

Reversed Phase

Advanced Features

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Home page:

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

Reversed Phase HPLC

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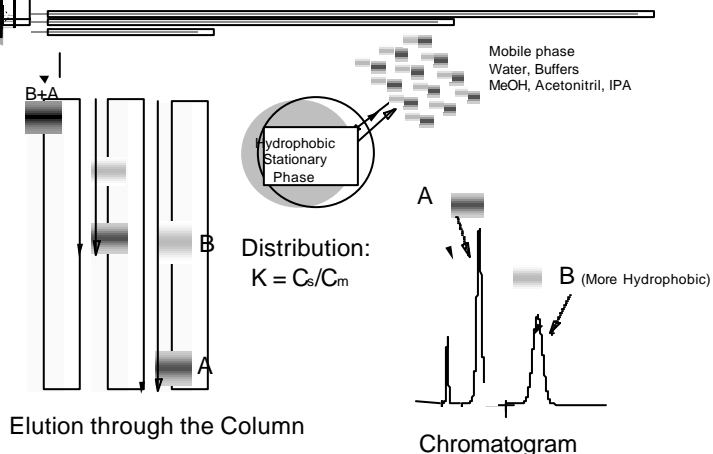
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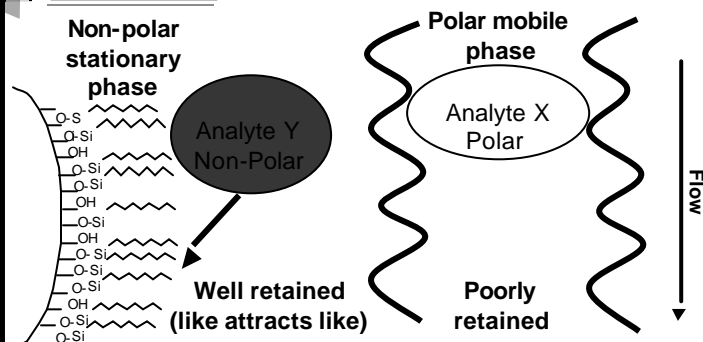
Home page:

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

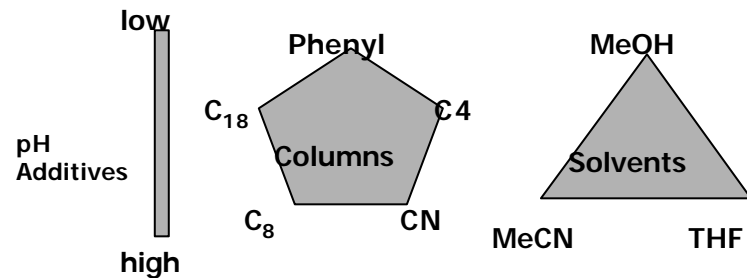
Chromatographic Process



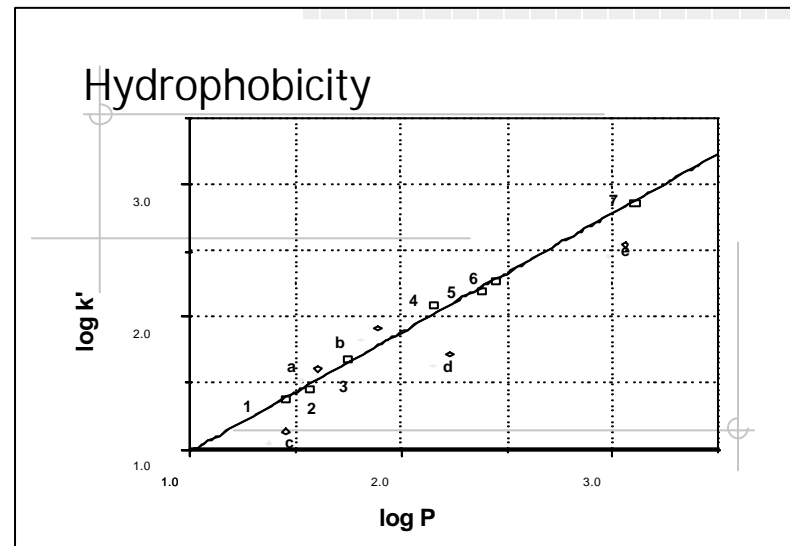
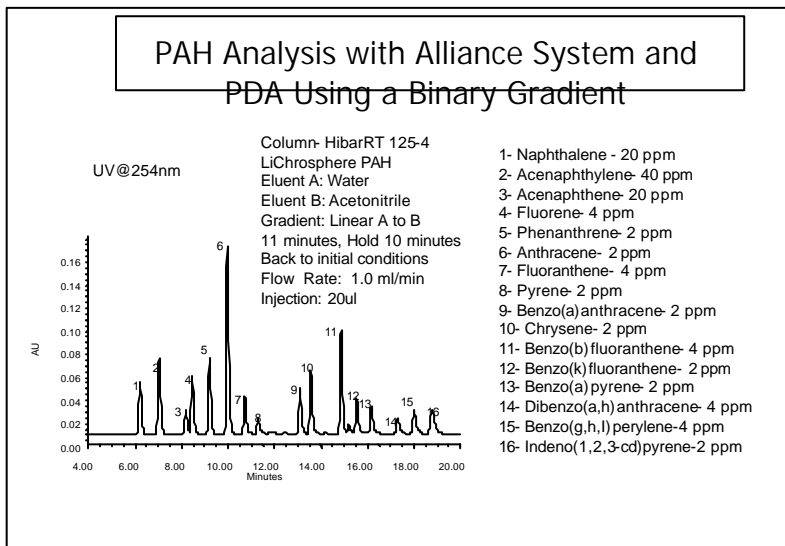
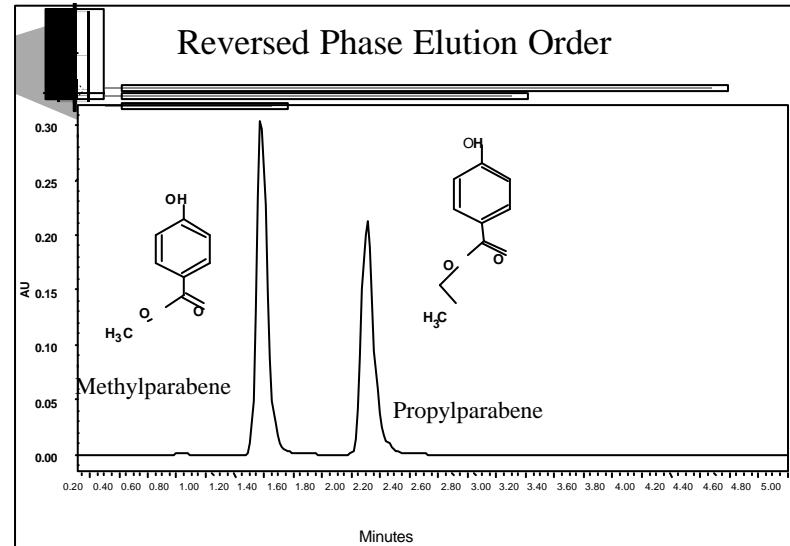
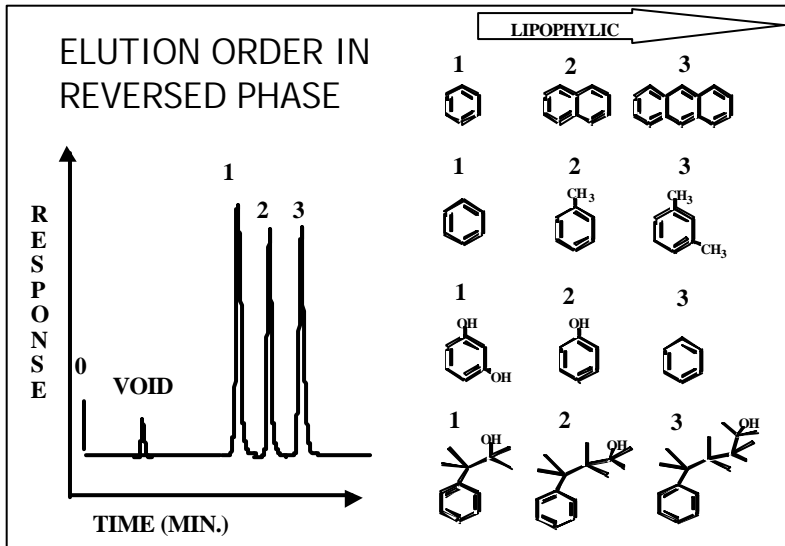
Reversed-Phase Chromatography



RP Method Development Tools

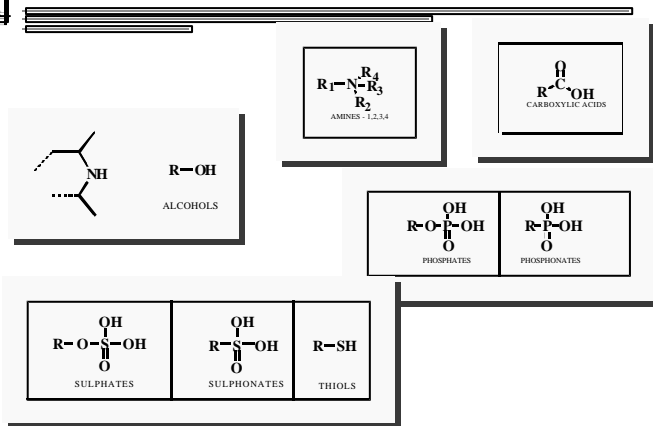


Reversed Phase HPLC



Reversed Phase HPLC

IONIZABLE



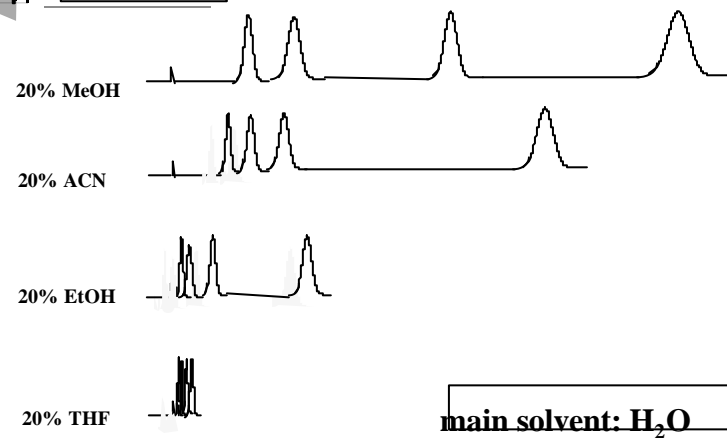
MOBILE PHASE

- * 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



Reversed Phase HPLC

MOBILE PHASE

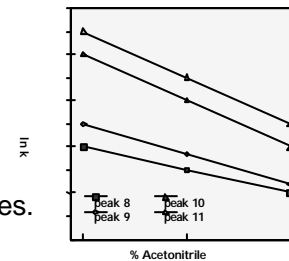
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SOLVENT STRENGTH

Analyte Retention as a Function of % Modifier

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

Thus, the resolution between peaks changes.



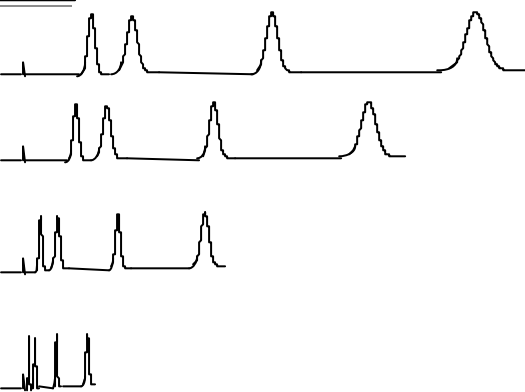
OPTIMIZATION: % SOLVENTS

20% MODIFIER

40% MODIFIER

60% MODIFIER

80% MODIFIER



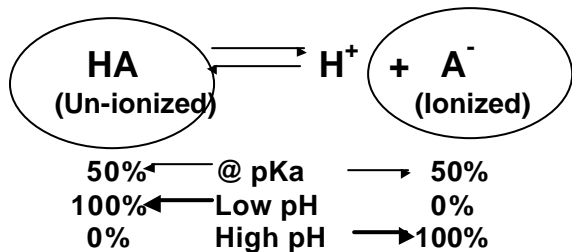
MOBILE PHASE

- * TYPE OF MODIFIER (MeOH, ACN)
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- * pH
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Ionization of Acids and Bases

Dissociation of Molecule

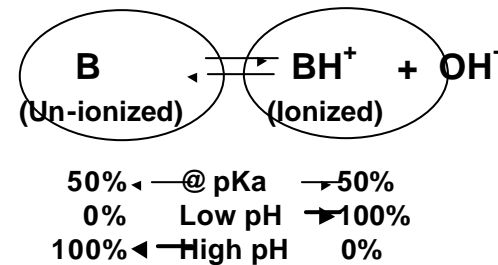
Acid



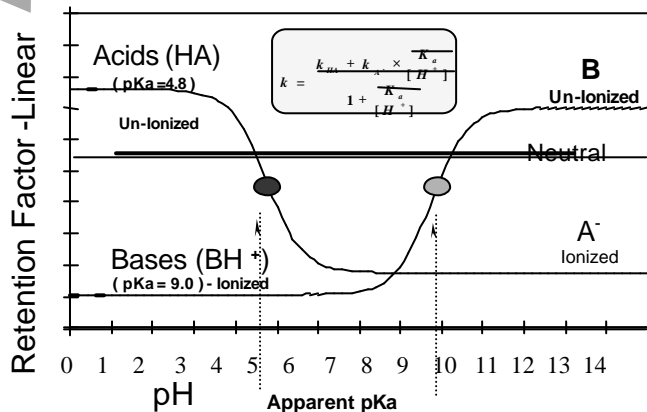
Ionization of Acids and Bases

Dissociation of Molecule

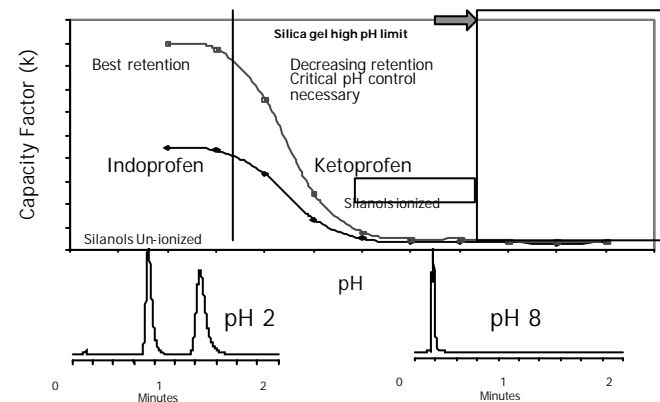
Base



Retention Factor versus pH for Acids, Bases and Neutrals

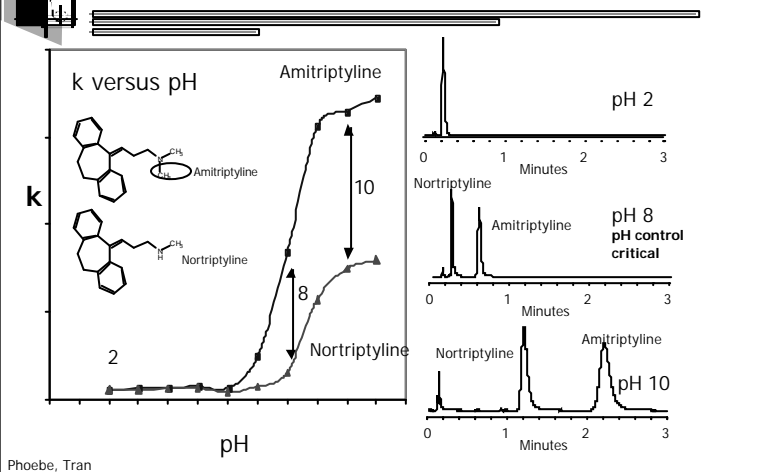


Resolution of Two Acidic Compounds at Different Mobile Phase pH's

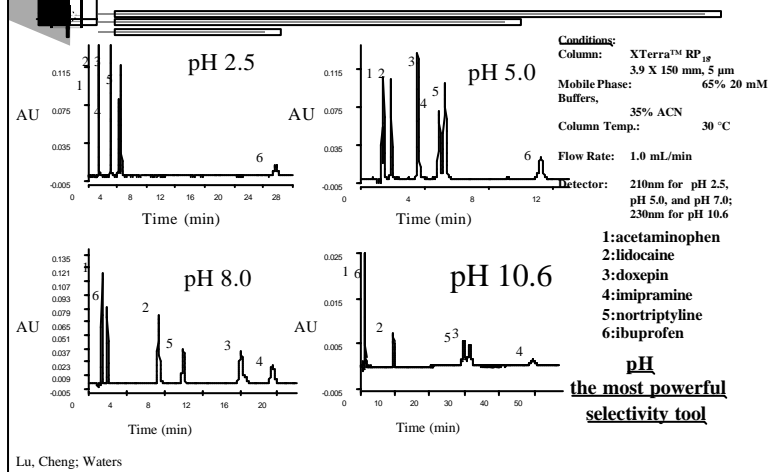


Reversed Phase HPLC

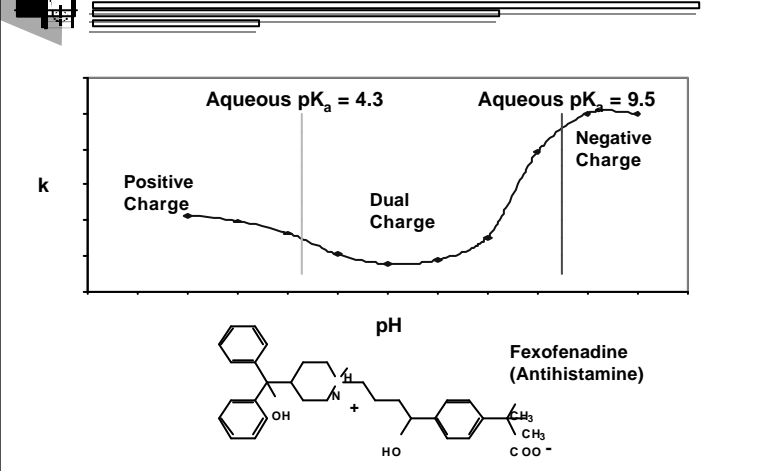
Enhanced Resolution of Basic Compounds at High pH



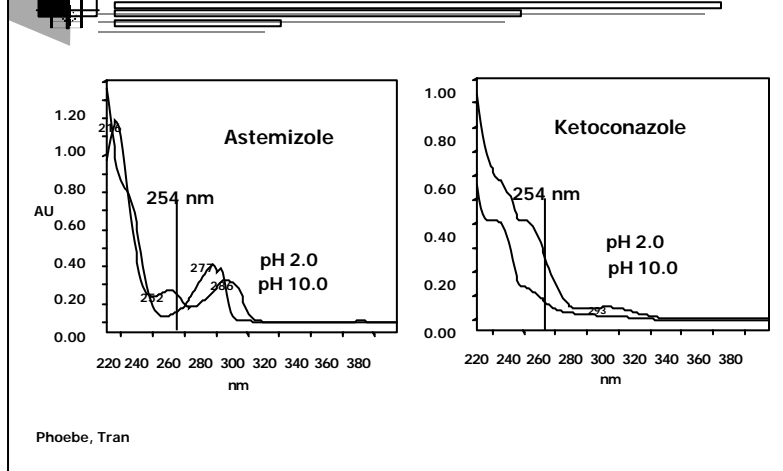
Dependence of Selectivity on pH



Impact of pH on the Retention of a Zwitterionic Compound

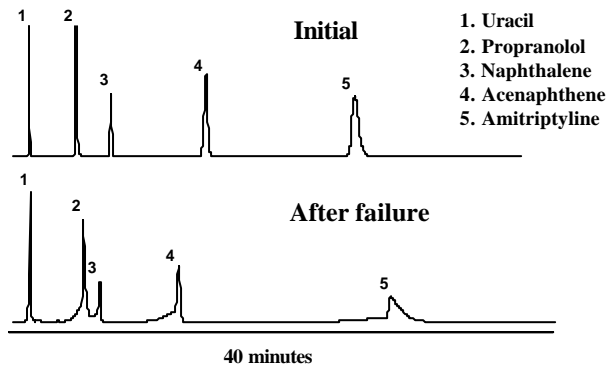


UV/Vis Spectral Change Between Ionized and Non-ionized Forms



Reversed Phase HPLC

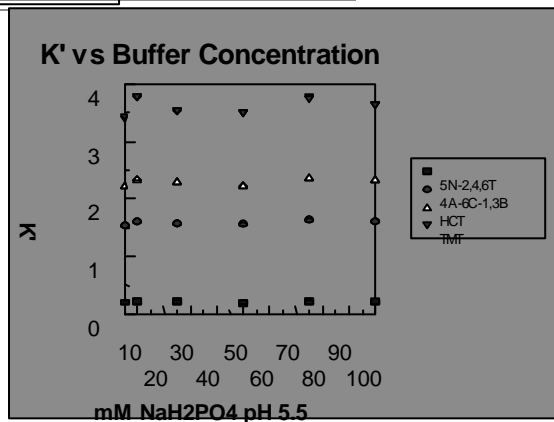
Typical Chromatograms for pH Failure of an Ordinary C₁₈-Silica Column



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

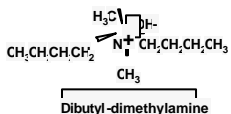
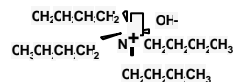
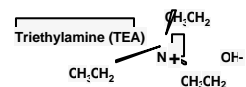
- **pH 10: Borate**
 - 20 mM H_3BO_3
- **pH 7: Phosphate**
 - 20 mM K_2HPO_4
- **pH 4-5: Acetate**
 - 10 mM CH_3COONH_4
 - 100 mM CH_3COOH
- **pH 2-3.5: Phosphate**
 - 20 mM $H_3PO_4 - KH_2PO_4$

MOBILE PHASE

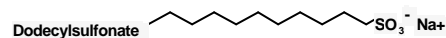
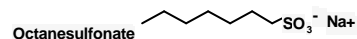
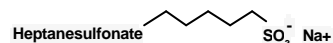
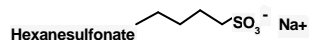
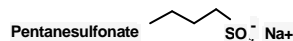
- * **TYPE OF MODIFIER (MeOH, ACN)**
- * **SOLVENT STRENGTH (% modifier)**
- * **pH**
- * **TYPE OF BUFFER (phosphate, acetate)**
- * **IONIC STRENGTH (Salts, buffer concentration)**
- * **ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)**

Ion Pair Reagent

Alkylamines



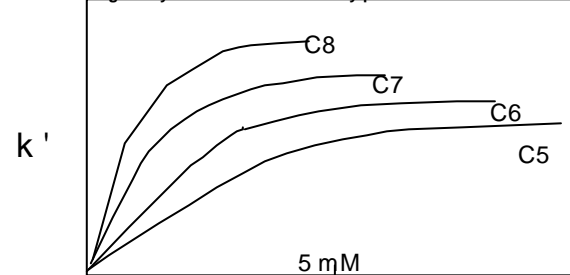
Alkylsulfonates



Concentration of Ion-Pair Reagent in the Mobile Phase

The larger the alkyl, the longer are retention times

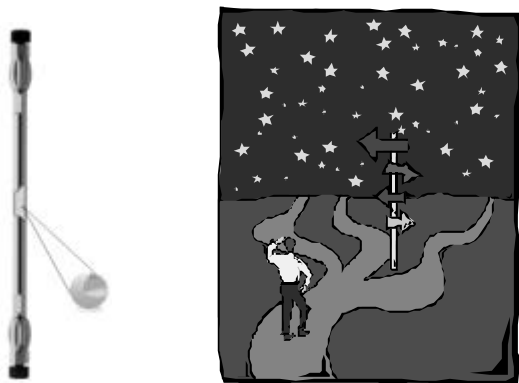
The larger alkyls saturate the stationary phase at lower concentrations



Conc. of Ion Pair Reagent in the Mobile Phase

Reversed Phase HPLC

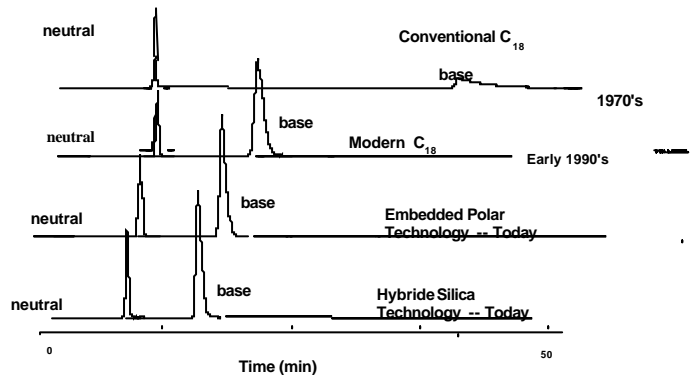
Stationary Phase Characterization



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

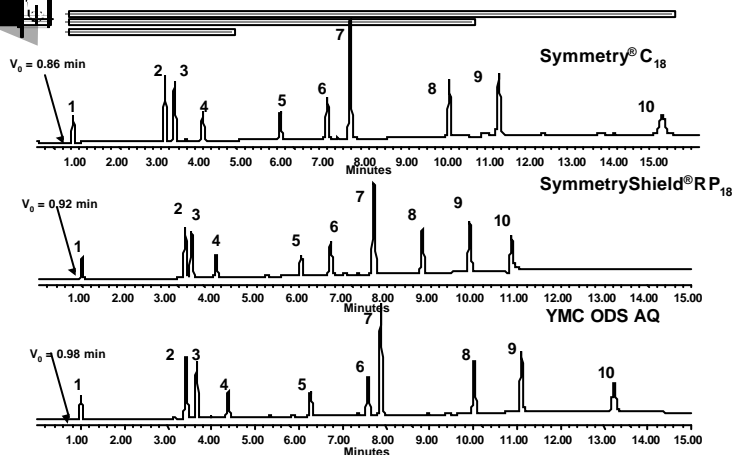
Improvement in Peak Shape for Bases



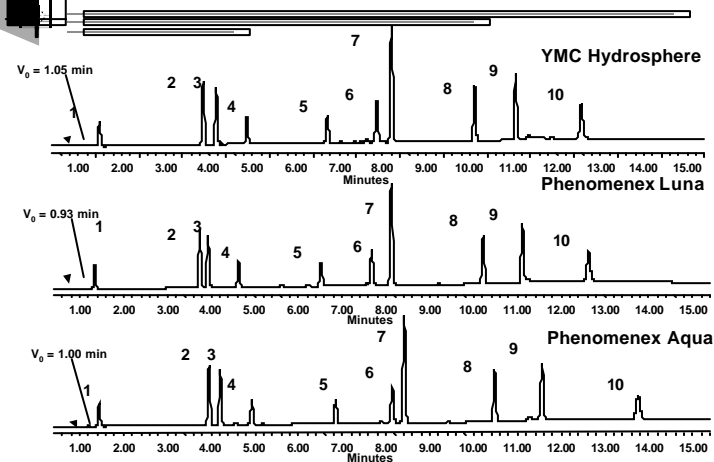
Not all C18's are the same!

Reversed Phase HPLC

Different Columns – Different Chromatograms



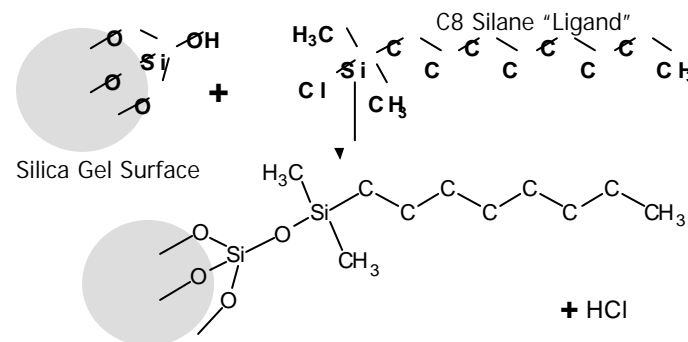
Different Columns – Different Chromatograms



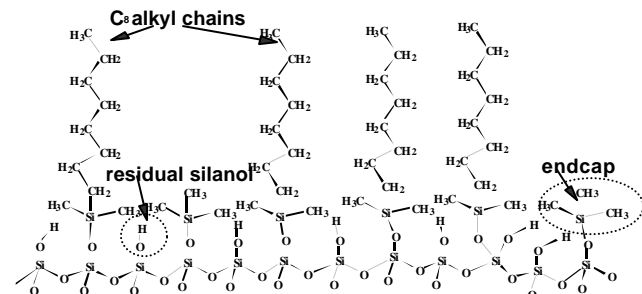
“Relative” Ranking of C18 Columns Using a Standardized Test

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

Making a Bonded Phase Material: Monofunctional Synthesis



Surface of a Silica Gel Bonded-Phase Packing Material

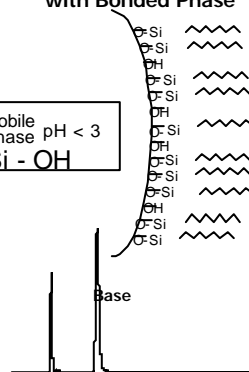


Note: ~50% of the surface silanols remain even with high bonding densities

Mixed-Mode Retention:

Hydrophobic Interaction
with Bonded Phase

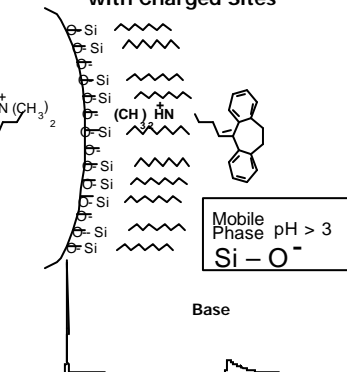
Mobile
Phase pH < 3
Si - OH



Base

Ion exchange Interaction
with Charged Sites

Mobile
Phase pH > 3
Si - O⁻



Base

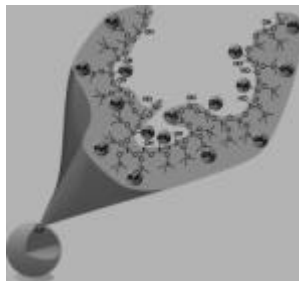
Stationary Phase Properties

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

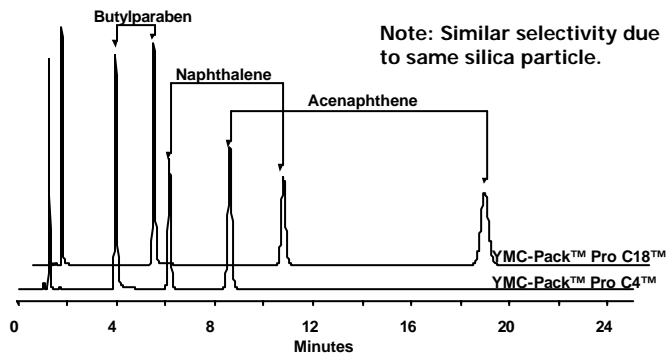
| | |
|-----------------|--|
| C ₁₈ | -Si(CH ₃) ₂ C ₁₈ H ₃₇ |
| C ₈ | -Si(CH ₃) ₂ C ₈ H ₁₇ |
| tC ₂ | -SiC ₂ H ₅ |
| Aminopropyl | -Si(CH ₃) ₂ NH ₂ |
| Cyanopropyl | -Si(CH ₃) ₂ (CH ₂) ₃ CN |
| Diol | -Si(CH ₃) ₂ OCH ₂ CH(OH)CH ₂ OH |

Retention time

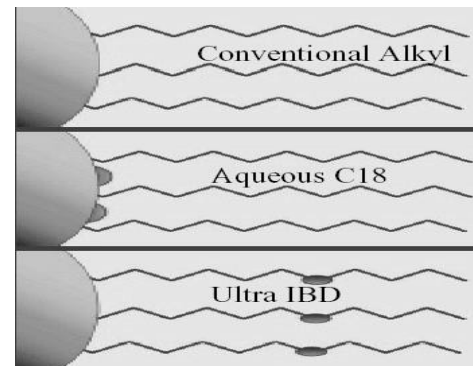
Chain length CN Phenyl NH₂ C₄ C₈ C₁₈

Reversed Phase HPLC

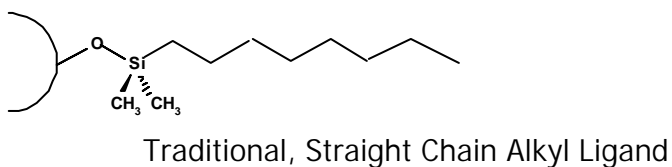
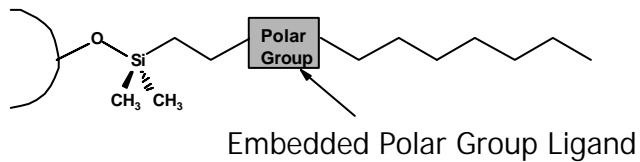
Neutral Compounds: C18 versus C4 (Same Brand - Different Ligands)



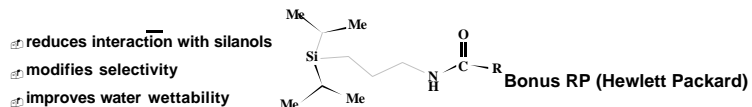
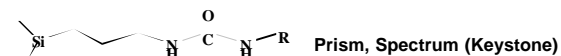
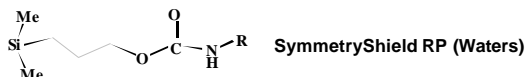
Type of Ligands



Reversed-Phase Packing with an Embedded Polar Ligand

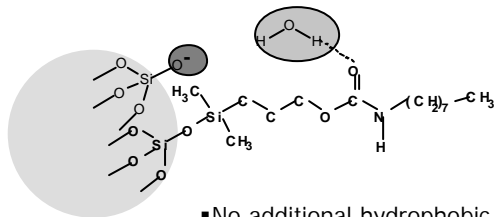


Commercial Phases with Embedded Polar Group



Embedded Polar Ligand: Possible Mechanism

Polar group increases water concentration in surface layer



Shields
Negative
Silanols

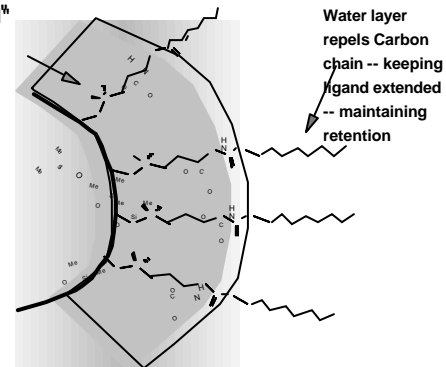
- No additional hydrophobic retention
- Reduced retention of bases
- Reduced peak tailing

Embedded Polar Groups

Embedded Polar Wetted Surface

Water "Shield"
Layer

Silica Surface



Water layer
repels Carbon
chain -- keeping
ligand extended
-- maintaining
retention

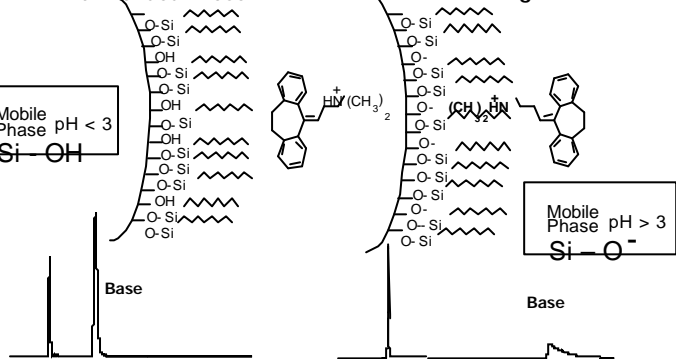
Mixed-Mode Retention:

Hydrophobic Interaction
with Bonded Phase

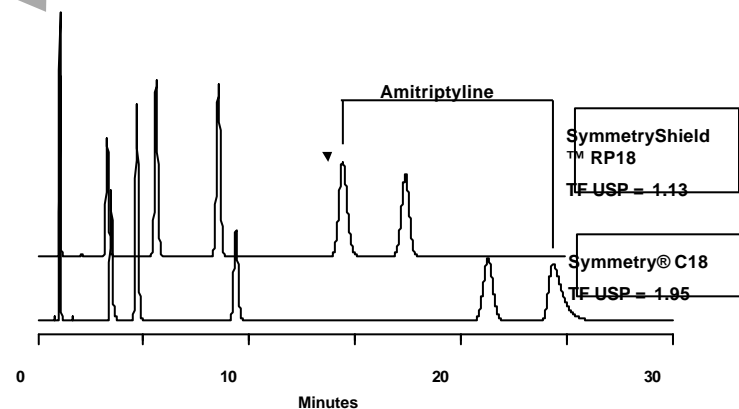
Ion exchange Interaction
with Charged Sites

Mobile
Phase pH < 3
Si-OH

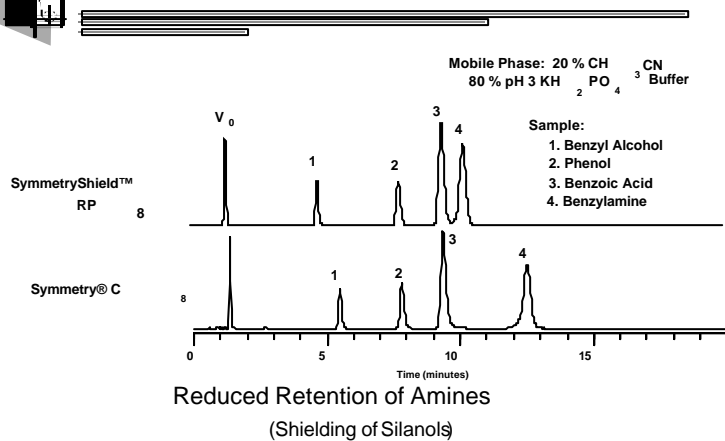
Mobile
Phase pH > 3
Si-O⁻



Embedded Polar Ligand versus Linear Alkyl Ligand on Silica Gel



Impact on Selectivity - Retention

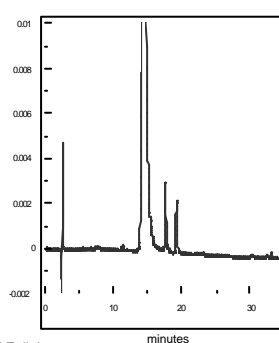


B. A. Alden

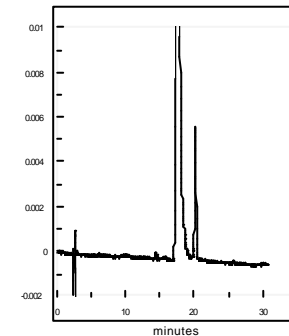
Selectivity Difference: Furazolidone Impurities

SymmetryShield™ RP₈

Symmetry® C₈



El Fallah



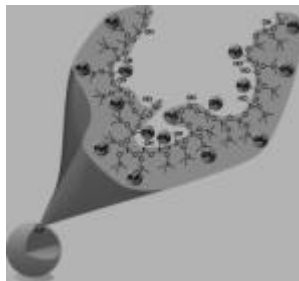
Stationary Phase Properties

CHEMISTRY:

- * BONDED HYDROCARBON:
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- * % COVERAGE
- * TYPE OF SILICA GEL

GEOMETRY

- * SPHERE-IRREGULAR
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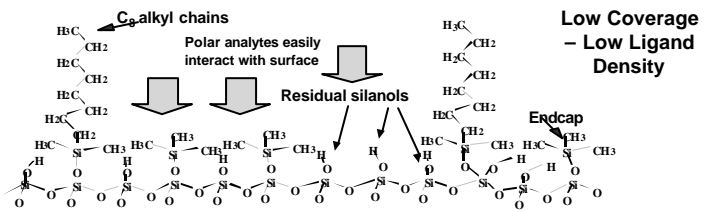
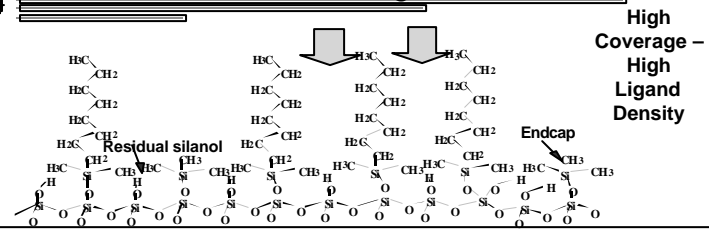
CARBON LOAD

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

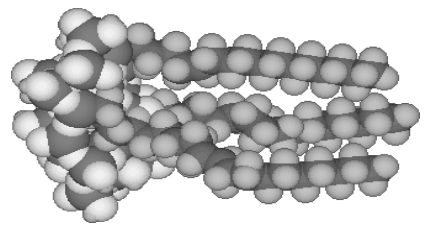
Retention time

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

Surface of a Silica Gel Bonded-Phase Packing Material

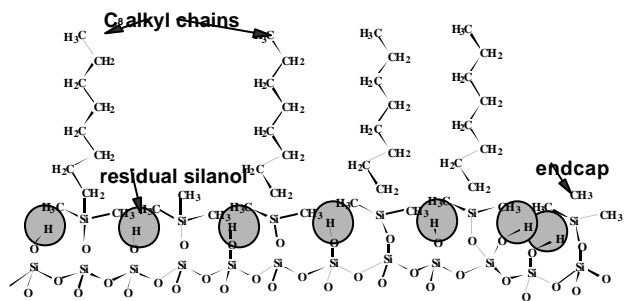


Silica based "bonded phases"



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)

| | $\mu\text{moles/m}^2$ |
|---------------------------------|-----------------------|
| 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)

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

SA - Specific Surface Area

%C % Carbon Load

MW - Molecular Weight of Ligand

nC - # of Carbon Atoms in Ligand

Ligand Density



Retention



Silanols



Surface Area



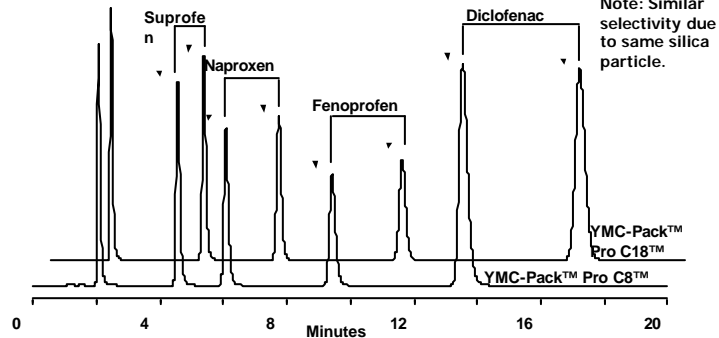
% C



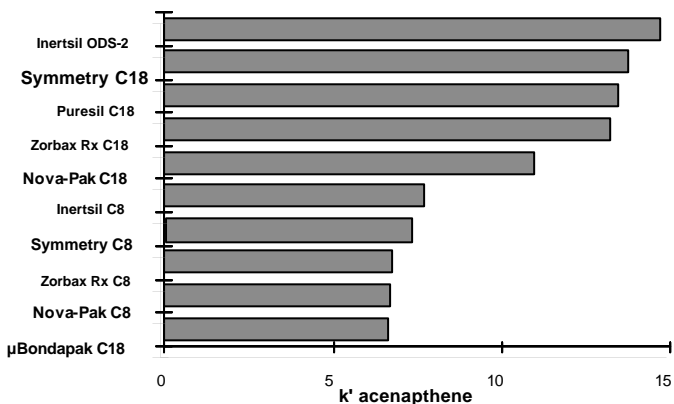
Ligand Density



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



Relative Hydrophobicities of General Purpose Analytical Packings



Stationary Phase Properties

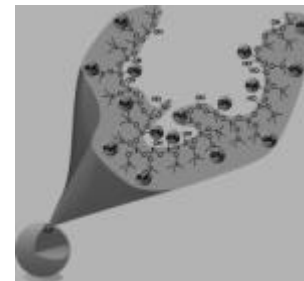
CHEMISTRY:

* BONDED HYDROCARBON:
C-18, C-8, C-4, C-1, CN, phenyl

* % COVERAGE
TYPE OF SILICA GEL
Native/Synthetic/Pure

GEOMETRY

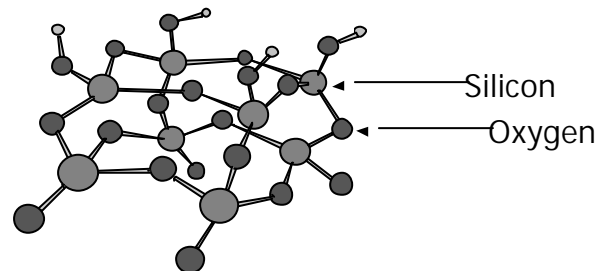
- * SPHERE- IRREGULAR
- * PARTICLE DIAMETER
- * POROSITY



Types of Silica

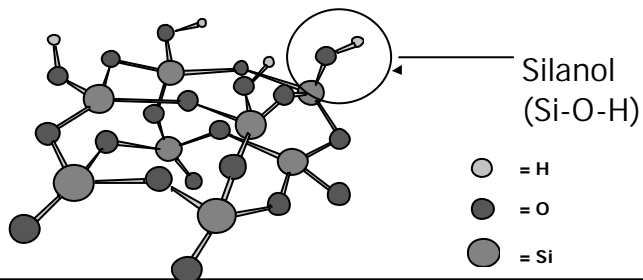
- ◆ Silanols
- ◆ pH stability
- ◆ Metal content
- ◆ Temperature stability

Structure of Silica Gel



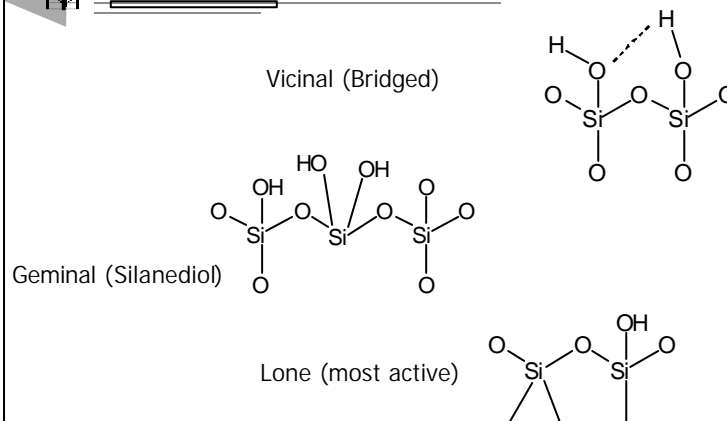
Amorphous, porous matrix of silicon atoms joined together with oxygen atoms to form "siloxane bonds" = (Si - O - Si)

What are Silanols?



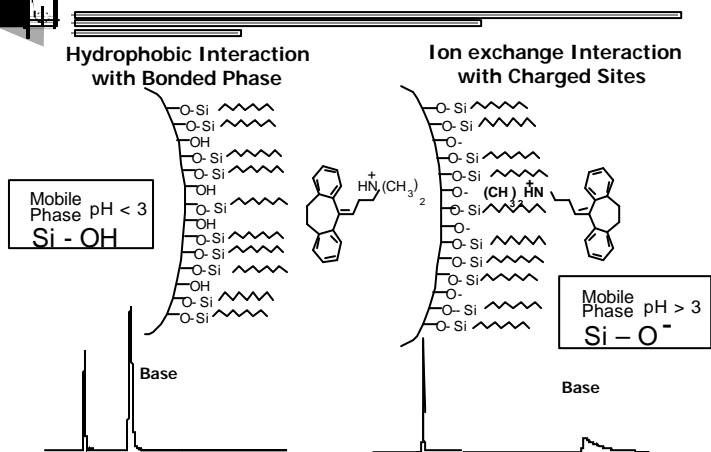
- Residual unreacted surface hydroxyl groups left over from polymerization
- Reactive sites for use in bonding ligands (C18) to the silica gel surface

Surface Silanols Found on Silica Gel

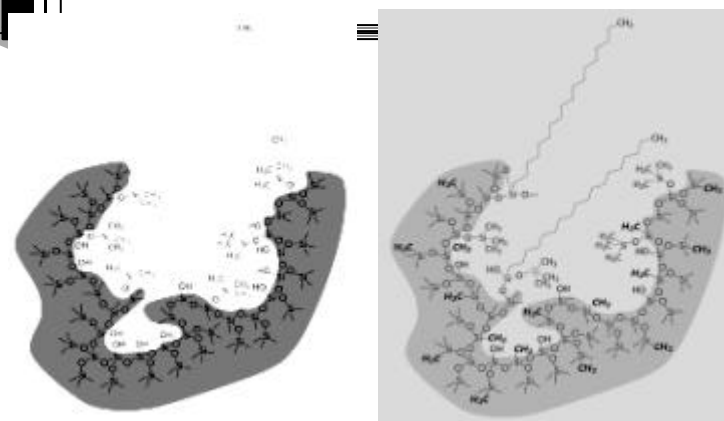


Reversed Phase HPLC

Mixed-Mode Retention:



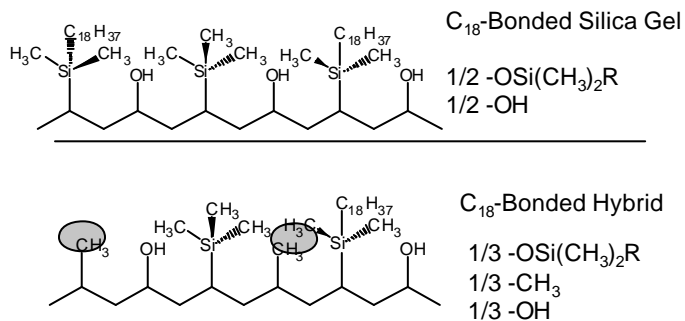
Bonded Phase on Particles



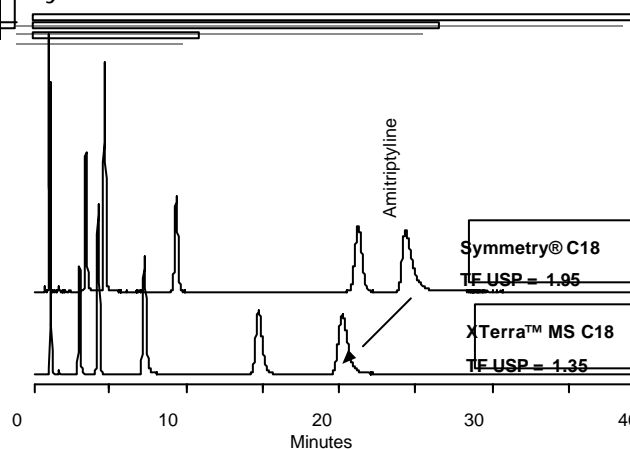
Silica Gel C18 Materials
1/2 free silanols

XTerra™ C18 Materials
1/3 free silanols

Bonded Hybrid versus Bonded Silica Gel Surfaces



Hybrid versus Silica Gel Particle



Reversed Phase HPLC

PERFORMANCE BY ONE PEAK

RETENTION FACTOR or CAPACITY RATIO

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

ASYMMETRY FACTOR

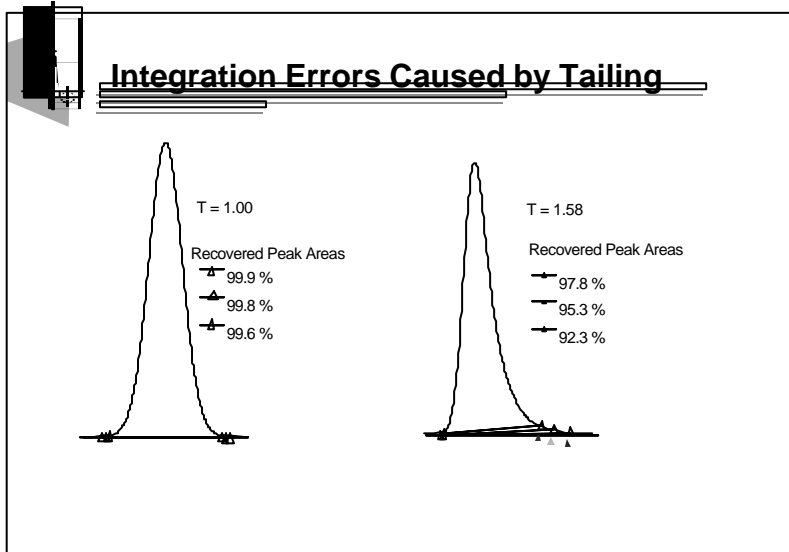
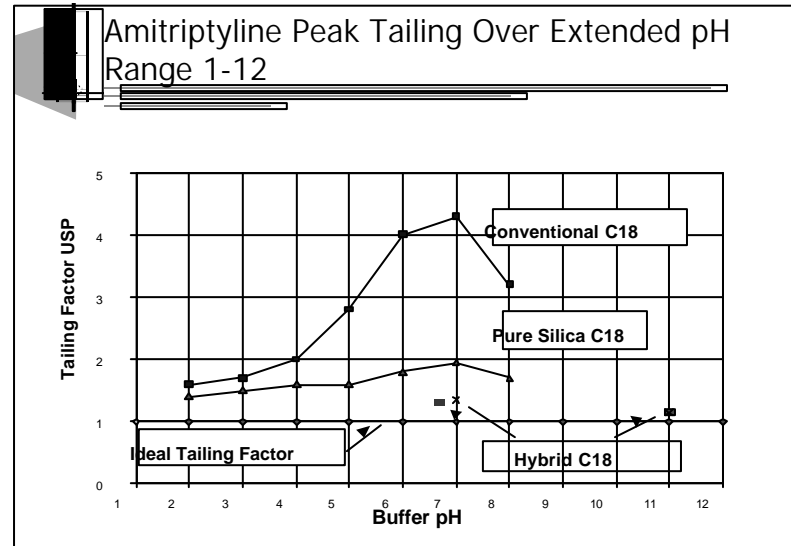
$$A_f = \frac{B_{(10\%h)}}{A_{(10\%h)}}$$

TAILING FACTOR

$$T_f = \frac{A + B}{2A} \quad (5\%h)$$

NUMBER OF THEORETICAL PLATES

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

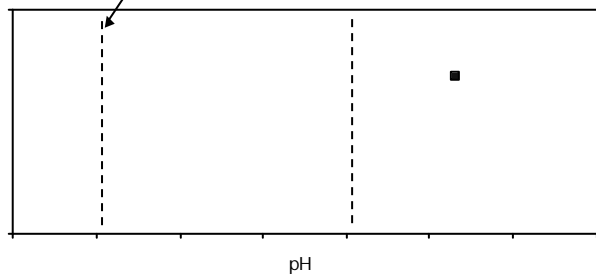


- ## Types of Silica
- ◆ Silanols
 - ◆ pH stability
 - ◆ Metal content
 - ◆ Temperature stability

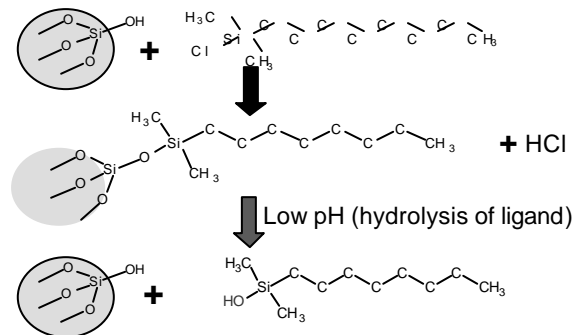
Reversed Phase HPLC

pH Limitations of Silica Based Packing Materials

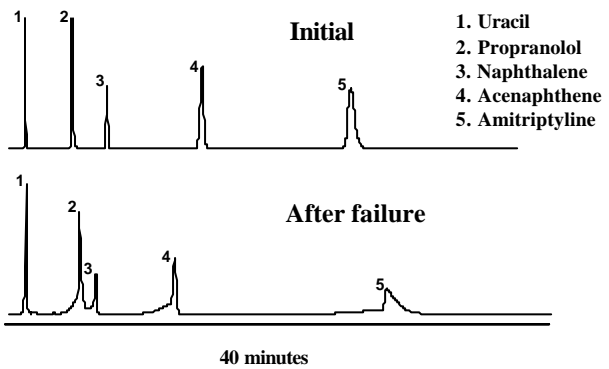
Hydrolysis of Bonded Ligand



Hydrolysis of a Bonded Phase Material: Monofunctional Ligand



Typical Chromatograms for Reference C₁₈-Silica Column



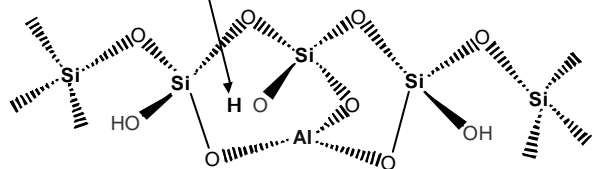
Types of Silica

- ◆ Silanols
- ◆ pH stability
- ◆ Metal content
- ◆ Temperature stability

Metal Content in Silica

Aluminum in the Silica Gel Lattice
Bronsted Acid

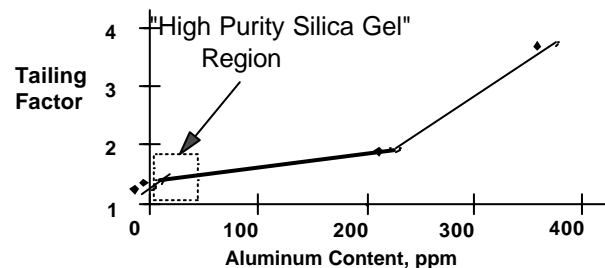
3D top view of silica particle surface with silanols pointing upward



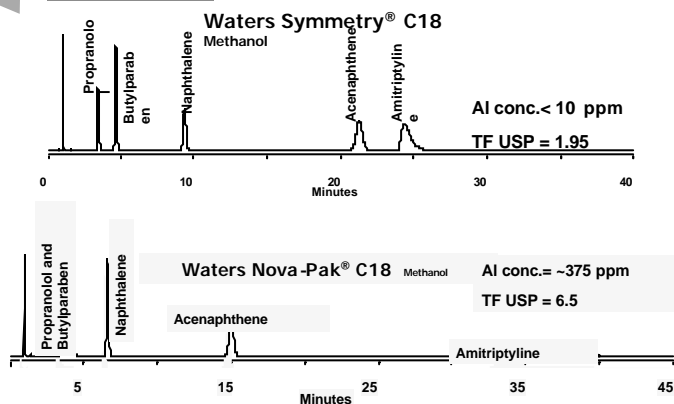
Metal available for chelation

Correlation Between Base Tailing and Aluminum Content of Silica Gel

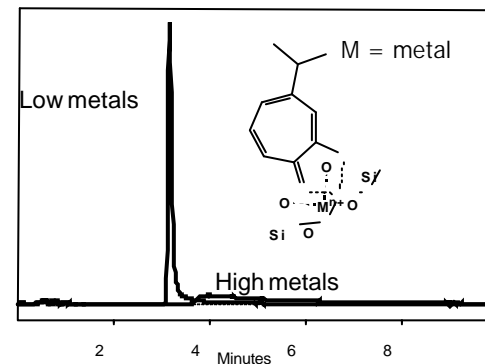
Analyte: Chlorpheniramine
Mobile Phase: Acetonitrile/ KH_2PO_4 pH 3.0 (20:80)



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



Peak Shapes of Chelating Agent (Hinokitiol)



Types of Silica

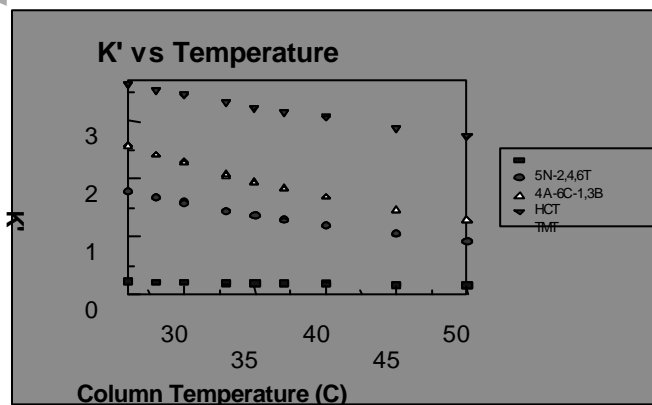
- ◆ Silanols
- ◆ pH stability
- ◆ Metal content
- ◆ Temperature stability

Temperature Effects on Resolution

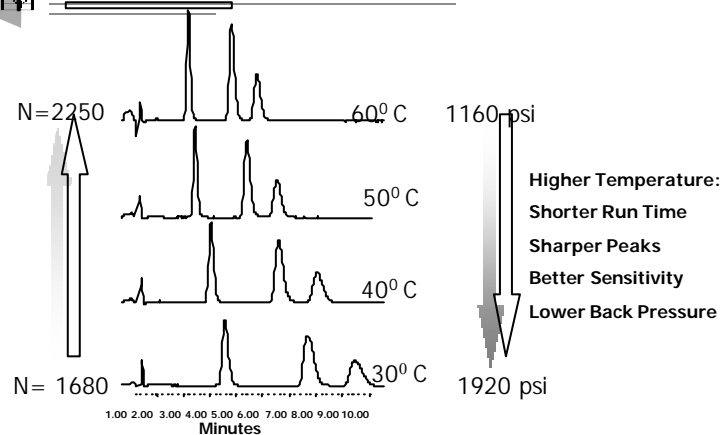
Resolution can be temperature dependent

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

K' vs Temperature

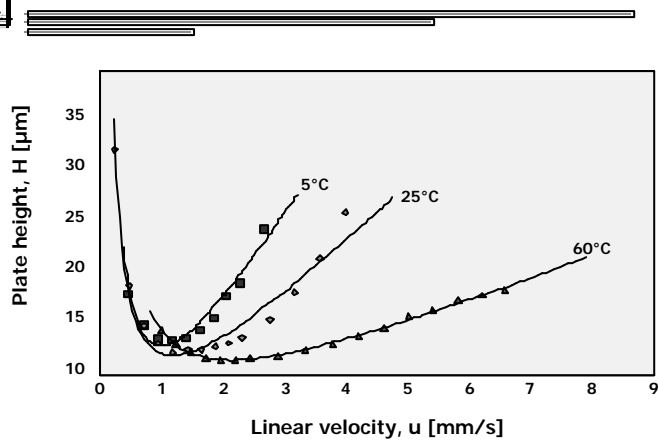


Effect of Temperature (Isocratic separations)

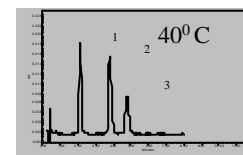
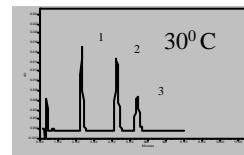


Reversed Phase HPLC

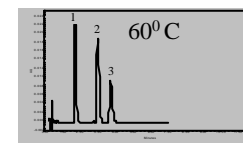
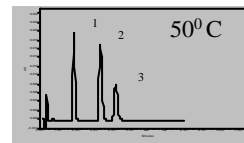
Effect of Temperature on Column Efficiency



Dependence of Retention on Temperature



Conditions:
 XTerra™ MSC₁₈
 Column: 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

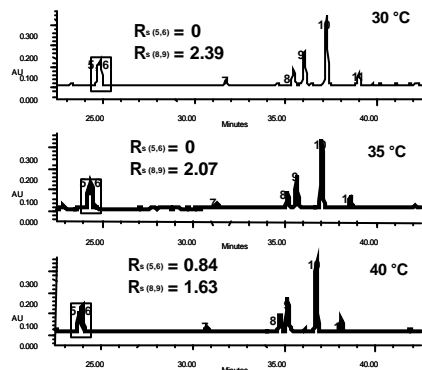


| Analyte | Conc. ($\mu\text{g/ml}$) |
|------------------|----------------------------|
| 1: doxepin | 0.5 |
| 2: imipramine | 1.0 |
| 3: amitriptyline | 3.0 |

Higher Temp.
Shorter Run Time
Higher Signal

Lu, Cheng

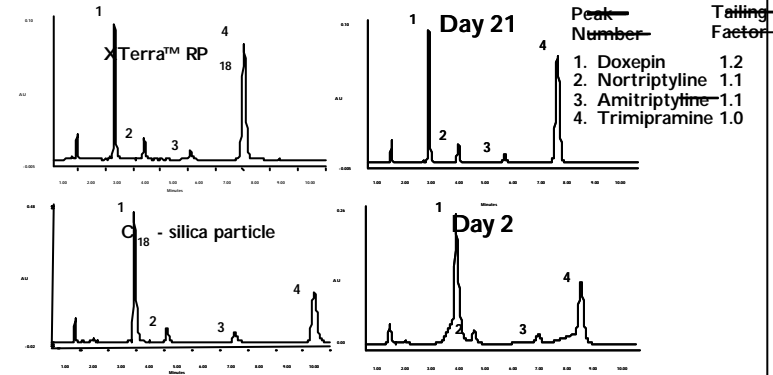
Temperature Effects on Resolution - Gradient



Conditions

- Column: Symmetry300™, C 5 μm , 3.9x150mm
- Sample: Tryptic digests of bovine cytochrome
- 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



Tricyclic Antidepressant Separation

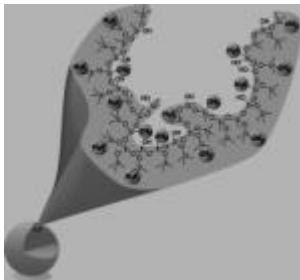
Stationary Phase Properties

CHEMISTRY:

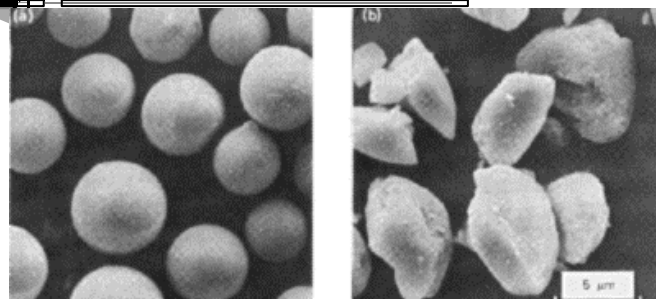
- * BONDED HYDROCARBON:
C-18, C-8, C-4, C-1, CN, phenyl
- * % COVERAGE
- * TYPE OF SILICA GEL

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]

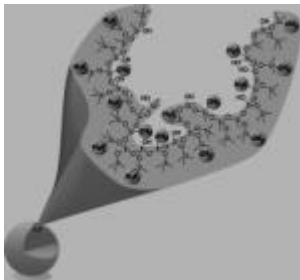
Stationary Phase Properties

CHEMISTRY:

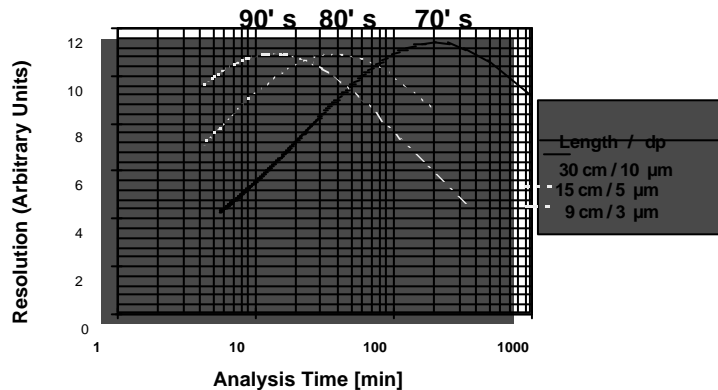
- * BONDED HYDROCARBON:
C-18, C-8, C-4, C-1, CN, phenyl
- * % COVERAGE
- * TYPE OF SILICA GEL

GEOMETRY

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



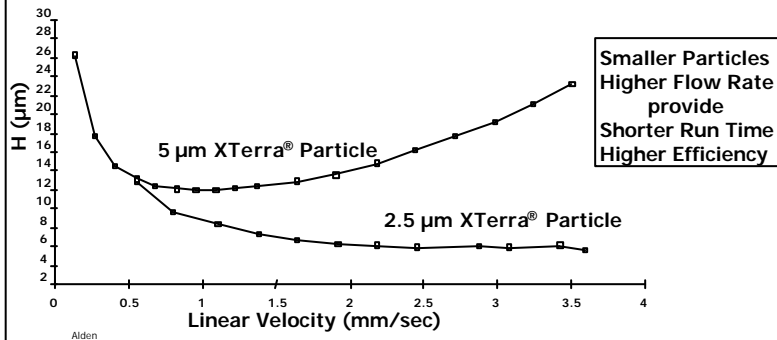
Resolution - Time Diagram



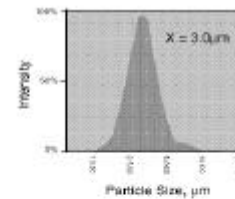
Reversed Phase HPLC

Comparison of the van Deemter Plots for 5 μm and 2.5 μm XTerra[®] MS C₁₈ Particles

(50/50, acetonitrile / water mobile phase)

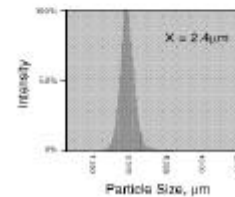


Challenge of producing smaller particles



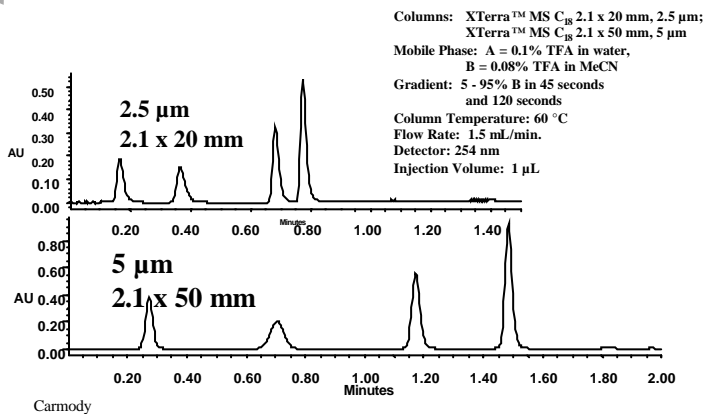
Contains a proportion of 2 μm particles

Both are commercial '2 μm ' packings

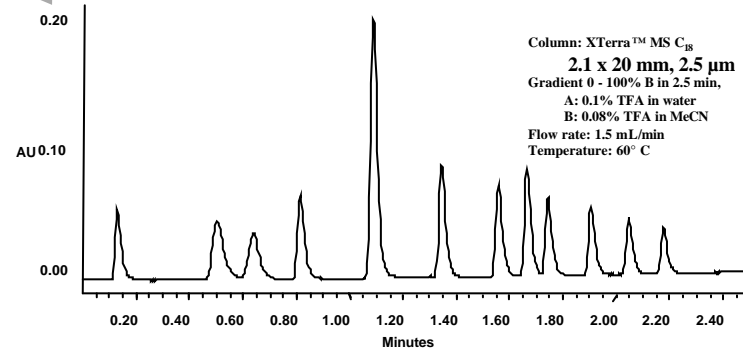


Centered at 2.4 μm
Narrower distribution
(Waters proprietary technology)

Fast Gradient Application



Fast Gradient of 12 Standards

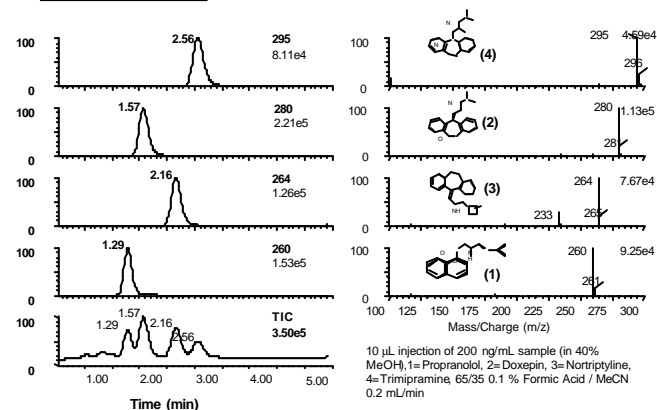


Fast LC/MS Applications

- ◆ 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: 175°C

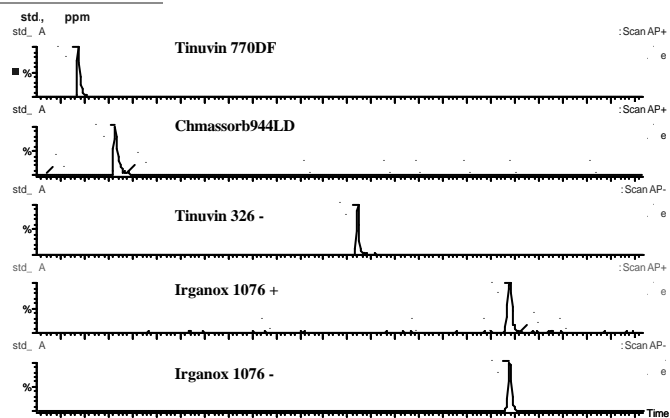
Fast LC-MS Analysis

XTerra™ MS C18, 2.1 x 50 mm (5 µm)



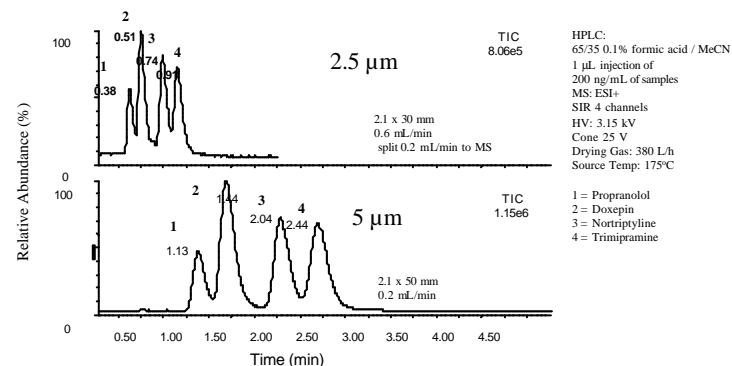
Ding

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



Fast LC-MS Analysis

XTerra™ MS C18: 5 µm vs.. 2.5 µm



Ding

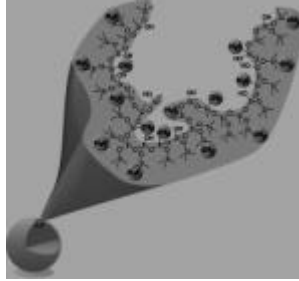
Stationary Phase Properties

CHEMISTRY:

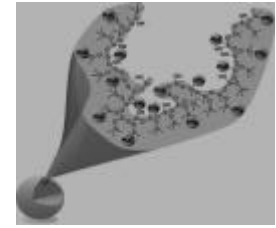
- * BONDED HYDROCARBON:
C-18, C-8, C-4, C-1, CN, phenyl
- * % COVERAGE
- * TYPE OF SILICA GEL

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 m²/gram)
 - ♦ (300 Å approx. 100 m²/gram)
 - "The greater the surface area, the greater the retention."
- * A typical 15 cm column holds a surface area of ~100-300 square meters

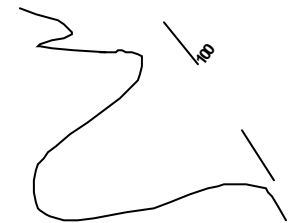
Silica Gel Pore Structure

- * Silica is Porous
- * Pore Size, or nm --distribution
- * Specific Pore Volume, mL/g

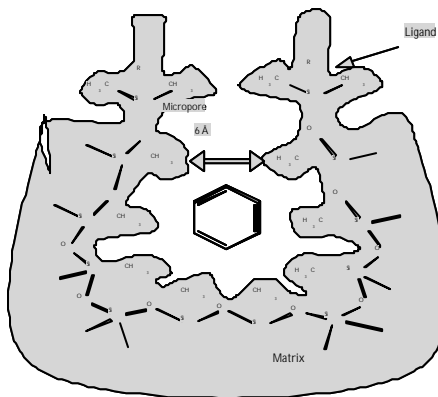
Range: 0.3 -- 1.3 mL/g

SV Particle Strength

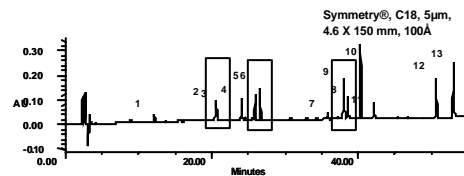
| Analyte MW | Pore Size Recommendation |
|----------------|---------------------------|
| < 3,000 | 60 - 130 (6 - 13 nm) |
| 3,000 - 10,000 | 100 (10 nm) |
| >10,000 | 300 - 1,000 (30 - 100 nm) |
| Very Large | non- porous |



Exclusion – Inclusion Effects

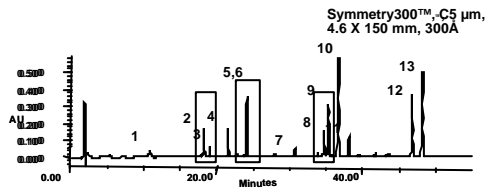


Pore Size Effects on Resolution



Conditions

- Sample: Tryptic digests of cytochrome (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

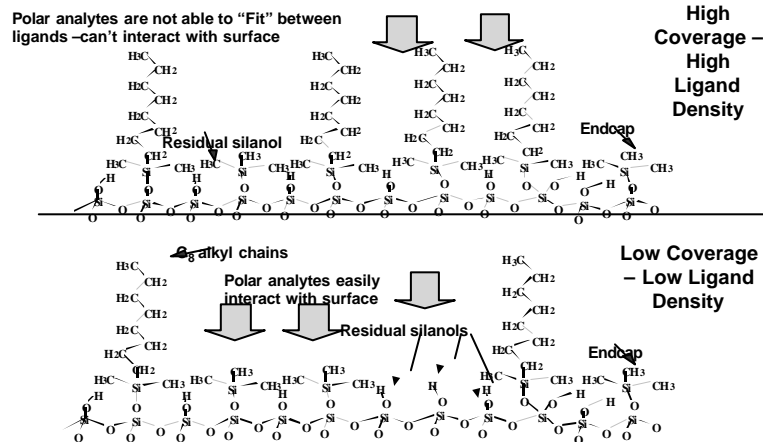


-Different pore sizes change selectivity.

Polarity/Aqueous Columns:

- Low ligand density
- High pore volumn

Mechanism of Retention of Polar Compounds on Aqueous Columns

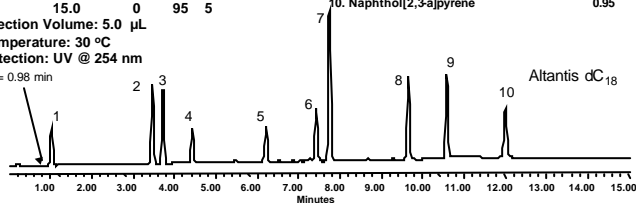


Polar Compounds - Aqua Columns

Polar and Non-Polar Compounds Test Mix

| Conditions | | | Compounds | | USP Tailing: |
|-----------------|-------------------------------------|--------------------|---|--|--------------|
| Columns: | 4.6 x 150 mm, 5 µm | | 1. Uracil | | 1.04 |
| Mobile Phase A: | H ₂ O | | 2. Acetanilide | | 0.95 |
| Mobile Phase B: | ACN | | 3. Triamcinolone | | 1.02 |
| Mobile Phase C: | 100 mM NH ₄ COOH, pH 3.0 | | 4. Hydrocortisone | | 1.03 |
| Flow Rate: | 2.0 mL/min | | 5. 2-Amino-7-chloro-5-oxo-5H-[1]-benzopyrano[2,3-b]pyridinecarbonitrile | | 1.01 |
| Gradient: | Time (min) | Profile (%A %B %C) | 6. 6a-Methyl-17a-hydroxyprogesterone | | 1.01 |
| | 0.0 | 80 10 10 | 7. 3-Aminofluoranthene | | 0.97 |
| | 10.0 | 0 95 5 | 8. 2-Bromofluorene | | 1.00 |
| | 15.0 | 0 95 5 | 9. Perylene | | 0.99 |
| | | | 10. Naphthol[2,3-a]pyrene | | 0.95 |

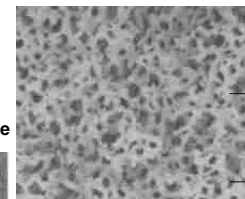
Injection Volume: 5.0 µL
 Temperature: 30 °C
 Detection: UV @ 254 nm
 V₀ = 0.98 min



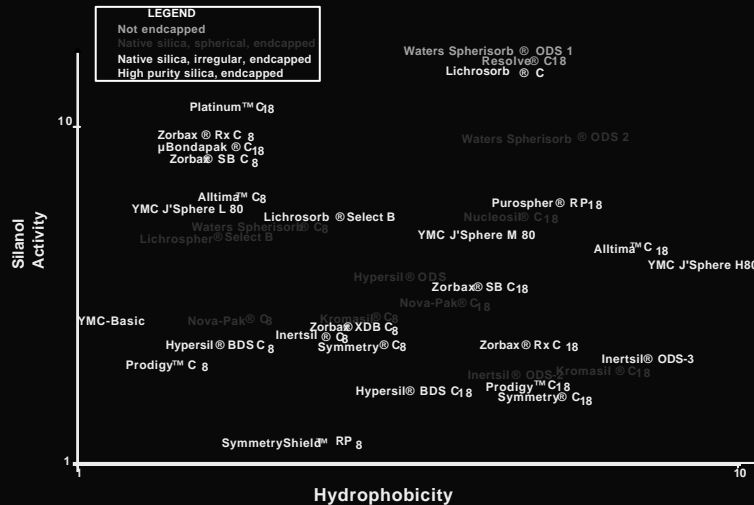
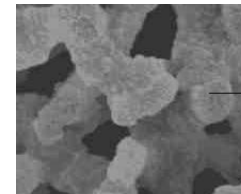
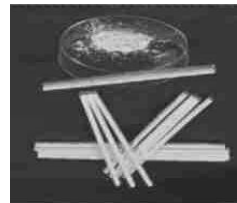
Chromolith Packing

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™ C₈ 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

