Grape aroma precursors in cv. Nebbiolo as affected by vine microclimate

GRAPE AROMA PRECURSORS IN CV. NEBBIOLO AS AFFECTED BY VINE MICROCLIMATE[1]

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Abstract

The influence exerted by bunch microclimate on some C_{13}-norisoprenoid precursors content was investigated for the first time in Nebbiolo grapes during ripening. Samples were collected, during two consecutive seasons, from two vineyards, which are characterized by different microclimatic conditions caused by vine vigour heterogeneity and different vineyard aspects. Enzymatic hydrolysis of the glycosides extracted from the grapes, and subsequent GC-MS determination of the aglycones, highlighted that the majority of norisoprenoid glycosides accumulated in Nebbiolo berries from pre-veraison until 3-4 weeks post-veraison. Vineyard aspect and vine vigour affected the timing of the maximum concentration of norisoprenoid precursors and their subsequent decrease at harvest. Low light in the vigorous blocks penalized norisoprenoids peak concentration. In the south less vigorous blocks, a decline of total norisoprenoids content during the pre-harvest period was observed. This decline appeared mainly regulated by the temperature. Vintage and/or microclimatic conditions affected the final content of some important norisoprenoids.

Keywords: *Vitis vinifera*; Nebbiolo; C_{13}-norisoprenoids; Ripening; Vineyard aspect; Vine vigour

1. INTRODUCTION

C_{13}-norisoprenoids represent key odoriferous compounds in wine due to their low olfactory threshold values (Etievant, 1991; Rapp, 1998), most of which are characterized by floral and fruity pleasant notes. In grapes, these compounds are formed by the enzymatic or photochemical breakdown of carotenoids (Isoe, Hyeon, Katsumura, & Sakan, 1972; Isoe, Katsumura, & Sakan, 1973; Lutz, & Winterhalter, 1992). During the former process, the hydrolysis by regiospecific dioxygenases and the eventual glycosylation of the reaction products are followed by acid-catalysed transformations of glycosides (Baumes, Wirth, Bureau, Gunata, & Razungles, 2002). The latter process, i.e., the degradation of carotenoids, occurs via photochemical reactions and involves the direct non-specific breakdown of carotenoids by light followed by a further degradation of apocarotenoids, which may be acid-catalysed or triggered by light, heat or oxygen (Mathieu, Terrier, Procureur, Bigey, & Günata, 2005).

The above-mentioned transformations in grapes result in a free fraction and a bound fraction of norisoprenoids. Those precursors that are in the non-glycosylated fraction, constitute the free C_{13} varietal aromas in grapes and future wine, whereas the glycoconjugated fraction (bound fraction) is stored and enzymatic and/or acid hydrolysis during crashing, fermentation, and bottle-ageing may result in cleavage of the bound sugar moiety releasing the free norisoprenoid aglycon (Mendes Pinto, 2009).

Both red and white simple-flavoured varieties including, Chardonnay, Semillon, Sauvignon Blanc, Cabernet Sauvignon and Syrah, are known to contain significant levels of bound norisoprenoids (Abbott, Coombe, & Williams, 1991; Marais, Van Wyk, & Rapp, 1992; Sefton, Francis, & Williams, 1993), while trace levels of free C_{13}-norisoprenoids are detectable in the grape

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juice. The majority of C_{13}-norisoprenoids in wine appear to derive from precursors, including non-volatile C_{13}-norisoprenoid glycosides, derived in turn from carotenoid cleavage, which can be released during winemaking or storage (Mathieu et al., 2005).

On the other hand, grape-derived aroma is extremely variable and complex; specific chemical compounds which define aroma vary depending on grape metabolism, and hence, on the grape variety and even clones, as well as the soil, climate, vineyard management and microclimate (Deluc, Quilici, Decendit, Grimplet, Wheatley, Schlauch, & Cramer, 2009; Ji & Dami, 2008; Kwasniewski, Vanden Heuvel, Pan, & Sacks, 2010; Lee, Seo, Riu, Cotta, Block, Dokoozlian, & Ebeler, 2007). Meteorological variables, in turn, play a key role in vine vegetative and productive responses and in grape quality, directly affecting the biosynthesis of primary and secondary metabolites and their accumulation in the berry (Cortell, Halbleib, Gallagher, Righetti, & Kennedy, 2007; Matus Loyola, Vega, Peña-Neira, Bordeu, Arce-Johnson, & Alcalde 2009; Tarara, Lee, Spayd, & Scagel, 2008). In particular, most aroma precursor synthesis seem to occur in warmer years and under more sun-exposed grapes, (Hernadez-Orte, Concejero, Astrain, Lacau, Cacho & Ferreira, 2015). Specifically, bunch exposure to sunlight is one of the parameters which predominantly influences norisoprenoid levels in grapes (Gerdes, Winterhalter, & Ebeler, 2002; Marais, Van Wyk, et al., 1992). According to literature in this regard, heterogeneity of the vine vigour and different aspect may lead to great variability in the microclimatic conditions of the bunch zone, such as light intensity and temperature. Actually, bunch sunlight exposure was found to increase grape norisoprenoids concentration (Lee et al., 2007; Razungles, Bayonove, Cordonnier, & Sapis, 1988), while bunch shading resulted in a decrease of glycoconjugate norisoprenoids (Bureau, Baumes, & Razungles, 2000). On the other hand, some research has highlighted that extreme heat and sunlight stress may cause a reduction of aroma precursors in grapes (terpenes, norisoprenoids, methoxypyrazines) (Marais, Calitz, & Haasbroek, 2001), as well as a negative effect on the glycosylated aromatic composition of grapes (Scafidi, Pisciotta, Patti, Tamborra, Di Lorenzo, & Barbagallo, 2013).

With regards to the evolution of norisoprenoids, an increase of C_{13}-norisoprenoids from carotenoids occurs during the ripening phase (Baumes et al., 2002; Mathieu et al., 2005). Among the variables that may promote the accumulation of norisoprenoids, such as light, temperature or soil water conditions, some favour carotenoid synthesis while others stimulate their degradation to norisoprenoids (Ryona & Sacks, 2013). The first effect mainly occurs during the herbaceous phase of berry growth, while the second occurs from veraison onwards (Razungles et al., 1988). Furthermore, the formation of norisoprenoid glycosides seems to be influenced by interconversions between the different carotenoids from which they derive (i.e. those linked with the xanthophylls cycle), which are in turn affected by modifying light conditions (Baumes et al., 2002; Mathieu et al., 2005; Ryona & Sacks, 2013).

Knowledge of the factors that influence the synthesis and degradation of secondary metabolites, including norisoprenoid flavour compounds, is essential in order to optimize the levels of aroma-relevant compounds in grapes; a balanced sugar/acidity ratio and colour optimum should be accompanied by the maximum amount of desired volatile components in order to obtain a premium wine (Coelho, Rocha, Delgadillo, & Coimbra, 2006). Thus, determination of the grape volatile composition, when its maximum potential is achieved, and how vineyard characteristics and management may affect them, could provide a useful tool for an estimation of wine aroma potential.

In particular, the contribution of norisoprenoid precursors to grape aroma could be important especially for red non-floral varieties such as Nebbiolo, destined to produce premium wines, such as Barolo and Barbaresco in Piedmont (north-west Italy), appreciated globally as aged wines. These wines require many years to reach a balance between the aromatic complexity and pronounced astringency and acidity. Nebbiolo is a very demanding cultivar in terms of soil and microclimate. To achieve the best grape-ripening conditions, to obtain wines with high alcohol content and colour intensity as well as a strong balance between tannins and acid, Nebbiolo vineyards require the best locations, usually facing south, and well-drained soils (Daniels, 2013). Nowadays, few studies regarding the characterization of the volatile compounds of Nebbiolo are carried out, and most of them are concerned with the organoleptic profile of wines (Gerbi, Zeppa, Minati, & Minetti, 1993). Grape aroma composition has not been extensively studied (Di Stefano, Bottero, Pigella, Borsa, Bezzo, & Corino, 1998), and only one recent study has investigated the

free varietal and pre-fermentative volatile accumulation in Nebbiolo grapes (Ferrandino, Carlomagno, Baldassarre, & Schubert, 2012). There is also little information regarding the glycosylated norisoprenoid precursors, or norisoprenoids released by chemical hydrolysis of Nebbiolo grapes at maturity (Di Stefano et al., 1998), but no information is available regarding their evolution during berry development. The impact of the vineyard's microclimatic characteristics on Nebbiolo grape development, ripening and colour quality, has been already investigated (Guidoni, Cavalletto, Bartolomei, Mania, & Gangemi, 2011; Guidoni, Ferrandino, & Novello, 2008); nevertheless, knowledge regarding the impact on the evolution of grape aroma precursors is still incomplete.

This research aimed to assess both the profile and content of norisoprenoid precursors in cv. Nebbiolo grapes during ripening, as well as the effect of vine vigour and vineyard aspects on their accumulation. To our knowledge, it is also the first time that bunch microclimate assessment has been linked to the evolution of aroma precursors during ripening. In order to achieve these aims solid phase extraction (SPE) and subsequent enzymatic hydrolysis were used, respectively, to isolate and release the glycosylated grape norisoprenoids prior to their determination via gas-chromatography mass spectrometry (GC-MS).

2. MATERIALS AND METHODS

2.1 Vineyard site and treatments

Data presented in this article concern the 2012 and 2013 growing seasons; grape samples of cv Nebbiolo (CVT 141/ 420A), were collected from 2 nearby commercial vineyards located in Sinio, Piedmont (44°36'04" N, 8°00'34" E; north-west Italy). Vine density was 5200 vine/ha (0.80 m x 2.40 m). Vines were vertical shoot positioned (VSP) and trained using the Guyot pruning system (10 bud cane plus 2 bud spur). The two vineyards, were homogeneous for average altitude (415-428 m a.s.l.) and slope (20 %) but the first vineyard was south-facing (EW row orientation) and the second was west-facing (NS row orientation). In addition, a vigour heterogeneity was evident in both vineyards. Blocks of different vigour were identified and sorted in each vineyard first on the basis of spring visual evaluation and then confirmed, at veraison time, by the Normalized difference vegetation index (NDVI), as already described (Guidoni, Asproudi, Mania, Cavaletto, Gangemi, Matese, Primicerio, Borsa, 2013). In 2012, two blocks were chosen in the south-facing vineyard (named SouthV–, V+) and one, more vigorous, in the vineyard facing West (WestV+). In 2013, a fourth block was identified (WestV–) in the west-exposed vineyard. Three replicates of 50 vines for each block were randomly identified to assess vine vigour and yield, and berry quality parameters.

2.2 Micrometeorological assessments

Photosynthetically Active Radiation (PAR) and air temperature were measured inside the foliage at the bunch zone level, from June 30 to October 12 of both years, equipping the vineyards according to an established protocol (Guidoni et al., 2013). The whole period was divided into three sub-periods, which were representative of the phenological phases more relevant for grape quality (pre-veraison, veraison and ripening). Furthermore, two indices were defined and calculated in each sub-period: 1) the Thermal Index (TI) was the number of hours of fruit exposure to different classes of temperature and 2) the Total PAR index (TPARI) was the integral of PAR radiation (mol m^{-2} on the number of days of each sub-period) reaching the fruit zone. As regards to TI, three classes of temperature were established: 1) below 25 °C, 2) between 25 °C and 35 °C, and 3) above 35 °C. The thresholds of 25 °C and 35 °C were chosen on the basis of vine physiology: temperatures below 25 °C may limit metabolic processes, such as photosynthesis, whereas temperatures above 35 °C may slow down some biosynthetic pathways and trigger degradation of many compounds, such as anthocyanins (Yamane, Jeong, Goto-Yamamoto, Koshita, & Kobayashi, 2006). Both indices were used to characterize, from a thermal and radiation point of view, the two seasons (2012 and 2013), aspects (south and west) and classes of vigour (V+ and V−). TPARI, assessed within the vine canopy, is also an indirect method of evaluating vine vigour: the higher its value, the less vigorous the parcels were. Conversely, TI is an indicator of the heat accumulation: the block with the maximum length of exposure to temperatures over 35 °C is the

hottest; conversely, the block with the maximum length of exposure to temperatures under 25 °C is the coldest.

2.3 Berry sampling and agronomical assessment

During the growing seasons, within each block, berries with their pedicels were collected at regular intervals of 15 to 20 days, starting 15 days after flowering until commercial maturity. Samples of 500 berries were randomly collected from different sides of the bunches, both shady and sunlit. The main analytical parameters of the must were assessed as follows: 50 or 100 berries, depending on berry size, were crushed to determine total soluble solids (expressed as degree Brix) using a PR-10 electronic refractometer (Atago, Tokyo, Japan); titratable acidity (TA, expressed as g/L of tartaric acid) was determined using the UE official method (*Commission Regulation (EEC) No. 2676/90).* At harvest, yield parameters were assessed, such as cluster number per vine, cluster weight and yield per vine; at pruning time the weight of the pruning wood was measured as an index of vine vigour.

2.4 Extraction and determination of aroma precursors from grapes

2.4.1 Extraction of glycosylated norisoprenoids from grapes: Previous research experience using non-floral varieties, including Nebbiolo, showed that the quantity of 100 berries is adequate to obtain detectable results, using a GC-MS method, regarding the determination of glycosylated precursors (Di Stefano et al., 1998). Thus, 100 berries of Nebbiolo, previously weighted, free of seeds and added of 100 mg of $Na_2S_2O_5$, were homogenized for 2 min using a Waring laboratory blender (Torringhton, USA). The suspension was then centrifuged (4000 g x 15 min) and the supernatant transferred to a 300 mL flask, washed and brought up to volume with tartaric acid buffer (pH=3.0, 0.04 M). Three replicates of all samples were analysed.

2.4.2 Glycoside isolation: The isolation of grape heterosides was performed as previously reported for a grape and wine matrix (Mateo, Gentilini, Huerta, Jiménez, & Di Stefano, 1997), after appropriate modifications for better extraction of target compounds from Nebbiolo grape extracts. Briefly, 250 mL of extract was passed through a 5 g C18-RP cartridge (Biotage AB, Uppsala, Sweden) previously activated with 20 mL of methanol and 50 mL of distilled water in sequence. After washing the cartridge with 50 mL of water and 30 mL of dichloromethane the glycosides were recovered with 25 mL of methanol (Sigma Aldrich Co., St. Louis, MO, USA).

2.4.3 Hydrolysis of glycosides by exogenous enzyme: The methanolic phase was evaporated to dryness under vacuum and the residue dissolved in 5 mL of citrate-phosphate buffer (pH 5.0) (51.5 %v/v of 0.2M sodium phosphate and 48.5 %v/v of 0.1M citric acid). 100 mg of polyvinylpolypyrrolidone (PVPP) was added and then the enzymatic hydrolysis was carried out with 0.2 mL of Pectinol (Genencor, Palo Alto , CA, USA) with glycosidase side activities at 40 °C for 24 h. After hydrolysis, 0.1 mL of ethyl-4-acetyl benzoate (100 mg/L), used as internal standard, was added and the hydrolyzed extract passed through a 1 g C18-RP cartridge previously activated with 5mL of methanol and 10mL of distilled water in sequence, to isolate the aglycons. After washing with 10 mL of water, the free compounds released by the action of the enzyme were eluted with 12 mL of dichloromethane. The organic layer was dried using anhydrous Na_2SO_4, transferred into a distillation flask and reduced to a small volume at room temperature. The analysis of the aglycons was carried out by GC-MS.

2.4.4 Gas chromatography-mass spectrometry (GC-MS) determinations: GC-MS analysis was carried out by an Agilent 7890A gas chromatograph, equipped with an Agilent 5975C Mass Selective Detector. The samples (2 µL of extract) were manually injected into the injector which was set at 250 °C and in splitless mode. The separation was achieved using a HP-INNOWAX, polyethylene glycol, 30 m x 0.25 mm x 0.25 µm (J&W Scientific, Folsom, CA, USA). The oven temperature was held at 45 °C for 2 min, then raised to 60 °C at a rate of 30 °C min^{-1}, from 60 to 230 °C at a rate of 2 °C min^{-1}, and held at 230 °C for 20 min. The carrier gas was helium with a constant flow of 1 mL/min. The transfer line was set at 230 °C. The ionization voltage was 70 eV, the quadrupole was set at 230 °C and the source at 250 °C. The acquisition of mass spectra for the analysis of compounds was carried out in total ion current mode (TIC) and a 29-300 m/z range

was recorded. Identification of volatile compounds was performed by comparing recorded mass spectra with those of the NBS75K and WILEY275 database and retention index with those of authentic standards, if available, or by comparison of the gas chromatographic retention index LRI (Bianchi, Careri, Mangia, & Musci, 2007) and mass spectrometric data with those reported in the literature (Table 1).

The quantification was carried out by comparing the areas of the chromatographic peaks with that corresponding to the internal standard thus, compound concentrations were calculated as equivalent of ethyl-4-acetylbenzoate. The results were expressed both as μg/kg of berries, frequently reported in the literature, and as μg/100 of berries to prevent the influence of the different berry size on the result interpretation.

Table 1. Volatile compounds identified by GC-MS (mass spectrum and LRI) after enzymatic hydrolysis of precursors from Nebbiolo grape extracts.

Compound	R.T. (min)	Calculated (LRI)[1]
3,4-dihydro-3-oxoactinidol (isomer II)	65.10	**2434**
3,4-dihydro-3-oxoactinidol (isomer III)	65.48	**2437**
3-hydroxy-β-damascone	68.05	**2510**
Unknown norisoprenoid[2]	69.49	**2559**
3-oxo-α-ionol	70.97	**2610**
4-oxo-β-ionol	71.23	**2613**
3,9-dihydroxy megastigma-5-ene	71.65	**2628**
4-oxo-7,8- dihydro-β-ionol	72.19	**2648**
Blumenol C	73.06	**2681**
trans-5,6-epoxy-β-ionone	73.70	**2699**
3-hydroxy-7,8-dehydro-β-ionol	74.97	**2749**
Vomifoliol	86.00	**3178**

[1]Linear Retention Index Calculated using an Innowax 30 m x 0.25 mm x 0.25 μm (J&W Scientific, Folsom, CA, USA).
[2]Mass spectrum of unknow norisoprenoid: MS (EI): m/z%: 43 (100), 55 (25), 69 (29), 81 (25), 85(32), 97(45), 109 (25), 112 (25), 123 (29), 136 (21), 138 (21), 151(5), 161(8), 179 (10), 194 (6).

2.5 Statistical analyses

The mean of three biological replicates and their standard errors are reported. With regards to chemical compounds, the analysis of variance (ANOVA) and Tukey's test at 5% significance ($P< 0.05$) were used to establish significant differences among blocks and sampling dates. All analyses were performed using SPSS software (SPSS Inc. Released 2006. SPSS for Windows, Version 15.0. Chicago, SPSS Inc.). In order to obtain more information regarding the influence of microclimatic variables on grape composition, a Principal Component Analysis (PCA) was also performed. Five variables related to the grape composition and two related to the temperature conditions explained an adequate level of model variance. For variables related to grape composition (soluble solid content and four norisoprenoids), new variables were calculated as the concentration difference between two subsequent samplings. TI>25°C and TI≥35°C values were calculated for the 27 days preceding the first sampling and for the periods between two subsequent samplings. The data submitted to the analysis were related to the first, third and fifth sampling dates of both years. PCA was performed using SAS 9.4 for Windows (SAS Institute, Cary, USA).

3. RESULTS

3.1 Bunch microclimate and agronomical assessment

In all sub-periods of both seasons, the TPARI value was the highest in the SouthV– block and, on average, its value was higher in the south, rather than the west, exposed vineyards. In addition, in 2012, the index was higher than in 2013, especially for the South blocks (Table 2).

Considering the average TI values over the two ripening seasons; in the south aspect, the percentage of hours below 25 °C was of 68.2 %, while 23.5 % was between 25 and 35 °C and 8.3% above 35 °C. The corresponding percentages in the west aspect were 72.1 %, 24.3 % and 3.6%, respectively; thus demonstrating that the south aspect was always the hottest. In fact, the major difference between the south and west aspects was evident in relation to the class of higher temperatures in 2013 when in the South 178 hours more than in the West were counted. With regards to the intermediate class of temperature, the ratio between the south and the west in 2013 was contrary to 2012; nevertheless, considering the number of hours over 25 °C, the south aspect proved to be the hottest. No other relevant differences between the ripening seasons were evident (Table 2).

Table 2. Integral of PAR (TPARI, above) and number of hours of fruit exposure to different thresholds of temperature (TI, below) depending on sub-period, season, vineyard aspect and class of vigour.

TPARI [mol m^{-2}]												
	Aspect	Class of vigour	2012					2013				
			periods			total	average	periods			total	average
			30/06 29/07	30/07 28/08	29/08 12/10			30/06 29/07	30/07 28/08	29/08 12/10		
	South	V-	376	531	622	1528	1169	171	178	343	692	586
		V+	190	287	332	809		147	146	187	480	
	West	V-	n.m.	n.m.	n.m.	n.m.	401	n.m.	n.m.	n.m.	n.m.	336
		V+	119	132	151	401		81	126	129	336	
TI [number of hours]												
Class of temperature	Aspect	Class of vigour	2012					2013				
			periods			total	average	periods			total	average
			30/06 29/07	30/07 28/08	29/08 12/10			30/06 29/07	30/07 28/08	29/08 12/10		
T < 25 °C	South	V-	417	373	907	1697	1713	421	438	832	1691	1727
		V+	428	382	919	1729		439	445	879	1763	
	West	V-	436	404	965	1805	1806	440	466	913	1819	1826
		V+	437	403	966	1806		442	468	922	1832	
25 °C ≤ T < 35 °C	South	V-	269	215	147	631	618	246	128	157	531	565
		V+	243	210	152	605		267	176	156	599	
	West	V-	222	218	108	548	582	272	233	161	666	645
		V+	267	238	110	615		267	218	138	623	
T ≥ 35 °C	South	V-	34	132	26	192	189	53	154	91	298	228
		V+	49	128	9	186		14	99	45	158	
	West	V-	62	98	7	167	133	8	21	6	35	50
		V+	16	79	4	99		11	34	20	65	

n.m. = not measured

In this context, some evaluations were possible: *i)* Different levels of fruit zone exposure to direct sunlight (TPARI) was determined by different vine vigour *ii)* The TPARI values showed the SouthV– block exhibited the lowest vigour, confirming the results obtained by weighing pruned wood and NDVI (Table 2 and Supplementary Table 1). In addition, the high values measured in 2012 in both south blocks underlined their lower vigour compared with 2013 and the west block. *iii)* Since the low vigour increased the exposure of the fruit zone to sunlight, higher temperatures at the bunch level were also expected, as occurred, in fact, in the south less vigorous blocks. The

south blocks always registered higher PAR and temperature values compared with the west exposed blocks, according to the data also reported for previous seasons (Guidoni et al., 2013).

As already observed (Guidoni et al*.,* 2013), all phenological stages in the south-exposed vineyards occurred 4 to 5 days earlier compared to the west in both seasons. In the south-exposed vineyards bud burst, bloom and veraison took place on 5 April, 1 June and 3 August in 2012, and on 15 April, 14 June and 14 August in 2013, respectively. Commercial harvest was performed on the same date in all blocks, on 5 October 2012 and 15 October 2013.

In 2012, a significantly lower berry weight was observed at harvest in the south blocks compared with the west-exposed blocks. Similar results were obtained for both cluster weight and yield per vine, with the lowest values found in the SouthV– block. The wood pruning weight was also the lowest in the SouthV–, confirming the visual spring evaluation and the NDVI assessments. In 2013, fewer differences were observed between the two vineyards, whereas an increase of berry size and a decrease of yield per vine were observed with respect to the previous year (Supplementary Table 1).

Supplementary Table 1. Mean values of yield components, vigour indexes, and juice composition at harvest in 2012 and 2013.

			Yield components				Vigour indexes		Juice composition	
Year	Aspect	Vigour	berry weight (g)	cluster weight (g)	cluster number/ vine	yield/vine (kg)	pruning weight (kg/vine)	NDVI	TSS (°Brix)	Titratable acidity (g/L)
2012	South	V −	1.61a[a]	170a[a]	7.1a	1.21a	0.47a	0.56a	25.0b	5.03a
	South	V +	1.64a[a]	245b	8.7a	2.12b	0.75b	0.59ab	24.3ab	5.40ab
	West	V +	1.84b	325c[a]	7.4a[a]	2.39b[a]	0.85b	0.63b	23.5a	6.15b
	Sig.		*	*	n.s	*	**	*	*	*
2013	South	V −	1.81a[b]	209a[b]	5.9a	1.24a	0.48a	−	24.9a	5.59a
	South	V +	1.88a[b]	254a	6.0a	1.54a	0.86b	−	24.7a	5.92ab
	West	V −	1.86a	273a	3.5a[b]	1.01a[b]	0.77b	−	24.7a	6.88b
	West	V +	1.87a	241a[b]	4.3a[b]	1.04a[b]	0.82b	−	24.6a	6.34ab
	Sig.		n.s.	n.s	n.s	n.s.	**	–	ns	*

Different letters indicate significant differences between treatments for each year (* = P ≤ 0.05, ** = P ≤ 0.01) Superscript different letters indicate significant differences between the two years for each treatment (P ≤ 0.05). No superscript letters indicate no significant differences between the two years for each treatment.

3.2 Evolution of glycosylated norisoprenoids

3.2.1. Year: 2012

The effect of both vine vigour and vineyard aspect on the evolution of the hydrolytically released C_{13}-norisoprenoids in Nebbiolo grapes during the 2012 ripening period was investigated (Fig. 1).

The accumulation of norisoprenoid glycosides in the berries started early, well before veraison, and continued intensively until 20-25 days post-veraison (dpV) (27 Aug). A slowdown was then noticed until about 53 dpV (25 Sep) in the south-exposed vineyards followed by an important decrease in the case of the SouthV– block, which led to significant differences at harvest compared with WestV+ block (Table 3). When the norisoprenoids concentration was expressed as μg/kg of berries, an almost plateau phase was observed in the south-exposed blocks from veraison until the pre-harvest date (25 Sep), thus during both the period of berry growth and when berry weight did not increase significantly.

In the WestV+ plot, a significantly delayed but continuous increase of norisoprenoids occurred until harvest; this delay was also due to the higher berry weight noticed for this block in this first year of study). At harvest time, the total norisoprenoids concentration decreased in the south blocks and was significantly lower in the SouthV– plot compared with the two vigorous SouthV+ and WestV+ plots, whose levels were quite similar.

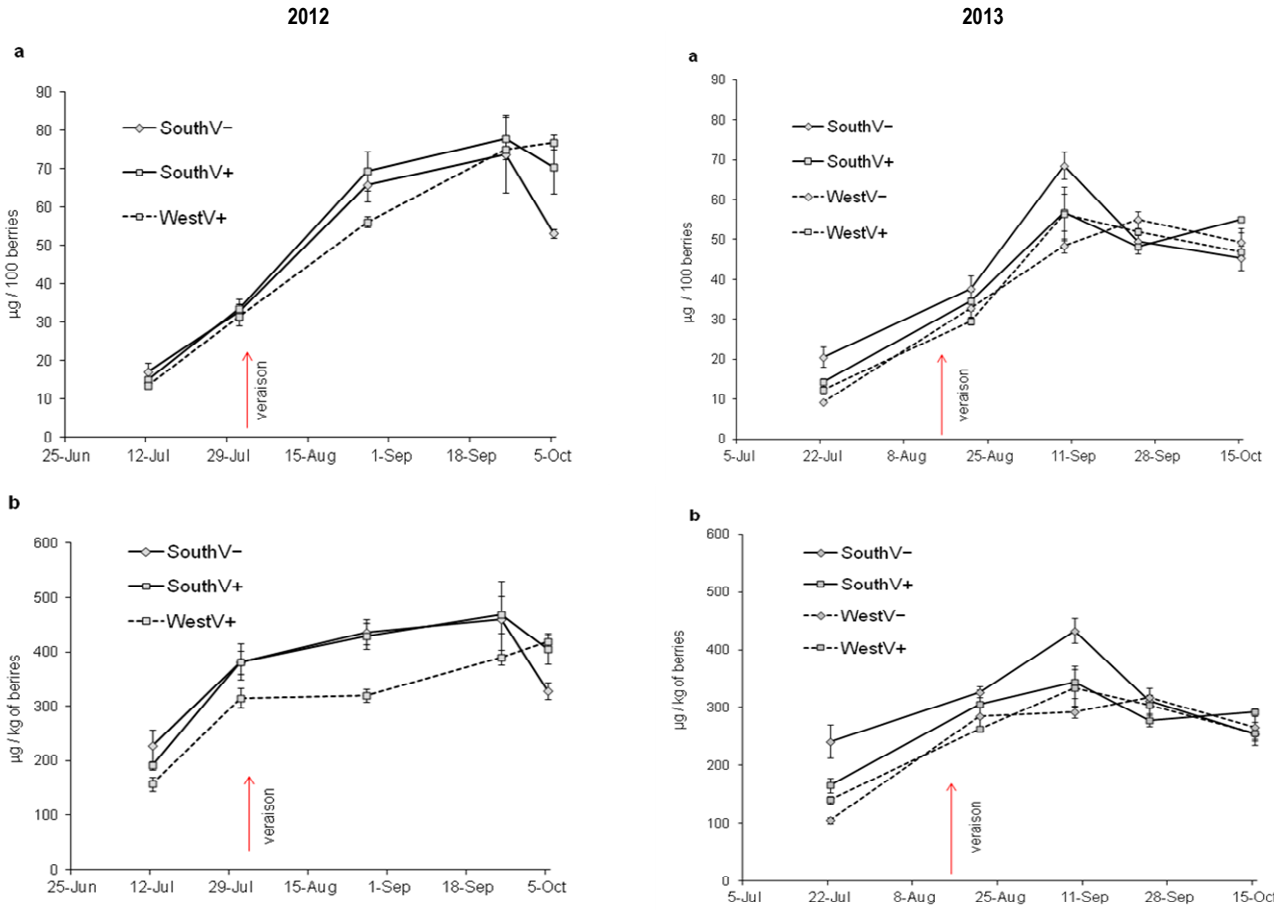

Fig. 1 **Evolution of the total amount of hydrolytically released C_{13}-norisoprenoids as µg/100berries (**a**) and as µg/ kg of berries (**b**) in Nebbiolo grapes, respectively in** 2012 **and in** 2013**, as a function of vine vigour and vineyard aspect, averages ± standard error (n=3).**

The compounds that mostly contributed to the total amount of norisoprenoids in Nebbiolo grapes during the 2012 vintagewere: 3-hydroxy-β-damascone, 3-oxo-α-ionol and the unknown norisoprenoid (tentantively identified as dihydroionone), followed by 3,9-dihydroxymegastigma-5-en, Blumenol C and 3-hydroxy-7,8-dehydro-β-ionol in lower concentration. Vomifoliol values were not included in the total amount of norisoprenoids because of their wide variability between dates and treatments, which has also been observed in previous research (Boido, Lloret, Medina, Fariña, Carrau, Versini, & Dellacassa, 2003; Borsa, 2003 personal communication).

The highest levels of 3-hydroxy-β-damascone were observed at the post-veraison date (25 Sep). The concentrations of Blumenol C and 3,9-dihydroxymegastigma-5-ene, after an increase in the pre-harvest stage, showed a constant trend. The pre-harvest decrease observed for almost all compounds in the SouthV– block grapes, was significant only for 3-oxo-α-ionol and 3-hydroxy-7,8-dehydro-β-ionol (Table 3).

3.2.2 Year: 2013

The effect of vine vigour and vineyard aspect on the evolution of the hydrolytically released C_{13}-norisoprenoids in Nebbiolo grapes during 2013 presented some peculiarity with respect to 2012 (Fig.1). The trend of early accumulation in the SouthV– block grapes was similar to 2012. An earlier norisoprenoid accumulation in the green berries (first sampling), was noticed for the less vigorous SouthV– block with respect to the others. The SouthV– block attained the highest values of total norisoprenoids at 26 days after veraison; a significant decrease of the total concentration was then observed, thus more earlier with respect to the previous year. The most vigorous blocks (SouthV+, WestV–, WestV+) showed similar concentrations during the last month without any significant decrease before harvest (Fig. 1, Table 4).

The differences among blocks, when compared at the same sampling date, were important in the first (22 July) and third (9 Sep) sampling dates, when the SouthV– block presented the highest

values of norisoprenoids with respect to the other three blocks. The differences were mainly due to 3-oxo-a-ionol, the unknown norisoprenoid, 3,9-dihydroxymegastigma-5-ene (22 July), 3-hydroxy-β-damascone and 3-hydroxy-7,8-dehydro-β-ionol (9 Sep), which exhibited significantly different levels among blocks (Table 4). Afterwards, as a consequence of the intense decrease of the total norisoprenoid concentration observed in the SouthV– block, no differences were observed between blocks at harvest; this decline, noticed after 26 dpV, was mostly due to the significant decrease of 3-oxo-α-ionol and 3-hydroxy-7,8-dehydro-β-ionol, as previously observed in 2012 (Table 4).

In 2013, the norisoprenoid compounds with important average contents at harvest were 3-oxo-α-ionol, Blumenol C and 3-hydroxy-β-damascone, followed by 3,9-dihydroxymegastigma-5-ene and the unknown norisoprenoid. The ratio between 3-hydroxy-β-damascone and 3-oxo-α-ionol was inverted with respect to the previous year, which was due to the lower content of 3-hydroxy-β-damascone (Table 4).

3.3 PCA analysis

The first principal component (Prin1) accounted for approximately 64% of the total variance and the second (Prin2) accounted for about 24%, which provided a good summary of the data. Following the eignevalues, Prin1 can be represented as a linear combination of the variables calculated as previously mentioned, and related to SSC (v1), 3,4-dihydro-3-oxoactinidols (v2), 3-oxo-α-ionol (v3), 3-hydroxy-7,8-dehydro-β-ionol (v5) and TI≥35°C (v7). Prin2 can be represented as a combination of the variables TI<25°C (v6) and 3,9-dihydroxy megastigma-5-ene (v4) that showed, respectively, a positive and negative loading on this component. TI≥35°C (v7) might well represent Prin3, however, it accounted for only 6% of the model variance (Supplementary Table 2). Both Prin1 and Prin2 contributed to the dispersion of the observations in the Cartesian plane (Fig. 2). As already observed (Tables 3 and 4), a general positive variation of norisoprenoids was confirmed between the first and third sampling, whereas the opposite trend was evident between the third and fifth sampling (Fig. 2, Tables 3 and 4). The variation of norisoprenoids representing Prin1 exhibited a high positive correlation with TI≥35°C (average R^2 = 0.71). Conversely, a negative correlation (R^2 = -0.50) emerged between TI<25°C and 3,9-dihydroxy megastigma-5-ene, which described the allocation of the observations along Prin2. This highlighted that the variation of the concentration of this latter norisoprenoid was influenced in a different manner by temperature when compared with the other compounds. Moreover, within each sampling, the observations were allocated and ordered consistently with vineyard aspect, vintage and vine vigour (Fig. 2); nevertheless, the relationships between the observations were not constant among samplings. In particular, a higher variation of norisoprenoid concentration was noticed for the south-exposed treatments with respect to the west treatments during ripening. This confirmed that the seasonal variations of the grape aroma composition depend on the microclimate conditions that are, in turn, determined by vineyard aspect and vine vigour.

Supplementary table 2. Eigenvectors of the three principal components (Prin1, Prin2, Prin3) with the loadings indicating the correlation between the variables (v1-v7) and the principal components.

		Prin1	Prin2	Prin3
V1	Soluble solid content	**0.420**	0.323	0.100
V2	3,4-dihydro-3-oxoactinidol (isomer II+III)	**0.456**	0.044	-0.120
V3	3-oxo-a-ionol	**0.448**	-0.004	0.315
V4	3,9-dihydroxy megastigma-5-ene	0.312	**-0.497**	0.432
V5	3-hydroxy-7,8-dehydro-b-ionol	**0.419**	-0.247	-0.037
V6	TI < 25°C	0.008	**0.719**	0.493
V7	TI ≥ 35°C	**0.377**	0.262	**-0.667**

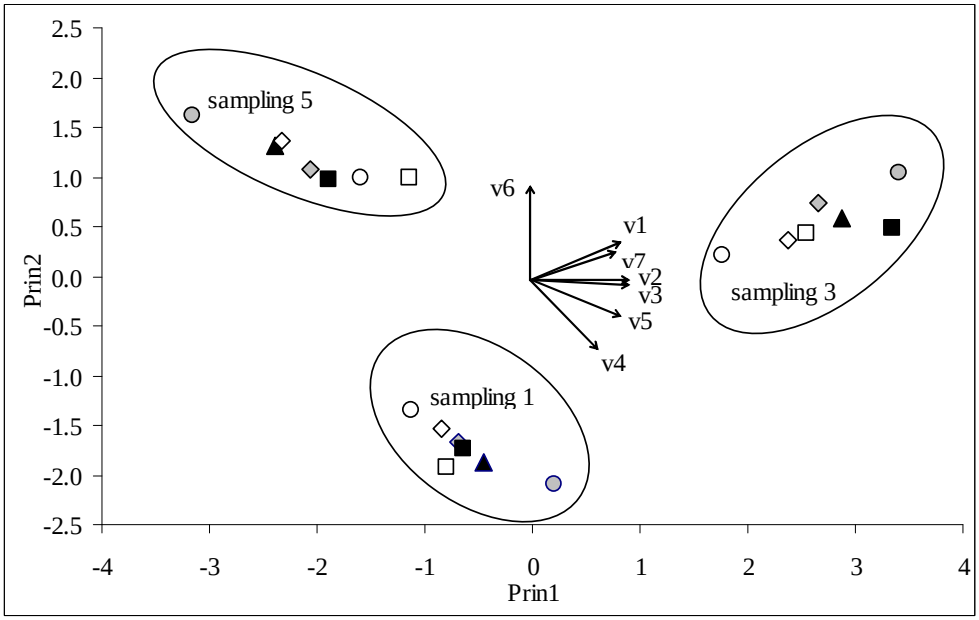

Fig. 2: Principal Component Analysis: x–y plot of the variables used for the analysis (v1 to v7) and distribution of the observations in the Cartesian coordinate system identified by the first two principal components (Prin 1 and Prin 2). ▲ = 2012southV-, ■ = 2012southV+, □ = 2012westV+, ⬤ = 2013southV-, ◆ = 2013southV+, ○ = 2013westV-, ◇ = 2013westV+.

4. DISCUSSION

The 2012 and 2013 seasons differed particularly in monthly pattern of rainfall and temperature. Seasonal average minimum and maximum temperatures were higher in 2012 (8.9 and 20.4 °C, respectively) than in 2013 (8.6 and 19.4 °C, respectively). This trend was confirmed by the annual value of the growing degree days, calculated by summing up the average temperatures exceeding 10 °C, which was more elevated in 2012 (2329 °C) than in 2013 (2129 °C). Conversely, the monthly rainfall was higher in 2013 than in 2012, (722 and 927 mm, respectively) especially in winter and spring; however, in summer, the rainfall was similar between seasons but more regularly distributed in 2013 than in 2012 (data supplied by Piedmont Agrometeorological Network). The 2013 meteorological situation impacted upon vine behaviour by enhancing vine vigor, reducing berry set and yield, avoiding water stress in the summer and delaying the timing of the phenological phases, such as bud burst, bloom, veraison and harvest. Nevertheless, the particularly dry and warm September allowed a level of sugar content to be reached which was comparable to that of the previous year, even if higher acidity was achieved in the west-exposed blocks compared with the south blocks and the previous year (Supplementary Table 2).

The results regarding the trend of glycosylated norisoprenoids, when expressed as μg/kg, confirmed in part the findings of Ryona and Sacks (Ryona & Sacks, 2013), regarding the behaviour of bound C_{13}-norisoprenoid during ripening over two different years, although the authors selected determination of acid hydrolysis instead of the enzymatic hydrolysis of glycosides. According to that study, glycosylated C_{13}-norisoprenoids showed a significant increase starting from 7 dpV (in the first year) or from 21 dpV (in the second), and showed a plateau at 30dpV or at 40-45dpV. Subsequently, a decrease in the first year and a slow continuous increase in the second, occurred until harvest.

Other research indicated that the first step in C_{13}-norisoprenoid glycoside production occurs via the degradation of carotenoids during veraison (Baumes et al., 2002). In this situation, bound forms of C_{13}-norisoprenoid are expected to plateau soon after veraison, once the carotenoid substrate is fully consumed by the carotenoid cleavage dioxygenase (Mathieu et al., 2005). However, other studies suggested that C_{13}-norisoprenoid glycosides increased dramatically after 40 dpV, but no statistical analyses were mentioned (Baumes et al., 2002; Marais, Versini, van Wyk, & Rapp, 1992). The expression of our results, as μg/100 berries, suggested that most of the C_{13}-norisoprenoid glycosides accumulation in Nebbiolo berries occurred early, from the pre-veraison period until 3-4 weeks after veraison, and this was confirmed over two consecutive years. A

subsequent slowdown until harvest was observed for the most vigorous vines, whereas for the less vigorous and warmest block (SouthV–) an important decrease was noticed in both years, earlier in the warmest year (Table 3 and 4). The PCA results (Fig. 2), confirmed that the variations in the concentration of aroma compounds during ripening were associated with the heat accumulation in the fruit zone of each block.

In 2012, total norisoprenoid accumulation occurred without any great differences between SouthV– and SouthV+ blocks, whereas in the WestV+ block, the accumulation after veraison was delayed but continuous until harvest, likely due to both vigour and vineyard aspect. In the most vigorous blocks (SouthV+ and WestV+), the total norisoprenoid levels at harvest were quite similar though their seasonal pattern accumulation showed differences. The important decrease of total norisoprenoids in the case of SouthV– grapes, led to significant differences between blocks at harvest.

In 2013, the SouthV+ and West V+ blocks exhibited similar vigour and were much more vigorous than SouthV–. As a consequence of the more relevant decrease of the total norisoprenoid concentration noticed in SouthV– in the pre-harvest period, no significant differences between blocks were noticed in the second year at harvest. These results indicated that vintage and vigour, more than vineyard aspect, could affect the seasonal accumulation trend and the peak of concentration of norisoprenoids.

The exposure to sunlight had an effect on the accumulation phase of the norisoprenoids (Figure 1, 2) which, in general, was more intense when bunch exposure was more prolonged (such as in 2012 *vs* 2013, in south-exposed vineyard *vs* west-exposed vineyard and in V– *vs* V+). The results obtained in 2013 indicated more clearly that increased light exposure promoted norisoprenoids accumulation until the post-veraison stage in the less vigorous vines, while in the vigorous ones, low light exposure as a consequence of the higher vigour penalized the maximum content of norisoprenoids.

On the other hand, the higher heat experienced by the south blocks may explain the decrease of the norisoprenoid concentration observed in the less vigorous south-exposed vines, during the last phase of ripening; this decrease occurred earlier in 2013, when September's temperatures were very high and consequently higher TI≥35°C were registered, evidencing the role of temperature in reducing norisoprenoid concentration.

According to the literature (Bureau et al., 2000; Morrison & Noble, 1990), glycoconjugates levels of C_{13}-norisoprenoids and changes in berry composition, as a result of leaf and cluster shading, are more closely related to the effect of light than to that of temperature. Increasing sunlight exposure leaded to an increase in norisoprenoid content, even though a different reaction was also highlighted depending on the variety (Bureau et al., 2000). On the other hand extreme heat and direct exposure to sunlight of Sauvignon blanc grapes reduced the content of aroma precursors, including norisoprenoids (Marais et al., 2001; Marais, Hunter, & Haasbroek, 1999). Moreover, in warm environmental conditions, heat and sunlight stress induced by early leaf removal in the cluster zone, negatively affected the grape flavour composition, causing a decrease of glycosylated compounds in the Grillo variety, in Sicily (Scafidi et al., 2013).

It is worth mentioning that, during our study, grapes harvested in the less vigorous and warmest SouthV– block presented more fragile skins as a consequence of the extreme microclimatic conditions, thus indigenous berry enzymes could probably hydrolyze part of the bound norisoprenoids at the harvest period.

Our results also showed significant quantitative differences between the two seasons, if we compare the content of the most important individual compounds, at the same phenological stage (Tables 3 and 4). The concentrations of 3-hydroxy-β-damascone and 3-hydroxy-7,8-dehydro-β-ionol were significantly influenced by the vintage as reduced concentrations were observed in the second year when a lower light intensity at the bunch zone was detected. 3-oxo-α-ionol and 3-hydroxy-7,8-dehydro-β-ionol were the compounds mostly affected by the microclimatic conditions in the near harvest period. This was particularly evident in the south-exposed vineyards where the temperature was higher as also confirmed by the PCA (Fig. 2). According to the literature (Baumes et al., 2002), exposing bunches to sunlight during ripening increases the proportion of certain compounds, such as 3-oxo-α-ionol, which are derived from non-epoxyxanthophylls, while higher proportions of compounds derived from epoxyxanthophylls, such as 3-hydroxy-7,8-dehydro-β-ionol, should be found in bunches ripening in the shade; but no distinction between light and

temperature impact was mentioned. In our case, significantly higher concentrations of 3-oxo-α–ionol were observed only at 21-26 days after veraison (9 Sep) in the SouthV- grapes, which were more exposed to both sunlight and higher temperature.

Although it was not possible to quantify and separate the impact of the high temperature and sunlight on the accumulation and degradation of norisoprenoids, the clear impact of the fruit zone temperature on the evolution of some grape norisoprenoids was confirmed by the PCA (Supplementary table 2, Fig. 2). An effect of the vine vigour was also highlighted but it is uncertain if this is to attribute on the light conditions of the fruit zone or on the size of the leaf surface. According to other research, foliage exposure to sunlight may also regulate fruit composition (e.g. pH) which could impact upon some aroma compounds, while critical environmental and cultural parameters, other than light and temperature, could also determine the amount of aroma precursors, such as norisoprenoids, in grapes (Kwasniewski et al., 2010; Lee et al., 2007).

5. CONCLUSIONS

Enzymatic hydrolysis of the glycosides extracted from grapes and the subsequent GC-MS determination of the aglycones highlighted that the accumulation of C_{13}-norisoprenoid glycosides in Nebbiolo berries starts before veraison and actively continues for 3-4 weeks after veraison. The observed differences in Nebbiolo grapes can be attributed to the microclimate conditions, which varied according to the vintage, vineyard aspect and vine vigour. The results indicate that all these variables may affect the timing of the peak concentration of norisoprenoid precursors and their eventual subsequent decrease prior to harvest. The high vigour, thus the low light intensity in the bunch zone, may have a negative impact on the peak concentration of the aroma precursors and hence, on their final concentration. On the other hand, the final concentration of norisoprenoid precursors is also influenced by the microclimate conditions during the last stages of grape ripening; high temperature, as a consequence of both vigour and aspect, evidently leads to a decrease of the total norisoprenoids.

Some of the most important individual compounds seem to be more affected by seasonal light conditions, while others are negatively influenced by extreme microclimate conditions close to the harvest period. A clear impact and different influence, especially of the temperature, on the accumulation and/or degradation of some norisoprenoids during ripening, was highlighted.

A further investigation to separate the impact of light and temperature on individual norisoprenoid compounds, as well as the contemporary evaluation of both free and glycosylated fractions, could be useful to better evaluate grape aroma potential.

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Table 3: Hydrolytically released C_{13}-norisoprenoids (μg/100 berries) in Nebbiolo berries at comparable phenological stages in 2012.

Sampling date	12-Jul			31-Jul			27-Aug			25-Sep			5-Oct		
Treatment	South V−	South V+	West V+	South V−	South V+	West V+	South V−	South V+	West V+	South V−	South V+	West V+	South V−	South V+	West V+
Phenological phase	22 d**b**V		26 d**b**V	3 d**b**V		7 d**b**V	24 d**p**V		20 d**p**V	53 d**p**V		49 d**p**V	63 d**p**V		59 d**p**V
Mean Berry Weight (g)	0.76a	0.78a	0.90b	0.85a	0.88ab	1.00b	1.50a	1.56ab	1.76b	1.61a	1.68a	1.93b	1.61a	1.64a	1.84b
°Brix	ND	ND	ND	7.2	8.2	5.8	22.0	20.6	18.1	25.0	23.8	23.3	25.0	24.3	23.5
Titratable acidity (g/L)	ND	ND	ND	ND	ND	ND	6.90	7.95	8.40	5.17	5.66	6.30	5.03	5.40	6.15
3,4-dihydro-3-oxoactinidol (isomer II)	0.37^{a}	0.43^{a}	0.28^{a}	1.32b^{ab}	1.3b^{ab}	0.98a^{a}	2.36^{b}	2.63^{b}	2.15^{b}	2.67^{c}	2.78^{b}	2.16^{b}	2.16^{c}	2.49^{b}	2.86^{b}
3,4-dihydro-3-oxoactinidol (isomer III)	0.63^{a}	0.54^{a}	0.50^{a}	1.76^{ab}	1.85^{ab}	1.22^{a}	2.83^{ab}	2.91^{ab}	2.54^{b}	3.84^{b}	3.22^{b}	2.94^{b}	2.63^{ab}	3.09^{b}	2.99^{b}
3-hydroxy-β-damascone	1.55^{a}	1.42^{a}	0.64^{a}	3.49b^{a}	4.39b^{a}	1.51a^{a}	16.1^{b}	17.6^{b}	15.6^{b}	18.2^{b}	18.7^{b}	21.9^{c}	13.1^{b}	17.8^{b}	16.0^{bc}
Unknown norisoprenoid *	ND	ND	ND	0.78^{a}	0.91^{a}	1.35^{a}	10.8^{b}	9.70^{b}	7.36^{b}	12.8^{b}	15.9^{bc}	11.2^{bc}	10.9^{b}	12.7^{c}	12.2^{c}
3-oxo-α-ionol **	1.83^{a}	1.50^{a}	1.90^{a}	6.14a^{b}	5.62a^{ab}	8.88b^{b}	9.59^{b}	11.5^{c}	13.2^{c}	13.7^{c}	13.82^{c}	12.4^{c}	7.12a^{b}	10.2b^{bc}	14.1b^{c}
4-oxo-β-ionol ***	1.78^{a}	2.04^{a}	0.70^{a}	2.28^{ab}	2.59^{a}	1.26^{a}	3.66b^{b}	4.38b^{b}	1.03a^{a}	3.51^{b}	1.19^{a}	1.20^{a}	4.08^{b}	5.55^{b}	6.71^{b}
3,9-dihydroxy megastigma-5-ene	3.06^{a}	2.53^{a}	3.61^{a}	4.56^{ab}	3.94^{ab}	6.05^{b}	5.30^{ab}	4.88^{ab}	5.27^{b}	6.30^{b}	6.31^{b}	5.03	3.47^{ab}	4.11^{ab}	5.14^{b}
4-oxo-7,8- dihydro-β-ionol	1.22	1.06	ND	1.65	1.22	ND	1.13	1.26	ND	ND	ND	ND	1.04	1.03	1.82
Blumenol C	4.29^{a}	3.94^{a}	4.40^{a}	7.51^{b}	7.51^{b}	7.74^{ab}	8.02^{b}	8.82^{b}	11.0 ^{b}	7.50^{b}	8.34^{b}	9.88^{b}	5.84a^{ab}	6.88a^{b}	9.26b^{b}
trans-5,6-epoxy-β-ionone	0.21	0.24	0.26	ND	ND	ND	0.54	0.51	ND	0.99	0.98	0.98	0.46	0.63	ND
3-hydroxy-7,8-dehydro-β-ionol	2.25^{a}	2.12^{a}	1.30^{a}	3.26^{a}	4.05^{ab}	3.41^{b}	6.05^{b}	6.93^{c}	5.41^{c}	6.67^{b}	7.11^{c}	7.47^{c}	4.60a^{a}	6.72b^{bc}	5.87b^{c}
Total	**17.1^{a}**	**15.0^{a}**	**13.6^{a}**	**32.8^{ab}**	**33.4^{b}**	**31.5^{b}**	**65.8^{c}**	**69.5^{b}**	**56.2^{b}**	**74.0^{c}**	**78.0^{b}**	**75.2^{c}**	**53.0a^{bc}**	**70.6ab^{b}**	**77.0b^{c}**
Vomifoliol	13.5^{a}	9.25^{a}	8.24^{a}	24.3^{ab}	27.2^{a}	19.1^{a}	63.9^{c}	66.6^{b}	57.7^{b}	70.5^{c}	68.0^{b}	68.6^{b}	36.9^{b}	56.4^{b}	55.9^{b}

Within the same sampling date averages of each compound were subjected to the analysis of variance and means were separated by the Tuckey test. Means followed by different letter are significantly different for *P≤0,05*. For each treatment superscript letters indicate differences between the sampling dates (*P≤0,05*). d**b**V= days before veraison, d**p**V=days post veraison, ND= Not Detectable. Where no letters are present no significant differences were revealed. *= tentatively identified as diidro-ionone, **,***= compounds at times coeluated with acetovanillone. All norisoprenoid concentrations are expressed as ethyl-4-acetylbenzoate equivalent.

Table 4: Hydrolytically released C_{13}-norisoprenoids (μg/100 berries) in Nebbiolo berries at comparable phenological stages in 2013.

Sampling date	22-Jul				21-Aug				9-Sep				24-Sep				15-Oct			
Treatment	South V–	South V+	West V–	West V+	South V–	South V+	West V–	West V+	South V–	South V+	West V–	West V+	South V–	South V+	West V–	West V+	South V–	South V+	West V–	West V+
Phenological phase	23 d**b**V		27 d**b**V		7 d**p**V		2 d**p**V		26 d**p**V		21 d**p**V		41 d**p**V		36 d**p**V		62 d**p**V		57 d**p**V	
Mean Berry Weight (g)	0.86	0.86	0.88	0.87	1.10	1.17	1.16	1.16	1.60	1.65	1.69	1.66	1.59a	1.73b	1.74b	1.71ab	1.81	1.88	1.86	1.87
°Brix	ND	ND	ND	ND	13.9	13.3	11.2	11.0	21.9	21.4	19.5	19.7	24.8	24.3	23.4	23.4	24.9	24.7	24.7	24.6
Titratable acidity (g/L)	ND	ND	ND	ND	ND	ND	ND	ND	9.03	9.07	11.0	10.2	7.11	7.92	8.81	8.79	5.59	5.92	6.88	6.34
3,4-dihydro-3-oxoactinidol (isomer II)	0.56^{a}	0.25^{a}	ND	0.21^{a}	1.49^{b}	1.43^{b}	1.18	1.13^{b}	2.34^{b}	1.89^{b}	1.25	1.75^{b}	1.42^{b}	1.69^{b}	1.72	1.47^{b}	2.06^{b}	1.65^{b}	1.63	2.30^{b}
3,4-dihydro-3-oxoactinidol (isomer III)	0.87^{a}	0.49^{a}	0.41^{a}	0.51^{a}	1.95^{b}	1.84^{a}	1.72^{b}	1.53^{b}	2.48^{b}	2.10^{b}	1.47^{b}	2.05^{b}	1.56^{b}	1.86^{b}	1.79^{b}	1.66^{b}	1.87^{b}	1.9^{b}	2.00^{b}	1.82^{b}
3-hydroxy-β-damascone	0.78ab^{a}	0.51a^{a}	0.91b^{a}	0.49a^{a}	3.43b^{b}	3.21ab^{a}	2.21ab^{a}	2.17a^{a}	7.99^{b}	10.15^{c}	8.63^{c}	8.29^{b}	6.79a^{b}	7.82ab^{b}	9.09b^{c}	6.94ab^{b}	6.67^{b}	6.61^{b}	6.93^{b}	6.82^{b}
Unknown norisoprenoid *	ND	ND	ND	ND	1.77b^{a}	1.46ab^{a}	0.96a^{a}	1.05a^{a}	7.76c^{b}	5.83bc^{b}	3.78a^{a}	4.59ab^{b}	5.39^{a}	5.27^{b}	4.95^{b}	5.05	6.02bc^{a}	6.76c^{b}	5.22ab^{b}	4.72a^{b}
3-oxo-α-ionol **	3.45c^{a}	2.64bc^{a}	1.29a^{a}	2.09ab^{a}	8.44b^{b}	7.10a^{ab}	6.72a^{b}	6.57a^{ab}	17.1b^{c}	11.0ab^{b}	10.4a^{b}	12.9ab^{b}	13.0b^{bc}	10.1a^{b}	13.1b^{b}	13.73b^{b}	9.85^{b}	13.93^{b}	11.74^{b}	9.8^{b}
4-oxo-β-ionol ***	0.81	0.75	0.31	0.57	1.08	0.76	0.87	0.78	3.93	3.69	2.82	3.76	3.06	3.59	2.91	2.55	2.59	3.88	2.82	3.33
3,9-dihydroxy megastigma-5-ene	5.72b^{a}	3.83a^{a}	2.36a^{a}	3.15a^{a}	6.53^{bc}	6.10^{a}	6.56^{b}	5.22^{ab}	9.89b^{b}	7.55ab^{b}	6.66a^{b}	7.39ab^{b}	6.87ab^{a}	5.92a^{b}	7.69b^{b}	6.68ab^{b}	6.03^{a}	7.14^{b}	7.08^{b}	6.21^{ab}
4-oxo-7,8- dihydro-β-ionol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Blumenol C	6.05b^{a}	4.21ab^{a}	3.12a^{a}	3.86a^{a}	8.17^{b}	8.41^{ab}	8.25^{b}	7.29^{ab}	11.48^{b}	9.31^{b}	8.22^{b}	10.1^{b}	8.94^{ab}	8.43^{b}	9.45^{b}	10.1^{b}	7.05^{a}	10.7^{b}	9.05^{b}	8.40^{b}
trans-5,6-epoxy-β-ionone	0.25	0.17	0.21	0.15	0.43	0.35	0.42	0.29	1.23	0.48	0.92	0.7	0.44	0.56	0.55	0.89	0.5	0.57	0.55	0.66
3-hydroxy-7,8-dehydro-β-ionol	2.29b^{a}	1.41a^{a}	0.67a^{a}	1.23a^{a}	4.44^{d}	3.93^{bc}	4.05^{c}	3.55^{bc}	4.36^{b}	4.83^{d}	4.28^{c}	4.95^{b}	2.05a^{a}	2.93ab^{c}	3.71b^{c}	2.9ab^{a}	2.60^{a}	2.23^{b}	2.35^{b}	2.91^{a}
Total	**20.6b^{a}**	**14.3ab^{a}**	**9.26a^{a}**	**12.3a^{a}**	**37.7^{b}**	**35.6^{b}**	**32.1^{b}**	**29.6^{ab}**	**68.6^{c}**	**56.8^{b}**	**48.5^{b}**	**56.4^{b}**	**49.5^{b}**	**48.2^{b}**	**55.0^{b}**	**51.9^{b}**	**45.4^{b}**	**55.1^{b}**	**49.4^{b}**	**47.0^{b}**
Vomifoliol	4.68	4.19	5.11^{a}	4.47	48.9^{b}	46.2^{b}	38.5^{b}	37.3^{b}	4.72	25.8	17.4^{b}	16.7	3.76	6.86	10.3^{ab}	7.96	15.8	4.54	4.86^{a}	16.7

Within the same sampling date averages of each compound were subjected to the analysis of variance and means were separated by the Tuckey test. Means followed by different letter are significantly different for *P≤0,05*. For each treatment superscript letters indicate differences between the sampling dates (*P≤0,05*). d**b**V= days before veraison, d**p**V=days post veraison, ND= Not Detectable. Where no letters are present no significant differences were revealed. *= tentatively identified as diidro-ionone, **,***= compounds at times coeluated with acetovanillone. All norisoprenoid concentrations are expressed as ethyl-4-acetylbenzoate equivalent.