Quantitative Measurement of Leaf Chlorophylls by Spectrophotometry of Their Pheophytins in Aqueous Alcoholic Extracts

J. L. Wickliff & S. Aronoff
Department of Biochemistry & Biophysics, & the Institute for Atomic Research, Iowa State University, Ames

Quantitative measurements of leaf chlorophylls generally utilize spectrophotometric or colorimetric assay following extraction of pigments from the tissue (3). However, all these methods require sampling of the leaf tissue, followed by extraction of the pigments. Both sampling and extracting introduce a variability into the results which may make the methods inadequate for detecting small changes in chlorophyll content. It is the purpose of this communication to evaluate the extent of the variability introduced into such methodology when assaying chlorophyll in leaves, as well as to estimate the precision of three types of instruments (2 spectrophotometers & a colorimeter) used in conjunction with these measurements.

Ideally, it would be desirable to determine chlorophyll in situ, and both reflectance and transmission methods have been proposed (S. Aronoff. 1962. Dimerization of chlorophyll a in concentrated solutions. Unpublished (5)). Unfortunately, these methods appear to lack the refinement necessary for detecting small differences.

While spectrophotometry of solutions of chlorophylls a and b at two wavelengths permits the calculation of the concentrations of the two components individually, colorimetric measurements, using incident light of relatively broad spectral range, allows quantitative estimation of total chlorophyll only. Determination of the chlorophylls as phyllins may yield variable results arising from uncontrolled alteration of the pigments during processing of the sample and measurement. For example, pheophytinization and allomerization are encountered commonly.

The method of determination of leaf chlorophylls reported here involves spectrophotometry of acidified 80%–ethanolic leaf extracts at two wavelengths. Chlorophylls a and b are thereby measured as their pheophytins. Thus, the variability arising from manipulations involving separations, pheophytinization upon extraction, and allomerization of the chlorophylls, is minimized.

Materials & Methods

Soybean plants, Glycine max L. var. Hawkeye, were grown in soil in the greenhouse, then sampled randomly by removing discs with a cork borer from the laminae of the first trifoliate leaflets. These samples were then extracted by one of two methods (see fig 1) and the resulting solutions acidified to convert the chlorophylls into pheophytins. The amounts of pheophytins a and b were then determined spectrophotometrically. For comparison, total pheophytin was also determined colorimetrically. The variability of results for a given set of samples, extracted by one of the two methods and analyzed by any one of the three instruments, then gives an estimate of the relative precision possible for a given analytical method. To compare the precision of the instruments themselves, aliquots of a homogeneous leaf extract solution were analyzed in sets with the two spectrophotometers and with the colorimeter.

Sampling: Each sample consisted of ten discs taken from an equal number of leaf blades with a 9 mm-diameter punch. The discs were taken randomly from the leaflets of the fully-expanded first trifoliate of a population of nine plants. No leaflet contributed more than one disc to each sample. A set of nine such samples was analyzed for each determination involving one of the extraction methods plus one method of instrumental assay.

To determine the precision of a particular instrument, six equal aliquots were taken from a homogeneous leaf extract. Three independent readings were averaged for each aliquot, so that the total assay for each instrument consisted of 18 measurements on 6 aliquots.

Preparation for Assay: The two methods of extraction are termed “frozen-simmer” and “dried-ground”. A flow sheet depicting the procedures is given in figure 1. All leaf extracts thus prepared showed absorbances in the range 0.2 to 0.6 at the pertinent wavelengths.

584
In both methods precautions were taken to keep the samples out of strong light. The short periods of storage were always in the dark. The extracted solutions were exposed to direct light in the laboratory only briefly during the extraction manipulations. Standing of the acidified solutions prior to assay was restricted to 5 minutes or less to minimize the possible photodecomposition of the pigments.

The frozen-simmer method of sample preparation was rapid—six samples were analyzed easily in 2 hours, whereas the dried-ground method required about 45 minutes for analysis of a single sample.

Spectrophotometry: A Beckman Model DU and a Cary (Model 14) were used.

Specific absorption coefficients for pheophytins a and b in acidified 80% ethanol were determined using pheophytins a and b, which had been prepared by triple chromatography on columns of confectioners' sugar, using Skellysolve B (bp range: 65 to 67 °C) containing 0.2% (v/v) n-propanol. Portions of these pigments were dissolved in a measured amount of absolute ethanol, then 1 N HCl and distilled water were added to make the final solution 80% in ethanol and 0.01 M in HCl. Equal amounts of the pigments were dissolved in anhydrous diethyl ether. Spectra of the solutions in the region 640 to 680 nm were determined with the Cary recording spectrophotometer. From the absorbance values of the ethereal solutions and the values for the specific absorption coefficients given by Smith and Benitez (6), the concentration of each pigment in acidified 80% aqueous ethanolic solution was calculated. These concentrations were then used to calculate specific absorption coefficients for the pigments in the acidified 80% aqueous ethanolic solutions. The averaged values of four determinations are shown on the second line of table I. However, when these coefficients were applied to calculations of pheophytin in acidified 80% aqueous ethanolic extracts from soybean leaves, abnormal a:b ratios were obtained, e.g., 6:1 to 11:1. That

---

### Frozen-Simmer Method

**Leaf Tissue**

- Freeze; store in liquid nitrogen

**Crude Chlorophyll Solution**

- Extract 3X with simmering 80% aqueous ethanol; decant

**Crude Pheophytin Solution**

- Acidify with HCl

**Crude Pheophytin Solution**

- Dilute to volume

- Measure spectrophotometrically or colorimetrically

---

### Dried-Ground Method

**Leaf Tissue**

- Dry at 70 °C, in vacuo for 24 hr; store in vacuo at -18 °C

**Crude Chlorophyll Solution**

- Grind with hot 80% aqueous ethanol; centrifuge; decant

**Crude Pheophytin Solution**

- Wash pellet 2X; centrifuge; decant

**Crude Pheophytin Solution**

- Dilute to volume

**Crude Pheophytin Solution**

- Measure spectrophotometrically or colorimetrically

---

Fig. 1. Flow sheet for the two methods of sample preparation.
these ratios were abnormal and, in fact, incorrect, was shown by transfer of the pigments from the acidified ethanolic extract into diethyl ether (followed by washing, drying, etc.) where spectrophotometry showed the pigments to exist in the usual approximately 3:1 ratio. Therefore operational absorption coefficients were calculated for the leaf extract by quantitative transfer of pigments from aliquots of the leaf extract into diethyl ether, followed by spectrophotometry in that solvent.

To calculate the operational absorption coefficients, two assumptions were made: First, it was assumed that the specific absorption coefficients for pheophytins a and b in diethyl ether solution (6) were the same for the crude pigments obtained by transfer from the ethanolic extracts. This assumption was believed to be reasonable, as the ratios of the pheophytins obtained from such solutions were approximately 3:1. Secondly, it was assumed that the amount and position of maximal absorption in the red region of the spectrum by pheophytins a and b were the same in the acidified extracts as in the pure acidified ethanolic solutions.

In figure 2 is shown the extent of deviation from this assumption and the basis for the calculation of operational absorption coefficients. Calculation of the amounts of pheophytin a and b from the observed curve, using the specific absorption coefficients, results in a:b ratios far in excess of that known to exist, as mentioned above. By transfer of the pigments to diethyl ether, and utilizing the two assumptions made above, the true concentrations of the pheophytins in the aqueous ethanolic extract could be obtained. These values were used to obtain the theoretical curves (dotted lines, fig 2). The difference spectrum was then obtained by subtraction of the combined theoretical spectra from the observed. The resulting curve is consistent with the concept of partial association of pheophytin a with co-pigmentation by a substance as yet unknown. Separate experiments have shown that it is not due to a residual independent pigment, e.g., an anthocyanin or an allomerized phyllin. Co-pigmentation association apparently does not exist in ether solutions, possibly because the unknown compound does not partition into the ether or because solvolysis by the ether disrupts the association. The difference spectrum suggests that all of the co-pigmentation is with pheophytin a. Consequently the operational constants, shown in table I, are calculated on this basis. Application of these operational absorption coefficients to calculations of pheophytin concentrations in acidified ethanolic extracts gave a:b ratios in a range of 2:1 to 3:1 for the different sets of samples, with

![Graph showing absorption spectrum](attachment:image.png)

**Fig. 2.** Observed spectrum for an acidified 80% aqueous-ethanolic leaf extract. The difference spectrum is obtained by subtracting the sum of the calculated contributions of pheophytins a and b from the observed spectrum (for details, see text).
the ratios being reasonably constant within a given set. As the operational absorption coefficients for soybean leaves at different seasons of the year are not precisely the same, the values given in table I cannot be considered definitive, even for soybean leaves, and probably reflect the amount of co-pigment present. It is therefore advisable for each investigator to determine the operational values for his system. These values, should then be reliable as long as the system is not changed.

- Colorimetry: Colorimetric measurements were made with a Klett-Summerson photoelectric colorimeter, employing a Corning No. 2408 red filter which transmits light only of wavelength exceeding 620 ma. Calibration curves for a set of samples were made by determining the colorimeter readings for a dilution series made from an acidified ethanolic leaf extract. The concentration of total pheophytins in the extract was then determined by quantitative transfer of the pigments from an aliquot into diethyl ether, where the concentration of pheophytins a and b were determined by spectrophotometry at two wavelengths, using the values of Smith and Benitez (6). Such determinations were done in triplicate for each calibration.

- Statistical Interpretation: The percentage coefficient of variation may be used to compare the variability or, conversely, the precision of the results of analysis of one set of samples with that of another set. The significance in the differences between the coefficients of variation of the various analytical procedures were estimated by analyses of variance, where coefficients of variation within samples (that is, the variation among the three assays on each sample) were used as the error term in the analysis of variance (7).

### Results & Discussion

The results of the calculations outlined above are shown in tables II and III. The coefficient of variation is given as a percentage, being accompanied by the sample standard deviation, s, and the sample mean, x. The diversity of the sample means demonstrates that the samples in one analysis set were not equivalent to the samples in any other set. In some cases the samples were not even from the same population of plants. This diversity in sample sets was necessary to avoid uncontrolled variability in analyses which might have occurred during long periods of storage (in this case, exceeding 7 days). Therefore, the coefficient of variation is a more valid indicator of variability in the different methods of analysis than the sample standard deviation.

The results in table II show that the least variability in values of total and individual chlorophylls was obtained when leaf samples were extracted by the frozen-simmer method and assayed by the Cary recording spectrophotometer. This method of sample preparation also yielded least variability for total chlorophyll when determined by the colorimetric assay. However, only in the determination of chlorophyll b did the difference in variability of the values from samples prepared by the two methods of extraction show any significance when the Beckman DU spectrophotometer was used for assay. These results indicated an inherent variability in this instrument which tended to mask sample variability and which did not exist in either the colorimeter or the recording spectrophotometer. An obvious source of variability with the Beckman DU spectrophotometer was the manual selection of wavelength.

The source of greater variability which occurred

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Total Chlorophyll</th>
<th>Chlorophyll a</th>
<th>Chlorophyll b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Statistic*</td>
<td>Frozen-simmer</td>
<td>Dried-ground</td>
</tr>
<tr>
<td>Cary recording spectrophotometer</td>
<td>C = 1.56 %, 4.08 %</td>
<td>0.00462</td>
<td>0.0121</td>
</tr>
<tr>
<td>Beckman DU spectrophotometer</td>
<td>C = 3.10 %, 5.95 %</td>
<td>0.00820</td>
<td>0.0166</td>
</tr>
<tr>
<td>Klett-Summerson colorimeter</td>
<td>C = 1.69 %, 2.87 %</td>
<td>0.00513</td>
<td>0.00825</td>
</tr>
</tbody>
</table>

* C = coefficient of variation in percent; s = sample standard deviation; X = mean sample value.

** The fraction of differences in the preceding values for C in the same row which will be significant, based on the variance ratio, F, computed by the analysis of variance.
in the values from analyses of samples prepared by the dried-ground method may be twofold: First, the actual extraction of the sample required more manipulation than did the frozen-simmer method. In principle, this might be expected to affect variability almost equally in determinations of total chlorophyll and of chlorophylls a and b. The figures in table II indicate, however, that the chlorophyll b values were rather more variable in samples prepared by the dried-ground method than in those prepared by the frozen-simmer method. This indicated a second source of variability, that is, a discrimination between components a and b. This may have been due to any or all of the following: A, enzymatic reactions which were not stopped immediately, but continued during the drying process; B, partial destruction or alteration of the b component by heat during the drying process, or C, differential incomplete extraction of the pigments from the dried sample. Some evidence for the last factor is the gray-green tinge which remains in the pellet of some dried samples after extraction and final centrifugation. The effects of storage upon the quantitative analysis of chlorophyll in leaf samples has been studied by Sestak (4), who found that exclusion of air, light, and temperatures above 2 to 3°C, were important for minimizing variability in results. He also noted that drying at 50°C resulted in increased difficulty of extraction of the chlorophyll pigments.

A homogeneous extract solution was used to indicate (table III) the contribution to variability by the instruments alone. The data indicate that all three instrumental methods were of practically equal precision for assays of total chlorophyll. However, the assays for the individual chlorophylls a and b with the Cary recording spectrophotometer yielded less variable results than those with the Beckman DU spectrophotometer.

The choice of the above methods of analysis for chlorophyll thus depends on the investigator's requirements and available equipment. Sensitivity can be sacrificed by using the colorimeter. Alternatively, greater sensitivity and precision can be attained, with somewhat more effort expended, by using the frozen-simmer method of sample preparation and spectrophotometry at two wavelengths with the Cary recording spectrophotometer. The main disadvantage of the frozen-simmer method of sample preparation is that storage of samples in liquid nitrogen for long periods of time is not practical. This disadvantage is overcome in the dried-ground method with some cost of effort and loss in precision of analysis.

An additional variable, implicit, but untested, is that of different instruments of the same manufacture. Thus, the results cannot be said to be definitive for these instruments as representative of classes of instruments.

From the values for s and x listed in table II for a given method of analysis, it is possible to estimate the resolving power of that method, i.e. to calculate the minimum difference between the means of values in two sets which would be significant with a given probability (7). Such a calculation for the frozen-simmer method of sample preparation and spectrophotometry with the Cary recording spectrophotometer shows this difference to be 0.9% of the lesser mean value at P = 0.05. Thus the difference between the means of results of two analyses for total chlorophyll, assuming the variability in each set of results to be similar in magnitude to that in the preceding data, would be significant if greater than about one percent of the lesser mean.

A disturbing feature of this methodology is the variation in the absorption coefficients by co-pigmentation of pheophytin a possibly due to polymerization. Indeed, preliminary experiments in our laboratory indicate that a similar co-pigmentation of chlorophyll a exists in 80% aqueous acetone leaf extracts. A comparison of the difference spectrum of figure 2 to a similar spectrum obtained by Brody and Brody (2) for aggregated chlorophyll a in vivo suggests that the co-pigmentation may be simply an aggregation of pheophytin a, which may or may not

<table>
<thead>
<tr>
<th>Statistic*</th>
<th>Total chlorophyll</th>
<th>Chlorophyll a</th>
<th>Chlorophyll b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cary recording spectrophotometer</td>
<td>C</td>
<td>1.18%</td>
<td>1.18%</td>
</tr>
<tr>
<td></td>
<td>s</td>
<td>0.00587</td>
<td>0.00629</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>0.498</td>
<td>0.330</td>
</tr>
<tr>
<td>Beckman model DU spectrophotometer</td>
<td>C</td>
<td>1.02%</td>
<td>2.20%</td>
</tr>
<tr>
<td></td>
<td>s</td>
<td>0.00434</td>
<td>0.00680</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>0.425</td>
<td>0.309</td>
</tr>
<tr>
<td>Klett-Summerson colorimeter</td>
<td>C</td>
<td>0.544%</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>s</td>
<td>0.00218</td>
<td>0.00434</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>0.401</td>
<td>0.309</td>
</tr>
</tbody>
</table>

** See footnotes to table II.
have been derived from chlorophyll a aggregated in vivo. Evidence for a dimerization of chlorophyll a in pure solution has recently been obtained by one of us (Aronoff, S. 1962. Dimerization of chlorophyll a in concentrated solutions. Unpublished.) and similar investigations of the properties of pheophytin a are in progress. However, by the method employed here the effects of co-pigmentation may be quantized for serial investigations, and do not vitiate the advantages of the method for assay of the chlorophylls.

Summary

Methods are described for analysis of leaf chlorophylls by spectrophotometry and colorimetry of their pheophytins in acidified 80% aqueous ethanolic extracts. Of two methods of sample preparation, that involving freezing in liquid nitrogen and extraction by simmering 80% aqueous ethanol was found to yield results with less variability than the method involving extraction of a dried sample by grinding with hot 80% ethanol. With a given method of sample preparation, the two spectrophotometric and the colorimetric methods of assay yielded results of practically equal precision, but the Cary recording spectrophotometer employed was the superior instrument with respect to sensitivity and precision of results. The data indicate that such analytical procedures can be employed to measure differences of 1% or greater in the chlorophyll content in leaves.

Acknowledgments

We gratefully acknowledge the assistance of Prof. O. Kempthorne and Prof. F. B. Cady of the Department of Statistics at Iowa State University, and the able technical assistance of Miss Cheryll Hermann. These investigations were supported, in part, by a grant from the National Science Foundation, G-7593.

Literature Cited