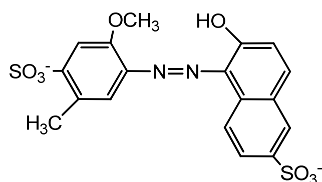
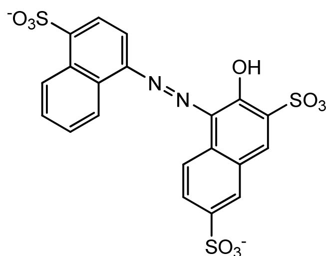
University of Wisconsin
Chemistry 524
Introduction to Separations Methods*

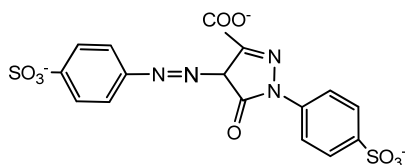
This experiment is designed to introduce you to five different separation techniques: thin layer chromatography, gel permeation, reverse phase liquid chromatography, gel electrophoresis and capillary electrophoresis. You will use these techniques to separate mixtures of food dyes and indicators. Colored compounds are used since they do not require special detection techniques and you will be able to watch the separations with your own eyes. As you go through the procedures, think about the important interactions or mechanisms occurring during the separation and **justify the order of elution in each case**. The molecular structures of the dyes used are shown below.



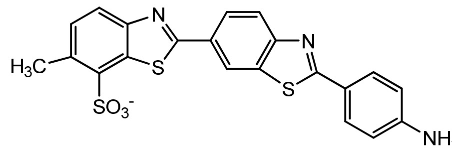
FD&C Red #40 M.W. = 496.4 g/mole



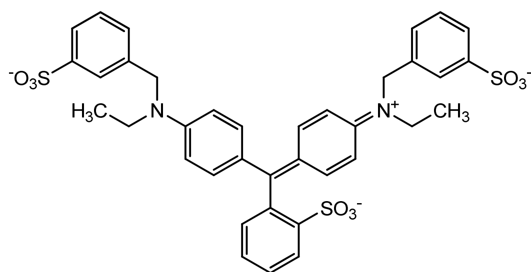
FD&C Red #2 M.W. = 604.5 g/mol



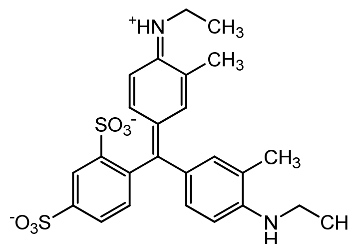
FD&C Yellow #5 M.W. = 534.4 g/mol



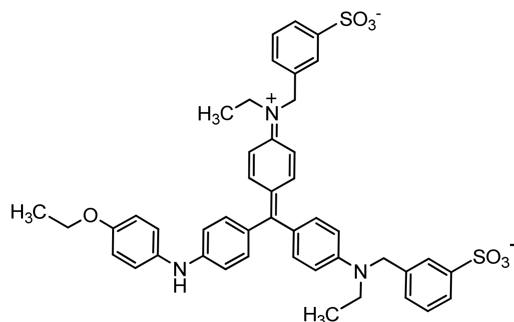
Direct Yellow #59 M.W. = 475.5 g/mol



FD&C Blue #1 M.W. = 792.9 g/mol



Acid Blue #147 M.W. = 538.6 g/mol



Brilliant Blue R M.W. = 826.0 g/mole

Procedural outline for accomplishing all five methods over two lab periods

Day 1.

1. Prepare your gel for electrophoresis and start the electrophoresis running.

2. Prepare your column for gel permeation and start eluting your sample.

3. Work on your reverse phase chromatography method while monitoring the electrophoresis and gel permeation experiments.

4. Analyze your data and summarize your Day 1 results.

Day 2

1. Make your microfluidic device for capillary electrophoresis. During the longer waiting periods of the device preparation, set up your TLC separations.

2. Run the CE separation.

3. Analyze your data and summarize your Day 2 results.

Thin Layer Chromatography (TLC) Thin-layer chromatography is one form of solid-liquid adsorption chromatography in which a solid adsorbent, about 100 to 300 μ thick, is coated on a piece of glass, plastic, or aluminum foil. In order to separate components in a solution, a drop of the solution is spotted near one edge of the plate, and the plate is placed upright in a developing chamber containing a layer of solvent as the liquid eluent. As the solvent migrates up the plate, components in the spot are carried at different rates, depending upon the strength of their adsorption to the solid layer. Differences in the distance of migration for components in the solution are used as the basis for the separation, and are expressed as values of R_f:

$$R_f = \frac{\text{(distance moved by compound)}}{\text{(distance moved by solvent)}}$$

Note that all values of R_f must be less than 1, and that a value of R_f=0 means that the compound has not moved from the original spot.

Common stationary phases include silica, with and without fluorescent indicator; alumina, cellulose, polyamide, and reverse-phase C_{18} on silica. Many solvents are used for developing the separations using TLC. Solvents are generally classified by their polarity into an eluotropic series, with more polar solvents having an increased ability to desorb polar molecules from the stationary phase.

Detection of the separated compounds can be accomplished using a variety of methods. If the compounds fluoresce under ultraviolet excitation, then a hand-held UV lamp can be used to see the spots. Chemical reagents can be used to visualize other compounds: ninhydrin spray reagent for amines; sulfuric acid spray for non-volatile carbon containing compounds; iodine. If the compounds are themselves colored, as in the case of the dye separation, then no detection reagent is necessary.

Procedure for TLC of the Dye Mixture containing Red #40, Yellow #5, and Blue #1

1. Cut three pieces of Silica TLC paper about 2cm by 7cm for the stationary phase. Mark a line (lightly) about 1 cm or so from the bottom of each plate. This is where you will spot samples.

2. Add mobile phase to the TLC developing jar so that the solvent is less than 1 cm deep (~5mL) at the bottom of the jar. You will set up three different jars with different mobile phases. The mobile phases that you are using are different mixtures of 1-butanol, methyl ethyl ketone, and water. Wet a piece of filter paper with the mobile phase and place it into the developing jar in contact with the solvent. This will help saturate the air in the developing chamber with mobile phase vapor to prevent solvent evaporation as it migrates toward the top of the plate.

3. Use a drawn capillary tube to spot the samples onto the plates. A few milligrams of compound in 0.1 mL of solvent is usually adequate for TLC analysis. Don't let the spot size on the plate become too large: 1-2 mm is typical.

4. Place the spotted plates into the developing chambers. Make sure that the height of the mobile phase is below the spot(s) on the plate. When the solvent front reaches a point about 2 cm below the top of the plate, remove the plate from the chamber and immediately mark the solvent front, since the mobile phase will evaporate quickly.

5. Let the plate dry. Measure the distance from the original spot to the solvent front, and from the original spot to each compound spot, then **calculate R_f for each compound in each mobile phase**.

Gel Permeation Chromatography Gel permeation chromatography is a noninteractive mode of chemical separation. Essentially a maze for molecules, a GPC column is packed with a stationary phase that has variously sized pores and pore networks. Sample molecules passing through the column migrate through some or all of the pores depending on their hydrodynamic volume (*i.e.* their size and shape). Large molecules cannot enter the pores of the stationary phase, so they elute very quickly from the column. Small molecules migrate into the pores created by the gel and elute much more slowly. Molecules are effectively separated according to molecular weight as they migrate through the column.

Procedure for Gel Permeation Separation of the Dye Mixture containing Brillant Blue R, Red #40, and Yellow #5

1. A pH 7.0 phosphate buffered saline (PBS) buffer will be used as the eluent for the column. Prepare 1 L of a 1X PBS (1mM) buffer by diluting 100 mL of the 10X buffer in 900 mL of water. You also have a slurry of 1.0 g of Sephadex™ G-25 (column packing material) in 25 mL of the 1X buffer. This slurry has been sitting for 3 hours to allow the gel to swell.

2. You will build the column out of a glass transfer pipet. Score and cut the narrow end of the pipet so that the narrow end extends only 1 " from the taper of the pipet. Attach a 3" piece of 1/16" I.D. tygon tubing to the end and place a pinch clamp on the tygon tubing. The pinch clamp will serve as your shutoff valve. Add a small amount of glass wool to the pipet then add enough sand to make a 1 cm layer at the bottom of the pipet. Use a 1" piece of ¼" I.D. tygon tubing to attached a plastic funnel to the top of the pipet and mount the apparatus on a ring stand. Use a transfer pipet to add the G-25 slurry to the column. You should notice a slow settling of the gel in the column. Open the column valve. Add more buffer as the buffer drains from the column and the gel settles. Add enough G-25 so that the packing fills the pipet up to the narrow in the pipet neck. Remember to make sure that the liquid in the column is high enough to keep the packing wet. Close the valve when the gel has settled and the buffer level is just at the top of the gel.

3. Gently apply a drop of the dye mixture to the top of the packing material. Open the stopcock briefly to place the sample in the gel. After the sample is below the top of the gel, add buffer to fill the column and the funnel.

4. GPC columns often employ single channel or multichannel visible or UV detectors. For this exercise, the eluent will simply be collected dropwise. Open the stopcock to start the separation. Try to collect as pure of fractions of the colors as possible into the 3 sample vials.

Reverse Phase Liquid Chromatography There are a great number of HPLC methods based on reverse phase liquid chromatography. A column is packed with a non-polar bonded phase, and a polar mobile phase is pumped through the column. It is "reverse" phase simply because historically the first separations were carried out using polar bonded phases and relatively non-polar solvents. The separation depends on the continual partitioning of the sample between these two phases as it is carried through a column by the flowing mobile phase. Compounds in the sample which are less soluble in the mobile phase are retained longer on the column, while those that are more soluble in the mobile phase are eluted first.

In this activity you are going to develop a reverse phase chromatography method suitable for the separation of a food dye mixture. The stationary phase you will be using is a C18 bonded phase of the type commonly used in HPLC applications. Your column is just a small amount of this material in a plastic tube with a syringe fitting at one end. This will allow you to “pump” mobile phase through the column using a small plastic syringe. You will make your own mobile phase by mixing water and n-propanol. The relative amount of n-propanol allows you to control the polarity of the mobile phase and hence the partitioning of the solutes between the phases. The less propanol, the more polar the mobile phase, and the larger difference in polarity between the mobile and stationary phases.

The trick in separating analytes by partition chromatography is to get the analytes to retain on the column long enough to achieve separation, but not have them retain so well that you cannot get them off. Sometimes this requires changing the mobile phase during the course of a run. This is called a gradient elution.

Procedure for Reverse Phase Separation of the Dye Mixture containing Red #40, Yellow #5, and Blue #1

In this activity you are going to develop a method suitable for the separation of the dye mixture using the small C18 column and water/isopropanol mixtures. Chromatography method development is done by insightful trial and error. You choose a mobile phase, try the separation, and alter the mobile phase based on your findings. The following steps outline the procedure for a single trial.

1. Prepare the stationary phase by flushing 3 mL of isopropanol, followed by 3 mL of 1% acetic acid, and 3 mL of the mobile phase through the column.

2. Gently push a small amount of air through the column so that the sample can be placed directly on the stationary phase. Add a drop of the mixture to the column and push air through the syringe until your mixture gets into the stationary phase.

3. Fill your syringe with the mobile phase and push through the column at a rate of steady drops. Observe the separation. Think.

4. Start at Step 1 with a new mobile phase.

Once you have accomplished a good separation**, describe your results.**

Agarose Gel Electrophoresis Electrophoresis is the separation of charged components by differences in mobilities when the components are subject to an electric field. Electrophoresis is a powerful separations technique having widespread use in biological applications because proteins and DNA are charged molecules at physiological pH's. Since the food dye molecules are also charged, electrophoresis should work for them as well.

Procedure for Gel Electrophoresis of the Dye Mixture containing Red #2, Acid Blue #147, and Direct Yellow #59.

1. A pH 7.0 phosphate buffered saline (PBS) buffer will be used as the buffer for the electrophoresis. A 10X PBS buffer has already been prepared for you. Prepare 1 L of a 1X PBS buffer by diluting 100 mL of the 10X buffer in 900 mL of water.

2. Weigh out 1.0 g of agarose and stir it into 50 mL of your 1X PBS buffer. Heat the mixture with stirring using a hot plate stirrer until the solution becomes clear. Wash the gel box and prepare it for pouring the gel. After the agarose is in solution remove the beaker from the hot plate and allow it to cool to 55°C to 60°C with frequent stirring. Pour the agarose solution into the gel box and place the comb into the agarose solution so that wells about 3 mm deep are formed near one end of the gel slab. When the gel has set remove the comb by pulling it out of the gel at an angle. Addition of buffer to the comb area reduces suction and facilitates comb removal.

3. Place the gel box into the electrophoresis chamber. The wells need to be placed near the negative terminal since the dyes are negatively charged and will move toward the positive terminal when the electric field is applied. Push the gel retainers down out of the way and add 1X PBS buffer to the chamber until the entire gel is submerged by about 2 mm.

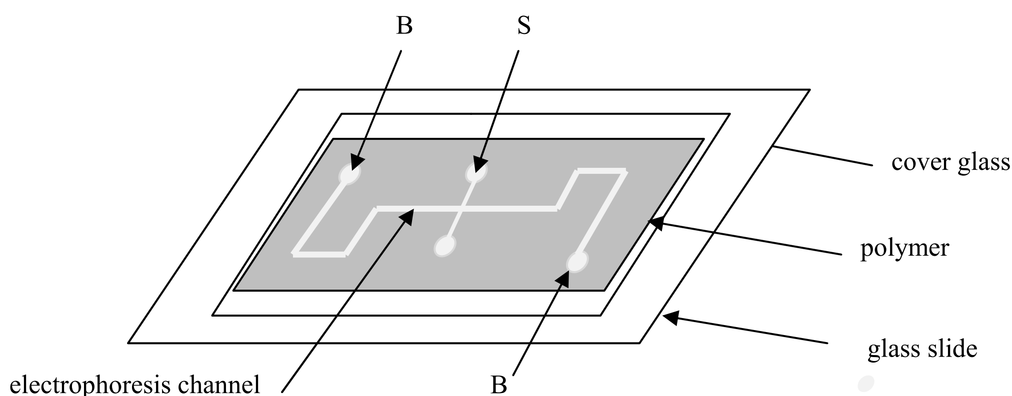
4. Carefully add 15µL of the dye mix (the dye mix is dissolved in glycerol) to 3 of the wells. The glycerol is used to make the sample solution denser than buffer so the solution settles into the wells of the gel.

5. HIGH VOLTAGES WILL BE USED. AS WITH ALL ELECTRICAL INSTRUMENTS, CAUTION IS REQUIRED WHEN POWER IS APPLIED TO THE GEL BOXES. Electrophorese the sample at 100 milliamp current flow. The voltage required may be higher than 100 volts. Be careful to limit the current to 100 milliamps because heat is generated at a rate proportional to the square of the current and too much heat will destroy your gel. Electrophorese the samples for 30 to 45 minutes or until you can see the separation of the dyes. Turn off the power supply before disconnecting the leads from the gel box.

6. **Measure the distance that each dye traveled and record the total electrophoresis time.**

Electrophoresis in a microfluidic device** Capillary electrophoresis (CE) is a separation technique that distinguishes between compounds based on their ion mobilities when an electric field is applied along the capillary. Molecules with different m/z ratios will migrate through the capillary with different velocities. CE provides high efficiency and reliability, small sample volumes and is used in a variety of separation applications.

Traditional CE uses quartz capillary tubing with an inner diameter near 50 µm and lengths up to 1 m for the separation. Instead of the quartz tubing, you are going to construct and use a µ-fluidic device consisting of a small fluid channel in a polymer matrix held between two glass slides.



Microfluidic device: B, buffer reservoirs; S, sample inlet. The electrophoresis channel is the main channel between the two buffer reservoir and the sample is introduced by drawing the sample across the main channel.

Chemicals & Materials Required

- monomer mixture (92 %wt isobornyl acrylate + 5 %wt tetraethyleneglycol dimethcrylate + 3 %wt 2,2 dimethoxy-2-phenyl acetophenone as the photoinitiator)
- mixed dye solution: Red #40, Yellow #5, and Blue #1
- glass slide (50x75 mm)
- 1X (1mM) phosphate buffered saline (PBS) solution with pH=7
- cover glass (35x50 mm)
- double sided adhesive (0.120 mm thick)
- photomask prepared on a overhead transparancy
- UV lamp
- High-voltage power supply ~1 kV, but current limited to less than 2 mA for Safety

Construction of the μ-fluidic device

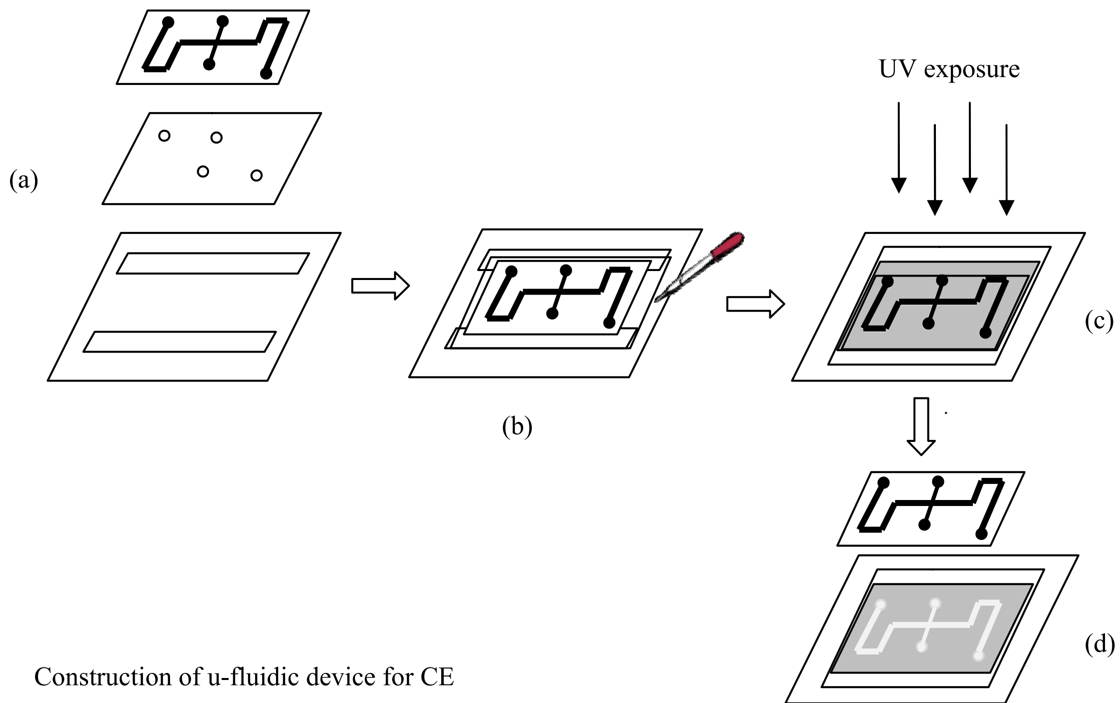
The pattern for the μ-fluidic channel is already printed out on transparency film (this is the photomask.) Drill the 1 mm holes in the cover glass followed by: (a) place two double-sided adhesive strips on the glass slide, place the cover glass onto the adhesive to form a space between the slide and cover glass, align the photomask and hold it in place with scotch tape; (b) add the monomer solution at the edge of the cover slide allowing capillary action to fill the space with the solution; (c) expose the device to UV light for 45 s to polymerize the material, remove the uncured polymer from the channel with an aspirator, retape the mask and expose to UV again for 15 min to harden the polymer; (d) finally, remove the mask and wash the channel with methanol.

Procedure for the CE Separation of the dye mixture containing Red #40, Yellow #5, and Blue #1

Dry off the glass surface of the device and place the 1 cm^2 adhesives with the small holes over the buffer reservoirs. Tape the two electrodes to the device so that the platinum ends will be in contact with the buffer reservoirs. Completely fill the main channel with the buffer making sure not to introduce any air bubbles. Put small drops of buffer solution on the adhesive to make the two buffer reservoirs.

HIGH VOLTAGES WILL BE USED. USE EXTREME CAUTION WHEN POWER IS APPLIED TO THE ELECTRODES. ONLY APPLY THE HIGH VOLTAGE DURING THE SEPARATION, OTHERWISE TURN THE VOLTAGE ALL THE WAY DOWN TO REDUCE THE CHANCE OF AN ACCIDENTAL SHOCK.

Connect the power supply to the electrodes after you make sure the supply voltage is set to the minimum output. Introduce the mixed dye solution into the system by placing a 3 μL drop on the cross channel entrance hole and pulling the drop through the channel by touching the opposite hole with a wet cotton tip applicator. If the sample preferentially flows to one of the buffer regions before applying a voltage, either add buffer or remove buffer to equalize the hydrodynamic forces. When you get a good injection, turn up the voltage so that you can see the separation occurring within the channel. Record the voltage and measure the time it takes for each color to reach the first bend in the channel.



Construction of u-fluidic device for CE

* R. McClain, November 2004.
** This experiment was developed by Saowapak Teerasong using microfluidic fabrication methods from David Beebe at the University of Wisconsin-Madison, May 2008.