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Influence of bunch exposure on anthocyanins extractability from grapes skins (*Vitis vinifera* L.)

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Summary

In relation to bunch exposure to solar irradiance (sun exposed *vs.* leaf shaded conditions), anthocyanin ripening and extractability were studied in two grape cultivars ('Croatina' and 'Pinot Noir') coming from three different vineyards in Northern Italy. Analysis of anthocyanin content were carried out by HPLC and spectrophotometry, and a simulated maceration process was developed. Pigments extraction occurred mainly in the first few hours of the maceration process. Anthocyanins with disubstituted B-ring showed a faster extractability than the trisubsituted ones. Bunch exposure to sunlight seemed to be important for pigment extractability timing in winemaking, showing a delay in pigments release. This delay was only partially explained by the different pigments profile, with higher percentage of disubstituted compounds in shaded berries, because all the molecules indicated a similar extraction trend during maceration.

K e y w o r d s : Grape, wine, color, anthocyanins, vineyard management, maceration process.

Introduction

The grapes anthocyanin profile varies depending on the variety (Revilla *et al.* 2001, Garcia-Beneytez *et al.* 2002, Mattivi *et al.* 2006) and it is influenced by vineyard management too. Therefore, bunch microclimate is an important factor determining the growing condition of berries. Leaf removal in the fruiting zone of the grapevine canopy, and, consequently, grape different exposure to solar radiation, may change the light and thermal microclimate around grapes with consequence on anthocyanin accumulation processes in term of quantity and profiling (Dokoozlian *et al*. 1996, Bergqvist *et al*. 2001, Spayd *et al.* 2002, Tomasi *et al.* 2003, Downey *et al.* 2004, Deloire *et al.* 2005, Rustioni *et al.* 2006, 2007) as well as in anthocyanin extractability (Cortell and Kennedy 2006, Ristic *et al.* 2007).

Therefore vineyard management could modify the raw material for wine production, and the derived changes should be considered in grape processing.

Maceration is a standard process in red wine production. It is essential for the diffusion of important molecules (including the phenolic ones) from the berry skins to wine, and it plays a fundamental role in obtaining the desired wine color. Nowadays it is well established that different maceration technique causes differences in the extraction of anthocyanins and in their stabilization in wine (Garcia-Beneytez *et al.* 2002), but some uncertainty still remains on the anthocyanic extraction dynamics.

The anthocyanin fingerprint in finished wines is quite different from that observed in the fresh grapes. Normally wine contains a higher relative amount of malvidin-3-O-glucoside (Revilla *et al*. 2001, Garcia-Beneytez *et al*. 2002, Romero-Cascales *et al*. 2005). Nevertheless, Romero-Cascales *et al.* (2005) and Fournand *et al.* (2006) have found that the rate of extraction seems to be similar for all the different pigments, in contrast with the results of García-Beneytez *et al.* (2002) which show that the anthocyanin fingerprint of fresh grapes is rather different from the one presented by crushed grape skins after fermentation. Contrarily, anthocyanin fingerprint seems to be maintained during wine aging (Revilla *et al.* 2001).

Following Garcìa-Beneytez *et al.* (2002), disubstituted pigments relative amount was higher in fresh grape skins than in the wine or in the skins after winemaking, in opposition with malvidin-3-O-glucoside behavior. Concerning the pigments acylation, Garcìa-Beneytez *et al.* (2002) found a decrease in *p*-coumaroyl and a stasis in acetyl derivatives. Revilla *et al.* (2001) instead, showed that the relative amount of acylated anthocyanins derived from malvidin (3-O-acetylglucoside and 3-O-*p*-coumaroylglucoside) slightly increased during the first eight months of wine maturation, and, on the other hand, the relative amount of malvidin-3-O-glucoside slightly decreased during that period.

Anyway, the largest changes in the anthocyanin profiling between wine and grape probably take place during the alcoholic fermentation, this may be due to several reasons: rate of anthocyanin extraction, degradation, polymerization during vinification or different capacity of adsorption of each anthocyanin by yeast cell wall (Revilla *et al.* 2001).

The influence of yeast on anthocyanin behavior during winemaking is demonstrated (Vasserot *et al.* 1997, Morata *et al.* 2003, Hayasaka *et al.* 2007). The bleaching activity of yeast cell wall is traditionally known, and some scientific works demonstrated its importance. In Champagne, beside to the use of charcoal, there is a marginal and traditional practice for the decoloration of 'Pinot Noir' and 'Pinot Meunier' grapes based on the use of lees recovered after alcoholic fermentation of 'Chardonnay' must by a physiochemical

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adsorption of anthocyanins on yeast walls (Vasserot *et al.* 1997). Vasserot *et al.* (1997) showed that all anthocyanins are easily adsorbed because of their high polarity, indicating that hydrogen bonding might be involved in the adsorption mechanism. In contrast, Morata *et al.* (2003) found a greater affinity of yeast wall for the more apolar acyl derivatives in respect to no-esterified glucosides, suggesting that adsorption involves a hydrophobic interaction. Peonidin, and at lower extent malvidin, were strongly adsorbed too (Morata *et al*. 2003).

To exclude yeast interference, as well as the most of the other variations in media conditions connected to a skin maceration process during fermentation, a simulated maceration process was used in this experiment. The objective was to study the anthocyanin extractability in relation to the modification provided by different bunch microclimate (sun exposed vs. leaf shaded bunches) obtained by a different canopy management. The experiments were conducted in Northern Italy in an area subjected to a temperate climate, testing two important cultivars, different for anthocyanin accumulation ability and profiling.

Material and Methods

Environmental conditions: experimental sites and vineyards management: The experiment was done in Oltrepò Pavese (Northern Italy). Three different sites were compared: "Zenevredo", positioned in the first hills next to the Po Valley; "Mornico", located in the bottom of a small valley; "Montalto", situated on the top of a hill. In each site, one vineyard of 'Croatina' and a vineyard of 'Pinot Noir' was selected. More information is shown in Tab. 1. In every vineyard two different grape exposures were obtained managing the canopy: exposed grapes and shaded bunches.

The experiments were conducted, in each vineyard, on one vine row selected according to correct vine balance (crop load *vs*. leaf area ratio), evaluated by visual inspection and able to assure a proper grape ripening according to the local experience (generally more than one square meter of exposed leaves per expected kilogram of grapes). In order to take into proper consideration the differences due to the individual physiological status of each vine, as well as to minimize the possible disturbance of the slight leaf removal around the exposed bunches, every treatment (*i.e.* sun exposed and leaf shaded) was replicated on each vine.

Grapes variety: cultivar anthocyanins: The cultivars chosen for the experiments were 'Croatina' and 'Pinot Noir'. They are both very commonly cultivated in Oltrepò Pavese. Whereas 'Pinot Noir' is a renowned French cultivar diffuse in many countries, 'Croatina' is an autochthonous variety of Oltrepò Pavese. For these reasons they were selected for this study.

Grape ripening and simulated maceration process: Grape phenolics ripening was monitored in three sampling times: post-veraison *i.e.* just after the onset of ripening, between 35 and 36 E-L phenophases following Eichorn and Lorenz (1977), mid ripening (between 36 and 37 E-L), full ripening (38 E-L) by determining, concerning anthocyanins, in 3 or 4 replications, anthocyanin content (Di Stefano *et al.* 1989) and anthocyanins profiling (Mattivi *et al.* 2006).

At ripening, a simulated maceration was made following Tab. 2. The method consist of the extraction of berry skins in an aqueous solution, progressively added of ethanol, to simulate the yeast fermentation.

All the liquid sample (extraction solution of simulated maceration) were analyzed directly, while the skins (during ripening and in the skin sampled during the simulated maceration process) were always extracted in a solution of 100 % methanol for 24 h, following the Mattivi *et al.* (2006) method.

Every simulated maceration was carried out in duplicate for each cultivar and for each experimental condition, therefore a total of 24 experimental processes were analyzed. Skins from 500 g of berries were placed in 625 mL of an acidic-aqueous solution (5 $g \cdot L^{-1}$ tartaric acid, 100 $mg \cdot L^{-1}$ sodium metabisulphite, buffered at pH 3.2 with NaOH), in dark condition, at room temperature, under continuous shaking. The pots were filled with CO_2, to prevent oxidation.

During maceration, at each sampling time, both liquid and solid phases were collected without changing their relative proportion in the bulk solution and were analyzed by HPLC (Mattivi *et al.* 2006) and by spectrophotometer (Di Stefano *et al.* 1989), for a total of 312 samples.

Malvidin-3-monoglucoside was purchased from Sigma Chemical Co (St. Louis, MO). Identification and quantifi-

Table 1

Main characteristics of the vineyards involved in the experiments

Site	Grape variety	Elevation m a.s.l.	Slope direction	Row direction	Training system	Phenological stages (day of the year)			
						05 E-L*	23 E-L	35 E-L	38 E-L
Zenevredo	Pinot noir	115	E	E-W	Simple Guyot	82	134	197	221
Zenevredo	Croatina	99	W	E-W	Simple Guyot	80	142	212	239
Mornico	Pinot noir	182	S-W	E-W	Spur pruned cordon	80	136	209	226
Mornico	Croatina	188	S-W	E-W	Simple Guyot	80	146	218	238
Montalto	Pinot noir	280	S	NE-SW	Simple Guyot	83	134	197	223
Montalto	Croatina	288	S	NE-SW	Simple Guyot	81	142	209	231

*E-L phenophases following Eichorn and Lorenz (1977).

Table 2

Simulated maceration process parameters

Sampling time	Maceration time	Progressive alcohol content (% vol.)
0	h 0 00’	0.00
1	h 1 00’	0.15
2	h 2 30’	0.54
3	h 4 30’	1.00
4	h 22 00’	1.75
5	h 28 00’	2.50
6	h 47 00’	4.00
	h 70 30’	5.50
7	h 121 30’	7.00
	h 141 30’	8.50
8	h 190 00’	8.50

cation of anthocyanins peaks were obtained by calibration curve of standard.

Following Di Stefano (1989), total anthocyanin concentration was evaluated by measuring the absorbance of the extract at a wavelength of 535 nm (using a Jasco 7800 spectrophotometer) and referring the values to a malvidin-3-glucoside calibration curve.

According to Mattivi *et al.* (2006), anthocyanin profile was determined at 520 nm using a Shimadzu HPLC LC-10 AD (Shimadzu Co. Tokyo, Japan) connected to a Shimadzu UV-VIS detector SPD-10 A. HPLC analysis of the extract was conducted within the following parameters: flow rate 0.45 ml·min^{-1}; temperature 40 °C; column Purospher RP18, 5 µm (250 x 4 mm) (Merck, Darmstadt, Germany); solvent A: methanol; solvent B: aqueous 0.3 % perchloric acid – Elution: linear gradient from 27 % to 43 % A in 32 min, from 43 % to 68.5 % in 13 min, from 68.5 % to 100 % in 2 min, then isocratically with 100 % A for 3 min; re-equilibrating time: 5 min; loop: 10 µl. A calibration curve was established using malvidin 3-monoglucoside and results were expressed as “malvidin 3-monoglucoside equivalent” (mg·g^{-1} of berry).

The anthocyanin profiles were outlined as the relative levels of delphinidin 3 glucoside; cyanidin 3 glucoside; petunidin 3 glucoside; peonidin 3 glucoside; malvidin 3 glucoside; and their acetic and *p*-coumaric esters.

Statistical data analysis: For each variety, the statistical significance of the effects of the experimental treatments have been evaluated following general linear models including “site”, “bunch exposure” and “sampling time” as main factors and their two and three way interactions; means were then separated by LSD test at P = 0.05. Trends in maceration processes have been described by fourth degree polynomial regression models.

Results and Discussion

The two varieties have a different anthocyanin accumulation behavior: full ripen 'Croatina' was much more colored than 'Pinot Noir' (1130 mg malvidin·kg^{-1} of grapes in the autochthonous variety and 395 mg·kg^{-1} in the second one) in the Oltrepò Pavese growing condition. According to previous data from our laboratory, both varieties have malvidin as the dominant pigment (70 % ca. in 'Croatina' and 60 % ca. in 'Pinot Noir'), and cyanidin is found only in very small amounts (1 %). The main differences in the B ring substitution concern the delphinidin, peonidin and petunidin percentage. In 'Pinot Noir', peonidin is definitely more concentrate than the others (34 %), while in 'Croatina' they have more or less the same percentage. The main difference between the two varieties concerns acylation: 'Pinot Noir' was characterized by the absence of acylated pigments, while in 'Croatina' they represent ca. 25 % of the total pigments content.

The grape cultivars and the cultivation site influenced the ripe grape composition by affecting the anthocyanin accumulation starting from the very first steps after véraison. The location of the experimental site significantly affected the anthocyanin accumulation pattern, especially for 'Pinot Noir', demonstrating that the ripening conditions were rather different (data not shown). Concerning to the bunch exposure, our results indicated a little color increase (related to a higher 535 nm absorbance measured by spectrophotometer) in the ripe exposed bunches, with significant differences between shaded and exposed bunches only in 'Pinot Noir'. Considering only the total monomeric pigments obtained by the HPLC from the same extracts, exposed bunches showed a lower content than shaded ones (Tab. 3). This means that there is a part of the color not explained by the monomeric anthocyanin, which is higher in the exposed berries. These differences between spectrophotometer and HPLC data could be related to some kind molecular linkages (polymeric pigments) or interactions, even if copigmentation should be excluded because absorbance measurement was done in an ethanolic solution (Hermosin-Gutierrez 2003). Fig. 1 shows the contents of anthocyanins monomers contained

Table 3

Grape anthocyanins content analyzed by HPLC and spectrophotometer

Cultivar	Vineyard	Bunch exposure	Grape anthocyanins content (mg malvidin/kg of grapes)			
			HPLC assay		Spectro-photometric analysis	
Croatina	Montalto	Exposed	457.78	b	1038.39	a
		Shaded	1288.33	a	910.34	a
	Mornico	Exposed	512.79	b	1008.04	a
		Shaded	1571.41	a	1096.27	a
	Zenevredo	Exposed	470.36	b	988.73	a
		Shaded	938.92	a	670.92	b
Pinot noir	Montalto	Exposed	280.07	b	691.33	a
		Shaded	583.60	a	522.48	b
	Mornico	Exposed	188.63	b	455.80	a
		Shaded	356.95	a	325.61	b
	Zenevredo	Exposed	212.47	b	524.34	a
		Shaded	512.32	a	448.35	a

For each variety and each vineyard, means followed by the same letter are not significantly different (p = 5 %).

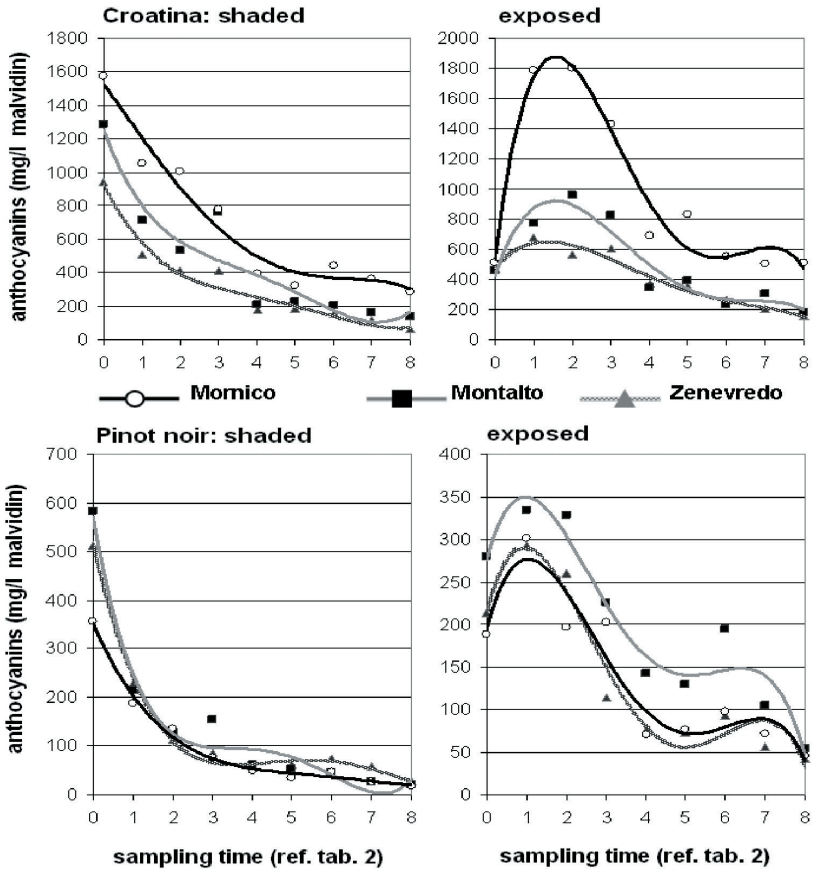

Fig. 1: Effect of bunch exposure in relation to the different cultivation sites. All the regression curves had a square R statistically significant ($P < 0.01$ or < 0.001).

in berry skins during the simulated maceration. The pigments content is derived from the amount of anthocyanins monomers extracted from samples of berry skins collected during the maceration process and analyzed by the method of MATTIVI *et al.* (2006). Anthocyanin levels are expressed in $mg \cdot L^{-1}$ of malvidin that potentially can pass from the skin to the solution. As expected, shaded bunches show a decrease in concentration of anthocyanin monomers in skin. Contrarily, the exposed grapes exhibited an unexpected increase in anthocyanin content in skin after some hours of maceration, to have a pigment content higher than the ripe sample (Tab. 3). This difference in anthocyanins behavior is repeated in all the analyzed experimental processes. Even if the Mattivi skin eduction (MATTIVI *et al.* 2006) provides the use of a strong extraction solution (24 h in 100 % Methanol), it is evident that in exposed grapes this alcohol is not enough to remove all the pigments from the skin. Only after some hours of maceration in aqueous solution, exposed bunches are capable of releasing all their anthocyanins.

By simulated maceration, it was possible to put in evidence that bunch exposure caused a delay in anthocyanin monomers extraction, instead of a lower accumulation (Fig. 1 and Tab. 3). Considering that a delay in pigments extraction can protect the molecules against oxygen, which is particularly concentrated during the first hours of fermentation, our data can support RISTIC *et al.* (2007) data.

In solution, anthocyanins concentration strongly increases during the first 4 samplings (22 h). After that, the extraction starts to slow down (data not shown).

Fig. 2 shows the behavior of the different kinds of pigments in 'Croatina' and 'Pinot Noir' in the extraction solution, relatively to the B ring substitution. The percentage of peonidin was nearly twice the relative average content in grapes ripen skins and it clearly decreased during the extraction process in both cultivars. Malvidin proportion increases during 'Pinot Noir' maceration, while in 'Croatina' did not show significant trends. Cyanidin decreased, while petunidin and delphinidin (the latter without significant trends) seemed to slow down after an initial percentage increase. While in the skins, specially 'Pinot Noir' shows a clear decrease of peonidin and an increase of malvidin.

Concerning acylated anthocyanins of 'Croatina' in the skin and in similar-wine solution, the proportion between the compounds does not show any particular trend in the extract solution. In the skin, the percentage of non esterified compounds suggest a slow decrease, in opposition to an increase of *p*-coumaroyl esters.

ROMERO-CASCALES *et al.* (2005) have found that the rate of extraction seems to be similar in all the different pigments, but they took the first sample after 3 d of maceration. Our data shows that, during the first few hours of maceration, the various anthocyanins have different extraction patterns, due to their different solubility and possibly cell compartmentalization (RIBEREAU-GAYON *et al.* 1998). Looking into the anthocyanin profile, our experimental results point out that pigments with disubstituted B-ring have the highest extractability, as demonstrated by the high ratios found in the first hours in the similar-wine solution. The lower extractability

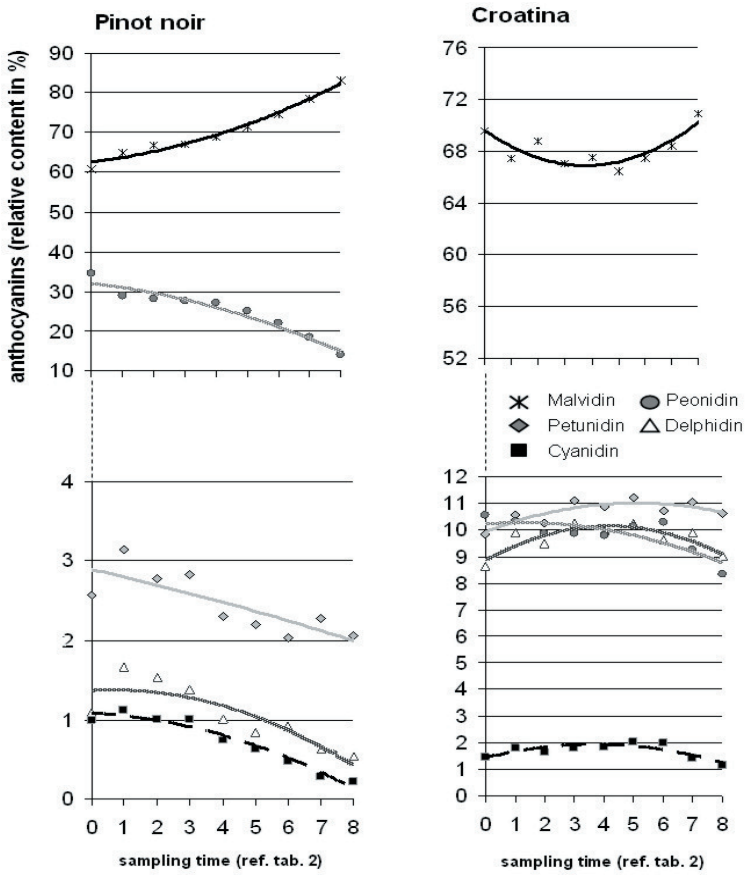

Fig. 2: B ring substitution: behavior of the pigments in 'Croatina' and 'Pinot Noir' in the solution. All the regression curves had a square R statistically significant ($P < 0.01$ or < 0.001), with the exception of the delphinidin ones that were not significant.

of malvidin is also confirmed by its relative increase in the 'Pinot Noir' skin, while in 'Croatina' the high concentration of this pigment does not permit to detect strong changes in the skin's pigment composition. The trisubstituted compounds are extracted later on in the process but, as the percentage of malvidin increases, delphinidin and petunidin showed a decrease after the first extraction hours. This may be due to the higher oxidability of these ortho-diphenolic compounds. García-Beneytez *et al.* (2002) explain their data hypothesizing that peonidin-3-O-glucoside and cyanidin-3-O-glucoside, after being readily extracted from grape skins, undergo some unknown degradation reaction during winemaking. The decrease of peonidin content in wine during fermentation can not be related only to yeast wall adsorption, because no microorganisms were involved in our experiment. Therefore, the decrease in concentration was possibly related to the high concentration of the compound in the first step of extraction, before any other antioxidant enters the solution. In berry skins, the proportion of non esterified compounds showed a slow decrease, in opposition to an increase of *p*-coumaroyl esters. This trend can be explained by a higher extractability of non acylated pigments. It was impossible to underline any particular behavior in the solution which may suggest the possibility of esterification or hydrolysis of these compounds.

The underlined delay in exposed bunches extraction could be partially related to a higher proportion of peonidin in shaded berries. Comparing the anthocyanin profile (Tab. 4), it is possible to note that peonidin percentage shows significant differences between exposed and shaded bunches, in both the two grape varieties.

In 'Pinot Noir' the significant difference in pigment profile, is related to a delay in the profiling change in exposed berries. Fig. 3 shows the increase in peonidin percentage

Table 4

Bunch exposure effect on the anthocyanins profile in the two cultivars

		Delphinidin	Cyanidin	Petunidin	Peonidin	Malvidin
Croatina	Exposed	8.61 a	1.23 a	9.74 a	9.41 b	71.00 a
	Shaded	7.74 a	1.45 a	8.99 a	12.34 a	69.46 a
Pinot noir	Exposed	1.40 a	0.97 a	3.06 a	27.95 b	66.59 a
	Shaded	0.77 b	1.04 a	2.08 b	41.47 a	54.62 b

For each cultivar means followed by the same letter are not significantly different ($p = 5$ %).

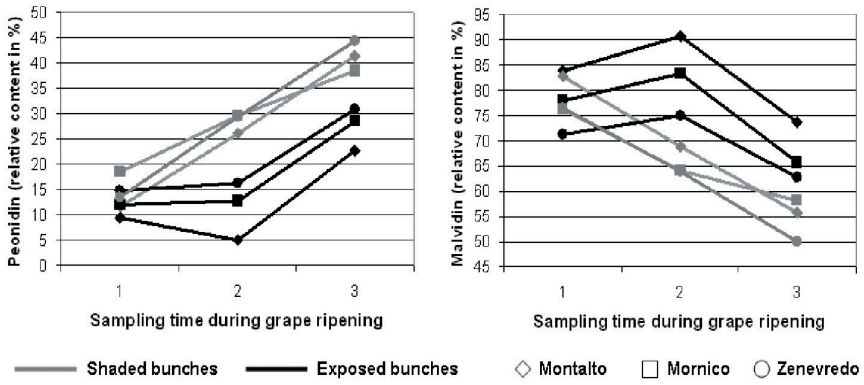

Fig. 3: Peonidin and malvidin percentage changes during ripening in 'Pinot Noir'. (LSD peonidin = 5.65; LSD malvidin = 5.88).

during ripening, balanced by the decrease in malvidin ratio. The different behaviour between the two bunch exposures is evident and reproducible in the three sites. In 'Croatina' grapes, it was impossible to underline a similar behavior, probably due to the low peonidin concentration.

It is important to note that the extractability trends were the same for all the single pigments (Fig. 4): in shaded bunches all the anthocyanins were extracted readily, while in exposed samples all the molecules were underestimated at ripening time, becoming extractable only after a short maceration in aqueous solution. This would indicate that the total anthocyanin extractability was quite independent from the differences in their ripening profiles. Consequently, according to the suggestion of RISTIC *et al.* (2007) the differences in extractability trends can be due to a different cell structure or anthocyanins compartmentalization in the two ripening conditions.

Conclusion

To improve the wine quality it's important to focus on the linkage between vineyard management and winemaking effect. In this work, we studied the grape anthocyanins composition in relation to the bunch exposure, demonstrating its effect also on pigments extractability pattern in winemaking. We underlined the connection between grapevine eco-physiological conditions, grape ripening and enological practices.

In particular, bunch exposure can produce an increase of the berries color (probably related to polymeric pigments or interactions), and a delay in the anthocyanins extraction. The pigments profile of grapes can only partially explain this delay, probably related to the skin tissue characteristics. This behavior can protect the pigments from oxidation during the first steps of winemaking, when phenolics can easily undergo degradations because of the oxidative conditions.

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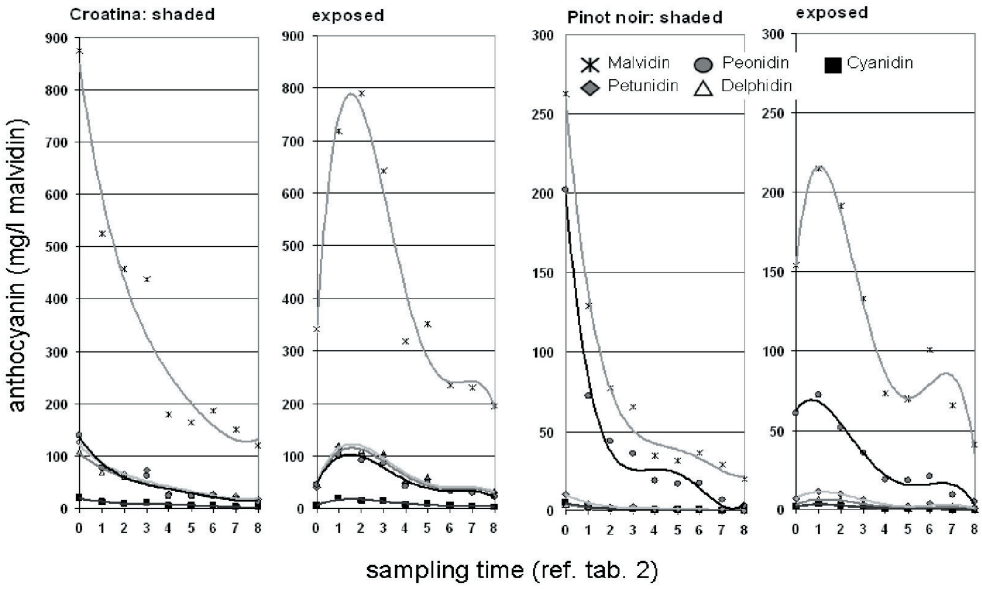

Fig. 4: Effect of bunch exposure in relation to the different monomeric pigments. All the regression curves had a square R statistically significant ($P < 0.01$ or < 0.001).

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