

## 301. EXPLORING LIGHT AND PIGMENTS THROUGH SPECTROPHOTOMETRY AND CHROMATOGRAPHY

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According to the second law of thermodynamics, organisms and cells must expend energy in order to maintain their steady-state condition. The universe and all closed systems tend to increase in disorder (entropy). To maintain order, and thus life, a constant supply of energy must be available. The ultimate source of energy for almost all living things on earth is the sun. The sun's energy is trapped by special light-absorbing molecules called **pigments**, and is transformed into chemical energy through the process of **photosynthesis**.

Light is composed of different wavelengths some of which we detect visually as different colors. The colors we see in an object depend on the wavelengths of light which it reflects or transmits. A red rose reflects the red wavelengths of light, absorbing blue and green wavelengths. A white dove looks white because it reflects all wavelengths and absorbs none, while a black cat appears black because it absorbs all wavelengths and reflects none.

Phototrophs contain a variety of light-harvesting and photosynthetic pigments. The absorption spectra of these pigments determine the wavelengths of light that can be used by the organism. Different phototrophs have different pigments. The pigments a phototroph uses depend on the nature of its photosynthetic process and reflect the environment to which it has adapted.

Two techniques useful in quantifying, characterizing and identifying pigments are chromatography and spectrophotometry. Chromatography is a valuable technique for separating complex mixtures of solutes, such as substances found in blood or cells. In chromatography, compounds are separated according to differences in *weight*, *size* and *solubility*. A small portion of a substance to be tested is placed near one end of the solid matrix such as filter paper. This end is then immersed in a solvent. As the solvent moves up the matrix, the compounds will be picked up and moved also, each at a different rate in accordance with its weight, size and solubility in the particular solvent used.

The function and use of the spectrophotometer is described in Appendix A. This instrument works by shining light of known wavelength through a sample. If a pigment that can absorb that wavelength is present in the sample, less light passes through the sample. The light that passes through is measured with a photometer (light meter). The greater the amount of pigment present, the more light will be absorbed, leaving less light to be detected by the photometer. Measuring the absorbance at different wavelengths provides an **absorption spectrum** for the pigment. As different pigments have different absorption spectra, pigments can be tentatively identified by their absorption spectra.

### Text References

POH p. 163-168, Light and Pigments; Box 8.B, Tools that Cracked the Calvin-Benson Cycle

### Study Questions

The second law of thermodynamics states that the disorder of the universe is always increasing. How can things as complex as organisms maintain and increase themselves?  
How does chromatography work?

## Purpose

To compare different phototrophs, using chromatography and spectrophotometry to characterize their pigments.

**Materials:** 20 ml acetone (**Caution:** Acetone is flammable. Do not use near open flame.); 6 ml 99.5% petroleum ether, 0.5% propanol (**Caution:** Ether is highly explosive. Confine it to the fume hood. Do not use ether in the same room with open flame.); mortar and pestle; 2 chromatography tubes with cork stopper and hook; 2 microcentrifuge tubes; two 13 x 100 mm test tubes; 2 strips chromatography (filter) paper; plant material (two species such as spinach and *Iresine*); spectrophotometer; cuvettes; microcentrifuge

Examine the plant material. *Note the differences in color between the plant species. Why are the plant leaves different colors?* Your answers to this "why" question are hypotheses. For example, you might answer that the plants are different colors because they have different photosynthetic pigments. Many other hypotheses are possible. A useful hypothesis is testable, leading to predictions we can test in experiments. *What predictions follow from the hypothesis that the two plant species have different photosynthetic pigments? What predictions follow from your hypotheses? How could you test these predictions?*

### Extraction of Pigments

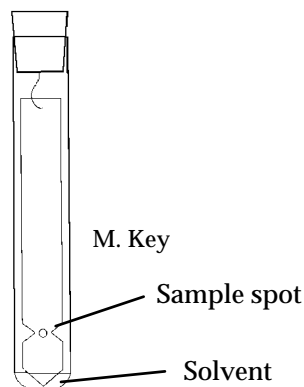
To release pigment from the plant cells, grind the leaves in a mortar and pestle with 3 ml of acetone. Do not over-grind, or you will end up with a pulpy mess that absorbs all of the solvent. Use one or two spinach leaves. For *Iresine*, use three to five leaves. After each extraction, clean the mortar and pestle with about 1 ml of acetone.

Transfer 1.5 ml of each sample to labeled microcentrifuge tubes. Spin the samples for 1 minutes at high speed. Pour the liquid supernatants to clean, labeled 13 x 100 mm test tubes, leaving behind the solid pellets of cellular debris. The pigment contents of the supernatants will be analyzed by chromatography and spectrophotometry.

### Chromatography

The chromatography solvent is **highly explosive**. Confine your work to the fume hood. Check the room to be sure that no one is using a Bunsen burner. For each pigment extract, place 3 ml of ether-propanol mixture (chromatography solvent) into a chromatography tube with a cork stopper. Keep the tube stoppered while you prepare the chromatography paper.

For each pigment sample, cut a 12 cm strip of filter paper. Handle the paper by the edges to avoid putting skin oils on it. Trim the filter paper strip is shown in Figure 1.



## Figure 1. Paper chromatography.

Stretch Pasteur pipettes (Figure 2) to form fine capillary tubes with which to spot the samples on the filter papers. With the large end of the Pasteur pipette held in one hand, and the tip of the pipette held with a pair of forceps in the other hand, heat the Pasteur pipette over a Bunsen burner. When the glass in the thin part of the Pasteur pipette is hot enough, pull your hands apart, stretching the pipette into a fine capillary. Then break the pipette in the narrow area.

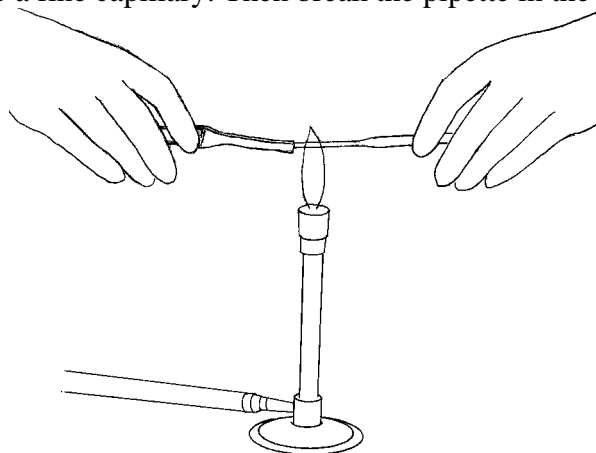


Figure 2. Making a capillary from a Pasteur pipette.

Apply the pigment extract as a spot 3 cm from the bottom of the strip of chromatography paper. To apply enough pigment so that the results will be visible, allow the paper to dry completely and then load more of the same sample onto the paper in the same place. Repeat this 10-20 times, allowing the spot to dry completely between applications. The spot should remain small, perhaps 2 mm in diameter. Impatience at this point will drastically reduce resolution.

Hang each piece of chromatography paper on a hook and place the stoppers in the chromatography tubes (Figure 1.). Make sure the extract spots do not enter the solution and that the tubes are tightly closed.

Before the solvent front reaches the top of the paper, remove the chromatography strip from the flask and restopper the flask. With a pencil, mark the area of each band before it fades. *Tape the chromatography strips onto the worksheet, recording the colors you observed.*

Other suitable chromatography solvents are water or 98% ether-2% ethanol; 90% ether-10% acetone; 90% acetone-10% ethanol.

### Spectrophotometry

Follow the instructions in the appendix for using the spectrophotometer. Use acetone to blank the spectrophotometer. Measure the absorbances of the pigment extracts every 25 nm from 350 to 700 nm. The maximum absorbance the spectrophotometer will read is 2.0 (equivalent to 0% transmittance). You will need to add acetone to the sample to obtain a final volume of at least 3 ml. (These spectrophotometers require 3 ml in the cuvette for accurate readings.) If the sample is too concentrated, dilute it further with acetone. *Record the absorbance values on the worksheet. Plot absorbance against wavelength for each sample. What color or colors of light did each of your sample extracts absorb and what colors were transmitted?*

## Clean-up

Pour the used extraction and chromatography solvents into the labeled containers in the fume hood. Rinse the mortar and pestle with acetone, then wash it with soapy water. Place the microcentrifuge tubes in a beaker on the discard cart. Return the chromatography tubes to the rack in the hood. Discard measuring pipettes in the tall pipette buckets. Discard Pasteur pipettes in the short pipette buckets. The stretched and broken Pasteur pipettes should be discarded in the broken glassware can. Gently wash the spectrophotometer cuvettes and invert them in the test tube rack.

### 301. SPECTROPHOTOMETRY AND CHROMATOGRAPHY OF PIGMENTS (17 PTS)

Name \_\_\_\_\_

Lab day and time \_\_\_\_\_

#### PRELAB PREPARATION:

1. Procedural outline:
2. Suggest possible reasons why *Iresine* has purple leaves, while spinach has green leaves.
3. What predictions follow from the hypothesis that the two plant species have different photosynthetic pigments? What predictions follow from your hypothesis (answer to Question 2)? How could you test these predictions?
4. What special precautions need to be taken when working with ether?

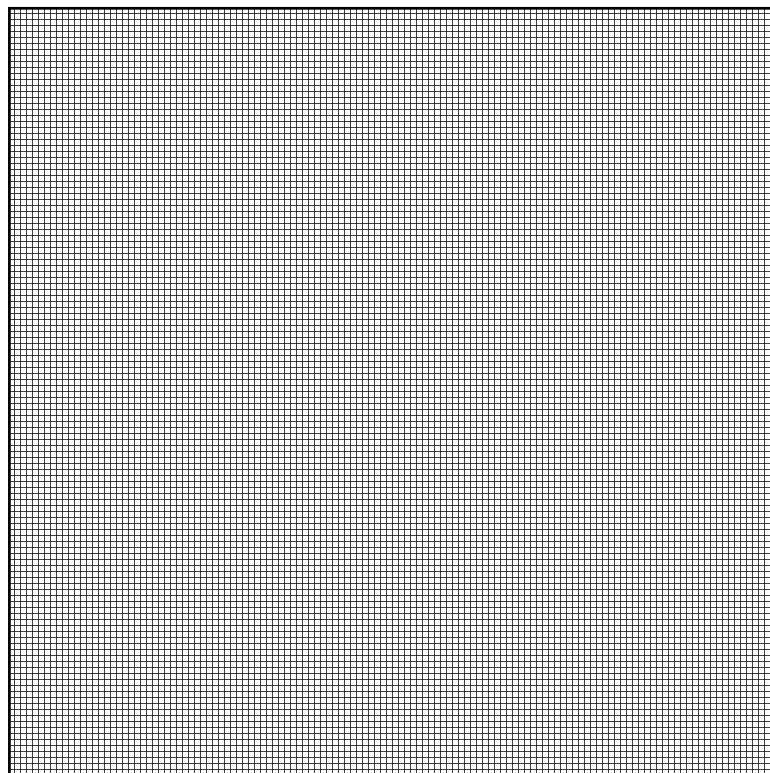
**RESULTS:**

5. Sketch your chromatography strips or tape them below. Identify and label each of the pigments found in the two plant species. Carotene is chrome yellow to orange, xanthophyll is pale yellow, chlorophyll *a* is blue-green, and chlorophyll *b* is pale yellow-green.

6. Spectrophotometry Data Table:

Color	Ultra-violet			Blue		Blue-Green		Green		Yellow		Orange		Red	
Wave-length	350	375	400	425	450	475	500	525	550	575	600	625	650	675	700
sample															
sample															

7. Plot the absorbance of your extract against wavelength



**QUESTIONS:**

8. How does the information obtained by using chromatography relate to the information obtained using spectrophotometry? Give specific examples using your data.

