Core practical 11: Investigate the presence of different chloroplast pigments using chromatography

Objective

- To separate and identify photosynthetic pigments from leaf chloroplasts

Safety

- Petroleum spirit is highly flammable, may cause severe lung damage if swallowed and is an irritant. Vapour may cause drowsiness or dizziness. Wear eye protection. Extinguish any naked flames close by. Do not inhale fumes, stopper containers and use in a well-ventilated area.
- Propanone is highly flammable and is an irritant to eyes and skin. Vapours may cause drowsiness and dizziness. Wear eye protection. Extinguish any naked flames close by. Do not inhale fumes. Use in small amounts in a well-ventilated area.

Specification links

- Practical techniques 1, 3, 7, 8
- CPAC 1a, 2a, 2b, 3a, 3b, 4a, 4b, 5b

Procedure

Chromatography is a separation process that relies upon the differential distributions of a mixture between a mobile liquid phase (the chromatography solvent) and a stationary solid phase (in this case, paper).

1. Take a piece of chromatography paper of a suitable size to fit the full length of a boiling tube without touching the sides. Draw a pencil line about 25 mm from the bottom edge.
2. Wearing eye protection, grind a few leaves in a mortar with a maximum of 10 cm^3 of propanone to extract the pigments. The extract should be as concentrated as possible. Work quickly to minimise evaporation.
3. Push any pieces of plant material to one side with a spatula. Use a fine pipette tip or capillary tube to take up a small amount of extract. Place one small drop of this extract in the centre of the pencil line and allow to dry before adding another drop on top. Build up a pigment spot that is as small as possible but dense enough that it contains sufficient pigment. Avoid touching the chromatography paper as fingerprints may interfere with the solvent movement. Begin step 4 while you wait for the spots to dry.
4. Wearing eye protection, carefully pour the chromatography solvent into a boiling tube to a depth of no more than 1 cm. Insert the bung.

Notes on procedure

- As an alternative to this practical, you could carry out an investigation using thin layer chromatography by following one of the many suitable protocols available online. This procedure instead uses paper chromatography, which relies on materials that are cheaper and more readily available.
- It is good practice to ask students to carry out some research and write a risk assessment for this practical, particularly regarding the use of the solvents.
- It is preferable for students to carry out the chromatography individually. The extraction stage can be done in groups as each extract should provide enough for several students. Class results are more interesting if each group uses a different type of leaf.
- It should be possible for students to take the pigment extract straight from the mortar, but if it proves difficult to prevent contamination with lumps of solid matter it may be necessary to filter the extract through muslin.
- Pigments may be more visible if the chromatogram is run in the dark or if tubes are covered with foil to avoid degradation by sunlight.
- Chromatography solvent is very toxic to aquatic organisms. Do not allow students to pour waste into the sink.

5. When the chromatography paper is ready, suspend it inside the boiling tube by pinning it to the underside of the bung or by trapping it in a split bung. The bottom of the paper should be dipped into the solvent but the pigment spot must not be immersed in the solvent at any time.
6. The solvent front should rise up the paper, evenly separating out different pigments. When it is close to the top of the tube after around 10 minutes, remove it from the solvent and quickly mark the position of the solvent front using a pencil. Allow the chromatogram to dry in a well-ventilated area, preferably in the dark.
7. When dry, examine the chromatogram. Calculate the R_f values for each pigment using the procedure shown in fig A.

Answers to questions

1. The R_f value of a particular substance should always be the same provided the chromatogram is treated in the same manner in each case. Different paper, solvent and running conditions may affect the R_f values. While the values in this table are for the same solvents, there may be slight differences.
2. It would be more accurate to use pure extracts of the pigments as reference standards and test them alongside the leaf extract in an identical chromatography procedure then compare R_f values.
3. Some substances do not move at all because they are insoluble in the mobile phase/solvent.
4. Each of the pigments absorbs and captures energy from light from particular areas of the spectrum. As a result, far more of the energy from the light falling on the plant can be used than if only one pigment was present.

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All the maths you need

- Use ratios, fractions and percentages.

Equipment

- pestle and mortar
- boiling tube and bung with pin
- boiling tube rack
- measuring cylinder
- spatula
- pencil and ruler
- fine tube for pigment loading
- a few leaves
- chromatography paper
- propanone (pigment extraction solvent)
- chromatography solvent (1 : 9 mix of propanone : petroleum spirit)

Diagram

$$R_f \text{ value} = \frac{\text{distance travelled by solute (photosynthetic pigment)}}{\text{distance travelled by solvent}}$$

R_f for (A) $= \frac{a}{x}$

R_f for (B) $= \frac{b}{x}$

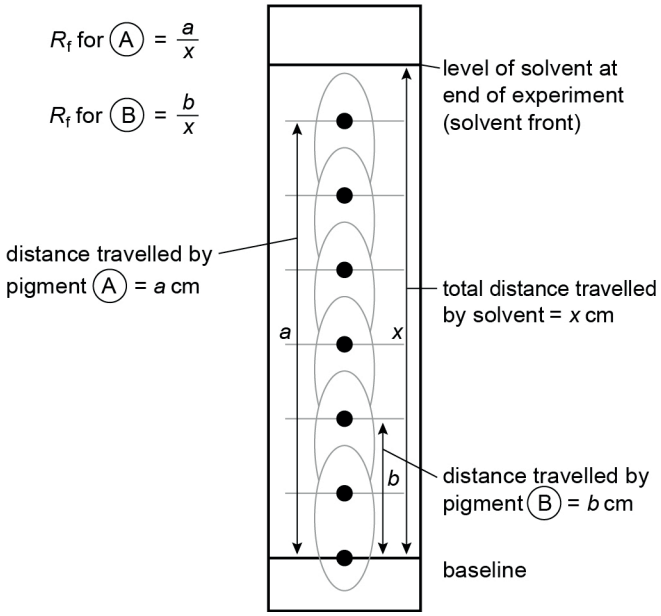

fig A An example of how R_f values are calculated.

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3. Push any pieces of plant material to one side with a spatula. Use a fine pipette tip or capillary tube to take up a small amount of extract. Place one small drop of this extract in the centre of the pencil line and allow to dry before adding another drop on top. Build up a pigment spot that is as small as possible but dense enough that it contains sufficient pigment. Avoid touching the chromatography paper as fingerprints may interfere with the solvent movement. Begin step 4 while you wait for the spots to dry.
4. Wearing eye protection, carefully pour the chromatography solvent into a boiling tube to a depth of no more than 1 cm. Insert the bung.
5. When the chromatography paper is ready, suspend it inside the boiling tube by pinning it to the underside of the bung or by trapping it in a split bung. The bottom of the paper should be dipped into the solvent but the pigment spot must not be immersed in the solvent at any time.
6. The solvent front should rise up the paper, evenly separating out different pigments. When it is close to the top of the tube after around 10 minutes, remove it from the solvent and quickly mark the position of the solvent front using a pencil. Allow the chromatogram to dry in a well-ventilated area, preferably in the dark.
7. When dry, examine the chromatogram. Calculate the R_f values for each pigment using the procedure shown in fig A.

Analysis of results

1. Record your results in a table, noting down a description of the colour of each pigment spot, distance from the baseline and R_f value. Use the table below to identify pigment spots that have a similar R_f value.

Pigment	Colour of spot	R_f value
carotene	yellow-orange	0.95
phaeophytin	grey-yellow	0.83
xanthophyll	yellow-brown	0.71
chlorophyll a	blue-green	0.65
chlorophyll b	light green	0.45

table A Appearance and R_f values for some common chloroplast pigments separated using propanone and petroleum spirit solvent.

Learning tip

- Remember that R_f values simply describe a ratio of the distance travelled by a pigment compared to the solvent front. So if a pigment travelled half the distance of the solvent front the R_f value will be 0.5. As they are ratios, R_f values are written without units.

Questions

1. It is likely that you obtained R_f values that were similar, but not identical, to published values or to the values provided. Explain why this might be the case.
2. What approach would be preferable to using published R_f values for comparison?
3. Explain why some substances do not move up the chromatography paper at all.
4. Explain why chloroplasts usually contain several different photosynthetic pigments.

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- Propanone undergoes dangerous reactions with organohalogens, nitric(V) acid and other oxidising agents.

Equipment per student/group	Notes on equipment
pestle and mortar	One per group
boiling tube and bung with pin	One per student. The stopper should be split or have a pin to allow vertical suspension of the chromatography paper underneath the stopper without touching the sides of the tube.
boiling tube rack	One per student
measuring cylinder	One 10 cm^3 measuring cylinder per student
spatula	One per student
pencil and ruler	One per student
fine tube for pigment loading	One fine pipette, very fine plastic pipette tip or piece of capillary tubing per student
a few leaves	Fresh leaves of plants such as nettle and spinach give good results, but leaves from plants with coloured or variegated leaves make an interesting comparison.
chromatography paper	One strip per student to fit the full length of a boiling tube without touching the sides. Time and paper are saved if this is cut to size in advance.
propanone (pigment extraction solvent)	90% propanone, approximately 10 cm^3 per student
chromatography solvent (1 : 9 mix of propanone : petroleum spirit)	90% propanone : petroleum spirit (100 °C–120 °C); 1 : 9 by volume. Approximately 5 cm^3 per student. Label as highly flammable. The solvent is very toxic to aquatic organisms and may cause long-term adverse effects in the aquatic environment. Collect waste solvent and dispose of it appropriately.
hairdryer	This will be useful for drying spots if available.
muslin	Have muslin available for students to filter the plant extract if necessary.

Notes