To close the yellow note, click once to select it and then click the box in the upper left corner.
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# #06 Investigating Plant Pigments: A Guided Inquiry Laboratory Experiment

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## INTRODUCTION

### Purpose of the Experiment

In this experiment, the guided inquiry format will be followed. An introduction to column separations, packing and using the column, actually separating a sample, and collecting fractions will be done as a directed experiment. Then, small groups will be assigned, and each group will study one of the variables in the experiment - packing material, solvent system (i.e. gradient vs. different solvents), length of column. The second part of the lab, the variable investigation, will be prompted by questions, and students will form hypotheses from testing as suggested by the questions. When the lab is completed, the data from each group will be combined and analyzed by the class. The small groups will first study the combined data and then come together as a class for a final collaboration. Based on the information gathered and the input of the groups to the discussion, an experiment will be written to test the conclusions of the class collaboration. During another lab period, the "new and improved" procedure will be tested.

### Description of Experiment

This experiment includes both traditional methods of laboratory instruction as well as guided inquiry. The first part of the experiment is designed to teach basic laboratory techniques of column chromatography, including the packing of a column, sample application, elution methods, and variations of these techniques. This portion is presented in a routine method of directed work to be sure the students understand the basic premise of column chromatography. After the students have successfully completed Part I of the experiment, the method switches to discovery or guided inquiry. In Part II, the students will be using a pre-prepared leaf extract and will design an experiment to separate the components of the extract. The students will be provided with a variety of packing materials, different solvents, and the necessary equipment. Four questions in Part II will be studied and will prepare the students for the task of actually writing the procedure for this experiment. The questions include identifying the variables in the experiment, determining how to test the variables, testing the variables, and finally writing the experiment. Part III has been included to extend the experiment to detection and identification of the components separated in Part II. Depending on the equipment available to your students, this can be as basic as using the Spec 20, or as complex as FTIR and GC-MS. Part IV gives the entire class an opportunity to collaboratively write and test a procedure after completing the first three parts of the experiment.

### Student Audience

The activities as written in this experiment would be most suitable for high school chemistry or biology classes, perhaps at the Advanced Placement level, or college chemistry classes. However, the concepts presented here could easily be adapted for lower levels. Middle school students could use some of the ideas presented in the bibliography articles. Forensic units are common in grades six through eight and most of these units include some type of

chromatography. Grade school classes, third through sixth, can also use many of the ideas presented in the bibliography.

## Goals for the Experiment

- To introduce separation techniques to first-year chemistry students.
- To encourage development of critical-thinking and problem-solving skills.
- To discover and develop a method for the separation of plant pigments in leaves.
- To test the developed procedures on different plant materials and discover any modification necessary for different samples.

## Recommended Placement in the Curriculum

This experiment, as written, would probably be appropriate any time midway or later in a term. Some background in the laboratory and the use of basic equipment would be helpful as well as strong safety orientation and habits. However, chromatography could be used at any time during a term. The parts of the experiment could be used at various times during the term when basic chromatography would be appropriate. Parts II, III, and IV, could be used at the end of a term as a cooperative assignment for lab assessment.

# STUDENT HANDOUT

## Investigating Plant Pigments: A Guided Inquiry Laboratory Experiment

## Objectives

1. To introduce a variety of separation techniques to first-year chemistry students.
2. To encourage development of critical-thinking and problem-solving skills.
3. To discover and develop a method for the separation of plant pigments in leaves.
4. To test the developed procedures on different plant materials and discover any modification necessary for different samples.

## Background Information

Techniques of separation, or chromatography in some form, have been known since early Greek writers documented methods in their writings. However, modern chromatography, as we know it, began in the very early 20$^{th}$ Century. It was developed by Mikal Tswett specifically for the analysis of chlorophyll in green plants. The separation of substances by chromatographic means is dependent on the interactions of a compound with both the **mobile phase**, which carries the sample, and the **stationary phase**, which may be a coating on the **support material** in a column, or the support material itself. These three terms identify the most important parts of any chromatography system, whether it has been designed to test for gas components as in **gas chromatography**, various sized particles as in **size exclusion chromatography**, or pigment separation as in **column chromatography** or **paper chromatography**. There are many other types of chromatography and variations of those different types. Substances often analyzed by chromatographic methods besides pigments, as in this experiment, include polymers for size distribution, components of a gas mixture, proteins in biological systems, ionic solutions, drug constituents, and many others.

The principle behind chromatographic separations is that different materials interact with both the mobile and stationary phases to varying degrees. This interaction is dependent on the "like dissolves like" concept. Components of the sample that are very similar in polarity to the stationary phase will be retained on the column for a longer period of time due to significant interaction of the sample and stationary phase. The components that are significantly different in polarity will move relatively more quickly through the column. The different rates of movement of molecules through the column result in the separation of different fractions of the sample. These fractions may then be further separated by varying the materials used for the stationary phase and the solvent system or characterized by using other analytical methods. Following are brief descriptions for several different types of chromatography.

## Paper Chromatography

Paper chromatography is a planar form of chromatography. Planar chromatography includes any form of separation that occurs on a flat surface or thin sheet, such as paper or coated glass plates, in which the mobile phase of the system moves across the sheet. Paper chromatography may be done horizontally or vertically, although most planar chromatography is run in the vertical direction. The paper is the solid, or stationary, phase and the eluent used is the mobile phase. Samples are carried by the mobile phase over the stationary phase allowing for the partitioning of the sample between the mobile and the stationary phases. This results in the separation of the component parts of the sample. Paper chromatography is commonly used for color separations in

food products and colored marking pens. This is frequently the first exposure students have to any type of chromatographic separations. Amino acid identification can also be done using paper chromatography. Separations may not always be visible to the naked eye and other forms of visualization may be necessary. Ultraviolet (UV) light is useful if the material being tested is fluorescent, while in the case of the amino acids, a chemical spray that reacts with the amino acids produces a colored product.

## Thin Layer Chromatography

Thin layer chromatography (TLC) is also a form of planar chromatography. Thin layer chromatography is run on plates constructed with an adsorbent layer of material, the solid phase, coated onto plastic, glass, or metal plates. Generally, thin layer chromatography is run in the vertical direction in a closed chamber to prevent evaporation of the mobile phase. The stationary phase is the coating material and again, the eluent is the mobile phase. Interactions similar to those in paper chromatography occur for separation of components in a sample. This form of chromatography is particularly useful for separation of organic components in materials, such as caffeine in tea or soft drinks, the components of pain relievers such as in Anacin or Bufferin, as well as color separation as in paper chromatography. As in paper chromatography, alternate methods of visualization may be necessary to see the separated components of a mixture.

## Gas Chromatography

Gas chromatography (GC), sometimes referred to as gas liquid chromatography (GLC), is a type of column chromatography. This type of separation is performed in a column, generally stainless steel, packed with a suitable solid support material that is coated with a liquid that acts as the stationary phase. The column is supported in a heated chamber and connected on one end to an injection port and on the other to a detector. The sample is introduced through the injection port into a stream of the carrier gas, the mobile phase, and is carried through the column where interactions cause separation to occur. The individual components finally pass over a detector giving a signal that is recorded. If the detector is a mass spectrometer, the individual components can be identified directly. For other detectors, such as thermal conductivity or flame ionization, standards must be run to aid in identification of the unknown components. Identification is then based on the amount of time it takes a sample to completely pass through the column. Quantitative analysis is also possible with gas chromatography by making a comparison of the areas under the peaks of the components of the mixture to standard reference samples.

## High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is another type of column chromatography most useful for relatively dilute solutions of complex mixtures. It is based on the use of high pressure to force an eluent (mobile phase) and sample through a closed column packed with a suitable support material (stationary phase), much like other types of column chromatography. The reason for the high pressure is that the stationary phase consists of densely packed spherical silica particles with diameters of 3, 5, or 10 mm. Depending on the polarity of the components to be separated, a column is chosen with an appropriate chemical compound bonded to the surface of the silica particles. The mobile phase for HPLC must be ultra pure to avoid degradation of the packing materials in the column and to provide the least interference for the detection systems. Both isocratic, pure solvent or fixed composition, and gradient elution, varying composition of two or three solvents, are used. Gradient elution is often used in HPLC to achieve the best possible separation of components of a sample mixture. Changing the polarity of the mobile phase over time allows for better separation of the sample components within a reasonable time frame. The separated sample components pass through a detection system as they elute from the

column. The ideal detection system gives sharp, well resolved peaks, is sensitive to all components, and gives a linear response. The detection system may measure the change in the refractive index of the eluent (RI detector), the change in conductivity of the eluent (TC detector), or the change in the absorbance of ultraviolet radiation (UV detector) all depending on the type of sample. Infrared (IR), nuclear magnetic resonance (NMR), and mass spectroscopy (MS) detectors are becoming more common, but are more expensive.

## Open Column Chromatography

Another type of column chromatography is the open packed column. A tube is packed with an adsorbent material (one to which a substance adheres) as the stationary phase, either dry or in a slurry, and a sample is applied to the top of the column. The sample is washed through the column with an appropriate solvent system, which is the mobile phase. As the sample travels through the column with the mobile phase it will begin to separate into distinct bands on the adsorbent material. The separation occurs because of the relative interactions between the sample and the stationary and mobile phases. The more attraction with the stationary phase, the longer the sample will take to elute from the column. It is possible to collect fractions from the original sample which can be further analyzed or processed. Sometimes the separation is not visible in the column, so measured fractions are collected and analyzed by other methods, often UV-VIS. A detector may also be placed to measure changes in the absorption of the UV-VIS light as the fractions are eluted from the column. These changes can be graphed and the information used for the further analysis of the samples.

## Experimental Procedure

*Before beginning any experiment, read MSDSs for all materials being used. Wear safety goggles and lab aprons. Gloves are optional for this experiment. Dispose of used reagents according to local ordinances.*

### Part 1: Introduction to Use of Packed Column

**Materials Needed**

- Baking soda (sodium bicarbonate, $NaHCO_3$)
- Pasteur pipet
- Ring stand and clamp
- One-hole stopper
- Test tubes and rack
- Glass wool
- Sand
- Dye sample
- Droppers
- Distilled water

1. Set up ring stand and clamp to support column to be packed. Insert the Pasteur pipet into the one-hole stopper and clamp the stopper to the ring stand. See Figure 1.

2. Place a **small** piece of glass wool in bottom of column. Do not pack too tightly. This is to prevent the stationary phase from falling through the bottom of the column.

3. Add a thin layer of sand, not more than 3-5 mm thick.

4. Fill the column to within about 2 cm of the top with baking soda, $NaHCO_3$. This is the adsorbent material, the stationary phase.

5. Cover the top of the adsorbent with another layer of sand about 3-5 mm thick. See Figure 2.

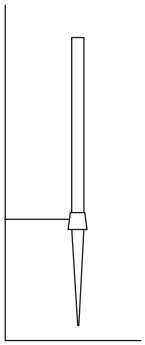

**Figure 1**

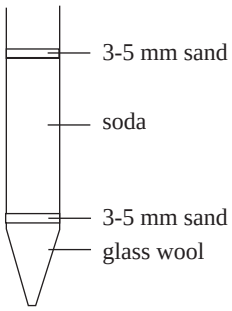


**Figure 2**

6. Add the mobile phase slowly, being sure to not disturb the sand or stationary phase. Continue adding solvent until the adsorbent packing is thoroughly wet and the solvent is dripping into a beaker placed below the column. Do not allow the solvent to drop below the top of the stationary phase.

7. After the column is thoroughly wet and the solvent has reached the top of the sand layer, with a dropper, place 10 drops of the dye solution to be separated on the column, allowing it to move through the sand to the top of the stationary phase.

8. Add water carefully to the column and observe what is happening. Do not disturb the sample when adding water. Continue adding water, keeping the level above the top of the sand. Collect any fractions that might separate in this column. These fractions will be visible and should be easy to collect.

9. Answer the following questions about the separation. This portion of the experiment is to acquaint you with the technique of using column separation techniques, packing the column, applying the sample, and extracting that sample.

   a. What is the stationary phase?

   b. What is the mobile phase of this experiment?

   c. How is the column packed and then supported?

   d. Why is it necessary to wait for the solvent to reach the top of the sand layer before adding the sample? Why wait for the sample to travel into the sand before adding eluent?

   e. Why is it necessary to keep the liquid level above the top of the sand layer at all times?

   f. What are your observations about the separation that occurs?

g. Explain why the separation occurs the way it does, or why do the colors separate in this particular order?

h. What do you think would happen if you had a longer column? Be specific in your answer.

i. What do you think would happen if the column had a larger diameter? Be specific in your answer.

j. What do you think would happen if you used a different solvent? Be specific in your answer.

10. After completing this portion of the experiment and answering the questions, bring your paper to the instructor to have your answers checked before going to the next portion of the laboratory exercise. You will be assigned to a small group to do further investigation of column chromatography.

## Part II: Investigation of Variables in Column Chromatography

In this portion of the experiment, you will be using pigment from leaves and devising a method for separation of the different substances making up that pigment. You will be provided with a variety of packing materials, several different solvents, and a sample. All groups will have the same leaf pigment for analysis. It will be the responsibility of each group to experiment and devise the best system for separating the substances in the pigment sample.

1. There are several variables that need to be considered in this experiment. What are they? When you have determined what these variables are, bring the list to the instructor for verification. After verification of these variables, your instructor will give you the next part of the experiment.

## Part II: Investigation of Variables in Column Chromatography

2. Now that you know what the variables are, how will you test them? Remember to consider each variable separately as well as in conjunction with the others. What are the considerations for determining the best combination of materials? Write an action plan with enough detail for your instructor to understand what you are planning to do. **Don't forget to include safety!** Bring the action plan to your instructor for approval before beginning any experimentation. When your plan has been approved, you will be given the next part of the experiment.

## Part II: Investigation of Variables in Column Chromatography

3. As you begin your experimentation with the variables, keep **precise** notes of your procedures and observations in your laboratory notebooks. Remember, your results will be combined with those of the rest of the groups and all groups will contribute to the final procedure. You may ask questions of the instructor as you proceed, but your questions will probably be answered with other questions. When you are satisfied with your results, take them to your instructor and turn them in. **Be sure you are wearing your safety goggles and lab aprons**.

## Part II: Investigation of Variables in Column Chromatography

4. The class has been given a summary of the work of all groups. Study these summaries, meet with your small group to discuss the summary. The class will design an experiment based on the results of the small group studies. If time permits, each group will run the "new" experiment and evaluate its effectiveness.

## Part III: Methods of Detection Available for Chlorophyll a and b and β-Carotene

After the first two parts of this experiment have been completed, study the reference materials available. Search for methods of detecting chlorophyll a and chlorophyll b as well as b-carotene and for the initial extraction of plant pigment from leaves. Write a short description of any methods that are available and determine which of these methods would be suitable to use in this laboratory. Be sure to include safety instructions when you are writing laboratory methods. You may ask your instructor if certain equipment is available for this class to use. This information will be submitted to your instructor for approval and then incorporated into the final written procedure.

**NOTE**: You may find clarifications and/or verifications in the reference materials for many of the conclusions you have reached during this project. Feel free to make notes or comments to yourself to use in the final phase of this project, the actual writing of the experiment.

## Part IV: Collaborative Experimental Procedure for Extracting and Separating Plant Pigments

NOTE: In this place will be a procedure written collaboratively by the class. It will include the initial extraction of the pigment from the leaves, the preparation of the column, the actual separation process, the collection of samples, and at least one form of detection of the presence of chlorophyll.

# INSTRUCTOR NOTES

## Investigating Plant Pigments: A Guided Inquiry Laboratory Experiment

## General Information

This exercise is designed for *three to four 3 1/2 hour laboratory sessions* such as in a traditional college chemistry course for a *total of 10 1/2 to 14 hours*. This time frame may vary depending on the depth of study and the grade level of the students. In a high school setting, Part I could be set up and run in two consecutive lab periods. If your school runs on a block schedule, one lab period would be sufficient. Each question for Part II could be assigned during one day, or possibly as an out-of-class assignment. *Small groups of three to five persons would be appropriate* to allow for adequate interaction and collaboration. One of the purposes of this investigation is to teach and encourage the development of collaboration as an important life skill.

## Materials List (per group)

This list is for Part I and could be for each student instead of each group. This would depend on how necessary it is for every student to develop the basic lab skills needed for this experiment.

- Baking soda (sodium bicarbonate, $NaHCO_3$)
- Pasteur pipet
- Ring stand and clamp
- One-hole stopper
- Test tubes and rack
- Glass wool
- Sand
- Dye samples*
- Droppers
- Distilled water

*NOTE: The dye samples may be FD&C food colors, the paste colors used in cake decorating, or any other known dye samples. A mixture should be run. The separations can be compared to elution of the pure colors if desired.

This list is for Parts II, III, and IV as needed. Not all of the materials may be necessary, but suggested for the discovery and development part of the experiment.

Same materials as above plus:

- Variety of leaf samples, tree plant, spinach, succulents
- Acetone
- Ethyl alcohol
- Alumina
- Acetic acid
- Petroleum ether
- Spectrophotometer - Spec 20
- Long wavelength UV light source
- Other eluents and support material as needed such as
  - FTIR
  - UV-VIS
  - GC-MS

## General Safety Information

Safety goggles and laboratory aprons should be worn at all times in the laboratory. Avoid breathing vapors of organic solvents. Use a fume hood and keep vapors away from ignition sources. Gloves may be necessary when handling solvents and desirable when handling dyes. Dispose of used reagents according to local ordinances.

## Part I: Investigation of Variables in Column Chromatography

Before going to the laboratory, some background information about chromatography in general, and column chromatography in particular, is necessary. A simple introduction to chromatography can be given by using paper chromatography of a black marking pen (Vis-a-Vis works very well.) Follow this with a discussion of the basic principles involved in separation science.

Part I of this experiment is in the traditional "cookbook" format. The students will be given a chance to learn how to pack a column, apply a sample, and use solvent or eluent properly. Due to time restraints in the community college schedule, it is not practical for this to be used in a strictly discovery mode. However, in an open lab situation or perhaps in an AP Chemistry class in high school, the entire experiment could be adapted to full discovery.

Provide the MSDSs either on reserve in the library, or make a computer assignment to the class to look up the needed information on the internet before the class period. Give points for being prepared. It would probably be a good idea to only have the MSDS for the first part in the beginning.

The dyes used in Part I of this experiment are from cake colorings and are standard FD&C colors. If you use brown, the colors will be a blue, a red, and a yellow. An extension to this experiment might be to identify which specific dyes are used by comparison to known pure dye samples. Students could also be encouraged to look at the dye content of colored beverages or candy samples.

The questions at the end of Part I are intended as the beginning point for the students to start using skills that will be needed in Part II to study variables and to begin development of a procedure for separating plant pigments.

## Answers to the Questions for Part I

a. The stationary phase in Part I is sodium bicarbonate, $NaHCO_3$.

b. The mobile phase in Part I is distilled water.

c. The column is supported in a one-hole rubber stopper or other appropriate holder clamped to a ring stand. A small glass wool plug is inserted first into the pipet followed by 3-5 mm of sand. The stationary support, $NaHCO_3$, is added and gently tapped to eliminate as many gaps in the support as possible. Another layer of sand, 3-5 mm thick, is added to the top of the column.

d. The sample should remain as concentrated as possible. This can be done by not mixing it with any eluent that would be above the sand layer or by adding eluent too quickly behind the sample. This also allows the sample to be in the narrowest band possible.

e. It is necessary to keep the mobile phase above the top of the sand layer to avoid getting any air pockets in the column, which may cause channeling and reduce the effectiveness of the column.

f. The separation of the food dye shows distinct bands of color: blue, red and yellow. (NOTE: this will depend on what dye is used.)

g. The dyes separate in a specific order due to the relative interactions occurring among the components of the mobile phase and the stationary phase. The dye component with the least attraction for the stationary phase will elute first because it spends less time interacting with that phase. The other colors will elute in an order dependent on increasing affinity for the stationary phase. The last component to elute will have the greatest affinity for the stationary phase.

h. If the column were longer the separation of the dyes would remain in the same order, but the distance between bands would be greater and the separation might be better defined.

i. If the column were larger in diameter, the separation would not change. The amount of solvent required would be greater, but the separation would not be affected.

j. The change in solvent could affect the order in which the dye components separate. This is dependent on the relative interactions between each dye component and the eluent and support material. (See answer to question g.)

## Part II: Investigation of Variables in Column Chromatography

In Part II the students will be guided by four questions, completed one at a time. When the first question has been answered satisfactorily, hand out the second and so on until all four questions have been presented. Depending on the class, a significant amount of "guiding" could be necessary. I plan to use this at the end of a semester after the classes have 10-12 weeks of lab experience and are comfortable with equipment and procedures.

The plant pigment that is used can be prepared ahead of time. Any green leaf is satisfactory - spinach is an excellent source as are many tree leaves. The leaf of the flowering red plum is very interesting to use. This experiment could also be used in a change over time study of leaves as the colors change in the fall. The pigments may be extracted using acetone, chopped leaves, and a mortar and pestle, or with ethyl alcohol as the extracting solvent. It might be a good idea to concentrate the pigment extract if a large amount of solvent is used. Remember, the students will need about 10 drops (established in Part I), or maybe less, per test run. Each group should need no more that 10-15 mL at the most.

## Answers to the Questions for Part II

1. The primary variables in this experiment are the column size (length and/or diameter), the packing materials (stationary phase), the mobile phase, the sample size and its application on the column. The students may add other items to this list such as temperature, humidity, amount of light and so on. Use your judgement as to which items on the list should be considered variable in this experiment.

2. The groups should tell you that only one variable should be changed at a time. Otherwise you won't be able to tell what caused any changes in results. Steer them in the right direction with prodding questions. The group is to write an action plan that explains how the experimentation will be handled, what will be varied, and how. Verify that only one variable is being changed at a time. **Be sure to check that safety considerations have been included.**

3. When the groups turn in their results of experimenting with the variables, look over the data to see that good records have been kept. They should have fairly detailed explanations of **HOW** the experiment was run (i.e. a procedure) and specific conclusions for each variable that was checked. This one is a judgement call on the part of the instructor.

4. After you have received the papers from all groups, combine and tabulate the information making copies available for each group to study. Work as small groups to begin writing a procedure for separation of the plant pigments based on the summary of all the small group information. After a preset time period, bring all groups together to consolidate the information.

### Part III: Methods of Detection Available for Chlorophyll a and b and β-Carotene

The students should find references to the use of UV-VIS and IR or FTIR spectra for identification and verification of the presence of the pigment fractions. There should also be reference to the use of the "black light" to check for fluorescence. Chlorophyll will fluoresce a brilliant red in the presence of long wave UV. The FTIR may be used to scan the collected fractions, but I was unable to find a reference spectra for chlorophyll. If the UV-VIS or Spec 20 is used, a scan can be used to find the maximum absorbance wavelength and that can be compared to known wavelengths. Good wavelengths to look at might be about 550, 430, and 340.

Ideally, after the "new" experiment is written, the class should be able to test the procedure and evaluate its effectiveness, based on the research that was done to develop this procedure. This is also a good time to find the problems with a procedure and work on refining those problems.

### Part IV: Collaborative Experimental Procedure for Extracting and Separating Plant Pigments

This portion is the "try it out" section of the exercise. At this point the groups will work independently for a short period of time, and then all groups will come together and collaborate to write a procedure for the extraction and separation of the pigments in leaves. Depending on the time available, it might be prudent to have the small groups meet independently prior to the lab time, so that collaborative writing could start at the beginning of the period. Once the experiment is agreed upon, the procedure will be typed and given to each group for actual use.

Grading is difficult in this type of experiment because there is no "number" from which to calculate "error" and then assign a grade. Instead, I would recommend assigning points to each part of the experiment, group points on the group assignments, and perhaps an overall class grade. I have also included my classes in determining the grading procedure for oral and written reports. The class has input as to what is important, how much is important, what weight should be given to mistakes or errors, etc. This has been very successful in the classes that have used it. Of course, individual credit should be given for creativity, or not given, if an individual does not put in a fair share of the work. The members of each small group could also be asked to evaluate their group, both as a whole and as individual members. Of course, a pass/fail could also be given for this type of exercise.

## Extension Activities

This experiment may be extended in several ways. If the instrumentation for IR, FTIR, and/or UV-VIS is available, the spectra for the collected extracts may be run and compared for different kinds of plant leaves or for changes in color during the fall. Grasses, flower petals, succulents (cacti), and other plants containing pigment may be used and compared to standard, if they are available. There are also suggested methods using mixed solvents that will separate the chlorophyll a, chlorophyll b, and the b-carotene on a column. Electrophoresis is another separation method that might be incorporated here as well. The comparison of the two methods

might provide interesting points of study. As mentioned in Part I of the Instructor Notes, candies, mouthwash (colored), and colored beverages may also be tested by these chromatographic methods. I have included a limited reference list that might be useful as supplemental information for you or for your students.

## References

The following textbook is a good general reference for analytical and instrumental techniques.

Harris, D. *Quantitative Chemical Analysis;* 4th ed; W. H. Freeman and Company: New York 1996.

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