

Chlorophyll Fluorescence Imaging for the Noninvasive Assessment of Anthocyanins in Whole Grape (*Vitis vinifera* L.) Bunches[†]

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ABSTRACT

The distribution of anthocyanins in grape (*Vitis vinifera* L.) bunches from the Sangiovese cultivar was measured nondestructively by chlorophyll fluorescence imaging using two excitation light bands at 550 and 650 nm in sequence. The pixel intensity in the derived logarithm of the fluorescence excitation ratio image was directly related, by an exponential function ($r^2 = 0.93$), to the anthocyanin concentration of berry extracts. The method will be useful for the assessment of the heterogeneity of anthocyanin accumulation in berries that is known to depend on physiologic and climatic factors. It can also represent a new, rapid and noninvasive technique for the assessment of grape ripening and the appropriate time of harvest.

INTRODUCTION

Grape phenolic maturity is now considered a fundamental parameter that has to be controlled for high-quality wine production (1). Monitoring the phenolic contents of grape berries, however, is difficult because of their large spatial and temporal heterogeneity among different vineyard sectors (2). Furthermore, at present, grape phenolic maturity has been determined by using destructive laboratory analysis (3), which is time-consuming, may produce certain artifacts due to pigment instability and loss of material, and requires an accurate sampling approach to be representative of the vineyard block considered. Recently, a new nondestructive optical method to assess berry skin anthocyanins on single berries has been presented (4). It is based on the sequential acquisition of chlorophyll (Chl) fluorescence under two excitation lights (green and red), which are differentially screened by anthocyanins in the berry skin. The excitation beam in the red for which the skin layers are almost transparent is used as a reference. Accordingly, the logarithm of the fluorescence excitation ratio (logFER) between red-excited and green-excited Chl fluorescence (ChlF) was found to be related to the absorbance of anthocyanins (Anth) in the fruit epidermal layers. The method was also recently applied to determine Anth in apple skins (5). Here, we

present an extension of the method by applying a Chl fluorescence imaging technique, which adds the information of the Anth spatial distribution within the whole grape bunch. Imaging provides important details needed for the scale transition of the logFER method from high-resolution spectroscopic measurements on single berries (4) to band-pass fluorescence measurements by optical sensors on whole bunches (6). The latter is the basis for nondestructive assessment of grape phenolic maturity.

MATERIALS AND METHODS

Whole bunches of grapes (*Vitis vinifera* L.) of the Sangiovese variety were sampled on August 3, 2006 (at véraison—when berries start changing color) from a vineyard in the northern part of the Chianti region (Tuscany, 43°47'N, 11°35'E). All measurements were performed on the next day after harvesting.

Images were collected, with a 14-bit dynamics, by a CCD camera (Chroma CX260, DTA, Italy), equipped with a motorized filter wheel carrying up to eight different interference filters, by using a 70–200 mm zoom lens (Centagon, Tokyo, Japan).

Chlorophyll fluorescence was excited by using a 180 W xenon lamp through a 10 mm diameter optical fiber bundle. The green and red excitation lights were selected by using 40 nm bandwidth interference filters (Andover Corporation, Salem, NH) centered at 550 (550FS40-25) and 650 nm (650FS40-25), respectively. Power density, measured by an Ophir PD300UV photodiode (Optronics Ltd, Jerusalem, Israel), was about 0.4 and 0.3 mW cm⁻² for the 550 and 650 nm excitation, respectively. Chl fluorescence images were detected at 740 nm with a 10 nm bandwidth interference filter (740FS10-25; Andover Corporation). The 740 nm emission is sufficiently shifted from chlorophyll absorption to be insensitive to reabsorption by the tissue. Color pictures of the bunches were obtained by RGB recombination of monochrome images acquired, under the white light of the xenon lamp, at the 680 (red), 546 (green) and 470 (blue) nm bands, selected by interference filters (680FS10-25, 546FS10-25 and 470FS10-25, respectively; Andover Corporation).

Image elaboration was performed at 16-bit resolution by using the Image-Pro Plus v.4.0 software (Media Cybernetics, Silver Spring, MD). Resulting images were converted to 8-bit (256 intensity levels) and presented in gray scale or pseudo colors.

By schematizing the grape skin as composed of two layers, an outermost layer, in which the Anth are accumulated, and an inner layer containing Chl (Scheme 1), the ChlF detected at 740 nm (at which no fluorescence reabsorption occurs) as function of the excitation wavelength λ is given by:

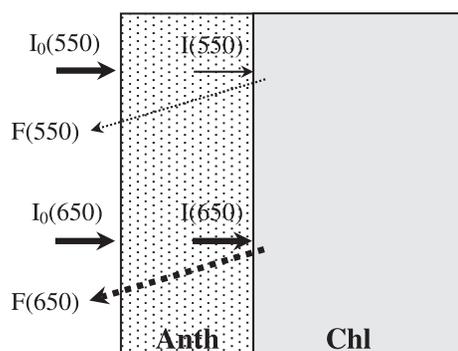
$$\text{ChlF}^{740}(\lambda) = I_0(\lambda) \cdot T_{\text{Anth}}(\lambda) \cdot (1 - 10^{-A_{\text{Chl}}(\lambda)}) \Phi_{\text{ChlF}} \cdot \gamma \quad (1)$$

where I_0 is the intensity of the excitation beam, T_{Anth} is the transmittance of the Anth layer, so that their product is equal to the

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Scheme 1. The grape skin is schematized as two layers: the outer layer with the Anth accumulation and the inner layer where Chl is present. I_0 and I are the light intensities impinging on the skin surface and reaching the Chl layer, respectively. The excitation beam at 650 nm is not attenuated by the Anth layer, then $I_0(650) = I(650)$, while $I(550)$ is reduced with respect to $I_0(550)$. Consequently, the detected Chl fluorescence with green excitation $F(550)$ is decreased with respect to red-excited fluorescence $F(650)$, proportionally to the skin Anth concentration. Thin and thick arrows represent lower and higher light fluxes, respectively.

intensity of light (I) reaching the Chl layer. A_{Chl} is the Chl absorbance, Φ is the ChlF quantum yield and γ is a factor depending on the detection system.

At 650 nm $T_{\text{Anth}}(650) = 1$, and then the Chl FER between red and green (550 nm) excitation is

$$\frac{\text{ChlF}^{740}(650)}{\text{ChlF}^{740}(550)} = \frac{1}{T_{\text{Anth}}(550)} \cdot \frac{I_0(650)}{I_0(550)} \cdot \frac{(1 - 10^{-A_{\text{Chl}}(650)})}{(1 - 10^{-A_{\text{Chl}}(550)})} \quad (2)$$

and by applying the Beer–Lambert's law ($A = \log T^{-1}$) we define the logarithm of FER ($\log \text{FER}$) as

$$\log \frac{\text{ChlF}^{740}(650)}{\text{ChlF}^{740}(550)} = A_{\text{Anth}}(550) + k \quad (3)$$

where

$$k = \log \frac{I_0(650) (1 - 10^{-A_{\text{Chl}}(650)})}{I_0(550) (1 - 10^{-A_{\text{Chl}}(550)})} \quad (4)$$

is due to the difference in the Chl absorbance, A_{Chl} , and light intensity, I_0 , at the two excitation bands, and is independent of the Anth content. Therefore, each pixel in the computed $\log \text{FER}$ image is related to the Anth concentration in the grape skin.

Immediately after detection of the fluorescence images, 10 berries per bunch with different skin color were selected. Caps of 5.5 mm diameter were cut from the imaged side of the berries with a cork borer and razorblade, frozen in liquid nitrogen and stored at -80°C until Anth extraction was performed.

Anth were extracted with acidified methanol-water (50% MeOH, 0.1% HCl) as described elsewhere (4). For each sample, the absorbance spectrum of the extract in a final volume of 3 mL was measured immediately upon extraction, from 300 to 800 nm, on a double beam Jasco spectrophotometer (mod. V560; Jasco, Tokyo, Japan). The absorbance at the maximal absorption band at about 530 nm, characteristic of the Anth compounds present in the Sangiovese cultivar, was used to quantitatively estimate the total Anth content. Absorbance values were converted in surface-based units and anthocyanin contents calculated on a skin surface basis by using the molar absorptivity at 530 nm of $28.5 \mu\text{mol}^{-1} \text{cm}^2$ of malvidin 3-*O*-glucoside (oenin), the major Sangiovese anthocyanin (7).

RESULTS AND DISCUSSION

The Chl fluorescence images of a whole grape bunch, acquired at 740 nm, under red and green excitations are reported in

Fig. 1A,B, respectively. For comparison, the color RGB image of the same bunch is shown in Fig. 1C. It can be noted that the fluorescence signal under red excitation is quite homogeneous among the different berries of the bunch (Fig. 1A), independently of the presence of Anth. On the contrary, the green excited fluorescence image shows large variation of intensity with higher and lower values for green and red berries, respectively (Fig. 1B vs 1C). As expected, the Chl fluorescence signal is weaker in berries with higher concentration of Anth due to a larger screening of green excitation light by these compounds. In order to give a quantitative distribution of

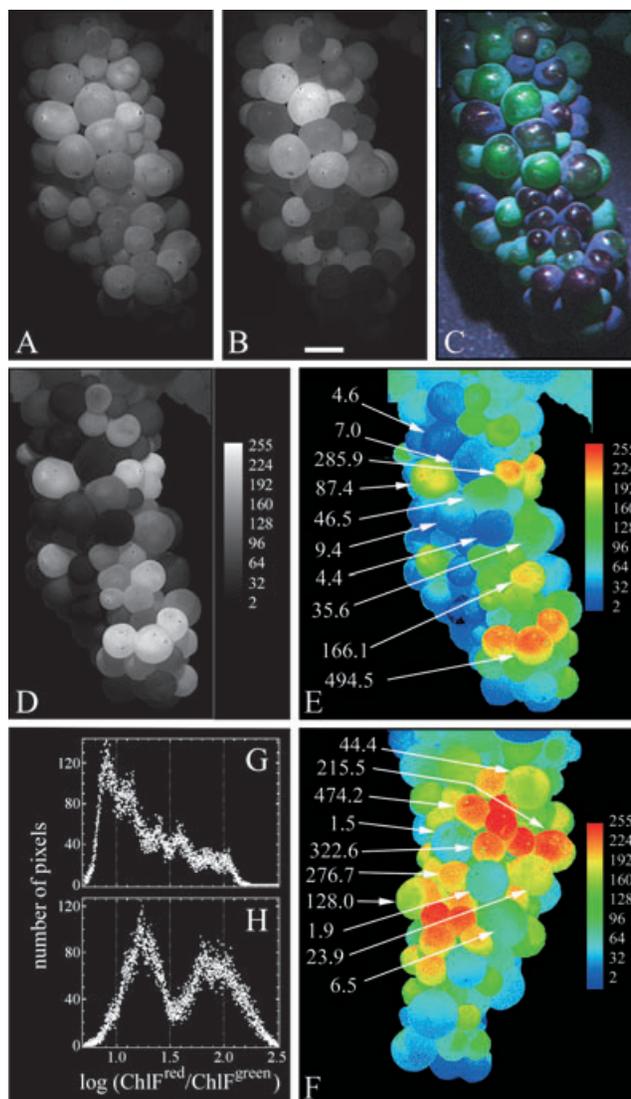


Figure 1. Chlorophyll fluorescence image of a grape bunch, acquired at 740 nm, under red (650 nm) (A) and green (550 nm) (B) excitation, bar = 1 cm. (C) RGB recombination of monochrome images acquired, under white light, at the 470, 546 and 680 nm bands. (D) Logarithm image of the ratio between the two red-excited and green-excited Chl fluorescence ($\log \text{FER}$) images of (A) and (B) respectively, converted to 256 intensity levels. (E) Pseudo-color representation of image in (D), intensity increases from blue to red, and Anth concentrations (nmol cm^{-2}) of selected berries determined on skin methanolic extracts of berries indicated by arrows (left-hand side). (F) $\log \text{FER}$ image, represented in pseudo-colors, of a second grape bunch. (G, H) Histograms of pixel distribution as function of $\log \text{FER}$ intensity for bunch in (E) and (F), respectively.

Anth throughout the grape bunch, the image of the logarithm of the ratio between red-excited and green-excited Chl fluorescence (logFER) was calculated and reported in Fig. 1D (for the theoretical basis of this calculation see also Agati *et al.* [8] and references therein). Further, in Fig. 1E the same logFER image is presented in false colors, with intensity increasing from blue to red, for a better visualization. Values of Anth concentration, expressed as nmol cm^{-2} , of selected berries determined by the spectrophotometric analysis of the skin methanolic extracts are also reported in Fig. 1E (left-hand side). The measured range of Anth skin content from 2 to 500 nmol cm^{-2} in surface-based units would correspond approximately to a concentration per berry mass of $5\text{--}1250 \text{ mg kg}^{-1}$.

For each selected berry, the average value of the logFER image intensity was used to calculate the average *in vivo* skin absorbance of Anth according to Eq. (3), in which k corresponds to the average logFER intensity of Anth-free greenest berries. The plot of skin absorbances against the Anth concentration determined from the corresponding skin extract resulted in a curvilinear relationship similar to that previously observed (4) in Pinot Noir and Pinot Meunier cultivars, using a different experimental approach (Fig. 2).

In Fig. 1F, the result of the fluorescence imaging analysis for a second grape bunch is presented. Again, there was a clear positive relationship between the logFER image intensity and the Anth content of berries. It is worth noting how the two logFER images of Fig. 1E,F demonstrate a marked difference in Anth content between the two grape bunches. In the second one (Fig. 1F), false color of berries was mostly in the yellow-red, rather than blue-green, indicating a larger content in Anth

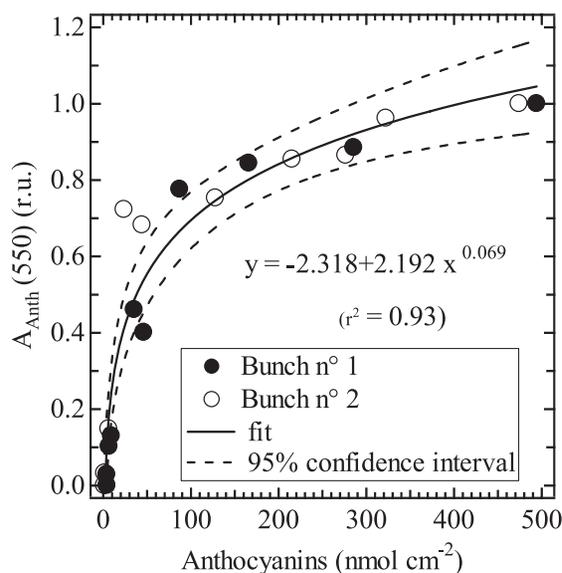


Figure 2. Relationship between the grape skin absorbance assessed nondestructively by the logFER imaging and the Anth concentration measured in the berry cap extracts. $A_{\text{Anth}}(550)$ was computed according to Eq. (3) (k was derived by the average logFER intensity over the Anth-free green berries for each bunch, and was 0.51 and 0.9 for bunches 1 and 2, respectively). Due to possible differences in the measuring distance and irradiance between the measurements of the two bunches, the recorded maximal $A_{\text{Anth}}(550)$ value of 1.667 for bunch 1 and of 1.768 for bunch 2, corresponding to 495 and 474 nmol cm^{-2} of anthocyanin, respectively, were normalized to the maximal values to obtain a single fit for both bunches.

with respect to the bunch represented in Fig. 1E. This can be also observed in the pixel distribution per intensity level reported in Fig. 1G,H, corresponding to bunches of Fig. 1E,F, respectively. The histogram relative to the grape bunch with the higher Anth content (Fig. 1H) is shifted toward higher logFER intensities and shows a second significant peak at around 1.8–2. The intensity analysis based on the pixel distribution of the logFER images can be therefore a suitable criterion to evaluate the precise stage of véraison and individual berry ripening dynamics. It was confirmed here that already at véraison individual berries could attain the level of Anth that will be present in the whole bunch at maturity. This accumulation is known to be under the control of water, light, temperature and sugar (9). These influences are still under debate at the local (berry) level. The accumulation of flavonols into the skin of white grape (*Vitis vinifera* L. cv. Pinot Blanc) berries exposed or nonexposed to sunlight was recently evaluated by the ChlF imaging technique, but using different excitation bands (10). So, the use of nondestructive imaging is a powerful means to classify berry samples for further clarification of the ripening physiological and biochemical processes in grape.

Image processing such as segmentation (11) can be also used to separate and measure areas of the grape bunch within a certain range of intensity. For example, applying this process to the two logFER images of Fig. 1E,F, we found that the relative bunch area with berry absorbances above a threshold of 1.5 was 25.7% and 52.8% for the first and second bunches, respectively.

We showed that the Chl fluorescence imaging method based on pigment screening of excitation is able to determine the distribution of Anth in whole grape bunches. On this basis, the development of suitable optoelectronic portable devices for the assessment of phenolic maturity directly in the vineyards can be foreseen.

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