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Non-Destructive Assessment of Chlorophyll Carotenoid and Anthocyanin Content in Higher Plant Leaves: Principles and Algorithms

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Non-destructive assessment of chlorophyll, carotenoid and anthocyanin content in higher plant leaves: Principles and algorithms

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Summary

Leaf pigment content provides valuable information about the physiological status of plants. Reflectance measurement makes it possible to quickly and non-destructively assess, *in situ*, the pigment content in leaves. Our objective was to investigate the spectral behavior of the relationship between reflectance and pigment content (total chlorophyll, carotenoids and anthocyanins) and to develop techniques for non-destructive pigment estimation in leaves using reflectance in a few spectral bands. Spectral reflectance of leaves of several non-related plant species from different climatic regions 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 from 695 to 705 nm related closely to the total pigment content (chlorophylls + carotenoids) in leaves of all species. Subtraction of near infra-red reciprocal reflectance, $(R_{NIR})^{-1}$, from $(R_\lambda)^{-1}$ made the index $[(R_\lambda)^{-1}-(R_{NIR})^{-1}]$ linearly proportional to the total chlorophyll content in spectral ranges λ from 525 to 555 nm and from 695 to 725 nm with a coefficient of determination $r^2 > 0.94$. To adjust for differences in leaf structure and thickness, the product of the latter index and NIR reflectance $[(R_{NIR}/R_\lambda)-1]$ 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.

The sensitivity of reciprocal reflectance to carotenoid content was maximal in a spectral range around 510 nm; however, chlorophylls also affect reflectance in this spectral range. To remove the chlorophyll effect from reflectance at 510 nm, a reciprocal reflectance at either 550 nm or 700 nm, which is linearly related to the chlorophyll content, was used. Indices in the form $[(R_{510})^{-1}-(R_{550})^{-1}]$ and $[(R_{510})^{-1}- (R_{700})^{-1}]*R_{NIR}$ were used for carotenoids estimation.

The main spectral feature of anthocyanin absorption *in vivo* was a peak around 550 nm where chlorophylls also absorb. To remove the chlorophyll effect from reflectance at 550 nm, a reciprocal reflectance in the range 700-710 nm was used. An index in the form $[(R_{550\text{-}570})^{-1}-(R_{700\text{-}710})^{-1}]*R_{NIR}$ allowed for an accurate estimation of anthocyanin accumulation, even in minute amounts, in intact senescing and stressed leaves.

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Introduction

The content of chlorophylls (Chl), carotenoids and anthocyanins, main pigments of a leaf, provides valuable information about plant physiological status. The chlorophylls 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, Chl content can directly determine photosynthetic potential and primary production (e.g., Curran *et al.* 1990). In addition, Chl gives an indirect estimation of the nutrient status because much of leaf nitrogen is incorporated in chlorophyll (e.g., Filella *et al*. 1995; Moran *et al*. 2000). Furthermore, leaf chlorophyll content is closely related to plant stress and senescence (e.g., Hendry 1987; Merzlyak and Gitelson 1995; Peñuelas and Filella 1998; Merzlyak *et al*. 1999).

Several specific physiological functions have been attributed to carotenoids because of their unique physicochemical and photophysical properties: a structural role in the organization of photosynthetic membranes, participation in light harvesting, energy transfer, quenching of chlorophyll excited states and singlet oxygen, and interception of deleterious free oxygen and organic radicals (e.g., Demmig-Adams *et al* 1996; Young and Britton 1990). The retention of carotenoids in the progress of Chl breakdown (Biswall, 1995) has been suggested as a mechanism of photoprotection during leaf senescence (Merzlyak and Gitelson 1995). The changes of leaf carotenoids content and their proportion to Chl are widely used for diagnosing the physiological state of plants during development, senescence, acclimation and adaptation to different environments and stresses (e.g., Demmig-Adams *et al.* 1996; Young and Britton 1990).

Anthocyanins (Anth) are water-soluble vacuolar pigments of higher plants abundant in juvenile and senescing plants. Anthocyanins are responsible for red coloration of plant tissues. Very often in leaves, a significant accumulation of anthocyanins is induced as a result of a number of environmental stresses such as strong light, UV-B-irradiation, low temperature, drought, wounding, bacterial and fungal infections, nitrogen and phosphorus deficiencies, certain herbicides and pollutants (e.g., Chalker-Scott, 1999). Some lines of evidence suggest that anthocyanins are able to protect plants against harmful effects induced by UV-radiation (Chalker-Scott, 1999) and visible light (Gould *et al.* 1995; Merzlyak and Chivkunova 2000). Since anthocyanins may serve as indicators of leaf senescence and stress in many plant species, their detection and quantitative assessment can provide important information about response and adaptation of plants to environmental stresses.

Traditionally, leaf extraction with organic solvents and spectrophotometric determination in solution is required for pigment analysis with wet chemical methods. Recently, alternative solutions of leaf pigment analysis with non-destructive optical methods have been developed (*chlorophylls:* Aoki *et al.* 1986; Buschmann and Nagel 1993; Gitelson and Merzlyak 1994a,b; 1996; 1977; Markwell *et al.* 1995; Gitelson *et al.* 1996; Lichtenthaler *et al.* 1996; Schepers *et al.* 1996; Blackburn 1998; Datt 1998; Gamon and Surfus 1999; Curren *et al.* 2001; Sims and Gamon 2002; Richardson *et al.* 2002; *carotenoids:* Thomas and Gausman 1977; Gamon *et al.* 1990; Blackburn 1998; Datt 1998; Gamon and Surfus 1999; Merzlyak *et al.* 1999; Zur *et al.* 2000; Sims and Gamon 2002; Gitelson *et al.* 2002; *anthocyanins*: Curran *et al.* 1991; Gamon and Surfus 1999; Gitelson *et al.* 2001; Sims and Gamon 2002). These newer methods are non-destructive, inexpensive, quick and now possible in the field.

The goal of this paper is to present new approaches to estimate total chlorophyll, carotenoids and anthocyanins content in higher plant leaves using reciprocal reflectance in a few spectral bands (Gitelson *et al.* 2001; 2002; 2003). The relationships between reflectance and pigment content were established and quantitative techniques for pigment estimation in leaves of

different non-related species with a wide range of pigment content and composition were developed. The first step was to find spectral ranges where total pigment content related closely to reciprocal reflectance $(R_\lambda)^{-1}$. Then, for total chlorophyll estimation, reciprocal reflectance in spectral bands with a minimal effect of carotenoids (540 to 570 nm and 695 to 740 nm) was used. For carotenoids and anthocyanin estimation, spectral bands sensitive to the content of these pigments were found (around 510 nm for carotenoids and 550 nm for anthocyanins) and a technique was developed to remove the chlorophyll contribution from reflectance in these regions. Finally, algorithms were validated by independent data sets.

Materials and Methods

For development of algorithms for Chl and carotenoids assessment Anth-free juvenile, mature and senescent leaves collected in 1992-2001 were used: Norway maple (*Acer platanoides* L.) and horse chestnut (*Aesculus hippocastanum* L.) leaves were from a park at Moscow State University, second-flush beech leaves (*Fagus sylvatica* L.) and wild vine shrub leaves (*Parthenocissus tricuspidata* L.) from the University of Karlsruhe campus. Maize leaves were collected in Sheldon, Nebraska, USA.

Anth-containing leaves of Norway maple, cotoneaster (*Cotoneaster alaunica* Golite) and dogwood (*Cornus alba* L. (*Swida alba* (L.) Opiz)) were collected in a park at Moscow State University in spring and fall. In maple, red pigmentation was especially expressed during cold seasons. Usually anthocyanin pigmentation was observed in sunlit leaves, whereas shaded leaves were green to yellow. *Pelargonium zonale* L'Herit (ex Soland) leaves were taken at Sede-Boker Campus of Ben-Gurion University of the Negev (Israel); green and red zones affording paired comparisons within the same leaf were used. Leaves healthy and homogeneous in color and without 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. Anthocyanin content was determined after extract acidification with concentrated HCl. Absorbance at 530 nm has been corrected for pheophytin contribution: pheophytins *a* and *b* were obtained from corresponding chlorophylls (Fluka Chemie AG) and their absorption coefficients at 530 nm in acid methanol were found to be 8.17 and 6.35 $mM^{-1}cm^{-1}$, respectively. Anth absorption coefficient of 30 $mM^{-1}cm^{-1}$ at 530 nm (21) was used. Pigment content was expressed on a leaf area basis. The methods are described in detail elsewhere (Gitelson and Merzlyak 1994a; 1994b; Gitelson *et al.* 2001; 2002a).

We used the SPAD 502 (Minolta Camera Co., Osaka, Japan) to estimate Chl content in maize leaves. SPAD has a 0.06-cm^2 measurement area, and calculates an index in "SPAD units" based on absorbance at 650 nm and 940 nm. Six separate measurements with SPAD were made on each leaf; the arithmetic mean of those measurements was used for all subsequent analyses.

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.

Spectral reflectance of maize leaves at wavelengths from 306 to 1138 nm was measured using a UniSpec Spectral Analysis System (PP Systems, Haverhill, Massachusetts, USA) with a 2.3-mm diameter (0.042 cm^2) foreoptic and an internal 6.8W halogen lamp. A Spectralon reflectance standard was scanned before every new leaf sample and scans were corrected for instrument's dark current. The reflectance spectrum for each scan was calculated as a ratio of leaf radiance to standard radiance at wavelength λ. Six separate measurements were made in a 1-cm diameter area and average of those spectra was used for subsequent analyses.

Results and Discussion

Reflectance versus total pigment content

To study the effect of chlorophylls and carotenoids on reflectance spectra, leaves were separated into several groups with different pigment content (Fig. 1A). The first and second groups can be considered to represent an advanced process of stress or senescence, when leaf color turns from completely yellow to yellowish green. Leaf groups 4 and 5 corresponded to different stages of stress and/or senescence when they were still green, but the suppression of biosynthesis and/or the increased degradation of the green pigments already had begun. Groups 6 and 7 represented green (both juvenile and mature) leaves at different stages of their development; this range ended with dark-green leaves represented by group 7.

In yellow leaves, reflectance was governed solely by carotenoids (Fig. 1B). In the blue range, reflectance was low (below 10%). A sharp increase in reflectance took place at 550 nm; at longer wavelengths, reflectance remained practically non-changeable in a wide spectral range including NIR. An increase in the total pigment content (groups 2 to 4) led to a slight decrease of reflectance in the blue range and a significant decrease in the green and especially in the red. A further increase in leaf greenness (groups 5 to 7) did not change the reflectance in the blue and the red. The only spectral ranges sensitive to pigment variation were between 510 nm to 650 nm and in the red edge around 700 nm.

The relationship between the infinite reflectance of an ideal layer, R_∞, in which a further increase in thickness results in no noticeable difference, and reflectance (R_0) and transmittance (T) of a real leaf measured against black background is as following (e.g., Kortüm 1969):

$$f(R_\infty) = (1+R_0^2-T^2)/2R_0 \propto k/s$$

Here k is absorption coefficient and s is scattering coefficient. For R_0 ranging from 0 to 50%, the relationship between $f(R_\infty)$ and $(R_0)^{-1}$ is linear, $f(R_\infty) = 0.4874*(R_0)^{-1}-0.707$ with a coefficient of determination $r^2=0.9998$ (Gitelson *et al*. 2003). Thus, reciprocal reflectance is closely related to ratio of leaf inherent optical properties, absorption and scattering coefficients, $(R_0)^{-1} \propto k/s$.

In the blue range, the spectra of reciprocal reflectance were fairly sensitive to the variation in pigment content between the leaf groups studied (Fig. 1C). Considerable changes also occurred in the spectral ranges of the green edge around 510-520 nm and the red edge around 700 nm. An increase in pigment content led to an increase in the reciprocal reflectance and to a shift of both green and red edges toward longer wavelengths.

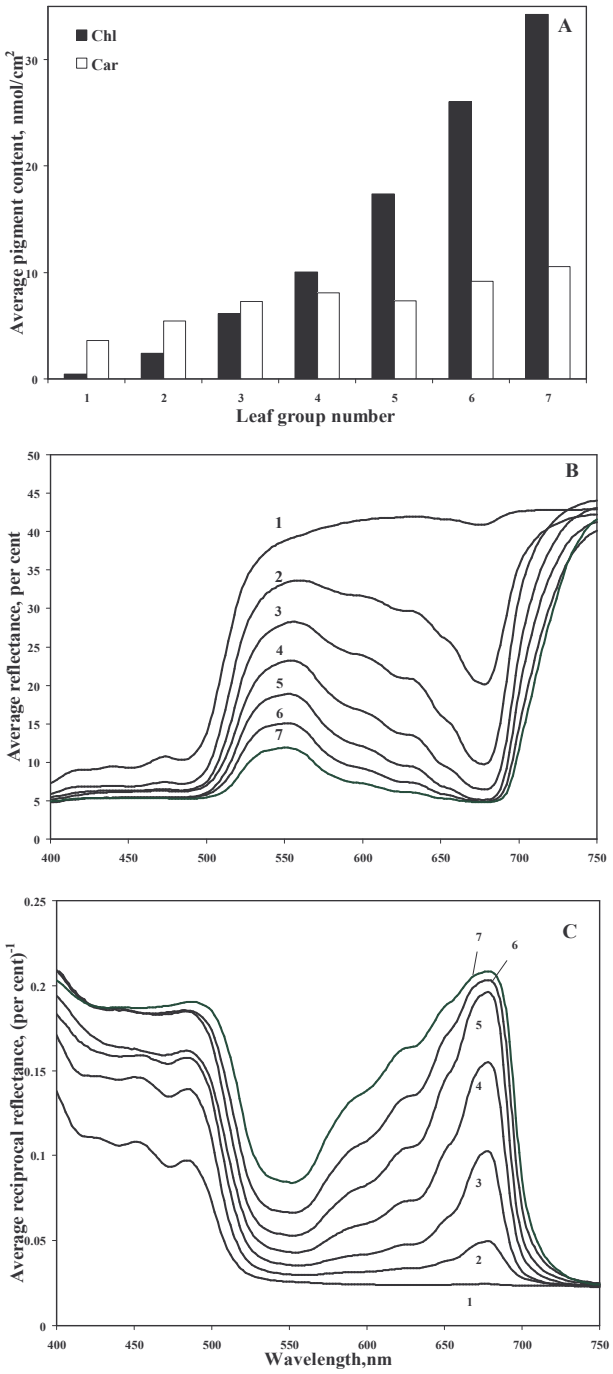

Figure 1. *Pigment content, reflectance and reciprocal reflectance spectra of different maple leaf groups. (a) Average total chlorophyll and carotenoids content in groups of yellow (group 1) to dark-green (group 7) leaves. (b) Average reflectance spectra for groups of yellow (group 1) to dark-green (group 7) leaves. (c) Average reciprocal reflectance spectra for groups of yellow (group 1) to dark-green (group 7) leaves.*

The first leaf group is best suited to evaluate general optical properties of carotenoids and to find their specific spectral features inherent in the reflectance, R_λ, and reciprocal reflectance, $(R_\lambda)^{-1}$. The second leaf group allowed us to understand how Chl affected absorption in this range. In this group, carotenoids increased slightly compared to the first group, whereas, Chl increased six-fold. The increase in Chl manifested itself as a sharp increase of $(R_\lambda)^{-1}$ around 680 nm. A significant increase in $(R_\lambda)^{-1}$ also occurred in the green edge range 480 to 510 nm; this range is a transition zone between the range with strong absorption by Chl and carotenoids (in the range 400 to 480 nm) and the green range (around 550 nm) where pigment absorption is weaker and relates mainly to the Chl content. Reciprocal reflectance $(R_\lambda)^{-1}$ in the green edge range related to content of both pigments, carotenoids and Chl (Fig. 2). Between 400 and 500 nm, the relationship $(R_\lambda)^{-1}$ vs. pigment content was essentially nonlinear; at 505 nm, the relationship became linear with a high sensitivity of $(R_\lambda)^{-1}$ to the total pigment content. Thus, reciprocal reflectance in a wide range from 505 to 550 nm, $(R_{505\text{-}550})^{-1}$, was found to be an accurate measure of the total pigment (chlorophylls+carotenoids) content.

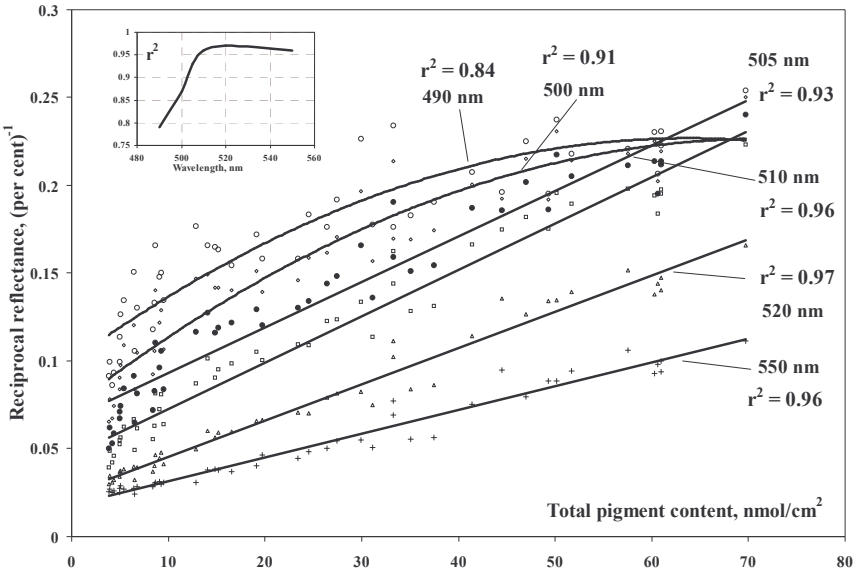

Figure 2. *Reciprocal reflectance in the range 490 nm to 550 nm plotted versus total pigment content in maple leaves. At wavelengths 490 and 500 nm, the relationship $(R_\lambda)^{-1}$ vs. [Chl+Car] was essentially non-linear. At 505 nm, the relationship became linear with a high sensitivity of $(R_\lambda)^{-1}$ to the pigment content. It remained linear in the range 505 to 550 nm with a decrease in the slope at longer wavelengths.* ***Insert:*** *the coefficient of determination for the linear relationship $(R_\lambda)^{-1}$ vs. [Chl+Car]. In the range 510 to 550 nm, r^2 was more than 0.96.*

Chlorophyll content estimation

At 550 nm and beyond, absorption by carotenoids is negligible (Fig. 1C, see also Zur *et al*. 2000); thus, we hypothesized that in the spectral range beyond 550 nm, reciprocal reflectance might be sensitive to Chl content solely. The relationship $(R_\lambda)^{-1}$ vs. total Chl showed two main spectral features (Fig. 3):

1. In the main bands of pigment absorption, the blue and the red, the relationship was essentially non-linear. $(R_\lambda)^{-1}$ was sensitive to Chl < 15 nmol/cm^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 and depended strongly on mean pigment content (Gitelson *et al.* 2003). In the green and red edge, the slopes were nearly the same for all species studied in a wide range of Chl content from 0.1 to 83 nmol/cm^2. In these spectral ranges, reciprocal reflectance was an accurate measure of Chl content, and the RMSE of the Chl estimation had minimal values.

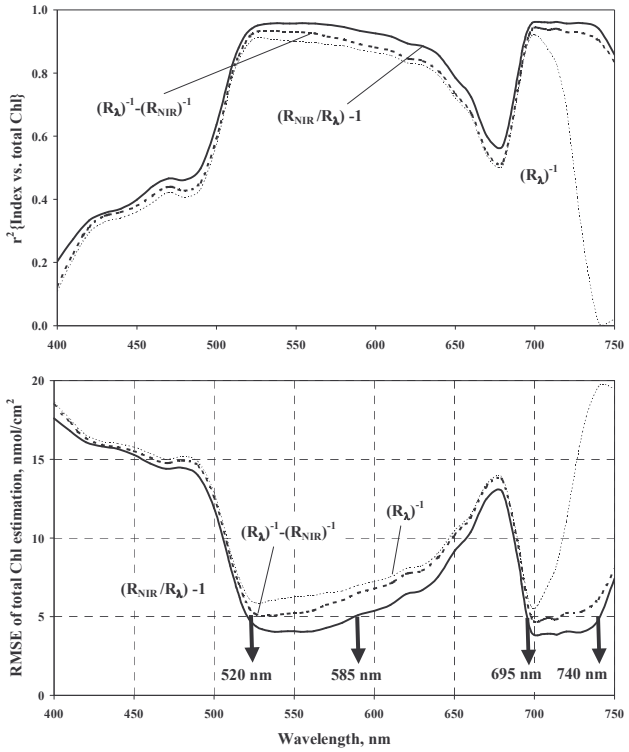

Figure 3. *(A) The coefficient of determination r^2 of the linear relationship index vs. total Chl, and (B) RMSE of total Chl estimation by three indices proposed for Chl assessment: $(R_\lambda)^{-1}$, $[(R_\lambda)^{-1}-(R_{NIR})^{-1}]$ and $(R_{NIR}/R_\lambda) - 1$. Spectral ranges where RMSE of Chl assessment dropped below 5 nmol/cm^2 are shown.*

For leaves containing trace amounts of Chl, reciprocal reflectance remained significantly above zero (Fig. 4). In the range beyond 550 nm, the intercept $(R_\lambda)^{-1}$ vs. Chl was almost equal to $(R_{NIR})^{-1}$ (not shown). To make the index linearly proportional to total Chl content, we subtracted the reciprocal reflectance in the NIR range, $(R_{NIR})^{-1}$, from $(R_\lambda)^{-1}$. 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 (Fig. 3B). The RMSE of Chl estimation by indices

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

was below 6 nmol/cm^2 (Fig. 3B).

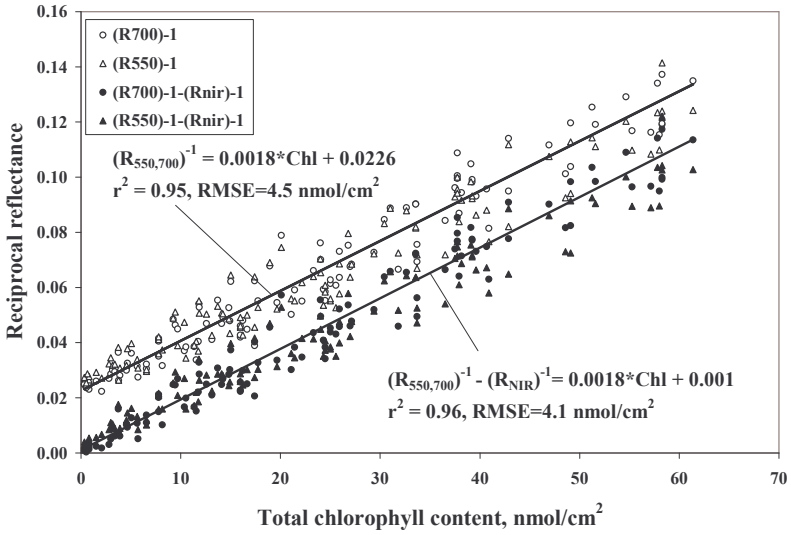

Figure 4. *Reciprocal reflectance at 550 and 700 nm, $(R_{550,700})^{-1}$, and the difference $(R_{550,700})^{-1}$-$(R_{750-800})^{-1}$ versus total chlorophyll content in maple, beech and chestnut leaves. Solid lines are best-fit functions. Subtraction of $(R_{750-800})^{-1}$ from $(R_{550})^{-1}$ and $(R_{700})^{-1}$ significantly (more than 20-fold) decreased the intercept; function $(R_{550})^{-1}$-$(R_{750-800})^{-1}$ and $(R_{700})^{-1}$-$(R_{750-800})^{-1}$ became linearly proportional to the Chl content with r^2 higher than 0.95 and RMSE of Chl estimation < 4.1 nmol/cm^2.*

To adjust for differences in leaf structure and thickness, the product of the index $[(R_\lambda)^{-1}$-$(R_{NIR})^{-1}]$ and R_{NIR} was used (Gitelson et al. 2003). It made it possible a further increase in the accuracy of Chl estimation:

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

The Chlorophyll Reflectance Index, (Chl)RI, is slightly different from R_{NIR}/R_{550} and R_{NIR}/R_{700}, suggested by Gitelson and Merzlyak (1994a; b). Importantly, however, the intercept of the function (R_{NIR}/R_λ)-1 vs. Chl was close to zero, which made the index linearly proportional to the Chl content. The RMSE of Chl estimation by the indices

$$(Chl)RI_{green} = (R_{750-800}/R_{520-585})-1$$
$$(Chl)RI_{red\ edge} = (R_{750-800}/R_{695-740})-1$$

was below 5 nmol/cm^2.

Leaf surface reflectance virtually did not change in leaves studied, so proposed indices worked accurately across the species. In the case when surface reflectance varies, incorporating reflectance in the blue range made 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:

$$(Chl)RI_{green} = [(R_{750-800}-R_{430-470})/(R_{520-580}-R_{440-480})]-1$$
$$(Chl)RI_{red\ edge} = [(R_{750-800}-R_{430-470})/(R_{695-740}-R_{440-480})]-1$$

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 Fig. 5, relationships between total chlorophyll content and the following indices are presented: $(R_{800}-R_{680})/(R_{800}+R_{680})$ and (R_{800}/R_{680}) – Blackburn (1998); (R_{675}/R_{700}) – Chappelle *et al.* (1992); $(R_{800}-R_{700})/(R_{800}+R_{700})$ – Gitelson and Merzlyak (1994a and b); $(R_{860}/R_{708}*R_{550})$ – Datt (1998); $(R_{750-800})/(R_{695-740})$ - 1 – this paper. In the range of total Chl variation from 0.1 to 83 nmol/cm^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 < 8 nmol/cm^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 < 3.9 nmol/cm^2.

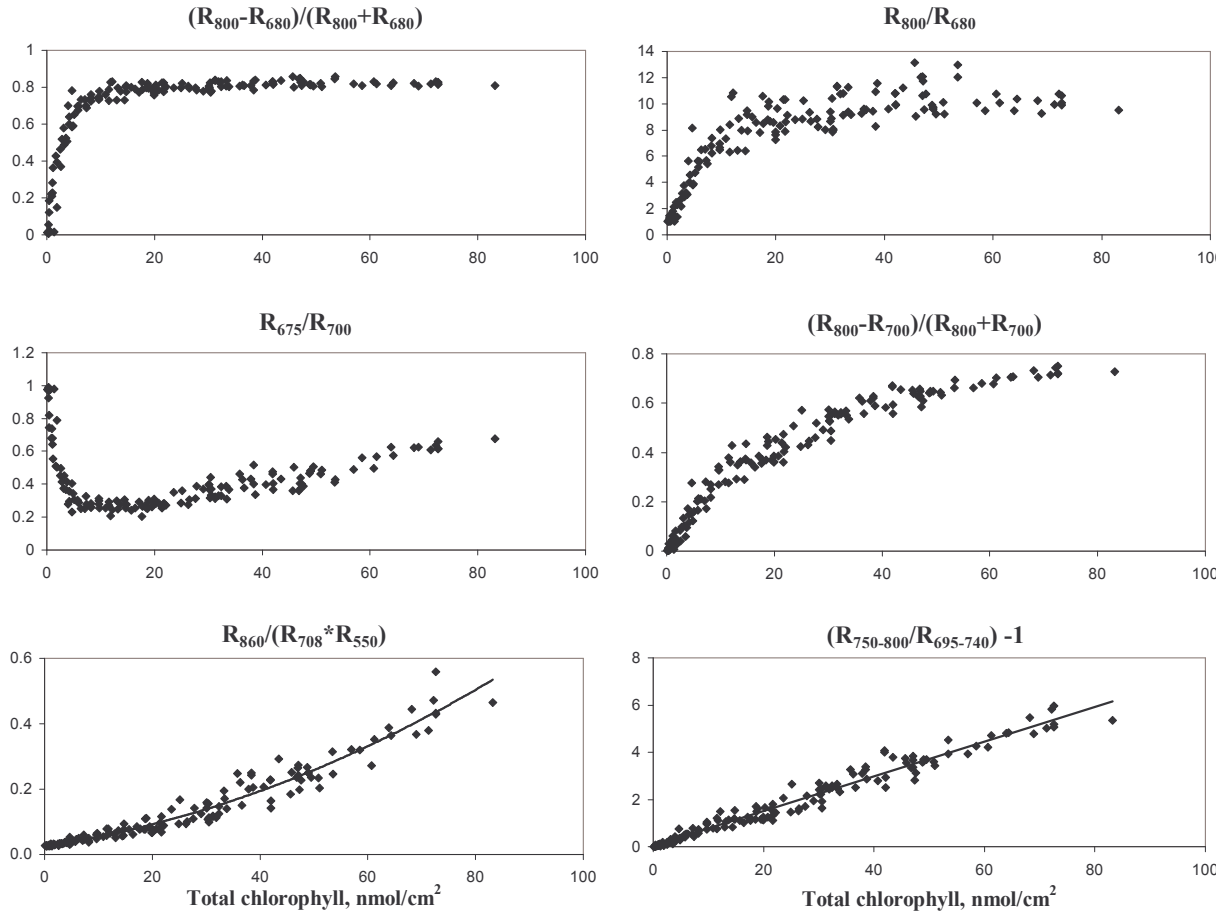

Figure 5. *Scatter plots indicating the relationship between various reflectance indices and total Chl content.*

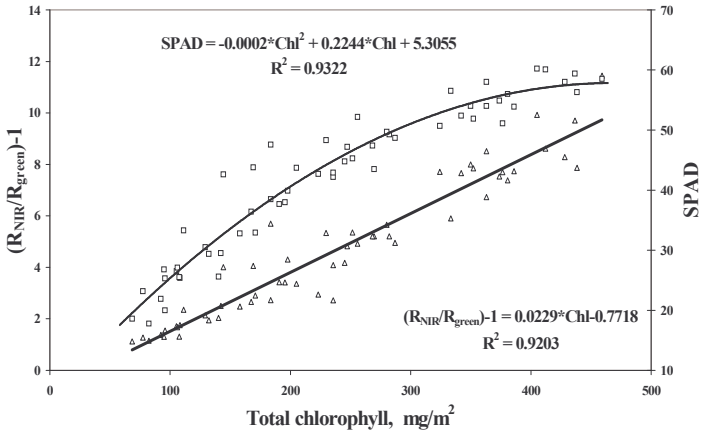

Figure 6. Chlorophyll assessment by SPAD and proposed index. Reflectance was measured by UniSpec with a clip.

Performance of $(Chl)RI_{green}$ index and SPAD chlorophyll meter was compared for maize leaves (Figure 6). SPAD reading linearly related to analytically measured Chl below 300 mg/m^2. When Chl content exceeded 350 mg/m^2, SPAD reading leveled off and became less sensitive to Chl. Index $(Chl)RI_{green}$ as well as $(Chl)RI_{red\ edge}$ (not shown) had fairly linear relation with analytically measured Chl in whole range of its variation.

Carotenoids content estimation

The Chl and carotenoids content in leaves were strongly dependent. In the yellow to dark-green maple leaves studied, $r^2\{[Car]$ vs. $[Chl]\}$ was higher than 0.81. Thus, to find a spectral band sensitive to the carotenoids content, one should analyze spectral properties of leaves with slightly dependent carotenoids and Chl. Fig. 6A shows the relationships $r^2\{(R_\lambda)^{-1}$ vs. $[Chl]\}$ and $r^2\{(R_\lambda)^{-1}$ vs. $[Car]\}$ for yellow to yellowish-green leaves (a total Chl content < 10 nmol/cm^2; groups 1-3 in Fig. 1A). For these leaves, $r^2\{[Car]$ vs. $[Chl]\} < 0.45$ and their spectral characteristics were governed mainly by carotenoids. Figure 6B shows $r^2\{(R_\lambda)^{-1}$ vs. $[Chl]\}$ and $r^2\{(R_\lambda)^{-1}$ vs. $[Car]\}$ for yellow to green leaves (groups 1-5 in Fig. 1A) with the total Chl < 20 nmol/cm^2; for these leaves, $r^2\{[Car]$ vs. $[Chl]\} < 0.26$. Spectral characteristics of the leaves were governed by both carotenoids and Chl. The behavior of r^2 was generally the same as for yellow to yellowish-green leaves, with maximal sensitivity of $(R_\lambda)^{-1}$ to carotenoids in the range around 510 nm. In Fig. 6C, $r^2\{(R_\lambda)^{-1}$ vs. $[Chl]\}$ and $r^2\{(R_\lambda)^{-1}$ vs. $[Car]\}$ for yellow to dark-green leaves (0.14 < total Chl < 54 nmol/cm^2) are shown. High $r^2\{(R_\lambda)^{-1}$ vs. $[Chl]\}$ values (>0.9) in the range 505 to 550 nm indicated a strong contribution of Chl to $(R_\lambda)^{-1}$.

Thus, $(R_\lambda)^{-1}$ was maximally sensitive to carotenoids content in the range 500 to 520 nm; however, Chl contributed significantly to reflectance in this range (Fig. 7, see also Lichtenthaller 1987; Merzlyak *et al.* 1999; Zur *et al.* 2000). Therefore, the challenge was to find a way to accurately subtract the Chl contribution from the reflectance in the range around 510 nm and apportion the remainder to carotenoids. It is especially important for estimation of the early stages of plant stress or senescence when a process of green pigment degradation begins in still-green leaves and the Chl absorption is much higher than that of carotenoids.

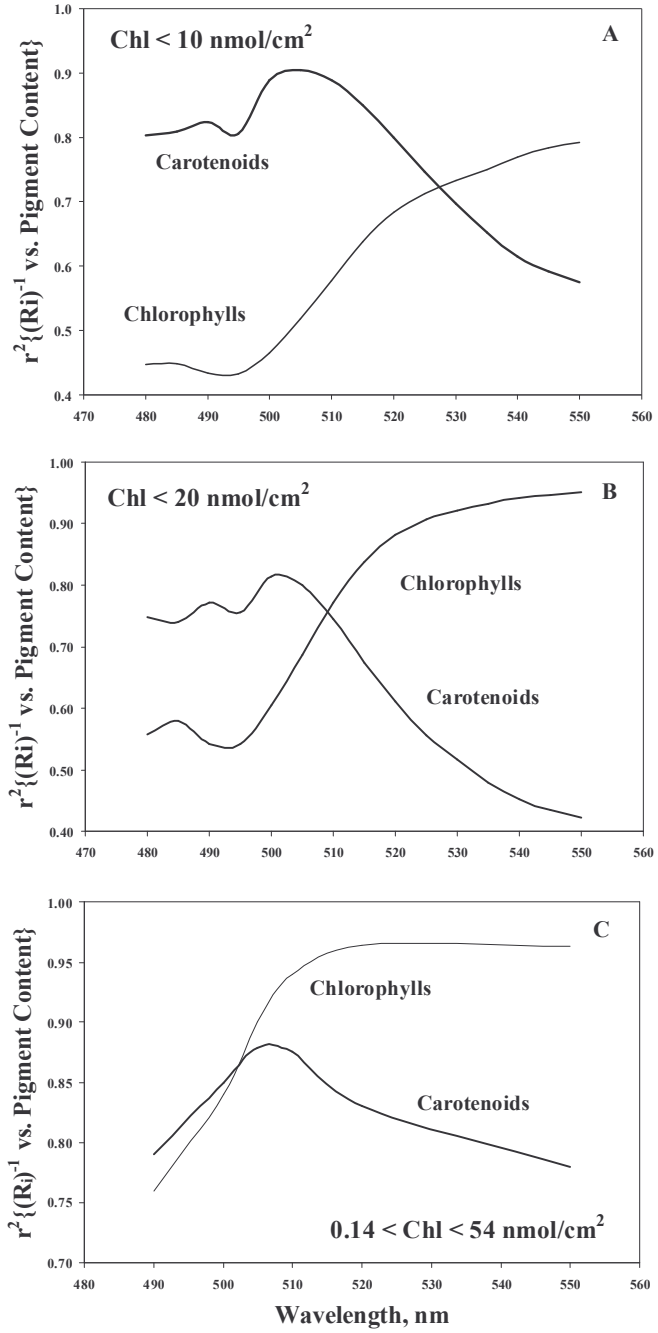

Figure 7. *The coefficient of determination for linear relationships $(R_\lambda)^{-1}$ vs. pigment content. (A) leaves with total Chl content < 10 nmol/cm^2; (B) leaves with total Chl content < 20 nmol/cm^2; (C) leaves with total Chl content ranging from 0.14 to 54 nmol/cm^2.*

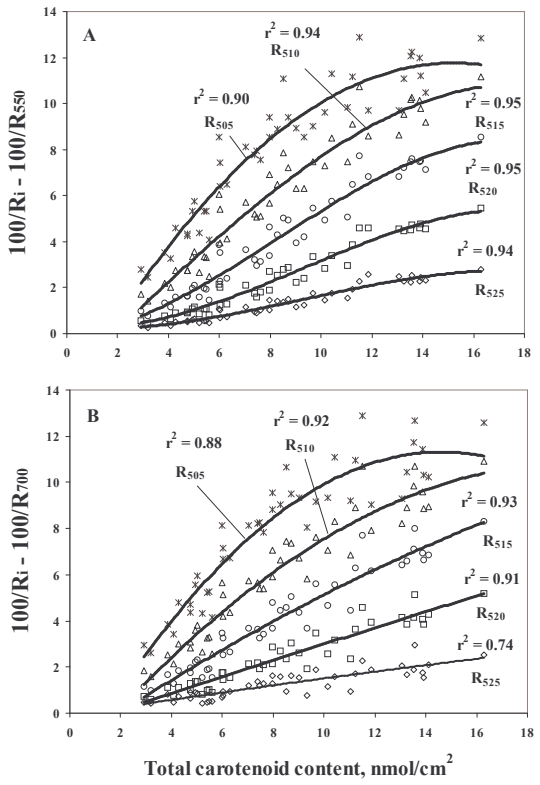

Figure 8. *Differences of reciprocal reflectance (A): $(R_\lambda)^{-1}$-$(R_{550})^{-1}$ and (B): $(R_\lambda)^{-1}$-$(R_{700})^{-1}$ versus total carotenoids content in maple leaves. R_λ is the reflectance in the range 505 to 525 nm. Solid lines represent best-fit functions.*

In the presence of trace amounts of Chl (< 0.3 nmol/cm^2) and considerable quantities of carotenoids (> 6 nmol/cm^2), there is no evidence of a carotenoids contribution to leaf absorption and reflectance at 550 nm; reflectance at 550 nm and longer wavelengths is governed by Chl absorption solely (Fig. 1B and 1C, the first leaf group, see also Gitelson and Merzlyak 1996; Zur *et al.* 2000). As shown above, a close linear correlation exists between $(R_{550\text{-}570})^{-1}$ and [Chl] and between $(R_{700\text{-}710})^{-1}$ and [Chl]. Thus, to remove the Chl contribution from the reciprocal reflectance in the green edge range, we used either $(R_{550\text{-}570})^{-1}$ or $(R_{700\text{-}710})^{-1}$. The differences $(R_\lambda)^{-1}$-$(R_{550\text{-}570})^{-1}$ and $(R_\lambda)^{-1}$-$(R_{700\text{-}710})^{-1}$ were calculated in the range of λ from 505 to 525 nm (Fig. 8). The closest linear correlation between the differences and carotenoids content and maximal sensitivity to the carotenoids was achieved in the range around 510 nm, where the product of the slope and the coefficient of determination reached the maximum for both indices (Gitelson *et al.* 2001). To adjust for variability in leaf structure and thickness, R_{NIR} in the range 750 to 800 nm was incorporated into the index. Carotenoids Reflectance Indices (CRI) were suggested as an accurate measure of carotenoids content:

$$CRI_{green} = [(R_{510})^{-1}\text{-}(R_{550\text{-}570})^{-1}]*R_{750\text{-}800}$$
$$CRI_{red\ edge} = [(R_{510})^{-1}\text{-}(R_{700\text{-}710})^{-1}]*R_{750\text{-}800}$$

In Fig. 9, the relationships between CRI_{green} and both pigments are plotted. The determination coefficient for the polynomial function CRI_{green} vs. [Car] was $r^2 = 0.94$, and it was a little bit lower (r^2=0.92) for the linear function.

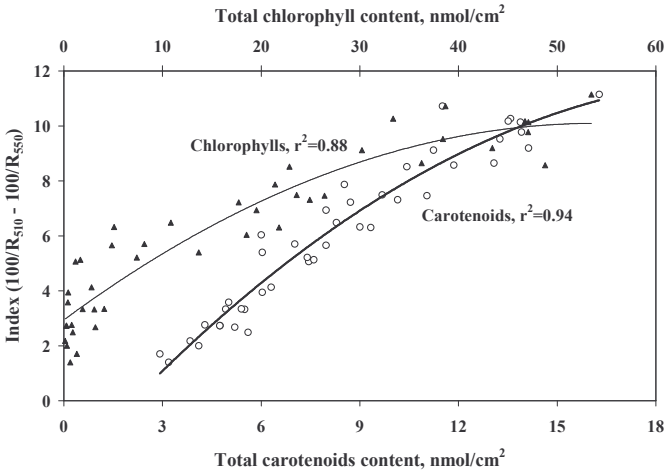

Figure 9. *The Carotenoid Reflectance Index, CRI = $(R_{510})^{-1}$-$(R_{550-570})^{-1}$, plotted versus total carotenoids and total chlorophyll content in maple leaves. The solid line is the best-fit function of CRI vs. carotenoids; the thin line is the best-fit function of CRI vs. Chl. The sensitivity of the CRI to the carotenoids content was much higher than to the chlorophyll content.*

Anthocyanin content estimation

To determine the effect of anthocyanin on reflectance, we compared reflectance spectra of leaves with almost the same Chl content but different anthocyanin content (Fig. 9). An increase in anthocyanin content resulted in lower reflectance in the green range (i.e., higher absorption), while reflectance in the blue, red and NIR remained virtually the same. Hence, that difference in reflectance of leaves in the green range could be attributed solely to anthocyanin absorption. Gitelson *et al.* (2001) found that *in vivo* anthocyanin absorption peak positioned around 550 nm, and its magnitude related closely to the anthocyanin content determined analytically. However, chlorophyll also strongly affects reflectance around 550 nm (Figs. 1-4); to retrieve anthocyanin content from reflectance, one should find a way to remove the contribution of Chl absorption from reflectance around 550 nm. To accomplish that, we used fundamental spectral features of leaves:

1. In leaves with minute quantities of anthocyanin, reflectance around 550 nm, $R_{550-570}$, is almost equal to reflectance in the range 700-710 nm, $R_{700-710}$, (Chappelle *et al.* 1992; Gitelson and Merzlyak 1994a,b; 1996; 1997), and both reciprocal reflectances $(R_{550-570})^{-1}$ and $(R_{700-710})^{-1}$ are closely related to Chl content (Figs. 3 and 4).

2. In anthocyanin-containing leaves, $(R_{700-710})^{-1}$ was closely (r^2 = 0.95, RMSE of Chl estimation < 3.7 nmol/cm^2) related to Chl content (Gitelson *et al.* 2001). Thus, anthocyanins did not affect leaf optical properties in the red edge range.

While $(R_{700-710})^{-1}$ depends solely upon Chl content, $(R_{550-570})^{-1}$ depends on both Chl and anthocyanins (Fig. 10). To remove the effect of Chl on reflectance around 550 nm, we subtracted $(R_{700-710})^{-1}$ from $(R_{550-570})^{-1}$. An Anthocyanin Reflectance Index (ARI) was proposed in the form (Gitelson *et al*. 2001):

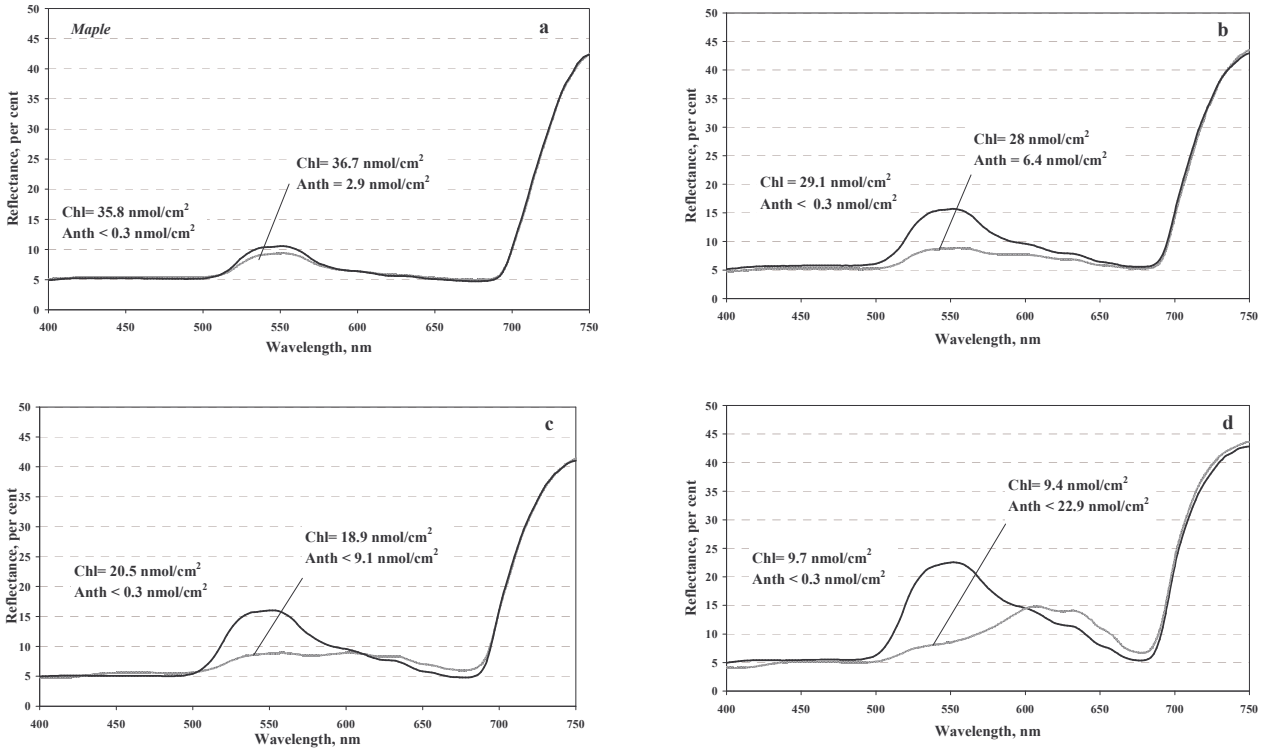

Figure 10. *Reflectance spectra of maple leaves with different anthocyanin content and almost the same chlorophyll content. The differences between reflectance of leaves could be attributed solely to anthocyanin absorption. An increase in anthocyanin content manifested itself in lower reflectance in the green range, while reflectance in the blue, red and NIR remained virtually the same.*

$$ARI = [(R_{550\text{-}570})^{-1} - (R_{700\text{-}710})^{-1}]*R_{750\text{-}800}$$

$R_{750\text{-}800}$ was incorporated into the index to adjust for variability in thickness and structure of leaves. The first term in brackets is responsible for both Chl and anthocyanins absorption and the latter term for Chl absorption. Fig. 11 compares the non-destructive estimate and the actually measured anthocyanin content in maple leaves. In the range of anthocyanin from 0.3 to 48 nmol/cm^2, RMSE of anthocyanin estimation was less than 3.9 nmol/cm^2.

Conclusion

Based on fundamental optical properties of the leaves and specific spectral features of chlorophylls, carotenoids and anthocyanins revealed in this study, indices for non-destructive estimation of pigment content in leaves were devised. Three spectral bands, either 550±20 nm or 715±20 nm, 450±20 nm and an NIR band above 750 nm, were found to be sufficient for total chlorophyll content estimation. For anthocyanin-containing leaves, spectral bands 715±20 nm, 450±20 nm and an NIR band above 750 nm are recommended. For carotenoids estimation, three

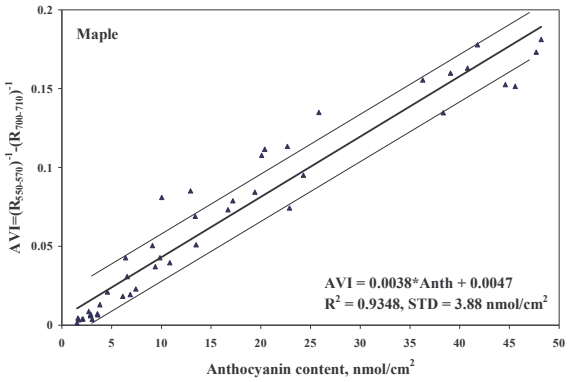

Figure 11. *Relationship between Anthocyanin Reflectance Index, ARI = $[(R_{550})^{-1} - (R_{700})^{-1}]$, and anthocyanin content for maple leaves with a wide range of pigment content and composition. The solid line represents the best-fit function, and the dotted lines represent root-mean square variation from best-fit function.*

spectral bands, 510±5 nm, either 550±15 nm or 700±7.5 nm and an NIR band above 750 nm are needed. Reflectances in two spectral bands, 550+/-15 nm and 705+/-5 nm, are sufficient to estimate anthocyanin content non-destructively.

Once the fundamental optical properties of chlorophylls, carotenoids and anthocyanins *in vivo* have been used, the same basic method would be applicable for other plant species. It should be stressed, however, that the applicability of the proposed algorithms to other plant species remains to be verified. Interestingly, the approaches described here are valid for estimation of chlorophylls, carotenoids and anthocyanin contents in apple fruit (Merzlyak *et al.* 2003).

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