



FRALIN LIFE SCIENCES INSTITUTE
VIRGINIA TECH™

COLUMN CHROMATOGRAPHY KIT

INFORMATION MANUAL

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Virginia Tech
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WHAT NEW IN 2008-2009?

New sponsor!!

The Fralin Outreach Program, specifically Biotech-in-a-Box, has received a grant from the Virginia Council on Advanced Technology Skills (VCATS), in partnership with the Virginia Biotechnology Association (VaBIO) and the Virginia Manufacturers Association (VMA). We are very grateful for their support, which will enable us to purchase new equipment and to expand the program.

Manuals on CD!

Instead of hard copies, this manual (and the other three as well) has been sent to you on a CD. Since there are few changes in the manual from year-to-year, this seemed like the right time to make a change. This should make it easier to print materials from the manual. If you would infinitely prefer a hard copy, please contact me with a request.

ACKNOWLEDGMENTS

Experiments 1 & 2 were developed by Dr. R.E. Ebel and Dr. E.M. Gregory, Dept. of Biochemistry, Virginia Tech, Blacksburg, VA. The student Observations & Conclusions sections for Experiments 1 & 2 were written by Ellen Lamb, formerly at Mills Godwin High School, Richmond, VA, and her collaboration was very important to the development of this kit. Experiment 3 was developed based on an Access Excellence article by Ted Lau of Northwest Community High School, Flint, MI, and on materials provided by the Waters Corporation. The Questions section for Experiment 3 was written by Kay Mikula, of Oakton High School, Vienna, VA. Thanks also go to Adrienne Warren, formerly of the Chesapeake Center for Science & Technology, Chesapeake, VA, for the information on using Mountain Dew in Experiment 3, to John McLaughlin, Lord Botetourt High School, Daleville, VA, for his careful reading of this manual and for his helpful suggestions. Thanks also to Erin Dolan for her excellent comments and suggestions for improving the manual.

Safety note: Please follow the safety guidelines set by your school district for liquid handling, especially in terms of safety glasses.

COLUMN CHROMATOGRAPHY KIT CONTENTS

Here is a list of the kit contents. The kit is designed for 12 student groups.

Item	Quantity
Overheads	1 pack
Gel filtration columns (G-75 Sephadex)	12
Ion exchange columns (CM-cellulose)	12
Reverse-phase columns (Sep-Pak® C-18 cartridges)	12
50-ml plastic beakers	60
transfer pipettes	50
microcentrifuge tubes	12/class
3-cc syringes	12
label tape & marking pen	1 ea
sample solution	4 ml
10X loading buffer	100 ml
10X NaCl (for elution buffer)	12 ml

INTRODUCTION TO THE COLUMN CHROMATOGRAPHY KIT

The column chromatography kit from the Fralin Biotechnology Center contains the materials and information to run three experiments that demonstrate three different types of column chromatography. The kit is designed for 12 student groups (2-3 students per group). In the first experiment, a mixture of samples is separated by size using gel filtration chromatography. One of the fractions collected in the first experiment is then further fractionated using ion exchange chromatography, which separates molecules based on their charge. In the third experiment, colored dyes found in grape soda are separated based on their polarity using reverse phase chromatography.

This manual is divided into sections. The first section covers the theoretical basis of chromatography. The instructor will have to decide the complexity of material to be presented to the students. A “nutshell” version of each type of chromatography is included that lists the main points. Overheads are included in the kit for most of the figures in this manual.

The second section of the manual is the experimental section. It includes descriptions of the advance preparation needed and some notes for the instructor. This section also contains the experimental procedures and student handouts (pages 16-17, 19-20, and 23-24). Feel free to reproduce these pages for use in your classroom, but further reproduction of these pages is not allowed.

The final section of the manual is the Appendix, which contains a scenario for the kit with both student and teacher guides. This section also contains practical information about pouring and running chromatographic columns, sources for the materials used, and directions for making the kit solutions. There are also some additional resources listed that you may be able to use.

Feel free to call Dr. Kristi DeCourcy at the Center with any questions about this material. Corrections and suggestions will be gratefully received. My phone number is (540) 231-7959 and my email address is decourcy@vt.edu.

INTRODUCTION TO COLUMN CHROMATOGRAPHY

Chromatography is a group of laboratory methods, based on selective adsorption, by which components of complex mixtures can be identified and/or purified. (Adsorption is the adherence of molecules to the surface of another substance.) Chromatography was first described in 1906, and the discoverer named it that because the plant pigments he was studying resulted in colored bands (Lewis 1993).

Column chromatography involves a “mobile” phase (liquid or gas) flowing over a “stationary” phase (solid or liquid). In the experiments to follow, the mobile phase is a buffer solution and the solid phase consists of tiny beads.

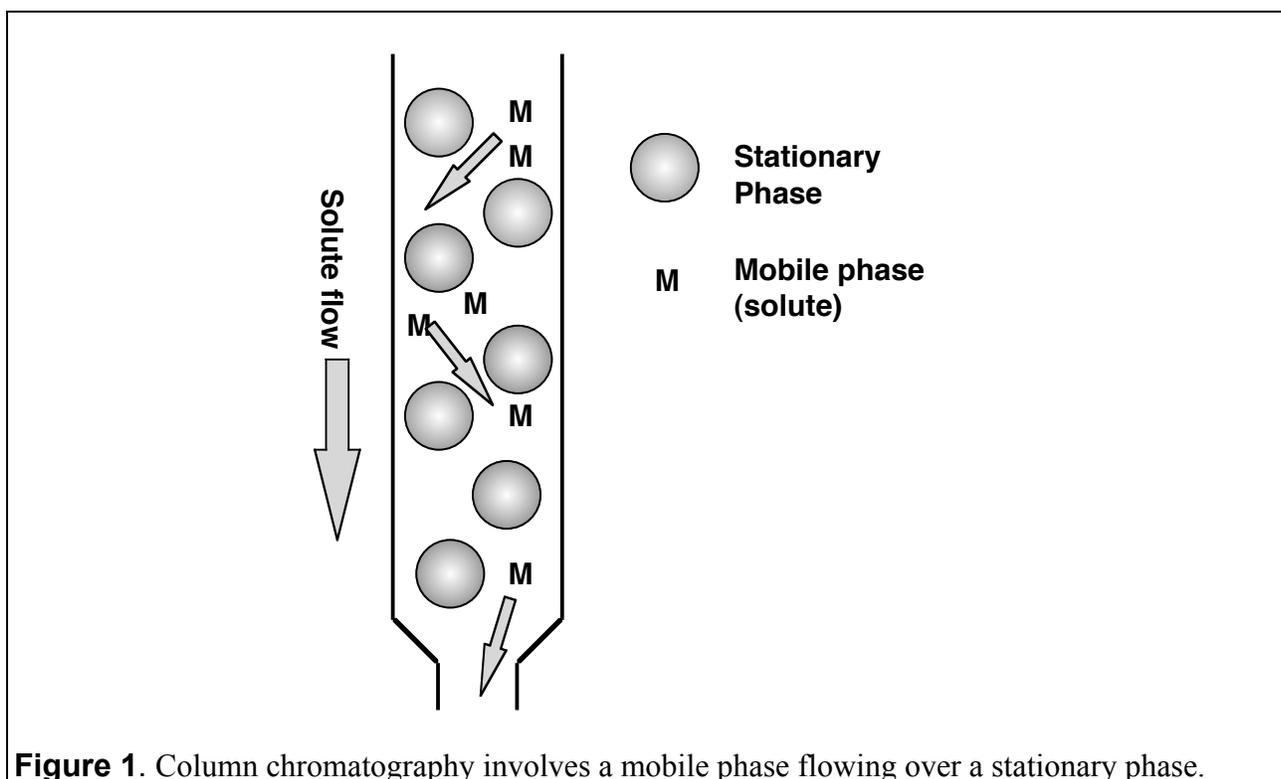


Figure 1. Column chromatography involves a mobile phase flowing over a stationary phase.

In column chromatography, a mixture of molecules is separated based on each molecule’s degree of affinity for the mobile and the stationary phases. If Molecule A has a stronger affinity for the stationary phase than Molecule B, then B will migrate through the column more rapidly than A.

There are many varieties of column chromatography. The three that will be covered in these labs are gel filtration (molecular exclusion) chromatography, ion exchange chromatography, and reverse-phase chromatography. Other common varieties include gas-liquid, hydrophobic interaction, affinity, and partition chromatography.

GEL FILTRATION CHROMATOGRAPHY

Nutshell overview of gel filtration chromatography

The big ideas are:

- Gel filtration separates molecules based solely on their size.
- Larger molecules elute from (i.e., flow off) the column first because they have a shorter path through the gel filtration beads. (They're too large to enter the beads.)
- Smaller molecules elute from the column later than large molecules. because they have a longer path through the gel filtration beads. (They enter the beads, which makes their path through the column longer than the path for molecules that cannot enter the beads.)
- Gel filtration chromatography is used primarily to purify proteins or other molecules of interest.
- Gel filtration chromatography can be used to estimate the size of unknown proteins.

The instructor can decide how much detail to convey to the students. The following discussion and overheads that accompany the chromatography kit can be used in whatever manner the instructor chooses.

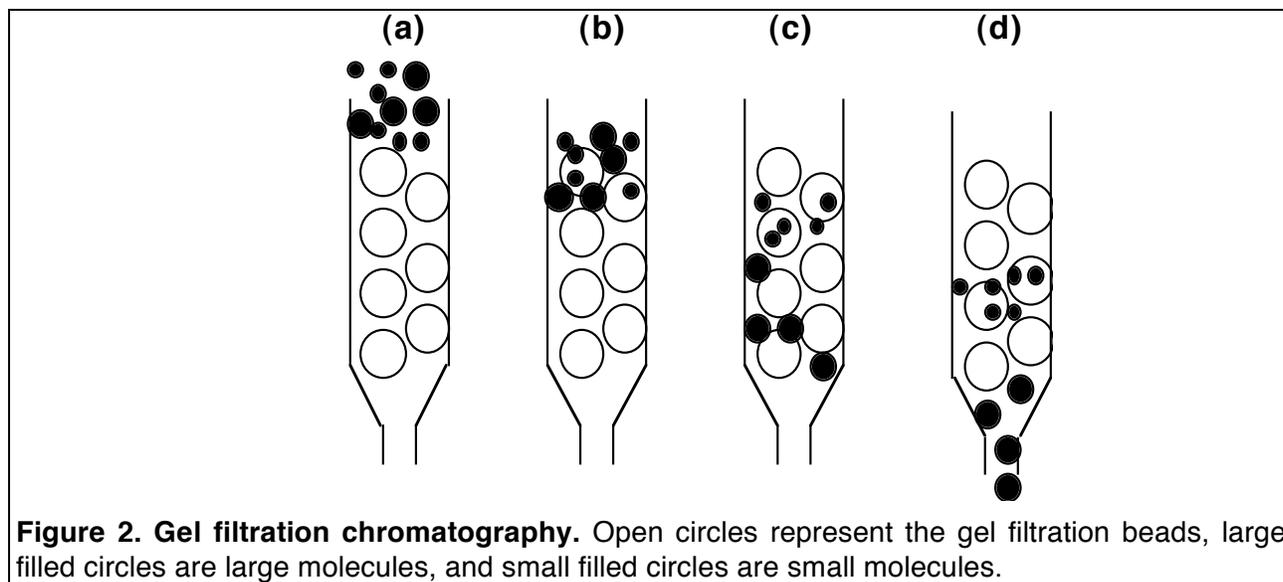
Principles of gel filtration chromatography

In gel filtration (also called molecular exclusion) chromatography, molecules in solution are separated by size as they pass through a column of cross-linked beads that form a three-dimensional network. These polymer beads (frequently made of dextran, agarose, or acrylamide) have pores of a specific size. The size of the pores depends on the particular type of bead being used (see below). (One way to try to help students visualize this is to compare the beads to little whiffleballs.)

As a sample passes through the column, there are two routes that molecules can take through the column, depending on their size and the size of the pores in the beads. Molecules that are larger than the pores will not enter the beads, staying in the solution surrounding the beads. Hence they elute first from the column. Smaller molecules will enter the pores in the beads and so move more slowly through the column (Figure 2). Molecules of intermediate size will enter the stationary phase to some extent, but will not spend as much time there as do the smaller molecules.

To summarize, larger molecules will elute from the column first, and the smallest molecules will elute last, with intermediate molecules strung out in between.

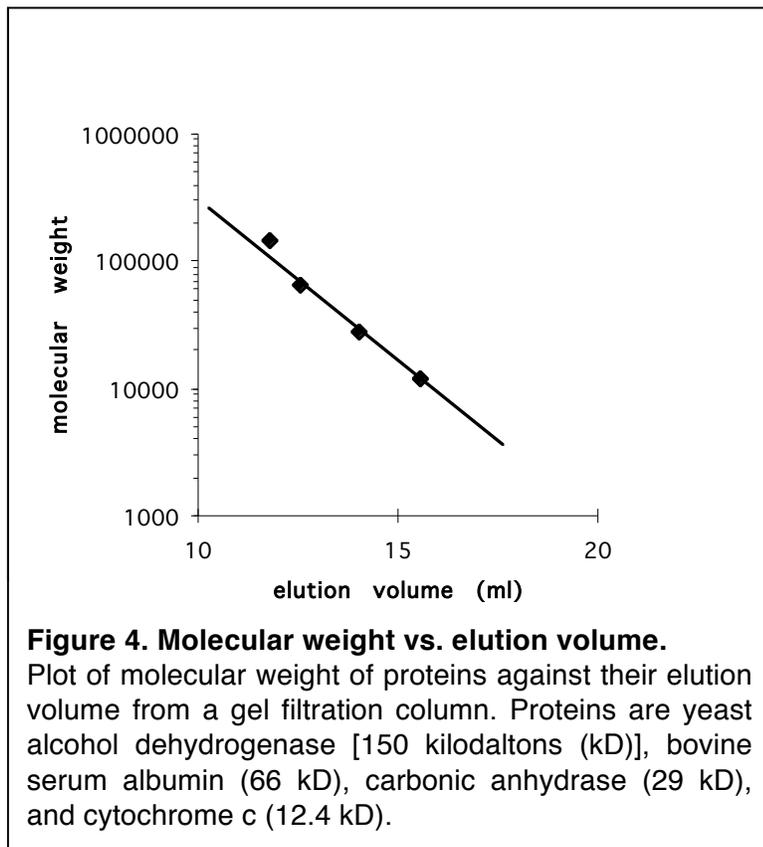
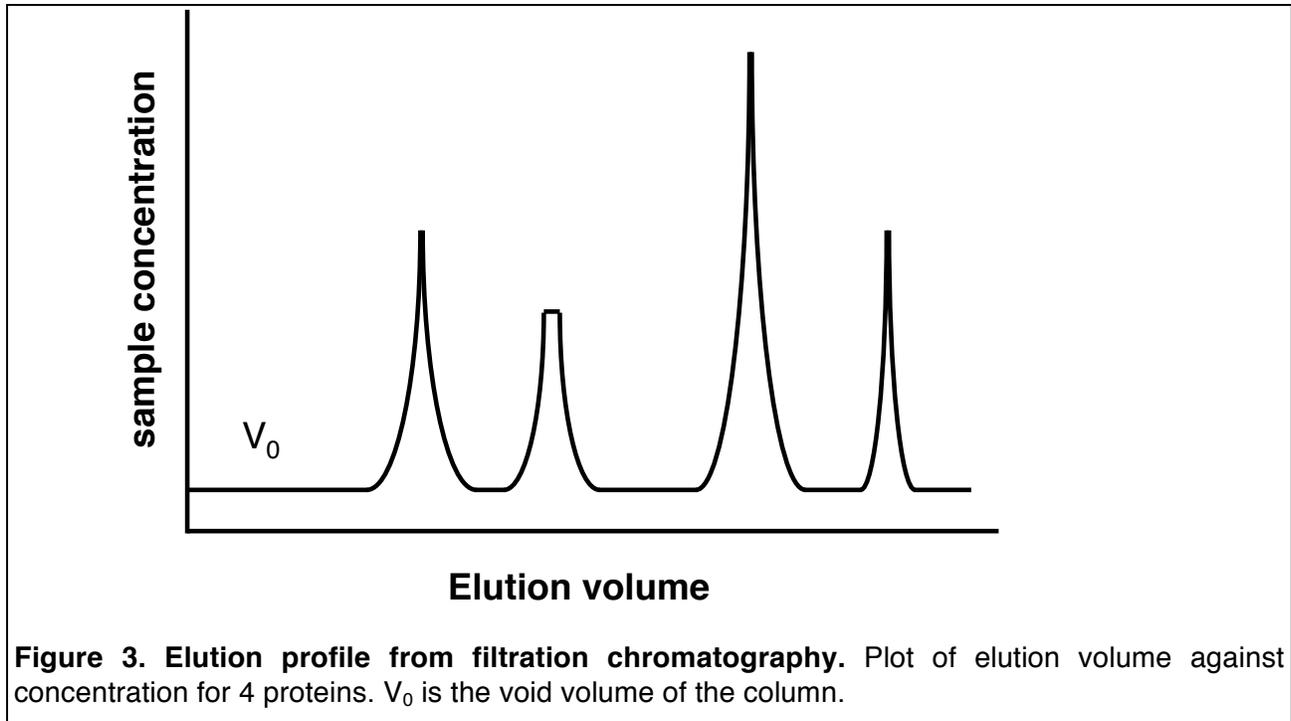
Putting gel filtration chromatography in terms that fit the general description of chromatography given above, the beads comprise the stationary phase and the solution is the mobile phase. Larger molecules have a greater affinity for the mobile phase than the stationary phase, so they migrate through the column more rapidly than smaller molecules, which have a greater affinity for the stationary phase.



As the samples elute from the bottom of the column, they are collected in tubes as fractions. Usually, fractions of a particular volume are collected. In the experiments in this kit, the samples are all colored, so no other detection method is needed to determine in which fractions particular molecules have eluted. In most cases, however, the eluted fractions must be tested to determine both what fractions contain the samples (usually proteins) and how much of the sample (protein) is in the fraction. Several methods commonly used are: (1) spectrophotometric examination of the fractions (proteins and other molecules can be quantified based on the amount of light they absorb at particular wavelengths); (2) electrophoresis of the fractions; and (3) functional assays (e.g., assays that detect enzyme activity or other functions of the sample).

Once the sample concentrations in the fractions have been determined, the concentrations can be graphed against the elution volume to create an elution profile (Figure 3). The first volume that elutes from the column before any sample is called the void volume (V_0) of the column. (Before you load a sample on the top of the column, there is already a volume of buffer in the column. When you start the column and load the sample, the volume of buffer already in the column will flow out of the column *before* any of the sample can flow out. That volume is the void volume.) Any proteins or other molecules that are too large to enter the pores of the beads will elute immediately after the void volume.

If the molecular weights of the proteins are already known, then these data can be used to create a standard curve to calculate the molecular weight of an unknown protein. The elution volume is plotted against the known molecular weights of the proteins on semilog graph paper (Figure 4) and a line derived. From this line and the elution volume of an unknown protein, the molecular weight of the unknown can be estimated.



There are many types of gel filtration materials. The choice of matrix depends on the range of size of molecules to be separated and the goal of the separation. Different bead types have pores of different sizes. Consult a reference book such as those listed in the Appendix (page 39) for more details on factors to be considered in choosing a matrix.

The gel filtration material that will be used in the experiment below is called Bio-Gel P-30 and it will separate molecules with molecular weights from 2,500 to 40,000. Molecules with molecular weights larger than 40,000 will be excluded from entering the beads. (Previously, the kit used Sephadex G-75, a very similar material from another company.)

ION EXCHANGE CHROMATOGRAPHY

Nutshell overview of ion exchange chromatography

The big ideas are:

- Ion exchange chromatography separates proteins based on their charges.
- Except at their isoelectric point, proteins are charged molecules.
- Charged proteins will bind to a column filled with beads of the opposite charge from the protein, but charged proteins will not bind to a column filled with beads of the same charge as the protein.
- A salt solution with high concentrations of ions, e.g., 0.5-1 M sodium or potassium chloride solution, is used to elute proteins that bind to the charged beads. (The ions compete for the charged sites on the beads.)
- Ion exchange chromatography is used primarily to purify proteins.

Principles of ion exchange chromatography

In ion exchange chromatography, molecules are separated based on their charges. In ion exchange, the stationary phase is a bead or resin with a fixed charge. If that charge is negative, then the process is called cation-exchange chromatography, and if the charge is positive, then it is anion-exchange chromatography. Two common resins are carboxymethyl (CM) cellulose (cation exchange) and diethylaminoethyl (DEAE) cellulose (anion exchange). In Experiment 2 below, CM cellulose will be used to separate two proteins.

The fixed charges on the beads will interact with molecules of opposite charge in the buffer solution (mobile phase). For example, the fixed negative charge of a cation exchange resin will always be associated with a positively charged species in the buffer solution (Figure 5a). If a protein of positive charge is passed through the column, it may bind to the matrix (Figure 5b), and it can subsequently be eluted by increasing the ion concentration of the buffer solution. (The elution can be looked on as a competition for binding sites on the matrix. As the ionic strength of the buffer increases, it becomes more likely that a salt ion from the buffer will take the site on the resin and the protein will be eluted from the column.)

The pH of the buffer solution is critical in ion exchange chromatography. The pH must be such that the ion exchange resin has the correct charge. For example, at very low pH (high number of free protons H^+), the carboxymethyl species would be protonated and, hence, would not have the required negative charge. Additionally, the protein that one wishes to bind the resin must be at the pH that will give it the appropriate charge, i.e., below the isoelectric point (pI) of the protein in order to have a net positive charge.

There are 2 common ways that ion exchange columns are eluted. In a gradient elution, the salt concentration increases constantly and gradually during the elution process, for example, gradually moving from no KCl to 1 M KCl over the course of the elution. The second method is a step-wise elution, where the change in salt concentration is abrupt, for example, switching from the no-salt loading buffer to the 1 M elution buffer with no intermediate stages.

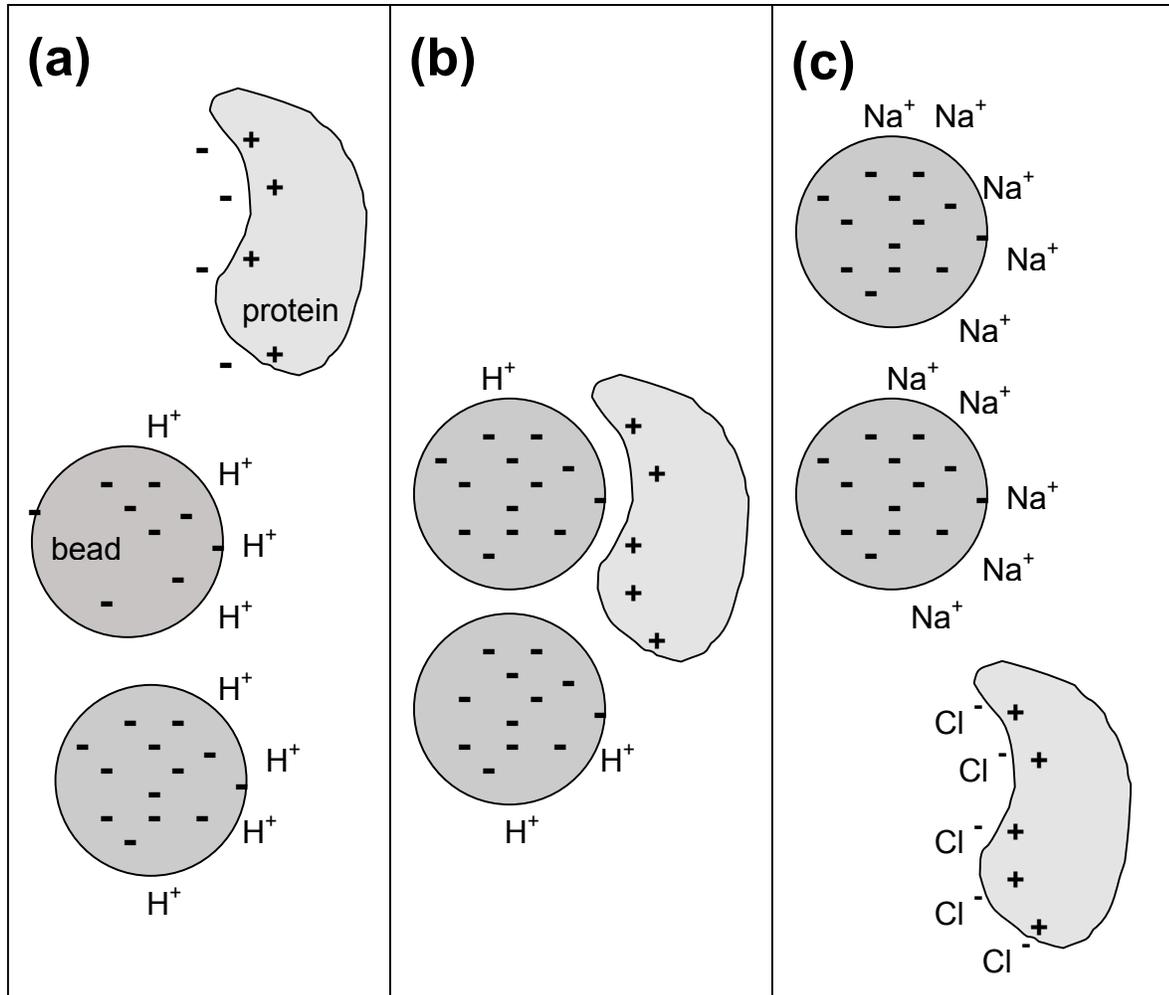
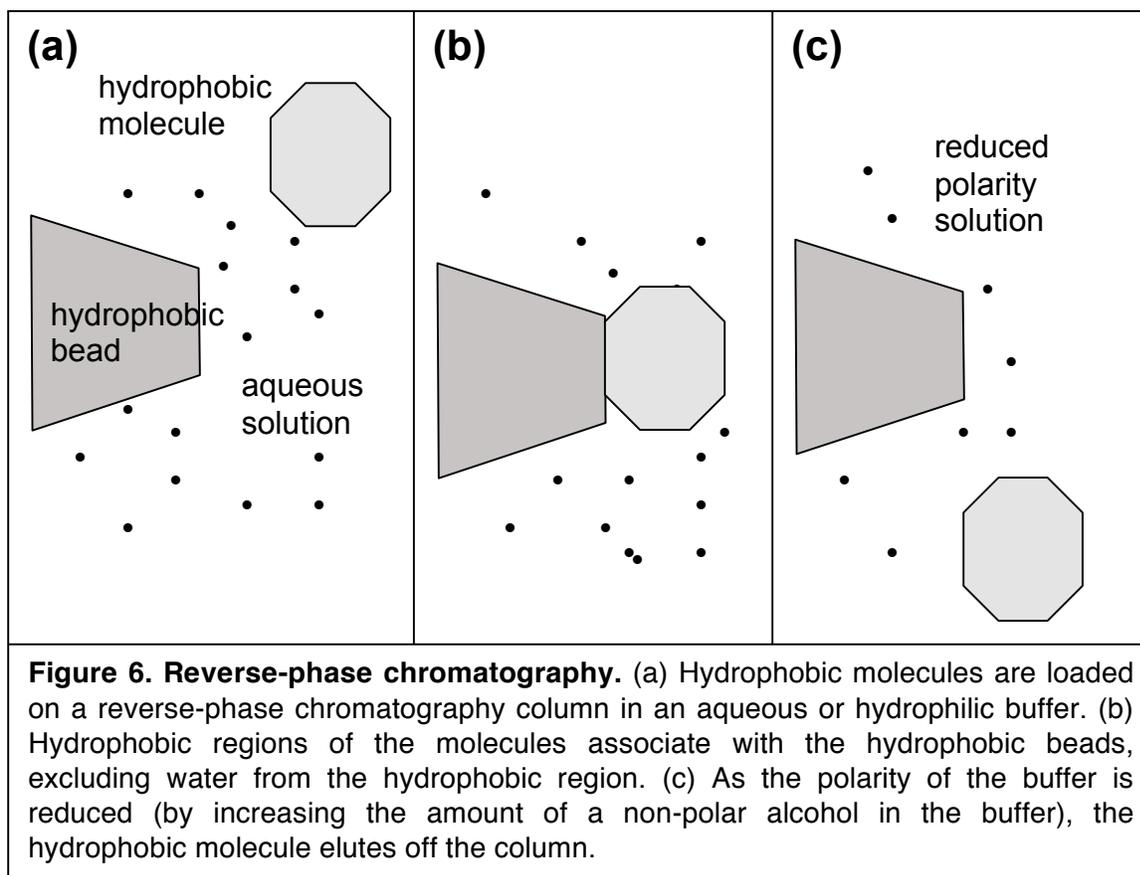


Figure 5. Protein binding to an ion exchange bead. (a) Negatively charged bead has positively charged counter ions from the buffer solution associated with it. The positively charged protein has negative counter ions associated with it. (b) When the protein binds to the bead, some of the counter ions are displaced from both the bead and the protein. (c) When the elution buffer (high ionic strength) is added to the column, the Na^+ and Cl^- ions bind to the beads, displacing the binding between the bead and the protein, and the protein elutes from the column.

In the ion exchange experiment below, a mixture of 2 proteins will be loaded onto a carboxymethyl cellulose column (negatively charged beads; cation exchange resin). One protein, myoglobin, will pass through the column without binding, but the second, cytochrome c, will bind to the beads. The cytochrome c will be eluted, using a step-wise elution, with 1 M NaCl.

REVERSE-PHASE CHROMATOGRAPHY

Reverse-phase chromatography can be viewed as a form of hydrophobic interaction chromatography, in which hydrophobic (nonpolar or antagonistic to water) molecules interact with each other rather than interacting with water molecules (Figure 6). In reverse phase chromatography, molecules are bound to hydrophobic matrix in a buffer of hydrophilic buffer (water-loving; of high polarity) and eluted from the column by reducing the polarity of the buffer (by adding alcohol to the water).



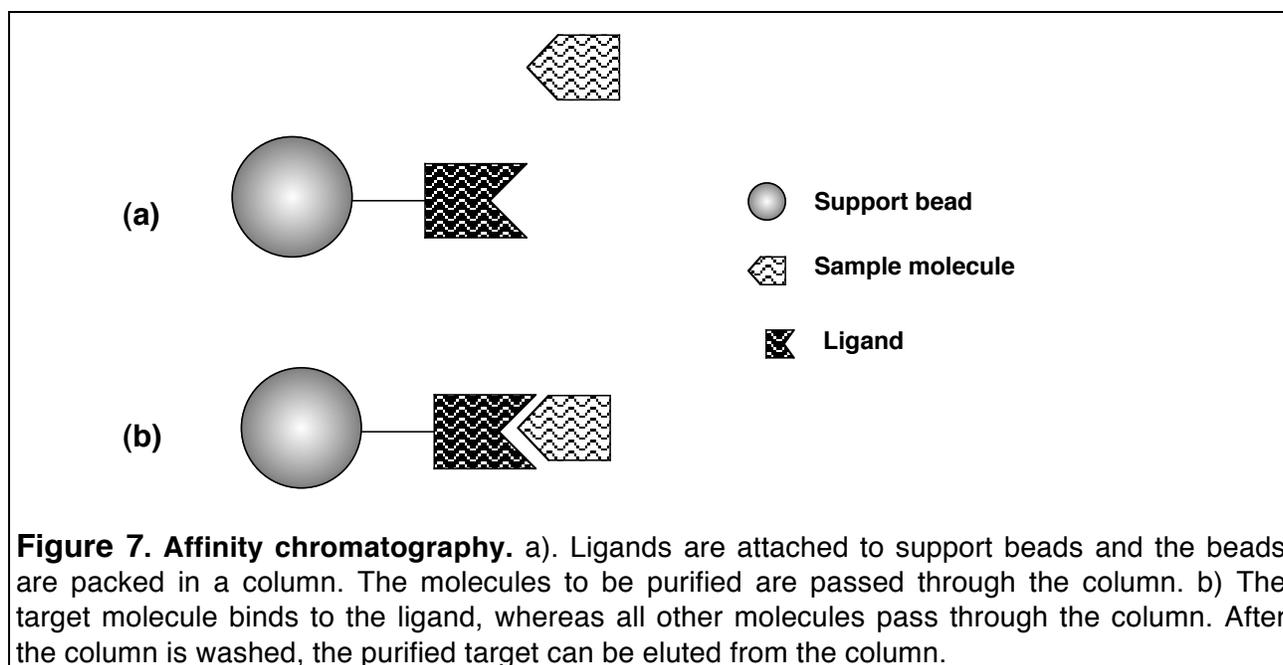
Reverse-phase chromatography is generally used to separate small molecules. The beads (stationary phase) consist of hydrophobic molecules, such as long chain hydrocarbons, bound to a silica matrix. Non-polar or hydrophobic molecules in an aqueous buffer (mobile phase) will bind the matrix. They can then be eluted by decreasing the polarity of the buffer, e.g., increasing the concentration of alcohol or another non-polar solvent in the buffer.

In the experiment below, grape soda is loaded on a reverse-phase chromatographic column. The column is a C-18 column, so the hydrophobic beads have 18-carbon chains. The two dyes in the grape soda (blue and red) will bind to the column. As the polarity of the buffer is decreased (by increasing the concentration of isopropyl alcohol), the dyes elute from the column.

AFFINITY CHROMATOGRAPHY

There are other types of column chromatography that are commonly used in research and industrial laboratories. One of the most important of these is **affinity chromatography**, in which molecules are separated based on very specific aspects of their structure or biological activity. Biological molecules function through binding a variety of other molecules called ligands, for example, antibodies, peptides, nucleic acids, other proteins, hormones, and enzyme substrates. These ligands can be used to purify proteins that bind to them.

The purification is accomplished by immobilizing the ligand on a support matrix (like a bead). A column is then packed with the beads. When the sample is run over the column, the protein of interest will bind the ligand on the column (Figure 7). The protein of interest is then eluted, either by changing the column conditions so that the protein will no longer bind the ligand (e.g., change of pH, salt concentration, etc.) or by using another biological molecule that will out-compete the molecule of interest for ligand binding sites.



Affinity chromatography is a very effective purification technique. It is possible to use affinity chromatography to purify a molecule of interest in a single step, for example, to purify an overexpressed protein from a complete bacterial extract, (containing thousands of other proteins and biological molecules) in one step. Some examples of common ligands used in affinity chromatography are given below.

Ligand	Specificity
2'5' ADP	enzymes with NADP ⁺ as cofactor
Concanavalin A	sugar residues on proteins
Cibacron Blue (dye)	enzymes with nucleotide cofactors
Protein A (from bacteria)	IgG molecules
lysine	ribosomal RNA
arginine	some proteases
metal chelation	histidine-tagged proteins
poly A or poly U	nucleic acids

TEACHER PREPARATION FOR EXPERIMENTS

Additional materials needed:

- For Experiments 1 and 2, **supports** for the gel filtration and ion exchange columns, ideally stands and clamps. If these are not available, it is possible to clamp or tape the columns to an improvised support, e.g., a stack of tube racks. Cover as little of the column bed as possible, so that the students will be able to see the colored bands separating on the column.
- For Experiment 3 (reverse phase chromatography): **isopropyl alcohol**. This is rubbing alcohol, widely available. Purchase the 70% rubbing alcohol (91% is also available) without added colors or fragrances. To prepare the 35% solution, dilute the rubbing alcohol 1:1 with water (e.g., 50 ml water + 50 ml alcohol). To prepare the 8% solution, dilute the rubbing alcohol 1:7.75 with water (e.g., 88.5 ml water + 11.5 ml alcohol). Each student group will need about 10-15 ml of each.
- For Experiment 3 (reverse phase chromatography): **grape soda**. Grape soda (and powdered grape drink mixes) contain the dyes FD&C Red #40 and FD&C Blue #1. Any grape drink with these dyes listed in the ingredients should work. I have used Faygo, Welsh's, and Slice grape soda.

The soda must be flat (no bubbles) to work. Leaving the lid off will allow the soda to flatten, or, if you're in a hurry, stir with a magnetic stirrer to speed things up.

- **Optional:** For Experiment 3 (reverse phase chromatography): **Clinistix** or Dia-stix glucose test strips. These should be available at drug stores, etc., with the diabetic supplies. (They were \$6-7 for a pack of 50 at the Wal-Mart pharmacy.) You will need 3 strips for each group.

Timing of the experiments

These three experiments can all be done in about 2 hours total (2 hours experimental time, not allowing for introduction of the material or discussion of the results). Experiment 1 takes the longest, about an hour. (Some teachers have their students run Experiment 3 while waiting for Experiment 1 to be completed.) Depending on the amount of class time that you want to devote to chromatography, one way would be to do the three experiments over 3 class/lab periods. Each class could be devoted to a separate type of chromatography. The instructor may wish to pre-run the experiments to get a feel for the time and effort involved. Feel free to call Kristi DeCourcy at the Fralin Biotech Center if you want to discuss the best way to time the experiments.

Teacher notes for experiments 1 and 2

- There is an accompanying scenario for experiments 1 and 2 on pages 25-36. Your students can use column chromatography to determine if Acme Widget Company is responsible for the dead fish appearing in a local river.
- The sample solution has 4 components. The concentrations of the components and how the solution was prepared are given in the Appendix.

Molecule	Molecular weight	color
blue dextran	>500,000	blue
myoglobin	16,900	brown
cytochrome c	12,400	red
DNP-glutamate	313	yellow

In Experiment 1, cytochrome c and myoglobin will elute from the gel filtration column as a single brown/orange-colored band. (Their molecular weights are too close for the proteins to be separated on this column under these conditions.) They will then be separated into the component red and brown proteins in Experiment 2 using ion exchange chromatography. Experience has shown that if you tell experimenters that there are 4 components to the sample solution, they will see 4 colored bands where only 3 exist in Experiment 1. It is recommended that information on the components and colors of the sample solution be withheld until the end of the experiment so that the experimental observations are not prejudiced by the knowledge.

- If Experiments 1 and 2 are to be done during different lab periods, each student group's Fraction 2 (brown fraction containing cytochrome c and myoglobin) should be stored in the refrigerator until the second lab period.
- Both the blue and yellow fractions from Experiment 1 can be discarded after the experiment.
- Some additional information about the components in the sample solution:

Blue dextran is a glucose polymer, a long chain of glucose molecules. The average molecular weight of the kind in the sample solution is 2,000,000!

Myoglobin is an iron-containing protein found in muscles (hence the myo- prefix). Myoglobin stores oxygen molecules in the muscle like hemoglobin does in the blood.

Cytochrome c is another iron-containing protein, and it is the heme group (iron portion) that gives the protein its red color. Cytochrome c is very important in cellular metabolism and electron transport.

DNP-glutamate is an amino acid (glutamic acid) with a dinitrophenyl (DNP) group attached. The DNP group gives the molecule its color.

Experiment 1 preparation

- The Sephadex or Bio-Gel gel filtration columns are prepacked, so no preparation of the columns is needed. Attach columns to the clamps in such a way that the column bed is visible.

Note: Occasionally the columns are damaged during shipment. If you notice that a column has cracks in the resin or has dried out, do not use it.

- Dilute the Loading Buffer. To save space and weight during shipment, the Loading Buffer is sent as a 10X stock solution. Dilute 1:10 in distilled water. For example, for 1 liter of Loading Buffer, add 100 ml of 10X stock buffer to 900 ml distilled water and mix. For 750 ml of Loading Buffer, add 75 ml of 10X stock buffer to 675 ml of distilled water, etc.

Note: You will need loading buffer stock solution (12 ml/class) to prepare Elution Buffer for Experiment 2, so do not dilute all of the stock solution now if you plan to do Experiment 2.

- Prepare student aliquots of Loading Buffer. Each student group will need approximately 30 ml of Loading Buffer in Experiment 1. A number of 50-ml plastic beakers are included in the chromatography kit that may be used for buffer aliquots. Please do not write on the beakers; use label tape instead.
- Prepare student aliquots of sample solution. Aliquot 300 microliters (μl) per student group into microcentrifuge tubes. Store these in the refrigerator and in the dark until ready to use. (One of the components is light-sensitive.) If you do not have a way to accurately measure 300 μl , then just divide the sample evenly among the tubes. (Four milliliters of sample solution is included for 12 student groups. Loading a little more than 300 μl will not affect the column run.)
- The plastic beakers can be used to collect all fractions in these experiments, but if you have test tubes and racks available, you may want to use them. The colors will appear more definitive in glass test tubes than they do in plastic beakers.

Note: If any of the columns run much more slowly than the rest, please mark them with tape, so we can replace them. As the columns are used repeatedly, they tend to run more slowly.

EXPERIMENT 1: GEL FILTRATION CHROMATOGRAPHY

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Materials (per student group):

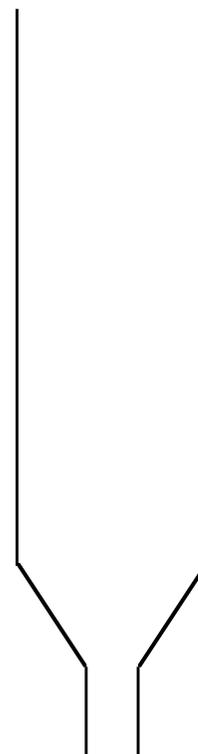
- Gel filtration column
- 0.3 ml sample solution
- 30 ml Loading Buffer (0.05 M imidazole buffer, pH 6.0)
- Small beakers or test tubes and rack (to collect fractions)
- Support stand and clamp
- 2 transfer pipettes

Experimental Procedure

1. Remove the stopper from the top of the column and place the column on the support stand. Place a beaker under the column to collect the effluent and remove the end cap from the bottom of the column. (The effluent is the solution that elutes from the column.) Allow the column to drain until it stops dripping. Note: If there is a lot of buffer in the column when you start, a portion of it can be removed using a transfer pipette, but allow the final cm of buffer to flow normally into the column.
2. Add the sample to the top of the column bed using a transfer pipette.
3. Allow the sample to enter the column. When the column has stopped dripping, add a small volume (~5-10 drops) of Loading Buffer and allow that to enter the column. When the column stops dripping, repeat with one more small volume of Loading Buffer.
4. Add 5 ml of Loading Buffer to the column and let the column flow. You should be able to see the separation of the colored bands almost immediately. Replenish the buffer volume as necessary, making sure that the column never stops flowing.
5. When the first colored band is about to elute from the column, switch to a new beaker (or to a test tube) to collect the sample. This will be Fraction 1.
6. After the first colored band has been eluted, switch back to the original beaker to collect the effluent.
7. When the second colored band is about to elute from the column, switch to a new beaker (or test tube) to collect the sample. This will be Fraction 2.
8. Collect fractions until all of the colored bands have eluted from the column. Put the end cap back on the bottom of the column. Make sure that there is buffer on top of the column and replace the stopper in the top of the column. **Save Fraction 2 for Experiment 2!**

Observations and Conclusions for Experiment 1

1. How many different colored bands appear as the sample separates through the column?
2. What colors are the bands in the column?
3. What color is eluted first? Second? Third?
4. In the space to the right, draw and label a diagram of how your column looked during the separation.
5. What conclusions can be drawn about the relative sizes of the molecules that make up each colored band?



Experiment 2 preparation

- The CM-cellulose columns are prepacked, so no preparation of the columns is needed. Attach columns to the clamps in such a way that the column bed is visible.
- If the columns have been used by students previously, you should add an initial column wash step with Loading Buffer. If there is Elution Buffer on the column from previous runs, cytochrome c will not bind to the beads. If the wash step is needed, have the students wash the column following Step 1, prior to loading the sample.
- For each class, you will need approximately 120 ml of Elution Buffer. To prepare 120 ml of Elution Buffer, place 96 ml of distilled water in a beaker. While stirring, add 12 ml of 10X loading buffer stock and 12 ml of 10X NaCl.

Note: if you have goofed and diluted all of the loading buffer stock solution already (without saving any 10X stock solution for making Elution Buffer), it's still okay! Just add 12 ml of 10X NaCl to 108 ml of (diluted, 1X) Loading Buffer. The slight difference in concentration will not affect the experiment.

- If you have not already done so, prepare Loading Buffer by diluting the 10X loading buffer stock (see page 15 for directions).
- Prepare student aliquots of Loading Buffer and Elution Buffer. (The Loading Buffer is the same buffer that was used in Experiment 1.) Each student group will need approximately 20 ml of Loading Buffer and 10 ml of Elution Buffer in Experiment 2. A number of 50-ml plastic beakers are included in the chromatography kit that may be used for buffer aliquots. Please do not write on the beakers; use label tape instead.
- The sample solution for Experiment 2 is Fraction 2 (brown fraction) collected in Experiment 1. [Fractions 1 and 3 (blue and yellow fractions) can be discarded after Experiment 1.]
- The plastic beakers can be used to collect all fractions in these experiments, but if you have test tubes and racks available, you may want to use them. The colors will appear more definitive in glass test tubes than they do in plastic beakers.

Note: The results of this experiment are not as dramatic as those in Experiment 1. The cytochrome c should bind to the top of the column and the red color should be clearly visible on the column. The myoglobin will run through the column without binding. It is a brown protein and will not be as obvious as the red color, especially as it is dilute. If the column flow-through is collected, perhaps only the first 2-3 ml, it should be seen that the solution is not clear. Also, you can point out that the material binding the column is clearly red, and not the orange brown that was loaded on the column.

The purpose of including this experiment is to demonstrate that multiple techniques must be used to purify some proteins, i.e., that proteins close in size must be separated based on some other facet of their structure or activity (in this case, charge).

EXPERIMENT 2: ION EXCHANGE CHROMATOGRAPHY

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Materials (per student group):

- Carboxymethyl (CM) cellulose column
- 20 ml Loading Buffer (0.05 M imidazole, pH 6.0)
- 15 ml Elution Buffer (0.05 M imidazole, pH 6.0, 0.5 M NaCl)
- Small beakers or test tubes and rack (to collect fractions)
- Support stand and clamp
- 3 transfer pipettes
- Fraction 2 (brown fraction) from Experiment 1

Experimental Procedure

1. Place a beaker under the ion exchange column. Open the bottom of the column and let the buffer elute from the column until it stops dripping. (This column runs very quickly.)
2. Place a new test tube or beaker under the column and gradually add Fraction 2 (the middle, brown fraction) from Experiment 1. Let the sample run completely into the column.
3. Wash the column twice with 0.5 ml volumes of Loading Buffer, continuing to collect the effluent in the same tube or beaker. This will be Fraction A.
4. Switch to the original beaker and continue to wash the column until the effluent looks colorless and the column has color concentrated only at the top.
5. Place a new test tube or beaker under the column and gently add 10 drops of Elution Buffer (0.05 M imidazole, pH 6.0, 0.5 M NaCl). Once the colored band is moving through the column, continue to add elution buffer until all color is removed from the column. This will be Fraction B.

Observations and Conclusions for Experiment 2

1. How many different colored bands were contained in Fraction 2 from Experiment 1?
2. What colors were they?
3. Why did these molecules separate in Experiment 2?
4. Describe the differences in chemical behavior of these molecules, based on your observations.
5. Identify the molecules found in each fraction from Experiments 1 and 2, given the information below.

<u>Molecule</u>	<u>Color</u>
blue dextran	blue
DNP-glutamate	yellow
myoglobin	brown
cytochrome c	red

6. List these molecules according to their relative size.
7. Why did myoglobin and cytochrome c **not** separate in Experiment 1?

Teacher notes for experiment 3

- Grape soda contains two dyes, FD & C Red #40 and FD & C Blue #1. In a strongly polar solution (water), both dyes will bind to a non-polar substrate (C-18 hydrocarbon molecules on a silane matrix). The dyes have structural differences and the red dye is more polar than the blue dye. Consequently, the red dye will detach from the substrate (beads) and elute from the column when the polarity of the surrounding liquid decreases (8% isopropyl alcohol in water is less polar than water alone). When the polarity of the solution is further reduced (35% isopropyl alcohol), the blue dye will elute from the column.
- Other experiments of the same sort can be done with these columns. (See page 22, for example.) Look at the ingredients of other sodas or drink mixes to determine what dyes are present. Try loading these samples on the columns. (Columns must have been washed with water first.) If other dyes bind, then the students will have to determine what percentages of isopropyl alcohol are necessary to elute these dyes. One way to do this would be to increase the concentration of alcohol incrementally, for example, starting with 5% alcohol, then 10%, etc.

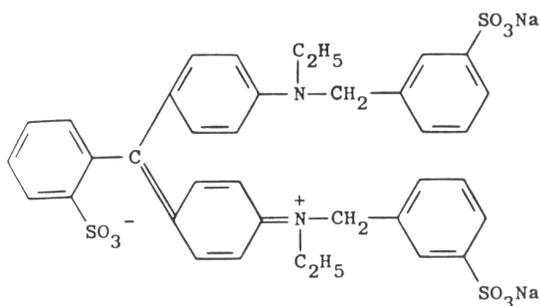


Figure 6. FD & C Blue Dye #1.

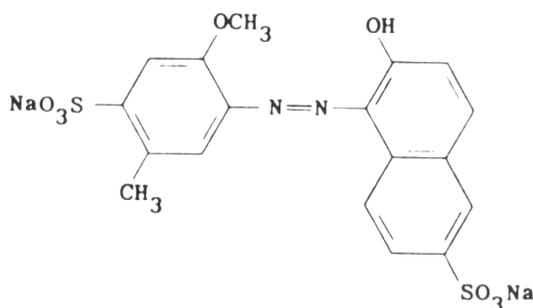


Figure 7. FD & C Red Dye #40.

Experiment 3 preparation

- The columns for reverse-phase column chromatography are small cartridge columns manufactured by the Waters Corporation. They are ready to use when you unpack them from the kit. For these columns, stands and clamps are not needed. The columns attach directly to the end of the syringes.
- Prepare student aliquots of water, 8% isopropyl alcohol, and 35% isopropyl alcohol. Plastic beakers are included in the chromatography kit that may be used for aliquots. Please do not write on the beakers; use label tape instead.

Each student group will need about 15 ml of 8% alcohol. Dilute isopropyl alcohol (70% rubbing alcohol) 1:7.75 with water (e.g., for 200 ml, mix 177 ml water + 23 ml alcohol).

Each student group will need about 15 ml of 35% alcohol. Dilute isopropyl alcohol (70% rubbing alcohol) 1:1 with water (e.g., for 200 ml, mix 100 ml water + 100 ml alcohol).

- Prepare grape soda aliquots. Each student will need 5 ml. The soda must be flat (no bubbles) to work. Leaving the lid off will allow the soda to flatten, or, if you're in a hurry, stir with a magnetic stirrer to speed things up.
- Syringes: If the syringes are new, take them from the packages and remove the caps at the ends. If this is not done, inevitably the students will try to attach the column to the syringe without removing this cap.
- Although the columns have a longer "tube" at one end than the other, there is no difference in the ends of the column. Either end can be attached to the syringe. You should be consistent, however, in the end that you attach; always put the same end onto the syringe during the experiment.

Note: Several years ago, Adrienne Warren (then at Chesapeake Center for Science and Technology, Chesapeake, VA) reported that there are 3 dyes in **Mountain Dew** that will bind the column. The dyes will elute from the column with 2.5% isopropyl, 5% isopropyl, and 15% isopropyl alcohol.

Real World Application: Sep-pak C-18 columns are widely used for sample preparation in the laboratory. According to the Waters Corporation website (<http://www.waters.com>), the columns are used to remove "analytes (substances being analyzed or measured) of even weak hydrophobicity from aqueous solutions; typical applications include drugs and their metabolites in serum, plasma or urine, desalting of peptides, trace organics in environmental water samples, and organic acids in beverages."



EXPERIMENT 3: REVERSE-PHASE CHROMATOGRAPHY

Materials (per student group):

- 1 reverse phase column (Sep-Pak® C-18 cartridge)
- 1 3-cc syringe
- 50 ml water
- 15 ml 8% isopropyl alcohol
- 15 ml 35% isopropyl alcohol
- 5 ml grape soda (flattened)
- 3 small beakers (to collect fractions)
- 3 Clinistix strips (optional)

Procedure

1. Fill the syringe with 35% isopropyl alcohol and attach the syringe to the column. Wash the column by injecting the alcohol through the column.
2. Equilibrate the column by washing with 2-3 syringe volumes of water. To do this, remove the column from the syringe. Fill the syringe with water, then reattach the column to the syringe. Inject the water through the column. Repeat these steps for the second and third wash.
3. Inject 2 ml of grape soda on the column, collecting the column flowthrough in a beaker. (The flowthrough from the column should be clear.) Then wash the column with 3 syringe volumes of water, collecting the flowthrough in the same beaker. Follow the same procedure as for the washes in Step 2. In other words, remove the column from the syringe, draw the solution into the syringe, then reattach the column to the syringe and inject the solution.
4. Elute the red dye by injecting 2 syringe volumes of 8% isopropyl alcohol, collecting the flowthrough in a second beaker. If there is still pink coming off the column, use a third syringe volume of 8% isopropyl alcohol.
5. Elute the blue dye by injecting 2 syringe volumes of 35% isopropyl alcohol, collecting the flowthrough in a third beaker. If there is still blue coming off the column, use a third syringe volume of 35% isopropyl alcohol.
6. **(Optional)** Test the 3 pools eluted from the column with Clinistix to determine in which fraction the sugar was eluted.

QUESTIONS FOR EXPERIMENT 3

1. What is the purpose of the reverse-phase chromatography experiment?
2. Which dye was collected first? Why?
3. What is the function of the alcohol?
4. Why are two different percentages of alcohol used?
5. What would have happened if you had eluted first with 35% alcohol?
6. Which fraction contained the sugar?
7. What does elute mean?
8. How does reverse-phase chromatography differ from gel filtration chromatography?

Optional:

Try two of the other available sodas or drink mixes. If other dyes bind, you will have to determine what percentages of isopropyl alcohol are necessary to elute these dyes.

SODA OR DRINK MIX USED	RESULTS

Teacher Guide: Mystery of the Dying Catfish

This teacher guide is provided to give discussion points and sample answers to questions. Students may have correct answers that are not included in this guide. Although the experiments are designed to provide a single answer, there may be variation in data and variation in student perception of their data. As long as students examine their data and can justify their answers based on their data, that's fine!

Purpose: Try to find what is causing the death of many freshwater fish in a nearby river.

Scenario:

In the Rio River, which runs near your school, large numbers of dead fish are suddenly seen floating on the surface. What is killing the fish?

What do you know? The floating fish are primarily benthic (bottom-dwelling) fish, like catfish and darters, and fish who feed on bottom dwellers, like sunfish and some minnows. The fish all appear to have skin lesions, reddening or inflammation of the fins, and raised scales. Since they all have the same symptoms, perhaps there is a common cause.

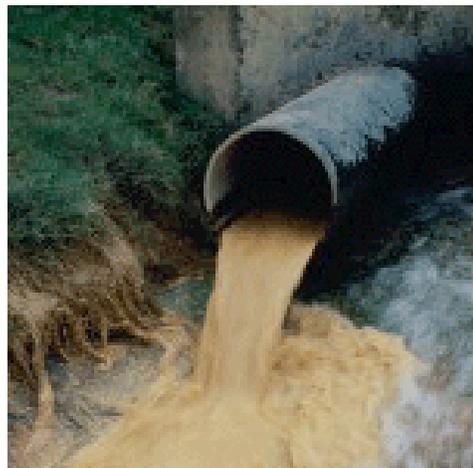
You conduct a quick search on fish kills on the internet, and find that these symptoms are very similar to those caused by Rufino toxin (RT). (Note to teachers- Rufino toxin is not a real toxin! I made it up.)

What do you find out about Rufino toxin in your search? RT is a protein that has been implicated in fish kills previously. It is known to bind to heavy metals, and hence tends to localize to the bottom of waterways. RT has a red color. (What can cause proteins to have a red color? Hint: think about hemoglobin.) It has a molecular weight of 13 kilodaltons (kDa), and its isoelectric point is 7 (see box at right).

So, are there any sources of RT near the Rio River fish kills?

Acme Widget Company is located upstream from where the dead fish have been found. Through further investigation, you find that RT is a by-product in the manufacturing of widgets. But Acme Widgets categorically denies that RT is in its effluent, and says that only harmless chemicals remain after they process the waste. As proof, they point to the color of the effluent, greenish brown, with no red color.

What is an isoelectric point? The isoelectric point (or pI) of a protein is the pH at which the protein has an equal number of positive and negative charges. This means that below the pI, the protein will have a positive charge and above the pI it will have a negative charge. So, for Rufino toxin with a pI of 7, the protein will carry a positive charge when it is in a solution below pH 7 and a negative charge when it is in a solution above pH 7.



Effluent: Municipal sewage or industrial liquid waste (untreated, partially treated, or completely treated) that flows out of a treatment plant, septic system, pipe, etc. www.deq.state.va.us/tmdl/glossary.html

Here are some questions to get you thinking about today's lab:

Can there be Rufino toxin, a red chemical, in the effluent, even though it looks greenish brown?

Yes. And that could lead to the question of what levels of toxins can cause problems. Suppose you've got 0.5 g of arsenic in a glass of milk. You might not be able to see the arsenic, but it would still be toxic. Students could look up lethal doses of toxins on the web: For example, arsenic is four times as toxic as mercury and a lethal dose to humans is a dose of 125 milligrams (http://www.thewaterpage.com/arsenic_factsheet.htm)

If RT could be there, what are some ways that you could use to separate the components of the effluent?

Chemical means- size, charge, polarity, etc.

Physical means, like centrifugation, to remove particulates from the effluent.

What do you know about RT?

Its molecular weight is 13 kDa, it has an isoelectric point of 7, and it is red.

How can we use the properties of RT to determine if Rufino toxin is in the effluent from the Acme Widget Company?

Any of the physical or chemical properties can be used to separate RT from molecules with different characteristics.

First, we will use the relative small size of RT to try to separate it from other components of the effluent.

Experiment 1: Separating Effluent Components by Size

The sample of effluent that you have been given has already been centrifuged, so that the particulate matter has been removed. It is still greenish brown, but transparent. (The original effluent was cloudy because of the particulate matter suspended in it.)

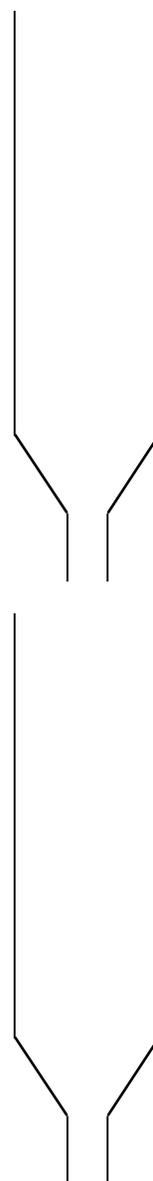
Materials (per student group):

gel filtration column	small beaker
effluent solution	support stand and clamp
Loading Buffer	transfer pipettes
test tubes	

Procedure

1. Remove the stopper from the top of the column and place a beaker under the column to collect the liquid that flows through. Remove the end cap from the bottom of the column. Allow the column to drain until it stops dripping.
2. Add the effluent sample to the top of the column bed using a transfer pipette.
3. Allow the sample to enter the column. When the column has stopped dripping, add a small volume (~5-10 drops) of Loading Buffer and allow that to enter the column. When the column stops dripping, repeat with one more small volume of Loading Buffer.
4. Add 5 ml of Loading Buffer to the column and let the column flow. Replenish the buffer volume as necessary, making sure that the column never stops flowing.
5. When the first colored band is about to elute from the column, switch to a new test tube to collect the sample. This will be Fraction 1.
6. After the first colored band has been eluted, switch back to the original beaker to collect the flow-through.
7. When the second colored band is about to elute from the column, switch to a new test tube to collect the sample. This will be Fraction 2.
8. Collect fractions until all of the colored bands have eluted from the column. Put the end cap back on the bottom of the column. Make sure that there is buffer on top of the column and replace the stopper in the top of the column.

While your column is running draw pictures of what you see.



Results:

What are the colors of the samples that you collected?

blue, brown, yellow

Does this mean that there is no Rufino toxin in the effluent from the Acme Widget factory?

Same answer as above- just because we cannot see it doesn't mean that it isn't there.

If the Rufino toxin is present, in which sample collected by gel filtration is it likely to be?

It is more likely to be in the brown band. Unlikely that it is in yellow or blue.

Can gel filtration separate RF from all other proteins?

Not if they are close in size,

Will gel filtration separate RT from a protein that is 15 kDa?

No, 13 kDa and 15 kDa are too close to separate using gel filtration.

What other characteristics of RF can we use to separate it from other proteins that are close to it in size?

What else do we know? Color. No help there. Charge! We can use charge to try and separate RF from other similarly sized proteins.

Experiment 2: Separating Effluent Components by Charge

Materials (per student group):

ion exchange column	small beaker
Loading Buffer	support stand and clamp
Elution Buffer	transfer pipettes
test tubes	
Fraction 2 (brown fraction) from gel filtration column.	

Procedure

1. Place a beaker under the ion exchange column. Open the bottom of the column and let the buffer elute from the column until it stops dripping.
2. Place a new test tube under the column and gradually add Fraction 2 from Experiment 1. Let the sample run completely into the column.
3. Wash the column twice with 0.5 ml volumes of Loading Buffer, continuing to collect the effluent in the same tube or beaker. This will be Fraction A.
4. Switch to the original beaker and continue to wash the column until the effluent looks colorless and the column has color concentrated only at the top.
5. Place a new test tube under the column and gently add 10 drops of Elution Buffer. Once the colored band is moving through the column, continue to add elution buffer until all color is removed from the column. This will be Fraction B.

Results:

Describe your results. Did the brown fraction contain more than one component?

A red chemical bound to the column and a brown one did not.

How do you know that the brown and the red components are not the same thing?

Since one bound to the column and the other did not, that means that they do not have the same charge at this pH.

Since the red protein bound to the negatively-charged ion exchange resin at pH 6, what is the charge of the red protein at pH 6?

Since the red protein bound to the negative resin at pH 6, it must have a positive charge at pH 6.

Do your results support the hypothesis that Acme Widgets is releasing Rufino toxin in their wastewater?

Yes, they do.

In what way?

Rufino toxin has an isoelectric point of 7, so below that pH, in more acidic conditions, the toxin has a positive charge. Since red protein has a positive charge at pH 6, it could be Rufino toxin.

So, there is evidence that Acme is releasing a chemical that has the same characteristic size, charge, and color as Rufino toxin. The fish kills, with the symptoms of RT poisoning are certainly evidence as well. At this point, you'll need to call in the experts for more tests to confirm that the red chemical that you have isolated is actually Rufino toxin.

What other experiments might the experts perform to prove or disprove the presence of RT in the effluent?

Perhaps they might sequence the purified protein, to see if the sequence is the same as RT. There might be an antibody to RT available, and a Western blot could be used to identify the protein.

Name _____
 Period _____

Mystery of the Dying Catfish

Purpose: Try to find what is causing the death of many freshwater fish in a nearby river.

Scenario:

In the Rio River, which runs near your school, large numbers of dead fish are suddenly seen floating on the surface. What is killing the fish?

What do you know? The floating fish are primarily benthic (bottom-dwelling) fish, like catfish and darters, and fish who feed on bottom dwellers, like sunfish and some minnows. The fish all appear to have skin lesions, reddening or inflammation of the fins, and raised scales. Since they all have the same symptoms, perhaps there is a common cause.

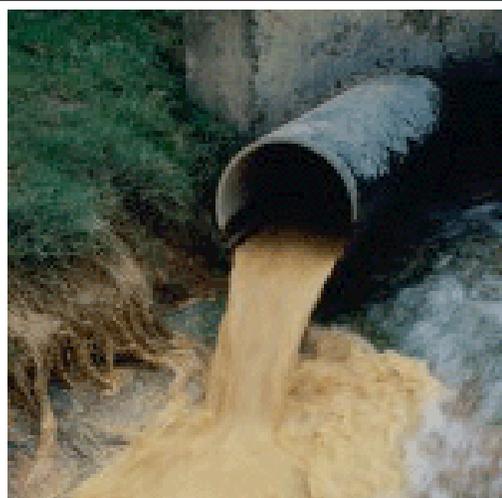
You conduct a quick search on fish kills on the internet, and find that these symptoms are very similar to those caused by Rufino toxin (RT).

What do you find out about Rufino toxin in your search? RT is a protein that has been implicated in fish kills previously. It is known to bind to heavy metals, and hence tends to localize to the bottom of waterways. RT has a red color. (What can cause proteins to have a red color? Hint: think about hemoglobin.) It has a molecular weight of 13 kilodaltons (kDa), and its isoelectric point is 7 (see box at right).

So, are there any sources of RT near the Rio River fish kills?

Acme Widget Company is located upstream from where the dead fish have been found. Through further investigation, you find that RT is a by-product in the manufacturing of widgets. But Acme Widgets categorically denies that RT is in its effluent, and says that only harmless chemicals remain after they process the waste. As proof, they point to the color of the effluent, greenish brown, with no red color.

What is an isoelectric point? The isoelectric point (or pI) of a protein is the pH at which the protein has an equal number of positive and negative charges. This means that below the pI, the protein will have a positive charge and above the pI it will have a negative charge. So, for Rufino toxin with a pI of 7, the protein will carry a positive charge when it is in a solution below pH 7 and a negative charge when it is in a solution above pH 7.



Effluent: Municipal sewage or industrial liquid waste (untreated, partially treated, or completely treated) that flows out of a treatment plant, septic system, pipe, etc. www.deq.state.va.us/tmdl/glossary.html

Here are some questions to get you thinking about today's lab:

Can there be Rufino toxin, a red chemical, in the effluent, even though it looks greenish brown?

If RT could be there, what are some ways that you could use to separate the components of the effluent?

What do you know about RT?

How can we use the properties of RT to determine if Rufino toxin is in the effluent from the Acme Widget Company?

First, we will use the relative small size of RT to try to separate it from other components of the effluent.

Experiment 1: Separating Effluent Components by Size

The sample of effluent that you have been given has already been centrifuged, so that the particulate matter has been removed. It is still greenish brown, but transparent. (The original effluent was cloudy because of the particulate matter suspended in it.)

Materials (per student group):

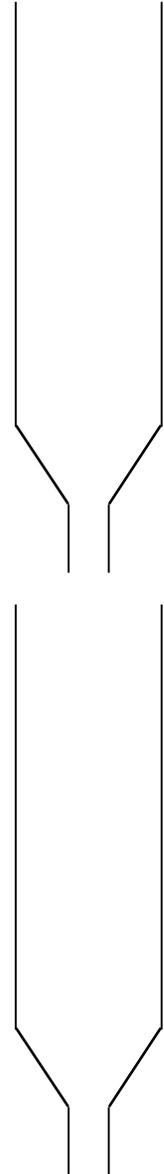
gel filtration column
effluent solution
Loading Buffer
test tubes
small beaker
support stand and clamp
transfer pipettes

Procedure

1. Remove the stopper from the top of the column and place a beaker under the column to collect the liquid that flows through. Remove the end cap from the bottom of the column. Allow the column to drain until it stops dripping.
2. Add the effluent sample to the top of the column bed using a transfer pipette.
3. Allow the sample to enter the column. When the column has stopped dripping, add a small volume (~5-10 drops) of Loading Buffer and allow that to enter the column. When the column stops dripping, repeat with one more small volume of Loading Buffer.
4. Add 5 ml of Loading Buffer to the column and let the column flow. Replenish the buffer volume as necessary, making sure that the column never stops flowing.
5. When the first colored band is about to elute from the column, switch to a new test tube to collect the sample. This will be Fraction 1.
6. After the first colored band has been eluted, switch back to the original beaker to collect the flow-through.
7. When the second colored band is about to elute from the column, switch to a new test tube to collect the sample. This will be Fraction 2.
8. Collect fractions until all of the colored bands have eluted from the column. Put the end cap back on the bottom of the column. Make sure that there is buffer on top of the column and replace the stopper in the top of the column.

Results:

While your column is running draw pictures of what you see.



What are the colors of the samples that you collected?

Does this mean that there is no Rufino toxin in the effluent from the Acme Widget factory?

If the Rufino toxin is present, in which sample collected by gel filtration is it likely to be?

Can gel filtration separate RF from all other proteins?

Will gel filtration separate RT from a protein that is 15 kDa?

What other characteristics of RF can we use to separate it from other proteins that are close to it in size?

Experiment 2: Separating Effluent Components by Charge

Materials (per student group):

ion exchange column
Loading Buffer
Elution Buffer
test tubes
small beaker
support stand and clamp
transfer pipettes
Fraction 2 (brown fraction) from gel filtration column.

Procedure

1. Place a beaker under the ion exchange column. Open the bottom of the column and let the buffer elute from the column until it stops dripping.
2. Place a new test tube under the column and gradually add Fraction 2 from Experiment 1. Let the sample run completely into the column.
3. Wash the column twice with 0.5 ml volumes of Loading Buffer, continuing to collect the effluent in the same tube or beaker. This will be Fraction A.
4. Switch to the original beaker and continue to wash the column until the effluent looks colorless and the column has color concentrated only at the top.
5. Place a new test tube under the column and gently add 10 drops of Elution Buffer. Once the colored band is moving through the column, continue to add elution buffer until all color is removed from the column. This will be Fraction B.

Results:

Describe your results. Did the brown fraction contain more than one component?

How do you know that the brown and the red components are not the same thing?

Since the red protein bound to the negatively-charged ion exchange resin at pH 6, what is the charge of the red protein at pH 6?

Do your results support the hypothesis that Acme Widgets is releasing Rufino toxin in their wastewater?

In what way?

So, there is evidence that Acme is releasing a chemical that has the same characteristic size, charge, and color as Rufino toxin. The fish kills, with the symptoms of RT poisoning are certainly evidence as well. At this point, you'll need to call in the experts for more tests to confirm that the red chemical that you have isolated is actually Rufino toxin.

What other experiments might the experts perform to prove or disprove the presence of RT in the effluent?

REPACKING AND SHIPPING THE KIT

COLUMNS

Gel filtration columns: Place the end cap on the column and twist to be certain it is secure. Fill the column with buffer. Place the stopper in the top of the column and, while holding the end cap in position, press down on the stopper until it's all the way in the column. Place columns back in the Styrofoam rack and place in Ziploc bag. **Important:** Please place the columns **upright** in the rack for return shipping; this protects the columns so that they can be re-used.

Ion exchange columns: Cap the bottom of the column, then place a couple of ml of Loading Buffer on the top of the column. Put the cap on and replace column in the box labeled "ION EXCHANGE COLUMNS."

Reverse-phase columns: Inject with 2 syringe volumes of water, then one volume of air. Place columns in small plastic box labeled "REVERSE-PHASE COLUMNS."

OTHER MATERIALS

Unused aliquots of sample solution and 10X NaCl. Please return any *unused* tubes of sample solution and 10X NaCl with the kit.

Bottles, transfer pipettes, and tubes: Please return any Nalgene bottles, as they are re-used. It is not necessary to return *used* microcentrifuge tubes, 15-ml tubes, or transfer pipettes. If they are unused, please return them. Please **do not** mix used and unused materials!

Beakers: Please return the plastic beakers. If they are still wet, please place them in a Ziploc or other plastic bag. Please remove any label tape from the beakers.

RETURN SHIPPING

Place everything back in the shipping trunk and mark items off the checklist in the repacked column. Please make a note of any problems or damaged items. **Note:** Please return the checklist with the kit!

Seal the trunk with the spare cable ties. Please be sure that the cable ties are fastened securely. Believe it or not, there is a right way and a wrong way to secure the trunks with the cable ties. We've had trunks come back totally unsecured, and this was probably due to the cable ties being put on incorrectly. Look at the end through which you put the tab. The tab should be put in from the side that is smooth with the tie, not the end that sticks out. If it is done the wrong way, the cable tie will open when you pull on it. Please test the cable tie by pulling to be sure that you've done it correctly.

Remove any old shipping labels, then follow the instructions for return shipping that came in the kit. Make sure that the shipping label is securely on the trunk.

Practical information for gel filtration chromatography.

- Never allow a gel filtration column to dry out. (The columns in the chromatography kit have filters on the top that should prevent this from happening.) If the column dries out, it must be re-poured. It is essential for good separation that the column be consistent from top to bottom.
- Good separation is also dependent on sample volume. The smaller the sample volume, the better the separation.
- Try to avoid diluting the sample when it is being loaded. Allow the buffer level to drop to the top of the column, then add the sample. The sample must be added gently to prevent disturbing the top of the column (this is not necessary with the kit columns, which have filters that protect the top of the resin bed). After the sample has run into the column, gently add a small amount of buffer. Repeat. After the sample has fully entered the column, additional buffer can be added to the top of the column.

If you would like to pour your own columns:

- See any of the references listed on the next page for details on choosing the correct gel filtration matrix and pouring columns.
- The columns in the chromatography kit are prepacked with gel filtration beads. However, matrix beads normally come in dry form and must be swollen before use. It is important not to use a magnetic stirrer when preparing the beads, as the beads can be fragmented by rough treatment. It takes several days to swell beads like the Sephadex in this kit. One shortcut, however, is to autoclave the solution, which causes the beads to swell more rapidly without damaging them.
- To prepare a column, pour or pipet a slurry of resin and buffer into a column. The resin is then packs **as the column is flowing**. It is important that the gel bed be consistent from top to bottom. This means that the column must be packed continuously until it is the desired height. If all the beads have packed and you then add additional slurry, there will be a discontinuity in the column.

Practical information for ion exchange chromatography.

- Ion exchange resin and columns are much more forgiving than gel filtration columns. The beads require very little time for swelling and have at least some tolerance for being allowed to dry. The bed does not have to be uniformly poured. Finally, unlike gel filtration chromatography, the sample volume is not important.
- When running an ion exchange column, it is best to run a small amount of elution (high salt) buffer over the column and then equilibrate the column thoroughly with loading buffer before loading your sample. This accomplishes two things: 1) it elutes sample that might remain on the column from a previous user; and 2) it confirms that the column is in the correct loading buffer. If there is **any** elution buffer left on the column, your sample will not bind the column as it should. (The remaining ions from the elution buffer will out-compete the protein samples for the binding sites on the beads.)
- When designing an experiment using ion exchange chromatography, there are no set rules for what types of ion exchange resin or buffers to use, or for what pH or ionic strength of buffer to use for either loading or eluting samples. The appropriate conditions must be determined experimentally.

REFERENCES

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Harris, E.L.V., and Angal, S. 1989 *Protein Purification Methods: A Practical Approach*. IRL Press, Oxford.

Lewis, Richard J. 1993 *Hawley's Condensed Chemical Dictionary*. Van Nostrand Reinhold Company, New York.

_____ 1991 *Ion Exchange Chromatography: Principles and Methods*. Pharmacia LKB Biotechnology, Uppsala

_____ 1991 *Gel Filtration: Theory and Practice*. Pharmacia LKB Biotechnology, Uppsala

SOURCES FOR MATERIALS USED IN EXPERIMENTS

CHEMICALS

All the chemicals used in this kit were purchased from Sigma, except for the Bio-Rad Bio-Gel P-30. The other chemicals should be available from most chemical supply sources.

Chemical	catalog number	Unit size	Cost
Blue dextran	D-5751	1 g	\$ 31.00
Myoglobin, horse heart	M-1882	250 mg	39.60
cytochrome c, bovine heart	C-3006	100 mg	73.70
DNP-glutamate	D-9129	100 mg	30.00
Imidazole	I-0125	100 g	22.20
CM-cellulose	C-0806	50 g	57.20
◆ Sephadex G-75	G-75120	10 g	68.30
Sodium chloride	S-9888	500 g	26.50
◆ Bio-Gel P-30 **	150-4150EDU	100 g	96.00

◆ Sephadex and Bio-Gel expand when hydrated. Each gram of resin will result in 12-15 ml of slurry.

** For cost reasons, the gel filtration columns are now prepared with Bio-Gel P-30 rather than Sephadex.

SUPPLIES

Columns. The 5" columns used for ion exchange are from Evergreen Scientific (800/421-6261). A package of 200 costs \$46.00 (catalog #208-3393-060).

The polypropylene columns used for gel filtration are from Pierce (800/874-3723; www.piercenet.com). A pack of 100 costs \$148.00 (catalog number 29924). They come with porous polyethylene disks for the top of the columns.

Reverse-phase (Sep-Pak C-18) columns. The supplier is Waters Corp. Phone: (800) 645-5476. A box of 50 columns costs \$157 (catalog # WAT052725). The columns are very hardy and can be used repeatedly.

Other chromatography kits can be purchased from Bio-Rad, Modern Biology, Flinn Scientific, Inc., Carolina Biological Supply Company, and Edvotek.

PREPARATION OF SOLUTIONS USED IN EXPERIMENTS

SAMPLE MIXTURE FOR EXPERIMENTS 1 AND 2

The sample mixture for the gel filtration and ion exchange chromatography includes blue dextran, myoglobin, cytochrome c, and DNP-glutamate. The final concentrations in the 300 μ l that are loaded on the Sephadex column are:

Blue dextran	2 mg/ml
myoglobin	4 mg/ml
cytochrome c	3 mg/ml
DNP-glutamate	0.4 mg/ml

Notes:

- Blue dextran and cytochrome c are dissolved in water. Myoglobin and DNP-glutamate are dissolved in 0.1 M sodium phosphate, pH 7.0.
- Blue dextran dissolves slowly. Shake *gently* to speed it up.
- If you want to make the sample solution on your own, please contact me for more specific details.

SAMPLE MIXTURE FOR EXPERIMENT 3

Buy soda containing red dye #40 and blue dye #1. Open and let go flat. (You can hasten this step by stirring.)

BUFFERS FOR EXPERIMENTS 1 AND 2

Loading Buffer (0.05 M Imidazole, pH 6.0)

For 1 liter, add 3.4 g of imidazole to ~700 ml of distilled water. Stir until dissolved, then adjust pH to 6.0 with hydrochloric acid. It takes *a lot* of HCl. I add 4 ml of 12.1 N HCl (concentrated), which brings the pH down to ~6.3, then I use 1 N HCl to bring it the rest of the way down to 6. Then add water until the total volume is 1 liter. (**Note:** If you want to make one liter of a 10X stock of loading buffer, as is being sent in the kit, add 34 g of imidazole to 700 ml water. After it is dissolved, adjust pH to 6 with hydrochloric acid. Add 35 ml of concentrated HCl to begin with, then smaller amounts. Bring to 1 liter with water.)

Elution Buffer (0.05 M Imidazole, pH 6.0, 0.5 M NaCl)

For 1 liter, prepare exactly as above through adjusting the pH to 6.0. Then add 29.2 g of sodium chloride. After the NaCl has dissolved, add water until the total volume is 1 liter. (**Note:** If you want to make 10X stock of sodium chloride buffer, as is being sent in the kit, add 146 g of sodium chloride to 400 ml water. After it is dissolved, bring to 500 ml with water.)