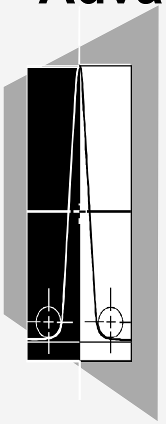
Reversed Phase Advanced Features



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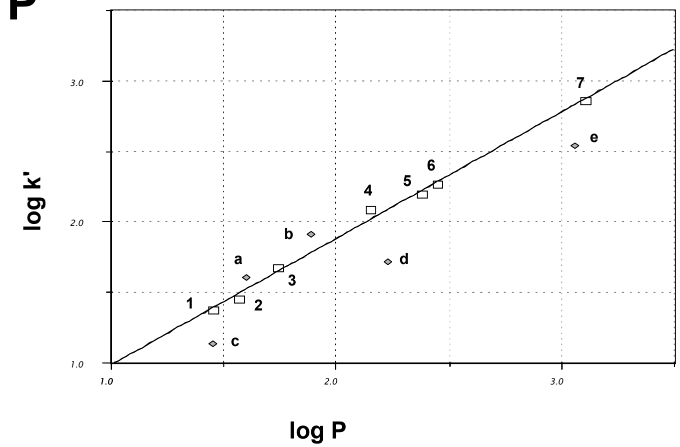
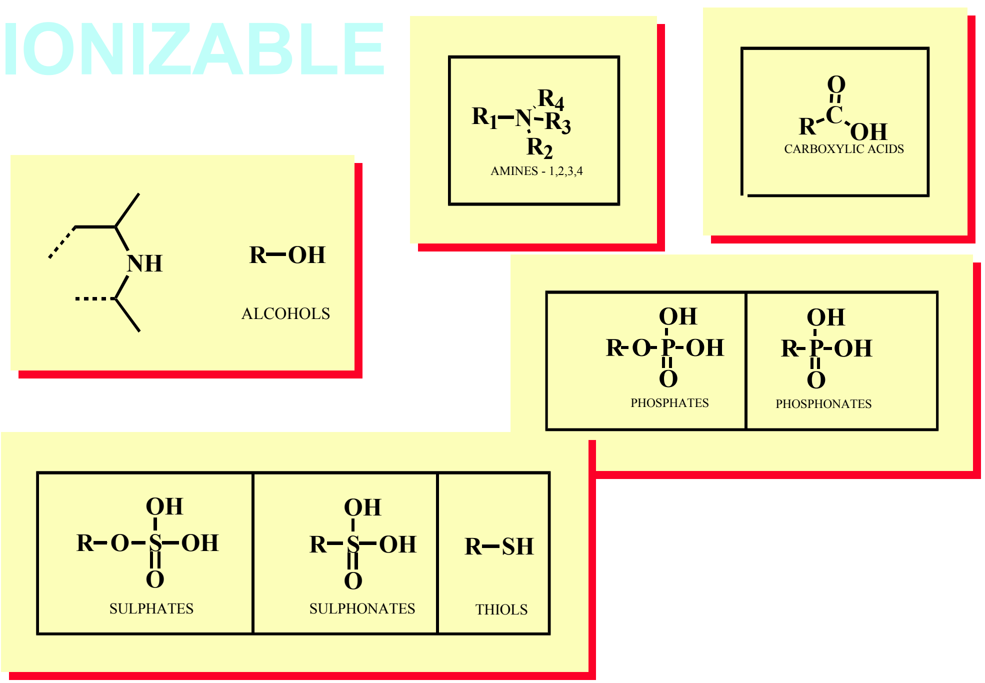
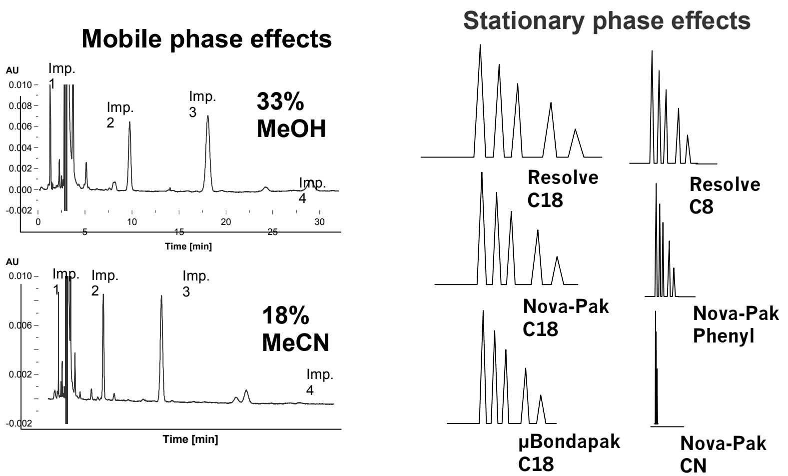
Log k' as function of log P



IONIZABLE



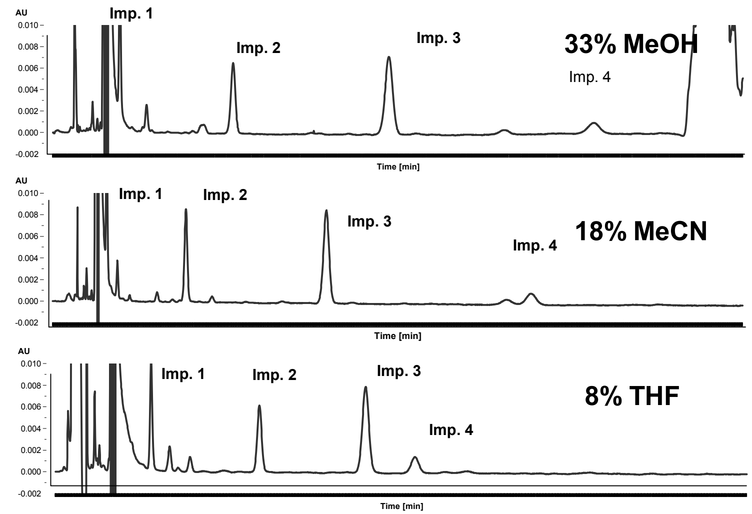


MOBILE PHASE EFFECTS in RP

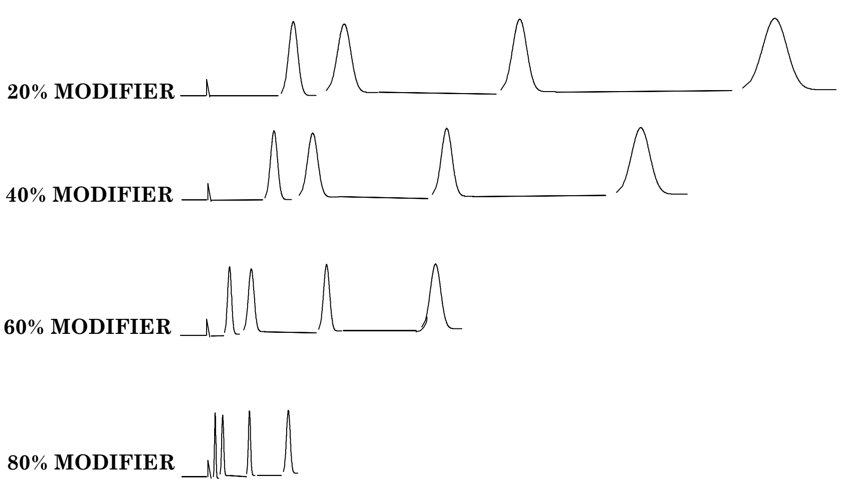
- TYPE OF MODIFIER (MeOH, ACN, THF, IPA)
- SOLVENT STRENGTH (% modifier)
- pH
- TYPE OF BUFFER (phosphate, acetate)
- IONIC STRENGTH (Salts, buffer concentration)
- ION-PAIRING REAGENTS (alkyl-amines, alkyl-sulfonates)
- TEMPERATURE (?)

TYPE OF MODIFIER

AZT: Solvent Selectivity at pH 7.0



OPTIMIZATION: % SOLVENTS

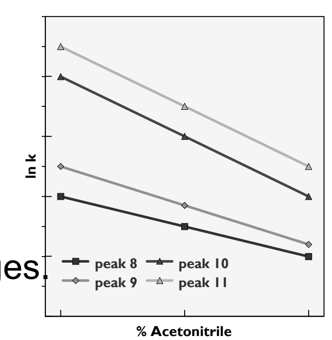


SOLVENT STRENGTH

Analyte Retention as a Function of % Modifier

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

Thus, the resolution between peaks changes.

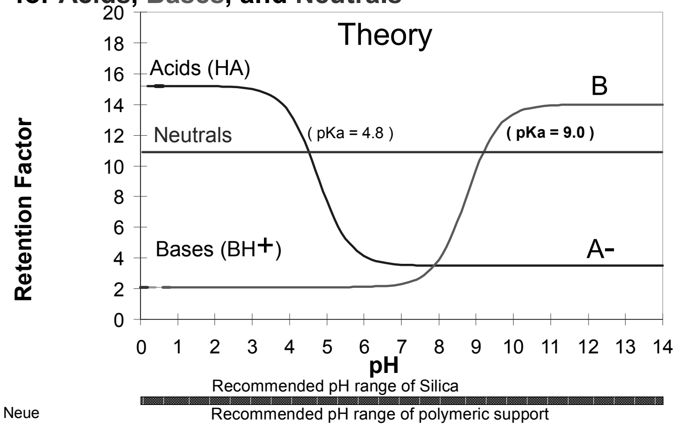


Carmody

pH

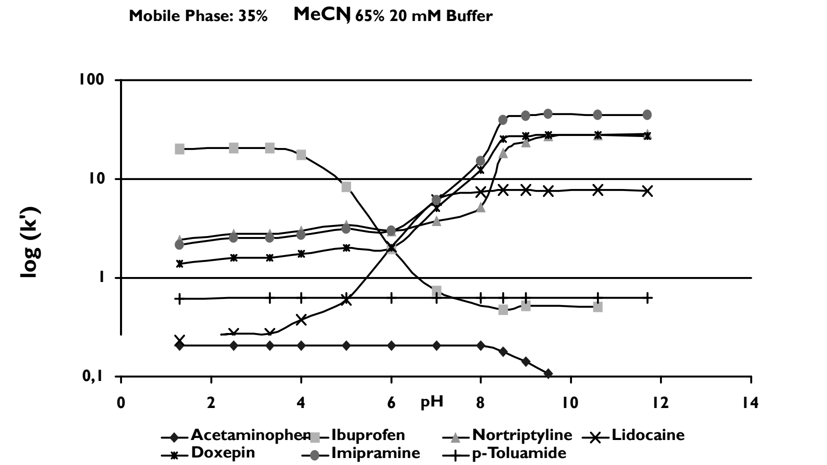
Retention Factor versus pH for Acids, Bases, and Neutrals

$$k = \frac{k_{HA} + k_{A^-} \times \frac{K_a}{[H^+]}}{1 + \frac{K_a}{[H^+]}}$$

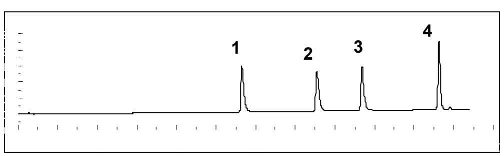


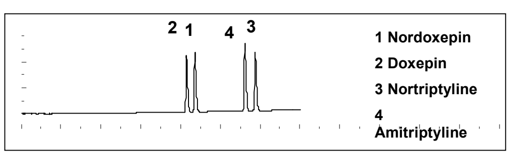
Neue

Dependence of Retention Factor on pH



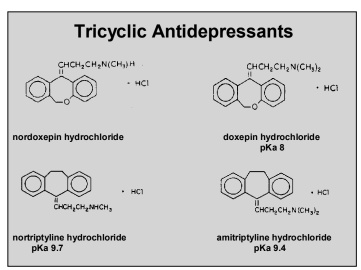
Antidepressants: Initial Gradients at pH 2.3 and 7.0



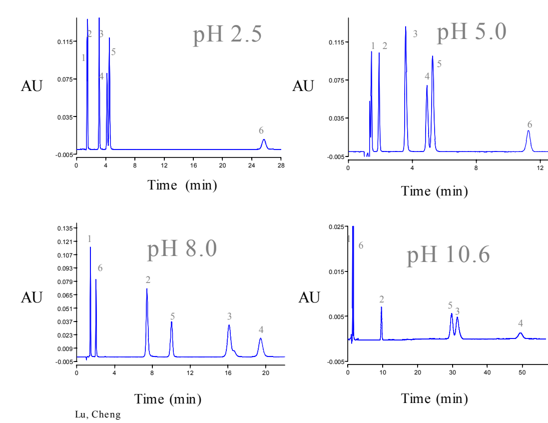


1 Nordoxepin
2 Doxepin
3 Nortriptyline
4 Amitriptyline

Column: Symmetry C18
3.9 mm x 150 mm
Temperature: 35 ºC
Gradient: from 20% MeOH/ 80% 20 mM potassium phosphate buffer to 80% MeOH/ 20% buffer in 60 min
Flow Rate: 1 mL/min
Detection: UV at 254 nm



Dependence of Retention on pH



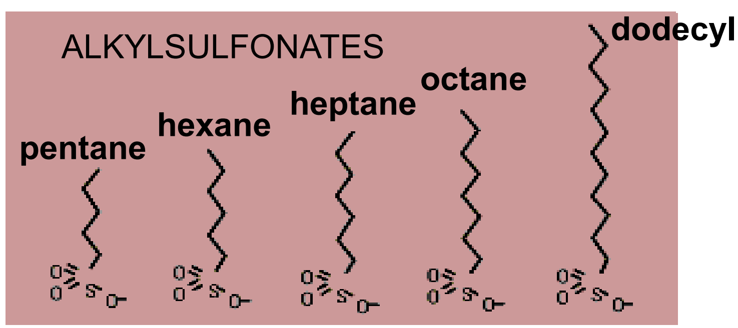
1: acetaminophen
2: lidocaine
3: doxepin
4: imipramine
5: nortriptyline
6: ibuprofen

Conditions:
Column: XTerra™ RP$_{18}$, 3.9 X 150 mm, 5 µm
Mobile Phase: 65% 20 mM Buffers, 35% ACN
Column Temp.: 30 °C
Flow Rate: 1.0 mL/min
Detector: 210nm for pH 2.5, pH 5.0, and pH 7.0; 230nm for pH 10.6

Types of Buffers and Ionic Strength

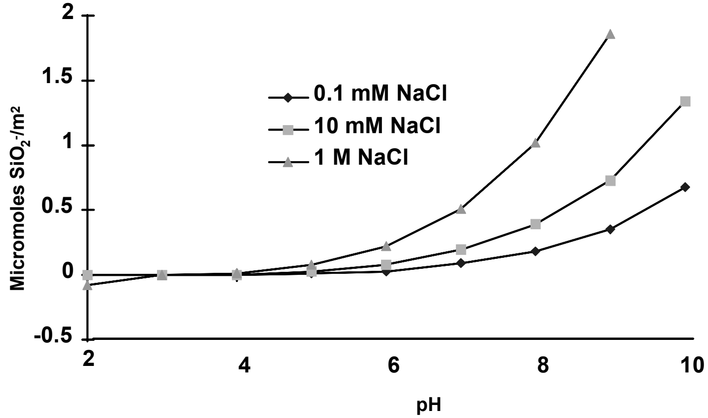
- pH 10: Borate
  - 20 mM H_3BO_3
- pH 7: Phosphate
  - 20 mM K_2HPO_4
- pH 4-5: Acetate
  - 10 mM CH_3COONH_4
  - 100 mM CH_3COOH
- pH 2-3.5: Phosphate
  - 20 mM H_3PO_4 - KH_2PO_4

ION PAIR REAGENTS



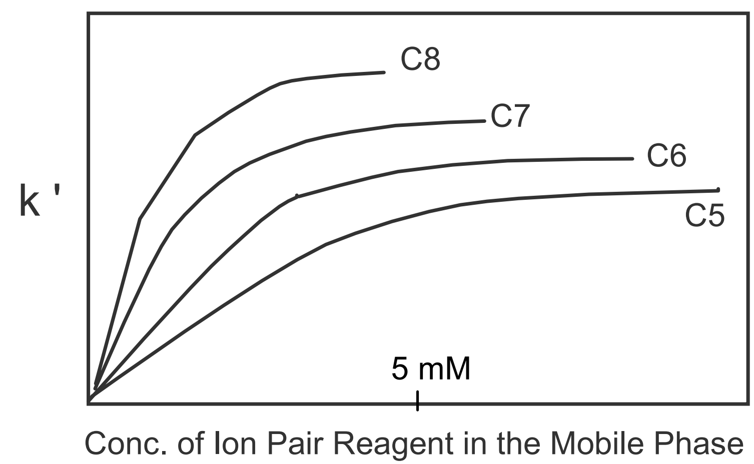
Silica Surface Charge

(Bolt, J. Phys. Chem. 61, 1166, 1957)



The larger the alkyl, the longer are retention times

The larger alkyls saturate the stationary phase at lower concentrations



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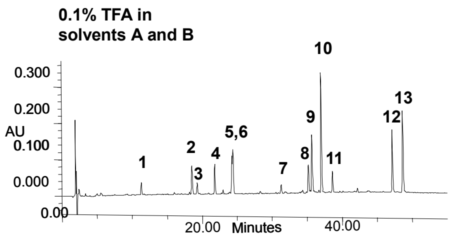
Effects of TFA Concentration on Resolution

- Typical gradient conditions

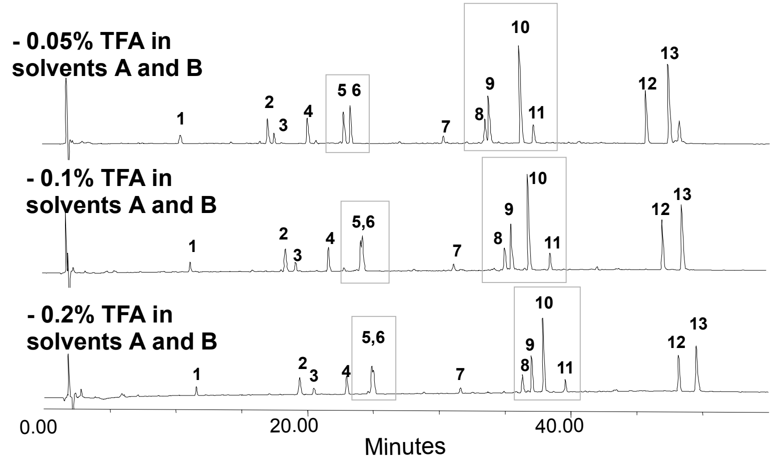


Conditions

- Column: Symmetry300™, C_{18}, 5 µm, 3.9x150mm,
- Sample: Tryptic digests of bovine cytochrome *c*
- Injection: 20 µL
- Mobile Phase:
  Solvent A: water
  Solvent B: acetonitrile
- Gradient: 0-45 min., 0-30%B
- Flow rate: 0.75 mL/min.
- Temperature: 35 ºC
- Detection: 214 nm

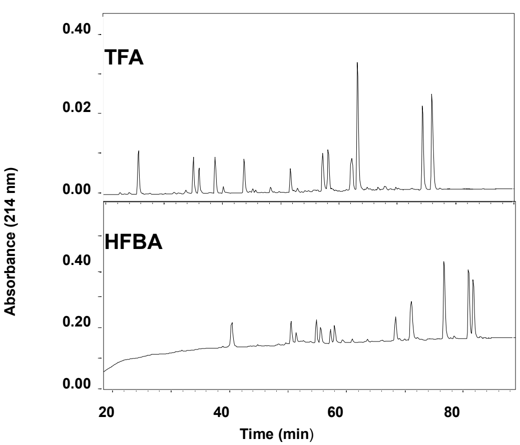
Alden

The Power of Different TFA Concentrations in Your Mobile Phase



Alden

Alternate Ion Pairing Reagents TFA and HFBA (Heptafluorobutyric Acid)



Sample: Rabbit cytochrome *c* tryptic digest, 500 pmol

Column: Delta-Pak™ C_{18}, 5µm, 300Å, 2.0 x 150 mm

Eluents: A=water/ 0.1% TFA or HFBA
B=acetonitrile/ 0.1% TFA or HFBA

Gradient: O-60 % B 120 min

Flow: 0.18 mL/min

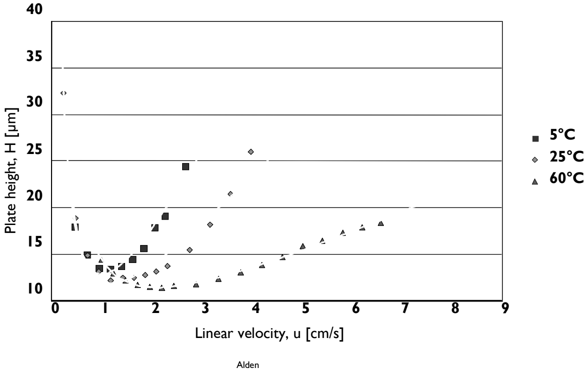
Temp: 35 ºC

Temperature Effects on Resolution

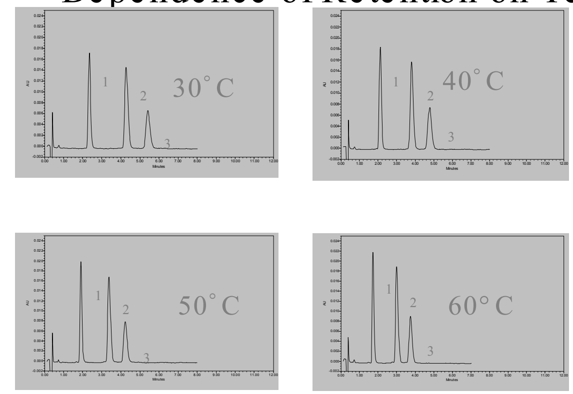
Resolution can be temperature dependent

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

Effect of Temperature on Column Efficiency



Dependence of Retention on Temperature



Conditions:

Column: XTerra™ MSC_{18}, 2.1 X 50 mm, 2.6 µm

Mobile Phase: % 25ACN/75% buffer (10 mM, pH5, NH_4AC)

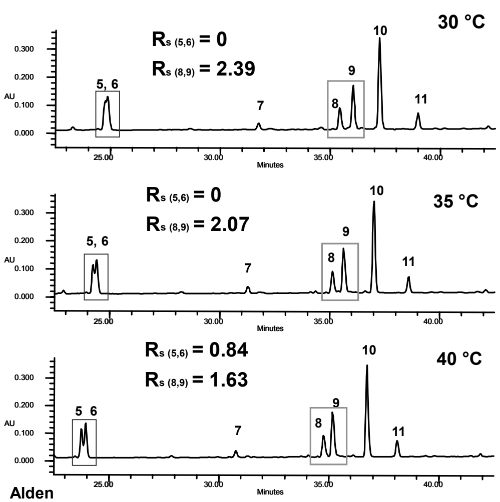
Flow Rate: 0.6 mL/min

Injection Vol. 3 µL

Detector: 210 nm

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

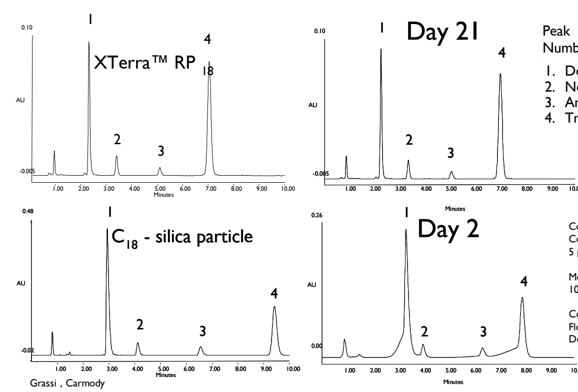
Temperature Effects on Resolution



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 Tricyclic Antidepressant Separation



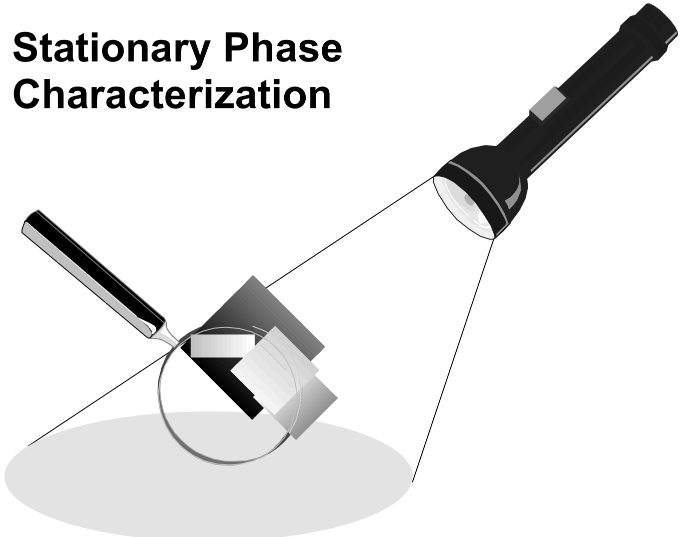
Peak Number	USP Tailing Factor
1. Doxepin	1.2
2. Nortriptyline	1.1
3. Amitriptyline	1.1
4. Trimipramine	1.0

Conditions :
Column: XTerra™ RP $_{18}$, 5 µm, 4.6 X 150 mm

Mobile Phase : 40% pH 7 10 mM Na_2PO_4, 60% ACN

Column Temp.: 60 °C
Flow Rate: 1.5 mL/min
Detector: 254 nm

Stationary Phase Characterization



Stationary Phase Supports

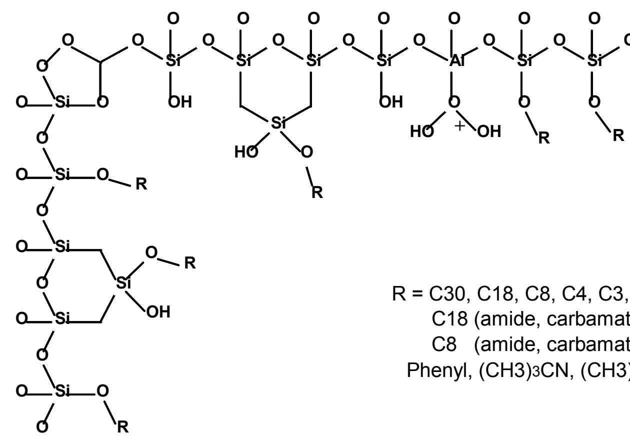
Stationary phase	Functionality
C_{18}	$-Si(CH_3)_2C_{18}H_{37}$
C_8	$-Si(CH_3)_2C_8H_{17}$
tC_2	$-SiC_2H_5$
Aminopropyl	$-Si(CH_2)_3NH_2$
Cyanopropyl	$-Si(CH_3)_2(CH_2)_3CN$
Diol	$-Si(CH_2)_3O\,CH_2CH(OH)CH_2OH$

STATIONARY PHASE PROPERTIES

CHEMISTRY:
* Type of Boded Hydrocarbon
* % Coverage
* Type of Silica Gel

GEOMETRY
* SPHERE- IRREGULAR
* PARTICLE DIAMETER
* POROSITY

Silica based Surface



R = C30, C18, C8, C4, C3, C1
C18 (amide, carbamate),
C8 (amide, carbamate),
Phenyl, $(CH3)_3CN$, $(CH3)_3NH_2$

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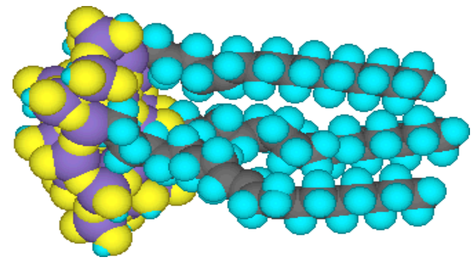
Stationary Phase Chemistry

- ❑ Hydrophobicity
- ❑ Silanol Activity
- ❑ Neutral pH
- ❑ Acidic pH
- ❑ Metal Contamination

Silica based "bonded phases"

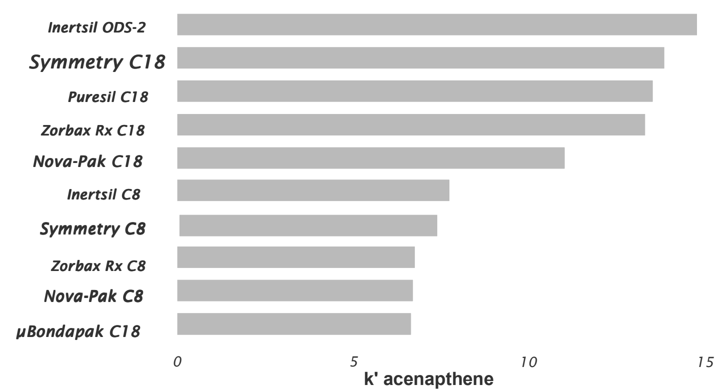


Bulky alkylsilane ligands can not react with all available silanols due to the steric hindrance.

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%]

Relative Hydrophobicities of General Purpose Analytical Packings



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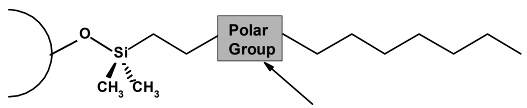
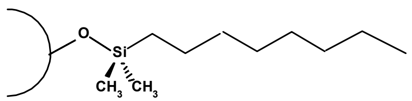
Reversed-Phase Packing with an Embedded Polar Group



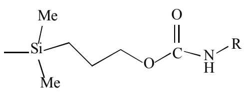
Embedded Polar Group Ligand



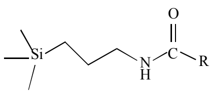
Traditional, Straight Chain Alkyl Ligand

Commercial Phases with Embedded Polar Group

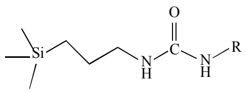
- reduces interaction with silanols
- modifies selectivity
- improves water wettability



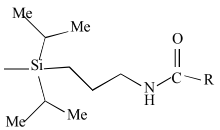
SymmetryShield RP (Waters)



Discovery RP Amide16 (Supelco)



Prism, Spectrum (Keystone)

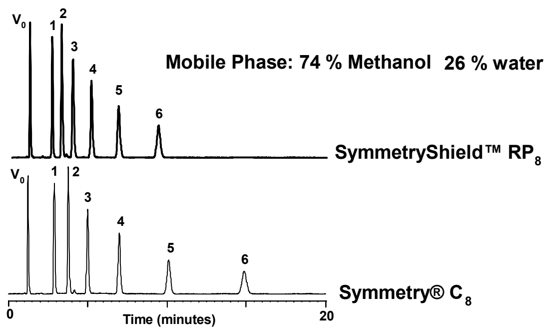


Bonus RP (Hewlett Packard)

Impact on Selectivity Hydrophobic Retention

Sample: n-Alkylbenzenes {Neutrals}

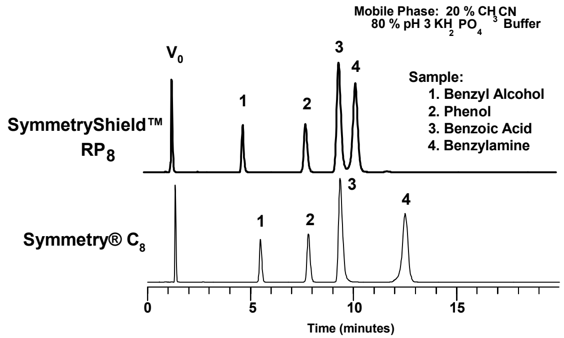
(benzene - C_6 to amylbenzene -- C_{11})



Reduced Methylene Group Selectivity

B. A. Alden

Impact on Selectivity Polar Retention



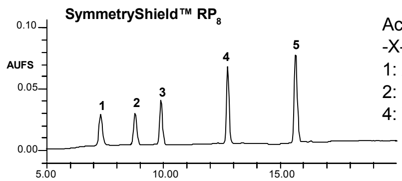
Reduced Retention of Amines

(Shielding of Silanols)

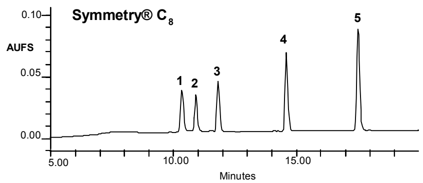
B. A. Alden

Selectivity and Retention Differences

Alberta Peptides on Symmetry® Reversed-Phase Columns



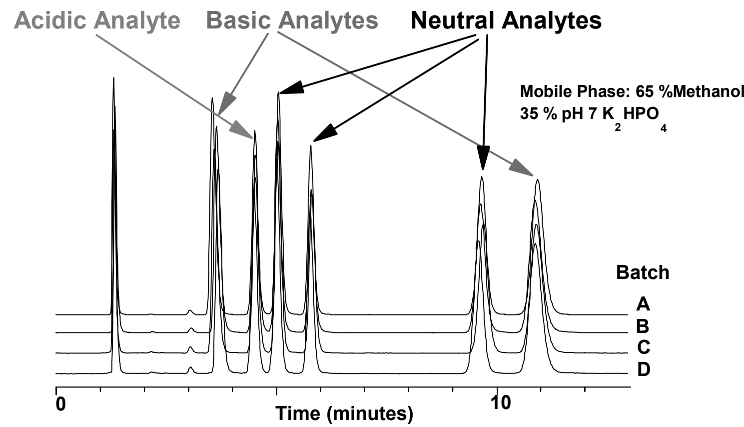
Ac-Arg-Gly-X-X-Gly-Gly-Leu-Gly-LysAmide
-X-X-:
1: Ala-Gly with free alpha amino group
2: Gly-Gly 3: Ala-Gly
4: Val-Gly 5: Val-Val



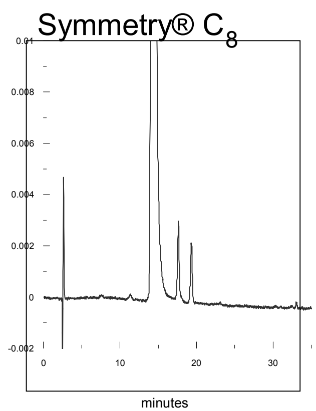
Conditions: Columns:	3.9 mm x 150 mm
Flow Rates:	0.8 mL/min
Mobile Phase:	A. 0.1% TFA aqueous, B. acetonitrile with 0.1% TFA
Gradient:	10% to 40% B in 30 minutes, step to 60% B for 5 min
Sample:	9 µL Alberta Peptides, mix with 5 decapeptides
Detector:	214 nm
Temperature:	35°C

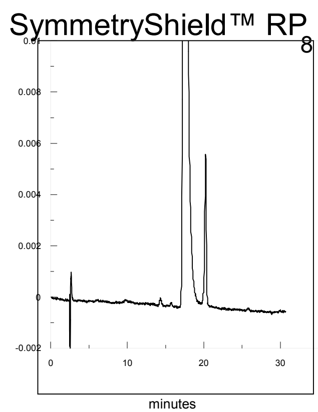
D. J. Phillips

Characterization of a New Surface Chemistry: SymmetryShield™ RP_8



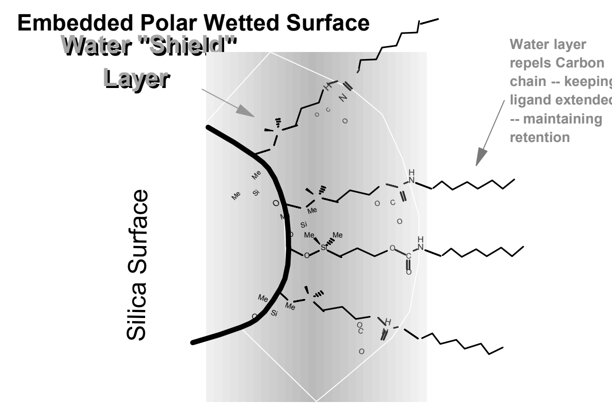
Selectivity Difference: Furazolidone Impurities





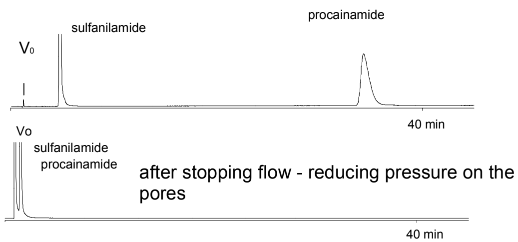
El Fallah

SymmetryShield™ Technology Provides Better Peak Shape

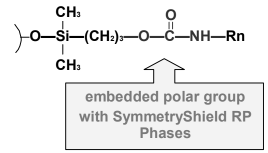


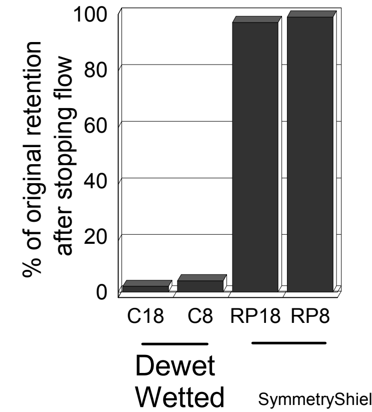
Effect of Bonding Chemistry on Wettability

Hydrophobic stationary phases can dewet in highly aqueous mobile phases
Extrusion of mobile phase from pore
Reduced retention



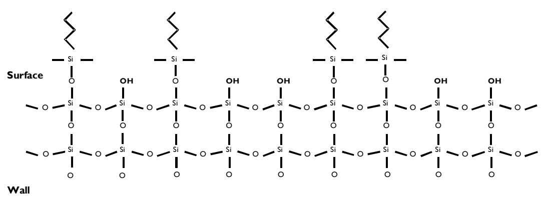
Effect of Embedded Polar Group on Stationary Phase Dewetting



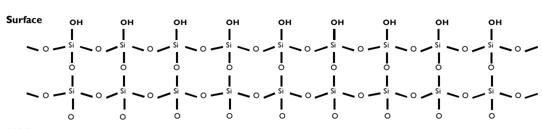


Mobile Phase:
20 mM K2HPO4, pH 6.0

Silica Particles



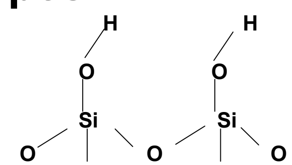
Bonded Silica Particle



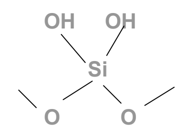
Unbonded Silica Particle

Silanol Types

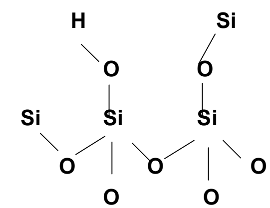
- Vicinal



- Geminal

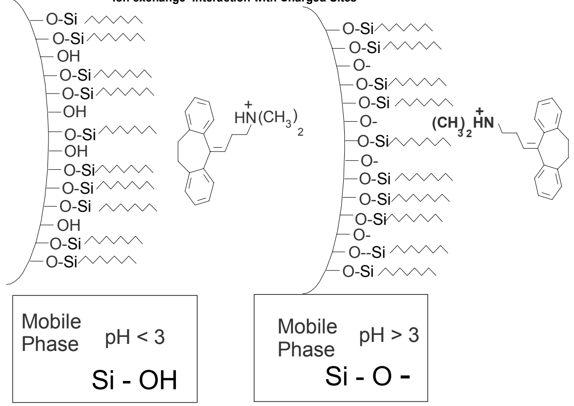


- Lone
  (Most Active)



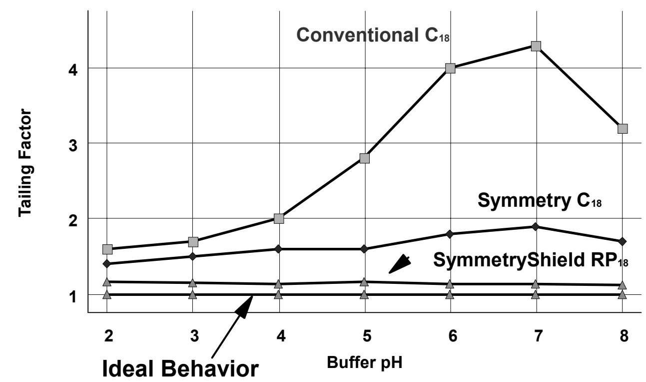
Mixed-Mode Retention:

-- Hydrophobic Interaction with Bonded Phase
-- Ion exchange Interaction with Charged Sites



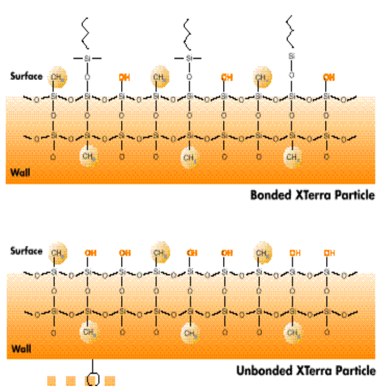
Peak Shape Over Wide pH Range

(Strong Base -- Amitriptyline pKa = 9.4)



Alden, Iraneta, Carmody, Andrews

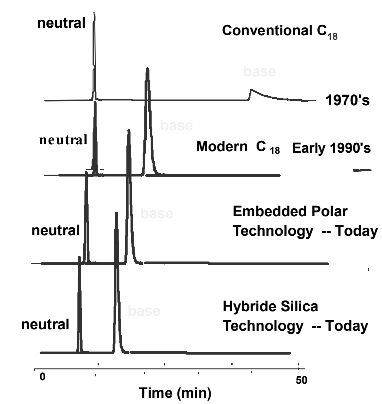
Organic/Inorganic Hybrid Technology: State-of-the-Art XTerra™ Particle



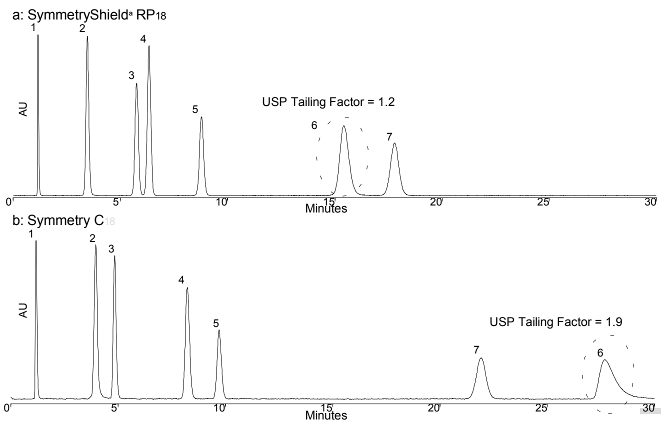
Best properties of silica
* mechanical strength
* high efficiency

Best properties of organic polymers
* high chemical stability
* no tailing for bases

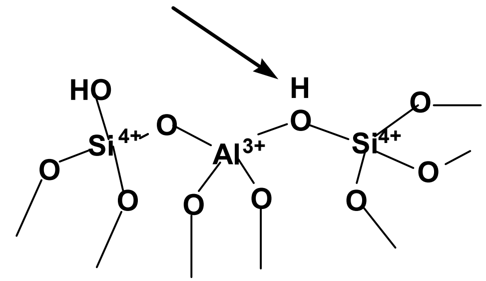
Improvement in Peak Shape for Bases



Embedded Polar Group vs Conventional RP
U.S.P. Tailing Factor Comparison



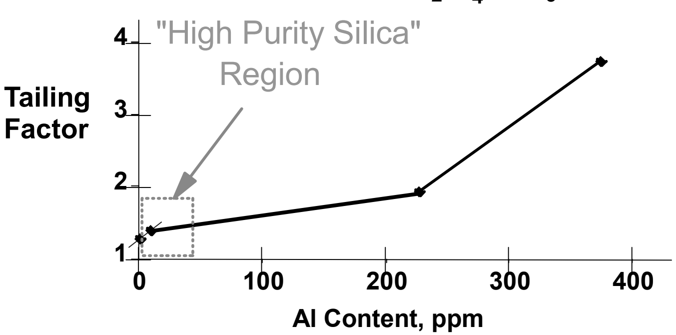
Al in the Silica Lattice



Correlation Between Base Tailing and Al Content of Silica

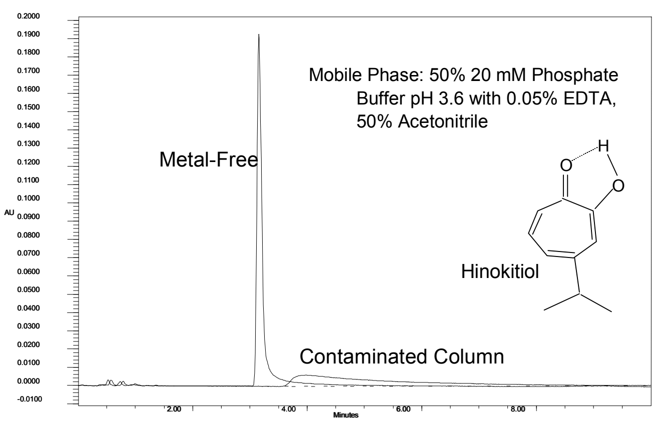
Analyte: Chlorpheniramine

Mobile Phase: pH 3 KH_2PO_4/CH_3CN 80:20

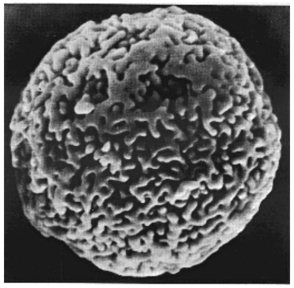


Note: Manufactures are reaching the limits of performance improvements achieved solely through reduced metals in the starting silica particle

Peak Forms of Complexing Agent (Hinokitiol)



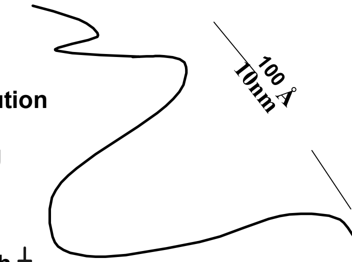
Pore size, shape and distribution



- 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.

Silica Structure



*** Silica is Porous**

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

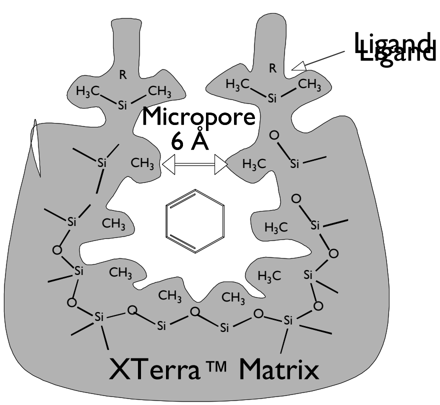
*** Specific Pore Volume, mL/g**

Range: 0.3 -- 1.3 mL/g

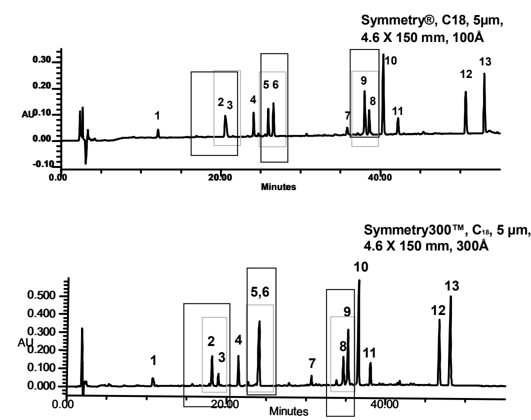
SV ↑ Particle Strength ↓

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

Exlusion - Inclusion Effects



Pore Size Effects on Resolution



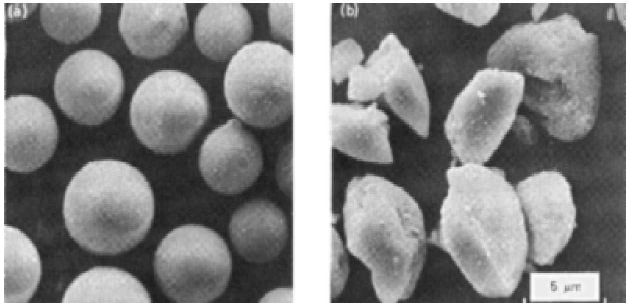
Conditions

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

-Different pore sizes change selectivity.

Carmody

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]

Total band broadening

$$H_p = 2\lambda d_p$$

$$H_d = 2\frac{\gamma D_m}{v}$$

$$H_m = \omega \frac{d_p^2}{D_m} v$$

Hetp = H p + H d + H m

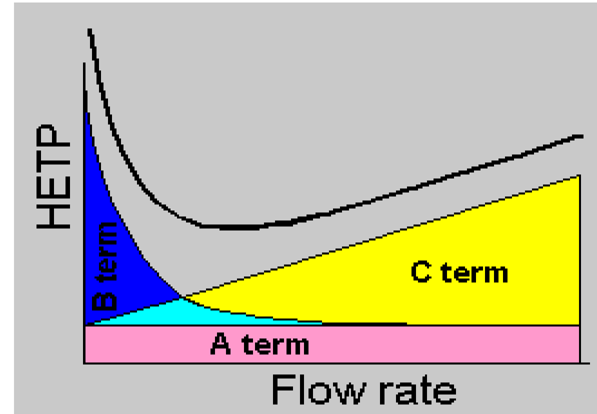
Each term discussed above introduces its part in the total band broadening, therefore the sum of all of them will give the total column plate height.

L/N = Hetp

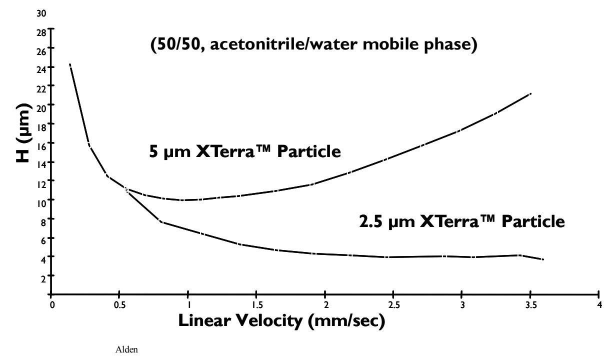
L = Column Length; N=Plate Number

Van Deemter Equation

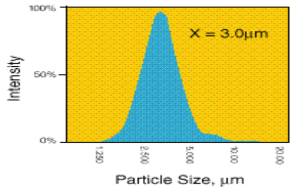
$$H = A + \frac{B}{\upsilon} + C\upsilon$$



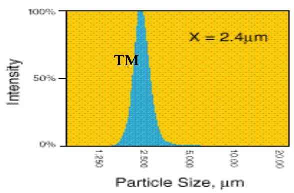
Comparison of the van DeemterPlots for 5 µm and 2.5 µm XTerra™ MS C_{18} Particles



Challenge of Making "2 µm" Packings



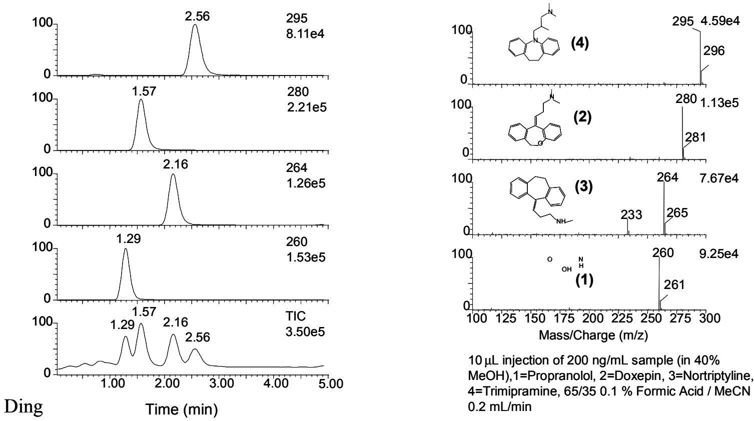
Centered at 3 µm
Wider distribution



Centered at 2.4 µm
Narrower distribution

Fast LC-MS Analysis

XTerra™ MS C18, 2.1 x 50 mm (5 µm)

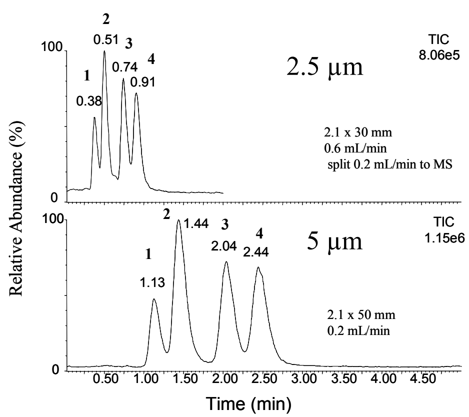


10 µL injection of 200 ng/mL sample (in 40% MeOH),1=Propranolol, 2=Doxepin, 3=Nortriptyline, 4=Trimipramine, 65/35 0.1 % Formic Acid / MeCN 0.2 mL/min

Ding

Fast LC-MS Analysis

XTerra™ MS C18: 5 µm *vs..* 2.5 µm

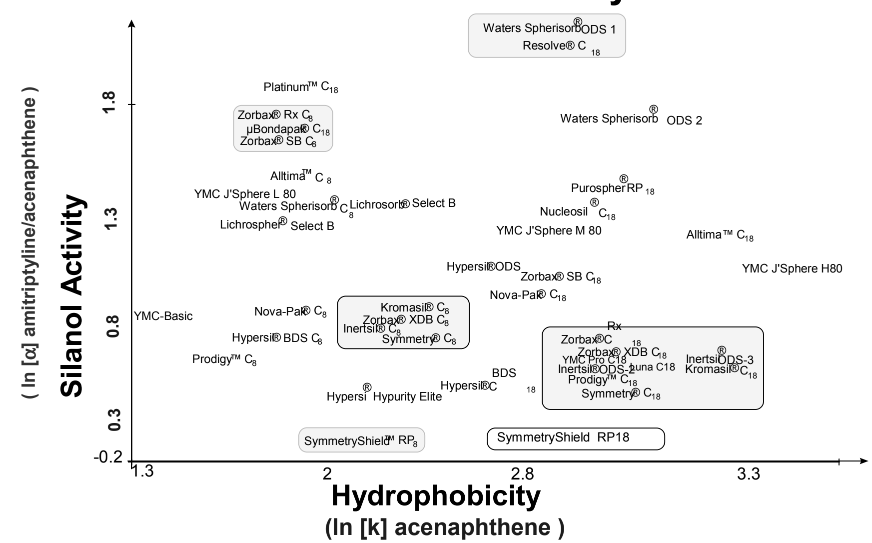


HPLC:
65/35 0.1% formic acid / MeCN
1 µL injection of
200 ng/mL of samples
MS: ESI+
SIR 4 channels
HV: 3.15 kV
Cone 25 V
Drying Gas: 380 L/h
Source Temp: 175 °C

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

Ding

Reversed-Phase Selectivity Chart



Dr. Shulamit Levin, Analytical Consulting, Medtechnica.

Interpretation of Column Selectivity Chart

Hydrophobicity - x-axis - log scale

increasing hydrophobicity from left to right
C_8 bonded phases to the left; C_{18} bonded phases to the right

Silanol Activity - y-axis - log scale

best peak shape achieved at the bottom of the chart (lowest silanol activity)
increasing silanol activity from bottom to top

Who needs guard columns?

- Dirty or Complex sample matrix
- Biological Fluids
- Environmental
- Food Samples
- Needs increased capacity