Separation Techniques – Reverse Phase Thin Layer Chromatography

Background: Reverse phase thin layer chromatography (RP-TLC) is a modification of the TLC technique in which the adsorbant material is reacted with long-chain fatty acids and thus made hydrophobic in nature. Inversally, the solvent mixture used as a mobile phase is mainly polar in nature. As a result the pattern of separation of pigments also changes as compared to the conventional TLC method.

Requirements:

Category	Particulars
Specimen/Biological sample	Green leaf
Chemicals	• Acetone • Distilled water • Ethyl acetate • Methanol
Apparatus/Facilities	• Glass capillary tube • Glass test tube • 250 ml glass beaker or Coplin jar (as chromatography chamber) • Microcentrifuge vial • Mortar pestle • Pipettes • Readymade, pre-coated RP-TLC plate (Silica gel 60 RP-18, 10×2 cm) • Test tube stand
Miscellaneous	• Aluminum foil • Forceps • Pencil • Scissors

Procedure:

Part 1: Preparing the chromatography chamber

1. Pour 15 ml solvent system (Water : Ethyl acetate : Methanol :: 0.5 : 2 : 1) into the beaker and cover it with aluminium foil.
2. Keep it aside for 15–20 minutes to allow the chamber to get saturated with the solvent vapours. *(For good chromatographic separation, the solvent must uniformly saturate the chamber.)*
3. Meanwhile, proceed with the next part of the experiment.

Part 2: Preparing the leaf extract

1. Cut the leaf into fine pieces and put them in the mortar.
2. Add 5 ml of 100% acetone and macerate well to extract the pigments into the solvent.
3. Add another 5 ml acetone and macerate further. *(Remember to be quick, as acetone is volatile.)*
4. Pour the prepared extract into a test tube and allow the leaf debris to settle.
5. After some time, you will see that the leaf debris has settled down.
6. Now carefully collect the supernatant in a vial and use it as the leaf extract.

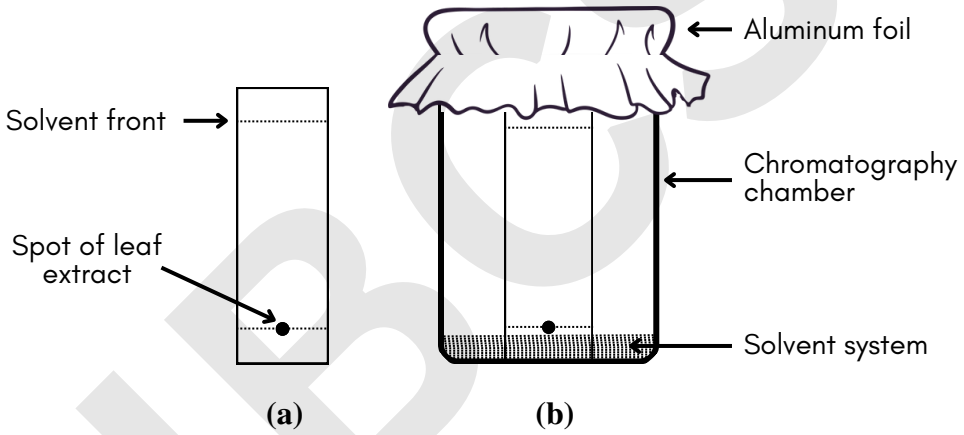

Fig. 1: Reverse phase thin layer chromatography setup

Part 3: Spotting the leaf extract

1. Take a strip of thin layer silica coated on an aluminum sheet, which we will be using for TLC. We can call it a 'RP-TLC strip' for ease.
2. With a pencil, make a faint line 1 cm from the base of the RP-TLC strip, as shown in Fig.1a. *(Avoid damaging the silica gel layer. Always hold the RP-TLC strip at the edges to avoid any contamination of the strip.)*
3. Now take the capillary tube and dip it in the extract.
4. The extract will rise in the tube due to capillary action.
5. Now briefly touch the capillary tube containing the plant extract at the center of the line on the strip to make a small spot, as shown in Fig.1a.
6. Allow the spot to dry completely.

7. Repeat the same process 10–15 times. *(Ensure that the spot's diameter remains the same until spotting is complete. Take care that no particulate matter is loaded.)*

Part 4: Separation of plant pigments

1. Carefully remove the foil covering half of the beaker.
2. Quickly place the RP-TLC strip in the chamber and immediately cover it with the foil, as shown in Fig.1b.
3. Ensure that the sides of the strip don't touch the walls of the beaker.
4. Allow the solvent to rise up to 1 cm below the upper edge of the strip, as shown in Fig.1a.
5. Then, remove the RP-TLC strip from the chamber and immediately mark the solvent front using a pencil. (Do not use an eraser, marker or a pen on the RP-TLC strip.)
6. Let the chromatogram dry completely, and then mark the positions of the separated plant pigments on the RP-TLC strip.

Part 5: Identifying the plant pigments

Using the formula,

R.f. value = Distance travelled by the pigment / distance travelled by the mobile solvent

calculate the R.f., i.e., retardation factor values of the various plant pigments.

(Since the pigments fade with time, one must calculate the R.f. values immediately.)

Points for Discussion:

1. Comment on the chemical nature of the solvent used as the mobile phase.
2. List out the pigments observed on the chromatography paper after separation.
3. Identify the predominant pigments separated on the chromatogram.
4. Comment on the polarity of chlorophylls (a & b), carotenoids and anthocyanins.
5. Perform chromatography using extracts from different leaves and compare the separation patterns obtained on the chromatograms.