Demystifying the Chromatographic Process

Agilent Technologies, Inc.

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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
- What Affects Resolution, R_S
- What Affects Retention, t_R
- What Affects Pressure, P
- Mis-use, Over-interpretation of Chromatographic Equations

Conclusions of Talk

Effect of Increasing	N	α	k	tR	P
Flow	Van Deemter	No	No	Decrease	Increase
% B	Slight	Varies	Exp Dec	Exp Dec	Varies
Temperature (T)	Increase	Varies	Decrease	Decrease	Decrease
Particle Size (d_p)	Decrease	No	No	No	Decrease
Col Length (L)	Increase	No	No	Increase	Increase
Col Diameter(d)	Slight	No	No	No*	No*

* At constant linear velocity

Chromatographic Process

- Partition between mobile phase and stationary phase
- Description of the separation:

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

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Topics

- Chromatographic Process
- What Affects Resolution, Rs
- What Affects Retention, t_R
- What Affects Pressure, P

Definition of Resolution

$$R_s = \frac{\Delta t_R}{\bar{w}}$$

Resolution is a measure of the ability to separate two components

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

Resolution …

Determined by 3 Key Parameters – Efficiency, Selectivity and Retention

The Fundamental Resolution Equation

$$R_S = \frac{\sqrt{N}}{4} \frac{(\alpha - 1)}{\alpha} \frac{k_2}{(\bar{k} + 1)} = \frac{\Delta t_R}{\bar{w}}$$

N = Column Efficiency – Column length and particle size

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

cf, Foley, *Analyst*, **116**, 1275 (1991)

Resolution …

Determined by 3 Key Parameters – Efficiency, Selectivity and Retention

The Fundamental Resolution Equation

$$R_S = \frac{\sqrt{N}}{4} \frac{(\alpha - 1)}{1} \frac{k_1}{(\bar{k} + 1)} = \frac{\Delta t_R}{\bar{w}}$$

N = Column Efficiency – Column length and particle size

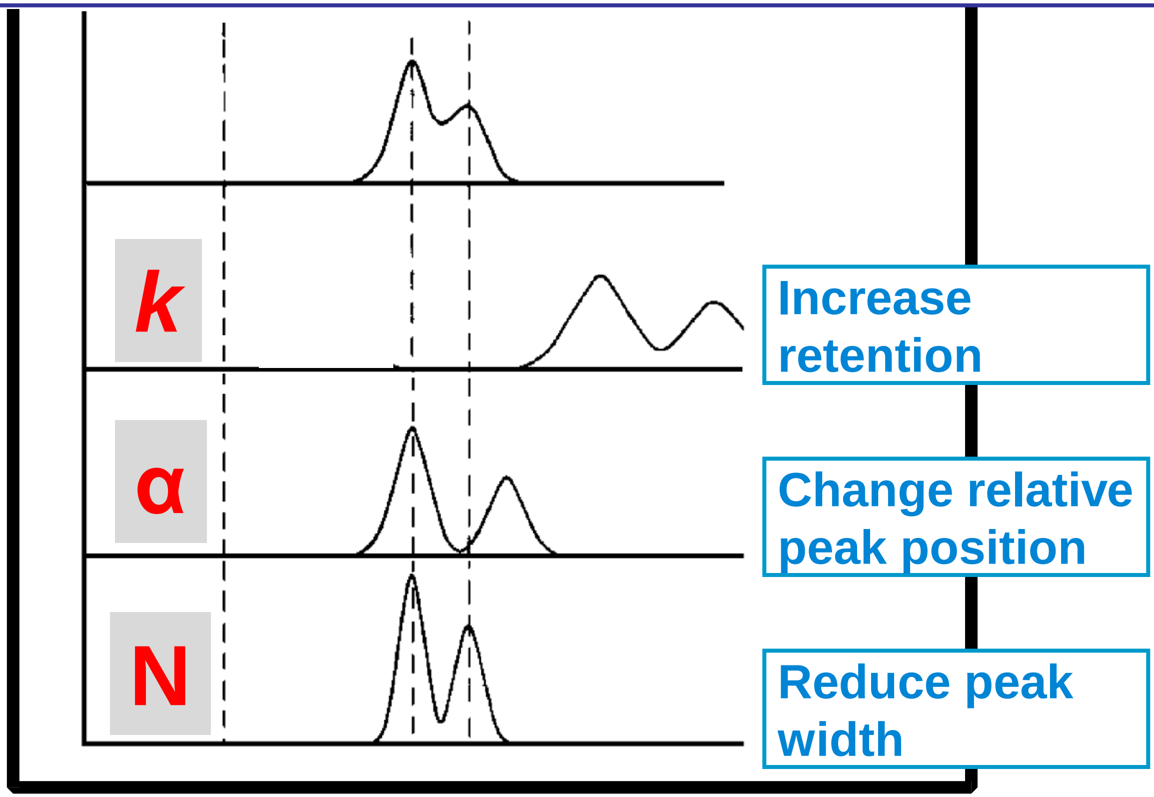
α = Selectivity – Mobile phase and stationary phase

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

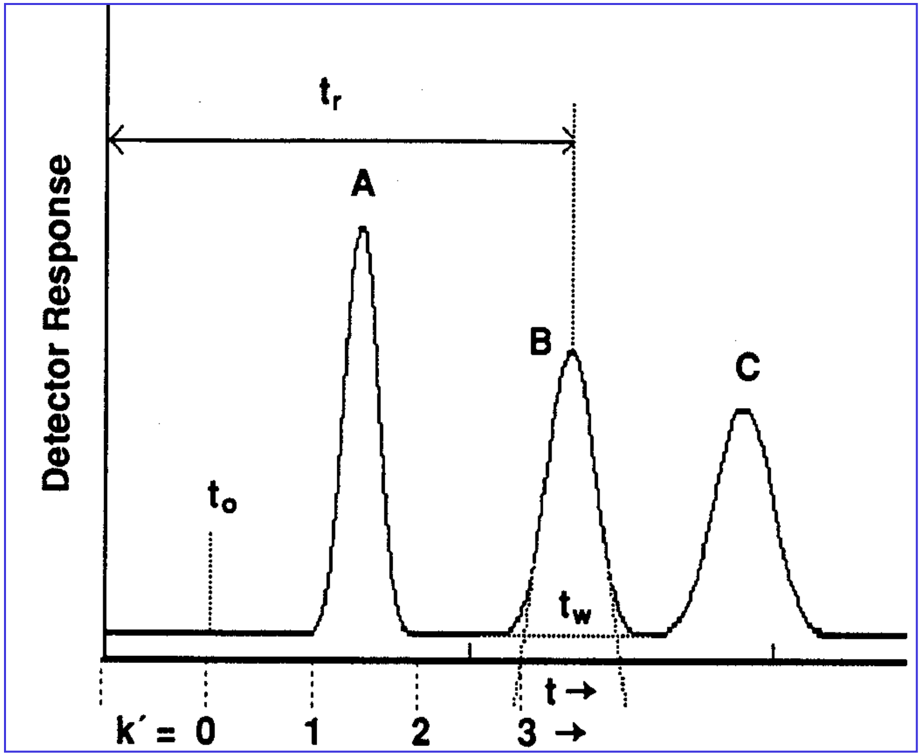
cf, Foley, *Analyst*, **116**, 1275 (1991)

Factors that Improve Resolution

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



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

$$N = 16(t_R / t_W)^2$$

Some Basic Chromatography Parameters

- Resolution (R_S)
- **Retention Factor (*k*), Capacity Factor (*k'*)**
- Selectivity or Separation Factor (α)
- Column Efficiency as Theoretical Plates (N)

Retention Factor (*k*), Capacity Factor (*k'*)

Chromatographic separation is an Equilibrium Process

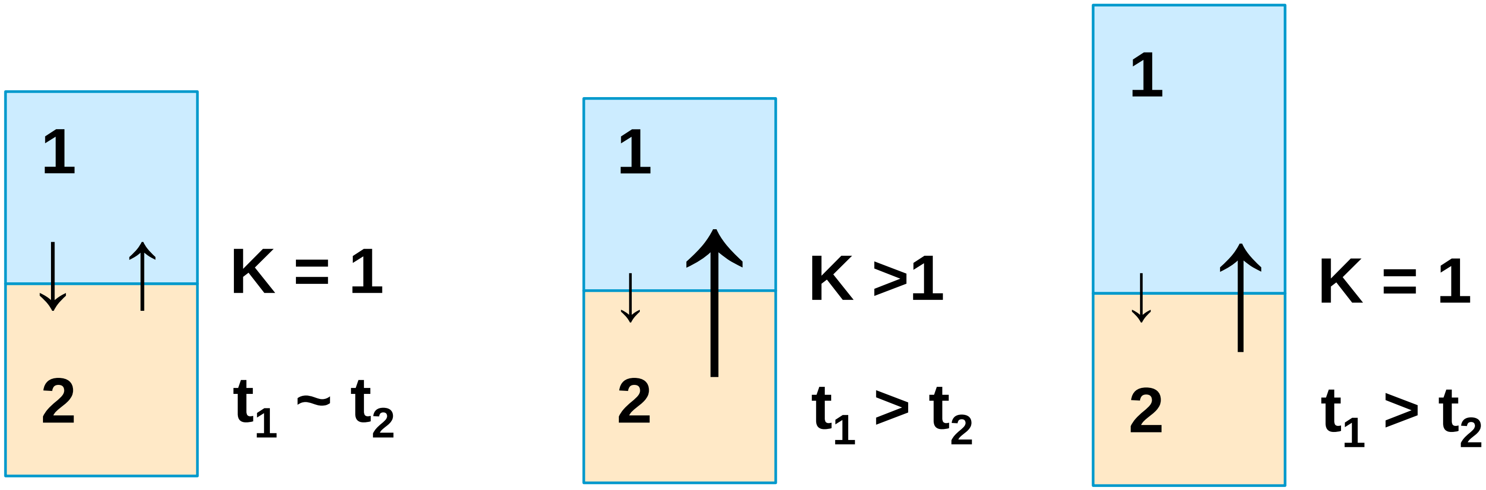
Sample partitions between Stationary Phase and Mobile Phase:

$$K = C_s/C_m$$

Compound moves through the column only while in mobile phase.

Separation occurs in <u>Column</u> <u>Volumes</u>.
(Flow is volume/time – mL/min)

Retention Factor (*k*), Capacity Factor (*k'*)



$$K = C_1 / C_2$$

$$K \propto t_1 / t_2$$

Retention Factor (*k*), Capacity Factor (*k'*)

$$K = C_s/C_m \Rightarrow\Rightarrow \quad k = \frac{t_S}{t_M} \qquad \boxed{k = \frac{t_R - t_0}{t_0}}$$

k is measure of number of column volumes required to elute compound (proportion of time in stationary phase).

Fundamental, dimensionless parameter that describes the retention (independent of flow rate, column length).

***k* = <u>1 to 20</u> - OK; *k* = <u>3 to 10</u> - Better; *k* = <u>5 to 7</u> - Ideal**

Retention Factor (*k*), Capacity Factor (*k'*)

$$K = C_s/C_m \Rightarrow\Rightarrow \quad k = \frac{t_S}{t_M}$$

$$t_R = t_M + t_S$$

k is measure of number of column volumes required to elute compound (proportion of time in stationary phase).

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Retention Factor (*k*), Capacity Factor (*k'*)

$$K = C_s/C_m \Longrightarrow\!\!\!\Rightarrow \quad k = \frac{t_S}{t_M}$$

$$t_R - t_M = t_S$$

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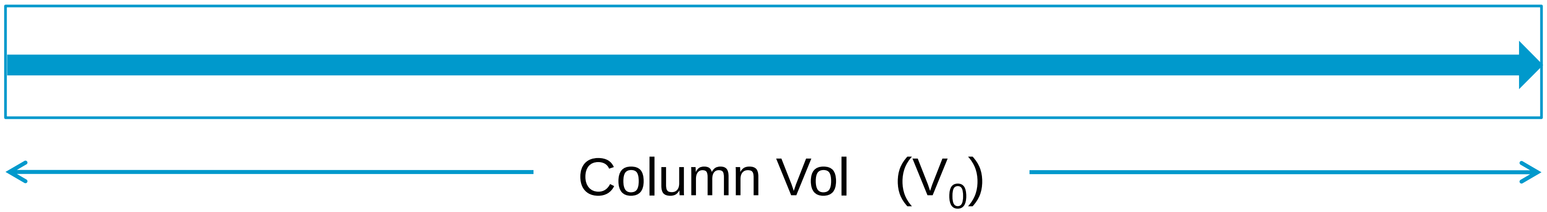
Retention Factor (*k*), Capacity Factor (*k'*)

$$k = \frac{(V_R - V_0)}{V_0} = \frac{(t_R - t_0)}{t_0}$$

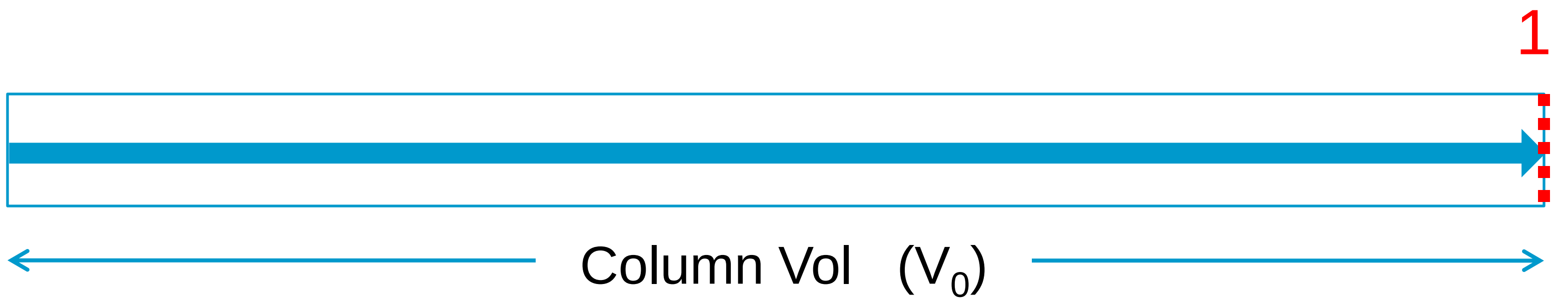
Measure of number of column volumes required to elute compound – fraction of time spent in SP (C_s)



Retention Factor (*k*), Capacity Factor (*k'*)

Un-retained component – elutes w/ solvent front, $C_s = 0$

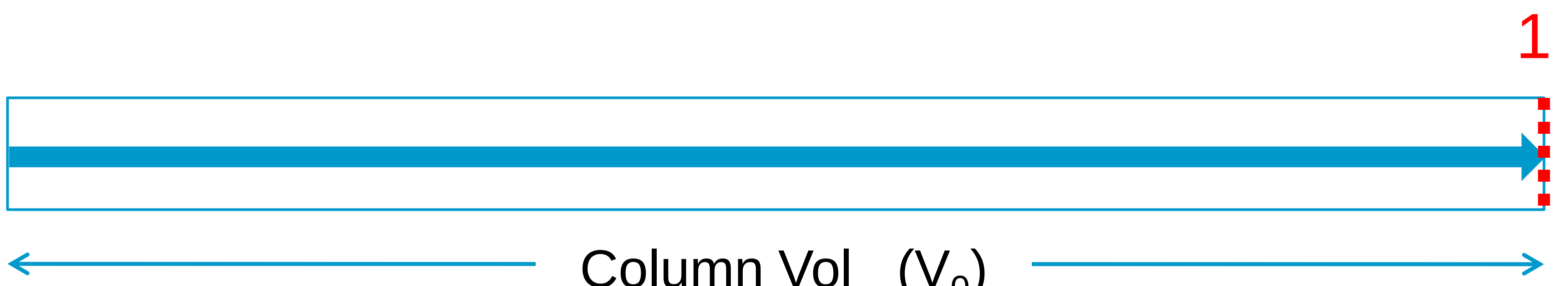
<u>**$C_s = 0 \Rightarrow k = 0$**</u>



Retention Factor (*k*), Capacity Factor (*k'*)

Un-retained component – elutes w/ solvent front

$k = (1 - 1) / 1 = 0$

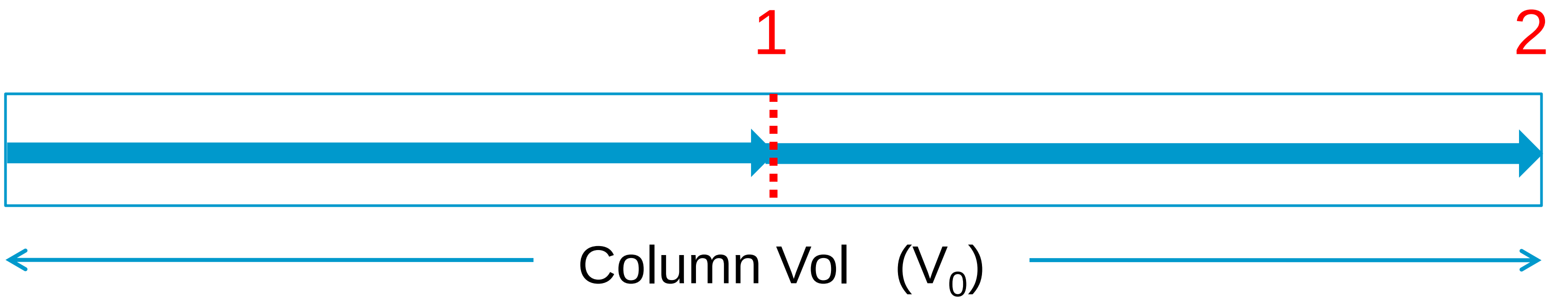


$$k = (t_R - t_0) / t_0$$

Retention Factor (*k*), Capacity Factor (*k'*)

Component retained – elutes in 1 add'l column volumes

$k = (2 - 1) / 1 = 1$

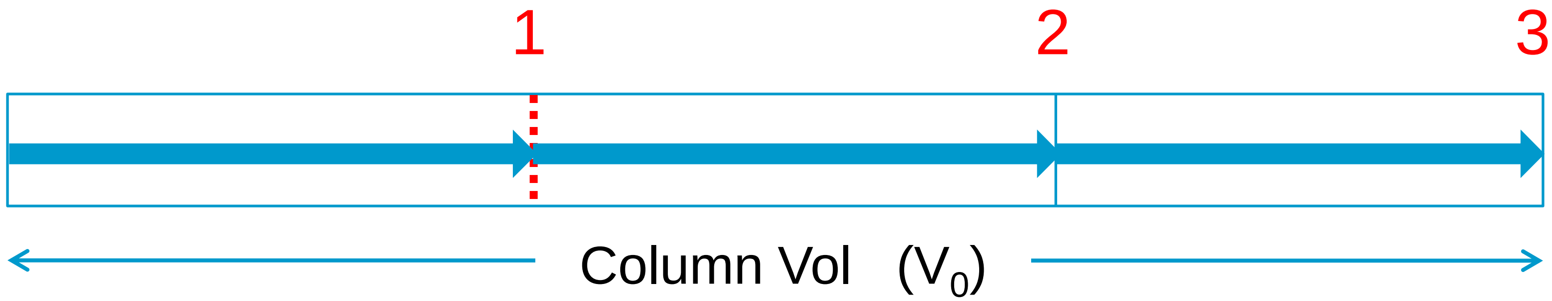


$$k = (t_R – t_0) / t_0$$

Retention Factor (*k*), Capacity Factor (*k'*)

Component retained – elutes in 2 add'l column volumes

$k = (3 - 1) / 1 = 2$

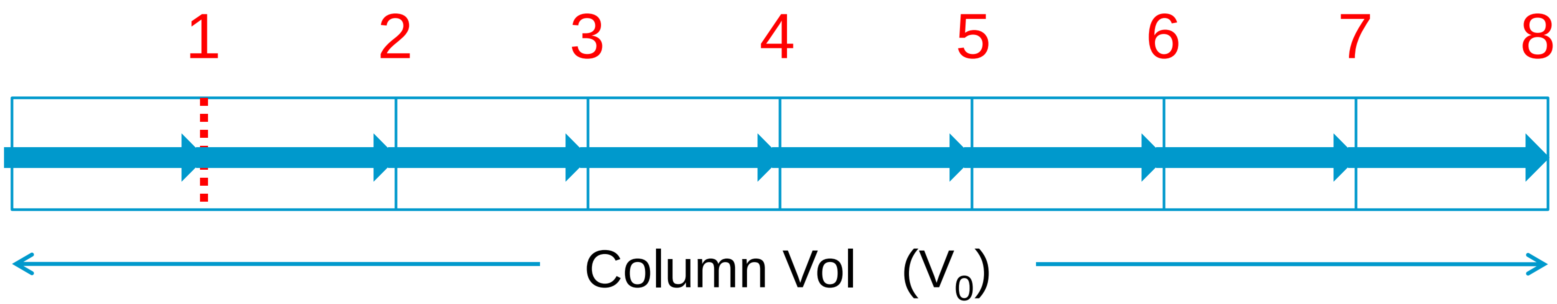


$$k = (t_R – t_0) / t_0$$

Retention Factor (*k*), Capacity Factor (*k'*)

Component retained – elutes in 7 add'l column volumes

k = (8 - 1) / 1 = 7



$$k = (t_R - t_0) / t_0$$

Retention Factor (*k*), Capacity Factor (*k'*)

Component retained – elutes in 7 add'l column volumes

$k = (8 - 1) / 1 = 7$

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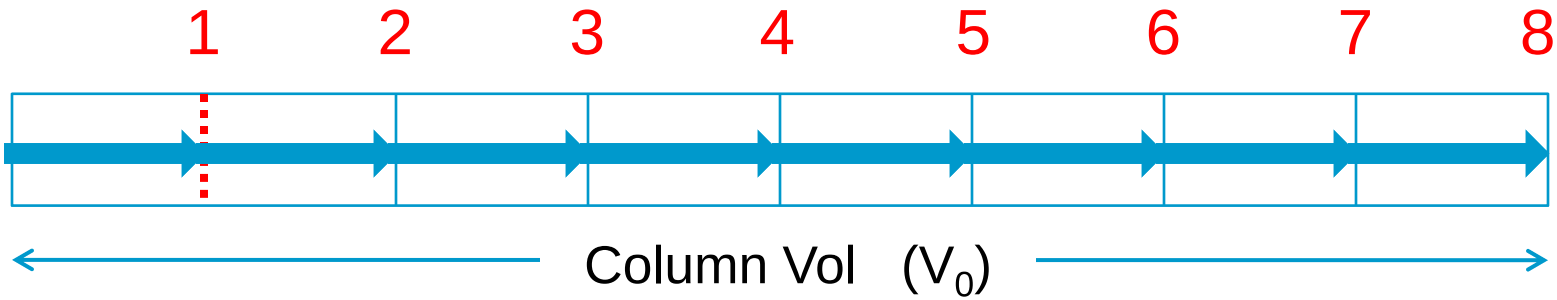
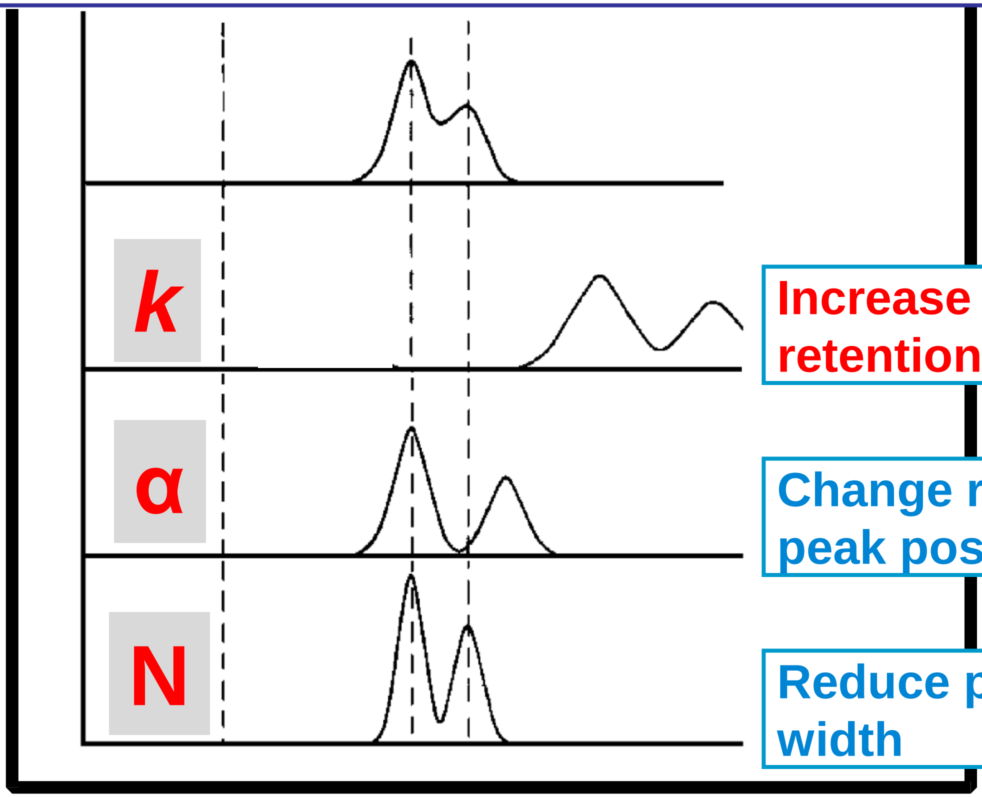


$k = (t_R – t_0) / t_0$

Factors that Improve Resolution

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

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



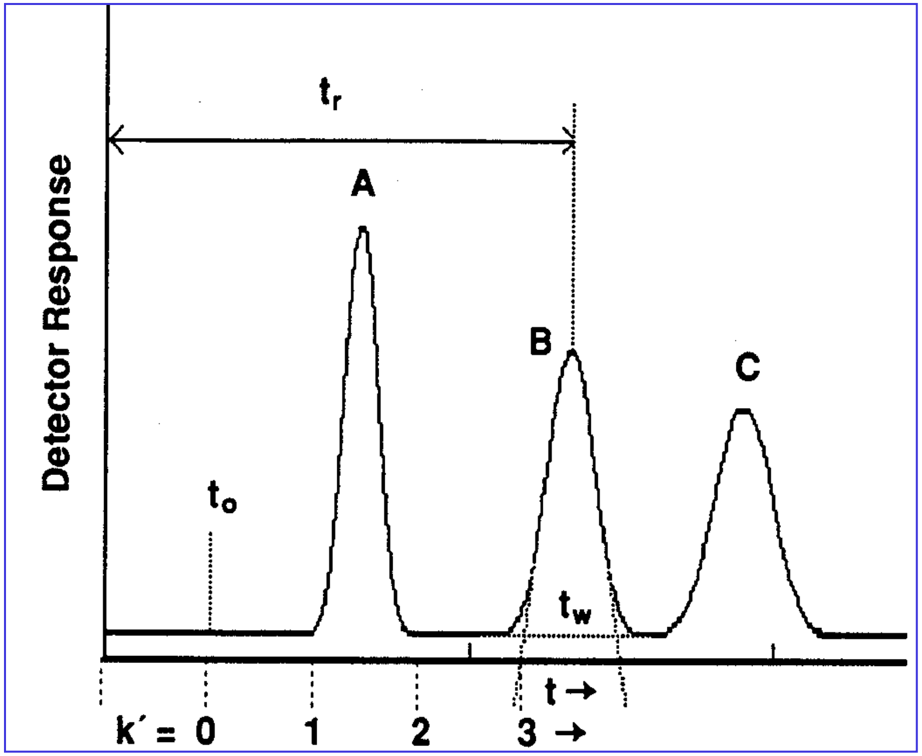
Increase retention

Change relative peak position

Reduce peak width

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

$$N = 16(t_R / t_W)^2$$

Some Basic Chromatography Parameters

- Resolution (R_S)
- Retention Factor (k), Capacity Factor (k')
- **Selectivity or Separation Factor (α)**
- Column Efficiency as Theoretical Plates (N)

Selectivity (*α*)

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

α is measure relative difference in retention

Selectivity (α)

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

α is measure relative difference in retention

$$k_1 = t_{s1}/t_m$$

$$k_2 = t_{s2}/t_m$$

Selectivity (α)

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

α is measure relative difference in retention

$$k_1 = t_{s1}/t_m$$

$$k_2 = t_{s2}/t_m$$

$$\alpha = \frac{t_{s2}}{t_{s1}}$$

Selectivity (α)

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

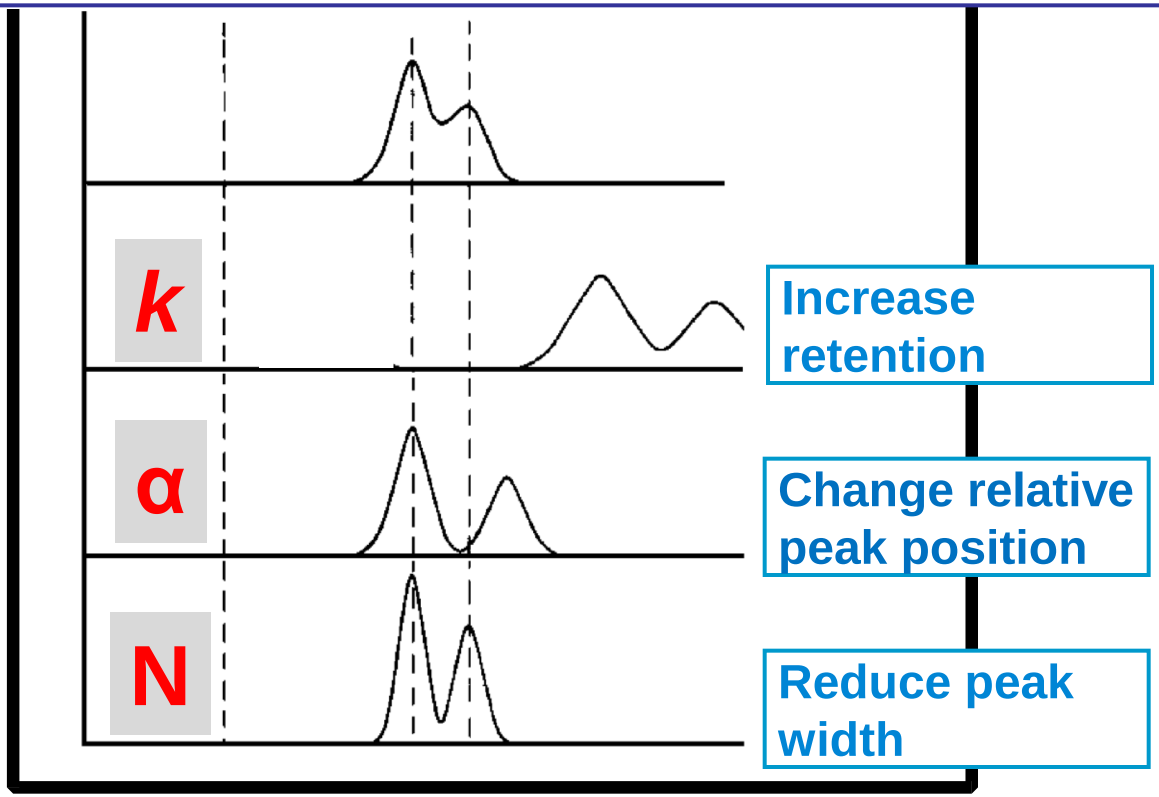
α is measure of relative difference in retention

By definition, k_2 is more retained component;
k_1 is less retained component, so α is always ≥ 1

To obtain separation, α must be > 1

Factors that Improve Resolution

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

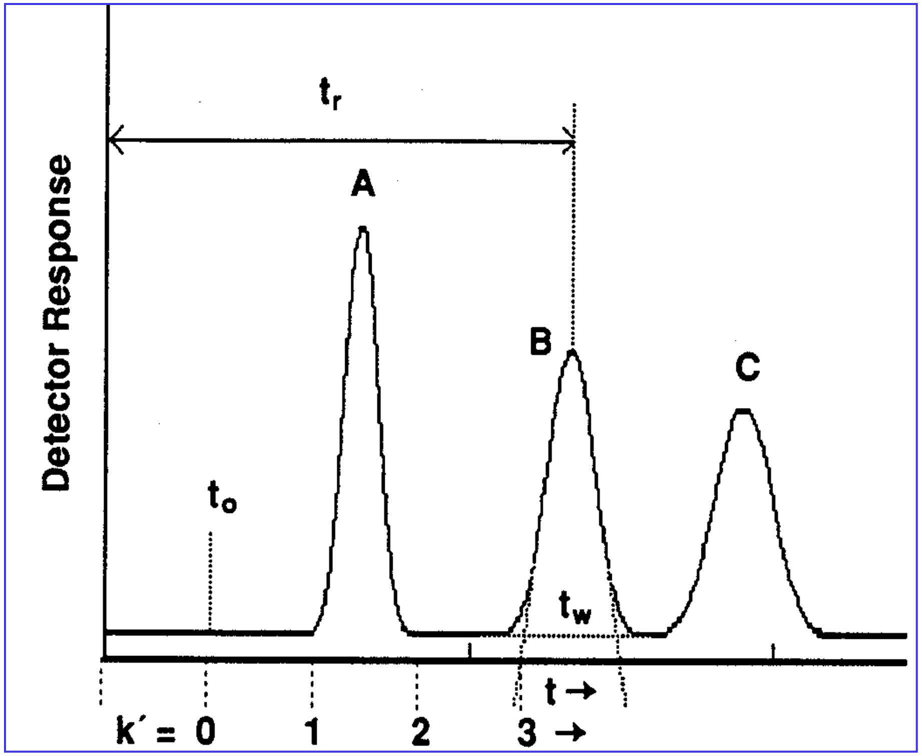


Selectivity

$\alpha = k_2/k_1$

Chromatographic Profile

Equations Describing Factors Controlling R_S



Retention Factor

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

Selectivity

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Theoretical Plates-Efficiency

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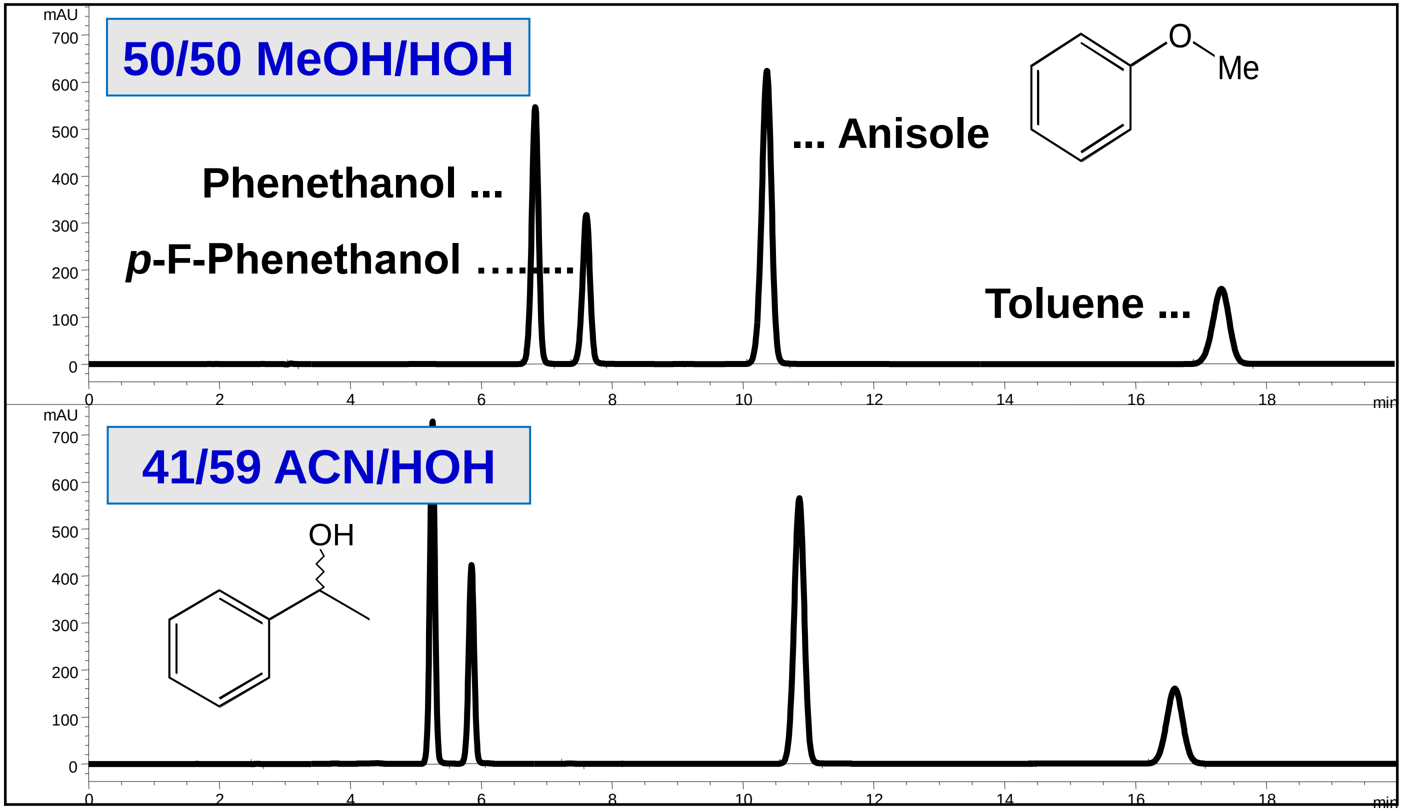
Selectivity

- Mobile Phase
- Stationary Phase

Selectivity

- **Mobile Phase**
- Stationary Phase

Different Mobile Phases May Give Different Selectivity



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

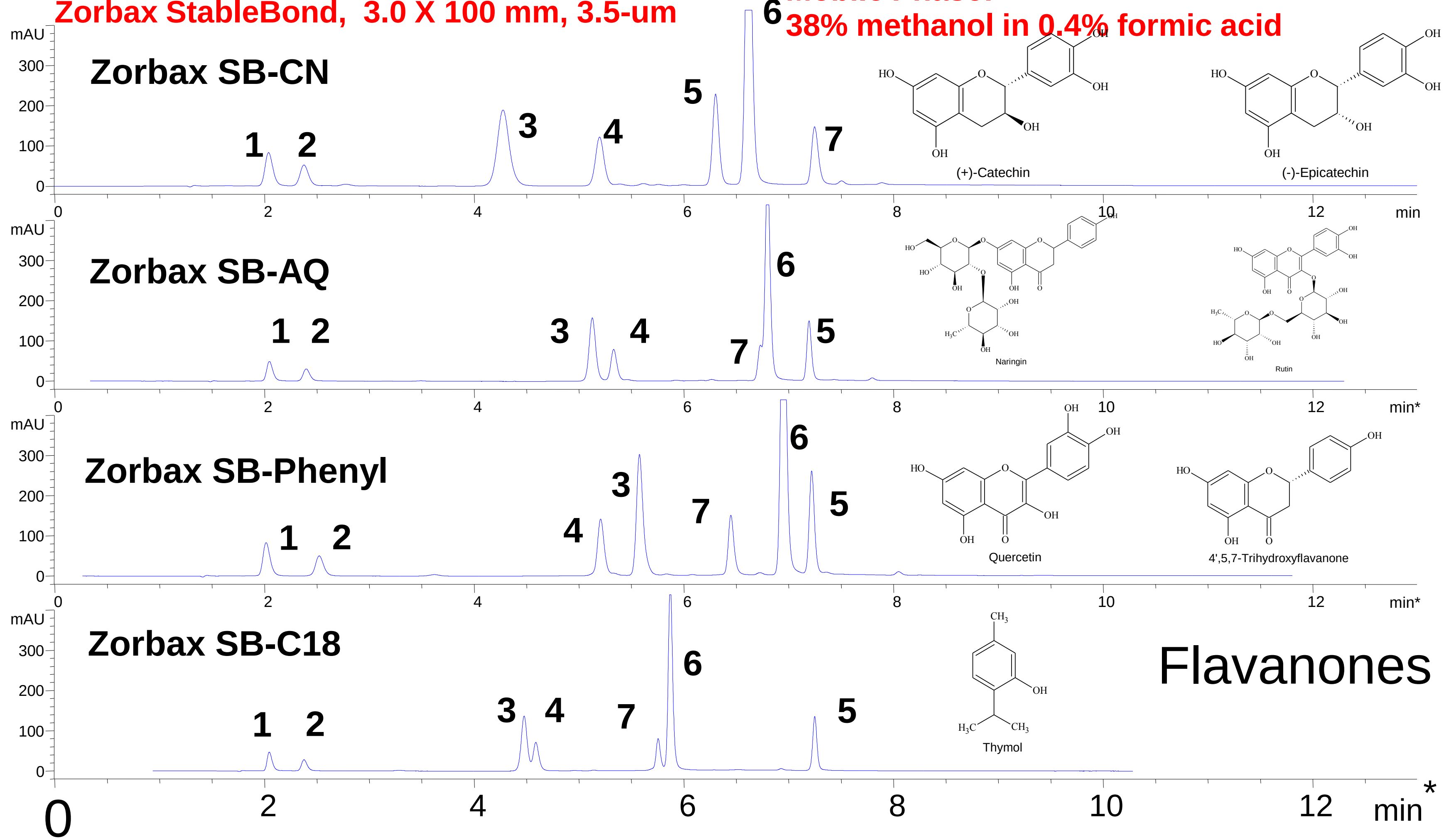
Selectivity

- Mobile Phase
- **Stationary Phase**

Different Stationary Phases May Give Significantly Different Selectivity

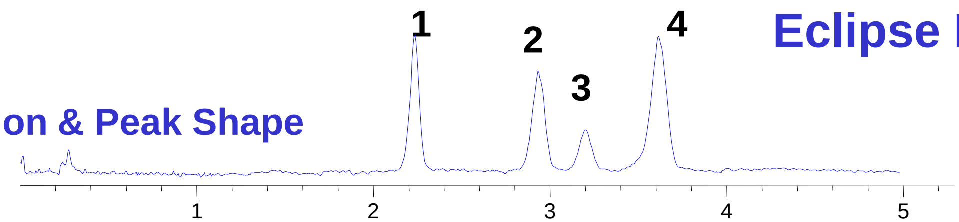
Columns:
Zorbax StableBond, 3.0 X 100 mm, 3.5-um

Mobile Phase:
38% methanol in 0.4% formic acid



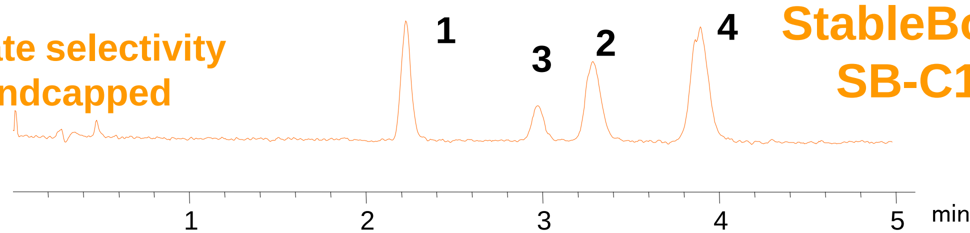
Similar Stationary Phases May Give Different Selectivity

1st choice
Best Resolution & Peak Shape



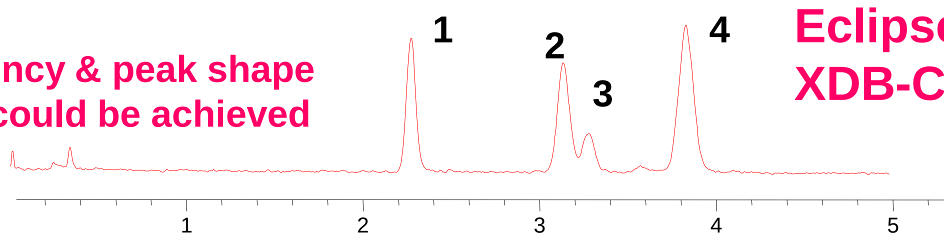
Eclipse Plus C18

2nd choice
Good alternate selectivity due to non-endcapped



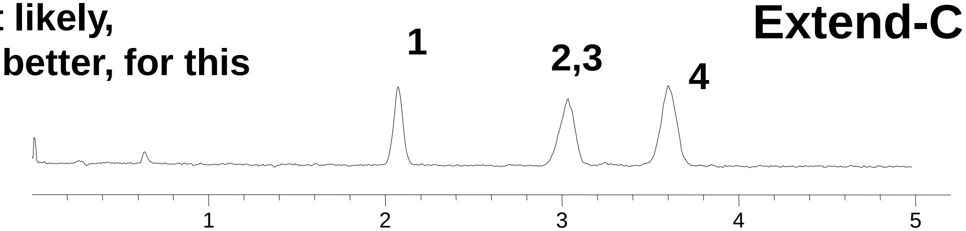
StableBond SB-C18

3rd choice
Good efficiency & peak shape
Resolution could be achieved



Eclipse XDB-C18

4th choice
Resolution not likely,
Other choices better, for this separation.



Extend-C18

Mobile phase: (69:31) ACN: water
Flow 1.5 mL/min.
Temp: 30 °C
Detector: Single Quad ESI positive mode scan
Columns: RRHT
4.6 x 50 mm 1.8 um

Sample:
1. anandamide (AEA)
2. Palmitoylethanolamide (PEA)
3. 2-arachinoylglycerol (2-AG)
4. Oleoylethanolamide (OEA)

Multiple bonded phases for most effective method development.
Match to one you're currently using.

Selectivity

- "Chromatography is an Experimental Science"
- α not as predictable as *k*, N – but more powerful

Some Basic Chromatography Parameters

- Resolution (R_S)
- Retention Factor (*k*), Capacity Factor (*k'*)
- Selectivity or Separation Factor (α)
- **Column Efficiency as Theoretical Plates (N)**

Column Efficiency (N)

N - Number of theoretical plates.

"Plates" is a term inherited from distillation theory. It is a measure of the relative peak broadening (or peak width) for an analyte in a separation – **w**

$$N = 16\left[\frac{t_R}{w}\right]^2$$

A Number of Theoretical Plates

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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)

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

L = column length
d_p = particle size

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)

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

L = column length
d_p = particle size

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)

$$N \approx 5{,}000 \times \frac{L}{d_p}$$

L = column length (mm)
d_p = particle size (µm)

cf: Snyder, Kirkland, Dolan, *Introduction to Modern Liquid Chromatography, 3rd Ed*, Wiley (2010), p244

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 #:

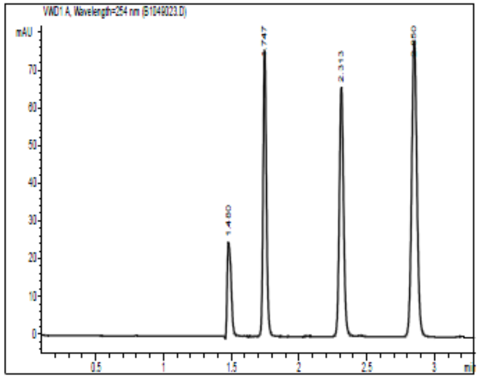
TEST CONDITIONS

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 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)

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

L = column length
d_p = particle size

Column Efficiency (N) – Effect of Particle Size

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)

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

L = column length
d_p = particle size

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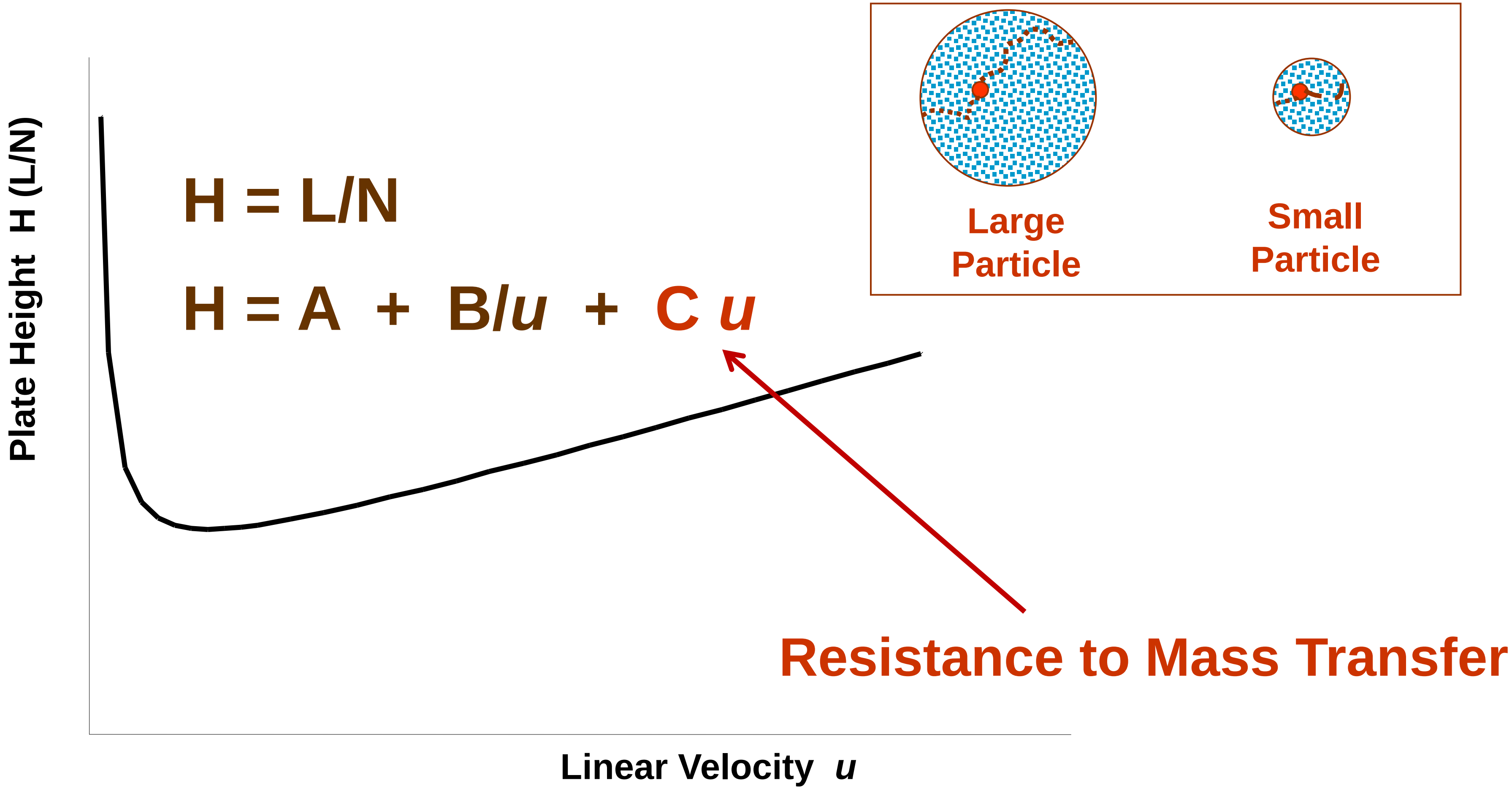
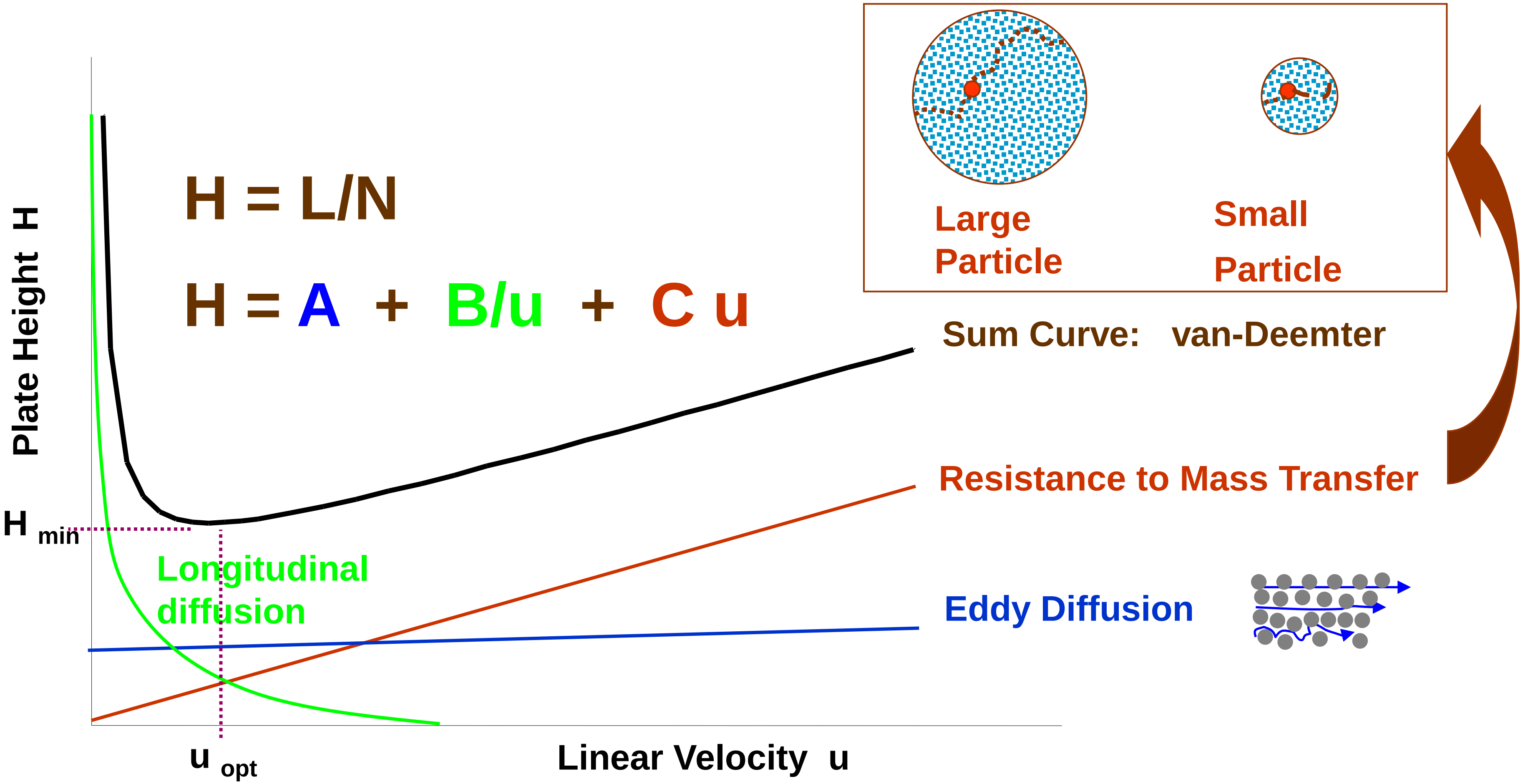
Van Deemter Curve
Factors Affecting N



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

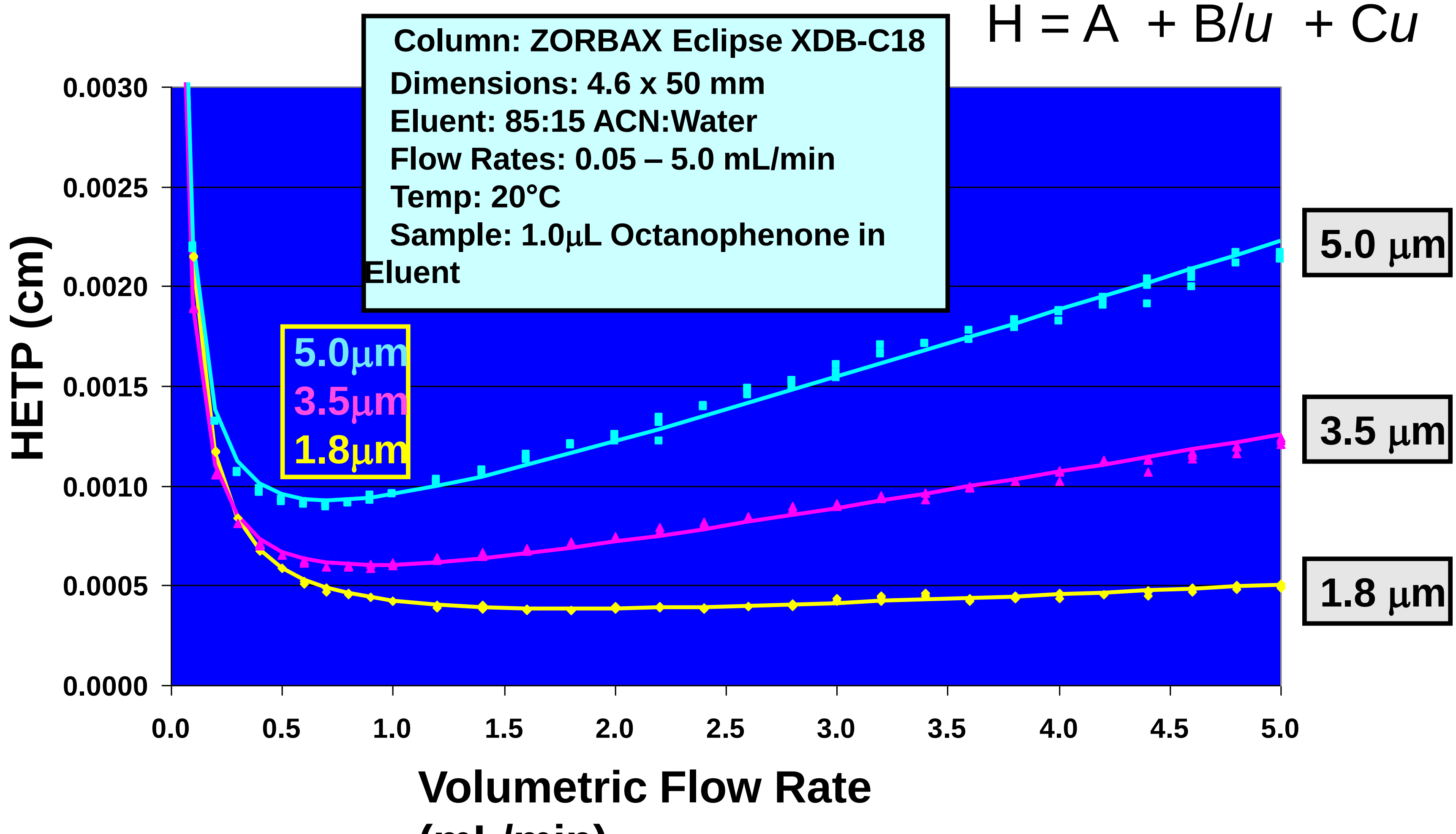
Putting it Together
The van Deemter Equation



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

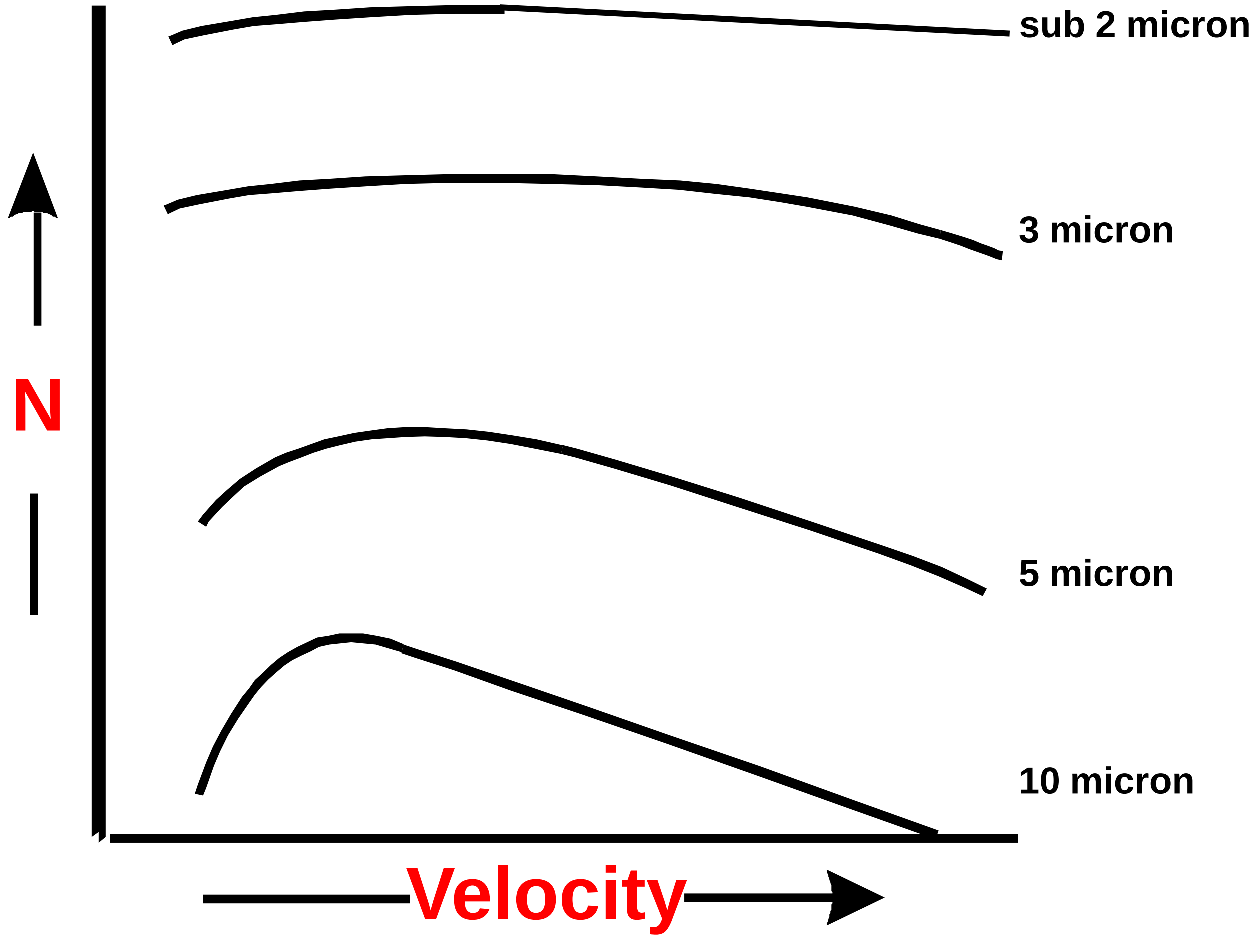
Van Deemter Curve
Effect of Particle Size

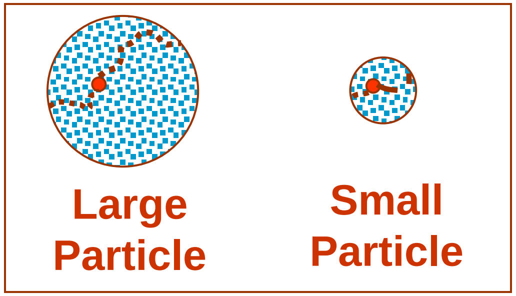
$$H = A + B/u + Cu$$



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

Columns Packed with Smaller Particles Provide Higher Efficiency

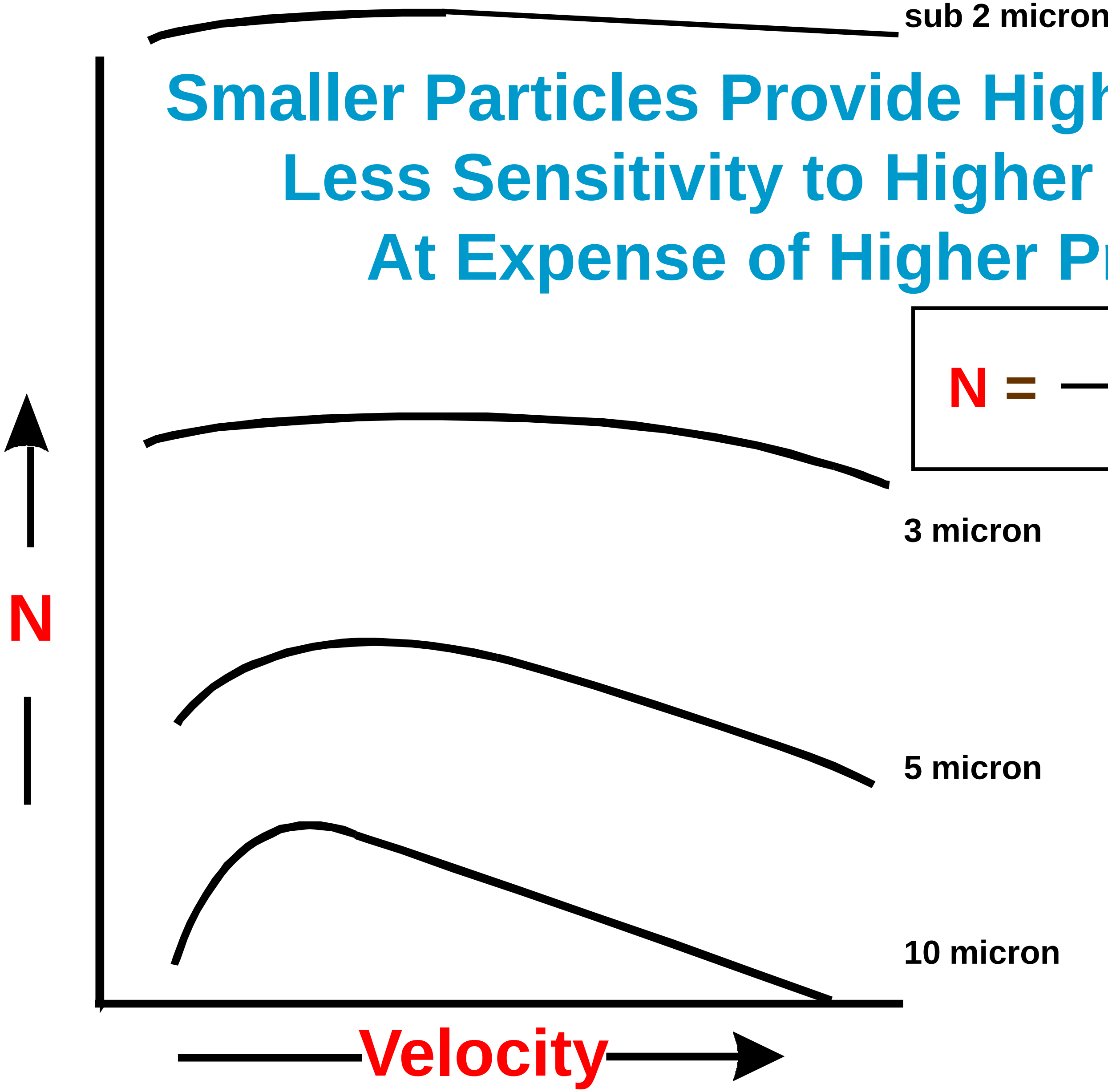




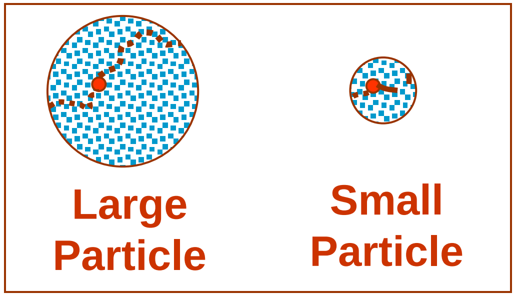
$$N \propto 1/(d_p)$$

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

Smaller Particles Provide Higher Efficiency, Less Sensitivity to Higher Flow Rate At Expense of Higher Pressure



$$N = \frac{1}{A + B/u + C\,u}$$



$$N \;\alpha\; 1/(d_p)$$

$$P \;\alpha\; 1/(d_p)^2$$

Decreasing Particle Size

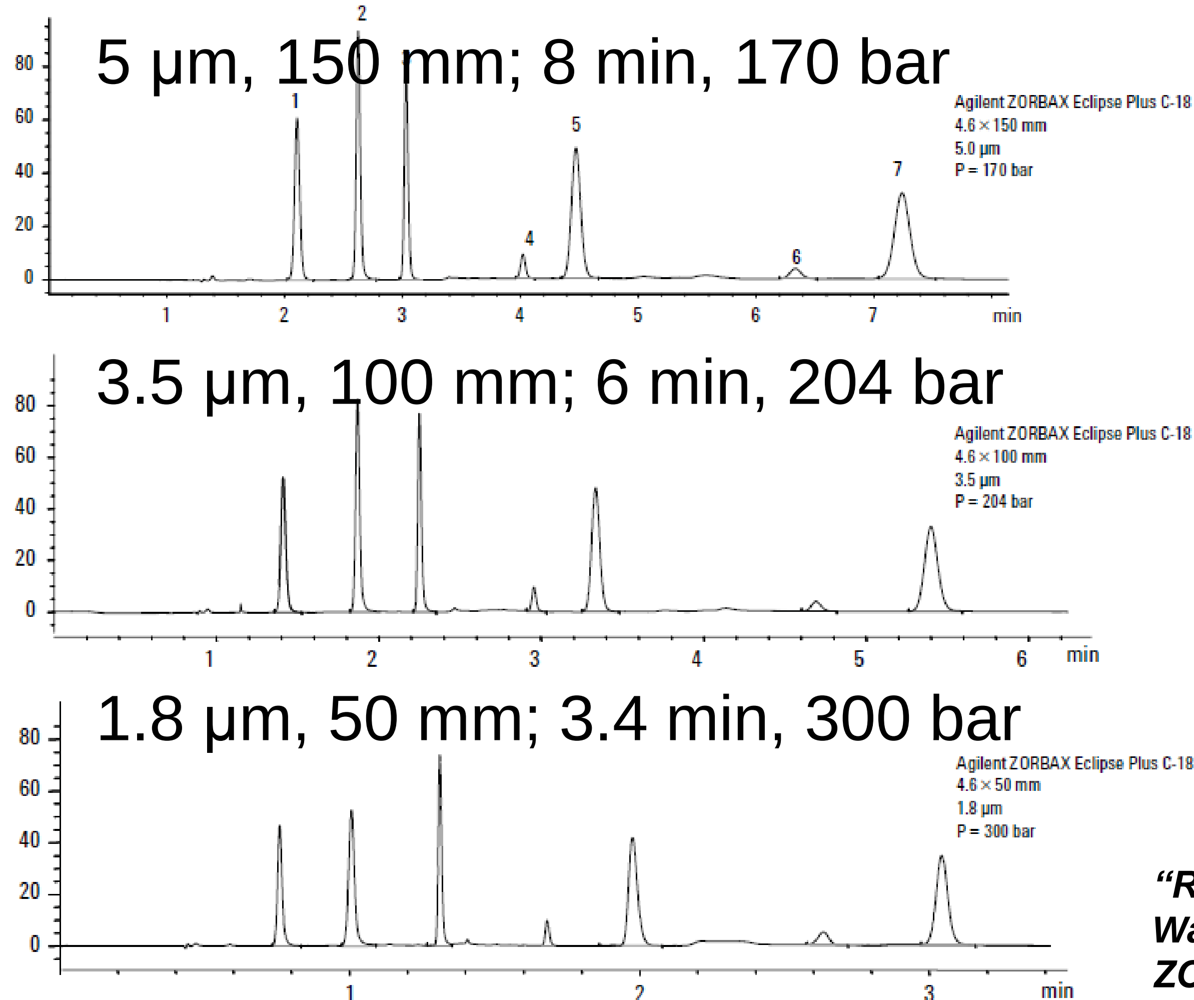


Table 2. Chromatographic Conditions

LC	Agilent 1200 SL
Mobile phase A	25 mM NaH_2PO_4 pH = 2.5
Mobile phase B	Methanol
Flow rate	1.00 mL/min
Column compartment temperature	35 °C
Detection	220 nm, no Reference
Response time	0.05 s
Injection volume	Adjusted for column size: 5 µm, 5 µL 3.5 µm, 3.3 µL 1.8 µm, 1,7 µL
Detector flow cell	Micro flow cell (2 µL)

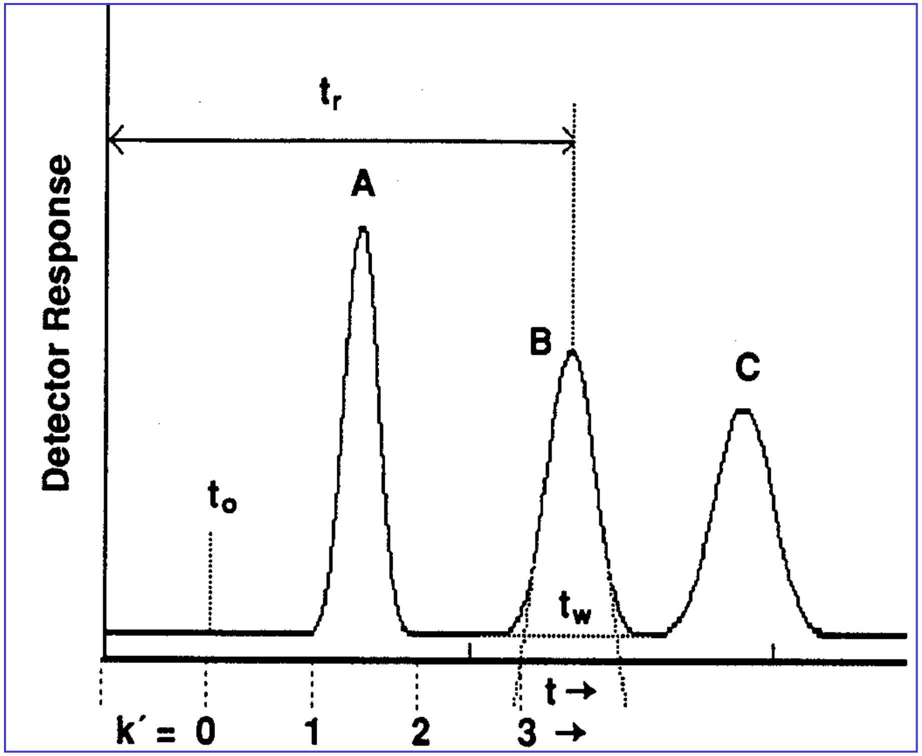
Table 3. Gradients for Equivalent k*

%B	5 µm		3.5 µm		1.8 µm
1	0.00 min	→	0.00 min	→	0.00 min
12	1.50 min		1.00 min		0.50 min
30	1.53 min		1.03 min		0.51 min

"Reversed-Phase HPLC Separation of Water-Soluble Vitamins on Agilent ZORBAX Eclipse Plus Columns",
5989-9313EN (2008)

Chromatographic Profile

Equations Describing Factors Controlling R_S



Retention Factor

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

Selectivity

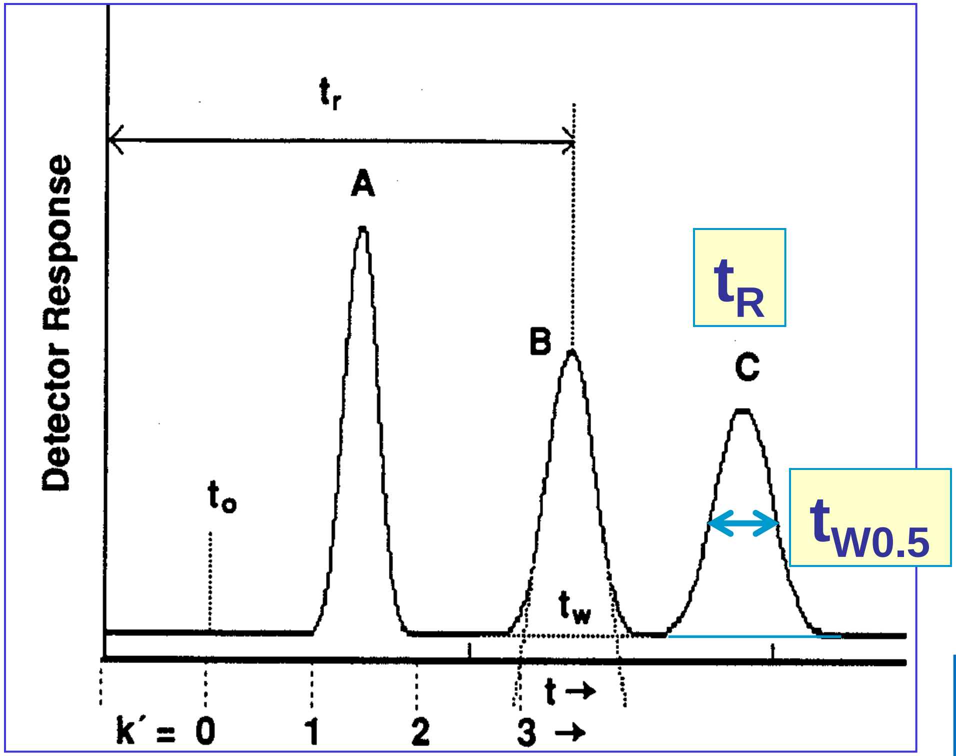
$$\alpha = k_2 / k_1$$

Theoretical Plates - Efficiency

$$N = 16(t_R / t_W)^2$$

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

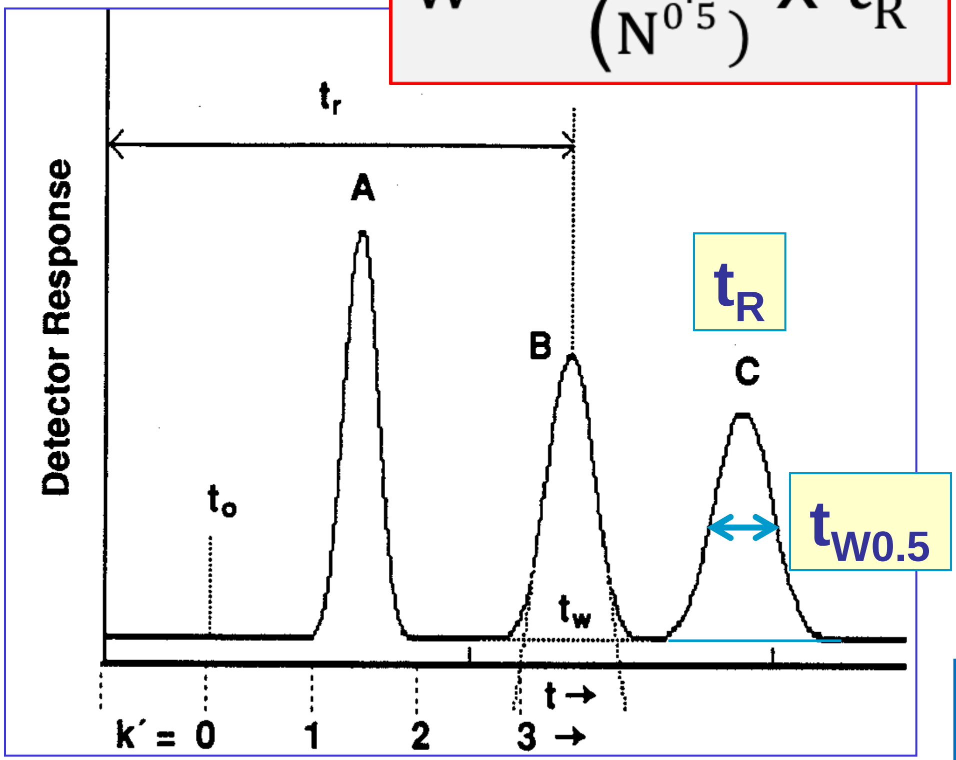
$$N = 16(t_R / t_W)^2$$

$$\mathbf{N = 5.54(t_R / t_{W0.5})^2}$$

Chromatographic Profile

Equations Describing Factors Controlling R_S

$$w = \frac{4}{(N^{0.5})} \times t_R$$



Retention Factor

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

Selectivity

$$\alpha = k_2/k_1$$

Theoretical Plates - Efficiency

$$N = 16(t_R / t_W)^2$$

$$\mathbf{N = 5.54(t_R / t_{W0.5})^2}$$

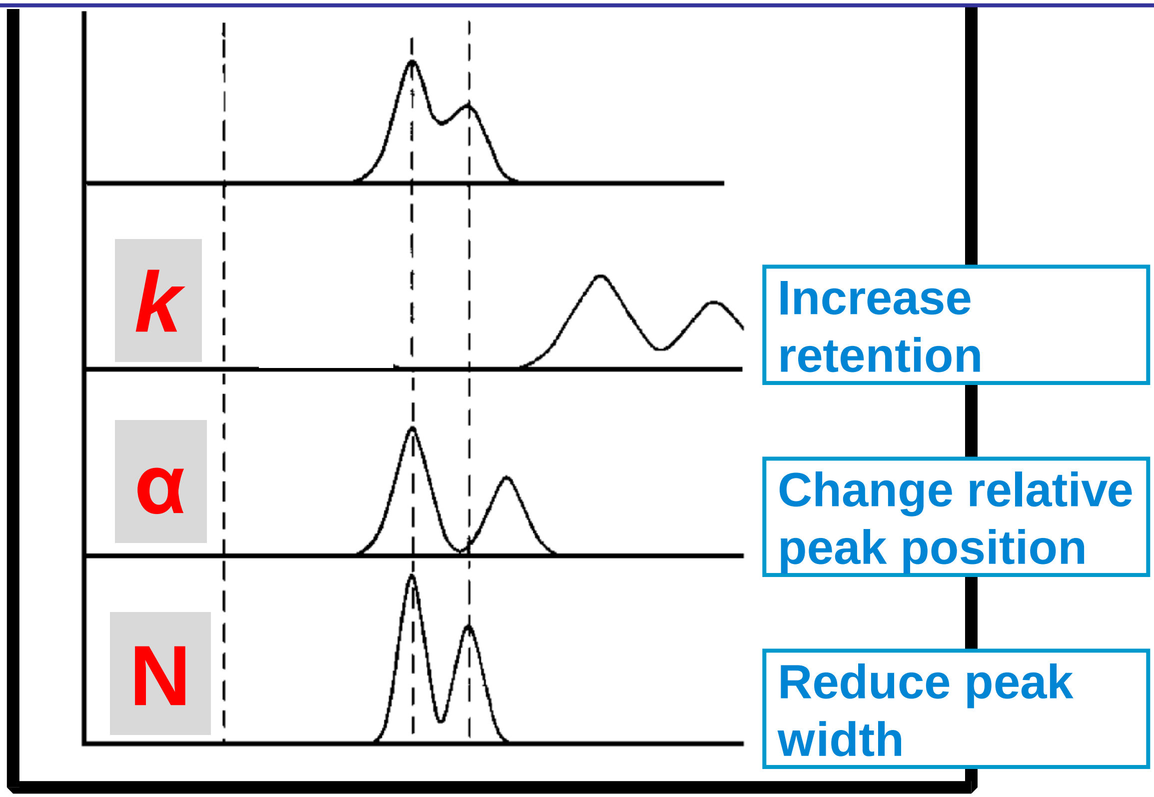
Agilent Technologies

Some Basic Chromatography Parameters

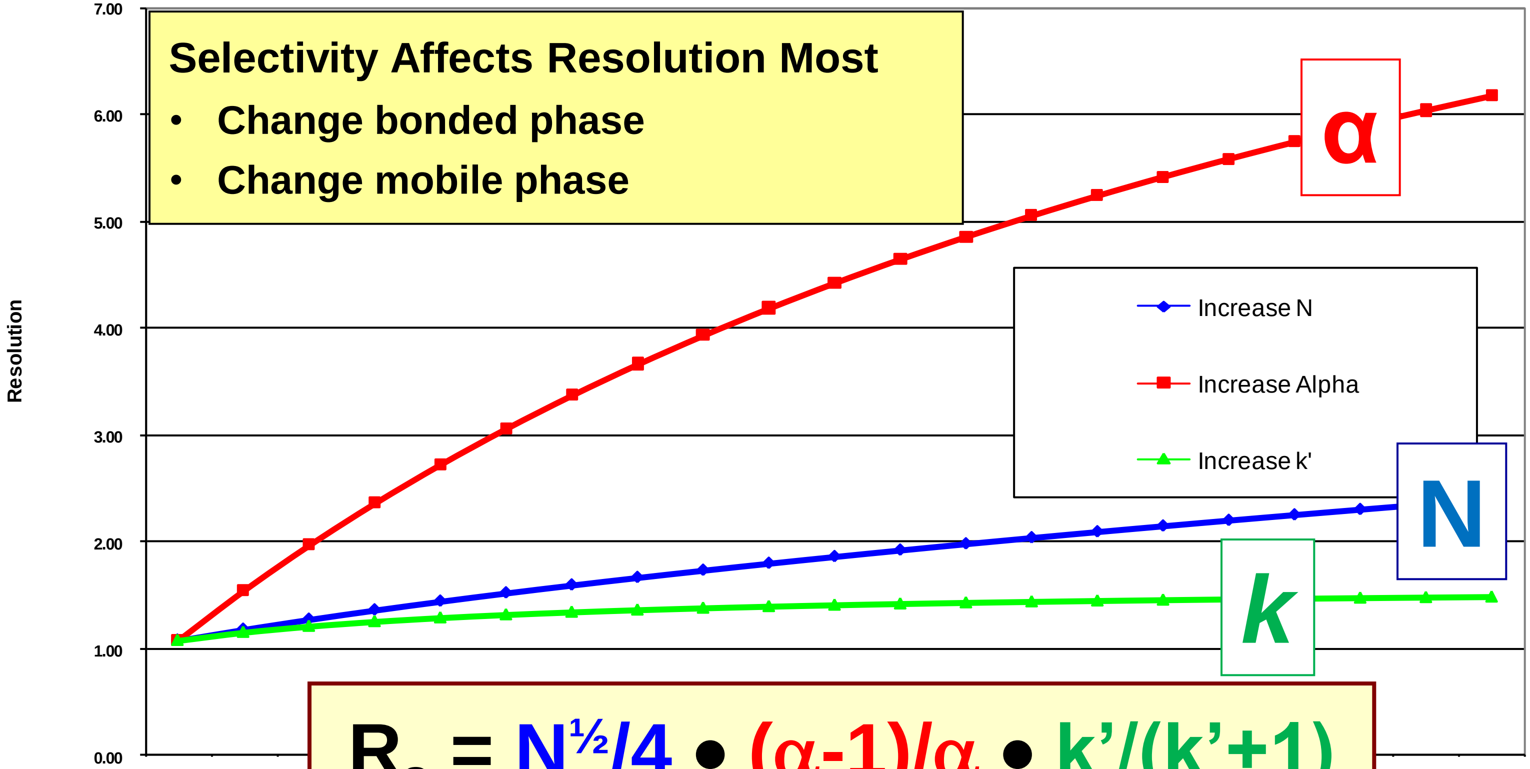
- Resolution (R_S)
- Retention Factor (*k*), Capacity Factor (*k'*)
- Selectivity or Separation Factor (α)
- Column Efficiency as Theoretical Plates (N)

Factors that Affect Resolution

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



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



$$R_s = N^{1/2}/4 \bullet (\alpha-1)/\alpha \bullet k'/(k'+1)$$

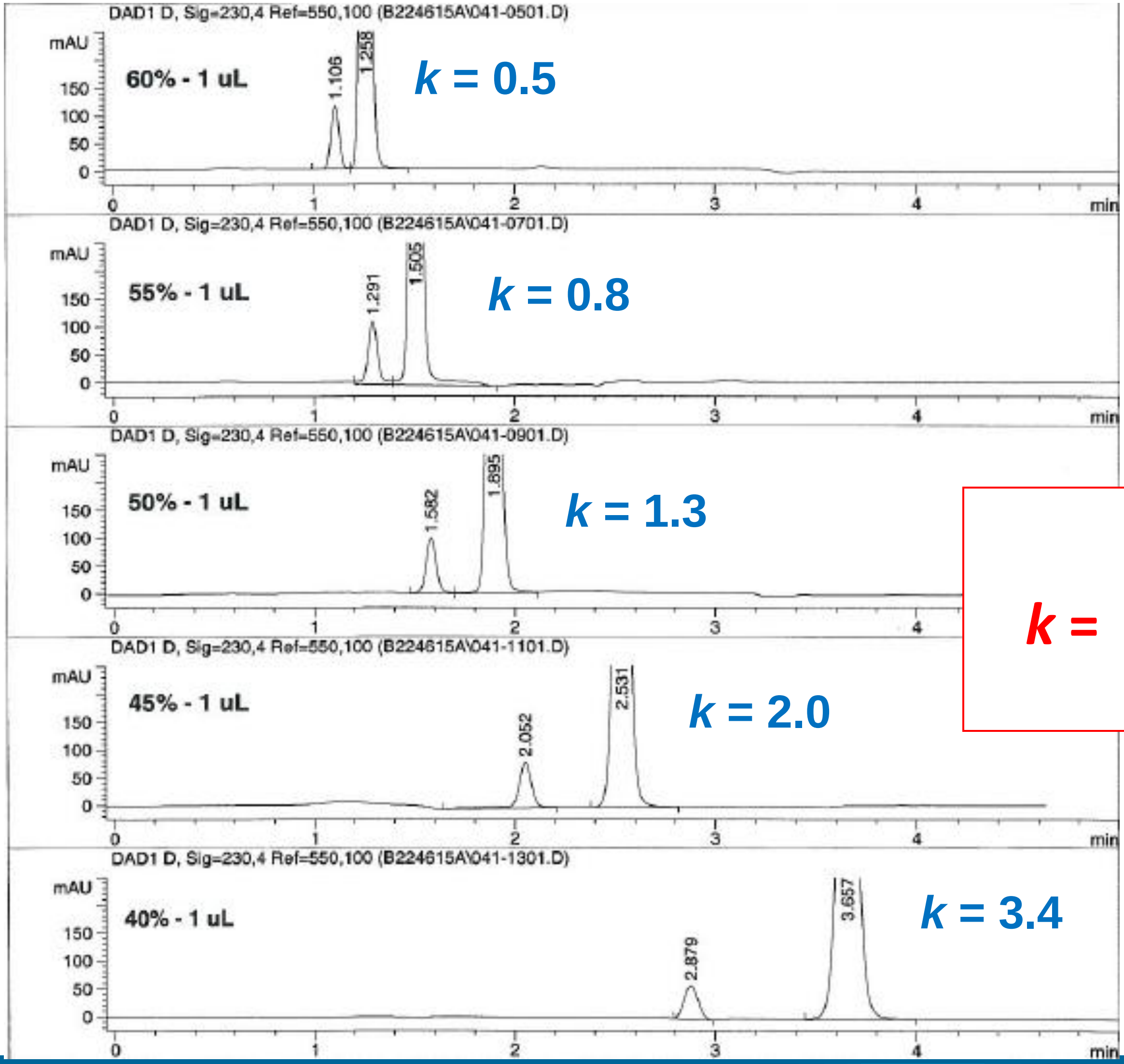
Topics

- **What Affects Resolution, R_S**
- **What Affects Retention, t_R**
- **What Affects Pressure, P**

Retention

- Mobile Phase Strength
- Temperature

Effect of Mobile Strength on Retention



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

Mobile Strength

%ACN	tR-1	tR-2	k(1)	k(2)	ln(k1)	ln(k2)	Sel (a)
60	1.11	1.26	0.33	0.52	-1.10	-0.66	1.58
55	1.29	1.51	0.56	0.81	-0.59	-0.21	1.45
50	1.58	1.90	0.91	1.28	-0.10	0.25	1.41
45	2.05	2.53	1.47	2.05	0.39	0.72	1.39
40	2.68	3.66	2.47	3.41	0.90	1.23	1.38

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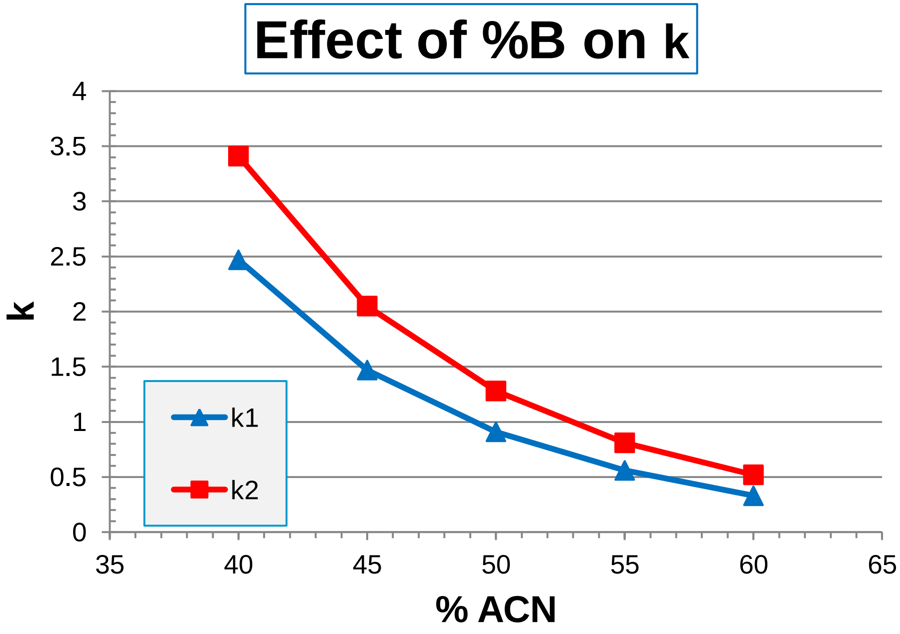

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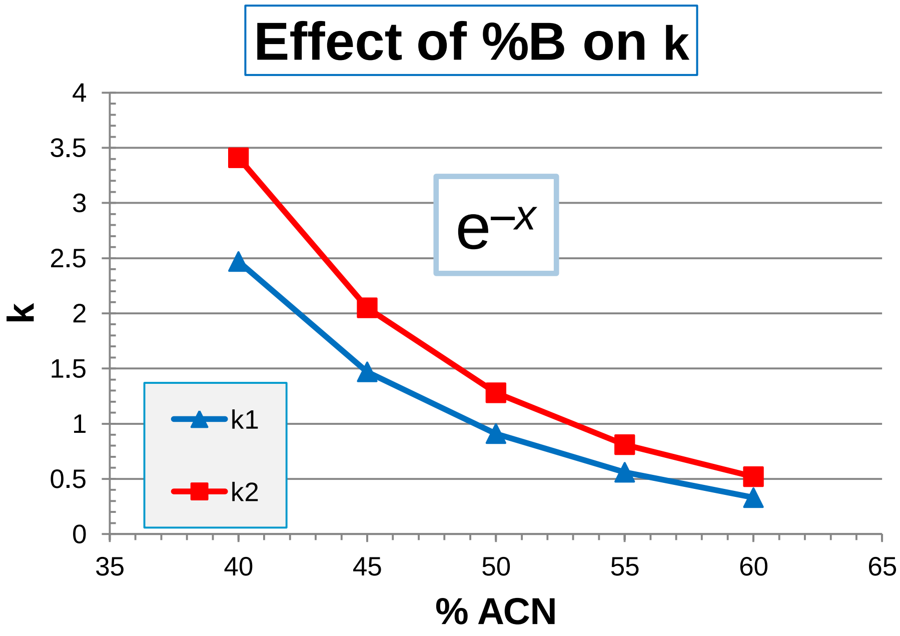

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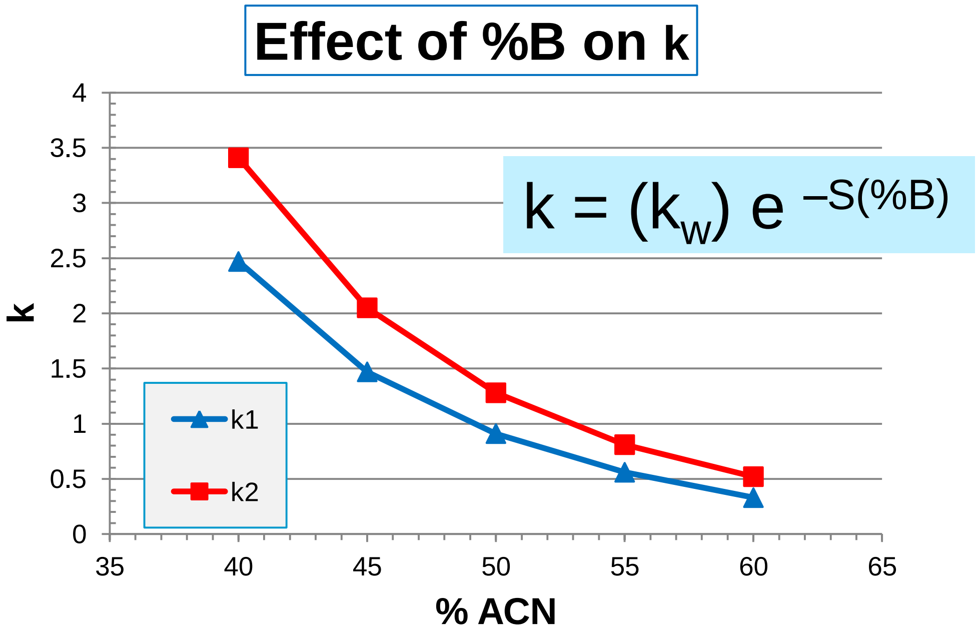

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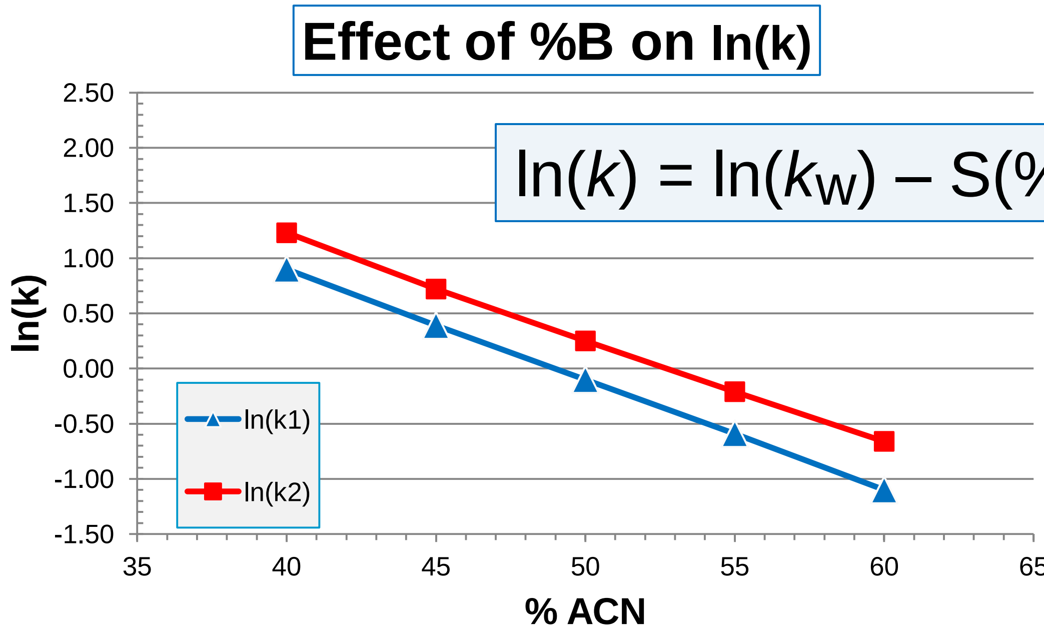

$$\ln(k) = \ln(k_W) - S(\%B)$$

Mobile Strength

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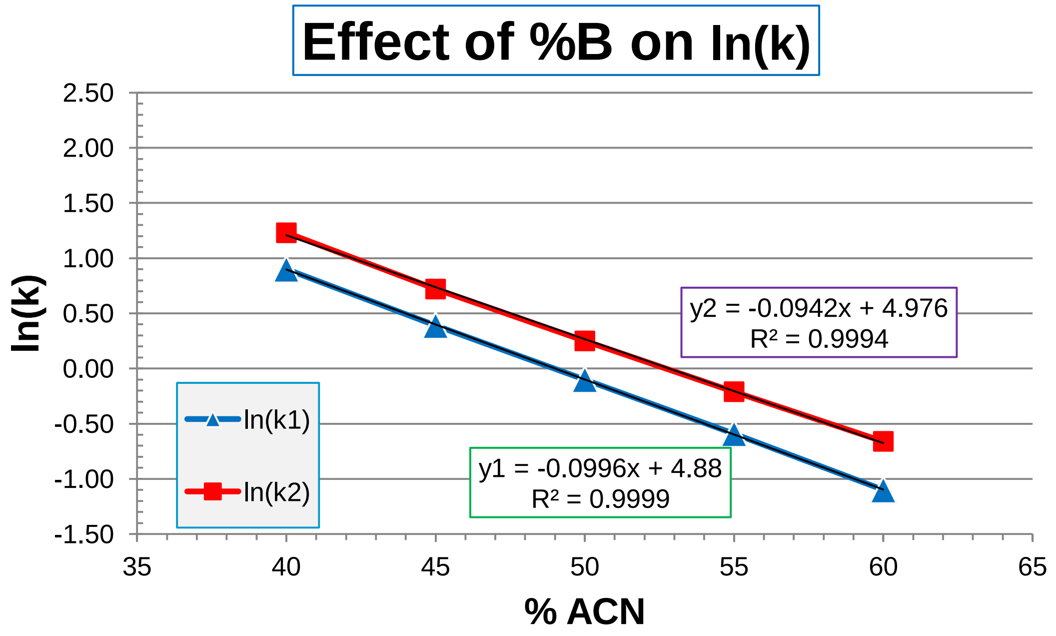

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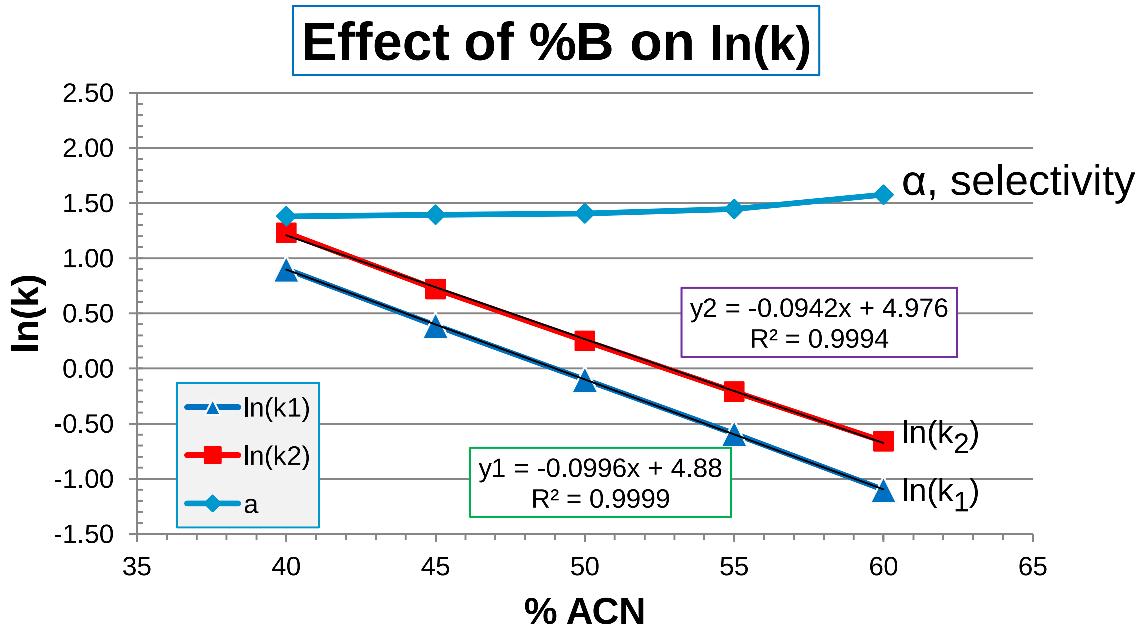
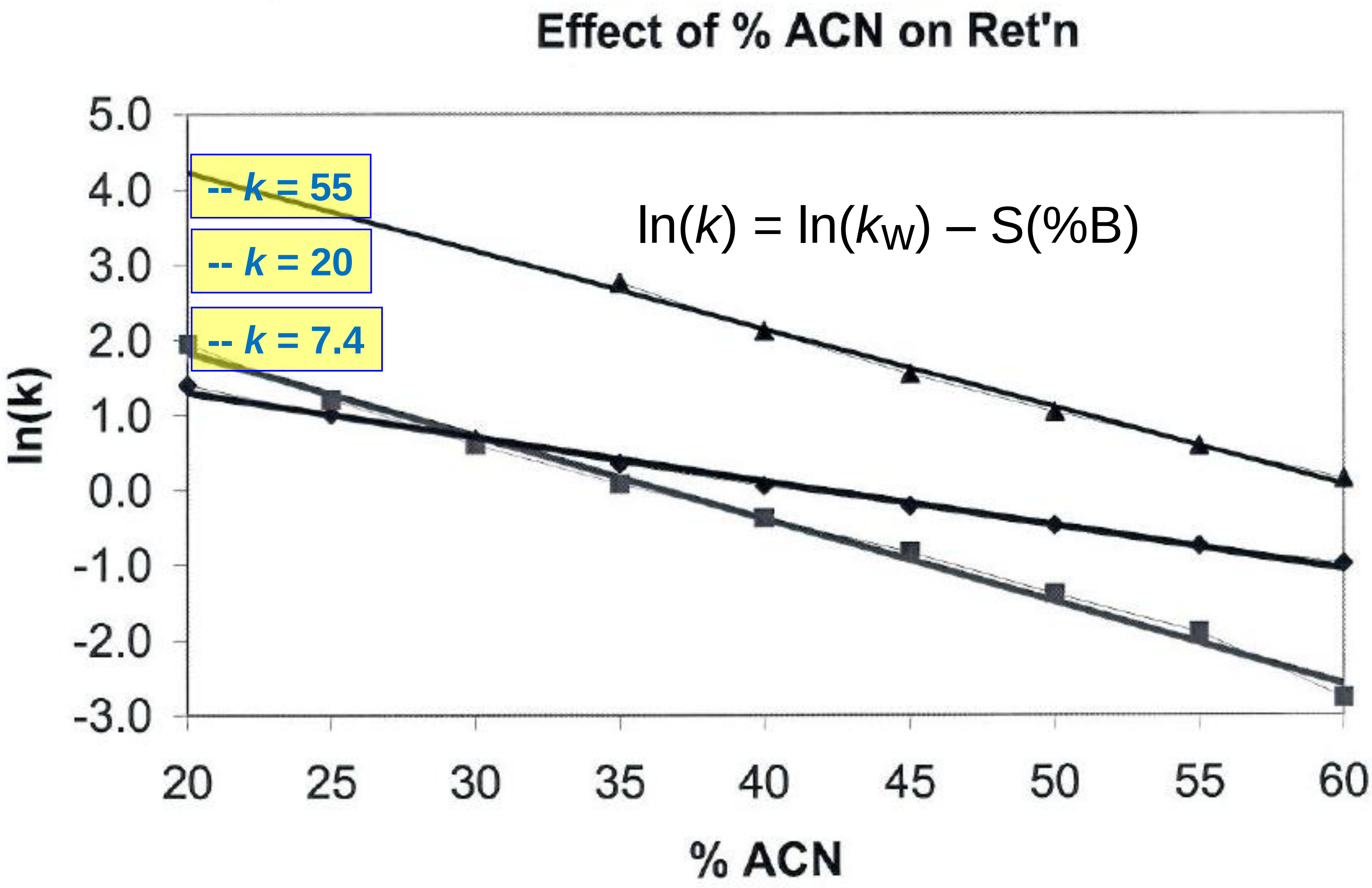
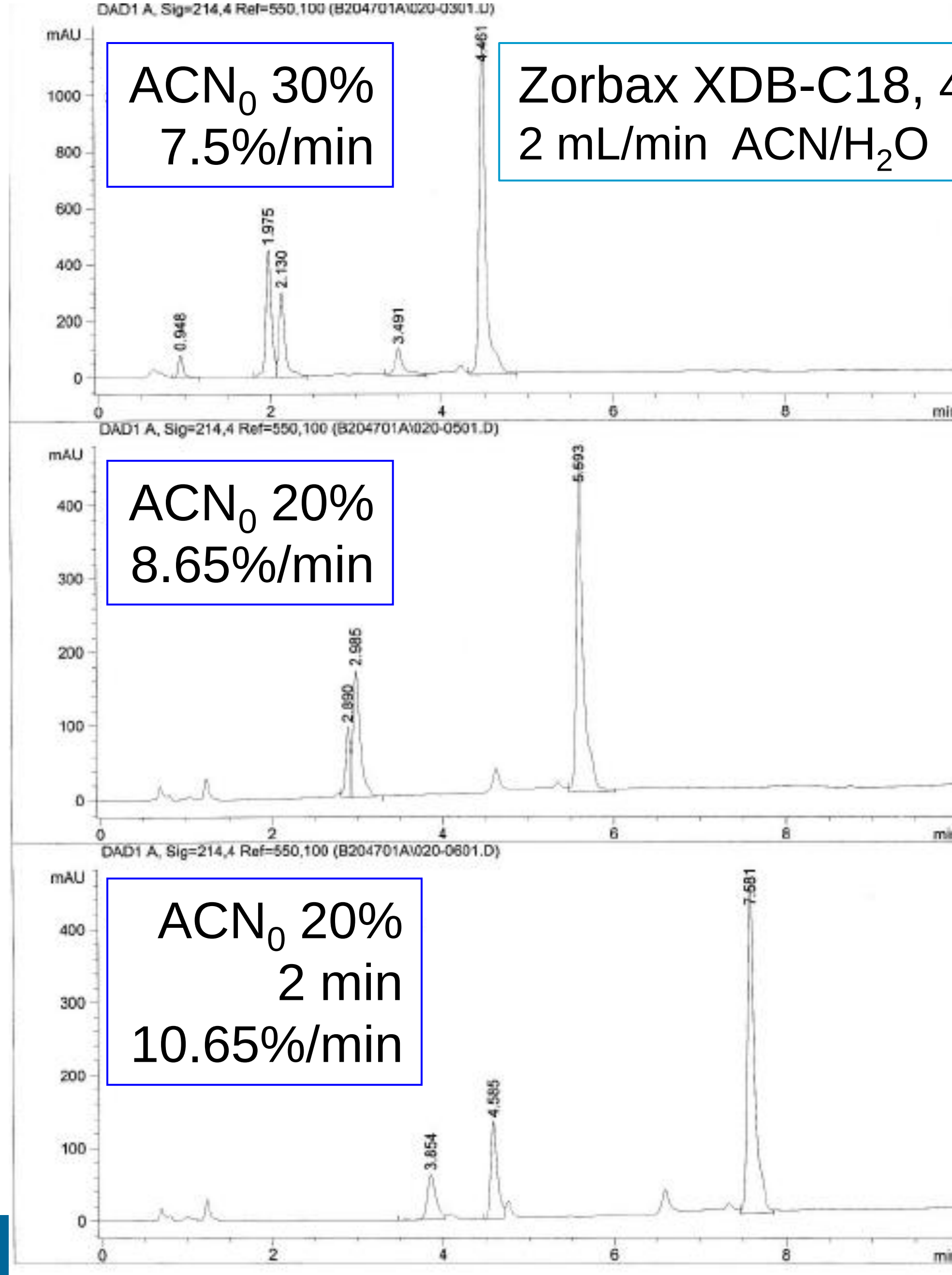


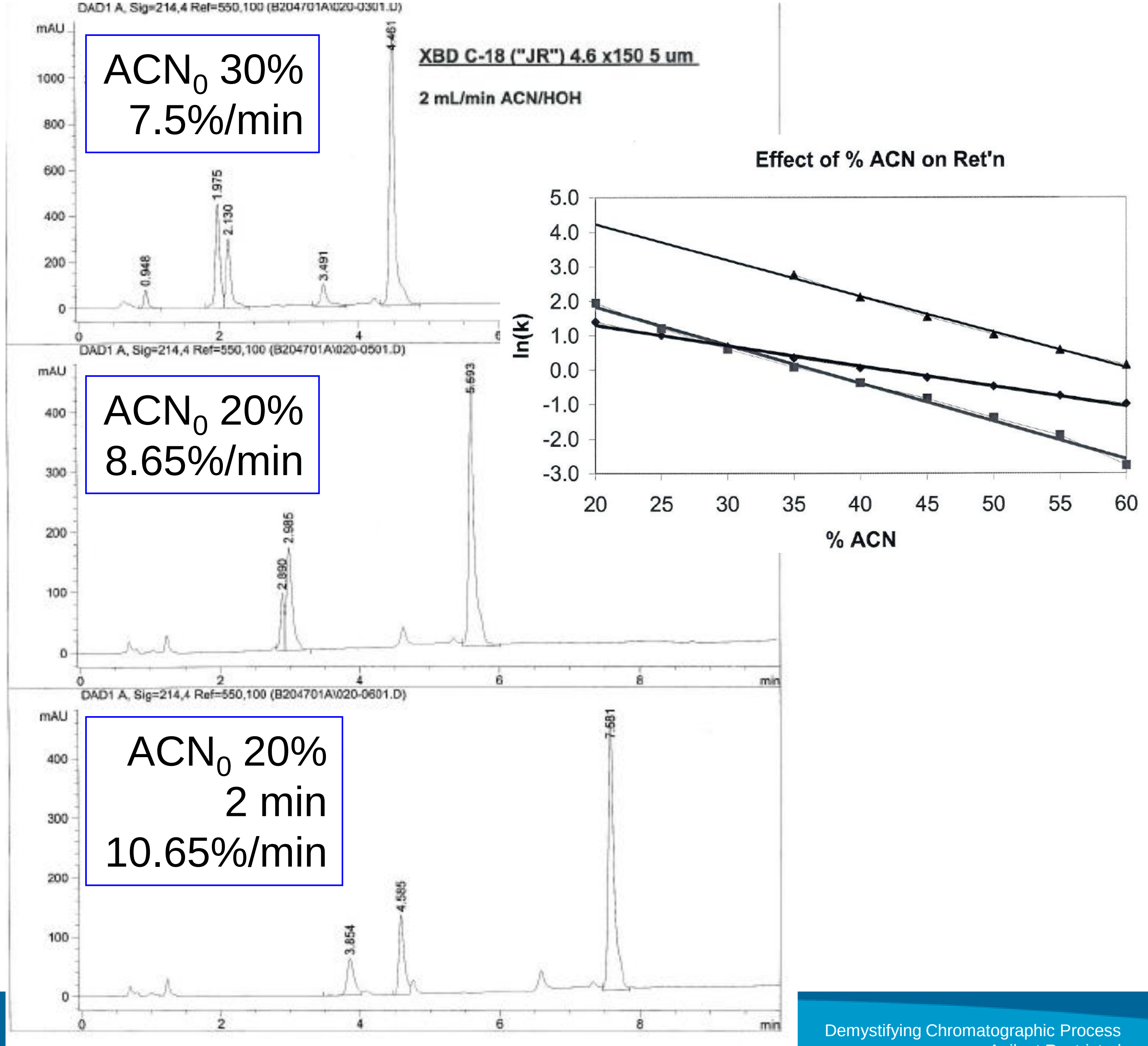
Change in Order of Elution



$$\ln(k) = \ln(k_W) - S(\%B)$$

Zorbax XDB-C18, 4.6 x 150 mm, 5 um
2 mL/min ACN/H_2O

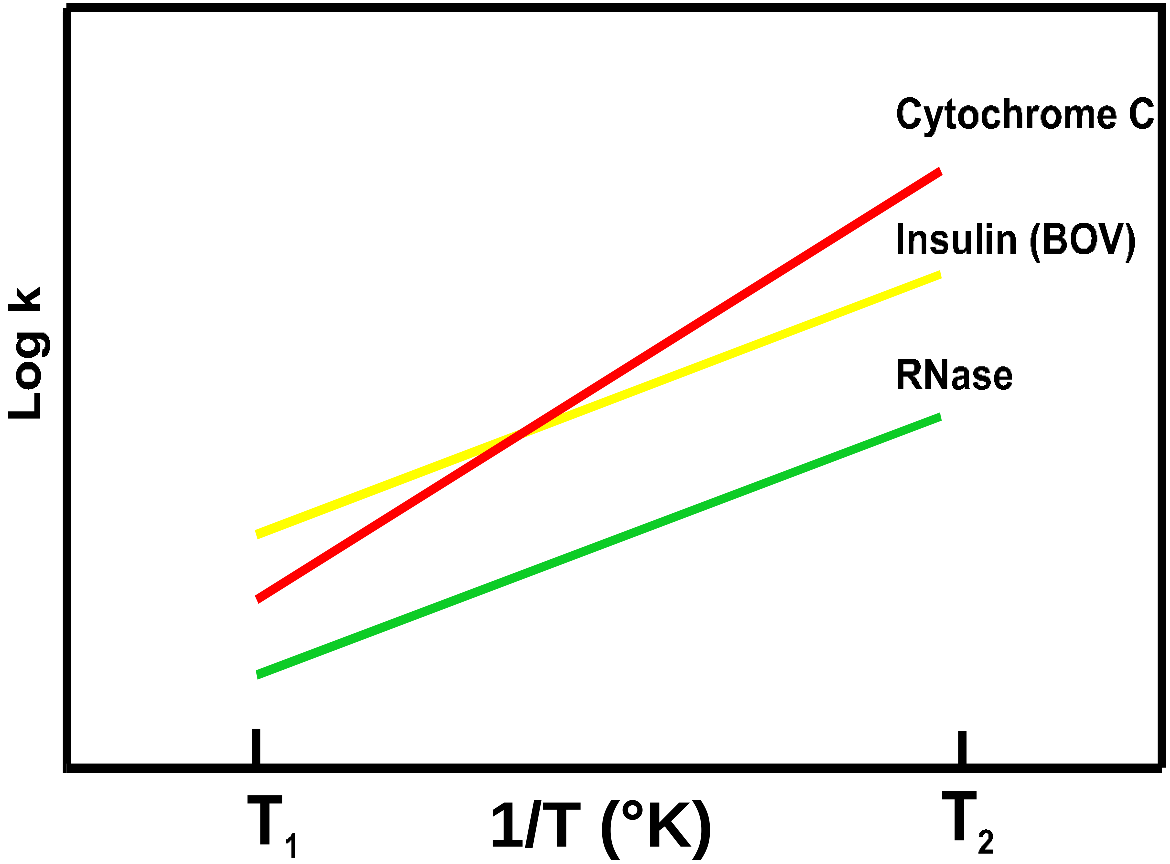




DAD1 A, Sig=214,4 Ref=550,100 (B204701A\020-0301.D)
ACN0 30%
7.5%/min
XBD C-18 ("JR") 4.6 x150 5 um
2 mL/min ACN/HOH
DAD1 A, Sig=214,4 Ref=550,100 (B204701A\020-0501.D)
ACN0 20%
8.65%/min
DAD1 A, Sig=214,4 Ref=550,100 (B204701A\020-0601.D)
ACN0 20%
2 min
10.65%/min
Effect of % ACN on Ret'n
ln(k)
% ACN

Effect of Temperature

- Reduces Analysis Time
- May Change Selectivity



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Topics

- **What Affects Resolution, R_S**
- **What Affects Retention, t_R**
- **What Affects Pressure, P**

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**

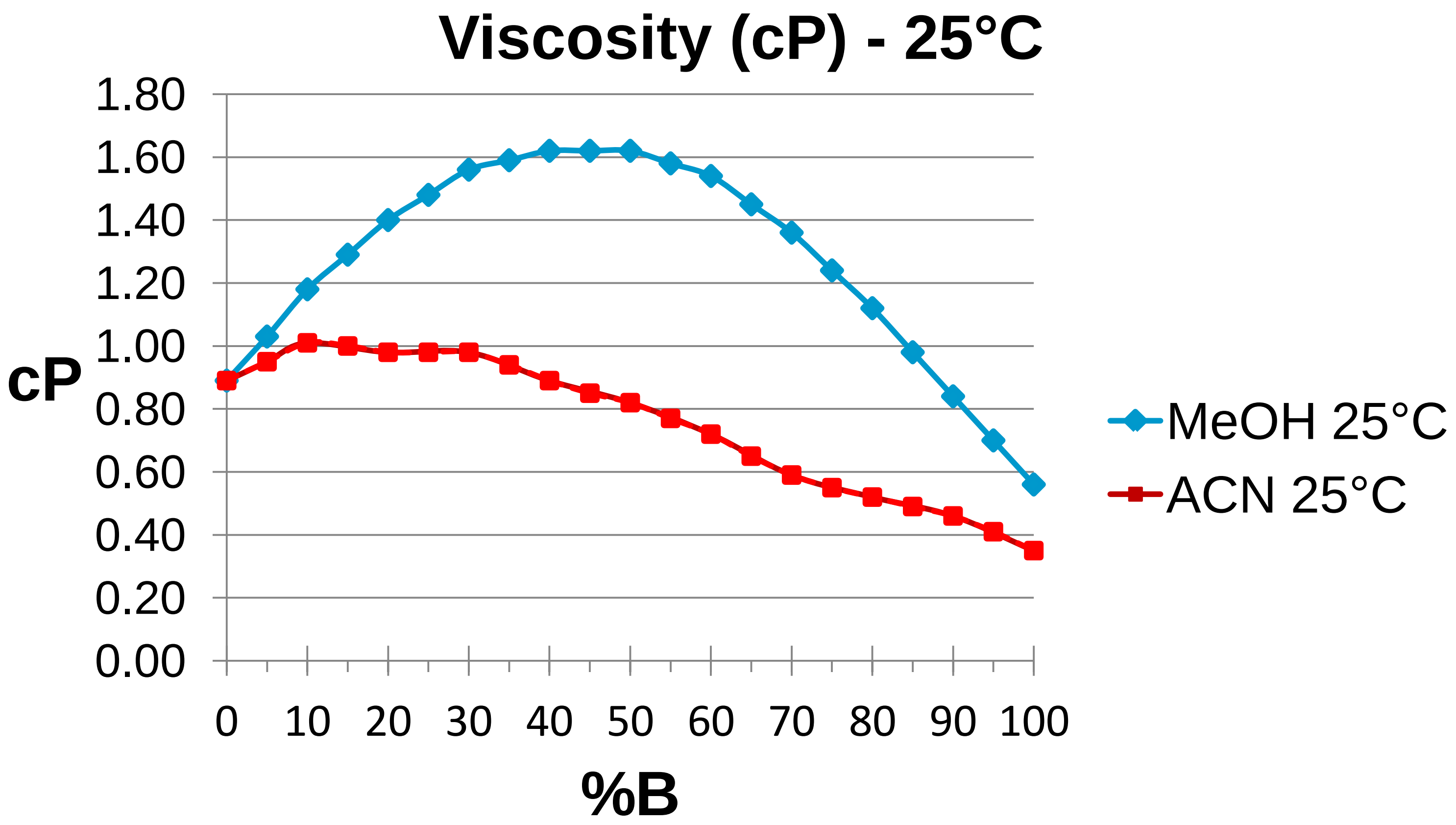
v = Flow Velocity

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

Comparison of Effect of Water/ACN and Water/MeOH on Viscosity



cf: Neue, *HPLC Column – Theory, Technology, and Practice*, Wiley (1997)

Topics

- What Affects Resolution, R_S
- What Affects Retention, t_R
- What Affects Pressure, P

“UHPLC” vs HPLC

Effect of Increasing	N	α	k	tR	P
Flow	Van Deemter	No	No	Decrease	Increase
% B	Slight	Varies	Exp Dec	Exp Dec	Varies
Temperature (T)	Increase	Varies	Decrease	Decrease	Decrease
Particle Size (d_p)	Decrease	No	No	No	Decrease
Col Length (L)	Increase	No	No	Increase	Increase
Col Diameter(d)	Slight	No	No	No*	No*

* At constant linear velocity

Pressure does not cause improved separation.

Pressure is the result of the conditions that cause improved separation.

Thank you – Questions?

Bill Champion
800-227-9770, opt 3, opt 3, opt 2
lc-column-support@agilent.com