A rapid extraction and fast separation of leaf pigments using thin layer chromatography

Stephen P Tomkins and M Barry Miller

INTRODUCTION

The extraction of leaf pigments and their separation by chromatography has been a long standing practical investigation in school science. This note describes a more rapid extraction and faster separation method. The technique described by Roberts [1] is that most commonly employed in schools and is based on the method described originally in Nuffield A Level Biology Laboratory Guide *Maintenance of the Organism* (1970)[2]. With these methods, pigment extraction and separation takes 1½ to 2½ hours. Using short run paper chromatograms, five pigments are clearly revealed. Valadon et al [3] described a large-scale paper chromatography technique that will separate clearly up to nine distinct pigments or pigment products. However, here the solvent running time is one to two hours and because of its large paper and tank size scale it could not realistically be done as an individual investigation.

These previously published procedures are protracted because of the large-scale grinding of plant material by pestle and mortar, the need to remove water from the extract, the time spent on pigment concentration and the relatively slow separation of pigment by paper chromatography. An additional problem is caused by the fact that large volumes of propanone necessitate the use of a fume cupboard. The procedure described here is one that has several notable advantages.

- It uses a fresh actively photosynthesizing green plant. For students this lends an immediacy to the activity, going from a living green plant to leaf pigment separation in just a few minutes.
- Extraction is done on a micro-scale and does not employ large volumes of propanone or a fume cupboard. No pestle and mortar nor acid washed sand are needed for the principal techniques outlined.
- Pigment concentration is done on a micro-scale, with a hair dryer, using a fine paint brush to deliver the extracted and concentrated pigments to the chromatogram.
- The chromatogram is run on a thin-layer plate of silica-gel in just four minutes or less; this is much faster than with paper. Silica gel TLC sheets are not cheap but this investigation is on a small enough scale to be economical.
- The chromatographic separation is vastly better than with a short run by paper chromatography. Each pigment separates out as a clear band within a few seconds.
- The whole process can be completed easily in the space of twenty minutes, thus allowing far more time for class discussion of the results, or indeed, extension work on the individual pigments themselves.

The investigation of leaf pigments is a challenging one for students. They may be led to asking such questions as - how many pigments are there in a leaf, whether they are all green, what the different pigment molecules are and what wavelengths of light they reflect and absorb etc.

In this investigation the juice squeezed out from fresh green leaves is analysed following the dissolving of the pigments with propanone. The few drops of propanone extract must be evaporated to dryness with any remaining water eliminated completely by evaporation before re-dissolving the pigments again in more propanone prior to spotting. The different distances run by the pigments on the chromatogram, relative to the solvent front (R_f values), may be calculated. R_f values are dependent on the chromatographic medium, solubility of the solute and on the solvent used. R_f values will remain constant if these variables are not altered. We recommend two possible solvents with slightly different R_f results and separations. Both produce clear separation of about seven pigment spots. For this small-scale work we strongly recommend the use of discarded 35 mm film canisters as stoppered containers. These are obtainable from high street photographic processing shops, free on request. The clear Fuji cans are best and their lids make excellent small watch glasses. We recommend either a very fine paint brush for spotting or a very fine capillary tube.

APPARATUS RECOMMENDED FOR EACH STUDENT

Two or three young wheat plants or some fresh green leaves of grass (wheat is easily grown from seed in under two weeks under good light)
2 glass microscope slides (to act as leaf scrapers)
1 flat-ended glass rod
1 glass teat pipette
2 cm^3 propanone (easily given out in a closed clear Fuji film cassette can)
2 very small watch glass (or ideally Fuji film canister lids)
1 electric hair-dryer (two or three to a class of twenty would do) for wheat extract, (or 1 pestle and mortar for nettle extract)
1 very fine watercolour paint brush (good ones with a fine point are expensive and, if not borrowable, retails at £1) or a fine glass capillary tube
1 or more TLC chromatography strips, 1.25 × 10 cm (eight of these may be cut from one 5 × 20 cm plastic sheet of thin-layer silica-gel chromatography plate, using good scissors or a Stanley knife and straight edge. POLYGRAM SIL G 0.25 mm layer from CAMLAB Ltd, Nuffield Road, Cambridge CB4 1TH, tel: 0223 420856 or Philip Harris at approximately 60p per sheet, making the unit price about 7p per chromatogram. Using strips cut even smaller to 1 × 6.7 cm further reduces this cost to 4p.)
1 glass specimen tube 2 × 10 cm
1 cork to fit the tube, with a horizontal V-slit below (see Figure 3)
1 marker pen and a pencil
5 cm^3 chromatography running solvent in a clear capped Fuji film can (because of differential evaporation of the solvents in the mixture this must be stored capped. Better still the solvent is mixed *immediately* before use).
Recommended solvents are:
Either:

1,1,1-trichloroethane($CH_3.CCl_3$)	5 parts
Propanone ($CH_3.CO.CH_3$)	3 parts
Petroleum ether (BP 40° to 60 °C)	2 parts

or,

Propanone	1 part
Petroleum ether (BP 30° to 40 °C)	2 parts

NOTE Warning

No perspex apparatus must be used since it is soluble in propanone. The solvents are all classified as HIGHLY FLAMMABLE (and open to solvent abuse).

Trichloroethane and propanone are classified as HARMFUL but are relatively safe and economical in the very small volumes used. A risk assessment is now needed for the use of highly flammable substances [4].

PROCEDURE

1 Have ready a pipette, two or three small wheat plants and the propanone solvent. Then take two glass slides and lay one of them on the bench.
2 Holding the end of one slide at an angle of 45° to the one on the bench use the hand-held slide to scrape the juice rapidly out of several wheat leaves. Build up a green mush on one end of the slide (see Figure 1).

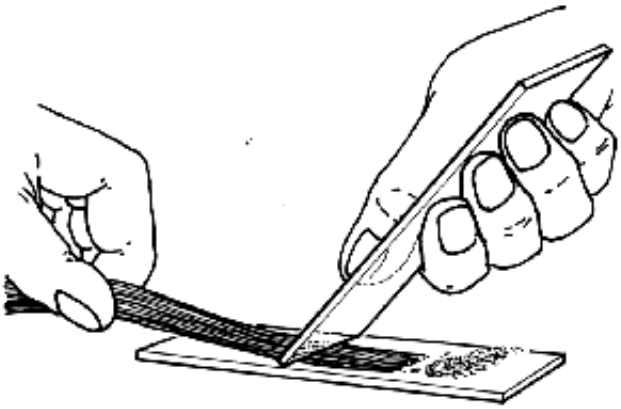

Figure 1 Making a leaf scrape extract

3 Transfer the mush to an inverted Fuji canister lid or watchglass. With a teat pipette add ten drops of propanone to the mush and reseal the propanone container. Mash in the solvent with the end of the glass rod. Gently tilt the lid and then suck up as much green liquid as you can. Place this in a second container (see Figure 2). Try to get as dark a green juice as you can.
4 Place the container under the hairdryer (it may be necessary to hold it still with a small piece of Blutak) to completely evaporate all the propanone and water to dryness. Do not let the container become too hot.
5 When the contents are completely dry (about 4-5 minutes), add two or three drops of propanone. With the paint brush mix all the pigments together.
6 As the propanone evaporates and when the pigment is at its most concentrated use the paint brush (or fine capillary tube) to make a small pigment spot on the matt surface of the TLC strip just 1 cm from one end (see Figure 3). Try to make the spot less than 2 mm wide.
7 Repeat, letting the spot dry completely between additions. Continue spotting until the

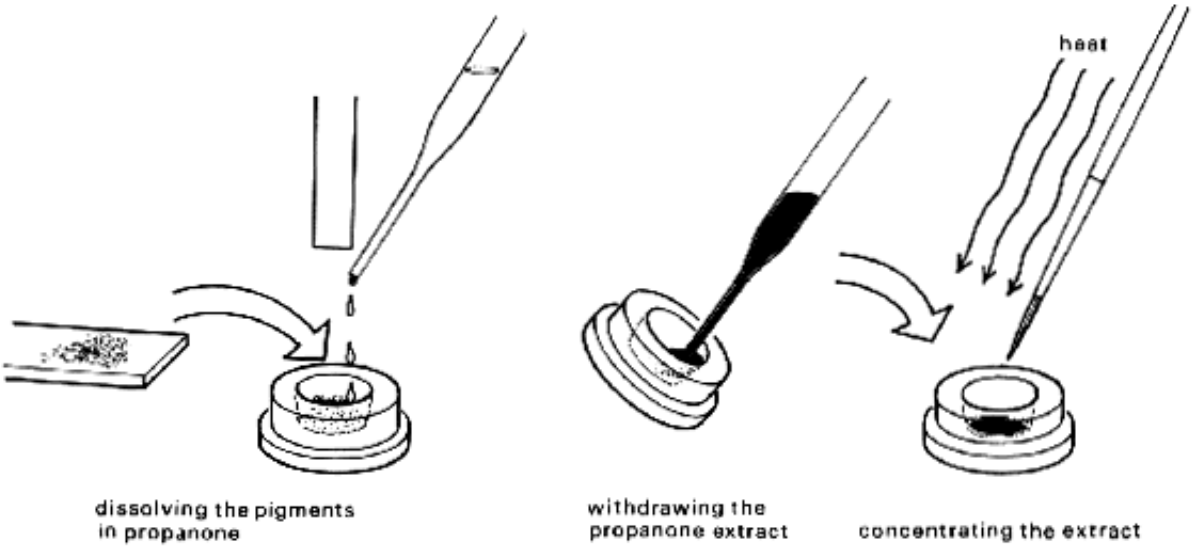

Figure 2

one small spot is very dark green - almost black. Make a base-line mark to one side of the spot with a pencil.

8 Fit the TLC strip to the split cork (Figure 4) and insert both into the empty glass specimen tube. With the marker pen, mark a level on the side of the tube just below the pigment spot. Remove the cork and strip.

9 Now shake the running solvent in its container to mix it. Remove the lid and add the running solvent up to the marked level. **Immediately** put the TLC strip and cork back into the tube before the running solvent evaporates. Be very careful not to allow the pigment spot to be splashed by the solvent solution or let the TLC strip touch the sides of the tube.

10 Watch as the chromatogram forms (Figure 4).

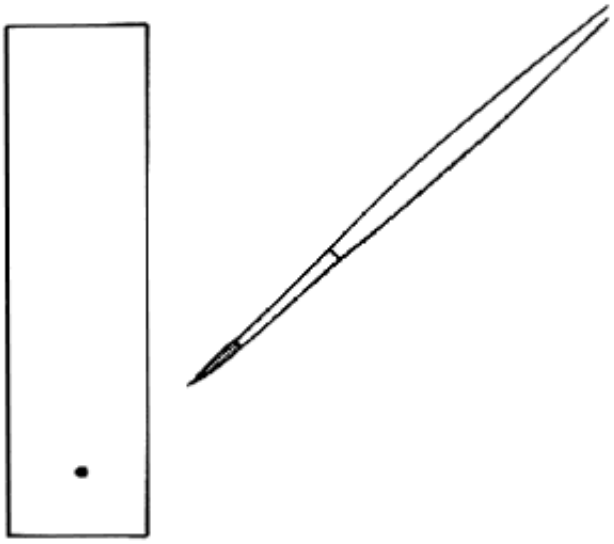

Figure 3 Spotting the thin layer

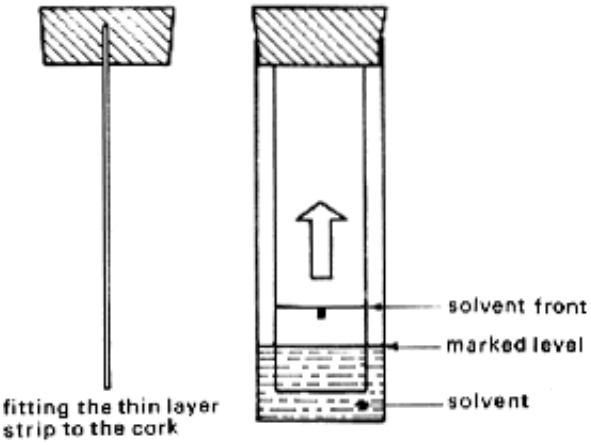

Figure 4

As soon as adequate separation has occurred (40 sec - 3 minutes), remove the strip and mark, with a pencil, the position finally reached by the solvent.

Allow the strip to dry and examine it in good white light

(If fresh leaves are not available dried nettle leaves may of course be used as an alternative. Fresh nettle leaves are dried at around 50 °C and can be stored indefinitely in sealed tubes. The best small-scale technique is to take 1 g of dried leaves and grind them to a powder in a pestle and mortar. The pigment is dissolved by adding 1-2 cm^3 propanone to the powder. This is concentrated by evaporation and applied directly to the TLC strip without filtration. The above procedure is then followed from step 6.)

RESULTS

If the distance to each pigment spot centre from the starting spot is measured, the R_f values may

Table 1

Pigment colour	Pigment name	R_f values for 2 different solvents	
		Trichloroethane propanone pet ether 40°-60°C	Propanone pet ether 30°-40°C
orange yellow	Carotene α and β	95	96
grey	Phaeophytin (a breakdown product)	80	82
blue green	Chlorophyll α	60	72
yellow green	Chlorophyll β	50	64
deep yellow	Lutein (a xanthophyll)	35	59

be calculated (R_f = distance run by pigment divided by distance run by solvent). Typical results for the five principal pigments are shown in the Table 1.

In addition to the five major pigments it is also possible to see very clearly some other minor carotene and xanthophyll pigments as distinct spots. These are well described by Valadon [3]. One of these, unidentified by us, turns from yellow to blue in a few minutes. The colours do not keep their brilliance for very long, fading altogether over a period of days and weeks. Colours should, therefore, be recorded by students in their write up, either in words or with colours by careful crayon colour matching.

EXTENSION WORK

The different pigments may be isolated by scraping the silica gel layer complete with the pigment bands off the plastic into several small tubes. If re-dissolved in a very small volume of propanone the pure pigment solutions may be viewed with a spectroscope to confirm the absorption of the different light wavelengths characteristic of each one. The pigments may also be viewed for their fluorescence under UV light from a shaded UV source. Chlorophyll fluoresces red quite strongly in UV light, whilst the carotenoids do not.

ACKNOWLEDGEMENTS

This work was carried out as part of the Science and Plants for Schools (SAPS) programme at Homerton College, Cambridge CB2 2PH and at the Royal Botanic Garden Edinburgh EH3 5LR. The wheat technique was first trialled at an ASE workshop at Loughborough University in January 1993 and the nettle technique at various 'Higher' workshops in Scotland during 1991-2. Helpful procedural suggestions on the technique described here were made by Colin Wood-Robinson, University of Leeds. Funding for the SAPS programme is provided by the Gatsby Charitable Foundation and is gratefully acknowledged.

REFERENCES

1 Roberts, MBV, *Biology a Functional Approach: Students' Manual*, (Nelson, 1974).

2 Nuffield Advanced Biological Science (1970), *Maintenance of the Organism* - a Laboratory Guide, (Penguin Books, 1970), chap 7.

3 Valadon, LRG and D Bendall, 'Text books can be misleading: an A-level exercise. The separation of chlorophyll pigments by paper chromatography', *SSR*, 1988, **69**(248), 512-14.

4 Management of Health and Safety at Work Regulation, (HMSO, Jan 1993).

S Tomkins is Director of Studies in Biological Sciences and Senior Lecturer in Science Education at Homerton College, Cambridge.
MB Miller is Research and Development Teacher on the Science and Plants for Schools programme based at the Royal Botanic Garden, Edinburgh.