Tips and Tricks of Faster LC Analysis Without Capital Investment

It may be easier than you think!

Agilent Technologies, Inc.

Fall 2008

There Are Many Reasons to Reduce Analysis Time

- Run More Samples/Day
- Reduce Mobile Phase Use
- Reduce Mobile Phase Waste Disposal
- Better Utilize Existing Resources in Instruments and Personnel
- Implement “HB5”* Work Schedule

(* “HB5” **H**ome **B**y **5,** Ray Lombardi, Agilent Technologies, Inc. 2007)

At What Cost and Difficulty?

That Depends on What You Want to Accomplish!

How Fast Do You Want To Go?

Your Answer Will Determine the Cost of Increased Speed

- 2 to 3 X increase in Speed – Easy
- 5-10 X Increase – May Require Instrument Tweak
- 10 X + Increase – Will Require Instrument Upgrade and Tweak

What Are the Paths to LC Method Speed Gains?

- Most Speed From Column and Particle Size Optimization
- Instrument Optimization Will Enable or Boost Those Gains

The Following Discussion is Designed to

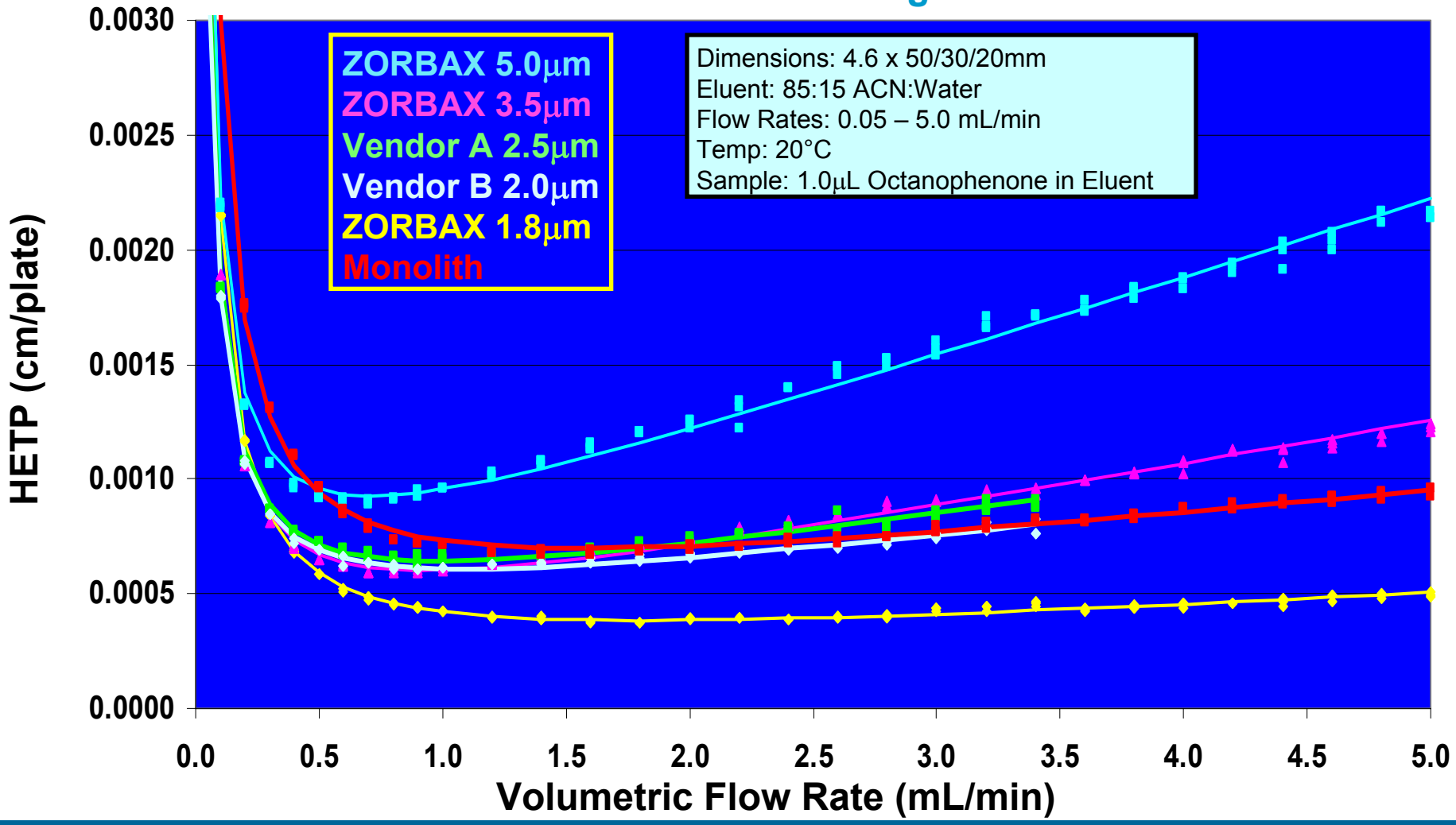
Simplify the Process - Often at No Cost!

How Do Small Particles Help Speed Up Methods?

- Increase Kinetics of the separation
- Increase Efficiency (N) in Same Length Column
- Allow Shorter Column to Match Efficiency of Longer Column
- Provide Expanded Flow Rate Range Without Loss of Efficiency

Trick: Use Smaller Particles For More Efficient Separations Across a Wide Flow Rate Range

Small Particle Columns including Monolith



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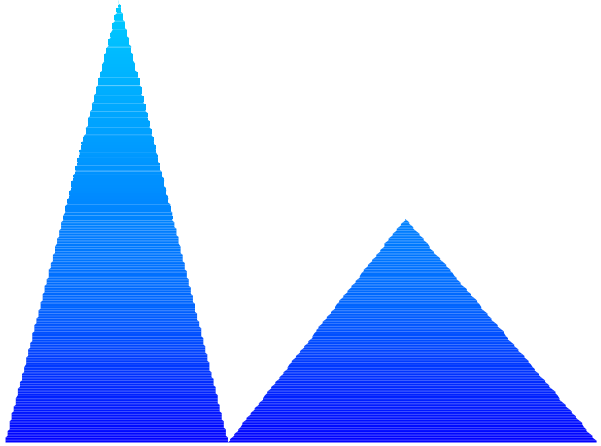


ISOCRATIC ELUTION

Tip: Short Column/Smaller Particle Maintains Rs Trick: Tailor Column Dimensions and Particle Size to Analysis Needs

Column Length (mm)	Resolving Power N(5 µm)	Resolving Power N(3.5 µm)	Resolving Power N(1.8 µm)	Typical Pressure Bar (1.8 µm)	Analysis Time*
150	12,500	21,000	32,500	>400	
100	8,500	14,000	24,000	>400	-33%
75	6000	10,500	17,000	320	-50%
50	4,200	7,000	12,000	210	-67%
30	N.A.	4,200	6,500	126	-80%
15	N.A.	2,100	2,500	55	-90%

Analysis Time ↓ Peak Volume ↓ Solvent Usage ↓

* Reduction in analysis time compared to 150 mm column

- **pressure determined with 60:40 MeOH/water, 1ml/min, 4.6mm ID**

Goal: Run Current Method 2X Faster

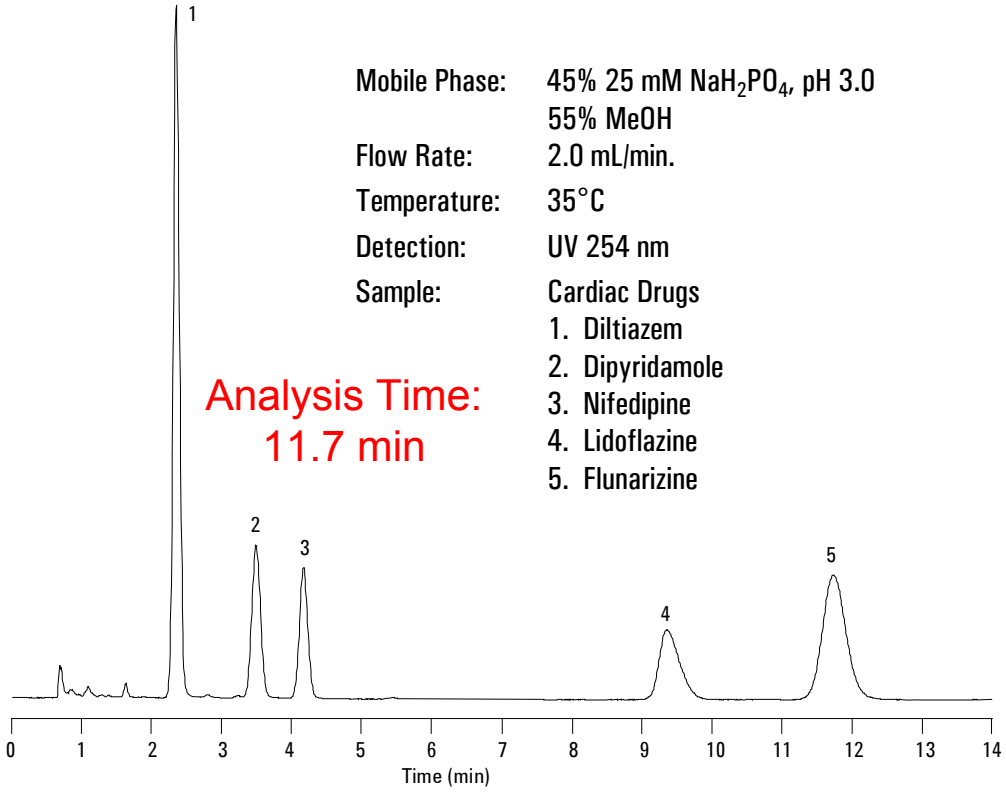
Trick: ½ Column Length and Smaller Particle = ½ Time

StableBond SB-C18

4.6 x 150 mm, 5 µm

Mobile Phase:	45% 25 mM NaH_2PO_4, pH 3.0 55% MeOH
Flow Rate:	2.0 mL/min.
Temperature:	35°C
Detection:	UV 254 nm
Sample:	Cardiac Drugs 1. Diltiazem 2. Dipyridamole 3. Nifedipine 4. Lidoflazine 5. Flunarizine

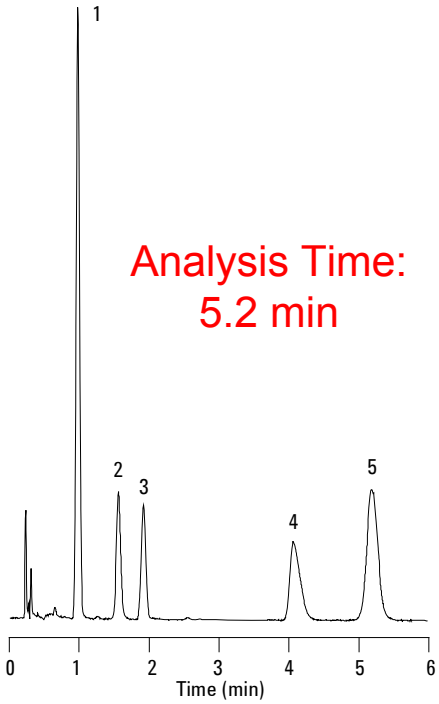
Analysis Time:
11.7 min



Rapid Resolution StableBond SB-C18

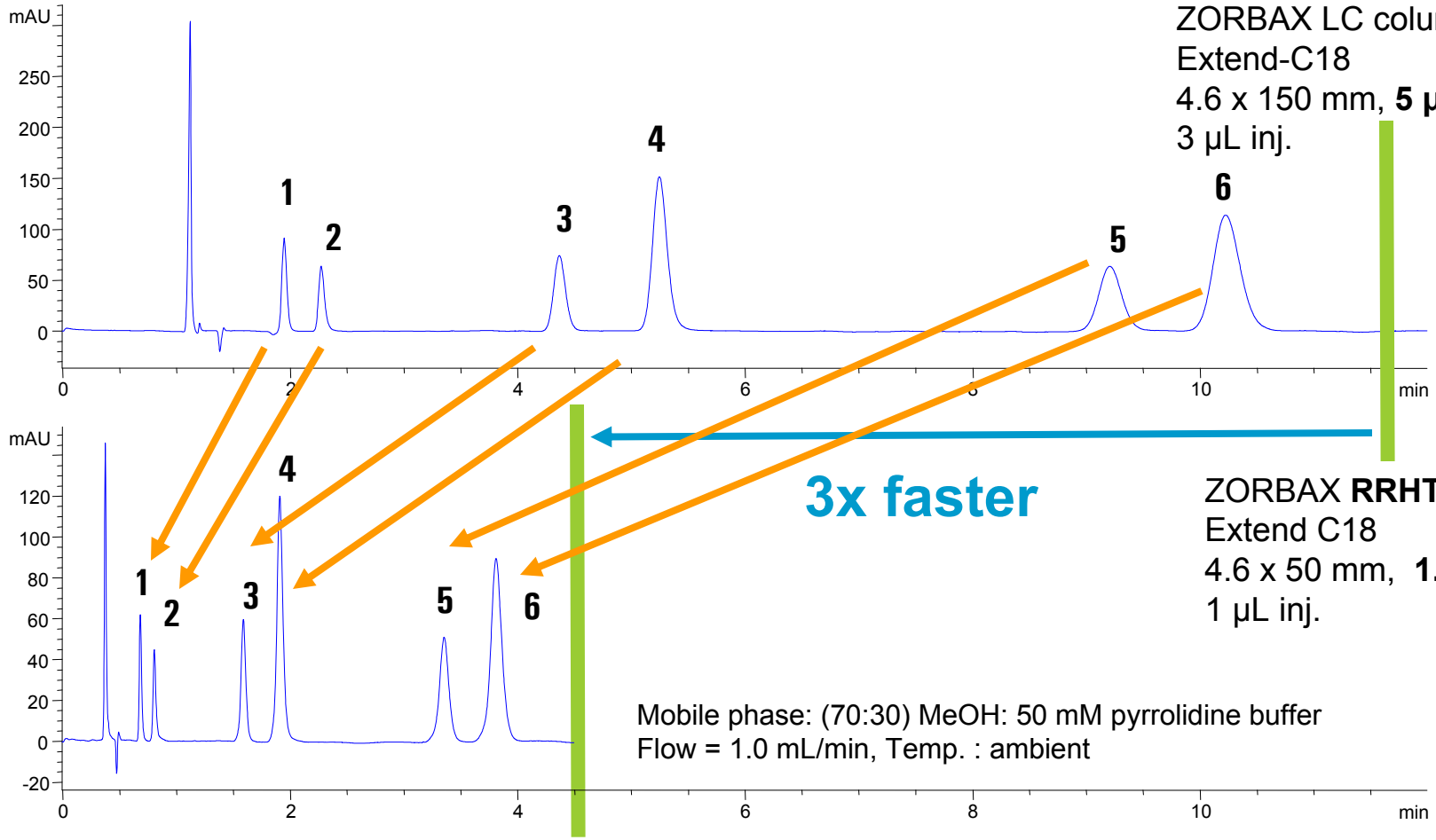
4.6 x 75 mm, 3.5 µm

Analysis Time:
5.2 min



Goal: Run Current Method 3X Faster

Trick: 1/3 Column Length and Smaller Particle = 1/3 Time



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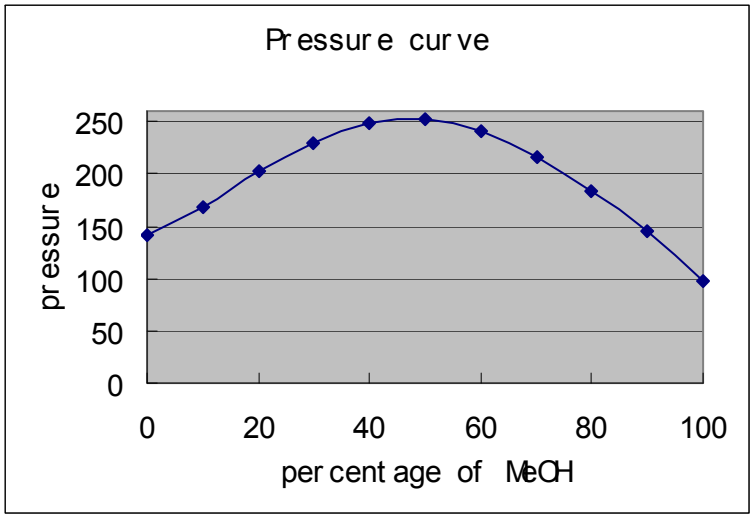
How Long Can the Column Be with MeOH?

System Pressure With MeOH/Water

4.6 x 50mm Column with 1.8um particles at 1 ml/min,30°C



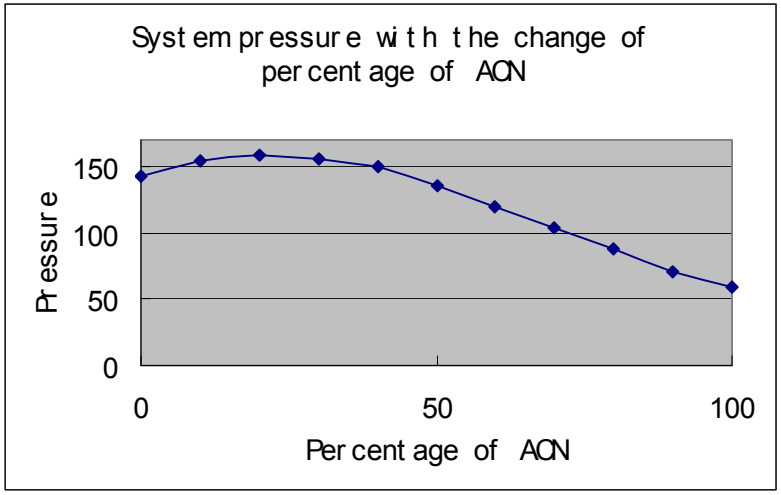
Percentage of **MeOH** (%)	Pressure (bar)
0	142
10	169
20	203
30	229
40	249
50	252
60	240
70	216
80	184
90	145
100	98

- **Use P of 4.6 x 50mm as baseline**
- **Multiply P by L_{col2} / L_{col1}**
- **If P is Lower than Max P of Instrument you can run that Length**

How Long Can the Column Be with ACN?

System Pressure with ACN/Water

4.6 x 50mm Column with 1.8um particles at 1 ml/min,30°C



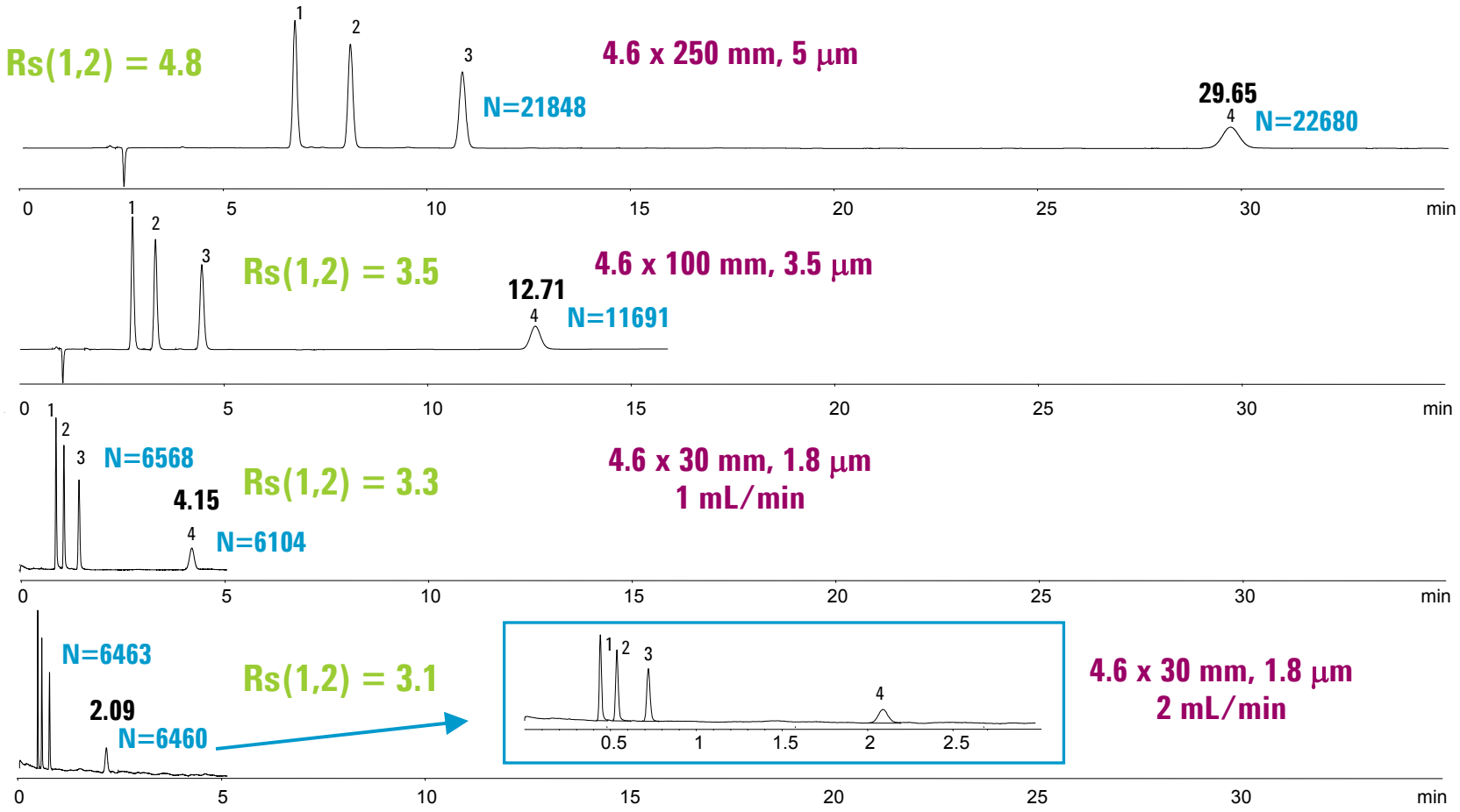
Percentage of ACN (%)	Pressure (bar)
0	142
10	154
20	159
30	156
40	150
50	135
60	120
70	104
80	88
90	70
100	59

- **Use P of 4.6 x 50mm as baseline**
- **Multiply P by Lcol2 / Lcol1**
- **If P is Lower than Max P of Instrument you can run that Length**

More Resolution in Original Method than Needed

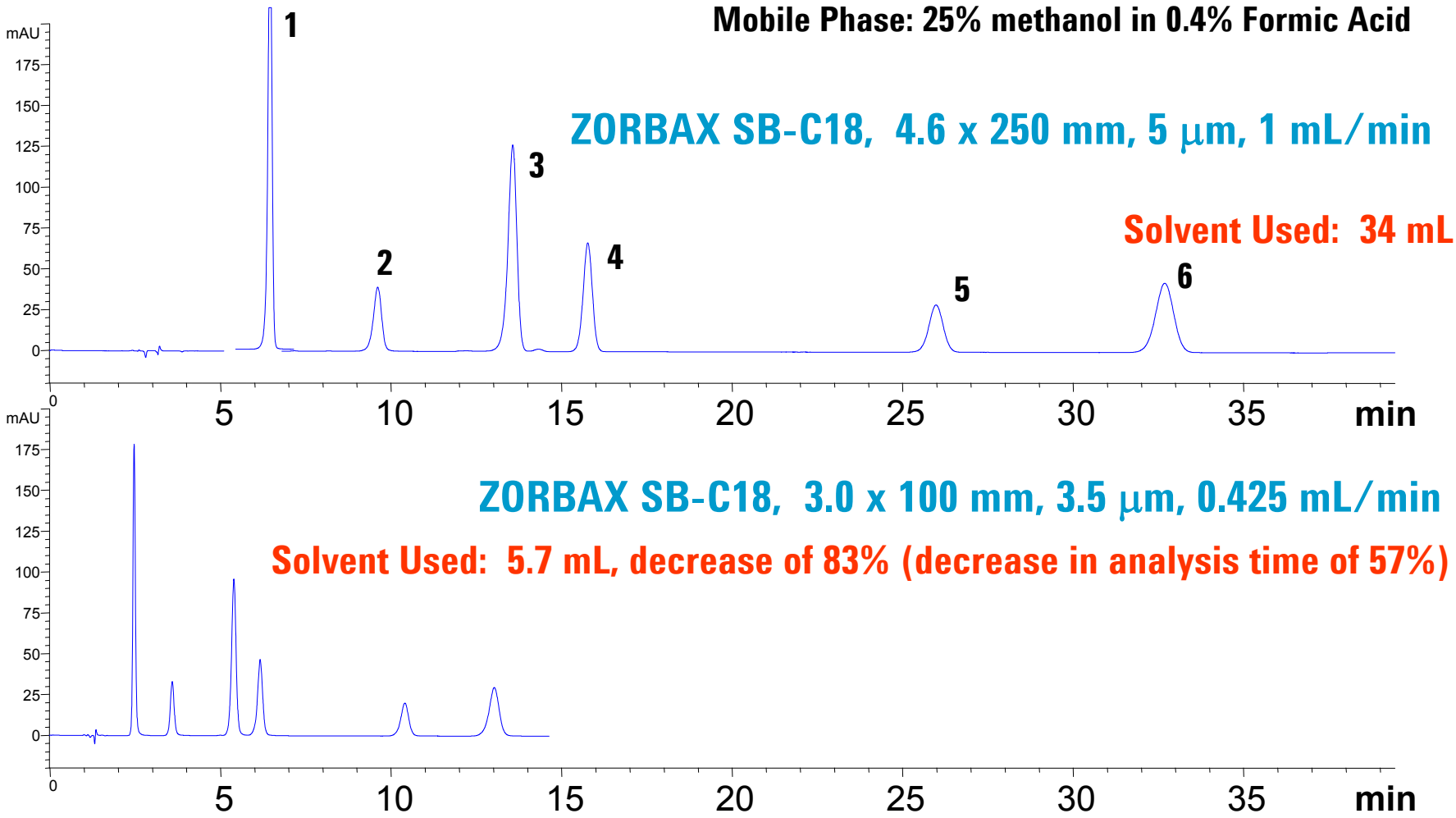
Tip: 1.8u, Very Short Column, Faster Flow 5-10X Faster

Isocratic on Instrument with 400bar Pressure Limit



Columns: ZORBAX SB-C18 Mobile Phase: 50% 20 mM NaH_2PO_4, pH 2.8: 50% ACN Flow Rate: 1 mL/min Temperature: RT
Detection: UV 230 nm Sample: 1. Estradiol 2. Ethynylestradiol 3. Dienestrol 4. Norethindrone

Tip: Constant Linear Velocity Maintains Efficiency
Trick: Reduce Flow Rate When Reducing Column Diameter



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Quick Reference for Changing to Common Column Diameters

Maintains Equivalent Linear Velocity for Different Column ID's:

Column Type	Column ID	Flow Rate
Analytical	4.6 mm	1.0 mL/min
Solvent Saver	3.0 mm	0.42 mL/min
NarrowBore	2.1 mm	0.21 mL/min
MicroBore	1.0 mm	47 µL/min
Capillary	0.5 mm	12 µL/min
Capillary	0.3 mm	4.2 µL/min
Nano	0.1 mm	472 nL/min
Nano	0.075 mm	266 nL/min

Flow rate column 2 = (diameter column 2)^2/(diameter column 1)^2 x Flow rate column 1

- **Maintain equivalent mobile phase linear velocity when changing column diameter.**

Isocratic Tips and Tricks Summary

- Increased Isocratic Speed Does Not Necessarily Require Sub-Two Micron Particles
- Particle Diameter and Column Length are Major Factors in Resolving Power
- Chose New, Shorter Column to Approximate Original Column Efficiency - Chose Length Based on Time Savings Goal

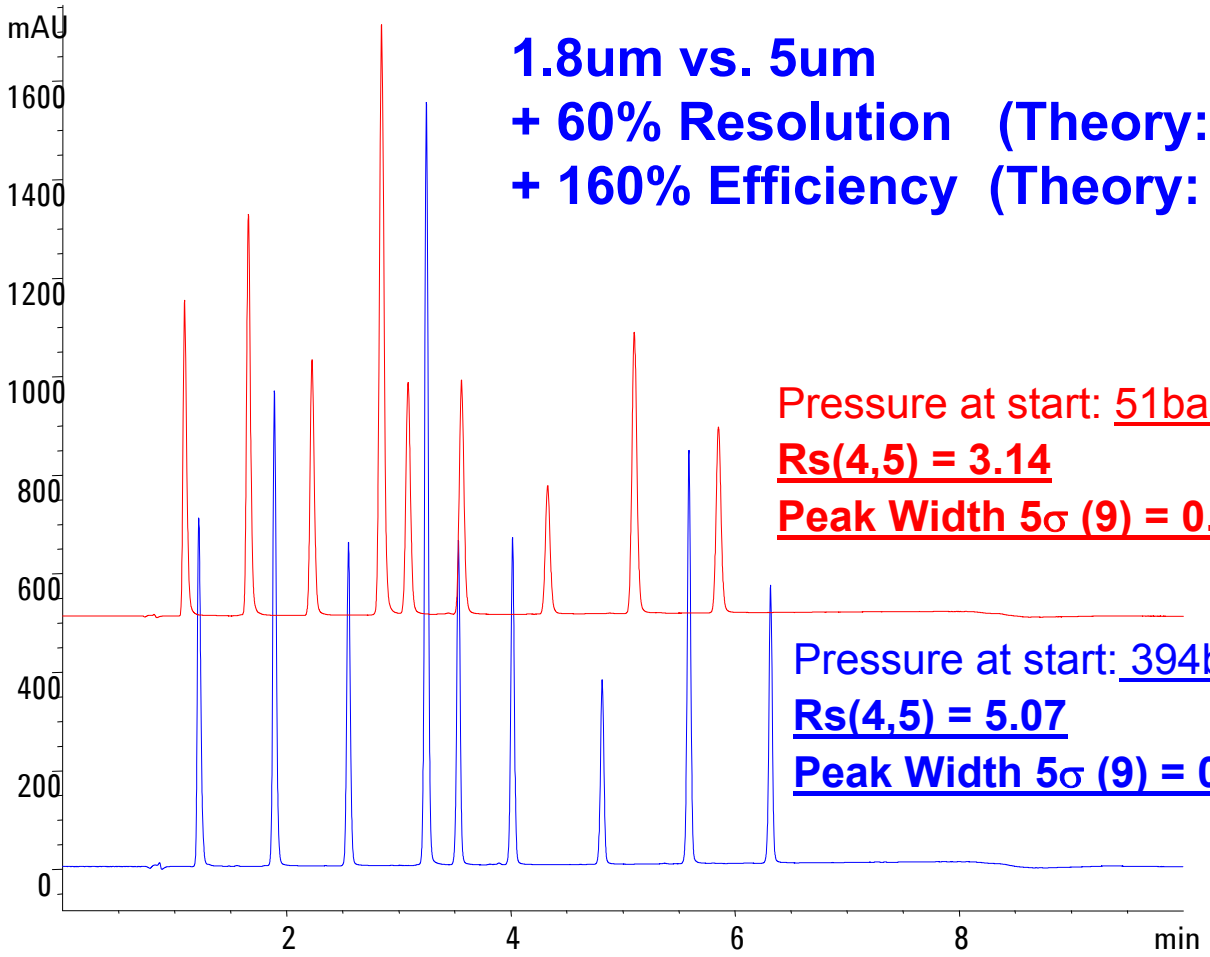
Example 3X: **150mm, 5µm (~12,000N) ~ 50mm, 1.8µm (~12,000N)**

- Usually Only Need to Reduce Col. Length and Particle Size – Not Necessary to Change Flow, Injection Vol. or Organic Content of M.P.
- Altered Column Diameter Requires Change in Flow and Injection Volume


GRADIENT
ELUTION

Reducing Particle Size Improves Kinetics

Reduces Peak Width, Increases Height- It Does NOT Change the Gradient!



1.8um vs. 5um
+ 60% Resolution (Theory: 67%)
+ 160% Efficiency (Theory: 177%)

Pressure at start: 51bar, 5µm
Rs(4,5) = 3.14
Peak Width 5σ (9) = 0.107

Pressure at start: 394bar, 1.8µm
Rs(4,5) = 5.07
Peak Width 5σ (9) = 0.074

➢**Chromatograms Offset for Better View**

Resolution Equation for Gradient Elution
Relationship of k* to Gradient Time, Flow and Column Dimensions

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

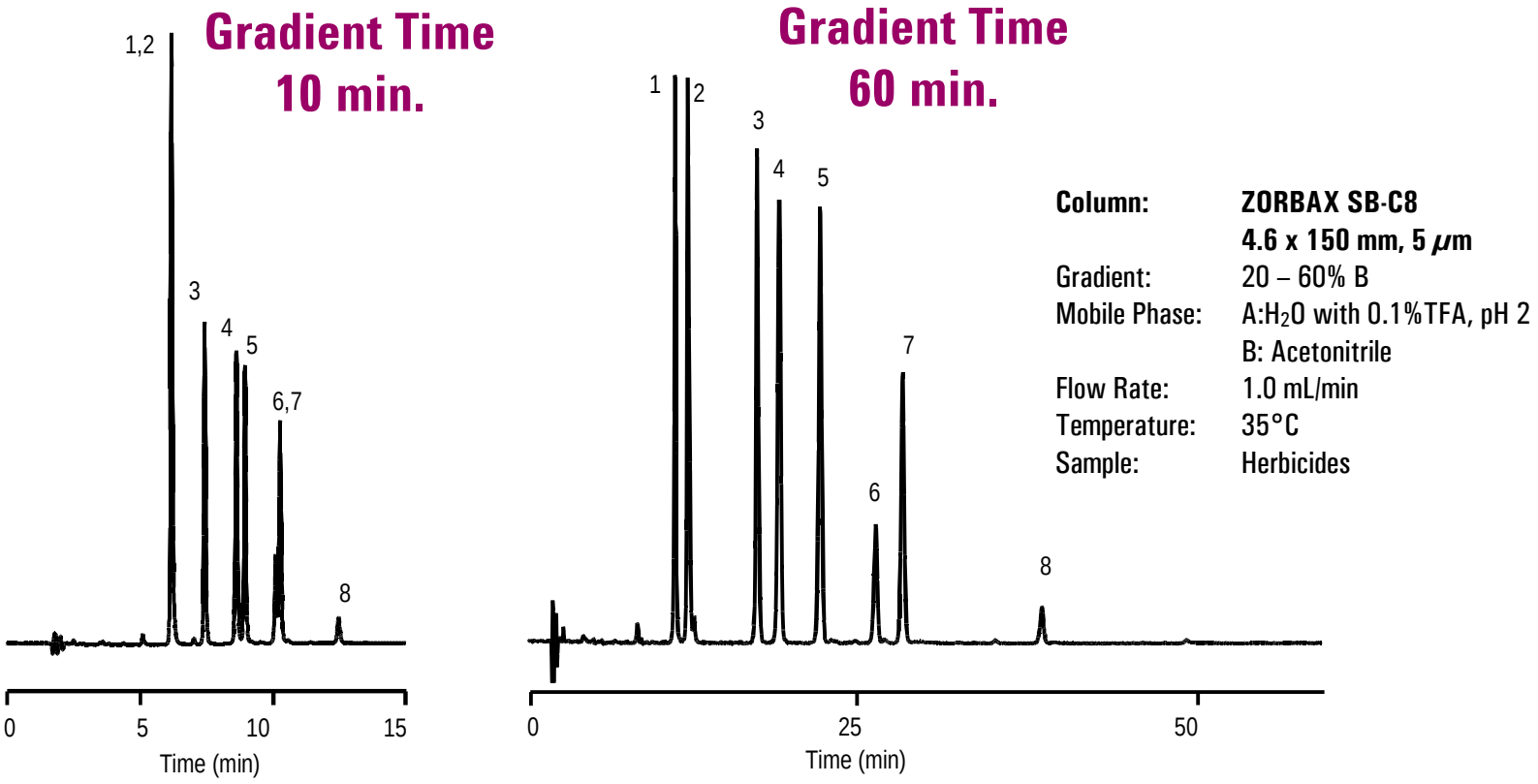
$$k^* \propto \frac{t_g F}{S\,(\Delta\%B)\,V_m}$$

$\Delta\%B$ = difference between initial and final % B
S = constant (≈ 4 for 100 - 500 Da)

F = flow rate (mL/min.)
t_g = gradient time (min.)
V_m = column void volume (mL)

Zorbax Vm= π x (Col Internal Radius)^2 x Length x 0.6

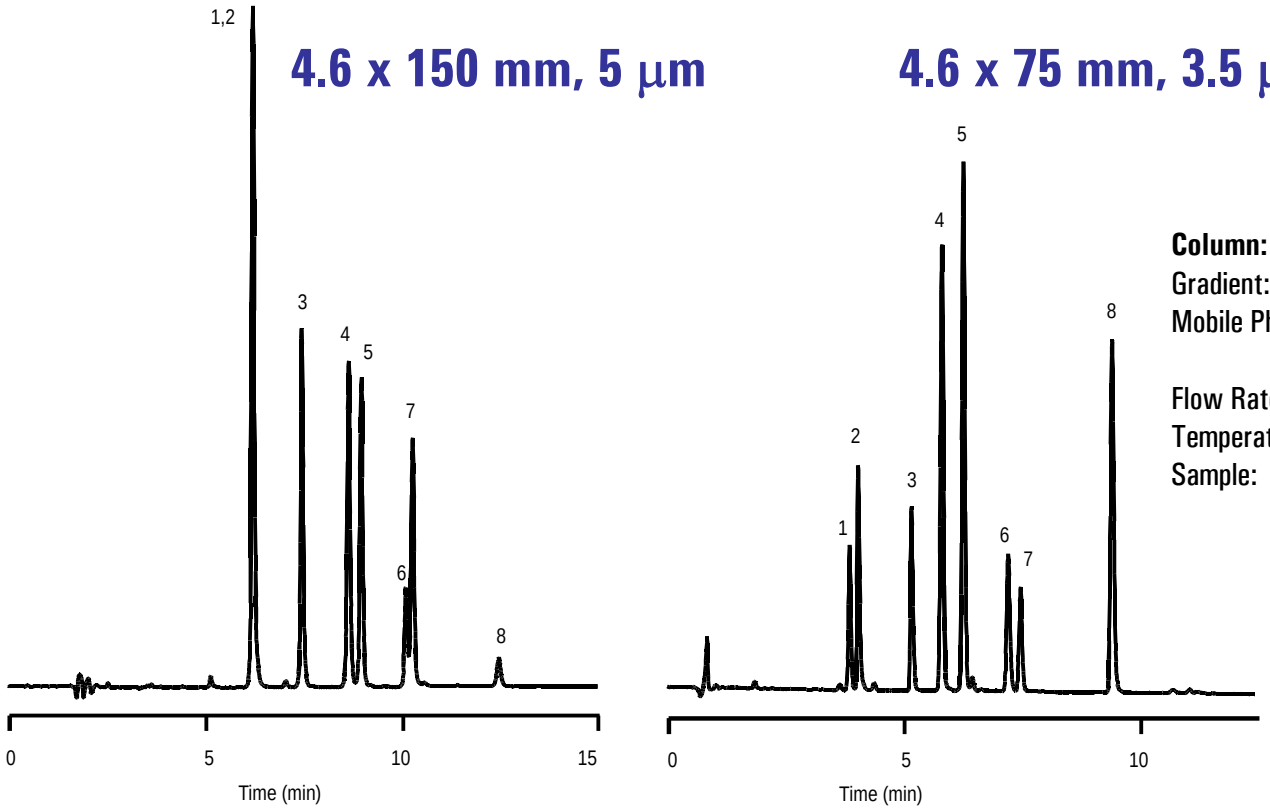
Expected: Use of a Longer Gradient Time Increases Gradient Retention



- **Increased gradient retention improves resolution of several peak pairs – 1,2 and 4,5.**

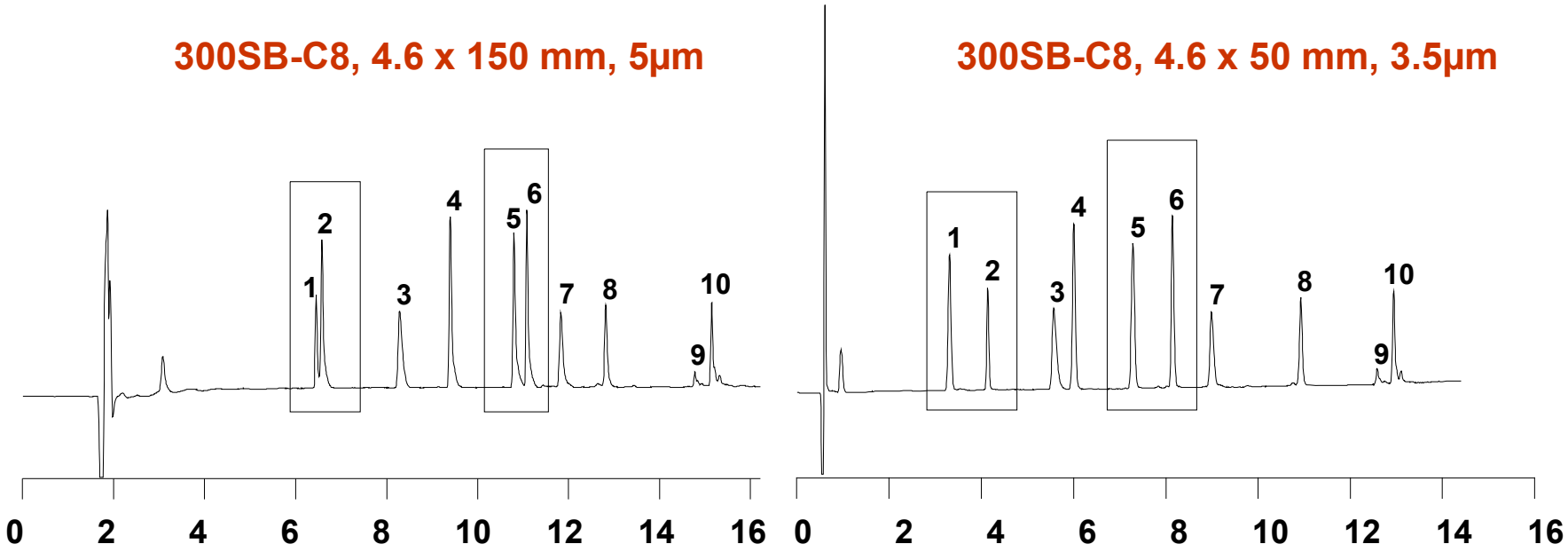
Tip: A Shorter Column (smaller Vm) Also Increases Gradient Retention and Rs

Increases Gradient Retention, Increases Overall Resolution



Column:	**ZORBAX SB-C8**
Gradient:	20 – 60% B
Mobile Phase:	A:H_2O with 0.1%TFA, pH 2
	B: Acetonitrile
Flow Rate:	1.0 mL/min
Temperature:	35°C
Sample:	Herbicides

Tip: Improve Resolution By Using Short Column Length (Vm) for BioMolecules



Mobile Phase: A: 95% Water : 5 % ACN, 0.1% TFA
B: 5% Water : 95% ACN, 0.085% TFA
Gradient: 10-60% B in 30 min.

Flow Rate: 1.0 mL / min.

Temperature: Ambient

Sample:
1. Gly-Tyr
2. Val-Tyr-Val
3. [Gln^{11}] Amyloid-β-Protein Fragm 1-16
4. (TYR8) Bradykinin
5. Met-Enk
6. Leu-Enk
7. Angiotensin II
8. Kinetensin
9. RNase
10. Insulin (Eq.)

Tip: Maintain k* To Keep Relative Peak Position in a Chromatogram Unchanged While Reducing Time

Any Decrease in	Can be Offset by a Proportional
Vm-Column length	Decrease in t_G or F
Vm-Column (i.d.)	Decrease in t_G or F

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

Trick: Change V_m and t_G by Same Proportion

Two Chromatograms Both Having the Same Gradient Steepness

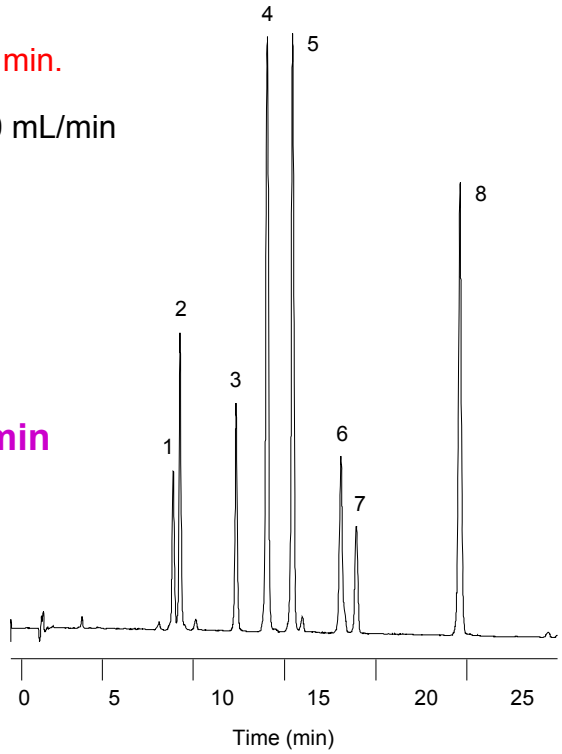
Sample: 1. Tebuthiuron 2. Prometon 3. Prometryne 4. Atrazine 5. Bentazon 6. Propazine 7. Propanil 8. Metolachlor

Column: **StableBond SB-C8**
4.6 x 150 mm, 5 µm

Gradient Time: 30 min.

Flow Rate: 1.0 mL/min

Analysis Time: 24 min

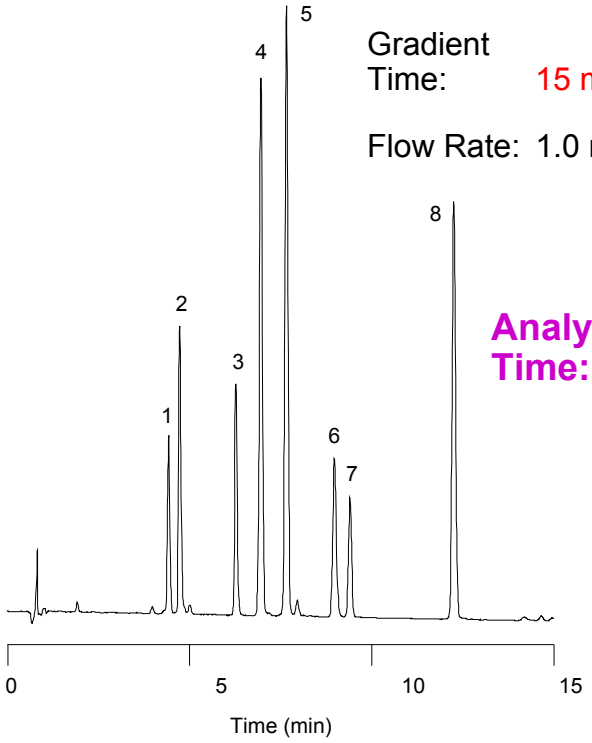


Column: **Rapid Resolution StableBond SB-C8**
4.6 x 75 mm, 3.5 µm

Gradient Time: 15 min.

Flow Rate: 1.0 mL/min

Analysis Time: 12 min



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Very Fast LC on Conventional 1100 HPLC

The Instrument

G1379 Degasser

G1311 Quaternary pump

G1313A ALS autosampler

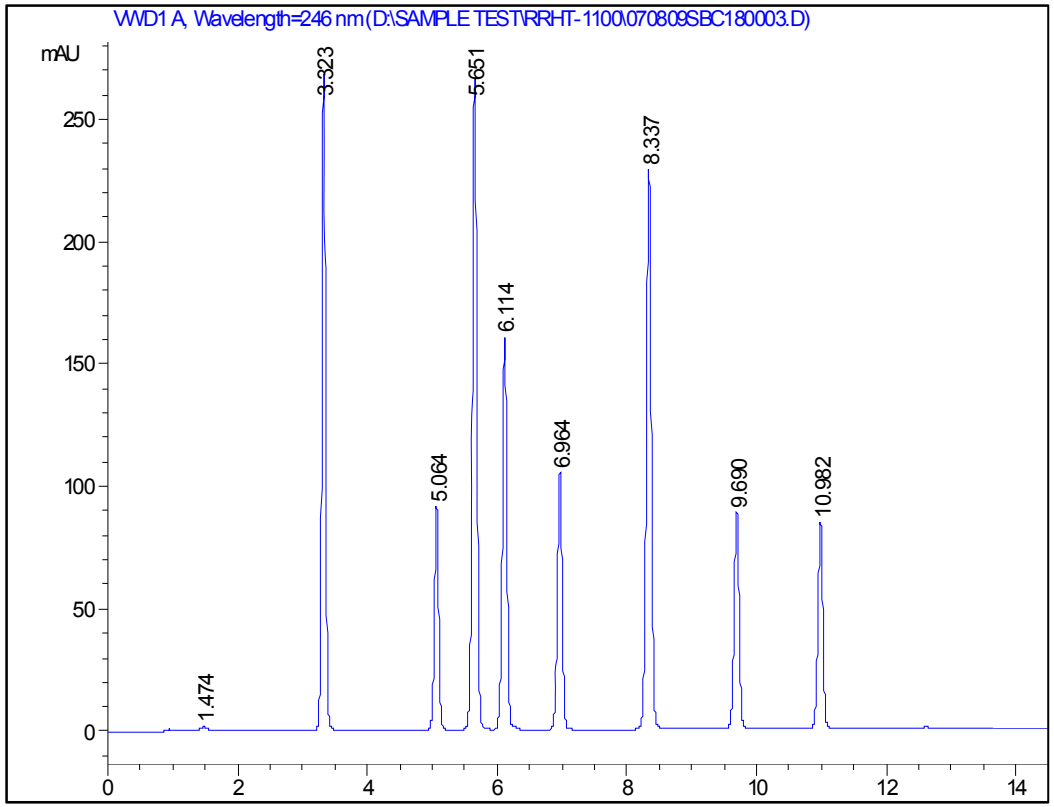
G1316A column compartment

G1314A VWD (standard cell G1314-60086, 10mm, 14uL)

The Sample

Acetophenone
Diethyl phthalate
Benzophenone
Butyrophenone
Valerophenone
Hexanophenone
Heptanophenone
Octanophenone

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

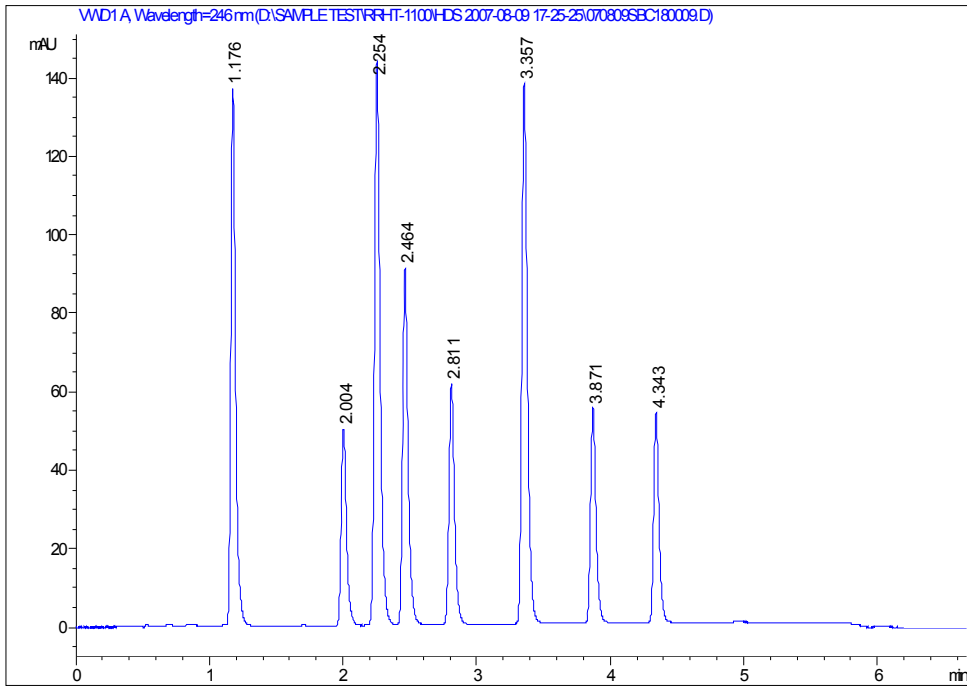
Initial Pressure: 69 bar

Final Pressure: 38 bar

Tip: Shorten Column and Gradient Time by Same Factor

1/3 Column Length- 1/3 Gradient Time

RRHT Column – 4.6 x **50mm**, 1.8µm, SB-C18



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

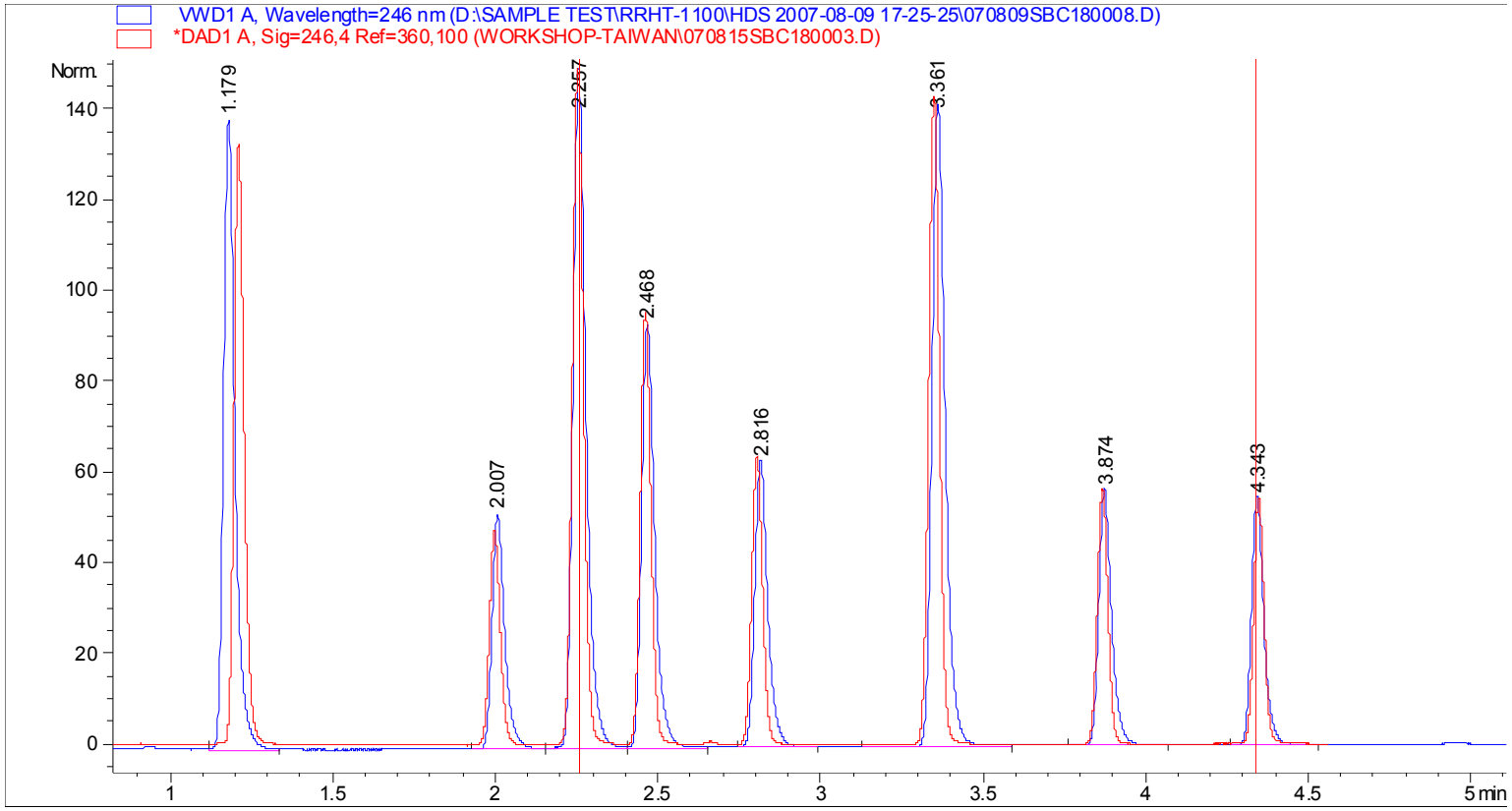
Time (min)	% Acetonitrile
0	50
3.33	90
4.5	90
4.53	50
5	50

Initial Pressure: 132 bar

Final Pressure: 74 bar

The Comparison of chromatogram with the same RRHT column in 1100 and 1200 SL

Overlay the two Chromatograms

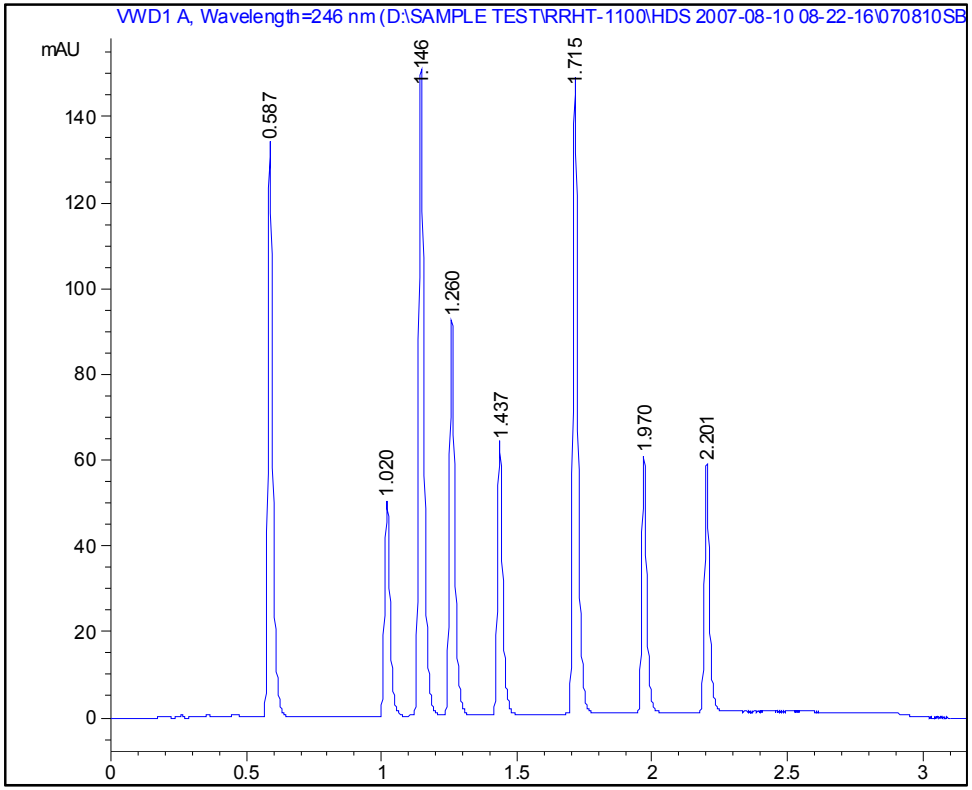


The red one is 1200, the blue one is 1100.

Tip: Increase Column Flow-Reduce Gradient Time

Double Flow (2mL/min) – ½ Gradient Time

RRHT 4.6 x 50mm, 1.8µm, SB-C18



Flow Rate	2.0 ml/min
Injection Volume	5uL
Temperature	30°C
Wavelength	246nm
Sample rate	13.74 Hz

Time (min)	% Acetonitrile
0	50
1.67	90
2.25	90
2.27	50
3.34	50

Initial Pressure: 266 bar

Final Pressure: 146 bar

Gradient Tips and Tricks Summary

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

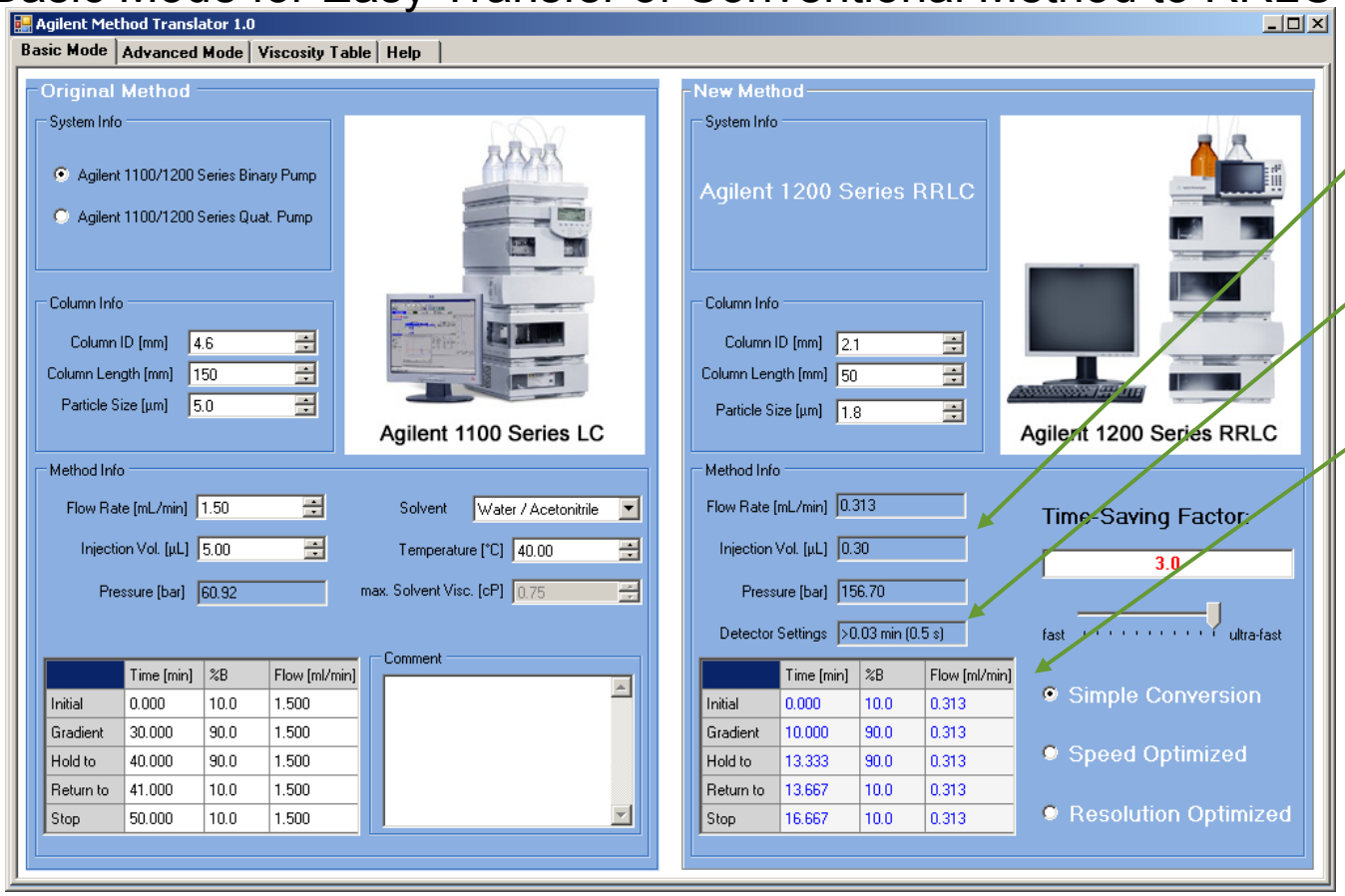
Gradient Retention Relationship Formula Is the Key to -

- Improving Resolution
  - Longer Gradient Time (t_g)
  - Smaller Column Volume (Vm) Length or Inner Diameter
  - *Keeping All other Parameters Constant*
- Increasing Speed Without Losing Resolution
  - Shorter Column with Proportionally Shorter Gradient Time
  - Shorter Gradient Time with Proportionally Faster Flow Rate

Tip: Method Translator Makes Changes Easy

Trick: Let Agilent Method Translator Do the Math

Basic Mode for Easy Transfer of Conventional Method to RRLC

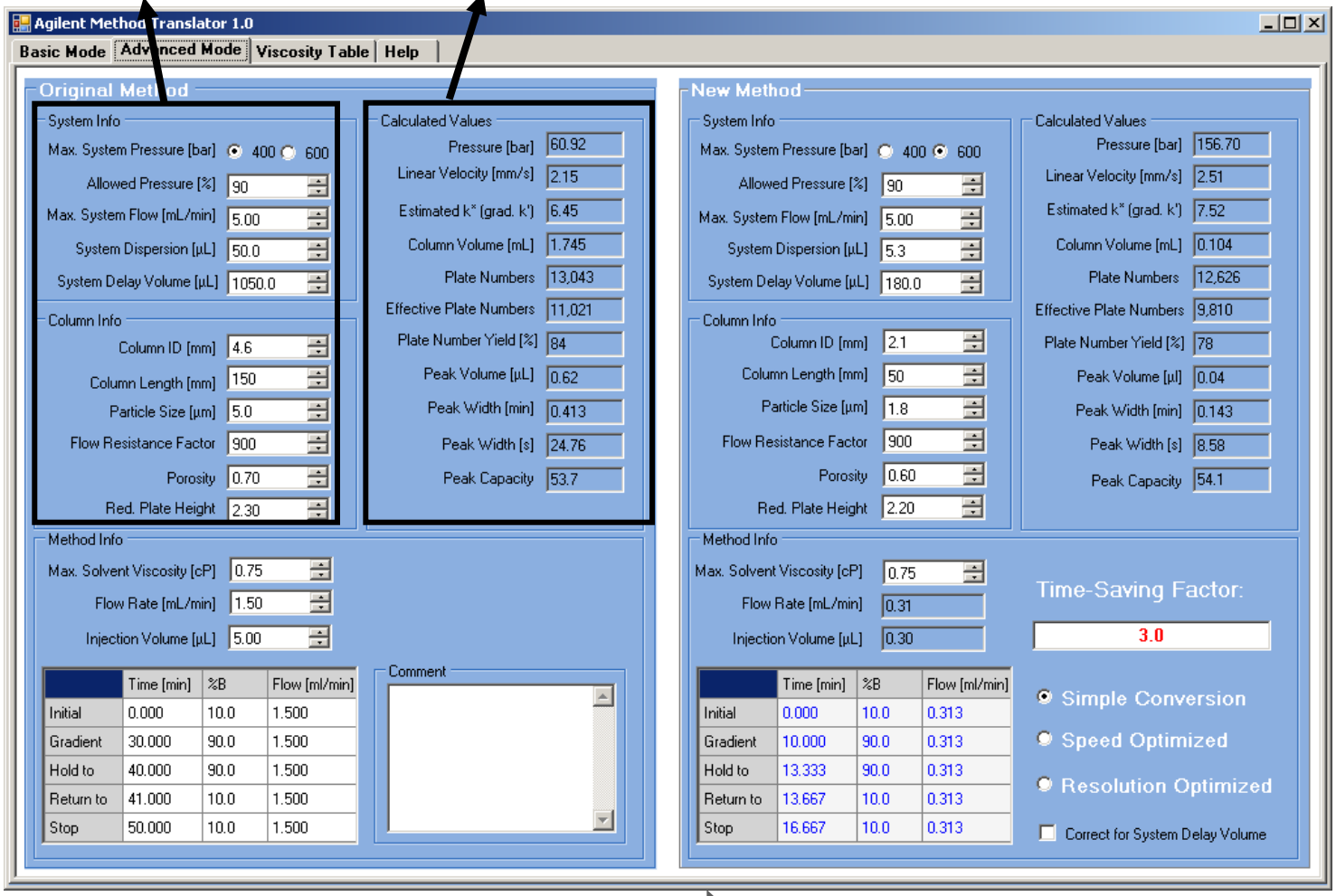


- Injection Volume Conversion
- Detector Settings recommendation
- Gradient and Isocratic Method Conversion (auto-detected)

Agilent Method Translator – Advanced Mode

More Detaled Information, But Still Easy to Use

Detailed Input Detailed Output



Original Method → **New Method**

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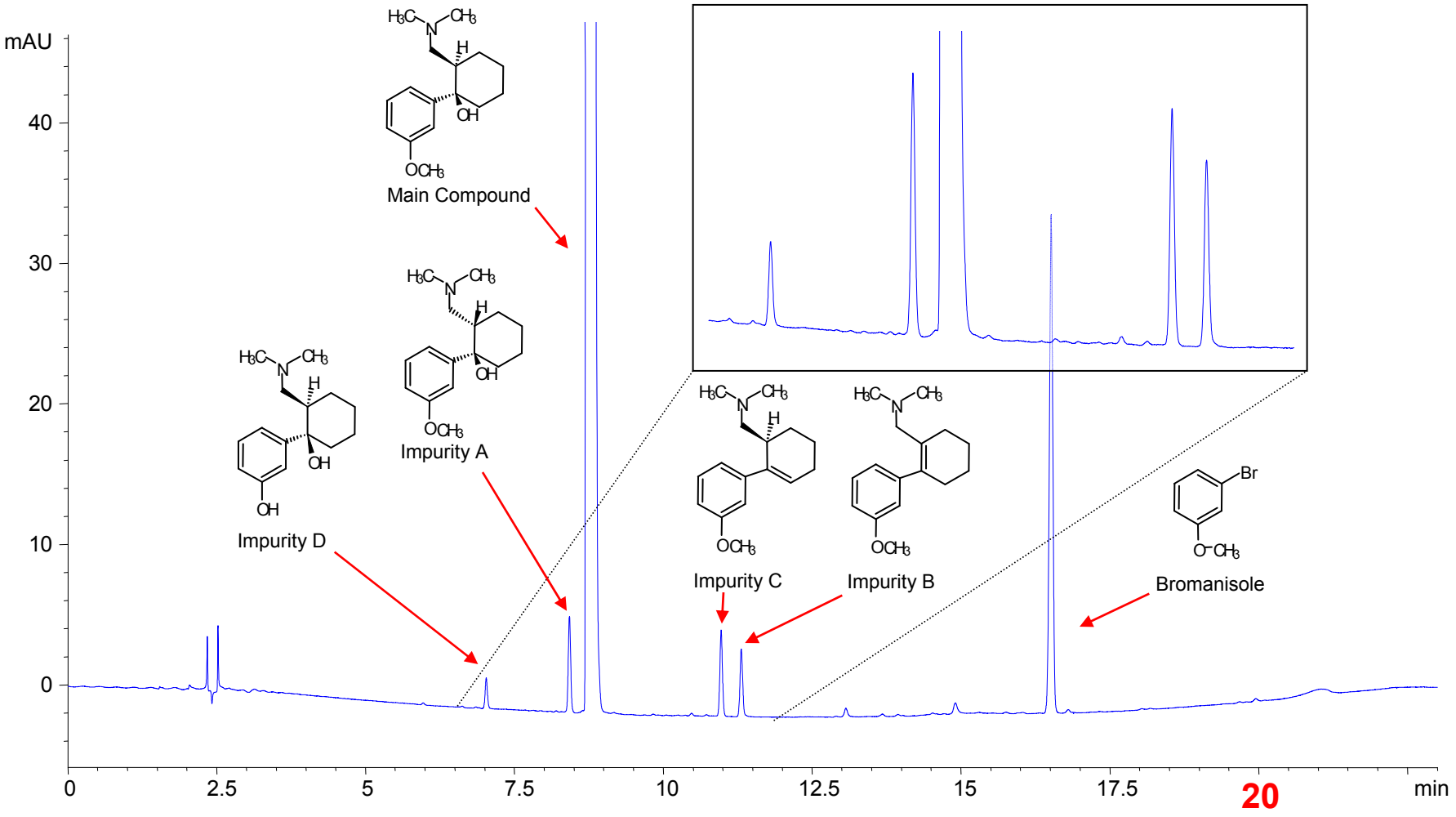
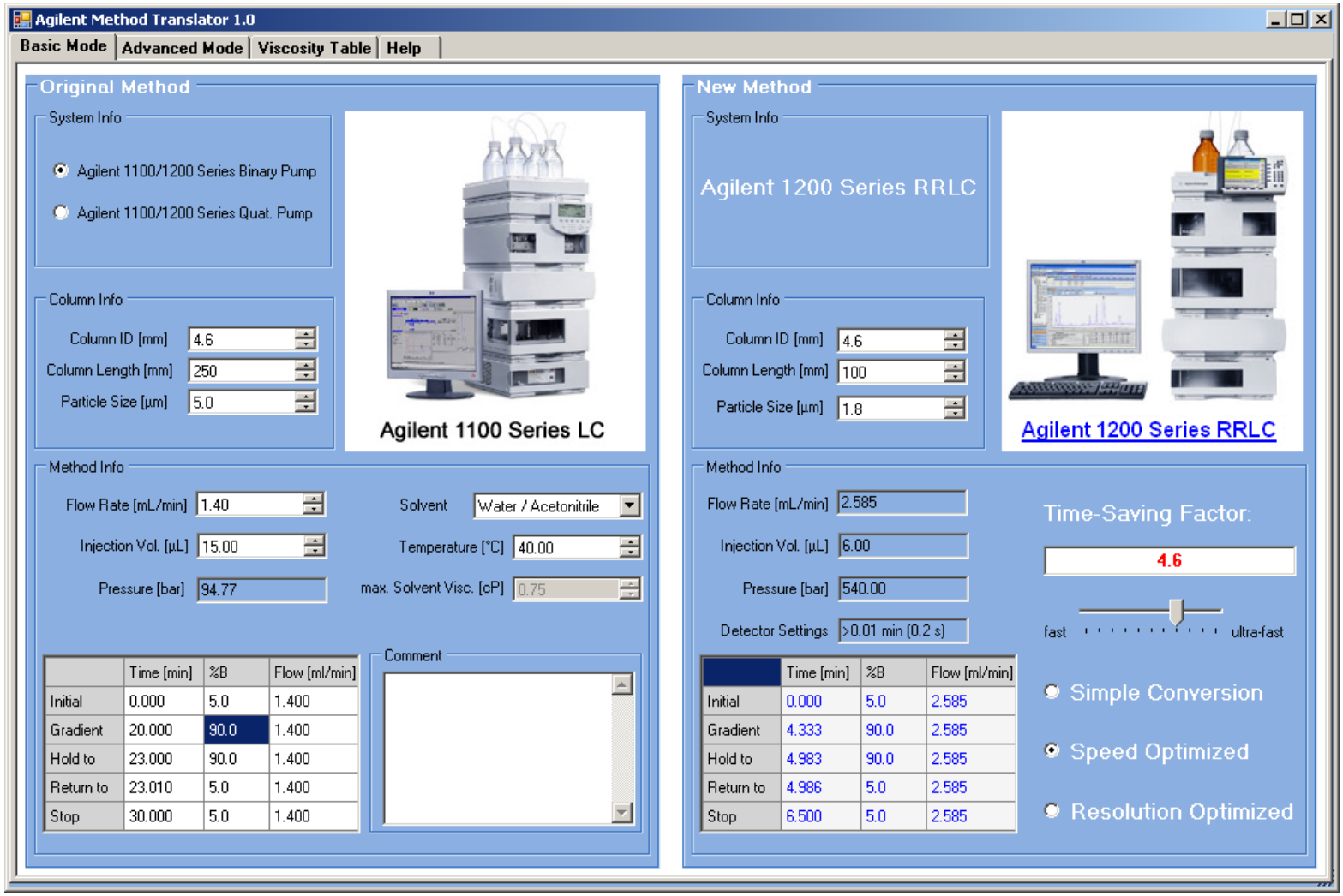
Does it work? - Example

Analysis of impurities of an active pharmaceutical ingredient by conventional HPLC (4.6mm ID x 250 mm, 5.0 µm):



Converting to a 4.6 mm ID x 100 mm, 1.8µm column:



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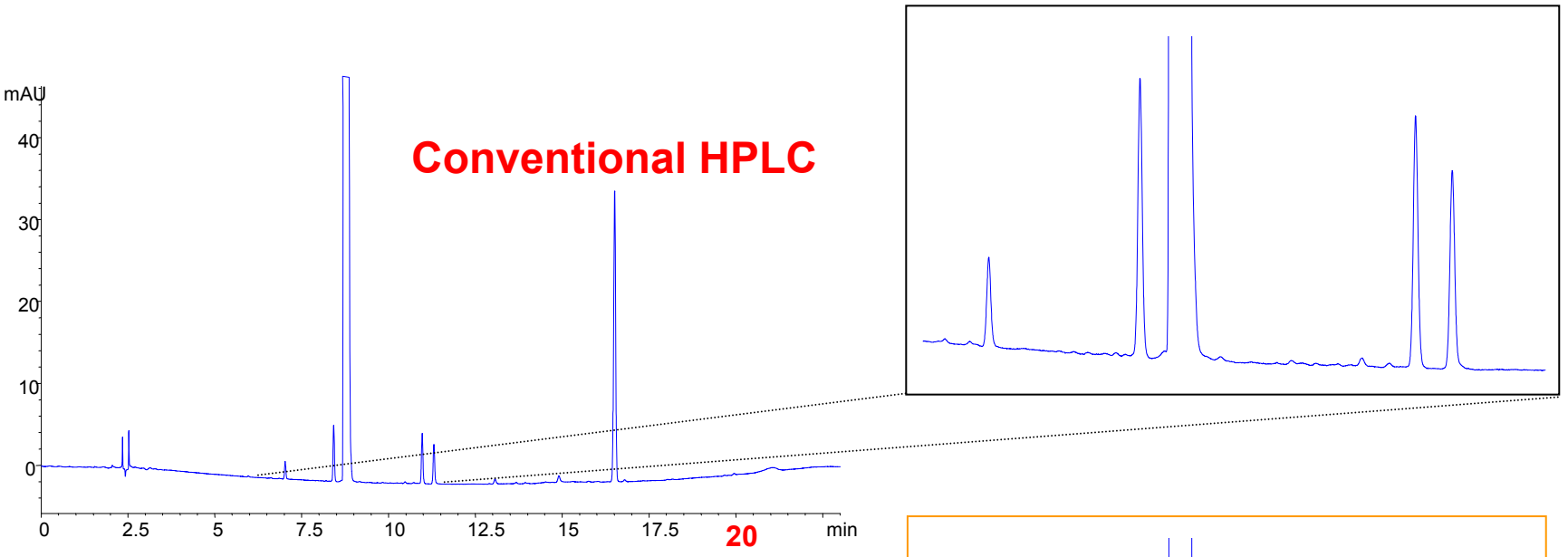
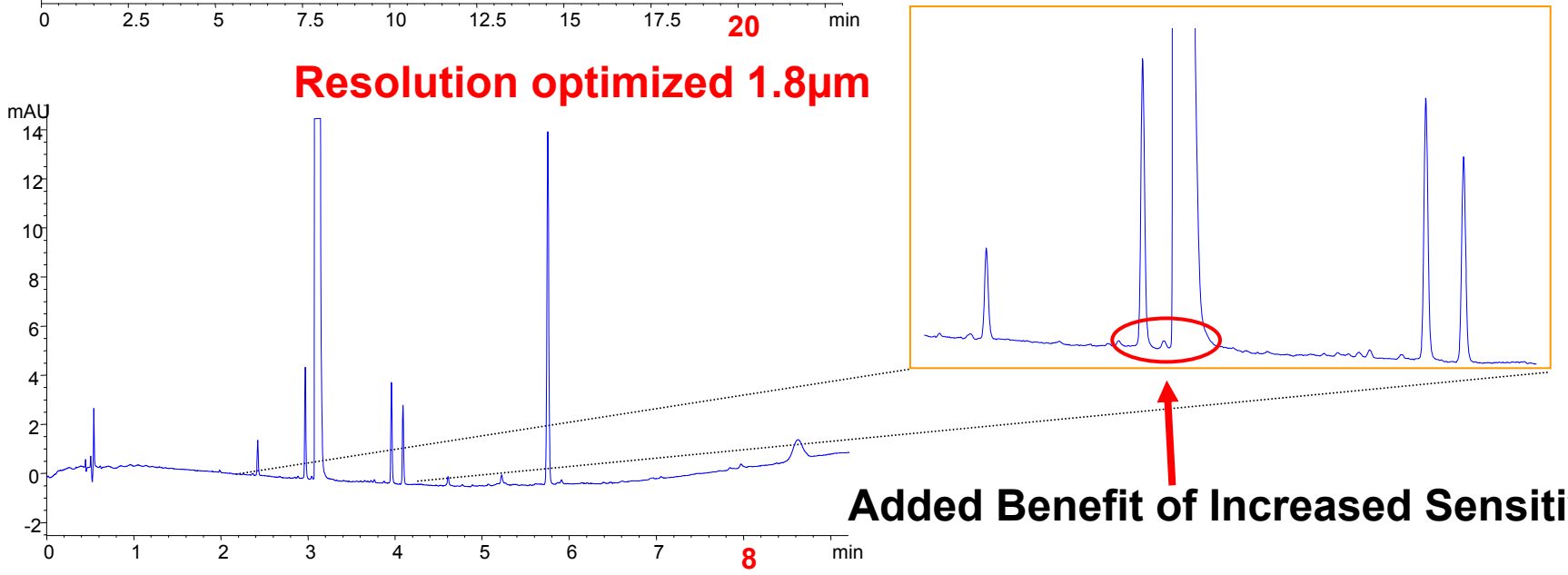
Does it work? Yes!



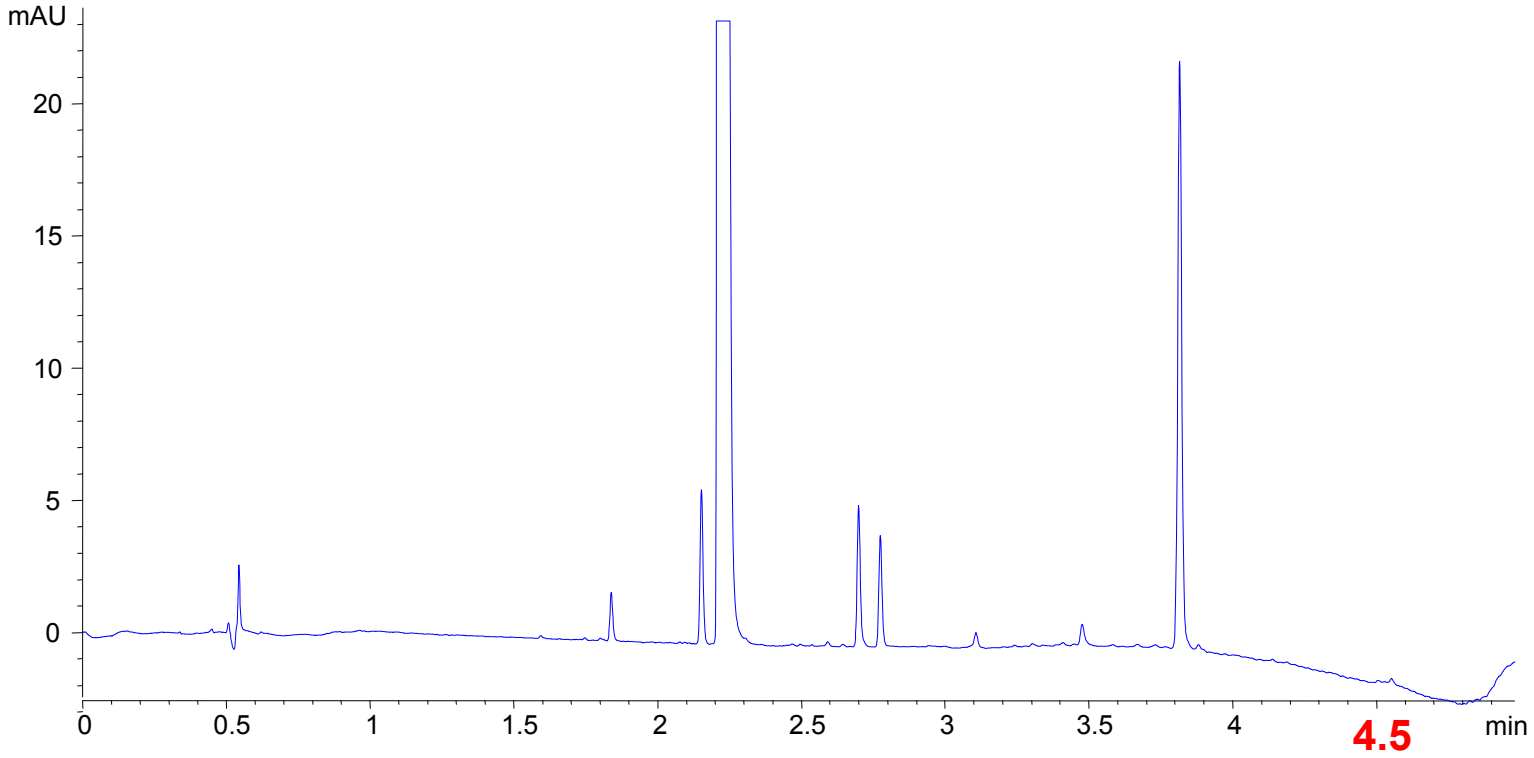
Conventional HPLC



Resolution optimized 1.8µm

Added Benefit of Increased Sensitivity!

Does it work?

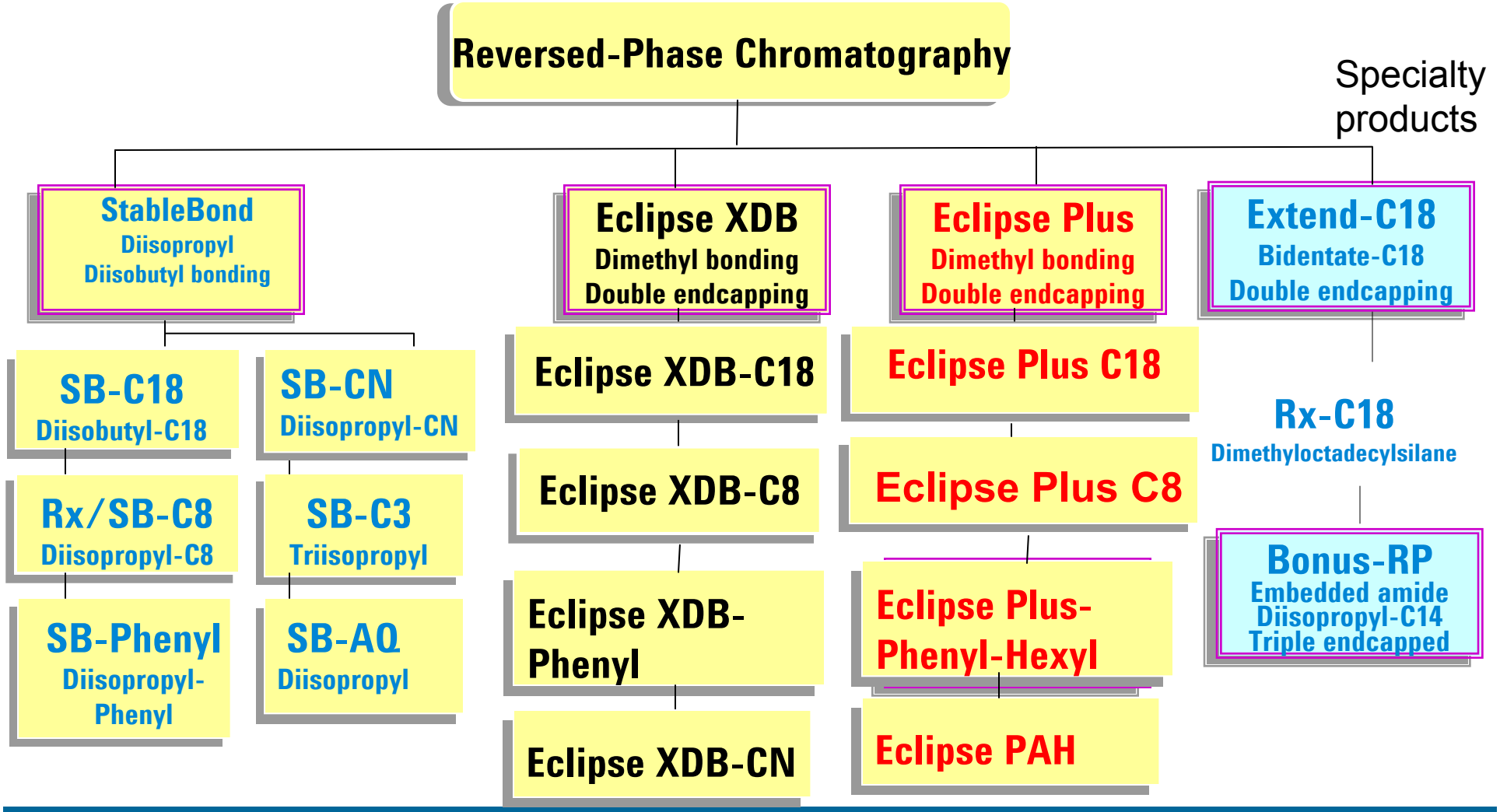


4.6 mmID x 100 mm, 1.8µm Zorbax SB C18

Speed Optimized

0.00 min	5%	B
4.33 min	90%	B
4.98 min	90%	B
4.99 min	5%	B
6.5 min	5%	B

ZORBAX Selectivity Choices
Options for Improved Speed and Resolution



Tip: Bonded Phase Can Change Selectivity
Improve Peak Shape and Shorten HPLC Assay Time

- Each ZORBAX phase available in matching 1.8µm, 3.5µm and 5µm choices, most in 7µm!

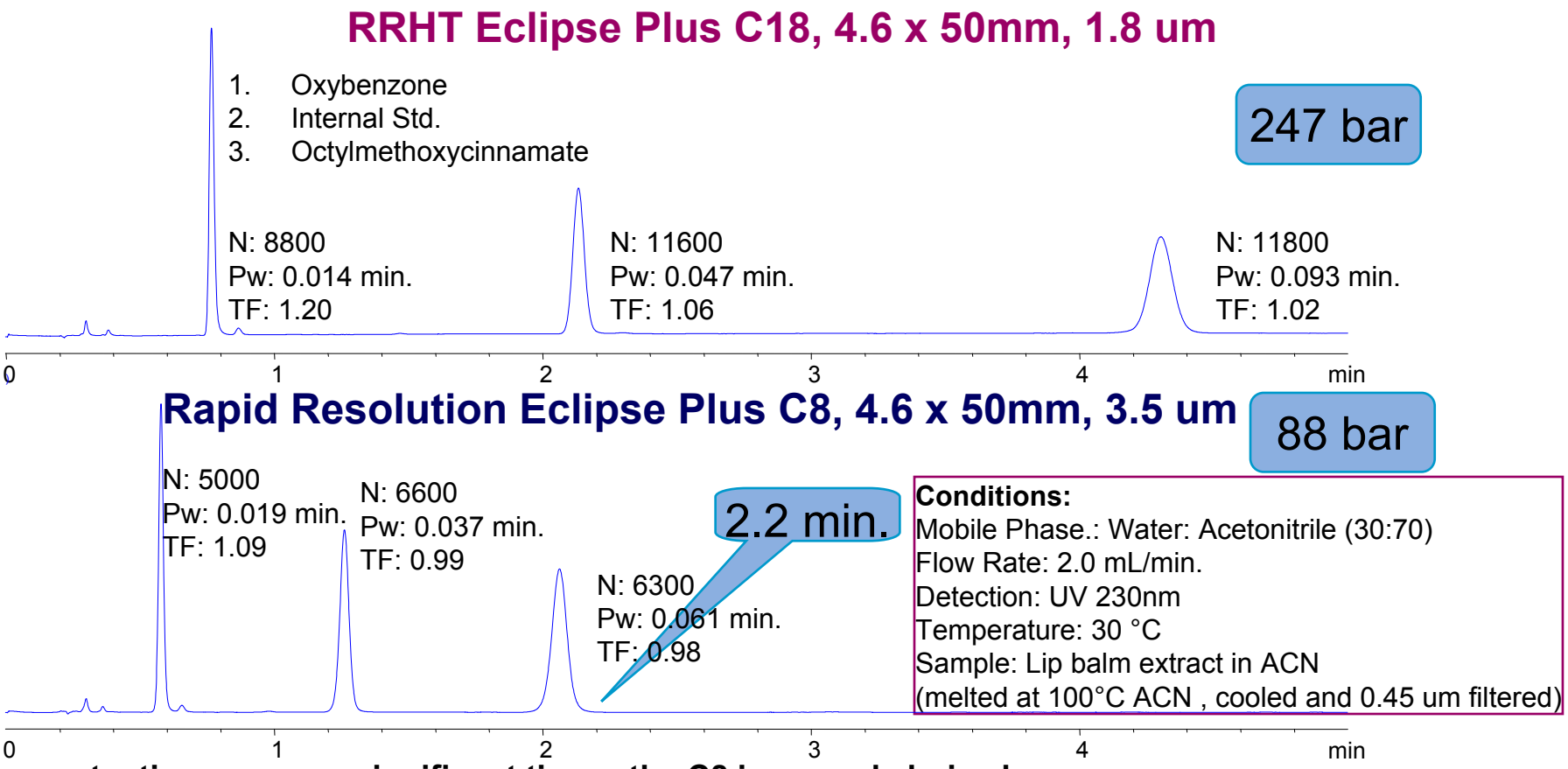
- Over 140 RRHT,1.8um 600 bar column choices available

- 14 Different Column Chemistries – 13 bonded phases and silica for HILIC use

- 7 column lengths (250*, 150, 100, 75, 50, 30 and 20 mm long) * 1.8µm as custom column

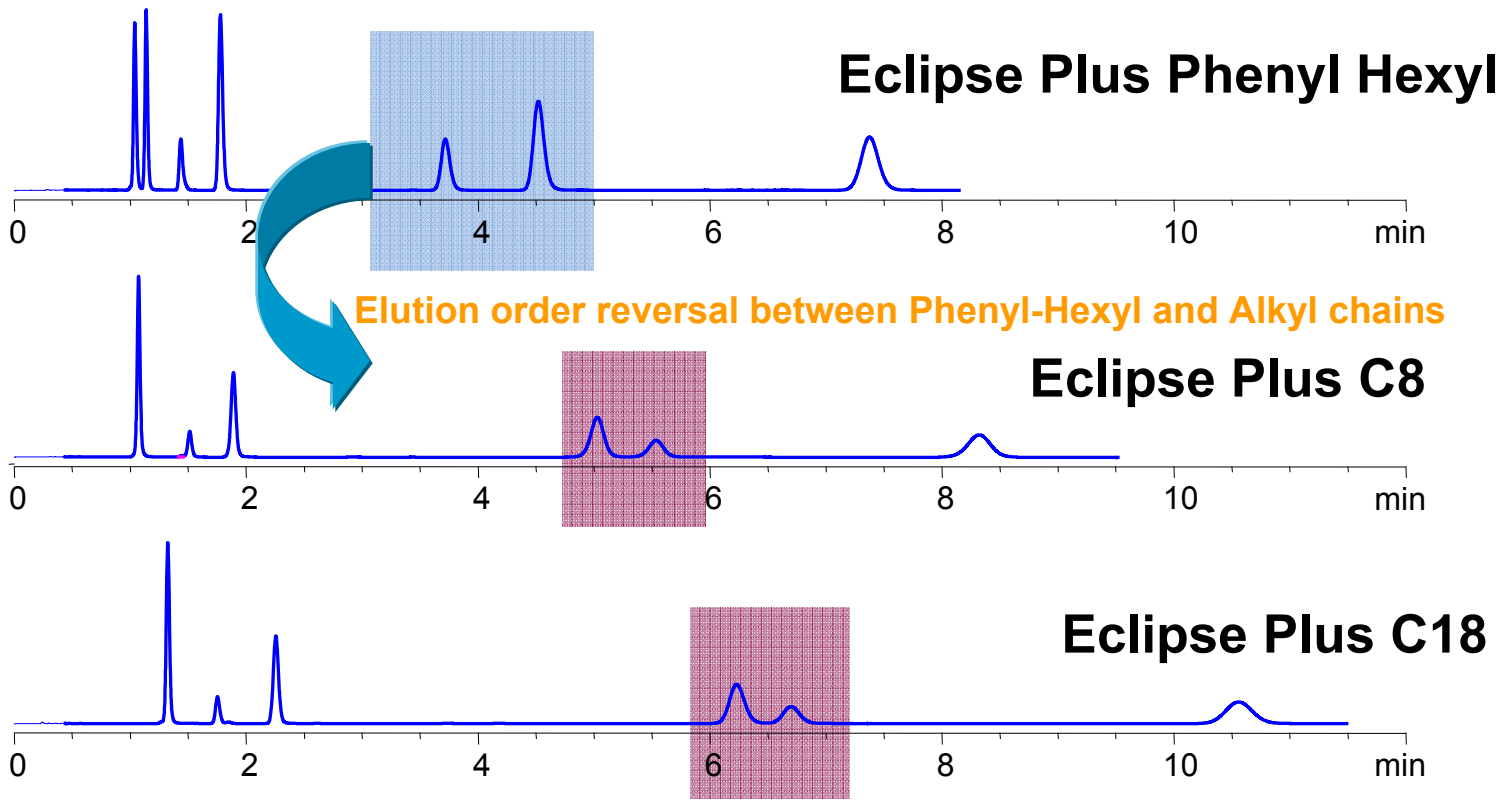
- 3 internal diameters (4.6, 3.0, 2.1 mm and Prep ID)

Tip: C8 Often Yields Same Peak Order in < Time Than C18 Trick: Evaluate Different Phases with Same M.P.



Less retention can save significant time – the C8 is a good choice here.
The RRHT column is delivering the efficiency and resolution expected, but the C8 bonded phase may be the best choice.

Tip: Polar Phases Can Save Analysis Time
Trick: Evaluate Non-Polar and Polar Phases with Same M.P.

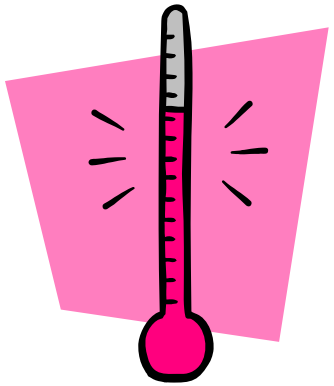


Mobile Phase 40 % ACN 60 % 25 mM Sodium Phosphate Buffer pH 2.4 Flow Rate: 1.5 ml/min 4.6 x 50mm UV 210 nm 2µl Elution order for Eclipse Plus Phenyl Hexyl: (1) Piroxicam, (2) Sulindac,(3) Tolmetin, (4) Naproxen, (5) Ibuprofen, (6) Diclofenac, (7) Celebrex (equal portions of approximately 1 mg/ml solutions

Tip: Higher Temperature Can Improve Speed and Resolution

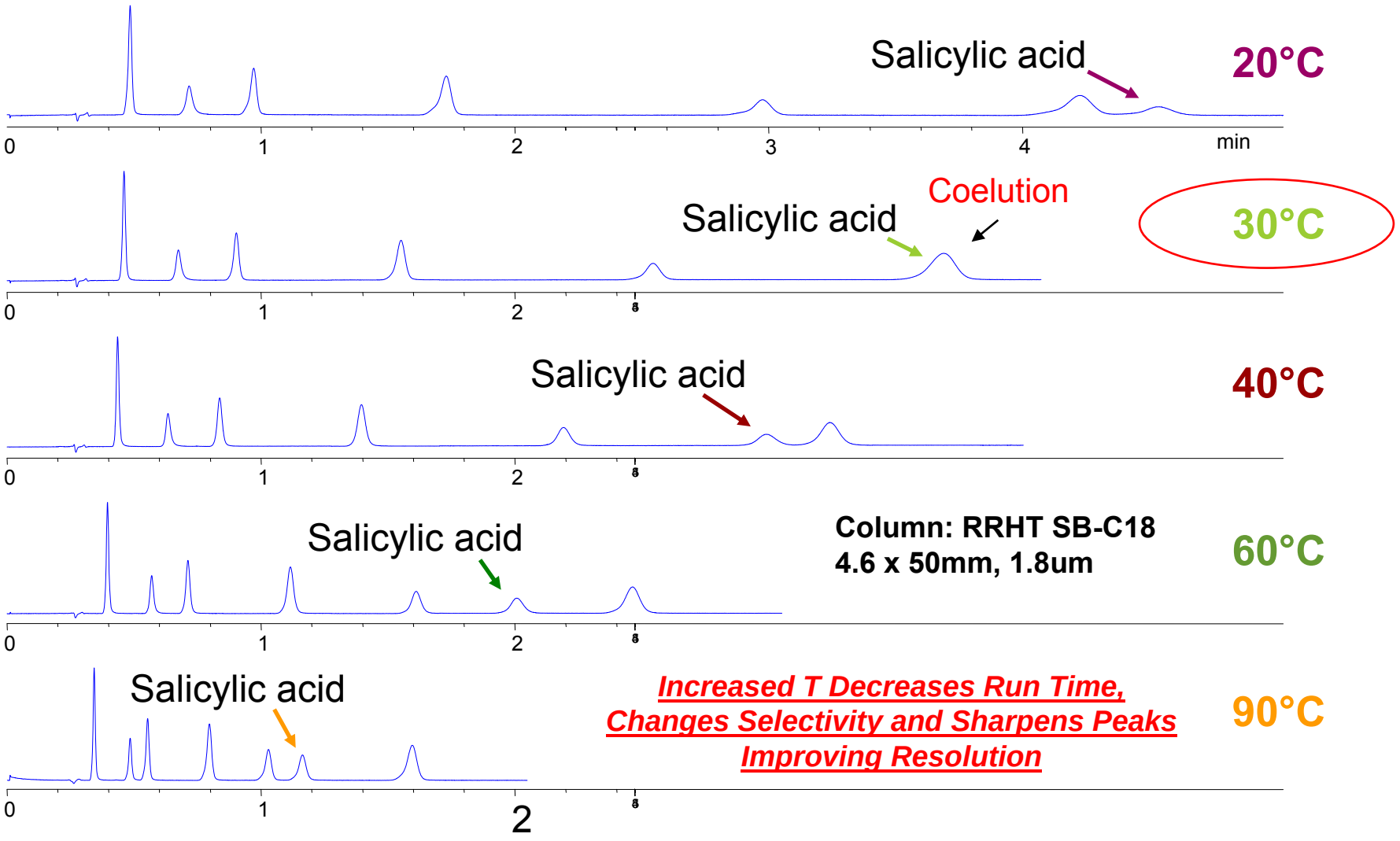
Higher Temperature should always be considered

as a parameter during speed optimization



- 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
- Can change selectivity – optimize resolution
- Not Necessary to Go to the Limit-Midrange Offers Benefits

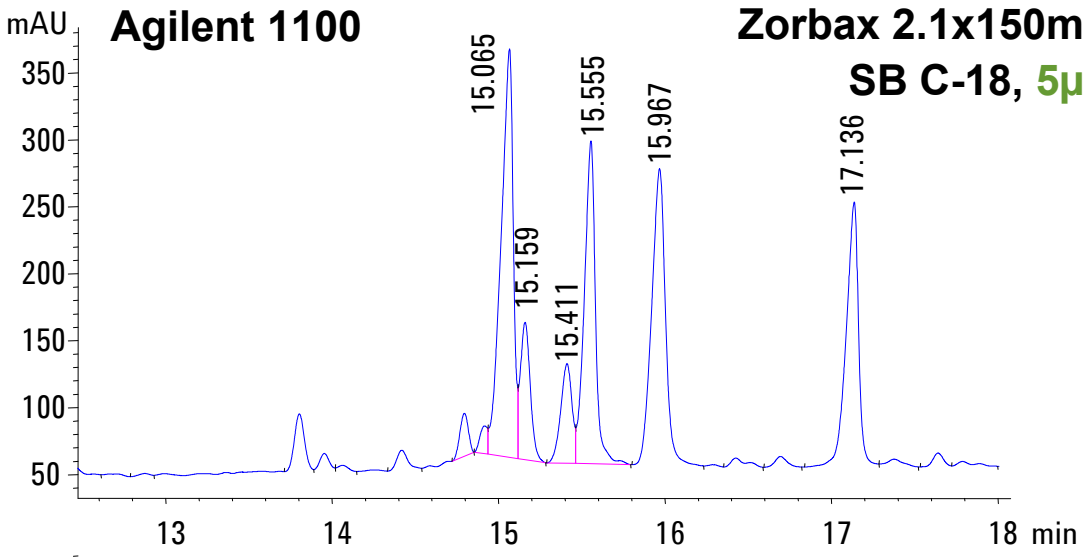
Tip: Temperature Changes Can Alter Selectivity
Trick: Optimize Col Temp For Best Speed/Rs

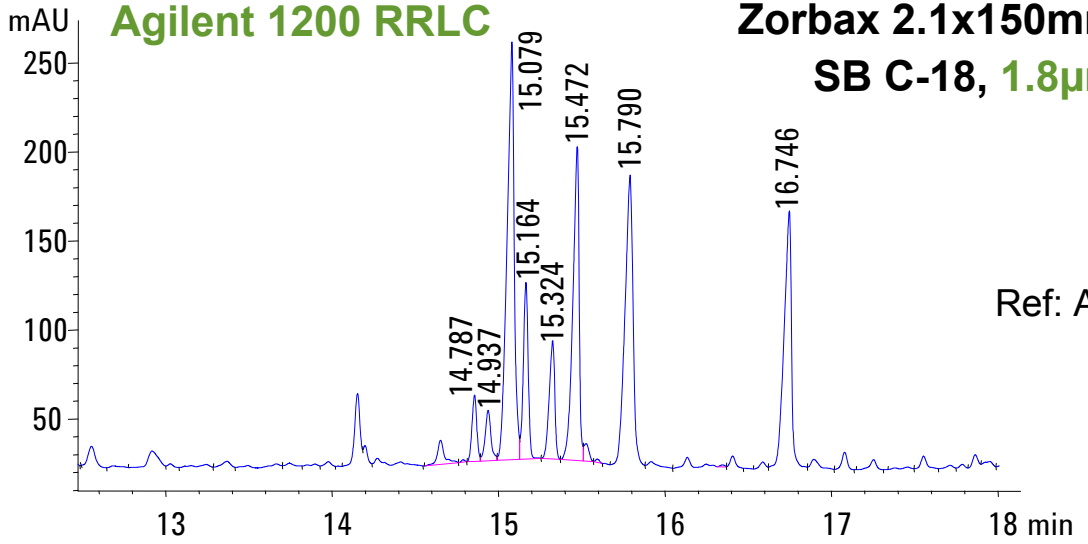


Tips on When You Should Move to the Higher Pressure and Performance 1200 SL (RRLC) System

- Very Fast Methods with High Velocity Mobile Phase That Increase Backpressure Above 400bar
- Resolution of Difficult Samples Requires Long Columns With 1.8µm Particles (>400bar Backpressure)
- Labs Required to Run Both Traditional and New, Higher Performance LC Methods
- Need Faster Data Acquisition for Narrow, High Efficiency Peaks Generated in Higher Speed Methods

Tip: 1.8µm Particle Increases Resolution (How?)
Trick: Substitute 1.8µm Particles in Same Length Column





Conditions for both experiments

Pumps

- Solvent A: H_2O + 0.1% TFA
  Solvent B: ACN + 0.1% TFA
- Gradient: 10% to 95% ACN in 40min, hold for 1min
- Flow Rate: 0.4ml/min

Autosamplers

- Injection volume: 3µl

Thermostatted Column Comp.

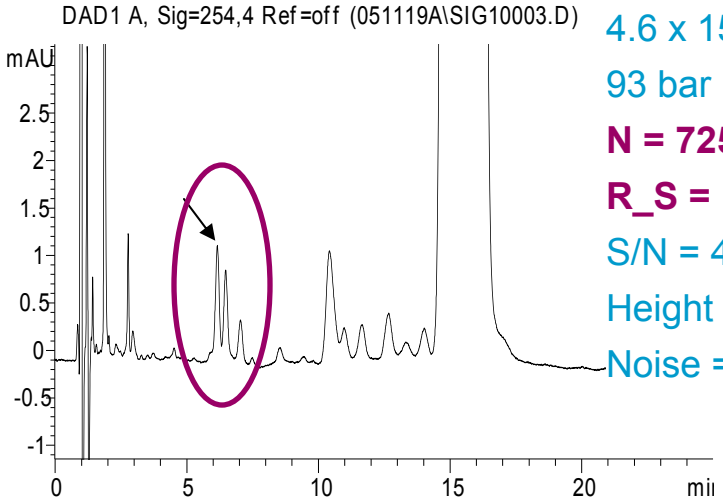
- Temperature: 50°C

Detectors

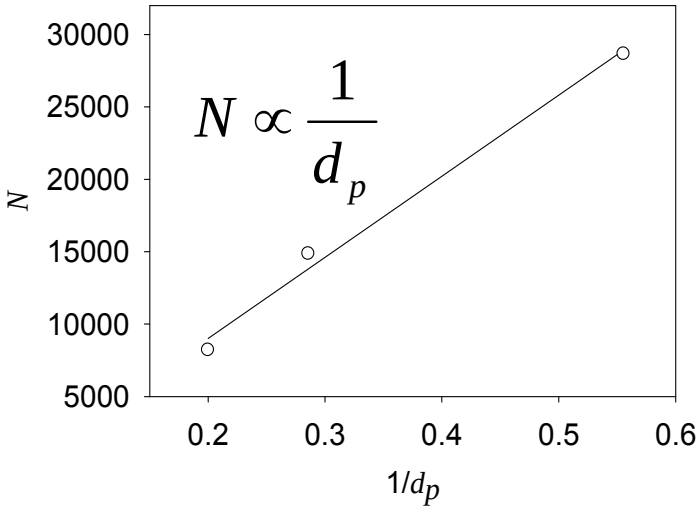
- DAD 2µl cell and **20Hz**, 220nm,

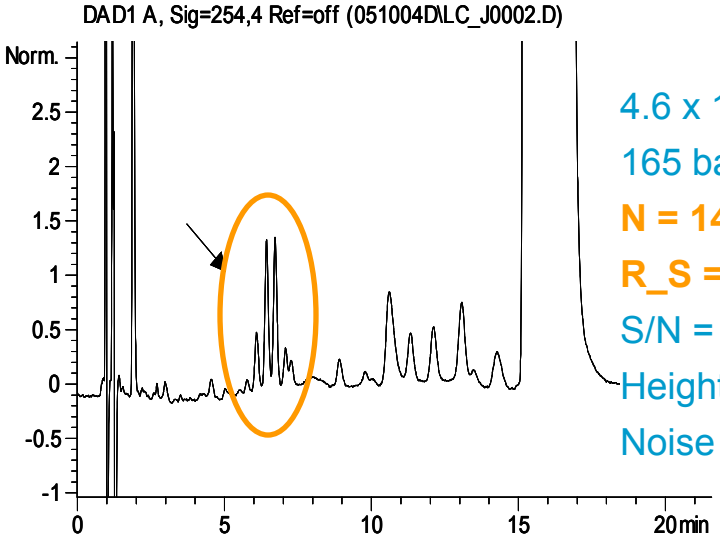
Ref: Appl. Note 5989-4506 by Edgar Naegele

Tip: Small Particles Improve Detection of Low Level Impurities

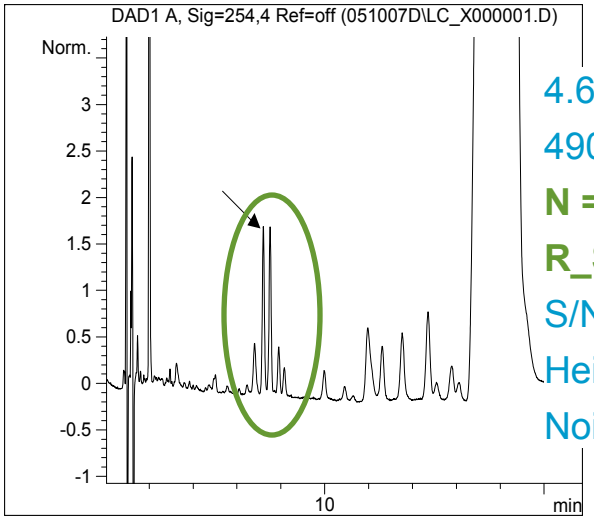


4.6 x 150, **5um**
93 bar
N = 7259
R_S = 1.15
S/N = 42
Height = 1.25
Noise = 24uAU





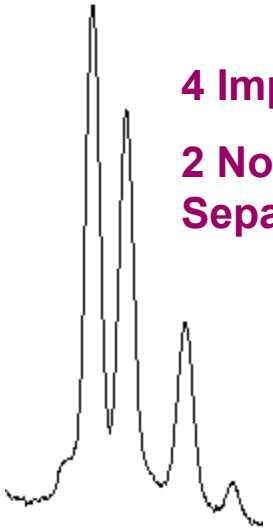
4.6 x 150, **3.5um**
165 bar
N = 14862
R_S = 1.37
S/N = 50
Height = 1.34
Noise = 20uAU



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

Particles Reveal More Information and Improve Detection and Integration

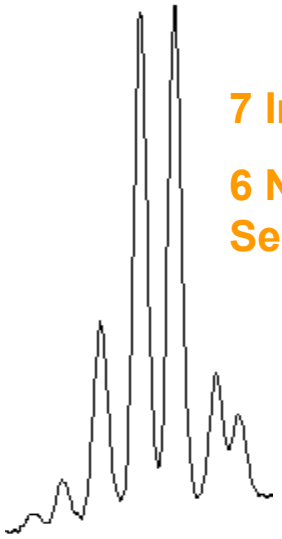
Customer Sample, Translation of Isocratic Impurity Methods, Zoom Critical Time Range (t = 7min)



4 Impurities

2 Not Baseline Separated!

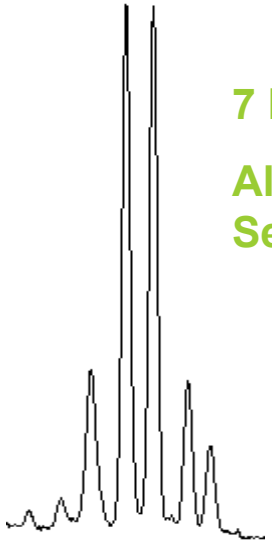
4.6 x 150, 5µm
93 bar
N = 7259
R_S = 1.15
S/N = 42



7 Impurities

6 Not Baseline Separated!

4.6 x 150, 3.5µm
165 bar
N = 14862
R_S = 1.37
S/N = 50



7 Impurities

All 7 Baseline Separated!

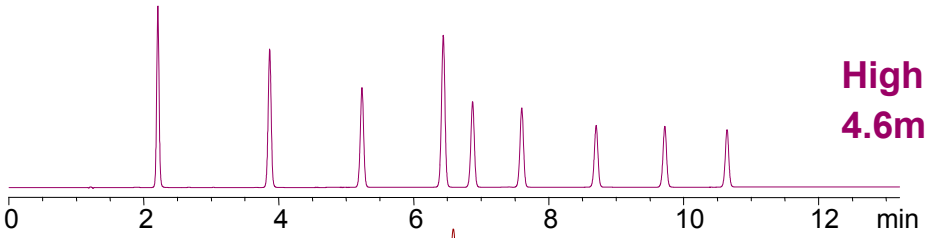
4.6 x 150, 1.8µm
490 bar
N = 28669
R_S = 1.80 (+57%)
S/N = 44

Up to 60% higher resolution without loss in sensitivity

Example of What Is Possible!

Shorter Column, Smaller Particle, > Flow Rate, High Temp

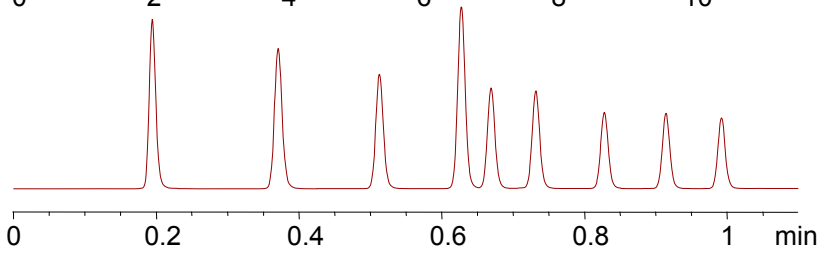
F= 1.20ml/min
T = 40°C
Analysis Time = 11min
Solvent Cons. = 13.2ml



Easy!

High Resolution:
4.6mm x 150mm 5.0µm

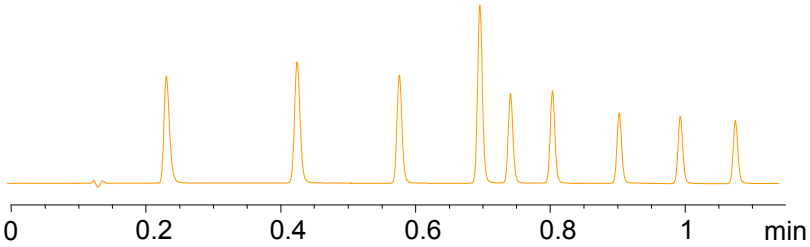
F = 4.80ml/min
T = 40°C
Analysis Time = 1.05min
Solvent Cons. = 5.1ml



Easy!

High Speed:
4.6mm x 50mm 5.0µm

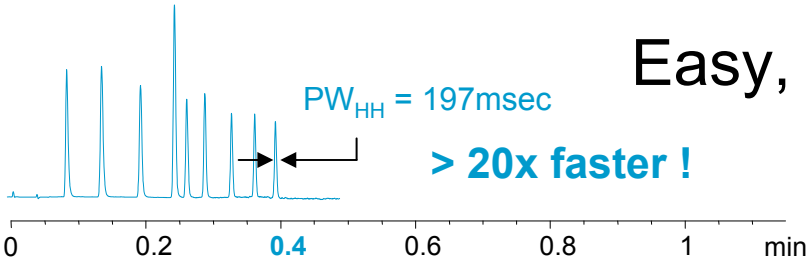
F= 1.00ml/min
T = 40°C
Analysis Time = 1.1min
Solvent Cons. = 1.1ml



Easy!

High Speed & Resolution:
2.1mm x 50mm 1.8µm

F= 2.40ml/min
T = 95°C
Analysis Time: 0.4min
Solvent Cons. = 1.0ml

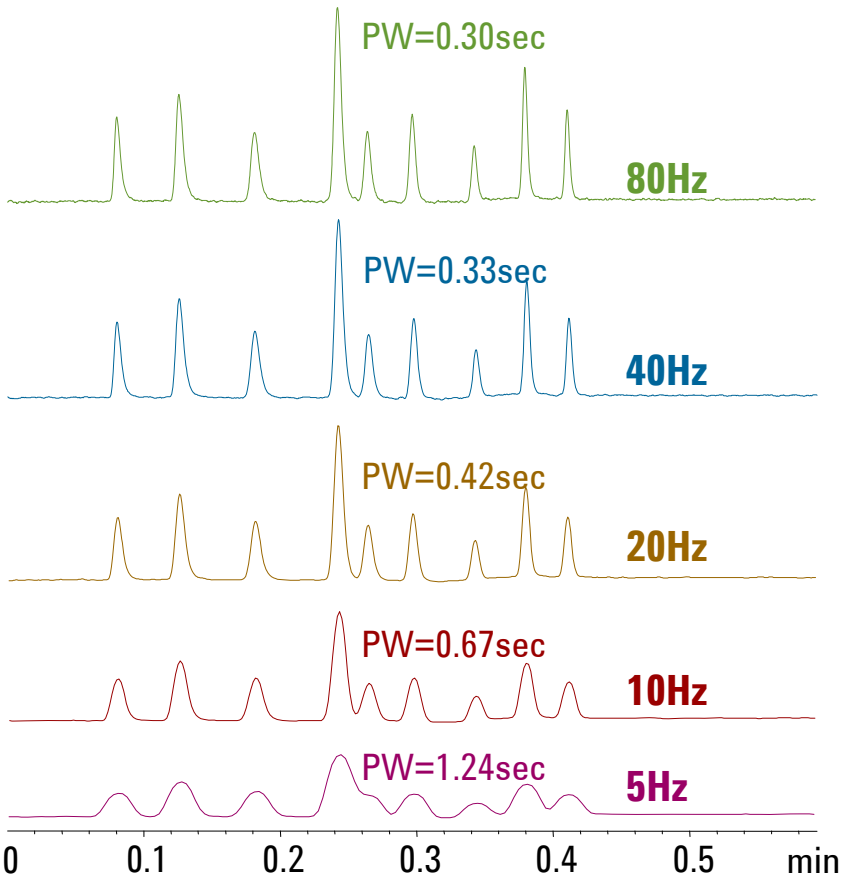


> 20x faster !

Easy, But You Will Need > 400bar!

Max Speed at T = 95°C
2.1mm x 50mm 1.8um

Tip: Higher Data Improves Response and Rs
Trick: Optimize Data Rate for Accurate Profile



80Hz versus 10Hz Data Rate

- Peak Width: – 55%
- Resolution: + 90%
- Peak Capacity: + 120%
- App. Column Eff.: + 260%

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

Moving From Conventional LC to RRLC:

"Doesn't this cost a fortune ?"

Moving to RRLC is simple and easy !

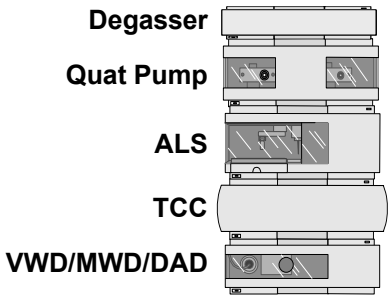
- **Same software (e.g. ChemStation, EZChrom)**
  - ⇨ **<u>No</u> additional training effort**
- **Limit capital investment by modular step-wise upgrade**
  - ⇨ **<u>Leverage</u> existing 1100 modules, existing method and stationary phase**
- **Configurable delay volume in 1200 SL pump**
  - ⇨ **<u>Easy</u> to also run legacy LC methods**
- **Simple LC to RRLC method translator**
  - ⇨ **<u>No</u> tedious method development**

"No - not at all !"

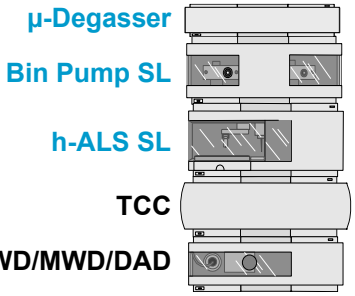
Stepwise Scale-up to Rapid Resolution LC

From 1100 to 1200 RRLC in two steps - Add What You Need When You Need It!

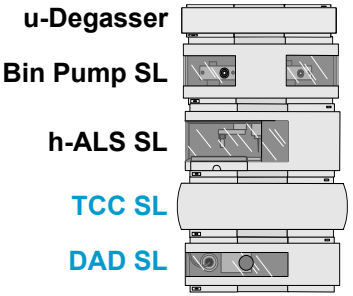
Actual: 1100 Quat System



Step 1: 1100/1200 „Bin SL“ System



Step 2: 1200 Rapid Resolution System



		+ Resolution + Speed + MS-Compatibility + Solvent Saving + Compatible with conv. HPLC	+ Speed + Resolution + Sensitivity + MS-Robustness + Data Security & Traceability + Qualification (Degasser, ACE) + Compatible with conv. HPLC
Analysis Time	> 5 min	> 1.5min	> 0.2min
Cycle times	> 6 min	> 2min	> 0.4min
Peak Width	> 3 sec	> 1.5 sec	> 0.2 sec
N	5 - 12,000	5 - 30,000	5 - 60,000
Column ID	4.6 mm	2.1 - 4.6 mm	2.1 - 4.6 mm
Column Length	50 mm	50 - 150 mm	20 - 150mm
Flow rates	0.2 - 10ml/min	0.05 - 5 ml/min	0.05 – 5 ml/min
Temperature	80 C	80 C	100 C
Pressure	400bar	600 bar	600bar

SUMMARY

- Increasing Speed of HPLC Separations Need Not be Difficult or Costly
- Changes in Columns and Equipment Depend on Your Goals
- Majority of Time Saved Will Be Based on Column and Particle Size
- Isocratic and Gradient 2X , 3X and 5X Times Savings are Easy
- Greater Time Savings May Require Instrument Modifications
- 10-20X Increase in Speed May Require Instrument Upgrade
- Long Columns with 1.8µm Particles May Require >400 bar Pump and Autosampler Capability