High Resolution Fast LC

Easier Than You Think

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What is High Resolution Fast LC?

- Maintain Resolution with Faster Run Time
- Increased Resolution with Faster Run Time
- Increasing Speed by 2X – 10X Original Run Time

Topics for this presentation

1. Explore benefits of reducing LC column length, column diameter, and packing particle size.
2. Provide practical examples of Fast LC, using existing HPLC hardware.
3. Instrument issues with Fast LC –higher pressure and smaller peak volumes.

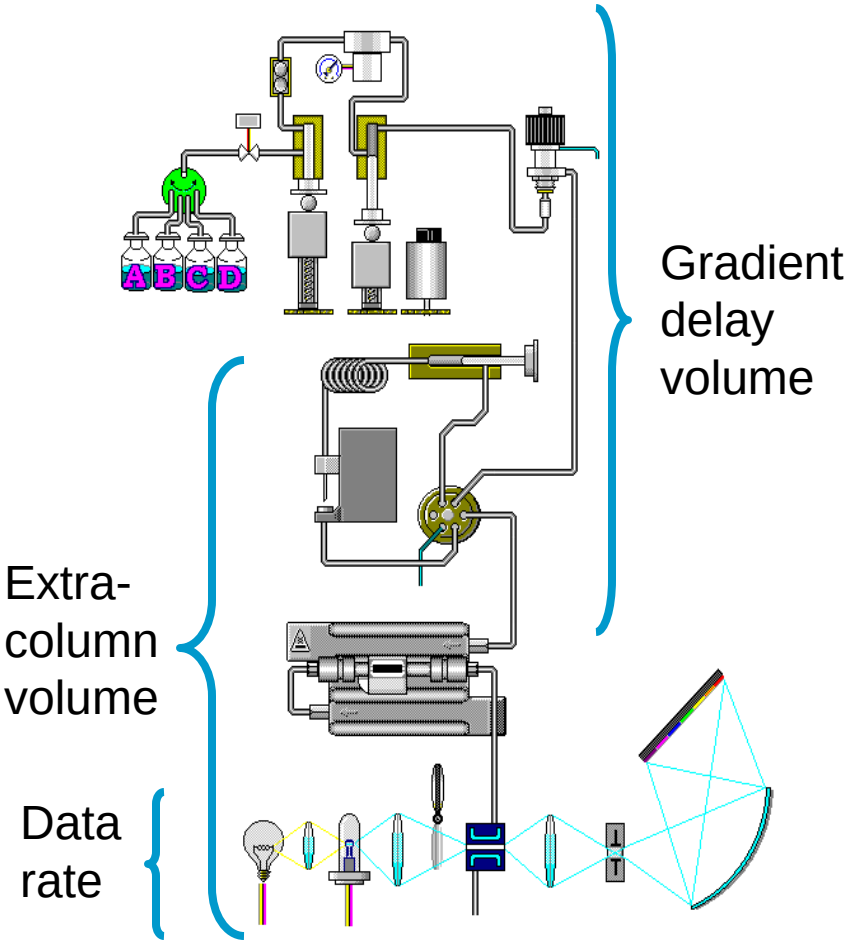
Fast LC Instrument Considerations:

Can the HPLC in your lab today handle fast LC ?

Data Rate – collect data fast enough to match expected peak widths for LC and LC/MS (i.e. 80 Hz DAD, 55Hz VWD, 6140 MS)

Extra-column volume – minimize with short capillaries, 0.12 mm ID (red); UV flow cell w/ smaller volume (needed with 2.1 mm ID columns)

Gradient delay volume – high-pressure mixing pump, optimized for columns to be used (e.g. RRLC 1200 SL, optimized 1100).



Practical Strategy for Reducing Analysis Time Using the HPLC in your lab today

1. Reduce column length (Match L to required R)
   a. i.e. 150 mm → 50 mm or 50 mm → 30 or 15 mm
2. Reduce particle size to maintain resolution in short column lengths
   a. i.e. 5 mm → 3.5 mm → 1.8 um
3. Increase mobile phase temperature (decrease P)
4. Increase flow rate
5. Combine any/all of the above parameters for the most reduction in analysis time – *faster* HPLC

Resolution …

Determined by 3 Key Parameters – Efficiency, Selectivity and Retention

The Fundamental Resolution Equation

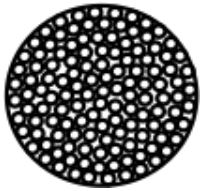
$$R_S = \frac{\sqrt{N}}{4}\,\frac{(\alpha-1)}{\alpha}\,\frac{k}{(k+1)} = \frac{\Delta t_R}{\overline{w}}$$

N = Column Efficiency – Column length and particle size

α = Selectivity – Mobile phase and stationary phase

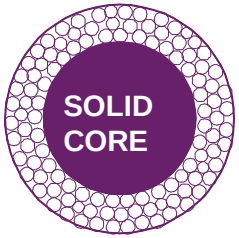
***k* = Retention Factor** – Mobile phase strength

Two Advances For Improved LC Performance



Sub 2um Porous Particle

Zorbax 1.8 um



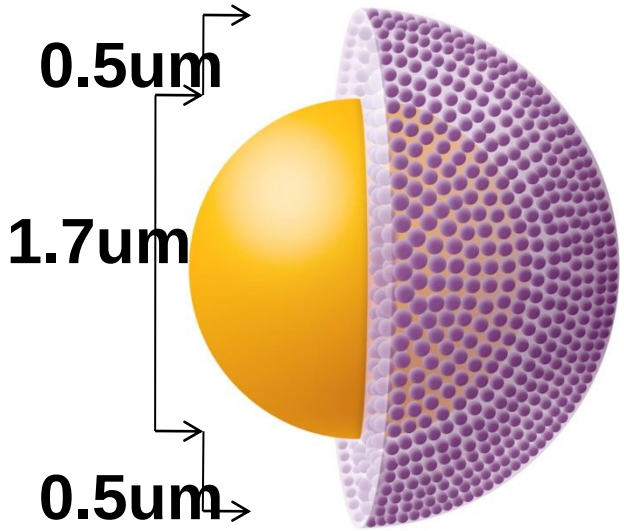
Superficially Porous Particle

Poroshell 120 2.7 um

Superficially Porous Column Technologies

Poroshell 120 columns:

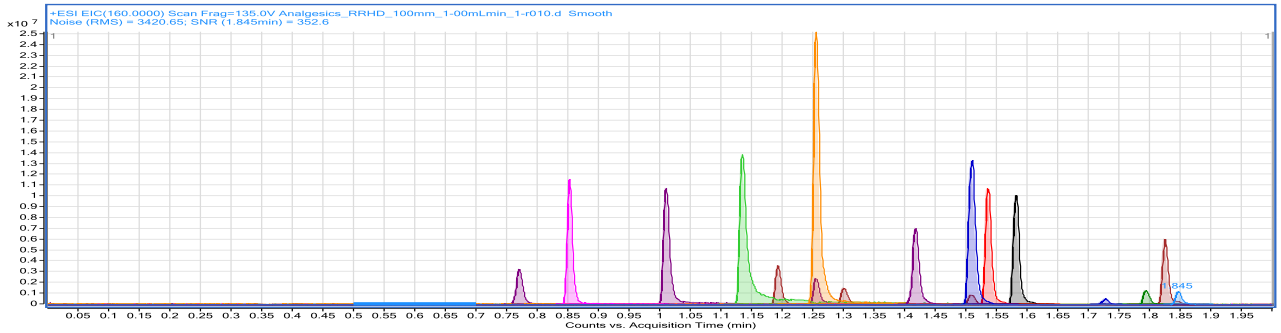
- Eficiency ≈ 90% of sub-2 µm
- Pressure ≈ 40-50% of sub-2 µm
- N ≈ 2X 3.5 µm (totally porous)
- d_p = 2.7µm
- 2 µm frit to reduce clogging
- P_{limit} = 600 bar for HPLC or UHPLC
- Particles
  - 1.7 µm solid core
  - 0.5 µm diffusion path
  - 2.7 µm total diameter



Analysis of 15 Analgesic Compounds

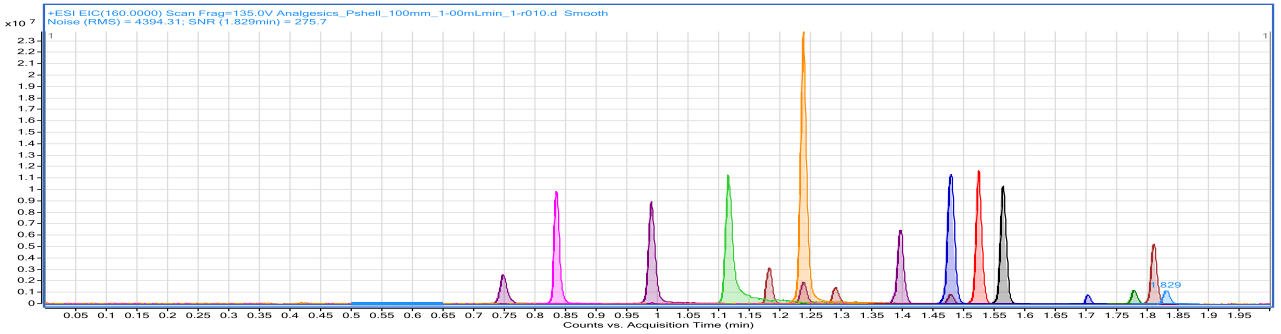
Same Method for both Columns

ZORBAX RRHD Eclipse Plus C18, 3 x 100 mm, **1.8 µm**



Ibuprofen:
$PW_{1/2}$=0.012
S/N=353
n_c=54

ZORBAX Poroshell 120 EC-C18, 3 x 100 mm, **2.7 µm**



Ibuprofen:
$PW_{1/2}$=0.012
S/N=256
n_c=56

2 min

How do we increase Efficiency (N)

$N \propto L/d_p$ so need to increase column length and/or decrease particle size, *but* …

1. Increasing L leads to longer analysis time

2. Decreasing d_p leads to higher back pressure

Reduce column length

Shorter Columns with Smaller Particles

Plates Selectivity Retention

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{\alpha-1}{\alpha} \cdot \frac{k'}{k'+1}$$

$$N \propto \frac{L}{d_p}$$

To Maintain R_S:

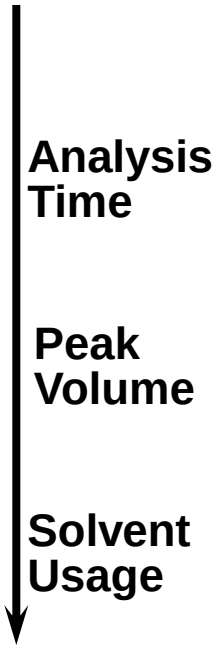
e.g.: L/2 ⇨ d_p/2

↓ Column Length	=	↓ N (& P)
↓ Particle Size	=	↑ N
↓ Particle Size	=	↑ P

Smaller Particle Size Increases Column Efficiency

Choose Column Dimensions to Meet Separation Needs

Column Length (mm)	Resolving Power N(5 µm)	Resolving Power N(3.5 µm)	Resolving Power N(1.8 µm)	Analysis Time*
150	12,500	21,000	32,500	
100	8,500	14,000	24,000	-33%
75	6000	10,500	17,000	-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%



* Reduction in analysis time compared to 150 mm column

Instrument Considerations - How To Use Small Volume Columns Effectively (Maintain N)

Shorter columns = smaller peak volumes

Critical instrument parameters include:

Isocratic and Gradient
- Data rate
- Flow cell size
- Tubing

Gradient only
- Gradient delay volume
- Column equilibration volume (10 x V_m)

Majors, LC/GC, Dec 2003, v 21 n 12, pp 1124-1133, “Are you getting the most out of your HPLC column?”

Joseph, et al, Agilent publ 5988-9251EN, “The Influence of Sub-Two Micron Particles on HPLC Performance”

Changing LC column dimensions
What else needs to be modified?

1. Isocratic Conditions:

- Column length - no changes required
- Column diameter - change flow rate and inj. volume by $(d_1/d_2)^2$
- Extra Column Volume (see appendix)

How conversion works for flow

Flow modification, for columns of different diameters

$$\text{Flow}_{\text{col.1}} \times \left(\frac{\text{Diam.}_{\text{column2}}}{\text{Diam.}_{\text{column1}}} \right)^2 = \text{Flow}_{\text{col. 2}}$$

$$\text{i.e.} \quad 1.0 ml/min \times \left(\frac{2.1 mm}{4.6 mm} \right)^2 = 0.21 ml/min$$

Conversion for injection volume

Keep Injection volume proportional to column volume

$$\text{Inj.Vol.}_{\text{col.1}} \times \left(\frac{\text{Volume}_{\text{column2}}}{\text{Volume}_{\text{column1}}} \right) = \text{Inj.Vol.}_{\text{col. 2}}$$

Zorbax column volume = 3.14 x r^2 x L x 0.6 (r and L in cm)

$$\text{i.e.} \quad 20\mu l_{\text{col.1}} \times \left(\frac{0.4ml_{\text{column2}}}{2.0ml_{\text{column1}}} \right) = 4\mu l_{\text{col. 2}}$$

Changing Column Diameters – Important Values to Know

Column ID	Column Volume	Peak Volume, k=1	Typical Injection Vol.*	Typical Inj Vol Range	Flow Rate for equivalent ν**
4.6 mm	1500 µL	148 µL	20 µL	5 – 50 µL	1.0mL/min
3.0 mm	640 µL	44 µL	10 µL	3 – 30 µL	0.42mL/min
2.1 mm	320 µL	22 µL	2 µL	0.5 – 15 µL	0.21mL/min
1.0 mm	70 µL	4 µL	0.5 µL	0.1 – 3 µL	47 µL/min
0.5 mm	15 µL	1 µL	150 nL	40 – 500 nL	12 µL/min
0.3 mm	6 µL	0.3 µL	50 nL	15 – 250 nL	4.2 µL/min
0.1 mm	700 nL	32 nL	10 nL	1 – 10 nL	472 nL/min
0.075 mm	400 nL	18 nL	2 nL	0.5 – 5 nL	266 nL/min

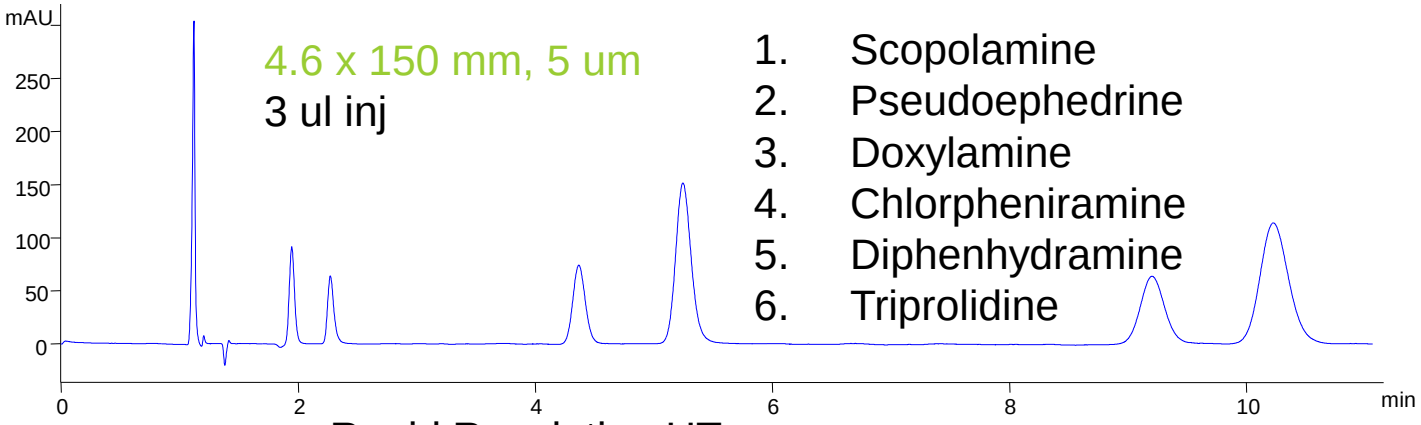
Column length = 150 mm, N =13,000

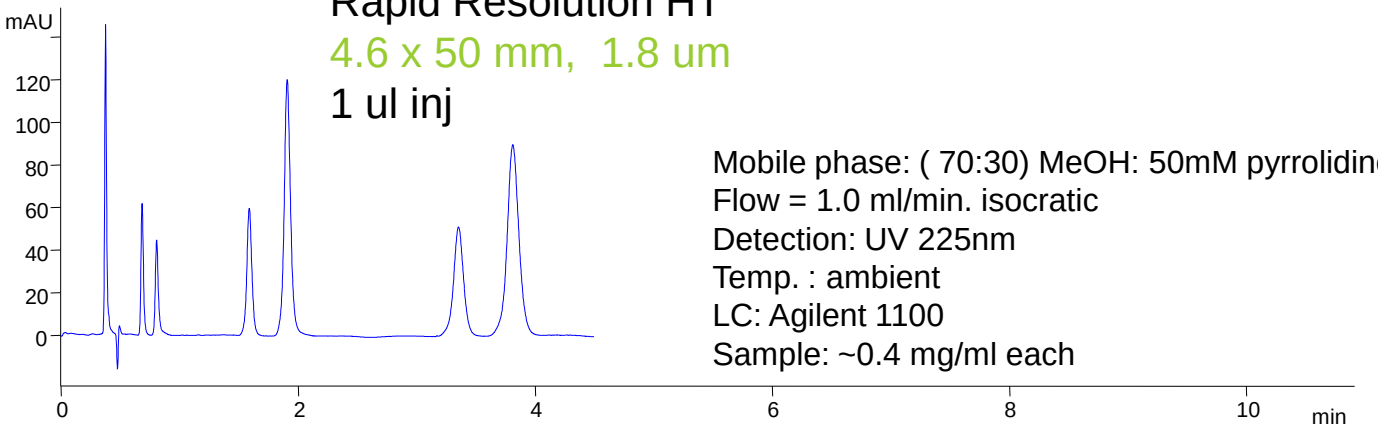
** Typical injection volume = 10 – 30% of peak volume of first eluting peak*

*** Maintain equivalent mobile phase linear velocity when scaling down in column diameter*

Maintain Chromatographic Performance

Assay of Antihistamines at High pH (pH 11.5) on 1.8 μm Extend-C18

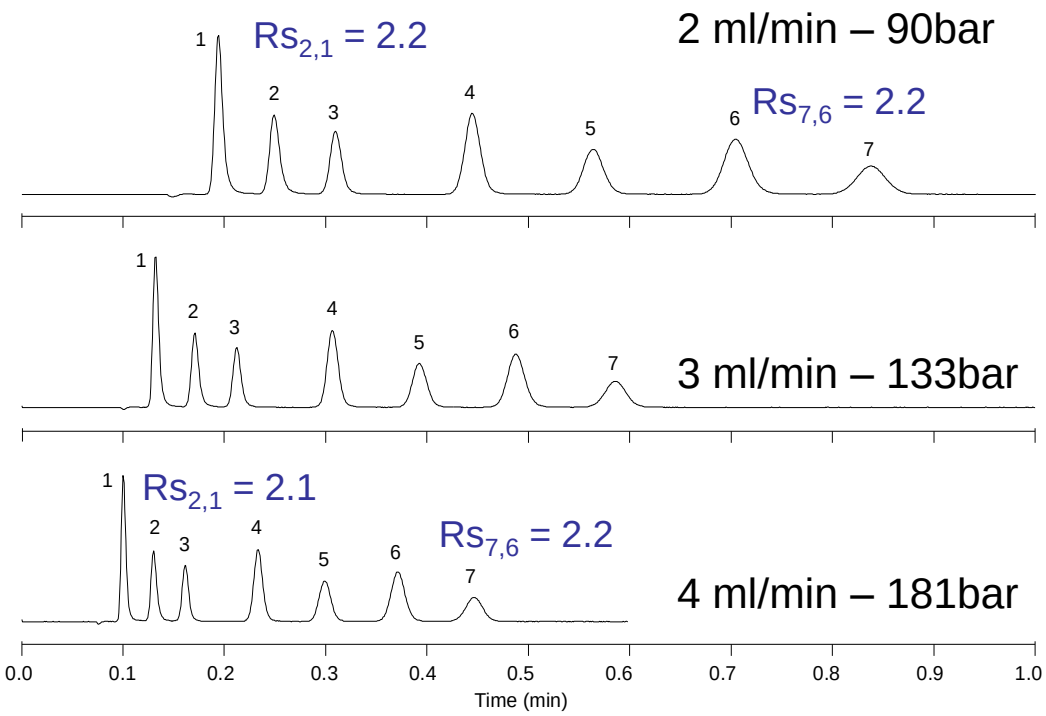




Mobile phase: (70:30) MeOH: 50mM pyrrolidine buffer, pH 11.5
Flow = 1.0 ml/min. isocratic
Detection: UV 225nm
Temp. : ambient
LC: Agilent 1100
Sample: ~0.4 mg/ml each

Gaining Speed – Ultra-Fast Isocratic Analysis with Zorbax 1.8 um RRHT:

Higher flow rate: higher speed – same resolution – higher sensitivity/precision



Sample: 7 Analgesics
Column: Zorbax RRHT SB-C18, 1.8 um, 4.6 x 30 mm

100 Bar = 1450 PSI

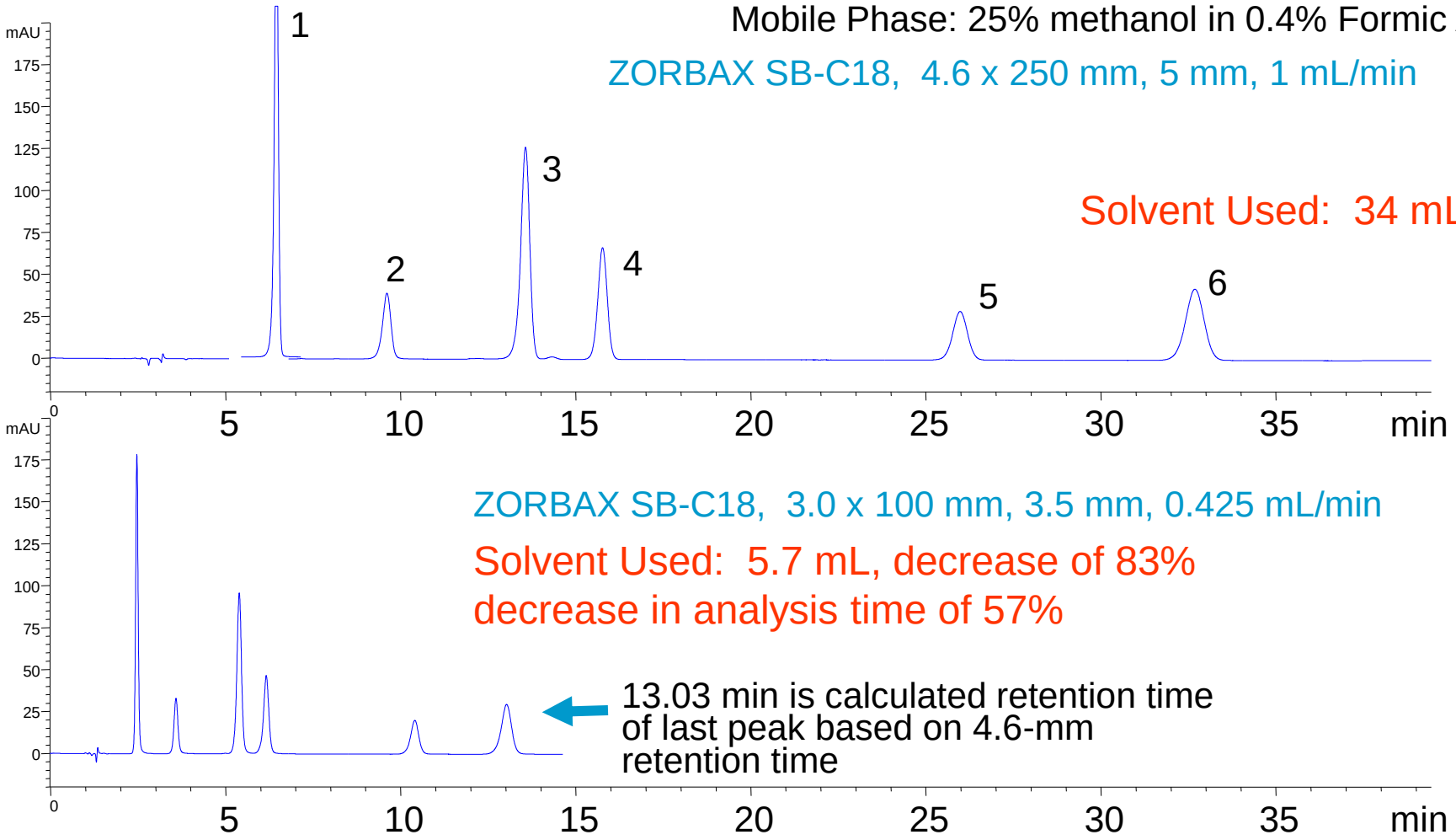
2x faster than 2ml/min without loss in resolution!

Ultra-fast analysis in 26 sec
4σ peakwidth ~ 0.6 sec
< 50 sec cycle time

7 analgesics separated in 21 sec for peaks 1,2,3
Total time Injection-to-Injection

Benefits of Changing *Both* Column Length and Diameter - Update of an ancient isocratic method:

Change from a 4.6 x 250 mm (5 um) to a 3.0 x 100 mm (3.5 um) Column.



Changing column dimensions
What else needs to be modified?

1. Isocratic:

- Column length - no changes required
- Column diameter - change flow rate and inj. volume by $(d_1/d_2)^2$
- Extra Column Volume (see appendix)

2. Gradient:

- Column length - need to change gradient time (t_G) proportional to change in column length
- Column diameter - change flow rate and inj. volume by $(d_1/d_2)^2$
- Extra column volume
- System dwell volume may complicate things further (see for example: Majors, LC/GC, Dec 2003, v 21 n 12, pp 1124-1133)

Recommendations for Lowest Delay Volume Gradient Separations on Low Volume Columns

Choose and configure lowest volume pump:

- High pressure mixing – lower delay volume
- Binary pump - lower delay volume than quaternary pump
- Solvent Mixer – no mixer or low volume 80 ul mixer

Minimize extra column volume:

- Tubing i.d. – 0.12 mm i.d. (particularly for 2.1 mm column)
- Flow cell size – DAD – 8 uL or 1.7 uL, VWD – 5 uL or 1 uL
- Injection size – < 5 uL
- Run e.g., 1100 autosampler program for “bypass” or use “ADVR” (Automatic delay volume reduction)

Gradient Retention (k*)

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

$\Delta\Phi$ = 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)

- S ≈ 4–5 for small molecules
- 10 < S < 1000 for peptides and proteins

- ***Selectivity in gradient elution is determined by the gradient retention factor***
- ***In gradient separation the effective value of k (k*) for different bands will be about the same.***

This Relationship Says
To Keep Relative Peak Position in the Chromatogram Unchanged

Any Decrease in	Can be Offset by a Proportional
• Column length	• Decrease in $\mathbf{t_G}$ 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 $\mathbf{t_G}$ or F

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

Very Fast LC on Conventional 1100 HPLC

The Instrument

G1379 Degasser

G1311 Quaternary pump

G1313A ALS autosampler

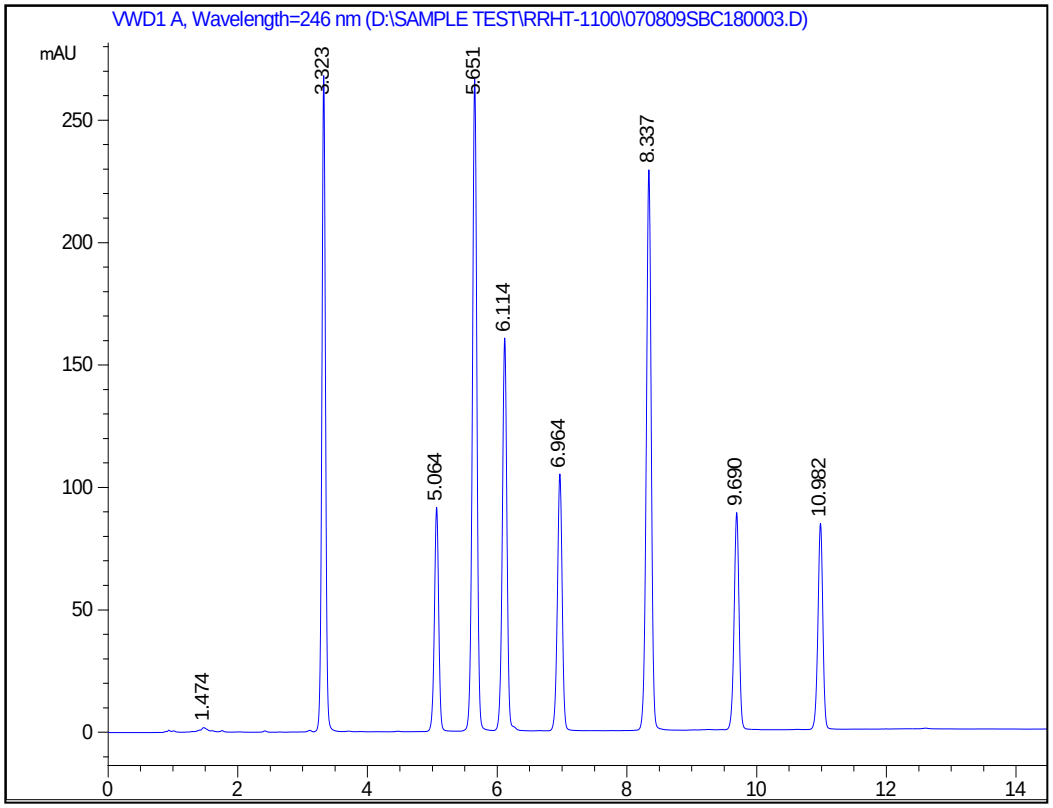
G1316A column compartment

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

The Sample - alkylphenones

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

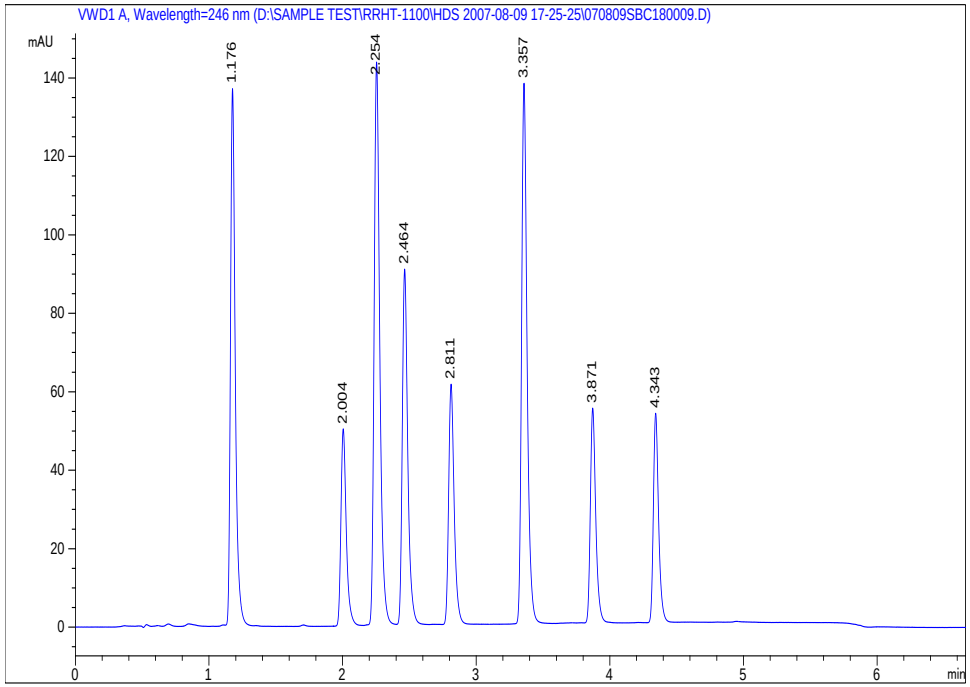
Final Pressure: 38 bar

Column Efficiency ~12,500

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

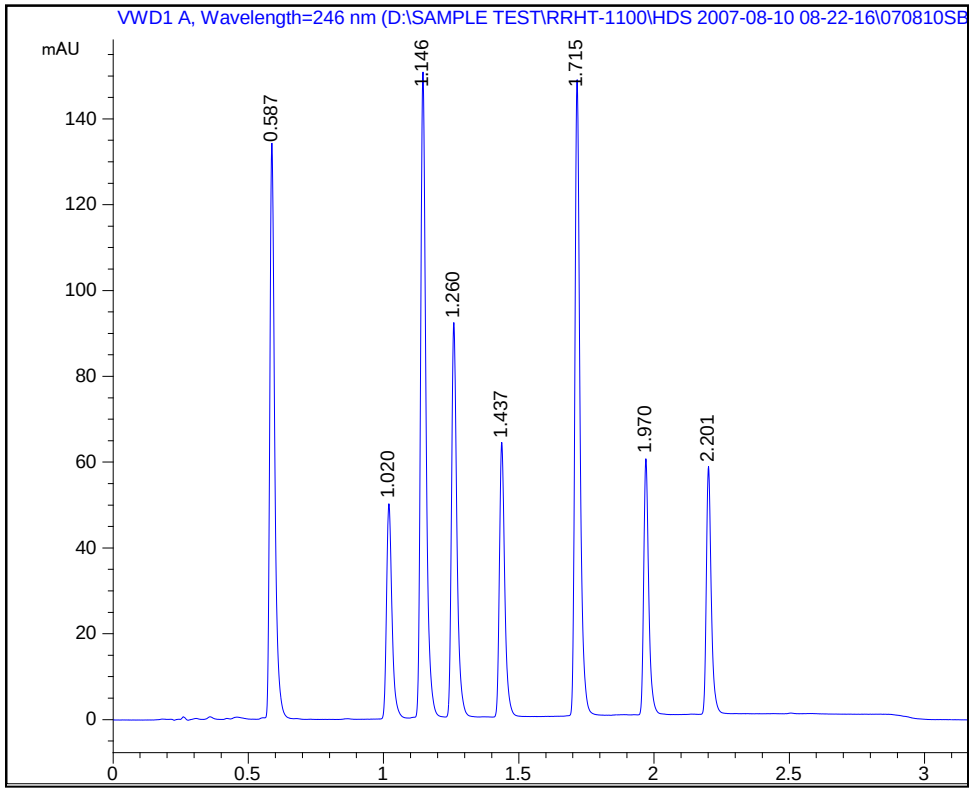
Final Pressure: 74 bar

Column Efficiency ~12,000

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

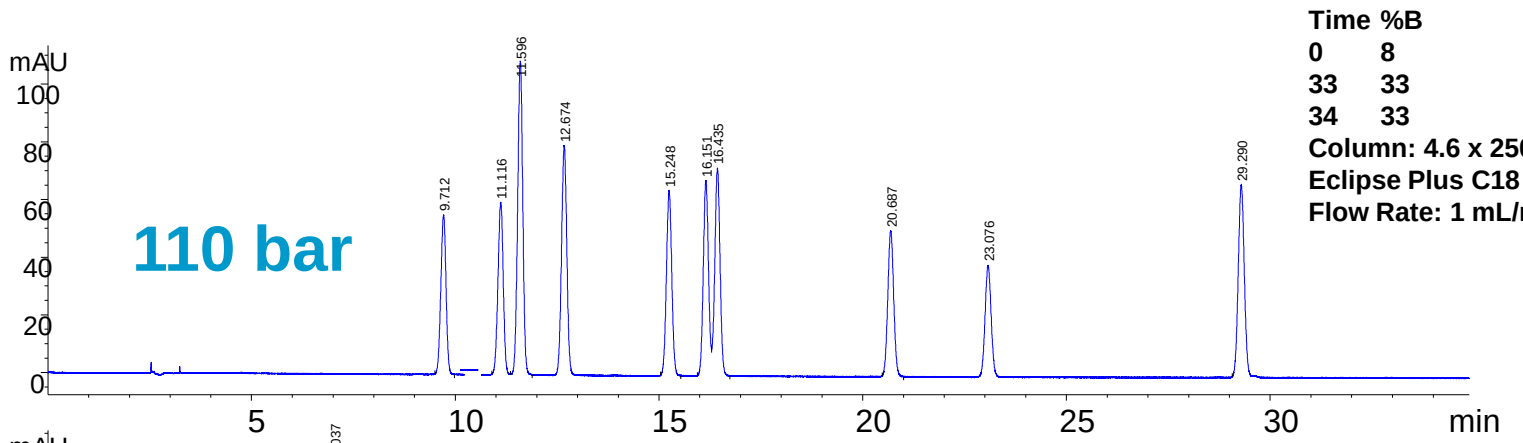
Initial Pressure: 266 bar

Final Pressure: 146 bar

Compare 4.6 x 250mm, 5um Eclipse Plus C18 to Poroshell 120 EC-C18 4.6 x 100mm, 2.7um

Mobile Phase: A: 0.1% formic acid in Water; B: 0.1% formic acid in ACN

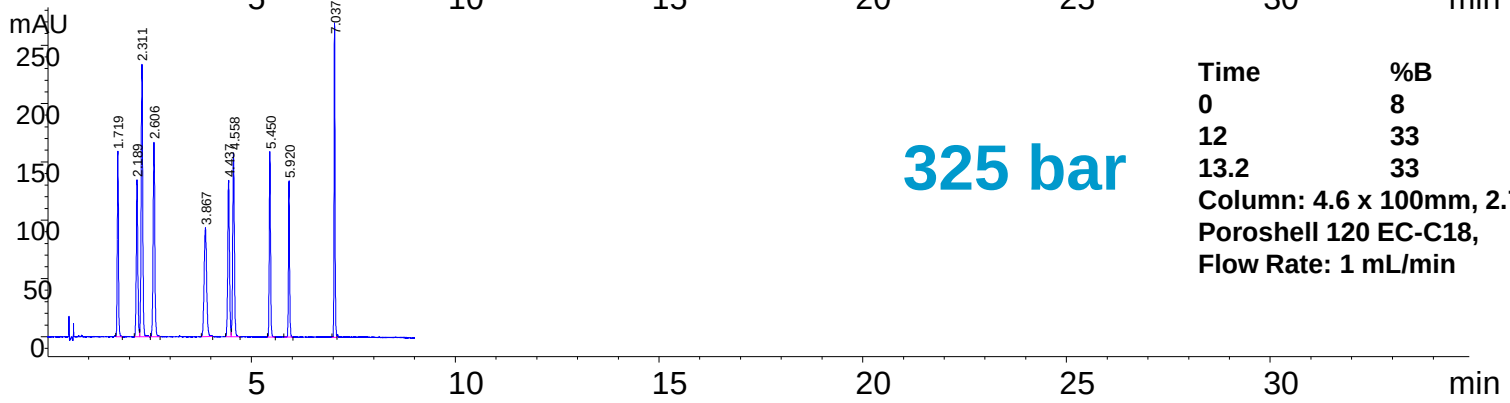
Sample: Sulfadiazine, Sulfathiazole, Sulfapyridine, Sulfamerazine, Sulfamethazine, Sulfamethazole, Sulfamethoxypyridazine, Sulfachloropyridazine, Sulfamethoxazole, Sulfadimethoxine



110 bar

Time	%B
0	8
33	33
34	33

Column: 4.6 x 250mm, 5um Eclipse Plus C18
Flow Rate: 1 mL/min

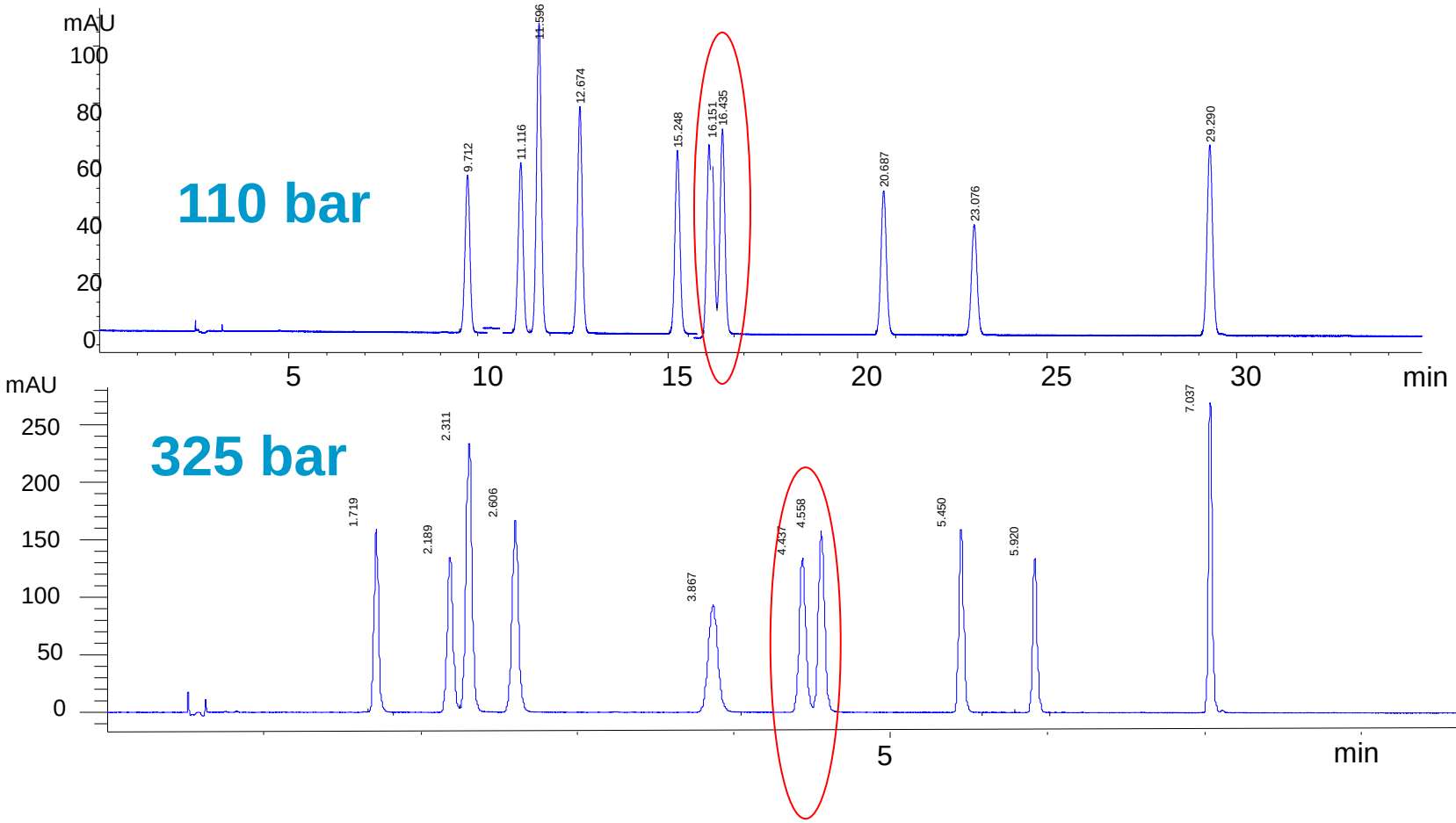


325 bar

Time	%B
0	8
12	33
13.2	33

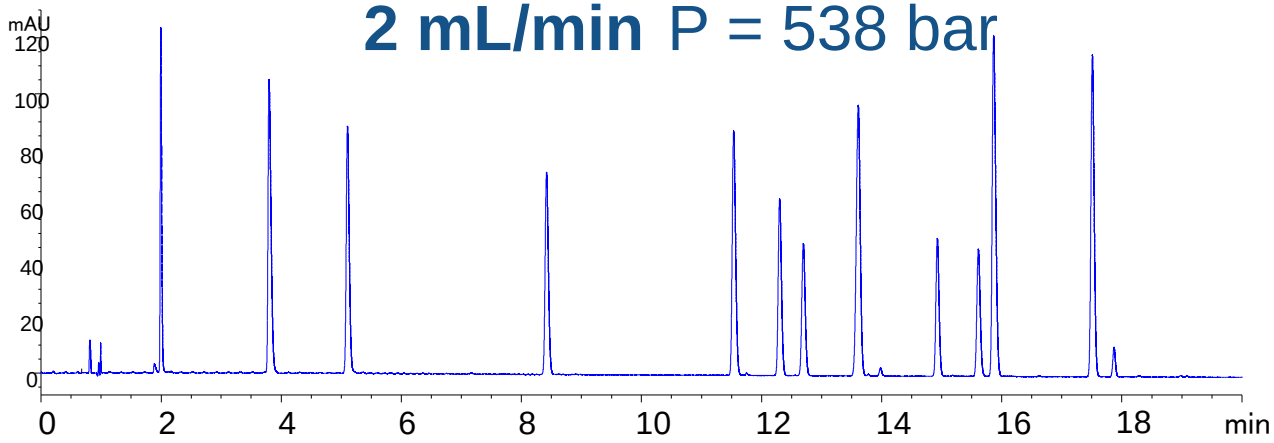
Column: 4.6 x 100mm, 2.7um Poroshell 120 EC-C18,
Flow Rate: 1 mL/min

Expand High Speed Chromatograms for True Comparison to Slower Separation

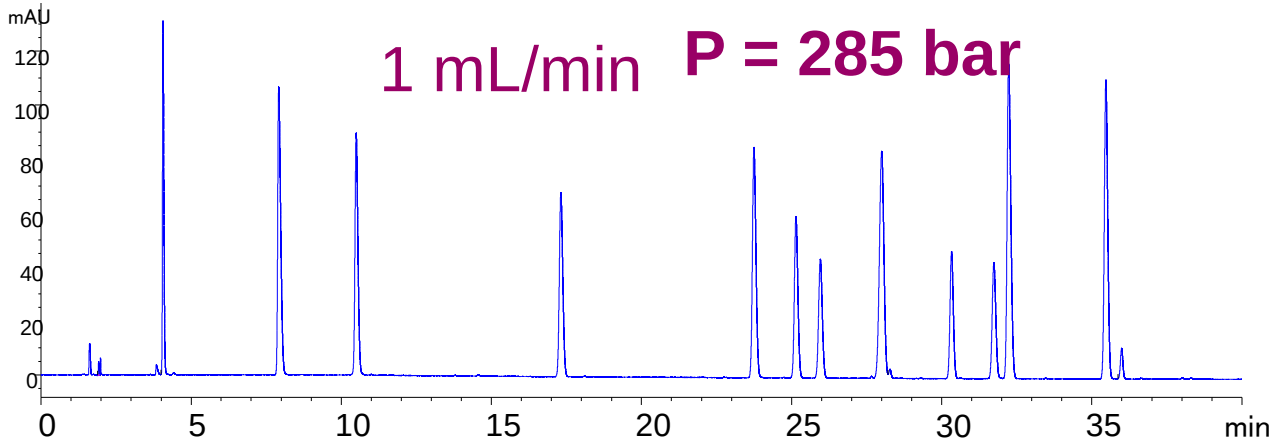


Poroshell 120 150mm Columns – HPLC or UHPLC

Conditions: Column: Poroshell 120 EC-C18, 4.6 x 150mm, 2.7um Mobile Phase: Solvent A: Water with 0.1% Formic Acid Solvent B: Acetonitrile 1200 SL controlled temperature at 25 °C 2 ul flow cell



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



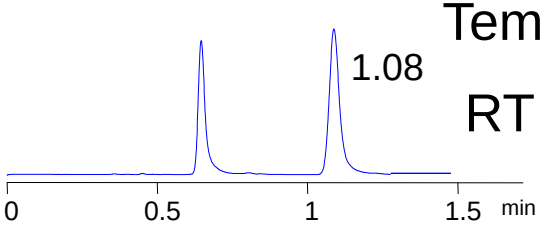
Gradient: 1mL/min

Time	**%B**
6.0	5%
51	60%

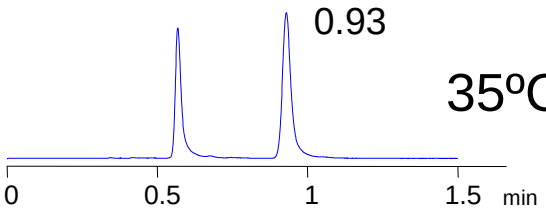
Gradient: 2mL/min

Time	**%B**
3.0	5%
25.5	60%

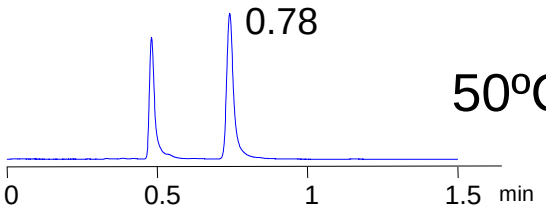
Increase Temperature to Reduce Analysis Time, Reduce P, and Improve Throughput



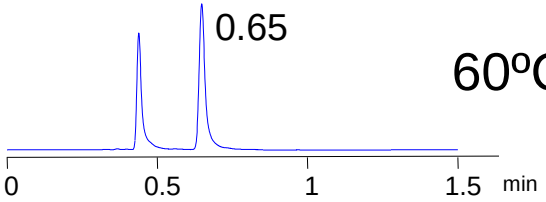
Temp. RT — Pressure 245 bar



35ºC — 225 bar



50ºC — 200 bar



60ºC — 189 bar

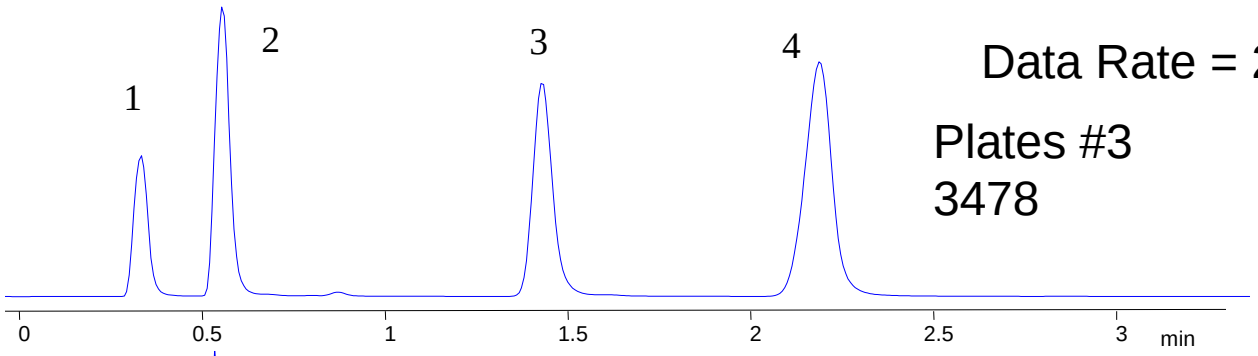
200 bar = 2900 psi

Column: RRHT SB-C18
4.6 x 30 mm, 1.8 μm
Mobile Phase: 40% water: 60% MeOH
Flow Rate: 1 mL/min
Detection: UV 254 nm
Temperature: As noted
Sample: 1. Triamcinolone
2. Hydrocortisone

Increasing temperature reduces analysis time by 40% or more and pressure by 25%.

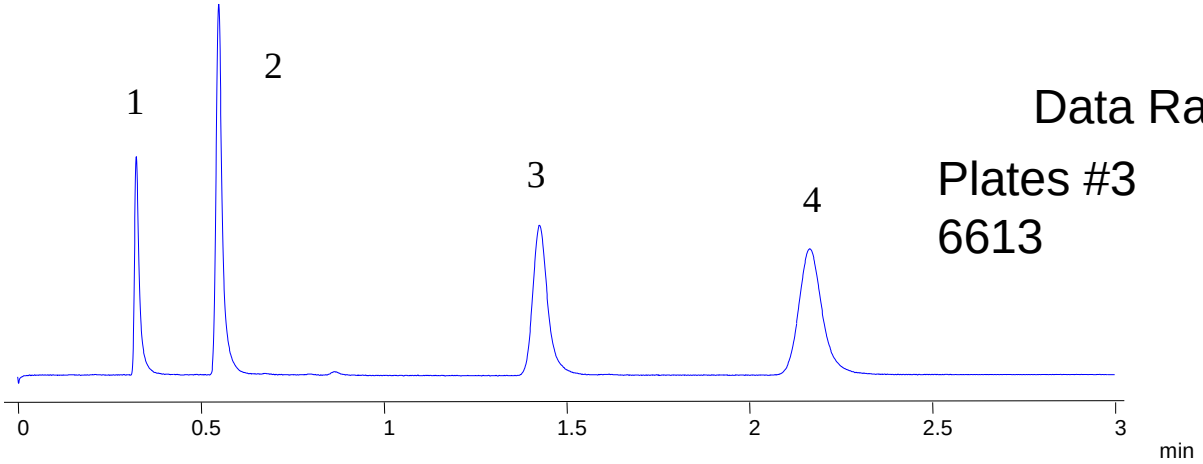
Detector Acquisition Rate

Column: ZORBAX SB-C18 4.6 x 30 mm, 1.8 um Mobile Phase: 60% Methanol: 40 Water Flow Rate: 1mL/min
Temperature: RT
Detection: UV 254 nm Sample: QC Test 1. Uracil 2. Phenol 3. 4-Cl-Nitrobenzene 4. Toluene



Data Rate = 2 sec

Plates #3	Plates #4
3478	4384



Data Rate = 0.1 sec

Plates #3	Plates #4
6613	6138

Fast data rate gives higher efficiency using 1.8 um column.

Instrument Considerations - How To Use Small Volume Columns Effectively (Maintain N)

Shorter columns = smaller peak volumes

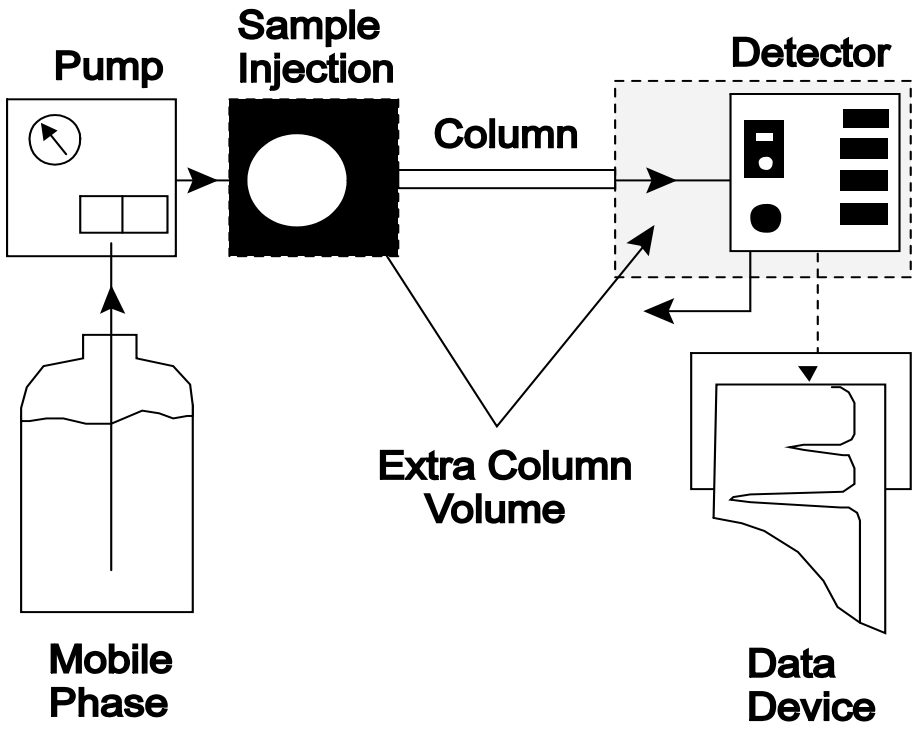
Critical instrument parameters include:

Isocratic and Gradient
- Data rate
- Flow cell size
- Tubing

Gradient only
- Gradient delay volume
- Column equilibration volume (10 x V_m)

The LC Handbook, Pub. No. 5990-7595EN

Critical Instrument Volumes – ECV and Gradient Delay volume:



ECV (band broadening) = sample volume + connecting tube volume + fitting volume + detector cell volume

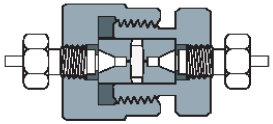
Dwell Volume/Delay Volume = volume from formation of gradient to top of column – an imposed isocratic hold (Delay Time = V_D x F)

Tubing volume effects to extra column volume and gradient delay?

With Fast LC peak volumes, every uL in extra-column volume counts!

ID (mm)	Length (cm)	Color Code	Volume (µl)	Part No.	Price ($)
0.12	10.5	Red	1.2	5021-1820	28
0.12	15	Red	1.7	5021-1821	28
0.12	20	Red	2.3	5065-9935	28
0.12	28	Red	3.2	5021-1822	28
0.12	40	Red	4.5	5021-1823	28
0.12	50	Red	5.6	5065-9964	28
0.17	10.5	Green	2.4	5021-1816	28
0.17	15	Green	3.4	5021-1817	28
0.17	20	Green	4.6	5065-9931	28
0.17	28	Green	6.4	5021-1818	28
0.17	40	Green	9.1	5021-1819	28
0.17	60	Green	13.6	5065-9933	32
0.17	70	Green	15.9	5065-9932	32
0.17	90	Green	20.5	5065-9963	32
0.25	28	Blue	13.8	5022-6508	28
0.25	80	Blue	39.3	5065-9930	32
0.50	10.5	None	20.6	5065-9927	28
0.50	15	None	29.5	5022-6509	28
0.50	28	None	55	5022-6510	28
0.50	80	None	157	5065-9926	66

LC capillary volumes:



Ref: Agilent pub 5988-2662ENUS, HPLC Maintenance Guide, page 55

Recommendations for using low dispersion columns with the HPLC in your lab today:

1. Determine the total extra-column volume in your system.
2. Determine the gradient dwell volume in your system.
3. Compare these results to the column dimensions and peak volumes you might expect to generate on the smaller column, to determine if your system is compatible with low dispersion HPLC.
4. Consult with instrument manufacturer to determine if instrument ECV and gradient dwell volumes may be reduced.
5. **Or, just try a column ½ the length packed with ½ the particle size you are currently using – and see what happens!**

Agilent 1.8um and Poroshell 120 Columns

- Make High Resolution, Fast LC Possible on All Instruments
- Reduce Analysis Time
- Speed Up Method Development
- Improves Methods on HPLC
- Provides Best Benefits on UHPLC
- Coupled with UHPLC Instruments Allow Higher Flow Rates for Higher Resolution Without Long Run Times

Quick Summary of Families and Bonded Phases

Poroshell 120

Start with EC-C18

Pore size: 2.7um

Add'l phases: EC-18, SB-C18, Phenyl-Hexyl, SB-AQ, SB-C8, Bonus-RP, HILIC, EC-CN

Eclipse Plus

Start w/Eclipse Plus C18

Pore sizes: 5, 3.5, 1.8um

Additional phases: C8, Phenyl-Hexyl, PAH, HILIC

StableBond

Choice for pH 1-2, alternate selectivity

Pore sizes: 7, 5, 3.5, 1.8um

Available phases: C18, C8, Phenyl, CN, AQ

Eclipse XDB

Pore sizes: 7, 5, 3.5, 1.8um

Available phases: C18, C8, Phenyl, CN

Bonus-RP

For changes in selectivity, particularly with acids and bases

Pore sizes: 5, 3.5, 1.8um

Extend-C18

Choice for high pH methods

Pore sizes: 5, 3.5, 1.8um

Summary

- High resolution fast LC is doable even on standard LCs
- Shorter 1.8um and 2.7um columns allow faster analysis with same resolving power as longer 5um columns
- Adjust run time and gradient to allow for the shorter column
- Change column diameter if necessary to allow for lower solvent consumption and higher accessible velocity
- Adjust the injection volume to maintain the same mass loading on the smaller column
- Speed up the flow rate until max flow, max pressure or see loss of resolution

Appendix

How to Estimate the Extra Column Volume of an HPLC System

One Way:

Remove HPLC column from instrument

Join injector and detector tubing with zero-dead-volume (ZDV) union

Inject (0.5 - 2 μL) of toluene in 100% acetonitrile

Make conc. about 1-5 mg/mL

Determine width of peak at base ($w_{instrument}$)

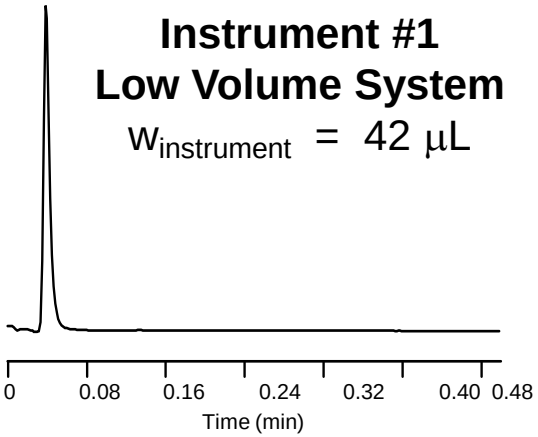
Peak bandwidth follows:

$$W^2_{tot} = W^2_{col} + W^2_{instrument}$$

How to Estimate the Extra Column Volume of an HPLC System

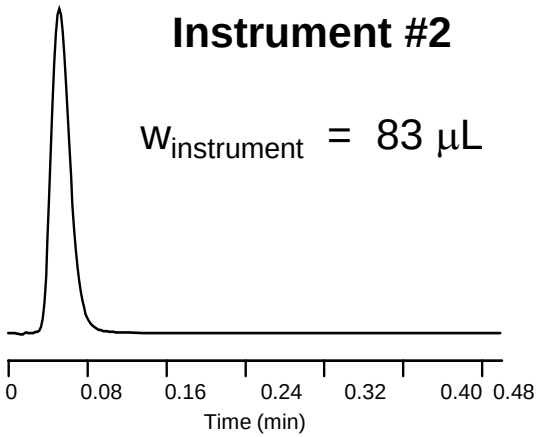
Toluene in Acetonitrile

Instrument #1
Low Volume System
$w_{instrument}$ = 42 µL



Instrument #2

$w_{instrument}$ = 83 µL



$$w^2_{tot} = w^2_{col} + w^2_{instrument}$$

For peak having a k′ = 2

4.6 x 150 mm, 5 µm

$w^2_{tot} = (180)^2 + (42)^2$
w_{tot} = 185 µL

↑ **3%**

$w^2_{tot} = (180)^2 + (83)^2$
w_{tot} = 198 µL

↑ **10%**

4.6 x 50 mm, 3.5 µm

$w^2_{tot} = (73)^2 + (42)^2$
w_{tot} = 84 µL

↑ **15%**

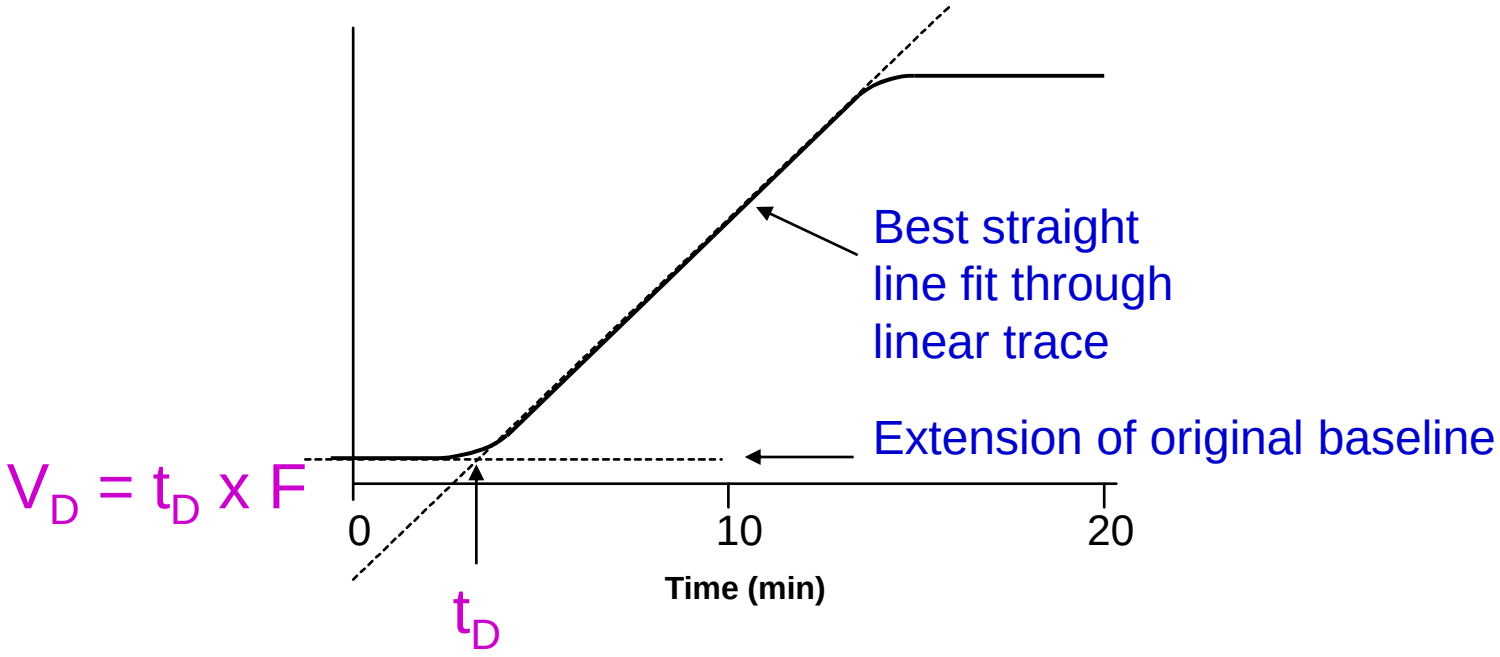
$w^2_{tot} = (73)^2 + (83)^2$
w_{tot} = 110 µL

↑ **51%**

Determine the Dwell Volume of Your System

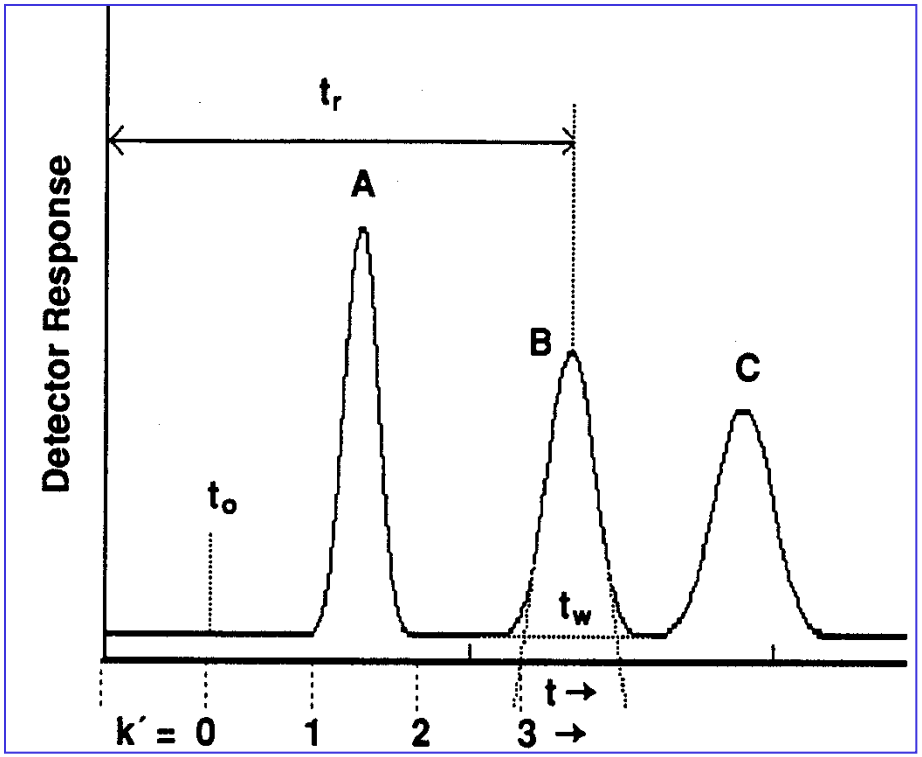
- ✓ Replace column with short piece of HPLC stainless steel tubing
- ✓ Prepare mobile phase components
  A. Water - UV-transparent
  B. Water with 0.2% acetone - UV-absorbing
- ✓ Monitor at 265 nm
- ✓ Adjust attenuation so that both 100% A and 100% B are on scale
- ✓ Run gradient profile 0 - 100% B/10 min at 1.0 ml/min
- ✓ Record

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.

Chromatographic Profile
Equations Describing Factors Controlling R_S



Retention Factor

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

Selectivity

$$\alpha = k_2/k_1$$

Theoretical Plates-Efficiency

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