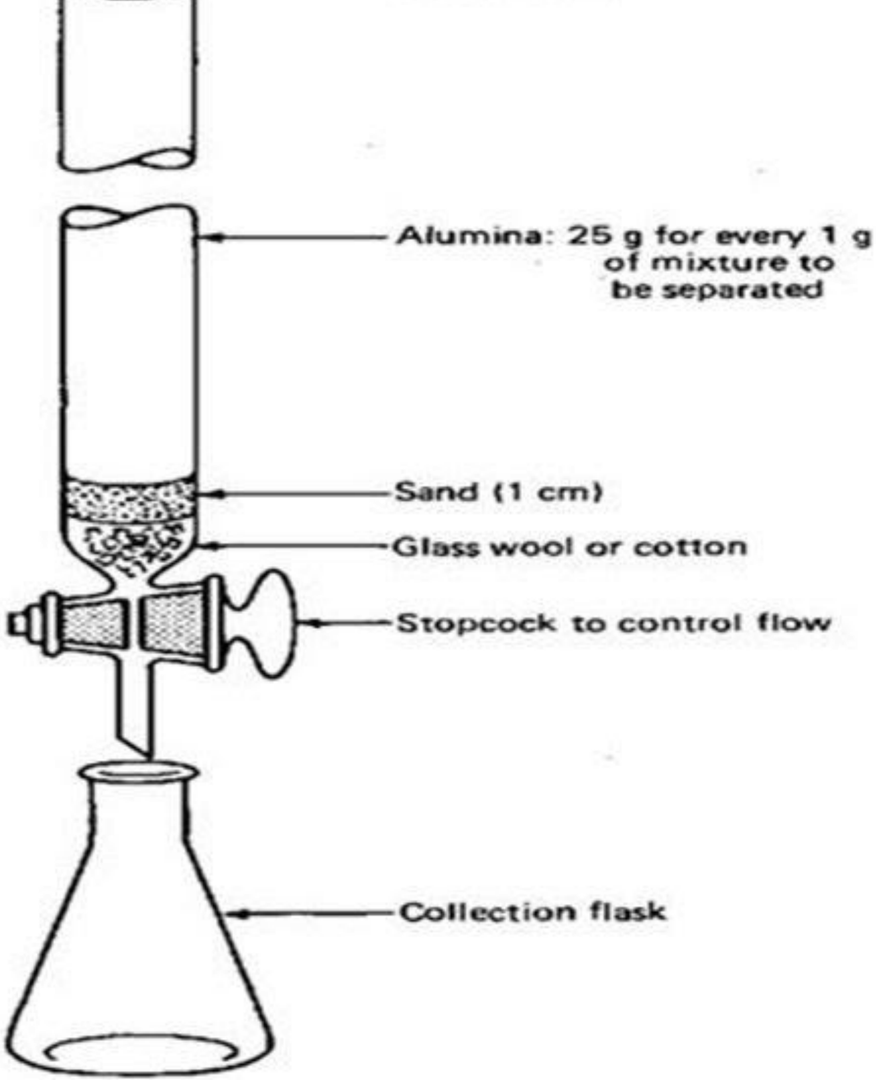
chromatography:

- **PREPARATION OF THE COLUMN:**
- At first a plug of cotton wool or glass wool is placed in the bottom of the column and pressed down evenly. Then some cleaned, washed sand can be poured on the top of the plug to form a thin layer. The sand serves to give a flat base to the column of adsorbent when cotton wool is used instead of sinter glass.

two ways:

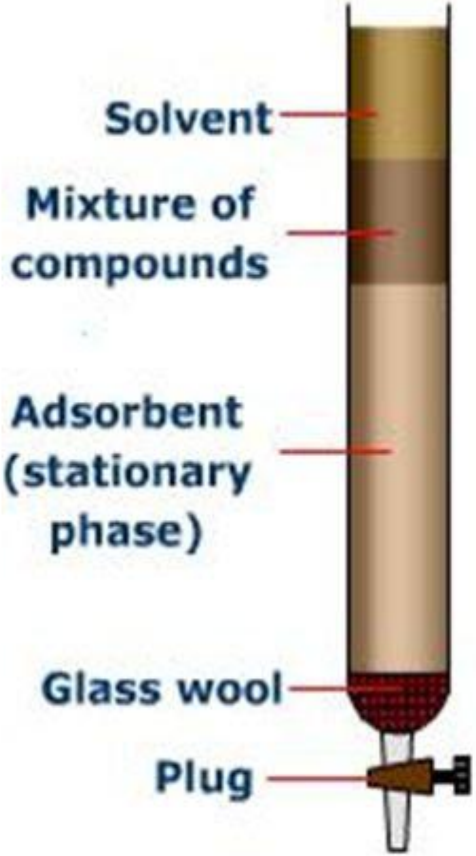
- 1. **Dry packing:** In this method the dry adsorbent is poured to the column directly. Vibration/vacuum pump is the applied to get rid of air bubbles then the mobile phase as passed through the adsorbent.

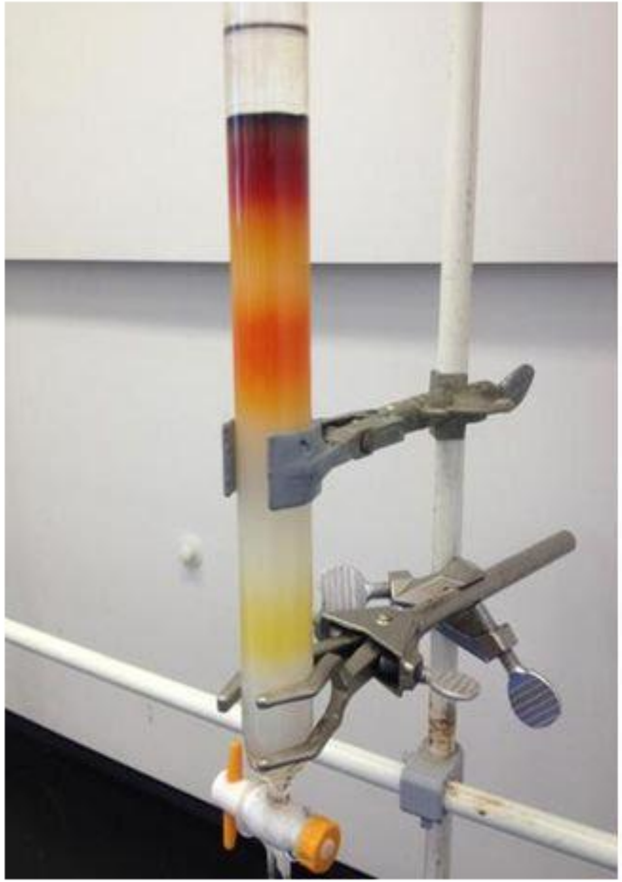
- Demerits:
- Air bubbles are entrapped between mobile phase and stationary phase
- Column may not be uniformly packed
- Cracks appear in the adsorbent present in the column.


Alumina: 25 g for every 1 g of mixture to be separated
Sand (1 cm)
Glass wool or cotton
Stopcock to control flow
Collection flask

solvent (PE) & mixed well in a beaker & then poured into the column. This process is continued for several times until the stationary phase is firmly packed in the column. Sand may be added after the slurry.

- **PRECAUTION:** After setting the adsorbent the excess solvent is allowed to drain out by the outlet duct but a “head” or layer of solvent should always cover the adsorbent. Otherwise cracks will develop in the column and becomes useless for chromatography because the solvent runs through the cracks rather than between the particles of adsorbent.


Solvent
Mixture of compounds
Adsorbent (stationary phase)
Glass wool
Plug



INTRODUCTION OF SAMPLE

- The sample is placed at the top of the column (stationary phase) as a band either by two ways:
- **1. Wet application:** Dissolve the sample in the initial mobile phase and apply by pipette to the top of the column. This is very good method but in most of cases the samples are not soluble in the initial mobile phase.
- **2. Dry loading:** Dissolve sample in any volatile solvent. The sample solution is then adsorbed on small weight of adsorbent (stationary phase) and the solvent is allowed to evaporate. The dry adsorbent loaded with the sample is then applied

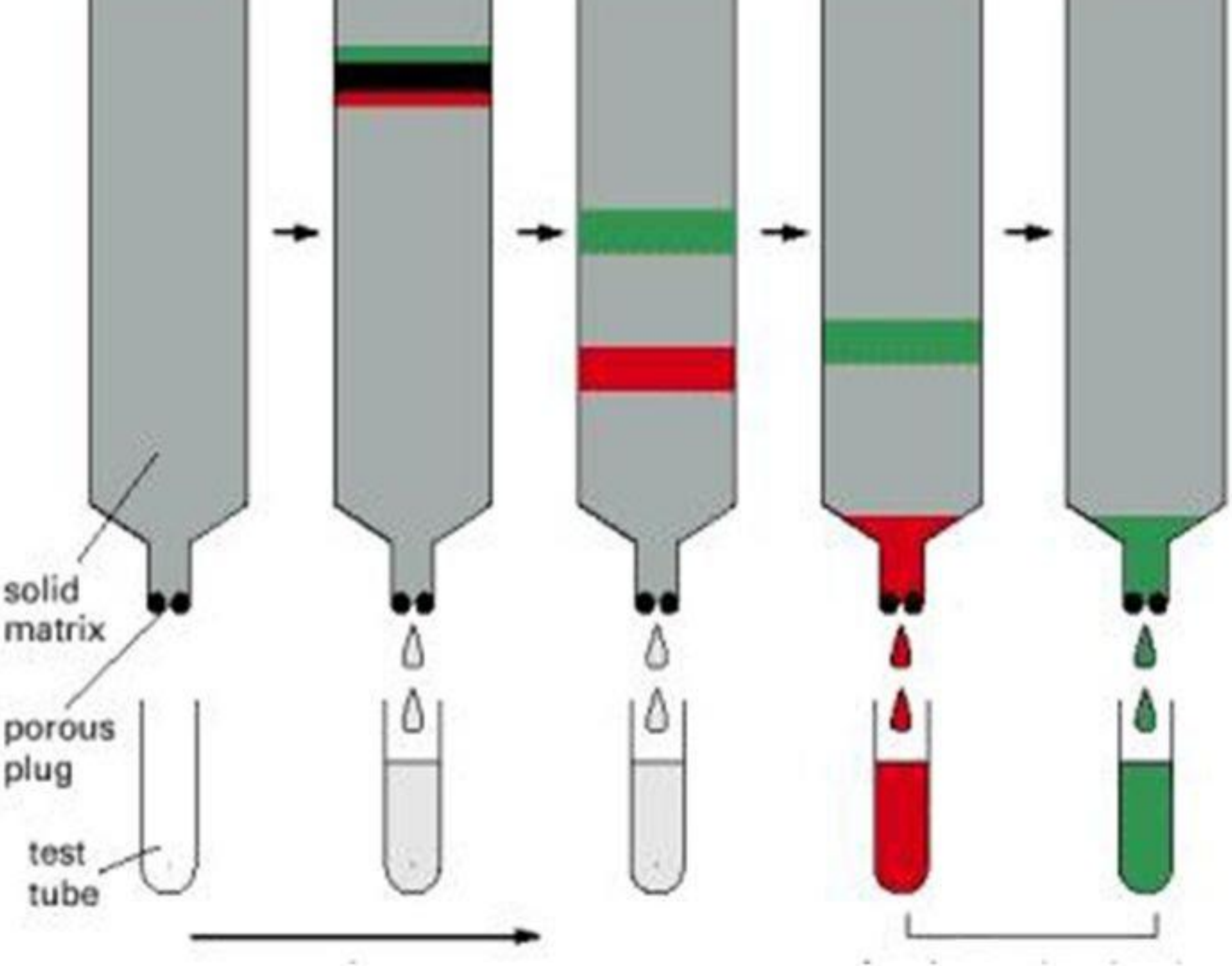
- Once the sample solution is added onto the silica, eluent is added to the top of the column at a rate sufficient to ensure a “Head” of liquid on the top layer of sand at any point of chromatogram development.

- The composition of the eluent can be changed as the column progresses.

- The constituents of the sample will pass down the column at varying rates.

column in small volumes of 5, 10 or 15ml test tubes.

- ***Analyzing the fractions:***
- 1. Then the fractions are analyzed by thin-layer chromatography.
- 2. Similar fractions are combined.
- 3. If the fraction contains a single compound the solvent is evaporated to obtain the pure compound.
- 4. But if the fraction contains more than one component they are separated using another suitable technique.


solid matrix
porous plug
test tube

same/similar. In this elution technique, the same solvent or solvent system of same polarity is used throughout the process of separation. E.g.: chloroform, petroleum ether etc.

- **Gradient/Stepwise elution technique:** The solvents of gradually increasing polarity or increasing elution strength are used during the process of separation. Initially low polar solvent is used followed by gradually increasing the polarity. E.g.: Initially benzene then chloroform, ethyl acetate, methanol etc.

developing the chromatogram by running the solvent, different bands of components are formed. If the bands are colored then we can detect the component visually if color of the component is known.

- **Eluting the various components with solvents:** It is however more convenient to complete the chromatogram by eluting the various components with solvents. The eluate is collected as a large number of fractions, each of small volume. The large number of fractions assists in obtaining better separation of components. Each fraction is examined appropriately for the presence of compounds by:
- **By simple spot tests by paper or TLC**
- **By passing UV light compounds can be detected visually.**
- **By addition of reagents**

- 1. Measuring changes in pH,
- 2. Measuring changes in conductivity,
- 3. Measuring changes in refractive index,
- 4. Measuring absorption of UV light,
- 5. Measuring fluorescence (for quinine).
- 6. Adding fluorescein (0.04%) to the adsorbent and then viewing the column under UV light. The background will fluoresce except in those areas where chemicals are present and they will appear as dark bands.
- 7. Adding dimethyl amino azobenzene with silica gel to identify alkaloids. The silica will appear red colored except where the alkaloids are present.
- 8. Adding ethereal solution of ferric chloride with alumina to identify organic compounds which react with ferric chloride to give colored compounds

such as:

- **1. Silica gel**
- **2. alumina**
- **3. Silicic acid**
- **4. Sodium carbonate**
- **5. Calcium carbonate**
- **6. Charcoal**
- **7. Fuller's earth**
- **8. Sucrose**
- **9. Starch etc.**
- The most commonly used adsorbents for column chromatography are **Silica gel (SiO_2) and**

as indicated by a number on the bottle label such as silica gel 60 or silica gel 230-400.

- The larger the mesh size, the smaller the adsorbent particles.

- □ **Silica gel 70–230 is used in gravity column chromatography &**

- □ **Silica gel 230–400 is used in flash chromatography.**

- Different solvents are used for this purpose such as:
- **1. Petroleum ether**
- **2. Carbon tetra chloride**
- **3. Chloroform**
- **4. Acetone**
- **5. Benzene**
- **7. Toluene**
- **8. Water**
- **9. Pyridine etc.**
- Choice of an eluting solvent is very important for the successful separation of a mixture of chemical compounds.
- Polar solvents elute polar molecules more rapidly and vice-versa.

- **Factors due to stationary phase:**
- Particle size of the stationary phase: **Reducing the particle size increases the surface area and improves separation.** However, reduction of the particle size will decrease the flow rate of the mobile phase.

In HPLC we use very fine particles to get very good separation. The flow rate problem is solved by the use high pressure pumps to push the mobile phase through the stationary phase. Columns are made of stainless steel to withstand the high pressure.

- Adsorbent activity: The choice of the suitable adsorbent is very important.
- Uniformity of packing of the column: If the stationary phase is not packed uniformly then the bands will be irregular and resulting in poor separation.
- Concentration of the mixture: the proper ratio between sample to be separated and the amount of stationary phase is very important. Too much samples resulted in poor separation.

- Selection of the proper mobile phase: Very polar mobile phase will wash out all components without any separation. On the other hand very non polar mobile phase will result in broad band and poor separation.
- Rate of flow: Slower flow rate usually resulted in a better separation and narrower bands.
- Consistency of flow: The continuous flow of the mobile phase during the whole experiment gives better separation.

- Column dimensions: Increasing the length of the column improve separation. However, that usually leads to slower flow rate.

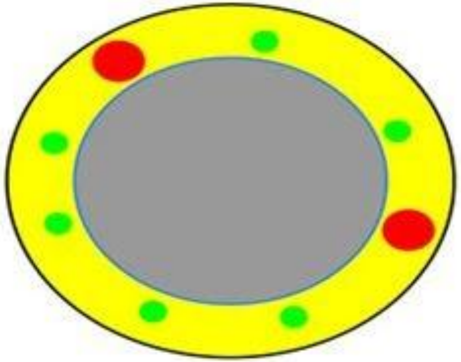
- Column temperature: Increasing the temperature usually reduces the adsorption power of the stationary phase and increase elution speed. This may leads to decrease in the separation efficiency.

- The separation of a component in partition chromatography is based on the partition co-efficient of the individual component of a mixture between a liquid stationary phase and gaseous or liquid mobile phase. But in case of column partition chromatography, the mobile phase is a liquid.

Parameter	Normal phase	Reversed phase
Stationary phase	Polar	Non-polar
Mobile phase	Non-polar	Polar
Compound eluted first	Non-polar	Polar
Compound eluted last	Polar	Non-polar
Example of stationary phase	Silica gel	C18, C8-bonded phase
Frequently used in	CC, TLC	HPLC

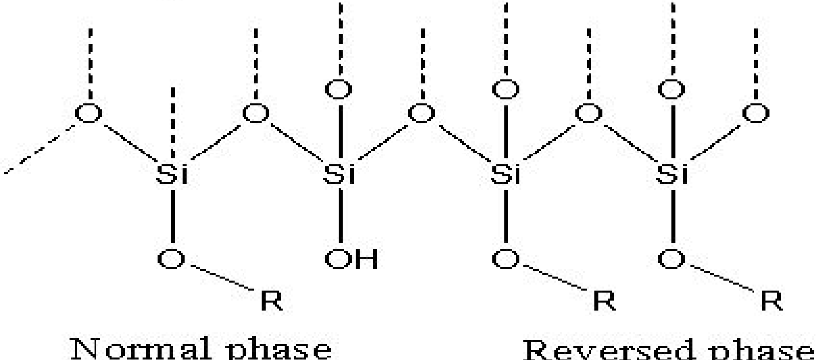
phase. Usually the liquids used as stationary phase is polar in nature. For example, water or aqueous buffer solution may be used as stationary phase. Silica gel is the most convenient supporting material for the liquid stationary phase because it is capable of retaining considerable volume of liquid phase. Cellulose powder can also be used as supporting material.

- Organic liquids or solvents may also be used as stationary phase. In this case, kieselguhr is commonly used as supporting material.



adsorption chromatography may also be used for partition column chromatography.

- **Reversed-phase C18 columns (ODS):** octadecyl carbon chain (C18) is bonded with. Because of steric effects, not all of the hydroxyl groups of the silica are derivatized by the ODS reagent, so the remainder are reacted with trimethylsilane in a process called capping to reduce adsorption effects.



purify substantial quantities of components from a mixture.

- ***Disadvantages:***
- 1. Packing of the column requires some technical skill.
- 2. The technique is time-consuming and tedious, especially for larger samples utilizing gravity column chromatographic technique.
- 3. Requires large amount of solvents.
- 4. It requires constant attention while the experiment is being performed:
- 5. Collection vessels must be frequently changed and solvent should be added continuously to the top of the column at a rate sufficient to cover the adsorbent.