Analytical chemistry

Lecture №32

Liquid chromatography
Part I

• **Chromatography** is a technique in which the components of a mixture are separated based on differences in the rates at which they are carried through a fixed or **stationary phase** by a gaseous or liquid **mobile phase.**

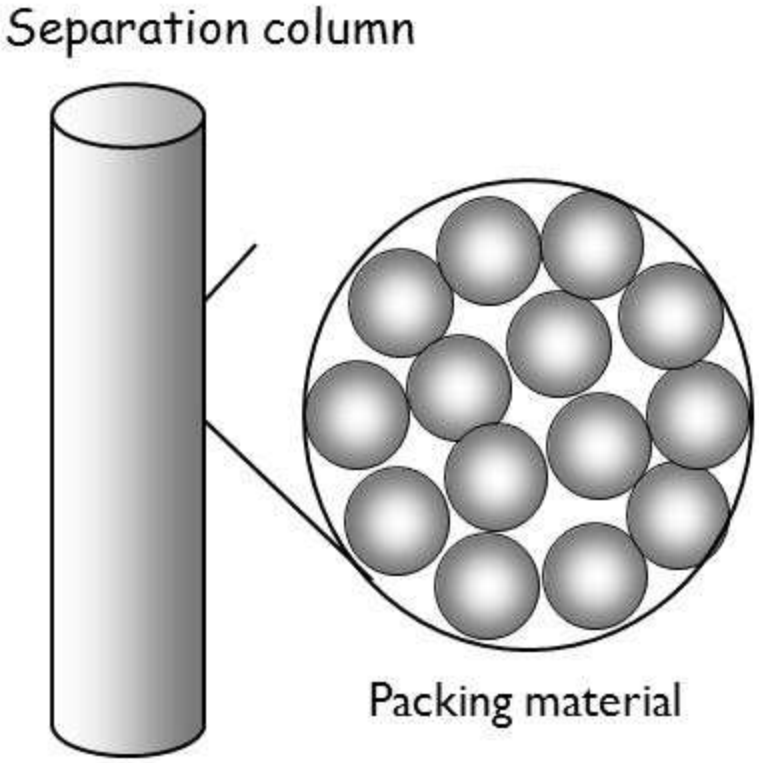
• The **stationary phase** in chromatography is a phase that is fixed in place either in a column or on a planar surface.

• The **mobile phase** in chromatography is a phase that moves over or through the stationary phase carrying with it the analyte mixture. The mobile phase may be a gas, a liquid, or a supercritical fluid.

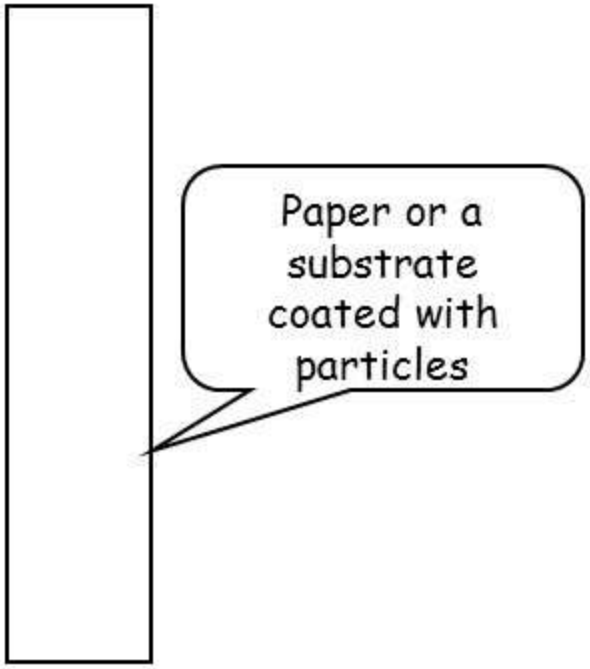
Classification of Column Chromatographic Methods

General Classification	Specific Method	Stationary Phase	Type of Equilibrium
1. Gas chromatography (GC)	a. Gas-liquid (GLC)	Liquid adsorbed or bonded to a solid surface	Partition between gas and liquid
	b. Gas-solid	Solid	Adsorption
2. Liquid Chromatography (LC)	a. Liquid-liquid, or partition	Liquid adsorbed or bonded to a solid surface	Partition between immiscible liquids
	b. Liquid-solid, or adsorption	Solid	Adsorption
	c. Ion exchange	Ion-exchange resin	Ion exchange
	d. Size exclusion	Liquid in interstices of a polymeric solid	Partition/sieving
	e. Affinity	Group specific liquid bonded to a solid surface	Partition between surface liquid and mobile liquid
3. Supercritical fluid chromatography (SFC) (mobile phase: supercritical fluid)		Organic species bonded to a solid surface	Partition between supercritical fluid and bonded surface

Column Chromatography and Planar Chromatography



Column Chromatography



Paper Chromatography
Thin Layer Chromatography (TLC)

Chromatography

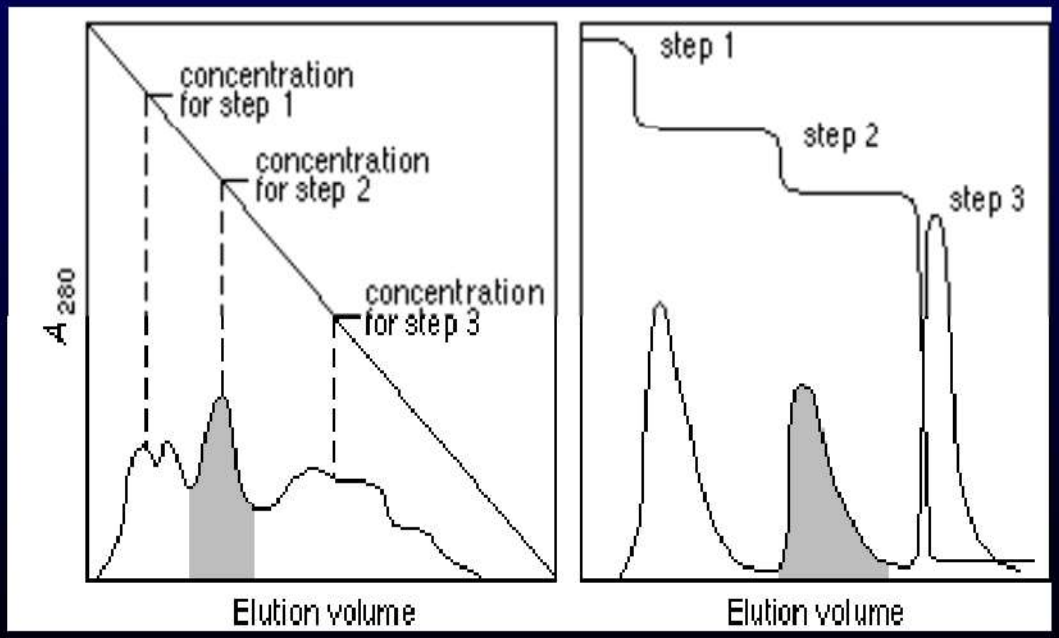
Elution mode

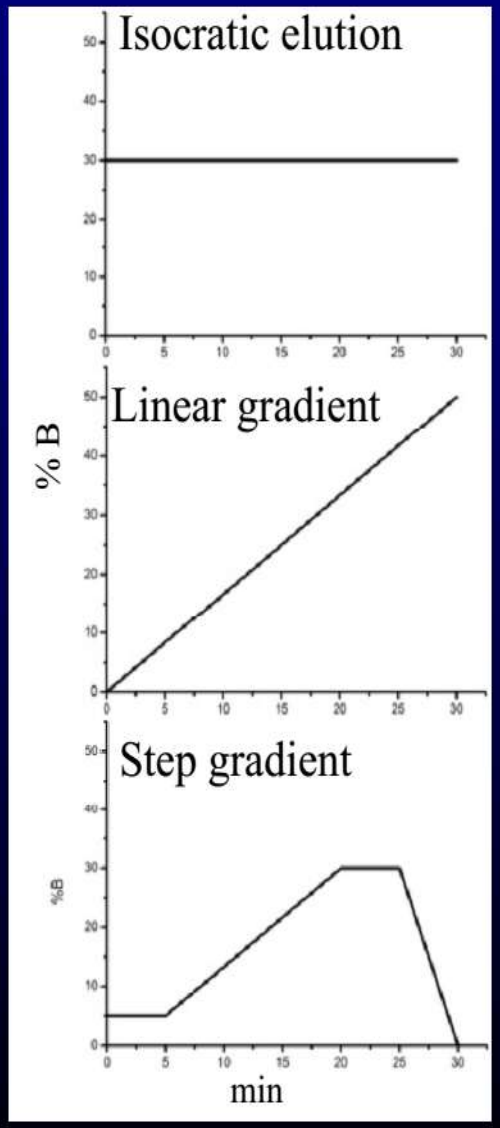
Isocratic elution :

The composition of the mobile phase kept constant through out elution

Gradient elution :

The composition of the mobile phase varied during elution





Isocratic and Gradient Elution

Isocratic elution

- Is performed with a single solvent (or constant mixture)

Gradient elution

- Increasing amount of solvent B are added to solvent A to create a continuous gradient; B (organic) and A (aqueous).
- Increased eluent strength (more polar) is required t elute more strongly retained solutes.
- In a reverse-phase separation, less polar solvent to elute more strongly retained solutes; more polar solvent to increase resolution.

Isocratic Elution

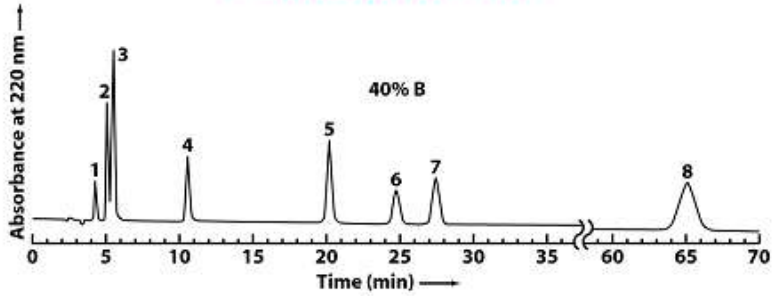


Figure 25-12 part 3
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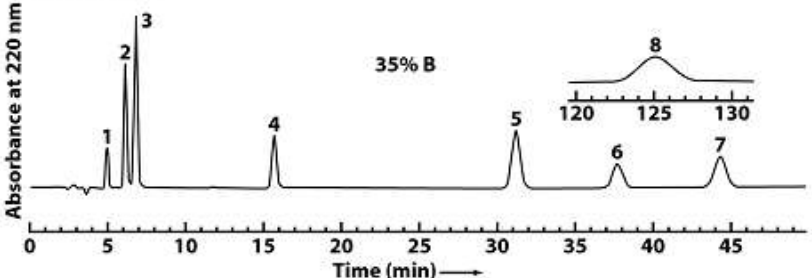


The Elute Process

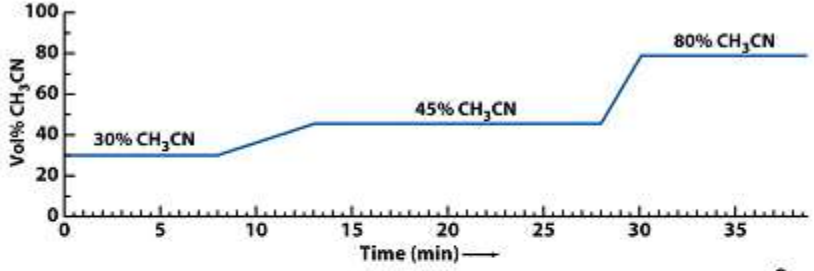
•Normal-phase chromatography:

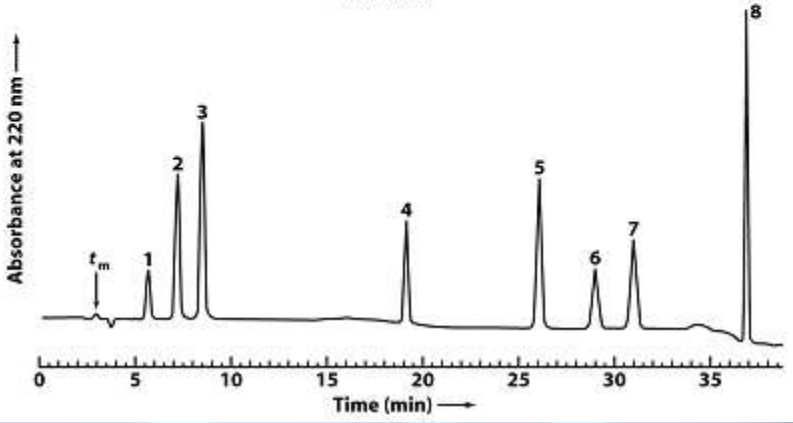
- Adsorption chromatography on bare silica
- Polar stationary phase (*e.g.* SiO) and a less polar solvent.
- A more polar the solvent has a higher eluent strength (•°).

•Reverse-phase chromatography:

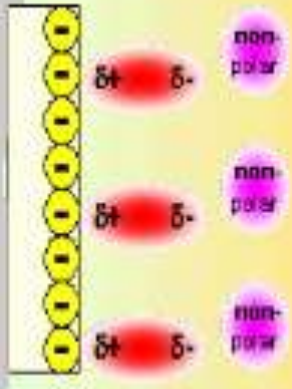
- Nonpolar stationary phase and more polar solvent.
- A less polar solvent has a higher eluent strength.
- More common, less sensitive to polar impurities (such as water) in the eluent.

Gradient Elution

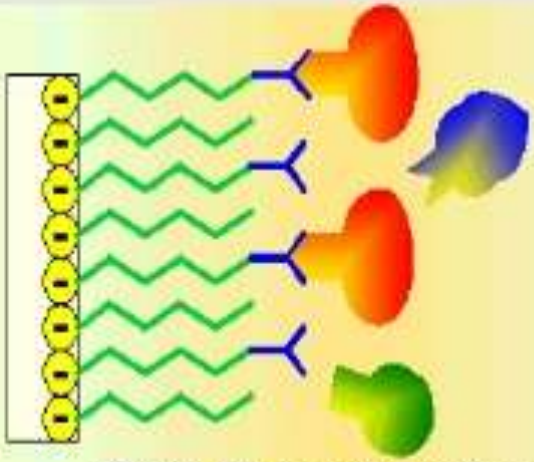




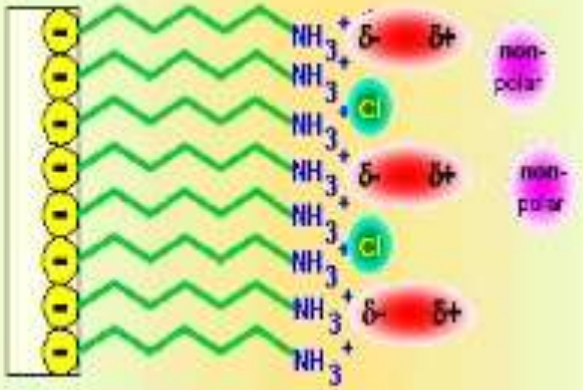
TYPES OF CHROMATOGRAPHY



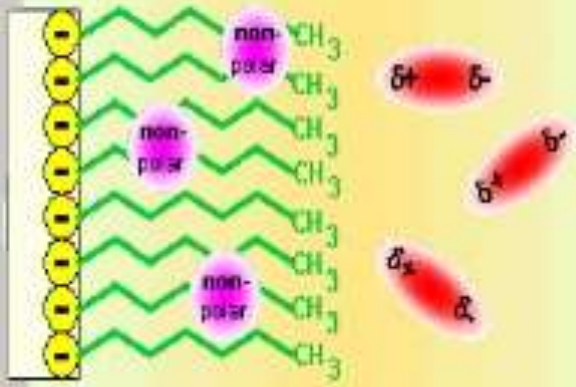
Adsorption Chromatography



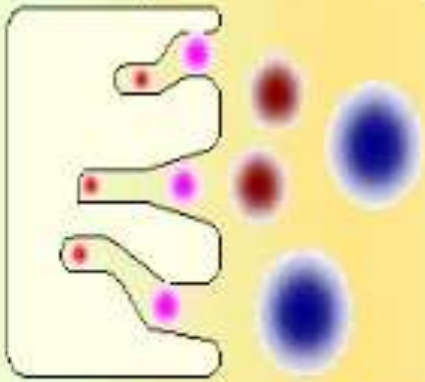
Affinity Chromatography
(covalent binding)



Ion-Exchange Chromatography
(cation or anion resins)

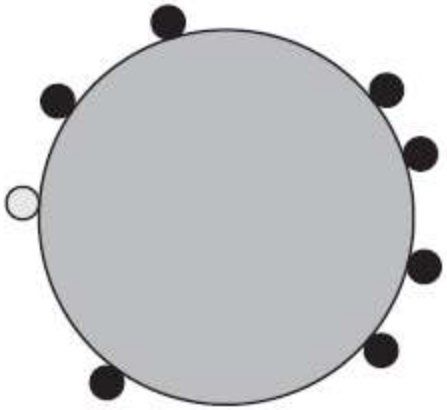


Partition Chromatography
(reversed phase)

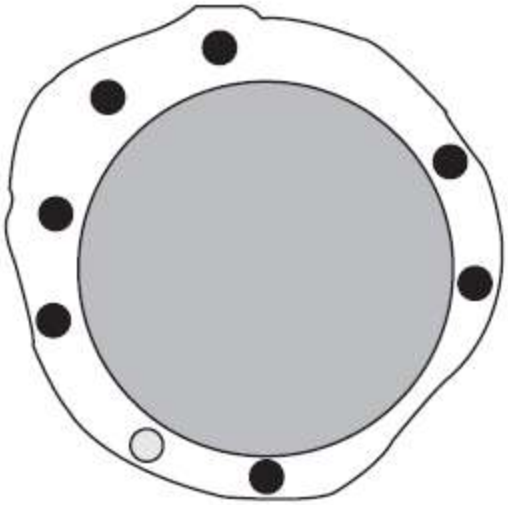


Molecular Exclusion Chromatography
(gel filtration, gel permeation,
molecular sieve)

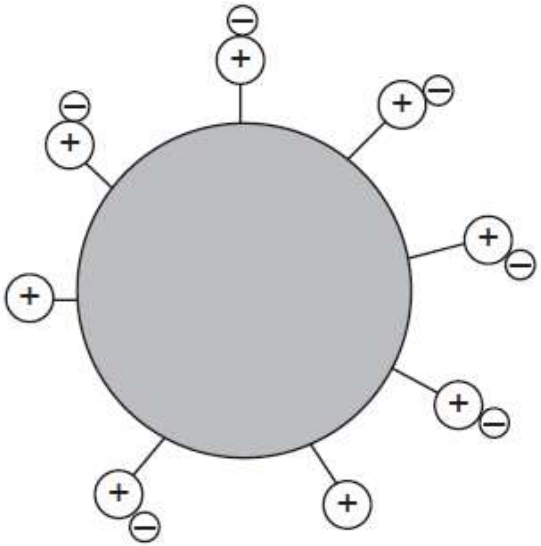
Ion-Pairing Chromatography
(liquid cation or anion resins)



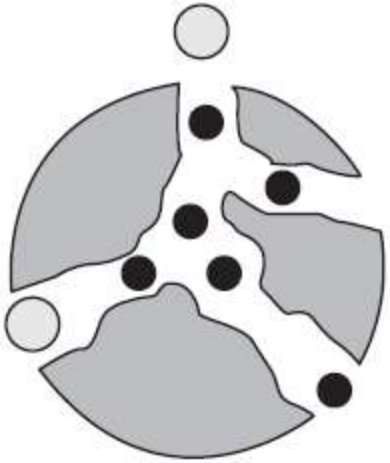
(a)



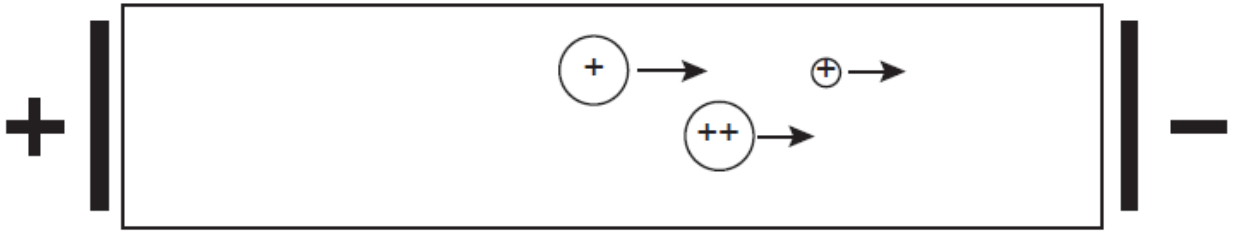
(b)



(c)



(d)



(e)

(a) adsorption chromatography, (b) partition chromatography, (c) ion-exchange chromatography, (d) size exclusion chromatography, and (e) electrophoresis. For the separations in (a), (b), and (d) the solute represented by the solid circle (•) is the more strongly retained.

Liquid chromatography (LC) refers to any chromatographic procedure in which the moving phase is a liquid, in contrast to the moving gas phase of gas chromatography. Traditional column chromatography (whether adsorption, partition, or ion-exchange), thin-layer and paper chromatography, and modern LC are each examples of liquid chromatography.

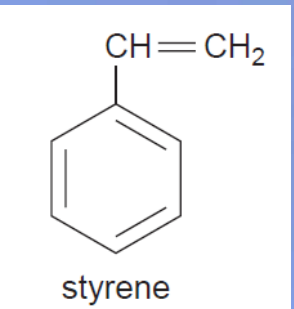
Different LC Methods

- Ion Exchange cromatography
- Size Exclusion chromatography
- Affinity chromatography
- Thin Layer chromatography
- HPLC

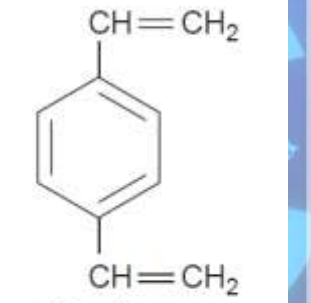
Separating Ions by Ion Exchange

Ion-exchange chromatography-A form of liquid chromatography in which the stationary phase is an ***ionexchange resin***.

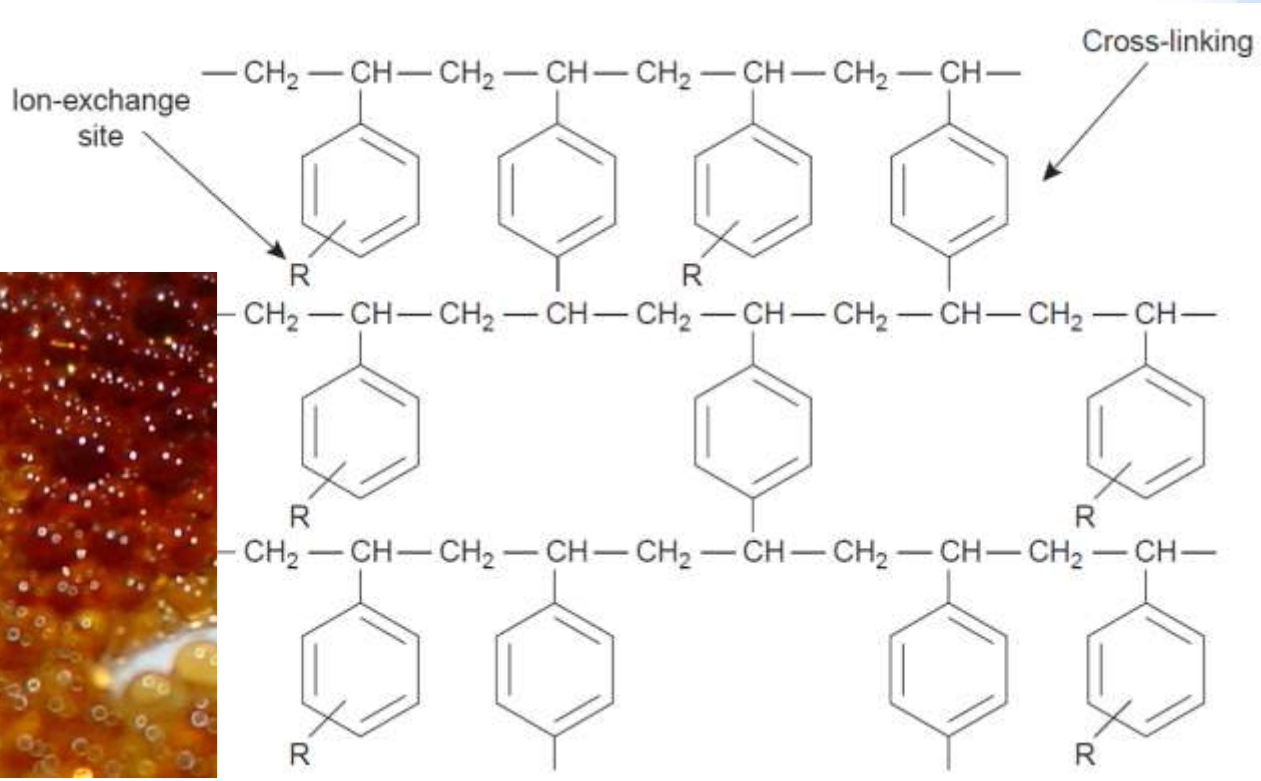
The stationary phase is a cross-linked polymer resin, usually divinylbenzene cross-linked polystyrene, with covalently attached ionic functional groups. Synthetic ion-exchange resins are high-molecular-mass polymers that contain large numbers of an ionic functional group per molecule. The counterions to these fixed charges are mobile and can be displaced by ions that compete more favorably for the exchange sites. Ion-exchange resins are divided into four categories: strong acid cation exchangers; weak acid cation exchangers; strong base anion exchangers; and weak base anion exchangers.

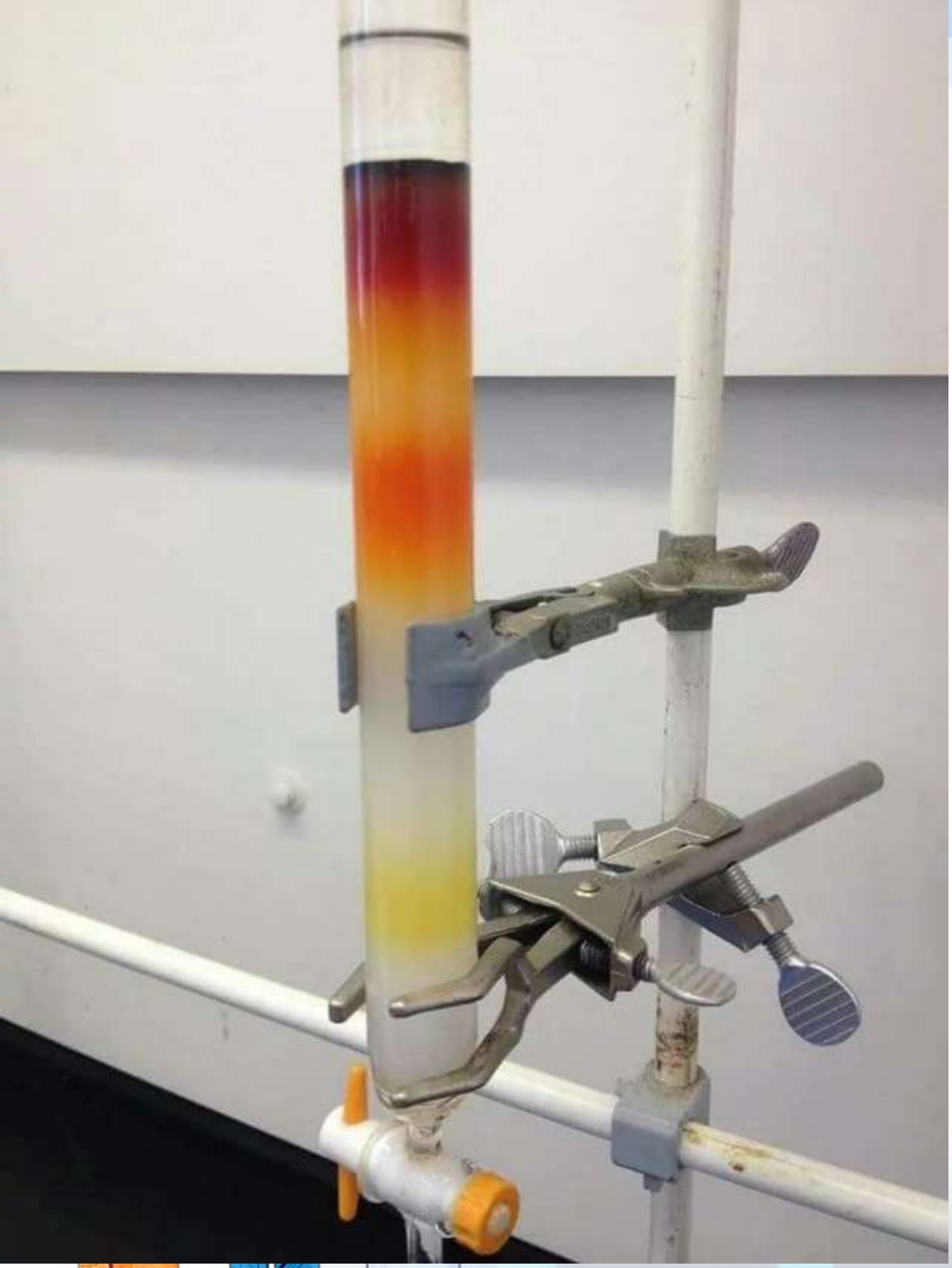


styrene



divinylbenzene





Examples of Common Ion-Exchange Resins

Type	Functional Group	Examples
strong acid cation exchanger	sulfonic acid	$-SO_3^-$ $-CH_2CH_2SO_3^-$
weak acid cation exchanger	carboxylic acid	$-COO^-$ $-CH_2COO^-$
strong base anion exchanger	quaternary amine	$-CH_2N(CH_3)_3^+$ $-CH_2CH_2N(CH_2CH_3)_3^+$
weak base anion exchanger	amine	$-NH_3^+$ $-CH_2CH_2NH(CH_2CH_3)_2^+$

Strong acid cation exchangers include a sulfonic acid functional group that retains its anionic form, and thus its capacity for ion-exchange, in strongly acidic solutions. The functional groups for a weak acid cation exchanger, however, are fully protonated at pH levels less then 4, thereby losing their exchange capacity. The strong base anion exchangers are fashioned using a quaternary amine, therefore retaining a positive charge even in strongly basic solutions. Weak base anion exchangers, however, remain protonated only at pH levels that are moderately basic. Under more basic conditions, a weak base anion exchanger loses its positive charge and, therefore, its exchange capacity.

The ion-exchange reaction of a monovalent cation, M+, at a strong acid exchange site is

$$-SO_3^-{-}H^+(s) + M^+(aq) \rightleftharpoons -SO_3^-{-}M^+(s) + H^+(aq)$$

$$x\mathrm{RN(CH_3)_3^+OH^-} + \mathrm{A}^{x-} \rightleftharpoons [\mathrm{RN(CH_3)_3^+}]_x\mathrm{A}^{x-} + x\mathrm{OH^-}$$

solid soln solid soln

Selectivity is somewhat dependent on whether the resin includes a strong or weak exchange site and on the extent of cross-linking. The latter is particularly important because it controls the resin's permeability and, therefore, the accessibility of the exchange sites. An approximate order of selectivity for a typical strong acid cation exchange resin, in order of decreasing *D, is*

$$Al^{3+} > Ba^{2+} > Pb^{2+} > Ca^{2+} > Ni^{2+} > Cd^{2+} > Cu^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+} > Ag^+ > K^+ > NH_4^+ > Na^+ > H^+ > Li^+$$

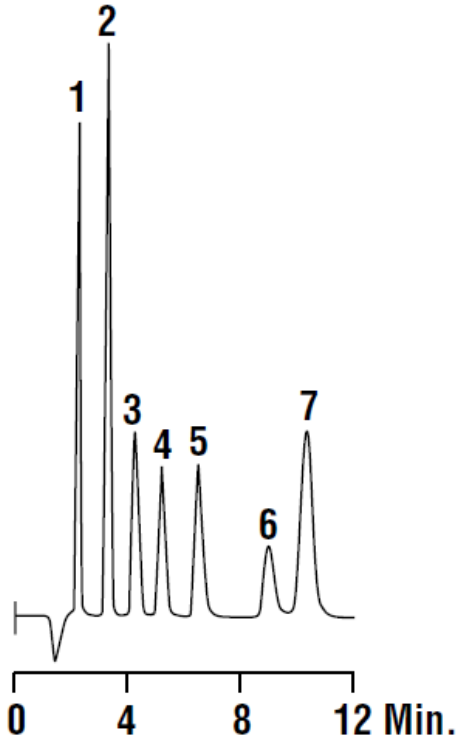
highly charged ions bind more strongly than ions of lower charge. Within a group of ions of similar charge, those ions with a smaller hydrated radius or those that are more polarizable bind more strongly. For a strong base anion exchanger the general order is

$$SO_4^{2-} > I^- > HSO_4^- > NO_3^- > Br^- > NO_2^- > Cl^- > HCO_3^- > CH_3COO^- > OH^- > F^-$$

Again, ions of higher charge and smaller hydrated radius bind more strongly than ions with a lower charge and a larger hydrated radius.

Ion-exchange chromatography has found important applications in water analysis and in biochemistry. For example, ion-exchange chromatography can be used for the simultaneous analysis of seven common anions in approximately 12 min. Before IEC, a complete analysis of the same set of anions required 1–2 days. Ion-exchange chromatography also has been used for the analysis of proteins, amino acids, sugars, nucleotides, pharmaceuticals, consumer products, and clinical samples.

Anion standards

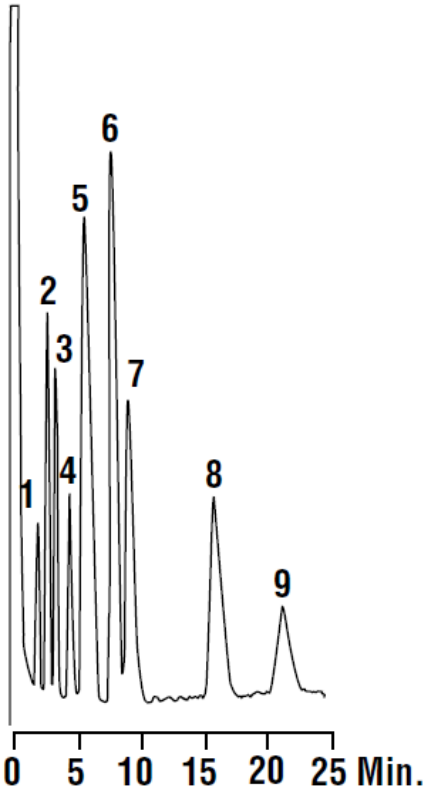


1. Fluoride, (2 ppm)
2. Chloride, (4 ppm)
3. Nitrite, (4 ppm)
4. Bromide, (4 ppm)
5. Nitrate, (4 ppm)
6. Phosphate, (6 ppm)
7. Sulfate, (6 ppm)

Column: Allsep™ Anion, 100 × 4.6 mm
Mobile Phase: 0.7 mM $NaHCO_3$:1.2 mM Na_2CO_3
Flowrate: 1.0 mL/min
Temperature: 40°C

Monovalent and divalent cations, and transition metals

CHROM 6355



1. Lithium (0.5 ppm)
2. Sodium (0.5 ppm)
3. Ammonium (0.5 ppm)
4. Potassium (0.8 ppm)
5. Nickel (5 ppm)
6. Zinc (5 ppm)
7. Cobalt (5 ppm)
8. Magnesium (0.35 ppm) and Manganese (0.35 ppm)
9. Calcium (0.7 ppm)

Column: Universal cation 100 × 4.6 mm
Mobile Phase: 2 mM Tartaric acid/1 mM oxalic acid
Flowrate: 1.0 mL/min

Applications of Ion-Exchange Methods

There are many uses for ion-exchange resins. They are used in many cases to eliminate ions that would otherwise interfere with an analysis. For example, iron(III), aluminum(III), and many other cations tend to coprecipitate with barium sulfate during the determination of sulfate ion. Passing the solution that contains sulfate through a cation-exchange resin results in the retention of these interfering cations and the release of an equivalent number of hydrogen ions. Sulfate ions pass freely through the column and can be precipitated as barium sulfate from the effluent.

Another valuable application of ion-exchange resins is to concentrate ions from a dilute solution. Thus, traces of metallic elements in large volumes of natural waters can be collected on a cation-exchange column and subsequently liberated from the resin by treatment with a small volume of an acidic solution. The result is a considerably more concentrated solution for analysis by atomic absorption or ICP emission spectrometry. The total salt content of a sample can be determined by titrating the hydrogen ion released as an aliquot of sample passes through a cation exchanger in the acidic form. Similarly, a standard hydrochloric acid solution can be prepared by diluting to known volume the effluent resulting from treatment of a cation-exchange resin with a known mass of sodium chloride. Substitution of an anion-exchange resin in its hydroxide form will permit the preparation of a standard base solution.

Ionexchange resins are also widely used in household water softeners as discussed. Ion-exchange resins are particularly useful for the chromatographic separation of both inorganic and organic ionic species.

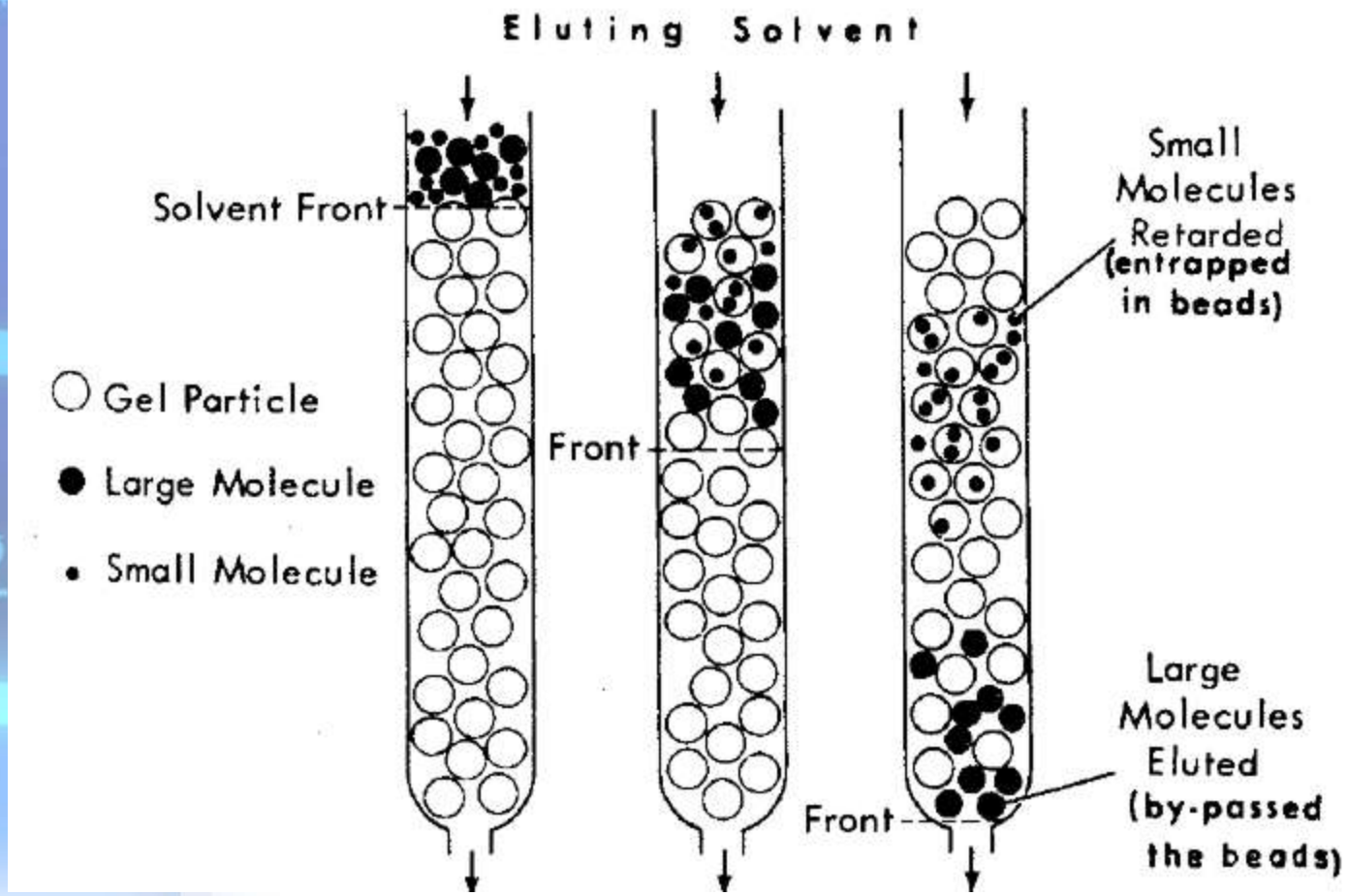
Size-Exclusion Chromatography

***Size-exclusion chromatography* -** A form of liquid chromatography in which the stationary phase is a porous material and in which separations are based on the size of the solutes.

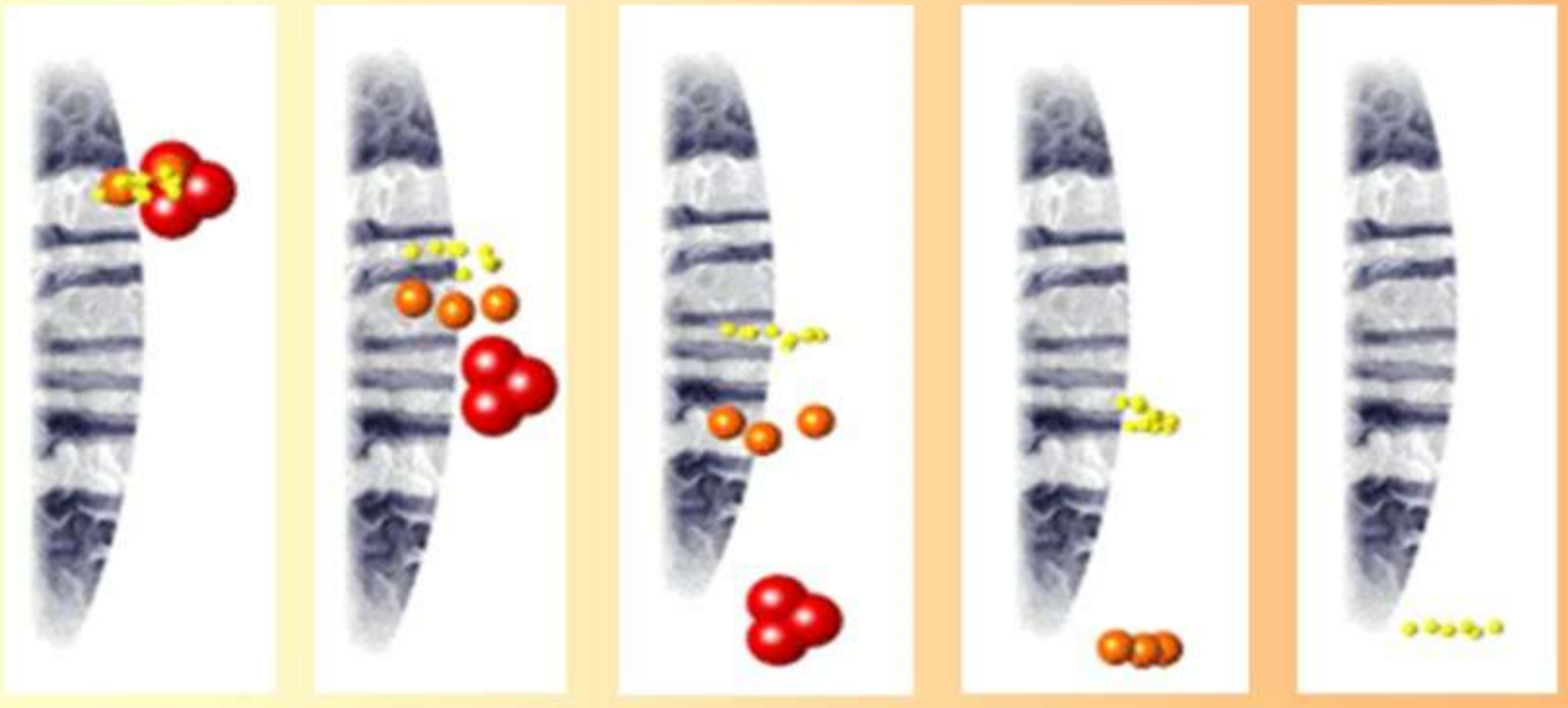
Gel filtration is a type of size exclusion chromatography in which the packing is hydrophilic. It is used to separate polar species.

Gel permeation is a type of size-exclusion chromatography in which the packing is hydrophobic. It is used to separate nonpolar species.

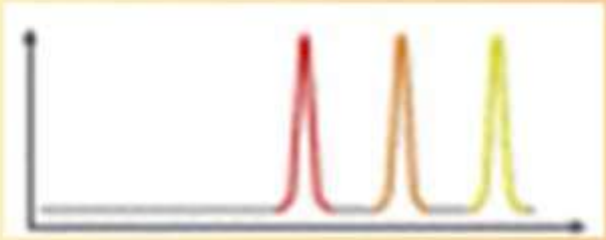
In ***size-exclusion chromatography, also called molecular-exclusion or gel-permeation*** chromatography, separation is based on the solute's ability to enter into the pores of the column packing. While in the pores, molecules are effectively trapped and removed from the flow of the mobile phase. The average residence time of analyte molecules depends on their effective size. Molecules that are significantly larger than the average pore size of the packing are excluded and thus suffer no retention, that is, they travel through the column at the rate of the mobile phase. Molecules that are appreciably smaller than the pores can penetrate throughout the pore maze and are thus entrapped for the greatest time; they are last to elute. Between these two extremes are intermediate-size molecules whose average penetration into the pores of the packing depends on their diameters. The fractionation that occurs within this group is directly related to molecular size and, to some extent, molecular shape.


Eluting Solvent
Solvent Front
Front
Front
Small Molecules Retarded (entrapped in beads)
Large Molecules Eluted (by-passed the beads)
Gel Particle
Large Molecule
Small Molecule

Retention mechansim

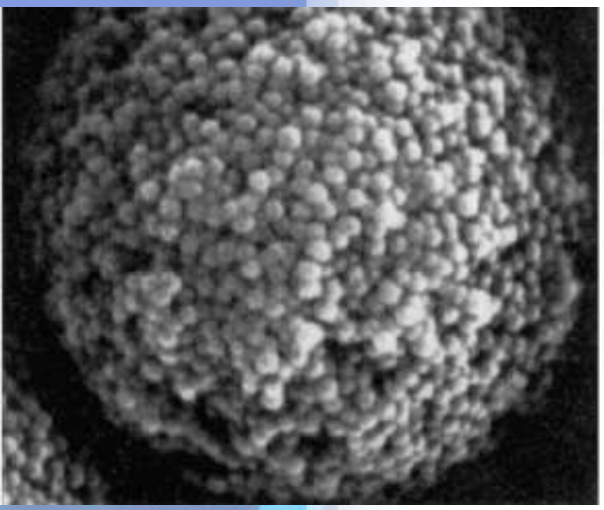


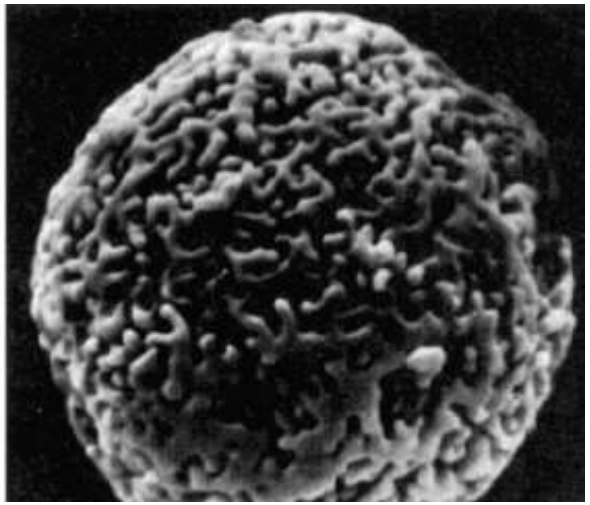
Large molecules cannot enter gel and are excluded.
They have less volume to traverse and elute sooner



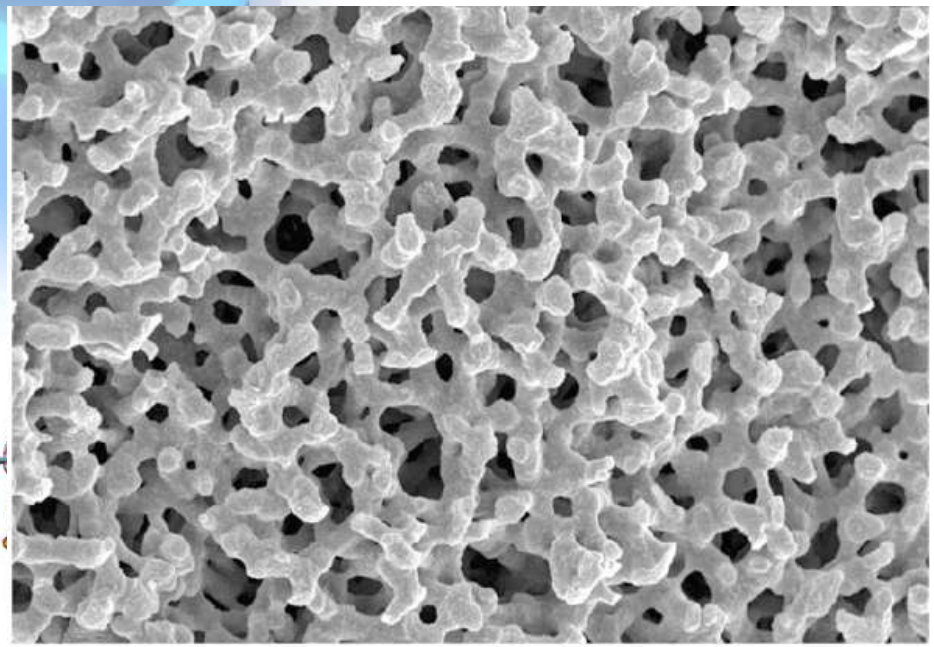
Smaller molecules can enter the pores, they are not excluded.
They have more volume to traverse and they elute later

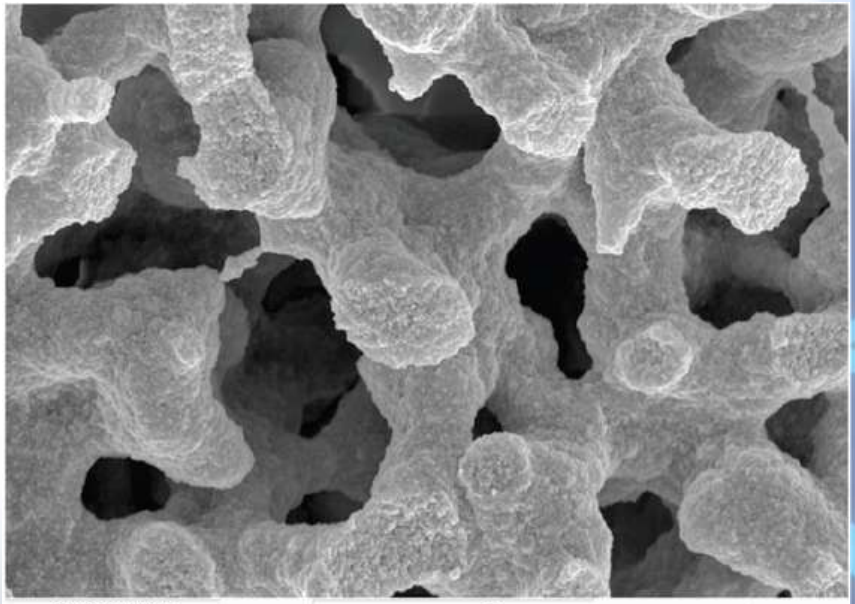
Two classes of micron-sized stationary phases have been encountered in this section: silica particles and cross-linked polymer resin beads.





Both materials are porous, with pore sizes ranging from approximately 50 to 4000 Å for silica particles and from 50 to 1,000,000 Å for divinylbenzene cross-linked polystyrene resins.

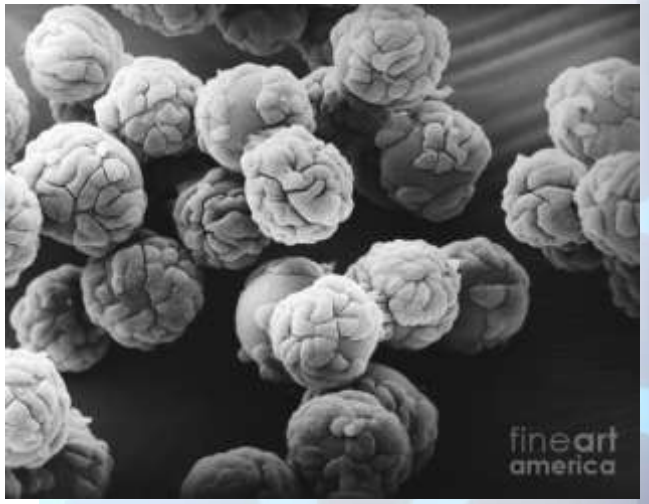




The timely adoption of the *cross-linked dextran gels (i.e.,* ***Sephadex)*** in late-fifties as a packing material for column chromatography opened an altogether new horizon of chromatographic separation whereby substances, in general, undergo separation more or less as per their molecular size. In actual practice, the inert gels of dextran (I)-a polyglucose or other types of polymers, for instance: agarose and polyacrylamides, wherein the macromolecules invariably are cross-linked to afford a reasonably porous 3D-structure*, served as the stationary phases in size-exclusion chromatography. Buffered aqueous solutions normally serve as mobile phases in size-exclusion chromatography. However, highly modified gel polymers are also available commercially (*e.g., Sephadex-LX) that* exclusively make use of organic solvents.

Dextran

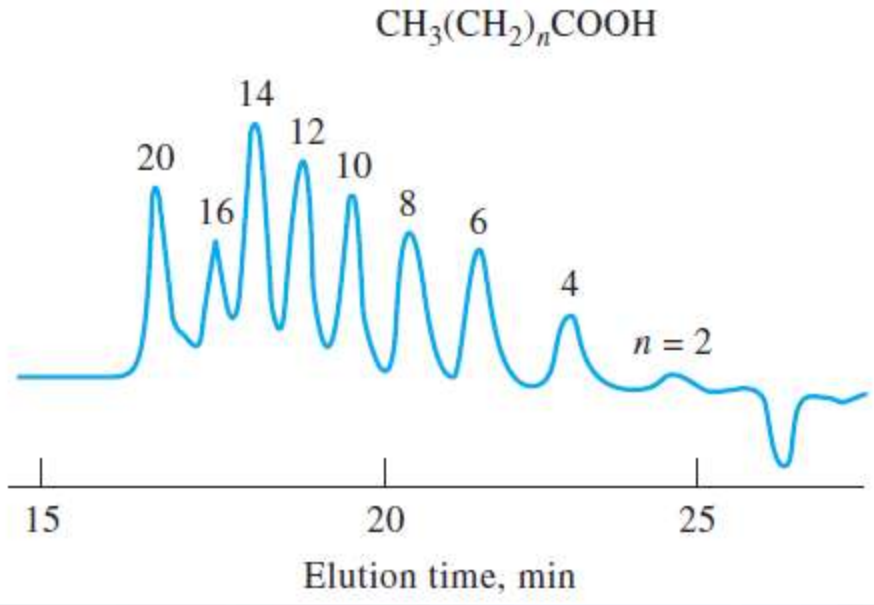
- A homopolysaccharide of glucose residues.
- It's prepared with various degrees of cross-linking to control pore size.
- It's bought as dry beads, the beads swell when water is added.
- The trade name is sephadex.
- It's mainly used for separation of small peptides and globular proteins with small to average molecular mass.



The apparatus for '**size-exclusion chromatography'** essentially comprises of a chromatographic column generally made up of glass having a diameter to height ratio of between 1 : 10 and 1 : 20, packed with an appropriate separation material (*e.g., different grades of* Sephadex) which is capable of fractionation in the suitable range of molecular size and may be adequately temperature controlled.

The size-exclusion-chromatography may be used for *two specific purposes in the analysis of pharmaceutical*
substances, such as:

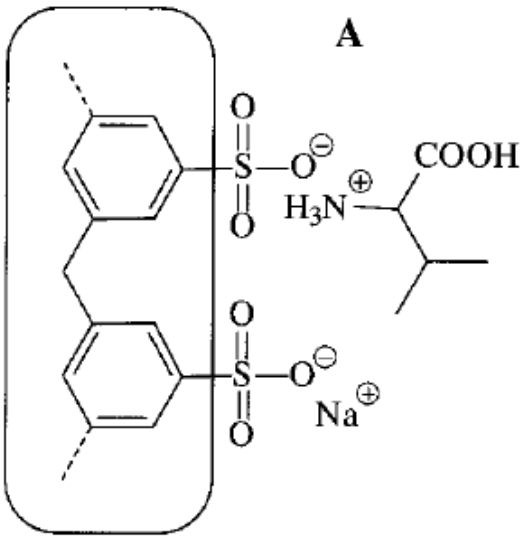
- *Determination of relative component composition, and*
- *Determination of molecular weight.*



Separation of fatty acids. Column: polystyrene based, 7.5 3 600 mm. Mobile phase: tetrahydrofuran.

Affinity Chromatography

In affinity chromatography, a reagent called an **affinity ligand** *is covalently bonded* to a solid support. Typical affinity ligands are antibodies, enzyme inhibitors, or other molecules that reversibly and selectively bind to analyte molecules in the sample. When the sample passes through the column, only the molecules that selectively bind to the affinity ligand are retained. Molecules that do not bind pass through the column with the mobile phase. After the undesired molecules are removed, the retained analytes can be eluted by changing the mobile phase conditions.

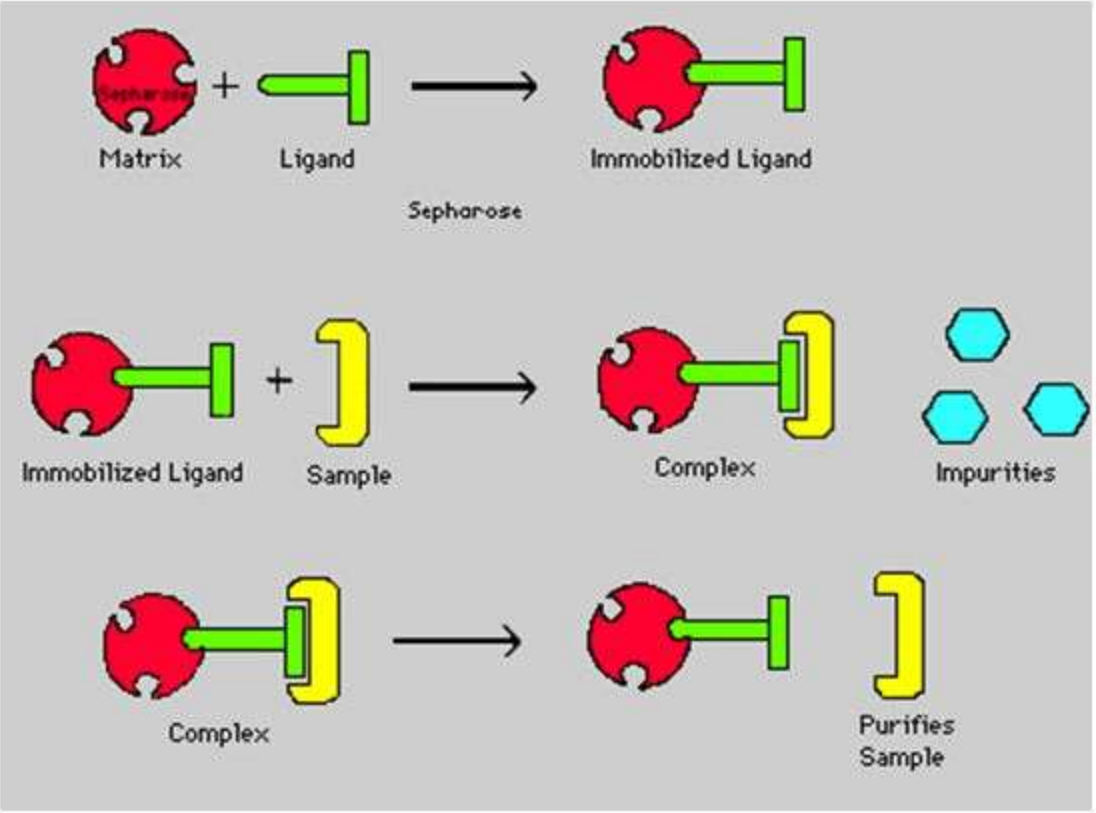


The stationary phase for affinity chromatography is a solid, such as agarose, or a porous glass bead to which the affinity ligand is immobilized. The mobile phase in affinity chromatography has two distinct roles to play. First, it must support the strong binding of the analyte molecules to the ligand. Second, once the undesired species are removed, the mobile phase must weaken or eliminate the analyte-ligand interaction so that the analyte can be eluted.

Affinity chromatography has the major advantage of extraordinary specificity. The primary use is in the rapid isolation of biomolecules during preparative work.

4.) Affinity Chromatography (AC)

Separates based on the use of immobilized biological molecules (and related compounds) as the stationary phase



Based on the selective, reversible interactions that characterize most biological systems

- binding of an enzyme with its substrate or a hormone with its receptor
- immobilize one of a pair of interacting molecules onto a solid support
- immobilized molecule on column is referred to as the *affinity ligand*

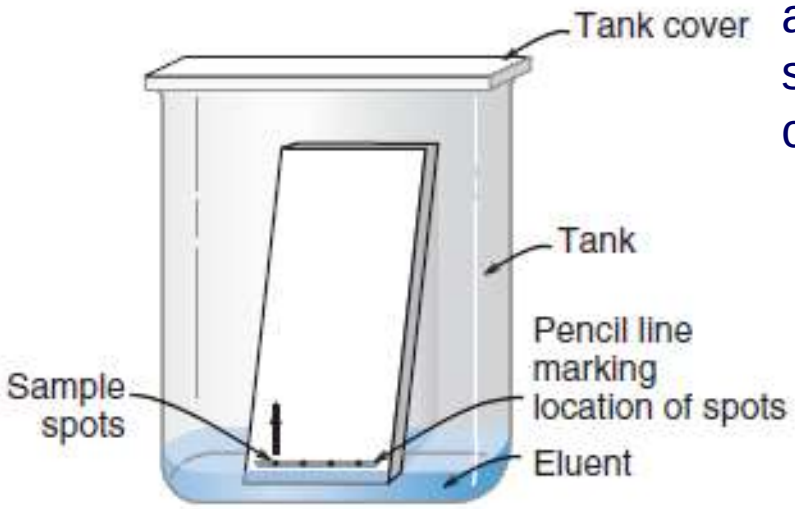
Thin Layer Chromatography

Thin-layer chromatography (TLC) is a ***planar*** form of chromatography widely used for rapid qualitative analysis. It can also be used in a high-performance mode (HPTLC). Quantitative analysis is also possible, although the technique is most widely used for rapid screening, e.g., in a synthetic organic chemistry laboratory to check if the desired compound is being made and how many impurities there are. The stationary phase is a thin layer of finely divided sorbent supported on a glass, metal (most commonly aluminum) or plastic sheet.

Subsequently, the mobile phase is permitted to move across the surface of the plate (usually by capillary action) and the chromatographic phenomenon may solely depend upon adsorption, partition, or a combination of both, depending on the adsorbent, its treatment, and the nature of the solvents employed. During the chromatographic separation procedure the TLC-plate is placed in a chromatographic chamber, mostly made up of glass to enable clear observation of the movement of the mobile phase up the plate, that is pre-saturated with the solvent vapour. The inert solid supports invariably employed are, namely: alumina, silica gel, kieselguhr and cellulose, to these may be added appropriate substances, for instance: calcium sulphate (gypsum) so as to provide adequate adhesion to the solid support, example: silica gel-G (G-stands for gypsum).

Thin-layer chromatography setup

The three stages of injection, separation and detection in HPLC correspond in TLC to sample spotting, plate development, and detection (quite often just visual examination). In the simplest case, a pencil line is drawn horizontally towards the bottom of the plate (ca. 5–10mm from the bottom). This is where one or more sample spots are applied. Samples (typically 0.5 − 5 *μL) are spotted onto the line at regular* intervals (ca. 20mm apart) with a micropipette. The chromatogram is "developed" by placing the bottom of the plate or strip in the developing solvent. The solvent is drawn up the plate by capillary action, and the sample components move up the plate at different rates, depending on their relative affinities for the mobile vs. the stationary phase.



Following development, the positions of the individual solute spots are noted. Different analytes move at a fraction of the rate of solvent movement; each analyte is thus characterized by the *Rf* ***value:***

$$R_f = \frac{\text{vertical distance solute moves}}{\text{vertical distance solvent front moves}}$$

where the distances are measured from the original position of the sample spot and the solvent front is a line across the plate.

Thin Layer Chromatography (TLC -1)

- Moving liquid phase, solid stationary phase
- TLC Procedure
  - Sample is dissolved in a solvent
  - Spotted onto the lower edge of the plate
  - The plate is placed into a closed chamber with liquid
  - The liquid slowly rises up by capillary action. Separation occurs as the components with the greatest affinity for the moving phase migrate faster
  - Visualized UV fluorescence or developed with a chemical reagent spray → color spots

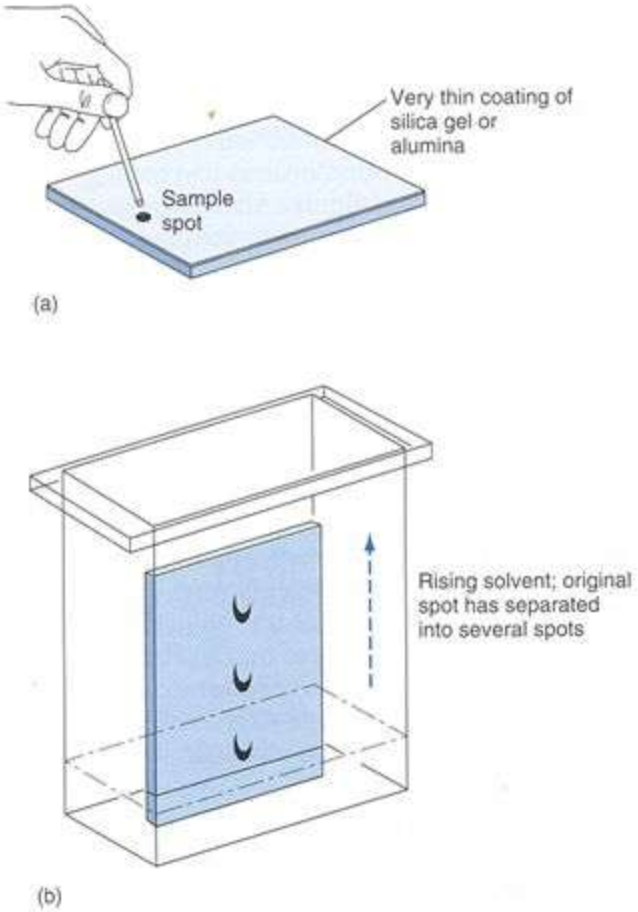
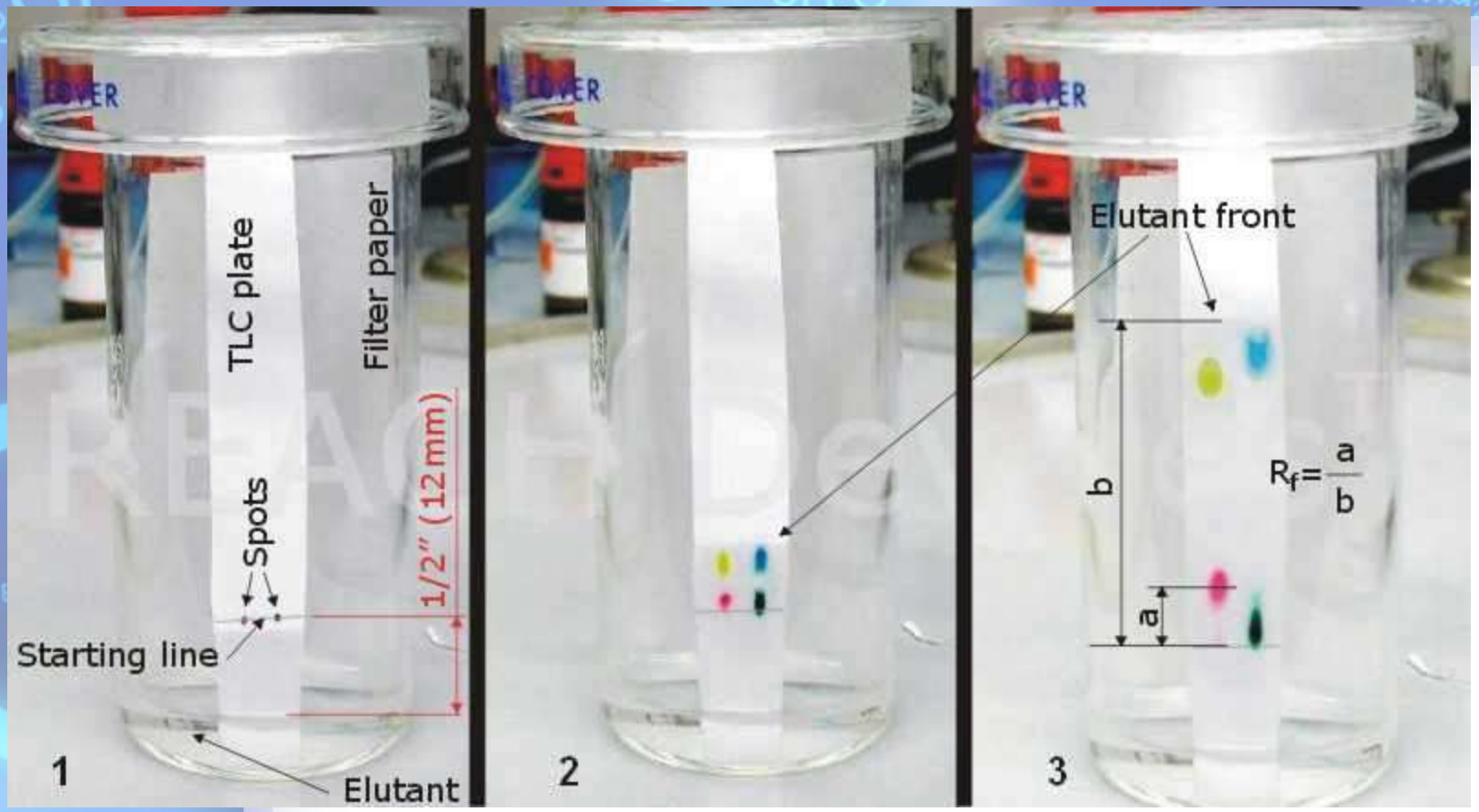


FIGURE 5–8 (a) In thin-layer chromatography, a liquid sample is spotted onto the granular surface of a gel-coated plate. (b) The plate is placed into a closed chamber that contains a liquid. As the liquid rises up the plate, the components of the sample will distribute themselves between the coating and the moving liquid. The mixture is separated, with substances having a greater affinity for the moving liquid traveling up the plate at a faster speed.



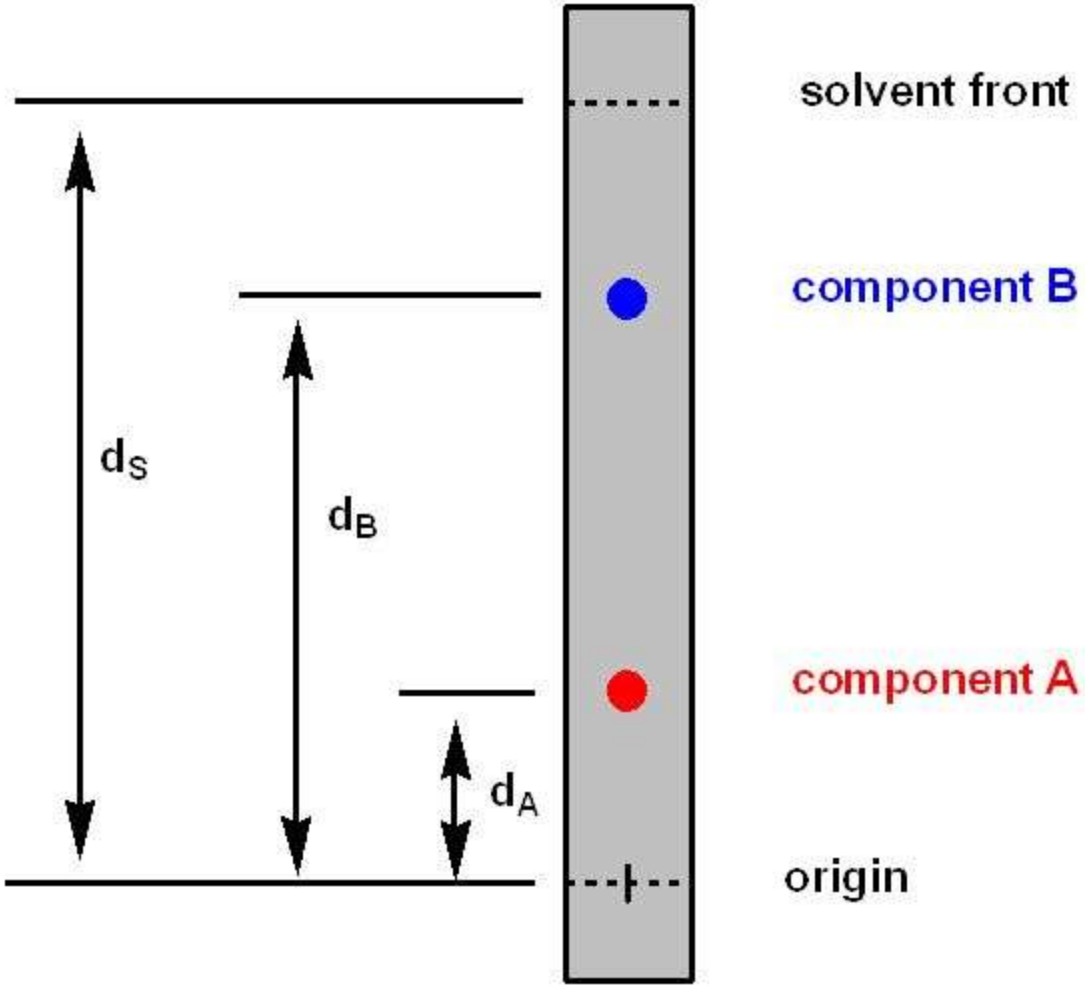
Thin Layer Chromatography (TLC), animation.mp4

Thin-Layer Chromatography: Determination of R_f Values

R_f of component A = $\frac{d_A}{d_S}$

R_f of component B = $\frac{d_B}{d_S}$

The R_f value is a decimal fraction, generally only reported to two decimal places



Adsorbents for TLC

Table 3.1 Adsorbents for thin layer chromatography

Solid	Used to separate
Silica gel	Amino acids [6, 7], alkaloids [10], sugars [11], fatty acids [12, 13], lipids [14], essential oils [15], inorganic anions and cations [16], steroids [17, 18], terpenoids [19]
Alumina	Alkaloids [10], food dyes [20], phenols [21], steroids [18, 22], vitamins [23], carotenes [24], amino acids [25]
Kiselguhr	Sugars [11], oligosaccharides [26], dibasic acids [27], fatty acids [28], triglycerides [29], amino acids [30], steroids [31, 32]
Celite	Steroids [33], inorganic cations [16]
Cellulose powder	Amino acids [34, 35], food dyes [36], alkaloids [37], nucleotides [38]
Ion exchange cellulose	Nucleotides [39], halide ions [40]
Starch	Amino acids [41]
Polyamide powder	Anthocyanins [42], aromatic acids [42], antioxidants [43], flavanoids [44], proteins [45]
Sephadex	Nucleotides [46], proteins [47, 48], metal complexes [49]

Common TLC Stationary Phases

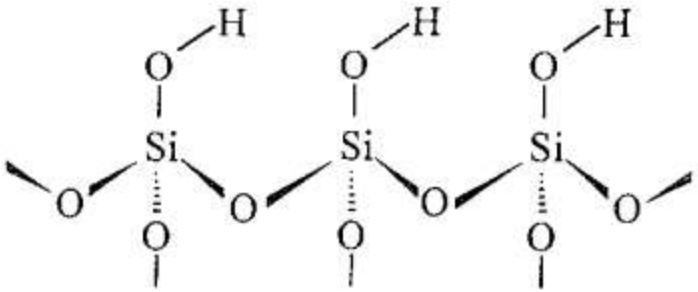


Figure 3.2 Silica gel matrix structure.

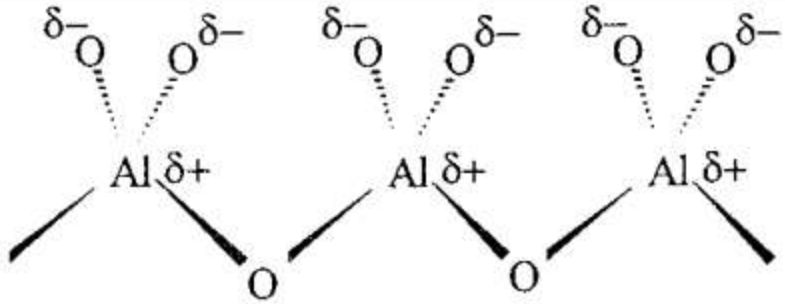


Figure 3.3 Partial structure of alumina.

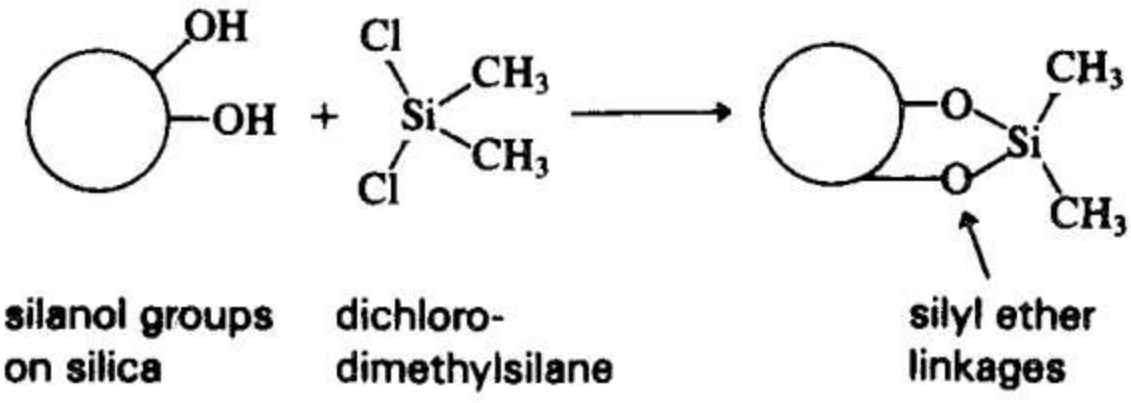
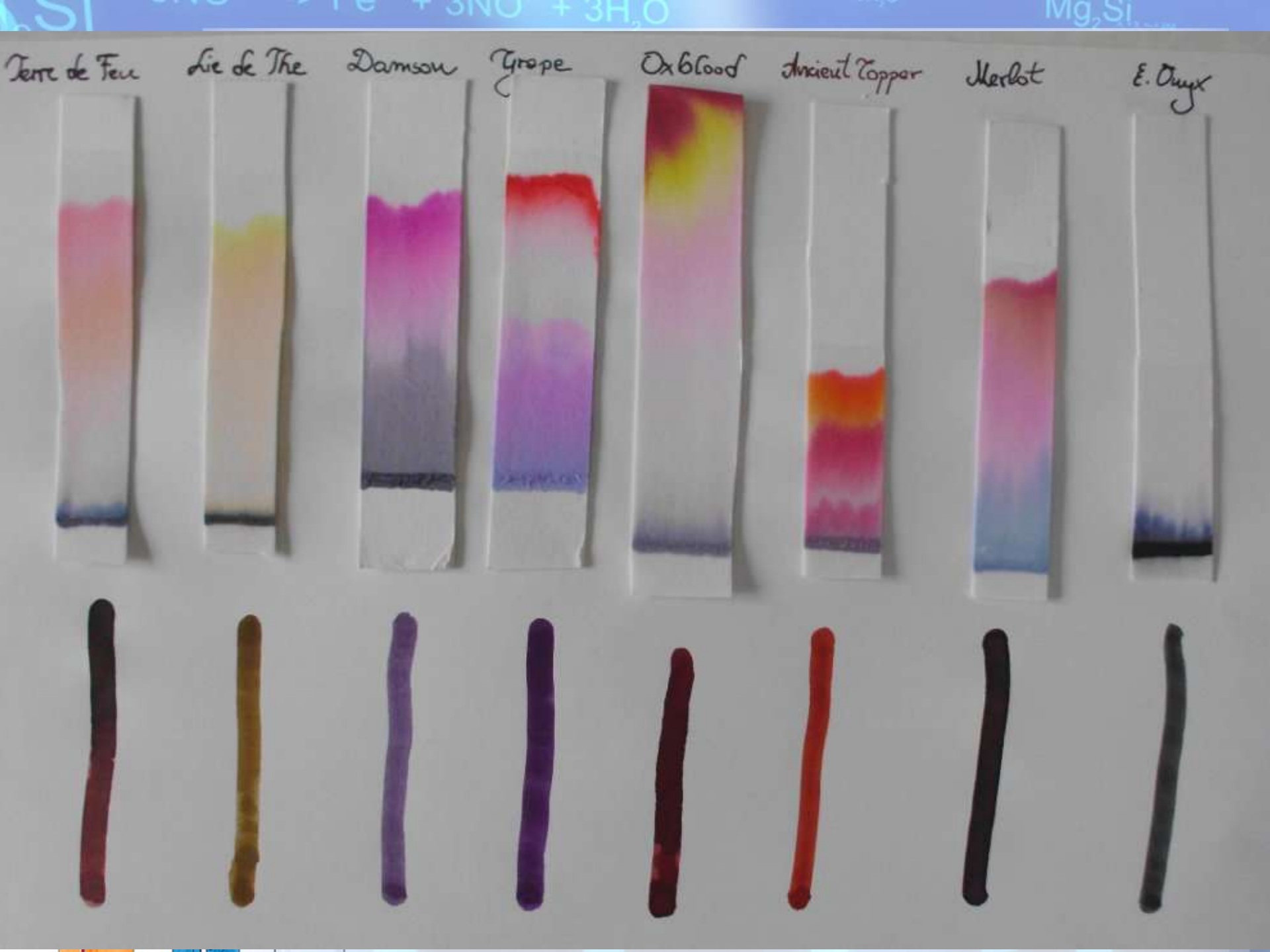


Figure 3.5 Preparation of silanised silica gel.


Terre de Feu
Lie de The
Damson
Grape
Ox Blood
Ancient Copper
Merlot
E. Onyx

The technique of thin-layer chromatography (TLC) has been used extensively in the domain of pharmaceutical analysis for a variety of specific and useful applications, for example:

- To identify the presence of undesirable specific organic compounds present as impurities in a number of pharmaceutical substances, namely: morphine in apomorphine hydrochloride; hydrazine in carbidopa ; 3-aminopropanol in dexampanthenol; etc.,
- *Related substances present* in official drugs, namely : related substances present in a wide number of potent pharmaceutical substances e.g., aminophylline; baclofen; chloramphenicol; carbamazepine etc.,
- Foreign alkaloids present in alkaloidal drugs, for instance: atropine sulphate; codeine;
- Foreign steroids present in steroidal drugs, for example: betamethasone valerate;
- Ninhydrin positive substances in official amino acids e.g., glutamic acid; leucine.

- Applications
  - Screening many samples very quickly
  - Analysis in cases where sample preparation is very difficult
  - Analysis of compounds in complex matrices
- Time per analysis: 3-15 min.
- Limitations
  - Non-volatile compounds
  - Low separating power