

## Absorption Spectrum of Chlorophyll

Group R1

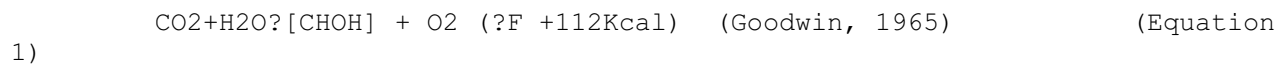
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The absorption spectra of chlorophyll from spinach leaves and okra pods were determined along with the relative amounts of chlorophyll a and chlorophyll b in each of the two plants. The chlorophyll was extracted using 100% acetone and the absorption spectrum was determined over a range of 400-700 nm using a Spectronic 20D spectrophotometer. The relative amounts of chlorophyll a and chlorophyll b were determined using equations from the literature based on the absorbance data. Plots of absorbance versus wavelength were generated and the location of the peaks determined the wavelengths at which the chlorophyll extracts maximally absorbed. The chlorophyll extracts from both spinach and okra had two absorbance peaks. The mean absorbance maxima for spinach were  $(430 \pm 3)$  nm and  $(662 \pm 3)$  nm while those of okra were  $(430 \pm 4)$  nm and  $(664 \pm 4)$  nm. Normalization of the spectra from the spinach and okra extracts did not show the spectra of the two plants to be statistically different. Use of the literature equations gave chlorophyll a to chlorophyll b ratios of  $1.49 \pm 0.18$  for spinach and  $1.67 \pm 0.45$  for okra, and a t-test at the 95% confidence level did not prove the two ratios statistically different. The ratio for spinach was significantly lower than the literature value of  $4.02 \pm 0.79$  while the single literature value available for okra falls just outside the uncertainty interval of the experimentally determined chlorophyll ratio. Degradation of chlorophylls to pheophytins during storage is a likely reason for the discrepancies observed in spinach.

The main classes of plant pigments in nature are chlorophylls, carotenoids, and

anthocyanins (Gross, 1991). Chlorophylls are the pigments responsible for photosynthesis, the fundamental process of life that converts light energy into chemical energy (Gross, 1991). The absorption of light by different pigments causes excitation of electrons from their ground state to an excited state. Light absorption takes place at the reaction centers of photosystems that contain accessory and primary pigments. Currently, photosystems I and II are the two accepted photosystem units (Green, 1984). Carbohydrates and other organic compounds are produced from water and carbon dioxide in the presence of light (Gross, 1991). In green plants, the photoreduction of carbon dioxide to an organic compound is accompanied by the liberation of oxygen from water. Photosynthesis occurs by adding light to the following equation.



This ability of green plants to synthesize organic compounds makes them the producers of the biosphere.

Chlorophylls are found in the chloroplasts of plant cells, more specifically, on the interface between hydrophilic and hydrophobic layers where they form bimolecular layers. The participation of chlorophylls a, b, c, d, e, protochlorophyll, bacteriochlorophyll and bacteriochlorophyll make photosynthesis possible. The pigments are concentrated in the chloroplast particles with an average concentration of  $10^{-1}$  M. The average distance between molecules in the grana is on the order of 50 Angstroms.

All green plants contain chlorophyll a and chlorophyll b in their chloroplasts. Chlorophyll b differs from chlorophyll a by having an aldehyde (-CHO) group in place of a methyl group (-CH<sub>3</sub>) (Gross, 1991). This aldehyde group is also the reason that chlorophyll b has a greater molecular weight than chlorophyll a. Along with chlorophylls, the chloroplast also contains a family of accessory pigments called carotenoids (Campbell, 1996). In higher plants, chlorophyll a is the major pigment and chlorophyll b is an accessory pigment, and the a/b ratio is generally around 3 to 1 (Gross, 1991).

The structural formula of Chlorophyll a is C<sub>55</sub>H<sub>72</sub>O<sub>5</sub>N<sub>4</sub>Mg (see figure 1) with a molecular weight of 893.48 g/mol. Chlorophyll b has a structural formula of C<sub>55</sub>H<sub>70</sub>O<sub>6</sub>N<sub>4</sub>Mg (see figure 2) and a molecular weight of 907.46 g/mol (Paech & Tracey, 1955). The differences in these structures cause the red absorption maximum of chlorophyll b to increase and lower its absorption coefficient (Goodwin, 1965). Both molecules are hydrophobic in the C<sub>20</sub>H<sub>39</sub>OH region and the remaining region is

hydrophillic. Chlorophyll pigments strongly absorb in the red and blue regions of the visible spectrum, which accounts for their green color.

Figure 1. Chlorophyll a (Paech & Tracey, 1955 )

Figure 2. Chlorophyll b (Paech & Tracey, 1955)

In diethylether, chlorophyll a has approximate absorbance maxima of 430 nm and 662 nm, while chlorophyll b has approximate maxima of 453 nm and 642 nm, as shown in Figure 3 below

(Gross, 1991). The exact positions of these maxima vary slightly with the nature of the solvents used

(Gross, 1991). Lichtenthaler and Wellburn found that the red peak maxima of the chlorophylls were

shifted to longer wavelengths with increasing polarity of the solvents. Since chlorophyll b can absorb at

slightly higher wavelengths in the blue region, it is present in increased proportions in plants that grow in

shade (Gross, 1991). It can capture light effectively at low intensity, partially filling the gap in the

chlorophyll a spectrum (Gross, 1991).

Figure 3. Absorption spectra of chlorophyll a and b in diethyl ether

The purity of chlorophyll can be measured by the ratio of the absorbencies of the "blue" and

"red" maxima. In an organic solution, chlorophyll has two absorption maxima in the visible spectrum

with wavelengths shorter than 720 nm. The short-lived fluorescent state is produced by the excitation of

either the blue or red absorption band. Electrons return to their ground states by emission of

fluorescence, dissipation of heat or by a chemical reaction. A possibility of transferring the energy from

one pigment molecule to another also exists. This is referred to as a homogeneous energy migration,

and it results in a depolarization and a quenching of the fluorescence. An excited chlorophyll molecule

transfers one electron to an acceptor molecule in the adjacent lipid layer and recovers it from the protein

layer. Transferring the electron causes the two reaction products (see Equation 1) to be in two different

phases. Enzymatic stabilization and conversion into a carbohydrate and oxygen without immediate

recombination is possible by this mechanism. This mechanism is comparable to electron migration in

crystalline photoelectric conductors (Gross, 1991).

The transfer of energy is highly efficient and can only occur from a pigment absorbing at a

shorter wavelength to one absorbing at a longer wavelength. The fluorescence of chlorophyll a may

correspond to the same yield of light absorbed by chlorophyll b. T.W. Goodwin suggests that the light

absorbed by pigments other than chlorophyll a is transferred to chlorophyll a which then sensitizes photosynthesis. Experiments have indicated that it is necessary to have the simultaneous excitation of chlorophyll a and another pigment in order to have efficient photosynthesis. Accessory pigments are believed to effect the efficiency of photosynthesis by transferring their excitation energy by this resonance transfer to fluorescent chlorophyll a (Goodwin, 1965).

Absorption spectra of the various chlorophylls depend on the solvent used. Kundt's rule states

that the longer the refractive index of the solvent, the longer the wave length of the absorption maxima

(Paech & Tracy, 1955). Lichtenthaler & Wellburn derived equations (2) and (3) in order to determine

the amounts of chlorophyll a and b in individual leaves using a 100% acetone extract. Similar

equations exist for other solvents such as methanol and diethyl ether. The coefficients in equations (2)

and (3) were determined for pure chlorophyll a and b in 100% acetone by finding the locations of their

peaks in the red region (645 nm for chlorophyll b, 662 nm for chlorophyll a) and the values of their

absorption coefficients at these peaks. Assuming that the absorbance by one pigment does not

influence the absorbance by the other, the total absorbance in a mixture of chlorophyll a and b at the

two peaks can be calculated as the sum of the individual absorbances of each pigment. This gives rise

to two equations which can be solved simultaneously to give the concentration of each pigment in ?g/ml

of extract as shown in equations (2) and (3) below. Ca is the concentration of chlorophyll a and Cb is

the concentration of chlorophyll b. These equations are valid for standard square cuvettes with a path

length of 1cm.

$$Ca = 11.75A_{662} - 2.35A_{645} \quad (\text{Equation 2})$$

$$Cb = 18.61A_{645} - 3.96A_{662} \quad (\text{Equation 3})$$

However since acetone extracts would eat through the 1cm-square plastic cuvettes, 1 cm-

circular glass cuvettes had to be used instead. Provided a constant conversion factor exists for

absorbances in square and round cuvettes, the absorbances in equations (2) and (3) measured with

round cuvettes will all be scaled by a constant factor. Thus, for calculating the ratio of chlorophyll a :

chlorophyll b, this factor cancels out and thus absorbance readings from round cuvettes will give the

same results for the ratio as those obtained from square cuvettes.

Leaves are composed of many membranes and cell organelles. The cell wall can easily be

destroyed by physically grinding the leaves, thus exposing the organelles of the plant. Chlorophylls

reside in the chloroplast and are separable by chemical reactions. They are soluble in most strong

alcohol solvents such as acetone (Paech & Tracey, 1955). Extraction of chlorophylls by acetone is adequate for spectrophotometer measurements because chlorophylls are the only light sensitive pigments.

Chromatography is a technique for separating chemical substances. The separation of colored compounds on paper strips requires the use of a porous material that absorbs liquids and solutions (Figure 4). This material is known as the adsorbent and includes materials such as paper, silica gel and alumina. The paper is a self-supporting adsorbent since it is relatively stiff and hangs together (Zubrick, 1992).

#### Figure 4. Paper Chromatography

The relative adsorption rate from a stationary phase to a mobile phase is analyzed for liquids or gases. Partition chromatography is a technique that involves the liquid-liquid phases. The stationary phase is water bounded to granules of the adsorbent and the mobile phase is a water-immiscible solvent (Gross, 1991). Solute molecules are partitioned between the stationary liquid phase and the mobile phase.

Paper chromatography is a widely used technique. Solvents are used in chromatography to separate the constituents by running through the adsorbent. Solvents that are used specifically for this purpose are called eluents. Polar eluents are not held strongly by the adsorbent and are preferable for timely trials. Acetone, ethanol and methanol are the more polar eluents used in paper chromatography. Less polar eluents include petroleum ether and cyclohexane. A problem that commonly arises in chromatography is that the eluent is too strong for the mixture. If the solvent is too polar, it may move the entire mixture with it. Trial and error is commonly used to determine the best eluent to use for a specific mixture. Complete separation of the components of the mixture allows the retention factor ( $R_f$ ) to be calculated. The  $R_f$  value is the distance traveled by the substance divided by the distance traveled by the solvent (Zubrick, 1992). However, running conditions greatly affect  $R_f$  values. Instead, a reference compound should relate the positions of the experimental compound with others. Mobility of individual pigments is affected by the degree of saturation of the tank with solvent, the type of paper used, whether it is equilibrated with the solvent, and the amount of pigment applied (Goodwin, 1965). For chlorophyll, up to eight colored spots can be observed. In order of decreasing  $R_f$  values they are

carotenes (two spots, orange), chlorophyll a (blue-green), the xanthophylls (four spots, yellow) and chlorophyll b (green).

During storage chlorophyll encounters many elements which cause degradation. Chlorophyll a is formed about five times faster than chlorophyll b. At room temperature in vitro chlorophylls will form isomeric chlorophylls a and b, which are formed in vivo by heating. An in vitro sample will experience isomerization in a few hours or rapidly if alkali is added at room temperature. Chlorophyll a and chlorophyll b each have three isomers with magnesium bonded to three different pairs of pyrrole nitrogen atoms. These isomers exist as tautomers rather than as resonant hybrids (Goodwin, 1965).

Also, the original chlorophyll sample may undergo oxidation and produce oxidized products. The isomers and oxidized products account for the chlorophyll-like products that are separable from chlorophyll on chromatographic analysis and they absorb at slightly different wavelengths. Oxygen attack on the isocyclic carbon C-10 also breaks down the pigment. This process causes oxidation of C-10 to hydroxy, then a breaking of the ring to form a variety of purpurins and chlorins. Further oxidation of these derivatives occurs through complete scission of the isocyclic ring, then oxidation of tetrapyrrole occurs (Goodwin, 1965). Other derivatives such as pheophytins a and b, pheophorbides a and b, and pyroderivatives are caused by acidification, hydrolyzation and heat treatment, respectively (Gross, 1991).

Reduction occurs not only by oxidation, but also by changes in temperature, pH and light. Heating degrades the structure and instigates chemical reactions as well as further oxidation. The pH changes during storage and processing leads to chlorophyll degradation. Chlorophyll stability varies as a function of pH. A pH between 6 and 7 is critical. For spinach the critical pH is between 6.7 and 7 (Gross, 1991). Strong light bleaches chlorophyll solutions (Paech & Tracey, 1955). In vitro, photobleaching causes chlorophyll breakdown where the chlorophyll solution is irreversibly bleached by light in the presence of oxygen. Chlorophyll a and b have reaction rates on the same order. The order of magnitude is larger than that of pheophytins and pheophorbides which are more stable. The difference relates to the magnesium attached to the chlorophyll a and b excited triple state molecules. Dim light studies during senescence show that the ratio of chlorophyll a to other chlorophylls decreases while held in darkness. In this environment, direct photochemical degradation of the pigment occurs.

Spinach is a commonly used sample for determining the absorption spectrum of chlorophyll.

Chromatography can separate spinach into its chlorophylls a and b and the following carotenoids: ?- carotene, lutein, violaxanthin, antheraxanthin, neoxanthin, zeaxanthin in order of increasing polarity (Gross, 1991). For fresh spinach samples, the chlorophyll a to b ratio is 4.02 ? 0.79. With storage, this ratio can be expected to change due to the faster degradation of chlorophyll a (Goodwin, 1965). A single study has found okra to have a rather low chlorophyll a : chlorophyll b ratio of 1.208 (Gross, 1991).

1. Spinach leaves
2. Okra Pods
3. Distilled Water
4. 100% Acetone
5. Chloroform
6. 100% Ethanol
7. Whatman Chromatography Paper (2cm x 100m reel, 3mmChr)
8. Circular glass cuvettes
9. Square polystyrene cuvettes
10. 50ml polypropylene tubes (with caps)
11. Micropipette
12. 100 ml measuring cylinder
13. Aluminum foil

1. Milton Roy Spectronic 20D Spectrophotometer ( range: 400-700 nm, wavelength accuracy = ? 3 nm, absorbance accuracy = 0.5 percent transmittance)
2. Lab View (Interface designed by Al Giandomenico)
3. IEC -7000 Centrifuge
4. Absocol Refrigerator
5. Ultraviolet Lamp

The spinach leaves were cut into small pieces which were then ground with a mortar and pestle. Okra pods were freed from seeds, cut into small pieces and similarly ground with a mortar and pestle. The plant pulp thus obtained was then shaken with the solvent in a 50 ml polypropylene tube, whose sides were wrapped with aluminum foil to minimize exposure to light. A volume of roughly 5 ml solvent per gram of plant pulp was used. With the okra, it was necessary to leave the sample standing for at least an hour to extract a substantial amount of chlorophyll. However, for spinach, shaking with the solvent for 1-2 minutes would yield a relatively large amount of chlorophyll. The extract was then

centrifuged in the IEC- 7000 at 12°C at 3000 rpm for 15 minutes (a longer time of 20 minutes was used for the okra extracts). The residue was discarded and the supernatant was transferred to another 50 ml tube (with the sides wrapped with aluminum foil) which was promptly shut to minimize evaporation of solvent. With spinach extracts, 20 fold dilutions usually gave absorbance peaks within the range of the spectrophotometer while no dilution was needed for the okra extracts. The cuvette containing the sample was sealed using aluminum foil and adhesive tape and absorbance readings were then taken over the 400-700 nm range with more data points being taken in the region of the peaks of chlorophyll a and b. Four separate extractions of both spinach and okra were performed and the resulting absorption spectra were recorded. The different spinach spectra were compared by normalizing the peaks in the blue (430 nm) region and a similar normalization procedure was used for the four okra extracts. One spinach extract and one okra extract were randomly chosen and the spectra were compared using normalization.

To verify that acetone would be the most appropriate solvent for chlorophyll extraction, the absorption spectra obtained with spinach extracts having water and acetone as solvents were recorded. Equations (2) and (3) were available to determine the absolute concentration of chlorophyll a and b from a 100% acetone extract using the absorbance readings obtained at 645 nm and 662 nm (Lichtenthaler & Wellburn, 1983). However, these equations were valid only for 1 cm square cuvettes, and only 1 cm circular glass cuvettes were available (acetone would dissolve the 1 cm square plastic cuvettes). A comparison between absorbance readings from the two types of cuvettes was performed using a spinach water extract to determine whether any conversion factor existed.

In order to verify whether the chlorophyll would degrade during storage in the refrigerator, a refrigerated spinach extract obtained using 100% acetone was tested one week later using once again a 20-fold dilution and the two spectra were compared.

Paper chromatography was performed on one of the spinach extracts. A line was drawn approximately 3 cm from the end of the chromatographic paper strip using a pencil. One drop of the chlorophyll extract was applied at the center of the line using a micropipette. Approximately 5 ml of eluent (both chloroform and ethanol were tested as eluents) was placed inside a 100 ml measuring cylinder, and the end of the chromatographic paper strip was dipped inside the eluent, with the drop of extract above the level of eluent. The cylinder was then sealed using aluminum foil and adhesive tape and the eluent was allowed to move up the strip until the solvent front was near the top end of the paper



(Zubrick, 1992). The resulting chromatogram was viewed using an ultraviolet lamp. With optimum separation, it is possible to see up to 8 bands showing the different chlorophylls, carotenes, and xanthophylls.

#### Absorption Spectra of Chlorophyll from Spinach Leaves

Figure 5 shows the absorption spectrum for one trial of spinach in 100% acetone. The ratio of spinach leaf mass to acetone volume used was roughly 1 g : 5 mL (Appendix 1), and the extract was diluted 20-fold for measurement taking.

Figure 5. Absorption Spectrum of Chlorophyll from Spinach Leaves

Figure 5 shows that the chlorophyll extracted from spinach has absorbance maxima in both the blue and red regions of the visible spectrum. In the blue region chlorophyll absorbs maximally at  $(430 \pm 3)$  nm, while in the red region it absorbs maximally at  $(662 \pm 3)$  nm. Also evident is a slight shoulder on the peak in the blue region at a wavelength around 452 nm. The uncertainty in the wavelength setting for all measurements with the Spectronic 20D is  $\pm 3$  nm, while the uncertainty in the absorbance measurements comes from the Spectronic 20D's uncertainty of 0.5 percent transmittance (Appendix 2).

Four separate extractions of chlorophyll from spinach leaves were performed, and one spectrum was run for each of the extractions. The last three extractions came from the same batch of spinach, while the first extraction was from a different batch of spinach. All four extracts were prepared using roughly a 1 g : 5 mL ratio of spinach leaf mass to acetone volume and were diluted 20-fold for measurement taking. The four absorption spectra are plotted in Figure 6.

Figure 6. Comparison of Spectra from Different Spinach Extracts

The range in absorbance values at a given wavelength for the four spectra indicates that varying amounts of chlorophyll were extracted. However, all four extracts had blue absorbance maxima of  $(430 \pm 3)$  nm and red absorbance maxima of  $(662 \pm 3)$  nm (Table 1). Extract 1 was performed first, before increasing the frequency of measurements, and thus it has fewer data points.

In order to accurately compare the spectra from separate extracts, it is necessary to normalize the absorbance values. The spectra in Figure 6 were normalized using Extract 4, the one with the largest absorbance values, as the basis. Normalization was done by multiplying the absorbance of each data point of a given extract by the ratio of the peak blue absorbance of the basis to the peak blue

absorbance of the extract being normalized. The normalized spectra are shown in Figure 7 with error bars omitted for visual clarity.

Figure 7. Comparison of Spinach Extract Spectra using Normalization

Figure 7 shows that the absorption spectra from the four different extracts of spinach agree very closely.

This indicates that the spinach data is very precise and that the acetone extraction procedure is consistent in its removal of chlorophyll pigments from spinach leaves. Though the error bars are not shown, the method for calculating the uncertainty of a normalized absorbance value is shown in Appendix 2.

Absorption Spectra of Chlorophyll from Okra Pods

Figure 8 shows the absorption spectrum for one extraction of chlorophyll from okra pods using 100% acetone. The ratio of okra pod mass to acetone volume was 1 g : 5 mL, the same as for the spinach leaves, however absorbance measurements were taken on the undiluted okra extract solution.

Figure 8. Absorption Spectrum of Chlorophyll from Okra Pods

Figure 8 shows that the chlorophyll pigments extracted from the okra pods for this trial absorb maximally at  $(430 \pm 3)$  nm and  $(662 \pm 3)$  nm, just like the chlorophyll extracted from the spinach leaves. Furthermore, the okra spectrum also has a slight shoulder in the vicinity of 450 nm, however it does not appear as prominent as that of the spinach spectrum.

As with the spinach, four separate extractions of chlorophyll were performed for the okra pods. The last three extractions were from the same batch of okra while the first extraction was from a different batch. All four extracts were prepared using a roughly 1 g : 5 mL ratio of okra pod mass to acetone volume, and the absorbance measurements were taken on the undiluted extract solutions. The

four absorption spectra that were obtained are shown in Figure 9.

Figure 9. Comparison of Spectra from Different Okra Extracts

The range of absorbance values at a given wavelength in Figure 9 shows that varying amounts of chlorophyll were extracted from okra by the acetone. This is the same thing that happened with the spinach. The first three extracts had blue absorbance maxima of  $(430 \pm 3)$  nm while the fourth extract had a maximum of  $(428 \pm 3)$  nm. The red absorbance maximum ranged from  $(662 \pm 3)$  nm to  $(665 \pm 3)$  nm for the four extracts.

The spectra in Figure 9 were normalized using Extract 3 as the basis and are shown in Figure

10. The error bars are again not shown for the sake of visual clarity.

Figure 10. Comparison of Okra Extract Spectra using Normalization

Figure 10 shows that the absorption spectra from the four different okra extracts agree very closely, as did the spinach extracts. This indicates that the okra data is very precise and that the acetone extraction procedure is consistent in its removal of chlorophyll pigments from okra pods.

Comparison of Spectra from Spinach and Okra

Table 1. Comparison of Absorption Maxima

Blue Absorption  
Maximum (nm)  
Uncertainty in Blue  
Absorption  
Maximum (nm)  
Red Absorption  
Maximum (nm)  
Uncertainty in Red  
Absorption  
Maximum (nm)  
Spinach

1  
430  
3  
662  
3  
2  
430  
3  
662  
3  
3  
430  
3  
662  
3  
4  
430  
3  
662  
3  
Mean  
430  
3  
662  
3

Okra

1  
430  
3  
663  
3  
2  
430  
3  
662  
3  
3  
430  
3  
665  
3  
4  
428  
3  
664  
3  
Mean  
430  
4  
664  
4

Table 1 shows that there is no significant difference in either the blue absorption maximum or the red absorption maximum for the spinach and okra chlorophyll extracts. The mean values for the blue absorption maximum were (430 ± 3) nm for the spinach and (430 ± 4) nm for the okra. These are identical to the blue absorption maximum of pure chlorophyll a. The mean values for the red absorption maximum were (662 ± 3) nm for spinach and (664 ± 4) nm for okra, compared to the red absorption maximum of 662 nm for pure chlorophyll a.

Since the four normalized spectra of spinach were virtually identical, and the four normalized spectra of okra were virtually identical, as shown by Figures 7 and 10 respectively, any one of each can be selected for comparing the spectra of the two plants. Figure 11 compares the second extracts of spinach and okra. Both extracts were prepared with plant mass to acetone volume ratios of 1 g : 5 mL. The spinach extract was diluted 20-fold for taking absorbance readings while the okra extract was not diluted.

Figure 11. Spectral Comparison of Spinach and Okra

Figure 11 shows that at a given wavelength, the absorbance of the spinach extract is greater than the absorbance of the okra extract even though the spinach extract had been diluted 20-fold and the okra extract was not diluted. At the blue maximum of 430 nm, the spinach had an absorbance of 0.885 while that of the okra was 0.311. This indicates that either spinach has an intrinsically higher chlorophyll content than okra does or else it is much easier for acetone to extract chlorophyll from spinach leaves than from okra pods. The spinach leaves were thin and could be ground up easily, while the okra pods had a tough, thick, fibrous skin, and could not easily be ground into small pieces.

The two spectra in Figure 11 were normalized using the spinach spectrum as the basis, and the new curves are shown in Figure 12 below.

#### Figure 12. Spectral Comparison of Spinach and Okra using Normalization

Figure 12 shows that there is no significant difference between the normalized spectra of spinach and okra. The points at 400 nm are significantly separated, however, this is at the lower bound of the usable range of the particular spectrophotometer that was used. As one moves along the curves, at no points does one curve deviate from the other. While it visually appears that the spinach spectrum has more of a shoulder around 460 nm than does the okra spectrum, there is no statistical difference since the error bars overlap. From the two spectra it can be inferred that the ratio of chlorophyll a : chlorophyll b is very similar for the two plants. If one of the plants had a relatively larger amount of chlorophyll b than the other, that plant's spectrum would have more prominent peaks around 453 nm and 645 nm, the two wavelengths at which chlorophyll b maximally absorbs.

In order to more accurately compare the relative amounts of the chlorophyll a and b pigments in spinach and okra, a more quantitative means of comparison is required. Thus, equations (2) and (3) were employed to determine the relative ratio of chlorophyll a to chlorophyll b using 100% acetone as the solvent.

$$Ca = 11.75 A_{662} - 2.35 A_{645}$$

(Equation 2)

$$Cb = 18.61 A_{645} - 3.96 A_{662}$$

(Equation 3)

where  $Ca$  = amount of chlorophyll a in ?g /ml extract  
 $Cb$  = amount of chlorophyll b in ?g /ml extract

These equations are valid for 1-cm square cuvettes, however, acetone degrades the square polystyrene cuvettes that were available in the laboratory. Thus, round glass cuvettes had to be used for measuring

the absorbances of the extracts in acetone. However, for a given sample, round cuvettes give different absorbance readings than square cuvettes since the round glass bends the light beam slightly. Since the absorbance readings obtained in the round cuvettes will be different than those that would be obtained using square cuvettes, equations (2) and (3) cannot be used to find absolute amounts of chlorophyll a and chlorophyll b. In order to determine whether or not the equations can be employed to find the ratio of chlorophyll a to chlorophyll b, an extraction from spinach using water as the solvent was measured in both round and square cuvettes. This comparison will determine whether a fixed conversion factor exists between each set of measurements.

Table 2. Comparison of Absorbance Readings in Round and Square Cuvettes

Wavelength	Absorbance using round 1cm-cuvettes	Absorbance using 1cm square cuvettes	Ratio of absorbances	Uncertainty in Ratio
550	0.273	0.233	1.17	0.04
600	0.272	0.239	1.14	0.03
650	0.359	0.302	1.19	0.03
682	0.564	0.492	1.15	0.03

Table 2 indicates that for all four wavelengths tested, the absorbance in the round cuvettes was significantly higher than the absorbance in the square cuvettes. Furthermore, the ratio of round to square is not statistically different at the wavelengths measured as indicated by the overlap of their uncertainty intervals (Appendix 2). Since equations (2) and (3) make use of absorbances at wavelengths which differ from each other by only 17 nm (A662 and A645), assuming the same conversion

factor for both absorbances is reasonable. Both absorbance measurements taken for the equations will be multiplied by the same factor to convert round absorbances to square absorbances. When  $C_a$  is divided by  $C_b$ , the conversion factor can be factored out of both the numerator and the denominator. Thus the ratio  $C_a : C_b$  obtained using the round cuvettes should be the same as that which would be obtained using square cuvettes.

Using equations (2) and (3), the relative ratio of chlorophyll a : chlorophyll b was calculated for each extract of spinach and okra, and the results are shown in Table 3 below. Table 3. Ratio of Chlorophyll a : Chlorophyll b in Spinach and Okra.

Chlorophyll a : Chlorophyll b ratio  
 Uncertainty in ratio  
 Spinach

1	
1.55	
0.08	
2	
1.46	
0.08	
3	
1.57	
0.10	
4	
1.39	
0.07	
Mean	
1.49	
0.18	
Literature Value	
for fresh leaves	
4.02	
0.79	

Okra

1	
1.43	
0.07	
2	
1.54	
0.16	
3	
1.63	
0.15	
4	
2.08	
0.15	
Mean	

1.67

0.45

Literature Value  
for fresh pods

1.208

only one study was  
found

The uncertainties in the experimental mean values are the sums of the standard deviation and the maximum uncertainty of one of the individual trials (Appendix 2). The literature value for spinach was calculated from the results of three separate studies; Yamauchi et al. (1985), Khachik et al. (1986), and Izaki et al. (1986) (Gross, 1991). The literature value for okra comes from a single study by Singh and Dankhar (1980) (Gross, 1991). A paired t-Test between the experimental values of the ratios for spinach and okra yielded a t-Stat value of -1.00, which is less than the t-Critical value of 2.35. This indicates that there is no significant difference between the mean chlorophyll a: chlorophyll b ratios of okra and spinach at the 95% confidence level. Table 3 shows that the experimental ratios are significantly different from the literature values. The spinach extracts had a percent error of 67.1% while the okra extracts had a percent error of 38.2%. However, it is very important to keep three points in mind regarding the literature values. The literature values: (1) are for fresh samples, (2) are scarce, and (3) show great variation. These points will be further discussed in the Discussion section.

Using equations (2) and (3) the absorbance values at 645 nm which are needed to obtain the mean chlorophyll a: chlorophyll b ratios cited by the literature can be calculated. These values can then be compared to the experimental absorbance values as shown in Table 4 below.

This allows verification of the changes needed in the absorbance values to cause significant changes in the chlorophyll a: chlorophyll b ratio.

Table 4. Comparison of Experimental Absorbances with Expected Absorbances at 645 nm Given the Literature Chlorophyll a: Chlorophyll b Ratio

Absorbance at 645  
nm Needed for Ratio  
from Literature.

Experimental  
Absorbance at 645  
nm

% Deviation in  
Absorbances  
Spinach (literature  
ratio = 4.02? 0.79)



1  
0.138  
0.221  
-37.3  
2  
0.118  
0.196  
-39.6  
3  
0.085  
0.135  
-37.0  
4  
0.143  
0.245  
-41.5  
Mean Deviation

-38.9

Okra (literature ratio  
= 1.208)

1  
0.222  
0.200  
10.9  
2  
0.080  
0.069  
15.8  
3  
0.099  
0.083  
19.5  
4  
0.075  
0.070  
7.5  
Mean Deviation

13.4

Note the consistently larger absorbance values and the rather large mean deviation of - 38.9 % in the case of spinach and the comparatively smaller mean deviation of 13.4% for okra.

Use of Paper Chromatography to Separate the Different Pigments in Chlorophyll

Separation of the different pigments present in the leaf extract was attempted using ethanol and chloroform as eluents. A good chromatogram would be valuable in confirming the presence of different pigments in the leaf extract. However, no separation was observed when the chromatogram was viewed with an ultraviolet lamp. This was due to the fact that both eluents were too polar such that they would strongly bind to the different pigments and carry them all the way to the solvent front without significant separation. Better results could be obtained using a less polar eluent such as petroleum ether.

#### Role of the Solvent in the Extraction Process

The choice of the solvent is a critical step in the extraction process. Chlorophylls being planar organic compounds, are expected to be well solvated by acetone, which is a polar organic molecule. Methyl alcohol or diethyl ether would also have made good solvents. In a very polar solvent such as water, chlorophyll molecules associate to form dimers and polymers; resulting in marked differences between the absorption spectrum as compared to the ones obtained using acetone. Figure 13 compares the absorption spectra of spinach extracts in water and in acetone. Figure 13. Comparison of Absorption Spectrums in Water and Acetone

The spectrum in water has no real peak in the blue region and furthermore, the maximum in the red region is shifted to the right to (678 ± 3) nm. This agrees well with the spectrum of chlorophyll observed in a medium with a relatively large concentration of water molecules (Gurinovich & Pavlovich, 1971). Thus, for a better approximation of the absorption spectrum of chlorophyll which occurs in vivo, using organic polar solvents is recommended since they solvate the chlorophyll molecules without causing association and thus more closely replicate the conditions occurring in the leaves (Gurinovich et al, 1971).

#### Storage

The chlorophyll content in plants is known to vary with time during storage with chlorophyll a being degraded at a faster rate (Goodwin, 1965). To test whether the extracted chlorophyll would remain stable over the span of a few days, a 100% acetone spinach extract was diluted using a dilution factor of 20 and the absorption spectrum was measured on the same day. The extract was stored in the refrigerator for one week and then tested one week later using the same dilution factor of 20.

#### Figure 14. Effects of Storage on Absorption Spectrum

Figure 14 shows that despite identical dilution factors, corresponding absorbance readings were consistently higher after one week. One reason for this trend could be the rapid evaporation of the acetone solvent (which has a relatively high vapor pressure) each time the tube containing the extract is opened. This leaves behind a more concentrated extract of chlorophyll, thus accounting for the higher absorbance readings. However, this was not an issue in subsequent tests with measurements taken shortly after extractions, in sealed cuvettes to minimize evaporation of the acetone. Figure 15 compares the two absorption spectra from Figure 14 using normalization.

#### Figure 15. Normalized Comparison of Storage Effects

Both graphs seem to agree at the blue and red peaks, which would suggest that the relative ratio of chlorophyll a : chlorophyll b (which determines the shape of the graph, especially in the region of the peaks) has shown little fluctuation over the one week of storage. However, higher absorbance readings were obtained in the 500-625 nm region which raises the possibility that by-products which could be formed from the breakdown of some of the pigments in the extract might be present after storage.

#### Discussion of Results

The absorption spectra of chlorophyll pigment extracts from both spinach and okra had absorption maxima in two regions of the visible spectrum over the range of 400 to 700 nm. For the four trials of spinach, the mean absorption maximum in the blue region was  $(430 \pm 3)$  nm and the mean absorption maximum in the red region was  $(662 \pm 3)$  nm. The two mean absorption maxima for the okra extracts were  $(430 \pm 4)$  nm and  $(664 \pm 4)$  nm. The small uncertainties indicate that there was an excellent agreement between trials for a given plant and thus that the data were very precise. These peaks are essentially the same as the absorption maxima of pure chlorophyll a, 430 nm and 662 nm. Some of the absorption spectra had slight shoulders on the side of the blue peaks around 450 nm. This is very close to the blue absorption maximum of pure chlorophyll b, 453 nm. Since the peaks of chlorophyll a were more prominent, the spectra qualitatively indicate that the chlorophyll extracts from both spinach and okra had relatively larger amounts of chlorophyll a than chlorophyll b.

The four extracts of spinach were all prepared similarly and diluted the same, yet for a given wavelength, a range of absorbance values was observed for the four extracts. This same relationship held true with the okra extracts. This indicates that for a given plant, varying amounts of chlorophyll were extracted in the four trials. The amount of chlorophyll extracted depends on how finely the plant sample is ground and on the length of time that the plant pulp spends in the acetone. The finer the plant is ground, the greater the surface area over which the acetone can act. The longer the pulp is in the acetone, the more time the acetone has to break the protein complex and remove the chlorophyll pigments.

Though the various extracts for a given plant showed a range of absorbance values at a given wavelength, when normalized, the spectra were statistically identical. At any given wavelength, the uncertainty intervals on the four data points (since there were four extracts) overlapped. When plotted, the points were basically on top of each other, but if they were not, their error bars overlapped. This relationship held for the chlorophyll pigment extracts from both the spinach and the okra. This indicates that for either plant, the acetone extraction procedure is consistent in its removal of chlorophyll pigments.

The comparison of one spinach extract with one okra extract indicated that the two spectra were not statistically different. It was expected that the two spectra would be significantly different since spinach was expected to have a higher ratio of chlorophyll a : chlorophyll b than okra. The experimental conclusion that the ratios were not different was supported by the results from employing equations (2) and (3). Spinach had a mean chlorophyll a : chlorophyll b ratio of  $1.49 \pm 0.18$  while okra had a mean ratio of  $1.67 \pm 0.45$ . A paired t-test showed that the two means were not statistically different at the 95% level of confidence. Thus, the spectra and equation results indicate that the ratios are the same for spinach and okra.

The results from the equations were significantly different than the literature values. The literature value for fresh spinach samples was  $4.02 \pm 0.79$  while that for okra was 1.208 (only one study found). Though the experimental values are very different than these values, it is important to keep in mind three points regarding the literature values. First, the literature values are for fresh samples. The research groups that performed these studies grew their own plants and tested the samples immediately after collecting them from the plants. The plants at a grocery store were the only ones available for testing. Thus the experiment was at the mercy of the grocer and the vegetable

wholesaler. Also the length of time that the plants had been stored was also unknown; a week or more is not an unreasonable time period. Chlorophyll pigments degrade significantly during storage, thus altering the ratios. Second, analytical literature data on the chlorophyll content of plants is scarce, and most texts on plant pigments are quick to point this out. The literature value for spinach was calculated from only three studies while that of okra came from only one study. Finally, the results of studies in the literature show great variation. The standard deviation of the calculated literature value for spinach was 20%. Furthermore, many studies only gave absolute amounts of total chlorophylls rather than breakdowns of chlorophyll a and chlorophyll b. Six such studies were found for spinach (Kaur and Manjerkar (1975), two from Yamauchi et al. (1985), Khachik et al. (1986), Izaki et al. (1986), and Ogura et al. (1987)) and they had a standard deviation of 20% (Gross 1991). For okra, two studies were found (Singh and Dankhar (1980) and Gupta and Mukherjee (1981)) and they had a standard deviation of 58%. Comparison of the experimental absorbances at 645 nm (used in equations (2) and (3) to calculate the relative ratio of chlorophyll a : chlorophyll b) with the expected absorbances, as shown in Table 3, revealed that the mean deviation in absorbances was -38.9 % for spinach. Such a large deviation strongly suggests that the ratios experimentally obtained and those from the literature are statistically different and probably not a result of the uncertainty in the spectrophotometer. The experimental ratio of chlorophyll a : chlorophyll b (1.67) for okra was relatively closer to the literature value and this trend is confirmed by the smaller mean deviation of 13.4% when the expected and experimental absorbances at 645 nm are compared. However in the case of okra, it must be noted that the absorbances in the red region were small (due to the relatively low concentration of chlorophyll) extracted and thus, small changes in absorbance have a greater potential to cause relatively large shifts in the ratio of pigments calculated. One way around this would be to use dilution factors (e.g. 10-fold rather than 20-fold as previously used) which would maximize the peak in the red region for spinach, aid resolution and thus minimize error in the calculation of the ratios. For okra, the problem lies in the length of time required to extract the pigments. Allowing the okra pulp to stay in solvent overnight would probably yield a higher concentration of extract.

#### Sources of error:

(1) The specific storage and growth conditions for the plants from which leaves (or pods) were taken are completely unknown to us.

(2) Pheophytins a and b formed by the decomposition of chlorophyll a and b respectively are always present in any extract (Paech & Tracey, 1955). Treatment of the chlorophyll with acid will speed up this decomposition. In each case, the result is a shift of the blue peak to higher wavelengths and a shift in the red peak to lower wavelengths.

(3) To accurately determine the chlorophyll ratio by spectrophotometry, the wavelength positions and absorption coefficients used must be checked under the conditions of analysis. This is in order to check any discrepancies in the coefficients found in the equation. This involves getting the spectrum for pure known concentrations of chlorophyll a and b and then determining the absorption coefficients at the wavelengths used in the equations.

(4) The derivation of equations (2) and (3) assumes that the absorbance of one of the chlorophyll pigments would not influence the absorbance of the other. One way to verify this assumption would be to mix known amounts of chlorophyll a and b and then determine whether the resultant spectrum is indeed the mathematical sum of the absorbances of the individual pigments.

#### Modifications:

1. 100% extractions of the samples can be performed by adding more solvent to the residue of the first extract and perform further extractions until no more chlorophyll is being removed from the sample.

Using square glass cuvettes, optimal use of the equations (2) and (3) can then be made to obtain the absolute amounts of chlorophyll a and b as opposed to the relative amounts of the two pigments in this case.

2. Similar equations as the ones shown for 100% acetone are available for other solvents such as diethyl ether or methanol. These solvents can be used to perform extractions on a given sample and the ratios obtained from the different equations can then be compared.

3. Equations (2) and (3) cannot be used to find the absolute concentrations of chlorophyll a and b since circular cuvettes were used rather than the standard 1 cm square cuvettes used in the derivation of (2) and (3). However the total chlorophyll content can be accurately determined by measuring the magnesium content of the extract using an Atomic Absorption Spectrophotometer

(Tracey, 1955). This value can then be used with the ratio of chlorophyll a: chlorophyll b to find the absolute concentration of each pigment. The total chlorophyll content in a leaf can also be measured by recording the absorbance at a given wavelength where chlorophyll a and b are known to have the same absorption coefficient (e.g. 602 nm for diethyl ether). This absorption

coefficient can be determined and absorbance readings can then be directly scaled to the total chlorophyll content for a given extract.

4. To build a larger database of results two things can be done:

? The absorbance spectra of other plant samples could be measured to verify how far they differ from okra and spinach.

? Further spectra of fresh samples of spinach and okra, grown under known conditions can be taken in order to determine the effects of changes in temperature and light intensity on the total chlorophyll content and the ratio of chlorophyll a: chlorophyll b.

5. Pure chlorophyll a and b can be mixed in the ratio determined for the given sample by equations

(2) and (3) and the resultant absorption spectrum obtained from this mixture can then be compared

to the one obtained for the sample. This procedure would serve as a check on the relevance of the equation and its coefficients.

6. For smaller uncertainties in the relative ratio of chlorophyll a:

chlorophyll b, dilutions, especially in

the case of spinach, can be performed such that the peak in the red region is optimized, even though

it is at the expense of having the peak in the blue region off-scale.

7. To show that the plant extract actually consists of a mixture of pigments, paper chromatography can

be performed with a less polar solvent such as petroleum ether (which was unavailable in The

Bioengineering Undergraduate Lab). With good separation up to eight spots corresponding to the

different pigments could be visible (Zubrick, 1992). Cutting the paper and eluting the separated

pigments would give extracts whose absorbances can be measured and compared to the known

spectra of the pure pigments in order to confirm identification.

Storage problems:

Chlorophyll a degrades faster than chlorophyll b to the corresponding pheophytin (Goodwin,

1965). The result is a lowering of the ratio of chlorophyll a: chlorophyll b with increasing length of

storage time of the plant. Furthermore, the total chlorophyll content of the plant will vary depending

upon environmental conditions such as amount of shading or season (Goodwin, 1965). From our

results, it did not appear that a storage time of a week would significantly alter the ratio of chlorophyll

a: chlorophyll b since the normalized plots for readings taken on the day of the extraction and one

week after the extraction were in close agreement with each other. However, evaporation of the solvent

appeared to be a problem since the extract became more concentrated. While this does not affect

determination of the ratio of pigments, it would significantly influence quantification of the total chlorophyll content in the extract.

#### Absorption Spectrum in-vivo and in-vitro

While the absorption spectrum of chlorophyll gives an insight in its function during the process of photosynthesis, it must be noted that it does differ from the spectrum in-vivo. For instance,

chlorophyll a has two peaks in-vivo in the red region, at 670 and 683 nm (compared to a broad 662 nm peak in acetone) while chlorophyll b absorbs at 650 nm in-vivo (compared to 643 nm in acetone).

These discrepancies are probably due to association between the individual chlorophyll molecules or the complex formation with proteins (Goodwin, 1965). The presence of these different peaks for

chlorophyll a is related to the biochemistry of the photosynthetic process. P690 and P700 are two

specialized forms of the chlorophyll a pigments, with the numbers reflecting the peak absorbances in

vivo. Note that the absorbance in-vivo mentioned for chlorophyll a is a combination of all the various

specialized chlorophyll a molecules. P690 and P700 function as the primary pigments where the light

reaction of photosynthesis is actually driven. The other pigments, however, are accessory pigments

which absorb at higher energy and then eventually pass their energy to the primary pigments (Green,

Stout & Taylor, 1985). The fact that energy at either 690 nm or 700 nm (the upper end of the visible

spectrum) is needed to drive the reaction ensures a broader range of light available for photosynthesis.

#### Practical Application.

Research in the absorption spectrum of plants has been instrumental in elucidating the

biochemical pathways governing the photosynthetic process.

Figure 16. Comparison of Action Spectrum for Photosynthesis with the Absorption Spectrum of Photosynthetic Pigments.

Figure 16, above, shows a comparison of the action spectrum for photosynthesis (which is a measure of

the rate of photosynthesis) with the absorption spectrum of photosynthetic pigments. It can be seen that

there is a direct correlation between absorption of light by the photosynthetic pigments and the rate of

photosynthesis. This effectively confirms that the pigments, in particular chlorophyll, are directly

involved in the photosynthetic process. Thus, with the rate of photosynthesis being dependent upon the

range of light available to plants, greenhouse owners can employ artificial lighting to ensure a continuous

supply of light to the plants from the appropriate region of the spectrum. For instance, budding,

flowering and fruiting in greenhouses are favored by a High Pressure Sodium Bulb which emits a



significant proportion of light from the red-orange-yellow region of the spectrum (Sierratech International Homepage). On the other hand, a metal-halide (blue-green) spectrum seems to favor healthy and strong vegetative growth (Sierratech International Homepage).

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#### Appendix 1. Extract Preparation

Table A1-1. Amounts Used in Preparing Extracts

Extract

Mass of Plant Pulp Used

(g)

Volume of Acetone Used

(mL)

Spinach 1

10.169 ? 0.001  
50 ? 2  
2  
6.231 ? 0.001  
31 ? 1  
3  
6.810 ? 0.001  
34 ? 1  
4  
6.072 ? 0.001  
30 ? 1  
Okra 1  
7.621 ? 0.001  
37 ? 1  
2  
7.338 ? 0.001  
37 ? 1  
3  
7.546 ? 0.001  
38 ? 1  
4  
7.523 ? 0.001  
38 ? 1

## Appendix 2. Error Calculations

### UNCERTAINTY IN ABSORBANCE MEASUREMENTS

The Spectronic 20D manual stated that the inaccuracy of percent transmittance readings was less than 0.5%. Since the accuracy of the machine was given in terms of percent transmittance, it must be converted to absorbance. Absorbance and percent transmittance are related as follows:

OR

Employing the differential approach to determine the maximum systematic uncertainty in the absorbance:

(Equation 4)

The uncertainty is calculated using  $\%T = 0.5$ .

### UNCERTAINTY IN NORMALIZED ABSORBANCE MEASUREMENTS

The normalization procedure employs the equation:

where AN is the normalized absorbance value

AB is the peak absorbance of the basis

AS is the peak absorbance of the spectrum being normalized

AO is the absorbance at the point being normalized

Employing the differential approach:

(Equation 5)

UNCERTAINTY IN RATIO OF CHLOROPHYLL a TO CHLOROPHYLL b

The ratio of Ca : Cb comes from equations (2) and (3):

$$\begin{aligned} \text{Ca} &= 11.75 A_{662} - 2.35 A_{645} = C1 A_{662} - C2 A_{645} \\ \text{Cb} &= 18.61 A_{645} - 3.96 A_{662} = C3 A_{645} - C4 A_{662} \end{aligned}$$

Thus, the ratio is given by:

(Equation 6)

UNCERTAINTY IN RATIO OF ROUND: SQUARE CUVETTE ABSORBANCE

The ratio is given by:

where AR and AS are the absorbances in the round and square cuvettes

(Equation 7)

Appendix 3. Absorption Spectra

Figure A3-1. Spinach Extracts in 100% Acetone

Figure A3-2. Okra Extracts in 100% Acetone

Figure A3-3. Spinach Extract in Water

Group R1-1