SEED PHENOLICS OXIDATION: DEVELOPMENT OF A NEW RIPENING INDEX•

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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 *De Re Rustica*, Columella (4 – 70 ca. AD) indicated the seed color change as the best grape ripening index. Through the centuries, in 2000 Kennedy *et al.* described visual seeds color change during ripening, and in 2005, Ristic *et al.* published a seed color chart. In spite of its importance, and despite the increasing in chemical and physiological knowledge, the observation of seed browning is still one of the best methods to evaluate the grape phenolic ripening, even though it's a subjective indicator, and it's not easy to discriminate the brown hue, because of the lack of homogeneity in the seed and between the seeds, in relation with the similarity of the brown colors of the last steps of ripening. At *véraison*, an oxidative burst, characterized by rapid accumulation of H_2O_2 starting from *véraison* and by the modulation of many ROS scavenging enzymes, was observed by Pilati *et al*. (2007). Phenolic oxidation could also explain some results related to the phloroglucinol assay yield. In an initial study using the phloroglucinol method to monitor the changes of grape seed procyanidin concentrations in 'Shiraz' grapes, the conversion yield varied considerably, and was related to fruit maturity. These observations suggest that procyanidins are oxidized after *véraison* (Kennedy, Jones, 2001). Tannins are very important quality factors in red wines. Their taste changes during grape ripening, but this is not related to a particular behavior of accumulation/degradation.

The objective of this work was to develop a new and useful seed tannin index related to phenolic oxidation, using simple and fast spectrophotometric assays.

2. MATERIALS AND METHODS

In 2009 we worked on two grape cultivars in Riccagioia experimental farm (Torrazza Coste - Oltrepò pavese): 'Pinot noir' and 'Merlot'. Starting from bunch closure, samples were collected weakly, in three replications. Before the extraction, berries and seeds were weighed and counted. The average seed color was obtained using the Ristic *et al.* (2005) color chart. The collected seeds were first extracted for 20 hours in 25 mL of methanol, and afterwards for other 4 hours in new 25 mL of methanol. Finally the two extracts were mixed, to obtain 50 mL. A number of assays were tested to develop new indexes related to phenolic oxidation: total polyphenol content (Di Stefano *et al*., 1989), proanthocyanidin index (Makkar, 2000), 280 nm absorbance, DPPH assay (Siddhuraju, Becker, 2003; Aqil *et*

• QUAD. VITIC. ENOL. UNIV. TORINO, 2009-2010

al., 2006), ortho-dihydroxylated proanthocyanidins index (Maestro Duran *et al*., 1991). Finally four extracts for each cultivar were selected to study the phenolic compositional changes by HPLC (Kammerer *et al*., 2004; Monagas *et al*., 2005).

3. RESULTS AND DISCUSSION

The change in color related to the Ristic *et al.* (2005) color chart (fig. 1) shows an important color change just after *véraison*, when the green seeds became yellow/brown. The main problems evidenced by this method were the subjectivity and the difficulty of the classification of the brown seeds.

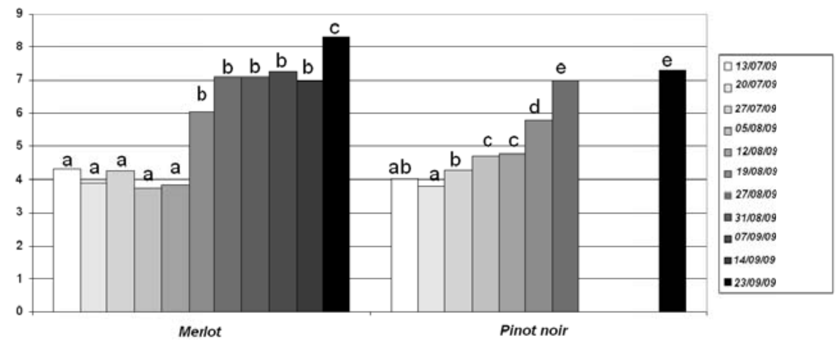

Fig 1. Seed color change obtained by the Ristic *et al.* (2005) color chart.

The objective of our study was to develop an index useful for different cultivars. It should evaluate the quality of seed phenolics, and for this reason we decided to standardize the results using ratios instead of direct contents. Using the correlation between the different indexes tested, and considering the performances of the indexes in the two cultivars and their chemical meaning, we selected two indexes:

- the ortho-dihydroxylated proanthocyanidins as structural index;
- the DPPH/280 absorbance for the antioxidant properties.

To develop chemical indexes, we followed two different ways. We tried to underline the changes in tannins structure (structural index), in relation to the different properties of the different linkages of tannins polymers. A classical assay for tannins is the proanthocyanidin index: by acid hydrolysis, the extension units C4-C8 or C4-C6 are converted into colored anthocyanidins (Xie, Dixon, 2005; Dixon *et al*., 2005). On the other hand, it's demonstrated that the ortho-dihydroxylated substitutions are the best site for oxidation in flavonoid molecules (Waterhouse, Laurie, 2006; Ribéreau-Gayon *et al*., 1998; Castañeda-Ovando *et al*., 2009). For this reason, we used the ability to complex metals of the ortho-dihydroxylated phenols in order to evaluate the quantity of catechol groups not involved in oxidation linkage. The assay proposed by Maestro Duran *et al*. (1991) utilizes the properties of molybdenum complex. The trend of the structural index during seed development (fig. 2) shows that the first increase can be related to the tannin synthesis, while the successive decrease can be related to the phenolic oxidation. This index has a significant linear regression with both the seed color and the sampling date. The other point of view was related to the antioxidant properties. We decided to relate the DPPH assay to the 280 nm absorbance.

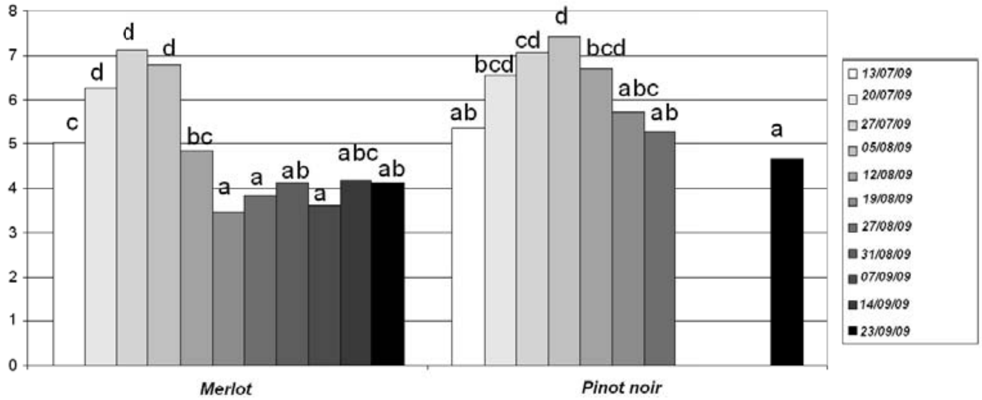

Fig 2. Structural index during seed development. After a first increase probably due to tannin synthesis, the index shows a decrease related to the phenolic oxidation.

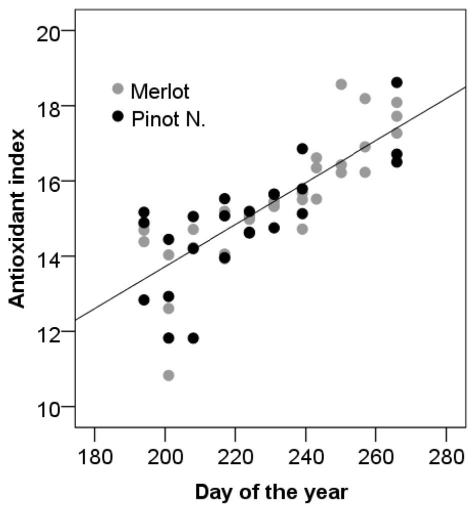

Fig 3 – Linear regression of the antioxidant index in relation to the day of the year.

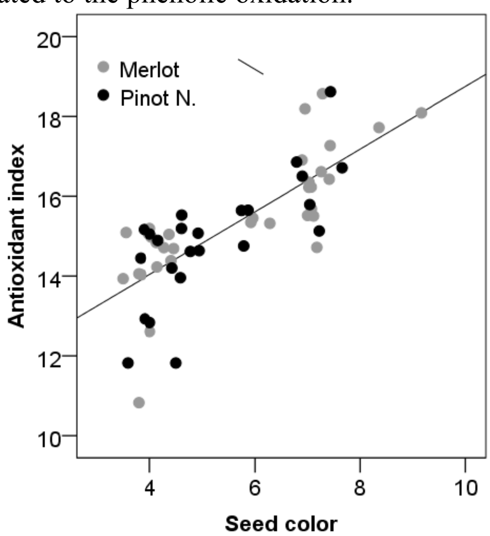

Fig 4 – Linear regression of the antioxidant index in relation to the seed color index.

With regard to the linear regression between the antioxidant index and seed color and sampling date (figg. 3 and 4), contrary to our expectations the antioxidant index progressively increased during seed development. A significant and negative correlation was also observed between our two indexes. This theoretically means that oxidation products have stronger antioxidant properties than their precursors. Trying to explain the compatibility of these results, we took in consideration the antioxidant properties of phenolic compounds. When phenolics react with ROS, the reaction rate of each phenolic depends on the ability to form a stable product radical. Compounds containing a 1,2,3-trihydroxyl group (pyrogallol), a 1,2-dihydroxyl aromatic ring (catechol), or a 1,4-dihydroxyl aromatic ring (absent in wine) are easiest to oxidize because the resulting phenoxyl semiquinone radical can be stabilized by a second oxygen atom (Ribéreau-Gayon *et al.*, 1998; Waterhouse, Laurie, 2006; Castañeda-Ovando *et al.*, 2009). An example of proanthocyanidin oxidation product (Allen, 1998) shows that the stabilization of the radical intermediate is related with a very high delocalization (fig. 5).

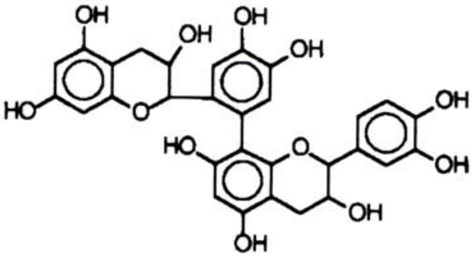

Fig. 5 – Example of oxidation product (Allen, 1998)

From this point of view it is possible to suppose that some oxidation products can be more antioxidant then their precursors. Finally we selected 4 samples of each cultivar representative of the trends of the indexes. After HPLC analysis, we found that the main change in flavanol composition was related to a decrease of the epigallocatechin-3-O-gallate, according with Kennedy *et al*. (2000) which found a decrease in epicatechin-3-O-gallate from 8 % at *véraison* to 1 % at harvest.

Abstract
During ripening seed tannins evolve, as demonstrated by the taste and color changes. In this work we tried to develop an objective, easy and fast index, useful for winemakers. In this direction we propose two different spectrophotometric indexes, one related to the molecular structure and tannin subunits linkages, and the other related to the antioxidant properties. Especially the second one gave very interesting and unexpected results. Even though seed phenolics are oxidized during ripening, their antioxidant power increases.
The results obtained in 2009 are very motivating, suggesting a validation next years.

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