

## INS Chemistry Lab 4, weeks 9 and 10

### Chromatography and Spectrophotometric Examination of Plant Pigments

**This lab is designed to be done by groups of 3-4.**

#### **Introduction**

This lab introduces two of the most commonly used techniques in chemistry and biology. Chromatography allows the separation of complex mixtures into the individual components that are present. This allows you to determine the number, and often the identity of the molecules present. Spectrophotometry (or spectroscopy) uses the light absorbing properties of material to provide information about the molecules present. This can also aid in the identification and quantification of the molecules present.

In biology you have seen how the types of light absorbing pigments vary in different photosynthetic organisms. The methods we use today are how these differences were discovered.

#### **Thin-layer chromatography**

**Thin-layer chromatography (TLC)** is one of the most widely used methods in chemistry and biology. All chromatography methods depend on a stationary phase and a mobile phase. Here, the stationary phase is a thin layer of silica (silicon dioxide) fixed on a plastic sheet. The sample is spotted near the bottom and then a solvent (the mobile phase) is allowed to diffuse up the sheet. Some molecules will stick very tightly to the silica and will not be attracted to the mobile phase solvent. These will move very little. Other materials will not stick so tightly to the silica and will be very soluble in the mobile phase; these compounds will move rapidly.

#### **Experimental procedure for TLC**

1. Use a PENCIL to draw a light line about 1 cm from the bottom of the TLC plate. (Be sure to do this on the silica side and not on the plastic backing.)
2. Apply the plant pigment solutions we will provide in small spots on this line. We will demonstrate how to apply the extracts. You want the spot to be as small as possible. You want to handle the silica surface as little as possible and be careful not to scratch the surface.
3. In a fume hood place a few mL of fresh TLC solvent (Be sure to note its composition) into a chromatography jar. **THE LEVEL OF THE LIQUID MUST BE BELOW THE STARTING LINE ON YOUR TLC PLATE.** Put your plate into the jar, close the lid, and observe at intervals. You want the diffusing solvent to near the top of the plate but not go off the edge.
4. When it reaches your stopping point, remove the plate from the jar and **IMMEDIATELY** mark the line where the solvent has stopped. Allow the slide to dry in the hood.
5. Sketch your plate in your notebook and indicate the color of each spot. The spots may fade with time and light exposure.
6. Measure the distance each individual spot has moved from the initial point and the total distance the solvent front has moved. Now determine the retention factor ( $R_f$ ) for each spot. ( $R_f = \text{distance a spot has moved} / \text{total distance the solvent front}$ )

- has advanced.) This value is characteristic for a given molecule with the same stationary phase and mobile phase.
7. The components you are likely to see, in order of decreasing  $R_f$  values, are carotenes (yellow-orange); pheophytin a (gray, may be as intense as chlorophyll b); pheophytin b (gray, may not be visible); chlorophyll a (blue green, more intense than chlorophyll b); chlorophyll b (green); xanthophylls (as many as three spots, yellow).
  8. Here, we are lucky-the spots are colored. Often, the plate would need to be examined under UV light or after chemical treatment. We will have a UV light to examine your plates. Do any molecules fluoresce?

### **Another TLC method. (OPTIONAL)**

This method allows the direct examination of the pigments from a plant leaf. This works reasonably well for plants that do not have thick cuticle or waxy layers.

1. As above, draw a thin pencil line at the bottom of a TLC plate.
2. Hold your leaf against the plate and use a coin to roll over the leaf along your pencil line. This should crush the leaf in a narrow area and transfer some pigment to your plate.
3. Develop and examine the plate by the procedure used above.

### **Column chromatography**

This is a similar process to TLC, except here the stationary phase is powdered material in a plastic or glass tube. Here we will use silica again. The material is soaked with the mobile phase, and the material to be separated is added to the top of the column. The mobile phase is then added and the material flows through the column with mobile phase. Again, some materials will stick tightly to the stationary phase and come out of the column very slowly. Other substances will not adhere to the stationary phase and will come out of the column very quickly. (The mobile phase here is petroleum ether, often called pet ether. It is a misnomer, as it really isn't an ether. It is a mixture of hydrocarbons that have a relatively low boiling point, similar to ether. It is mostly alkanes with 5-7 carbon atoms.)

### **Experimental for Column Chromatography**

1. Set up your column in the hood as demonstrated.
2. Add the plant pigment solution to your column and elute with the mobile phase solvent (again, note its composition)
3. Collect fractions of solvent as they pass through the column. Whenever a new color band comes off of the column, collect it in its own clean test tube. Estimate the volume of liquid that has eluted as you collect each fraction.

### **Spectrophotometric examination of plant pigments.**

We will demonstrate to groups how to use our diode-array spectrometers. Run an absorbance spectrum of each plant pigment solution we have, being sure to blank the instrument with the appropriate solvent. You want to cover all of the visible range of light. For each extract note the wavelengths of light most strongly absorbed. What colors

of light do these absorbance peaks correspond to? (A helpful figure here is figure 6.4 in the chem. text.)

### **Examination of column fractions**

When you have collected your fractions from your column chromatography, they can be further characterized. For example, you should do an absorbance spectrum of each collected pigment solution and note the differences. How do these absorbances compare to that of the starting pigment mixture? If there is time, you can also run a TLC plate of the pigments and see how many components are present in each of your column fractions. How cleanly did you separate the materials present?