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Laboratory Experiment

How Should Apples Be Prepared for a Fruit Salad? A Guided Inquiry Physical Chemistry Experiment

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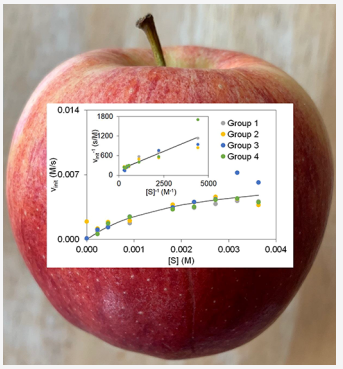
ABSTRACT: In this guided inquiry experiment, students extract catecholase enzyme from apples to catalyze the oxidation of catechol. They follow the reaction using the UV−vis absorbance of the *p*-benzophenone produced to determine the Michaelis−Menten kinetic parameters. Students make selected experimental choices within a structured framework such as selecting the apple varietal, the pH of the reaction mixture, and the reaction inhibitor. The experiment has been tested at multiple universities in physical chemistry laboratory courses with both large and small enrollments. We describe the experiment and its implementation in both synchronously and asynchronously taught courses.



KEYWORDS: *Laboratory Instruction, Physical Chemistry, Inquiry-Based/Discovery Learning, Kinetics, Enzymes, Upper-Division Undergraduate*

INTRODUCTION

Enzyme kinetics is a topic central to the study of biological systems. In a physical chemistry context, enzyme kinetics represent a unique opportunity for students to be introduced to the physicochemical phenomena that underpin the functioning of enzyme−substrate systems. According to a recent survey of instructors to assess the state of physical chemistry courses across the U.S., the topic of "rate laws" was the fifth most covered of 16 topics in thermodynamics/kinetics with 69% of respondents reporting a "great" level of coverage of the topic.[1] This finding indicates that the topic is one that experts in physical chemistry designate as important for students to explore. One approach to making this topic amenable to student exploration is to situate the learning experience in the context of a system that is both familiar and relevant to students.[2,3]

Enzymatic browning of fruits, such as apples, is mediated by the enzyme catechol oxidase. Pyrocatechol is the substrate of the enzymatic reaction and is an antiseptic compound released by the fruit once its outer layer is penetrated. The enzyme interacts with pyrocatechol and oxidizes the catechol to benzoquinone—the compound whose electronic absorption properties are responsible for the brown color observed in oxidized fruit.[4] This natural enzymatic browning process is estimated to be responsible for up to 50% of commercial losses and therefore has significant economic impact.[5] We chose the browning of apples as a highly relevant system for students to investigate in an inquiry-based, physical chemistry laboratory setting.

Kinetic studies of enzyme/substrate reaction systems have proven fruitful as subjects for a wide variety of undergraduate experiments. Targeted courses for these experiments include general chemistry, physical chemistry, organic chemistry, biochemistry, biophysical chemistry, and even middle and high school science.[6] The majority of the inquiry-based experiments were project-oriented experiments that required multiple lab meetings (even up to an entire semester). For example, in these project-oriented experiments, the students determine the Michaelis−Menten kinetic parameters as a part of a larger biochemical study of the enzyme and substrate[7−10] or as part of a comparison of enzymatic reactors.[11] Different measurement techniques have been used to determine the concentration of products with UV−vis spectroscopy being the most common.[8−10,12,13] Other experimental apparatus used include ion-selective electrodes,[14] pressure sensors,[15] displaced volume apparatuses,[6] fluorescence spectroscopy,[16] and blood glucometers.[17] An important feature of all these experiments is their appeal to student interest, including the use of yeast,[6] ethanol,[9] urease,[12] and the production of vanilla.[7] The enzyme in most reports may be purchased commercially, while in a few

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reports the enzyme extraction or production is part of the experimental procedure.[12,16]

Physical chemistry experiments typically emphasize data modeling and often rely on applying linear models to data. The Michaelis−Menten mechanism yields a nonlinear relation that may be linearized as a reciprocal−reciprocal plot. Earlier reports of enzyme kinetics papers tend to emphasize this linear method of data analysis (for example, see Lewis et al.[15]). As spreadsheet programs or other software become more ubiquitous, experiments reported in the *Journal* now use nonlinear modeling of the Michaelis−Menten equation to arrive at the kinetic parameters.[18,19] Some experiments use advanced modeling techniques. Barton[20] reports using Eadie−Hofstee linearization to extract kinetic parameters from the data. Her et al.[21] describe an experiment in which students employ the Lambert-W function and progress curve analysis to extract the Michaelis−Menten kinetic parameters from a single experiment at one substrate concentration by monitoring the change in the NMR signal as a function of time. This analysis requires tools not found in a typical spreadsheet program, so the analysis could be a limiting factor depending on institutional resources.

Another aspect of reviewing existing experiments is the level of inquiry expected of students. We adopt a perspective inspired by the work by Bruck et al.[22] along with the Next Generation Science Standards[23] to define the inquiry levels in the cited enzyme kinetics experiments. An example of a confirmation experiment is the report by Lewis et al.;[15] their experiment provides to the student all aspects of carrying out the experiment. Silverstein[9] describes a structured or guided inquiry experiment focused on alcohol dehydrogenase. In this 6 week enzyme kinetic experiment, students select which enzyme inhibitor to test, and they must determine all solution concentrations and volumes needed for their experiment. The semester-long project-oriented experiment described by Sarisky et al.[8] includes some aspects of a confirmation experiment in the early weeks but builds to authentic inquiry by the final weeks of the experiment. Students make and test hypotheses concerning the role of certain amino acids in the enzyme, and they also make all decisions related to solution preparation (concentrations and volumes). Notably, it is possible for students to experience a research "failure" in the project, yet all students will be able to achieve all the desired experimental outcomes including the kinetic analysis.

The original experiment described here was developed as part of the Physical Chemistry On Line (PCOL) Project[24] and was then adapted to the pedagogical framework for active-learning in the laboratory (Process Oriented Guided Inquiry Learning—POGIL) for physical chemistry.[25,26] The POGIL−PCL (physical chemistry laboratory) project is an NSF-funded effort to grow and sustain a community of instructors who develop, test, refine, and publish inquiry-based laboratory experiments for physical chemistry.

Figure 1 illustrates the experiment cycle developed by faculty participants in the POGIL−PCL project. For any such experiment, the title is a question; students complete the experiment in order to answer the question, rather than to confirm a numerical value. To investigate the first question, students must proceed through the cycle in Figure 1 by addressing pre-experiment questions, making initial predictions, carrying out the investigation in the laboratory, responding to "thinking about the data questions" that guide students to focus and reflect on relevant features of the data,

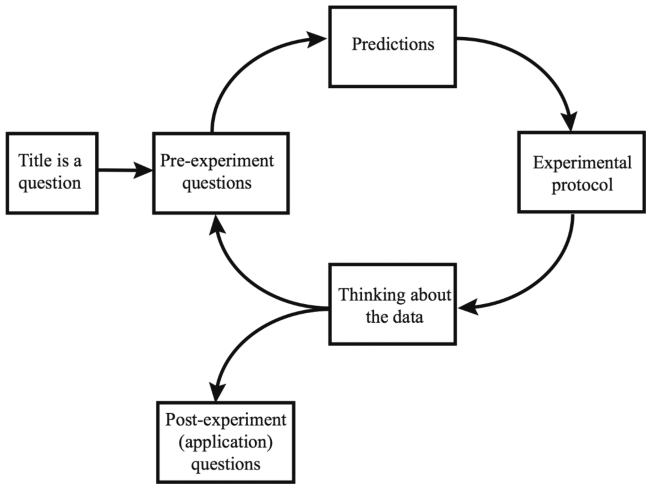

Figure 1. Learning cycle from POGIL−PCL25 (reproduced with permission by the American Chemical Society).

and come back to pre-experiment questions for the next part of the experiment (thus repeating the cycle). Students are led to apply mathematical models during the subsequent runs through the same cycle. A set of postexperiment questions prompt students to apply concepts learned to new situations or to delve more deeply into theoretical aspects of the current experiment. In the scheme described by Bruck et al., the experiment falls into the guided level of inquiry-based experiments.

Most published descriptions of inquiry-based laboratory experiments are designed and implemented in laboratory environments where all students in each laboratory section complete the experiment simultaneously. This allows for a distribution of experimental conditions and opportunities for whole class discussion to more fully explore issues related to experiment design and analysis. However, upper level laboratory courses in many institutions are implemented using a rotation model where student teams rotate through the experiments (typically due to limitations in available instrumentation). Only one or two groups carry out a particular experiment at any given time, and multiple experiments are being conducted during the laboratory period. The rotation model creates challenges for implementing inquiry-based experiments because there are minimal opportunities to compare data and because the instructor (often a graduate teaching assistant) monitors several different experiments simultaneously. Our goal was to ensure that the experiment described here could be modified to function in a rotation style laboratory environment as well as in the more easily facilitated laboratory environment where all students complete the experiment simultaneously.

EXPERIMENT OVERVIEW

Learning Outcomes

The experiment has intended learning outcomes that address both content knowledge related to enzyme kinetics and laboratory skills related to experimental design and data analysis. Specifically, the experiment is designed to facilitate students being able to do the following:

(1) analyze and manipulate equations and graphical representations to appropriately model experimental data/results

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(a) fit a nonlinear function to data
(b) convert a nonlinear relationship to a linear form
(c) extract relevant information from the results (i.e., slope, intercept)

(2) choose experimental conditions to obtain appropriate data for analysis
(3) describe the Michaelis−Menten mechanism for enzyme catalysis, including the meaning of the Michaelis−Menten parameters
(4) determine which apple has the slowest rate of browning, making it the best choice for a fruit salad
(5) determine the mechanism of inhibition for a particular inhibitor based on changes in Michaelis−Menten parameters

Description of the Experiment

The experiment discussed in this paper is rooted in these two questions:

- Which apple would be best for a fruit salad?
- What is the best way to keep cut apples from browning?

The experiment has four cycles, each of which follows the format in Figure 1. Each cycle focuses on the kinetics of the oxidation of catechol to quinone under different reaction conditions. The heart of the experimental protocol is the same for each cycle: Students monitor the time-dependent UV−vis absorbance at 540 nm of mixtures of catechol with catecholase extracted from apples. Any spectrometer may be used; a spectrometer with a kinetics package is preferable because the data are recorded in tabular form. The quinone produced in the oxidation of catechol is colored, so the data collected represent the rate of change in the concentration of quinone. Students are required to make a prediction of an experimental outcome in each cycle. They also have opportunities to make decisions about how to carry out the experiment. For each cycle, we summarize the pre-experiment questions, the predictions made by the students, the variations of the basic protocol, and the "Thinking About The Data" questions; the steps in each cycle are made bold.

Detailed options for timing for this experiment are described in the Supporting Information (instructor handbook). Completing all four cycles is expected to require three to four 3 h lab periods. If solutions are prepared for students, the first two cycles can be completed through the second round of data collection in one 3 h lab period. The modeling for Cycle Two and prelab experiment questions for design Cycles Three and Four could then be completed out of class or in a second lab period. Alternatively, a single 4 h lab period is sufficient to carry out the first two cycles, including the modeling. Data collection for Cycles Three and Four can be completed in a single 3 h lab period, but students must be well-prepared and efficient in order to obtain quality data.

Cycle One

The experiment handout begins with a brief introduction to the chemical basis of the browning of fruit. Students then answer **Pre-Experiment questions** designed to get them thinking about the oxidation of catechol to produce quinone and about enzyme kinetics. They are prompted to suggest a method for monitoring the reaction progress over time, and they review the protocol. The students are required to **predict** the appearance of the absorbance versus time graph for a solution of catechol and enzyme. To test this prediction, students **monitor the reaction progress** of three mixtures each containing catechol:

- 2.0 mL of catechol in water with 0.5 mL of water
- 2.0 mL of catechol in water with 0.5 mL of apple juice extract (the enzyme)
- 2.0 mL of catechol in buffer with 0.5 mL of apple juice extract (the enzyme)

During this phase, the instructor may choose to give the students the opportunity to make some experimental decisions. For example, the students can make the catechol solution and design and make the buffer solution, or the instructor can prepare the buffer in advance to save time. The students can decide how to best extract the catecholase from the apple (i.e., juice the apple) or be provided with a protocol. If the experiment is carried out with the whole class simultaneously, the students can decide how to divide up the necessary tasks, such as solution preparation or which students will test which mixtures.

After collecting the kinetic data, the **Thinking About the Data questions** lead the students to describe the effect of the enzyme on the oxidation reaction and to predict how to inhibit the enzymatic catalysis of the oxidation. Students are asked to decide whether or not to repeat the experiment using buffered or unbuffered mixtures based on their results from Cycle One; the question foreshadows enzyme inhibition (Cycles Three and Four). Data modeling takes place in Cycle Two, described next. Thus, instructors could implement Cycle One as a standalone inquiry experiment for an entry level chemistry laboratory course.

Cycle Two

Data modeling using the Michaelis−Menten reaction scheme is introduced in Cycle Two. In answering the **Pre-Experiment questions**, students **predict** the appearance of a graph of the initial reaction rate versus substrate concentration after examining the Michaelis−Menten equation, which is provided to students without derivation. The derivation of this equation from the mechanism is done by students in the Post-Experiment questions.

To test their prediction, the students **repeat the protocol** from Cycle One but with varying amounts of substrate. Students may also be given the option of choosing which apple varietals to test. The students decide which substrate concentrations to test given a range of concentrations; instructors should encourage students to be sure to obtain initial rates for enough trials at low substrate concentrations. As noted in Cycle One, students can choose to run the experiment in unbuffered mixtures, but if the apple varietals are acidic, there may be little to no browning observed.

The **Thinking About the Data** questions in Cycle Two guide students through linear and nonlinear data analysis to arrive at the Michaelis−Menten kinetic parameters, K_M and v_{max}. Students are guided to linearize the Michaelis−Menten equation and construct a double-reciprocal plot of $1/v_{init}$ vs $1/[S]$. This Lineweaver−Burk plot is used to obtain a reasonable estimate of the K_M and v_{max}. An example of a Lineweaver−Burk plot based on pH ∼ 7 data from multiple student groups is displayed Figure 2. All student groups used the same buffered apple solution.

Students use the resulting K_M and v_{max} estimates to fit the full (nonlinear) Michaelis−Menten equation to the data, refining their parameters to get the best fit curve. The nonlinear analysis requires a minimization routine such as

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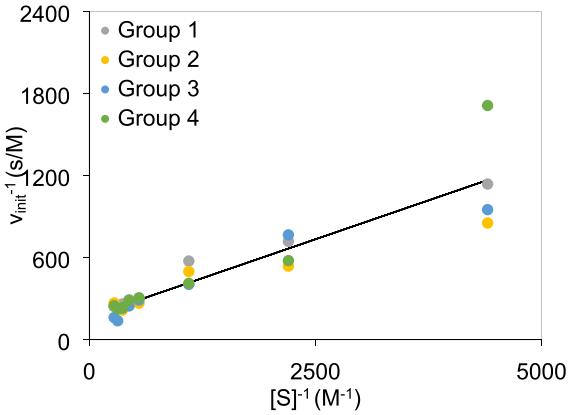

Figure 2. Typical Lineweaver–Burk plot based on student data (uninhibited). Results from four groups were combined to give K_M and v_{max} of 1.37×10^{-3} M and 6.03×10^{-3} M s^{-1}, respectively. These parameters were extracted via a simple linear regression (i.e., using the Excel LINEST function).

Microsoft Excel's solver, and the Instructor's handbook includes a sample spreadsheet. Figure 3 shows the data from

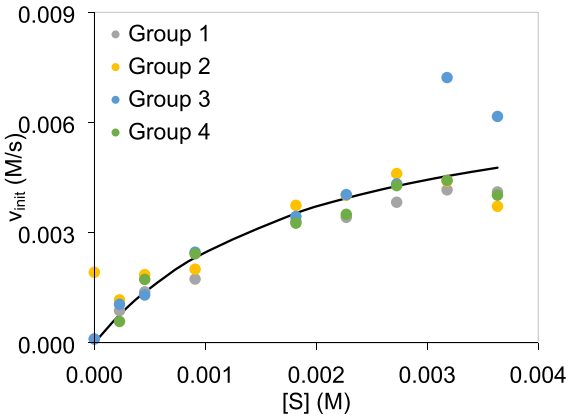

Figure 3. Typical Michaelis–Menten plot resulting from student data (uninhibited) for apple solutions buffered at pH = 7. The data from four student groups were combined. The extracted K_M and v_{max} via the nonlinear regression were 1.97×10^{-3} M and 7.36×10^{-3} M s^{-1}.

Figure 2 plotted as v_{init} versus [S], including the nonlinear fitted curve. Using the M–M equation rather than its linearized cousin results in a fit that does not overemphasize low substrate concentrations (large 1/[S], where the initial rates have a larger relative error compared to high substrate concentrations).

The data presented in Figures 2 and 3 were collected using the *same* apple extract due to the simultaneous (as opposed to rotational) structure of the laboratory course. Thus, it is possible for different teams of students to directly compare their data for validity.

This cycle closes with an open-ended question, "Suppose you want to make a fruit salad with apples. What factors would you consider when preparing the apples and the salad? Discuss based on your kinetics results." Thus, instructors in a physical chemistry laboratory course could choose to only carry out Cycles One and Two, which would answer the question of the experiment title and include data modeling.

Cycles Three and Four

The experiment is extended in these two cycles by including enzyme inhibition. The two cycles are motivated by reminding students that all apples brown over time, so cooks may try to find ways to slow the browning process. **Pre-Experiment questions** guide students to select an inhibitor, recalling the effect of acidity on browning from the first two cycles. Next, students must decide (**predict**) the appropriate substrate and inhibitor concentrations before going on to determine the Michaelis–Menten kinetic parameters experimentally. The inhibitor concentration must be selected so that the browning reaction is slowed but not stopped. Students repeat the same **protocol** to find the best inhibitor concentration and analyze the results by answering the next set of **Thinking About the Data** questions. Students have more difficulty determining the best concentrations than with any other aspects of this experiment; they often say something like, "just give me the instructions." Although it would be straightforward to simply provide appropriate concentrations, the point is to facilitate students' development of the skills that are more authentic to research environments. The goal is to communicate to students that learning the process of experimental design is important, not just getting the "right" answer.

Inhibition mechanisms are introduced in the Cycle Four **Pre-Experiment** questions, which come after students select their inhibitor and determine its appropriate concentration. A series of questions guide students to match the inhibition reaction mechanisms to the mechanism type (competitive, uncompetitive, or noncompetitive) and the corresponding equilibrium expression. Ultimately, students predict how their initial rate versus substrate concentration graphs will be affected by each of the inhibition mechanisms. At this point, they set up the appropriate concentrations of enzyme, inhibitor, and substrate in order to determine the mechanism for their selected inhibitor. The students repeat the **protocol** from Cycle Two to determine the Michaelis–Menten kinetic parameters in the presence of the inhibitor; the final **Thinking About the Data** questions guide the students to determine the inhibition model from their results. The selection of inhibitors has been guided by those commonly seen in commercial food products or recipes (citric acid, ascorbic acid, sodium benzoate, and lemon juice). Citric acid and lemon juice show competitive inhibition, while ascorbic acid and sodium benzoate show noncompetitive inhibition. Student data are not always of sufficiently high quality to determine the mode of inhibition, but students are able to reflect on why their experimental design was insufficient and what changes they could make to achieve better outcomes.

Students observe inhibition as shown in Figure 4a; Figure 4b demonstrates that these students were able to determine that citric acid inhibited browning through a competitive mechanism. However, some students do find that their collected data make it challenging to clearly distinguish between mechanisms. One important reason for this is that the system studied is an "apple", not a well-controlled system. Another issue is that students frequently neglect to collect uninhibited data when collecting the inhibited data and therefore compare their inhibitor results to prior results with a different apple, albeit the same type of apple. This result—or rather, that lack of a clear result—helps students learn to justify their conclusions as well as recognize some of the challenges in experimental design and inherent to working with natural samples.

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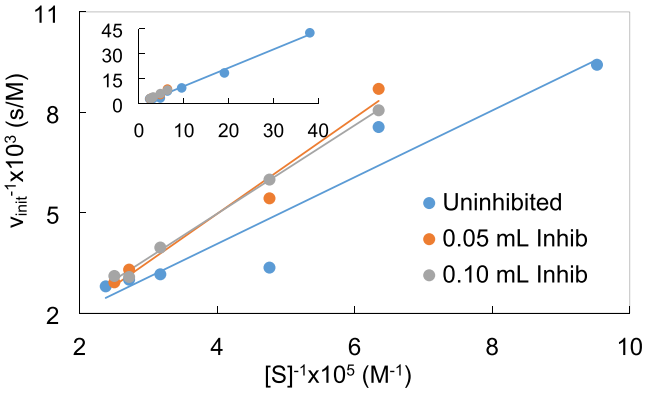

Figure 4. Typical Lineweaver–Burk plot based on student data (uninhibited and inhibited with citric acid). The main graph is enlarged to focus on data within a similar substrate concentration range; the inset shows the full data set.

HAZARDS AND SAFETY PRECAUTIONS

Students should be aware that catechol (MSDS 04360) and benzoquinone (MSDS 96461) are acute eye, skin, lung, and gastrointestinal irritants. In addition, benzoquinone is an environmental toxin. Appropriate PPE should be worn by all individuals in the laboratory, including goggles and nitrile gloves, to limit exposure to catechol and quinone during the experiment. Waste should be disposed of properly to avoid releasing benzoquinone into environmental water.

Although apple contents are benign, students should be reminded that they are not to eat, drink, or ingest any material in the laboratory environment irrespective of its role as a food product in a context outside the lab.

IMPLEMENTATION

Different variations of the experiment were implemented at multiple institutions over the last 15 years. The POGIL–PCL version was used at over five institutions with over 400 students. It was implemented multiple times both with all students in a section completing the experiment simultaneously and in a rotation style where different student teams complete the experiment in different weeks throughout the semester. In addition, this experiment was tested with about 50 faculty at three different POGIL–PCL workshops.

This experiment can be implemented in full, which takes three or four laboratory sessions (each typically about 3 h in length) depending on how much time students are given in the laboratory to work on data analysis. The first cycle could be used in an introductory laboratory course as long as the instructor provides the buffer solution. The first and second experiment cycles could be implemented as a standalone experiment in one or two lab-class periods as part of a standard physical chemistry laboratory sequence. The instructor handbook, included in the Supporting Information, provides suggestions based on different institutional constraints.

Although not always possible, carrying out the experiment with the whole class simultaneously has some advantages. The instructor leads a discussion of the pre-experiment questions and guides students as they brainstorm experimental choices, especially making the buffer and arriving at a consensus decision regarding buffering in the second cycle. Doing the experiment with the whole class simultaneously makes it easier for students to study different apples of their choosing. Students can be prompted to share their predictions. Including the inhibitor cycles is a good option for simultaneous mini-research projects; different student teams may select the inhibitor of their choice and present their results to the whole class.

Students are prompted to share results in several instances in the experiment handout, as is typical for POGIL–PCL experiments.[25] However, we intentionally wrote this experiment so that it could also be implemented in courses using experiment rotation. To facilitate comparisons of parameters from different apples, instructors required students to share data in a common spreadsheet that was updated by students over the course of the semester or by posting results on a discussion board using the course management system. In this way, each team of students obtains quality data to analyze, and the data set builds as new teams rotate through completing the experiment. Scheduling was done so that all teams, including the first team, had access to data from a different apple varietal to compare parameters for different species in order to complete the required analysis, which was included in their laboratory reports. The instructor also provided oversight to ensure different apple varieties and different inhibitors were selected by students at different times. Although the opportunity for whole class discussion was lost, completion and submission of the prelaboratory questions in advance of the laboratory provided TAs the opportunity to respond to student ideas and ask further questions as needed.

ASSESSMENT OF LEARNING OUTCOMES

The primary mode of evaluation of student achievement of outcomes was through analysis of submitted lab reports using a detailed grading rubric (two examples are included in the Supporting Information). The initial rubric was based on an Excel rubric published in this *Journal*.[27] The published rubric was modified using categories from the ELIPSS rubrics[28,29] to add criteria to reflect explicitly the focus on critical thinking and argumentation. The grading rubric has also been imported into the learning management system to facilitate scoring and feedback to students. Instructors' evaluation of student reports, in both course settings, showed that students were generally successful with respect to achieving the desired process and content skills.

Students successfully analyzed and manipulated equations and graphical representations to model experimental results, including using the parameters extracted from the Lineweaver–Burk plot to inform their initial guesses for the nonlinear fit of the data to the Michaelis–Menten equation. Students were able to successfully choose experimental conditions to obtain appropriate data for analysis most of the time, although they often struggled to articulate the rationale for their choices. It was also observed that students tend to want to use serial dilutions, even when cued to consider that this approach will not generate the most useful data set.

One area of concern, based on student reports, was the tendency of students to neglect explicitly addressing the guiding question of the activity. The students' focus on equations is problematic given that a fundamental practice in science is to ask questions to inform investigations. The National Academy of Sciences recommends that students build competence in asking questions to inform investigation, listing this competence as one of eight science and engineering essential practices.[23] Thus, instructors should tailor their assessment tools (i.e., rubrics) to guide students to explicitly

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use their results to address the guiding questions. This experiment and associated assessment of student work may be considered part of the broader effort to lead students away from viewing the purpose of a laboratory activity to "get a number" or a set of numbers and toward viewing it in a way that is conducive to using, and interpreting, evidence to address research question(s).

CONCLUSIONS

The experiment we describe here is *guided inquiry* (with some elements of *open inquiry*); can be completed in two to four class periods for a physical chemistry laboratory course; and applies nonlinear modeling that can be carried out using spreadsheets. It has been tested at multiple institutions, and it can be carried out in classes that have a synchronous or rotational format. Extracting the enzyme system from apples increases the experiment's appeal to students, but the enzyme extraction is not onerous. This experiment requires students to think about experimental parameters and make decisions about what data to collect. When students make their own choices for the experimental parameters, the resulting data are sometimes insufficient for drawing scientifically meaningful conclusions. However, such results provide opportunities for students to reflect on their choices and the impact they have on the quality of data. Students gain skill in how to implement procedures from the literature that are less defined than they typically encounter in most undergraduate laboratory experiments.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available at https://pubs.acs.org/doi/10.1021/acs.jchemed.0c00517.

Student handout for the experiment (PDF, DOCX)

Instructor handbook for the experiment (PDF, DOCX)

Scoring rubric 1 used for grading student reports (PDF)

Scoring rubric 2 used for grading student reports (PDF)

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Notes

The authors declare no competing financial interest.

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