November 26, 2016
The Artistry of Animal Coloration

Activity: Analyzing Plant Pigments Using Paper Chromatography

Class time: 1 to 4 class periods, depending on parts of the experiment performed and depth of analysis
Day 1: Paper chromatography experiment to learn the general procedure
Day 2: Students design their own experiment
Days 3 and 4: Students perform their experiments and analyze data

Purpose: To extract pigments from one or more plants, beginning with spinach, and use this technique in the study of plant pigments. Also, to develop, test and analyze related hypotheses.

Notes to the teacher: Students can explore the basics of this technique and then develop their own experimental design, based on their background knowledge, to test a different hypothesis. Creating the spinach solution before class begins will decrease the amount of in-class time needed to perform the initial chromatography experiment.

General background on technique: Chromatography (Greek for "to write in color") is the physical process of separating compounds in a mixture based on each compound's ability to move within a mobile phase in a definite direction through a stationary phase. In 1903, Russian botanist Michael Tsvet used a column of calcium carbonate to extract plant pigments from a petroleum ether and chlorophyll solution, creating the chromatography process. The same method can be applied to extract plant pigments using paper chromatography. Plants have many pigments, each of which is a molecule that absorbs and reflects light. When a slurry of pigments is prepared and placed on the paper (the stationary phase) and the paper is placed in an acetone-vinegar solution (the mobile phase), the pigment molecules are carried up the paper and distributed. Separation is based on their attraction to the stationary phase (paper) and the mobile phase (solvent), as well as on their molecular weight. Generally, the least massive molecules will travel the fastest and those with the greatest mass will travel at a relatively slower rate. By measuring the distance a pigment travels under controlled conditions (the R_f-value), that pigment may be identified.

Color-producing pigments like eumelanin and pheomelanin can be found in animals, as described in "Color me dino." Plants also have color-producing pigments, such as chlorophyll a (blue-green) and xanthophyll (pale yellow-green), that are crucial for the light reactions of photosynthesis. Plants have various types of pigment groups and these produce the varied colors we see in fruits, vegetables and greenery. Other plant pigments such as flavonoids often play a role in the chemical defense of plants and are toxic to many herbivores and pathogens. Some block ultraviolet (UV) radiation that can damage cell proteins and DNA. Carotenoids are a category of pigments known for their bright red, yellow and orange colors and have antioxidant properties.

Materials for each pair of students:

- Blackline Master 4
- Chromatography or filter paper
- Scissors
- Ruler
- 600 mL cup or beaker
- Pencil
- About 5 fresh spinach leaves, finely chopped
- Cheesecloth or strainer
- Mortar and pestle
- 3 mL disposable pipette or dropper
- Acetone (5 mL)
- Small beaker or small vial with lid
- Solvent of 92% vinegar 8% acetone
- Gloves and safety goggles
- Calculator (optional)

Directions:

1. Introduce students to the categories of pigments. If time allows, students might research the colors that various pigments create and where they are found:

   Some familiar plant pigments of interest in nutritional and pharmaceutical research:

Pigment	Color	Found in
Anthocyanins	blue/purple/red	berries, grapes, red peppers, beets, eggplant, plums
Beta-carotene	orange/yellow	carrots, pumpkin, sweet potatoes, citrus, papaya, melon, squash
Curcumin	yellow	turmeric
Lutein	yellow/orange	kale, broccoli, spinach
Lycopene	red	tomatoes, watermelon, red grapefruits
Zeaxanthin	yellow	corn

2. Cut chromatography paper: Show students how to prepare their chromatography or filter paper. If your paper doesn't come in strips, then show students how to cut the paper into strips that are long enough to reach from the top of their beaker to just above the bottom, wrapping around the pencil to hang freely. Be sure edges are straight and try not to touch the paper with bare hands, as that will transfer body oils.
3. Pause for a safety moment: Check student allergies and moderate materials accordingly. Remind students not to taste any substances and to use materials only as directed. Students should wear safety goggles and gloves. After handling the materials, students should wash their hands thoroughly. Be sure there is proper room ventilation because acetone is a volatile substance.
4. Prepare the spinach: Place the finely chopped spinach leaves into a mortar and add about 5 mL of acetone. Thoroughly grind with the pestle. Add more acetone as needed, but your goal is a dark concentrated solution. Pour the solution through a funnel lined with cheesecloth and drain into a small vial or beaker (squeeze the cloth to get the highly concentrated portion of the solution).
5. Pipette transfer: Using a pencil, draw a reference line about 1 to 2 cm from the bottom of the chromatography paper. Create a dot of spinach solution on the middle of the reference line (you should have an intense green color) by adding one drop at a time, allowing it to dry, then adding another until the dot is highly concentrated.
6. Remind students to read the remaining directions on Blackline Master 4 carefully. If you want to show them the next steps (this takes the moment of discovery away, but provides clear direction), add some solvent solution (92% vinegar 8% acetone) to the bottom of the beaker so the tip of the chromatography

paper is submerged but your dot is not (Step 5 on Blackline Master 4). Leave the beaker untouched for about 10 to 20 minutes. Point out that students should stop their experiment before the solvent reaches the very top of their filter paper. Give them time to practice their technique.

7. Demonstrate how to calculate the R_f-value (measure the distance from the reference line to the middle of the new location of the pigment and divide by the distance from the reference line to the solvent near the top of the paper). Since everyone did the same basic process, you can have students post their results and compare. Ask why some of their results might be different [*experimental error, different amount of time for solution to separate out, etc.*]. Discuss which pigment traveled farthest and why students think so. Which pigments could they identify based on their R_f-values? For those pigments not listed on the reference chart provided on Blackline Master 4, how could students identify them? How did the pigments look after they were separated versus when they started? Why use a solvent instead of water to separate the pigments? Why stop the chromatogram before it reaches the top of the paper? What could make it difficult to identify each pigment [*pigments overlap on the paper, for example*]? What could you do to fix such a problem [*change the ratio of vinegar to acetone in the solvent, for example*]?

8. Now students can make the experiment their own: Based on the background and initial test, students may have questions that they'd like to answer. Ask students to list variables they might like to test in a different chromatography experiment [*type of plant, part of plant, temperature of sample and whether it is cooked or fresh, solvent type, solvent strength, type of paper, distance traveled over time*]. Discuss the importance of isolating variables and have students work in teams to identify how they will test a new variable. Depending on the time you'd like to invest, a matrix of variables can be set up and teams can be assigned to tests so that each variable is tested at least three times.

Example matrix with possible variables to test:

Variable to test:	**Team 1**	**Team 2**	**Team 3**	**Team 4**	**Team 5**
Fresh vs. cooked spinach	x	x			x
Spinach leaf vs. oak tree leaf		x	x	x	
Spinach leaf vs. spinach stem	x	x	x		
Solvent (vinegar and acetone): 92% vinegar vs. 50% vinegar			x	x	x
Chromatography paper vs. coffee filter	x			x	x

9. Students should formulate a hypothesis based on the variable they are testing.

10. Students need to consider how many times they need to repeat their experiment in order to be confident of their results. They can use any of a variety of power analysis calculators to help them or you can print out a table for students to use manually. Match the table with the statistical analysis you'd like your students to use, such as the t-test.

11. Give students time to design their experiment using Part 2 of Blackline Master 4 and identify what materials they'll need. You might want to check their written work before they proceed or have groups orally share their experimental designs before giving them the go-ahead.

12. Give students time to perform their own experiments, collect data and analyze it. Groups can then share their data orally, as group posters or lab reports.

Analyzing Plant Pigments Using Paper Chromatography

PART 1: INTRODUCTION TO SPINACH CHROMATOGRAPHY

Directions: Practice the paper chromatography process

1. Prepare a data table similar to the one shown in Step 8 below.
2. Put on your gloves so you don't transfer the oils from your skin to the chromatography paper. (This is known as contaminating the sample and will affect your results.) Cut your chromatography paper so it is the right length for the beaker or cup you will be using.
3. Place about 5 leaves of chopped, fresh spinach in a mortar and add 5 mL acetone. Use the pestle to release the pigment from the leaf. You want a very intense color solution.
4. Using a pencil, draw a reference line about 1 to 2 cm from the bottom of the chromatography paper.
5. Use a pipette or dropper to transfer a drop of solution to your chromatography paper so that the middle of the dot is on the reference line. Let the drop dry, then add another to the same spot. Repeat until the spot is very dark (about 20 drops). Only add the next drop when the last is dry (you can blow on it gently).
6. Prepare your chromatography paper so it hangs from a pencil (or dowel) just above the bottom of your beaker (as shown in Figure 1).
7. When you're ready to go, add solvent solution (92% vinegar 8% acetone) to the bottom of the beaker so the tip of the chromatography paper is submerged, but your dot is not. Leave the beaker untouched for about 10 to 20 minutes. Watch as the solvent (mobile phase) moves up the paper (stationary phase) and observe what happens to the pigment dot over time. Remove the chromatography paper from the solution just before the solution reaches the top of the paper (where it touches the pencil). Using a pencil, mark the final solvent line and the location of each pigment as seen in Figure 2 (these get harder to read later when the paper dries).
8. Use your ruler to measure the distance each pigment traveled compared to the reference line and record your data on a table like this one:

FIGURE 1: BEAKER AND PAPER SETUP

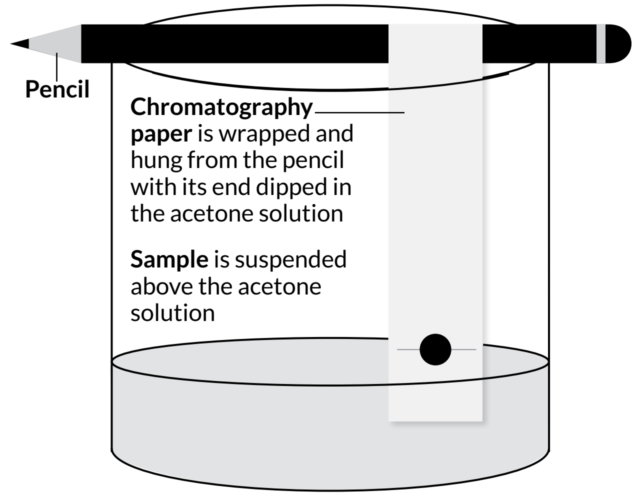
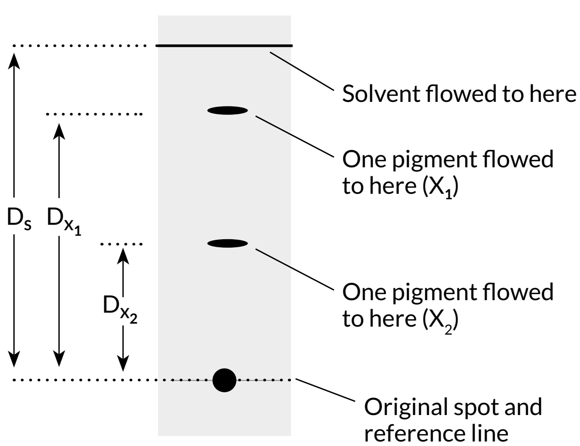


FIGURE 2: LABEL YOUR PAPER



Pigment band	Band color	Distance solvent traveled (mm)*	Distance pigment traveled (mm)	Calculate R_f-value	Possible identity of pigment
1					
2					
3					
Continue as needed					

* this value will be the same for all entries

9. Calculate your R_f-value:
   R_f = distance pigment traveled (mm) / distance solvent traveled (mm)

10. Use the table below to help you identify the pigments in your sample:

R_f value	Visible color	Pigment type
.98	Yellow	Carotene
.86	Yellow	Xanthophyll
.80	Red	Xanthophyll
.67	Dark gray	Phaeophytin a
.60	Light gray	Phaeophytin b
.50	Yellow	Xanthophyll
.48	Light blue-green	Chlorophyll a1
.46	Dark blue-green	Chlorophyll a
.30	Light yellow-green	Chlorophyll b1
.25	Dark yellow-green	Chlorophyll b
.15	Yellow	Xanthophyll

Source: http://www.dpcdsb.org/NR/rdonlyres/7799127C-23A2-4E9B-9E0E-7E8A9095428D/105157/pigmentslab.pdf

PART 2: PRE-LAB: DESIGN YOUR OWN EXPERIMENT

Directions: Use the prompts below to help you plan your experiment. Once you've planned the experiment, have your design checked as directed by your teacher and then get started.

1. What do you want to focus on? Do you want to compare the results of different plants, the same plant under different conditions or a different composition of acetone and vinegar solvent?

2. What is the question you want to explore?

3. Identify the variables you want to test in order to answer your question:

4. Write a hypothesis based on your variables and question.

5. What materials will you need to perform your test?

6. Data collection: What data will you collect? How will you collect it? How many times will you need to repeat your test? How will you organize your data table? Make your data table:

7. What statistical analysis will you use on your data?

8. What procedure will you use? Be as specific as possible.

PART 3: POST-LAB: ANALYZE YOUR DATA

Directions: Use the prompts below to analyze your data.

1. Based on your data and the statistical test you selected, what story does your data tell about your plant's pigments? Explain.

2. Did you prove or disprove your hypothesis?

3. How will you visually display your data and results? Be sure to label any visuals and provide a key for necessary information.

4. What errors do you believe existed in your experiment? How could you minimize or remove the potential error in future experiments?

5. As you examine your data, what new questions arise? How could you test for those?