LEARNING ABOUT PLANT COLORS AND PIGMENTATION **Developed by Julia Dupin, 2017**

This activity was designed to showcase the diversity of pigment types in plants and show how they can be 'extracted' from plant tissues using simple solvents.

Plants colors come from different types of chemical compounds, called pigments. The most abundant plant pigment is chlorophyll, the compound that makes plant leaves appear green to our eyes. However, this is just one of many pigments that plants naturally manufacture. Other photosynthetic pigments include carotenoids, yellow-orange compounds that are similar in chemical structure and stored with chlorophyll in organelles called plastids. Additional colors arise from anthocyanin and betalain pigments. These water soluble pigments are red to purple, depending on pH, and are found in the vacuole, the large storage pool at the center of plant cells. Anthocyanins are more common than betalains, which are only found in small group of plant species that includes beets. Both anthocyanins and betalains are principally important for coloration, although anthocyanins are involved in response to UV light, acting like a sort of plant sunscreen. They also serve as antioxidants in our bodies, and have major health benefits when eaten.

This activity is focused on the chemical extraction of anthocyanins. The suggested samples, red apple, blackberry, eggplant, and radish, have different types of anthocyanins.

EXTRACTION OF ANTHOCYANIN PIGMENTS FROM RED APPLE SKIN, EGGPLANT SKIN, RADISH SKIN, AND BLACKBERRIES, AND SEPARATION WITH PAPER CHROMATOGRAPHY

Materials/steps marked with a ★ should be handled only by the instructor, or by a student in close supervision

Materials (see Fig. 1):
- mortar and pestle (the kitchen one works just fine)
- fruit/root samples
- 1/2 teaspoon measuring spoon
- disposable plastic pipette (to measure 0.5 and 1 mL of isopropanol)
- dropper bottle
- containers with lid for sample storage
- small glass vial (for blackberry solution)
- coffee filters
- funnel
- plastic pipette tips (volume up to 200µL)
- glass jars (e.g. Mason jars of pint size)
- chromatography paper (there are multiple types of chromatography paper, we have tested this method using the 'Whatman™ Grade 3MM Chr' Chromatography Paper. Other types of paper of similar grade recommended for chromatography use should yield the same quality results)
- lemon juice
- ★ isopropanol (rubbing alcohol), ~91% (this seemed to work slightly better than the 70%)
- ★ acetone 100% (some brands of nail polish remover have this concentration)
- ★ single edge blade
- ★ vegetable peeler
- ★ regular table knife

For each student/small group working with one sample you will need:
- one mortar and pestle set
- one pipette tip (minimum)
- mason jar with acetone

- if the sample is blackberry, they will also need one coffee filter, one funnel, and one small glass vial

All other materials can be prepared in large amounts and shared in a large classroom. For an estimate of the amount of plant sample to prepare given a class size see the information under "Preparing the skin samples" and the details under the extraction of pigments from blackberry.

Preparations for Pigment extraction

----------Before class----------

Selecting the fruits/roots

- when buying the vegetables go for those that have the darkest colors, especially in the case of apples

Preparing the skin samples

- before going into the details, here is some important information
    - first, as a heads up, know that the easiest sample to work with is the radish skin because it lacks the waxy layer present both on the apple and eggplant skins
    - second, you will need about 1/2 teaspoon of sample skin per person for this experiment
    - third, if you need to prepare samples for a large number of people do so a couple of days in advance, these can be stored in the fridge in sealed containers and still look fresh after 3 to 5 days. This way you can avoid having to peel a large amount of things on the day before, your fingertips will be thankful.
    - ***Amount of sample -*** I found that if students are working individually one radish has enough skin for about 4 people, one apple likely 10 people, and one eggplant many people :), for the blackberries, use one per person

    - ★ peeling process

    for any of the samples here the final product should be small pieces of skin that are easier to grind than long strip of peeled skin; this is especially important for the apple and the eggplant; these pieces should be about 0.5cm x 0.5cm

        Radish - it is very easy to remove skin samples just by using a regular single edge blade

        Apple and eggplant - it will be tempting to just use a vegetable peeler to remove the skins but the use of it will generate peels that have a fair amount of white fruit flesh on the under sides. So a follow up step needed is to use a regular table knife to scrap off the fruit flesh and have a final product that is cleaner (see Fig. 2 to check the ideal final look of your samples)

        Blackberry - there is no peeling step for the blackberries, so just keep them intact

    - storage

    if the samples are not going to be used immediately you can place these in a sealed container in the fridge for about 3 days (never in the freezer)

Lemon juice storage

- to make things easier you can store the lemon juice in the dropper bottle

★ *Chromatography chamber preparation*

- given how volatile acetone is, it is safer to have all mason jars that will be used already filled with enough acetone to fill up 1cm at the bottom of each jar

Chromatography paper preparation

- cut the paper into 2cm wide strips, these strips should not be longer than the glass jar used as chamber
- using a pencil, measure 2cm of height and make a horizontal line on one of the sides (see Fig. 3)

General comment about the preparation of samples and solutions: if you are doing this activity with middle and high school students, and there is no time rush, the students can be the ones collecting the samples as long as there is enough supervision for using the blades. They can also do the marking of the chromatography paper preparation step in class.

Pigment extraction and paper chromatography

----------During class----------

Extracting the pigments

For apple, eggplant and radish

- in the mortar add 1/2 teaspoon of a chosen sample skin, 0.5 mL of rubbing alcohol and 3 drops of lemon juice, and grind the skin pieces
  - for younger kids, the radish is the best choice because it's easier to grind
  - apple and eggplant is recommended for older kids
- you will be done grinding once you have a pigmented, likely thick solution; you may add more of the lemon juice and alcohol if the tissue was dry and no liquid remains after grinding. Still it should be concentrated and darkly colored.

For blackberries

- place one blackberry in the mortar along with 1mL of rubbing alcohol and 6 drops of lemon juice, and grind all
- because there is now too much solid material in this final mix, you will need to transfer a filtered solution to a small glass vial. To do that use a funnel and put a coffee filter in the funnel, place those two items on your vial, and gently pour the solution on the coffee filter. It might at first just soak the filter and not really go through it, so you can slowly squeeze the solution to accelerate the filtering process.

Paper chromatography technique

- you will now need to a strip of chromatography paper and a chromatography chamber already filled with 1cm of acetone
- make sure your strip of paper is marked on one side with a horizontal **pencil** line at 2 cm (do not use pen) (Fig. 3).
- you will now apply small amounts of your solution to the chromatography paper using a pipette tip. Grab one pipette tip and touch the pigment solution with its very tip and hold it there for a couple of seconds. Due to capillary action, the solution will naturally start moving up the tip. Once you have collected some of your solution (not too much, see Fig. 3 for reference) gently tap the chromatography paper to transfer it to the paper, you want to tap exactly on the middle point of that reference pencil line (Fig. 3). You will have to repeat this step three to four times to ensure that there is enough sample, always tapping the paper on the same middle line spot. It is important to wait for the paper to dry a bit in between steps to keep the size of the spot small.
- now place your strip of paper in your chromatography chamber, being careful to open and close the chamber quickly. There is no need to screw the lid on, just place it on top of the jar.
- if students are working on different samples, it is important, for comparison sake, to keep the paper strips in the chambers for the same amount of time, so the samples are developed equally (Fig. 4). Another point to keep an eye on is that you need to allow enough time for the pigment to migrate but not too much time so that it doesn't runs off the paper. A good distance is about 2/3 of the paper, and this usually takes between 5 and 10 minutes.

- what you should see is a faint color blob moving up along with the acetone that is now soaking the paper.
- once the pigment reaches about 2/3 of the paper length you can remove it from the chamber (again quickly), being careful not to touch the wet part.
- make a horizontal line with a pencil to indicate where the solution stopped, this is called the front of the solution (i.e., the point between the wet part and the dry part) (Fig. 4)
- place your paper strip on a countertop or desk and let it dry for at least 5 minutes. Once it starts drying you should see the pigment more clearly being able to tell how much it migrated on the paper.

How to interpret the results

- Each sample presents pigments that have different affinity for the solvent (acetone) so the pigments will then travel different distances on the chromatography paper.
- Different pigments have different colors on paper like they do in the plant tissues. The pigment in radishes skin (called pelargonidin) is bright red while the one in eggplant skin (delphinidin) is purple.
- this protocol mimics the real protocol used by researchers that study plant colors, here we have the three main components of the pigment extraction and separation protocol: an alcohol (isopropanol), an acid solution (lemon juice) and the medium for pigment separation (chromatography paper and acetone solution).

Acknowledgements
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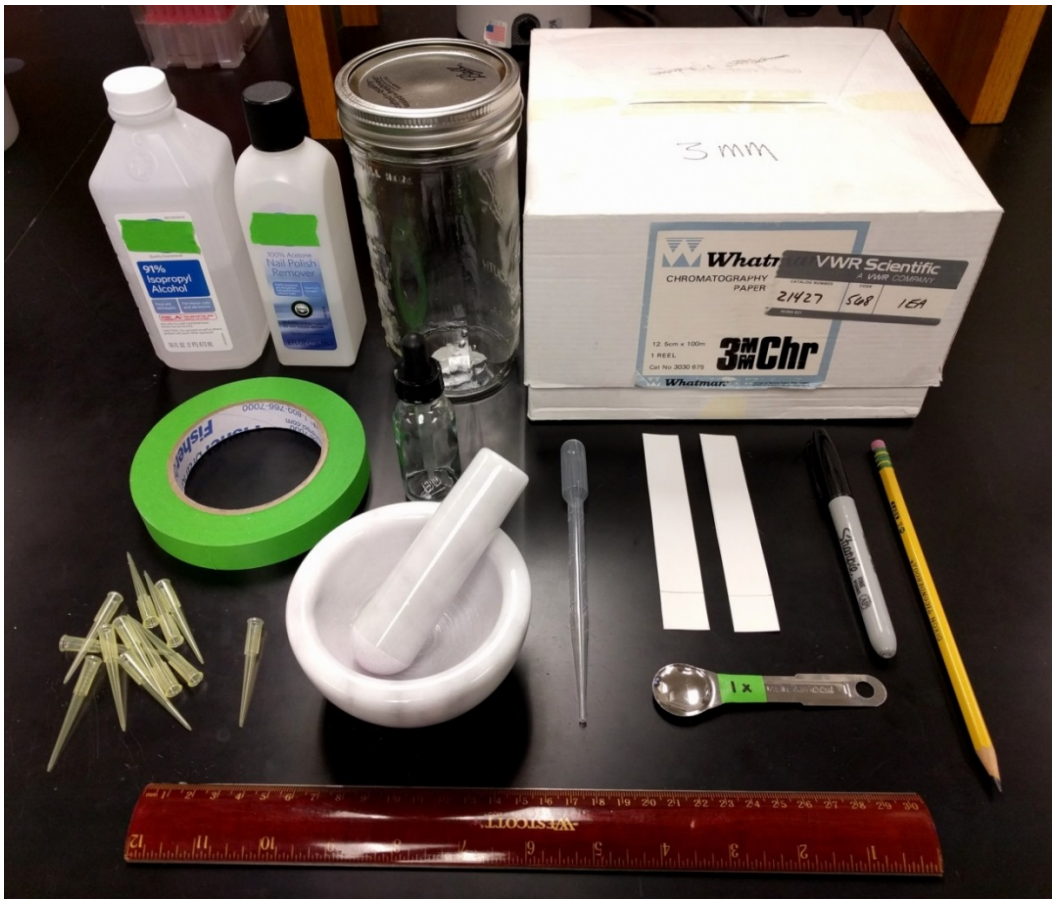

Figure 1. Most materials used in this activity. Not included in this picture, coffee filters, funnel and glass containers for storage.

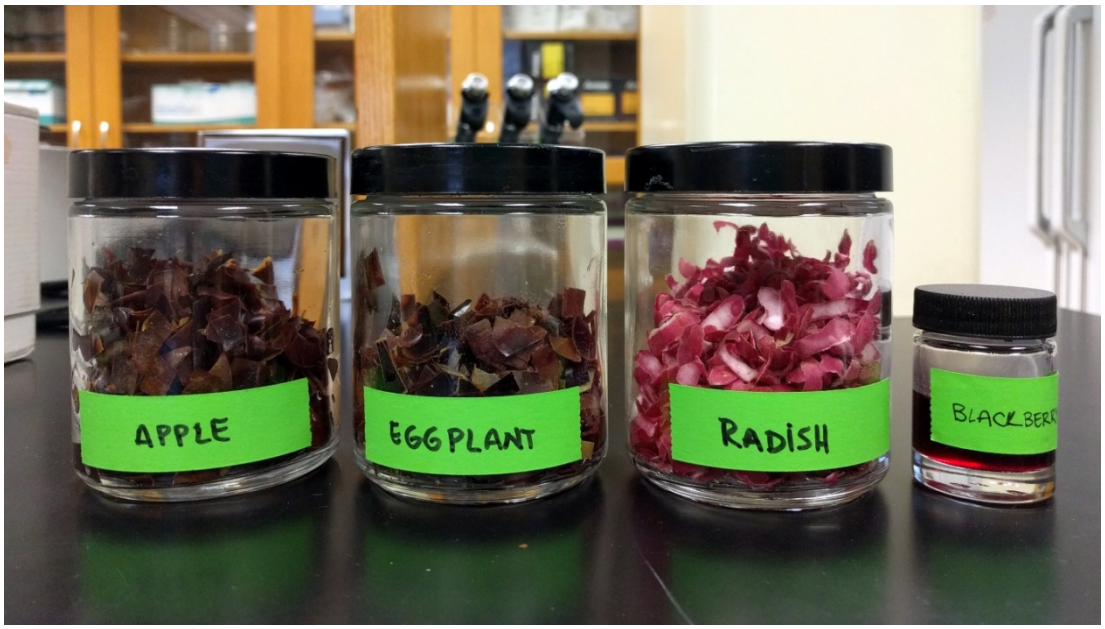

Figure 2. Skin samples for apple, eggplant and radish, and extraction solution for blackberry after filtering. Notice that the skin samples have little to none fruit flesh (white).

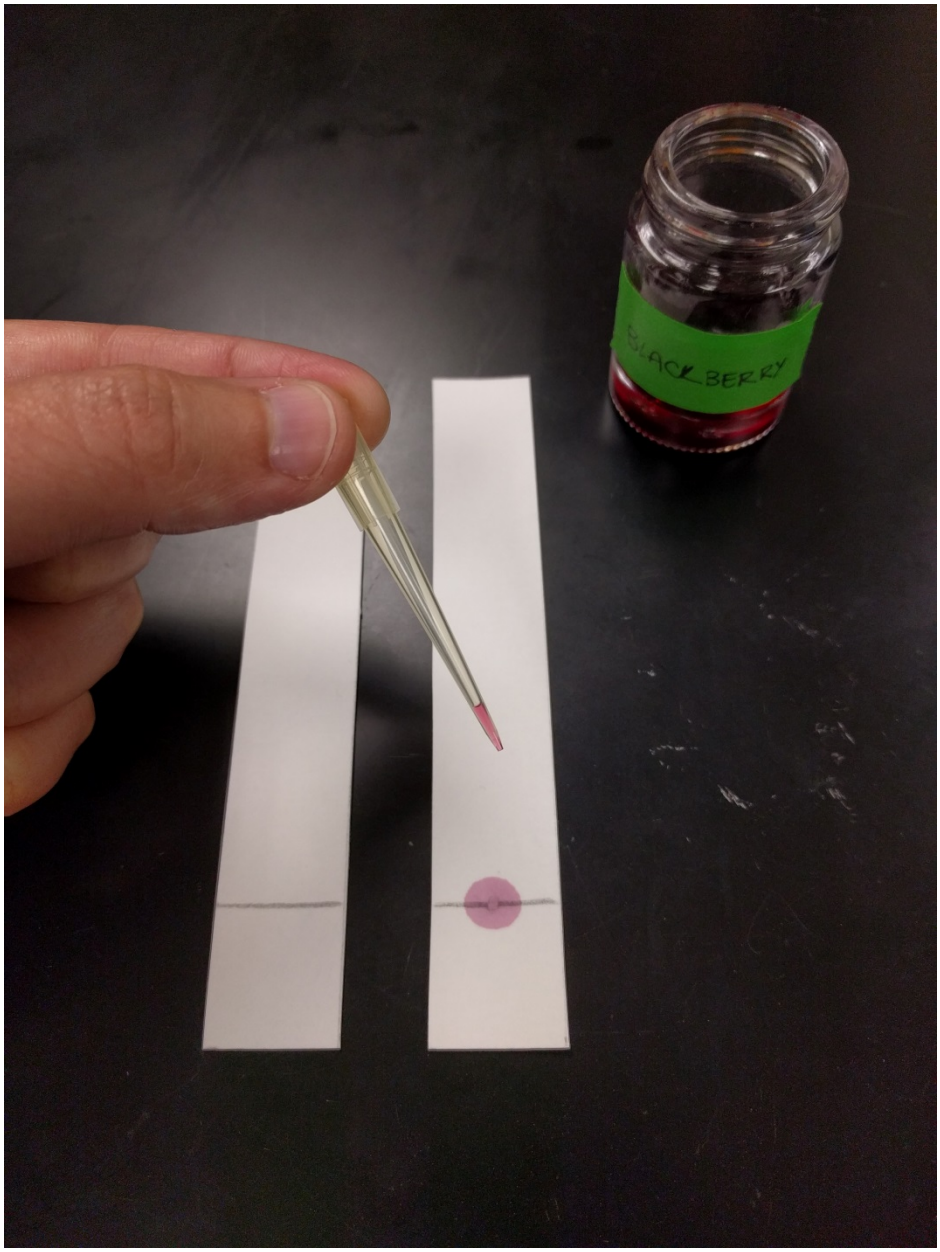

Figure 3. Strips of chromatography paper with a pencil mark at 2 cm. The strip on the right already received some of the blackberry solution. We recommend collecting roughly the amount of solution indicated on the pipette tip shown here, for each time the solution is transferred to the paper. Collecting too much solution each time will generate dots that are too big and not ideal.

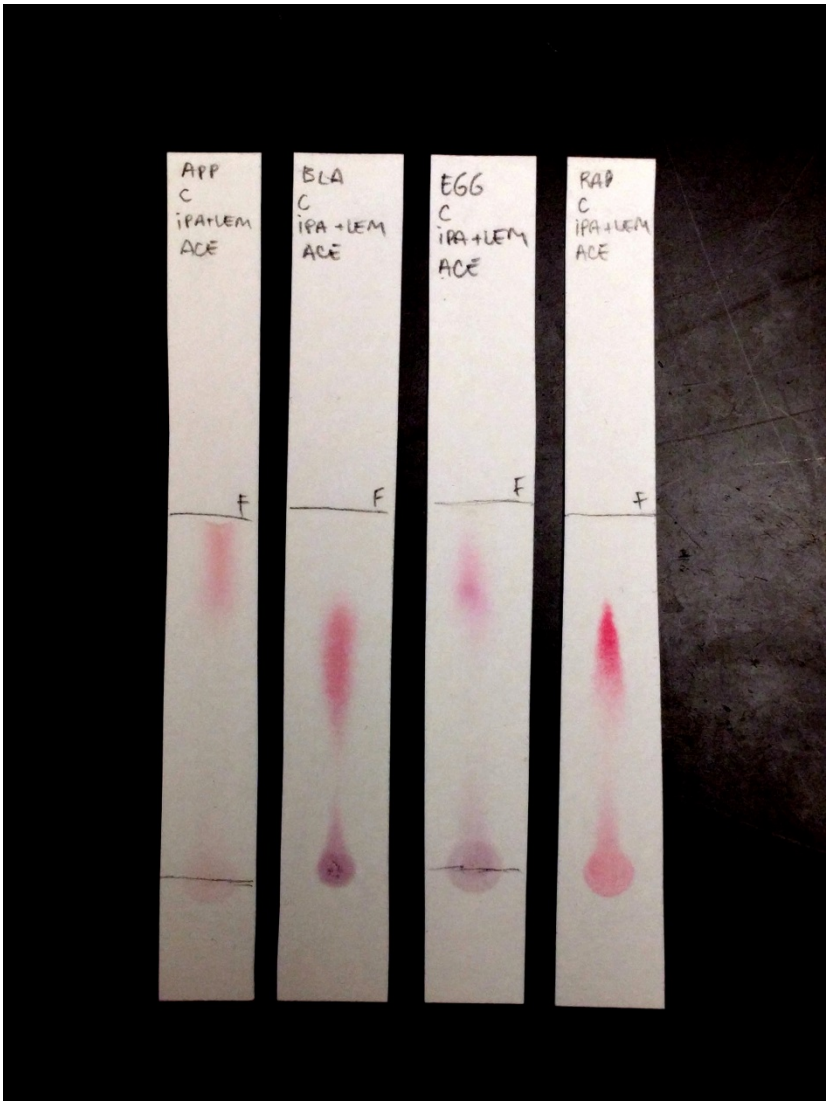

Figure 4. Here we see the results for all the samples, from left to right: apple, blackberry, eggplant, and radish. The lines identified as F represent the length covered by the solution while the paper was in the chamber.