

## Laboratory Exercises

# Using a Homemade Spectrophotometer in Teaching Biosciences

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**In this article a homemade spectrophotometer that can be made of inexpensive components available to everyone is presented. This apparatus can be used to implement several low cost standard biochemical and biological experiences like quantitation of biomolecules, the study of biological pigments, and following the time plot of an enzymatic reaction or the growing kinetics of microorganisms.**

Spectrophotometers are usually at hand at University teaching laboratories, but this is not always the case for high school institutions. The same is true of substances that are commonly available in biochemical teaching experiments. In this work, our aim is to show that basic biochemical colorimetric experiments and demonstrations can be done with a homemade spectrophotometer that is easy and inexpensive to build. In the same spirit, we provide examples of experiments that can be performed with ordinary and fairly inexpensive substances.

A spectrophotometer is an apparatus intended to measure the degree of absorbance of light in specific wavelength ranges. It consists of three essential elements, a light source, a monochromator, and a detector. The light source and detector define the limits of wavelength and sensitivity of the apparatus, whereas the monochromator separates the light produced by the light source into different small ranges, usually in the nanometer scale. The sample, placed between the monochromator and the detector (usually in a transparent container called a cuvette), is illuminated by a specific range of wavelengths, and the intensity of the light not absorbed by the substances present in the sample can be quantified by the detector. A more detailed discussion on spectrophotometers and spectroscopy can be found in Refs. 1 and 2.

### THE LAMBERT-BEER LAW

Several substances follow Lambert & Beer's law,  $\ln(I_0/I) = A = \epsilon_\lambda \times l \times c$ , where  $I_0$  is the intensity of the light after it has passed through a sample of medium without the substance under study, and  $I$  is the same except when the sample contains the substance of interest at concentration  $c$ .  $\epsilon$  is a constant for the given substance in a certain medium using the selected wavelength  $\lambda$ , and  $l$  is the length of the light path through the sample.  $A$  is the absorbance of the sample, and  $I/I_0$  is the transmittance of the sample (usually expressed as a percent value).

This simple but useful relationship is used to construct

calibration curves of absorbance *versus* concentration that are subsequently applied to quantitate substances at unknown concentrations. Lambert & Beer's law is valid (*i.e.* the calibration curve is a straight line) within a certain concentration range whose limits are determined empirically. Sometimes the absorbance response in an extended concentration range is slightly curved. In these cases the whole range of concentrations can still be measured by using two lines (*i.e.* two  $\epsilon$  values) to cover two different subranges, as shown below.

### APPARATUS SET-UP

The spectrophotometer is made up of the following elements.

- A light source (a four-battery flashlight (torch) with a halogen bulb is recommended).
- A compact disc (CD).<sup>1</sup> This element will function as the diffraction grating of the monochromator.
- A standard plastic cuvette for visible spectroscopy. If one is not available, it can be constructed as described below.
- A light-dependent resistor (LDR). It can be acquired in electronic supply stores and is inexpensive. This device will act as the light detector. The LDR contains cadmium sulfide in its light-sensitive zone, and it exhibits a variable electric resistance that is a function of the intensity of the light received; it has a greater resistance when less light is received.
- An electronic multimeter that can measure resistance in the range of 1 to 500 K $\Omega$  (a multimeter is a device used to measure different electrical quantities such as current, voltage, resistance, capacitance, etc. It can be obtained in hardware stores; it is also inexpensive).

In constructing the spectrophotometer, the light source must be held fixed and provided with a stable power source. Then, the CD is mounted on a pivoting axis, by attaching it to a screw or other element that will allow the

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<sup>1</sup> The abbreviations used are: CD, compact disc; LDR, light-dependent resistor.

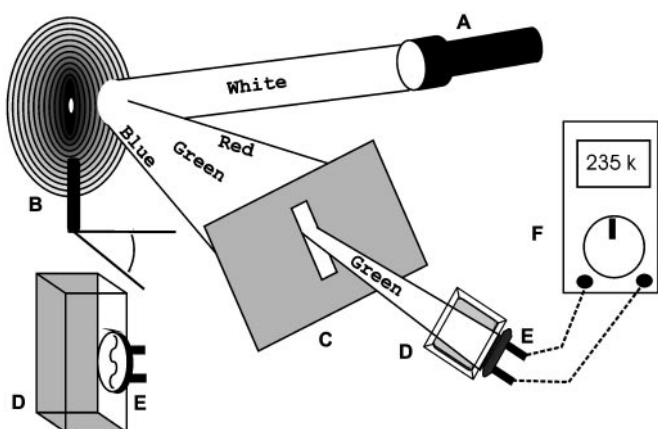


FIG. 1. Schematics of the homemade spectrophotometer and lateral view of the cuvette-LDR assembly (bottom left) are shown. A, flashlight (light source); B, CD (diffraction net) with a protractor under its base; C, slit (two are required); D, cuvette; E, LDR (detector); F, multimeter. See text for details.

operator to rotate it. It is convenient to add a protractor to the CD base, to measure its angular movement. The center of the light beam should impinge on the CD in the middle of its left or right half face, on the side of the grooves.

The plastic cuvette can be borrowed from a lab or made from transparent plastic strips glued with methacrylate. For an LDR of 1-cm diameter or less, a cuvette constructed as a box 4-cm high with a base of 1 cm × 1 cm is a convenient size.

The LDR is a flattened cylinder that has two connectors on its back and a face that must be exposed to the light beam. This face should be glued to one of the faces of the cuvette with methacrylate, with only two drops on each side, avoiding the S-shaped, light-sensitive zone.

The LDR-cuvette assembly is placed in a cardboard box, and two holes are made in the box, a circular hole on its lid just over the cuvette to introduce the samples and a slit that is about 0.5-cm wide, so it can act as the first element of a collimator of the diffracted light. A second cardboard slit is placed inside the box, between the first one and the cuvette (obviously, the LDR-cuvette assembly and the two slits must be aligned). A white paper strip is placed outside of the box, over the first slit, to assess the color of the light entering the detector.

Finally the connectors of the LDR are connected to the multimeter, which is set to measure in the range of one to several hundred K $\Omega$ . The mounting of the apparatus is displayed in Fig. 1. To introduce and remove the sample, a dropper or transfer pipette is used.

The apparatus was preliminarily tested with methylene blue. In Fig. 2A, calibration curves generated with the same concentration standards using an ordinary commercial spectrophotometer and the homemade spectrophotometer are displayed. In Fig. 2B, the readings of both devices are compared. It can be seen that there is a strong linear relationship among readings between 0 and 1.25 absorbance units for this substance at the indicated wavelength.

#### EXPERIMENTAL PROCEDURES

The experiments described below are simple variations on a large list of similar ones that are readily available in the

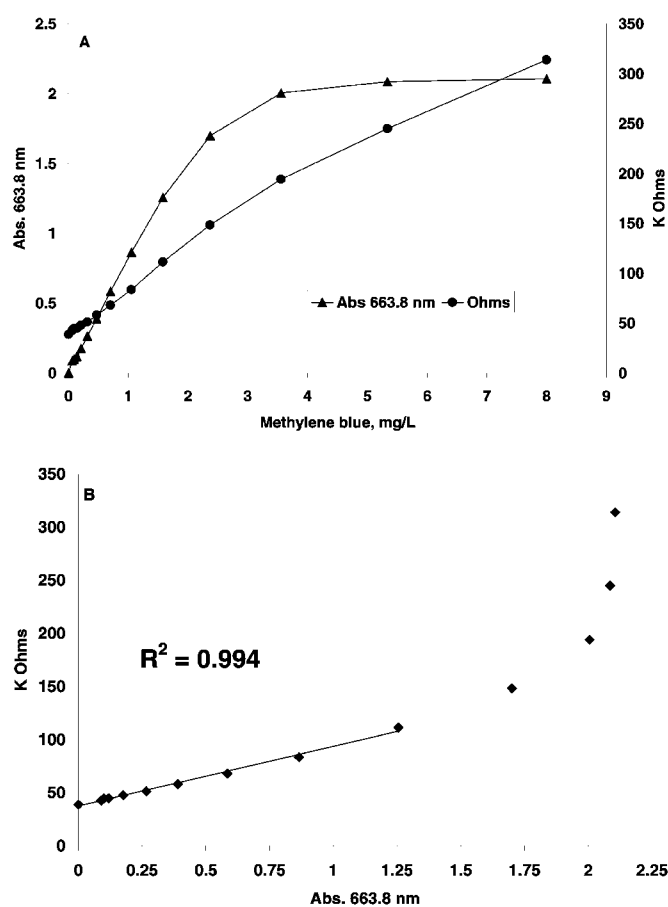


FIG. 2. Readings from a calibration curve of methylene blue made with a commercial spectrophotometer (in absorbance units, Abs) and with the homemade spectrophotometer (in Ohms). Comparison of both responses (A) and range of linear relationship between them (B).

literature. They are included only to provide tested examples that the reader can implement in his/her apparatus without further modification.

These experiments were designed to cover some important biochemical and biological topics with inexpensive and easily obtainable materials. They also constitute an introduction to the use of the spectrophotometer. Last but not least, they can help the students to develop laboratory skills, because they will have to carry out serial dilutions, timed sampling, etc.

Because this device is so simple, it is also useful for showing students how spectrophotometers work. Indeed, commercial spectrophotometers, which should not be opened by the user, have many ancillary devices that complicate appreciating the working principle of the spectrophotometer.

#### Study of Biological Pigments

To stress the relationship between the green color of chlorophyll and its biological function in photosynthesis, a spectrum of its absorbance in the visible range is included as a laboratory experiment in many basic biochemistry and biology courses.

Leaves from Swiss chard (spinach can also be used), whose dominant pigment is chlorophyll, were cut in slices and crushed, then water was added and mixed, and the

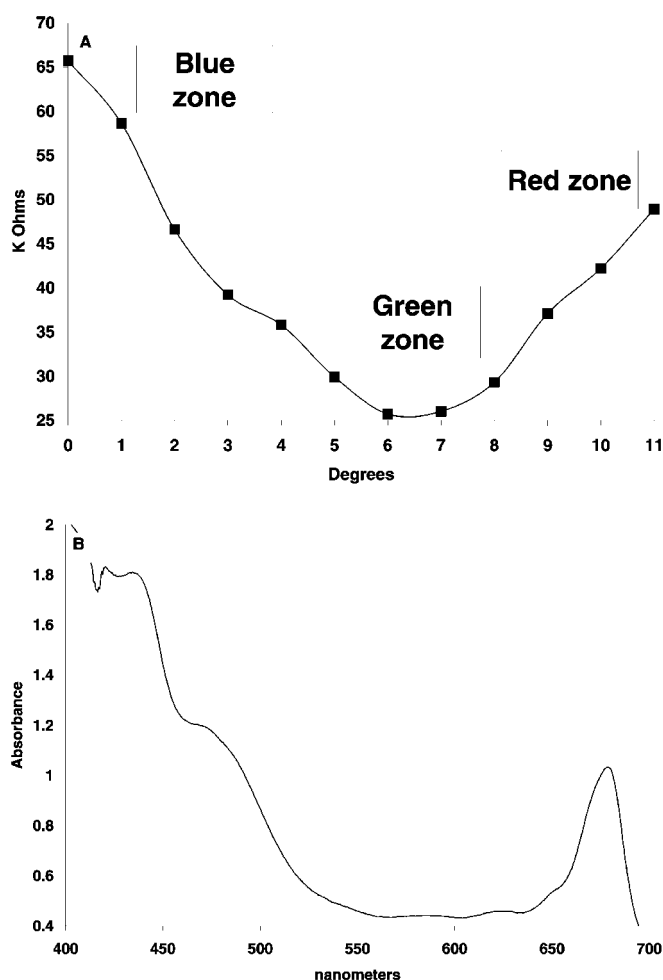


FIG. 3. Spectrum of chlorophyll made with ten readings from the homemade spectrophotometer (A). Compare with the central zone of a spectrum in the visible range made with a commercial spectrophotometer (B).

extract was filtered through cotton or fiberglass. The resulting solution was kept on ice until readings were made. The CD was placed at such an angle that the red extreme of the diffracted light spectrum passed through the monochromator. Then a reading of only water and another of the chlorophyll solution was made. After that, the CD was twisted  $1^\circ$ , and the process was repeated until measurements were made through the entire available spectrum. Results are shown in Fig. 3.

#### Quantitation Using a Standard Curve

These exercises are intended to introduce students to the generation and use of a standard curve. First, the students should be introduced to the most relevant properties of light and the characteristics of its interaction with matter, especially Lambert & Beer's law (see above). The functioning of a spectrophotometer and the reasons to use a blank in spectrophotometry should also be explained to them. Finally, the principle of color development of the assay to be performed should be addressed.

**Starch Curve**—Triiodide ion ( $I_3^-$ ) has a light yellow/red-dish color that becomes dark black/bluish when it forms a complex with starch, which dramatically increases its absorbance at long wavelengths (red). The reagents required

for generating this curve are as follows.

- Potassium iodide stock solution, dissolve 24 g of potassium iodide in 2 liters of water.
- Iodinated iodine solution, dilute 1 g of  $I_2$  in 1.65 liters of the potassium iodide stock solution. If solid iodine or potassium iodide are not available, they can be replaced by properly diluted Lugol solution or iodinated iodine solution from a pharmacy.
- Starch stock solution, 10 g per liter of water. It has to be boiled until the starch dissolves completely. Pure starch is sold for cooking or ironing, but if it is not available, flour can be used instead; in this case, after a half-hour of boiling the solution must be filtered through ordinary absorbent paper to remove the undissolved material.

To generate the standard curves, 3 ml of starch stock solution was diluted with water to a final volume of 25 ml, yielding a concentration of 1.2 g/liter. This solution was subsequently diluted in nine serial dilutions of 2 ml of the standard solution added to 1 ml of water to make the next standard. One ml of the iodinated iodine stock was added to 1 ml of each standard. The blank was prepared by adding 10 ml of the iodinated iodine stock to 10 ml of water.

As a preliminary experiment, several readings were taken using the most concentrated sample and a blank in the red zone, twisting the CD between readings. The angle of maximal reading (taken as the subtraction of the resistance value exhibited by the blank from the value of the standard) was selected, and the CD was fixed in this position. To generate the standard curve, the blank was measured in the apparatus, followed by successive standard solutions, from the most diluted to the most concentrated. As the data points followed a slight curve, two lines were fitted to different concentration ranges to cover all the concentrations measured. To fit the lines, ordinary curve-fitting software can be used, but in the laboratory experiment, students just drew the data points on graph paper and obtained eye-fitted approximate lines that subsequently were employed for quantitation of the samples. Their estimations were not substantially different from those obtained using a computer. Results are shown in Fig. 4.

Finally, samples of starch solutions of different and unknown concentrations, in the range of the calibration curve and above, were provided. Some estimated dilutions were made from them and processed as described above. Dilutions fell in the range of the standard curve, as judged by visual inspection, measured in the apparatus, always using the aforementioned blank.

**Protein Curve**—When proteins are placed in an alkaline system containing  $Cu^{2+}$ , a colored complex can form between the peptide bonds and the copper ions. This is the basic Biuret reaction that can be used to quantitate proteins in the milligram range. To find out more about spectrophotometric methods for measuring proteins, see Ref. 3.

The recipe to prepare the Biuret reagent is given below, although this low cost reagent can be readily obtained from laboratory suppliers. The method of Lowry *et al.* [8] could also be used if the reagents are available, but we have found that Biuret is good enough and simpler for

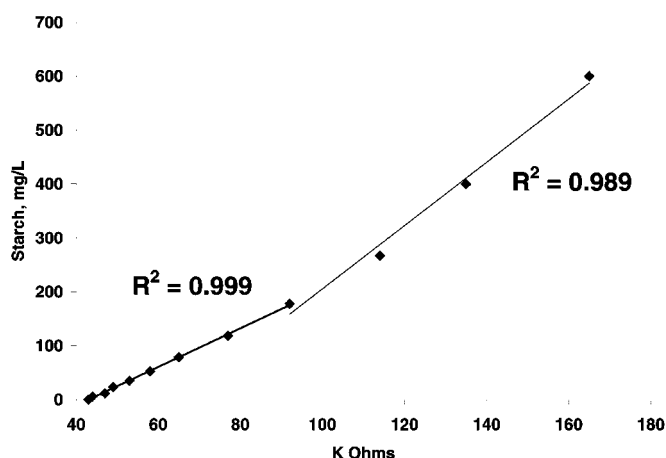


FIG. 4. **Starch standard curve.** Points represent final concentration in the cuvette, and lines are linear regression fittings adjusted to two contiguous concentration ranges.

demonstration purposes. The required reagents are as follows.

- Gelatin stock solution, commercial dry gelatin; dissolve 3 g in 1 liter of boiling water.
- Gelatin standard solutions, make dilutions of the gelatin stock to cover the range 0.25–3 g/liter.
- Biuret reagent, place 45 g of sodium potassium tartrate and 15 g of  $\text{CuSO}_4 \cdot \text{H}_2\text{O}$  in a 1-liter volumetric flask. Fill it about 3/4 full with 0.2N NaOH, and shake to dissolve. Add 5 g of potassium iodide, let it dissolve, and then bring the total volume to one liter with 0.2 N NaOH. Biuret reagent does not degrade with time.

As before, a preliminary experiment was performed to determine the absorbance maximum of the Biuret reagent acting as a protein stain. One ml of the gelatin stock and 2 ml of the Biuret reagent were mixed and allowed to stand for 5 min. Then the sample was read (against a blank of Biuret reagent diluted with water in the same 2:1 ratio) several times, twisting the CD between readings. The grating angle of maximum reading was chosen, and all the standards were processed as described above and read at that angle (results can be seen in Fig. 5). Finally, unknowns made of gelatin, egg white, and meat extract were processed in a way similar to that described for the starch standards.

**Time Course of an Enzyme-catalyzed Reaction**—The kinetics of enzyme-catalyzed reactions can be illustrated using the human salivary amylase. The required reagents are the starch and iodine solution described previously and an amylase solution, which is prepared just before the experiment is to be done, by taking a sip of water and trying to secrete saliva for 5 min. Then this liquid is filtered through ordinary absorbent paper to obtain a clear saliva solution.

The time course of the enzyme-catalyzed reaction was obtained as follows: 3ml of saliva solution were added to 60 ml of the starch stock solution and mixed thoroughly. Then, 3-ml aliquots of this reaction mixture were withdrawn at 1-min intervals and placed in a 90 °C bath for 5 min to stop the enzymatic reaction. Then, the solution was mixed with 25 ml of iodine solution and kept on ice until the

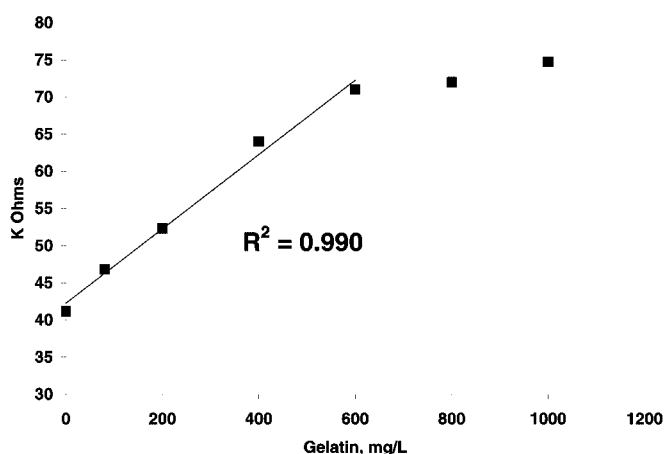


FIG. 5. **Protein standard curve.** Points represent the final concentration in the cuvette, and the line is a first order polynomial adjusted to the first five points, because the response shows saturation for higher concentrations.

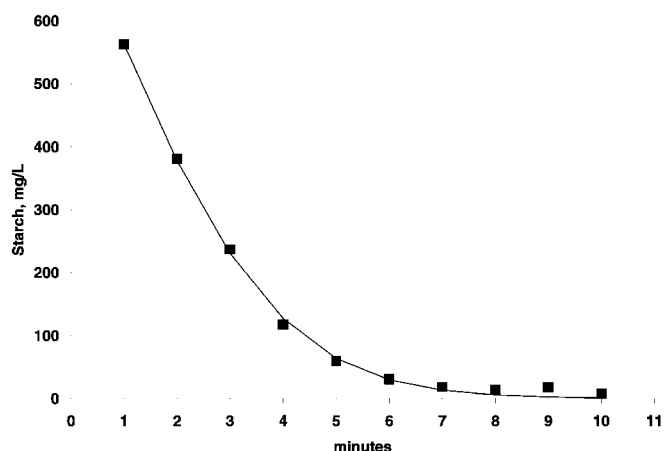


FIG. 6.  **$\alpha$ -Amylase kinetic plot with readings translated to concentration units using the standard curve of Fig. 4.**

measurements were made. Finally, readings were converted to concentration units using the starch curve described previously. Results are shown in Fig. 6.

#### FINAL REMARKS

The spectrophotometer described in this article is inexpensive and easy to construct and use. Probably the most important fact to be aware of when figuring out how to assemble it is that its pieces should be well fixed and not move at all during the experiments, except when the operator wants to change the CD angle. It is also critical to provide the light source with a very stable power source.

The main drawbacks of this apparatus, if compared with a commercial spectrophotometer, are as follows: its lack of resolution (turning the CD a single degree corresponds to around 25 nm), its lack of identification of the wavelength in use (you can select green light, but you cannot select 600-nm light), and that it does not provide absorbance but resistance values. Nevertheless, resolution can be improved with narrower slits and a longer CD to LDR distance (this, in turn, will require a stronger light source). Lack of resolution is not important for the experiments proposed here if they are performed as described, and

resistance values can be used instead of absorbances for all practical means as long as their concentration dependence is found to be linear (as Lambert & Beer's law predicts).

The experiments described here were selected to suggest the diversity of biological phenomena that can be addressed in the laboratory using a spectrophotometer. They are very cheap and easy to implement and provide satisfactory results, even in unskilled hands. They were devised to be introductory and semi-quantitative, and besides their didactical value, they also may serve to train students in laboratory and cognitive skills.

Even more advanced experiments can be performed using this apparatus. For example, the kinetic parameters ( $K_m$  and  $V_{max}$ ) of human salivary amylase can be estimated from Fig. 6. The homemade spectrophotometer can also be used to follow the exponential growth kinetics of yeast and bacteria in liquid media under non-limiting conditions. Microorganisms do not really form a solution or absorb light; they form a suspension that scatters light. Nevertheless, a linear response to biomass concentration can be obtained from a spectrophotometer if an adequate biomass range is used [7]. This apparatus we have described

can be a valuable asset where commercial spectrophotometers are unavailable. However, many students and even faculty who have access to a commercially built spectrophotometer would find building this device to be an educational experience in itself.

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