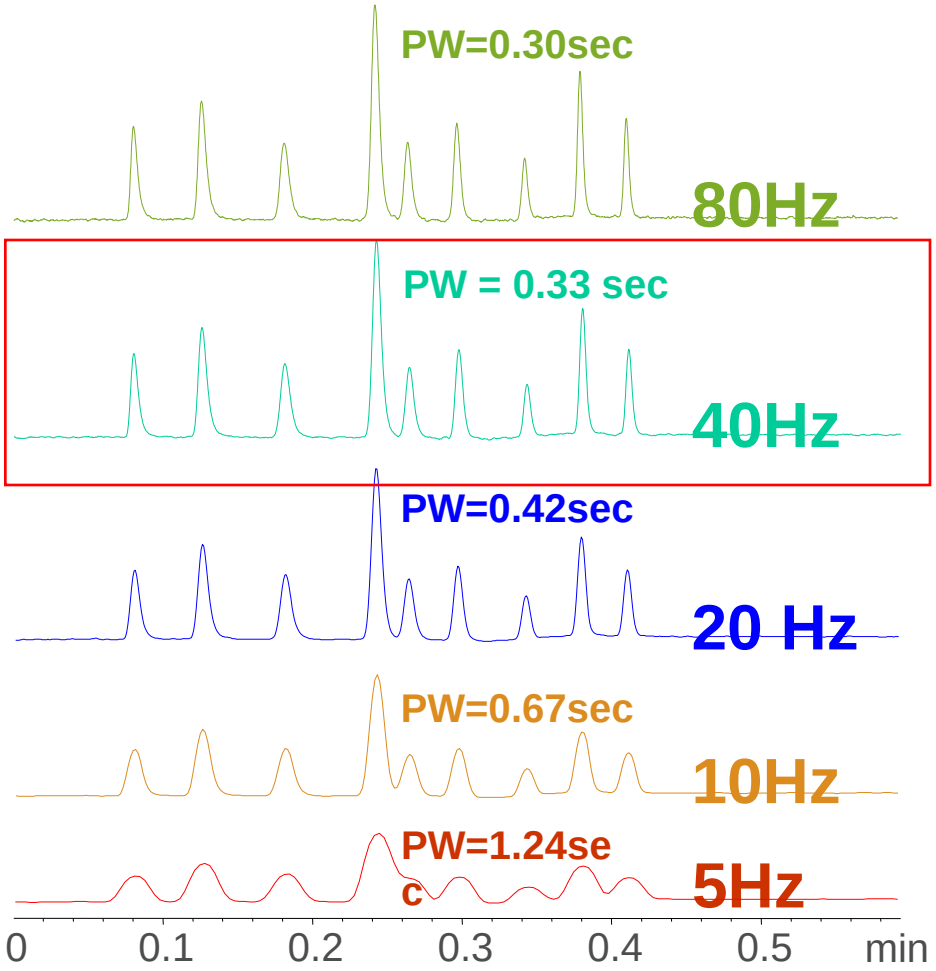


Number of Scans or points

Data Sampling (or Acquisition) Rate

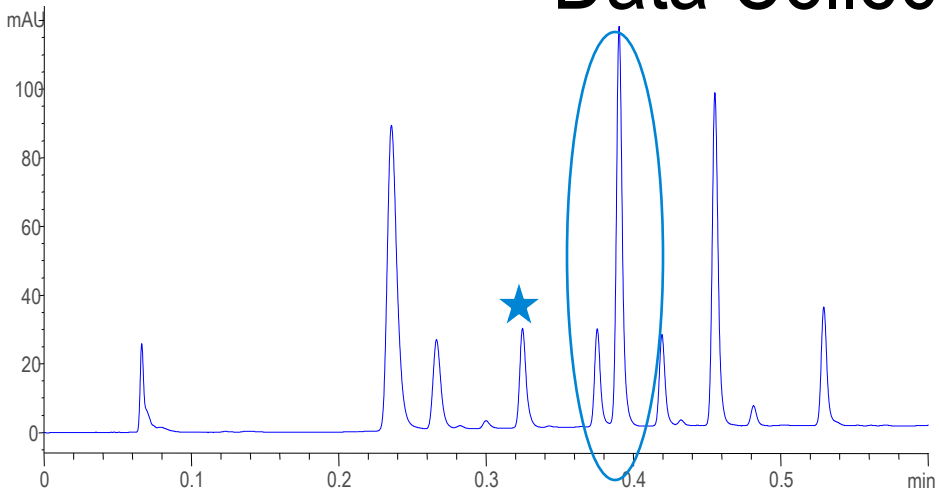
Effect of Data Acquisition Rate (time constant)

High definition UHPLC Requires High Definition Chromatogram

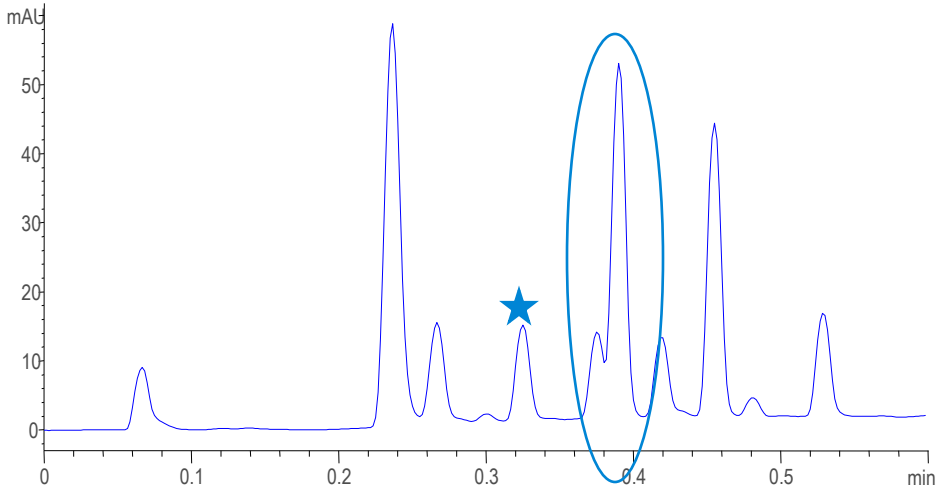


- Increased Data Rate
- More Accurate " Picture"
- Make Sure Rate is Adequate
- Faster Rates Generate More Noise and Take up More Memory

Solve Apparent Rs Problems with Optimum Data Collection Rate



★ *Peak width = 0.017min at 80Hz*

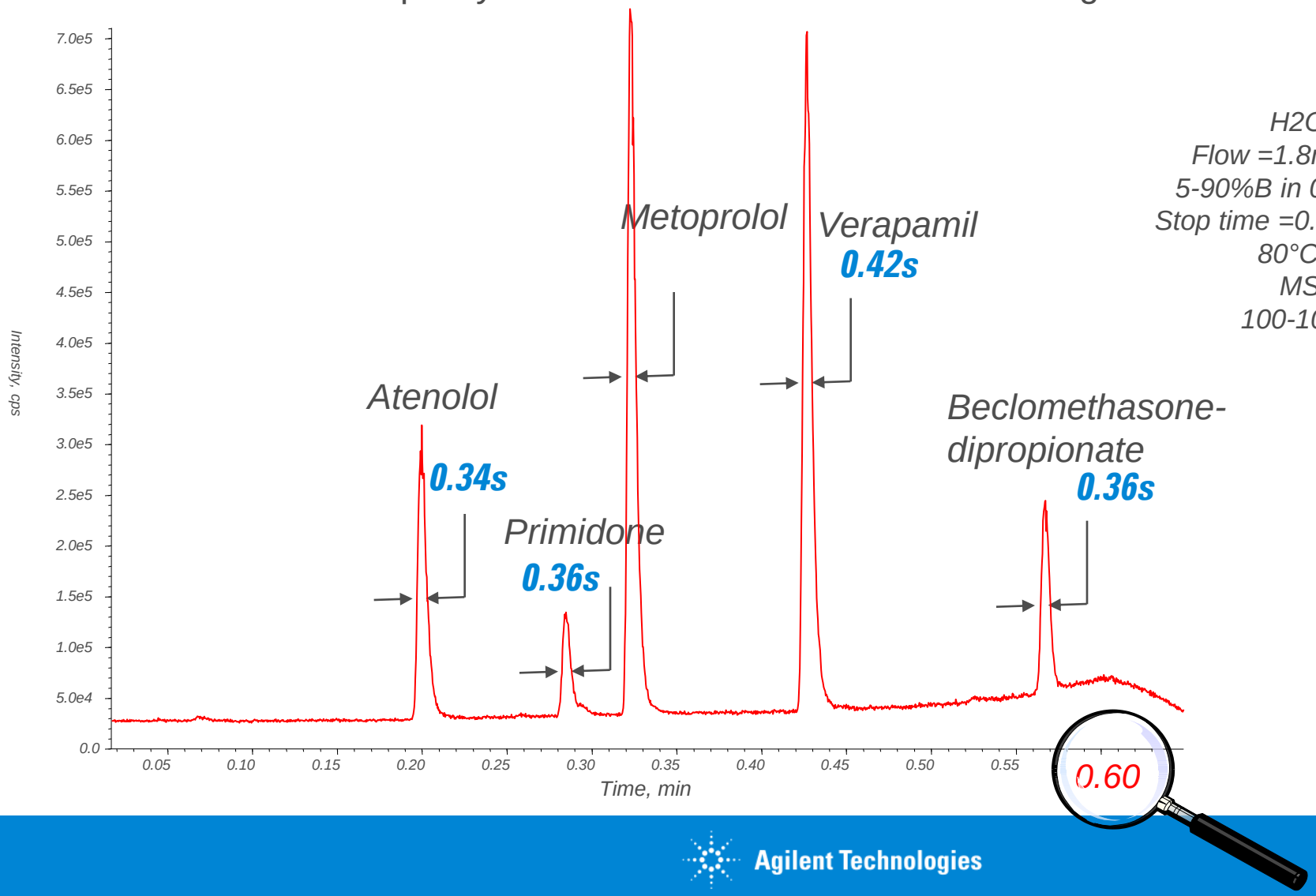


★ *Peak width = 0.021min at 10Hz*

Very Fast Gradients Benefit From Very Fast Scan

MS with 40 Cycle/s, 5-90%B Gradient in 0.65min

Peak capacity of >40 in 39 sec in the MS chromatogram



H2O/ACN
Flow =1.8ml/min
5-90%B in 0.5min
Stop time =0.65min
80°C, ACR
MS 40Hz
100-1000Da

Why Use Short, Low Volume Columns for Gradient Analysis

Fast analyses with fast gradients can be done on short, low volume columns

Fast re-equilibration can be achieved, so gradient separations take no additional time

Short columns with small particle sizes do not compromise resolution (N)

High-throughput, screening separations are possible with unknown samples

Good Habits for Gradient Separations on Low Volume Columns (Short, Efficient)

High-throughput separations require minimal extra-column and dwell/delay volume

- Minimize dwell volume
- Minimize extra column volume

High-throughput separations require optimal performance of all instrument components

Fast data acquisition rate (response time of the detector) – 0.1 sec

Optimize gradient column parameters with short, fast columns

- High flow rate for fastest gradient and shortest re-equilibration time
- Low starting organic in gradient for maximum sample focusing range

Putting it All Together – High Performance Gradient Separations

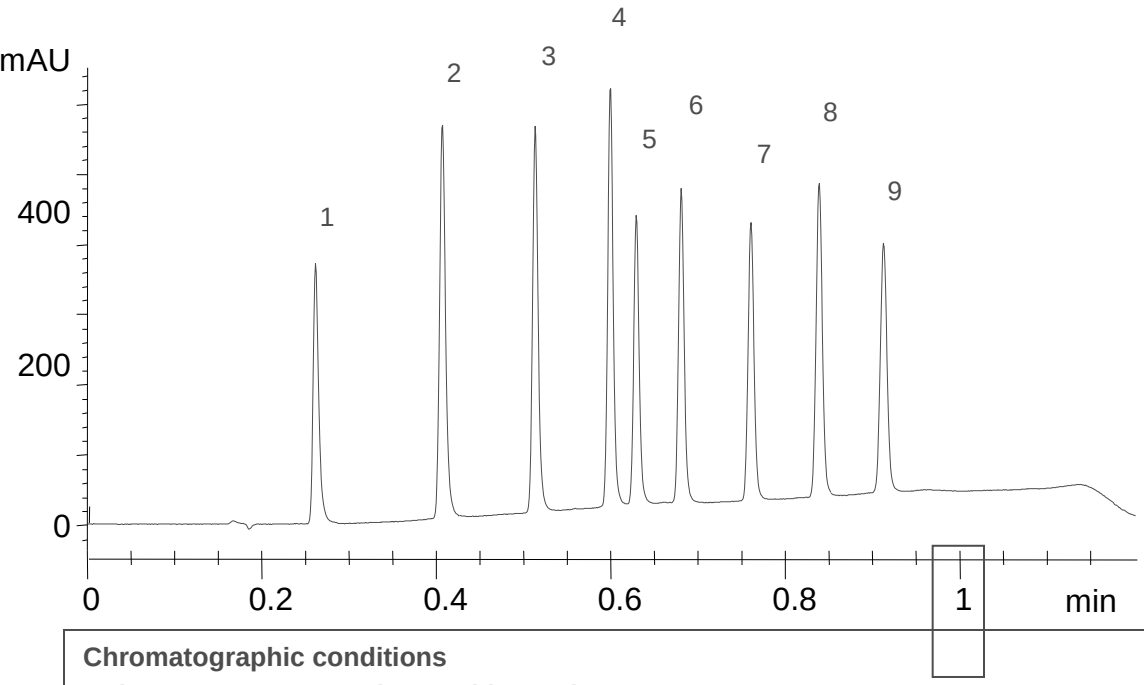
Short, high efficient columns

Optimize your instrumentation

High flow rates

Result = high performance gradients

Ultra-fast Gradient Analysis of 9 Alkylphenones



Flow Rate	2.6ml/min
Temp.	32°C
Pressure	346 bar
CycleTime	1.5 min
Run Time	1.2 min
Analysis T.	0.912 min
Rs (4,5)	2.76
RT% RSD	<0.2%.

Chromatographic conditions

Column: 4.6x50mm Zorbax StableBond-C18, 1.8µm

Injection: 1 µL

Separation: Mobile phase: A: water + 0.1% HCOOH; B: acetonitrile + 0.1% HCOOH.

Gradient: from 50% B to 100% B in 0.65 min, hold over 0.2 min.

Stop time = 1.2 min.

Sample: alkylphenones and acetanilide (100 ng/µL each) consisting of: 1 acetanilide, 2 acetophenone, 3 propiophenone, 4 butyrophenone, 5 benzophenone, 6 valerophenone, 7 hexanophenone, 8 heptanophenone and 9 octanophenone

Temperature: 32 °C

DAD detection: UV signal = 245 nm, 10 nm Reference = 360 nm, 80 nm

Slit: 8 nm

Peak width (response time): < 0.01 min (0.1 sec), i.e. 20 Hz data acquisition rate

Good Habits for Successful Gradient Separations

- Maximizing gradient efficiency – balancing speed and resolution
- Optimizing LC system performance for gradients
- Transferring gradients from one column or LC to another