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An evaluation of noninvasive methods to estimate foliar chlorophyll content

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Summary

• Over the last decade, technological developments have made it possible to quickly and nondestructively assess, *in situ*, the chlorophyll (Chl) status of plants. We evaluated the performance of these optical methods, which are based on the absorbance or reflectance of certain wavelengths of light by intact leaves.

• As our benchmark, we used standard extraction techniques to measure Chl*a*, Chl*b*, and total Chl content of paper birch (*Betula papyrifera*) leaves. These values were compared with the nominal Chl index values obtained with two hand-held Chl absorbance meters and several reflectance indices correlated with foliar Chl.

• The noninvasive optical methods all provided reliable estimates of relative leaf Chl. However, across the range of Chl contents studied (0.0004–0.0455 mg cm^{-2}), some reflectance indices consistently out-performed the hand-held meters. Most importantly, the reflectance indices that performed best (Chl NDI = $(R_{750} - R_{705})/(R_{750} + R_{705})$, RII = $\int_{705}^{750}(R_\lambda/R_{705} - 1)\,d\lambda$) were not those most commonly used in the literature.

• We report equations to convert from index values to actual Chl content, but caution that differences in leaf structure may necessitate species-specific calibration equations.

Key words: absorbance, chlorophyll, Chl*a* : Chl*b*, leaf optical properties, pigment, red edge, reflectance, spectral index.

Introduction

In photosynthesis, antenna pigments in leaf chloroplasts absorb solar radiation, and through resonance transfer the resulting excitation is channeled to the reaction centre pigments, which release electrons and set in motion the photochemical process. The chlorophylls, Chl*a* and Chl*b*, are the most important of these pigments, and are thus virtually essential for the oxygenic conversion of light energy to the stored chemical energy that powers the biosphere. From a physiological perspective, leaf Chl content (for example, how it varies both between and within species) is therefore a parameter of significant interest in its own right. However, from an applied perspective, leaf pigmentation is important to both land managers and ecophysiologists. There are several reasons for this. First, the amount of solar radiation absorbed by a leaf is largely a function of the foliar concentrations of photosynthetic pigments, and therefore low concentrations of chlorophyll can directly limit photosynthetic potential and hence primary production (Curran *et al.*, 1990; Filella *et al.*, 1995). Second, much of leaf nitrogen is incorporated in chlorophyll, so quantifying Chl content gives an indirect measure of nutrient status (Filella *et al.*, 1995; Moran *et al.*, 2000). Third, pigmentation can be directly related to stress physiology, as concentrations of carotenoids increase and chlorophylls generally decrease under stress and during senescence (Peñuelas & Filella, 1998). Fourth, the relative concentrations of pigments are known to change with abiotic factors such as light (e.g. sun leaves have a higher Chl*a* : Chl*b* ratio; Larcher, 1995) and so quantifying these proportions can provide important information about relationships between plants and their environment.

The amount of chlorophyll in a leaf is normally expressed in terms of either concentration (i.e. μg Chl g^{-1} tissue) or content (i.e. μg Chl cm^{-2} tissue); preference for one over the other may depend on the researcher's objectives. Sometimes, Chl

concentration or content is expressed in terms of moles per amount of leaf mass or area, since photon flux and carbon assimilation rates are usually expressed in similar units, and this permits better understanding of physiological processes. The molecular weights of Chl*a* and Chl*b* are 892 and 906, respectively.

Traditionally, wet chemical methods have required Chl extraction in a solvent, followed by the spectrophotometric determination of absorbance by the chlorophyll solution, and conversion from absorbance to concentration using standard published equations (e.g. those of Arnon (1949) and modifications thereof). Although long considered the standard method for Chl determination, extraction requires destructive sampling (which precludes, for example, developmental studies of single leaves) and is relatively time consuming.

More recently, nondestructive optical methods, based on the absorbance and/or reflectance of light by the intact leaf, have been developed. Optical methods generally yield a 'chlorophyll index' value that expresses relative chlorophyll content but not absolute Chl content per unit leaf area, or concentration per gram of leaf tissue. These newer methods are nondestructive, very quick, and now possible in the field (Markwell *et al.*, 1995; Gamon & Surfus, 1999). In this paper, we evaluate both nondestructive absorbance and reflectance methods.

Hand-held Chl absorbance meters, of which several are commercially available, measure absorbance by the leaf of two different wavelengths of light: *c.* 660 nm (red) and *c.* 940 nm (near-infrared). The red light is strongly absorbed by Chl; the near-infrared light is a 'reference wavelength' that is used to adjust for differences in leaf structure. The theoretical principles on which these meters are based are described in detail by Markwell *et al.* (1995).

Portable reflectometers, which essentially allow the application of remote sensing technology at the leaf or branch level, record reflectance at many closely spaced wavelengths across an entire spectrum. This spectrum typically spans ultraviolet, visible, and near-infrared wavelengths. Mathematical indices have been developed which reduce complex spectra to a single value; these indices, which are based on knowledge of the reflectance properties of the biochemical components in leaves, can be targeted to estimate (among other things) pigment content. More complete reviews of some of the practical and theoretical considerations of reflectance spectroscopy are given by Curran *et al.* (1990), Adams *et al.* (1999), Datt (1999), and Gamon & Surfus (1999).

Compared to hand-held Chl absorbance meters, which yield just a single index value, one of the benefits of reflectance spectroscopy is the wealth of information that can be obtained from each leaf scan. A typical spectrum, containing 256 or more data points, can be transformed in an almost infinite number of ways. However, a key problem for the researcher is to choose an appropriate transformation index from among the vast array of those available. The application of reflectance spectroscopy to the estimation of leaf Chl content has recently received considerable attention in the literature, and many of these papers have presented new indices which are well-correlated with Chl (Curran *et al.*, 1990; Gitelson & Merzlyak, 1994, 1996; Gitelson *et al.*, 1996; Blackburn, 1998; Datt, 1998, 1999; Adams *et al.*, 1999; Gamon & Surfus, 1999). The researcher's choice of index is not made any easier by the fact that some of these authors have failed to test the applicability of the proposed index by using a second, independent, data set. In some cases, the results of such tests are not presented in a way that allows meaningful comparison of the indices across different studies. Finally, these indices have rarely been tested using data from species other than those used in the formulation of the index. Notwithstanding the fact that reflectance spectroscopy can provide considerably more data than hand-held Chl absorbance meters, there is no evidence that more is actually better. Although two studies have tested hand-held Chl absorbance meters (Monje & Bugbee, 1992; Markwell *et al.*, 1995), we are not aware of any studies that attempted a comparative test of absorbance meters and different reflectance indices, to determine which noninvasive method produces the index best correlated with leaf Chl.

Thus, the purpose of this study is to compare the performance of two commercially available hand-held Chl absorbance meters with that of several reflectance indices for leaf-level Chl (other indices may be more appropriate for remote sensing applications at the canopy or stand level; e.g. Datt, 1999; Gamon & Surfus, 1999). As our standard against which the noninvasive methods would be judged, we measured leaf Chl using standard extraction techniques.

Methods

Overview

Over the course of the study, 10 leaves were processed every afternoon as follows. First, leaf samples were brought from the glasshouse to the laboratory, and then five measurements were made on each leaf sample using each of the two hand-held Chl absorbance meters, and then the reflectometer. Once these measurements were complete, three circular disks were punched from each leaf using a cork borer, and Chl extractions from all 10 leaves were conducted simultaneously. Finally, the Chl concentration of the extracts was measured with a spectrophotometer.

Plant material

We used leaves from paper birch (*Betula papyrifera* Marsh.) grown from seed in a glasshouse. One hundred leaf samples, spanning as wide a range of Chl contents as possible, from very pale yellow to very dark green, were used. Approximately one-half of the low Chl leaves were yellowed as a result of stress or senescence; the other half we subjected to illumination with UV light to induce chlorophyll degradation.

Hand-held chlorophyll meters

We used two hand-held Chl meters, the CCM-200 (Opti-Sciences, Tyngsboro, Massachusetts, USA), and the SPAD-502 (Minolta Camera Co., Osaka, Japan). The CCM-200 weighs 180 g, has a 0.71-cm^2 measurement area, and calculates a chlorophyll content index (CCI) based on absorbance measurements at 660 and 940 nm. The claimed accuracy of the CCM-200 is ±1.0 CCI units. The SPAD-502 weighs 225 g, has a 0.06-cm^2 measurement area, and calculates an index in 'SPAD units' based on absorbance at 650 and 940 nm. The claimed accuracy of the SPAD-502 is ±1.0 SPAD units.

Five separate measurements with each hand-held meter were made on each leaf; we used the arithmetic mean of these measurements for all subsequent analyses.

Leaf reflectance

Spectral reflectance at wavelengths from 306 to 1138 nm was measured using a UniSpec Spectral Analysis System (PP Systems, Haverhill, Massachusetts, USA) with a 2.3-mm diameter (0.042 cm^2) foreoptic and an internal 6.8 W halogen lamp. The instrument was allowed to warm up for 20–30 min before use. A Spectralon reflectance standard was scanned before every new leaf sample, and scans were corrected for the instrument's dark current. Individual leaves were held in a black plastic polyvinyl chloride (PVC) leaf clip at a 60° angle relative to the foreoptic. Each leaf scan represented the average of six passes, and the instrument's integration time was set at 65 ms. The reflectance spectrum for each scan was calculated as R_λ = leaf radiance at wavelength λ/reflectance standard radiance at wavelength λ, and was averaged across the five separate scans made on each leaf. We transformed the average raw reflectance spectrum for each leaf to a first-difference spectrum, calculated as $D_\lambda = (R_n - R_{n-1})/(\lambda_n - \lambda_{n-1})$, where n is the band number of wavelength λ, between 1 and 256. The first-difference spectrum measures how much reflectance changes from one wavelength to the next: it is an approximation of the slope, or first derivative, of the raw spectrum.

We transformed the reflectance spectra using published indices which have been recommended as excellent indicators of foliar Chl. We began with two standard indices used in remote sensing, the vegetation index (VI, also known as the simple ratio, SR) and the normalized difference vegetation index (NDVI) (Gamon *et al.*, 1995; Peñuelas & Filella, 1998). Through difference normalization, the VI is converted to a scale from −1.0 to +1.0, and the effects of confounding external factors, such as solar elevation (especially important in remote sensing work) and leaf structure, are reduced. However, difference normalization also results in a highly non-linear relationship between NDVI and Chl, whereas the relationship between VI and Chl is quite linear. The vegetation index was calculated as $VI = R_{NIR}/R_{Red}$, and the normalized difference vegetation index was calculated as $NDVI = (R_{NIR} - R_{Red})/(R_{NIR} + R_{Red})$; we used R_{750} for R_{NIR}, and R_{680} for R_{Red}.

Some authors have suggested the use of wavelengths other than 750 and 680 nm when calculating NDVI, claiming improved sensitivity to a wider range of Chl concentrations. A revised version of the this index was therefore calculated as $\text{Chl NDI} = (R_{750} - R_{705})/(R_{750} + R_{705})$ (Gitelson & Merzlyak, 1994; Gamon & Surfus, 1999).

The reflectance integral index proposed by Gitelson & Merzlyak (1994), which we denote RII, was calculated using a discrete summation approximation to the following integral:

$$\text{RII} = \int_{705}^{750} (R_\lambda / R_{705} - 1)\, d\lambda$$

Total chlorophyll content is known to be correlated with the red edge position (Curran *et al.*, 1990), which is the wavelength λ (nm) of the maximum slope of the reflectance spectrum at wavelengths between 690 and 740 nm. The red edge position is considered to be highly robust to confounding factors, and has a good signal-to-noise ratio (Adams *et al.*, 1999), although some research has suggested that it may be somewhat sensitive to variation in leaf structure (Gitelson *et al.*, 1996). Red edge λ (λRE), measured in nm, was taken to be the wavelength (between 690 and 740 nm) at which the first-difference spectrum reached a local maximum.

The yellowness index (YI) was formulated to indicate chlorosis in stressed leaves by measuring changes in the shape or concavity of reflectance spectra around 600 nm (Adams *et al.*, 1999). We calculated the index as a finite approximation to the second derivative of the reflectance spectrum between 580 and 668 nm: $YI = -10(R_{\lambda_{-1}} - 2R_{\lambda_0} + R_{\lambda_{+1}})/\Delta\lambda^2$, with λ_{-1} = 580 nm, λ_0 = 624 nm, λ_{+1} = 668 nm, and $\Delta\lambda$ = 44 nm. The scaling factor of −10 was included to indicate increasing yellowness within increasingly positive values (Adams *et al.*, 1999).

Several indices targeted directly at either Chl*a* or Chl*b* were also tested. First, we used R_{680}, which is the absorption peak of Chl*a*. For Chl*b*, we used R_{635}, which is somewhat lower than the absorption peak (645–650 nm) of Chl*b*, but is reported by Blackburn (1998) to be the individual wavelength best correlated with Chl*b* content. Second, we used the pigment specific simple ratios for Chl*a* and Chl*b*, $PSSR\,a = R_{800}/R_{675}$, and $PSSR\,b = R_{800}/R_{650}$ (Blackburn, 1998). Note that there is a near-perfect linear correlation between PSSR *a* and VI, because of the similarity of the wavelengths used in each index.

Chlorophyll extraction

We used the dimethyl sulphoxide (DMSO) Chl extraction technique of Hiscox & Israelstam (1979). This method,

which has also been recommended by others (Barnes *et al.*, 1992; Monje & Bugbee, 1992), has two principal advantages over other extractions (e.g. methanol, ethanol, or acetone). First, the method is faster, largely because grinding and centrifuging is not required. Second, the Chl extracts are more stable in DMSO, and do not break down as quickly as those in acetone: Hiscox & Israelstam (1979) reported that DMSO extracts are stable for up to 5 d, whereas with acetone extracts the measured level of Chl begins to fall off immediately.

For the extractions, glass centrifuge vials containing 7 ml DMSO were preheated to 65°C in a water bath. Chl was extracted from three disks (each 3.038 cm^2; approx. 100 mg f. wt total) from each leaf sample. In preliminary trials, we found that extraction at 65°C was complete within 15–20 min and no loss of Chl occurred in the heated DMSO during the first hour; we therefore ran our extractions for 30 min When the extractions were complete, samples were removed from the water bath and each graduated vial was topped up to exactly 10 ml with DMSO using a Pasteur pipette; 3 ml of each extract were then transferred to disposable polystyrene cuvettes with a reported standard deviation between cuvettes of $< \pm 0.005$ extinction units, and a transmission between 600 and 700 nm of 85% or better (catalogue 14-385-985, Fisher Scientific, Pittsburgh, PA, USA). The spectrophotometer (range 200–1100 nm, spectral bandwidth 5 nm, wavelength accuracy ±1 nm, and wavelength setting repeatability of ±0.3 nm; model U-1100, Hitachi Ltd, Tokyo, Japan), was calibrated to zero absorbance using a blank of pure DMSO. Absorbance of both blank and sample were measured at 645 and 663 nm. The elapsed time between removal from the water bath and completion of spectrophotometer measurements was in the order of 20 min.

Hiscox & Israelstam (1979) demonstrated that the absorption spectrum (600–680 nm) for Chl extracted in DMSO was virtually identical to that for extracted in 90% acetone. They therefore recommended the use of Arnon's (1949) equations: Chl*a* ($g\ l^{-1}$) = $0.0127\ A_{663} - 0.00269\ A_{645}$; Chl*b* ($g\ l^{-1}$) = $0.0229\ A_{645} - 0.00468\ A_{663}$; tot Chl ($g\ l^{-1}$) = $0.0202\ A_{645} + 0.00802\ A_{663}$. The Chl concentration of the extract calculated from these equations was then converted to leaf Chl content (mg Chl cm^{-2} leaf area).

Data analysis

To arrive at a ranking of the best methods for estimating relative Chl, we first randomly separated the data set of 100 leaves into two equal halves of $n = 50$. The first half was used to develop calibration equations relating the different indices to Chl*a*, Chl*b*, and total Chl. The calibration equations we used were generally second-order polynomials, but other functional forms (e.g. power or exponential functions) were used where appropriate. The difference between the fitted and actual measured Chl (i.e. the regression residual) is the calibration error (ε_c). We used the root mean square calibration error (calculated as the square root of the mean of the squared calibration errors, i.e. $RMS\varepsilon_c = \sqrt{\sum \varepsilon_c^2 / n}$) to assess the goodness-of-fit of the calibration equation. We then tested these calibration equations on the second half of the data, by generating predicted levels of Chl based on the measured index values. The difference between the predicted and actual measured Chl is the prediction error (ε_p), and we used the root mean square prediction error ($RMS\varepsilon_p$, calculated analagously to $RMS\varepsilon_c$) as an indicator of the actual success of the index to estimate Chl content. For each index, we then scaled the $RMS\varepsilon_p$ by the average level of Chl across all 100 leaf samples to arrive at a relative measure (in percentage terms) of the accuracy of each method.

Results

Actual chlorophyll content

Total Chl content ranged from 0.0004 to 0.0455 mg cm^{-2}; the average Chl*a* and Chl*b* contents were 0.0127 mg cm^{-2} and 0.0040 mg cm^{-2}, respectively. Leaf concentrations (mg Chl g^{-1} leaf f. wt) of total Chl ranged from 0.04 to 3.87 mg g^{-1}, with a mean of 1.33 mg g^{-1}. There was generally a close, linear relationship between Chl*a* and Chl*b* (Chl$b = 0.2897$ Chl$a + 0.004$, $r^2 = 0.97$, $n = 100$), and for most samples the Chl*a* : Chl*b* ratio was in the range of 2.5–4.0.

Comparison of SPAD-502 and CCM-200

There was a close curvilinear relationship between readings from the SPAD-502 and CCM-200 meters (Fig. 1). The correlation was tightest at lower levels of Chl (i.e. lower SPAD-502 and CCM-200 readings). The relationship was best explained by a third order polynomial ($r^2 = 0.97$, $n = 100$):

$$CCI = 0.9640 + 0.1850SPAD - 0.0020SPAD^2 + 0.0002SPAD^3$$

General relationships between reflectance spectra and Chl concentrations

With decreasing total Chl, reflectance from 500 to 700 nm increased noticeably (Fig. 2a), ultimately causing the disappearance of the reflectance peak around 550 nm, the trough around 680 nm, and the red edge around 700 nm. Changes in the position and shape of the red edge with decreasing Chl were especially obvious in the first difference spectra (Fig. 2b). Not only did the local maximum of the first difference spectra (λRE) shift to shorter wavelengths at lower Chl, but there was also a steady decrease in the slope of the reflectance spectrum at the 'shoulder' of the red edge (i.e. around 720 nm). Although changes in the original reflectance spectra were most pronounced at low levels of Chl, changes in

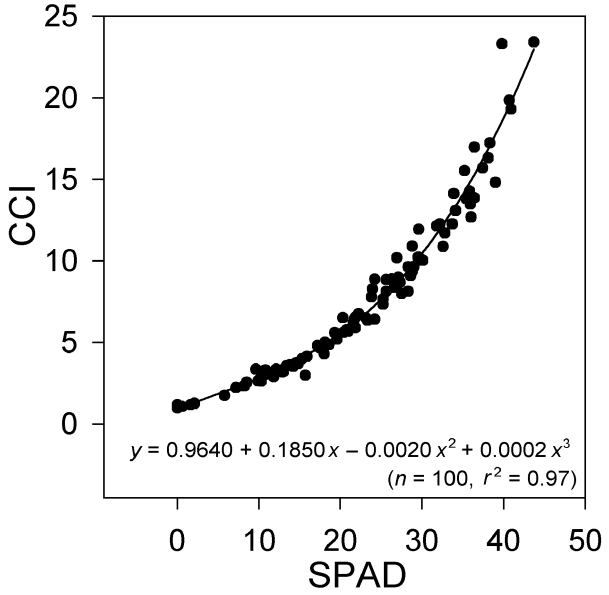

Fig. 1 Comparison of chlorophyll index readings from the Minolta SPAD-502 (SPAD units) and Opti-Sciences CCM-200 (chlorophyll content index (CCI), units) hand-held absorbance meters. Each point represents the mean of five measurements taken on an individual paper birch leaf.

the first difference spectra appeared to be more sensitive across a wide range of Chl contents.

Correlograms showing the linear correlation between reflectance at different wavelengths and the measured total Chl content (Fig. 3a) indicated that reflectance in the visible wavelengths was negatively correlated with measured total Chl. Thus leaves with more Chl pigments were able to absorb more radiation at the wavelengths important for photosynthesis (i.e. PAR). Between 700 and 713 nm, the correlation between total Chl and reflectance was strongest, and consistently better than $r = -0.90$. Approx. 730–740 nm, the correlation between reflectance and Chl content was approx. zero; above 750 nm, reflectance was positively correlated with Chl. The linear correlation between reflectance and the Chl*a* : Chl*b* ratio generally followed a similar pattern (Fig. 3a), although the correlation coefficient was usually somewhat smaller.

Total Chl was highly correlated ($r \geq 0.95$) with the first difference at wavelengths between 721 and 744 nm. Using the 50 observations in our calibration set, we determined that D_{730} was the single wavelength best correlated with total Chl, and so we added this to our list of indices to be tested.

The Chl*a* : Chl*b* ratio was reasonably well correlated ($|r| = 0.50$) with the first difference between 632–672, 678–691 and 704–750 nm (Fig. 3b). Within the range of values of our study, D_{681} was related to the Chl*a* : Chl*b* ratio as ($r^2 = 0.52$, $n = 88$, Fig. 4):

$$\frac{\text{Chl}a}{\text{Chl}b} = 3.50 - 122.42 D_{681}$$

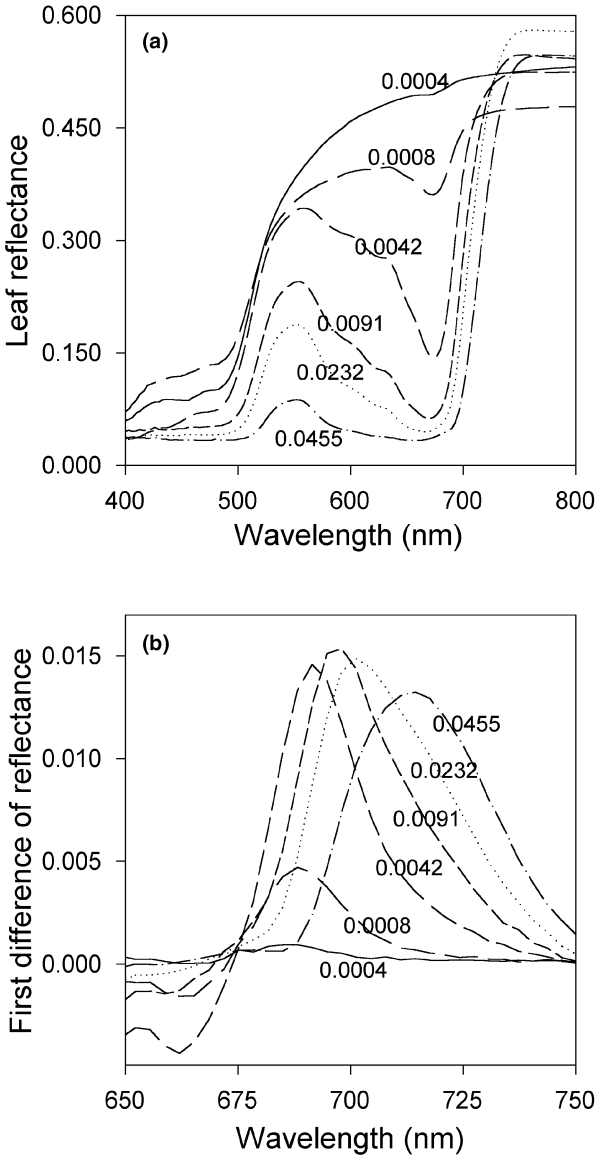

Fig. 2 Reflectance spectra indicating changes in (a) percent reflectance and (b) the first difference (slope of the original reflectance spectrum) across a range of chlorophyll contents. Each curve represents the arithmetic mean of five separate scans taken on a single leaf sample. Numbers next to each curve indicate the total Chl content (mg cm^{-2}) of the leaf, as measured by dimethyl sulfoxide extraction and spectrophotometric determination.

Relationships between Chl indices and measured Chl content

In Table 1, we list the 10 best indices for estimating Chl*a*, Chl*b*, and total Chl content, sorted according to our assessment (relative RMSε_p) of the accuracy of each method. Generally the best reflectance indices out-performed those of the hand-held absorbance meters. Chl NDI consistently performed the best, ranking first for Chl*a*, second for Chl*b*, and first for total Chl. For Chl*a* and total Chl, Chl NDI had

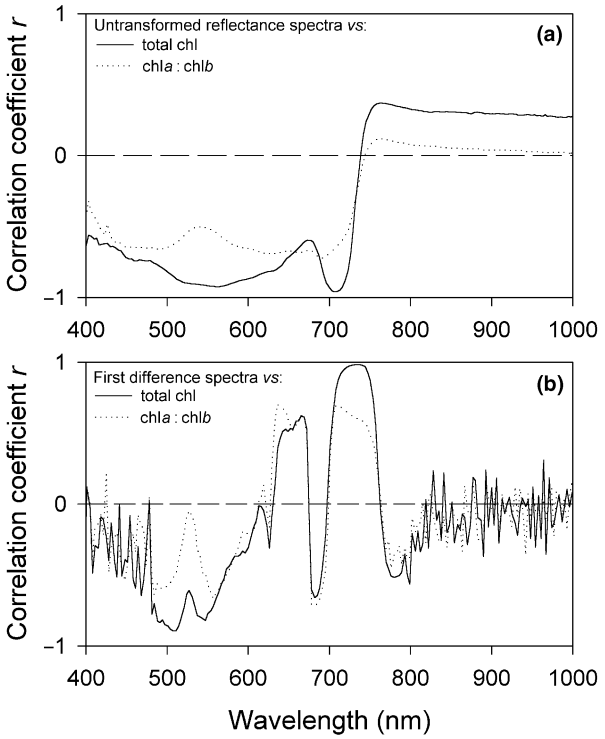

Fig. 3 Correlograms indicating the linear correlation coefficient, *r*, between total Chl and the Chl*a* : Chl*b* ratio, and leaf reflectance at different wavelengths. (a) Correlation with the untransformed reflectance spectra; (b) correlation with the first difference spectra. Curves are based on $n = 97$ leaf samples from paper birch.

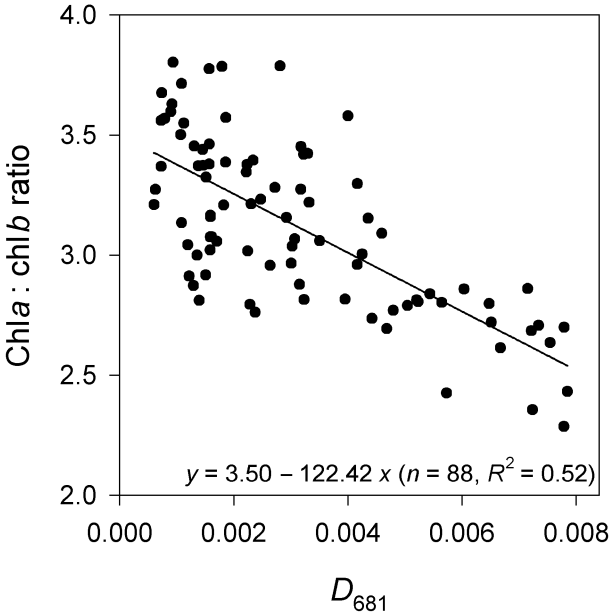

Fig. 4 The linear correlation between D_{681} and the Chl*a* : Chl*b* ratio for leaves of paper birch. Chl*a* : Chl*b* ratio calculated following dimethyl sulfoxide extraction and spectrophotometric determination of Chl*a* and Chl*b* content.

Table 1 Ranking of 10 different indices for estimating leaf chlorophyll. Indices sorted by the root mean square error of predicted values ($RMS\varepsilon_p$), expressed as a percentage of mean chlorophyll content across all 100 leaves studied. Calibration equations were derived using a randomly selected subset of the data (50 leaves). $RMS\varepsilon_p$ calculated using data for the 50 leaves which were not used for calibration. Actual Chl content determined by extraction in dimethyl sulfoxide followed by spectrophotometric determination

Rank	Chl*a*	Chl*b*	Total Chl
1	Chl NDI (11.9%)	D_{730} (14.6%)	Chl NDI (12.1%)
2	RII (12.1%)	Chl NDI (15.2%)	RII (12.5%)
3	D_{730} (13.2%)	SPAD (15.6%)	D_{730} (12.9%)
4	λRE (13.7%)	RII (16.1%)	R_{706} (16.7%)
5	R_{706} (16.5%)	CCI (17.7%)	SPAD (18.6%)
6	SPAD (20.3%)	R_{706} (18.9%)	CCI (20.0%)
7	CCI (22.3%)	PSSR*b* (20.7%)	λRE (23.8%)
8	VI (29.9%)	VI (30.0%)	VI (29.8%)
9	PSSR*a* (30.8%)	R_{635} (32.3%)	NDVI (29.8%)
10	R_{680} (32.4%)	λRE (36.2%)	YI (38.0%)

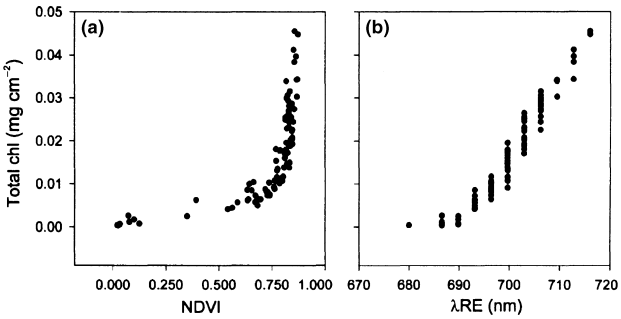

Fig. 5 Correlation of two standard reflectance indices (a) normalized difference vegetation index (NDVI) and (b) red edge λ (λRE), with total Chl.

a $RMS\varepsilon_p$ that was some 40% lower than that of either SPAD or CCI. On the other hand, for Chl*b*, Chl NDI was only 2% better than SPAD but 14% better than CCI. However, several indices commonly used in the literature, such as λRE and NDVI, performed surprisingly poorly (Fig. 5): $RMS\varepsilon_p$ of NDVI was consistently more than double that of Chl NDI. Some indices that have been found to perform well in other species, such as YI and PSSR, performed very poorly for birch.

In Fig. 6 we show scatter plots relating SPAD, CCI, Chl NDI, RII and D_{730} index measurements to the measured Chl*a*, Chl*b*, and total Chl concentrations. The plots indicate a close positive correlation between measured Chl and all five indices. The corresponding calibration equations we derived are given in Table 2. We caution that these equations were developed using leaves of only one species, and they may not accurately predict the Chl content of leaves of other species. Furthermore, as there may be differences between instruments, it seems wise to caution that equations derived for one

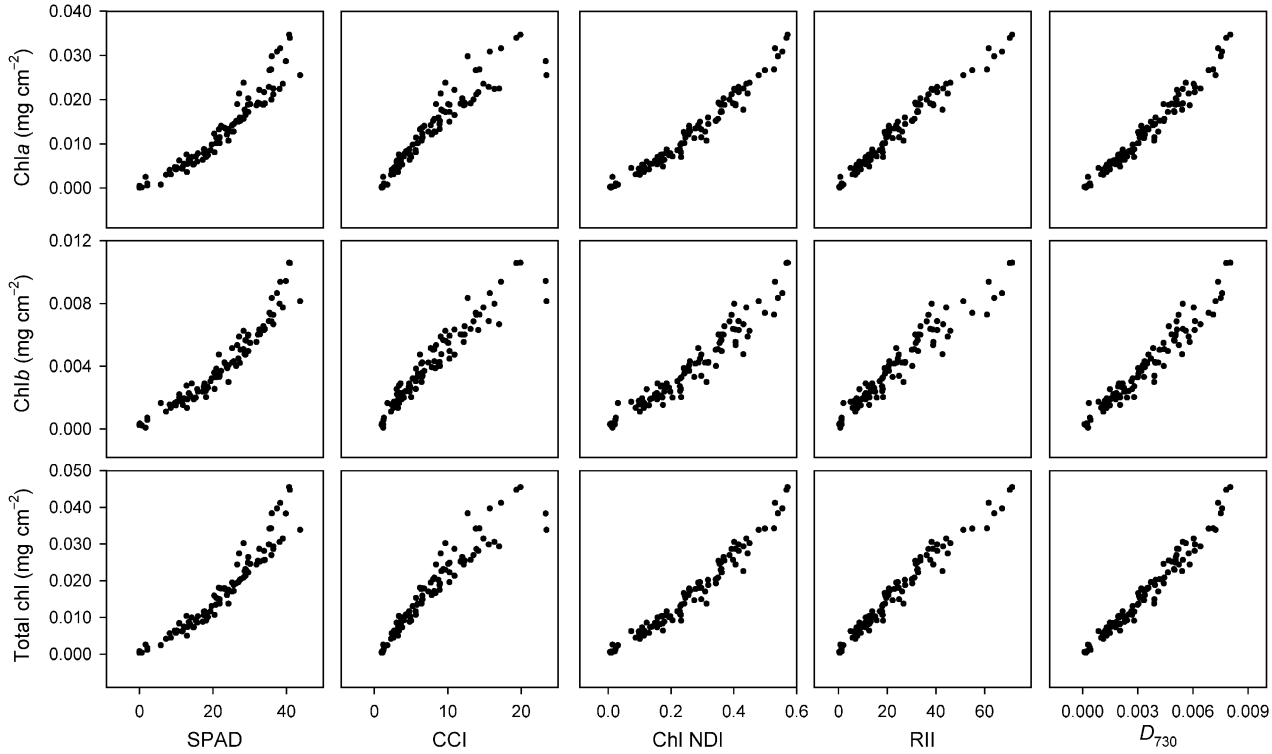

Fig. 6 Scatter plots indicating the relationship between various absorbance and reflectance indices, and Chl *a*, Chl *b*, and total Chl. SPAD and chlorophyll content index (CCI) calculated by the Minolta SPAD-502 and Opti-Sciences CCM-200 hand-held chlorophyll meters, respectively. Chl NDI (chlorophyll normalized difference index), RII (reflectance integral index), and D_{730} (first difference of the reflectance spectrum at 730 nm) indices calculated from reflectance spectra.

Table 2 Calibration equations for five different chlorophyll indices. The calibration equation converts from the index value to the chlorophyll (Chl*a*, Chl*b*, or total Chl) content. Calibration equations derived using 50 randomly selected leaf samples from the 100 paper birch (*Betula papyrifera*) leaves studied. r^2 is the coefficient of determination of the calibration equation. RMSε_c is the root mean square error of the calibration equation residuals. RMSε_p is the root mean square of the prediction errors when the calibration equations were tested against the remaining 50 leaves. SPAD and CCI calculated by the Minolta SPAD-502 and Opti-Sciences CCM-200 hand-held chlorophyll meters, respectively. Chl NDI (chlorophyll normalized difference index), RII (reflectance integral index), and D_{730} (first difference of the reflectance spectrum at 730 nm). Indices calculated from reflectance spectra

Method	*y*	r^2	RMSε_c	RMSε_p	Calibration equation to convert from index value to chlorophyll content (mg cm^{-2})
SPAD	Chl*a*	0.952	0.0018	0.0026	y = 1.56E-06+3.33E-04x+9.03E-06x^2
	Chl*b*	0.964	0.0005	0.0006	y = 5.46E-04+6.89E-05x+3.37E-06x^2
	total Chl	0.960	0.0021	0.0031	y = 5.52E-04+4.04E-04x+1.25E-05x^2
CCI	Chl*a*	0.952	0.0018	0.0028	y = −2.12E-03+2.44E-03x − 4.66E-05x^2
	Chl*b*	0.955	0.0005	0.0007	y = −6.68E-05+6.41E-04x − 9.54E-06x^2
	total Chl	0.958	0.0022	0.0034	y = −2.20E-03+3.09E-03x − 5.63E-05x^2
Chl NDI	Chl*a*	0.978	0.0012	0.0015	y = −1.35E-04+3.48E-02x+4.16E-02x^2
	Chl*b*	0.920	0.0007	0.0006	y = 3.13E-04+1.11E-02x+9.39E-03x^2
	total Chl	0.969	0.0018	0.0020	y = 1.81E-04+4.60E-02x+5.12E-02x^2
RII	Chl*a*	0.978	0.0012	0.0015	y = −4.82E-04+6.39E-04x − 2.39E-06x^2
	Chl*b*	0.920	0.0007	0.0006	y = 1.89E-04+1.97E-04x − 9.90E-07x^2
	total Chl	0.970	0.0018	0.0021	y = −2.93E-04+8.40E-04x − 3.39E-06x^2
D_{730}	Chl*a*	0.984	0.0010	0.0017	y = −2.25E-04+3.39E+00x+8.84E+01x^2
	Chl*b*	0.932	0.0006	0.0006	y = 2.73E-04+1.06E+00x+1.10E+01x^2
	total Chl	0.978	0.0015	0.0022	y = 4.98E-05+4.48E+00x+9.98E+01x^2

instrument may not be exactly correct for other instruments, even of the same make and model.

The indices we tested were generally better indicators of Chl*a* and total Chl than Chl*b*. For example, for our Chl NDI calibration equations, the coefficients of determination for Chl*a* (r^2 = 0.97) and total Chl (r^2 = 0.97) were both somewhat higher than for Chl*b* (r^2 = 0.92).

The relationship between the index values and measured Chl was generally much tighter at low levels of Chl. The increasing scatter with increasing Chl was especially pronounced for both SPAD and CCI, and occurred for both Chl*a* and Chl*b*, as well as total Chl.

The linearity of the relationship between the different index values and the measured Chl varied from index to index. Chl NDI, RII, and D_{730} were all close to linearly related to the different Chl measurements, but both SPAD and CCI exhibited some nonlinearity. Certain popular indices, such as NDVI (Fig. 5a), R_{680}, R_{635}, and YI, all showed a highly nonlinear relationship with Chl content. This nonlinearity indicates saturation of the indices at moderate levels of Chl, and consequently these indices were generally unable to differentiate between different levels of Chl, except when Chl contents were exceptionally low (e.g. below 0.010 mg cm^{-2} total Chl for NDVI).

Discussion

Comparison of methods

When light hits a leaf, it can either be reflected from, absorbed by, or transmitted through the leaf. Because the function of Chl pigments is to absorb quanta of incident light, it could be hypothesized that instruments that estimate Chl content by directly measuring the amount of radiation absorbed should be able to give better estimates of Chl content than those relying on reflectance measures. However, the results presented in this paper indicate the opposite, namely that relative Chl content is best estimated by reflectance rather than absorbance (Table 1).

It could also be hypothesized that an instrument that samples a larger leaf area would give a more accurate measure of leaf Chl than one that samples a smaller leaf area. However, of the two hand-held absorbance meters, the Minolta SPAD was superior, despite its measuring area being just one-tenth that of the CCM-200. Furthermore, the fibre-optic probe of the Unispec had a sampling area that was even smaller than that of either of the two absorbance meters, and yet the best reflectance indices were all more highly correlated with actual leaf Chl than either CCI or SPAD. Related to this, a significant problem with the hand-held absorbance meters was that estimates of leaf Chl appeared to be less accurate as the Chl content increased (Fig. 5). A possible explanation is that the distribution of Chl in high-Chl leaves is less uniform than in low-Chl leaves (Terashima & Saeki, 1983). Thus the 'sieve effect' associated with the irregular distribution of Chl has been used to explain the poor performance of absorbance meters at high levels of Chl (Monje & Bugbee, 1992). This may be an inherent limitation of absorbance meters that cannot be overcome, even with a large sampling area. Another possible explanation is that at high levels of Chl, so much of the 660 nm light is absorbed by the leaf that little remains to be transmitted and measured on the far side; use of a slightly longer wavelength (for example, 695 nm instead of 660 nm) might improve the sensitivity of hand-held absorbance meters.

Furthermore, although all methods tested resulted in index values that were reasonably well correlated with actual leaf Chl content, our results suggest that researchers need to consider using reflectance indices to estimate Chl content at the leaf level because they can be more accurate than the absorbance meters. In addition, the best reflectance indices were not the ones most commonly used in the literature. For Chl*a*, Gitelson & Merzlyak (1994) reported RMSε_c of 0.0019 mg cm^{-2} for Chl NDI and 0.0013 mg cm^{-2} for RII, and our data set confirmed the high reliability of both of these indices. These indices were both more highly correlated with chl content than the widely used NDVI and λRE.

Generalization of results

An important objective of future investigations into the relationships between leaf optical properties and pigment concentrations should be to find indices that hold across a wide range of species and functional groups. However, this may prove difficult to achieve. Gamon & Surfus (1999) demonstrated that the relationship between NDVI and total Chl is markedly different for the coniferous *Pseudotsuga menziesii* and the herbaceous *Helianthus annuus*; they suggest this may be due to differences in leaf morphology and structure. Indeed, differences in leaf structure, and the associated effects of this on leaf reflectance, appear to severely impair our ability to use many indices across a wide range of vegetation types. In addition, such differences make it unlikely that calibration equations from one study can be directly applied to leaves with different structural attributes. For example, we tested the exponential equation presented by Markwell *et al.* (1995) for converting SPAD values to total Chl of soybean and maize, and calculated a RMSε_p of 0.0038 mg cm^{-2}. This was surprisingly low – only slightly worse than the RMSε_p of 0.0031 mg cm^{-2} for our second-order polynomial calibration equation. On the other hand, the SPAD calibration equation presented by Demarez *et al.* (1999) for oak and beech leaves performed very poorly – it consistently over-estimated total Chl of our birch leaves by nearly 50%. Similarly, we found that the equations proposed by Curran *et al.* (1990) for λRE, Blackburn (1998) for PSSR and PSND, and Gitelson & Merzlyak (1996) for R_{750}/R_{700}, produced poor Chl estimates when applied to our data.

The work on Australian eucalyptus species by Datt (1998, 1999) may give some insights into this matter, as his results help us to appreciate the ways in which relationships between Chl and reflectance vary between species. For example, although it is believed that in many northern temperate species there is little relationship between Chl content and NIR reflectance above 750 nm (see Fig. 3a), Datt (1998) demonstrated that this is not the case for certain eucalyptus. Consequently, indices such as Chl NDI, which use reflectance at 750 nm as structural 'reference wavelengths' supposedly uncorrelated with Chl content, can be expected to perform poorly when applied to the structurally dissimilar foliage of other biomes. Furthermore, whereas we found a strong positive correlation between D_{730} and Chl, Datt's results (Datt, 1999) indicate that in eucalyptus the correlation is much weaker and may in fact be negative. On the other hand, the wavelengths used to calculate YI are though to be little affected by either leaf water content or leaf structure (Adams *et al.*, 1999). Therefore, although YI did not perform as well as some of the other indices we tested, it might have broader applicability when the objective is to assess pigment concentrations across a range of different leaf morphologies. However, the high $\mathrm{RMS}\varepsilon_c$ values reported by Datt (1999) for many indices might also be interpreted as indicating that, for some species, such as eucalyptus, the accuracy of reflectance indices as indicators of leaf Chl content may be somewhat limited.

Effects of nonlinearity

Many of the indices studied exhibited a curvilinear response to increasing Chl content, as demonstrated by the almost universally significant x^2 term in our calibration equations. One implication of this nonlinearity is that caution must exercised when means are taken of index values. When a function is curvilinear, the mean value of a function is not the same as the function expressed at the mean. In mathematical terms this can be written as:

$$\frac{f(x_1) + f(x_2)}{2} \neq f\left(\frac{x_1 + x_2}{2}\right)$$

As an example of this, consider two leaves, one with NDVI = 0.900 and 0.040 mg cm^{-2} total Chl, the other with NDVI = 0.600 and 0.005 mg cm^{-2} total Chl (data drawn from Fig. 5a). The average NDVI is 0.750, which, in our data set corresponded to approx. 0.010 mg cm^{-2} total Chl. However, the actual average Chl content of the two leaves is 0.023 mg cm^{-2}. Using NDVI as a proxy for total Chl may result in significant misestimation of leaf Chl if NDVI is averaged across a sample of leaves. Note that if the relationship between the index value and Chl content is linear then there is no problem. Generally the indices that performed best in our study were also those that were close to linear, such as RII and Chl NDI (Fig. 6).

Future developments

It is possible that as research progresses, new indices will be created which outperform any of the Chl indices developed to date. Alternatively, new technology may offer other possibilities. For example, Gitelson *et al.* (1999) recently described their use of a spectrofluorometer to measure the fluorescence ratio $F_{735} : F_{700}$. Although the $\mathrm{RMS}\varepsilon_p$ (0.0042 mg cm^{-2}) of this method was higher than that for many of the methods we tested here, future developments and improvements may prove the application of fluorescence techniques to the quantification of leaf Chl content to be highly useful.

Conclusion

We used traditional extraction techniques to measure Chl*a*, Chl*b*, and total Chl of 100 paper birch leaves. We found that some reflectance-based indices, such as the Chl NDI proposed by Gitelson & Merzlyak (1994), were much better indicators of Chl content than some of the more commonly used indices, such as λRE or NDVI. Furthermore, the best reflectance indices were better indicators of Chl content than the indices given by either of the hand-held Chl absorbance meters we tested. However, these noninvasive optical methods for quantifying leaf pigmentation all performed quite well as a whole: they are fast, easy to use, and produce reliable estimates of relative leaf Chl. Nevertheless, differences in leaf structure may make index comparisons between species difficult. Furthermore, it seems essential to derive species-specific calibration equations for the different indices if estimates of absolute Chl content are desired.

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