15. PAPER CHROMATOGRAPHY (PC)

- ☞ Introduction - Paper Partition Chromatography
  - Paper Adsorption Chromatography
- ☞ Principle of Separation
- ☞ Practical Requirements
  - ☆ Stationary Phase & Papers used
  - Application of sample
  - Mobile phase
  - Development Technique
    - Ascending development
    - Descending development
    - Ascending-Descending development
    - Circular / Radial development
    - Two dimensional development
  - Detecting or visualising agents
    - Non-specific & specific methods
    - Destructive & Non-destructive technique
- ☞ Quantitative analysis
  - ☆ Direct and Indirect technique
- ☞ Qualitative analysis - R_f, R_x, R_M
- ☞ Applications

INTRODUCTION

Paper chromatography is defined as the technique in which the analysis of unknown substances is carried out mainly by the flow of solvents on specially designed filter paper. There are two types of paper chromatography. They are

Paper adsorption chromatography: in which paper impregnated with silica or alumina acts as adsorbent (stationary phase) and solvent as mobile phase.

Paper partition chromatography: in which moisture/water present in the pores of cellulose fibres present in filter paper acts as stationary phase and another mobile phase is used as solvent.

In general, Paper chromatography refers to paper partition chromatography only since most separations are based on partition type only.

PRINCIPLE OF SEPARATION

The ***principle of separation*** is mainly ***partition*** rather than adsorption. Cellulose layers in filter paper contains moisture which acts as stationary phase. Organic solvents or buffers are used as mobile phases. Instead of water as stationary phase, other organic solvents can be used by suitable modification.

PRACTICAL REQUIREMENTS

1. Stationary phase and Papers used
2. Application of sample
3. Mobile Phase
4. Development technique
5. Detecting or visualising agents

1. STATIONARY PHASE AND PAPERS USED

Paper of chromatographic grade consists of α-cellulose - 98-99%, β-cellulose 0.3-1%, pentosans - 0.4-0.8%, ether soluble matter - 0.015 -

0.02%, ash - 0.01 - 0.07%. Whatman filter papers of different grade like No.1, No.2, No.3, No.3MM, No.4, No.17, No.20 etc are used. These papers differ in sizes, shapes, porosities and thickness.

- ☞ Choice of filter paper depends upon thickness, flow rate, purity, technique, etc.

- ☞ **Modified papers** - Acid or base washed filter paper, glass fibre type paper.

- ☞ **Hydrophilic papers** - Papers modified with methanol, formamide, glycol, glycerol etc.

- ☞ **Hydrophobic papers** - Acetylation of OH groups leads to hydrophobic nature, hence can be used for reverse phase chromatography. Silicone pretreatment and organic non-polar polymers can also can be impregnated to give reverse phase chromatographic mode.

- ☞ Impregnation of silica, alumina or ion exchange resins can also be made.

- ☞ **Size of the paper used:** Paper of any size can be used. Paper should be kept in a chamber of suitable size.

2. APPLICATION OF SAMPLE

The sample to be applied is dissolved in the mobile phase and applied using capillary tube or using micropipette. Very low concentration is used to avoid larger zone.

3. MOBILE PHASE

Pure solvents, buffer solutions, or mixture of solvents are used. Some of the examples of ***Hydrophilic mobile phases***:

Isopropanol : Ammonia : Water - 9:1:2
n-Butanol : glacial acetic acid : water - 4:1:5
Methanol : water - 3:1 or 4:1
t-Butanol : water : Formic acid - 40:20:5

Examples of Hydrophobic mobile phases

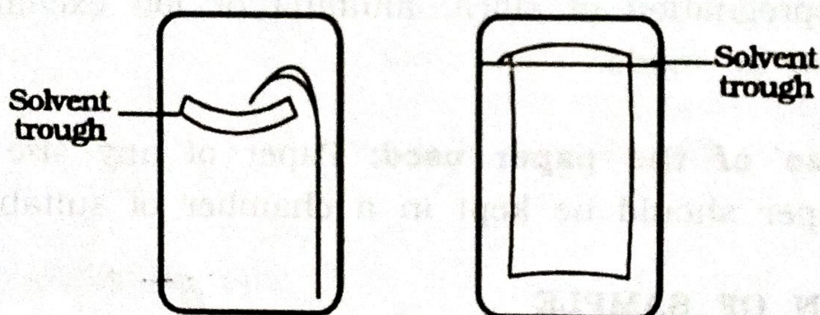
Kerosene : 70% Isopropanol
Dimethyl ether : cyclohexane
Single/two phase or three phase solvent systems are also used.

4. DEVELOPMENT TECHNIQUE

Since paper is flexible when compared to glass plate used in TLC, several types of development are possible which increases the ease and efficiency of operation. They are

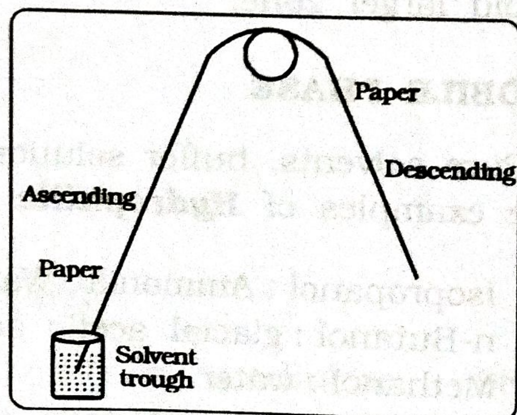
i. **Ascending development:** Like conventional type, the solvent flows against gravity. The spots are kept at the bottom portion of paper and kept in a chamber with mobile phase solvent at the bottom. (same as Fig 14.2 & 14.3 in TLC chapter)

ii. **Descending development:** This is carried out in a special chamber where the solvent holder is at the top. The spot is kept at the top and the solvent flows down the paper. The advantage is that the flow of solvent is assisted by gravity and hence the development is faster.



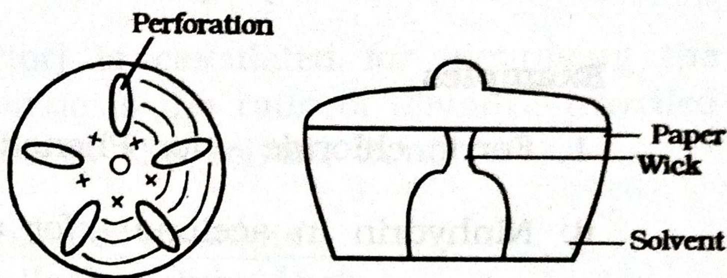
Development chamber (lateral & posterior view)

iii. **Ascending-Descending development:** This is a combination of ascending and descending type. Only the length of separation is increased by using a combination of techniques. First ascending takes place followed by descending development.



Ascending cum descending development

iv. **Circular/ radial development (Horizontal):** Here, the spot is kept at the centre of a circular paper. The solvent flows through a wick at the centre and spreads in all directions uniformly. Hence the individual spots after development look like concentric circles. By making perforations radially, number of quadrants can be created allowing more number of samples to be spotted.



Radial development (top view and side view)

v. **Two dimensional development:** This technique is similar to 2-Dimensional TLC. The paper is developed in one direction and after development, the paper is developed in the second direction allowing more compounds or complex mixtures to be separated into individual spots. In the second direction, either the same solvent system or different solvent system can be used for development. (same as Fig 14.5 in TLC chapter)

5. DETECTING OR VISUALISING AGENTS

After the development of chromatogram, the spots should be visualised. Detecting coloured spots can be done visually. But for detecting colourless spots, any one of the following techniques can be used.

a. **Non specific methods:** Where the number of spots can be detected, but not the exact nature or type of compound.

Examples

i. **Iodine chamber method:**, where brown or amber spots are observed when the TLC plates are kept in a tank with few iodine crystals at the bottom.

ii. **UV chamber for flourescent compounds:** When compounds are viewed under UV chamber, at 254nm (short λ) or at 365nm (long λ), flourescent compounds can be detected. Bright spots are seen against a dark background.

b. **Specific methods:** Specific spray reagents or detecting agents or visualising agents are used to find out the nature of compounds or for identification purposes.

Examples

i. Ferric chloride - for Phenolic compounds and tannins

ii. Ninhydrin in acetone - for amino acids

iii. Dragendroff's reagent - for alkaloids

iv. 3,5 - Dinitro benzoic acid - for cardiac glycosides

v. 2,4 - Dinitrophenyl hydrazine - for aldehydes and ketones

The detecting techniques can also be categorised as

i. **Destructive technique:** eg. Specific spray reagents, etc where the samples are destroyed before detection. eg. Ninhydrin reagent.

ii. **Non-Destructive technique:** Like UV chamber method, Iodine chamber method, densitometric method, etc where the sample is not destroyed even after detection.

For radioactive materials, detection is by using autoradiography or Geiger muller counter.

For antibiotics, the chromatogram is layed on nutrient agar inoculated with appropriate strain and the zone of inhibition is compared.

QUANTITATIVE ANALYSIS: (Direct and Indirect techniques)

Direct technique: Densitometer is an instrument which measures quantitatively the density of the spots. When the optical density of the spots for the standard and test solution are determined, the quantity of the substance can be calculated. The papers are neither destroyed nor eluted with solvents to get the compounds. This method is also called as in-situ method.

Indirect technique: In this technique, the spots are cut into portions and eluted with solvents. This solution can be analysed by any conventional techniques of analysis like spectrophotometry, electrochemical methods, etc.

QUALITATIVE ANALYSIS

Rf VALUE

The Rf value (Retardation factor) is calculated for identifying the spots i.e. in Qualitative analysis. Rf value is the ratio of distance travelled by the solute to the distance travelled by the solvent front.

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent front}}$$

The Rf value ranges from 0 to 1. But ideal values are from 0.3 to 0.8. Rf value is constant for every compound in a particular combination of stationary and mobile phase. When the Rf value of a sample and reference compound is same, the compound is identified by its standard. When the Rf value differs, the compound may be different from its reference standard.

R_x value

R_x value is nothing but the ratio of distance travelled by the sample and the distance travelled by standard. R_x value is always closer to 1.

R_m values

R_m value is used in qualtitative analysis to find out whether the compounds belong to a homologous series. If they belong to a homologous series, the ΔR_m values are constant. The ΔR_m values for a pair of adjacent member of a homologous series is determined by using the formula:

$$R_m = \log\left(\frac{1}{R_f} - 1\right)$$

APPLICATIONS

The applications are wider and there is no limitation to the compounds that can be analysed by paper chromatography. Paper chromatography is more useful for the analysis of polar compounds like amino acids, sugars, natural products, etc. The different types of applications are listed below.

1. Separation of mixtures of drugs of chemical or biological origin, plant extracts, etc

2. Separation of carbohydrates (sugars), vitamins, antibiotics, proteins, alkaloids, glycosides, aminoacids, etc

3. Identification of drugs

Drug	Mobile phase	Detecting agent
Erythromycin estolate	Isobutyl methyl ketone	Nutrient agar containing Bacillus pumilus
Gentamycin	Chloroform : Methanol : Ammonia : Water (10:5:3:2)	Ninhydrin in pyridine - acetone mixture
Vancomycin	t-Amyl alcohol : Acetone : Water (2:1:2)	Nutrient agar containing Bacillus subtilis

4. Identification of impurities

Drug	Mobile phase	Detecting agent
Hydroxocobalamin	s-Butyl alcohol : acetic acid : Potassium cyanide	Elution and measurement of absorbance at 361nm

5. Identification of related compounds

Drug	Mobile phase	Detecting agent
Phenformin HCl	Ethyl acetate : ethanol : water (6:3:1)	Potassium ferricyanide, Sodium nitroprusside & NaOH
Ergotamine injection	Chloroform : methanol	p-dimethyl amino benzaldehyde reagent
Vitamin A	Dioxan : methanol : water with BHA (70:15:5)	UV 366nm

6. Identification of foreign substances in drugs

7. Identification of decomposition products

8. Analysis of metabolites of drugs in blood, urine etc.