Quantification of Chlorophyll A Using UV-Vis Absorbance Spectroscopy

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Introduction

Students in Hamline's *Advanced Laboratory Techniques* course will learn about UV-Vis Absorbance Spectroscopy by quantifying chlorophyll, which they will extract from green plant material. To determine the concentration of their extract, students must identify that they need to create a calibration curve using standard chlorophyll A solutions. The work presented here is the preparation for this experiment, including extraction, standard generation, and instrument calibration.

Chlorophyll A quickly degrades when exposed to oxygen and light. When extracting chlorophyll from plant material, it is important to minimize degradation by working in low light and limiting exposure to oxygen. Rate of degradation also depends on the type of solvent used to extract the chlorophyll. Because chlorophylls are fat-soluble, organic solvents are ideal for their extraction.[1] Previous experiments have found that using ethanol creates a more stable extract than acetone or methanol.[2,3] For this experiment, students will use measurements at 666 nm, as chlorophyll A has a characteristic absorbance in the mid-660 nm range, which differentiates it from other species in solution.[2,4]

(a) (b)

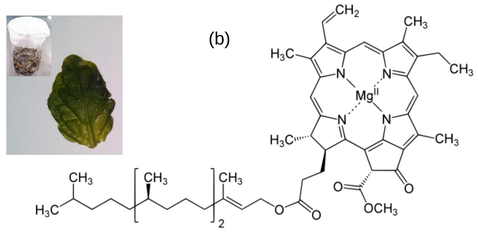
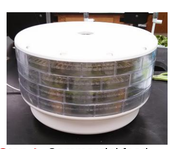


Figure 1. (a) Spinach and Extract, (b) Chlorophyll A Structure

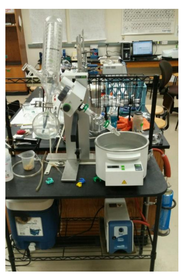
Methods

Preparation of Chlorophyll Extract Sample

The flowchart below illustrates the steps taken to extract chlorophyll A from baby spinach. This extract will be used to make standards for the *Advanced Laboratory Techniques* course.



Step 1: Commercial food dehydrator used to dehydrate about 900 g of baby spinach.



Step 4: Ethanol removed by rotary evaporation at 26°C to 30°C.

Step 2: Dehydrated spinach and ethanol placed in a commercial blender to macerate sample.

Step 3: Solid particles removed by vacuum filtration, resulting in 75 mL of solution.

Step 5: Remaining extract is a mixture of plant oils, chlorophylls, carotenoids, and plant material.

Calibration Curve Preparation

A 25.00 mL stock solution containing 1 mg of pure chlorophyll A (Sigma Aldrich) and 190-proof ethanol was prepared. Seven standard solutions were made from 1.79×10^{-7} M to 1.79×10^{-5} M. Two UV-Vis spectrometers were used: HP 5452A Diode Array and Ocean Optics Fiber Optic USB 4000. Three spectra were collected per standard solution, and this data was used to create calibration curves for each instrument at 666 nm. Solutions were stored by wrapping ParaFilm-sealed flasks in aluminum foil to minimize evaporation and photodegradation.

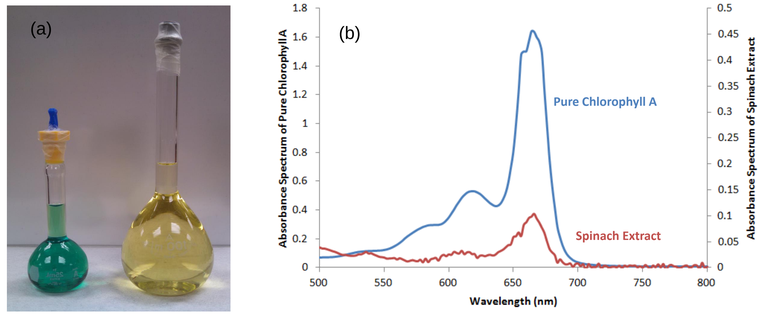


Figure 2. (a) 40 μg/mL chlorophyll A and 40 μg/mL spinach extract (b) Absorbance spectra of 40 μg/mL pure chlorophyll A (—) and 40 μg/mL spinach extract (—).

Quantification of Chlorophyll A in Extract

Fifty milliliters of ethanol were added to 8 mg of chlorophyll extract. Three solutions of this concentration were made, and three absorbance spectra were taken per solution. This data was averaged and then used to quantify the amount of chlorophyll in the extract.

Results

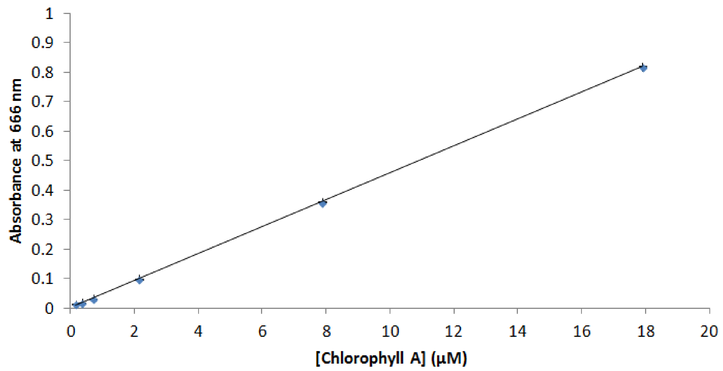


Figure 3. Calibration Curve for Quantification of Chlorophyll A (Chl A) Using the HP 5452 A Diode Array Spectrophotometer. A 1-cm quartz cuvette was used in the collection of the spectra.
Calibration Curve Equation: $A_{666} \pm 0.002_6 = (0.0457_0 \pm 0.0001_7)(\mu M^{-1})[\text{Chl A}] + (0.000_4 \pm 0.001_3)$;
$R^2 = 0.99995$

Key Points from Figure 3

- The relationship between Absorbance and [Chl A] is linear between 1.79×10^{-7} M to 1.79×10^{-5} M.
- The limit of detection for chlorophyll A on this instrument is 0.164 μM.
- The molar absorptivity for chlorophyll A at 666 nm is 45700 ± 200 $M^{-1}cm^{-1}$.
- Using the absorbance from the extract and the calibration curve, it was determined that the concentration of chlorophyll A in the extract solution was $(10.43_3 \pm 0.03_6) \times 10^{-6}$ M and $5.82_7 \pm 0.02_0$% (w/w) of the extract was chlorophyll A.

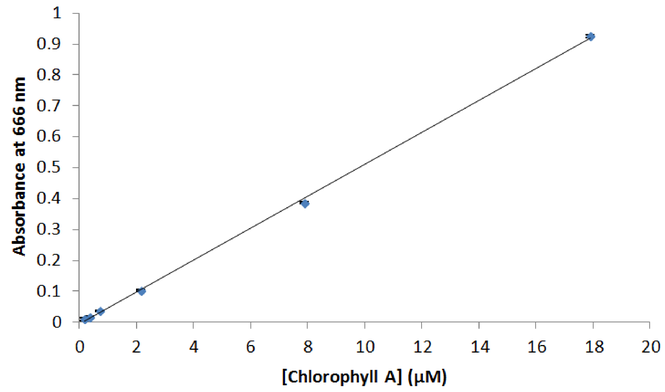


Figure 4. Calibration Curve for Quantification of Chlorophyll A Using the Ocean Optics USB4000 Fiber Optic Spectrophotometer. A 1-cm quartz cuvette was used. Calibration Curve Equation: $A_{666} \pm 0.009_4 = (0.0516_2 \pm 0.0006_0)(\mu M^{-1})[\text{Chl A}] + (-0.004_4 \pm 0.004_8)$; $R^2 = 0.99948$

Key Points from Figure 4

- The relationship between Absorbance and [Chl A] is linear between 1.79×10^{-7} M to 1.79×10^{-5} M.
- The limit of detection for chlorophyll A on this instrument is 0.546 μM.
- The molar absorptivity for chlorophyll A at 666 nm is 51620 ± 600 $M^{-1}cm^{-1}$.
- The concentration of chlorophyll A in the extract solution was $(10.2_2 \pm 0.1_4) \times 10^{-6}$ M and $5.70_9 \pm 0.07_8$%((w/w) of the extract was chlorophyll A.

A paired t-test of the weight percent of chlorophyll A in the extract from both instruments indicated their difference was not statistically significant at the 95% confidence level.

Conclusions and Future Directions

Absorbance peaks at 666 nm were consistent for both instruments through all of the trials using both the pure chlorophyll A sample and the chlorophyll extracted from spinach. When the calibration curves for both instruments were used to quantify the amount of chlorophyll A in the spinach extract, it became apparent that the process did not extract a pure sample.

It would be beneficial for future experiments to run the solution through vacuum filtration twice and/or using varying grades of filter paper to minimize the amount of larger plant material left upon evaporation. In addition, the extract should only be taken from the bottom third of the round-bottomed flask after rotary evaporation. A paper wick might be used to remove plant oil prior to the removal of the extract from the flask.

Literature Cited

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4. Martin, James L. *Hydro-Environmental Analysis: Freshwater Environments.* Texas: CRC Press, 2013: 425-426.

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