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An improved method for extraction and separation of photosynthetic pigments

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The method for extracting and separating hydrophobic photosynthetic pigments proposed by Katayama *et al.* (*Japanese Journal of Phycology*, **42**, 71-77, 1994) has been improved to introduce it to student laboratories at the senior high school level. Silica gel powder was used for removing water from fresh materials prior to extracting pigments by a mixture of organic solvents that was also used for chromatographic separation of the pigments. A small silica gel thin-layer plate or a paper strip was used for separating the pigments. The improved method may be applicable to all kinds of plant materials including algae, is easier than most other methods, and can lead to more successful results in separating these pigments by both thin-layer chromatography and paper chromatography. The method has been carried out in student laboratories in some senior high schools and universities in Japan. The results indicate that this laboratory exercise is effective for students to recognise the unity and diversity of plants. Therefore, this laboratory seems to be useful for teaching plant systematics as well as for teaching photosynthesis.

Key words: Photosynthetic pigments, Secondary biology, Students' laboratory, Thin-layer chromatography.

Introduction

Photosynthesis is a fundamental topic studied in biology at the secondary school level. In all the present biology textbooks for senior high schools in Japan, an experiment involving the extraction and separation of hydrophobic photosynthetic pigments appears as a student laboratory exercise. The most popular experimental procedure in the textbooks is to use a 3:1 (v/v) mixture of methanol and acetone, known as propanone in England, for extracting photosynthetic pigments and then to use this extract directly for separating pigments by paper chromatography (PC) or thin-layer chromatography (TLC). This procedure does not in fact result in clear separation of the pigments.

Storey (1980) recommended using alcohol for extracting chlorophyll and other hydrophobic photosynthetic pigments because of student safety. Klein (1981) described a method similar to Storey's and showed similar unclear PC results. Foote (1984) introduced an experimental procedure using a hand-made, thin-layer plate for TLC to separate chlorophyll and other pigments. He used acetone extracts from some fresh materials for TLC, but the resulting chromatography presented in his report seems unclear. Why, then, is the separation of pigments unclear when the alcohol or acetone extract is used for PC or TLC? A major reason is the trace of water, which is transferred from fresh material, contaminating the pigment solution used for chromatography. Since alcohol and acetone are hydrophilic organic solvents, they might contain some traces of

water when used for extracting pigments from fresh materials. The water contaminant prevents clear chromatographic separation of the pigments. In order to separate the pigments clearly, the water must therefore be removed from the extract using an aspirator (Misonou and Yokohama, 1984) or a hair dryer (Tomkins and Miller, 1994).

Compared to the method described by Misonou and Yokohama (1984), the method developed by Tomkins and Miller (1994) is simple and timesaving for extraction and separation of pigments from a terrestrial plant leaf. In the latter method no mortar and pestle are needed. However, since a hair dryer is required for removing the water from the pigment extract, the number of hair dryers required may restrict individual student activity. Moreover, the method is applicable for soft leaves, but not for hard leaves or seaweeds.

The procedure developed by Katayama *et al.* (1994) is another simple method for qualitative analysis of photosynthetic pigments from algal materials. The features of this method were (1) silica gel powder was used for removing water from the material, (2) diethyl ether, a hydrophobic organic solvent, was used instead of a hydrophilic organic solvent such as methanol, and (3) the number of steps for the extraction was reduced. As in the method developed by Tomkins and Miller (1994), small-scale TLC was used for the separation of pigments. As a result, both the amount of organic solvents used for the extraction of pigments and the time required for the extraction and separation



of pigments could be reduced. We thought this method might be suitable for a student laboratory exercise in senior high school biology and therefore improved it. Laboratory exercises using the improved method have already been carried out in some senior high school laboratories (Kaga, 1998) as well as in university laboratory classes (Katayama *et al.*, 2000). These trials were successful, and we have obtained many data useful for improving experimental results. In this article, the improved method, recommendations and expected results are described.

Extraction of photosynthetic pigments from seaweeds

The following procedure may be used for all types of plant materials and should take less than 10 minutes:

1. Cut an algal frond or a leaf into small pieces with scissors. Put the pieces into a mortar.
2. Add one small spatula full of dried silica gel powder to the mortar.
3. Grind frond pieces or leaf together with silica gel powder. If the ground material does not result in smooth powder, add some more silica gel powder and grind.
4. Scrape the resulting mixture out of the mortar using a spatula.
5. Transfer the mixed powder into a 1.5 cm³ disposable microtube, which is made of polypropylene.
6. Into the microtube, pour a mixture of petroleum ether (BP 30 to 60°C, PE) and acetone, which is used for PC or TLC as the running solvent.
7. Mix the powder and solvent well using a small spatula or a stirring rod.
8. Leave the microtube to stand in a holder for a few minutes to let the contents separate into two layers.

The upper dark green layer, the organic solvent extract, is used as a pigment solution for PC or TLC.

Recommendations

- Usually 0.1 – 0.2 g of fresh material or one-tenth of this weight in dried material is an appropriate amount except when a frond piece of red alga, which contains lesser amounts of chlorophyll and carotenoids, is used.
- Cut the material as small as possible to reduce the time required for grinding.
- If using a fresh algal sample, remove the excess water on the frond; if not, a large amount of silica gel powder must be used to generate the powdered material. This results in a lower concentration of extracted pigments.
- Coarse silica gel powder might be better to use. An electric coffee mill can be used to make the powder, but to avoid inhaling the fine dust of silica gel, a clean mask containing a dust filter should be used. The silica gel powder should be dried well in a drying oven at around 90 °C and left it in a desiccator before use.
- The appropriate volume of PE and acetone mixture is about 1 cm³. Any mixing ratio of these organic solvents can be used.

Separation of photosynthetic pigments

The small-scale PC or TLC is suitable for separating chlorophyll and carotenoids in student laboratories. A 1 cm x 10 cm strip of ordinary chromatography paper is appropriate for PC and a

1 cm x 10 cm strip of TLC plate cut from a Merck Silica gel 60 TLC Plastic Sheet (20 cm x 20 cm: No.5748-1M or 5735-1M) is most appropriate for TLC. For a chromatographic chamber, a 17.5 mm x 150 mm test tube (or boiling tube) sealed with a silicone plug is useful. The running solvent is a mixture of PE and acetone. The recommended mixing ratio of PE : acetone (v/v) is 10:1 or 9:1 for PC (Valadon and Bendall, 1988) and 7:3 or 6:4 for TLC.

Procedure

1. Put the pigment solution repeatedly on the point previously marked 2 cm from the lower end on the chromatography paper strip or the TLC plate using a capillary tube (a disposable pipette tip is convenient to use for this).
2. Put the paper strip or TLC plate into a test tube containing about 0.5 cm³ of running solvent. Seal the test tube with a silicone plug and leave to stand.
3. When the running solvent reaches the point previously marked 1 cm below the top end on the paper strip or the TLC plate (i.e. the distance run by solvent is 7 cm), remove the paper strip or the TLC plate from the test tube.
4. Mark each of the pigment spots on the paper strip or the TLC plate with a pencil as soon as possible.

When the room temperature is about 20 °C, it takes approximately 15 minutes to separate pigments with this procedure, although separation adequate for detecting major pigment spots occurs within several minutes after starting the chromatography.

Recommendations

- The diameter of the pigment solution put on the paper strip or the TLC plate should be less than 1 cm so that part of the pigment solution put on the strip/ plate dissolves out into the running solvent.
- The chromatography chamber should be saturated by the running solvent vapour.
- Use a pair of forceps when putting the paper strip or the TLC plate into the test tube.
- After taking the paper strip or the TLC plate out of the test tube, mark pigment spots as soon as possible. It is easier to detect the pigment spots on a light box.

Expected results

Typical results of chromatographic separation of photosynthetic pigments extracted from terrestrial plants and seaweeds using a TLC plate and the 7:3 mixture of PE : acetone as running solvent are shown in Figure 1.

- When a green leaf or a green alga is used as a material, six major pigment spots are observed on the TLC plate as shown in Figure 1 (A, B). From the top, these pigments are carotenes, chlorophyll *a*, chlorophyll *b*, lutein, violaxanthin — this pigment turns from yellow to blue in a few minutes after taking the TLC plate out of the test tube (the phenomenon was pointed out by Tomkins and Miller, 1994) — and neoxanthin. Sometimes a few other minor carotenoids can be observed depending on the materials used.
- When a brown alga is used as a material, four major pigment spots are observed as shown in Figure 1 (C). From the top

these pigments are carotenes, chlorophyll *a*, fucoxanthin and chlorophyll *c*.

- In the case of red algae in general, three major pigments spots are observed on the chromatogram as shown in Figure 1 (D). From the top these pigments are carotenes, chlorophyll *a* and lutein.
- Sometimes one or more grey spots occur above chlorophyll *a*. The pigment(s) contained in the spot(s) is phaeophytin(s), a degraded by-product of chlorophyll. The phaeophytin spot is particularly apparent when the material is old. Sometimes pigments might be degraded in manipulated material such as dried seaweeds. Teachers should confirm whether pigments in the material are degraded prior to the student exercise.
- The sequence of separated pigments on the paper chromatogram is somewhat different from that on the thin-layer chromatogram mentioned above. Whereas xanthophylls occur above chlorophyll *a* on the paper chromatogram, they occur below chlorophyll *b* on the thin-layer chromatogram (Foote, 1984, made a mistake in indicating the phaeophytin spot as xanthophylls in his TLC result).

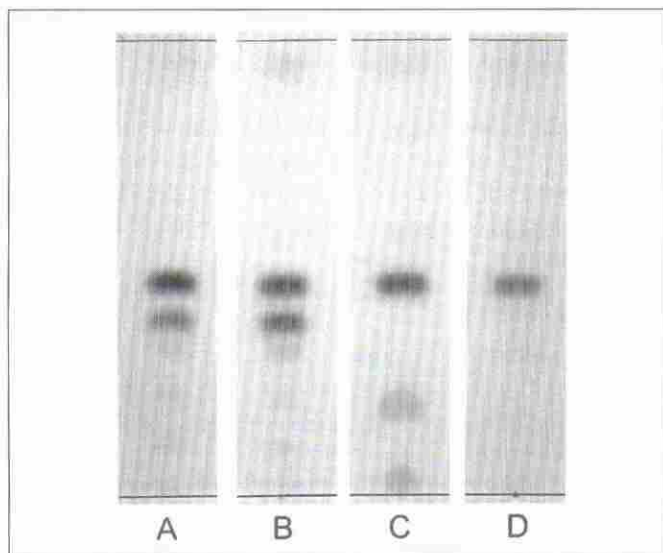


Figure 1 Typical patterns of thin-layer chromatography of the pigments extracted from the leaf of *Camellia japonica*, a terrestrial green plant (A), the frond of *Ulva pertusa*, a green alga (B), the frond of *Sargassum horneri*, a brown alga (C) and *Grateloupia filicina*, a red alga (D). The names of pigments in each spot is mentioned in the text.

Discussion

Neil Reese in 1997 introduced reverse phase chromatography for separating photosynthetic pigments. Although it gives us a clearer separation of the pigments, the method does not seem to be appropriate for introduction into a student laboratory exercise at the secondary level, because the procedure is not easy for secondary students and the cost for laboratory equipment is quite high. At secondary school level, PC and TLC seem to be appropriate for separation of pigments.

The use of diethyl ether (Katayama *et al.*, 1994) instead of the mixture of PE and acetone for extracting pigments gives a better result in separating the pigments by both PC and TLC. However, PE and acetone are less volatile and less harmful than diethyl ether and are therefore more suitable for use in secondary school laboratories. In addition, if we use the mixture of PE and acetone for extracting pigments, the number of organic solvents required for this exercise can be

reduced, making the cost for the exercise less.

As the separation of pigments by TLC is clearer than that by PC, many kinds of TLC plates were tested. Of the thin-layer material, silica gel resulted in better separation of pigments than cellulose powder (Avicel). Of the thin-layer substratum, there are three types: glass plate, plastic plate and aluminium plate. A large sized glass plate is not useful because to cut it into small strips is very difficult. There is a small sized glass plate, which is the same size as microscopic slide glass. This one is very convenient because a vessel for slide staining can be used as the chamber for chromatography, but the cost is rather high. The plastic plate and aluminium plate are easy to cut with a box cutter (a cutter for paper craft), and the former is better than the latter because pigment spots can be detected on the chromatogram by penetrating light. Among the silica gel TLC plastic plates provided, Merck's TLC plate is the best one because thin-layer silica gel does not come off easily when the plate is cut into small strips.

Katayama *et al.* (1994) and Takakuwa (2001) implied that the laboratory exercise on the extraction and separation of photosynthetic pigments from terrestrial plant leaves and algal fronds might be useful to allow students to realize both the unity and diversity of plants. So, the laboratory exercise described in the present paper was implemented in some biology classes at senior high schools and in biology laboratories at some universities. Seaweeds collected previously and kept in a freezer and fresh leaves were provided as materials. Each student selected one of them for the experimental material. By sharing their results obtained from different materials, students could therefore recognise the unity and diversity of plants. It might allow students to understand the systematics and evolution of plants.

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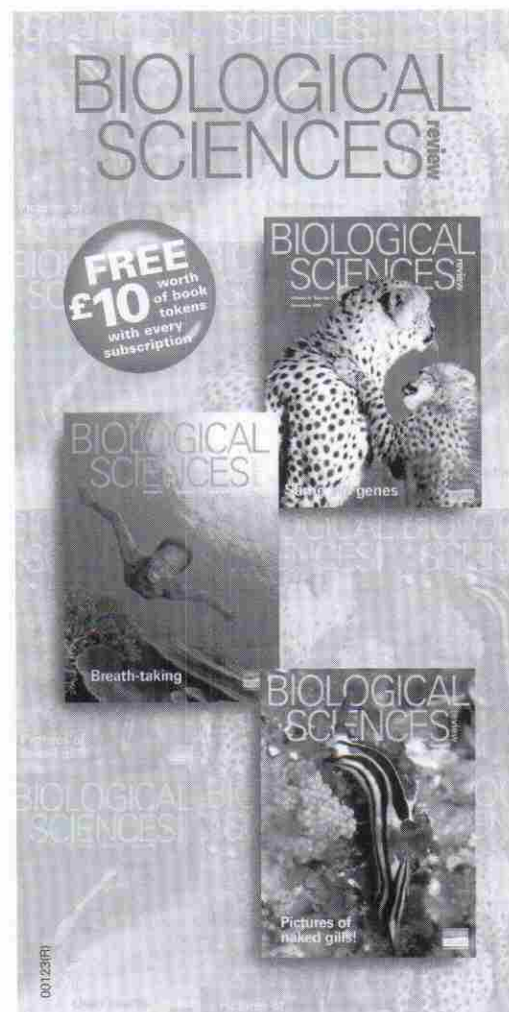
Websites

The following website is useful to know the experimental method described by Tomkins S and Miller M B (1994): www.saps.plantsci.cam.ac.uk/worksheets/ssheets/ssheet10.htm

Appendix

Suppliers

Merck Silica gel 60 TLC Plastic Sheet (No.5748-1M or 5735-1M), a product of Merck Co. Ltd., can be purchased from a laboratory ware supplier in each country.



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