CHEM 2219: Exp.# 2 Microscale Column Chromatography
Separation of Methylene Blue from Fluorescein Dyes

Objective: In this experiment you will learn how to determine appropriate solvents to separate the two components of a dye solution by column chromatography and how to take measurements of the retention time and band width in order to calculate "Theoretical Plates" for the two dyes. The effect of pH on the fluorescence of fluorescein will also be determined.

***** Column chromatography is a useful method for separating components of a mixture of compounds based on their polarity.

Reading Assignment:

MTOL, pp. 119-125 (column chromatography); and, OCLT, pp. 119-139 (column chromatography) and pp. 370-371 (column chromatography techniques).
Also on **CANVAS** read and answer prelab questions for **– Column Chromatography**

Concepts:

Adsorbent, Band Width, Chromatography, Eluent, Fluorescence, Mobile Phase, pH, Polarity, Retention Time, Stationary Phase, Theoretical Plates

Chemicals:

Alumina (Al_2O_3), Ethanol (C_2H_5OH), Fluorescein, Hydrochloric Acid, Methylene Blue, Sodium Hydroxide and Water

Safety Precautions:

Wear chemical splash-proof goggles and appropriate attire at all times.
Ethanol is a flammable liquid.
Hydrochloric acid is highly corrosive. Sodium hydroxide is extremely caustic.
If you spill an acid or a base on the counter or floor, call for a TA or an instructor to neutralize the spill.
If you spill an acid or a base on your skin, immediately walk to the nearest sink and wash thoroughly with cold water. Strong bases dissolve the fats in your skin to produce a soapy feeling. Keep rinsing with cold water until long after the "soapy" feeling is gone. Do *not* attempt to neutralize a spill on your skin.

Materials: balance, beakers (50 ml, 100ml, 150 ml), burette clamp, column (plastic pipet with funnel and stopcock), disposable pipets & bulbs, graduated cylinder (10 ml), ringstand, ruler, test tube rack, 2 test tubes with rubber stoppers and a UV light.

Background:

The word **chromatography** is derived from two Greek words meaning color (*chroma*) and to write (*graphein*). The term chromatography was coined in 1903 by the Russian chemist Michael Tswett to describe a new technique he had invented to separate the pigments in green plant leaves. When Tswett ran the liquid extracted from green leaves through a column containing a clay-like adsorbent solid, he discovered that even green leaves contained red and yellow pigments in addition to the main green pigment, chlorophyll.

Since Tswett's discovery, chromatography has evolved into one of the most important tools chemists have for separating the components of a mixture. In addition to column chromatography, there are many other types of chromatography: gas chromatography, gel-permeation chromatography and ion-exchange chromatography and high performance liquid chromatography.

Column chromatography is a simple form of chromatography based on adsorption. The column contains a **solid stationary phase** (such as aluminum oxide, Al_2O_3 or silica gel $mSiO_2 \bullet nH_2O$) which acts as the **adsorbent** (surface to which the molecules adhere). A thin layer of the mixture to be separated is placed on top of the stationary phase in the column.

The **mobile phase** consists of a flow of a liquid **eluent** (or solvent) which is washed through the column. It carries (or mobilizes) the components of the mixture down the solid column and they separate based on their relative affinity (preference) for the adsorbent versus the eluent. The affinity of a molecule for the stationary phase is dependent on its intermolecular forces. The types of intermolecular forces present depend on both the adsorbent and the molecules in the mixture to be separated. **Nonpolar molecules** normally only have weak dispersion forces; whereas, **polar molecules** may have the stronger dipole-dipole forces and hydrogen bonding forces in addition to the weak dispersion forces. Traditionally, the stationary phase is a relatively polar compound and the mobile phase is rather nonpolar. Therefore, the more polar the compound in the mixture, the stronger the intermolecular attraction will be for that molecule to the stationary phase. So those molecules that have a greater affinity for the adsorbent will adhere to the stationary phase longer and remain in the column longer, traveling at a slower rate. The molecules that have lesser affinity for the adsorbent will not interact with the adsorbent. In other words, these molecules will not adhere as strongly to the stationary phase and will travel through the column at a faster rate along with the mobile phase.

In column chromatography, the choice of solvent is also important for the success of the separation of the compounds in the mixture. Usually a single solvent is unable to all the components in a mixture. Often a single solvent will not move the mixture at all or it will move all of the components together. In order to offset this difficulty, the composition of the mobile phase is altered by varying the solvents throughout the process. Generally, a nonpolar solvent is used first to move all of the nonpolar compounds through the column. This is followed by solvents with gradually increasing polarity until all of the components of the mixture have travelled through the column.

So as the components of the mixture travel down the column, they begin to separate based on their interaction with the stationary phase and their rate of travel. Ideally, if the column is long enough, the components will begin to separate into distinct bands which contain only a single component from the original mixture. The successful separation of the components of a mixture using column chromatography then is based on two factors: the absorptivity of the component to the stationary phase and the solubility of the component in the mobile phase.

In this experiment, a mixture of fluorescein and methylene blue will be separated. Fluorescein was named because it fluoresces under basic conditions (**pH** ≥ 9). When fluorescein is dissolved in an alkaline solution of NaOH, a water-soluble salt forms. The sodium salt of fluorescein absorbs electromagnetic radiation and immediately emits light at a longer wavelength, which has a lower energy than the absorbed radiation. In other words, it has the property of **fluorescence**. The light emitted is in the UV spectrum, so the alkaline fluorescein solution will appear to glow green when viewed under a black light.

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Procedure for Packing Column:

1. Assemble your column. (See Figure 1.) Using a burette clamp, clamp the column (pipet) to a ringstand. Place a 100 ml beaker below the tip of the column. Make sure the column stopcock (drain valve) is closed.*

> ***Note:** Similar to a burette, the stopcock attached to the pipet is open when it is parallel to the pipet and closed when it is perpendicular to the pipet.

2. Make a slurry. In a 150 ml beaker, add 7.5-8.0 g of alumina. Acquire ~20ml of 95% ethanol in a 50ml beaker. Add only enough ethanol* to the alumina to make the slurry "*soupy*." Stir until the slurry is a consistent texture and is thin enough to be pourable.

> ***Do *not* use all 20 ml of the ethanol.**
> Most of it will be used for rinsing later.

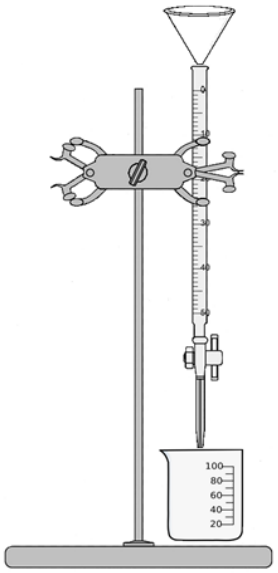

Figure 1: Column Assembly.

3. Quickly pour $^1/_3{}^{rd}$ of the slurry into the column while stirring. Allow the alumina to settle. Drain off excess solvent* by opening the valve and draining the excess ethanol or pipetting into the slurry beaker.

> ***Note:** Do not allow the alumina to become dry. Make sure the ethanol level never drops below the top of the alumina (i.e., maintain **1-3 mm** or $^1/_{16}$ **-** $^1/_8$ **inch** of solvent above the slurry).

4. Add the second $^1/_3{}^{rd}$ and allow it to settle. Drain excess ethanol as needed. Add remaining $^1/_3{}^{rd}$ slowly until the column of alumina is about **13 cm** or **5"** (inches). (*Rulers may be found in your drawer*.) Drain off any excess solvent.*

> ***Note:** Pressure may be applied by a plastic syringe to the funnel to speed up drainage. You may also remove excess solvent from above the alumina using a disposable pipet.

5. Carefully rinse any powder off the walls of the column above the liquid. Allow the column to drain until there are no gaps in the alumina* and the liquid level is just even with the top of the alumina.

> ***Note:** Verify that there are not any fissures (*cracks*) in the column packing. If there are, remove the funnel and use your microspatula to break up any pockets of air or cracks in your alumina.

6. The final alumina column should be **2-3cm** or **~1"** (*or 1-2 finger widths*) from the top of the pipet. Once the column is prepared, **1-2 mm** or ~ $^1/_{16}$**"** of solvent should remain above the packing. If necessary, drain off any excess ethanol.

Chromatography Procedure:

1. Acquire a bottle of the green dye (fluorescein/methylene blue mixture) and its corresponding disposable pipet and rubber bulb from the trays near the balances.

2. With the pipet carefully*, add 3-4 drops of the green dye directly above the column packing. Return the dye mixture bottle and pipet to the trays by the balances.

> ***Note:** Be careful not to get any dye in the rubber bulb of the pipet. The tip of the pipet should be just above, but not in, the alumina. Add the drops slowly, so that they do not splash up on the sides of the column or on the funnel. If dye does get on the sides or the funnel, you will need to clean them before you start draining your column. If it is necessary to clean the funnel or the sides of the column, remove the funnel. To clean the funnel, wash it with acetone over the waste container. To clean the column, run a Q-tip along the inside of the column. Repeat if necessary. The Q-tips and acetone can be found in the waste hood.

3. Open the stopcock to allow the dye to start soaking into the packing. When the dye drops below the top of the packing, add a couple of drops of EtOH. Repeat until there is a definite blue ring along the top of the alumina packing. Close the stopcock and proceed to step 4.

4. Acquire a disposable pipet and bulb from the supply cart. With the disposable pipet, carefully add 95% ethanol (EtOH) down the side of the column (so as not to disturb the packing) until the solvent is **1-3 cm** or **½"- 1"** above the level of the alumina.

5. Open the stopcock and allow the eluent to drain into the waste beaker. Drain until the EtOH is **1-2 mm** or ~ **$^1/_{16}$"** above the alumina. While the EtOH drains, the methylene blue band should be observed to be moving down the column. The yellow fluorescein should remain at the top of the column. (See Figure 2.) The flowrate of the mobile phase (solvent) should be about 1 drop every 2-5 seconds.*

> * Air pressure may be carefully applied via syringe to increase the flow rate. Press the plastic syringe into the funnel and apply pressure to speed up drainage. Do not drain off the solvent too quickly as this will cause the bands to become wide. The narrower the band width, the more efficient the separation.

6. Repeat the addition of EtOH and drainage steps until the front (bottom) of the blue band is **0.5 – 1.5 cm** or **¼"** to **½"** from the bottom of the column (*above where the column tapers*). Close the stopcock. Measure and record in cm the **retention time**, $\mathbf{t_R}$, as the distance from the top of the alumina to the center of the blue band and the **band width**, $\mathbf{w_B}$, as the width of the blue band in the vertical direction. (See Figure 3.) You will use these values to calculate the number of theoretical plates, $\mathbf{N=16(t_R/w_B)^2}$ for the blue dye.

7. Restart the flow. Stop when the blue dye is at the tip of the column. Replace the waste beaker with a 10 ml graduated cylinder. Restart the flow and collect the column eluate until all of the methylene blue has eluted (i.e., the blue color disappears). Close the stopcock. Record the volume in ml of methylene blue/EtOH collected. Dispose of the solution. Clean the graduated cylinder and the 50 ml beaker.

8. Place the waste beaker under the column. If necessary, apply air to drain any excess ethanol. **Do *not* allow the solvent to go below the packing.** Once the solvent is level with packing, go to the next step.

9. In the 50 ml beaker, acquire ~20 ml of 1M HCl.

10. Carefully add **1-3 cm** or **½"** – 1" of acid to the column to begin the elution of the fluorescein. **Wait 5 minutes.** Repeat the procedure (Steps 3-6) used for methylene blue for fluorescein using 1M HCl instead of EtOH. Always keep the level of the solvent above the alumina. Stop the yellow band near the bottom to measure and record in cm, the $\mathbf{t_R}$ and $\mathbf{w_B}$. Collect the yellow dye. Measure and record the volume in ml. **Do *not* discard the yellow dye.**

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Effect of pH on Dyes

1. Test the pH of the fluorescein dye eluate. Acquire pH paper and 2 small labels from the supply cart. Using a glass stirring rod, dip the stirring rod in the solution and then touch it to the pH paper. Match the color of the paper with a swatch on the side of the bottle and record the corresponding pH value. (Since 1M HCl was added, the pH is expected to be low.)

2. Acquire 2 test tubes and a test tube rack from your drawer. Add 2.5 cm or ~1"of yellow solution to two of the test tubes. Label one HCl or acid and place a rubber stopper on it. Label the other one NaOH or base. To the base test tube add 1 ml of 3M NaOH. Shake the test tube. Test the pH of the solution in this test tube. If the pH < 9, add more 3M NaOH and retest the pH. If the pH ≥ 9, record the pH. Place a rubber stopper on the NaOH test tube.

3. Compare the fluorescence of the dye solutions under black light (UV) and the color of the solutions in ordinary light at the different pHs. Tabulate the results for pH vs. color and fluorescence.

Cleanup

1. Add excess water to the column. Shake the column with water to suspend the alumina. Quickly invert the column over a waste beaker and shake out packing. Repeat as needed. Rinse out any remaining alumina with water. Rinse the column with acetone. Return the clean and dry column to the buret clamp.

2. Dispose of all chemicals in the appropriate containers. Clean and return all glassware and equipment.

Post Lab:

1. **Calculations:** Calculate Theoretical Plates for Blue and Yellow Dyes.
2. **Tabulate data for both dyes:** volumes, t_R, w_B and **N.**
   Tabulate for yellow dye only: results for pH vs. color and fluorescence.
3. **Discussion:** In your conclusion, discuss which dye was more efficiently eluted. (Efficiency is based on theoretical plates and volume. The higher the theoretical plates and the lower the volume the more efficient the elution. If the data is inconsistent, base efficiency on theoretical plates alone.) Also discuss whether the experiment was successfully performed and / or any problems that arose during the experiment and your recommendation for how to avoid those problems if you were to redo the experiment.

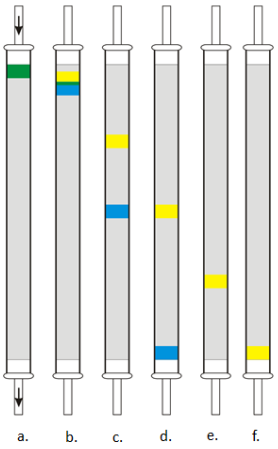

Figure 2: Separation of dyes.

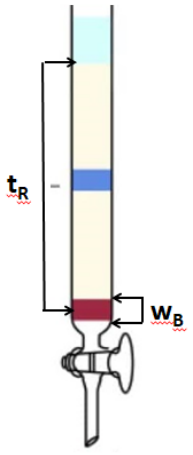

Figure 3: For the calculations, t_R and w_B are measured vertically, where t_R is from the top of the alumina to the center of the band being measured and w_B is the width of the band from top to bottom.