JOURNAL OF CHEMICAL EDUCATION
Cite This: J. Chem. Educ. 2018, 95, 1198−1204
Laboratory Experiment
pubs.acs.org/jchemeduc

Protein Colorimetry Experiments That Incorporate Intentional Discrepancies and Historical Narratives

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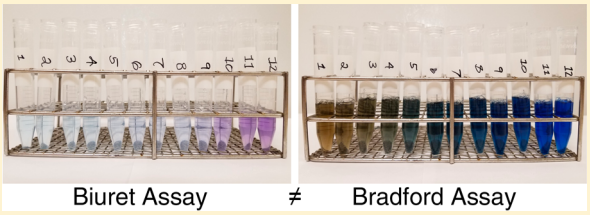
Supporting Information

ABSTRACT: A set of biochemistry experiments based on the technique of protein colorimetry is described. Students used the Biuret and Bradford assays to qualitatively and quantitatively assess solutions for protein. Two unique features of these experiments are (1) the use of intentional discrepancies within each experiment to promote critical thinking and (2) the use of authentic historical narratives to introduce critical concepts in the interpretation of assays and demonstrate the relevance of laboratory work to "real-world" scenarios. Details on the implementation and the evaluation of these experiments are discussed.



KEYWORDS: *Upper-Division Undergraduate, Continuing Education, Biochemistry, Laboratory Instruction, Inquiry-Based/Discovery Learning, Misconceptions/Discrepant Events, Constructivism, Proteins/Peptides, Spectroscopy, UV−Vis Spectroscopy*

INTRODUCTION

Laboratory activities are widely accepted as an integral component of undergraduate biochemistry education.[1,2] Ideally, laboratory experiments should offer students the opportunity to strengthen their understanding of the scientific method, develop critical inquiry skills, and address authentic scientific problems.[3,4] However, many traditional biochemistry experiments focus on the acquisition of manual laboratory skills and learning of specific techniques. The emphasis on techniques and obtaining the "correct" experimental results, as opposed to understanding the broader relevance of the results, and how they are obtained, may lead to frustration and attenuated interest, particularly on the part of the significant number of students who enroll in biochemistry courses to fulfill graduate health school prerequisites, as opposed to an authentic interest in biochemistry and research methods.[5,6] A technique-centric approach may sacrifice the opportunity for students to grasp the significant principles that underlie the lab experiment, and how these concepts extend beyond biochemical research into areas of public health and public policy.[7]

A suite of biochemistry experiments based on the colorimetric detection and quantification of proteins was developed to address the need for lab experiences that require critical thinking and promote understanding the "real-world" relevance of the biochemistry lab class. The new laboratory module consists of three experiments: (1) qualitative detection of unknowns (to detect whether protein is present in a solution), (2) quantitative analysis of an unknown (to measure the concentration of protein in solution), and (3) comparison of the selectivity of two different protein colorimetric reagents. Both qualitative and quantitative protein colorimetric assays are relatively straightforward to perform, making it possible for most students to obtain high-quality data. Two distinguishing features of these new experiments are (1) the use of intentional discrepancies to provoke intellectual arousal and engage critical thinking, and (2) the incorporation of authentic, historical events into the laboratory module to increase student interest and awareness of the "real-world" relevance of laboratory experimentation. The overall objectives are for students to understand the basic principles of assay design and interpretation, and to appreciate the relevance of this information to their future professional and societal responsibilities.

BACKGROUND

Biuret and Bradford Assays

The addition of a colorimetric reagent to a protein-containing solution results in a color change in the solution.[8] Two commonly used protein colorimetric tests are the Biuret assay and the Bradford assay. The Biuret reagent turns from light blue to purple in the presence of protein due to the chelation of copper ions, present in the Biuret solution, by the polypeptide backbone.[9] The Bradford reagent turns from an orange-brown color to blue when mixed with protein as a result of the aromatic dye Coomassie Brilliant Blue G-250 (CBB) binding to aromatic and positively charged amino acid residues in a protein.[10] Both assays can be performed either qualitatively (to

Received: August 17, 2017
Revised: May 9, 2018
Published: June 4, 2018

DOI: 10.1021/acs.jchemed.7b00633
J. Chem. Educ. 2018, 95, 1198−1204

determine if protein is present in an unknown solution) or quantitatively (to determine the concentration of protein in the unknown solution). Due to overlap of the absorption spectra of the protein-free and protein-bound CBB dye, and depletion of free CBB due to binding with protein, the Bradford assay has a lower upper-range limit than the Biuret assay, resulting in a hyperbolic standard curve.[10−13] The Bradford assay is also significantly more sensitive than the Biuret assay. False positive or false negative results of both Biuret and Bradford assays occur in the presence of (distinct) interfering substances. For example, a false positive Biuret, but not Bradford, assay is obtained in the presence of tris(hydroxymethyl)aminomethane (Tris base), commonly used to prepare biological buffers.[14,15]

Intentional Discrepancies

Exposure to intentionally confusing or discrepant information during the learning process is positively correlated with favorable learning outcomes and a deeper understanding of the relevant concepts.[16,17] Impasses (such as those that occur when encountering ambiguous or conflicting data) engage critical analysis and thinking skills necessary to explain or account for the discrepancy. Intentional confusion may also promote learning by confronting students with the actual limitations of their knowledge. In one study, students' mastery of physics concepts was significantly better when encountering an impasse than those of students who did not experience *intentional* confusion during the learning process.[18] The potential for using discrepancies to engage higher-order thinking in the chemistry laboratory has also been established.[19,20]

In each stage of the laboratory module, students encounter a discrepancy between the anticipated results and their actual data, or between the results of the two different assays. They must then use information from the biochemistry lecture and the laboratory handout to explain the source of the discrepancy. The three intentional discrepancies follow.

(1) During the qualitative analysis of unknowns, one assay indicates the presence of protein while the other indicates that protein is absent. This is due to the presence of an interfering substance in the Biuret assay (Tris base), giving rise to a false positive.
(2) During the quantitative analysis of an unknown, the results of Biuret and Bradford assays differ. This is due to actual concentration of protein exceeding the upper-range limit of the Bradford assay, such that the protein concentration obtained is lower than that obtained using the Biuret assay.
(3) During the quantitative analysis of three proteins, all three proteins respond equally well to the Biuret assay but show a differential response to the Bradford assay. This is a result of the Biuret assay interacting with the polypeptide backbone while the Bradford assay interacts with select types of amino acids that vary in number between proteins. As a result, the standard curves obtained for three different proteins nearly superimpose when obtained with the Biuret assay, but vary significantly when obtained using the Bradford assay.

Historical Events

The perceived lack of personal or professional relevance is one limitation on student learning in science classes.[21] Passive, disinterested students are unlikely to engage in deep analysis of the subject material. It has been demonstrated that persistence and success in academic tasks that are perceived as tedious or uninteresting are enhanced when these tasks are *explicitly* coupled to a "purpose for learning" which incorporates both intrinsic (self-beneficial) and extrinsic (self-transcendent, or societally important) objectives.[22] Illustrating important concepts through the use of authentic real-world scenarios is an established approach to enhance the perceived relevance of laboratory coursework to students' lives, and their professional and social aspirations.[7,21]

The following historical or current events were used to illustrate the consequences of failing to appreciate potential limitations in the design or interpretation of assays or analyses. In each scenario, a discrepancy between the result of an assay or test and the actual result had significant, life-altering consequences. The topic of each narrative was intentionally chosen to illustrate the broad societal relevance of the key concepts encountered in the laboratory.

(1) The Chinese Milk Scandal was used to introduce the concepts of false positives and interfering substances.[23] In an attempt to boost profits, Chinese dairy plant owners diluted their milk and other dairy products with water. As a result, the concentration of milk proteins fell below accepted standards, as determined by the Kjedhal protein assay. To conceal their deceit, the diluted milk was intentionally adulterated with melamine, a toxic chemical with no nutritional value that produces a false positive in the Kjedhal assay.
(2) The Apollo 13 accident was used to introduce the concept of the upper-range limit.[24] The accident involved the explosion of a spacecraft oxygen tank en route to the moon, nearly leading to the death of the three onboard astronauts. The explosion was the result of damaged wiring within the oxygen tank, a consequence of abnormally high temperatures melting the wire insulation within the tank during a prelaunch test. A technician monitoring the test failed to notice the unsafe temperatures because the thermocouple installed in the tank had an upper-range limit that was significantly lower than the actual temperature within the tank.
(3) The case of Amy Albritton was used to introduce the concepts of false positives and selectivity.[25] A police officer, who stopped Albritton for a routine traffic violation, noticed a suspicious substance in her car. Using the roadside colorimetric Scott test, the substance was determined to be cocaine. Albritton was arrested and jailed, losing her home and job. Subsequent laboratory testing determined that the substance was likely a food crumb. The failure to appreciate the broad spectrum of chemicals that cause false positives in the Scott test cost Albritton her job, her home, and, for some time, her freedom.
(4) Drug masking agents were used to introduce the concepts of interfering substances, false negatives, and sensitivity (lower-range limits).[26] The use of masking agents or diuretics by athletes and users of recreational drugs results in drug concentrations falling below the detection limit (sensitivity) of regular drug screening assays.

Both discrepancies and historical narratives were integral components of the laboratory module. Each new concept was introduced in the context of one of the relevant historical circumstances. As students proceeded through the laboratory

DOI: 10.1021/acs.jchemed.7b00633
J. Chem. Educ. 2018, 95, 1198−1204

exercises and write-ups, they had to interpret their observations and data in the context of both basic principles and the appropriate historical events. This approach was meant to both increase student interest in the experiments and help establish an understanding of the "real-world" implications of the concepts encountered in the laboratory.

Learning Objectives

The suite of experiments had four main learning objectives:

(1) Use colorimetric assays to ascertain the presence and concentration of protein in solution.
(2) Describe the chemical basis of protein detection by both Biuret and Bradford assays.
(3) Examine the fundamental concepts of assay design and interpretation, including interfering substances, false negatives, false positives, upper-range limits, lower-range limits (sensitivity), and selectivity.
(4) Weigh the origin of discrepancies between different protein colorimetric assays, and the pernicious "real-world" consequences of failing to appreciate the potential limitations of an assay.

Students' learning was assessed at three points in time: (1) the written responses to inquiry-style worksheet report questions that were distributed at the start and collected at the end of each lab session; (2) laboratory report questions that were completed at home at the end of the three-week laboratory sequence; and (3) questions on an closed-book, in-class exam. The laboratory handout, inquiry worksheets, at-home and in-class exam questions are presented in the Supporting Information (pages S3, S29, S57, and S58, respectively).

Participants and Context

The experiments were conducted as part of a combined Biochemistry lab and lecture course for upper-level undergraduates and continuing education students. All students in the course had intentions to pursue graduate education in the health sciences (e.g., to become a physician, physician assistant, dentist, nutritionist, or veterinarian). The experiment was run a total of three times with lab sections ranging in size from 14 to 18 students. Unless otherwise noted, all data described here were obtained from 12 students from a single lab section (total enrollment of 16 students) who provided signed informed consent forms.

The experiment was conducted over three laboratory periods of approximately 2.5 h each. Students worked individually (Part I) or in groups of two (Parts II and III). Prior to the first laboratory, students were given a handout that contained background information, the historical narratives, and the laboratory protocol. Separate worksheets were distributed at the start of each lab session. The worksheets included tables to record data and were interspersed with questions that encourage students to examine their data and engage in critical thinking throughout the laboratory session. Incorporating inquiry questions directly into the laboratory session has been demonstrated to improve learning outcomes in the biochemistry laboratory.[27] The worksheets were collected at the end of each lab session and returned to students at the start of the following lab session. The previous week's worksheet questions formed the basis of a prelab discussion in week 2 and week 3. Following the completion of each laboratory period, each group of students submitted their data and graphs electronically prior to the start of the following lab session.

EXPERIMENTAL OVERVIEW

Week 1: Qualitative Analysis

The lab session commenced with an introduction to the principles of protein colorimetry, a discussion of the historical narratives, and the week 1 laboratory protocols. In the first experiment, students had to determine if protein was present in two samples, Unknown-A and Unknown-B. Students were informed that the only possible protein present in the two unknowns was bovine serum albumin (BSA) and were given the protein sequence. Students were provided with a 10 mM solution of BSA in water to serve as a positive control (distilled water served as a negative control). Students were also provided Biuret and Bradford reagents. Students performed a qualitative analysis for each reagent, including both positive and negative controls and recorded their observations on the worksheet. Unbeknownst to students, Unknown-B contained 4.5 mg/mL BSA, while Unknown-A contained 1 M Tris-HCl, pH 7.5, an interfering substance that produces a false positive Biuret test.[14]

Week 2: Quantitative Analysis

Prior to the start of the second week's experiments, students discussed their data and conclusions from week one. Next, there was a brief introduction to the protocols for the experiments to be performed during the second lab session, including a review of the Beer−Lambert law and the standard curve.

In the second experiment, students had to determine the concentration of protein in Unknown-B using both quantitative Biuret and Bradford assays. Students were provided with a sample of 10 mM BSA as well as Unknown-B. Following the protocol outlined in the lab manual, students prepared the appropriate protein standards. After addition of the appropriate colorimetric reagent, students measured the absorbance of their samples, including Unknown-B. At home, students used this information to construct standard curves and find the concentration of Unknown-B. The standard curves and protein concentration values were submitted electronically prior to the next lab session.

Week 3: Selectivity

At the start of the third lab session, students reviewed their experiments and conclusions from week two, recording their answers in the worksheets. Prior to the start of experiments in week three, students formulated a hypothesis as to which assay is more *selective*. The hypothesis required students to understand the principle by which each assay interacts with proteins, through the backbone (for Biuret) or through a subset of amino acids (for the Bradford assay). As part of this experiment, students hand-drew ideal standard curves for assays that were selective and nonselective (Supporting Information page S66). Next, students tested their hypotheses by establishing Biuret and Bradford standard curves using three different proteins.

Students were given tubes containing BSA (10 mg/mL, in distilled water), chicken egg albumin (OVA) (10 mg/mL, in distilled water), and bovine gamma globulin (BGG) (5 mg/mL, in 0.1 M NaCl). Students had to prepare a range of standards necessary for constructing three standard curves (one for each protein) for each assay.

DOI: 10.1021/acs.jchemed.7b00633
J. Chem. Educ. 2018, 95, 1198−1204

HAZARDS

Lab coats, gloves, and protective eyewear (safety glasses or goggles) must be worn throughout the laboratory session. Biuret assay reagent contains concentrated sodium hydroxide, which is corrosive and can damage skin and eyes. Biuret assay also contains copper sulfate that is a skin and eye irritant that will stain skin and clothes. Bradford assay reagent contains phosphoric acid that is corrosive and can damage skin and eyes. In addition, Bradford reagent contains methanol, which is flammable. Bradford assay also contains the dye Coomassie Brilliant Blue G-250 that is a skin and eye irritant that will stain skin and clothes. Solutions containing BSA, OVA, BGG, and buffers are nonhazardous. The UV−vis spectrophotometer may emit dangerous UV light, even when not actively scanning.

RESULTS AND DISCUSSION

Students submitted laboratory data including graphs, answers to worksheet questions, home lab report questions, and exam questions. These data were analyzed to determine if students had met the learning objectives of the laboratory experiments (as outlined on page S57 in the Supporting Information).

Qualitative Analysis

The worksheet questions were designed to determine whether students could properly set up a qualitative analysis for both colorimetric reagents, interpret the results, and provide plausible explanations for the discrepancies that occurred between the two assays. A representative photograph of data from Part I (qualitative analysis) is shown in Figure 1.

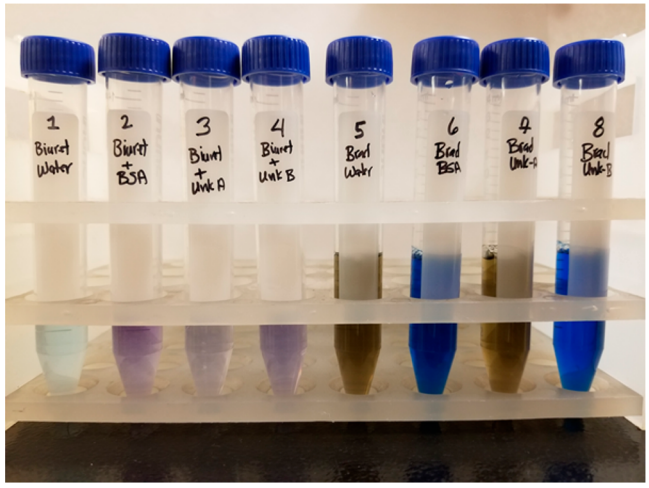


Figure 1. Qualitative analysis of protein using Biuret and Bradford assays (Supporting Information page S16). From left: Biuret + water (negative control), Biuret +10 mM BSA (positive control), Biuret + Unknown-A, Biuret + Unknown-B, Bradford + water (negative control), Bradford +10 mM BSA (positive control), Bradford + Unknown-A, Bradford + Unknown-B. Note that Biuret is positive for both Unknown-A and Unknown-B, while Bradford is positive only for Unknown-B. Each student obtained results identical to the author-generated figure shown above.

All 12 students properly set up the qualitative analysis and generated the appropriate colors in the negative and positive controls (blue/purple) and (orange-brown/blue) for the Biuret and Bradford assays. Each student also correctly noted on the worksheets that while both assays indicated that Unknown-B contained protein, the assays gave *contradictory results* with Unknown-A, with Biuret indicating that protein was present while Bradford indicated the absence of protein. The initial response of students to this result while in lab was surprise, followed by the hypothesis that their experiment might have been performed incorrectly and a request that they be allowed to repeat the experiment.

An examination of students' in-class writings revealed that all 12 students proposed at least one reasonable explanation for the discrepancy and were able to relate their results to one or more of the historical circumstances. The two most common explanations given by students were false positives due to interfering substances (the Chinese milk scandal and the case of Amy Albritton) or differences in the sensitivity of the assay. The question of relative sensitivity of the two assays was evaluated using data that were obtained in week 2.

Students' understandings of the mechanisms of the assays and their potential for discrepancies were also assessed through at-home lab report questions and in-class exam questions. Eleven of the 12 students received perfect scores on both the at-home report questions and the in-class question, indicating that overall they (1) understood the mechanism of protein detection by both colorimetric assays, (2) understood the nature of potential discrepancies between the assays, and (3) could relate the discrepancies observed in lab to authentic "real-world" scenarios.

Quantitative Analysis

In the second experiment, students prepared standard curves for both Biuret and Bradford assays and used these standard curves to obtain the concentration of protein in Unknown-B (Figure 2). All six groups of two students successfully submitted standard curves and protein concentration values for Biuret and Bradford assays, respectively (data from one lab section are presented on Supporting Information pages S48−S49). The standard curve for Biuret is linear to 5 mg/mL, and students obtained an average value of 4.51 ± 0.4 mg/mL from the standard curve. By comparison, the standard curve of the Bradford assay is hyperbolic; the data are no longer linear at concentrations greater than 0.2 mg/mL. When the data are plotted, they demonstrate a clear hyperbolic relationship with concentration (Figure 2, right). Despite the clear nonlinearity of the data, and the reading beyond the upper-range limit, all 12 students fit the entire data set to a straight line (as shown in Figure 2, right). Their attempts to fit the data using a linear model were unsuccessful, and their resulting (incorrect) standard curves produced a value of 1.32 ± 0.08 mg/mL.

When prompted to write (during lab session 3) why a linear fit over the entire data range was used, despite the obvious hyperbolic shape of the data, *every* group for two consecutive semesters provided a similar response such as the following: "The Beer−Lambert law demonstrates a linear relationship between absorbance and concentrations." Thus, despite the discussion of upper-range limits in the laboratory discussion and handout, students continued to presume the Beer−Lambert law is a physical relationship to which the data must conform, as opposed to a description of a phenomena that is valid only under appropriate conditions. It is possible that this misconception arose from students' prior experiences with the Beer−Lambert law both in this course, as well as in two prerequisite courses, where the absorbance versus concentration data were always ideally linear.

However, when prompted for a potential explanation, 11 of 12 students did subsequently correctly interpret the discrep-

DOI: 10.1021/acs.jchemed.7b00633
J. Chem. Educ. 2018, 95, 1198−1204

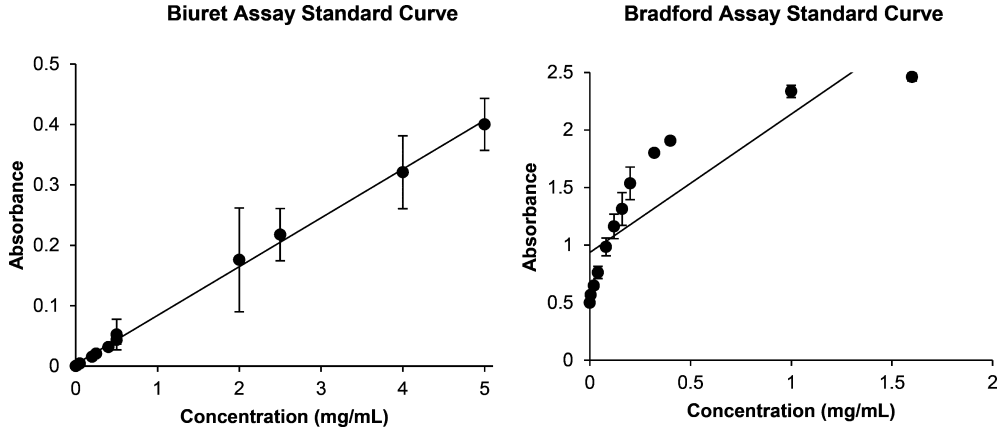


Figure 2. Standard curves for Biuret (left, Supporting Information page S19) and Bradford (right, Supporting Information page S21) prepared with BSA. The data points represent the average and standard deviation of the class data (Supporting Information pages S49−50). The class data grouped by group and experiment are presented on page S67 of Supporting Information.

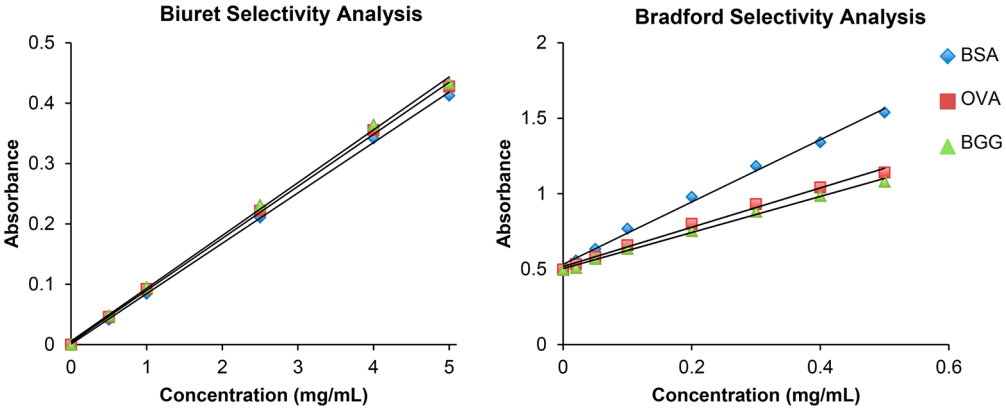


Figure 3. Selectivity of the Biuret (left, Supporting Information pages S25−S26) and Bradford (right, Supporting Information pages S26−S27) assays. The data points represent the average of the class data (Supporting Information pages S51−S52); error bars have been omitted for clarity but are within the same range as those in Figure 2. The class data graphed by group and experiment are presented on page S67 of the Supporting Information.

ancy in concentration measurements as due to the lower upper-range limit of the Bradford assay, and also noted similarities to the underlying cause of the Apollo 13 accident. One lab report question and one exam question directly addressed the question of upper-range limits and their relationship to the Apollo 13 accident; 11 of 12 students correctly identified upper-range limits as a possible cause of discrepancies between the assays and as a prime cause of the Apollo 13 accident, both in their home lab report and exam questions.

Sensitivity

One potential explanation for the discrepancy between the qualitative Biuret and Bradford assay (Part I) was that the Bradford assay was less sensitive than the Biuret assay, and so it did not detect the protein that was, in fact, present. The data obtained in week 2 were used to address this hypothesis. Students were instructed to find the relative slopes of the two assays, using only the linear region of the Bradford data. Initially, only four of 12 students successfully understood that the slope could be used as a measure of assay sensitivity. Following a class discussion of the lab data, nine of 12 students concluded, correctly, that the Bradford assay was, in fact, more sensitive, on the basis of a steeper slope (the other two students also thought the Bradford was more sensitive, but did so on the basis of a higher initial offset than the Biuret assay). Thus, it was concluded that the cause of the discrepancy in week 1 was most likely due to an interfering substance, not the inability of the Bradford assay to detect small quantities of protein. One lab report question and one exam question assessed student learning regarding this. Nine of 12 students correctly ascertained the relationship between slope and sensitivity on the lab report, and 11 did so successfully on the exam.

Selectivity

In the third week of the laboratory, students tested their hypotheses regarding the selectivity of each assay. Prior to beginning the experiment, the importance of assay selectivity was discussed, both in the context of protein colorimetric assays and also, more generally, in the context of "real-world" assays, such as drug and diagnostic tests. Initially, several students incorrectly suggested that a more selective colorimetric assay is desirable, since one could identify the protein on the basis of its response to the Bradford assay. Most students noted, however, that an "ideal" protein assay should respond equally well to all proteins (nonselective), since many interesting samples are undefined mixtures of proteins. During the discussion, one student (correctly) expressed the view that the ideal selectivity is a function of the application of an assay. A drug assay that is not sufficiently selective might give rise to many false positive drug tests (citing the case of Amy Albritton). The student

DOI: 10.1021/acs.jchemed.7b00633
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noted, however, that it might also be possible for an assay to be too selective and be unable to detect different "...types of the same drug, so maybe the drug can be changed a little or so it isn't detectible".

After the discussion, student groups were provided with three proteins, and they prepared standard curves using each protein for both Biuret and Bradford assays. All 12 students correctly hypothesized that the Bradford assay was more selective than the Biuret assay due to the Biuret reagent interacting with the protein backbone (which is shared by all proteins) while the Bradford reagent interacts with a subset of side chains (which differ in number and type between proteins). The result, as shown in Figure 3, was that the three protein standard curves nearly superimposed for the Biuret but not for the Bradford reagent. Eleven of 12 students correctly utilized these data to confirm their initial hypotheses on their worksheets while all 12 students correctly did so on both the lab reports and the exam.

Student Response

The student response to the laboratory sequence, as assessed by written comments, was mostly favorable. (Specific questions answered by each student are found on Supporting Information pages S33 and S58. Each student was also given the opportunity to comment further on the assignment.) The major concern of students was the initial discrepancy in the qualitative analysis and the discomfort they experienced with seemingly "incorrect" results. This was not surprising, as encountering ambiguous or discordant information is known to provoke a measure of discomfort that varies with an individual's tolerance of ambiguity.[28] Students exhibited less concern with the apparent discrepancies in Parts II and III of the sequence, possibly since these discrepancies were both anticipated after the discussion of Part I and resolved as part of the laboratory experiments.

All 12 participating students agreed that the historical narratives contributed to making the experiments more interesting and more relevant, and that the focus on discrepancies was important, citing one or more of the historical narratives. As one student noted, "I agree that discussing historical circumstances is (sic) important. It makes the concepts easier to understand. It also reminds us that in the real world, scientific tests could help and/or harm other peoples' lives. Having these real-life examples also made the lab discussion more fun and engaging."

The emphasis on understanding the mechanism by which the test results were obtained was also received enthusiastically. While several students (6 of 12) suggested the lab module was more complicated than others, all students were in agreement that this deeper level of understanding provided by the experiments was worthwhile. As one student noted, "It is important to understand the mechanism behind an assay as well as potential limitations, as this helps to avoid errors that could have disastrous consequences". This is particularly significant in light of the course being composed almost entirely of students planning healthcare careers.

CONCLUSION

A report on curricular reform in premedical education called for undergraduate academic experiences to incorporate cross-disciplinary synthesis of knowledge from the humanities, physics, chemistry, biology, and mathematics. According to this report, undergraduate premed coursework should emphasize critical thinking skills with the objective of understanding both how knowledge is acquired as well as its relevance to addressing real-life problems.[29] More generally, the desirability of incorporating authentic current and historical events into undergraduate science coursework for all students has been extensively discussed.[21] The learning objectives of this laboratory sequence were intended for students to understand how protein colorimetric assays were performed, the chemical basis for these assays, and their intrinsic limitations. The analysis of the experimental results required students to use critical thinking skills to integrate important concepts from chemistry, physics, biology, and mathematics, and relate each of those to an authentic "real-world" scenario. By tying the fundamental concepts of assay chemistry to authentic historical and current events, students were made aware of the "real-world" relevance of the laboratory learning objectives and the pernicious consequences of failing to account for the limitations of an assay or test. A holistic examination of students' worksheets, lab reports, and examinations indicated that, for the majority of students in the course, each of the learning objectives was met.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.7b00633.

Laboratory handouts (including protocols), inquiry worksheets, sample data, assessment questions, laboratory preparation, and ordering information (PDF, DOCX)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors wish to acknowledge Nina Cheprasova (New York City College of Technology) for the preparation of reagents and students enrolled in Biology 3601 Biochemistry (Fall 2016−Fall 2017) for participating in these laboratory experiments and contributing their data, comments, and suggestions. We thank Angelika Breckman for photographing the experiments. The anonymous reviewers are acknowledged for their thoughtful comments and suggestions on the manuscript. We also thank Louse Hainline and Lori Simms (Brooklyn College) for facilitating this collaboration. The collection and presentation of student data have been approved by the CUNY Institutional Review Board (Protocol 2016-0922).

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DOI: 10.1021/acs.jchemed.7b00633
J. Chem. Educ. 2018, 95, 1198−1204