Improved fluorescence-based evaluation of flavonoid in red and white winegrape cultivars

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Short running title: Non-destructive anthocyanin and flavonol measures

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Abstract

Background and Aims: Modern viticulture requires robust, fast, non-destructive methods to assess berry composition. We tested a chlorophyll fluorescence screening method to estimate berry phenolic substances.

Methods and Results: We focused on anthocyanin and flavonol in red and white cultivars. The ANTH_RG index was dependent on the cultivar anthocyanin profile. In Nebbiolo, in which di-hydroxylated anthocyanins prevail, ANTH_RG was 2.4 times higher than in Barbera, in which tri-hydroxylated anthocyanins prevail. Considering the profiles of the two cultivars at similar anthocyanin concentration and their relative in vitro absorbance, a bathochromic shift of 10 nm emerged, which can explain the different

screening effect exerted by anthocyanin on chlorophyll fluorescence. We propose the calibration of a new spectroscopic index, the FLAV_UV, in coloured and white berries, finding good correlation with flavonol concentration determined analytically (R^2 higher than 0.7).

Conclusions: Spectroscopic indices can estimate the concentration of anthocyanin and flavonol in grape berries.

Significance of the Study: A calibration curve for Nebbiolo, which has a distinctive anthocyanin profile, and the calibration of a new index, the FLAV_UV, able to estimate flavonol concentration in both red and white cultivars, are described for the first time. These indices can effectively be applied for non-destructive assessment of grape flavonoid.

Keywords*: anthocyanin, flavonol, non-destructive measurements,* Vitis vinifera, *TSS*

Introduction

In coloured grapes the anthocyanin concentration is an important technological parameter to define harvest time and procedure. In the vineyard, the variability in the concentration of berry anthocyanin is higher than that due to sugar and acid concentration (Bramley 2005). *Vitis vinifera* cultivars have a genetically determined capacity to accumulate a high or low quantity of anthocyanin, however, the concentration of individual anthocyanins (the profile) can vary moderately as a function of the environmental conditions, one of which is microclimate (Guidoni et al. 2008, Chorti et al. 2010). These factors can contribute to considerable variability that can result in the application of different types of technological techniques in winemaking to influence the final wine composition. Grapegrowers and vineyard consultants have been aware for some time of the importance of detailed determination of berry anthocyanin concentration to plan harvest time and to choose the most appropriate winemaking techniques. In addition, the number of cooperatives or of associated producers interested to pay viticulturists based on berry composition is increasing. Sampling and routine laboratory analysis required to cover the wide within-vineyard variability are time consuming, thus expensive, even in small vineyards. The possibility of estimating the anthocyanin concentration quickly and contextually to the measurement itself greatly helps in taking decisions at harvest. Portable optical sensors can help in this direction, also by providing an immediate estimation of anthocyanin through on-going measurements (Bramley et al. 2011). For research purposes, the use of a portable instrument able to measure in a few minutes a large number of berries greatly increases the coverage and significance of the experimental sampling, as well.

Chlorophyll fluorescence screening methods have been recently developed and applied in viticulture (Cerovic et al. 2008, Ben Ghozlen et al. 2010). The theoretical principles were developed and applied by Agati and co-workers in 2007 to Pinot Noir berries that accumulate a limited quantity of anthocyanin. Briefly, the anthocyanin measurement is an indirect measure, based on the interference effect exerted by anthocyanin absorbance over chlorophyll fluorescence excitation in the green and in the red spectral regions. A commercial instrument has been available since 2008 (Cerovic et al. 2008) and since 2010, an improved version of the sensor was described (Ben Ghozlen et al. 2008) and commercialised. The sensor was tested on Shiraz grapes able to achieve an anthocyanin concentration of 2.5 mg/g (Bramley et al. 2011), that is a higher concentration than that reported for Pinot Noir. The studies devoted to the use of the sensor for measurement of grape anthocyanin concentration proposed several calibration curves to fit spectroscopic to analytical measurements. Most authors related the ANTH_RG index—the logarithm of the ratio of the far red fluorescence under red and that under green excitation, the log FER = log (FRF_R/FRF_G)—to anthocyanin concentration measured analytically. In 2011 Tuccio and co-workers observed that in Aleatico grapes the best fitting was obtained with an exponential curve ($R^2 = 0.88$), even though a linear regression would fit anthocyanin concentration measured analytically to spectroscopic data with $R^2 = 0.86$, as well. Bramley and co-workers (2011) proposed a second-degree polynomial curve ($R^2 = 0.78$) to fit anthocyanin concentration to spectroscopic measurements in Shiraz. A significant correlation ($R^2 = 0.74$) with an exponential curve function was obtained in Tempranillo (Baluja et al 2012). Different authors (Bramley et al. 2011; Baluja et al. 2012) obtained a significant correlation between anthocyanin concentration and the FERARI spectroscopic index (log

5000/FRF_R), as well. In addition, the chlorophyll fluorescence screening method was successfully applied to evaluate anthocyanin concentration in tablegrapes (Bahar et al. 2012). From these studies, it emerged that the predictive value of the calibration curves may differ as a function of the cultivar studied and this may depend on the anthocyanin profile specific for each cultivar. For this reason, in the present work we validated the use of the sensor to measure the anthocyanin accumulation in the skins of two *V. vinifera* Italian cultivars, Barbera and Nebbiolo, that differ widely in both anthocyanin concentration and profile. Barbera accumulates anthocyanin up to 2 g/kg with the prevalence of tri-hydroxylated forms (Ferrandino and Guidoni 2010), similar to Shiraz, Pinot Noir and Aleatico. Nebbiolo accumulates a reduced quantity of anthocyanin (up to 0.8 g/kg) with the prevalence of di-hydroxylated forms, peonidin 3-O-glucoside being the main anthocyanin (Ferrandino et al. 2012).

Flavonol is present in berry skins of both coloured and white berries, and ranges in concentration from few milligrams per kg up to about 200 mg/kg depending on the cultivar (Mattivi et al. 2007, Castillo-Muñoz et al. 2010, Ferrandino et al. 2012), on light exposure (Downey et al. 2004), and on vintage and water availability (Zarrouk et al. 2012). The anti-scavenging and antioxidant properties of berries, particularly of white berries, largely rely on flavonol; quercetin glycosides, in particular, are among the strongest antioxidants known in grapes. Flavonol accumulates in plant epidermal organs, included berry skins, in response to abiotic stress to protect the underlying tissues against UV-induced damage (Kolb et al. 2003). Recent work has shown that flavonol is involved in the plant response to biotic stress as well, contributing to the control of leaf sensitivity to *Plasmopara viticola* (Agati et al. 2008, Latouche et al. 2013).

The monitoring of plant response to biotic and abiotic stress conditions through the assessment of the trend in flavonol accumulation over the season could become a valuable, early tool to predict the state of vine health and to study the kinetics of flavonol accumulation. Flavonol is also important in winemaking, contributing to colour stabilisation through co-pigmentation phenomena of anthocyanin (Boulton 2001); in white wine processing, flavonol contributes to increase the concentration of phenolic substances in the resulting wines, with a positive effect on storage and antioxidant properties (Hernanz et al. 2007). Thus, the rapid assessment of flavonol could become an important feature to add to the routine parameters of juice evaluation, such as °Brix, titratable acidity and pH, particularly in white cultivars. The spectroscopic FLAV index, the logarithm of the ratio between the far-red fluorescence under red and that under UV excitation (Agati et al. 2011), was calibrated for estimating berry flavonol concentration in a limited number of cultivars. Among the studies dealing with the detection of flavonol in grape berries by spectroscopy (Kolb et al. 2003, Lenk et al. 2007) only a few were performed with a commercially available instrument on the coloured grape Aleatico (Tuccio et al. 2008) and on the white Vermentino (Agati et al. 2013). For all these reasons, in the present work we investigated the possibility of assessing non-destructively the flavonol accumulation in the coloured grape Barbera and in several white cultivars. Furthermore, we propose and calibrate here for the first time a new spectroscopic index, the FLAV_UV, calculated as the decadic logarithm of the inverted far-red fluorescence signal under UV excitation, which is related to the berry flavonol concentration.

Nowadays, spectroscopic methods in viticulture increasingly require multi-parametric instruments able to measure simultaneously as many parameters as possible. For this reason, the simple fluorescence emission ratio (SFR_R, equal to the far-red-

fluorescence_R/red-fluorescence_R ratio) was successfully tested to estimate TSS, being linearly and inversely correlated to TSS in grapes from Pinot Noir, Pinot Meunier and Chardonnay with $R^2 = 0.85$ (Ben Ghozlen et al. 2010). In the present work, we measured the SFR_R in white Nascetta and Chardonnay berries and we related the temporal changes of this index to the beginning of ripening (start of chlorophyll degradation in berries) and to TSS accumulation.

Materials and methods

In 2008 and 2009, Barbera and Nebbiolo anthocyanin was evaluated by the fluorescence excitation screening method with the Multiplex sensor (ForceA, Paris, France). In 2008, measurements on Barbera berries were taken starting 20 days after veraison in a vineyard located at Camporotondo, Agliano (Asti Province, Piedmont, north-west Italy), known to produce high quality grapes (the so called Super Barbera, Table 1). At commercial harvest, berries from ten other Barbera vineyards in the area, representing a wide range of grape composition (from low to high anthocyanin concentration at harvest) were measured spectroscopically and analytically. In 2009, measurements were repeated in the vineyard of Camporotondo (from early veraison onwards, three dates) and in another six vineyards chosen among those studied during the previous year (Table 1). Measurements for Nebbiolo were taken following the same timing (from 20 days after veraison onwards in 2008 and at early veraison in 2009) on berries from the Cannubi hill [Damilano cellar, Barolo, Cuneo Province (CN), Italy]. In 2008, spectroscopic data (FLAV index) of Barbera grapes from the Camporotondo vineyard and from the other ten vineyards sampled were correlated to flavonol concentration analytically measured. In 2010, the FLAV index and the newly proposed FLAV_UV index were measured with a Multiplex3 sensor on berries of several white berry cultivars from the collection vineyard of Grinzane

Cavour (CN; 44° 39'09.0" N; 7° 59 '41.0" E). In 2011, measurements were made on Chardonnay and Nascetta grapes. Chardonnay grapes were collected in a Barolo (CN) vineyard whereas Nascetta grapes were collected in a Sinio (Serralunga d'Alba, CN) vineyard (Table 1). In each vineyard, three rows, located at the top, in the middle and at the bottom of the hill, were chosen. On each row, measurements were taken on the bunches of 25 consecutive vines (10 vines in the Grinzane Cavour collection vineyard) from the two sides of the row and from the upper, the central and the basal part of the bunch. About 96 bunches (32 for each replicate) were flashed with the optical sensor, using the 8 cm diameter mask. From the flashed bunches, groups of two–three berries were collected and stored in portable refrigerators; once taken to the laboratory, subgroups of 20 berries each were immediately extracted for phenolic substances and stored at -20°C until further analysis. The remaining berries were crushed to measure TSS (ATAGO digital refractometer, Atago, Tokyo, Japan).

Calculation of spectroscopic indices

Spectroscopic indices were calculated in accordance with previously published papers (Table 2). The indices ANTH_RG and FERARI were used to estimate anthocyanin concentration; FLAV and SFR_R were related to flavonol and to TSS, respectively.

In addition, we calculated a new index, the FLAV_UV accordingly to the following formula:

$$FLAV\_UV = \log (1/FRF\_UV).$$

Similar to the FERARI index, which is a simplified spectroscopic estimation of anthocyanin as it exclusively takes into account the far-red chlorophyll fluorescence after

180 excitation in the red, we propose for the first time an index exclusively based on the
181 screening effect of flavonol on the chlorophyll far-red fluorescence after excitation in the
182 UV, to estimate the berry flavonol concentration. Before calculations, signals were
183 corrected for residual electronic noise and normalised to a fluorescence standard (blue
184 plastic foil, Force-A).

185 *Fluorescence spectroscopy of red grape berries*

186 Excitation spectra of chlorophyll fluorescence of intact grape berries were acquired by a
187 spectrofluorimeter equipped with an optical fiber (FluorMax II, Horiba Jobin Yvon,
188 Edison, NJ, USA) with emission set at 685 nm. Measurements were taken from the berry
189 apex and repeated, on the same area, after removing the skin. The in situ absorption
190 spectrum of the berry skin was calculated as the logarithm of the fluorescence excitation
191 ratio (FER) between skin-devoid berries and whole berries, as previously described
192 (Agati et al. 2005).

193

194 *Measurement of anthocyanin and flavonol concentration*

195 All spectroscopic measurements obtained in the field were fitted against laboratory
196 analysis of the same berries measured by the sensor, following previously published
197 methods used in our laboratory, by spectrophotometry (anthocyanin) or by HPLC-diode
198 array detector (DAD) (flavonol) (Ferrandino and Guidoni 2010). In brief, skin was
199 separated from pulp and seeds and immersed in a pH 3.2 buffer containing 2 g/L of
200 $Na_2S_2O_5$; after extraction of skin phenolic substances (at 30°C for 3 days), anthocyanin
201 was measured by spectrophotometry, by diluting the grape skin extract with ethanol:
202 water: HCl (70:30:1). Absorbance units at 520 nm were converted into mg/L according
203 to a calibration curve prepared for malvidin 3-O-glucoside chloride (Extrasynthèse,

Genay, France). Anthocyanin concentration was expressed as mg/kg of berries which is the best way to express measurements for applicative purposes. The anthocyanin profile of grape skin extracts was assessed by prior separation of anthocyanin on a 300 mg C18 cartridge. Anthocyanin was analysed by HPLC-DAD (Perkin-Elmer series 200-L pump) equipped with a Puropsher STAR RP-100 RP-18 5 µm (25 x 0.4 cm ID) and a pre-column (Puropsher RP-18, 5 µm), all from Merck (Darmstadt, Germany). Formic acid:water (10:90 v/v) and formic acid:methanol:water (10:50:40 v/v/v) were solvents A and B, respectively, with a flow rate of 1 ml/min and a gradient between 28 and 90% of B over 53 min (Di Stefano and Cravero 1991).

For flavonol measurement, the grape skin extracts were diluted 1.1 with 1 mol/L phosphoric acid, filtered through 0.2 µm GHP membrane filters (Pall Corporation, New York, NY, USA) in dark glass vials and injected into a HPLC-DAD (Perkin-Elmer) equipped with a LiChrosphere 100 RP-18 5 µm (25 x 0.4 cm ID) column with a LiChrocart C18 guard column (Merck). The chromatographic analysis followed a previously published procedure (Ferrandino et al. 2012); briefly, peaks were separated with solvent A (phosphoric acid 10^{-3} mol/L) and solvent B (CH_3OH 100%), establishing a gradient between 5 and 100% of solvent B over 49 min at a flow rate of 0.48 mL/min. The DAD was set at an acquisition range of 200–700 nm. Flavonol was detected at 360 nm and identified using pure standards [quercetin 3-O-glucopyranoside, myricetin 3-O-glucopyranoside and kaempferol 3-O-glucoside (Extrasynthèse)] when available and by comparing retention times and UV-VIS absorption data with those found in the literature for quercetin 3-O-glucuronide (Kammerer et al. 2004, Downey and Rochfort 2008). The concentration of each flavonol was calculated through the external standard method and expressed as mg equivalents of quercetin 3-O-glucoside per kilogram of berries. The

concentration of flavonol was obtained by summing the concentration of the individual flavonols.

Absorbance spectra simulation of grape anthocyanin extracts

The absorbance spectra of the anthocyanin mixtures of the two cultivars were calculated by taking into account their relative composition at a similar concentration of about 750 mg/kg of berries. The shape of the absorbance spectrum of individual anthocyanins and acylated-anthocyanins in formic acid:methanol:water (10:50:40, v/v/v) was derived by the HPLC-DAD analysis of skin berry extracts. Each spectrum, normalised to the maximum, was multiplied by the corresponding extinction coefficient at pH 3.1, as reported by Cabrita et al. (2000). For acylated compounds, the hyperchromic shift induced by acylation (Davies and Mazza 1993, Gris et al. 2007) was considered. Moreover, spectra of individual anthocyanins were weighted considering the proportion of each anthocyanin over the anthocyanin total concentration (the profile, as reported in Table 3). At each wavelength from 400 to 700 nm absorbance of all compounds was summed to obtain the total absorbance of the extracts.

Results and discussion

Calibration of spectroscopic indices with chemical analysis **Spectroscopic index calibration in Barbera and Nebbiolo for anthocyanin.** As previously described, the relation between ANTH_RG and anthocyanin concentration in Barbera berries was studied in an interval of 18–1800 mg/kg of fresh berries; it was represented by a complex curve, the difference between two exponential equations: ANTH_RG = 0.0597 – 8.85 exp (-0.0026x) – exp (0.0024x) (Ferrandino et al. 2012). This mathematical relation showed that within 350 mg/kg the correlation between the analytical data and spectroscopic index

252 was positive, whereas after this threshold it became negative, suggesting a non-univocal
253 relation between the two sets of data, as two different values of anthocyanin concentration
254 corresponded to a unique spectroscopic index value. Above an anthocyanin concentration
255 of 325 mg/kg, however, a second degree equation (ANTH_RG = 7.75E-08x^2 - 0.0003x +
256 0.4337; R^2 = 0.72) fitted the Barbera set of data (Figure 1). This second-degree equation
257 expressed a relationship between analytical and spectroscopic data similar to that
258 proposed for Shiraz (Bramley et al. 2011).
259
260 We examined the relationship between ANTH_RG and anthocyanin
261 concentration measured spectrophotometrically in Nebbiolo in a concentration interval of
262 270–770 mg/kg of berries (Figure 1). In this cultivar, above 270 mg/kg, the relation
263 between spectroscopic measurement and chemical analysis was also represented by a
264 second-degree equation (ANTH_RG = 3.77E-08x^2 -0.0004x + 0.9146; R^2 = 0.55).
265 The two fitting curves differed markedly as to the constant term value (Figure 1),
266 suggesting that the anthocyanin profile could influence the relation existing between
267 spectroscopic measurement and chemical analysis. Indeed, Barbera grapes, besides
268 showing an anthocyanin concentration higher than that of Nebbiolo grapes, also showed
269 a prevalence of tri-hydroxylated anthocyanins, in line with most *V. vinifera* cultivars.
270 Among the numerous grapevine cultivars, the anthocyanin profile of Nebbiolo grapes is
271 distinctive: in fact, it shows the uncommon prevalence of di-hydroxylated anthocyanins,
272 with peonidin 3-O-glucoside as the main form (Guidoni et al. 2008, Ferrandino et al.
273 2012). Moreover, the incidence of acylated forms as a proportion of the anthocyanin is
274 different, as in Barbera acylated anthocyanins account for about 20% of the anthocyanin

275 whereas in Nebbiolo they account for about 8% at harvest [Table 3 (Ferrandino et al.
276 2012)].

277 The absorption spectrum of tri-hydroxylated anthocyanins shows a bathochromic
278 shift of about 10 nm with respect to that of the di-hydroxylated anthocyanins (Cabrita et
279 al. 2000). The simulation of absorbance spectra of extracts of Barbera and Nebbiolo grape
280 skin (Figure 2a) confirmed the presence of a bathochromic shift of about 10 nm (peak
281 wavelength at 526 nm for Barbera and 516 nm for Nebbiolo). Although the calculation
282 refers to an in vitro approximation, it can provide an interpretation for the large shift (2.4
283 times on average) in the anthocyanin index between the cultivars shown in Figure 1.
284 According to Figure 2a, at similar anthocyanin concentration the filtering effect of
285 anthocyanin compounds on the red excitation light relative to the green is higher for
286 Barbera than for Nebbiolo, resulting in a lower value of ANTH_RG for Barbera compared
287 to that of Nebbiolo. This was confirmed by comparing the in situ absorption spectra of
288 berry skins of the two cultivars measured by the chlorophyll fluorescence excitation
289 method (Agati et al. 2005) (Figure 2b). The spectra showed that skin absorbance in the
290 red relative to that in the green for Barbera was higher than that for Nebbiolo, determining
291 a value of ANTH_RG in Barbera lower than that in Nebbiolo. Therefore, the difference
292 in the anthocyanin profile of the two cultivars could explain the different correlations
293 detected between the ANTH_RG spectroscopic index and anthocyanin concentration.
294 Due to the variability of the anthocyanin profile of *V. vinifera* cultivars, present results
295 suggest that the instrument should be calibrated for each cultivar or, alternatively, to use
296 different calibration curves in relation to the expected profile of the grape berry
297 anthocyanin. Some calibration curves are available at present for grapevine cultivars
298 whose anthocyanin profile is dominated by tri-hydroxylated compounds such as

299 Tempranillo [Baluja et al. 2012), see Nuñez et al. (2004) for profiles], Shiraz [see Downey
300 and Rochefort (2008) for profiles)], Aleatico [Tuccio et al. (2011), see Bellincontro et al.
301 (2006) for profiles], Cabernet Sauvignon and Merlot (Agati et al. 2013), in line with data
302 obtained in the present work for Barbera. To our knowledge, this is the first time a
303 calibration of a spectroscopic index has been proposed for a cultivar such as Nebbiolo
304 whose anthocyanin profile is dominated by di-hydroxylated compounds.

305 The Multiplex sensor system was calibrated from veraison, as well for the
306 application of the FERARI index (Figure 3). In Barbera, analytical data were related to
307 the spectroscopic index through an exponential curve with R^2 = 0.81, whereas for
308 Nebbiolo the same relationship was linear (R^2 = 0.61). Considering both cultivars taken
309 together, a good exponential fitting curve of data, $y = 0.2372 + 2.6285\ (1-e^{-0.0004x})$ with
310 R^2 = 0.85, was obtained (Figure 3). It is worth noting that the FERARI index, being mono-
311 parametric and referring exclusively to the far-red fluorescence under red excitation
312 (FRF_R), was not influenced by the anthocyanin profile of the cultivar. Figure 3 shows
313 that in the range 270–1800 mg/kg of anthocyanin the FERARI index can be used to
314 estimate the concentration of grape berry anthocyanin, regardless of the cultivar. More
315 improved calibration curves for the FERARI index can be obtained including pre-
316 veraison data (not shown).

317

318 **Spectroscopic index calibration for flavonol in Barbera and in white cultivars.** In
319 2008 we related the flavonol concentration of Barbera berries with the FLAV
320 spectroscopic index and with the newly proposed FLAV_UV index, using the Multiplex
321 (first version) sensor (Figure 4). The FLAV spectroscopic index did not correlate with
322 analytical data (R^2 = 0.04) in line with results obtained in Aleatico (Tuccio et al. 2011),

323 which could be due to the large change on the FRF_R signal induced by the anthocyanin
324 accumulation while the FRF_UV was slightly affected. In contrast, by plotting the
325 FLAV_UV index (that does not imply FRF_R in the calculation) against flavonol
326 concentration, we found a good correlation between the two sets of data with $R^2 = 0.74$
327 (Figure 4). Thus, this index can be proposed as a non-destructive proxy of flavonol
328 concentration in red grape berries.
329
330 In 2011, we studied the correlation between the Multiplex3 flavonol indices and
331 the flavonol concentration analytically determined in two white grape cultivars, Nascetta
332 and Chardonnay, chosen for their similarity in flavonol concentration and profiles (Table
333 4) and for being important for the Province (Nascetta) and as an international reference
334 (Chardonnay). Considering the two sets of data separately, we observed a good
335 correlation between flavonol analytically measured and both the FLAV and FLAV_UV
336 indices (Figure 5a,b). In both cultivars flavonol concentration fitted with spectroscopic
337 measurements through linear correlations with R^2 values always higher than 0.8 (Figure
338 5a,b). Taking data from the two cultivars together, it emerged that the FLAV_UV index
339 was still well correlated with flavonol concentration, with R^2 equal to 0.86, in a
340 concentration range from about 30 to about 150 mg/kg of flavonol in berries, regardless
341 of the cultivar (Figure 5c). Moreover, we compared the FLAV_UV versus flavonol
342 calibration curve defined for Chardonnay and Nascetta with data collected in 2010 for
343 several white cultivars whose flavonol concentration at harvest ranged between 50–220
344 mg/kg of berries and which had different flavonol profiles (Table 4). Figure 5c shows that
345 for Albarola, Cortese, Leiseret and Sauvignon Blanc the relationship between FLAV_UV
346 and flavonol concentration fitted the calibration curve well. In contrast, the data for Arneis

and Timorasso were markedly outside of it. At the moment, we do not know the reason for that. Since these two cultivars were those with the highest concentration of flavonol (Table 4), we can hypothesise that a saturation effect on the optical method occurs when the flavonol concentration in the berries is higher than 150 mg/kg. Further investigation collecting more experimental data is required to clarify this point. It is worth noting that the Nascetta data collected in 2010 were also well represented by the calibration curve built in 2011 (Figure 5c).

SFR_R as index of veraison and technological maturity in white berries. The simple fluorescence ratio index, representing the chlorophyll degradation, decreased over the season (Figure 6a) but offset in time between Chardonnay and Nascetta. In Chardonnay, a plateau phase was detected until day 190, whereas this plateau was prolonged until day 210 in Nascetta. Afterwards, in both cultivars the fluorescence ratio constantly declined over the remainder of the season. The beginning of ripening measured by TSS matched the start of the decrease in SFR_R, as shown by symmetric trends of the TSS accumulation curves (Figure 6a). This confirms that in white cultivars the SFR_R spectroscopic index is a valuable tool to estimate the start of ripening, well correlated with the TSS accumulation (Figure 6b), and accords with results previously found for Pinot Noir, Pinot Meunier and Chardonnay (Ben Ghozlen et al. 2010) and for Cabernet Sauvignon, Merlot and Vermentino (Agati et al. 2013).

Conclusions

Spectroscopic indices can effectively estimate the concentration of some important classes of grape berry skin phenolic substances (anthocyanin and flavonol) in a fast, reliable and non-destructive way. For ANTH_RG measurements applied to estimate anthocyanin, an important cultivar effect tied to the anthocyanin profile emerged. Actually, considering the anthocyanin profile of two cultivars, Barbera and Nebbiolo, at similar concentration, and the relative in vitro absorbance, a bathochromic shift of about 10 nm emerged that can contribute to the different screening effect exerted on the chlorophyll fluorescence excitation in the red relative to the green. As to cultivars such as Barbera, in which tri-hydroxylated anthocyanins are more prevalent, some calibration curves have already been published. As to Nebbiolo, which is a di-hydroxylated prevalent cultivar, this is the first time a calibration curve has been proposed. To assess spectroscopically the concentration of berry anthocyanin regardless of the anthocyanin profile, the FERARI index was found to be effective.

We presented here for the first time the FLAV_UV index, the logarithm of 1/FRF_UV. This index is suitable for non-destructive flavonol assessment of coloured grapes as it is not affected by the interference effect due to anthocyanin in the red. In white cultivars, both the FLAV and the FLAV_UV indices were suitable for flavonol estimation (R^2 always higher than 0.8) even though a saturation effect at concentration greater than 150 mg/kg was observed. As such high concentration is uncommon in *V. vinifera* berries we deem the FLAV_UV index as an efficient estimation of berry flavonol accumulation. Moreover, the simple fluorescence ratio (SFR_R) was confirmed to be highly and inversely related to TSS accumulation.

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510 **Table 1.** Geo-localisation of vineyards hosting the trials with the indication of the type of

511 wine produced.

512

Grape variety and type of wine	Municipality area	Geographic coordinates
'Super Barbera'†	Agliano, Camporotondo (AT)	44°46'29.8"N 8°16'01.3"E
Barbera 'Cuore di Gamma'	Agliano (AT)	44°46'32.9"N 8°15'58.5"E
Barbera 'Cuore di Gamma'	Agliano (AT)	44°46'36.4"N 8°15'54.4"E
Barbera 'Terre Rosse'†	Cassine (AL)	44°47'58.9"N 8°32'30.2"E
Barbera 'Cuore di Gamma'	Castel Rocchero (AT)	44°43'01.9"N 8°24'53.2"E
Barbera 'Cuore di Gamma' high yield†	Nizza Monferrato (AT)	44°45'53.9"N 8°23'33.7"E
Barbera 'Cuore di Gamma' low yield	Nizza Monferrato (AT)	44°45'51.6"N 8°23'27.9"E
Barbera 'Storico'	Mombaruzzo (AT)	44°46'04.8"N 8°26'41.0"E
Barbera 'Selezione'†	Ricaldone (AL)	44°43'55.3"N 8°28'02.1"E
Barbera standard, high yield†	Rivalta Bormida (AL)	44°41'36.5"N 8°33'42.8"E
Barbera standard, low yield†	Rivalta Bormida (AL)	44°41'35.3"N 8°33'44.8"E
Barbera standard, high yield†	Tortona (AL)	44°50'29.7"N 8°54'36.2"E
Nebbiolo 'Cannubi'	Barolo (CN)	44°36'50.1"N 7°56'37.5"E
Chardonnay	Barolo (CN)	44°36'51.8"N 7°56'24.1"E
Nascetta	Sinio (CN)	44°36'01.5"N 8°00'32.0"E

513 † Represents vineyards where grapes were collected in 2008 and 2009. AL, Alessandria

514 Province; AT, Asti Province; CN, Cuneo Province.

515

Table 2. Spectroscopic indices used to estimate berry anthocyanin (ANTH_RG and FERARI) and flavonol concentration (FLAV).

Spectroscopic index	Formula	Reference
ANTH_RG	log(FRF_R/FRF_G)	Agati et al. (2007)
FERARI	log (1/FRF_R)	Ben Ghozlen et al. (2010)
FLAV	logFRF_R/FRF_UV	Agati et al. (2011)
SFR_R	FRF_R/RF_R	Ben Ghozlen et al. (2010)

FRF_R, far-red fluorescence in the red; FRF_G, far-red fluorescence in the green; FRF_UV, far-red fluorescence in the UV; RF, red fluorescence.

529 **Table 3.** Average concentration of anthocyanin and anthocyanin profile in Barbera and
530 Nebbiolo grapes at harvest, and anthocyanin profile ofBarbera at a concentration of 750
531 mg/kg.

532

	Anthocyanin (mg/kg)	Non-acylated di-hydroxylated form (%)	Non-acylated tri-hydroxylated form (%)	Acylated forms (%)
Barbera at harvest	1200	7.4	71.7	20.9
Nebbiolo at harvest	750	62.5	29.1	8.4
Barbera at 750 mg/kg	-	5.0	66.6	28.4

533

534

535 **Table 4.** Flavonol concentration and flavonol profile of berries of white cultivars
536 collected 1 week before harvest (first row for each cultivar) and at harvest (second row
537 for each cultivar) in 2010 and of Nascetta and Chardonnay in 2011.

Cultivar	Flavonol (mg/kg)	Quercetin† glycosides (%)	Kaempferol‡ glycosides (%)
2010			
Albarola	53.6 ± 5.9	84.1	15.9
	-	-	-
Arneis	161.1 ± 8.8	85.9	14.1
	181.3 ± 11.0	82.5	16.5
Cortese	76.4 ± 11.3	82.5	17.6
	88.3 ± 7.7	83.9	16.1
Leiseret	125.9 ± 20.6	83.7	16.3
	118.2 ± 4.3	78.4	21.6
Nascetta	145.5 ± 8.3	79.5	20.4
	148.9 ± 15.8	73.1	26.9
Sauvignon Blanc	55.7 ± 2.2	84.8	15.2
	95.0 ± 19.9	84.1	15.9
Timorasso	221.6 ± 24.0	72.4	27.6
	211.6 ± 10.0	72.4	27.6
2011			
Chardonnay	152.5 ± 8.2	76.4	23.5
	101.5 ± 5.6	86.0	14.0
Nascetta	147.3 ± 8.9	75.4	24.7
	106.6 ± 5.2	94.2	5.8

538

539 †Quercetin glycosides, quercetin 3-O-glucuronide + quercetin 3-O-glucoside;
540 ‡kaempferol glycosides, kaempferol 3-O-glucuronide + kaempferol 3-O glucoside.

541

Figure captions

Figure 1. Relationship between the ANTH_RG spectroscopic index measured 'in field' and the anthocyanin concentration analytically measured in Barbera (◊) and Nebbiolo (●) grapes. Fitting curves are both second degree polynomial functions: $y = 7.75E\text{-}08x^2 - 0.0003x + 0.4337$; $R^2 = 0.72$ for Barbera ; $y = 3.77E\text{-}08x^2 - 0.0004x + 0.9146$; $R^2 = 0.55$ for Nebbiolo .

Figure 2. (a) Simulated absorbance spectra of extracts of Barbera (——) and Nebbiolo (——) berry skin anthocyanin calculated taking into account the profile (according to Table 3) measured at the same total anthocyanin concentration (750 mg/kg). (b) In vivo absorbance spectra of Barbera and Nebbiolo berry skins measured by the chlorophyll fluorescence excitation method with a spectrofluorimeter with emission set at 685 nm.

Figure 3. Relationship between the FERARI [log(1/FRF_R)] index measured 'in field' and the anthocyanin concentration analytically measured in Barbera (◊) and Nebbiolo (●) grapes. Exponential fitting curve for both cultivars taken together [$y = 0.2372 + 2.6285\ (1\text{-}e^{-0.0004x}$, $R^2 = 0.85$ (——)], exponential fitting curve for individual Barbera [$y = -0.0092 + 2.3061\ (1\text{-}e^{-0.0007x}$, $R^2 = 0.81$ (——)] and linear fitting curve for individual Nebbiolo [$y = 0.3815 + 0.0008x$, $R^2 = 0.61$ (——)].

Figure 4. Relationship between the FLAV (◊) and FLAV_UV (♦) indices measured 'in field' and the flavonol concentration in Barbera grapes (2008). Fitting curves are both linear functions: $y = 0.0008x + 0.0267$; $R^2 = 0.04$. $y = 0.0034x + 0.8744$; $R^2 = 0.74$.

Figure 5. Calibration curves for the FLAV and the FLAV_UV indices describing the relationship with flavonol concentration analytically measured. Measures were taken with the Multiplex3 instrument implemented in the UV emission in the white cultivars (a) Nascetta [for FLAV_UV $y = 0.0063x + 0.169$, $R^2 = 0.82$; for FLAV $y = 0.002x + 0.4385$, $R^2 = 0.8056$] and (b) Chardonnay [for FLAV_UV $y = 0.0055x + 0.2447$, $R^2 = 0.92$; for FLAV $y = 0.0018x + 0.3808$, $R^2 = 0.83$], in 2011. (c) The flavonol calibration curve defined for Nascetta (▲) and Chardonnay (■) has been used as reference for data related to the white cultivars Leiseret (●), Nascetta (Δ), Cortese (◊), Albarola (○), Sauvignon Blanc (♦), Arneis (□)and Timorasso (♦), measured in 2010 [$y = 0.0059x + 0.2087$, $R^2 = 0.86$].

Figure 6. (a) Simple fluorescence ratio after red excitation (SFR_R) and TSS accumulation [SSC (°Brix)] in Nascetta (▲) and Chardonnay (■) berries over the season. (b) Relationship between SFR_R and TSS (°Brix) in Nascetta (▲) and Chardonnay (■) berries [$y = -22.575x + 36.696$; $R^2 = 0.94$].

581