

# Gradient Design and Development

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12 March 2020

# Gradient Design and Development

Introduction:  
What and  
Why

System  
Configuration:  
Measure  
Delay Volume

Development:  
Scouting  
Gradient to  
Analytical  
Method

Transferring  
Gradients to  
Different  
Columns

# Gradient Design and Development

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What and  
Why

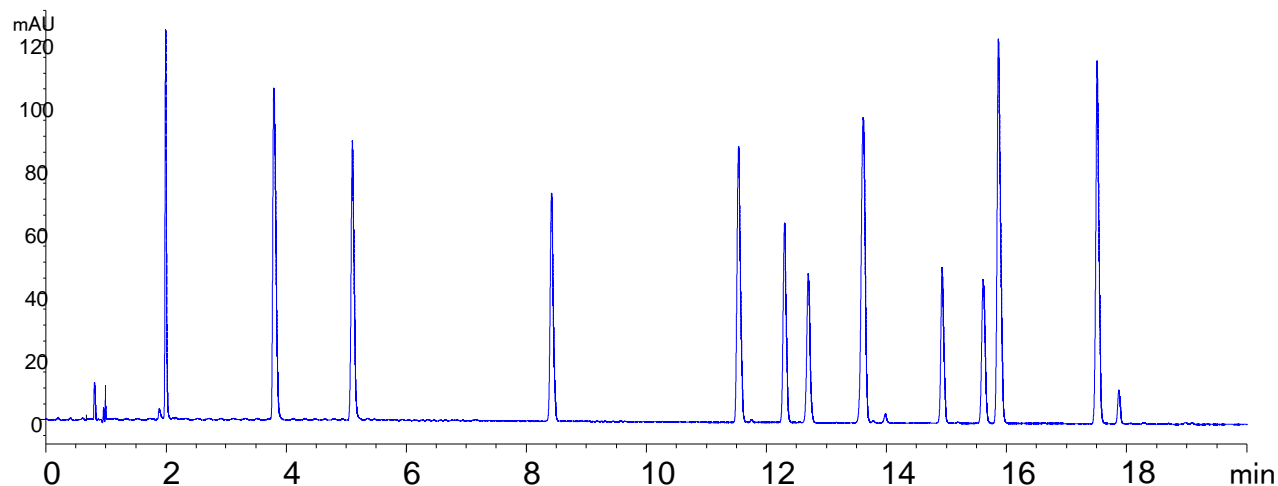
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# What is Gradient Elution?

A separation that occurs by continuously increasing the solvent strength of the mobile phase



1. Hydroquinone
2. Resorcinol
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-naphthol

**Column: Poroshell 120 EC-C18, 4.6 x 150 mm, 2.7  $\mu$ m**

**Mobile phase:** Solvent A: Water with 0.1% formic acid, Solvent B: Acetonitrile

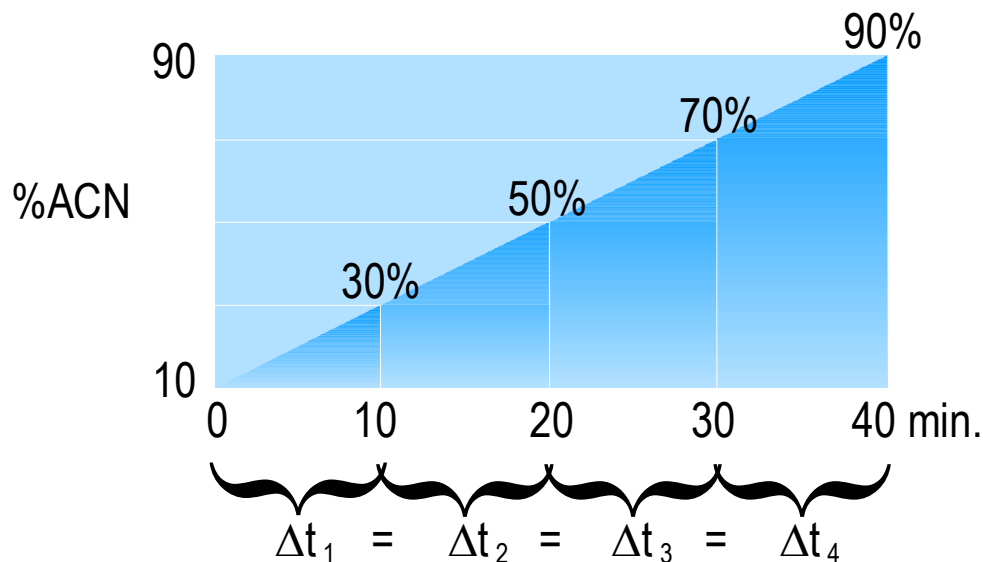
**Gradient: 0–3 min 5% B, 5%-60% B over 22 minutes**

Agilent 1200 SL controlled temperature at 25 °C, 2  $\mu$ l flow cell

# Gradient Elution for Reversed-Phase HPLC

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

Linear solvent strength gradient = % per min is a constant



$$\Delta\phi = 80\%$$

$$t_G = 40 \text{ min.}$$

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

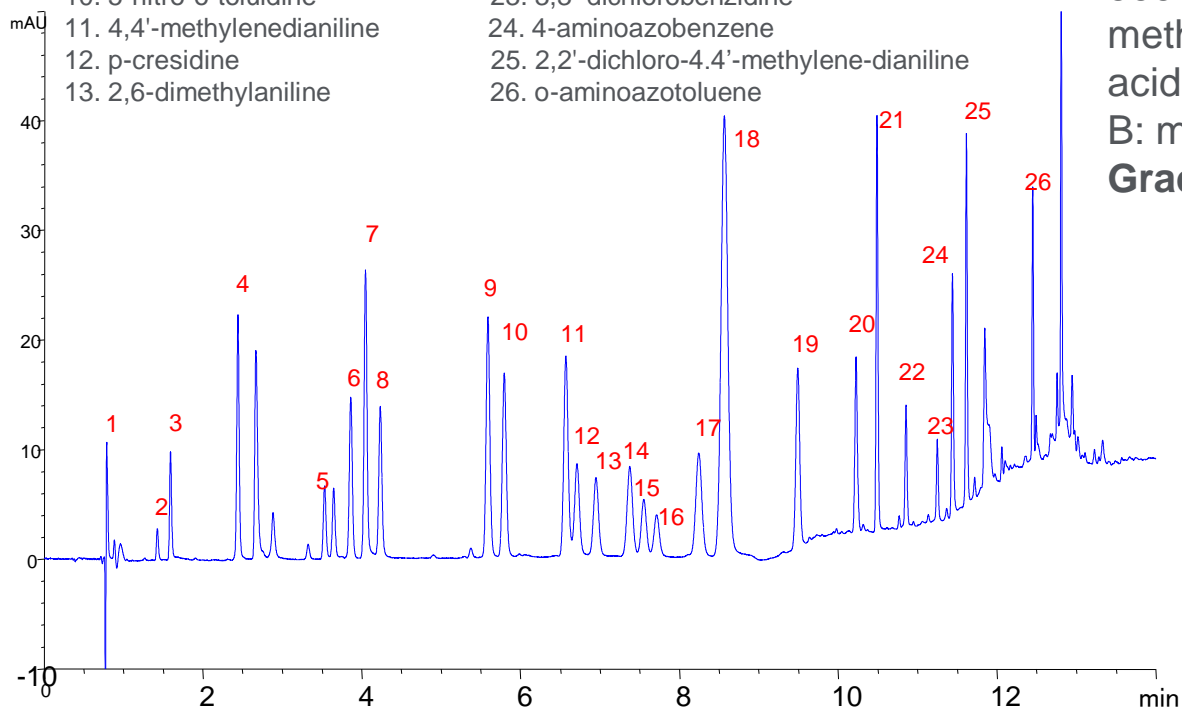
For every 20% change in ACN,  $\Delta t$  is 10 min.

# Gradient Elution Analysis of a Complex Sample

## EU banned Azo colorants in textiles

Sample:

- |                                 |  |
|---------------------------------|--|
| 1. 1,4-phenylenediamine         | 14. 2,4-dimethylaniline                    |
| 2. 4-methoxy-m-phenylenediamine | 15. o-dianisidine                          |
| 3. 4-methyl-m-phenylenediamine  | 16. 4,4'-bi-o-toluidine                    |
| 4. Aniline                      | 17. 4,4'-thiodianiline                     |
| 5. benzidine                    | 18. 2-naphthylamine                        |
| 6. o-anisidine                  | 19. 4-chloro-o-toluidine                   |
| 7. 4,4'-oxydianiline            | 20. 2,4,5-trimethylaniline                 |
| 8. o-toluidine                  | 21. 4,4'-methylenedi-o-toluidine           |
| 9. 4-chloroaniline              | 22. biphenyl-4-ylamine                     |
| 10. 5-nitro-o-toluidine         | 23. 3,3'-dichlorobenzidine                 |
| 11. 4,4'-methylenedianiline     | 24. 4-aminoazobenzene                      |
| 12. p-cresidine                 | 25. 2,2'-dichloro-4,4'-methylene-dianiline |
| 13. 2,6-dimethylaniline         | 26. o-aminoazotoluene                      |



**Column:** Poroshell 120 EC-C18,  
3.0 x 150 mm, 2.7  $\mu\text{m}$  (p/n: 693975-302)

**Column temperature:** 40  $^{\circ}\text{C}$

**Flow rate:** 0.8 mL /min

**Mobile phase:**

A: 0.575 g Mono ammonium phosphate,  
0.7 g Disodium hydrogen phosphate in  
900 mL water added with 100 mL  
methanol and adjusted using phosphoric  
acid to pH 6.9

B: methanol

**Gradient:** See table

Time (min)	B%
0	14
4.5	29
8	29
9	50
10.5	65
12	90
14	100
15	14

# Three Major Reasons to Choose Gradient Elution

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

2. To separate mixtures having a large number of components

3. Consistent, better peak shape throughout the run

# Gradient Separation Reduces Analysis Time and Improves Resolution – Even with the Shortest Columns

**Columns: Poroshell 120 EC-C18, 3.0 x 30 mm, 2.7  $\mu$ m**

Mobile Phase: A: 10 mM ammonium acetate, pH= 6.8, B: Acetonitrile. Temperature: 30 °C

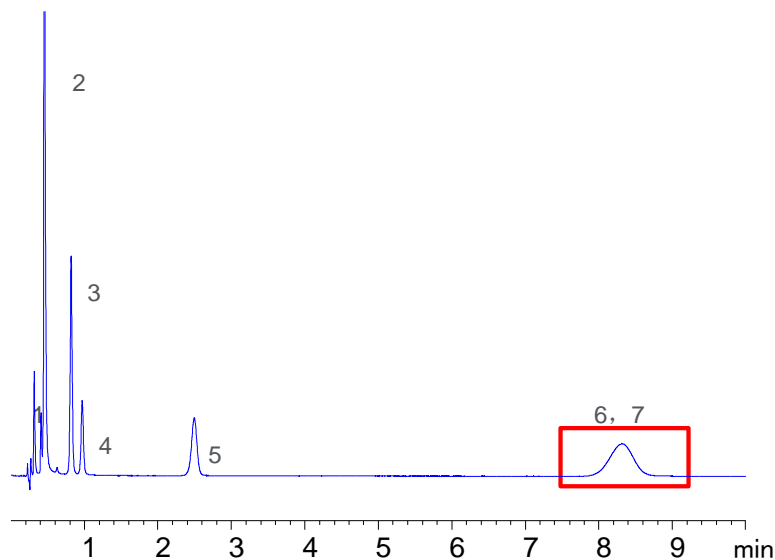
Mobile Phase: A: 20 mM Phosphate buffer, B: Acetonitrile. Temperature: 30°C. Detection: UV 245 nm.

Sample: Acetaminophen impurities.

## Isocratic elution:

90% aqueous/10% ACN

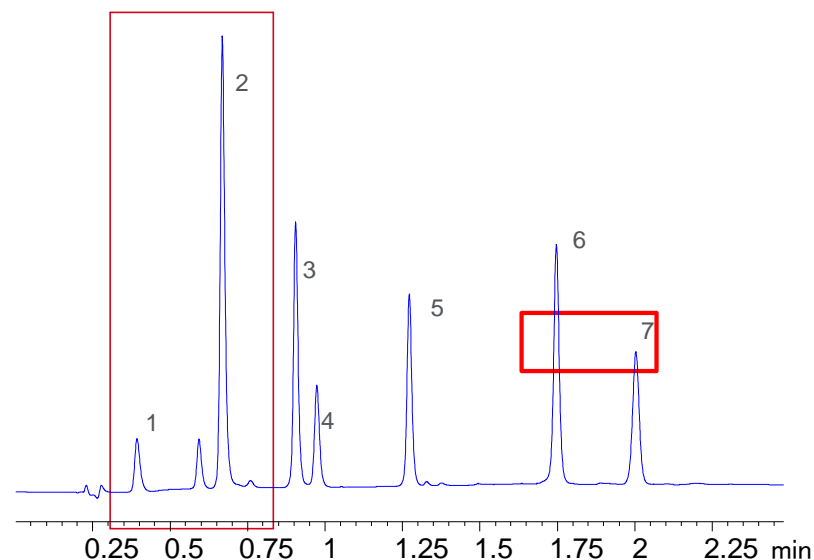
Flow Rate: 0.65 mL/min



## Gradient elution:

5–50% ACN in 2 min.

Flow rate: 0.65 mL/min



- The gradient analysis is much faster using the same 30 mm Poroshell 120 column.
- And resolution is dramatically improved for all peak pairs!



# Gradient Separation is Faster than Isocratic

## Separation of acetaminophen impurities on Poroshell 120 EC-C18

Isocratic elution:  
85% aqueous/15% ACN

Gradient elution:  
5–50% ACN in 5 min

**Column:** Poroshell 120 EC-C18, 4.6 x 50 mm, 2.7  $\mu\text{m}$

**Mobile Phase:**

A: 10 mM ammonium acetate, pH 6.8

B: Acetonitrile

**Flow Rate:** 1.5 mL/min

**Temperature:** 30  $^{\circ}\text{C}$

**Sample:**

1.4-aminophenol

2.Acetaminophen

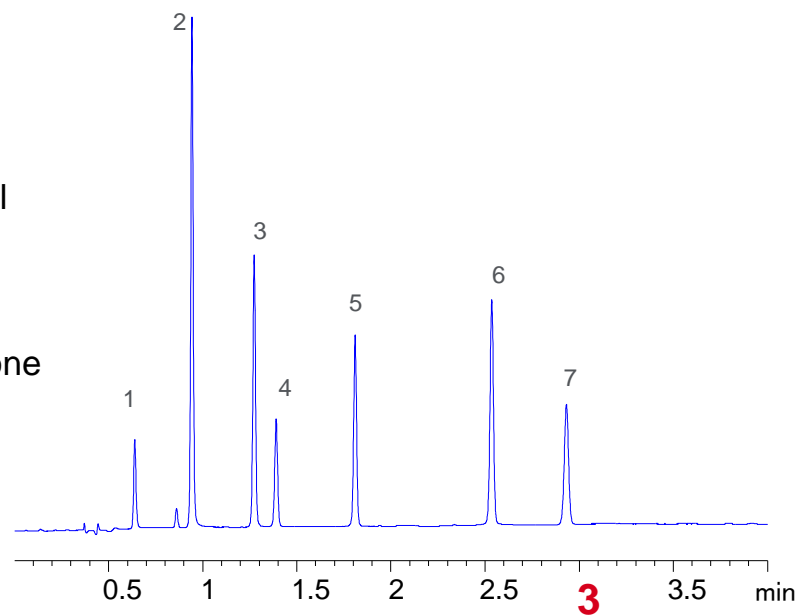
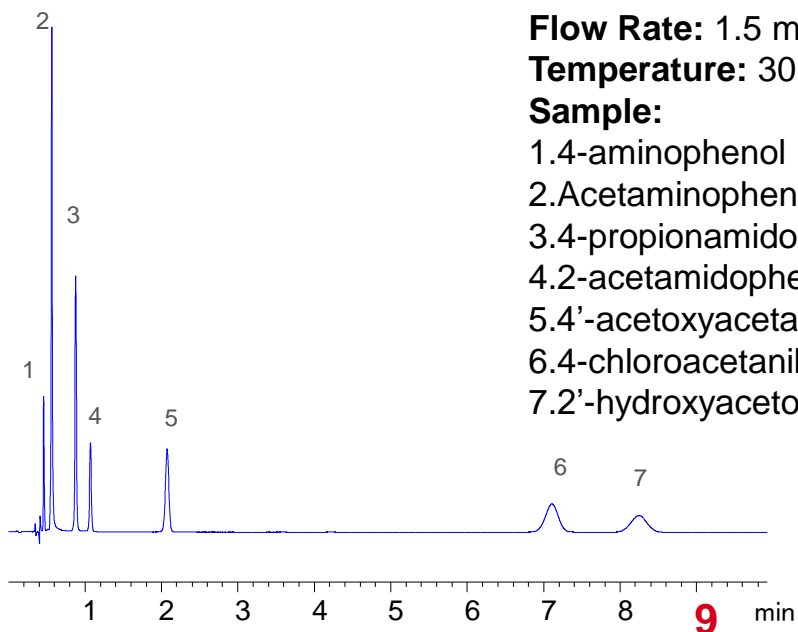
3.4-propionamidophenol

4.2-acetamidophenol

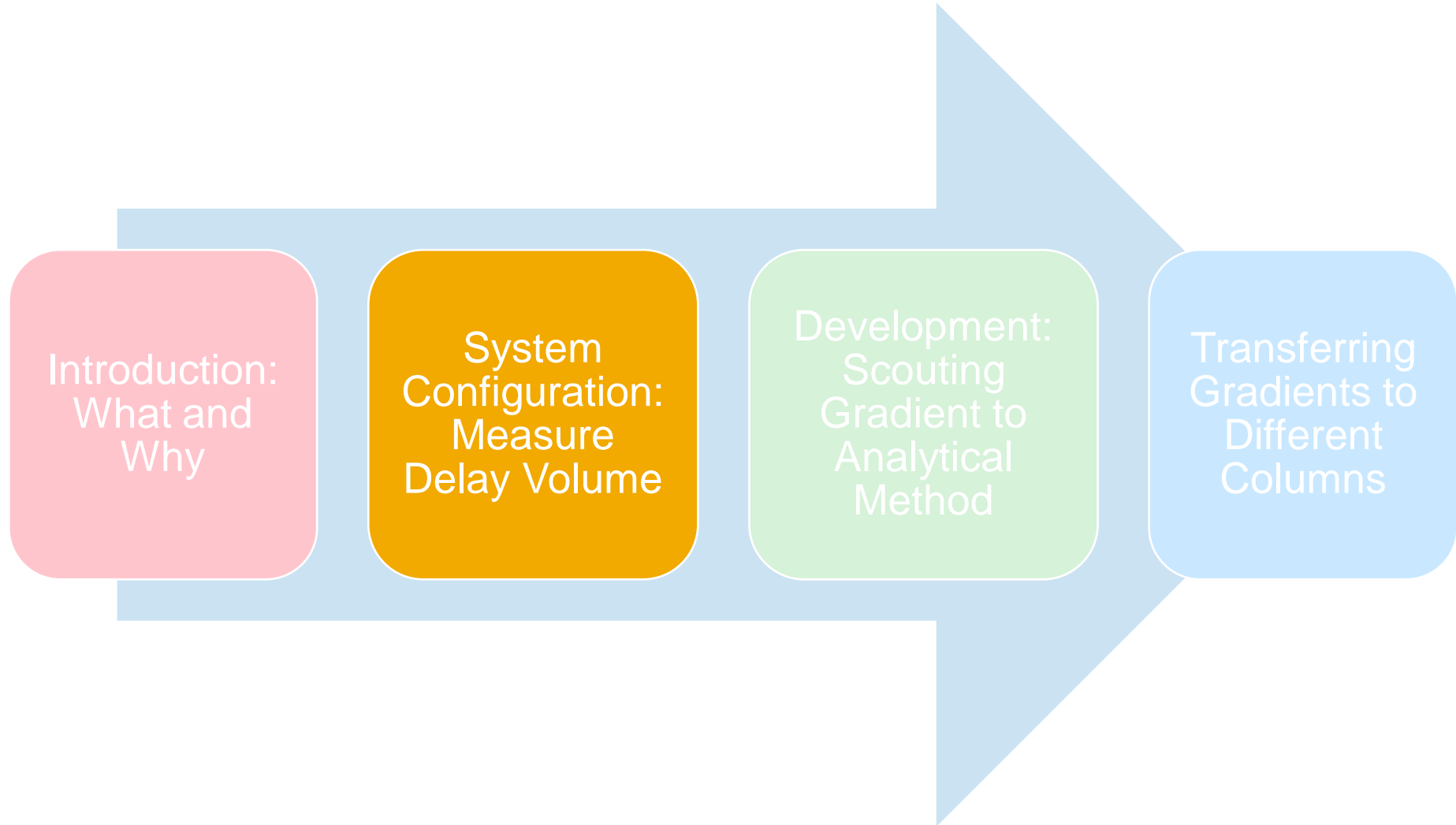
5.4'-acetoxyacetanilide

6.4-chloroacetanilide

7.2'-hydroxyacetophenone

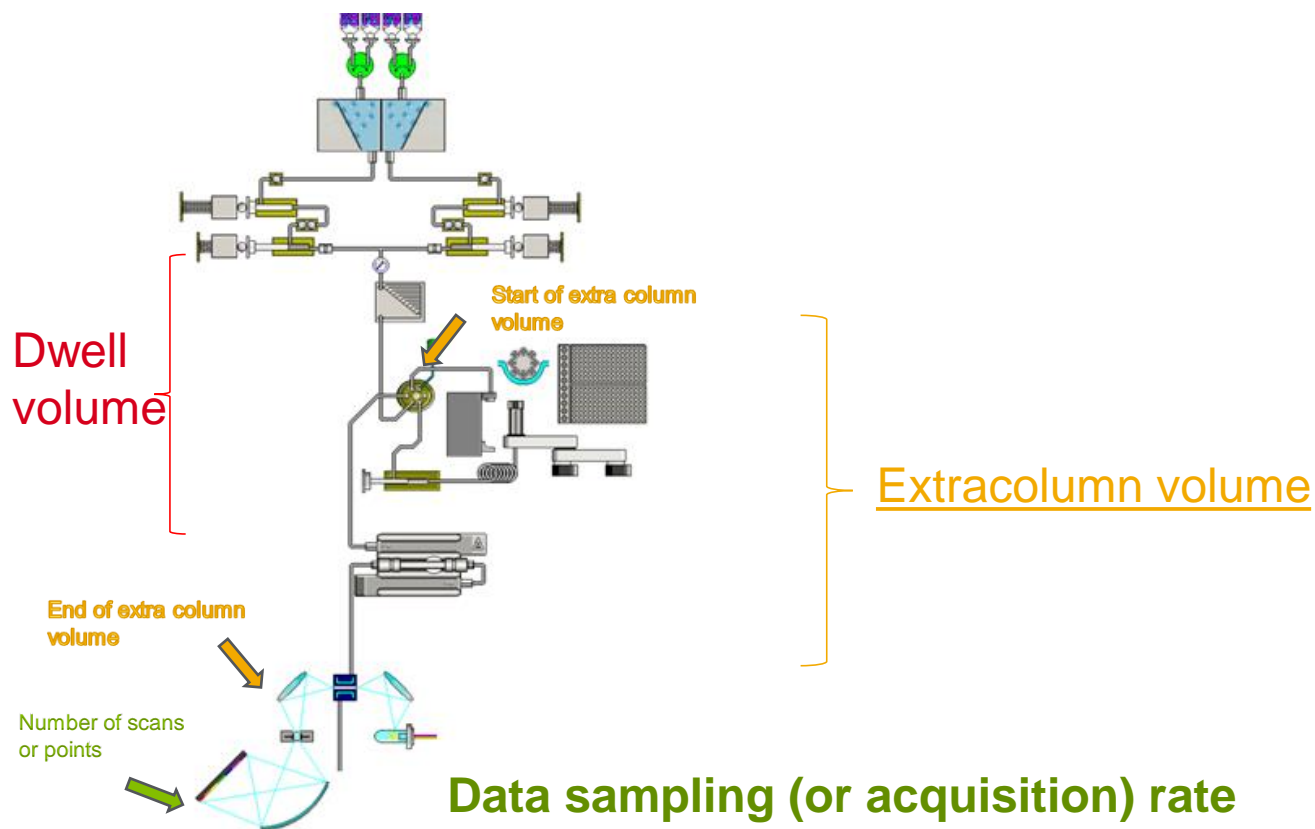


# Gradient Design and Development



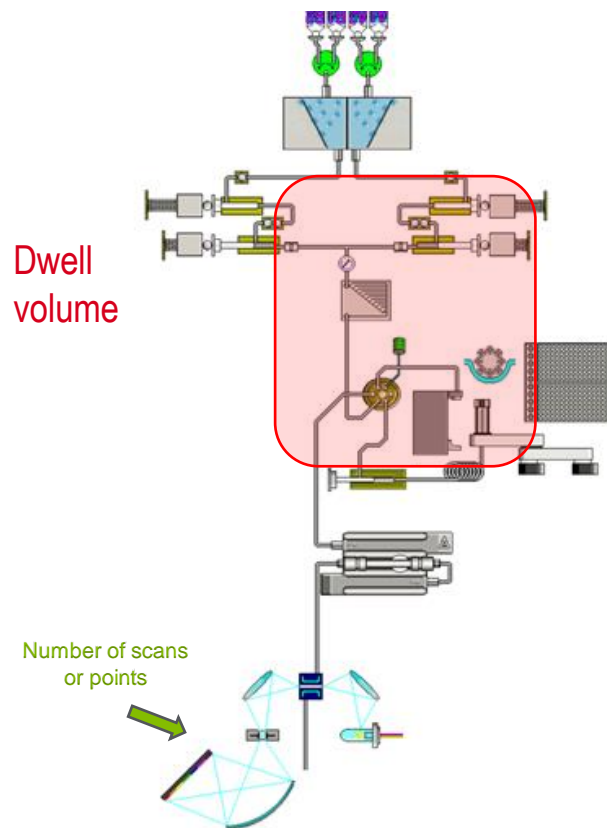
# Gradient Separations

## Instrument Impact on Column Performance



# Instrument Impact on Column Performance

## Dwell volume

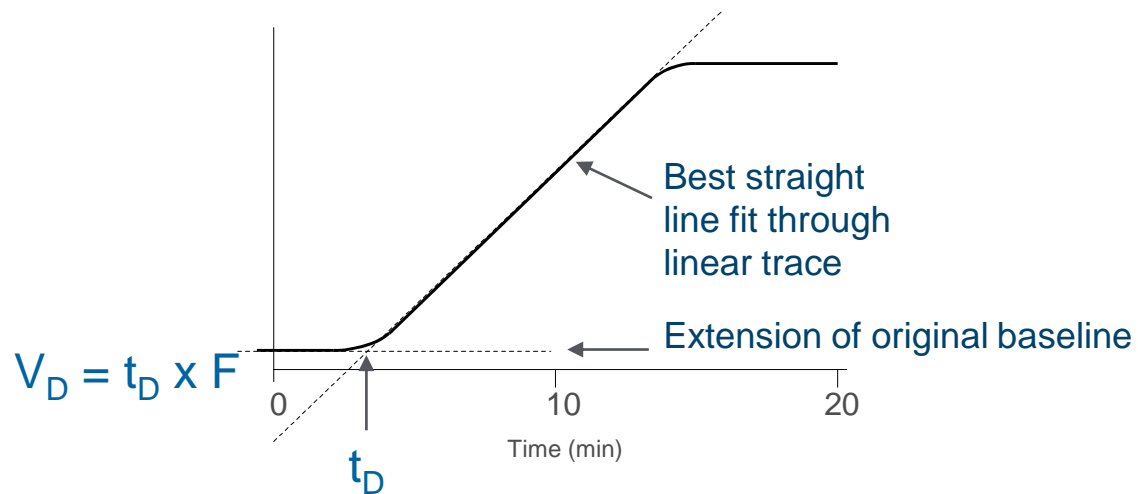


- Dwell volume = volume from formation of gradient to the column

# Determining the Dwell Volume of Your System

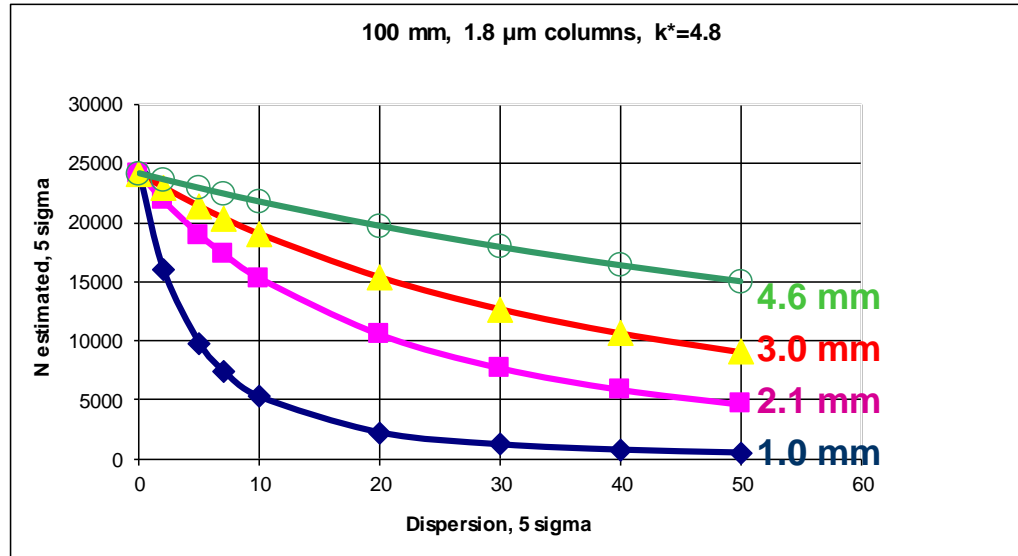
- Look it up in the LC manual or follow the procedure below
- Replace column with short piece of HPLC stainless steel tubing
- Prepare mobile phase components
  - A. Water - UV-transparent
  - B. Water with 0.2% acetone - UV-absorbing
- Monitor at 265 nm
- Run gradient profile 0–100% B/10 min at 1.0 mL/min
- Record
- Expected dwell volume in UHPLCs –  $\mu\text{L}$  range!

# Measuring Dwell Volume ( $V_D$ )



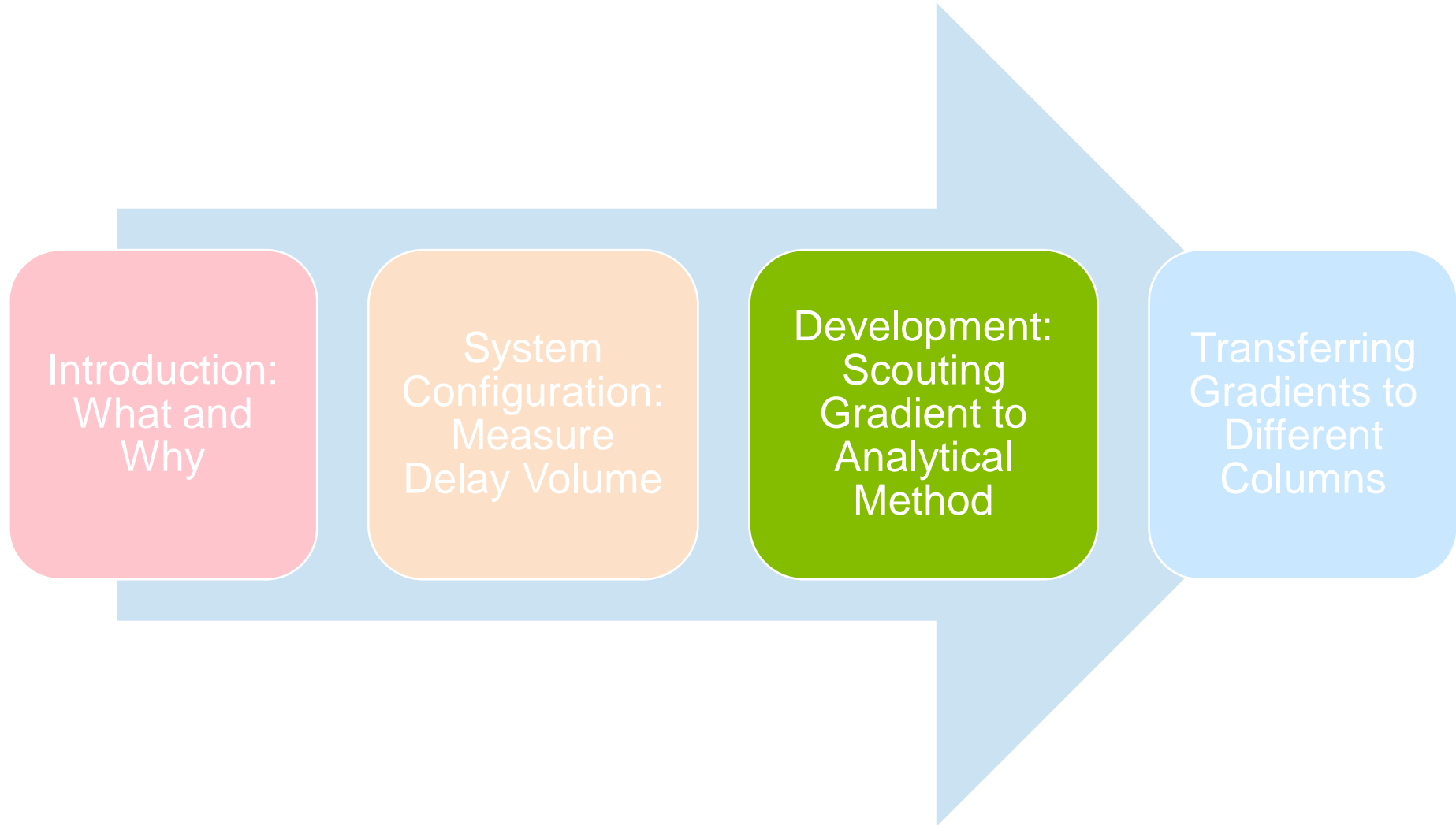
- Intersection of the two lines identifies dwell time ( $t_D$ )
- Dwell volume is equal to product of the flow rate and the dwell time.

# Efficiency Yield vs. Dispersion in LC Systems



Use the largest columns suitable for the application requirements -- they are less affected by extracolumn contributions. At the same time, consider solvent consumption and detector (ELSD, MS, etc.) compatibility.

# Gradient Design and Development





# Starting Point Scouting Gradient

A good starting point for work is a scouting gradient.

The conditions recommended by John Dolan are 5–95% acetonitrile, low pH, and are dependent on the column length.

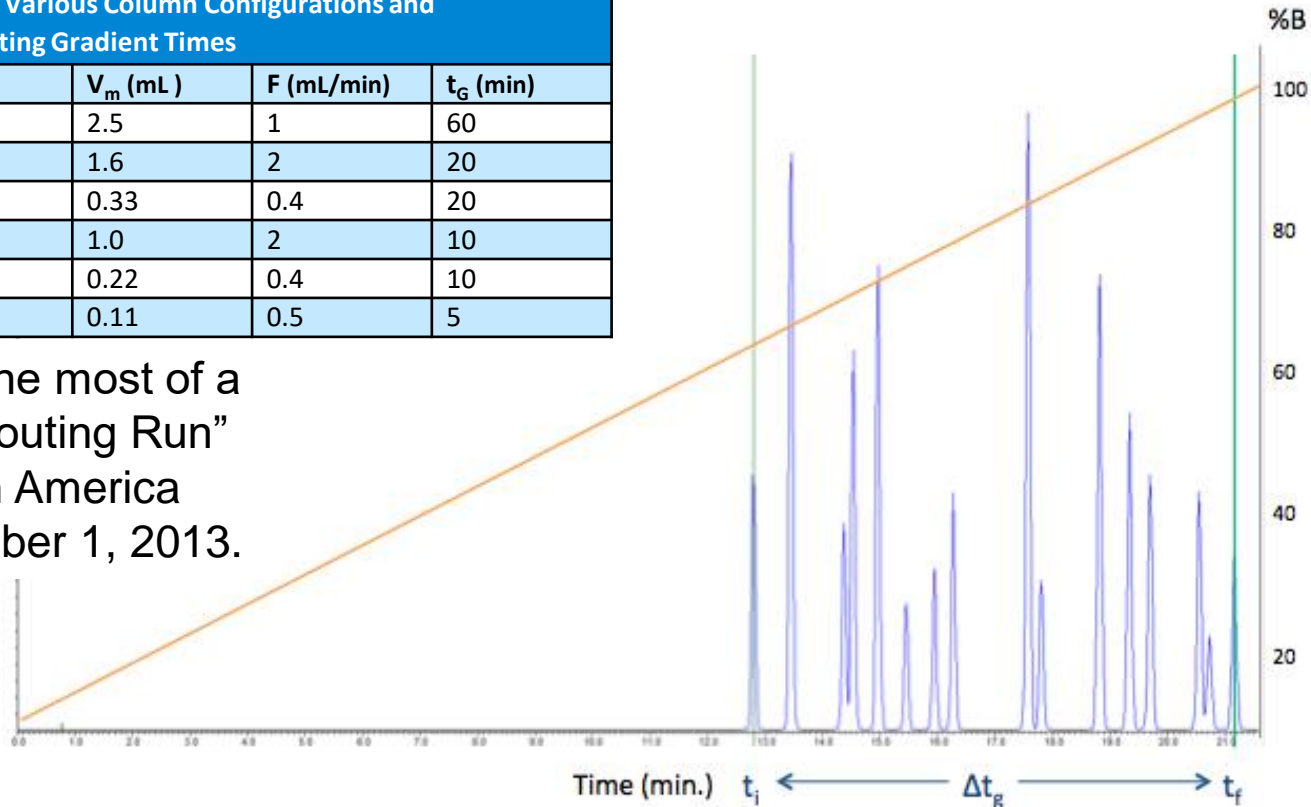
Where 10 cm columns are chosen, use a 10 minute gradient.

This example shows a 150 mm column.

**Column Volumes for Various Column Configurations and Recommended Scouting Gradient Times**

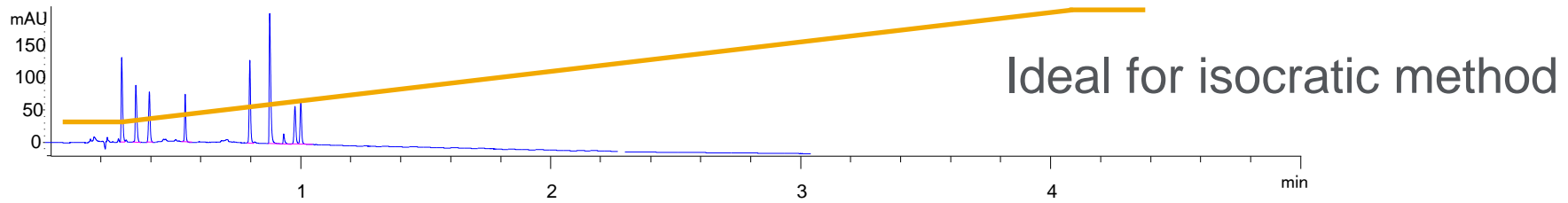
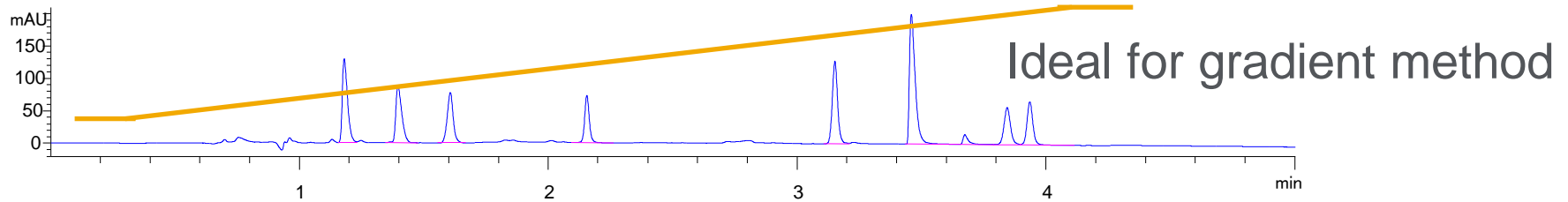
L (mm)	d <sub>c</sub> (mm)	V <sub>m</sub> (mL)	F (mL/min)	t <sub>G</sub> (min)
250	4.6	2.5	1	60
150	4.6	1.6	2	20
150	2.1	0.33	0.4	20
100	4.6	1.0	2	10
100	2.1	0.22	0.4	10
50	2.1	0.11	0.5	5

“Making the most of a Gradient Scouting Run”  
 LCGC North America  
 Vol. 31, Number 1, 2013.



# Gradients are Critical Tools for Faster Methods

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



# Step 1: Choose, Shorter Efficient Column

## Perform Gradient Scouting from 5%B-100% in 15 min

Column: InfinityLab Poroshell 120 EC-C18,  
4.6 x 100 mm, 2.7  $\mu\text{m}$

Gradient time: 15 min

Mobile phase:

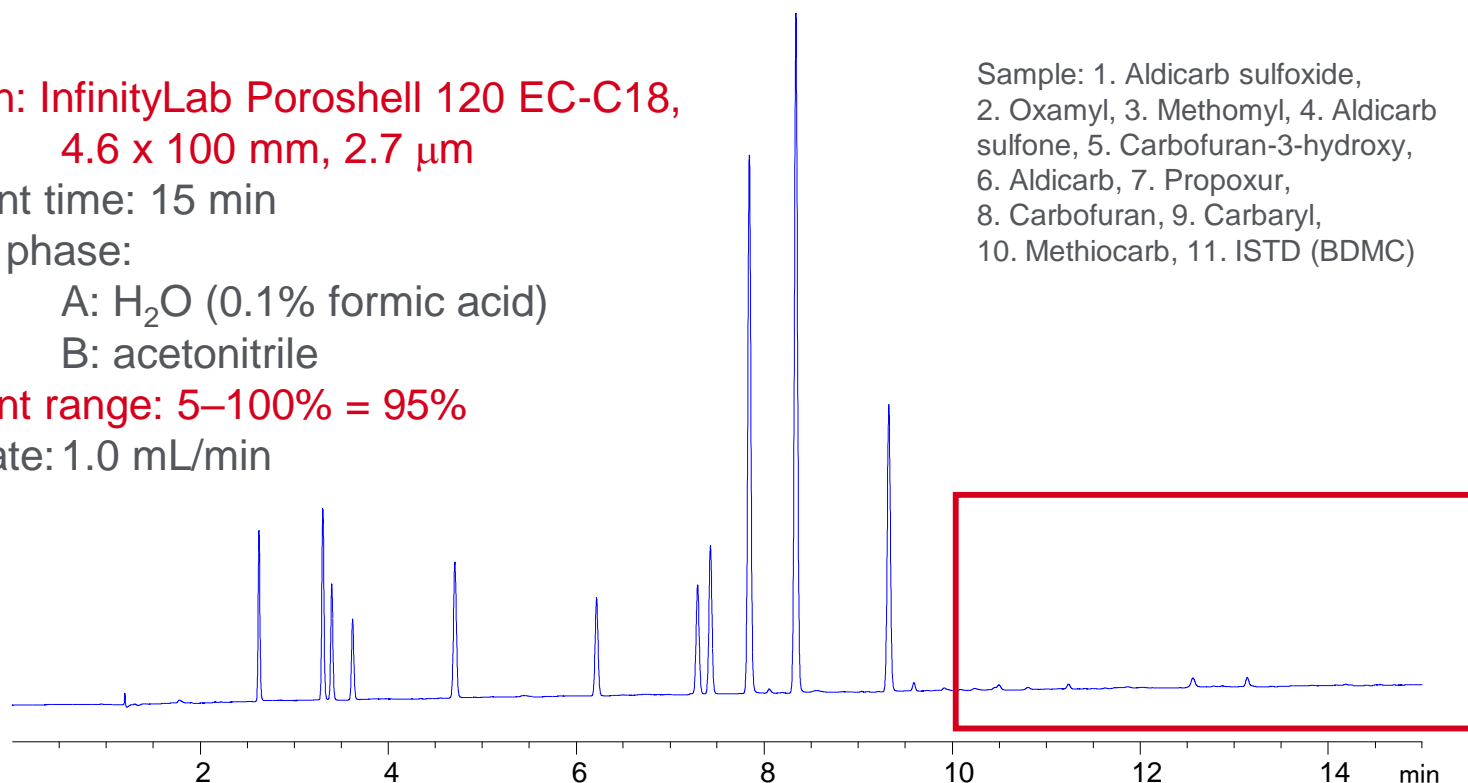
A: H<sub>2</sub>O (0.1% formic acid)

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 to Minimize Time

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

Column: InfinityLab Poroshell 120 EC-C18,  
4.6 x 100 mm, 2.7  $\mu\text{m}$

Gradient Time: 10 min

Mobile phase:

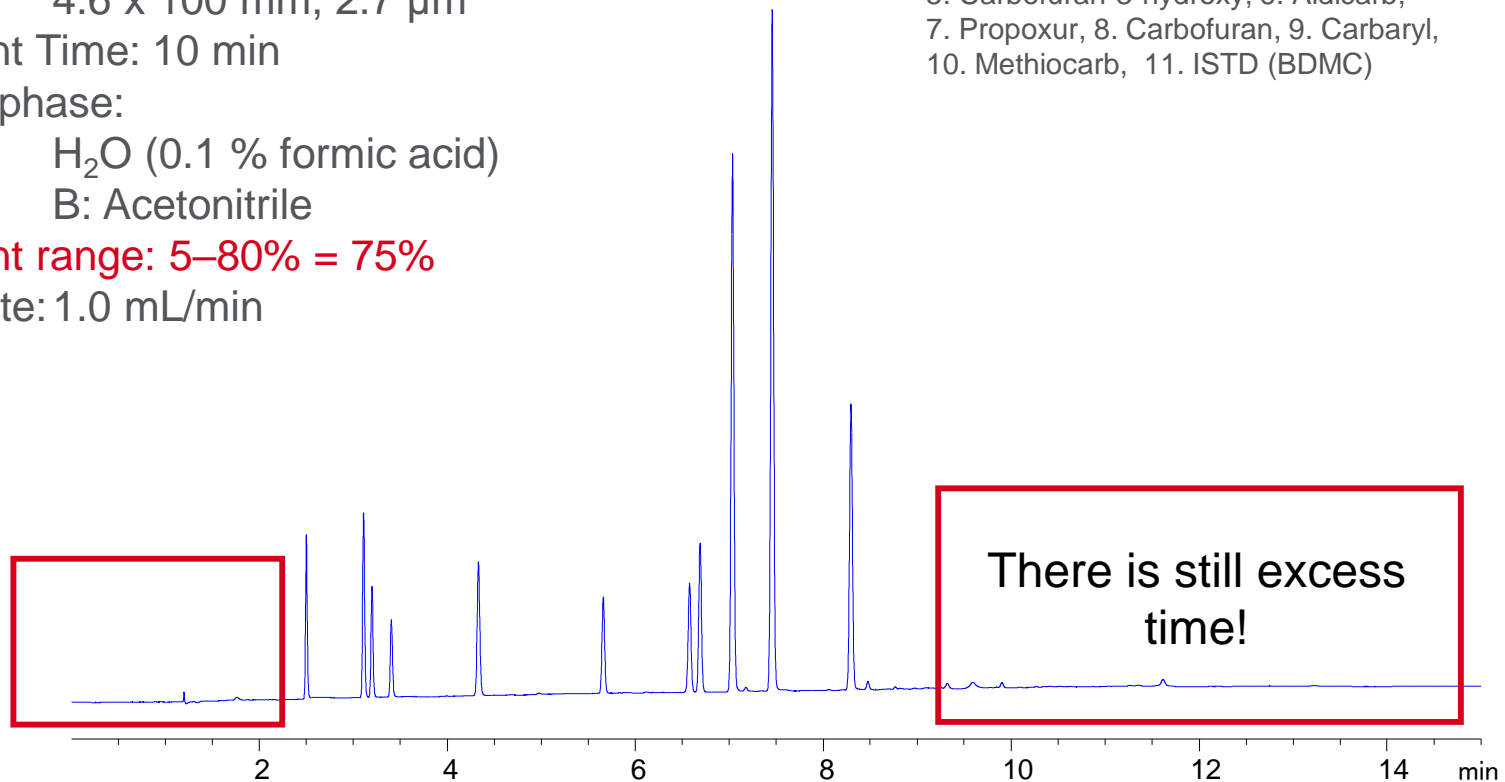
H<sub>2</sub>O (0.1 % formic acid)

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: InfinityLab Poroshell 120 EC-C18,  
4.6 x 100 mm, 2.7  $\mu$ m

**Gradient: 15 – 80%**

**B = 65% in 5 minutes**

Mobile phase:

H<sub>2</sub>O (0.1 % formic acid)

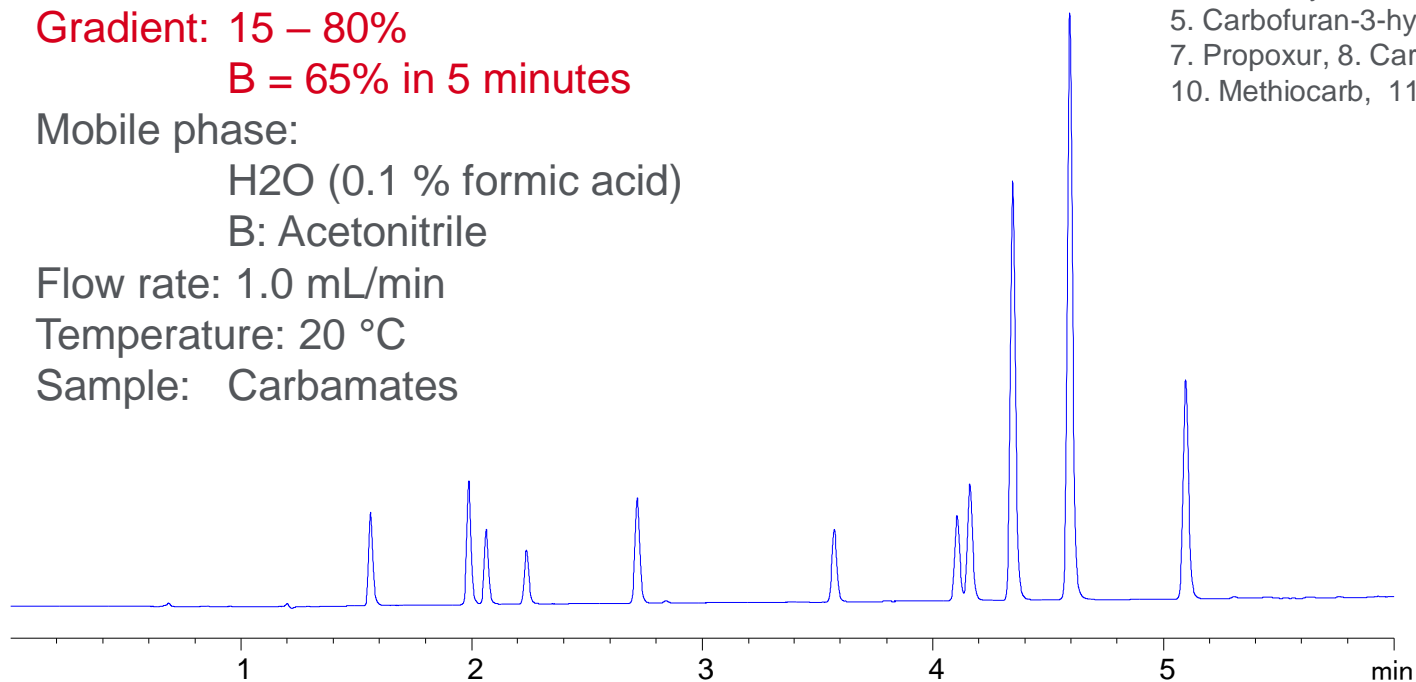
B: Acetonitrile

Flow rate: 1.0 mL/min

Temperature: 20 °C

Sample: Carbamates

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



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

# Gradient Scouting Works for Any Sample

## Gradient from 5%–100% in 10 min for acetaminophen

**Column: Poroshell 120 EC-C18, 4.6 x 50 mm 2.7  $\mu$ m**

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

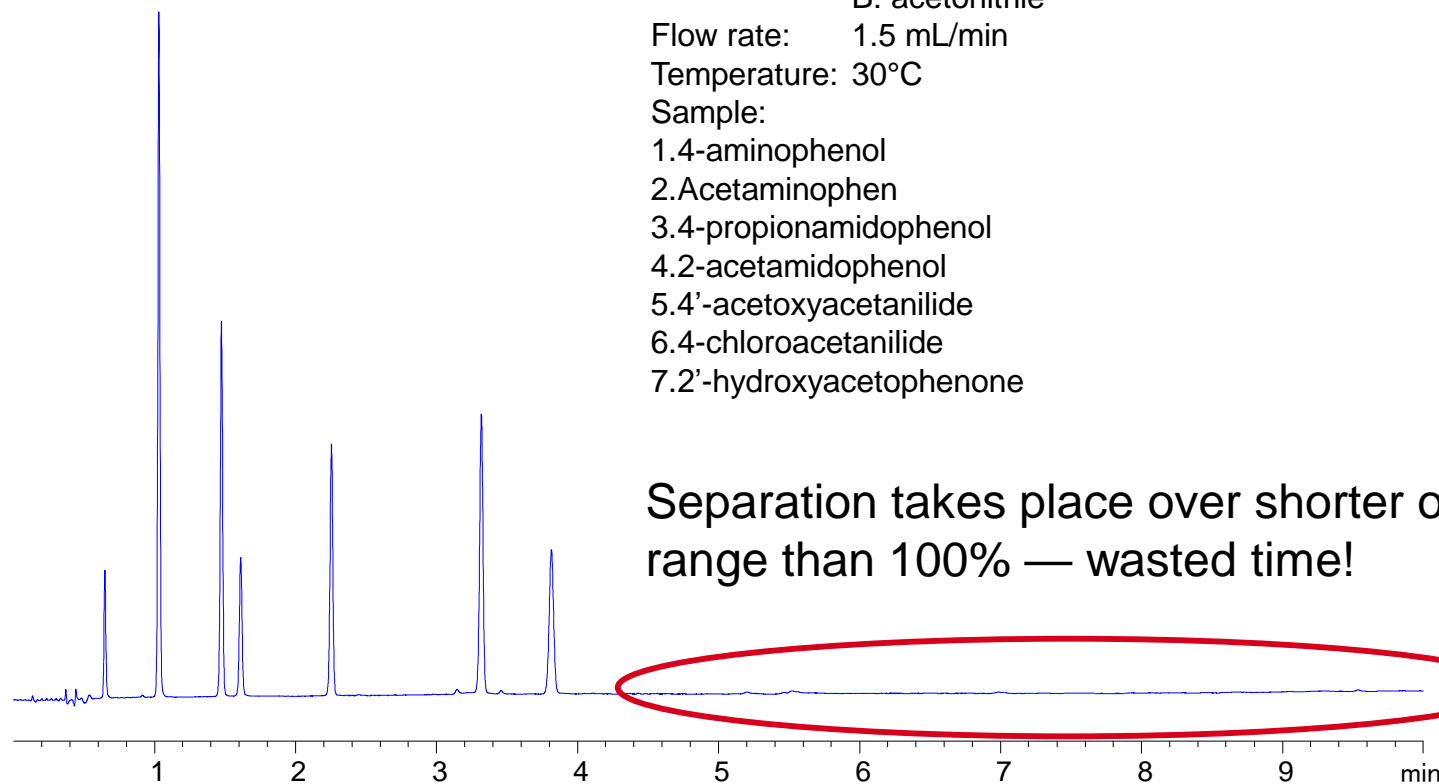
3.4-propionamidophenol

4.2-acetamidophenol

5.4'-acetoxyacetanilide

6.4-chloroacetanilide

7.2'-hydroxyacetophenone



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

**Column: Poroshell 120 EC-C18, 4.6 x 50 mm, 2.7  $\mu$ m**

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

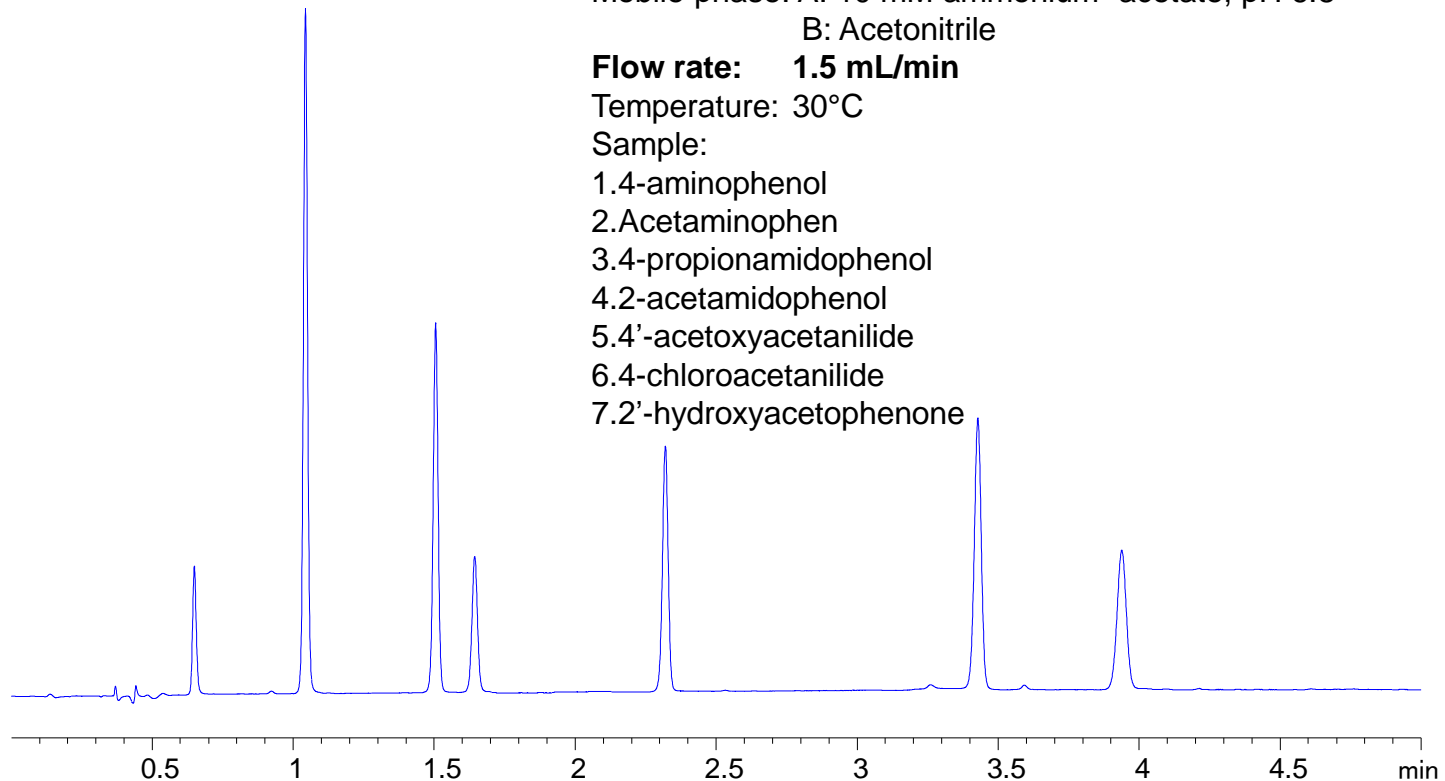
3.4-propionamidophenol

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, 2.7 $\mu$ m**

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

B: Acetonitrile

**Flow rate: 2.0 mL/min**

Temperature: 30 °C

Sample:

1.4-aminophenol

2.Acetaminophen

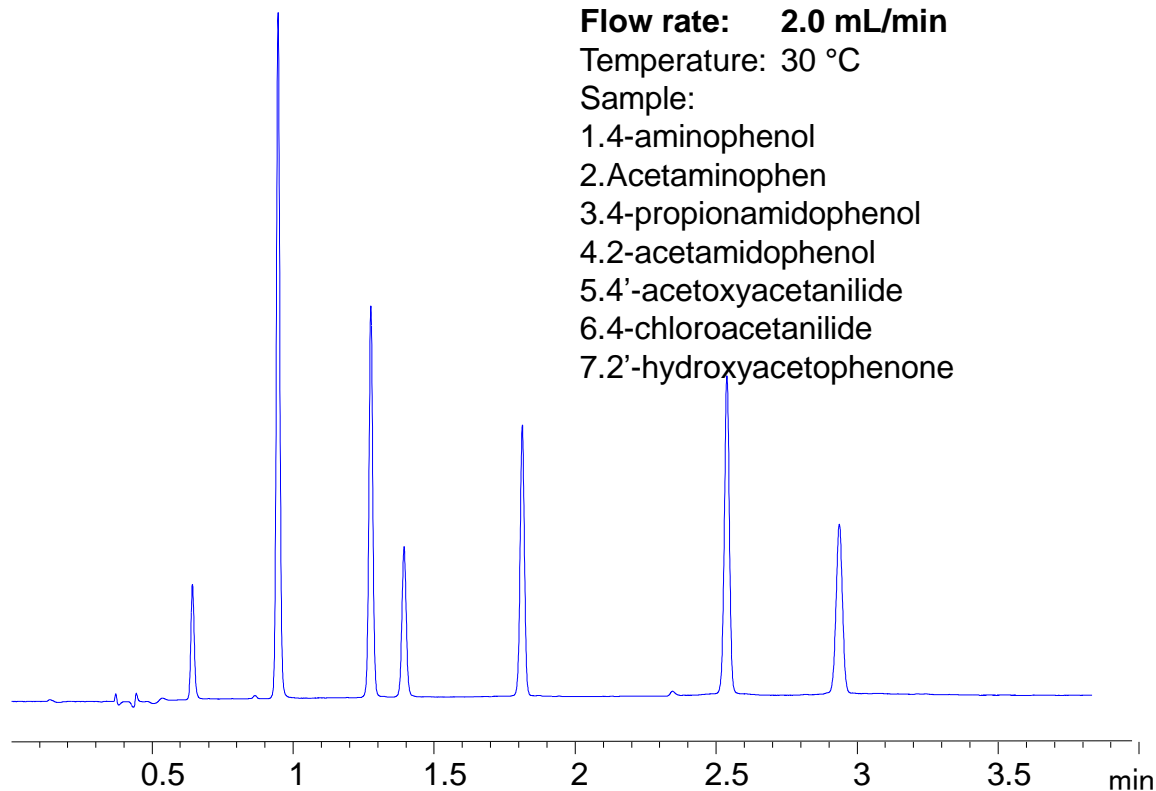
3.4-propionamidophenol

4.2-acetamidophenol

5.4'-acetoxyacetanilide

6.4-chloroacetanilide

7.2'-hydroxyacetophenone





# Gradient Design and Development

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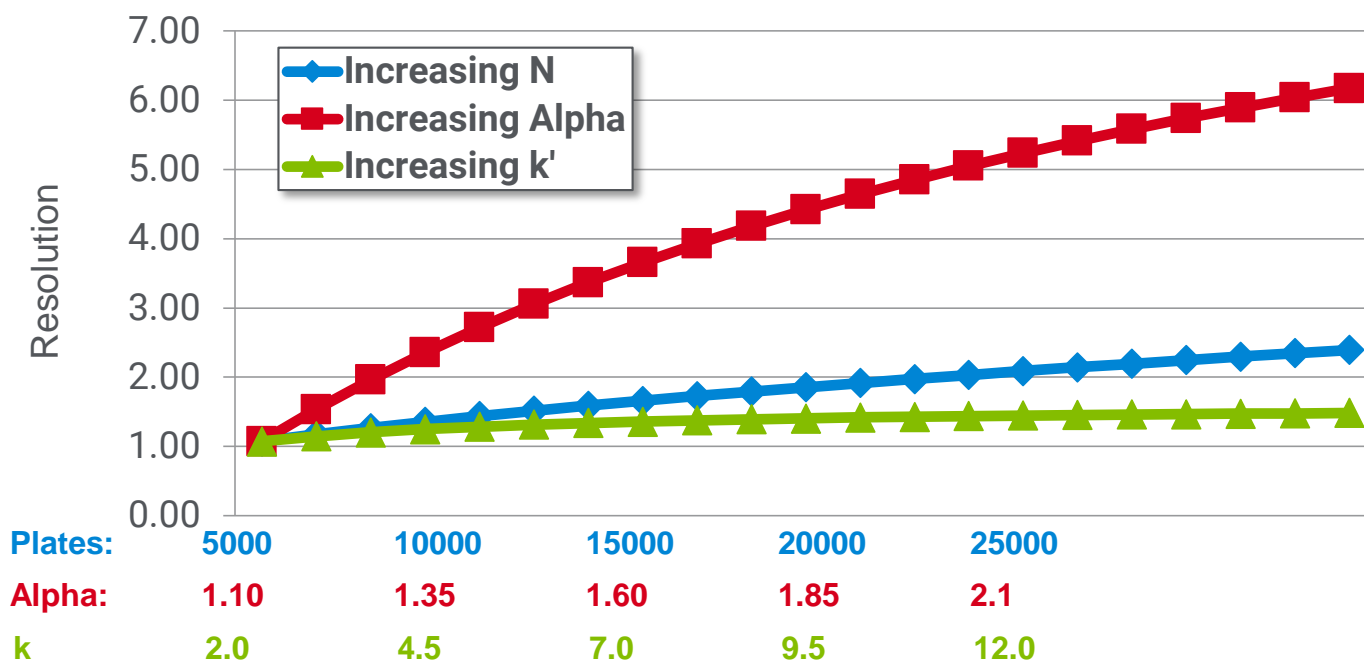
Development:  
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Columns

$$R_s = \left(\frac{1}{4}\right) N^{0.5} \left(\frac{\alpha - 1}{\alpha}\right) \left(\frac{k}{1 + k}\right)$$

Resolution    Efficiency    Selectivity    Retention

## Factors that Affect Isocratic Resolution



### Selectivity impacts resolution the most

- Change bonded phase
  - Change mobile phase
- } Typical Analytical Method Development Parameters

# Resolution Relationship for Gradient Elution

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

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

$$k^* = \frac{87 t_g F}{S (\Delta\%B) V_m}$$

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

# Maintaining $k^*$

To keep relative peak position unchanged while changing analysis parameters

Any decrease in:

- Column length



Can be offset by a proportional:

- Decrease in  $t_G$  or  $F$
- Increase in  $\Delta\%B$

- Column volume (i.d.)



- Decrease in  $t_G$  or  $F$
- Increase in  $\Delta\%B$

- $\Delta\%B$  (same column)



- Decrease in  $t_G$  or  $F$

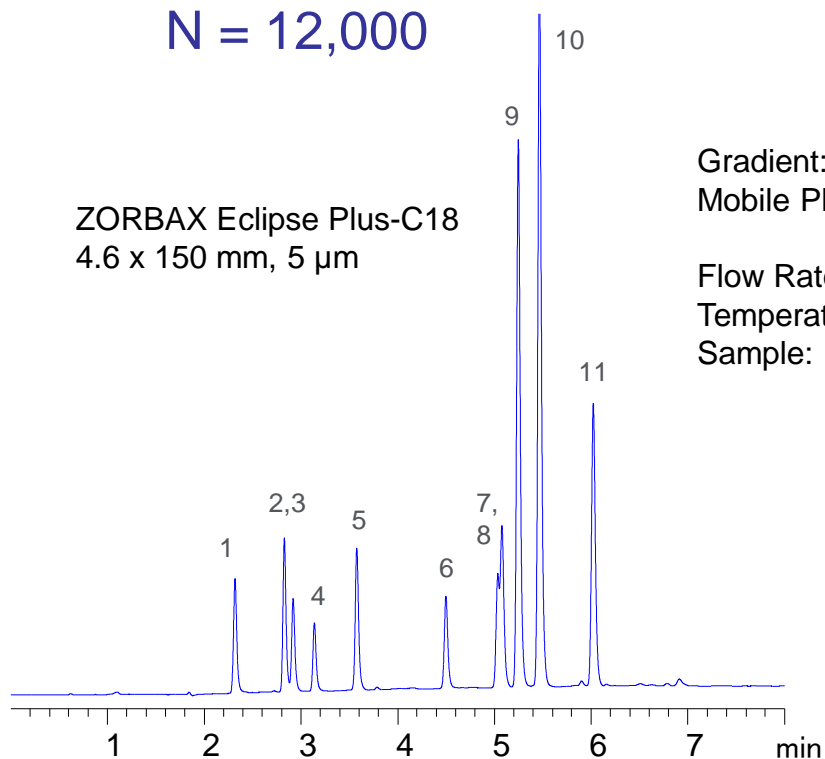
$$k^* \propto \frac{t_G \cdot F}{S \cdot \Delta\Phi \cdot V_m}$$

# A Shorter Column (smaller $V_m$ )

Reduce run-time and improve resolution while maintaining constant N

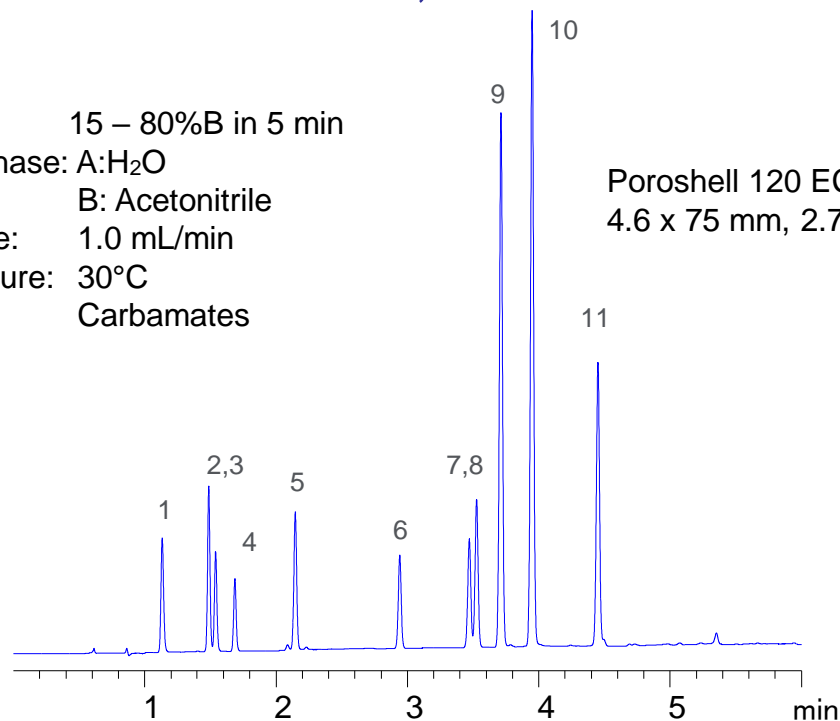
4.6 x 150 mm, 5  $\mu$ m  
N = 12,000

ZORBAX Eclipse Plus-C18  
4.6 x 150 mm, 5  $\mu$ m



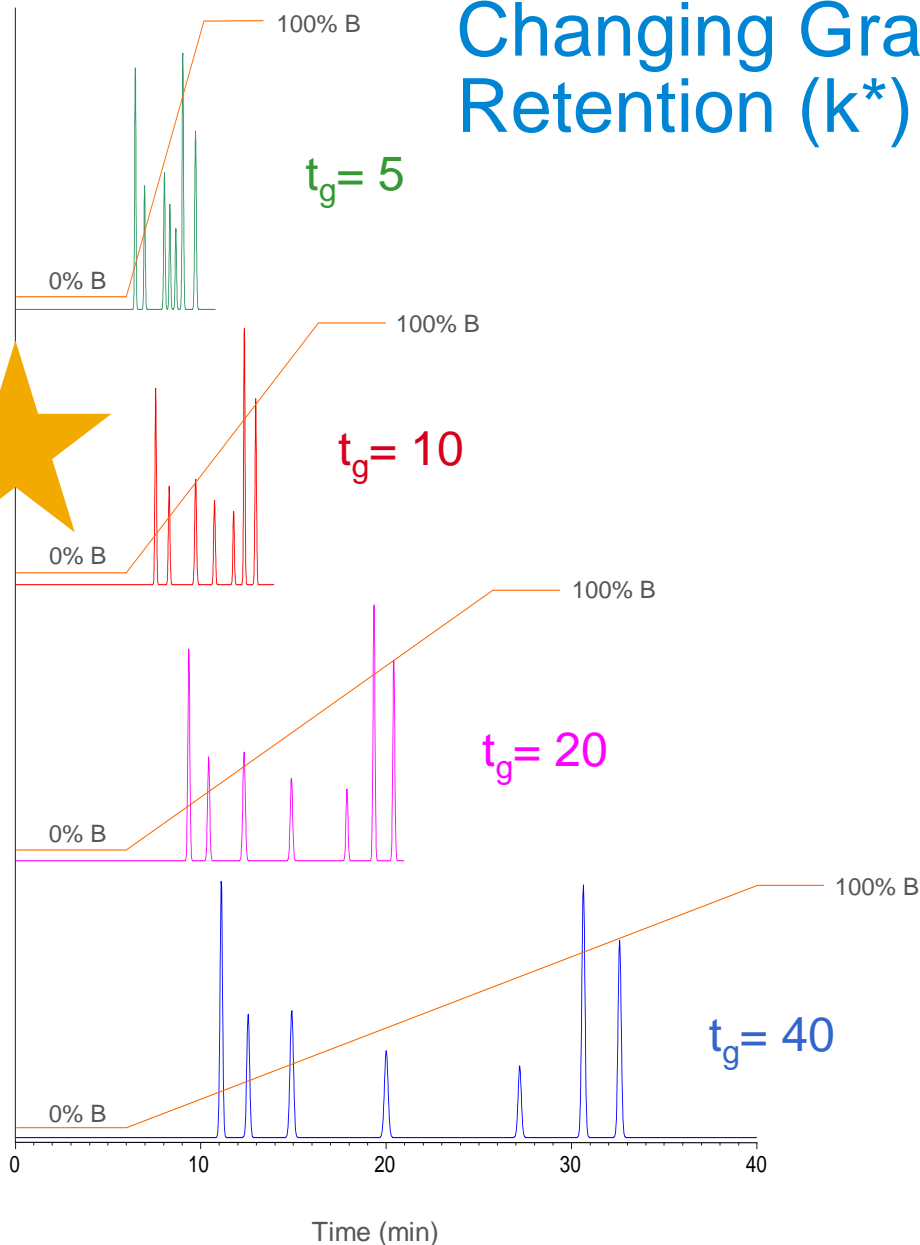
4.6 x 75 mm, 2.7  $\mu$ m  
N = 16,000

Poroshell 120 EC-C18  
4.6 x 75 mm, 2.7  $\mu$ m



Gradient: 15 – 80%B in 5 min  
Mobile Phase: A:H<sub>2</sub>O  
B: Acetonitrile  
Flow Rate: 1.0 mL/min  
Temperature: 30°C  
Sample: Carbamates

# Changing Gradient Time to Affect Retention ( $k^*$ ) and Resolution



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

$1/k^* = \text{gradient steepness} = b$

$\Delta\Phi$  = 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)

- $S \approx 4-5$  for small molecules
- $10 < S < 1000$  for peptides and proteins

# Adapting Gradient Methods to Different Column Dimensions

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

$$1/k^* \propto \text{gradient Steepness} = b = \frac{S \cdot \Delta\Phi \cdot V_m}{t_G \cdot F}$$

S = constant

$\Delta\Phi$  = change in % organic  
during the gradient run

$V_m$  = void volume of column

F = flow rate

$t_G$  = gradient time

$k^*$  = k of solute at mid point  
of column

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

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

## 4.6 x 150 mm

$$\Delta\Phi = 40 \text{ (20\% - 60\%)}$$

$$V_m = 1.5 \text{ mL}$$

$$F = 1.0 \text{ mL/min}$$

$$t_G = 15 \text{ min}$$

## 2.1 x 100 mm

$$\Delta\Phi = 40 \text{ (20\% - 60\%)}$$

$$V_m = 0.2 \text{ mL}$$

$$F = 0.2 \text{ mL/min}$$

$$t_G = ? \text{ (10 min)}$$

$$b = \frac{\Delta\Phi_1 \cdot V_{m1}}{F_1 \cdot t_{G1}} = \frac{\Delta\Phi_2 \cdot V_{m2}}{F_2 \cdot t_{G2}}$$

$$t_{G2} = t_{G1} \cdot \frac{\Delta\Phi_2}{\Delta\Phi_1} \cdot \frac{V_{m2}}{V_{m1}} \cdot \frac{F_1}{F_2}$$

$$t_{G2} = 15 \cdot \frac{40}{40} \cdot \frac{0.2}{1.5} \cdot \frac{1}{1.5} = 10$$



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

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

for constant %B

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

4.6 x 150 mm

F = 1.0 mL

2.1 x 100 mm

F = 0.2 mL

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

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

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

for constant %B

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

4.6 x 150 m

F = 1.0 mL

2.1 x 100 mm

F = 0.2 mL

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

# Contact Agilent Chemistries and Supplies Technical Support



1-800-227-9770 Option 3, Option 3:

Option 1 for GC/GCMS Columns and Supplies

Option 2 for LC/LCMS Columns and Supplies

Option 3 for Sample Preparation, Filtration and QuEChERS

Option 4 for Spectroscopy Supplies

**Available in the USA & Canada 8-5 all time zones**



[gc-column-support@Agilent.com](mailto:gc-column-support@Agilent.com)

[lc-column-support@agilent.com](mailto:lc-column-support@agilent.com)

[spp-support@agilent.com](mailto:spp-support@agilent.com)

[spectro-supplies-support@agilent.com](mailto:spectro-supplies-support@agilent.com)

