BHARATHIDASAN UNIVERSITY

Tiruchirappalli – 620024,
Tamil Nadu, India.

Programme: M.Sc., Botany

Course Title : CELL BIOLOGY AND BIOINSTRUMENTATION
Course Code : 22PGBOT104

Unit – V

SEPARATION TECHNIQUES

Topic: Chromatography

(Paper & Thinlayer)

Dr. M. SATHIYABAMA
PROFESSOR
Department of Botany

Chromatography

(Paper and Thin Layer)

- used for separation of mixtures.

- **Chromatography** is a physical method of separation in which the components to be separated (partition of solute) are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction.

- Mobile phase – liquid, gas
- Stationary phase – solid (porous), liquid
- Interaction between solid and mobile phases results in separation of compound from a mixture.
- - interaction include physico chemical principles such as adsorption, ion exchange, molecular sieving and affinity.

History

- Michael Tswett is credited as being the father of liquid chromatography. Tswett developed his ideas in the early 1900's.
- He separated plant pigments using chalk powder. (chroma- colour; graphy- to write)

- 1938:- Izmailov & shraiber described basic principle and used it for separation of plant extracts.

- 1944:- Consden, Gorden & Martin started using filter papers for separation of amino acid.

- 1950:-Kirchner who used impregnated glass plate coated with alumina, identified terpenes.

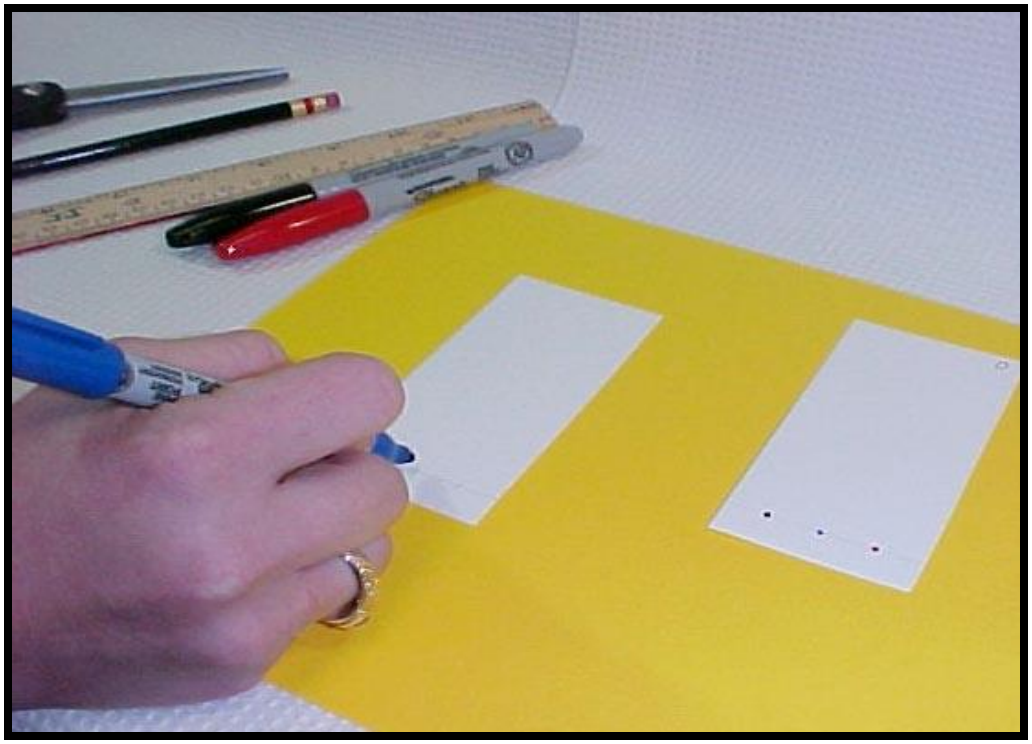
- 1958:- Ergon stahl introduced a standard equipment for preparing uniform thin layers of known thickness

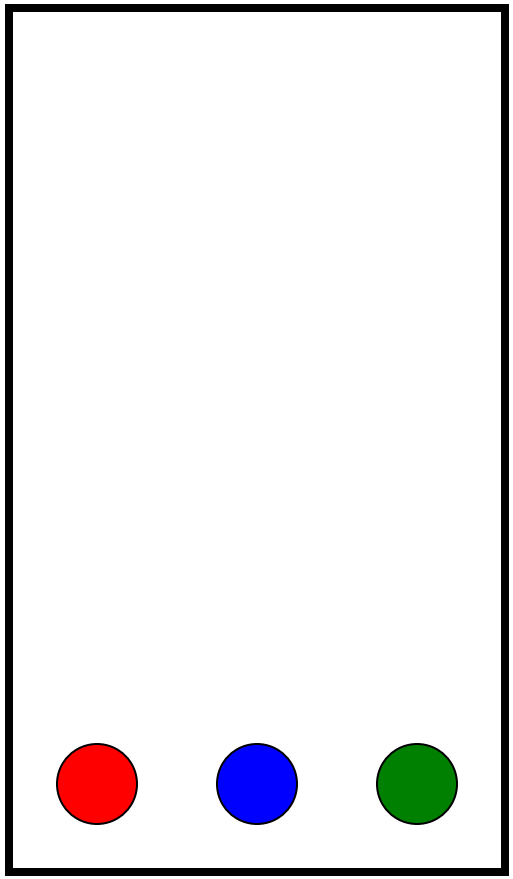
- Based on the interaction between sample and stationary phase :
- Partition
- Adsorption
- Ion exchange
- Gel filtration
- Affinity

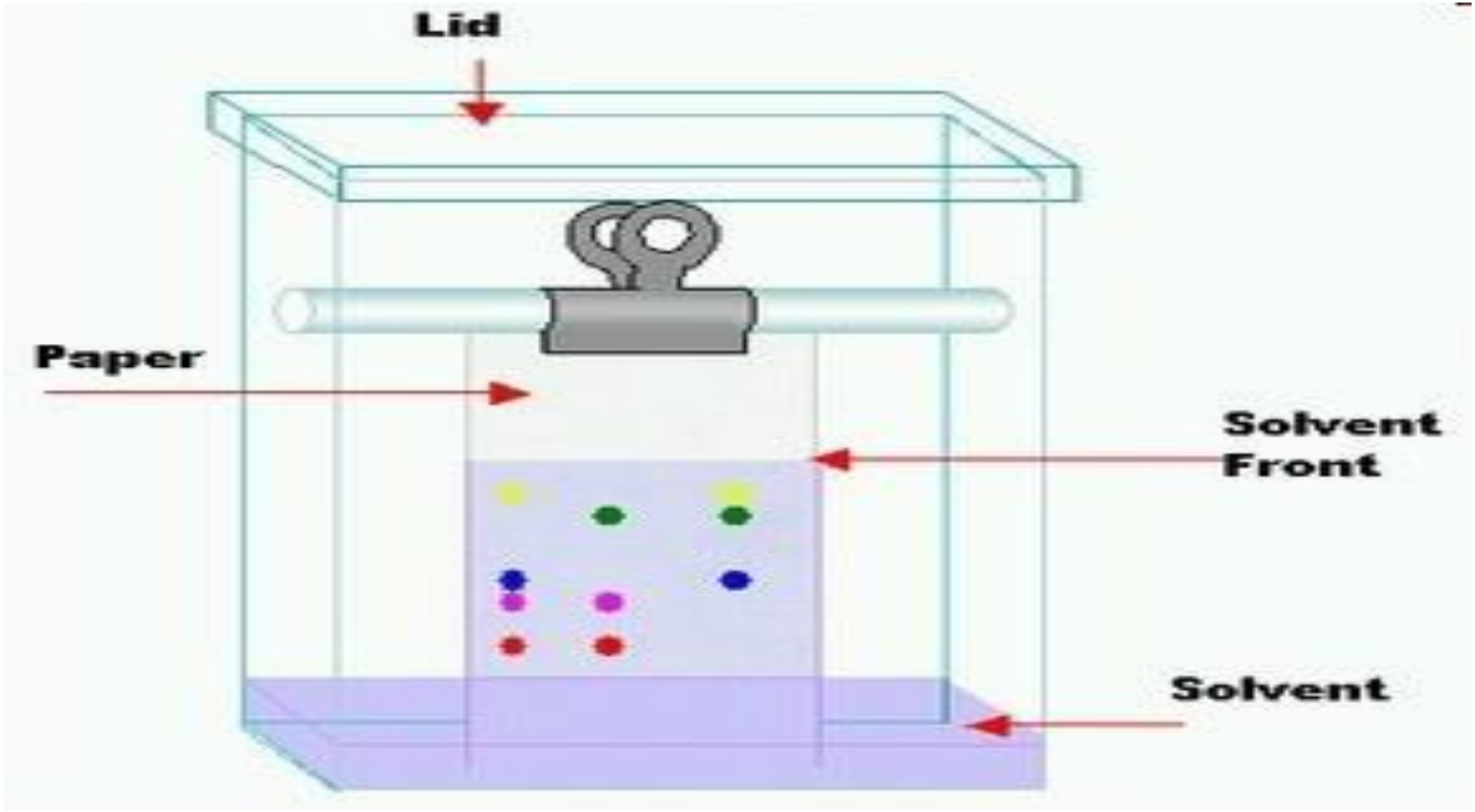
Type	Stationary phase	Mobile phase
Adsorption	Solid	Liquid
Partition	Liquid	Liquid
Gas liquid	Liquid	Gas
Gas solid	Solid	Gas
Ion exchange	Matrix with charged groups	Salt in liquid
Gel permeation	Gel with porous structure (solid)	Liquid
Affinity	A matrix with a ligand for a macromolecule	Small molecule in liquid

Paper Chromatography

- Most popular and simple procedure
- Materials – Whatman No. 1 or 3 (stationary phase) are used for chromatography.
- Chromatographic tank
- Mobile phase/solvent system
- Detecting agents
- Adsorption/partition chromatography.






Lid
Paper
Solvent
Front
Solvent

- Adsorption: Adsorption of solutes to cellulose fibers and desorption of solutes by mobile phase
- Partition: between the stationary aqueous (water molecules bound to cellulose) phase to mobile phase

- Principle: separation is mainly based on partition/ adsorption.
- Cellulose fibers with moisture act as stationary phase

- The chromatographic chamber are made up of many materials like glass, plastic or stainless steel.
- Glass tanks are preferred most. They are available in various dimensional size depending upon paper length and development type.
- The chamber atmosphere should be saturated with solvent vapor.

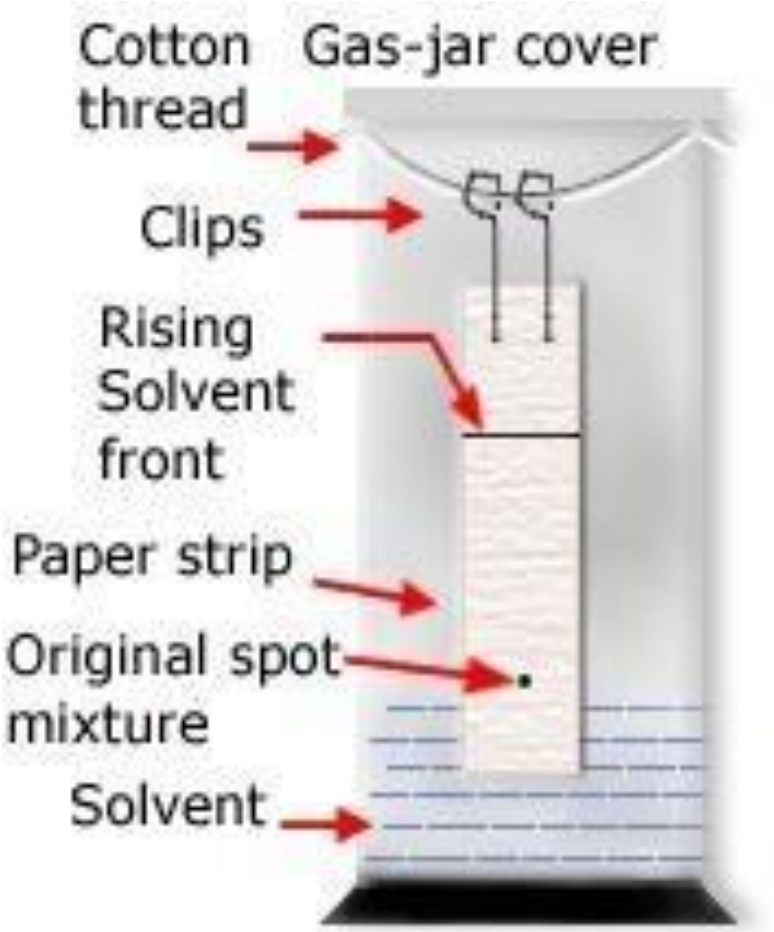
- Sample applied to the stationary phase above 2 cm from the bottom side. Different samples can be applied as a small spot (@1.5 cm distance) using capillary tube and dried.
- Kept inside the saturated chromatographic chamber and allowed to separate.

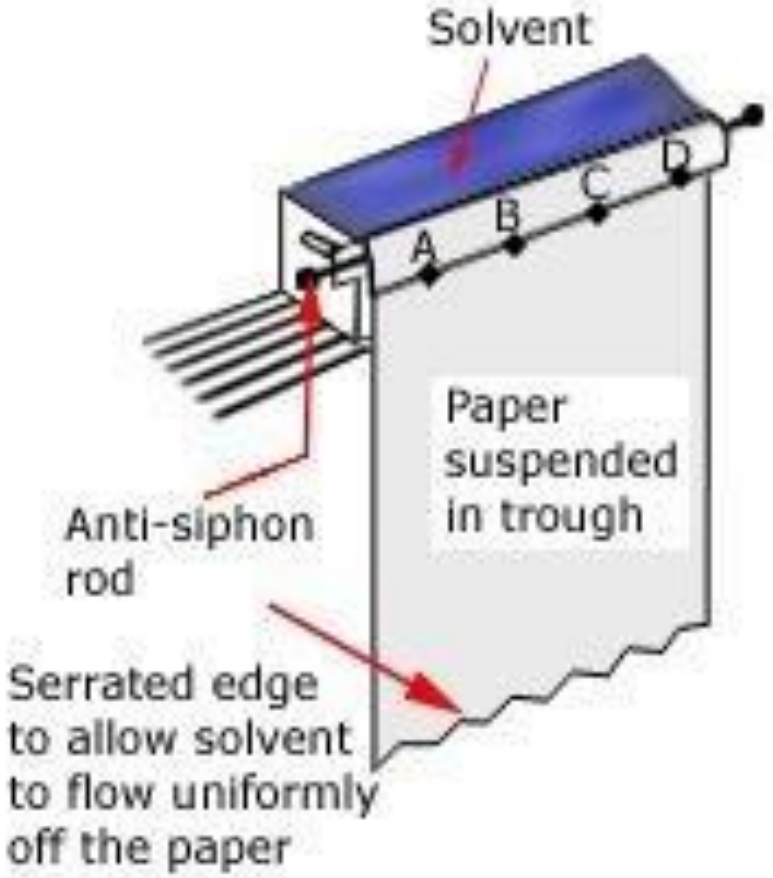
- Different compounds in the sample mixture travel at different rates due to differences in solubility in the solvent and due to differences in their adsorption on to the membrane.

- For amino acids:
- Butanol/acetic acid/water (40/10/50)
- Butanol/pyridine/water (33/33/34)
- Methanol/pyridine/water (25/12/63)
- For Mono/disaccharides:
- Butanol/pyridine/water (50/28/32)
- Butanol/ethanol/water (52/33/15)
- For Chlorophyll and carotenoids:
- Propanol/petroleum ether (4:96)
- Chloroform/petroleum ether (30:70)

- Separation:
- Ascending
- Descending
- Ascending: bottom edge of the paper dipped in the solvent. Mobile phase moves up on the paper through capillary action.
- Descending: The paper is placed at the top of the chamber (solvent holder at top) -Mobile phase moves downwards (i.e top to bottom) – Development is faster.

Single dimension





2-dimension

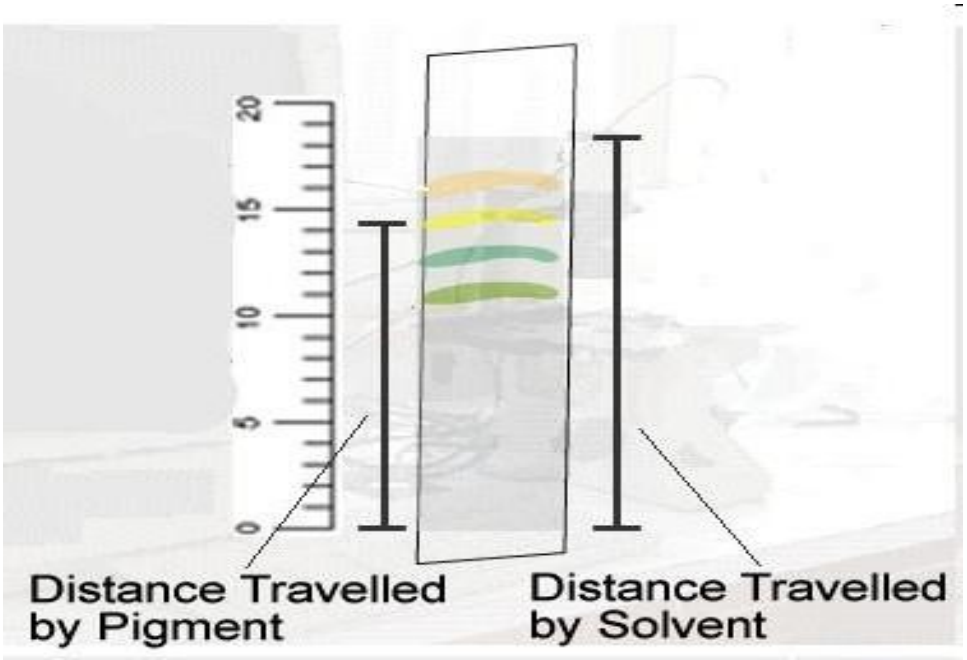
- Paper is run in one direction and then placed in another solvent system by placing the same paper in another direction (90°)

Detection of compounds

- If the substances are coloured then it can be easily visualized. Colourless substances can be viewed by Physical/ chemical methods
- Physical: under UV light (non-destructive)
- Chemical method: by spraying specific reagents (destructive)
- Amino acids: detected using ninhydrin
- Sugars: dipped in alkaline silver

Retention factor

- Rf value: Distance travelled by solute/Distance travelled by solvent



TLC

TLC is one of the simplest, fastest, easiest and least expensive of several chromatographic techniques used in qualitative and quantitative analysis to separate organic compounds and to test the purity of compounds.

TLC is a form of liquid chromatography consisting of:

- A mobile phase (developing solvent) and
- A stationary phase (a plate or strip coated with a form of silica gel, aluminium oxide, cellulose)

- **Thin Layer Chromatography** can be defined as a method of separation or identification of a mixture of components into individual components by using finely divided adsorbent solid / (liquid) spread over a glass plate and liquid as a mobile phase by capillary action

- Adsorbents:
- Silica gel - amino acids, peptides, fatty acids, steroids, phospholipids
- Aluminium oxide – amino acids, steroids, vitamins
- Cellulose – aminoacids

- Non-volatile/low volatile substance
- Cost-effective
- Corrosive agents can be used in TLC.

PREPARATION OF CHROMATOPLATES

- Glass plates or flexible plates are commonly used for adsorbent. Size used depends on type of separation to be carried out, the type of chromatographic tank and spreading apparatus available.
- The standard sizes are 20 x 5 cm, 20 x 10 cm or 20 x 20 cm .
- The surface should be flat without irregularities.
- The standard film thickness is 250um

Methods for application of adsorbent.

- Pouring
- Dipping
- Spraying
- Spreading.

- **Pouring:** The adsorbent of finely divided and homogeneous particle size is made into slurry and is poured on a plate and allowed to flow over it so that it is evenly covered.

- **Dipping :** This technique is used for small plates by dipping the two plates at a time, back to back in a slurry of adsorbent in chloroform or other volatile solvents. Exact thickness of layer is not known and evenness of layer may not be good.

- **Spraying :** Slurry is diluted further for the operation of sprayer. But this technique is not used now a days as it is difficult to get uniform layer.

- **Spreading :** All the above methods fail to give thin and uniform layers. Modern methods utilize the spreading devices for preparation of uniform thin layers on glass plates. Commercial spreaders are of two types (a) Moving spreader, (b) Moving plate type.

  It gives layer thickness from 0.2 to 2.0 mm.

ACTIVATION OF PLATES

- After spreading plates are allowed to dry in air and further dried and activated by heating at about 100^0c for 30 mins.

- By removing the liquids associated with layer completely, the adsorbent layer is activated.

- Thank You