# 3 SEPARATION OF LEAF PIGMENTS BY CHROMATOGRAPHY

## 3.1 INTRODUCTION

Living things contain thousands of different molecules in a single cell. If we want to study a single molecule it is necessary to separate it from the rest of them. Chromatography is one of the most effective and widely used technique for separation and identification of biomolecules. Different kinds of chromatography have been evolved and improved during the last fifty years. The technique can also be used for large scale separation of compounds after making some technical modifications.

Chloroplasts of higher plants contain photosynthetic pigments, predominently chlorophylls *a* and *b* and the two carotenoids β-carotene and xanthophyll. These are located in the thylakoid membranes in the form of pigment—protein complexes in the lipid bilayer. Carotenoids are also present in chromoplasts which are present in colourful petals of flowers and in fruits. The pigments can be extracted with organic solvents.

In this exercise you will learn to extract the pigments from leaves, separate them by chromatographic technique and identify certain pigments by their chromatographic behaviour and colour.

### Objectives

After performing this experiment you should be able to:

- extract pigments from leaves
- separate leaf pigments by paper chromatography and
- use the technique of paper chromatography for the separation of other organic compounds such as amino acids and sugars.

## 3.2 MATERIALS REQUIRED

fresh lawn grass (*Cyanodon*)

separating funnel (25 ml)

water bath

filter paper (Whatman 5 MM or Whatman 1)

petroleum ether,

solid sodium sulphate

distilled water

capillary tube (used for finding M.P.) or fine painting brush

specimen jars with lid or gas collecting jars with a cover

## 3.3 PROCEDURE

**A. Extraction of Pigments**

(Your counsellor will demonstrate this part of the experiment)

1. Take 10 grams of fresh or 0.5 gram of dry leaves (10 leaves about 1 inch long) in a small flask and add 5 ml. of 90% acetone to it. Add less than a pinch of $CaCO_3$. Keep it in a boiling water bath for extraction. Strain the liquid to get a

clear extract. Take the extract in a small separating funnel and add 10 ml. of petroleum ether. Mix by gentle rotation for 30 seconds. Wash it with 10 ml. of distilled water. Place the funnel on a stand to let it settle. Remove the aqueous acetone layer and discard it. Take petroleum ether layer in a test tube and add 1 gm of solid sodium sulphate and let it settle for a few minutes. Drain the liquid in a small beaker and use it for chromatography.

Save the pigment extract for the observation of fluorescence and determination of absorption spectra of the pigments.

**B. Running the Chromatogram**

1. Fill the specimen jar up to a height of 1 cm with a mixture of petroleum ether : acetone (9:1). Close it with the lid and let it saturate with vapours of the solvent.
2. Cut 20 cm long 2.5 cm wide (or according to the length of the specimen jar) strip of Whatman 1 filter paper. Draw a line at about 2.5 cm with a pencil. Apply the sample on this line carefully using a fine painting brush or by a capillary. Once it is dry apply again at the same line. Repeat the procedure until a fairly good concentration of the sample (a dark green line) is built up. Take care not to spread the sample. It should be a fine green line.
3. To place the strip in the centre of the jar, take a thick square size paper, a few inches bigger in size than the mouth of the jar. Fold it and place the Wharman paper strip between the folds perpendicular to the line of fold. Pin them together with a paper clip. Open the folded side. Now the strip can be hanged in the jar keeping the thicker paper above the mouth. You may use some other device to hang the paper or stick it with gum paper.
4. Open the jar and hang the strip carefully. Make sure that its lower edge is dipped in the solvent layer but the pigment spot remains well above it. The paper should not touch the walls of the jar. Leave the jar undisturbed. Note the solvent front from time to time and allow it to run until the solvant front reaches almost to the top edge of the paper or till the individual components are separated as four sharp bands.
5. Take out the chromatogram carefully and dry it. Mark the solvent front and circle the pigments immediately, because they tend to fade rapidly when dry.

**C. Observation of Fluorescence**

1. Take the acetone extract in a test tube and observe it against transmitted light. What is the colour of the solution?

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2. Now see this solution with light behind you, that is, in reflected light. What colour do you observe?

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**D. Determination of absorption spectra of pigments**

(This will be demonstrated by your counsellor)

## 3.4 RESULTS

Identify the pigments by their colours. Make the diagram of the chromatogram and show the pigments using appropriate colours. (Use pencil or crayons).

Measure the distance from base line to the centre of each spot. Calculate Rf value for each spot.

$$Rf = \frac{\text{distance travelled by the pigment from the start}}{\text{distance travelled by the solvent}}$$

| Pigment | Colour | Rf value |
|---|---|---|
| Chlorophyll *a* | blue green | |
| Chlorophyll *b* | green | |
| β-carotene | Yellow | |
| Xanthophyll | Yellow brown | |

If you obtain any extra bands they could be due to chlorophyllide (green) and phaeophytin (yellow grey) the breakdown products of chlorophyll formed during isolation procedure. Discuss below the problems faced if any in performing the experiment. Make a tracing of the chromatogram in the space provided below.

## 3.5 PRECAUTIONS

1. Hold the chromatogram from an edge only so as to avoid finger marks.
2. Try to get a very small but concentrated line of the pigment.
3. Choose a dim corner for working. Keep pigment solution and the chromatogram away from light because the pigments break down on exposure to light.
4. The jar must be saturated with solvent. Therefore, do not leave it without lid except while you hang the chromatogram. Moreover petroleum ether evaporates very fast, so it would disappear in no time.
5. Do not let solvent mixture run beyond the end of paper.

**SAQ**

1. Why do we add $CaCO_3$ during extraction of pigments.

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2. Why do we add $Na_2SO_4$ in pigment extract?

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3. Predict from the colour of pigments in which region of light they would show absorption peak?

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4. What is the role of carotenes in photosynthesis?

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5. What is the chemical difference in chlorophyll *a* and chlorophyll *b*?

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6. Why are the pigments not soluble in water?

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7. When the aqueous methanol is added to the petroleum ether extract chlorophyll *b* and xanthophyll dissolve in this layer but chlorophyll *a* and carotenes remain in the petroleum ether. What does it show?

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8. Which of the following colours of light works best for photosynthesis?

   i) Green

   ii) Blue and red

   iii) Yellow

   iv) Violet and yellow.

9. The red, orange and yellow brown colours of autumn leaves are due to the presence of:

   i) Chlorophyll *a*

   ii) Chlorophyll *b*

   iii) Carotenoids

10. Why do you observe red colour of the extract with light behind you?

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