Chlorophyll and carotenoid quantifications in white grape (Vitis vinifera L.) skins by reflectance spectroscopy

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Summary

In white grapes, chlorophylls and carotenoids play important roles in berry color and environmental interactions (e.g. radiative stresses). In this paper, easy, fast and low cost non-invasive reflectance methods have been tested and developed. Previously published indexes showed good performances for chlorophyll quantifications. However, in this work, new formulas able to discriminate chlorophylls a and b were also proposed. The wavelengths of major interest for the absorption detection were identified. Formulas based on the Gaussians half heights were proposed. In general, chlorophyll quantifications were obtained directly from reflectance spectra, while carotenoid absorption bands did not allow good reflectance correlations. However, the chlorophyll/carotenoid ratio (due to the pigments physiological linked roles) could be used to estimate carotenoid content. Their proportion changes during berry development, thus the index coefficients should be adapted in relation to the BBCH phenological phase. The obtained indexes demonstrated good correlations with the destructive quantifications. These methods could support further researches concerning cultivar classification and physiological studies.

Key words: photosynthetic pigments; non-invasive quantification; indexes; optical properties; grapevine.

Introduction

Chlorophylls and carotenoids are pigments of major importance in white grapes. The chlorophyll fundamental role in photosynthesis is well known, and the implication of this pigment class in berry sunburn symptoms has been recently suggested (Rustioni et al. 2014). While carotenoids achieve two main complementary and indispensable functions in the photosynthetic pathway of higher plants: light harvesting and photo-protection (Rustioni et al. 2015). In white grape berries, the skin color clearly change during ripening from green to yellow. The amount of chlorophylls and carotenoids and their proportion, as well as the melanin-like pigments and the catabolic products, mostly define the final color (Rustioni et al. 2015).

From the ecophysiological point of view, chlorophyll and carotenoid concentrations in plant tissues are also considered to be an indirect information concerning plant water stress and its ability to tolerate it (Filella et al. 1995, Lashbrooke et al. 2010, Moran et al. 2000).

Conventionally pigment determination requires tissue extraction by solvents and spectrophotometric determination. Nowadays nondestructive optical methods based on spectral indices and formulas have been developed and used (Gitelson et al. 2002). Spectroscopy techniques have been adopted to investigate fruit quality and plant physiological status for different varieties of grapes, apples, bananas, oranges and potatoes (Knee 1980, Merzlyak et al. 1999, Merzlyak and Chivkunova 2000, Rustioni et al. 2013, 2014). In grape skins the interaction of the radiation with cuticle, exocarp tissue and pigments, should be considered. It results finally in the pigment absorptions at specific bands in the visible part of the spectrum (Richer and Fukshansky 1996). In order to develop indices for nondestructive pigment content estimations, it is necessary to find spectral bands where reflectance is maximally sensitive to the compounds of interest and minimally sensitive to others (Zur et al. 2000). Considering chlorophylls...
and carotenoids, the first ones show two intense absorption bands in the blue (400-500 nm) and red (600-700 nm) regions while carotenoids and the xanthophylls mainly absorb into the green-blue wavelengths.

Different authors demonstrated that normalized reciprocal reflectance, in leaves (Gitelson and Merzlyak 1996, Gitelson et al. 2003, 2006, Merzlyak et al. 2003a) and apple fruits (Merzlyak et al. 1999, Merzlyak and Chivkunova 2000, Merzlyak et al. 2003b), at certain wavelengths, relate to pigment concentrations. For instance the indices for chlorophyll estimation based on the reflectance ratios $R_{500}/R_{700}$ and $R_{600}/R_{400}$ showed good quality performances in total chlorophyll quantification.

The aim of this work was to define easy, fast and low cost non-destructive indexes for chlorophyll and carotenoid quantifications in grape white berries. Published methods has been tested and new formulas are proposed in this paper.

**Material and Methods**

**Plant material and growth conditions:** The *Vitis vinifera* L. accessions used in this study are all grown in the same germplasm collection vineyard, located in the Lombardy region (Northern Italy), already described in a previous article (Rustioni et al. 2013). Sixteen white wine and table grape cultivars were selected: ‘Chardonnay’, ‘Italia’, ‘Matilde’, ‘Moscat Giallo’ (syn. ‘Goldmuskateller’), ‘Pedro Ximenez’, ‘Perle of Csaba’, ‘Pizzutello’ (syn. ‘Cronichon Blanc’), ‘Regina’ (syn. ‘Afu Ali’), ‘Regina dei Vigneti’ (syn. ‘Muscat Queen of Vineyards’), ‘Ribolla Gialla’, ‘Riesling’, ‘Rkatsiteli’, ‘Sultanina’, ‘Verdeho Blanco’, ‘Verdicchio’, ‘Zibibbo’ (syn. ‘Muscat of Alexandria’). One bunch for each accession was harvested at different BBCH phenological stages (Lorenz et al., 1995): pre-veraison (77 BBCH), veraison (81 BBCH) and ripening (89 BBCH). Four berries of each accession in each phenophase were studied in 2013 (a total of 192 berries were analyzed). On each berry we measured: the weight, the length and width, the skin chlorophyll and carotenoid concentrations (by wet chemistry analysis), and the reflectance spectra in four different positions, measured after epicuticular wax removal (Rustioni et al. 2012). The average of the four reflectance spectra of each berry was than correlate with the individual berry pigment composition.

**Berries optical properties detection:** On the whole 768 reflectance spectra were obtained using a customized spectrometer Jaz System (Ocean Optics, B.V.), completed with a channel with aDPU module and ILX511b detector, OFLV-3 filter, L2 lens and 50 mlst as installed options. A reflection probe QR600-7-VIS125 was coupled to the spectrophotometer. Each spectrum was set up to be the average of 20 spectra, directly calculated by the instrument. The spectra were calculated in percentage of reflectance (%R). A calibration with a reference spectrum was achieved by taking the light source switch on and by using a PTFE blank (WS-1. Diffuse Reflectance Standard, Ocean Optics, B.V.). Also a dark spectrum was taken into account with the light path blocked. Collected spectra ranged between 341 and 1025 nm and had a spectral resolution of about 0.3 nm. In this work, the visible spectral changes (450-750 nm) will be presented and discussed.

**Reflectance spectra elaborations:** For each spectrum, published indexes for carotenoid and chlorophyll estimations were tested. In particular, concerning chlorophylls, the formulas tested were:

\[
\text{Chl} = (R_{500}/R_{900}) \quad (\text{Merzlyak et al. 2003b});
\]

\[
\text{Cl}_1 = (1/R_{520}-1/R_{700})*R_{800} \quad (\text{Gitelson et al. 2003});
\]

\[
\text{Cl}_2 = (1/R_{640}-1/R_{500})*R_{800} \quad (\text{Gitelson et al. 2003});
\]

Concerning carotenoids, the considered indexes were:

\[
\text{CRI}_1 = (1/R_{520}-1/R_{700})*R_{800} \quad (\text{Merzlyak et al. 2003b});
\]

\[
\text{CRI}_2 = (1/R_{520}-1/R_{550})*R_{800} \quad (\text{Merzlyak et al. 2003b}).
\]

Visible spectra were also entirely considered (450-750 nm) with the purpose of developing new formulas able to distinguish chlorophylls a and b concentrations. The Table reports a summary of the indexes considered in this work.

**Reagents:** HPLC degree ethanol (95 %) and calcium carbonate were all Sigma.

**Preparation of extracts of chlorophylls, carotenoids and xanthophylls:** Berry fresh weights and diameters were measured. Pigment contents were determined from the same berry samples used for reflectance measurements. Four berries of each accession and each phenophase were squeezed to separate the exocarp from the pulp. Each exocarp was singularly grounded under liquid nitrogen, in a mortar with pestle, to a fine powder and 1.5 mg of calcium carbonate salt were

<table>
<thead>
<tr>
<th>References</th>
<th>Indexes</th>
<th>Formulas</th>
<th>Pigments</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Merzlyak et al. 2003b</td>
<td>Chl Index</td>
<td>R800/R678</td>
<td>Total chlorophylls</td>
<td>0.594</td>
</tr>
<tr>
<td>Gitelson 2003</td>
<td>Cl 1</td>
<td>(1/R700-1/R800)*R800</td>
<td>Total chlorophylls</td>
<td>0.214</td>
</tr>
<tr>
<td>Gitelson 2003</td>
<td>Cl 2</td>
<td>(1/R640-1/R800)*R800</td>
<td>Total chlorophylls</td>
<td>0.482</td>
</tr>
<tr>
<td>Merzlyak et al. 2003b</td>
<td>CRI 1</td>
<td>(1/R520-1/R700)*R800</td>
<td>Carotenoids</td>
<td>0.051</td>
</tr>
<tr>
<td>Merzlyak et al. 2003b</td>
<td>CRI 2</td>
<td>(1/R520-1/R550)*R800</td>
<td>Carotenoids</td>
<td>0.021</td>
</tr>
<tr>
<td>Present work</td>
<td>Car Index</td>
<td>R800/R530</td>
<td>Carotenoids</td>
<td>0.073</td>
</tr>
<tr>
<td>Present work</td>
<td>Car</td>
<td>a*C+b</td>
<td>Carotenoids</td>
<td>0.726</td>
</tr>
<tr>
<td>Present work</td>
<td>C_1</td>
<td>log(R800/R675)-(R800/R660)</td>
<td>Chlorophyll a</td>
<td>0.661</td>
</tr>
<tr>
<td>Present work</td>
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<td>log(R800/R650)-(R800/R630)</td>
<td>Chlorophyll b</td>
<td>0.402</td>
</tr>
<tr>
<td>Present work</td>
<td>C_1</td>
<td>C_1 + C_1</td>
<td>Total chlorophylls</td>
<td>0.626</td>
</tr>
</tbody>
</table>
added during tissue grinding to prevent the formation of pheophytins, during chlorophyll extraction and to neutralize acids in tissue samples to avoid cis/trans isomerization when extracting carotenoids (Van Den Berg et al. 2000; Kamffer et al. 2010).

The grounded powder, combined with three washings of the pestle and the mortar (each of about 1.5 mL of ethanol 90%) were poured in a Falcon tube and extracted by a total of 5 mL of solvent (ethanol 95%).

All procedures were carried out in subdued green light to minimize light associated degradations of chlorophylls and carotenoids (Kamffer et al. 2010). After 20 min the homogenate was centrifuged at 10000 r.p.m. for 10 minutes to make the extract fully transparent. The supernatants were poured in a new Falcon tube and adjusted to a final volume of 5 mL with ethanol 95%. The spectrum was recorded at 470 nm, 648.6 nm and 664.2 nm by a Jasco 7,800 spectrophotometer. Specific absorption coefficient and formulas reported by Lichtenthaler (1987) were used to calculate chlorophyll \( a \), \( b \), \( a+b \) and carotenoid + xanthophyll concentrations.

The berry surface was calculated by the measured diameters. Berries were compared to prolate or oblate spheroids (\( a=b>c \) in oblate ellipsoid of revolution and \( a=b<c \) in prolate ellipsoid of revolution, where \( a \), \( b \) and \( c \) are the semi-principal axes length). The surfaces (\( S \)) were calculated as follow:

\[
S\text{ (oblate)} \approx 2\pi \left[ a^2 + (c^2 - a^2 \arctanh \left( \frac{a}{c} \right)) / \sin \left( \frac{\pi}{2} \right) \right] \\
S\text{ (prolate)} \approx 2\pi \left[ \frac{c^2 + a^2 \arctanh \left( \frac{c}{a} \right)}{\sin \left( \frac{\pi}{2} \right)} \right] \\
\phi = \arccos \left( \frac{c}{a} \right)
\]

Pigment concentrations were converted in \( \mu g \cdot cm^{-2} \) to be compared with the optical properties of the berry surface.

**Theoretical absorbance bands:** The theoretical absorbance bands in Fig. 1, were calculated as Gaussian functions. The absorption maxima were fixed at the experimental wavelength peaks and the intensity was calculated considering the molar extinction coefficient proposed by Lichtenthaler (1987). Each obtained Gaussian, was then multiplied by the average experimental quantification of chlorophyll \( a \) and \( b \) respectively. In this way, it was possible to hypothesize the absorption contribution of each pigment in the red spectra region.

**Statistical analysis:** Each berry was singularly considered. The pigment quantification obtained by wet chemistry analysis was compared with the average of the four related spectra measured on the same berry by regression models by using SPSS® software (PASW Statistics 21 version, SPSS Inc. Chicago, Illinois).

**Results and Discussion**

**Grape pigment composition:** In pre-veraison, chlorophyll \( a \) content ranged between 0.98 and 3.97 \( \mu g \cdot cm^{-2} \), with a medium value of 2.12 \( \mu g \cdot cm^{-2} \). During veraison the concentration ranged between 0.27 and 2.91 \( \mu g \cdot cm^{-2} \), with a medium value of 1.35 \( \mu g \cdot cm^{-2} \). At ripening, pigment content ranged between 0.01 and 2.89 \( \mu g \cdot cm^{-2} \); the average value was 0.97 \( \mu g \cdot cm^{-2} \). Chlorophyll \( b \) was less concentrated. In pre-veraison the average content was 1.4 \( \mu g \cdot cm^{-2} \). During the other phenological stages chlorophyll \( b \) dropped below about 0.6 \( \mu g \cdot cm^{-2} \). A general decrease in total chlorophyll was visible from pre-veraison 3.5 \( \mu g \cdot cm^{-2} \) to harvest 1.5 \( \mu g \cdot cm^{-2} \). This result agrees with other research works (Baumes et al. 2002; Downey et al. 2004). Also carotenoids decreased during berry development. At the pre-veraison phenological stage "berries beginning to touch" (=BBCH 77) the average content among the samples was 0.575 \( \mu g \cdot cm^{-2} \), at stage 81, the average was 0.467 \( \mu g \cdot cm^{-2} \); and at ripening (=BBCH 89) the average decreased until 0.381 \( \mu g \cdot cm^{-2} \).

**Development of Chlorophyll index:** All reflectance (R) spectra collected in this work were converted in their reciprocal (1/R), considering that the reciprocal reflectance at certain wavelengths relates to pigment concentrations (Solovchenko et al. 2010). To obtain a scale normalization, facilitating the data comparison, \( R_{670}/R_{800} \) spectra were calculated to better analyse the maximum peaks of both chlorophylls \( a \) and \( b \). The normalization wavelength (800 nm) was selected in agreement with literature (Merklyak et al. 2003b, Gitelson 2003) because plant pigments do not absorb at this wavelength. A particular detail of the spectral range 600-750 nm (obtained by the average values of all the 768 spectra) is shown in figure 1 (dark area). To clarify the spectrum interpretation, the same figure also shows the theoretical absorbance contributions of chlorophyll \( a \), \( b \) and their sum (total chlorophyll), which are drawn in the figure as dashed, dotted and solid grey lines respectively.

The chromophore concentration should be proportional to the area under the Gaussian curve. Gaussian's area is proportional to the semi width, in correspondence of half of its height. However, the reflectance band width was quite similar between our samples. Thus, our aim was to find the Gaussian's half height cleaned from the baseline, as main indicator of the light absorption intensity. \( R_{670}/R_{800} \) was found to be the maximum reflectance edge of chloro-

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Fig. 1: Chlorophyll indexes explanation. The dark area represents the average (768 measurements) reciprocal reflectance multiplied per the 800 nm reflectance value. The gray lines draw the expected absorption bands of the pigments. The wavelengths of interest for the half height are indicated.
Chlorophyll a and R\textsubscript{800}/R\textsubscript{650} was established to be that one of chlorophyll b. The absorbance at 660 nm and 630 nm were the best descriptors of the half height for chlorophylls a and b respectively. The differences of these values should then be related to the pigment concentrations. Thus we calculated the half height as (R\textsubscript{800}/R\textsubscript{575})-(R\textsubscript{900}/R\textsubscript{800}) for chlorophyll a and (R\textsubscript{600}/R\textsubscript{650})-(R\textsubscript{900}/R\textsubscript{600}) for chlorophyll b.

It has been demonstrated that, in the visible spectral range, the relation between reflectance and leaf chlorophyll content is non linear (Buschmann and Nagel 1993, Gittelson and Merzlyak 1994). The logarithm of the reciprocal reflectance has been proven to be a good predictor for chlorophyll in fresh leaves (Yoder and Pettigrew-Crossby 1995). Chlorophyll indexes were, thus, calculated as follows:

\[
C_1I = \log[(R_{600}/R_{575})-(R_{900}/R_{800})]
\]
\[
C_2I = \log[(R_{650}/R_{600})-(R_{600}/R_{530})]
\]
\[
C_3I = C_1I + C_2I
\]

C\textsubscript{1}I: chlorophyll a index; C\textsubscript{2}I: chlorophyll b index; C\textsubscript{3}I: total chlorophylls index; R\textsubscript{x}: reflectance at x wavelength.

\textbf{Indexes performance evaluation:} In Fig. 2a the comparison is described between C\textsubscript{1}I and the measured chlorophyll a content (µg·cm\textsuperscript{-2}) during pre veraison, veraison and harvest. A linear model was able to define this relationship, with an r\textsuperscript{2} = 0.66. Thus, it is possible to estimate the chlorophyll concentration by the formula:

\[
C_E = 1.7235 * C_I + 1.3185
\]

C\textsubscript{1}I: chlorophyll a index; C\textsubscript{E}: estimated chlorophyll a concentration. We used the same approach to estimate the chlorophyll b (Fig. 2b). A lower coefficient of determination was detected (r\textsuperscript{2} = 0.40), probably due to the lower pigment concentration.

\[
C_E = 0.9591 * C_I + 0.9385
\]

C\textsubscript{1}I: chlorophyll b index; C\textsubscript{E}: estimated chlorophyll b concentration. The sum of C\textsubscript{1}I and C\textsubscript{2}I provided a good estimation of the total chlorophyll contents. The C\textsubscript{3}I appeared to be linearly correlated to the total chlorophyll concentrations with an r\textsuperscript{2} = 0.63 (Fig. 2c).

Other indexes proposed by the literature were also tested (Table). Among them the index proposed by Merzlyak et al. (2003b) was the most sensitive for total chlorophyll estimation (r\textsuperscript{2} = 0.59). The others were not able to provide high correlations between reflectance spectra and pigment content. It is worth noting that these indexes were developed on apple fruits and on leaves. In grapes, a general shift in pigments absorption bands, as well as possible interferences by different compounds should be the reasons for the correlation lack. Saturation at certain wavelengths (e.g. in the carotenoids absorption region) should be also taken into account.

\textbf{Carotenoids estimation:} For carotenoid estimation, we tested different formulas. As an example, we calculated an index based on the same model of Merzlyak et al. (2003b). Thus, the ratio R\textsubscript{600}/R\textsubscript{530} was calculated, but no correlations were obtained (r\textsuperscript{2} = 0.07). In this case, we trust that the main problem is related to the carotenoid absorption region, which overlaps the one of many other grape pigments. Moreover, carotenoid absorption bands are closer to the lamp emission tail and, thus, the reflectance measurement is less precise with the selected equipment. For these reasons it was not possible to identify direct relations between reflectance spectra and carotenoid concentrations. Thus an alternative approach was followed by studying the relationship between carotenoids and chlorophyll a. In fact, chlorophyll and carotenoid concentrations are expected to be linked due to their photosynthetic role, as well as to their common cell localization. Also considering different cultivars, the correlation between these two pigments resulted very high. At the BBCH phenophases 77, 81 and 89 r\textsuperscript{2} were 0.73, 0.70 and 0.52 respectively (Fig. 3a, b, c). On this base it was possible to obtain a carotenoid index (Carl), calibrated by the regression curve:

\[
Carl = a * C_I + b
\]

Carl: carotenoid index; C\textsubscript{1}I: Chlorophyll a Index; a: slope; b: y-intercept. It is interesting to note that during fruit development, the ratio between chlorophylls and carotenoids decrease, as demonstrated by the slope values in the regression curves (Fig. 3). This finding is in agreement with our previous research concerning the effect of anthocyanin absence on white berry grape (Rustioni et al. 2015).

Fig. 2: Chlorophyll indexes performances. Correlations between the pigment indexes (average of 4 replications/berry) and the measured concentration (192 berries in total) of respectively: (a) chlorophyll a; (b) chlorophyll b; (c) total chlorophylls.
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it is possible to calculate the best coefficients (slope and y-intercepts) at each BBCH phenophase in the range from 77 (pre-veraison: "berries beginning to touch") to 89 (ripening: "berries ripe for harvest"):

\[
a = -0.0004 \times PP_{BBCH}^2 + 0.0562 PP_{BBCH} - 1.5433 \\
b = 0.0001 \times PP_{BBCH}^2 + 0.0224 PP_{BBCH} - 1.4189
\]

a: slope; b: y-intercept; \(PP_{BBCH}\): BBCH phenophase. Introducing these phenological dependent coefficients, it is possible to obtain a carotenoid index (CarI) based on the chlorophyll \(a\) index calculated on the reflectance spectra. The correlation between CarI and the carotenoid concentrations quantified by wet chemistry analysis is shown in Fig. 5 and it has an \(r^2 = 0.73\).

**Conclusions**

Chlorophylls and carotenoids play important roles in berry color and eco-physiology. The reflectance indexes are non-invasive methods which allow a fast data collection during the short period of phenological interest. The new formulas, as well as the published index performanc-es, presented in this paper could give alternative methods to support further studies concerning cultivar classification and/or physiological roles in abiotic stresses (Rustioni et al. 2015).

Fig. 3: Correlation between the chlorophyll \(a\) index (average of 4 replications/berry) and the carotenoid quantification (192 berries in total) among berry development. (a): BBCH 077; (b): BBCH 081; (c): BBCH 089. During ripening, the regression line becomes flatter, indicating a decrease in the chlorophyll/carotenoid ratio.

Fig. 4: Carotenoid index coefficients. Changes in slope and y-intercept during berry development. Dots represent the slopes and y-intercepts shown in Fig. 3. By using the regression curve it is possible to calculate the best coefficient at each BBCH phenological stage.

Fig. 5: Carotenoid index (CarI) performances. Correlation between the pigment index (average of 4 replications/berry) and the experimental quantification (192 berries in total).
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