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Original Article

In situ measurement of leaf chlorophyll concentration: analysis of the optical/absolute relationship

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ABSTRACT

***In situ* optical meters are widely used to estimate leaf chlorophyll concentration, but non-uniform chlorophyll distribution causes optical measurements to vary widely among species for the same chlorophyll concentration. Over 30 studies have sought to quantify the *in situ*/*in vitro* (optical/absolute) relationship, but neither chlorophyll extraction nor measurement techniques for *in vitro* analysis have been consistent among studies. Here we: (1) review standard procedures for measurement of chlorophyll; (2) estimate the error associated with non-standard procedures; and (3) implement the most accurate methods to provide equations for conversion of optical to absolute chlorophyll for 22 species grown in multiple environments. Tests of five Minolta (model SPAD-502) and 25 Opti-Sciences (model CCM-200) meters, manufactured from 1992 to 2013, indicate that differences among replicate models are less than 5%. We thus developed equations for converting between units from these meter types. There was no significant effect of environment on the optical/absolute chlorophyll relationship. We derive the theoretical relationship between optical transmission ratios and absolute chlorophyll concentration and show how non-uniform distribution among species causes a variable, non-linear response. These results link *in situ* optical measurements with *in vitro* chlorophyll concentration and provide insight to strategies for radiation capture among diverse species.**

Key-words: CCM-200; Chl*a*/Chl*b*; leaf optical properties; SPAD-502.

INTRODUCTION

Leaf chlorophyll concentration is most accurately measured by extraction of chlorophyll in a solvent followed by *in vitro* measurements in a spectrophotometer. However, non-destructive, *in situ*, optical techniques have become widely used to provide a relative indication of leaf chlorophyll concentration. Two commercially available meters are widely used (Minolta, model SPAD-502 (Spectrum Technologies, Plainfield, Ill.); and Opti-Sciences, model CCM-200 (Opti-Sciences, Inc., Hudson, NH)) and results from these meters have been reported in over 30 studies (Table 1). Neither meter has a linear relationship with chlorophyll concentration, and the reported optical/absolute chlorophyll concentration relationship has varied widely, sometimes even within the same species.

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Measurement of absolute chlorophyll concentration *in vitro*

The extraction method, extraction solvent, spectrophotometric equation and spectrophotometer resolution must match to accurately determine chlorophyll *in vitro* (Wellburn 1994). More than 30 studies have been conducted, but few have used the appropriate combination of analytical procedures.

Seven organic solvents have been widely used for chlorophyll extraction: acetone, methanol, ethanol, chloroform, diethyl-ether, dimethyl-formamide (DMF) and dimethyl sulphoxide (DMSO). Acetone has been the most widely used solvent because it has sharp chlorophyll peaks, but it is considered to be less efficient at chlorophyll extraction than methanol and ethanol (Holmhansen & Riemann 1978; Ritchie 2006). Acetone, methanol and ethanol require grinding of leaf tissue for complete extraction of chlorophyll. DMF and DMSO have an advantage over other solvents in that they allow for immersion of intact leaf tissue for chlorophyll extraction. However, immersion may not be effective for all plant tissues. Schaper & Chacko (1991) were not able to completely extract chlorophyll from Cashew and Mango leaf discs using DMSO. DMSO is less toxic than DMF, and extracted solutions are stable up to 7 d in the dark at 4 °C (Barnes *et al*. 1992). These advantages have led to increasing use of DMSO as an extraction solvent, but it is absorbed through the skin and gloves should be worn when handling it (Barnes *et al*. 1992).

Matching extraction solvent with spectrophotometric equation to convert absorption values to chlorophyll concentration

Wellburn (1994) emphasized the importance of using spectrophotometric equations that have been derived from accurate extinction coefficients determined in a reliable reference solution. Extinction coefficients from Smith & Benitez (1955) derived for diethyl-ether are generally accepted as accurate and are recommended for use in deriving extinction coefficients for other extraction solvents using the procedures described in Porra *et al*. (1989). Based on the

Table 1. Summary of publications on the optical/absolute chlorophyll concentration relationship

Meter type/Author (year)	Species
SPAD-501	
Yadava (1986)	Twenty-two unrelated species
Marquard & Tipton (1987)	Twelve unrelated species
Schaper & Chacko (1991)	Eight tropical and subtropical fruit-tree species
Dwyer *et al.* (1991)	Maize
Fanizza *et al.* (1991)	Twelve wine-grape cultivars
SPAD-502	
Gratani (1992)	Six Sclerophyllous species
Monje & Bugbee (1992)	Rice, soybean, wheat
Markwell *et al.* (1995)	Soybean and maize
Xu *et al.* (2000)	Sorghum
Bindi *et al.* (2002)	Potato
Richardson *et al.* (2002)	Paper birch
Netto *et al.* (2002)	Papaya
Yamamoto *et al.* (2002)	Sorghum and pigeon pea
Esposti *et al.* (2003)	Four citrus species
Wang *et al.* (2004)	Peace lily
Netto *et al.* (2005)	Coffee
Jifon *et al.* (2005)	Six citrus species
Cartelat *et al.* (2005)	Wheat
Uddling *et al.* (2007)	Birch, wheat and potato
Marenco *et al.* (2009)	Six Amazonian tree species
Naus *et al.* (2010)	Tobacco
Imanishi *et al.* (2010)	Flowering cherry
Coste *et al.* (2010)	Thirteen tree species of tropical rainforest
Ling *et al.* (2011)	*Arabidopsis thaliana*
Cerovic *et al.* (2012)	Kiwi, grape, wheat, and maize
CCM-200	
Richardson *et al.* (2002)	Paper birch
van den Berg & Perkins (2004)	Sugar maple
Jifon *et al.* (2005)	Six citrus species
Goncalves *et al.* (2008)	Four tropical wood species
Cerovic *et al.* (2012)	Kiwi, grape, wheat and maize

magnesium concentration of a known chlorophyll a and b solution, Porra *et al.* (1989) confirmed the extinction coefficients of Smith & Benitez (1955) for both chlorophyll a and b in diethylether. They found that the error in the original Smith & Benitez (1955) equation was less than 1%. Several equations developed for DMSO and DMF solvents have failed to follow the appropriate Porra *et al.* (1989) procedure (Moran & Porath 1980; Moran 1982; Inskeep & Bloom 1985; Barnes *et al.* 1992).

The equations developed by Arnon (1949) have often been used to quantify chlorophyll a and b concentration in higher plants and green algae. These equations were developed for use with 80% acetone in water. Several authors (Lichtenthatler & Wellburn 1983; Barnes *et al.* 1992; Porra 2002) have reported that equations from Arnon (1949) are inaccurate because they used the less accurate extinction coefficients of Mackinney (1941). Also, the chlorophyll a/b ratios obtained from the equations of Arnon (1949) underestimate the true a/b chlorophyll ratio (Porra *et al.* 1989; Wellburn 1994). Porra *et al.* (1989) developed an equation to convert a/b chlorophyll ratios determined by the equations of Arnon (1949) to correct values.

Several authors have used DMSO as an extracting solvent, but used spectrophotometric chlorophyll equations developed for 80% acetone (Monje & Bugbee 1992; Richardson *et al.* 2002). This has been justified by citing other publications that suggest that the absorption spectra for chlorophylls a and b are identical for 90% acetone and DMSO (Shoaf & Lium 1976; Hiscox & Israelstam 1979; Ronen & Galun 1984). However, equations from Arnon (1949) were developed for 80% (not 90%) acetone. Furthermore, Barnes *et al.* (1992) showed that the peak absorption wavelength for chlorophylls a and b is at a longer wavelength in DMSO than 80% acetone and found that equations from Arnon (1949) underestimated chlorophyll concentration using DMSO extracts by approximately 10%.

Matching spectrophotometric chlorophyll equations with instrument resolution

Wellburn (1994) discussed differences in chlorophyll measurement among spectrophotometers with differing spectral bandwidth resolution. Early spectrophotometer models used to derive equations were capable of only 1–4 nm resolution. High-quality modern spectrophotometers have a resolution of 0.1–0.5 nm and have been used to derive recently developed spectrophotometric chlorophyll equations. Wellburn (1994) compared three types of spectrophotometers (Uvikon model 941 Plus (Kontron, [U.K] Ltd.), 0.5 nm resolution;

Hewlett-Packard model HP8452A (Hewlett Packard Corporation, Palo Alto, CA), diode array 2 nm fixed resolution; and Pye Unicam model SP30 (Pye Ltd., Cambridge England), 1–4 nm variable resolution) and determined chlorophyll concentrations in six solvents. He omitted data from the diode array spectrophotometer because it had values that almost always deviated more than 10% from values of the other two instruments. Wellburn (1994) concluded that diode array spectrophotometers are not appropriate for use with equations derived by non-diode array spectrophotometers, and emphasized that equations derived with one spectrophotometer should not be used with a spectrophotometer with a different spectral resolution.

Although the goal of previous studies has been to develop standard curves to convert optical measurements to absolute chlorophyll concentration, measurement techniques vary widely. Predicted chlorophyll concentration from optical measurements of wheat leaves, measured with the same model of meter, has varied up to 80% among studies (Monje & Bugbee 1992; Uddling *et al*. 2007). These differences have not been widely acknowledged in the literature.

Optical meters used to determine chlorophyll concentration

The two most widely used chlorophyll concentration meters are the Konica Minolta, model SPAD-502 (Konica Minolta Sensing, Inc., Sakai, Osaka, Japan) and the Opti-Sciences, model CCM-200 (Opti-Sciences, Inc., Hudson, NH, USA). Both meters measure the transmission of two wavelengths of radiation through plant leaves: red at approximately 650 nm, and near infrared (NIR) at approximately 900 nm. Increased chlorophyll concentration increases the absorption of red radiation. All plants transmit a high fraction of NIR radiation as these wavelengths are not absorbed by photoreceptors and this transmission is used as a reference wavelength.

Another hand-held, optical chlorophyll meter was recently introduced, the Dualex 4 Scientific (Dx; FORCE-A, Orsay, France). This meter measures the transmission of radiation at 710 and 850 nm and converts the measurement into a value of chlorophyll in μg cm^{-2}.

The sampling area differs between meters. The CCM-200 samples 71 mm^2, the SPAD-502 samples 6 mm^2 and the Dx4 samples 20 mm^2. Larger areas provide a larger spatial average, but smaller areas can measure narrower leaves.

Description of the optical differences between meters

The output of the CCM-200 is the ratio of transmission of radiation from a light emitting diode (LED) centred at 931 nm to transmission of radiation from an LED cantered at 653 nm (CCM-200 user manual). This ratio is defined as the chlorophyll content index (CCI).

$$CCI = \frac{\%\ \text{transmission 931 nm}}{\%\ \text{transmission 653 nm}} \quad (1)$$

The SPAD-502 measures radiation centred at 940 and 650 nm (Minolta Manual), but the equation to convert these measurements to a 'SPAD' value has been reported differently in four publications. The most complete equation is given by Naus *et al*. (2010):

$$SPAD = k \times \log\left(\frac{\%\ \text{transmission 940 nm}}{\%\ \text{transmission 650 nm}}\right) + C \quad (2)$$

where k is a confidential slope coefficient and C is a confidential offset value. Three other publications have reported less complete equations to calculate the SPAD value. Uddling *et al*. (2007) reported this equation, but without the C offset. Cerovic *et al*. (2012) and Markwell *et al*. (1995) reported the equation without either k or C. As the slope and offset values are confidential, it is not possible to derive SPAD values from transmission measurements, and it is not possible to mathematically derive a conversion equation between meters. However, as both the SPAD values and the CCI are based on a ratio of the transmission at two closely related wavelengths:

$$SPAD \approx k \times \log(CCI) + C \quad (3)$$

Studies on the optical/absolute chlorophyll concentration relationship

Four studies have reported empirical relationships that relate optical measurements to absolute chlorophyll concentration for a meter (model SPAD-501) that was a predecessor to the SPAD-502 (Yadava 1986; Marquard & Tipton 1987; Fanizza *et al*. 1991; Schaper & Chacko 1991). The SPAD-501 used slightly different wavelengths and is thus not directly comparable with the SPAD-502.

Monje & Bugbee (1992) appear to have been the first to develop an equation that relates the output from the SPAD-502 to absolute chlorophyll concentration in mg m^{-2}. Since then, numerous other relationships for a range of species have been proposed (Schaper & Chacko 1991; Markwell *et al*. 1995; Xu *et al*. 2000; Bindi *et al*. 2002; Netto *et al*. 2002, 2005; Richardson *et al*. 2002; Yamamoto *et al*. 2002; Esposti *et al*. 2003; Wang *et al*. 2004; Cartelat *et al*. 2005; Jifon *et al*. 2005; Uddling *et al*. 2007; Marenco *et al*. 2009; Coste *et al*. 2010; Imanishi *et al*. 2010; Naus *et al*. 2010; Ling *et al*. 2011; Cerovic *et al*. 2012). The acronym 'SPAD' refers to the division of Minolta that developed the meter, special products analysis division. As the acronym implies, SPAD has no direct relationship to chlorophyll concentration.

Like SPAD, CCI values returned by the CCM-200 are only relative indicators of chlorophyll concentration, as CCI has no direct relationship to chlorophyll concentration. Several studies have also developed chlorophyll prediction equations using CCI measurements from the CCM-200 meter (Richardson *et al*. 2002; van den Berg & Perkins 2004; Jifon *et al*. 2005; Goncalves *et al*. 2008; Cerovic *et al*. 2012).

Variation in experimental techniques among studies

Extraction and measurement techniques have not been consistent among studies. Because chlorophyll concentration can have significant spatial variation it is important to remove the leaf disk from exactly the same location as the optical measurement. This precaution has not always been described in experimental procedures. Multiple extraction solvents, measurement wavelengths, spectrophotometric equations and instruments with varying resolution have been used to measure absolute chlorophyll. Sampling and measurement differences likely have caused significant variation among studies.

Most studies that have sought to determine the optical/absolute relationship have used only a single meter with the assumption that all meters of the same model are uniform. In an early study, Marquard & Tipton (1987) found 5% differences between two SPAD-501 meters. Markwell *et al*. (1995), mentioned that three SPAD-502 meters at the same university differed by ±5% and recommended that separate equations be developed for individual meters, but they did not indicate if optics in the meters had been cleaned before use. A comprehensive evaluation of uniformity among replicate meters has not been done. Two studies have attempted to estimate the prediction error associated with an individual measurement. Richardson *et al*. (2002) examined the error associated with individual optical measurements for paper birch leaves. They compared CCM-200 and SPAD-502 meters and found similar errors for both meters (19% for the SPAD meter and 20% for the CCM-200 meter). This relative error was calculated by dividing the root mean square error (RMSE) by average chlorophyll concentration across all samples. Cerovic *et al*. (2012) compared the Dx4 meter to SPAD-502 and CCM-200 meters and reported similar RMSEs for all three meters.

Differences among plant groups and species

Related species may share leaf optical properties. Monocots have a larger fraction of vascular tissue per unit surface area and dicots have a thicker adaxial cuticle with more palisade and spongy tissue. Cerovic *et al*. (2012) measured two monocot and two dicot species, and suggested that optical/absolute chlorophyll relationships could be grouped into separate monocot and dicot categories.

Chlorophyll a/b ratio

Considering that chlorophyll a and b can be easily distinguished *in vitro*, there has been a surprising lack of literature reporting differences among species. Few of the 30 studies on the optical/absolute relationship have reported the a/b chlorophyll ratio. Chang and Troughton (1972) pointed out that the chlorophyll a/b ratio can be affected by the species, environment, phase of leaf and plant growth and nutrient status on the chlorophyll a/b ratio. Their data indicate that chlorophyll a/b ratios are higher in C_4 than C_3 plants.

Chlorophyll a/b ratios are known to decrease during leaf senescence (Watts & Eley 1981; Castro & Sanchez-Azofeifa 2008), but several studies have found that drought stress has no effect on the chlorophyll a/b ratio (Martin & Warner 1984; Mafakheri *et al*. 2010). Several authors have suggested that chlorophyll a/b ratio should increase as leaf nitrogen content decreases, and the data of Kitajima & Hogan (2003) support this conclusion.

Cultivar differences within a species

Markwell *et al*. (1995) developed a single optical/absolute chlorophyll relationship for multiple strains of soybeans and maize, Uddling *et al*. (2007) found that a single curve could be used for multiple wheat cultivars grown over multiple seasons, and Dwyer *et al*. (1991) found that six maize (corn) hybrids had similar relationship curves. However, significantly different relationships were observed among citrus cultivars (Jifon *et al*. 2005). Cate & Perkins (2003), Richardson *et al*. (2002), and van den Berg & Perkins (2004) have all cautioned against treating a single optical/absolute chlorophyll relationship as universal.

The objectives of this study were: (1) estimate the magnitude of differences associated with the use of non-standard combinations of solvents and equations; (2) to implement the most correct methods for chlorophyll measurement to provide improved equations for conversion of optical measurements to absolute chlorophyll concentration; (3) to examine uniformity among two meter models (Opti-Sciences, model CCM-200; and Minolta, SPAD-502) manufactured from 1992 to 2013; (4) to develop equations for interconverting between units (CCI and SPAD units) from the two most common chlorophyll meters (Opti-Sciences, model CCM-200; and Minolta, SPAD-502); (5) estimate environmental effects on the optical/absolute chlorophyll concentration relationship; and (6) use optical and mathematical principles to better understand the underlying causes of nonlinearity in the optical/absolute chlorophyll concentration relationship.

MATERIALS AND METHODS

Collection and extraction of samples

Leaves of multiple ages and intensity of green colour were measured and sampled from 22 plant species (five monocots and 17 dicots, 11 deciduous species, and 11 annual crop plants) grown in greenhouse and field environments. Leaves were visually selected for a wide range of the intensity of greenness, which varied due to leaf age, position on the plant, and nutrient deficiencies. A common nutrient deficiency was lack of either nitrogen or iron, which was caused by high root-zone pH. Measurements were made near midday to minimize potential effects of light intensity on chloroplast movement.

CCI, using a CCM-200 meter, was measured at least three times in the same location on each leaf and averaged. A leaf disk was extracted from the exact same location as the measurement. Leaf disks were immediately extracted using a

number 4 cork borer with an area of 63.6 mm^2 to replicate the area measured by the chlorophyll meter and placed in a vial containing 10 mL of DMSO. Vials were incubated in an oven at 65 °C until all of the chlorophyll was in solution and the disk became transparent. This extraction occurred in less than 30 min for some species, but required 3 h for other species. After incubation, a 3 mL aliquot was transferred to an optical-grade analysis cell to measure light absorbance at 646.6 and 663.6 nm (Porra, 1989 acetone equation), and at 649.1 and 665.1 nm (Wellburn 1994; DMSO equation) using a Shimadzu UV-2401PC (Shimadzu Corporation, Kyoto, Japan) spectrophotometer with a resolution of 0.1 nm. Chlorophyll a and b concentrations were determined from spectral measurements using the equations developed by Wellburn (1994) for DMSO and for 0.1–0.5 nm spectral resolution:

$$\text{Chlorophyll a}\,(\mu g\ \text{mL}^{-1}) = 12.47 \times A(665.1\,\text{nm}) - 3.62 \times A(649.1\,\text{nm}) \quad (4)$$

$$\text{Chlorophyll b}\,(\mu g\ \text{mL}^{-1}) = 25.06 \times A(649.1\,\text{nm}) - 6.5 \times A(665.1\,\text{nm}) \quad (5)$$

where A is the absorption at the referenced wavelength and chlorophylls a and b are summed to obtain the total chlorophyll concentration.

Because several publications have extracted with DMSO, but incorrectly used the equation of Porra *et al.* (1989) that was developed for 80% acetone, chlorophyll was calculated using both procedures to determine the magnitude of error between equations.

Uniformity among meters

Five replicate Minolta SPAD-502 meters, manufactured from 1992 to 2008, and 25 replicate Opti-Sciences CCM-200 meters, manufactured from 2007 to 2013, were examined for uniformity of output by making replicate measurements on six coloured filters. These filters provided a consistent, uniform standard over a range of readings from 2 to 72 CCI units and from 6 to 62 SPAD units. The filters were Roscolux filters: #88, 'Light Green'; #3204, 'Half Blue'; #86, Pea Green; #92, 'Turquoise'; #89, 'Moss Green'; and #4490, 'CalColor 90 Green'.

Conversion between meters

Optical measurements were made in multiple identical locations on leaves of 10 plant species using a SPAD-502 and a CCM-200 meter. These measurements were supplemented with measurements made on 16 Roscolux filters to provide a wide range of SPAD and CCI values. Measured SPAD values were plotted against corresponding CCI measurements to obtain a relationship curve for the output of the two meters.

Multiple wheat cultivars

Four diverse wheat cultivars (Golden Spire, Lewjain, Greenville and Wanser) were grown in a greenhouse under three nutrient treatments: optimal nutrient availability, nitrogen deficient and iron deficient to determine relationships among cultivars and environmental conditions.

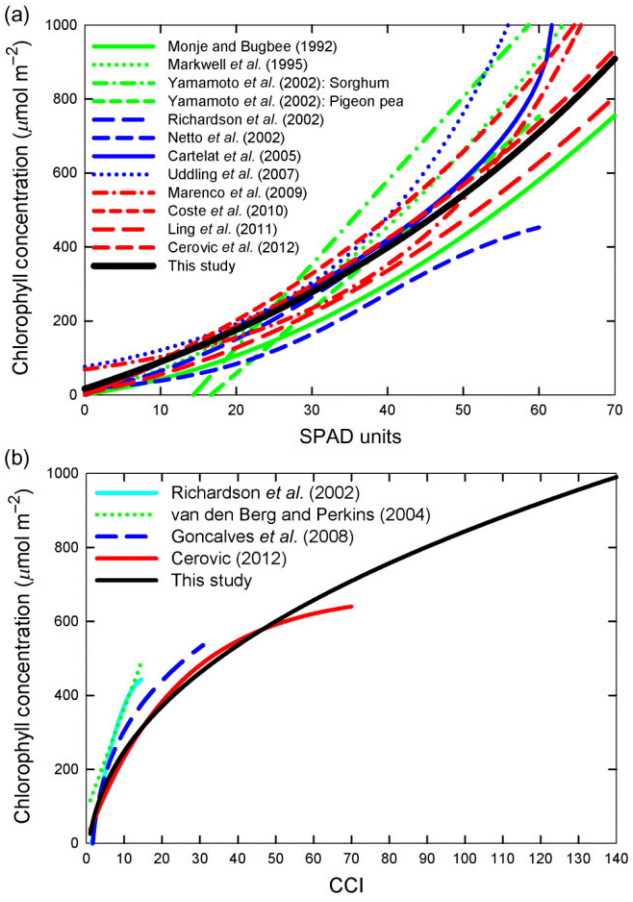

Figure 1. Relationship between meter output and chlorophyll concentration (μmol m^{-2}). (a) Twelve representative studies using special products analysis division (SPAD) units, and (b) four studies and this study using chlorophyll content index (CCI). Species and analytical methods differed among studies.

RESULTS

Summary of previous studies

Relationships between SPAD-502 and CCM-200 meters and absolute chlorophyll concentration from 17 previous studies indicate a wide range of relationships among species (Fig. 1a,b).

Relationships among similar species in different studies

Wheat is the most widely studied species with four SPAD-502 curves reported in four studies (Monje & Bugbee 1992; Cartelat *et al.* 2005; Uddling *et al.* 2007; Cerovic *et al.* 2012). The difference in the optical/absolute relationship among studies was as high as 80% between Uddling *et al.* (2007) and Monje & Bugbee (1992) (Fig. 2).

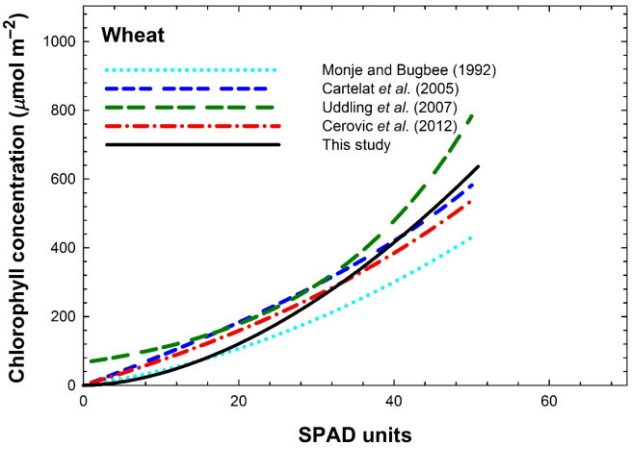

Figure 2. Relationship between special products analysis division (SPAD) units and chlorophyll concentration (μmol m^{-2}) for wheat from four prior studies and this study. The chlorophyll content index relationship from this study was converted for use with SPAD units using the equation in Fig. 7a.

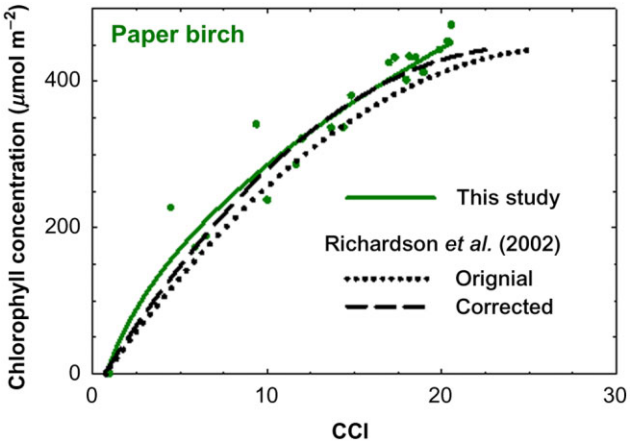

Figure 3. Relationship between chlorophyll content index (CCI) and chlorophyll concentration (μmol m^{-2}) for paper birch (*Betula papytifera*) leaves from two studies. The original relationship from Richardson *et al.* (2002) was corrected for the underestimation of chlorophyll concentrations derived from the equation of Porra *et al.* (1989) for dimethyl sulphoxide extractants.

Some of our measurements on wheat were made with the SPAD-502 meter; others were made with the CCM-200 meter. All data were converted to SPAD units to develop a comprehensive curve for wheat (Fig. 2). No significant difference among cultivars or nutrient stress treatments was found in the optical/absolute chlorophyll relationship. Our measurements were close to the average of the other studies across all chlorophyll concentrations.

Mean percentage difference between relationship curves of this study and others was also calculated for soybean (29%) (Markwell *et al.* 1995) and sorghum (40%) (Yamamoto *et al.* 2002; data not shown).

Paper birch was the only species that was common among studies using the CCM-200 meter. Richardson *et al.* (2002) used DMSO as the extractant, and the equation of Porra *et al.* (1989) that was developed for acetone extractants. We determined the magnitude of the error associated with this incorrect match of extraction solvent and spectrophotometric equation. Based on calculations for each of the 22 species in this study, we found that the mean difference between absolute chlorophyll concentrations calculated for a DMSO extractant using the DMSO equation of Wellburn (1994) and the acetone equation of Porra *et al.* (1989) is 7.84% (SD 0.28%; data not shown). We thus corrected the equation from Richardson *et al.* (2002) for paper birch by multiplying it by 7.84%. This correction resulted in a nearly identical fit to our derived equation for paper birch (Fig. 3).

Differences among species

The 22 species in this study had a wide range of optical/absolute chlorophyll relationships (Fig. 4a). A single universal relationship for all species was derived (Fig. 4b), along with individual equations for each species (Table 2; Fig. 5).

Although it appears that some cultivars within a species can be expressed by a single relationship, we found significantly different optical/absolute chlorophyll concentration relationships between two lettuce cultivars (cv. Waldman's Green and cv. Buttercrunch; *Lactuca sativa*; Fig. 5). However, our data indicate that the monocots barley, wheat, and rice have a similar optical/absolute chlorophyll concentration relationship (Fig. 5).

Uniformity of replicate meters

Output from each individual meter was plotted against the mean of all meters of the same type to determine variation among studies because of variation among replicate meters (Fig. 6). Mean coefficient of variation was 2.60% for the CCM-200 meter and 1.10% for the SPAD-502 meter.

Inter-conversion between units

Our results indicate that that universal relationships can be used to inter-convert between CCI and SPAD units (Figs 7A & 3B; $r^2 = 0.98$, 0.99). A similar relationship was developed by Richardson *et al.* (2002) for converting SPAD units to CCI units ($r^2 = 0.97$). However, the meter conversion relationship created by Richardson *et al.* (2002) was based on measurements on paper birch leaves with a narrow range of chlorophyll (SPAD units of 0–40). It was also developed for a prototype CCM-200 meter, which had a different wavelength for the red absorption wavelength. This meter was replaced with the current version in late 2002. The meter conversion curves for this study were developed from multiple species over a wide range of chlorophyll concentrations.

Monocot and dicot species differences

The absolute/optical relationships between CCI and chlorophyll concentration for the mean of five monocot

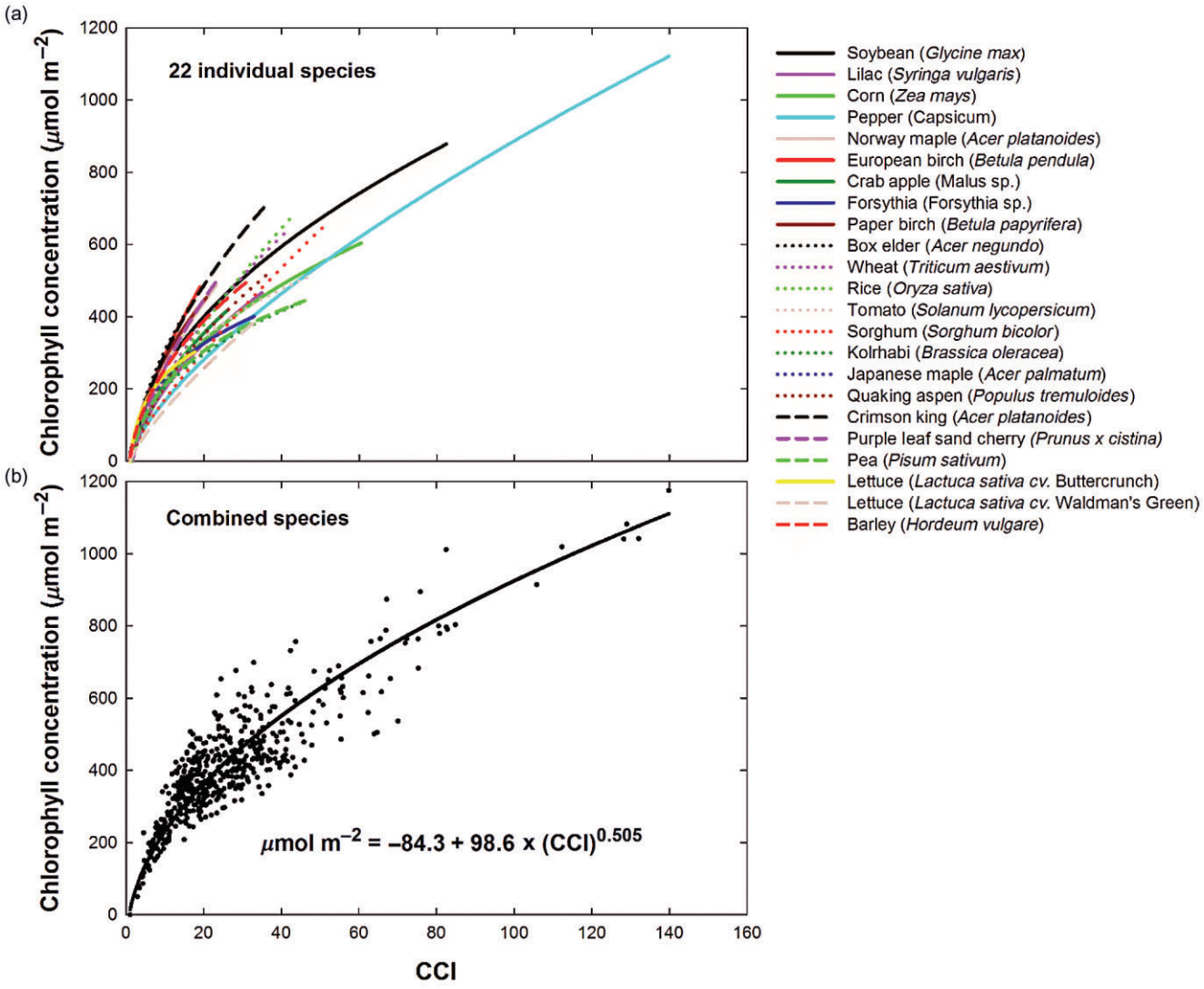

Figure 4. Relationship between chlorophyll content index (CCI) and chlorophyll concentration (μmol m^{-2}) for (a) 22 individual species and (b) all 22 species combined. The molar mass of the chlorophyll molecule is about 900 grams per mole. These measurements can easily be converted to mass per unit area.

species and the mean of 17 dicot species were not significantly different as indicated by the 95% prediction intervals (Fig. 8).

Chlorophyll a/b ratio

The mean chlorophyll a/b ratio for C_3 and C_4 plants was 3.2 and 6.3, respectively (Table 2). These results are similar to the values of Chang and Troughton (1972) when corrected for the underestimation of the Arnon (1949) equation (C_3: 3.9 and C_4: 5).

There was a small positive relationship between chlorophyll concentration and the a/b ratio. The coefficient of determination between absolute chlorophyll concentration and a/b ratio was 0.68 for Lilac, 0.48 for Japanese Maple and less than 0.20 for all other species (data not shown).

DISCUSSION

Relationship between transmission and absolute chlorophyll and cell wall content of leaves

Output of both Minolta SPAD-502 and Opti-Sciences CCM-200 meters is based on the ratio of transmission of NIR to red wavelengths. Transmission of radiation is non-linearly related to the amount of absorbing compound in leaf tissue and linearly related to the absorbance of compound (Atkins 1990). Absorbance is the negative log of transmittance.

Non-chlorophyll compounds (primarily cell walls) absorb radiation similarly at both red and NIR wavelengths, so transmission of red light is similarly affected by both compounds. Transmission of NIR radiation is not affected by chlorophyll and is thus primarily determined by the amount of non-chlorophyll compounds. Assuming a uniform

Table 2. Equations to determine chlorophyll concentration (μmol m^{-2}) from chlorophyll content index (CCI), r^2 values for each equation and mean chlorophyll a/b ratio for 22 species

	Conversion Equation (μmol m^{-2} from CCI)	r^2	Mean Chlorophyll a/b ratio	Standard Deviation of a/b ratio
Deciduous Species				
European Birch	$-76 + 85*(CCI)^{0.64}$	0.89	3.3	0.5
Lilac	$-98 + 93*(CCI)^{0.51}$	0.95	2.6	0.5
Norway Maple	$-95 + 96*(CCI)^{0.57}$	0.94	3.9	0.7
Quaking Aspen	$-128 + 106*(CCI)^{0.50}$	0.92	3.3	0.3
Purple Leaf Sand Cherry	$-144 + 113*(CCI)^{0.55}$	0.96	2.5	0.7
Crab Apple	$-124 + 117*(CCI)^{0.47}$	0.93	4.4	1.4
Paper Birch	$-120 + 135*(CCI)^{0.48}$	0.94	2.5	0.4
Crimson King Maple	$-160 + 144*(CCI)^{0.50}$	0.90	2.6	0.3
Japanese Maple	$-150 + 150*(CCI)^{0.43}$	0.97	1.9	0.1
Boxelder	$-191 + 182*(CCI)^{0.38}$	0.92	2.7	0.3
Forsythia	$-486 + 477*(CCI)^{0.18}$	0.93	2.6	0.5
Annual Crop Plants				
Sorghum (C_4)	$-8 + 29*(CCI)^{0.80}$	0.90	6.9	2.0
Pepper	$-19 + 39*(CCI)^{0.69}$	0.92	3.7	0.7
Rice	$-64 + 57*(CCI)^{0.68}$	0.82	5.0	1.5
Wheat	$-84 + 79*(CCI)^{0.60}$	0.87	4.3	0.4
Soybean	$-103 + 123*(CCI)^{0.47}$	0.95	4.2	0.6
Maize (C_4)	$-121 + 129*(CCI)^{0.42}$	0.84	5.7	1.4
Barley	$-132 + 146*(CCI)^{0.43}$	0.95	3.1	0.7
Kohlrabi	$-150 + 162*(CCI)^{0.34}$	0.83	3.1	0.8
Tomato	$-328 + 304*(CCI)^{0.26}$	0.87	2.9	0.7
Pea	$-334 + 316*(CCI)^{0.24}$	0.84	3.8	0.9
Lettuce				
Waldman's Green	$-2204 + 2204*(CCI)^{0.04}$	0.98	2.7	0.2
Buttercrunch	$-29 + 32*(CCI)^{0.74}$	0.98	2.5	0.2

distribution of chlorophyll in leaves, the absolute amount of cell wall and chlorophyll in leaves can be determined from the ratio of percentage transmission by the following relationship based on the Beer–Lambert law:

$$CCI = \frac{\%\ \text{Transmission NIR}}{\%\ \text{Transmission RED}} \approx \frac{e^{-(\text{cell wall})}}{e^{-(\text{chlorophyll+cell wall})}} = \frac{e^{(\text{chlorophyll+cell wall})}}{e^{(\text{cell wall})}} \quad (6)$$

$$CCI = e^{(\text{chlorophyll+cell wall})} - e^{(\text{cell wall})} \quad (7)$$

$$\ln(CCI) = \ln\left[e^{(\text{chlorophyll+cell wall})}\right] - \ln\left[e^{(\text{cell wall})}\right] \quad (8)$$

$$\ln(CCI) = (\text{chlorophyll} + \text{cell wall}) - (\text{cell wall}) \quad (9)$$

$$SPAD \approx \ln(CCI) = (\text{chlorophyll}) \quad (10)$$

As shown by the final equation, if chlorophyll is uniformly distributed, SPAD values would be linearly related to chlorophyll concentration of leaves and CCI values would be related to chlorophyll concentration as a logarithmic function. Chlorophyll, however, is not uniformly distributed in leaves and this causes concentration estimates based on transmission measurements to deviate from the equations shown earlier. The optical changes caused by non-uniform distribution are caused by the sieve and detour effects.

The sieve effect and the detour effect

The transmission of light through a leaf is affected by pigment concentration and pigment spatial distribution in leaves. Non-uniform chlorophyll distribution (clumping of chlorophyll molecules) decreases transmission of light at lower chlorophyll concentrations and increases transmission of light at higher chlorophyll concentrations. Distribution of chlorophyll within a leaf is influenced by structural organization of grana within chloroplasts, chloroplasts within cells, and cells within tissue layers (Fukshansky *et al*. 1993). When light passes through leaf tissue without encountering an absorber it is known as the sieve effect, which increases with increasing non-uniformity of chloroplasts. As chloroplast uniformity increases, efficiency of red light absorption increases.

The detour effect (light scattering) increases the optical path-length through the leaf, which reduces light transmission. The leaf reflectance at the reference NIR wavelength is much higher than the leaf reflectance at the red chlorophyll absorption wavelength. This causes the detour effect to be more pronounced for the reference NIR wavelength. The detour effect reduces transmission per unit chlorophyll (Monje & Bugbee 1992; Uddling *et al*. 2007; Naus *et al*. 2010). Differing optical/absolute chlorophyll relationships among species are likely due to different chlorophyll distribution patterns, and thus differing sieve and detour effects.

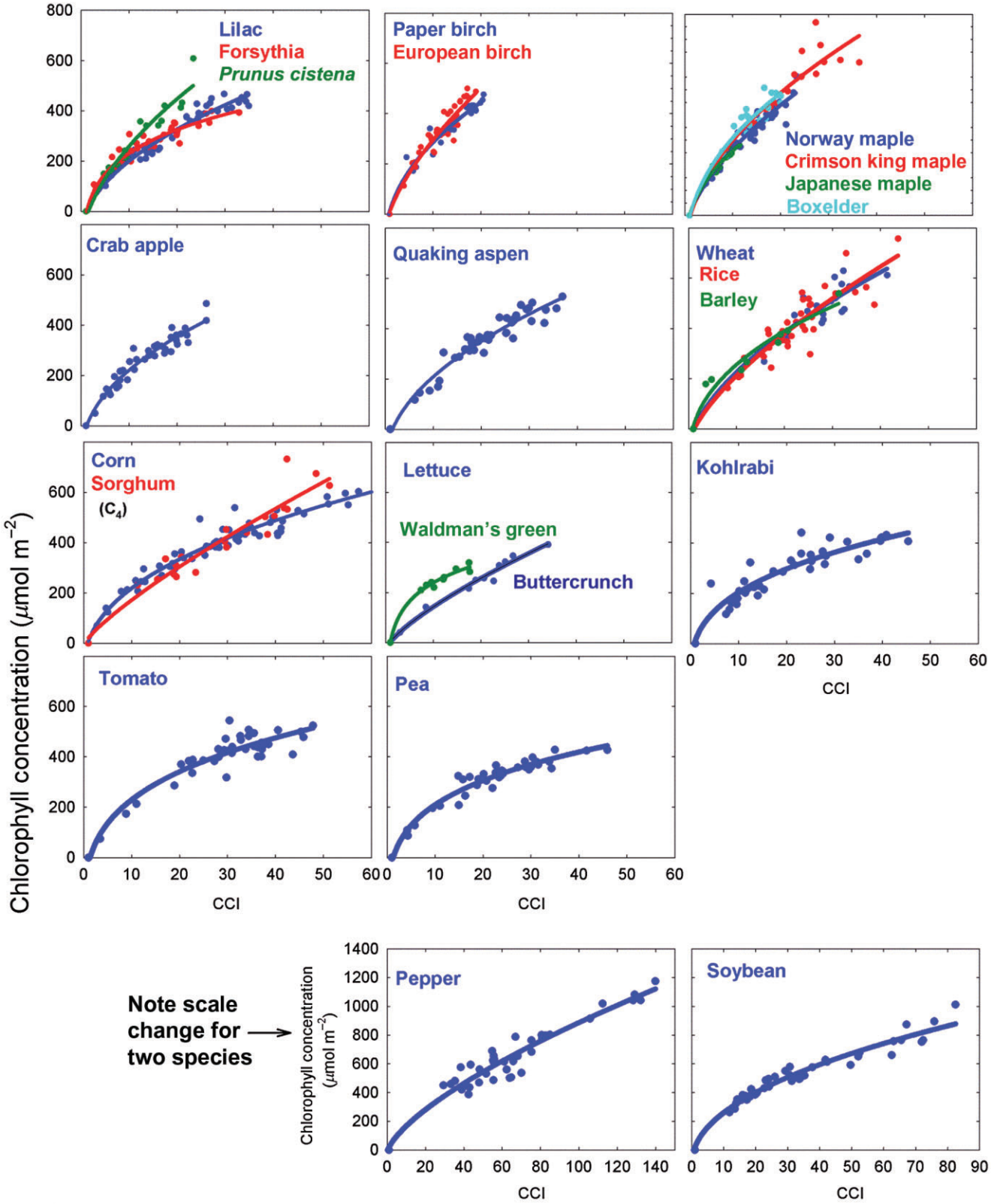

Figure 5. Relationship between chlorophyll content index (CCI) and chlorophyll concentration (μmol m^{-2}) for 22 species. Equations for each relationship are provided in Table 2.

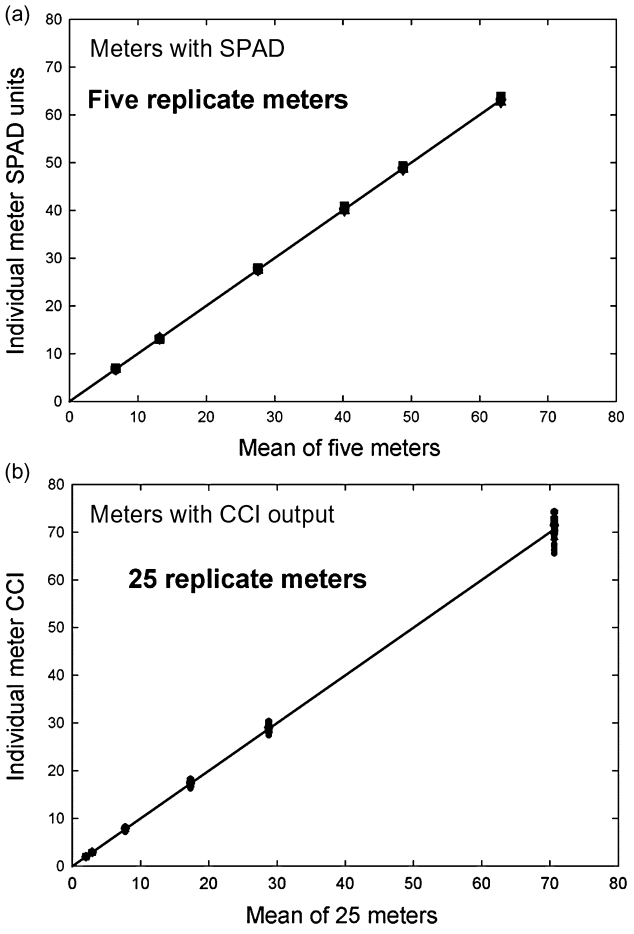

Figure 6. Uniformity of the two most common chlorophyll meters. The output of individual meters was compared with the mean of all meters of the same type. Measurements were made on coloured filters to provide a uniform reference. (a) Five Minolta model SPAD-502 meters with special products analysis division (SPAD) output manufactured from 1992 to 2008. (b) 25 Opti-Sciences model CCM-200 meters with chlorophyll content index (CCI) output manufactured from 2007 to 2013. The coefficient of variation (SD/mean) was 1.1% among meters with SPAD unit output, and 2.6% among meters with CCI output. Both types of meters were highly uniform and differences among meters are much smaller than differences in genetic, environmental and extraction/analytical techniques.

The sieve effect causes transmission to increase and thus the optical chlorophyll measurement is lower than a sample with uniform chlorophyll distribution (Monje & Bugbee 1992; Richardson *et al*. 2002; Jifon *et al*. 2005; Uddling *et al*. 2007; Marenco *et al*. 2009). The detour effect decreases transmission of light compared with a sample with uniform chlorophyll distribution and thus increases the optical chlorophyll measurement (Uddling *et al*. 2007). Uddling *et al*. (2007) observed a noticeable deviation caused by the sieve affect above a SPAD value of 20 and a relatively larger deviation caused by the detour effect below a SPAD value of 20. The combined effects of these relationships on the optical/absolute chlorophyll relationship cause a predictable deviation from the theoretical relationship (Fig. 9).

Environmental effects on optical measurements

Changes in leaf environment have the potential to alter leaf morphology, leaf thickness and chloroplast distribution. Changes in specific leaf area, often caused by water or temperature stress, have the potential to alter the optical/absolute chlorophyll relationship. Light scatter is higher in thicker leaves (Naus *et al*. 2010); however, unlike other studies (Campbell *et al*. 1990; Jifon *et al*. 2005), we did not find a different optical/absolute chlorophyll relationship between leaves of the same species (tomatoes, peppers, maize, peas) grown in greenhouse versus outdoor environments. Our data for paper birch leaves match the corrected data of Richardson *et al*. (2002), in spite of measurements made on seedlings in a greenhouse (Richardson *et al*. 2002), and our measurements on mature trees in an arid environment in Utah. Collectively, these findings do not suggest a significant environmental effect on the optical/absolute chlorophyll concentration relationship.

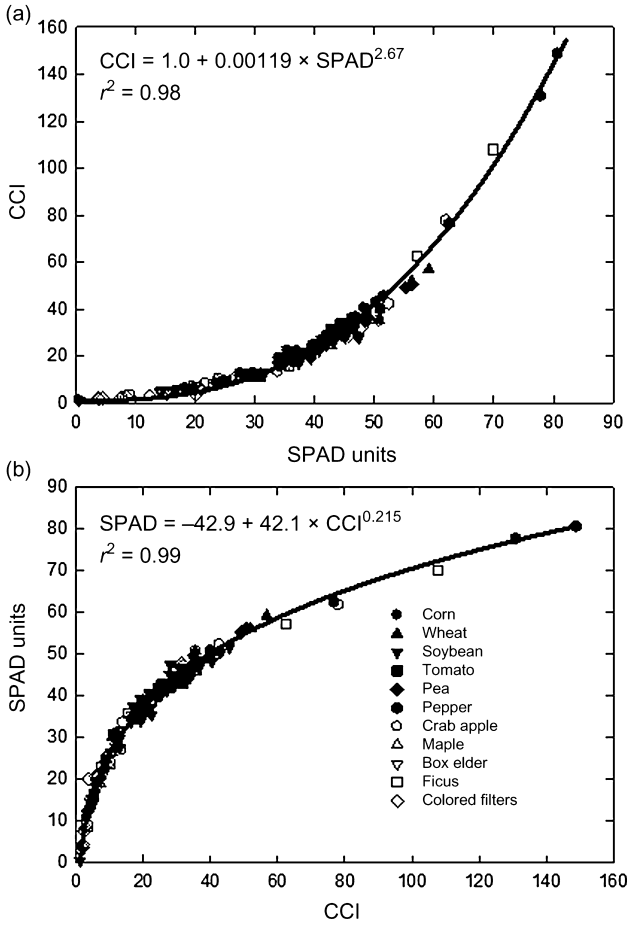

Figure 7. Equations to convert (a) special products analysis division (SPAD) units to chlorophyll content index (CCI) and (b) CCI to SPAD units. Data are from replicate measurements of multiple species. Each comparison measurement was made on the same spot on each leaf.

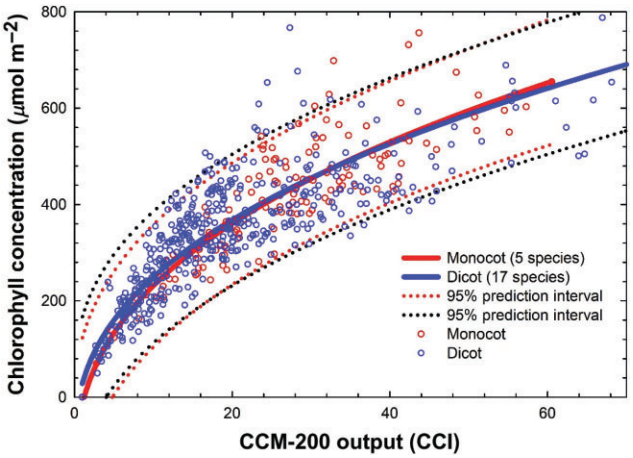

Figure 8. Relationship between chlorophyll content index (CCI) and chlorophyll concentration (μmol m^{-2}) for the mean of five monocot species and 17 dicot species. In spite of leaf anatomical differences among species there was no significant difference between these diverse plant groups.

Light-dependent chloroplast movement

Light intensity can alter chloroplast orientation (Hoel & Solhaug 1998; Naus *et al.* 2010), which can affect the optical/absolute chlorophyll relationship. Davis *et al.* (2011) found that the effects of chloroplast movement were greatest in shade species and found that mean maximum percentage change in red light transmission between low and high light acclimation was 6.3% (SD 4.7%) for shade-grown leaves and 2.1% (SD 1.6%) for sun-grown leaves. This change in chloroplast orientation in response to light is small, but potentially significant in the optical/absolute chlorophyll relationship.

Davis *et al.* (2011) hypothesized that the amount of chlorophyll movement was correlated with cell diameter. Narrower, more columnar cells of sun leaves may have a greater restriction on chloroplast movement than shade leaf cells. Leaf cell size and shape differ greatly with species (Lee *et al.* 2000), which may explain varying degrees of chloroplast movement among species. All measurements in this study were made in high light to minimize effects from light-dependent chloroplast movement.

Differences among replicate meters

Previous studies on differences among meters have not provided a comprehensive test of meter variability (Marquard & Tipton 1987; Markwell *et al.* 1995). Our results indicate that differences among replicate meters were minimal, suggesting differences among studies in the optical/absolute chlorophyll concentration relationship are not caused by different meters.

Most of the variability among optical/absolute chlorophyll concentration relationships of similar species is likely due to the variability of extraction methods, extraction solvents, chlorophyll concentration equations and the resolution of spectrophotometers. Some studies have determined chlorophyll concentration using diode array spectrophotometers with methanol extinction coefficients from Porra *et al.* (1989) (e.g. Cerovic *et al.* 2012). This is contrary to the recommendations of Wellburn (1994) and would likely lead to errors in determination of absolute chlorophyll concentration. Porra *et al.* (1989) used a Hitachi 3200 spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan) with a spectral resolution of 0.1–0.5 nm over the visible spectrum for extract extinction coefficient determination. Spectrophotometers with similar resolution should be used for best accuracy.

Differences among cultivars of the same species

Many studies have shown that cultivars within species have similar optical/absolute chlorophyll concentration relationships, but this is not always the case. There were significant

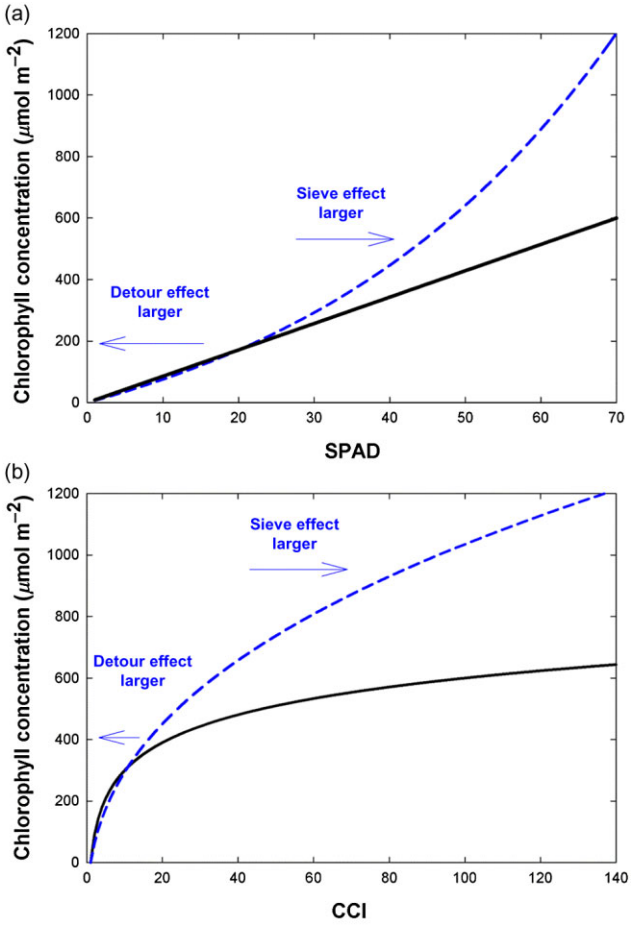

Figure 9. Impact of the detour (light scattering) and sieve effects (non-uniform chlorophyll distribution) on the optical/absolute chlorophyll concentration relationship for (a) special products analysis division (SPAD) units and (b) chlorophyll content index (CCI). The black line indicates the theoretical relationship if chlorophyll was uniformly distributed in the leaf. The range of units and shapes of the blue dashed lines are from the measured data in Fig. 1.

differences in the optical/absolute chlorophyll relationship for the two lettuce cultivars in this study. This difference can most likely be attributed to the difference in leaf morphology and anatomy in these two cultivars.

Relationship between monocots and dicots

On the basis of measurements in two monocot and two dicot species, Cerovic *et al*. (2012) suggested that there may be a difference between monocots and dicots. However, no significant difference was found between monocot and dicot curves for the five monocot and 17 dicot species in this study (Fig. 8). In spite of anatomical differences, it does not appear that monocot and dicot species have different optical/absolute chlorophyll concentration relationships.

Chlorophyll a/b ratio

Chlorophyll a/b ratios are often reported to be a 3 to 1 ratio, but this ratio has not been widely studied. Chang and Troughton (1972) reported typical ratios of C_3 plants as 3 to 1; and ratios in C_4 plants as 5 to 1. They suggest that the a/b ratio is affected by both genetics and by biotic and abiotic factors. We found a similar difference in the ratios for C_3 and C_4 plants (Table 2). We did not find a difference in the optical/absolute chlorophyll relationship between C_4 and C_3 plants in spite of the anatomical difference between these plant groups, and a significant difference in the a/b chlorophyll ratio.

The slope of the optical/absolute relationship indicates differences in chlorophyll distribution and radiation capture

Species with a steep slope in the optical/absolute relationship poorly intercept light per unit chlorophyll; species with a low slope efficiently intercept light per unit chlorophyll. It is likely that increasing non-uniformity of chlorophyll leads to a steeper slope of this relationship. This study highlights the enormous differences in chlorophyll distribution among species and even within species. The lettuce cultivar (Buttercrunch) had one of the lowest slopes and the other (Waldman's Green) had one of the highest slopes.

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DISCLOSURE OF CONFLICT OF INTEREST

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