Reversed Phase Advanced Features

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http://www.forumsci.co.il/HPLC

Reversed Phase HPLC

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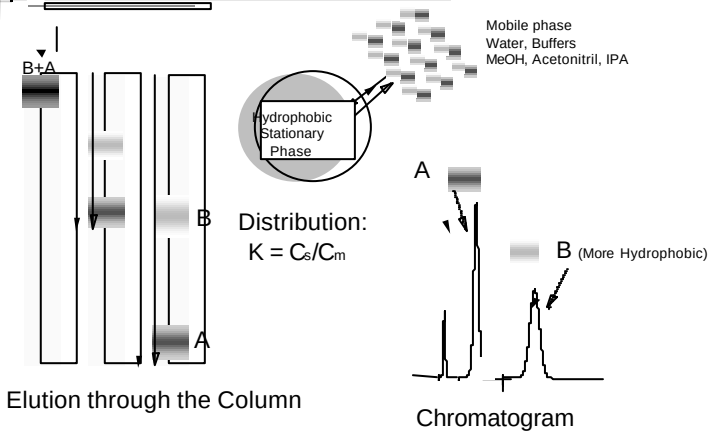
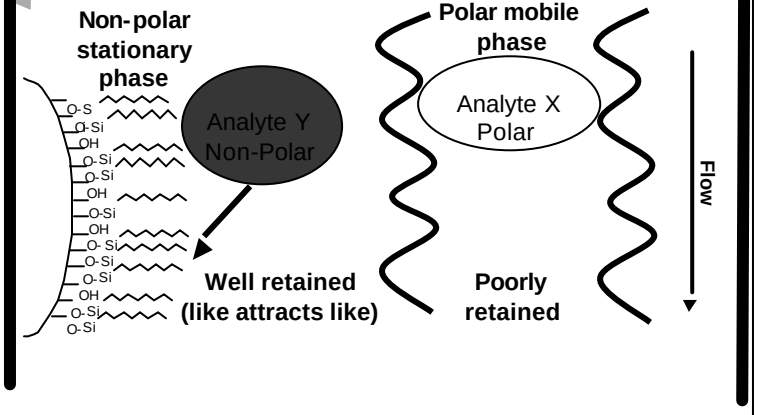
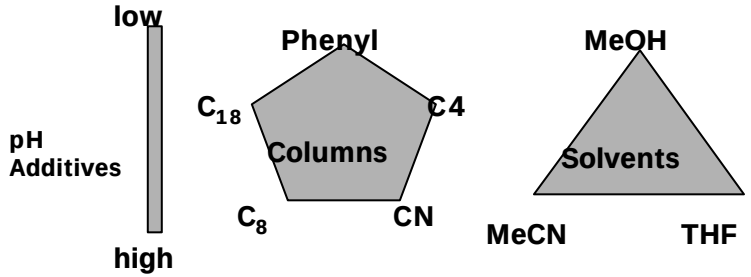
Chromatographic Process



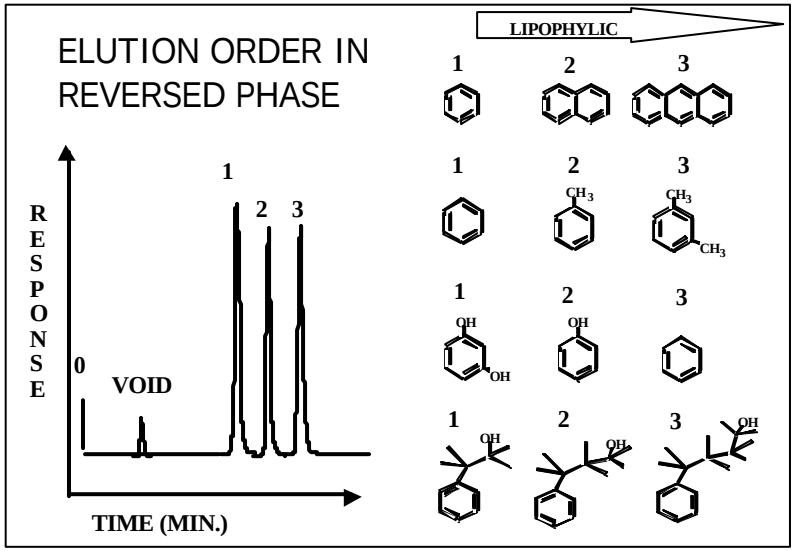
Reversed-Phase Chromatography

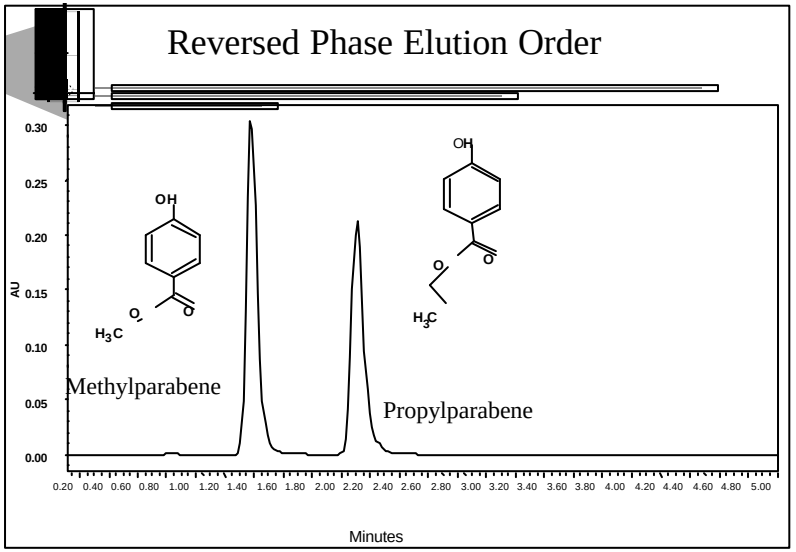


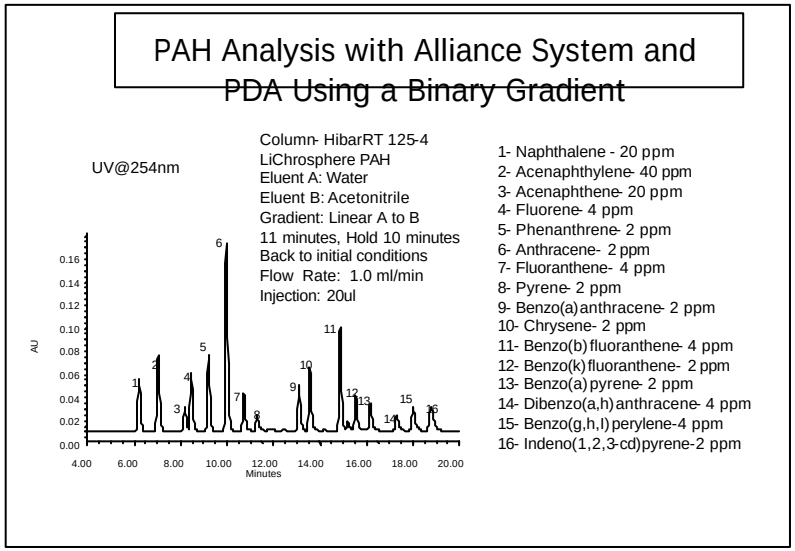
RP Method Development Tools

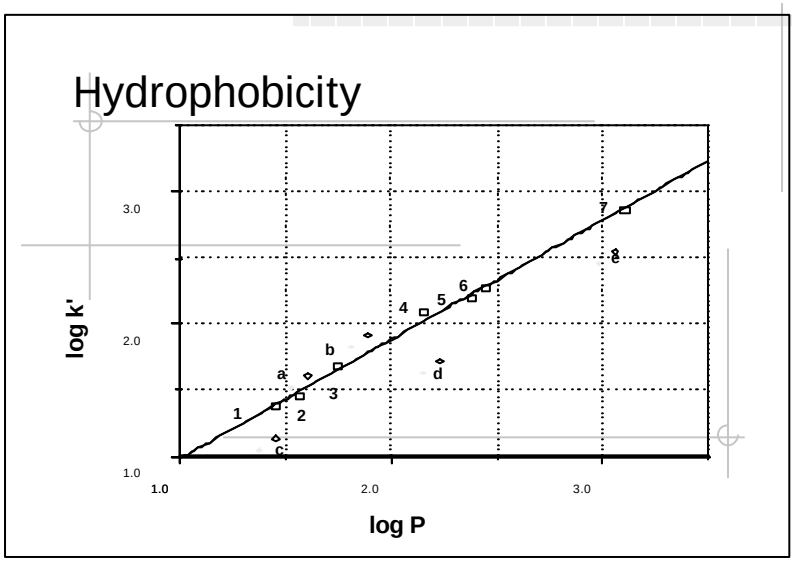


Reversed Phase HPLC



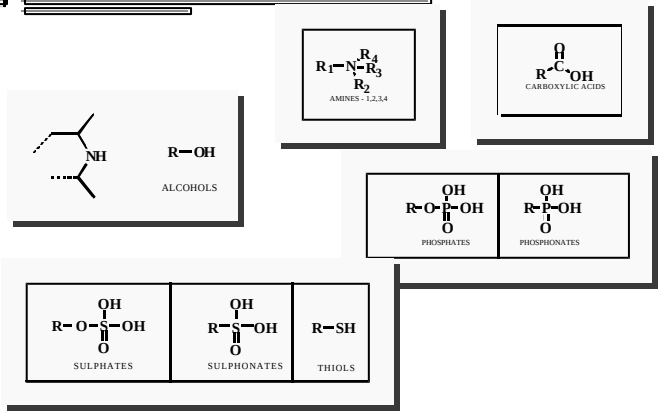






Reversed Phase HPLC

IONIZABLE



MOBILE PHASE

- **TYPE OF MODIFIER (MeOH, ACN)**
- **SOLVENT STRENGTH (% modifier)**
- **pH**
- **TYPE OF BUFFER (phosphate, acetate)**
- **IONIC STRENGTH (Salts, buffer concentration)**
- **ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)**

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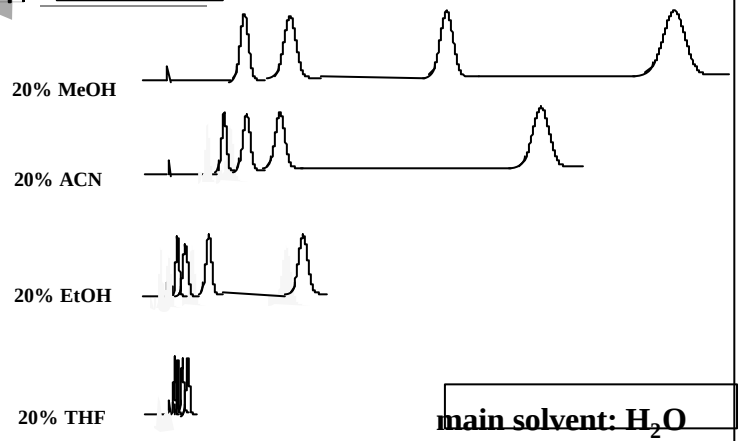
OPTIMIZATION: CHOICE OF SOLVENTS



main solvent: H_2O

Reversed Phase HPLC

MOBILE PHASE

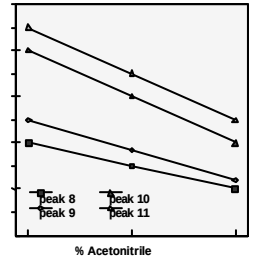
- TYPE OF MODIFIER (MeOH, ACN)
- **SOLVENT STRENGTH (% modifier)**
- pH
- TYPE OF BUFFER (phosphate, acetate)
- IONIC STRENGTH (Salts, buffer concentration)
- ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)

SOLVENT STRENGTH

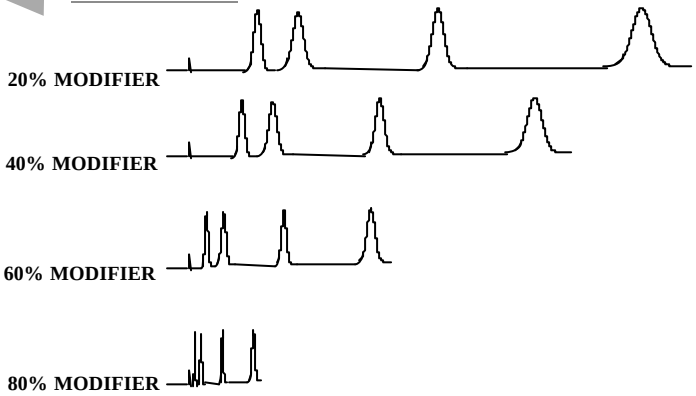
Analyte Retention as a Function of % Modifier

k (retention) for each analyte changes independently as % Modifier changes.

Thus, the resolution between peaks changes.



OPTIMIZATION: % SOLVENTS



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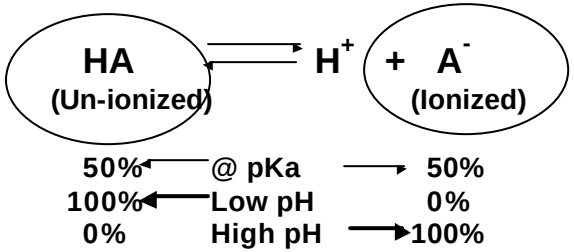
- SOLVENT STRENGTH (% modifier)
- **pH**
- TYPE OF BUFFER (phosphate, acetate)
- IONIC STRENGTH (Salts, buffer concentration)
- ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)

Reversed Phase HPLC

Ionization of Acids and Bases

Dissociation of Molecule

Acid

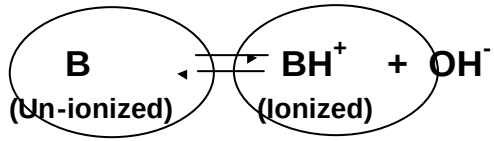


50%	@ pKa	50%
100%	Low pH	0%
0%	High pH	100%

Ionization of Acids and Bases

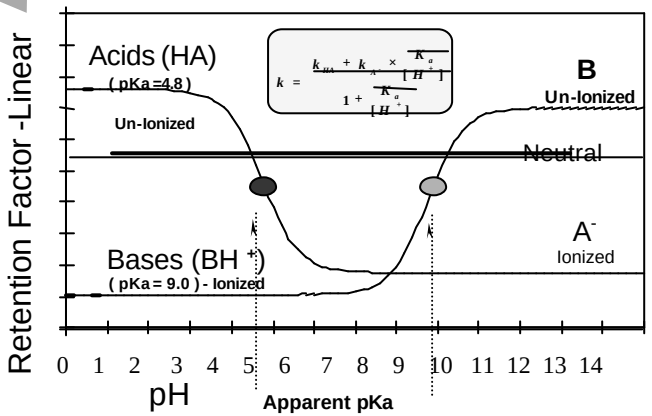
Dissociation of Molecule

Base

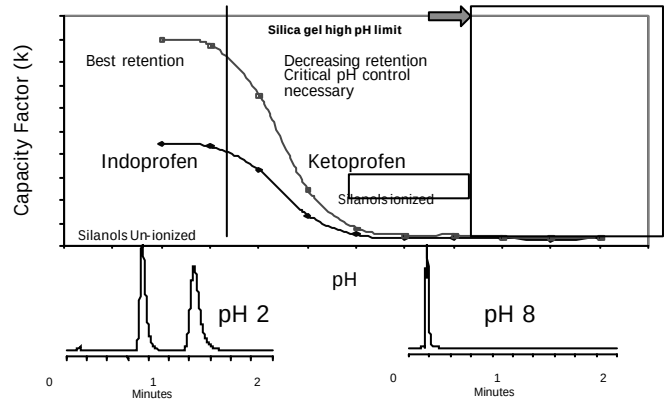


50%	@ pKa	50%
0%	Low pH	100%
100%	High pH	0%

Retention Factor versus pH for Acids, Bases and Neutrals

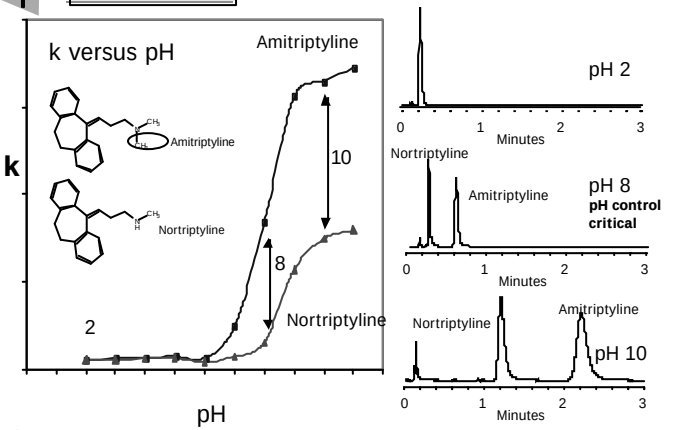


Resolution of Two Acidic Compounds at Different Mobile Phase pH's



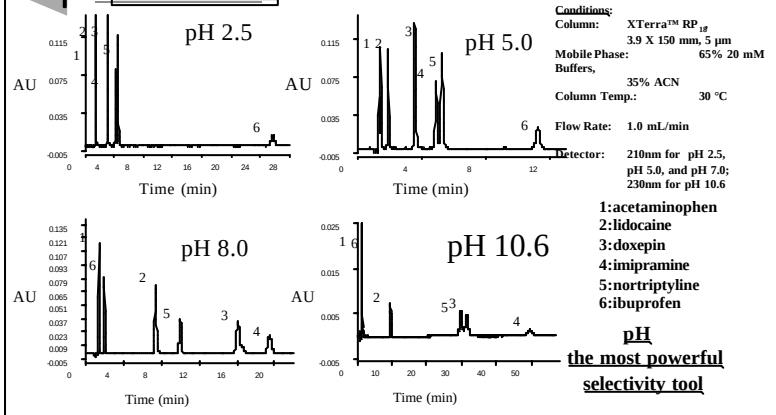
Reversed Phase HPLC

Enhanced Resolution of Basic Compounds at High pH



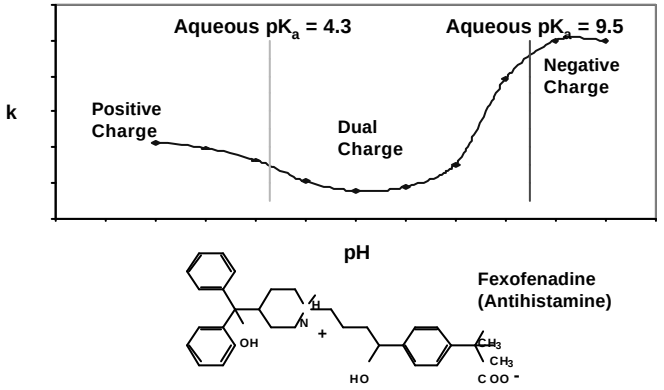
Phoebe, Tran

Dependence of Selectivity on pH

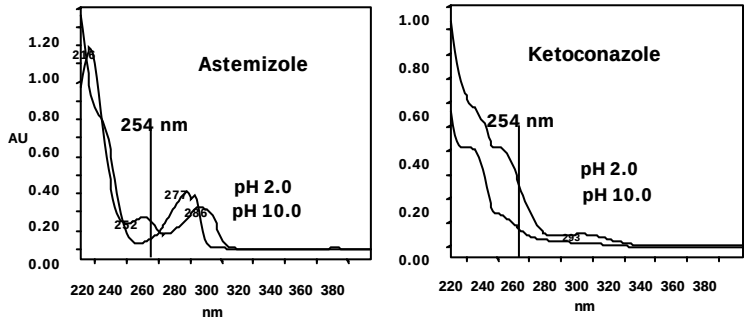


Lu, Cheng; Waters

Impact of pH on the Retention of a Zwitterionic Compound



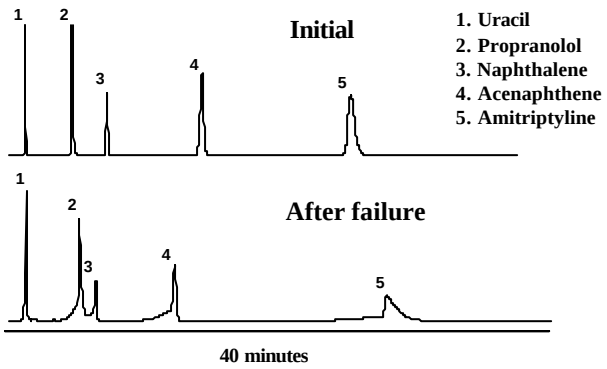
UV/Vis Spectral Change Between Ionized and Non-ionized Forms



Phoebe, Tran

Reversed Phase HPLC

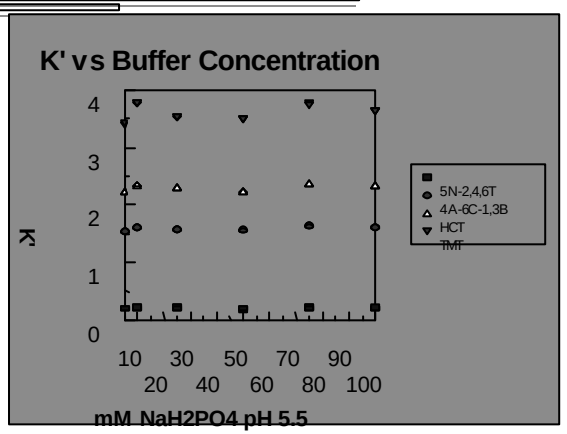
Typical Chromatograms for pH Failure of an Ordinary C_{18}-Silica Column



MOBILE PHASE

* TYPE OF MODIFIER (MeOH, ACN)
* SOLVENT STRENGTH (% modifier)
* pH
* TYPE OF BUFFER (phosphate, acetate)
* IONIC STRENGTH (Salts, buffer concentration)
* ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)

k' Versus Temperature and Buffer Concentration



Recommended Buffers for pH's 2-7

Additive or Buffer	pK_a	pH range (± 1 pH unit)	Volatile or Non-Volatile	Recommended for use with Extended pH Packings
TFA	0.3		Volatile	Yes (0.02-0.1%)
Acetic Acid	4.76		Volatile	Yes (0.1-1.0%)
Formic Acid	3.75		Volatile	Yes (0.1-1.0%)
Acetate	4.76	3.76 – 5.76	Volatile/Non-volatile	Yes (1-10mM) NH_4, Na, K
Formate	3.75	2.75 – 4.75	Volatile/Non-volatile	Yes (1-10mM) NH_4, Na, K
Phosphate	2.15	1.15 – 3.15	Non-volatile	Yes
	7.20	6.20 – 8.20	Non-volatile	No for pH's >7.0 (lower the temperature the longer the column lifetime)

Types of Buffers and Ionic Strength

- **pH 10: Borate**
  - **20 mM H_3BO_3**
- **pH 7: Phosphate**
  - **20 mM K_2HPO_4**
- **pH 4-5: Acetate**
  - **10 mM CH_3COONH_4**
  - **100 mM CH_3COOH**
- **pH 2-3.5: Phosphate**
  - **20 mM H_3PO_4 - KH_2PO_4**

MOBILE PHASE

* **TYPE OF MODIFIER (MeOH, ACN)**
* **SOLVENT STRENGTH (% modifier)**
* **pH**
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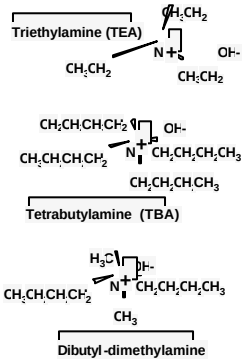
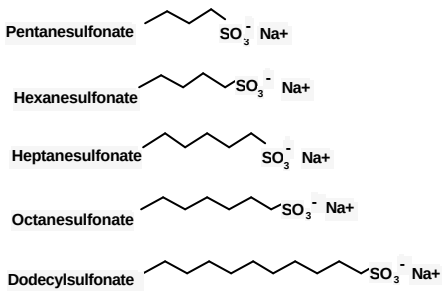
Ion Pair Reagent

Alkylamines



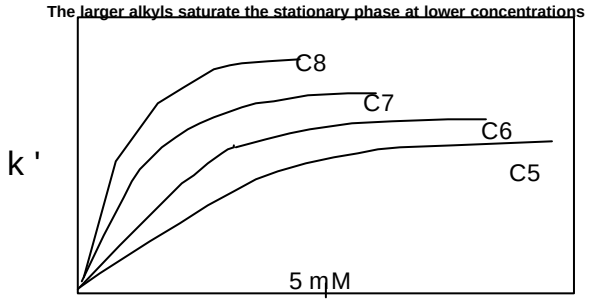
Alkylsulfonates



Concentration of Ion-Pair Reagent in the Mobile Phase

The larger the alkyl, the longer are retention times

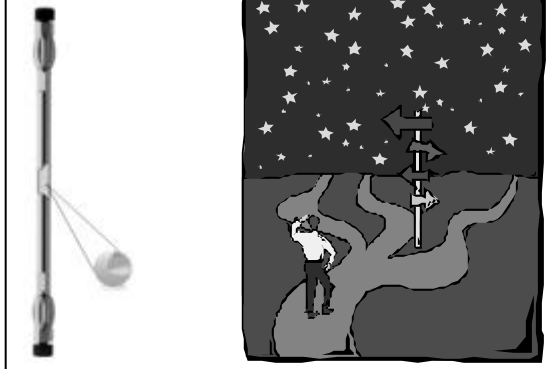
The larger alkyls saturate the stationary phase at lower concentrations



Conc. of Ion Pair Reagent in the Mobile Phase

Reversed Phase HPLC

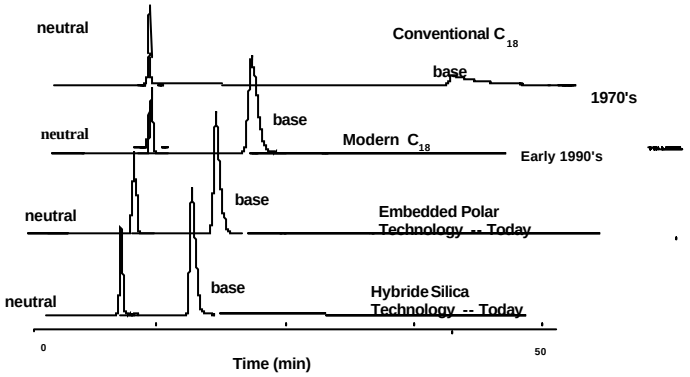
Stationary Phase Characterization



The Evolution of the Silica Gel Particle Platform

- 1960's Pellicular native silica
- 1970's Irregular 10 µm native silica
- 1980's Spherical 5 µm native silica
- 1990's Spherical 3-5 µm high purity silica
- 2000's Hybride Silica-Gel (co-polymer organic/Inorganic) high purity silica

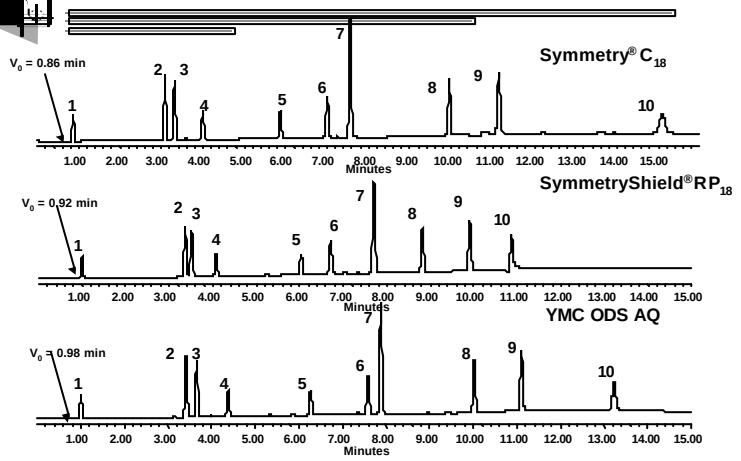
Improvement in Peak Shape for Bases



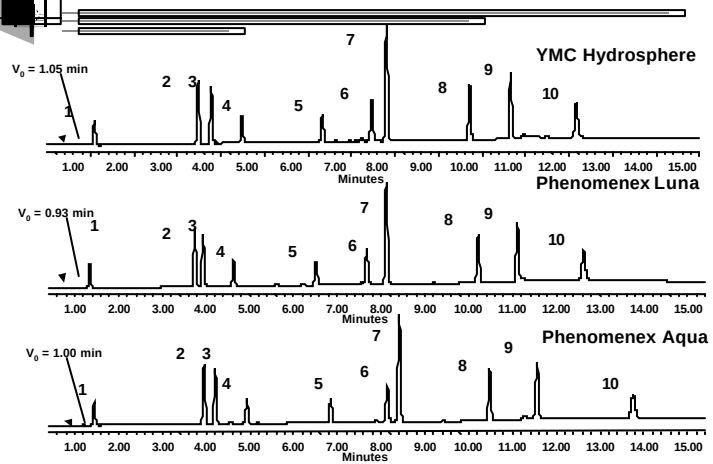
Not all C18's are the same!

Reversed Phase HPLC

Different Columns – Different Chromatograms



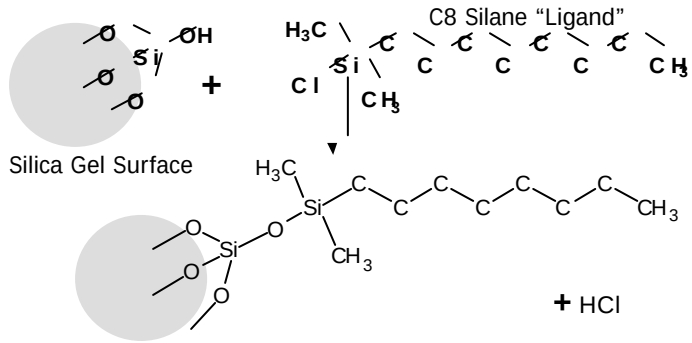
Different Columns – Different Chromatograms



"Relative" Ranking of C18 Columns Using a Standardized Test

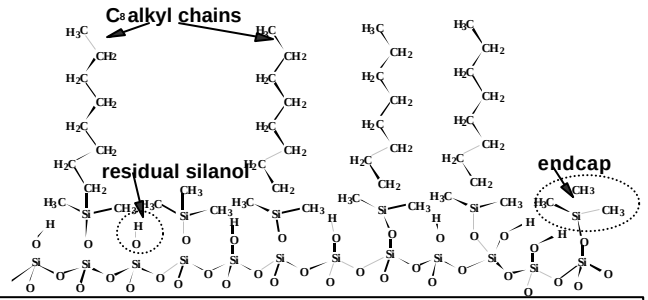
- There are no bad C18 columns.
- There are only different C18 columns.

Making a Bonded Phase Material: Monofunctional Synthesis



Reversed Phase HPLC

Surface of a Silica Gel Bonded-Phase Packing Material

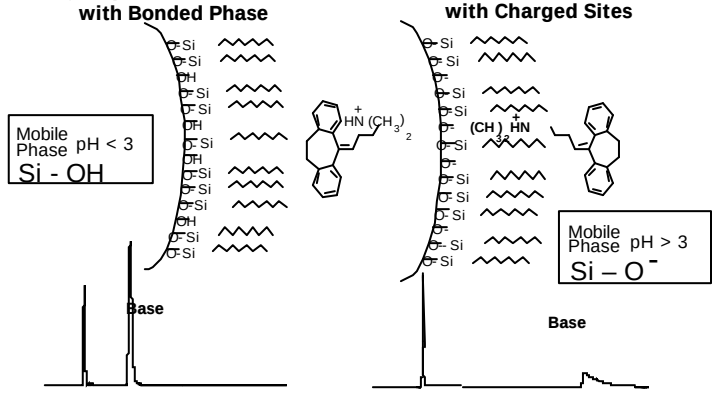


Note: ~50% of the surface silanols remain even with high bonding densities

Mixed-Mode Retention:

Hydrophobic Interaction with Bonded Phase

Ion exchange Interaction with Charged Sites



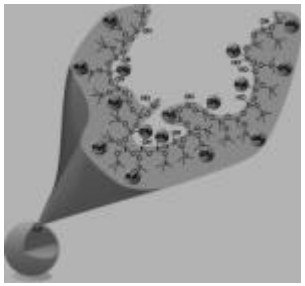
Stationary Phase Properties

CHEMISTRY:

* **BONDED HYDROCARBON: C-18, C-8, C-4, C-1, CN, phenyl**
* **% COVERAGE**
* **TYPE OF SILICA GEL**

GEOMETRY

* **SPHERE- IRREGULAR**
* **PARTICLE DIAMETER**
* **POROSITY**



Stationary Phase Ligands

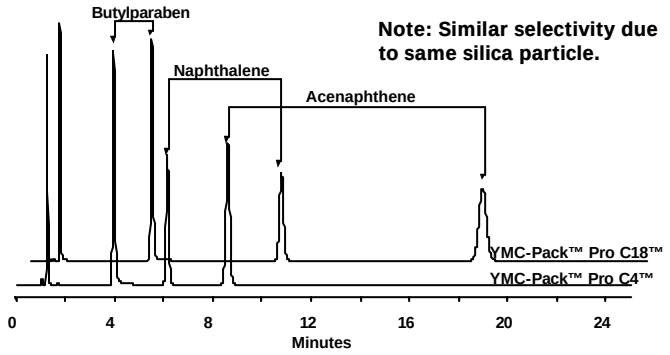
Stationary phase	Functionality
C_{18}	$-Si(CH_3)_2C_{18}H_{37}$
C_8	$-Si(CH_3)_2C_8H_{17}$
tC_2	$-SiC_2H_5$
Aminopropyl	$-Si(CH_2)_3NH_2$
Cyanopropyl	$-Si(CH_3)_2(CH_2)_3CN$
Diol	$-Si(CH_2)_3OCH_2CH(OH)CH_2OH$

Retention time

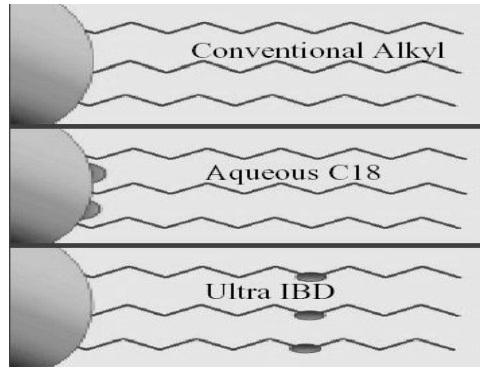
Chain length CN Phenyl NH2 C4 C8 C18

Reversed Phase HPLC

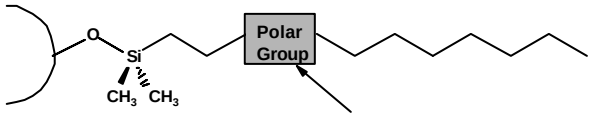
Neutral Compounds: C18 versus C4 (Same Brand - Different Ligands)



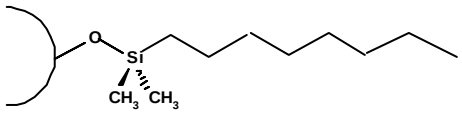
Type of Ligands



Reversed-Phase Packing with an Embedded Polar Ligand

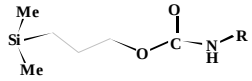


Embedded Polar Group Ligand

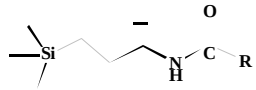


Traditional, Straight Chain Alkyl Ligand

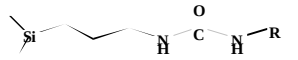
Commercial Phases with Embedded Polar Group



SymmetryShield RP (Waters)

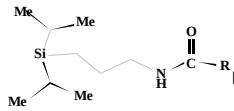


Discovery RP Amide16 (Supelco)



Prism, Spectrum (Keystone)

- reduces interaction with silanols
- modifies selectivity
- improves water wettability

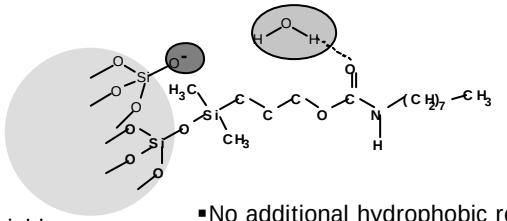


Bonus RP (Hewlett Packard)

Reversed Phase HPLC

Embedded Polar Ligand: Possible Mechanism

Polar group increases water concentration in surface layer

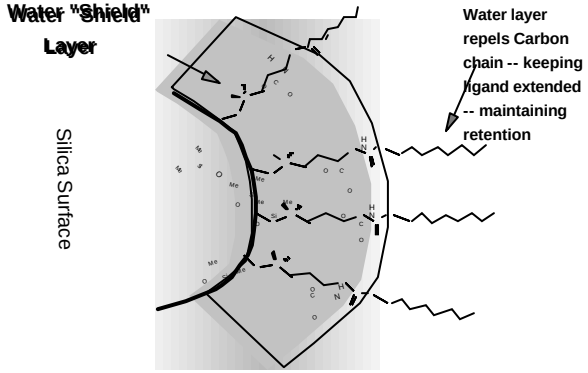


Shields Negative Silanols →

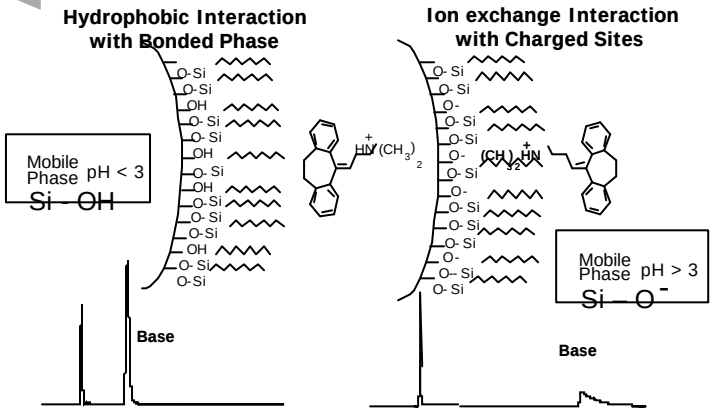
- No additional hydrophobic retention
- Reduced retention of bases
- Reduced peak tailing

Embedded Polar Goups

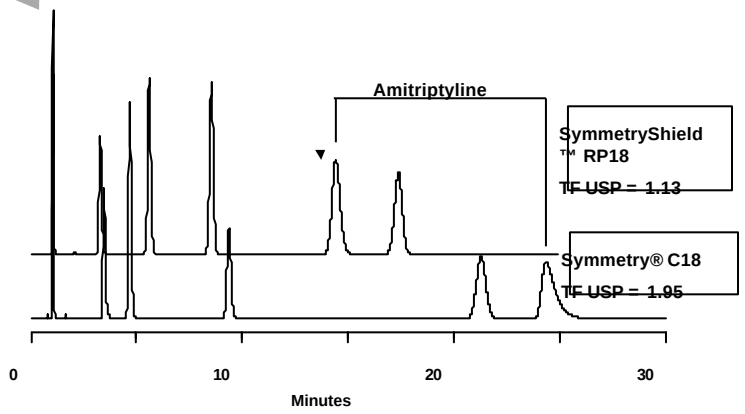
Embedded Polar Wetted Surface



Mixed-Mode Retention:

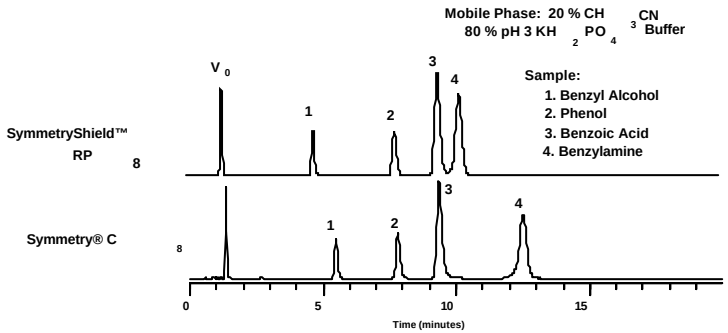


Embedded Polar Ligand versus Linear Alkyl Ligand on Silica Gel



Reversed Phase HPLC

Impact on Selectivity - Retention

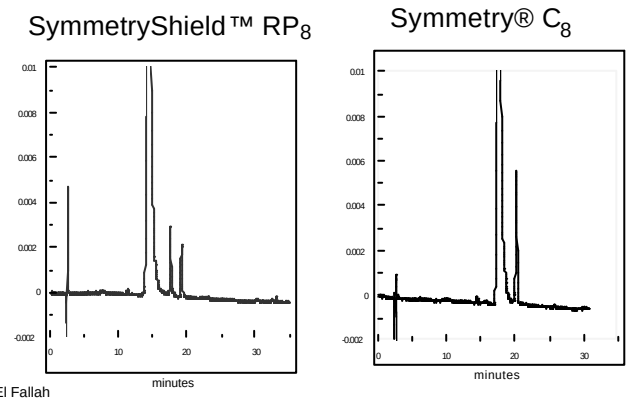


Reduced Retention of Amines

(Shielding of Silanols)

B. A. Alden

Selectivity Difference: Furazolidone Impurities



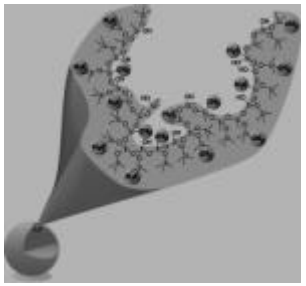
El Fallah

Stationary Phase Properties

CHEMISTRY:

*** BONDED HYDROCARBON:**
C-18, C-8, C-4, C-1, CN, phenyl
*** % COVERAGE**
*** TYPE OF SILICA GEL**

GEOMETRY
*** SPHERE- IRREGULAR**
*** PARTICLE DIAMETER**
*** POROSITY**



CARBON LOAD

Increasing carbon load on a particles increases retention. **similar geometrical shaped**

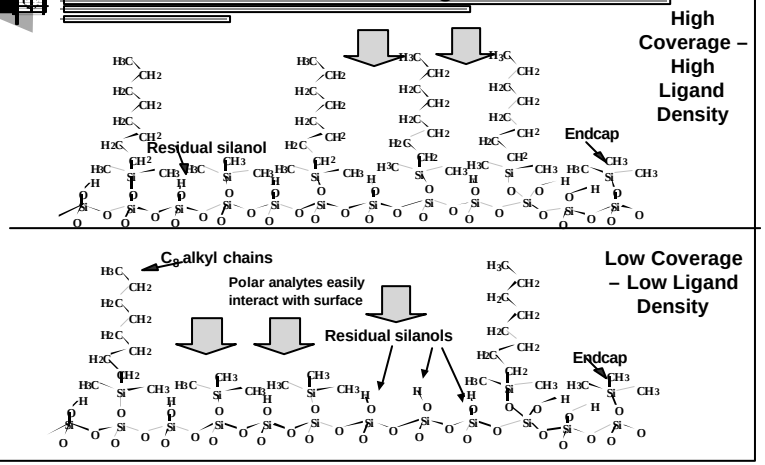
Retention time

Carbon load 5% 7% 9% 12% 15% 17%

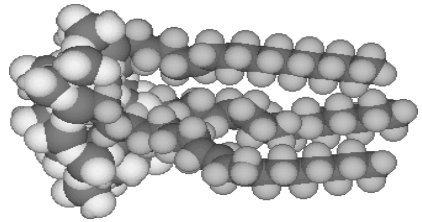
Dr. Shulamit Levin, Medtechnica

Reversed Phase HPLC

Surface of a Silica Gel Bonded-Phase Packing Material

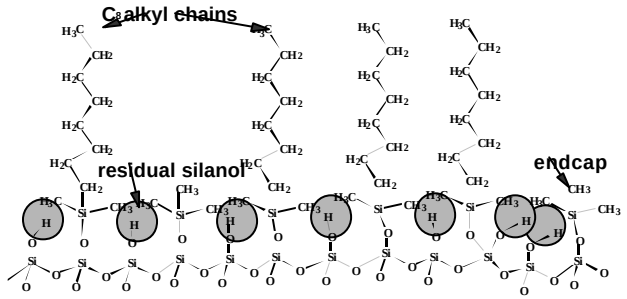


Silica based "bonded phases"



Bulky alkylsilane ligands can not react with all available silanols due to the steric hindrance.

Surface of a Silica Gel Bonded-Phase Packing Material



Note: ~50% of the surface silanols remain even with high bonding densities

Ligand Density (Surface Coverage)

Ligand Density (Surface Coverage)

		μmoles/m²
Silica Silanols :		6 - 8
Highest Bonding Reported	:	4.2
Residual Silanols (Best Case)	:	2.0 [~ 30%]
Residual Silanols (Typical)	:	> 3.5 [> 50%]

Reversed Phase HPLC

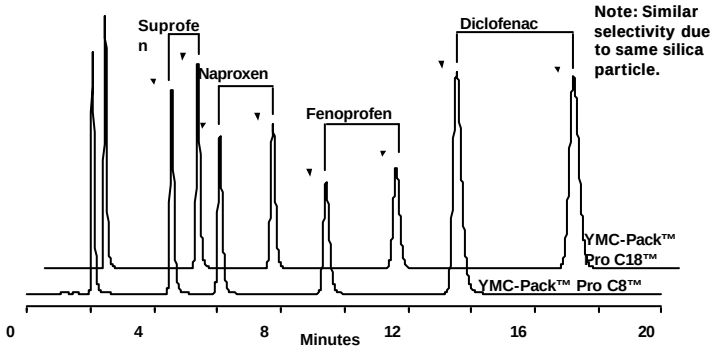
Better Way to Compare: Ligand Density (Surface Coverage)

$$C = \frac{\%C}{100 \bullet SA \bullet nC \bullet 12 \bullet \left[1 - \frac{\%C}{100} \bullet \frac{MW - 1}{nC \bullet 12} \right]} = \text{mmoles/m}^2$$

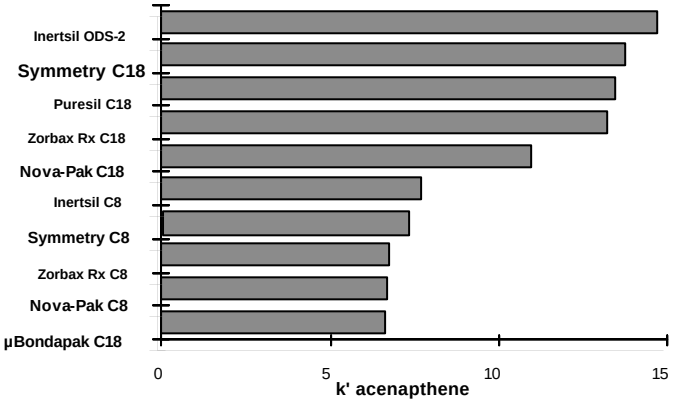
SA - Specific Surface Area
MW - Molecular Weight of Ligand
%C % Carbon Load
nC - # of Carbon Atoms in Ligand

Ligand Density	⇧	Retention	⇧	Silanols ⇩
Surface Area ⇧		% C ⇨		Ligand Density ⇩

Acidic Compounds:C18 versus C8 (Same Brand - Different Ligand)



Relative Hydrophobicities of General Purpose Analytical Packings



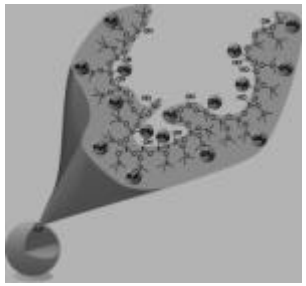
Stationary Phase Properties

CHEMISTRY:

* BONDED HYDROCARBON: C-18, C-8, C-4, C-1, CN, phenyl
* % COVERAGE
* TYPE OF SILICA GEL Native/Synthetic-Pure

GEOMETRY

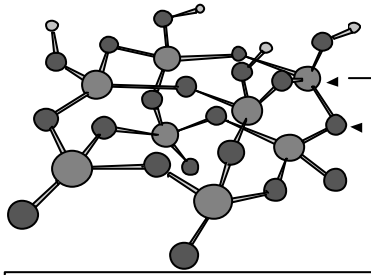
* SPHERE- IRREGULAR
* PARTICLE DIAMETER
* POROSITY



Types of Silica

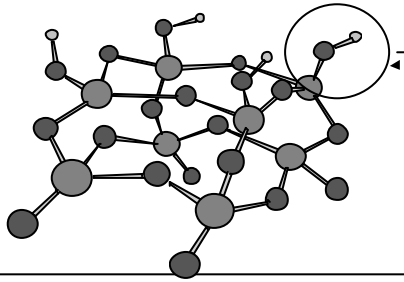
- Silanols
- pH stability
- Metal content
- Temperature stability

Structure of Silica Gel



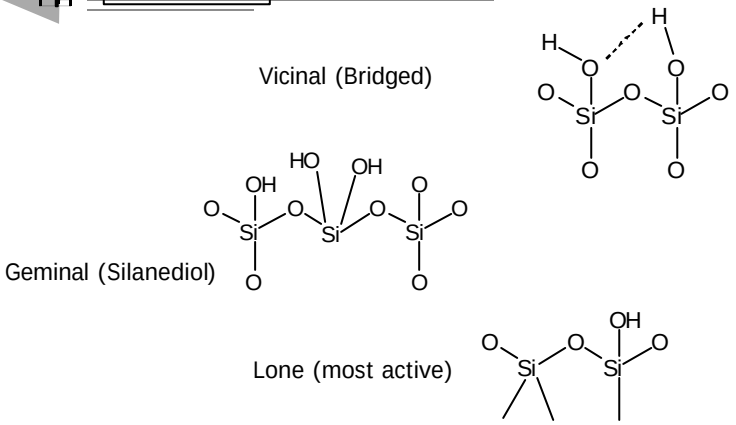
- **Amorphous, porous matrix of silicon atoms joined together with oxygen atoms to form "siloxane bonds" = (Si – O – Si)**

What are Silanols?



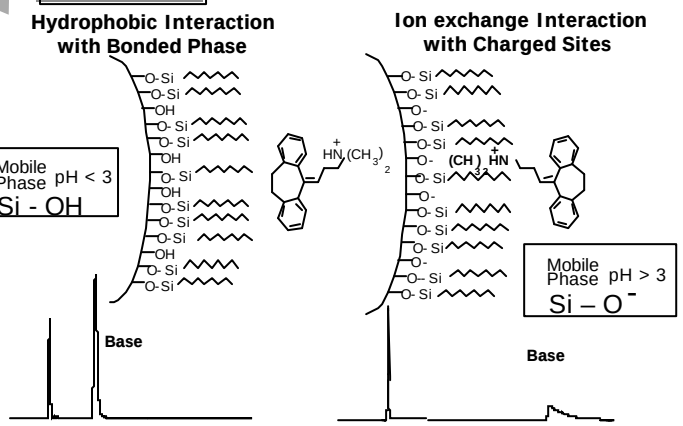
- **Residual unreacted surface hydroxyl groups left over from polymerization**
- **Reactive sites for use in bonding ligands (C18) to the silica gel surface**

Surface Silanols Found on Silica Gel

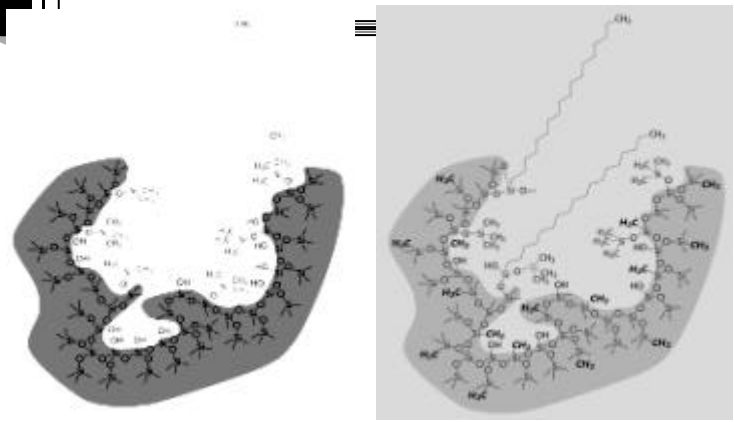


Reversed Phase HPLC

Mixed-Mode Retention:



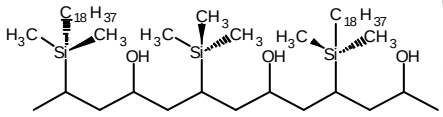
Bonded Phase on Particles



Silica Gel C18 Materials
1/2 free silanols

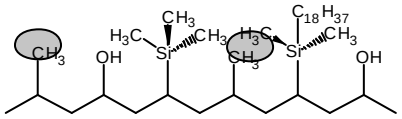
XTerra™ C18 Materials
1/3 free silanols

Bonded Hybrid versus Bonded Silica Gel Surfaces



C_{18}-Bonded Silica Gel

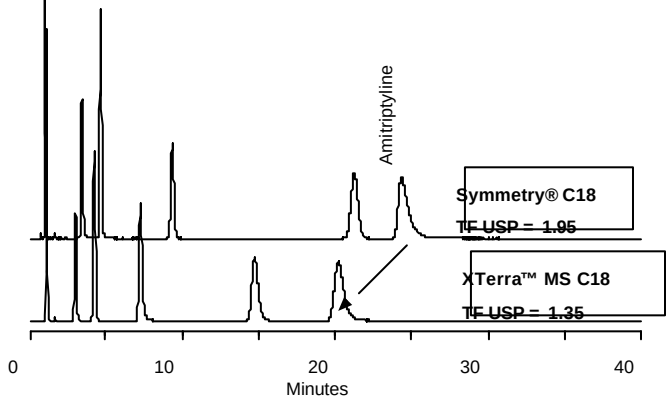
1/2 $-OSi(CH_3)_2R$
1/2 -OH



C_{18}-Bonded Hybrid

1/3 $-OSi(CH_3)_2R$
1/3 $-CH_3$
1/3 -OH

Hybrid versus Silica Gel Particle



Reversed Phase HPLC

PERFORMANCE BY ONE PEAK

ASYMMETRY FACTOR

$$A_f = \frac{B_{(10\%\ h)}}{A_{(10\%\ h)}}$$

RETENTION FACTOR or CAPACITY RATIO

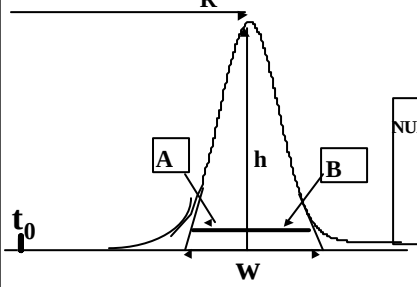
$$k' = \frac{t_R - t_0}{t_0 t_R} \qquad k' = f\,\frac{C_s}{C_m}$$

TAILING FACTOR

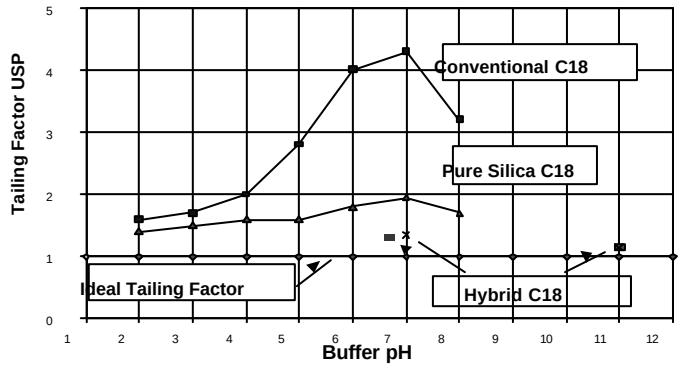
$$T_f = \frac{A + B}{2\,A}$$

NUMBER OF THEORETICAL PLATES

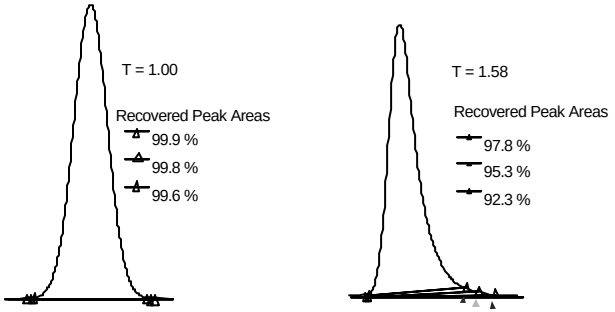
$$N = 16\left(\frac{t_R}{W}\right)^2$$



Amitriptyline Peak Tailing Over Extended pH Range 1-12



Integration Errors Caused by Tailing

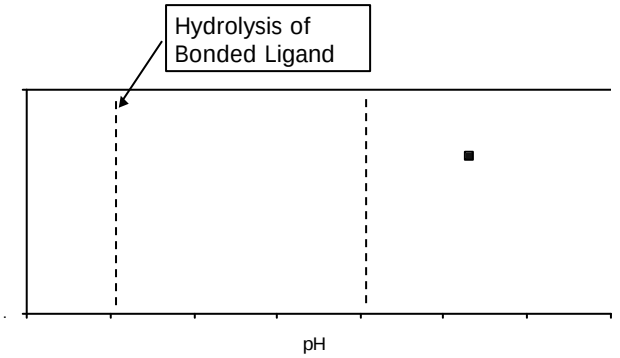


Types of Silica

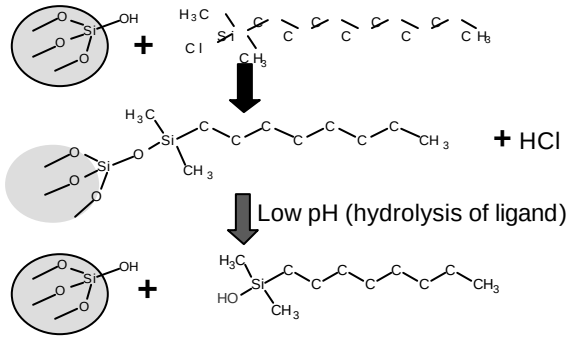
- Silanols
- pH stability
- Metal content
- Temperature stability

Reversed Phase HPLC

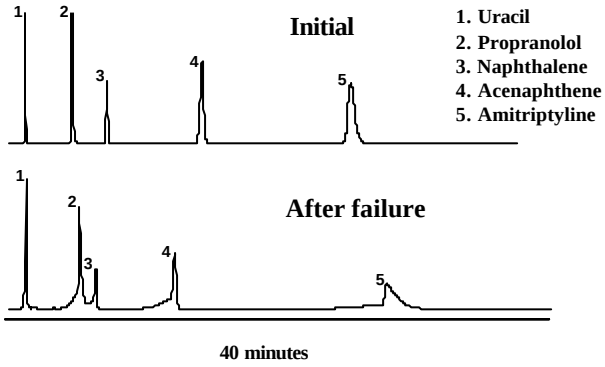
pH Limitations of Silica Based Packing Materials



Hydrolysis of a Bonded Phase Material: Monofunctional Ligand



Typical Chromatograms for Reference C_{18}-Silica Column



Types of Silica

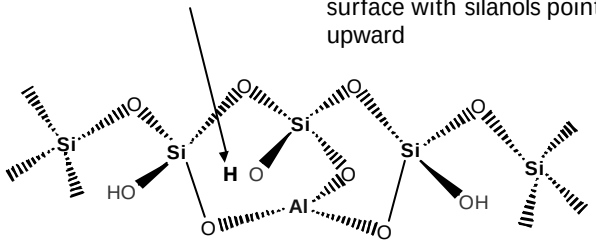
- Silanols
- pH stability
- Metal content
- Temperature stability

Metal Content in Silica

Aluminum in the Silica Gel Lattice

Bronsted Acid

3D top view of silica particle surface with silanols pointing upward

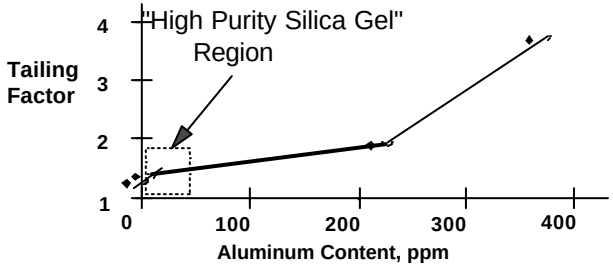


Metal available for chelation

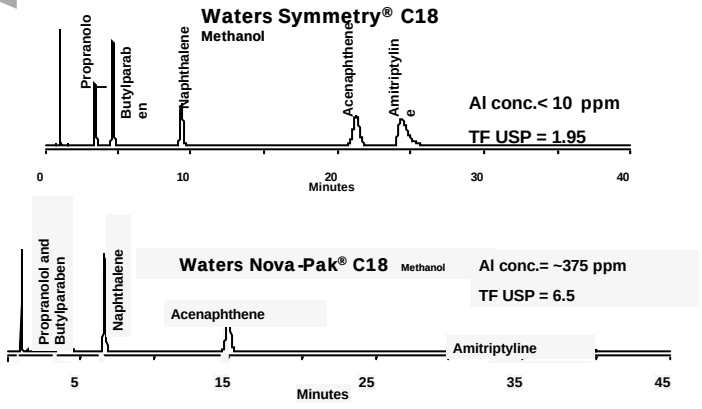
Correlation Between Base Tailing and Aluminum Content of Silica Gel

Analyte: Chlorpheniramine

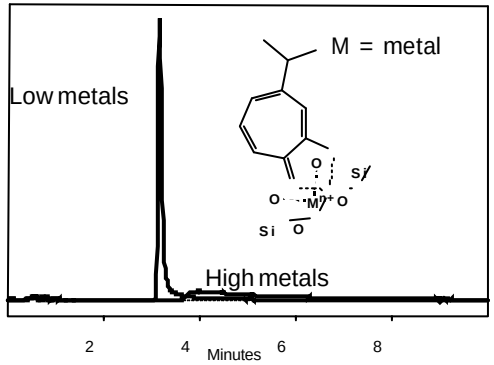
Mobile Phase: Acetonitrile/KH_2PO_4 pH 3.0 (20:80)



Correlation between Metal Content of Silica Gel and Peak Retention and Shape



Peak Shapes of Chelating Agent (Hinokitiol)



Types of Silica

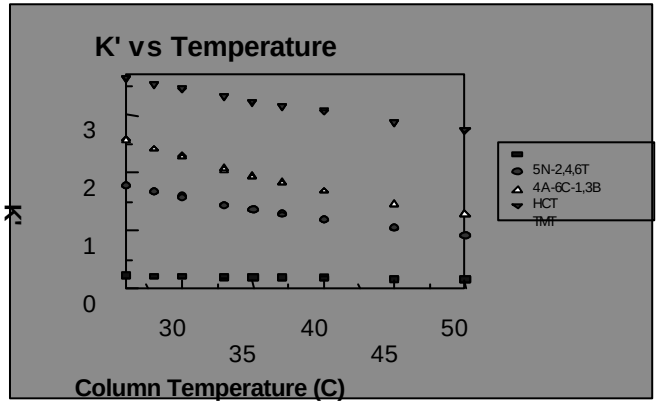
- Silanols
- pH stability
- Metal content
- Temperature stability

Temperature Effects on Resolution

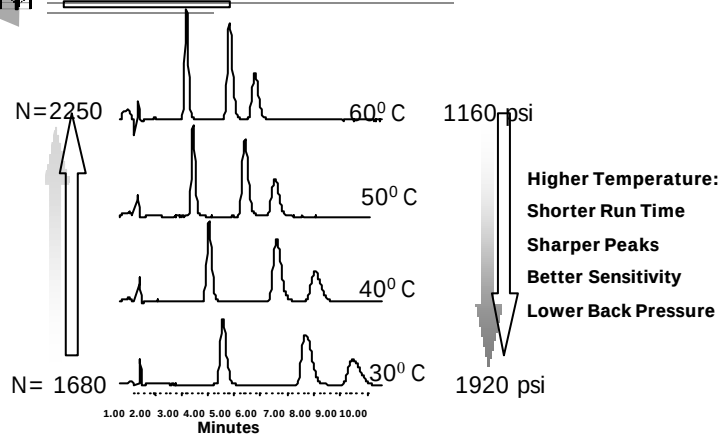
Resolution can be temperature dependent

Temperature can be a critical parameter to control in order to achieve reproducible separations.

K' vs Temperature

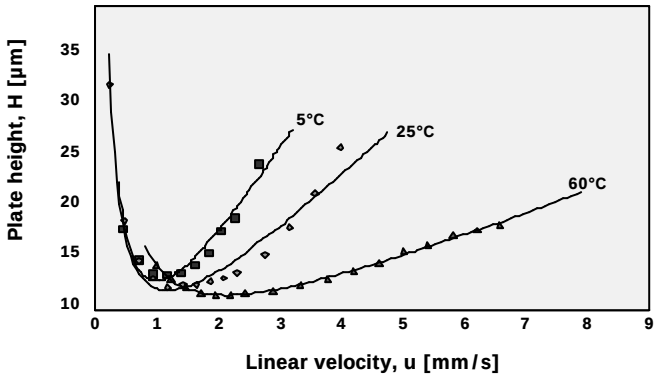


Effect of Temperature (Isocratic separations)

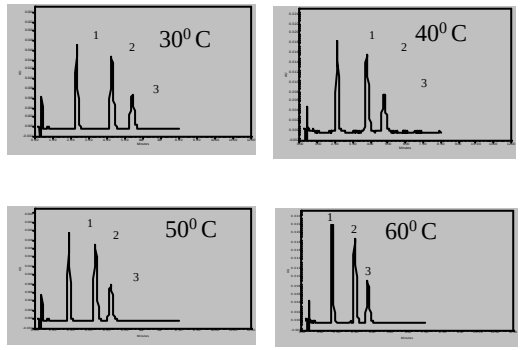


Reversed Phase HPLC

Effect of Temperature on Column Efficiency



Dependence of Retention on Temperature



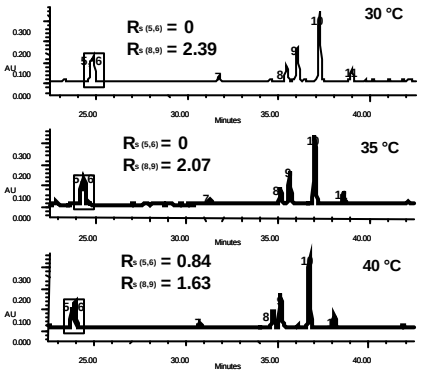
Conditions:
Column: XTerra™ MSC$_{18}$, 2.1 X 50 mm, 2.6 µm
Mobile Phase: 25% ACN/75% buffer (10 mM, pH5, NH4AC)
Flow Rate: 0.6 mL/min
Injection Vol. 3 µL
Detector: 210 nm

Analyte	Conc. (µg/ml)
1: doxepin	0.5
2: imipramine	1.0
3: amitriptyline	3.0

Higher Temp.
Shorter Run Time
Higher Signal

Lu, Cheng

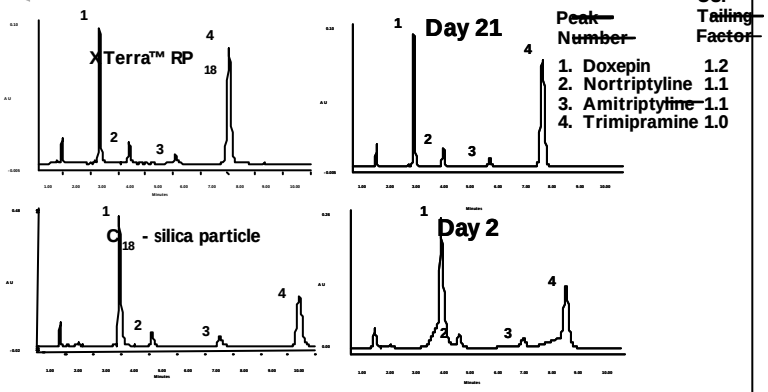
Temperature Effects on Resolution - Gradient



Conditions

- Column: Symmetry300™, C 5 µm, 3.9x150mm
- Sample: Tryptic digests of bovine cytochrome c
- Injection: 20 µL
- Mobile Phase:
  Solvent A: 0.1% TFA in water
  Solvent B: 0.1% TFA in acetonitrile
- Gradient: 0-45 min., 0-30%B
- Flow rate: 0.75 mL/min.
- Detection: 214 nm

High Temperature Phosphate Buffer Test



Peak Number	USP Tailing Factor
1. Doxepin	1.2
2. Nortriptyline	1.1
3. Amitriptyline	1.1
4. Trimipramine	1.0

Tricyclic Antidepressant Separation

Reversed Phase HPLC

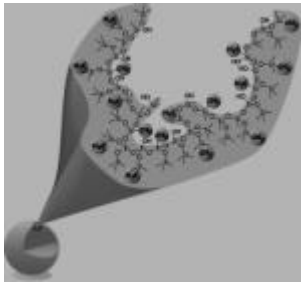
Stationary Phase Properties

CHEMISTRY:

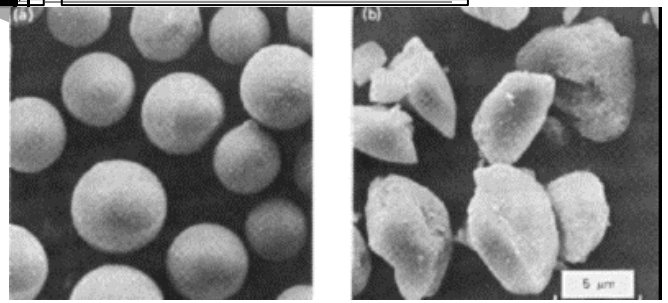
* **BONDED HYDROCARBON:**
  C-18, C-8, C-4, C-1, CN, phenyl
* **% COVERAGE**
* **TYPE OF SILICA GEL**

GEOMETRY

* **SPHERE- IRREGULAR**
* **PARTICLE DIAMETER**
* **POROSITY**



Spherical and Irregular particles



Electron microphotograph of spherical and irregular silica particles. [W.R.Melander, C.Horvath, Reversed-Phase Chromatography, in HPLC Advances and Perspectives, V2, Academic Press, 1980]

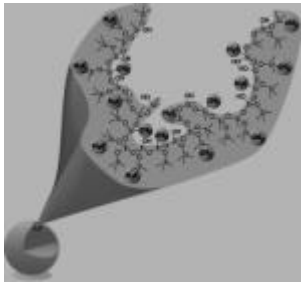
Stationary Phase Properties

CHEMISTRY:

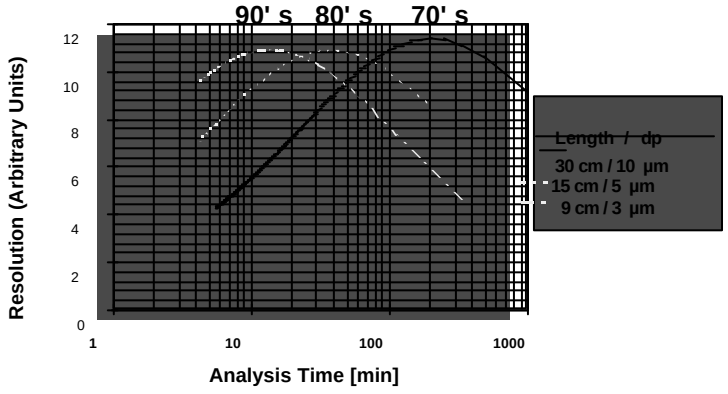
* **BONDED HYDROCARBON:**
  C-18, C-8, C-4, C-1, CN, phenyl
* **% COVERAGE**
* **TYPE OF SILICA GEL**

GEOMETRY

* **SPHERE- IRREGULAR**
* **PARTICLE DIAMETER**
* **POROSITY**



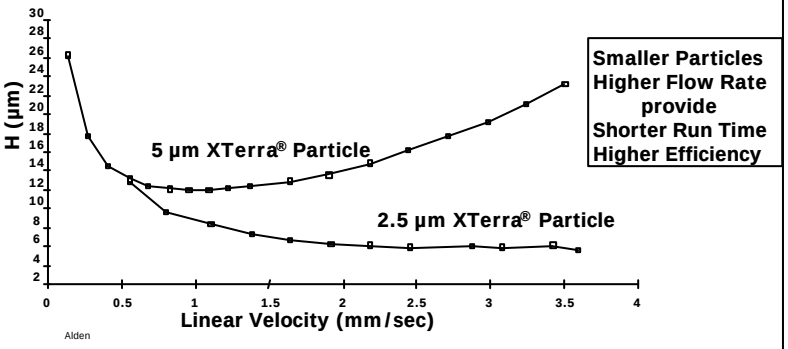
Resolution - Time Diagram



Reversed Phase HPLC

Comparison of the van Deemter Plots for 5 µm and 2.5 µm XTerra® MS C_{18} Particles

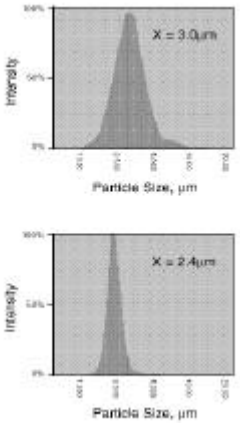
(50/50, acetonitrile / water mobile phase)



Smaller Particles
Higher Flow Rate provide
Shorter Run Time
Higher Efficiency

Alden

Challenge of producing smaller particles



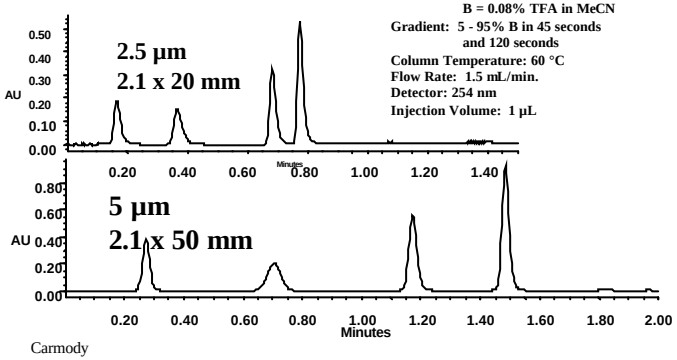
Contains a proportion of 2µm particles

Both are commercial '2µm' packings

Centered at 2.4 µm
Narrower distribution
(Waters proprietary technology)

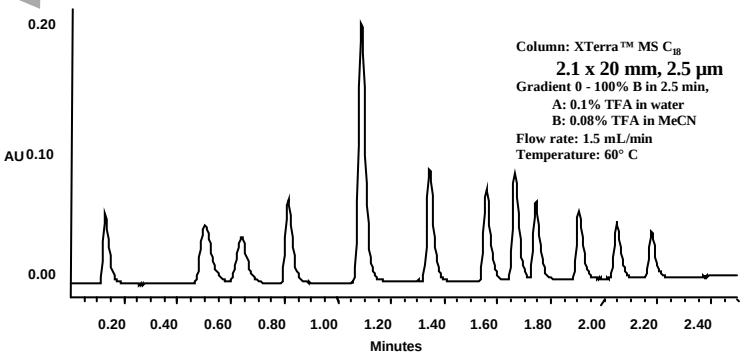
Fast Gradient Application

Columns: XTerra™ MS C_{18} 2.1 x 20 mm, 2.5 µm;
XTerra™ MS C_{18} 2.1 x 50 mm, 5 µm
Mobile Phase: A = 0.1% TFA in water,
B = 0.08% TFA in MeCN
Gradient: 5 - 95% B in 45 seconds and 120 seconds
Column Temperature: 60 °C
Flow Rate: 1.5 mL/min.
Detector: 254 nm
Injection Volume: 1 µL



Carmody

Fast Gradient of 12 Standards



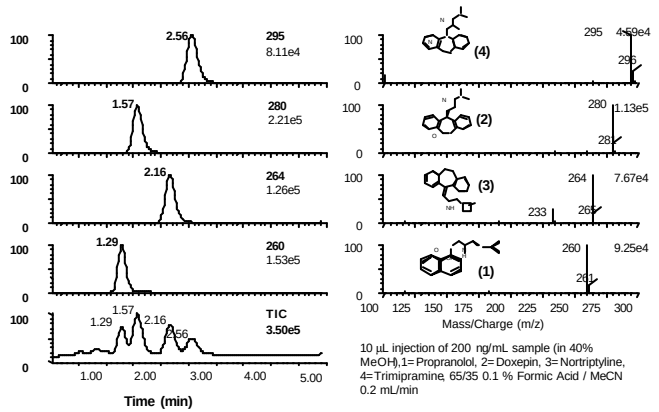
Reversed Phase HPLC

Fast LC/MS Applications

- 2.1 mm 5 µm and 2.5 µm columns
- length: 5 cm and 3 cm
- flow rates 0.2 and 0.6 mL/min
- Conditions:
  - **HPLC:**
  - **65/35 0.1% formic acid / MeCN**
  - **1 mL injection of 200 ng/mL of samples**
  - **MS:**
  - **ESI+; SIR 4 channels**
  - **HV: 3.15 kV, Cone 25 V**
  - **Drying Gas: 380 L/h**
  - **Source Temp: 175ºC**

Fast LC-MS Analysis

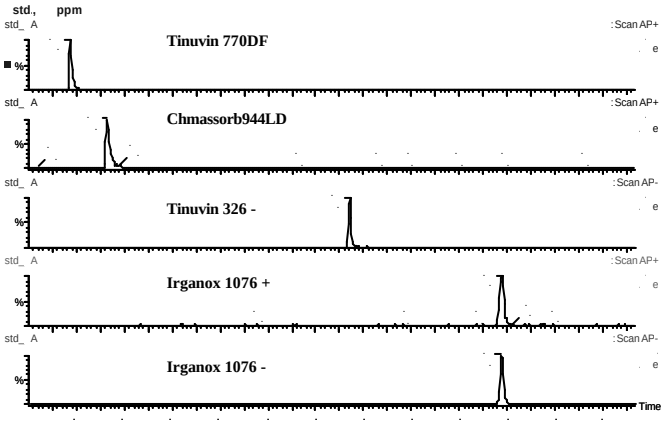
XTerra™ MS C18, 2.1 x 50 mm (5 µm)



10 µL injection of 200 ng/mL sample (in 40% MeOH),1= Propranolol, 2= Doxepin, 3= Nortriptyline, 4= Trimipramine, 65/35 0.1 % Formic Acid / MeCN 0.2 mL/min

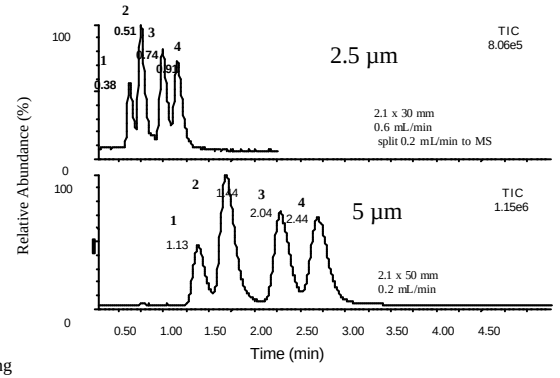
Ding

Masschromatograms of Std.10ppm (APcI +/-)



Fast LC-MS Analysis

XTerra™ MS C18: 5 µm *vs.*. 2.5 µm



HPLC:
65/35 0.1% formic acid / MeCN
1 µL injection of
200 ng/mL of samples
MS: ESI+
SIR 4 channels
HV: 3.15 kV
Cone 25 V
Drying Gas: 380 L/h
Source Temp: 175ºC

1 = Propranolol
2 = Doxepin
3 = Nortriptyline
4 = Trimipramine

Ding

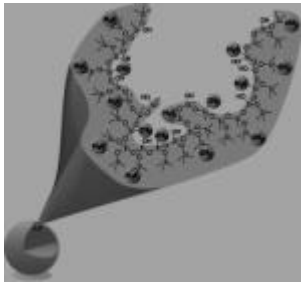
Stationary Phase Properties

CHEMISTRY:

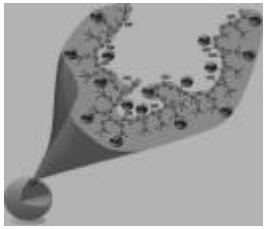
* **BONDED HYDROCARBON:**
  C-18, C-8, C-4, C-1, CN, phenyl
* **% COVERAGE**
* **TYPE OF SILICA GEL**

GEOMETRY

* **SPHERE- IRREGULAR**
* **PARTICLE DIAMETER**
* **POROSITY**



Pore size, shape and distribution



Macroporous spherical silica particle. [K.K.Unger, Porous silica, Elsevier, 1979]

Pore size defines an ability of the analyte molecules to penetrate inside the particle and interact with its inner surface. This is especially important because the ratio of the outer particle surface to its inner one is about 1:1000. The surface molecular interaction mainly occurs on the inner particle surface.

Pore Size

- Most silica gel packings are porous
  - >99% of the surface area is contained within the particle (not on the surface)-"Where the chromatography happens."
- Rules of Thumb
  - **"The smaller the pore size, the greater the surface area."**
    - **(100 Å approx. 300 m^2/gram)**
    - **(300 Å approx. 100 m^2/gram)**
  - **"The greater the surface area, the greater the retention."**
- A typical 15 cm column holds a surface area of ~100-300 square meters

Silica Gel Pore Structure

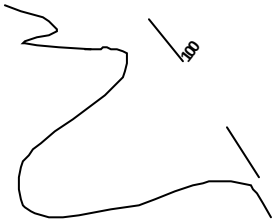
*** Silica is Porous**

*** Pore Size, or nm -- distribution**

*** Specific Pore Volume, mL/g**

Range: 0.3 -- 1.3 mL/g

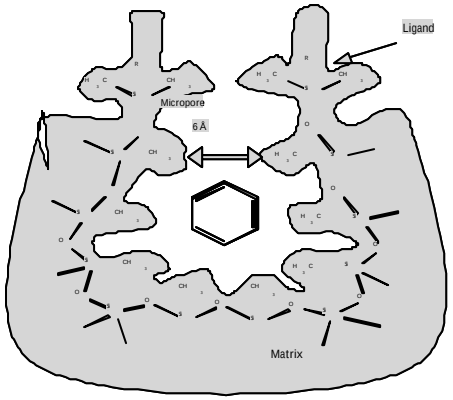
SV Particle Strength



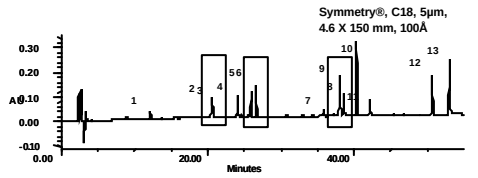
Analyte MW	Pore Size Recommendation
< 3,000	60 - 130 (6 - 13 nm)
3,000 - 10,000	100 (10 nm)
>10,000	300 - 1,000 (30 - 100 nm)
Very Large	non- porous

Reversed Phase HPLC

Exclusion – Inclusion Effects

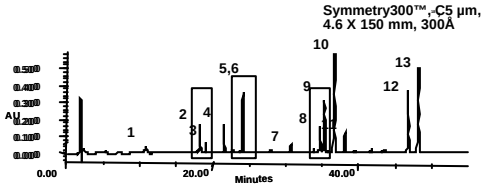


Pore Size Effects on Resolution



Conditions

- Sample: Tryptic digests of cytochrome c (bovine)
- Injection: 20 µL
- Mobile Phase:
  Solvent A: 0.1% TFA in water
  Solvent B: 0.1% TFA in acetonitrile
- Gradient: 0-50 min., 0-30%B
- Temperature: 35 °C
- Flow Rate: 0.75 mL/min.
- Detection: 214 nm



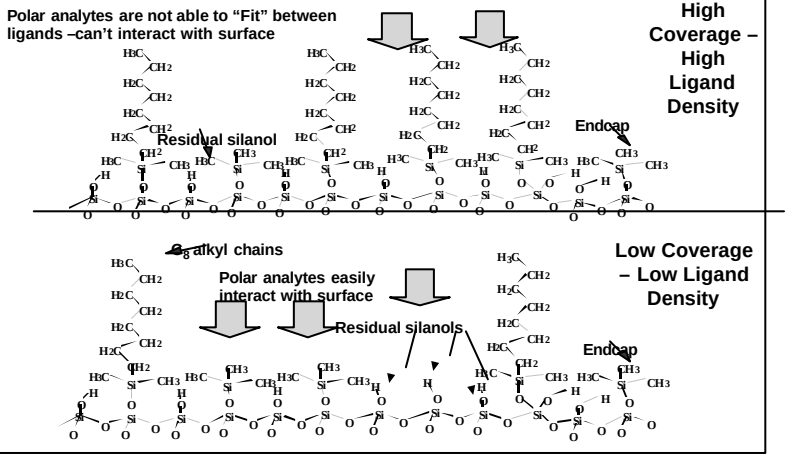
-Different pore sizes change selectivity.

Carmody

Polarity/Aqueous Columns:

- Low ligand density
- High pore volumn

Mechanism of Retention of Polar Compounds on Aqueous Columns



Reversed Phase HPLC

Polar Compounds - Aqua Columns

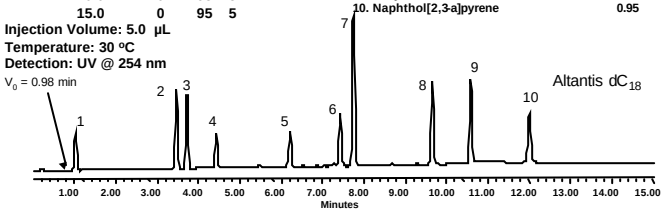
Polar and Non-Polar Compounds Test Mix

Conditions
Columns: 4.6 x 150 mm, 5 µm
Mobile Phase A: H_2O
Mobile Phase B: ACN
Mobile Phase C: 100 mM NH_4COOH, pH 3.0
Flow Rate: 2.0 mL/min
Gradient:

Time (min)	%A	%B	%C
0.0	80	10	10
10.0	0	95	5
15.0	0	95	5

Injection Volume: 5.0 µL
Temperature: 30 °C
Detection: UV @ 254 nm

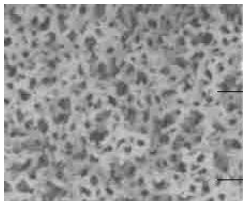
Compounds	USP Tailing:
1. Uracil	1.04
2. Acetanilide	0.95
3. Triamcinolone	1.02
4. Hydrocortisone	1.03
5. 2-Amino-7-chloro-5-oxo-5H-[1]-benzopyrano[2,3-b]pyridinecarbonitrile	1.01
6. 6a-Methyl-17a-hydroxyprogesterone	1.01
7. 3-Aminofluoranthene	0.97
8. 2-Bromofluorene	1.00
9. Perylene	0.99
10. Naphthol[2,3-a]pyrene	0.95



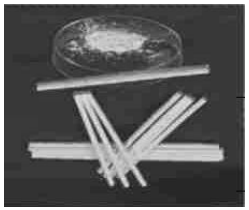
Chromolith Packing

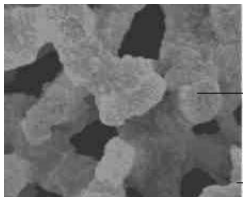
By utilising an innovative new "Gel-Sol" technology, a silica gel polymer is formed, which after ageing, is dried into the required form of a straight rod of highly porous silica with a bimodal pore structure.

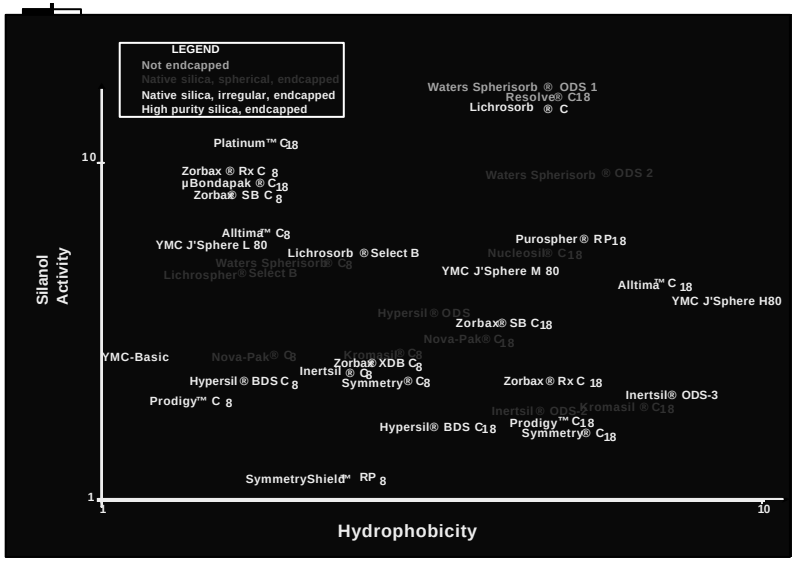
Chromolith macropore structure



Chromolith mesopore structure

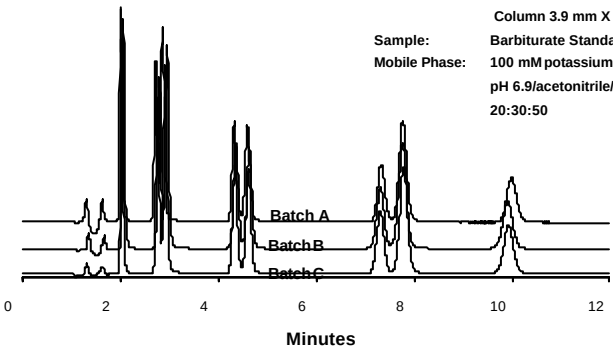






Batch-to-Batch Reproducibility of Columns

Columns: Symmetry ™ C_8 3.9 mm X 150 mm with Sentry ™ Guard Column 3.9 mm X 20 mm
Sample: Barbiturate Standard
Mobile Phase: 100 mM potassium phosphate, pH 6.9/acetonitrile/water 20:30:50



Reversed Phase HPLC