The Equations Behind Your HPLC Separation

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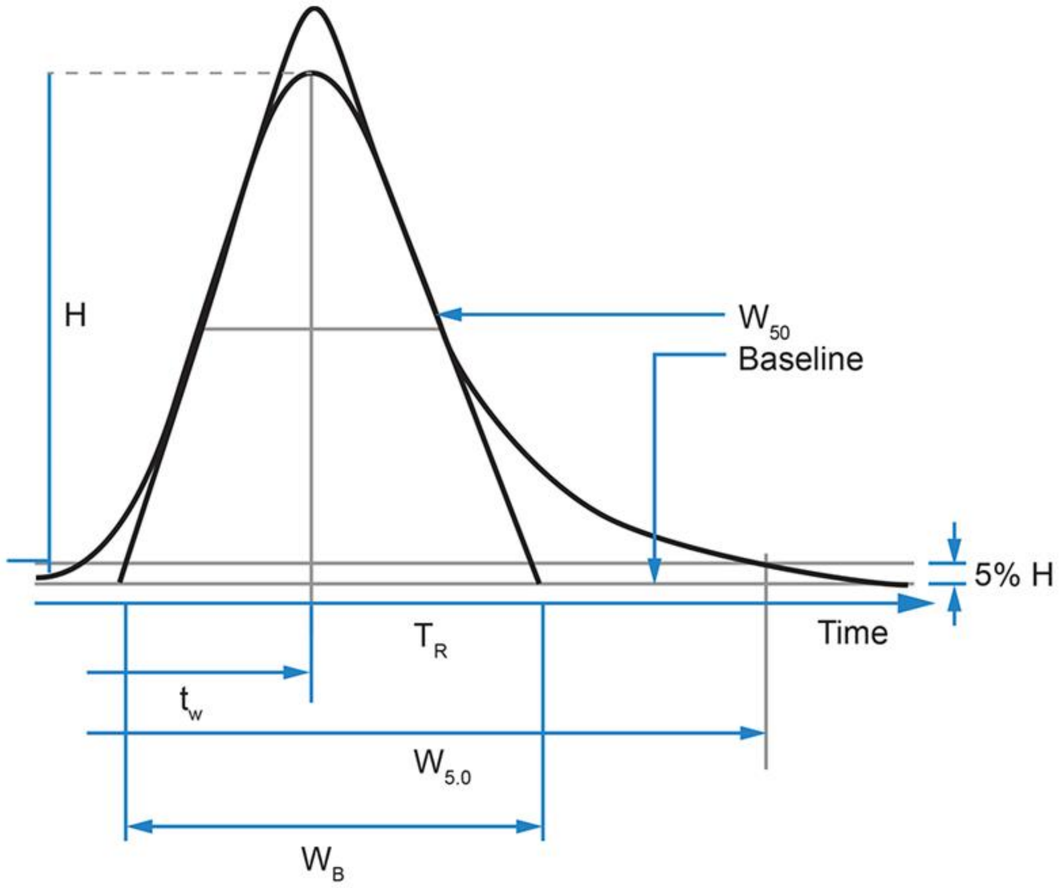
Applications Engineer

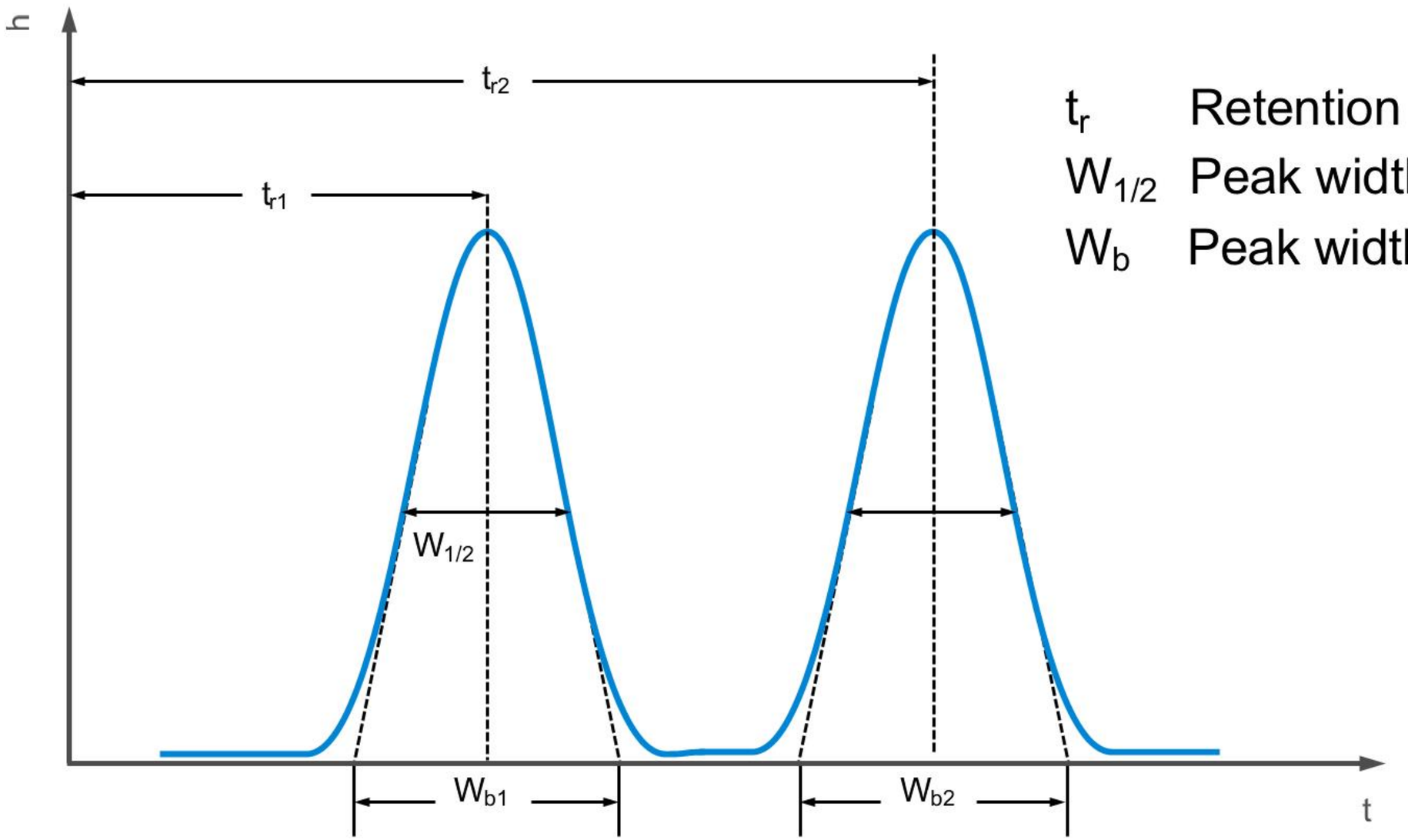
Columns and Supplies Technical Support

Key Parameters

- Efficiency (N)
- Retention (k)
- Selectivity (α)
- Resolution (R_s)
- van Deemter Equation
- Gradient Equation



Key Parameters
Retention Time & Peak Width



t_r Retention time compound
$W_{1/2}$ Peak width at half height
W_b Peak width at baseline

Efficiency

$$N = 16(t_R/\mathrm{w}_b)^2$$

Or

$$N = 5.54\left(t_R/\mathrm{w}_{1/2}\right)^2$$

Columns with high plate numbers are more efficient. A column with high N will have a narrower peak at a given retention time than a column with a lower N number.

N = Efficiency
t_R = Retention time
w_b = Peak width at base
$\mathrm{w}_{1/2}$ = Peak width at ½ height

Efficiency

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

Parameters influencing column efficiency:

- Column length (increasing column length increases efficiency)
- Particle size (decreasing particle size increases efficiency)

Retention Factor

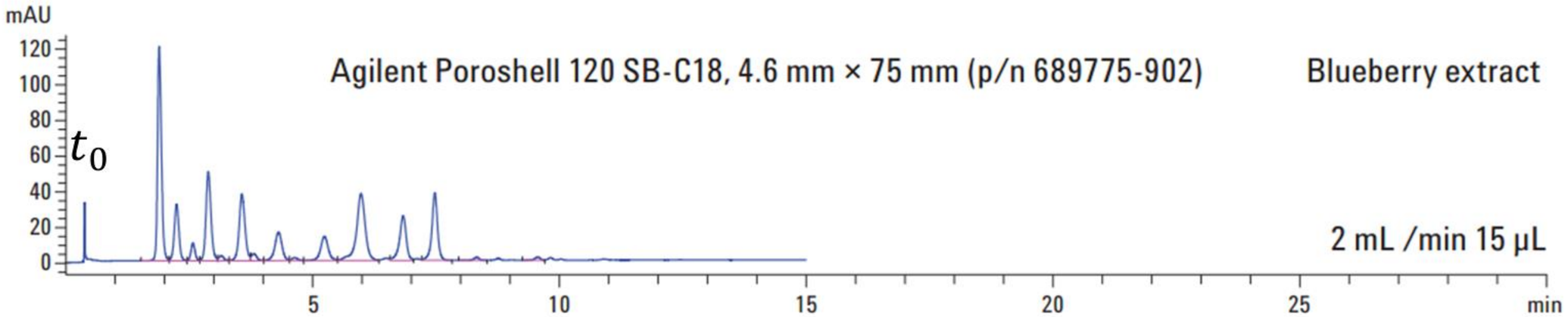
$$k = \frac{(t_R - t_0)}{t_0}$$

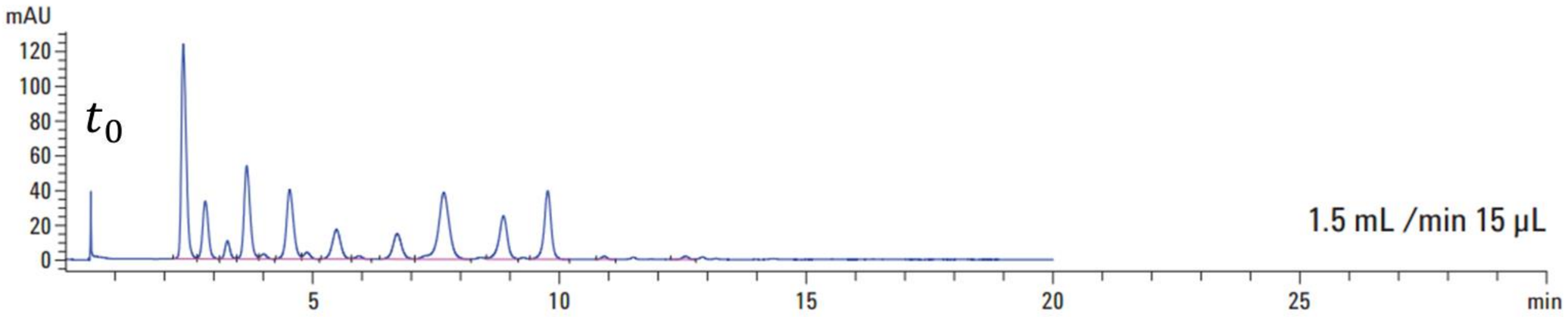
t_R = retention time for sample peak

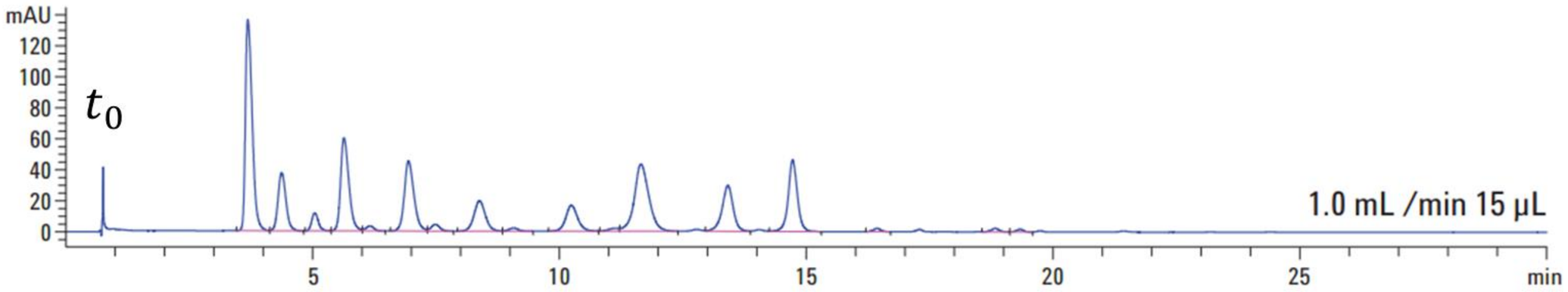
t_0 = retention time for unretained peak

The retention factor measures the period of time that the sample component resides in the stationary phase relative to the time it resides in the mobile phase. It is calculated from the retention time divided by the time for an unretained peak.

Void Time







$$t_0 \approx V_m * F$$

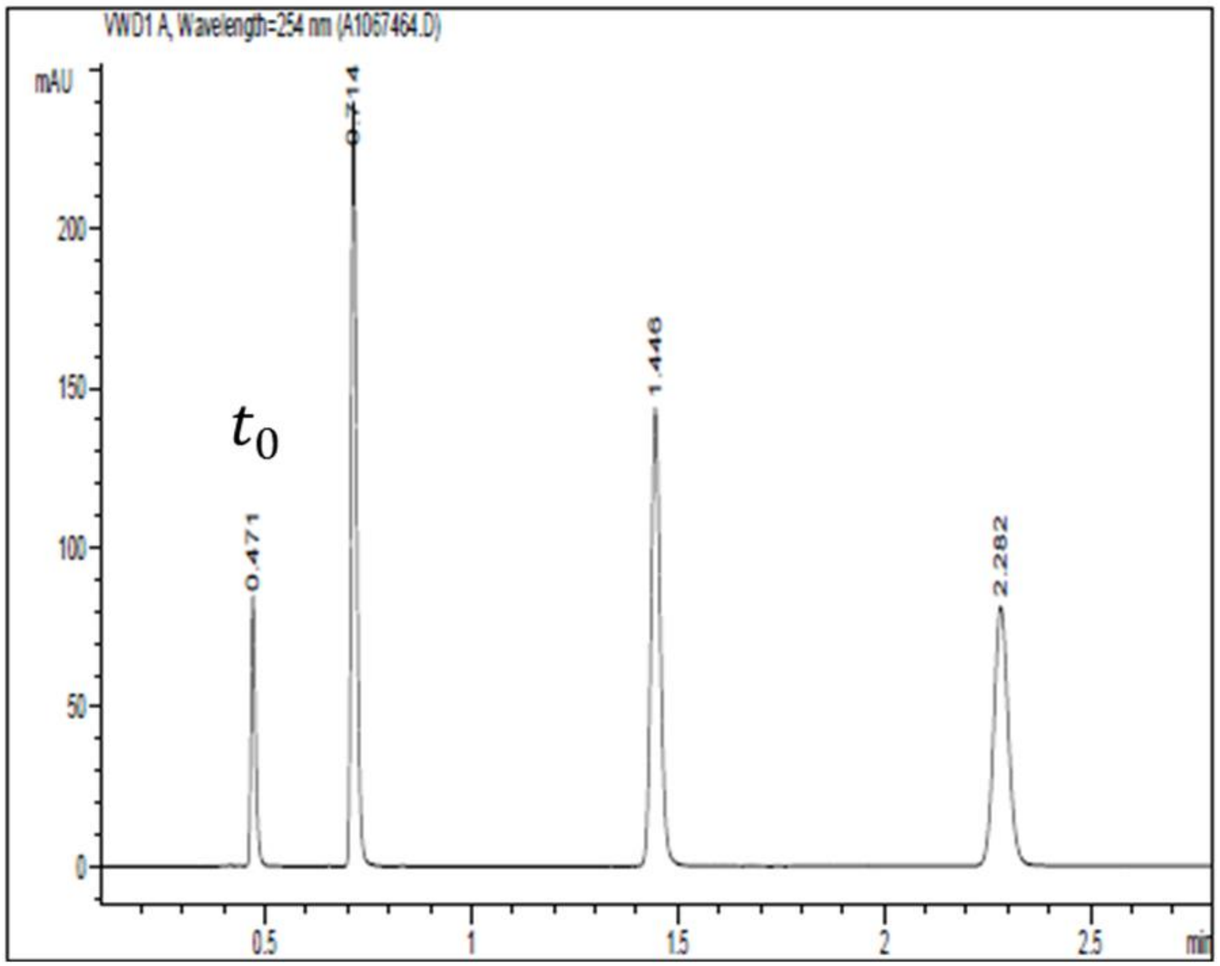
t_0 is also estimated by column volume * flow rate

Void Time

PART NUMBER: 695975-302
COLUMN TYPE: Poroshell 120 EC-C18 3 x 100 mm, 2.7 µm

TEST CONDITIONS

MOBILE PHASE	=	60% Acetonitrile / 40% Water
COLUMN PRESSURE	=	274.8 Bar
COLUMN FLOW	=	0.80 ml / min
LINEAR VELOCITY	=	0.354 cm / sec
TEMPERATURE	=	AMBIENT (Nominally 23 °C)
INJECTION VOLUME	=	2 µl



Sample components with concentrations diluted in mobile phase in the following elution order.

Peak #	Conc (ug/ml)	Sample Component
1	10	Uracil
2	400	Phenol
3	50	4-Chloro Nitrobenzene
4	80	Naphthalene

Column Volume

$$V_m = \pi \cdot r^2 \cdot L \cdot \sim 0.6$$

Column volume is calculated as the volume of a cylinder less the space occupied by the packing material. As an example, Agilent ZORBAX Eclipse Plus C18 packing material occupies 40% of the column, the remaining 60% of the cylinder would be considered as column volume.

Separation Factor or Selectivity

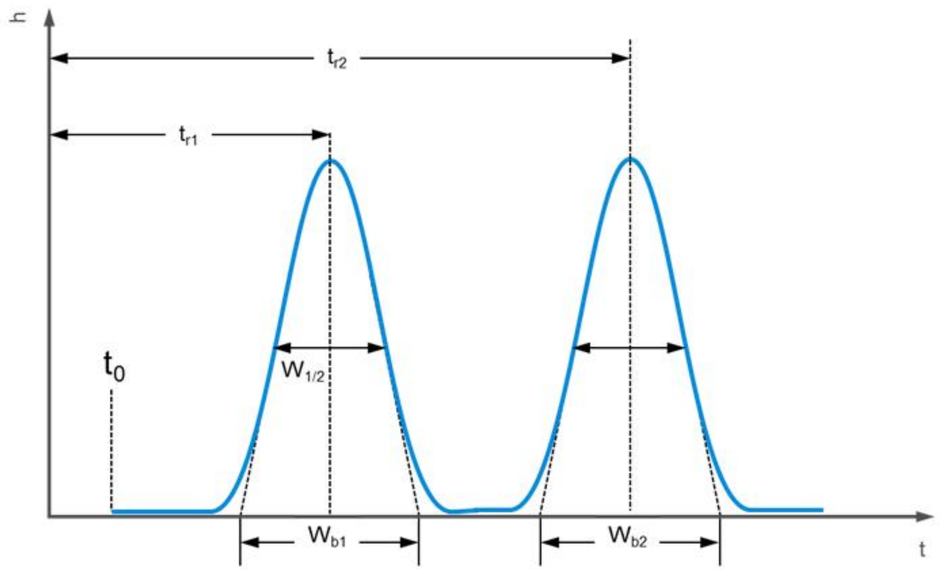
$$\alpha = \frac{k_2}{k_1}$$

α	Selectivity
k_1	Retention factor of 1st peak
k_2	Retention factor of 2nd peak

Selectivity is a measure of the time or distance between the maxima of two peaks. If α = 1, the two peaks have the same retention time and co-elute.

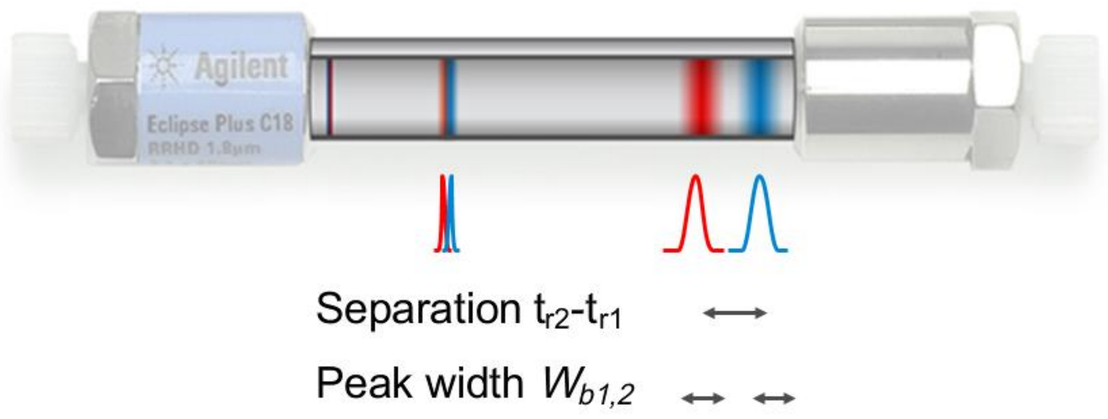
Parameters influencing selectivity:

- Stationary phase
- Mobile phase
- Temperature



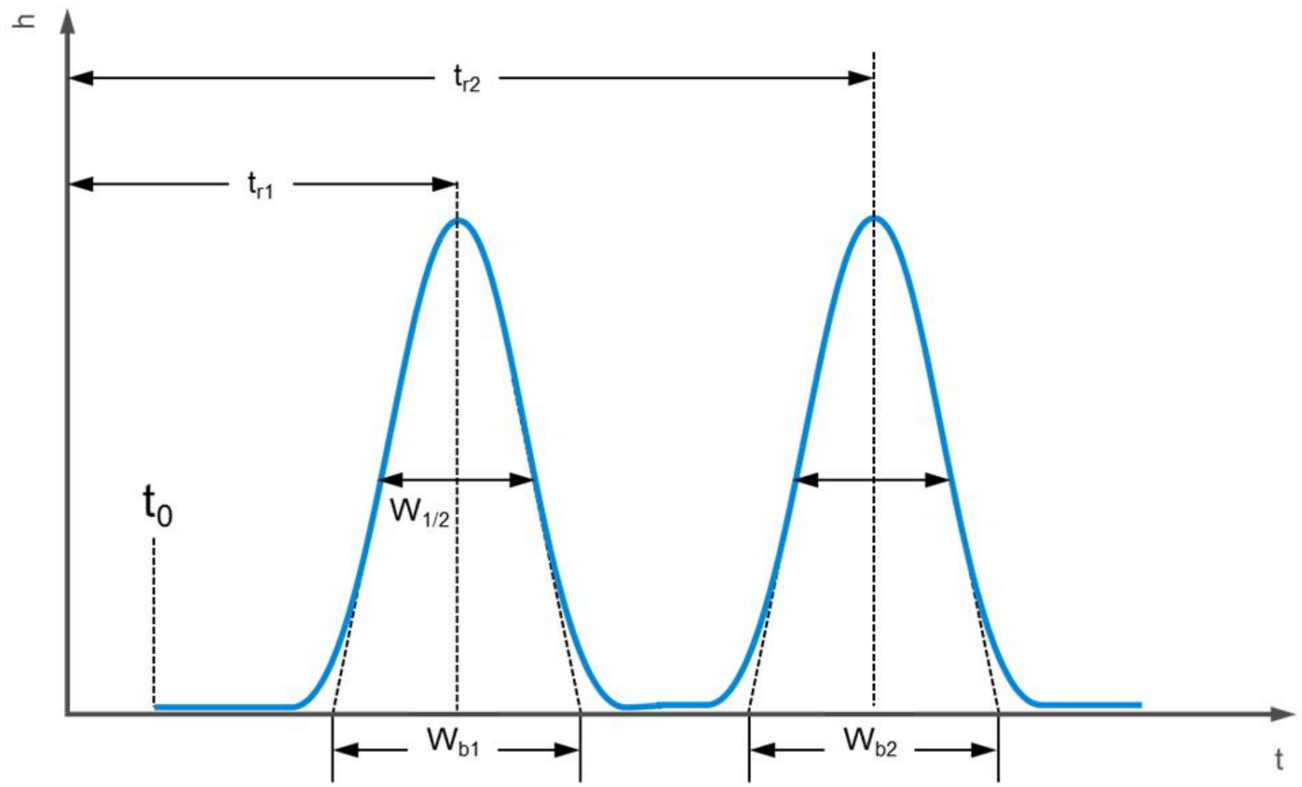
Resolution

$$R_s = \frac{t_{r2} - t_{r1}}{1/2 \cdot (W_{b2} + W_{b1})}$$



Resolution describes the ability of a column to separate the peaks of interest.

Resolution describes whether you have achieved base line separation or not.



Resolution Equation

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

Efficiency **Selectivity** **Retention**

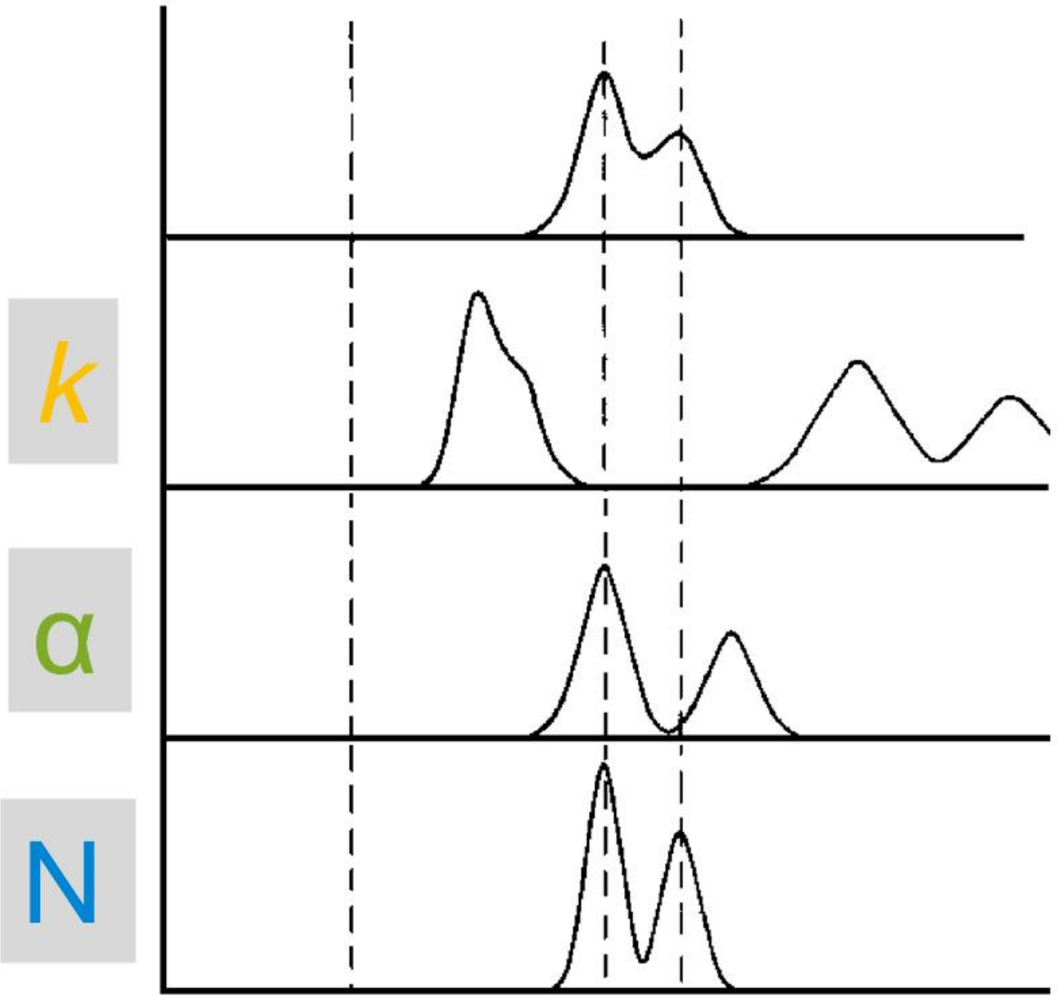
Improve resolution by improving any of these parameters:

- Selectivity has the highest influence on the resolution. Small changes in selectivity can lead to big changes in resolution
- Retention has only a significant influence at small k values
- Efficiency describes the separation power of the column

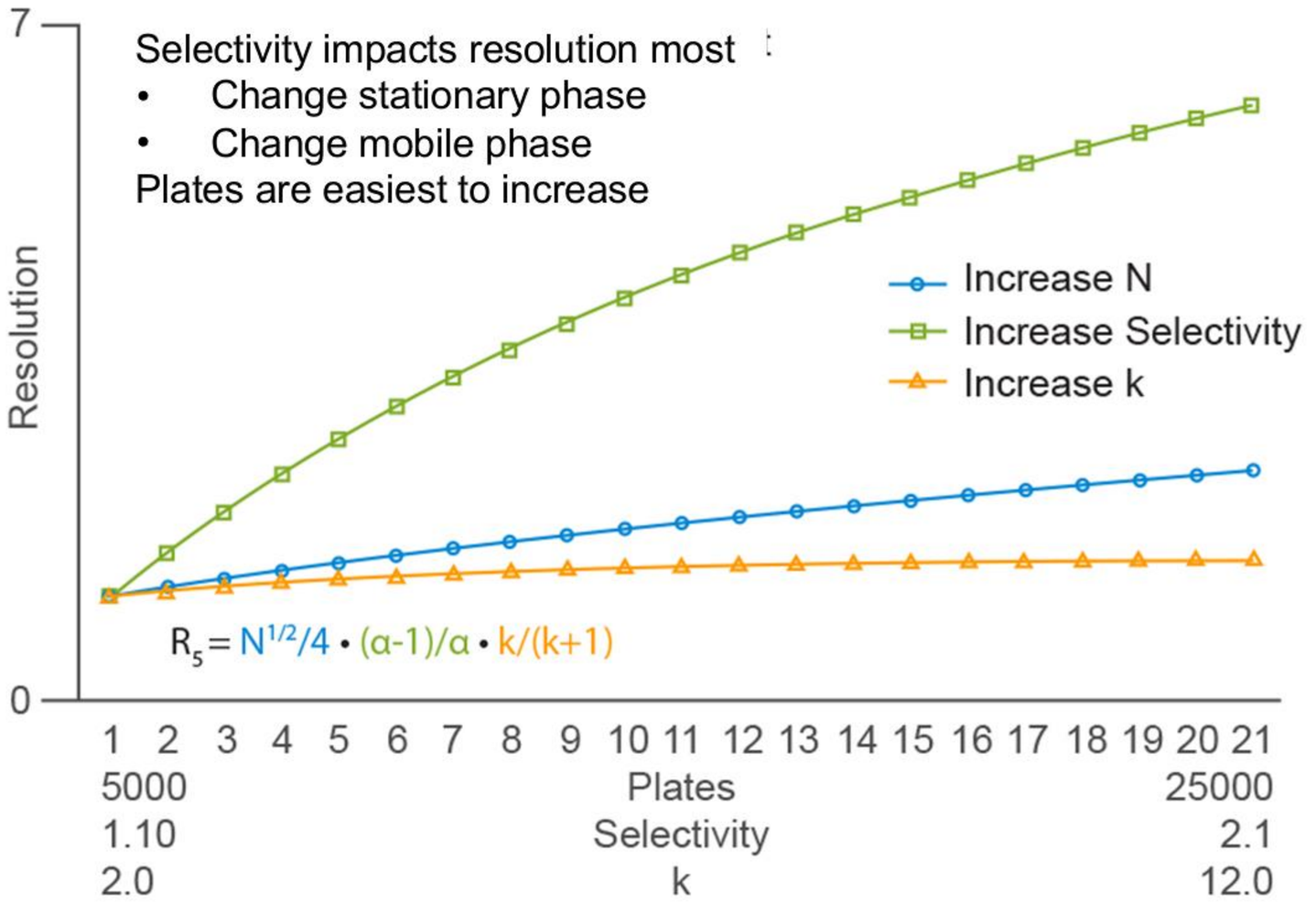
Factors that Affect Resolution

$$R_s = \tfrac{1}{4}\sqrt{N} \cdot \left(\frac{\alpha - 1}{\alpha}\right) \cdot \left(\frac{k}{1+k}\right)$$

Efficiency Selectivity Retention

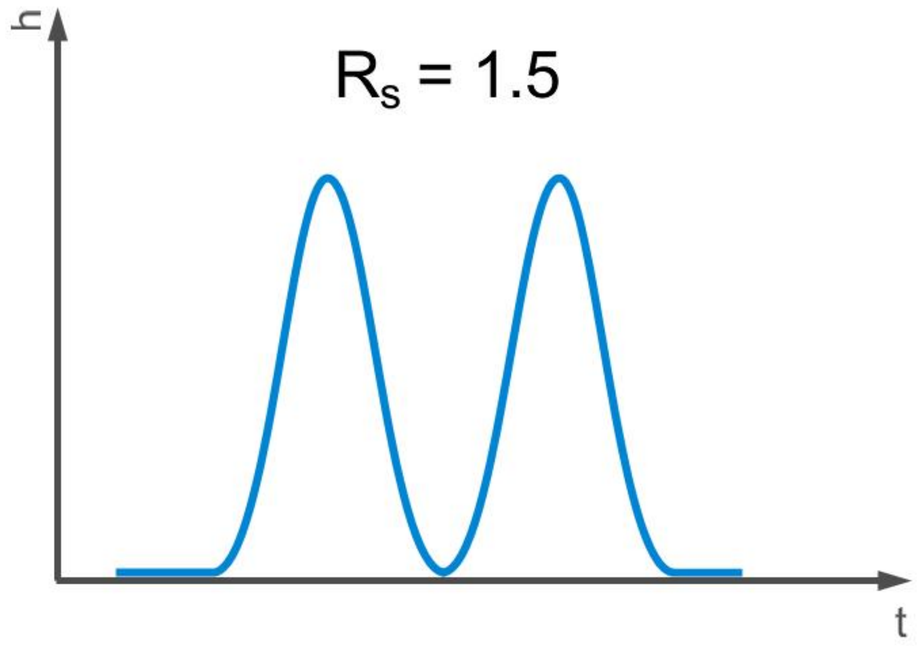


Resolution



- Resolution as a function of selectivity, column efficiency or retention
- If you double the column length, you will obtain more theoretical plates
- But you only get a square root of 2, or 1.4x improvement in the resolution

Resolution
Baseline Separations



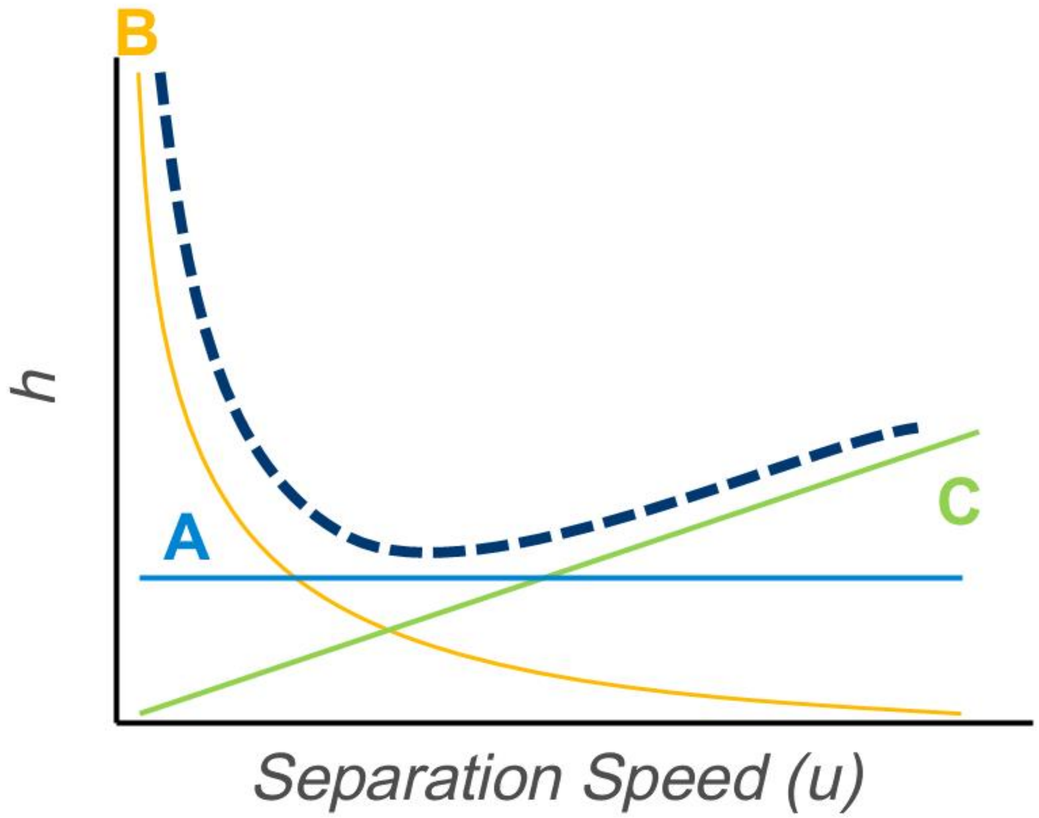
If we consider peaks of equal height:

- 1 - minimum for a measureable separation
- 0.6 - required to discern a valley between two equal-height peaks
- 1.5 - considered to be a baseline separation
- 1.7 or greater - desirable for rugged methods

Van Deemter Equation

$$h = L/N$$

$$h = A + B/u + C \cdot u$$



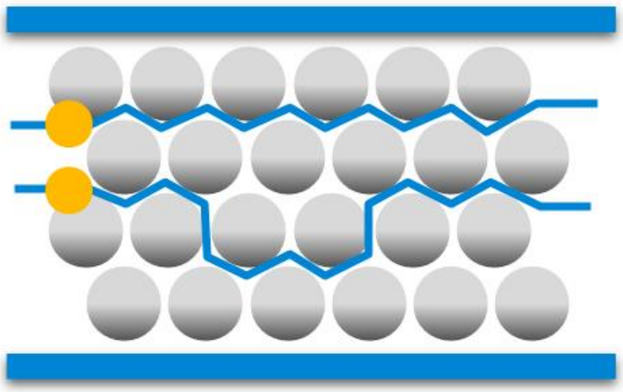
- **A term: eddy diffusion and flow distribution**
  - particle size & packing quality important
  - narrow particle size distribution
- **B term: longitudinal diffusion**
  - Diffusion in the mobile phase
- **C term: mass transfer**
  - shorter diffusion paths
  - better with superficially porous particles
  - more effect on large molecules
- ***u*: linear velocity**
  - velocity of mobile phase through column
  - $u = L/t_0$ in cm/sec

Lower *h (reduced plate height)* = higher efficiency

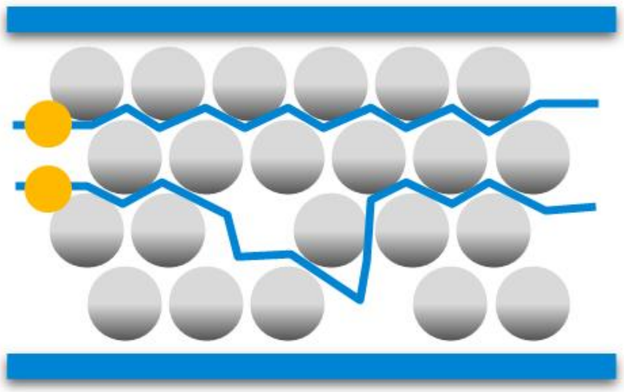
Van Deemter Equation
Eddy Diffusion

$w_{eddy} \sim \lambda\ d_p$ λ: Quality of column packing

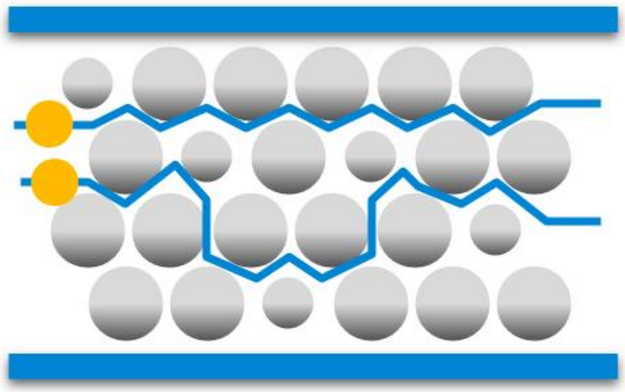
Differences in diffusion paths due to:



Different paths



Poor column packing



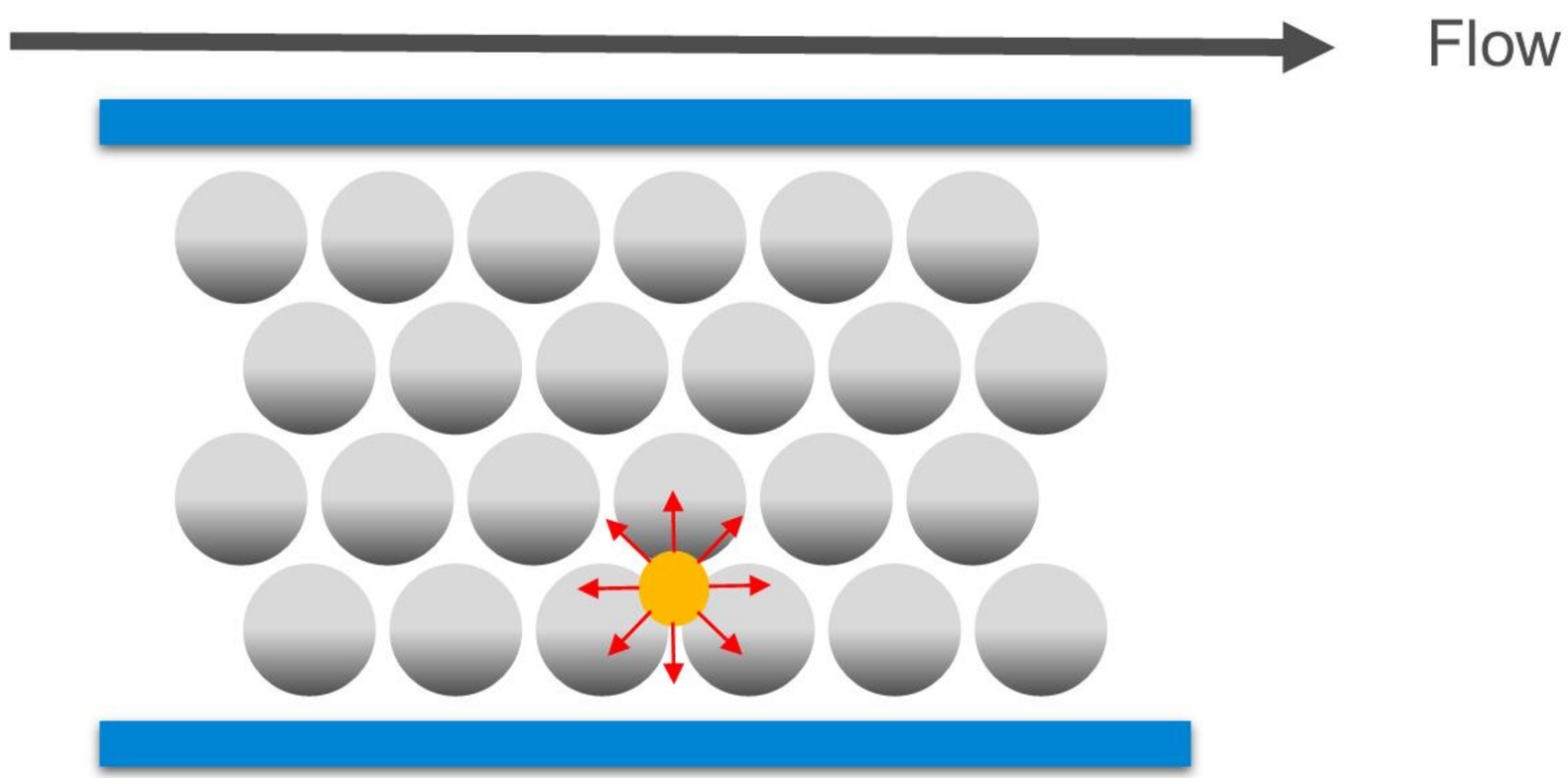
Broad particle size distribution

Van Deemter Equation
Axial or Longitudinal Diffusion

Increase in peak width due to self-diffusion of the analyte

At low flow the analyte remains in the mobile phase for a long time

- High increase in peak width
- Increased height of a theoretical plate



Van Deemter Equation
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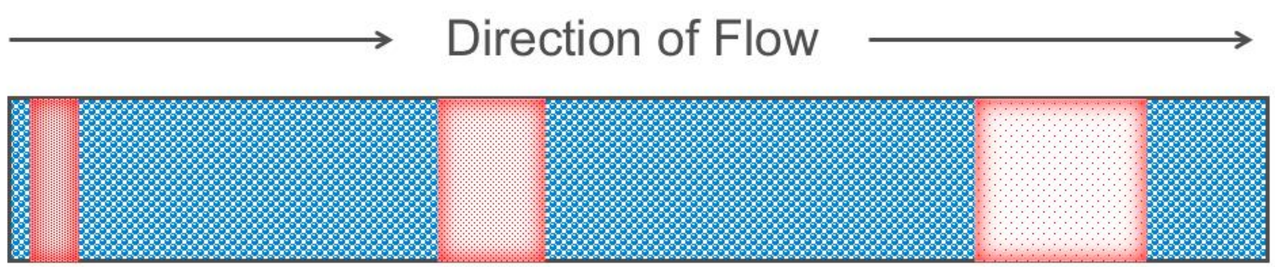
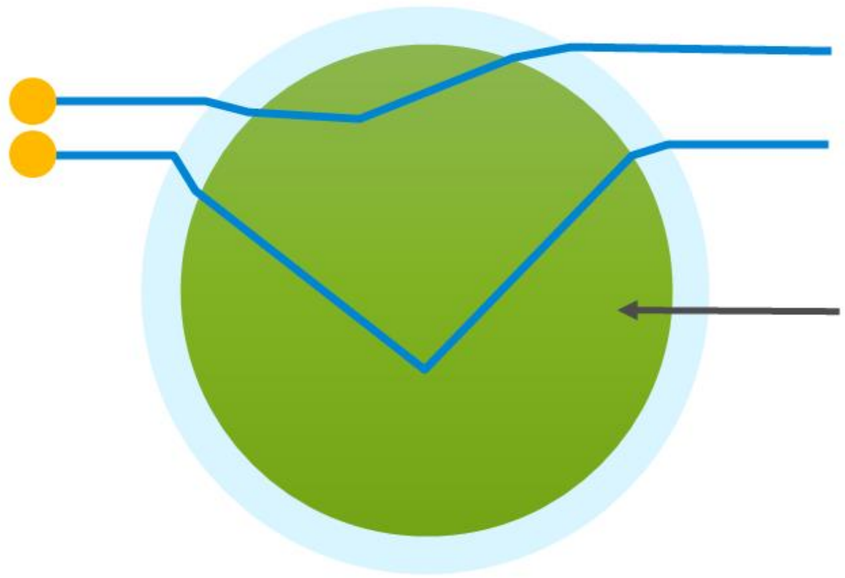


Van Deemter Equation
Resistance to Mass Transfer

$$w_C \sim d_p^2$$

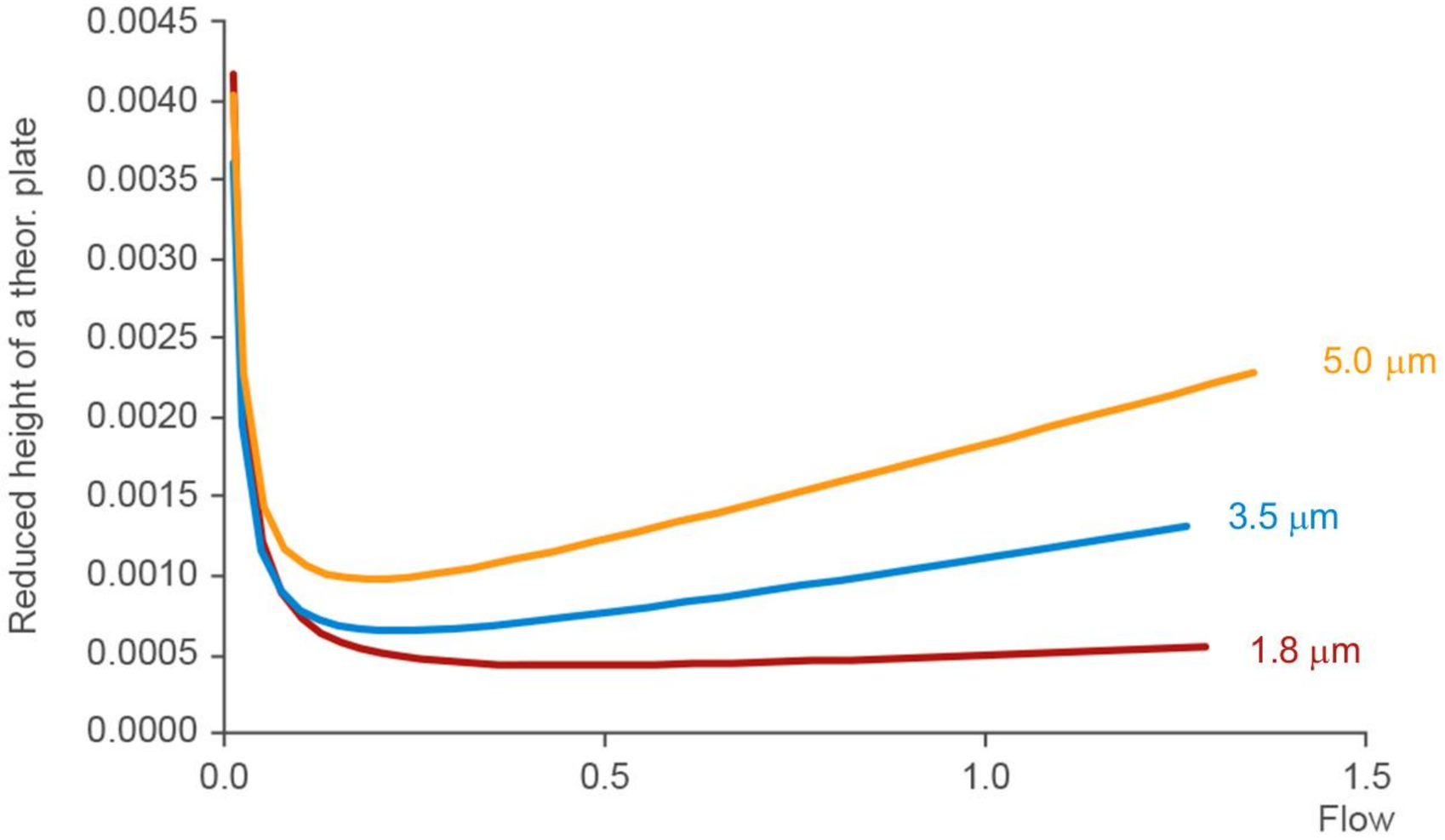


Different diffusion paths

Porous particle

Van Deemter Equation

Measured for Different Particle Sizes



- Small particles lead to lower plate heights and therefore higher separation efficiency
- For smaller particles the separation efficiency suffers less when increasing the flow

Pressure Drop across an HPLC column

$$\Delta P = \frac{\eta \cdot L \cdot v}{\theta \cdot d_p^2}$$

Shorter column length and larger particle diameter reduce column pressure

ΔP = Pressure Drop

η = Fluid Viscosity

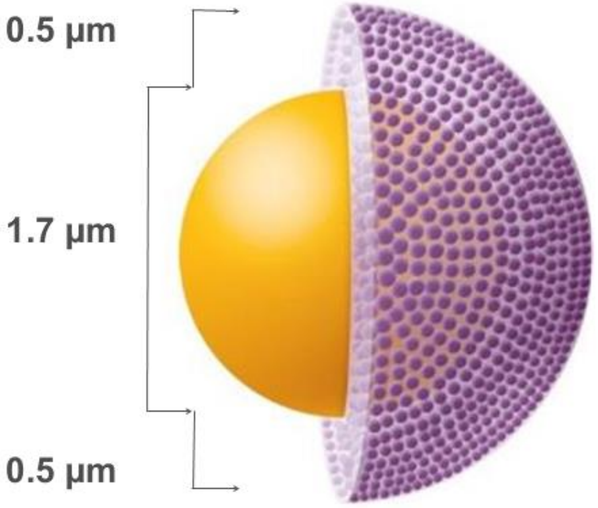
L = Column Length

v = Flow Velocity

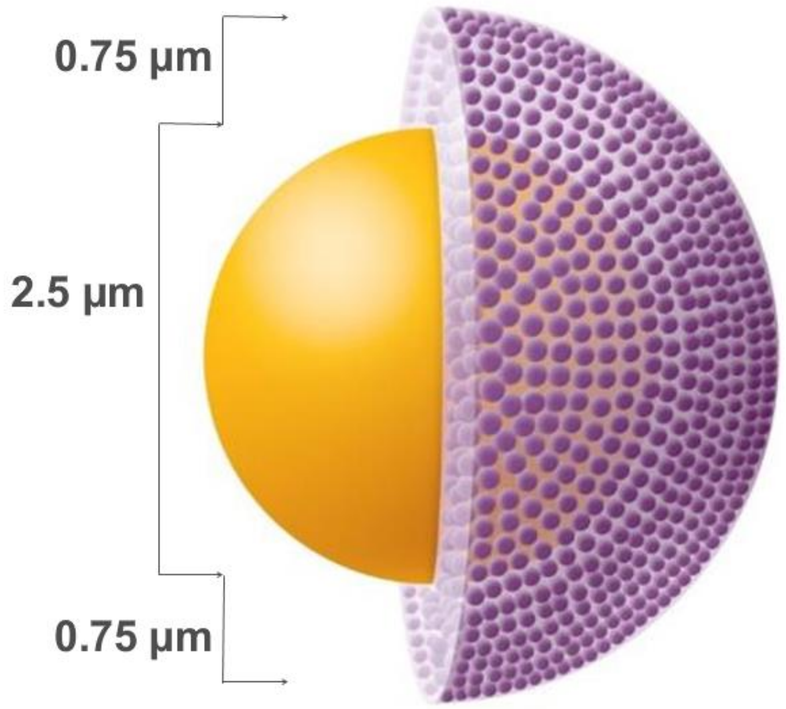
d_p = Particle Diameter

θ = Dimensionless Structural Constant
~ 600 For Packed Beds in LC

Superficially Porous Particles for LC



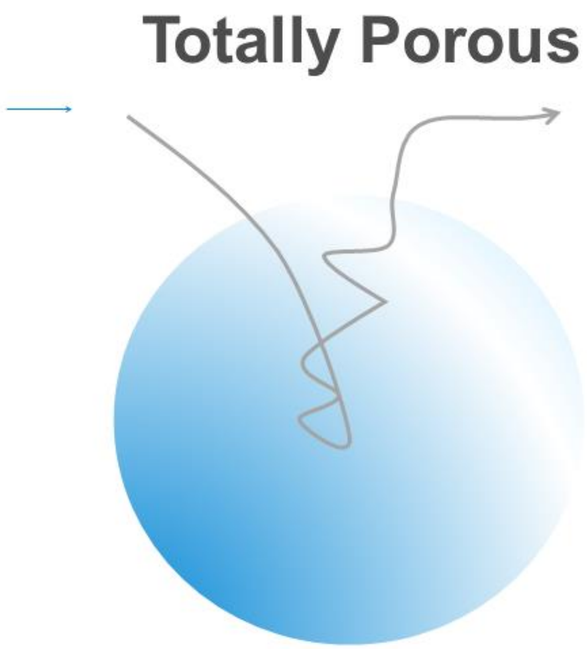
Poroshell 120 2.7 µm



Poroshell 120 4 µm

www.agilent.com/chem/discoverporoshell

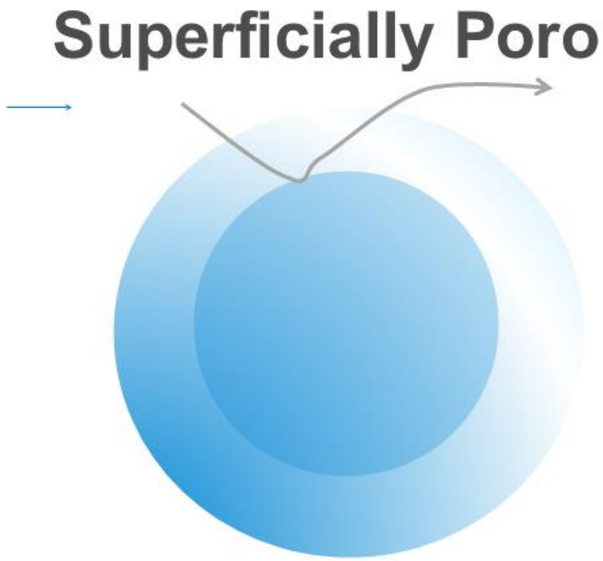
Analyte Mass Transfer Improvements



- **Totally porous particles**
  - **diffusion throughout particle**
- **Poroshell 120**
  - **diffusion limited to outer shell**

van Deemter equation:

$$h = A + B/\nu + C \cdot \nu$$



- **Results:**
  - **Lower C term**
  - **Higher efficiency**
- **And**
  - **Higher flow rate with**
  - **Minimal impact on efficiency**

Increase Resolution

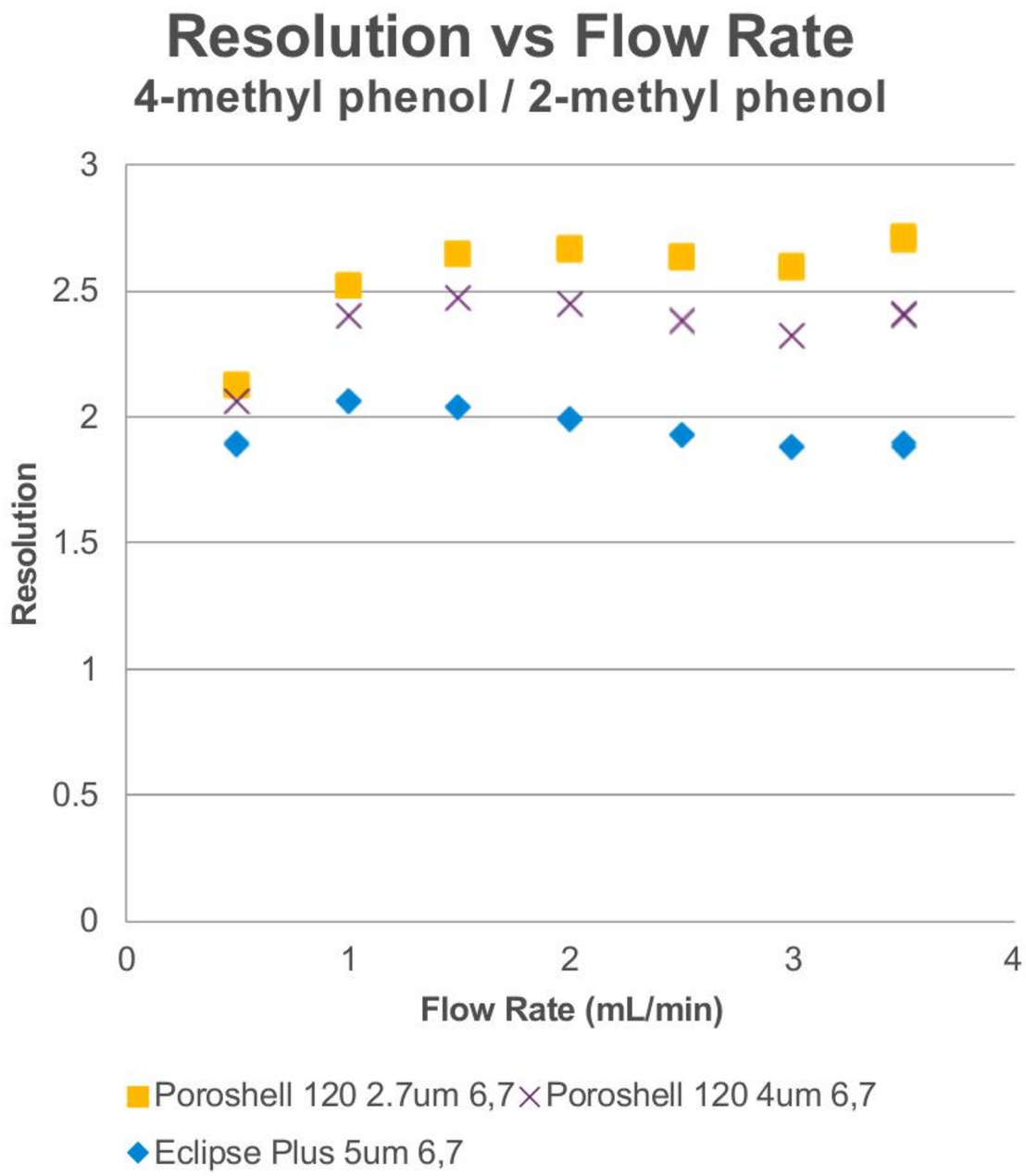
High efficiency with Poroshell 120

Particle Size/Type	Pressure	Efficiency	LC Compatibility
5 µm Totally Porous	80 bar	5,000	HPLC (up to 400 bar)
 4 µm Superficially Porous	120 bar	**10,000**	HPLC/UHPLC (up to 600 bar)
3.5 µm Totally Porous	123 bar	7,800	HPLC (up to 400 bar)
 2.7 µm Superficially Porous	180 bar	**12,000**	HPLC/UHPLC (up to 600 bar)
1.8 µm Totally Porous	285 bar	12,500	UHPLC (up to 1200 bar)

Columns: 4.6 x 50mm, Mobile Phase: 60% ACN:40% Water Flow Rate: 2 mL/min

Increase Resolution

4 µm - good resolution at low pressure



Column: 4.6 x 100 mm

Mobile phase A: 0.1 % formic acid in water

Mobile phase B: 0.1 % formic acid in acetonitrile

Temperature: 35°C

% B	Time (min)						
5	4	2	1.33	1	0.8	0.67	0.34
40	34	17	11.33	8.5	6.8	5.67	2.84
40	40	20	13.33	10	8	6.67	3.34
5	42	21	14	10.5	8.4	7	3.5
5	50	25	16.67	12.5	10	8.34	4.17
Flow rate (mL/min)	0.5	1	1.5	2	2.5	3	3.5

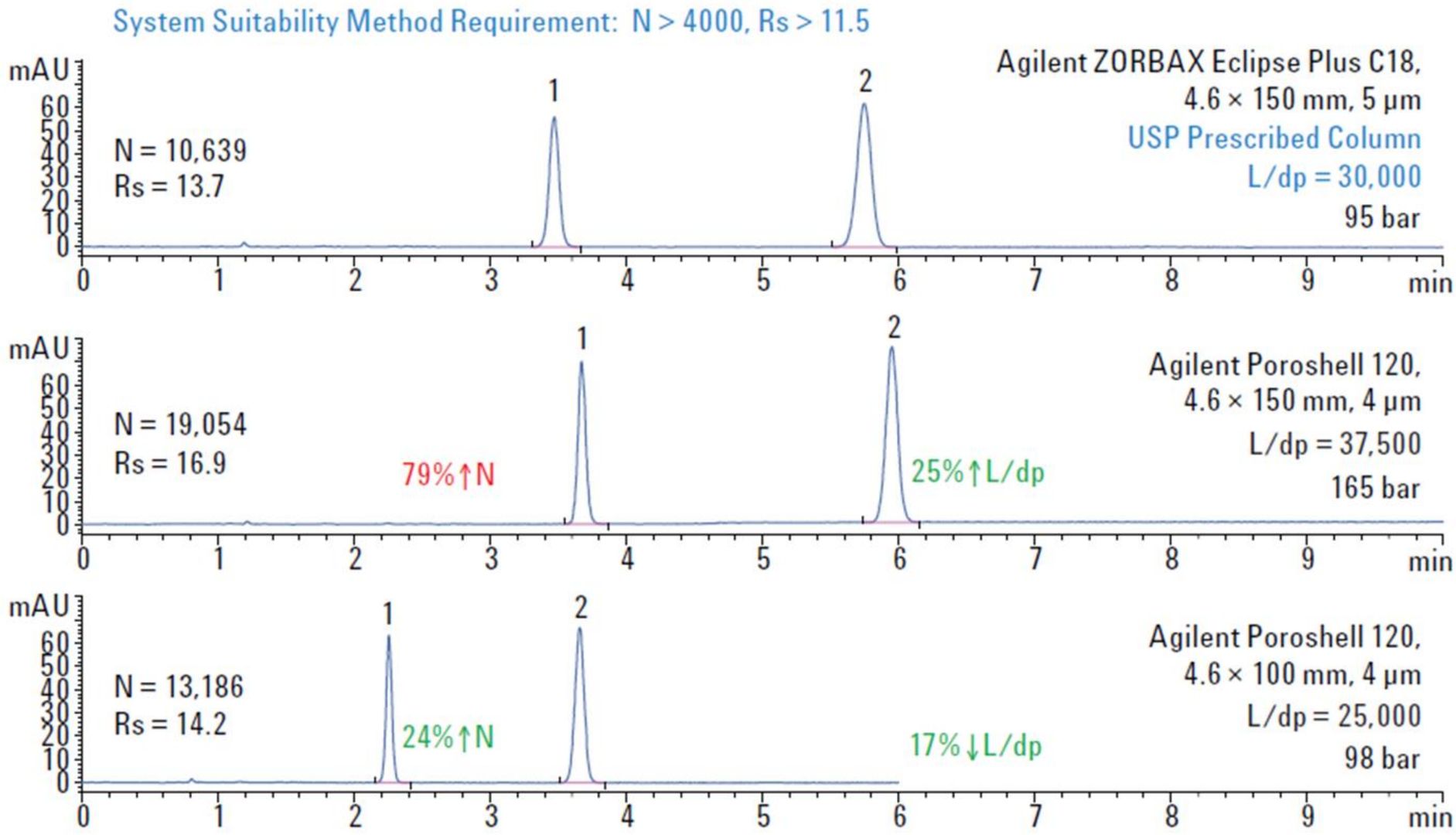
Results at 2mL/min	R_s	P (bar)
Poroshell 120 EC-C18, 2.7 µm	2.7	293
Poroshell 120 EC-C18, 4 µm	2.5	169
Eclipse Plus C18, 5 µm	2.0	103

Application note 5991-5510EN

Run Fast with High Resolution

Shorter 4 µm column gives fast, high resolution runs

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



Mobile phase: 50:49:1 MeCN:H_2O:Acetic acid
Flow rate: 1.2 mL/min

Peak ID

1. Naproxen
2. Butyrophenone

Application note 5991-5408EN

How to Improve Resolution?

High plate number (***N***) provides:

- Sharp and narrow peaks
- Better detection
- Ability to resolve complex samples

But **resolution** increases only with the square root of the plate number

- $R_S \sim \sqrt{N}$

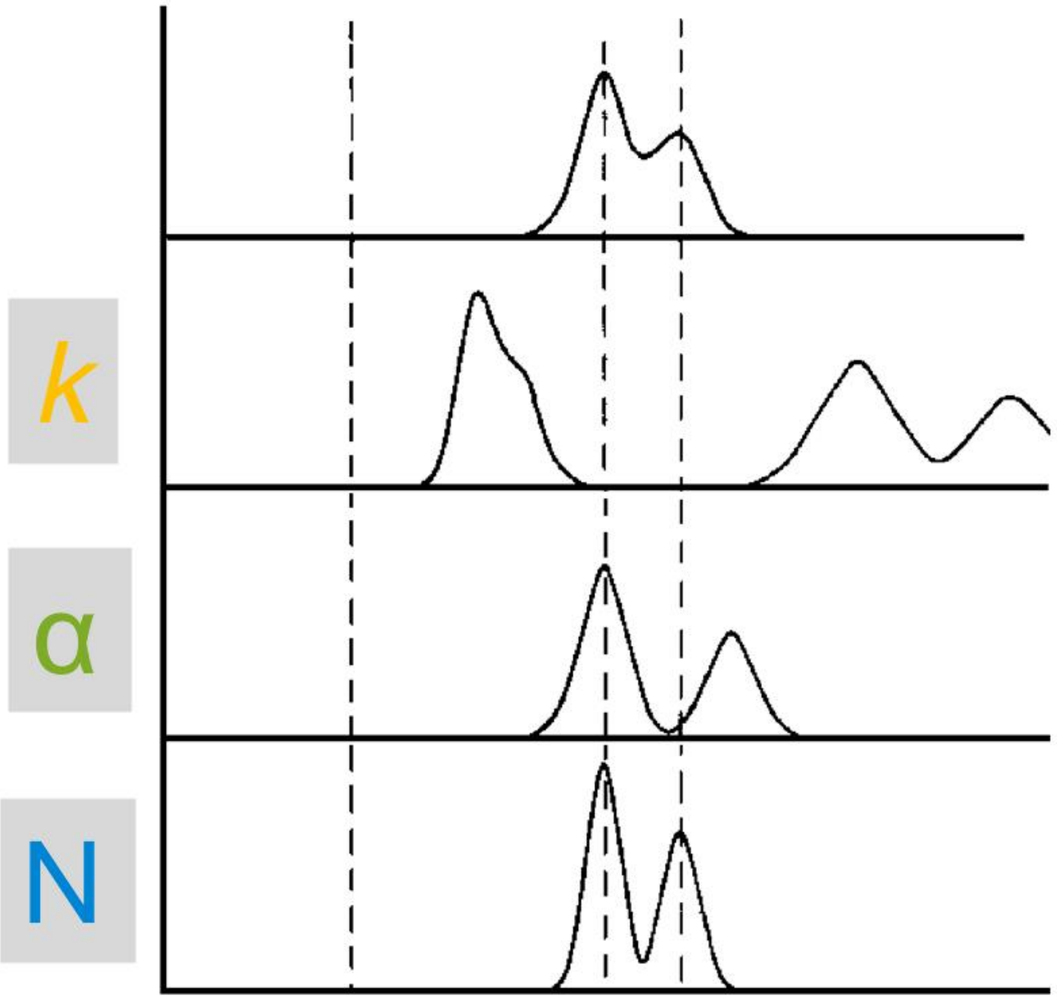
Plate number increase is limited by experimental conditions

- **Time**
- **Pressure**

Factors that Affect Resolution

$$R_s = \tfrac{1}{4}\sqrt{N} \cdot \left(\frac{\alpha - 1}{\alpha}\right) \cdot \left(\frac{k}{1+k}\right)$$

Efficiency Selectivity Retention



Changing Selectivity to Improve Resolution

Mobile phase

- Mobile phase – organic modifier (ACN, MeOH etc.)
- Mobile phase – pH – over a wide pH range – pH 1-12 if needed

Bonded phase

- Phases other than C18/C8
- Phenyl-Hexyl, Polar-embedded, CN, PFP
- Differences in interactions between polar and non-polar compounds
- Other types of interactions with a bonded phase (π-interactions, etc.)

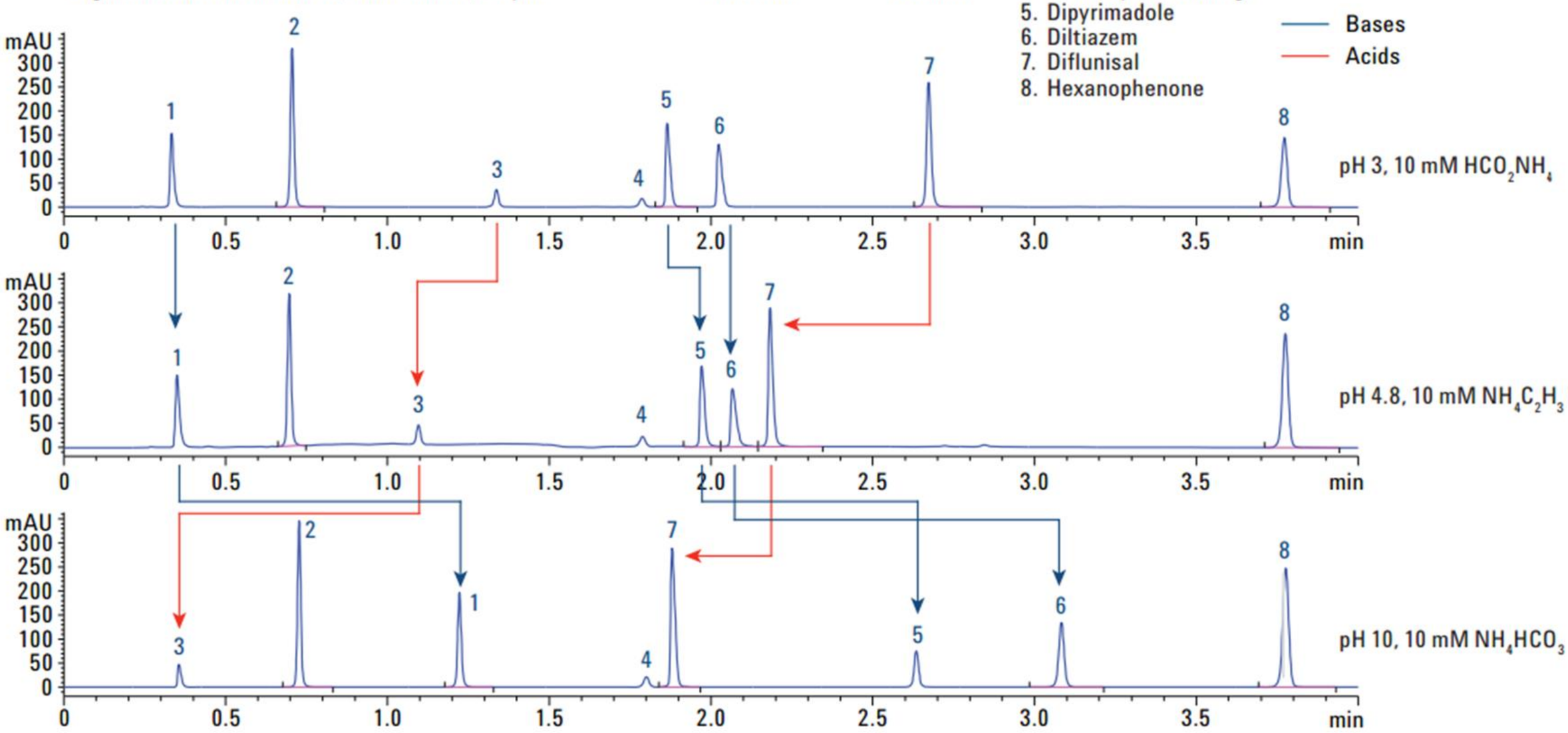
Comparing Low, Mid, & High pH

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

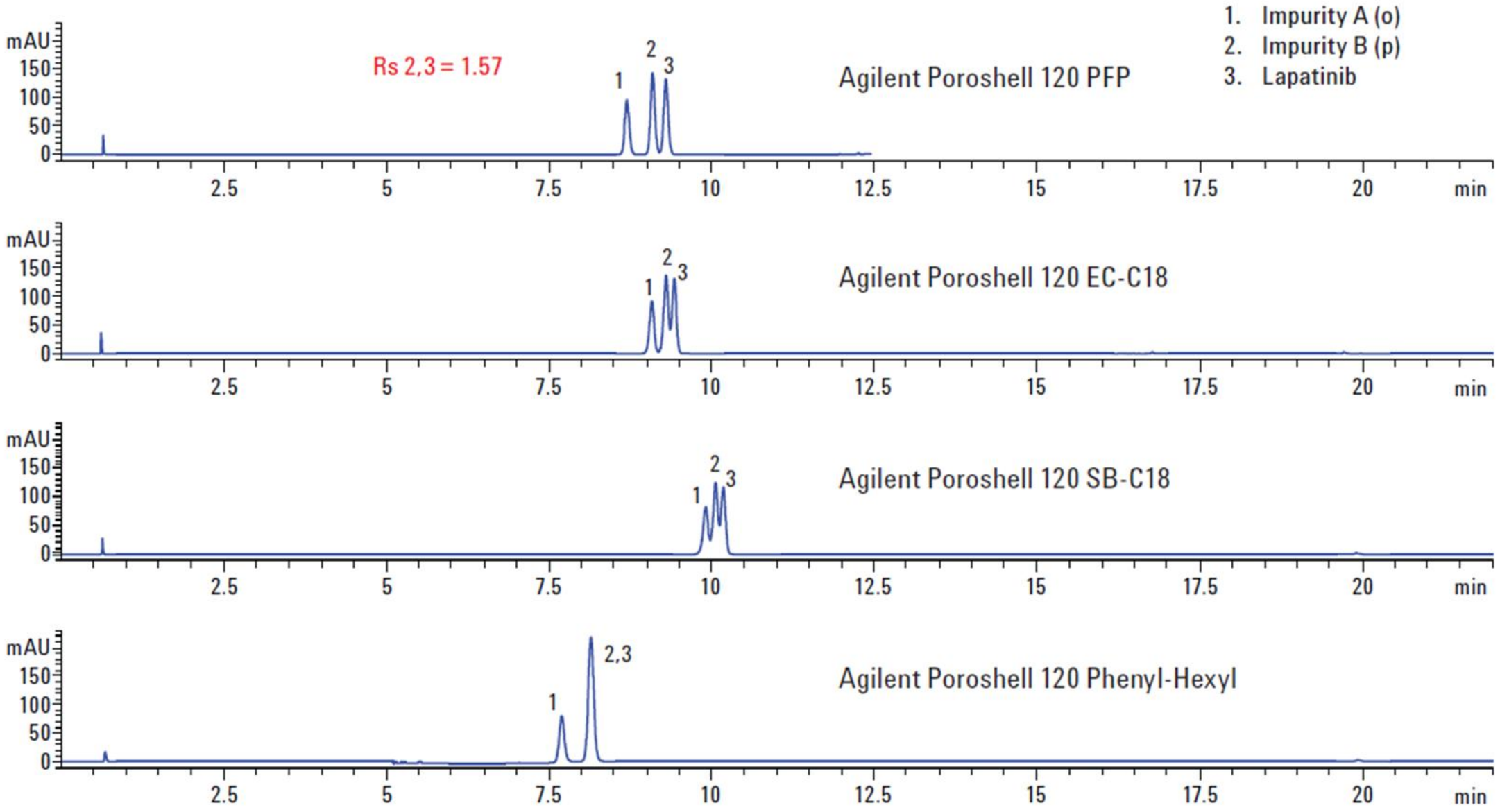
Peak ID

1. Procainamide
2. Caffeine
3. Acetyl salicylic acid
4. Hexanophenone deg.
5. Dipyrimadole
6. Diltiazem
7. Diflunisal
8. Hexanophenone

Agilent Poroshell HPH C-18, 4.6 × 50 mm, 2.7 µm

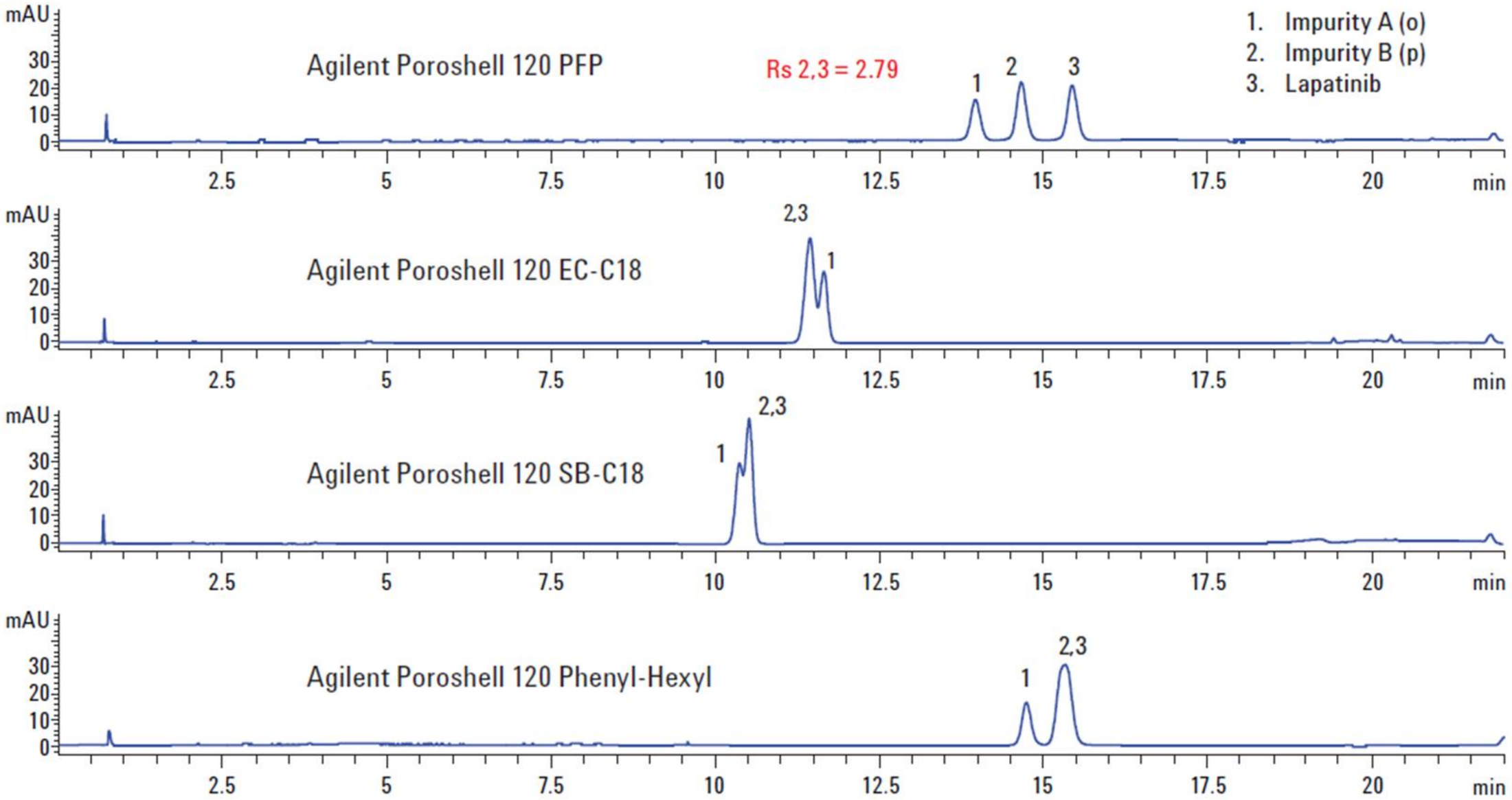


Changing Selectivity to Improve Resolution



With acetonitrile

Changing Selectivity to Improve Resolution



With methanol

Retention Factor - Gradients

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

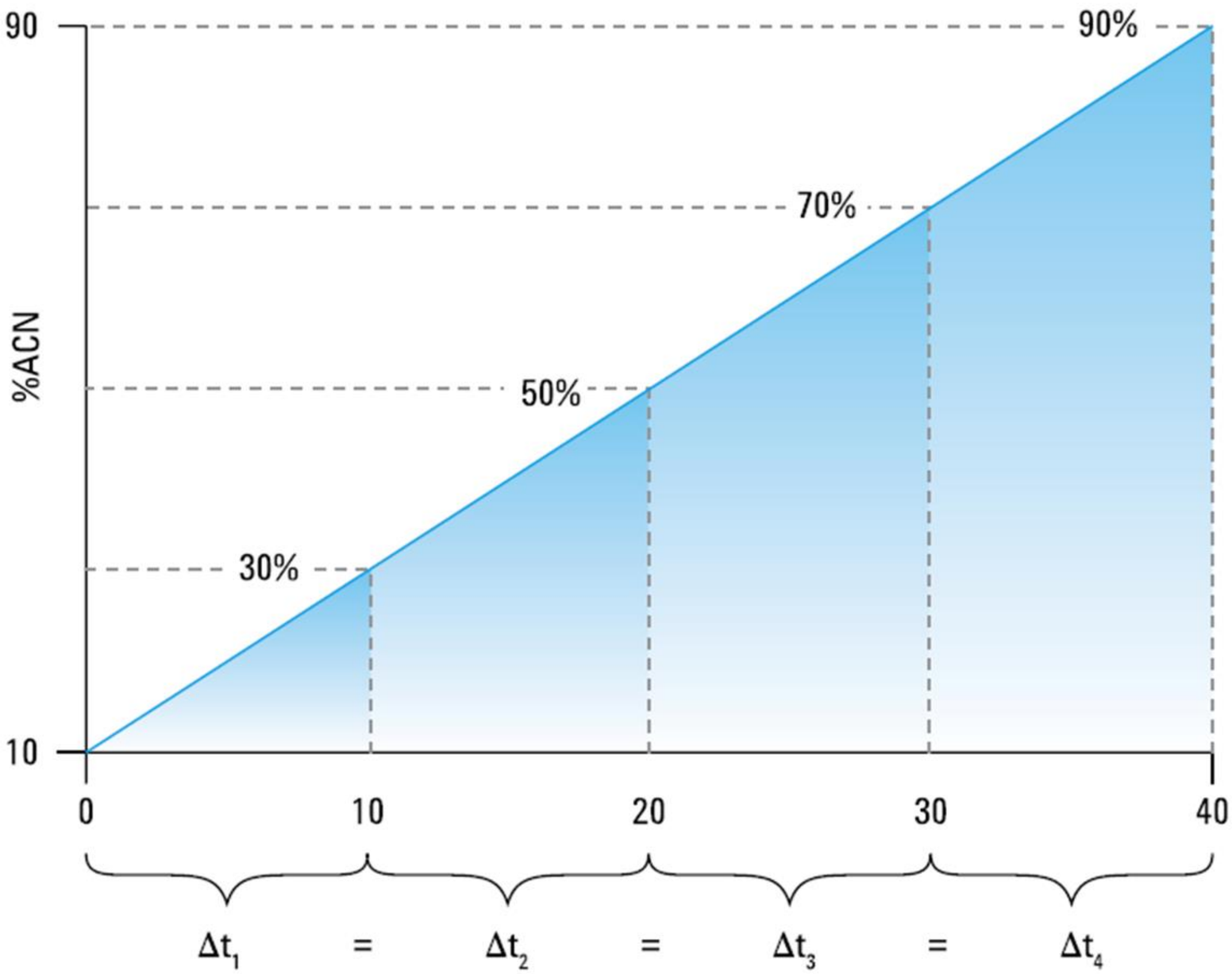
With gradient separations, the retention factor is influenced by

- F = flow rate
- t_G = gradient time (minutes)
- $\Delta\Phi$ = change in volume fraction of B mobile phase
- V_m = column volume
- S = constant (4 - 6 for small molecules, 10 - 1000 for peptides and proteins)

To keep the retention factor constant, changes in the denominator need to be offset by proportional changes in the numerator, and vice versa.

Retention Factor - Gradients

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



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

Linear solvent strength gradient = % per min is a constant

$\Delta\Phi = 80\%$

$t_G = 40$ min

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

Maintaining k*
Keep Relative Peak Position 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

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

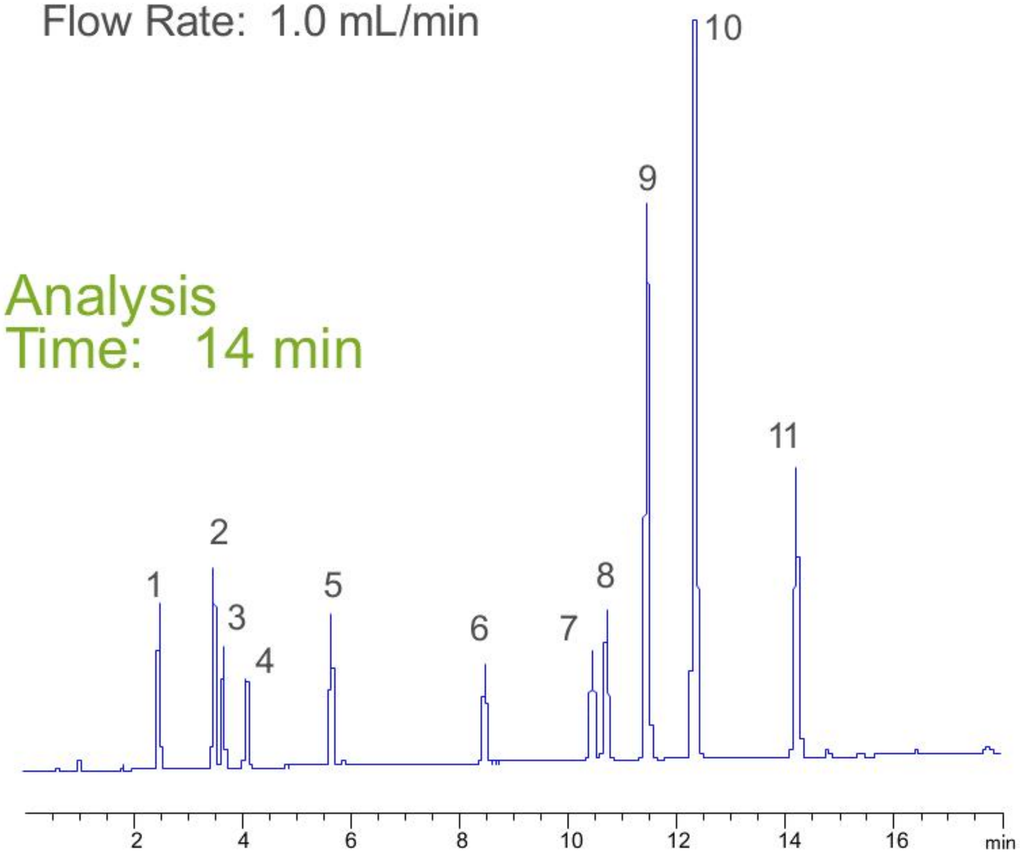
Reduce Analysis Time
Keep Gradient Steepness the Same

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

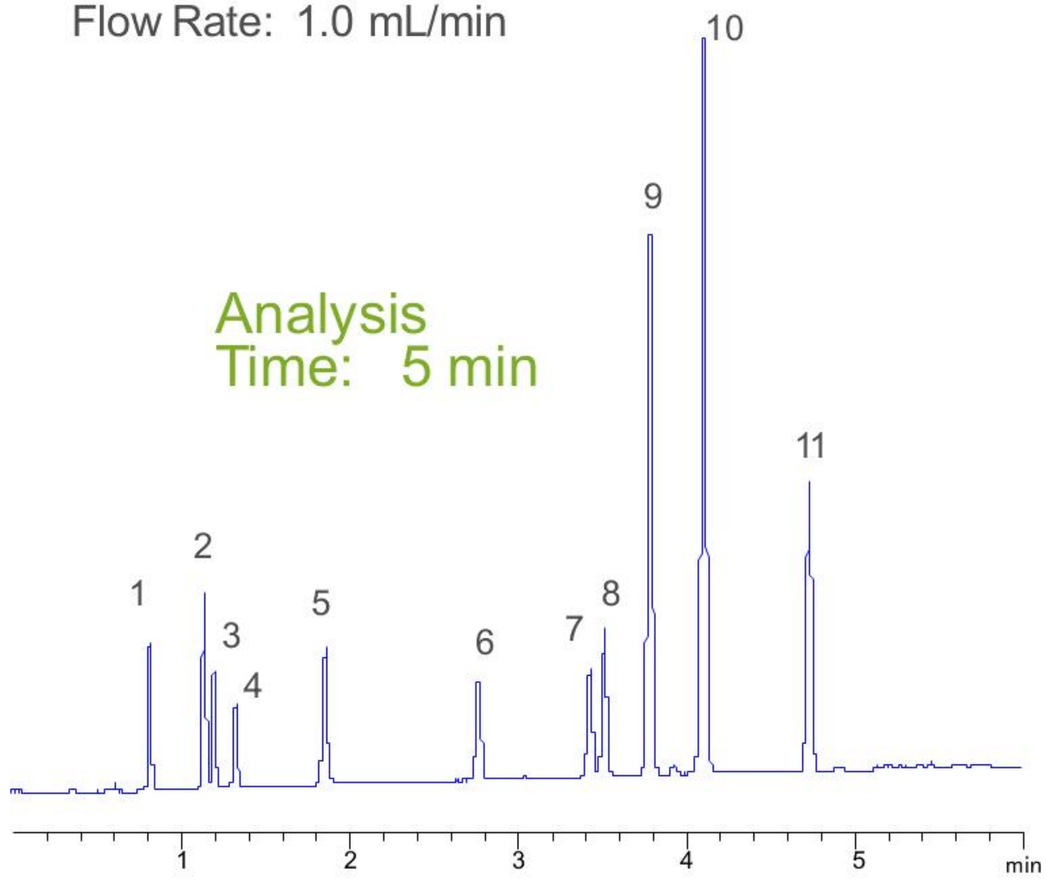
Analysis
Time: 14 min



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

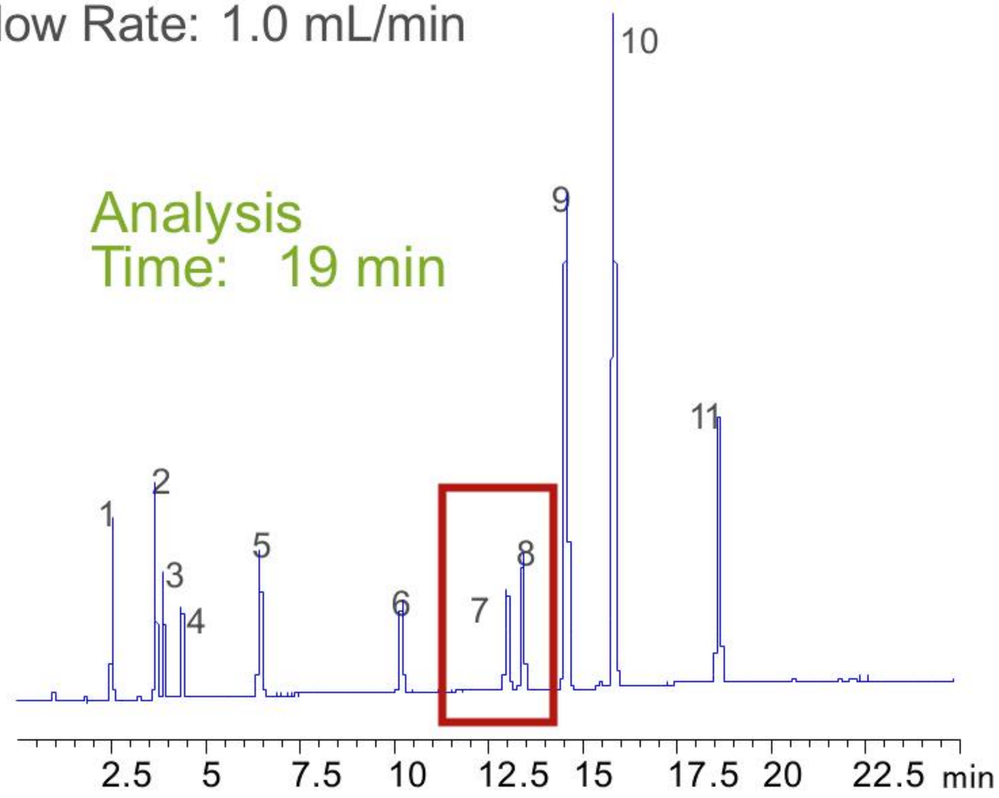


Reduce Analysis Time More
Keep Gradient Steepness the Same

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)

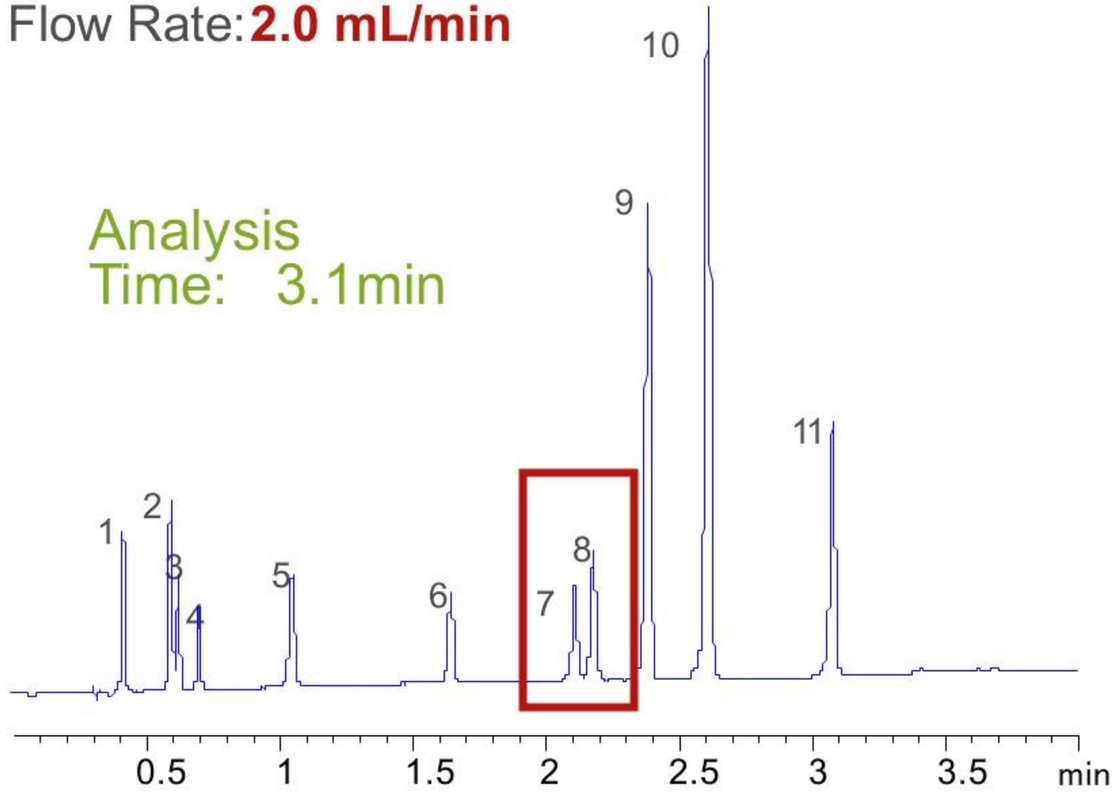
Column: Poroshell 120 EC-C18
4.6 x 150 mm, 2.7 µm

Gradient
Time: 30 min
Flow Rate: 1.0 mL/min



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

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

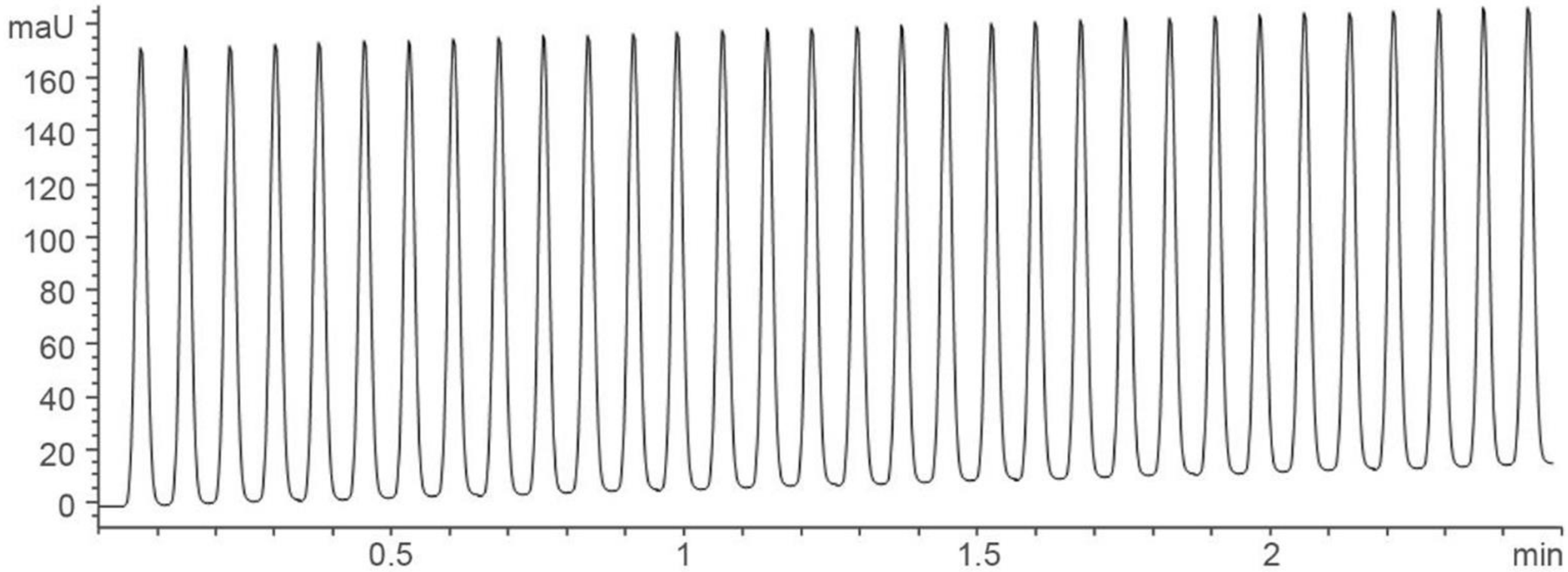


Multiple gradient parameters can be changed to maintain resolution and reduce analysis time.

Peak Capacity
Definition

Peak capacity is the number of peaks (n) that can be separated in a given time with a given resolution (Rs = 1).

The peak capacity depends on different factors like column length and particle size.



Peak capacity: 32 peaks in 2.5 min

Peak Capacity

Calculation of Peak Capacity

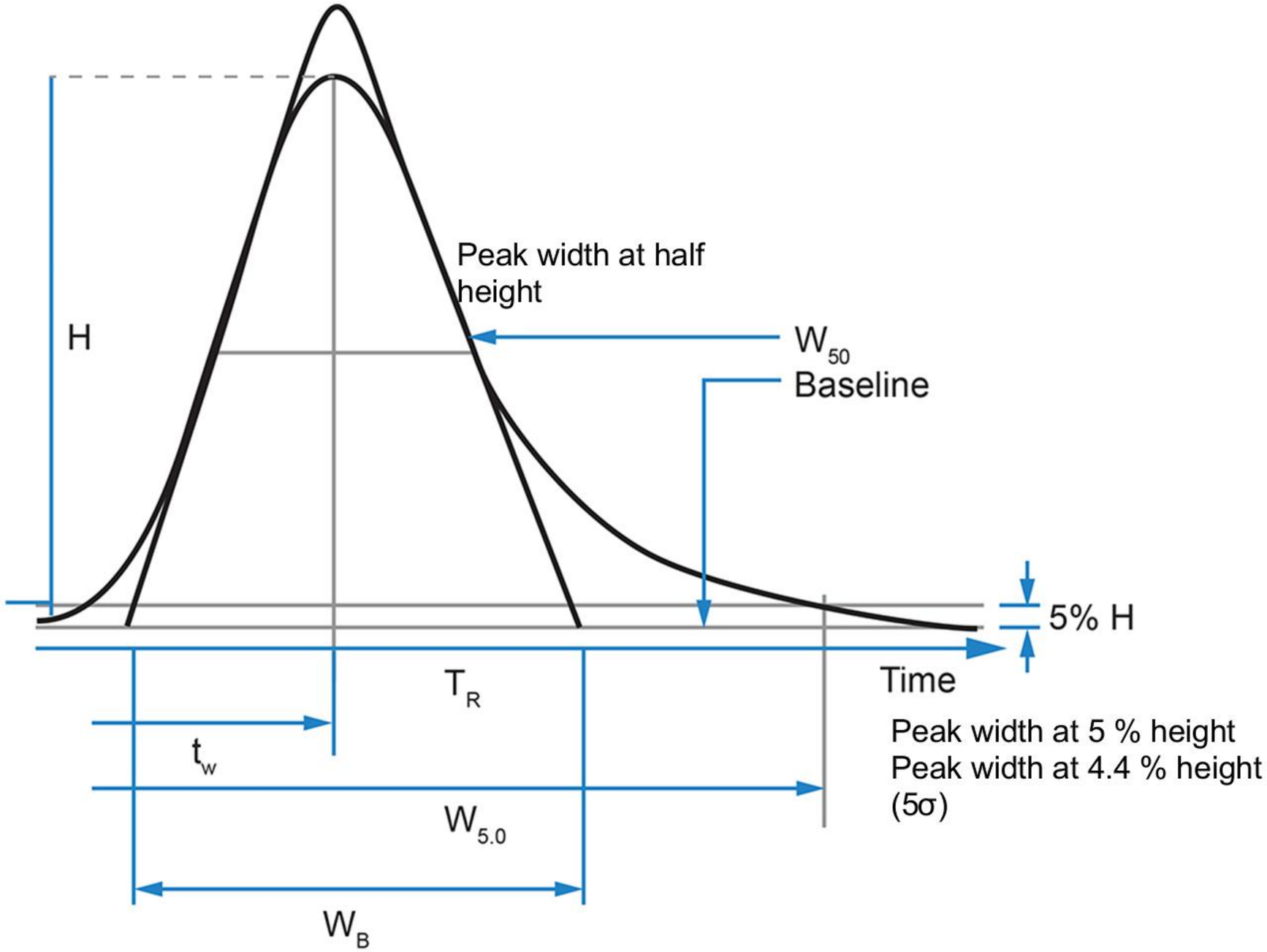
$$P = 1 + \frac{t_G}{\frac{1}{n}\sum_{1}^{n} w} = 1 + \frac{t_G}{w_{av}}$$

w_{av}	Average peak width
n	Number of peaks
t_G	Gradient time
w	Peak width of selected peak

Simplification: $$P = 1 + \frac{t_G}{w}$$

Peak Capacity

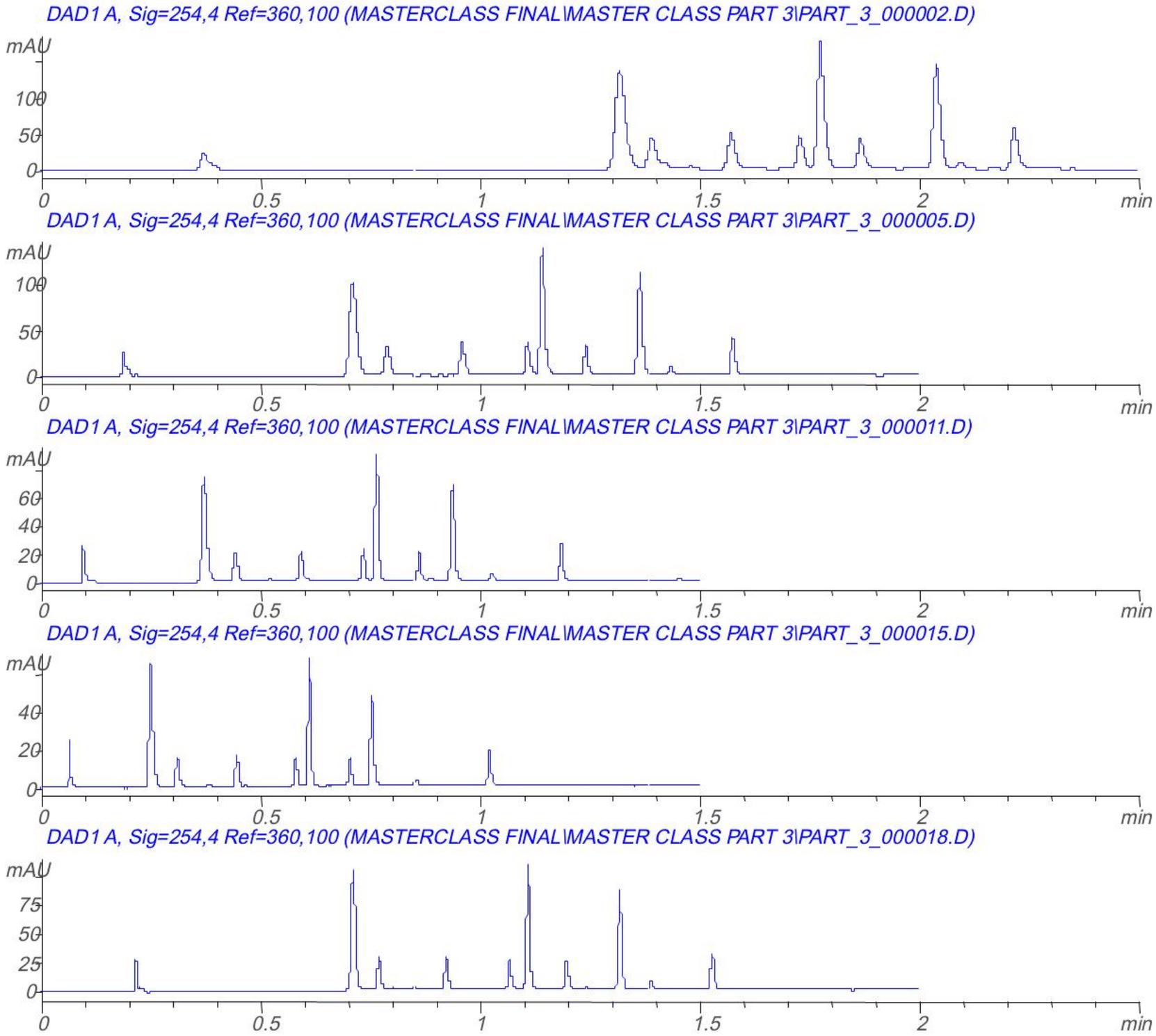
Peak Width



Peak width according to tangent method

Peak width at 5 % height
Peak width at 4.4 % height
(5σ)

UHPLC Durability Allows Enhanced Performance at High Flow



Column: 2.1x50Eclipse plus C18
Flow: 0.3ml/min
Peak Capacity: 35 (PW= 5sigma)
204bar

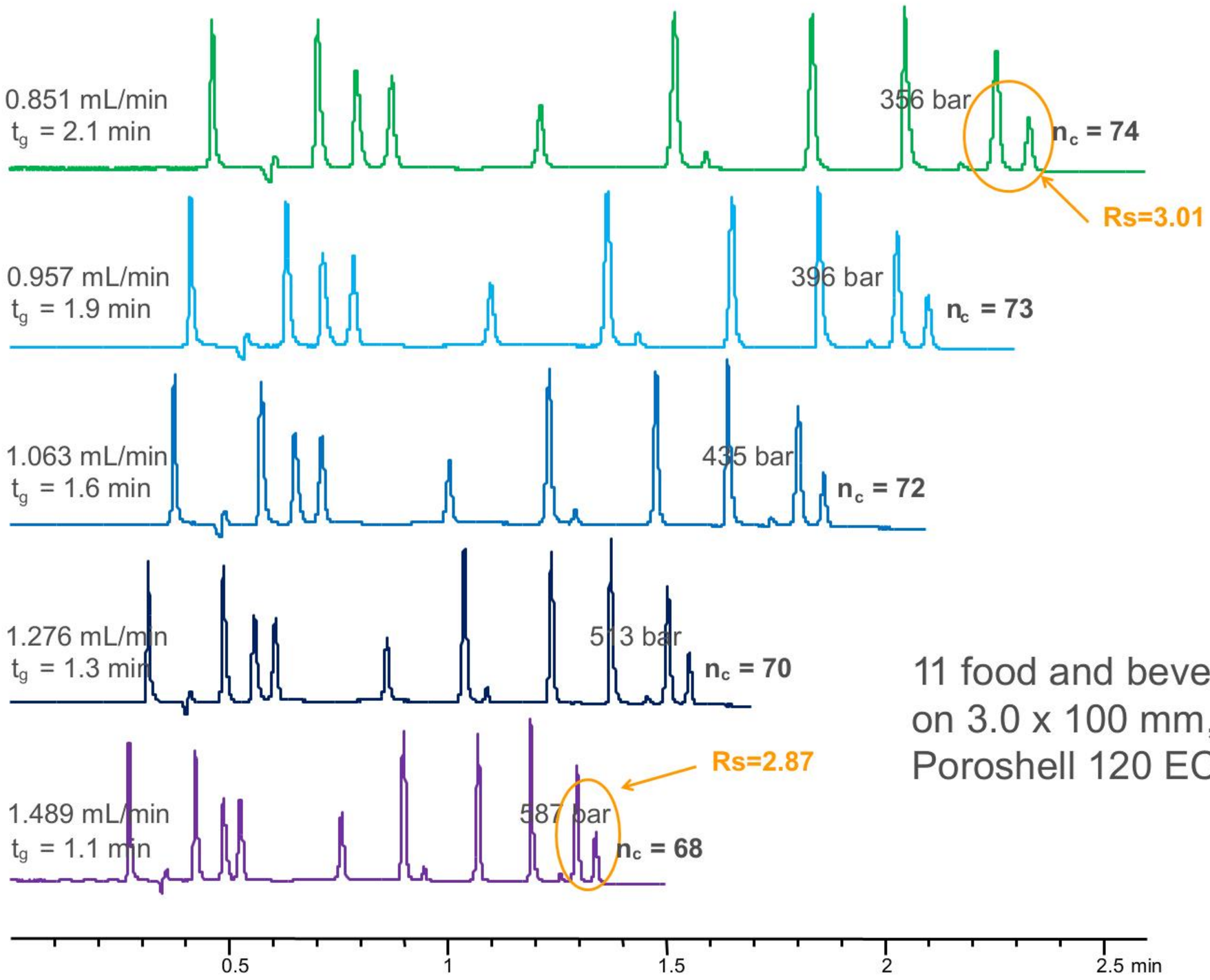
Column: 2.1x50Eclipse plus C18
Flow: 0.6ml/min
Peak Capacity: 53
395bar

Column: 2.1x50Eclipse plus C18
Flow: 1.2ml/min
Peak Capacity: 72
735bar

Column: 2.1x50Eclipse plus C18
Flow: 1.8ml/min
Peak Capacity: 84
1050bar

Column: 2.1x100Eclipse plus C18
Flow: 0.6ml/min
Peak Capacity: 69
862bar

High Flow Rates on Poroshell

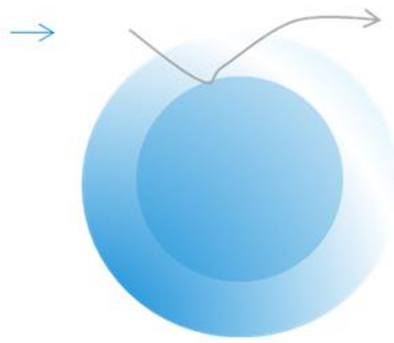


11 food and beverage additives on 3.0 x 100 mm, 2.7 µm Poroshell 120 EC-C18

Summary

$$N \propto \frac{L}{\mathrm{d}_p}$$

$$h = A + B/u + C \cdot u$$



$$R_s = \underbrace{\tfrac{1}{4}\sqrt{N}}_{\text{Efficiency}} \cdot \underbrace{\left(\frac{\alpha - 1}{\alpha}\right)}_{\text{Selectivity}} \cdot \underbrace{\left(\frac{k}{1+k}\right)}_{\text{Retention}}$$

LC Handbook

5990-7595EN