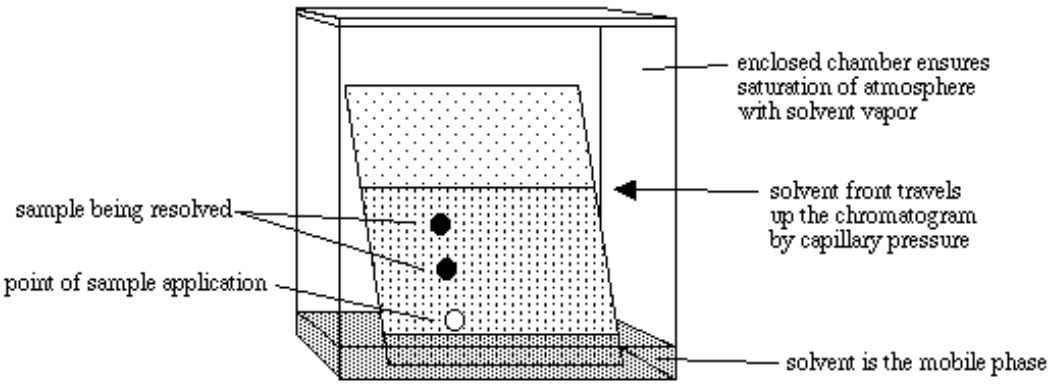
Overview
You and your partner will choose your favorite citrus fruit from a selection and isolate its free carbohydrates. After isolation, the carbohydrates will be separated and identified using TLC (thin layer chromatography) by comparison with carbohydrate standards. When you isolate something you take advantage of its chemical and physical characteristics, such as solubility, ionic charge, hydrophobicity, or size. If the compound you want to isolate is very similar to other compounds, you have to choose a technique that emphasizes the characteristics that are different. Chromatography is one of several procedures for isolating compounds.

The definition of chromatography is a technique that separates compounds by differential migration during passage through a porous medium. Another way to describe this is that the compounds are separated as a result of differential affinity for a **stationary phase** (solid or liquid) or for a **mobile phase** (gas or liquid). Several properties of the stationary and mobile phases determine how strongly the compounds interact with those phases: adsorption, ion exchange, its relative solubility in the stationary versus the mobile phases, and many other properties. The different chromatographic procedures, such as adsorption chromatography, gas chromatography, gel-filtration chromatography, ion-exchange chromatography, thin-layer chromatography and many others, usually take advantage of two or more of these properties.



Thin layer chromatogram in process of development

Thin layer chromatography (TLC) is one the simplest of the chromatography methods to use and understand. Since TLC cannot accept large amounts of sample but is fast, it is used as an analytical technique in the identification of unknowns. It is an example of adsorption chromatography and the adsorbent is usually silica (sometimes alumina). The adsorbent is coated onto a plastic sheet in a layer about 1-2 mm thick. The sample is spotted near the bottom of the sheet, high enough that it won't be submerged in the solvent. The sheet is placed on edge in a chamber to which the mobile phase has been added to a depth of a

few millimeters. From that edge, the mobile phase slowly covers the rest of the plate by capillary flow. The sample is carried upward with the mobile phase solvent. Development of the chromatogram is stopped when the leading edge of the mobile phase, known as the solvent front, gets near the top of the plate. Because the sample interacts somewhat with the silica, it does not migrate as fast as the solvent front. By measuring how far your sample moved in comparison to standards it is possible to identify what your sample consists of.

The stationary phase

Thin layer chromatography separates molecules on the basis of how well they adsorb to silica. Silica, silicic acid and silica gel all refer to acidified silicate solutions with the empirical formula of H_2SiO_3. Silica is popular because it has a large surface area, meaning it can bind a large number of molecules at once. Its surface consists of Si-OH groups that are very closely spaced and it is these groups that are the active sites. Silica is polar and the oxygens can hydrogen bond to the sample compound and to water. The activity of a given batch of silica depends on the number and availability of these sites. These sites can easily be masked when there is surface-adsorbed water on the silica; you can re-activate these groups by drying the gel with heat to drive off the water. The white silica powder is insoluble in water and remains bound to the plastic backing.

The mobile phase

We take advantage of solvent composition when working out a chromatographic procedure with TLC. This is obviously because we can't change the silica adsorbent or the sample compound we are isolating. The solvent carries out two functions: 1) to carry the sample through the chromatographic bed, and 2) to compete with the sample for space in the adsorbed layer of molecules. The choice of solvent depends on working these two solvent functions against one another. If the solvent is very polar, it will prevent the adsorption of the sample to the silica. In this case, the sample will travel through the chromatographic bed rapidly. With a less polar solvent, the sample spends some time adsorbed to the silica and moves slower than the bulk solvent. The object is to choose a solvent in which your compound is soluble but that emphasizes differences with compounds of similar chemical composition.

R_f value

The distance traveled by each compound from the origin, or base line, relative to the solvent front is defined as the Rf:

$$R_f = \frac{\text{Distance compound traveled from origin}}{\text{Distance solvent traveled from origin}}$$

Every compound has a particular R_f value when measured under specified conditions, such as solvent, temperature, whether ascending or descending chromatography, and the adsorbent used. Because there are so many variables, the R_f of a substance can only give you a rough indication of the identity of your

unknown. Therefore, a sample of known material (a positive control) is resolved along with the unknown. Of course, this assumes that you already have an idea as to the identity of your unknown.

Before Lab:

1. Use the USDA Nutritional Database to determine the % mass of fructose, glucose, and sucrose in honey.

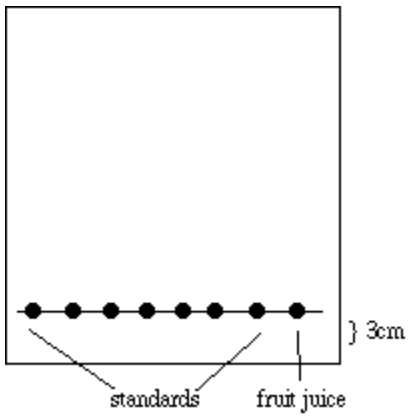
2. Use the USDA Nutritional Database to determine the % mass of sugars in light corn syrup.

Isolation of soluble sugars from honey and corn syrup

Prepare 1 mL each of a 1:20 dilution of honey and corn syrup using water. To remove any protein, take 0.25 ml of the liquid and add 0.75 ml of ethanol in a microfuge tube. Most proteins are insoluble in ethanol. Spin balanced tubes for 2 min @ 14,000 rpm. Place 100 uL of supernatant in a clean microfuge tube and evaporate the ethanol.

Resolving the sugars by TLC

Use a blunt pencil to draw a very light line 1.5 cm from one of the short sides of the thin layer sheet (see diagram below). On this line at regular intervals, indicate where you are going to spot the six different sugar standards and the food extracts. Spot samples using a glass capillary. Allow the spot to dry. Adding only a little at a time keeps the spot small and concentrated. Remember to use a new glass capillary for each sample that you apply to the sheet. You only need to spot once.

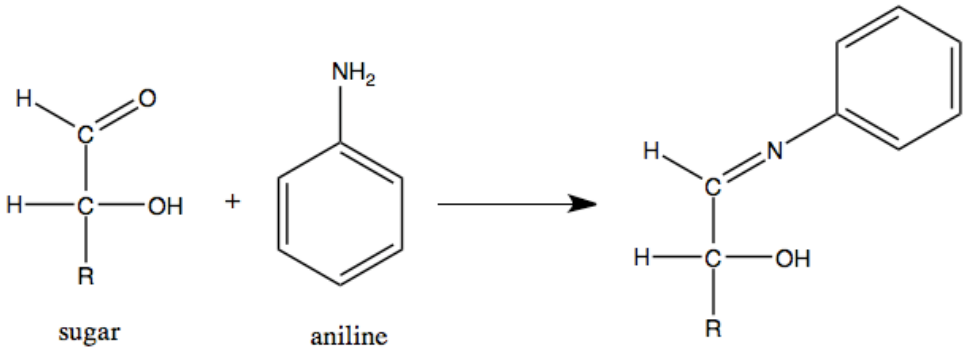


IN A HOOD:

Pour enough solvent (n-butanol/acetic acid/ water (2:1:1)) into the bottom of the chromatography tank so that it is 1 cm deep. Place your thin layer plate into the solvent (sample edge down). Allow the solvent to migrate up the plate until it stops moving. You will need to watch the plate for the solvent front stalling out. Remove the plate and draw a line across the plate with a pencil to indicate the

solvent front. Wait for the plate to dry before removing the plate from the hood and proceeding to the detection step.

Detection of sugars on TLC by aniline-acid-diphenylamine reagent
Do this in the hood and wear gloves! **Aniline is toxic if you breath it, ingest it, or get it on your skin.** Very quickly dip your TLC plate in the dye reagent. Use a fan to dry the plate. Heat the chromatogram at 100-105 °C for 5 min to develop the colored spots that form by reaction of the reducing sugar with the reagent. Alternately, try using a hair drier.



sugar aniline

Make sure to **note the color of the spots when they are fresh!** All of the colors eventually darken to brown. Aldohexoses are brownish and aldopentoses are reddish, but there will be colors ranging from black to green to gold. Take a picture of your TLC plate.

1.) **Staple in a photo of your TCL plate.**
2.) **Open your TLC plate image in ImageJ (free software to dowload https://imagej.nih.gov/ij/).**
3.) **Orient the image with the origin line at the bottom and the solvent front at the top.**
4.) **Select the *straight* line tool.**
5.) **Starting at the bottom of the for the first sample, click and drag a line up to the solvent front transversing the migration lane. A yellow line should be visible on your image which goes through the spots for that sample, the spotting line, and the solvent front line.**
6.) **In the "Analyze" menu, select "Plot Profile". A second window reporting that gray scale across the line on your image will appear.**
7.) **Move the cursor over the middle of each peak and record the distance in pixels for each major spot. Record the distance of the spotting line and the solvent front in pixels.**
8.) **Repeat for each sample lane on the TLC plate.**
9.) **Staple in a well-labeled table summarizing the pixel distances of the two lines and major spots for each sample. Indicate the color for each major spot in the table.**

10.) **Subtract the solvent front line pixel distance from the spotting line pixel distance for each sample. This is the distance of solvent migration for the denominator in the R_f calculation.**
11.) **Determine the R_f value for each of the major spots for each sample.**
12.) **Staple in a well-labeled table summarizing the R_f values for the major spots for each sample. Indicate the color for each major spot in the table.**
13.) **Does your chromatogram support the information in the USDA Nutritional Database for honey? Explain your reasoning.**
14.) **What are the major sugars present in corn syrup? Explain your reasoning.**