Tips and Tricks of HPLC Separations

Agilent Technologies, Inc.

LC Tips And Tricks Seminar Series

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Topics

- **Chromatographic Process**
- **Improving Separations**
- **Troubleshooting**

Chromatographic Process

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

  R_s – Resolution
  N – Column Efficiency, Plates
  k, k' – Retention Factor, Capacity Factor
  α – Selectivity

Some Basic Chromatography Parameters

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

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

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

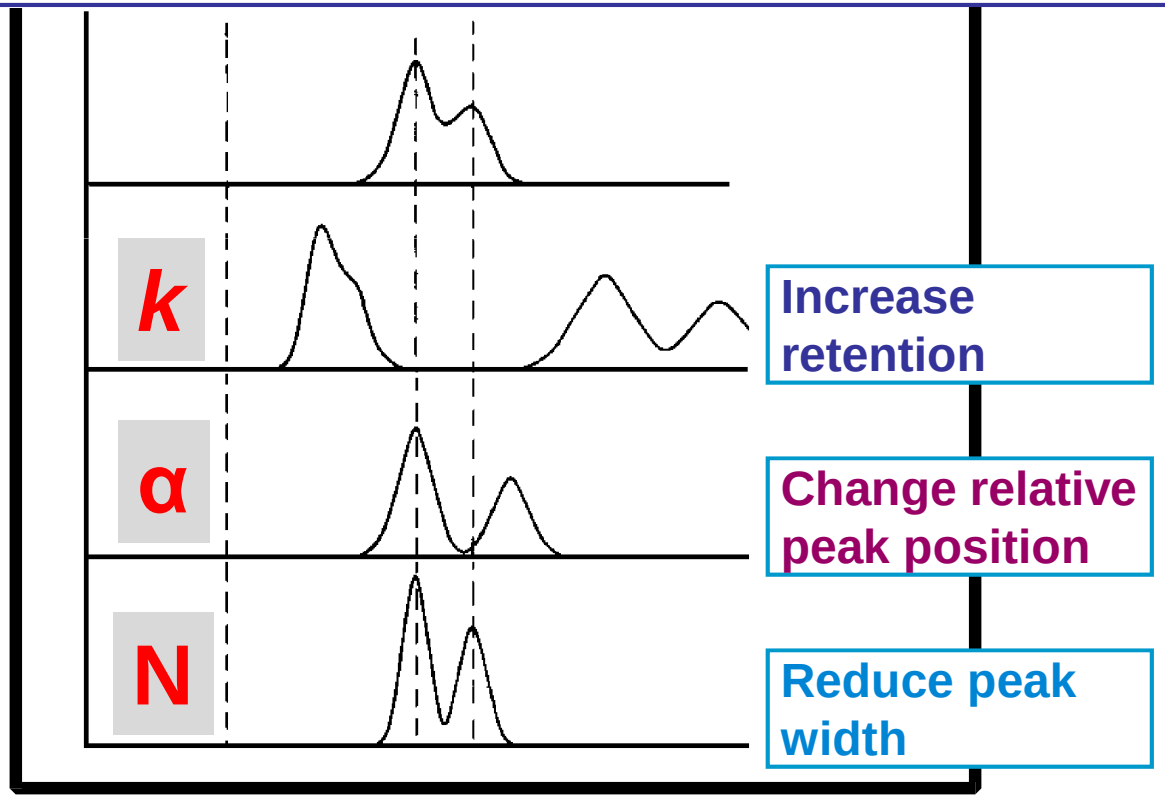
N = Column Efficiency – Column length and particle size

α = Selectivity – Mobile phase and stationary phase

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

Factors that Improve Resolution

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



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 Column Volumes.
(Flow is volume/time – mL/min)

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

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

k is measure of number of column volumes required to elute compound.

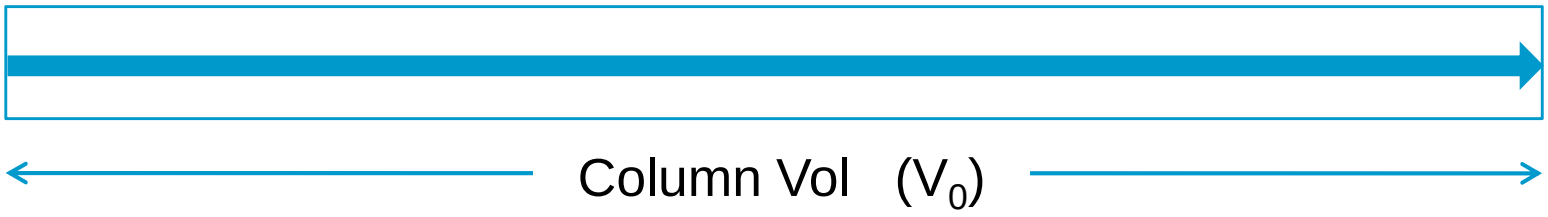
Fundamental, dimensionless parameter that describes the retention.

***k* = 1 to 20 - OK; *k* = 3 to 10 - Better; *k* = 5 to 7 - Ideal**

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

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

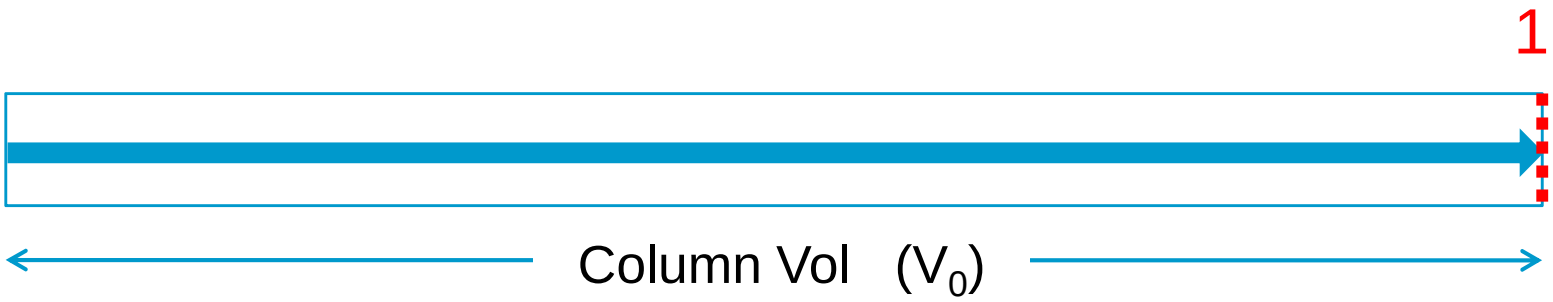
Measure of number of column volumes required to elute compound



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

Un-retained component – elutes w/ solvent front

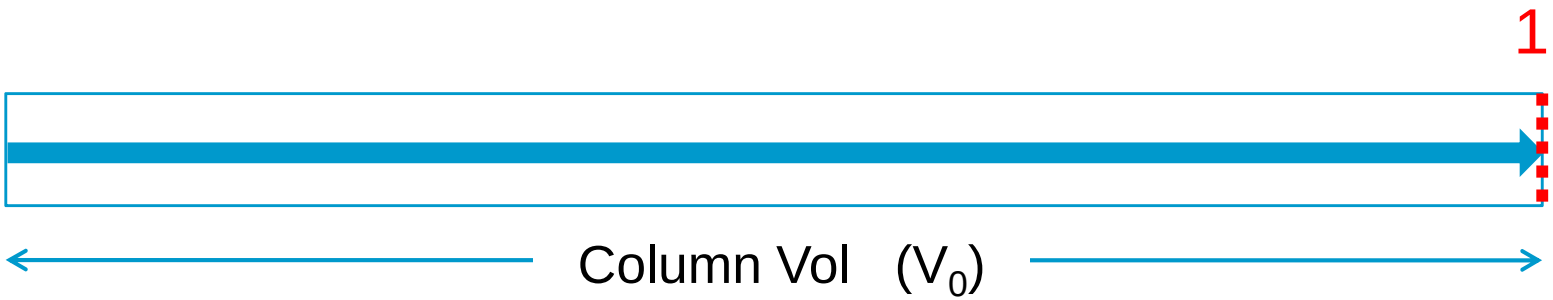
k = 0



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

Un-retained component – elutes w/ solvent front

k = (1 - 1) / 1 = 0

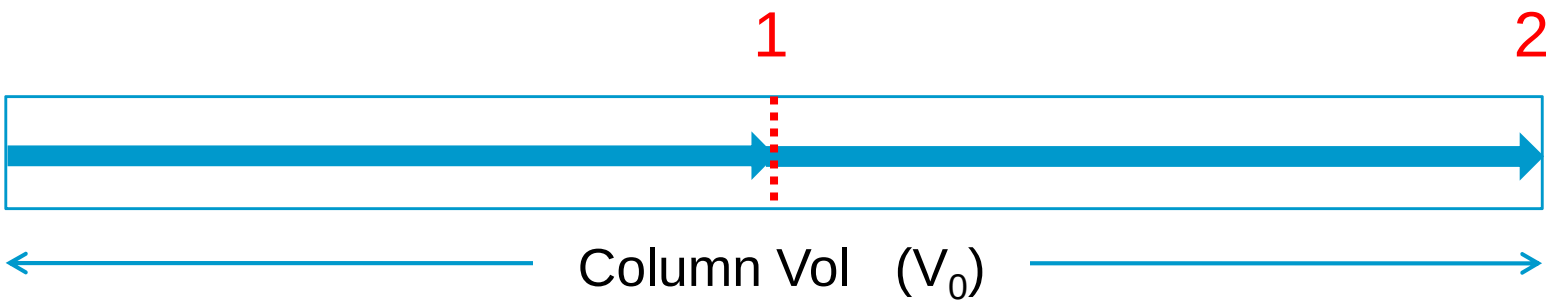


$k = (t_R - t_0) / t_0$

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

Component retained – elutes in 1 add'l column volumes

k = (2 - 1) / 1 = 1

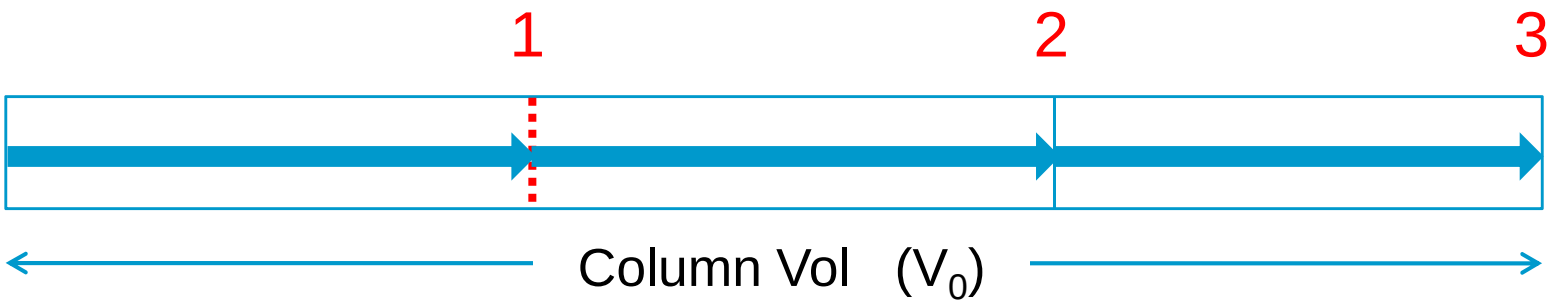


$$k = (t_R - t_0) / t_0$$

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

Component retained – elutes in 2 add'l column volumes

k = (3 - 1) / 1 = 2

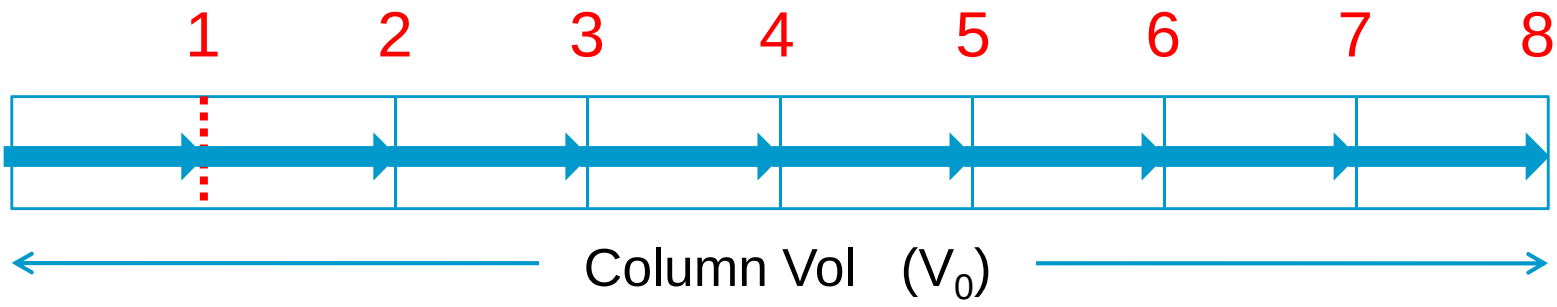


$k = (t_R - t_0) / t_0$

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

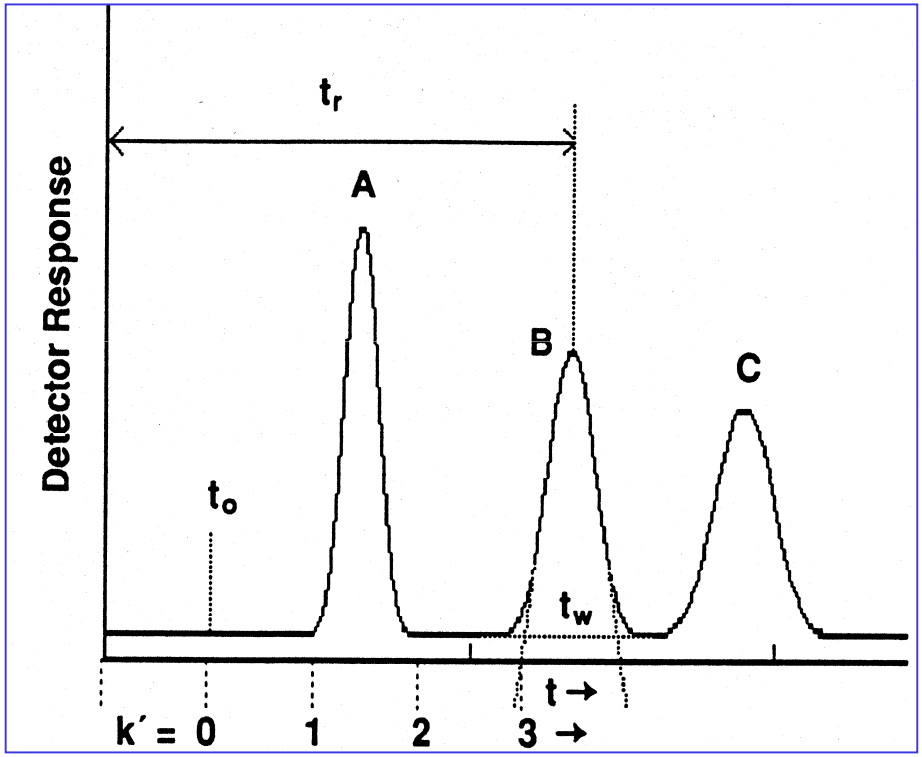
Component retained – elutes in 7 add'l column volumes

k = (8 - 1) / 1 = 7



$k = (t_R - t_0) / t_0$

Chromatographic Profile
Equations Describing Factors Controlling R_S



Retention Factor

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

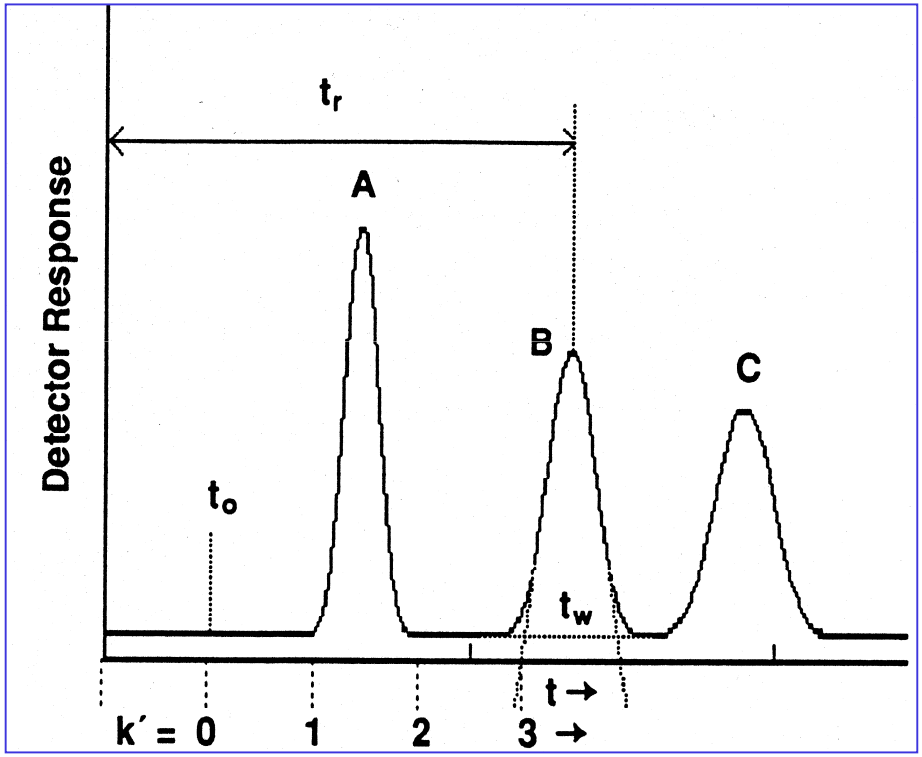
Selectivity

$$\alpha = k_2/k_1$$

Theoretical Plates-Efficiency

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

Chromatographic Profile
Equations Describing Factors Controlling R_S



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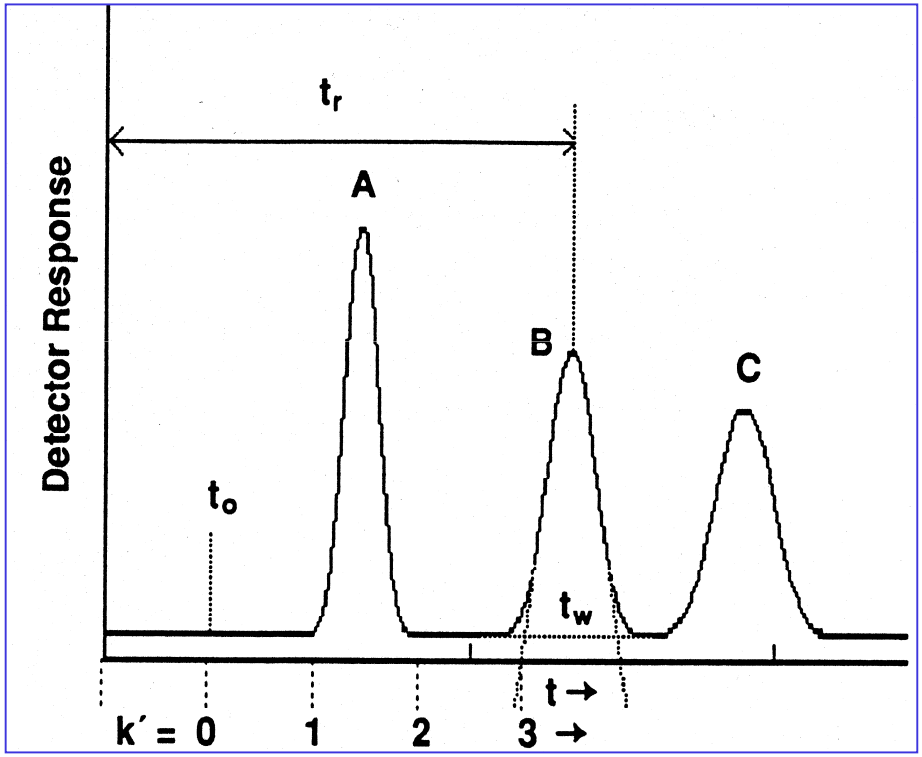

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Selectivity (α)

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

α is measure relative difference in retention

Selectivity (α)

$$\alpha = \frac{k_2}{k_1} = \frac{(t_{R2} - t_0)/t_0}{(t_{R1} - t_0)/t_0}$$

α is measure relative difference in retention

Selectivity (α)

$$\alpha = \frac{k_2}{k_1} = \frac{(t_{R2} - t_0)}{(t_{R1} - t_0)}$$

α is measure relative difference in retention

Selectivity (α)

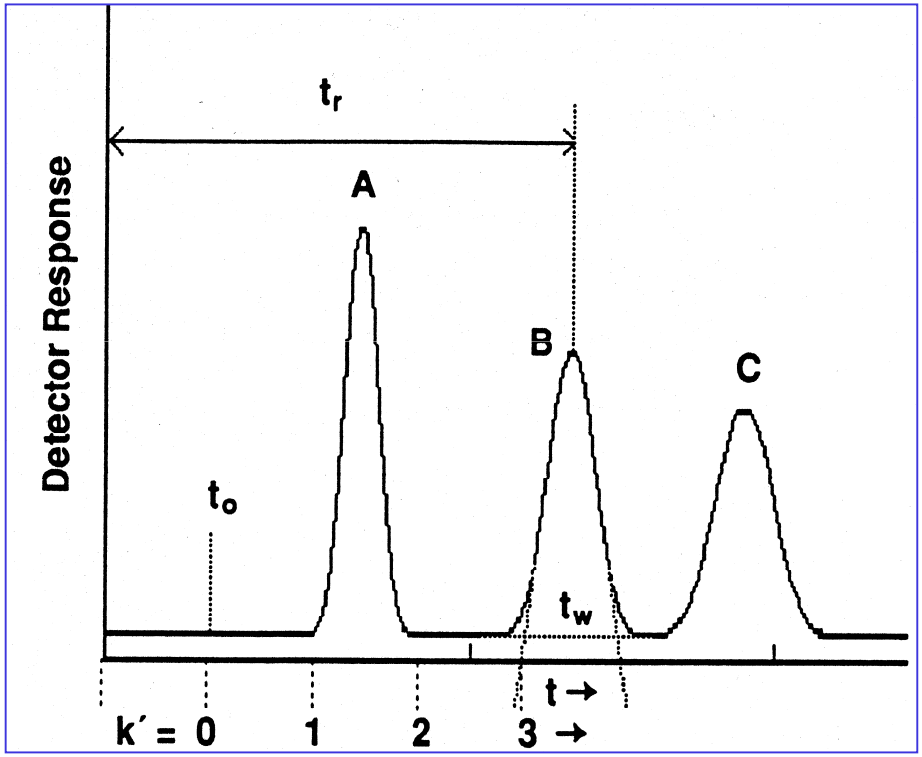
$$\alpha = \frac{k_2}{k_1}$$

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

Chromatographic Profile
Equations Describing Factors Controlling R_S



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Theoretical Plates - Efficiency

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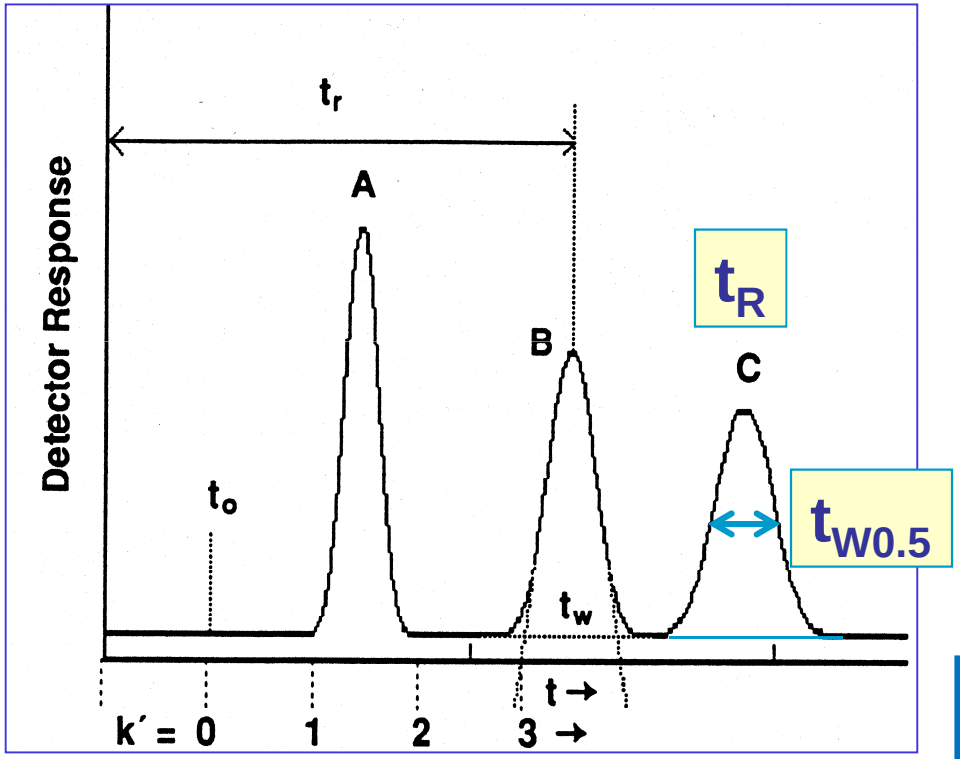
Equations Describing Factors Controlling R_S



Retention Factor

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

Selectivity

$$\alpha = k_2/k_1$$

Theoretical Plates - Efficiency

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

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

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

Column Efficiency (N)

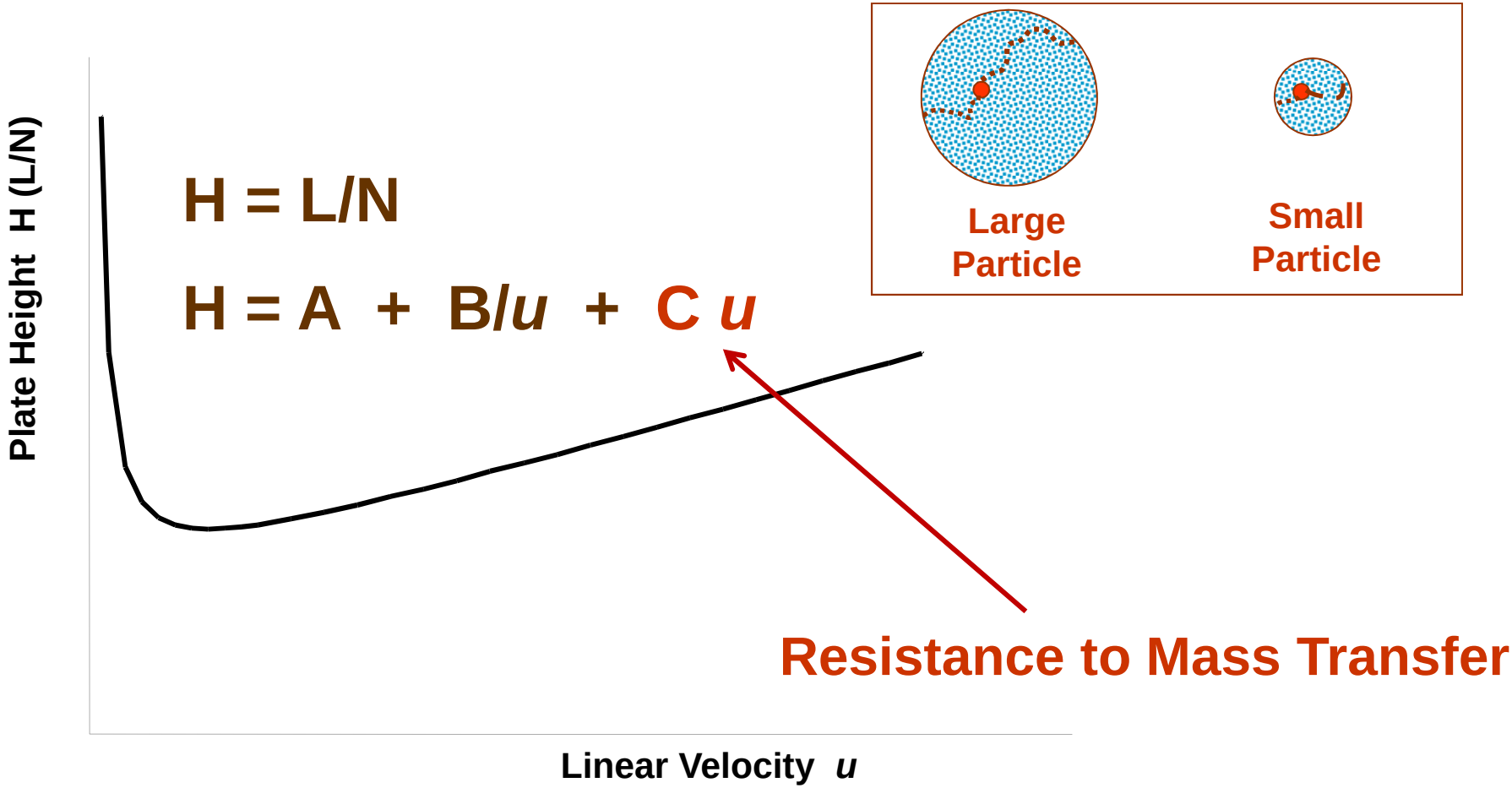
N - Number of theoretical plates.

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

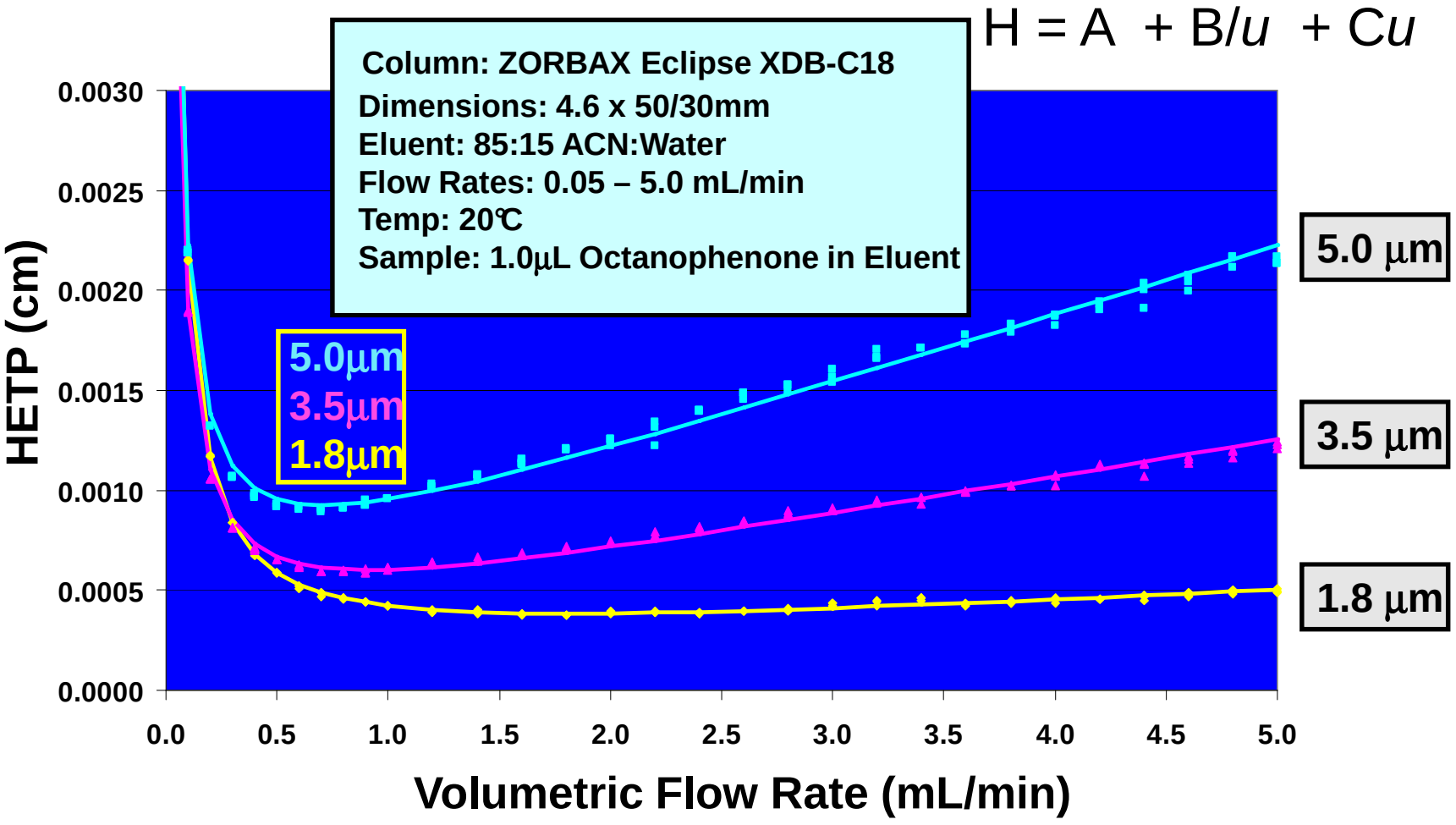
Van Deemter Curve
Factors Affecting N



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

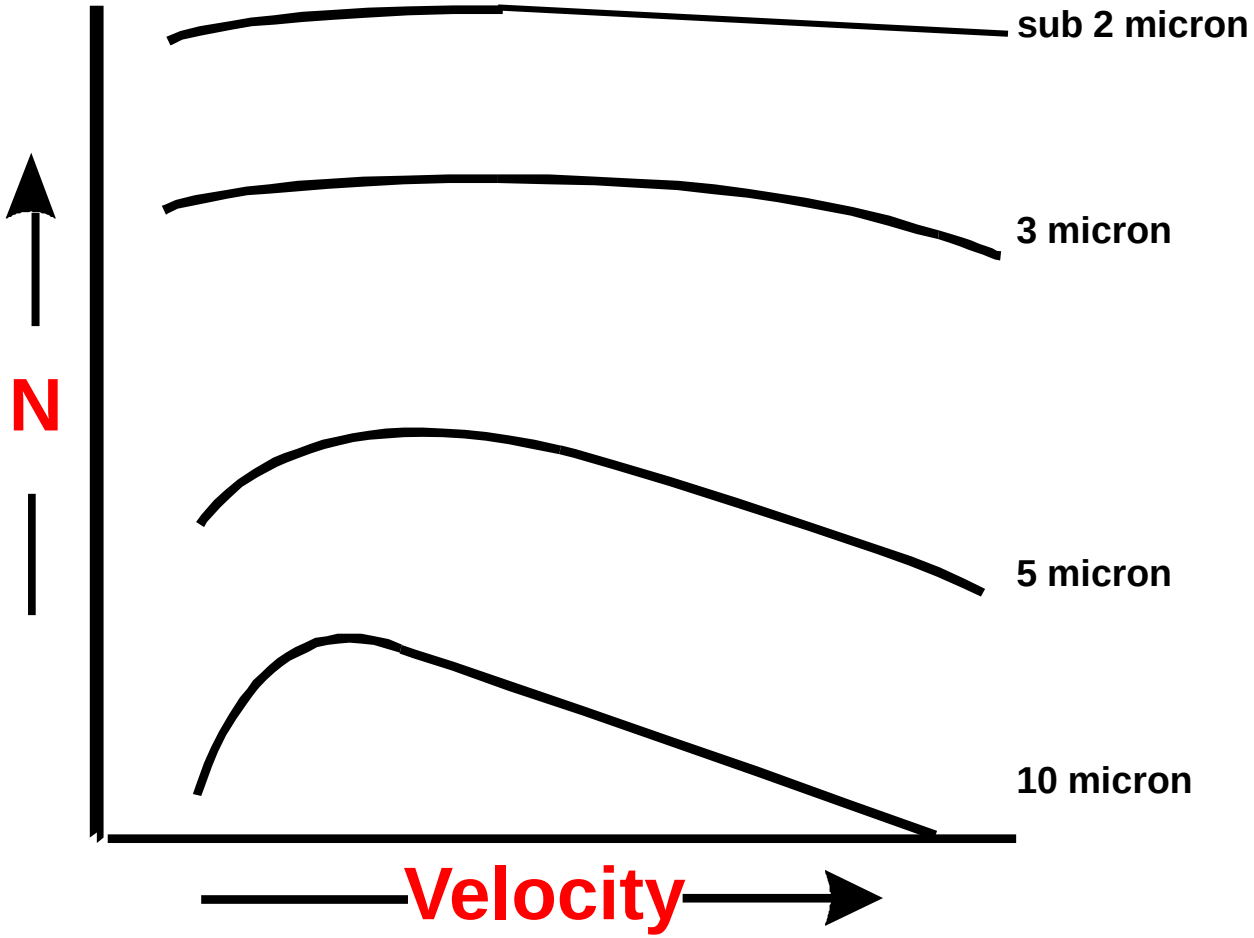
Van Deemter Curve
Effect of Particle Size

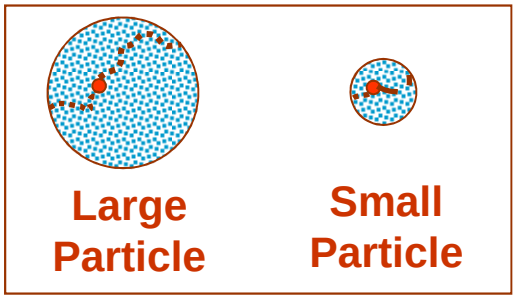
$$H = A + B/u + Cu$$



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

Columns Packed with Smaller Particles Provide Higher Efficiency





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

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

Topics

- Chromatographic Process
- **Improving Separations**
- Troubleshooting

Improving the Separations

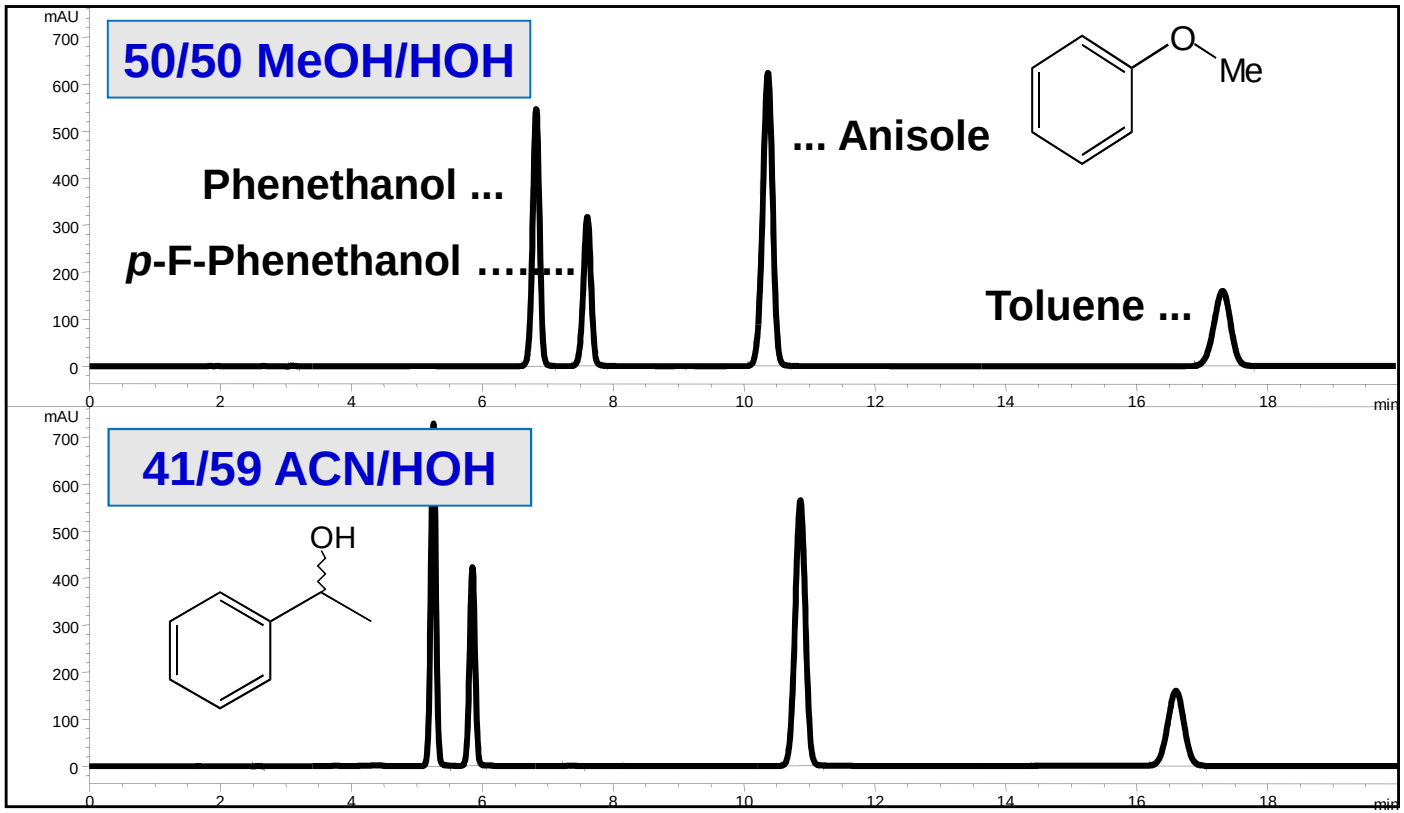
- Improve Selectivity (α)
- Improve Column Efficiency (N)
- Improve Chromatography Choices

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

Selectivity

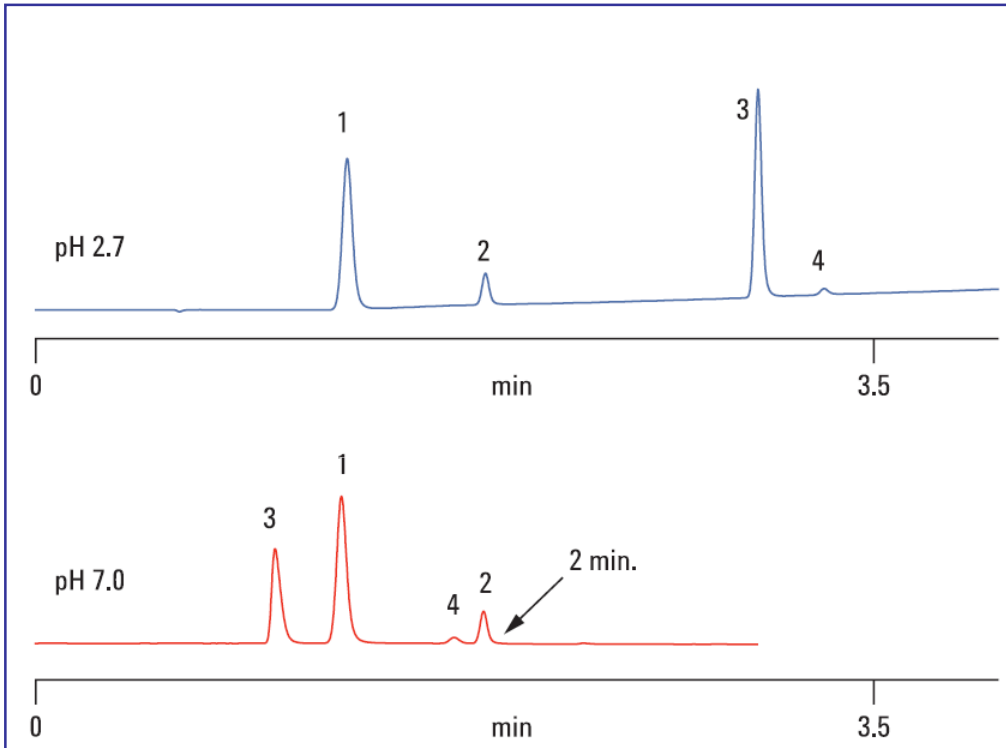
- Mobile Phase
- Stationary Phase

Different Mobile Phases May Give Different Selectivity

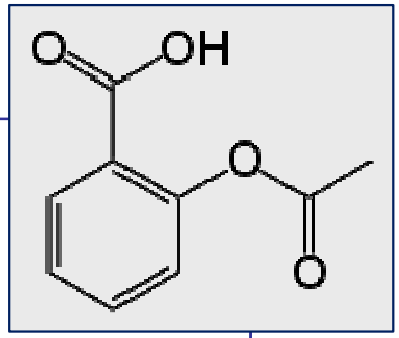


ZORBAX® SB-C18 4.6 x 250 mm
1 mL/min, 40°C, 225 nm

Effect of pH on Retention



Selectivity differences at pH 2 and pH 7 can be dramatic



Peak identification
1. Acetaminophen
2. Caffeine
3. Acetylsalicylic acid
4. unknown

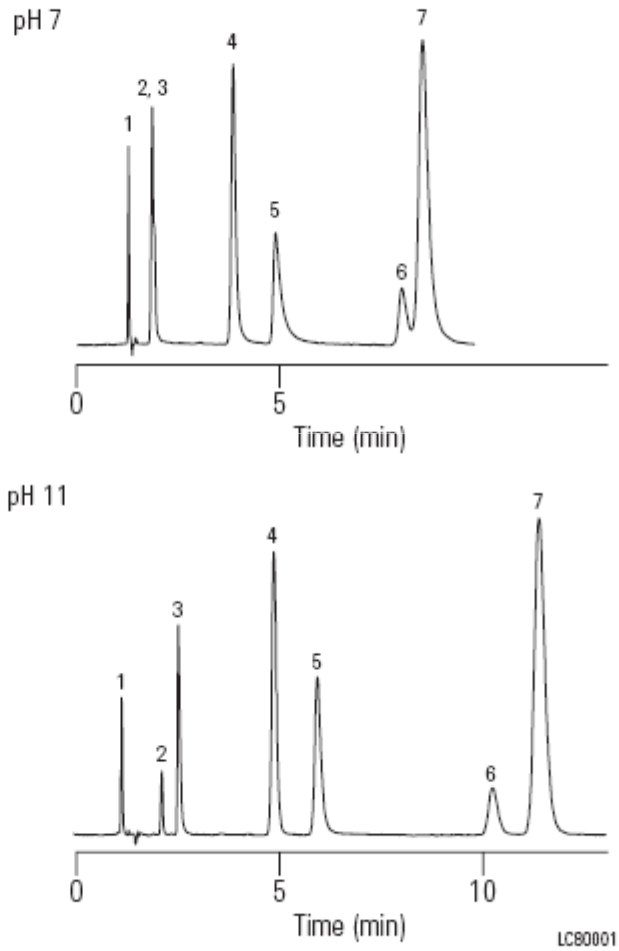
Conditions

Column:	Eclipse Plus C8 4.6 x 50mm, 5 µm, PN 959946-906
Gradient:	10-60% B/3 min.
pH 2.7 –	A: 0.1% formic acid B: 0.1% FA in ACN
pH 7.0 –	A 20 mM Na phosphate adjusted to pH 7.0 with phosphoric acid B: ACN
Sample:	"generic Excedrin tablet"

Effect of pH on Retention, Peak Shape

Basic Antihistamines on Extend-C18 at High pH

Column:	**ZORBAX Extend-C18** **773450-902** **4.6 x 150 mm, 5 µm**
Mobile Phase:	pH 7: 30% 20 mM Na_2HPO_4 70% MeOH pH 11: 30% 20 mM TEA 70% MeOH
Flow Rate:	1.0 mL/min
Temperature:	Ambient
Detector:	254 nm
Sample:	Antihistamines

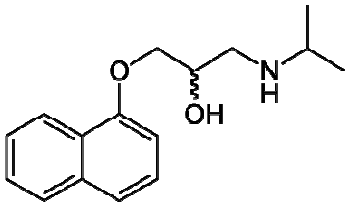


1. Maleate
2. Scopolamine
3. Pseudoephedrine
4. Doxylamine
5. Chlorpheniramine
6. Triprolidine
7. Diphenhydramine

Pseudoephedrine and scopolamine are difficult to retain at low and mid pH. Pseudoephedrine is often analyzed by ion exchange methods. The Extend-C18 column retains these compounds in a noncharged form at high pH and improves resolution.

Test for pH Robustness

Column: ZORBAX Rapid Resolution Eclipse XDB-C8, 4.6 x 75 mm, 3.5 µm
Mobile Phase: 44% 25 mM phosphate, pH 7.00 : 56% methanol Flow Rate: 1.0 mL/min Temperature: 25°C
Detection: UV 250 nm
Sample: 1. ketoprofen 2. ethyl paraben 3. hydrocortisone 4. fenoprofen 5. propyl paraben 6. propranolol 7. ibuprofen



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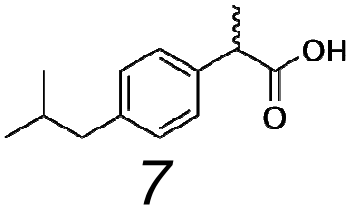

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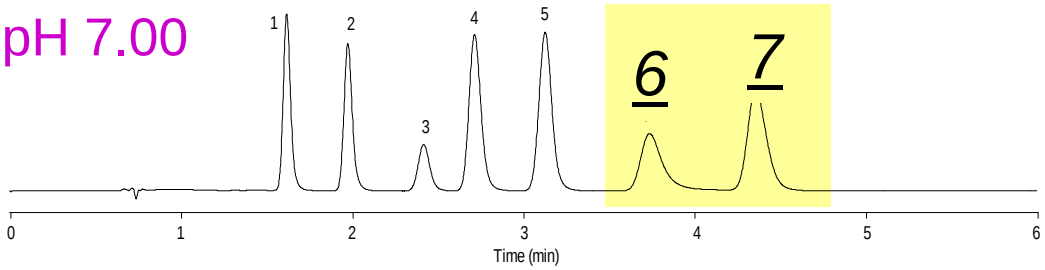
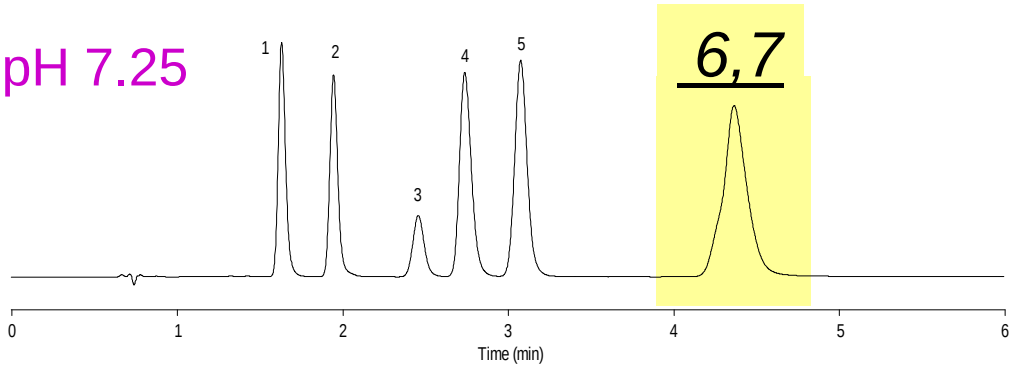


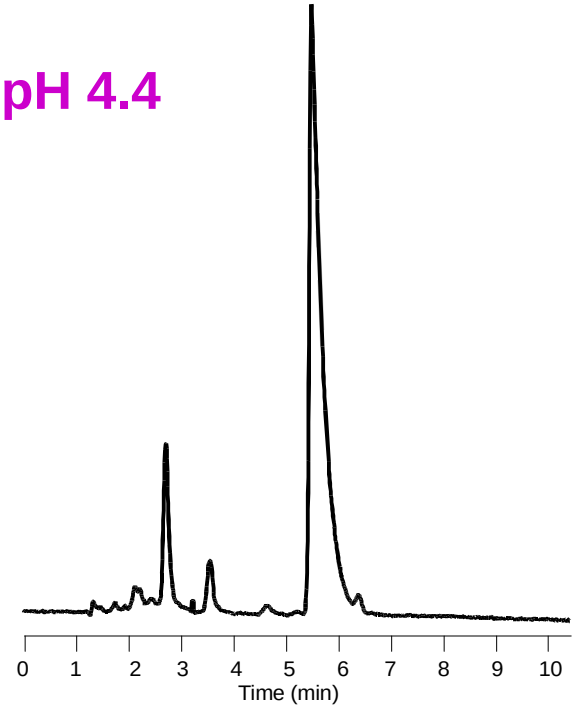


- The resolution of ionizable compounds can change markedly with pH changes—even as small as 0.05–0.25 pH units.

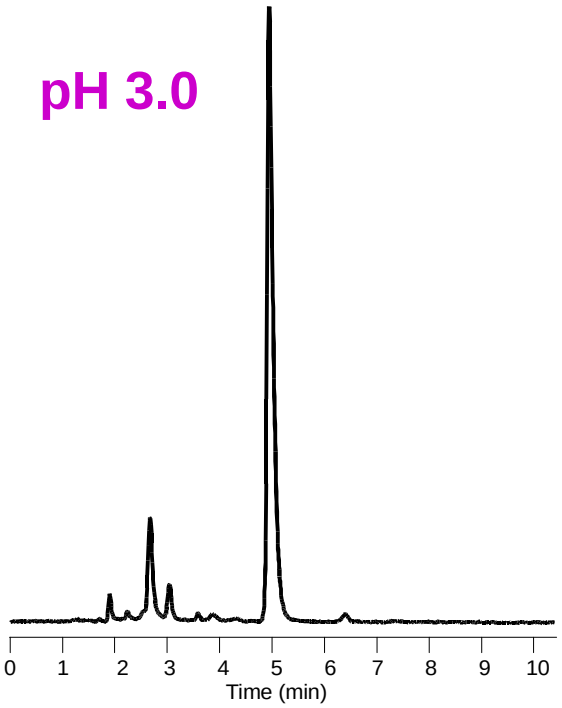
Effect of pH on Peak Shape at or Near the Sample pK_a

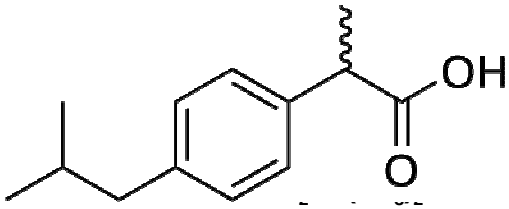
Column: ZORBAX SB-C8 4.6 x 150 mm, 5 mm
Mobile Phase: 40% 5 mM KH_2PO_4: 60% ACN
Flow Rate: 1.0 mL/min
Temperature: RT

pH 4.4



pH 3.0





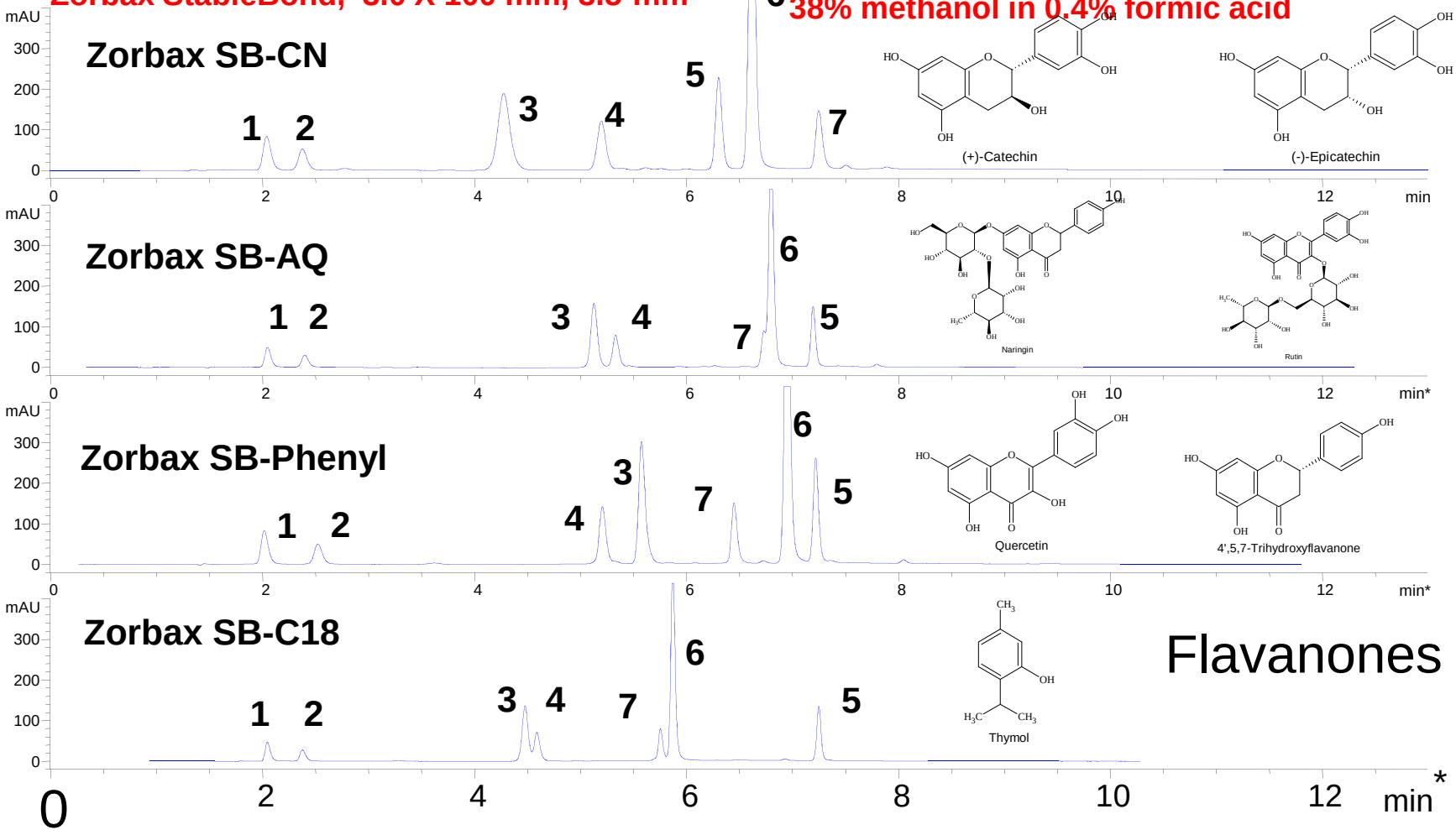
Ibuprofen
pK_a = 4.4

- Inconsistent and tailing peaks may occur when operating close to an analyte's pKa and should be avoided.

Different Stationary Phases May Give Significantly Different Selectivity

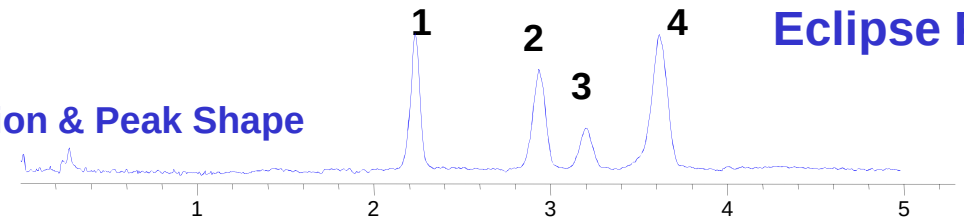
Columns:
Zorbax StableBond, 3.0 X 100 mm, 3.5-mm

Mobile Phase:
38% methanol in 0.4% formic acid



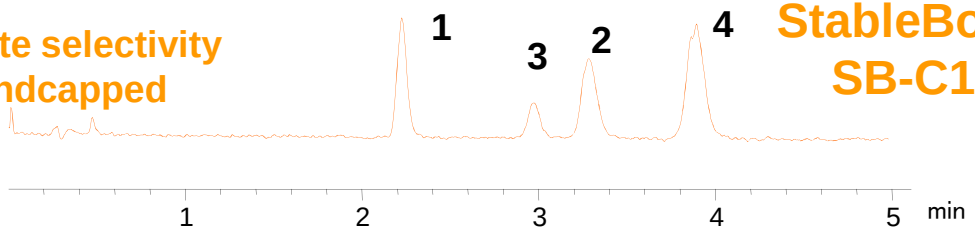
Similar Stationary Phases May Give Different Selectivity

1st choice
Best Resolution & Peak Shape



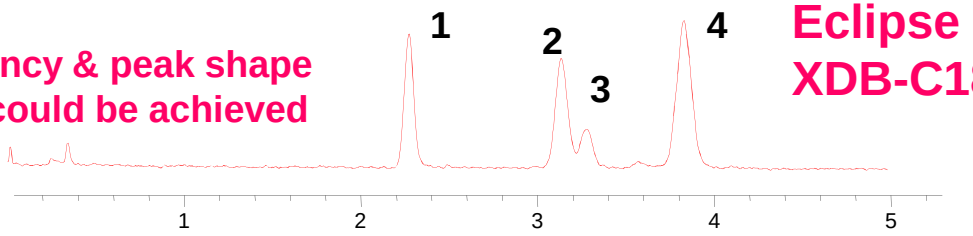
Eclipse Plus C18

2nd choice
Good alternate selectivity due to non-endcapped



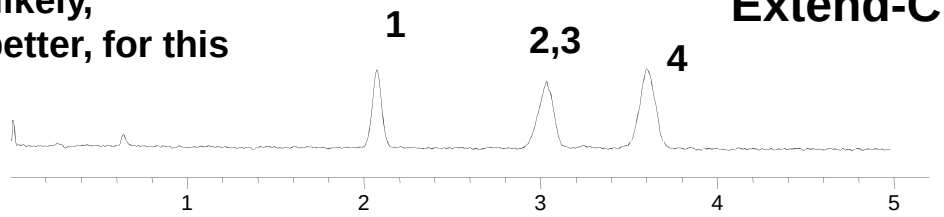
StableBond SB-C18

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 ℃
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)

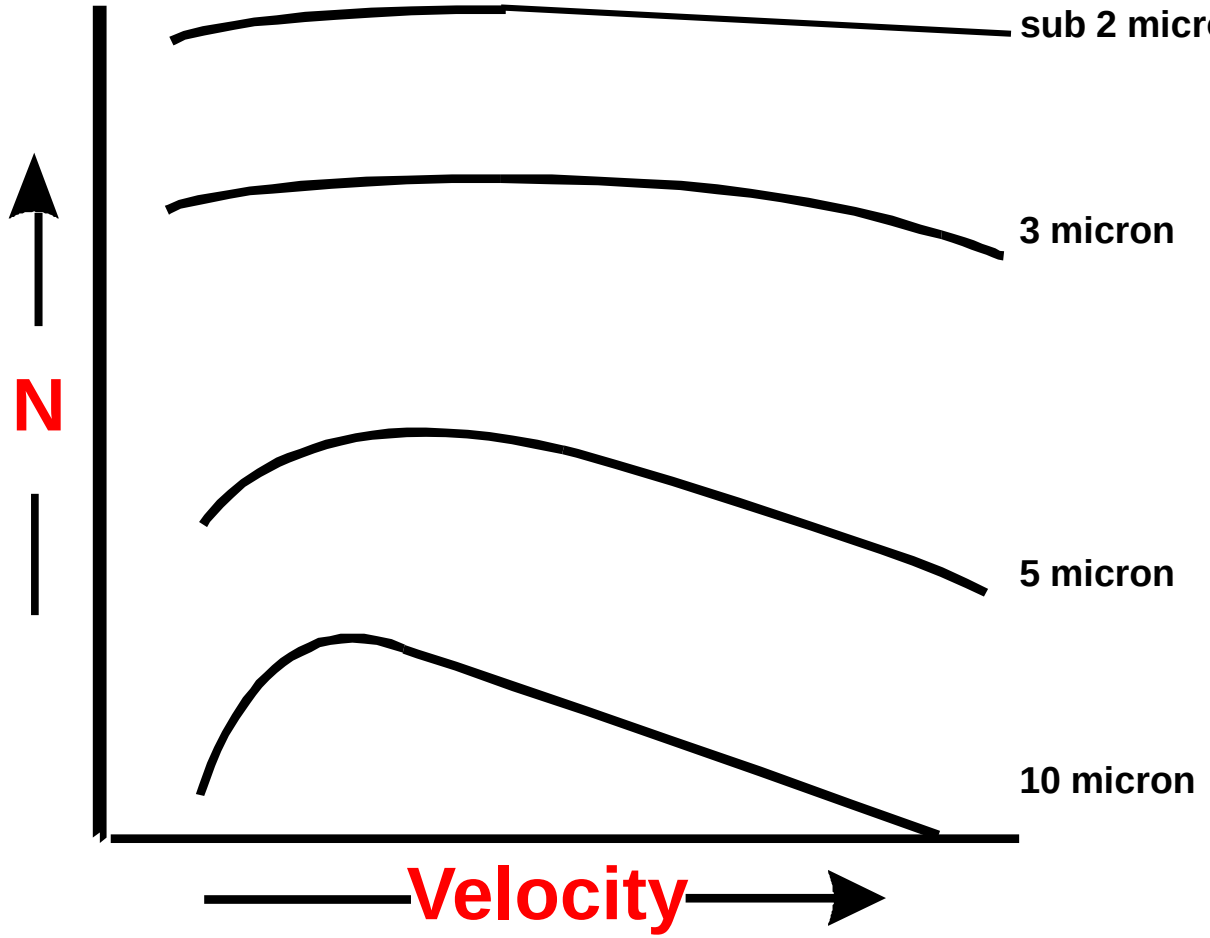
Multiple bonded phases for most effective method development.
Match to one you're currently using.

Improving the Separations

- Improve Selectivity (α)
- Improve Column Efficiency (N)
- Improve Chromatography Choices

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

Columns Packed with Smaller Particles Provide Higher Efficiency



$N \propto 1/(d_p)$

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

Decreasing Particle Size

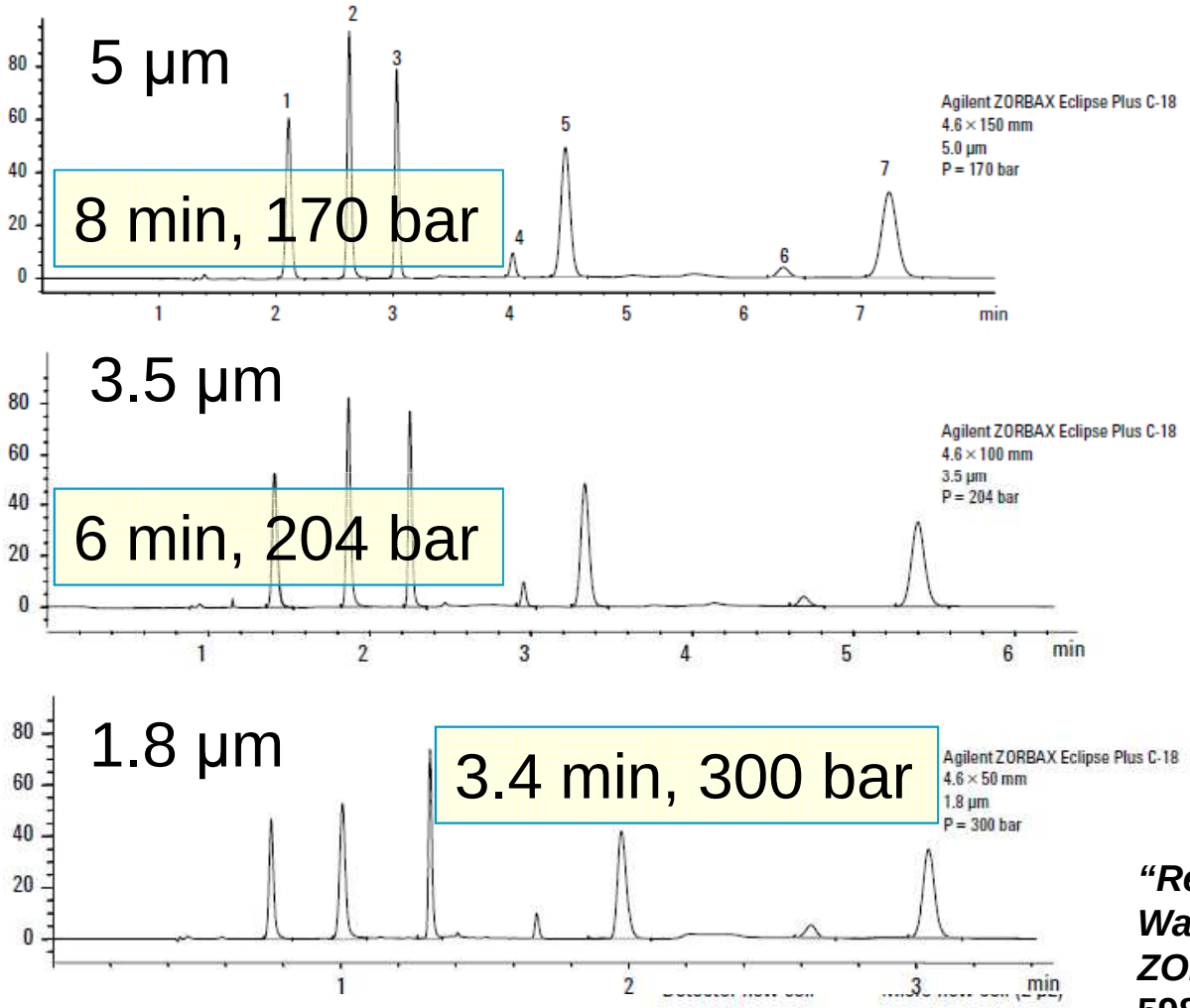


Table 2. Chromatographic Conditions

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)**

Improving the Separations

- Improve Selectivity (α)
- Improve Column Efficiency (N)
- **Improve Chromatography Choices**

Improve Chromatography Choices

- Shorten analysis time:
  reduce column length,
  increase flow rate
- Sample Preparation

Improve Chromatography Choices

- Shorten analysis time:
  reduce column length,
  increase flow rate
- Sample Preparation

Reduce analysis time

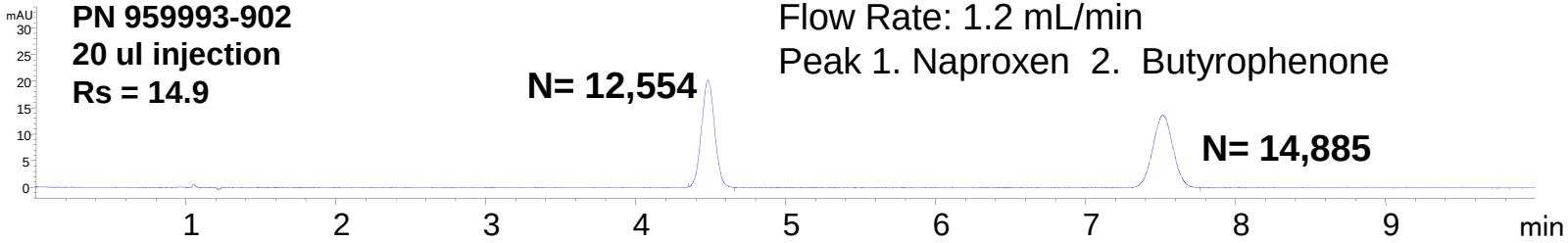
- 250 mm, 5 um ~ 150 mm, 3.5 um – 60%
- 2 mL/min vs 1 mL/min – 50%
- Reduce 25 min run to 7.5 min run

USP Method for Naproxen Tablets – 4X Faster Analysis on Poroshell 120

Method Requirement N > 4000, Rs better than 11.5

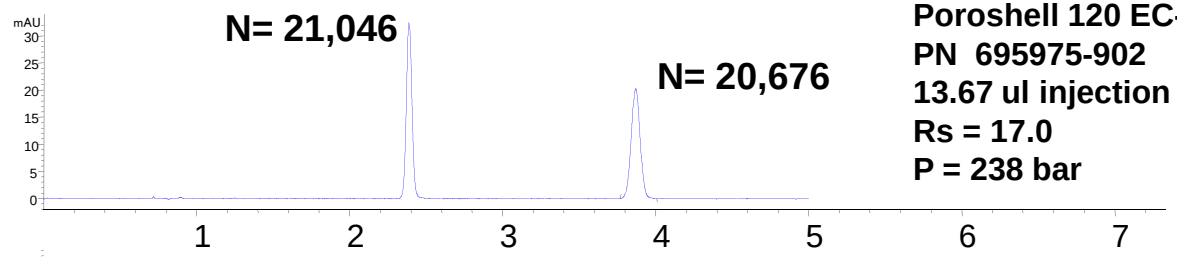
4.6 x 150 mm (L1)
Eclipse Plus C18, 5um
PN 959993-902
20 ul injection
Rs = 14.9

Common Conditions:
Mobile Phase: 50:49:1 MeCN:H2O Acetic Acid
Flow Rate: 1.2 mL/min
Peak 1. Naproxen 2. Butyrophenone



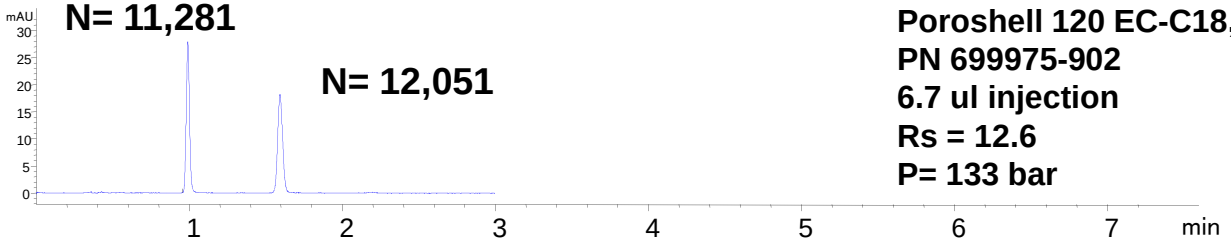
4.6 x 100 mm (L1)
Poroshell 120 EC-C18, 2.7 um
PN 695975-902
13.67 ul injection
Rs = 17.0
P = 238 bar

2X Faster



4.6 x 50 mm (L1)
Poroshell 120 EC-C18, 2.7 um
PN 699975-902
6.7 ul injection
Rs = 12.6
P= 133 bar

4X Faster



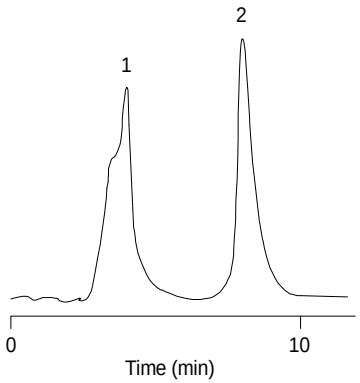
Improve Chromatography Choices

- Shorten analysis time:
  reduce column length,
  increase flow rate
- Sample Preparation

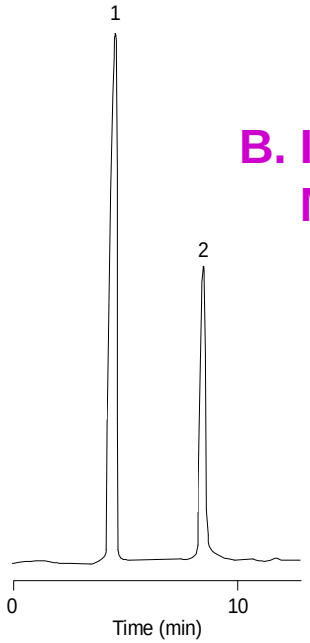
Split Peaks from Injection Solvent Effects

Column: StableBond SB-C8, 4.6 x 150 mm, 5 µm; Mobile Phase: 82% H_2O : 18% ACN; Inj Vol: 30 µL
Sample: 1. Caffeine 2. Salicylamide

A. Injection Solvent 100% Acetonitrile



B. Injection Solvent Mobile Phase



Tip: Injecting in a solvent stronger than the mobile phase can cause peak shape problems such as peak splitting or broadening

Trick: Keep Organic Concentration in Sample Solvent ≤ Mobile Phase

Columns Die from the Sample
Prevention Techniques - A Better Choice!

- Use column protection
  - In-line filters
  - Guard columns
- Filter samples
- Filter buffered mobile phases

Easy

- Sample clean-up (i.e. SPE)
- Appropriate column flushing

Not as Easy

Column cleaning: R. Majors, *LCGC* (2003) Vol **21** p19.

Column Cleaning

Flush with stronger solvents than your mobile phase

Reversed Phase Solvent Choices
In Order of Increasing Strength

Use at least 25 mL of each solvent for analytical columns

This Is time consuming

Often performed offline

- Mobile phase without buffer salts
- 100% Methanol
- 100% Acetonitrile

- 75% Acetonitrile:25% IPA
- 100% Isopropanol

- 100% Methylene Chloride*
- 100% Hexane*

Must reverse to Re-equilibrate

Tip: When using either Hexane or Methylene Chloride; The column must be flushed with Isopropanol before returning to your reverse phase mobile phase.

Topics

- Chromatographic Process
- Improving Separations
- **Troubleshooting – Poor Peak Shape**

Peak Tailing, Broadening and Loss of Efficiency

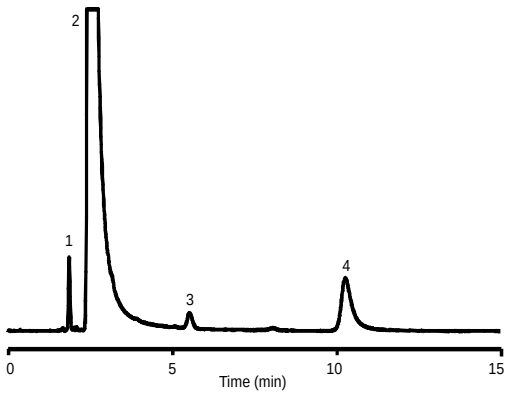
May be caused by:

- Column “secondary interactions”
- Column contamination
- Column aging
- Column loading
- Extra-column effects

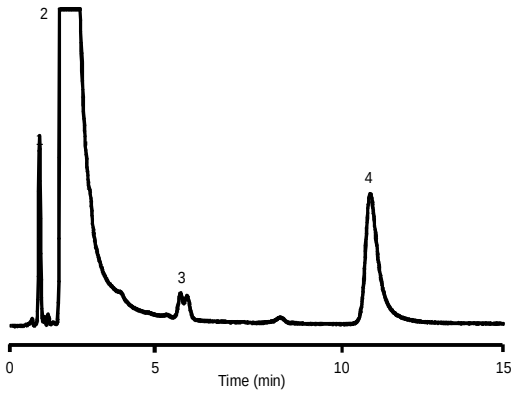
Split Peaks from Column Contamination

Column: StableBond SB-C8, 4.6 x 150 mm, 5 µm Mobile Phase: 60% 25 mM Na_2HPO_4, pH 3.0 : 40% MeOH Flow Rate: 1.0 mL/min
Temperature: 35℃ Detection: UV 254 nm Sample: Filtered OTC Cold Medication: 1. Pseudoephedrine 2. APAP 3. Unknown 4. Chlorpheniramine

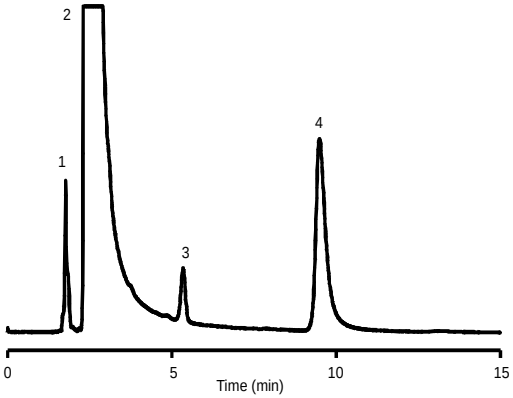
Injection 1



Injection 30



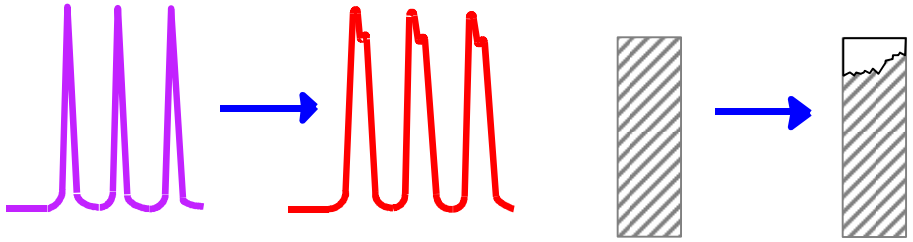
Injection 1 After Column Wash with 100% ACN



Tip: Column washing eliminates the peak splitting, which resulted from a contaminant on the column
How could this be prevented? (Guard Column, SPE clean up of samples, Periodic column wash)

Peak Splitting Caused By Disrupted Sample Path

- **Flow path disrupted by void**
- **Sample allowed to follow different paths through column**
- **Poorly packed bed settles in use**
- **High pH dissolves silica**

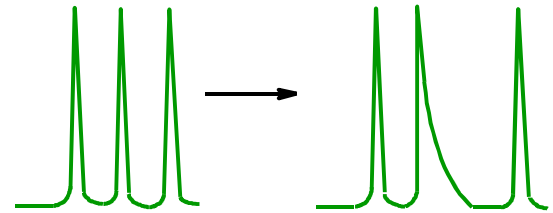


Normal **Double Peaks**

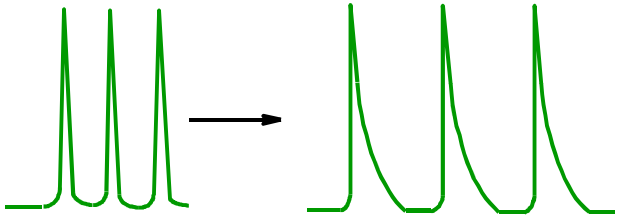
Tip: Similar Effect Can be Caused by Partially Plugged Frit

Peak Shape: Tailing Peaks

Symmetry > 1.2



Normal Tailing



Normal Tailing

Causes

Some Peaks Tail

- Secondary - Retention Effects.
- Residual Silanol Interactions.
- Small Peak Eluting on Tail of Larger Peak.

All Peaks Tail

- Extra-Column Effects.
- Build up of Contamination on Column Inlet.
- Heavy Metals.
- Bad Column.

Peak Tailing - Column Contamination

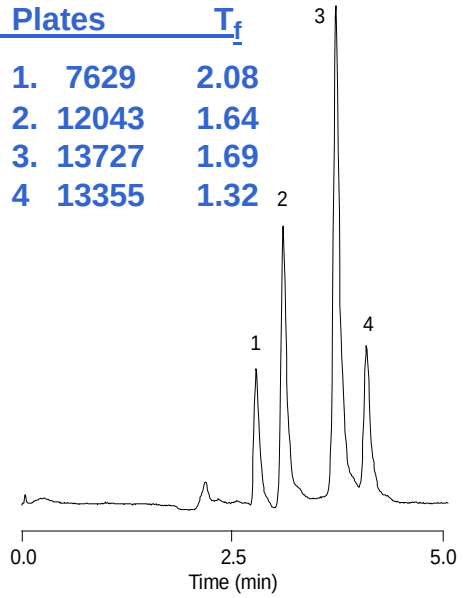
Tip: Quick test to determine if column is dirty or damaged

Trick: Reverse column and run sample

- If improved; Possible cleaning will help
- No improvement; Column damaged and needs to be replaced

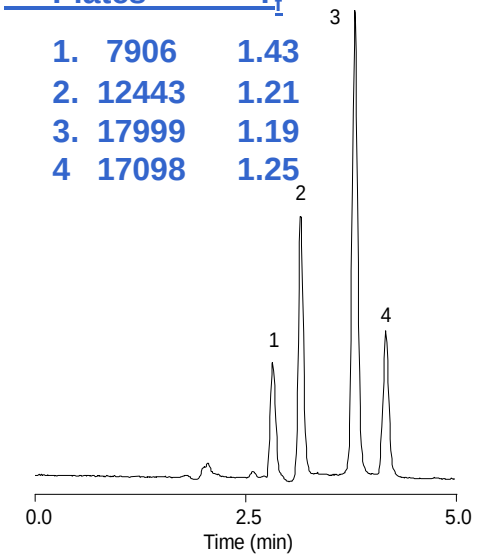
QC test forward direction

	Plates	T_f
1.	7629	2.08
2.	12043	1.64
3.	13727	1.69
4	13355	1.32



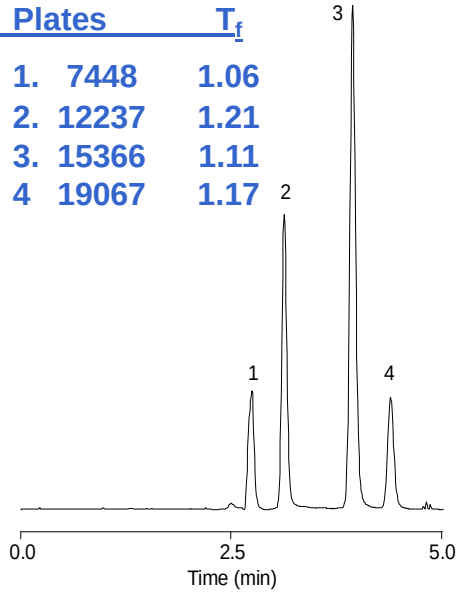
QC test reverse direction

	Plates	T_f
1.	7906	1.43
2.	12443	1.21
3.	17999	1.19
4	17098	1.25



QC test after cleaning 100% IPA, 35℃

	Plates	T_f
1.	7448	1.06
2.	12237	1.21
3.	15366	1.11
4	19067	1.17



Column: StableBond SB-C8, 4.6 x 250 mm, 5µm Mobile Phase: 20% H_2O : 80% MeOH Flow Rate: 1.0 mL/min
Temperature: R.T. Detection: UV 254 nm Sample: 1. Uracil 2. Phenol 3. 4-Chloronitrobenzene 4. Toluene

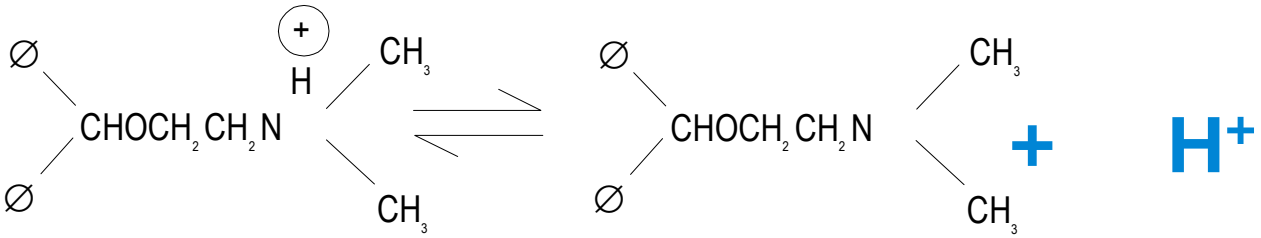
Why Worry About pH?
pH, pKa and Weak Bases

$$R_3NH^+ \rightleftharpoons R_3N + H^+$$

$$K_a = \frac{[R_3N][H^+]}{[R_3NH^+]}$$

$K_a = 1 \times 10^{-9}$

$pK_a = 9$

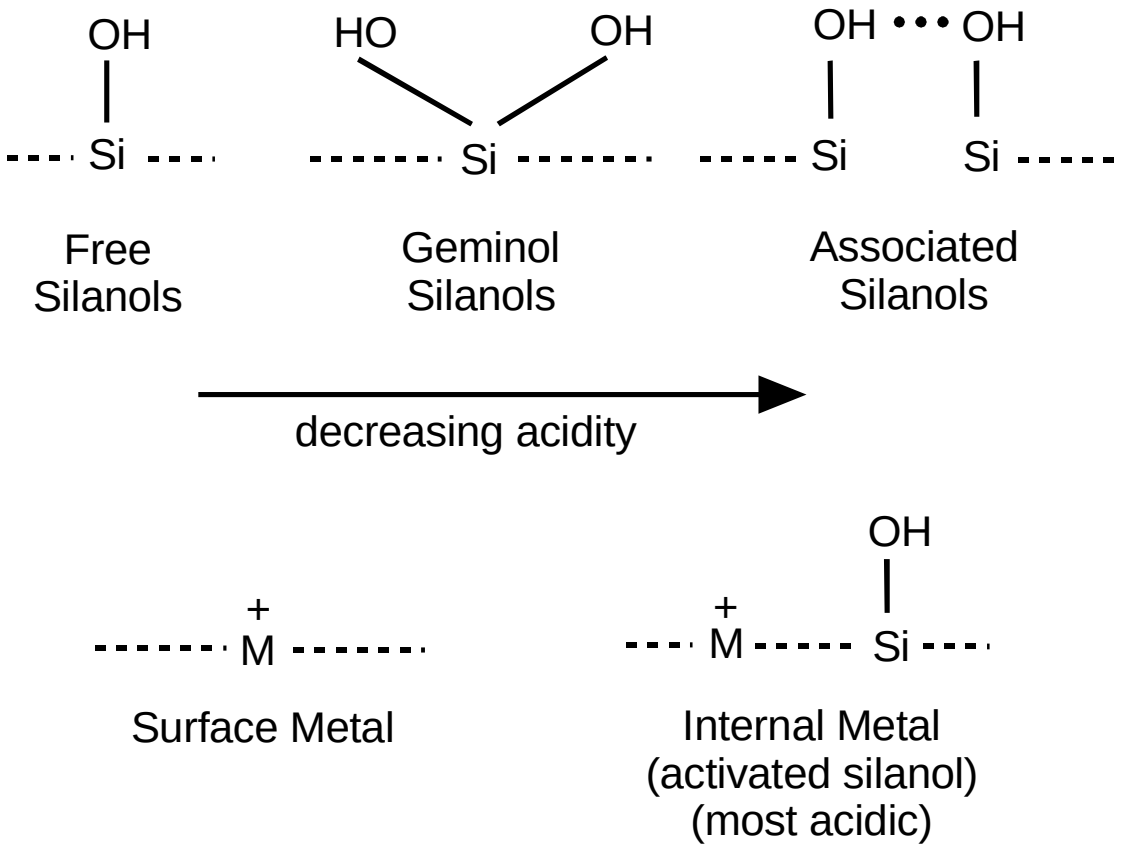


At pH 9 – the sample exists as protonated and unprotonated diphenhydramine in a ratio of 1:1. Peak shape can be poor.

At pH 10 – 91% of the sample exists as unprotonated diphenhydramine.

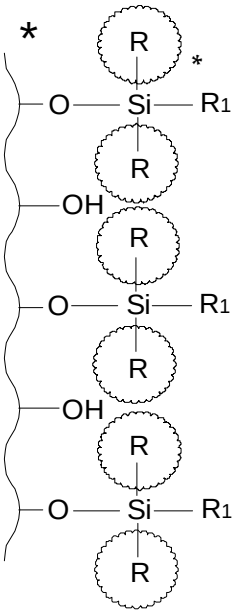
At pH 8 – 91% of the sample exists as protonated diphenhydramine.

The Surface of Silica Supports for HPLC

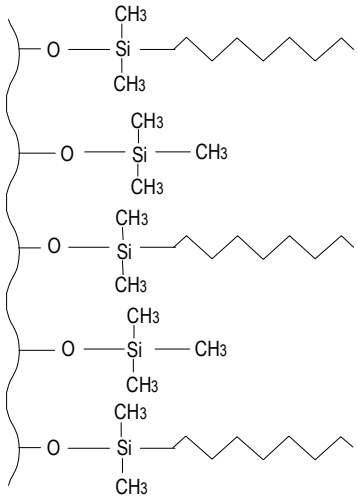


Choose the Best Bonded-Phase for Each pH Range

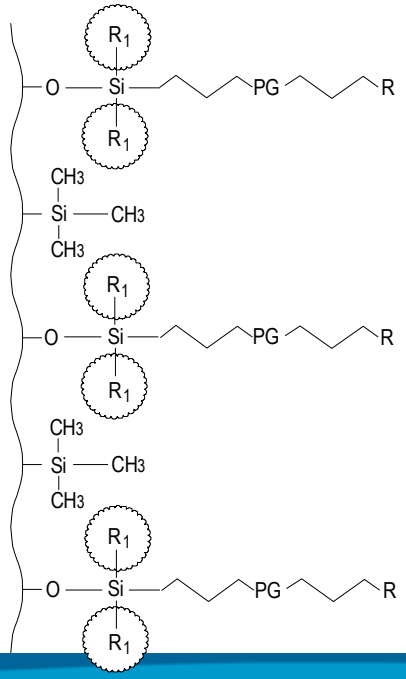
StableBond, pH 1-6
Use at Low pH
1. Uses bulky silanes
2. Non-endcapped



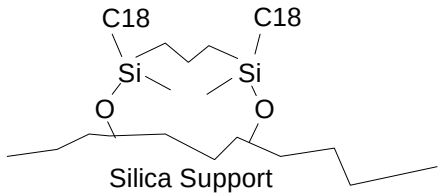
Eclipse Plus, Eclipse XDB, pH 2-9
Low and Mid pH
1. Densely Bonded dimethylalkylsilanes
2. proprietary double-endcapping



Bonus-RP, pH 2-8
Use at Low and Mid pH
1. polar alkyl phase
2. triple endcapped
3. uses bulky silanes



Extend-C18, pH 2-11.5
Use at High pH
1. unique bidentate structure
2. double endcapped

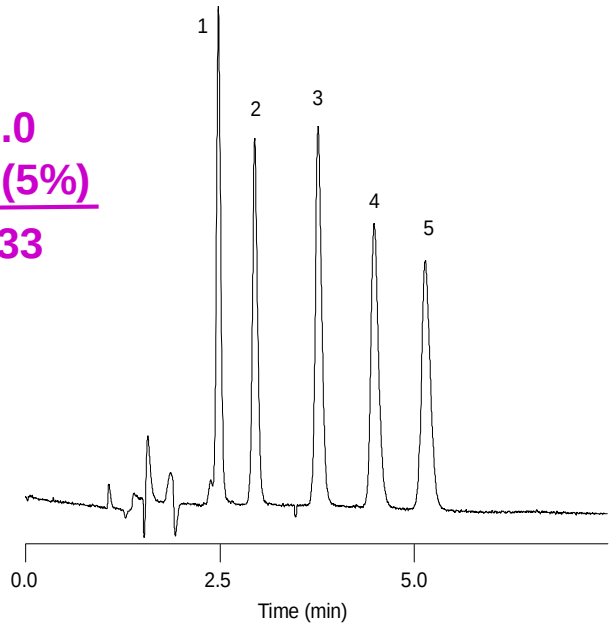


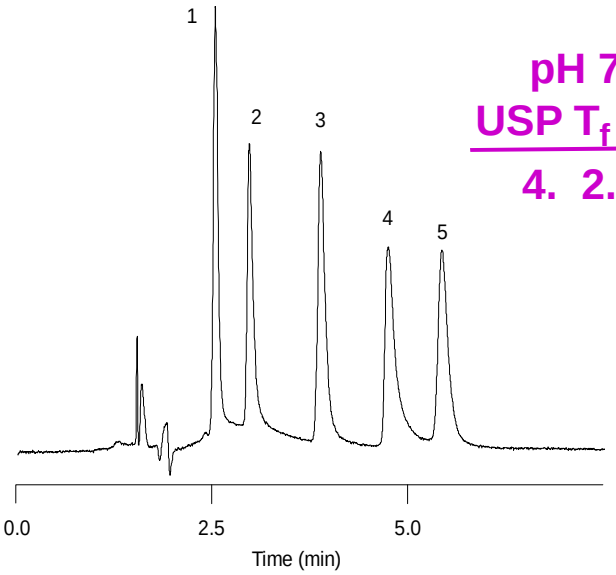
Peak Tailing

Low pH Minimizes “Secondary Interactions” for Amines

Column: Alkyl-C8, 4.6 x 150 mm, 5µm Mobile Phase: 85% 25 mM Na_2HPO_4 : 15% ACN Flow Rate: 1.0 mL/min
Temperature: 35℃ Sample: 1. Phenylpropanolamine 2. Ephedrine 3. Amphetamine 4. Methamphetamine 5. Phenteramine

pH 3.0
USP T_f (5%)
4. 1.33





pH 7.0
USP T_f (5%)
4. 2.35

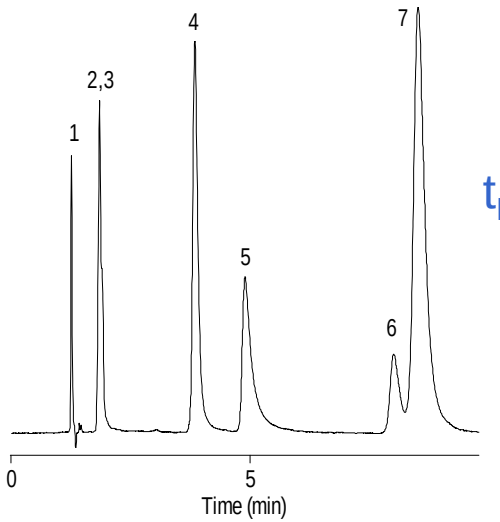
Tip: Reducing mobile phase pH reduces silanol interaction and peak tailing.

Peak Tailing

High pH Eliminates “Secondary Interactions” for Amines

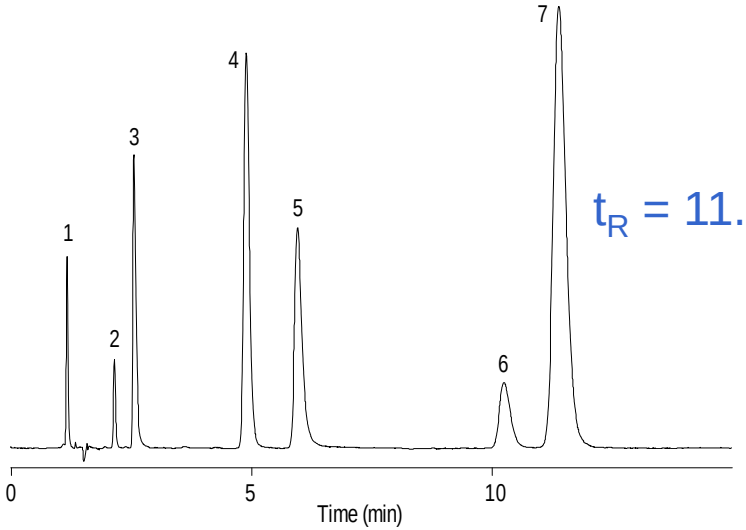
Column: ZORBAX Extend-C18, 4.6 x 150 mm, 5 m m
Mobile Phase: See Below , Flow Rate: 1.0 mL/min , Temperature: RT, Detection: UV 254 nm
Sample 1. Maleate 2. Scopolamine 3. Pseudoephedrine 4. Doxylamine 5. Chlorpheniramine 6. Triprolidine 7. Diphenhydramine

pH 7
30% 20 mM Na_2HPO_4
70% MeOH



t_R = 8.5

pH 11
30% 20 mM TEA
70% MeOH



t_R = 11.4

Peak shape and retention of this sample of basic compounds improves at high pH where column has high IEX activity. *Why?*

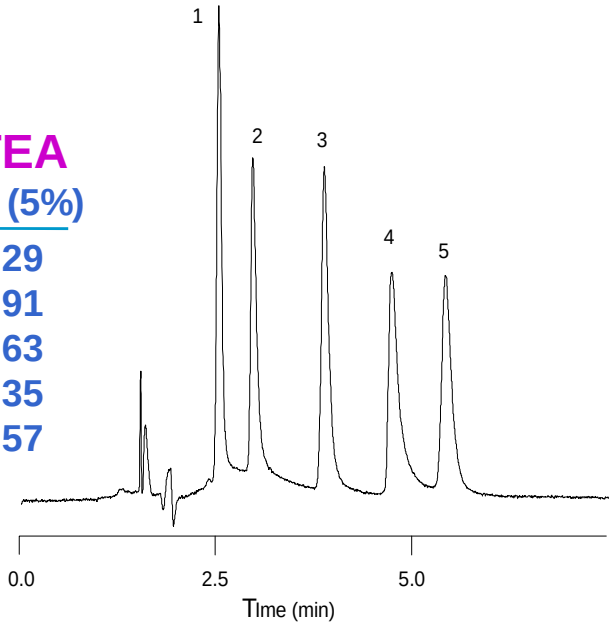
Peak Tailing
Identifying Column “Secondary Interactions”

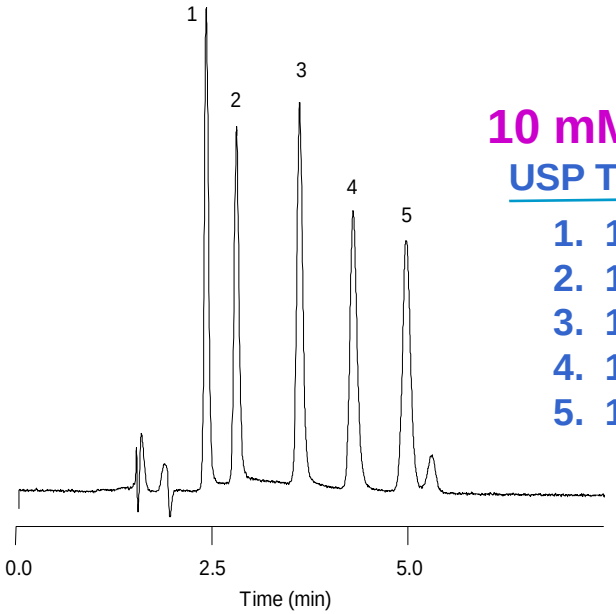
Column: Alkyl-C8, 4.6 x 150 mm, 5µm Mobile Phase: 85% 25 mM Na_2HPO_4 pH 7.0 : 15% ACN Flow Rate: 1.0 mL/min
Temperature: 35℃ Sample: 1. Phenylpropa nolamine 2. Ephedrine 3. Amphetamine 4. Methamphetamine 5. Phenteramine

No TEA

USP T_f (5%)

1. 1.29
2. 1.91
3. 1.63
4. 2.35
5. 1.57





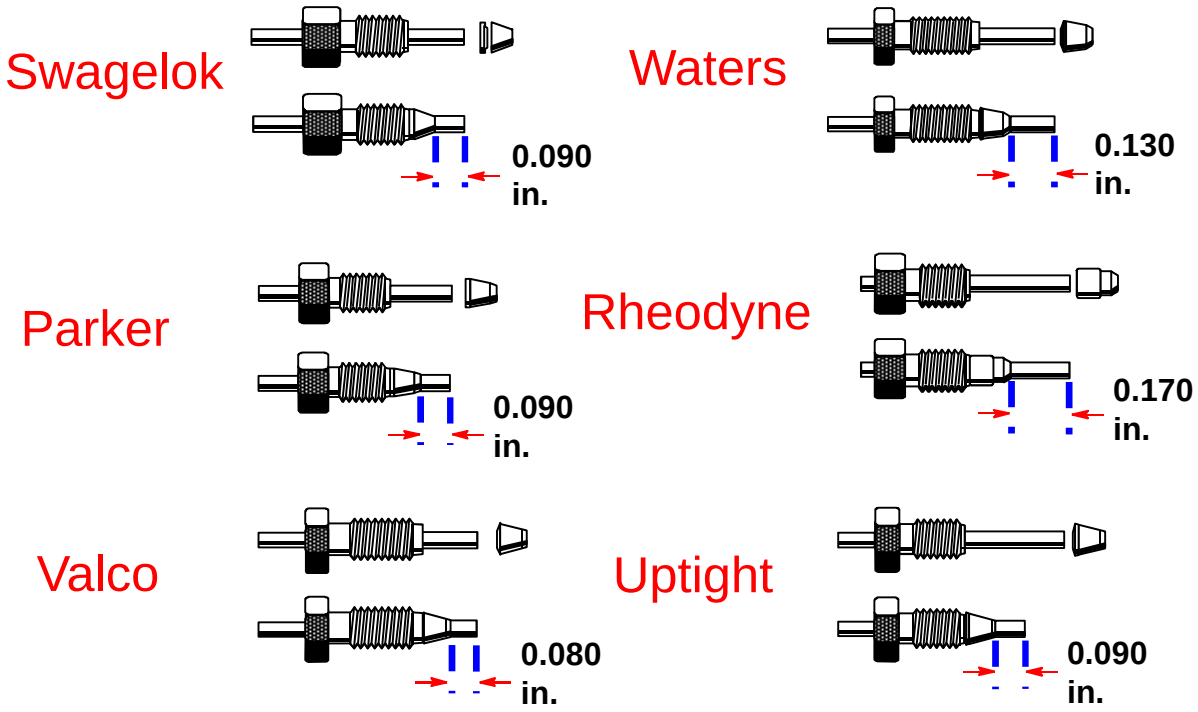
10 mM TEA

USP T_F (5%)

1. 1.19
2. 1.18
3. 1.20
4. 1.26
5. 1.14

Tip: Mobile phase modifier (TEA) competes with sample for surface ion exchange sites at mid-range pH values

Column Connectors Used in HPLC

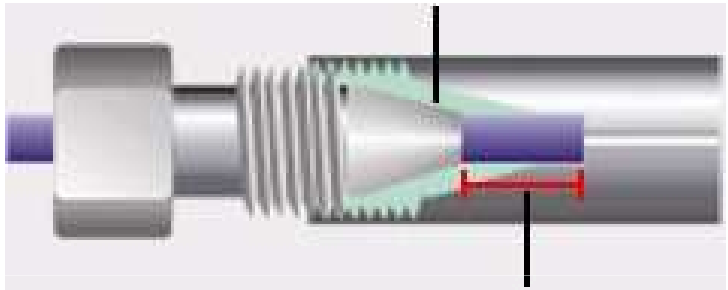


Troubleshooting LC Fittings, Part II. J. W. Dolan and P. Upchurch, LC/GC Magazine 6:788 (1988)

What Happens If Connections Are Poorly Made?

Wrong … too long

Ferrule cannot seat properly



X

If Dimension X is too long, leaks will occur

Wrong … too short

Mixing Chamber

X

If Dimension X is too short, a dead-volume, or mixing chamber, will occur

Topics

- **Chromatographic Process**
  - Separation occurs in column volumes
- **Improving Separations**
  - Selectivity
  - Column efficiency
  - Control pH
- **Troubleshooting**
  - Sample clean-up
  - Secondary interaction

Thank you – Questions?

Bill Champion
800-227-9770, opt 3, opt 3, op2
william_champion@agilent.com

Retention vs. pH for Ionizable Compounds

Effects are Compound Dependent