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Bunch Microclimate Affects Carotenoids Evolution in cv. Nebbiolo (*V. vinifera* L.)

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Abstract: This study investigates the impact of bunch microclimate on the evolution of some relevant carotenoids in Nebbiolo grapes. Four bunch-zone microclimates, defined by different vineyard aspect and vine vigor, were characterized by radiation and temperature indices. Berry samples were collected from green phase up to harvest, during two consecutive seasons and carotenoid determination was assessed by High-Performance Liquid Chromatography (HPLC). High carotenoid concentrations were highlighted in Nebbiolo. Lutein and neoxanthin contents ($\mu\text{g berry}^{-1}$) varied similarly in both seasons achieving a concentration peak after veraison especially in the cooler plots while a variety effect on the lutein seasonal trend was presumed. Conversely, β -carotene content remained generally constant during ripening, with the exception of the south plots showing dissimilar evolution between the seasons. Furthermore, higher temperature in the less vigorous and south facing vineyards led to lower amounts of carotenoids, both during ripening and at harvest. Bunch zone temperature and light condition may affect both synthesis and degradation of grape carotenoids determining their amount and profile at harvest. These findings add further knowledge about the influence of climate changes on grape aroma precursors, and are useful to adapt cultural strategies and preserve grape quality consequently.

Keywords: vineyard aspect; vineyard topography; vine vigor; heat accumulation; temperature; photosynthetically active radiation; lutein; neoxanthin; β -carotene

1. Introduction

Plant carotenoids are essential for photosynthesis and photoprotection due to their multiple functions as potent free radical quenchers, singlet oxygen scavengers and lipid antioxidants. They are present in the photosynthetic tissues as part of photosystem II [1]. Carotenoids also give rise to the formation of numerous biologically active cleavage products such as aroma compounds, vitamins, phytohormones, and apocarotenoid pigments [2].

Grape carotenoids were identified as precursors of certain key odorants in wine, namely C_{13} -norisoprenoids, which are low threshold aroma compounds characterized by floral and fruity pleasant notes strongly linked to increases in wine quality, especially for non-floral varieties [3]. The formation of norisoprenoids is thought to occur from the biodegradation of the parent carotenoid, followed by enzymatic conversion to the aroma precursor (e.g., a glycosylated or other polar intermediate), and finally by the acid-catalyzed conversion to the aroma compound [4], which may be then subjected to further acid reaction during wine aging [5]. A family of region-specific carotenoid

cleavage dioxygenase (CCD) enzymes is implicated in the initial biodegradation and oxidative cleavage of carotenoids to form plant apocarotenoids, e.g., C13-norisoprenoids [6–8]. The expression of a CCD capable of producing C13-norisoprenoids from lutein and zeaxanthin (*VvCCD1*) was reported to increase at veraison [6], while the reported increase in expression of a CCD4 gene (*VvCCD4*) after veraison is suggestive of a possible role of this enzyme on norisoprenoid formation during the late stage of berry ripening [8]. Carotenoids could also be precursors of norisoprenoids during fermentation and wine aging [9–11].

Lutein and β -carotene represent nearly 85% of the total carotenoids in grapes and they are mostly involved in degradation reactions in grapes, juice, and wine. The carotenoids directly involved in the aroma of wine are β -carotene, generating β -ionone, and neoxanthin generating β -damascenone. Lutein and violaxanthin also undergo breakdown reactions that may produce norisoprenoid compounds in wines [3,12]. Lutein, for instance, is reported to be an important precursor of 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) [13] while the formation of megastigmane-3,9-diol and of 3-oxo- α -ionol glucosides from the ϵ -cycle of lutein has also been illustrated [14]. Moreover, the involvement of lutein epoxide (Lx) cycle, an additional slower and reversible mechanism of photoprotection that supplements the violaxanthin cycle (violaxanthin and zeaxanthin), was recently demonstrated [8,15]. Authors observed a higher accumulation of lutein 5,6-epoxide in shaded berries [15] that is de-epoxidized to lutein following normal light aspect [8]. Because of the activation of the xanthophyll and Lx cycles at the end of the ripening period [14], processes of bioconversion between different carotenoids, also induced by modified light conditions, may take place, and influence the formation of norisoprenoids [6,14,16].

Generally, carotenoids are thought to be mostly synthesized between berry set and veraison. For this reason, the aromatic profile of the wine also depends on the carotenoid composition of immature berries while the end of veraison (and not the beginning as thought in the past) appears to be a key moment for the changes in the ratio carotenoid norisoprenoid [17]. Several variables may promote the norisoprenoid final content in grapes: some favor carotenoid synthesis during the herbaceous phase of berry growth, some others stimulates their degradation to norisoprenoids occurring from veraison onwards [16,18]. Therefore, carotenoid evolution during ripening can be considered as an indicator of grape aromatic potential [2,19,20].

As reported in many studies, the concentration of carotenoids in ripe grapes depends on cultivars [21], ripening stage, climate region [22], altitude [23], soil water retention capacity [21], and degree of bunch exposure to sunlight [15]. The highest carotenoid concentration occurred in the hot regions, likely due to the higher amount and intensity of the received solar radiation [22,24]. In warm climates, the level of β -carotene at harvest resulted as three–six-fold higher than that of lutein [18,19,25]. In particular, light is the main factor responsible for the changes in the biosynthesis of carotenoids [4] promoting both their accumulation before veraison and causing their degradation during ripening [26]. The degree of the bunch exposure to sunlight appeared to influence the ratio epoxyxanthophylls: non-epoxyxanthophylls whereas high UV-B levels favored carotenoid degradation [23,27], actually, higher rates of degradation emerged during the hotter period of grape ripening [28].

Furthermore, vineyard topographic features, such as slope gradient and aspect or altitude, along with the heterogeneity of vineyard vigor and different vine management may generate a great variability in microclimatic conditions (light, temperature, and humidity) within vineyards, canopy and bunch zone, likely influencing grape ripening and quality. The impact of the vineyard microclimatic characteristics on Nebbiolo grape development, ripening and anthocyanin accumulation, as well as on the evolution of grape norisoprenoid precursors has already been investigated [29–31]. Until now, only one study regarding the evolution of the carotenoid compounds in Nebbiolo grapes has been carried out [28].

Many studies explored the impact of artificial regulation of the bunch exposure to sunlight, by leaf removal or other canopy manipulation, on grape metabolic composition [15,22,23,32]. Nevertheless,

integrated studies focusing on the impact of vineyard aspect and natural vine vigor on bunch microclimate and grape aroma precursors are lacking.

Therefore, the aim of this study was to complete a previous research by assessing the concentration and seasonal accumulation pattern of the most relevant carotenoids in Nebbiolo grapes as well as to study the link between bunch microclimate and carotenoid evolution during ripening.

2. Materials and Methods

2.1. Vineyard Site and Treatments

This study is complementary of a previous research on Nebbiolo grapes [31]. The experimental vineyard, site and treatments are widely described in the cited article. Briefly, the study was performed during 2012 and 2013 in two commercial vineyards located in North-West Italy (44°36′04″ N, 8°00′34″ E; 428 m above sea level). Four vineyards differing in terms of slope aspect (South and West) and natural vine vigor (two level of vigor in each vineyard: V+ and V−) were identified by assessing the Normalized Difference Vegetation Index of the parcel, as previously described [33]. In 2012, the vineyards compared were SouthV+, SouthV−, WestV+; in 2013, the WestV− vineyard was included. In each vineyard, three replicates of 50 vines were used for berry sampling.

2.2. Microclimate Assessment

The thermopluviometric characterization of the two seasons was assessed by bioclimatic indices calculated by the observation of an agrometeorological station belonging to the Regione Piemonte network. In order to characterize the four microenvironments in terms of radiation and thermal conditions, Photosynthetically Active Radiation (PAR) and air temperature inside the bunch zone were measured at intervals of 20′ each, from pea size stage to harvest as described in the previous research and according to an established protocol [31,33]. Then, the integral of daily amount of PAR (SPAR) and maximum daily temperatures (ST) and other cumulative thermal indices, such as Normalized Sum of the Degrees Celsius (SD) and Number of Hours (NH), were calculated over four periods corresponding to different phases of berry growth. The daily values of SPAR [$\text{MJ m}^{-2} \text{d}^{-1}$] were obtained by the cumulative sum of the hourly mean values of PAR irradiance [$\text{J m}^{-2} \text{s}^{-1}$] multiplied by 3600 s. NHs were the number of hours over the considered period in which the mean hourly maximum temperature (T_{max} , [°C]) met three established ranges: $T_{\text{max}} \geq 15 \text{ °C}$ and $T_{\text{max}} < 25 \text{ °C}$ (NH15 - 25); $T_{\text{max}} \geq 25 \text{ °C}$ and $T_{\text{max}} < 35 \text{ °C}$ (NH25 - 35); $T_{\text{max}} \geq 35 \text{ °C}$ (NH ≥ 35). The same thresholds were used to calculate the SD indices by adding together the mean hourly maximum temperature (T_{max} , [°C]) that were simultaneously higher than the minimum value of the threshold and lower than the maximum one, thus, SD15 - 25, SD25 - 35 and SD ≥ 35 respectively were assessed. To eliminate the influence of the period (from early summer to early autumn) in which the data were recorded, and of the different length of each period, the values of all variables were normalized by Equation (1):

$$\text{Normalized value} = (\text{VALUE} - \text{MEAN})/(\text{MAX} - \text{MIN}) \quad (1)$$

where VALUE was the value of the variable in a specific vineyard in a specific period; MEAN, MAX, and MIN, were, respectively, the mean, the maximum, and the minimum values of the variable calculated over all the vineyards in the considered period.

2.3. Berry Sampling

Four subsequent samplings of 400 berries were carried out randomly on each vineyard replicate, from about BBCH code75 (five–six weeks after bloom) until harvest [31]. In more detail:, in 2012, samplings were conducted on 12 July (about 24 days before veraison: dbV), 31 July (about 5 dbV), 27 August (about 22 days post veraison: dpV), 5 October (about 60 dpV); in 2013, 22 July (about 25 dbV),

21 August (about 5 dpV), 9 September (about 24 dpV), 15 October (about 60 dpV). For carotenoid analysis, three replicates of 50 g of berries were analyzed as described below.

2.4. Extraction and Determination of Carotenoids

2.4.1. Extraction from Grape Material

The procedure of carotenoid extraction was adapted from the method of Oliveira and others [21], as optimized by Crupi and collaborators [34]. Approximately 50 g of fresh berries, without seeds, added of 100 mg Na₂S₂O₅, were homogenized for 5 min in presence of magnesium carbonate basic. The homogenate was spiked with 200 µL of 183.2 mg/L of β-apo-8-carotenal (Fluka, Porto, Portugal, ref. 10,810) as internal standard, and diluted with 40 mL of water (Milli-Q, Millipore). Extraction was first carried out with 40 mL of ether/hexane (1:1, v/v), agitating for 30 min, then repeated twice with further 20 mL of ether/hexane. The upper layer was separated each time. The final extract was concentrated to dryness at 20 °C (Laborota 4001, Heidolph instruments) and resuspended in 1 mL of acetone/hexane (1:1, v/v) for High Performance-Liquid Chromatography (HPLC)/DAD determination. Each sample was injected in duplicate. Sample handling, homogenization, and extraction were carried out on ice under dim yellow light to minimize light-induced isomerization and oxidation of carotenoids.

2.4.2. High Performance-Liquid Chromatography (HPLC) Determinations

An Agilent Model 1200 quaternary solvent system equipped with quaternary pump solvent delivery and a UV-visible photodiode array detector was used. The absorption spectra were recorded at 447 nm and the sample injection volume was of 20 µL. The reversed stationary phase employed was a Lichrospher 100 RP C18 (5 µm) LichroCART (250 × 4 mm i.d.). Mobile phase was performed with solvent A: acetone/water (70:30 v/v), solvent B: acetone 100% (Sigma pure-grade), flow rate = 1 mL min⁻¹. The analytical gradient was: 0–20 min (from 100% to 0% of A), then from 20 to 30 min isocratic with 100% of B [25].

2.4.3. Identification and Quantification

The most relevant carotenoids were identified by comparison of UV-visible spectra with those of commercially available standards, β-carotene (Sigma 95%, synthetic,) (C-9750), lutein (Sigma 70%, from alfalfa) neoxanthin (0234.1) from CaroteNature GmbH (Erlenauweg 17, 3110 Münsingen, Switzerland), matching also different information such as position of absorption maxima (λ_{max}) and the degree of vibration fine structure (% III/II) (Table 1) [34].

Table 1. High Performance-Liquid Chromatography (HPLC)/DAD characteristics of carotenoids found in Nebbiolo grapes.

Compound	k'	λ _{max} (nm)	% (III/II) a
(9' Z)-Neoxanthin	4.38	414; 436; 464	
(all-E)-Lutein	7.23	(422); 445; 472	42
β-Apo-8'-carotenal	9.28	460	
β-Carotene	11.82	(428); 452; 478	25

a % III/II is the ratio of the height of the longest-wavelength absorption peak, designated III, and that of the middle absorption peak, designated as II, taking the minimum between the 2 peaks as baseline.

Quantification of individual compounds was done by calibration curves using the respective standards for lutein and β-carotene with R² = 0.9997 and R² = 0.9991, respectively, whereas neoxanthin was expressed as lutein equivalent because of the unavailability of a fresh neoxanthin standard. The results were expressed in terms of concentration (mg kg⁻¹ of berries) and content (µg berry⁻¹) to avoid an overestimation of the changes that may be the result of an altered berry surface area to volume ratio.

2.5. Statistical Analyses

Data were statistically analyzed by ANOVA (SPSS 15.0 for Windows, Chicago, USA and SAS 9.4 SAS Institute, Cary, USA), Tukey's test was used to assess the differences as regards microclimatic variables and carotenoids both among treatments for each sampling date and between sampling dates for each treatment; the general effect of factors such as vineyard microenvironments (by treatment) and seasons (by year) and their possible interaction (year*treatment) were assessed too. Moreover, a 3-way-ANOVA, as regards carotenoid data of South treatments, was assessed, in order to evidence the vigor effect and interactions between year, sampling date and vigor level.

A preliminary ANOVA on normalized microclimatic variables (SD, NH, ST:SPAR) assessed the differences among sampling periods and the opportunity that these latter could be used as replicates for the comparison of vineyard microclimates and seasons. No differences among the periods emerged for none of the microclimatic variables; thus, the periods were used as replicates when microenvironments and seasons were compared. A Hierarchical Clustering Analysis (HCA) was also carried out using the method of centroid distance to evaluate the clustering of the vineyards based on the microclimatic conditions.

With the aim of identifying a model able to describe the impact of microclimate on berry composition, several Principal Component Analysis (PCA) were also performed on both microclimate (NH, SD, ST:SPAR) and carotenoids related variables including concentration (mg kg^{-1}), content ($\mu\text{g berry}^{-1}$), proportion (%) of lutein, β -carotene and neoxanthin, lutein: β -carotene ratio, and sum of carotenoids (lutein+ β -carotene+neoxanthin) as mg kg^{-1} of berries and $\mu\text{g berry}^{-1}$. The results reported here, refer to the data set of variables that explained the higher amount of the model variance. HCA and PCA were performed by SAS 9.4 (SAS Institute, Cary, USA).

3. Results

3.1. Seasonal Meteorological Trends

The two seasons presented some peculiarities from the meteorological point of view. In terms of annual values, the mean minimum and maximum temperatures were higher in 2012 than in 2013, as well as the average of the maximum monthly temperature that in 2012 exceeded the 2013 value by 2.7 °C. In the summer of 2012, the maximum temperatures exceeded 30 °C for 78 days, whereas in 2013 for 66. The hot condition of 2012 was also certified by the cumulative amount of Growing Degree Days (GDD) which exceeded the value of the 2013 by approximately 10%. Moreover, 2012 was drier than 2013, both in terms of rainfall amount and number of rainy days (> 1 mm). The differences between the years were also reflected on the growing period (Table 2). The warmest condition of 2012 affected the timing of the phenological phases that occurred earlier in 2012 than in 2013 [31]. In 2013, in fact, a delay of around 10 days for bud burst, a couple of weeks for bloom, a week for veraison and 10 days for commercial harvest was observed in comparison to 2012.

3.2. Bunch Microclimate

The thermal and radiative normalized indices calculated for each sampling date, were able to separate the two vineyard aspects when a cluster analysis was carried out, whereas clear separations between the levels of vigor, between seasons and among sampling dates were not evident (Figure 1 HCA). In more detail, ANOVA analysis, showed that differences among the four environments emerged for singular variables in both years (Table 3). When negative, the normalized indices indicated a negative difference compared to the average value calculated for all vineyards, and vice versa when positive. The higher the absolute values of the index, the higher were the differences. In 2012, the year with higher temperatures and lower rainfall SD15 - 25, NH15 - 25, and ST:SPAR were negative in the vineyards facing south, therefore, lower than the average calculated on all the vineyards, and they were significantly different from those of the vineyards facing west. In addition the vigor of the plants affected NH15 - 25 and ST:SPAR indices of the south facing vineyards showing both a lower value in

the less vigorous condition (V−). No differences among vineyards emerged for the indices referring to the other temperature ranges.

Table 2. Meteorological characterization of the two seasons (the values are calculated both for the entire year and for the grapevine vegetative period: April–October). Data were registered by Serralunga Boscareto’s meteorological station (Agrometeorological Network, Regione Piemonte).

	Tmax Mean (°C)	Tmin Mean (°C)	Average of the Monthly Maximum Values of Tmax (°C)	Total Rainfall (mm)	Growing Degree Days (Base 10 °C)	Days with T > 30 °C (Number)	Rainy Days ¹ (Number)
Annual Values							
2012	20.4	8.9	28.3	722	2329	78	63
2013	19.4	8.6	25.6	971	2119	66	83
Values of April–October Period							
2012	26.0	13.8	32.2	491	2147	78	36
2013	25.6	13.4	31.8	563	2046	66	49

¹ Days with precipitation over 1 mm.

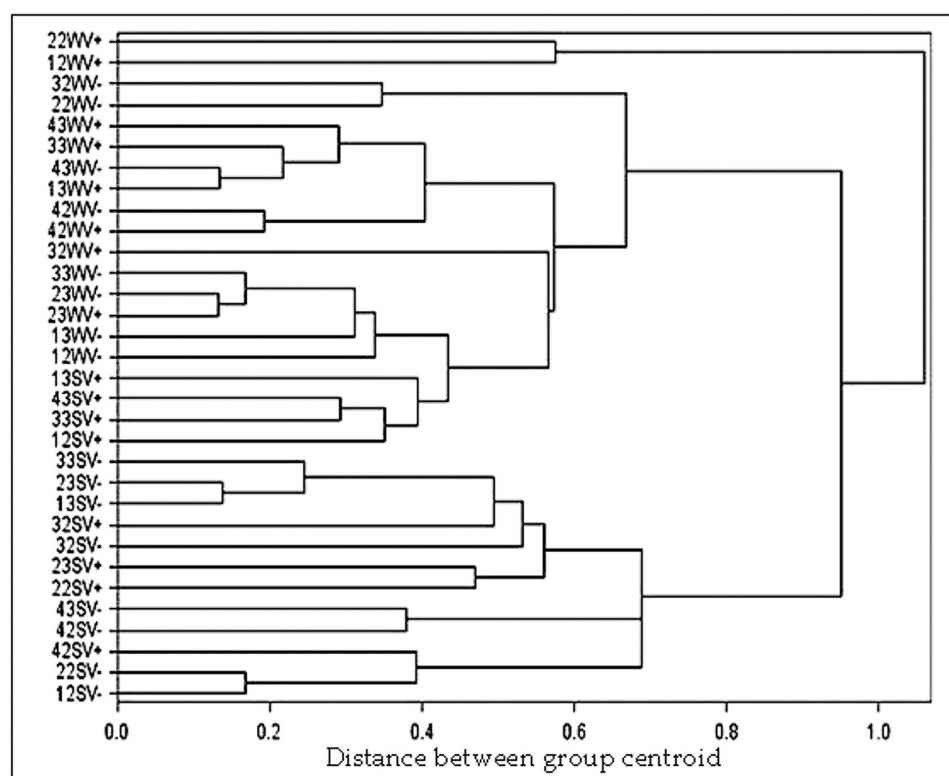


Figure 1. Dendrogram of hierarchical clustering analysis of the four microenvironment (SV−, SV+, WV−, and WV+) obtained by analyzing the meteo-climatic indices reported in Table 2. (1, 2, 3, and 4, as the first digit in the treatment acronym, correspond to the phenological period; 2, 3, as the second digit, correspond to the season 2012 and 2013, respectively; W and S represent the West and South vineyard aspect, respectively; V+ and V− indicate a higher or lower plot vigor, respectively).

Table 3. Normalized Sum of the Degrees Celsius (SDs), number of hours (NHs) related to the temperature ranges (15– 25; 25– 35; ≥ 35) and ST:SPAR; ST=summation of the maximum daily temperatures; SPAR=summation of the daily integrals of PAR.

Treatment		SDs Celsius (°C)			NHs Number of Hours NHT			ST:SPAR
					Number of Hours NHT			
		15–25	25–35	≥35	15–25	25–35	≥35	
2012	SouthV–	–0.54 a	0.18 a	0.12 a	–1.20 a	0.50 a	0.58 a	–0.56 a
	SouthV+	–0.10 ab	–0.06 a	–0.05 a	–0.37 b	–0.02 a	0.20 a	–0.18 b
	WestV–	0.40 b	0.22 a	–0.43 a	0.78 c	0.45 a	–1.05 a	0.29 c
	WestV+	0.23 b	–0.34 a	0.36 a	0.80 c	–0.92 a	0.27 a	0.44 c
		***	ns	ns	***	ns	ns	***
2013	SouthV–	–0.59 a	–0.23 a	0.66 c	–0.59 a	–0.34 a	0.63 c	–0.59 b
	SouthV+	0.03 b	0.28 a	–0.07 b	–0.08 b	0.12 a	–0.01 b	–0.22 b
	WestV–	0.23 b	0.22 a	–0.34 a	0.29 b	0.28 a	–0.37 a	0.40 c
	WestV+	0.32 b	–0.27 a	–0.24 ab	0.37 b	–0.06 a	–0.26 a	0.41 c
		***	ns	***	***	ns	***	***
Treatment		***	ns	***	***	ns	***	***
Year		ns	ns	ns	ns	ns	ns	ns
Year*Treatment		ns	ns	***	***	ns	ns	ns

For the same year, means followed by different letters are significant different for $p < 0.05$. ns—not significant; * $p < 0.05$, *** $p < 0.001$ indicate the significance of differences among treatments.

In 2013, the coolest and wettest year, the differences emerged in the first year were confirmed. Furthermore, $SD \geq 35$ and $NH \geq 35$, were higher in the vineyards facing south and, in particular, in the less vigorous ones. On the contrary, in the vineyards facing west, the influence of vigor did not emerge. Therefore, differences among vineyards were evident for the indices relating to the lowest and highest temperature ranges whereas for the intermediate range (25–35) both SD and NH were similar in all the vineyards. For none of the indices, differences between the years emerged while the year * vineyard interaction was significant only for $SD \geq 35$ and NH_{15-25} .

3.3. Evolution of Carotenoids in Nebbiolo Grapes

The compounds that mostly contributed to the total amount of carotenoids in Nebbiolo grapes were lutein and β -carotene. The evolution of each compound during ripening, both as $\mu\text{g berry}^{-1}$ (Figure 2a, Figure 3a, and Figure 4a) and as mg kg^{-1} of fresh berries (Figure 2b, Figure 3b, and Figure 4b), is shown for both years 2012 and 2013 (Table 4).

• Lutein

In 2012, at the first sampling date, at 24 days before veraison (dbV) similar amounts of lutein were detected in green berries from both west and south exposed vineyards (Figure 2a). Lutein concentration (mg kg^{-1} of berries) and content ($\mu\text{g berry}^{-1}$) remained then constant until the beginning of veraison (5 dpV, second sampling) (Figure 2a,b and Table 4). In 2013, at the first sampling (25 dbV), the lutein concentration (mg kg^{-1}) was significantly higher in SouthV+ when compared to the other treatments and to the previous year. Afterwards, at the second sampling (5 dpV), lutein content decreased significantly only in south-exposed vineyards (Figure 2a,b, Table 4).

Table 4. Seasonal changes of the carotenoid concentration and of the ratio Lutein: β -carotene in Nebbiolo grapes during 2012 and 2013.

Year	Treatment	2012					2013					sig. §		
Sampling Date		12 Jun	31 Jul	27 Aug	5 Oct	sig. §	22 Jul	21 Aug	9 Sep	15 Oct		sig. §	sig. §	
Phenological Phase		24dbV	5dbV	22dpV	60dpV	date	25dbV	5dpV	24dpV	60dpV	date	T	Y	Y*T
Lutein mg kg ⁻¹	SouthV–	4.84b	4.52b	4.37b	1.65a	**	6.19b	4.09a	3.53a	2.52a	*			
	SouthV+	5.29b	4.73b	5.60b	2.68a	*	9.69b	4.13a	5.64a	3.70a	*	***	***	ns
	WestV–	-	-	-	-		5.39a	5.60a	5.59a	3.95a	ns			
	WestV+	4.42ab	3.76a	5.76b	3.75a	**	5.41ab	5.09ab	6.55b	4.25a	*			
Sig. among treat.		ns	ns	ns	*		**	ns	*	**				
Lutein μ g berry ⁻¹	SouthV–	3.56a	3.81a	6.80 b	2.78a	**	5.35a	4.76a	5.70a	4.40a	ns			
	SouthV+	4.21a	4.18a	9.07b	4.66a	**	6.90b	4.89a	9.30c	7.04b	**	***	***	ns
	WestV–	-	-	-	-		4.68a	6.58ab	9.51b	7.41ab	**			
	WestV+	3.94a	3.76a	10.1c	6.75b	**	4.80a	5.84a	11.3b	7.91ab	*			
Sig. among treat.		ns	ns	*	*		ns	ns	*	**				
β -carotene mg kg ⁻¹	SouthV–	8.11b	5.37ab	3.60a	2.53a	**	10.3b	2.32a	3.08a	3.18a	**			
	SouthV+	9.57b	6.37ab	4.46a	3.64a	**	13.7c	2.49a	4.43b	3.51ab	**	***	ns	ns
	WestV–	-	-	-	-		10.1c	5.55b	4.17ab	3.48a	**			
	WestV+	7.47b	5.91ab	3.92a	4.07a	**	10.4b	5.64a	5.18a	4.22a	**			
Sig. among T		ns	ns	ns	*		ns	***	ns	*				
β -carotene μ g berry ⁻¹	SouthV–	5.97a	4.53a	5.61a	4.24a	ns	8.91b	2.70a	4.97a	5.56a	**			
	SouthV+	7.61a	5.63a	7.21a	6.26a	ns	11.4c	2.95a	7.31b	6.70ab	**	***	**	ns
	estV–	-	-	-	-		8.70a	6.52a	7.08a	6.52a	ns			
	WestV+	6.66a	5.91a	6.91a	7.33a	ns	9.24a	6.47a	8.98a	7.88a	ns			
Sig. among T		ns	ns	ns	*		ns	**	*	*				
Neoxanthine mg kg ⁻¹	SouthV–	nd	0.14a	0.41b	0.11a	**	0.06a	0.24a	0.15a	0.07a	ns			
	SouthV+	nd	0.12a	0.55b	0.17a	**	0.18ab	0.25b	0.26c	0.10a	**	*	ns	ns
	WestV–	-	-	-	-		0.08a	0.15ab	0.34c	0.23b	**			
	WestV+	nd	0.03a	0.51c	0.25b	**	0.06a	0.14ab	0.41c	0.18b	**			
Sig. among T		-	**	ns	*		ns	ns	ns	**				
Neoxanthine μ g berry ⁻¹	SouthV–	nd	0.12a	0.64b	0.18a	**	0.08a	0.28ab	0.25b	0.12ab	*			
	SouthV+	nd	0.11a	0.89b	0.30a	**	0.15a	0.30a	0.43b	0.19a	**	**	ns	***
	WestV–	-	-	-	-		0.07a	0.17a	0.58b	0.44b	**			
	WestV+	nd	0.03a	0.91c	0.46b	**	0.06a	0.16a	0.70c	0.34b	**			
Sig. among T		-	*	ns	*		ns	ns	ns	**				
Lutein: β -carotene	SouthV–	0.60a	0.84a	1.21b	0.65a	**	0.60a	1.75b	1.15ab	0.79a	***			
	SouthV+	0.55a	0.75a	1.25b	0.73a	**	0.72a	1.65b	1.28ab	1.05ab	**	**	***	***
	WestV–	-	-	-	-		0.52a	0.91ab	1.27b	1.01a	*			
	WestV+	0.59a	0.63a	1.49c	0.93b	**	0.54a	1.01b	1.34b	1.13b	*			
Sig. among T		*	***	ns	*		ns	***	*	***				
¹ Sum of carotenoids mg kg ⁻¹ of berries	SouthV–	13.0c	10.0bc	8.38b	4.30a	**	16.6b	6.6a	6.84a	5.81a	**			
	SouthV+	14.9b	11.2ab	10.6ab	6.49a	*	23.5c	6.9a	10.5b	7.31a	***	***	**	ns
	WestV–	-	-	-	-		15.5b	10.9a	10.1a	7.67a	*			
	WestV+	11.9a	9.7a	10.2a	8.07a	**	15.9b	11.3ab	12.1ab	8.65a	*			
Signif. among T		ns	ns	ns	*		**	**	*	*				
Sum of carotenoids μ g berry ⁻¹	SouthV–	9.53ab	8.46ab	13.1b	7.21a	**	14.3b	7.73a	11.0ab	10.1ab	*			
	SouthV+	11.9ab	9.91a	17.2b	11.2ab	**	18.5c	8.14a	17.3bc	13.9b	**	***	***	ns
	WestV–	-	-	-	-		13.4a	12.5a	17.2a	14.4a	ns			
	WestV+	10.6ab	9.67a	18.0c	14.5bc	**	14.1a	13.3a	21.0a	16.1a	ns			
Sig. among T		ns	ns	ns	*		ns	*	*	**				

For the same line and year means followed by different letters indicate significant differences among sampling dates for 2012 and 2013 respectively. *, **, *** indicate, respectively, significant differences for $p < 0.05$, $p < 0.01$, and $p < 0.001$. nd—not detectable, ns—not significant, sign §—statistical differences by date, by T—Treatment; by Y—year; T*Y—interactions between Treatment and Year; ¹—Lutein+ β -carotene+neoxanthin.

Concurrently to the increase of the berry weight and sugar concentration [31], a significant increase of lutein content per berry was noticed in both years reaching a peak at about 4 weeks after veraison, with the exception of SouthV– in 2013. This increase of lutein content was proportional to the vine vigor, thus more important for the most vigorous vines. After veraison, in SouthV– a minor increase was observed in 2012 and a constant trend in 2013, thus, at the time of the peak, significant differences were noticed between WestV+ and SouthV– in both seasons. At the final stage of ripening, a significant lutein degradation was observed for all treatment in 2012; significant differences between SouthV– (1.66 mg kg⁻¹ of berries or 2.78 μ g berry⁻¹) and WestV+ parcels (3.75 mg kg⁻¹ of berries or 6.78 μ g

berry⁻¹) were found at harvest (Figure 2a,b and Table 4). The concentration decline observed in 2013 was less remarkable for all vineyards compared to 2012, resulting significant only for the most vigorous plots, WestV+ (mg kg⁻¹ of berries) and SouthV+ (µg berry⁻¹). The content of lutein in the less vigorous SouthV– at harvest was significantly lower (4.4 µg berry⁻¹ or 2.52 mg kg⁻¹ of berries) than in the other vineyards. Thus, the warmest vineyard had the lowest lutein concentration at harvest in both years. Lower amounts of lutein were detected at harvest in 2012 for all treatments, but significantly lower only for SouthV–, when compared to 2013 (average at harvest, 2.69 mg kg⁻¹ and 4.73 µg berry⁻¹ in 2012; 3.61 mg kg⁻¹ and 6.7 µg berry⁻¹ in 2013). The interaction year*treatment was not significant regardless of the unit (Table 4).

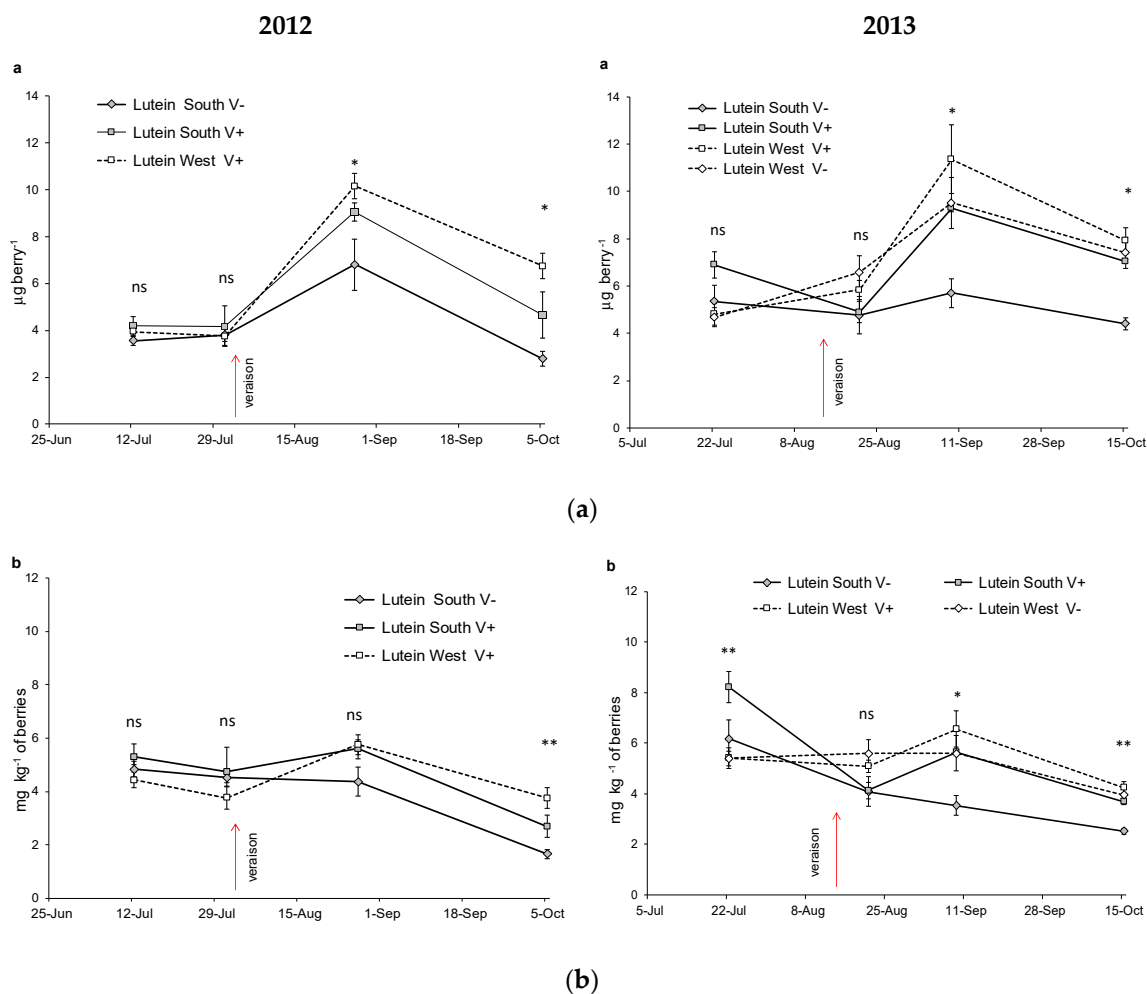


Figure 2. Seasonal changes of lutein as µg berry⁻¹ (a) and mg kg⁻¹ of berries, (b) in Nebbiolo grapes in 2012 (on the left) and 2013 (on the right), as a function of vine vigor and vineyard aspect. Averages ± standard error ($n = 3$). * $p < 0.05$, ** $p < 0.01$ indicate the significance of differences among treatments.

• β-Carotene

In 2012, the β-carotene concentrations (mg kg⁻¹ of berries) decreased during the season with no significant differences among treatments at any sampling point except at harvest, when significantly higher amounts were noticed in the WestV+ (4.07 mg kg⁻¹ or 7.33 µg berry⁻¹) than in the SouthV– grapes (2.54 mg kg⁻¹ or 4.24 µg berry⁻¹). In 2013, the β-carotene decline between the first and second sampling was significant for south-exposed vineyards while the post veraison increase of β-carotene content in 2013, was significant only for the SouthV+ grapes (Figure 3a,b, Table 4). β-carotene content (µg berry⁻¹) and concentration (mg kg⁻¹ of berries) were significantly lower in SouthV– than in

WestV+ both at the third sampling and at harvest (3.2 mg kg^{-1} or $5.6 \mu\text{g berry}^{-1}$ for SouthV– and 4.2 mg kg^{-1} or $7.9 \mu\text{g berry}^{-1}$ for WestV+). Moreover, differences between the two years at harvest, were not significant when comparing the values as mg kg^{-1} (averagely 3.41 versus 3.60, in 2012 and 2013, respectively) but they were significant when comparing the values as $\mu\text{g berry}^{-1}$ (5.94 versus 6.7, in 2012 and 2013, respectively). The interaction year*treatment was not significant regardless the unit (Table 4).

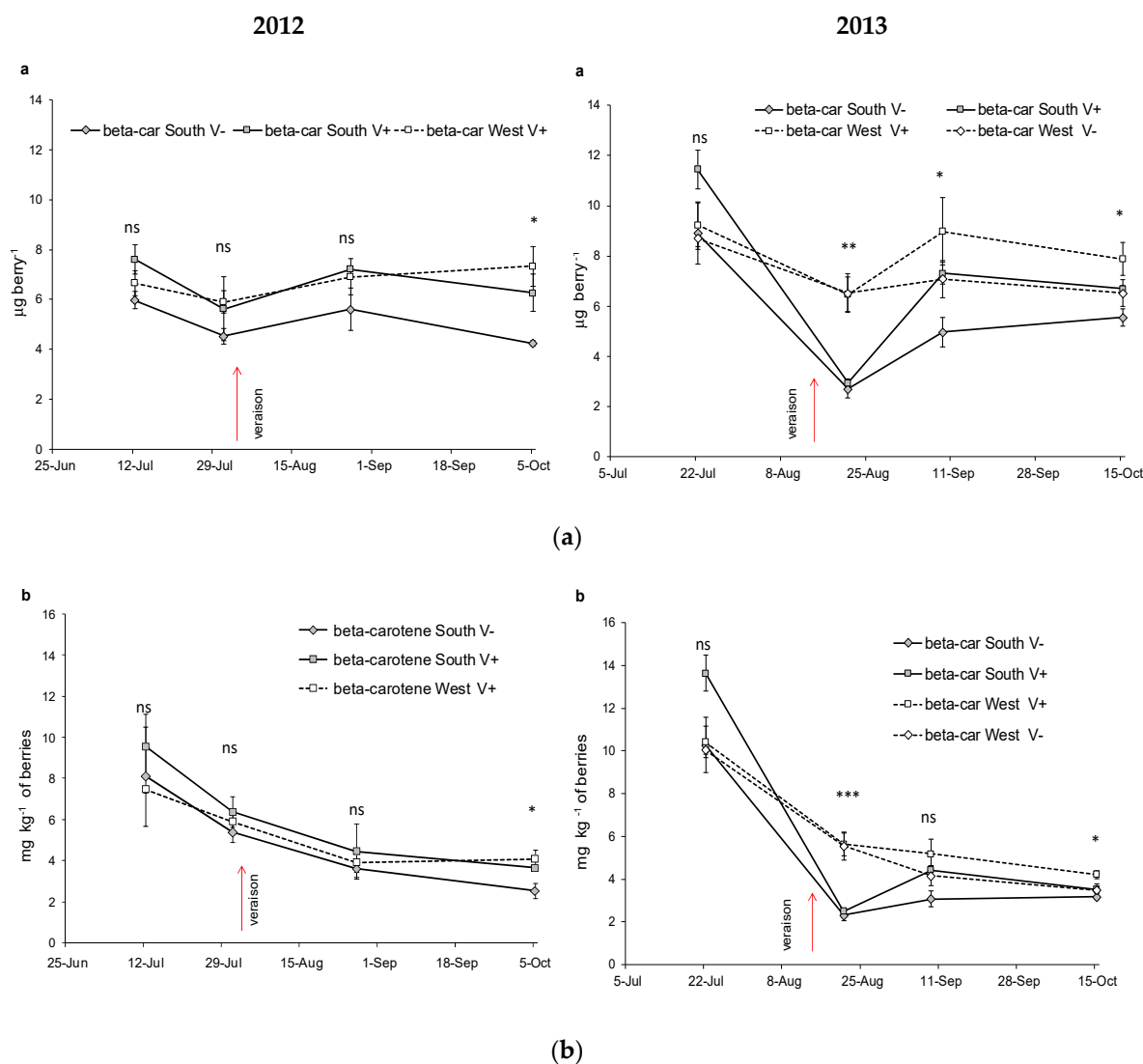


Figure 3. Seasonal changes of β -carotene as $\mu\text{g berry}^{-1}$ (a) and mg kg^{-1} of berries (b) in Nebbiolo grapes in 2012 (on the left) and 2013 (on the right), as a function of vine vigor and vineyard aspect. Averages $\pm$ standard error ($n = 3$). * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ indicate the significance of differences among treatments. b.

• Neoxanthin

In the green berries, undetectable amounts (2012) or traces (2013) of neoxanthin were observed for all treatments. The important increase after veraison, observed for the most vigorous plots was more remarkable in 2012 than in 2013, and was then followed by a significant degradation of this compound until harvest. In 2012, as for lutein and β -carotene, significant differences were observed at harvest between SouthV– (0.11 mg kg^{-1} of berries or $0.18 \mu\text{g berry}^{-1}$) and WestV+ grapes (0.25 mg kg^{-1} of berries or $0.46 \mu\text{g berry}^{-1}$). Similarly to lutein, a peak of concentration was achieved 4 weeks after

veraison but, differently from lutein and β -carotene, was higher in 2012 (averagely $0.49 \mu\text{g kg}^{-1}$ and $0.81 \mu\text{g berry}^{-1}$) than in 2013 (averagely $0.30 \mu\text{g kg}^{-1}$ and $0.49 \mu\text{g berry}^{-1}$) (Figure 4).

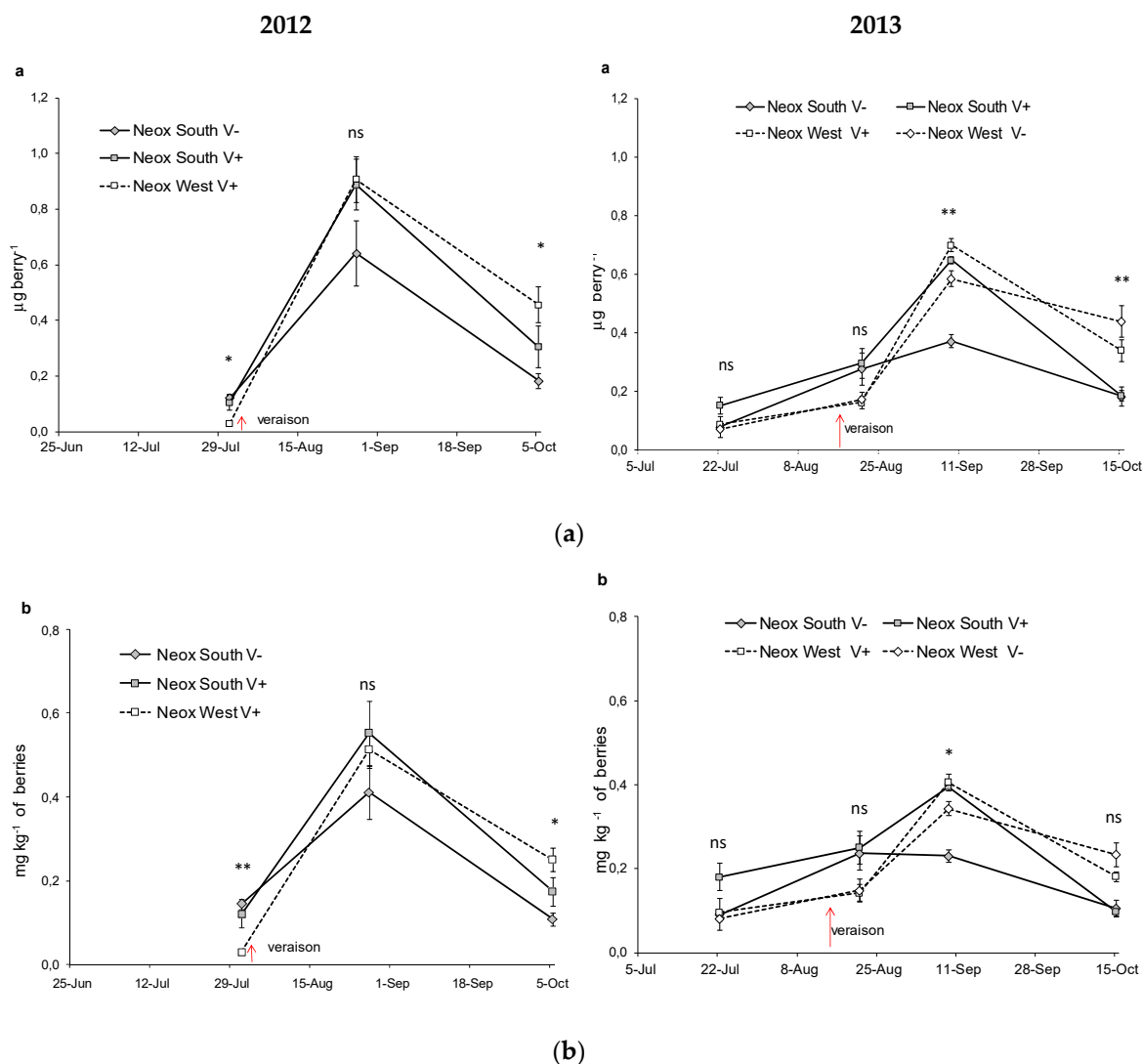


Figure 4. Seasonal changes of neoxanthine as $\mu\text{g berry}^{-1}$ (a) and mg kg^{-1} of berries (b) in Nebbiolo grapes in 2012 (on the left) and 2013 (on the right), as a function of vine vigor and vineyard aspect. Averages $\pm$ standard error ($n = 3$). * $p < 0.05$, ** $p < 0.01$ indicate the significance of differences among treatments.

In both years, this peak was followed by a significant degradation of neoxanthin until harvest period in SouthV+, WestV−, and WestV+ (Figure 3a). Moreover, in both years, a high degradation rate was highlighted especially for the most vigorous SouthV+ that influenced neoxanthin amount at harvest time. In SouthV−, instead and similarly to lutein, neoxanthin levels followed a more flattened evolution showing a minor peak mostly in 2013. In both years after veraison, SouthV− attained the lowest values compared to the other treatments especially when $\mu\text{g berry}^{-1}$ were considered. The vine vigor (V−, V+) did not affect the neoxanthin content at harvest. Differences between the years were not significant; the interaction year*treatment was significant when values were expressed as $\mu\text{g berry}^{-1}$ (Figure 4, Table 4).

• Lutein: β -Carotene Ratio and Sum of Carotenoids

The ratio lutein: β -carotene increased between green phase (first sampling) and complete veraison (third sampling), whereas a decrease was evident during the weeks preceding commercial harvest. In 2012, the ratio was in favor of β -carotene whereas in 2013 the ratio was often in favor of lutein. Comparing both years, the ratio varied at harvest from 0.65 to 0.79 in the warm and more exposed plots (SouthV−) and from 0.93 to 1.13 in the cooler and more vigorous WestV+. In average, the difference between years was significant as well the interaction years*treatments (Table 4). In general, the sum of the concentration (mg kg^{-1}) of the three considered carotenoids, decreased from the first to the last sampling, whereas, when expressed as $\mu\text{g berry}^{-1}$, a peak was observed at the third sampling in both years. However, at harvest in 2012 and at all sampling dates in 2013, the sum of the carotenoids (concentration and content) was lower in SouthV−, than in WestV+.

Summarizing, the season significantly influenced all the variables except the neoxanthine and β -carotene concentration. Regardless the season, the treatment significantly influenced the content of all compounds. Nevertheless, the interaction year*treatment was significant only for neoxanthine ($\mu\text{g berry}^{-1}$) and lutein: β -carotene ratio. Focusing on the results of the 3-way ANOVA, carried out on the south facing vineyards, significant differences between the two levels of vine vigor emerged for all compounds, but neither the interaction year*vigor nor year*vigor*sampling date were significant, regardless the unit of measurement of carotenoids (Supplementary Table S1).

Performing a PCA on a data matrix including microclimatic indices and berry carotenoids an effective distribution of the vineyards along the first component (PC1), and of the sampling dates along the second component (PC2), emerged (Figure 5).

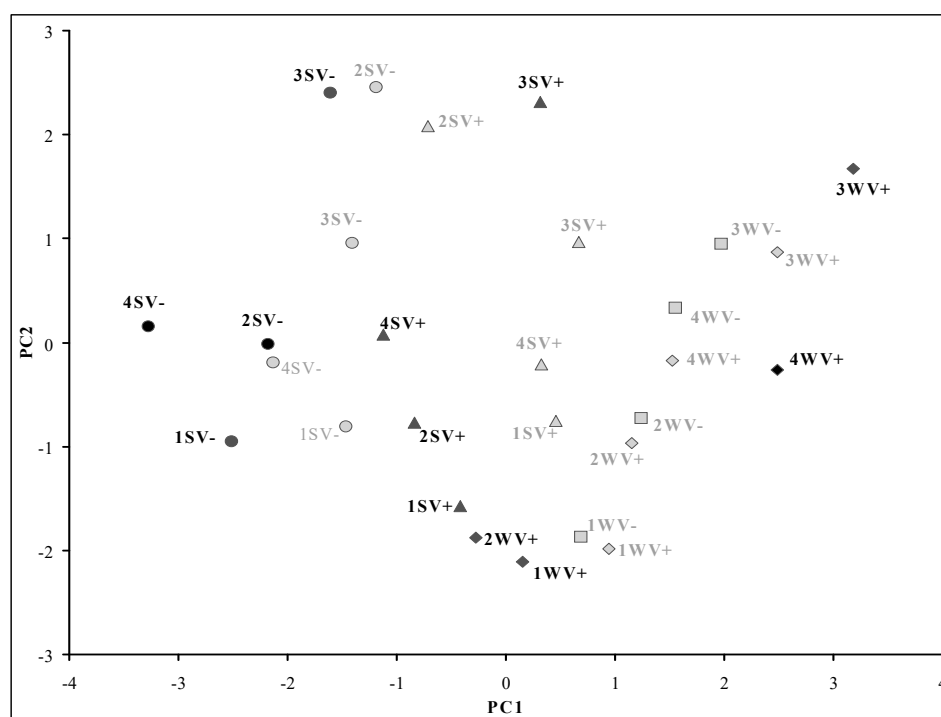


Figure 5. Score plot of the observations in the Cartesian coordinate system identified by Principal Component Analysis: analysis including variables related to carotenoids and microclimatic indexes (the variables included in the analysis are reported in Table 4). 1, 2, 3, and 4 refer to the data sampling; S—South, W—West, V+—high vigor, V—low vigor. Symbols in black color—2012; symbols in grey color—2013; ●—SouthV−, ▲—SouthV+, ■—WestV−, and ◆—WestV+.

Prin1 and Prin2 explained, respectively, 39% and 27% of the total variance. The case distribution along Prin1 was well represented by a linear combination of the variables NH15 - 25, ST:SPAR and

sum of carotenoids (positively correlated with Prin1). The case distribution along Prin2 was well represented by a linear combination of the variables Neoxanthine (%) and lutein: β -carotene ratio (positively correlated with Prin2) (Table 5).

Table 5. Principal Component Analysis (PCA): Percentage of variance explained by the three principal components (Prin1, Prin2, and Prin3), eigenvalues and loadings indicating the correlation between the variables and the three principal components.

	Prin1	Prin2	Prin3
Explained variance (%)	39	27	20
Eigenvalues	2.7	1.9	1.4
Loadings of the variables			
Neoxanthin (%)	0.15	0.67	0.02
Sum of carotenoids ($\mu\text{g berry}^{-1}$)	0.46	0.09	−0.12
Lutein: β -carotene	0.18	0.65	0.04
NH15–25	0.53	−0.20	0.23
NH25–35	−0.13	−0.02	−0.79
NH35	−0.41	0.05	0.54
ST:SPAR	0.51	−0.26	0.09

4. Discussion

The two seasons of the study were different from a weather point of view. In fact, 2012 was generally warmer and drier than 2013 (Table 1). The conditions of 2013 enhanced vine vigor avoided summer water stress and delayed the timing of all phenological phases. Nevertheless, dry and warm condition from mid-September to mid-October prolonged vine metabolic activity, and allowed it to reach an optimal level of berry ripening.

The thermal and radiative microclimate indices allowed separating south facing vineyards from the west facing ones, but neither the sampling dates, nor the vigor levels (Figure 1). In general, the southern vineyards were the hottest even if, as evidenced by the significant interaction year*treatment for SD $\geq$ 35 and NH15–25, the differences between the south and west vineyards were amplified in the cooler and wetter season (2013) whereas they were mitigated in the warmer season (2012). ST:SPAR was smaller in the southern than in the western vineyards in both years. This index is an expression of two synergistic effects. Firstly, in hilly conditions such as those of the study, the southern aspect intercepted higher amount of solar radiation (higher values of SPAR). Secondly, the west side of the hill registered the maximum daily temperature (thus, the high value of ST) when, in the afternoon, the Sun's rays were perpendicular to the slope. This index showed a good potential in characterizing the microclimates and it contributed to their separation when inserted into the dataset analyzed by PCA.

The PCA analysis conducted on both microclimatic indices and carotenoid compounds, effectively separated the west facing vineyards from the south facing ones and, in the latter case, also the two levels of vigor (Figure 5). The vineyards separation along Prin1 was determined by the sum of the carotenoids and by both the microclimatic indices NH15–25 and ST:SPAR, whose values were greater in the west facing plots. All these variables were positively correlated with Prin1 (Table 5), thus, the overall amount of the carotenoids was favored by cooler conditions. The differences between the vintages and between the levels of vigor were evident in the warmer vineyards where the intercepted radiation was greater (South) but not in the fresher ones (West) whose conditions were evidently less favorable for the degradation of carotenoids (Figure 5). This observation led to think that the negative effect of higher temperatures prevails over the positive effect of a higher interception of radiation on grape carotenoid concentration at harvest. In the first part of this study [31], higher amount of C13 norisoprenoids was found in the sunniest year and in the more sunlit vineyards. The decrease in the concentration of those compounds in the warmer SouthV– plot in the last ripening stage was likely attributable to the effect of high temperatures. This decrease was more evident for some compounds and less for others,

thus microclimate had an effect on both concentration and profile of C13-norisoprenoids in Nebbiolo grapes [31] confirming, at least in part, results already issued [14].

The higher carotenoid content found in Nebbiolo grapes when compared to other varieties [35–38], agrees with the levels found so far for cv. Nebbiolo [28]. A peak of lutein concentration, that was proportional to the vine vigor, was noticed after veraison in both seasons. A similar peak was reported for Touriga Franca [21], Nebbiolo, and Barbera [28] and, more recently, for Merlot [36]. Therefore, it is possible that cultivar differences exist in the timing of lutein synthesis and degradation thus, a probable delay in VvCCD1 expression can be hypothesized for these varieties [8]. In the present study, the lutein peak after veraison was more evident in the most vigorous plants. This result may be attributable to the high vigor which often reflects a greater synthesis of chlorophyll so, probably, of carotenoids and/or to the lower degradation of this compound under the cooler conditions of these plants. Furthermore, a higher content of lutein-5,6-epoxide (not quantified in this study) in shaded berries prior the veraison can be presumed; the accumulation of this compound has been shown to be a plant early response to shade conditions [8,15]. Therefore, a higher transformation of this compound in lutein may occur after veraison. In both years, the peak of lutein after veraison was less evident in SouthV–; high temperatures and a higher exposure to sunlight probably promoted the carotenoid degradation in post veraison, as proposed in literature [39]. Lutein is also reported to be more efficient than violaxanthin in preventing ROS formation, thus, it could be further used by grapevines for photoprotection under stress conditions [40]. This could also explain the lower lutein concentration and its higher rate of degradation under more stressing condition, such as in south facing vineyard and in the warmer periods of the season, where high radiation and temperature ($>35^{\circ}\text{C}$) were achieved for many hours a day in both years [31]. As regards the other treatments, the west-exposed and the more vigorous plots maintained the highest amounts at harvest. Our findings agree with previous research reporting that grapes grown in shaded conditions [21,41] had higher carotenoid levels. On the contrary, under high UV-B levels, lower concentrations of total carotenoids were found [27].

In 2012, constant levels for β -carotene were observed during ripening in all experimental plots when results were expressed in $\mu\text{g berry}^{-1}$, while a significantly lower amount was registered in SouthV– in both years at harvest. In 2013, instead, higher contents of β -carotene than in 2012 were highlighted in green berries (three weeks before veraison) and for the south facing vineyards a notable decrease was registered thereafter during veraison. The decline of β -carotene during that period was already observed for Nebbiolo and Barbera grapes and was attributed to the high temperature [28]. According to other studies, instead, β -carotene content in grapes, shows an increase from preveraison to veraison, and a decline thereafter until harvest [23,25,36]. In our study, the condition of west facing vineyards, less sunlit and lower temperature than in south ones, likely led to a lower degradation of this compound allowing the maintenance of a constant content ($\mu\text{g berry}^{-1}$) in both years. Other research reported also a greater impact of microclimatic variations on lutein more than on β -carotene [42]. Our results showed a different impact of microclimate on β -carotene if compared to the two xanthophylls since high temperatures of south vineyards in the second year led to a higher degradation of β -carotene during veraison more than at the final stages of ripening.

The warmer conditions registered 4 weeks after veraison in the second year of the study, probably penalized also the concentration ($\mu\text{g kg}^{-1}$) and content ($\mu\text{g berry}^{-1}$) of the neoxanthin that resulted particularly lower at the supposed peak moment, in the south-facing plots. The behavior of this compound was similar to lutein.

The lutein: β -carotene ratio is an indicator of flux to the a- and b-branches, respectively, of the carotenoid metabolic pathway [15]. In literature is reported an influence of the variety on this ratio; in some cultivars indeed (i.e., Syrah, Sauvignon, Pinot Noir, and Merlot), the lutein level was almost twice than that of β -carotene. In Chardonnay and Carignan, the concentration of the two carotenoids was very similar while a higher level of β -carotene was found in Grenache, Gamay, and Sauvignon blanc [15,25,26,38]. In addition, the growing region and topographic features of the site [34,38], as well as cultural practices, such as leaf removal [15], may also affect this ratio. The lutein: β -carotene ratio in

Nebbiolo grapes, varied during berry development also depending on season and vineyard aspect (Table 4). Lutein prevailed on β -carotene after veraison. However, this did not happen, neither in the early stages nor at harvest, nevertheless, in both years, the ratio in the later phases was lower in the warmest nor more exposed plot in accordance with previous research [15,19].

Recent research on the effect of bunch zone leaf removal on Sauvignon blanc [15] concerned mostly the light effect since irrelevant temperature differences within the bunches were registered. Under these conditions, the concentration of the major carotenoids decreased during berry development, following the behavior of chlorophylls, whereas specific xanthophylls, such as lutein, resulted more abundant during the early stages of berry development in berries more exposed to sunlight. In addition, as already reported, the intensity of solar radiation exerts a great role on the degradation of grape carotenoids [26]. Nevertheless, another study highlighted that the carotenoid concentration from veraison to harvest was positively correlated with temperature but less correlated with both rainfall and radiation [19]. According to our study, individual carotenoids respond in a different way depending on the microclimatic conditions of each specific period during ripening. Nevertheless, differently from the previous studies, a major synthesis of lutein occurred after veraison in the vigorous plots where both radiation and temperature were lower whereas in the warmer and more exposed plots (SouthV–), lutein accumulation and final content were penalized (Figure 2). β -carotene content was lower in the warmer period and in the more sunlit south plots with respect to the cooler and more shaded west plots, as well. Actually, the differences emerged between the growing seasons and between vineyard aspects highlighted the influence of the temperature on the rate of synthesis and/or degradation of β -carotene, (Figure 3, Table 4). In any case, the positive effect of radiation did not clearly emerge in our study since the highest peaks were recorded in the less sunlit vineyards (Figure 2).

According to the literature, the amount of berry carotenoids at harvest seemed to be more dependent on the condition of the earlier developmental stage that impact on their synthesis, rather than on the condition of the final phases that impacts their degradation [26]. As a result of the current study, the lower temperature of the early phases of 2013, favored the amounts of carotenoids in the green berries, even in conditions of lower radiation [31]. The particularly warmer condition of the period after veraison in the less vigorous south plots, promoted a higher degradation even with different rate depending on the compound. Nevertheless, a higher total content ($\mu\text{g berry}^{-1}$) of carotenoids was averagely measured at harvest in 2013 than in 2012, confirming the importance of the accumulation phase on the final content at harvest [26].

5. Conclusions

This study confirms most results of previous studies and illustrates the effect of vineyard aspect and bunch microclimate on both synthesis and degradation of the most relevant grape carotenoids during berry development in Nebbiolo grapes. Normalized microclimate indices appeared useful to characterize growing seasons and the microenvironments and to explain the compositional differences between the examined environments.

Lutein and neoxanthin responded in a similar way to environment variability having a similar peak after veraison in both years, whereas a variety effect can be presumed as regards lutein trend during ripening. A different trend was observed for β -carotene content depending on season and vineyard aspect. Generally, the warmer conditions of the most sunlit south facing vineyards led to low amounts of all compounds at harvest. On the other hand, less warm conditions, like those of west-exposed vineyards or more vigorous vines, likely favored the synthesis and/or induced a lower degradation of carotenoid compounds. Nevertheless, carotenoids seemed to respond to microclimate variability differently depending on the compound. In addition, the amount of radiation accumulated in specific periods, and mainly the prolonged high temperatures during the last stages of grape ripening, determined the evolution of carotenoids during season and their profile and quantity at harvest. Furthermore, a repeatability of these results can be expected since the relationships observed

among the environments were maintained in both years and despite the overall differences between them from a meteorological point of view.

Our results highlight also that in earlier vintages, driven by the climate warming, the grapes ripen in a warmer period when high temperature determine a higher degradation influencing considerably both berry carotenoid profile and concentration. Nowadays, winegrowers are called to face such warmer climatic conditions, therefore, our study add further knowledge to target vineyard cultural strategies in order to modulate aroma potential of grapes.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2076-3417/10/11/3846/s1>, Table S1: Supplementary Table S1. Results of the 3-way ANOVA, carried out on the carotenoid content ($\mu\text{g berry}^{-1}$) and concentration (mg kg^{-1}) of the South facing vineyard considering the two levels of vigour (V+ and V-), the two seasons and the four sampling dates as factors of variability.

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