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How-To-Do-It

A Rapid, Simple & Inexpensive Experiment in Gel Filtration Chromatography

Roger A. Sauterer Jody Jones

We have developed a simple and inexpensive experiment using gel filtration chromatography that can be performed in less than 90 minutes. This procedure not only provides students with hands-on experience in both the preparation and use of chromatography columns, but also uses colored molecules to provide dramatic visual confirmation of the process of size-exclusion chromatography. Additionally, the column can be calibrated using molecules of known molecular weights and can then be used to estimate the molecular weights of unknown samples. Our gel filtration experiment costs only a fraction of that of commercially marketed laboratory exercises.

A basic understanding of methods and techniques in modern life science research is important in understanding the way that life scientists develop new methods for answering biological questions and the potentials and limitations of these modern experimental methods. On both the introductory college and high school levels, many students are performing basic exercises using electrophoresis and recombinant DNA. Unfortunately, few of these students are exposed to chromatography, the major means of purifying proteins and other biological molecules. This is partly because of the expense and complexity of many chromatographic procedures.

Our experiment involves a simplified version of the analytical methods used by research laboratories to fractionate proteins and estimate molecular weights by gel filtration. This exercise was modified from such procedures performed by one of the authors during his postdoctoral training. This experiment illustrates the concepts and mechanisms of gel filtration and gives a simplified understanding of both chromatography column preparation and typical data analysis. It is currently used as a lab exercise in the introductory Cell Biology course at Jacksonville State University.

Our experiment offers significant advantages over commercially marketed chromatography experiments. These kits often provide pre-packed columns while our protocol allows students to actually prepare the column. Additionally, many commercial gel filtration experiments require the use of an expensive, ultraviolet-capable spectrophotometer to monitor the elution profile, while our experiment requires only an inexpensive visible light spectrometer. Finally, our experiment costs less than a dollar per student (after an initial purchase of columns and gel filtration media) compared with the $10 per student cost through commercial competitors. Although initial purchases of needed supplies will cost slightly over a hundred dollars, reagent and material costs for repeated exercises are very low. The hardware may be rinsed and reused, and the Sephadex can be re-used as well.

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Simplified Theory of Gel Filtration

Gel filtration or size-exclusion chromatography is a method of separating macromolecules in their native state by size using non-denaturing conditions. Gel filtration is widely used as a later stage in protein purification and is commonly used to estimate molecular weights of native molecules (see Voet & Voet 1990, pp. 87–89, for examples). The gel filtration media is made of precisely shaped beads of modified polysaccharides or plastic resins. These beads have networks of internal pores approximating the size of biological macromolecules. Large molecules, larger than the pore size or ***exclusion limit*** of the beads (which depends on their composition and degree of crosslinking as set by the manufacturer), percolate around the spaces between the beads without entering them (Freifelder 1982; Reiland 1971). Since the majority of the buffer volume is contained within the beads, molecules unable to enter the beads are confined to the limited ***void volume*** of buffer surrounding the beads and will percolate out of the column first. Small molecules, by contrast, are smaller than the pores inside the beads and enter the beads. They diffuse inside the internal pore network inside the beads and are therefore retarded. Figure 1 illustrates the principles of size-exclusion chromatography.

The probability of a molecule diffusing into the beads increases with decreasing molecular size (Freifelder 1982), so the smaller molecules are retarded the most. Therefore a mixture of molecules separated on a gel filtration column will elute from the column in order of decreasing size. The longer a column is, the more pronounced the separation is, and research-grade gel filtration columns are usually 100 cm long.

Gel filtration differs from SDS-PAGE electrophoresis, which also separates molecules by size, because gel filtration is performed under mild, non-denaturing conditions. SDS-PAGE, by contrast, requires the use of the denaturing detergent SDS in order to sepa-

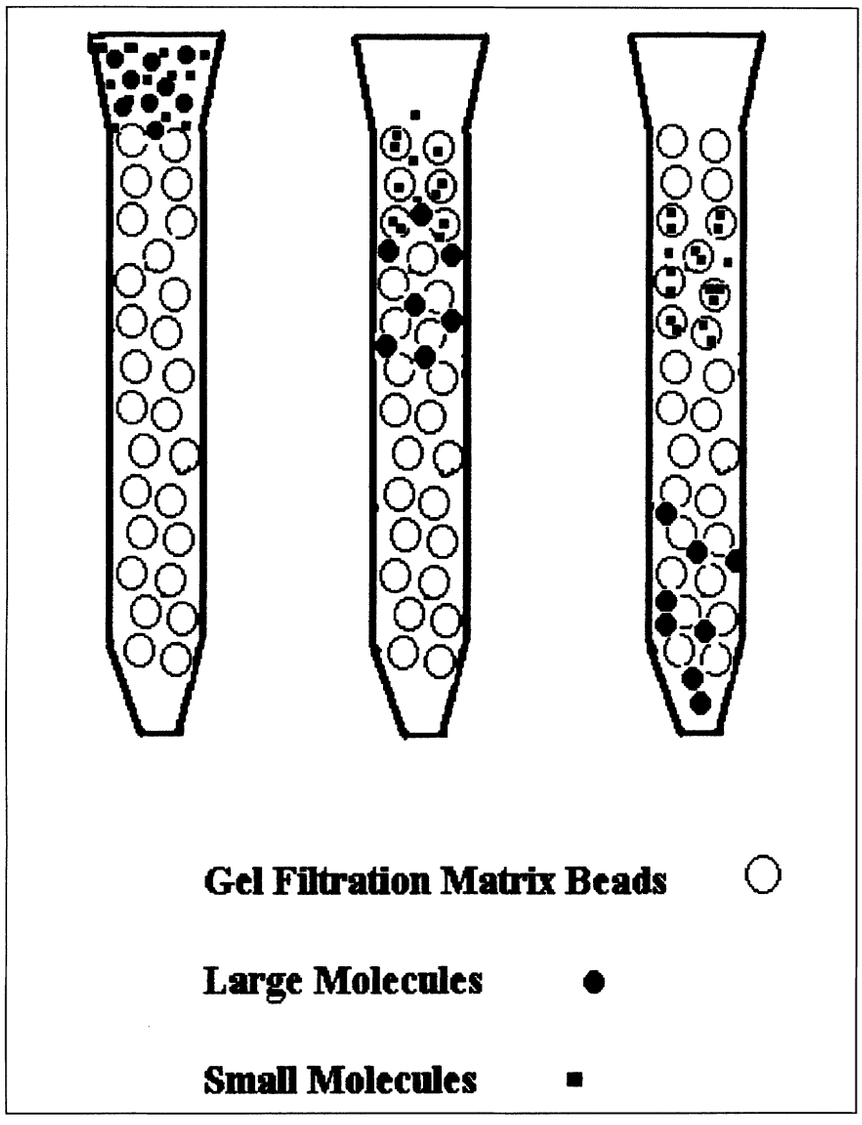

Figure 1. Principles of Gel Filtration
Diagram illustrating a gel filtration run in progress. Large molecules (filled circles) are confined to the void volume and elute first, while small molecules (filled squares) enter into the gel filtration beads (open circles) and are retarded.

rate molecules by size. Therefore, gel filtration is a widely used step to purify macromolecules in their native, functional state. Additionally, gel filtration does not dissociate multi-subunit functional proteins into their constituent amino acid chains as does SDS-PAGE. For example, SDS-PAGE electrophoresis of hemoglobin would show two closely spaced bands of 16,000 daltons, corresponding to the alpha- and beta-chains, while gel filtration would show a single, functional native molecule with a molecular weight of 64,000.

The laboratory exercise described below introduces, on a simplified level, the principles and applications of gel filtration chromatography in the estimation of molecular weights of macromolecules. It can be used as a simple demonstration for high school and perhaps even middle school courses, or be increased in sophistication for advanced high school and college-level courses.

Materials & Suppliers

1. Disposaflex Columns 0.8 X 20 cm with bed supports, on-off valves, and column extenders. Kontes, Inc. (1022 Spruce St., Vineland, NJ 08360). Columns + extenders, cat. #420160-0000, $41 per 100; 20 um bed supports, cat. #420162-0200, $12 per 100; valves, cat. #420163-0000, $57 for 50.

Note: The Dispo-Flex columns are highly recommended because of their ease of use, however, a comparable column can be made by cutting the top off of a 10 ml pipet, loosely packing about 0.5 cm of glass wool at the bottom of the tapered end, and attaching a length of tubing at the tapered end. A hemostat or clamp is then used to regulate buffer flow. Some difficulty in packing the column and some decrease in separation is expected when this method is used.

2. Sephadex G-100 filtration media, normal flow. Sigma (P.O. Box 14508, St. Louis, MO 63178) cat. #G-100-120, $117 for 50 g, enough for 150 experiments.
3. Catalase. Sigma cat. #C-9332, $11 per 1 g, enough for 150 experiments.
4. Cytochrome C. Sigma cat. #C3131, $59 per 100 mg, enough for 50 experiments.
5. Blue Dextran. Sigma cat. #D-5751, $22 per 1 g, enough for 250 experiments.
6. Gel Filtration (GF) buffer (2 liters): 0.2 M NaCl + 0.01 M Na_2HPO_4, pH 7.5. 0.05% sodium azide can be added to inhibit bacterial growth for long term storage but **azide is highly toxic.**
7. 1.0 ml pipets with 0.01 ml graduations and pipet bulbs or micropipettors.
8. Visible spectrometer (e.g. Bausch and Lomb Spectronic 20).
9. 13 X 100 mm test tubes and test tube racks, 60 tubes per student team.
10. Graduated disposable plastic transfer pipets or Pasteur pipets.
11. Ring stands and clamps.
12. Sodium Azide. Sigma cat. #S2002, $13 for 25 g.

Instructor Preparation

The Sephadex G-100 gel filtration medium is supplied as a dry powder that must be hydrated before use. For a class of 30 students working in pairs, make a 30% slurry by adding 10 g Sephadex to 600 ml GF buffer (Sephadex absorbs 20 times its dry weight in buffer once hydrated). If the hydrated Sephadex is to be re-used or stored for more than a week, add 0.05% (0.3g) sodium azide to inhibit bacterial growth **(Warning! Azide is highly toxic!).** Gently swirl the dry Sephadex into the buffer and let it sit a few days in the refrigerator before use.

Never stir with a stir bar or other mechanical aid as this will shatter the fragile Sephadex beads and generate "fines" that clog columns and decrease separation of molecules. Hydrated Sephadex is stable for over five years as long as it is stored refrigerated and is in the presence of azide to inhibit bacterial growth. If possible, put the Sephadex slurry under vacuum for 10 minutes prior to use. The removal of dissolved gasses will prevent bubble formation during the experiment.

Prepare the macromolecule solution (0.2—0.3 ml per student team) by dissolving 15 mg/ml Blue Dextran, 10 mg/ml cytochrome C, and 30 mg/ml catalase in GF buffer + 0.05% azide. Considerable mixing may be required to dissolve the macromolecules completely. Store in the refrigerator for up to 2 weeks or freeze until needed. *Azide is not necessary if the solution is frozen until use.*

Safety

If sodium azide is used in any of the solutions, warn students about its toxic nature. Ingestion of even a few milliliters of the buffers will cause slight but detectable effects, so warn students not to mouth-pipet any solutions and to wash off any spills. Additionally, students should wear gloves if azide is used. Note that *use of azide is not necessary if solutions and Sephadex are not intended to be stored long-term or re-used.*

Laboratory Procedure

Prior to performing the lab, the instructor should explain the principles and applications of gel filtration and contrast it to SDS-PAGE.

A. Assemble and pour column (10 to 15 minutes required).

(See Figure 2 for a view of the parts and the fully assembled column.)

1. Insert the porous plastic bed supports into the bottom piece, and then attach the valve.
2. Place this assembly on the column and attach the column extender.
3. Gently swirl the Sephadex slurry to resuspend it and pour the slurry into the column extender until the column and extender are nearly full. ***Caution students never to stir the Sephadex slurry with a pipet or any mechanical aid to avoid generation of "fines" that will clog the column and decrease separation.***

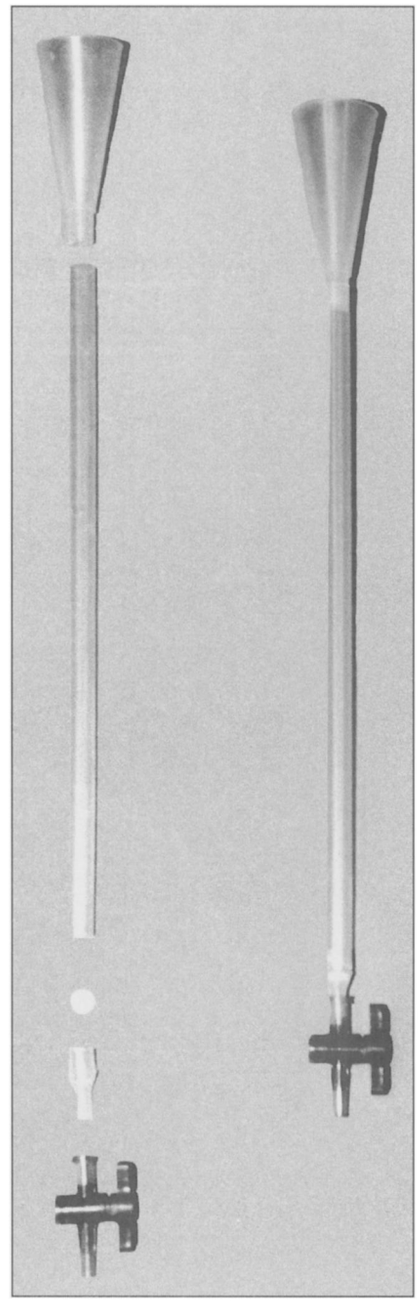

Figure 2. Gel filtration column components and fully assembled column. An unassembled view of the column components in position for assembly is on the left (top to bottom: column extender, column, porous bed support, outflow piece, and on-off valve). The fully assembled column is on the right. (Digitally enhanced image)

4. Open the valve to allow the buffer to drain.
5. Observe the clear region of Sephadex-free buffer that forms at the top of the column. This occurs because the Sephadex settles faster than the buffer drains. This clear region will increase to approximately 1 cm in height.
6. Below the clear region, you will note the translucent Sephadex layer. This layer should extend most of the height of the column once it is nearly packed. Either remove or add more of the Sephadex slurry until the bed is the appropriate level. To make this adjustment, use a plastic pipet to gently stir the Sephadex bed before adding or removing slurry. ***This must be done prior to the bed becoming fully settled or the column will have discontinuities in it that will decrease separation of molecules.***
7. The column is fully packed when the Sephadex-free clear zone just reaches the Sephadex layer. At the point the column is packed, ***immediately close the valve.*** Failure to quickly shut the valve will result in buffer draining from the packed column and cause large cracks to develop in the bed. A cracked column bed must be repoured.
8. It is useful for the instructor to let the demonstration column run dry to show how quickly a column bed will crack under such conditions.

B. Loading the column (1 to 3 minutes required).

1. Gently add about 0.2 ml of the macromolecule solution to the top of the column and re-open the valve, allowing the solution to enter the bed.
2. Close the valve once the macromolecule solution enters the bed, but prior to the column running dry. This prevents dilution of the sample by added GF buffer.
3. Gently add GF buffer until the column extender is full. Take care not to disturb the bed.
4. Once the buffer is added, open the valve and start counting the number of drops. Caution students to add more buffer if the column is about to run dry. The buffer should be allowed to run into a waste container until the colored solution nears the bottom of the column.
5. Observe the separation of molecules that starts almost immediately. You should note a blue fringe of Blue Dextran appearing at the bottom of the sample and a red fringe of cytochrome C appearing at the top. The fringes develop into distinctly colored

bands as the separation proceeds. The catalase, which is located slightly below the cytochrome C, is a brownish color and is not visible as a distinct band. The bands will be broad but should be even. Tilted bands or "channeling" (bands with extensions above or below the main part of the band) indicate a poorly settled and uneven Sephadex bed that will decrease the separation and resolution of the column. Figure 3 shows a typical separation in progress, and Table 1 indicates typical elution values.

C. Collecting the purified molecules (20 to 30 minutes required).

1. Allow the buffer to drain out into a waste container, counting and recording the number of drops until the first colored band (Blue Dextran) reaches the bottom of the column.
2. Once the first traces of Blue Dextran start to percolate off the column (about 40 to 55 drops), collect 5 drop fractions into successive test tubes.
3. Continue collecting 5 drop fractions until the cytochrome C band is completely off the column (about 130 to 140 drops).
4. Pour the Sephadex into buffer for re-use. Disassemble and rinse the columns.
5. If time is limited, samples may be covered and stored overnight before the next portion of the experiment is conducted.

D. Data Collection (10 to 15 minutes required).

1. Transfer 0.1 ml of each 5 drop fraction into fresh test tubes, then add 3 ml of GF buffer to each tube. Using a micropipettor to perform the 0.1 ml aliquotting enhances the accuracy of the data.
2. If a visible range spectrophotometer is available, measure and record the absorbance of each tube at both 400 nm and 600 nm. Cytochrome C and catalase absorb poorly at 600 nm. For those lacking a spectrophotometer, placing the tubes in front of white paper, "eyeballing" the intensity, and scoring each tube from 1 to 4 will allow generation of a crude absorbance curve.
3. Construct a graph of Absorbance vs. Number of Drops for the data collected at 400 nm and 600 nm. Students should plot the cumulative number of drops on the X-axis and the absorbance at 400 nm and 600 nm on the Y-axis. Two separate curves will be generated.

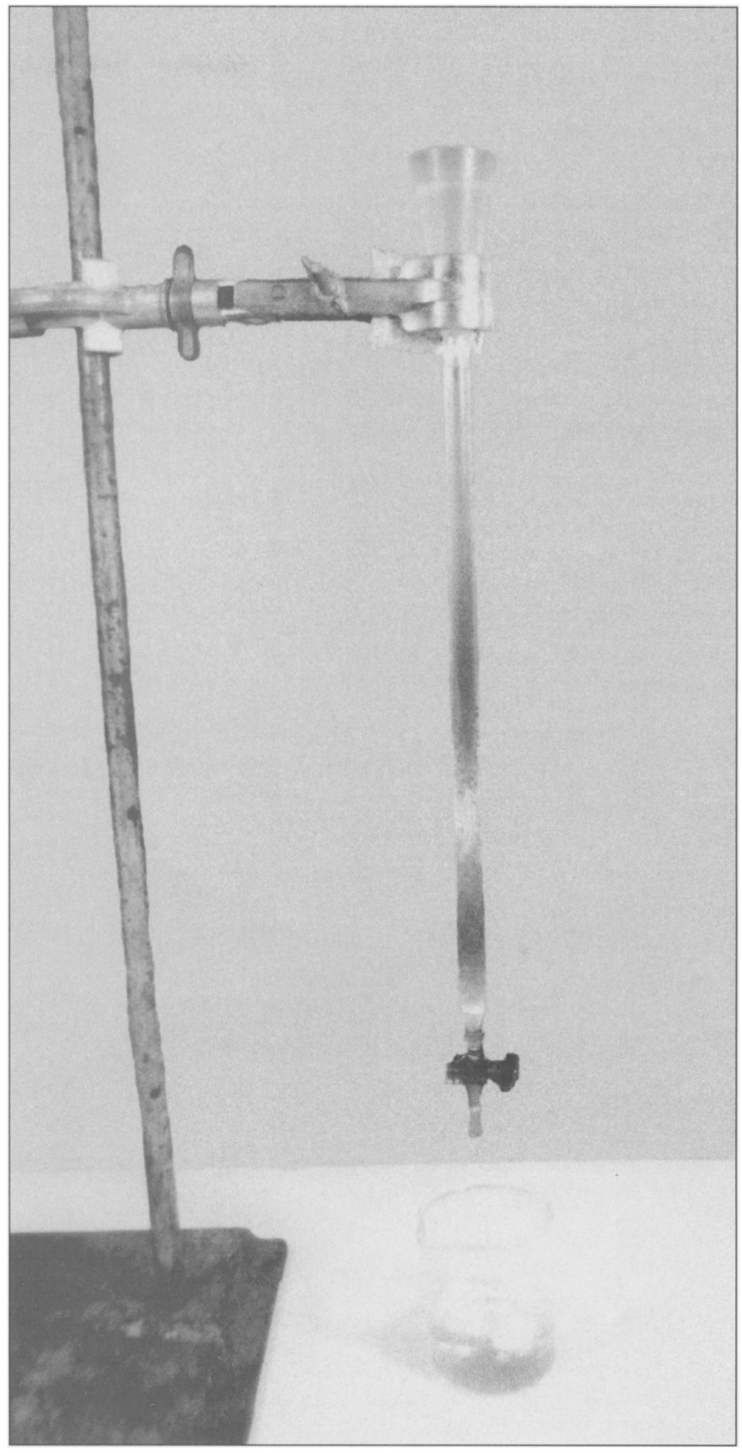

Figure 3. Gel filtration run in progress.
The column is attached to a clamp on a ring stand to keep it vertical. Visible are bands of Blue Dextran (bottom) and catalase/cytochrome C (top). Catalase is difficult to distinguish from the cytochrome C visually. (Digitally enhanced image)

E. Analysis of Data

1. The students should see evidence of three overlapping "humps" of absorbance on their curves, especially if the data are "smoothed" showing the separation of the

Table 1. Typical elution values for gel filtration column.

Molecule	Molecular Wt.	Color	Elution Volume (drops)
Blue Dextran	3×10^6	Blue	65–80
Catalase	250,000	Brown	90–100
Cytochrome C	12,500	Red	110–120

three macromolecules in the sample solution. Figure 4 shows data from a typical experiment. Three distinct peaks of absorbance corresponding to Blue Dextran, catalase, and cytochrome C are shown respectively. Note that catalase and cytochrome C cannot be distinguished at 600 nm, while all three molecules are detected at 400 nm. Typical student data will probably show more scatter.

2. Students should determine from their absorbance curves the cumulative number of drops to the center of each peak of absorbance. Using semilog paper, plot the molecular weight of the molecules (12,600, 250,000 and 3×10^6) on the Y-axis (logarithmic axis) and the number of drops to peak center on the X-axis. By fitting a straight line through the data points, this plot can be used as a calibration curve of molecular weight vs. elution volume for that column. The results, shown in Figure 5, show that the elution volumes of each molecule, when plotted on a log scale of molecular weight, fall nearly on a straight line. It should be noted that although the Blue Dextran at a molecular weight of 3×10^6 exceeds the exclusion limit (the largest molecule that can enter into the beads) of the gel, it clearly separates from the other two molecules, and because of the low resolution of these short columns, it still plots close to a straight-line curve of log (molecular weight) vs. elution volume.

F. Expanded Experiment: Estimation of the molecular weight of an unknown protein.

1. After the cytochrome C from the previous experiment has completely eluted off, students should drain the residual buffer from the column until the remaining buffer just touches the surface of the column bed. Load 0.2 ml of a 10 mg/ml solution of hemoglobin in GF buffer on to the column and run the column as before. To save

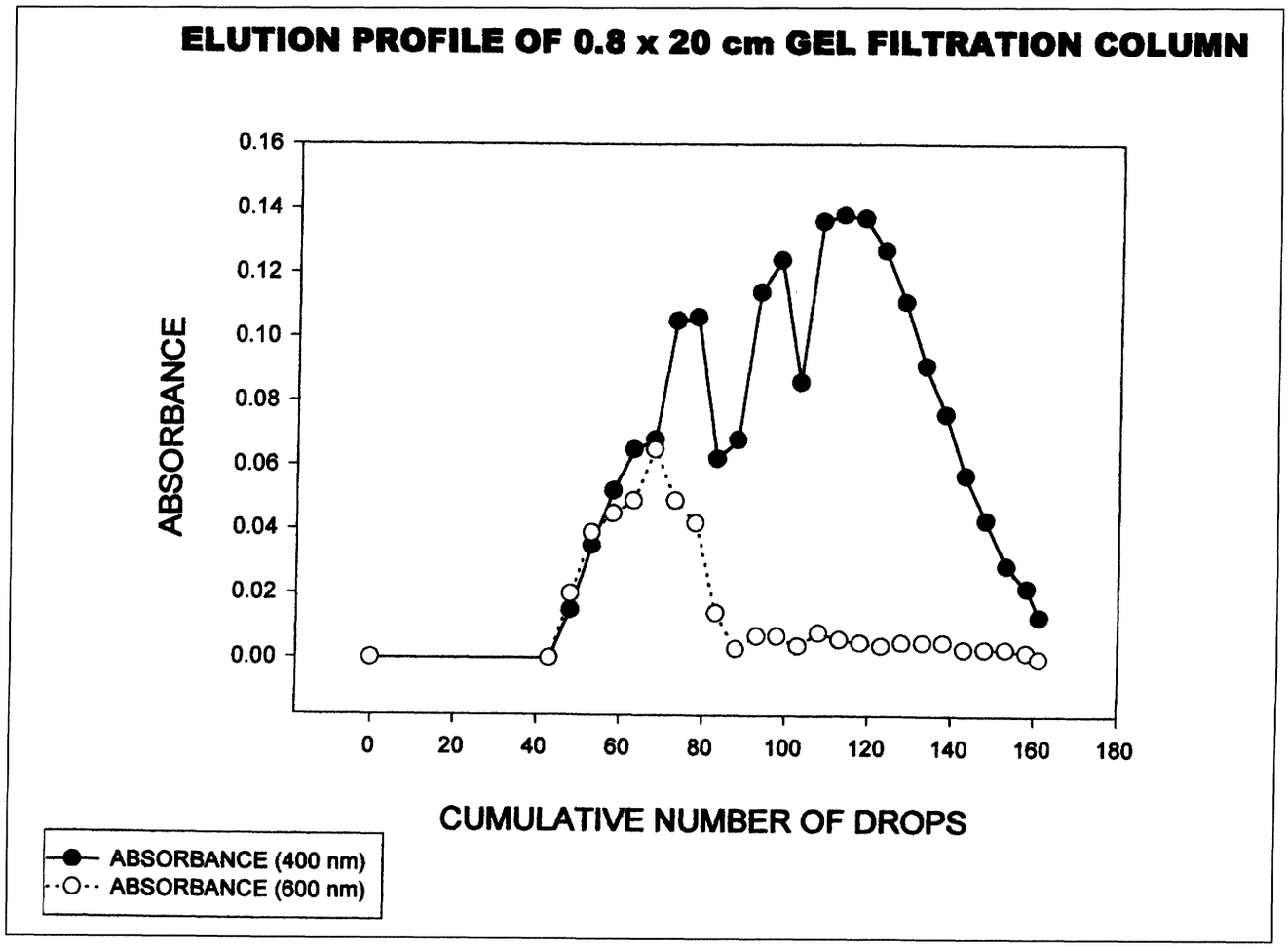

Figure 4. Elution profile of gel filtration column.
Shown are the elution profiles at 400 (filled circles) and 600 nm (open circles) from a typical run as previously described.

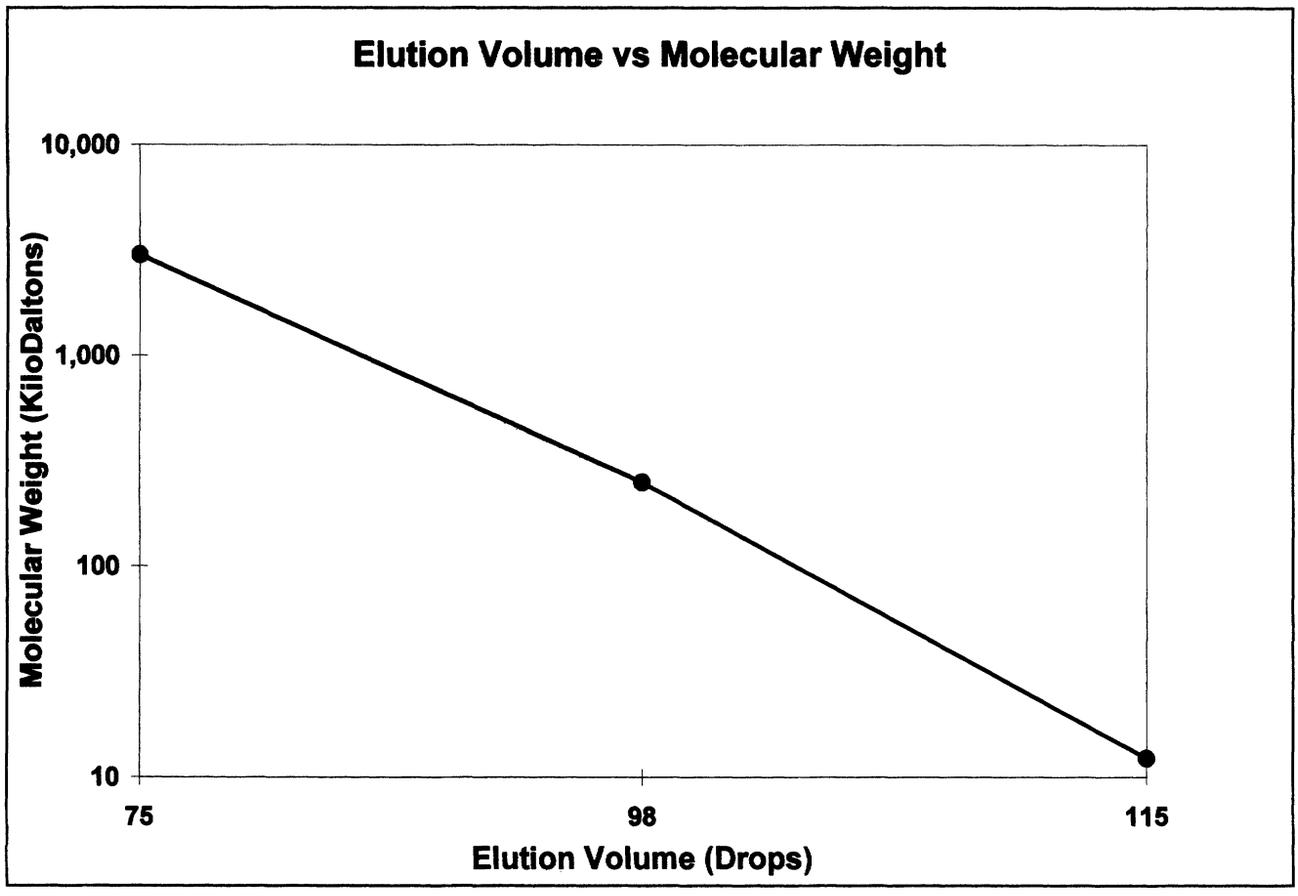

Figure 5. Plot of molecular weight vs. elution volume of each molecule.
Elution volume of the center of each peak in drops is plotted against molecular weight on a logarithmic scale.

time, one student in a team can perform the run of the hemoglobin while the other student(s) are analyzing and plotting the elution profile and plotting the molecular weight vs. elution volume curve from the previous experiment.

2. The remaining loading, running, and sample collection and analysis are performed just as previously described in Parts B, C and D.
3. Students should then plot the elution volume of the center of the hemoglobin peak on the molecular weight vs. elution volume curve from Part E to estimate the molecular weight of hemoglobin. With the low resolution of the column, students will estimate the molecular weight of hemoglobin as roughly 50,000 to 100,000. If students are given the molecular weights of the globin subunits (approximately 16,000) or have previously calculated it from SDS-PAGE gels, they should clearly see that native hemoglobin consists of multiple subunits and some students may be able to see that four subunits is the likeliest composition of native hemoglobin.

Conclusions

Our gel filtration experiment is both more versatile and considerably less expensive than commercially marketed student experiments in gel filtration. It can be modified to suit a wide range of students, from high school labs to undergraduate labs in cell biology.

The experiment described is intended for a two- to three-hour college laboratory in general biology or introductory cell biology, though it can be used for advanced placement courses in high schools as well. For high school labs, especially those without equipment such as spectrometers, this lab can be performed either as a demonstration or student teams can run their own columns to gain the experience of pouring and loading columns and watching the separation.

By "eyeballing" the collected fractions and scoring the color on a 1 to 4 scale, students can develop a crude elution profile. However, the cytochrome C and catalase peaks overlap and are of similar color, so they are difficult to impossible to distinguish visually. These two peaks are distinguishable by spectrometry at 400 nm.

Aside from the initial costs of the columns and the Sephadex, which can support hundreds of students, cost per student is less than a dollar each. The macromolecules chosen for separation in this experiment were selected for minimal cost as well as for separation and visibility characteristics. This again makes our experiment well-suited for a wide range of instructional settings and budgetary conditions.

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