

Biology 3B LABORATORY

Quantitative determination of chlorophyll using spectroscopy

Objectives

- Gain an understanding of the Beer-Lambert Law
- Use this principle to quantify the concentration of total chlorophyll in a solution
- Investigate the effect of light on the concentration of chlorophyll in leaves
- Learn to use the Beckmann DU 700 spectrophotometer

Introduction

When a liquid appears colored, some wavelengths of the visible light passing through it are absorbed and others are transmitted. It is possible to measure the amount of light absorbed (or transmitted) using a spectrophotometer. Last semester you used the spectrophotometer many times. For instance, you measured the rate of diffusion of potassium permanganate into an aqueous solution by taking periodic measurements of the optical density at a specific wavelength. In this lab we will use the spectrophotometer to accurately determine the chlorophyll concentration in leaves.

First, we need to consider how spectroscopy works. Absorbance is a measure of the light absorbed by a solution. Generally, we specify the wavelength of the absorbance in nanometers (nm) as a subscript to the capital letter "A." For instance, the absorbance at 645 nm would be A_{645} . Absorbance is defined by the Beer-Lambert law:

$$A = k L C \quad (1)$$

where k is the molar extinction coefficient (L/mole·cm), L is the path length (cm), and C is the concentration (mole / L)¹¹. This law allows us to use the spectrophotometer in a quantitative way. If we know k and L , and we measure A , we can calculate the concentration. For most measurements, the path length will be 1.0 cm (observe one of the cuvettes we are using). Thus, we can simplify our equation to

$$C = A / k \quad (2)$$

where concentration equals absorbance divided by the extinction coefficient. Deriving the extinction coefficient is time-consuming work. One needs to make up known concentrations of the molecule of interest and then measure the

¹ In most of today's chemistry textbooks the term *extinction coefficient* has replaced the term molar extinction coefficient. However, it is worth noting that the measurement is related to molar concentration. In addition, I am using the variable "k" for extinction coefficient since our Beckmann spectrophotometers and the cited references use that variable name. You may see the Greek letter ϵ (lower case epsilon) used in your chemistry or physics book.

absorbance of these various concentrations. However, many extinction coefficients are already known. Luckily, for us that is, chlorophyll is well documented.

MacKinney (1941) measured the extinction of acetone extracted chlorophyll at wavelengths between 425 and 680 nm. From his work we can determine the molar extinction coefficients for both of the common chlorophyll molecules (chlorophyll a and chlorophyll b). When two molecules are present and contributing to the absorbance we can add their absorbances together to get the total absorbance. For instance, the absorbance of a chlorophyll a solution with a path length of 1 cm would be

$$A = k C_a \quad (3)$$

where A is the absorbance and k is the absorbance coefficient and C_a is the concentration of chlorophyll a. If chlorophyll a and chlorophyll b are both present in the solution we can simply add the absorbances

$$A_{a+b} = (k_a C_a) + (k_b C_b) \quad (4)$$

Note that the k is specific to the molecule and the wavelength.

In MacKinney's (1941) article you will find the extinction coefficients in Table 2. MacKinney uses some older terminology in his paper. The wavelengths are stated in angstroms (Å), a unit that is no longer used. A nanometer (nm), the preferred metric unit, is $0.1 \times \text{Å}$. Also, MacKinney uses the letter k to indicate the extinction coefficient (k). Inspection of the table will show that the highest absorbance by chlorophyll a occurs at 663 nm (column 4 in Table 2) and the highest absorbance of chlorophyll b occurs at 645 nm (column 5). Thus you now have two formulas, one at each wavelength (663 and 645 nm), for absorbance of chlorophyll. One formula favors the chlorophyll a and one favoring the chlorophyll b:

$$A_{645} = (k_{a645} C_a) + (k_{b645} C_b) \quad (5)$$

and

$$A_{663} = (k_{a663} C_a) + (k_{b663} C_b) \quad (6)$$

We can look up the extinction coefficients (k values in columns 2 and 3 of Table 2 in MacKinney, 1941) and write equations 5 and 6 with their respective values. I'll do the first one for you (absorbance equals 645 nm):

$$A_{645} = (16.75 \cdot C_a) + (45.60 \cdot C_b) \quad (7)$$

You can complete the second equation

$$A_{663} = (\text{_____} \cdot C_a) + (\text{_____} \cdot C_b) \quad (8)$$

Now, it's time for a little algebra. Here you have two equations (7 and 8) each with two variables. You can solve for C_a and C_b . You solve one of the equations for C_a and substitute this solution into the other equation. Then solve for C_b . Write that equation below. Next substitute this into the other equation and solve for C_a . Write this equation below.

$$C_a = \quad (9)$$

$$C_b = \quad (10)$$

Okay, we're almost there. The total concentration of chlorophyll will equal the contribution from C_a and the contribution from C_b . So add the two equations and write the result here

$$C_a + C_b = C_{\text{total}} = \quad (11)$$

We will use this equation in our determination of chlorophyll in this experiment.

Procedure

Preparation of leaf discs

In this experiment we will test the hypothesis that the chlorophyll concentration in leaves on a plant held in the dark will differ from that of leaves of a plant held in the light. The plant we will use is the common zonal geranium, *Pelargonium x hortorum*. The "x hortorum" refers to the hybrid nature of this plant. It is the result of crossing several species of *Pelargonium*. You will need two equal sized test leaves, one from the dark adapted plant, one from the plant exposed to the light. Be sure to find out how long the plant was held in the dark. From each test leaf, we will obtain sixteen 6.1 mm leaf discs using a punch. Place two discs into scintillation vial and add 5 ml of 80 % (v/v) acetone. Label each vial. Place all sixteen vials into the refrigerator (4° C) until the next lab period. The extraction will take place during this time.

Measurement of chlorophyll

You will find five Beckman DU 700 spectrophotometers in the lab. There should be no more than three groups using each spectrophotometer. On the left rear of the machine you will find the power switch. Turn the machine on and wait as it goes through the self test procedure.

When this is complete and the main menu is visible, you may insert your flash drive into the port on the right rear of the machine.

The screen on this machine is active, meaning you will press it to make your selections. Here's the procedure:

1. Choose **Fixed Wavelength** from the **Main Menu**.
2. Touch the **Options** button.
3. Make sure the **Store on/off** is in the “on” position.
4. Touch the **More** button in the **Options** screen
5. Touch the **Concentration** button
6. Choose “**on**”
7. Touch the **Concentration Formula** button
8. Touch the **Formula** button again
9. Select **$K_1A_1 + K_2A_2$**
10. Touch λ_1 and key in 645
11. Touch λ_2 and key in 663
12. Touch **K_1** and key in your coefficient for 645 nm
13. Touch **K_2** and key in your coefficient for 663 nm
14. Touch **OK**
15. Touch **Return**

You are now ready to begin reading the samples. Transfer about 3 ml of acetone into one of your cuvettes. This will be the blank. Transfer about 3 ml of one of your sample into the other cuvette. Place the blank into the cuvette holder and close the lid. Press **Blank**. Open the lid and remove the blank and insert the cuvette containing the unknown. Close the lid and press **Read**. The concentration will be displayed. I would write this down, if I were you, since you might not get the store mode to work on your first try. Open the lid and remove the cuvette. Pour the contents of the cuvette back into the scintillation vial and fill the cuvette with the next unknown. Repeat the reading steps for all of your samples. Now press **Exit**. This forced the machine to save these samples as a single file.

Save data to your flash drive

From the **Main Menu**, touch **Recall Data**. Touch **Options**. Touch the computer icon. Choose the **All Data** radio button. Then touch **OK**. Assuming you have followed directions, the data sets are now stored on your flash drive, with the actual absorbance values, and the time and date of each sample. You may now touch **Recall Data**, again. Touch **Options**. Choose **Delete**. Choose the **All Data** radio button. Then touch **OK**. The spectrophotometer is now cleared of all stored data. If you are the last group to use the spectrophotometer, please turn it **Off**.

Calculations and Abstract

From this experiment you will complete an abstract and a figure. The values you have calculated are the concentration of total chlorophyll in mg/L. However, your total volume was only 5 ml for two leaf discs. You should calculate the total leaf surface area in each vial. You should calculate the chlorophyll as $\mu\text{g}/\text{mm}^2$. Your figure should be a bar graph with two bars, one showing the concentration

of chlorophyll in the plant held in the dark, one showing the concentration of chlorophyll in the plant kept in the light. Error bars should show the 95% CI.

You must complete an **abstract** and a **figure** in the standard format. This is a collaborative project, so one abstract and one figure will be submitted from each research team (two people, maximum). Please print the abstract and figure on a single piece of paper.