1. **What is chromatography?**

Chromatography is a laboratory method that is widely used for the separation, identification and determination of chemical components of a complex mixture. More specifically, chromatography separates compounds based on differences in their structure, size and/or composition. It is used to conduct qualitative analysis (identify the components) and quantitative analysis (determine the concentration) of unknown substances. No other separation method is as powerful and generally applicable as chromatography.

2. **What is paper chromatography?**

Paper chromatography is a technique for analysing mixtures by chromatography in which the stationary phase is absorbent phase. A spot of the mixture to be investigated is placed near one edge of the paper and the sheet is suspended vertically in a solvent, which rises through the paper by capillary action carrying the components with it. The components move at different rates, partly because they absorb to different extents on the cellulose and partly because of partition between the solvent and the moisture in the paper. The paper is removed and dried and the different components form a line of spots along the paper. Colourless substances are detected by using ultraviolet radiation or by spraying with a substance that reacts to give a coloured spot (e.g. Ninhydrin gives a blue coloration with amino acids). The components can be identified by the distance they move in a given time.

3. **Why has paper chromatography retained its applicability in the face of emergence of advanced instrumental techniques?**

Paper chromatography is economical, simple, reproducible, accurate and immensely versatile technique for the separation and identification of many organic and inorganic compounds from the mixture. It requires less maintenance, minimum operation and less solvent compared to other techniques. It provides graphical, clear and quick results. It is used as an analytical chemistry technique for identifying and separating coloured and

colourless mixtures. Sugars, amino acids, lipids, nucleic acids and other biomolecules can be easily identified and quantified by employing this technique.

4. What is the basic principle of paper chromatography?

Paper chromatography is a form of liquid chromatography where the components of a mixture of organic compounds get separated as unique spots by unidirectional flow of the developing liquid mobile phase solvent mixture over the filter paper to which a spot of the sample is applied. The distance travelled by each component is specific under the given set of operational conditions.

5. Why is it necessary to cover the developing chamber during the paper development?

During the chromatogram development chamber is covered. This is essential as the environment inside the chamber should remain saturated with the solvent vapour. Development times can vary from about an hour to several hours and a saturated environment prevents losses due to evaporation.

6. What are the common techniques for detecting colourless spots?

It is easy to distinguish coloured spots visually but for colourless compounds, alternate techniques need to be adopted which can be specific or non-specific. A common non-specific method is suspension of developed chromatogram in iodine vapour. Most organic compounds show up as brown spots.

The sheet is viewed in a UV viewing cabinet under 366 nm and 254 nm wavelength lamp illumination. On observation the spots need to be marked carefully with a pencil for calculating R_f values. Under specific methods amines and amino acids are observed by spraying heated paper on development with 0.2% hydrazine. Deep blue or purple spots begin to appear. Alkaloids- Dragendroff's reagent spray results in orange or orange yellow spots.

Aldehydes and Ketones- 2, 4 Dinitrophenylhydrazine (DNPH) spray in methanol and sulphuric acid results in orange or yellow spots.

7. **Why should the samples have reasonable solubility which is neither too high nor too low in the developing solvent mixture?**

The samples should have a medium solubility in the developing solvent mixture. Too high a solubility will lead to transfer of the component along with the solvent front and on the other hand if the solubility is too low the component will not be carried by the solvent mixture and will remain close to the initial applied spot. In either case, the resolution of the mixture components will be low. Thus, reasonably good resolution can be obtained for medium solubility of compounds in the solvent mixture.

8. **What are the essential criteria for selection of suitable solvents for paper chromatography?**

Solvents are selected on the basis of solubility of the sample components. In general, it is advisable to keep in mind:

a. Solvents are not toxic or carcinogenic.
b. Solvent constituents of mixture should not react with any of the sample constituents
c. Solvents selected should not interfere in detection of separated spots.
d. Solvents should not be highly volatile as loss of components can result in change of mixture composition.

9. **What is double-way paper chromatography?**

Double-way paper chromatography also called two-dimensional chromatography involves using two solvents and rotating the paper 90° in between. This is useful for separating complex mixture of compounds having similar polarity, for example, amino acids. If a filter paper is used, it should be of a high quality. The mobile phase is developing solutions that can travel up to the stationary phase carrying the sample along with it.

10. What is retention factor (R_f)?

After running the chromatogram, each separated band can be assigned a retention factor (R_f) which is a characteristic of each specific dye(s). The R is a ratio of the distance the band travels to the distance the solvent travels. It is calculated by dividing the band distance by the solvent distance. This ratio should be a constant that is characteristic of the dye (s) in a particular spot under a particular set of chromatographic conditions (i.e., paper chromatogram, solvent, etc.).

11. How are R_f values expressed and what factors affect them?

R_f values are usually expressed as a fraction of two decimal places. If R_f value of a solution is zero, the solute remains in the stationary phase and thus it is immobile.

If R_f value= 1, then the solute has no affinity for the stationary phase and travels with the solvent front.

The R_f values are affected by

(i) Temperature,

(ii) Concentration,

(iii) Impurities in solvent system,

(iv) Homogeneity of paper,

(v) Quantity of sample etc.

(vi) Adsorption of solution by paper will also affect the R_f values.

12. What are the limitations of paper chromatography?

Paper chromatography has certain limitations such as:

a. Semi-quantitative nature.

b. Overlapping of spots of components having close Rf values.

c. Higher concentration of components leading to streaking instead of well-defined spots.

d. Errors in R_f calculations due to uneven flow of solvent front.

e. Improper sample spotting or spotting below the marked line resulting in dipping into the solvent or accidental dipping of spot into solvent while inserting the paper into the solvent chamber.

Q. What is the major difference between isocratic elution and gradient elution? Why gradient elution is often advantageous over isocratic elution?

In the isocratic elution method, the mobile phase used is of the same composition during the separation process. But in the gradient method, the polarity or strength of the mobile phase is changed time to time to make analysis time shorter or to get a better resolution of components.

The main advantage of using gradient elution is the improved resolution of the solutes. In this process, the weak eluting solvent is used initially, followed by the addition of the strong eluting solvent. This is done by changing the polarity of the solvent by mixing.

Q. What is HPLC? Describe the different components used in HPLC?

High Performance Liquid Chromatography is an excellent analytical tool used to separate, identify as well as quantify the components of wide range of mixtures. This technique was earlier known as High pressure liquid chromatography and can be distinguished from conventional liquid chromatography on the basis of its high operational pressure which is near about 400 bar.

The major components in an HPLC system consist of a solvent reservoir or sometimes multiple reservoirs, a high-pressure pump, an injection port, a column and a detector

The mobile phase is contained in the solvent reservoir and usually two reservoirs are used reach of having about 1000 mL of volume capacity. The composition of the mobile phase renders two different modes of elution process viz. isocratic and gradient.

In standard HPLC system a pump pressure of around 50 to 400 bar has been applied to facilitate the separation of components.

The column seems to be the heart of the whole separation process. The diameter and length of the column as well as the nature and particle size of the stationary phase packed inside the column actually determines the efficiency of separation of the sample's ingredients. In standard HPLC, the column particle size ranges from 3 to 5 µm whereas in µHPLC, the particle size falls below 1.7 µm.

In HPLC, various types of detectors are used based on the structural characteristics of the analytes. Some of the important detectors are UV-vis absorbance detector, refractive index detector, fluorometric detectors, conductivity detector etc.

Q Define normal phase and reverse phase HPLC system.

In normal phase HPLC system, the column is packed with tiny silica particles which are polar in nature while the mobile phase is non-polar such as hexane. Through this column, the non-polar compounds are passed more quickly than the polar ones as because the polar components get stick to the stationary phase.

In reverse phase HPLC system, non-polar modified silica particles are used to fill the column and a polar solvent such as water or alcohol is used as the mobile phase. Through this column the polar molecules tend to elute more quickly than the non-polar ones. Reversed phase HPLC is the most frequently used form of HPLC rather than the normal phase HPLC.

Q. Describe the qualitative and quantitative aspects of HPLC.

In HPLC techniques, the detector systematically records the eluted components transported by the mobile phase in the form of retention peak. The peak signals are displayed in the form of Gaussian bell-shaped curve and thy provide both qualitative and quantitative information of the analyte. Each signal of the respective component is compared with the corresponding signal peak produced by the authentic sample. Very often HPLC is also

combined with a mass spectrometer (MS) which enables to have qualitative information of complex analyte mixture.

Moreover, quantitative information can be evaluated by using chromatographic data management software since the area under each peak represents the concentration of the analyte. We can calculate the concentration of each separated component by integration using the software.

Q. What is thin layer chromatography? In what way TLC is superior to paper chromatography?

Thin layer chromatography is a form of liquid-solid chromatography based on the principal of adsorption or partition chromatography or sometimes a combination of both.

This technique is much preferred over paper-chromatographic technique as because it is faster, sensitive and more reproducible. Better resolution can be achieved through TLC because the sorbent particles used on the plate are smaller and more regular than the paper fibres.

Q. Describe the components of Thin Layer Chromatography briefly?

Thin layer chromatography technique requires three basic components to carry out the separation

process. They include:

(a) Stationary phase

(b) Mobile phase

(c) Developing chamber

(a) **Stationary phase:** Usually, a TLC plate is prepared by spreading an aqueous slurry of the finely divided adsorbent solid onto the clean surface of a glass plate. The coated plates are then allowed to stand for some time at room temperature so that the sorbent

layer has set and adheres tightly to the surface followed by heating in an oven for several hours. The most commonly used sorbent particles are silica gel, alumina, and powdered cellulose.

(b) Mobile phase: The selection of mobile phase in TLC procedure is not specific and an extremely wide range of solvents can be employed for this purpose**.** The choice of the mobile phase or the developing solvent depends only on the nature of the components of the analytes or the nature of the sorbents layer on the stationary phase.

(c) Development of Chromatogram. At first, the sample mixture under analysis is dissolved in an appropriate solvent. The sample solution is then spotted on a performed TLC plate manually with the help of a capillary tube at height of about 1 cm from the bottom edge of the plate. The spitted TLC plate is then kept in a closed glass chamber saturated with the vapours of the of the mobile phase. The mobile phase is then starts migrating slowly over the stationary phase and the process needs to be stopped before reaches the other end of the plate.

Q. Briefly discuss the qualitative and quantitative aspects of HPLC?

The TLC technique is very much helpful in both qualitative and quantitative analysis of non-volatile organic mixture, which is generally inexpensive. For qualitative analysis, the separated components of a mixture are matched with the authentic counterparts by running all of them simultaneously on the same TLC plate.

For quantitative evaluation using TLC, usually the following procedure is followed

After the developing the TLC plate, the separated components can be quantified by scraping off each individual zone or band followed by eluting the component from the sorbents and finally analysing the resultant solution.

Advantages of TLC over paper and column chromatography.

The main advantages of TLC are:

1. Requires simple equipment.

2. Short development time: TLC takes one hour for development but paper and column chromatography take several hours to days.

3. Wide choice of stationary phase: Method may be employed for adsorption, partition (including reverse phase chromatography or ion exchange chromatography).

4. Easy recovery of separated compounds: While removing the Powderly coating of the plates by scraping with a knife or scrapper, the separated components can be recovered easily. The spot or zone may be removed quantitatively.

5. Preparation effect: superior than paper chromatography

6. Thickness of the layer: Variable thickness is available from preparative to analytical.

Comparison between Thin Layer Chromatography and Paper chromatography:

Thin Layer Chromatography	**Paper chromatography**
Principle of separation is adsorption	Principle of separation is partition
More quantity of sample is required	Minimum quantity of sample is required
Consuming less time (15-45 min.)	Consuming more time (1-3 h)
Plates may be heated in oven for long time	Paper cannot be heated for long time
Very sharp separation	Less sharp separation
Physical strength of the plate is high (Ascending/Descending)	Physical strength of the paper is low (Descending/Small paper only ascending)
Plates are prepared as thin coat with adsorbents	Paper is used as such since coating can't be done on paper.
Corrosive reagent can be used	Corrosive agents can't be used since it destroys it.
Compound can be detected under UV	Can't be detected under UV
Sensitivity of detection is high	Less sensitivity of detection
Spots are less diffused	Spots are easily diffused.