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PAPER

GRAPE SEED RIPENING EVALUATION BY *ORTHO*-DIPHENOL QUANTIFICATION

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ABSTRACT

Two millennia of viticulture recognize the seed browning importance on tannin ripening and, thus, on grape and wine quality. This color change was recently attributed to phenolic oxidations. However an objective chemical index able to quantify the oxidation status of seed tannins was missing, probably due to the heterogeneous oxidation polymerizations. This work suggests the adoption of the *ortho*-diphenol quantification as indication of the tannin ripening process, because *ortho*-dihydroxylated substitutions are highly susceptible to oxidation. The method proposed is based on the *ortho*-diphenol characteristic complexation with molybdenum. Different cultivars (Merlot, Pinot noir, Croatina, Aladasturi, Alexandrouli, Odjaleshi and Tavkveri) were studied during three vintages in Oltrepò pavese, Italy. The color darkening correlated with the *ortho*-diphenol decrease. We believe this index could find useful applications in viticulture, supporting harvesting time decisions.

Keywords: *Vitis vinifera*, tannins, seed browning, viticulture, wine quality

1. INTRODUCTION

"*… Naturalis autem maturitas est, si cum expresseris vinacea, quae acinis celantur, iam infuscata et nonnulla praeter modum nigra fuerint. Nam colorem nulla res vinaceis potest adferre nisi naturae maturitas …*". In his treatise "*De Re Rustica*", Columella (4-70 A.D.) suggested to use the seed darkening as the best grape ripening index. In 2000, Kennedy, Matthews and Waterhouse described the seed color change during berry development, and, in 2005, RISTIC and ILLAND, published a color chart to define the grape seed ripening. Two millennia of grapevines cultivation confirmed the importance of grape seed color. Also winegrowers are aware of the importance of this character: traditionally they check the seed color to evaluate the grape ripening status. Despite the increased knowledge in grape chemistry and physiology, the visual observation of the seed color is still one of the best available methods to evaluate the grape phenolic ripening. This method has some disadvantages: i) it is a subjective evaluation; ii) seeds have not an homogeneous color; iii) considering the color chart published by RISTIC and ILLAND (2005), it is not easy to discriminate the different brown tonality, especially in the last phenological steps (the most important for ripening estimation). For decades, a number of researchers focused their attention on phenolic content and polymeric subunit composition (KENNEDY *et al*., 2000) or extractability during winemaking (ROLLE *et al*., 2013). They payed attention to the phenolic concentration, overlooking this evident physiological color change. It should be noted that ROLLE *et al*. (2013) proposed an interesting acoustic method based on the physiological seed hardening during ripening, but the instruments required for this analysis are not widely diffused among grape and wine analytical laboratories.

Traditionally, winemakers consider seed lignification as the main responsible of this color variation. In their belief, lignin deposition would act as a barrier against tannins extraction, resulting also in a decrease in the detectable total phenolics (RIBEREAU-GAYON *et al*., 1998). However, the grape seed hardening is due to the lignification of the inner layers of the outer integument (RISTIC and ILAND, 2005), whereas nearly all of the soluble seed phenolics are localized in the thin-walled cells between the epidermis and the inner lignified layers (ADAMS, 2006). Adams (2006) also suggests that the seed browning characteristic of fruit ripening is the result of tannins and flavan-3-ols oxidation. Also KENNEDY *et al*. (2000) proposed seed polyphenol oxidation as the best explanation for their ripening study results. PILATI *et al*. (2007) described a rapid accumulation of H_2O_2 and an activation of the ROS scavenging enzymes at veraison. Thus, the hypothesis of a characteristic oxidative burst during ripening seems to be agreed among researchers.

In wine industries, tannins play a fundamental role, affecting the product quality in terms of astringency, body and bitterness. Their organoleptic feature change during ripening. At the moment, winegrowers usually assess the seed tannin ripening status by: i) a subjective visual color evaluation; and/or ii) a subjective organoleptic estimation by tasting; and/or iii) an approximate relationship between the anthocyanin accumulation or total phenolic content and the seed tannins evolution. Thus, an objective and accessible method to describe seed tannin ripening is still missing.

The aim of this work is to develop an index representative of the oxidative status of the seed phenolic compounds.

2. MATERIALS AND METHODS

Grapevines were all cultivated in the same germplasm collection located in Oltrepò Pavese (Lombardy region, northern Italy) already described in RUSTIONI *et al*. (2013). Plant material was collected during 3 growing seasons: 2009, 2010 and 2011. In the first

experimental year, Merlot and Pinot noir grapes were studied. In 2010, also Croatina (a local cultivar) was analyzed, together with Merlot and Pinot noir. In 2011, other 4 cultivars were included in the study: Aladasturi, Alexandrouli, Odjaleshi and Tavkveri (all of them are Georgian varieties). The list of cultivars and sampling dates is reported in SI 1. All samples were collected in 3 biological replications and the fresh seeds were extracted. A number of berries per replication were analyzed (depending on the seed number) as shown in SI 1. Berries were weighed and seeds were separated, counted, weighed and the color class was attributed following RISTIC and ILLAND (2005). Seeds were then extracted in 25 ml of methanol for 20 hours, and, then, in other 25 ml of methanol for 4 hours. All the extractions were performed in dark conditions and under continuous shaking. Finally the two extracts were mixed and kept at -20°C until analysis.
Within 3 months samples were analyzed following the method proposed by Maestro DURÁN *et al*. (1991). Two solutions were prepared: sol. A (water:ethanol 50:50) and sol. B (5% sodium molybdate in sol. A). 10 ml of diluted sample were added by 2 ml of sol. A (blank) and with sol. B (reacted) for 15 minutes. The reacted sample was then read against the blank at 370 nm by a JASCO 7800 spectrophotometer (JASCO, Mary's Court, Easton, Maryland). Dilutions were set up to optimize the absorbance range. A calibration curve was obtained by using standard caffeic acid solutions. Samples were also reacted with the Folin Ciocalteu solution to quantify the total phenolic content following Di Stefano, CRAVERO and GENTILINI (1989).
Data were statistically analyzed using the SPSS® statistical software (Version PASW Statistics 19, SPSS Inc, Chicago, Illinois).

3. RESULTS AND CONCLUSIONS

The list of samples analyzed, together with some general information, are reported in SI1. The number of berries considered depended on the expected number of seeds of each cultivar. Generally, in 50 ml of methanol, about 20-50 seeds were extracted. However, taking into account the seeds and berries number and weights, it was possible to elaborate the data concerning total phenolic content and *ortho*-diphenols concentrations in relation to the sampled grapes (e.g.: mg/seed; mg/kg of grapes).
Information concerning the number of seeds per berry as well as their phenolic content could be a useful support for winemaking technique optimization (maceration timing, seeds separation, aging expectations …) (Table 1).
These data could also be interesting for phenotypic characterization (RUSTIONI *et al*., 2014). However the important environmental effect on these parameters should not be forgotten. As an example, in our experimental conditions, Pinot noir generally had a high number of seeds/berry, and these seeds had a high phenolic concentration. In the opposite situation we found Odjaleshi. Nevertheless, intermediate characteristics were also recorded (e.g.: Aladasturi had a lot of seeds but they were not particularly concentrated in phenolic compounds).
Seed browning was studied following the method proposed by RISTIC and ILLAND (2005).
Fig. 1 reports the obtained data. In Fig. 1a, each point represents the average value of the three biological replications: the seed color level generally increased during berry development. In Fig. 1b each dataset (cultivar and year) is reported in a different color. It appears that the trend was consistent in all the varieties. Nevertheless, an important variability among the samples was recorded. The explanation could be found in the vintage and cultivar diversity (phenological shifts) as well as in the ripening degree variability among seeds.

Table 1: Cultivar characteristics: seed phenolic content and number of seeds per berry.

	Cultivar	Average	Standard deviation	Minimum	Maximum
Seed phenolic content mg/seed	Pinot noir	1.520	0.698	0.373	2.922
	Merlot	1.022	0.519	0.220	2.311
	Croatina	1.002	0.583	0.287	2.617
	Tavkveri	0.746	0.402	0.206	1.606
	Alexandrouli	0.596	0.144	0.347	0.844
	Aladasturi	0.505	0.319	0.226	1.358
	Odjaleshi	0.422	0.233	0.179	1.139
Number of seeds per berry	Aladasturi	3.2	0.2	2.9	3.5
	Pinot noir	2.3	0.4	1.6	3.3
	Tavkveri	2.2	0.6	1.3	3.4
	Alexandrouli	1.8	0.2	1.4	2.2
	Merlot	1.7	0.3	1.1	2.8
	Croatina	1.6	0.3	1.1	2.9
	Odjaleshi	1.5	0.2	1.1	2.1

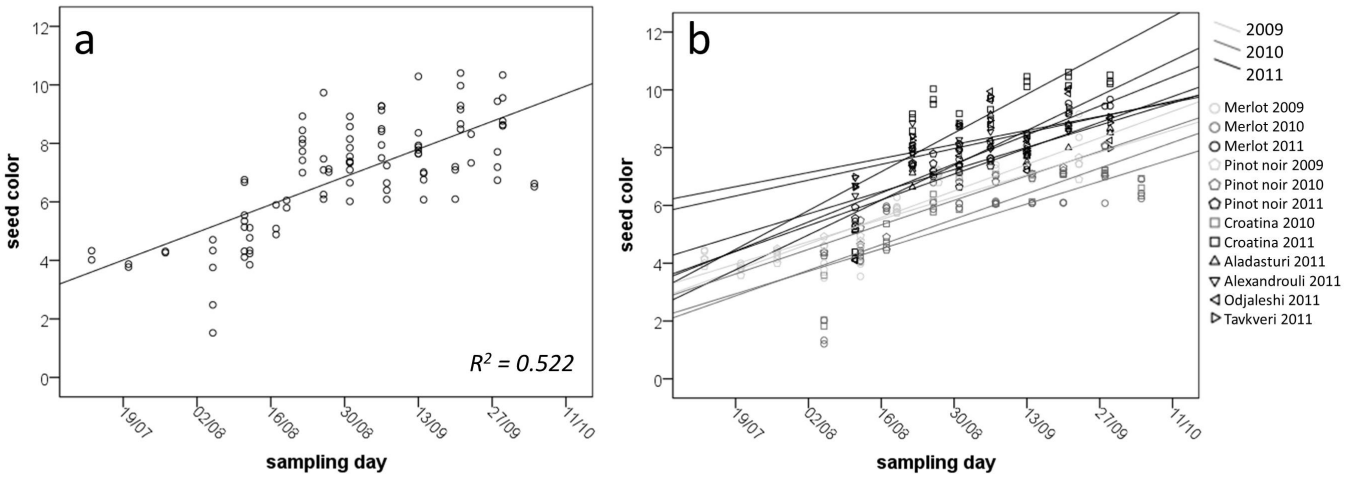

Figure 1: Seed color change during the growing season. In figure 1a each point represents the average value of the three biological replications. In fig. 1b each dataset (cultivar and year) is labeled by a different symbol. The obtained regression lines are classified depending on the sampling year (2009: light grey; 2010: dark grey; 2011: black).

A similar variability was found in the *ortho*-diphenol content decreasing trends during berry development (Fig. 2). Also in this case we attribute this heterogeneity to vintage, cultivar and seed variability. However, we observed a consistent decrease in the *ortho*-diphenol concentration in seeds (Fig. 2b), and we propose to adopt this value as an indicator of the grape phenolic ripening status. Recently, the central role of phenolic

oxidation in seed browning process has been underlined (KENNEDY *et al*., 2000; RISTIC and ILAND, 2005; ADAMS, 2006; PILATI *et al*., 2007).

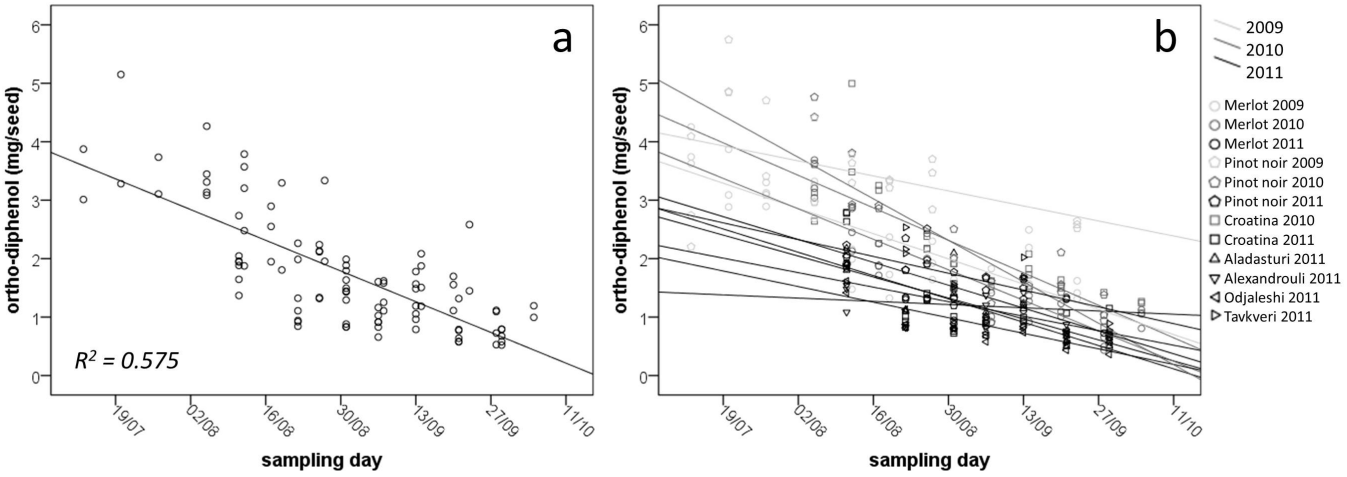

Figure 2: *Ortho*-diphenol concentration decrease during the growing season. In fig. 2a: each point represents the average value of the three biological replications. In fig. 2b each dataset (cultivar and year) is labeled by a different symbol. The obtained regression lines are classified depending on the sampling year (2009: light grey; 2010: dark grey; 2011: black).

However, in our knowledge, an "in deep" assay able to quantify tannin oxidation does not exist: via-radical polymerizations produce heterogeneous products due to the high reactivity of the instable reaction intermediates. *Ortho*-diphenol could be easily oxidized because the resulting phenoxyl semi quinone radical is stabilized by a second oxygen atom, while *meta*-diphenol are less susceptible to oxidation (WATERHOUSE and LAURIE, 2006). Thus, the catechol (*ortho*-diphenol) moieties are probably the most oxidizable groups in flavan-3-ols and proanthocyanidins. For that reason, it is possible to correlate the decrease in *ortho*-diphenol concentration to the oxidation processes characteristic of seed ripening. A number of studies have been carried out to clarify the role of anthocyanins-metal complexes on fruits and foods colors, and the importance of the *ortho*-diphenol substitutions are well known (KONDO *et al*., 1992; BOULTON 2001). Of course, complex solutions such as grape and wine could encourage further studies considering multiway interactions (RUSTIONI, 2015). Nevertheless, in our knowledge, considering non pigmented phenolics, any paper reports interferences in the *ortho*-dihydroxylated quantification through Molybdenum complexation by copigments.

Fig. 3 reports the correlation between the *ortho*-diphenol quantification and the seed color level (RISTIC and ILAND, 2005). The data dispersion should be attributed to the fundamentally different approaches of the tested methods. Nevertheless, the trend clearly appears: a decrease in *ortho*-diphenol content corresponds to the browning process characteristic of seed ripening.

The majority of the tannin synthesis occur before veraison. However, winegrowers are aware of the quality impact produced by the phenolic evolution during fruit ripening. Traditionally they use a visual inspection of the seed browning as signal of the phenolic ripening status, which results from proanthocyanidin oxidation. Thus, the method proposed by RISTIC *et al*. (2005) improved this qualitative evaluation. However, this technique is limited by the color inhomogeneity and by the subjectivity of the records.

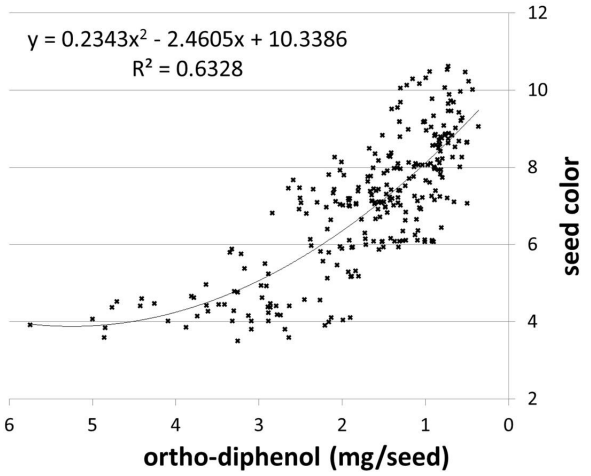

Figure 3: Correlation between *ortho*-diphenol concentration and seed color.

Thus, the aim of the present work is the production of an objective chemical index able to describe the tannin seed ripening. It does not require exclusive equipment and it is easy and fast to achieve. For these reasons we hope it will be a useful and practical support for the grape and wine industry. Moreover, this index could be adopted by researchers to characterize fruit quality in relation to treatments or vineyard managements.

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