For many centuries, people believed that the increase in the size of a plant was caused by the intake of material from the soil. It was not until a Belgian physician, Jan Baptista van Helmont (circa 1577-1644), performed an experiment that demonstrated conclusively what we accept today: the increase in the size of a plant is not due simply to the plant obtaining a mystery substance from the soil; plants gain what they require through the process of photosynthesis.

Photosynthesis uses energy from light captured by photosynthetic pigments, and splits water molecules in the process. The plants fix carbon from carbon dioxide into glucose and fructose chains and oxygen is released as a byproduct. In many plants the sugars then combine to form long chains known as starches. Many plants store their photosynthetic products this way.

Most plants produce their own organic molecules through photosynthesis and do not need to take them from another organism; these plants are called autotrophs. However, not all plants are able to carry out photosynthesis. Many parasitic plants that don’t have photosynthetic pigments rely entirely on other host species for nourishment; these plants are called heterotrophs.

Photosynthetic pigments include chlorophyll $a$, chlorophyll $b$, and the carotenes. Green plants usually have high chlorophyll content; in a typical plant, approximately three-quarters of the chlorophyll is chlorophyll $a$ and the rest is chlorophyll $b$. In some plants, the presence of other pigments becomes apparent in the fall when the chlorophyll no longer masks their presence. Other plants are high in pigments that mask the chlorophylls during the whole growing season (e.g., red cabbage remains red because of the presence of anthocyanin). Each pigment absorbs a specific range of wavelengths. Chlorophyll $a$, the primary pigment used in photosynthesis, absorbs blue and red light. Chlorophyll $b$ absorbs light in the blue-green and orange-red portions of the spectrum. Carotenoids absorb light in the blue and blue-green regions.

The first step in the conversion of light to chemical energy is the absorption of light by a pigment system. In all photosynthetic cells,
except photosynthetic bacteria, the pigment system includes chlorophyll $a$. In vascular plants, bryophytes, green algae, and euglenoid algae, chlorophyll $b$ is also found and functions as an accessory pigment. In the leaves of green plants, chlorophyll $b$ generally constitutes about one-fourth the total chlorophyll content. It extends the range of light that can be used for photosynthesis because it absorbs wavelengths different from chlorophyll $a$. At the same time, it shares the ability to absorb light energy and produce an excited state in the molecule with chlorophyll $a$. The excited molecule of chlorophyll $b$ transfers its energy to a molecule of chlorophyll $a$, which then transforms it into chemical energy. Chlorophyll $c$ or chlorophyll $d$ takes the place of chlorophyll $b$ in other groups of plants.

Certain carotenoids are also accessory pigments involved in the capture of light energy in photosynthesis. Carotenoids are red, orange, or yellow fat-soluble pigments found in all chloroplasts and also, in association with chlorophyll $a$, in the prokaryotic blue-green algae. Carotenoids are not found free in the cytoplasm, but like the chlorophylls are bound to proteins within the plastids. There are two classes of carotenoids: those that do not contain oxygen are called carotenes, and those that do contain oxygen are called xanthophylls. In green leaves, the color of the carotenoids is masked by the much more abundant chlorophylls; in some tissues, such as those of a ripe tomato or the petals of an orange flower, the carotenoids predominate. During autumn, chlorophyll begins to break down as the leaf begins to senesce, allowing the carotenes and xanthophylls to display the brilliant colors we associate with fall.

To measure the percentage of each wavelength of light absorbed by a pigment (the absorption spectrum), a spectrophotometer is used. A spectrophotometer directs a beam of light of a specific wavelength at the object to be analyzed, and records what percentage of the light of each wavelength is absorbed by the pigment or pigment system. The absorption spectrum is different from the action spectrum, which graphs the efficiency of different wavelengths of light in promoting a given photoresponse, as in photosynthesis or phototropism.

A spectrophotometer, or a similar instrument known as a colorimeter, can be employed to measure the rate of photosynthesis. In the dye-reduction technique, the compound DPIP (2,6-dichloroindophenol) is substituted for NADP (nicotinamide adenine dinucleotide phosphate), the primary electron-accepting compound of photosynthesis. As DPIP is reduced by chloroplasts in the presence of light, it changes from blue to colorless. Either a spectrophotometer or a colorimeter can measure the increase in light transmittance over time, and thus indicate the rate of photosynthesis.

One technique that can be used to separate extracted plant pigments is called chromatography. It separates liquid components into individual components based on their specific affinity, or attraction, for a solid surface, known as the stationary phase, and a specific solubility.
in a chromatography solvent, known as the mobile phase. Initially, an extract of the plant is placed on the bottom of a strip of chromatography paper, which is then placed in a vial with solvent covering the bottom. The chromatography strip and solvent are then placed in a vial. The paper acts as a wick, drawing the solvent upward by capillary action and dissolving the mixture as it passes over it. Different components of the mixture interact differently with the two phases. Some components will be more strongly attracted to the stationary phase and adsorb to the filter paper while others will be more attracted to the mobile phase and will migrate with the solvent. As the mobile phase passes over the stationary phase, the components more strongly adsorbed to the stationary phase will travel more slowly than those soluble in the mobile phase. The final result is that different pigments in the mixture show up as colored streaks or bands on the strip. The pattern formed on the paper is called a chromatogram.

To establish the relative rate of flow for each pigment, the $R_f$ value of each pigment is calculated. The $R_f$ value represents the ratio of the distance a pigment moved on the chromatogram relative to the distance the solvent front moved. It is calculated using the following formula:

$$ R_f = \frac{\text{Distance pigment traveled}}{\text{Distance solvent traveled}} $$

For example, the following sample chromatogram displays four different pigments: A, B, C, and D as well as the solvent front.

In order to determine the $R_f$ for pigment B, for instance, the distances that pigment B and the solvent front traveled would be placed into the $R_f$ formula:

$$ R_f = \frac{42 \text{ mm}}{80 \text{ mm}} $$

$$ R_f = 0.525 $$

Any molecule in a given solvent matrix has a uniquely consistent $R_f$, so the $R_f$ value can be used by scientists to identify molecules.
OBJECTIVES

- Measure the color changes brought about through photosynthesis by using a colorimeter
- Compare the rates of photosynthesis for plants in various light conditions
- Perform and explain the process of paper chromatography
- Separate and identify various pigments in photosynthetic plants
- Calculate the relative rate of flow (R_f) for each pigment
- Analyze results from collected data
- Explain the role of plant pigments

MATERIALS

PART A: MEASURING THE RATE OF PHOTOSYNTHESIS

MATERIALS NEEDED PER GROUP
LabPro
Computer with Logger Pro installed
Vernier colorimeter
Disposable pipets
Lamp
Beaker
Cuvettes w/caps
Timer
Lens tissue

SHARED MATERIALS
Aluminum foil
Masking tape
DPIP/Phosphate buffer solution
Boiled chloroplast suspension
Unboiled chloroplast suspension

PART B: CHROMATOGRAPHY OF PLANT PIGMENTS

MATERIALS NEEDED PER GROUP
Vial with cap
Chromatography strip
Disposable pipet
Metric ruler
Coin
Fresh spinach leaf

SHARED MATERIALS
Chromatography solvent

DID YOU KNOW?
Many scientists believe that chloroplasts and mitochondria have evolved from prokaryotic “trespassers” that invaded other, larger cells, a speculation known as the endosymbiotic theory.
1. Prepare the computer for data collection by opening the file “Exp 07 Photosynthesis” in the Experiment folder of Biology with Computers. A meter window will display the absorbance readings from the colorimeter.

2. You are now ready to calibrate the Colorimeter. Prepare a blank by filling a cuvette with distilled water. To correctly use a Colorimeter cuvette, remember:
   - All cuvettes should be wiped clean and dry on the outside with lens tissue.
   - Handle cuvettes only by the top edge of the ribbed sides.
   - All solutions should be free of bubbles.
   - Always position the cuvette with a clear, non-ribbed surface facing toward the white reference mark at the right of the cuvette slot on the Colorimeter.

3. Calibrate the Colorimeter.
   a. Holding the cuvette by the upper edges, place it in the cuvette slot of the Colorimeter.
   b. If your Colorimeter has a CAL button, set the wavelength on the Colorimeter to 635 nm (Red), press the CAL button, and proceed directly to Part A. If your Colorimeter does not have an CAL button, continue with this step to calibrate your Colorimeter.
      First Calibration Point
      c. Choose Calibrate from the Experiment menu and then click PERFORM NOW.
      d. Turn the wavelength knob on the Colorimeter to the “0% T” position.
      e. Type “0” in the edit box.
      f. When the displayed voltage reading for Input 1 stabilizes, click KEEP.

      Second Calibration Point
      g. Turn the knob of the Colorimeter to the Red LED position (635 nm).
      h. Type “100” in the edit box.
      i. When the displayed voltage reading for Input 1 stabilizes, click KEEP, then click OK.
PART A: MEASURING THE RATE OF PHOTOSYNTHESIS

1. Obtain a flood lamp and a 600 mL beaker. Fill the beaker with water and set it directly in front of the lamp. Doing this will prevent the samples from warming up too much. The lamp should not be turned on until indicated. Place three small pieces of masking tape on the bench in a row directly behind the beaker. Label the pieces: “B” for boiled, “U” for unboiled, and “D” for dark.

2. Obtain three cuvettes with caps and one piece of foil that is approximately 3” x 6”. Tear off a small piece of foil and wrap it around a cuvette cap. Wrap one of the cuvettes with the remaining piece of foil so that no light can pass through it. Make sure that this foil can easily slip on and off of the cuvette. Mark the foil cap “D”, another cap “B”, the last cap “U”.

3. Bring all of the cuvettes and their caps over to the chloroplast suspension bottles that have been prepared by your instructor. Gently swirl the bottles to re-suspend any chloroplasts that may have settled out. Dispense 0.25 mL of the unboiled chloroplast solution into the foil-covered cuvette and cover it with the foil-covered cap. Dispense another 0.25 mL of this solution into one of the empty cuvettes and cover it with the cap labeled “U”. Dispense 0.25 mL of the boiled chloroplast solution into the remaining cuvette and cover it with the cap labeled “B”.

4. Add 2.5 mL of DPIP/phosphate buffer solution to each of the three cuvettes. At this point each cuvette should be ¾ filled.

5. Take absorbance readings for each cuvette. Invert each cuvette two times to resuspend the chloroplast before taking a reading. If any air bubbles form, gently tap on the cuvette lid to knock them loose.

   a. Cuvette U: Place the cuvette in the cuvette slot of the Colorimeter and close the lid. Allow 10 seconds for the readings displayed in the Meter window to stabilize, then record the absorbance value in Table 1. Remove the cuvette and place it in its original position in front of the lamp.

   b. Cuvette D: Remove the cuvette from the foil sleeve and place it in the cuvette slot of the Colorimeter. Close the Colorimeter lid and wait 10 seconds. Record the absorbance value displayed in the Meter window in Table 1. Remove the cuvette and place it back into the foil sleeve. Place the cuvette in its original position in front of the lamp.
c. Cuvette B: Place the cuvette in the cuvette slot of the Colorimeter and close the lid. Allow 10 seconds for the readings displayed in the Meter window to stabilize, then record the absorbance value in Table 1. Remove the cuvette and place it in its original position in front of the lamp.

6. Turn on the lamp.

7. Repeat Step 5 again when 5, 10, 15, and 20 minutes have elapsed.

PROCESSING THE DATA

1. Prepare the computer for data entry by opening “Page 2”. This is done by going to the toolbar, clicking on the ‘down arrow’ to the right of “Page 1” and selecting “Page 2”.

   If your experiment “07 Photosynthesis” does not have a column on “Page 2” for ‘Boiled’ you will need to add that column.

   **To Add column:**

   1. Select ‘New column’ from the Data menu on the toolbar.

   2. Enter ‘Boiled Chloroplasts’ for “Name”, enter ‘Boiled’ for “Short Name”, enter ‘A’ for “units” under the ‘Column definition’ tab.

   3. Choose Display every ‘1’ points, choose Decimal places, and select Displayed Precision ‘3’ under the ‘Options’ tab.

   4. Choose a color under the ‘Options’ tab.

   5. Click on the Data Sets tab and ‘uncheck’ the box next to ‘latest’.

   6. Click ‘Done’.

2. Enter the data recorded in Table 1 into the appropriate column in the Data Set window. To type click on the table cell with the mouse pointer. The table cell will be highlighted and you will see a blinking cursor in the cell. Type your data point and press ENTER. The graph will update after each data point is entered.

   If your “Page 2” does not list ‘Boiled’ along the Y-axis, you will need to add it.
To add ‘Boiled’ to the Y-Axis
1. Right-click on the graph.
2. Choose Graph Options.
3. Click on the ‘Axes Options’ tab.
4. Check the box next to “Boiled Chloroplasts”.
5. Click Done.

3. Calculate the rate of photosynthesis for each of the three cuvettes tested.
   a. Click the Linear Fit button (Figure 1) to perform a linear regression. A dialog box will appear. Select the three data sets you wish to perform a linear regression on and click OK. A floating box will appear with the formula for a best fit line for each data set selected. You can adjust the position of each box by clicking on the box and dragging it to an appropriate location.

   b. In Table 2, record the slope of the line, $m$, as the rate of photosynthesis for each data set. Click on the Printer icon on the toolbar to print your results.

   c. Close the linear regression floating boxes.
PART B: CHROMATOGRAPHY OF PLANT PIGMENTS

1. Obtain a chromatography vial from your teacher and label it with your initials using a permanent marker.

2. Go to a fume hood or a well ventilated area and remove the cap from a chromatography vial. Using a disposable pipet, add 1 ml of chromatography solvent to the vial. Replace the cap and allow the chamber to sit undisturbed until needed in Step 9. This will ensure that the atmosphere within the vial is saturated with solvent vapors (equilibration).

3. Obtain a chromatography strip from your instructor. Be sure to handle the chromatography strip by the edges. Do not touch the surface of the strip. The oils from your fingers can interfere with the chromatogram.

4. Measure 1.5 cm from one end of the chromatography strip and draw a pencil line across the width of the strip.

5. Use a pair of scissors to cut two small pieces below the pencil line to form a pointed end (Figure 2). The pointed end will be referred to as the bottom end of the chromatogram.

6. Obtain a fresh piece of spinach and place it over the line on the chromatography strip. Rub the ribbed edge of a coin (dime or quarter) over the spinach leaf to extract the pigments. Repeat 5 to 10 times with different portions of the spinach leaf, making sure you are rubbing the coin over the pencil line (Figure 3).

Protective gloves, goggles, and aprons should be worn throughout this activity.

When working with the chromatography solvent, use a chemical hood or proper ventilation. Refer to the enclosed MSDS for disposal instructions for the solvent.

Step 2 should be performed under a chemical fume hood or with proper ventilation.

Steps 5-9 should be performed as quickly and as carefully as possible.
7. Remove the cap from your chamber and carefully place the chromatography strip into the vial so that the pointed end is barely immersed in the solvent. Make sure not to immerse the pigments in the solvent (Figure 4).

8. Cap the vial and leave it undisturbed. Observe as the solvent is drawn up the chromatography strip by capillary action. You will be able to see the plant pigments separating along the strip. Notice the different colors that you see during this process.

9. When the solvent reaches approximately 1 cm from the top of the strip, remove the cap from the vial. Using forceps, remove the strip from the vial. This is a chromatogram.

10. Immediately mark the location of the solvent front. The solvent will evaporate quickly.

11. In Table 2 in the Analysis section, list the pigment colors that you observe. Once the strip has dried, mark the middle of each pigment band on your chromatography strip with a pencil.

12. Using a metric ruler, measure the distance from the original pencil line with the spinach extract to the solvent front and each mark you have made for each pigment band (Figure 5). Record these distances in millimeters in Table 3 in the Analysis section.

13. Calculate the \( R_f \) value for each pigment on your chromatogram using the following formula and record your answers in Table 3.

\[
R_f = \frac{\text{Distance pigment traveled}}{\text{Distance solvent traveled}}
\]

14. Follow your teacher’s instructions for proper disposal of all materials.

Refer to the MSDS for proper disposal of chromatography solvent.
**WARD’S**  
**AP Bio/LabPro Lab 4: Plant Pigments and Photosynthesis Lab Activity**

**ANALYSIS**

**Table 1**

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Unboiled</th>
<th>Dark</th>
<th>Boiled</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2**

<table>
<thead>
<tr>
<th>Rate of Photosynthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unboiled</td>
</tr>
<tr>
<td>Dark</td>
</tr>
<tr>
<td>Boiled</td>
</tr>
</tbody>
</table>

**Table 3**

<table>
<thead>
<tr>
<th>Band Number</th>
<th>Band Color (Pigment)</th>
<th>Pigment Migration Distance (mm)</th>
<th>Solvent Front Migration (mm)</th>
<th>R_f Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>3</td>
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<td></td>
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</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1. In this experiment, a blue dye (DPIP) was used to replace NADP in light reactions. What did you observe with regard to the intensity of the blue color as the experiment progressed? Why do you think this was the case?

2. How would you expect the results of this lab to change if a dim light was used in place of a flood light?

3. What advantages did the CBL 2 have in processing this data?

4. What was the reason for using boiled chloroplasts in this experiment? What information was gained from using this solution?
5. In what way is the spectrophotometer used to measure the rate of photosynthesis?

6. On your chromatogram, which pigment migrated the farthest? Why?

7. During summer, leaves are generally bright green. What would you hypothesize that this indicates about the role of green light wavelengths, chlorophyll, and the photosynthetic process?

8. Design an experiment to test your hypothesis from the question above. Describe your experiment or draw a picture of your experimental setup. If you draw your setup, be sure to label each component and its purpose.

9. Why do leaves change color in autumn?
10. Shown below is a strip of chromatography paper and a list of five molecules and their various R_f values. Assuming the solvent front traveled 54 mm, place each molecule where it would be found on the finished chromatogram.

![Solvent front](image)

```
<table>
<thead>
<tr>
<th>Molecule</th>
<th>R_f value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N,N-fictionol</td>
<td>.41</td>
</tr>
<tr>
<td>cis-2,4-pretendium</td>
<td>.20</td>
</tr>
<tr>
<td>dl-made-upelene</td>
<td>.72</td>
</tr>
<tr>
<td>d(+)-tetra-iminase</td>
<td>.91</td>
</tr>
<tr>
<td>polysynthetic acid</td>
<td>.78</td>
</tr>
</tbody>
</table>
```

11. What is the absorption spectrum?
13. Below is graphic representing the steps in the process of photosynthesis. Fill in the blanks with the proper step.

Photosynthesis

Water is split

Electrons are energized and passed down

reduce NADP⁺ to

CO₂ is fixed then rearranged to

generated given off rising

in which consists of captures stored in

in which in which