Gradient Design and Development

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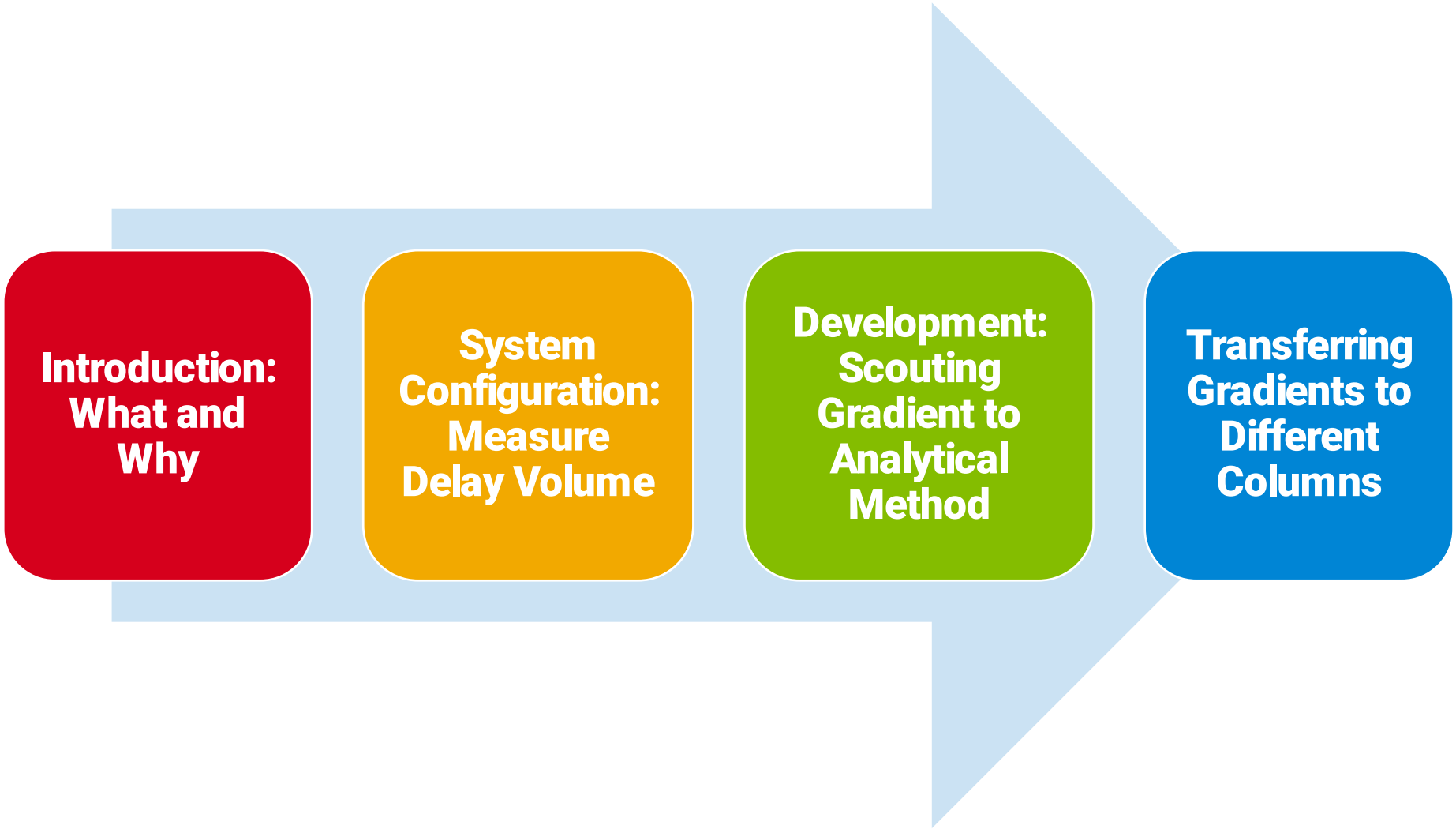

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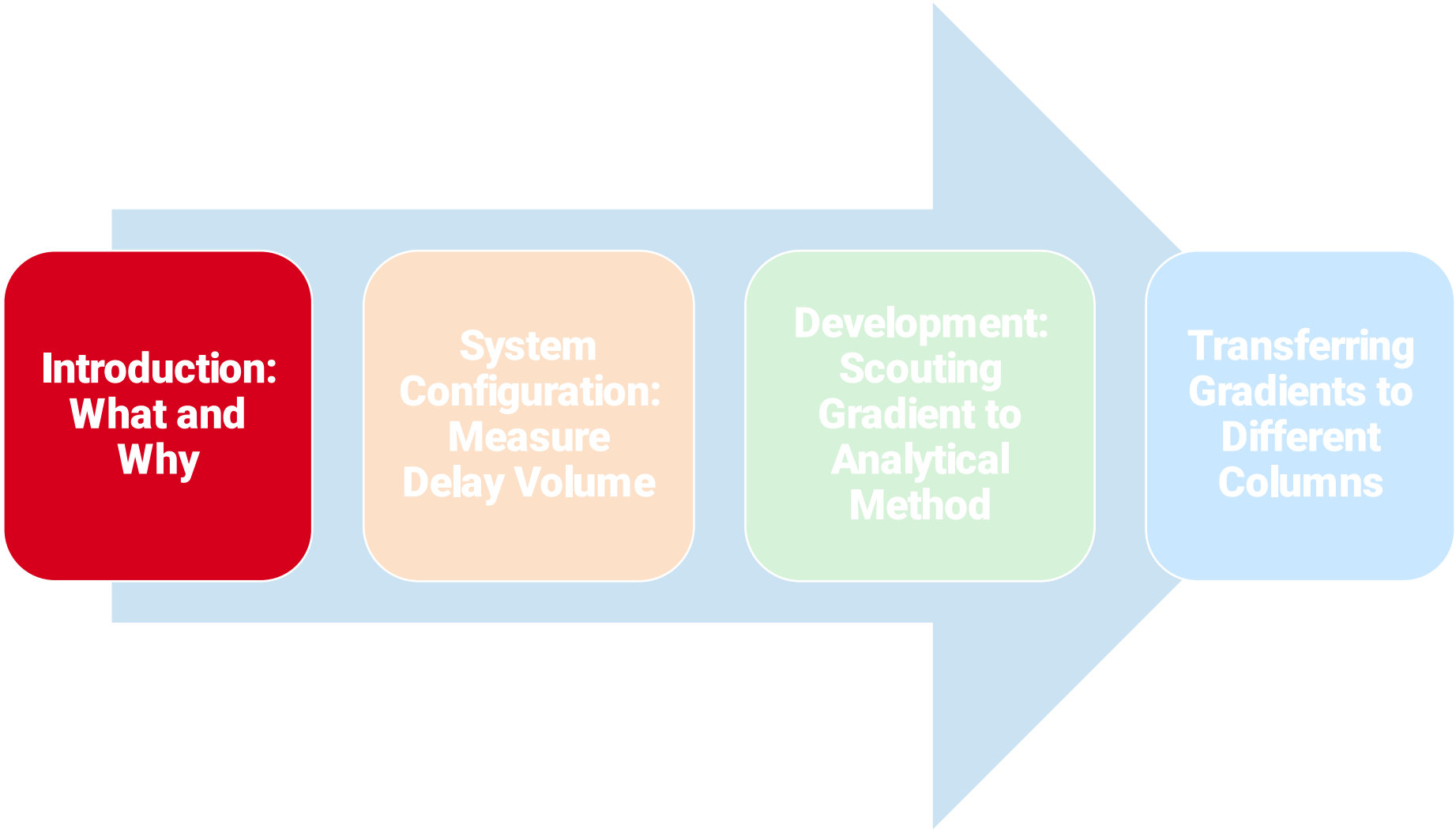
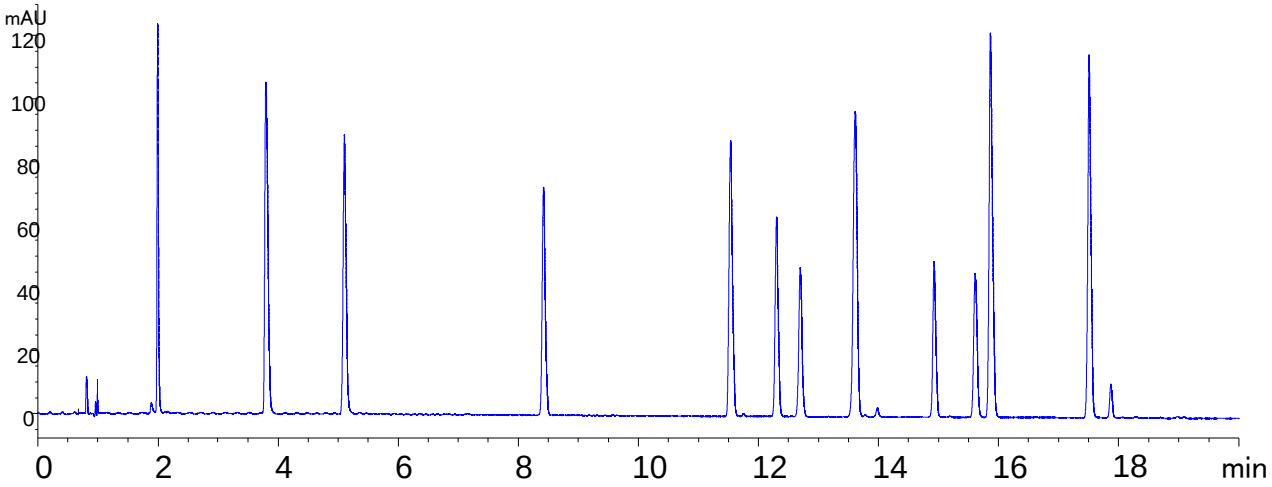


What is Gradient Elution?

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



1. Hydroquinone
2. 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

Column: Poroshell 120 EC-C18, 4.6 x 150 mm, 2.7 µm

Mobile Phase: Solvent A: water with 0.1% formic acid, Solvent B: acetonitrile

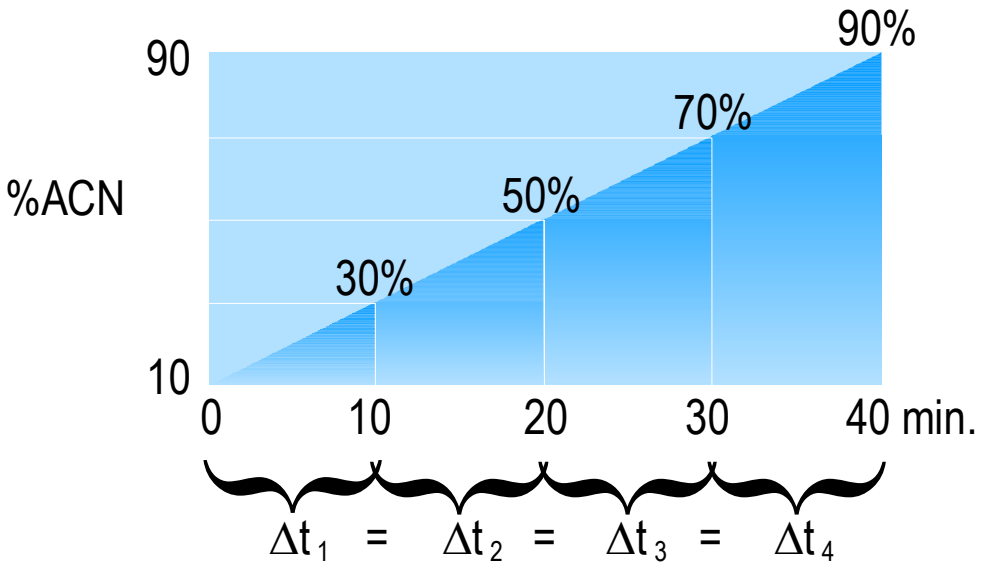
Gradient: 0–3 min 5% B, 5%-60% B over 22 minutes

Agilent 1200 SL controlled temperature at 25 °C, 2 µl 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



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

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

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

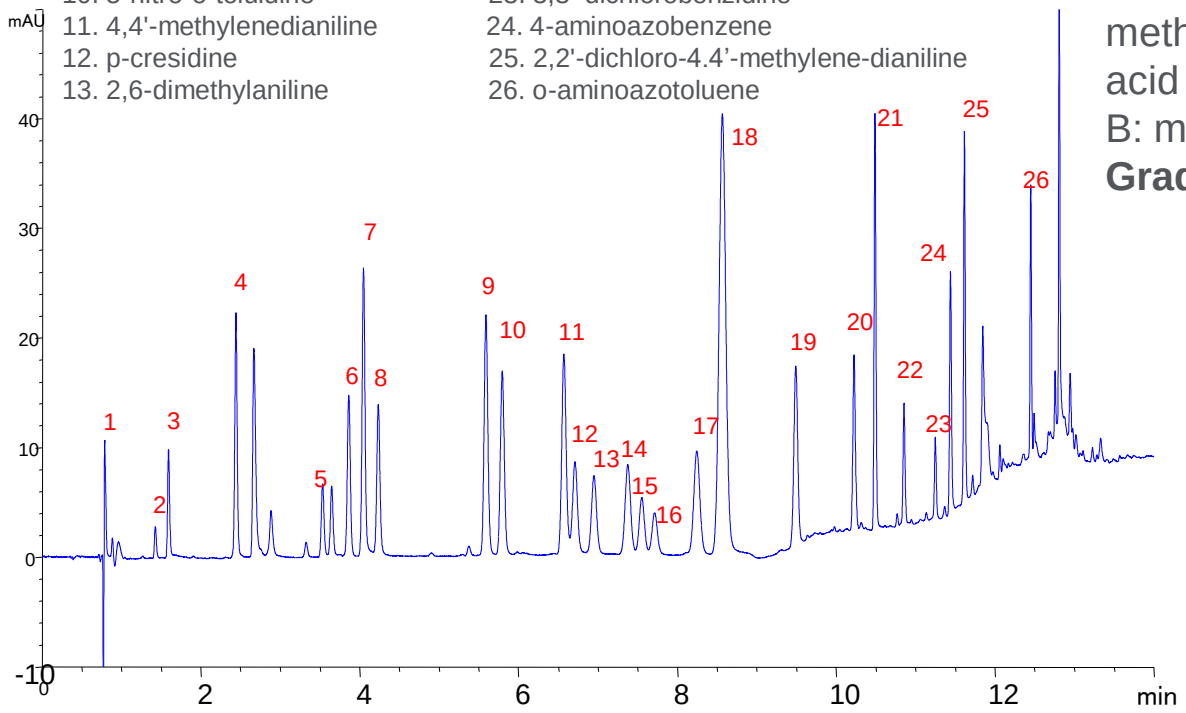
For every 20% change in ACN, △t is 10 min.

Gradient Elution Analysis of a Complex Sample:

EU banned Azo Colorants in textiles

Sample:

1. 1,4-phenylendiamine
2. 4-methoxy-m-phenylenediamine
3. 4-methyl-m-phenylenediamine
4. Aniline
5. benzidine
6. o-anisidine
7. 4,4'-oxydianiline
8. o-toluidine
9. 4-chloroaniline
10. 5-nitro-o-toluidine
11. 4,4'-methylenedianiline
12. p-cresidine
13. 2,6-dimethylaniline
14. 2,4-dimethylaniline
15. o-dianisidine
16. 4,4'-bi-o-toluidine
17. 4,4'-thiodianiline
18. 2-naphthylamine
19. 4-chloro-o-toluidine
20. 2,4,5-trimethylaniline
21. 4,4'-methylenedi-o-toluidine
22. biphenyl-4-ylamine
23. 3,3'-dichlorobenzidine
24. 4-aminoazobenzene
25. 2,2'-dichloro-4.4'-methylene-dianiline
26. o-aminoazotoluene



Column: Poroshell 120 EC-C18, 3.0 x 150 mm, 2.7 µm (p/n: 693975-302)
Column temperature: 40 °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.

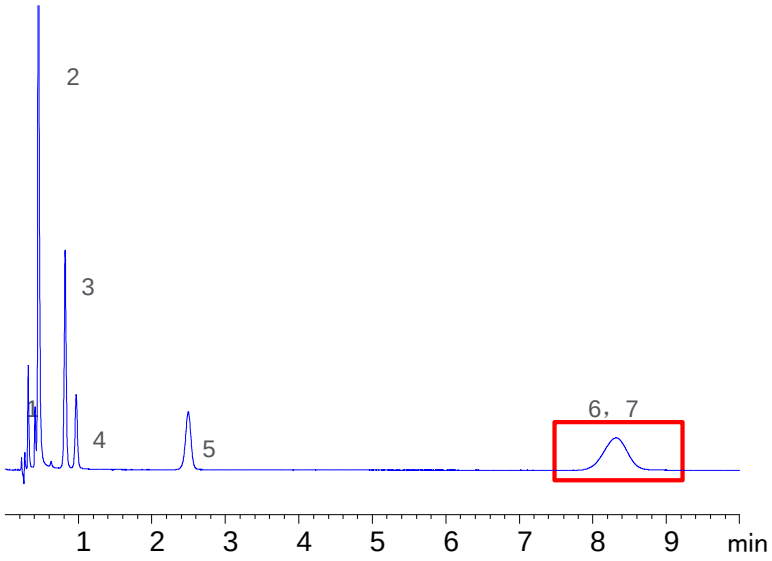
2. To separate mixtures having a large number of components

3. To separate high molecular weight mixtures (i.e., large molecules, peptides and proteins)

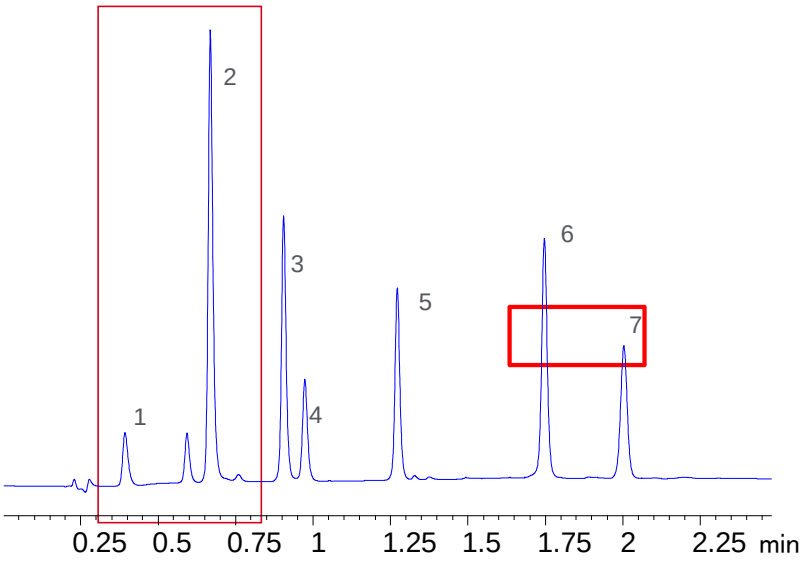
Gradient Separation Reduces Analysis Time and Improves Resolution – Even with the Shortest Columns

Columns: **Poroshell 120 EC-C18, 3.0 x 30 mm, 2.7 µm**
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!

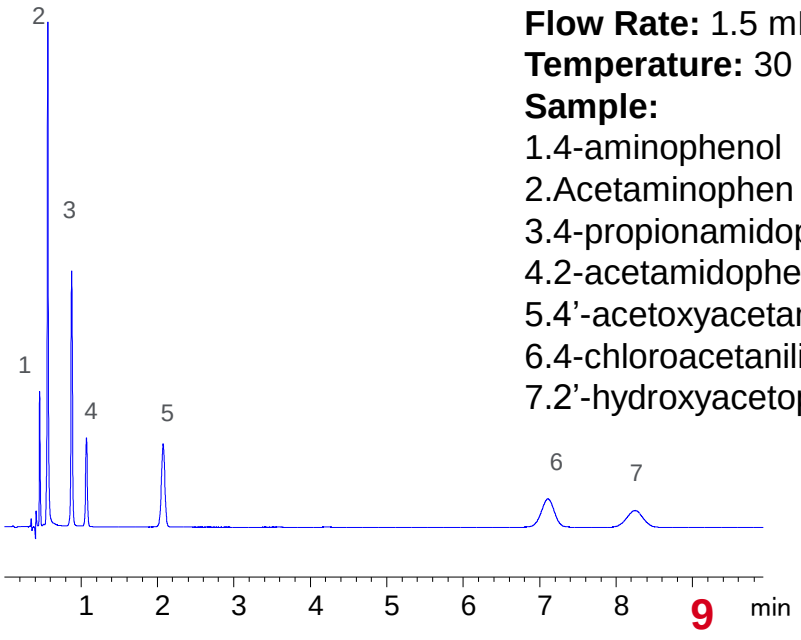
Gradient Separation is Faster than Isocratic

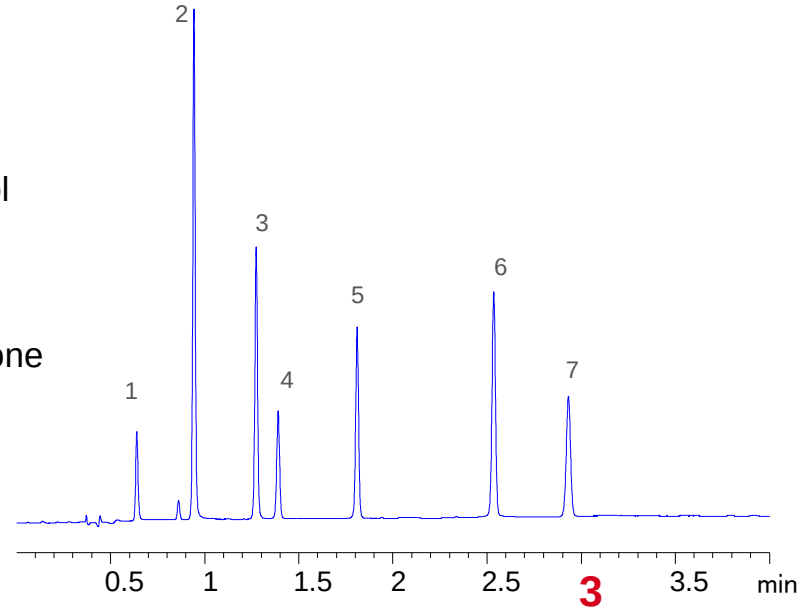
Separation of acetaminophen impurities on Poroshell 120 EC-C18

Isocratic elution:
85% aqueous/15% ACN

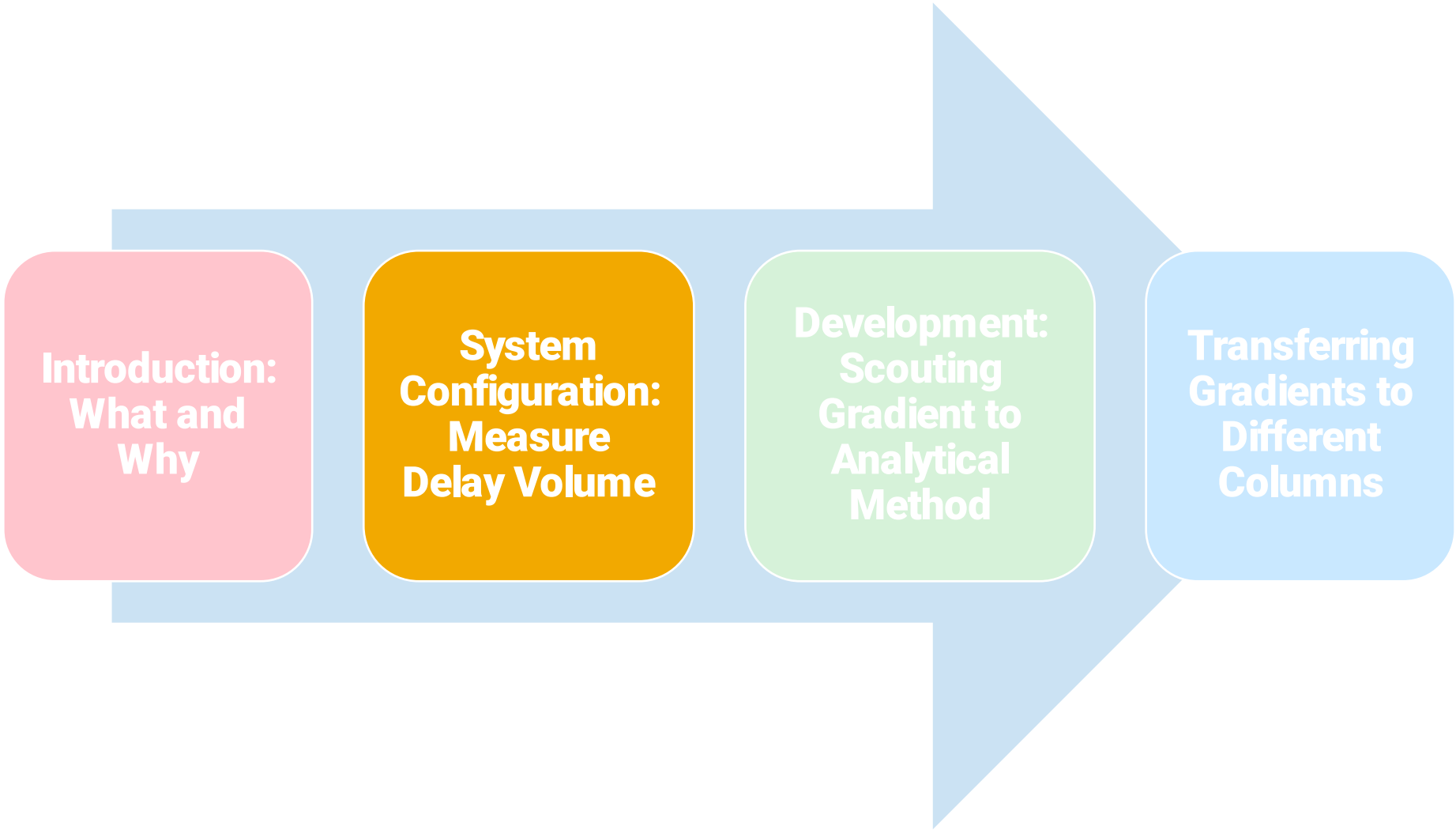
Gradient elution:
5–50% ACN in 5 min

Column: Poroshell 120 EC-C18, 4.6 x 50 mm, 2.7 µm
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



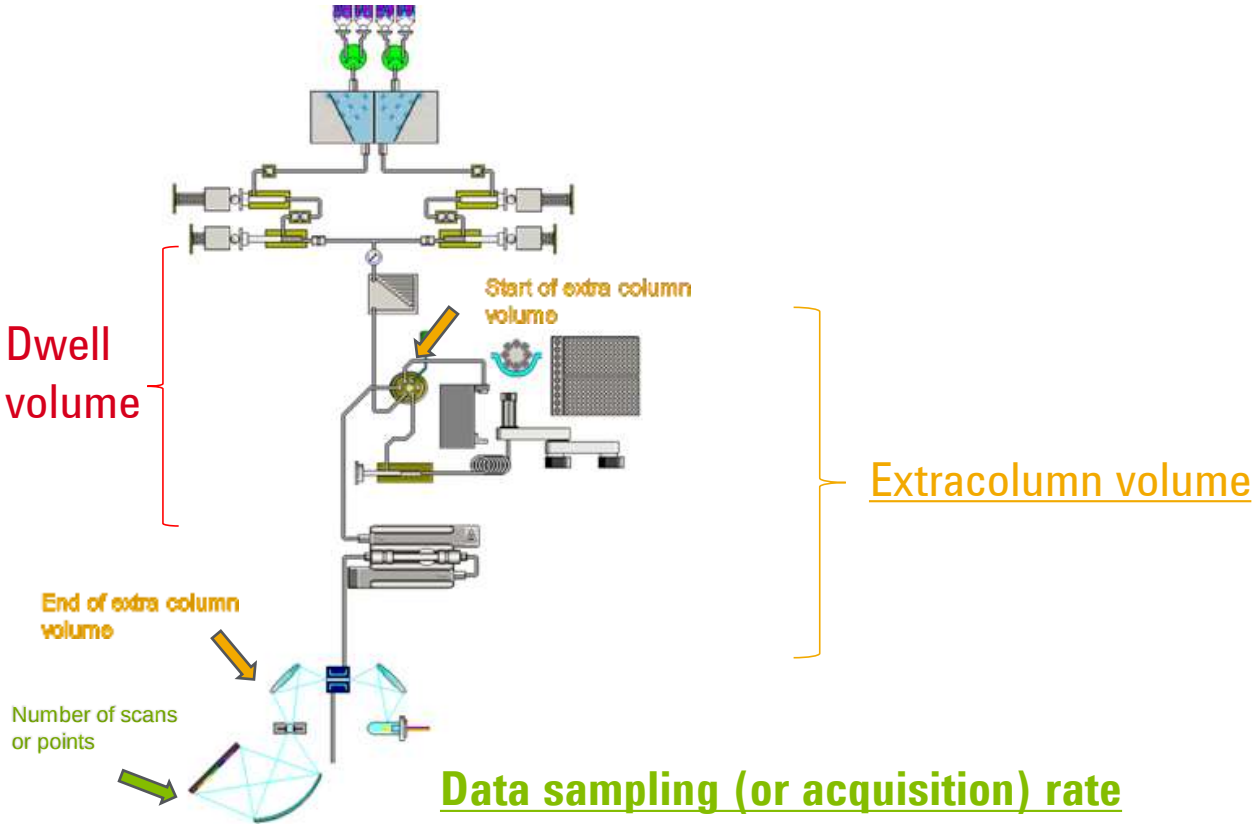


Gradient Design and Development



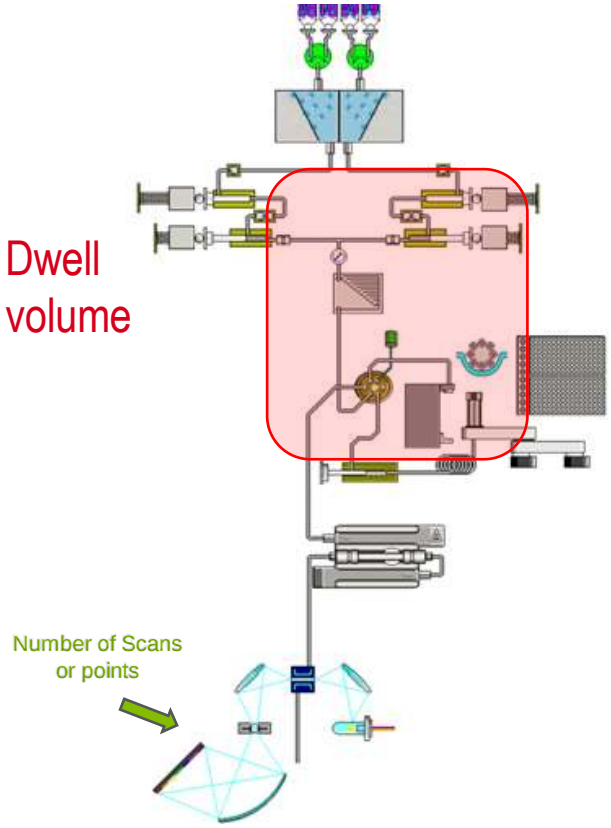
Gradient Separations

Instrument Impact on Column Performance



Instrument Impact on Column Performance

Dwell volume

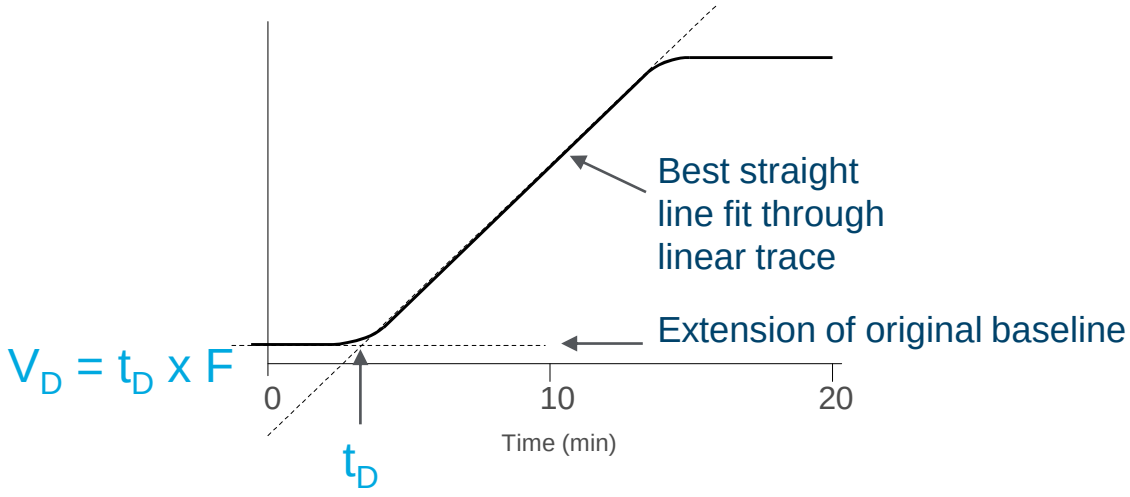


- Dwell volume = volume from formation of gradient to the column

Determining the Dwell Volume of Your System

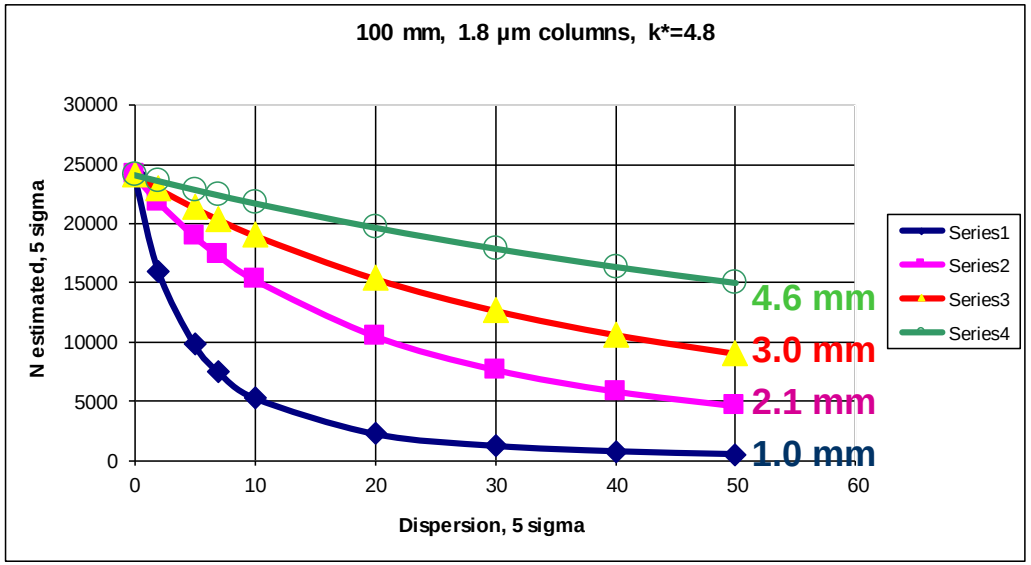
- Look it up in the LC manual or follow the procedure below
- Replace column with short piece of HPLC stainless steel tubing
- Prepare mobile phase components
  A. Water - UV-transparent
  B. Water with 0.2% acetone - UV-absorbing
- Monitor at 265 nm
- Run gradient profile 0–100% B/10 min at 1.0 mL/min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

Measuring Dwell Volume (V_D)



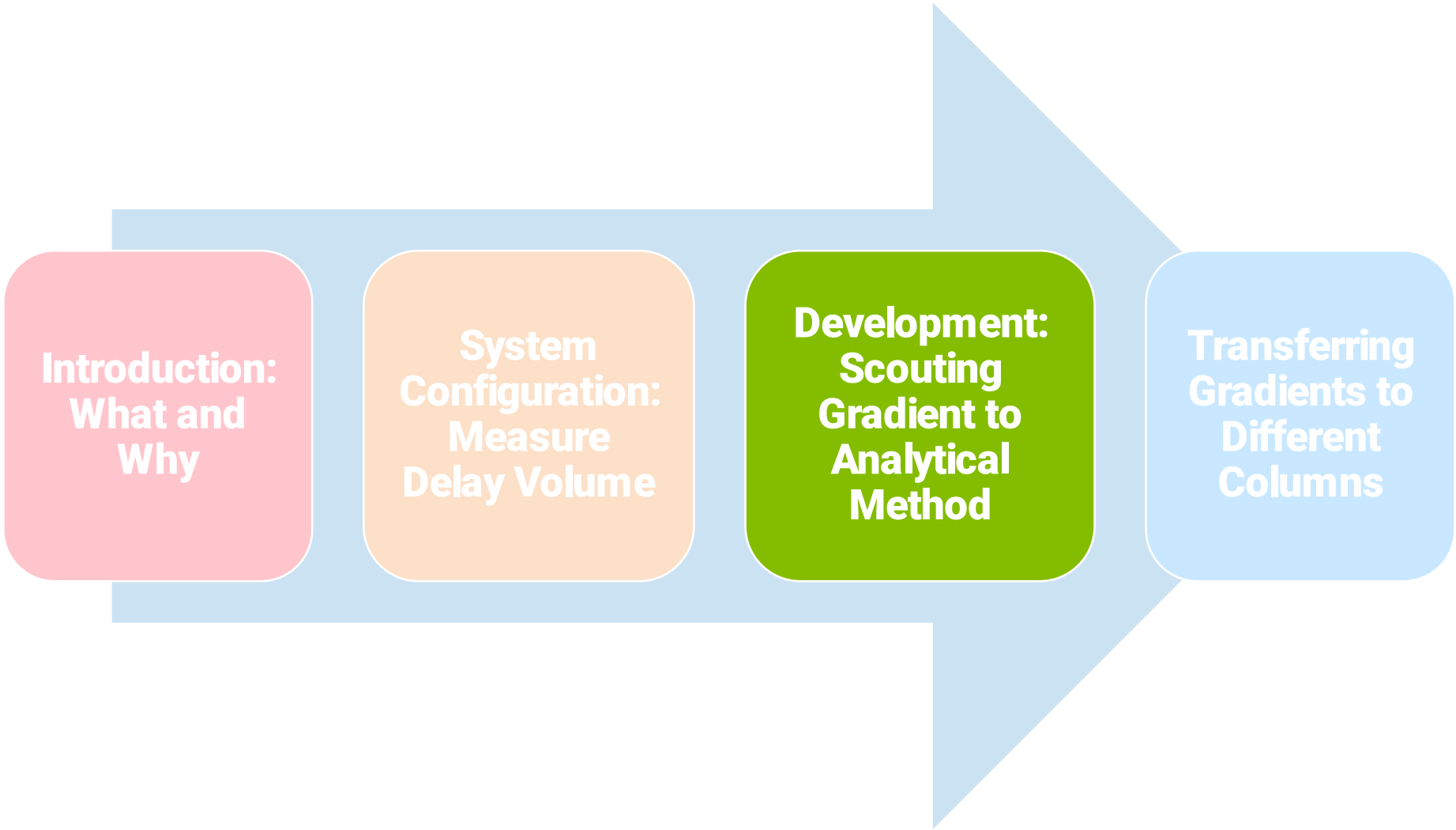
- Intersection of the two lines identifies dwell time (t_D)
- Dwell volume is equal to product of the flow rate and the dwell time.

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

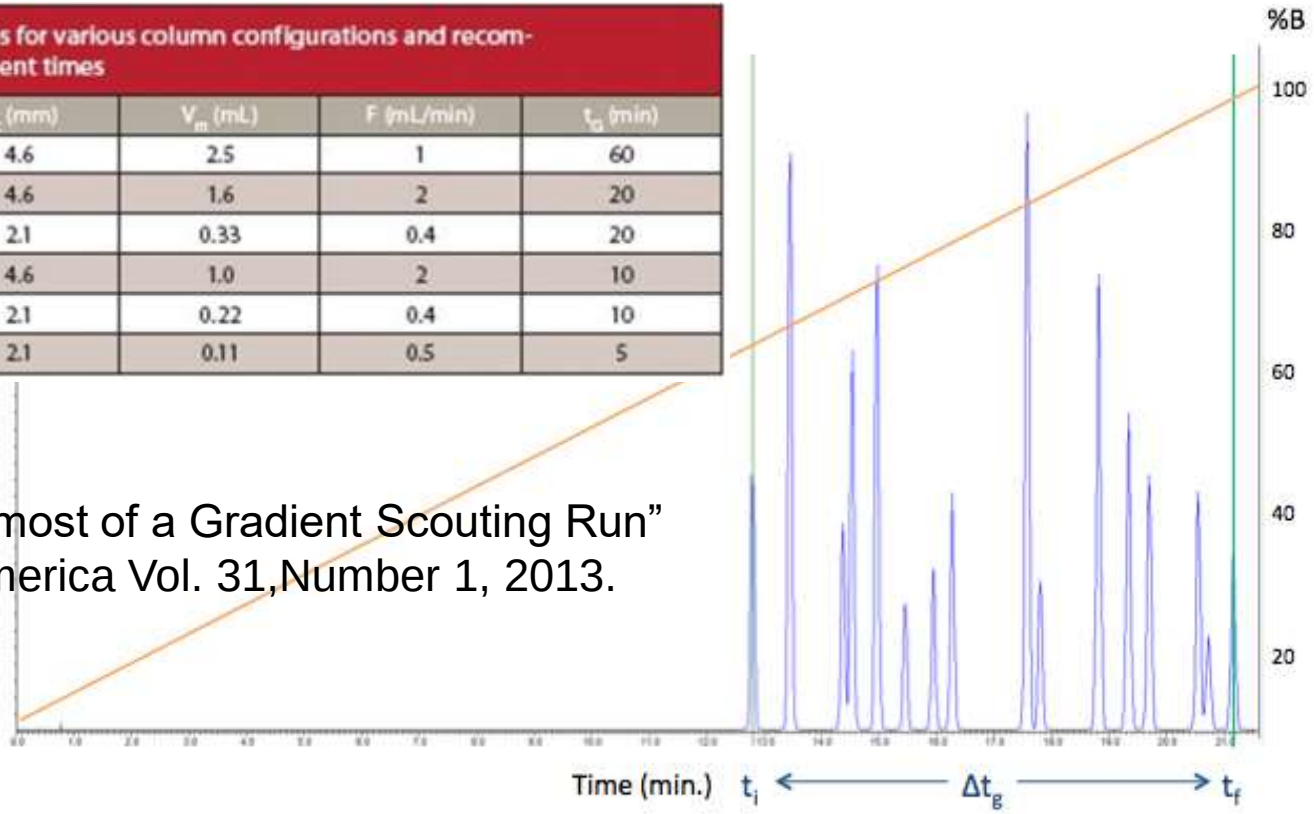


Starting Point Scouting Gradient

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.

Table I: Column volumes for various column configurations and recommended scouting gradient times

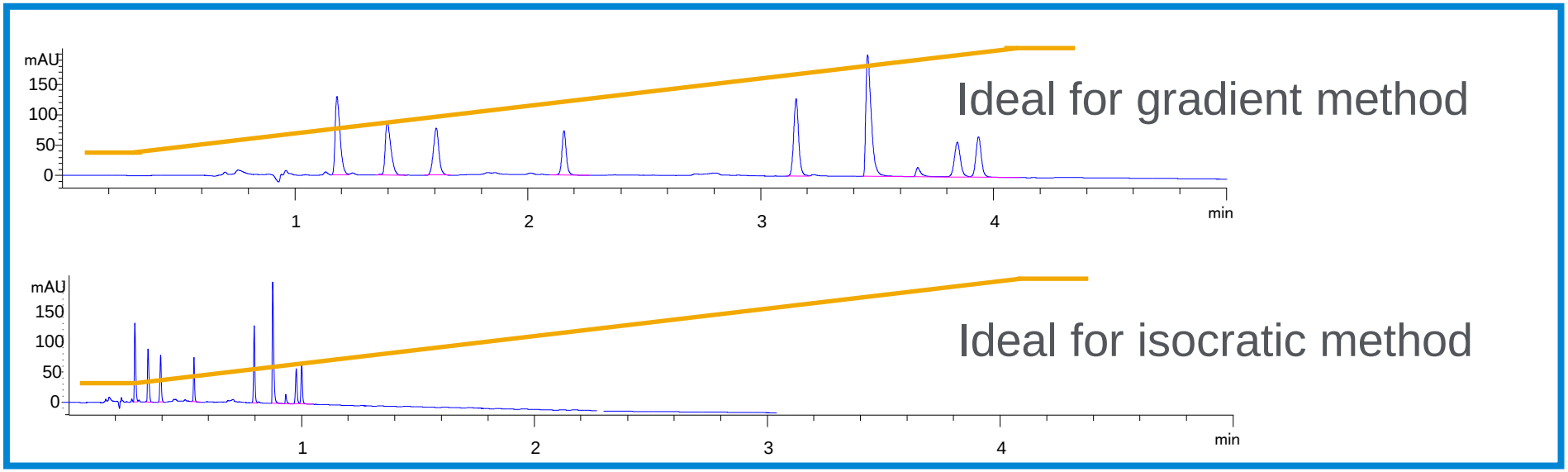
L (mm)	d_c (mm)	V_m (mL)	F (mL/min)	t_G (min)
250	4.6	2.5	1	60
150	4.6	1.6	2	20
150	2.1	0.33	0.4	20
100	4.6	1.0	2	10
100	2.1	0.22	0.4	10
50	2.1	0.11	0.5	5



"Making to 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

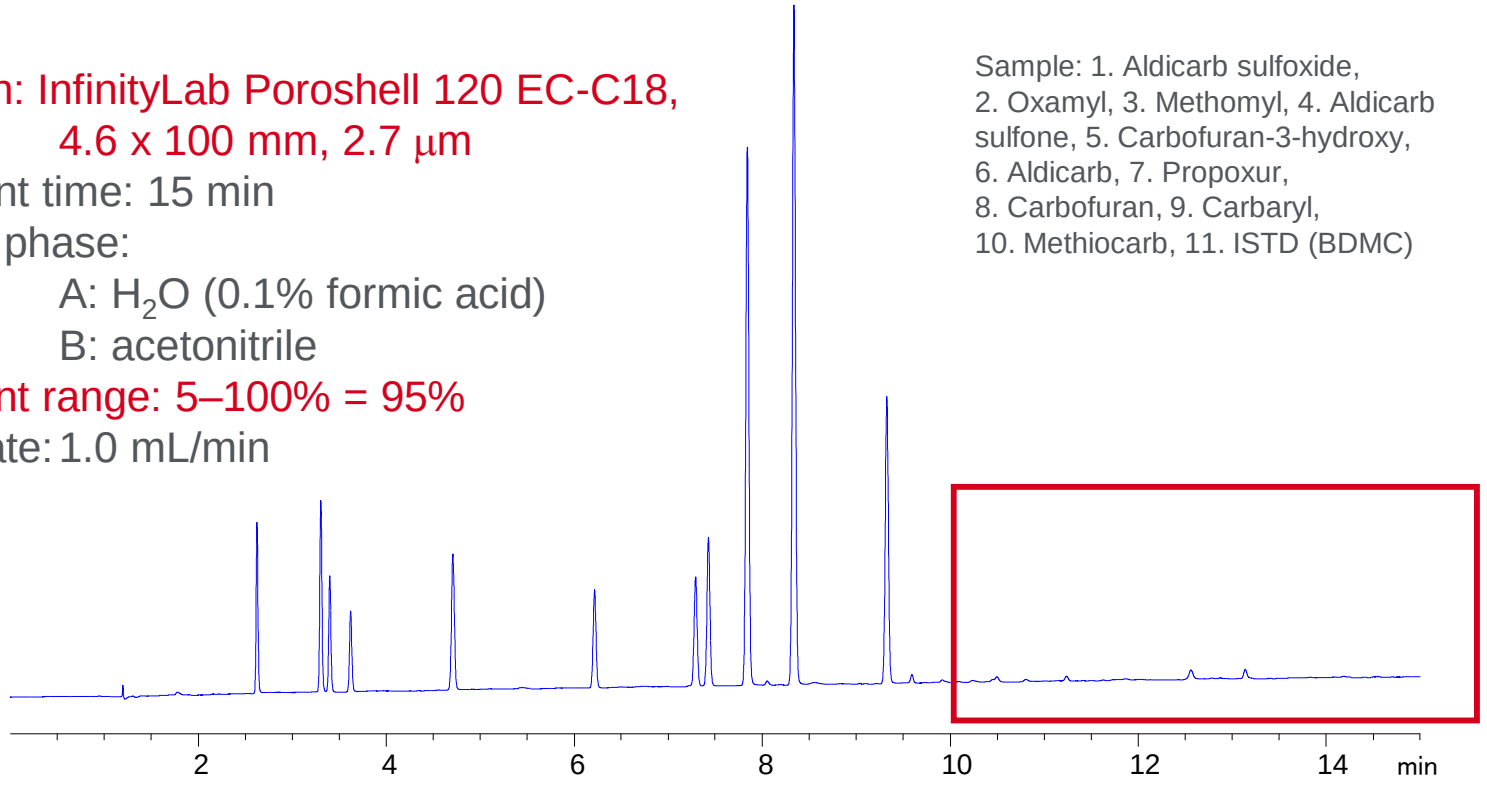


Step 1: Choose, Shorter Efficient Column

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

Column: InfinityLab Poroshell 120 EC-C18,
4.6 x 100 mm, 2.7 µm
Gradient time: 15 min
Mobile phase:
A: H_2O (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)



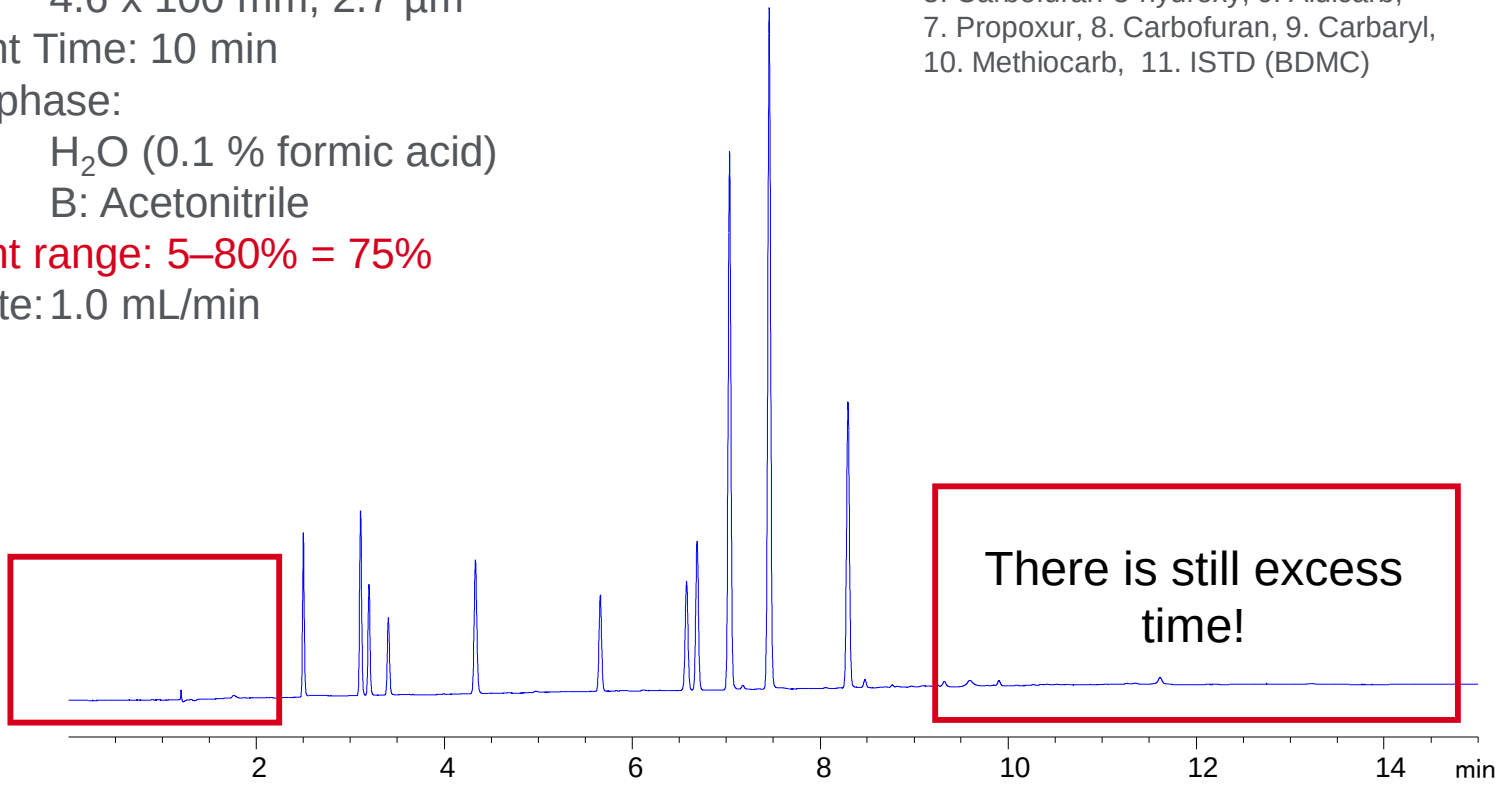
The scouting shows that there is wasted time in this chromatogram and resolution of all components can be achieved. Optimization possible!

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 µm
Gradient Time: 10 min
Mobile phase:
H_2O (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)

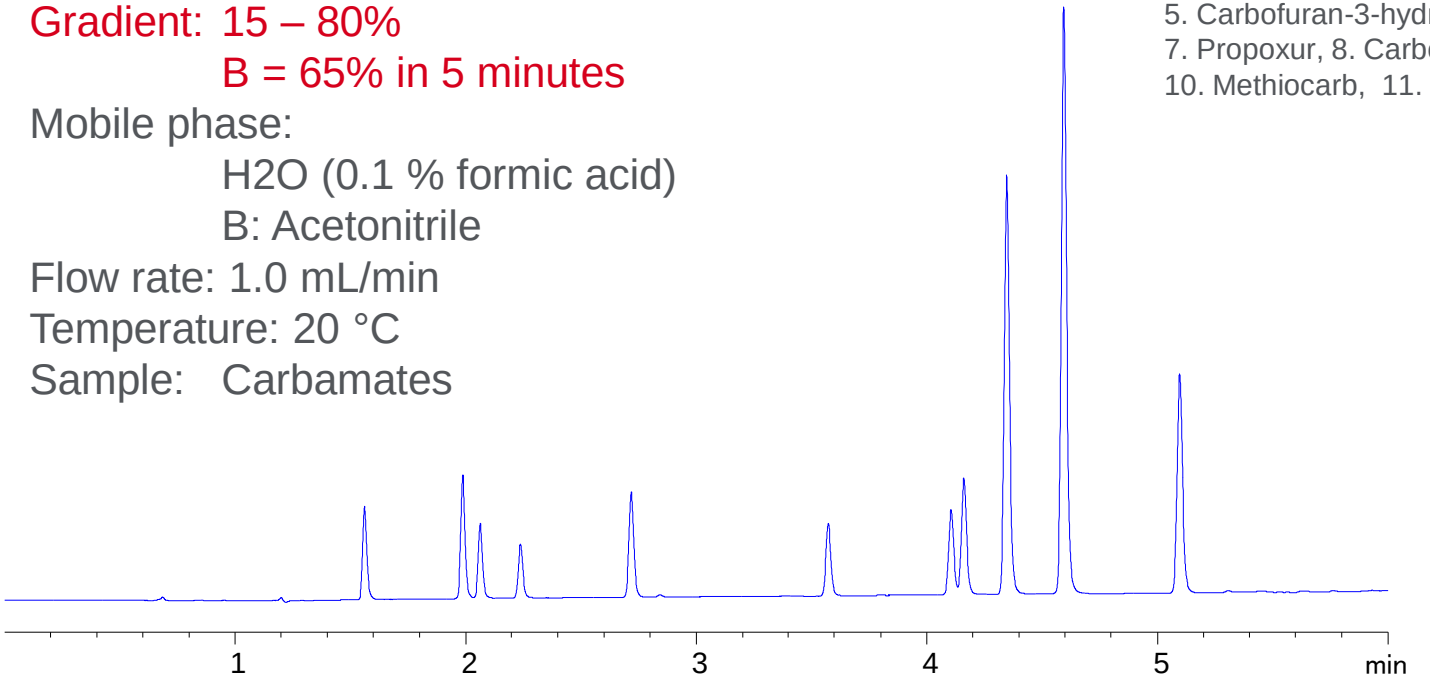


Step 3: Finalize Your Results

Increase starting % organic and reduce time

Column: InfinityLab Poroshell 120 EC-C18,
4.6 x 100 mm, 2.7 µm
Gradient: 15 – 80%
B = 65% in 5 minutes
Mobile phase:
H2O (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)



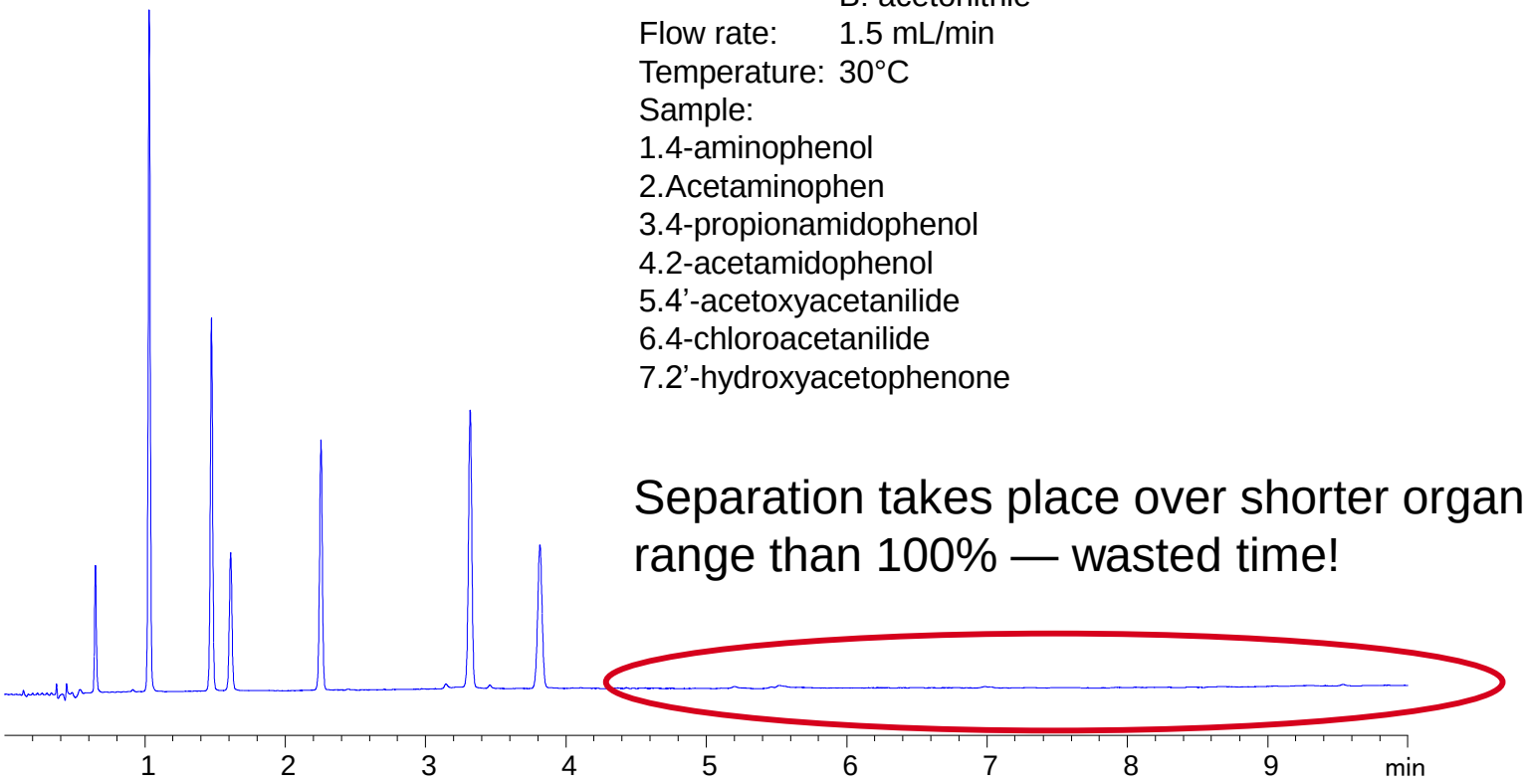
Saved 50% of the time with method optimization. Used Poroshell 120 for high efficiency and resolution.

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 µm

Mobile phase: A: 10 mM ammonium acetate, pH 6.8
B: acetonitrile
Flow rate: 1.5 mL/min
Temperature: 30°C
Sample:
1.4-aminophenol
2.Acetaminophen
3.4-propionamidophenol
4.2-acetamidophenol
5.4'-acetoxyacetanilide
6.4-chloroacetanilide
7.2'-hydroxyacetophenone

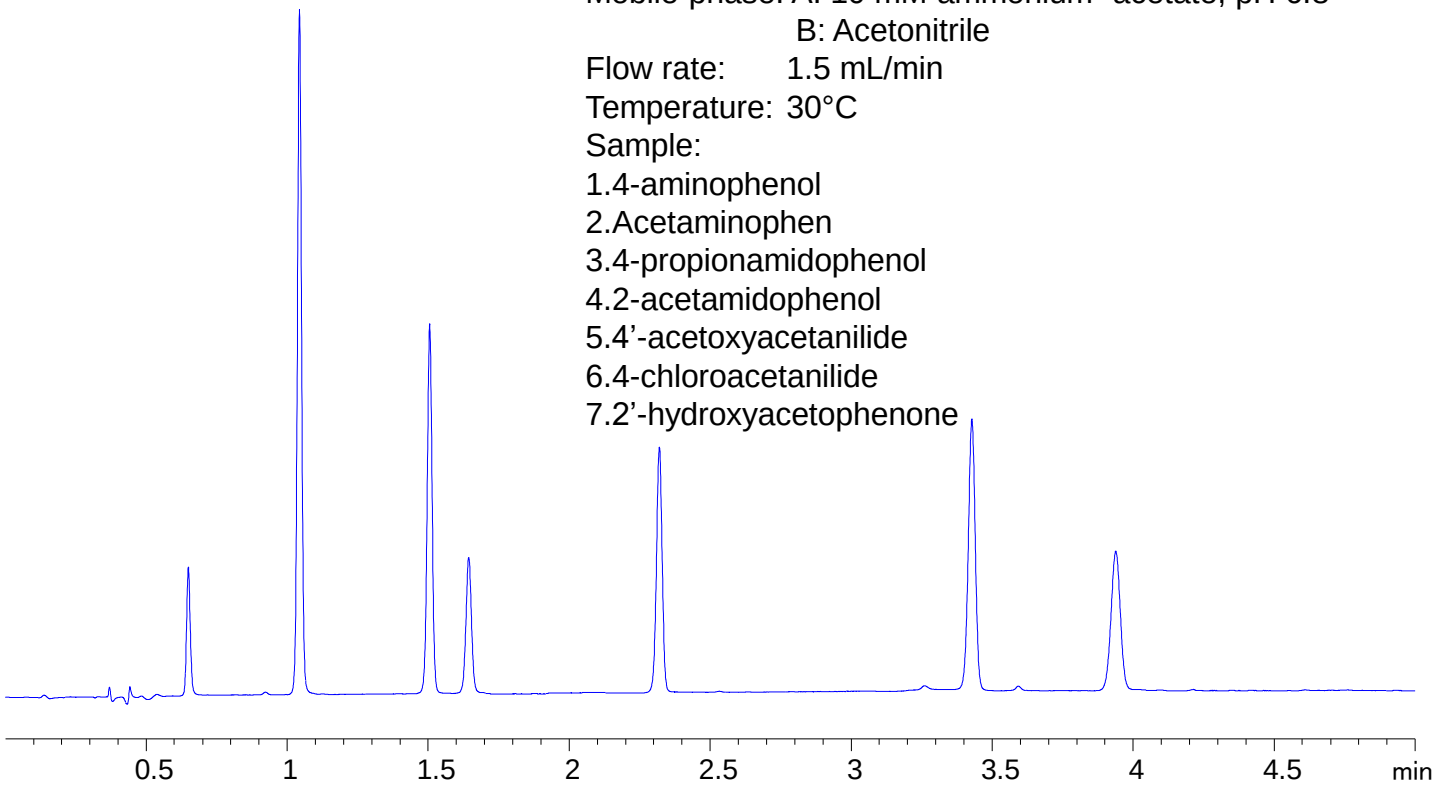


Separation takes place over shorter organic range than 100% — wasted time!

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

Column: Poroshell 120 EC-C18, 4.6 x 50 mm, 2.7 µm

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



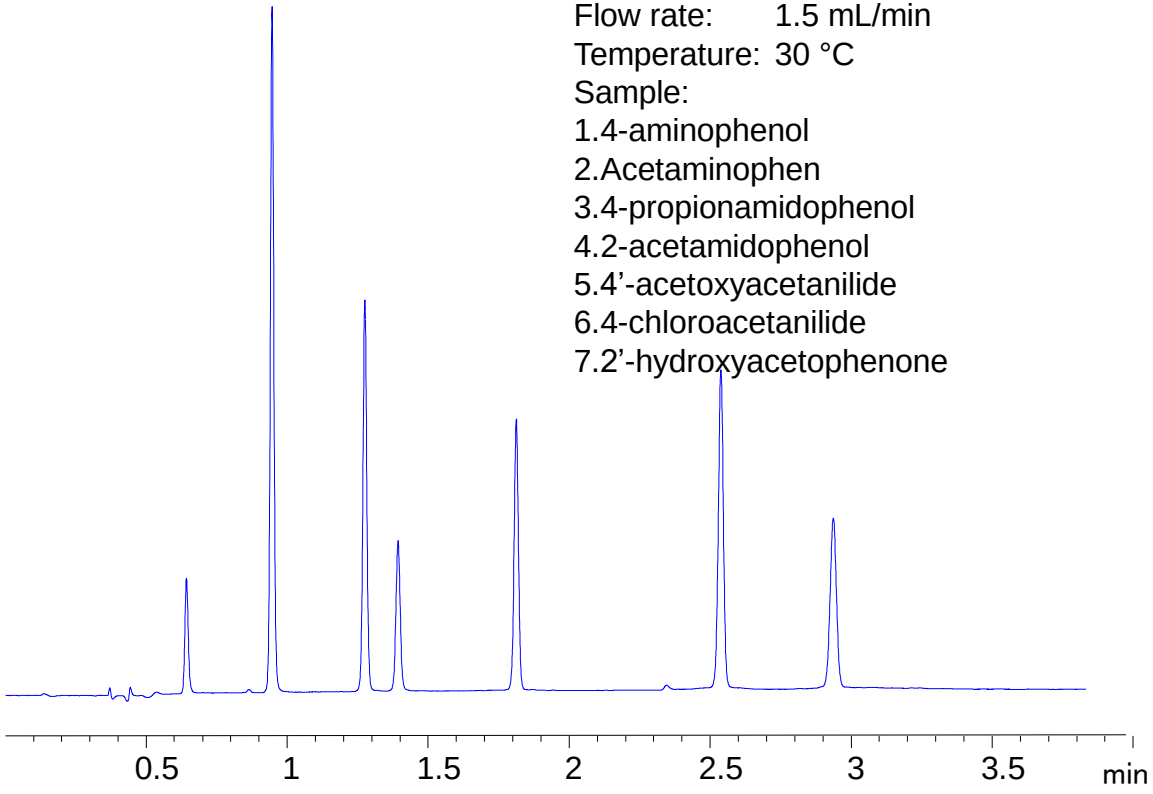
Excellent resolution and distribution of peaks in the gradient – within 5 minutes.

Final Optimization to Reduce Time

Gradient from 5%–50% in 3 min

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

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



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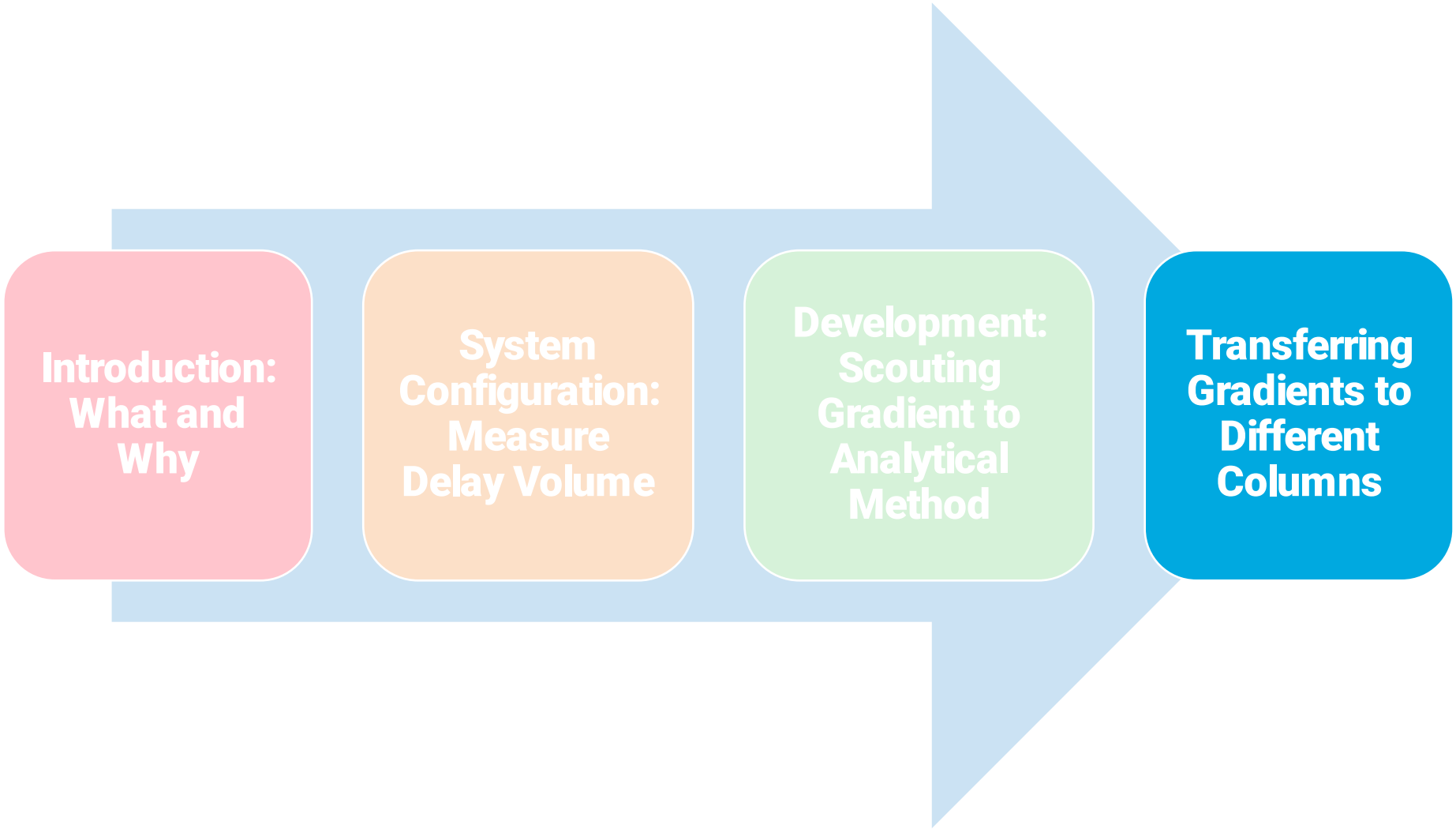


Resolution Relationship for Gradient Elution

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

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 (≈ 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	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

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

A Shorter Column (smaller Vm)

Reduce run-time and improve resolution while maintaining constant N

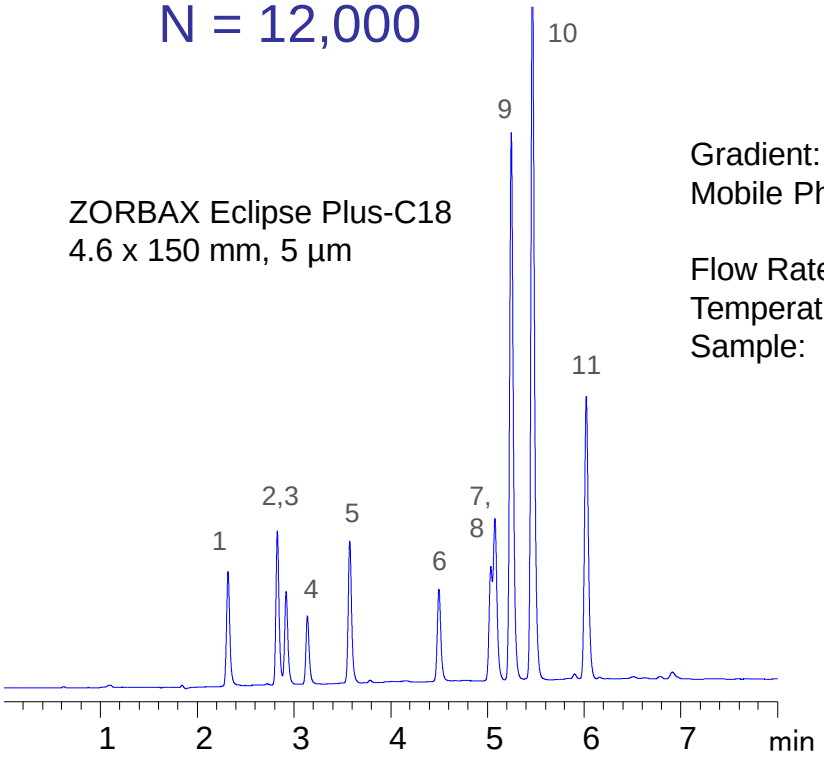
4.6 x 150 mm, 5 µm
N = 12,000

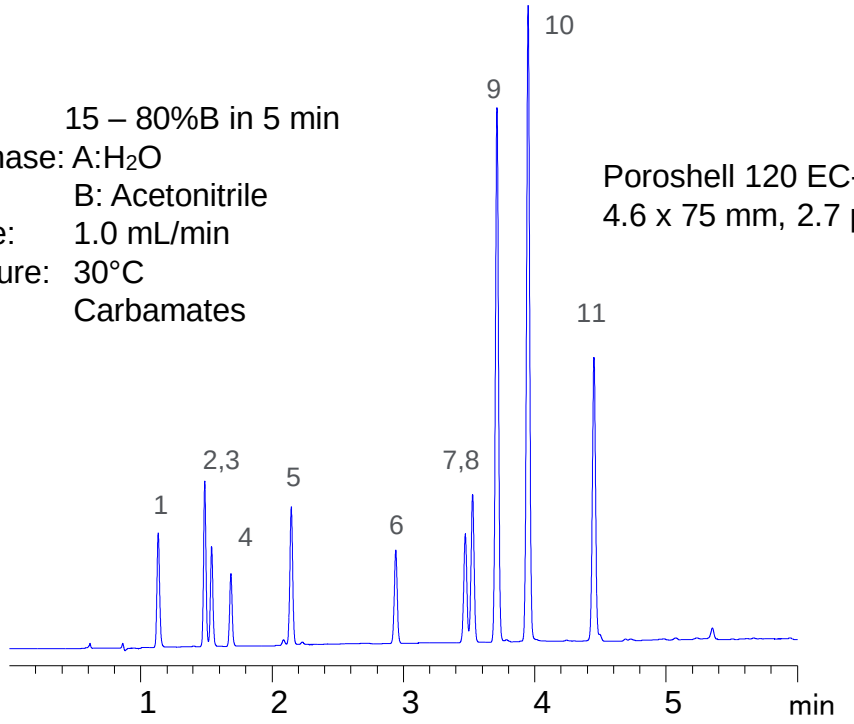
ZORBAX Eclipse Plus-C18
4.6 x 150 mm, 5 µm

Gradient: 15 – 80%B in 5 min
Mobile Phase: A:H_2O
B: Acetonitrile
Flow Rate: 1.0 mL/min
Temperature: 30°C
Sample: Carbamates

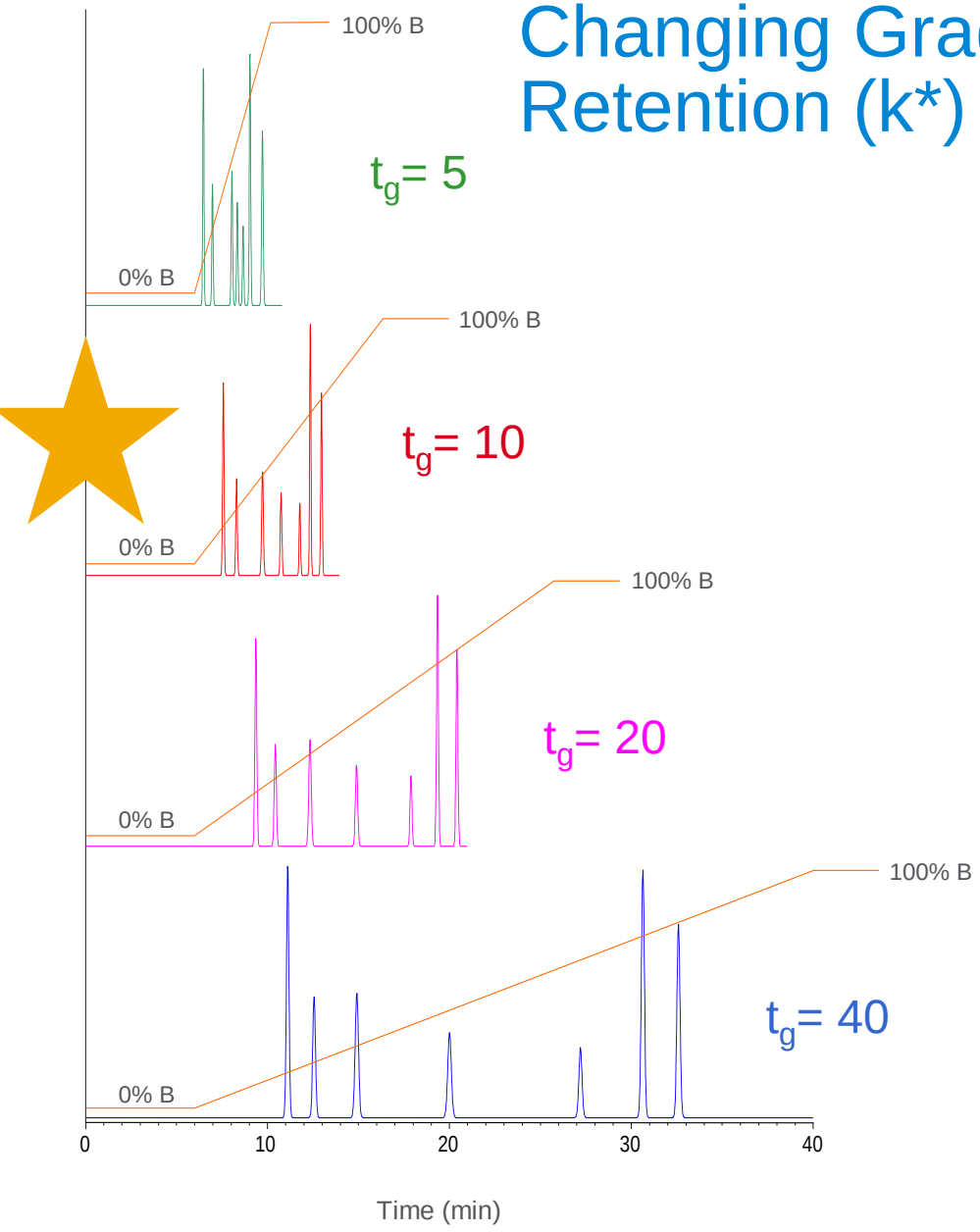
4.6 x 75 mm, 2.7 µm
N = 16,000

Poroshell 120 EC-C18
4.6 x 75 mm, 2.7 µm





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



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

$1/k^* =$ 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 ≈ 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.

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

S = constant
$\Delta\Phi$ = change in % organic during the gradient run
Vm = void volume of column
F = flow rate
t_G = gradient time
k* = k of solute at mid point of column

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

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

4.6 x 150 mm

ΔΦ = 40 (20% - 60%)
Vm = 1.5 mL
F = 1.0 mL/min
t_G = 15 min

2.1 x 100 mm

ΔΦ = 40 (20% - 60%)
Vm = 0.2 mL
F = 0.2 mL/min
t_G = ? (10 min)

$$b = \frac{\Delta\Phi_1 \cdot V_{m_1}}{F_1 \cdot t_{G_1}} = \frac{\Delta\Phi_2 \cdot V_{m_2}}{F_2 \cdot t_{G_2}}$$

$$t_{G_2} = t_{G_1} \cdot \frac{\Delta\Phi_2}{\Delta\Phi_1} \cdot \frac{V_{m_2}}{V_{m_1}} \cdot \frac{F_1}{F_2}$$

$$t_{G_2} = 15 \cdot \frac{40}{40} \cdot \frac{0.2}{1.5} \cdot \frac{1}{1.5} = 10$$

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

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

	%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

4.6 x 150 m

F = 1.0 mL

2.1 x 100 mm

F = 0.2 mL

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

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

	%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

4.6 x 150 m

F = 1.0 mL

2.1 x 100 mm

F = 0.2 mL

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

lc-column-support@agilent.com

spp-support@agilent.com

spectro-supplies-support@agilent.com