Chemistry 4631

Instrumental Analysis

Lecture 27

High Performance Liquid Chromatography (HPLC)

Instrumentation
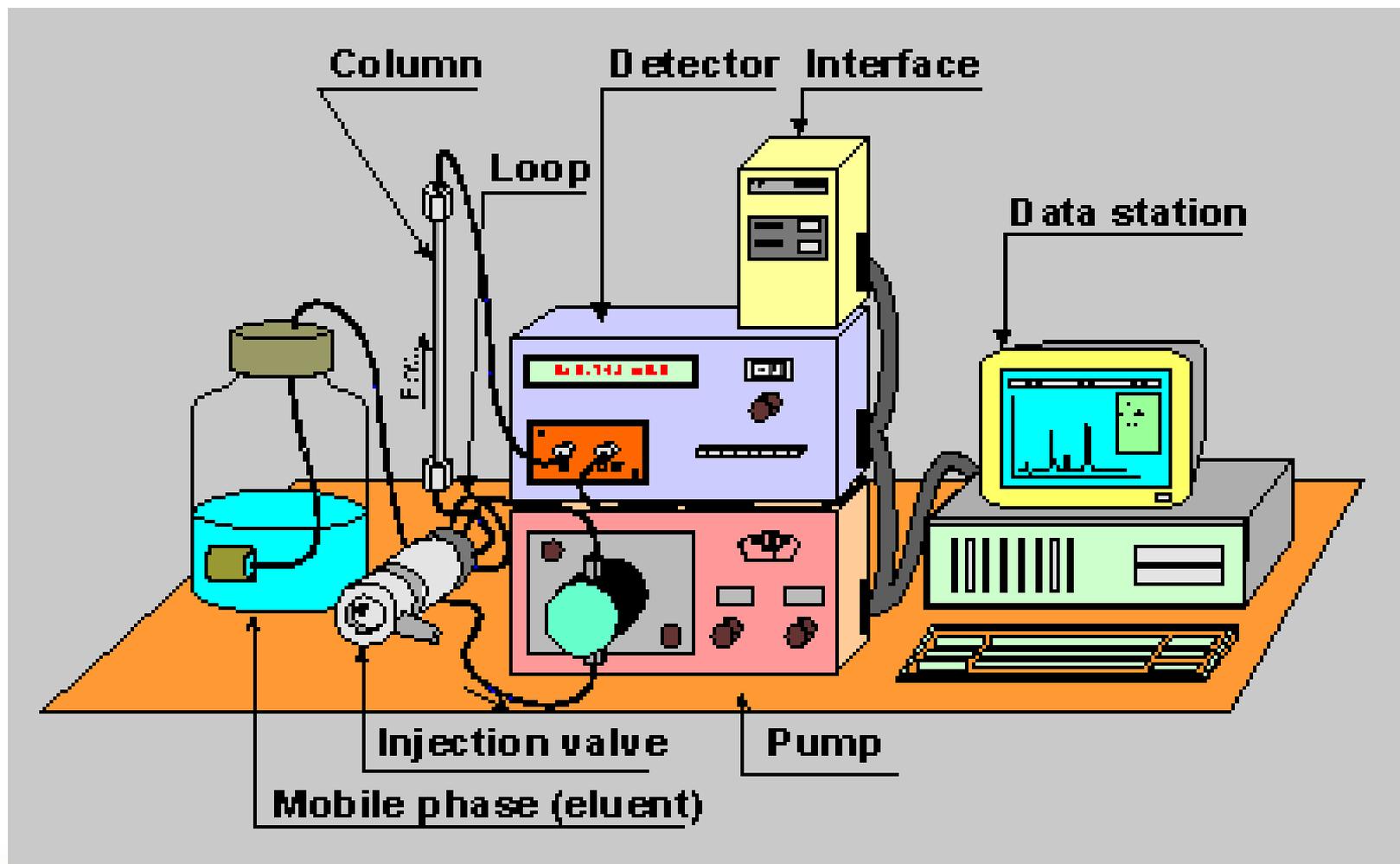
HPLC
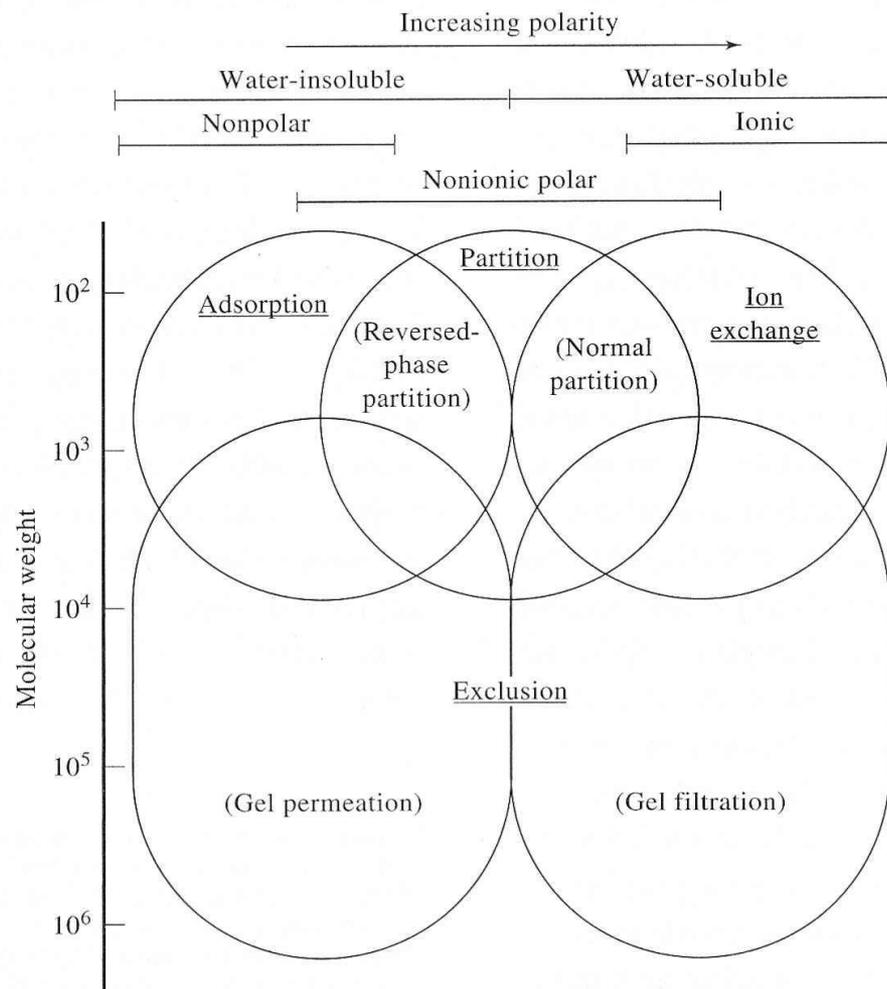
Figure 28-1 Applications of liquid chromatography. *(From D. L. Saunders, in Chromatography, 3rd ed., E. Heftmann, Ed., p. 81. New York: Van Nostrand Reinhold, 1975. With permission.)*

Chem 4631

HPLC

Normal Phase Chromatography

Normal Phase - a polar stationary phase with a less polar mobile phase.

Neutral solutes are separated on the basis of their polarity. More polar solute, longer retention time.

HPLC

Normal Phase Chromatography

Mechanism of Retention

Dominant retention mechanism is adsorption. Also called adsorption chromatography.

Selectivity depends on relative strength of the polar interaction.

The spatial configuration - how the solute is positioned on the stationary phase is important (sensitive to spatial or steric differences).

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Normal Phase Chromatography

Stationary Phase for Normal-Phase

Wide range of stationary phase materials have been used:

sucrose	cellulose
starch	silica gel
fluorisil	charcoal
magnesium oxide	alumina

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Normal Phase Chromatography

Stationary Phase for Normal-Phase

Most common - silica or alumina that have polar hydroxyl groups on the surface.

Silica is preferred - available, low cost, allows higher sample loadings, less likely to catalyze decomposition of sample components.

Alumina is used for basic compounds (i.e. amines) which are strongly retained on silica.

In general, both retain more polar compounds strongly.

HPLC

Normal Phase Chromatography

Stationary Phase for Normal-Phase

Silica adsorption sites (-Si-OH, -OH groups) can be controlled by pretreatment of column.

Usually silica for HPLC treated between 200-300 $^{\circ}$C

Drawback of normal phase - lack of separation selectivity between packing materials despite difference in particle shape, size, or surface area.

(Compounds elute in same order regardless of column selected).

So selectivity must be achieved through changing the mobile phase.

HPLC

Normal Phase Chromatography

Mobile phase - solvent strength

Variation in retention is generally achieved by altering the mobile phase.

The interaction of a solvent molecule with an analyte depends on several forces: dispersion, dipole, hydrogen bonding and dielectric interactions (electrical insulator).

The extent of a molecule interacting in these four ways is referred to as the polarity of the compound.

The chromatographic strength of a solvent is directly related to its polarity.

HPLC

Normal Phase Chromatography

Mobile phase - solvent strength

Biggest problem with normal phase is the effect that water has on the activity of polar adsorbents.

Water adsorbs onto the strongest adsorption sites - leaving weaker sites open to retain the sample – decreasing solute retention.

HPLC

Normal Phase Chromatography

Mobile phase - solvent strength

Difficult to control amount of water present in mobile phase - easy to pick up water from atmosphere.

Generally add small amount of water to mobile phase to give less variation in sample retention from run to run.

free silanol   bonded silanols (vicinal)   geminal silanols   siloxane   bound water
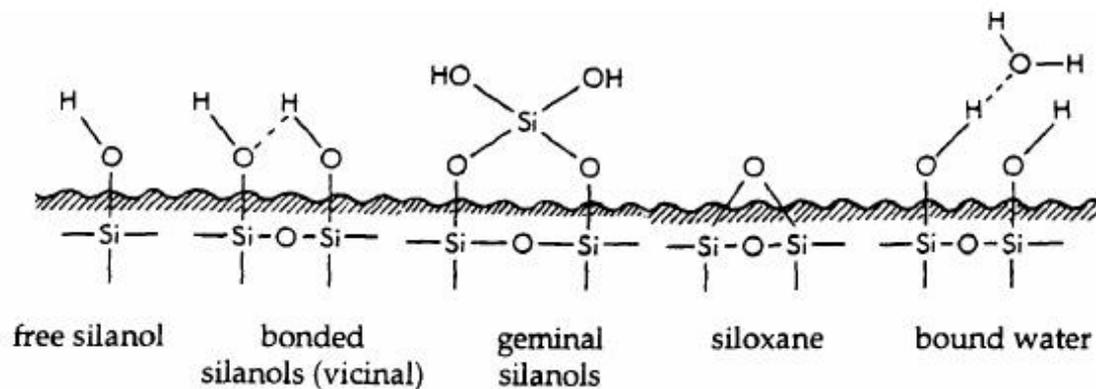
HPLC

Normal Phase Chromatography

Applications

Normal phase used less than other HPLC techniques.

Used for analysis of samples that are soluble in non-polar solvents and for isomer separation.

Examples: fat & water – soluble vitamins
   Less-polar plant pigments –porphyrins
   Pesticides- still better with GC
   Separation of isomers (6 isomers of trinitrotoluene)
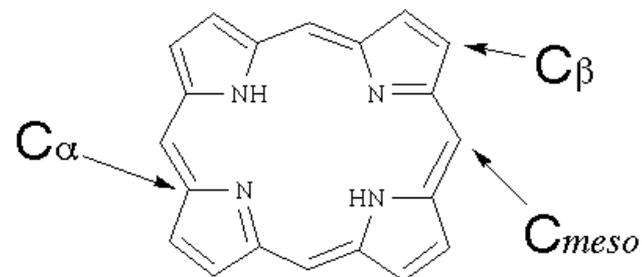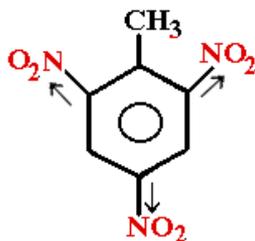
HPLC

Reverse-Phase Chromatography

Most widely used of LC modes (over 75%)

Uses a non-polar stationary phase with a polar mobile phase.

More polar the solute, the lower the retention time.

HPLC

Reverse-Phase Chromatography

Mechanism of separation

More complex than for normal-phase.

Interaction between solute molecule and non-polar stationary phase is too weak to explain the degree of solute retention observed.

Mechanism - combination of partition and adsorption.

HPLC

Reverse-Phase Chromatography

Mechanism of separation

Solvophobic theory
Assumes:
- **using aqueous mobile phases with low organic modifier**
- **stationary phase is uniform layer of a non-polar ligand**
- **solute binds to the stationary phase and reduces surface area of solute exposed to mobile phase.   (Solute binds to stationary phase because it is solvophobic)**

HPLC

Reverse-Phase Chromatography

Mechanism of separation

Solvophobic theory

Retention occurs because of interaction between mobile phase and solute - mobile phase composition influences separation selectivity more than stationary phase (behaves as a solid).

HPLC

Reverse-Phase Chromatography

Mechanism of separation

Partitioning theory

Solute is thought to fully interact with stationary phase and partitions between mobile phase and stationary phase (behaves as a liquid).

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Reverse-Phase Chromatography

Mechanism of separation
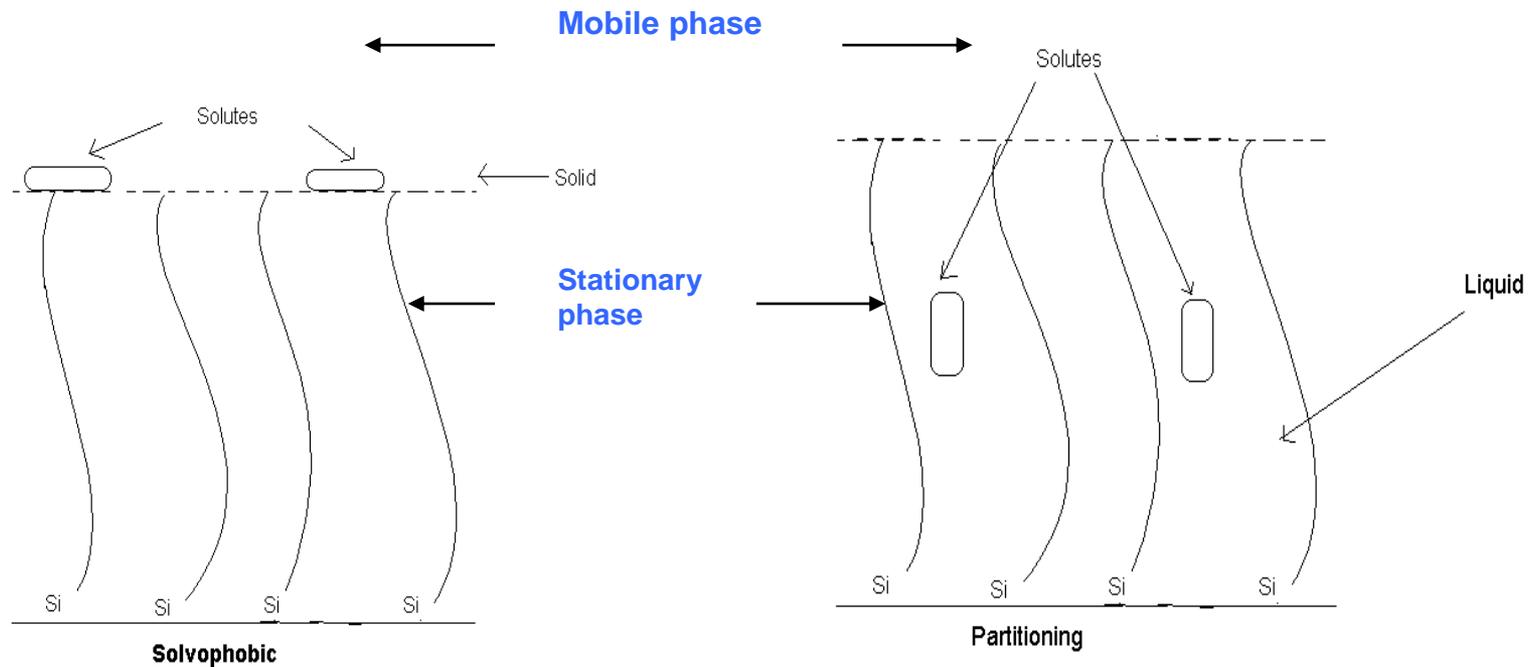
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Reverse-Phase Chromatography

Mechanism of separation

Partitioning theory

As the chain length of the bonded material increases the retention mechanism is more like partitioning.

Short chain lengths - more like solvophobic (adsorption).

HPLC

Reverse-Phase Chromatography

Stationary Phases - Bonded phase silica columns

Functional group is chemically attached to a silica support (like used for normal phase).

Attaching groups to the silica depends on reaction with the surface silanol groups.

Most common method - reaction of silica with organochlorosilanes to produce siloxane (Si-O-Si-R_3) packings.

HPLC

Reverse-Phase Chromatography

Stationary Phases - Bonded phase silica columns

$$\overset{\diagdown}{\underset{\diagup}{}}Si-OH + Cl-\overset{\overset{R}{|}}{\underset{\underset{R}{|}}{Si}}-R' \dashrightarrow \overset{\diagdown}{\underset{\diagup}{}}Si-O-\overset{\overset{R}{|}}{\underset{\underset{R}{|}}{Si}}-R'$$

$$\begin{array}{c} \overset{\diagdown}{\underset{\diagup}{}}Si-OH \\ \overset{\diagdown}{\underset{\diagup}{}}Si-OH \end{array} + \begin{array}{c} Cl \diagdown \quad \diagup R \\ \quad Si \\ Cl \diagup \quad \diagdown R' \end{array} \quad \begin{array}{c} \overset{\diagdown}{\underset{\diagup}{}}Si-O \diagdown \quad \diagup R \\ \qquad\qquad Si \\ \overset{\diagdown}{\underset{\diagup}{}}Si-O \diagup \quad \diagdown R' \end{array}$$
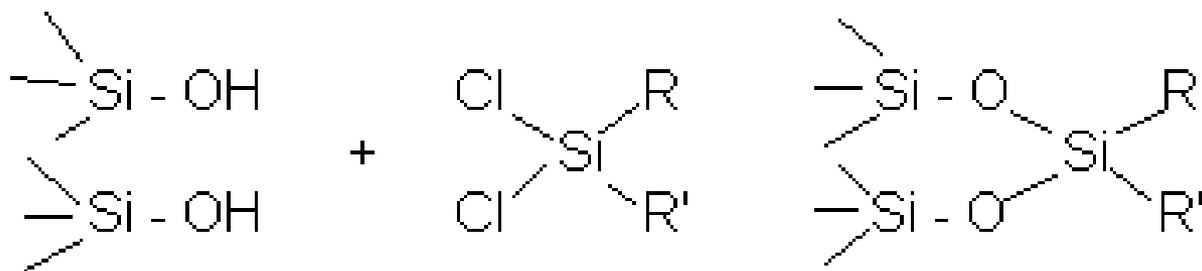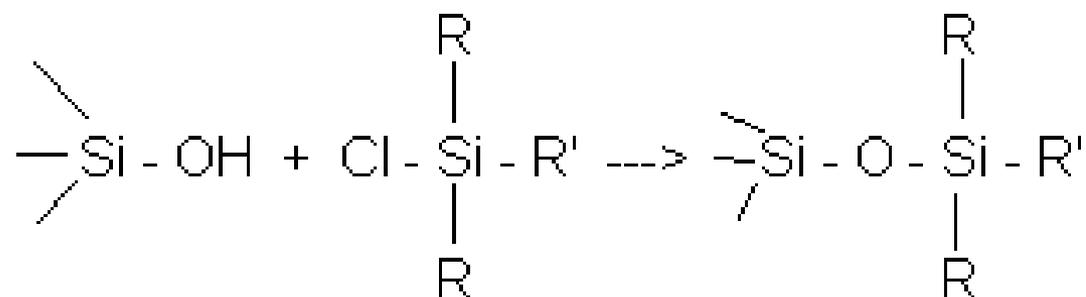
HPLC

Reverse-Phase Chromatography

Stationary Phases - Bonded phase silica columns monomeric type
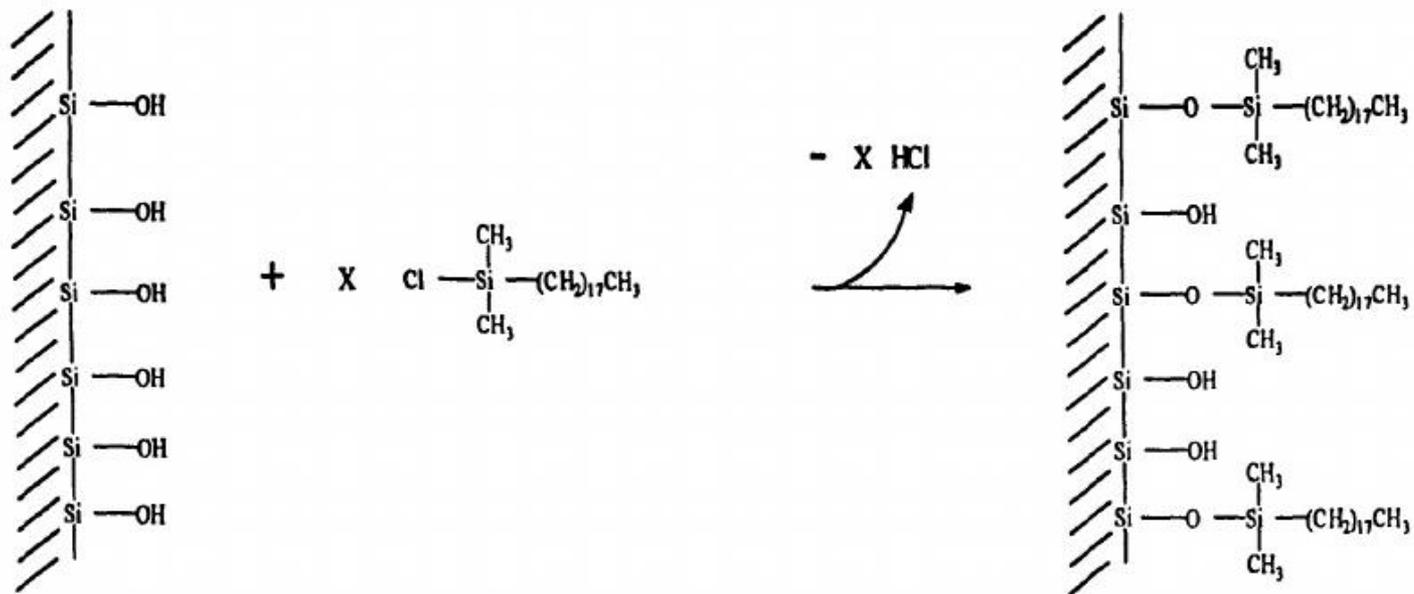
HPLC

Reverse-Phase Chromatography

**Stationary Phases - Bonded phase silica columns
 polymeric type**
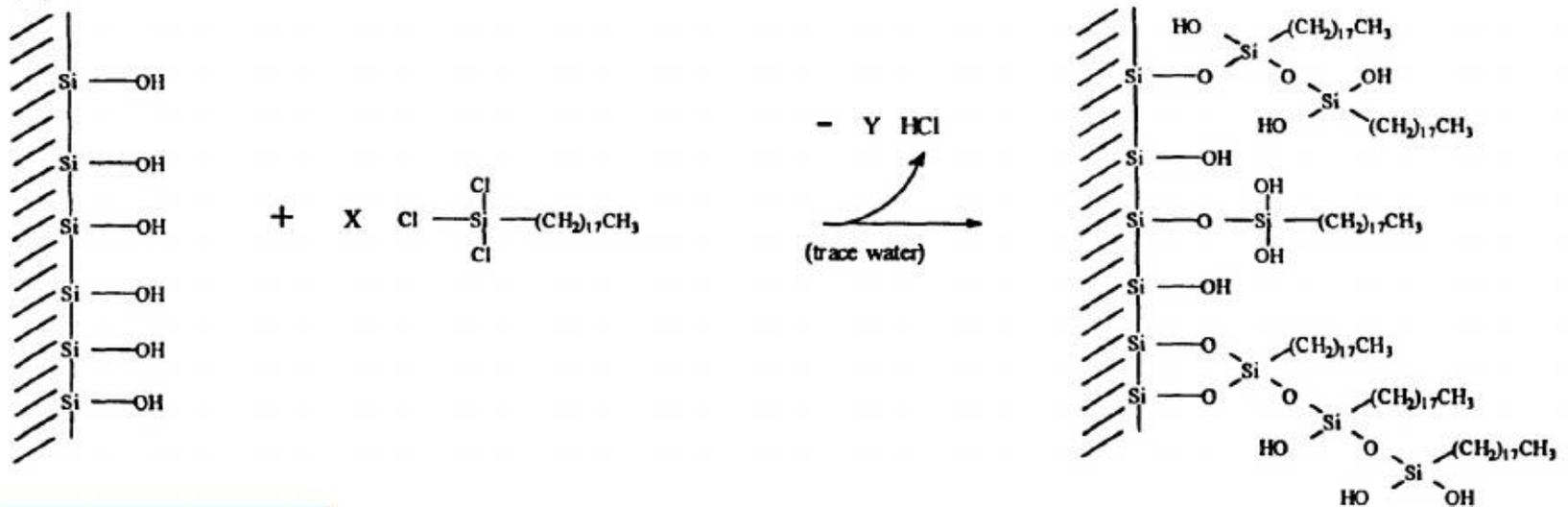
HPLC

Reverse-Phase Chromatography

Stationary Phases - Bonded phase silica columns

Amount of material bonded to the silica is described by the term carbon load - amount of carbon as a weight % of bulk silica packing.

Example: Monofunctional C_{18} - carbon load = 7-15 % (w/w)

Monofuntional preferred - easier to control - less batch to batch variation.

Higher the carbon load, more hydrophobic the column.

HPLC

Reverse-Phase Chromatography

Stationary Phases - Bonded phase silica columns

Identify the column by the nature of the bonded R' group. C_{18} - most common.

Others – C_8, phenyl, C_6, C_4, C_2, CN, NH_2, and NO_2.

Retention increases exponentially as alkyl chain length increases.

HPLC

Reverse-Phase Chromatography

Stationary Phases - Bonded phase silica columns

Not all silanol groups are functionalized.

These residual acidic silanol groups can cause tailing of basic compounds.

So usually after functional groups are added - an end-capping procedure is done.

This procedure uses chlorotrimethylsilane to cap open groups.

$$\underset{CH_3}{\overset{CH_3}{H_3C-Si-Cl}}$$
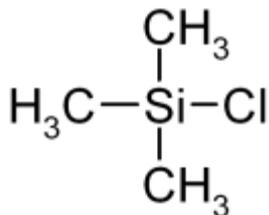
HPLC

Reverse-Phase Chromatography

Stationary Phases - Bonded phase silica columns

Stability of bonded phases are largely limited by the silica support - mobile phase pH must be kept between 2-8.

Below pH 2 - hydrolysis of the bonded functional groups - decreasing retention.

Above pH 8, silica dissociates and the support starts to dissolve - creating voids in the packing material decreasing column efficiency.

HPLC

Reverse-Phase Chromatography

Mobile Phases - Solvent classification

Classification scheme developed by Snyder uses a triangle - the apexes represent solvent properties such as proton donor, proton acceptor, dipole interaction.

There are eight groups of solvents.

HPLC

Reverse-Phase Chromatography

Mobile Phases - Solvent classification

Table 6.6 Classification of solvents according to their groups.

Group	Solvents
I	Aliphatic ethers, hexamethylphosphoric acid amide, trialkyl amines
II	Aliphatic alcohols, methanol
III	Pyrine derivatives, amides, glycol ethers, sulfoxides, tetrahydrofuran
IV	Glycols, benzyl alcohol, acetic acid, formamide
V	Methylene chloride, ethylene chloride
VI	Tricresyl phosphate, aliphatic ketones and esters, polyethers, dioxane, sulfones, nitriles, polypropylene carbonate, acetonitrile
VII	Aromatic hydrocarbons, nitro compounds, aromatic ethers
VIII	Fluoroalkanols, *m*-cresol, chloroform, water

Data from Snyder [17].

proton acceptor

proton donor   dipole interaction
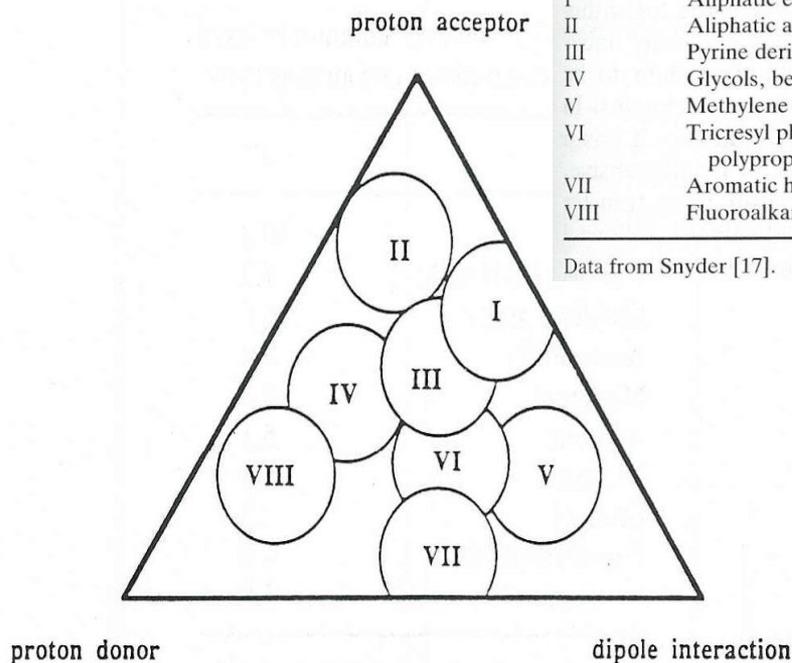
Fig. 6.15. The solvent classification triangle which illustrates the eight selectivity groups determined by polarity and selectivity. Modified with permission from Snyder (1974) *J. Chromatogr.*, **92**, 223.

HPLC

Reverse-Phase Chromatography

Mobile Phases - Solvent Strength

Expressed as a function of polarity.

Solvent polarity parameter – P′

Lower the value of P′ - stronger the solvent.

Water is generally used as the base solvent and mobile phase strength determined by mixing water with another solvent (organic modifier).

HPLC

Reverse-Phase Chromatography

Mobile Phases - Solvent classification

The eluotropic series are common solvents placed in order of relative chromatographic polarity.

- Water is generally used as the base solvent and mobile phase strength determined by mixing water with another solvent (organic modifier).

Polar Molecule

Non-Polar Molecule

Mobile Phases

Water   Alcohol   Acetronitrile   THF   Hexane

HPLC

Reverse-Phase Chromatography

Mobile Phases - Solvent strength

Generally solute retention decreases by a factor of 2 for every 10% volume addition of solvent to water.

Example:
50:50 H_2O/ CH_3OH nitrobenzene t_r= 2 min
45:55 H_2O/ CH_3OH nitrobenzene t_r= 1 min
55:45 H_2O/ CH_3OH nitrobenzene t_r= 4 min

HPLC

Reverse-Phase Chromatography

Mobile Phases - Solvent strength

Methanol is most widely used organic modifier since it has low u.v. cut-off (205 nm), reasonable eluting strength and is inexpensive.

Acetonitrile has a lower u.v. cut-off (190 nm), better mass transfer properties, more expensive.

Tetrahydrofuran next most used.

HPLC

Reverse-Phase Chromatography

Mobile Phases - Solvent strength

To find optimal mobile phase strength start with solvent-rich mobile phase (more organic modifier) and decrease organic modifier content until desired retention is obtained.

Once a binary mixture of a desired strength is found - selectivity can be altered by changing the organic modifier - while still maintaining a constant eluotropic strength.

Example: 3 mobile phases: 30:70 CH_3OH/H_2O or 22:78 ACN/H_2O or 16:84 THF/H_2O - all give equal retention but different selectivity.

There are many different equations to calculate this but a simple rule to remember is:

$\phi ACN = 0.77\ \phi CH_3OH$ ϕ_M – represents the mobile phase volume fraction.

$\phi THF = 0.66\ \phi CH_3OH$

HPLC

Reverse-Phase Chromatography

For HPLC, the separation is described by 4 major concepts:

- **Capacity, efficiency, selectivity and resolution**

- **Capacity and selectivity of a column are controlled by the manufacturer.**

- **Efficiency and resolution are controlled by the user.**

HPLC

Reverse-Phase Chromatography

Capacity Factor

For effective LC separations, a column must have the capacity to retain samples.

Capacity factor – k′ - of a column is a direct measure of the strength of the interaction of the solute with the packing material.

$$k' = \frac{t_r - t_m}{t_m} = \frac{V_r - V_m}{V_m}$$

t_r – ret. time of the analyte
t_m - ret. time of unretained solute (marker compounds NaI)

HPLC

Reverse-Phase Chromatography

Capacity Factor

Capacity factor of a column is mostly a function of the packing material but can be manipulated to some degree by varying the solvent strength.

Higher k′ - the greater the ability of the column to retain solutes, longer analysis time.

k′ values range 1-10

Usually k′ between 2-5 represents a good balance between analysis time and resolution.
k′ - too low - solutes not resolved
k′ - too high - analysis time too long

HPLC

Reverse-Phase Chromatography

Selectivity

Selectivity or separation factor, α , describes the separation of two peaks relative to each other.

$$\alpha = \frac{k_2{}'}{k_1{}'} = \frac{t_{r_2} - t_m}{t_{r_1} - t_m}$$

Selectivity is a function of packing material.

Value of α ranges from unity (1) when $t_{r2} = t_{r1}$, $\alpha \geq 1$.

Easy way to increase α is to change the composition (components) of the mobile phase.

HPLC

Reverse-Phase Chromatography

Resolution

Resolution, R or R$_s$- degree of separation between neighboring solute peaks. R$_s$ is affected by selectivity (α), efficiency (N), and capacity (k') of the column.

$$R = \frac{1}{4}\frac{\alpha-1}{\alpha}(N^{1/2})\frac{k'}{1+\text{k}'}$$

$$R_s = \frac{2(t_{r_2} - t_{r_1})}{wb_1 + wb_2}$$

HPLC

Reverse-Phase Chromatography

Resolution

When the two peaks are just resolved at the baseline R = 1.5.

Increasing efficiency (double theoretical plates, longer columns) only gain 1.4 in resolution since $R \propto N^{1/2}$.

Better to change selectivity, α, by varying the mobile phase or capacity factor, k′ by varying mobile phase strength

HPLC

Reverse-Phase Chromatography

Efficiency – can be defined as "mechanical separation power"

Determined by the column length and the particle size

Efficiency (N) describes peak broadening as a function of retention, and is described in terms of number of theoretical plates. More efficient the column - the less band spreading.

$$N = 16(\frac{t_r'}{w})^2 \qquad\qquad n = 5.54(\frac{t_r'}{w_{1/2}})^2$$

Typical ODS-C18 column ~ 12.5 cm long and 5 um packing
N ≈ 6250   H = 0.02 mm

HPLC

Reverse-Phase Chromatography

Efficiency

    For a given particle size, more mechanical separation power is gained by increasing column length. However, the trade-offs are longer chromatographic run times, greater solvent consumption, and higher backpressure. Shorter column lengths minimize all these variables but also reduce mechanical separation power.
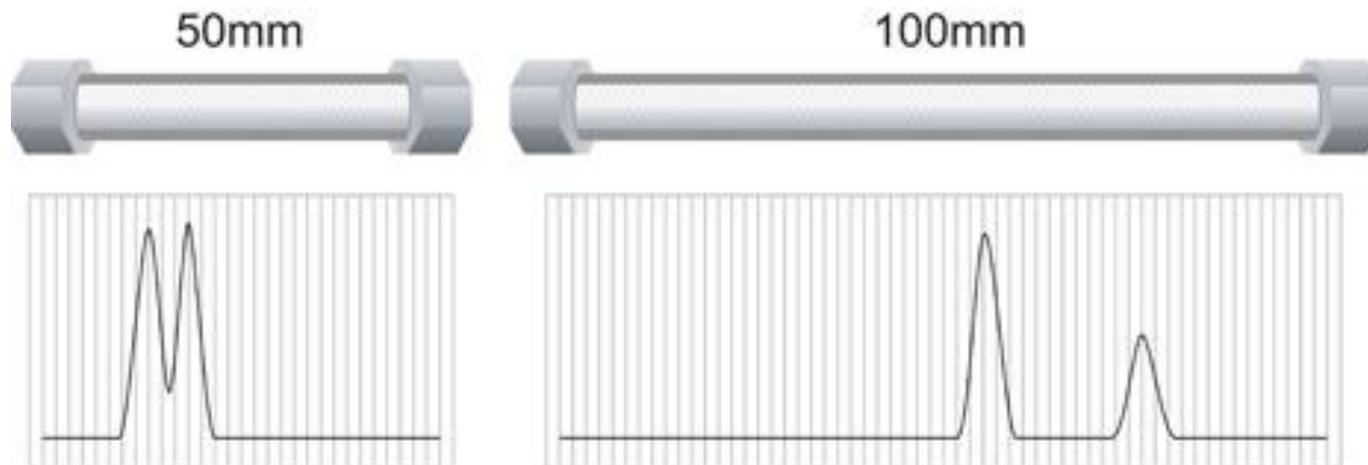
HPLC

Reverse-Phase Chromatography

Efficiency

A column of the same length and i.d., but with a smaller particle size, will deliver more mechanical separation power in the same time.

However, its backpressure will be much higher.
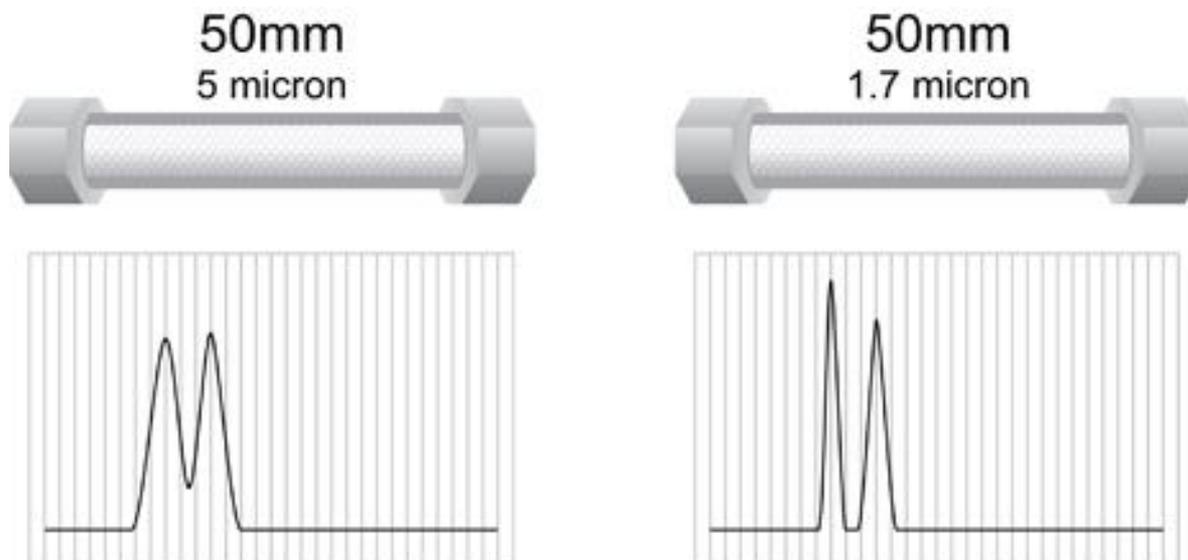
HPLC

Reverse-Phase Chromatography

The Fundamental Resolution Equation

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

- **α = Selectivity (influenced by mobile and stationary phase)**
- **N = Column Efficiency (influenced by length and particle size)**
- **k = Capacity Factor (retention) (influenced by stationary and mobile phase)**

HPLC

Reverse-Phase Chromatography

Applications

With RPLC best separations are of neutral solutes that are soluble in water or other relatively polar solvents with molecule weights less than 2000-3000.

Hydrocarbons was the first group separated with reverse phase.

Non-polar solutes or fatty acids resolved on the basis of their carbon chain length.

HPLC

Reverse-Phase Chromatography

Applications

Largest HPLC reverse phase industry is pharmaceutical for drug analysis.

Clinic labs - analysis of plasma and urinary catecholamines with EC detection.

Also amino acids, proteins, peptides.

Analysis of food - beverage, agriculture, carbohydrates, food additives, aflatoxins, sweetners.

Environmental samples - pesticides, pollutants

Assignment

- Read Chapter 28
- Homework Chapter 28: 2-12, & 15
- HW16 Chapter 28 Due 4/19/24