Research Note

Nondestructive Estimation of Anthocyanin Content in Grapevine Leaves

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Abstract: The anthocyanin (Anth) content in leaves provides valuable information about the physiological status of plants. Thus, there is a need for accurate, efficient, practical methodologies to estimate this biochemical parameter. Reflectance measurement is a means of quickly and nondestructively assessing leaf Anth content in situ. The objective of this study was to test the overall performance and accuracy of nondestructive techniques for estimating Anth content in grapevine leaves. Relationships were established between Anth content and four vegetation indices: NIR (near-infrared)/green, red/green, anthocyanin reflectance index (ARI, based on reflectances in bands within the green and the red-edge regions), and a modified anthocyanin reflectance index (MARI, based on reflectances in green, red edge, and NIR). The algorithms for Anth retrieval were calibrated. The accuracy of Anth prediction was evaluated using an independent data set containing sampled leaves from two field-grown grape cultivars (Saint Croix and Saint Pepin) with no adjustment of the coefficients after initial calibration. Although Anth in the validation data set was widely variable, from 3 to 45 nmol cm^{-2}, the ARI and MARI algorithms were capable of accurately predicting Anth content in grapevine leaves with a root mean square error below 3 nmol cm^{-2} and 2.3 nmol cm^{-2}, respectively. Such an approach has potential for developing simple hand-held field instrumentation for accurate nondestructive Anth estimation and for analyzing digital airborne or satellite imagery to assist in making informed decisions regarding vineyard management.

Key words: anthocyanins, grapes, leaves, nondestructive, reflectance

Recent interest in red anthocyanin (Anth) pigments is partially due to their pharmacological and nutritional effects and possible role in prevention of common chronic diseases, including atherogenesis, coronary heart diseases, thrombosis, and cancer. The polyphenols in red wine are believed to be the main contributors to the beneficial health effects of this beverage. The well-known French paradox is related to the low incidence of cardiovascular problems among the populace of France, despite their consumption of rich foods. The broad range of biological activities of anthocyanins is often described as their antioxidant properties (see review, Borkowski et al. 2005).

Anthocyanin biosynthesis is genetically determined, and plant leaves differ widely, according to species and cultivar, in their ability to synthesize Anth. In many plants, the bright red Anth coloration is abundant in young and senescing leaves (Lee 2007). Anth biosynthesis is often initiated due to drought, insect pests, potassium deficiency, extreme temperature, and excessive light. This behavior may allow the pigment to serve as an indicator of plant stress (e.g., Neill and Gould 1999). It is generally accepted that one key physiological function of Anth in higher plants is its photo-protective role. The pigment, which is localized in vacuoles of epidermal cells, serves as a filter: an internal light trap for excessive solar radiation (Chalker-Scott 1999, Close and Beadle 2003, Steyn et al. 2002). In particular, the photo-protective role of epidermal Anth in young grapevine leaves has been reported (Liakopoulos et al. 2006).

Anthocyanins have traditionally been determined through wet-chemical methods, including pigment extraction in a solvent, spectrophotometric determination of absorbance by the Anth solution, and conversion from measured absorbance to Anth content (e.g., Gitelson et al. 2001). However, laboratory procedures are laborious, time-consuming, and destructive to leaves. Spectral reflectance may provide viticulturists with an efficient, nondestructive method of monitoring Anth content and the associated physiological status of the fruit crop. Various models (called vegetation indices) have been developed for retrieving pigment content in leaves via spectral information (Richardson et al. 2002). Such indices, when applied to measurements acquired by hand-held field instrumentation or aerial imagery, should enhance the ability to make informed decisions regarding vineyard management. Recently, vegetation indices were designed to estimate Anth content in leaves nondestructively (Gamon

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and Surfus 1999, Gitelson et al. 2001, 2006, van den Berg and Perkins 2005). These indices are based on relationships involving reflectance at several specific wavelengths with varying sensitivity and response to changes in Anth content.

Gamon and Surfus (1999) used a ratio of reflectances at red (ρ_{red}) and green (ρ_{green}) wavelengths as a proxy of Anth content with the following:

$$\text{Red/green} = \rho_{red}/\rho_{green} \quad (1)$$

Anth absorbs in situ at ~550 nm (Gitelson et al. 2001), and the red peak of chlorophyll (Chl) absorption in situ is ~670 nm, but absorption by Chl also occurs in the green range of the spectrum (Gitelson et al. 2003). Thus, the ρ_{red}/ρ_{green} estimates Anth content by comparing reflectance in the red band of Chl absorption to reflectance in the green band where both Chl and Anth absorb.

Van den Berg and Perkins (2005) developed and tested a modified chlorophyll content meter (CCM-200) for measuring Anth in sugar maple leaves displaying fall colors. The device was optimized for Anth detection by replacing the light emitting diode centered at 655 nm with one centered at 530 nm. The authors used the ratio of absorbance (α) in the near-infrared band (940 nm) and the green (530 nm) as a proxy of Anth. An Anth content index (ACI) was suggested in the form: $ACI = \alpha_{green}/\alpha_{NIR}$.

An anthocyanin reflectance index (ARI) for estimating Anth content is based on hemispherical reflectance measurements of various types of leaves, compiled using an integrating sphere (Gitelson et al. 2001):

$$ARI = (\rho_{green}^{-1} - \rho_{red\ edge}^{-1}) \quad (2)$$

ARI compares the reciprocal of reflectance in the green with that in the red-edge region and attributes the difference to absorption by Anth. The ARI was later modified (labeled MARI) to include NIR reflectance, ρ_{NIR}, as a means of accounting for variability in leaf thickness (Gitelson et al 2001, 2006):

$$MARI = [\rho(\lambda_{green})^{-1} - \rho(\lambda_{red\ edge})^{-1}] \times \rho(\lambda_{NIR}) \quad (3)$$

The goal of the current study was to investigate the performance of several reflectance-based nondestructive techniques for estimating Anth content in grapevine leaves, specifically, (1) to establish the relationship between the Anth content measured analytically and four selected vegetation indices (ACI, red/green, ARI, and MARI) and to calibrate the algorithms for Anth estimation; and (2) to validate the algorithms using an independent data set for two grape cultivars (Saint Croix and Saint Pepin), thus determining the accuracy of Anth-content prediction without adjustment of the coefficients after initial calibration. The reflectance measurements described in this work were carried out by means of a fiber optic attached to a leaf clip (i.e., no expensive integrating sphere). Our interest was in developing and testing a simple method for making pigment measurements in a routine, efficient manner in a vineyard setting.

Materials and Methods

Forty-two leaves were sampled during four field campaigns in the summer of 2006. Leaves were selected based on visual characteristics: selected leaves ranged from dark green with little red to completely red with little green. Field campaigns took place on: (1) 1 June 2006 (10 Saint Croix leaves); (2) 9 June 2006 (10 Saint Pepin leaves); (3) 19 Sept 2006 (12 Saint Croix leaves); and (4) 20 Sept 2006 (10 Saint Croix leaves). Both Saint Croix and Saint Pepin are French-hybrid wine cultivars grown in the Midwest where *vinifera* vines are not well-suited to climatic conditions. The selected leaves were detached from the vine, immediately sealed in a plastic bag with a small amount of water, and placed in a cooler with ice.

After field sampling was completed, the leaves were transported to the lab. When the coloration of the entire leaf was not uniform (as often occurred during and after veraison), areas of homogeneous color on each leaf were identified and delineated with a marker. Six thickness measurements per leaf were made with an Absolute ID-S 1012 Digimatic Indicator (Mitutoyo, Aurora, IL) and mean thickness was determined for each leaf. Care was taken to restrict the measurements to areas between veins.

Reflectance measurements. Spectral-reflectance measurements for the leaves were collected for each of the two grape cultivars noted above using a clip with a 2.3-mm diam bifurcated fiber optic attached to an Ocean Optics USB2000 radiometer (Dunedin, FL) and an Ocean Optics LS-1 tungsten halogen light source. The USB2000 radiometer measures radiance with a spectral resolution of ~1.5 nm in a wavelength range from 350 to 1000 nm. The LS-1 light source uses a regulated power supply and a tungsten halogen filament bulb. The light source was turned on 15 minutes prior to scanning to allow the bulb and filament to warm and stabilize.

The plastic leaf clip, which positioned the fiber against individual grapevine leaves, consisted of a black polyvinyl chloride (PVC) attachment and the bifurcated glass fiber optic (transmissive between 400 and 1000 nm). The black PVC clip held each leaf at a 60° angle relative to the fiber to reduce specular reflectance from the surface of the leaf. The clip also held a black foam background, with a nominal reflectance of 3% within the spectral range of the instrument, upon which each leaf was placed during spectral sampling. The low reflectance properties of the background minimized extraneous reflectance from the reflected light being transmitted through the leaf.

The radiometer was calibrated before each data-collection session using a Labsphere Spectralon reference panel (North Sutton, NH) with a nominal reflectance of 99%. The reference panel was held tightly against the fiber optic, and a spectral scan was recorded. The sensor was operated by the CALMIT Data Acquisition Program (University of Nebraska-Lincoln), which uses

a single calibration scan collected at the time nearest to the acquisition of the target scans to compute reflectance. The reflectance spectra were calculated as a ratio of leaf radiance to the radiance of the reference panel.

For accurate representation of reflectance of the marked area, six reflectance measurements were acquired for each leaf. The locations of spectral measurements were carefully distributed throughout the entire marked area superimposed on the leaf. The average of the six scans per sample was calculated to establish a single representative reflectance spectrum per leaf from which index values were calculated.

Pigment extraction. After reflectance measurements were acquired, two or three discs (1 cm diam) were cut from the marked area on each leaf. Pigment contents were calculated in a two-step procedure. The punches were weighed and ground in 100% methanol using a mortar and pestle until the pulp turned white in color and all pigments were extracted. The resulting homogenate was centrifuged in test tubes for 6 min. Absorption spectra of the supernatants were recorded, and Chl-*a*, Chl-*b,* and carotenoid contents were calculated using absorption coefficients (Lichtenthaler 1987).

Once chlorophyll-absorption spectra were collected, HCI (final concentration ~0.1 %) was added to the solution, resulting in the appearance of the characteristic Anth coloration of the extract. Next, absorption spectra of the acidified extracts were taken and absorbance at 530 nm was corrected for the contribution of pheophytins (Lichtenthaler 1987). Anth concentrations were quantified using an absorption coefficient of 30 mM cm^{-1} at 530 nm (Strack and Wray 1989). Pigment content was expressed as a function of leaf area.

Calibration and validation. Reflectance spectra and the corresponding measured pigment contents were combined and split into two data sets: calibration and validation. The data sets were arranged in ascending order, with odd samples assigned to calibration groups and even samples to validation groups.

ACI was tested using reflectances in green ρ_{green} (530 nm) and NIR ρ_{NIR} (940 nm) spectral bands. Thus, the modified ACI had the following form:

$$\text{Modified ACI} = \rho_{NIR}/\rho_{green} \quad (4)$$

Red/green, ARI, and MARI were calculated using average reflectance values for each leaf in following bands (Gamon and Surfus 1999; Gitelson 2006): λ_{green} = 540–560 nm; λ_{red} = 660–680 nm; $\lambda_{red\ edge}$ = 690–710 nm; and λ_{NIR} = 760–800 nm. The index values from the calibration data set were regressed against the corresponding measured Anth contents to calibrate the algorithms. The calibrated algorithms were applied to predict Anth content using the validation data set. The predicted Anth contents were compared to Anth values determined analytically as part of the validation data set, and the root mean square error (RMSE) of Anth prediction was calculated.

Results and Discussion

Analytical laboratory measurements yielded a wide variety of pigment contents, with Anth ranging from 3.90 to 44.43 nmol cm^{-2} and total Chl ranging from 5.1 to 47.6 nmol cm^{-2}, similar to ranges observed in other studies (Gamon and Surfus 1999, Gitelson et al. 2001, 2006). Data for the two sampled cultivars at various growth stages and for both the calibration and validation data sets are shown (Figure 1). There was a wide variation in content and overall composition of both pigments in the sampled leaves. A Chl to Anth ratio for each data set was calculated to underscore that variation: 0.11 to 6.08 for the data set acquired on 1 June; 0.11 to 8.7 for 9 June; 0.07 to 8.7 for 19 Sept; and 0.21 to 10.8 for 20 Sept. The best fit functions for the relationship between Chl and Anth for each data set and all data taken together are power functions of the form $Chl = k \times Anth^{-n}$. The determination coefficients for these relationships were all high: 0.73 for the 1 June data set; 0.82 for 9 June; 0.86 for 19 Sept; and 0.84 for 20 Sept. When all data were combined, the relationship was $Chl = 90.5Anth^{-0.67}$ with $R^2 = 0.67$. For individual and combined data sets, moderate-to-high Chl content (>25 nmol cm^{-2}) corresponded to low Anth (<10 nmol cm^{-2}) while leaves with high Anth had much lower Chl (<20 nmol cm^{-2}).

The leaves studied were between 10 and 90 days old with leaf thickness ranging between 0.25 and 0.6 mm. Leaves with moderate-to-high Anth content were younger and thinner (between 0.25 and 0.35 mm) than mature leaves with high Chl content (thicker than 0.4 mm). NIR reflectance, a proxy of leaf thickness and density (Slaton et al. 2001), was much higher in green mature leaves

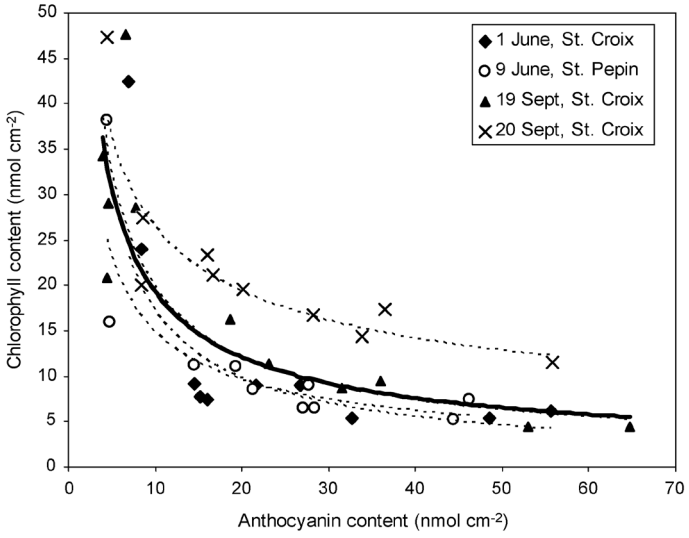

Figure 1 Relationship between anthocyanin and total chlorophyll content in grapevine leaves. Data correspond to leaves from the two cultivars at different growth stages. Note the wide variation in pigment content and composition for leaves comprising each data set. The best fit functions of the relationship Chl vs. Anth for each data set (dashed lines) and all data combined (solid line) are shown.

with low Anth content and lower in red-tinted to reddish leaves with higher Anth content (Figure 2).

While reflectance in the red-edge region ~700 nm remains insensitive to changes in Anth content, reflectance in the green range (~550 nm) notably decreases (Figure 2) with increase in Anth because that pigment absorbs in-situ in the green range (Gitelson et al. 2001). However, the green reflectance is also affected by Chl content as it decreases with an increase in Chl. This overlapping of Chl and Anth absorption in the green spectral region was the main challenge in developing algorithms for nondestructive Anth estimation.

The modified ACI (Eq. 4) was related to Anth content in neither the calibration (Figure 3A, $R^2 = 0.06$) nor validation ($R^2 = 0.0005$, not shown) data sets. Relationships between red/green, ARI, and MARI vegetation indices (Eqs. 1, 2, and 3) and the analytically determined Anth content were established; all three co-varied in a statistically significant manner. These relationships yielded calibration of three algorithms for nondestructive Anth estimation.

Red/green ratio ($R^2 = 0.54$) (Figure 3B):

$$Anth_{red/green} = 51.36 \times \rho_{red}/\rho_{green} - 2.88 \quad (5)$$

Anthocyanin reflectance index ($R^2 = 0.91$) (Figure 3C):

$$Anth_{ARI} = 370.5 \times (\rho_{green}^{-1} - \rho_{red\ edge}^{-1}) + 6.46 \quad (6)$$

Modified anthocyanin reflectance index ($R^2 = 0.93$) (Figure 3D):

$$Anth_{MARI} = 13.41 \times [\rho(\lambda_{green})^{-1} - \rho(\lambda_{red\ edge})^{-1}] \times \rho(\lambda_{NIR}) + 5.13 \quad (7)$$

Algorithms (Eqs. 1, 2, 3) were also calibrated separately for Saint Pepin leaves. The calibrations yielded relationships in which slopes and offsets were not statistically significant from Eq. 5 to 7 (not shown).

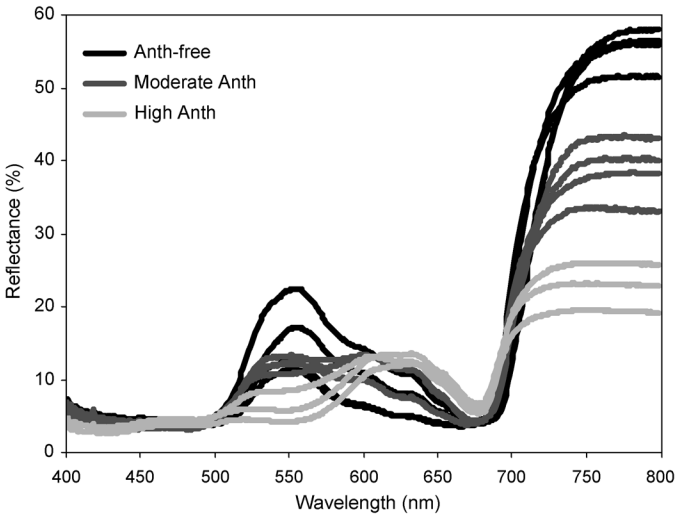

Figure 2 Reflectance spectra of leaves with different anthocyanin contents.

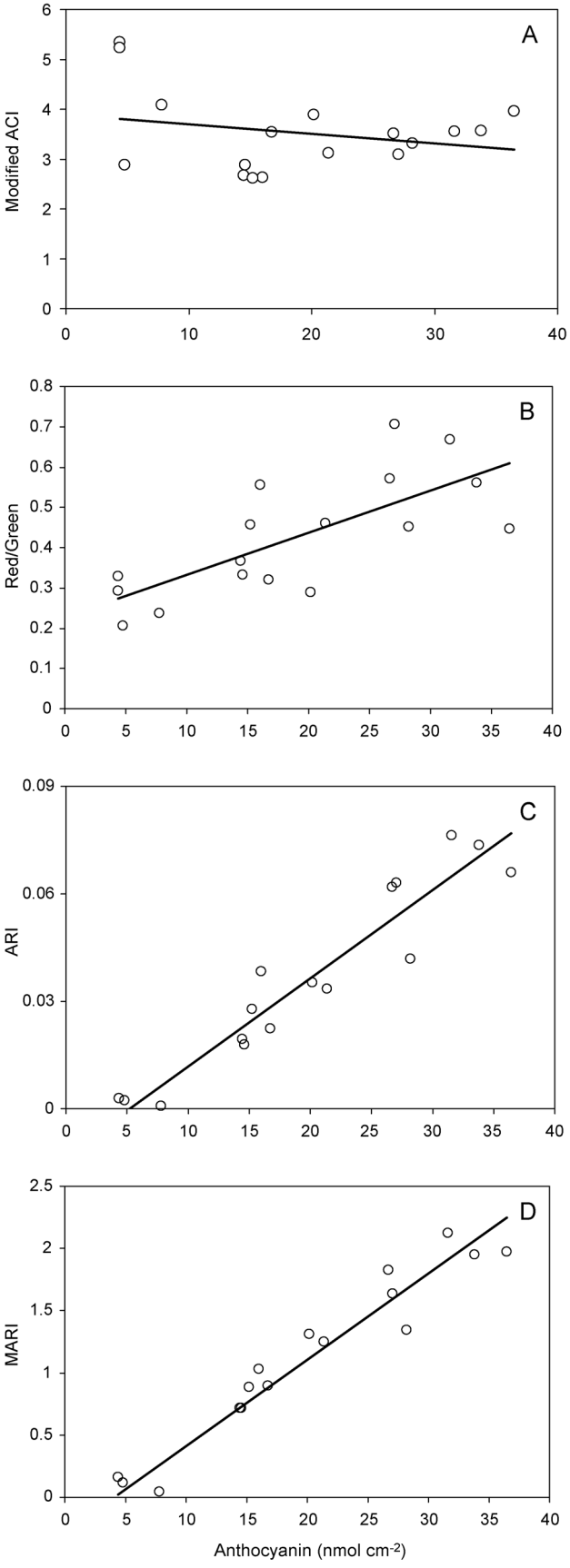

Figure 3 Relationships between reflectance indices and anthocyanin content measured analytically in the lab for leaves comprising the calibration data set.

Because of the absence of a reliable close relationship between the modified ACI and Anth, we did not validate this index. The results of validating the other three algorithms (Figure 4) were consistent with the findings in the calibration phase. Anth prediction by the red/green was the poorest with the lowest determination coefficient (R^2 = 0.72) and highest RMSE (7.63 nmol cm^{-2}) (Figure 4A). The derived model was:

$$Anth_{red/green} = 1.02 \times Anth_{meas} + 1.48 \quad (8)$$

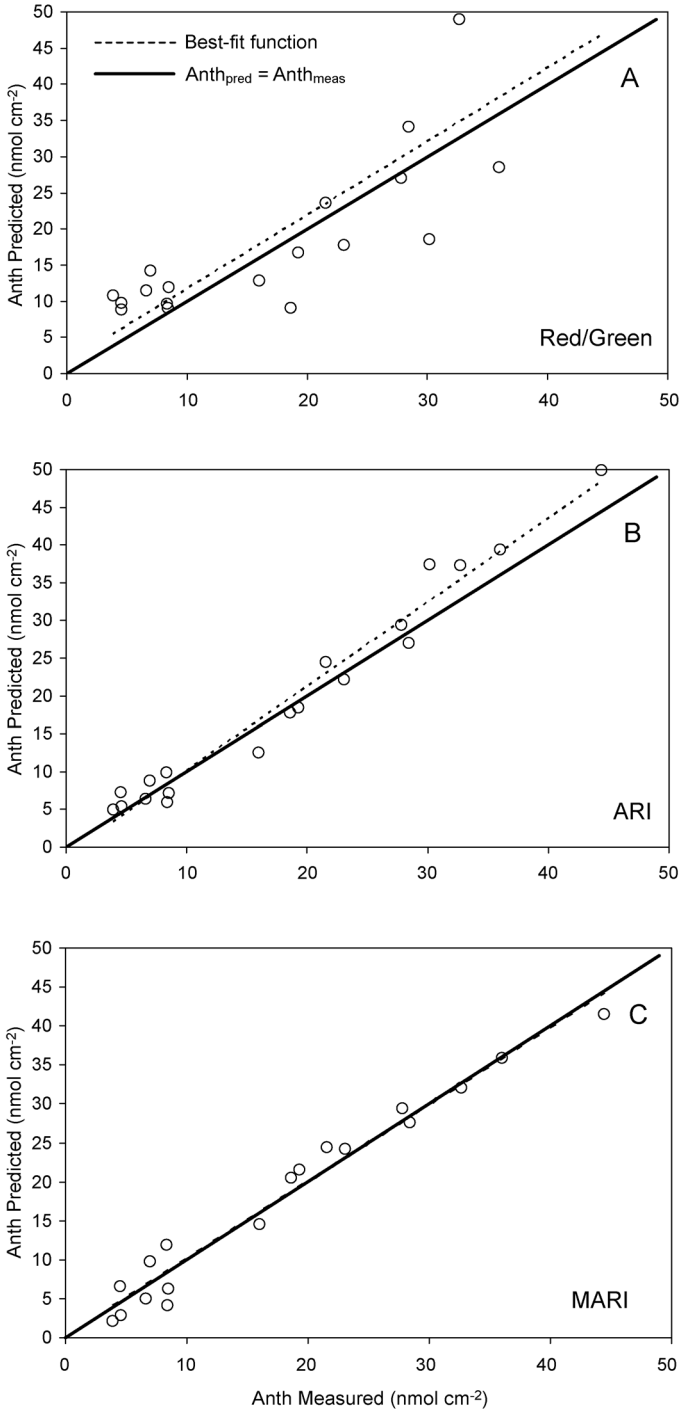

Figure 4 Anthocyanin prediction by vegetation indices.

Anth prediction by ARI was much more accurate than the red/green, with an increase in accuracy more than two-fold (R^2 = 0.96, RMSE < 2.93 nmol cm^{-2}) (Figure 4B). The model was:

$$Anth_{ARI} = 1.11 \times Anth_{meas} + 0.97 \quad (9)$$

MARI was the most accurate in Anth prediction (R^2 = 0.97, RMSE < 2.23 nmol cm^{-2}) (Figure 4C), and a model of the form:

$$Anth_{MARI} = 0.99 \times Anth_{meas} + 0.18 \quad (10)$$

Importantly, the slope of the best-fit function of the relationship between Anth content predicted by MARI and the analytically measured was almost equal to one and the intercept was close to the origin (Figure 4C).

The poor performance of the modified ACI is understandable. In the modified ACI = ρ_{NIR}/ρ_{530}, the reflectance ρ_{530} is the term sensitive to content of both pigments, Anth and Chl, and it decreases as their contents increase. In addition, NIR reflectance, ρ_{NIR}, is affected by leaf thickness (Slaton et al. 2001). Thus, the modified ACI depends upon three variables (Chl, Anth, and leaf thickness) and when they vary independently, it becomes insensitive to Anth. The close relationship between ACI and Anth, as found by others (van den Berg and Perkins 2005), may be explained by the very close relationship between Chl and Anth in the autumn sugar maple leaves studied. Analyzing table 1 in van den Berg and Perkins (2005), we found that Chl and Anth contents were related with $R^2 > 0.96$. Thus, ACI was an accurate proxy of Anth as well as Chl (the determination coefficient of the polynomial relationship ACI vs. Chl was greater than 0.95). It appears that ACI is an effective tool for Anth estimation only under specific conditions: when Chl and Anth are closely related and leaf thickness does not vary.

The red/green ratio has the same limitation as ACI. When Chl and Anth contents are closely and inversely related, the ratio can be used to measure Anth content. However, when Chl and Anth contents are slightly related or not related at all, the red/green ratio depends upon pigment composition rather than on Anth content alone (Gitelson et al. 2001).

Conclusions

ARI and MARI were effective in the nondestructive prediction of anthocyanin in the leaves of two French-hybrid grapevine cultivars despite differences in pigment composition, leaf thickness, age, and pubescence. Spectral bands selected for this study were 10 nm in width and both algorithms produced very accurate prediction of Anth, ranging from 3.90 to 45 nmol cm^{-2}, with root mean square error below 3 nmol cm^{-2} by ARI and below 2.25 nmol cm^{-2} by MARI. Thus, a radiometer with only two spectral bands (540–560 nm and 690–710 nm) is required for nondestructive Anth estimation using ARI. For precise Anth estimation by MARI, one additional spectral band in the NIR range (760–800 nm) is required. ARI

and MARI were validated with two hybrid cultivars of grapes, but it does not appear that the algorithms are cultivar-specific among the two varieties examined. Thus, the algorithms are likely to allow accurate Anth determination in leaves of the pure *V. vinifera* vines used for wine production worldwide. More work should be done to test the algorithms in various plant species.

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