Liquid Chromatography

1. Introduction and Column Packing Material

2. Retention Mechanisms in Liquid Chromatography

3. Method Development

4. Column Preparation

5. General Instrumental aspects

6. Detectors

(Chapter 4 and 5 in The essence of chromatography)

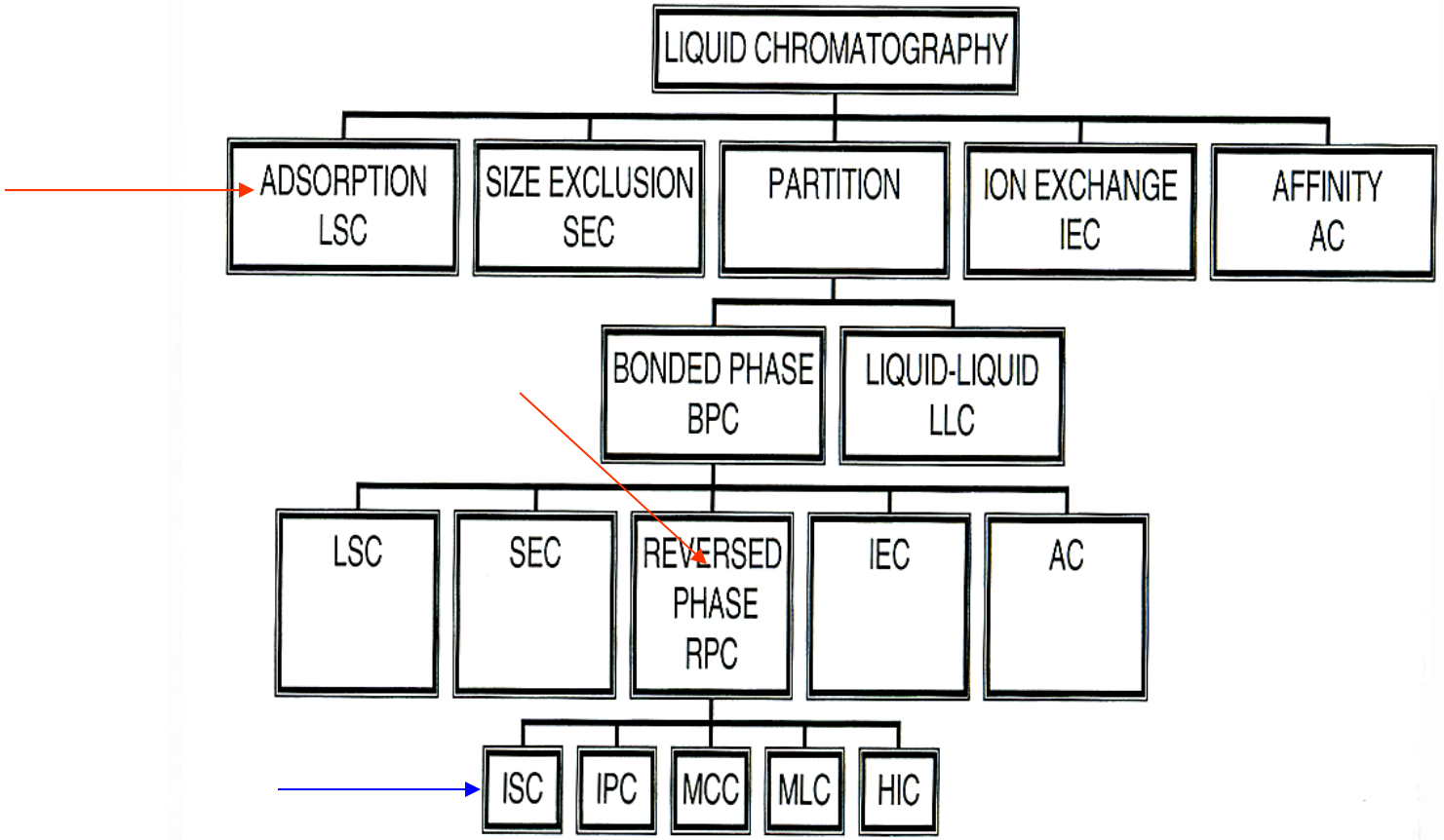
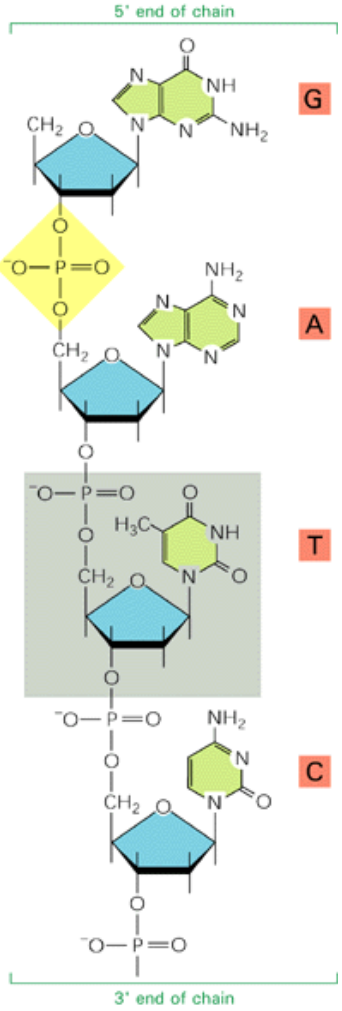
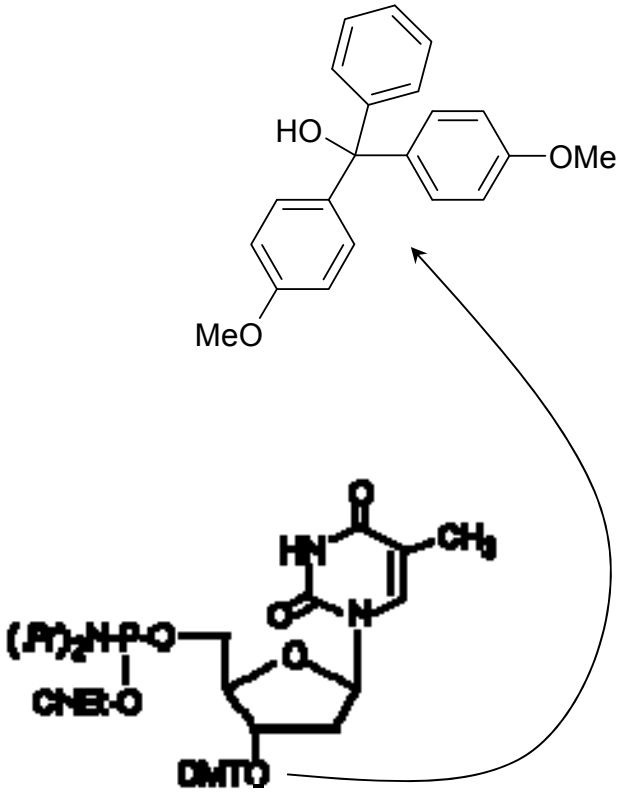


Figure 4.1. Family tree of liquid chromatographic separation modes. LSC = liquid-solid (or normal-phase) chromatography; SEC = size-exclusion chromatography; IEC = ion-exchange chromatography; AC = affinity chromatography; BPC = bonded-phase chromatography; LLC = liquid-liquid chromatography; RPC = reversed-phase chromatography; ISC = ion-suppression chromatography; IPC = ion-pair chromatography; MCC = metal-complexation chromatography; MLC = micellar-liquid chromatography; and HIC = hydrophobic-interaction chromatography.

Separation of synthetic Oligonucleotide using RPLC





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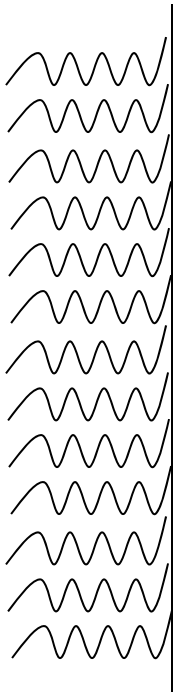


Column

A. Ion-Suppression Chromatography

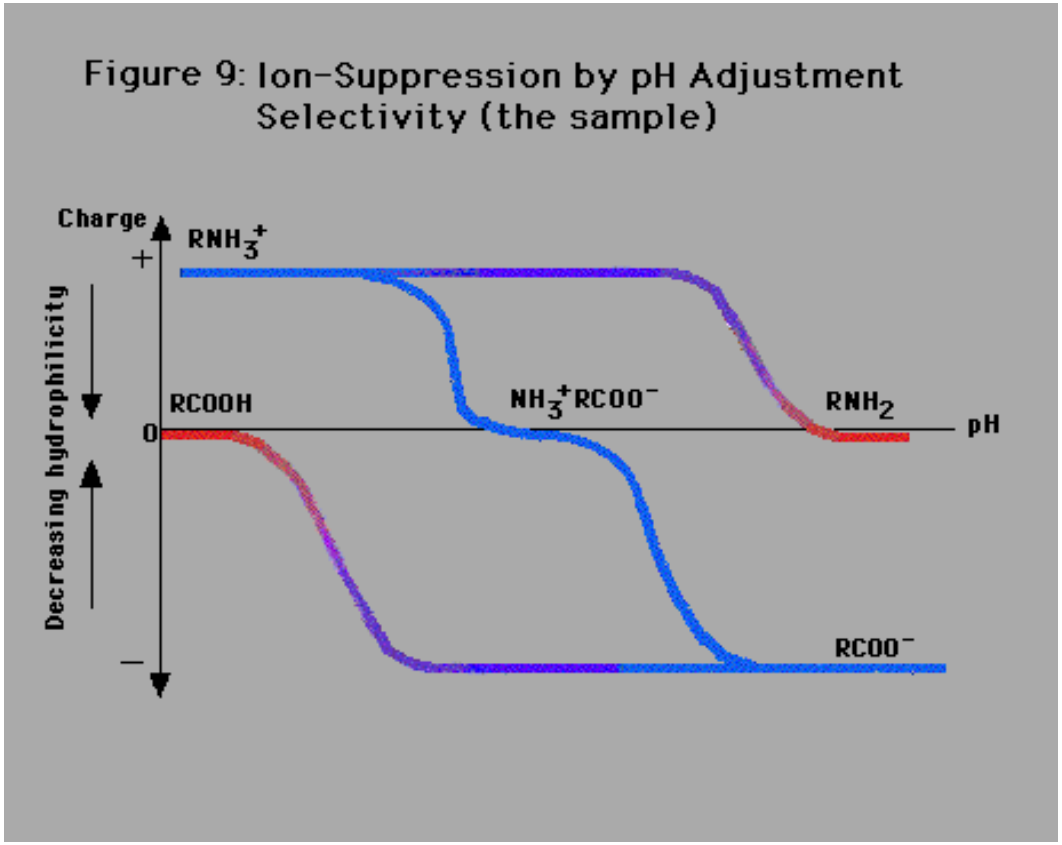
1. Ion-Suppression Chromatography is used fro the separation of weak acid and base by reversed-phase chromatography.

2. Mechanism: the difference properties between neutral and ionic substance.

$$HAc \underset{}{\overset{pH}{\rightleftharpoons}} Ac^-$$

Ionic components show low solubilities in the lipid layer of the particles making up the stationary phase, because of the highly hydrophilic character of their charged groups. If the charged group is weakly acidic (R-COO-) or basic (R-NH3+), it may be rendered neutral by adding a buffering substance to the mobile phase. Acidic buffers neutralize weak acids, while alkaline buffers neutralize weak bases in terms of net charges.

3. careful adjustment of the mobile phase pH to result in a nonionized analyte.

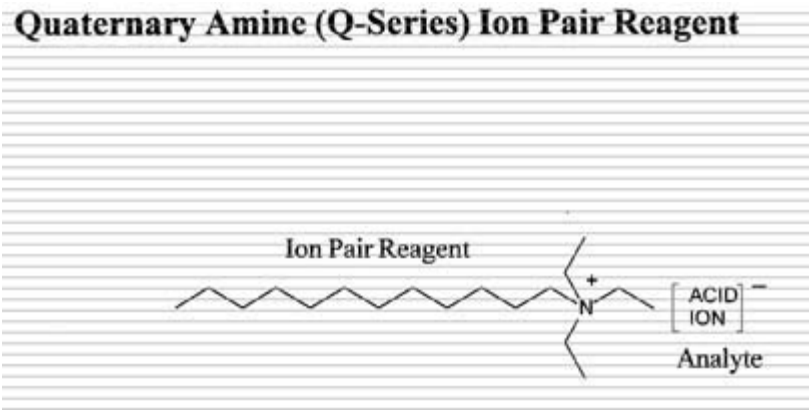


B. Ion-pair Chromatography

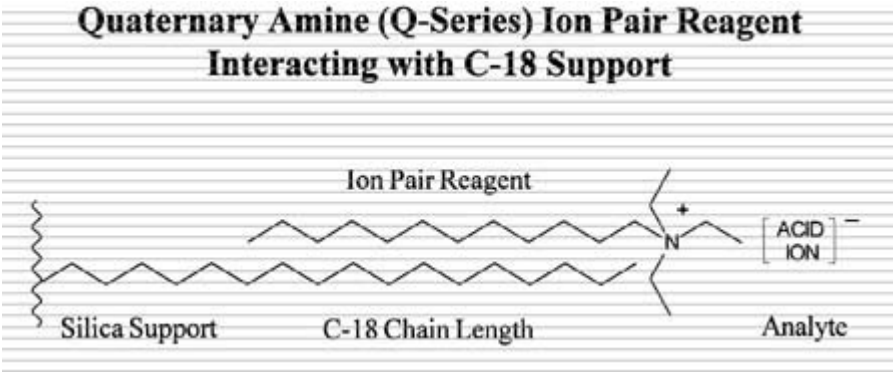
1. Ion-pair chromatography is used for the separation of ionic and ionizable compounds and mixtures of neutral and ionic compounds.

2. In this method, counterions (species of opposite charge to the solutes) thereby regulate the retention. Typically alkyl amines or tetra alkyl amines are added to ion pair with acids whereas alkyl sulfates, sulfonates, or phosphates are used to ion pair with bases

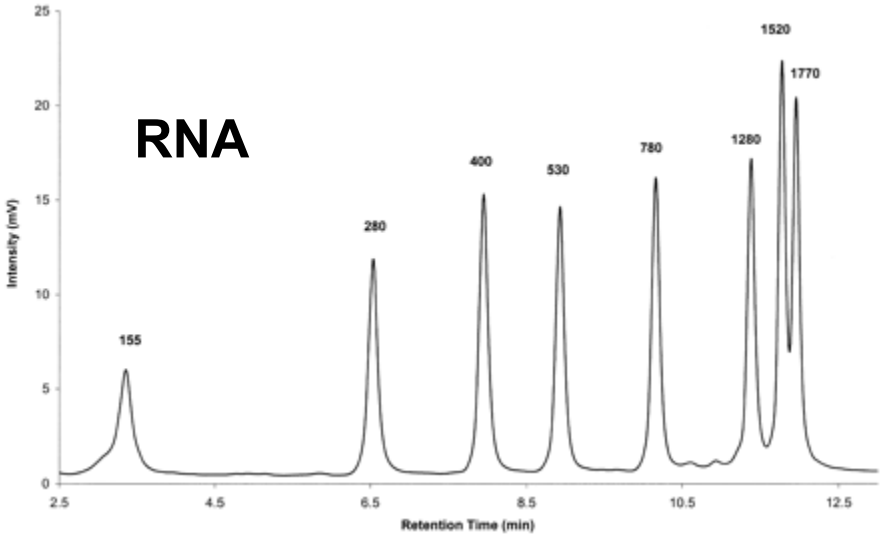
$$A^- + B^+ \longrightarrow A^-B^+$$

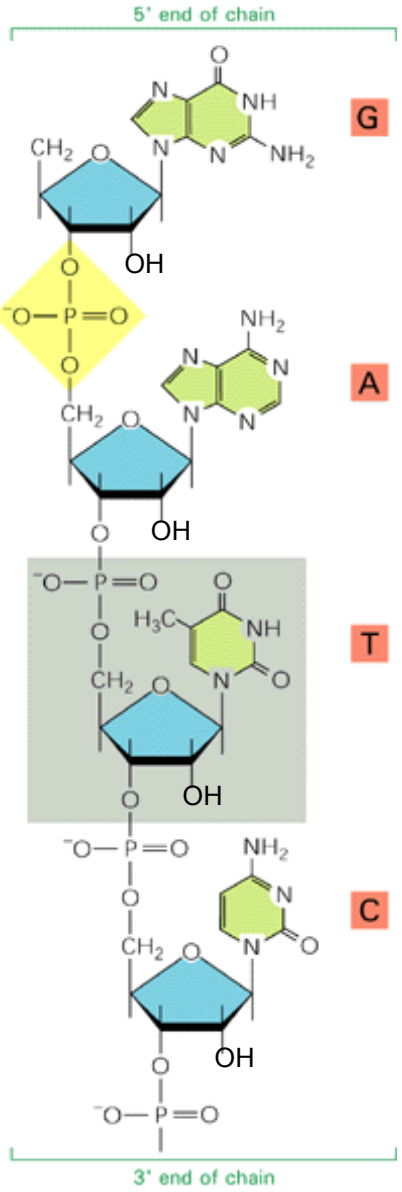


3. Retention of ion pairs



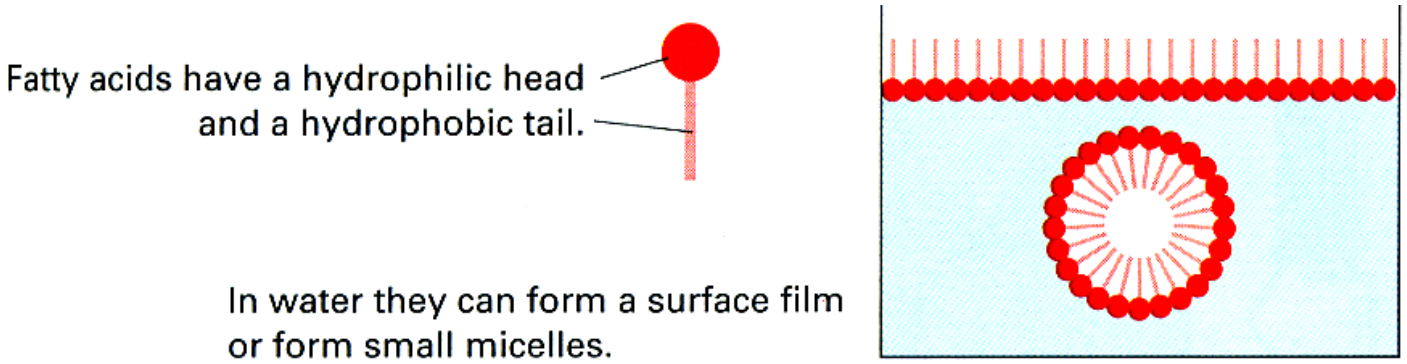
4. An example of separation of RNA





C. Micellar Liquid Chromatography

1. Micellar liquid chromatography is a reversed-phase technique that uses an aqueous-organic solvent mobile phase containing a surfactant above its critical micelle concentration.

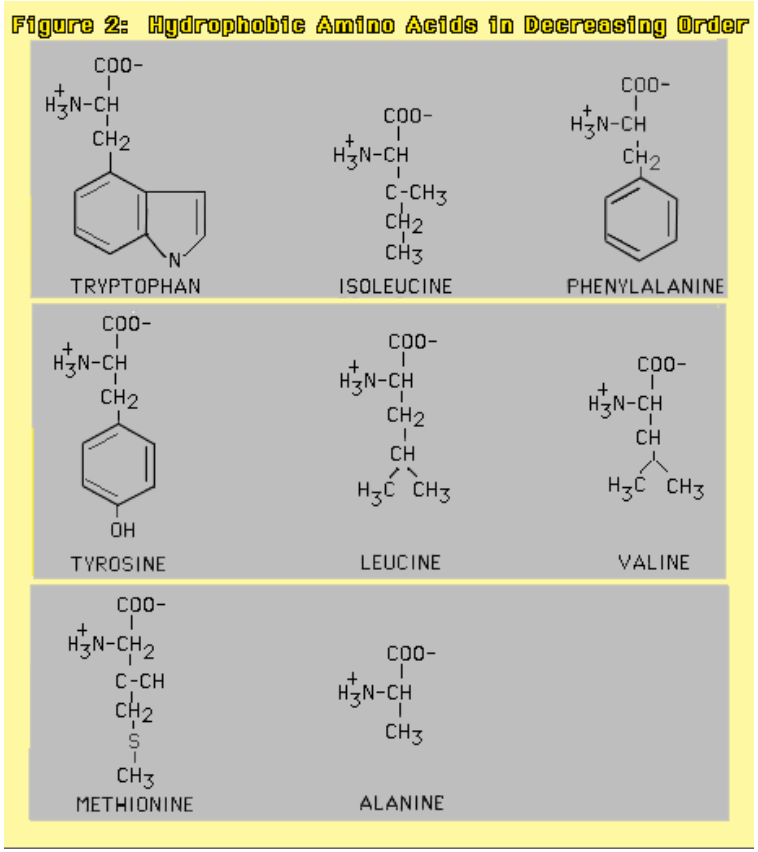


2. Mechanism: $nA^- + B_n^+ \longrightarrow nA^-B_n^+$

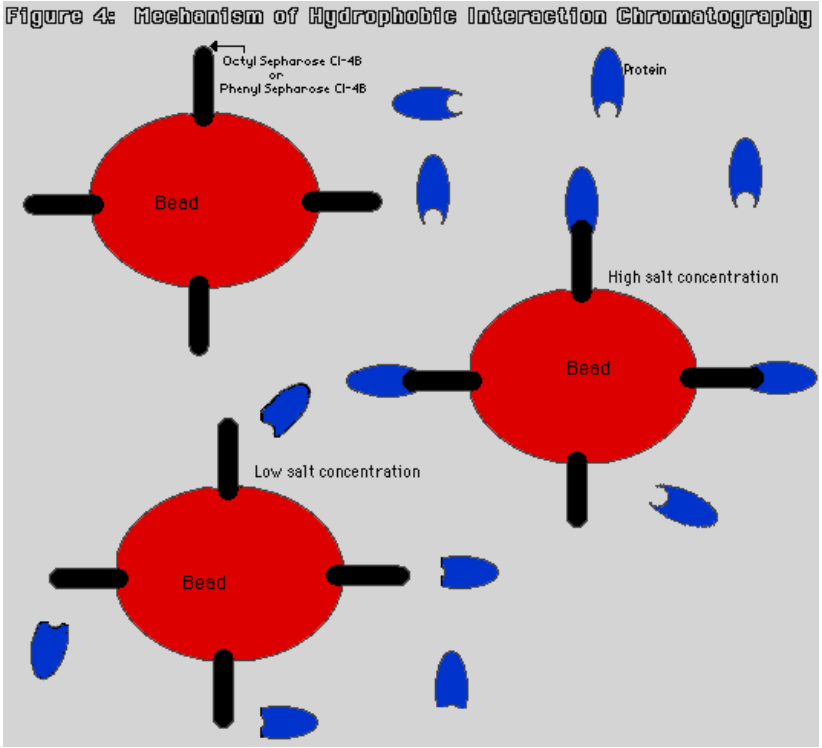
D. Hydrophobic interaction Chromatography (HIC)

1. Hydrophobic interaction chromatography is a reversed-phase separation technique that uses a weakly hydrophobic phase and a negative ionic strength gradient in a buffered aqueous mobile phase for the separation of proteins.

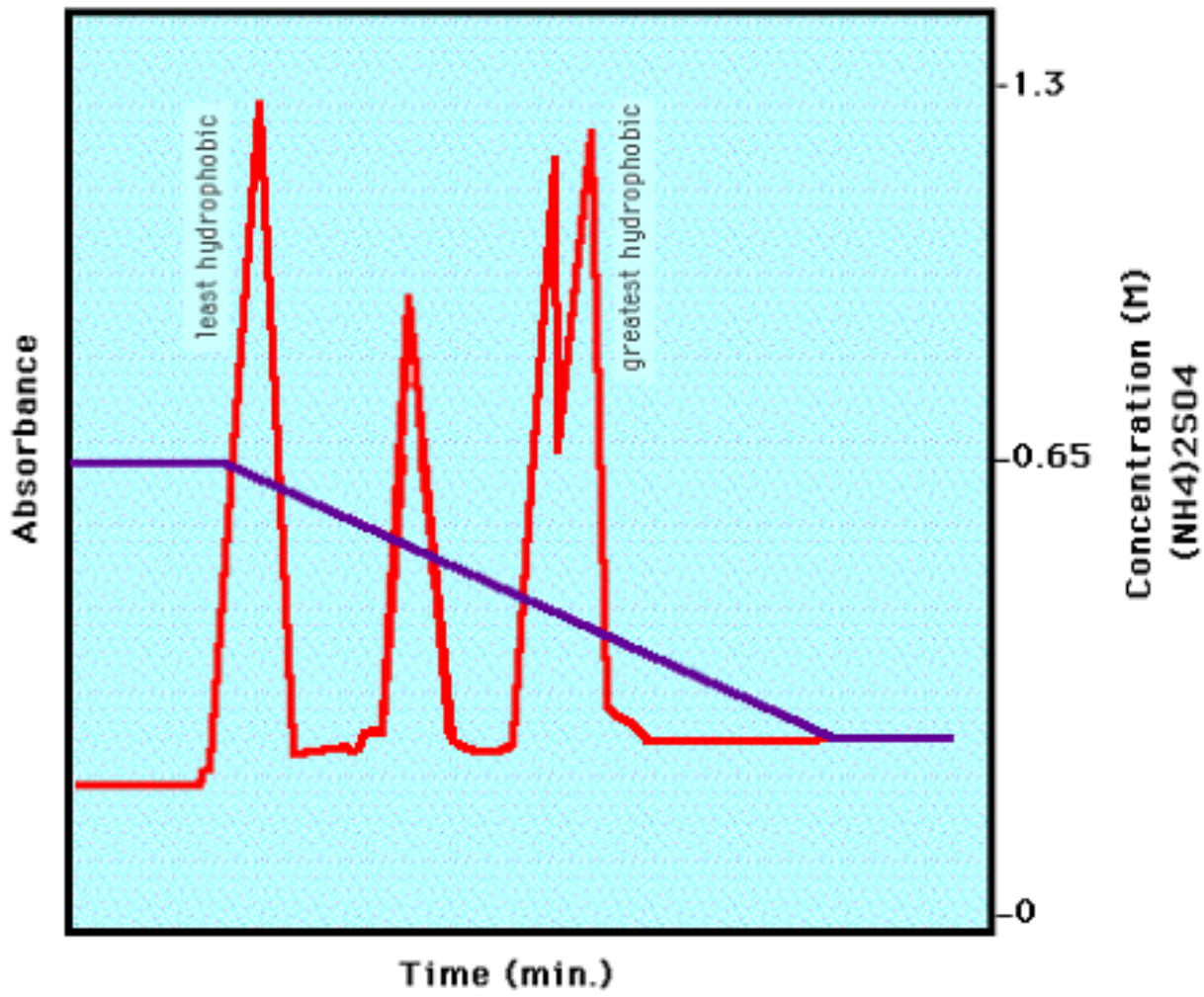
2. Mechanism:
hydrophobicity differences



3. Protein separation

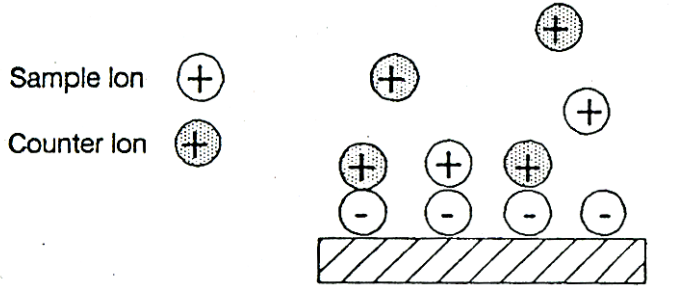


Separation on HIC matrices (red bead) are usually done in aqueous salt solutions which generally are non denaturing. Samples (blue) are loaded onto the matrix in a high-salt buffer and elution is by a descending salt gradient. HIC depends on surface hydrophobic groups and is carried out under conditions which maintain the integrity of the protein.


least hydrophobic
greatest hydrophobic
Absorbance
Time (min.)
1.3
0.65
0
Concentration (M)
(NH4)2S04

Ion-Exchange Chromatography

1. Ion-exchange chromatography is a liquid chromatography technique in which solutes are separated by their adsorption onto a support containing fixed charge onto a support containing fixed charges on its surface.



2. Ion-exchange is a fairly common technique used in water softners and in the industrial removal or replacement of ionic compounds for products. Ion-exchange is used in chromatography for separation of a wide variety of charged compounds, including inorganic ions, organic ions, and biological compounds (such as amino acids, proteins and nucleic acids)

3. Mechanism

$$n(\text{support}—A^-B^+) + C^{n+} \rightleftharpoons (\text{support}—A^-)_nC^{n+} + nB^+$$

$$K = ([B^+]^n[\overline{C^{n+}}]/[\overline{B^+}]^n[C^{n+}])$$

4. Stationary phase:

a. There are two general types of stationary phases used in IEC

i. Cation-exchange

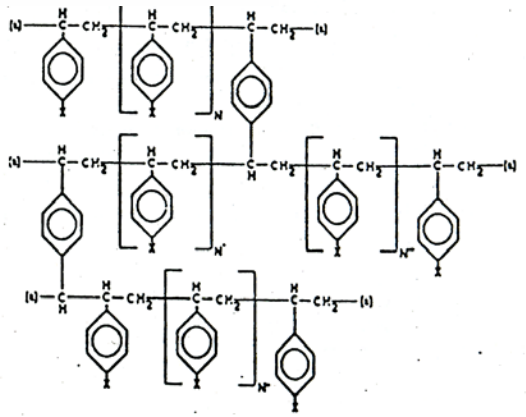
S-cation exchanger CM-cation exchanger

ii. Anion-exchange

Q-anion exchanger DEAE-anion exchanger

b. Three types stationary phases

i. Cross-linked polystyrene resins



ii. Carbohydrate-based resins

(Polymeric carbohydrates, Agarose, dextran, cellulose)

These are especially useful in the separation of biomolecules.

iii. Silica-based supports

5. Ion selectivity

$$n(\text{support—}A^-B^+) + C^{n+} \rightleftharpoons (\text{support—}A^-)_nC^{n+} + nB^+$$

$$K = ([B^+]^n[C^{n+}]/[B^+]^n[C^{n+}])$$

a. For strong-acid resins (e.g. $-SO_3H$), the binding strength of a cation is related to its charge and radius. This is described by the polarizing power (P).

$P = Z^2/r$, **Where: Z=charge on the ion, r=radius of the ion solvent cavity**

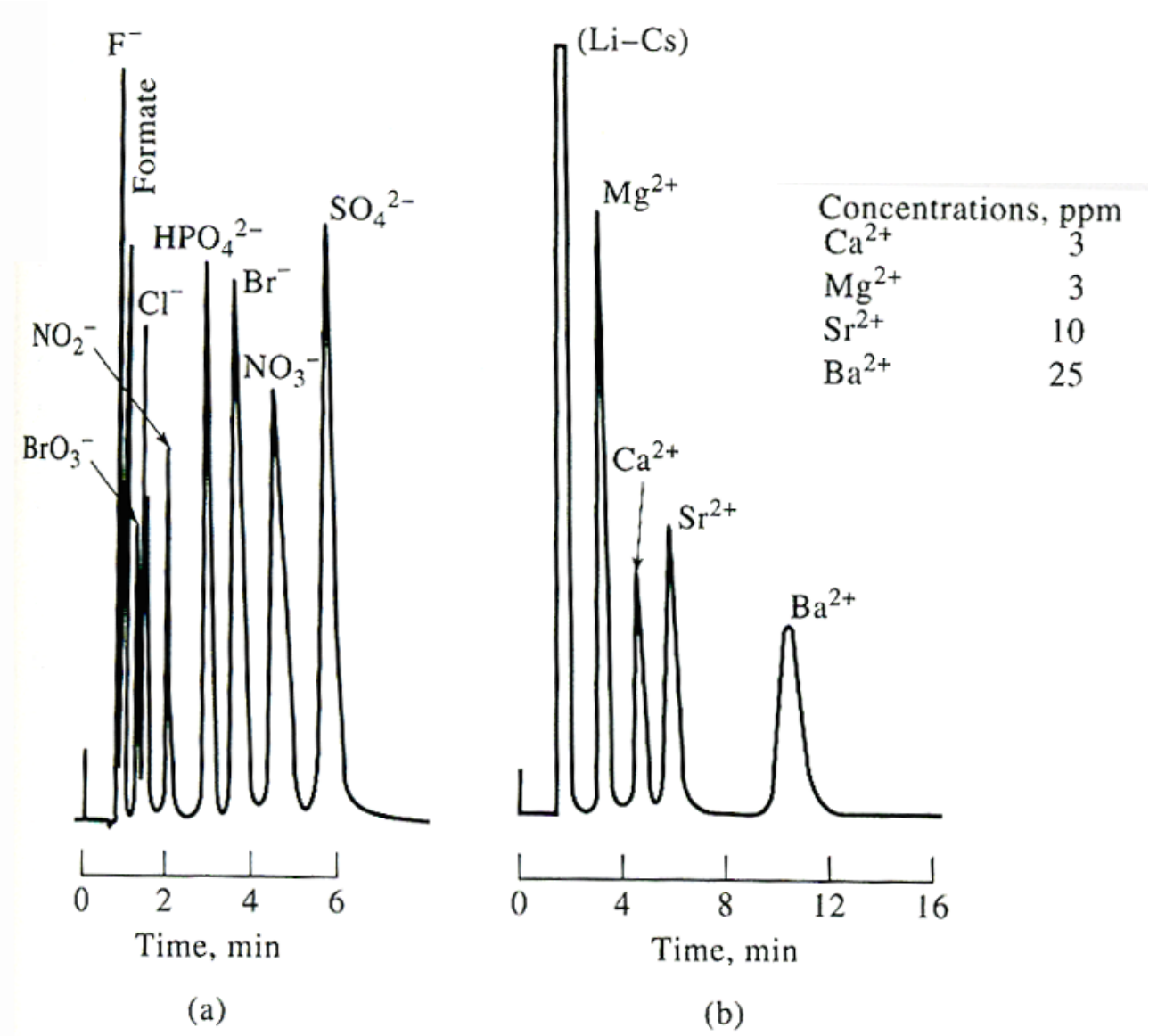
b. The order of binding strengths of various cations on a strong cation exchanges resin.

$$Li^+ < H^+ < Na^+ < NH_4^+ < K^+ < Rb^+ < Cs^+ < Ag^+ < Mg^{2+} < Zn^{2+} < Co^{2+} < Cu^{2+} < Ca^{2+}$$

c. The binding of anions to strong anion exchange resins.

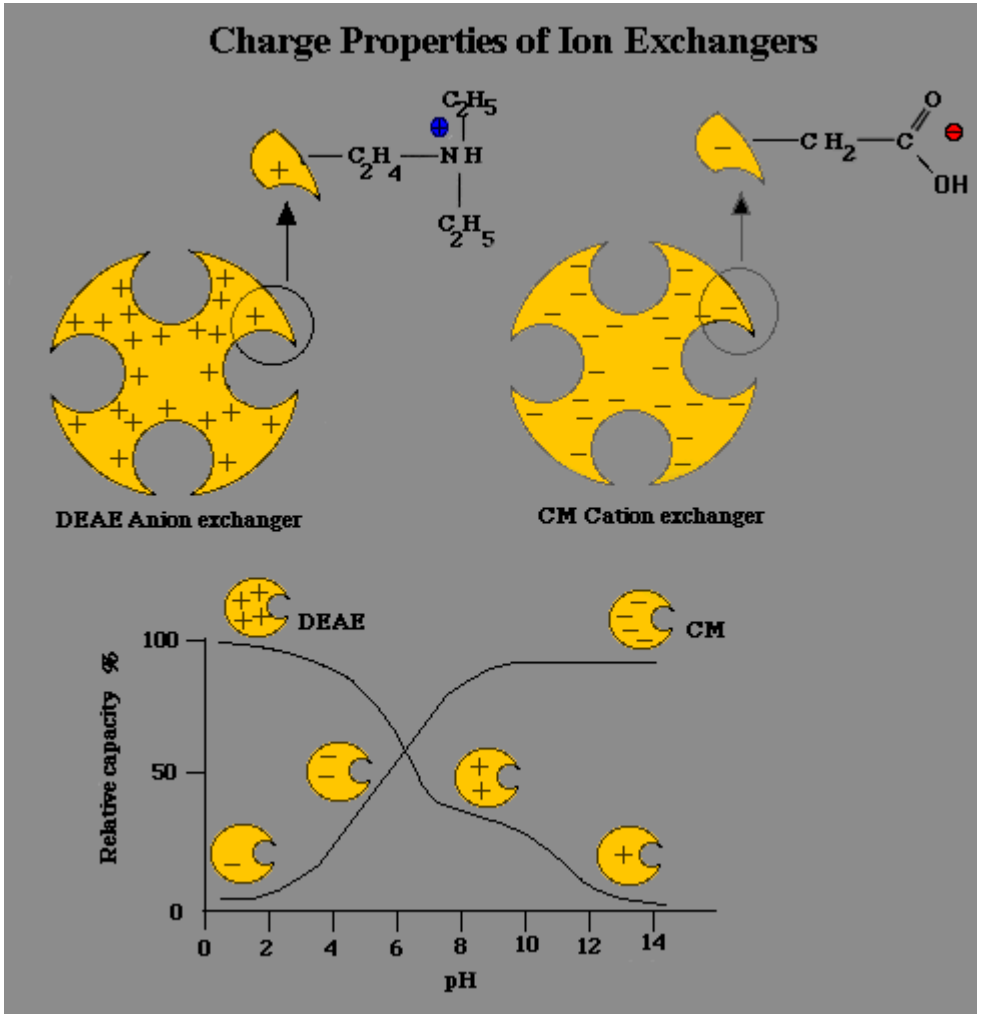
$$F^- \sim OH^- < CH3COO^- < H_2PO_4^- < HCO_3^- < Cl^- < NO_2^- < HSO_3^- < CN^- < Br^- < NO_3^- < HSO_4^- < I^-.$$

Concentrations, ppm	
F^-	3
Formate	8
BrO_3^-	10
Cl^-	4
NO_2^-	10
HPO_4^{2-}	30
Br^-	30
NO_3^-	30
SO_4^{2-}	25

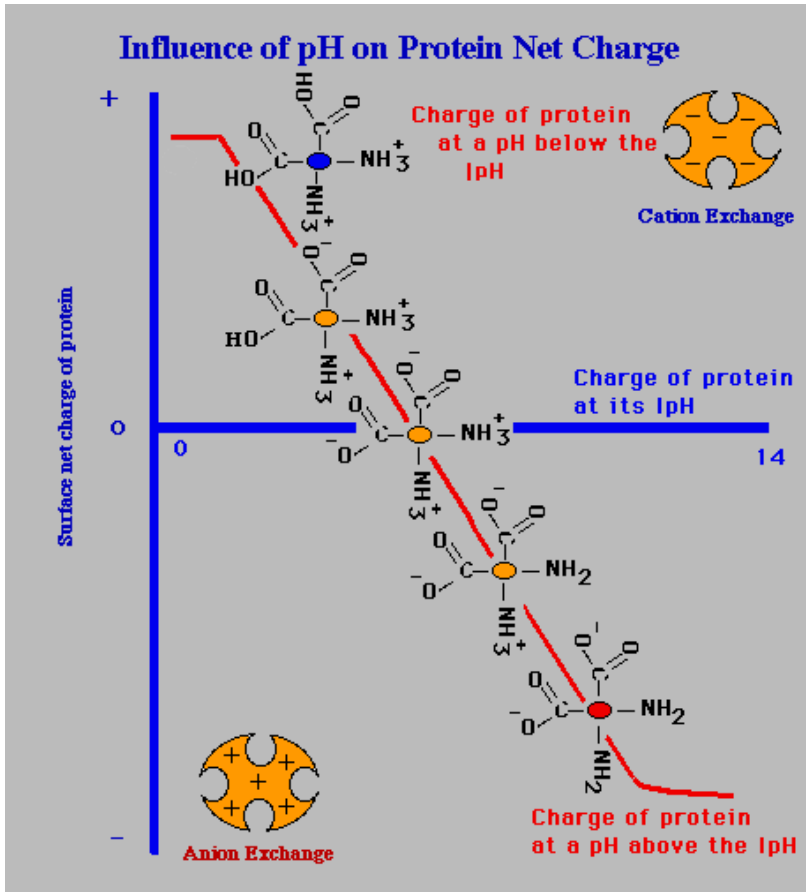


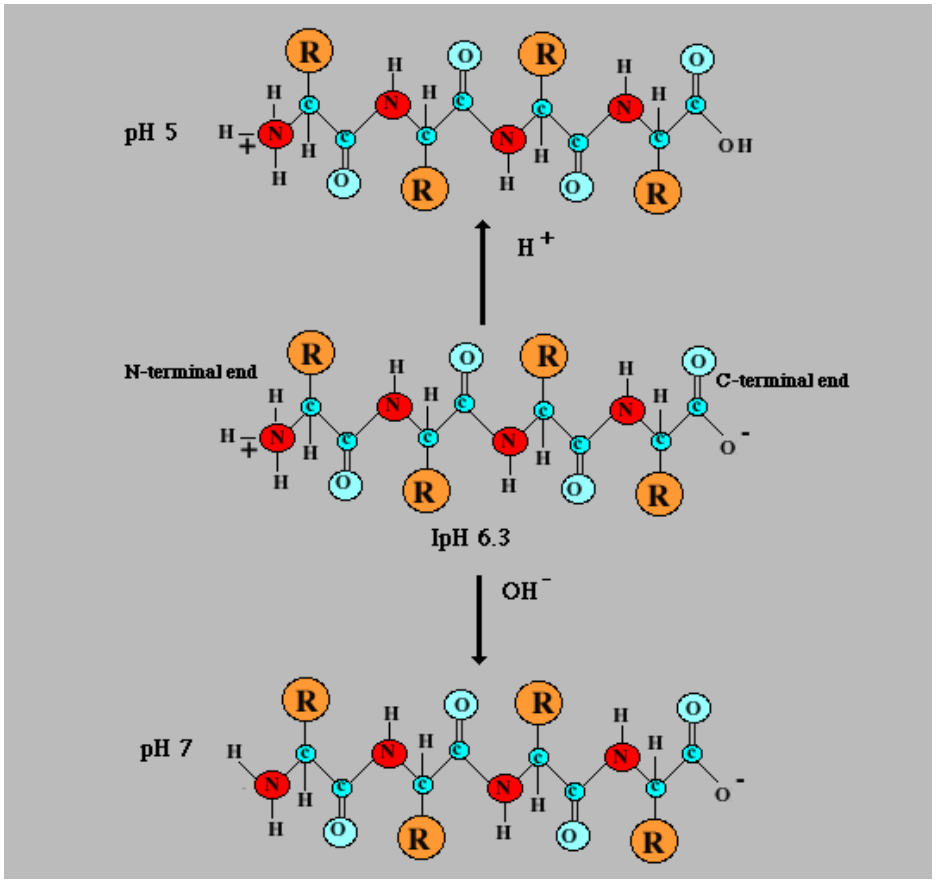
Concentrations, ppm	
Ca^{2+}	3
Mg^{2+}	3
Sr^{2+}	10
Ba^{2+}	25

6. Weak ion Exchanger with pH



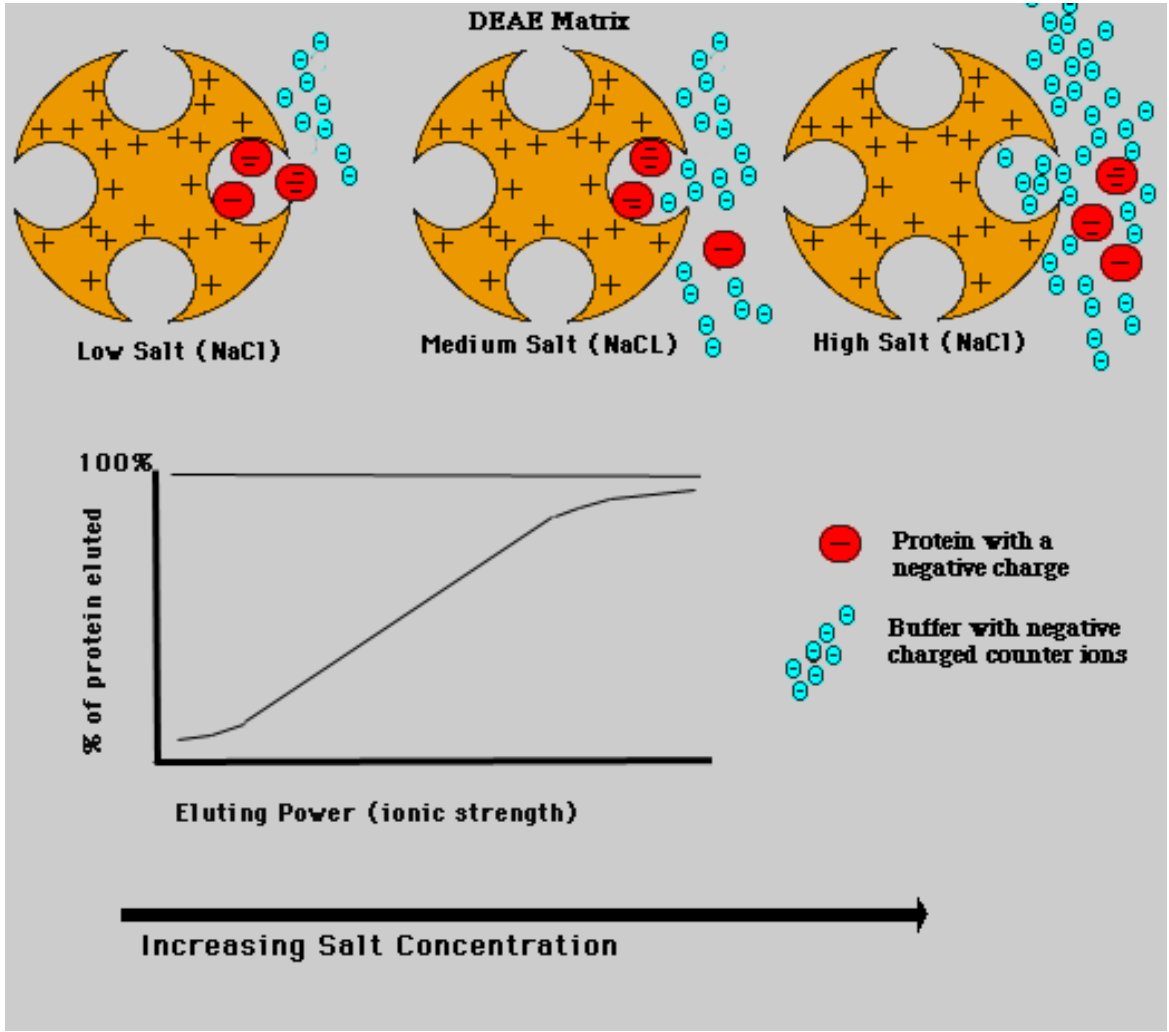
6. Protein and pH





7. Strong and weak mobile phase

Strong mobile phase contains a high concentration of an ion that competes with sample ions for charged groups in the stationary phase.



Ion-exclusion Chromatography

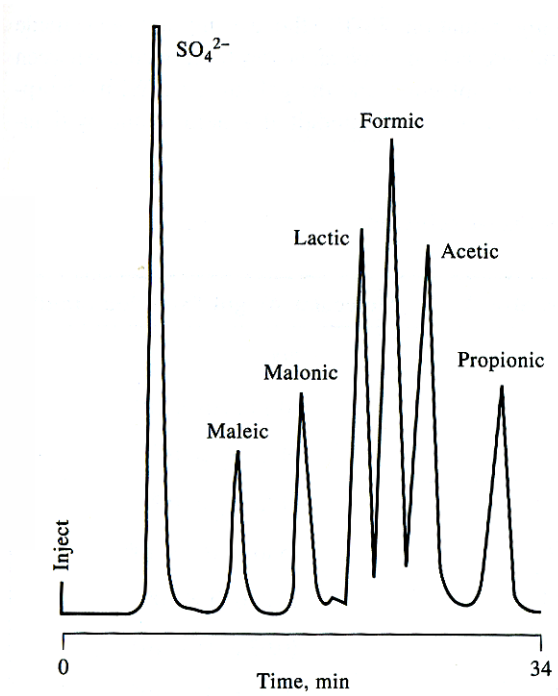
1. **Ion-exclusion Chromatography is used for the separation of low molecular weight ions and neutral substances by a combination of partition, adsorption and ion repulsion.**

2. The stationary phase is a high capacity ion exchange with same type of immobilized ionic groups as the sample ions.

3. Donnan exclusion: same charge as the stationary phase repelled and not allowed to enter the stagnant mobile phase, but the ions with opposite charges or neutral do enter.

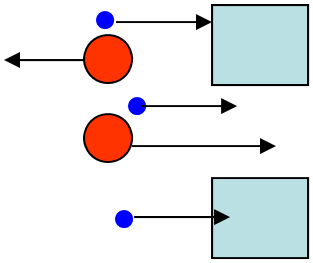
3. Separation of acidic species

S-cation exchanger CM-cation exchanger



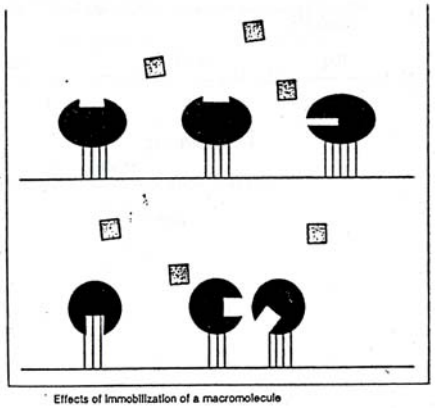
Size exclusive chromatography

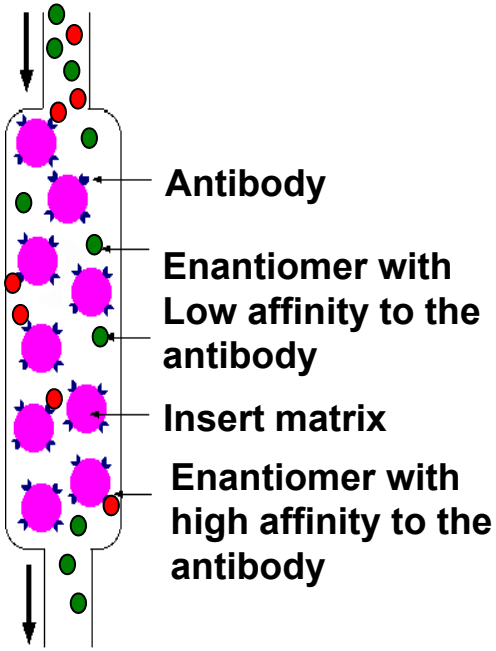
Standard entropy effect



Affinity chromatography

Antibody-antigen





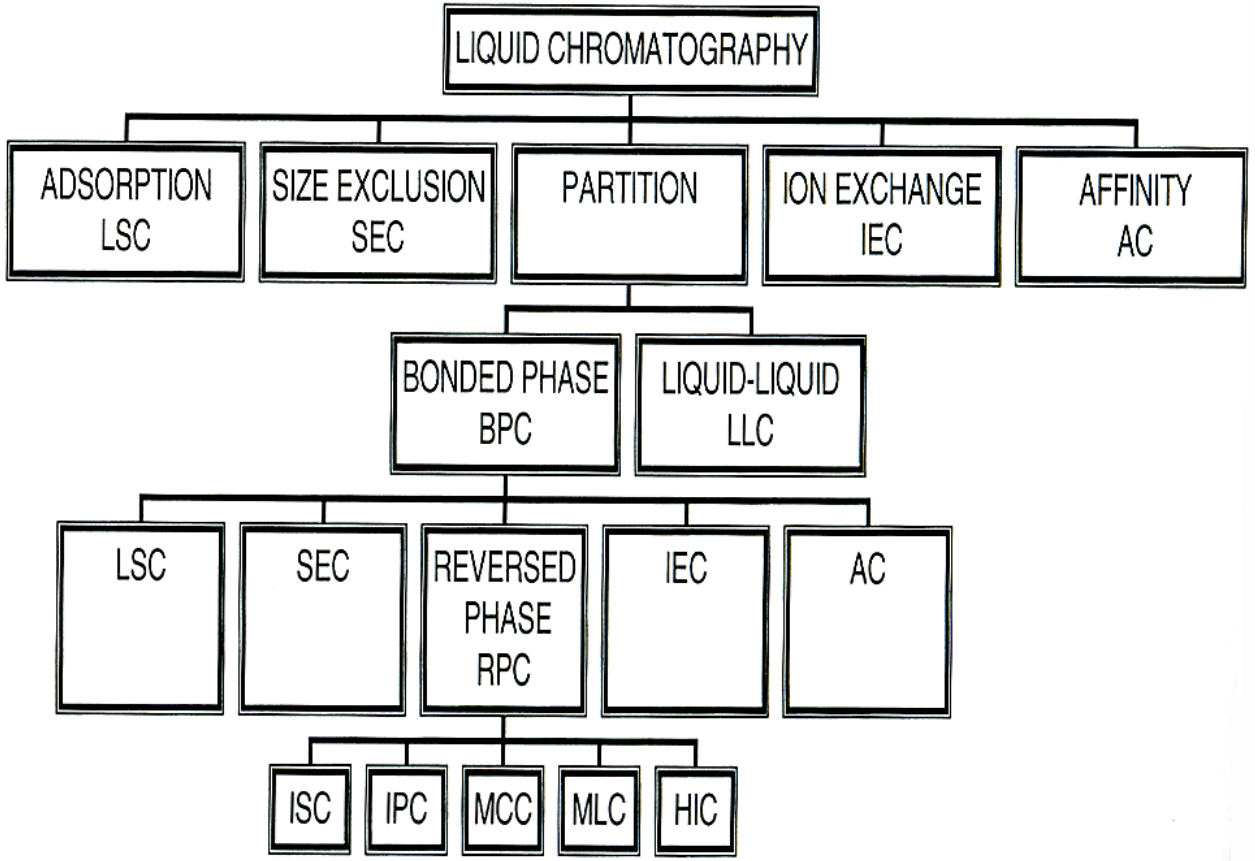


Figure 4.1. Family tree of liquid chromatographic separation modes. LSC = liquid-solid (or normal-phase) chromatography; SEC = size-exclusion chromatography; IEC = ion-exchange chromatography; AC = affinity chromatography; BPC = bonded-phase chromatography; LLC = liquid-liquid chromatography; RPC = reversed-phase chromatography; ISC = ion-suppression chromatography; IPC = ion-pair chromatography; MCC = metal-complexation chromatography; MLC = micellar-liquid chromatography; and HIC = hydrophobic-interaction chromatography.

Next Class: 3. Method Development