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GENOTYPE X ENVIRONMENT INTERACTION IN GRAPES RIPENING METABOLIC TRAITS

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to my husband

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FLAVANOL ACCUMULATION IN GRAPEVINE BERRIES: ECOPHYSIOLOGICAL AND ENOLOGICAL ASPECTS

GENOTYPE X ENVIRONMENT INTERACTION IN GRAPEVINES: ECOPHYSIOLOGICAL BASIS AND GRAPES RIPENING EFFECTS

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The genotype is the genetic constitution of an individual where the potential is encoded, but cannot define alone the phenotypic expression rather interacts with the environment that modulates the response and determines the level of this potential. As a result, the quantity and quality of the grapes depends on the interaction between genotype and environment including the viticultural techniques. It is therefore arguable that the same varieties grown in different areas have different performance, and this is due to the different degree of interaction between genotype and environmental conditions as summarized in the French concept of *terroir*. This concept include all the factors present in a homogeneous agro-ecosystem, in which the various elements such as ecological (geology, topology, climate, etc.), biological (grape variety, rootstock) and human (agricultural techniques and winemaking practices) contribute to the quality of the final product. The so-called local grape varieties are believed to have limited adaptive capacity, meaning less plasticity versus environmental and cultural changes in regard to so-called international varieties.

An improvement in the knowledge of the eco-physiological basis of the genotype x environmental interaction, in terms of grape ripening processes, would have important scientific and practical outputs. In fact to shed light on the differences in the berry metabolic expressions according to the changes in environmental conditions would allow to develop the scientific knowledge to optimize the cultural techniques suitable to obtain the expected grapes quality in agreement with the environmental conditions.

This report consists of two parts, where are studied the grapevine response on different environments and some techniques in the vineyard.

Objectives of the first part of the research program are the effects of some environmental and physiological aspects on the intensity of flavonoid synthesis. Today an increased attention is addressed to polyphenolic compounds, as a relevant part of quality, mainly in red varieties. They have determinant role in the quality of final product, on the other hand for their nutraceutical properties on human health.

Two grapevine cultivars were selected with different ability in flavonoid accumulation. Vranac, with moderate accumulation of tannins (native variety grown in Montenegro and in some parts of Serbia, Bosnia and Macedonia) was compared with the international variety Cabernet Sauvignon, usually designed with very good accumulation of polyphenols.

In particular the following experimental treatments were applied: different bud load per vine, to modify the plant source vs. sink ratios; early leaf removal (flowering time) to induce a light, thermal and nutritional stresses to the berry and cluster thinning (veraison time), to modify the source vs. sink ratio.

The second part of research included the study of the Sangiovese grapes, a variety of great cultural and economic significance and characterized by a particular response to changing environmental conditions, and Cabernet Sauvignon grapes, a so called international cultivar characterized by more stable features. The study sites were selected on the basis of their representativeness of important areas of cultivation of Sangiovese and by differences in soil and climatic conditions. It was operated within the framework of Appellations of Origin Bolgheri (Tuscany coast), Montalcino (Tuscany Apennines) and Misano (Romagna coast). To highlight the phenotypic effects that arise in the berries specific interaction between environment and genotype was essential analyze the same variety grown in different environments, and simultaneously analyzed grapes growing in different environments as similar as possible.

Since the carotenoids could be potentially precursors of aromatic norisoprenoid compounds, important for the future wine, the purpose of this investigation was to evaluate the different cultivars response in diverse climatic regions on the evolution of these compounds.

FLAVANOL ACCUMULATION IN GRAPEVINE BERRIES: ECOPHYSIOLOGICAL AND ENOLOGICAL ASPECTS

Introduction

Viticulture and wine production in Montenegro dates back from before the Roman period and even today it is mainly based on grape and wine production of autochthonous varieties (Vranac, Kratosija, Krstac and Zizak).

Until The First World War, Vranac was the most cultivated variety in Crmnica area so it is called Crmnica`s variety (1). Due to the excellent quality of grape and wine, the Vranac variety was expanded from Crmnica to the coastal area of Montenegro and in 1950 it was transferred and planted in Macedonia (2). After this it spread to other areas of former Yugoslavia where today it is grown to a lesser or greater extent. However, the best characteristics of the Vranac variety are expressed in the Crmnica subregion. Many authors have written about quality of Vranac and Crmnica's wine (3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19). Vranac is a Montenegrin autochthonous grapevine variety, belonging to the ecological-geographical group *Proles pontica* (*Convarietas pontica*). DNA analysis (20) shows that Vranac is closely related to Kratosija and that there is probably a first-degree relationship (the closest relationship) between these two varieties, since for the old Montenegrin grapevine variety Kratosija was determined to have the same DNA profile as Zinfandel – Primitivo.

Wine produced from grape of this variety is pleasant, harmonious, with a specific varietal aroma and taste with a percentage of alcohol from 11-14%vol. and 5-6 g/l of total acids. It is recognized by its intensive color. According to a group of experts from Milan's weekly magazine "Il Mondo" in 1991, Vranac wine was ranked among the 100 best red wines of Europe.

The vine of Vranac variety is vigorous, the flower is hermaphrodite, the berry is medium large to large, with an oblong shape, thin skinned, blue color and with abundant bloom. The bunches are medium large to large, cylindrical shaped, medium dense, rarely loose. Vranac variety has high yielding potential and the grapes ripen in the third epoch (medium-late variety). To reach a good quality, yield it should be cultivated in a sunny position and on warm, loose – gravelly, permeable and moderately fertile soil. Vranac typically reach a good technological maturation and high levels of anthocyanins while the accumulation of tannins may be insufficient to give high longevity to the wine in term of body and color stability.

The aim of the present work was to investigate the effect of early leaf removal and cluster thinning treatments in the Mediterranean climate on the berry growth and how these two techniques affect phenolic profile (especially proathocyanidins) and colour characteristics for later wine production. Two grapevine cultivars with different ability in flavonoid accumulation were compared: Vranac, with moderate accumulation of tannins and an international variety, Cabernet Sauvignon, usually

designed with very good accumulation. For cultivar Vranac, characterized by high yield and compact cluster, especially beneficial effects from the two agronomic techniques are expected.

The effect of leaf removal depends on the phase in which the measure is carried out. Early leaf removal may induce a light, thermal and nutritional stress to the berry and causes photosynthetic shock, which in such manner can cause a halt in the grape's development. Cluster thinning with winter pruning is the most widely used tool in viticulture for yield reduction which modifies the source vs. sink ratio and does not affect the canopy microclimate. Cluster thinning advances grape maturity, improves grape quality and also influences the chromatic characteristic of the wine (21, 22).

In the Sangiovese variety, characterized by highly compact clusters, early defoliation significantly reduced: the fruit set, yield per shoot, cluster weight, number of berries per cluster and cluster compactness. The berry size was unaffected (23, 24, 25). The berry size and weight were unaffected because the remaining leaves were able to compensate; therefore, carbohydrate availability was not limited by leaf removal treatments during berry development (24). Other studies reported that the influence of leaf removal on yield components depended on the variety. Leaf removal decreased yield per vine and cluster weight in Merlot and Sangiovese, while the berry size was unaffected in both varieties. Only in Merlot number of berries per cluster and cluster compactness decreased, while in Cabernet Sauvignon the effect of leaf removal was restrained by berry size (38). The timing of leaf removal had a marked effect on berry maturity, wine composition and the sensory properties of Grenache wines made from grapes grown under dry-farmed conditions (39).

Increased exposure of fruit to sunlight improves wine composition. Generally speaking, grape color depends on the degree of cluster exposure and the resulting berry temperature (30, 31, 32). In the warm growing region of the Central San Joaquin Valley (California) it was shown that anthocyanins and total phenolics in Grenache and Cabernet-Sauvignon increased linearly to increasing levels of sunlight exposure on the north side of the canopy, but declined with increasing levels of cluster exposure on the south side. From these findings, the authors suggested that the effects of light on fruit composition are heavily dependent on berry temperature (31). Sunlight-exposed clusters have shown higher levels of sugar content, anthocyanins, and phenolics, and lower values of titratable acidity, malate content, K concentration and pH compared to shaded fruits (26, 27, 28, 29).

Materials and Methods

Experimental design and treatments: The trial was carried out during the 2011 growing season in the commercial vineyard of the Plantaze firm in the Cemovsko area in Podgorica (Montenegro), planted with *V. vinifera* L. cvs., Vranac and Cabernet Sauvignon. The study was conducted in vineyards within a uniform zone. The treatments of variety Vranac were established in ten-year-old vineyard, grafted onto Kober 5BB rootstock, trained to a modified double Guyot training system, rows spaced 2.8 m apart and with 0.9 m between plants in the row. The grapevines of Cabernet Sauvignon were planted in 2005 (clone R5), grafted onto 1103P rootstock and trained to a Guyot training system. The vine had a between-row and within-row spacing of 2.60 m x 0.70 m.

Winter pruning, for both varieties, was carried out leaving 14 buds per vine. In the first week of May, when the shoots reached 20 cm to each vine, shoot thinning was applied and 10 shoots per plant were retained (all waterspouts were pulled out). The vineyard was managed according to standard viticultural practices of the company.

In the experimental design four treatments were compared: a) not defoliated - not thinned (NLR-NCT), leaf removal - not cluster thinning (LR-NCT), not leaf removal - cluster thinning (NLR-CT) and leaf removal - cluster thinning (LR-CT).

Defoliated treatment was applied in full bloom on DOY 152 corresponding to the phenophase 23 according to the grapevine growth stage classification proposed by Coombe 1995 (33), which consisted of manual removal of the first eight basal leaves of each shoot. All lateral shoots were retained. Cluster thinning was conducted on DOY 200, at mid veraison, at stage 35 (33), where the distal cluster was removed leaving one cluster per shoot. The elementary experimental plot was composed of 20 consecutive vines; each treatment was replicated in three elementary plots, randomly positioned in the vineyard.

Meteorological data. The meteorological conditions, daily air temperatures (°C) and daily rainfall (mm) from June to August were measured by a meteorological station located in the vineyard (Boreas Ltd. Budapest, Hu). The thermal status of the berries in the different conditions (exposed clusters and shadow clusters) was monitored by specific hypodermic thermocouple thermometers (HOBO) inserted into the berry center and connected to a data logger.

Yield components. At harvest, cluster number as well as total yield was recorded on 15 tagged vines per treatment. In the laboratory, the following variables of 25 randomly selected clusters for each treatment were estimated: cluster weight, cluster and berry length and width, berry number and weight. Compactness index (*Ci*), an index representing the compactness degree of the cluster was estimated with equation 1.

$$Ci = V_{eb}/V_{tc} \quad \text{(Equation 1)}$$

V_{eb} is the volume that berry material effectively takes up in the whole cluster volume and V_{tc} is the estimated volume of the cluster. V_{eb} was estimated by multiplying n_{bc} (the mean number of berries

per cluster) by the mean berry volume calculated assuming an ellipsoidal shape of berries with width and height radius of "*a*" and "*b*", respectively (as shown in equation 2).

$$V_{eb} = n_{bc} \text{ x } 4/3\ \pi^2 b \quad \text{(Equation 2)}$$

V_{tc} was estimated assuming a conical shape of clusters, where *R* is the half of the cluster width and *H* is the cluster height (Equation 3).

$$Vtc = 1/3\ \pi R^2 H \quad \text{(Equation 3)}$$

Ratio skin/berry is expressed in %, and was obtained weight of skins/berry weight.

Grape juice analysis. The soluble solids of grape juice were determined by refractrometry, pH values achieved by pH meter and titratable acidity (TA) with 0.1N NaOH and bromothymol blue as indicator (expressed as g/L of tartaric acid equivalents).

Grape skin analysis. For the two grape varieties studied, on day 161, 172, 185, 200, 213, 222 and harvest time 231, 20 berries were sampled and weighed for each treatment in triplicate. Berry skins were removed manually from the pulp using a laboratory scalpel, weighed, and quickly placed in 50 ml hydro-alcoholic buffer at pH 3.2, containing 2 g/L $Na_2S_2O_5$ and 12% of ethanol. The samples were stored at - 20°C until analysis for phenolic compounds. The total phenolic content of skins extracts of grapes was determined using the Folin–Ciocalteu method (34). Determination of total anthocyanins was performed using the method described by Di Stefano et al. (1989) (35) and total proanthocyanidins described by Di Stefano e Cravero (1991) (36). Total anthocyanins, phenolics and proanthocyanidins were expressed in mg per kg grapes and mg per berry. All analyses were run in triplicate.

Wine analysis. Eight microvinifications were carried out to study influence of the agricultural treatments to wine composition and quality. All treatments were individually harvested manually on August 19 (DOY 231) just before the harvest of company. For each treatment, approximately 100 kg of grapes were stored overnight in a cool chamber (4°C) and the following day warmed to room temperature before being slightly crushed. All musts were immediately inoculated with selected *Saccharomyces cerevisiae* yeast (Lalvin BM 4X4, Lallemand Inc., Montreal, Canada). Fermentations were conducted in tanks at 25°C for 10 days. The total polyphenols (34), total anthocyanins (35) and total proanthocyanidins (36) of the wine were determined by UV-VIS spectrophotometry. Spectrophotometric measurements of absorbance at 420, 520 and 620 nm were made using a 1 mm quartz cuvette. Colour intensity was calculated by adding absorbance values at 420, 520 and 620 nm (37). The tonality of the wine is defined as the ratio of absorbance at 420 and 520 nm (37).

Statistical analysis. Within each variety analysis of variance ANOVA was used to test the main effect using SPSS software (IBM SPSS version19). Comparison of means was performed using Duncan test at $p < 0.05$.

Results and discussion

Meteorological data. The season 2011 was dry and hot. The average temperature in June was 21.8°C, in July 25.4°C and in August 27.1°C. Days with extreme temperatures (> 35°C) were: in June 1 day, in July 9 days and in August 13 days (all in the second half of the month).

Total rainfall for the three months was 374.5 mm referred: in June 353.5 mm, July 21mm and without registered precipitation in August. High temperatures with no precipitation in the second half of August caused dehydration of berries and accelerated (anticipated) harvesting.

Berries growth. In the first phase of berry growth, with the Cabernet Sauvignon variety, early defoliation affected the berry growth, causing delay in development compared to those of the control. However, before veraison, defoliated treatment showed a higher average berry weight with no significant differences (Figure 1A). During the ripening stage the cluster thinning treatment (NLR-CT) did not modify the growth of the berry in confront to the control. The treatment previously defoliated (LR-NCT) showed a delay in growth after veraison, even if at harvest it did not showed difference by control and thinned treatment. The treatment defoliated and then thinned (LR-CT) stopped in advance the growth and showed an early transition in over ripening. As a result at harvest only the treatment LR-CT showed an average weight of berry significantly inferior to the other treatments, which did not significantly differed among them (Figure 1B).

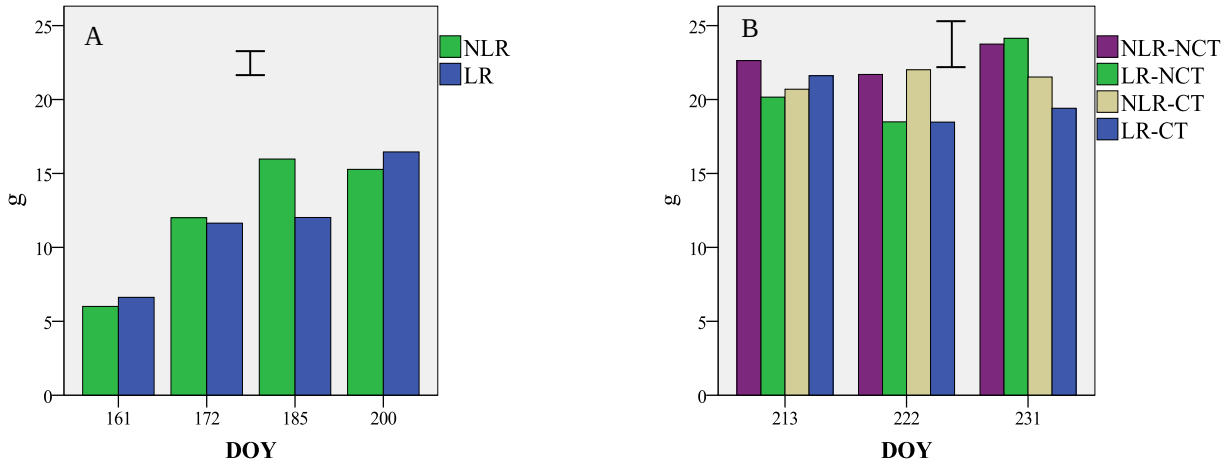

Figure 1. Berry weight in Cabernet Sauvignon in 2011 from berry set to before veraison (A) and after veraison to harvest (B). Treatments: leaf removal (LR) not leaf removal (NLR), leaf removal-not cluster thinning (LR-NCT), not leaf removal-cluster thinning (NLR-CT), leaf removal-cluster thinning (LR-CT) and control (NLR-NCT). LSD from berry set to before veraison (A)=1,56 g and from veraison at harvest (B)=3,06 g.

In cultivar Vranac during the first part of the berry growth, until veraison, in response to defoliation there were not significant differences in the berry weight (Figure 2A). However, the treatment defoliated presented before veraison a higher berry weight. In the course of ripening the cluster thinning did not affect the berry growth. The defoliated treatment (LR-NCT), similar to Cabernet Sauvignon, had a developmental delay, but reached similar values at harvest to the other treatments.

Overall, all featured treatments (o types of treatments) showed the maximum weight of the berries in the DOY 213 and decrease in the last 20 days (Figure 2B).

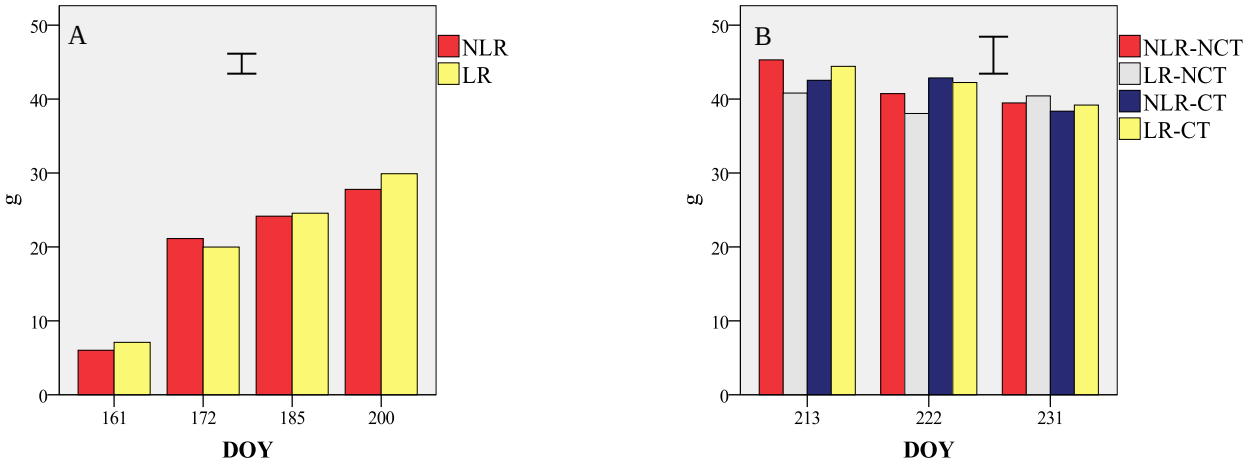

Figure 2. Berry weight in Vranac in 2011 from berry set to before veraison (A) and after veraison to harvest (B). Treatments: leaf removal (LR) not leaf removal (NLR), leaf removal-not cluster thinning (LR-NCT), not leaf removal-cluster thinning (NLR-CT), leaf removal-cluster thinning (LR-CT) and control (NLR-NCT). LSD from berry set to before veraison (A)=2,65 g and from veraison at harvest (B)=5,06 g.

Yield components. Table 1 shows the yield production per vine of four experimental conditions for two varieties. The defoliation significantly reduced yield in both variety. The impact of this practice on yield is higher in Cabernet Sauvignon: defoliation reduced yield by 36% and defoliation with subsequent cluster thinning reduced it by 63%. Reduction was less in the Vranac variety: defoliation reduced yield by 23%, while defoliation followed by cluster thinning reduced it by 46% compared to the control.

Table 1. Yield components and cluster and berry characteristics at harvest recorded in 2011 in Cabernet Sauvignon and Vranac. Vines subjected to early defoliation (LR-NCT), cluster thinning (NLR-CT), early defoliation and cluster thinning (LR-CT) or control (NLR-NCT).

		Clusters / Vine	Yield/vine (kg)	Cluster wt (g)	Berry wt (g)	Total berries/ cluster	Compactness index	Skins/berry %
Cabernet Sauvignon	NLR-NCT	18	1.48 c	134 b	1.13 b	113 b	0,18 a	17.17 a
	LR-NCT	17	0.96 b	117 b	1.13 b	97 ab	0,18 a	20.48 a
	NLR-CT	10	0.91 b	163 c	1.08 b	152 c	0,28 b	18.59 a
	LR-CT	9	0.56 a	86 a	0.94 a	89 a	0,22 a	17.18 a
Vranac	NLR-NCT	13	2.35 b	176 a	1.96 a	89 a	0,45 a	16.10 a
	LR-NCT	11	1.82 a	161 a	1.96 a	80 a	0,42 a	19.86 a
	NLR-CT	9	1.64 a	170 a	1.94 a	89 a	0,47 a	14.73 a
	LR-CT	9	1.27 a	147 a	1.87 a	75 a	0,32 b	16.73 a

a,b,c-different letters within each column differ significantly according to Duncan Test at $p < 0.05$

In Cabernet Sauvignon the defoliation reduced the average weight of the bunch and total berry numbers per cluster. On the other hand, the cluster thinning had no influence on the berry weight. The treatment "defoliated-cluster thinning" had lower bunch weight and berry weight. Both treatments defoliated showed the lowest number of berries per cluster, while the treatment "cluster thinning" recorded the highest number of berries per bunch. As a consequence, the density of the cluster is higher in the treatment NLR-CT. There are no significant differences in the ratio skin / berry (Table 1).

In cultivar Vranac early leaf removal slightly but not significantly reduced the cluster weight. There are not the differences in the berry weight and in the number of berry per cluster among treatments. Anyway, the treatment "leaf removal-cluster thinning" had a lower berry weight and number of berry which determine the lowest average cluster weight. The index of compactness is lower in the treatment LR-CT. The treatment LR-NCT recorded a higher (but not significant) effect in the relationship skin / berry.

Grape juice analysis. Both varieties and all treatment show good accumulation of sugars. However, the control treatment showed the lowest value in content of sugar. The best accumulation of soluble solids in Vranac was achieved by the treatment defoliated-thinned (LR-CT), while the cluster thinning treatment (NLR-CT) had a greater value in the variety Cabernet Sauvignon (Table 2). None treatment induced significant change in titratable acidity and pH, that always resulted in agreement with the expected values.

Table 2. Must composition at harvest recorded in 2011 in Cabernet Sauvignon and Vranac. Vines subjected to early defoliation (LR-NCT), cluster thinning (NLR-CT), early defoliation and cluster thinning (LR-CT) or control (NLR-NCT). The reported values are from must just crushed for microvinification.

		TSS (Brix)	Titratable acidity (g tartaric acid/l)	pH
Cabernet Sauvignon	NLR-NCT	22.40	7.40	3.59
	LR-NCT	24.40	8.90	3.53
	NLR-CT	25.60	7.57	3.59
	LR-CT	24.80	7.95	3.58
Vranac	NLR-NCT	22.00	6.77	3.57
	LR-NCT	22.80	6.60	3.62
	NLR-CT	22.80	6.75	3.61
	LR-CT	24.60	6.53	3.59

Total polyphenols in berry skins. Early defoliation in Cabernet Sauvignon, in the early stages of berry growth leads to increase the total berry polyphenols (Figure 3A). Before veraison there were no significant differences in the total polyphenols content between the treatments. Increased content in mg/kg grapes was due to the less berry weight and not of increase of synthesis (Figure 3B).

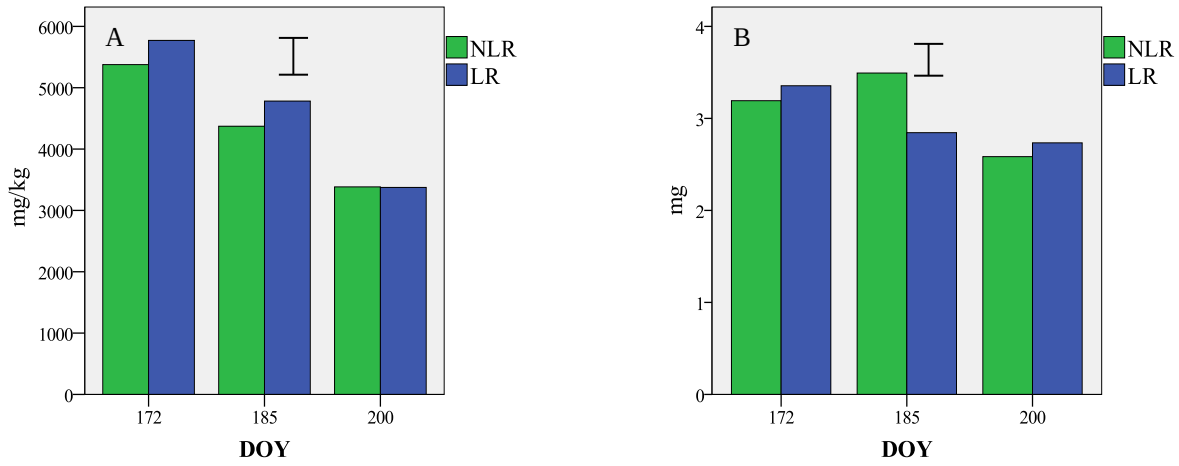

Figure 3. Effect of leaf removal on levels of total polyphenols (A) and polyphenols per berry (B) in Cabernet Sauvignon in 2011 from berry set to before veraison. Treatments: leaf removal (LR) and not leaf removal (NLR). LSD for total polyphenols (A)=604 mg and for polyphenols per berry (B)=0,34 mg.

The total phenols in the variety Vranac, up to veraison, increased due to the effect of early leaf removal (Figure 4A). Polyphenols content in mg per berry was also increased in defoliated treatment, due to increased synthesis in the berries (Figure 4B).

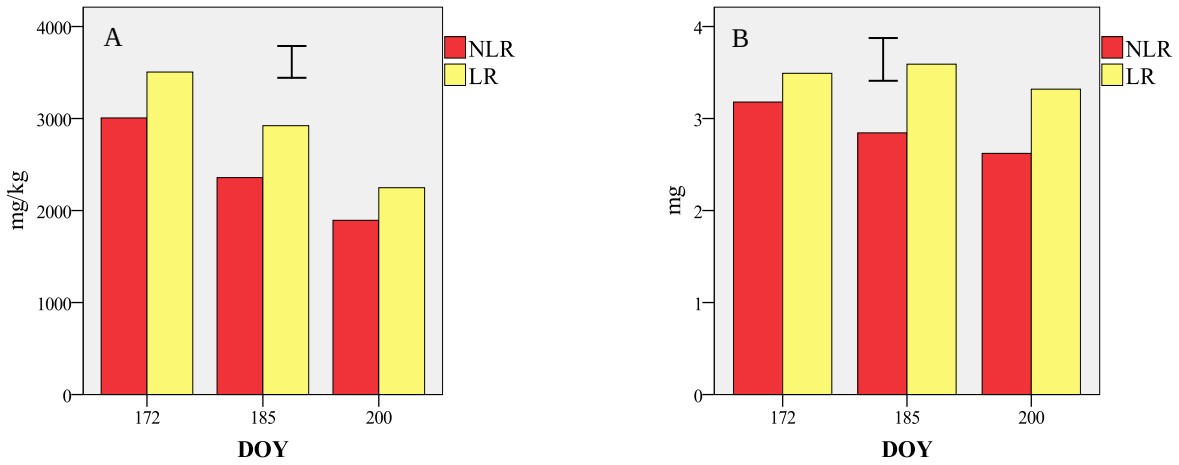

Figure 4. Effect of leaf removal on levels of total polyphenols (A) and polyphenols per berry (B) in Vranac in 2011 from berry set to before veraison. Treatments: leaf removal (LR) and not leaf removal (NLR). LSD for total polyphenols (A)=347 mg and for polyphenols per berry (B)=0,45 mg.

Total anthocyanins in berry skins. Figure 5A shows the total anthocyanin content (mg/kg grapes) in Cabernet Sauvignon from veraison to harvest. No significant differences between the treatments were found, except at harvest time. The control (NLR-NCT) and defoliated treatment (LR-NCT) differs from cluster thinning (NLR-CT) and defoliated –thinned (LR-CT) treatments which had higher athocyanins concentration. This effect seems to be related to berry growth and not to the increase of the synthesis, as can be seen in figure 5B.

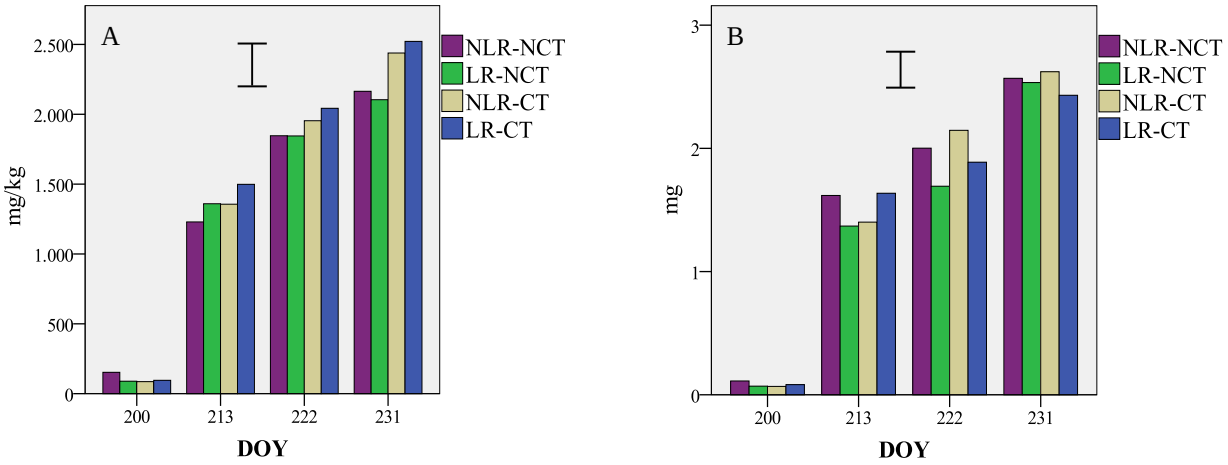

Figure 5. The total anthocyanins (A) and anthocyanins per berry (B) in Cabernet Sauvignon in 2011 from veraison to harvest. Treatments: leaf removal-not cluster thinning (LR-NCT), not leaf removal-cluster thinning (NLR-CT), leaf removal-cluster thinning (LR-CT) and control (NLR-NCT). LSD for total anthocyanins (A)=302 mg and for anthocyanins per berry (B)=0,29 mg.

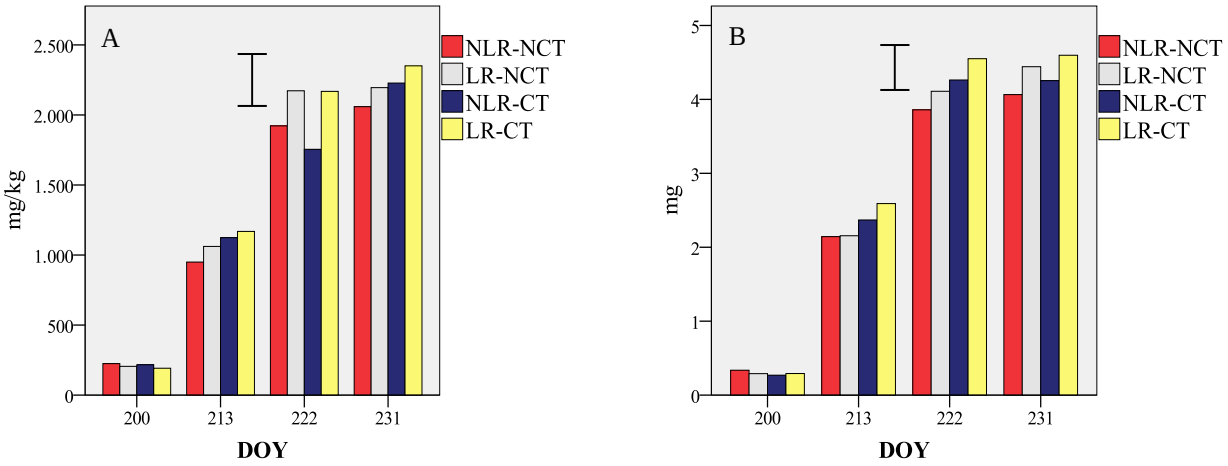

Figure 6. The total anthocyanins (A) and anthocyanins per berry (B) in Vranac in 2011 from veraison to harvest. Treatments: leaf removal-not cluster thinning (LR-NCT), not leaf removal-cluster thinning (NLR-CT), leaf removal-cluster thinning (LR-CT) and control (NLR-NCT). LSD for total anthocyanins (A)=373 mg and for anthocyanins per berry (B)=0,62 mg.

No significant differences between the treatments during maturation were found in the total anthocyanin content for the cultivar Vranac, except that at DOY 222 is observed lowest content in the thinned treatment (Figure 6A). However, the highest concentration was in the treatment defoliated-thinned. The concentration of anthocyanins per berry is not associated to berry growth albeit, to the increase in their synthesis (Figure 6B). Besides, agricultural practices defoliation and

cluster thinning have had an impact to the content of anthocyanins, increasing it compared to the control.

Total proanthocyanidins in berry skins. The content of proanthocyanidins in Cabernet Sauvignon, from berry set to before veraison, reacts in the same way as polyphenols: defoliation causing retardation of the berry growth and increased content of proanthocyanidins (Figure 7A). Even in this case, the effect was not due to the increased synthesis per berry (Figure 7B).

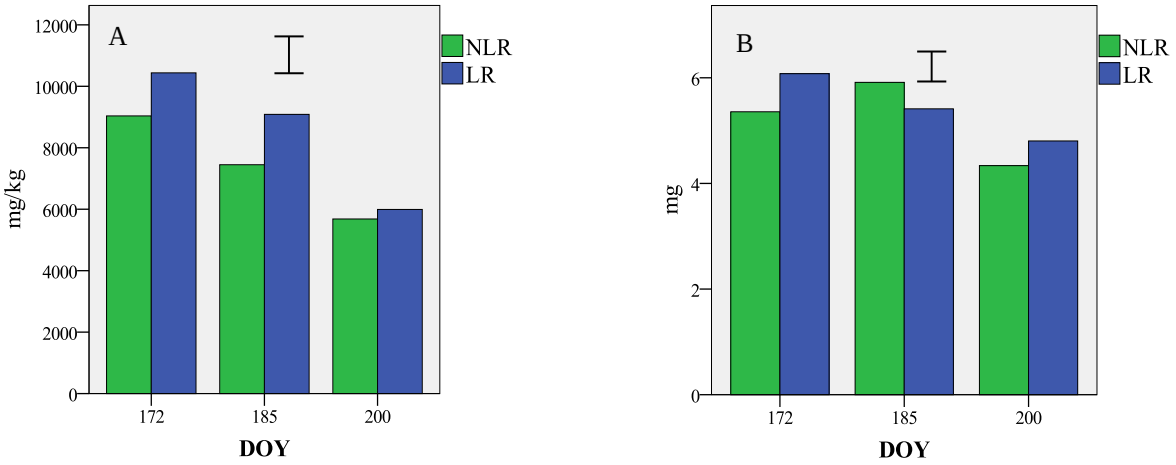

Figure 7. Effect of leaf removal on levels of total proanthocyanidins (A) and proanthocyanidins per berry (B) in Cabernet Sauvignon in 2011 from berry set to before veraison. Treatments: leaf removal (LR) and not leaf removal (NLR). LSD for total proanthocyanidins (A)=1200 mg and for proanthocyanidins per berry (B)=0,58 mg.

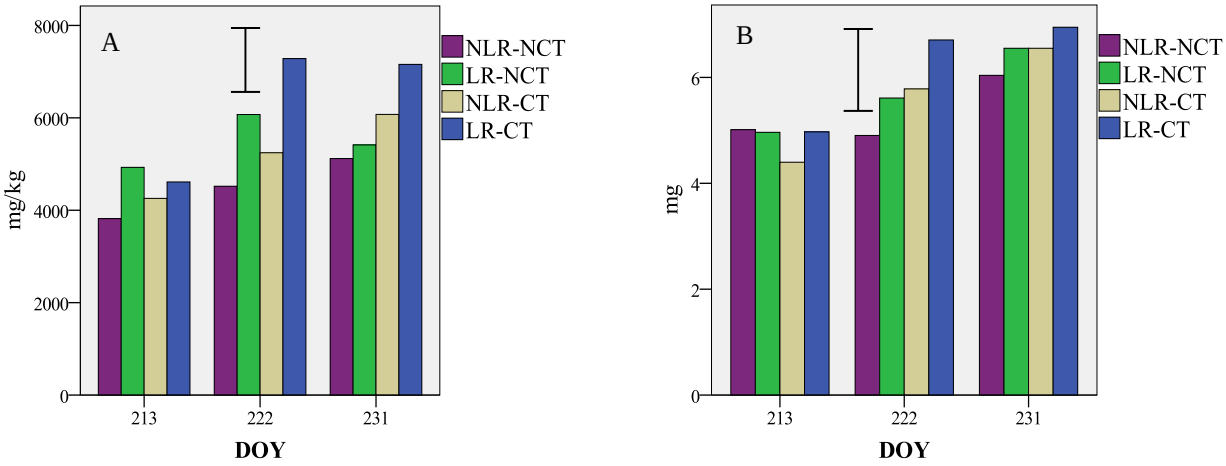

Figure 8. The total proanthocyanidins (A) and proanthocyanidins per berry (B) in Cabernet Sauvignon in 2011 from veraison to harvest. Treatments: leaf removal-not cluster thinning (LR-NCT), not leaf removal-cluster thinning (NLR-CT), leaf removal-cluster thinning (LR-CT) and control (NLR-NCT). LSD for total proanthocyanidins (A)=1387 mg and for proanthocyanidins per berry (B)=1,56 mg.

After veraison, for all treatments a further increase in the content of proanthocyanidins was observed, which could be due to the persistence in the synthesis or different extraction of proanthocyanidins from the skins (different compartmentation in the cell). All viticultural practices

led to higher content of proanthocyanidins at harvest, without significant differences (Figure 8A and 8B).

In Vranac, before veraison, greater accumulation of total proanthocyanidins and proanthocyanidins per berry in the treatment defoliated was observed (Figure 9A and 9B). At harvest the highest contents per berry and in mg/kg grapes was observed in the treatment defoliated-cluster thinned. However, treatments defoliation and cluster thinning enhanced proanthocyanidins concentration compared to the control (Figure 10A and 10B).

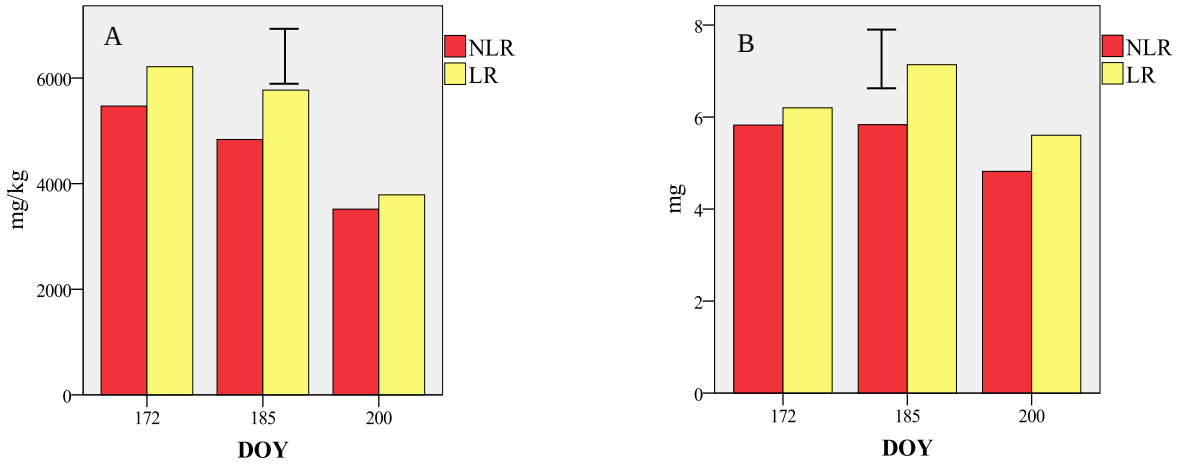

Figure 9. Effect of leaf removal on levels of total proanthocyanidins (A) and proanthocyanidins per berry (B) in Vranac in 2011 from berry set to before veraison. Treatments: leaf removal (LR) and not leaf removal (NLR). LSD for total proanthocyanidins (A)=1044 mg and for proanthocyanidins per berry (B)=1,30 mg.

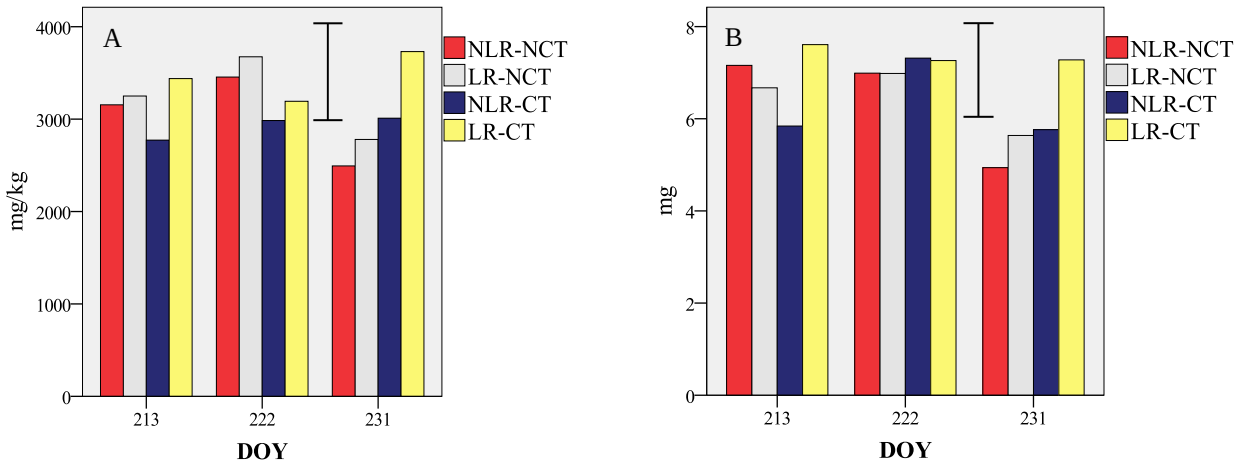

Figure 10. The total proanthocyanidins (A) and proanthocyanidins per berry (B) in Vranac in 2011 from veraison to harvest. Treatments: leaf removal-not cluster thinning (LR-NCT), not leaf removal-cluster thinning (NLR-CT), leaf removal-cluster thinning (LR-CT) and control (NLR-NCT). LSD for total proanthocyanidins (A)=1026 mg and for proanthocyanidins per berry (B)=2,03 mg.

Wine analysis. Table 3 shows descriptors of wines made from grapes of four experiments for two varieties. In the wines of cultivar Cabernet Sauvignon, higher alcohol content was found in the cluster thinning treatment (as a result of the best accumulation of sugar) and lowest in the control. All the wines present similar color hue. The value of total anthocyanins, polyphenols, proanthocyanidins and color intensity was highest in the treatment defoliated-cluster thinned followed by the treatment defoliated. The lowest values of all parameters were found in the control treatment. The ethanol content in Vranac wines, in accordance with sugar accumulation, was same for the treatment "defoliated" and treatment "cluster thinning", highest in the treatment "defoliated-cluster thinned" and lowest in the control. The values of color intensity and color hue were very similar for all the treatments, even though some slightly higher value was found in the treatment "defoliated-cluster thinned". The content of total anthocyanins, polyphenols and proanthocyanidins is highest in the treatment "defoliated-cluster thinned" followed by the treatment "defoliated". The best wine characteristics were found in products from the plots where defoliation was applied. These results could be due to better extraction of polyphenolic compounds in wine.

Table 3. Wine composition at harvest recorded in 2011 in Cabernet Sauvignon and Vranac. Vines subjected to early defoliation (LR-NCT), cluster thinning (NLR-CT), early defoliation and cluster thinning (LR-CT) or control (NLR-NCT).

		Alcohol content (% vol)	Colour intensity	Colour hue	Total anthocyanins (mg/l)	Total polyphenols (mg/l)	Total proanthocyanidins (mg/l)
Cabernet Sauvignon	NLR-NCT	13.43	1.42	0.63	295	1897	872
	LR-NCT	14.59	1.76	0.62	333	2311	1112
	NLR-CT	15.36	1.69	0.64	318	2028	910
	LR-CT	14.94	1.89	0.64	353	2749	1224
Vranac	NLR-NCT	13.31	1.86	0.57	389	1532	308
	LR-NCT	13.67	1.86	0.59	392	1711	314
	NLR-CT	13.68	1.85	0.59	344	1548	292
	LR-CT	14.68	2.06	0.61	467	1842	555

Conclusion

Objectives of the research program were the effect of some environmental and physiological aspects on the intensity of flavonoid synthesis.

The study was conducted in 2011 in Podgorica, Montenegro. Two grapevine cultivars were selected to confront different ability in flavonoid accumulation: Vranac, with moderate accumulation and Cabernet Sauvignon with good accumulation of polyphenols. In particular the following experimental treatments were compared: early leaf removal (flowering time), cluster thinning (veraison time) and combination of both treatments.

The early defoliation reduced yield per vine in Cabernet Sauvignon and Vranac. In Cabernet Sauvignon defoliation initially delayed berry growth, but at harvest only the treatment "defoliation-cluster thinning" had significantly lower berry weight. In cultivar Vranac defoliation did not modify the berry growth and berry weight. In the both varieties, cluster thinning had no affect on the berry weight. In the treatments "defoliated-thinned" is observed reduction of the cluster weight, berry weight and berry number per cluster. This is probably the consequence of a lower fruit set, where the defoliation had a greater impact on the first cluster. At harvest, no damaged bunches (caused by sunburn) were found in defoliated treatment.

Early defoliation and cluster thinning in both varieties raised the concentration of anthocyanins and proanthocyanidins. The enhanced contents of these compounds per berry in variety Vranac is the result of increased synthesis.

Defoliation and cluster thinning led to the better soluble solids accumulation than in the control. The skins extracts contained the highest content of anthocyanins and proanthocyanidins in the treatment defoliated - thinned followed by the treatment thinned, while these contents were higher in wines from the vineyards where was applied defoliation. It could be due to better extraction of these compounds during winemaking.

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GENOTYPE X ENVIRONMENT INTERACTION IN GRAPEVINES: ECOPHYSIOLOGICAL BASIS AND GRAPES RIPENING EFFECTS

Introduction

Genotype x environment interaction represents a dominant aspect of the viticulture. The qualitative expression of most of wine cultivars is assumed to be strongly affected by the soil and climatic growing conditions. The specific environmental conditions are considered to have an effect of paramount importance in comparisons with the possible role played by the cultural techniques in determining the quality potential of grapes and wine.

The aim of this study was to evaluate the effect of genotipe x environment interaction on the evolution of carotenoids, chlorophylls, C-13 norisoprenoids and flavonols through ripening of grape berries from unripe (green) to ripe (red) stage in two cultivars: Sangiovese and Cabernet Sauvignon. Study was carried out in two growth seasons 2011-2012 in three different sites: Bolgheri, Montalcino and Misano Adriatico. In each site was therefore identified consistent features of the experimental plots and clonal rootstock, matched for age, details of the planting and management, and representative of the prevailing conditions of the regions concerned.

Furthermore, the study aimed to explore the potential relationship, if any, between carotenoid content and norisoprenoid aromatic compounds.

Chlorophylls in grapes

Chlorophylls are the pigments responsible for photosynthesis, the fundamental light-driven process in which carbon dioxide is fixed to yield carbohydrates and oxygen. All green plants contain chlorophyll a (major pigment) and chlorophyll b (accessory pigment). Chlorophylls are located in small subcellular organelles, the plastids, called chloroplast. In the chemical structure, chlorophyll b differs from chlorophyll a, only by having an aldehyde group (-CHO) in place of the methyl group at position 3. Small differences in the structures of the two chlorophylls produce differences in the absorption maxima of chlorophyll a and chlorophyll b. Since chlorophyll b absorbs strongly in the 450-480 nm range, it can captures light at low intensity effectively, partially filling the gap in the chlorophyll a spectrum.

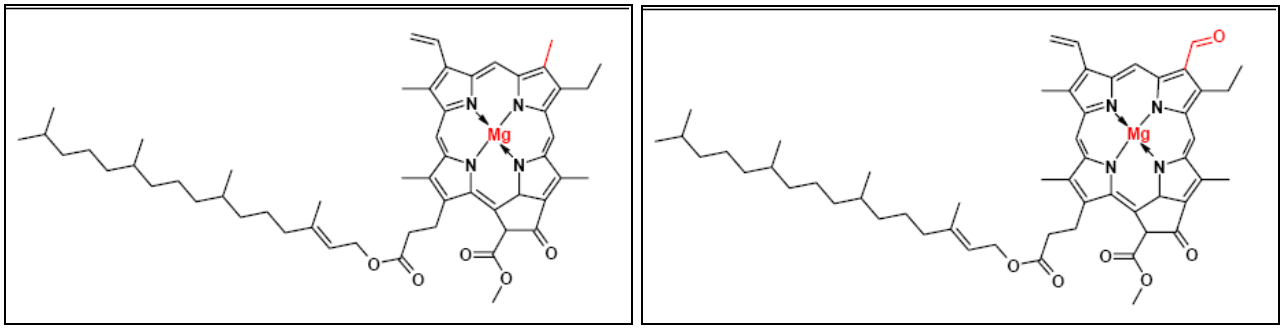


Figure 1. The structure of chlorophyll a and chlorophyll b

Chlorophyll content of grape berries decreases with ripening, some authors found that chlorophyll concentration (mg/g fresh weight of berry) starts to decrease from two weeks after veraison until the fourth week post veraison to approximately 50% of the original concentration and remains at this level until harvest (1). Plant species exposed to sun tend to have a greater content in chlorophyll a while in shaded plants higher content of chlorophyll b is due to it absorption properties. The less chlorophyll content in shaded berries throughout the ripening season compared to berries exposed to sunlight showing that chlorophyll synthesis in grape berries is light-induced (1).

Chlorophylls are susceptible to degradation either by means chemical (weak acids, oxygen, light and heat) or enzymatic. Chlorophyll a is more rapidly degraded than chlorophyll b. Chemical degradation of chlorophylls leads to the formation of a large number of degradation products. Pheophytins are easily obtained from chlorophylls by the action of dilute acids, where the central magnesium atom is replaced with hydrogen. Pheophytins are reported in grapes, but it is unsure if these breakdown products of chlorophylls do exist in grape berries or if they are artefacts of berry sample processing (2).

Carotenoids in grapes

The carotenoids are isoprenoid polyenes with 40 carbon atoms, synthesized only in plants. They are important natural yellow, orange, and red pigments. The carotenoids have several important roles in plants: to absorb light at different wavelengths from the chlorophylls, transfer the absorbed light energy to the chlorophylls and protect photosynthetic apparatus from photo-oxidative damage. Under excess of light, violaxanthin is converted rapidly via the intermediate antheraxanthin to zeaxanthin dissipating excess energy, and this reaction is reversed under low light levels. Carotenoids are located in leaves and many non-photosynthetic plant organs, such as: fruit, roots, seeds, pollen and petals. They are split into two classes: xanthophylls (which contain oxygen, *e.g.,* Lutein) and carotenes (which are purely hydrocarbons, *e.g,.* β-carotene).

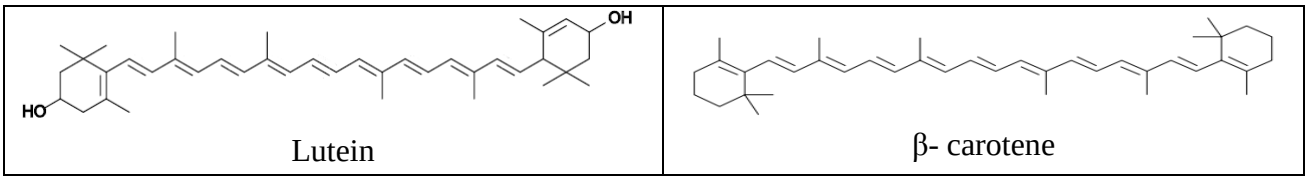


Figure 2. Chemical structures of Lutein and β-carotene

Like chlorophylls, carotenoids are located and synthesized in chloroplasts, but unlike chlorophylls are also synthesized in chromoplasts. During the maturation of many fruit (like tomatoes, peppers and paprika) chloroplasts transform into chromoplasts when chlorophylls disappear, new carotenoids are synthesized and fruit colour changes. Grape carotenoids are synthesized mostly from the first stage of fruit formation until veraison. The level of carotenoids decreases from veraison until end of maturity (5). This decline in carotenoids from veraison to harvest is probably due to the disappearance of chloroplasts which are not transformed into chromoplasts able to synthesize carotenoids. Lutein-5,6-epoxide, violaxanthin, and luteoxanthin have a particular behavior during the maturation period with consistent rises around the end of veraison. Their evolutions do not follow those of β-carotene, lutein, flavoxanthin and neoxanthin, which decrease in that period (11).

Levels of carotenoids in the grapes are higher in skin than in pulp; probably photosynthetic activity is higher in the skins (4). The carotenoids found in mature berries probably remain from the synthesis attained during the green stage of the fruit (4).

In grapes the most common carotenes are β-carotene and lutein (almost 85%) accompanied by minor xanthophylls such as neoxanthin, violaxanthin, lutein-5,6-epoxide, zeaxanthin, neochrome, flavoxanthin and luteoxanthin (3). Carotenoids exist in the cis- or trans- isomer configurations, most of them in berries are in the all-trans-configuration. Isomerisation of all-trans-carotenoids to cis-isomers is promoted by contact with acids, heat treatment and exposure to light. However, for cis-isomers of lutein, β-carotene and neoxanthin reported in grapes is not certain if these exist in grape berries or if it is an artefact of sample processing (2).

The combination and interactions of several factors are strongly considered to be responsible for the qualitative and quantitative profiles of carotenoids in grapes including: plant variety, climatic conditions, maturity stage, soil characteristics and viticulture practices.

Light is one environmental factor with the greatest influence on the growth and development of higher plants, essential for photosynthesis. It seems that light promotes the increase of carotenoids in the first stage of berry growth in the sun exposed grapes compared to shaded grapes. Instead, from verasion to the end of maturity, grapes exposed to sunlight show a significant decrease of carotenoids compared to grapes under shade conditions (6, 7). The temperature effect on grape carotenoids composition is complex, and is associated to the different degree of sunlight exposure. In general the highest carotenoid levels occurred in hot regions (7). Though, in the Douro Valley, the high-elevation terraces which presented a lower temperature and higher humidity during the maturation period, produced grapes with higher carotenoid values (6). In high vigour vines the amount of sunlight which infiltrates to the bunch zone is less. Grapes grown with higher vegetative height seem to have higher carotenoid levels (6). Some studies indicate that the carotenoids content in higher water retention capacity soil was similar for nonirrigated and irrigated treatment, while in the lower water retention soil capacity the levels of carotenoids was lower in irrigated treatment (8).

Carotenoids are unstable compounds. Because, from veraison to maturity they are liberated into the cell and it start to degrade with chemical and enzymatic reactions that involve their typical conjugated double-bond structure. This generates several compounds, some of which have powerful aroma properties, glycosylated C13-norisoprenoids. Considering that carotenoids are precursors of norisoprenoids, the knowledge of the biogenetic pathway for the formation of these compounds was already interpreted as a useful tool for the prediction of potential volatilities in wine (9,10), but are few studies in literature concerning a quantitative relationship between carotenoids level and potential norisoprenoid aroma in grapes. Wine aroma is one of the most important aspects of wine quality, and aromas originating from the grape berries make a paramount contribution of the final product.

Norisoprenoids in grapes

The C13-norisoprenoids in wines can derive from direct carotenoid degradation by enzymatic reactions (catalysed by dioxygenases) or can be released during winemaking or storage by nonenzymatic mechanisms (light, oxygen, temperature and acid hydrolysis). These compounds typically have low sensory thresholds and therefore present interesting flavour aroma properties even at very low concentrations. The C-13 norisoprenoids can be divided into: (a) compounds with the megastigmane structure (the family of ionones and damascones with oxygen at different positions) and (b) compounds with the megastigmane structure but without oxygen in the lateral chain ((E,E)-megastigma-4,6,8-triene). Compounds such as 2,2,6-trimethylcyclohexen-1-one, β-cyclocitral and DHA (dihydroactinidiolide) are examples of C9, C10, C11 norisoprenoids, respectively (9). The norisoprenoids in grapes are present in free (non-glycosylated) and mainly in bound (glycoconjugated) fraction.

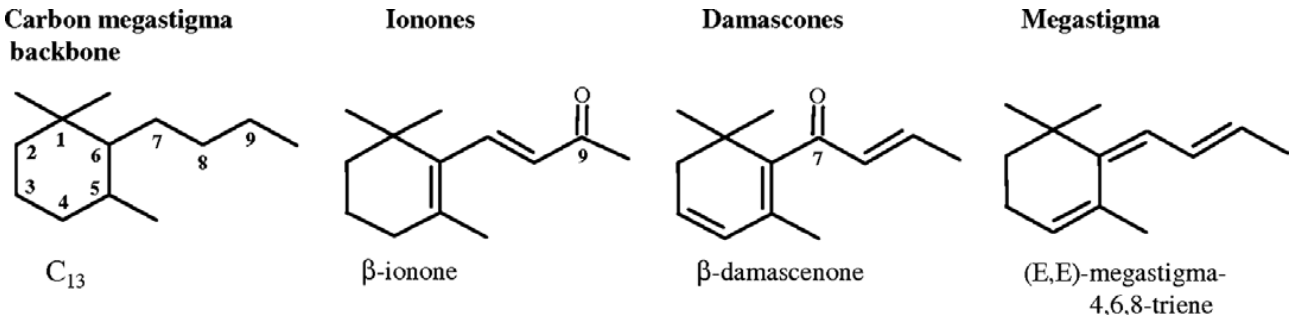


Figure 3. Chemical structures of norisoprenoids with the megastigma carbon backbone

The one of the most potent odorants known is norisoprenoid β-damascenone [(2,2,6-trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one], with extremely low odour threshold in water of 2 ng/L (12), described as honey, flowery, cooked apple, quince like aroma. The odour thresholds in red wines depending on the wine matrix and can reach concentrations of 4000 ng/L (13,14).). One of the important C13-norisoprenoids which contributes to wine aroma is β-ionone [(2,2,6-trimethyl-1,3-cyclohexen-1-yl)-3-buten-2-one] with a low threshold value of 90 ng/L (in a model base wine) (15) has a violet, woody, raspberry like aroma. TDN (1,1,6-trimethyl-1,2-dihydronaphthalene) has received particular interest due to a kerosene-petrol like aroma and gives a characteristic bottle-aged aroma to many wines, particularly Rieslings. Reported aroma threshold is 20 μg/L and the aged Riesling wines can reach 200 μg/L. Acinidiol it has a descriptor of camphoraceous or woody and resinous and the odour threshold is still undefined. Vitispirane (2,10,10-trimethyl-6-methylene-1-oxaspiro[4.5]dec-7-ene) has a eucalyptus or camphoraceous odour with an aroma threshold in wine of 800 μg/L (16). Numerous other individual norisoprenoids have been identified that also contribute to complex aroma of red and white wines such as: riesling acetal (2,2,6,8-tetramethyl-7,11-dioxatricyclo [6.2.1.$0^{1,6}$]undec-4-ene), 4- buta-1,3-diene (TPB), vomifoliol, β-cyclocitral and α-ionone.

It was reported that norisoprenoids could originate from direct degradation of carotenoid molecules such as β-carotene, lutein, neoxanthin, and violaxanthin (17). Recently, it was demonstrated that β-

damascenone can be formed directly from 9-cis-neoxanthin by chemical oxidation using high temperatures (18). Other researchers reported that the carotenoid lutein might be the original precursor of TDN in their study of the breakdown of lutein in a heated model wine solution (19). β-ionone can be formed as a cleavage product of the carotenoid β-carotene (20) and zeaxanthin (21).

Although the aromatic compounds are typical for the grape variety, they are also closely related to the soil, climate, viticultural practices and the conditions of production. Several works were carried out to understand how the content of norisoprenoids depends on these factors.

The carotenoid profile of 8 grapes variety at harvest date was related to the C13- norisoprenoids from Duoro region. The cultivars with low carotenoid content correspond to wines with higher levels of the grape-derived C13-norisoprenoid volatiles β-ionone, TDN and vitispirane (9).

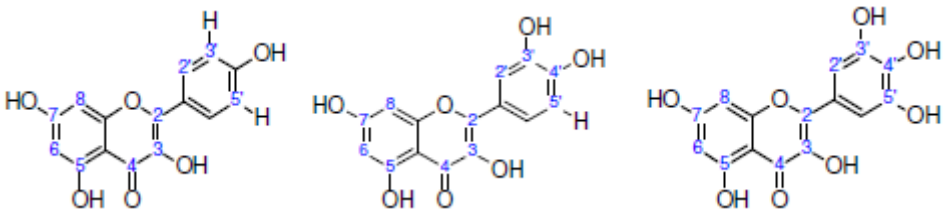
The study of effect of the light environment to the aroma of cv. Muscat shows that artificial shading decreased the levels of the free and glycosylated C13-norisoprenoids when compared to naturally shaded and sun-exposed berries, which had similar levels of C13-norisoprenoids (22). The same authors reported similar results for the effect of bunch shading in cv. Syrah, adding that total levels of bound C-13 norisoprenoids were not modified by cluster thinning (23).

However, another study of the effect of sun-exposed and natural shaded grape bunches on the C13-norisoprenoid content of cvs. Chenin blanc and Weisser Riesling confirms that with a exceptions for β-damascenone, norisoprenoids concentrations were significantly higher in sun-exposed grapes than in the shaded grapes (24).

The study of the effect of different *terroirs* in the Rhone Valley on the volatile compounds of cv. Grenache wines, suggested two major groups of wine. The wines from the southern zone with warmer climate where grape maturation occurs early, contains the highest amount of β-damascenone. The other group consists of wines from soils producing grapes that mature later and wines with higher amounts of β-ionone (25).

Flavonols in grapes

The flavonols are a class of flavonoid compounds and they act as UV protectors and free radical scavengers in the plants. The flavonols are yellow pigments which contribute to the colour in white wine, while for red wine are important as copigments for the anthocyanins. Flavonols in grapes exist only as 3-glycosides. In grapes have been found kaempferol (4'-hydroxy flavonol), quercetin (3',4'-dihydroxy flavonol), and myricetin (3',4',5'-trihydroxy flavonol) together with isorhamnetin the methoxylation product of the 3'-OH of quercetin.



Kaempferol Quercetin Myricetin

Figure 4. Chemical structures of flavonols

The corresponding free aglycones can be found in wines as a result of acid hydrolysis that occurs during winemaking and aging. The content of isorhamnetin depends on the variety, can be present only in trace amounts, while in the white grape cultivars are present only kaempferol and quercetin. The content of flavonols in grapes varies from 10 to 120 mg/kg, while their content in red wines is equal to 100 mg/L in both forms: aglycones and glycosides.

The flavonols in grapes are synthesized in the first phase of berry growth and after veraison. The content of these compounds is higher in the grapes exposed to the sunlight. The bunch shading in Syrah berries significantly decreased flavonol synthesis in the grape skin. In the exposed fruit, flavonol concentration was highest around flowering then declined during berries grew, but there was an increase in flavonols per berry during ripening. When the shading was applied before flowering, grapes had much lower levels of flavonols throughout berry development and at harvest than in the exposed fruit (1). However, also the other authors report that the exposure to solar radiation increased concentrations of the total flavonols, while temperature had little to no effect on their concentrations (26).

Materials and Methods

Grape material and sampling procedure. The study was carried out in the vineyards located in: Bolgheri (Tuscany coast), Montalcino (Tuscany Apennines) and Misano (Romagna coast). The experiment was conducted during the two growing seasons (2011-2012). Two *Vitis vinifera* L. red wine cultivars were selected: Sangiovese and Cabernet Sauvignon. The environmental conditions were the same for the two vineyards to avoid the differences in soil that can lead to an accumulation of different metabolites. In all locations cultivar Sangiovese were VCR23clone and they were grafted onto 420A rootstocks. All Cabernet Sauvignon were R5 clone, rootstock in locality Bolgheri was 161-49, in Montalcino SO4 and in Misano K5BB.

During grapes growing the samplings were carried out at 4 different times: pea size (PS), lag phase (LPh), 20 °Brix (20B) and in the ripening phase (RP). Three biological replicates of 400 berries each were randomly collected at each sampling date. The berries were harvested with pedicels, in the central and lateral cluster area, on the sun exposed and shaded side. Berries were immediately frozen after collection in liquid nitrogen to prevent any enzymatic or thermal degradation and were kept at -20 °C prior to analysis.

Ecophysiological data. The influence of weather condition was assessed with measure of maximum and minimum temperatures at each site, degree days (base of 10°C), global radiation, rainfall, available water content and midday stem water potential.

Air temperature of the vineyard above the canopy layers has been monitored during 2011 and 2012 growing seasons in all three experimental sites. Daily mean, maximum and minimum values have been extracted from hourly values. The growing degree days (GDD) at base 10°C, were calculated adding the average daily temperature subtracting each day 10°C for June through September. If the value is negative the value is equal to zero.

Daily global solar radiation has been reconstructed applying the Heargreaves formula to daily maximum and minimum temperatures of the three experimental sites (33). The Heargreaves radiation formula becomes:

$$R_s = k_{Rs}\sqrt{(T_{max}-T_{min})}\, R_a$$

where: R_a is extraterrestrial radiation ($MJm^{-2}d^{-1}$), T_{max} is maximum air temperature (°C), T_{min} is minimum air temperature (°C) and k_{Rs} is adjustment coefficient (0.16..0.19)($°C^{-0.5}$).

The rainfall data represent and essential input variable for the computation of the Available Water Content based on a tank approach model (29). In a tank model, the water present in a soil (Available Water Content, AWC) can be divided into the following general characteristics thresholds: maximum water capacity (CIM) is the water content in the soil in saturated conditions (when all the interstices are filled with water), field capacity (FC) is the water content of the soil (when all the excess water is percolated) and wilting point (WP) is the water content of the limit below which the plants are no longer able to extract water from the soil.

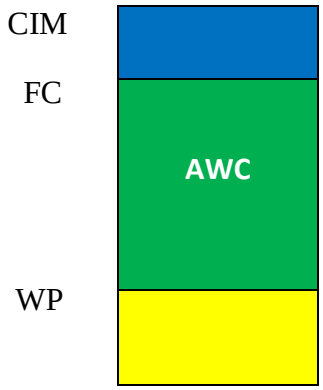


Figure 5. The scheme of tank approach model: maximum water capacity (CIM), field capacity (FC) and wilting point (WP)

The thank dimension and thresholds are influenced by the soil type. Knowing sand and clay values, AWC can be computed by means of Saxton and Rawls function (29). In particular:

INPUT:

Sand, Clay = % in weight (range 0-1)

OM = % organic matter (0-100)

Gravel = skeleton (% 0-1)

OUTPUT:

TETA_1500=1500 kPa moisture [% volume] -> water content at wilting point (WP)

TETA_33=33 kPa moisture [% volume] -> water content at field capacity (FC)

TETA_S= 0 kPa moisture [% volume] -> Water content at saturation.

AWC = WP-FC

In order to estimate the tank reservoir state at time t_0+1 must be considered the following terms: Input: Rainfall (*R*), Irrigation (*I*), Evapotranspiration (*Et*), runoff (*Rs*) Infiltration (*Ip*).

$$AWC[t_0+1]=AWC[t_0]+R+I-Et-Rs-Ip$$

Midday stem water potential was monitored with a Scholander-pressure chamber (32). Mature, undamaged, sun-exposed leaves were selected for measurements which were taken between 12:30 and 13:30 h (local time) in 10 replicates. Leaves were placed into a plastic bag wrapped with aluminum foil. The pressure was determined after one hour, immediately after removal from the canopy, with the pressure chamber.

Yield components and fruit composition. Just few days before commercial harvest, 6 vines per variety in each site were harvested. The following parameters were measured: number of cluster per vine, yield (kg/vine), total soluble solids of grape juice (°Brix) by refractrometry, pH values achieved by pH meter and titratable acidity with 0.1N NaOH and bromothymol blue as indicator. Titratable acidity was expressed as grams tartaric acid per liter juice.

Carotenoid extraction. Carotenoids extraction procedure was adapted from the method of Mendes-Pinto and others (27) with some modifications. Preparation of samples for carotenoids analysis was done in darkness at all times and under ice to avoiding oxidation and to prevent photoisomerization and degradation of carotenoids. While berries were still frozen, their seeds were removed. Approximately 50 g of fresh berries, without seeds, were homogenized using a commercial blender for 3 min. This procedure provided 40 g of sample that was diluted with 40 mL of a 50 mM Tris-HCl (pH 7.5) solution containing 1 M NaCl (28).

Extraction was carried out with 40 mL of diethyl ether/hexane (1:1, v/v), and agitated for 30 min. The resulting upper layer separated was taken and put into the Erlenmeyer flask. The extraction was repeated two more times with 20 mL of diethyl ether/hexane (30 min each), picking up every time the supernatant. The organic phases pooled were dried with anhydrous Na_2SO_4, and evaporated to dryness in a rotatory evaporator under 20-23 °C. The final extract was resuspended in 4 mL of acetone/hexane (1:1, v/v) for HPLC determination. Carotenoid extraction was performed in triplicate; a sample volume of 20 µL was injected into the HPLC. The absorbance was recorded at 447 nm.

HPLC–DAD analysis of carotenoids. HPLC quantification of carotenoids was performed with a Thermo Surveyor Plus consists of a pump (LC pump plus) four-channel, an autosampler (plus Autosampler) and a diode array detector (PDA plus). The C18-reversed-phase column choice was a Nova-Pak C18 (300 mm x 3.9 mm, 4 µm), Waters. The detector module was set to scan from 270 to 700 nm and two fixed channels to 447 nm and 460 nm.

The mobile phase consisted of (A) ethyl acetate and (B) acetonitrile/water (9:1 v/v), resulting in the following gradient: 0–28 min, 0–40% B; constant for 3 min; 31–35 min, 40-0% B; 35–40 min, 100% B. Flow rate was 1mL min^{-1}. Four different calibration curves were done with Lutein, β-carotene, chlorophyll a and chlorophyll b for samples quantification. Neoxanthin, neochrome/a, neochrome/b, violaxanthin, and flavoxanthin concentrations were calculated from the calibration curve of lutein. Pheophytin A was calculated from calibration curve of chlorophyll a and pheophytin b from chlorophyll b. Due to the extract acidification some compounds changed to the isomeric form, respectively the content of neoxanthin is the sum of neoxanthin, neochrome/a and neochrome/b. The content of chlorophyll a is the sum of chlorophyll a and pheophytin a, instead chlorophyll b is the sum of chlorophyll b and pheophytin b. *β*-carotene is the sum of *β*-carotene and *Z-β*-carotene. In the Table 1 are presented identified compounds with their retention times and spectral data.

The carotenoids composition was determined by comparison of HPLC retention times of standards solution and UV spectra with references obtained from commercial standards (*β*-carotene and lutein) and published data (27).

Table 1. Chromatographic and spectroscopic characteristics used for carotenoid identification. The retention times are average values.

Peak	Compound	Retention-time	λ max (nm)
1	Neochrome/a	4.657	400, 423, 449
2	Neoxanthin	5.292	413, 422, 448
3	Neochrome/b	5.505	400, 423, 449
4	Violaxanthin	6.042	419, 441, 470
5	Flavoxanthin	6.465	402, 424, 449
6	Unknown	9.035	403, 428, 471
7	Unknown	12.295	432, 450
8	Lutein	14.710	422, 447, 474,
9	Chlorophyll b	20.007	458, 600, 646
10	Chlorophyll a	22.337	413, 430, 663
11	Pheophytin b	26.032	410, 436, 528, 600, 653
12	Pheophytin a	28.463	408, 504, 535, 600, 665
13	*β*-carotene	30.227	424, 455, 481, 665
14	(*Z*)-*β*-carotene	30.787	340, 425, 453, 475, 655

Flavonols extraction. From sampling of 400 berries were weighed 50 berries. Each berry was cut into two parts, removing the seeds and pulp, and the skins were immediately placed in a plastic container containing 50 mL of tartaric buffer at pH 3.2 (5 g of tartaric acid, 22 mL of NaOH 1N, 2 g of $K_2S_2O_5$, 125 mL of 95% ethanol, water up to 1 L). Samples were stored in the freezer until analysis. Before analysis extract was, homogenized in a blender for 3 minutes and centrifuged (15 minutes at 4000 rpm and 15 °C). The liquid phase was collected in a volumetric flask, while the solid residue was resuspended in buffer at pH 3.2 and recentrifuged. The liquid phase obtained was added to the previous and brought to a final volume of 150mL with the same buffer.

The skins extract was acidified with phosphoric acid 1M (4.5 mL of extract + H_3PO_4 1M up to 5 mL), filtered with a membrane (0.45µm) and 0.20 µL injected for HPLC-DAD analysis (31).

HPLC–DAD analysis of flavonols. Analysis was performed in the same liquid chromatograph as for the carotenoids. Separations of the flavonols were performed on a reversed-phase C18 Econosphere column (250x4.6mm, 5µm) using a 10 µL injection volume and detected at 360 nm. The mobile phases were consisted of phosphoric acid 0.001M (A) and methanol (B). The solvent flow rate in the pump was 0.48 mL min^{-1}. The following binary linear gradient method was used: A from 95% to 90% from 0 to 5 min, A from 90% to 70% from 5 to 20 min, A from 70% to 40% from 20 to 30 min, A from 40% to 0% from 30 to 40 min and A from 0% to 95% from 40 to 50 min (31).

Identification of individual flavonols was made with HPLC retention time of standard solution and with references data. Concentrations were calculated by using calibration curves of standards: myricetin, quercetin and kaempferol. For comparison purposes, reported data are the sum of all flavonols.

Norisoprenoid extraction. The content of norisoprenoids was determined for two stage period: 20 Brix (20B) and ripening phase (RP). From sampling of approximately 400 berries, carried out in the vineyard, randomly were select 50 berries and determined the total weight. While the berries were still frozen, their seeds were removed. Defrosted skins and pulps were homogenate for 3 minutes using a commercial blender.

Free aroma compounds: 5g of homogenate was placed into a 20 mL headspace vials to which was added 1g of NaCl to increase the ionic strength and 1µl of internal standard 2-octanol for quantification. Samples were analyzed by SPME fiber GC-MS technique.

Glycosylated aroma compounds: to 15g of homogenate was added 50 mL of tartaric acid buffer pH 3.2 (12% ethanol) and vortexed per 24 hours. After centrifugation, 45mL of extract was passed on 1 g SPE C18 cartridge (WAT 036 795). Cartridge was previously activated with 5 mL of methanol followed by 10 mL of distilled water. Afterwards, was washed with 10 mL of water to remove the hydrophilic compounds (sugars, acids, salts) and eluted the free varietal compounds with 6 mL of dichloromethane. The bound varietal compounds were subsequently eluted from the C18 cartridge with 10 mL of methanol, collected in a distillation flask of 100 mL and taken to dryness under vacuum. The dry residue was resuspended with 5 mL of citrate-phosphate buffer at pH 5.0 in the same distillation flask, added a high enzyme activity glycosidase (Lallzyme Beta, Lallemand) and placed in thermostat at 36 °C for 24 hours. At the end, to the product of the enzymatic reaction was added 1µl 2-octanol internal standard, and placed into a 20 mL headspace vials for SPME analysis (30).

GC–MS analysis of norisoprenoids. A DVB/CAR/PDMS, 50/30 µm SPME fibre was used for aromatic analysis. The fibre was preconditioned at 35°C for 2 minutes and then exposed to the sample for 30 min at a temperature of 35°C degrees. Desorption of aromatic compounds were done at 250°C into the injection port for 6 min in splitless mode.

GC-MS analysis was performed on an Agilent 7890A GC- system MS 5975C inert xl MSD with Triple Axis Detector and COMBI PAL CTC ANALYTICS for fiber technique. Aromatic compounds were separated on a TR-WAX capillary column (30 m x 0.25mm x 0.25µm). Helium carrier gas was used with a total flow of 1 mL min^{-1}. Injection and interface temperatures were respectively 250°C and 230°C. Following temperature program was applied: 36°C for 3 min, 36-160°C at 2°C/min, 160-230 °C at 3°C/min and then 230°C for 5 min.

For analysis of peaks was utilized an Agilent Chem Station E.20.00.493.The identification of volatile aroma compounds was achieved by mass spectra comparison with NIST library 2009 spectra database. Quantification was calculated from the GC peak area of volatile compound relative to the internal standard area. For comparison purposes, all data reported are the sum of free and bound norisoprenoids.

Statistical analysis. For each sample, after the exclusion of out groups data, analysis of variance (ANOVA) was used to test the main effects (cultivar, site and year) and their interactions using SPSS software (IBM SPSS version19). Comparison of means was performed using Duncan test at $p < 0.05$.

Results and Discussion

Ecophysiological data. Analyzing the temperature behavior, from pea size phase to ripening, it is evident regarding mean temperature that Misano had higher values in both 2011 and 2012 seasons. Bolgheri had lower mean temperatures in 2012 season, in particular from pea size to 20 Brix phase. Anyway, the daily mean temperatures until 20 Brix phase in all sites were higher in 2012 seasons (Figure 6A and 6B). Daily maximum temperatures were very similar each other during 2011 (Figure 7A), some differences were detectable in 2012 season. Montalcino had a constantly higher daily maximum temperature respect to Bolgheri and Misano, even if the temperatures were highest in all sites during 2012 season compared to 2011 (Figure 7B).

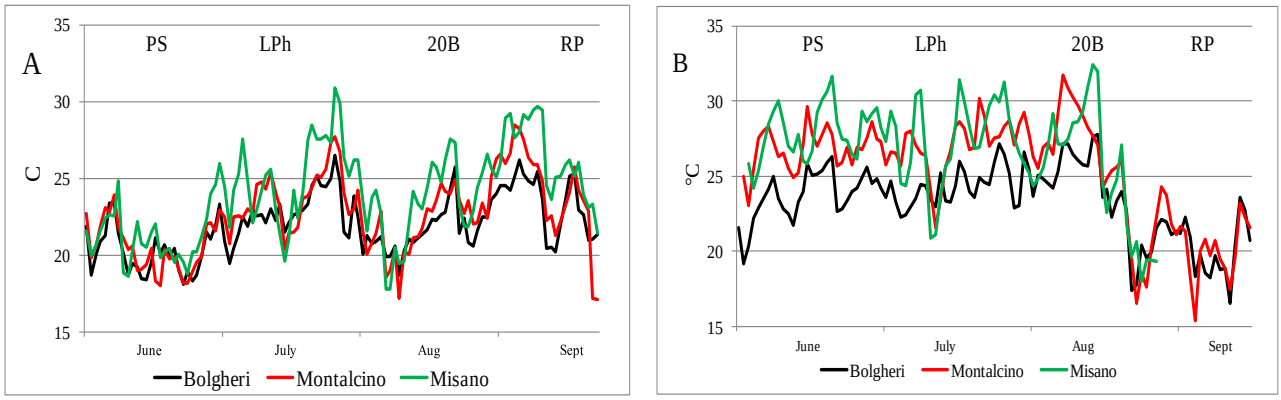


Figure 6. Daily mean temperature from June to September for 2011 (A) and 2012 (B) in three experimental sites (Bolgheri, Montalcino and Misano) and four ripening stages: pea size (PS), lag phase (LPh), 20 °Brix (20B) and ripening phase (RP).

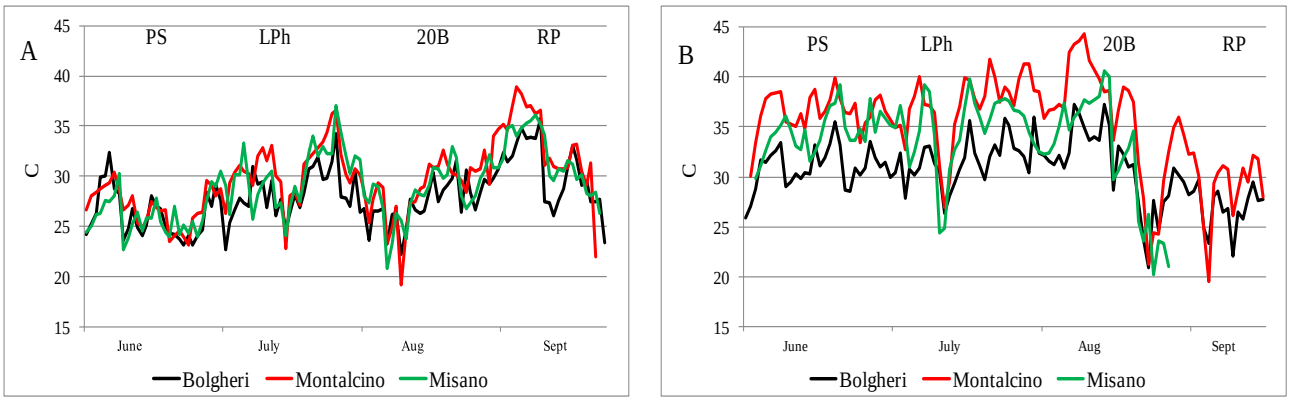


Figure 7. Daily maximum temperature from June to September for 2011 (a) and 2012 (b) in three experimental sites (Bolgheri, Montalcino and Misano) and four ripening stages: pea size (PS), lag phase (LPh), 20 °Brix (20B) and ripening phase (RP).

Regarding Growing Degree Days (GDD) in 2011 season Misano cumulated more thermal resources than the other sites (Figure 8A). In 2012, Montalcino and Misano showed the same trend in GDD sum (Figure 8B). On the other hand, Bolgheri cumulated less thermal resources than the other two sites in both seasons.

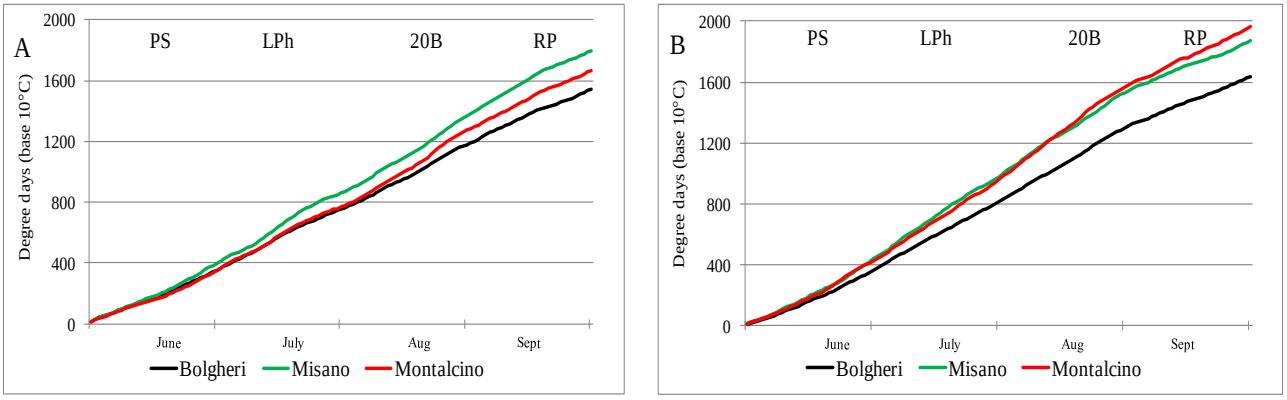


Figure 8. Growing degree days (GDD, base temperature 10°C) from June to September for 2011 (A) and 2012 (B) at three experimental sites (Bolgheri, Montalcino and Misano) and four ripening stages: pea size (PS), lag phase (LPh), 20 °Brix (20B) and ripening phase (RP).

Reconstructed values of daily global solar radiation expressed in MJ m-2 day-1 are directly correlated to daily thermal range, which in Montalcino was higher than in the other two sites. Analyzing two seasons (2011 and 2012), global solar radiation data, it's evident that radiative resources available in Montalcino was higher than in the other two sites, especially respect to Misano (Figure 9A and 9B).

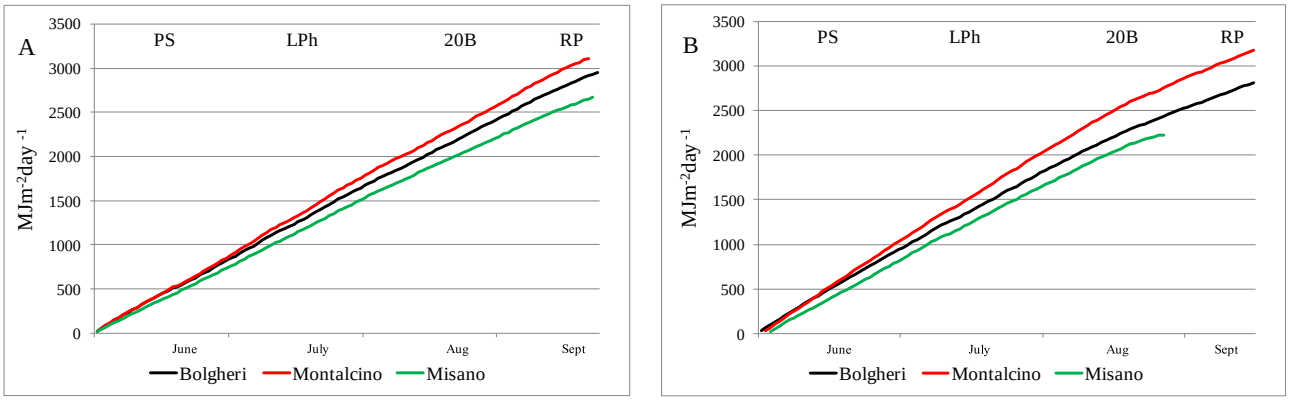


Figure 9. Estimate Global Solar Radiation ($MJm^{-2}day^{-1}$) from April to September for 2011 (A) and 2012 (B) in three experimental sites (Bolgheri, Montalcino and Misano) and four ripening stages: pea size (PS), lag phase (LPh), 20 °Brix (20B) and ripening phase (RP).

Considering monthly rainfall data, it can be noted that 2011 and 2012 had been characterized by different monthly values. In particular spring (April and May) 2012 was more rainy respect to 2011, but during June and July 2011, for example Montalcino had higher rainfall compared to 2012. Worthy of note that during August 2011 precipitations were completely absent. Their levels in 2012 were very low in June, July and August in all three sites (Figure 10A and 10B).

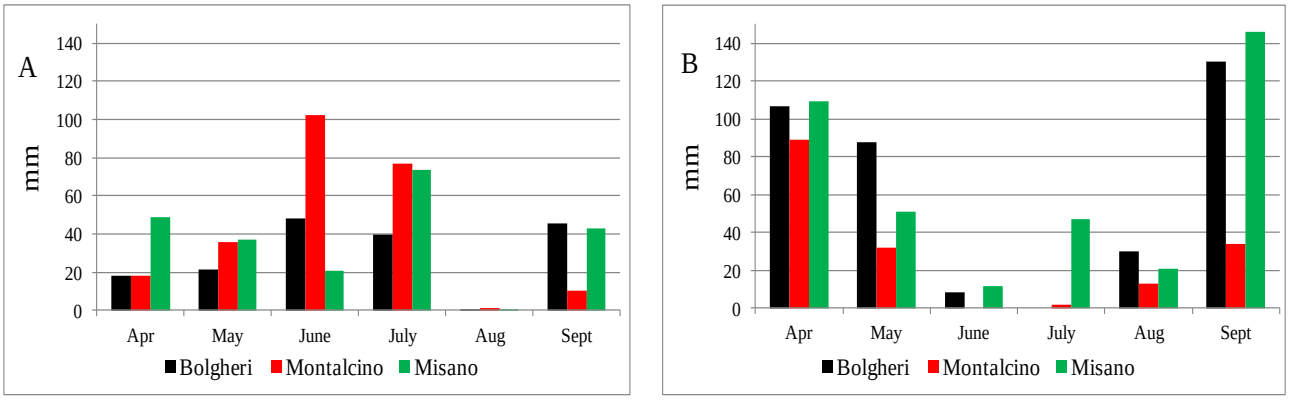


Figure 10. Monthly sum of precipitation (mm) from April to September for 2011 (A) and 2012 (B) in three experimental sites (Bolgheri, Montalcino and Misano).

The soils of Montalcino and Misano have similar structures represented by 43-44% of clay, 38-42% of silt and 14-19% of sand. The soil of Bolgheri instead contains 16% of clay, 12% of silt and 72% of sand. Analyzing available water content (AWC, calculated from soil type and rainfall) during 2011 is evident that since July the water reservoir was under wilting point all over summer, evidencing a stress situation for all vineyards. In summer 2012 this stress situation has been recovered during August thanks to some rainfalls (Figure 11A and 11B).

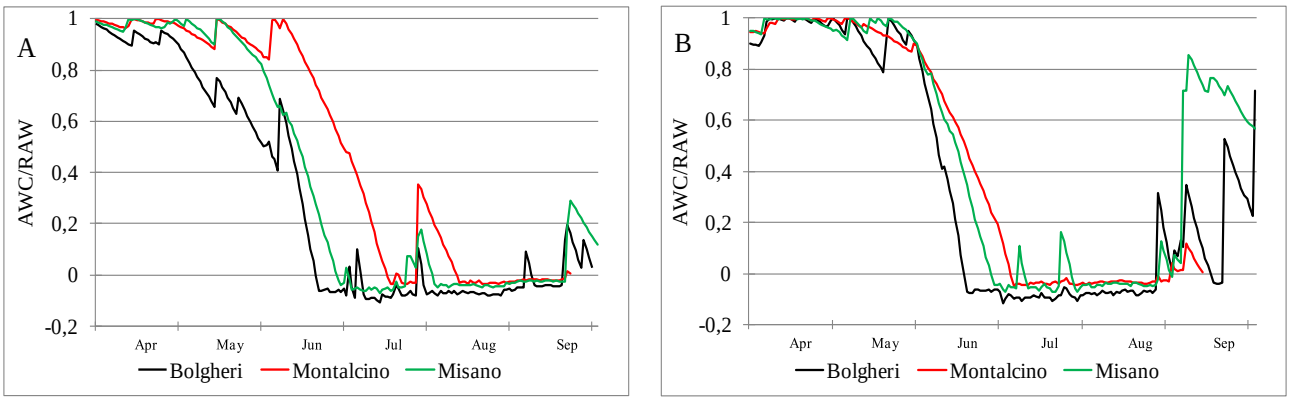


Figure 11. Estimate Available Water Content (AWC) from April to September for 2011 (A) and 2012 (B) in three experimental sites (Bolgheri, Montalcino and Misano).

The lower values of midday stem water potential in Cabernet Sauvignon variety throughout the growing season 2011 were at Montalcino, while the highest values were at Misano (Figure 12A). In the variety Sangiovese for the two measurements (pea size and lag phase) the lowest values were registered in Montalcino, while at phase 20 brix data were similar for all three sites. The values at ripening phase are difficult to interpret (Figure 12B).

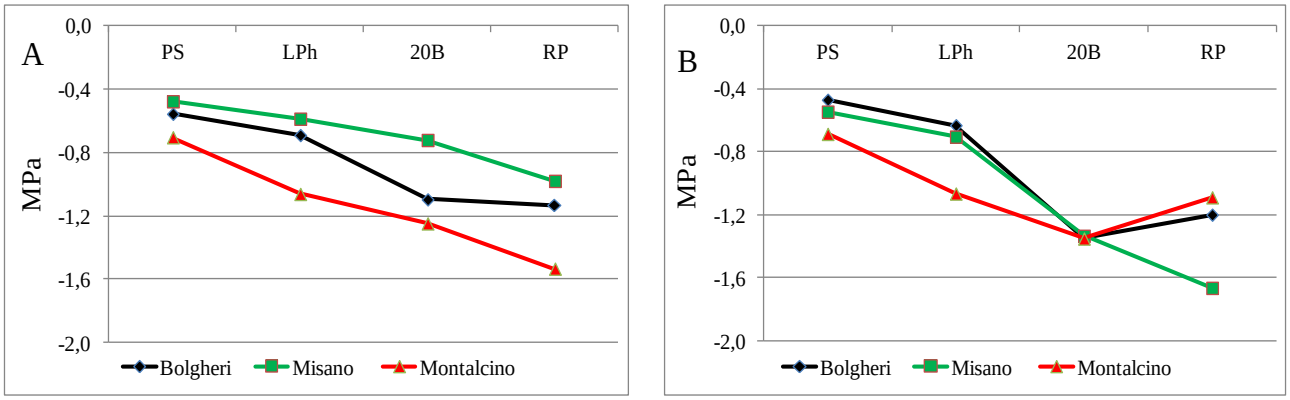


Figure 12. Midday stem water potential of Cabernet Sauvignon (A) and Sangiovese (B) measured in 2011 at three experimental sites (Bolgheri, Montalcino and Misano) and four ripening stages: pea size (PS), lag phase (LPh), 20 °Brix (20B) and ripening phase (RP).

During the growing season 2012 for variety Cabernet Sauvignon the lower values of midday stem water potential were measured at Montalcino and the highest in Misano (Figure 13A). The lowest values were also registered in Sangiovese at Montalcino (Figure 13B).

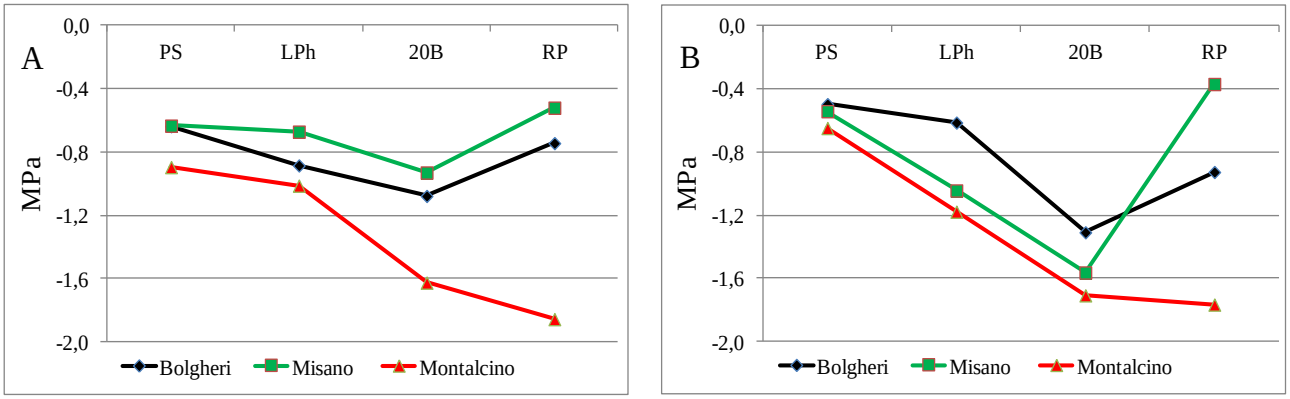


Figure 13. Midday stem water potential of Cabernet Sauvignon (A) and Sangiovese (B) measured in 2012 at three experimental sites (Bolgheri, Montalcino and Misano) and four ripening stages: pea size (PS), lag phase (LPh), 20 °Brix (20B) and ripening phase (RP).

Yield components, fruit composition and berry weight. Cabernet Sauvignon grapes in 2011 at Montalcino had the highest soluble solids, while other two sites had similar sugar concentration. Misano had the highest grape juice acidity, but Montalcino had the highest pH although had similar titratable acidity as Bolgheri. Misano had the highest yield per vine and number of cluster. The sugar level in 2012 was higher in Misano followed by Montalcino. A similar trend as 2011 season was observed for titratable acidity, pH, yield per vine and clusters number (Table 2).

The top accumulation of soluble solids in Sangiovese in 2011 was achieved in locality Montalcino instead in 2012 was greater at Misano. The higher titratable acidity and lower pH are recorded at Misano in 2012. Yield per vine and clusters number were higher in Misano in both years, although in 2012 there are no significant differences between sites (Table 3). The year did not significantly affect the fruit composition.

Table 2. Yield components (yield per vine, number of clusters) and fruit composition (soluble solids, titratable acidity and pH) of variety Cabernet Sauvignon recorded at harvest for two years (2011-2012) in three experimental sites (Bolgheri, Montalcino and Misano).

Year	Site	Soluble solids (°Brix)	Titratable acidity (g/L)*	pH	Yield/vine	Number of clusters
2011	Bolgheri	22.5 a	5.68 a	3.46 a	1.4 a	12.2 a
	Montalcino	26.2 b	5.64 a	3.79 b	1.2 a	8.8 a
	Misano	22.8 a	6.96 b	3.46 a	2.6 b	25.7 b
2012	Bolgheri	23.7 a	4.32 b	3.40 a	1.2 a	8.7 a
	Montalcino	24.9 ab	3.86 a	3.70 b	0.6 a	10.2 a
	Misano	26.2 b	5.08 c	3.39 a	2.3 b	24.2 b

* Titratable acidity is expressed as g/L of tartaric acid
a,b,c-different letters within each column differ significantly according to Duncan Test at $p < 0.05$

Table 3. Yield components (yield per vine, number of clusters) and fruit composition (soluble solids, titratable acidity and pH) of variety Sangiovese recorded at harvest for two years (2011-2012) in three experimental sites (Bolgheri, Montalcino and Misano).

Year	Site	Soluble solids (°Brix)	Titratable acidity (g/L)*	pH	Yield/vine	Number of cluster
2011	Bolgheri	20.9 a	5.24 a	3.36 a	1.4 a	5.8 a
	Montalcino	26.7 b	7.15 b	3.54 b	1.3 a	7.2 a
	Misano	21.6 a	5.24 a	3.36 a	4.9 b	21.7 b
2012	Bolgheri	21.0 a	5.37 a	3.15 a	1.6 a	7.8 a
	Montalcino	23.3 ab	4.84 ab	3.37 b	1.5 a	7.7 a
	Misano	24.1 b	4.36 b	3.38 b	2.6 a	10.7 a

* Titratable acidity is expressed as g/L of tartaric acid
a,b,c-different letters within each column differ significantly according to Duncan Test at $p < 0.05$

At phase pea size, berry weight of Cabernet Sauvignon in 2011 was highest at Misano. From lag phase to ripening phase Montalcino had the significantly lowest berry weight (Figure 14A). The berries of Sangiovese in 2011 reached higher weight in all sites respect to Cabernet Sauvignon, even though exist a similarity trends for the two varieties. Berry weight at pea size was higher in Misano and from lag phase to ripening, the lowest berry weight was registered at Montalcino. The highest berry weight at 20 Brix and at harvest it was in Sangiovese variety of Bolgheri (Figure 14B).

In 2012 no significant differences was observed in the berry growth and in berry weight in Cabernet Sauvignon between Bolgheri and Misano in comparisons to Montalcino in which the lowest berry weight in both varieties was noticed (Figure 14C and 14D). From pea size to lag phase the highest berry weight had Sangiovese of Misano, while from 20 Brix to ripening phase the berry weight was significant the highest at Bolgheri (Figure 14D).

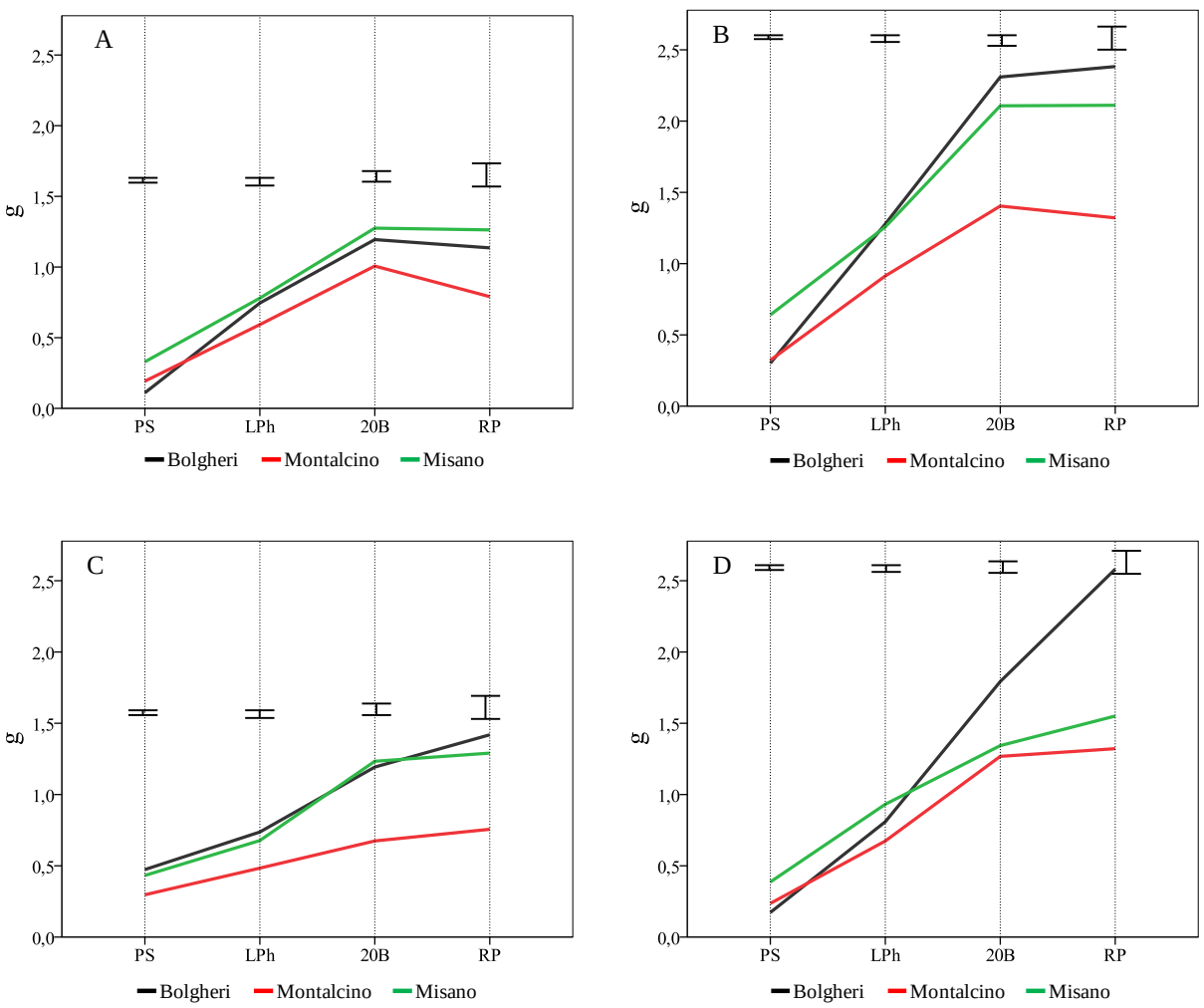


Figure 14. Berry weight of Cabernet Sauvignon 2011 (A), Sangiovese 2011 (B), Cabernet Sauvignon 2012 (C) and Sangiovese 2012 (D) at four ripening stages. LSD for pea size (PS)=0,030 g; lag phase (LPh)=0,048 g; 20 Brix (20B)=0,078 g and ripening phase (RP)= 0,158 g.

Carotenoids and Chlorophylls. The effect of the environment x genotype interaction on the total carotenoids and chlorophylls content as well as the individual carotenoids and chlorophylls of berries were evaluated in the two seasons (2011 and 2012).

From the results, in both years and for both varieties the amounts of total carotenoids decreased through ripening. The carotenoids content (mg/kg) at pea size and in lag phase in Cabernet Sauvignon of Misano was significantly lower compared with other two sites. However, no significant differences were observed at ripening phase between sites (figure 15A). Similar results were obtained in Sangiovese and Cabernet Sauvignon during maturation, with exception of Montalcino where Sangiovese had the lowest content of total carotenoids in lag phase (Figure 15B). In the 2012 season for both varieties was even more evident that the carotenoids content was lowest in Misano during all the maturation. Cabernet Sauvignon of Montalcino at pea size had the highest carotenoids content decreasing strongly up to lag phase. No significant differences were observed between sites at harvest (Figure 15C). The carotenoids content for Sangiovese in Bolgheri was significantly higher than in Montalcino at pea size, but after, they had similar trend up to the harvest. Sangiovese of Misano had significantly lower content at ripening stage (Figure 15D).

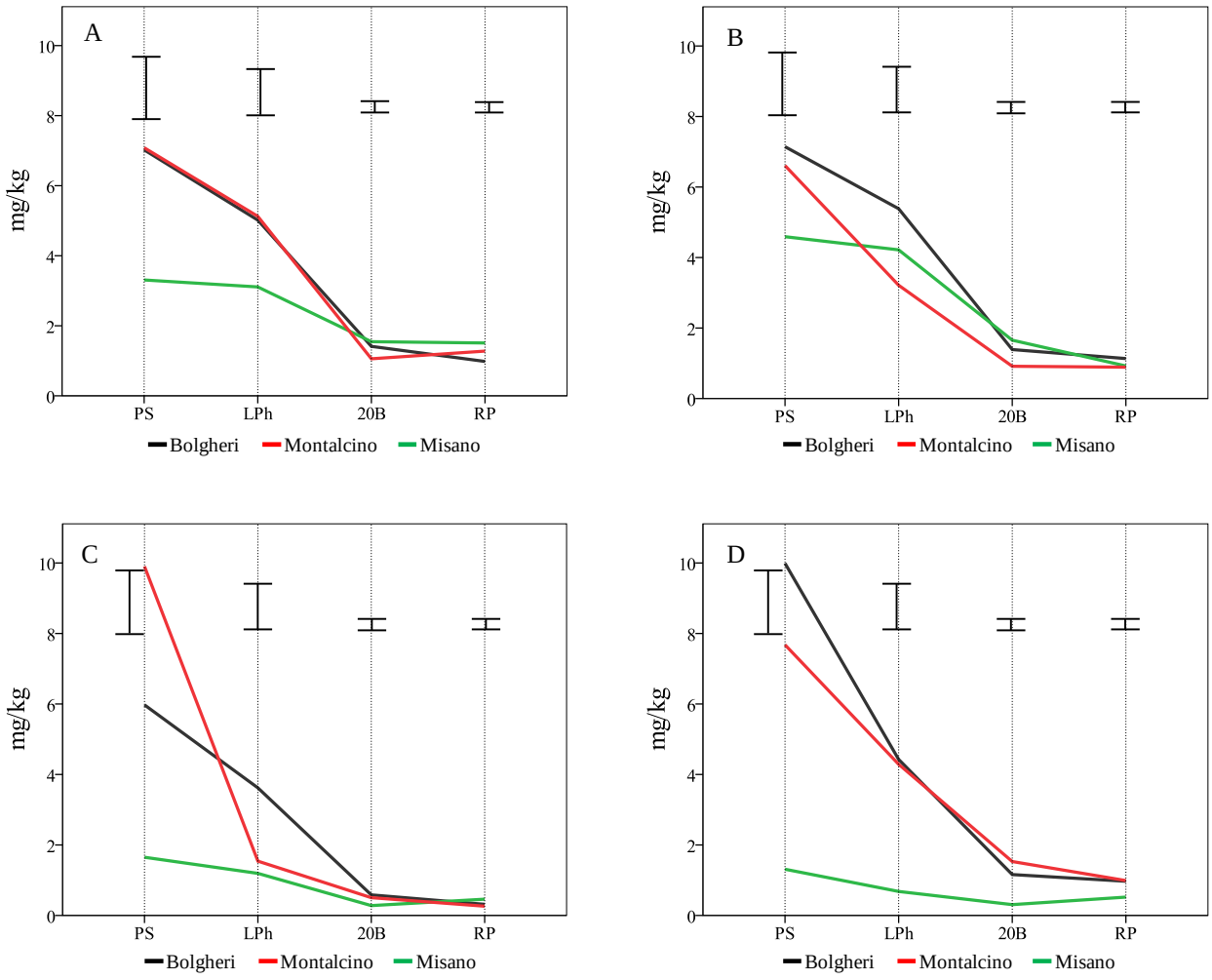


Figure 15. Carotenoids contents (mg/kg of grapes) of Cabernet Sauvignon 2011 (A), Sangiovese 2011 (B), Cabernet Sauvignon 2012 (C) and Sangiovese 2012 (D) at four ripening stages. LSD for pea size (PS)=1,749 mg; lag phase (LPh)=1,280 mg; 20 Brix (20B)=0,312 mg and ripening phase (RP)= 0,292 mg.

The carotenoids concentrations per berry (µg) of the two varieties during the seasons 2011 and 2012 at three experimental sites are shown in Figure 16. It can be observed for both varieties, that generally the trend of the concentration of carotenoids per berry (µg) is different from the trend of total carotenoids content expressed as mg/kg. It is visible an increase that can be due to a more intense synthesis of carotenoids per berry from the pea size until lag phase, a decrease of content up to 20 Brix, and then the values remain almost unchanged until the harvest in comparison to the previous stage. In 2011 at lag phase, where the maximum carotenoids concentration per berry is observable, for variety Cabernet Sauvignon no differences were noticed between sites. In the cultivar Sangiovese significant differences are evident between the localities at lag phase and ripening, where the highestcontent was of Bolgheri and the lowest of Montalcino (Figure 16A and 16B). In 2012 in Misano the carotenoids content per berry was lowest in all sampling stages for both varieties. In Cabernet Sauvignon, at lag phase it should be noted an unexpected decrease in Montalcino and no significant difference were observed between sites at harvest (Figure 16C). In Sangiovese, the significantly lowest amounts at all phases were observed at Misano and at ripening stage the highest amounts at Bolgheri (Figure 16D).

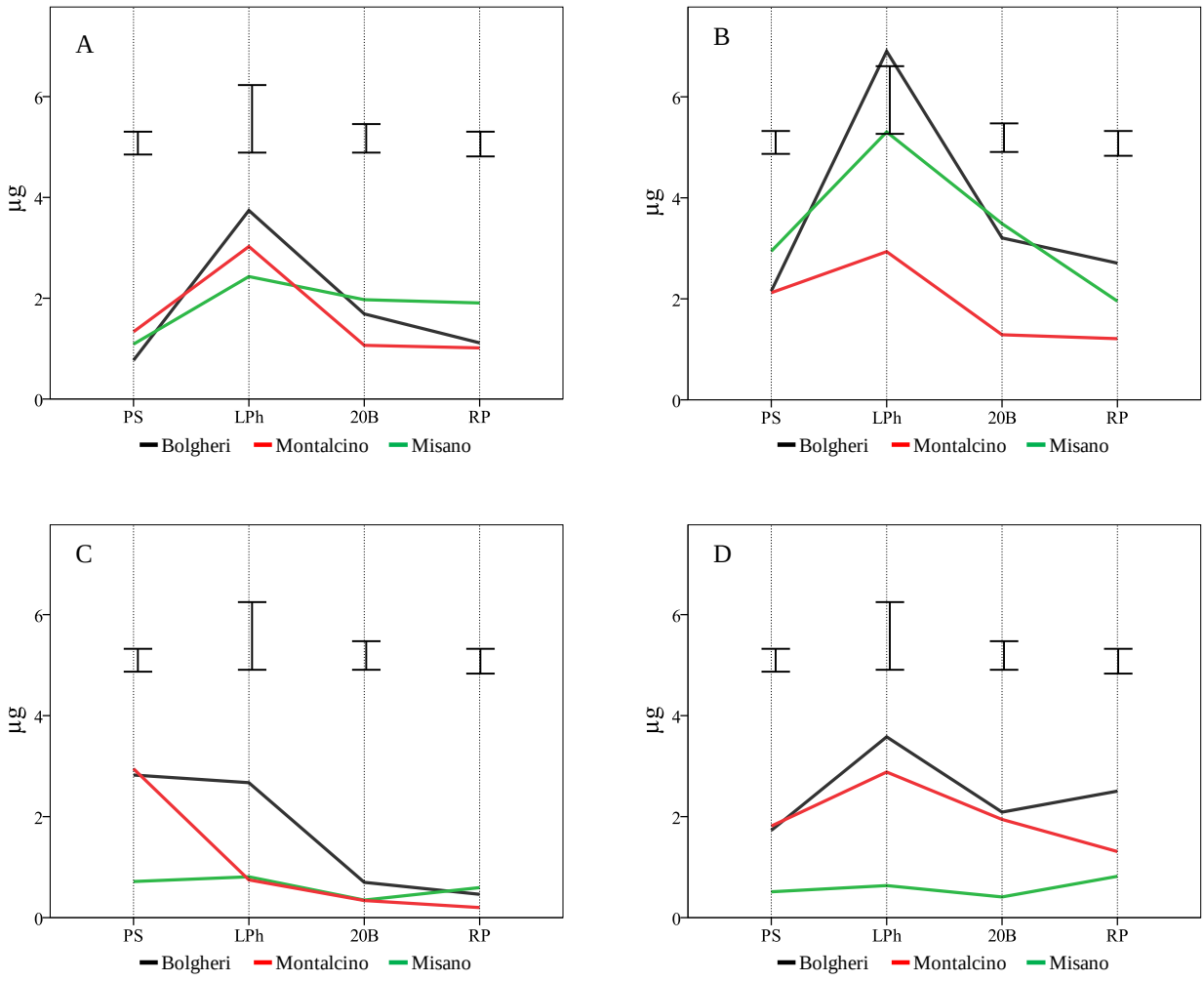


Figure 16. Carotenoids per berry (µg) of Cabernet Sauvignon 2011 (A), Sangiovese 2011 (B), Cabernet Sauvignon 2012 (C) and Sangiovese 2012 (D) at four ripening stages. LSD for pea size (PS)=0,636 µg; lag phase (LPh)=1,894 µg; 20 Brix (20B)=0,785 µg and ripening phase (RP)= 0,687 µg.

The Chlorophylls content (mg/kg) of Cabernet Sauvignon and Sangiovese in 2011-2012 at the four sampling stages is shown in Figure 17. As expected, also the total chlorophylls content was decreasing during maturation. In cultivar Cabernet Sauvignon in 2011, at pea size, the sites were significantly different in chlorophylls content. Bolgheri had the higher amounts and the lower content was registered at Misano, which maintained it until lag phase, but at ripening reached a higher concentration than Montalcino (Figure 17A). In Sangiovese in 2011 at pea size, significantly differed Bolgheri to Misano, while at lag phase significantly differed Bolgheri and Montalcino (Figure 17B). The chlorophylls content in Cabernet Sauvignon in 2012 at all phases was significantly higher in locality Montalcino, while Bolgheri and Misano had similar trends (Figure 17C). The chlorophylls content in cultivar Sangiovese in same year at pea size, were lower in Misano in comparison the other two sites. From lag phase to ripening, the chlorophylls content was similar for Bolgheri and Misano. Montalcino had a higher content during this period, although was with significant differences only at 20 Brix (Figure 17D).

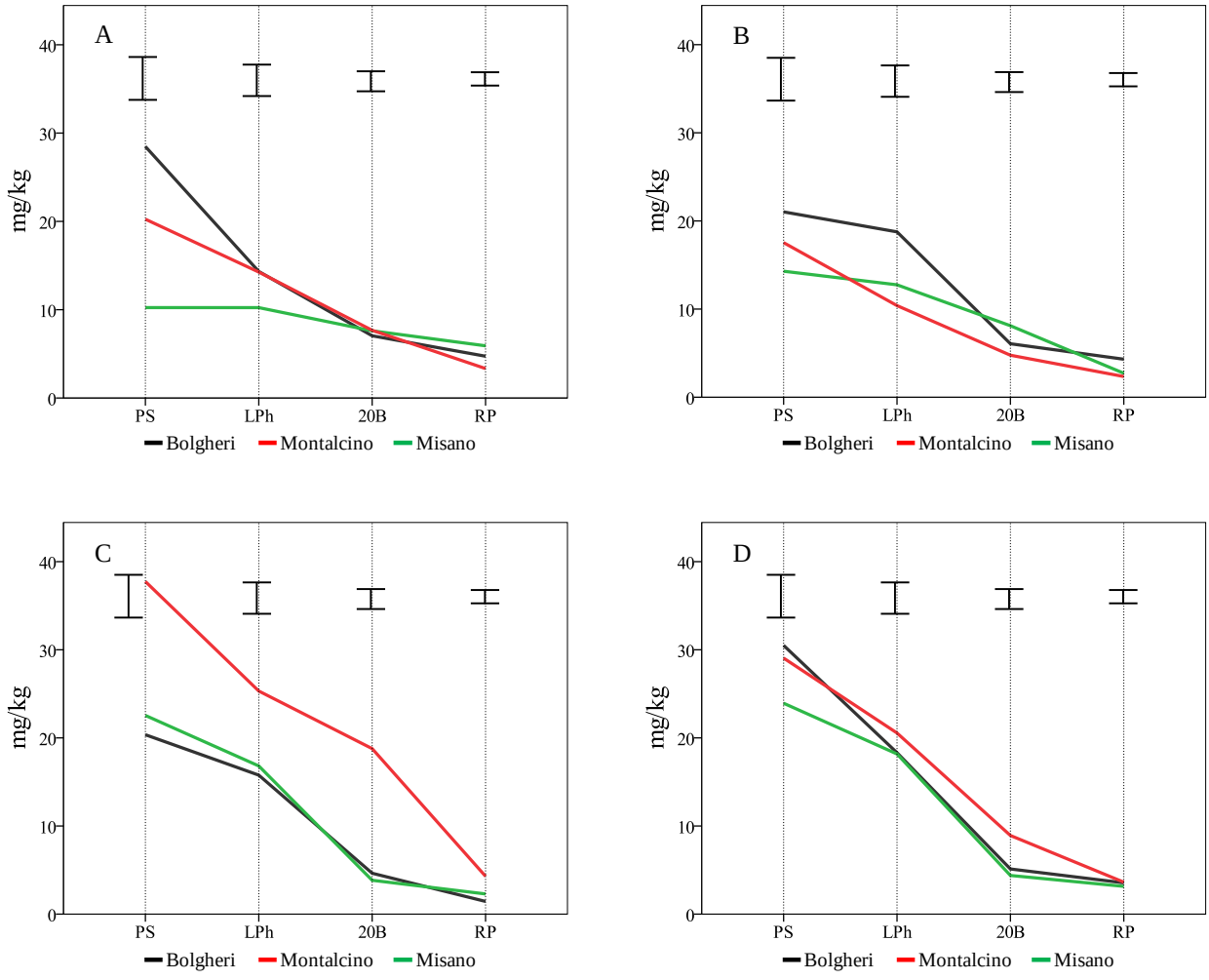


Figure 17. Chlorophylls contents (mg/kg of grapes) of Cabernet Sauvignon 2011 (A), Sangiovese 2011 (B), Cabernet Sauvignon 2012 (C) and Sangiovese 2012 (D) at four ripening stages. LSD for pea size (PS)=4,765 mg; lag phase (LPh)=3,511 mg; 20 Brix (20B)=2,171 mg and ripening phase (RP)= 1,585 mg.

Below are reported all the data for Chlorophylls per berry, summarized in four different graphics (Figure 18) for the two years and varieties during all phases. Some trends of chlorophylls per berry result to be shifted toward the ripening in comparison to those of the carotenoids per berry. The decrease of content due to degradation, seem to have lesser slope from lag phase to 20 Brix, than carotenoids per berry.

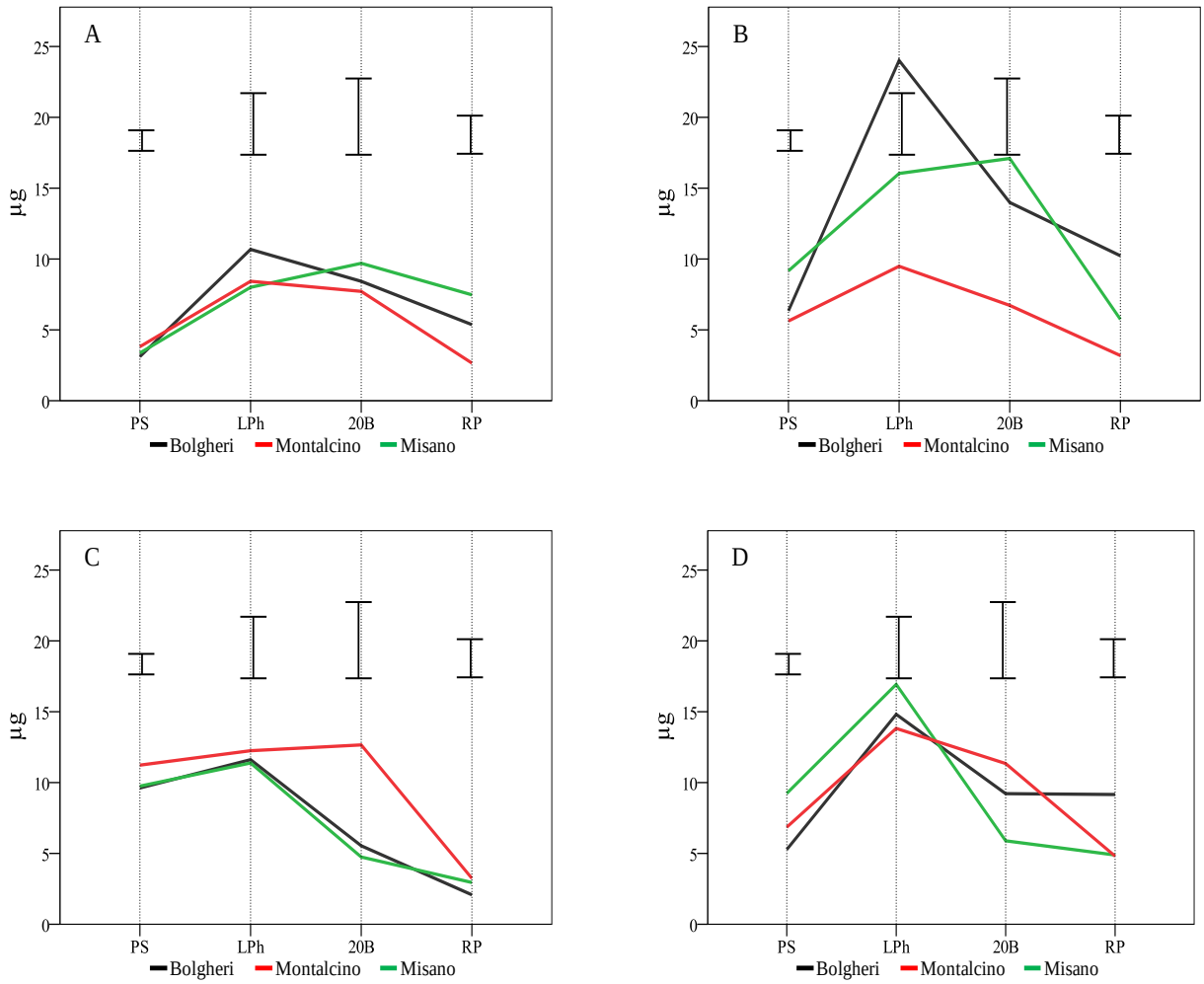


Figure 18. Chlorophylls per berry (µg) of Cabernet Sauvignon 2011 (A), Sangiovese 2011 (B), Cabernet Sauvignon 2012 (C) and Sangiovese 2012 (D) at four ripening stages. LSD for pea size (PS)=1,445 µg; lag phase (LPh)=4,294 µg; 20 Brix (20B)=5,301 µg and ripening phase (RP)= 2,638 µg.

The carotenoids profile was same for the varieties, years and sites studied. The carotenoid more representative was β-carotene with 69% followed by lutein (25%), neoxantin (5%) and flavoxantin plus violaxanthin around 1%.

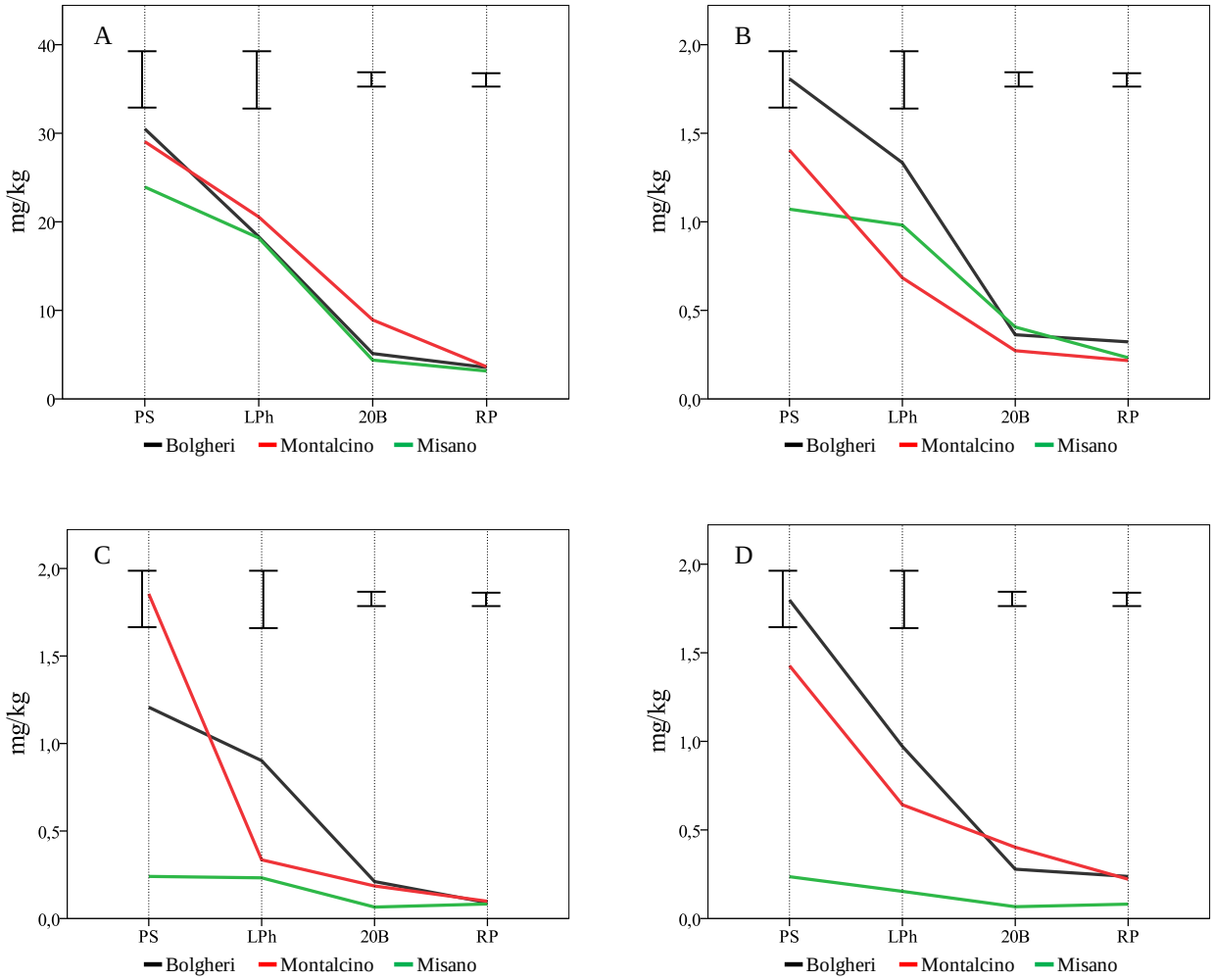


Figure 19. Lutein contents (mg/kg of grapes) of Cabernet Sauvignon 2011 (A), Sangiovese 2011 (B), Cabernet Sauvignon 2012 (C) and Sangiovese 2012 (D) at four ripening stages. LSD for pea size (PS)=0,319 mg; lag phase (LPh)=0,324 mg; 20 Brix (20B)=0,082 mg and ripening phase (RP)= 0,075 mg.

The lutein content (mg/kg) of Cabernet Sauvignon and Sangiovese in 2011-2012 at four ripening stages is shown in Figure 19 and that of β-carotene (mg/kg) in Figure 20. The trends during ripening for two carotenoids compounds were similar to the trend of total carotenoids (mg/kg) for the two varieties in two studied years.

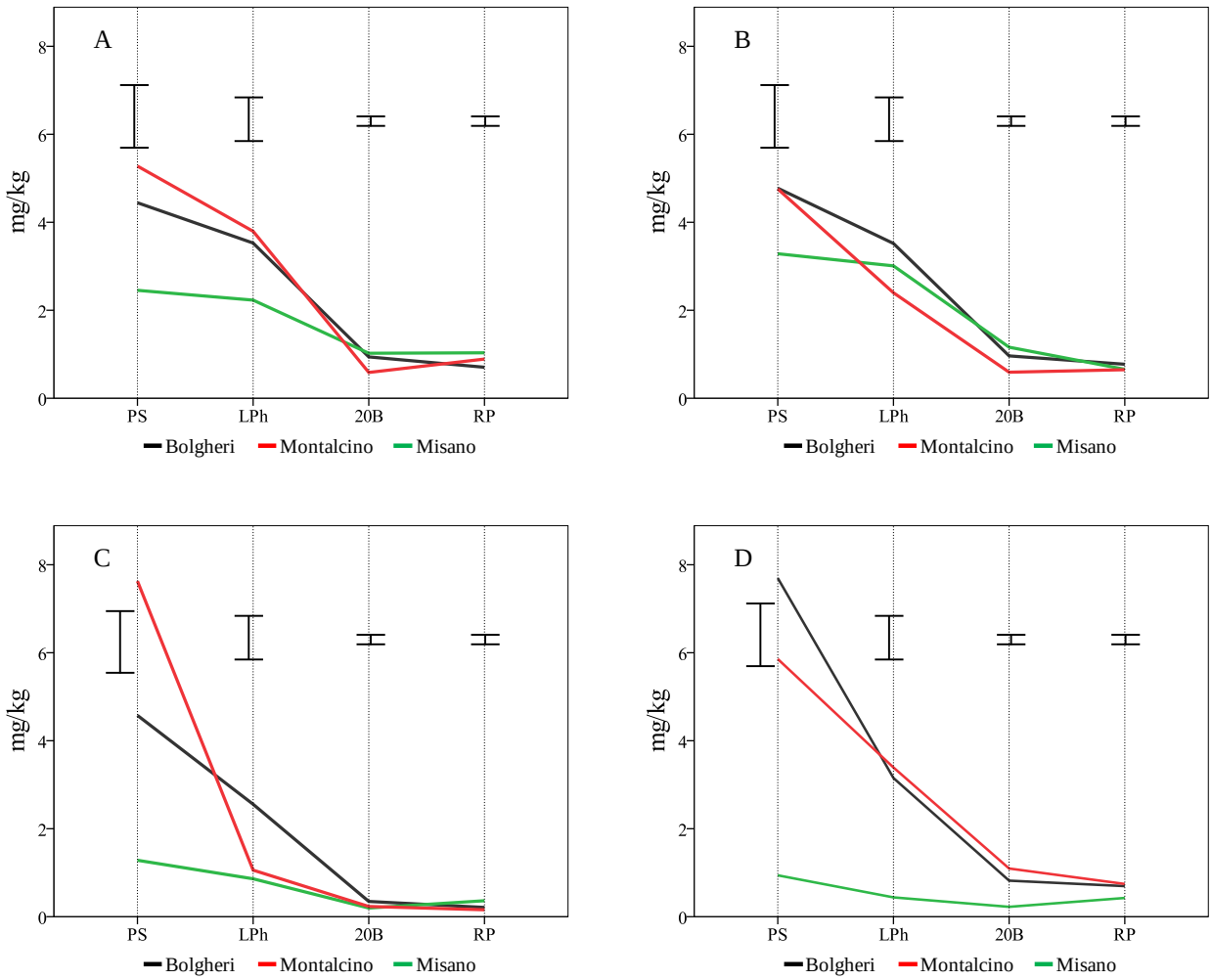


Figure 20. β-carotene contents (mg/kg of grapes) of Cabernet Sauvignon 2011 (A), Sangiovese 2011 (B), Cabernet Sauvignon 2012 (C) and Sangiovese 2012 (D) at four ripening stages. LSD for pea size (PS)=1,422 mg; lag phase (LPh)=1,001 mg; 20 Brix (20B)=0,229 mg and ripening phase (RP)= 0,226 mg.

The content of neoxanthin (mg/kg) was higher in 2011 in comparison to 2012 both for the varieties that for sites. In 2011, at pea size and lag phase, Cabernet Sauvignon had the highest content at Bolgheri and lowest values in Misano. No significant differences were observed at 20 Brix, while at ripening stage significantly higher content was observed in Misano (Figure 20A). In cultivar Sangiovese, Bolgheri at pea size and lag phase had significantly higher content. The lower content at pea size was in Misano, while in the lag phase was recorded in Montalcino. However, no significant differences were observed at harvest between the three sites (Figure 20B). In Cabernet Sauvignon 2012, in locality Montalcino the values of neoxanthin were higher during maturation. Misano and Bolgheri had similar trend and values (Figure 20C). In Sangiovese 2012 no significant differences in total neoxanthin content were observed between sites through ripening. Anyway, the highest content was achieved in Montalcino and lowest in Misano at all the phases (Figure 20D).

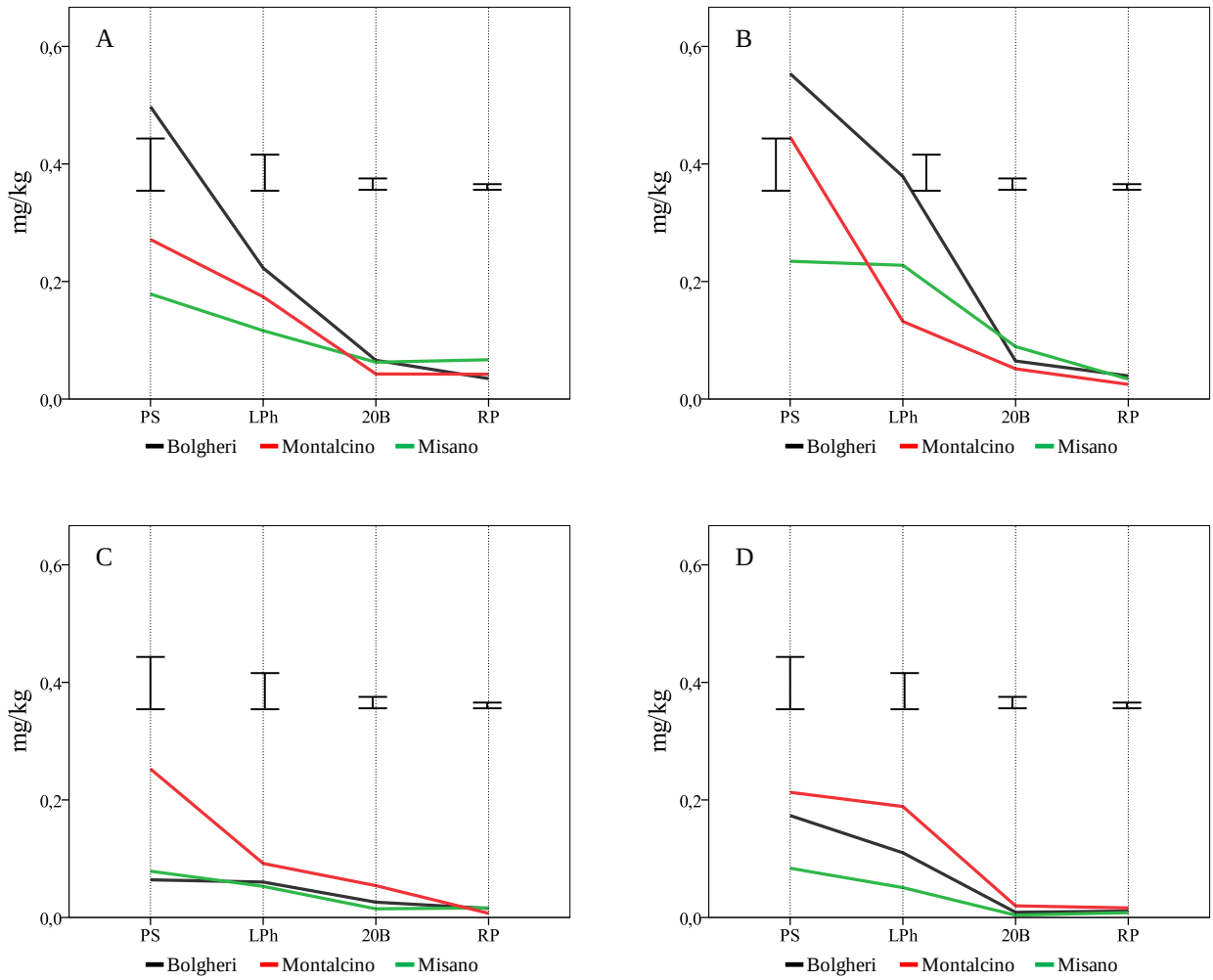


Figure 20. Neoxanthin contents (mg/kg of grapes) of Cabernet Sauvignon 2011 (A), Sangiovese 2011 (B), Cabernet Sauvignon 2012 (C) and Sangiovese 2012 (D) at four ripening stages. LSD for pea size (PS)=0,088 mg; lag phase (LPh)=0,060 mg; 20 Brix (20B)=0,020 mg and ripening phase (RP)= 0,010 mg.

Flavonols. Total flavonols (mg/kg) reported in Figure 21, decreased from pea size until veraison, after that again are increased up to the harvest. In any case, the concentrations in both varieties were higher in 2011 respect to 2012. In Cabernet Sauvignon in 2011, during all stages Bolgheri and Montalcino which did not differ between them had the greater concentrations than Misano (Figure 21A). In variety Sangiovese in 2011, instead, only Montalcino had significantly highest content confronted to the other sites (Figure 21B). Similarly to the first year of study, in 2012 in Cabernet Sauvignon, Misano had the lowest flavonols content during maturation but with no evident differences (Figure 21C). In cultivar Sangiovese in 2012 similar results were obtained as in precedent year, with lesser amounts (Figure 21D).

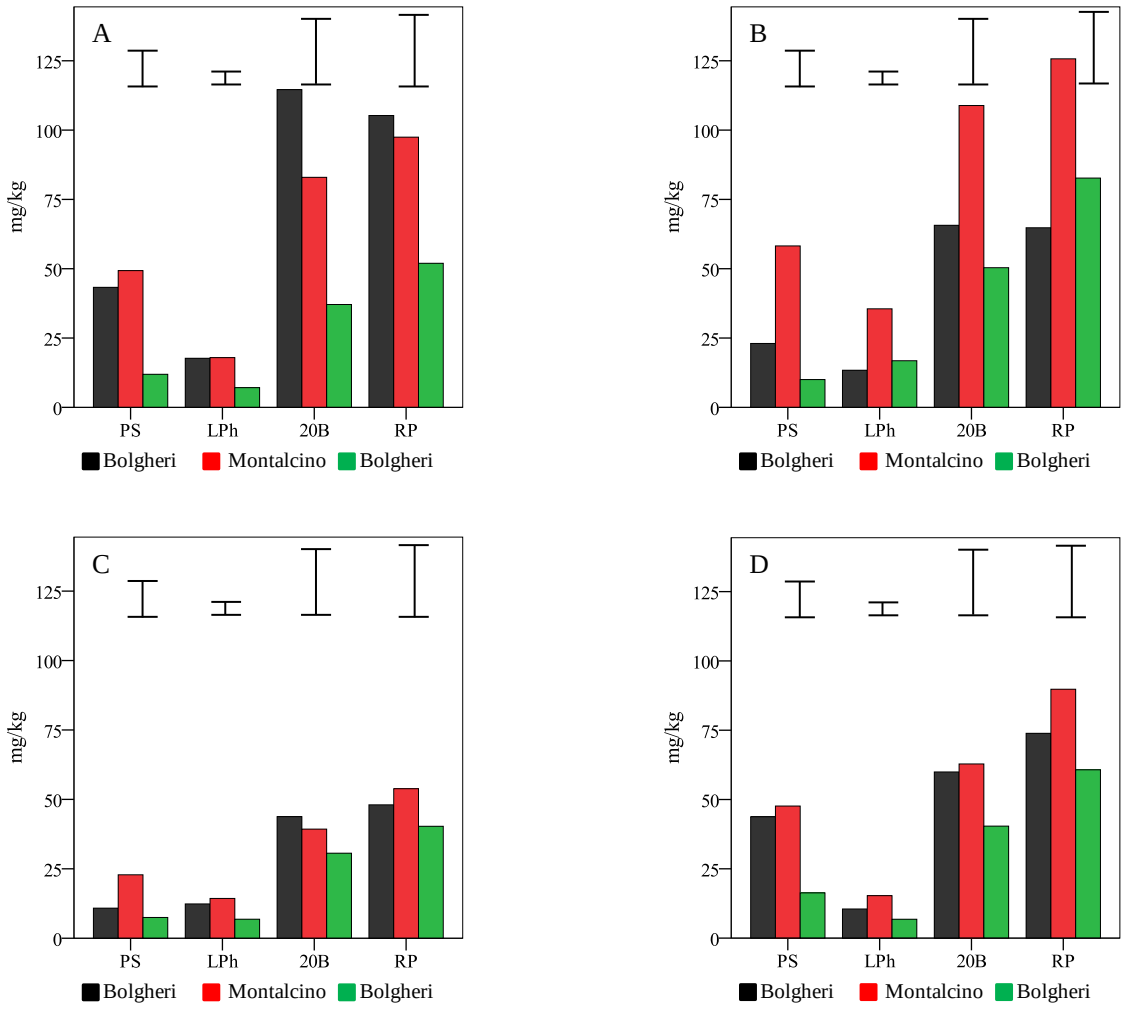


Figure 21. Flavonols contents (mg/kg of grapes) of Cabernet Sauvignon 2011 (A), Sangiovese 2011 (B), Cabernet Sauvignon 2012 (C) and Sangiovese 2012 (D) at four ripening stages. LSD for pea size (PS)=13,197 mg; lag phase (LPh)=4,545 mg; 20 Brix (20B)=23,408 mg and ripening phase (RP)= 25,882 mg.

Norisoprenoids. Considering that carotenoids are precursors of norisoprenoids, the carotenoid levels at lag phase and norisoprenoids at ripening have been compared. The lag phase in this study was the stages with the highest content of carotenoids per berry. Also the differences of carotenoids content between lag phase and ripening and norisoprenoids were compared. In two years of study at three different sites of two cultivars, no correlation was evident between carotenoid precursors and norisoprenoids content at ripening.

Comparing carotenoid levels at lag phase and norisoprenoids, in Cabernet Sauvignon in 2011, Misano had lower carotenoids content but differences between sites in norisoprenoids were not found (Figure 21A). In the same year in variety Sangiovese, the highest carotenoids concentration had Bolgheri, lowest Montalcino but they had the similar values of norisoprenoids. The highest concentration was registered at Misano (Figure 21B).

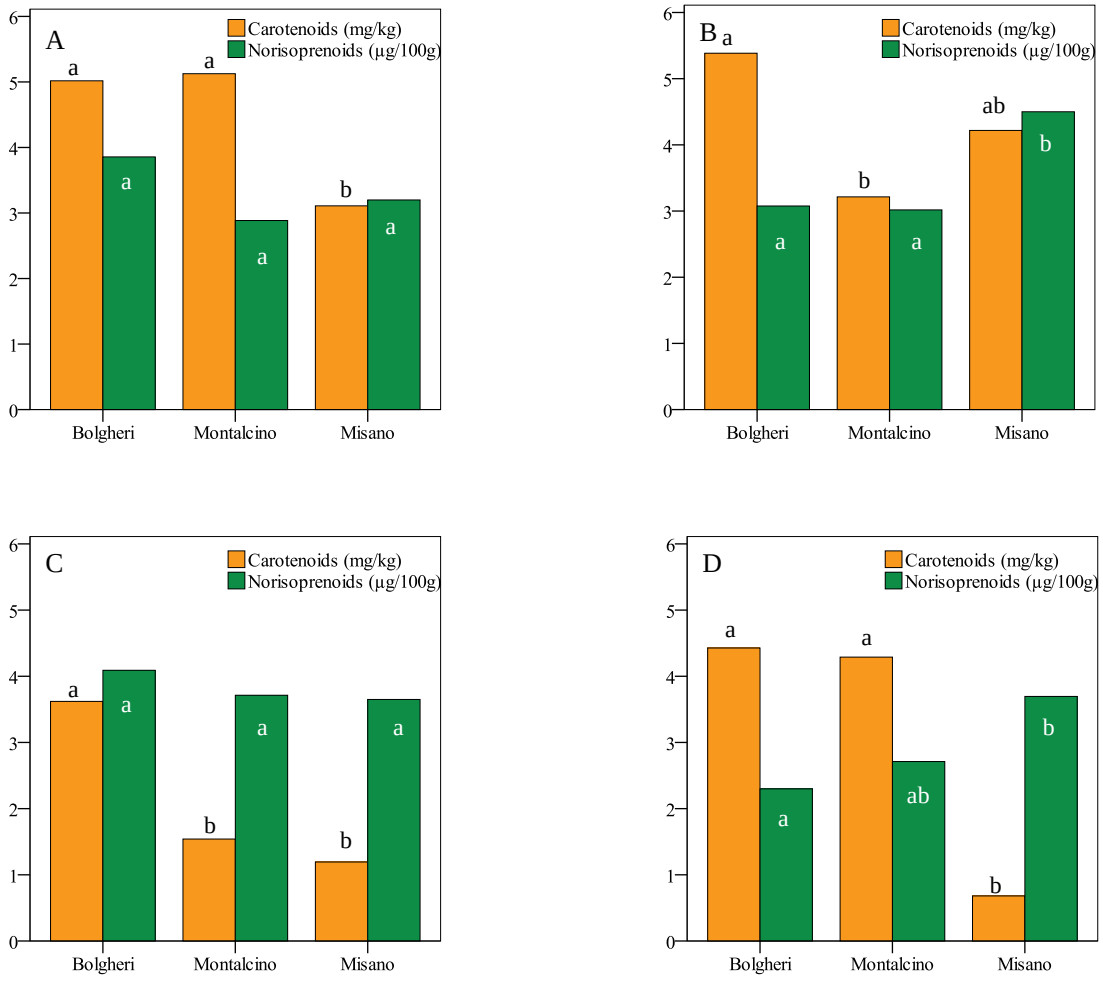


Figure 21. Carotenoids content (mg/kg of grapes) at lag phase and norisoprenoids content (µg/100g of grapes) of Cabernet Sauvignon 2011 (A), Sangiovese 2011 (B), Cabernet Sauvignon 2012 (C) and Sangiovese 2012 (D) in three sites. Norisoprenoids content was calculated from six biological repetitions: three at 20Brix and three at ripening stage; because the values of two samplings were not significantly different. In each graph and for each variable the letters linked to each column follow the Duncan Test at $p < 0.05$.

Though, in Cabernet Sauvignon in 2012 the significantly higher carotenoids content was in Bolgheri, no significant differences were observed between sites in norisoprenoid contents (Figure 21C). In Sangiovese, the locality Misano with lowest carotenoids contained the highest norisoprenoid concentrations (figure 21D).

In order to determine a better correlation between carotenoids and norisoprenoids content, was calculated the difference of total carotenoids concentration between lag phase and full maturity. The results showed only the lower values of carotenoids, but did not change their tendency and did not improve the correlation with norisoprenoids (Figure 22).

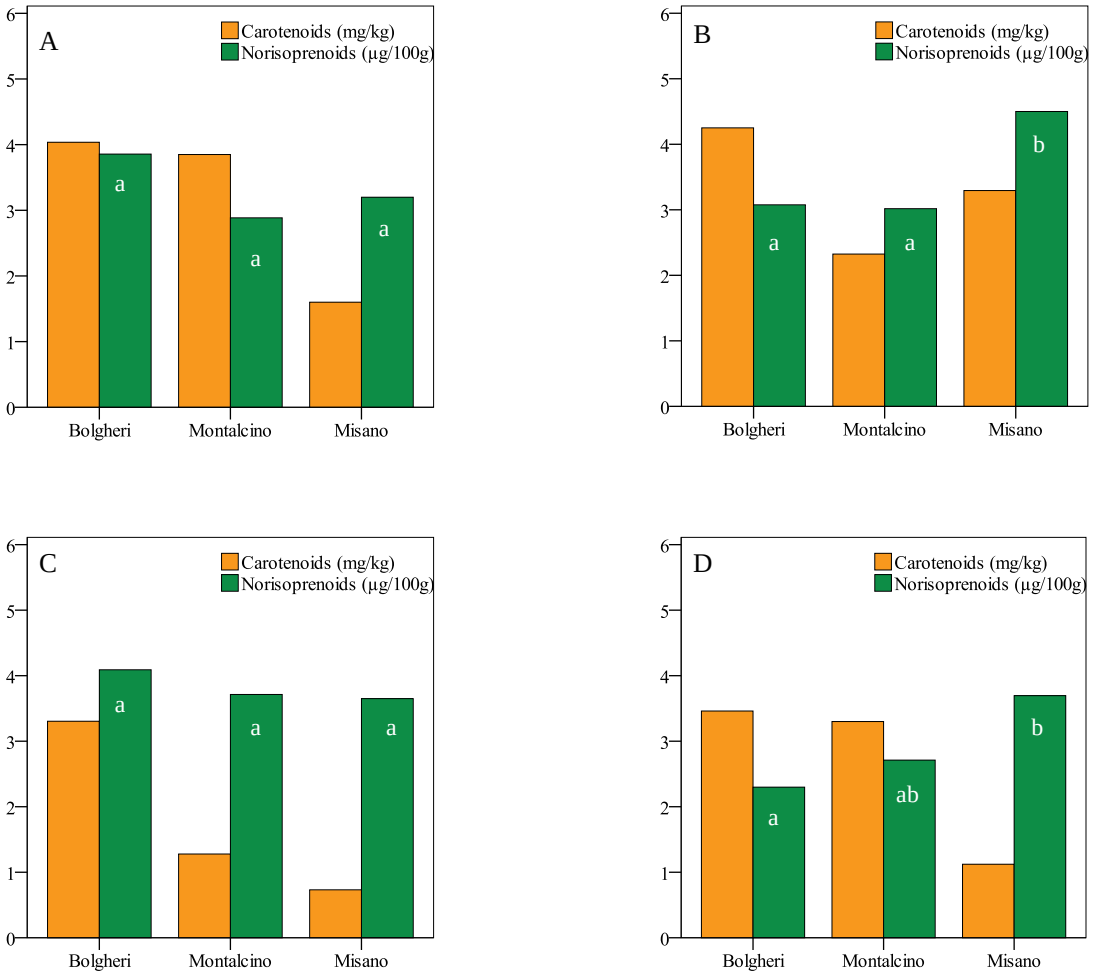


Figure 22. The carotenoids difference between lag phase and ripening phase (ΔLPh-RP) and norisoprenoids content (µg/100g of grapes) of Cabernet Sauvignon 2011 (A), Sangiovese 2011 (B), Cabernet Sauvignon 2012 (C) and Sangiovese 2012 (D) in three sites. Norisoprenoids content was calculated from six biological repetitions: three at 20Brix and three at ripening stage; because the values of two samplings were not significantly different. In each graph the letters linked to each column follow the Duncan Test at $p < 0.05$.

Comparing the values of total norisoprenoids and norisoprenoids per berry, in two years of study at three sites for two varieties, it is observed that there are differences between the two varieties. In Cabernet Sauvignon contents per berry corresponds to the total contents, except small deviation in 2012 for site Montalcino due to the significantly lower berry weight (Figure 21A and 21C). Sangiovese of Bolgheri, due to the highest berry weight in two years, showed lower total content in mg / kg of grapes. However, it appears that at Misano occurred greater synthesis of norisoprenoids (Figure 21B and 21D).

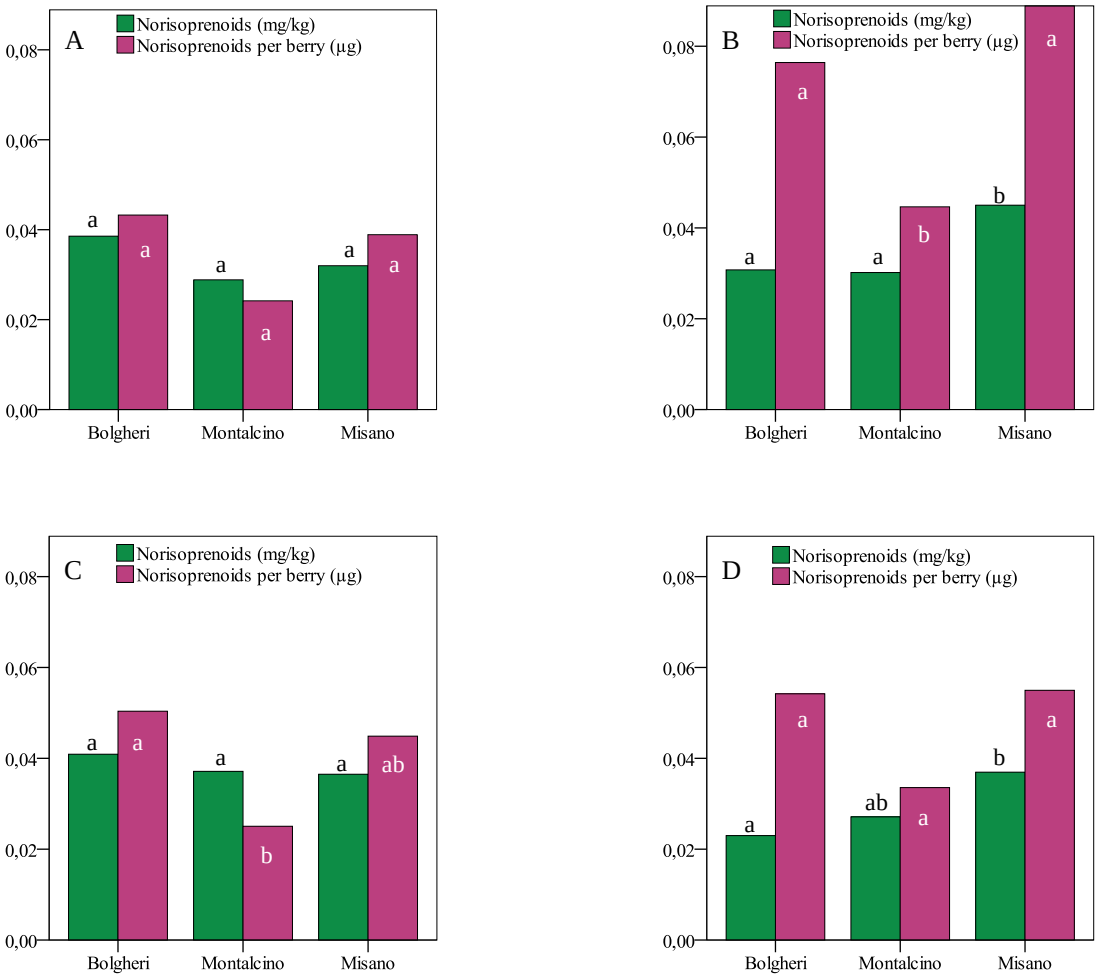


Figure 21. Norisoprenoids content (mg/kg of grapes) and norisoprenoids per berry (µg) of Cabernet Sauvignon 2011 (A), Sangiovese 2011 (B), Cabernet Sauvignon 2012 (C) and Sangiovese 2012 (D) in three sites. Content of Norisoprenoids and Norisoprenoids per berry was calculated from six biological repetitions: three at 20Brix and three at ripening stage; because the values of two samplings were not significantly different. In each graph and for each variable the letters linked to each column follow the Duncan Test at $p < 0.05$.

Conclusion

The objective of this study was to improve the knowledge of the eco-physiological basis of the genotype x environmental interaction, in terms of grape ripening processes, which would have important scientific and practical outputs. In fact to shed light on the differences in the berry metabolic expressions according to the changes in environmental conditions would allow to develop the scientific knowledge to optimize the cultural techniques suitable to obtain the expected grapes quality in agreement with the environmental conditions.

The experimental plan included the study of the Sangiovese grapes, a variety by a particular responsiveness to changing environmental conditions, and Cabernet Sauvignon grapes, a so called international cultivar characterized by more stable features. It was operated within the framework of Appellations of Origin Bolgheri (Tuscany coast), Montalcino (Tuscany Apennines) and Misano (Romagna coast). To highlight the phenotypic effects that arise in the berries specific interaction between environment and genotype was essential analyze the same variety grown in different environments, and simultaneously analyzed grapes growing in different environments as similar as possible. In each site were therefore identified consistent features for the experimental plots including clones, rootstocks, vine age, details of the planting design and vineyard management, also to represent the prevailing conditions of the different vinicultural areas.

The experiment was conducted during two growing seasons (2011-2012) through grape development and ripening from fruit set to ripe stage. Since carotenoids are the precursors to C13-norisoprenoid aroma compounds in wine, relationship between carotenoid and norisoprenoid levels in grapes was investigated.

In the first phase of berry ripening, before veraison, sites with greater global solar radiation (Montalcino and Bolgheri) showed positive correlation with the intensity of total carotenoids accumulation. Location Montalcino was also characterized by a greater water stress.

Correlation between carotenoids accumulation prior to veraison and accumulation of norisoprenoids were not observed. Sangiovese showed a greater reactivity to the environment compared to Cabernet Sauvignon. The norisoprenoids levels in variety Sangiovese largely varied between different years and sites. The synthesis of norisoprenoids appears to be more related to the specific ecophysiological conditions that occur during maturation, rather than to the carotenoids content at veraison.

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