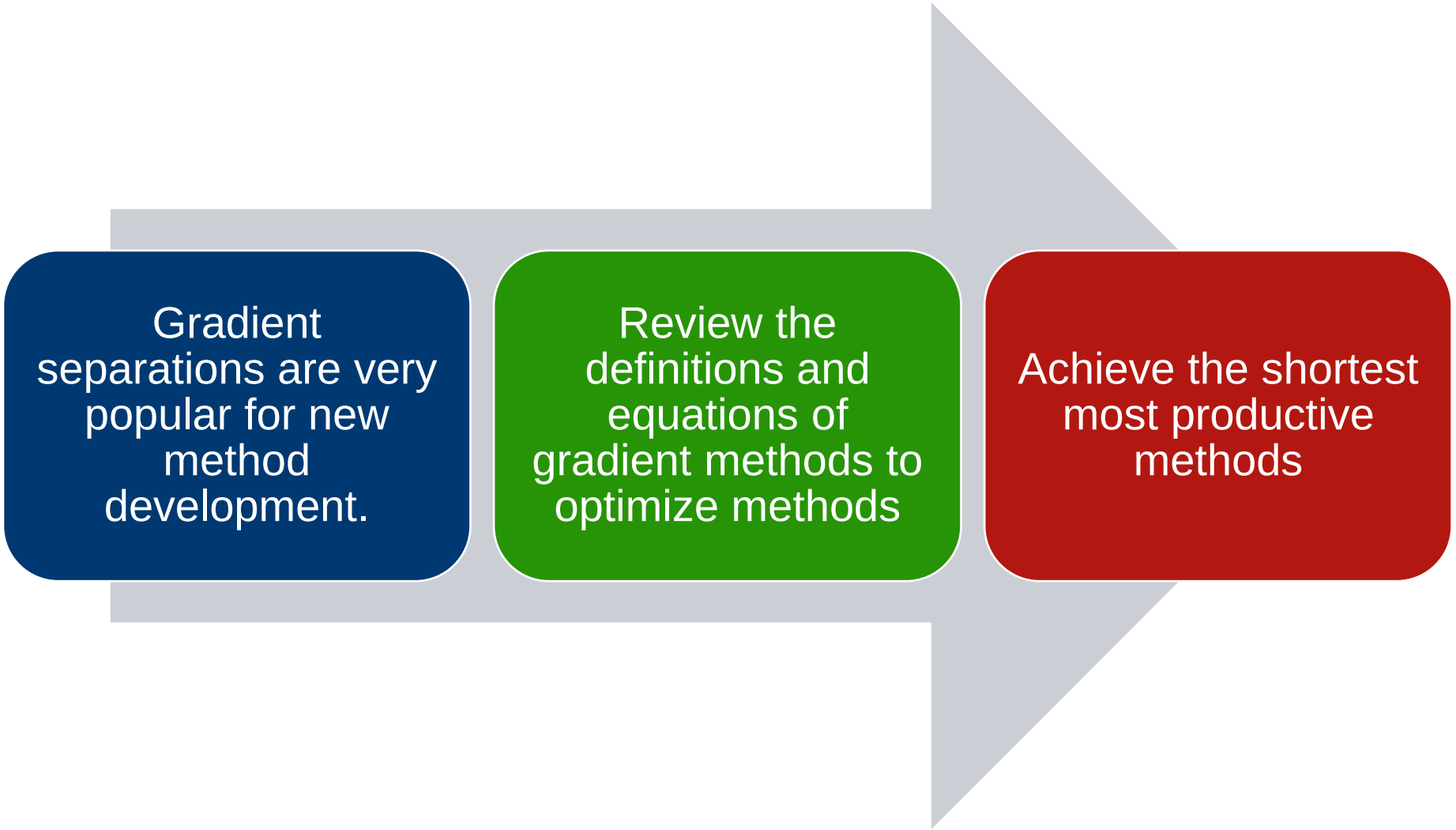
Gradient Design and Development

Breaking the Bad Gradient Cycle

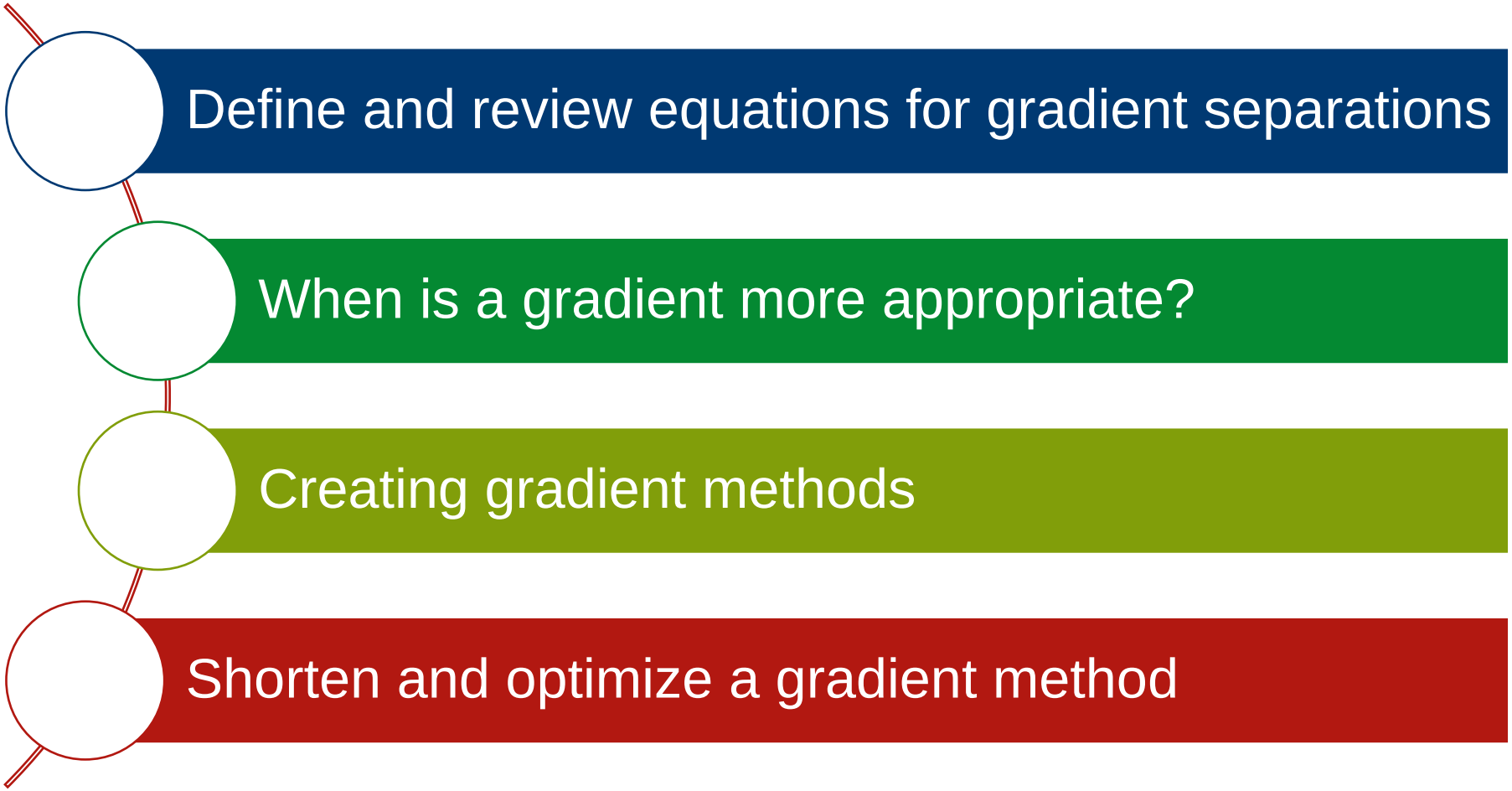
Gradient Design and Development – Breaking the Bad Gradient Cycle

In this session we will define gradient separations and review equations that define the gradient process. From there we will move to a discussion on determining when a gradient is more appropriate than isocratic and using a simple experimental process for that determination. The next step is to present a logical process for creating a gradient method. Since many gradient methods are longer than needed we will also explore techniques for shortening existing methods.

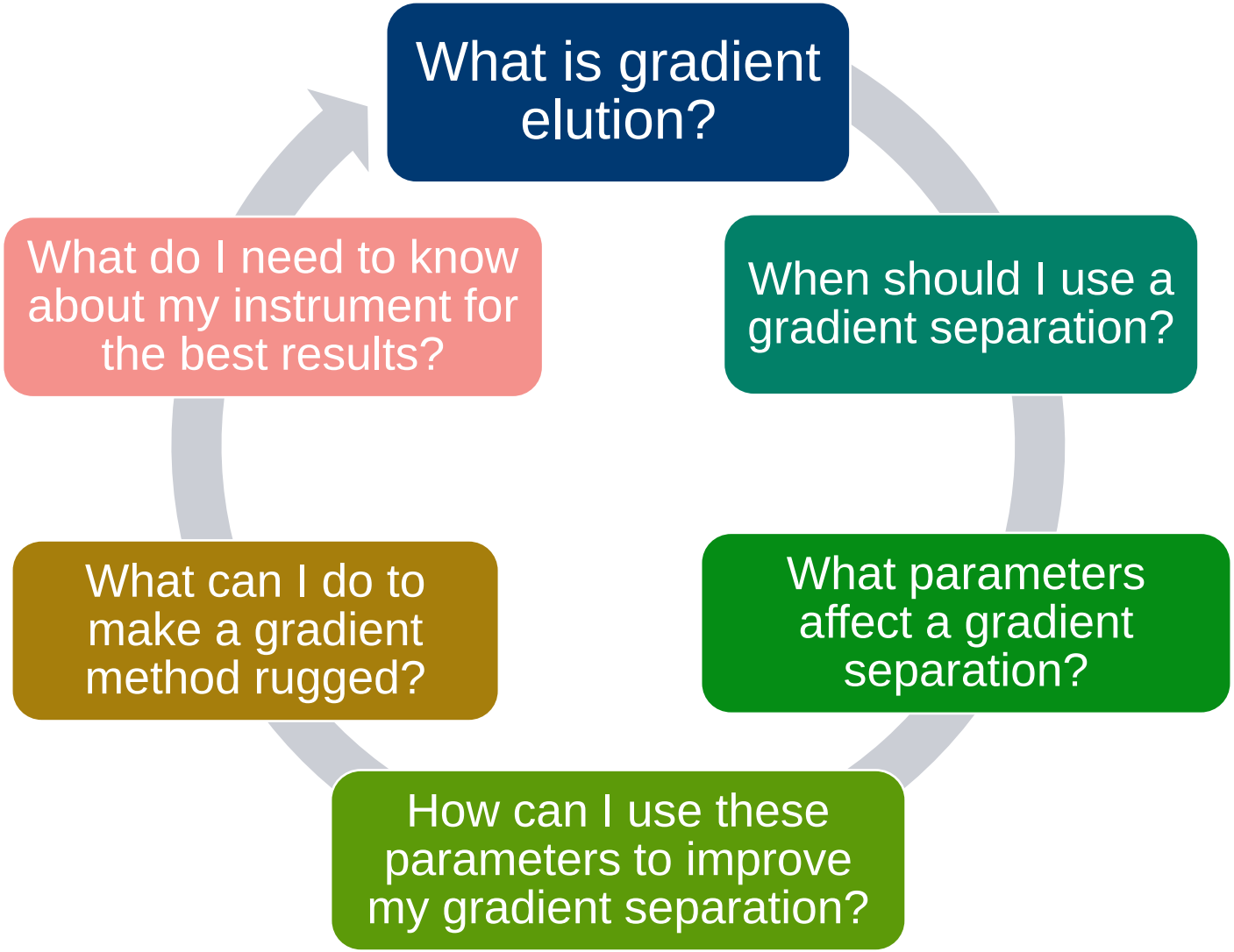
Gradient Design and Development



Gradient Design and Development – Breaking the Bad Habits

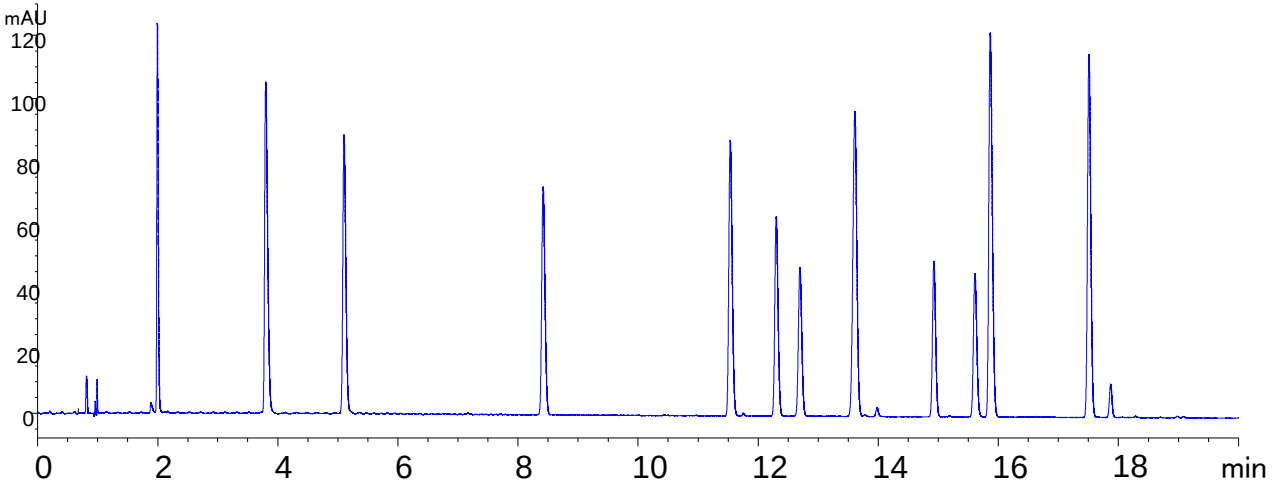


Gradient Elution – So Many Questions!



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 150mm, 2.7um

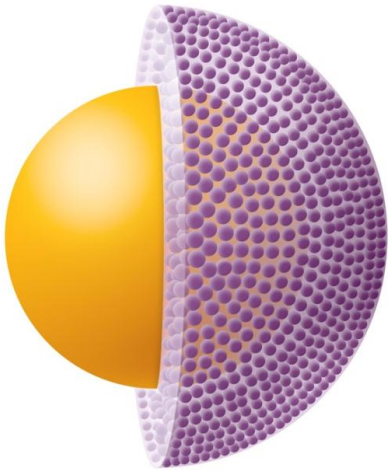
Mobile Phase: Solvent A: Water with 0.1% Formic Acid Solvent B: Acetonitrile

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

Agilent 1200 SL controlled temperature at 25 °C 2 ul flow cell

Benefits of Superficially Porous Column Technologies

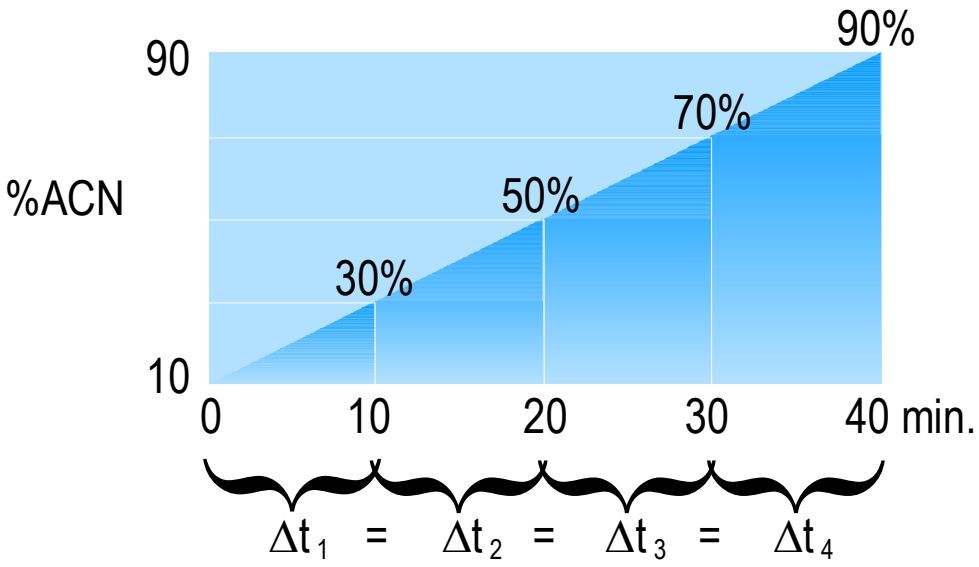
- Increased separation speeds - reduce your analysis time for isocratic and gradient methods
- Use with any instrument - HPLC or UHPLC
- Reduced solvent & waste costs – through shorter methods
- Poroshell 120
  - scalability
  - ease of method transfer
  - similar phases to totally porous ZORBAX columns



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.

3 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)

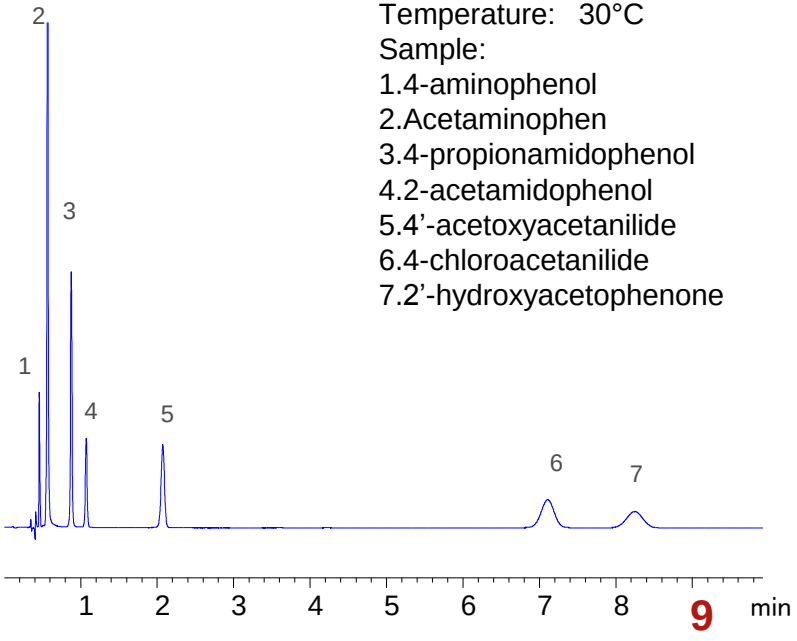
1. Gradient Separation is Faster than Isocratic

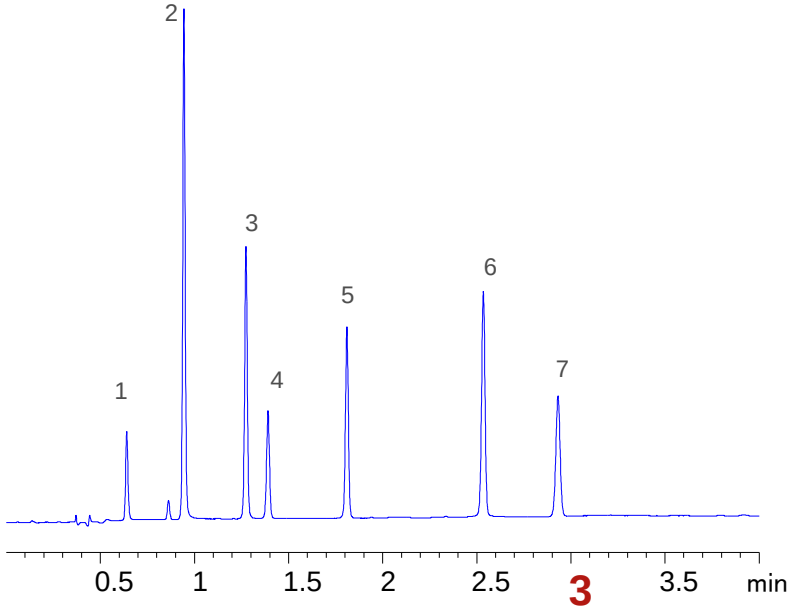
Separation of Acetaminophen Impurities on Poroshell 120 EC-C18

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

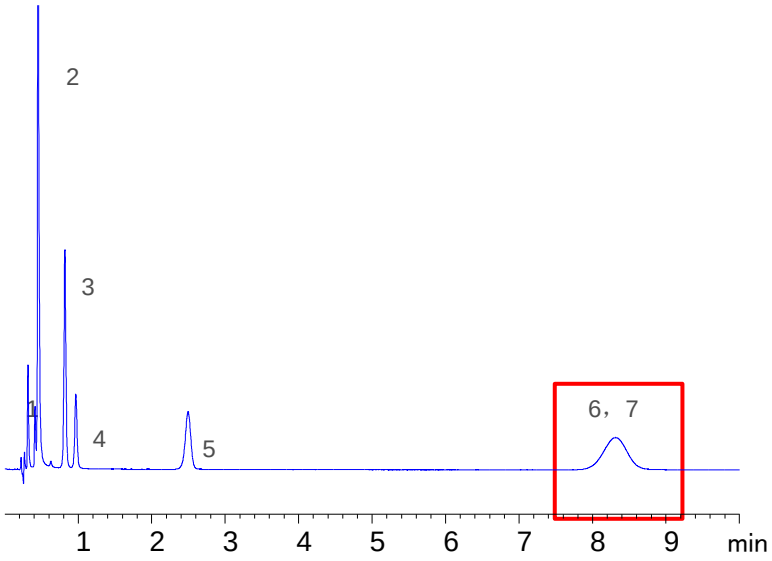




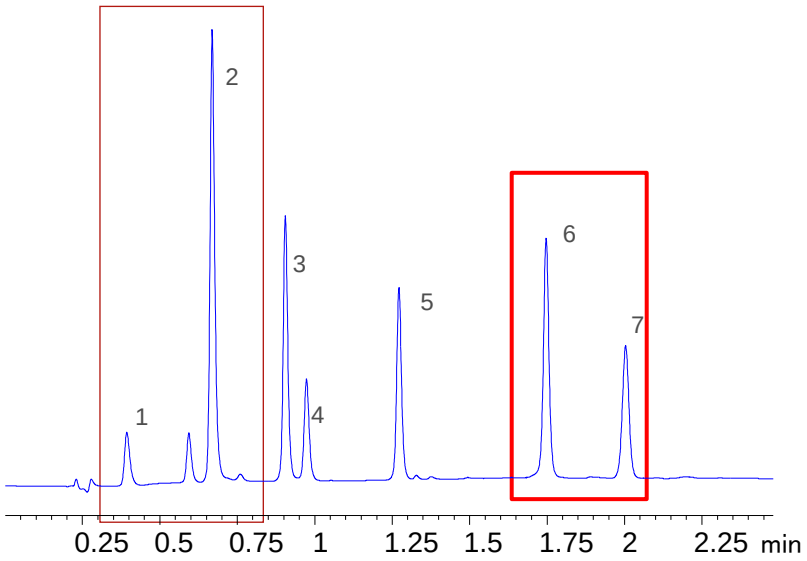
1. Gradient Separation Reduces Analysis Time and Improves Resolution – Even with the Shortest Columns

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

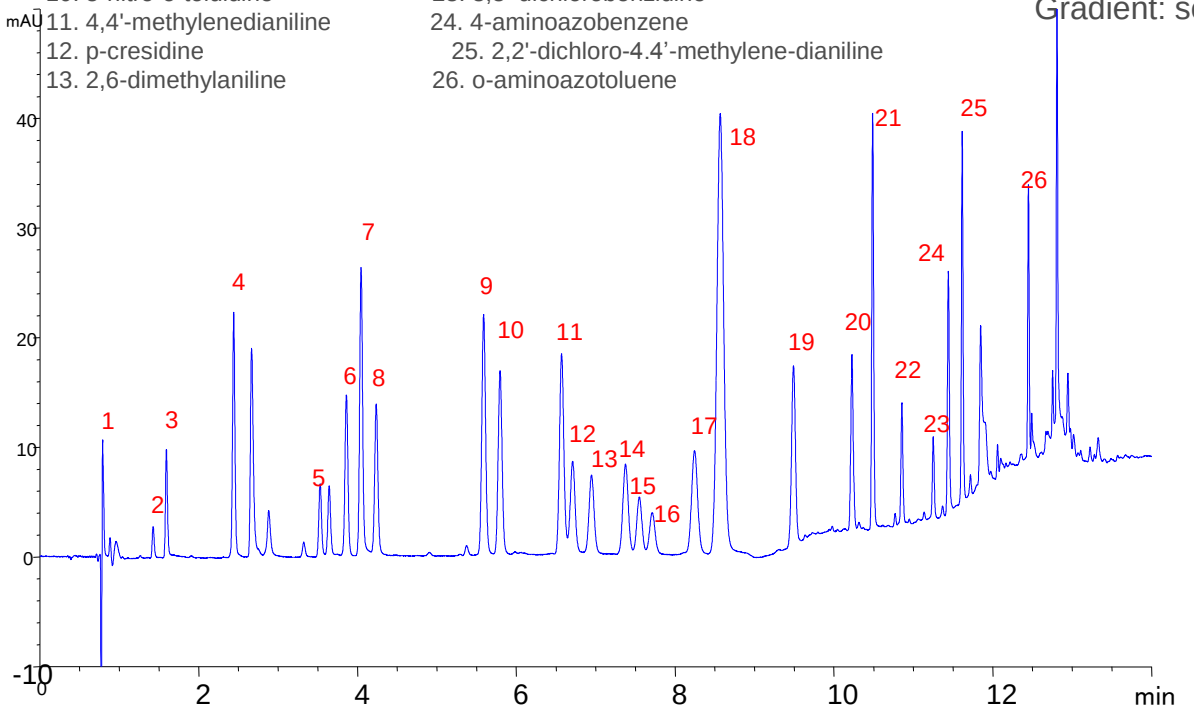


- The gradient analysis is much faster using the same 30mm Poroshell 120 column.
- And resolution is dramatically improved for all peak pairs!

2. Gradient Elution Analysis of a Complex Sample –
EU banned Azo Colorants in Textiles

Sample:

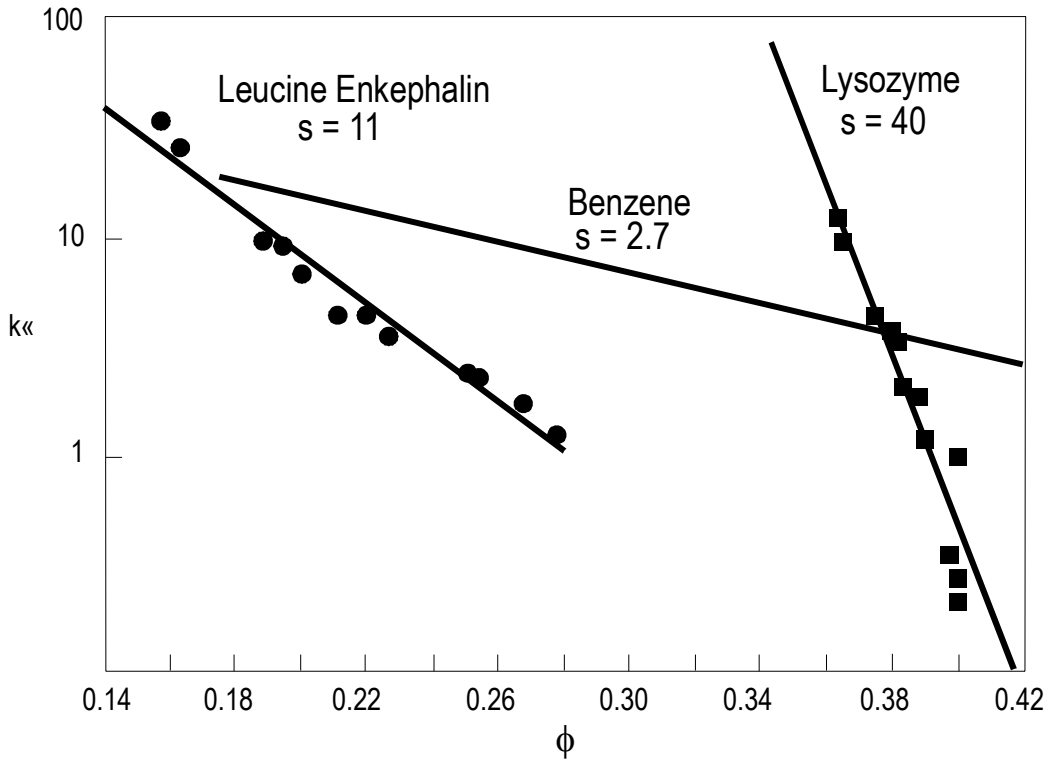
1. 1,4-phenylendiamine
2. 4-methoxy-m-phenylenediamine
3. 4-methyl-m-phenylenediamine
4. Aniline
5. benzidine
6. o-anisidine
7. 4,4'-oxydianiline
8. o-toluidine
9. 4-chloroaniline
10. 5-nitro-o-toluidine
11. 4,4'-methylenedianiline
12. p-cresidine
13. 2,6-dimethylaniline
14. 2,4-dimethylaniline
15. o-dianisidine
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, 3.0×150 mm, 2.7µm (p/n: 693975-302);
Column Temperature：40℃
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 100mL 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

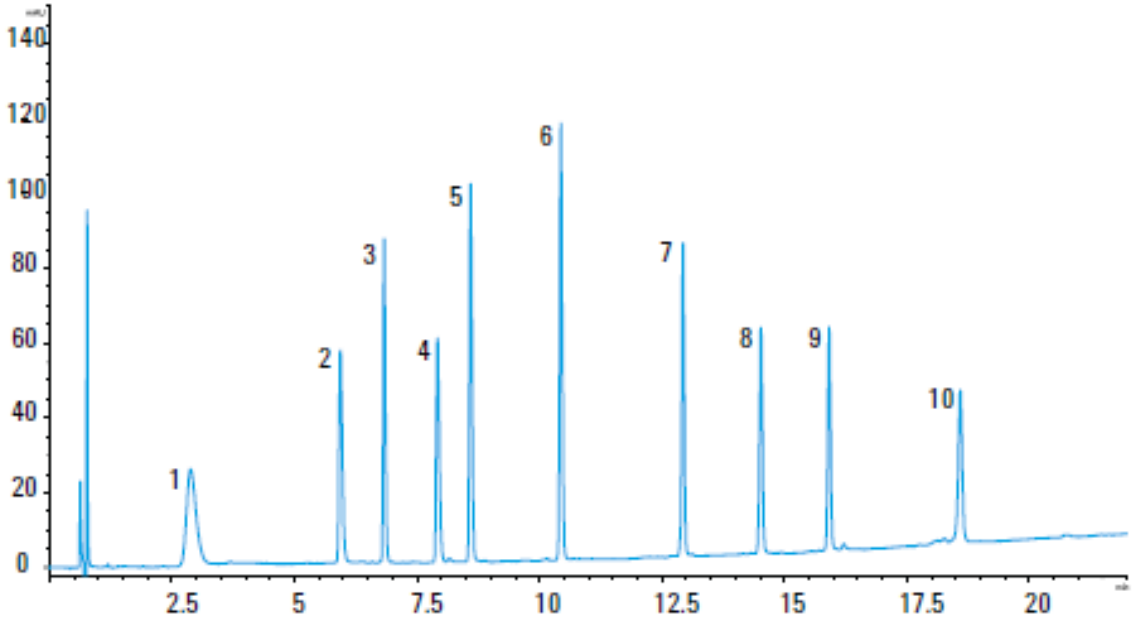
Larger Molecules are More Sensitive than Small Molecules to Changes in % Organic



- Lysozyme is 15X more sensitive to changes in organic modifier than benzene and 4X more sensitive than leucine enkephalin.

3. Gradient Separation of High Molecular Weight Compounds -Peptides on AdvanceBio Peptide Mapping Column

Column: Advance Bio Peptide Mapping 2.1 150 mm, 2.7 µm Gradient: 15-65% B in 25 min.
Mobile Phase: A: 95 : 5 H_2O : ACN with 0.1% TFA B: 5 : 95 H_2O : ACN with 0.1% TFA
Temperature: 55°C Detection: UV 220 nm Sample: Peptides



1. Bradykin fragment (1-7)
2. Angiotensin
3. RNase A
4. Insulin
5. Cytochrome C
6. Lysozyme
7. Myoglobin
8. Carbonic Anhydrase

- Gradient conditions are required for the separation of this 10 peptide mix.
- Molecular weights range from 757 to 2845 Daltons

Advantages of Gradient Elution

- Complex samples are analyzed in a single HPLC run
- Analysis time is reduced
- All peaks elute with the same bandwidth
- More peaks can be baseline resolved per unit time
- Sensitivity and LOD are unchanged during a gradient run

Understanding Gradient Resolution

Quick review of isocratic resolution

What is the gradient resolution equation?

What factors affect gradient resolution?

How do I optimize gradient resolution based on these factors?

Fundamental Resolution Equation – Isocratic Separations

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

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

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

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

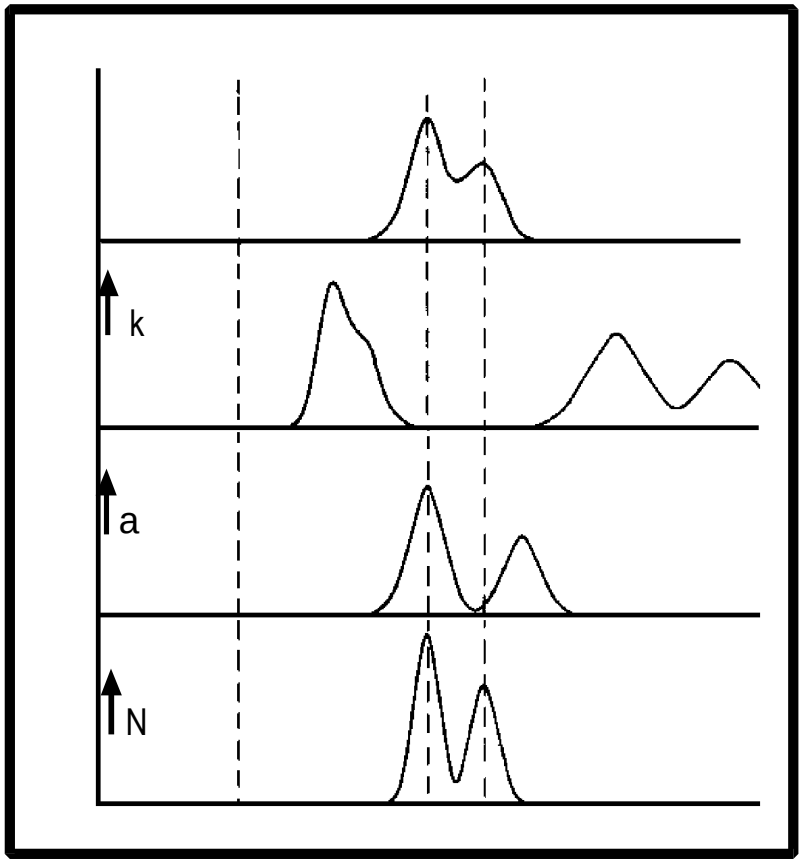
does not improve Rs above k ≈10

Factors that Maximize Isocratic Resolution Between Peaks

Increase retention

Change relative peak position

Reduce peak width

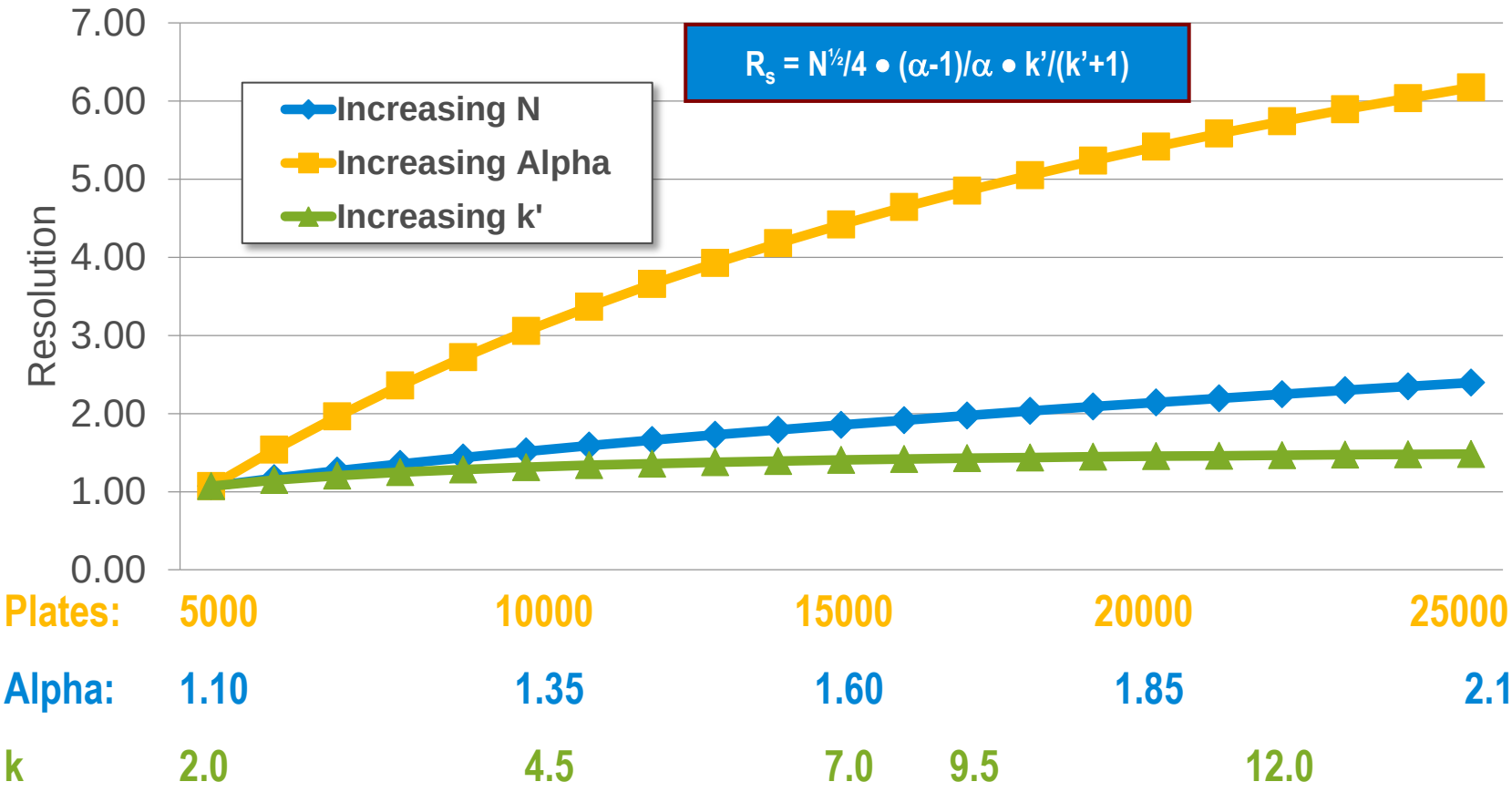


- Decrease % organic

- Change the chemistry of the mobile or stationaryphase
- Change % organic

- Increase column length
- Decrease particle size or flow rate

Selectivity Impacts Resolution the Most



Selectivity Impacts Resolution Most

- Change bonded phase
- Change mobile phase

} Typical Method Development Parameters

- Plates are easiest to increase

Resolution Relationship for Gradient Elution

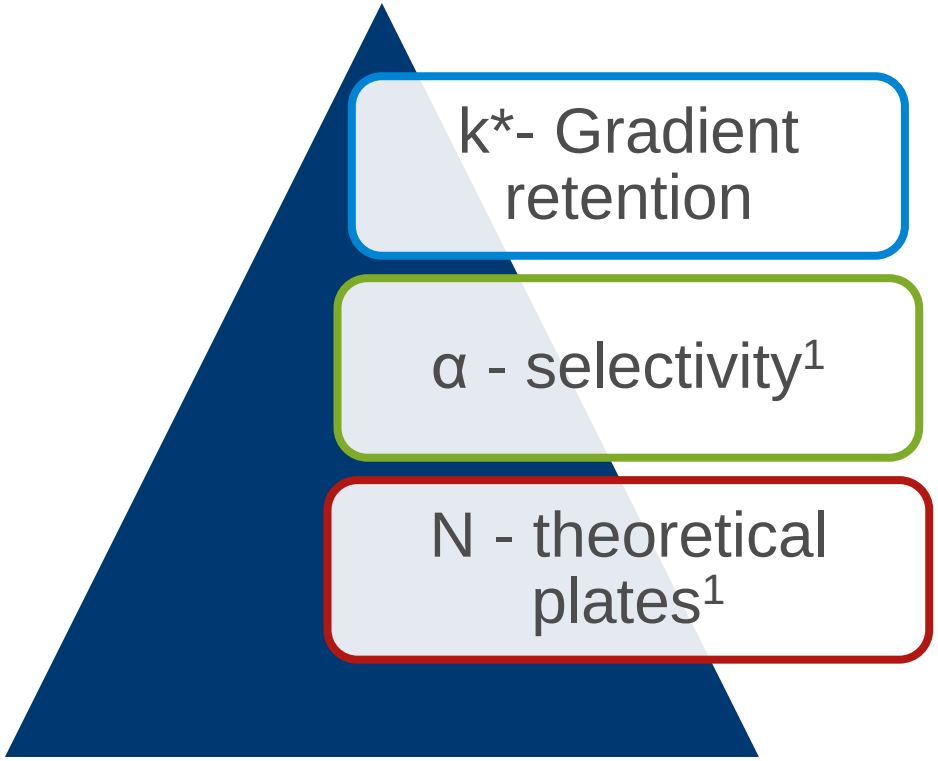
$$R \approx \frac{\sqrt{N}}{4} \alpha k^*$$

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

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

$\Delta\%B$ =	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)

To Maximize Gradient Resolution Between Peaks INCREASE one or more of the following:



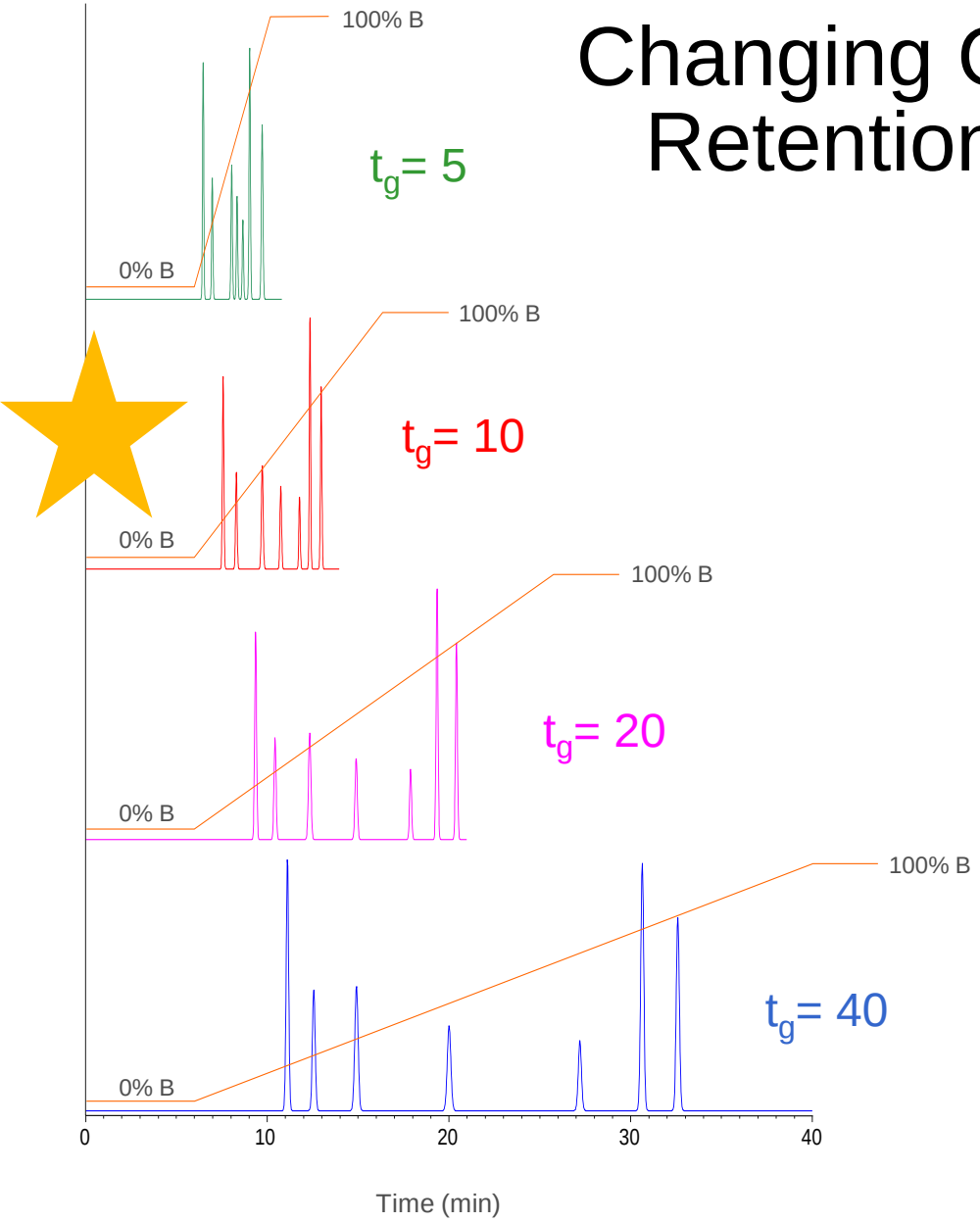
[1]similar to isocratic elution

To Increase Gradient Resolution by Changing Retention (k*) Use:

t_G	• A longer gradient time
Vm	• A shorter column
F	• A higher flow rate
△%B	• A shorter organic range

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

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)

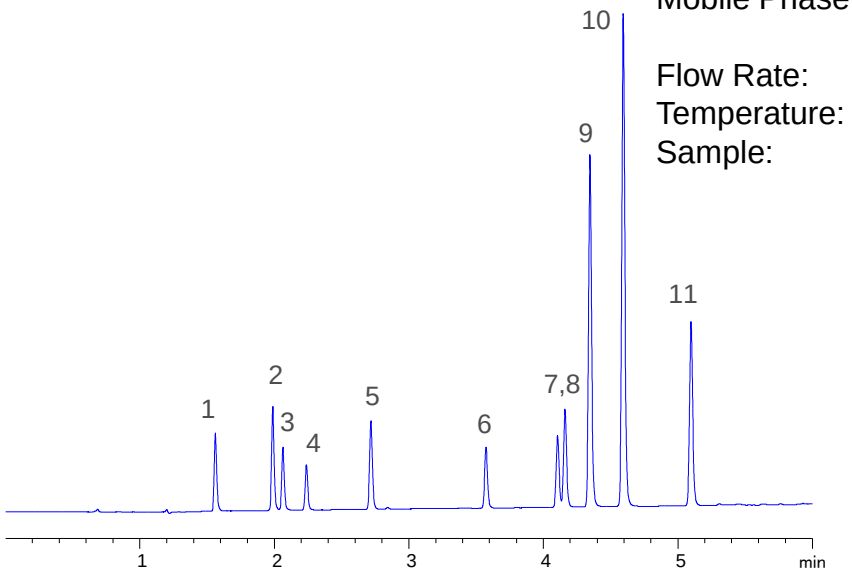
- S ≈ 4–5 for small molecules
- 10 < S < 1000 for peptides and proteins

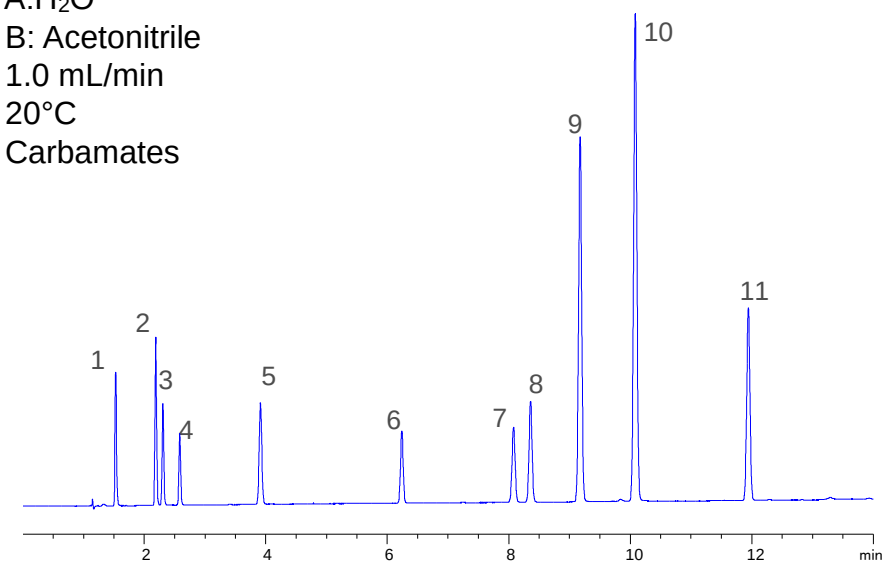
Use of a Longer Gradient Time Increases Gradient Retention

Gradient Time
5 min.

Gradient Time
20 min.

Column: Poroshell 120 EC-C18
4.6 x 100 mm, 2.7 µm
Gradient: 15 – 80% B
Mobile Phase: A:H_2O
B: Acetonitrile
Flow Rate: 1.0 mL/min
Temperature: 20°C
Sample: Carbamates





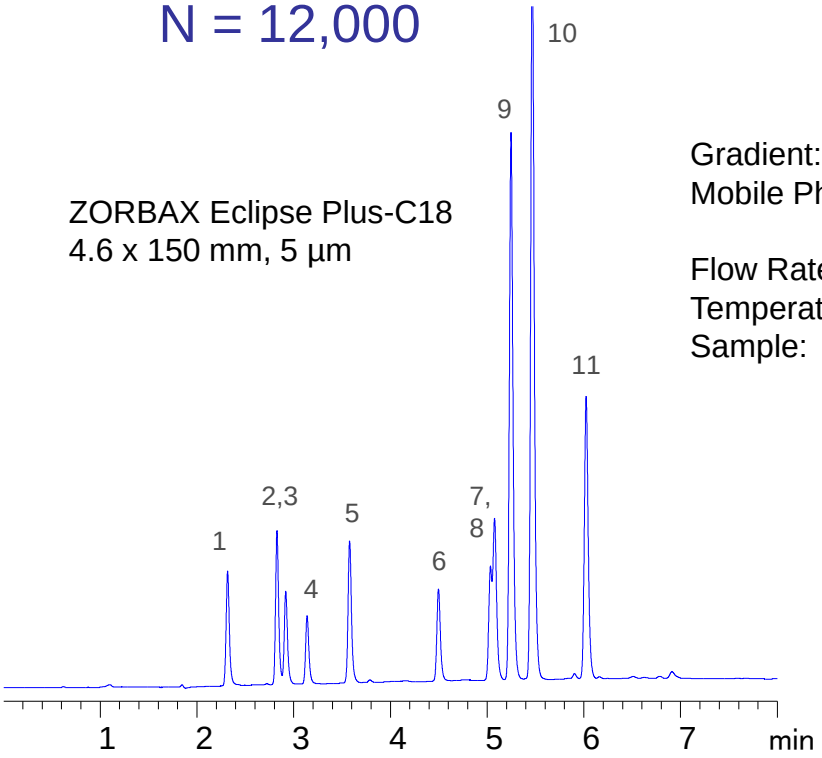
- Increased gradient retention improves resolution of several peak pairs – 2,3 and 7,8.

A Shorter Column (smaller Vm):

Increases Gradient Retention, Increases Overall Resolution, Assumes Nearly Constant N

4.6 x 150 mm, 5 µm
N = 12,000

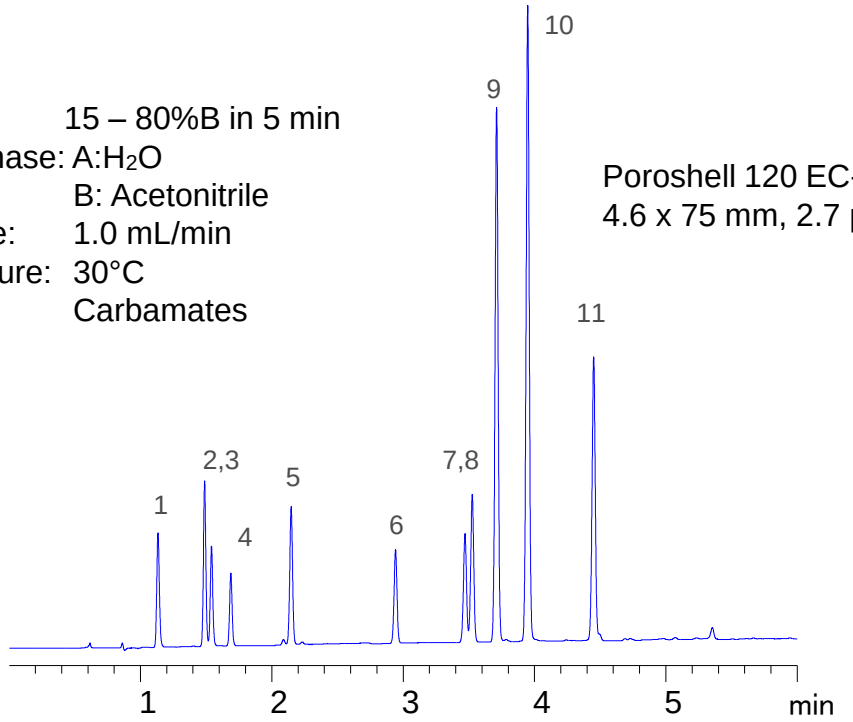
ZORBAX Eclipse Plus-C18
4.6 x 150 mm, 5 µm



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

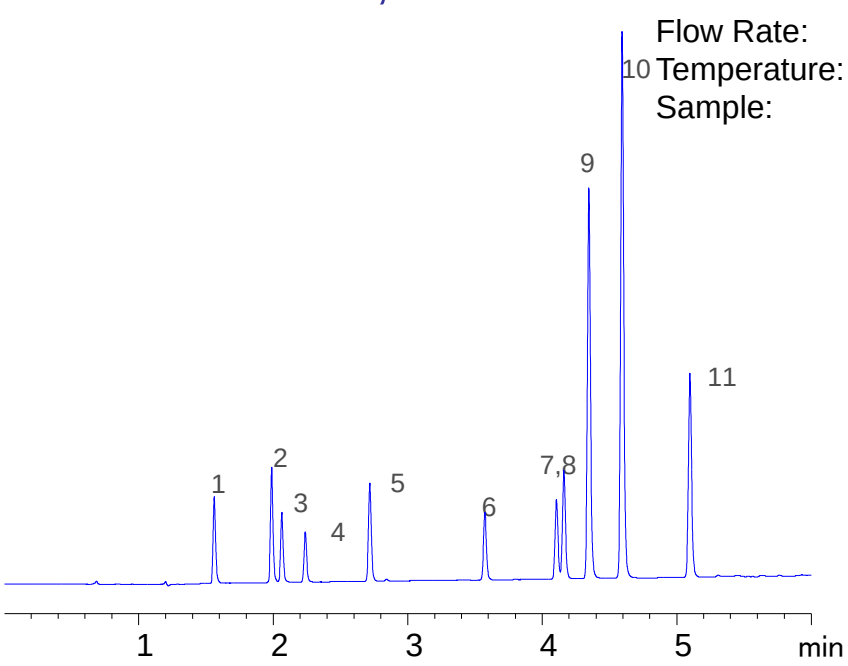


A Shorter Column (smaller Vm):

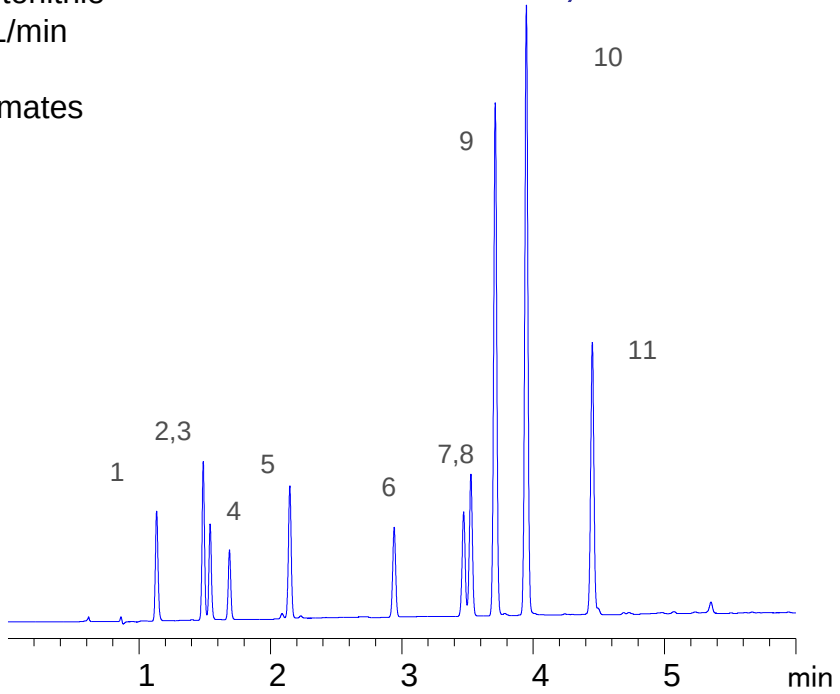
Increases Gradient Retention, Decrease N, getting the same overall resolution

Poroshell 120 EC-C18
4.6 x 100 mm, 2.7 µm
N = 22,000

Gradient: 15 – 80%B in 5 min
Mobile Phase: A:H_2O
B: Acetonitrile
Flow Rate: 1.0 mL/min
Temperature: 20°C
Sample: Carbamates



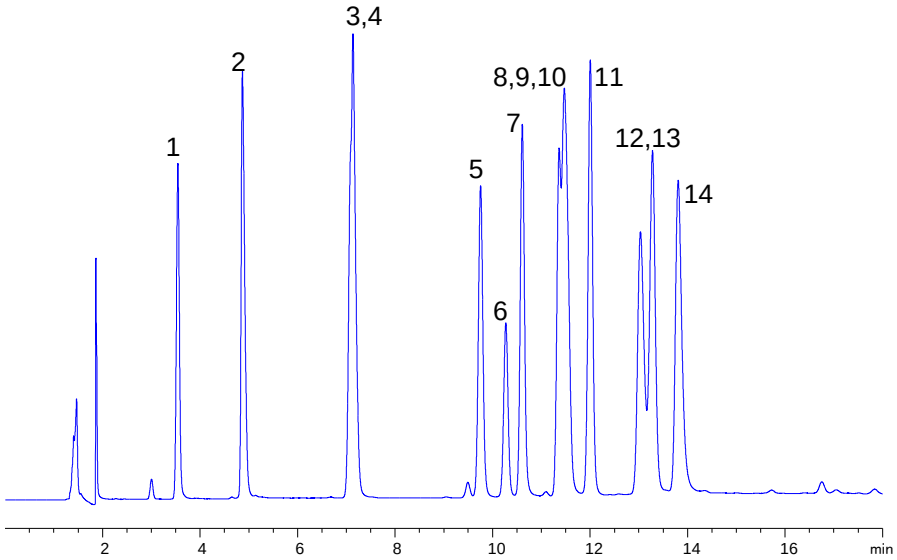
Poroshell 120 EC-C18
4.6 x 75 mm, 2.7 µm
N = 16,000



Improving Resolution Using Short Column Length (Vm) and Particle Size (N)

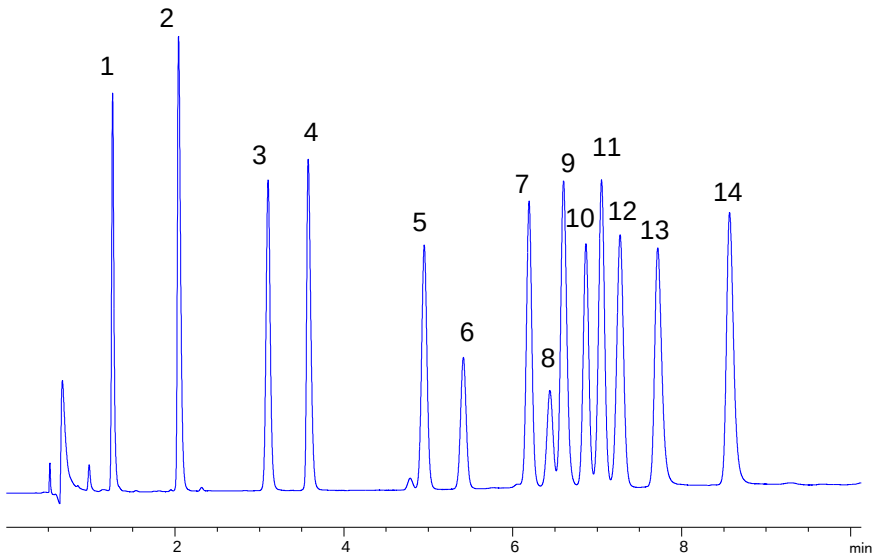
Updating methods with new column types

ZORBAX SB-C18, 4.6 x 150 mm, 5µm



Mobile Phase: A: 0.1% Phosphoric acid in Water
B: ACN
Gradient: 20-60% B in 20 min.
Flow Rate: 1.0 mL / min.
Temperature: 30°C

Poroshell 120 SB-C18, 4.6 x 50 mm, 2.7µm



Sample: 1. Barbital, 2. Chlordiazepoxide, 3. Phenobarbital, 4. Midazolam maleate salt, 5. Nitrazepam, 6. Amobarbital, 7. Oxazepam, 8. Estazolam, 9. Secobarbital, 10. Lorazepam, 11. Clonazepam, 12. Alprazolam, 13. Diazapam, 14. Triazolam

Maintaining k* - To Keep Relative Peak Position in a Chromatogram Unchanged and Shorten Analysis

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

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

Gradient Retention and Gradient Steepness

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

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)

Gradient Steepness “b”:

- Analogous to % organic modifier in isocratic separations
- Gradient steepness decreases, like % organic, retention increases
- When gradient steepness increases, like % organic, retention decreases

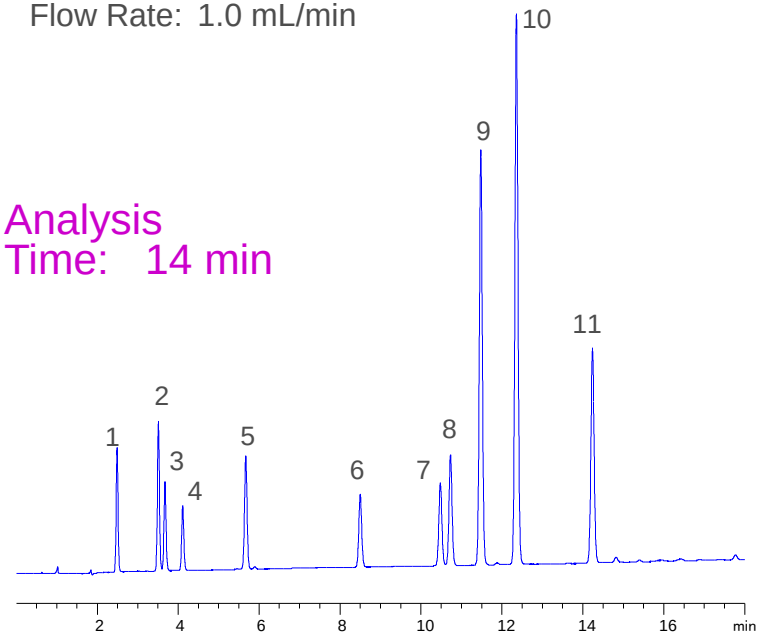
Reduce Analysis Time and Keep Gradient Steepness the Same

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

Gradient Time: 20 min
Flow Rate: 1.0 mL/min

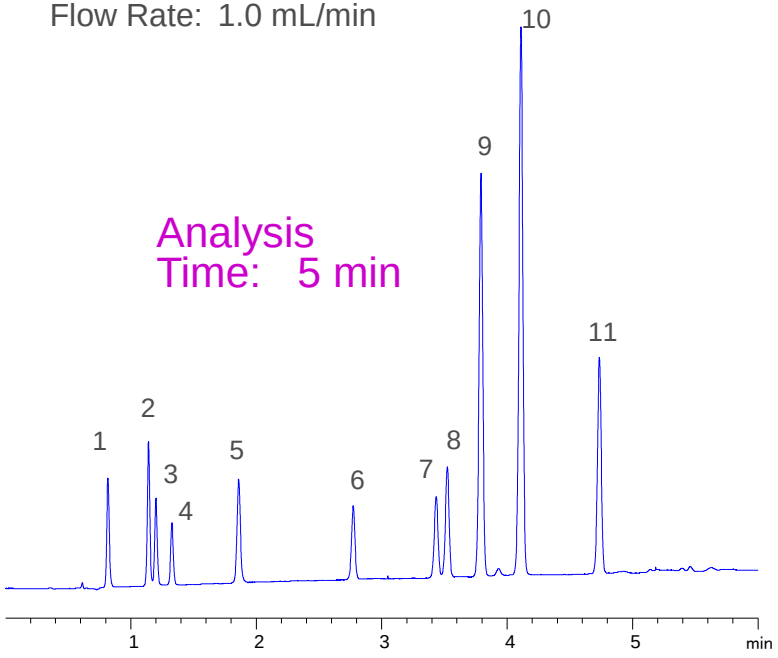
Analysis
Time: 14 min



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

Gradient Time: 6.7 min
Flow Rate: 1.0 mL/min

Analysis
Time: 5 min



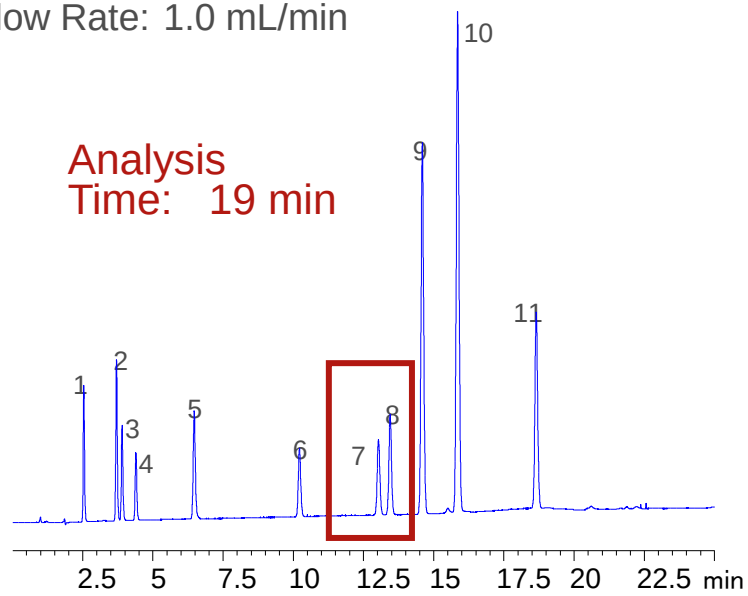
Reduce Analysis Time More! and Keep Gradient Steepness the Same

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

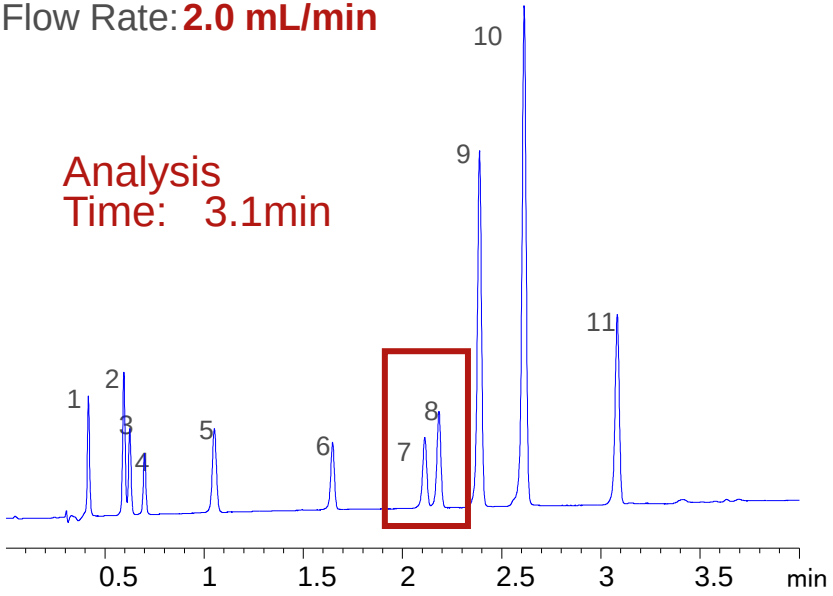
Analysis
Time: 19 min



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

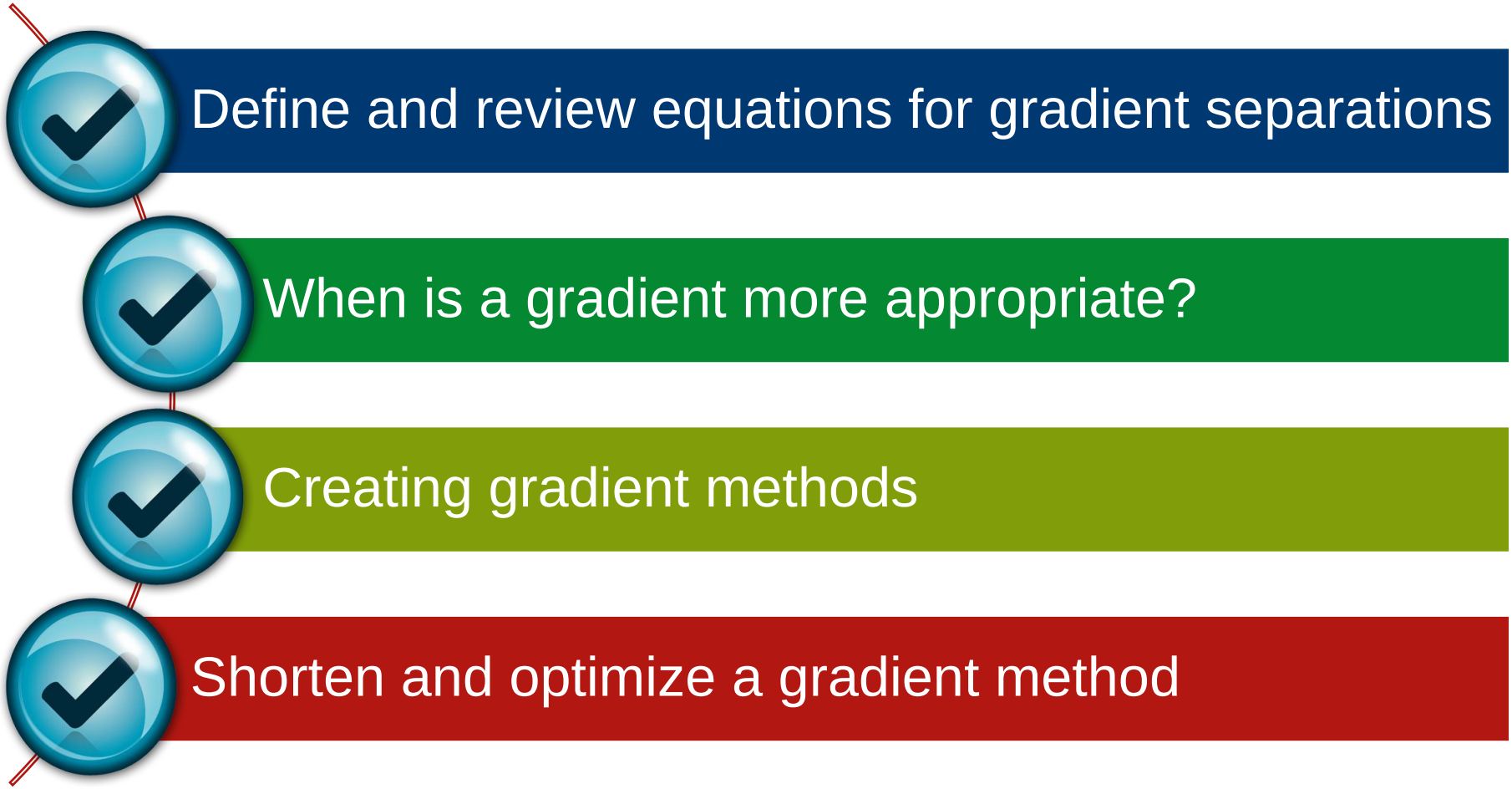
Gradient
Time: **5 min**
Flow Rate: **2.0 mL/min**

Analysis
Time: 3.1min



- Multiple gradient parameters can be changed to maintain gradient resolution and reduce analysis time.

Gradient Design and Development – Breaking the Bad Habits



Conclusions – Gradient Elution Method Development

There are many reasons to use gradients – faster analysis, better separations of complex mixtures

Gradient method development is done by adjusting “gradient parameters” - gradient time, column length, flow rate and gradient range, before changing column and mobile phase

You can start by evaluating the organic range for the separation and optimizing based on the distribution of the peaks.

Short columns with smaller particle sizes (1.8 μm, Poroshell 120 2.7 μm) are used for high-throughput, high resolution gradients