Experiment 9

CHROMATOGRAPHY

Fig. 9-1

Mikhail Tswett (1872 - 1917)

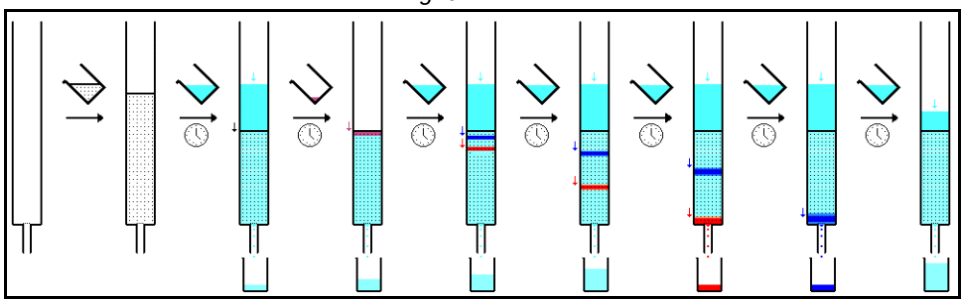
Text Topics and New Techniques

Column and paper chromatography, visible spectroscopy http://en.wikipedia.org/wiki/Mikhail_Tsvet

Discussion and Techniques

One of the very important pursuits of chemistry is the analysis of mixtures. Usually, a mixture must be separated into pure compounds before chemical analysis can be attempted. Polluted water often contains several pesticides as well as chemicals such as PCBs. Analytic techniques usually cannot identify one pesticide (e.g., DDT) when another is present unless a separation is first performed. Many mixtures are separated using some variation of a technique discovered by Michael Tswett (Russian botanist) early in the 20th century.

Fig. 9-2



Tswett allowed a mixture of pigments extracted from plants to percolate down through a column of calcium carbonate. Solvent was added from the top as needed to cause continuous movement of the pigments down the column. Pigments that were more strongly attracted to the stationary phase (calcium carbonate) and had less affinity for the solvent, moved down the column more slowly than pigments that had greater affinity for the solvent and had weaker attraction for the stationary phase. Tswett observed that the pigments had separated into several differently colored bands as a result of their movement down the column at different rates. The term "chromatography" was coined to describe the phenomenon. In this experiment, you will perform a column chromatogram similar to Tswett's and paper chromatographic separations of food colorings and natural pigments.

As indicated in the previous experiment in a discussion of gas chromatography, chromatography can be described as a dynamic extraction where the components of a mixture have the continuous ability to partition between a stationary phase and a moving phase. If the two phases are selected appropriately, different substances will spend different amounts of time in each phase and move through the system at different rates and separate. For column chromatography, the moving phase is the solvent that is added continuously from the top. The stationary phase is the absorbent that has been used to pack the column. Alumina and silica gel are the most commonly used absorbents. For paper chromatography, the paper (cellulose) is the stationary absorbent phase and the solvent moves up the paper and is the moving phase. Selection of the appropriate phases is the challenging and time intensive part of chromatography. Once appropriate moving and stationary phases have been found, running the chromatograms can become routine.

For column chromatography, it is common procedure to start with a non-polar solvent and gradually increase the polarity of the solvent as the substances move down the column. Non-polar substances usually move rapidly down with non-polar solvents and are eluted first. Polar substances do not move much until the solvent polarity is increased. For paper chromatography, many solvents are usually tested until one is found that separates the mixture into pure substances. Often, mixtures of solvents are needed to accomplish this purpose.

The paper chromatography experiments you will perform today also utilize similar principles to those Tswett employed. A piece of paper, spotted with pure compounds and mixtures, is placed in a solvent as illustrated. Assume spots 1, 2, and 3 are pure compounds and spot 4 is an unknown mixture of the compounds. The solvent (moving phase) will move up the paper (stationary phase). When the solvent reaches the spots, the components of each spot have two options. They can dissolve in the solvent and progress up the paper or they can stay absorbed on the paper and resist movement. The choice depends on several factors including the polarities of the compounds, the solvent and the paper. The solvent and the stationary phase are selected so that the components spend some time in each phase and do not move right along with the solvent front or stay at the origin. If the solvent and the stationary phase are selected properly, different compounds will move up the paper at different rates and separate.

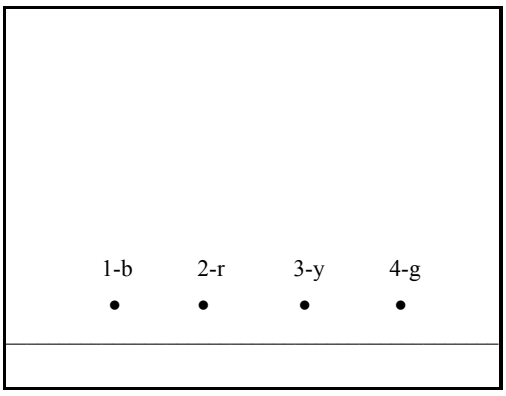


Figure 9-3

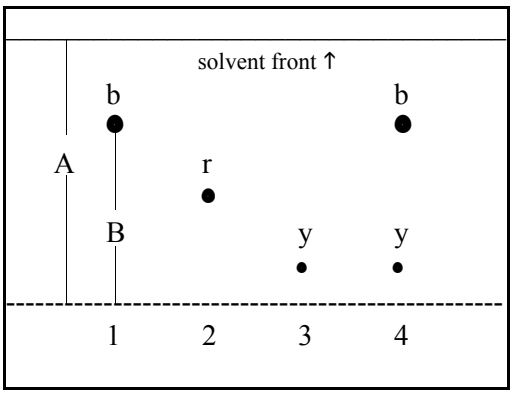


Figure 9-4

b = blue r = red y = yellow g = green

The spots of compounds that are less strongly adsorbed on the paper will move up the paper faster than the spots of the more strongly adsorbed compounds. When the solvent front nears the top of the paper, the chromatogram is removed from the solvent and the solvent front marked with a pencil. To find out the composition of the fourth spot, two factors are considered, color and relative distance moved. To quantify the relative distances, the R_f (ratio to front) value of each spot is calculated.

$$R_f = \frac{\text{distance from origin to center of spot}}{\text{distance from origin to solvent front}} = \frac{B}{A} \text{ for spot 1}$$

In the chromatogram on this page, it can be seen that spot 4 (the green spot) has compounds with the same color and R_f values as pure compounds 1 and 3. This provides strong evidence (but not proof) that spot 4 contains compounds 1 and 3 and does not contain compound 2. Paper chromatography then serves as a separation technique and can also assist with identification if the possible compounds in a mixture are available for determination of R_f values.

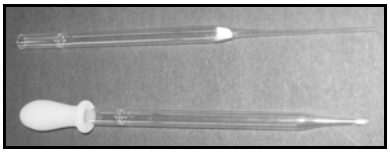
Procedure

Column Chromatography. For this part of the experiment, some pigments will be extracted from spinach and separated in a silica gel column packed in a disposable (Pasteur) pipet or a buret . Visible spectra will be run on the separated pigments. Note that the solvent "hexanes" refers to a mixture of isomeric hexanes. *n*-Hexane also works but is more expensive. Low boiling petroleum ether or ligroin should also work.

Using a mortar and pestle, grind about 5 g of spinach leaves and 2 g of anhydrous magnesium sulfate in 10 mL of an 80/20 mixture of hexanes and acetone. Grind until the solvent is dark green. Decant the liquid into a centrifuge tube and centrifuge the mixture until the top liquid portion is clear. Carefully extract the top liquid portion from the tube with a large Beral pipet and transfer abut 3/4 of it to a clean test tube or vial for use in the column chromatography (label it S1). Transfer the remaining portion to another vial or test tube to use for the paper chromatography (S2). Do not transfer any of the solids or the water that should be in the bottom of the centrifuge tube. Stopper the test tubes or vials and save the solutions until the chromatography column and paper chromatograms have been prepared.

Techniques for the use of two different sized columns are described below. A Pasteur pipet affords the advantages of the use of much smaller amounts of silica gel and considerably shorter use of time. The buret has the advantages that it provides more product and has a method for controlling and stopping flow rates.

Pasteur Pipet Method *Fig. 9-5*



1. Using a 3 fingered clamp, align a Pasteur pipet vertically (important) on a ring stand with the tip slightly inside the top of a 50 mL beaker. Use the tip of another Pasteur pipet to insert a very small plug of glass wool into the narrowing portion of the supported pipet. Add about a 5 mm layer of sand to the top of the glass wool plug.

2. Using weighing paper as a funnel, while gently tapping the supported pipet with a rubber hose, add about a 5 cm layer of silica gel to the pipet. Add another 0.5 cm layer of sand to the top of the silica gel while tapping to make it level.

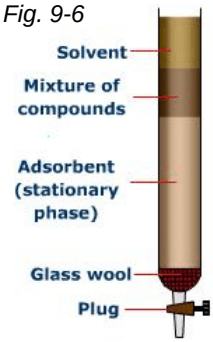
3. The next step is to thoroughly prepare for the chromatographic process. Most columns such as the buret have a method (stopcock) for interrupting the flow of solvent. However, there is no easy way to stop the flow through the Pasteur pipet. Since the top of the column should not be allowed to go dry, once flow is started with the introduction of solvent, the procedure should be carried through continuously to its conclusion. The following solvents should be within your reach before you begin the procedure: 15 ml hexanes, 5 mL 90% hexane-10% acetone, 10 ml 70% hexanes-30% acetone solution (by volume), 10 ml acetone. In addition you should also have several vials or test tubes with stoppers labeled 1, 2, 3, etc., and a beaker to hold waste chromatography solvent.

4. If your spinach extract has dried out, add about 10 drops of hexanes to dissolve it. Place a small beaker to collect solvent under the Pasteur pipet and carefully add (try not to disturb the top of the sand layer) hexanes to within about 1 cm from the top of the Pasteur pipet. Allow the hexanes to move down the column until the top of the mobile layer is very slightly above the top of the sand. The rate of flow can be increased by putting slight pressure on the top of the Pasteur pipet with a rubber bulb. **Now move quickly on to the next step.**

5. Carefully add about 5 drops of the spinach extract to the top of the column. Allow the extract to barely soak into the sand layer, and then add about 0.5 mL of hexanes to the top of the pipet. Allow the liquid layer to just reach the sand again, and add another 0.5 mL of hexanes. As the extract drains, the pigments should begin to separate. the yellow carotene bands should move faster than the green chlorophyll band. Continue collecting solvent in your waste solvent beaker until the yellow band has reached the bottom of the column. Replace the solvent beaker with vial number 1 and collect the yellow band as it drips from the column. The yellow band is sometimes very narrow and should require only about 5 mL of hexanes to move it out of the column. After the yellow band has eluted or you have already used several mL of hexanes, add the 90% hexane-10% acetone as needed to finish eluting the yellow band. If vial number 1 fills before elution of the yellow band is complete, collect the remaining yellow in vial number 2. When colorless solvent resumes eluting, switch the collector back to the solvent beaker. Now switch solvent to the 70/30 mixture of hexanes and acetone. The green band should move fairly rapidly down the column. As soon as it begins to elute from the bottom of the column, switch to the next vial (probably number 2 or 3) and collect the green band. Add solvent to make sure the top of the column does not go dry and to completely elute the green band If not all of the green has eluted after about 10 mL of the 70/30 mixture of hexanes and acetone has been added, switch to 100% acetone to finish eluting the green. After most of the of the green band has been collected, replace the latest vial with your waste solvent beaker and allow the residual solvent to drain out of the pipette. Run near uv - visible spectra of the yellow and green bands as instructed below.

Buret (or similar) Column

Fig. 9-6



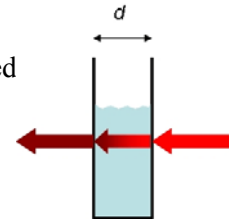
1. The column will be a 25 or 50 mL buret held with a buret clamp on a ring stand. With the aid of a glass rod, carefully place a small plug of glass wool in the bottom of the buret immediately above the stopcock. Be careful handling the glass wool as it can result in annoying tiny splinters that cause minor itching. Also make sure the plug is not too big or packed so tightly that it inhibits flow down the column. Pour a small layer of sand (<0.5 cm) on top of the glass wool. Add sufficient silica gel so that the height of the column is **about** 8 times its diameter. While adding the silica gel, tap the column with vacuum hose continually to assure even packing. Top the silica gel with another layer of sand (<0.5 cm).

2, Add a few mL of hexanes to the column and open the stopcock and allow the hexanes to flow through until the top of the hexanes layer is slightly above the top sand layer (~0.2 cm).

3. Using a Beral or disposable pipet, carefully add about 2 mL of your pigment solution directly above the sand layer. Slightly open the stopcock and allow the pigment solution to enter the column but **do not ever let the top of the sand go dry.** Now add 1 mL of hexanes to the top of the column and let it run down to the top of the sand. Repeat the addition of 1 mL of hexanes once more. Now fill the top of the column, open the stopcock and begin the elution process. Collect the elutant and transfer it to a waste solvent bottle until the first colored band nears the stop cock. At this point, change receivers and collect the yellow band and save it in a stoppered container. After the yellow band has eluted, change receivers and continue to elute with several mL of hexane. Now elute with about 10 mL of a 50/50 mixture of hexanes and acetone and then change to elution with pure acetone. The second band should begin moving down the column as soon as acetone is added. Collect the second band in a new receiver, stopper the receiver and save the solution. Run near uv - visible spectra of the yellow and green bands as instructed below.

Visible absorption spectrum. Please refer first to page E7-4. The two colored fractions should be transferred to 1 cm path length cuvettes.

Fig. 9-7



Run uv-visible spectra of the two collected colored bands from about 300 nm to 700 nm. The range will depend on the capabilities of your instrument and the cells used. If your cells are pyrex, the high energy limit (short wavelength limit) will be about 310 nm as pyrex absorbs at higher energies. If the absorption values are too high (>2), dilute a portion of your sample with hexanes and rerun the spectrum. Compare your spectra to those presented.

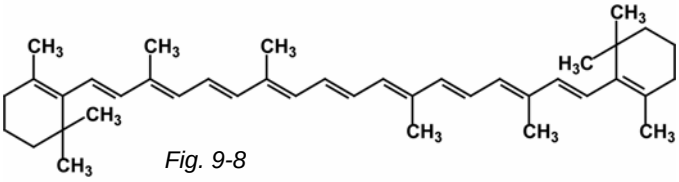


Fig. 9-8

β-carotene

Visible absorption spectrum of β-carotene - broad absorption between 400 nm and 500 nm with peaks at 450 nm and 480 nm.

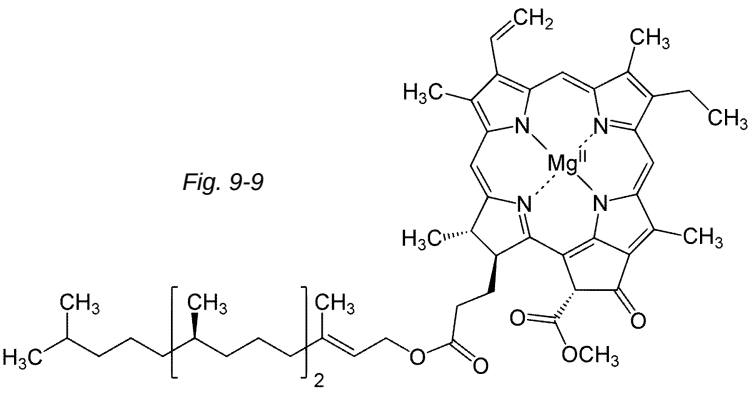


Fig. 9-9

chlorophyll a

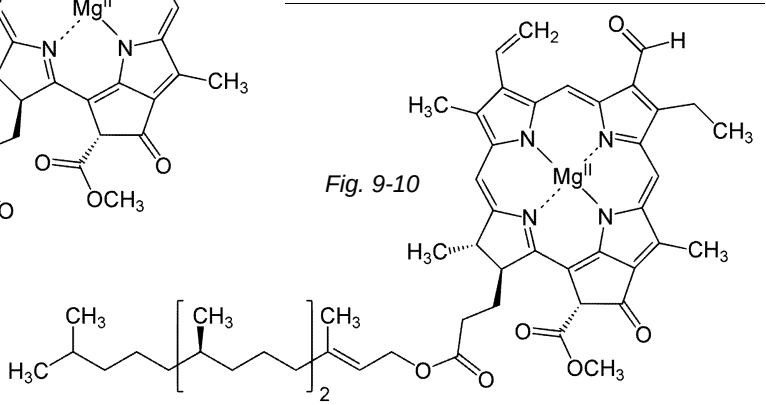
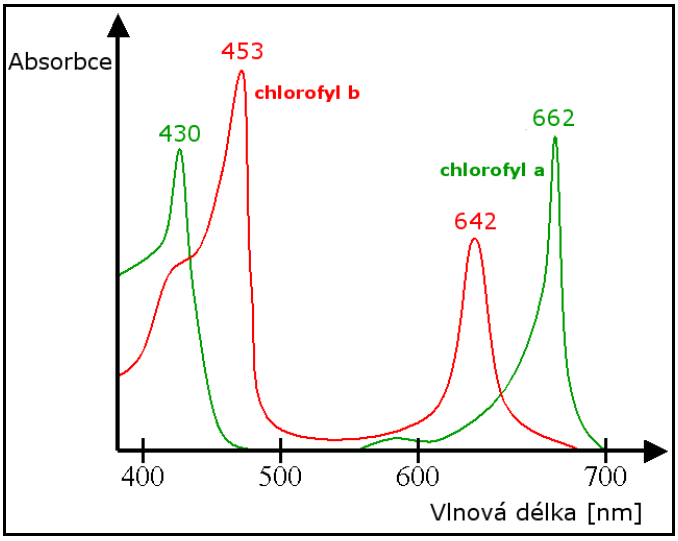


Fig. 9-10

chlorophyll b

Visible absorption spectra of chlorophyll a and chlorophyll b.

Fig. 9-11

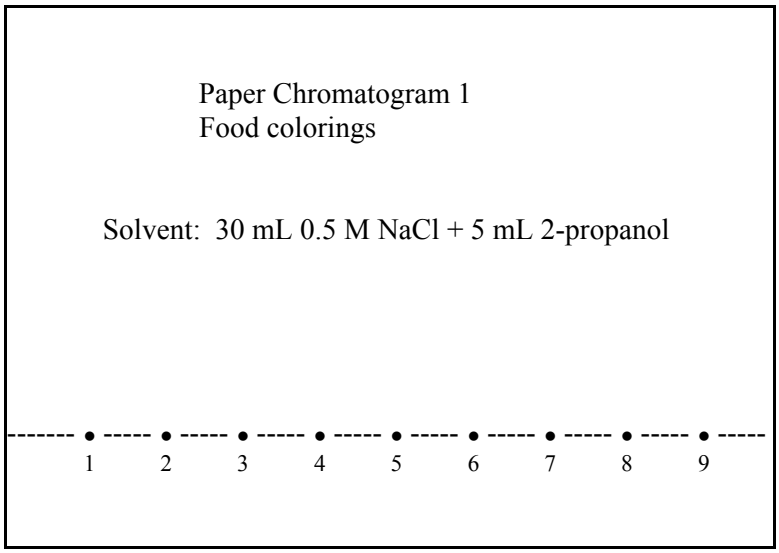


Paper chromatography. Food coloring chromatogram. Following *Figure 9-12*, draw a straight pencil line 2 centimeters from the bottom of a 11x20 cm piece of Whatman #1 chromatography paper and place pencil dots numbered from 1 to 9 at 2 cm intervals along the lines and 2 cm from each edge.

Dilute solutions of 4 food coloring knowns will be available in vials. Dip the end of an open ended capillary tube into the Red # 3 solution and apply it quickly to a scrap piece of chromatography paper. Practice this until you can make spots about this size ●. Now use the capillary to put a spot of the Red # 3 on to spot 1. Use new capillary tubes to spot Red # 40, Blue # 1 and Yellow # 5 on their respective spots.

Put a drop each of red, blue, green and yellow food coloring into 4 depressions in a spot plate. Keep them in that order so you will know which color is in which depression. Dip the end of capillary tubes into each of the food colorings and spot them on the appropriate places on the paper.

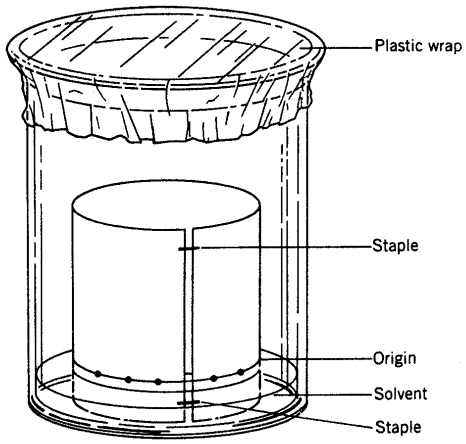
For the ninth spot, take two brown M & M's and gently shake them in a test tube with 1 mL of a 50/50 mixture of water and ethanol. Before the solvent exposes the chocolate, decant the solvent into a clean test tube. Use a new capillary to spot the solution on spot # 9. Because the coloring in this spot will be considerably more dilute than the previous 8 spots, you should allow the solvent to dry and re-spot at least eight times.



1 - Red # 3 (erythrosine)
2 - Red # 40 (allura red)
3 - Blue # 1 (brilliant blue FCF or erioglaucine)
4 - Yellow # 5 (tartrazine)
5 - Red food coloring
6 - Blue food coloring
7 - Green food coloring
8 - Yellow food coloring
9 - Brown M & M shell

Figure 9-12

Figure 9-13



After the spotting (*Figure 9-12*) is complete, roll the paper into a cylinder and staple it so that there is a small gap between the two ends (*Figure 9-13*). The ends of the paper should **not** overlap. Add 30 mL 0.5 M NaCl + 5 mL 2-propanol to a 600 mL beaker **and mix**. Gently put the paper cylinder (spotted edge down) into the beaker and cover the beaker with plastic wrap. Do not move or turn the beaker again. At this point, you should begin your second chromatogram but keep your eye on the first one. When the solvent front reaches about 2 cm below the top of the paper, remove the chromatogram. Mark the solvent front with a pencil and dry the paper in the hood, with a hot air blower. Outline every spot with a pencil. Measure the distance to the solvent front and to the center of each spot and calculate and record each R_f value and the color of each spot.

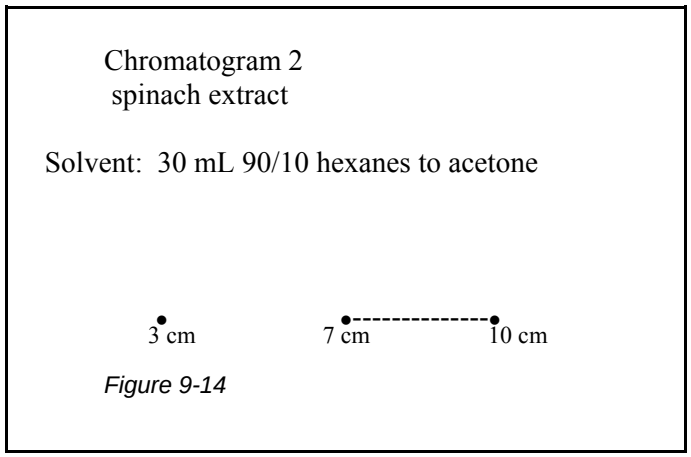


Figure 9-14

Spinach extract chromatogram. For the second chromatogram, a 9.5 x 14 cm piece of filter paper will be used. Draw a pencil line 2 cm from the bottom edge. Put a pencil dot 3 cm in from the edge and put two more dots at the 7 and 10 cm points. Using an open ended capillary, spot the 3 cm spot about 5 times with the spinach extract prepared for the column chromatography. Let the paper dry between spot applications. Put a spinach leaf down covering the 7 and 10 cm dots and lay a ruler down over the leaf on the line that is 2 cm up from the bottom. Use a quarter to roll over the leaf with pressing from approximately the 7 to 10 cm mark several times. Roll the paper into a cylinder and staple as with the previous chromatogram. Add 30 mL of a 90/10 hexanes to acetone solution to a 400 mL beaker. Insert the paper cylinder, cover with plastic wrap and do not disturb until the solvent reaches about 1 cm from the top of the paper. Withdraw the paper, mark the solvent front with a pencil and either allow it to dry or use a hot air blower to dry it. Be careful not to char the paper. Outline every spot or band with a pencil. Measure the distance to the solvent front and to the center of each spot and calculate and record each R_f value and the color of each spot or band.

Origin to solvent front dist._____

pigments

spot #		color	dist.	R_f	color	dist.	R_f	color	dist.	R_f	color	dist.	R_f
1	red # 3												
2	red # 40												
3	blue # 1												
4	yellow # 5												
5	red f. c.												
6	blue f. c.												
7	green f. c.												
8	yellow f. c.												
9	brown M&M												

References

For food coloring information, see: http://www.fda.gov/food/foodingredientspackaging/ucm094211.htm
http://www.fda.gov/forindustry/coloradditives/coloradditiveinventories/ucm115641.htm
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References cont.

Additional chromatography options including information that might be useful for the chromatographic analysis of plant pigments:
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Galloway, K. R.; Bretz, S. L.; Novak, M. *J. Chem. Educ.*, **2015**, *92*, 183-188.
http://pubs.acs.org/doi/full/10.1021/ed101188q

Prelaboratory Preparation - *Experiment 9*

First, be sure to list all the goals of the experiment. Read the references on food coloring listed above. Include a statement about yellow # 5 (tartrazine) in your introduction and comment on the significance if any if you find tartrazine in the food colorings or the M & M. Look up the structure of heme and discuss the similarities and differences between the structure of heme and the structures of the chlorophylls. Also briefly discuss the functions of heme and chlorophyll in animals and plants respectively. Both heme and chlorophyll have extremely important but very different biochemical roles yet significant similarities in structure. Does this seem strange to you? Explain your answer.

Observations

Report all relevant observations including the color and R_f of each spot, the order of elution on the column chromatograph and the spectra of the bands eluted.

Conclusions

This section should include the following:

1. Were the goals of the experiment achieved? Explain your answer.
2. Did you collect at least two different colored bands from the column chromatograph? Were you able to identify the bands using spectroscopy? Explain your answer.
3. Were the conditions used adequate for separating the different compounds used in food colorings? Were the four F,D&C dyes used in the food colorings and the brown M&M? Were any other dyes used? Explain your answer.
4. Do the food coloring bottles and the M&M package give adequate warnings about the presence of tartrazine? Explain your answer?
5. Were the conditions used adequate for separating the different compounds present in the spinach extract? The R_f value for β-carotene should have been very high and the values for the chlorophylls considerably lower. Were you able to separate β-carotene from the chlorophylls and from other dyes if they appeared to be present? Explain your answer.
6. Comment on the applicability of column chromatography and paper chromatography. Are they good procedures for separating, purifying and identifying? Is sample size an important consideration?
7. Suggest changes you would make to any part of this experiment to improve it.