HPLC Separation Fundamentals

LC Columns and Consumables

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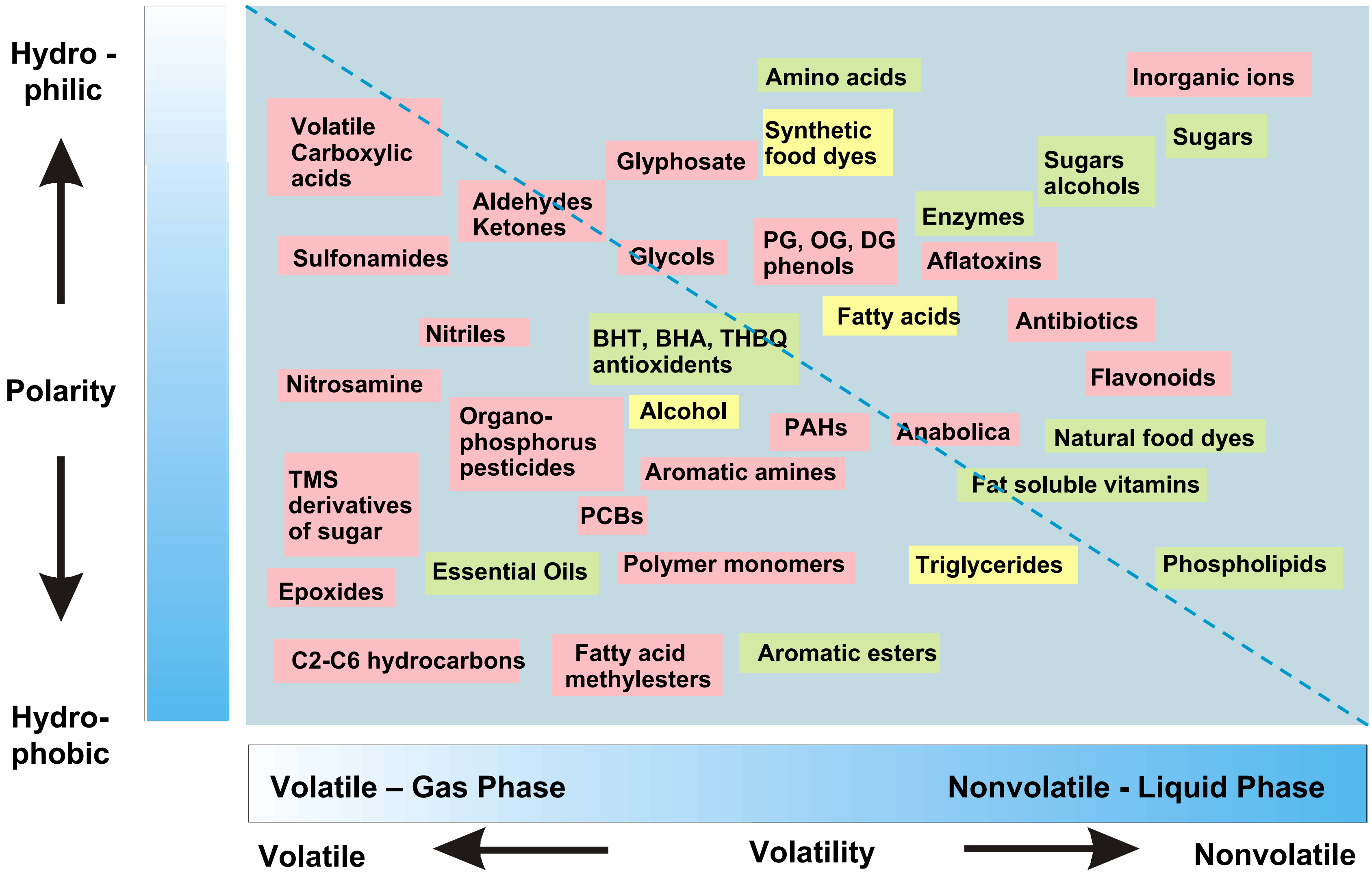
Presentation Outline

- Major HPLC modes
- Key Equations
  - Resolution
  - van Deemter
- Common terms & definitions
- Key parameters & conditions that affect them
  - efficiency, selectivity, and retention
- Role of pressure
  - Sub-2um

Separation Techniques



Major Separation Modes of HPLC
A Review

There are four major separation modes that are used to separate most compounds:

- Reversed-phase chromatography (most popular)
- Normal-phase and adsorption chromatography
- Ion exchange chromatography
- Size exclusion chromatography

….Let's look briefly at each mode

Normal Phase or Adsorption Chromatography

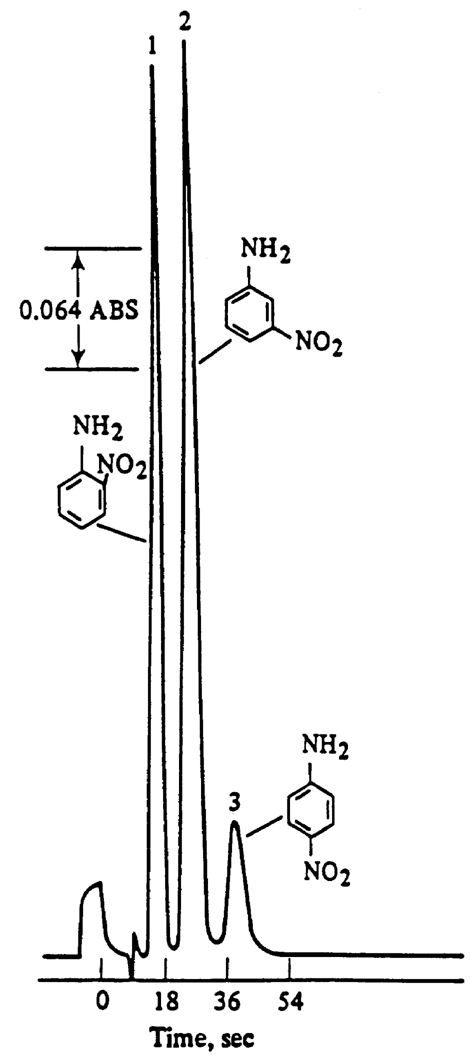
Key Points

- **Column packing is polar**
  - → silica (strongest)>amino>diol>cyano (weakest)
- **Mobile phase is non-polar**
  - hexane, iso-octane, methylene chloride, ethyl acetate, etc.
- **Retention decreases as polarity of mobile phase increases**

Reasons to choose normal phase

- **Resolve strongly retained hydrophobic samples.**
- **Isomer separation.**
- **Sample injection solvent is non-polar.**
- **Recovery in non-polar solvents is desirable.**

Separation of Nitroanilines on HPLC Column packed with silica gel using hexane (mobile phase component A) mixed with methylene chloride (mobile phase component B)



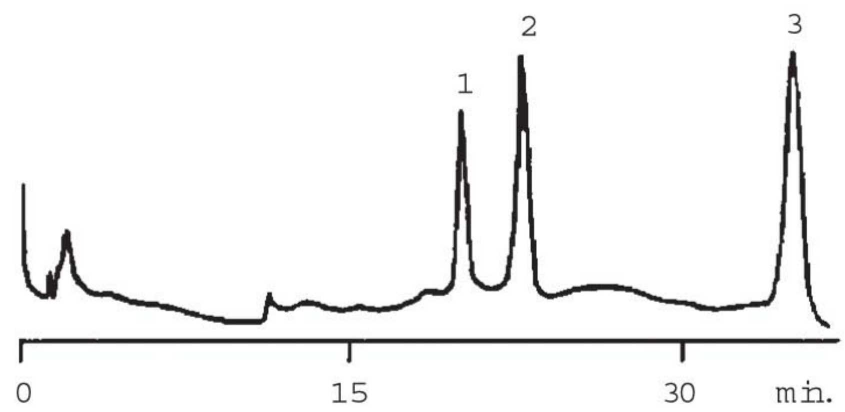
Ion Exchange Chromatography

In ion exchange:

- Column packing contains ionic groups, e.g. sulfonic, tetraalkylammonium
- Mobile phase is an aqueous buffer (e.g. phosphate, formate, etc.)
- Used infrequently
- Similarities to Ion-pair chromatography
- Well suited to the separation of inorganic and organic anions and cations in aqueous solution.

Basic proteins on strong cation exchanger ($-SO_3^-$)

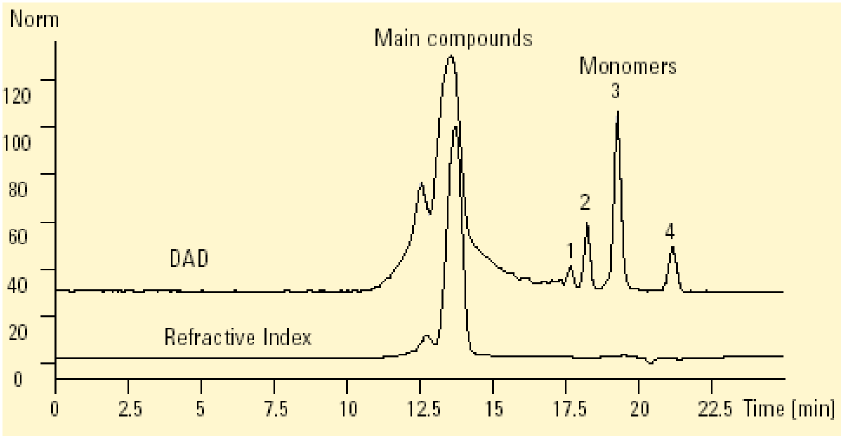
1. **RNA polymerase**
2. **Chymotrypsinogen**
3. **Lysozyme**



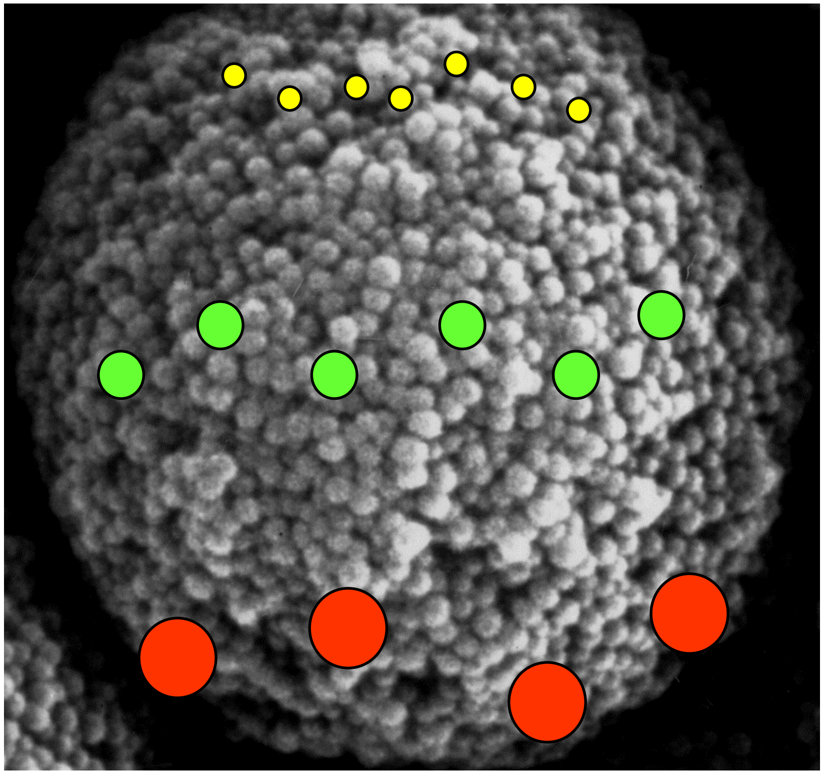
Size Exclusion Chromatography (SEC)

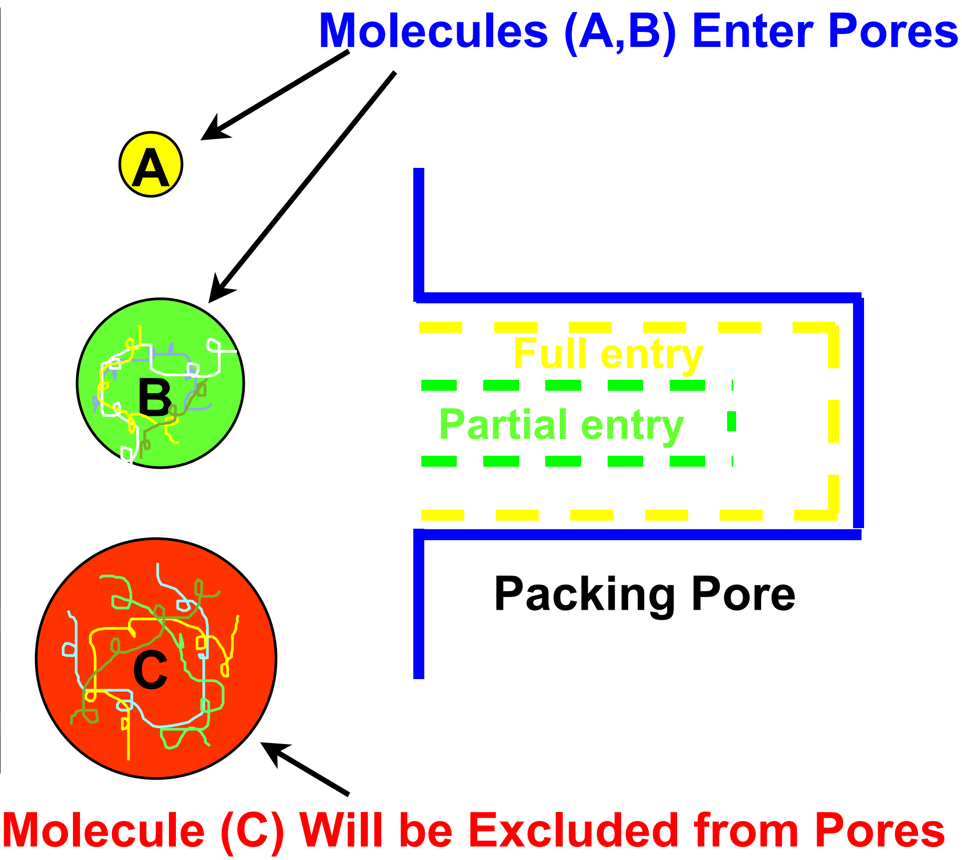
- There are two modes:
  - non-aqueous SEC [sometimes termed Gel Permeation Chromatography (GPC)]
  - aqueous SEC [sometimes referred to as Gel Filtration Chromatography (GFC)]
- No interaction between the sample compounds and packing material
  - Molecules diffuse into pores of a porous medium
  - Molecules are separated depending on their size relative to the pore size
    - ✓ molecules larger than the pore opening do not diffuse into the particles while molecules smaller than the pore opening enter the particle and are separated
    - ✓ large molecule elute first, smaller molecules elute later
- The mobile phase is chosen mainly to dissolve the analyte
- Used mainly for polymer characterization and for proteins.

Gel Permeation Chromatogram of Polybutadiene polymer on non-aqueous SEC (GPC) column;
The monomers elute after the polymer.
Column: PLgel mixed-D gel
Mobile phase: Tetrahydrofuran (THF)

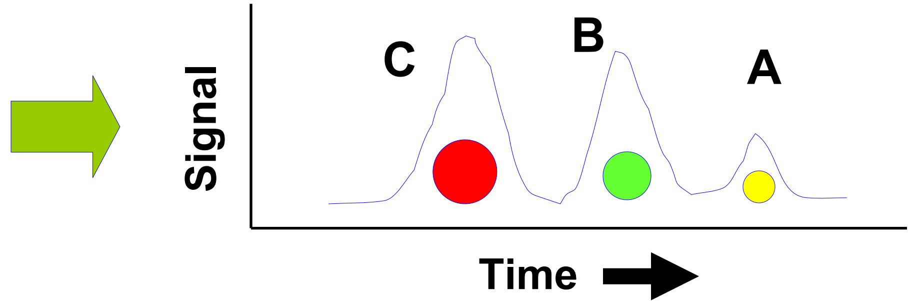


Mechanism of SEC





Molecules must freely enter and exit pores to be separated. Largest molecules elute first, followed by intermediate size molecules and finally the smallest molecules elute last.



Reversed-Phase Chromatography (RPC)

Principle: Partition of analytes between mobile phase and stagnant phase inside the pore space + adsorption on the surface of bonded phase

- Nonpolar (nonspecific) interactions of analyte with hydrophobic adsorbent surface
  - C18, C8, Phenyl, C3, etc.
- Different sorption affinities between analytes results in their separation
  - More polar analytes retained less
  - Analytes with larger hydrophobic part are retained longer
- Mobile phase; water (buffer) + water-miscible organic solvent, e.g. MeOH, ACN*
  - water (buffer) + water-miscible organic solvent, e.g
- Can be used for non-polar, polar, ionizable and ionic molecules
- Gradient elution is often used

*Non-aqueous reverse phase

Chromatography Terms are All Around Us
But what do they mean…..

Gradient Steepness

Efficiency

Plates

Particle Size

Peak Capacity

3.5µm

Gradient Retention

RRLC

Peak Shape

Resolution

1.8µm

Selectivity

Retention Factor

UPLC

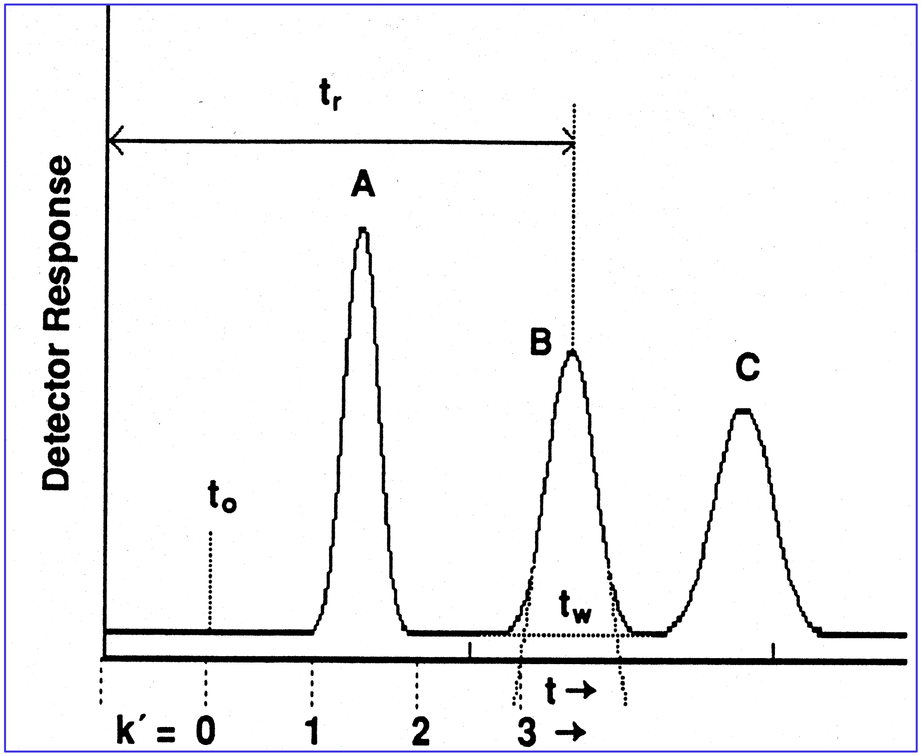
Tailing Factor

Rapid Resolution HT
RRHT

Rapid Resolution

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

$$N = 5.54\,(t_R / W_{1/2})^2$$

Chromatographic Terms
Retention Factor

Two compounds can be separated if they have sufficiently different retention factors – k' values

$$k' = \frac{t'_R}{t_0} = \frac{t_R - t_0}{t_0}$$

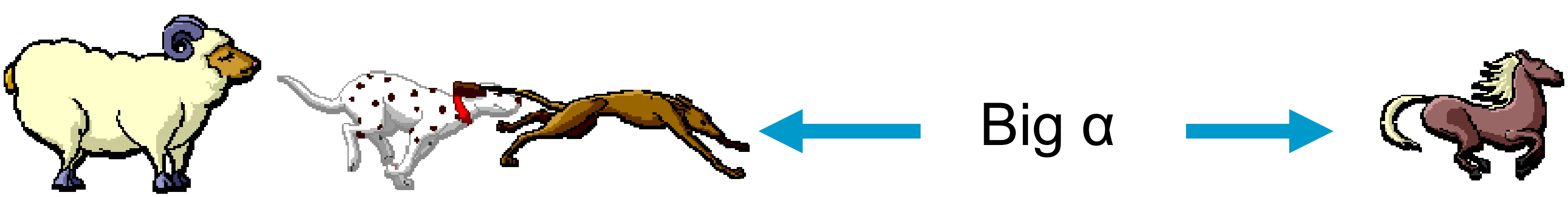
Chromatographic Terms

Selectivity factor

Alpha, α (separation factor, relative retention, capacity factor) – this is used to measure how far apart the k’ values of two peaks are and if the separation can be achieved

$$\alpha = \frac{k'_2}{k'_1}$$

$k'_2 > k'_1$ $\qquad$ $\alpha > 1$ for a separation to take place



Gradient Retention (k*)

Selectivity in gradient elution is determined by the gradient retention factor

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

$\Delta\Phi$ = 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)

- $S \approx 4\text{–}5$ for small molecules
- $10 < S < 1000$ for peptides and proteins

In gradient separation the effective value of k (k*) for different bands will be about the same same.

`

This Relationship Says that to Keep Relative Peak Position in the Chromatogram Unchanged

Any Decrease in	Can be Offset by a Proportional
Column length	Decrease in $\mathbf{t_G}$ or F
	Increase in $\Delta\Phi$
Column volume (i.d.)	Decrease in t_G or **F**
	Increase in $\Delta\Phi$
$\Delta\Phi$ (same column)	Decrease in $\mathbf{t_G}$ or F

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

More Chromatographic Terms
Efficiency

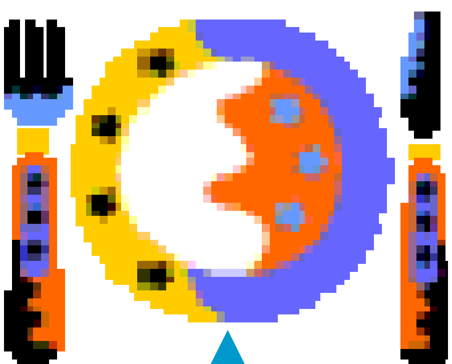
N - Number of theoretical plates – This is one case where more is better! “Plates” is a term inherited from distillation theory. For LC, it is a measure of the relative peak broadening for an analyte during a separation – **w** (from our chromatogram).

Column length

$$N = 16\left[\frac{t_R}{w}\right]^2 \quad \text{or} \quad N = \frac{L}{H}$$

HETP

A Number of Theoretical Plates



A Single Real Plate

More Chromatographic Terms

Resolution

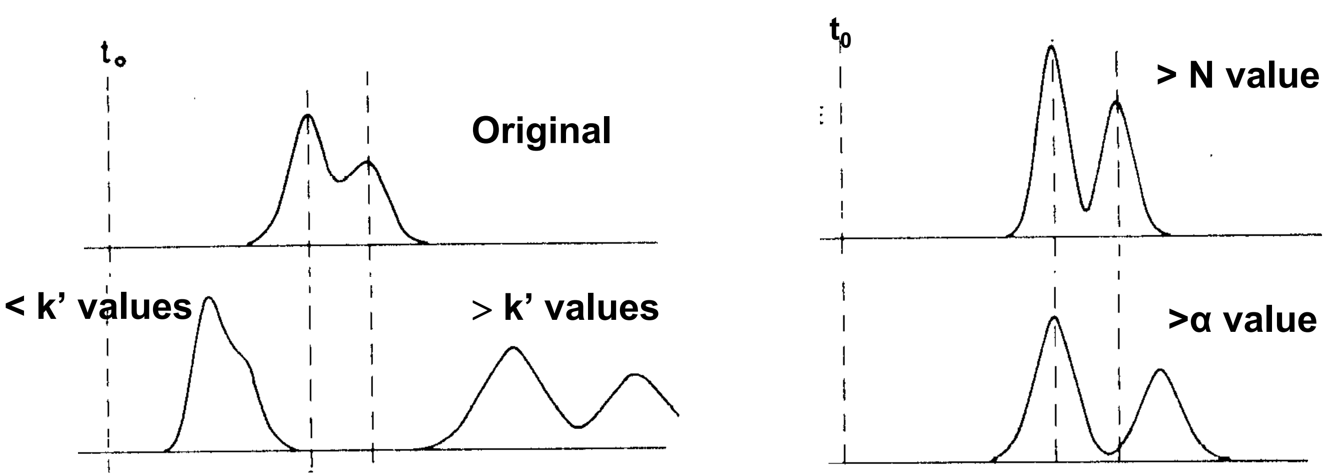
The distance between two neighboring peaks

R = 1.5 is baseline resolution

R = 2 is highly desirable during method development

$$R = 2\,\frac{(t_{R2} - t_{R1})}{(w_1 + w_2)}$$

Resolution is reduced based on how fat or wide the peaks are, so **thin is better!**



Resolution …

Determined by 3 Key Parameters – Efficiency, Selectivity and Retention

The Fundamental Resolution Equation

Resolution can be expressed in terms of the components we have discussed thus far.

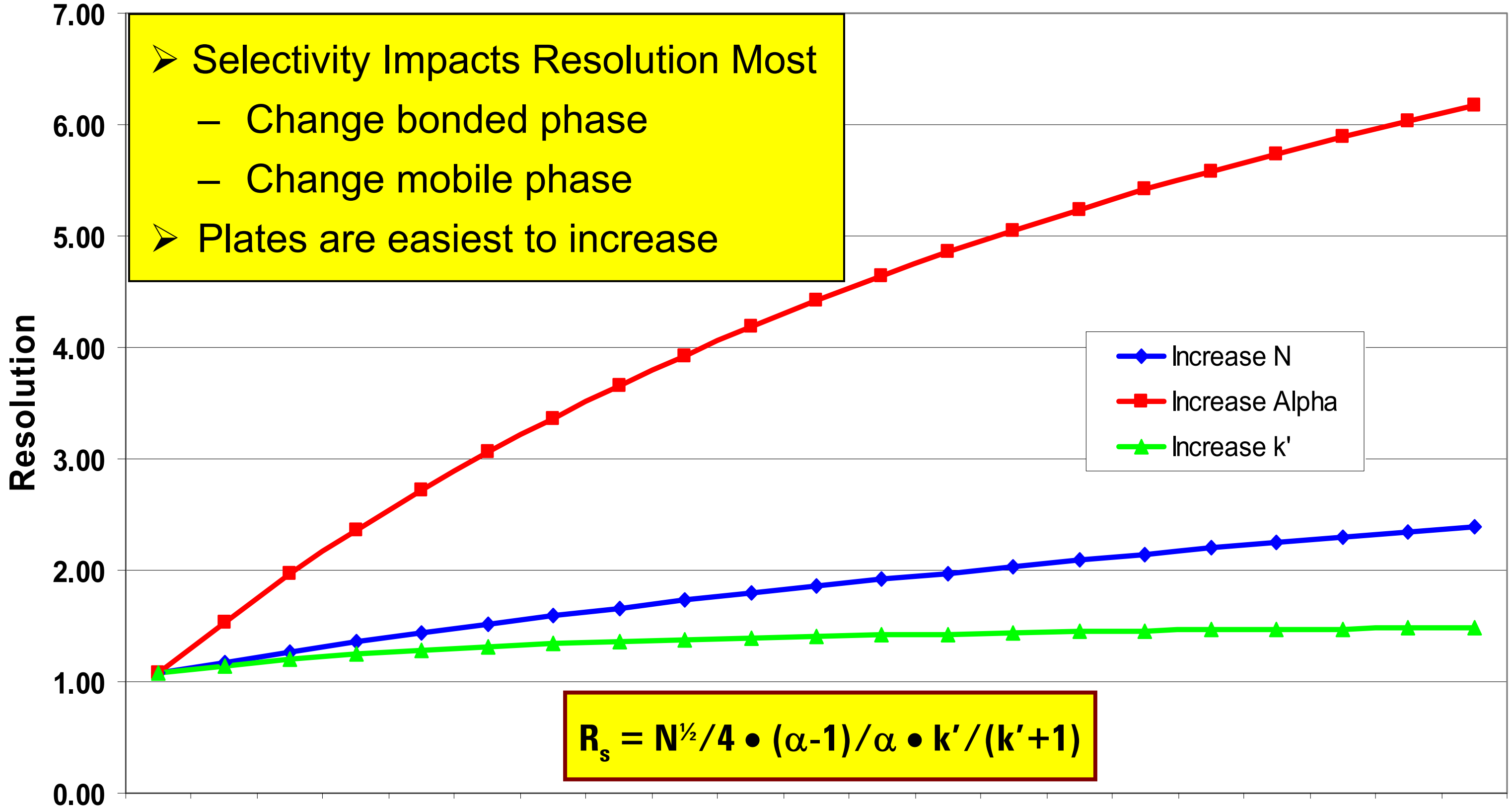
$$R_S = \frac{\sqrt{N}}{4} \frac{(\alpha - 1)}{\alpha} \frac{k}{(k + 1)}$$

α = **Selectivity** – influenced by mobile and stationary phase

N = Column Efficiency – influenced by length and particle size

k = Capacity Factor (retention) – influenced by stationary and mobile phase, gradient slope and dwell volume (gradients)

Resolution—Effects of Selectivity, Efficiency, and Retention

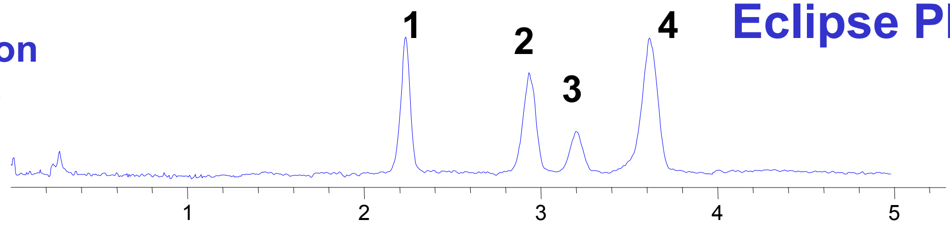


$$R_s = N^{1/2}/4 \bullet (\alpha-1)/\alpha \bullet k'/(k'+1)$$

Plates:	**5000**	**10000**	**15000**	**20000**	**25000**	
Alpha:	**1.10**	**1.35**	**1.60**		**1.85**	**2.1**
k′:	**2.0**	**4.5**	**7.0**		**9.5**	**12.0**

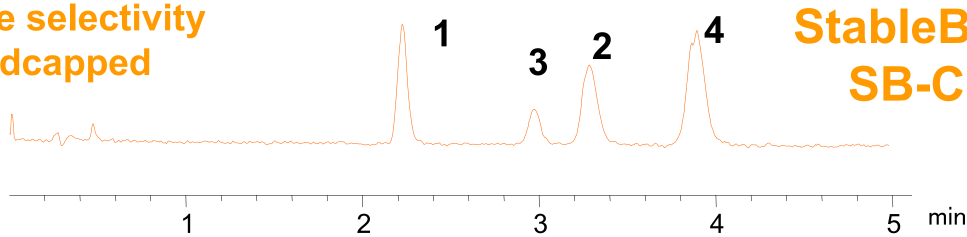
Start Method Development with RRHT Columns: Different ZORBAX C18 Bonded Phases for Max Selectivity

1st choice
Best Resolution & Peak Shape



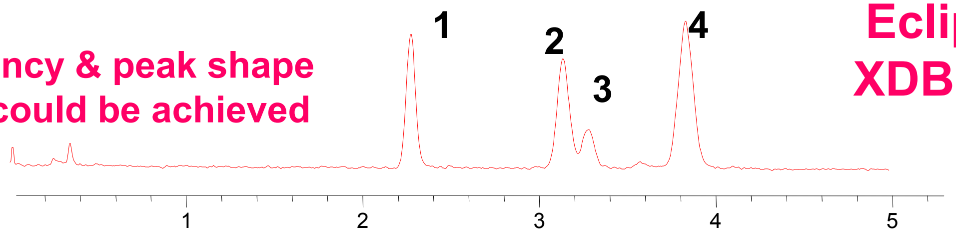
Eclipse Plus C18

2nd choice
Good alternate selectivity due to non-endcapped



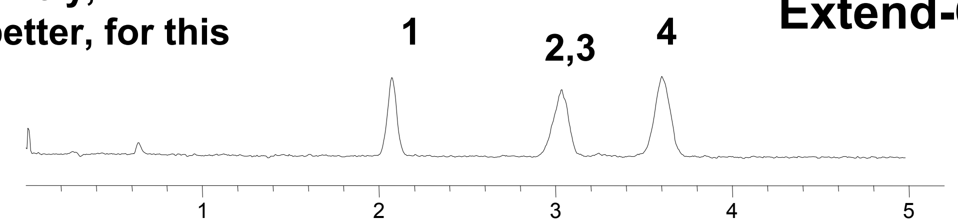
StableBond SB-C18

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)

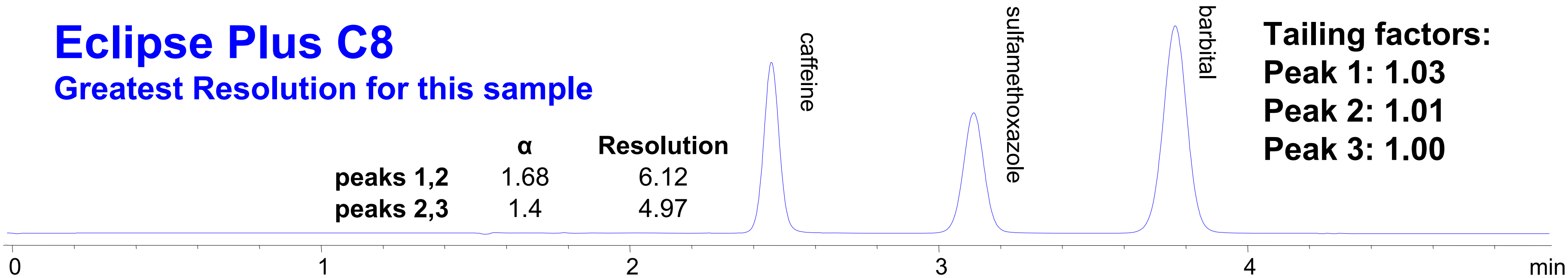
Multiple bonded phases for most effective method development. Match to one you're currently using.

Small Differences in α Can Provide Large Differences in Resolution

Eclipse Plus C8

Greatest Resolution for this sample

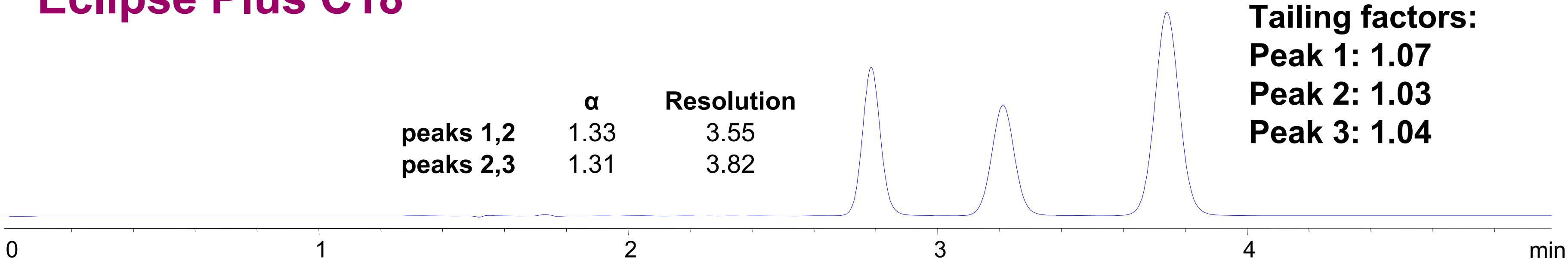
	α	Resolution
peaks 1,2	1.68	6.12
peaks 2,3	1.4	4.97



Tailing factors:
Peak 1: 1.03
Peak 2: 1.01
Peak 3: 1.00

Eclipse Plus C18

	α	Resolution
peaks 1,2	1.33	3.55
peaks 2,3	1.31	3.82

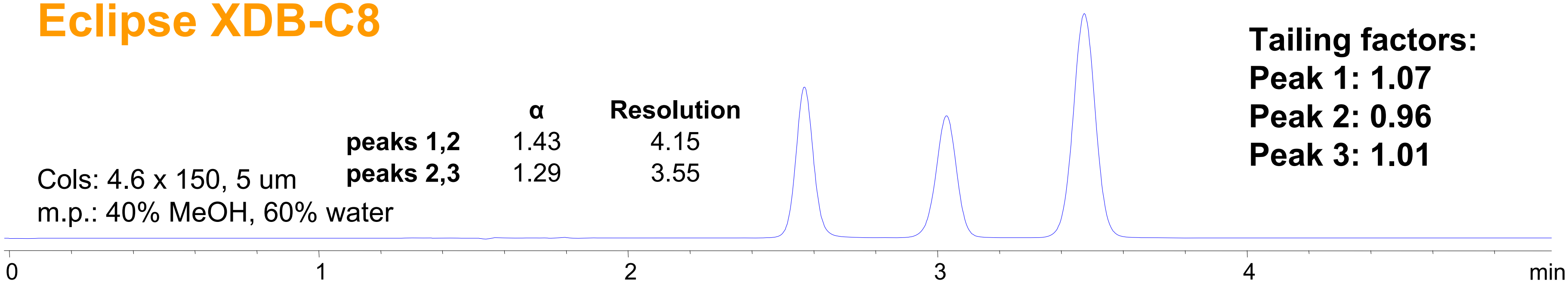


Tailing factors:
Peak 1: 1.07
Peak 2: 1.03
Peak 3: 1.04

Eclipse XDB-C8

	α	Resolution
peaks 1,2	1.43	4.15
peaks 2,3	1.29	3.55

Cols: 4.6 x 150, 5 um
m.p.: 40% MeOH, 60% water



Tailing factors:
Peak 1: 1.07
Peak 2: 0.96
Peak 3: 1.01

If α Has the Most Impact, Why Focus on N? It's Easy

High plate number (N) provides:

- **Sharp and narrow peaks**
- **Better detection**
- **Peak capacity to resolve complex samples**

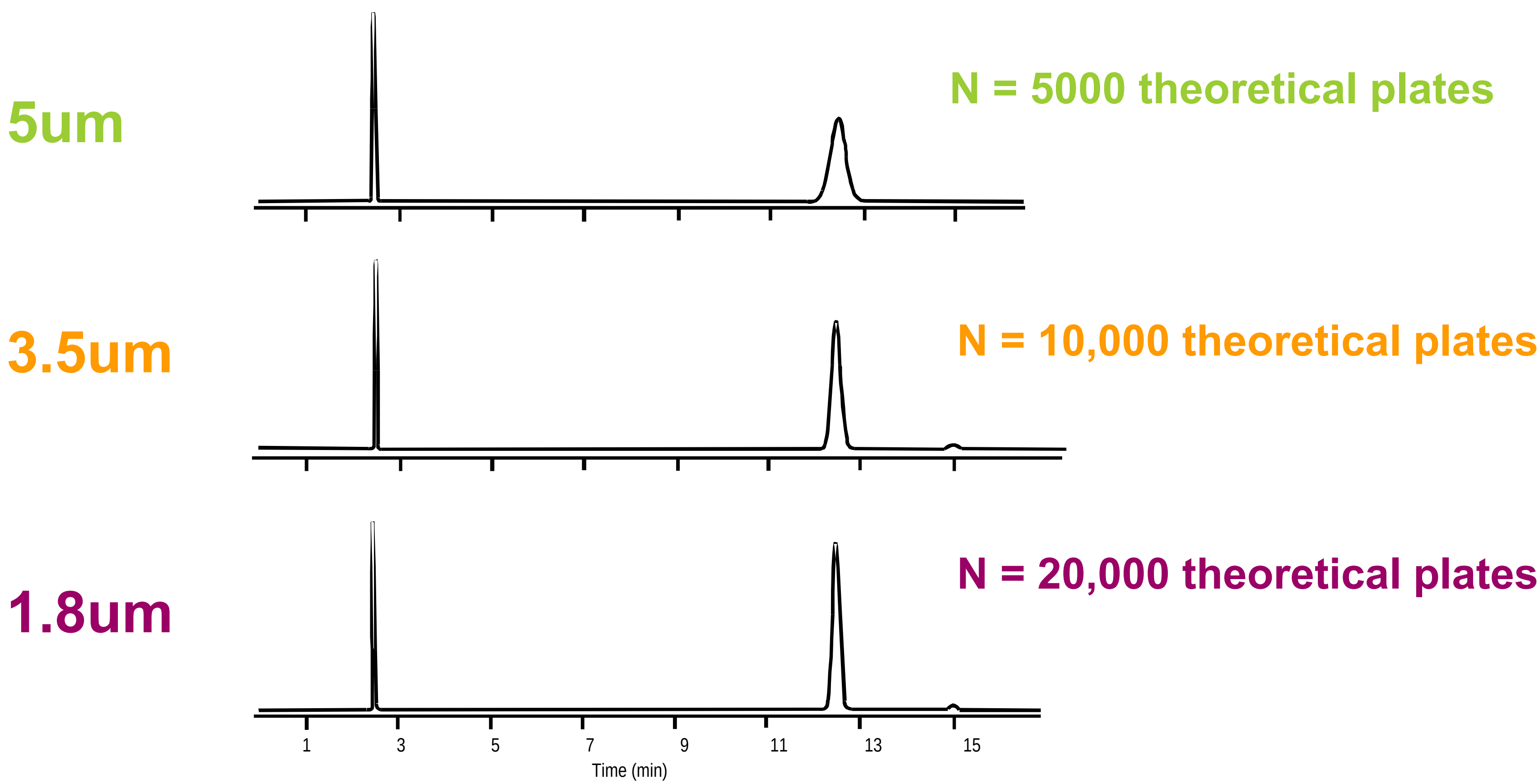
But…

- Resolution increases only with the square root of the plate number.
- Plate number increase is limited by experimental conditions (analysis time, pressure

Selectivity (α) helps best but… Is difficult to predict, so method development is slower (experience helps, model retention)

Note: Software supported, optimization for separation of multi-component mixtures can reduce method development time (ChromSword, DryLab)

Peak Width Decreases with Increasing N – Column Efficiency

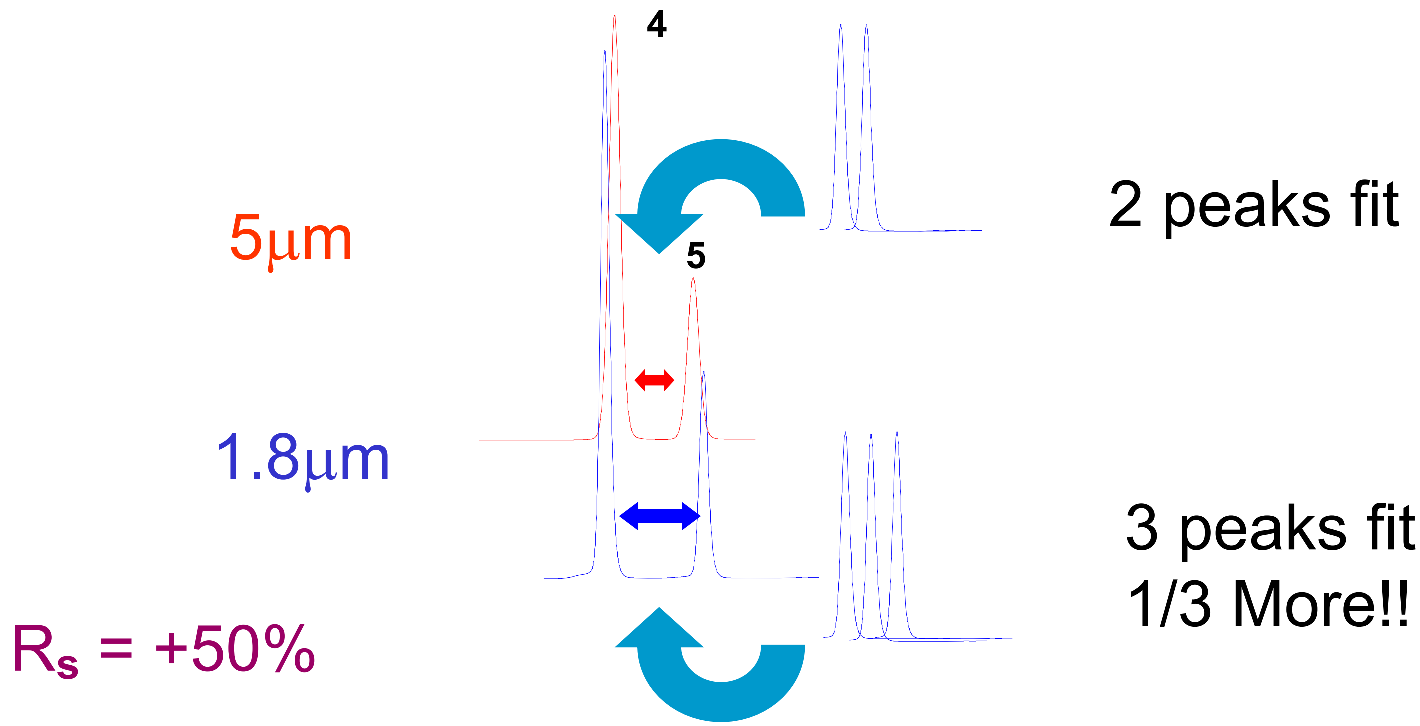


Peak Capacity

Better Peak Capacity – More Peaks Resolved

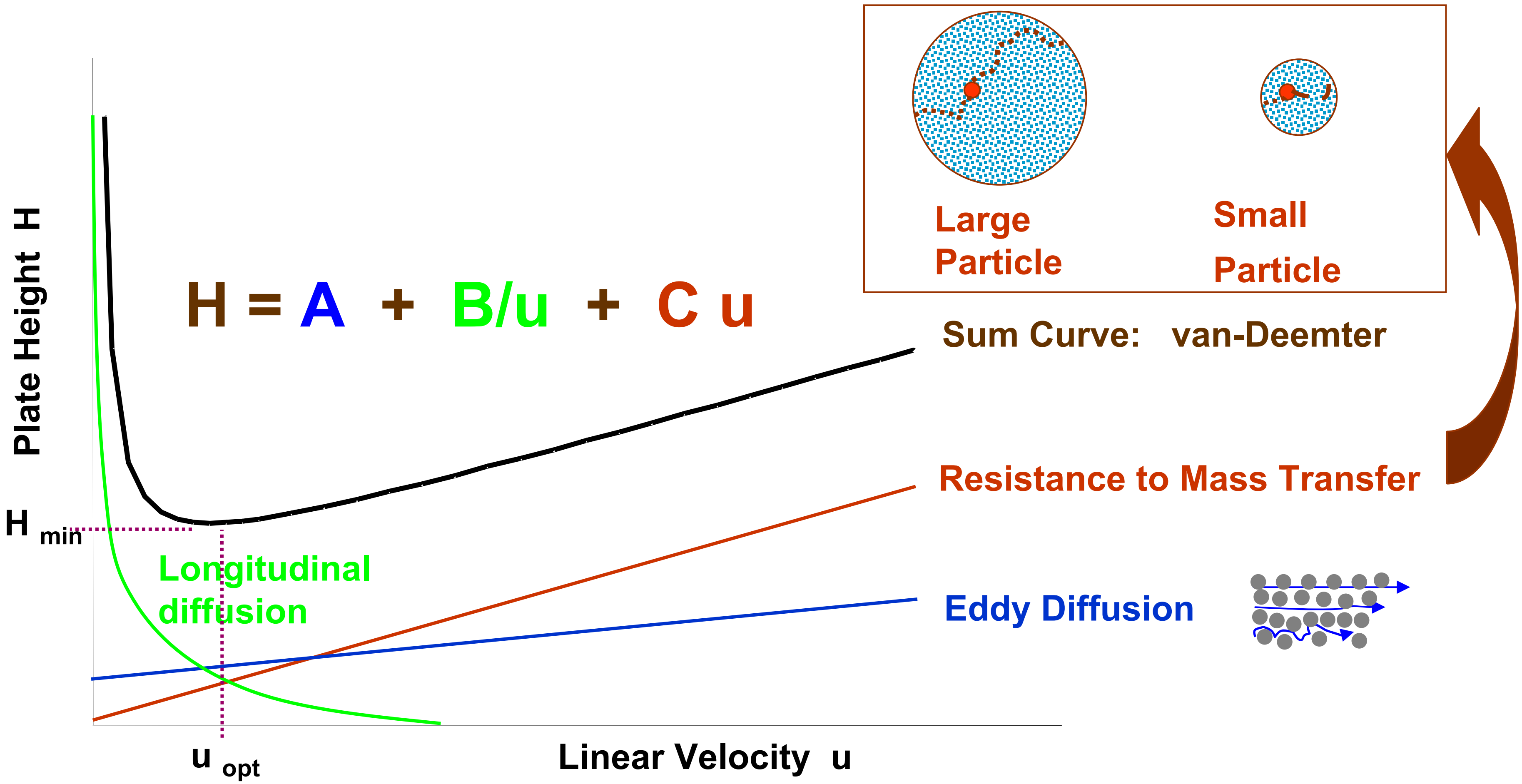
Peak capacity is the number of peaks that can be separated (at a specified resolution, example R=1) by a given system (think column length, particle size) in a given amount of time.

It is another measure of efficiency.



Putting it Together

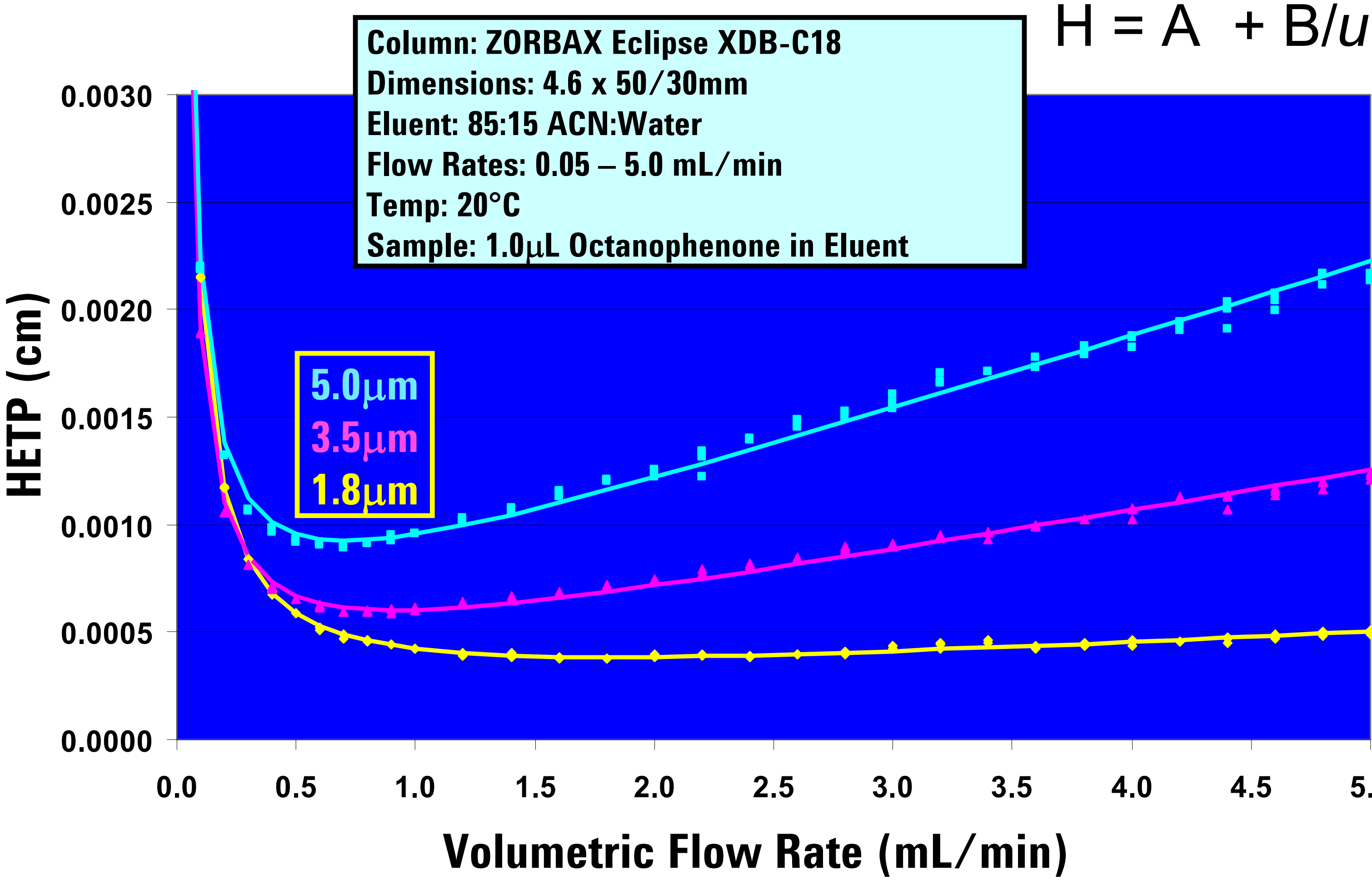
The van Deemter Equation



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

Van Deemter Curve : HETP vs. Volumetric Flow Rate

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



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

Key Chromatographic Parameters Impacted by Particle Size

Optimum
Particle Diameter

Efficiency

Pressure

Analysis Time

Peak Volume

What About Pressure?

Pressure Increases Exponentially with Decreasing Particle Size

Equation For Pressure Drop Across an HPLC Column

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

- ✓ 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

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

Calculated Effect of Particle Size on Key Chromatographic Parameters

dp µm	Flow mL/min	L cm	Vm mL	P psi	P bar	t_R Min	t_0 Min
2	1.0	6	0.6	2500	172	3.6	0.6
3	1.0	10	1.0	1850	128	6.0	1.0
5	1.0	15	1.5	996	69	9.0	1.5
10	0.2*	30	3.0	116	8	90	3.0

N = 13,000 $k_{(\text{last peak})}$ = 5 column i.d. = 4. 6 mm mobile phase = 100% water

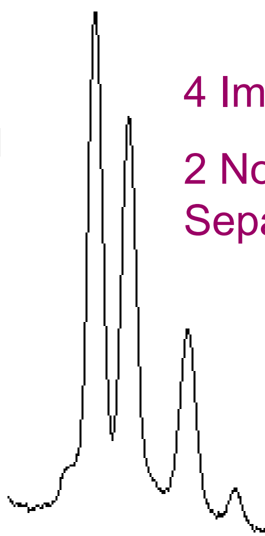
***To achieve N= 13,000 on a 10 mm, 30 cm column, flow rate must be reduced to 0.2 mL/min**

Smaller Particle Size Columns Improve Resolution – But Pressure Increases

Up to 60% higher resolution than in conventional HPLC

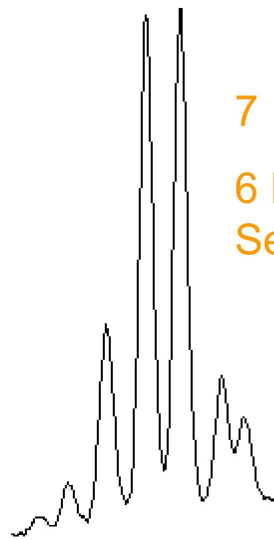
Customer Example
Isocr. Impurity Method

Zoom of critical time range @ 7min



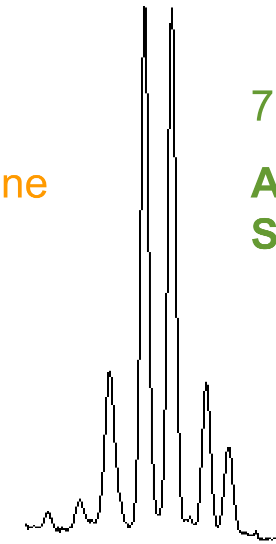
4 Impurities

2 Not Baseline Separated!



7 Impurities

6 Not Baseline Separated!



7 Impurities

All 7 Baseline Separated!

4.6 x 150, 5um
93 bar
N = 7259
Rs = 1.15
S/N = 42

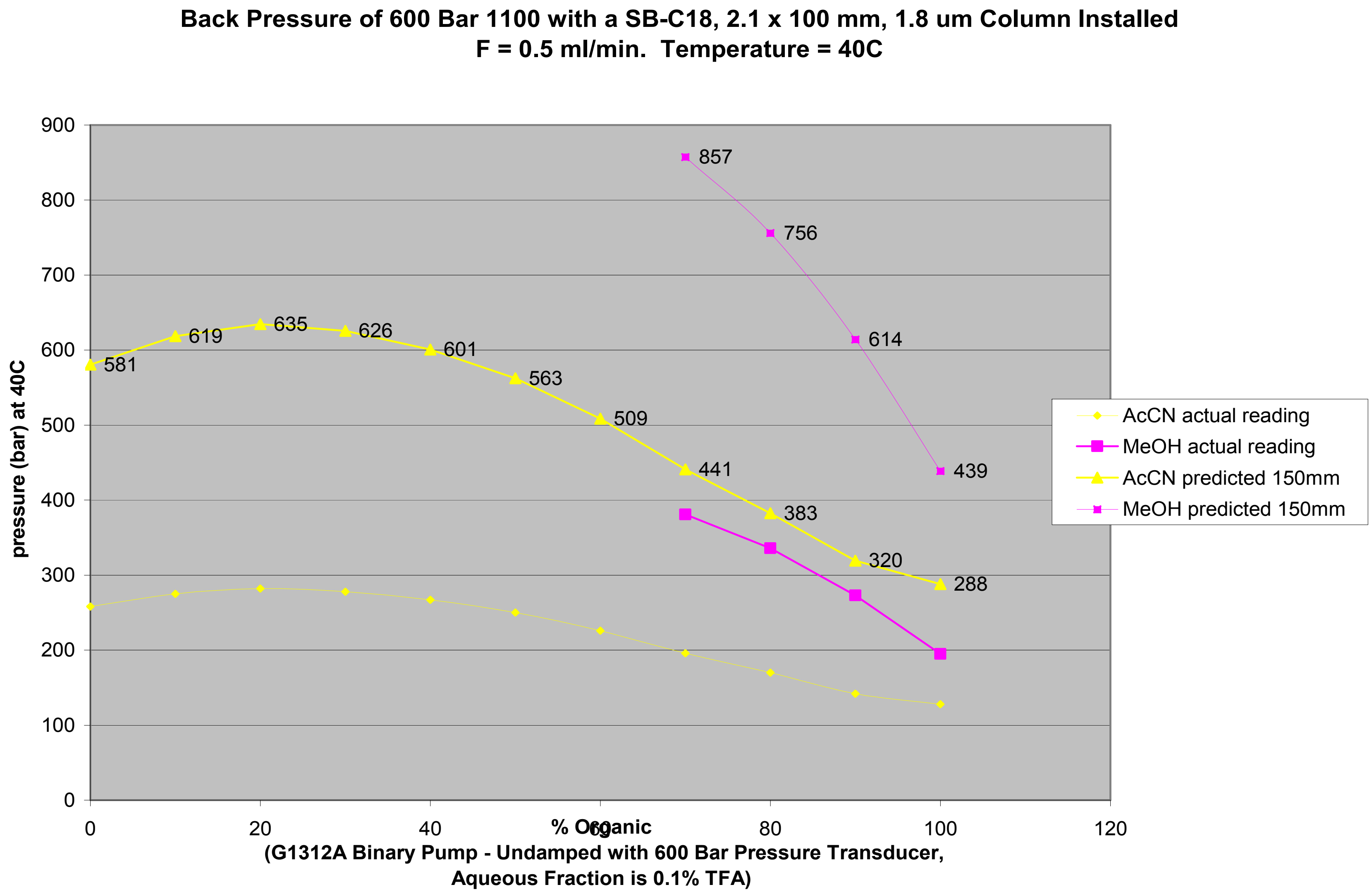
4.6 x 150, 3.5um
165 bar
N = 14862
Rs = 1.37
S/N = 50

4.6 x 150, 1.8um
490 bar
N = 28669
Rs = 1.80 (+57%)
S/N = 44

Mobile Phase
How Does it Impact Pressure

1. Solvent viscosity – lower viscosity results in lower pressure
   - **Acetonitrile < Methanol** The difference between MeOH and ACN can be dramatic and is the first thing to change if lower pressure is needed.
   - **Water < Buffer** While buffers increase viscosity, the organic selected is more critical. Make sure the buffer is soluble in the organic at all points in the run (gradient).
2. % of organic solvent – there is a pressure maximum and minimum for organic:aqueous mobile phases and it differs depending on the organic
   - A 2.1 x 100mm column can be used with ACN below 400 bar, especially with slightly elevated temperature.
   - But with MeOH you will need the 600 bar RRLC systems for almost all MeOH water mobile phases.

Pressure on 2.1 x 100 mm
ACN vs. MeOH

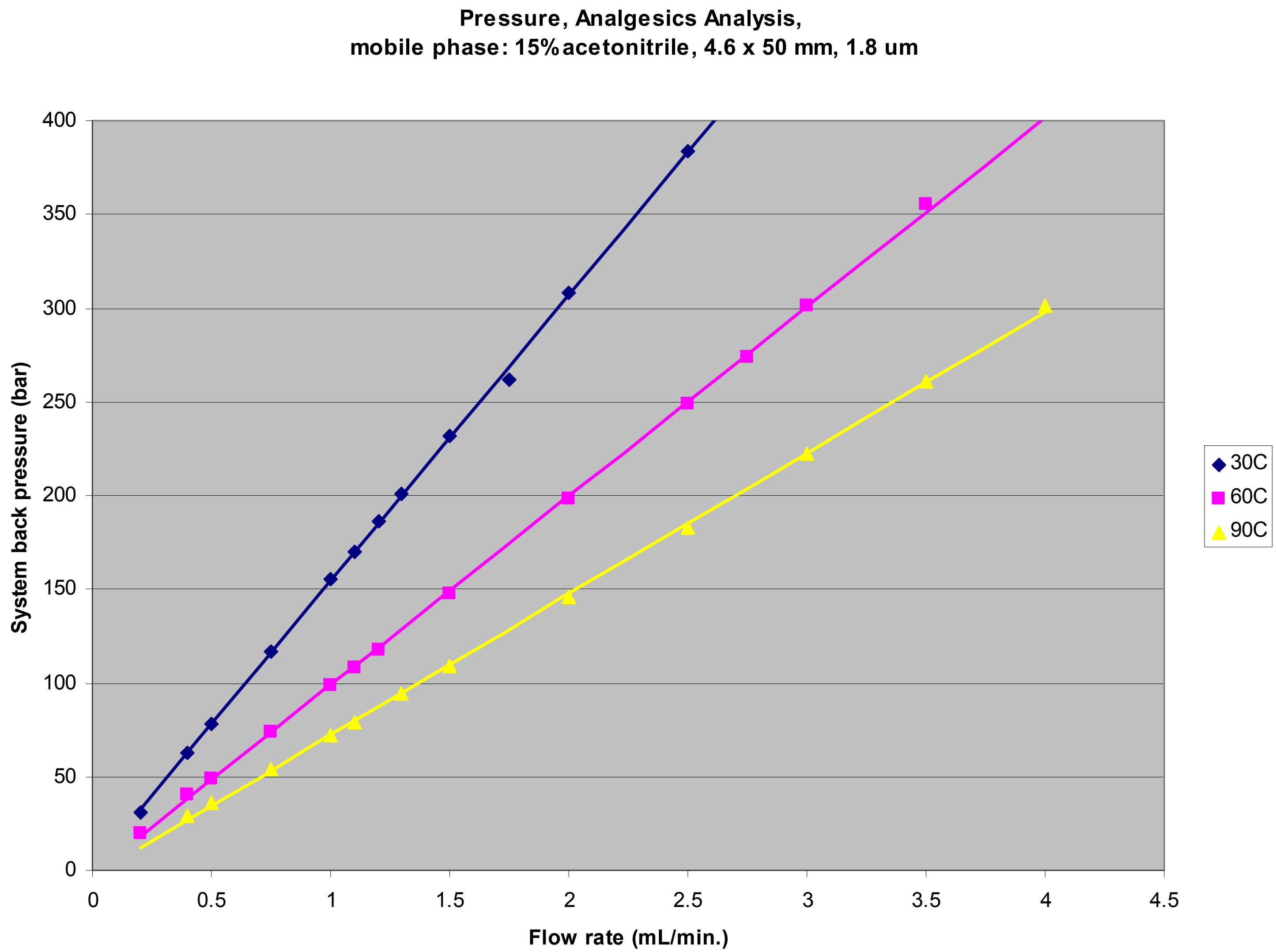


Some Examples of the Pressure On RRHT Columns (4.6 mm ID)

Length	50 mm	30mm	50mm	50mm	150mm
Mobile Phase	85% MeOH: 15% H_2O	60% MeOH: 40% H_2O	15% ACN, 0.1% FA:85%, 0.1% FA	70% NaAc: 30% ACN	5% ACN: 95% H_2O w/ 0.1%TFA
Pressure	208 bar	245 bar	384 bar	200 bar	418 bar
Temp.	Ambient	Ambient	30°C	25°C	70°C
Flow Rate	1 mL/min	1 mL/min	2.5 mL/min	1.5 mL/min	2 mL/min

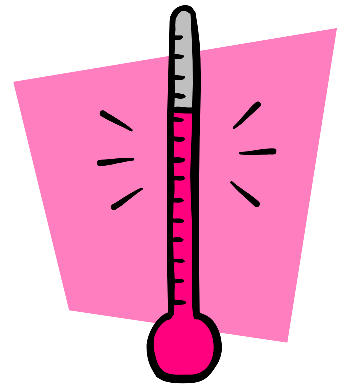
Short columns can run below 400 bar, longer columns can not.
Acetonitrile runs at lower pressure as expected, and organic choice is critical.

Increasing Temperature Lowers Operating Pressure



Higher Temperature as an Aid to Method Development and Faster Operation

Higher Temperature:



Temperature should always be considered as a parameter during method development

Decreases Mobile Phase Viscosity

- Lowers backpressure – allows for higher flow rates, faster separations, greater efficiency and use of sub 2-micron columns

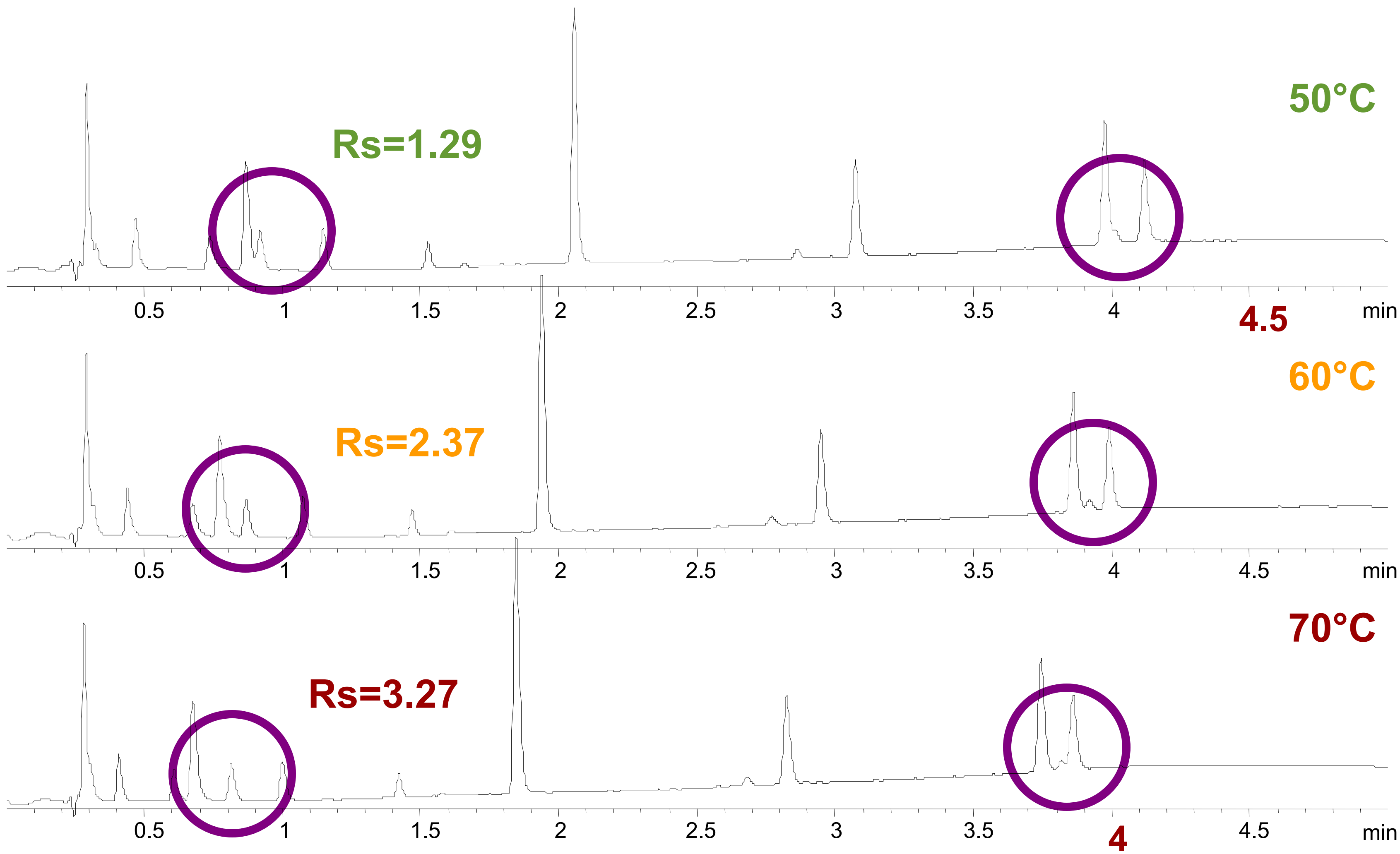
Provides more rapid mass transfer:

- Improves Efficiency – enhances resolution
- Decreases analysis time – faster separations with no loss in resolution

•**Can change selectivity – optimize resolution**

Use Elevated Temperature to Optimize Resolution, Selectivity

Gradient of Ten Cardiac Drugs on SB-C18 RRHT



Conclusion

HPLC is a powerful analytical tool

There are a variety of separation modes

- Most applications are RP

It's important to know what the terms mean

- We have introduced a number of chromatographic terms here
- Many build up from simple measurements taken on a chromatogram

Key Equations

- Resolution
  - Many parameters can affect resolution
- van Deemter

Other things like pressure can affect our separation as well

- Does not have to be a limitation

Appendix

What Extra Precautions Do You Need to Use RRHT Columns Successfully?

1. At column installation
   - ✓ Solvent changes
   - ✓ LC System cleanliness
   - ✓ Column Equilibration
2. Column use
   - ✓ Are you operating at the pressure you anticipate?
   - ✓ Check your gradient
3. Mobile phase
   - ✓ Are there limitations?
4. Sample considerations
   - ✓ Particulate free samples
   - ✓ Injection solvent

RRHT Column Installation Recommendations

1. Purge the pumps (connections up to the column) of any buffered mobile phases. Flush at least 5 mL of solvent before attaching the column to instrument.

   Goal: Eliminate any dried out or precipitated buffer from the system so it doesn't wash onto the column and plug the frit.

2. Flush the column with your mobile phase (compatible with the solvents the column was shipped in) starting slowly at 0.1 mL/min for a 2.1 mm ID column, 0.2 mL/min for a 3.0 mm ID column, and 0.4 mL/min for 4.6 mm ID.

   Goal: Avoid a pressure spike when the new mobile phase reaches the column. This occurs when the different solvents mix. The low flow rate allows this to happen without causing an unanticipated pressure change.

3. Increase the flow rate to the desired flow over a couple of minutes.

   Goal: anticipate the final operating pressure

4. Once the pressure has stabilized, attach the column to the detector.

5. Equilibrate the column and detector with 10 column volumes of the mobile phase prior to use.

   Goal: reproducible chromatography from the 1st run

RRHT Column Installation Recommendations (cont.)

6. If you are running a gradient, check that the pressure range of the gradient – which may be 100 – 130 bar or more, will not cause the system to overpressure, before starting any sequence.

   Goal: no surprises, good unattended operation over 100's or 1000's of injections.

7. Once the pressure has stabilized, attach the column to the detector.

8. Equilibrate the column and detector with 10 column volumes of the mobile phase prior to use.

   Goal: reproducible chromatography from the 1st run

9. If you are running a gradient, check that the pressure range of the gradient – which may be 100 – 130 bar or more, will not cause the system to overpressure, before starting any sequence.

   Goal: no surprises, good unattended operation over 100's or 1000's of injections.

10. Avoid overtightening fittings/replace fitting to column periodically – use polyketone fittings for quick changes.

    Goal: good connections, no extra volume, no overtightening of fittings.

Mobile Phase and Sample Recommendations to Avoid High Pressure

If the system has been sitting with buffer in it, flush the injector as well as the column. This prevents any bacterial growth in the injector from transferring to the column and plugging the frit.

Replace bottles of mobile phase buffer every 24 – 48 hours. Do not top off the bottle with more mobile phase, replace the buffer with a fresh bottle

Do not use a high buffer salt mobile phase (>50mM) in combination with high ACN concentrations due to possible precipitation.

Filter all aqueous buffers prior to use through a 0.2 um filter.

Use solvents that are high quality chromatography grade solvents (HPLC or MS grade).

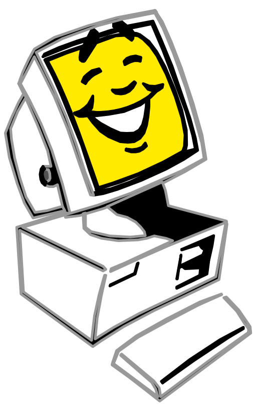
Filter all samples with particulates through an appropriate 0.2um filter. Particulates can clog the inlet frit on the column and cause high pressure and short column lifetime.

Install an in-line filter to catch particulates before they get to the column.

Agilent LC Columns and Agilent J&W GC Columns Scientific Technical Support

800-227-9770 (phone: US & Canada)*

302-993-5304 (phone)



For LC columns

Select option 4, then option 2

For GC Columns

**** Select option 4, then option 1.***

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January 22, 2008 – 1:00 p.m. EST