

Cell Biology Laboratory Exercise – Chromatography of Photosynthetic Pigments

The sun's energy is critical to the maintenance of life on earth and there are several mechanisms that organisms have evolved to capture and utilize portions of this energy. Photosynthesis, occurring at the level of the cell, is the most important of these mechanisms. In plants and many algae, photosynthesis is carried out in chloroplasts, organelles whose structure is specialized for the process. Within the chloroplast is a series of pigments that possess the ability to absorb photons of light with wavelengths in the visible spectrum (ca. 400 – 700 nm) and to transfer this energy among molecules comprising two photosystems. The end result is that water is split, yielding oxygen, and ATP is generated for plant growth and maintenance. In addition to the nutritive value animals gain from consuming plant tissues, they also benefit from the molecular oxygen generated by photosynthesis and needed in their metabolic pathways.

The photosynthetic pigments in plant leaves are varied functionally and, consequently, structurally. The most familiar are chlorophylls a and b, and they are central to the operation of the photosystems. Their structure includes a long phytol chain with saturated carbon to carbon bonds and a porphyrin ring. This ring resembles the heme group in hemoglobin and the cytochromes and contains a magnesium atom instead of the iron found in the others. Chlorophyll a absorb light in the red and blue regions of the spectrum and transmit or reflect (and, therefore, appear) green. Absorption maxima for chlorophylls are 645 nm for a and 663 nm for b. The other (accessory) pigments in higher plants are carotenoids, classified as either carotenes or xanthophylls. Carotenes are made entirely of long chains of carbon and hydrogen atoms; xanthophylls have similar structures but also contain some oxygen. Xanthophylls include lutein, violaxanthin, neoxanthin, and fucoxanthin. Carotenoids absorb in the blue region and transmit light that is yellow or orange. Having multiple pigments with different absorption spectra broadens the range of light energies available for photosynthesis. In addition to using absorbed energy for photosynthesis, the chlorophylls can also re-emit light of longer wavelength than that absorbed; this process is called fluorescence.

The mixture of pigments present in cells can be extracted with solvents and can be separated into individual components. A simple and effective means of doing this is by chromatography, a process that involves partitioning pigments between a stationary and a mobile phase. In the system we will use, thin-layer chromatography, the stationary phase of the system is water present on a support medium – silica gel here (paper can also be used) – and the mobile (organic) phase ascends through it. Pigment molecules will move at different speeds through this system, depending on their relative solubility in the mobile phase and their affinity for the water and the support medium. The different positions of pigments on the chromatogram when separation is complete are represented by the R_f (ratio of fronts) values or factors. Each is defined as the relative distance traveled by a pigment as compared to that traveled by the solvent front.

Separated pigments can be characterized by measuring their absorption spectra – the pattern representing absorption of light of different wavelengths. This is a constant for a pigment and is based on its molecular structure. Since solvents can change this structure and also have absorption spectra of their own, considering the solvent is critical to interpreting data generated by a spectrophotometer, the instrument designed to measure absorption/transmission of light of certain wavelengths impinging on a sample.

The exercise has several objectives:

- to extract chloroplast pigments from spinach leaves,
- to separate individual pigments from each other by means of chromatography, and
- to determine the absorption spectra of the pigment mixture and each individual pigment following their separation on the chromatogram.

Procedure:

Before beginning pigment extraction,

1. pour 1/2 inch of developing solvent (petroleum ether: acetone:methanol, 9:1:0.5) into the chromatography chamber and replace the cover, allowing 30 minutes for equilibration of the air and the solvent.
- 2.
3. Extract the chloroplast pigments from spinach leaves into acetone by grinding in a mortar with a pestle. Use approximately 1 g. of leaf tissue from which the major veins have been removed; extract in about 5 ml ice-cold acetone. Keep everything cold during extraction. Filter the resulting extract to remove solid material.
4. Mix the acetone extract with petroleum ether in a separatory funnel or burette; allow them to separate, then remove and discard the bottom layer of acetone.
5. Save an aliquot of the petroleum ether extract in a test tube for spectrophotometry, allowing it to evaporate to dryness in the hood; the remainder will be used for chromatography.
6. Draw a line 1 inch from the bottom edge of a chromatography sheet. (Paper may be substituted for a plastic sheet with silica gel.) Use a brush to apply a narrow line (or series of spots) of pigment extract along the line, being careful not to extend the line to the edges of the sheet. Allow the line to dry and repeat the procedure until the line is dark. This may require 10 or more applications.
7. Place the chromatography sheet into the chamber and quickly replace the lid. Allow to develop in darkness until the solvent line has reached the top edge of the sheet or until no further movement is evident. **When removing the developed chromatogram from the chamber, quickly mark the level of the solvent front on the sheet.** The solvent evaporates almost immediately and you will need this information to calculate an R_f value for the pigments separated.
8. On your developed chromatogram, identify the individual pigments by their color: chlorophyll a - blue-green; chlorophyll b - yellow-green; carotene - orange-yellow; phaeophytin - gray; xanthophylls (lutein, violaxanthin, fucoxanthin, neoxanthin) - yellow. Carefully record the positions and the sizes of each band. Measure the distances from the origin to the solvent front

and to the middle of each band of pigment. Examine the sheet with UV light to detect natural fluorescence in any of the pigments. **Wear UV-absorbing goggles when using the lamp!**

9.

10. Carefully scrape the portions of silica gel support containing each of the pigments from the plastic backing of the chromatography sheet. The gel containing each pigment is to be placed into a separate test tube containing a **very small** quantity of alcohol to elute the pigment. Pool your sample with those of the other members of the class to increase the amount of pigment available, **making sure that you place your samples into the correct test tube**. Centrifuge to separate the silica gel and decant the clear extract. Dissolve the dehydrated complete extract (from step 3) in alcohol as well. Run absorption spectra for the unseparated extract and for each of the isolated pigments.

Data Presentation

Include in your report a copy of the spectrum for the whole extract as well as spectra for each of the individual pigments. Your instructor will provide copies made from the original data. Calculate an R_f value for each of the pigments. You can present your chromatographic data either as a table or drawing indicating the R_f values of each of the pigments separated. Indicate which pigments were fluorescent.

Points to Ponder:

1. What is the physical basis of the chromatographic procedures we used?
2. What could you do to alter the pattern of pigments observed on the chromatography sheet in another exercise?
3. What is the practical usefulness of the absorption spectrum to students of cell biology?
4. How could you draw an **action spectrum** (a graph of some physiological process vs. wavelength for the spinach pigments)? Would you ever find a peak in the action spectrum where you don't have one in the absorption spectrum? Why or why not?