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Laboratory Experiment

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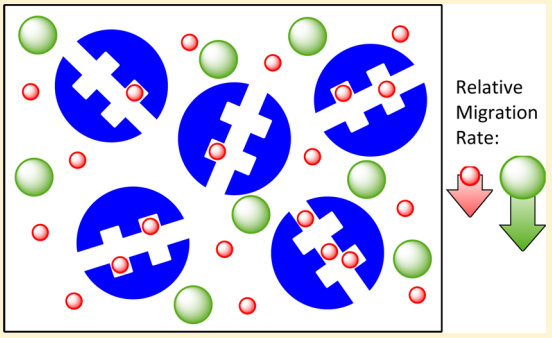
A Size Exclusion Chromatography Laboratory with Unknowns for Introductory Students

Edward J. McIntee,* Kate J. Graham, Edward C. Colosky, and Henry V. Jakubowski

Department of Chemistry, College of Saint Benedict and Saint John's University, Saint Joseph, Minnesota 56374, United States

Supporting Information

ABSTRACT: Size exclusion chromatography is an important technique in the separation of biological and polymeric samples by molecular weight. While a number of laboratory experiments have been published that use this technique for the purification of large molecules, this is the first report of an experiment that focuses on purifying an unknown small molecule from a protein. Students must then determine the structure of the unknown using spectral determination.



KEYWORDS: *First-Year Undergraduate/General, Second-Year Undergraduate, Laboratory Instruction, Problem Solving/Decision Making, Chromatography*

INTRODUCTION

Size exclusion chromatography separates compounds on the basis of molecular size as illustrated in the graphical abstract. Size exclusion (or gel permeation) is used in a variety of chemical applications including polymer synthesis, natural product isolation, and purification of biomolecules. This technique has become a mainstay in the field of chromatography for the separation of analytes.[1−6] Many experiments have been reported that demonstrate exciting applications of this technique.[7−10] Due to the importance of this method, many more biochemistry and polymer laboratory experiments include experiments that rely on at least one purification step involving size exclusion chromatography.[11−17]

Many experiments have been developed to specifically train students in size exclusion chromatography,[18−21] yet few of these reported experiments incorporate the use of unknowns.[22] The use of unknowns in technique labs can increase individual work, reinforce individual spectral analysis, and provide a more challenging problem-solving aspect to the experiment.[23−26] There have been a few recent publications on laboratory experiments that employ unknowns for normal phase chromatography and unknowns for HPLC reverse phase, but there is not a similar experiment for gel permeation chromatography especially for lower division laboratory experiments.[27−32] A first-year laboratory experiment that introduces an unknown laboratory experiment is reported here. Students must separate an unknown small peptide or amino acid from a larger protein and then identify the unknown compound through various spectral techniques.

EXPERIMENTAL OVERVIEW

A detailed student handout is provided in the Supporting Information. Students are given a frozen sample in 0.5 mL of ammonium acetate (10 mM) buffer that contains a mixture of unknown small peptide/amino acid (approximately 30 mg/mL, MW ranges from 180 to 264 g/mol), bovine serum albumin (BSA) (2.0 mg/mL, MW 66.5 kDa), and Blue Dextran (0.266 mg/mL, MW ∼ 2000 kDa). Students use a 4−5 mL Bio-Gel P-4 gel column (Bio-Rad) to separate their mixture. Students wash the column with 8 mL of buffer and then elute the mixture to collect 30 (0.3 mL) fractions in a 96-well cell plate.

Students determine which fractions to combine by looking at absorbance readings at 280 (containing Trp) and 260 nm (containing Phe or Tyr). Typically, the protein elutes in fractions 5−10, with an absorbance reading of around 0.4 au. As the Blue Dextran coelutes with the protein, students can visually see when the protein is eluting. Typically, the peptides elute much later in fractions 23−29, with an absorbance reading of around 0.2 au. The solvent is removed from the combined peptide fractions (∼1−2 mL) under reduced pressure. The resulting sample is then used to make NMR and GC/MS samples.

For optimal fraction collection, the liquid level should always remain constant. Students can mark a point on the column above the gel and keep the buffer solution approximately at that height for the entire procedure. The flow rate should be about 1 drop every 10 s.

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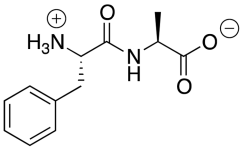
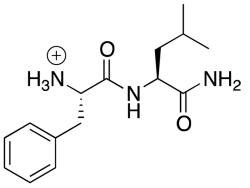
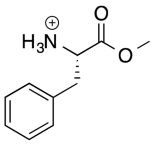
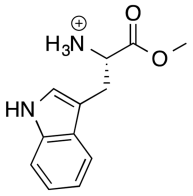
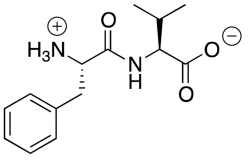
Table 1. Unknowns Used in Size Exclusion Experiment

Structure	Name	Formula Weight (g/mol)	GC/MS Retention Time (min)
	Phe-Ala	236	16.3
	Phe-Leu Amide	277	19.3
	Phe methyl ester	180	9.2
	Trp methyl ester	219	15.3
	Phe-Val	264	17.9

Unknown Considerations

Several criteria were employed in the selection of small molecules to be used as unknowns in this experiment. Specifically, student unknowns needed to contain an amino acid residue that was easily detectable via UV (Trp, Phe, Tyr) and that were relatively low cost. In addition, samples needed to be relatively volatile and contain a variety of different retention times in order to be analyzed by GC−MS. A list of unknowns that did not work has been included in the Supporting Information.

A list of unknowns used in this experiment is provided (Table 1).

Column Considerations

The Bio-Rad Bio-Gel P-4 gel (fine particle size, 45−90 μm) was used for this lab because of the polyacrylamide beads' 800−4000 MW fractionation range. The gels are extremely hydrophilic and essentially free of charge. Polyacrylamide gel was chosen rather than carbohydrate-based gels in order to minimize nonspecific polar interactions. For this experiment, the P-4 beads are packed into 10 mL Bio-Rad columns with 10 mM ammonium acetate as the eluent. When not in use, columns were stored in a cold room or refrigerator to retard microbial growth. The columns are robust allowing for up to 10 samples before needing to be repacked.

Instrumentation

A Molecular Devices SpectraMax M2 plate reader was utilized in the analysis of fractions. Peptide fractions with similar UV absorbance and retention time were combined and analyzed by Varian Saturn 2000 GC/MS. An Agilent Technologies DB-5MS column (30 m × 0.250 mm, 0.5 u) was utilized under constant flow (1 mL/min) and a temperature gradient (130 °C hold for 5 min, followed by a ramp at 15 °C/min to 250 °C with a 10 min hold at that temperature; total run time was 23 min). A solvent delay (4.5 min) was employed for the mass spectrometer.

■ HAZARDS

Many of the small molecules and buffers are irritants. The methanol and deuterated methanol are flammable and toxic. Eye protection should be worn at all times while in the laboratory, and care must be taken when handling laboratory glassware and chemicals. All chemicals should be disposed of according to your departmental and state policy as outlined in your chemical hygiene plan.

■ RESULTS AND DISCUSSION

This experiment has been used successfully for several semesters in an introductory first-year laboratory course. This laboratory course serves approximately 300+ students per year in sections of 16 students each. The goal of the course is to

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introduce students to a variety of chromatographic techniques commonly used for the purification of different inorganic, organic, and biochemical samples. Students were exposed to the theory of different chromatographic separations in a recitation period prior to these experiments. For each experiment, each student is given an unknown to promote independent problem-solving skills. In addition, students utilize a variety of spectral techniques for analysis of samples throughout the semester.

The learning goals for this size exclusion gel chromatograph experiment are to develop an understanding of the theory behind size exclusion chromatography and to reinforce the spectral analysis of molecular compounds. The experiment emphasizes chromatographic technique as well as the analysis of UV, ^{1}H NMR, and mass spectral data. Thus, this experiment is both a technique and puzzle-solving experiment that is an alternative to cookbook-style laboratory experiments. This experiment could easily be adapted to an organic chemistry course or an introductory biochemistry course.

The size exclusion chromatography is easily completed within a 4 h laboratory period. The students can complete the chromatography and the UV analysis of fractions during this time so that samples can be submitted for GC/MS and ^{1}H NMR analysis at the end of the period. The use of autosamplers on both the GC/MS and ^{1}H NMR instruments allows the students to obtain data electronically quickly and with minimal TA involvement. Schools without autosamplers may need to balance the amount and type of spectral data required.

Samples contain an unknown small peptide and BSA, a protein impurity. Originally, students would often have trouble deciding which fractions contained their protein and which contained their peptide. Blue Dextran is a colored marker of high molecular weight that signals the exclusion volume from column and would, thus, coelute with proteins whose molecular weights exceed the fractionation range of the resin used. Use of the Blue Dextran dramatically improved students' ability to correctly combine like fractions. Students are able to use visual cues to determine when the protein has eluted and know that the smaller peptide component is eluting later. Although this approach may not showcase the subtle separation power of size exclusion chromatography, it does serve as a simple illustration of the underlying concept of elution based on size.

After completing the separation, students use UV absorption of the fractions to verify the presence of protein and unknown in each fraction. If a plate-reader is not available, the experiment can be easily modified to use a channeled UV cell. Students typically report recoveries of ∼75% for each unknown peptide using the directions in the student handout (Supporting Information).

Once students have combined the fractions, they must analyze the ^{1}H NMR and mass spectral data to determine the identity of their small molecule. For confirmation of the unknown identities, students compare their data to previously published data in the NIST mass spectral library database and to ^{1}H NMR data of standards provided (Supporting Information). This is a more complicated NMR analysis due to diastereotopic protons on the unknown resulting in doublets of doublets. In our curriculum, students complete this experiment in the second semester of a laboratory sequence that relies heavily on NMR interpretation so our students have been able to handle the interpretation of these peaks. However, a reader who is adapting this for a more introductory type experiment might choose to have the students select the unknown's identity by simply matching the NMR spectra to the reference spectra and identifying a few key peaks based on chemical shift only.

To assess how successful students were in developing an ability to use size exclusion chromatography to separate an unknown and subsequently determine the identity of their separated compounds, the faculty instructors gathered success rates. Presently, 70% of the students identify their unknown components correctly. Most of the errors associated with this lab are due to poor data being collected. Approximately 25% of the students collected data that instructors deemed as poor quality or was not sufficient to determine the structure of the unknown. One common reason for poor data was an overwhelming water peak in the ^{1}H NMR that made student interpretation of the data difficult. Students should be cautioned to evaporate their samples to dryness.

Students must complete a report that includes spectral analysis and conclusions about purity and percent recovery (Supporting Information). In the most recent semester, several faculty compared student performance on the report. The average grade for the experiment was 34/42 points (81%), suggesting that most students were able to interpret and communicate their results effectively.

In the spring of 2015, student understanding of size exclusion technique was also probed with a question on the laboratory final exam. This question required students to predict an order of elution of molecules (laboratory exam questions are available upon request). Eighty-three percent of the students (n = 179) were able to correctly answer a multiple-choice question on the laboratory final indicating that a large majority of the students retained an understanding of the basic concept of this experiment.

CONCLUSION

A discovery based purification experiment using size exclusion column chromatography with a range of potential unknowns (Table 1) for an introductory lab course has been developed. Students successfully purify a small organic compound from a mixture containing an unknown and protein. Students use a variety of spectral data to correctly identify their unknown. While this experiment has been utilized in an introductory laboratory sequence, this laboratory experiment could readily be implemented into a sophomore organic chemistry laboratory or an introductory biochemistry course. This experiment introduces students to key introductory concepts in size exclusion chromatography while reinforcing spectral analysis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.5b00396.

Instructor preparatory notes, student handouts, and a grading rubric (PDF, DOCX)

AUTHOR INFORMATION

Corresponding Author

*E-mail: emcintee@csbsju.edu.

Notes

The authors declare no competing financial interest.

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