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Optical Properties and Nondestructive Estimation of Anthocyanin Content in Plant Leaves¶

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ABSTRACT

Absorption and reflectance spectra of maple (*Acer platanoides*), cotoneaster (*Cotoneaster alaunica*), dogwood (*Cornus alba*) and pelargonium (*Pelargonium zonale*) leaves with a wide range of pigment content and composition were studied in visible and near-infrared spectra in order to reveal specific anthocyanin (Anth) spectral features in leaves. Comparing absorption spectra of Anth-containing and Anth-free leaves with the same chlorophyll (Chl) content, absorption spectra of Anth in leaves were derived. The main spectral feature of Anth absorption *in vivo* was a peak around 550 nm; the peak magnitude was closely related to Anth content. A quantitative nondestructive technique was developed to subtract Chl contribution to reflectance in this spectral region and retrieve Anth content from reflectance over a wide range of pigment content and composition. Anth reflectance index in the form ARI = $(R_{550})^{-1} - (R_{700})^{-1}$, where $(R_{550})^{-1}$ and $(R_{700})^{-1}$ are inverse reflectances at 550 and 700 nm, respectively, allowed an accurate estimation of Anth accumulation, even in minute amounts, in intact senescing and stressed leaves.

INTRODUCTION

Anthocyanins (Anth)† are water-soluble vacuolar pigments of higher plants abundant in juvenile and senescing plants. Anth are responsible for red coloration of plant tissues (1–6). Cyanidin (as the 3-glucoside) is the characteristic and dominating pigment in the leaf in all stages of its development; other Anth are less frequent (1,4). Very often, in plant leaves, significant accumulation of Anth 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 (1,3,6). Some lines of evidence suggest that Anth are able to protect plants against photoinhibition (5) and harmful effects induced by UV-radiation (1,6–8) and visible light (6,9). In addition some other protection mechanisms not involving photochemical reactions have been attributed to these pigments (6). Since Anth 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.

Anth, as well as other pigments, including chlorophylls (Chl) and carotenoids (Car) participate in light absorption in particular bands and can readily be assessed with absorption and reflectance spectroscopy. Spectral absorption and reflectance offer an alternative to destructive and time-consuming chemical analysis. Considerable progress in development of nondestructive methods for estimating physiological state of vegetation has been achieved during the last decade. While much attention was given to the estimation of Chl and Car content (10–18), not much is known about Anth estimation in intact leaves. The effect of red amaranthin on red edge position of leaf reflectance spectra was studied by Curran *et al.* (19). They found a strong effect of this pigment on the relationship between red edge position and Chl concentration. Gamon and Surfus (16) suggested using red (500–600 nm) to green (600–700 nm) reflectance ratio for Anth estimation. This Anth index worked well for newly emerging to mature oak leaves, when Anth content decreased and Chl content concurrently increased. However, it is not clear whether this index will be applicable and robust over a wide range of pigment composition, when Chl and Car contents in leaves change.

The absorption spectra of Anth-containing plant tissues indicate a strong overlapping of Anth, Chl and Car absorption (*e.g.* Lee and Graham [2] and Merzlyak *et al.* [9]). Up till now Anth effect on the optical properties of intact leaves with a wide range of pigment composition has not been investigated. To develop a technique for nondestructive estimation of Anth in leaves and to understand Anth functions in leaves better, inherent (absorption) and apparent (reflectance) optical properties of Anth *in vivo* should be studied in greater detail. Then, particular bands sensitive to Anth content should be found and relationships 'Anth *vs* absorption and reflectance' in these particular wavebands should be established.

In this paper, optical properties of the leaves of four plant

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†*Abbreviations:* Anth, anthocyanin; Chl, chlorophyll; Car, carotenoids; NIR, near infrared.

Table 1. Pigment content (nmol/cm^2) in anthocyanin-free and anthocyanin-containing leaves of the plant species studied

Plant	Chl *a*	Chl *b*	Total Chl	Car	Anth
Maple* (n = 61)					
Min	0.2	0.1	0.3	1.46	<0.3
Max	42.0	15.0	57.0	8.26	<0.03
Median	14.1	5.1	18.9	4.12	<0.3
Maple† (n = 51)					
Min	0.04	0.03	0.16	3.2	1.5
Max	26.0	10.7	36.7	18.2	48.2
Median	5.1	1.7	6.8	7.5	10.4
Dogwood† (n = 21)					
Min	0.03	0.04	0.1	0.73	1.94
Max	36.6	17.1	53.7	16.4	50.4
Median	9.1	4.1	13.2	5.5	13.6
Cotoneaster† (n = 26)					
Min	0.1	0.1	0.4	5.1	0.03
Max	47.9	14.9	62.8	26.3	97.2
Median	17.4	4.5	21.9	13.0	14.4
Pelargonium‡ (n = 10)					
Min	—	—	23.8	—	<0.03
Max	—	—	35.6	—	10.88
Median	—	—	33.0	—	6.2

*Anthocyanin-free leaves.
†Anthocyanin-containing leaves.
‡Green, green-red and red zones of leaves (only total Chl and anthocyanin content have been determined).

species showing the ability to produce high amounts of Anth pigments have been investigated. The first step in our research was to evaluate the optical properties of Anth-containing leaves in order to find specific Anth features inherent in absorption and reflectance spectra. Secondly, having found the spectral band around 550 nm that was sensitive to Anth content, a technique was developed to subtract Chl effect on reflectance in this spectral range. An index for estimating Anth content was devised and validated by a few independent data sets.

MATERIALS AND METHODS

Plants. Green and red leaves of Norway maple (*Acer platanoides* L.), cotoneaster (*Cotoneaster alaunica* Golite) and dogwood (*Cornus alba* L. (*Swida alba* (L.) Opiz)) were collected in a park at Moscow State University (1992–2000) in spring and during fall. In maple red pigmentation was especially expressed during cold seasons. Usually Anth pigmentation was observed in sunlit leaves, whereas shaded leaves were green to yellow. *Pelargonium zonale* L'Herit (ex Soland) leaves were taken in Sede-Boker Campus of Ben-Gurion University of the Negev, Israel; green and red zones affording paired comparisons within the same leaf were used. Healthy and homogeneously colored leaves without visible symptoms of damage were used in the experiments.

Pigment analysis. One or two discs (1.6 cm in diameter) were cut from leaves and ground with mortar and pestle in *ca* 5–8 mL of methanol in the presence of $CaCO_3$ to prevent pheophytization. Homogenates were centrifuged for 3–4 min in glass tubes at 3000 ***g***. The resulting extracts were immediately assayed spectrophotometrically. Specific absorption coefficients of Chl *a*, Chl *b* and total Car reported by Lichtenthaler (20) were used. A molecular weight of 570 for Car was recognized. Anth 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 the corresponding Chl (Fluka Chemie AG, Seelze, Germany) and their absorption coefficients, at 530 nm, in acid methanol were found to be 8.17 and 6.35 mM^{-1} cm^{-1}, respectively. An Anth absorption coefficient of 30 mM^{-1} cm^{-1} at 530 nm (21) was used. The pigment content was expressed on a leaf area basis.

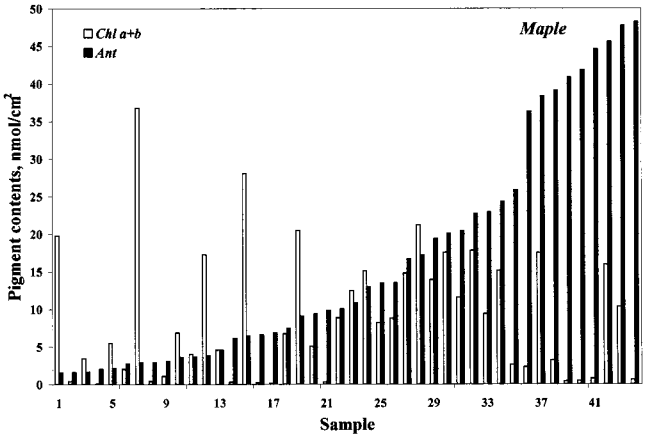

Figure 1. Chl and Anth content in Anth-containing maple leaves studied. Pigment content varied significantly; the study included leaves with high Chl content and very low to moderate Anth content, and high Anth content *vs* background of low Chl content.

Spectral measurements. Reflectance (R) and transmittance (T) spectra of maple, dogwood and cotoneaster leaves were measured with a 150-20 Hitachi spectrophotometer equipped with an 150 mm integrating sphere attachment (part 150-0901) and interfaced to a personal computer. Adaxial reflectance spectra of the leaves were recorded against barium sulfate as a standard with a spectral resolution of 2 nm. Black velvet with reflectance of less than 0.5% over the whole spectral range studied was used as a background in leaf reflectance measurements. Reflectance and transmittance spectra of *P. zonale* leaves were taken with Licor LI-1800 spectroradiometer equipped with an integrating sphere LI-1800-IS. Leaf light absorption (A) was calculated as $1 - R - T$.

RESULTS AND DISCUSSION

Pigment content and composition

Chl, Car and Anth content and composition varied widely in all four species studied (Table 1). As an example, Anth and Chl contents in Anth-containing maple leaves are presented in Fig. 1. It can be seen from Fig. 1 and Table 1 that a wide variety of leaves were studied: leaves with high Anth content, against a background of low and high Chl content, as well as those with low Anth quantities with high Chl content.

Absorption and reflectance

To investigate the spectral properties of Anth in leaves, we studied the absorption spectra of leaves with negligible Anth quantities (less than 0.3 nmol/cm^2) (Fig. 2a) and leaves with Anth content ranging from 1 to around 50 nmol/cm^2 in maple (Fig. 2b) and dogwood leaves and up to 100 nmol/cm^2 in cotoneaster leaves. When Anth was present in leaves in minute amounts (Fig. 2a) an increase in Chl content led to an increase of absorption in the visible range of the spectrum, especially in the green–orange range between 520 and 650 nm. In Anth-containing leaves an increase in Anth content caused an increase of absorption in the green range around 550 nm. In leaves with Anth content more than 6 nmol/cm^2 absorption in the green range was higher than 80% even when Chl content was low. In the blue, red and near-

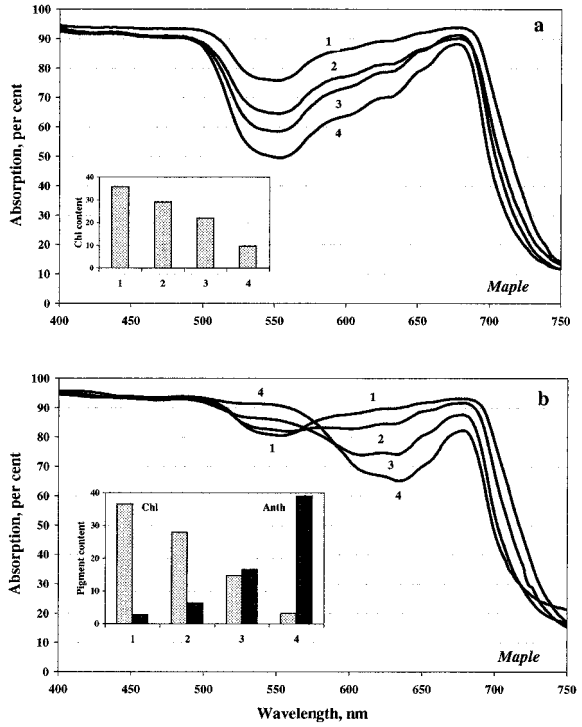

Figure 2. Absorption spectra of maple leaves with: (a) negligible Anth quantities; and (b) wide range of Anth variation. Chl and Anth contents (nmol/cm^2) in leaves are shown in insets.

infrared (NIR) regions, absorption remained almost the same as in Anth-free leaves.

Chl affected absorption spectra significantly (Fig. 2a) and made it difficult to retrieve information on Anth spectral properties present in the absorption spectra. To solve this problem we suggest determining the effect of Anth on leaf absorption comparing absorption spectra of leaves with equal Chl content but different Anth content. The difference in spectral behavior of absorption of Anth-free and Anth-containing maple leaves could be seen clearly in Fig. 3, where spectra of leaves with almost the same Chl content but different Anth content were compared. An increase in Anth content from 2.9 (Fig. 3a) to 22.9 nmol/cm^2 (Fig. 3d) resulted in higher absorption in the green spectral range, while absorption in the blue, red and NIR remained virtually the same. Hence, this difference in absorption by leaves could be attributed solely to Anth absorption. Thus, the spectra of Anth absorption could be obtained as a difference between absorption of Anth-containing leaves and Anth-free leaves with the same Chl content. The calculated Anth absorption spectra for the leaves of three plant species are shown in Fig. 4. The spectral feature common to all species with Anth ranged from 0.8 to almost 40 nmol/cm^2 was an absorption peak near 550 nm. The magnitude of the peak increased with increase in Anth content. The peak position of Anth absorption in the plant leaves studied coincided with that reported for the leaves of rainforest plants (2) and for apple fruits (9). *In vivo* Anth absorption peak position is shifted significantly toward longer wavelengths from that in solution (just near 525 nm acid methanol for plant species studied).

In vivo Anth absorption in leaves at 550 nm related close-

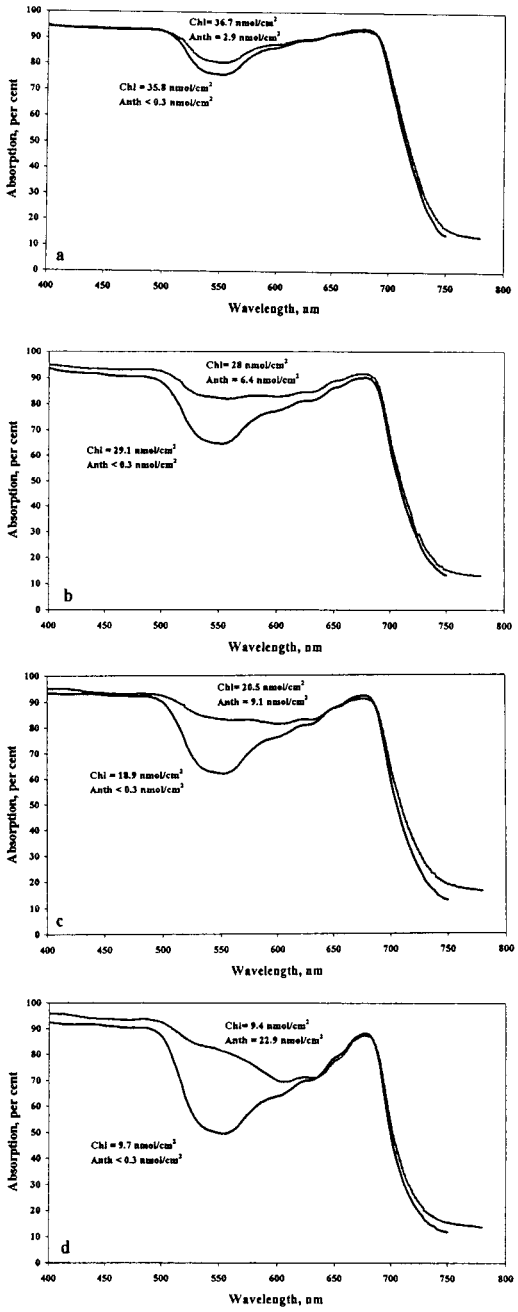

Figure 3. Four examples of absorption spectra of maple leaves with different Anth content and almost the same Chl content. (a) Anth-containing leaf: Anth = 2.9 nmol/cm^2; Chl = 36.7 nmol/cm^2; Anth-free leaf: Anth $<$ 0.3 nmol/cm^2; Chl = 35.8 nmol/cm^2; (b) Anth-containing leaf: Anth = 6.4 nmol/cm^2; Chl = 28 nmol/cm^2; Anth-free leaf: Anth $<$ 0.3 nmol/cm^2; Chl = 29.1 nmol/cm^2; (c) Anth-containing leaf: Anth = 9.1 nmol/cm^2; Chl = 20.5 nmol/cm^2; Anth-free leaf: Anth $<$ 0.3 nmol/cm^2; Chl = 18.9 nmol/cm^2; (d) Anth-containing leaf: Anth = 22.9 nmol/cm^2; Chl = 9.4 nmol/cm^2; Anth-free leaf: Anth $<$ 0.3 nmol/cm^2; Chl = 9.7 nmol/cm^2.

ly to Anth content determined analytically (Fig. 5). The function 'Anth absorption *vs* Anth content' was found to be linear with determination coefficient R^2 = 0.94 and root mean square deviation from the line of less than 3.11 nmol/cm^2. The linear relationship of 'absorption *vs* Anth content' indicates that Anth-specific absorption coefficient (absorp-

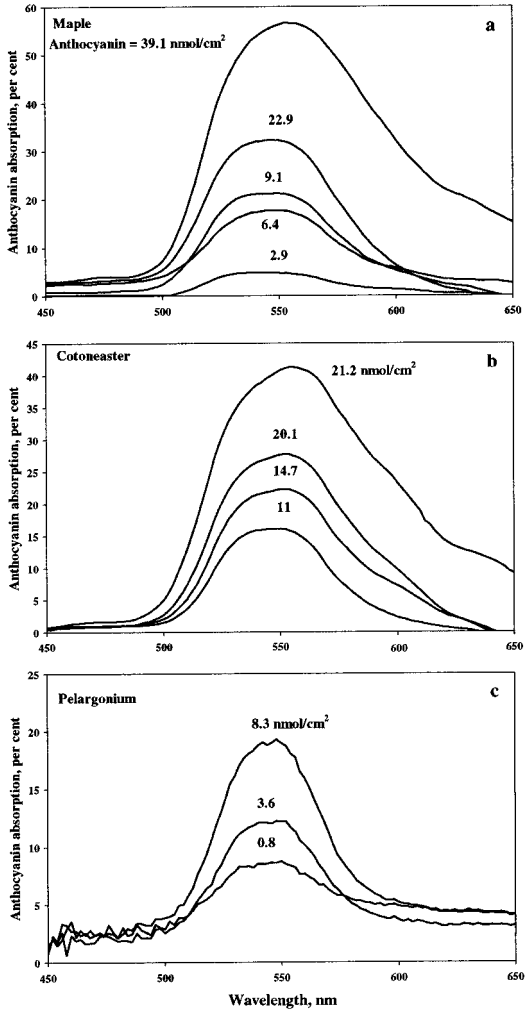

Figure 4. Anth absorption spectra for three plant species: (a) maple; (b) cotoneaster; and (c) pelargonium obtained as a difference between absorption spectra of leaves with almost the same Chl content but different in Anth content. Absorption spectrum of leaf with negligible Anth quantities was subtracted from absorption spectrum of Anth-containing leaf. For all plant species studied absorption spectrum of Anth peaked around 550 nm. Magnitude of the peak depended on Anth content.

tion per nmol/cm^2) remained fairly constant, 1.4% cm^2/nmol, for different, unrelated plant species. It suggests that Anth content in leaves can be determined accurately through absorption measurements with estimation error within 3.1 nmol/cm^2. Thus, comparison analysis of absorption spectra of Anth-free and Anth-containing leaves allowed the determination of a specific spectral feature of Anth in leaves, absorption in the green near 550 nm, that was found to be closely related to Anth content up to 40 nmol/cm^2.

Having found the specific spectral feature of Anth absorption in leaves, we studied how this feature manifests itself in reflectance spectra, trying to find a way to retrieve the Anth content from measured reflectance. We used the same approach as with absorption spectra, comparing reflectance spectra of leaves having close Chl content but different Anth content (Fig. 6). In Anth-containing leaves reflectance in the green was considerably lower than that in Anth-free leaves with the same Chl content. Even in dark-green leaves

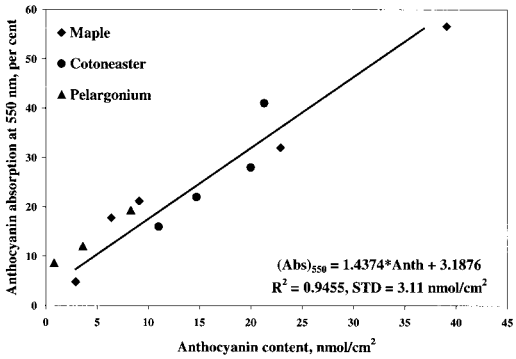

Figure 5. Anth absorption at 550 nm plotted *vs* Anth content determined analytically for three plant species. Fairly linear relationship with determination coefficient $R^2 = 0.94$ shows that for the species studied specific absorption by Anth (percent per nmol/cm^2) remained almost invariable.

with high Chl content (Fig. 6a) the presence of Anth in small quantities (less than 3 nmol/cm^2) resulted in a decrease of reflectance. An increase in Anth content caused a further decrease in the green reflectance; the difference between spectra, near 550 nm, with and without Anth increased significantly. In Anth-free and Anth-containing leaves reflectance in the blue, red and NIR ranges remained practically the same.

Algorithm development and validation

In order to develop a technique for Anth content estimation using reflectance spectra, one should find a way to retrieve the contribution of Anth absorption (which was found to be closely related with Anth content [Fig. 5]) from reflectance spectra. The task is complicated by the fact that absorption of Anth peaks around 550 nm (Fig. 4), where Chl has a strong impact on leaf absorption and reflectance (Fig. 2) (11,12,17). Chl absorption in the green was found to be so pronounced that the inverse reflectance near 550 nm, $(R_{550})^{-1}$, has been used as an accurate measure of Chl content in a wide range of Chl and Car contents in leaves with minute amounts of Anth (Fig. 7a) (11–14,16,22). In Anth-containing leaves both Chl and Anth contribute to absorption and reflectance in the green range. The problem is to find a way to accurately subtract Chl contribution to green reflectance and apportion the remainder to Anth.

To determine the Chl contribution to reflectance around 550 nm, we suggest using fundamental spectral features of leaves with minute quantities of Anth: (1) reflectance at 550 nm, R_{550}, is almost equal to that at 700 nm, R_{700}, (10–14,22). For 61 Anth-free maple leaves studied the determination coefficient of the relationship 'R_{550} *vs* R_{700}' was $R^2 = 0.985$; and (2) both inverse reflectances $(R_{550})^{-1}$ and $(R_{700})^{-1}$ are closely related to Chl content in all species studied (Fig. 7 for 61 maple leaves) (11–14,22).

In this study we found that $(R_{700})^{-1}$ was closely related to Chl content also in Anth-containing leaves (Fig. 7b). The relationship $(R_{700})^{-1}$ *vs* Chl was linear with $R^2 = 0.95$ and estimation error of Chl as small as 3.7 nmol/cm^2 in a wide range of Anth, Chl and Car contents (Table 1). Very close correlation between reflectance in the red edge range near 700 nm and Chl content over a wide range of Anth content

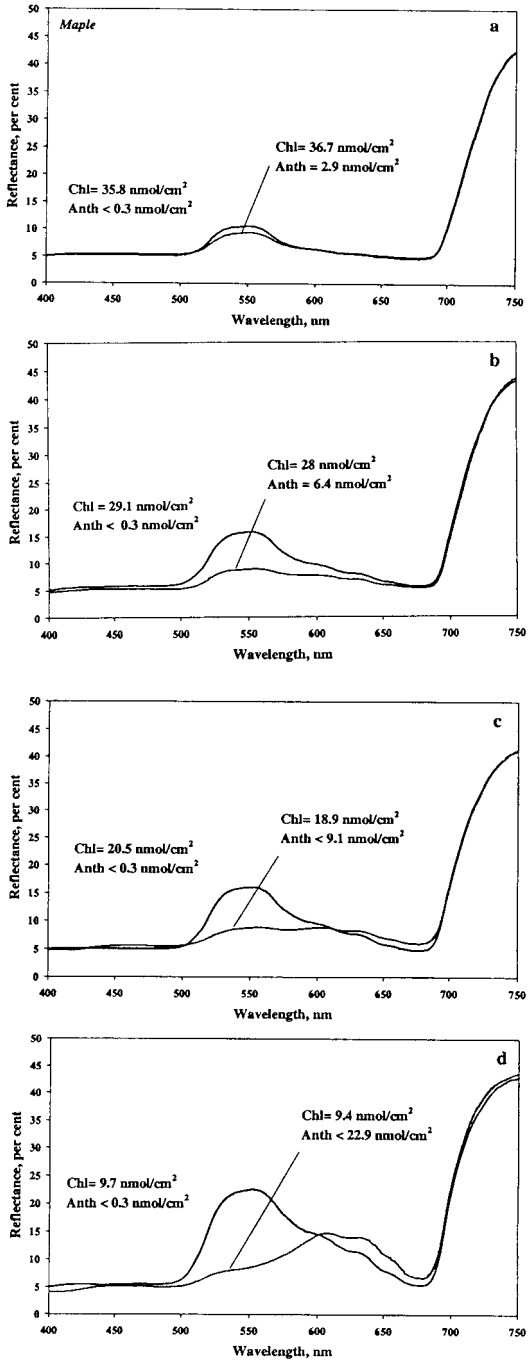

Figure 6. Four examples of maple leaf reflectance spectra with various Anth content and almost the same Chl content. (a) Anth-containing leaf: Anth = 2.9 nmol/cm²; Chl = 36.7 nmol/cm²; Anth-free leaf: Anth < 0.3 nmol/cm²; Chl = 35.8 nmol/cm²; (b) Anth-containing leaf: Anth = 6.4 nmol/cm²; Chl = 28 nmol/cm²; Anth-free leaf: Anth < 0.3 nmol/cm²; Chl = 29.1 nmol/cm²; (c) Anth-containing leaf: Anth = 9.1 nmol/cm²; Chl = 20.5 nmol/cm²; Anth-free leaf: Anth < 0.3 nmol/cm²; Chl = 18.9 nmol/cm²; (d) Anth-containing leaf: Anth = 22.9 nmol/cm²; Chl = 9.4 nmol/cm²; Anth-free leaf: Anth < 0.3 nmol/cm²; Chl = 9.7 nmol/cm². Chl content for spectra presented in each figure was almost the same. Thus, as for absorption spectra, differences between reflectance of leaves could be attributed solely to Anth absorption. An increase in Anth content manifested itself in lower reflectance in the green range, while reflectance in the blue, red and NIR remained virtually the same.

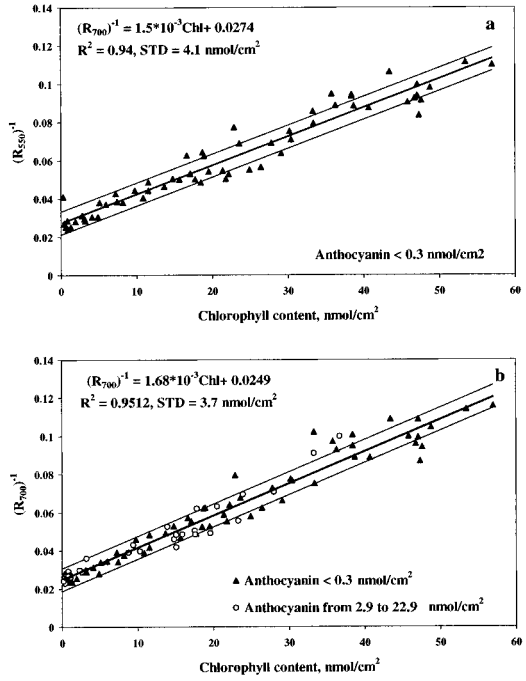

Figure 7. Inverse reflectance at: (a) 550 nm; and (b) 700 nm *vs* Chl content in maple leaves. Solid lines represent the best-fit functions, dotted lines represent root mean square variation from best fit function. R_{700} was proportional to Chl content in both Anth-free and Anth-containing leaves, while R_{550} correlated closely with Chl content in Anth-free leaves only; it was affected also by Anth content.

(up to 100 nmol/cm²) is evidence that Anth do not affect leaf optical properties in the red edge range. It is opposite to the very strong impact of red amaranthin on the red edge position that was found by Curran *et al.* (19).

While $(R_{700})^{-1}$ depends solely upon Chl content (Fig. 7b), R_{550} depends on both Chl and Anth content (Fig. 6). Thus, in Anth-containing leaves, the linear relationship between $(R_{700})^{-1}$ and $(R_{550})^{-1}$ was disturbed. In Fig. 8 $(R_{550})^{-1}$ was plotted *vs* $(R_{700})^{-1}$ for Anth-containing and Anth-free leaves. The solid line represents a best fit function for 61 maple leaves with $R^2 = 0.985$, coefficient of variation <5.2%. In Anth-containing leaves $(R_{550})^{-1}$, representing absorption by both Chl and Anth, was higher than $(R_{700})^{-1}$ and points corresponding to these leaves were above $(R_{550})^{-1} = (R_{700})^{-1}$ line. Thus, for sample A (Fig. 8), total absorption at 550 nm was proportional to inverse reflectance $(R_{550})^{-1}$, length AB, in which length OB was due to Chl absorption and length OA was due to Anth absorption. To determine Anth absorption at 550 nm one should subtract Chl absorption (proportional to length OB) from total absorption at 550 nm (proportional to length AB). Thus, in spectral space $[(R_{550})^{-1}, (R_{700^{-1}})]$, distance of sample from line $(R_{550})^{-1} = (R_{700})^{-1}$, length OA, is a measure of Anth absorption. As it was shown Anth absorption and Anth content were closely related to each other (Fig. 5). So, Anth content would be proportional to difference $[(R_{550})^{-1} - (R_{700})^{-1}]$. Taking into account these findings an index for Anth estimation, Anth reflectance index (ARI), was suggested as:

$$\mathrm{ARI} \propto (R_{550})^{-1} - (R_{700})^{-1}.$$

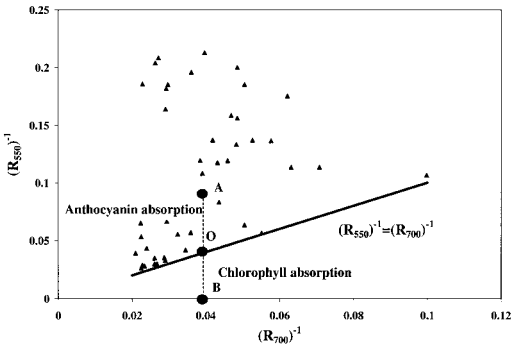

Figure 8. Inverse reflectance at 550 nm *vs* inverse reflectance at 700 nm. Solid line represents the best-fit function for Anth-free leaves ($R^2 = 0.985$, coefficient of variation < 5.2%). In leaves with measurable Anth content reflectance at 550 nm depended on both Anth and Chl contents decreasing with increase of Anth. R_{700} depended solely upon Chl content (Fig. 7) and was equal to R_{550} in Anth-free leaves. For sample A total absorption at 550 nm was proportional to inverse reflectance at 550 nm, length AB, in which length OB is due to Chl absorption and length OA is due to Anth absorption. To determine Anth absorption at 550 nm one should subtract Chl absorption (proportional to length OB) from total absorption (proportional to length AB). So, the distance of sample in spectral space $[(R_{550})^{-1}, (R_{700}^{-1})]$, length OA, is a measure of Anth absorption.

The first term is responsible for both Chl and Anth absorption, and the latter one for Chl absorption.

To test the model, we used the ARI to calculate Anth content in maple leaves with a wide range of pigment variation (Fig. 1, Table 1) and compared it to actually measured Anth content (Fig. 9a). The distance from line $(R_{550})^{-1} = (R_{700})^{-1}$, length OA in Fig. 8, was found to be linearly proportional to Anth content, and the relationship between the ARI and Anth was linear with determination coefficient $R^2 = 0.93$. For Anth ranged from 2 to 48 nmol/cm^2, standard error of Anth estimation of less than 3.9 nmol/cm^2 was achieved.

To validate the model, the ARI was used to estimate Anth in dogwood and cotoneaster leaves (Fig. 9b,c). For dogwood leaves, the ARI correlated closely with Anth content with $R^2 = 0.89$ and standard error of Anth estimation of less than 5.9 nmol/cm^2. In cotoneaster leaves, Anth ranged even wider from 0.2 to 98 nmol/cm^2. The index allowed the estimation of Anth with standard error within 11.2 nmol/cm^2.

It should be noted, however, that in all the species studied, the slope of the relationship 'ARI *vs* Anth' for Anth > 25 nmol/cm^2 became smaller and the ARI appeared to be less sensitive to high Anth content. Usually, Anth is located in the upper cell layer of the leaf (6), and is able to produce a special gradient in the leaf that particularly protects the more deeply located chloroplasts within the leaf (5). When Anth absorption is small to moderate (up to 30%) light interacts with both Chl and Anth. When Anth absorption becomes higher than 30% no further light interaction with Chl is likely. A simple two-flow approximation model shows that when Anth > 30%, contribution of Chl to leaf reflectance declines drastically, and for Anth absorption > 40% (that corresponds to Anth content about 25 nmol/cm^2 [Fig. 5]), Chl contribution becomes negligible. Thus, for Anth content > 25 nmol/cm^2 the second item in the ARI, $(R_{700})^{-1}$, almost does not work; reflectance at 550 nm is governed solely by Anth absorption. For maple leaves with Anth > 22 nmol/cm^2, a close linear correlation between $(R_{550})^{-1}$ and Anth did occur with $R^2 = 0.8$ and standard error of Anth estimation of less than 3.9 nmol/cm^2 (data not shown). Close linear correlations between $(R_{550})^{-1}$ and high Anth have also been found for dogwood and cotoneaster leaves. So, for high Anth content, $(R_{550})^{-1}$ may be used for nondestructive Anth detection.

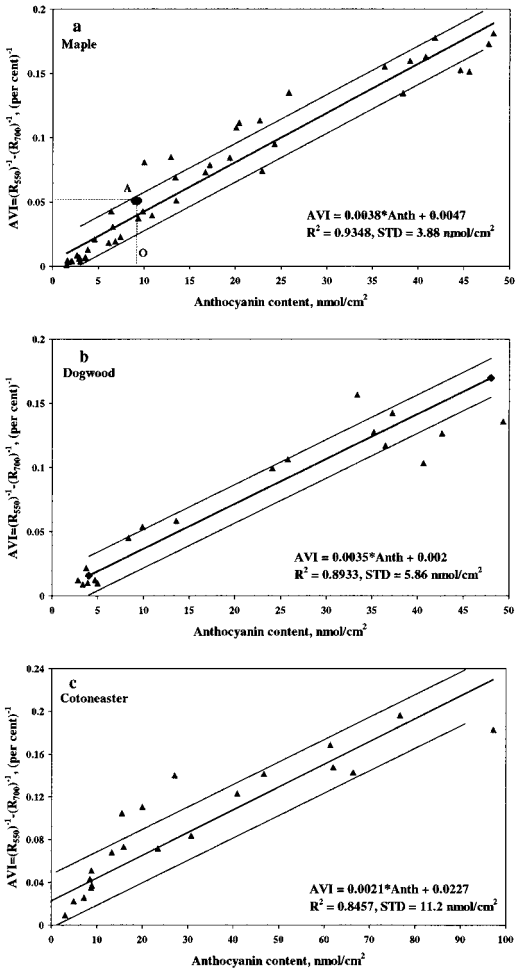

Figure 9. Relationship between ARI = $[(R_{550})^{-1} - (R_{700})^{-1}]$ and Anth content for: (a) maple; (b) dogwood; and (c) cotoneaster leaves with a wide range of pigment content and composition (Fig. 1, Table 1). Solid lines represent the best fit functions, dotted lines represent root mean square variation from best fit function.

Significantly, that the sensitivity of the ARI to Anth content, ranging from low to moderate values (up to 25 nmol/cm^2) was almost equal for diverse plant species studied. For maple, dogwood, cotoneaster and pelargonium, the slope of the relationship ARI *vs* Anth remained almost the same and equal to 0.006 cm^2 (nmol%)$^{-1}$. It is in accordance with our findings that specific Anth absorption remained practically invariant for different unrelated plant species (Fig. 5).

Ratios R_{NIR}/R_{550} and R_{NIR}/R_{700}, where R_{NIR} is reflectance in the NIR range above 750 nm, were found to be proportional to Chl content in Anth-free leaves (11,12). R_{NIR} is not affected by pigment absorption and depends upon leaf density and thickness. Thus, ratios of reflectance, where numerator depends on leaf thickness and density and denominator depends upon pigment concentration, allow the estimation

of Chl content in a wide range of leaf thickness. Thus, following this finding, we suggest using R_{NIR} in ARI to make it less dependent on leaf thickness and density.

In the range of Anth from 0.3 to 100 nmol/cm^2, we suggest using ARI in the form of either

$$(R_{550})^{-1} - (R_{700})^{-1} \quad \text{or}$$

$$R_{NIR} \cdot [(R_{550})^{-1} - (R_{700})^{-1}]$$

In the range of Anth 0.3–25 nmol/cm^2 the standard error of Anth estimation by both indices did not exceed 3 nmol/cm^2, and in the whole range 0.3–100 nmol/cm^2, it was within 11 nmol/cm^2.

For Anth $>$ 25 nmol/cm^2, when ARI $>$ 0.1, we suggest using either

$$(R_{550})^{-1} \quad \text{or} \quad R_{NIR}/R_{550}$$

It allowed the Anth estimation with an error of less than 3.9 nmol/cm^2.

We have also tested the accuracy of the index red/green (16) to assess Anth content in leaves studied. Relationship 'red/green *vs* Anth' for maple leaves was found to be low ($R^2 = 0.55$) and standard error of Anth estimation was 10.2 nmol/cm^2, which was 2.5 times higher than that with the ARI estimation. For dogwood and cotoneaster leaves scattering of points from the linear relationship was even higher (not shown). The red (600–700 nm) reflectance depends solely upon Chl content while the green (500–600 nm) depends upon three pigments: Chl, Car and Anth (Fig. 6) (11,15–18,23,24). This can explain why the index red/green is strongly influenced not only by Anth content but also by pigment composition. For leaves with Anth ranging widely against the background of high and small Chl and Car contents, the index red/green varied drastically regardless of the Anth content (data not shown).

Anth absorption in plant tissues is strongly dependent on the Anth species present, the pigment aggregation, pH, copigmentation effects by flavonols and metal complexing (1). Therefore, there are uncertainties between analytical determination of Anth content and their contribution to light absorption by leaves. Taking into account these circumstances the accuracy of Anth assessment by the technique developed here is satisfactory.

For nondestructive Anth estimation two spectral bands, the green at 550±15 nm and the red edge at 700±7.5 nm were found to be sufficient. The simulated reflectances in these spectral bands were used to calculate ARI; as a result the error in Anth estimation did not exceed those for the narrow channels mentioned above.

CONCLUSIONS

Optical properties of Anth were investigated in leaves of four unrelated plant species over a wide range of Anth, Chl and Car contents and pigment composition. Specific spectral feature of Anth in leaves was an absorption peak around 550 nm. *In vivo* absorption of Anth was strongly dependent upon Anth content. A specific absorption coefficient (per Anth content) was found. This is the first reported study on specific Anth absorption in leaves.

Based on fundamental optical properties of the leaves and specific spectral features of Anth revealed in this study, an index for nondestructive estimation of Anth content in leaves was devised. Reflectances in two spectral bands, 550±15 and 700±7.5 nm, are sufficient to estimate nondestructively the Anth content with high accuracy.

Once the optical properties of Anth *in vivo* have been investigated and 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. More studies are required to broaden the models offered in this work in order to devise comprehensive algorithms for monitoring Anth pigments by remote sensing.

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