Spectrometry and Chromatography of Plant Pigment Extracts

(adapted with permission from R. Griffin, City College of San Francisco)
WARNING: The petroleum ether used in this experiment is highly explosive. There must be NO FLAME OR SPARK in the vicinity when petroleum ether is being used. Ethanol is less hazardous but is still flammable, so it must also be used with care.

A. Objectives:

On completion of this exercise you should be able to:

1. Use a grating spectrometer to obtain the data necessary to plot the approximate transmittance spectrum of a given solution and plot it in standard scientific format.
2. Use those same data to plot the approximate absorbance spectrum of that same solution in standard scientific format.
3. Diagram the components of a spectrophotometer and briefly explain how it works.
4. Explain briefly the physical principles on which chromatography operates.
5. Describe and demonstrate how to carry out ascending paper chromatography and calculate the Rf for each substance isolated.
6. Comparing your results with the published Rfs for plant pigments and the absorption spectrum in Fig. 10.9 on page 187 in Campbell et al. *Biology*, hypothesize which spot on your chromatography paper corresponds to which pigment.
7. Briefly explain the value of chromatography to a scientist.
8. Explain the phenomenon of fluorescence.

B. Before coming to lab

1. Read this laboratory section.
2. Study Figures 10.8 and 10.9 on p 187 in Campbell et al. *Biology.*
3. Look at Figures 1a) and 1b) and predict which pigment is more soluble. Justify your answer in your lab notebook.
4. Write a concise protocol for the chromatography part of your investigation. Be sure to use numbered steps, put each step on a new line, and have only one action in each step.

C. During lab

Note: Some groups should start with the chromatography, others with the spectrometry part of this lab.

1. Working in groups of two, observe the ethanol extract of chloroplast pigments with the spectrometer, recording the significant data in you laboratory notebook as explained in Part E, Note 2.
2. Compare your data with Fig. 10.9 in Campbell et al., and generate a hypothesis about which pigments are present in the spinach extract. Justify you hypothesis.
3. Observe the demonstration of fluorescence of that same extract and record your observations.
4. Working by yourself, spot your chromatogram with the pigment extract as described in Part E, Note 5.
5. Place your chromatogram into the tube to start the elution and record the starting time in your laboratory notebook.
6. Just before the eluent reaches the hook in the chromatography tube, remove the chromatogram and record the time.

7. As soon as the eluent evaporates from your chromatogram (20 - 30 seconds), immediately outline the pigment spots in pencil, starting with the palest ones. The pale ones react with air and often become colorless in a minute or two.
8. Glue your chromatogram into your notebook and record your best description of the original color next to each pigment spot.
9. Identify the pigments using the key provided in Part E.

D. After lab:

- In your laboratory notebook plot a transmission spectrum of the pigment extract, plotting percent transmittance on the ordinate and wavelength on the abscissa. You may plot it directly in your notebook or on a piece of graph paper which you glue into your notebook. Be sure to use glue. Cellophane tape doesn't last, and staples tear through three or four pages ahead and behind when you close your notebook.
- Calculate the absorbance of the observed extract at each wavelength which marks a change in percent transmittance and plot an absorption spectrum for that extract in your lab notebook under "Results".
- Calculate the R_f for each pigment. Show your calculations in your notebook, clearly labeled. (You may do these calculations in the laboratory if you have time, but you must be sure to finish first all the things which can be done ONLY in the laboratory. Calculations can easily be done at home.)
- Identify the pigments on your chromatography paper in your lab notebook.
- Answer the review questions in Part F.

E. Background

Note 1: Theory and vocabulary on spectrometry

A **spectroscope** is a device which allows a narrow beam of light to enter it and pass through either a prism or a diffraction grating to disperse the light into a spectrum. A spectrometer is a spectroscope with some method of indicating the wavelengths or of selecting a narrow band of wavelengths to pass through a specimen. A spectrophotometer adds an electrical or electronic photometer to the system to measure light intensity much more accurately than the human eye can judge it and displays it on a meter or, on the more expensive models, prints it out on a graph.

The first investigation involves a **grating spectrometer**. The light passes through an adjustable slit and then through a diffraction grating in the eyepiece. The light is dispersed into a spectrum, which is projected onto the retina of the eye, giving the observer the impression that the spectrum is inside the instrument, although it is purely a virtual image. The instrument also projects an image of a wavelength scale into the eye, superimposing it on the spectrum so the observer can read what wavelength each color corresponds to. Most light sources provide a fairly complete spectrum with virtually all of the wavelengths present. If a solution is placed between the light source and the spectrometer, only those wavelengths transmitted by the solution will be seen. Wavelengths which are absorbed by the solution will be either significantly dimmer or absent, leaving a black space in the spectrum.

Transmittance means exactly what it implies. It is a measure of the light that is transmitted. It is the decimal fraction of the incident light which is transmitted through a sample of solution. Percent transmittance is simply the expression of that fraction as a percent instead of a decimal fraction. In spectrometry, we are concerned primarily with the differences in transmittance of different wavelengths. For example, if a given solution absorbs half of the light of a given

wavelength and allows the other half of that light, then its transmittance for that wavelength is 0.5 and the percent transmittance for that wavelength is 50%.

Absorbance is the opposite of transmittance; that is, it is a measure of the amount of light of a particular wavelength that is absorbed by the sample solution and therefore not transmitted. Absorbency is measured in absorbance units, which are related to transmittance inversely and logarithmically, as indicated in the equation below. In older reference books you may see the term "optical density" and "optical density units". You may also see in chemistry books the term "extinction". Both of these mean the same as absorbance, and the units are identical to absorbance units.

Math Formulas

A solution with 50% transmittance of a certain wavelength has an absorbance at that wavelength of:

$$\log_{10} 1/\text{transmittance} = \log_{10} (1/0.5) = 0.30$$

In case you are wondering why anyone would invent a logarithmic unit, the reason is that absorbance is directly proportional to the concentration of the solute in the solution, and therefore, absorbance units are extremely useful for determining concentrations and changes in concentrations.

An absorption spectrum is the portion of the spectrum that is absorbed by a given substance. The term is also applied to a graph of that spectrum, with absorbance plotted against wavelength. In a chemistry class you may have used an expensive spectrophotometer which automatically measures the absorbencies at numerous wavelengths and plots the graph for you. In this exercise you are asked to make your best estimate by eye of the percent transmittance and then, after class, convert those estimates to absorbance units and plot them against wavelength to obtain an absorption spectrum graph.

Remember that a graph is a form of communication, and communication is always clearer when a standard format is followed. In science, the standard format is that the independent variable is plotted on the horizontal axis (X-axis or abscissa) and the dependent variable is plotted on the vertical axis (Y-axis or ordinate). The independent variable is most easily remembered as the one that you can be independent about. In this case, if you do not feel like observing the percent transmittance at 550 nm, you can express your independence by observing it at 520 nm or 560 nm. The wavelengths, therefore, is your independent variable. The percent transmittance you observe, however, is dependent upon the wavelength you choose. Percent transmittance is the dependent variable and so is the absorbance you calculate from it.

Your transmission spectrum should be plotted as in Reed, R. et al., Fig. 21.2, page 113. Do not forget the title, legend, and labels for your figure. You are welcome to use a program like Excel to generate your graph. You can find instructions on how to use spreadsheet programs in Reed, R. et al., Chapter 50.

After plotting your transmission spectrum you need to calculate the absorbance at each wavelength where there is a significant change and plot a rough absorbance spectrum on a graph like the one below. Be aware that the range of absorbance units is from zero (at 100% transmittance) to infinity (at 0 % transmittance). (If you use your electronic calculator to calculate the base 10 logarithm of zero, it will probably give you an answer of ERROR. Few calculators will give you the correct answer of "Infinity". This is why you have to plot this graph by hand.)

Fig. 10.9 in Campbell et al. shows the absorbance spectra of different plant pigments.

Absorption: The action of absorbing.
Absorption spectrum: The portion of the spectrum that is absorbed by a particular substance; OR a graph showing absorbance plotted against wavelength.
Excitation: The process by which electrons absorb energy (often light or other forms of radiant energy) and become elevated to higher energy levels.
Fluorescence: The process in which excited electrons drop INSTANTLY back to the ground state, releasing the energy previously absorbed, emitting most of it as radiant energy (which must have a longer wavelength than the energy absorbed, since some energy is always lost as heat).
Optical density: A synonym for absorbance (Often abbreviated as O. D.)
Percent transmittance: The percent of light of a given wavelength that passes through a particular transparent or translucent substance.
Phosphorescence: The process in which excited electrons drop back to the ground state a few at a time, GRADUALLY releasing the energy previously absorbed, emitting most of it as radiant energy over a period of time long after the exciting energy has stopped.
Spectrum: The band of colors produced when visible light is diffracted into its different wavelengths by a prism or diffraction grating; OR, the band of different wavelengths of radiant energy produced in the same way, even if not visible to the human eye.
Wavelength: The physical length of one complete wave of radiant energy, usually measured in nanometers or Angstroms.

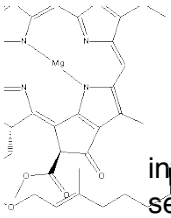
Note 2: How to use the grating spectrometer

In this investigation you will observe the absorption of light by pigments extracted with boiling ethanol from the chloroplasts of leaves. Light is passed through the extract (solution of pigments) and then passed through a grating spectrometer. The spectrometer diffracts the light passing through the pigment extract to form a spectrum which you can see when you look through the eyepiece.

If you compare that spectrum with the spectrum that results when the same light is passed through the spectrometer without passing through the pigment extract, you can see that certain colors of light are absorbed by the pigment extract.
The differences between the two spectra are due to absorption by the pigment extract, and the questions we want to answer are:

- What wavelengths are absorbed by the pigment?
- What is the approximate amount of absorbance?

Observe the demonstration spectrometer set up with an ethanol extract of chloroplast pigments. Adjust the flask of pigment extract so that its surface bisects the slit at the left rear of the spectrometer, making two spectra appear in the eyepiece, the upper one from light which passes above the surface of the pigment extract and the lower one from light which passes through the pigment extract. Record in your notebook how the sample is set up in relation to the spectrometer. (A diagram is best for this.)
For the purposes of this exercise you should compare the upper and lower spectra every 25 nanometers within the visible spectrum (400 nm through 700 nm). At each wavelength which marks a change in percent transmittance, estimate what percent of the light of that wavelength is transmitted through the pigment. There will be some entire ranges of wavelengths in which the percent transmittance will be zero, some in which it is 100 and some in which it will be some



intermediate value. You will probably find it useful to tip the spectrometer slightly up or down to see the upper or the lower spectrum more clearly.

Later you will need to convert these percent transmittance values into absorbance units, but absorbance units are logarithmic and can not realistically be estimated by eye. Therefore, the best approach is to estimate percent transmittance and then calculate the conversion to absorbance units afterward.

Pay special attention to recording the wavelengths at which there is a conspicuous increase or decrease in the percent transmittance. Your eye is reliable to only the nearest 25% transmittance, so do not try to estimate any closer than that. After the laboratory period you can calculate the absorbance and plot your absorption spectrum.
Observe also the fluorescence demonstration. Make a sketch of the apparatus and the direction of viewing relative to the direction of the light path. Record what you observe.

Note 3: Theory of chromatography

The technique of **chromatography** is used widely to separate the individual components of a mixture of related substances -- for example a mixture of amino acids, or one of sugars. The term chromatography refers to the fact that after separation, the individual substances may be visualized as spots of color, either because of their own natural color, or more commonly by causing them to react with reagents to yield a colored product.

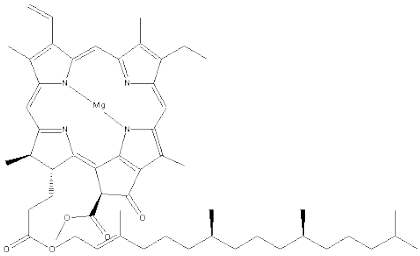
Chromatographic procedures have been developed for a variety of specific purposes. In general, a sample of a solution containing the mixture of substances to be separated is applied to a supporting medium. A suitable solvent is then made to move through the medium, and carries with it the mixture of components to be separated. Different components of the mixture travel at different speeds, depending on their affinity for the medium and their solubility in the solvent. Molecules are characterized by their R_f value, which is specific for a given solvent.

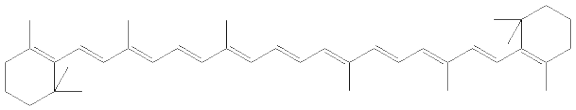
$$R_f = \text{distance traveled by molecule/ distance traveled by solvent}$$

One of the simplest chromatographic techniques is that of Paper Chromatography, in which the supporting medium is filter paper. In today's laboratory you will separate plant pigments by paper chromatography.

Identification of Pigments

The predominant pigment in the leaves of green plants is chlorophyll, which occurs in two slightly different chemical forms called **chlorophyll a (see Fig. 1 a)** and **chlorophyll b**. Most leaves contain at least two additional types of pigments, carotenes (see Fig. 1b) and xanthophylls, which are ordinarily not visible because they are masked by the more abundant chlorophyll.





b) Beta-carotene

Fig. 1: a) Chlorophyll a

The pigments you are working with were extracted from spinach leaves. Spinach leaves were cut into small pieces and placed in ethanol. The liquid was saved and tissue debris was discarded. Most students are able to separate and identify four pigments from spinach (Fig. 2).

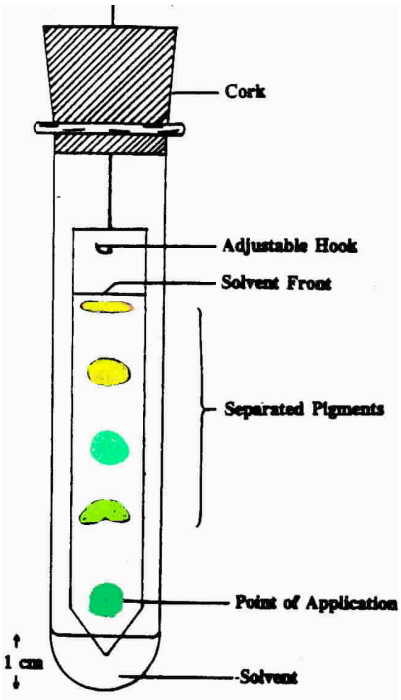


Figure 2: Chromatography set-up

Note 4: How to perform paper chromatography

Before doing anything else, obtain a chromatography tube with a cork and bent paper clip for suspending the paper. If there is any liquid inside the tube, be careful not to shake or tilt it. The inside walls of the tube must not be wet when you insert the paper strip. If they do get wet, let the tube stand for a minute or so: the petroleum ether-acetone solution will drain down to the bottom and the residue on the walls will evaporate quite rapidly.

Before you do anything else, be sure that there is approximately one centimeter of the (92 % petroleum ether - 8 % acetone) in the bottom of the tube. Also be sure the cork seals the tube well. If you fail to do this first, the atmosphere inside the tube will not become saturated with eluent vapors, and your chromatogram will not elute properly. If your tube looks as if someone washed it out with water, get a different tube. WATER IS A CONTAMINANT FOR THIS EXPERIMENT, and must be avoided.

Next, obtain a strip of chromatography paper, **using a pair of forceps to carry it.** When you lay it down, **do so on a very clean piece of paper,** a fresh piece of paper towel, or an untouched page in your notebook. Check that the strip is of the proper length and the hook is in the proper position to allow your strip to dip into the eluent in the bottom of the tube to a depth of at least 3 mm, but not more than 5 mm. The paper clip should go all the way through the cork so you can lower the paper strip into the eluent after you have everything assembled. Punch a hole in the strip where it is to be hung on the hook. A sharp pencil is ideal for this; any pencil lead that gets on the paper will not influence your results. The point of a pair of scissors will also work well. Be sure not to handle any part of the paper below the hook except with forceps.
Draw a pencil line across the paper strip 20 mm from the lower end. (The end opposite the one you just punched a hole into.) This line is called the "origin" and will mark the position for application of the pigment extract.

Obtain a capillary tube and fire-polish one end by touching it to a Bunsen burner flame for about one second. The object is to get the glass to the point where it gets soft so the rough edges smooth up and the opening of the tube is constricted by about 1/4 to 1/3 of its original diameter.

Obtain one of the small tubes of ethanol extract of pigments. (The extract was prepared by boiling the leaves (usually spinach leaves) briefly in water to rupture the cells, then blotting them dry and boiling them in ethanol for several minutes to extract the pigments.)

Touch the capillary tube to the pigment extract to draw some of it up into the tube. Then lightly touch the end of the tube to the origin on the line you drew 20 mm from the lower end of your paper strip and WITHDRAW IT INSTANTLY. The object is to transfer enough pigment to the paper to make a spot 5-7 mm in diameter and no larger. If you hold the capillary tube in contact with the paper the slightest bit too long, your spot will get too large. Wave your strip gently in the air to evaporate all the ethanol before applying more sample.

You may handle the chromatography paper with your fingers on the part above the hook, since you will stop the process before the eluent reaches that far. When you are sure that all the ethanol has evaporated, apply another sample of the extract directly on the preceding spot, and evaporate the ethanol again. Repeat this process until you have at least ten (10) samples superimposed on one another. If you have time and want to experiment a little, apply 15 - 20 superimposed applications. In any event, record the actual number of applications you made. You must evaporate all the ethanol from each sample before attempting to apply more, or your spot will grow too large. The object is to get the maximum amount of pigment in a spot 5 - 7 mm in diameter.

When you have finished applying the extract to the paper, carefully make a pencil mark at the uppermost edge of the area containing the applied sample. Check that the paper is of the right length and the hook is in the right position so that when you hang the paper strip in the tube it will dip 3-5 mm into the eluent, but your entire spot of pigment will be well above the surface of the eluent.

When everything is ready, *check that there is no open flame nearby and no apparatus capable of producing a spark in the vicinity of your tube of eluent.* Then, without leaving the tube open to the air any longer than absolutely necessary, remove the cork, attach the strip to the hook, and lower the strip into the tube, resealing the tube with the cork. The eluent will quickly climb up the paper to begin the chromatographic process. (If you dip your pigment spot into the liquid eluent in the bottom of the tube it will instantly wash off and you will get no chromatogram.)

Be sure your tube is in the middle of the table so it will not be disturbed during the elution. (It will take 20-30 minutes.) DO NOT TOUCH, MOVE, OR PICK UP YOUR APPARATUS DURING THE ELUTION PROCESS.

Just before the solvent reaches the hook, remove the strip and immediately lay it on a clean paper towel. Replace the cork in the tube immediately to minimize the contamination of the air with eluent vapors. If you fail to stop the elution before the eluent reaches the hook, your chromatogram will be irretrievably invalidated. **IMMEDIATELY mark the position of the eluent front with a soft pencil** (ink will run) before the eluent evaporates entirely. As soon as the paper is dry (20 - 30 seconds), use a pencil to outline every pigment spot, doing the faintest pigments first, since they sometimes disappear within a minute or so. Examine the strip very carefully in light from various angles so you will not overlook the very faint pigments.

Record in your notebook the color of each pigment before it fades. This is subjective, of course, but, nonetheless, it is useful information. At least four separate colors should be visible, unless you failed to apply enough pigment at the beginning, or the atmosphere within the chamber was not kept saturated with eluent vapor.
Glue your chromatogram into your notebook. (Otherwise you risk losing it.)

For each pigment, measure and record the distance the pigment traveled from the upper edge of the origin spot to the leading edge of the pigment. Measure and record also the distance from the upper edge of the original spot to the eluent front. Compute the R_f for each pigment. Record clearly in your notebook each calculation and each Rf obtained.

Note on safety…

- ! *Discard the eluent only in the flask labeled "used eluent".*
- ! *Do not ever return eluent (or any reagent) to the original flask.*
- ! *Do not pour eluent in the sink.*
- ! *Do not wash the tube out with water - water is a contaminant in this experiment.*
- ! *Return the tube with its cork for use by the next class.*

F. Review questions

1. From the results of your spectrometry and Fig. 10.9 in Campbell et al. **predict** which pigments might be present in the spinach leaf extract. Justify your answer.

2. Compare your chromatography data with your above prediction. Explain discrepancies.

3. Why is the plant extract green?

4. What does a small R_f value tell you about the characteristics of the moving molecules?

5. Which are more soluble in the chromatography solvent, xanthophylls or chlorophyll a?

6. Would you expect the R_f value of a pigment to change if we altered the composition of the solvent? Why or why not?

7. What color of light is least effective for plant growth? Why?

8. What color of light did the plant pigment fluoresce?

9. What happens if pigments in the extract absorb blue light?

10. What happens if pigments in the extract absorb red light?

11. Could a chlorophyll extract fluoresce blue light? Why or why not?