EFFECT OF DIFFERENT LEVELS OF OXYGENATION ON PHENOLIC AND VOLATILE COMPOUNDS OF NEBBIOLO WINE DURING BOTTLE STORAGE

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Introduction

Oxygen plays a pivotal role in wine evolution and consequently on its organoleptic characteristics. Numerous studies have taken place over the past few decades with the aim of understanding and quantifying these effects. These researches underlined the delicate and ambivalent role of this gas in enology: on the one hand, the oxygen main role as oxidant and the main factor of the oxidation spoilage sometimes evident during conservation, while, on the other hand, a vector of transformations and compositional changes essential for the positive evolution of bottled wine including increased color stability, improved mouthfeel and aroma complexity as well as elimination of reductive off-odors.

From a chemical point of view the action of oxygen is exerted primarily on the most oxidizable compounds, and on polyphenols leading to the formation of quinones and hydroxide peroxide. In turn, these molecules oxidize the compounds most present in wine, starting from the ethyl alcohol which is oxidized to acetaldehyde. Furthermore, thanks to these oxidation processes, polyphenols form polymers that modify the perception of color, bitterness and astringency, while new odorants are formed.

However, the optimal oxygen intake to wine is not easy to assess and depends strictly on the grape material under winemaking and therefore on their main compositional characteristics, including the presence of antioxidant molecules such as glutathione, sulfur dioxide level, total polyphenol content and the relationship between the various polyphenolic fractions.

Given the economic and qualitative importance of Nebbiolo-based wines, this research sought to explore the effect of increasing oxygen doses on four wines made from Nebbiolo grapes hand-picked in Barolo and Barbaresco areas. As known, Nebbiolo is distinguished mainly by the other grape varieties due to its good polyphenolic content but also by its low level of extractable anthocyanins from grapes (250 to 600 mg/kg in average, Mattivi, 2003). The anthocyanin profile shows a dominance of di-substituted anthocyanidins, which are the most easily oxidizable, peonidin above all (Rio Segade et al., 2015; Torchio et al., 2016), the rest consists of malvidin and cyanidin, while the quantities of delphinidin and petunidin are quantitatively negligible

Given that peonidin-3-glucoside and cyanidin-3-glucoside can easily undergo oxidation processes, a rational use of oxygen in winemaking is decisive to obtain an appreciable and stable color of the wine promoting the formation of colored polymers while avoiding high oxidation. Previous studies have evaluated the effect of microoxygenation on Nebbiolo-based wines (Gerbi et al., 2006)., while the effect of macrooxygenation on color and aromas has not been investigated even if high intakes in oxygen can be reached during winemaking.

Materials and methods

Experimental plan and wines treatment

Four Nebbiolo-based wines, two from the Barolo area and two from the Barbaresco area, with a content of about 25 mg/L of free sulfur dioxide were subjected to subsequent oxygenation cycles (Petrozziello et al., 2018). For each cycle, a portion of wine was separated and bottled without undergoing further oxygenations (Figure 1). Oxygen was applied by air stirring in sequential cycles each one involving the consumption of about 7 mg/L of oxygen (saturation level); each oxygenation was performed only after the complete oxygen consumption by each sample. The wines were therefore oxygenated up to a maximum total value of 28 mg/L of oxygen. Wines were then stored in the absence of oxygen, and their compositional traits were carried out after 60 days (T60) and 300 days (T300)

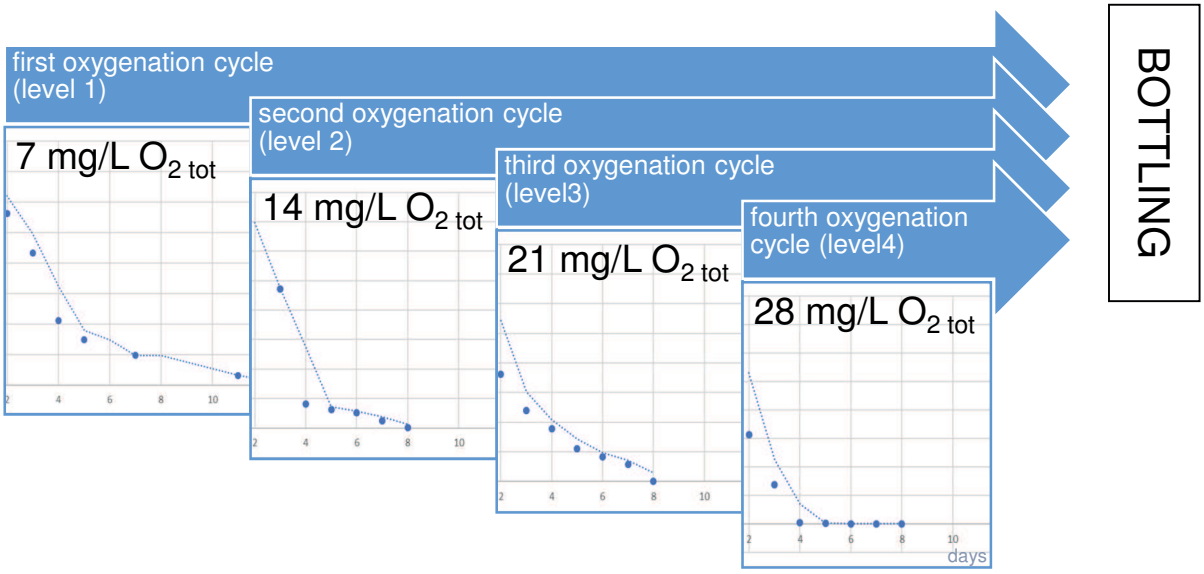

Figure 1: Experimental plan and mean oxygen consumption trend of subsequently oxygenation steps.

Dissolved oxygen

The dissolved oxygen content was assessed by a luminescence-based measurement using a portable analyzer (NomaSense O_2 P300, Nomacorc, Zebulon, NC, USA). Data has been expressed in mg/L (Figure 1). The evaluation of oxygen in bottled samples was monitored until the complete dissolved oxygen consumption (< 0.5 mg/L of O_2).

Color analysis and polyphenolic profile of wines

Wine color as intensity and hue as well as CIEL*a*b* parameters (OIV, 2016) have been measured by spectrophotometry. The contribution to wine colour was divided into non-bleachable structures (dTAT%), sensitive to discoloration pigments (dAT%), and free anthocyanins (dAI%) following the method proposed by Glories (1984) and simplified by Di Stefano and Cravero (1989). The copigmentation colour indexes (copigmented anthocyanins, free anthocyanins and polymeric pigments fractions) were also determined according to the method proposed by Boulton (1996).

Total polyphenol content was determined spectrophotometrically according to Singleton and Rossi (1965). Flavan-3-ols were evaluated by the proanthocyanidins index, using the Bate-Smith reaction by heating wine in acidic medium (Glories, 1984; Di Stefano et al. 1989), flavans were also characterized by their reactivity towards vanillin in accordance with the method proposed by Di Stefano and Cravero (1991), by the total anthocyanins index measuring the maximum absorbance at 536-540 nm after dilution with hydrochloric ethanol, while

anthocyanins in their monomeric form were determined using a SPE (Solid Phase Extraction) preparative step (Di Stefano et al., 1989).

Volatile aldehydes by GC-MS

2 mL of wine were extracted by MLLE (Micro Liquid/Liquid Extraction) in a single step using 200 µL of dichloromethane. Hexenal, *t*-2-octenal, *t*-2-nonenal, phenylacetaldehyde, and methional, were derivatised by means of O-(2,3,4,5,6- pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA) as described by Petrozziello et al. 2018. Adducts were separated and detected by gas chromatography-mass spectrometry (GC-MS). The GC-MS analysis was performed on an Agilent 6890 Series gas chromatograph equipped with a single quadrupole Agilent 5973N mass detector (Agilent Technologies, Santa Clara, CA, US) and spectrum were acquired in Single Ion Monitoring (SIM) mode. The identification of aldehydes was carried out by comparing the retention index of the compounds of interest with those of a pure derivatized standard and analysed under the same conditions. Quantitative data were obtained by interpolating the peaks areas relative to the calibration graphs constructed by the analysis of synthetic wines containing known amounts of the analytes. Each sample was analysed in duplicate.

Major aldehydes by HPLC

Acetaldehyde, glyceraldehyde and glyoxylic acid were analysed by HPLC after derivatization of 1 mL of wine with 2,4-dinitrophenylhydrazine (DNPH) as proposed by Nishikawa and Sakai (1995), adapted by Elias et al. (2008). The analyses were carried out on an Agilent 1100 HPLC equipped with a LiChrosper 250×4 mm (5µm particle size) column.

Results and discussion

The following table shows the main chemical-physical parameters of the wines before oxygenation steps. The alcoholic and malolactic fermentations were successfully completed for all wines. The metals content was low in all wines, with a slightly higher copper concentration for wine BR 3 (0.16 mg/L).

Wine	Alcohol % v/v	Reductive sugars g/L	pH	Total acidity g/L as tartaric acid	Malic acid g/L	Cu mg/L	Fe mg/L	A	TPI	PC
BB 01	14.24	3.10	3.39	5.74	0.06	0.10	0.86	126	2833	3847
BB 02	14.15	1.10	3.48	4.96	0.04	0.10	1.06	165	3215	4128
BR 01	13.60	2.00	3.55	5.27	0.00	0.16	1.01	118	3119	3595
BR 02	13.82	1.30	3.39	5.63	0.00	0.10	1.48	179	3635	4171
mean±σ	13.95±0.30	1.88±0.90	3.45±0.08	5.4±0.36	0.025±0.03	0.12±0.03	1.10±0.27	147	3201	3935

Table 1: General composition of Nebbiolo wines. BB: wines obtained from Barbaresco zone Nebbiolo grapes; BR: wines obtained from Barolo zone Nebbiolo grapes. A: Total anthocyanin index [mg malvidin-3-glucoside chloride/L]; TPI: Total polyphenols index [mg (+)-catechin/L]; PC: Proanthocyanidins index (PC) [mg cyanidin chloride/L]

Oxygen consumption

The dissolved oxygen decrease in wine is related to some important reactions involving polyphenolic compounds, which take place during its storage and complete throughout the wine shelf life. The measurements of dissolved oxygen carried out during macro-oxygenation have shown that the rate of oxygen consumption tends to increase as the oxygenation cycles proceed (Figure 1): complete consumption of dissolved oxygen took 10 days during the first cycle while 8 and only 4 days are needed during subsequently oxygenation steps.

Overall Polyphenolic profile and colour during storage

Wines obtained from Nebbiolo grapes are characterized by a low content of anthocyanins and a quite high content of polyphenols. Experimental wines had mean contents of 147 mg/L as total anthocyanins and 3201 mg/L as total polyphenols, respectively. Total polyphenols (Folin-Ciocalteu assay) in the wines before oxygenation varied from a minimum of 2833 mg/L to a maximum of 3635 mg/L, while condensed tannins (proanthocyanidins, PC) ranged from 3595 to 4171 mg/L. Flavans with a low molecular weight (FRV) ranged from 1931 to 2994 mg/L (Table 1).

Storage time (days)	oxygenation level	Total anthocyanin index	Monomeric anthocyanins	Total polyphenols index	Proanthocyanidins index (PC)	Vanillin assay (FRV)	FRV/PC ratio	L*	a*	b*	Color hue	Color intensity	dTAT [%]	dAL [%]	dAT [%]	Copigmentation fraction [%]	Free anthocyanins fraction [%]	Polymeric pigments fraction [%]
T0	**No Ox**	**147**	**56**	**3201**	**3935**	**2475**	**0,63**	**25,4**	**53,8**	**46,2**	**0,86**	**6,3**	**22,4**	**26,3**	**51,3**	**18,1**	**37,3**	**44,6**
T60	**Ox 1**	135	33	3552	3932	2532	0,64	15,1	43,3	30,8	1,12	7,6	25,9	17,4	56,7	13,6	38,4	48,0
	Ox 2	130	29	3574	3864	2538	0,65	14,2	42,9	30,0	1,10	7,9	27,1	16,0	57,0	11,6	39,8	48,6
	Ox 3	128	27	3609	3862	2507	0,64	12,9	42,0	28,6	1,08	8,2	27,9	14,7	57,4	11,1	39,4	49,4
	Ox 4	124	24	3497	3732	2524	0,67	12,3	41,4	27,8	1,06	8,3	28,3	13,2	58,2	11,3	38,8	49,9
	Mean	**129**	**28**	**3558**	**3848**	**2525**	**0,56**	**13,6**	**42,4**	**29,3**	**0,91**	**8,0**	**27,3**	**15,3**	**57,3**	**11,9**	**39,1**	**49,0**
T300	**Ox 1**	102	19	3548	3898	2206	0,53	25,6	53,9	48,1	0,90	6,5	32,0	22,0	46,1	8,5	34,5	57,0
	Ox 2	100	16	3493	3930	2106	0,54	24,3	53,3	46,9	0,90	6,7	34,3	20,3	45,4	11,2	31,6	57,2
	Ox 3	99	14	3562	3923	2133	0,55	23,3	52,7	45,9	0,89	6,9	37,6	18,6	43,8	8,6	33,3	58,0
	Ox 4	98	13	3569	3880	2133	0,63	22,5	52,1	45,0	0,86	7,1	38,8	16,6	44,7	8,2	32,2	59,6
	Mean	**100**	**16**	**3543**	**3908**	**2145**	**0,56**	**23,9**	**53,0**	**46,5**	**0,89**	**6,8**	**35,7**	**19,4**	**45,0**	**9,1**	**32,9**	**58,0**

Table 2: Average polyphenolic composition of wines during bottle storage. Total and monomeric anthocyanin are expressed as mg malvidin-3-glucoside chloride/L; Total polyphenols index is expressed as mg (+)-catechin/L. Proanthocyanidins and vanillin indexes are expressed as mg cyanidin chloride/L.; color intensity is referred to A.U. - O.P. 10 mm.

During the first 60 days of storage an overall increase in color intensity was observed for all wines (table 2). Then, this value decreased after 300 days of bottle storage. Similarly, colour hue reached a maximum after two months of storage showing an increase in yellow colour, then steadily decrease. CIEL*a*b* parameters confirmed this trend showing a general decrease of a*, b*, and L* values for all wines in the early stages of storage that was followed by an increase after 300 days. This behaviour could have caused by the SO_2 combination with anthocyanins at T0 and its subsequent release during storage.
A quite complete depletion of monomeric anthocyanins was observed during storage. Their average concentration decreased from 56 mg/L (T0) to 16 mg/L (300 days storage). This evolution may be explained by both polymerization and oxidation phenomena, as we evidence an increase of the dTAT percentage (stable polymeric pigments) and a decrease in the total anthocyanin index and in the dAl percentage (free anthocyanins).

The amount of low molecular flavans (FRV) decreased for all wines, following a slight increase observed after 60 days of storage. A similar behaviour was observed for the FRV/PC ratio, which showed an increase during the first months of storage (T60), followed by a general decrease after 300 days of storage. The proanthocyanidins index decreased after 60 days of storage, then it increased during aging: oxidation and depolymerization processes of polymeric phenols can explain this complex behaviour. A moderate increase over time in the total polyphenol index was observed for all wines, especially between the first and second storage time (T60 and T300).

Effect of oxygen intake on the color and polyphenolic profile

Focusing on macro-oxygenation trial, the amount of oxygen supplied in the early stages of storage to young Nebbiolo wines influenced significantly their color. As mentioned above, after 60 days of bottle aging an increase in yellow tones for all wines, corresponding to a general decrease of a*, b*, L* (CIEL*a*b* color space parameters), was evidenced. On the one hand, the yellowness is strictly related to oxygen intake: the higher the amount in dissolved O2, the higher the hue value. On the other hand, the wines with higher oxygen intake gained in color intensity. As observed for T60, after 300 days of storage (T300), significant differences were related to the degree of oxidation: the most oxygenated wines had the lowest values of luminance, a* and b* parameters.

Polyphenolic composition of wines clearly explains color differences among wines: lower content in monomeric anthocyanins (dAI%) was observed in higher oxygenation level, while an increase of pigments not bleachable by SO_2 (dTAT%) and polymeric structures was measured in higher oxygen intakes.Therefore, the dTAT% parameter significantly increased by increasing the level of oxygen both after 60 days of storage and after 300 days of storage. At the same time a decrease of dAI% was observed both after 60 and 300 days of storage.

Finally, the effect of oxygenation on copigmentation and on the polymeric pigments was modest; only after 60 days of storage the polymeric pigments fraction significantly increased by increasing the dose of oxygen in two wines out of four (data not reported). No significant differences were noticed after 300 days of storage.

During aging, a progressive decrease in total and free anthocyanins was observed: by increasing the amount of supplied oxygen, the content of anthocyanins decreased. Nevertheless, the decrease was modest and did not concern all the thesis compared among them. As regards flavanols (FRV, PC parameters) and their derivative ratio FRV/PC, the higher the dose of supplied oxygen, the higher the decrease in the FRV/PC ratio.

Aldehydic compounds

The concentration of glyceraldehyde and glyoxylic acid increased over time in all wines (Table 3). Glyceraldehyde concentration increased significantly only after two months of storage (T60) and the differences between theses, subjected to different doses of oxygen, were not significant. At ten months of storage (T300) the differences among oxygenation levels remained not significant. Only acetaldehyde, whose concentration after 60 days of storage increased together with the oxygen level, changed rapidly in all wines. Differences of this compound among wines with different oxygen intake were shown at 60 days: concentrations above 10 mg/L were observed in Barolo wines subjected to the highest oxygen exposure (Ox4). From two until ten months of storage, acetaldehyde levels decreased rapidly and reached their minimum at the end of the monitored period. This trend was observed in all wines except those less exposed to oxygen (Ox1) where no accumulation was observed.

Like acetaldehyde, glyoxylic acid content increased together with the oxygen intakes, but after 300 days of storage only in one wine out of four significant differences between the theses with different doses of oxygen were noticed.

With regards to minor volatile aldehydes (quantities less than 50 µg/L) a slight decrease was observed until 60 days of storage, then aldehydes concentration tended to increase overall, and significant differences between T60 and T300 were observed. Highest concentrations were reached for phenylacetaldehyde (Table 3). This compound has honey-like, sweet, rose, green, grassy nouances and a very low odour threshold. It is plausible that, at the concentrations measured after 300 days, phenylacetaldehyde could play an important role contributing to the formation of evolution notes of honey in wines stored in bottles. However, this study highlights how its formation is not strictly dependent on the supply of oxygen in the early stages of winemaking, but rather on storage time.

Storage time (days)	oxygenation level	Glyceraldehyde [mg/L]	Glyoxylic acid [mg/L]	Acetaldehyde [mg/L]	*t*-2-Hexenal [µg/L]	Methional [µg/L]	*t*-2-Nonenal [µg/L]	Phenylacetaldehyde [µg/L]
0		**17,8**	**0,8**	**4,1**	**0,8**	**2,9**	**0,9**	**29,4**
60	**Ox 1**	17,3	1,2	3,9	0,4	2,0	1,2	18,1
	Ox 2	16,7	1,3	5,6	0,5	2,1	1,2	17,0
	Ox 3	16,8	1,5	7,4	0,4	2,2	1,3	16,7
	Ox 4	17,4	1,8	9,5	0,4	2,3	1,2	18,6
	Mean	**17,1**	**1,5**	**6,6**	**0,4**	**2,2**	**1,2**	**17,6**
300	**Ox 1**	25,2	1,8	2,3	2,4	9,1	10,2	128,6
	Ox 2	24,9	1,9	3,6	1,5	9,1	6,3	109,8
	Ox 3	24,7	2,0	3,6	2,0	7,1	7,7	132,0
	Ox 4	24,9	2,2	3,9	1,8	8,3	5,8	112,8
	Mean	**24,9**	**2,0**	**3,4**	**1,9**	**8,4**	**7,5**	**120,8**

Table 3: Main aldehyde compounds (as oxidation markers) of Nebbiolo wines during the trial.

Conclusion

In this study we have described the chemical and physical changes of Nebbiolo wines subjected to 4 different levels of air exposure. The amount of oxygen supplied in the early stages of storage to these wines favorably influenced the evolution of their color during 300 days of bottle storage. The wines with higher oxygen intake had a higher color intensity and a slightly lower hue. Moderate gas absorption results to the partial oxidation of polyphenolic compounds. This process leads to hydrogen peroxide formation that, in the presence of metallic catalysts, such as iron and copper, is capable of oxidizing ethanol and tartaric acid to form acetaldehyde and glyoxylic acid. Regarding aldehydes content, even high oxygen intakes during the first phases did not markedly change their concentration in wine at the end of the studied period, except for acetaldehyde. As previously reported, this latter compound allows the formation of complex, colored and stable molecules, starting from anthocyanins and tannins present in wine: this last passage is most concerning by the enologists for color stabilization. This study showed as moderate oxygenations (up to 28 mg / L total dissolved oxygen) could be beneficial to improving the quality of young Nebbiolo wines in terms of intensity and color stability. The acetaldehyde content, however, given its reactivity, is strongly conditioned by the presence of free sulfur dioxide, which limits the condensation process. Moderate oxygenation during refining contributes to color stabilization, while strong oxidation of the product compromises the sensory qualities irreversibly.

References

Gerbi, V., Caudana, A., Zeppa, G., and Cagnasso, E. (2006). Micro-oxygenation effect in connection with the phenolic composition of red wines. Industrie delle Bevande 35, 462–468.

Mattivi, F.; Prast, A.; Nicolini, G.; Valenti, L. (2003). Il potenziale polifenolico delle uve rosse e la sua applicazione in enologia. L'Enologo, 39 (10): 105-114.

OIV (2016). Compendium of International Methods of Wine and Must Analysis. Organisation Internationale de la Vigne et du Vin, Paris, France.

Boulton, R. B. (1996). A method for the assessment of copigmentation in red wines. in 47th annual meeting of the American Society for Enology and Viticulture, Reno, NV. June 1996.

Di Stefano, R., and Cravero, M. C. (1991). The grape phenolic determination. Riv. Vitic. Enol. 49, 37-45.

Di Stefano, R., Cravero, M. C., and Gentilini, N. (1989). Metodi per lo studio dei polifenoli dei vini. L'Enotecnico 25, 83–89.

Elias, R. J., Laurie, V. F., Ebeler, S. E., Wong, J. W., and Waterhouse, A. L. (2008). Analysis of selected carbonyl oxidation products in wine by liquid chromatography with diode array detection. Anal. Chim. Acta 626, 104–110. doi:10.1016/j.aca.2008.07.048

Glories, Y. (1984). La couleur des vins rouges: 2e. Partie: mesure, origine et interpretation. Connaissance Vigne Vin 18, 253–271.

Nishikawa, H., and Sakai, T. (1995). Derivatization and chromatographic determination of aldehydes in gaseous and air samples. J. Chromatogr. A 710, 159–165. doi:10.1016/0021-9673(94)01006-Z

OIV (2016). Compendium of International Methods of Wine and Must Analysis. Organisation Internationale de la Vigne et du Vin, Paris, France.

Petrozziello, M., Torchio, F., Piano, F., Giacosa, S., Ugliano, M., Bosso, A., & Rolle, L. (2018). Impact of Increasing Levels of Oxygen Consumption on the Evolution of Color, Phenolic, and Volatile Compounds of Nebbiolo Wines. Frontiers in chemistry, 6.

Rio Segade, S., Pace, C., Torchio, F., Giacosa, S., Gerbi, V., and Rolle, L. (2015). Impact of maceration enzymes on skin softening and relationship with anthocyanin extraction in wine grapes with different anthocyanin profiles. *Food Res. Int.* 71, 50–57. doi:10.1016/j.foodres.2015.02.012

Singleton, V. L. (1987). Oxygen with Phenols and Related Reactions in Musts, Wines, and Model Systems: Observations and Practical Implications. Am. J. Enol. Vitic. 38, 69–77.

Torchio, F., Urcan, D. E., Lin, L., Gerbi, V., Giacosa, S., Río Segade, S., et al. (2016). Influence of different withering conditions on phenolic composition of Avanà, Chatus and Nebbiolo grapes for the production of 'Reinforced' wines. *Food Chem.* 194, 247–256. doi: 10.1016/j.foodchem.2015.08.009