ADVANCES IN CHROMATOGRAPHIC TECHNIQUES

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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….

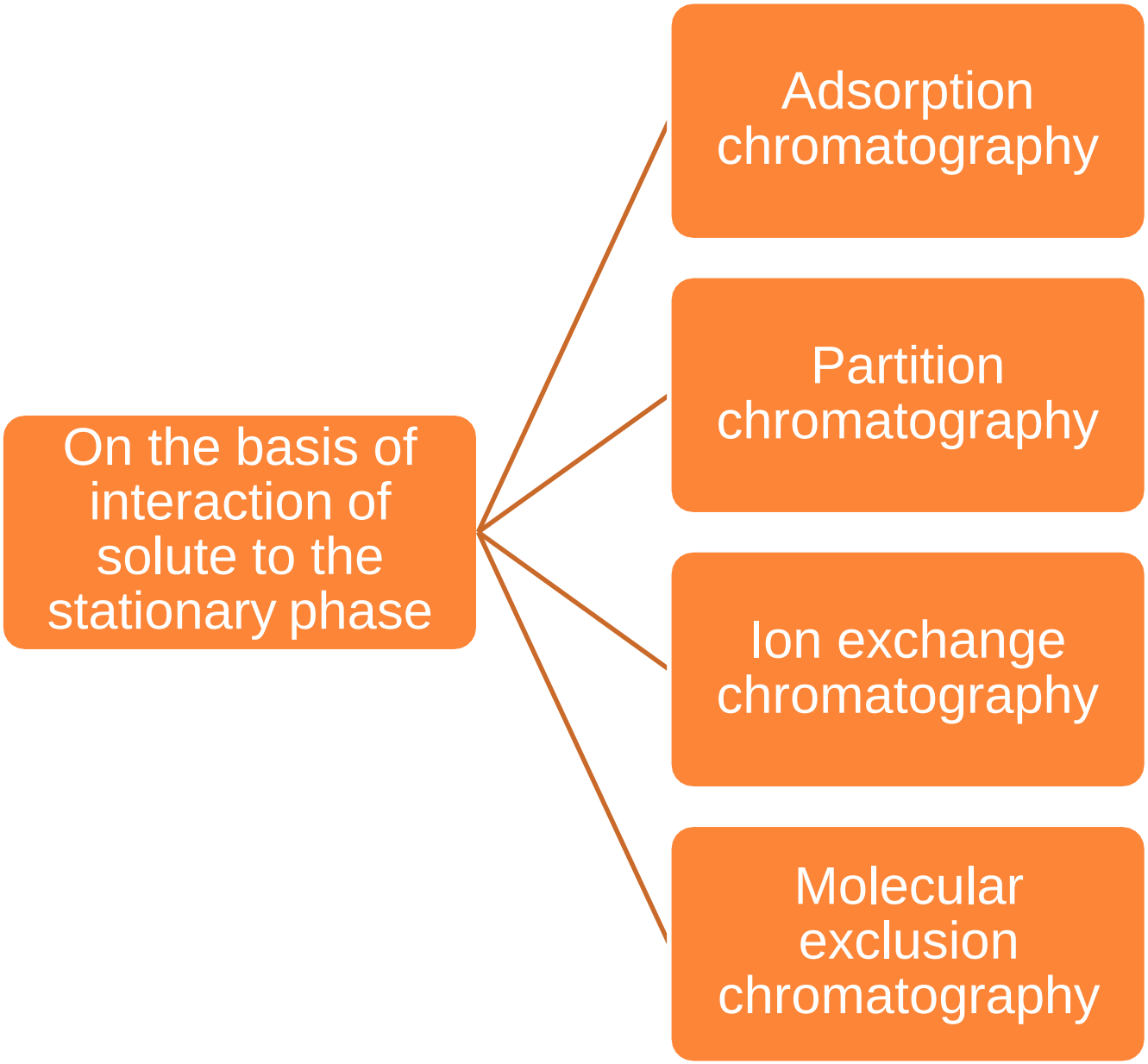
- **Adsorbtion:** Interaction of solute molecules with the surface of the stationary phase
- **Eluent:** The mobile phase
- **Elution:** Motion of the mobile phase through the stationary phase
- **Elution time:** The time taken for a solute to pass through the system. A solute with a short elution time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

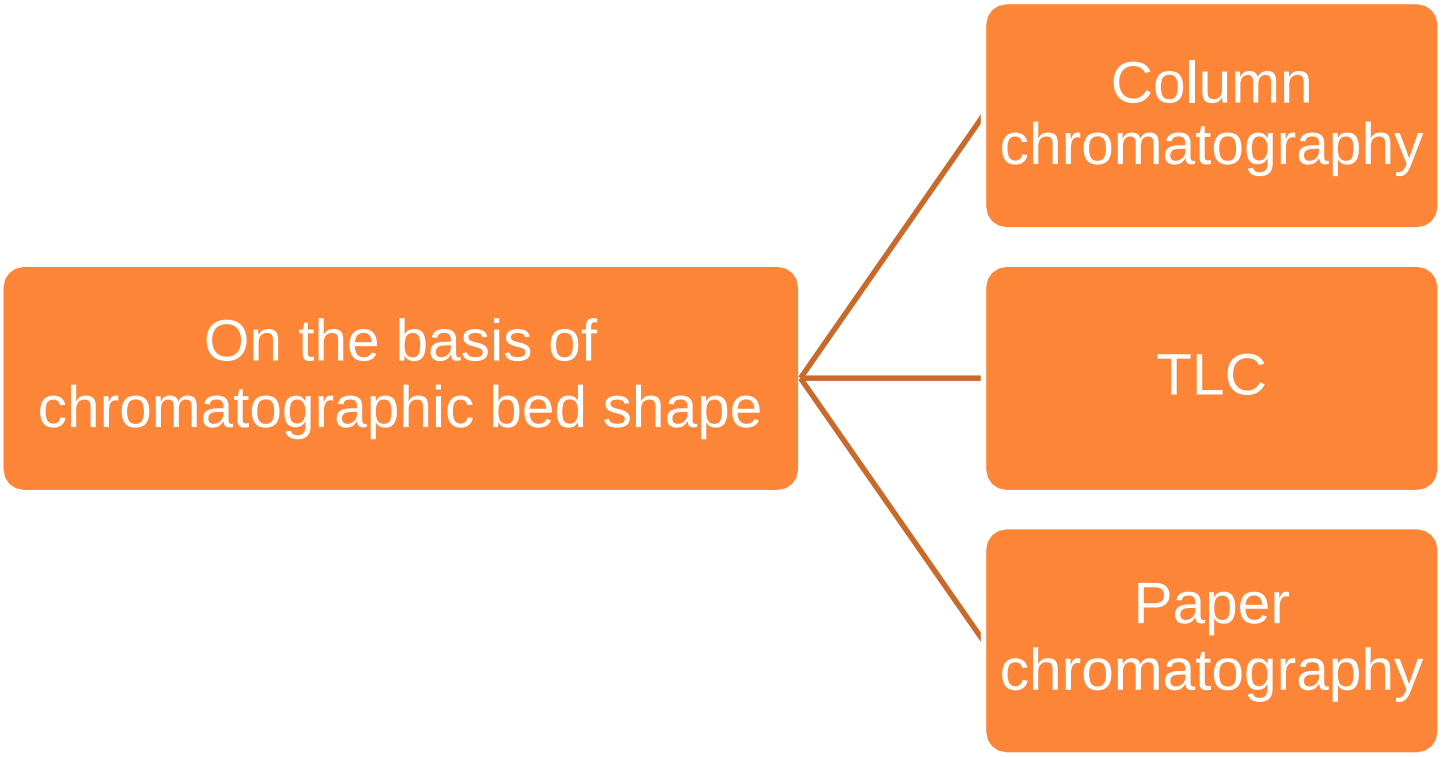
- **Normal phase:** “Unmodified” stationary phase where POLAR solutes interact strongly and run slowly
- **Reverse phase:** “Modified” stationary phase where POLAR solutes run fast *i.e. reverse order*
- **Resolution:** Degree of separation of different solutes. In principle, resolution can be improved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

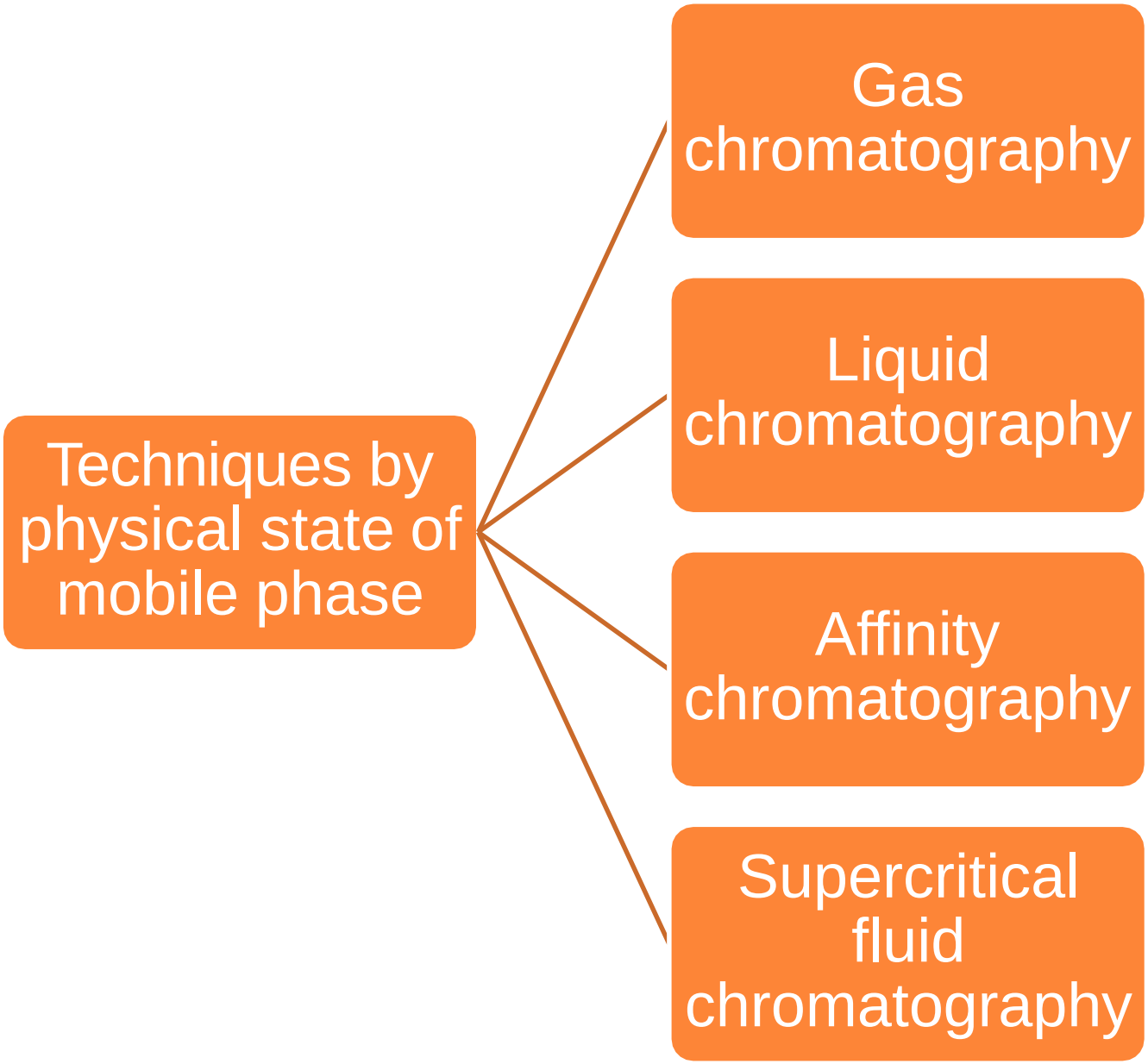
  distance travelled by solvent
- **Rf =** retardation factor. The Rf value has to be between 0 and 1, and it is typically reported to two decimal places.

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

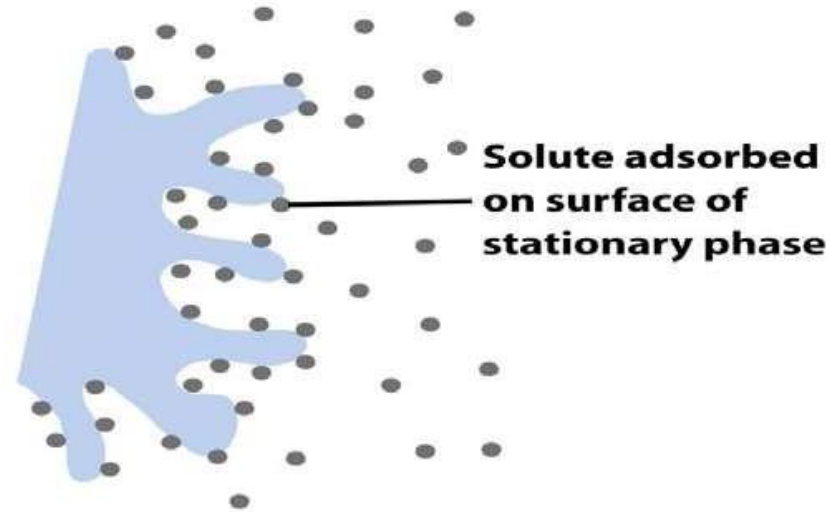

Techniques by physical state of mobile phase
Gas chromatography
Liquid chromatography
Affinity chromatography
Supercritical fluid chromatography

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

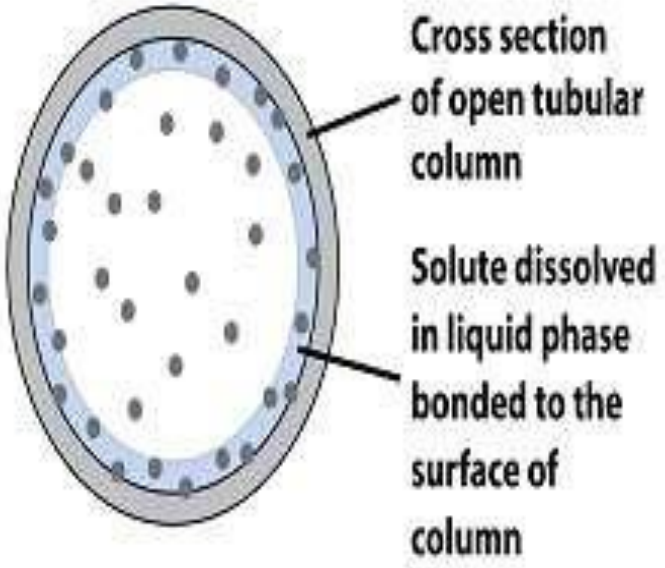
- One of the oldest type
- solute molecules bond directly to the surface of the stationary phase
- Stationary phases may contain a variety of adsorption sites differing in the tenacity with which they bind the molecules and in their relative abundance

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

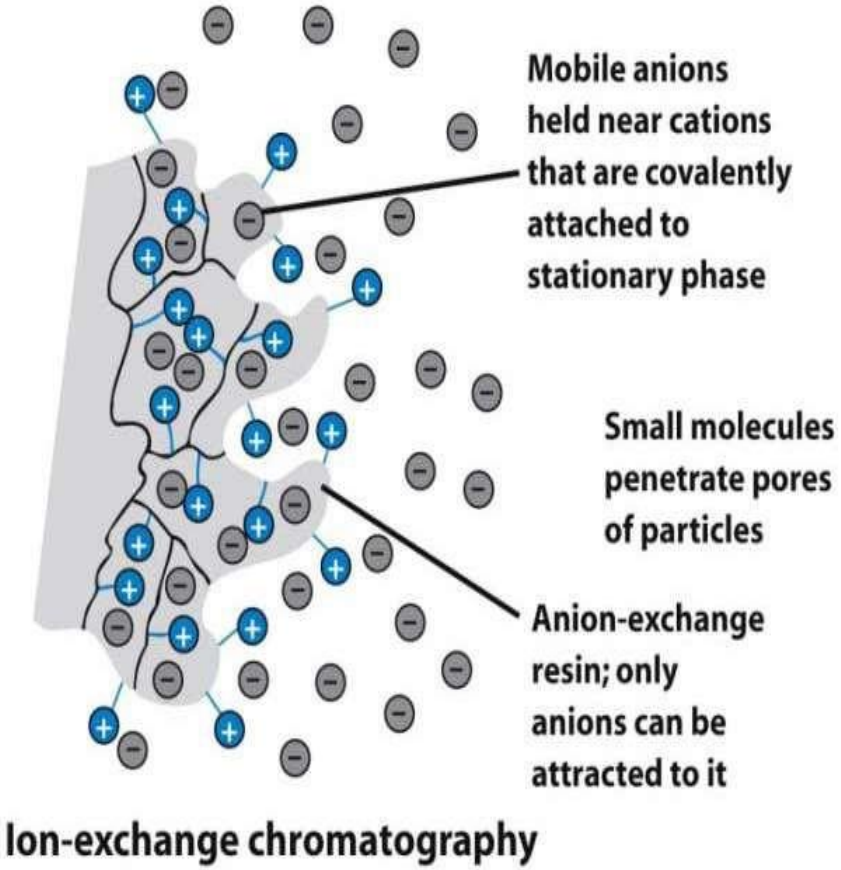
- Based on thin film formed on the surface of a solid support by a liquid stationary phase
- Solute equilibrates between mobile phase and stationary liquid phase



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

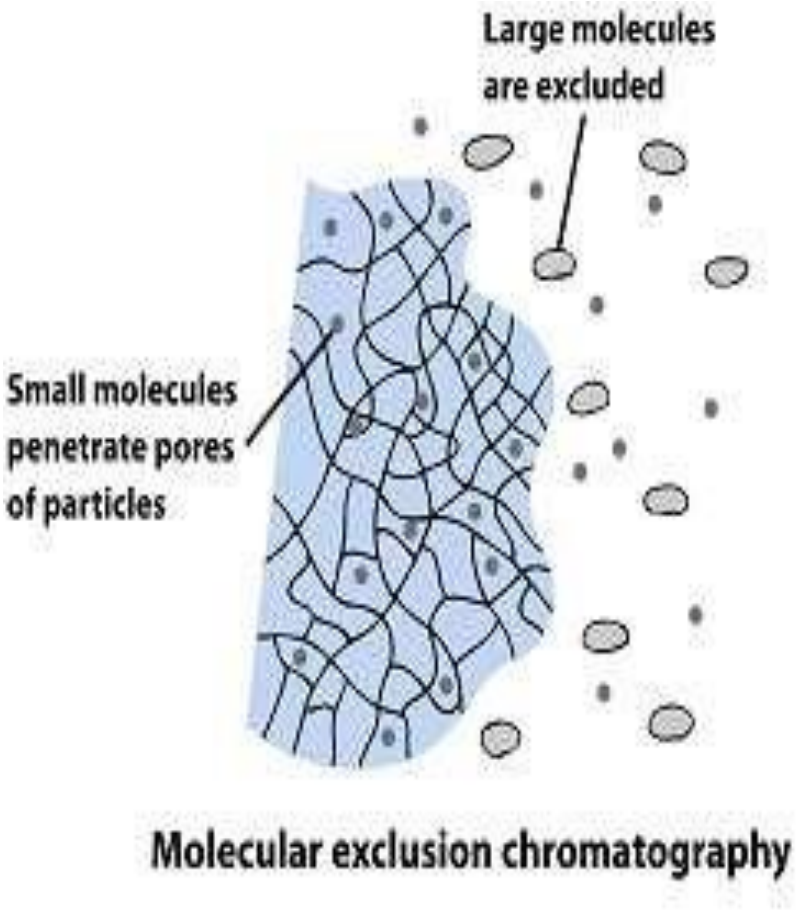


Mechanism

- 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

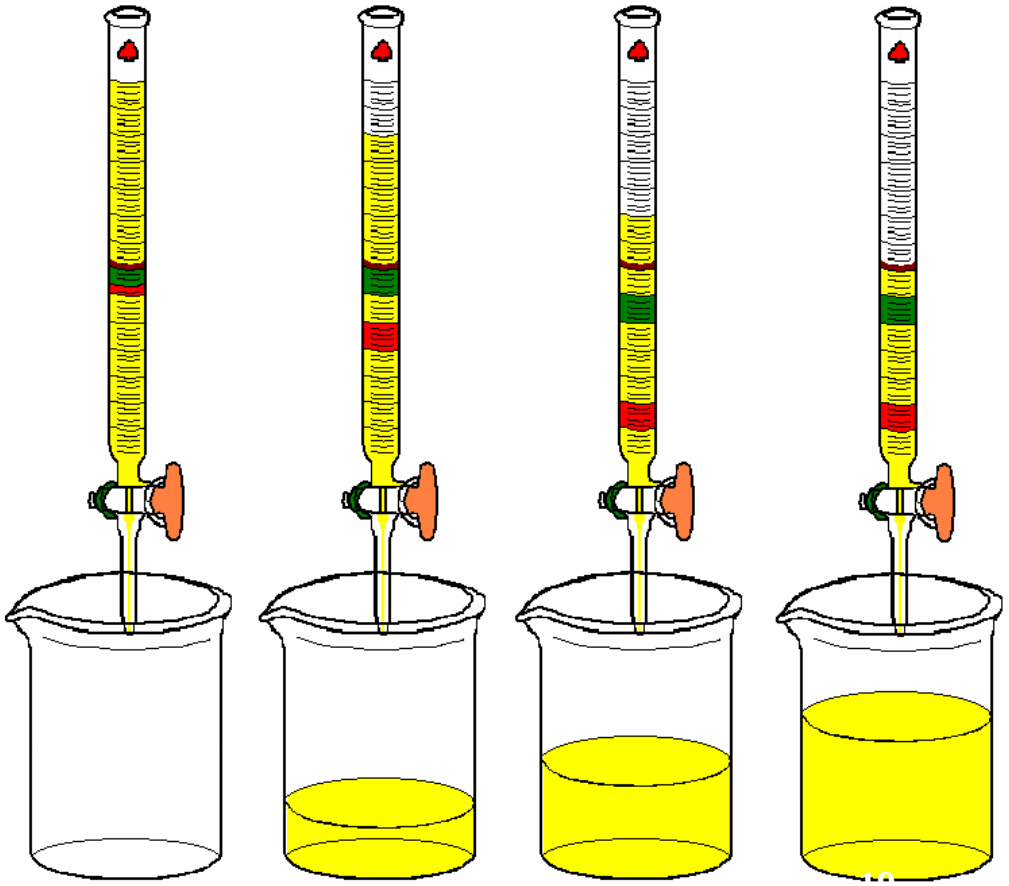
- Also known as gel permeation or gel filtration chromatography
- Lacks attractive interaction between solute and stationary phase



- 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.

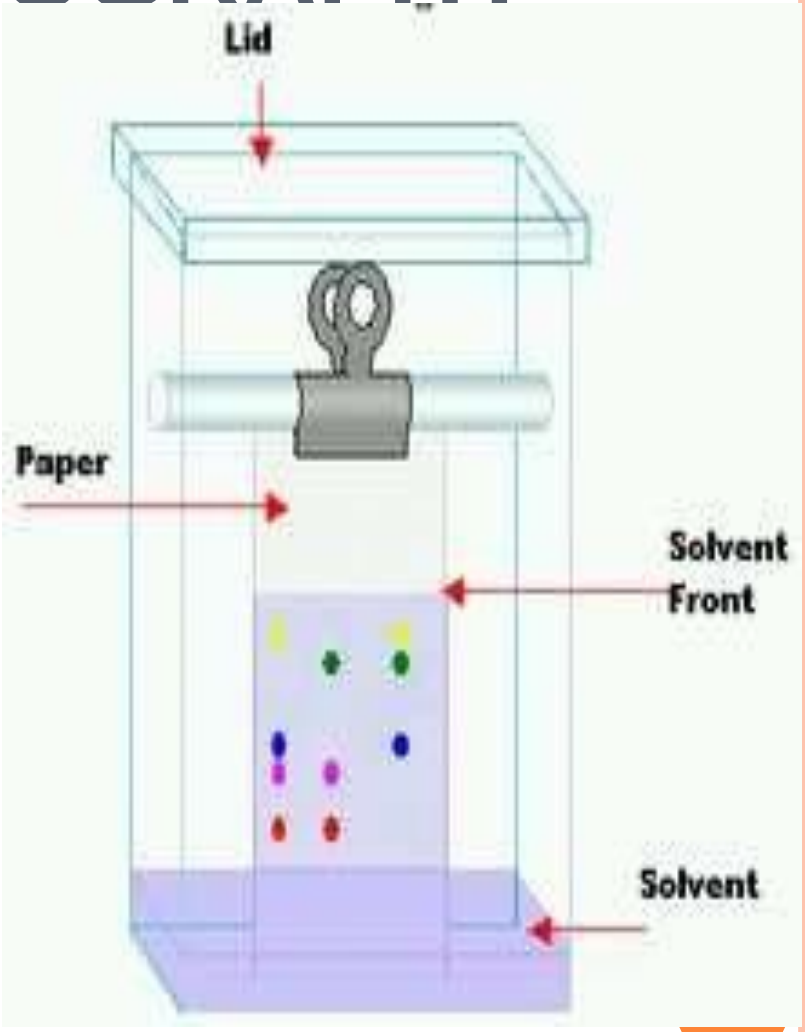
PAPER CHROMATOGRAPHY

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

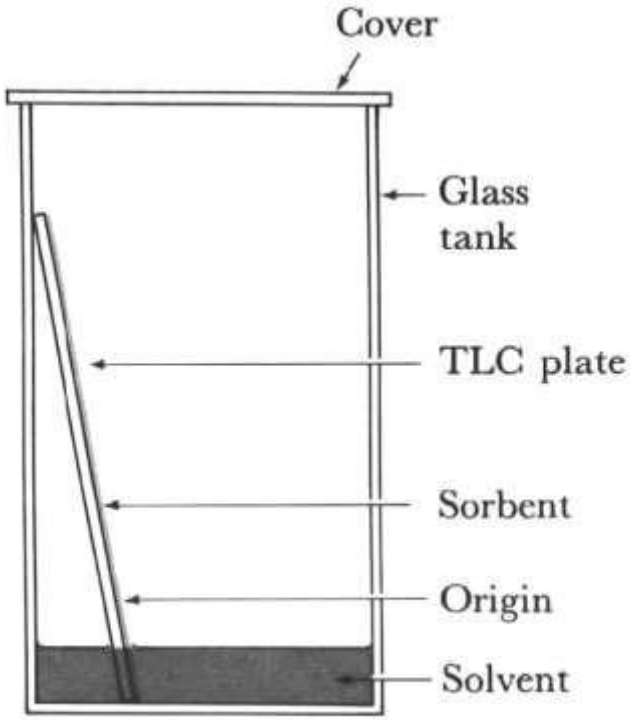
- a small dot or line of sample solution is placed onto a strip of chromatography paper
- The paper is placed in a jar containing a shallow layer of solvent and sealed
- As the solvent rises through the paper, it meets the sample mixture which starts to travel up the paper with the solvent.
- This paper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

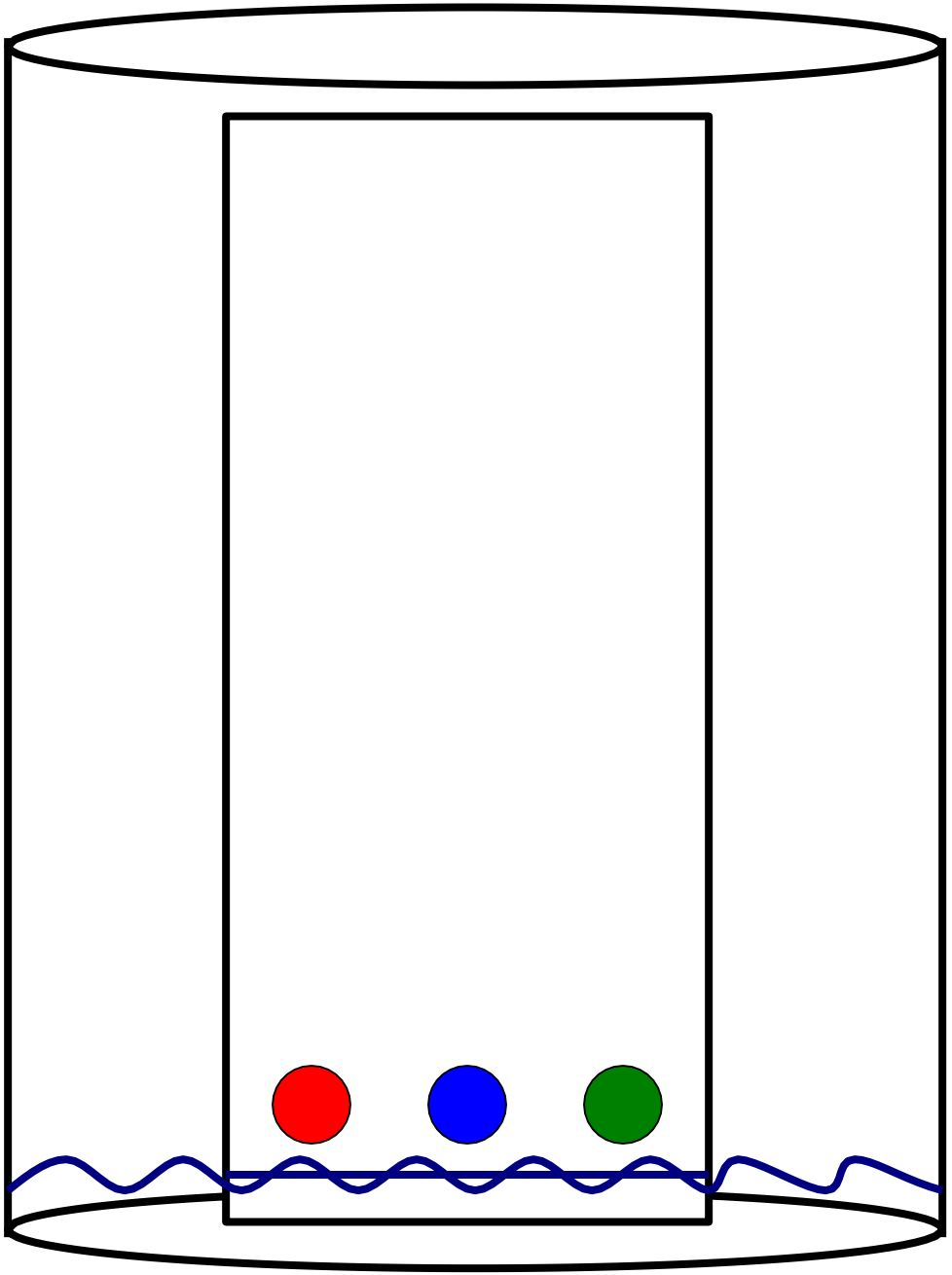
are non-polar

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

Sample Preparation

Selection of chromatography layer

Pre-washing

Pre-conditioning

Application of sample

Chromatography development

Detection of spots

Scanning & documentation

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.

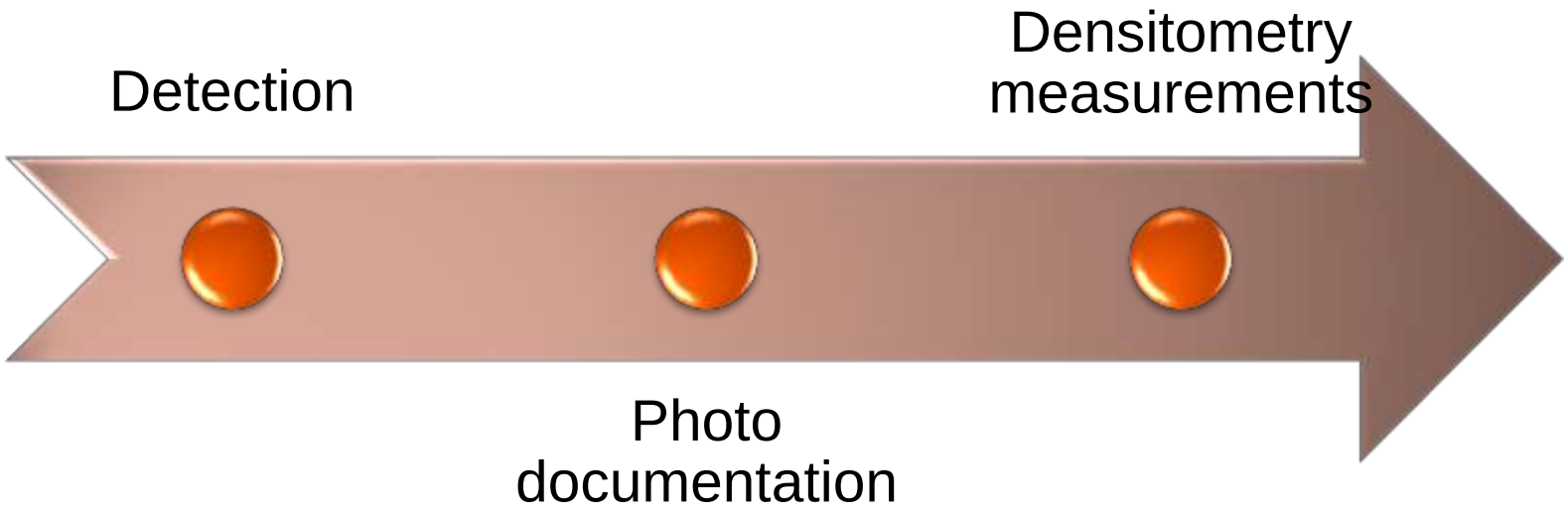
Pre-conditioning

- 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

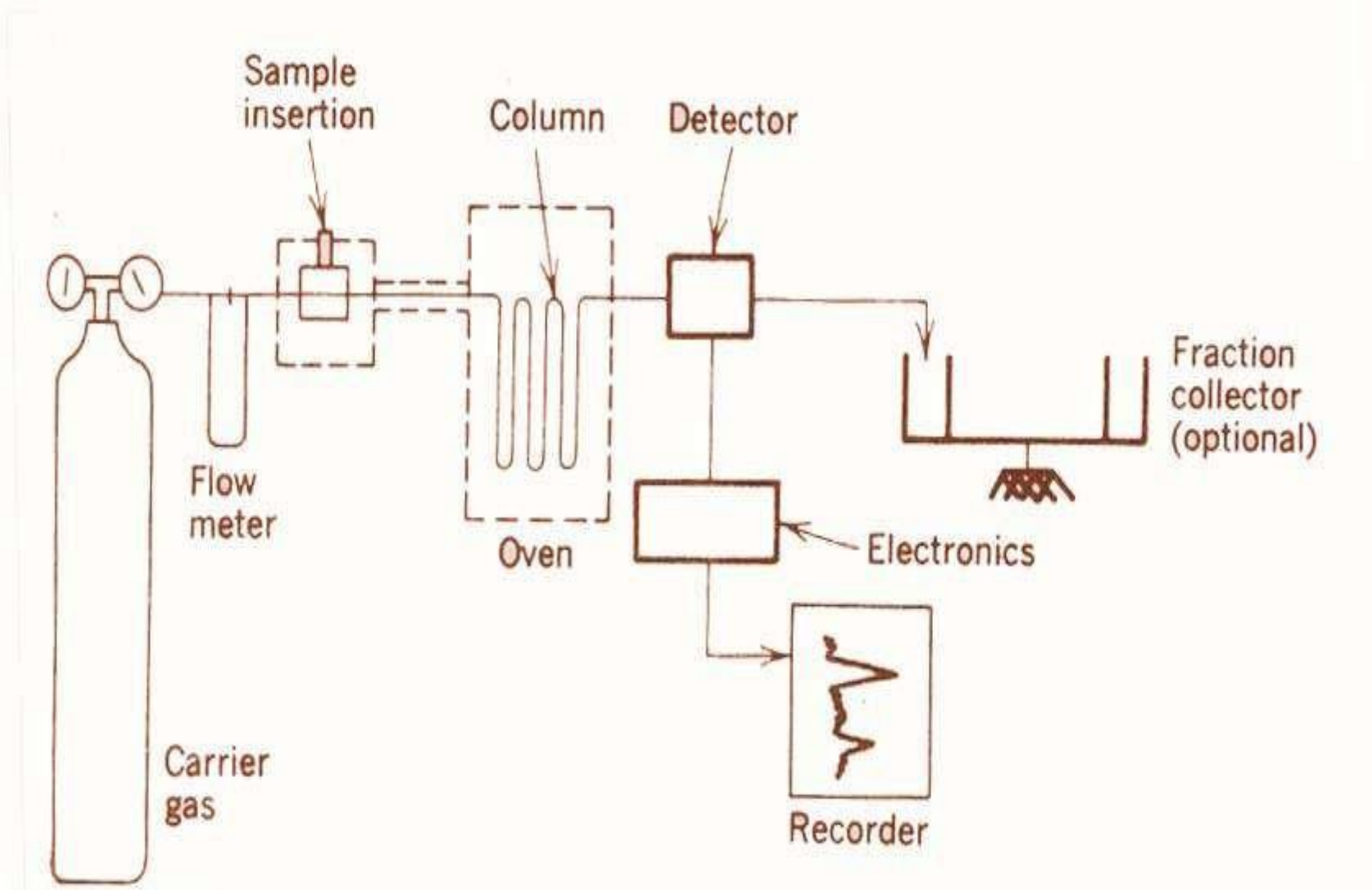
DISPLACEMENT CHROMATOGRAPHY

- The basic principle of displacement chromatography is, “A molecule with a high affinity for the chromatography matrix (the displacer) will compete effectively for binding sites, and thus displace all molecules with lesser affinities”
- Displacement chromatography has advantages over elution chromatography
- components are resolved into consecutive zones of pure substances rather than “peaks”.
- Because the process takes advantage of the nonlinearity of the isotherms, a larger column feed can be separated on a given column with the purified components recovered at significantly higher concentrations

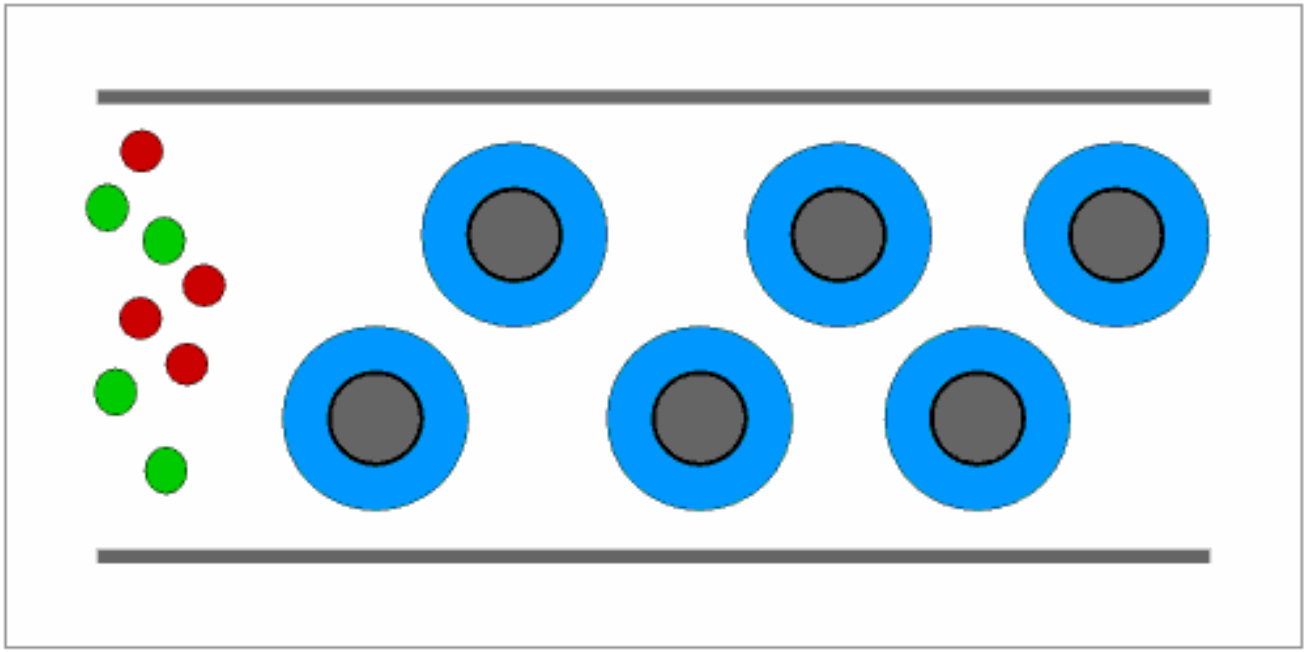
GAS CHROMATOGRAPHY (GC)

- 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

In the animation below the red molecules are more soluble in the liquid (or less volatile) than are the green molecules.



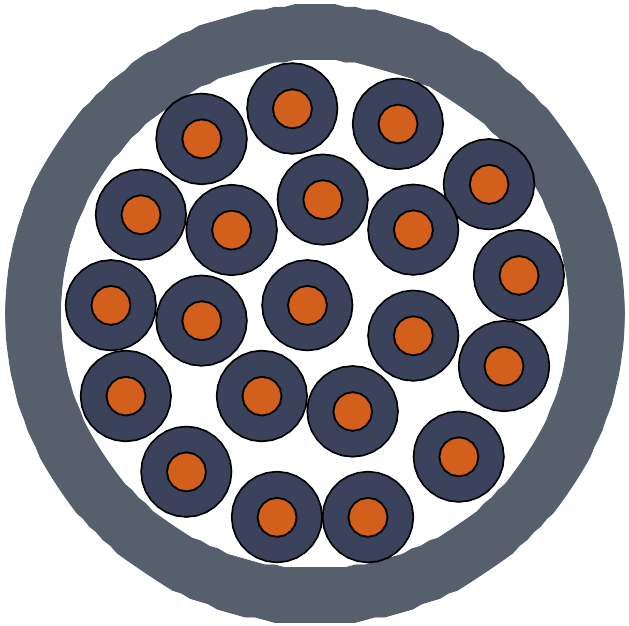
INSTRUMENTATION:

- 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

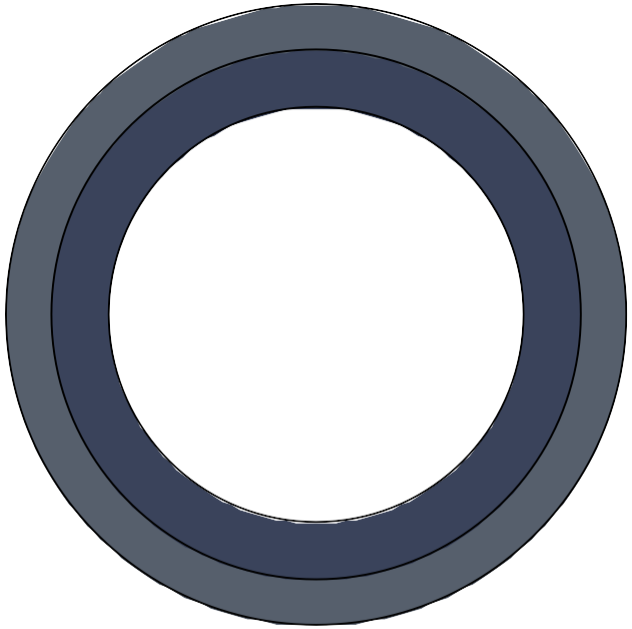
COLUMNS:

Columns in GC are two types

1) packed column
2) capillary column



Packed column



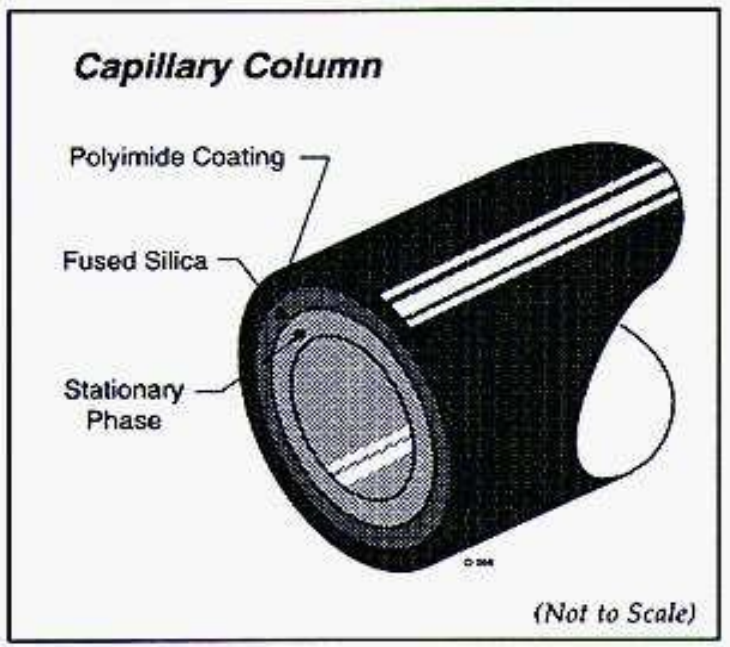
Open tubular column (capillary column)

Packed column:

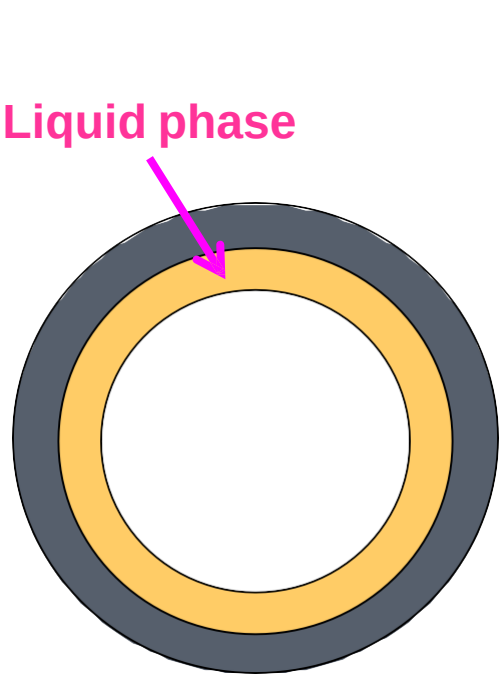
Glass or metals
2-3 m long, 2-4 mm I'd.
Densely packed with packing materials or solid support coated with thin layer of stationary liquid phase
Diatomaceous earth
Size: 60-80 mesh (250-170 μm) or 80-100 mesh (170-149 μm)

Open tubular column

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

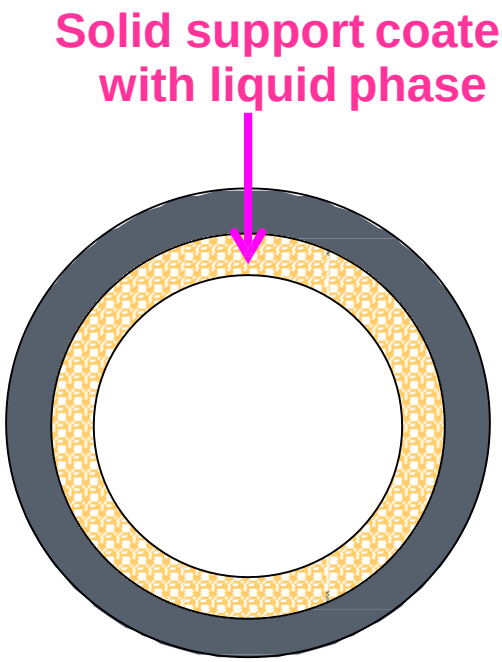


Types of open tubular column:



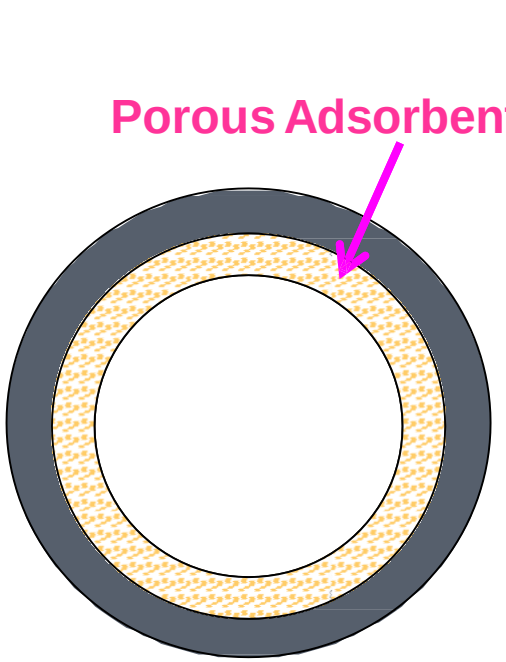
Wall-coated Open Tubular

(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

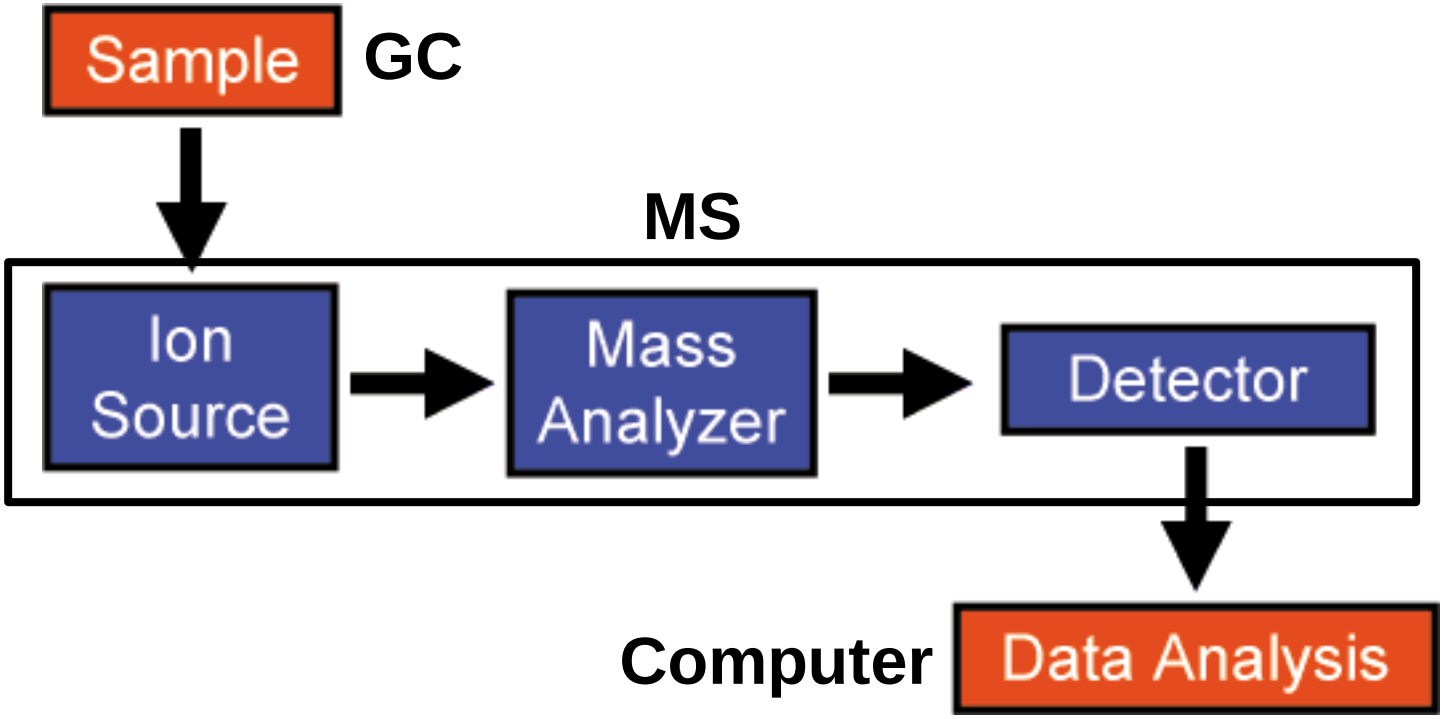
- Gas chromatography-Mass spectroscopy (GC-MS) is one of the so-called hyphenated analytical technique
- As the name implies, it is actually two techniques that are combined to form a single method of analyzing mixtures of chemicals
- Gas chromatography is a technique capable of separating, detecting 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

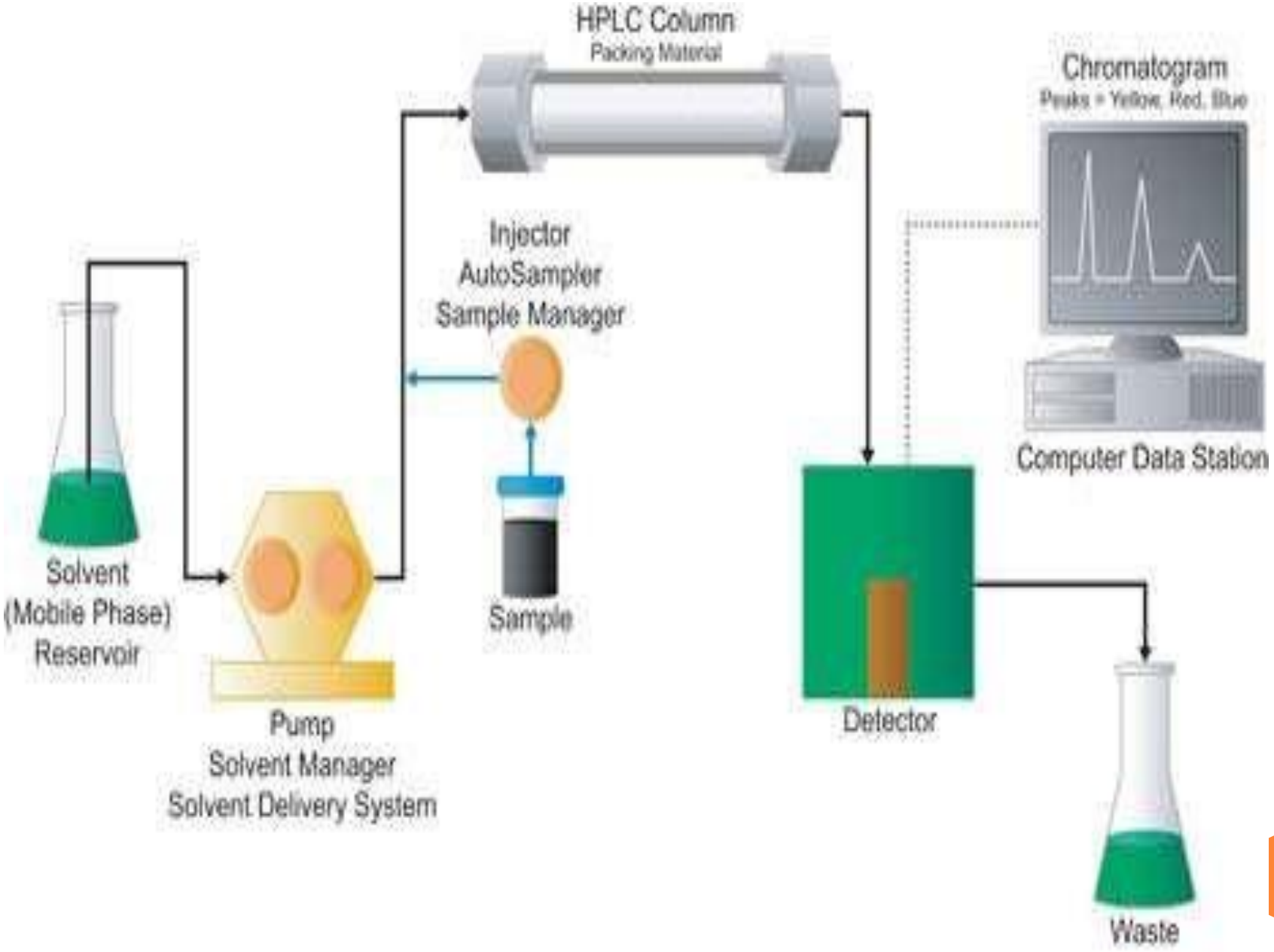
- Highly specific and selective
- Molecular recognition groups (affinity ligands) covalently attached to agarose or cellulose beads
- Affinity ligand binds selectively and reversibly the analyte
- Mechanism of retention: interaction with highly specialized molecular recognition systems which are attached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

Normal-Phase HPLC

- Adsorption of analytes on the polar, weakly acidic surface of silica gel
- Stationary Phase.: Silica (pH 2-8), Alumina (pH 2 - 12), Bonded Diol and NH2
- Mobile Phase: Non-polar solvents (Hexane, $CHCl_3$)
- Applications: Non-polar and semi-polar samples; hexane soluble; positional isomers
- Polar solutes elute later than non-polar lyophilic ones

Reversed-Phase HPLC

- Partition of analytes between mobile phase and stagnant phase inside the pore space + adsorption on the surface of bonded phase
- Stationary Phase: **Hydrophobic surfaces of moieties** bonded on silica (C18, C8, C5, Phenyl, CN)
- Mobile phase: Methanol or Acetonitrile and Water
- Applications: ~80% of all separations done on RP HPLC
- organic molecules are separated based on their degree of hydrophobicity

UPLC

- In 2004, further advances in instrumentation and column technology were made to achieve very significant increase in:
  - RESOLUTION
  - SPEED
  - SENSITIVITY
  - Increase separation EFFICIENCY
- Columns with smaller particles [<1.7um]
- Mobile phase delivery is done at >15,000psi

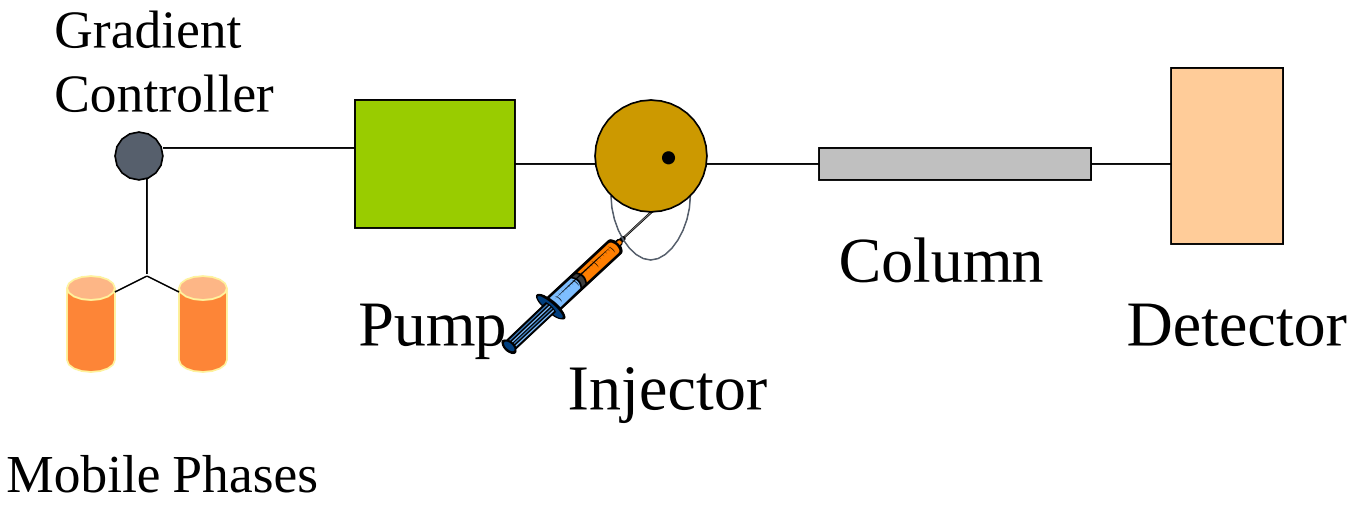
Contrasting HPLC and UPLC

- UPLC gives faster results with better resolution
- UPLC uses less of valuable solvents like acetonitrile which lowers cost
- The reduction of solvent use is more environmentally friendly

Instrumentation

- Autosampler – Reduced Cycle Time and niglisible carryove
- Flow cell
- Van guar column – for protecting column performance
- Tubings – Special for UPLC, reduced volume, optimized connection
- Mobile Phase Filters – To prevent system damage
- column
- Detectors

Schematic Presentation of UPLC



Tubing and Fitting

- Tubing and fittings are carefully designed to provide leak-free connections, with low dead volume, and minimal band spreading. For best performance, these components must be clean, and designed to work together.

Mobile Phase Filters

- Good practice to always filter your solvents
- As it prevent system damage
- Filters should be changed periodically depending on usage and mobile phase

Detectors

- Evaporative Light Scattering (ELS) Detector
- Fluorescence (FLR) Detector
- Photodiode Array (PDA) Detector
- Tunable UV (TUV) Detector
- Single Quadrapole Detector (SQD)

ADVANTAGES OF UPLC

- Decreases run time and increases sensitivity
- Provides the selectivity, sensitivity, and dynamic range of LC analysis
- Maintaining resolution performance.
- Expands scope of Multiresidue Methods
- UPLC's fast resolving power quickly quantifies related and unrelated compounds
- Faster analysis through the use of a novel separation material of very fine particle size
- Operation cost is reduced
- Less solvent consumption
- Reduces process cycle times, so that more product can be produced with existing resources
- Delivers real-time analysis in step with manufacturing processes
- Assures end-product quality, including final release testing

Disadvantages OF UPLC

Due to increased pressure requires more maintenance and reduces the life of the columns of this type.

THANK YOU