

Practical Booklet 8
Separation of leaf pigments by chromatography
Cambridge International AS & A Level
Biology 9700

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Introduction

Practical work is an essential part of science. Scientists use evidence gained from prior observations and experiments to build models and theories. Their predictions are tested with practical work to check that they are consistent with the behaviour of the real world. Learners who are well trained and experienced in practical skills will be more confident in their own abilities. The skills developed through practical work provide a good foundation for those wishing to pursue science further, as well as for those entering employment or a non-science career.

The science syllabuses address practical skills that contribute to the overall understanding of scientific methodology. Learners should be able to:

1. plan experiments and investigations
2. collect, record and present observations, measurements and estimates
3. analyse and interpret data to reach conclusions
4. evaluate methods and quality of data, and suggest improvements.

The practical skills established at AS Level are extended further in the full A Level. Learners will need to have practised basic skills from the AS Level experiments before using these skills to tackle the more demanding A Level exercises. Although A Level practical skills are assessed by a timetabled written paper, the best preparation for this paper is through extensive hands-on experience in the laboratory.

The example experiments suggested here can form the basis of a well-structured scheme of practical work for the teaching of AS and A Level science. The experiments have been carefully selected to reinforce theory and to develop learners' practical skills. The syllabus, scheme of work and past papers also provide a useful guide to the type of practical skills that learners might be expected to develop further. About 20% of teaching time should be allocated to practical work (not including the time spent observing teacher demonstrations), so this set of experiments provides only the starting point for a much more extensive scheme of practical work.

Guidance for teachers

Aim

To separate and identify the pigments present in leaves using chromatography.

Outcomes

Syllabus sections 13.1 (e)

Skills included in the practical

A Level skills	How learners develop the skills
Analysis	Calculate R_f values
Evaluation	Evaluate the methods used and their effect on the accuracy and reliability of the results
Conclusions	Draw conclusions about the pigments shown in leaves of different species and different colours Use scientific knowledge to explain why leaves have these pigments

This practical provides an opportunity to build on essential skills introduced at AS Level.

AS Level skills	How learners develop the skills
MMO collection	Measure distance moved by pigment
PDO recording	Record quantitative results appropriately in a table

Method

Safety glasses must be worn when preparing the slide.

The room should be well ventilated and an extraction cabinet used if available.

- Leaves appear green due to the presence of chlorophyll but contain other pigments that are hidden by the colour of chlorophyll. Chromatography is a technique used to separate mixtures of chemicals by their solubility. There is a support layer, the paper, on which the mixture to be separated is placed. Chemicals in the mixture dissolve in the solvent, propanone, and are carried through the support layer. The support layer interacts with the solvent and the dissolved chemicals, attracting the chemicals in the solvent onto the support layer. The least soluble chemicals are left on the support first, the most soluble last.
- During this investigation learners will use chromatography to separate and identify the pigments in leaves.
- Learners should be introduced to the principle of chromatography by:
 - asking questions about their experience of what happens when water is spilt against the edge of dry paper or cloth
 - demonstrating what happens when a spot of black ink (not biro) is placed at one end of a piece of filter paper and then dipped into water.

Guidance for teachers, *continued*

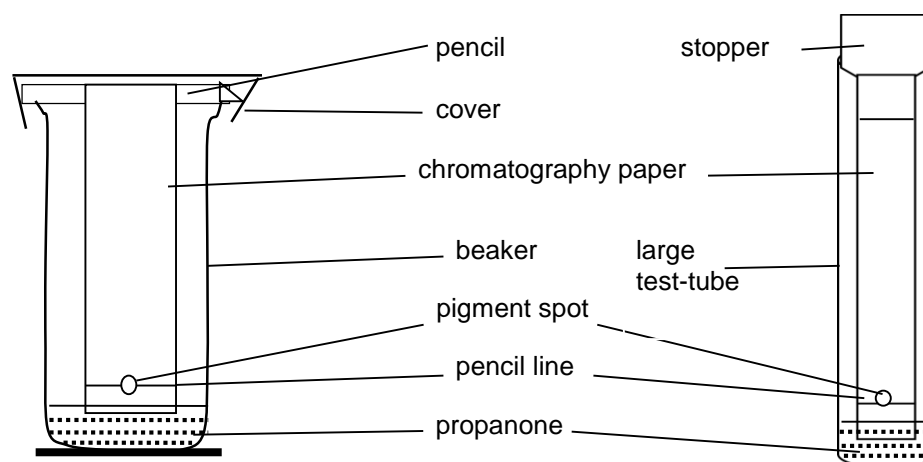
- Learners are asked to make a leaf extract by cutting leaves into small pieces and then grinding in a mortar and pestle with a small quantity of propanone (acetone). A variety of leaves can be used. Soft leaves are easier to grind and dark green leaves usually give better extracts. If there are local species that have red or orange coloured leaves these can be used for comparison. This activity provides an opportunity for learners to experience the difficulty of breaking open plant cells and to understand why it is necessary. If the leaves are very thick, a small quantity of sand can be added to the mortar.
- Learners will then filter their extract. If too much propanone has been used the extract will be too dilute and will need to be concentrated. This can be done in hot water, but as propanone is flammable and has irritant vapour this should only be done in an extractor cabinet and with no naked flames in the room.
- Learners will then place one or more samples of their leaf extract onto paper. Chromatography paper (Watman no.1) gives a good separation, but standard filter papers used in filter funnels or coffee filters can be used. For small scale chromatography, strips of filter paper that fit inside a large test-tube can be used. These are suitable for one pigment sample. Wider pieces of filter paper that fit inside a 250 cm³ beaker can be used for two or three samples. To place samples onto filter paper learners should:
 - draw a line in pencil about 15 mm from one end of the filter paper
 - make a pencil mark on this line where the samples are going to be placed. This is the origin. For single samples this should be in the centre. For two or more samples these should be at least 20 mm from the edge and evenly spaced, at least 20 mm apart
 - use a capillary tube to place a spot of extract on the marked places. Spots should be no more 2 mm in diameter
 - leave the spot to dry and then place another spot on top of the first and leave to dry. At least 5 spots are needed, more if the extract is dilute.

Thin layer chromatography can also be used for comparison if facilities are available for making thin layers of cellulose powder onto one surface of glass microscope slides.

- After spotting the chromatography paper learners will place it into the solvent to run. To do this, learners should place propanone into a container to a depth of 10 mm. The paper is then placed into the container so that the end with the leaf sample is just touching the propanone. Learners must be instructed to make sure that the propanone does not go above the level of the pencil line. If a large test-tube is used, the top edge of the paper can be folded over the edge of the tube and a tightly fitting stopper inserted. If a beaker is used, the free edge of the paper can be folded over a pencil laid across the beaker and then covered by metal foil or parafilm.

Guidance for teachers, *continued*

Diagram of apparatus ready to use



- The apparatus should then be left to run so that the propanone rises up the paper and carries the different pigments with it. Learners should be instructed to watch the separation and to observe the order in which each of the different colours appear. They should not move the container during this time. Learners should also be instructed to make sure that the propanone does not run off the end of the paper. When they see the rising edge of the propanone (the solvent front) about 10 mm from the end of the paper, they should lift the paper from the solvent and draw a pencil line at the solvent front. The waiting time provides an opportunity to discuss why pencil is used for marking chromatography paper, why the spots must be concentrated and how the propanone (solvent) is able to separate out the pigments.
- The separation will take between 20 – 30 minutes depending on the type of paper used for separation. Coffee filters will be the fastest, but give the least separation.
- Removing the chromatograms must be done in a well-ventilated area and the paper left to dry. This is best carried out in an extractor cabinet, but if this is not available then the papers can be hung to dry in an open, well-ventilated space. The containers with propanone should be recovered and left in a safe place for safe disposal.
- Propanone can be flushed down a sink using a lot of water. Crushed leaves can be wrapped in paper and left outside or in an extractor cabinet until all the propanone has evaporated and then disposed of with normal waste.

Guidance for teachers, *continued*

Results

- Once the paper is dry, learners are instructed to use pencil to draw around the shapes of the coloured spots and to record the colours. This needs to be done immediately as the colours often fade within 24 hours. At least three spots should be seen, but a good separation should give up to six. Dark yellow or orange carotenes should be close to the solvent front, blue-green chlorophyll a and green chlorophyll b should be next in sequence, yellow xanthophyll is closest to the starting line. A good separation will give two xanthophyll spots and a grey-green phaeophytin spot between carotene and the chlorophylls.
- Learners are then instructed to calculate the R_f (retention values). This provides an opportunity for discussion about R_f values and how they can be used to identify specific compounds because every compound has a specific R_f value in every specific solvent.

Learners are instructed to measure from the origin to the solvent front and then to the centre of each pigment spot and use these to calculate the R_f value.

$$R_f = \frac{\text{distance moved by pigment spot}}{\text{distance moved by solvent (propanone)}}$$

Measurements of R_f are likely to be inaccurate as spots tend to spread and 'trail' at the edges. Learners are then instructed to measure to the front of a spot and to the back of the same spot and take the average of these as the position of the spot.

- If different leaves are used then R_f values can be compared. There should be the same pigments, whatever the type of leaf. Leaves with a colour other than green may have additional pigments, but carotene and chlorophylls should be present.

This could also be an opportunity to introduce the idea of 2-dimensional chromatography to separate compounds that have the same solubility in one solvent but a different solubility in a different solvent.

Interpretation and evaluation

There is an opportunity to discuss results and the reason why learner's results may not match the expected results. For propanone these are:

<i>Carotene</i>	0.95
<i>Chlorophyll a</i>	0.60
<i>Chlorophyll b</i>	0.50
<i>Xanthophyll</i>	0.35

Learners should discuss possible improvements, such as better application of spots, longer chromatography paper to give more time and distance for separation and different supporting material. This provides an opportunity to introduce other types of chromatography, for example, silica gel (thin layer chromatography), column chromatography using cellulose powder, solvents that are mixtures and two-way chromatography.

Other uses of chromatography could also be discussed, for example using two-way chromatography to work out the sequence of reactions in the light independent stage of photosynthesis.

Information for technicians

Each learner will require:

- propanone in closed containers, labelled **Highly Flammable Irritant.[H]** Nail varnish remover can be used as an alternative to propanone. The volume required will depend on the container used for chromatography. **[F]**
- 5 – 10 fresh leaves.
- 1 × pair of scissors
- 1 × mortar and pestle
- 2 or 3 × 10 cm length capillary tubing or ignition tubes
- large test-tube or small beaker. Transparent plastic drinking glasses can be used Expanded polystyrene should not be used.
- 1 x Pasteur pipette
- Watman® no.1 chromatography paper, or filter paper or coffee filter
- metal foil or parafilm for covering chromatography container
- support for large test-tube if required, e.g. a rack or clamp stand, boss and clamp.
- 1 × pair of safety glasses
- 1 × 15 or 30 cm ruler, marked in mm
- 1 x pencil
- 1 x container for disposal of leaves and used propanone
- Access to hot water if required

Additional instructions

An extractor cabinet or fume cupboard should be used if possible to run the chromatograms. Otherwise a well-ventilated room with windows that open is necessary. Air conditioning should not be used. There should not be any naked flames in the area.

Capillary tubes can be made from glass tubing by heating the centre of a length of glass tubing until it softens and then pulling out. The thin tubing and then cut into sections. These should be made before the practical. Heat proof gloves and eye protection should be worn.

Hazard symbols

C = corrosive substance

F = highly flammable substance

H = harmful or irritating substance

O = oxidising substance

N = harmful to the environment

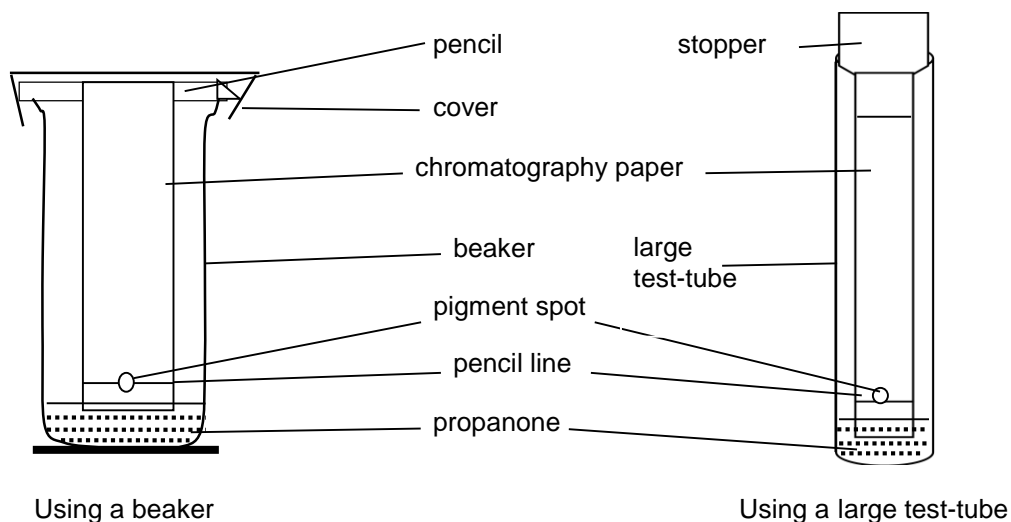
T = toxic substance

Worksheet

Aim

To separate and identify the pigments present in leaves using chromatography.

Apparatus



Method

You are using propanone, which is a highly flammable and irritant chemical. You must make sure there are no naked flames in the room at any time. You should try not to breathe too many fumes and keep containers with propanone covered.

Safety glasses must be worn when preparing the slide.

1. Cut up some leaves into small pieces.
2. Put the leaf pieces into a mortar to about 2 cm depth.
3. Add about six drops of propanone using a Pasteur pipette.
4. Grind the mixture with a pestle for at least 3 minutes. If the leaves are very thick, add a small quantity of sand to help the cells to break.
5. Cut a strip of chromatography paper to fit into a large test-tube or small beaker.
6. Draw a pencil line 15 mm from one end of the chromatography paper.
7. Using a pencil, mark places on this line where the leaf extract will be placed. This is the origin.
8. Use a capillary tube to draw up liquid from around the crushed leaves.
9. **Touch** the capillary on to the origin mark on the chromatography paper. The leaf extract will flow onto the paper. You must keep the spot as small as possible (no more than 2 mm).
10. Allow the spot to dry and then add another spot on top.
11. Add 5 more spots of solution, letting each one dry before putting on the next. This should give a very concentrated small spot on the paper.
12. Put propanone into a large test-tube or small beaker until there is a 10 mm depth.

Worksheet, *continued*

13. If you are using a large test-tube:

- place it into a rack or other support. Lower the chromatography paper into the test-tube with the origin towards the propanone. The end of the paper should just touch the propanone. Do not allow it to rise above the origin. Fold the other end of the paper over the edge of the test-tube and insert a stopper. Try not to move the test-tube.

If you are using a beaker:

- fold the top end of the paper over a pencil and lower the paper into the beaker with the origin towards the propanone. The end of the paper should just touch the propanone. Do not allow it to rise above the origin. Cover the beaker with metal foil or parafilm. Try not to move the beaker.

14. Leave the apparatus to allow the propanone to rise up the paper and separate the pigments in the leaf extract. This is called 'running a chromatogram'.

15. Observe the order in which the different colours of pigment appear.

16. When the propanone is about 10 mm from the top of the paper, remove the paper and mark the position of the solvent. This is the solvent front.

17. Leave the chromatogram to dry.

18. The crushed leaves and the used propanone must be disposed of safely.

Results

1. Outline the shape of each spot you can see in pencil and label the colour. The colours fade very quickly so you should do this as soon as the chromatogram is dry.

2. Identify each pigment by its colour.

3. Find the R_f value of each pigment on your chromatogram.

- Measure from the origin to the solvent front
- Measure from the origin to the centre of each pigment spot
- Calculate the R_f value.

$$R_f = \frac{\text{distance moved by pigment spot}}{\text{distance moved by solvent (propanone)}}$$

If your spots have spread, to find the centre measure to the front of a spot and to the back of the same spot. The average of these is the centre of the spot.

Compare your results to the results from other species of leaf and describe the similarities.

Interpretation and evaluation

1. Draw conclusions about the different pigments observed in your leaf and those shown in the leaves of different species. Explain why leaves have these pigments.

2. Review the procedure used and identify sources of error. Make suggestions about improvements. This could include using other solvents and other types of chromatography.

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