Reversed Phase Advanced Features

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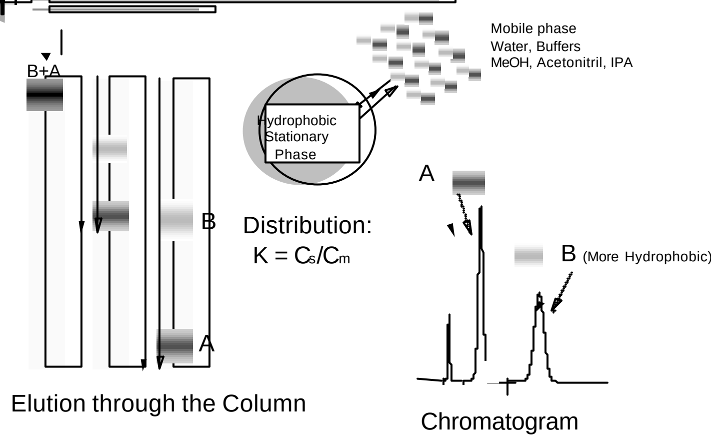
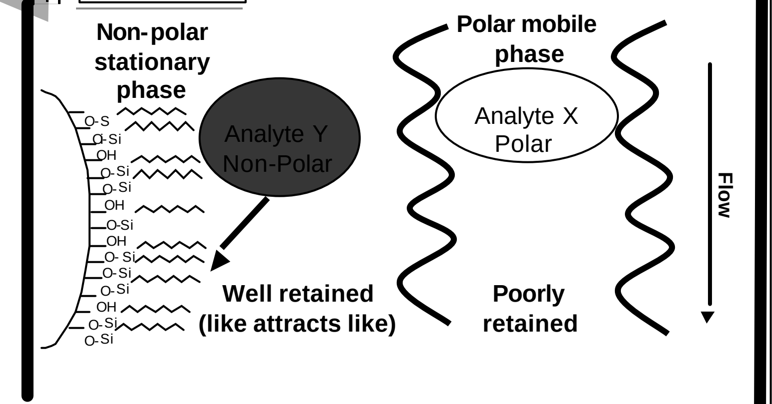
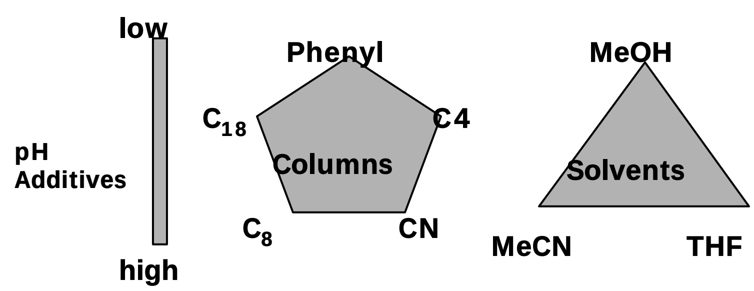
Chromatographic Process



Reversed-Phase Chromatography

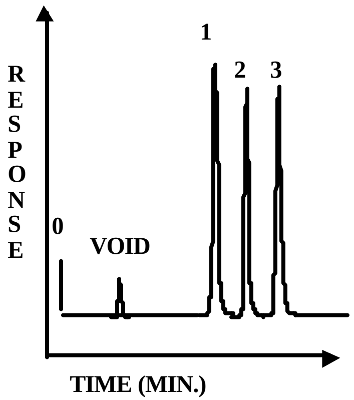


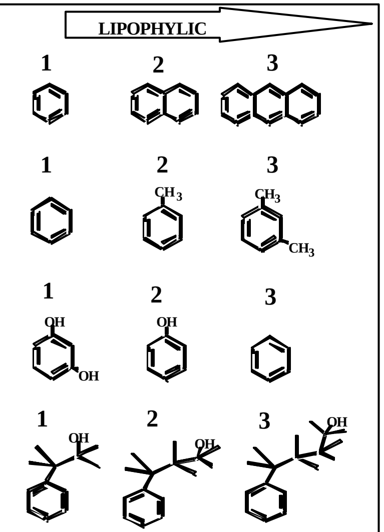
RP Method Development Tools



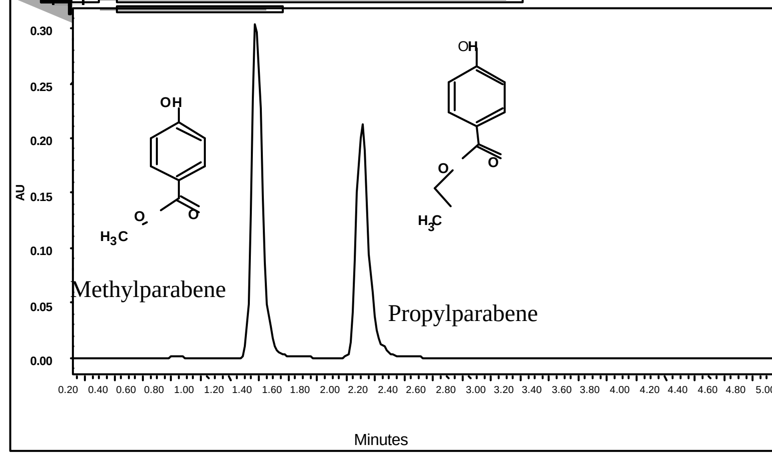
Reversed Phase HPLC

ELUTION ORDER IN REVERSED PHASE





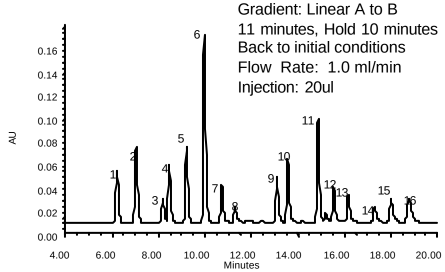
Reversed Phase Elution Order



PAH Analysis with Alliance System and PDA Using a Binary Gradient

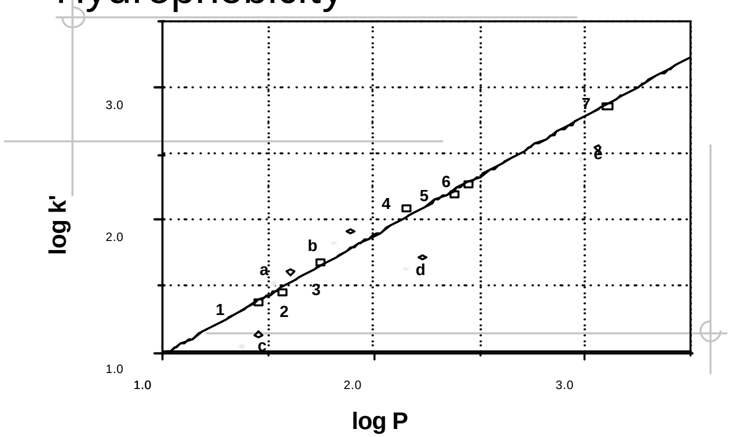
UV@254nm

Column- HibarRT 125-4
LiChrosphere PAH
Eluent A: Water
Eluent B: Acetonitrile
Gradient: Linear A to B
11 minutes, Hold 10 minutes
Back to initial conditions
Flow Rate: 1.0 ml/min
Injection: 20ul



1- Naphthalene - 20 ppm
2- Acenaphthylene- 40 ppm
3- Acenaphthene- 20 ppm
4- Fluorene- 4 ppm
5- Phenanthrene- 2 ppm
6- Anthracene- 2 ppm
7- Fluoranthene- 4 ppm
8- Pyrene- 2 ppm
9- Benzo(a)anthracene- 2 ppm
10- Chrysene- 2 ppm
11- Benzo(b)fluoranthene- 4 ppm
12- Benzo(k)fluoranthene- 2 ppm
13- Benzo(a)pyrene- 2 ppm
14- Dibenzo(a,h)anthracene- 4 ppm
15- Benzo(g,h,I)perylene-4 ppm
16- Indeno(1,2,3-cd)pyrene-2 ppm

Hydrophobicity



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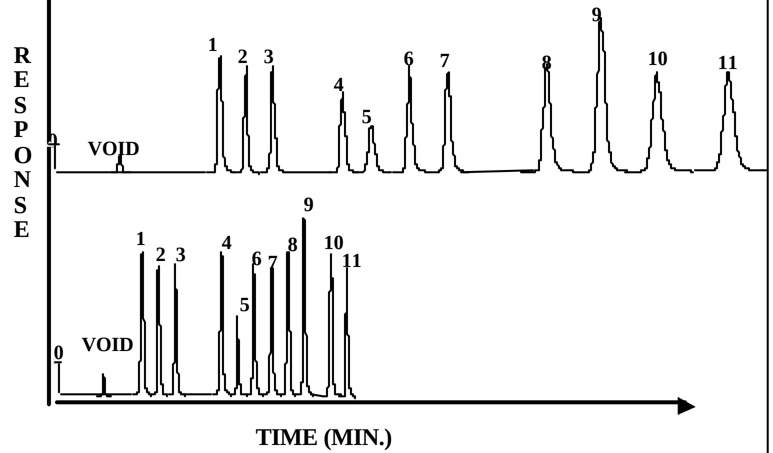
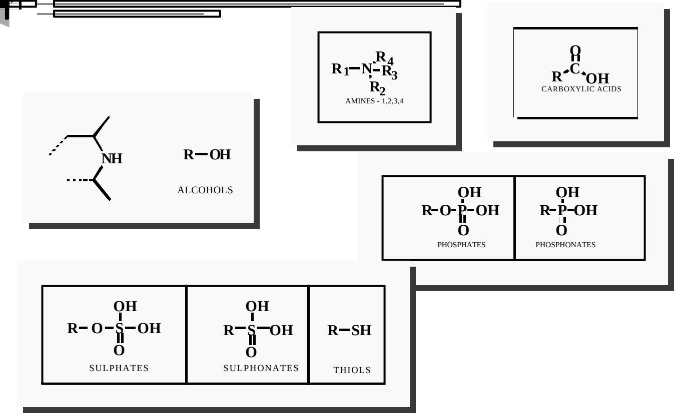
ISOCRATIC vs GRADIENT



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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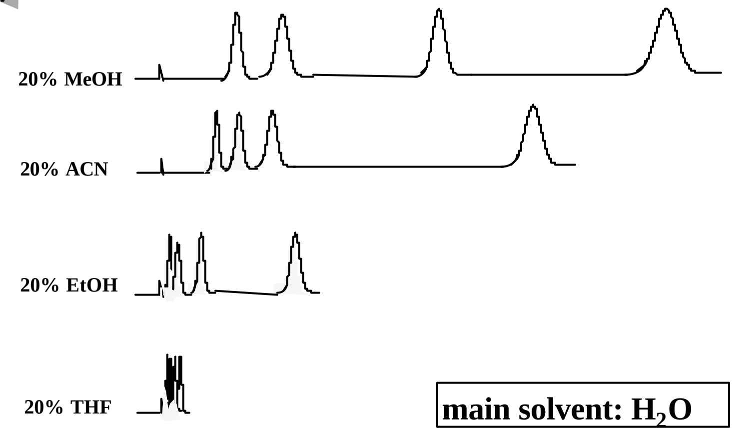
OPTIMIZATION: CHOICE OF SOLVENTS



main solvent: H_2O

MOBILE PHASE

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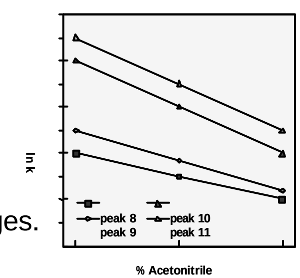
- ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)

SOLVENT STRENGTH

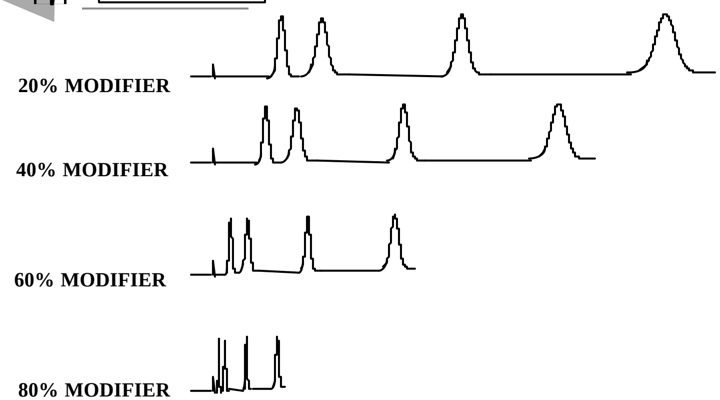
Analyte Retention as a Function of % Modifier

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

Thus, the resolution between peaks changes.



OPTIMIZATION: % SOLVENTS



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Ionization of Acids and Bases

Dissociation of Molecule

Acid

HA (Un-ionized) ⇌ H^+ + A^- (Ionized)

HA		A^-
50%	@ pKa	50%
100%	Low pH	0%
0%	High pH	100%

Ionization of Acids and Bases

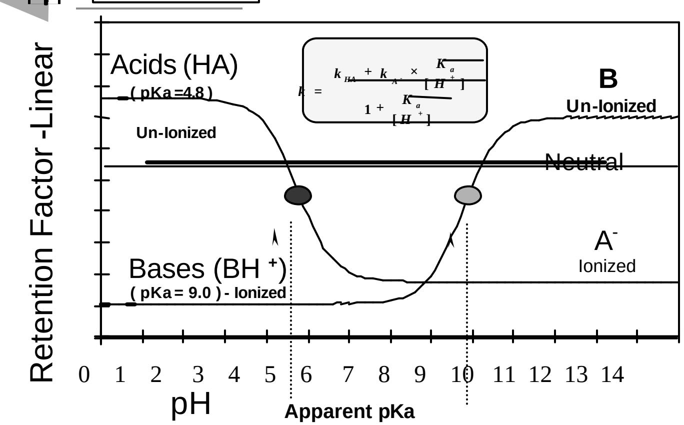
Dissociation of Molecule

Base

B (Un-ionized) ⇌ BH^+ (Ionized) + OH^-

B		BH^+
50%	@ pKa	50%
0%	Low pH	100%
100%	High pH	0%

Retention Factor versus pH for Acids, Bases and Neutrals



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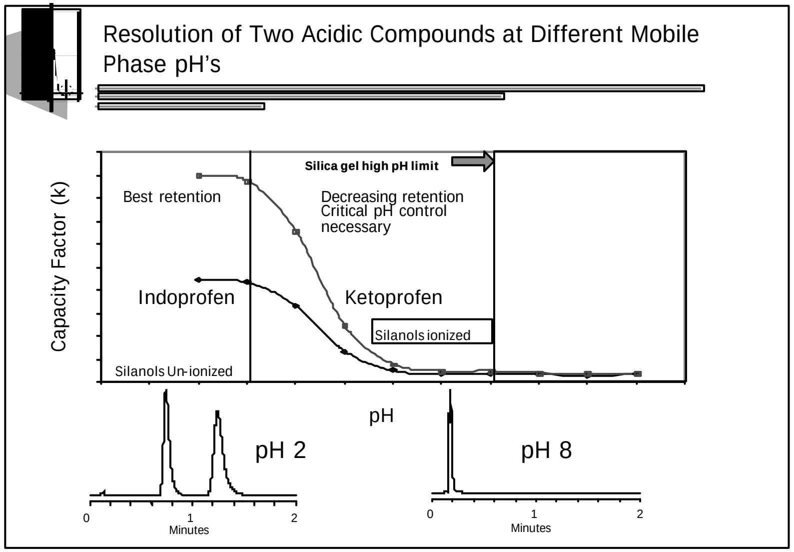
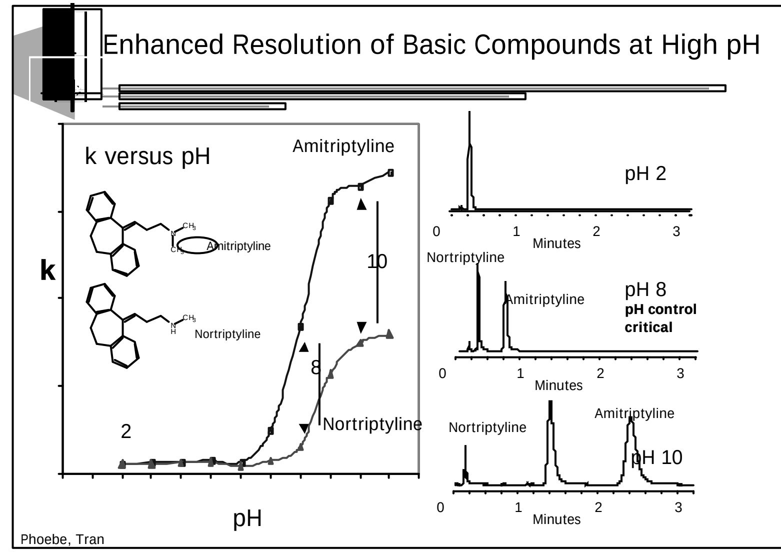
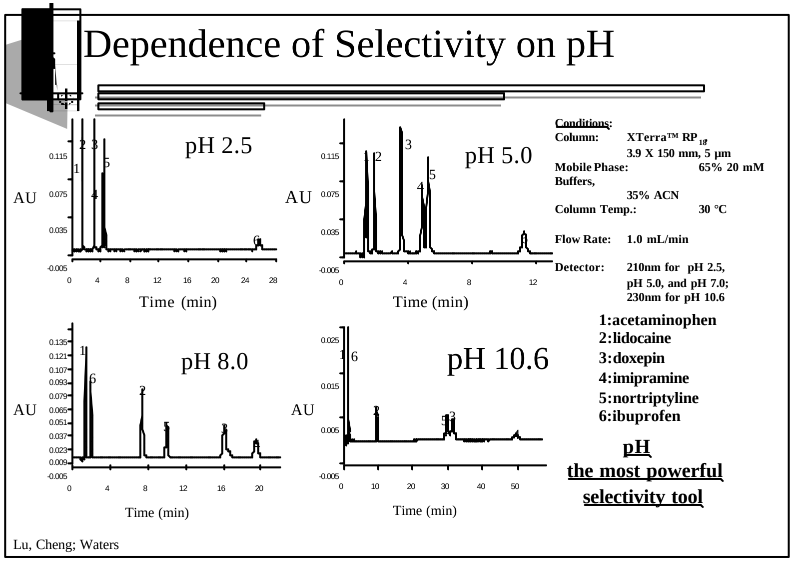
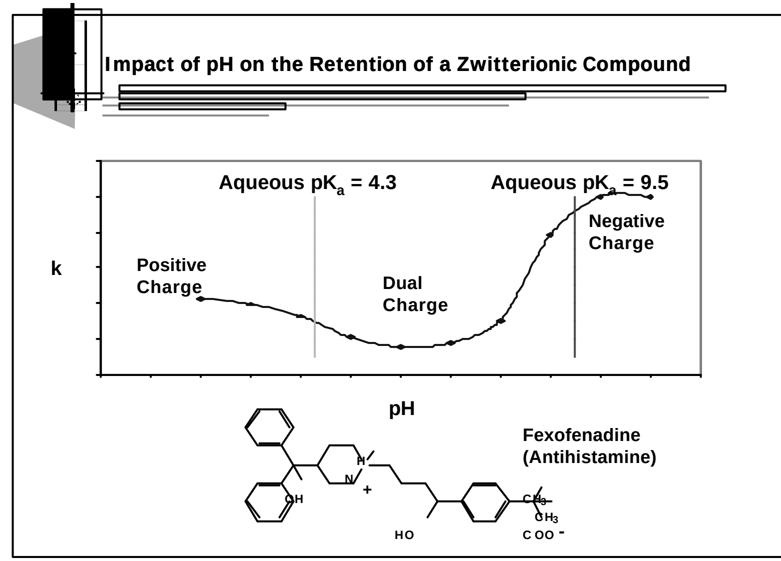
Reversed Phase HPLC









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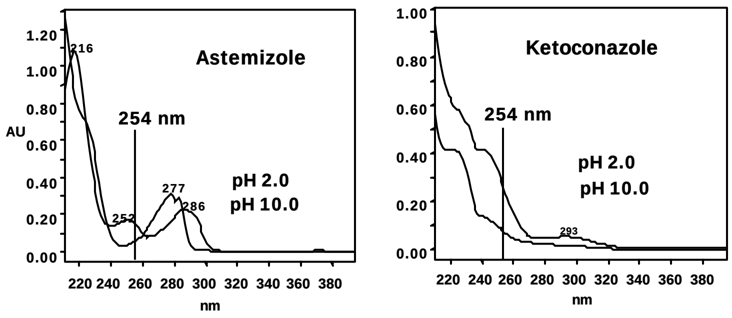
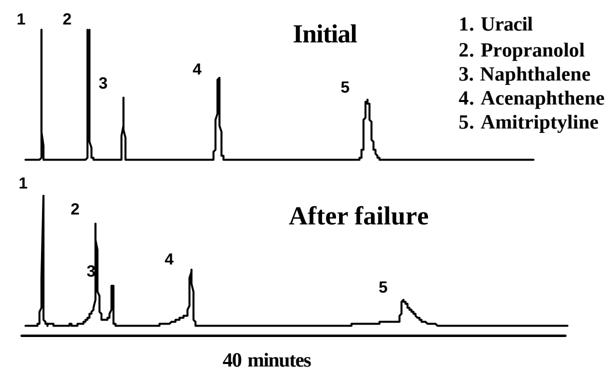
UV/Vis Spectral Change Between Ionized and Non-ionized Forms



Phoebe, Tran

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



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- * ION-PAIRING REAGENTS (alkyl-amines, -sulfonates)

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)

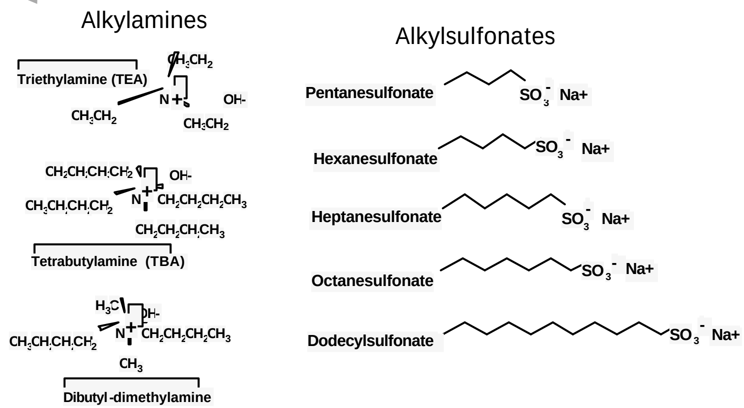
Types of Buffers and Ionic Strength

- 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)

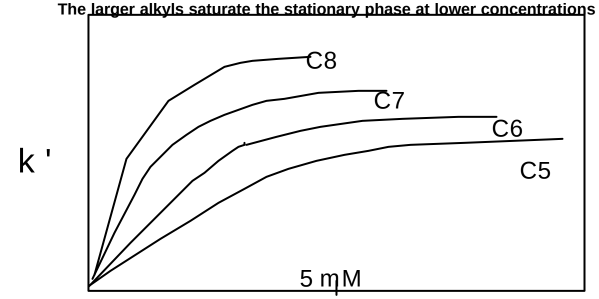
Ion Pair Reagent



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

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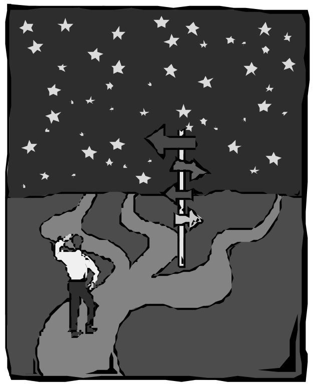
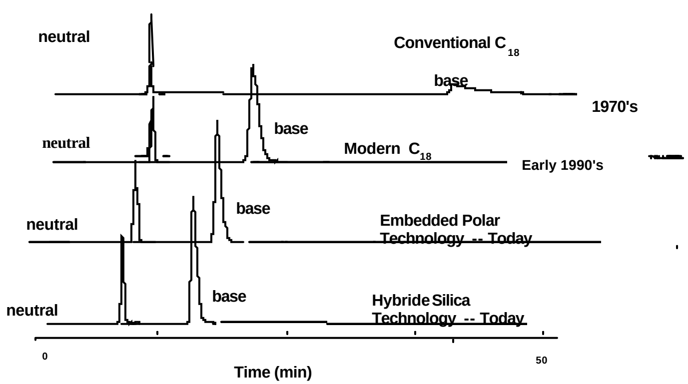
Stationary Phase Characterization



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!

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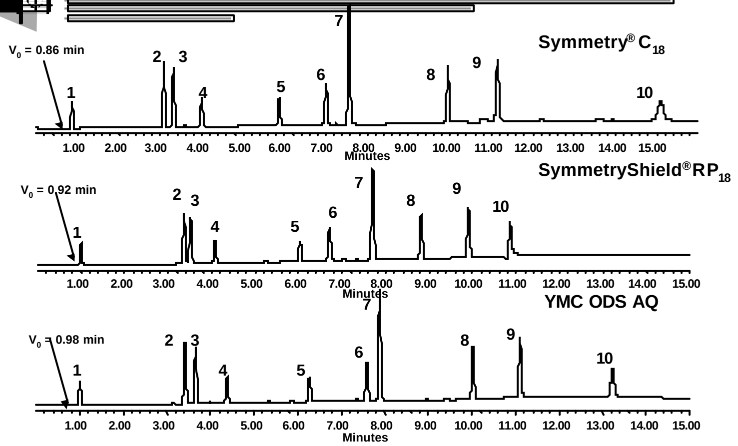
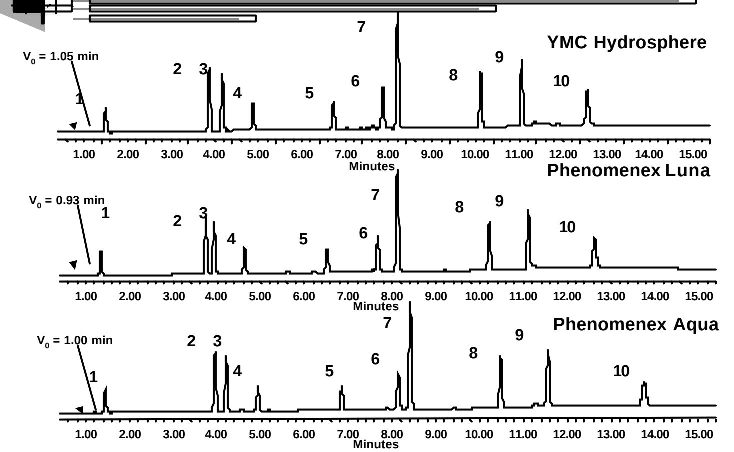
Different Columns – Different Chromatograms



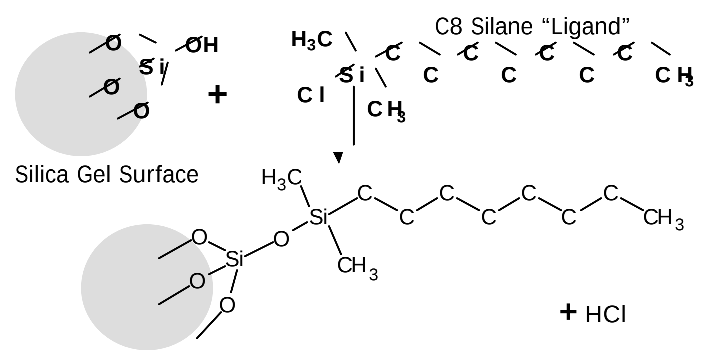
Different Columns – Different Chromatograms



"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



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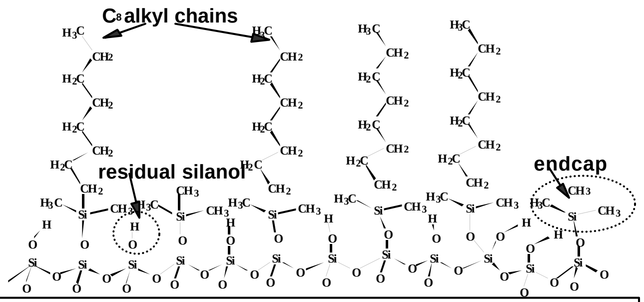
Surface of a Silica Gel Bonded-Phase Packing Material



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

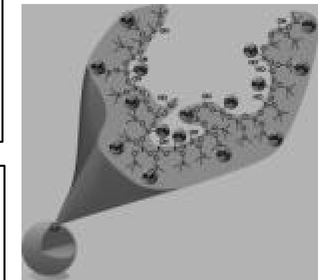
Stationary Phase Properties

CHEMISTRY:

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

GEOMETRY

* SPHERE- IRREGULAR
* PARTICLE DIAMETER
* POROSITY



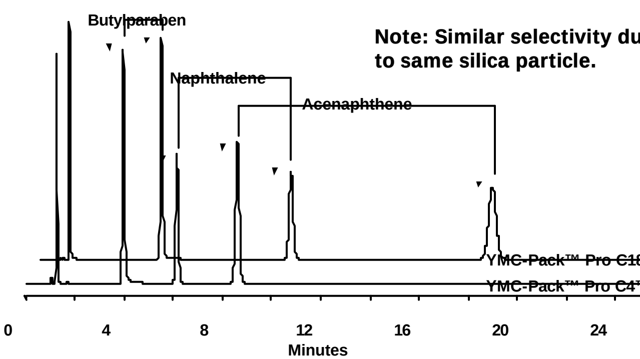
Stationary Phase Ligands

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	$-Si\ (CH_2)_3O\ CH_2CH(OH)CH_2OH$

Retention time

Chain length CN Phenyl NH2 C4 C8 C18

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



Note: Similar selectivity due to same silica particle.

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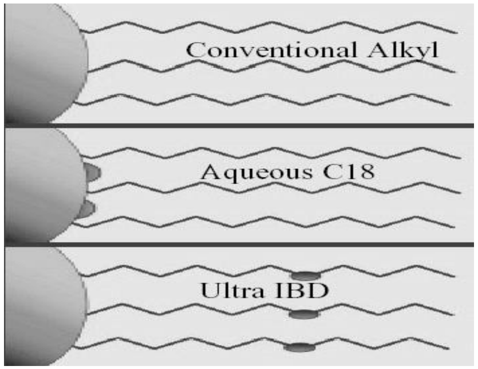
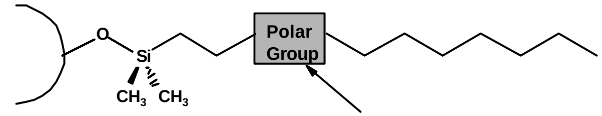
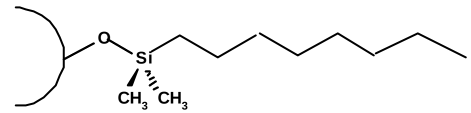
Type of Ligands



Reversed-Phase Packing with an Embedded Polar Ligand

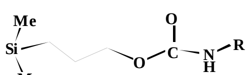


Embedded Polar Group Ligand

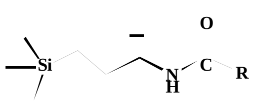


Traditional, Straight Chain Alkyl Ligand

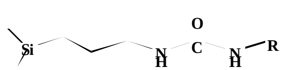
Commercial Phases with Embedded Polar Group



SymmetryShield RP (Waters)

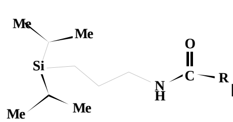


Discovery RP Amide16 (Supelco)



Prism, Spectrum (Keystone)

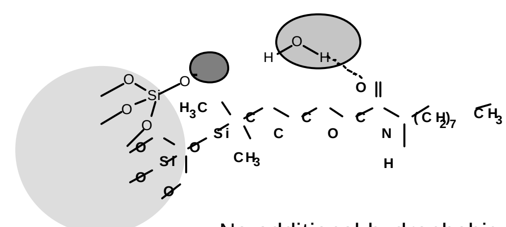
- reduces interaction with silanols
- modifies selectivity
- improves water wettability



Bonus RP (Hewlett Packard)

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

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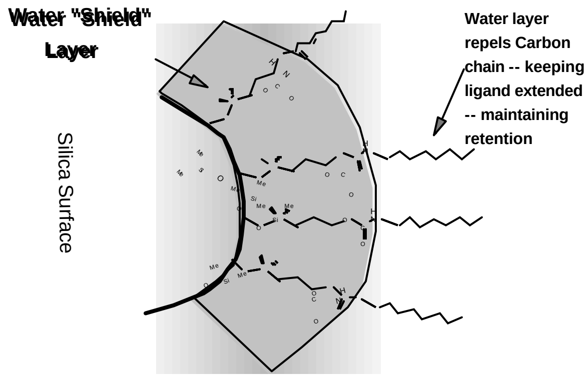
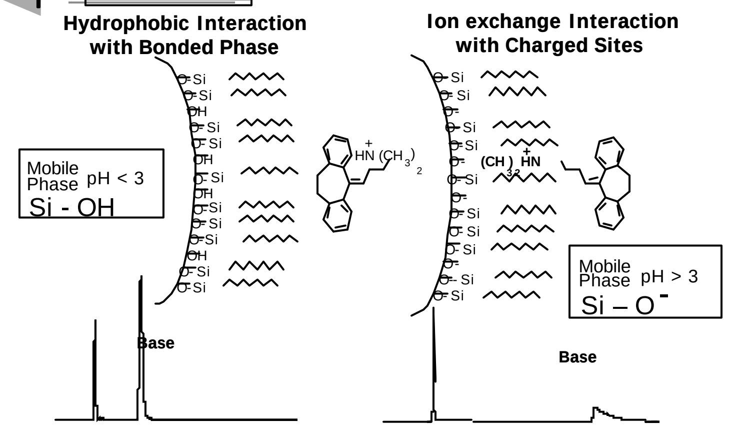
Embedded Polar Goups

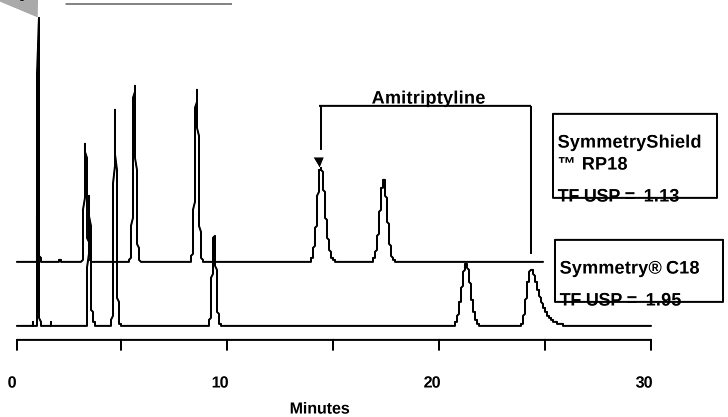
Embedded Polar Wetted Surface



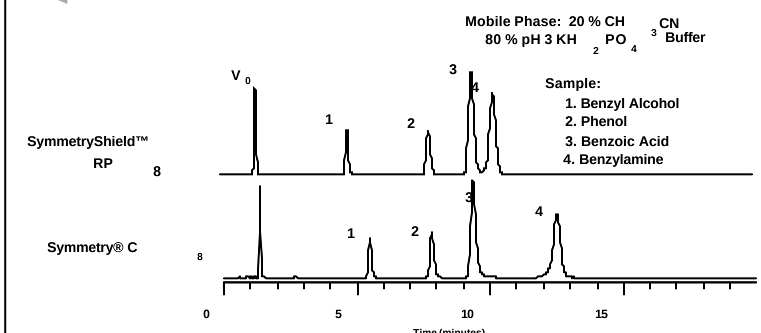
Mixed-Mode Retention:



Embedded Polar Ligand versus Linear Alkyl Ligand on Silica Gel



Impact on Selectivity - Retention



Reduced Retention of Amines

(Shielding of Silanols)

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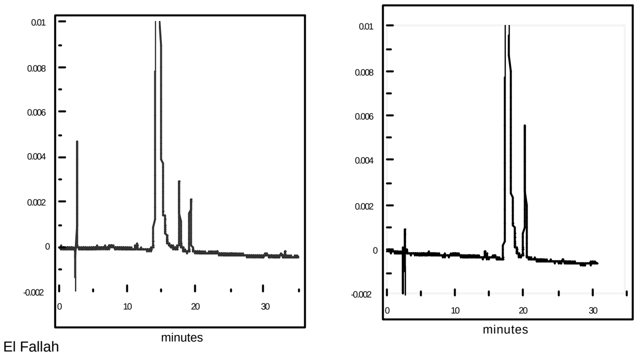
Selectivity Difference: Furazolidone Impurities

SymmetryShield™ RP_8 Symmetry® C_8



El Fallah

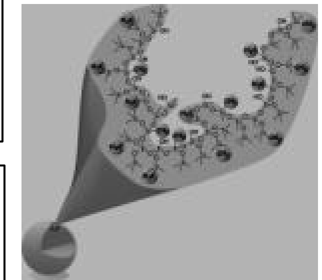
Stationary Phase Properties

CHEMISTRY:

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

GEOMETRY

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



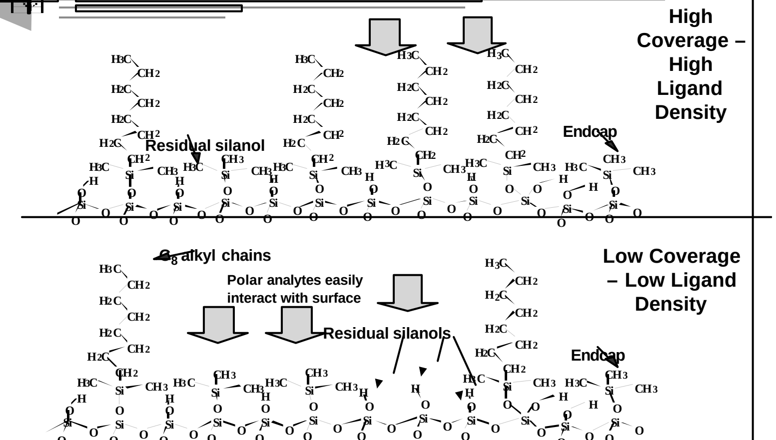
CARBON LOAD

Increasing carbon load on a <u>similar geometrical shaped</u> particles increases retention.

Retention time

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

Surface of a Silica Gel Bonded-Phase Packing Material



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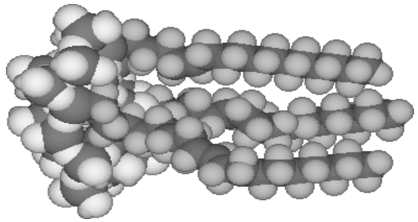
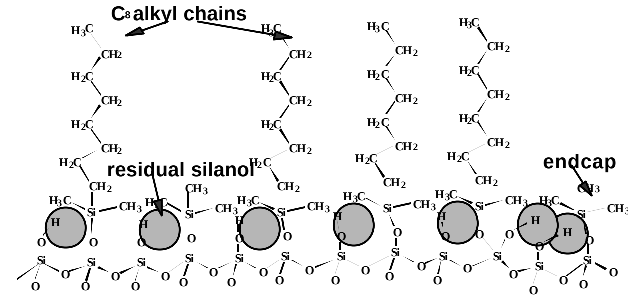
Silica based "bonded phases"



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/m^2
Silica Silanols :	6 - 8
Highest Bonding Reported :	4.2
Residual Silanols (Best Case) :	2.0 [~ 30%]
Residual Silanols (Typical) :	> 3.5 [> 50%]

Better Way to Compare: Ligand Density (Surface Coverage)

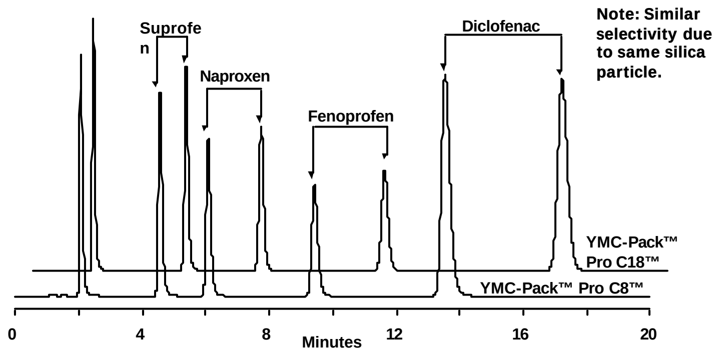
$$C = \frac{\%C}{100 \cdot SA \cdot nC \cdot 12 \left[1 - \frac{\%C}{100} \cdot \frac{MW - 1}{nC \cdot 12} \right]} = \mu moles/m^2$$

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

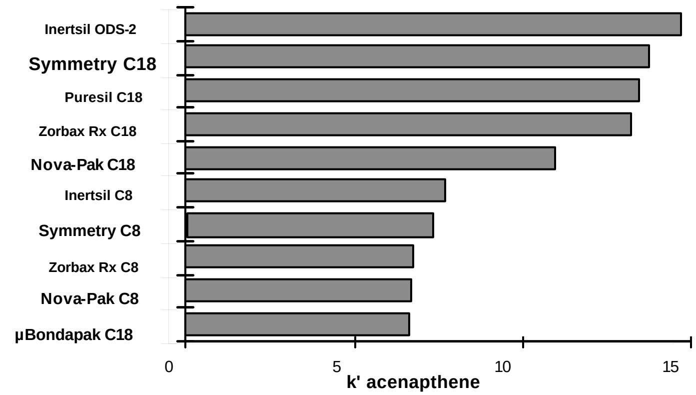
Ligand Density ↑ Retention ↑ Silanols ↓

Surface Area ↑ % C → Ligand Density ↓

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



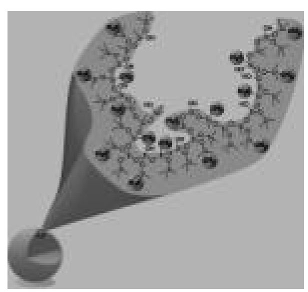
Relative Hydrophobicities of General Purpose Analytical Packings



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

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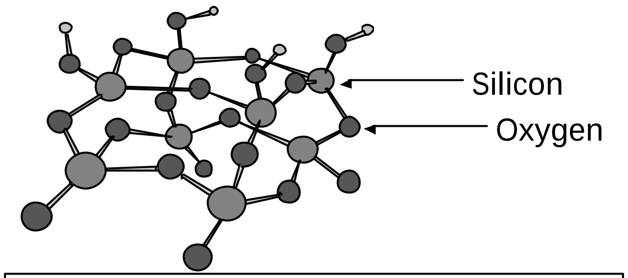
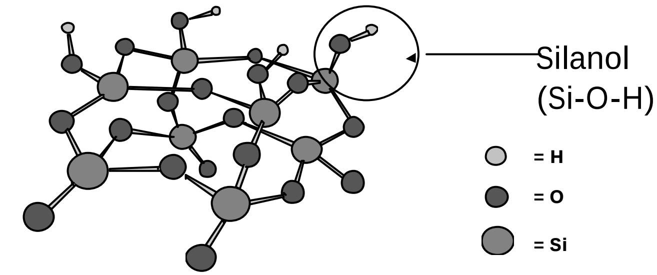
Structure of Silica Gel



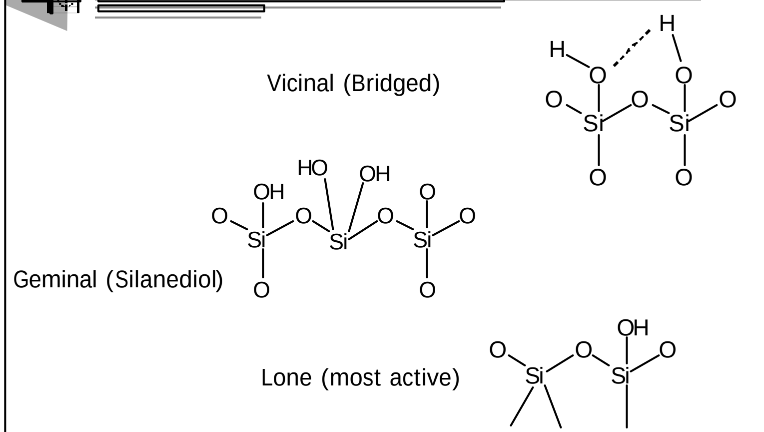
- **Amorphous, porous matrix of silicon atoms joined together with oxygen atoms to form “siloxane bonds” = (Si – O – Si)**

What are Silanols?

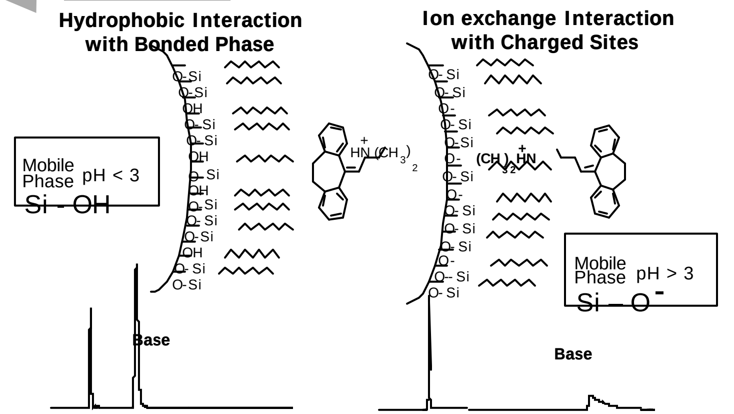


- **Residual unreacted surface hydroxyl groups left over from polymerization**
- **Reactive sites for use in bonding ligands (C18) to the silica gel surface**

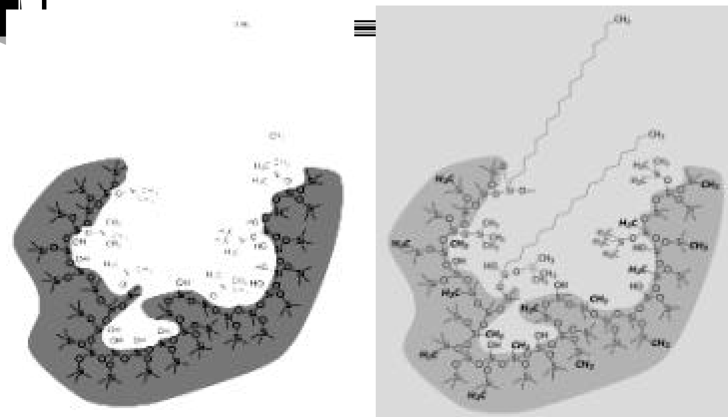
Surface Silanols Found on Silica Gel



Mixed-Mode Retention:



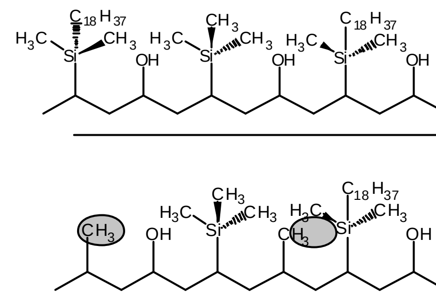
Bonded Phase on Particles



Silica Gel C18 Materials
1/2 free silanols

XTerra™ C18 Materials
1/3 free silanols

Bonded Hybrid versus Bonded Silica Gel Surfaces



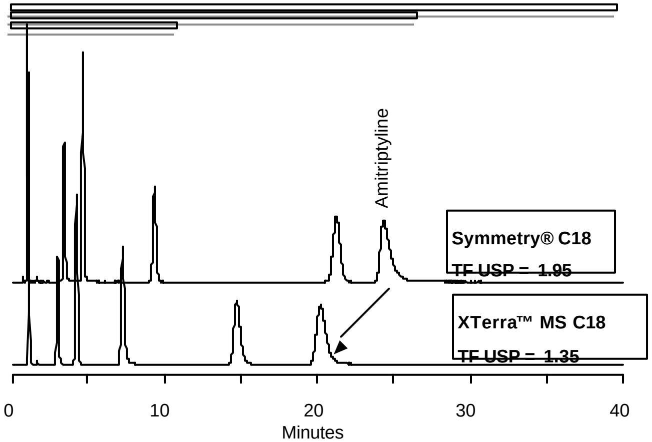
C_{18}-Bonded Silica Gel

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



PERFORMANCE BY ONE PEAK

ASYMMETRY FACTOR

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

RETENTION FACTOR or CAPACITY RATIO

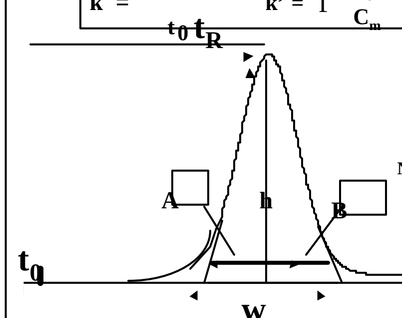
$$k' = \frac{t_R - t_0}{t_0} \qquad k' = f\ \frac{C_s}{C_m}$$

TAILING FACTOR

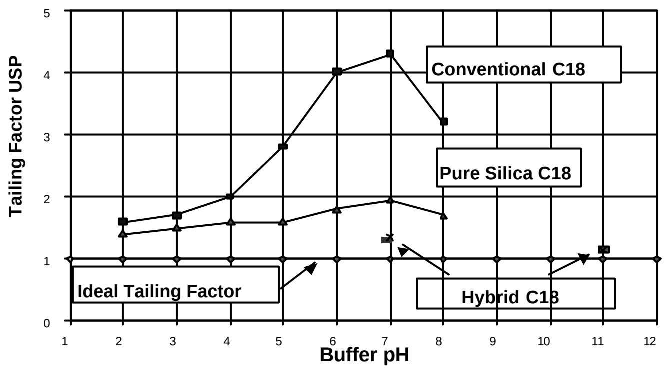
$$T_{f\ (5\%\ h)} = \frac{A + B}{2\,A}$$

NUMBER OF THEORETICAL PLATES

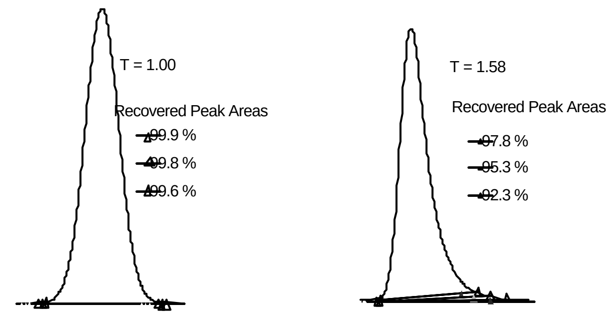
$$N = 16\left(\frac{t_R}{w}\right)^2$$



Amitriptyline Peak Tailing Over Extended pH Range 1-12



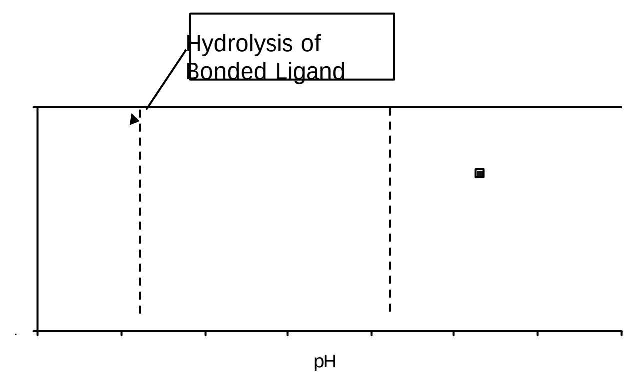
Integration Errors Caused by Tailing



Types of Silica

- Silanols
- pH stability
- Metal content
- Temperature stability

pH Limitations of Silica Based Packing Materials



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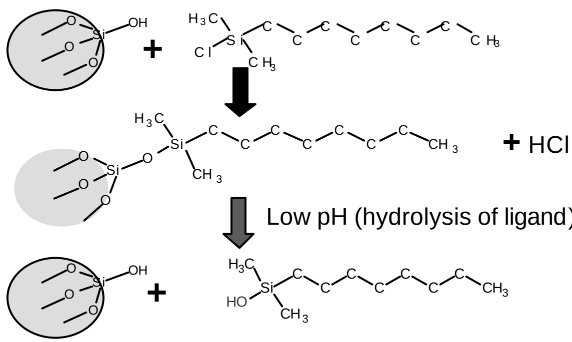
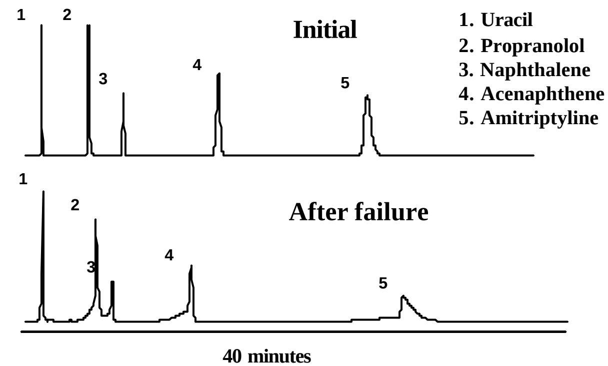
Hydrolysis of a Bonded Phase Material: Monofunctional Ligand



Typical Chromatograms for Reference C_{18}-Silica Column

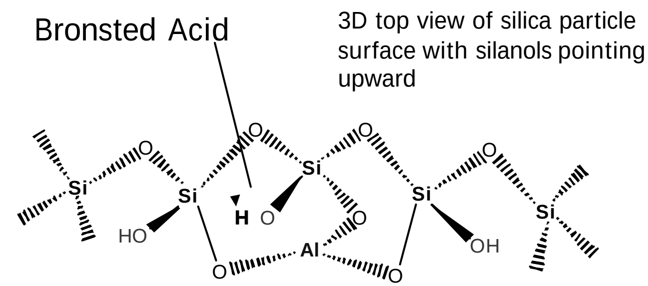


Types of Silica

- Silanols
- pH stability
- Metal content
- Temperature stability

Metal Content in Silica

Aluminum in the Silica Gel Lattice



Metal available for chelation

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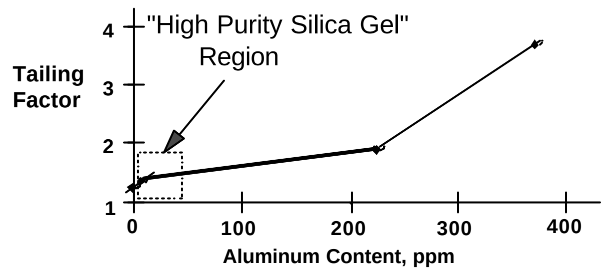
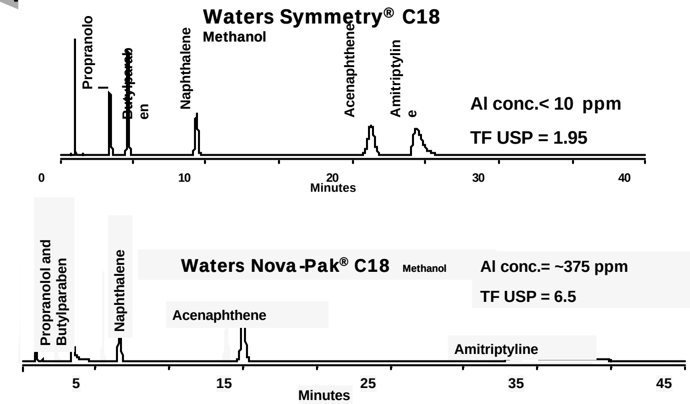
Correlation Between Base Tailing and Aluminum Content of Silica Gel

Analyte: Chlorpheniramine

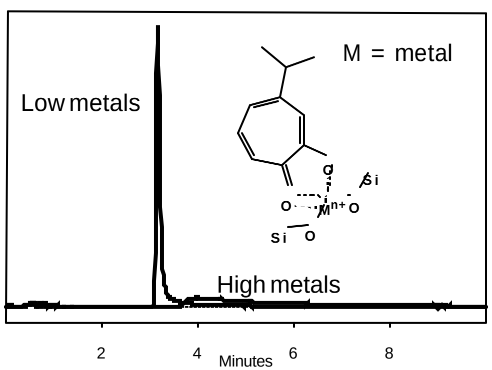
Mobile Phase: Acetonitrile/KH_2PO_4 pH 3.0 (20:80)



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

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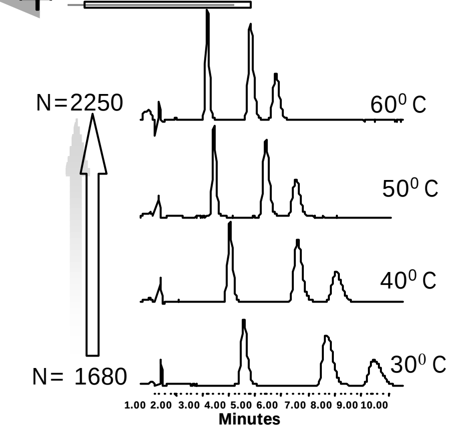
Temperature Effects on Resolution

Resolution can be temperature dependent

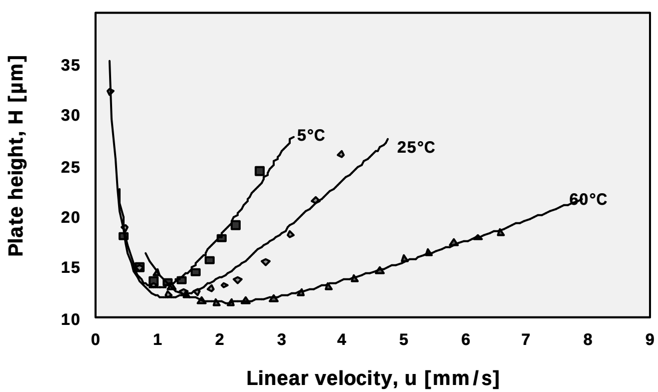
Temperature can be a critical parameter to control in order to achieve reproducible separations.

Effect of Temperature (Isocratic separations)

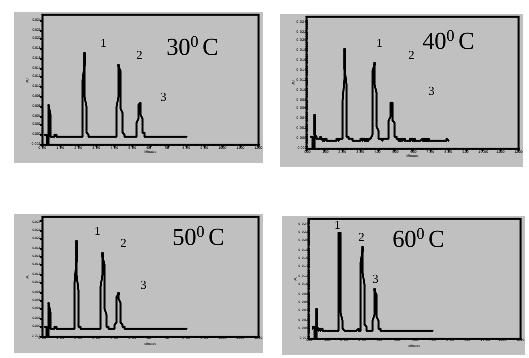


Higher Temperature:
- **Shorter Run Time**
- **Sharper Peaks**
- **Better Sensitivity**
- **Lower Back Pressure**

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

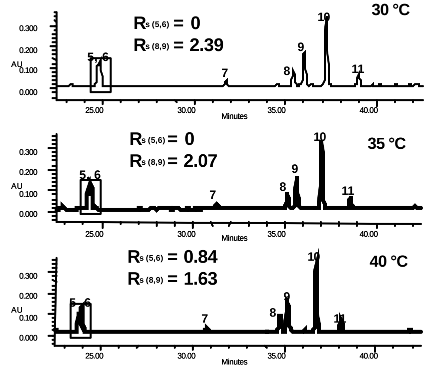
Detector: 210 nm

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

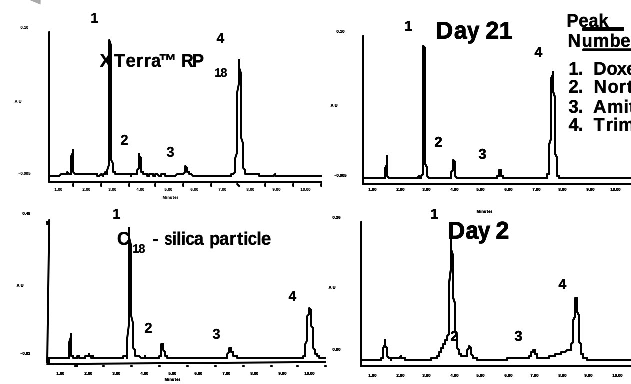
Temperature Effects on Resolution - Gradient



Conditions

- Column: Symmetry300™ C_{18}, 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



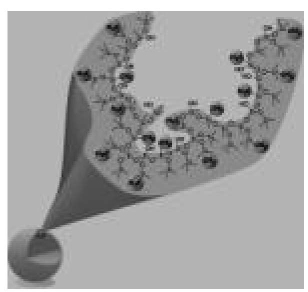
Peak Number	USP Tailing Factor
1. Doxepin	1.2
2. Nortriptyline	1.1
3. Amitriptyline	1.1
4. Trimipramine	1.0

Tricyclic Antidepressant Separation

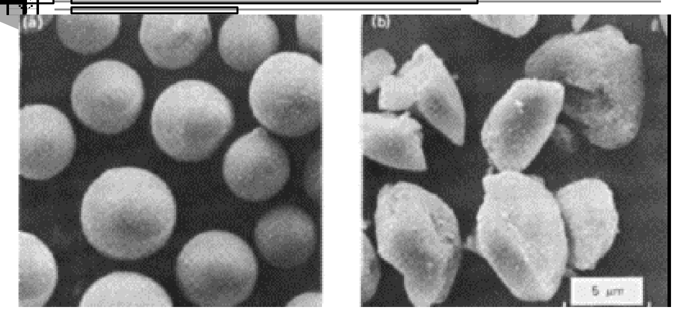
Stationary Phase Properties

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

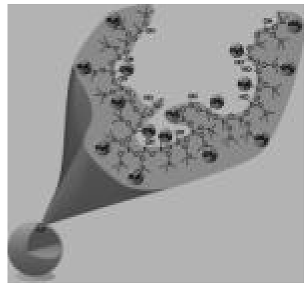


Electron microphotograph of spherical and irregular silica particles. [W.R.Melander, C.Horvath, Reversed-Phase Chromatography, in HPLC Advances and Perspectives, V2, Academic Press, 1980]

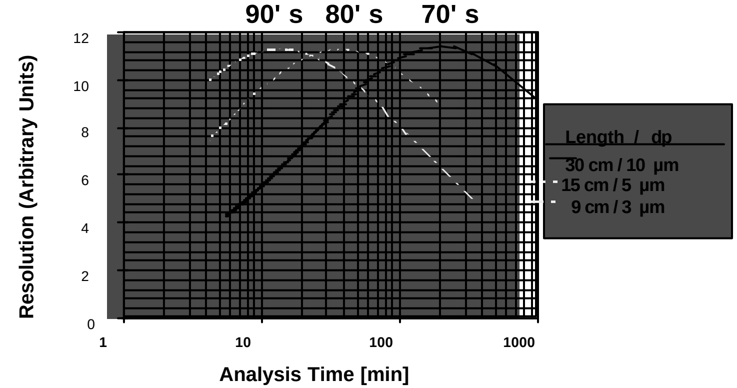
Stationary Phase Properties

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

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

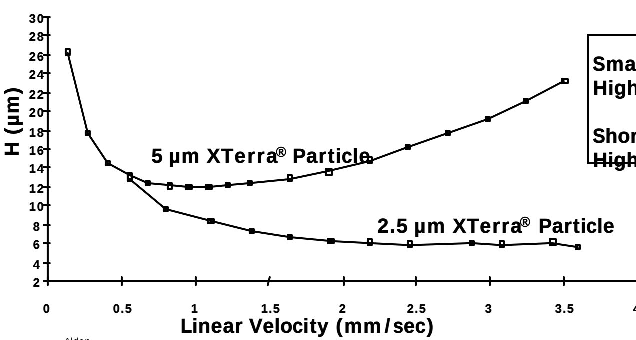


Resolution - Time Diagram



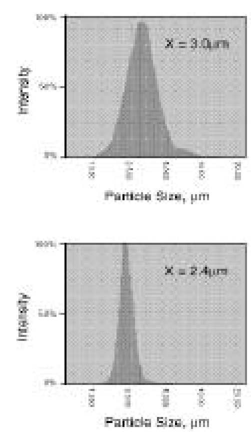
Comparison of the van Deemter Plots for 5 µm and 2.5 µm XTerra® MS C_{18} Particles

(50 / 50, acetonitrile / water mobile phase)



Smaller Particles
Higher Flow Rate
provide
Shorter Run Time
Higher Efficiency

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)

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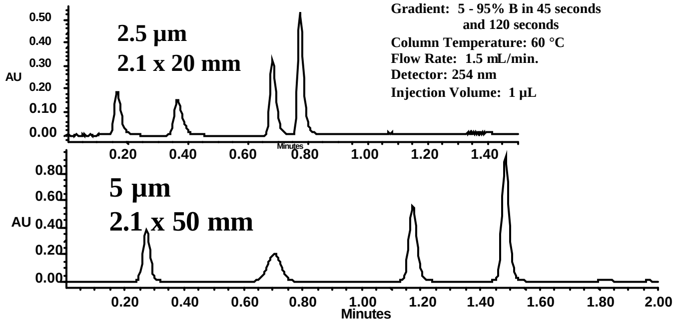
Fast Gradient Application

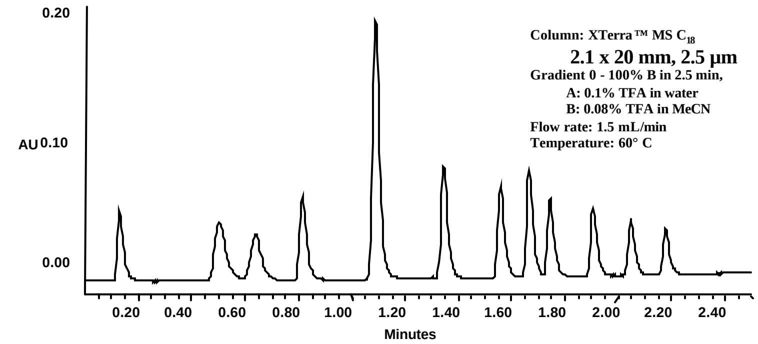
Columns: XTerra™ MS C_{18} 2.1 x 20 mm, 2.5 µm;
XTerra™ MS C_{18} 2.1 x 50 mm, 5 µm
Mobile Phase: A = 0.1% TFA in water,
B = 0.08% TFA in MeCN
Gradient: 5 - 95% B in 45 seconds
and 120 seconds
Column Temperature: 60 °C
Flow Rate: 1.5 mL/min.
Detector: 254 nm
Injection Volume: 1 µL



Carmody

Fast Gradient of 12 Standards

Column: XTerra™ MS C_{18}
2.1 x 20 mm, 2.5 µm
Gradient 0 - 100% B in 2.5 min,
A: 0.1% TFA in water
B: 0.08% TFA in MeCN
Flow rate: 1.5 mL/min
Temperature: 60° C

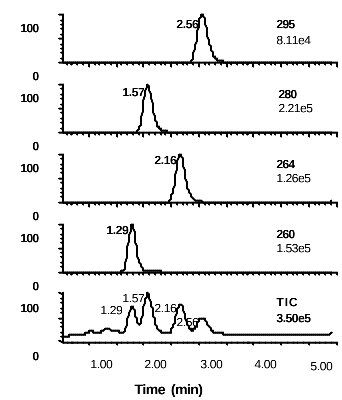


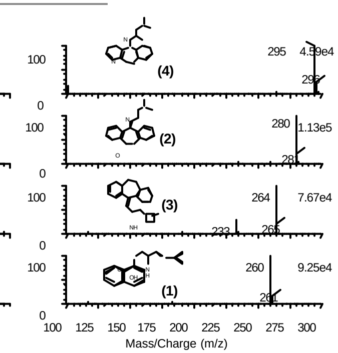
Fast LC/MS Applications

- 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

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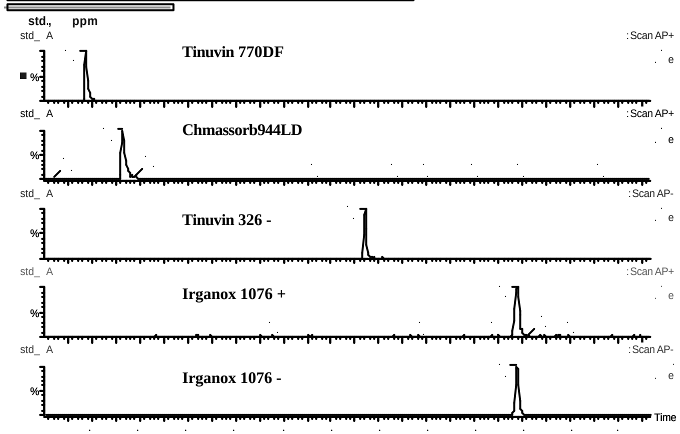
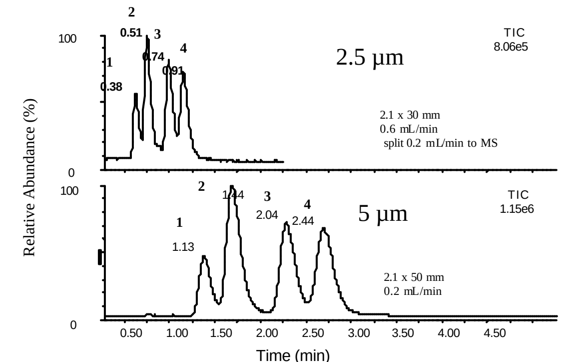
Masschromatograms of Std.10ppm (APcI +/-)



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

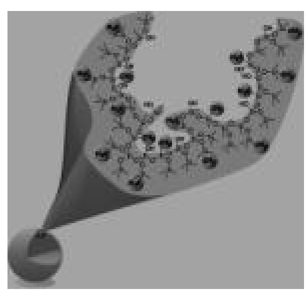
1 = Propranolol
2 = Doxepin
3 = Nortriptyline
4 = Trimipramine

Ding

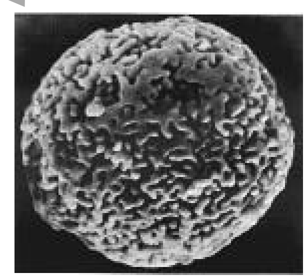
Stationary Phase Properties

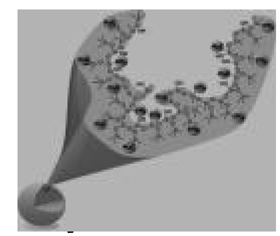
CHEMISTRY:
* BONDED HYDROCARBON:
C-18, C-8, C-4, C-1, CN, phenyl
* % COVERAGE
* TYPE OF SILICA GEL

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.

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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

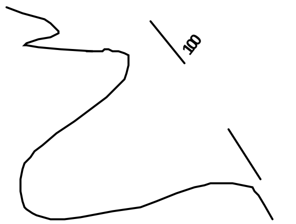
*** Silica is Porous**

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

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

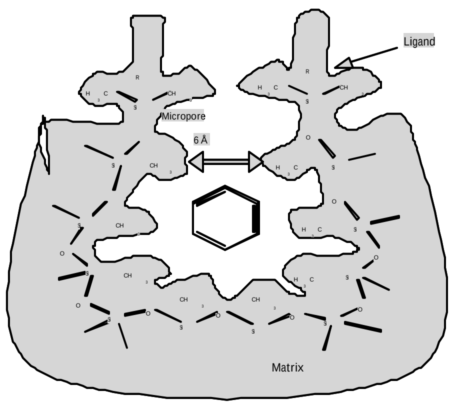
Range: 0.3 -- 1.3 mL/g

SV Particle Strength



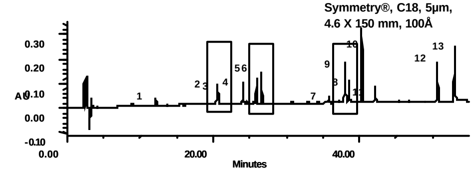
Analyte MW	Pore Size Recommendation
< 3,000	60 - 130 (6 - 13 nm)
3,000 - 10,000	100 (10 nm)
>10,000	300 - 1,000 (30 - 100 nm)
Very Large	non- porous

Exclusion – Inclusion Effects

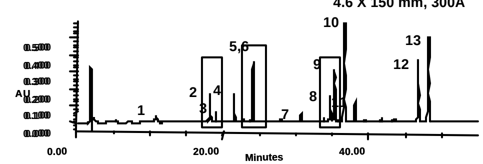


Pore Size Effects on Resolution

Symmetry®, C18, 5µm, 4.6 X 150 mm, 100Å



Symmetry300™, C5 µm, 4.6 X 150 mm, 300Å



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 ºC
- Flow Rate: 0.75 mL/min.
- Detection: 214 nm

-Different pore sizes change selectivity.

Carmody

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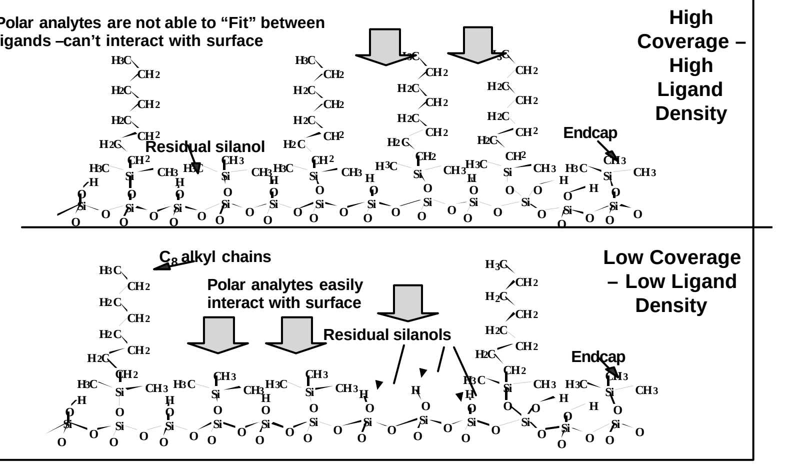
Polarity/Aqueous Columns:

- Low ligand density
- High pore volumn

Mechanism of Retention of Polar Compounds on Aqueous Columns



Polar Compounds - Aqua Columns

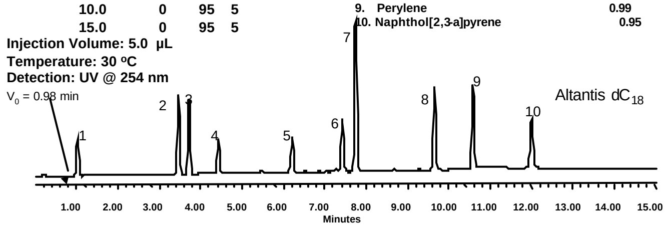
Polar and Non-Polar Compounds Test Mix

Conditions
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 ºC
Detection: UV @ 254 nm

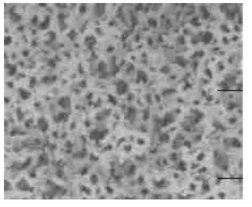
Compounds	USP Tailing:
1. Uracil	1.04
2. Acetanilide	0.95
3. Triamcinolone	1.02
4. Hydrocortisone	1.03
5. 2-Amino-7-chloro-5-oxo-5H-[1]-benzopyrano[2,3-b]pyridinecarbonitrile	1.01
6. 6a-Methyl-17a-hydroxyprogesterone	1.01
7. 3-Aminofluoranthene	0.97
8. 2-Bromofluorene	1.00
9. Perylene	0.99
10. Naphthol[2,3-a]pyrene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



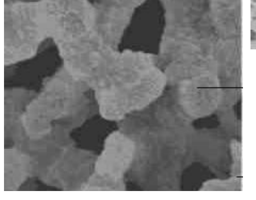
Chromolith Packing

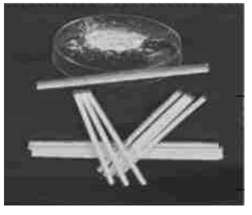
By utilising an innovative new "Gel-Sol" technology, a silica gel polymer is formed, which after ageing, is dried into the required form of a straight rod of highly porous silica with a bimodal pore structure.

Chromolith macropore structure



Chromolith mesopore structure





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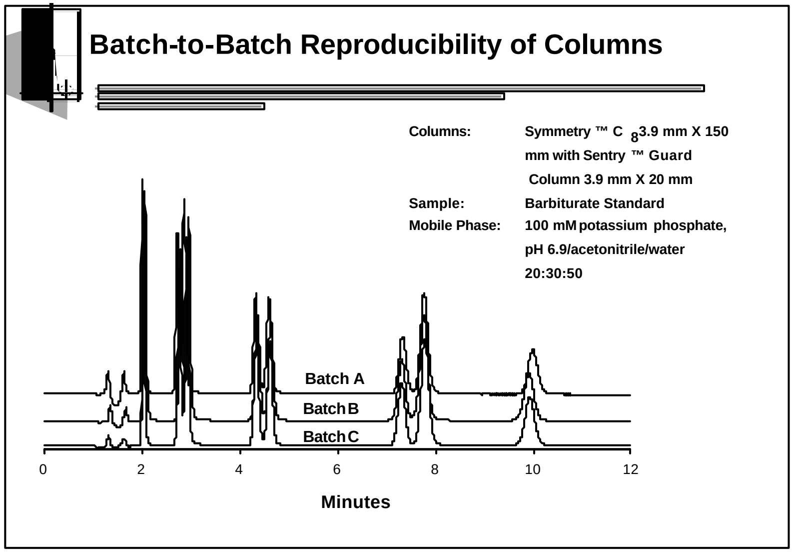
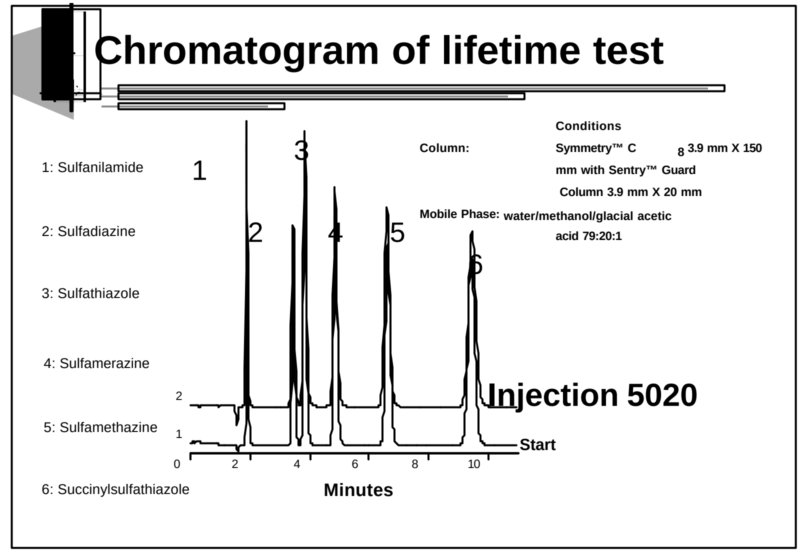
Reversed Phase HPLC





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