A Comparison of Two Techniques for Nondestructive Measurement of Chlorophyll Content in Grapevine Leaves

Mark R. Steele, Anatoly A. Gitelson,* and Donald C. Rundquist

Notes & Unique Phenomena

ABSTRACT

Traditional methods for chlorophyll (Chl) measurement include wet chemical extractions and handheld Chl meters. The recent availability of small and affordable radiometers has provided means to estimate Chl from reflectance measurements. This paper compares the performance of a handheld SPAD Chl meter and a recently developed Red Edge Chlorophyll Index ($CI_{red\ edge}$) for Chl estimation in grapevine (*Vitis* spp.) leaves. Leaves were sampled and Chl was quantified using these two methods. Both techniques were compared against grapevine-leaf Chl as analyzed using standard laboratory procedures. Both SPAD and $CI_{red\ edge}$ were equally accurate in measuring Chl < 300 mg/m2. However, at higher Chl, SPAD sensitivity declined and the $CI_{red\ edge}$ accuracy was much higher than that of SPAD. The Chl index was found to be capable of accurately estimating pigment contents across a greater range than the SPAD, thus it can be used for quantitative assessment of early stages of plant stress.

Leaf chlorophyll content is a key indicator of the physiological status of a plant. Leaves contain chlorophyll, Chl *a* and Chl *b*, as essential pigments for the conversion of light energy to stored chemical energy, and the amount of solar radiation absorbed by a leaf is a function of the photosynthetic pigment content. Thus, Chl content is linked directly to photosynthetic potential and primary production (e.g., Curran et al., 1990). Additionally, Chl gives an indirect estimation of the nutrient status as considerable leaf N is incorporated in that pigment (e.g., Filella et al., 1995). Leaf Chl amounts are affected directly by plant stress and senescence (Hendry et al., 1987). Thus, in grapevines, leaf Chl serves as an integrator of all the environmental and nutritional variables ultimately affecting the quality of a fruit crop and the wine.

The traditional approach to Chl measurement is to employ wet chemical methods requiring pigment extraction in a solvent, the spectrophotometric determination of absorbance of the Chl solution, and conversion from the absorbance to Chl content (e.g., Porra et al., 1989). This approach has long been considered the standard method for Chl determination. However, it requires destructive sampling (thus preventing developmental studies of single leaves) and is time consuming.

The Minolta SPAD-502 leaf chlorophyll meter (Minolta Camera Co., Osaka, Japan), one of several hand-held Chl meters on the market today, measures leaf transmittance at two wavelengths in the red (approximately 660 nm) and near infrared, NIR (approximately 940 nm). The former spectral location is associated with absorption by Chl and the latter is used as reference. The theoretical principles of this technique are described by Markwell et al. (1995). In that study, the authors established a relationship between SPAD readings and analytically measured (i.e., in a laboratory setting) Chl content for soybean [*Glycine max* (L.) Merr.] and maize (*Zea mays* L.) leaves. The relationships were essentially nonlinear showing, however, high coefficients of determination: $r^2 = 0.94$ for an exponential and $r^2 = 0.96$ for second-order polynomial approximation.

The application of reflectance spectroscopy to the estimation of leaf pigment content has recently received considerable attention. Compared to hand-held Chl meters, which yield a single index value, reflectance spectroscopy offers a wealth of information. Besides Chl, the many wavelengths of reflectance spectroscopy provide the basis for calculating the content of other pigments. However, a key problem is selection of an appropriate index from among the vast array of those available. Richardson et al., (2002) compared the performance of two commercially available hand-held Chl meters with that of several reflectance indices for leaf level Chl estimation and found that the indices based on reflectance, either in the red edge (around 700 nm) or the green bands (around 550 nm), and the near-infrared (Gitelson and Merzlyak, 1994; Gitelson et al., 1996), were better indicators of Chl content than the two hand-held Chl meters (the CCM-200, Opti-Sciences, Tyngsboro, MA, and the SPAD-502) tested.

Subsequently, an improved Chl retrieval technique using the green and red edge spectral regions has been documented (e.g., Gitelson and Merzlyak, 1994; Gitelson et al., 1996), and recently a conceptual model to estimate total Chl, carotenoids and anthocyanin content in higher plant leaves was developed and tested for leaves from a number of crop and tree species (Gitelson et al., 2003, 2006). That model relates reflectances

M.R. Steele,Center for Advanced Land Management Information Technologies (CALMIT), School of Natural Resources, Univ. of Nebraska-Lincoln, Lincoln, NE 68583-0517; and Agricultural Research and Development Center, Univ. of Nebraska-Lincoln, Ithaca, NE 68033-2234; A.A. Gitelson, and D.C. Rundquist, Center for Advanced Land Management Information Technologies (CALMIT), School of Natural Resources, Univ. of Nebraska-Lincoln, Lincoln, NE 68583-0517. Received 24 July 2007.
*Corresponding author (agitelson2@unl.edu).

Published in Agron. J. 100:779–782 (2008).
doi:10.2134/agronj2007.0254N

AJ AGRONOMY JOURNAL 1908–2008

Abbreviations: Chl, chlorophyll; $CI_{red\ edge}$, Red Edge Chlorophyll Index.

in three wavebands to content of the pigment of interest ($C_{pigment}$). The formulation is:

$$C_{pigment} \propto [R(\lambda_1)^{-1} - R(\lambda_2)^{-1}] \times R(\lambda_3) \quad [1]$$

where R is reflectance and λ is wavelength.

Reflectance in the first band (λ_1) is maximally sensitive to the pigment of interest. However, $R(\lambda_1)$ is also affected by absorption of other pigments and leaf scattering. Reflectance $R(\lambda_2)$ should be maximally sensitive to absorption by pigments other than the pigment of interest (e.g., Chl) and should not be affected by pigment of interest. Thus, the difference $R(\lambda_1)^{-1} - R(\lambda_2)^{-1}$ is related to the pigment of interest (e.g., Chl) but is also affected by leaf scattering (Gitelson et al., 2003, 2006). Therefore, the third reflectance term $R(\lambda_3)$ should be closely related to leaf scattering. Tuning the specific wavelengths selected in accord with the spectral properties of the pigment of interest, resulted in selection of the optimal bands (λ_1, λ_2, and λ_3).

It was found that for Chl estimation in anthocyanin-containing leaves, the optimal λ_1 was in the red edge region around 700 nm and optimal $\lambda_2 = \lambda_3$ was in the NIR range beyond 760 nm (Gitelson et al., 2003, 2006). Consequently, the conceptual model was simplified and the $CI_{red\ edge}$ (Gitelson et al., 2003) was suggested in the form:

$$CI_{red\ edge} = (R_{NIR}/R_{red\ edge}) - 1 \quad [2]$$

The objective of this study is to compare the performances of the hand-held SPAD-502 chlorophyll meter and the $CI_{red\ edge}$ for Chl estimation in grapevine leaves. The $CI_{red\ edge}$ was calculated with reflectances measured by a radiometer (Model USB2000, Ocean Optics, Inc., Dunedin, FL) and both techniques were tested and compared against grapevine-leaf Chl content as analyzed using standard laboratory procedures.

MATERIALS AND METHODS

To compare results of Chl estimation by means of the SPAD and the $CI_{red\ edge}$ with laboratory derived Chl content, 20 Edelweiss (*Vitis labrusca* hybrid) leaves were sampled on 1 June 2006. Edelweiss is a white *Vitis labrusca* hybrid (Minnesota 78 × Ontario) developed by Wisconsin grapevine breeder Elmer Swenson and introduced by the University of Minnesota in 1980. Chl content was measured using each of the three techniques: SPAD readings, reflectance measurements with $CI_{red\ edge}$ calculation, and analytical Chl determination using standard laboratory procedures.

Individual leaves were selected in the field based on various levels of greenness; that is, they were chosen carefully to ensure a range in color from dark green to pale green. The selected leaves were cut from the canopy, immediately sealed in a plastic bag with a small amount of water, and placed in a cooler with ice. After field sampling was completed, the leaves were transported to the lab, and areas of homogeneous pigmentation on each were visually identified and delineated with a permanent marker.

Using the Minolta SPAD-502 chlorophyll meter, three readings were acquired from each of delineated areas of the 20 Edelweiss grapevine leaves sampled. The mean of the three SPAD readings per leaf was then used for comparison to lab-derived Chl and $CI_{red\ edge}$ values.

Spectral-reflectance measurements of the same leaves were collected using a leaf clip with a 2.3-mm diam. bifurcated fiber optic attached to both an Ocean Optics USB2000 radiometer and to an LS-1 tungsten halogen light source. The USB2000 uses a charged coupled device (CCD) to measure radiance with a spectral resolution of approximately 1.5 nm across 2024 individual spectral channels ranging from 350 to 1010 nm in wavelength. The LS-1 light source uses a regulated power supply and a tungsten halogen filament bulb burning at 3100 K to output a steady beam of light with a spectral range between 260 and 2500 nm. In this study, the light source was turned on at least 15 min before scanning to allow the bulb and filament to warm and stabilize.

The plastic leaf clip, used to position the Ocean Optics fiber against individual grapevine leaves, consisted of a black polyvinyl chloride (PVC) attachment and a bifurcated glass fiber optic with a diameter of 2.3 mm. This fiber optic, transmissive between 400 and 1000 nm, was positioned at a 60° angle relative to the leaf surface to minimize specular reflectance. A black foam background, with a nominal reflectance of 3% within the spectral range of the instrument, was held against the abaxial side of the leaf to reduce extraneous reflectance from the reflected light being transmitted through the leaf.

The radiometer was calibrated before each data-collection session using a Labsphere Spectralon reference panel (North Sutton, NH) with a nominal reflectance of 99% between 250 and 2500 nm. First, the reference panel was held tightly against the fiber optic, and a spectral scan was recorded. Then, the panel was rotated and the procedure repeated.

The sensor was operated by means of the CALMIT Data Acquisition Program, which uses a single calibration scan collected at the time nearest to the acquisition of the target scans to compute reflectance. To ensure that a calibration scan used for reflectance calculations was accurate, at least three additional reference-panel scans were recorded for each dataset. The reflectance was calculated as a ratio of leaf radiance to the radiance of the calibration standard at wavelength λ.

A total of six reflectance measurements were acquired within the marked homogeneous areas of each leaf. Care was taken to distribute the locations of the spectral scans throughout the entire marked area to acquire an accurate representation of Chl content. No data were taken at leaf edges or at locations where staining was present due to ink from the marker. The average of the six scans per sample was calculated to establish a single representative reflectance spectrum per leaf.

For each of the leaves sampled as part of this 20 leaf Edelweiss dataset, two or three 1-cm diam. disks were cut from the delineated areas using a standard leaf punch. The disks were then weighed and ground in an 80% aqueous acetone solution using a mortar and pestle. The tissue was ground until the pulp turned white in color and all pigments were suspended in the solution. The resulting homogenate was centrifuged in test tubes for 6 min. Absorption spectra of the solution were recorded using a Cary Spectrophotometer, which was configured to measure absorption of the sample at 1 nm intervals between 400

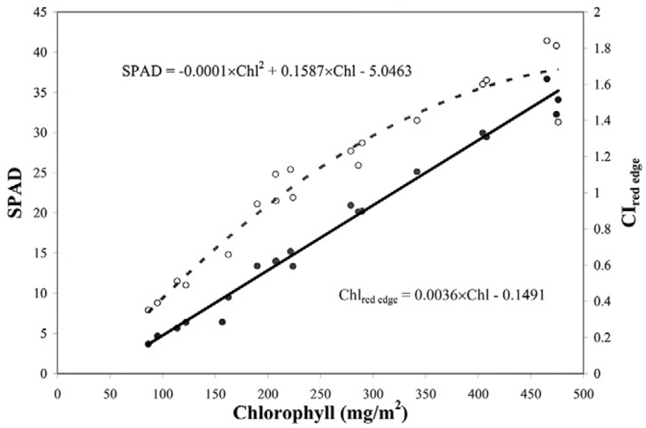


Fig. 1. Relationships of the SPAD readings (left y axis) and Red Edge Chlorophyll Index ($CI_{red\ edge}$) (right y axis) versus the laboratory-determined chlorophyll content for the 20 Edelweiss leaves.

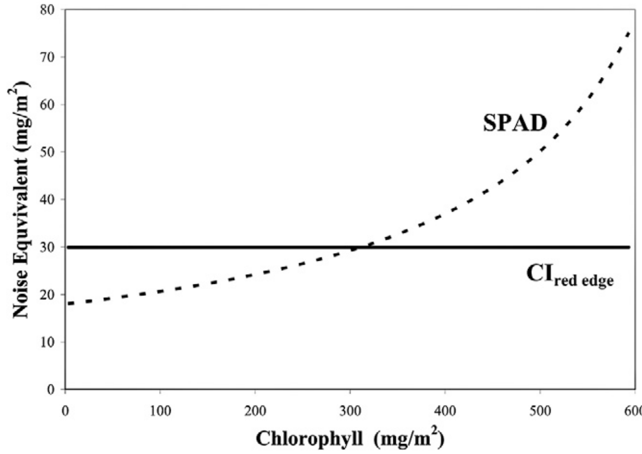


Fig. 2. Noise equivalent of chlorophyll estimation by SPAD and Red Edge Chlorophyll Index ($CI_{red\ edge}$) plotted vs. Chl content. As chlorophyll <300 mg/m^2 (yellowish to green leaves), noise equivalent of SPAD was slightly less than noise equivalent of $CI_{red\ edge}$. When chlorophyll >300 mg/m^2, noise equivalent of SPAD increases drastically indicating sharp decrease in accuracy of estimation of moderate-to-high chlorophyll content by SPAD. Noise equivalent of $CI_{red\ edge}$ remained constant in a wide range of chlorophyll variation.

and 800 nm. Chlorophylls *a* and *b* were calculated from the spectra using coefficients described by Porra et al. (1989).

To compare the sensitivity of the SPAD and $CI_{red\ edge}$ to change in Chl content, the noise equivalents of Chl estimation were calculated as (Viña and Gitelson, 2005):

$$NE_{CI}\ \Delta Chl = RMSE(CI_{red\ edge}\ vs.\ Chl)/[d(CI_{red\ edge})/d(Chl)] \qquad [3]$$

and

$$NE_{SPAD}\ \Delta Chl = RMSE(SPAD\ vs.\ Chl)/[d(SPAD)/d(Chl)] \qquad [4]$$

where RMSE($CI_{red\ edge}$ vs. Chl) and RMSE(SPAD vs. Chl) are the root mean squared errors of the relationship $CI_{red\ edge}$ vs. Chl and SPAD vs. Chl, respectively; d($CI_{red\ edge}$)/d(Chl) and d(SPAD)/d(Chl) are the first derivatives of $CI_{red\ edge}$ vs. Chl and SPAD vs. Chl relationships with respect to Chl, respectively. Defined in this way the noise equivalent allows the direct comparison among different techniques, with different scales and dynamic ranges (Govaerts et al., 1999).

To further compare the two methods of Chl retrieval, SPAD readings and reflectance measurements were collected from 144 Edelweiss and 144 St. Croix (*Vitis riparia* hybrid) leaves sampled during eight field campaigns between 23 May and 28 August during the 2006 growing season. St. Croix is a red *Vitis riparia* hybrid (ES-283 × ES-193) developed by Elmer Swenson and introduced by the University of Minnesota in 1981. These 288 leaves were sampled in the field using the same methods described previously. Reflectance and SPAD measurements were again recorded using the techniques implemented on the 20 Edelweiss leaf dataset and $CI_{red\ edge}$ was calculated using measured reflectance. The Chl content was not retrieved using wet-chemical methods; instead it was derived from the following equation:

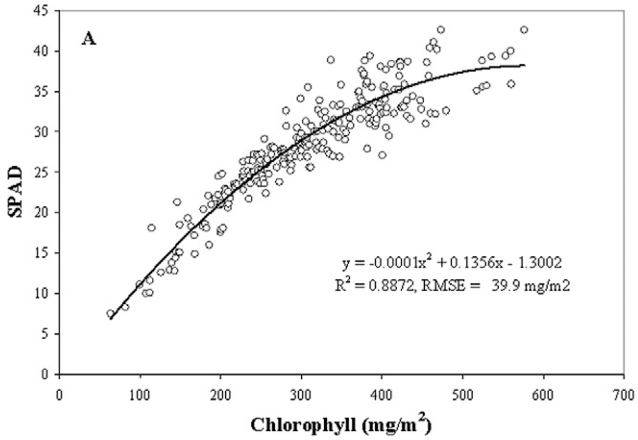
$$Chl,\ mg/m^2 = 29.97 + 322.26 \times CI_{red\ edge} \qquad [5]$$

Equation [5] was verified and documented in a thesis by M.R. Steele (2007) (Non-destructive estimation of leaf pigments and monitoring phenology of grapevines. M.A. thesis. Dep. of Geography, Univ. of Nebraska-Lincoln) using an extended database containing 93 grapevine leaves. In that study, $CI_{red\ edge}$ explained 96.8% of Chl variability and the RMSE of Chl prediction was lower than 28 mg/m^2 in the range of Chl from 3 to 515 mg/m^2; the coefficient of variation was below 13%. The SPAD readings for the 288 leaves sampled in the current study were plotted vs. Chl content as retrieved from reflectance measurements by means of Eq. [5].

RESULTS AND DISCUSSION

Relationships of the SPAD readings (left *y* axis) and $CI_{red\ edge}$ (right *y* axis) versus the laboratory-determined Chl content for the 20 Edelweiss leaves is presented in Fig. 1. The SPAD vs. Chl relationship was asymptotic, with decrease in the slope as the pigment content exceeds 300 mg/m^2. Thus, the SPAD instrument exhibits limitations in Chl estimation at moderate-to-high Chl content, similar to what was found for the relationship between NDVI and other NDVI-like vegetation indices and leaf Chl content (Gitelson et al., 2003). While the best fit function for the SPAD vs. Chl relationship was a second order polynomial, the $CI_{red\ edge}$ vs. Chl relationship was linear with a coefficient of determination $R^2 > 0.98$ and RMSE of Chl estimation <16.4 mg/m^2 for Chl ranging from 86 to 476 mg/m^2.

The noise equivalents of Chl estimation by both the SPAD and $CI_{red\ edge}$ are shown in Fig. 2. For Chl <300 mg/m^2, the SPAD NE ΔChl was between 18 and 30 mg/m^2 while the $NE_{red\ edge}$ ΔChl was 30 mg/m^2; thus, SPAD was slightly more accurate (less noisy) than $CI_{red\ edge}$ in Chl estimation. When Chl exceeded 300 mg/m^2, however, the NE_{SPAD} ΔChl increased exponentially, reaching 100 mg/m^2 for Chl = 650 mg/m^2 (not shown in Fig. 2). Thus, with increase in Chl beyond 300 mg/m^2, $CI_{red\ edge}$ became much more accurate than the SPAD in Chl estimation.



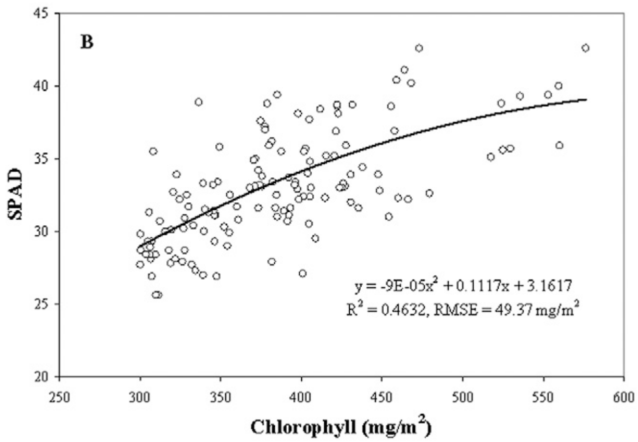


Fig. 3. Comparison of Minolta SPAD-502 chlorophyll meter readings with chlorophyll content derived from Eq. [5] with measured reflectance for 288 Edelweiss grapevine leaves. (A) Chlorophyll contents ranged from 63 to 576 mg/m^2: 288 yellowish to dark green leaves; (B) Chlorophyll exceeded 300 mg/m^2: 148 green to dark green leaves.

The relationship between the SPAD and Chl is shown in Fig. 3A. This relationship was asymptotic as in Fig. 1, with significant scattering of points from a second order polynomial line when Chl exceeded 300 mg/m^2. It is noteworthy that Chl content beyond 300 mg/m^2 is typical for green vegetation and thus, it was important to document in great detail the performance of the SPAD meter in this region. Therefore, in Fig. 3B we presented the SPAD vs. Chl relationship within the Chl range 300 to 576 mg/m^2 for 148 leaves. The SPAD explained <47% of the variation in leaf Chl content and the RMSE of Chl estimation was higher than 49 mg/m^2. This prevents an accurate estimation of Chl exceeding 300 mg/m^2, which is of great interest for early detection of plant stress, especially at times in the growing season when the plant canopy is fully developed (Stamatiadis et al., 2006).

CONCLUSIONS

The SPAD meter showed adequate sensitivity to Chl contents in yellowish-green to green leaves with Chl below 300 mg/m^2. Above that level, however, the sensitivity of the SPAD to Chl became considerably diminished. This decrease in sensitivity takes place in the range of Chl that is typical for green vegetation and it prevents using SPAD for indication of early (pre-visual) stages of plant stress. The loss of sensitivity can likely be attributed to the red band (650 nm) used and the associated saturation in transmittance vs. Chl relationship for moderate to high Chl content (Gitelson and Merzlyak, 1994, 1996; Gitelson et al., 2003).

The $CI_{red\ edge}$, which employed reflectance in the red-edge and NIR region of the spectrum, is linearly related to Chl in the grapevine leaves studied and also in the range of Chl well beyond 600 mg/m^2 (e.g., Gitelson et al., 2003; 2006). The chlorophyll index was found to be capable of accurately estimating pigment contents across a much greater Chl range than the SPAD meter; thus, the index can be used for quantitative assessment of the early stages of plant stress.

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