Why They Matter

An Introduction to Chromatography Equations

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Objectives of Talk

- **Chromatography is a physical process**
- **Much can be described with simple equations**
- **Understanding the process simplifies Method development, Troubleshooting, Predicting behavior, etc.**

Topics

- Chromatographic Process
- Isocratic Resolution
- Particles and Pressure
- Van Deemter Equation
- Gradient Resolution
- Method Conversions

Chromatographic Process

- Partition between mobile phase and stationary phase ($K = C_s/C_m$)
- Description of the separation:
  - R_s – Resolution
  - N – Column Efficiency, Plates
  - *k, k'* – Retention Factor, Capacity Factor
  - α – Selectivity
  - t_{ret} – Retention time

Definition of Resolution

$$R_s = \frac{t_{R-2} - t_{R-1}}{(w_2 + w_1)/2} = \frac{\Delta t_R}{\bar{w}}$$

Resolution is a measure of the ability to separate two components

Resolution …

Determined by 3 Key Parameters –
Efficiency, Selectivity and Retention

The Fundamental Resolution Equation

$$R_S = \frac{\sqrt{N}}{4}\,\frac{(\alpha-1)}{\alpha}\,\frac{k}{(k+1)} = \frac{\Delta t_R}{\overline{w}}$$

N = Column Efficiency – Column length and particle size

α = Selectivity – Mobile phase and stationary phase

***k* = Retention Factor** – Mobile phase strength

Parameters Affecting Resolution (R_s)

- **Retention Factor (*k*):** describes how well an analyte is retained by the stationary phase, expressed as a ratio of column volumes, can be adjusted by making changes to the organic strength of the mobile phase

- Selectivity or Separation Factor (α)

- Column Efficiency as Theoretical Plates (N)

Parameters Affecting Resolution (R_s)

- Retention Factor (*k*)

- **Selectivity or Separation Factor (α):** This is the ratio of retention factors for two adjacent peaks. Larger α values indicate better separation. Selectivity can be adjusted by changes to either the mobile phase or the stationary phase.

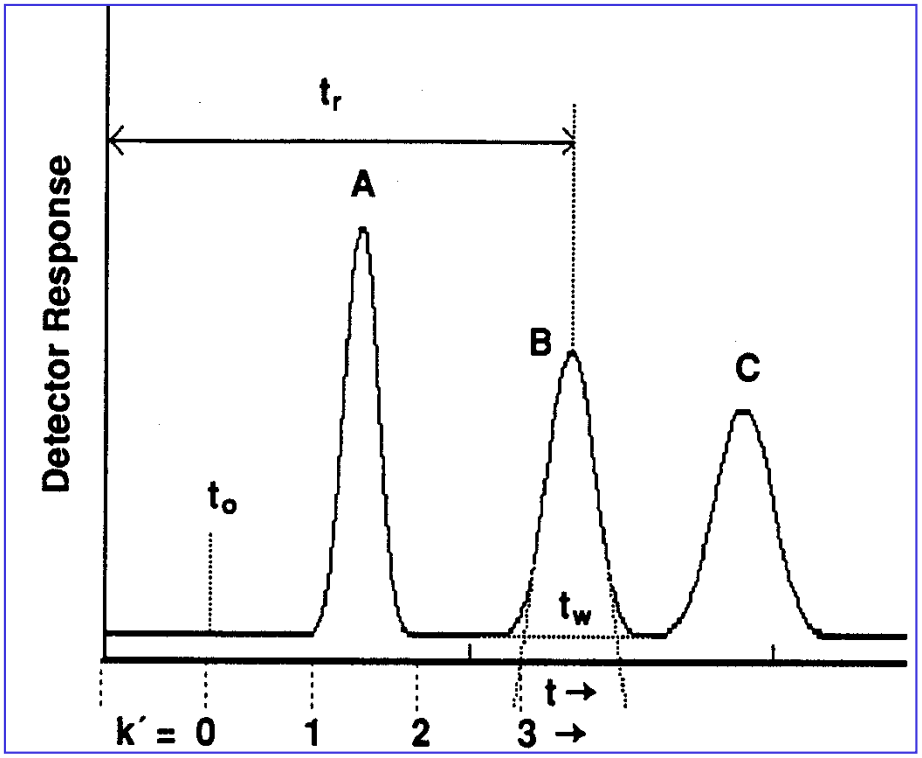
- Column Efficiency as Theoretical Plates (N)

Parameters Affecting Resolution (R_s)

- Retention Factor (*k*)
- Selectivity or Separation Factor (α)
- **Column Efficiency as Theoretical Plates (N):** As the number of plates increase, peaks become thinner and sharper, which improves resolution. Plates are often described by their height (H),or Height Equivalent to the Theoretical Plate (HETP). Number of plates and plate height are inversely proportional, i.e. H = L/N

Chromatographic Profile

Equations Describing Factors Controlling R_S



Retention Factor

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

Selectivity

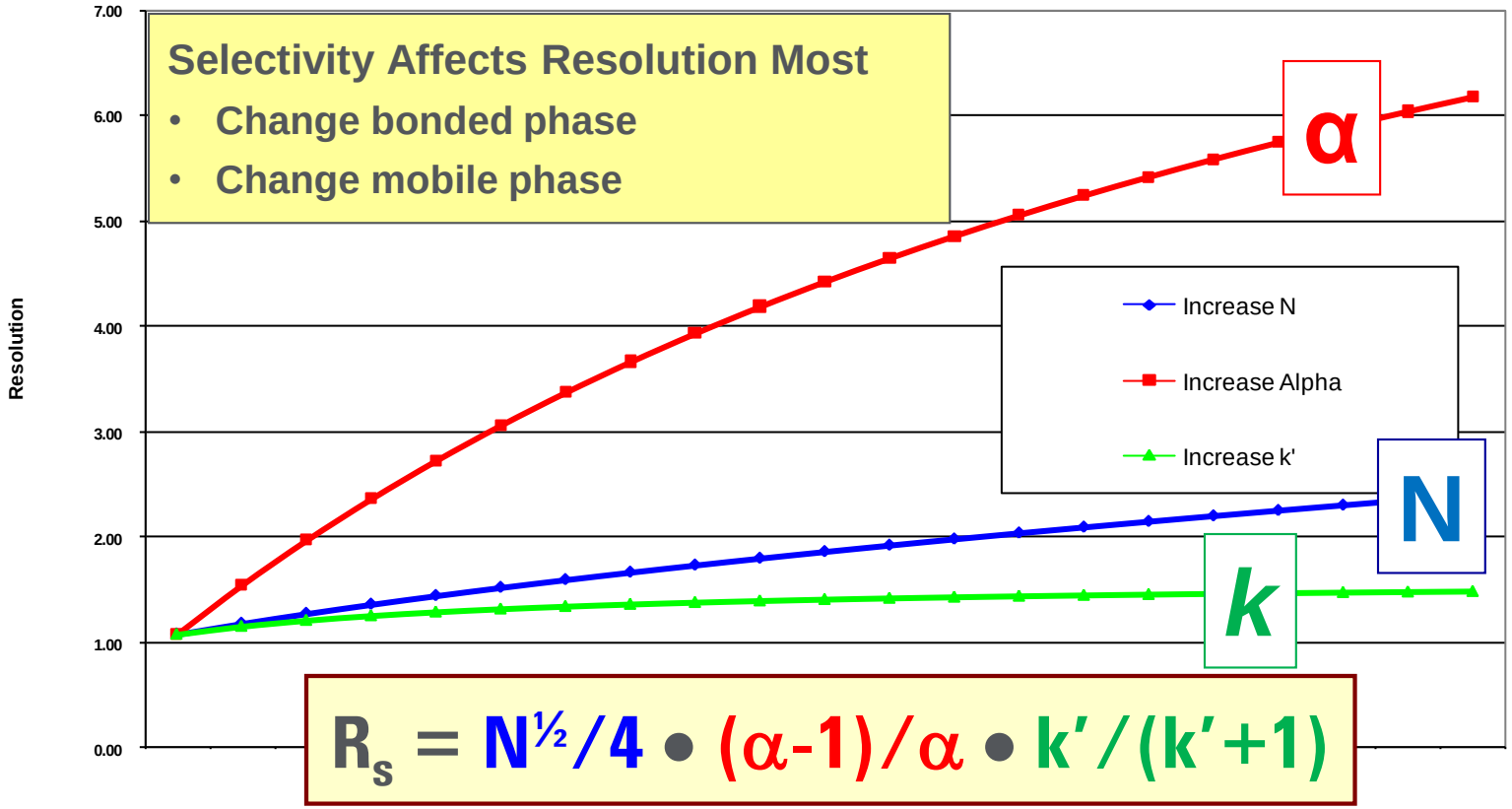
$$\alpha = k_2/k_1$$

Theoretical Plates-Efficiency

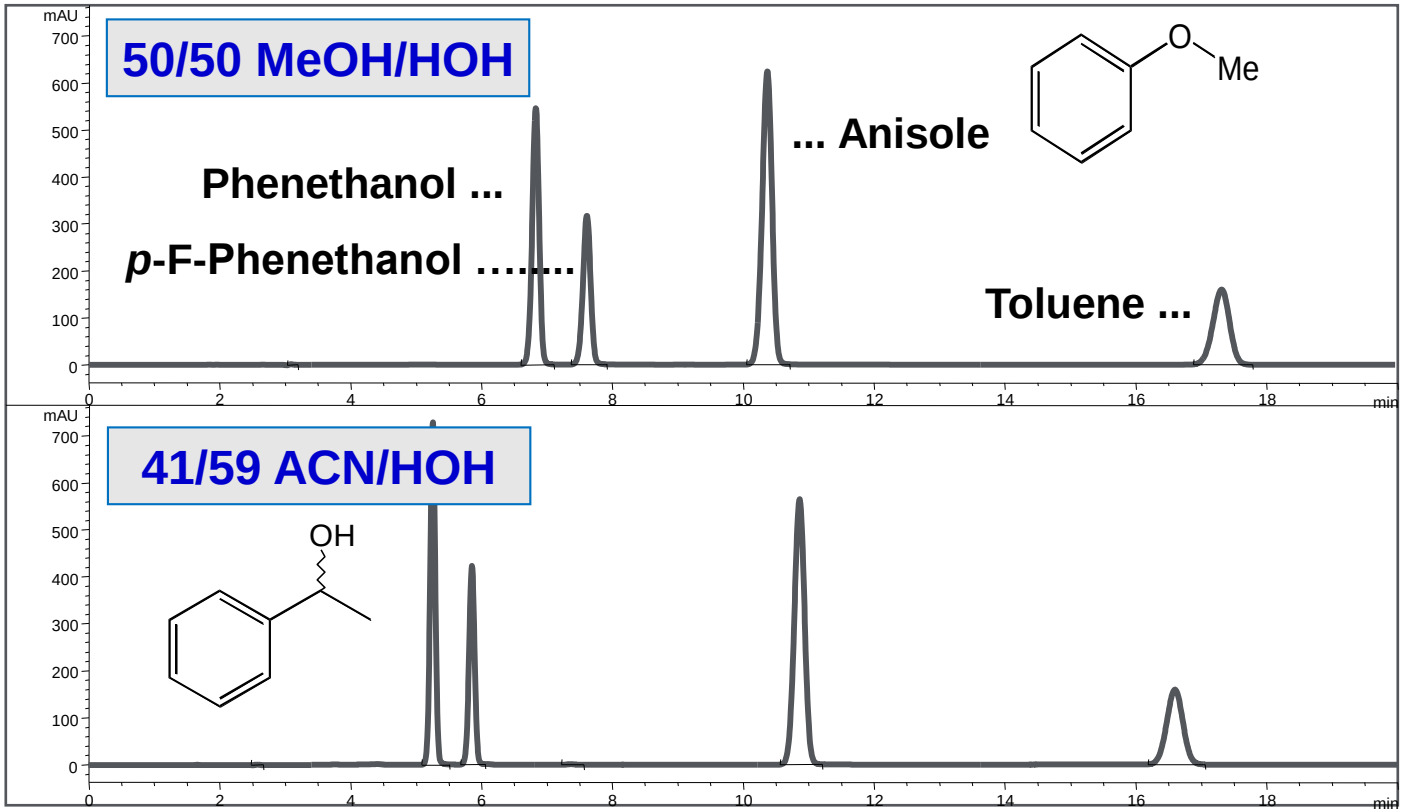
$$N = 16(t_R / t_{W\text{-}base})^2$$

$$N = 5.56(t_R / t_{W\text{-}1/2})^2$$

Resolution as a Function of Selectivity, Column Efficiency, or Retention

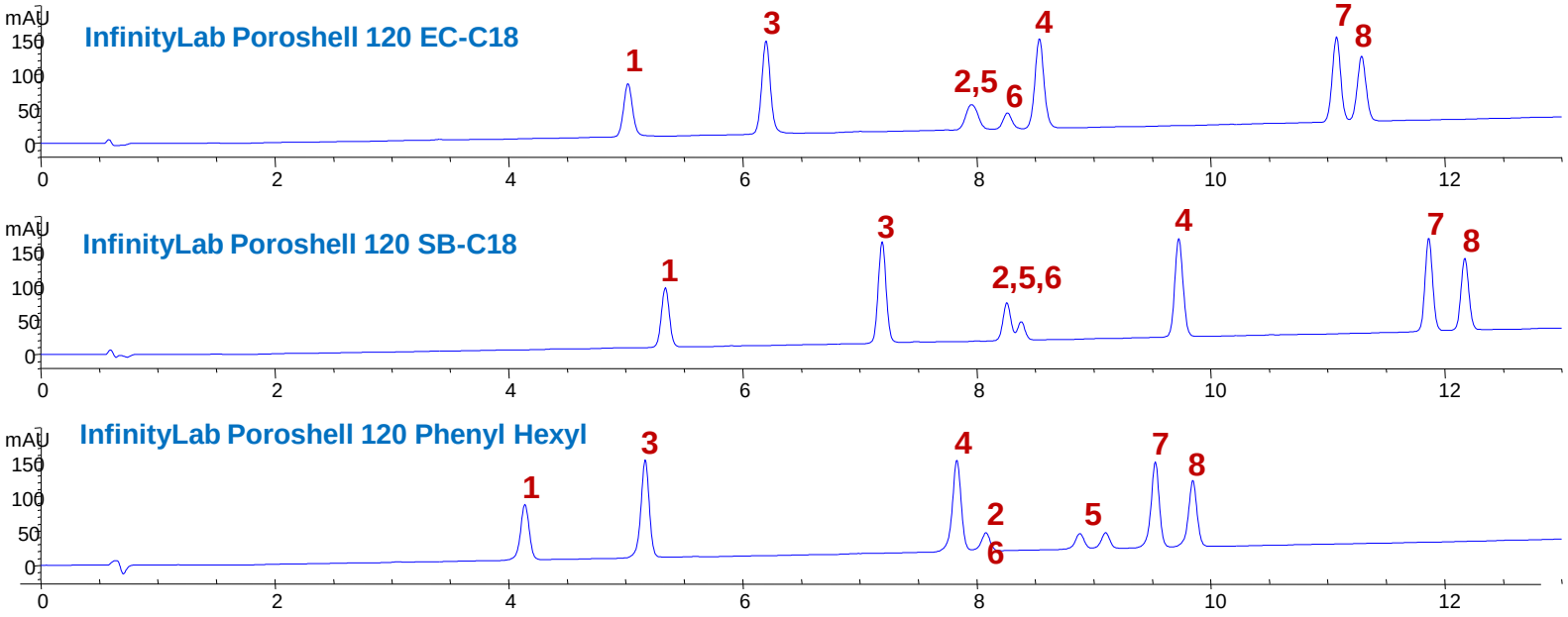


Different Mobile Phases May Give Different Selectivity



ZORBAX® SB-C18 4.6 x 250 mm
1 mL/min, 40°C, 225 nm

Selectivity Differences Across InfinityLab Poroshell Bonded Phases



1. Hydrocortisone 2. B Estradiole, 3. Andostadiene 3. 17 dione, 4. Testosterone
5. Ethyestradione 6. Estrone 7. Norethindone acetate 8. Progestreone

40-80 % Methanol in 14 min, DAD 260, 80 nm 0.4 ml/min, 2.1 x 100 mm column, 40 C, 0.1% Formic Acid in Water and Methanol, Agilent 1260 Method Development Solution

Column Efficiency (N)

N - Number of theoretical plates.

We can increase N by increasing the length of the column or decreasing the size of the stationary phase particles.

(1.8 µm > 2.7 µm > 3.5 µm > 5 µm > 10 µm)

$$N = 16\left[\frac{t_R}{W}\right]^2 = f(L, 1/d_p)$$

L = column length

d_p = particle size

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

L = column length

d_p = particle size

What About Pressure?

Pressure Increases with Decreasing Particle Size

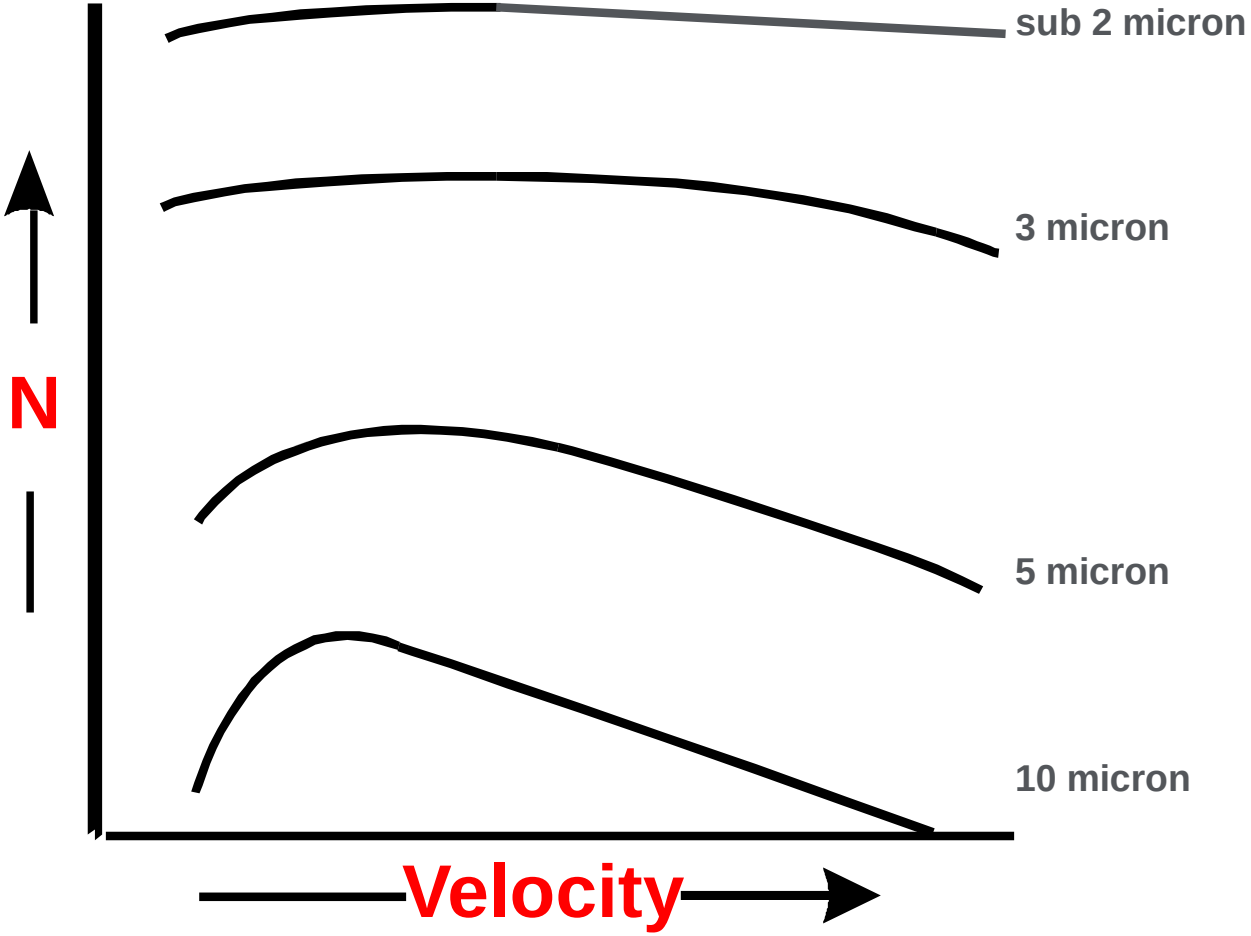
Equation For Pressure Drop Across an HPLC Column

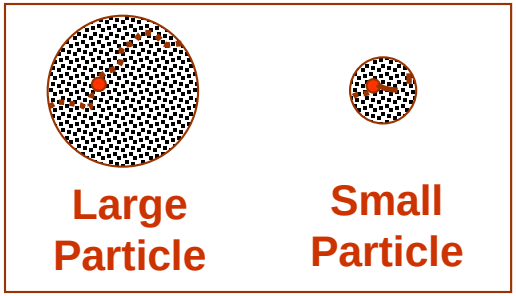
$$\Delta P = \frac{\eta \cdot L \cdot v}{\theta \cdot d_p^2}$$

- ΔP = Pressure Drop
- η = Fluid Viscosity
- L = **Column Length**
- v = Flow Velocity
- d_p = **Particle Diameter**
- θ = Dimensionless Structural Constant of Order 600 For Packed Beds in LC

✓ Many parameters influence column pressure
✓Particle size and column length are most critical
✓Long length and smaller particle size mean more resolution and pressure
✓ We can now handle the pressure

Columns Packed with Smaller Particles Provide Higher Efficiency

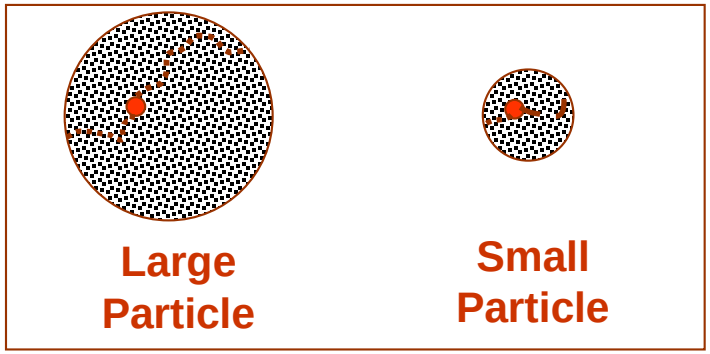




$$N \propto 1/(d_p)$$

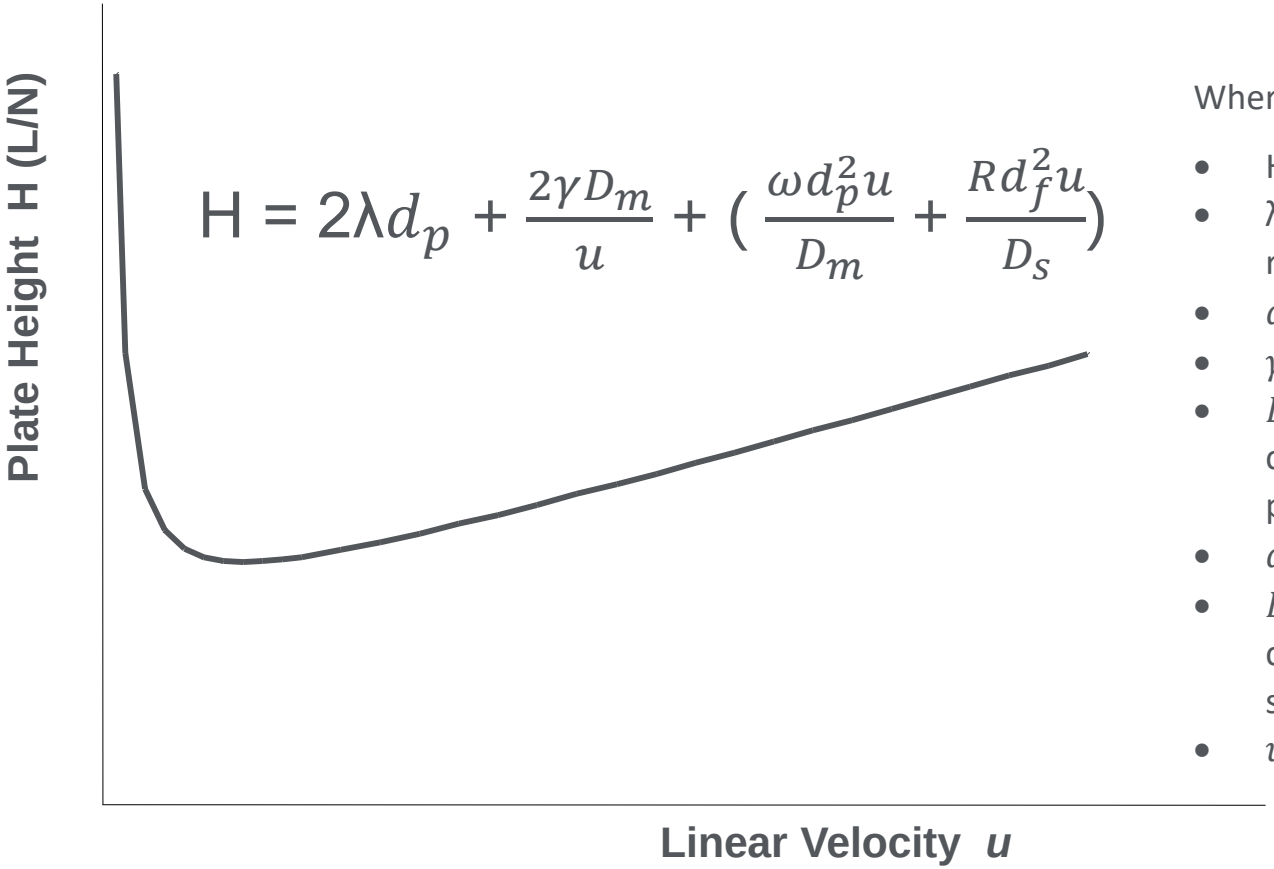
$$P \propto 1/(d_p)^2$$

Van Deemter Curve
Factors Affecting N



The smaller the plate height, the higher the plate number and the greater the chromatographic resolution

Van Deemter Equation, Expanded



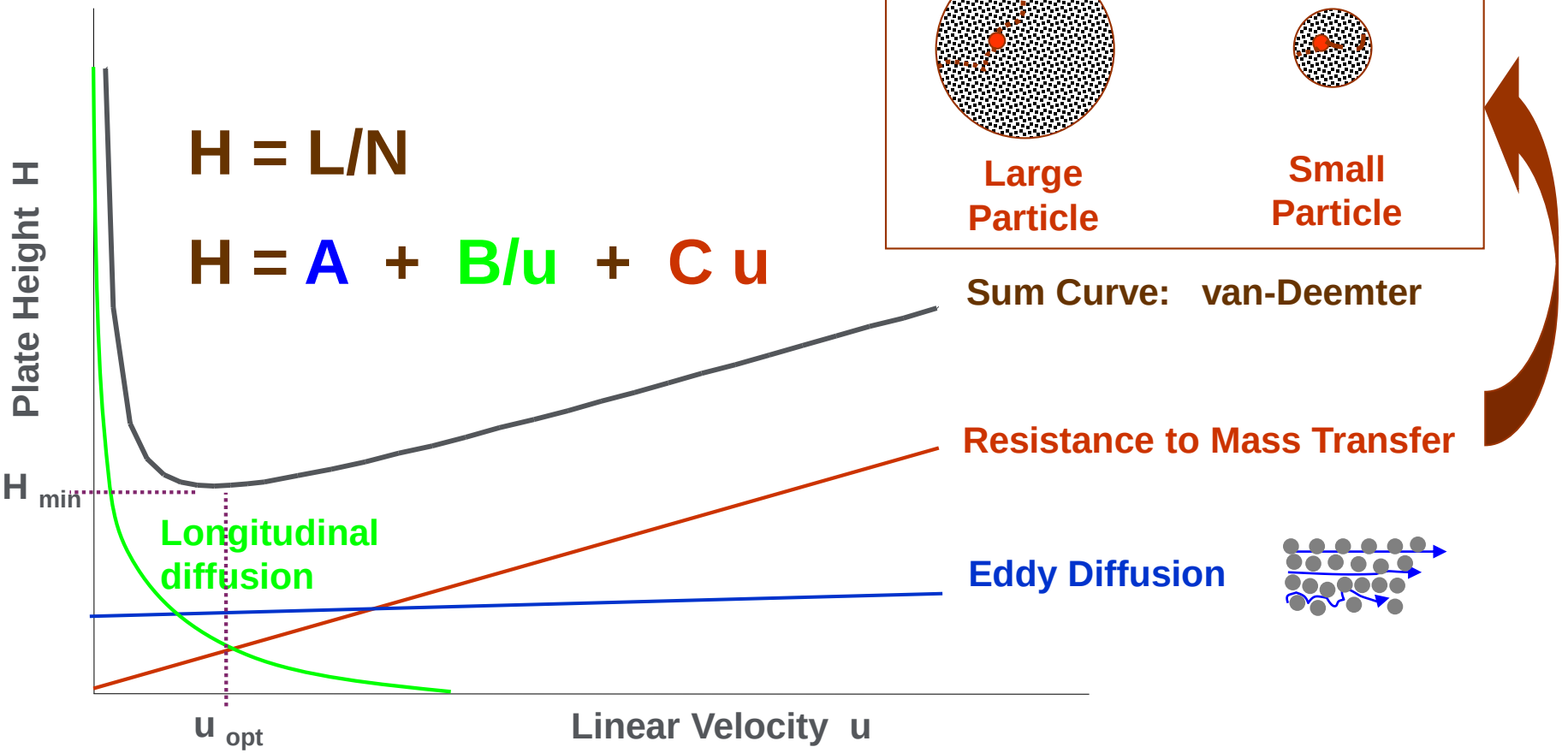
Where:

- H is plate height
- λ is particle shape with regard to the packing
- d_p is particle diameter
- γ, ω, and R are constants
- D_m is the diffusion coefficient of the mobile phase
- d_f is the film thickness
- D_s is the diffusion coefficient of the stationary phase
- u is the linear velocity

From Wikipedia, which references

Kazakevich, Yuri. "Band broadening theory (Van Deemter equation)". Seton Hall University. Retrieved 5 February 2014.

Putting it Together
The van Deemter Equation



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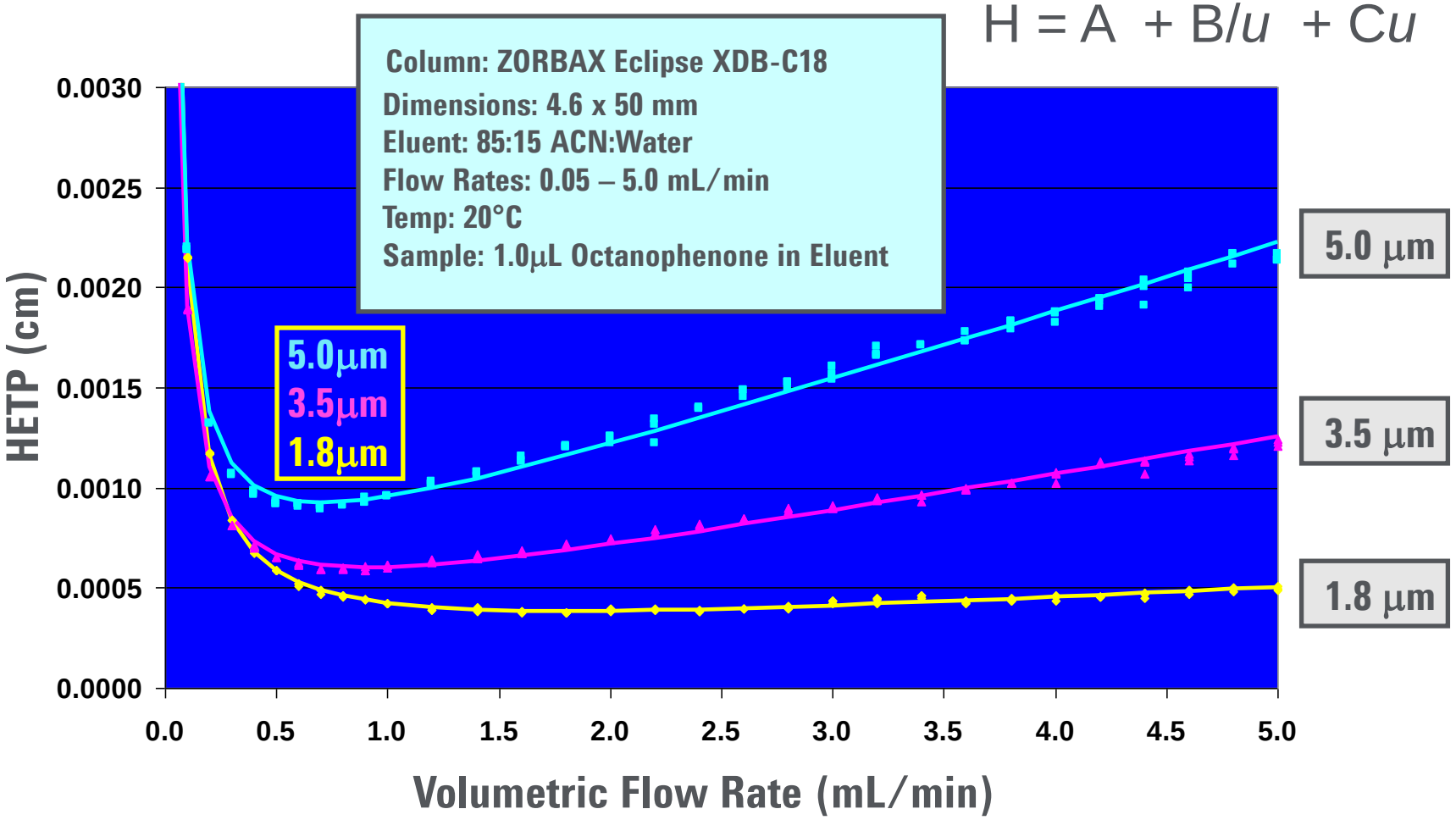
Van Deemter Curve
Effect of Particle Size

$$H = A + B/u + Cu$$

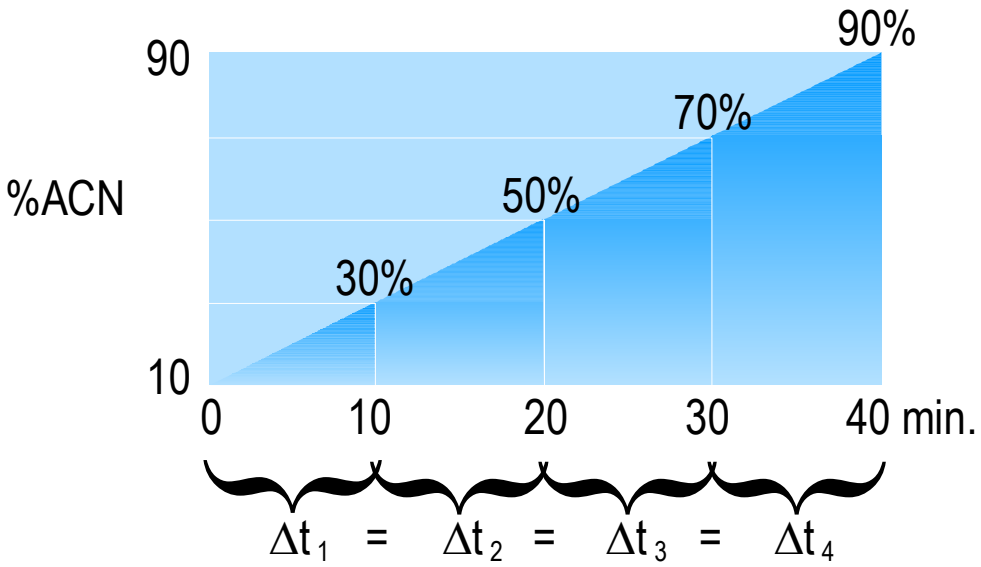


Smaller particle sizes yield flatter curves, minima shift to higher flow rates

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.

Resolution Relationship for Gradient Elution

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

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

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

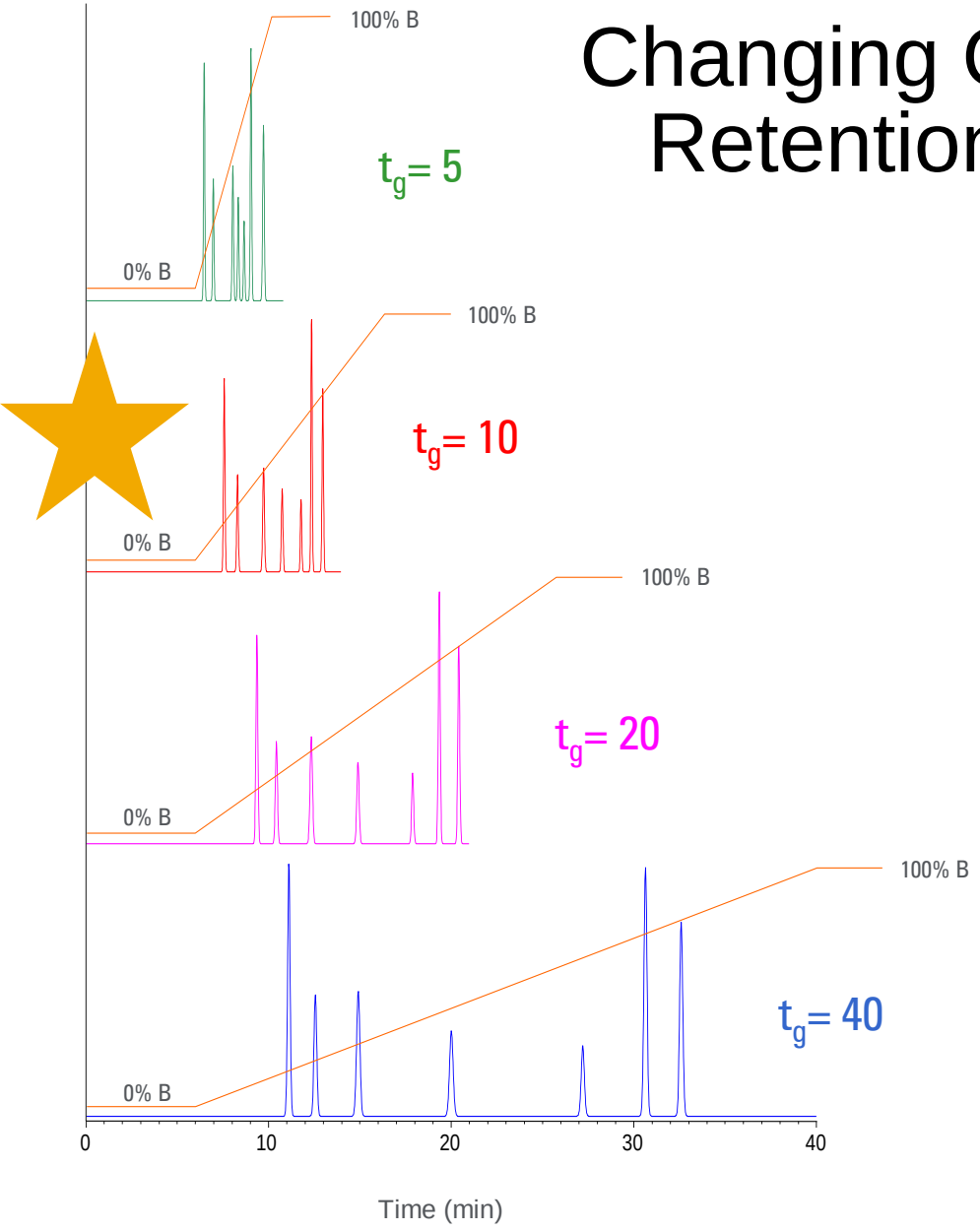
$\Delta\%B$ =	difference between initial and final % B values
S =	constant
F =	flow rate (mL/min.)
t_g =	gradient time (min.)
V_m =	column void volume (mL)

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

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

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

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

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

Any Decrease in	Can be Offset by a Proportional
• Gradient time	• Decrease in Δ%B or V_m • Increase in F
• 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 Transfer Considerations

•Keeping k^* constant, substituting for the volume of the column and cancelling out constants we can establish the equality:

- $$t_{new}\left(\frac{F_{new}}{L_{new}d_{new}^2}\right) = t_{old}\left(\frac{F_{old}}{L_{old}d_{old}^2}\right)$$

Gradient Transfer Considerations

- Rearranging to solve for the new time we get

$$\bullet t_{new} = t_{old} \cdot \frac{F_{old}}{F_{new}} \cdot \frac{L_{new}}{L_{old}} \cdot \left(\frac{d_{new}}{d_{old}}\right)^2$$

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

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Gradient Transfer Considerations

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$$F_{new} = F_{old} \cdot \frac{t_{old}}{t_{new}} \cdot \frac{L_{new}}{L_{old}} \cdot \left(\frac{d_{new}}{d_{old}}\right)^2$$

Gradient Transfer Considerations

- Rearranging to solve for the new time we get
- $$F_{new} = F_{old} \cdot \left(\frac{d_{new}}{d_{old}}\right)^2$$

Column Volume

- When developing methods, we regularly recommend flushing the column at a high %B for at least two column volumes. We also talk about re-equilibrating our columns for 3-6 column volumes at the end of our gradients.
- But how do you calculate column volume?
- Column volume can be calculated either from a checkout chromatogram with a void marker
- Or geometrically

Column Volume from Test Chromatogram

LC Column
Performance Report

SERIAL NUMBER:

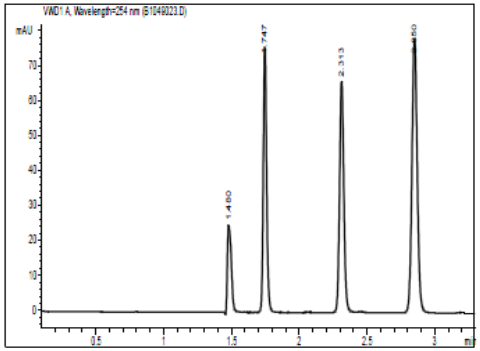
PART NUMBER: 959963-902
COLUMN TYPE: ZORBAX Eclipse Plus C18 4.6 x 150 mm, 3.5 µm
PACKING LOT #:

TEST CONDITIONS

MOBILE PHASE	=	85% Methanol / 15% Water
COLUMN PRESSURE	=	126.4 Bar
COLUMN FLOW	=	1.00 ml / min
LINEAR VELOCITY	=	0.168 cm / sec
TEMPERATURE	=	AMBIENT (Nominally 23 °C)
INJECTION VOLUME	=	5 µl

QUALITY CONTROL PERFORMANCE RESULTS FOR TOLUENE

	TEST VALUES	SPECIFICATIONS
THEORETICAL PLATES =	25116	MIN = 18000
SELECTIVITY =	1.65	RANGE = 1.61 - 1.71
USP TAILING FACTOR = (@ 5% Peak Height)	1.07	RANGE = 0.98 - 1.20
k' =	0.93	



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

Peak #	Conc (ug/ml)	Sample Component
1	5	Uracil
2	200	Phenol
3	25	4-Chloro Nitrobenzene
4	850	Toluene

Column Volume by Geometry

- Starting with the equation for the volume of a cylinder
- $V_{cyl} = \pi r^2 d$
- Then adjust to allow for the space taken up by the particles and call it column volume
- $V_{col} = (\pi r^2 d) \cdot 0.6$

Summary

- Chromatographic Process
- Isocratic Resolution
- Particles and Pressure
- Van Deemter Equation
- Gradient Resolution
- Method Conversions

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

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spp-support@agilent.com

spectro-supplies-support@agilent.com