EXTRACTION AND SEPARATION TECHNIQUES

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Extraction:

- Extraction is the method of removing active constituents from a solid or liquid by means of liquid solvent.
- The separation of medicinally active portions of plant or animal tissues from the inactive or inert components by using selective solvents.
- In this method the wanted components are dissolved by the use of selective solvents known as menstrum & undissolved part is a marc.

After the extraction unwanted matter is removed.

Extracts are prepared by using ethanol or other suitable solvent.

- **Extract :** Extracts can be defined as preparations of crude drugs which contain all the constituents which are soluble in the solvent.

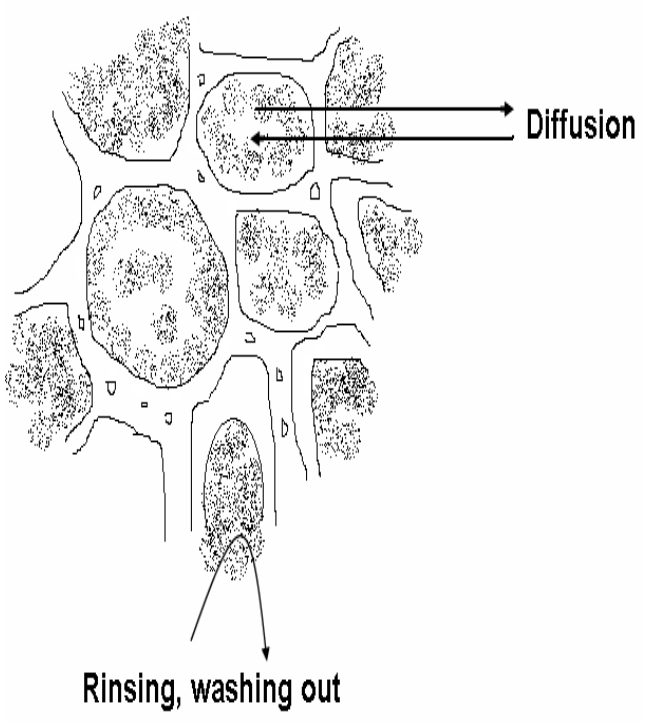
Marc: Solid residue obtain after extraction

Menstruum: Solvent used for extraction

Type of extracts

- **Dry extract (Tab, cap.)**
  E.g. belladonna extract
- **Soft (Ointment, suppository**)
  E.g. glycerrhiza extract.
- **Liquid :**
  As tincture.

- Dissolution of extractive substances out of disintegrated cells.
- Dissolution of extractive substances out of intact plant cell by diffusion (requires steeping and swelling)



- Penetration of the solvent into the plant cells and swelling of the cells.
- Diffusion of the dissolved extractive substances out of the cell.

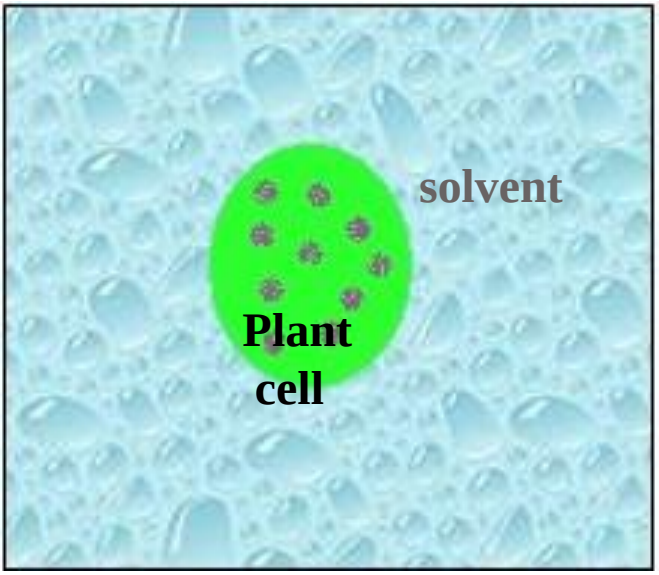
•Plant constituents are usually contained inside the cells. Therefore, The solvent used for extraction must diffuse into the cell to dissolve the desired compounds whereupon the solution must pass the cell wall in the opposite direction and mix with the surrounding liquid.

•An equilibrium is established between the solute inside the cells and the solvent surrounding the fragmented plant tissues

Ideal properties of the solvents :

1. Be highly selective for the compound to be extracted.
2. Not react with the extracted compound or with other compounds in the plant material
3. Have a low price.
4. Be harmless to man and to the environment.
5. Be completely volatile.
6. Should not mix up with water.
7. Should have the big capacity in relation to extractive.
8. The density of solvent should be difference from water density.
9. Should have the minimum viscosity.

Mechanism of Extraction :



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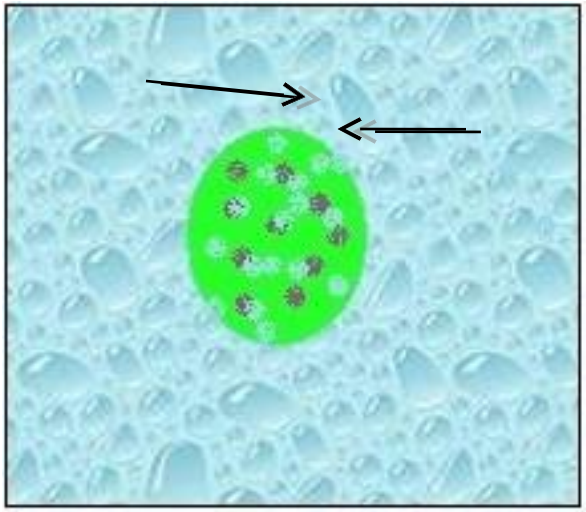

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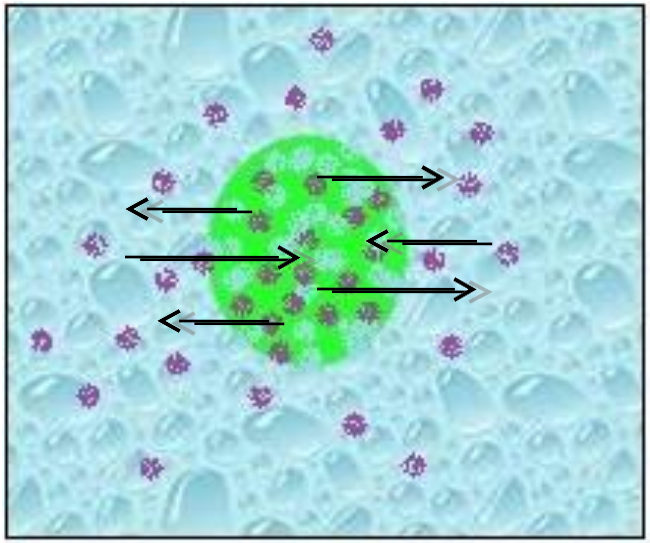

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Factors affecting extraction process :

- Nature of drug
- Solvent
- Temperature
- p H
- Particle size

Methods of extraction

Infusion
Decoction
Digestion
Maceration
Percolation
Continues hot extraction
Supercritical fluid extraction
Counter current extraction
Microwave assisted extraction
Ultrasonication-Assisted Extraction

Infusion :

Fresh infusions are prepared by macerating the crude drug for a short period of time with cold or boiling water. These are dilute solutions of the readily soluble constituents of crude drugs.

Types of Infusion :

Fresh Infusion : e.g. Infusion of orange

Concentrated Infusion : e.g. Concentrated infusion of Quassia

Decoction :

In this process, the crude drug is boiled in a specified volume of water for a defined time; it is then cooled and strained or filtered. This procedure is suitable for extracting water-soluble, heat stable constituents.

e.g. Tea , Coffee

Digestion :

This is a form of macerationin which gentle heat is used during the process of extraction.

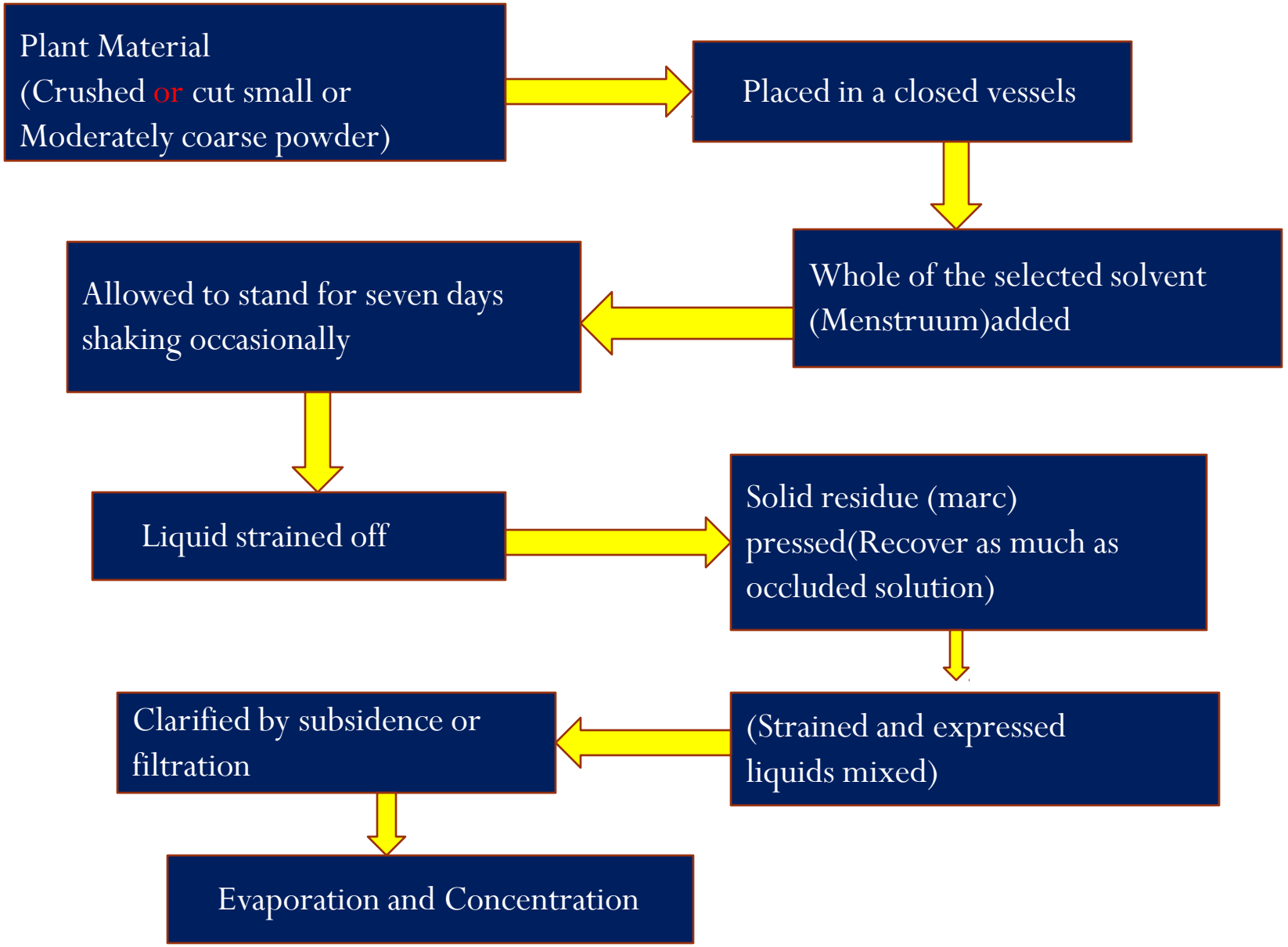
It is used when moderately elevated temperature is not objectionable. The solvent efficiency of the menstruum is thereby increased.

e.g. Extraction of Morphine

Maceration :

In this process solid ingredients are placed in a stoppered container with the whole of the solvent and allowed to stand for a period of at least 3 days (3 - 7 days) with frequent agitation, until soluble matter is dissolved. The mixture is then strained (through sieves / nets), the marc pressed and the combined liquids clarified (cleaned by filtration) or by decantation, after standing.

Process of maceration :



Types of maceration :

- Simple maceration: for organized and unorganized Crude drug

  e.g. i) Tincture of Orange

  ii) Tincture of Lemon

  iii) Tincture of Squill

- Double maceration : Concentrated infusion of orange
- Triple maceration: The maceration process may be carried out with help of heat or stirring

e.g. i) Concentrated infusion of Quassia

ii) Concentrated infusion of Senna

Factors affecting Maceration

Concentration gradient (C1-C2) is affected by several factors

1.Solid/solvent ratio: Yield decreases with constant quantity of solvent and increasing proportion of drug material.

2.Dissolution from disintegrated cells: Particle size

3.Steeping and swelling of plant material: Capillary dilation and increase in diffusion rate (Mucilage)

4.Diffusion from intact plant cell: Solvent must be able to solublilize substances

5.Temperature: increase solubility (diffusion coefficient), and decrease the viscosity

6.pH value: Influence the selectivity of extraction (qualitative and quantitative)

7.Interaction of dissolved constituents with insoluble support material of plant

8.Degree of lipophilicity

9.Effect of addition of surfactants, salts and co-solvents

Merits

- Small sample size.
- Strong swelling properties or high mucilage.
- Energy saving process.

Demerits

- Not exhaustively extract the drug.
- It is very slow process.
- Solvent required is more.

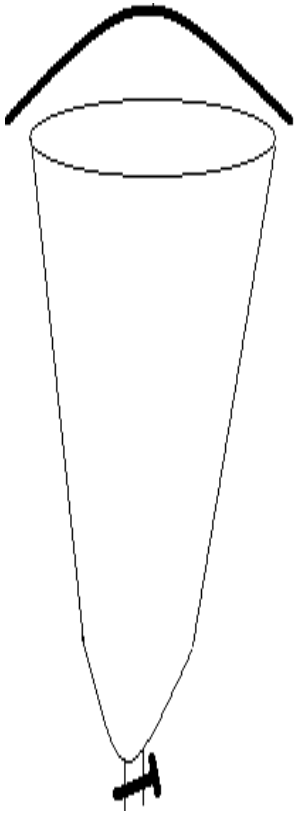
Percolation

It is continuous downward displacement of the solvent through the bed of crude drug material to get extract.

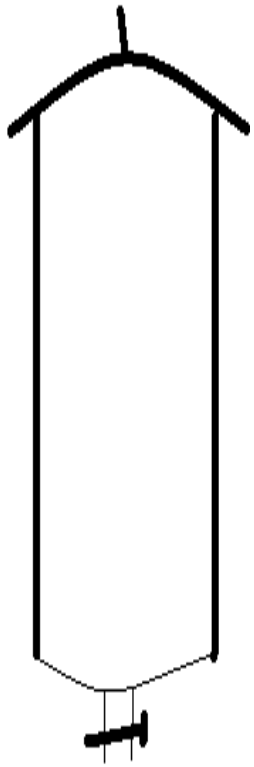
Most frequently used to extract active ingredients in the preparation of tinctures and fluid extracts.

It is the method of short successive maceration or process of displacement

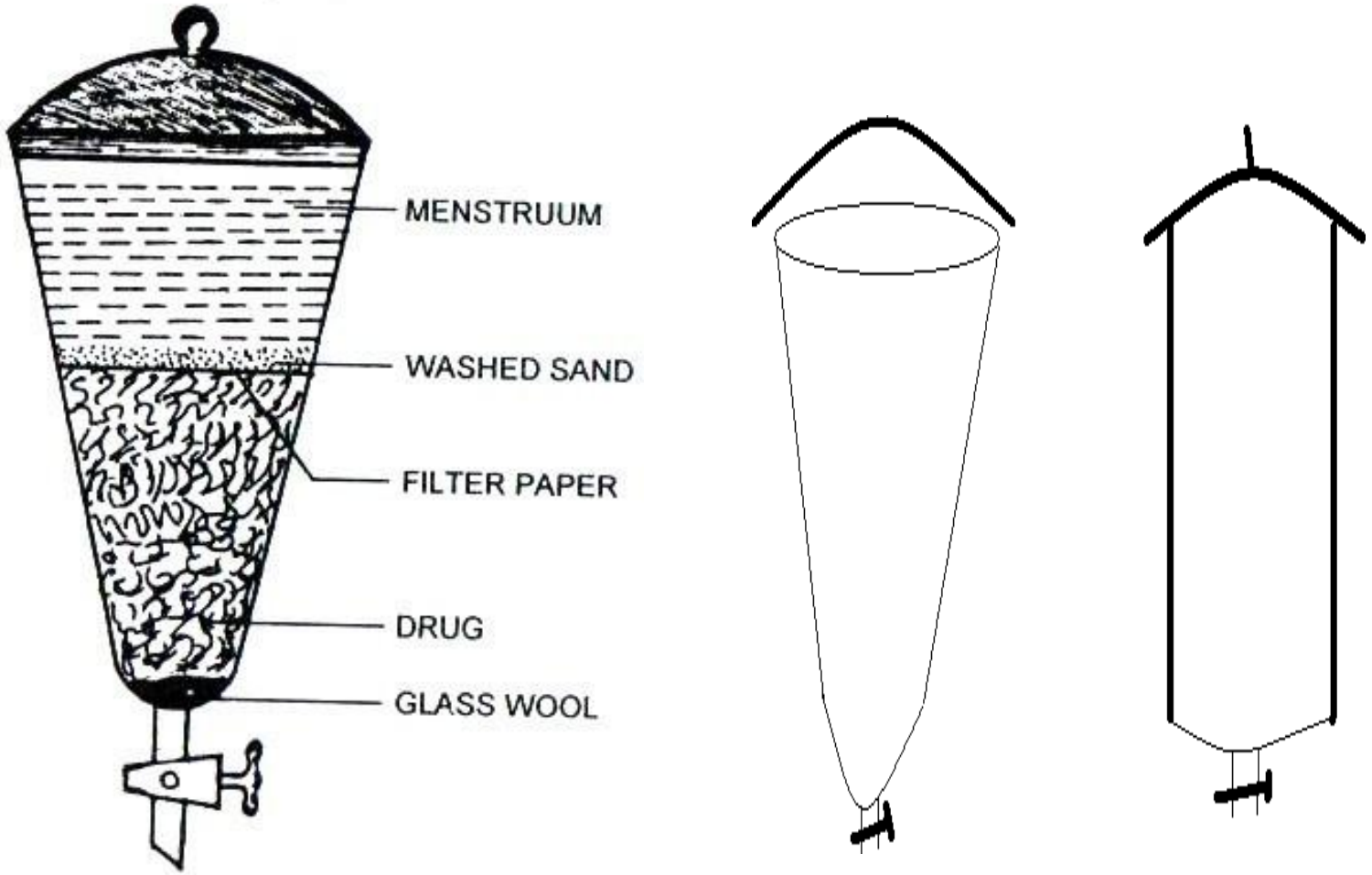
A percolator (a narrow, cone-shaped vessel open at both ends) is generally used.



conical



cylindrical


MENSTRUUM
WASHED SAND
FILTER PAPER
DRUG
GLASS WOOL

Steps in percolation

1. **Size reduction:** The drug to be extracted is subjected to suitable degree of size reduction, usually from coarse powder to fine powder.
2. **Imbibition:** During imbibition the powdered drug is moistened with a suitable amount of menstruum and allowed to stand for four hours in a well closed container.
3. **Packing:** After imbibition the moistened drug is evenly packed into the percolator.
4. **Maceration:** After packing sufficient menstruum is added to saturate the material. The percolator is allowed to stand for 24 hours to macerate the drug.
5. **Percolation:** The lower tap is opened and liquid collected therein is allowed to drip slowly at a controlled rate until 3/4th volume of the finished product is obtained.

Types of Percolation

1.Simple Percolation :

Drug → Imbibition → Maceration → Percolation and collect the percolate i.e.3/4th of the volume of finished preparation

200gms — For 4 hours — For 24 hours

e.g. i) Tincture of Belladonna

ii) Compound tincture of cardamom

2. Modified Percolation :

-Repeated maceration is more effective than simple.

-Multiple maceration – Solvent divided into equal multiple time considering the solvent retained by plant tissue.

-Used to prepare concentrated preparation.

Merits :

- Requires less time than maceration.
- Extraction of thermolabile constituents can be possible.

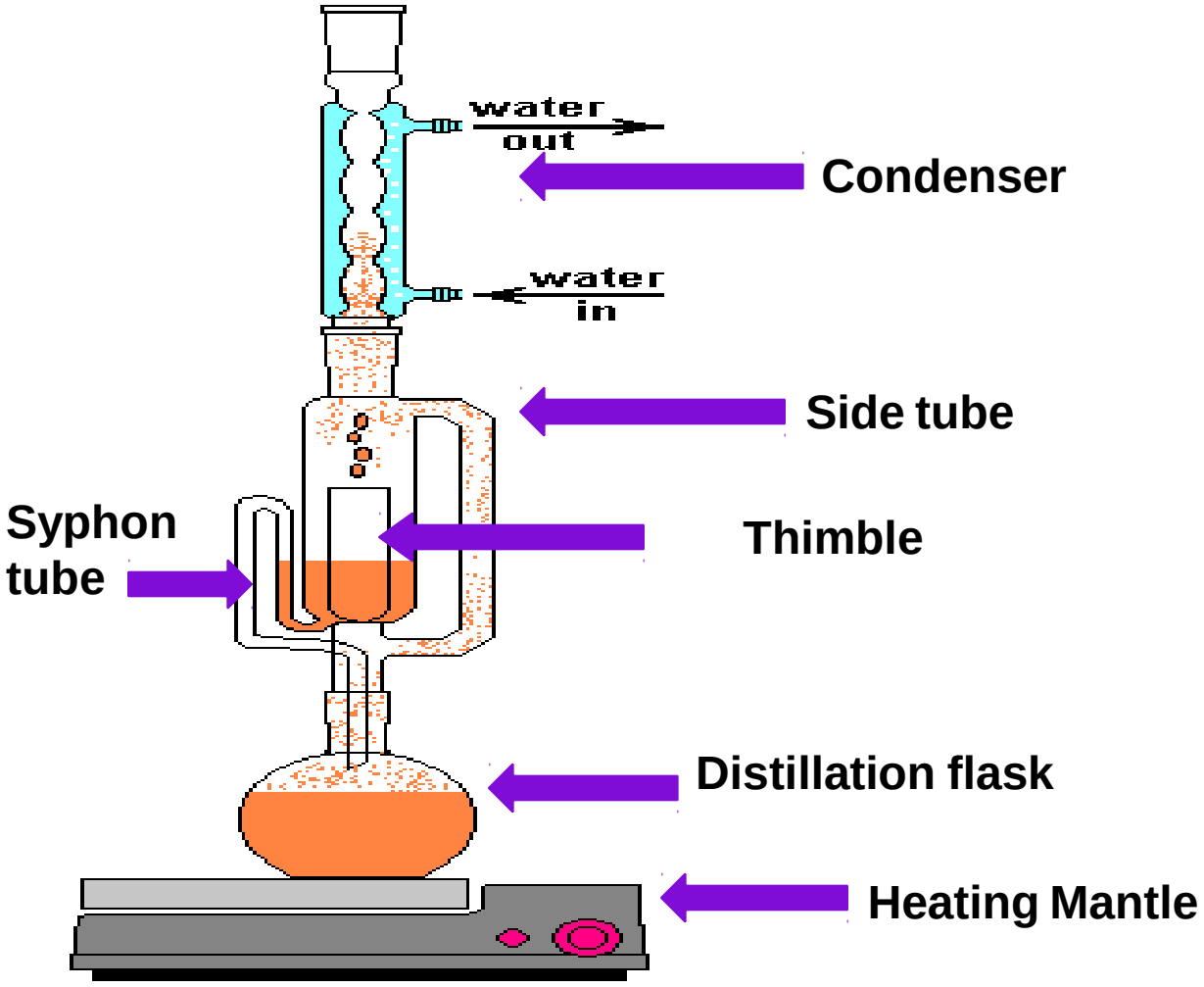
Demerits :

- Requires more time than soxhalation.
- More solvent is required.
- Skilled person is required.

Maceration Vs Percolation

Maceration	Percolation
•Time consuming and also extraction is not complete	• short time and more complete extraction
•Not required skilled operator	•Skilled operator is required
•For certain substances which are very less soluble in solvent and requires only prolonged contact with solvent.	•Special attention should be paid on particle size of material and throughout process.
•Suitable method for less potent and cheap drugs	•Suitable method for potent and costly drugs

Soxhalation



THE SOXHLET EXTRACTOR Continuous extraction of a component from a solid mixture.

Boiling solvent vapors rise up through the larger side- arm. Condensed drops of solvent fall into the porous cup, dissolving out the desired component from a solid mixture.

When the smaller side-arm fills to overflowing, it initiates a siphoning action.

The solvent, containing the dissolved component, is siphoned into the boiler below residual solvent then drains out of the porous cup, as fresh solvent drops continue to fall into the porous cup and the cycle repeats .

Merits

- Large amount of drug can be extracted with much smaller quantity of solvent.
- Tremendous economy in terms of time, energy & ultimately financial inputs.
- Small scale used a batch-process.
- Becomes more economical when converted into continuous extraction.
- Procedure on large scale.

Demerits

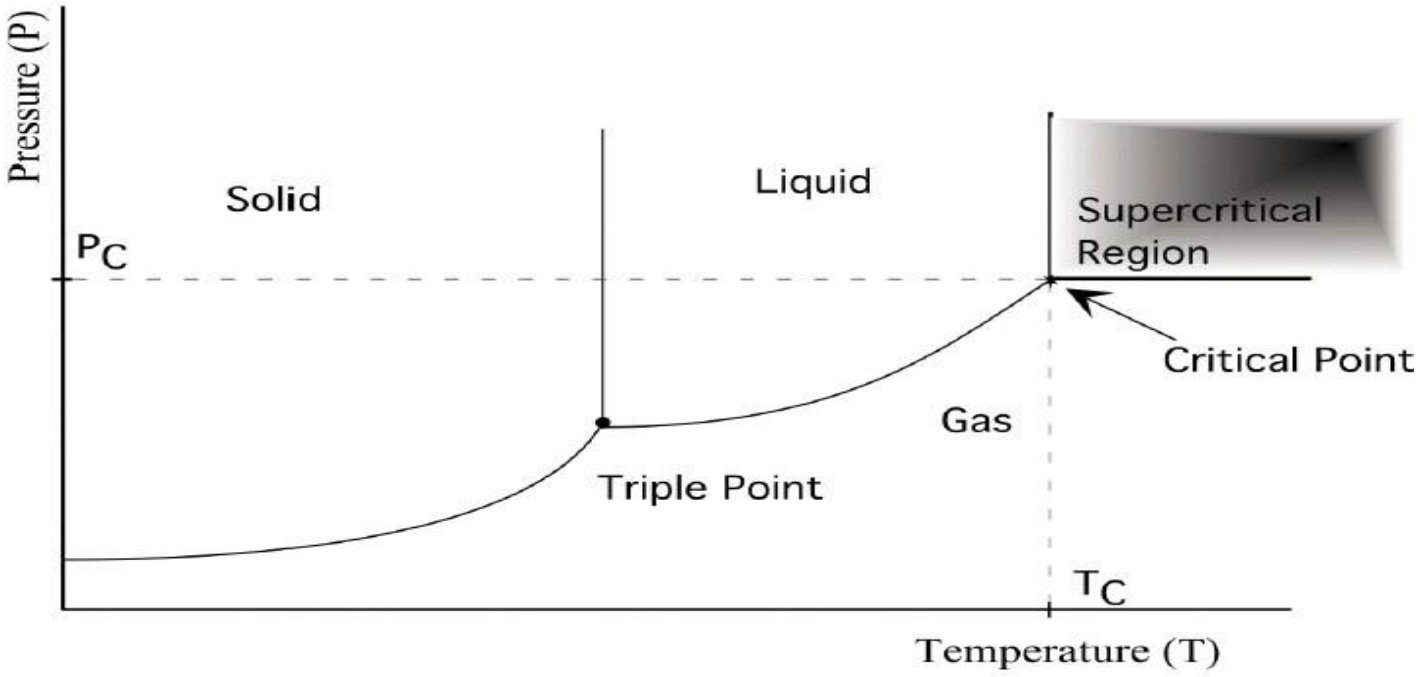
- Physical nature of drug.

Solvent.

- Chemical constituent of drug.

Supercritical Fluid Extraction

For every substance, there is a critical temperature (Tc) and pressure (Pc) above which no applied pressure can force the substance into its liquid phase. If the temperature and pressure of a substance are both higher than the Tc and Pc for that substance, the substance is defined as a **supercritical fluid.**



Properties of SCFs

At the critical point, the density of the gas and liquid phases is the same; there is no distinction between the phases. i.e. between those of the pure liquid and gas.

Supercritical posses densities that are liquid-like and

Transport properties that are gas-like.

These offers good penetrative ability and good extractive ability.

Choice of SCFs solvent

- Good solving property
- Inert to the product
- Easy separation from the product
- Cheap
- Low CP because of economic reasons
- Carbon dioxide is the most used SCF, due primarily to its low critical parameters (31.1°C, 73.8 bar)
- non-toxicity.

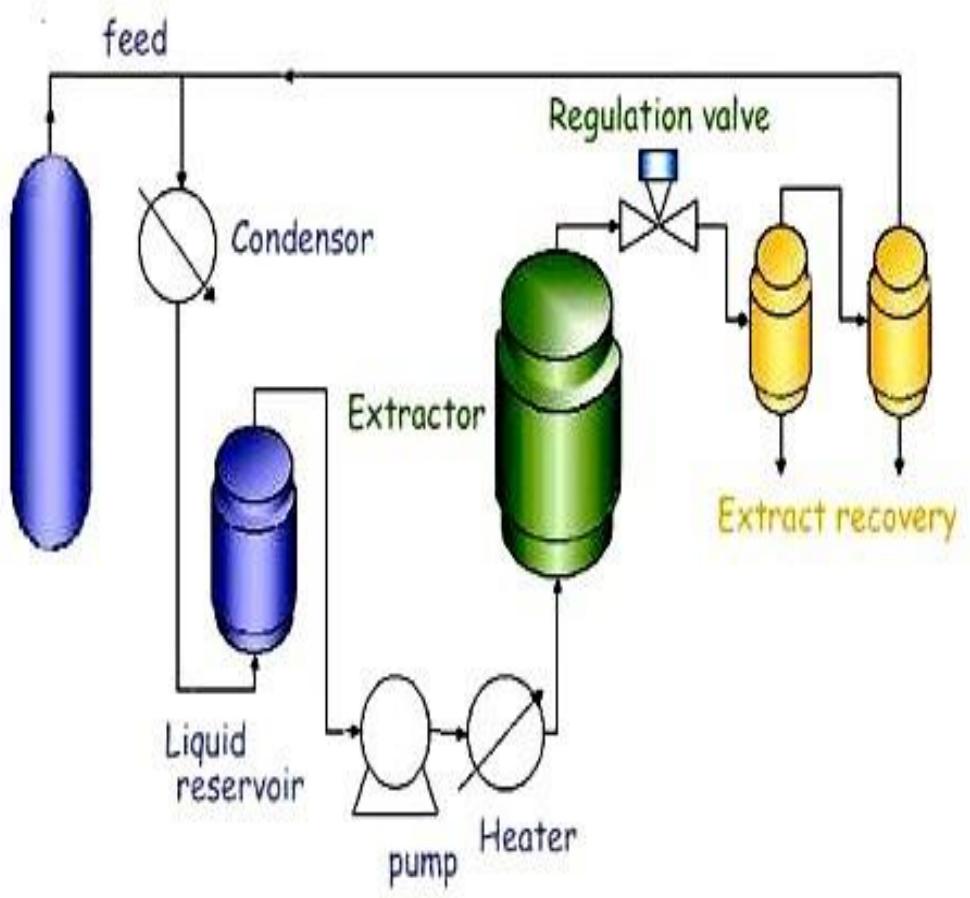
However, several other SCFs have been used in both commercial and development processes. The critical properties of some commonly used SCFs are -

Fluid	Critical Temperature (K)	Critical Pressure (bar)
Carbon dioxide	304.1	73.8
Ethane	305.4	48.8
Ethylene	282.4	50.4
Propane	369.8	42.5
Propylene	364.9	46.0
Trifluoromethane (Fluoroform)	299.3	48.6
Chlorotrifluoromethane	302.0	38.7

Supercritical Fluid Extraction Process :

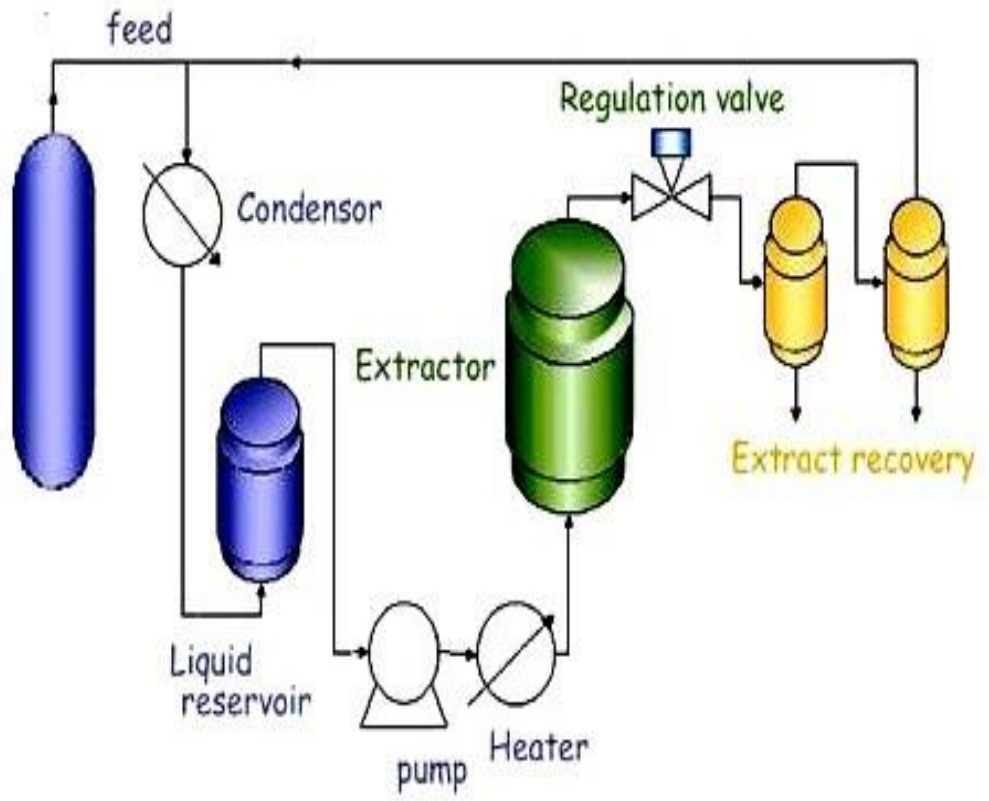
- The oldest typical and most common supercritical fluid extraction from solids is performed as a batch process, with a continuous flow of SCF.

- An extraction medium (going to be SCF) stored in the feed tank and liquid SCF is pumped from a reservoir ; it is heated and pressurized to reach the supercritical conditions



Supercritical Fluid Extraction Process :

- Supercritical SCF enters the extraction chamber where contact with crude drug bed occurs and the more volatile substances are dissolved into the supercritical fluid.
- Solute and SCF leave extractor and extract is precipitated in separators, where SCF becomes gaseous.
- Gas is recycled by condensation before returning to liquid reservoir.



Advantages of SFE

- Dissolving power of the SCF is controlled by pressure and/or temperature.
- SCF is easily recoverable from the extract due to its volatility.
- Non-toxic solvents leave no harmful residue.
- High boiling components are extracted at relatively low temperatures.
- Separations not possible by more traditional processes can sometimes be effected.
- Thermally labile compounds can be extracted with minimal damage as low temperatures can be employed by the extraction.

Disadvantages of SFE

- Elevated pressure required.
- Compression of solvent requires elaborate recycling measures to reduce energy costs.
- High capital investment for equipment.

Applications of Supercritical Fluid Extraction :

- Recovery of organics from oil shale
- Separations of biological fluids
- Bioseparation
- Petroleum recovery
- Crude dewaxing
- Coal processing (reactive extraction and liquefaction)
- Selective extraction of fragrances, oils and impurities from agricultural and food products
- Pollution control
- Combustion and many other applications.

Counter-Current Extraction

- A liquid-liquid extraction process in which the solvent and the process stream in contact with each other flow in opposite directions.

- Screw extractors and carousel extractors are the two type of equipments used for Counter-Current Extraction.

Counter-Current Extraction Process

- In counter-current extraction (CCE), wet raw material is pulverized using toothed disc disintegrators to produce fine slurry.

- The material to be extracted is moved in one direction (generally in the form of fine slurry) within a cylindrical extractor where it comes in contact with extraction solvent.

- The further the starting material moves, the more concentrated the extract becomes.

- Finally, sufficiently concentrated extract comes out at one end of the extractor while the marc (practically free of visible solvent) falls out from the other end.

Advantages

1. A unit quantity of the plant material can be extracted with much smaller volume of solvent as compared to other methods like maceration, decoction, and percolation.
2. CCE is commonly done at room temperature, which spares the thermolabile constituents from exposure to heat which is employed in most other techniques.
3. As the pulverization of the drug is done under wet conditions, the heat generated during comminution is neutralized by water. This again spares the thermolabile constituents from exposure to heat.
4. The extraction procedure has been rated to be more efficient and effective than Continuous hot extraction.

Applications :

1. DNA purification.

2.Food Industry.

eg. Citrus oils, Unsaturated fatty acids, and squalene tocopherol.

3. An important application is citrus oil processing,
An important subject in perfumes and food industries.

Microwave-assisted Extraction

- Microwaves are electromagnetic radiations with a frequency from 0.3 to 300 GHz (Camel, 2001).

- In order to avoid interferences with radio communications, domestic and industrial microwaves generally operate at 2.45 GHz (Fig. 1). Owing to their electromagnetic nature, microwaves possess electric and magnetic fields which are perpendicular to each other.

- The electric field causes heating via two simultaneous mechanisms, namely, dipolar rotation and ionic conduction

- Microwave-assisted extraction offers a rapid delivery of energy to a total volume of solvent and solid plant matrix with subsequent heating of the solvent and solid matrix, efficiently and homogeneously.
- Components of the sample absorb microwave energy in accordance to their dielectric constants.
- When plant material is immersed inside a microwave transparent solvent, the heat of microwave radiation directly reaches to the solid without being absorbed by the solvent, resulting in instantaneous heating of the residual moisture in the solid.
- Heating causes the moisture to evaporate and creates a high vapour pressure that breaks the cell wall of substrate and releases the content into solvent.
- The extracting selectivity and the ability of the solvent to interact with microwaves can be modulated by using mixtures of solvents.
- One of the most commonly used mixtures is hexane-acetone.

Collection of the essential oil. The oil is separated from water simply by decantation.

High definition video system for visual control of process.

Icon-driven programs provide full control of the extraction method parameters.

Samples are placed in dedicated easy-to-handle glass modules. Loading/unloading of samples are immediate and easy.

Microwave labstation, microprocessor controlled with infrared automatic temperature system.

Special lab-grade water chiller for optimal extraction performance (option)

Mobile module for flexibility of use (option)

Advantages of Microwave Assisted Extraction

- It reduces solvent consumption.
- It has a shorter operational time.
- It possess moderately high recoveries.
- Has a good reproducibility and minimal sample manipulation for extraction process.

Disadvantages of Microwave Assisted Extraction

- An additional filtration or centrifugation is necessary to remove the solid residue during MAE.

- Furthermore, the efficiency of microwaves can be very poor when either the target compounds or the solvents are non-polar, or when they are volatile.

Applications of Microwave-Assisted Extraction

- MAE can extract nutraceuticals products from plant sources in a faster manner than conventional solid–liquid extractions.

- MAE (80% methanol) could dramatically reduce the extraction time of ginseng saponin from 12 h using conventional extraction methods to a few seconds.

Biologically active compounds extracted by MAE

- Extraction of taxanes from *Taxus brevifolia* needles,
- Azadiractin related limonoids from **Azadirachta indica** seed kernels,
- Extraction of glycyrrhizic acid from *Glycyrrhizia glabra* roots,
- Extraction of artemisinin from *Artemisia annua*.

- A higher microwave temperature and a short extraction time are more effective in extracting anti-oxidative phenolic compounds from tomato using MAE.
- MAE was proven as a potential alternative to traditional methods for extraction of phenols such as chlorogenic acids from green coffee beans.

Ultrasonication-Assisted Extraction (UAE)

The procedure involves the use of ultrasound waves, which have frequencies higher than 20 kHz, have great effects on extraction yield and kinetics.

- UAE involves ultrasonic effects of acoustic cavitations. Under ultrasonic action solid and liquid particles are vibrated and accelerated and, because of that solute quickly diffuses out from solid phase to solvent

- Ultrasound assisted extractors are ultrasonic baths or closed extractors fitted with an ultrasonic horn transducer. The mechanical effects of ultrasound induce a greater penetration of solvent into cellular materials and improve mass transfer.

Advantages of Ultra sonicated extraction:

- It is an inexpensive, simple and efficient alternative to conventional extraction technique.
- It include the increase of extraction yield and faster kinetics.
- It reduce the operating temperature allowing the extraction of thermolabile compounds.
- Compared with other novel extraction techniques such as microwave-assisted extraction, the ultrasound apparatus is cheaper and its operation is easier.

Disadvantages of Ultra sonicated extraction:

- The active constituents of medicinal plants through formation of free radicals and consequently undesirable changes in the drug molecules.

Applications

- Used to extract nutraceuticals from plants such as essential oils and lipids dietary supplements.

  e.g. oils from almond, apricot and rice bran

- Extraction of saponinfrom ginseng, the observed total yield and saponin yield increased by 15 and 30%, respectively

- extracts. It was found that rice bran oil extraction can be efficiently performed in 30 min under high-intensity ultrasound either using hexane or a basic aqueous solution.

- Extraction rates of carvone and limonene by ultrasound-assisted extraction with hexane were 1.3–2 times more rapid than those by the conventional extraction depending on temperature

Conclusion:

- Extraction is essential for isolation of different chemical constituent from crude drug material.
- Extraction depends on properties of material to be extracted. Hence it is necessary to study extraction methods in detail.

SOLVENTS

- *Petroleum ether* :- Fixed oils, Phytoglycerols.
- *Benzene*:- Fixed oils, Phytosterols.
- *Chloroform*;- Alkaloids
- *Acetone:-* Phytosterols
- *Ethanol:-* Carbohydrates, Glycosides
- *Saponin:-* Phenolics, Tannins, proteins, Amino acids
- *Water:-* Proteins, Amino acids, Glycosides, Gums, Mucilages, Carbohydrates

Properties of ideal solvent:

1.Be highly selective for the compound to be extracted.
2.Have a high capacity for extraction in terms of coefficient of saturation of the compound in the medium.
3.Not react with the extracted compound or with other compounds in the plant material.
4.Have a low price.
5.Be harmless to human being and to the environment.
6.Be completely volatile.

DIFFICULTIES:-

- Different active constituents like alkaloids, glycosides, tannins, terpenoids, resins oils etc requires advanced knowledge of phytoconstituents which help in selection of method.
- Different forms of insoluble matters may affect the extraction process.

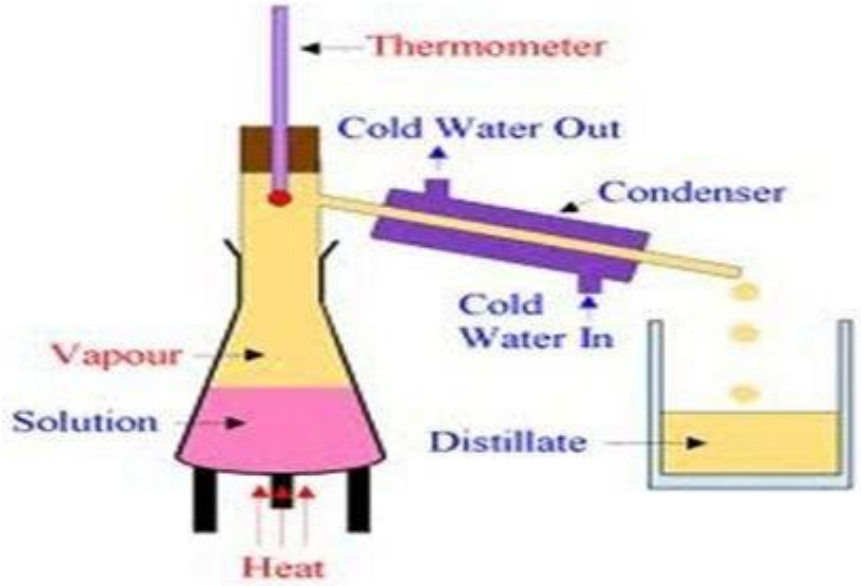
  eg. Cellulose, proteins etc. in many drugs only the active constituent is not soluble material but along with it large proportion of unwanted material is solubilized. In such situations, a solvent chosen is as selective as possible.
- Wet vegetable material is an excellent medium for microbial growth and it may leads to loss of active substances and solvent must have suitable preservative action.

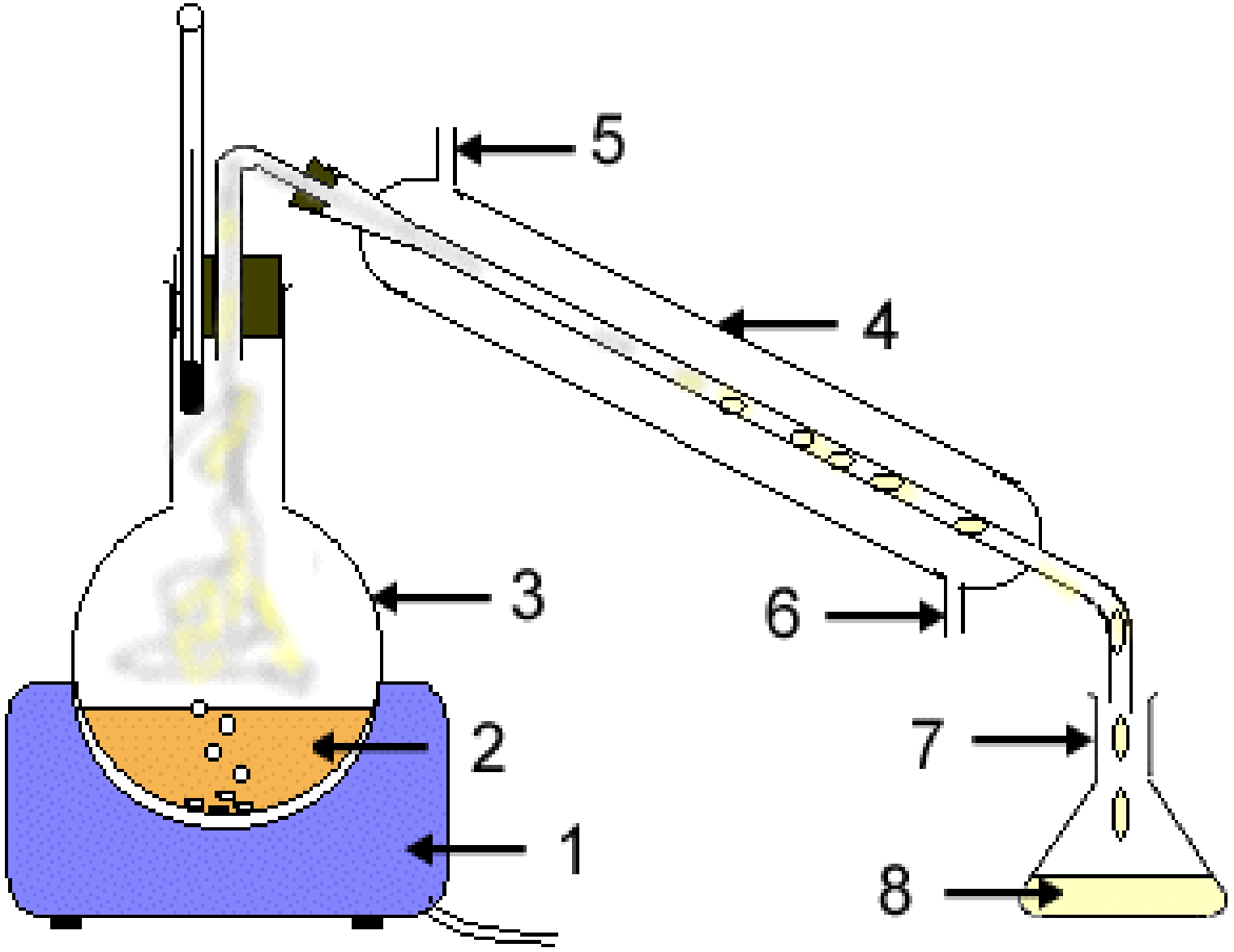
- ***Non Chromatographic***

Separation Techniques

Distillation

- Distillation is a method of separating mixtures based on differences in their boiling points
- Distillation is a **physical separation** process and not a chemical reaction

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Different Parts of Distillation Unit

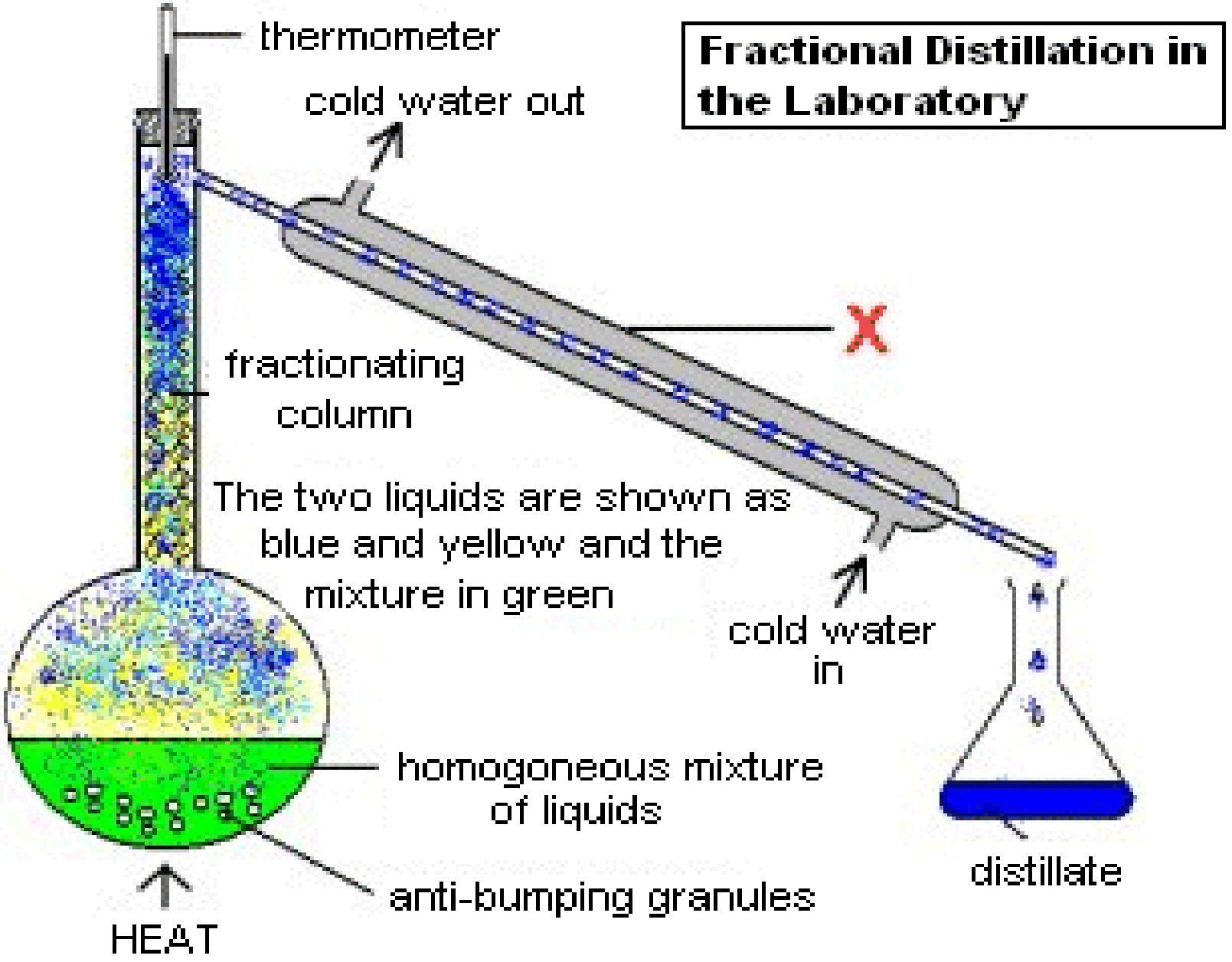
- ✓ **Still**
- ✓ **Condenser**
- ✓ **Receiver**
- ✓ **Distillate**

Fractional Distillation

- This technique of the distillation process, used to separate two liquids having close (near) boiling points (Lower than 25 °C).Example:-Separation of ethanol (78) °C from water (100) °C.

Fractional distillation system composed from the following main parts:-

1. **Heating source.**
2. **Distillation flask.**
3. **Fractional column.**
4. **Thermometer.**
5. **Condenser.**
6. **Receiving flask .**


Fractional Distillation in the Laboratory
thermometer
cold water out
X
fractionating column
The two liquids are shown as blue and yellow and the mixture in green
cold water in
homogoneous mixture of liquids
anti-bumping granules
HEAT
distillate

Uses of Fractional Distillation

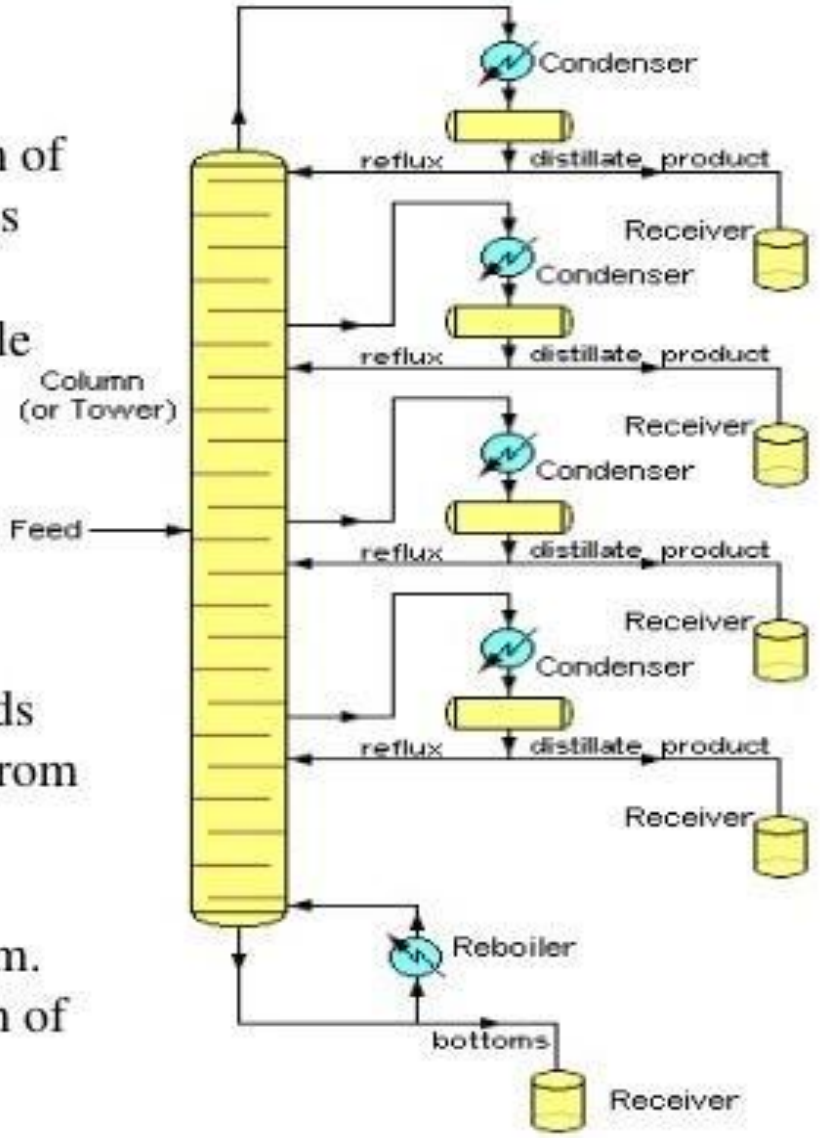
- Distillation of crude oil to remove impurities.
- Petroleum refining industries and chemical industries.
- Large-scale alcoholic fermentation in order to purify alcoholic beverages like beer and wine.
- To purify wines and other alcoholic drinks.
- It is used for solvent recycling.
- It is used for extraction of essential oils.
- It is used in the purification of fragrances in perfume industries.
- It is also used in hydrogen isotopes research

FRACTIONAL DISTILLATION

This method is used for the separation of the components from volatile mixtures
Largely using in the separation of hydrocarbons from oxygenated volatile oil eg citral, eucalyptol

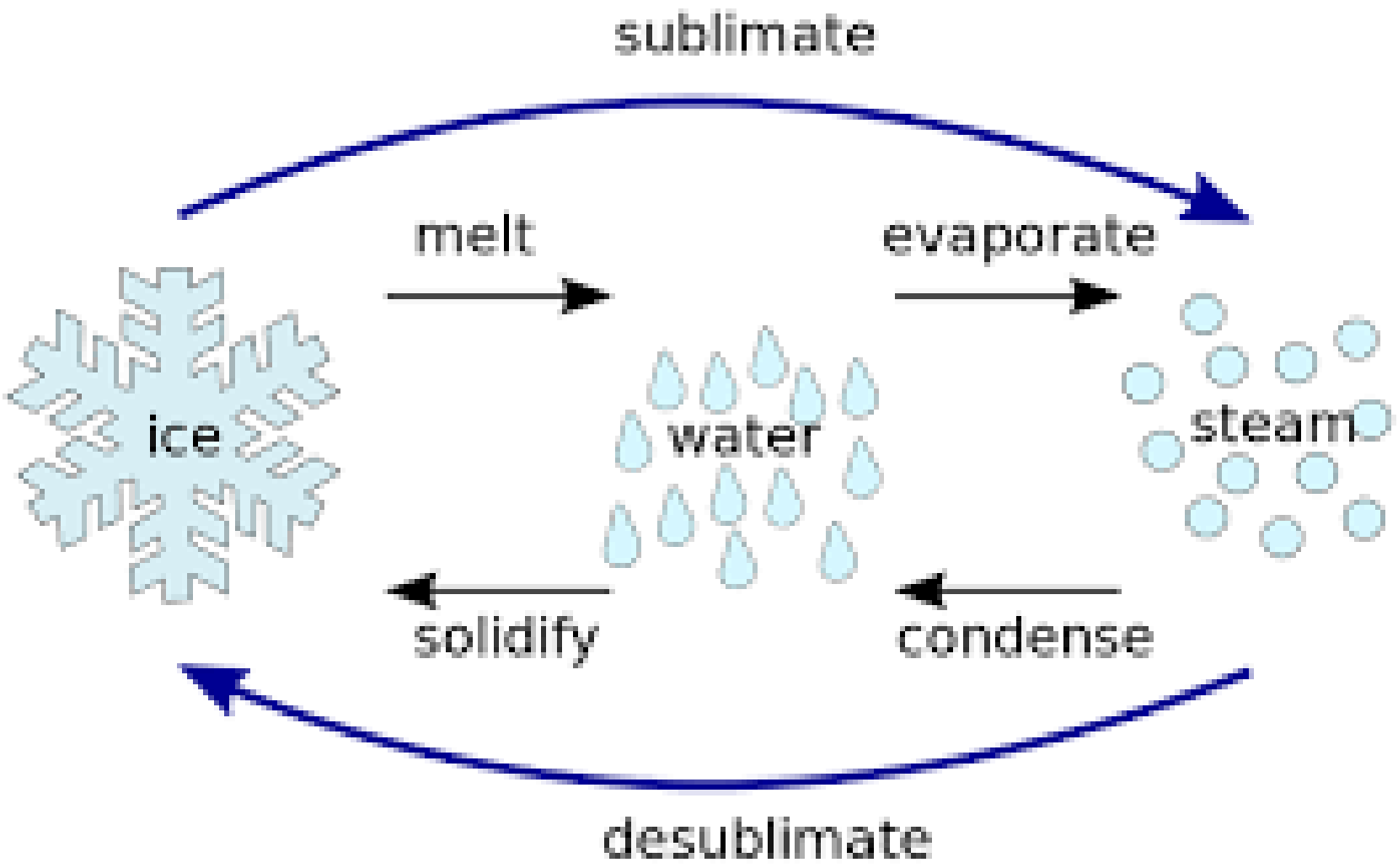
FRACTIONAL LIBERATION

In this proces the groups of compounds having the tendency of precipitation from the solution.
Incertain cases the compounds may modified by converting to its salt form.
This proces is often used in separation of cinchona alkaloids, morphine etc.



SUBLIMATION

- **Sublimation** is the transition of a substance directly from the solid to the gas phase, without passing through the intermediate liquid phase.
- **Sublimation** is an endothermic phase transition that **occurs** at temperatures and pressures below a substance's triple point in its phase diagram.
- When energy is transferred to dry ice, the solid carbon dioxide does not melt to liquid carbon dioxide.


sublimate
melt
evaporate
ice
water
steam
solidify
condense
desublimate

Three Types Of Sublimed Substances

- ✓ **Cake Sublimate**
- ✓ **Powder Sublimate**
- ✓ **Crystalline Sublimate**

DERIVATIZATION.

- **Derivatization** is a **chemical** reaction where a polar group in a molecule (e.g. a carboxyl or hydroxyl group) is **chemically** converted to a non-polar group in order to make the molecule volatile so that it can be analyzed by GC/MS.

Goals of derivatization.

- To increase the volatile nature
- Elimanation of Polar Groups like OH, NH & SH groups.
- Reduction of Reactivity
- Increase Stability
- Improvement of the Chromatographic Behavior

Types Of Chemical Derivatization

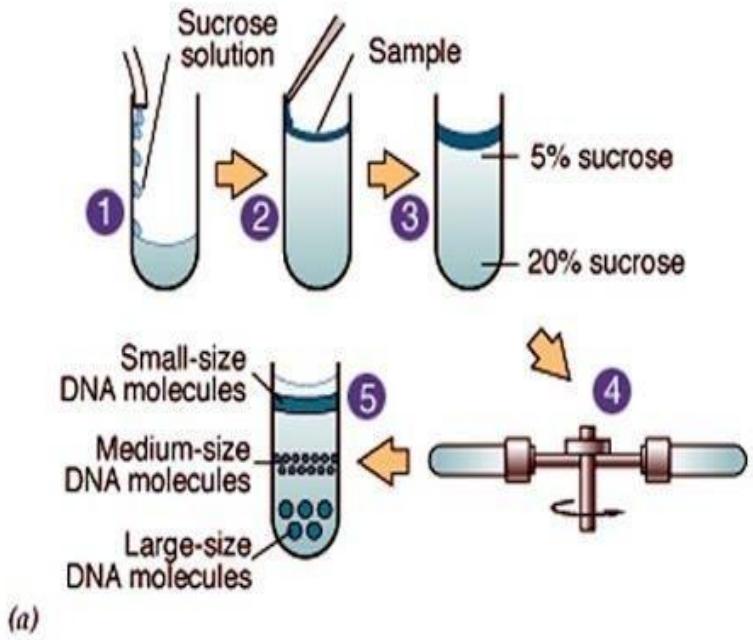
- Esterification – For carboxylic Acid
- Perfluoroacylaytion – increases mol. Wt of sample
- Condensation- For Aldehydes or Ketones
- Alkylation – Replacement of Active Hydrogen By aliphatic or benzyl group
- Acylation- Reduce the polarity by Acetic Anhydride
- Silylation - e.g Dimethylsilyl, t-butyl Dimethylsilyl
- Chiral Derivatization - Convert specific functional group into individual diastereomers
- Pre- column Derivatization- involve the reaction of analyte before Chromatographic separation
- Post column Derivatization – extracts are derivatized before chromatographic analysis

FRACTIONAL CRYSTALLIZATION

Is a method of Purification of substances based on differences in Solubility.

It is carried out by preparing conc. Sol of Compounds. When the sol gets cool, few crystals are observed.

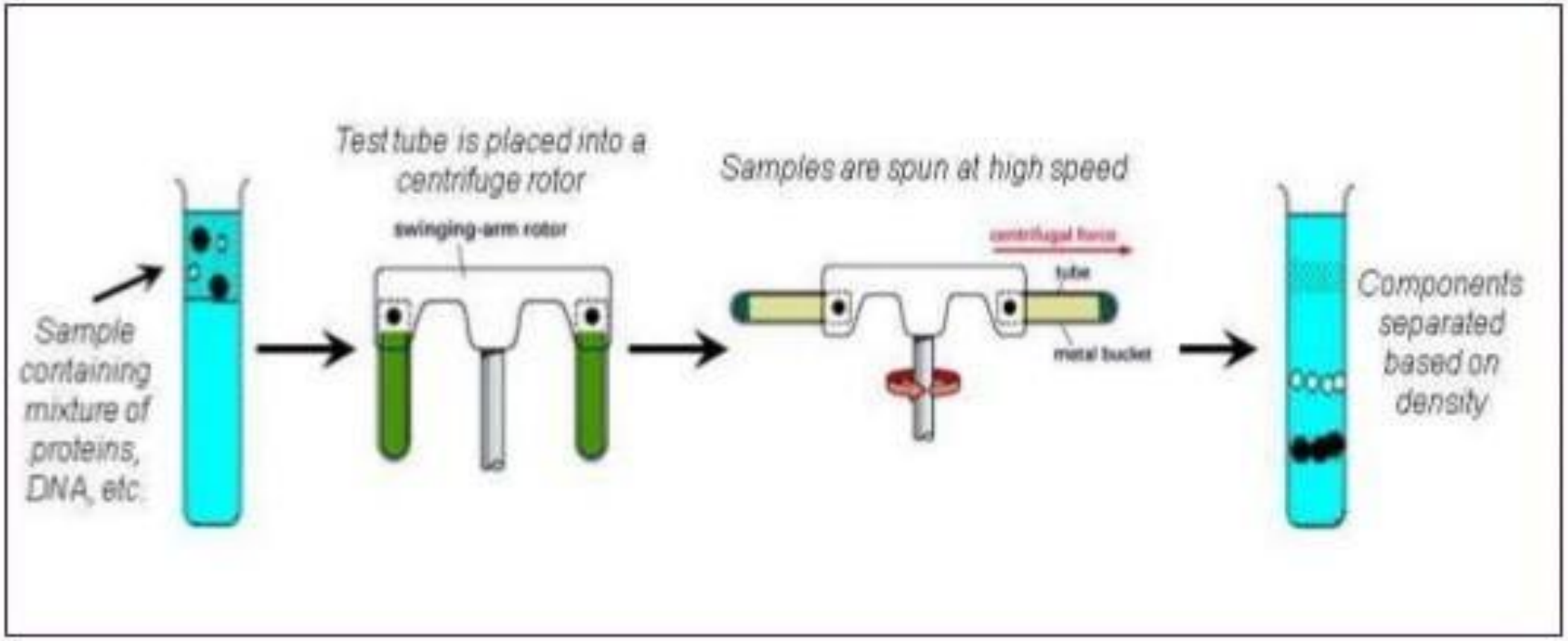
CENTRIFUGATION



DEFINITION

Centrifugation is a process used to separate or concentrate materials suspended in a liquid medium. The theoretical basis of this technique is the effect of gravity on particles (including macromolecules) in suspension. Two particles of different masses will settle in a tube at different rates in response to gravity.

- To take advantage of even tiny differences in density to separate various particles in a solution, gravity can be replaced with the much more powerful "**centrifugal force**" provided by a centrifuge.



Principle of centrifugation

A particle whether it is a precipitate a macromolecule or a cell organelle is subjected to a centrifugal force when it is rotated at a high rate of speed. The centrifugal force F is denoted by equation

$F=m\omega^2 r$

Where

F= intensity of the centrifugal force

m= effective mass of the sedimenting particle

ω= angular velocity of rotation

r= distance of the migrating particles from the central axis of rotation

CENTRIFUGE ROTOR

- A centrifuge rotor is the rotating unit of the centrifuge, which has fixed holes drilled at an angle. Test tubes are placed inside these holes and the rotor spins to aid in the separation of the materials.

TYPES OF ROTOR

swing-bucket Rotor

fixed-angle Rotor

vertical rotor

APPLICATION IN WATER TREATMENT

OTHER APPLICATIONS

- Separating chalk powder from water
- Removing fat from milk to produce skimmed milk
- Separating textiles
- Removing water from lettuce after washing it in a salad spinner
- Separating particles from an air-flow using cyclonic separation

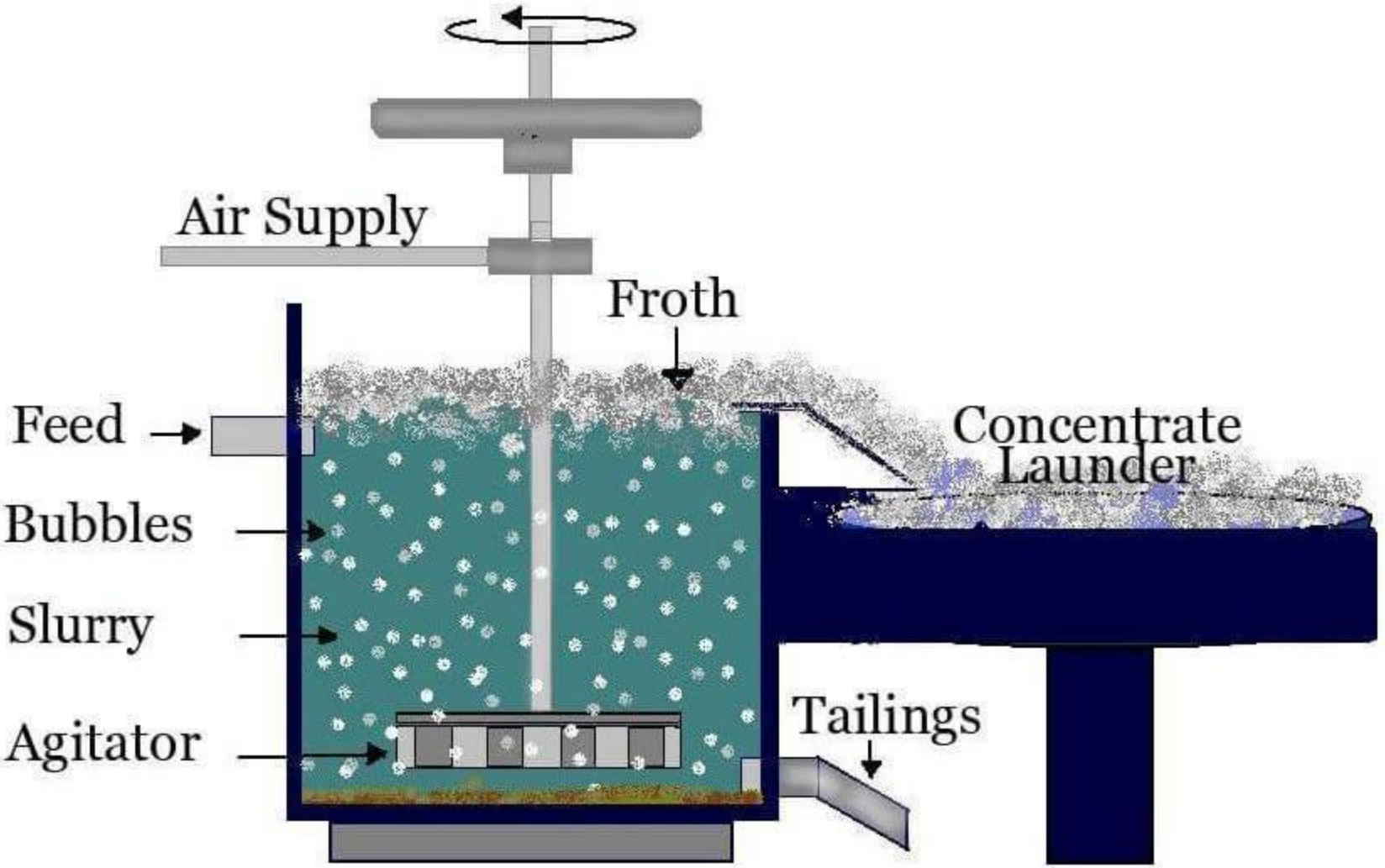
- The clarification and stabilization of wine
- Separation of water particles from clothes while spin-drying in washing machines
- Separation of urine components and blood components in forensic and research laboratory



Froth flotation

- **Froth flotation** is a process for selectively separating hydrophobic materials from hydrophilic.
- It occurs on gas-liquid interfaces.
- Hydrophobic Particles get selectively adsorbed or remain attached to the surface of air bubbles rising through Suspension, & hence get separated from the suspension in the form of forth.

FLOTATION PROCESS



Mechanics of flotation

- Reagent conditioning to achieve hydrophobic surface charges on the desired particles.
- Collection and upward transport by bubbles in an intimate contact with air or nitrogen
- Formation of a stable froth on the surface of the flotation cell
- Separation of the mineral laden froth from the bath (flotation cell)

Basic elements/reagents used in Froath Floatation

- Collector
- Frother
- Depressents & Activator
- PH modifier

Chromatographic Separation Techniques

HISTORY

- Tswett used chromatography to separate plant pigments (1906)
- colorful separation of plant pigments was done using a column of calcium carbonate(chalk)
- the new technique was called chromatography because the result of the analysis was **written in color**.(Chroma means **color** and graphein means to **write**)

Mikhail Tswett, Russian Botanist, 1872-1919

CHROMATOGRAPHY

Technique used to separate and identify the components of a mixture

PRINCIPLE: Works by allowing the molecules present in the mixture to distribute themselves between a stationary and a mobile medium. Molecules that spend most of their time in the mobile phase are carried along faster.

Components:

Mobile phase: a solvent that flows through the supporting medium

Stationary phase: a layer or coating on the supporting medium that interacts with the analytes

Supporting medium: a solid surface on which the stationary phase is bound or coated

BASIC TERMS

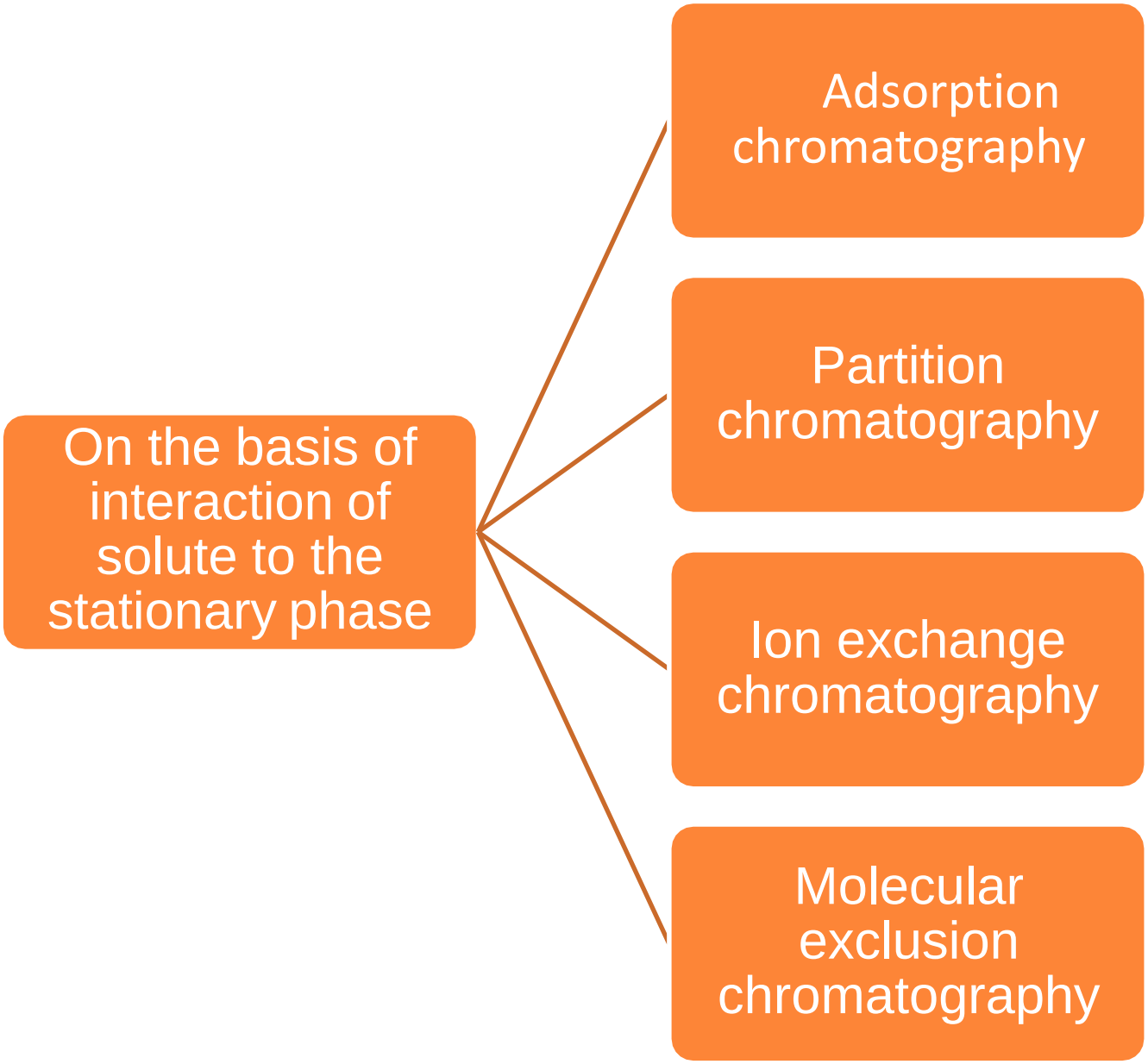
- **Adsorbtion:** Interaction of solute molecules with the surface of the stationary phase
- **Eluent:** The mobile phase
- **Elution:** Motion of the mobile phase through the stationary phase
- **Elution time:** The time taken for a solute to pass through the system. A solute with a short elution time travels through the stationary phase rapidly, *i.e. it elutes fast*
- **Stationary phase**: The part of the chromatography system that is fixed in place

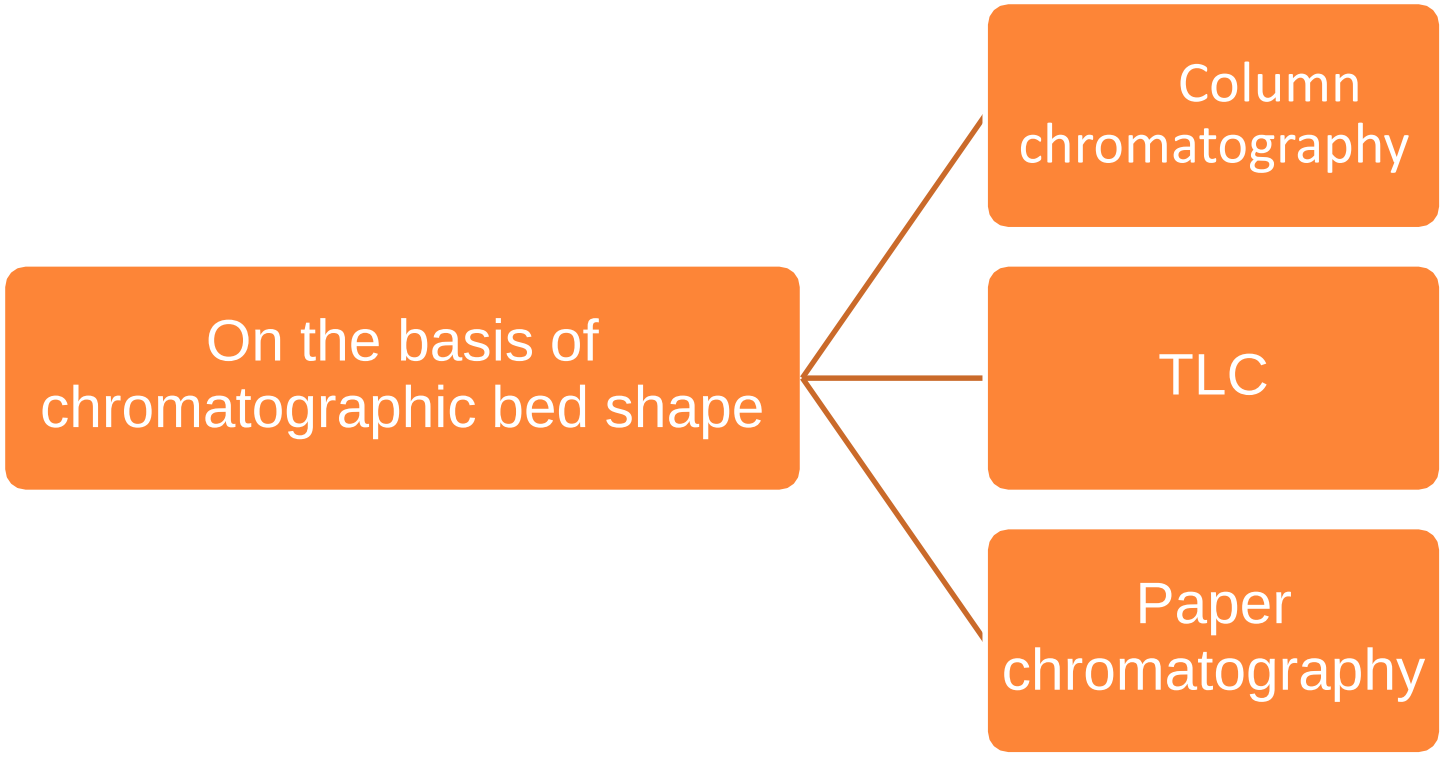
- **Normal phase:** “Unmodified” stationary phase where POLAR solutes interact strongly and run slowly
- **Reverse phase:** “Modified” stationary phase where POLAR solutes run fast *i.e. reverse order*
- **Resolution:** Degree of separation of different solutes. In principle, resolution can be improved by using a longer stationary phase, finer stationary phase or slower elution.
- **Rf value**: distance travelled by solute

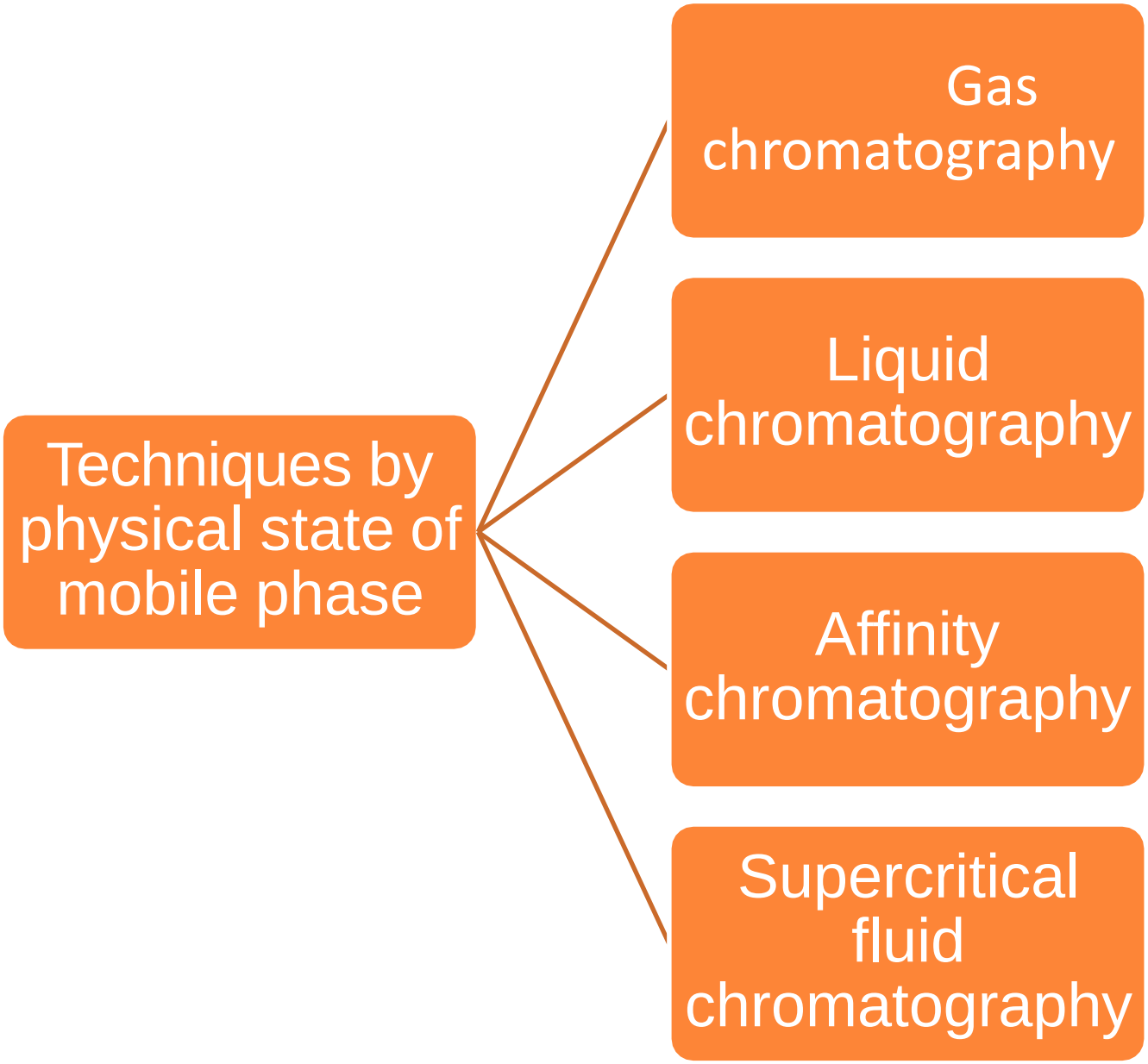
  distance travelled by solvent
- **Rf =** retardation factor. The Rf value has to be between 0 and 1, and it is typically reported to two decimal places.

CLASSIFICATION

- On the basis of interaction of solute to the stationary phase
- On the basis of chromatographic bed shape
- Techniques by physical state of mobile phase


On the basis of interaction of solute to the stationary phase
Adsorption chromatography
Partition chromatography
Ion exchange chromatography
Molecular exclusion chromatography


On the basis of chromatographic bed shape
Column chromatography
TLC
Paper chromatography

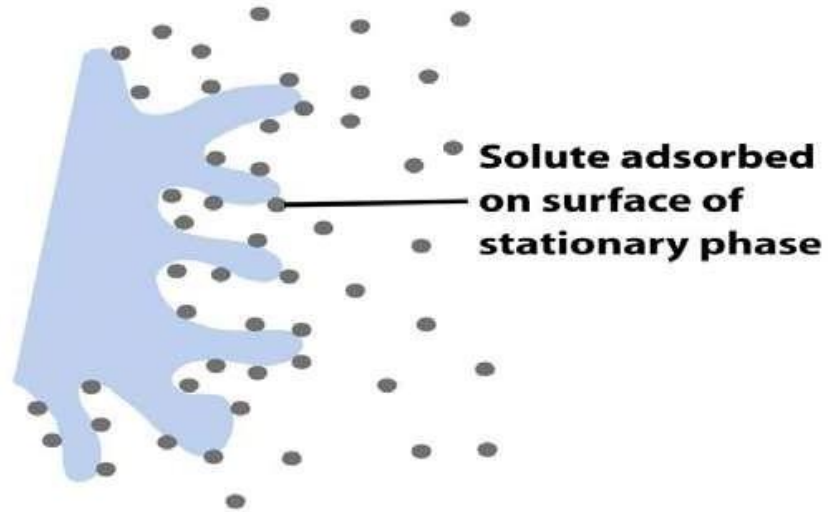

Techniques by physical state of mobile phase
Gas chromatography
Liquid chromatography
Affinity chromatography
Supercritical fluid chromatography

ADSORPTION CHROMATOGRAPHY

- Principle of separation:

utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase

- Stationary phase: adsorbent filled in a tube (column)
- Mobile phase: various solvents (eluents)



Adsorption chromatography

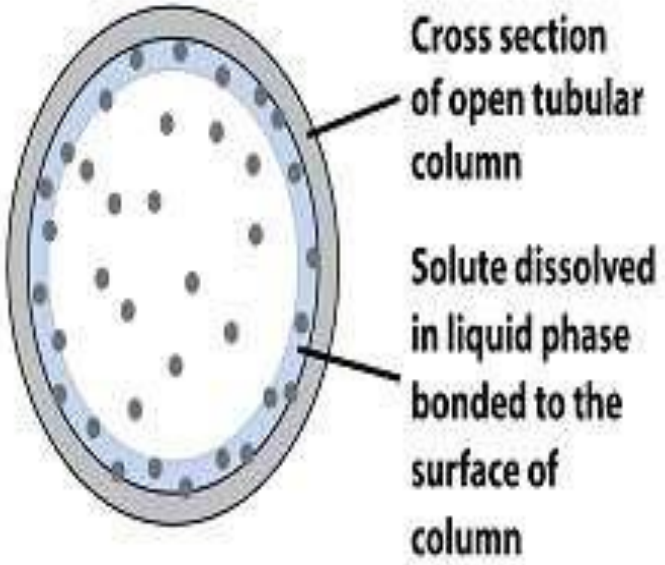
- One of the oldest type
- solute molecules bond directly to the surface of the stationary phase
- Stationary phases may contain a variety of adsorption sites differing in the tenacity with which they bind the molecules and in their relative abundance

PARTITION CHROMATOGRAPHY

PRINCIPLE:

Partiton of component of sample between sample and liquid/gas stationary phase retard some components of sample more as compared to others. This gives the basis of separation

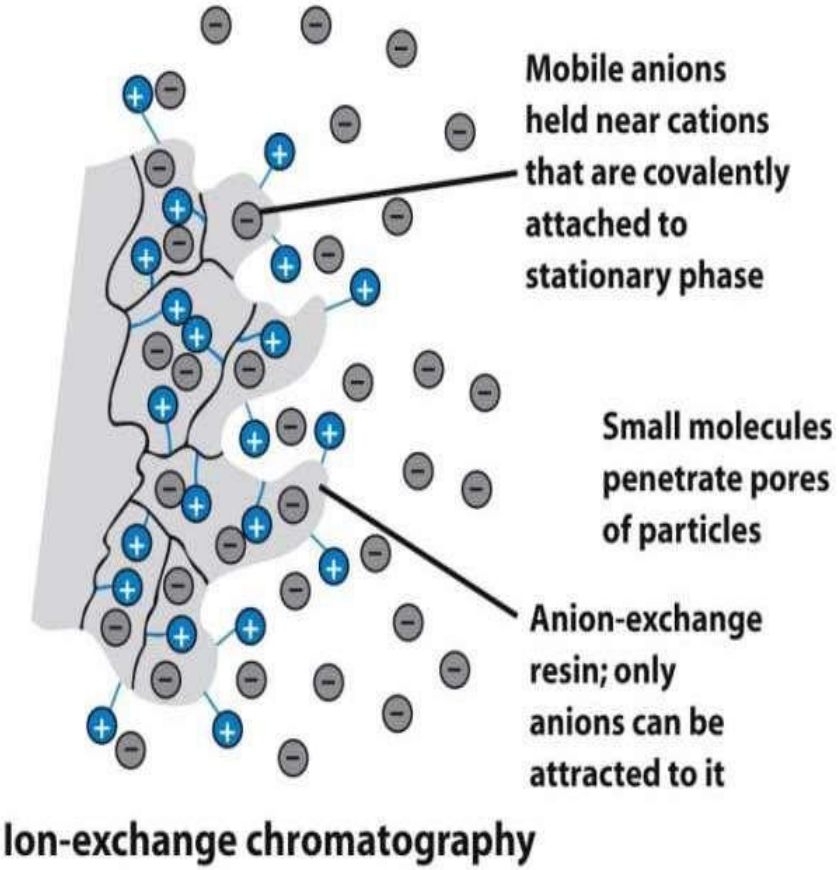
- Based on thin film formed on the surface of a solid support by a liquid stationary phase
- Solute equilibrates between mobile phase and stationary liquid phase



Partition chromatography

ION EXCHANGE CHROMATOGRAPHY

- Resin(stationary phase) used to covalently attach anions or cations onto it
- Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces
- Ion exchange mechanism separates analytes based on their respective charges



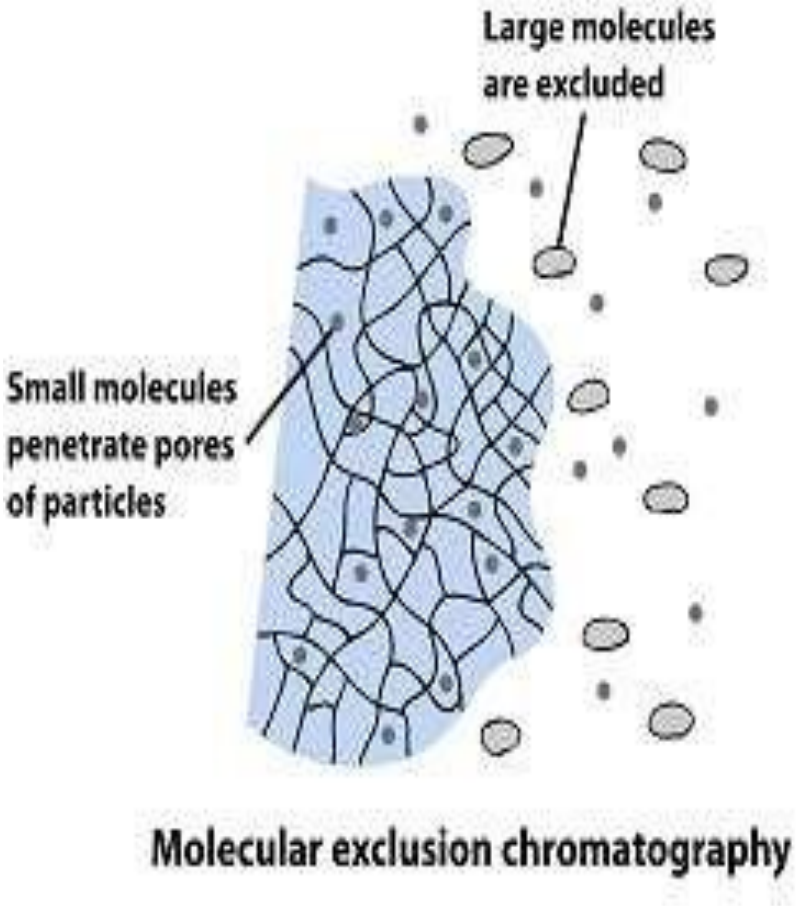
Ion-exchange chromatography

MECHANISM

- To optimize binding of all charged molecules, the mobile Phase is generally a low to medium conductivity (i.e., low to medium salt concentration) solution.
- Adsorption of the molecules to the solid support is driven by the ionic interaction between the oppositely charged ionic groups in the sample molecule and in the functional ligand on the support.
- The strength of the interaction is determined by the number and location of the charges on the molecule and on the functional group.
- By increasing the salt concentration the molecules with the weakest ionic interactions start to elute from the column first.
- Molecules that have a stronger ionic interaction require a higher salt concentration and elute later in the gradient.
- The binding capacities of ion exchange resins are generally quite high.

MOLECULAR EXCLUSION CHROMATOGRAPHY

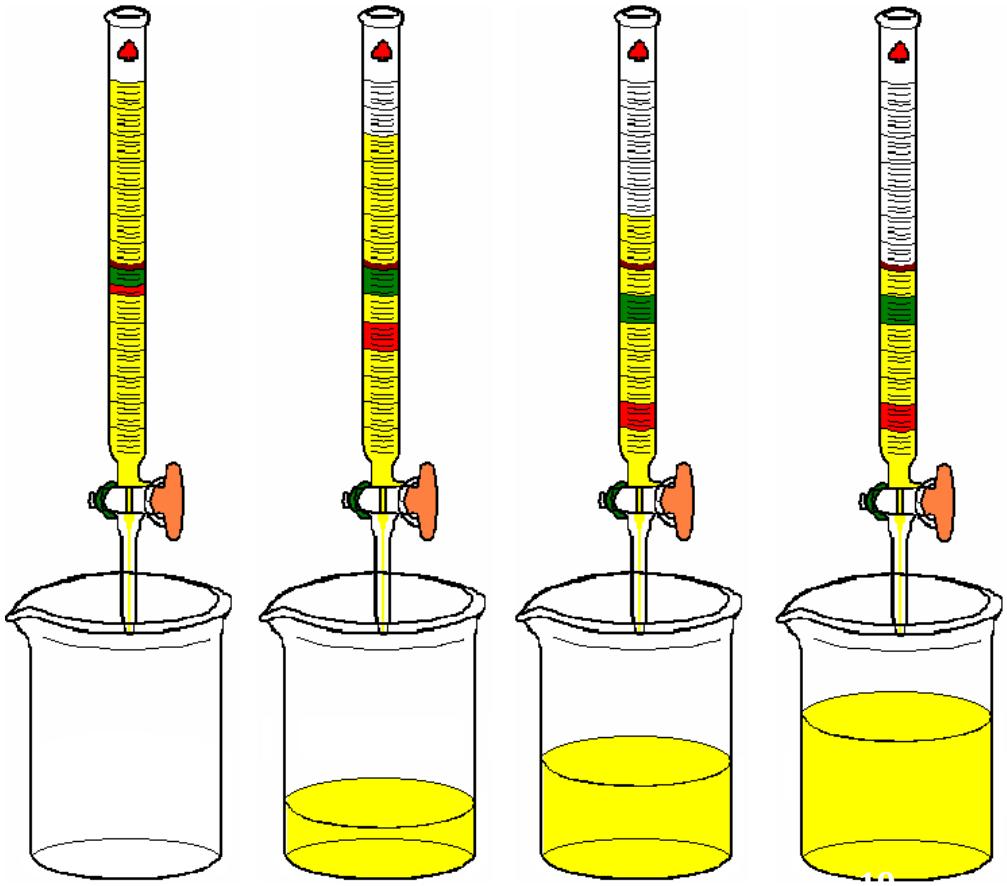
- Also known as gel permeation or gel filtration chromatography
- Lacks attractive interaction between solute and stationary phase



- Liquid or gaseous phase passes a porous gel which separates the molecule according to its size
- The pores are normally small and exclude the larger solute molecules, but allows the smaller molecules to enter the gel, causing them to flow through a larger volume. This causes the larger molecules to pass through column at faster rate than smaller ones
- It is generally low resolution technique and thus it is often reserved for the final “polishing” step of a purification

Column Chromatography

Stationary phase is held in a narrow tube through which the mobile phase is forced under pressure or under the effect of gravity



Factors affecting solutes separation in CC
(Factors affecting column efficiency)

Factor	Effect
Particle size of solid stationary phase (or of support)	Decrease of size improves separation (but very small particles need high pressure).
Column dimensions	Efficiency increases as ratio length / width increases.
Uniformity of packing	Non uniform packing results in irregular movement of solutes through column & less uniform zone formation, (i.e. band broadning or tailing).
Column temperature	Increase in column temperature results in speed of elution but does not improve separation (tailing).
Eluting solvent	Solvents should be of low viscosity (to give efficient resolution) & h igh volatility (to get rapid recovery of the substances).
Solvent flow rate	Uniform & low flow rate gives better resolution.
Continuity of flow	Discontinuous flow disturbs resolution
Condition of adsorbent	Deactivation of adsorbent decreases separation.
Concentration of solutes	Substances of high concentration move slowly.

Elution techniques

Technique	Procedure
Isocratic elution	**Addition of solvent mixture of fixed composition during the whole process.**
Gradient elution	**<u>Continuous or linear elution</u>: in which there is continuous change in the composition of the mobile phase over a period of time (e.g. polarity, pH or ionic strength).**
	<u>Step wise or fractional elution</u>: in which the change is not continuous i.e. a sudden change in the composition of the mobile phase is followed by a period where the mobile phase is held constant.

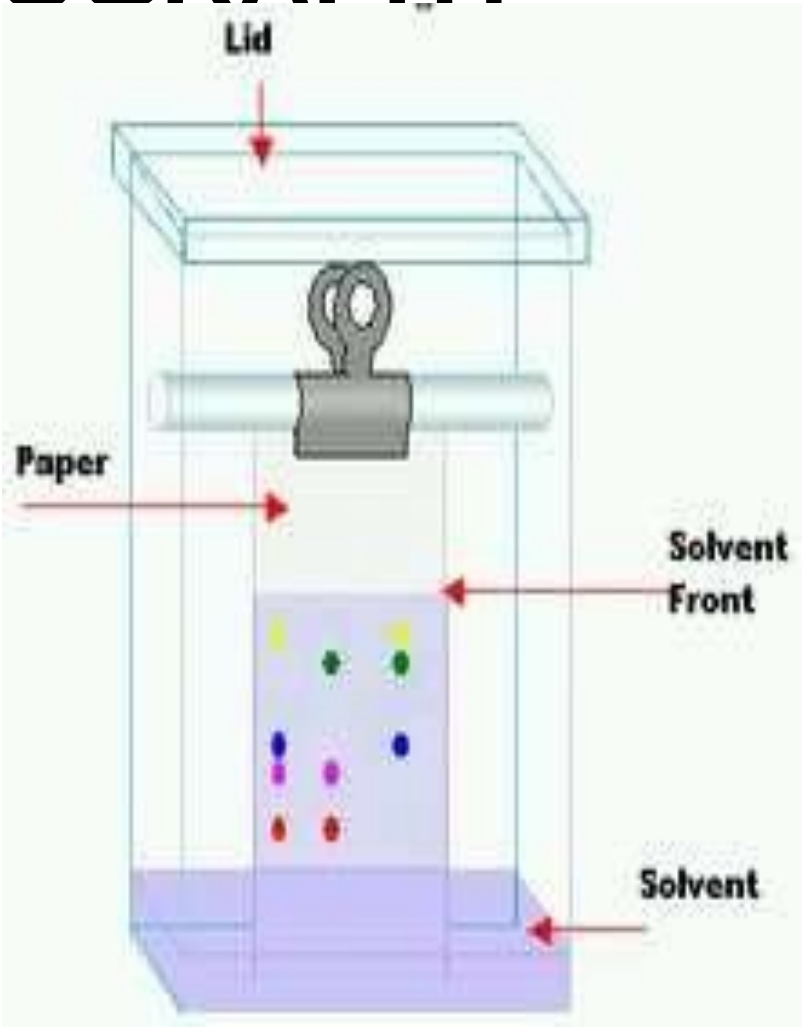
PAPER CHROMATOGRAPHY

Uses filter paper strips as carrier or inert support.

The factor governing separation of mixtures of solutes on filter paper is the partition between two immiscible phases.

One is usually water adsorbed on cellulose fibres in the paper (stationary phase).

The second is the organic solvent flows past the sample on the paper (stationary phase).



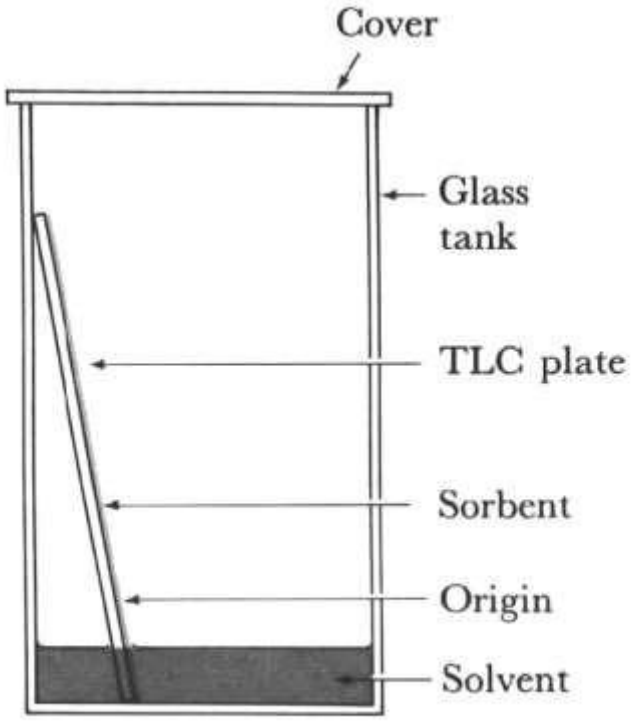
PAPER CHROMATOGRAPHY

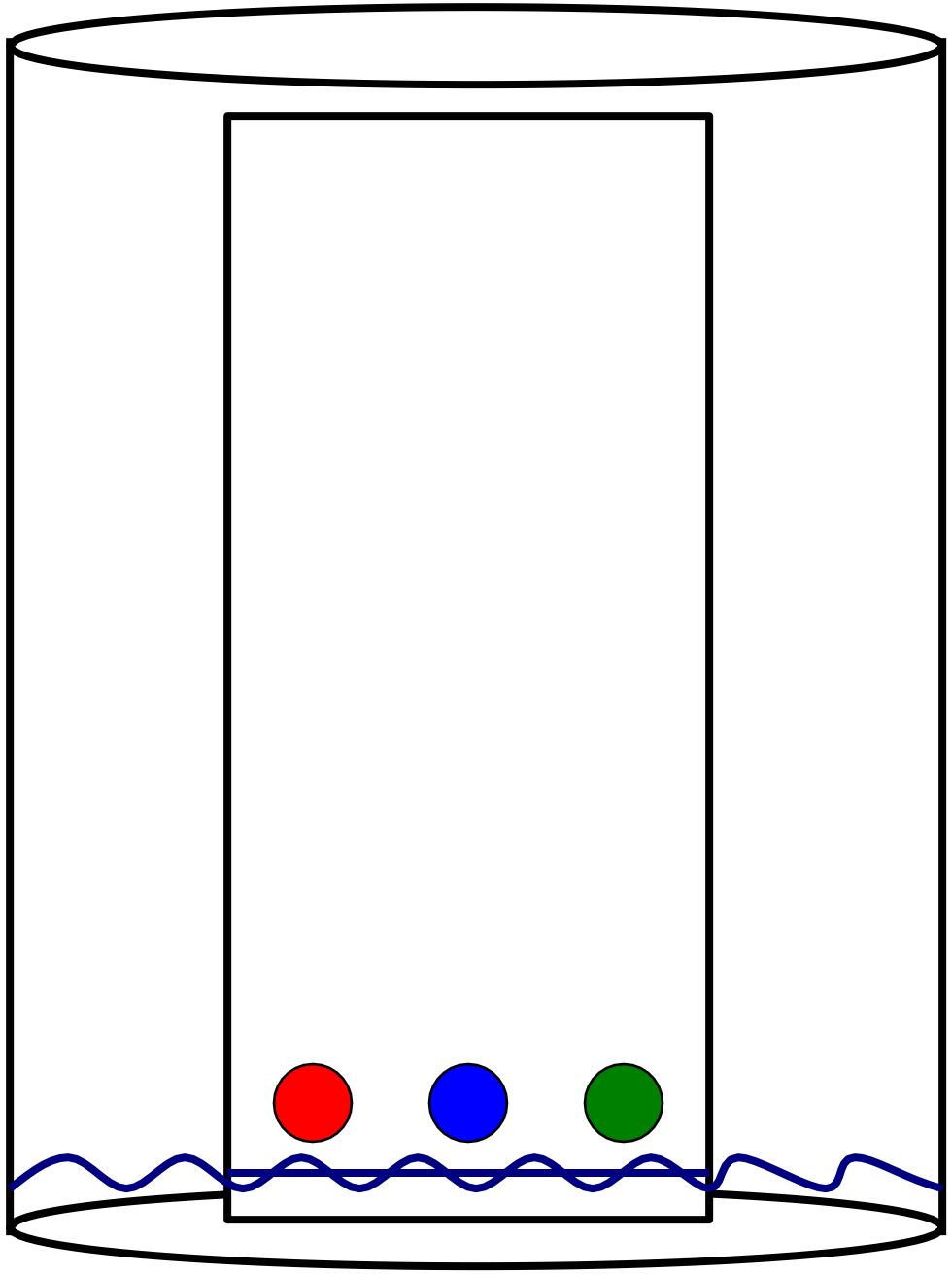
- a small dot or line of sample solution is placed onto a strip of chromatography paper
- The paper is placed in a jar containing a shallow layer of solvent and sealed
- As the solvent rises through the paper, it meets the sample mixture which starts to travel up the paper with the solvent.
- This paper is made of cellulose, a polar substance,
- and the compounds within the mixture travel farther if they are non-polar

More polar substances bond with the cellulose paper more quickly, and therefore do not travel as far

THIN LAYER CHROMATOGRAPHY (TLC)

Involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or cellulose on a flat, inert substrate





HPTLC

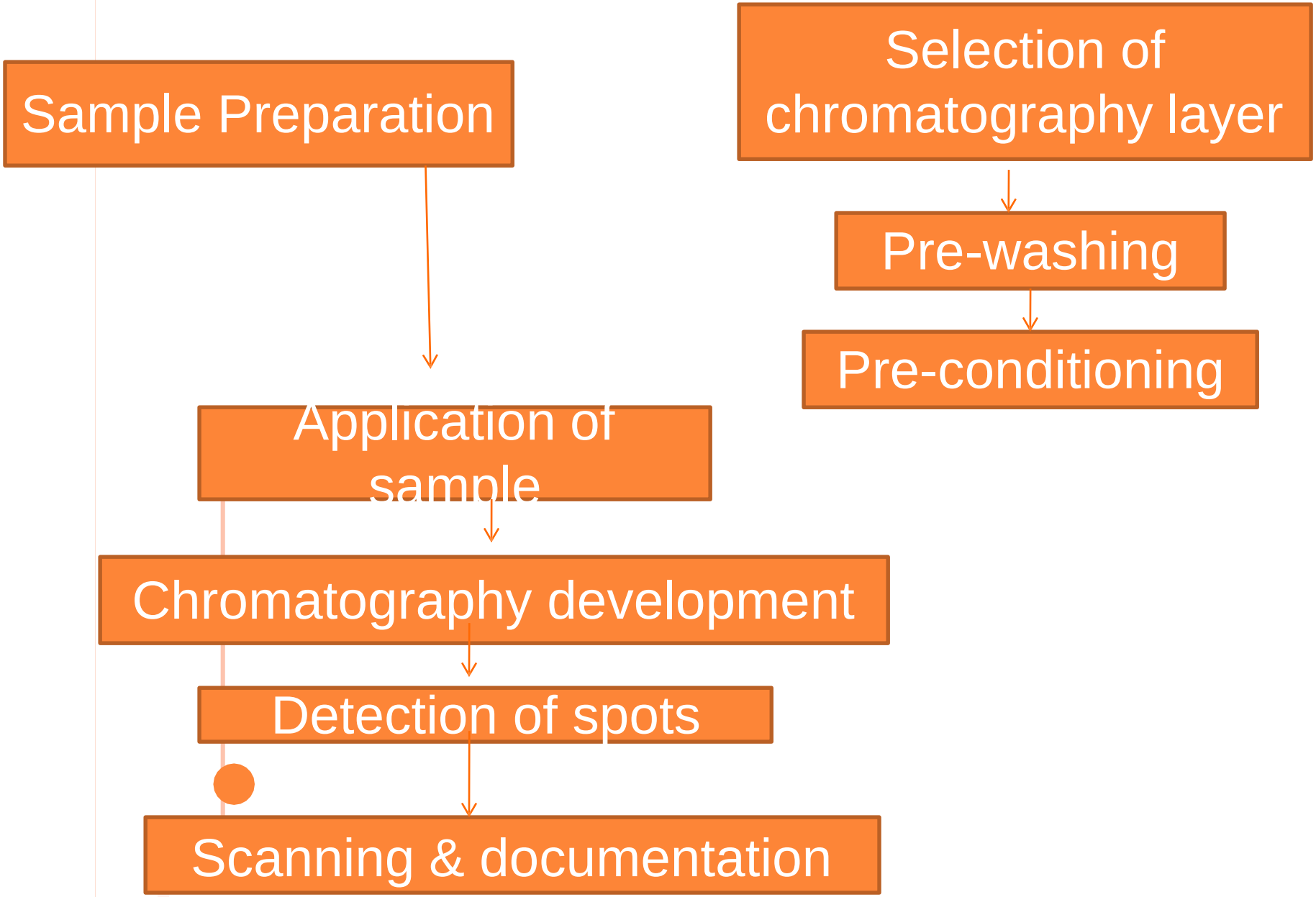
- Sophisticated form of thin layer chromatography. It involves the same theoretical principle of thin layer chromatography.
- It is also known as planar chromatography or Flat-bed chromatography.
- Traditional Thin Layer Chromatography & its modern instrumental quantitative analysis version HPTLC are very popular for many reasons such as
  - visual chromatogram
  - simplicity
  - multiple sample handling
  - low running and maintenance costs, disposable layer etc.

PRINCIPLE

- HPTLC have similar approach and employ the same physical principles of TLC (adsorption chromatography) i.e. the principle of separation is adsorption.
- Solvent flows through because of capillary action
- Components move according to their affinities towards the adsorbent Component with more affinity towards the stationary phase travels slower
- Component with lesser affinity towards the stationary phase travels faster

Thus the components are separated on a chromatographic plate

STEPS INVOLVING IN HPTLC



Pre washing of pre coated plates

Main purpose of the pre-washing is to remove impurities which include water vapours and other volatile substances from the atmosphere when they get exposed in the lab environment.

Silica gel 60F is most widely used sorbent

Major disadvantage of this sorbent is that it contain iron as impurity

This iron is removed by using Methanol : water in the ratio of 9:1.This is the major advantage of the step of pre-washing.

ACTIVATION OF PLATES

- Freshly opened box of HPTLC plates doesn't need activation.
- Plates exposed to high humidity or kept in hand for long time require activation.
- Plates are placed in oven at 110^{o}-120^{o}c for 30 min prior to the sample application.

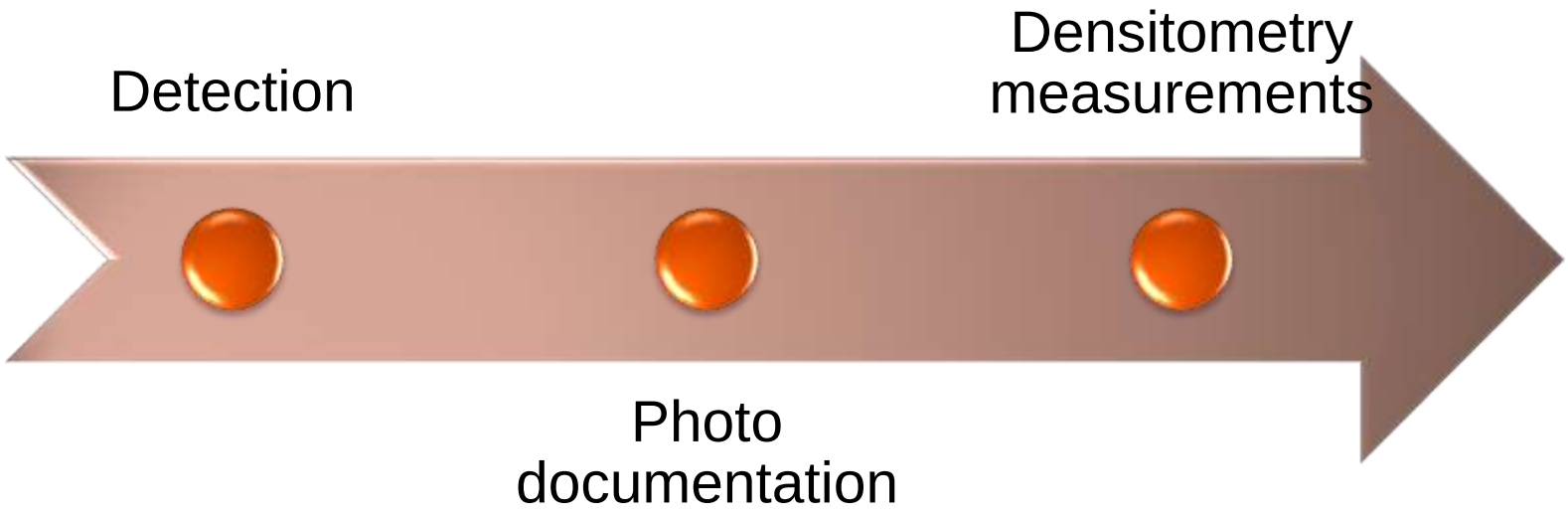
PRE-CONDITIONING

- Also called Chamber Saturation
- Un- saturated chamber causes high Rf values

Sample application

- Usual concentration range is 0.1-1µg / µl
  Above this causes poor separation
- sample and standard application from syringe on TLC plates as bands
- Band wise application - better separation

POST CHROMATOGRAPHY STEPS



DETECTION

Detection under UV light is first choice - non destructive

-Spots of fluorescent compounds can be seen at 254 nm (short wave length) or at 366 nm (long wave length)

-Spots of non fluorescent compounds can be seen - fluorescent stationary phase is used - silica gel GF

UV CABINET

DENSITOMETRY MEASUREMENTS

- Measures visible, UV absorbance or Fluorescence.
- Convert the spot/band into chromatogram consisting of peaks

Instrumentation of HPTLC consists of following:

- Lamp selector
- Entrance lens slit
- Monochromator entry slit
- Grating
- Mirror
- Slit aperture disc
- Mirror
- Beam splitter
- Reference photo multiplier
- Measuring photo multiplier
- Photo diode for transmission measurements.

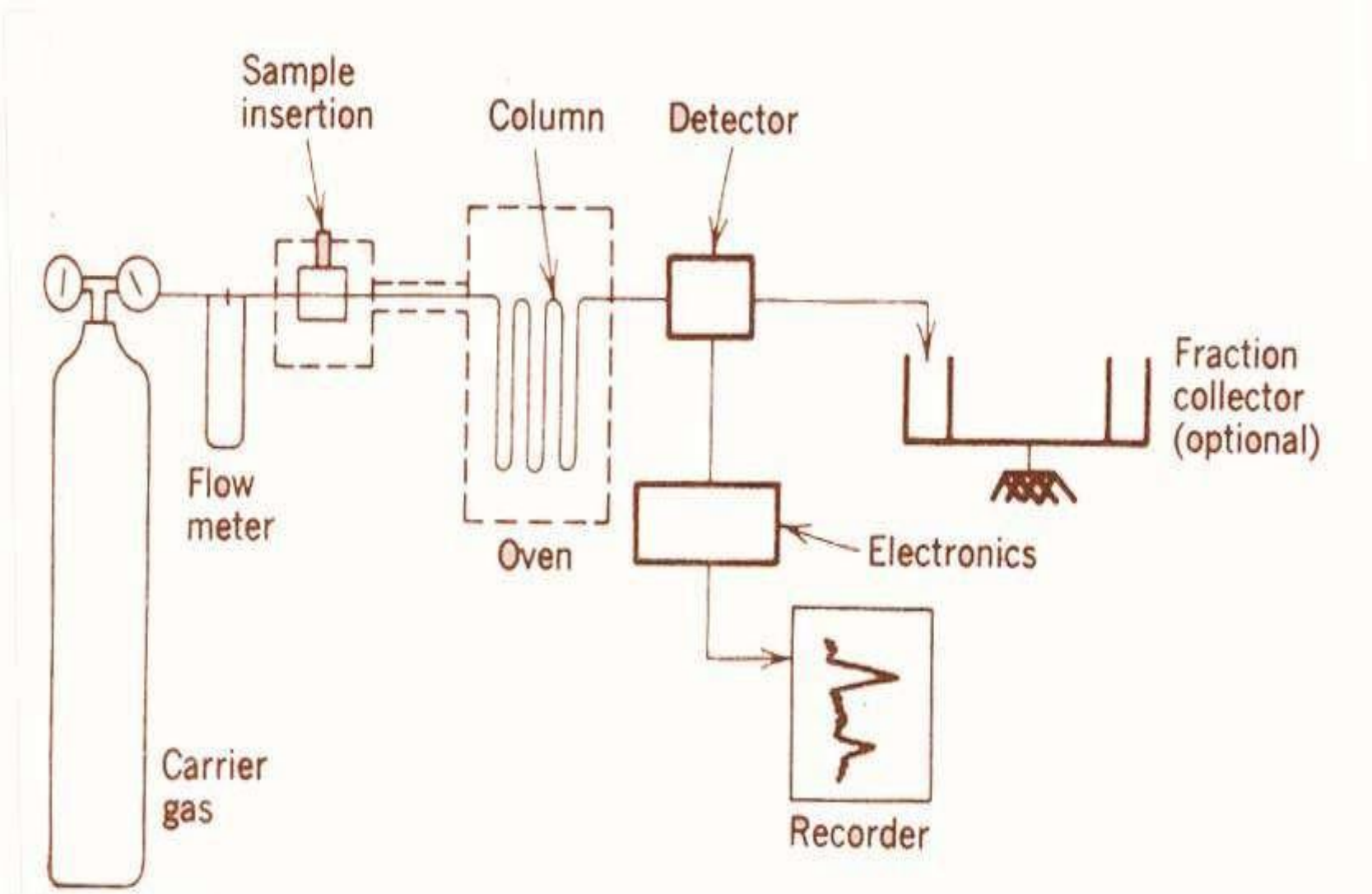
DIFFERENCES BETWEEN TLC AND HPTLC

Parameter	TLC *Qualitative*	HPTLC *Quantitative*
Chromatographic plate used	Hand made /pre-coated	Pre-coated
Sorbent layer thickness	250 µm	100-200µm
Particle size range	5-20µm	4-8µm
Pre-washing of the plate	Not followed	Must
Application of sample	Manual/Semi automatic	Semi automatic/Automatic
Shape	Spot	Spot/Band
Spot size	2-4mm	0.5-1mm
Sample volume	1-10µl	0.2-5µl
Application of larger volume	Spotting which leads to over loading	Can be applied as bands
No. of samples/plate (20X20)	15-20	40-50
Optimum development distance	10-15 cm	5-7 cm
Development time	Depends on mobile phase	40% Less than TLC
Reproducibility of results	Difficult	Reproducible

GAS CHROMATOGRAPHY (GC)

- Also called as Gas liquid chromatography (GLC)
- based on a partition equilibrium of analyte between a solid stationary phase (often a liquid silicone-based material) and a mobile gas (most often Helium).
- The stationary phase is adhered to the inside of a small-diameter glass tube (a capillary column) or a solid matrix inside a larger metal tube (a packed column).

- high temperatures used in GC make it unsuitable for high molecular weight biopolymers or proteins (heat will denature them)
- Well suited for use in the petrochemical, environmental monitoring, and industrial chemical fields. It is also used extensively in chemistry research.
- Here the mobile phase is an unreactive gas (e.g. Nitrogen) flowing through a tube.
- And the stationary phase is an involatile liquid held on particles of a solid support.


Sample insertion
Column
Detector
Fraction collector (optional)
Flow meter
Oven
Electronics
Carrier gas
Recorder

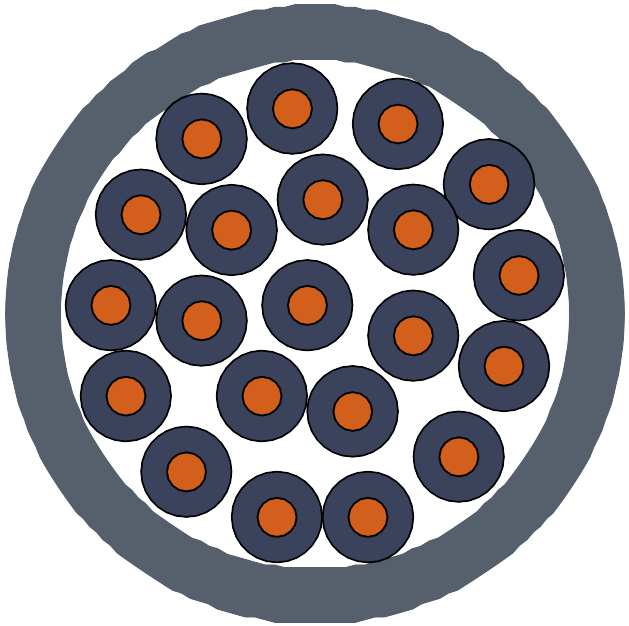
INSTRUMENTATION:

- Mobile Phase: He, Ar, N_2, H_2
- Flow regulators & flow meters:
- Injection Port: Rubber septum barrier (usually maintained at a higher temperature than the boiling point of the least volatile component in the sample mixture)
- Column: (fused silica with a thin coating of stationary phase on the inner surface)
- Oven: Thermostat controlled forced air oven
- Detector:
- Data System: recorders & integrators

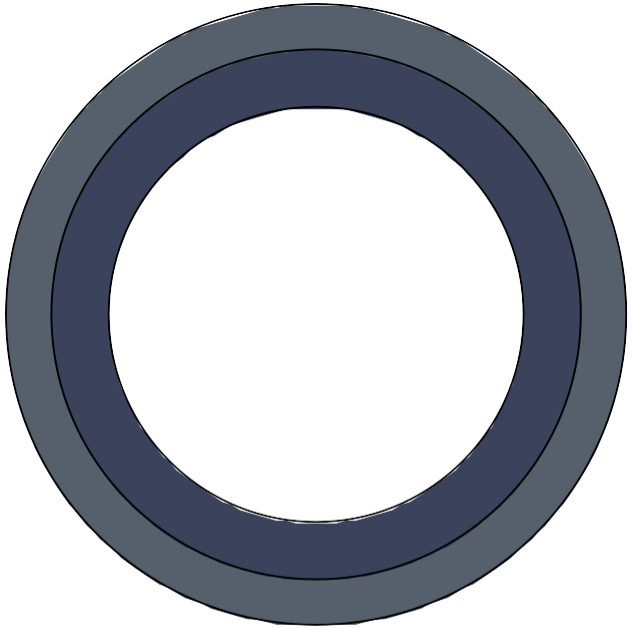
COLUMNS:

Columns in GC are two types

1) packed column
2) capillary column



Packed column



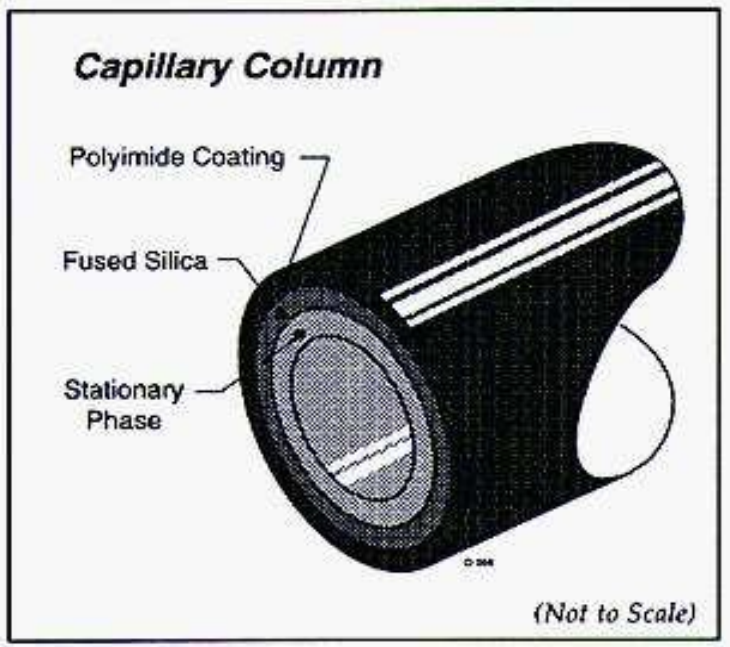
Open tubular column (capillary column)

Packed column:

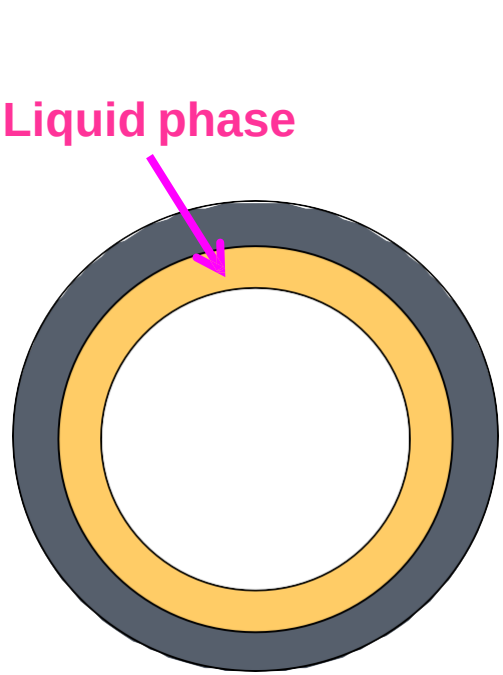
Glass or metals
2-3 m long, 2-4 mm I'd.
Densely packed with packing materials or solid support coated with thin layer of stationary liquid phase
Diatomaceous earth
Size: 60-80 mesh (250-170 μm) or 80-100 mesh (170-149 μm)

Open tubular column

Better resolution – efficient mass transfer between gas and SP
Tubing – fused silica, glass, copper, stainless steel

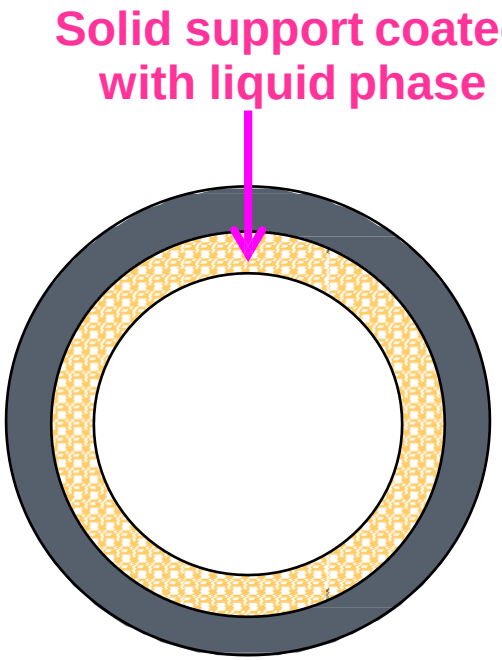


Types of open tubular column:



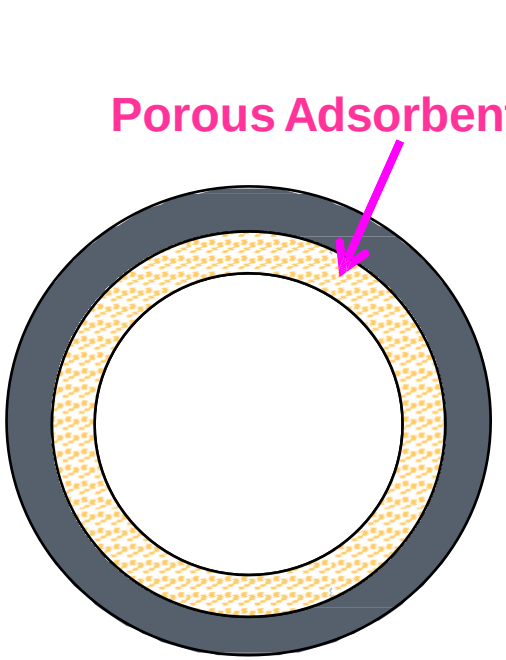
Wall-coated Open Tubular

(WCOT)



Support-coated Open Tubular

(SCOT)



Porous Layer Open Tubular

(PLOT)

GC DETECTORS

- TCD (thermal conductivity detector)
- FID (Flame ionization Detector)
- MSD (Mass Selective Detector)
- ECD (Electron Capture Detector)
- NPD (Nitrogen Phosphorus Detector)
- FTIRD (Fourier transformation infrared detector)
- AED (Atomic emission detector)

GC-MS

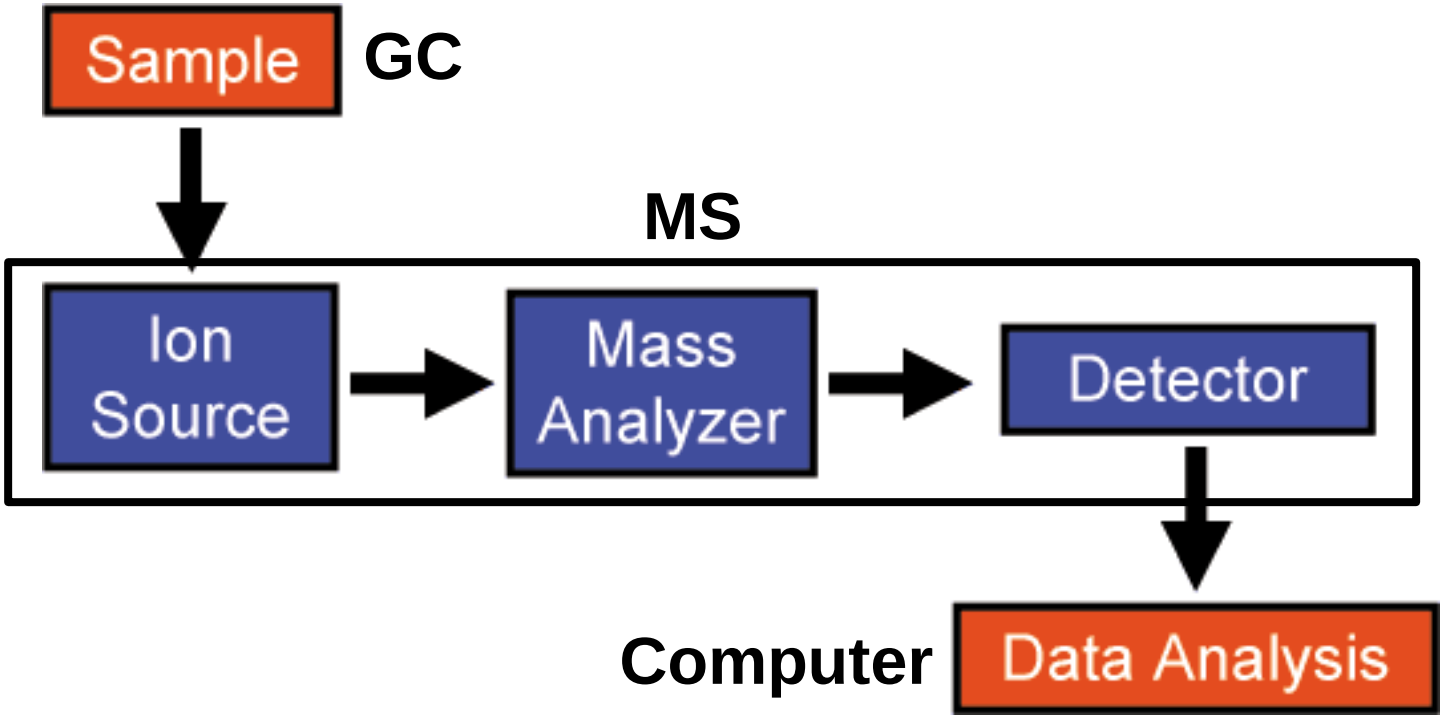
- Gas chromatography-Mass spectroscopy (GC-MS) is one of the so-called hyphenated analytical technique
- As the name implies, it is actually two techniques that are combined to form a single method of analyzing mixtures of chemicals
- Gas chromatography is a technique capable of separating, detecting and partially characterizing the organic compounds particularly when present in small quantity.
- Mass spectroscopy provides some definite structural information from in small quantity.

- Combination of GC-MS provides extremely powerful tool because it permits direct and effectively continuous correlation of chromatographic and mass spectroscopic properties
- The separation and identification of the components of complex natural and synthetic mixture are achieved more quickly than any other technique with less sample

PRINCIPLE OF GC-MS

- The sample solution is injected into the GC inlet where it is vaporized and swept onto a chromatographic column by the carrier gas (usually helium).
- The sample flows through the column and the compounds comprising the mixture of interest are separated by virtue of their relative interaction with the coating of the column (stationary phase) and the carrier gas (mobile phase).
- The latter part of the column passes through a heated transfer line and ends at the entrance to ion source where compounds eluting from the column are converted to ions.

GC-MS


Sample
GC
MS
Ion Source
Mass Analyzer
Detector
Computer
Data Analysis

GC-MS INSTRUMENT

The insides of the GC-MS, with the column of the gas chromatograph in the oven on the right.

LIQUID CHROMATOGRAPHY

- separation technique in which the mobile phase is a liquid.
- can be carried out either in a column or a plane.
- Present day it utilizes very small packing particles and a relatively high pressure is referred as high performance liquid chromatography (HPLC).
- In the HPLC technique, the sample is forced through a column that is packed with irregularly or spherically shaped particles or a porous monolithic layer (stationary phase) by a liquid (mobile phase) at high pressure.

SUPERCRITICAL FLUIDCHROMATOGRAPHY

- Supercritical fluid chromatography is a separation technique in which the mobile phase is a fluid above and relatively close to its critical temperature and pressure

AFFINITY CHROMATOGRAPHY

Based on selective non-covalent interaction between an analyte and specific molecules

Stationary Phase

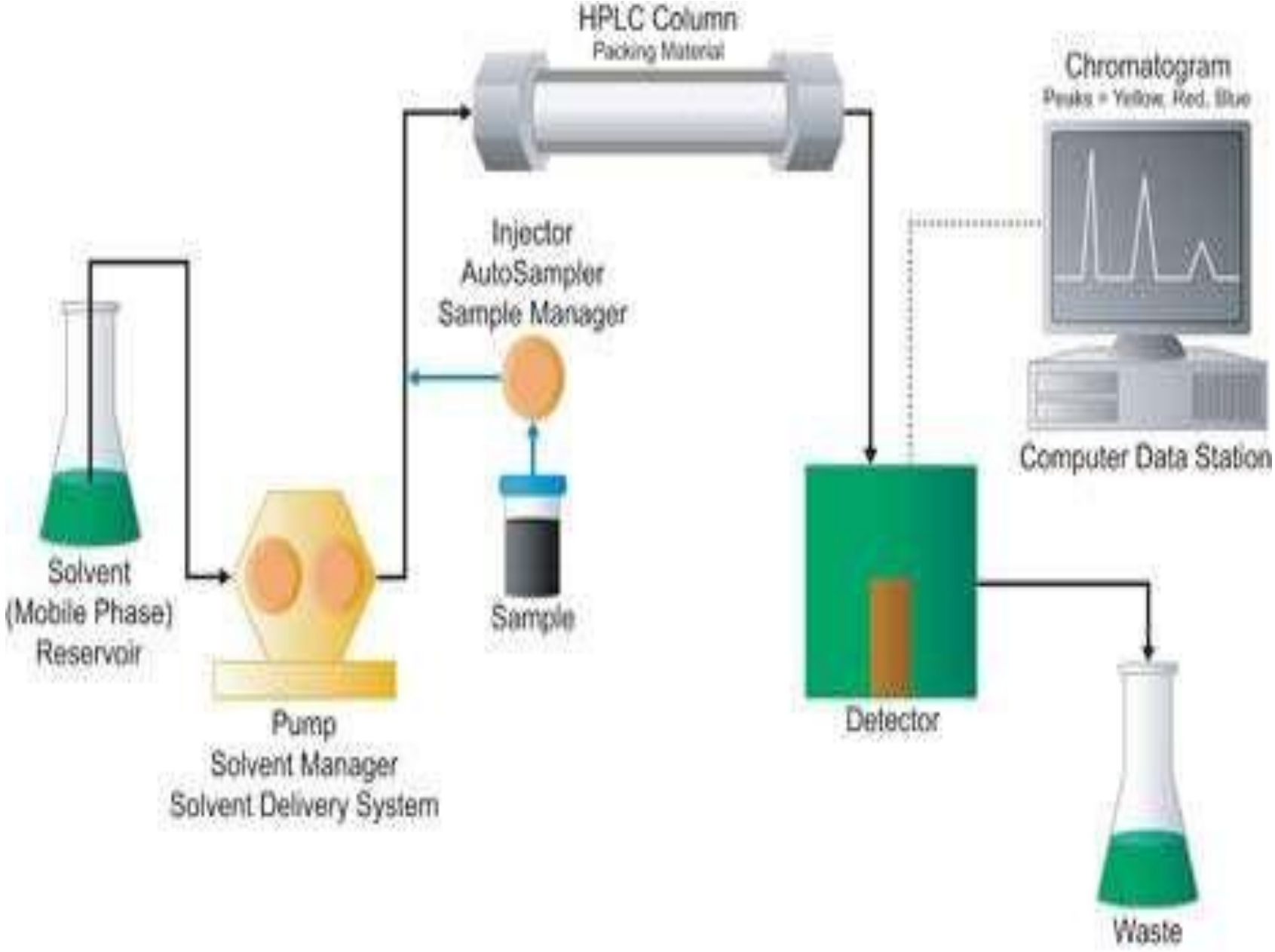
- Highly specific and selective
- Molecular recognition groups (affinity ligands) covalently attached to agarose or cellulose beads
- Affinity ligand binds selectively and reversibly the analyte
- Mechanism of retention: interaction with highly specialized molecular recognition systems which are attached to the stationary phase

Mobile Phase

- Two distinct roles
  – Support the strong binding of the analyte molecule to the ligand
  – Weaken and eliminate the analyte-ligand interaction

HPLC

- one of the most powerful tools in analytical chemistry
- has the ability to separate, identify, and quantitate the compounds that are present in any sample that can be dissolved in a liquid
- Similar to gas chromatography but uses a liquid mobile phase
- The stationary phase is usually an inert solid or a liquid held on the inert solid
- Mobile phase travels through the column forcibly with the aid of the high pressure pump
- Solutes of the sample separated on column and eluted with mobile phase
- The technique is applicable to thermally fragile samples, e.g. amino acids, proteins, nucleic acids, hydrocarbons, antibiotics, steroids, drugs, inorganic and may organic substances.


HPLC Column
Packing Material
Chromatogram
Peaks = Yellow, Red, Blue
Injector
AutoSampler
Sample Manager
Computer Data Station
Solvent
(Mobile Phase)
Reservoir
Sample
Pump
Solvent Manager
Solvent Delivery System
Detector
Waste

COLUMNS

- Stainless steel, or heavily walled glass tubing of 10-30 cm, capable of withstanding 6000 psi, 4 to 10 mm inside dia meter, 0.5 µm particles provide 40-60000 plates/meter.
- 1 - 4.6 mm dia, 3.5 mm particles, 3-5 cm length columns offer 1, 00,000 Plates/meter. Such columns have low solvent consumption and speed up the separations

GUARD COLUMN:

Filter and separate irreversibly bonding compounds.

The particles have similar composition to that of the analytical column but slightly larger particle size to minimize pressure drop.

Column thermostats must be capable of controlling ± 0.10 C. This is accomplished by using column heaters or water jackets

DETECTION METHODS

UV – Ultraviolet light--- most popular

– Lamp

– Grating/Lens - Wave length 190-350 nm

– FlowCell

– PhotoDiode - Differential Light Output

RI – Refractive Index

– Universal analyte detector

– Solvent must remain the same throughout separation

– VERY temperature sensitive

– Sometimes difficult to stabilize baseline

FD – Fluorescence-greater sensitivity, not so popular

– Excitation wavelength generates fluorescence emission at a higher wavelength

– Analytes must have fluorophore group---not very common

– Very sensitive and selective

MS – Mass Spectrometry

– Mass to charge ratio (m/z)

– Allows specific compound ID

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THANK YOU