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Relationships between leaf chlorophyll content and spectral reflectance and algorithms for non-destructive chlorophyll assessment in higher plant leaves

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Summary

Leaf chlorophyll content provides valuable information about physiological status of plants. Reflectance measurement makes it possible to quickly and non-destructively assess, *in situ*, the chlorophyll content in leaves. Our objective was to investigate the spectral behavior of the relationship between reflectance and chlorophyll content and to develop a technique for non-destructive chlorophyll estimation in leaves with a wide range of pigment content and composition using reflectance in a few broad spectral bands. Spectral reflectance of maple, chestnut, wild vine and beech leaves in a wide range of pigment content and composition was investigated. It was shown that reciprocal reflectance $(R_\lambda)^{-1}$ in the spectral range λ from 520 to 550 nm and 695 to 705 nm related closely to the total chlorophyll content in leaves of all species. Subtraction of near infra-red reciprocal reflectance, $(R_{NIR})^{-1}$, from $(R_\lambda)^{-1}$ made index $[(R_\lambda)^{-1}\text{-}(R_{NIR})^{-1}]$ linearly proportional to the total chlorophyll content in spectral ranges λ from 525 to 555 nm and from 695 to 725 nm with coefficient of determination $r^2 > 0.94$. To adjust for differences in leaf structure, the product of the latter index and NIR reflectance $[(R_\lambda)^{-1}\text{-}(R_{NIR})^{-1}]*(R_{NIR})$ was used; this further increased the accuracy of the chlorophyll estimation in the range λ from 520 to 585 nm and from 695 to 740 nm. Two independent data sets were used to validate the developed algorithms. The root mean square error of the chlorophyll prediction did not exceed 50 µmol/m^2 in leaves with total chlorophyll ranged from 1 to 830 µmol/m^2.

Key words: chlorophylls – non-destructive assessment – leaf optics – reflectance

Abbreviations: Chl = Chlorophyll. – NIR = Near Infra Red. – RMSE = Root Mean Square Error

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Introduction

The chlorophylls, Chl *a* and Chl *b*, are virtually essential pigments for the conversion of light energy to stored chemical energy. The amount of solar radiation absorbed by a leaf is a function of the photosynthetic pigment content; thus, chlorophyll content can directly determine photosynthetic potential and primary production (Curran et al. 1990, Filella et al. 1995). In addition, Chl gives an indirect estimation of the nutrient status because much of leaf nitrogen is incorporated in chlorophyll (Filella et al. 1995, Moran et al. 2000). Furthermore, leaf chlorophyll content is closely related to plant stress and senescence (Hendry 1987, Merzlyak and Gitelson 1995, Peñuelas and Filella 1998, Merzlyak et al. 1999).

Traditionally, leaf extraction with organic solvents and spectrophotometric determination in solution is required for pigment analysis with wet chemical methods (e.g., Lichtenthaler 1987). Recently, alternative solutions of leaf pigment analysis (i.e., chlorophyll, carotenoids and anthocyanins) with non-destructive optical methods have been developed. These newer methods are non-destructive, inexpensive, quick and now possible in the field (Buschmann and Nagel 1993, Gitelson and Merzlyak 1994 a, b, Markwell et al. 1995, Gamon and Surfus 1999, Gitelson et al. 2001, 2002).

Transmittance and reflectance spectroscopy is applied extensively for non-destructive estimation of leaf Chl (Adams and Arkin 1977, Aoki et al. 1986, Curran et al. 1990, Gitelson and Merzlyak 1994 a, b, 1996, 1977, Markwell et al. 1995, Gitelson et al. 1996, Blackburn 1998, Datt 1998, Gamon and Surfus 1999, Richardson et al. 2002). Relationships between reflectance in the visible range and leaf Chl content are essentially nonlinear (e.g., Buschmann and Nagel 1993, Gitelson and Merzlyak 1994 a). First-difference transformation of the apparent absorbance, the logarithm of reciprocal reflectance $(R)^{-1}$, was found to be the best predictor for nitrogen and chlorophyll in fresh bigleaf maple leaves (Yoder and Pettigrew-Crosby 1995). Gitelson and Merzlyak (1994 a and b) have found that reflectances in the spectral bands located quite far from the main absorption bands of pigments near 550 nm and 700 nm were closely hyperbolically related to Chl for a variety of plant species and in a wide range of pigment content and composition. Indices based at these spectral bands were proposed and used to estimate Chl content in the leaves of various plant species (Chappele et al. 1992, Gitelson and Merzlyak 1994 a, b, 1996, 1997, Gitelson et al. 1996, Lichtenthaler et al. 1996, Gamon and Surfus 1999).

Richardson et al. (2002) evaluated both non-destructive absorbance and reflectance methods for Chl assessment. They compared the performance of two commercially available hand-held Chl absorbance meters with that of several reflectance indices for the estimation of leaf-level Chl and found that some indices based on reflectance in the red edge region (Gitelson and Merzlyak 1994 a) were much better indicators of Chl content than some of the more commonly used indices. The best reflectance indices were found to be better indicators of Chl compared with those based on absorbance measurements (Richardson et al. 2002).

Sims and Gamon (2002) examined hundreds of leaves of non-related plant species and proved that reflectance in the spectral channel around 700 nm was the most sensitive indicator of Chl and that indices R_{750}/R_{700} and $(R_{750}-R_{705})/(R_{750}+R_{705})$, Gitelson and Merzlyak (1994 a), could be used as a measure of Chl content. However, the correlation of these indices with Chl was weaker when applied across a wide range of species. The modified indices $(R_{750}-R_{445})/(R_{700}-R_{445})$ and $(R_{NIR}-R_{705})/(R_{NIR}+R_{705}-2R_{445})$, which were developed to eliminate the effect of variability in surface reflectance between species by incorporating reflectance at 445 nm, R_{445}, produced substantially better correlation with total Chl content.

Although a leaf consisting of cuticula, epidermis, palisade and spongy parenchyma, etc. with numerous boundaries and containing high amounts of pigments is very complicated from an optical point of view (Gates et al. 1965, Heath 1969, Fukshansky 1981, Vogelmann 1993, Richter and Fukshansky 1996), it was found that reciprocal reflectance alone at certain wavelengths could be used for chlorophyll quantification (Gitelson et al. 1996). Based on that finding, algorithms for non-destructive estimation of carotenoids (Gitelson et al. 2002) and anthocyanins (Gitelson et al. 2001) were developed in which reciprocal reflectances at 550 nm and 700 nm were used to eliminate the Chl contribution in spectral ranges with overlapping absorption by Chl and other pigments. However, the mechanisms responsible for close relationships between reciprocal reflectance and pigment content needs more detailed investigations.

The indices for chlorophyll estimation based on reflectances in narrow spectral bands (usually 1 to 2 nm wide) at 550 nm and/or around 700 nm have been tested (Aoki et al. 1986, Gitelson and Merzlyak 1997, Datt 1998, Gamon and Surfus 1999, Carter and Knapp 2001, Richardson et al. 2002, Sims and Gamon 2002) and showed good performance in Chl estimation. However, to provide sufficient sensitivity to a small variation of Chl, broad spectral range is required. It is also desirable to use an algorithm that is minimally sensitive to the differences in leaf structure to avoid species-specific calibration if the estimation of absolute Chl content is desired. We are not aware of any studies that have attempted to assess how the accuracy of Chl estimation depends on the width of spectral bands and how wide spectral bands must be to provide the required accuracy.

The goal of this research was to investigate the spectral behavior of the relationship between reflectance and Chl content and to develop a technique for Chl estimation in leaves with a wide range of pigment content and composition using reflectance in a few broad spectral bands. The first step was to find spectral ranges where Chl related closely to reciprocal reflectance $(R_{\lambda})^{-1}$ for all species studied. We found that reciprocal reflectances in narrow bands in the green and red edge spectral regions were sensitive and closely related to the Chl content. Subtraction of $(R_{NIR})^{-1}$ from $(R_\lambda)^{-1}$ made index $(R_\lambda)^{-1}$-$(R_{NIR})^{-1}$ linearly proportional to the total Chl content in wide spectral bands 525 to 555 nm and 695 to 725 nm. To correct for differences in leaf structure, we suggested the product of the above index and NIR reflectance $[(R_\lambda)^{-1}\text{-}(R_{NIR})^{-1}] * R_{NIR}$. This algorithm made it possible to reach a high accuracy of Chl estimation with Root Mean Square Error (RMSE) < 40 µmol/m^2 in broad spectral bands (520–585 nm and 695–735 nm). Finally, algorithms were validated by independent data sets.

Materials and Methods

Juvenile, mature and senescent leaves of Norway maple (*Acer platanoides* L.) and horse chestnut (*Aesculus hippocastanum* L.) were collected in a park at Moscow State University in the spring, summer and fall of 1992–2000. Second-flush beech leaves (*Fagus sylvatica* L.) grown on the University of Karlsruhe campus were taken in August and September 1996 and in August 2000, and wild vine shrub (*Parthenocissus tricuspidata* L.) leaves were collected in August and September 1996. Leaves were visually selected according to their difference in color. Leaves healthy and homogeneous in color without anthocyanin pigmentation or visible symptoms of damage were used in the experiments.

The leaf pigment content was determined from the same leaf samples used for reflectance measurement. Circular pieces were cut from the leaves and extracted with 100 % acetone or methanol using a mortar. The pigment extracts were centrifuged for 3–5 min in glass tubes to make the extract fully transparent. The resulting extracts were immediately assayed spectrophotometrically. Specific absorption coefficients of Chl *a*, Chl *b* and total carotenoids reported by Lichtenthaler (1987) were used. The accepted molecular weight of carotenoids was 570.

Adaxial reflectance (R) and transmittance (T) spectra of the leaves were taken in a spectral range between 400 and 800 nm with a spectral resolution of 2 nm with a Hitachi 150–20 spectrophotometer (measurements of maple and chestnut leaves) and a Shimadzu 2101 PC spectrophotometer (measurements of beech and wild vine leaves) equipped with an integrating sphere. Leaf reflectance spectra were recorded against barium sulphate as a standard; black velvet was used as a background.

Four data sets containing 140 leaves (maple 1992–1998, chestnut, beech 1996 and wild vine) were used for model development. Two independent data sets (23 maple-1999 and 34 beech-2000 leaves) were used to validate the models. Relationships between the developed indices and Chl content obtained for the model development data sets were inverted, and reflectance data from the model validation data sets were used to calculate the predicted Chl content. Finally, the predicted Chl content was compared with analytically measured Chl values, and the RMSE of the Chl prediction was determined.

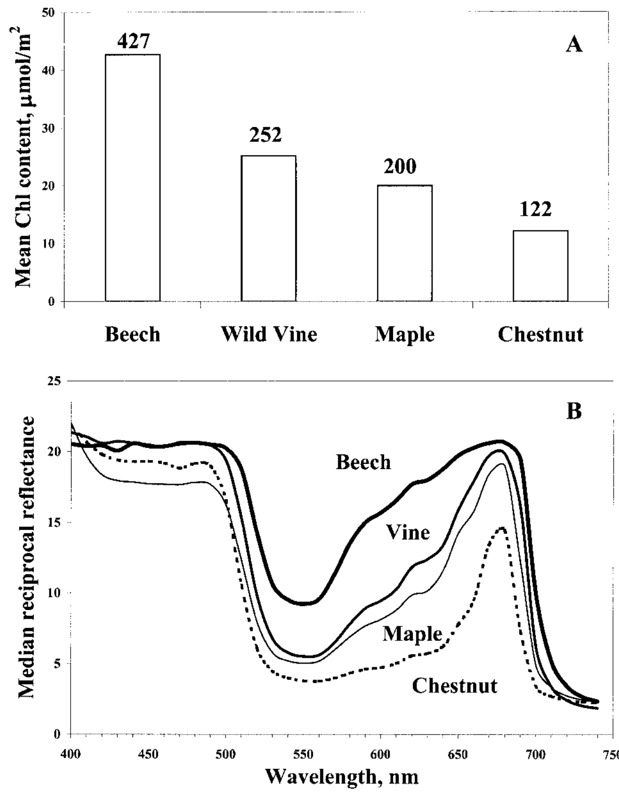

Figure 1. (A) Mean Chl content in four leaf groups used for model development. (B) Median reciprocal reflectance spectra for each leaf group. Maximal reciprocal reflectance in the range from 500 nm to 700 nm was in beech leaves with maximal Chl content, and minimal reciprocal reflectance was in chestnut leaves with minimal Chl. In the blue range, reciprocal reflectance depends on both chlorophylls and carotenoids content.

Table 1. Pigment content and composition in leaves used for model development and model validation. Pigment content in µmol/m^2, *n* is the number of leaves in each data set.

Data Set	*n*	Total Chlorophyll	Carotenoids
Model development			
Beech – 1996	38	97–832	53–240
Wild Vine	19	47–530	21–180
Maple – 92–98	65	1–570	29–170
Chestnut	20	10–470	53–170
	142	1–832	21–240
Model validation			
Maple – 1999	23	1–460	16–120
Beech – 2000	34	14–670	36–210
	57	1–670	16–210
Total	199	1–832	16–240

Results

Pigment content and composition

Pigment content and composition in the leaves varied widely (Table 1). Carotenoids were the dominant pigments in leaves

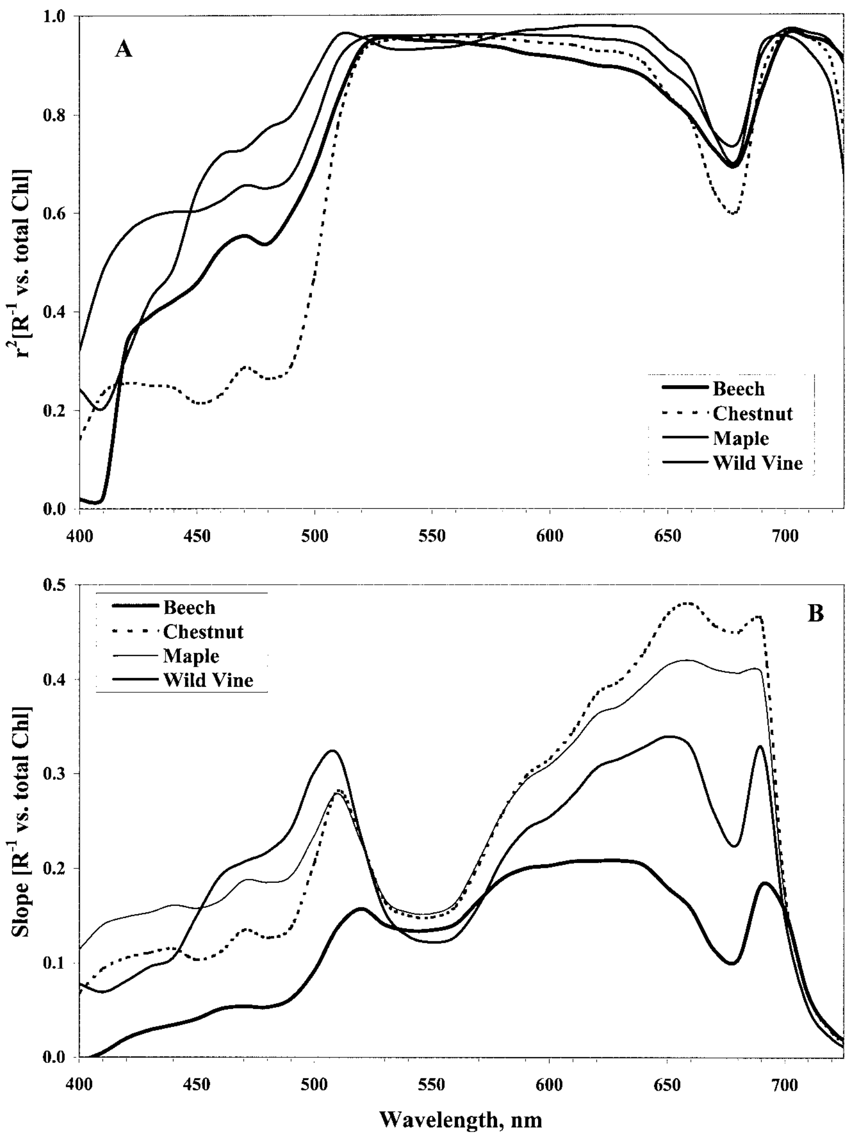

Figure 2. (A) Spectra of the coefficient of determination, r^2, and (B) the slope of the linear relationship between reciprocal reflectance $(R_\lambda)^{-1}$ and total Chl content for four leaf group studied (Table 1: model development data sets).

with a total pigment content (chlorophylls + carotenoids) of less than 50 µmol/m^2; they constituted more than 97 % of the total pigment content. With an increase in total pigment content, the carotenoids fraction decreased sharply; Chl presented the major portion of pigments in the leaves with a total pigment content > 200 µmol/m^2. With a further increase in leaf greenness, the ratio of carotenoids to total pigment content decreased only a little: from 30 % in slightly green leaves to 20 % in dark-green leaves. The relationship between carotenoids and total Chl was linear with a coefficient of determination $r^2 = 0.87$.

Leaves of four plant species, used for model development, were quite different with respect to total Chl content. Mean Chl content ranged from a little bit higher than 120 µmol/m^2 in chestnut leaves to more than 400 µmol/m^2 in beech leaves (Fig. 1A).

Spectral characteristics of leaves and their relations with total Chl content

The difference in mean Chl content in the four leaf groups studied manifested itself in different reciprocal reflectance, $(R)^{-1}$, spectra (Fig. 1B). In the blue range from 400 to 500 nm, $(R)^{-1}$ was the highest in beech and wild vine leaves and slightly lower in chestnut and maple. In this range, $(R)^{-1}$ was affected by both Chl and carotenoids. Considerable changes of $(R)^{-1}$ occurred in the range between 500 and 700 nm. With an increase in Chl content, both green edge (around 530 nm) and red edge (around 700 nm) shifted toward longer wavelengths and the reciprocal reflectance increased. The highest reciprocal reflectance was in beech leaves with maximal mean Chl, and the lowest one was in chestnut leaves with minimal mean Chl.

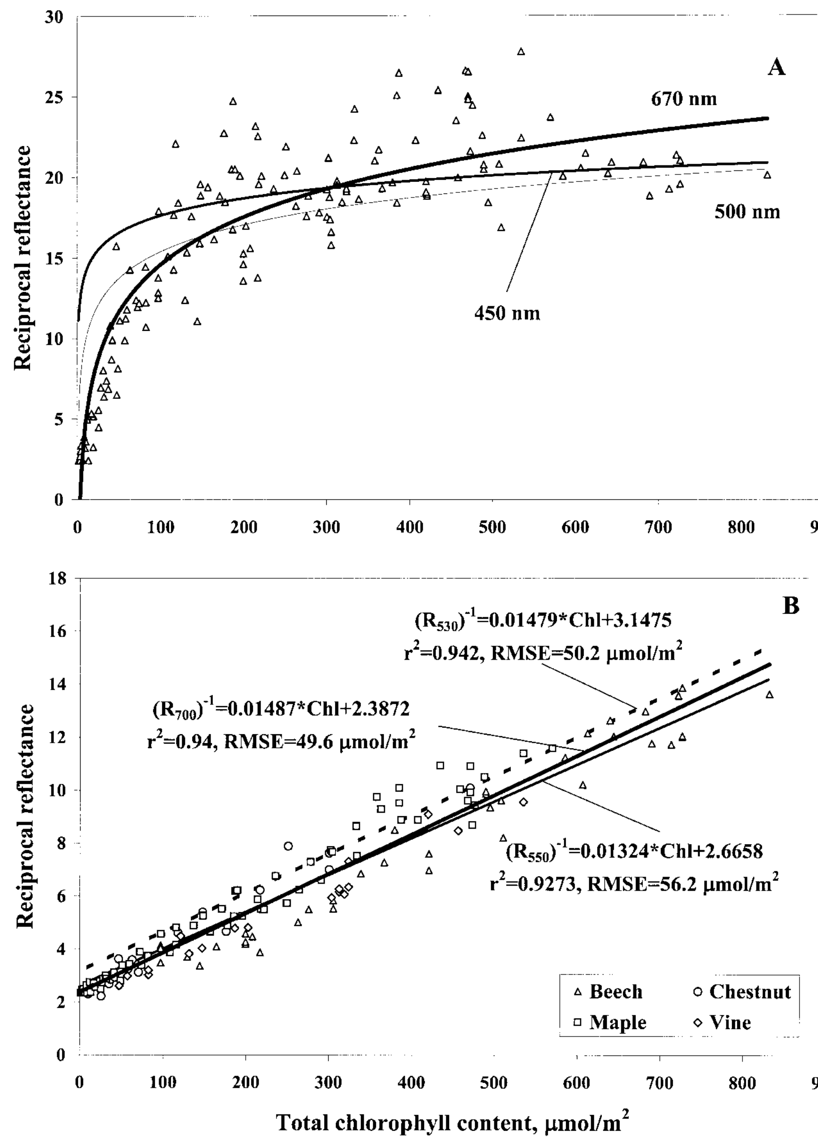

Figure 3. Relationship between reciprocal reflectance and total chlorophyll content (A) in the blue (450 and 500 nm) and the red (670 nm), and (B) in the green (530 and 550 nm) and red edge (700 nm).

To find spectral bands where reciprocal reflectance was sensitive to total Chl content, we studied the spectral behavior of the coefficient of determination, r^2, and the slope of the linear relationship $(R_\lambda)^{-1}$ vs. total Chl content (Fig. 2). In the blue (400–500 nm) and red (around 680 nm), r^2 was minimal (Fig. 2 A). In these spectral ranges, the relationship $(R_\lambda)^{-1}$ vs. total Chl was essentially non-linear (Fig. 3 A). For Chl $<$ 100 μmol/m², the sensitivity of $(R_\lambda)^{-1}$ to Chl was high; for Chl $>$ 150 μmol/m², the relationship leveled off, and $(R_\lambda)^{-1}$ became virtually insensitive to Chl. In the spectral range between 510 and 620 nm and near 700 nm, the function $(R_\lambda)^{-1}$ vs. Chl was linear for all species with $r^2 > 0.9$ (Figs. 2 A and 3 B).

The slope of the relationship $(R_\lambda)^{-1}$ vs. Chl reached maximal values around 510–520 nm, at 650 nm and at 690–695 nm, spectral ranges located far from main peaks of Chl absorption (Fig. 2 B). In the blue range, the slope depended upon the content of Chl and carotenoids and varied widely between species with a minimal slope for beech, which had a maximal mean Chl content. In the orange and red ranges, the slopes were inversely related to mean Chl content: the higher Chl content, the lower the slope; so, the absorption per Chl unit was higher for species with lower Chl than for species with higher Chl. In the green (between 520 and 580 nm) and red edge (around 700 nm), the slopes of the relationships $(R_\lambda)^{-1}$ vs. Chl remained fairly similar for different species.

The high linear correlation between Chl content and $(R_\lambda)^{-1}$ in a wide range from 510 to 650 nm and in a narrow range around 700 nm (Figs. 2 A and 3 B) suggested the use of $(R_\lambda)^{-1}$ as a measure of total Chl content. However, to be applied for variety of species, relationship $(R_\lambda)^{-1}$ vs. Chl should have minimal variation in the slope between species. The coefficient of variation of the slope between species was the highest (more

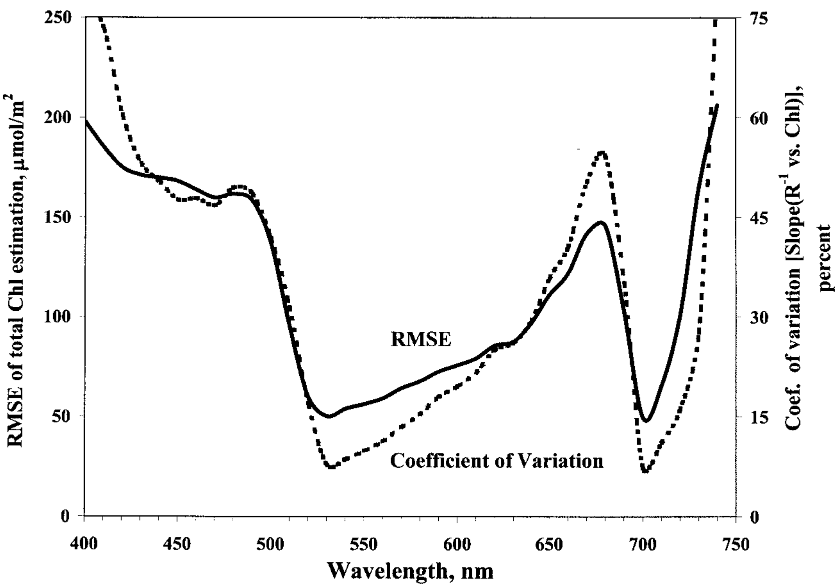

Figure 4. The coefficient of variation of the slope of the linear relationship between $(R_\lambda)^{-1}$ and total Chl content (right axis), and the RMSE of Chl estimation by reciprocal reflectance (left axis) for the four species studied.

than 45 %) in the blue range between 400 and 490 nm and in the red range near 675 nm (Fig. 4). It reached minimal values (below 10 %) in the green range between 520 and 580 nm, and in the red edge around 700 nm; in these spectral ranges, the RMSE of Chl estimation had minimal values (50 to 60 μmol).

Thus, the relationship $(R_\lambda)^{-1}$ vs. Chl showed several notable spectral features.

1. In the main bands of pigment absorption, the blue and the red, the relationships were essentially non-linear. $(R_\lambda)^{-1}$ was sensitive to Chl < 150 μmol/m^2, but insensitive to moderate-to-high Chl.
2. In the blue and the red, the slopes of the relationship $(R_\lambda)^{-1}$ vs. Chl varied widely between species. In the green and red edge, the slopes remained nearly the same for all species studied. In these spectral ranges, reciprocal reflectance was a measure of Chl content, and the RMSE of the Chl estimation had its minimal values.

Algorithm development and validation

For leaves containing trace amounts of Chl, reciprocal reflectance remained significantly above zero (Fig. 3 B). In the range beyond 550 nm, the intercept $(R_\lambda)^{-1}$ vs. Chl was almost equal to $(R_{NIR})^{-1}$ (not shown). Thus, we subtracted the reciprocal reflectance in the NIR range, $(R_{NIR})^{-1}$, from $(R_\lambda)^{-1}$ to make the index

$$[(R_\lambda)^{-1}\text{-}(R_{NIR})^{-1}]$$

linearly proportional to total Chl. At 700 nm, the intercept of the relationship $[(R_{700})^{-1}\text{-}(R_{NIR})^{-1}]$ vs. Chl dropped 16-fold and became very close to zero. The intercept $[(R_{550})^{-1}\text{-}(R_{NIR})^{-1}]$ vs. Chl also decreased significantly (six-fold), but nevertheless, remained positive (Fig. 5 A); it became negligible beyond 550 nm. Remarkably, the subtraction of $(R_{NIR})^{-1}$ made it possible to extend significantly the spectral range of an accurate Chl estimation. It was especially evident in the wide ranges from 525 to 555 nm and 695 to 735 nm. The RMSE of Chl estimation by indices

$$[(R_{525-555})^{-1}\text{-}(R_{NIR})^{-1}]$$
$$[(R_{695-725})^{-1}\text{-}(R_{NIR})^{-1}]$$

was below 50 μmol/m^2 (Fig. 5 B).

A further increase in the accuracy of Chl estimation was achieved by using the product of the index $[(R_\lambda)^{-1}\text{-}(R_{NIR})^{-1}]$ and R_{NIR}:

$$[(R_\lambda)^{-1}\text{-}(R_{NIR})^{-1}] * R_{NIR} = (R_{NIR}/R_\lambda)\text{-}1.$$

This algorithm is slightly different from R_{NIR}/R_{550} and R_{NIR}/R_{700}, suggested by Gitelson and Merzlyak (1994 a, b). Importantly, however, the intercept of the function $(R_{NIR}/R_\lambda)^{-1}$ vs. Chl was quite close to zero, which made the index linearly proportional to the Chl content. The main differences in the spectral behavior of all three indices, $(R_\lambda)^{-1}$, $[(R_\lambda)^{-1}\text{-}(R_{NIR})^{-1}]$ and (R_{NIR}/R_λ)-1, were in the green and red edge ranges (Fig. 6). The RMSE of Chl estimation by the indices

$$(R_{NIR}/R_{520-585})\text{-}1$$
$$(R_{NIR}/R_{695-740})\text{-}1$$

was minimal among indices, remaining below 50 μmol/m^2.

Validation of the proposed technique has been carried out for two independent data sets: maple-1999 and beech-2000 with a wide variation of pigment content (Table 1). The relationship index vs. Chl obtained for model development data sets was inverted, and reflectance data from the model validation data sets were used to calculate the predicted Chl content. The predicted Chl content was compared with ana-

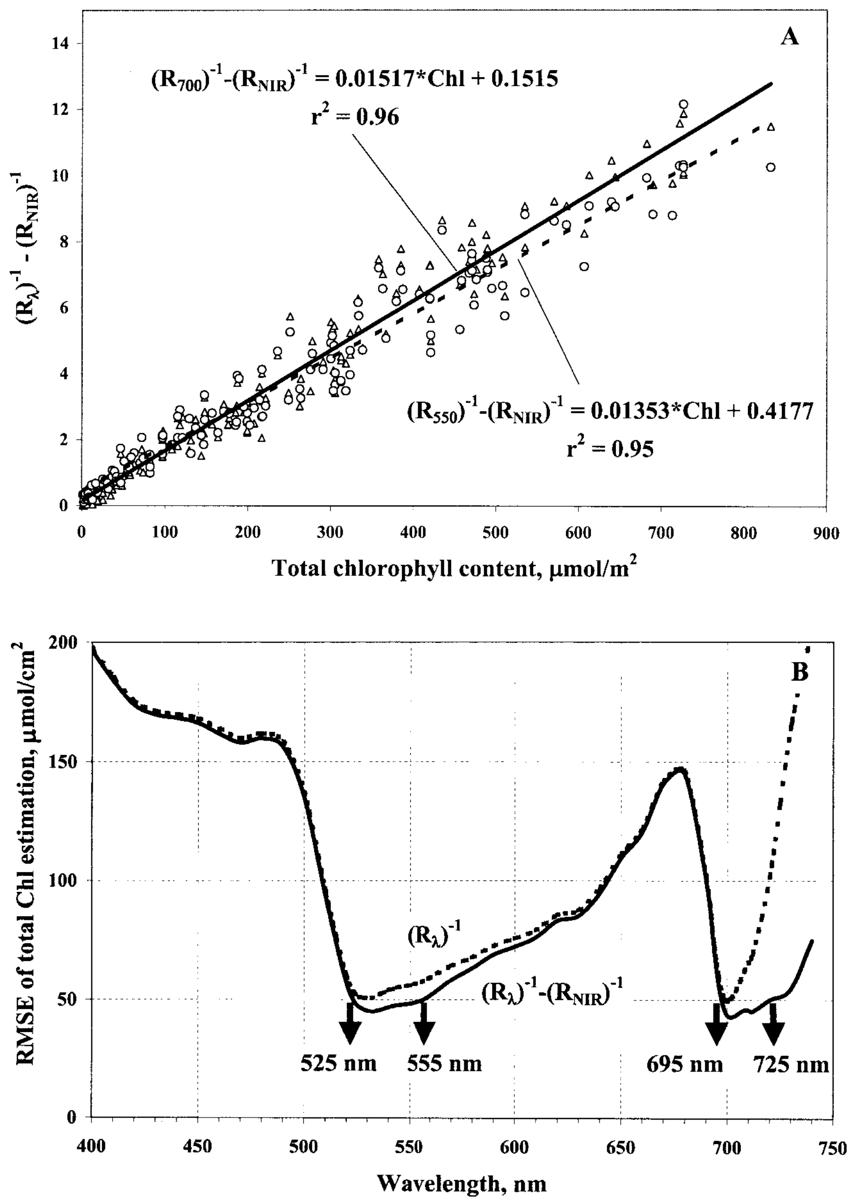

Figure 5. (A) Relationship between index $[(R_\lambda)^{-1}$-$(R_{NIR})^{-1}]$ and total Chl content in the green (550 nm) and red edge (700 nm) regions for the four leaf groups studied; (B) RMSE of total Chl estimation by $(R_\lambda)^{-1}$ and index $[(R_\lambda)^{-1}$-$(R_{NIR})^{-1}]$. Spectral ranges where the RMSE of total Chl estimation dropped below 50 µmol/m^2 are shown.

lytically measured Chl, and the RMSE of the Chl prediction was determined (Table 2). For all 57 leaves examined, the RMSE of the total Chl prediction by index (R_{NIR}/R_λ)-1 in spectral ranges λ from 520 to 585 and from 695 to 740 nm was less than 49 µmol/m^2.

Discussion

In the blue and red spectral ranges, specific absorption coefficients of pigments are very high (e.g., Heath 1969, Lichtenthaler 1987) and the depth of light penetration into the leaf is very low (Kumar and Silva 1973, Cui et al. 1991, Vogelmann et al. 1991, Vogelmann 1993, Fukshansky et al. 1993, Merzlyak and Gitelson 1995). As a result, even low amounts of pigments are sufficient to saturate absorption. When Chl exceeded 150 µmol/m^2, total absorption reached maximal values (more than 90 %), the depth of light penetration drastically decreased, and a further increase in pigment content did not cause an increase in total absorption. Thus, the relationship $(R_\lambda)^{-1}$ vs. total Chl leveled off, and $(R_\lambda)^{-1}$ became virtually insensitive to Chl (see also, Thomas and Gausman 1977, Gausman 1982, Chapelle et al. 1992, Buschmann and Nagel 1993, Gitelson and Merzlyak 1994 a, b, 1997, Gamon and Surfus 1999). In the spectral regions located closer to the main absorption bands, the slope $(R_\lambda)^{-1}$ vs. Chl depended strongly on mean pigment content in leaf group (Fig. 2 B). The closer the spectral band was to the main absorption band of pigments, the lower the Chl content at which saturation of $(R_\lambda)^{-1}$ vs. Chl relationship appeared.

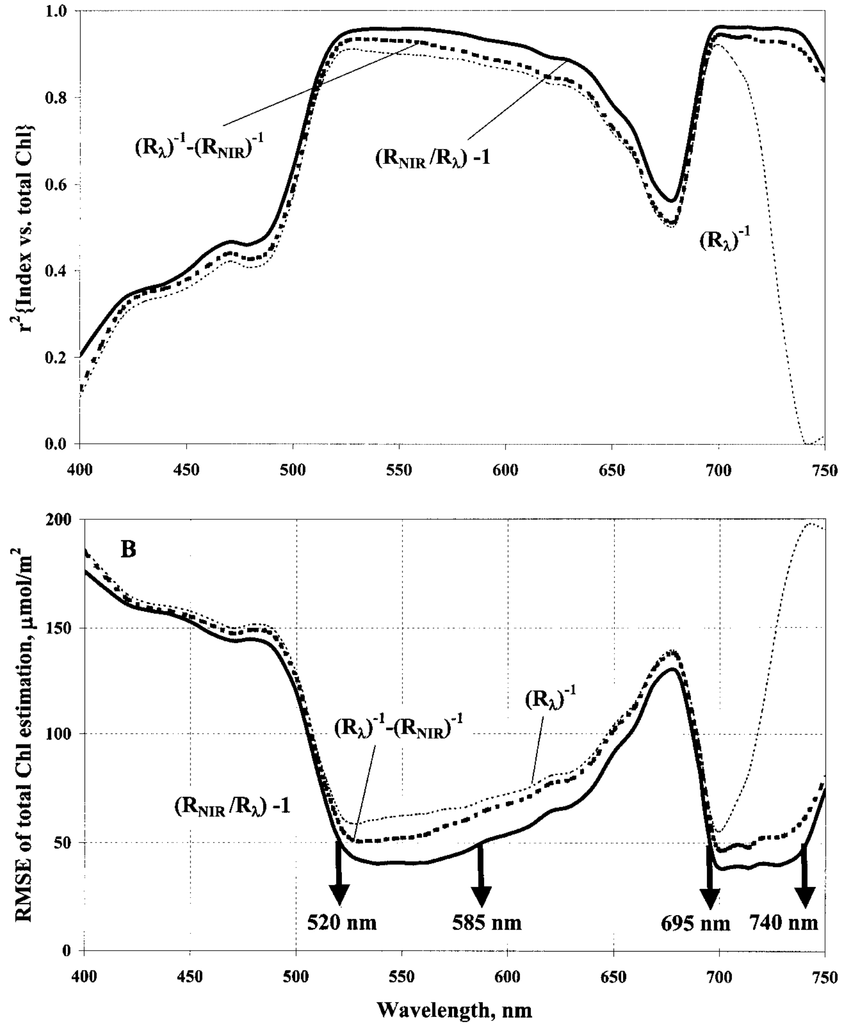

Figure 6. (A) The coefficient of determination r^2 and (B) RMSE of total Chl estimation by three indices proposed for Chl assessment: $(R_\lambda)^{-1}$, $[(R_\lambda)^{-1}\text{-}(R_{NIR})^{-1}]$, and (R_{NIR}/R_λ)-1. Spectral ranges where RMSE of Chl assessment dropped below 50 µmol/m² are shown.

Table 2. Root mean square error of leaf chlorophyll prediction (in µmol/m²) for model validation data sets: beech–2000 and maple–1999. The relationship index vs. Chl obtained for model development data sets was inversed, and reflectance data from the model validation data sets were used to calculate the predicted Chl content. Then, the predicted Chl content was compared with analytically measured actual Chl values, and the RMSE of Chl prediction was determined. Note: Chl content in beech leaves ranged from 14 to 670 µmol/m²; whereas, it ranged from 1 to 460 µmol/m² in maple leaves.

Waveband λ (nm)	Index	Beech–2000	Maple–1999
520–550	$(R_\lambda)^{-1}$	63	59
525–555	$(R_\lambda)^{-1}$-$(R_{NIR})^{-1}$	59	49
520–585	$(R_{NIR}/R_\lambda)-1$	48	35
695–705	$(R_\lambda)^{-1}$	64	58
695–725	$(R_\lambda)^{-1}$-$(R_{NIR})^{-1}$	61	49
695–740	$(R_{NIR}/R_\lambda) - 1$	49	33

In the green and in the red edge (around 700 nm), specific absorption coefficient of chlorophylls in extract is very low; it does not exceed even 6 % of that in the blue and red (e.g., Heath 1969, Lichtenthaler 1987); however, green leaves absorb more than 80 % of incident light in these spectral ranges (e.g., Moss and Loomis 1952, Heath 1969, Gausman et al. 1969, Gausman and Allen 1973, Gitelson and Merzlyak 1994 a). In these spectral ranges, depth of light penetration into the leaf was found to be four- to six-fold higher than in the blue and red (e.g., Fukshansky et al. 1993, Fig. 2 in Merzlyak and Gitelson 1995). Therefore, in the green and red edge absorption of light is high enough to provide high sensitivity of $(R_\lambda)^{-1}$ to Chl content and much lower than in the blue and red to avoid saturation of the $(R_\lambda)^{-1}$ vs. Chl relationship for moderate to high Chl.

The Kubelka-Munk remission function, which is proportional to the ratio of the absorption coefficient, *k*, to the scat-

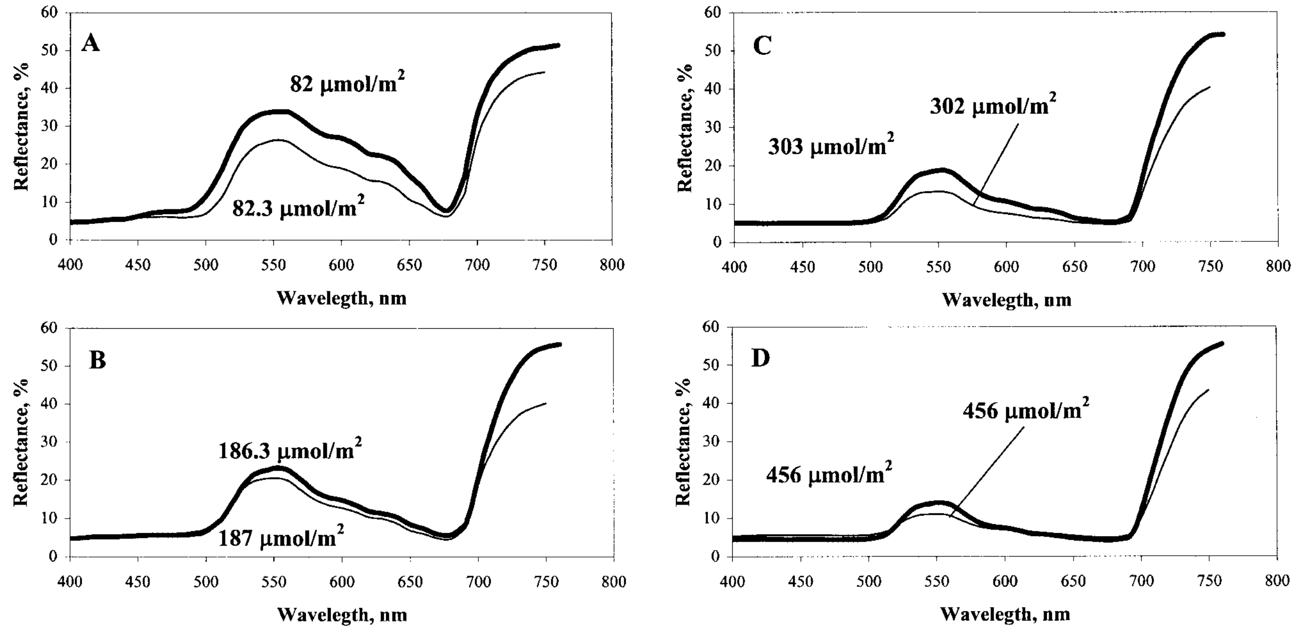

Figure 7. Reflectance spectra of leaves with almost the same total Chl content (indicated as numbers near the curves) and different NIR reflectance. For the same Chl content, higher NIR reflectance values correspond to higher reflectance in the green (around 550 nm) and in the range of the red edge (near 700 nm).

tering coefficient, *s* (e.g. Wendlandt and Hecht 1966) relates very closely to leaf reciprocal reflectance (Appendix 1). Therefore, in the green and red edge ranges, the ratios $(k/s)_{green}$ and $(k/s)_{red\ edge}$ were closely and linearly related to the total Chl content (Fig. 3 B). The *k/s* ratio per total Chl units (specific ratio in m^2/µmol) remained virtually invariant (0.0129 ± 0.003 m^2/µmol) in leaves with Chl from 1 to 830 µmol/m^2 and a wide range of pigment composition.

In an attempt to understand why the indices (R_{NIR}/R_{550})-1 and (R_{NIR}/R_{700})-1 were effective in improving the accuracy of Chl assessment, we compared reflectance spectra of leaves with almost the same Chl content but different R_{NIR}. We found with no exception that the higher the reflectance in the NIR range, the higher the reflectances in the range around 550 nm and 700 nm (Fig. 7). The processes that govern behavior of reflectance in the NIR range and the visible spectrum are very different. In the NIR range, an increase in reflectance might be caused by an increase in leaf thickness or/and density; in the visible range, an increase in reflectance indicates a decrease in pigment content (surface reflectance of adaxial surface for all leaves studied was virtually the same). In leaves with the same Chl content, an increase in leaf thickness might lead to an increase in R_{NIR} and to a decrease in Chl concentration (per volume); the latter caused an increase in reflectances in the green and red edge that was observed (Fig. 7). A decrease in leaf thickness might cause a decrease in R_{NIR} and an increase of Chl concentration, which caused a decrease in reflectance in the green and red edge. With a variation in leaf thickness, reflectances $(R_{520-585})^{-1}$, $(R_{695-740})^{-1}$, and R_{NIR} varied in the same direction, and, thus, indices $(R_{NIR}/R_{520-570})$-1 and $(R_{NIR}/R_{695-735})$-1 became less sensitive to this factor. As a result, these indices correlated with total Chl much more closely than $(R_{520-570})^{-1}$ and $(R_{700})^{-1}$ (Fig. 6).

Leaf surface reflectance virtually did not change in leaves studied, so, indices worked accurately across the species. In the case when surface reflectance varies, incorporating reflectance in the blue range makes it possible to eliminate the effect of surface reflectance (Sims and Gamon 2002). We recommend using the reflectance in the range between 430 and 470 nm in modified indices:

$$[(R_{520-555}-R_{440-480})^{-1}\text{-}(R_{NIR}-R_{440-480})^{-1}]$$
$$[(R_{695-725}-R_{440-480})^{-1}\text{-}(R_{NIR}\text{-}R_{440-480})^{-1}]$$

and

$$[(R_{NIR}\text{-}R_{440-480})/(R_{520-580}-R_{440-480})]\text{-}1$$
$$[(R_{NIR}\text{-}R_{440-480})/(R_{695-740}-R_{440-480})]\text{-}1$$

Comparison with previously developed indices

Using our data sets, we tested the accuracy of the indices that had been previously developed for Chl estimation and compared it with the performance of the indices proposed in this paper. In Figure 8, relationships between total chlorophyll content and the following indices are presented:

$(R_{800}-R_{680})/(R_{800}+R_{680})$ – Blackburn 1998,
(R_{800}/R_{680}) – Blackburn 1998,

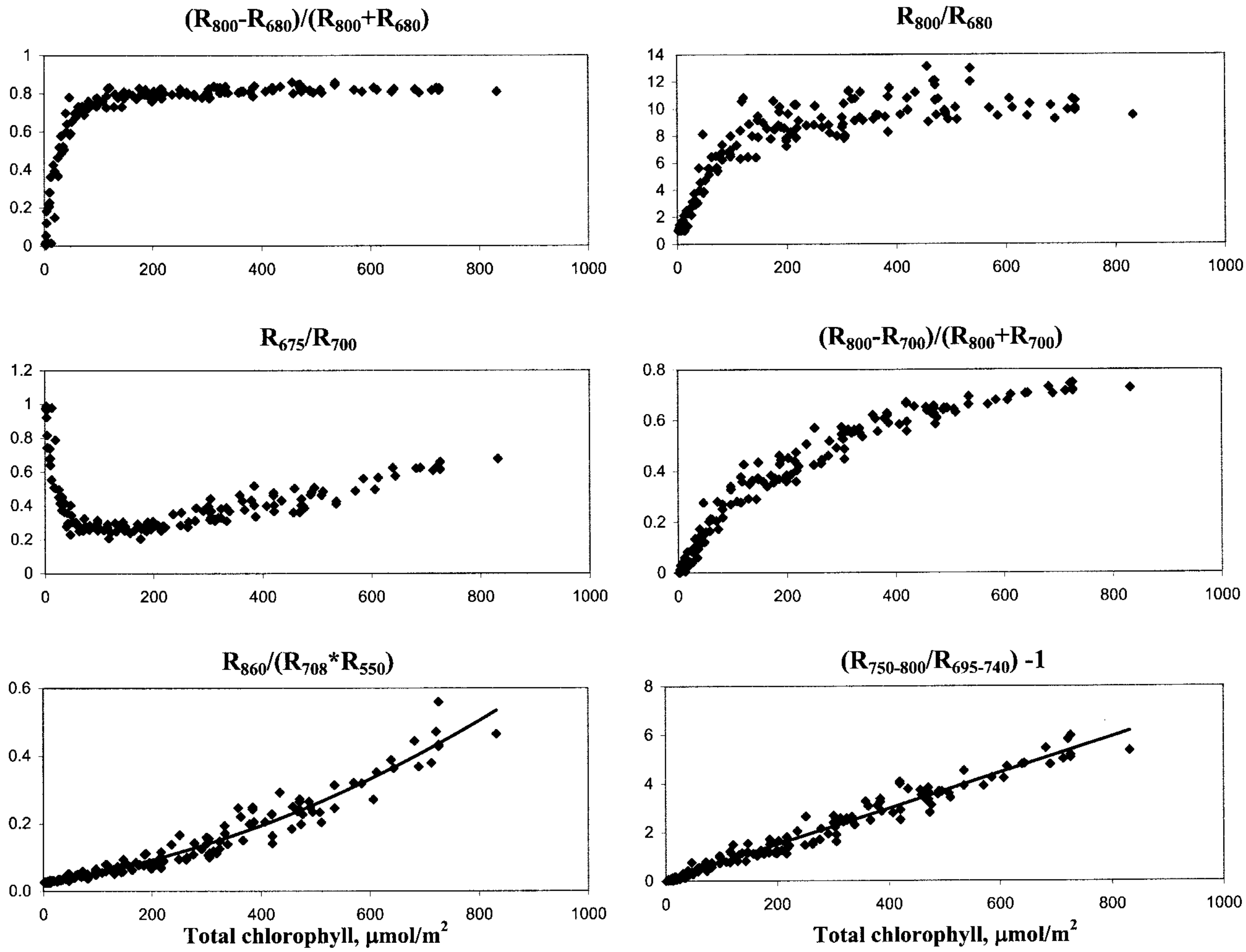

Figure 8. Scatter plots indicating the relationship between various reflectance indices and total Chl content.

(R_{675}/R_{700}) – Chappelle et al. 1992,
$(R_{800}-R_{700})/(R_{800}+R_{700})$ – Gitelson and Merzlyak 1994 a and b,
$(R_{860}/R_{708} * R_{550})$ – Datt 1998,
$(R_{750-800})/(R_{695-740})$-1 – this paper.

Indices that used reflectance in the red range around 680 nm were sensitive to only very low Chl and non-sensitive to moderate to high Chl. Thus, the index $(R_{800}-R_{680})/(R_{800}+R_{680})$ became invariant with respect to Chl > 100 µmol/m^2 (Fig. 8 A), and the index (R_{800}/R_{680}) became insensitive to Chl > 200 µmol/m^2 (Fig. 8 B).

The index R_{675}/R_{700} decreased sharply with an increase in total Chl up to 70 µmol/m^2, then, it was almost invariant with respect to Chl between 70 and 200 µmol/m^2 and had a quite linear relationship with Chl higher than 200 µmol/m^2. The indices $[R_{800}-R_{700}]/[R_{800}+R_{700}]$, $[R_{860}/R_{708} * R_{550}]$ and $[(R_{750-800})/(R_{695-740})$-1] were much better indicators of Chl content. The index $[R_{800}-R_{700}]/[R_{800}+R_{700}]$ was shown to be a good predictor of Chl for a variety of plant species (Gitelson and Merzlyak 1994 a, b, Gamon and Surfus 1999, Richardson et al. 2002, Sims and Gamon 2002), however, the sensitivity of the index to high total Chl (more than 400 µmol/m^2) was four-fold lower than to low-to-moderate Chl. In the range of total Chl variation from 1 to 830 µmol/m^2, indices $[R_{800}-R_{700}]/[R_{800}+R_{700}]$ and $[R_{860}/R_{708} * R_{550}]$ provided estimation of total Chl with a RMSE < 80 µmol/m^2. Indices with broad spectral bands $[(R_{750-800})/(R_{695-740})$-1] and $[(R_{750-800})/(R_{520-585})$-1], proposed in this work were the best Chl predictors with a RMSE < 39 µmol/m^2 (Table 3).

Three spectral bands, either 550 ± 20 nm or 715 ± 20 nm, 450 ± 20 nm, and NIR band above 750 nm were found to be sufficient for non-destructive Chl estimation. For anthocyanin-containing leaves (see Gitelson et al. 2001), spectral bands 715 ± 20 nm, 450 ± 20 nm and a NIR band above 750 nm are recommended. The use of wide band filters in reflectometers for Chl estimation allows for a significant increase in sensitivity and the signal-to-noise ratio and decreases the cost of the reflectometers.

Table 3. Root mean square error of total Chl, Chl *a* and Chl *b* estimation calculated for 142 maple, chestnut, beech and wild vine leaves included in the model development data set. Total Chl content ranged between 1 and 832 μmol/m^2, Chl *a* from 1 to 620 μmol/m^2 and Chl *b* from 1 to 210 μmol/m^2.

RMSE	$(R_{800}-R_{680})/(R_{800}+R_{680})$	R_{780}/R_{680}	R_{675}/R_{700}	$(R_{800}-R_{700})/(R_{800}+R_{700})$	$R_{860}/(R_{708}*R_{550})$	$(R_{750-800}/R_{695}-740)$-1
Chl *a*+*b*	168	139	63*	80	60	39
Chl *a*	123	102	49*	58	47	29
Chl *b*	46	39	20*	24	16	14

* The RMSE of Chl estimation in the range of total Chl > 200 μmol/m^2, Chl *a* > 148 μmol/m^2 and Chl *b* > 60 μmol/m^2.

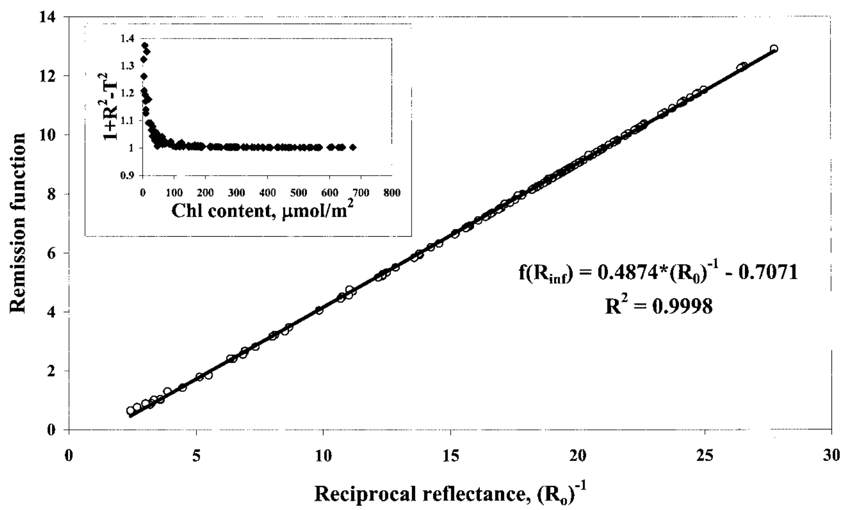

Figure 9. Remission function $f(R_\infty)$ plotted versus reciprocal reflectance $(R_0)^{-1}$. Linear best-fit function describes this relationship with $r^2 > 0.9998$. **In insert:** numerator of equation 2 (Appendix 1) is very close to unity in a wide range of total Chl. Both R^2 and T^2 decrease virtually synchronously with an increase in Chl content.

Appendix 1

The relationship between the infinite reflectance of an ideal layer, R_∞, in which a further increase in thickness results in no noticeable difference, and inherent optical properties (absorption coefficient, *k*, and scattering coefficient, *s*) is as following (e.g., Kortüm 1969):

$$f(R_\infty) = (1 - R_\infty^2)/2R_\infty = k/s \quad (1)$$

To find the relationship between reflectance of a real leaf and its inherent optical properties, one should find a relationship between $f(R_\infty)$ and the measured leaf reflectance. In our work, a leaf was placed on a black velvet background, and the reflectance was measured using an integrating sphere. In terms of the Kubelka-Munk theory, the measured reflectance is defined as R_o where the index ‹0› is used to designate the ideal black background with reflectance of the background equal to 0. The relationship between R_0 and $f(R_\infty)$ may be retrieved from the equation (Kortüm 1969, p. 120):

$$f(R_\infty) = (1 + R_o^2 - T^2)/2R_o - 1 \quad (2)$$

where T is leaf transmittance.

The numerator $(1 + R_o^2 - T^2)$ is very close to unity (insert in Fig. 9), and $f(R_\infty)$ is the hyperbolic function of R_o, therefore, $f(R_\infty) \propto (R_o)^{-1}$. For R_o ranging from 0 to 50%, the relationship between $f(R_\infty)$ and $(R_o)^{-1}$ is linear, $f(R_\infty) = 0.4874*(R)^{-1} - 0.707$ with $r^2 = 0.9998$ (Fig. 9).

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