Gradient Reversed Phase HPLC

The Why, What, When, and How

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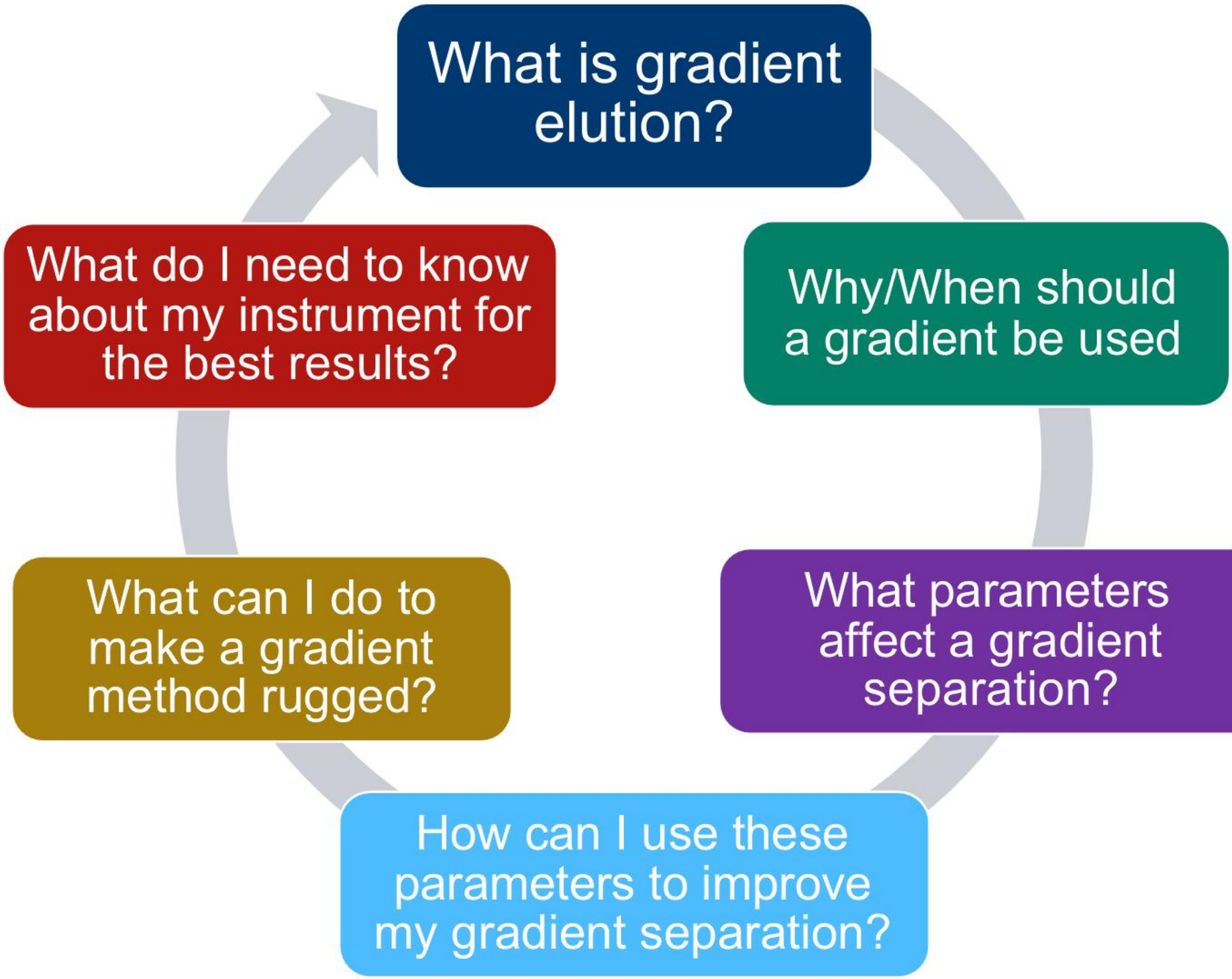
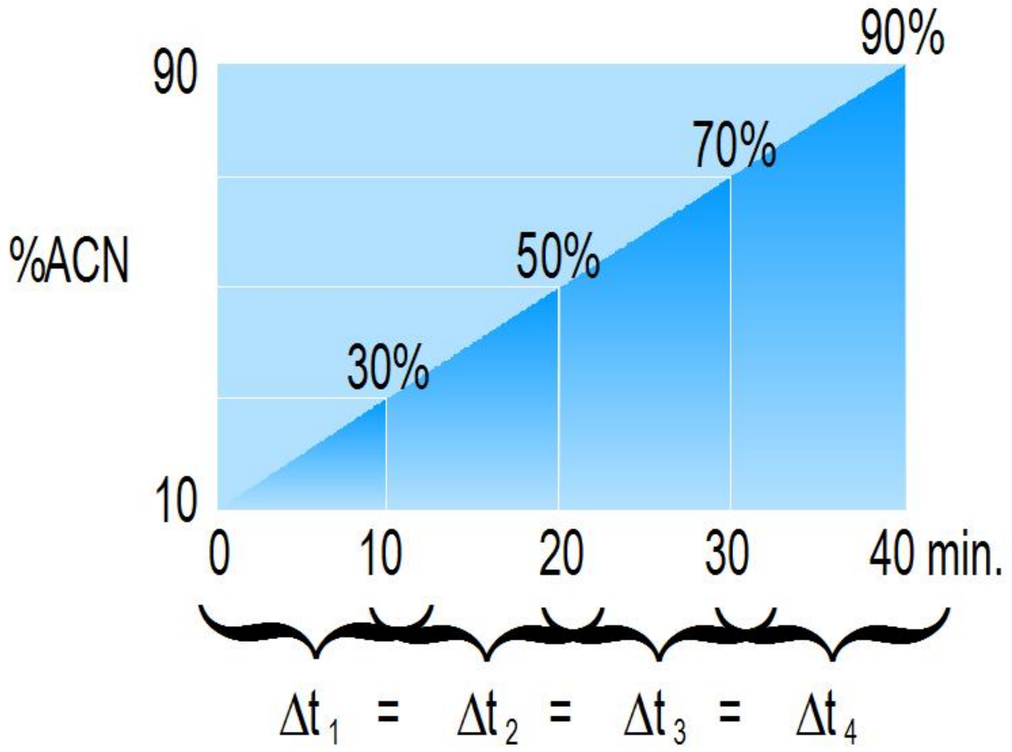
Gradient Elution – So Many Questions!



What is Gradient Elution

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

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.

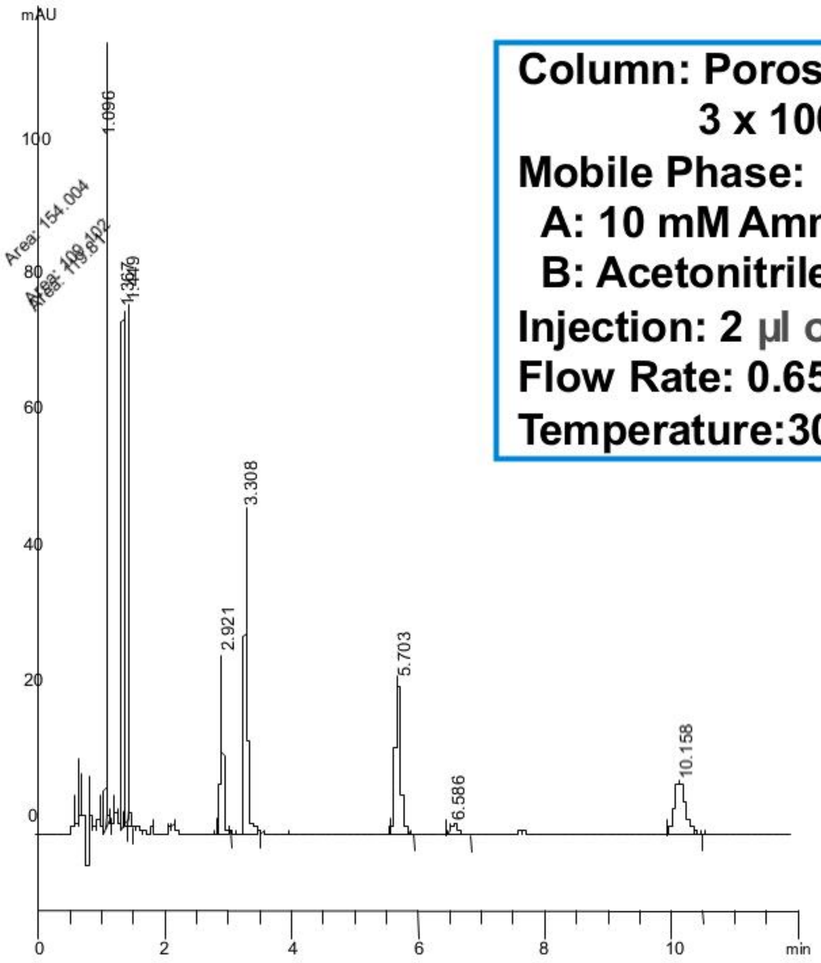
Why/When

- Samples where no isocratic solution is found
- Separate mixtures with a large number of components (may be unknowns)
- Separate high molecular weight mixtures (i.e., peptides and proteins)

Sample Components Vary Widely in Polarity

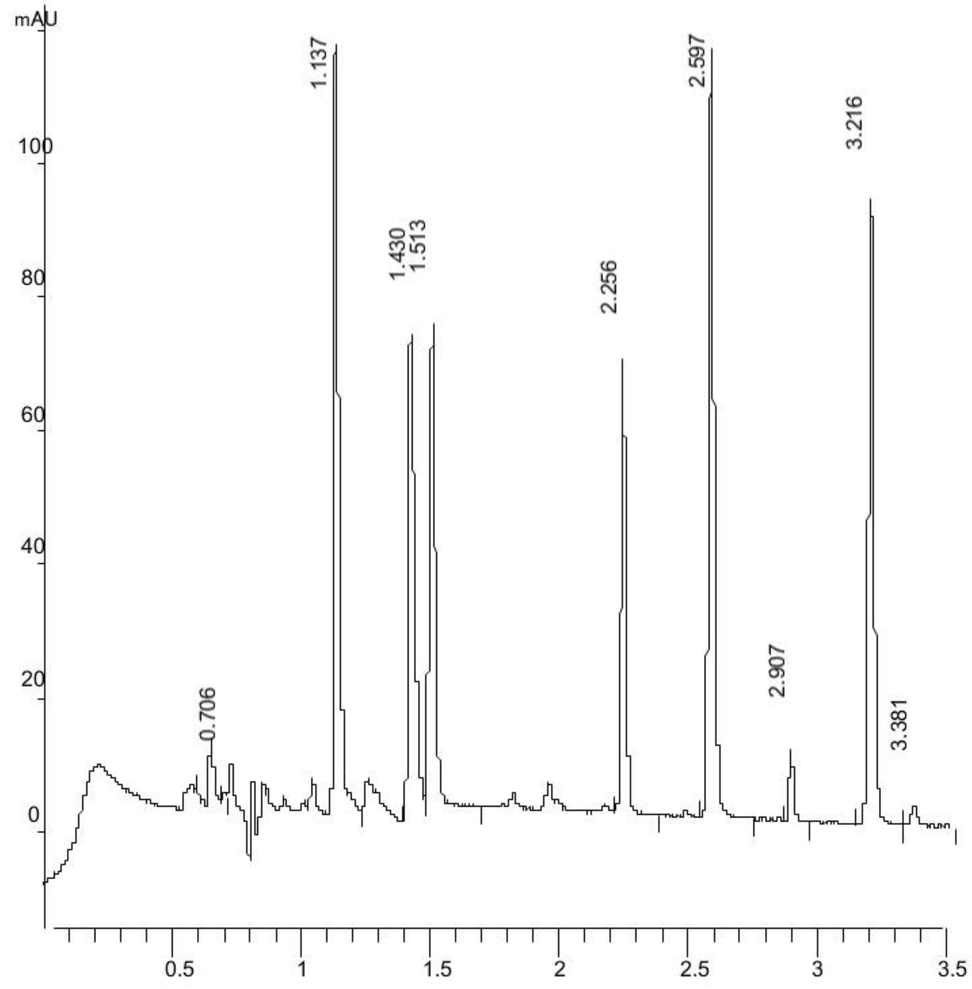
Separation of Soft Drink Additives on Poroshell 120 EC-C18

Isocratic Elution



Column: Poroshell 120 EC C18
3 x 100 mm, 2.7 µm
Mobile Phase:
A: 10 mM Ammonium Acetate, pH 4.8
B: Acetonitrile
Injection: 2 µl of ~0.06 mg/ml
Flow Rate: 0.65 ml/min
Temperature:30°C

Gradient Elution



Isocratic
90%A:10%B

Sample: ascorbic acid, acesulfame k, saccharin, p-hydroxybenzoic acid, caffeine, benzoic acid, aspartame, sorbic acid;

Gradient
10-40%B over 4 min

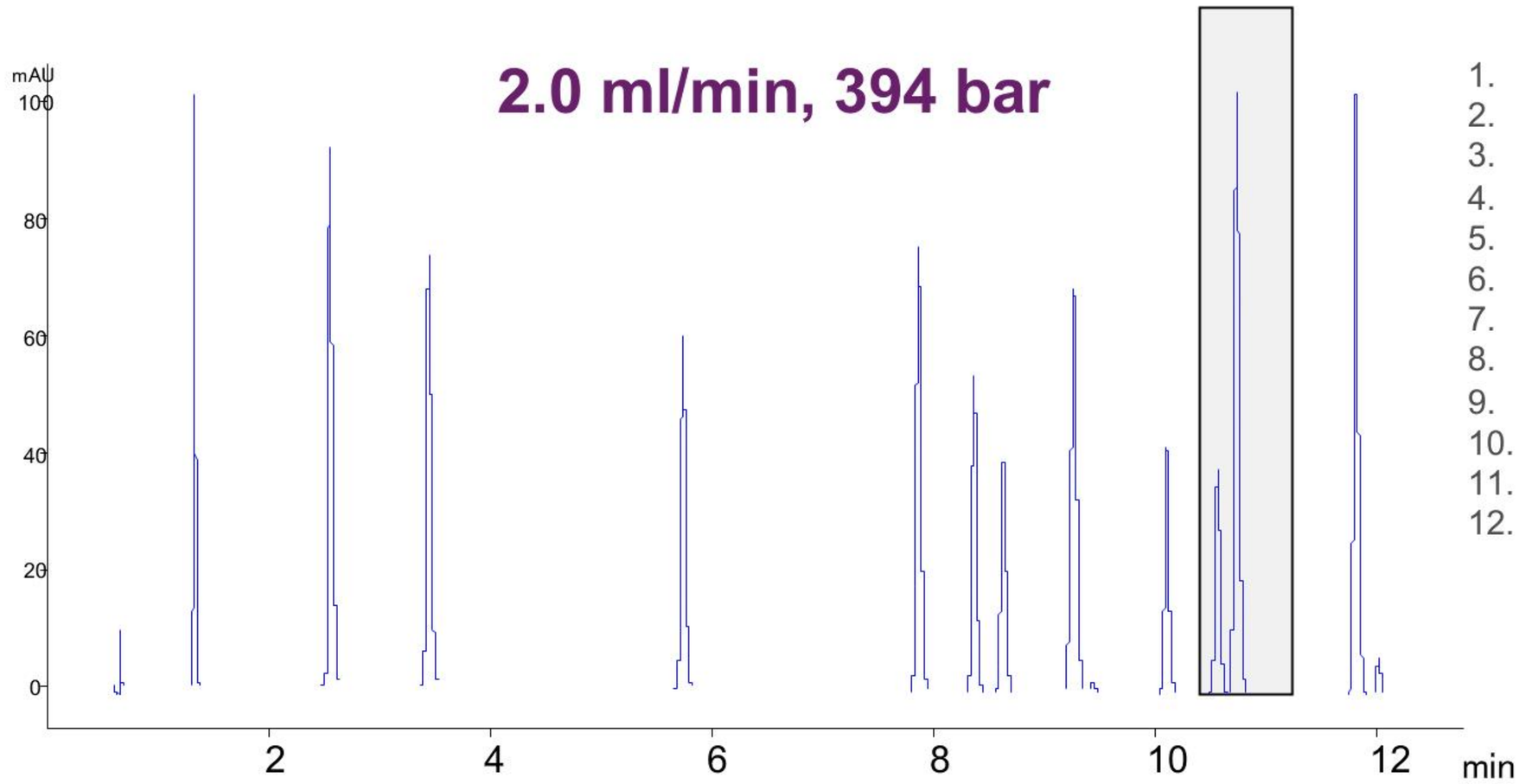
Sample With Large Number of Components

Gradient Separation of 12 Phenols on Poroshell 120 EC-C18

Column: Poroshell 120 EC-C18, 4.6 x 100mm, 2.7um
Mobile Phase: Solvent A: Water with 0.1% Formic Acid; Solvent B: Acetonitrile

Gradient:	Time	%B
	2.0	5%
	17	60%

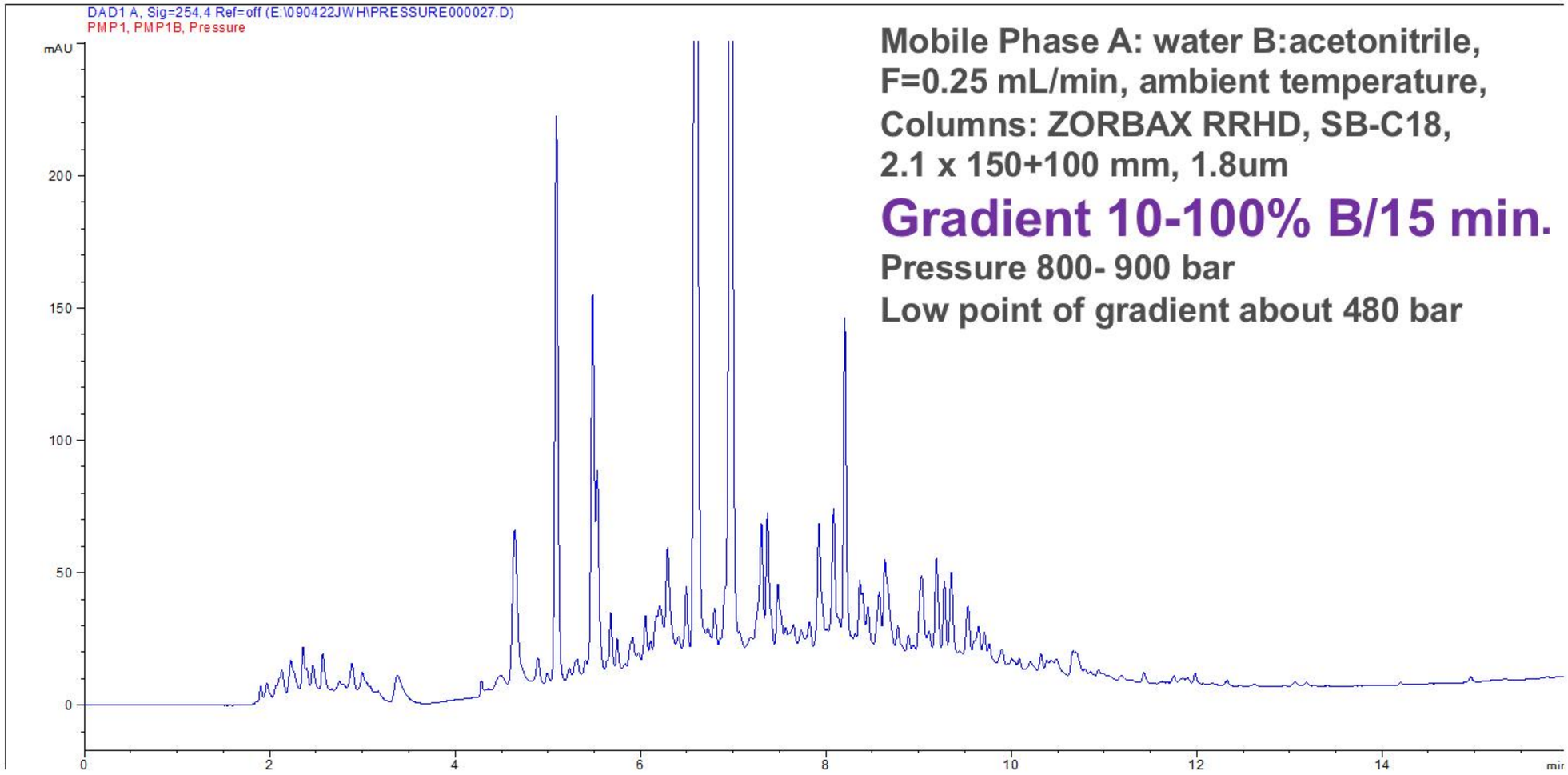
Temperature, 25 °C; 2 mm flow cell



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

Complex Sample

Analyzed in a Single HPLC Run



Mobile Phase A: water B:acetonitrile,
F=0.25 mL/min, ambient temperature,
Columns: ZORBAX RRHD, SB-C18,
2.1 x 150+100 mm, 1.8um

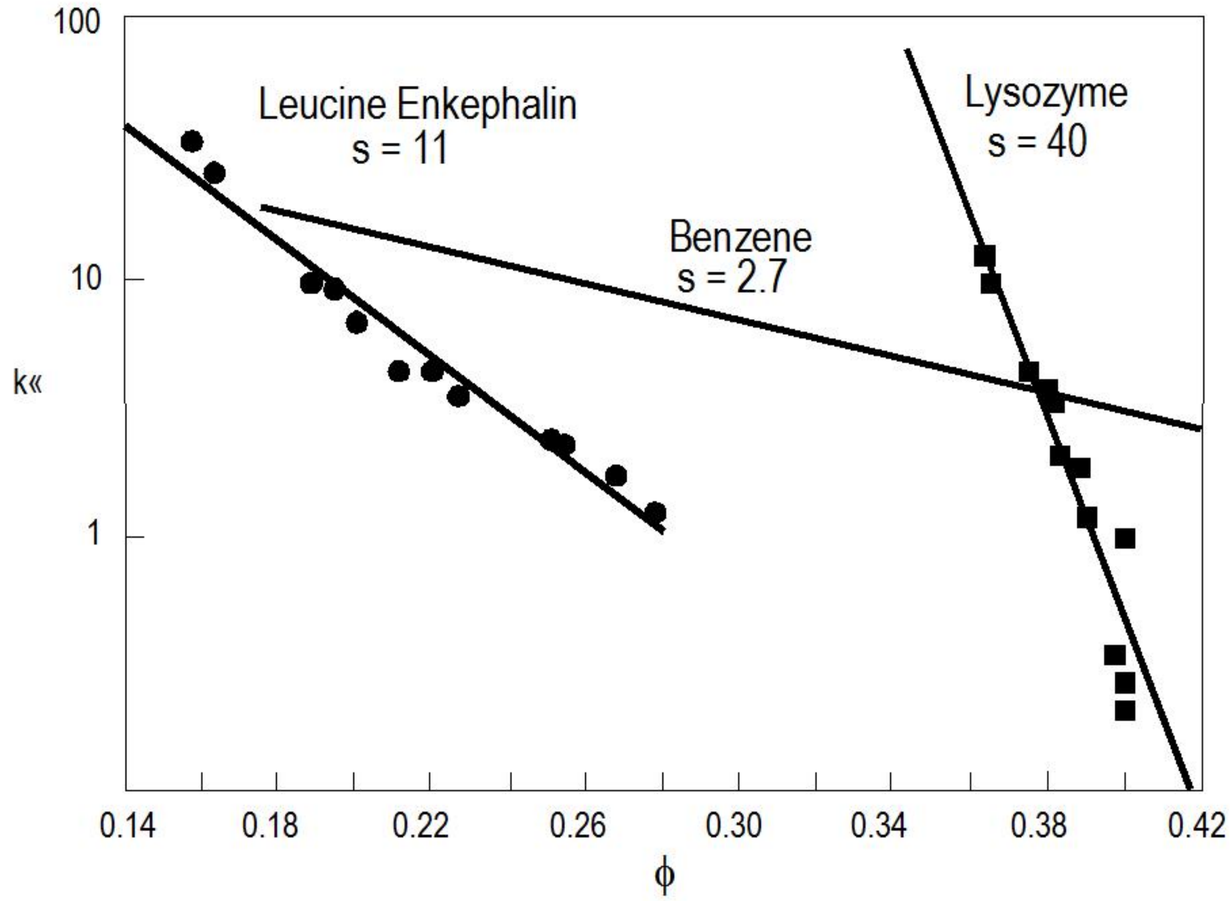
Gradient 10-100% B/15 min.

Pressure 800- 900 bar
Low point of gradient about 480 bar

Smoke food flavoring mix is a very complex sample.
Requires gradient separation for good resolution.

Separate High MW Mix (e.g., peptides/proteins)

Larger molecules are more sensitive to changes in % organic than small are



- S is a constant based on the response of a molecule to changes in organic
- Lysozyme (14.3 kDa) is 15X more sensitive to changes in organic modifier than benzene and 4X more sensitive than leucine enkephalin (555.6 Da)

Gradient Separation of Peptides and Proteins

Columns: Poroshell 300SB-C18
2.1 x 75 mm, 5 um

Mobile Phase: A: 0.1% TFA
B: 0.9% TFA in ACN

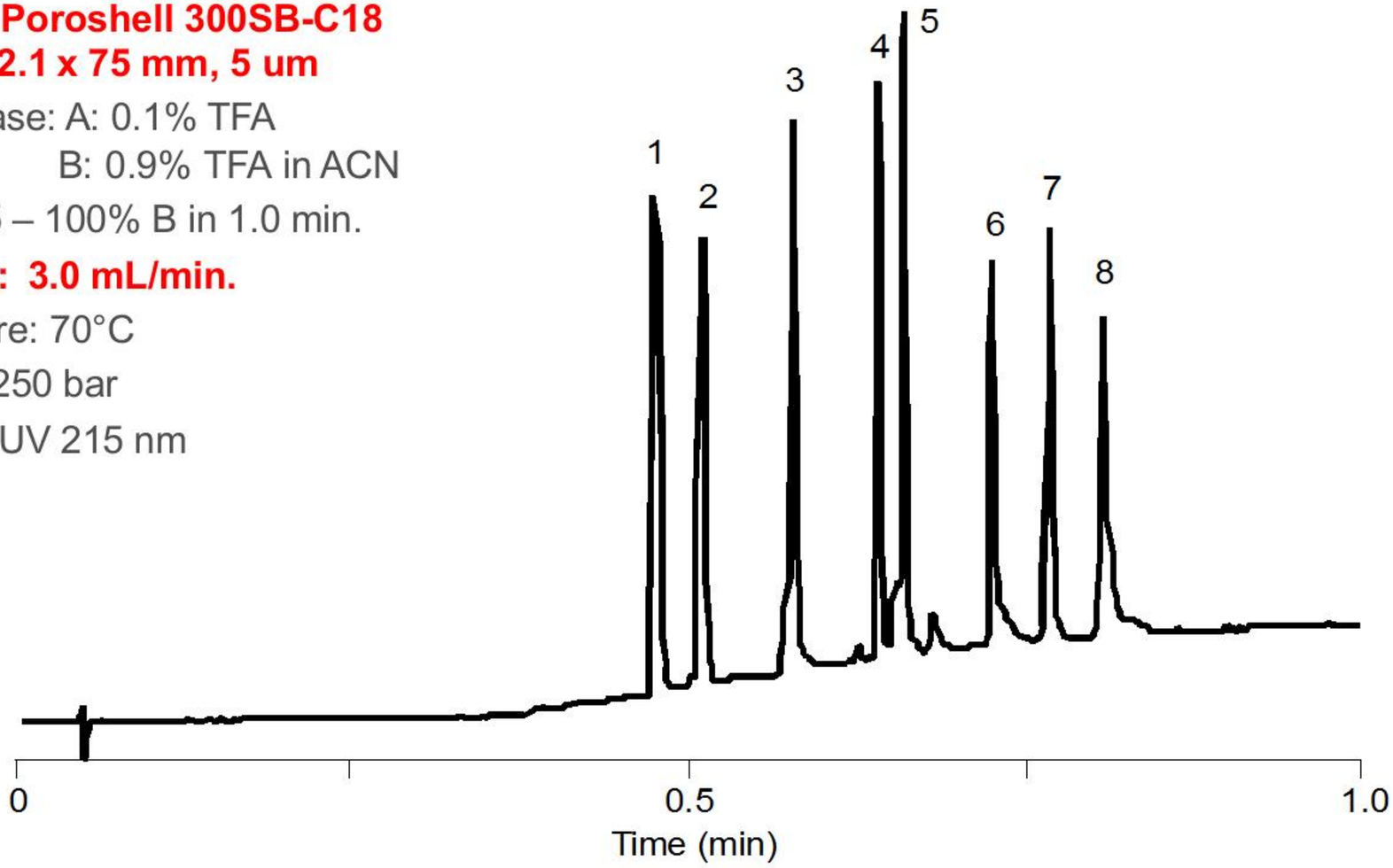
Gradient: 5 – 100% B in 1.0 min.

Flow Rate: 3.0 mL/min.

Temperature: 70°C

Pressure: 250 bar

Detection: UV 215 nm



Sample:
1. Angiotensin II
2. Neurotensin
3. Rnase
4. Insulin
5. Lysozyme
6. Myoglobin
7. Carbonic Anhydrase
8. Ovalbumin

- **Poroshell can provide high efficiency at higher flow rates = fast pro/pep gradient**
  - Rapid mass transfer of the superficially porous particle

Key Gradient Parameters

Quick review of isocratic resolution

What is gradient retention

What factors affect gradient resolution

How to build a gradient

Fundamental Resolution Equation – Isocratic

$$R_S = \tfrac{1}{4}(N)^{1/2}\left(\frac{(\alpha - 1)}{\alpha}\right)\left(\frac{k}{1 + k}\right)$$

α= selectivity – increase by changing bonded phase or mobile phase

N = plates – increase by using longer column or reducing particle size

k = retention – increase by changing bonded phase and/or mobile phase

Factors that Maximize Isocratic Resolution

Increase retention

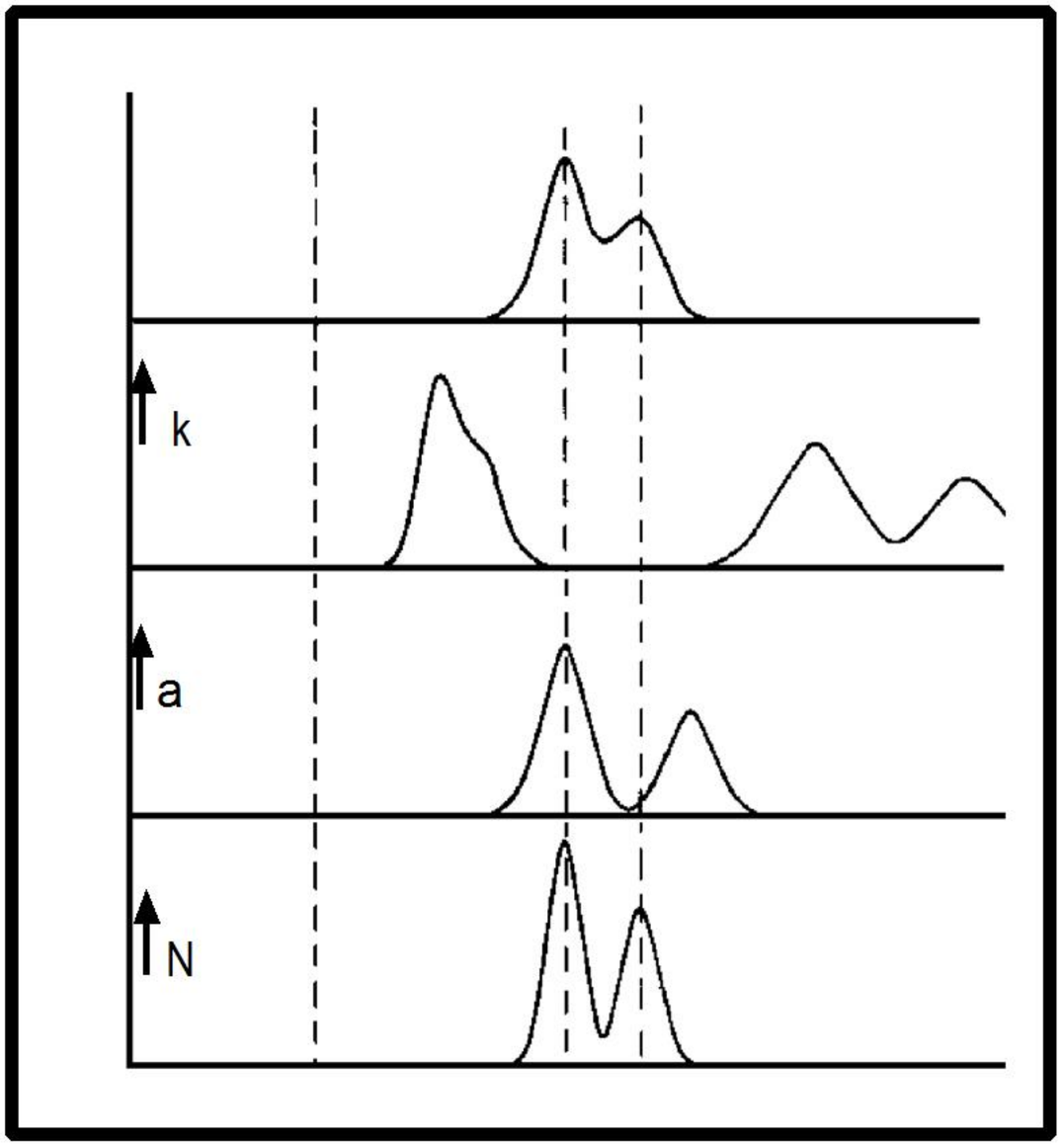
- Decrease % organic

Change relative peak position

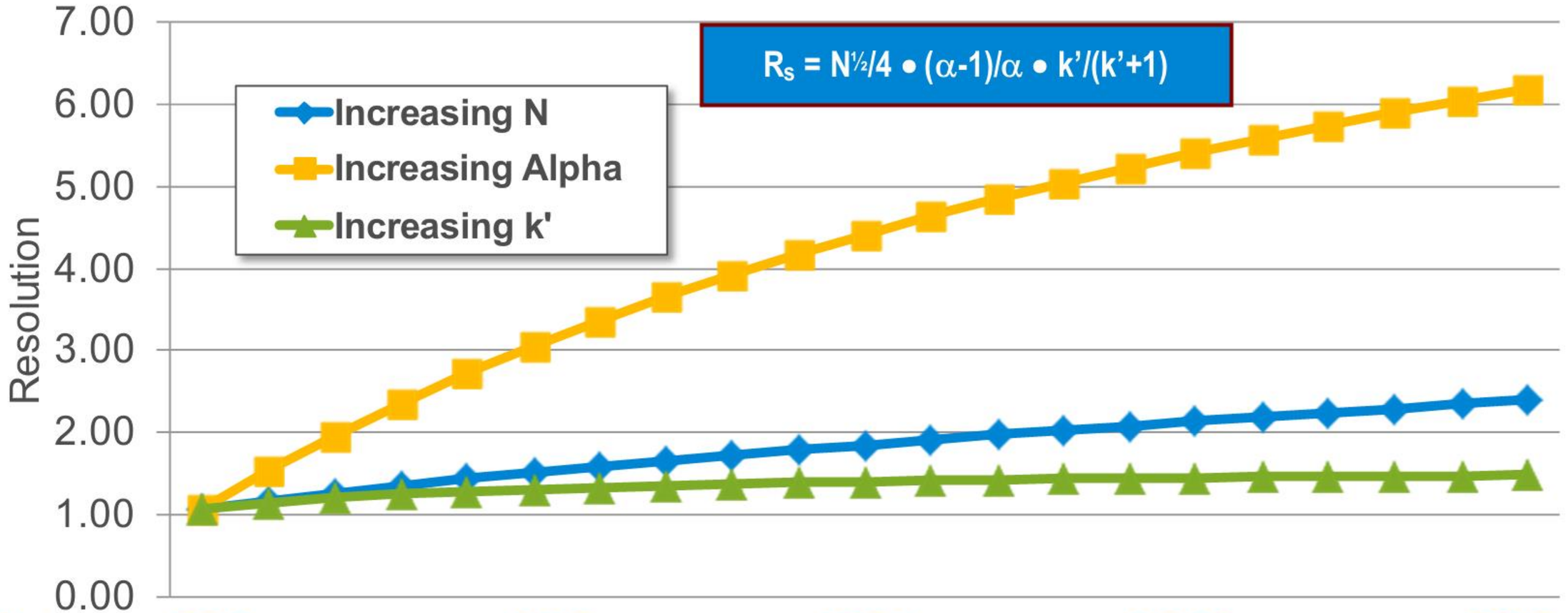
- Change chemistry of the mobile or stationary phase
- Change % organic

Reduce peak width

- Increase column length
- Decrease particle size



Selectivity Impacts Resolution the Most



Plates:	5000	10000	15000	20000	25000
Alpha:	1.10	1.35	1.60	1.85	2.1
k	2.0	4.5	7.0	9.5	12.0

Selectivity impacts resolution most

- **Change bonded phase**
- **Change mobile phase**

} **Typical Method Development Parameters**

Plates are easiest to increase

Resolution Relationship for Gradient Elution

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

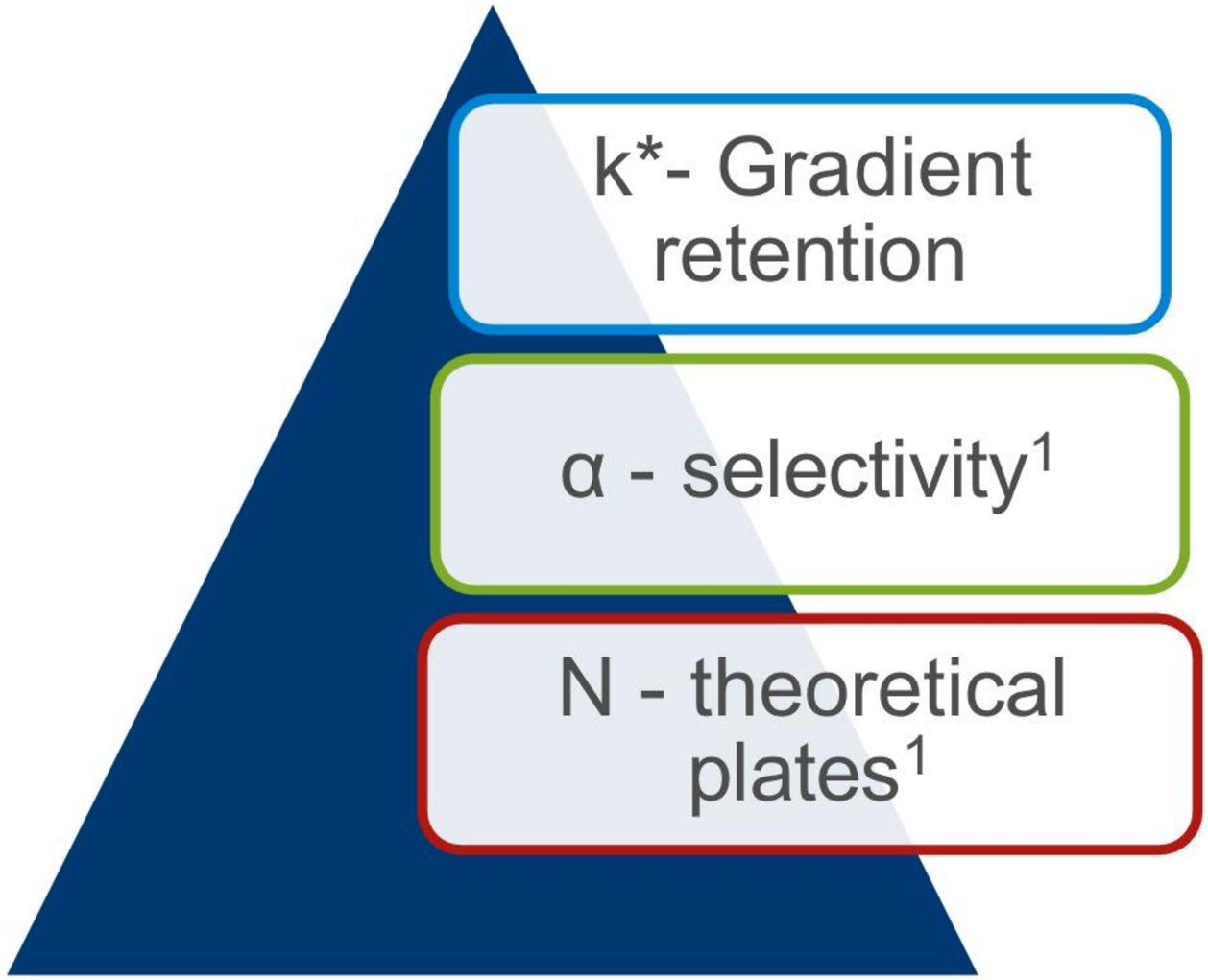
k* - represents the fact that k changes constantly during a gradient

Gradient Retention

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

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

To Maximize Gradient Resolution Between Peaks *INCREASE* One or More of the Following



[1]similar to isocratic elution

All of the Following Increase Gradient Retention (k*)

A longer gradient time	t_G
A shorter column	V_m
A higher flow rate	F
A shorter organic range	$\triangle\Phi$

Because:

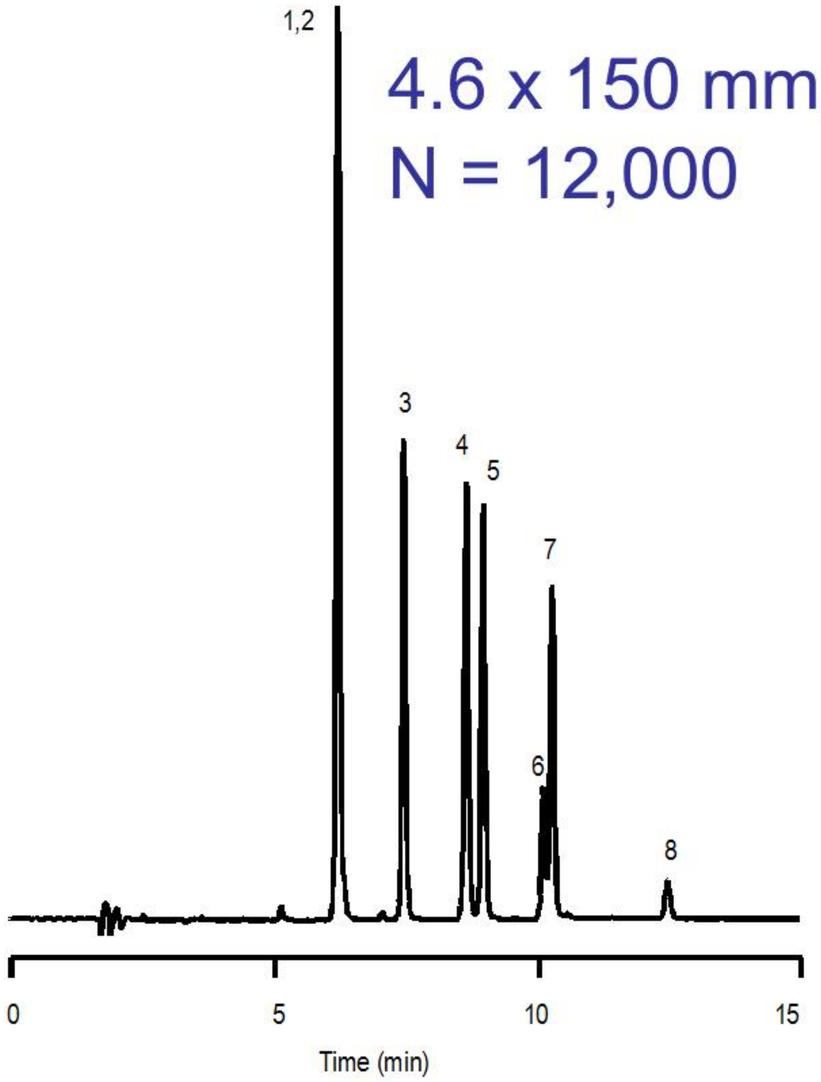
$$1/k^* \propto \text{Gradient steepness} = b = \frac{S \bullet \triangle\Phi \bullet V_m}{t_G \bullet F}$$

- Before changing the stationary phase or mobile phase to generate changes in α (selectivity), explore increasing gradient retention, k*

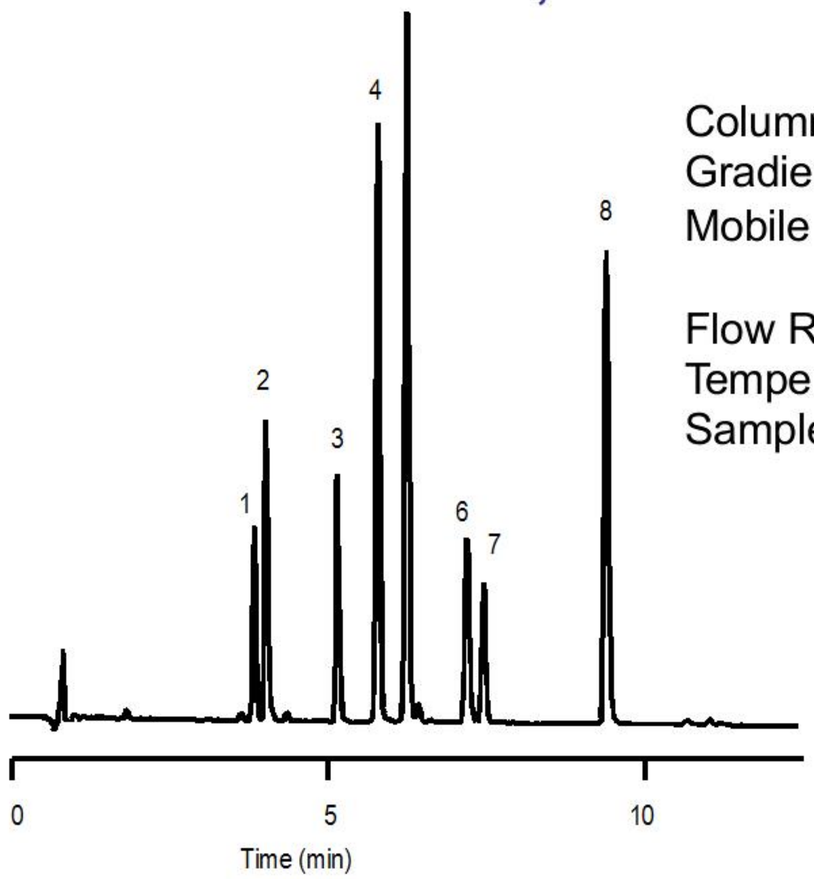
Shorter Column

Increases gradient retention and overall resolution; Assumes constant N

4.6 x 150 mm, 5 μm
N = 12,000

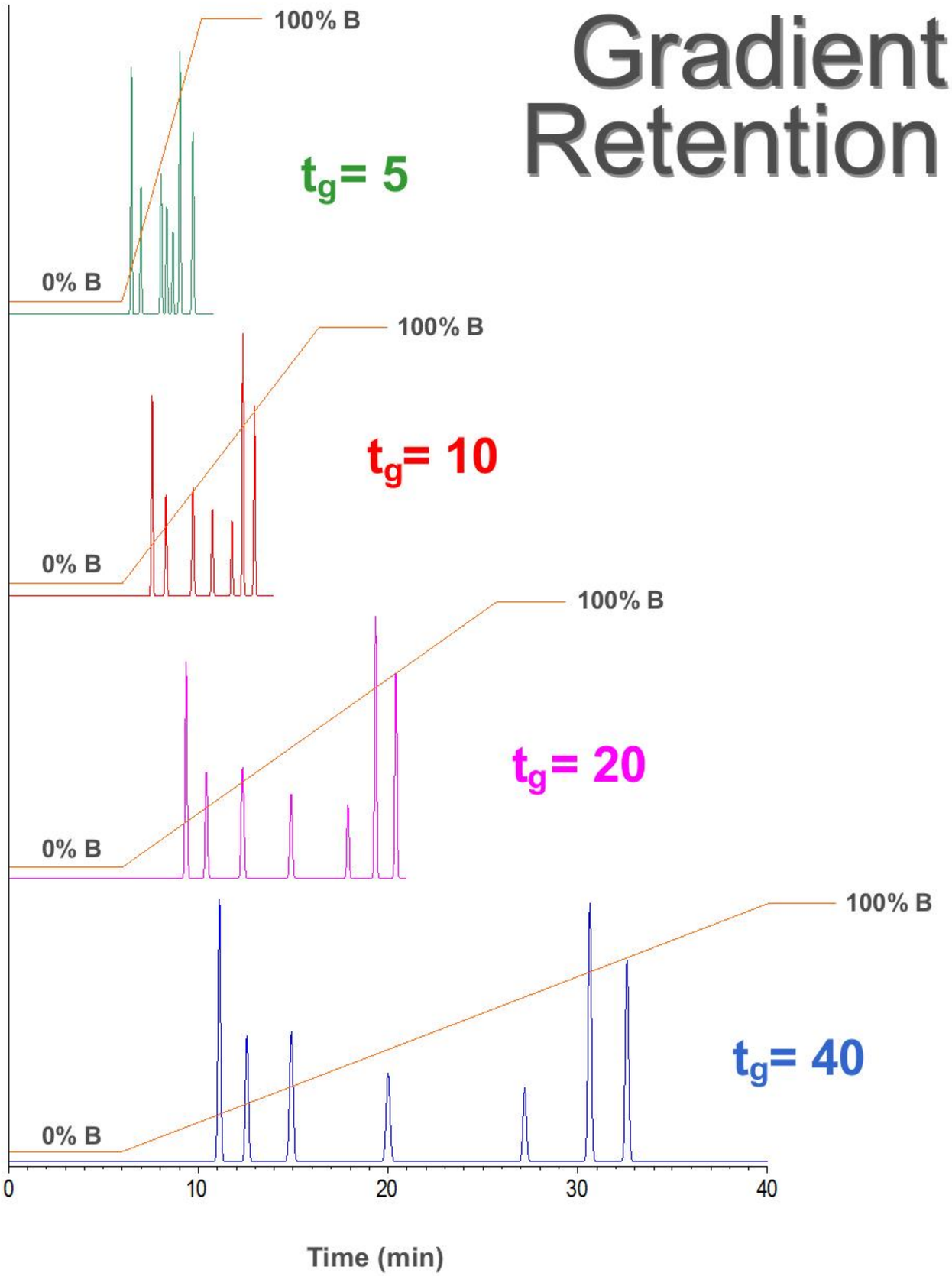


4.6 x 75 mm, 3.5 μm
N = 10,000



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

Gradient Steepness Affects Retention (k*) and Resolution



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

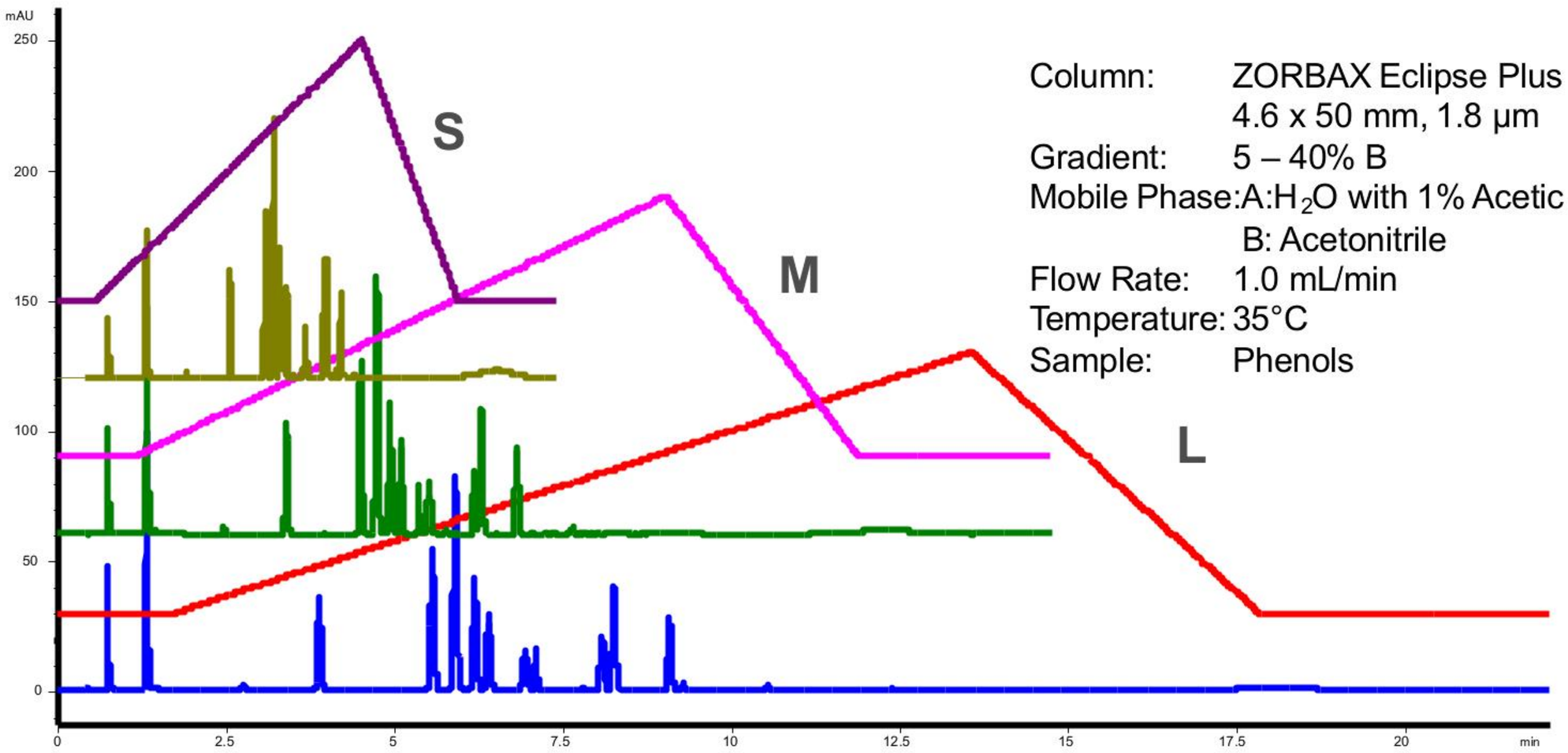
1/k* = gradient steepness = b[a]

$\Delta\Phi$ = change in volume fraction of B
S = constant
F = flow rate (ml/min.)
t_g = gradient time (min.)
V_m = column void volume (ml)

[a]If "b" is kept constant from run-to-run peaks will elute in the same relative pattern.

Longer Gradient Time Increases Gradient Resolution

Gradient	S	M	L
$Rs_{4,5}$	3.29	3.25	3.29
$Rs_{9,10}$	1.14	1.45	1.64



Column: ZORBAX Eclipse Plus C18
4.6 x 50 mm, 1.8 µm
Gradient: 5 – 40% B
Mobile Phase: A: H_2O with 1% Acetic Acid,
B: Acetonitrile
Flow Rate: 1.0 mL/min
Temperature: 35°C
Sample: Phenols

- Increased gradient retention improves resolution of several peak pairs

If "b" is Kept Constant From Run-to-Run
Peaks Will Elute in the Same Relative Pattern

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

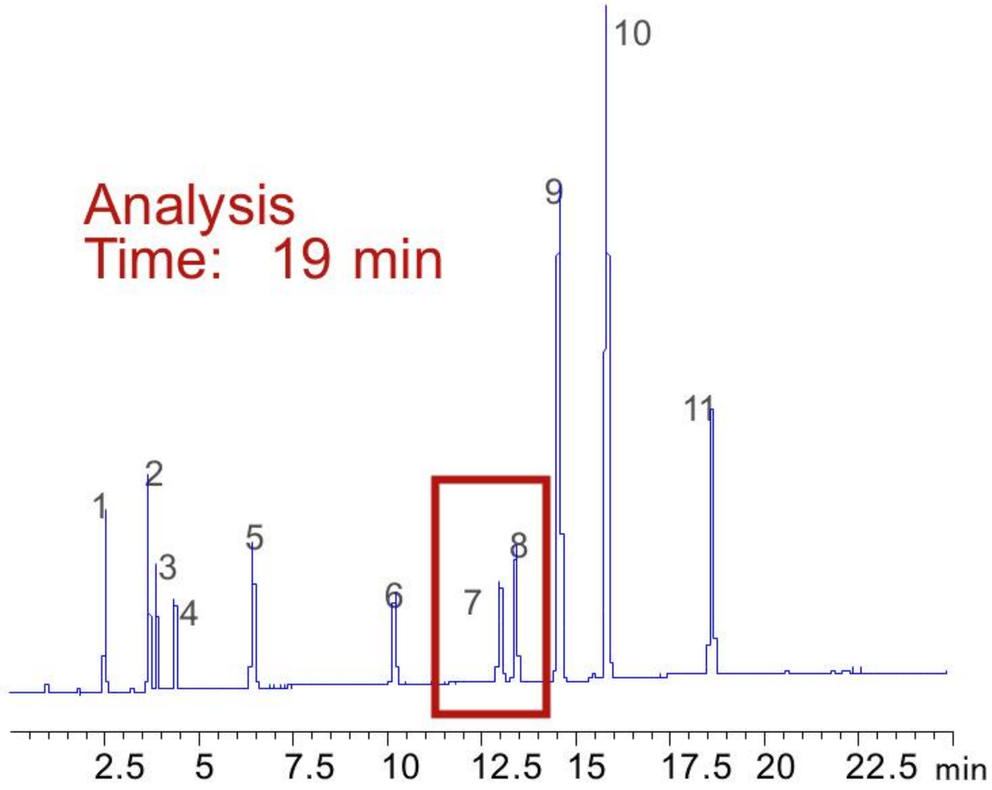
$$k^* \propto \frac{t_G \bullet F}{S \bullet \Delta\Phi \bullet Vm}$$

Keep Gradient Steepness the Same

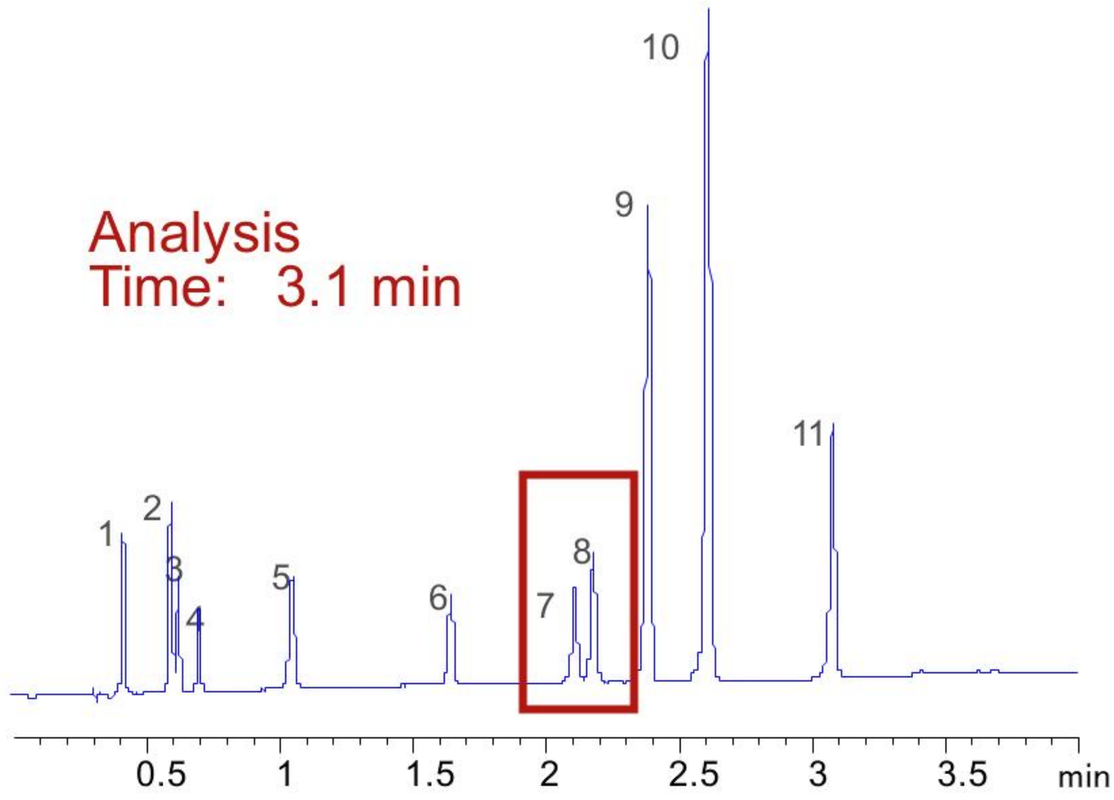
Relative Peak Position Unchanged & Analysis Time Reduced

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)

Column: Poroshell 120 EC-C18
4.6 x 150 mm, 2.7 µm
Gradient Time: 30 min
Flow Rate: 1.0 mL/min



Column: Poroshell 120 EC-C18
4.6 x 50 mm, 2.7 µm
Gradient Time: **6 min**
Flow Rate: **2.0 mL/min**



- Multiple gradient parameters can be changed to maintain gradient resolution while reducing analysis time.

Gradient Method Development

What to Consider

Column selection - More time efficient column – shorter, smal

- Shorter, smaller particle size
  - Reduce analysis time
  - Reduce re-equilibration time

Optimize conditions

- Mobile phase components & pH – Easiest so try first
- Bonded phase – Lots of optimization potential

Optimize the LC

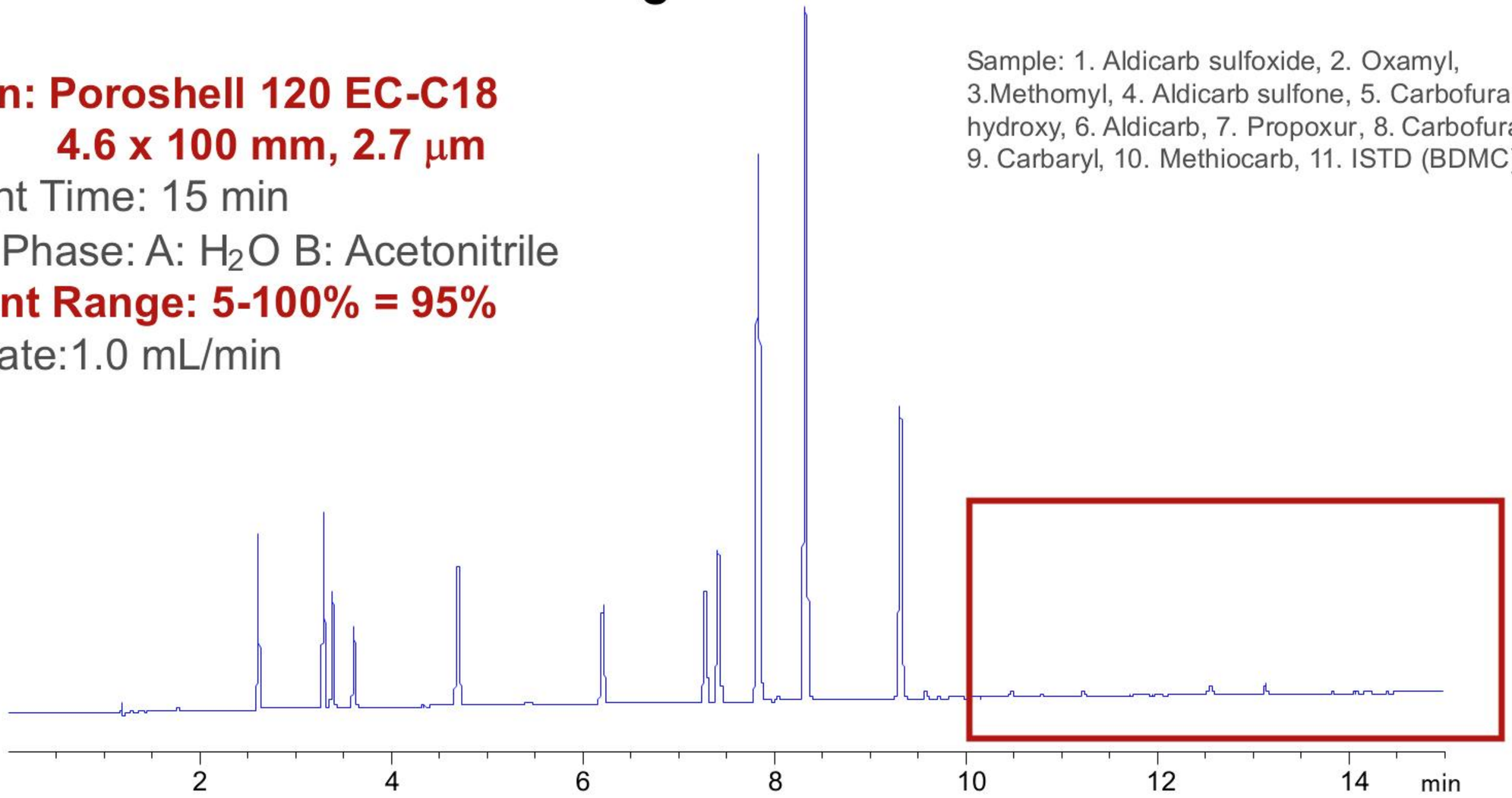
- Is LC configured to deliver the best results
- Are parameters set appropriately, e.g. data acquisition rate

Step 1: Choose, Shorter Efficient Column

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. ISTD (BDMC)



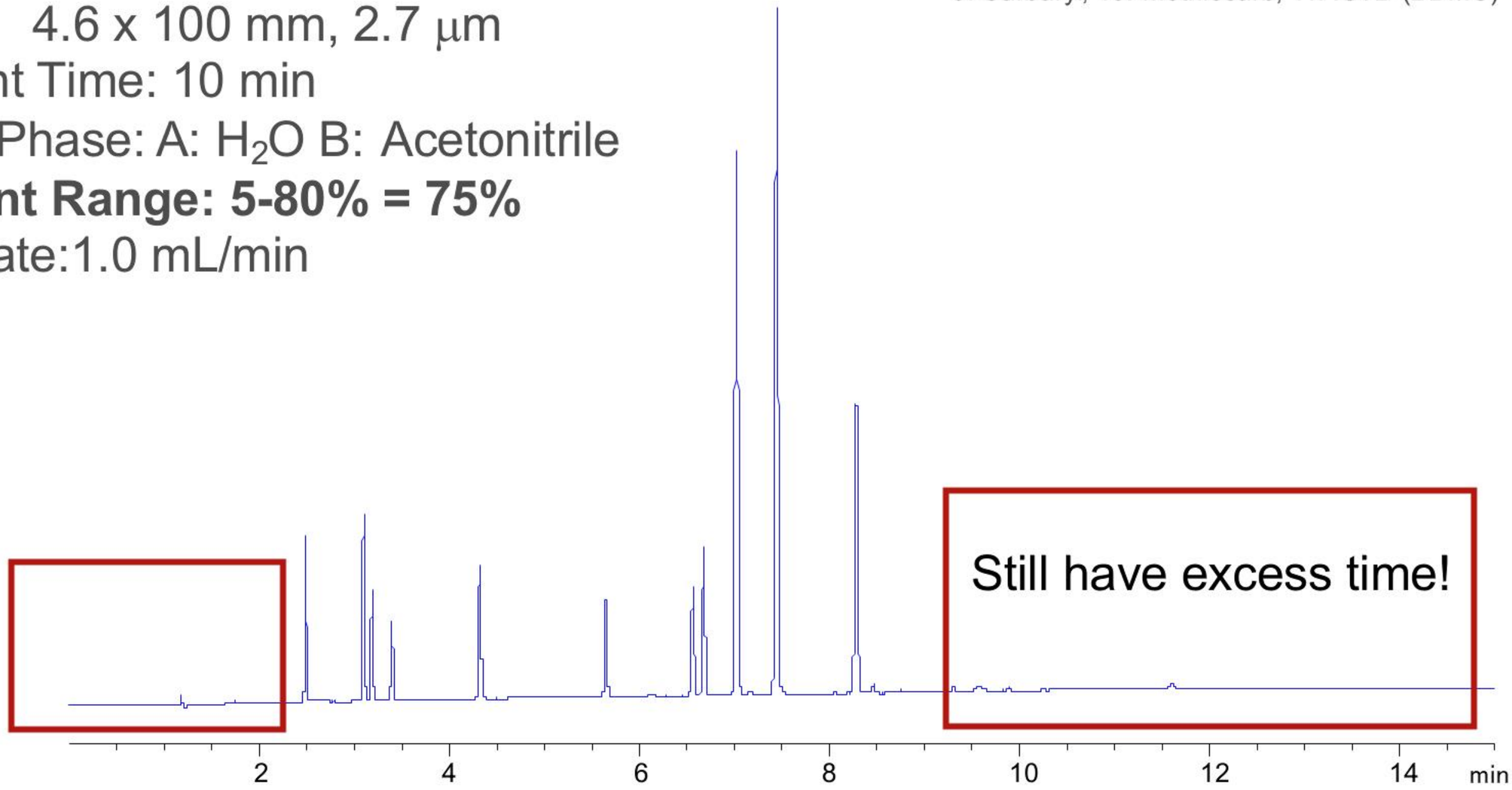
The scouting shows that there is wasted time in this chromatogram and resolution of all components can be achieved. Optimization possible!

Step 2: Reduce Gradient Range and Time

Adjust gradient - 5%-80% B in 10min

Column: Poroshell 120 EC-C18
4.6 x 100 mm, 2.7 μm
Gradient Time: 10 min
Mobile Phase: A: H_2O B: Acetonitrile
Gradient Range: 5-80% = 75%
Flow Rate:1.0 mL/min

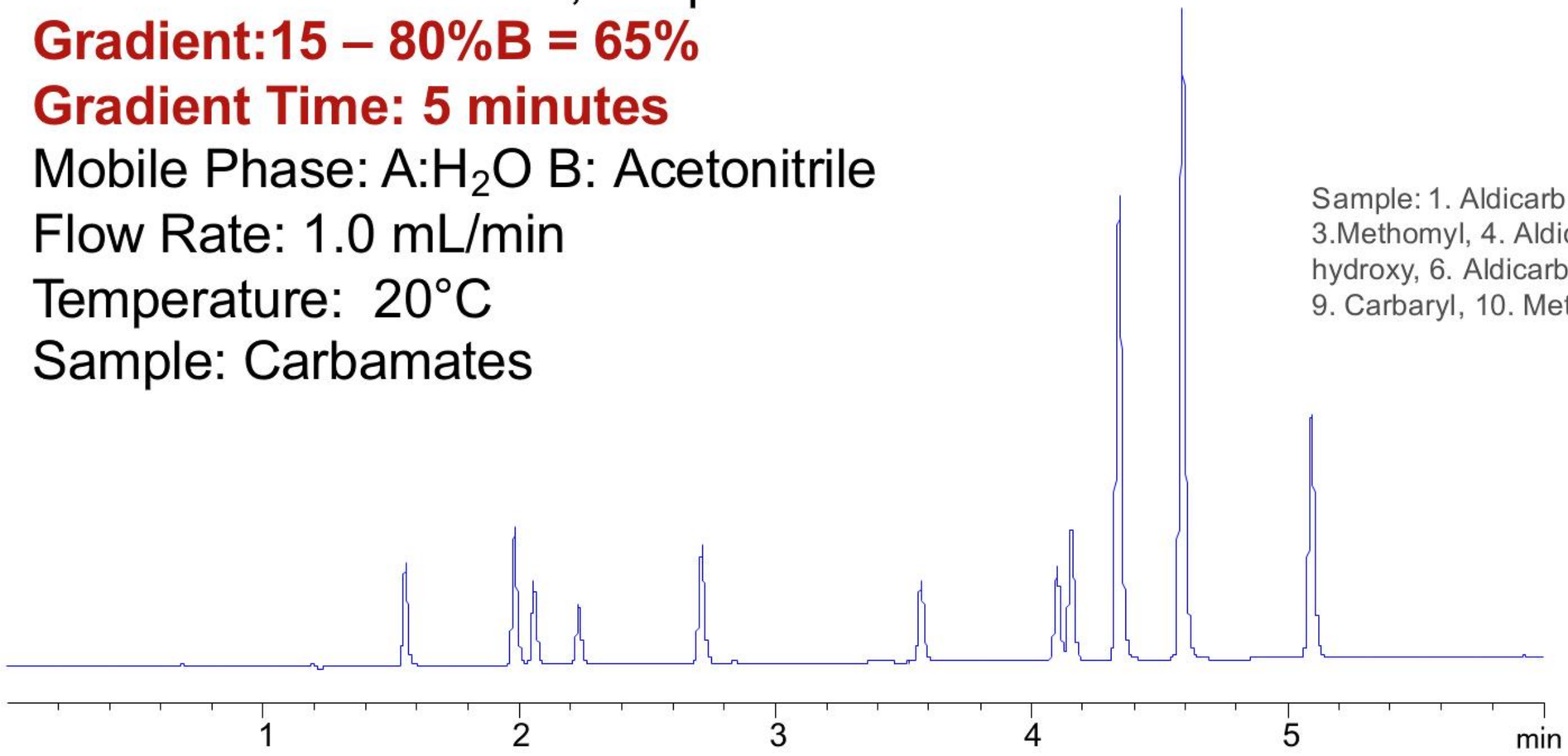
Sample: 1. Aldicarb sulfoxide, 2. Oxamyl, 3.Methomyl, 4. Aldicarb sulfone, 5. Carbofuran-3-hydroxy, 6. Aldicarb, 7. Propoxur, 8. Carbofuran, 9. Carbaryl, 10. Methiocarb, 11. ISTD (BDMC)



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%
Gradient Time: 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.

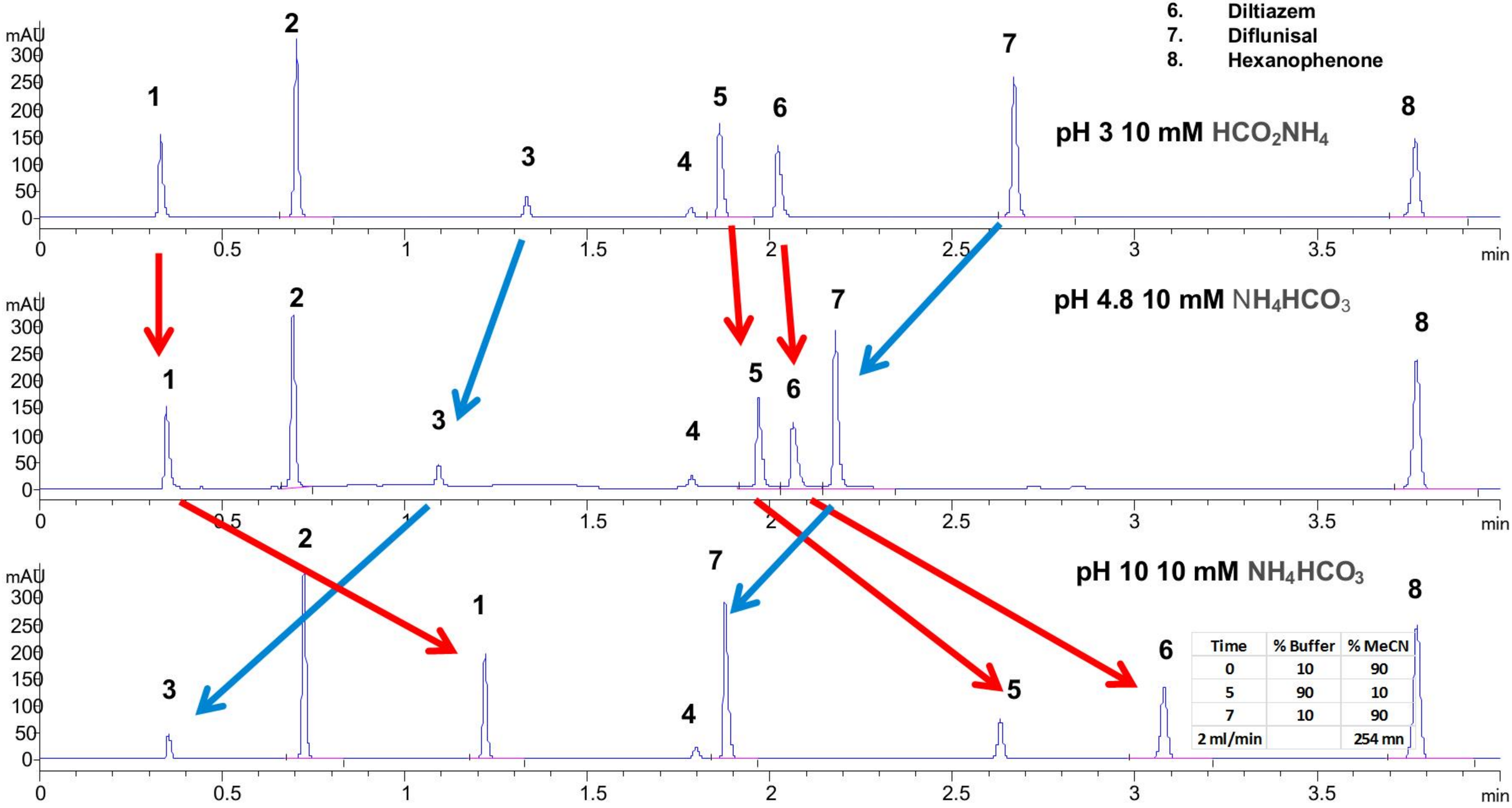
pH
Use to Adjust Peak Spacing

- **Are compounds pH sensitive?**
- **How does pH affect retention and resolution?**
- **How does pH influence column choice?**

Selectivity Can Be Controlled by Changing pH

Poroshell 120 HPH C18 4.6x50 mm, 2.7µm

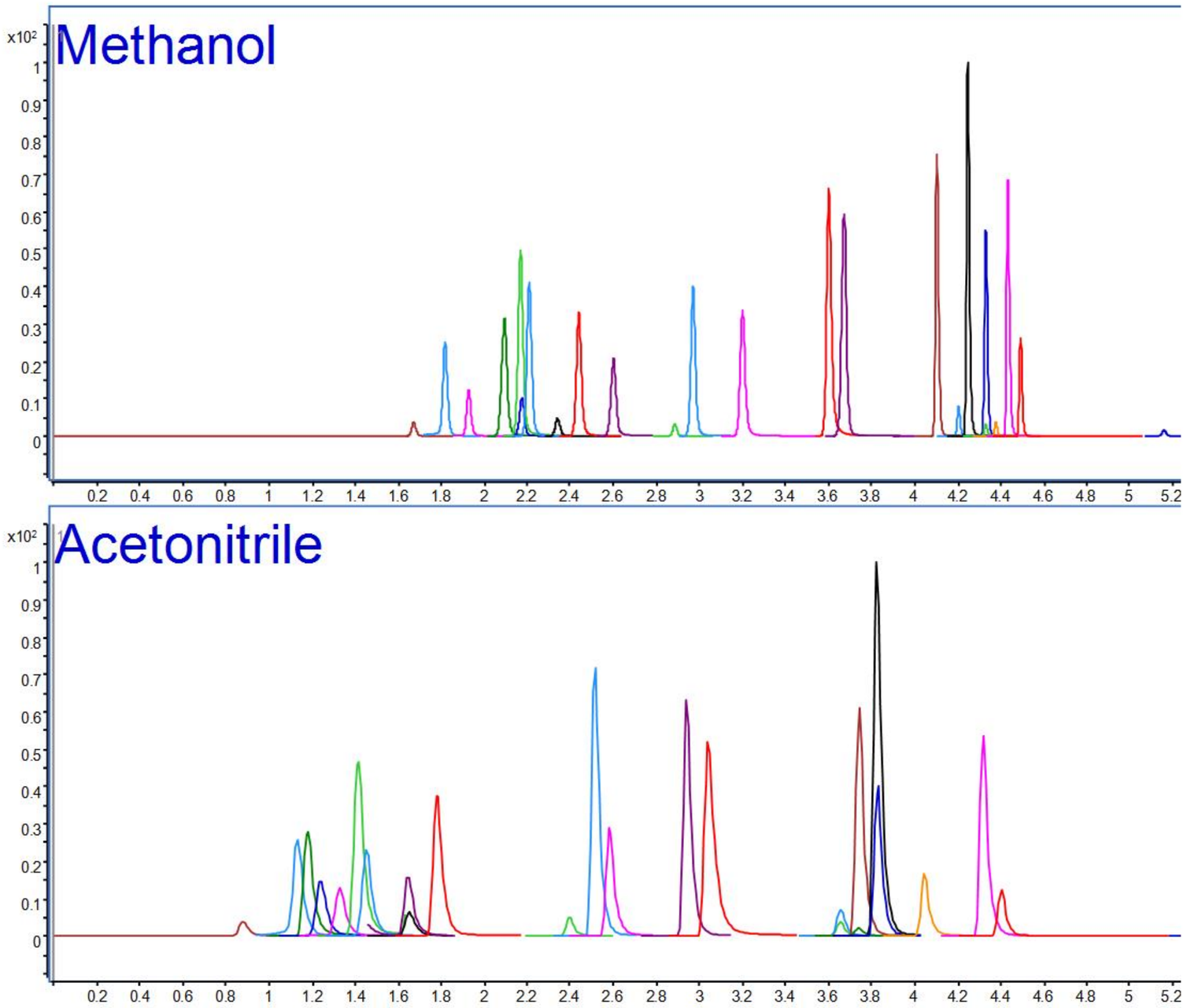
1. Procainamide
2. Caffeine
3. Acetyl Salicylic Acid
4. Hexanophenone Deg.
5. Dipyrimadole
6. Diltiazem
7. Diflunisal
8. Hexanophenone



Time	% Buffer	% MeCN
0	10	90
5	90	10
7	10	90
2 ml/min		254 mn

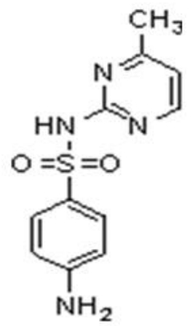
Methanol or Acetonitrile as the Mobile Phase

Comparison of 25 Component Mixture

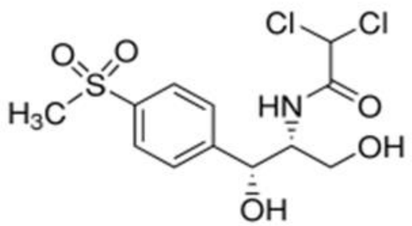


- **ACN vs MeOH**
  - Selectivity differences
  - Consider a blend

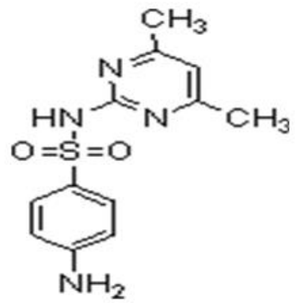
Structures of Selected Antibiotics



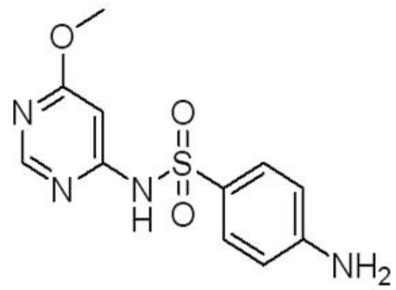
Sulfamerazine
(SMR)



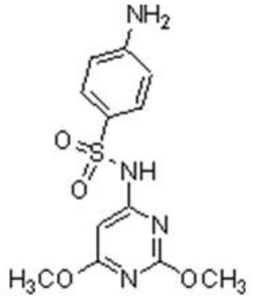
Thiamphenicol
(TPC)



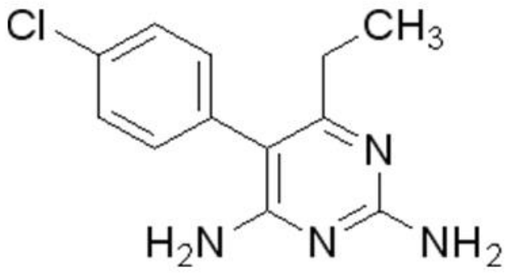
Sulfadimidine
(SDD)



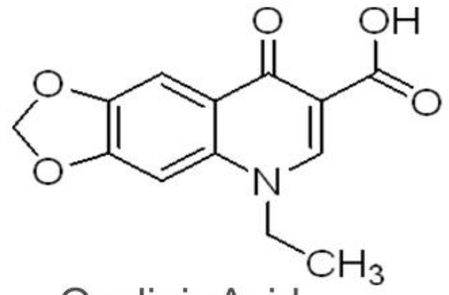
Sulfamonomethoxine
(SMMX)



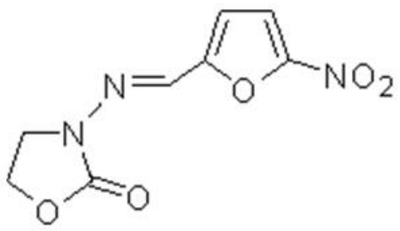
Sulfadimethoxine
(SDMX)



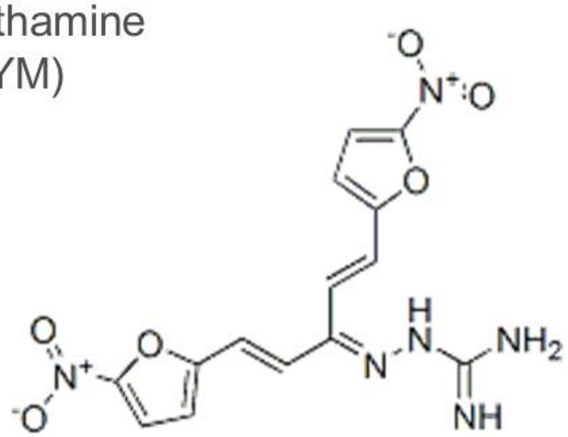
Pyrimethamine
(PYM)



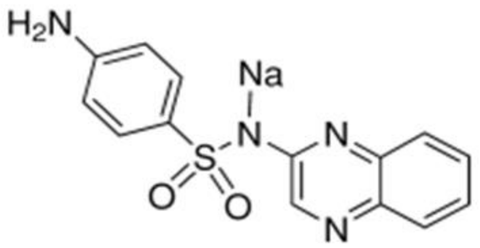
Oxolinic Acid
(OXA)



Furazolidone
FZD



Difurazone
DFZ

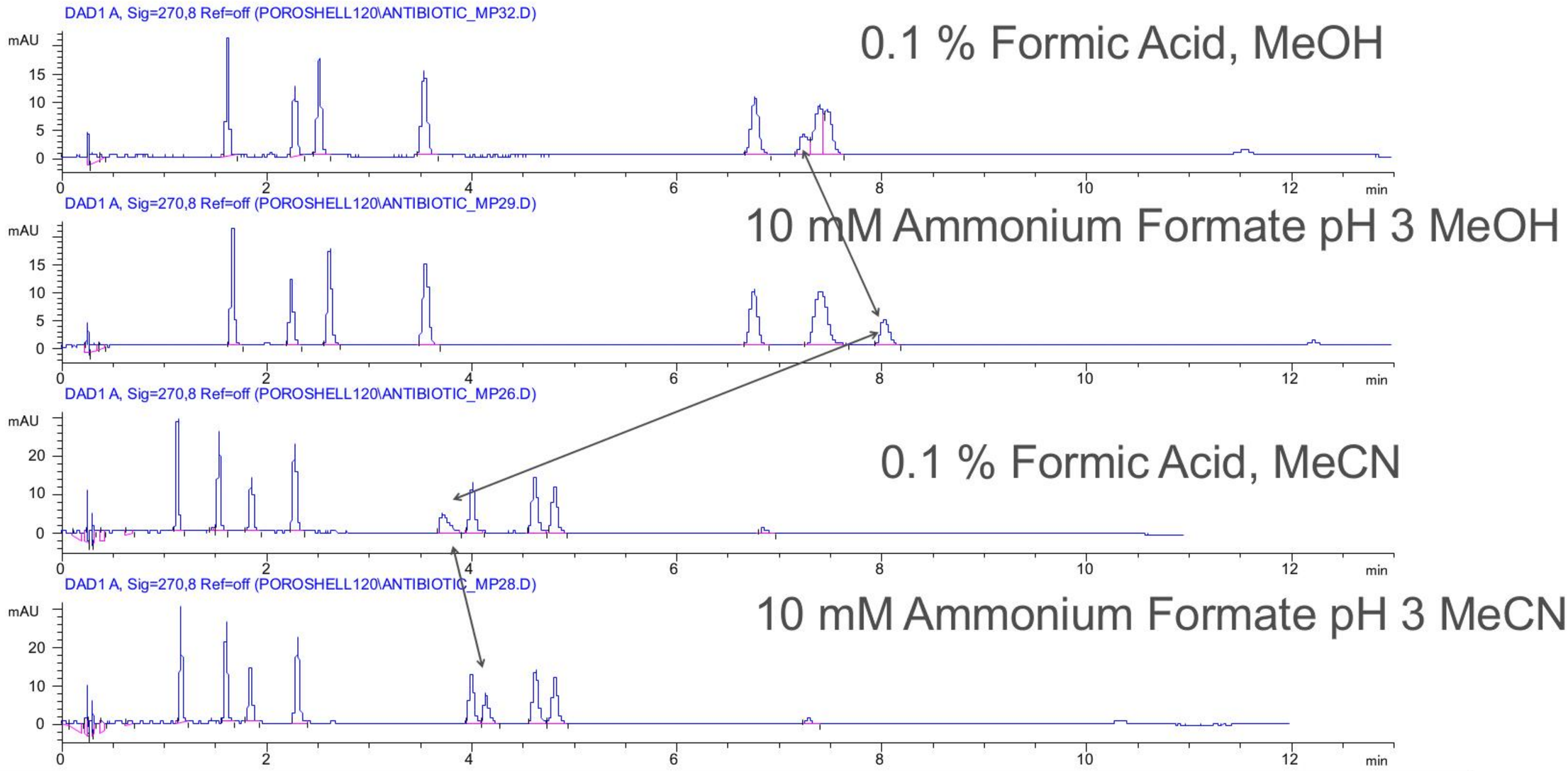


Sulfaquinoxaline
(SQX)

Compare Solvent and Mobile Phase Additive

Mobile Phase Buffer or 0.1 % Formic Acid; MeOH or MeCN

10 mM Ammonium Formate buffer gives better peak shape and selectivity than 0.1% FA at similar pH



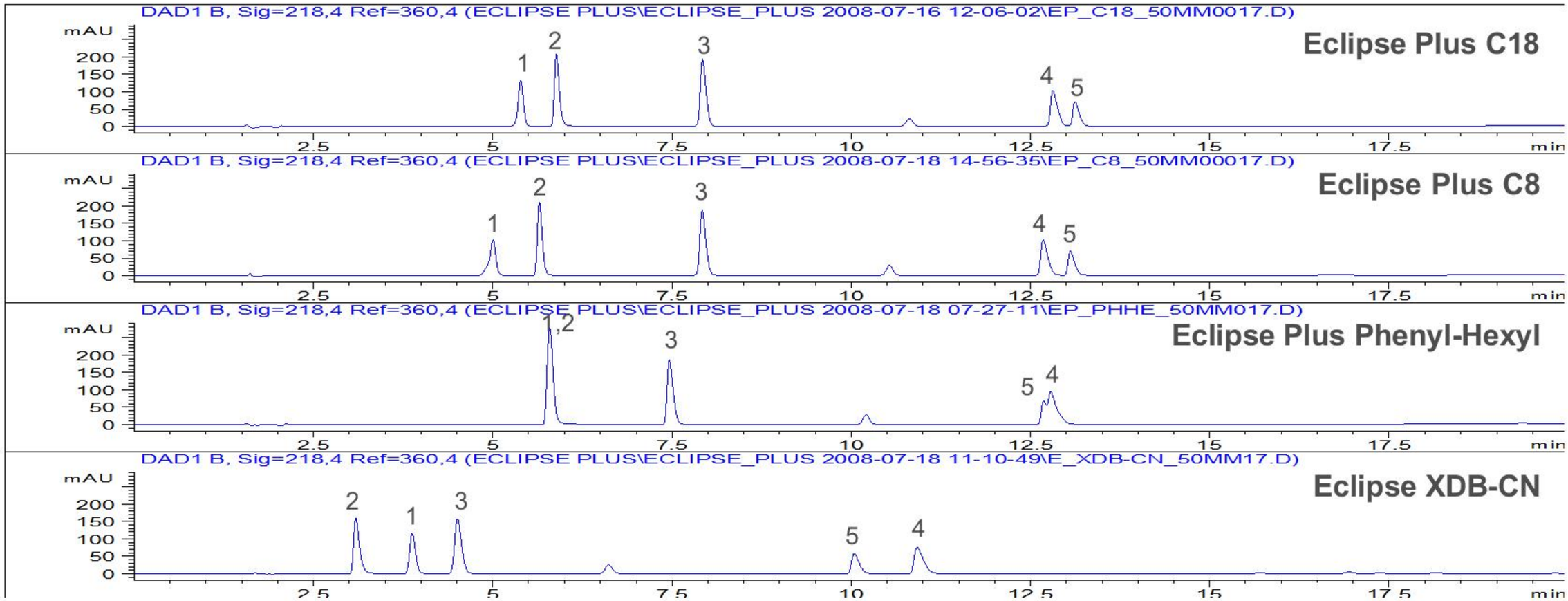
Column: Poroshell 120 EC-C18, 4.6 x 50 mm, 2.7um
Gradient Conditions: 10-40 %B/12 min; Flow: 2 mL/min; Injection: 0.5 ul injection, 0.1 mg/ml each

Bonded Phase

Different Phase Can Vary Selectivity

Mobile Phase A: 50mM NaH_2PO_4 ,pH 2.5 in 95% Water / 5% Acetonitrile, Mobile Phase B: 50mM NaH_2PO_4 pH 2.5 in 47% Water / 53% Acetonitrile

Gradient: 0-100% B; Gradient Time: 18.8 minutes; Flow 1 ml /min,; 218nm; Injection: 4ul; Temperature: 26° C



Sample: 1. Procaine, 2. d-Cinchonine, 3. Lidocaine, 4. Butacaine, 5. Tetracaine

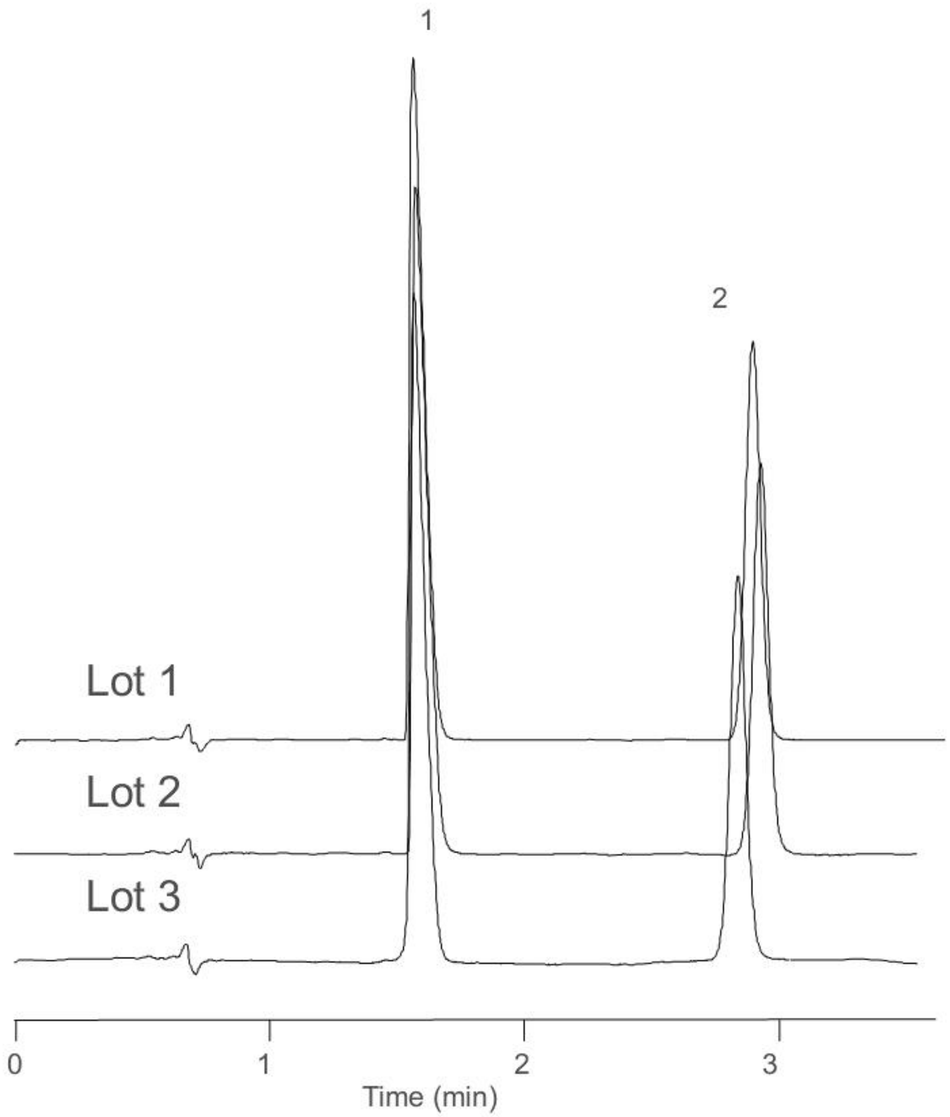
- ✓ C18 - Adequate separation and peak shape (min. R=1.82)
- ✓ C8 - Separates compounds well; Procaine has poor peak shape & tailing (min. R=2.20)
- ✓ Phenyl-Hexyl - Does not separate Procaine and d-Cinchonine; Poorly separates Butacaine and Tetracaine
- ✓ Eclipse XDB-CN - Different selectivity than C18, C8, or Phenyl-Hexl; Best resolution of Butacaine and Tetracaine (min. R=3.84)

Multiple Lots

- Compare three current lots of material for consistency of retention (k) and selectivity (α)
- Method validation kits

Three Lot Summary

	Mean	SD	RSD
k (D)	1.1	0.01	1.0%
k (B)	2.9	0.06	2.1%
α	2.6	0.05	1.8%



Column: SB-CN, 4.6 x 75 mm, 3.5 μm
Mobile Phase: 55% CH_3COONH_4 (pH 4.5)/0.5% TEA : 45% ACN
Flow Rate: 1.0 mL/min
Temperature: RT
Sample: 1. Diphenhydramine 0.5 mg/mL 2. Benzophenone .005 mg/mL
Injection Volume: 10 uL

What Do I need to Know About My Instrument

Isocratic Separations

Sample load; V_{inj}, analyte

Sample solvent strength

Extra column volume

- Flow cell volume
- Injection volume
- Tubing volume

Injector precision

- Can vary with V_{inj}

Data Rate

- Too fast, too much noise
- Too slow, loss of N

Gradient Separations

Same as isocratic separations plus…

Delay Volume

- Same instrument (different pressures)
- Different instrument (for example, Capillary vs. Binary)

Gradient Time

- Adjust relative to equation for gradient retention
- Keep k* constant

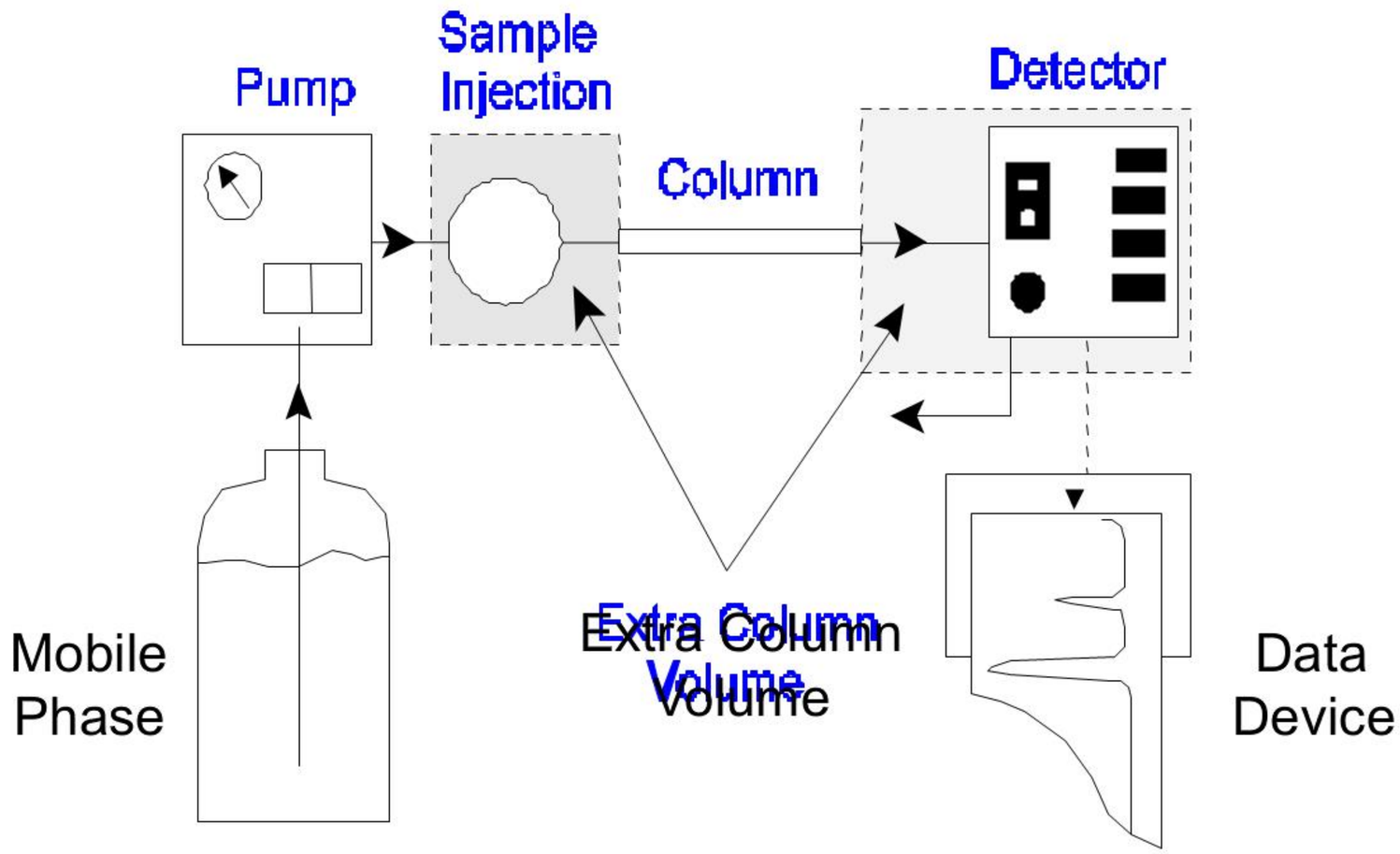
Gradient Delay Time

- Gradient delay time must be same as for larger column separation
- Ratio of gradient volume/column volume must be same as for larger column

Column Equilibration Time (Post Time)

Extra Column Volume =

Sample volume + Connecting tube volume + Fitting volume + Detector cell volume

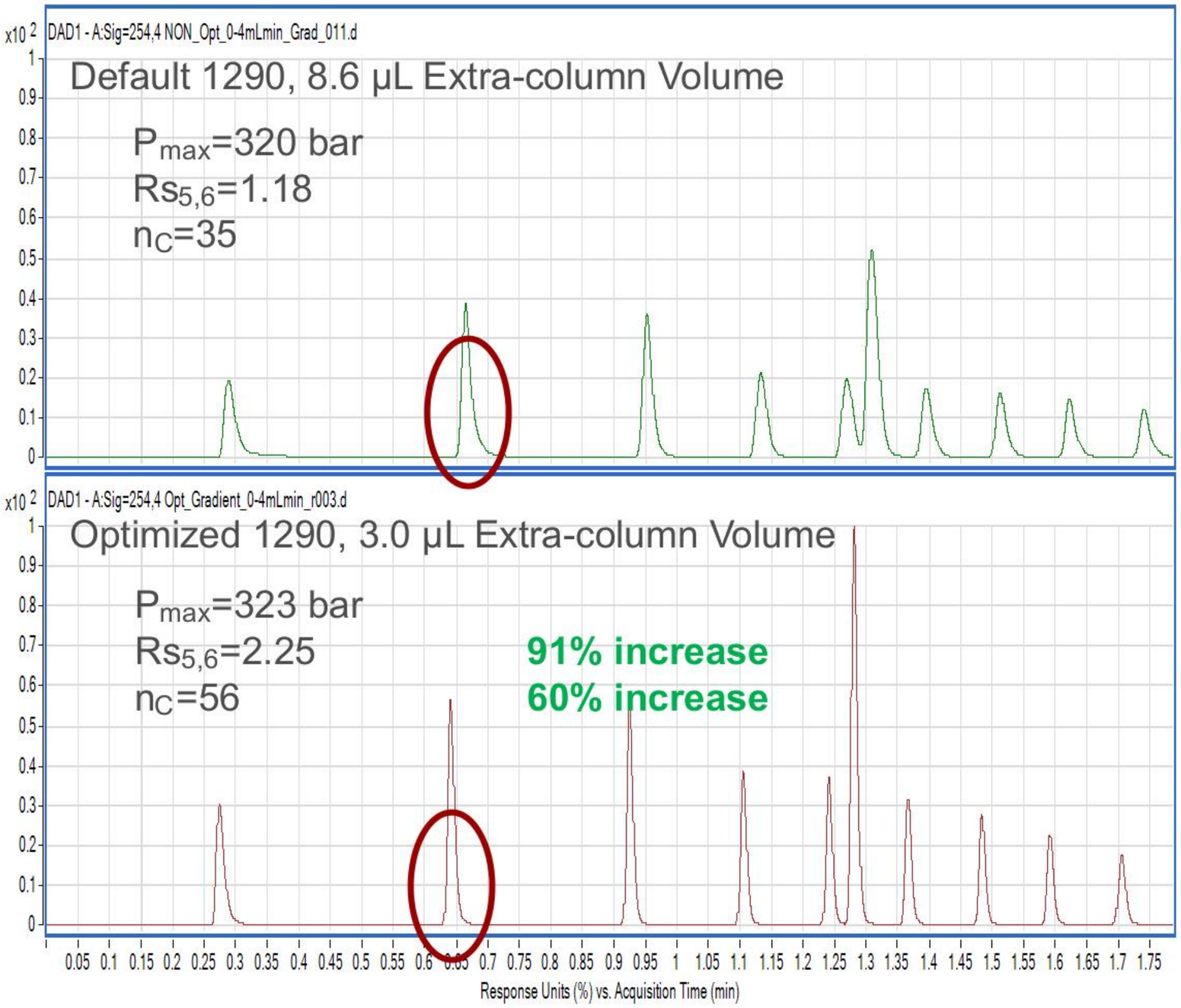


The instrument schematic above depicts where extra-column volume can occur – affects instrument and column performance.

For more information, see LC Column Troubleshooting recorded e-seminar, http://www.chem.agilent.com/en-US/Training-Events/eSeminars/14736B/Pages/default.aspx

Effect of ECV on Gradient Analysis of Alkylphenones

Efficiency and Tailing



Agilent ZORBAX RRHD Eclipse Plus C18
2.1 mm x 50 mm, 1.8 µm, 959757-902

LC Rack System, 5001-3726

0.08 x 220 mm Capillary Tubing

V(σ)0.6 µL Flow Cell

A: H_2O; B: CH_3CN
0.4 mL/min

t (min)	0	1.2
%B	25	95

1 µL injection of RRLC Checkout Sample

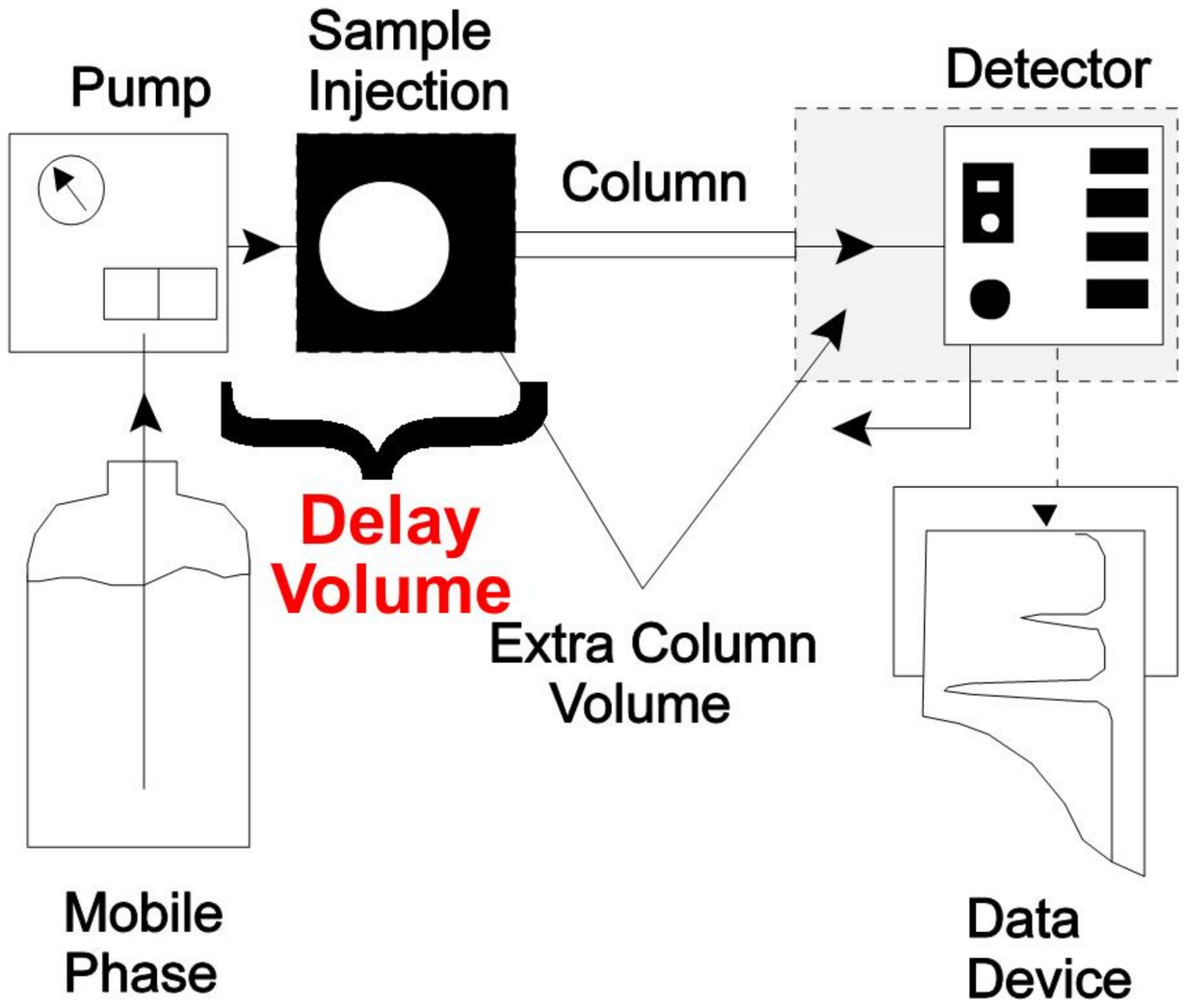
(PN 5188-6529) spiked w/ 50 µL 2 mg/mL

Thiourea in water/acetonitrile

TCC: ambient

DAD: Sig=254,4nm; Ref=Off

Gradient Separations – What is Delay Volume?

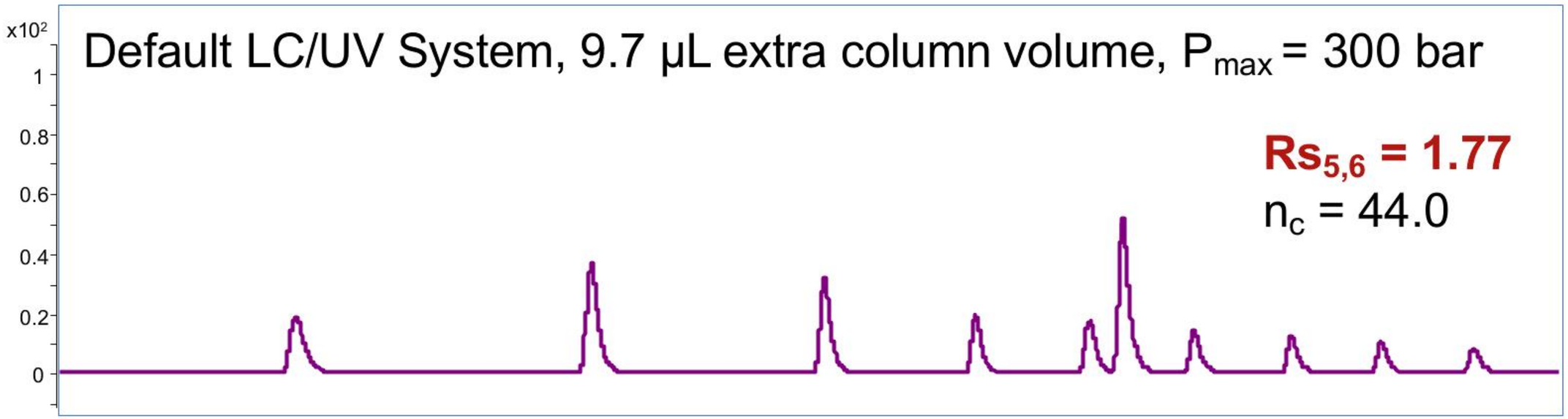


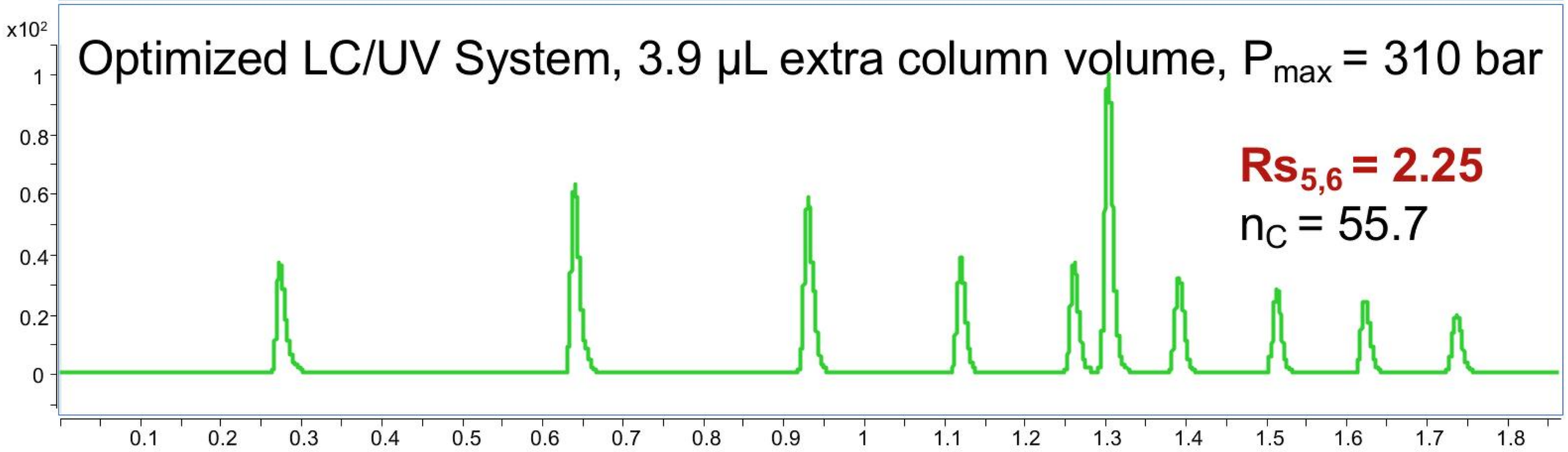
Also known as Dwell Volume

- Delay Volume = volume from formation of gradient to the column
- Behaves as isocratic hold at the beginning of gradient
- Determine Dwell Volume (see appendix)

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

Delay Volume Comparison:

1100/1200 Series Binary Pump vs. 1200 Series Binary Pump SL

Binary pump SL (pressure range up to 600 bar):

Standard delay volume configuration: 600-800µL (incl. damper and mixer)

Low delay volume configuration: 120µL (virtual damper)

Damper volume: 80-280µl

Binary pump (pressure range up to 400 bar):

Standard delay volume configuration: 600-900µL (incl. damper and mixer)

Reduced delay volume configuration: ~200µL (damper needed)

Damper volume: 180µl + 1µl per bar

Conclusions

Gradients Are a Useful Item in the Chromatography Tool Kit

- Why/When to use a gradient
  - Late eluters
  - Unknowns
  - Large molecules
- What factors can maximize gradient resolution
  - Gradient retention
  - Selectivity
  - Theoretical plates
- How to make a rugged/reproducible method
  - Don’t forget to try multiple lots – Try a method validation kit
- What do you need to know about your instrument
  - ECV
  - Dwell volume

Agilent Technical Support

800-227-9770 (US & Canada)

Options 3, 3, 2

Email: lc-column-support@agilent.com

www.agilent.com/chem

Still have questions?

Follow-up calls

Appendix

Extra Column Volume

Use 0.12 mm Tubing Instead of 0.17 mm Tubing

Inside Diameter (mm)	Length (mm)	Material	Color	Connections	Part Number	Volume (ul)
0.12	180	SS	Red	1 end pre-swaged	G1313-87304	2.0
0.12	280	SS	Red	1 end pre-swaged	01090-87610	3.2
0.12	105	SS	Red	1 end pre-swaged	01090-87611	1.2
0.12	150	SS	Red	pre-swaged	G1315-87312	1.7
0.12	105	SS	Red	Without fittings	5021-1820	1.2
0.12	150	SS	Red	Without fittings	5021-1821	1.7
0.12	280	SS	Red	Without fittings	5021-1822	3.2
0.12	400	SS	Red	Without fittings	5021-1823	4.5
0.17	180	SS	Green	1 end pre-swaged	G1313-87305	4.1
0.17	280	SS	Green	1 end pre-swaged	01090-87304	6.4
0.17	130	SS	Green	1 end pre-swaged	01090-87305	2.9
0.17	90	SS	Green	1 end pre-swaged	G1316-87300	2.0
0.17	105	SS	Green	Without fittings	5021-1816	2.4
0.17	150	SS	Green	Without fittings	5021-1817	3.4
0.17	280	SS	Green	Without fittings	5021-1818	6.4
0.17	400	SS	Green	Without fittings	5021-1819	9.1

Use lower volume *RED* tubing when possible

GREEN tubing has 2x volume of RED tubing of same length

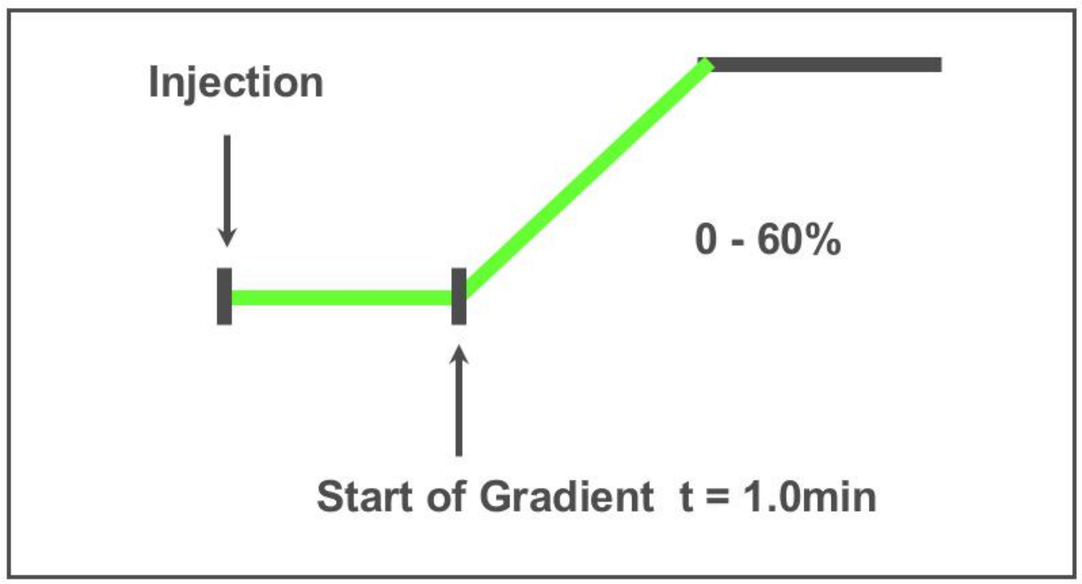
Determining the Dwell Volume of Your System

- 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
- λ 265nm
- Adjust attenuation such that both 100% A and 100% B are on scale
- Run gradient profile 0 - 100% B / 10 min at 1.0 mL / min
- Record

Correcting for Dwell Volume

1. Measure the Dwell Volume of your HPLC System
   V_D = 1.0 mL
2. Draw Effective Gradient Profile at First Flow Rate
   Calculate the time delay (imposed isocratic hold) caused by dwell volume
   $V_D = t_D \bullet F$ 1.0 mL = $t_D \bullet$ 1.0 mL / min
   where F = 1.0 mL / min for 4.6 x 150 mm column
   V_D = 1.0 mL

$t_D = F/V_D$ t_D = 1.0 mL / min / 1.0 mL
t_D = 1.0 min



To Accommodate Different Column Sizes

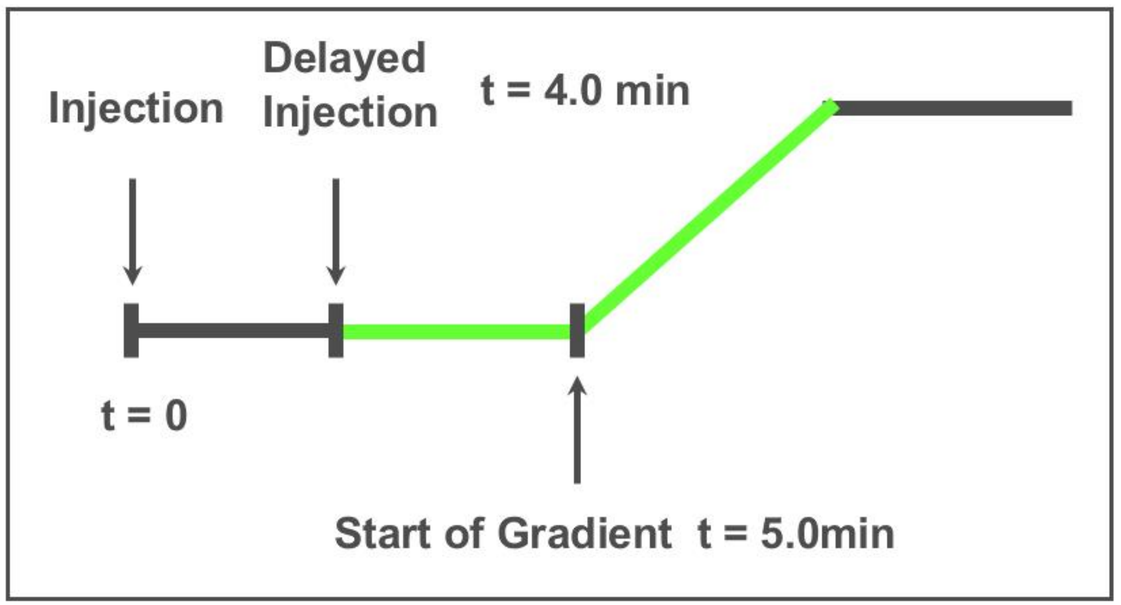
3. Draw Effective Gradient Profile at Second Flow Rate

$t_D = F / V_D$ $t_D = (0.2\ mL / min) / 1.0mL$

$t_D = 5.0\ min$

where $F = 0.2\ mL / min$ for 2.1 x 150 mm column

$V_D = 1.0\ mL$ (same for HPLC system)



Delay injection on the 2.1 x 150 mm column by 4.0 min (5.0 min - 1.0 min) so that the gradient profile is the same on both columns

Correcting for Dwell Volume

If $V_{D1} > V_{D2}$

Compensate for longer V_{D1} by adding an isocratic hold to V_{D2}, such that Hold + V_{D2} = V_{D1}

If $V_{D1} < V_{D2}$

Delay injection, such that V_{D2} - delay = V_{D1}

(very difficult to accomplish in practice)