STRATEGIES TO REDUCE SO_2 USE IN EARLY PHASES OF WINEMAKING

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YEASTS – LACTIC BACTERIA CO-INOCULATION

The fundamental role that selected micro-organisms play in the behaviour of both alcoholic and malolactic fermentation is well known.
Yeasts - lactic bacteria co-inoculation is a recent technique which is aimed at optimising the management of the malolactic fermentation (MLF) by reducing the risks related to the incomplete transformation of malic acid as well as the production of toxic compounds, such as biogenic amines or ethyl-carbammate.
This practice consists of the simultaneous development in the must of both yeasts and lactic bacteria (MLB) by adding a starter culture of selected MLB just few hours (e.g. 12 hours) after the inoculation of selected yeasts.

Co-inoculation and reduction of sulphur dioxide

Principles
According to Masqué and co-workers[1], the co-inoculation is not only useful in reducing the risk of incomplete malolactic fermentations or in avoiding the development of microbial alterations (formation of biogenic amines or other toxic compounds), but, due to the faster behaviour of the MLF it means that the wine can be left without sulphur dioxide protection for long periods of time. Thus co-inoculation can be considered as a useful technique to optimise the management of SO_2 in wine-making.
This observation was also confirmed by the results obtained during the experimental trials that were performed during the first two years of ORWINE Project.

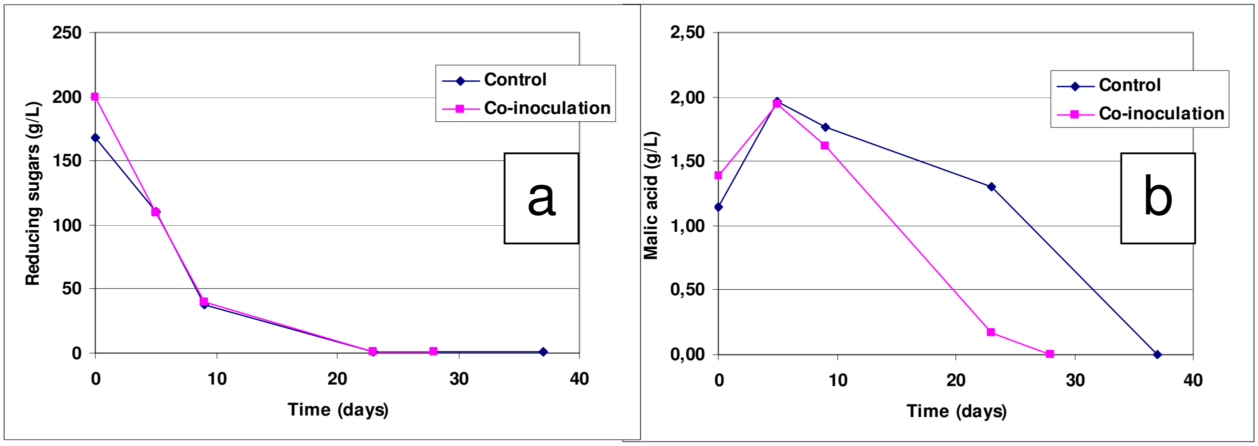
Description of the trials
In different trials the co-inoculation technique was compared with the conventional usage of malolactic bacteria which is the late addition of MLB at the end of alcoholic fermentation. Sulphites were avoided when co-inoculation was used.

Main results
The results confirmed that co-inoculation does not affect the behaviour of alcoholic fermentation (Figure 1a), but it can be helpful in reducing the time needed for MLF: the total consumption of malic acid was faster in the co-inoculated samples than in control wines, being malic acid almost totally consumed just at the end of alcoholic fermentation (Figure 1b).

In 2007, the chemical composition of the final wines was very similar, with a very low volatile acidity (0,21 g/L), and acetaldehyde levels (4-5 mg/L).
However the co-inoculated samples obtained in 2006 showed a remarkably lower level of volatile acidity (table 1). Moreover, co-inoculation demonstrated the ability to control biogenic amine formation even when sulphur dioxide was not used before alcoholic fermentation (table 2).

[1] Masqué et al., 2008. Co-inoculation of yeasts and lactic bacteria for the organoleptic improvement of wines and for the reduction of biogenic amine production during the malolactic fermentation. Rivista Internet di Viticoltura ed Enologia (www.infowine.com)

Fig. 1: Effect of co-inoculation on the behaviour of alcoholic (a) and malolactic (b) fermentations in Merlot wines (harvest 2007).



Control: classic inoculation of MLB, in the final stages of alcoholic fermentation (12th day)
Co-inoculation: inoculation of MLB 12 hours after selected yeasts addition (2nd day)

Table 1: Analytical parameters of some experimental Merlot wines from harvest 2006 (alcoholic degree: 12,00 % v/v)

MERLOT	Volatile acidity (g/L)	Malic acid (g/L)	Lactic acid (g/L)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Acetaldehyde (mg/L)
Classic inoculation SO_2 *	0,51	0,08	1,60	3	14	2
Co-inoculation NO SO_2	0,31	0,06	2,04	*n.d.*	1	*n.d.*

n.d. = not detectable
** 30 mg/L before alcoholic fermentation*

Table 2: Biogenic amines in some experimental Merlot wines in different moments of the vinification process (harvest 2006)

MERLOT	Histamine (mg/L)	Tyramine (mg/L)	Putrescine (mg/L)
Classic inoculation SO_2 *	*n.d.*[a] – *tr.*[b]	0,2[a] - 0,8[b]	1,4[a] - 1,9[b]
Co-inoculation NO SO_2	*n.d.*[a] – *tr.*[b]	0,2[a] - 0,8[b]	1,2[a] - 2,8[b]
Classic inoculation NO SO_2	*n.d.*[a] – *tr.*[b]	0,2[a] - 1,3[b]	1,4[a] - 5,2[b]

[a] end of alcoholic fermentation (October 2006); [b] élevage sur lies (January 2007)
*n.d. = not detectable; tr. = traces; * 30 mg/L before alcoholic fermentation*

With regards to the sensory point of view, co-inoculation, in comparison with SO_2 addition before alcoholic fermentation, led to wines with less buttery, vegetal and volatile acidity notes. The analyses of aromatic compounds in these wines highlighted a higher level of volatile esters (basically connected to fruity and flowery sensations) in the samples obtained by co-inoculation.

Conclusions

The reduction of sulphur dioxide in the early stages of wine-making certainly is a sustainable practice for both organic and conventional producers but its practicality is dependant on the particular care in the management of the fermentations.
With regards to red wines, some simple practices, such as yeasts - lactic bacteria co-inoculation can be helpful tools in managing MLF even when reduced SO_2 amounts are used.

HYPER-OXYGENATION

The concept of hyper-oxygenation was introduced by Müller-Späth in 1977[2], and it is based on the treatment of the must with an excess of oxygen, with the aim to completely eliminate from the must itself all the oxidisable substances. The products of the oxidation of these compounds (particularly phenolic substances) are completely eliminated with a simple racking at the end of the hyper-oxygenation treatment.
Oxygen can be added as gaseous O_2 or air from a cylinder (with the aid of a microporous diffuser) or simply by pumping over.
If the treatment is performed in the early phases of vinification (e.g. just after pressing), it is possible to obtain chemical stabilisation of the must by the elimination of the unstable phenolic substances (e.g. hydroxycinnamyltartaric acids) without damaging the volatile compounds which are at this moment should be protected in form of “precursors”. In the fresh juice just after pressing, aromatic compounds are mainly present as glycosides, bound to sugars such as glucose. It is in this form that certain substances which are sensitive to oxidation, such as terpenols (Muscat-like aroma), are relatively stable and are poorly affected by the excessive injection of oxygen .

Hyper-oxygenation and reduction of sulphur dioxide

Principles

As outlined above the injection of oxygen is able to produce the elimination (by oxidation and polymerization) of the unstable phenolic fraction, poorly affecting varietal aroma compounds..
Sulphites must be avoided if hyper-oxygenation is selected as a wine-making practice as due to its antioxidant activity, sulphur dioxide reacts strongly against O_2 activity.
Thus hyper-oxygenation can have a role in the reduction of SO_2 as it requires the total elimination of sulphites before alcoholic fermentation hence the interest in this practice in organic wine-making.

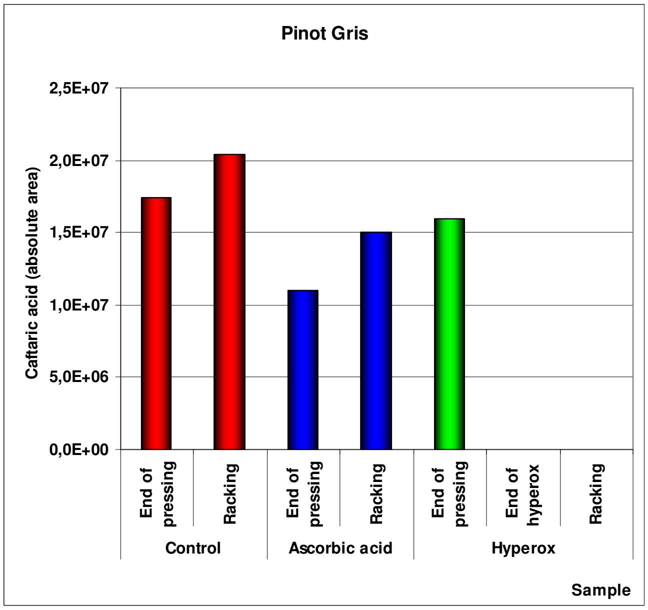
Description of the trials

The application of hyper-oxygenation on organic musts was subject of investigation during the three years of ORWINE Project.
The trials were at first related to the comparison between the traditional use of SO_2 during crushing and destemming (e.g. 30 mg/L addition), and its total replacement by using hyper-oxygenation.
Results demonstrated that hyper-oxygenation can give a good stabilisation of musts and wines, lowering the levels of oxidable phenolic substances (Figure 2).

Nevertheless, this technique can be sometimes problematic for processing certain aromatic grape varieties whose aroma is particularly sensitive to oxidation (e.g. Sauvignon blanc). For such wines a significant loss in some varietal notes (e.g. “box tree” attributes) was highlighted during sensory evaluation (Figure 3).

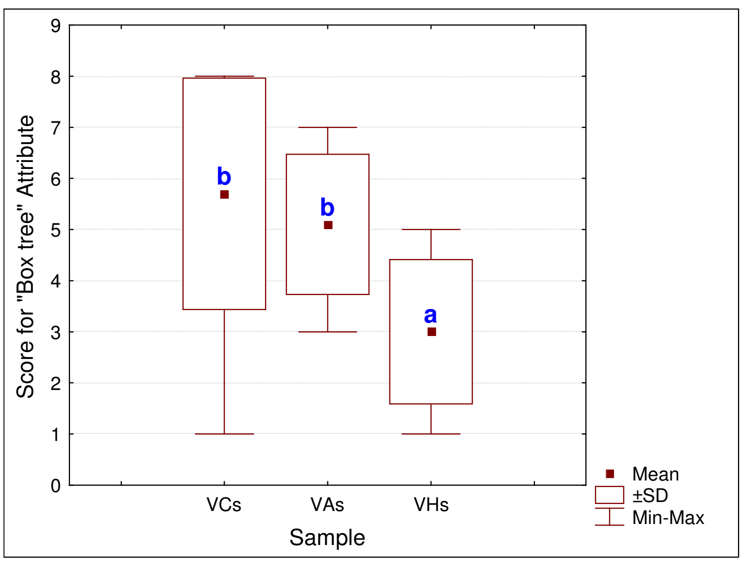
[2] H. Müller-Späth, 1977. Neueste Erkenntnisse über den Sauerstoffeinfluss bei der Weinbereitung – aus der sicht der Praxis. Weinwirtschaft, 113: 144-157.

Fig. 2: Caftaric acid[3] levels detected in different pre-fermentative steps. Three trials are compared (harvest 2006).



Control: conventional vinification (30 mg/L of SO_2 added during crushing - destemming)
Ascorbic acid: replacement of SO_2 with a mix of ascorbic acid (50 mg/L) and grape tannin (50 mg/L)
Hyperox: elimination of SO_2 using hyper-oxygenation

Fig. 3: Results of a Sensory Attribute Difference Test carried out on Sauvignon blanc wines.



VCs: conventional vinification (30 mg/L of SO_2 added during crushing - destemming)
VAs: replacement of SO_2 with a mix of ascorbic acid (50 mg/L) and grape tannin (50 mg/L)
VHs: elimination of SO_2 using hyper-oxygenation

Three trials are compared and the results of a Least Significant Difference Test, subsequent to a two factors (samples and panelists) ANOVA, are presented; different letters mark significant differences among samples at $p < 0,05$.

The use of hyper-oxygenation in some cases brought out a slower alcoholic fermentation and as a consequence a slight increase of wine volatile acidity resulted. This fact was related to an excessive delay between hyper-oxygenation itself and the racking which normally follows the treatment. If the time between these two steps was too long, a rapid increase in the population of wild yeasts (non *Saccharomyces* ssp.) was observed (table 3), and the development of these micro-organisms led unavoidably to a rapid consumption of assimilable nitrogen (in table 3, almost the 80 % of the must original value).

[3] Caftaric acid is one of the most oxidizable phenolics in must; it is the most important substrate for the enzymatic oxidations (polyphenoloxydases), and for this reason it is involved in the browning reactions of white wines. Caftaric acid disappears after hyper-oxygenation treatment.

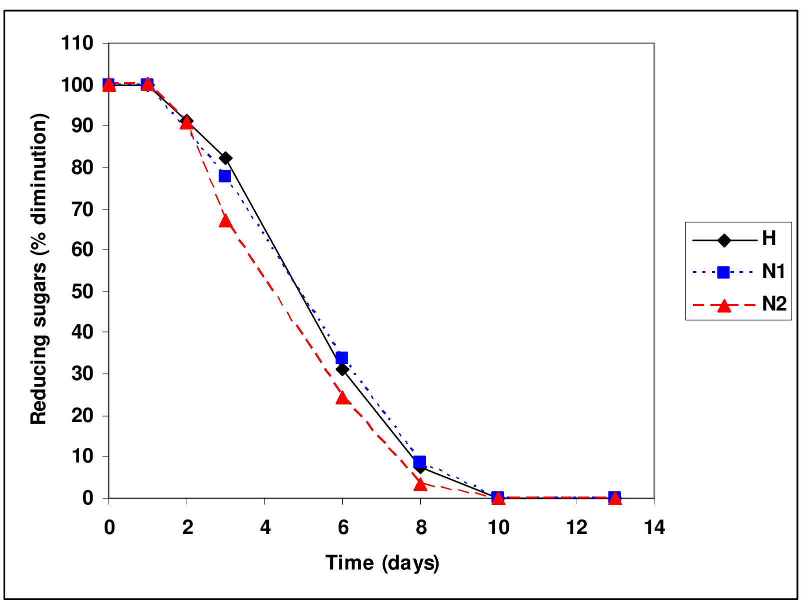
Table 3: Development of *Saccharomyces* and non *Saccharomyces* populations before selected yeasts inoculation in a hyper-oxygenated must; the levels of free amino acids are also reported.

Sample	Date	Free amino acids (mg/L)	*Saccharomyces* (CFU/mL)	Non *Saccharomyces* (CFU/mL)
Must	03-set	94	$1,3 \times 10^6$	$3,7 \times 10^5$
After Hyperox	03-set	87	$1,1 \times 10^6$	$3,6 \times 10^5$
After Racking	04-set	21	< 10	$1,0 \times 10^6$
After SYI	04-set	20	$3,0 \times 10^5$	$1,9 \times 10^6$

SYI: Selected Yeasts Inoculation

This fact means that when the selected yeasts are added after the racking, they will find very little assimilable nitrogen in the must, and for this reason the behaviour of alcoholic fermentation will be conditioned by this lack of nitrogen sources, with a higher risk of a stuck or sluggish fermentation.
To avoid these problems, the preparation of an active *pied de cuvée* (selected yeasts starter culture) is fundamental. This process must be carried out as early as possible even using some unsedimented must issuing from the pressing plant, instead of the racked must (as usually done). These precautions, together with a nitrogen supplementation (particularly ammonium salts, as di-ammonium phosphate) during *pied de cuve* addition, are shown to be useful strategies to increase the fermentation rate and to avoid fermentation sluggishness (Figure 4).
Finally, to reduce the lag between hyper-oxygenation and racking, a treatment with pectolytic enzymes could be recommended.

Fig.4: Behavior of alcoholic fermentation in hyper-oxygenated musts treated in different ways with regards nitrogen supplementation and *pied de cuvée* preparation:
No fermentation problems were highlighted in musts from harvest 2008, but trial N2 showed a slightly higher fermentation rate.



H: *pied de cuve* prepared with cleaned must (after racking); nitrogen supplementation for *pied de cuve* (during preparation) [4]

N1: *pied de cuve* prepared with uncleaned must (from the pressing plant); nitrogen supplementation for *pied de cuve* (during preparation) and for the whole must before addition [5]

N2: *pied de cuve* prepared with uncleaned must (from the pressing plant); nitrogen supplementation for *pied de cuve* (during preparation) and for the whole must before addition [6]

[4] Yeast walls (400 mg/L) and thiamine (0,6 mg/L) during *PdC* preparation

[5] Yeast walls (400 mg/L) and thiamine (0,6 mg/L), a half on *PdC* at preparation, and a half on the whole lot at *PdC* addition

[6] Yeast walls (400 mg/L) and thiamine (0,6 mg/L), a half on *PdC* at preparation, and a half on the whole lot at *PdC* addition; diammonium phosphate (150 mg/L) also added

[7] Yeast walls (400 mg/L) and thiamine (0,6 mg/L) during *PdC* preparation

Conclusions

In conclusion, the hyper-oxygenation of the must can be helpful to avoid the use of SO_2 in the pre-fermentation steps of wine-making process. Nevertheless the opportunity to use this technique should be carefully evaluated for the musts of certain grape varieties whose typical aroma is particularly sensitive to oxidation (e.g. Sauvignon blanc).

When using this practice, special precautions should be taken in the addition of selected yeasts and their management (e.g. nutrients supply, yeast acclimatization) as well as in ensuring a rapid must clarification after the addition of oxygen. These precautions are critical for the reduction of non-*Saccharomyces* growth, before selected yeasts addition, and in avoiding sluggish fermentations.

ALTERNATIVE ADDITIVES TO SO_2

The increase in knowledge which has characterized oenological sciences in the last decades has indicated that there are different additives and practices which can partially replace sulphites in some basic functions.

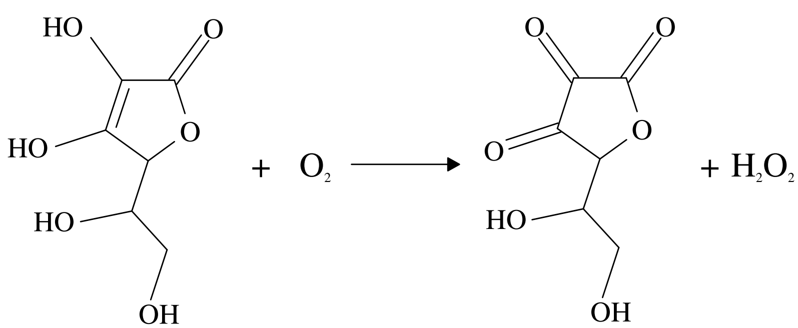
When considering alternatives to sulphur dioxide, it must be emphasised that, even today, the total elimination of SO_2 is still not possible without a risk of compromising wine quality. Nevertheless, the overall reduction in quality by using some alternative technologies or additives is definitely feasible and the concept of sulphite reduction is becoming particularly important not only for organic wine-making but also in the production of conventional wines.

Ascorbic acid and reduction of sulphur dioxide

Ascorbic acid (AA, vitamin C) is one of most important alternative additive to SO_2.

According to Rigaud and co-workers[10] it reduces the risk of enzymatic oxidations in the must (preservation of caftaric acid) and, for its antioxidant activity, it is able to scavenge oxygen and reactive oxygen molecules (e.g. some free radicals) even in wine and reducing the oxidation of phenolic compounds (Figure 5).

Fig. 5: Oxidation of ascorbic acid to dehydroascorbic acid



ascorbic acid **dehydroascorbic acid**

With regards this last point of view, AA acts faster than sulphur dioxide thus being more useful in reducing the problems connected with a sharp oxygenation (e.g. during racking or bottling) For this reason it is often used on the wines just before bottling. Despite this faster reactivity, however, its

[8] Yeast walls (400 mg/L) and thiamine (0,6 mg/L), a half on *PdC* at preparation, and a half on the whole lot at *PdC* addition

[9] Yeast walls (400 mg/L) and thiamine (0,6 mg/L), a half on *PdC* at preparation, and a half on the whole lot at *PdC* addition; *PdC* addition: di-ammonium phosphate (300 mg/L) also added on the must

[10] Rigaud et al., 1990. Mécanismes d'oxydation des polyphenols dans les môuts blancs. R.F.Œ., 124: 27-31.

action is less durable with respect to that of SO_2 so these two additives are mostly used in combination.

Another important reason why wine-makers mix SO_2 and AA, is the evidence reported in figure 5: the oxidation of ascorbic acid produces hydrogen peroxide (H_2O_2), which is itself a powerful oxidant; sulphites are able to scavenge H_2O_2, giving an underlying contribution to the antioxidant properties of the mix itself.

This last consideration is an important concept. If the wine-maker wants to replace SO_2 by using ascorbic acid, it is not possible to avoid the use of sulphites without suitable alternative additives, which are able to replace the fundamental scavenging activity of sulphur dioxide against hydrogen peroxide.

Description of the trials

The approach of ORWINE programme to this problem consisted of using grape tannin as an "alternative scavenger". It is well known that tannins are able to reduce the activity of free radicals (such as superoxide or hydro peroxide)[11], and for this reason they can be used in combination with AA to replace one of the traditional uses of sulphites viz. their addition during crushing (in white wine-making).

The results obtained during the harvest 2006 showed that a mix of ascorbic acid and grape tannin was able to reduce the oxidation of phenolic compounds (in figure 2 the behaviour was similar to that of the SO_2 added must). Thus this sort of hyper-reductive technology demonstrated its ability to stabilise the must on the basis of a principle which is opposite to that of hyper-oxygenation, i.e. the protection of the must itself from oxidations (table 4).

Moreover, hyper-reduction was also able to preserve the typical smell of certain varietal wines such as Sauvignon blanc (figure 3). During the sensory evaluation of such wines, no significant differences were noted as regards the attributes related to these varietal notes between the samples produced using sulphites and those obtained by adding the mix AA + tannins.

One of the problems related to the hyper-reduction technique is the higher susceptibility of the resulting wines to oxidation during storage. The POM Test, an index related to the susceptibility of the wine to oxidation was higher in the wines obtained by the mix AA + tannin as opposed to those obtained by hyper-oxygenation or by the classic SO_2 addition during crushing.

Table 4: Summary of the main aspects related to some alternative practices in the use of sulphur dioxide

	HYPEROXYGENATION	**HYPER-REDUCTION**
Basic principle	Total oxidation of the unstable substances	Total protection of oxidisable substances
Specific treatment	Massive oxygen addition on must after pressing	Ascorbic acid + tannins addition on must during crushing
Relationship with sulphites	No SO_2: alternative practice	No SO_2: alternative additives
Effects on O_2 sensitive phenolic compounds	Elimination by oxidation and precipitation	Preservation
Effects on O_2 sensitive volatile compounds	Partial loss	Preservation
Effects on the stability of the final wines	Higher stability to oxidation compared to that observed by the traditional use of SO_2 before alcoholic fermentation	Lower stability to oxidation compared to that observed by the traditional use of SO_2 before alcoholic fermentation
Effects on wine sensory characters	For certain varieties: partial loss of specific varietal notes	Preservation of specific varietal notes

[11] Vivas, 1997. Composition et propriétés des préparation commerciales de tanins à usage œnologique. R.F.Œ., 84: 15-21.

For this reason, when hyper-reductive techniques are used, special care should be taken in the management of any operation which could affect the uptake of oxygen in the wine (e.g. racking, bottling, filtration, transfers of wine from one tank to another). Additional precautions, such as the saturation of tubing, tanks and connections with carbon dioxide, nitrogen or other inert gases can be useful to manage these oxygen sensitive products and to avoid any further oxidation without the necessity of a massive use of sulfites.

In conclusion it is possible to question the use of grape tannin as alternative scavenger to replace sulphites as it can affect wine sensory characters causing wood-like notes in the sensory profile of the treated wines. However in the trials carried out in this ORWINE project and for the amounts used no evidence was found concerning any sensory effect of the added tannin.

Table. 5: Analytical parameters of some experimental wines obtained during harvest 2006; two varieties and three trials are compared

PINOT GRIS (FINAL WINE – JAN 07)						
Sample code	Date	DO 420	DO 320	DO 280	**POM Test** [12]	Catechins (mg/L)
VC	23-gen	0,1273	7,2	8,7	**3**	20
VA	23-gen	0,1545	7,1	8,4	**20**	14
VH	23-gen	0,1314	5,8	7,2	**0**	8

SAUVIGNON (FINAL WINE – JAN 07)						
Sample code	Date	DO 420	DO 320	DO 280	**POM Test** [9]	Catechins (mg/L)
VC	23-gen	0,0951	5,3	8,9	**36**	15
VA	23-gen	0,1078	6,4	10,4	**52**	13
VH	23-gen	0,1204	5,2	7,9	**0**	9

VC, conventional vinification; **VA**, use of AA + grape tannins; **VH**, hyper-oxygenation

Conclusions

The use of ascorbic acid as an alternative additive to sulphur dioxide requires the replacement of SO_2 with other free radical scavengers. The use of a mix of AA and grape tannins gave good results in white musts, preserving oxygen-sensitive phenolic compounds as well as the typical notes of certain varietal wines whose aroma is susceptible to oxidation.

However when hyper-reduction technology is used special care is necessary to avoid massive oxygen application to the final wine which become more sensitive to oxidation with their higher content of phenolic compounds.

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DISCLAIMER

[12] The higher the POM Test value, the higher the susceptibility to oxidation of the wine