

## IDENTIFICATION OF UNKNOWN SOLUTIONS USING TLC CHROMATOGRAPHY

### Purpose:

To become familiar with the principles and terminology of TLC chromatography to identify an unknown molecule based on comparisons with known laboratory standards and  $R_f$  factors.

### Background

#### **Thin Layer Chromatography:**

Chromatography is a sophisticated method of separating and identifying mixtures of two or more compounds. The separation is accomplished by the distribution of the mixture between two phases: one that is stationary and one that is moving or mobile. Chromatography works on the principle that different compounds will have different solubilities and adsorption to the two phases, which will allow for their separation.

Thin Layer Chromatography (TLC) is a solid-liquid technique in which the two phases are a solid, stationary phase and a liquid, mobile phase. The solid phase you will be using in today's lab is a plastic plate covered with an adsorbent, in this case, silica gel. Aluminum is another common solid phase used. Because silica is a polar molecule, the components of the solution we will use in lab today will be separated based on their relative polarities. The more polar a molecule, the higher affinity it will have for the more polar silica plate and will therefore spend less time in the mobile phase. As a result, it will move up the plate more slowly. Conversely, a less polar molecule will spend more time in the mobile phase and will therefore move up the plate more quickly. The speed at which the molecules will move up the plate thus depends on the relative difference in polarity between the stationary and mobile phases, and will vary depending on the nature of the stationary and mobile phases used for separation.

The following are some common uses of Thin-Layer Chromatography:

1. To determine the number of components in a mixture.
2. To determine the identity of two substances.
3. To monitor the progress of a reaction

The difference each molecule travels along the adsorbent in relation to how far the mobile phase has traveled is called the Retention Factor ( $R_f$ ) and can be used to identify molecules, as the value is molecule specific. The  $R_f$  for any given molecule will vary depending on the mobile and stationary phases used.

### Materials per group:

2 TLC Plates

2 Developing Chambers (beaker, plastic wrap, rubber band)

Ruler

Pencil

7 Microcapillary tubes

UV lamp

Tweezers

Vials containing prepared solutions of:

- Adenine
- Cytosine
- Guanine

Unknown solution 1 (generated from the DAMN reaction)

Unknown Solution 2  
Unknown Solution 3  
Unknown Solution 4  
Distilled Water (will serve as the mobile phase)  
A pencil  
A ruler

### Procedure for TLC

#### **READ ALL INSTRUCTIONS BEFORE BEGINNING EACH STEP IN THE LAB**

##### *Step One: Preparation of the Developing Chamber*

1. Obtain two developing chambers from the front of the room. A developing chamber consists of a beaker, a piece of saran wrap, a piece of filter paper and a rubber band
2. Place a piece of filter paper into the beaker, as demonstrated by the image below. This will ensure that the TLC plate will remain saturated with the vapor from the aqueous mobile phase, so that the plate will run correctly.



3. Carefully pour water into the beaker, to a depth of approximately 1 cm, making sure that the entire filter paper is saturated, swirling if necessary.

##### *Step Two: Preparation of the TLC plate.*

Plate one will be used to identify an unknown solution by comparing the distance it travels on the TLC plate to the distance traveled by the stock solutions. On plate two you will be running the remaining unknown solutions, and will be used for identification of the unknown by calculation of the R<sub>f</sub> factors.

1. Using a ruler and a **pencil**, draw a across the TLC plate 2.0 cm from the bottom, as indicated in the picture below **pressing the pencil lightly as to not damage the coating on the TLC plate**. This line will serve as the **origin line** on which you will spot the plate, or use the capillary tubes to place the stock and unknown solutions. Repeat this step for both plates.



2. All three stock solutions and unknown A will be run on plate one, the remaining three unknowns will be run on plate two. Again, taking care to press gently down on the TLC plate, mark the location you will be placing or spotting each solution by adding a hash mark to the line you have already drawn. Evenly space out the marks along the plate, starting and ending no less than 0.5 cm from the edge of the plate.

Label each hash mark below your origin line, using the symbols

- a. Plate one: A,C,G, U1
- b. Plate two: U2,U3,U4

### ***Step Three: Spotting the TLC plate***

You will be using the capillary tubes to spot both the known and unknown solutions to your TLC plates. Each solution will require a separate capillary tube to prevent cross-contamination. Using a piece of tape wrapped around the top of the tube, carefully identify each tube, using the same symbols you used to label the plates. Use caution with the capillary tubes, as they are fragile and very sharp when broken.

1. Take a capillary tube labeled A and place the pointed end into stock solution A. You should be able to see the solution rise up into the capillary, through capillary action.
2. Next, using plate #1, touch the end of the capillary tube **gently** to on the origin line at the spot indicated for that solution. Your goal is to make only a small spot. **DO NOT let all of the contents of the capillary tub run onto the paper. You will not use all of the solution inside the capillary tube.** Repeat this process on plate 3, spotting on the mark that says "A".
3. You will now repeat this process for the remaining solutions, putting one spot at the indicated spot on each TLC plate, until each solution has been spotted on its appropriate plate.
4. After each spot is dry, you will repeat the entire process 2 more times, so that each solution has been spotted 3 times on every plate. It is **VERY IMPORTANT** that you allow each spot to fully dry before re-spotting the solution. Blowing on the spot, or fanning the TLC plates may help to speed drying time.
4. Repeat the spotting procedure for each marked solution for both plates.
5. Proceed to step four when **all the spots on both plates are dry.**

#### ***Step Four: Developing the TLC plate***

Before placing your TLC plates into its developing chamber, measure and compare the height of the water in relation to the line you have drawn on your TLC plate. If it appears that the water may cover the line, then remove some of the water from the beaker. This step is critical! **If the water covers the line when you place the TLC plate into the developing chamber, you will have to start the lab over from the beginning.** If you are unsure if the water will cover the line, then err on the side of caution, and remove some of the water. You can always add water back with no negative effects if there isn't enough water in the beaker to develop the plate.

1. Using the gloves, carefully place the prepared TLC plate in the developing beaker, so that it is sitting on the bottom of the beaker, and leaning against the side of the beaker that is not covered by the filter paper. Be very careful! If the plate falls into the water, you will have to start the lab over from the beginning! Make sure you record what time you first placed the plate into the chamber.
2. Cover the beaker with the saran wrap, and carefully secure the saran wrap with a rubber band, making sure not to dislodge the TLC plate within the chamber. **DO NOT** pick up the chamber!
3. Repeat this process for the remaining plate.
4. Watch as the mobile phase runs up the TLC plate. When the mobile phase is between 2- 4 cm from the top of the TLC plate, remove it from the chamber using the tweezers, and place it on a paper towel. Make sure you remove the plate before the mobile phase runs off the end of the plate. If this happens, you will have to start the lab over from the beginning.
5. Using a pencil, trace the line of the mobile phase on your plate. This is a critical step for the calculation of the R<sub>f</sub> factor.
6. Leave the plate on the paper towel until it is completely dry.
7. Proceed to step 5 **only when the plates are totally dry.**

#### ***Step Five – Visualizing the spots***

**Warning:** UV light is damaging both to your eyes and to your skin. Make sure you are wearing your goggles and do not look directly into the lamp. Protect your skin by wearing gloves.

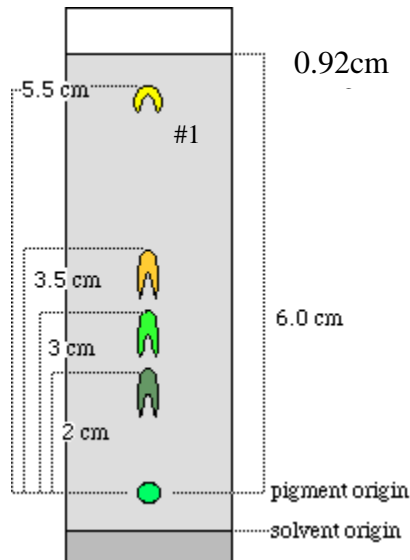
1. With a lab partner, hold the UV lamp over the plate, facing down, and mark any spots which you see lightly with a pencil, tracing around their outline. Ideally, you should see individual dots for each compound in the mixture.
2. Now that you can visualize the molecules that are on the plate, you can begin collecting the data which will allow you to determine the identity of the unknown solutions.

#### ***Step Six – Evaluating the data***

**Plate #1** - The purpose of plate one is to compare the distance travelled by the spot with known standards. Examine the spot and compare its location on the plate with the location of the known standards, and record your information in the student data sheet. In addition, plate one allows you to calculate the R<sub>f</sub> factor for the known solutions.

**Plate # 2** - The purpose of plate two is to identify the unknown standards using a comparison of R<sub>f</sub> values. To do this, you will need to calculate the R<sub>f</sub> value for each spot on both plates, using the formula provided below, and record your information on the student work sheet.

**Sample Calculation:** This calculation is based upon a sample that is made up of a combination of substances, and therefore has more than one spot. The procedure for determining the R<sub>f</sub> value for each spot within one sample is the exact same as the procedure for determining the R<sub>f</sub> value for many individual samples on one plate



The **solvent front** is the distance the mobile phase traveled on the plate, and is what you recorded with your pencil when you took your plates out of the developing chamber C

The formula for calculating the R<sub>f</sub> values is

$R_f = \text{Distance moved by the molecule (location of the spot)} / \text{Distance moved by the mobile phase (solvent front)}$

The R<sub>f</sub> value for the substance indicated by #1 would be:

$$R_f = 5.5 \text{ cm} / 6.0 \text{ cm} = 0.92 \text{ cm}$$

### **Student Solution Sheet:**

What is your prediction for the chemical identity of:

Unknown 1: \_\_\_\_\_

Unknown 2: \_\_\_\_\_

Unknown 3: \_\_\_\_\_

Unknown 4: \_\_\_\_\_

Justify your answer for each unknown using as much evidence as you can. Evidence may be either qualitative or quantitative in nature. Make sure you use the correct terminology when referring to the components of the experiment!! Use your justification to convince me that you understand how the TLC process works.

### **Additional Post Lab Questions:**

1. Which method did you think was more useful in identifying the unknown solution?
2. On a scale from 1 – 5, how confident are you that your unknown's were identified correctly (5 being most confident)
3. What applications might this process have in other areas of science?
4. What were potential sources for error in this experiment?

## Student Work Sheet

<b>Sample</b>	<b>Distance Traveled</b>	<b>Solvent Front</b>	<b>Rf Value</b>
Adenine Standard			
Thymine Standard			
Cytosine Standard			
Unknown 1			
Unknown 2			
Unknown 3			
Unknown 4			

Provide a sketch of your TLC Plates below, making sure to include the Rf Values on the sketch.

TLC Plate #1 contains 3 known solutions and 1 unknown solution and has 2 important pieces of information, relevant to this experiment.

What are they?

- 1.
- 2.

TLC Plate #2 contains 3 unknown solutions only. What useful information can you obtain from TLC plate # 2?

### Pre-Lab Questions:

1. What is the name of the technique you will be using in today's lab?
  - a. Thin Layer Chromatography
  - b. Mass Spectrometry
  - c. Thick Liquid Calorimetry
  - d. Liquid Chamber Chromatography
  
2. How many plates will you be using in today's lab?
  - a. 1
  - b. 2
  - c. 3
  - d. 4
  
3. What is the purpose of the lab?
  - a. To measure the rate of the movement of molecules in a solution
  - b. To identify the composition of unknown substances
  - c. To determine the speed of a chemical reaction
  - d. To isolate and separate different DNA nucleotides
  
4. Which of the following is a piece of equipment you will be using in today's lab?
  - a. A Bunsen Burner
  - b. A calorimeter
  - c. A UV Lamp
  - d. A triple beam balance
  
5. TLC plates separate molecules on the basis of:
  - a. Polarity
  - b. Color
  - c. Size
  - d. Bond orientation

## TEACHER INFORMATION SHEET

### Preparing the solutions:

- The Adenine and Cytosine standards can be made by dissolving approximately 1 gram at a time into 5 mL of water, until the solution is saturated. Continue mixing until no more solute will dissolve in the water.
- The Guanine standard can be made by following the above procedure, but using KOH as the solvent instead of water. The solution may get warm while you are mixing it as guanine is a strong base.
- The unknown solutions 2,3 and 4 are just the stock solutions, relabeled
- Unknown solution 1 is a product of the DAMN reaction
- All of the solutions can be disposed of by rinsing them down the drain, and will store in a freezer for at least a year.
- Capillary tubes can be prepared in advance with Bunsen burners

### Timing for Class

- There is a fair amount of 'wait time' associated with running the chromatography plates (about 35-40 min), which gives flexibility to the teacher about how to best fill the time.
- On block schedule, on class should be sufficient to run the lab, but you will need to leave the plates over night to dry before reading them.
- With a regular bell schedule (50 min. classes), this lab would take a segment of 3 days, as divided below
  - Day 1 – full day; discuss lab and spot plates
  - Day 2 – run plates and leave overnight to dry (approx. 10 min.)
  - Day 3 – read plates and answer questions (flexible timing, depending on how much in/out of class)

### Safety Considerations

This lab has relatively few safety concerns. Students should wear goggles when dealing with the chemicals in the lab, and wear gloves to protect their skin from the UV lights and from the chemicals used. The capillary tubes are fragile and sharp. For clean-up, the chemicals can be disposed of down the drain.

### Support and Materials

This lab was developed with funding from the NSF and the Origins Project at Emory and Georgia Tech. Teacher support and funding for lab equipment is available at a substantially reduced cost through the center to interested teachers. Please contact Dr. Nick Hud at [hud@chemistry.gatech.edu](mailto:hud@chemistry.gatech.edu) for more information about obtaining material or participating in future development opportunities.

### State Science Standards:

#### **SCSh1. Students will identify and investigate problems scientifically.**

- a. Suggest reasonable hypotheses for identified problems.
- b. Develop procedures for solving scientific problems.
- c. Collect, organize and record appropriate data.
- d. Graphically compare and analyze data points and/or summary statistics.
- e. Develop reasonable conclusions based on data collected.

Evaluate whether conclusions are reasonable by reviewing the process and checking against other available information.

**Biology:**

**SB2. Students will evaluate the role of natural selection in the development of the theory of evolution.**

- a. Explain how fossil and biochemical evidence support the theory.