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Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages

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Abstract

Leaf pigment content can provide valuable insight into the physiological performance of leaves. Measurement of spectral reflectance provides a fast, nondestructive method for pigment estimation. A large number of spectral indices have been developed for estimation of leaf pigment content. However, in most cases these indices have been tested for only one or at most a few related species and thus it is not clear whether they can be applied across species with varying leaf structural characteristics. Our objective in this study was to develop spectral indices for prediction of leaf pigment content that are relatively insensitive to species and leaf structure variation and thus could be applied in larger scale remote-sensing studies without extensive calibration. We also quantified the degree of spectral interference between pigments when multiple pigments occur within the same leaf tissue. We found that previously published spectral indices provided relatively poor correlations with leaf chlorophyll content when applied across a wide range of species and plant functional types. Leaf surface reflectance appeared to be the most important factor in this variation. By developing a new spectral index that reduces the effect of differences in leaf surface reflectance, we were able to significantly improve the correlations with chlorophyll content. We also found that an index based on the first derivative of reflectance in the red edge region was insensitive to leaf structural variation. The presence of other pigments did not significantly affect estimation of chlorophyll from spectral reflectance. Previously published carotenoid and anthocyanin indices performed poorly across the whole data set. However, we found that the photochemical reflectance index (PRI, originally developed for estimation of xanthophyll cycle pigment changes) was related to carotenoid/chlorophyll ratios in green leaves. This result has important implications for the interpretation of PRI measured at both large and small scales. Our results demonstrate that spectral indices can be applied across species with widely varying leaf structure without the necessity for extensive calibration for each species. This opens up new possibilities for assessment of vegetation health in heterogeneous natural environments. © 2002 Elsevier Science Inc. All rights reserved.

1. Introduction

Pigments are integrally related to the physiological function of leaves. Chlorophylls absorb light energy and transfer it into the photosynthetic apparatus. Carotenoids (yellow pigments) can also contribute energy to the photosynthetic system. However, when incident light energy exceeds that needed for photosynthesis, the carotenoids that compose the xanthophyll cycle dissipate excess energy, thus avoiding damage to the photosynthetic system (Demmig-Adams & Adams, 1996). Anthocyanins (pink, purple, and red pigments) may also protect leaves from excess

light (Barker, Seaton, & Robinson 1997; Dodd, Critchlet, Woodall, & Stewart, 1998; Gould, Kuhn, Lee, & Oberbauer, 1995) or from UV light (Burger & Edwards, 1996; Klaper, Frankel, & Berenbaum, 1996; Mendez, Gwynn, & Manetas, 1999). However, a study of the location of anthocyanins in leaves of a wide range of species found that anthocyanins were more common in the mesophyll and lower epidermis than in the upper epidermis where they would be most effective as light screens (Gould & Quinn, 1999). Anthocyanins may also serve as scavengers of reactive oxygen intermediates (Sherwin & Farrant, 1998; Yamasaki, 1997) or as antifungal compounds (Coley & Kusar, 1996).

Because of the importance of pigments for leaf function, variations in pigment content may provide information concerning the physiological state of leaves. Chlorophyll tends to decline more rapidly than carotenoids when plants are under stress or during leaf senescence

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(Gitelson & Merzlyak, 1994a, 1994b; Merzlyak, Gitelson, Chivkunova, & Rakitin, 1999). Anthocyanin content tends to be high in young leaves that also have low photosynthetic rates (Gamon & Surfus, 1999), in leaves of plants where growth has been limited by low temperature or other stresses (Chalker-Scott, 1999; Cobbina & Miller, 1987; Pietrini & Massacci, 1998), and in senescing leaves of certain species.

Traditional methods of pigment analysis, through extraction and spectrophotometric or HPLC measurement, require destruction of the measured leaves and thus do not permit measurement of changes in pigments over time for a single leaf. In addition, the techniques are time consuming and expensive, thus making assessment of the overall vegetation health of landscapes and ecosystems impractical. In contrast, measurement of spectral reflectance is nondestructive, rapid, and can be applied across spatial scales (Gamon & Qiu, 1999). Some good theoretical models have been developed for prediction of leaf reflectance from pigment, water content, and structure variables (Dawson, Curran, & Plummer, 1998; Jacquemoud et al., 1996). However, these theoretical models are difficult to invert and require information about leaf structure that may not be available. Consequently, most relationships between leaf reflectance and pigment contents have been derived empirically.

To be most useful in ecological studies, these relationships should be generalizable across species and leaf developmental stages. However, most of the relationships between leaf reflectance and pigment content have been developed and tested for only one or at most a few closely related species (e.g., Blackburn 1998a, 1998b; Chappelle, Kim, & McMurtry, 1992; Gamon & Surfus, 1999; Gitelson & Merzlyak 1994a). Structural differences (i.e., leaf thickness, density, number of air water interfaces, cuticle thickness, and pubescence) between leaves may have significant effects on these relationships. Light reflected directly from the leaf surface never enters the leaf cells and thus is not influenced by pigment and water content. Surface reflection can be greatly enhanced by leaf hairs (Billings & Morris, 1951; Ehleringer, Björkman, & Mooney, 1976) or surface waxes (Cameron, 1970; Clark & Lister, 1975; Reicosky & Hanover, 1978). Hairs increase reflectance throughout the visible region of the spectrum but their effect in the near infrared is variable (Grant, 1987; Slaton, Hunt, & Smith, 2001). Waxes increase surface reflectance throughout the visible and NIR regions of the spectrum although the effect is often greatest at shorter wavelengths due to Rayleigh scattering (Clark & Lister, 1975; Reicosky & Hanover, 1978). Light that does enter the leaf follows a complex and unpredictable path due to internal reflection and scattering. Fukshansky et al. (1993) calculated the mean path length of light through a leaf to be two to four times the leaf thickness. This results in the observed broadening of pigment absorption peaks in vivo as compared to in vitro

measurements (Fukshansky et al., 1993). Finally, pigments are not distributed homogeneously in leaf tissue so that even light that travels through an equal path length may encounter different amounts of pigment (Fukshansky et al., 1993; Rabinowitch, 1951).

Our objective in this study was to develop spectral indices for prediction of leaf pigment content that are relatively insensitive to species and leaf structure variation. We collected young, mature, and senescent leaves from a wide range of species and functional types that varied greatly in structural characteristics and quantified the effects of these structural variations on the relationship between pigment content and spectral indices. We tested several published spectral indices using this data set, and developed a new set of indices that were less sensitive to leaf structural variation.

We also quantified the degree of spectral interference between pigments when three pigment classes, chlorophylls, carotenoids, and anthocyanins occur within the same leaf tissue. Mature and senescent leaves of sweetgum (*Liquidambar styraciflua*) proved ideal for this purpose since it was possible to find almost all possible combinations of these three pigment classes in leaves of this species.

2. Theory and definition of indices

2.1. Chlorophyll

The chlorophylls have strong absorbance peaks in the red and blue regions of the spectrum (Fig. 1a). Since the blue peak overlaps with the absorbance of the carotenoids, it is not generally used for estimation of chlorophyll content. Maximal absorbance in the red region occurs between 660 and 680 nm. However, reflectance at these wavelengths has not proved as useful for prediction of chlorophyll content as has reflectance at slightly longer or shorter wavelengths. This is because relatively low chlorophyll contents are sufficient to saturate absorption in the 660–680 nm region, thus reducing the sensitivity to high chlorophyll contents of spectral indices based on these wavelengths. Consequently, empirical models for prediction of chlorophyll content from reflectance are largely based on reflectance in the 550 or 700 nm regions where higher chlorophyll contents are required to saturate the absorbance (Buschman & Nagel, 1993; Datt, 1998, 1999; Gitelson & Merzlyak, 1994a, 1994b, 1996; Lichtenthaler, Gitelson, & Lang, 1996; Schepers, Blackmer, Wilhelm, & Resende, 1996; Thomas & Gausman, 1977; Yoder & Waring, 1994). Since anthocyanin also absorbs around 550 nm (Fig. 1a), we chose to work only with chlorophyll indices based on absorbance around 700 nm.

Three primary types of indices (simple ratio [SR], normalized difference, and red edge) have been developed for estimation of chlorophyll content (see Table 1 for summary of indices used in this study). An SR index typically divides

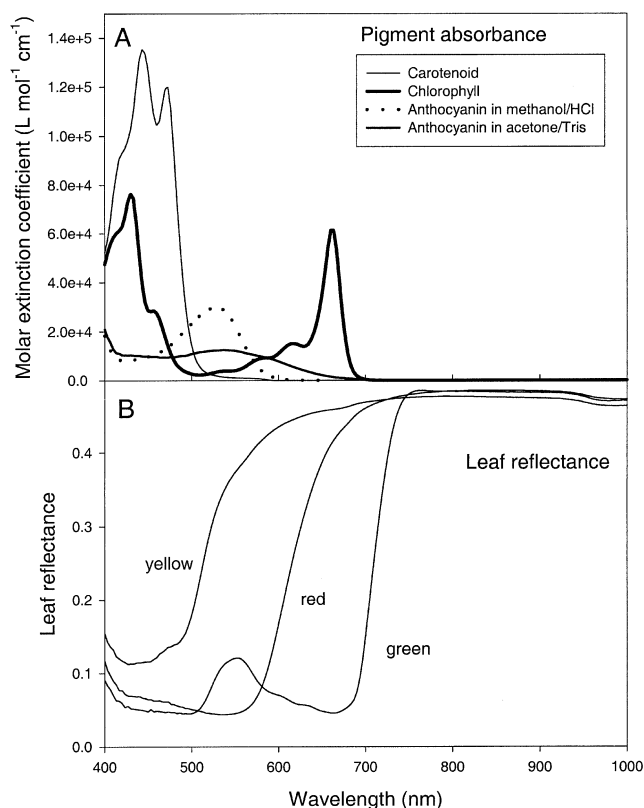


Fig. 1. The upper panel (A) shows molar extinction coefficient spectra for mixed carotenoids, chlorophyll *a+b* and anthocyanin. Carotenoids and chlorophylls were extracted from a corn leaf, spectra measured in 80% acetone solutions and scaled to published extinction coefficients for specific wavelengths (Lichtenthaler, 1987). Anthocyanins were extracted from senescing *L. styraciflua* leaves and measured in either 80% acetone (pH = 7.8) or acidic methanol (pH < 1). Spectra were scaled to an extinction coefficient of 30,000 l mol⁻¹ cm⁻¹ at 529 nm for the anthocyanin in acidic solution. Lower panel (B) shows representative leaf reflectance spectra for yellow, red and green *L. styraciflua* leaves.

reflectance at a reference wavelength, (R_{ref} , typically between 750 and 900 nm) by an index wavelength (R_{index} , typically between 660 and 720 nm) although the inverse of this relationships is also possible (Eq. (1)).

$$\text{SR}_{\text{index}} = \frac{R_{\text{ref}}}{R_{\text{index}}} \quad (1)$$

In this paper, we use two different sets of wavelengths for R_{index} and R_{ref} ; 680 and 800 nm are in the middle of the broad bands typically used for normalized difference vegetation index (NDVI) calculations from satellite data, the other set of wavelengths, 705 and 750 nm, are based in the chlorophyll index developed by Gitelson & Merzlyak (1994a). The different indices will be designated with a subscript based on the R_{index} wavelength, i.e., SR_{680} or SR_{705} .

Indices based on NDVI use the same wavelengths as the SR but subtract, rather than divide, R_{index} from R_{ref} . Then

the value is normalized through division by the sum of the reflectance at the same two wavelengths. In this paper, we use several indices with this mathematical form but with different R_{index} and R_{ref} wavelengths. To distinguish these indices from the commonly used forms of NDVI from remote sensing, we will designate these indices as ND_{index} where “index” is the reflectance index wavelength. These indices have the following general form (Eq. (2)) (see Table 1 for a full listing of these indices).

$$\text{ND}_{\text{index}} = \frac{(R_{\text{ref}} - R_{\text{index}})}{(R_{\text{ref}} + R_{\text{index}})} \quad (2)$$

We made further modifications of these indices to compensate for high leaf surface (specular) reflectance, which tends to increase reflectance across the whole visible spectrum. Adding a constant to all reflectance values reduces both SR and ND even when there is no change in the absorbance of tissues below the epidermis. To remove this effect, we chose R_{445} as a measure of surface reflectance. Combined absorbance by chlorophyll and carotenoids results in minimal reflectance in this region of the spectrum (Fig. 1b). Preliminary results for *L. styraciflua* (sweetgum) leaves showed that R_{445} was constant until total chlorophyll content dropped below 0.04 mmol m⁻² (less than 4% of maximal chlorophyll content, see Fig. 3d). Thus, R_{445} should be a good reference for all but the lowest pigment content leaves. We defined two new modified indices as follows (Eqs. (3) and (4)):

$$\text{mSR}_{\text{index}} = \frac{(R_{\text{ref}} - R_{445})}{(R_{\text{index}} - R_{445})} \quad (3)$$

$$\text{mND}_{\text{index}} = \frac{(R_{\text{ref}} - R_{\text{index}})}{(R_{\text{ref}} + R_{\text{index}} - 2R_{445})} \quad (4)$$

The equations are simply the result of subtracting R_{445} from all terms in the SR and ND equations. The ND equation was then simplified to combine terms. Datt (1999) developed an index based on a similar principle but used R_{680} as the reference. Our results suggest that R_{445} is a more reliable reference than R_{680} (Fig. 3c and d). R_{445} is lower and more stable than R_{680} because carotenoids absorb at 445 nm but not at 680 nm (Fig. 1a).

Red edge indices are based not on reflectance per se but rather on the wavelength position of the transition between low reflectance in the red region of the spectrum and high reflectance in the near infrared (Horler, Dockray, & Barber, 1983). Fitting of reflectance in this region to an inverted Gaussian model results in two parameters with units of wavelength (the point of minimum reflectance and the inflection point of the transition between red and infrared regions) in addition to the minimum and maximum (shoulder) reflectance parameters (Bonham-Carter 1988; Miller,

Table 1
Summary of the spectral reflectance indices used in this study

Pigment estimated	SR indices	Normalized difference indices	Others
Chlorophyll	$SR_{680} = \frac{R_{800}}{R_{680}}$	$ND_{680} = \frac{(R_{800} - R_{680})}{(R_{800} + R_{680})}$	$\lambda_{re} = \lambda_{\max \frac{dR}{d\lambda}}$
	$SR_{705} = \frac{R_{750}}{R_{705}}$	$ND_{705} = \frac{(R_{750} - R_{705})}{(R_{750} + R_{705})}$	$mSR_{705} = \frac{R_{750} - R_{445}}{R_{705} - R_{445}}$
Carotenoid/chlorophyll		$PRI = \frac{(R_{531} - R_{570})}{(R_{531} + R_{570})}$	$mND_{705} = \frac{(R_{750} - R_{705})}{(R_{750} + R_{705} - 2R_{445})}$
			$SIPI = \frac{(R_{800} - R_{445})}{(R_{800} - R_{680})}$
Anthocyanin	$\frac{Red}{Green} = \frac{\sum_{i=600}^{699} R_i}{\sum_{i=500}^{599} R_i}$		$PSRI = \frac{(R_{680} - R_{500})}{R_{750}}$

R_{xxx} refers to leaf reflectance at wavelength xxx in nanometers. See text for full names and sources for the indices.

Hare, & Wu, 1990). The inflection point is the parameter most often used as a spectral index and is the parameter we chose for use in this study. The inflection point can also be estimated from linear and Lagrangian models (Dawson & Curran, 1998), polynomial models (Baret, Jacquemoud, Guyot, & Leprieur, 1992), or from determining the position of the peak in the first derivative of the spectrum when high spectral resolution data are available (Curran, Windham, & Gholz, 1995). Since our data employed high spectral resolution, we found that the latter technique provided sufficient resolution without the need for use of a mathematical fitting routine. This red edge parameter (λ_{re}) can be defined as the wavelength of the maximum in the derivative of reflectance with wavelength (Eq. (5)):

$$\lambda_{re} = \lambda_{\max \frac{dR}{d\lambda}} \quad (5)$$

where λ is limited to the range 670–740 nm.

One advantage of the red edge parameter over spectral indices such as SR and ND is that it is not affected by additive constants such as leaf surface reflectance. Thus, it would be expected to be more reliable when the data includes a wide range of leaf structures. Red edge parameters derived from the second derivative of reflectance have also been found to be useful at the canopy scale since they are less sensitive to canopy structure and background reflectance effects (Demetriades-Shah, Steven & Clark, 1990).

2.2. Carotenoids

Estimation of leaf carotenoid content from reflectance is much more difficult than estimation of chlorophyll because of the overlap between the chlorophyll and carotenoid absorption peaks (Fig. 1a) and because of the higher concentration of chlorophyll than carotenoid in most leaves. Consequently, reflectance indices have proved more suc-

cessful for the estimation of the ratio of carotenoid to chlorophyll, than in the estimation of the absolute carotenoid content (Merzlyak et al., 1999; Peñuelas, Baret, & Filella, 1995). Attempts have been made to estimate total carotenoids from reflectance (Chappelle et al., 1992; Datt 1998) but these indices have not shown good generality when applied to other data sets (Blackburn, 1998b). Most indices for estimation of carotenoid/chlorophyll ratios are based on the comparison of reflectance in the region of the carotenoid absorption peak (400–500 nm) with reflectance in the red region, which is influenced only by chlorophyll. In this study, we tested the “structure-insensitive pigment index” (SIPI), which was developed by Peñuelas et al. (1995), the “plant senescence reflectance index” (PSRI), which was developed by Merzlyak et al. (1999) and the photochemical reflectance index (PRI), which was originally developed by Gamon, Peñuelas, and Field (1992) to estimate rapid changes in the relative levels of xanthophyll cycle pigments and thus serves as an estimate of photosynthetic light use efficiency (Gamon, Filella, & Peñuelas, 1993; Gamon, Serrano, & Surfus, 1997; Peñuelas et al. 1995). Since PRI measures the relative reflectance on either side of the green hump (550 nm), it also compares the reflectance in the red and blue regions of the spectrum. These indices are defined as follows (Eqs. (6)–(8)):

$$SIPI = \frac{(R_{800} - R_{445})}{(R_{800} - R_{680})} \quad (6)$$

$$PSRI = \frac{(R_{680} - R_{500})}{R_{750}} \quad (7)$$

$$PRI = \frac{(R_{531} - R_{570})}{(R_{531} + R_{570})} \quad (8)$$

2.3. Anthocyanins

Anthocyanin absorption also overlaps with chlorophyll (Fig. 1a) and thus its estimation suffers from the same difficulties as carotenoids. Gamon and Surfus (1999) found that a ratio of reflectance in the red and green regions of the spectrum was effective for estimation of the ratio of anthocyanin to chlorophyll during leaf development. Here we define the red/green ratio as (Eq. (9)):

$$\frac{\text{Red}}{\text{Green}} = \frac{\sum_{i=600}^{699} R_i}{\sum_{i=500}^{599} R_i} \quad (9)$$

3. Methods

3.1. Plant material

A total of nearly 400 leaf samples were collected and analyzed. These leaves were selected to represent as large a range of plant functional types, leaf structures and leaf developmental stages as possible and included 53 species (Table 2). To compare the variability within a species (i.e., minimal structural variation) to that between species, 123 leaves spanning a wide range of pigment contents and leaf developmental stages were collected from a single species, *L. styraciflua*. The leaves were collected from plants growing on the California State University, Los Angeles campus, or from natural habitats in Southern California and Southern Nevada (Nevada Test Site, Mercury, NV).

3.2. Reflectance measurements

All spectral measurements were made with a field portable spectrometer (Unispec, PP Systems, Haverhill, MA). The Unispec has a nominal spectral range from 350 to 1100 nm with approximately 3 nm nominal bandwidth (10 nm full width, half maximum). Thus, for each measurement, the spectrometer program automatically collects 256 data points covering the entire spectral range. A linear interpolation routine was used to estimate values at 1-nm intervals prior to calculation of indices.

All leaves collected in the field were placed in plastic bags and kept cool until they were brought back to the laboratory for measurements. Leaf reflectance was measured with a bifurcated fiber optic cable and a leaf clip (models UNI410 and UNI501, PP Systems, Haverhill, MA). The leaf clip held the fiber at a 60° angle to the adaxial leaf surface (30° from normal). Leaf illumination was provided through one side of the bifurcated fiber from a halogen lamp in the spectrometer. Leaves had been exposed only to low room illumination prior to the measurements. Three replicate measurements were made on each leaf and the results

averaged. For a subset of 140 leaves, leaf clip measurements were compared to measurements made with an integrating sphere (model 1800, LI-COR Lincoln, NE) on the same leaf. Although there were some differences in the absolute values of reflectance between these two measurement systems (see Discussion), these differences were proportional across the spectrum. Consequently, the spectral indices were highly correlated (on 1:1 line with $r^2 > .98$) and we were able to use the leaf clip for all further measurements.

3.3. Leaf structural characteristics

Immediately following the leaf spectral measurements, punches (0.64 cm²) were taken from each leaf. Some of these were frozen at –50 °C for later determination of pigment content (see below). Others were immediately weighed to determine fresh mass, dried for 48 h at 60 °C and then weighed again to determine dry mass. Leaf water content per unit leaf area was calculated as the difference between leaf fresh and dry mass. We chose four parameters to quantify variation in leaf structural characteristics; leaf fresh mass per unit area (related to thickness), leaf dry mass as a percentage of fresh mass (related to structural investment and toughness), reflectance at 445 nm (related to leaf surface reflectance), and reflectance at 800 nm (an estimate of light scattering and a function of internal leaf structure). The observed ranges of these parameters for each species are listed in Table 2.

3.4. Quantification of chlorophylls and carotenoids

For chlorophyll/carotenoid measurement, the tissue samples (0.64 cm² in most cases) were ground in 2 ml cold acetone/Tris buffer solution (80:20 vol:vol, pH=7.8), centrifuged to remove particulates, and the supernatant diluted to a final volume of 6 ml with additional acetone/Tris buffer. The absorbance of the extract solutions was measured with the Unispec spectrometer reconfigured as a spectrophotometer using an external cuvette holder (model 664.000, Hellma Plainview, NY). This allowed better comparisons between the leaf reflectance and pigment absorbance measurements since both were made with the same type of spectral detector. Initial tests showed that the spectrophotometer response was linear with pigment concentration up to an absorbance of one. When the peak absorbance of the extract solutions exceeded one, the solutions were diluted further and remeasured.

Since this study included leaves containing anthocyanins, we developed and tested equations for spectrophotometric determination of chlorophyll and carotenoids in solutions also containing anthocyanins. Anthocyanins are unstable in neutral and basic solutions, such as the acetone/Tris buffer solution used for chlorophyll extracts, and rapidly convert to forms with lower, flatter absorption peaks. For this reason, anthocyanin assays are generally done in highly acidic medium (i.e., 1% HCl). To assess the contribution of

Table 2
List of the species used in this study

No. of samples	Species	Plant functional type	Leaf developmental stages	SLFW (g m ⁻²)	% dry mass	R ₄₄₅ (green leaves only)	R ₈₀₀ (all leaves)
<i>Thin leaves (herbaceous)</i>							
3	<i>Amsinckia intermedia</i>	A	M	348–466	13–16	0.07–0.08	0.27–0.28
4	<i>Aspidistra elatior</i>	EP	M	225–288	36	0.03–0.04	0.43–0.50
3	<i>Brassica nigra</i>	A	M	328–416	24–28	0.04–0.09	0.35–0.47
3	<i>Eriogonum fasciculatum</i>	DD	M	333–388	34–47	0.06–0.11	0.42–0.49
2	<i>Fraxinus</i> sp.	WD	Y	158–164	34–35	0.05	0.48–0.49
2	<i>Ginkgo biloba</i>	WD	S	300–336	NA	NA	0.53–0.54
3	<i>Hedera helix</i>	EP	Y,M	165–298	21–43	0.02–0.03	0.49–0.52
3	<i>Helianthus annuus</i>	A	M	241–260	22–24	0.05–0.06	0.33–0.38
123	<i>L. styraciflua</i>	WD	Y,M,S	116–285	24–59	0.02–0.08	0.39–0.60
1	<i>Liriodendron tulipifera</i>	WD	S	186	18	NA	0.34
6	<i>Marah macrocarpus</i>	H	M	258–344	14–15	0.06–0.08	0.36–0.43
2	<i>Nicotiana tabacum</i>	A	M	133	13	0.09	0.43
16	<i>Phaseolus vulgaris</i>	A	M,S	109–175	10–26	0.04–0.08	0.51–0.57
10	<i>Populus fremontii</i>	WD	Y,M,S	180–279	24–31	NA	0.35–0.50
2	<i>Prunus</i> sp.	WD	M	99–105	35	0.04	0.44–0.50
9	<i>Ribes aureum</i>	WD	M	146–225	36–40	0.06–0.14	0.47–0.66
6	<i>Rosa</i> sp.	WD	Y,M	144–197	28–37	0.02–0.04	0.45–0.59
8	<i>Sambucus mexicana</i>	WD	M	286–401	22–32	0.05–0.09	0.25–0.47
3	<i>Vicia villosa</i>	A	M	156–170	23–27	0.06–0.07	0.37–0.46
6	<i>Vitis girdiana</i>	WD	M	171–197	24–30	0.03–0.10	0.23–0.51
<i>High dry mass leaves (sclerophyllous)</i>							
3	<i>Adenostoma fasciculatum</i>	E	M	505–603	59–64	0.07–0.09	0.46–0.56
2	<i>Ceanothus megacarpus</i>	E	M	528–628	49–54	0.08	0.34–0.59
5	<i>Eriodictyon crassifolium</i>	E	Y,M	346–605	33–48	0.04–0.06	0.11–0.49
7	<i>Heteromeles arbutifolia</i>	E	Y,M	278–381	31–50	0.03–0.07	0.34–0.56
2	<i>Larrea tridentata</i>	E	M	365–500	53–57	0.04–0.05	0.49–0.50
2	<i>Mahonia aquifolium</i>	E	M,S	204–251	48–52	0.03	0.48–0.52
3	<i>Malacothamnus fasciculatus</i>	DD	M	189–218	38–46	0.07–0.09	0.43–0.50
9	<i>Malosma laurina</i>	E	Y,M	289–442	39–48	0.03–0.08	0.19–0.50
2	<i>Nerium oleander</i>	E	M	380–410	29–48	0.02–0.03	0.53–0.59
8	<i>Photinia serratifolia</i>	E	Y,M	214–383	32–50	0.01–0.04	0.49–0.60
26	<i>Quercus agrifolia</i>	E	Y,M	161–280	54–57	0.02–0.10	0.23–0.46

9	<i>Quercus lobata</i>	WD	Y,M	178–230	33–50	0.01–0.03	0.30–0.51
10	<i>Rhaphiolepis indica</i>	E	Y,M	248–440	26–48	0.01–0.04	0.43–0.56
3	<i>Salvia mellifera</i>	DD	M	406–492	41–47	0.05–0.08	0.43–0.71
<i>Succulent leaves</i>							
1	<i>Aloe vera</i>	EP	M	9634	2	0.04	0.27
2	<i>Chlorophytum comosum</i>	EP	M	666–728	7	NA	0.39–0.41
2	<i>Crassula argentea</i>	EP	M	2784–3227	6–8	0.03–0.04	0.11–0.12
2	<i>Lycium pallidum</i>	DD	M	507–910	20–30	0.11	0.29–0.32
4	<i>Hoya sp.</i>	EP	M	1590–2003	8–10	0.02–0.03	0.15–0.33
2	<i>Rheo discolor</i>	EP	M	803–892	7	0.03	0.33–0.38
2	<i>Setcreasea purpurea</i>	EP	M	920–1014	4	0.03	0.30
<i>Grasses</i>							
3	<i>Avena fatua</i>	A	M	165–185	27–30	0.11–0.12	0.45–0.49
3	<i>Lolium multiflorum</i>	A	M	135–148	22–25	0.11	0.48–0.50
2	<i>Pleuraphis rigida</i>	H	M	277–340	50–61	0.16–0.19	0.50–0.52
<i>Pubescent leaves</i>							
2	<i>Ambrosia dumosa</i>	DD	M	506–684	42–56	0.13–0.14	0.44–0.50
7	<i>Artemisia californica</i>	DD	M	339–352	47–48	0.04–0.13	0.18–0.48
2	<i>Krameria parvifolia</i>	WD	M	504–541	40–55	0.11–0.17	0.29–0.33
3	<i>Marrubium vulgare</i>	H	M	289–394	33–34	0.07–0.11	0.33–0.42
3	<i>Salvia apiana</i>	DD	M	334–372	29–31	0.19–0.21	0.46–0.49
3	<i>Tetradymia spinosa</i>	DD	M	429–538	24–25	0.24–0.32	0.35–0.44
<i>Waxy leaves</i>							
1	<i>Aechmea fasciata</i>	EP	M	577–617	23	0.35	0.56–0.57
7	<i>Eucalyptus sp.</i>	E	Y,M	248–375	39–47	0.08–0.19	0.44–0.58
2	<i>Nicotiana glauca</i>	E	M	333–361	22	0.12–0.13	0.51–0.52

The number of leaf samples measured for each species, the plant functional type (A=annual, DD=drought deciduous, E=woody evergreen, EP=evergreen perennial, H=herbaceous perennial, WD=winter deciduous), the leaf developmental stages that were measured (Y=expanding leaf, M=mature, S=senescing), the range of SLFWs, the range of leaf dry mass as percent of fresh mass, the range of leaf reflectance at 445 nm (R_{445}) and the range of leaf reflectance at 800 nm (R_{800}) are also given for each species. R_{445} was not available (NA) in cases where only nongreen and/or senescing leaves were measured. The leaf structure groups were defined as follows: thin leaves=median SLFW less than 400 g m⁻² and median dry mass less than 40%, high dry mass leaves=median % dry mass greater than 40%, succulent=median SLFW greater than 600 g m⁻², pubescent=leaves with a fairly dense pubescence visible to the eye, waxy=leaves with a waxy coating resulting in a visibly bluish or whitish leaf surface.

anthocyanin breakdown products to the overall absorption of chlorophyll extract solutions, we added a known quantity of purified anthocyanin (from senescing *L. styraciflua* leaves) to the chlorophyll extraction buffer and measured its absorption spectra. The quantity of anthocyanin added was determined by spectrophotometric assay (see next section) of another sample of the same pigment diluted in methanol/HCl. This value was then used to calculate an absorption coefficient spectrum for anthocyanin in neutral buffer (Fig. 1a). Since anthocyanins are a fairly diverse group of compounds with differing absorption spectra (Harborne, 1967), we also measured anthocyanin absorption spectra in neutral buffer for a range of flowers with different anthocyanin types (data not shown). The shapes of these curves were quite similar and we calculated that the maximum error that would be introduced into the chlorophyll calculations because of an assumption of a constant shape of the anthocyanin absorption spectra in neutral buffer, even for the maximum observed ratios of anthocyanins to chlorophylls in leaves, would be less than 3%. However, although the shape of the anthocyanin absorption spectrum in neutral buffer was fairly constant, its absolute value was not. Thus, estimation of anthocyanin content, rather than just the interference with the chlorophyll content estimation, from these equations is not reliable. The *L. styraciflua* anthocyanin data were used along with Lichtenthaler's (1987) values for absorption coefficients of chlorophyll *a* (Chl_a) and chlorophyll *b* (Chl_b) to derive equations for determination of three pigments in a mixture. The equations derived were (Eqs. (10)–(12)):

$$\text{Anthocyanin} = 0.08173A_{537} - 0.00697A_{647} - 0.002228A_{663} \quad (10)$$

$$\text{Chl}_a = 0.01373A_{663} - 0.000897A_{537} - 0.003046A_{647} \quad (11)$$

$$\text{Chl}_b = 0.02405A_{647} - 0.004305A_{537} - 0.005507A_{663} \quad (12)$$

where A_x is the absorbance of the extract solution in a 1-cm path length cuvette at wavelength x . The neutral buffer anthocyanin absorption coefficient spectrum was also used to determine anthocyanin absorbance at 470 nm for modification of Lichtenthaler's total carotenoid equation (Eq. (13)):

$$\text{Carotenoids} = \frac{(A_{470} - (17.1 \times (\text{Chl}_a + \text{Chl}_b) - 9.479 \times \text{Anthocyanin}))}{119.26} \quad (13)$$

The units for all the equations are micromoles per milliliter ($\mu\text{mol ml}^{-1}$). The following molecular weights were used to convert from gram units in Lichtenthaler

(1987) to mole units: Chl_a = 893.5 g mol⁻¹, Chl_b = 907.5 g mol⁻¹ and total carotenoid = 550 g mol⁻¹. Leaf pigment contents in millimoles per square meter (mmol m⁻²) (leaf area) were a function of the calculated solution concentrations, the total volume of extraction solution and the total leaf area extracted.

A subset of the samples was also measured with HPLC (model LC-10AS with detector SDP-10AV, Shimadzu, Kyoto, Japan) using the method of Thayer and Björkman (1990). These results were compared with the spectrophotometric results from our equations and with Lichtenthaler's (1987) equations.

3.5. Quantification of anthocyanins

Although anthocyanin contents can be calculated from the equations above, such results are unreliable given the wide variation in chemical structure of anthocyanins and the time dependence of anthocyanin degradation in neutral buffer. All anthocyanin contents reported in this paper were measured using a standard anthocyanin spectrophotometric assay (Murray & Hackett, 1991). Additional disks from the same leaves as those used for chlorophyll and carotenoid measurements were extracted with the same procedure as that for the chlorophyll measurements except that cold methanol/HCl/water (90:1:1, vol:vol:vol) was used in place of acetone/Tris buffer. The extract solution was then measured with the same spectrophotometer used for the chlorophyll and carotenoid measurements. Based on preliminary measurements we found that the red peak absorbance for degraded chlorophyll in these extracts occurred at 650 nm and has a tail that overlaps with the anthocyanin peak at 529 nm. To correct for the effect of chlorophyll we used the following empirically derived equation (Eq. (14)):

$$\text{AA} = A_{529} - (0.288A_{650}) \quad (14)$$

where AA is corrected anthocyanin absorbance. Total anthocyanin content was then calculated using this corrected absorbance and a molar absorbance coefficient for anthocyanin at 529 nm of 30,000 l mol⁻¹ cm⁻¹ (Murray & Hackett, 1991).

3.6. Statistical analysis

Curve fitting was performed in Sigmaplot (SPSS, Chicago, IL) using linear, polynomial, or rectangular hyperbolic models. The model that gave the highest correlation coefficient was chosen for use in the figures.

The multiple regression analyses in Table 3 were performed in Statview (SAS Institute, Cary, NC). Three separate multiple regressions were performed. In each one, total chlorophyll content was the independent variable and there were four dependent variables. Three of the dependent variables (the structural variables R_{445} , R_{800} , and leaf water content per unit area) were the same in all three analyses. The only factor that varied was the fourth

Table 3

Three separate multiple regression analyses of factors explaining variation in total leaf chlorophyll

First analysis		Second analysis		Third analysis	
Factors	<i>P</i> value	Factors	<i>P</i> value	Factors	<i>P</i> value
SR ₇₀₅	<.0001	mSR ₇₀₅	<.0001	Red edge	<.0001
<i>R</i> ₄₄₅	<.0001	<i>R</i> ₄₄₅	.24	<i>R</i> ₄₄₅	.87
<i>R</i> ₈₀₀	.0002	<i>R</i> ₈₀₀	.15	<i>R</i> ₈₀₀	.26
Water content	<.0001	Water content	<.0001	Water content	<.0001

This analysis systematically explores the ability of different index formulations to account for the effects of three leaf structural characteristics; surface reflectance (*R*₄₄₅), light scattering (*R*₈₀₀), and leaf water content per unit area (g m^{-2}) on the relationship between spectral reflectance and chlorophyll content. Leaf total chlorophyll content was the independent variable in each analysis. The three structural parameters (*R*₄₄₅, *R*₈₀₀ and water content) are included as dependent variables in each analysis. The only parameter that is varied between the three analyses is the spectral reflectance index (the SR index with an index wavelength of 705 nm (SR₇₀₅), the modified SR index (mSR₇₀₅), or the red edge parameter). Lack of a significant relationship between total chlorophyll content and *R*₄₄₅ or *R*₈₀₀ in the second two analyses suggests that the indices mSR₇₀₅ and red edge successfully account for the variation in the relationship associated with these leaf structural parameters. Since the red edge parameter is not meaningful for leaves lacking chlorophyll, the data set for this analysis excluded leaves with less than 0.1 mmol m^{-2} chlorophyll. Succulent leaves were also excluded since when they were included the water content effect was nonsignificant in all cases.

dependent variable (SR₇₀₅, mSR₇₀₅, or the red edge parameter). These analyses allowed us to examine in a quantitative fashion the extent to which the different spectral reflectance indices accounted for variation related to the leaf structural parameters.

4. Results

4.1. Chlorophyll

Both Lichtenthaler's (1987) chlorophyll equations and our equations modified to include the effect of anthocyanins produced results that correlated closely with HPLC chlorophyll determinations ($r^2 > .96$, Fig. 2). However, the anthocyanin correction did slightly improve the accuracy of the estimations. This effect was only a few percent at high chlorophyll contents but increased to as high as 20%

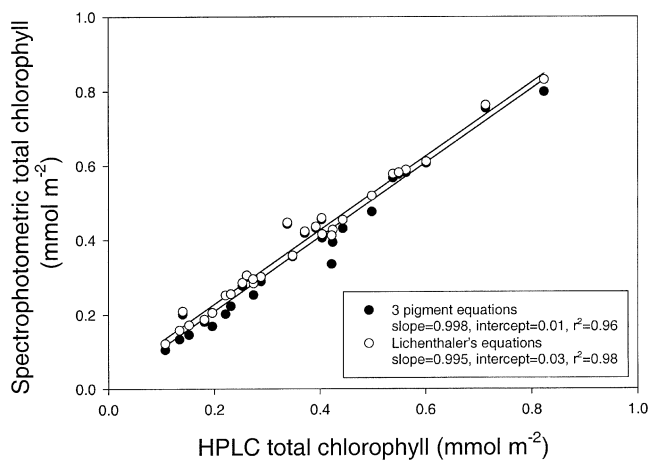


Fig. 2. Comparison of spectrophotometric and HPLC techniques for measurement of total chlorophyll content. Spectrophotometric calculations were based either on Lichtenthaler's (1987) equations for 80% acetone solution (open circles) or our modified equations for three pigments (chlorophyll *a* and *b* plus anthocyanin) in 80% acetone (closed circles). See text for details.

for leaves with very low chlorophyll and high anthocyanin contents.

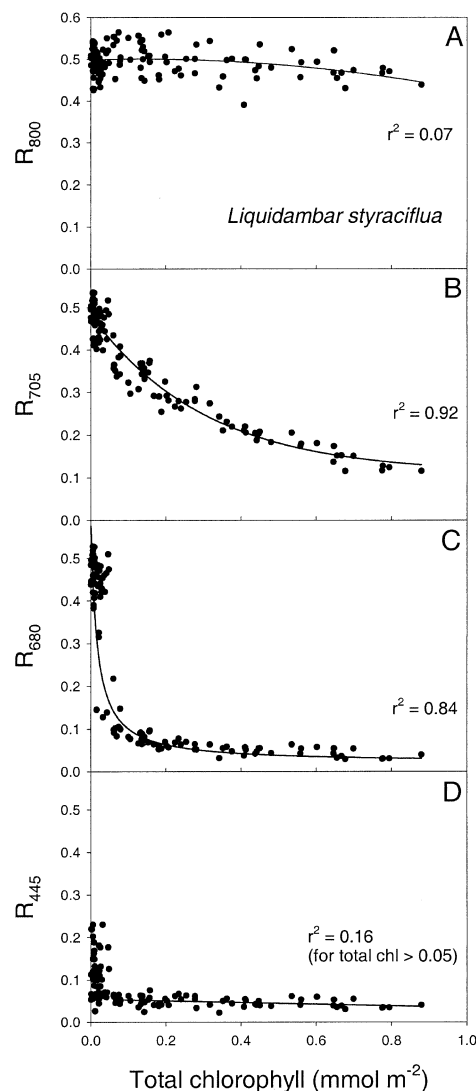


Fig. 3. Reflectance at four wavelengths as a function of total chlorophyll content for *L. styraciflua* leaves.

We used the data for *L. styraciflua* leaves, where leaf structure was relatively constant, to examine the relationship between total chlorophyll content and reflectance at four wavelengths (445, 680, 705, and 800 nm, Fig. 3). Chlorophyll content was best correlated with R_{705} and was only weakly related to R_{800} and R_{680} . R_{445} was insensitive to chlorophyll content until total chlorophyll dropped below about 0.04 mmol m^{-2} .

As expected from the individual wavelength analyses, indices based on reflectance at 680 nm were largely insensitive to variation in chlorophyll content (Fig. 4a and b). Use of reflectance at 705 nm substantially improved the correlations but considerable variation remained when data from all species were combined (Fig. 4c and d). Multiple regression analysis showed that this variability was primarily

related to three factors; leaf water content per unit area, R_{800} (a measure of light scattering), and R_{445} (a measure of leaf surface reflectance)(Table 3). Note that specific leaf fresh weight (SLFW) and percent dry mass also explained some variation but their effects were smaller and appeared to be explained by correlations between SLFW, percent dry mass, and water content, with water content being the primary causal factor. Increases in surface reflectance (R_{445}) and water content tended to reduce SR_{705} at a given chlorophyll content whereas increased light scattering (R_{800}) tended to increase SR_{705} . Of these factors, leaf surface reflectance (R_{445}) explained the largest portion of the variability. The modified indices mSR_{705} and mND_{705} , which were developed to eliminate the effect of surface reflectance by incorporating R_{445} , produced substantially

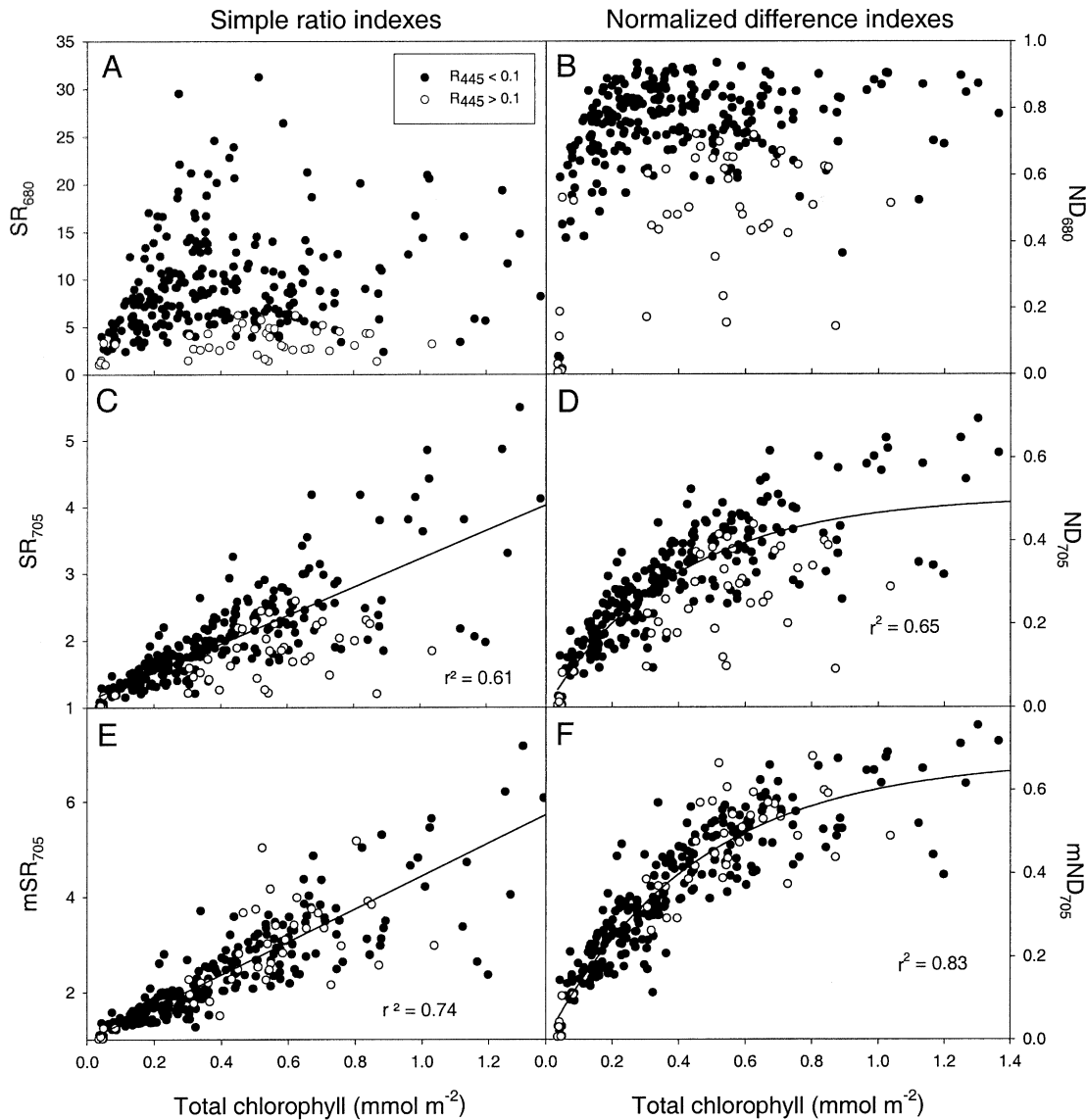


Fig. 4. SR and normalized difference indices with index wavelengths of 680 nm (SR_{680} and ND_{680}) or 705 nm (SR_{705} and ND_{705}) plus our modified indices (mSR_{705} and mND_{705}) as a function of total leaf chlorophyll. Closed symbols represent leaves with reflectance at 445 nm (R_{445}) less than 0.1 and open symbols represent leaves with $R_{445} > 0.1$. Regressions are linear for the SR indices and rectangular hyperbolic for the ND indices.

Table 4
Summary of the correlation coefficients (r^2) between the spectral reflectance indices (see text for definitions) and leaf pigment contents

	Linear model		Nonlinear models
Total chlorophyll indices	All data	Total chlorophyll less than 0.6 mmol m^{-2}	All data
SR ₆₈₀	.03	.04	—
SR ₇₀₅	.61*	.59*	—
mSR ₇₀₅	.74*	.71*	—
ND ₆₈₀	—	.08	.33*
ND ₇₀₅	—	.62*	.65*
mND ₇₀₅	—	.78*	.83*
Red edge	—	.66*	.73*
Carotenoid to chlorophyll ratio indices	All data	Carotenoid to chlorophyll ratio less than 1	
PRI	.003	.39*	
SIPI	.000	.000	
PSRI	.002	.000	
Anthocyanin index	All data		
Red/green ratio	.012		

Nonlinear models are rectangular hyperbolic except for the red edge parameter, which was fit to a polynomial model. Significant relationships are indicated with a *.

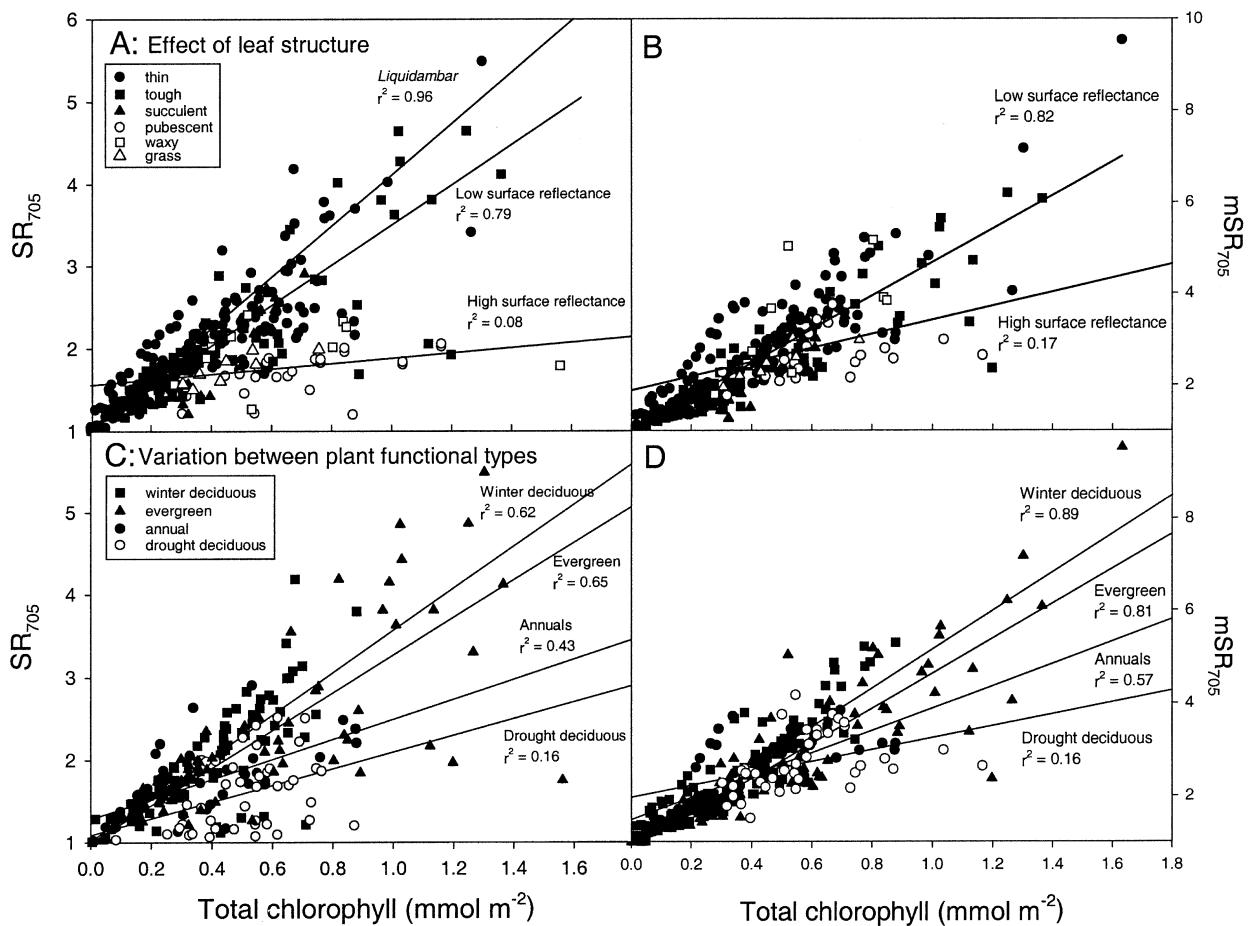


Fig. 5. SR and modified SR indices with index wavelength 705 nm (SR₇₀₅ and mSR₇₀₅) as a function of total leaf chlorophyll. In the top two panels, data are separated by leaf structure (see Table 2 for definitions of these groups). Closed symbols represent leaves with low surface reflectance (thin, tough, and succulent) and open symbols represent leaves with higher surface reflectance (pubescent, waxy and grass). Separate linear regressions are shown for *L. styraciflua* leaves, and the low and high surface reflectance groups. In the bottom two panels, the same data are separated by plant functional type. To simplify this figure, a few of the functional types listed in Table 2 have been lumped together. Evergreen in this figure includes both woody evergreens and evergreen perennials and drought deciduous in this figure includes both drought deciduous and herbaceous perennials.

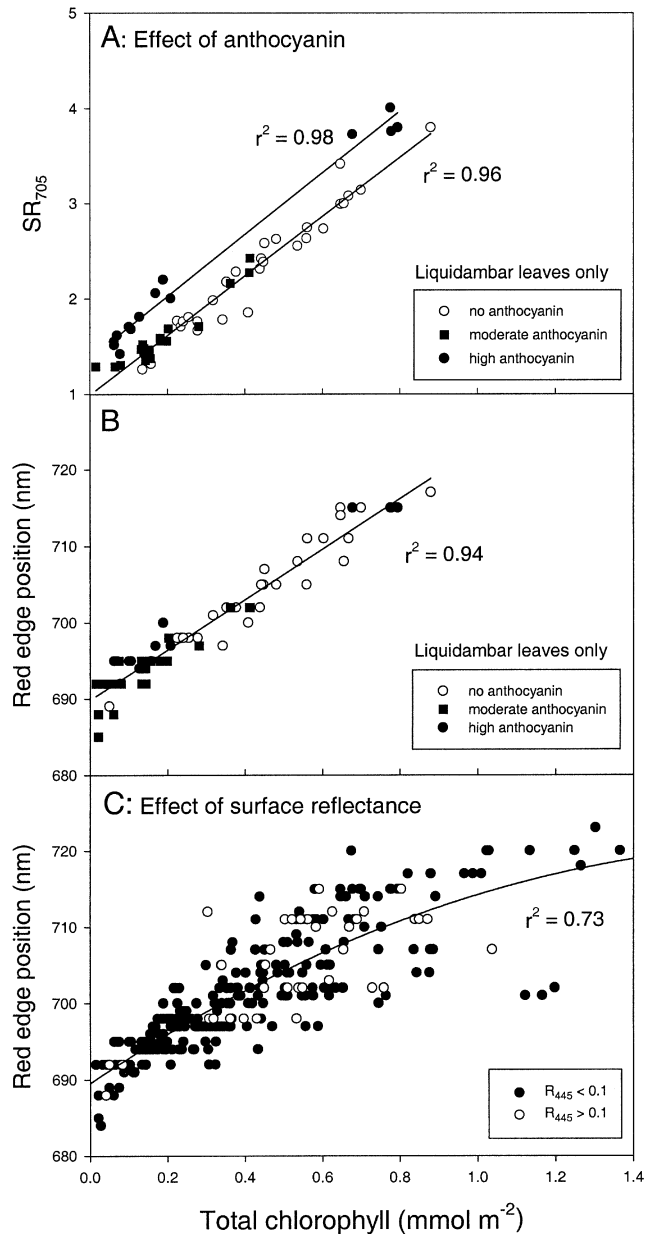


Fig. 6. The top two panels (A & B) show the effect of variation in leaf anthocyanin content (no anthocyanin (open circles) includes leaves with less than $0.1\ mmol\ m^{-2}$ anthocyanin, moderate anthocyanin (closed squares) is 0.1 to $2\ mmol\ m^{-2}$ anthocyanin and high anthocyanin (closed circles) is greater than $2\ mmol\ m^{-2}$ anthocyanin) on the relationship between the SR index (SR_{705}) or the red edge parameter and total chlorophyll content of *L. styraciflua* leaves. The bottom panel (C) shows the effect of low (closed symbols) or high (open symbols) leaf reflectance at $445\ nm$ (R_{445}) on the relationship between the red edge parameter and total chlorophyll for all the green leaves in the data set.

better correlations with total chlorophyll (Fig. 4e and f) and eliminated the effect of surface reflectance (R_{445}) in the multiple regression analysis (Table 3). The modified index also eliminated the effect of light scattering (R_{800}), but not the effect of water content (Table 3). For both the modified and unmodified indices, the correlations were better for ND_{705} than for SR_{705} . Since comparisons of linear and

nonlinear correlation coefficients are problematic, we also compared the linear correlations for leaves with total chlorophyll below $0.6\ mmol\ m^{-2}$ (where the relationships for both types of indices are essentially linear) and found that the ND_{705} indices still produced significantly higher correlations than the SR_{705} indices (Table 4).

Variation in leaf surface reflectance also explained some of the variation between leaf structure and plant functional types. Leaf types with low surface reflectance produced good correlations between SR_{705} and chlorophyll that were not substantially different from the relationship for *L. styraciflua* alone (Fig. 5a). In contrast, leaf types with high surface reflectance failed to show any correlation between SR_{705} and chlorophyll. This variation was greatly reduced by use of the mSR_{705} index (Fig. 5b). When the species were classified into functional types based on leaf and plant phenology, the winter deciduous and evergreen groups produced good correlations between SR_{705} and chlorophyll whereas the annuals and drought deciduous groups produced relatively poor correlations (Fig. 5c). Again, the latter two groups contain most of the species with highly reflective epidermal surfaces and some of this variation was eliminated by use of the modified index (Fig. 5d).

One source of variation not corrected for by the modified indices was anthocyanin content. Moderate amounts of anthocyanins had no effect on the relationship between SR_{705} and chlorophyll (Fig. 6a). However, extremely high anthocyanin contents (greater than $2\ mmol\ m^{-2}$, sometimes found in senescing *L. styraciflua* leaves that appear almost black in color) did increase SR_{705} for a given chlorophyll content. However, leaves with this high an anthocyanin content are relatively rare. Unlike the SR and ND indices, the red edge parameter was unaffected by differences in anthocyanin content (Fig. 6b) or leaf epidermal reflectance (Fig. 6c). However, since mND_{705} was better correlated with chlorophyll than was the red edge parameter (r^2 of .83 vs. .73) and the red edge parameter is

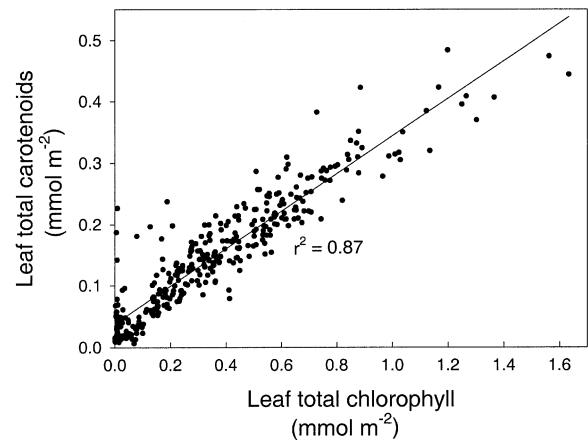


Fig. 7. The relationship between leaf total carotenoid content and total chlorophyll for all leaves.

more difficult to calculate than mND_{705} , the red edge parameter does not appear to offer an advantage in the context of leaf structural variation.

4.2. Carotenoids and anthocyanins

Total carotenoid content was closely related to total chlorophyll content (Fig. 7). Consequently, the chlorophyll indices were also the best predictors of total carotenoid content. However, within the general relationship between carotenoid and chlorophyll there was substantial variation in the carotenoid to chlorophyll ratio. Of the three carotenoid to chlorophyll indices we tested, none provided a significant correlation across the whole range of carotenoid/chlorophyll ratios (Table 4). However, PRI was significantly correlated with carotenoid/chlorophyll ratio for values between 0.2 and 0.8 mol/mol (Fig. 8c). This range included healthy and stressed leaves but excluded the highly senescent leaves. Unlike previous reports, neither SIPI nor PSRI were significantly correlated with carotenoid/chlorophyll ratio in this range (Fig. 8a and b). We did not find any significant variation in the carotenoid/

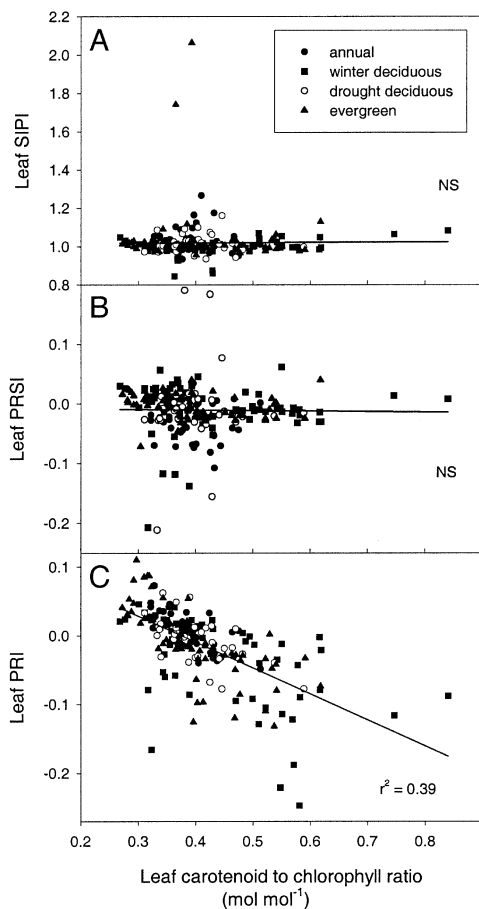


Fig. 8. The SIPI (Peñuelas et al., 1995), PSRI (Merzlyak et al., 1999) and the PRI (Gamon et al., 1992) as functions of leaf carotenoid to chlorophyll ratio for leaves containing no anthocyanin and excluding leaves with carotenoid to chlorophyll ratios greater than 1.

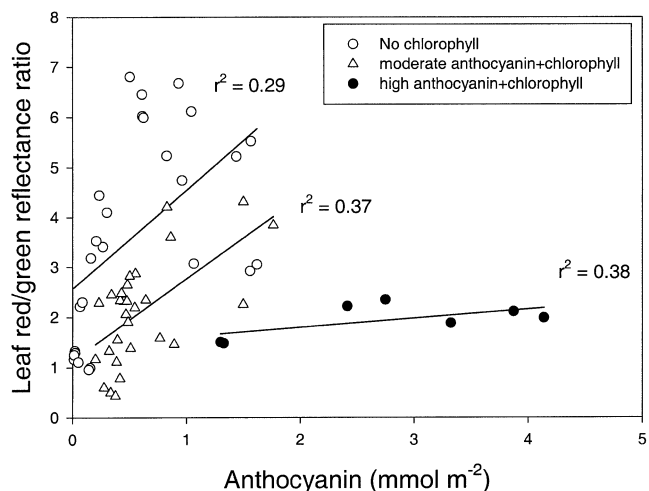


Fig. 9. The relationship between leaf red/green reflectance ratio and leaf anthocyanin content for all species. Open circles represent leaves containing anthocyanin but no chlorophyll. Open triangles represent leaves for which the total of anthocyanin plus chlorophyll is below 2 mmol m^{-2} and closed circles represent leaves with anthocyanin plus chlorophyll contents greater than 2 mmol m^{-2} .

chlorophyll ratio between the leaf structure and functional type groups.

Unlike previous reports for single-species data sets, the red/green ratio was not correlated with anthocyanin content when all data were considered (Table 4). There were some weak relationships between red/green ratio and anthocyanin content when the data were separated into groups based on absolute concentrations of anthocyanin and chlorophyll (Fig. 9). The red/green ratio was not correlated with the ratio of chlorophyll to anthocyanin (data not shown).

5. Discussion

5.1. Leaf structure effects

When applied across a wide range of species, leaf structures, and plant functional types, previously published spectral indices provided poor correlations with leaf chlorophyll content. Our analysis suggests that leaf surface reflectance was the most important factor in this variation. Our modified indices, mSR_{705} and mND_{705} , which were designed to eliminate the effect of leaf surface reflectance, were substantially better correlated (r^2 as high as .83) with chlorophyll content across all the leaf types than were the original indices. Datt (1999) also developed an index to correct for leaf surface reflectance and scattering. However, we found that his index produces unstable results for leaves with very low or no chlorophyll content. In contrast, our modified indices perform relatively well even at very low chlorophyll content since carotenoid content generally declines less quickly than chlorophyll and these carotenoids contribute to strong absorption at 445 nm.

Light scattering (R_{800}) and water content per unit leaf area also explained some of the variation in the relationship between SR_{705} and chlorophyll content. If internal light scattering had an equal multiplicative effect on reflectance at all wavelengths, it would not have been expected to have any effect on the spectral indices. However, this was not the case and there are two possible explanations for the apparent variation in the scattering effect with wavelength. First, increased scattering of visible wavelengths would increase the path length of light through the leaf and might thus increase absorption by pigments in visible regions but not in the near infrared. An alternative explanation is that leaf surface reflectance, which represents a constant additive effect, is a larger percentage of total reflectance in the visible than in the infrared. The latter effect would explain why the light scattering effect was eliminated for the modified indices.

Light scattering is primarily related to differences in internal leaf structure, e.g., number of air–water interfaces and cell and organelle particle sizes (Grant, 1987; Knipling, 1970). However, for our data there were no significant relationships between light scattering (R_{800}) and any of the leaf structural variables (thickness, water content, specific leaf mass, or percent dry mass), except for the extreme case of the succulent species where there was a reduction in measured light scattering. Measurements of stacks of leaves, thus increasing total optical thickness, show increases in near infrared reflectance up to thickness of about eight leaves (Blackburn, 1999; Lillesaeter, 1982) and light scattering is often modeled as a function of the number of distinct “layers” of cells separated by air spaces (Dawson et al., 1998; Jacquemoud et al., 1996). This would appear to suggest that light scattering should increase with thickness of individual leaves. However, this was not the case in our study and results of other studies are inconsistent. Knapp and Carter (1998) found a positive relationship between R_{850} and leaf thickness for 26 species but a recent study of 48 species by Slaton et al. (2001) found that R_{800} was not related to leaf thickness but rather was a function of the percentage of intercellular air space and the ratio of mesophyll surface area to leaf area (A_{mes}/A). Thus, across multiple species with variable leaf structures there is no consistent relationship between R_{800} and thickness per se.

The reduced light scattering for the succulent leaves may have been an artifact of the measurement system. When measured in an integrating sphere, as opposed to the leaf clip, light scattering of the succulent leaves was similar to that of the other leaves. The fiber optic in the leaf clip exposes a much smaller area (1–2 mm diameter) of the leaf to light than does the integrating sphere (14 mm diameter). When leaf thickness becomes greater than the diameter of the illuminated area, as was the case for the succulent leaves in the leaf clip, loss of light due to side scattering may become significant, leading to lower overall reflectance. However, in spite of the lower leaf clip reflectance from the succulent leaves, we found that the relationship between

pigment content and the modified spectral indices calculated from leaf clip data was not significantly different between the succulent leaves and the overall data set, suggesting that the leaf clip data can be reliably used for pigment estimation even for very thick leaves.

The effect of leaf water content per unit area on the chlorophyll/spectral index relationships was present only for the thin and moderately thick leaves. Inclusion of the succulent leaves eliminated this effect. It remains unclear what is responsible for moderately high water content leaves having lower values of the chlorophyll indices than low-water-content leaves. We attempted to use information from the 970-nm water band to correct for this effect but this did not substantially improve the prediction of chlorophyll content. Since leaf chlorophyll content was positively correlated with water content, a reduction in chlorophyll concentration did not appear to explain this result. A more likely explanation is that high water content leaves tend to be thicker, thus reducing light penetration. Chlorophyll detection would then tend to be limited to the upper leaf layers, missing the portion of total leaf chlorophyll that was in the lower leaf layers.

5.2. Interactions between chlorophyll and anthocyanin

The relationship between spectral indices and chlorophyll content may also be influenced by the presence of other pigments. Curran, Dungan, Macler, and Plummer (1991) found that amarantin, a red pigment found in *Amaranthus tricolor* leaves, significantly shifted the red edge to longer wavelengths. However, we did not find a similar effect for anthocyanin in this study. The reason for this difference is unclear, although it is possible that the amarantin pigment in that study absorbed at somewhat longer wavelengths than did the anthocyanins in our leaves and thus overlapped with chlorophyll absorbance to a greater extent. There was a small effect of anthocyanins on the SR and ND indices in this study but only for leaves with extremely high anthocyanin contents that are rarely observed. Carotenoids had no effect on the chlorophyll spectral indices used in this study since the wavelengths used were in the red and infrared regions of the spectrum where carotenoids do not absorb. Consequently, we did not find that either carotenoids or anthocyanins significantly affected the in vivo measurement of chlorophyll using spectral indices.

The red/green ratio was correlated with anthocyanin content for leaves containing both anthocyanin and chlorophyll but this relationship was weak and appeared to change depending on the absolute contents of anthocyanin and chlorophyll. This is in contrast to previous studies of the relationship between red/green ratio and anthocyanin content that have shown much better relationships (Gamon & Surfus, 1999). However, that study examined leaves of only a single species with a more limited range of pigment contents. Thus, while the red/green ratio may be suitable

for single-species studies, it does not work across many species with varying structure and pigment levels.

These relationships may be further complicated by changes in the color of anthocyanin pigments depending on their chemical environment within the leaf. We found that different species appeared to have different relationships between anthocyanin content and reflectance even when chlorophyll was not present. In flowers, similar anthocyanins can produce a wide range of colors depending on the chemical environment within the cells (Asen, 1976). Metal ions and pH are known to affect anthocyanin color *in vitro* but appear to play only a minor role *in vivo*. Copigmentation, the association of other flavonoid pigments with anthocyanins, appears to play the greatest role in determining flower color (Asen, 1976). The extent to which similar cofactors are important in the color of leaves does not appear to be known.

5.3. Carotenoid/chlorophyll ratios

Of the three carotenoid/chlorophyll ratio indices we tested, only one (PRI) produced a significant correlation across all species. This index measures the relative reflectance on either side of the green hump, one side being affected only by chlorophyll absorbance and the other by chlorophyll + carotenoid absorbance.

Although this relationship between PRI and the carotenoid/chlorophyll ratio was relatively weak in this study, its existence has important implications for the use of PRI as an estimate of photosynthetic light use efficiency. PRI was originally developed by Gamon et al. (1992) to estimate rapid changes in the relative levels of xanthophyll cycle pigments that are related to changes in photosynthetic light use efficiency (Gamon et al., 1993, 1997; Peñuelas et al., 1995). This relationship becomes more complicated if the carotenoid/chlorophyll ratio also influences PRI. The significance of changes in PRI now appears to depend on the time scale of the measurements. Interconversions between the xanthophyll cycle pigments occur on a time scale of minutes whereas changes in total carotenoid and chlorophyll contents occur over periods of days to weeks. Thus, PRI variation over the diurnal cycle will primarily be a function of xanthophyll cycle pigment changes whereas PRI variation over weeks or months may be a combined function of the xanthophyll cycle and changes in the total pools of carotenoids and chlorophylls. This effect can be seen in the data of Gamon, Field, Fredeen, and Thayer (2001) where there was a good correlation between diurnal changes in PRI and xanthophyll cycle pigment epoxidation state for young leaves with high chlorophyll contents, but this correlation broke down when older, senescing leaves (which had more variable carotenoid to chlorophyll ratios) were included. Ongoing measurements in the California chaparral suggest that evergreen shrubs undergo large seasonal changes in their leaf carotenoid/chlorophyll ratios that result in larger seasonal than diurnal changes in PRI (Sims, unpublished

data). When sampled across space, PRI may indicate either variation in xanthophyll cycle pigments between plants with different capacities for photosynthetic utilization of the ambient photon flux density or variation in chlorophyll/carotenoid ratios that have developed in response to longer term acclimation of the plants to their local environment. PRI may still be effective as a measure of changes in photosynthetic light-use efficiency during senescence, or in response to stress, to the extent that photosynthetic light-use efficiency is correlated with carotenoid/chlorophyll ratio. Such a relationship appears likely since senescence and stress result in increased carotenoid/chlorophyll ratios and decreased photosynthetic light use efficiency.

5.4. Ecological applications

To our knowledge, this is the first study to test spectral indices for pigment estimation across such a wide range of species and leaf structures. The relatively good correlations we obtained for chlorophyll content suggest that these indices could be applied across species without extensive calibration for each species. Since reflectance measurements can be made quickly and nondestructively, this would make possible broad-scale (in both time and space) surveys of plants under field conditions. The spectrometer and leaf clip, as described in this study, are easily field portable and leaf measurements can be made in seconds. This could be quite useful in ground truthing for remote sensing campaigns where researchers wish to separate the effects of leaf pigment changes from those due to canopy structure. Recent work in our laboratory has also documented correlations between drought induced seasonal variation in leaf photosynthetic rates and both ND₇₀₅ and PRI for evergreen chaparral species in California (D. A. Sims, unpublished data). Variations in leaf chlorophyll content detectable by spectral reflectance have also been shown to be related to leaf development and senescence (Carter & Knapp, 2001; Gamon & Surfus 1999; Gitelson & Merzlyak 1994a, 1994b), soil fertility (Carter & Knapp, 2001; Chappelle et al., 1992; Mariotti, Ercoli, & Masoni, 1996; McMurtrey, Chappelle, Kim, Meisinger, & Corp, 1994; Yoder & Pettigrew-Crosby, 1995), soil contamination (Chang & Collins, 1983; Collins, Chang, Raines, Canney, & Ashley, 1983; Horler, Barber, & Barringer, 1980; Jago, Cutler, & Curran 1999; Milton, Collins, Chang, & Schmidt, 1983), chilling (Savé, Pañuelas, Filella, & Olivela, 1995), air pollution (Rock, Hoshizaki, & Miller, 1988), and insect attack (Carter & Knapp, 2001).

The extent to which leaf chlorophyll concentration can be estimated from reflectance measurements at canopy and landscape scales remains uncertain. Correlations between spectral indices and leaf chlorophyll have been demonstrated for uniform canopies (Blackburn, 1998a; Jago et al., 1999). However, most chlorophyll indices are sensitive to both canopy structure and leaf characteristics, making it difficult to distinguish changes in chlorophyll content of

leaves when canopy structure is variable (Matson, Johnson, Billow, Miller, & Pu, 1994; Pinar & Curran, 1996). Demetriades-Shah et al. (1990) found that second-derivative parameters calculated from the red edge region of the spectrum were largely insensitive to canopy structure and background effects, making them much more effective than SR or NDVI indices for estimation of leaf chlorosis at the canopy scale. However, the generality of those results is unclear since only one species was measured. Ongoing work in our lab confirms the utility of red edge parameters for prediction of leaf chlorophyll content from canopy reflectance but also suggests that the relationship is affected by variation in leaf thickness between species as well as changes in leaf thickness during leaf out and subsequent leaf development. Recent studies linking leaf and canopy radiative transfer models have had some success at estimation of chlorophyll contents from canopy reflectance (Jacquemoud, Bacour, Poilve, & Frangi, 2000; Jacquemoud, Baret, Andrieu, Danson, & Jaggard, 1995; Zarco-Tejada, Miller, Harron et al., 2002; Zarco-Tejada, Miller, Noland, Mohammed, & Sampson, 2001). However, these model inversions typically require inputs of many canopy parameters that are not readily estimated from remote sensing data. Remote estimation of canopy structure from indices independent of leaf chlorophyll content, e.g., water content indices (Serrano, Ustin, Roberts, Gamon, & Peñuelas, 2000) and synthetic aperture radar (Ferrazzoli et al., 1997), should be further explored as methods to estimate the canopy parameters needed for model inversions.

5.5. Conclusions

We found that correction for leaf surface reflectance eliminates much of the variation in the relationships between leaf spectral reflectance indices and chlorophyll content across a wide range of species and leaf structures, suggesting that it may be possible to use a single calibration equation over a fairly wide range of species and leaf structures. Estimation of carotenoid and anthocyanin contents remains more difficult than estimation of chlorophyll content. However, we found a general relationship between PRI and carotenoid/chlorophyll ratio. The implications of this relationship for the use of PRI in photosynthetic light-use efficiency estimation needs to be further investigated. Much work also remains to be done to scale these pigment estimation relationships to canopy and larger scale remote sensing applications.

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