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Thin layer chromatography

Photosynthetic pigments from kiwi fruit

Aim

The aim of this practical activity is to extract, separate and identify photosynthetic pigments from kiwi fruit. Unlike similar methods, it uses a very small volume of solvent to extract the chloroplast pigments, and is therefore safer and cheaper to carry out.

Introduction

Many fruits contain chloroplasts but these, and the chlorophylls they contain, are usually broken down as the fruit ripens. Green-fleshed kiwi fruit of the variety 'Hayward' (the main variety sold in the UK) are unusual however, because even when they are ripe, their chloroplasts remain intact. In contrast, the newer yellow-fleshed cultivars (so-called 'Golden kiwi fruit') degrade the chlorophyll during ripening and the consequent loss of the green color uncovers the underlying yellow carotenoid pigmentation.

Photosynthetic pigments can be extracted from kiwi fruit chloroplasts by breaking up the fruit tissue in a suitable solvent. The different pigments can then be separated by thin layer chromatography, using a different solvent mixture.

You should be able to separate five or more different pigments, and by calculating R_f values, it should be possible to identify each pigment.

FROM CROWHURST, ET AL (2008)



Fruit diversity in the genus Actinidia. Only green-fleshed varieties retain chloroplasts and photosynthetic pigments in the ripe fruit.

From: Crowhurst, et al (2008)

Rf values

Rf values can be used to identify the pigments on a chromatogram. An Rf value is a ratio, calculated as follows:

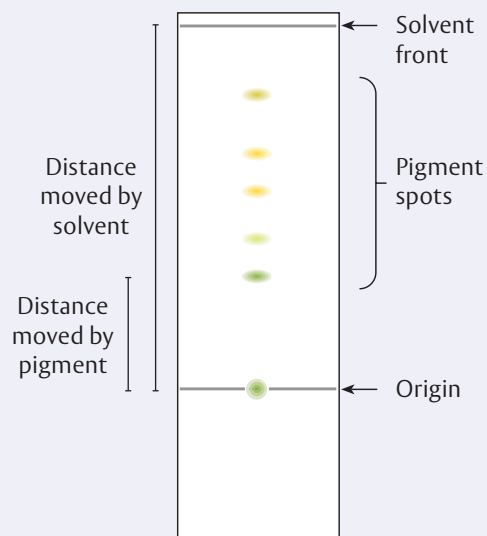
$$\frac{\text{distance moved by pigment}}{\text{distance moved by solvent}}$$

Rf values always lie between 0 and 1 (0 being a pigment that doesn't move at all and 1 being a pigment that is so soluble, it moves the same distance as the solvent).

Because for a given pigment, the Rf value will vary according to the solvent (or mixture of solvents) used, Rf values are often written with the name(s) of the solvent(s) and the proportions of them after the number. For example:

Rf = 0.24 (60% ethanol)

Rf = 0.78 (cyclohexane : propanone : ethoxyethane [5 : 3 : 2])



Safety guidelines

The extraction and chromatography solvents are both highly flammable, so they **MUST NOT** be used near naked flames.

Splashes of the solvent can cause **SEVERE EYE DAMAGE**, so you **MUST** wear eye protection throughout the practical procedure.



Some people are allergic to kiwi fruit. If you are, you should **NOT** carry out this practical activity. Alternative suggestions are given by *Science and Plants for Schools* (see Teacher's and Technician's notes).

Equipment and materials

Needed by each person

Equipment

- Eye protection (safety spectacles or goggles)
- Plastic pipette tip OR a fine-tipped glass Pasteur pipette
- 1 cm³ plastic pipette (not a syringe)
- 1.5 cm³ microcentrifuge tube
- *Plasticine*[®], *BluTack*[®] or a rack for standing the microcentrifuge tube vertically
- Knife
- Pair of forceps
- Ruler
- Sharp pencil with a fine point

Materials

- Slice of kiwi fruit, in a plastic bag to stop it from drying out
- Approximately 1 cm³ of extraction solvent in a closed glass container
- Approximately 2 cm³ of chromatography solvent in a closed glass container
- Strip of thin layer chromatography (TLC) sheet, cut to fit inside the bottle that contains the chromatography solvent.

The **extraction solvent** consists of 3 parts propanone (also called propan-2-one or acetone) plus 2 parts ethoxyethane (also called diethyl ether or ether).

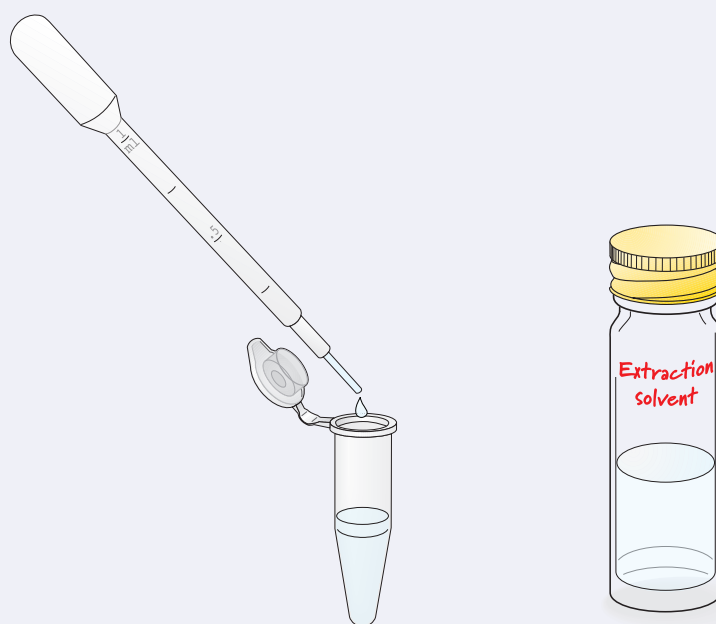
The **chromatography solvent** consists of 5 parts cyclohexane, 3 parts propanone (also called propan-2-one or acetone) and 2 parts ethoxyethane (also called diethyl ether or ether).

Procedure



- 1 Before you start, put on the eye protection (safety glasses or goggles).
- 2 Using the 1 cm³ pipette, carefully add **extraction solvent** to the microcentrifuge tube to the level shown in Figure 1 (just above the tapered part of the tube).

Fig. 1



DANGER

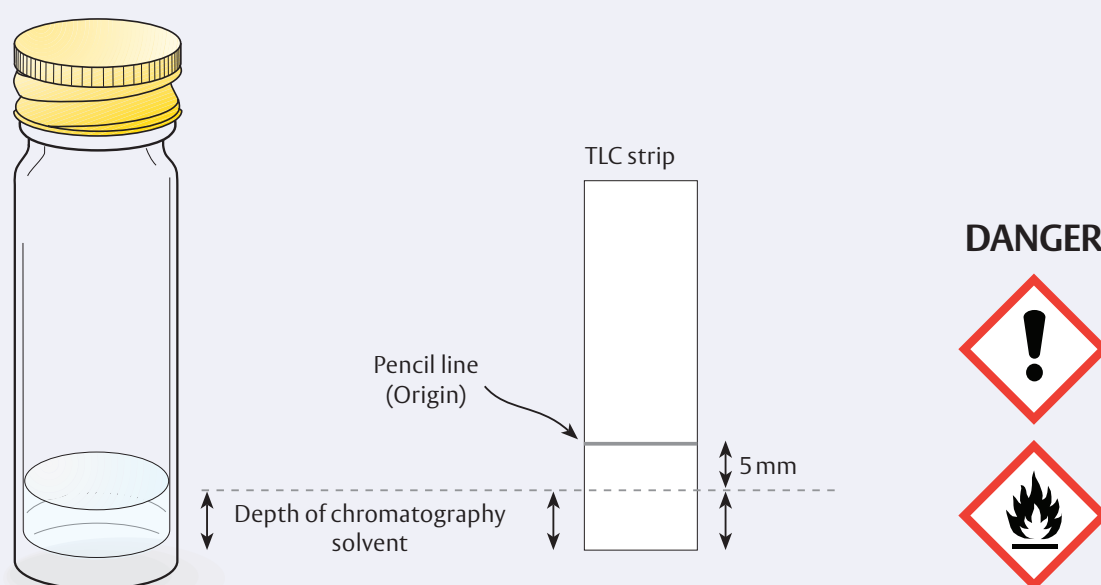


- 3 Close the microcentrifuge tube and the extraction solvent bottle tightly to prevent evaporation of the solvent.
- 4 Take the kiwi fruit out of the plastic bag. Place the kiwi fruit on the bag then use the knife to cut, from the kiwi fruit, a strip of skin at least 30 mm long with a layer of green fruit flesh attached. *The fleshy layer attached to the skin should be approximately 1 mm thick.*
- 5 Cut the kiwi fruit strip into two rectangles, each approximately 5 mm x 15 mm.
- 6 Use the forceps to place the two pieces of kiwi fruit in the microcentrifuge tube.
- 7 With the forceps, squeeze the skin and flesh against the side of the tube. Also squeeze the kiwi fruit pieces between the forceps. *The liquid will slowly develop a green colour.*
- 8 After approximately a minute of squeezing, use the forceps to push the fruit pieces to the bottom of the tube.
- 9 Close the tube and stand it upright on a piece of *Plasticine®* *BluTack®* or in a rack. *After about a minute the denser liquid will sink to the bottom of the tube and a less dense, bright green layer will rise to the top of the tube. Between the two layers of liquid you will see debris from the kiwi flesh.*

- 10 Stand the bottle of chromatography solvent on a level surface. Without opening the bottle, measure and record the depth of the solvent in the bottle.
- 11 **IMPORTANT: In this step, you must be VERY careful not to damage the surface of the thin layer chromatography (TLC) strip. Treat it with TLC (tender, loving, care). If you do damage the surface, however, ask for a new TLC strip.**

Try not to touch the white layer on the TLC strip with your fingers — hold it by its edges. Very carefully, without pressing, draw the pencil tip across the strip, 5 mm higher than the depth of the chromatography solvent, making a line as shown in Figure 2.

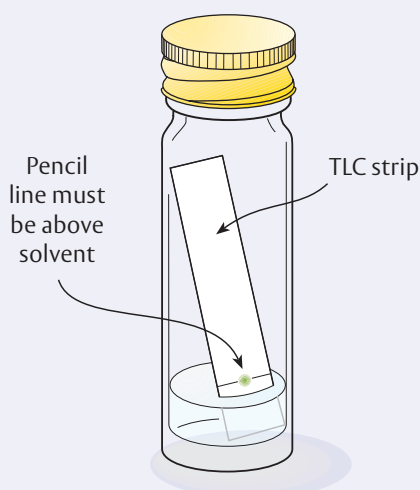
Fig. 2



- 12 Next, take the Pasteur pipette or plastic pipette tip. Open the microcentrifuge tube and place the pipette tip just below the surface of the top green layer of solvent. Some of the green liquid will be drawn into the tip. **Do not let the tip enter the liquid below the top green layer.** Close the microcentrifuge tube.
- 13 Hold the tip of the pipette vertically and briefly touch the middle of the pencil line on the TLC strip with the pipette tip so that a single, small drop soaks onto the white coating. Remove the tip immediately.
- 14 When the spot has dried, place a second drop of the green kiwi extract on the same spot. Continue adding spots of liquid, **waiting for each spot to dry after application** until you have a small green ring 3–5 mm diameter. *You will need to apply about 60–100 drops of kiwi extract.*

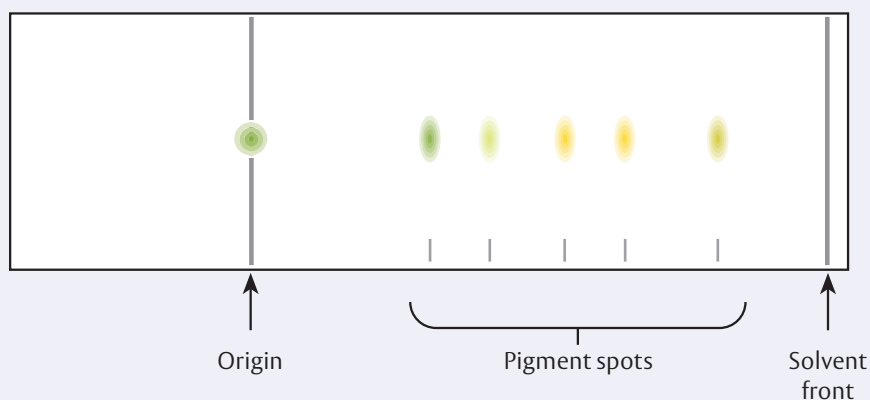
- 15 Remove the cap from the bottle of chromatography solvent. Holding the top of the TLC strip (the end furthest from the green ring you have produced) with forceps, lower the strip very carefully into the bottle. As you do so, check that the ring and pencil line will lie above the level of the solvent (see Figure 3). If it looks as if the ring will be submerged, **stop** and use the plastic pipette to remove a small amount of the chromatography solvent and try again.

Fig. 3



- 16 Ensure that the TLC strip is not touching the sides of the bottle, except where it leans against the bottle at the top. Replace the cap on the bottle and check that the solvent is soaking up the strip.
- 17 When the solvent has reached approximately 2 mm from the end of the strip (this will take several minutes), use forceps to remove the strip from the bottle and **immediately** mark the exact position of the solvent front on the TLC strip with a pencil.
- 18 Replace the cap on the bottle to prevent the solvent from evaporating.
- 19 As quickly and accurately as you can, use a sharp pencil to mark the position of the centre of each different pigment on the TLC strip (see Figure 4).

Fig. 4




- 20 Measure the distance from the origin to the solvent front. Record your result to the nearest 0.5 mm. Record your answer to **1 decimal place**.
- 21 Measure the distances from the origin to the **centre** of each pigment spot to the nearest 0.5 mm. Record the colour of each spot as well. Write your results in a copy of Table 1; if you see more pigment spots than there is space for, extend the table appropriately.

Table 1

Pigment code	Pigment colour	Distance from origin (mm)
A (closest to origin)		
B		
C		
D		
E		

Increasing
distance from
origin



- 22 From your results, calculate the R_f values for each pigment as follows:

$$R_f = \frac{\text{distance moved by pigment}}{\text{distance from origin to solvent front}}$$

- 23 Record your results in a copy of Table 2, below, to **2 decimal places** (extend the table if necessary).

Table 2

Your pigment code	R _f value
A (closest to origin)	
B	
C	
D	
E	

- 24 Table 3 shows Rf values for several different photosynthetic pigments. These values were obtained using the same chromatography solvent as the one you have used. Use the Rf values in Table 3 and your own calculated Rf values to complete a copy of Table 4.

Table 3

Pigment name	Rf value
Xanthophyll	0.40
Chlorophyll B	0.42
Chlorophyll A	0.47
Phaeophytin	0.55
Carotene	0.93

Table 4

Pigment code	Your Rf value	Suggested pigment
A (closest to origin)		
B		
C		
D		
E		

Additional investigations

- 1 Compare chromatograms produced by the green variety of kiwi fruit 'Hayward' with that produced by a golden variety such as 'Gold' or 'SunGold'.
- 2 The *Science and Plants for Schools* (SAPS) website has numerous suggestions for thin layer chromatography of plant pigments (including anthocyanins from coloured leaves, *etc*) see: 'TLC of plant photosynthetic pigments': www.saps.org.uk/secondary/teaching-resources/1347-a-level-set-practicals-tlc

Teacher's and technician's notes

Timing

This practical activity can easily be completed in 40–60 minutes.

Troubleshooting

To obtain good results, it is important that students produce a small, concentrated spot of pigment on the TLC sheet. They will also need to mark the solvent front immediately on removing the TLC sheet from the bottle, as the solvent will evaporate quickly. The pigment spots can also fade rapidly, so the position of these should be marked as soon as possible after marking the solvent front.

Safety

Solvents



In contrast to similar protocols, only small volumes of the solvents are used here, and almost always in containers that are or can be closed.

The solvents used in this activity are highly flammable however and there is a serious risk of the liquid or vapour catching fire. Therefore the solvents **MUST NOT** be handled where there are naked flames.

Ensure that the room is well ventilated and that teachers and technicians are familiar with appropriate ways of putting out fires.



In addition, the solvent can cause severe eye damage, so eye protection **MUST** be worn. Teachers must assess whether students should wear eye protection at all times while in the laboratory (for example, if there is a risk of students who are still carrying out the practical activity splashing solvent onto those who have finished the work).

The solvent bottles supplied to students should be marked with the signal word 'DANGER', a 'Flammable' pictogram (GHS02) and an 'Exclamation mark' pictogram (GHS07).

After the activity, the solvents should be disposed of in accordance with the institution's usual procedures. The solvents **MUST NOT** be emptied down a drain.

Schools in England, Wales and Northern Ireland should refer to CLEAPSS Hazcards 45A and 85 for further guidance on handling and disposing of the solvents. Similar advice is offered to schools in Scotland by SSERC.

Kiwi fruit allergy

Some people are allergic to kiwi fruit. Such people should **NOT** carry out this practical activity. Alternative suggestions are given on the *Science and Plants for Schools* (SAPS) website (see page 10 of this document).

Anaphylaxis campaign has more information about kiwi fruit allergy: www.anaphylaxis.org.uk (look under 'food' in the 'Factsheet' section).



Equipment and materials

Needed by each student

Equipment

- a glass, screw-capped 'universal' bottle (sometimes called a 'wide-mouthed McCartney bottle') or similar. *Note: some plastics will dissolve in the solvent used for chromatography, so it is important to use a glass container, with a lid made of metal or a suitable plastic (one that does not dissolve in the solvent).*
- a newly-sharpened pencil with a fine point (HB is suitable).
- a pair of blunt/coarse forceps (metal, not plastic).
- a plastic (polypropylene) pipette, for handling the chromatography solvent. *Note: A 1 cm³ pipette or similar is suitable: candidates do not need to measure out exact volumes. The tip of the pipette must be able to fit inside the container of chromatography solvent. A plastic syringe is unsuitable for this task because the solvent will dissolve the ink markings on the syringe barrel.*
- a clean 1.5 cm³ microcentrifuge tube ('Eppendorf' type).
- a piece of Plasticine® or BluTack® or a rack so that the microcentrifuge tube can be stood vertically.
- a clean plastic (polypropylene) micropipette tip (e.g., a non-graduated 100 µL tip) OR a clean fine-tipped glass Pasteur pipette OR a drawn melting point tube.
- a knife (a plastic 'disposable' picnic-type knife is suitable).
- a ruler for measuring millimetre lengths.
- eye protection (safety spectacles or goggles).

Materials

- a transverse slice of freshly-cut, ripe, kiwi fruit, approximately 6 mm thick, provided in a plastic bag. *Note: This MUST be of the species *Actinidia deliciosa*, var. 'Hayward'. Almost all kiwi fruit sold in the UK are of the 'Hayward' variety, and several supermarkets label them as such. It is essential to use this particular variety as it is the only one that contains intact chloroplasts in the fruit. Once the fruit has been cut, the photosynthetic pigments in the tissue degrade rapidly, so it is important to slice the fruit no more than an hour or so before the practical task starts. Recently-bought kiwi fruit or those that have been stored in a fridge for a week or so will be fine for this task, but if the uncut kiwi fruit are very hard to the touch, they should be left for a few days at room temperature to ripen.*
- a strip of thin layer chromatography (TLC) sheet, cut to fit inside the glass screw-capped container. *Note: Typically, a rectangle of TLC sheet 15 mm x 75 mm is required. The long edges of the strip must NOT touch the inside of the glass container. The white coating on the TLC plate can easily be scratched off, so it must be cut and handled with care. It is an important precaution to wear disposable gloves when cutting the sheet, to prevent oils from your skin from contaminating it. (The students do not need gloves: they will be told to handle the strip by its edges only.)*
- approximately 1 cm³ of freshly-prepared extraction solvent in a closed glass container labelled with appropriate safety warnings*. The extraction solvent consists of 3 parts propanone (also called propan-2-one or acetone) plus 2 parts ethoxyethane (also called diethyl ether or ether). *Note: No alternative solvents should be used, as the procedure will not give the expected results if they are. For example, **do not** use petroleum ether.*
- approximately 2 cm³ of freshly-prepared chromatography solvent in a closed glass container, labelled with appropriate safety warnings*.

The chromatography solvent consists of 5 parts cyclohexane, 3 parts propanone (also called propan-2-one or acetone) and 2 parts ethoxyethane (also called diethyl ether or ether).

* it is advisable to use paper labels on the bottles, as the solvent may wash away ink applied directly to the glass or lid of the bottles. See page 8 of this document for safety guidance.

Specimen results

The photograph on the right shows a typical chromatogram obtained using this method.

Suppliers

TLC Sheets

To obtain the same R_f values as those in Table 3, it is essential to use *Macherey-Nagel DC Polygram*[®] SIL G/UV254 TLC sheets. These are available from Fisher Scientific in packs of 50 sheets (Product code: 10640493). Each sheet measures 50 x 200 mm.

Fisher Scientific UK Ltd
Bishop Meadow Road
Loughborough
LE11 5RG

T: 01509 555 500
W: www.fisher.co.uk

NCBE TLC Pack

The NCBE provides a pack of materials with TLC sheets, microcentrifuge tubes and pipette tips that can be used for this practical activity. See: www.ncbe.reading.ac.uk

Additional information

Crowhurst, R.N. *et al* (2008) Analysis of expressed sequence tags from *Actinidia*: applications of a cross-species EST database for gene discovery in areas of flavor, health, color and ripening. *BMC Genomics* **9**, 351. The electronic version of this article can be accessed free-of-charge at: www.biomedcentral.com/1471-2164/9/351

Tomkins, S.P. and Miller, M.B. (1994) A rapid extraction and fast separation of leaf pigments using thin layer chromatography. *School Science Review* **75** (273) 69–72.

Science and Plants for Schools (SAPS) has numerous suggestions for TLC of pigments from other plant materials: 'TLC of plant photosynthetic pigments': www.saps.org.uk/secondary/teaching-resources/1347-a-level-set-practicals-tlc

