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Laboratory Experiment
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Colorimetric Measurements of Amylase Activity: Improved Accuracy and Efficiency with a Smartphone

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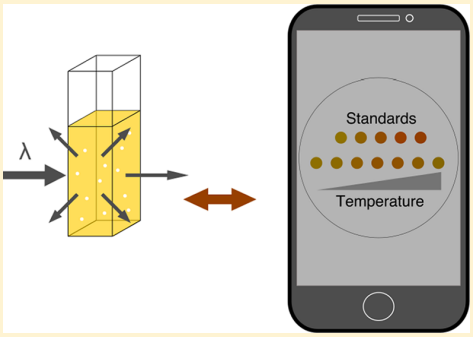
Supporting Information

ABSTRACT: Routinely used in quantitative determination of various analytes, UV−vis spectroscopy is commonly taught in undergraduate chemistry laboratory courses. Because the technique measures the absorbance of light through the samples, losses from reflection and scattering by large molecules interfere with the measurement. To emphasize the importance of light scattering in UV−vis spectroscopy, a laboratory experiment is described that guides students to study the hydrolysis of starch by amylase as a function of temperature. The reducing sugar produced from the hydrolysis is determined by dinitrosalicylic colorimetric assay via two methods: UV−vis spectroscopy and a smartphone-based colorimetry, which is capable of quantifying the concentration of a chromogenic compound in an opaque sample. From this experiment, students can directly learn to measure the activity of amylase, use a smartphone to perform colorimetry, observe the effect of light scattering, and gain a better understanding of UV−vis spectroscopy.



KEYWORDS: *Analytical Chemistry, Biochemistry, UV−Vis Spectroscopy, First-Year Undergraduate/General, Second-Year Undergraduate, Enzymes, Laboratory Instruction, Hands-On Learning/Manipulatives, Quantitative Analysis, Carbohydrates*

Quantitation of analytes by UV−vis spectroscopy is commonly taught in undergraduate chemistry laboratory courses. A UV−vis spectrophotometer is typically employed to measure the absorbance of light passing through the analytes, which absorb light at specific wavelengths. The absorbance is linearly related to the concentration of analytes as given by Beer's law, $A = \varepsilon bc$, where A is the absorbance, ε is the molar absorptivity coefficient, b is the path length of the cuvette, and c is the analyte concentration. Students generally recognize that the measured absorbance depends on the concentration of the analyte, the amount of light that each molecule absorbs, and the distance that light travels through the samples—parameters explicitly stated in Beer's law. However, the underlying principles of absorption spectroscopy seem to be missing from students' understanding.

As light travels through the sample-containing cell, its intensity is lost by reflection and scattering.[1] To compensate for these losses, an identical cell containing only solvent is used as a blank. If the sample contains suspended particles, the incident light is further reflected and scattered by the particles. This interference can result in artificially high absorbance values, which lead to drastic errors in the measurement. Therefore, it is important to emphasize the effect of light scattering on UV−vis spectroscopy in teaching laboratories. Prior to spectroscopic measurements, the particles must be removed by filtration or centrifugation. Otherwise, a different technique that is amenable to opaque samples must be employed.

One colorimetric assay that suffers from light scattering is the measurement of amylase activity, using starch as a substrate. The activity of amylase is quantified through a well-established dinitrosalicylic acid (DNS) assay.[2−5] Under alkaline conditions, reducing sugars reduce 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid, resulting in a color change from yellow to reddish brown, which can be monitored at 540 nm (Figure 1A,B). Because the substrate is a turbid starch slurry, any starch that remains from incomplete hydrolyses will interfere with subsequent spectroscopic measurements.

In addition to UV−vis spectroscopy, a smartphone and color intensity analysis have been previously reported as effective and accessible methods for colorimetric measurements.[6] Smartphone colorimetry has been employed in various experiments involving both a single color with a colorless blank[7−13] and color changes.[14] However, the uses of smartphone colorimetry to study samples containing particles have not been explored. For smartphone colorimetry to be effective, an image needs to be acquired under uniform lighting.[15] Previous experiments have employed various platforms, including a flatbed office scanner,[16,17] a computer screen,[6,7,14] or even ambient light.[11]

A simple, cost-effective lighting setup and imaging platform are described that allow for quantitative colorimetric analysis

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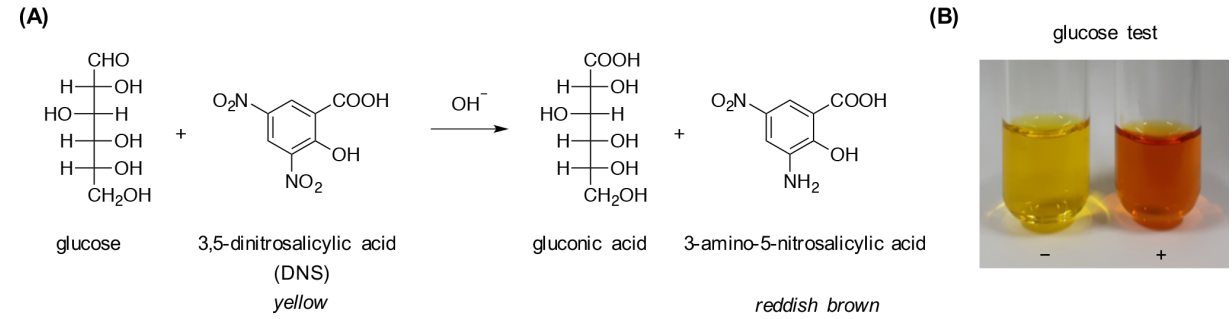

Figure 1. Reaction scheme and a sample image of DNS-reacted solution. (A) Under alkaline conditions, glucose reduces 3,5-dinitrosalicylic acid (yellow) to 3-amino-5-nitrosalicylic acid (reddish brown). (B) A sample image of the DNS reagent with water (left) and DNS reagent with 0.50 mg/mL glucose (right).

even in the presence of starch particles. Herein, an integration of this colorimetric method with the DNS assay to study the hydrolysis of starch by amylase is reported as a function of temperature, in addition to the standard UV–vis spectroscopy. A comparison of the results of these methods allows students to discern the effect of light scattering in UV–vis measurements directly from the experiment.

STUDENT LEARNING GOALS

This laboratory experiment directs students to quantify the temperature-dependent activity of amylase via two methods: traditional UV–vis spectroscopy and smartphone-based colorimetry. After completing this experiment, students should be able to (1) perform an enzyme activity assay, (2) use a smartphone colorimetric method to measure the concentration of a colored compound, and (3) identify light-scattering particles as an interference in the UV–vis measurements. These learning goals were evaluated from written laboratory reports and answers to postlaboratory questions.

This laboratory experiment has been incorporated into the biochemistry laboratory course for second- or third-year undergraduate students majoring in biology, chemistry, and food science. The experiment was carried out by students four times; the class sizes ranged from 18 to 21 students. Groups of 2 or 3 students were able to complete the experiment in 4 h. The study was approved by the Institute for Population and Social Research's Institutional Review Board (IPSR-IRB COA. No. 2017/06-154).

EXPERIMENTAL SECTION

Preparation of Calibration and DNS Assay

In the laboratory, DNS reagent and a standard glucose solution (1.00 mg/mL) were provided by the stockroom (see the Instructors' Notes in the Supporting Information for preparation procedures).[5] Students prepared calibration standards by diluting the standard glucose solution. The diluted standards were reacted with the DNS reagent and heated in a boiling water bath for 5 min (see the Student Handout in Supporting Information for detailed procedures). The solutions were diluted 6-fold before the absorbance was measured at 540 nm using a spectrophotometer. The undiluted solutions were kept for further measurements via smartphone colorimetry.

Temperature Dependency of Amylase Activity

Amylase was acquired from students' saliva and diluted 50-fold with distilled water. Students prepared reactions by mixing corn starch solution and pH 6.0 phosphate buffer in small test tubes. The starch substrate was equilibrated in water baths at temperatures 0–60 °C at 10 °C intervals for a few minutes. Diluted amylase solution (0.50 mL) was pipetted into the reactions. After a 5 min reaction time, the samples were reacted with DNS reagent (see the Student Handout in the Supporting Information for detailed procedures).

Image Acquisition

For cellphone-based colorimetry, a cost-effective uniform illumination platform was set up using a desk lamp with a 9 W LED light bulb, illuminating through a 2 mm thick solid white acrylic sheet, which is positioned about 40 cm away from the light bulb (Figure 2). This low-cost setup provides a window of illumination that is sufficiently uniform for illuminating samples arranged on a Petri dish. Any modern digital or cellphone camera can be used to acquire an image. Aliquots of 20 μL of undiluted DNS-reacted standards and samples were pipetted on 0.6 cm diameter #4 Whatman filter paper (punched from a regular office hole-puncher), arranged on a Petri dish (Figure 2). Ceiling lights were dimmed while students capture an image with their smartphones.

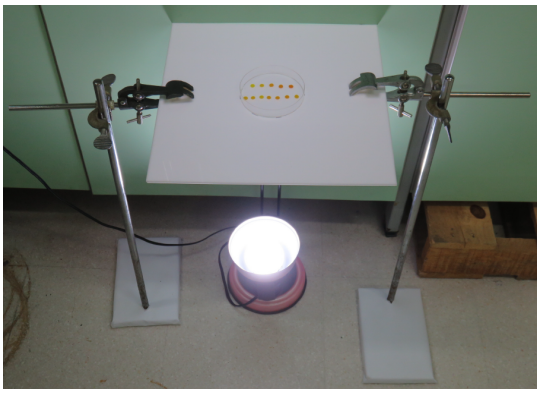

Figure 2. Simple setup to create uniform lighting for smartphone colorimetric analysis

Data Analysis and Comparison

Color intensity analysis from the acquired image was done using ImageJ, a free image analysis software that is widely used in biological image analysis.[18] The color image was separated into R, G, and B channels by selecting image, color, and split channels. The green channel was chosen because it shows a linear trend with different glucose concentrations. Alternatively,

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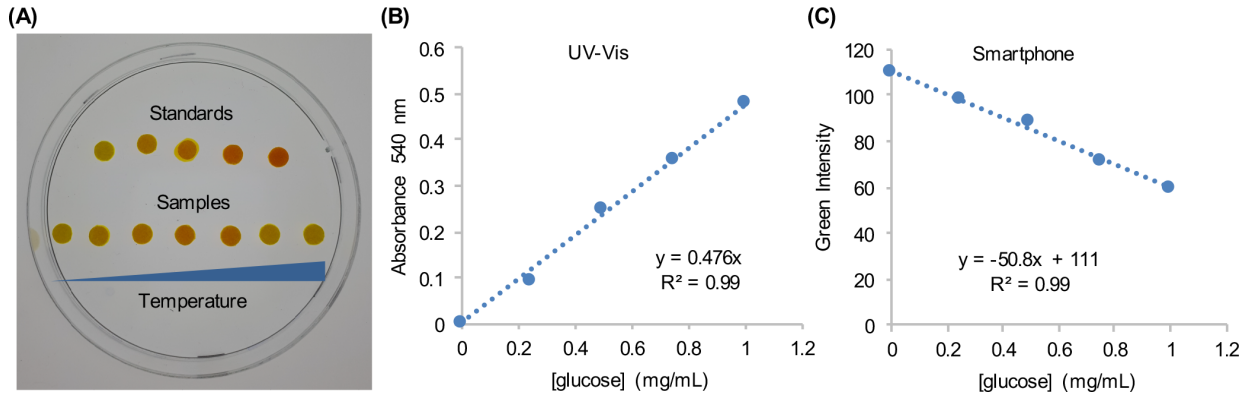

Figure 3. Sample acquired image and calibration curves. (A) An example of student data of the standards and the samples. (B) A UV−vis spectroscopy calibration curve of absorbance at 540 nm as a function of glucose concentration. (C) A smartphone colorimetry calibration curve of green channel intensity as a function of glucose concentration from the image in part A.

the RGB measure plugin in ImageJ can also be used. The measurement was carried out by selecting most of the filter paper (avoiding the edges) with the circle function and measuring the area averaged intensity from each spot. In addition to ImageJ, a variety of software (e.g., Adobe Photoshop and Color Code Picker)[6] and mobile applications (e.g., Color Grab and Colormeter)[12] can also perform similar color intensity analysis.

The green color intensity of the standard solutions was plotted as a function of glucose concentrations and fitted to a linear trendline to generate a calibration curve. The amount of reducing sugar produced in each reaction is directly related to the activity of the enzyme. For amylase, one unit of activity is defined as the enzyme producing reducing sugar equivalent to 1 μmol of glucose from the reaction of 1 mL of saliva in 1 min, as shown in the following equation:

$$\text{activity} = [\text{glucose}]\left(\frac{\text{mg glucose}}{\text{mL reaction}}\right) \times \frac{1\ \text{mmol}}{180.16\ \text{mg}} \times \frac{10^3\ \mu\text{mol}}{1\ \text{mmol}} \times \frac{3\ \text{mL reaction}}{0.5\ \text{mL dil saliva}} \times 50\ \text{dil factor} \times \frac{1}{5\ \text{min}}$$

HAZARDS

Dinitrosalicylic acid is corrosive and harmful if inhaled or swallowed. The DNS reagent also contains 0.40 M NaOH, which is an irritant. The reduced product, 3-amino-5-nitrosalicylic acid, is toxic if swallowed and causes eye irritation. The DNS reagent must be handled with care; laboratory gloves and eye protection must be worn at all times. DNS-containing waste must be separated for proper disposal. Phosphate buffer may cause eye and skin irritation. Saliva is a body fluid; thus, students should handle their own saliva, including disposal and rinsing equipment. Hot water can cause burns. Use heat resistant gloves when handling test tubes in a hot water bath. Do not heat test tubes on a direct flame.

RESULTS AND DISCUSSION

Standard glucose solutions and samples were evaluated by both the UV−vis absorbance measurement and the smartphone colorimetry. Calibration curves of both methods show good linearity (Figure 3). As the concentration of glucose increases, the glucose standards become more red (Figure 3A). This color trend corresponds to the increasing absorbance in the UV−vis measurement and the decreasing intensity of the green channel in the smartphone colorimetry (Figure 3B,C).

From the calibration curves (Figure 3B,C), the values of absorbance and the green intensity of the amylase-digested samples were converted to reducing sugar concentrations and, correspondingly, to the activity of the enzyme. Visually, the colors of the samples change from yellow to red, as the temperature increases from 0 to 40 °C, but at higher temperatures, the intensity of red color decreases (Figure 4A). These observations indicate that the activity of the enzyme increases with temperature to 40 °C and decreases at higher temperatures, in agreement with the literature.[19] The activity profile quantitated by the smartphone method agrees with these observations, but the results from the UV−vis spectroscopy do not show such a trend (Figure 4B). The activity of the enzyme at extreme temperatures appears artificially high, due to the remaining starch particles from incomplete hydrolysis in those samples.

As expected, different saliva samples contain different amounts of amylase and different level of activities. However, the temperature-dependent activity profiles, which peak at 30−40 °C, are generally observed (Figure 4B). The authors' data, showing the error bars (Figure S1), are included in the Instructors' Notes in the Supporting Information.

A smartphone can be used for quantitative colorimetric analysis effectively, as previously reported.[6−12,14,15] The result of this experiment illustrates that the smartphone colorimetric method even has an advantage over the traditional UV−vis method in analyzing complex samples, such as those containing scattering particles. In a spectrophotometer, the incident light is reflected and scattered by starch particles; therefore, the light intensity reaching the detector decreases (Figure 5A,B). On the other hand, the presence of starch particles does not significantly interfere with the color of the samples spotted on filter paper disks (Figure 5C,D). The color of the samples is caused by diffused reflection of light to the observer's eyes and the camera. Starch particles blend in with the white background of the filter paper. The thin layer of samples also minimizes the interference from the starch. Therefore, in this assay, the smartphone method provides a more accurate colorimetric measurement than traditional UV−vis spectroscopy.

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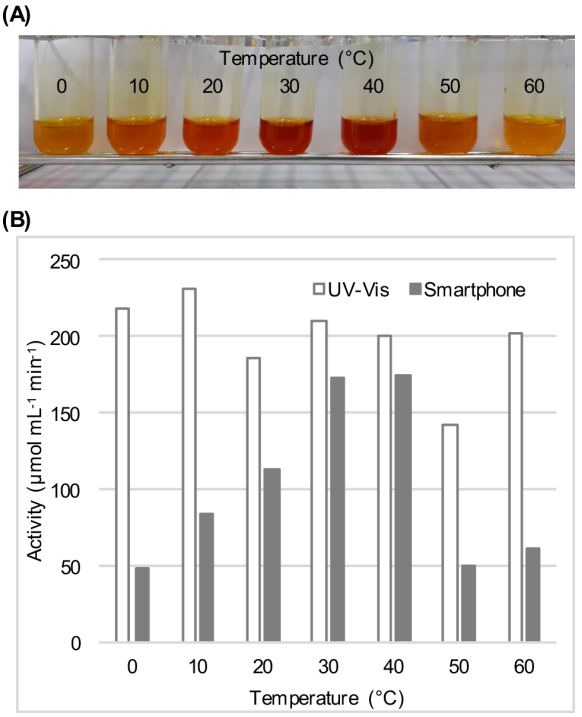

Figure 4. Activity of amylase at different temperatures. (A) An image of DNS-reacted samples after hydrolysis of starch by salivary amylase as a function of temperature. (B) An example of student data of the activity of amylase as a function of temperature as obtained from UV−vis spectroscopy (white bar) and smartphone colorimetry (gray bar).

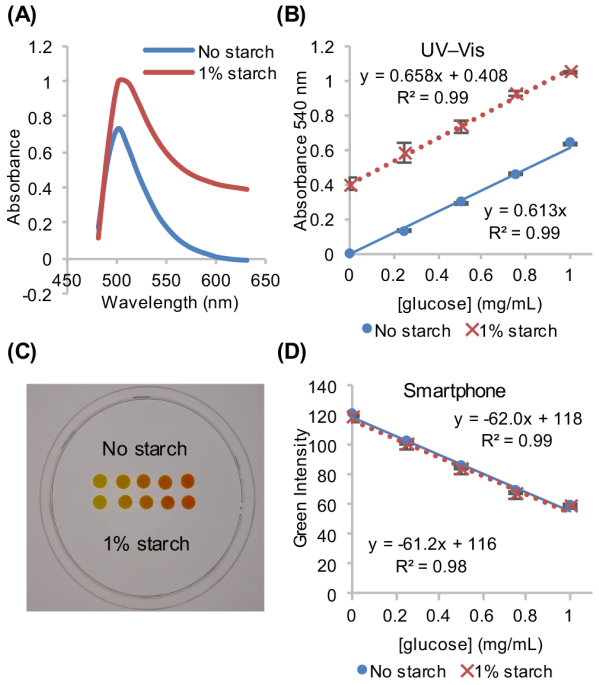

Figure 5. Comparison of UV−vis and smartphone colorimetric measurements. (A) Example UV−vis spectra of DNS-reacted glucose solutions with (red) and without (blue) starch. (B) The absorbance at 540 nm of solutions with (red) and without (blue) starch as a function of glucose concentration. Error bars are standard deviations from three trials. (C) An image of solutions without starch (top) and with 1% starch (bottom). The concentration of glucose is increased from left to right. (D) The intensity of green color as a function of glucose concentration. Error bars are standard deviations from three trials.

Additionally, spotting samples on filter papers also eliminates the risks of spilling of samples on the imaging platform. The low-cost lighting setup provides an alternative method to the existing imaging platforms: a flatbed scanner,[15−17,20] a computer screen,[6] or a light box,[6] previously reported.

During the laboratory experiment, many students were surprised by an artificially high absorbance of the reaction at 0 °C. This observation prompted the discussion of the principles of UV−vis spectroscopy, which led the students to think about light−particle interactions, specifically reflection and scattering by starch particles. The instructor can also demonstrate the light-scattering effect outside the spectrophotometer by shining a laser pointer through a starch-containing cuvette and a standard-containing cuvette.

To evaluate the students' perception of the experiment, students answered a survey at the end of the laboratory session (see Student Evaluation Survey in the Supporting Information). The survey data show that the experiment improves the students' understanding of UV−vis spectroscopy and enzyme activity measurements. The students also enjoyed using their smartphones to determine the color intensity of the samples. Not only does it bring a novel approach to scientific experiments, the method is also less time-consuming. An image of all samples can be acquired in one frame, while the UV−vis absorbance can only be measured one sample at a time, and the cuvette must be thoroughly rinsed when changing samples.

Analysis of students' data and laboratory reports showed that 20 out of 22 groups obtained calibration curves with good linearity ($R^2 > 0.95$) from the smartphone colorimetric method. For the rest, the discrepancy arose from errors in diluting the glucose standard, as their UV−vis results show similar deviations. Despite different amounts and activity levels of amylase in each student's saliva, a similar temperature-dependency profile, with a maximum activity at 30−40 °C, was generally obtained from the smartphone method. In the postlab assignments, students also identified that light scattering from starch particles introduced errors in the UV−vis measurements.

The smartphone colorimetric approach described here can also be adapted to other samples to emphasize the effect of light scattering and reflection in UV−vis spectroscopy and the importance of appropriate sample preparations. Experiments involving different starch substrate concentrations, such as kinetic studies of amylase, should benefit from the discussed method.

CONCLUSION

The smartphone colorimetry described here can effectively quantify the concentration of a colored compound in opaque samples, which interferes with the standard UV−vis measurement. Through the measurement of the activity of amylase, students learned the principles of UV−vis spectroscopy with an emphasis on light scattering. The students were also motivated by learning that their smartphone can be used as an effective analytical device.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.7b00468.

Student laboratory handout, instructors' notes, and student evaluation survey (PDF, DOCX)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Skoog, D. A.; Holler, F. J.; Crouch, S. R. *Principles of Instrumental Analysis*; Thomson Brooks/Cole: Belmont, CA, 2007; pp 336−342.

(2) Miller, G. L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* **1959**, *31* (3), 426−428.

(3) Rungruangsak, K.; Panijpan, B. The Mechanism of Action of Salivary Amylase: A Simple Undergraduate Experiment. *J. Chem. Educ.* **1979**, *56* (6), 423−424.

(4) Gusakov, A. V.; Kondratyeva, E. G.; Sinitsyn, A. P. Comparison of Two Methods for Assaying Reducing Sugars in the Determination of Carbohydrase Activities. *Int. J. Anal. Chem.* **2011**, *2011*, 1−4.

(5) Coughlan, M. P.; Moloney, A. P. Isolation of 1,4-β-D-Glucan 4-Glucanohydrolases of Talaromyces Emersonii. *Methods Enzymol.* **1988**, *160*, 363−368. (Accessed Sep 2017.)

(6) Kehoe, E.; Penn, R. L. Introducing Colorimetric Analysis with Camera Phones and Digital Cameras: An Activity for High School or General Chemistry. *J. Chem. Educ.* **2013**, *90* (9), 1191−1195.

(7) Knutson, T. R.; Knutson, C. M.; Mozzetti, A. R.; Campos, A. R.; Haynes, C. L.; Penn, R. L. A Fresh Look at the Crystal Violet Lab with Handheld Camera Colorimetry. *J. Chem. Educ.* **2015**, *92* (10), 1692−1695.

(8) Campos, A. R.; Knutson, C. M.; Knutson, T. R.; Mozzetti, A. R.; Haynes, C. L.; Penn, R. L. Quantifying Gold Nanoparticle Concentration in a Dietary Supplement Using Smartphone Colorimetry and Google Applications. *J. Chem. Educ.* **2016**, *93* (2), 318−321.

(9) Montangero, M. Determining the Amount of Copper(II) Ions in a Solution Using a Smartphone. *J. Chem. Educ.* **2015**, *92* (10), 1759−1762.

(10) de Morais, C. de L. M.; Silva, S. R. B.; Vieira, D. S.; Lima, K. M. G. Integrating a Smartphone and Molecular Modeling for Determining the Binding Constant and Stoichiometry Ratio of the Iron(II)−Phenanthroline Complex: An Activity for Analytical and Physical Chemistry Laboratories. *J. Chem. Educ.* **2016**, *93* (10), 1760−1765.

(11) Moraes, E. P.; da Silva, N. S. A.; de Morais, C. de L. M.; das Neves, L. S.; Lima, K. M. G. de Low-Cost Method for Quantifying Sodium in Coconut Water and Seawater for the Undergraduate Analytical Chemistry Laboratory: Flame Test, a Mobile Phone Camera, and Image Processing. *J. Chem. Educ.* **2014**, *91* (11), 1958−1960.

(12) Kuntzleman, T. S.; Jacobson, E. C. Teaching Beer's Law and Absorption Spectrophotometry with a Smart Phone: A Substantially Simplified Protocol. *J. Chem. Educ.* **2016**, *93* (7), 1249−1252.

(13) Moraes, E. P.; Confessor, M. R.; Gasparotto, L. H. S. Integrating Mobile Phones into Science Teaching To Help Students Develop a Procedure To Evaluate the Corrosion Rate of Iron in Simulated Seawater. *J. Chem. Educ.* **2015**, *92* (10), 1696−1699.

(14) Gee, C. T.; Kehoe, E.; Pomerantz, W. C. K.; Penn, R. L. Quantifying Protein Concentrations Using Smartphone Colorimetry: A New Method for an Established Test. *J. Chem. Educ.* **2017**, *94* (7), 941−945.

(15) Christodouleas, D. C.; Nemiroski, A.; Kumar, A. A.; Whitesides, G. M. Broadly Available Imaging Devices Enable High-Quality Low-Cost Photometry. *Anal. Chem.* **2015**, *87* (18), 9170−9178.

(16) Mathews, K. R.; Landmark, J. D.; Stickle, D. F. Quantitative Assay for Starch by Colorimetry Using a Desktop Scanner. *J. Chem. Educ.* **2004**, *81* (5), 702−704.

(17) Cochran, B.; Lunday, D.; Miskevich, F. Kinetic Analysis of Amylase Using Quantitative Benedict's and Iodine Starch Reagents. *J. Chem. Educ.* **2008**, *85* (3), 401−403.

(18) Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W. NIH Image to ImageJ: 25 Years of Image Analysis. *Nat. Methods* **2012**, *9* (7), 671−675.

(19) Bernfeld, P. Amylases, α and β. *Methods Enzymol.* **1955**, *1*, 149−158. (Accessed Sep 2017.)

(20) Poce-Fatou, J. A.; Bethencourt, M.; Moreno-Dorado, F. J.; Palacios-Santander, J. M. Using a Flatbed Scanner To Measure Detergency: A Cost-Effective Undergraduate Laboratory. *J. Chem. Educ.* **2011**, *88* (9), 1314−1317.

DOI: 10.1021/acs.jchemed.7b00468
J. Chem. Educ. 2018, 95, 141−145