LISBON, PORTUGAL,
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UNDERSTANDING VARIETAL AROMAS DURING ALCOHOLIC AND MALOLACTIC FERMENTATIONS

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LALLEMAND

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PROCEEDINGS
OF

THE *XXIV^es ENTRETIENS SCIENTIFIQUES LALLEMAND*

LALLEMAND

FOREWORD

At the *XXIVes Entretiens Scientifiques Lallemand*, researchers presented the most recent findings regarding varietal aromas, and the impact of alcoholic and malolactic fermentations on their release into wine.

For this occasion, the Lallemand – Institute of Masters of Wine research bursary was awarded to Clare Tooley of France, a first-year student in the Master of Wine program, for her essay, "In the context of the current market, how do viticultural practices influence the varietal aromas?" In addition, the winners of the ML Wine competition (Madrid, 2013) received their awards from Dr. Sibylle Krieger, the director of the Lallemand Malolactic Fermentation School.

The meeting opened with Eduardo Agosin, one of the most respected researchers in wine aromas. Professor Agosin, from the Pontificia Universidad Católica de Chile, discussed potential varietal aromas. This potential arises from glycosidic and cysteinylated conjugates, which can contribute significant aromas to wine once freed from the bound fraction, either by enzymatic or acid hydrolysis during fermentation and aging, respectively.

Coming all the way from the University of Auckland in New Zealand, and famous for his research on Sauvignon Blanc, Dr. Mat Goddard gave an impressive talk on research to better understand the microbial ecology of vines and wines, and to harness beneficial strains more reliably. He presented recent findings that show New Zealand harbours a distinct population of yeasts.

Touriga Nacional (TN) is a varietal typical of Portugal, characterized by a bergamot-like, fruity-citric-floral aroma and is attributed to terpenol, linalool and its acetate. Dr. Frank Rogerson of Symington Family Estates in Portugal presented on the possible modulation of key odorants responsible for the bergamot aroma in TN, while investigating the effect of a commercial pectolytic enzyme preparation rich in beta-glucosidase activity.

The research done by the NYSEOS group and presented by Dr. Laurent Dagan is very interesting. Dimethyl sulphide (DMS) is a versatile aroma compound that can have significant effects on the sensory properties of wine. Depending on its concentration and the type of wine, DMS can be responsible for various aromas, including truffle, herbaceous notes, undergrowth, cabbage and fruity sensations. He presented the results of the research on precursors for DMS and how to modulate the concentration in wine.

Engela Kritzinger presented the results of her Master's thesis done at the University of Stellenbosch with Dr. Wessel du Toit on the role of glutathione in wine. Recent research has come to the fore explaining the role different levels of oxygen and sulphur dioxide, yeast strains and commercial glutathione-enriched inactivated dry yeast preparations (GSH-IDYs) play on GSH concentrations in wine. GSH-IDY additions to juice have been shown to increase the GSH levels of wine when used correctly.

Wine bacteria also play an important role with varietal aromas. Dr. Maret du Toit, also from the University of Stellenbosch, has shown that different malolactic fermentation (MLF) inoculation strategies can be used to change the wine style – a major trend for the fresh and fruity wine styles. Her work demonstrated that the wine matrix, pH and alcohol concentration affect MLF and the final volatile aroma profile. The changes in volatile aroma composition can also be driven by using different lactic acid bacteria strains.

To conclude the *XXIVes Entretiens Scientifiques Lallemand*, Dr. Ana Escudero from the Universidad de Zaragoza in Spain presented a review of her group's knowledge and understanding of the roles played by different aroma chemicals in the positive aroma attributes of wine, and also presented a systematic approach to classifying the different aroma chemicals of wine.

The more we know about wine aroma, and the more we realize that such a complex matrix, with so many factors influencing the equilibrium of this environment, still has much to reveal. Current research offers a fascinating perspective, and understanding the impact of wine microorganisms and their derivatives on wine varietal aromas provides useful tools to help winemakers shape and master the final wine style.

CONTENTS

UNDERSTANDING VARIETAL AROMAS DURING ALCOHOLIC AND MALOLACTIC FERMENTATIONS

THE AROMATIC POTENTIAL OF WARM CLIMATE WINES

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Abstract

As far as consumers are concerned, the aroma and flavour of wine are among the main characteristics that determine its quality and value. The aroma of a wine is a unique mixture of volatile compounds originating with the grapes (varietal aromas), and secondary products formed during the fermentation of the wine (fermentative aromas) and during aging (post-fermentative aromas). The composition of the grape – which is unique to the variety and the *terroir* – makes a significant contribution to each wine, distinguishing one wine from another.

The potential varietal aroma is also important. Such potential arises from glycosidic and cysteinylated conjugates, which can contribute significant aromas to the product once they are freed from the bound fraction, either by enzymatic or acid hydrolysis during fermentation and aging, respectively.

In this paper, I will review our research of recent years on the characterization of the aromatic potential of red (Carménère and Malbec) and white (Sauvignon Blanc and Muscat) wine grape varietals grown in Chile and Argentina, and the evolution of this potential during fermentation and aging. An attempt to quantify the recovery yield of these potential aromas and their sensory impact on the final wine will also be presented.

1. Introduction

The aromas of a wine are linked to the different stages of its elaboration and to the *savoir-faire* of the oenologist, but fundamentally the bouquet of a wine is a reflection of the potential of the initial grape, the varietal and the *terroir*. Although a grape varietal may be grown in a remote geographic zone and is turned into wine through different techniques, the resulting wine will possess certain qualities inherent to the typicity of this varietal. Consequently, the identification and quantification of the aroma compounds present in the grape are vital, as they define, in large part, the quality of the final product (Ribéreau-Gayon et al. 1998).

Wines are made up of about 800 volatile compounds, present in concentrations that range from a few nanograms to hundreds of micrograms per litre. They make up the free fraction of the aroma of wine, and include the odour compounds. There are other groups of compounds as well, from the grape varietal, called precursors (the bound fraction in the final aroma), which constitute the aromatic potential of the wine. This is formed by non-volatile compounds, which, consequently, can never be perceived by the nose. Nevertheless, they are likely to liberate varietal aromas after hydrolysis during vinification or aging, depending on the nature of the precursor (Bayonove et al. 2000). This fraction of the wine aroma is the focus of this study, in which we present the results obtained by our team over the past few years.

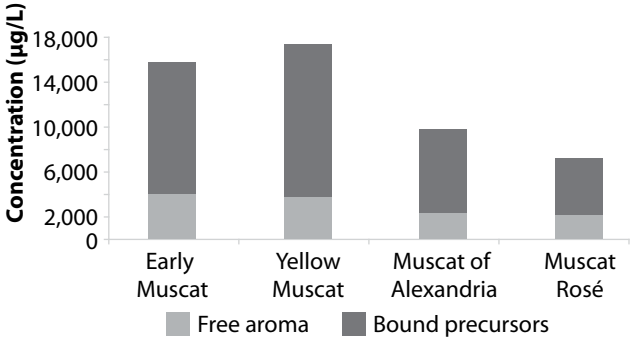
2. Glycosylated Aroma Precursors in Muscat Varietals

Terpineols are the basis for the typicity of Muscat grapes (Baumes et al. 1994). In the case of varietal aromas, terpineols contribute significantly to the typicity of wines through their aroma characteristics and thanks to a relatively low detection threshold.

The compounds responsible for fruity/floral aromas specific to these varietals are principally linalool, nerol, geraniol and, to a lesser degree, citronellol, α-terpineol, linalool oxides, alcohols (phenylethanol, hexanol, etc.), volatile phenols and C13-norisoprenoids. These compounds are present in part in a free form, but also bound to sugars, principally disaccharides. While all grape varietals have this type of precursors, Muscat grapes have the most, having, in general, a much greater quantity of glycosylated precursors than free aromas. These constitute the main part of varietal aroma and form the "aromatic potential" (Baumes et al. 1994, Bayonove et al. 2000). Acid or enzymatic hydrolysis of these precursors permits the freeing of these volatile compounds that increases the aromatic characteristics of the final product (Bayonove et al. 1992, Günata et al. 1990 and 1993).

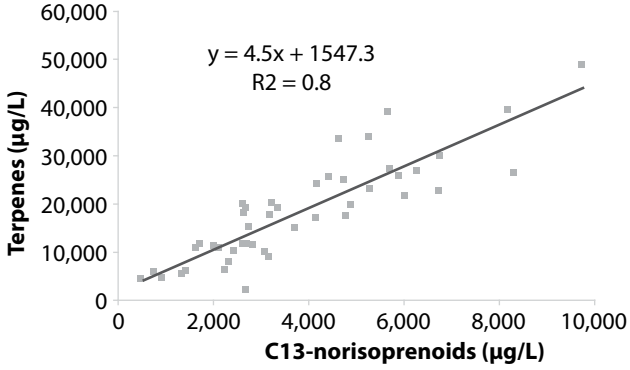
In Chile, Muscat grapes are mainly utilized to make Pisco, a young brandy with a fruity nose. The vineyards – about 12,000 hectares in total – are concentrated in the north of the country. The wine is distilled there and the alcohol possesses an *appellation d'origine*. Gas chromatography/mass spectrometry (GC/MS) analysis of the composition of free and bound terpenes in different Muscat grapes from the conservatory of the National Institute for Agrarian Research (INIA) in Chile has revealed the existence of varieties particularly rich in free and bound terpenes, in particular Yellow Muscat and Early White Muscat, as shown in figure 1 (Agosin et al. 2000). Paradoxically, both these varietals are found in small quantities in the field – less than 100 ha in the Pisco production zone. However, in Argentina the Yellow Muscat varietal, best known as *Torrontés riojano*, is much more present. As for the Muscat Rosé and Muscat of Alexandria (2,000 ha under plantation for each type), they are present in intermediate concentrations, comparable to similar varietals in Europe. Austria Muscat – present in a little more than 2,000 ha – has the least terpenes.

Figure 1. Concentrations of free and bound terpenes in some Muscat grape varietals in northern Chile (Agosin et al. 2000)



The study of the aromatic potential of more than 50 samples / varietals of Muscat of Alexandria and Muscat Rosé harvested in different sectors of the Pisco winegrowing region during the 2006 and 2007 harvests shows that the aglycones belonging to the terpene families and the C13-norisoprenoids represent more than 80% of the total compounds identified in each sample. Despite a great variability of concentrations of bound compounds in both these families, there is a strong correlation (R2 = 0.81) between the total concentration of terpenes and that of the C13-norisoprenoids, nearly 4.5 (figure 2) in both varietals, which could be related to their common ancestor – both are terpenes derived from the isoprenoid pathway – and to their accumulation in cell plastids and vacuoles in a water-soluble form.

Figure 2. Relation of glycosylated aroma precursors with C13-norisoprenoids and monoterpenes in a Muscat Rosé



The main terpenes, present in similar concentrations of about 7,000 µg/L in Muscat Rosé and Muscat of Alexandria, correspond to 3,7 diol and 2,6 dimethylocta-1,7-dien-1,6-diol. However, the Muscat of Alexandria presents linalool and geraniol concentrations 10 times greater than that of Muscat Rosé, which accentuate its floral character (table 1). As for the C13-norisoprenoids, they are present in the Muscat Rosé in concentrations from 500 to 10,000 µg/L, and double that in Muscat of Alexandria. The C13-norisoprenoids most abundant in the two varietals are 3-oxo-α-ionol, 3-hydroxy-7,8-dihydro-ß-ionol and vomifoliol, which confer floral, sweet and fresh wood notes to the wine (table 1).

3. Glycosylated Aroma Precursors in *Vitis vinifera* cv. Carménère

Vitis vinifera cv. Carménère is the emblematic varietal of Chile, which is not found in any other vineyards in the world. It is grown on about 10,000 ha. This cultivar was thought to have disappeared after the phylloxera epidemic in Europe, during the latter half of the 19th century. However, this varietal had already been present for a long time in Chile, but was confused with Merlot. In 1994, Dr.

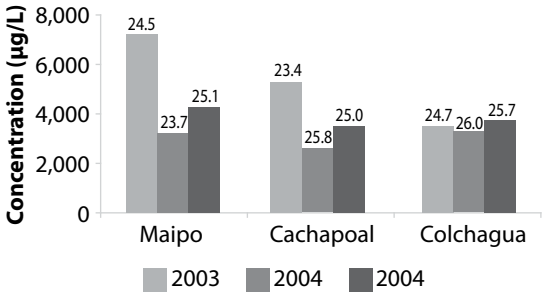
Table 1. Average concentrations of terpenes and C13-norisoprenoids in Muscat Rosé and Muscat of Alexandria grapes (northern Chile)

	Muscat Rosé			Muscat of Alexandria		
	Minimum µg/L	Maximum µg/L	Average n=50	Minimum µg/L	Maximum µg/L	Average n=50
NORISOPRENOIDS						
3,4-dihydro-3-oxo-actinidol IV	55	199		50	522	
3,4-dihydro-3-oxo-actinidol III	78	263		46	850	
3-hydroxy-beta-damascone	NA	267		62	761	
3-oxo-alpha-ionol	NA	1529		58	3577	
3-oxo-alpha-ionone	NA	NA		NA	89	
4-oxo-beta-ionol	78	261		31	1397	
3,4-dihydro-beta-ionol	NA	NA		NA	244	
3-hydroxy-7,8-dihydro-beta-ionone	35	1366		NA	58	
3-hydroxy-7,8-dihydro-beta-ionol (BIL3H)	176	835		114	2556	
4-oxo-7,8-dihydro-beta-ionol	NA	NA		NA	439	
3-OH-beta-ionone	NA	NA		NA	41	
3-oxo-7,8-dihydro-alpha-ionol	NA	162		NA	NA	
3-oxo-alpha-retro-ionol	NA	109		74	290	
3-hydroxy-7,8-dihydro-beta-ionol (BIL3D)	125	305		NA	868	
4,5-dihydro-vomifoliol	NA	NA		137	2450	
vomifoliol	NA	4315		351	7682	
TOTAL	**546**	**9611**	**3662**	**922**	**21825**	**4595**
TERPENES						
trans-linalool oxide	57	3832		114	1807	
cis-linalool-oxide	NA	369		36	102	
linalool	78	3246		780	20496	
neral	NA	NA		43	531	
alpha-terpineol	174	492		65	1824	
geranial + LOP-cis	NA	NA		NA	2255	
LOP-cis	74	2495		NA	115	
LOP-trans	NA	478		46	317	
citronerol	NA	NA		NA	73	
nerol	NA	541		166	3009	
geraniol	31	1438		534	10045	
diol-3,7	440	14759		629	13143	
3,7-dimethyloct-1-ene-3,7-diol	99	1017		68	988	
diol-3,6 (3,7-dimethyl-1,7-octadiene-3,6-diol)	NA	3051		98	3002	
Citronelool hydrate (3,7-dimethyloctan-1,7-diol)	NA	130		NA	2184	
3,7-dimethyloctan-1,7-diol	NA	NA		NA	273	
3,7-dimethyloct-1-ene-3,8-diol	55	1565		76	1548	
nerol hydrate	NA	151		38	850	
(Z)-2,6-dimethylocta-2,7-diene-1,6-diol	NA	10016		408	8777	
geraniol hydrate	79	1665		684	11611	
(2E,5E)-3,7-dimethylocta-2,5-diene-1,7-diol	NA	NA		79	1377	
(6Z)-2,6-dimethylocta-1,6-diene-3,8-diol	NA	NA		NA	505	
4-hydroxy-geraniol	NA	NA		NA	156	
(2Z)-3,7-dimethyloct-2-ene-1,8-diol	NA	NA		NA	500	
(2Z,6E)-3,7-dimethylocta-2,6-diene-1,8-diol	NA	NA		147	3082	
(E)-2,6-dimethylocta-2,7-diene-1,6-diol (diol-3.8)	236	2900		NA	NA	
(2E,5E)-3,7-dimethylocta-2,5-diene-1,7-diol	NA	131		NA	NA	
2,6-dimethyloct-1-ene-3,8-diol	NA	108		NA	NA	
(6E)-2,6-dimethylocta-1,6-diene-3,8-diol	NA	109		50	1185	
(2E)-2,6-dimethyloct-2-ene-1,8-diol	NA	132		32	574	
p-1-menthane-7,8-diol	259	694		65	594	
(2E,6E)-3,7-dimethylocta-2,6-diene-1,8-diol	NA	304		82	1619	
TOTAL	**1582**	**49624**	**17988**	**4237**	**92543**	**27628**
PHENOLIC DERIVATIVES						
4-vinylguaiacol	30	101		NA	NA	
zingerone	63	721		72	868	
guaiacol ethanol	77	544		83	1010	
4-vinyl-2-methoxyphenol	NA	NA		33	168	
3,4-dimethoxyphenol	NA	NA		67	338	
zingerol	NA	NA		105	619	
syringic acid	NA	NA		240	504	
TOTAL	**171**	**1367**	**841**	**600**	**3508**	**2108**

J. M. Boursiquot, from INRA Montpellier, and Professor P. Pszczólkowski, of the Pontificia Universidad Católica de Chile in Santiago, demonstrated that a large portion of the Merlot grapes in Chile were in fact the Carménère cultivar, an ancestral varietal from Bordeaux of great quality.

The Carménère has a high aroma potential. A study carried out over three consecutive years in three different valleys of south-central Chile quantified the glycosylated precursors at maturity. They varied from 3,000 to 8,000 µg/kg of grapes. The largest family is the C13-norisoprenoids, representing 50% to 60% of total precursors (figure 3). Among the 20 or so compounds identified in this family, the most abundant are derivatives of α-ionol (30% of total). The others are derivatives of β-ionol, β-ionone and 3-hydroxy-β-damascone, as well as vomifoliol.

Figure 3. Concentration of bound C13-norisoprenoids in Carménère grapes grown in three valleys of South-Central Chile measured over three consecutive years (2003, 2004 and 2005)



The numbers on the histograms indicate the °Brix of the grapes at harvest.

In order to verify the potential impact of these bound aromas on the quality of the wine during its development, we simulated the development of the precursors while bottled by utilizing a synthetic wine enriched with aroma precursors extracted from 3 litres of a Carménère wine. The synthetic wine was stored at 45°C for four weeks, which is roughly equivalent to two years of aging in a bottle at cellar temperature (15° to 17°C) (Schneider et al. 2001). After this period of incubation, we used GC/MS to quantify the aromas liberated during the accelerated aging process. In parallel, we studied the olfactory impact of these compounds through GC-sniffing (table 2). This is a very powerful technique as it allows us to determine, within the universe of compounds present, what the real impact of a given molecule is on the aromatic quality of the final product.

The results show that the accelerated aging resulted in a major release and formation of C13-norisoprenoids, volatile phenols, terpenes and lactones. Within the first group, the major release of β-damascenone, vitispiranes (spicy notes), 3-oxo-α-ionol, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) (kerosene notes), vomifoliol and its derivatives, as well as the derivatives ionone and ionol, should be noted. Also interesting to note is the appearance of Riesling acetal (fruity scent), a compound that is not found as a precursor, but comes from the transformation of a dihydro-β-ionone. Lastly, we found norisoprenoids that could not be formally identified, but are responsible for certain candied orange, herbaceous and fruity notes.

Table 2. Concentrations and descriptions of aromas in a synthetic wine enriched with precursors from Carménère grapes and submitted to accelerated aging (Belancic and Agosin, not published)

COMPOUNDS	Concentration µg/L	Descriptor GC-0
C13-norisoprenoids		
β-damascenone	40.6	Floral, fruity, baked quince, sweet grass
Vitispirane cis	50.3	Spicy, woody, herbal, green tea
Vitispirane trans	53.4	Spicy, woody, herbal, green tea
TDN	16.4	Kerosene, pharmaceutical, resinous
Actinidol ethyl ester (Isomer I)	67.3	Citrus, fruity
Actinidol ethyl ester (Isomer II)	87.3	Herbaceous, eucalyptus, floral
4,5-Dihydrovomifoliol	42.9	Cooked mint
3-oxo-α-ionol	38.8	Honeysuckle, apricot marmalade, tobacco
Riesling acetal	52.1	Fruity, herbal, sweet
3-hydroxy-β-ionone + 3-oxo-α-retro ionol	8.2	Fruity
3-keto-α-ionone or ketoisophorone	5.4	---
β-isomethyl ionone	21.3	---
α-ionone/ 3-hydroxy-7.8-dihydro-β-ionol	12.8	Floral
TDN derivative	13.7	---
TTN	9.0	Earthy, humidity, herbal
Vomifoliol derivative	17.4	Cooked fruit, prunes
Epoxy-α-ionone derivative (51.33 min)	13.6	Spicy, clove
Unk x192 (43.2 min)	9.5	Baked orange, orange marmalade
Unk 126 + X1 Norisop (51.1 min)	48.2	Fruity
Norisop X2 (58.1 min)	23.9	Straw, dry grass
Norisop X3 (60.4 min)	18.5	Herbal
TOTAL	**650.5**	

4. S-conjugate Precursors in Sauvignon Blanc Wines

Sauvignon Blanc wines present characteristic aromas the experienced tasters define as green pepper, tomato leaf, box tree, blackcurrant bud, grapefruit and exotic fruit. The compounds responsible for the grapefruit, exotic fruit and tomato leaf notes are thiols resulting from 3-mercaptohexanol (3MH), acetate from 3-mercaptohexanol (3MHA) and 4-methyl-4-mercaptopentanone (4MMP) (Darriet et al. 1993, Dubordieu and Darriet 1993, and Tominaga et al. 1996 and 1998a). The olfactory threshold of these compounds is very low: 20 ng/L for 3MH and 0.8 ng/L for 4MMP.

The fact that the Sauvignon Blanc grape has a relatively neutral taste that does not compare with the aromatic complexity of its wines led us to suppose the presence of aroma precursors in the grape, which are then revealed during alcoholic fermentation. The existence of these precursors could explain the phenomenon of "aromatic return" or lingering finish, described by various oenologists.

For the initial research, the presence of glycosylated precursors was presumed. However, Darriet (1993) showed that the utilization of glycosidase enzymes did not encourage the freeing of 4MMP. On the other hand, the utilization of a β-liase led to positive results, which leads us to suppose that the thiols are bound to the cysteine (Tominaga et al. 1995).

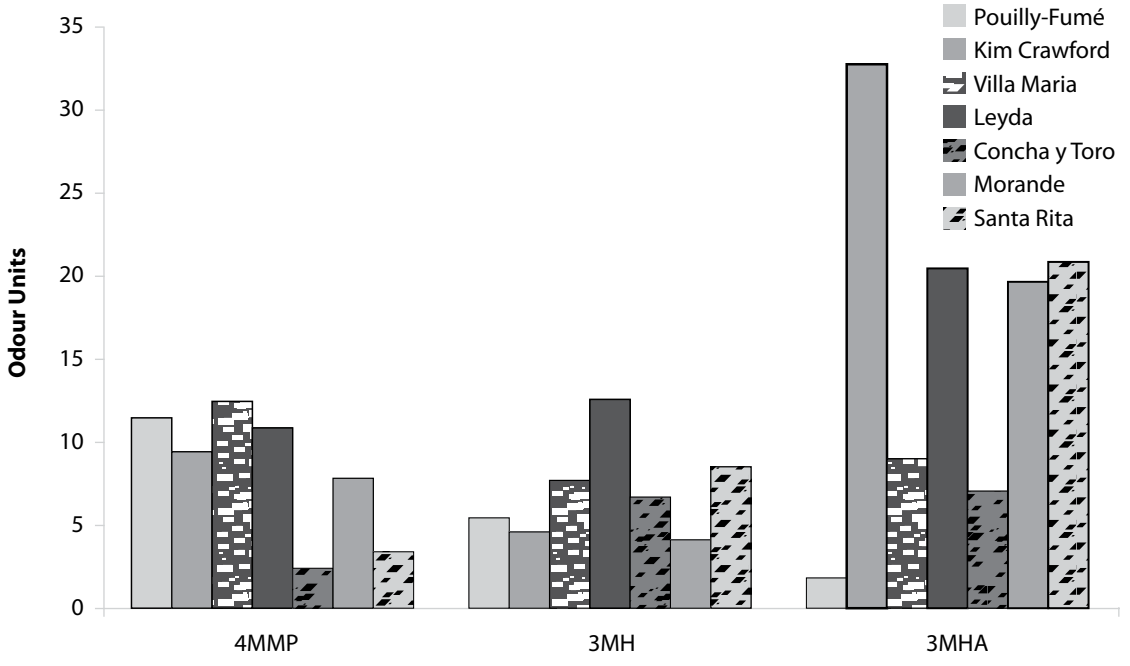
A few years later, Tominaga et al. (1998b) demonstrated the presence of precursors S-conjugated with a cysteine. The analysis of the fraction of these precursors identified the cysteine derivatives 4MMP, 3MH and 4-mercapto-4-methylpentan-2-ol (4MMPOH) (Peyrot des Gachons et al. 2000). More recently, the major presence of precursors S-conjugated with glutathione was shown (Peyrot des Gachons et al. 2002, Roland et al. 2010 and 2011, Capone et al. 2011, and Peña-Gallego et al. 2012).

4.1 Free thiol levels in Chilean and international Sauvignon Blanc wines

As no data were available for Sauvignon Blanc wines produced in Chile, in 2005 we analyzed the levels of 4-methyl-4-mercaptopentanone (4MMP), 3-mercaptohexanol (3MH) and 3-mercaptohexyl acetate (3MHA) in the Sauvignon Blanc wines from three vineyards in the Casablanca valley and one from the Leyda valley, as well as three other international wines: one from France and two from New Zealand (figure 4).

The results show that, in general, Chilean wines have high levels of thiols, greater than French wines and similar to New Zealand wines – more fruity and more floral. The greatest concentrations of 4MMP and 3MHA were measured in New Zealand wines, corroborating the results published in the literature, but the greatest concentration of 3MH was registered in the wine from Leyda valley (temperate climate and very close to the ocean).

Figure 4. Odour units for the 3MH and 3MHA thiols in Chilean and international (France and New Zealand) Sauvignon Blanc wines



4.2 Managing key variables to optimize thiol content in Sauvignon Blanc wines

4.2.1 Vine growing practices. We followed the accumulation of thiol precursors in the grape, mainly the cysteine precursors, P-3MH and P-4MMP. We evaluated the effect of the *terroir* (table 3), the harvesting method (figure 5) and the yield (figure 6).

Table 3. Influence of the *terroir* on the levels of thiol precursors in a Sauvignon Blanc grape

Precursors according to the valley of origin of the grape						
	Odour Units					
	Casablanca			Curicó		
	Min	Max	Average	Min	Max	Average
P-4MMP	240	980	600	150	770	370
P-3MH	170	960	400	28	270	90

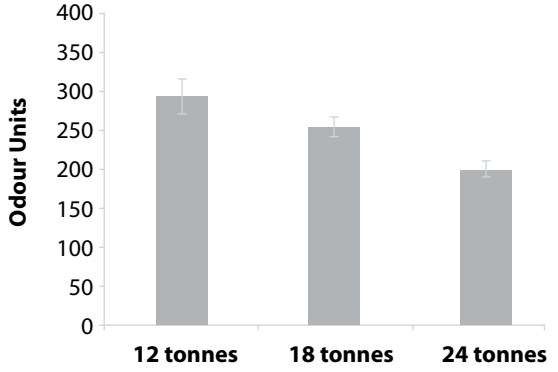
The effect of the *terroir* on the aromatic potential of Sauvignon Blanc is clear when comparing the grapes of Casablanca valley (temperate climate and later harvest) with those of Curicó valley, where it is very hot and vines are planted very densely (table 3). On average, the Casablanca valley produced grapes that were twice and four times higher in the 4MMP and 3MH precursors.

4.2.2 Studying the type of harvest – whether manual or mechanical – was particularly interesting because of its influence on the conservation of the potential of the precursors was ignored. Today, the type of harvest is essential to determine the production costs and, therefore, the competiveness of businesses. In both cases, an initial sample was taken in the vineyard in the morning of the harvest day, and a final sample was taken at the loading dock, directly from the truck that had transported the grapes to the weighing in.

In the case of the mechanical harvest, a significant loss of 55% of the P-4MMP precursor and 30% of the P-3MH precursor was recorded (figure 5), compared to the levels recorded in the vineyard. On the other hand, the loss was less for the manual harvest: down 14% for P-4MMP and 8% for P-3MH.

4.2.3 Studying the impact of yield (12, 18 or 24 tonnes per hectare) on the aromatic potential of Sauvignon Blanc grapes, carried out in Curicó valley – a hot climate zone, 200 km south of Santiago – showed a limited increase (33%) of P-3MH levels when the yield doubled, going from 12 to 24 tonnes/ha (figure 6).

Figure 6. Impact of grape yield (in tonnes per hectare) in the vineyard on the level of the S-cysteine precursor of 3MH (in odour units) in Sauvignon Blanc grapes grown in Curicó valley (2009)

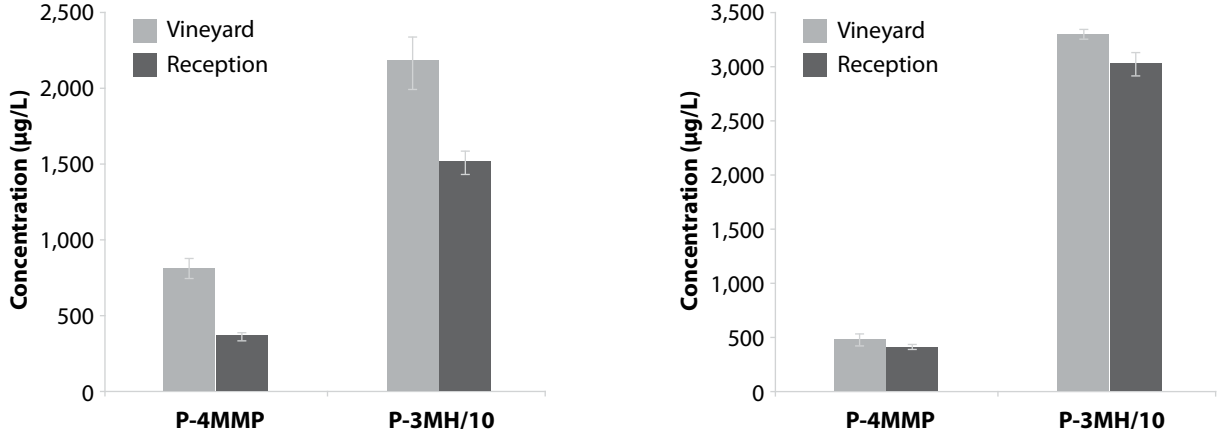


4.3 Oenological factors

On the oenological level, we evaluated the impact of the complete process (table 4), the specific steps of pre-fermentation and fermentation (figure 7), and he strain (figure 8) on the final concentration of thiol compounds in Sauvignon Blanc wines.

To do so, we weighed for the thiol compounds at the beginning and end of vinification, i.e., between delivery of the grape to the winery and the final dry, just after alcoholic fermentation. A very small part of the initial level of free thiols (2% to 5%) were still active and odour-produc-

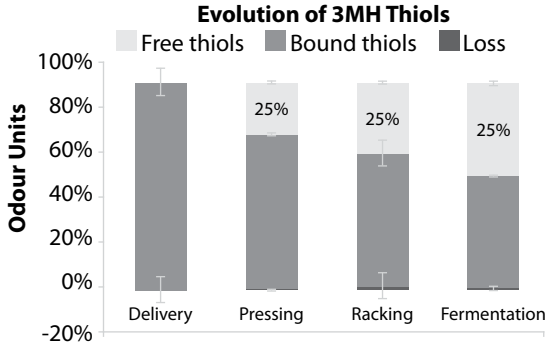
Figure 5. Impact of the type of harvest (manual/mechanical) on the aromatic potential of Sauvignon Blanc grapes



ing free thiols in the finished wine (table 4), both in the literature (research by Tominaga) and in our own research on thiols in vinification. These results are truly surprising and show that, for thiol compounds, a huge gap exists today between the beginning and the end of vinification, and, thus, show the opportunities that exist to recover a much larger quantity of these compounds through careful work in the winery.

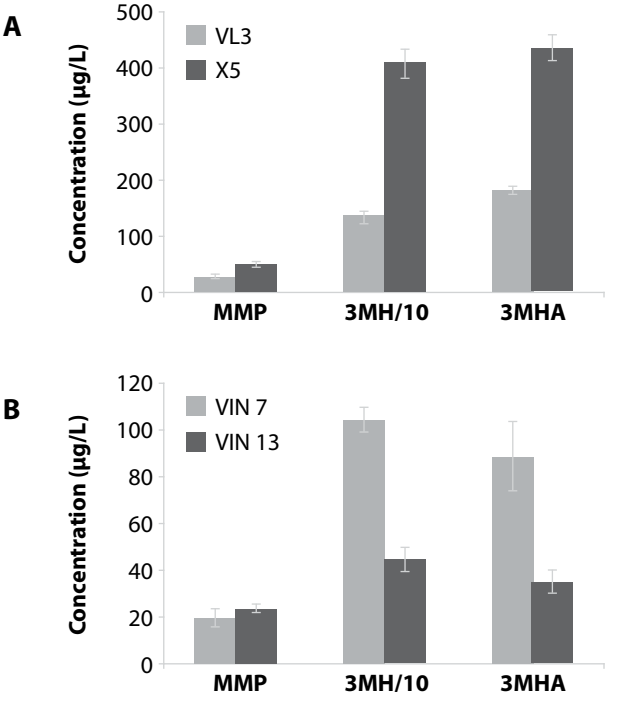
Later, by utilizing the same methodology, we researched the critical points in the loss of thiols during vinification. The results (figure 7) indicate that the pre-fermentation stage is the most important regarding the massive loss of thiol precursors, evaluated for three wineries and three clones of Sauvignon Blanc grapes. Indeed, losses vary from 45% to 92% for P-4MMP (results not shown) and 40% to 78% for P-3MH, while for the fermentation stage itself the results are less significant. Note that a major portion of the precursors remain non-hydrolyzed, and that the free 3MH is already present in the decantation, perhaps due to the action of the indigenous flora on these musts.

Figure 7. Evolution of free and bound 3MH (as percentage of total 3MH at delivery to the winery) during different stages of winemaking with Sauvignon Blanc grapes from Casablanca (2006 harvest)



Major differences were obtained with the different strains, as shown in figure 8.

Figure 8. Impact of the strain (VL3 vs. X5; wine 7 vs. wine 13) on the freeing of cysteinated thiols during the fermentation of Sauvignon Blanc grape must from Concha y Toro (A) and Morandé (B), respectively; each trial was carried out in duplicate in 5,000-litre vats (2006 harvest)



5. Conclusions

Regarding glycosylated precursors, we have shown there are Muscat varietals with particularly high levels of terpene precursors, such as Yellow Muscat (*Torrontés riojano*) and Early Muscat, which present great potential for development and are currently underused. Regarding the grape harvest, from a quantitative perspective there is an on-going relationship between the total level of C13-norisoprenoids and terpenes, which may be related to their common origin, the isoprenoid pathway.

As for the Carménère, the emblematic varietal of the Chilean vineyard, it has been shown to be particularly rich in C13-norisoprenoid precursors, which leaves us to pre-

Table 4. Free thiols in Sauvignon Blanc wine compared to the initial concentration of precursors in the grape at harvest

Thiol	Free thiols in Sauvignon Blanc wine according to the literature* (as a percentage of the initial precursor)			Free thiols in Sauvignon Blanc wine made in the Casablanca valley, Chile (as a percentage of the initial precursor)		
	Minimum	Maximum	Average	Minimum	Maximum	Average
4MMP	0.1%	4.1%	1.4%	0.1%	2.0%	0.9%
3MH	2.3%	9.5%	4.2%	0.9%	3.6%	2.0%
*Tominaga et al. 1998						

sume that the wines from this varietal have great potential for development, at least in terms of aroma.

Lastly, we can affirm that a great number of factors exist at the vineyard level that are likely to influence the levels of cysteinated precursors in Sauvignon Blanc grapes. However, we have shown that the rate of transformation of bound thiol precursors initially present in the grape into free thiols in the actual wine is very low, generally less than 5% to 10%. Consequently, future research into these precursors during the pre-fermentation stages and during alcoholic fermentation is fundamental to the final quality of Sauvignon Blanc wines and to their mastery, certainly more important than the research to attain the maximum level of precursors in the vineyard.

Acknowledgments

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MERGING ECOLOGY AND WINE MICROBIOLOGY

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Abstract

The field of wine science has a long history, with many excellent discoveries regarding the chemistry of wine and the biochemistry and genetics of the microbes involved in the process. However, the ecology of microbes associated with vines and wines has been studied comparatively little. I will briefly introduce concepts and techniques from the field of ecology, show how they may be used in wine science, and provide some appropriate examples.

1. Introduction

In many ways, the history of wine science follows the history of science, especially for the microbiological aspects of wine (Chambers and Pretorius 2010). Arguably the first biotechnology application by humans, the unwitting use of microbes to ferment grain to bread and beer, and fruit to wine, likely started around the dawn of civilization (McGovern et al. 2004, and McGovern et al. 1996). It was only relatively recently that Pasteur showed the process of fermentation was not magic, but due to microbial actions (Chambers and Pretorius 2010). Since then, microbiologists have increasingly scrutinized the yeasts responsible for fermentation, and it has transpired that these single-celled fungal organisms are extremely amenable for experimentation. Because of the desire to know more about the organisms that drive fermentation, and the ease with which experimenters can handle and manipulate yeasts, these organisms quickly became model research tools (Zeyl 2006, Landry et al. 2006, Greig 2007, and Replansky et al. 2008). We have learned a great deal about the genetics, biochemistry, molecular biology and cell biology of eukaryotic organisms due to work on the species that is principally involved in fermentation: *Saccharomyces cerevisiae*. Indeed, this species was the first eukaryotic organism for which we derived a whole genome sequence (Goffeau et al. 1996), and Nobel prizes have been awarded for work with yeast cell division as this relates to understanding cancer (www.nobelprize.org/nobel_prizes/medicine/laureates/2001/nurse-bio.html).

It is fair to say that we know an enormous amount about the biochemistry, genetics and molecular biology of the handful of *S. cerevisiae* strains that have been scrutinized. However, we have comparatively little understanding of the genetic or phenotypic diversity of yeasts, the distribution of yeasts in space and time, nor the ecology of these organisms in general, associated with vines and wines or otherwise. This is true at both the species level, and within species at the population level. For example, we still do not have adequate answers to the simplest of questions: Do we find the same yeast species associated with vines and wines across the globe? Do yeast communities differ from place to place? If they do differ, at what scale does this difference manifest? Within the region, the country, the continent? Equally, our knowledge of diversity within the species is poor. For example, do we find the same strains of *S. cerevisiae* in different places, or are there different strains in different regions? What about diversity within other species? There has been a long debate over the degree to which the *S. cerevisiae* associated with vines and wines is “domesticated.” What is the evidence for this?

These questions are fundamental to understanding the diversity and distribution of organisms generally, and, perhaps, to how human environmental manipulations in the form of agriculture may modify this. However, there is another aspect to rigorously understanding diversity that is commercially relevant, stemming from the abundant literature showing that different yeast species and strains within species may produce different ferment products that affect wine aroma and flavour (Swiegers and Pretorius 2005, and Pretorius 2000). As this is the case, it appears imperative to understand the population and ecological diversity of yeasts associated with vines and wines. Not least, this might inform us if there are any consistent patterns that would aid strain screening, i.e., is it worth gathering a diversity of strains from different places or not? Possibly not, if there is no evidence for regional differences in yeast communities or populations. The other aspect of obvious relevance is connected to the elusive but compelling (and marketing genius) idea that wine has a sense of place as it reflects the region where it was grown and made, as well as the vintage. The concept of *terroir* usually refers to the soil and climate of a region, but if microbes are also region-specific then they may also affect how vines develop, the fruit quality and composition (with the obvious example of how pathogenic fungi affect fruit quality), and, if the wine is spontaneously fermented, the flavour and aroma. Perhaps there is a microbial aspect to the *terroir*, but this has yet to be proven objectively. Many believe that different regions harbour different and unique microbes, but it is the role of science to determine if this is the case.

2. The Application of Population Biology and Ecology to Wine Microbiology

The field of wine microbiology, like the field of microbiology generally, has its roots in the discipline of biochemistry. The excellent and insightful work from this perspective has made large and significant advances in our understanding of the workings of cells – the biochemical reactions that allow microbes to exist and exploit certain niches, the genetics that support this, etc. This approach, by definition, focuses on characterizing the properties of the individual cells/clonal populations of a specific genotype, but is not focused on and does not provide the tools for quantifying the variance of traits within and among populations and species. To do the latter, we need to employ tools developed in the fields of ecology and evolution.

The crux of an ecological approach acknowledges that when samples are taken, only sub-samples from very large populations or communities are taken. Thus, to determine whether populations or communities from different sample points of interest differ, we need to calculate whether the variance between samples is any greater than one would expect to see by chance. This means that 1) we need replicate samples in order to calculate variance, and 2) we must then apply an appropriate statistical test to determine if any differences seen are more or less than one would expect to see by chance under a null hypothesis of no difference. From here on, I will primarily consider communities of yeasts (incidence of species), but similar analyses and considerations hold for bacteria, with the acknowledgement that these are haploid asexual organisms and, thus, the concept of species here is circumspect. Similar concepts underlie the population genetic analyses of individual populations of species (in this case *S. cerevisiae*), but the tools needed here are those drawn from population genetics and there is not space here to review that vast subject. For wine-specific studies that have analysed *S. cerevisiae* populations, please see Goddard et al. 2010, Gayevskiy and Goddard 2012, and Cubillos et al. 2009.

2.1 Analysing communities

Imagine we want to test whether the types and abundances of fungal species, i.e., fungal communities, associated with vineyards differ among different major wine-growing regions. One tempting response is that there are likely billions of cells and tens to hundreds of species, and it is therefore impossible to determine this. The same would be true for estimating differences in the collections of any species in most niches. However, if we take replicate random sub-samples from regions, this will allow us to estimate the variance in the species present and their incidence, which can then be compared to estimates derived from a similar number of random samples from another region, and determine whether there is greater variance between samples from different regions. Replicate and random are key here. Without replication we cannot estimate variance: single samples do not allow us to estimate the effect of sampling error. If these sub-samples are not random, the bias introduced prevents a determination of true differences between regions. In addition, we need an adequately sized sub-sample of individuals for each replicate. Too small a sub-sample will lead to larger variance estimates and a lack of power to reject the null hypothesis. Larger samples are preferable, but too large may be a wasted effort. The question of sample size is, unfortunately, a function of the diversity and incidence of species present and thus unknown before the samples are taken. As a rule of thumb, a minimum of six sub-samples per region should be aimed for, with about 100 individuals in each sub-sample. So, if one wished to compare just

two regions, this would require 6 × 2 ×100 = 1,200 individual yeast colonies to be identified.

The classic way to proceed is to, first, identify each individual in the sub-sample to species level (see section below), and then produce a matrix of species and their incidence in each sub-sample. Tabulating summary statistics of these species incidences, such as alpha diversity and the Shannon/Simpson's index, is informative, but does not allow an objective evaluation of whether any difference observed among regions is more or less than one would expect by chance. The null hypothesis that there is no difference in the communities from each region, thus any difference observed is due to chance (sampling error) and must be tested. Conceptually, this null hypothesis could be imagined as a very large (in fact, an infinitely large) jar containing a number of species at different incidences. The first sub-sample draws 100 individuals from this jar, the second another 100 and so on. The null hypothesis assumes that all samples from all regions are drawn from the same jar, and thus all samples are drawn from communities with the same underlying types and proportions of species. Of course, each sub-sample will be different due to sampling error, but on average this will be about the same difference for all samples. Thus, no matter how various sub-samples are partitioned, there will be on average no difference between samples within and between partitions.

There are many ways in which similarities between individual samples may be assessed, but the Bray-Curtis dissimilarity is a classic statistic used to quantify the compositional dissimilarity between two different samples, based on counts in each (Clarke 1993). If the number of individuals analyzed differs between samples, they should be normalized before comparison. It is also usual to square root transform the normalized counts to reduce the effect of common or rare species. The Bray-Curtis statistic is bounded between zero, where both samples have identical species incidence, and one where samples do not share any species.

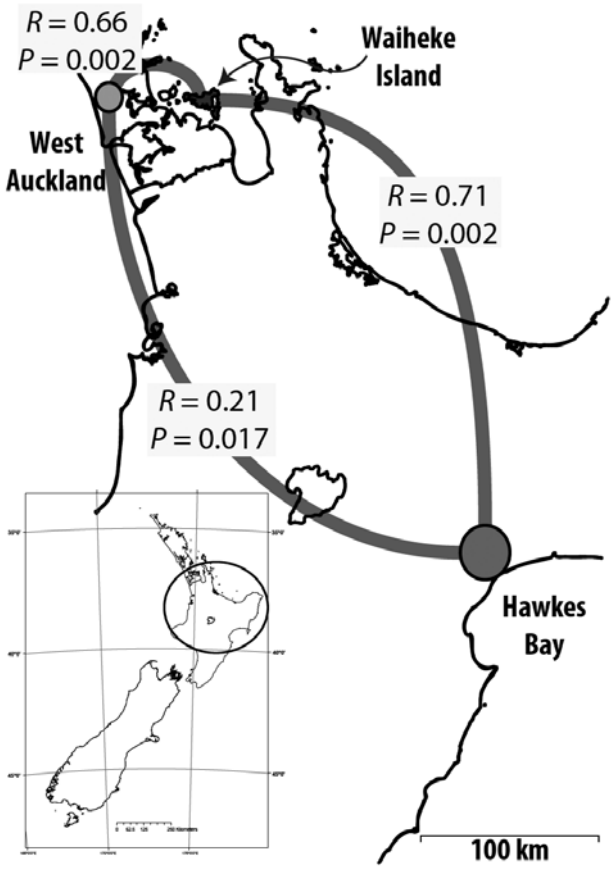
Calculations of similarities are interesting, but one really needs to determine if such dissimilarity measures are different between samples from different regions compared to samples from within regions. One way to achieve this is to employ an analysis of similarities (ANOSIM) test (Clarke 1993). Any dissimilarity statistic may be used for this test, and an ANOSIM first calculates all possible pairwise similarities between community samples, then ranks them. If groups of samples from different regions are really different in their species composition, then compositional dissimilarities between the groups ought to be greater than those within the groups, and the rank of comparisons will reflect this. If communities between regions are not different, then the within- and between-group sample comparisons will be intermingled in their rank. This comparison of ranks generates an *R* statistic based on the difference of mean ranks between groups and within groups. The statistic ranges from -1 to +1: a value of zero indicates the samples are collected into groups (regions) randomly, with respect to their dissimilarity; a value of +1 means there is complete delineation between samples from groups (regions); and negative values mean samples from different groups are more closely related than within groups. Whether the observed *R* statistic is any greater or less than might be seen by change given the data is determined by a permutation approach. The grouping of each sample is permuted and *R* recalculated a number of times (typically 1,000 or more) to obtain the empirical distribution of *R* under the null-model of no difference between groups (regions). The ANOSIM is quick and robust in that it makes no assumption about the distribution on the data, i.e., it is non-parametric. This may be achieved using the anosim function in the R (2011) vegan (2012) package. A more user-friendly implementation of an ANOSIM may be found in PAST (folk.uio.no/ohammer/past). Recently, some concerns about the performance of the ANOSIM have led to advice that a similar approach, which employs Permutational Multivariate Analysis of Variance Using Distance Matrices should be used. This may be achieved using the adonis function in the R vegan package and it is directly analogous to multivariate analysis of variance (MANOVA) (McArdle and Anderson 2001). Indeed, the vegan package is specifically written for analyses in community ecology, and may be used to perform a wide range of simple and advanced calculations and tests using the functions within.

2.1.1 An example

Gayevskiy and Goddard (2012) used precisely the above approach to analyze the fungal communities isolated from ripe Chardonnay fruit from six vineyards in each of three regions on the North Island of New Zealand (see figure 1). They identified 1,566 colonies from Chardonnay fruit, and an ANOSIM analysis shows that pairwise comparisons among all three regions produced positive *R* values that are significant ($R>0.22$; $P<0.017$), with regional comparisons with Waiheke Island showing the largest difference ($R>0.66$; $P=0.002$), as shown in figure 1.

These data and analyses objectively show that in the 2010 vintage randomly sampled fungal communities from ripe Chardonnay fruit showed a much greater difference between the New Zealand regions than would be expected

Figure 1. Three regions on the North Island of New Zealand



by chance. That is to say, they differ. This statement is clearly based on the assumption that these samples are randomly drawn from the fungal communities on ripe Chardonnay from these regions. If these samples were not a random draw from these communities, this estimate would be biased. The point to note is that any mechanisms behind any bias here would have to be consistent by region for a signal of community differentiation to be inferred. As this is a sample at one time point only, clearly no statement can be made about how this observation of difference may translate to other years. Table 1 in Gayevskiy and Goddard (2012) shows that *Rhodotorula glutinis* is the dominant species in West Auckland and the Hawkes Bay, but that *Aureobasidium pullulans* is dominant on Waiheke Island; this will contribute the significant differences inferred. Further analyses may be conducted to objectively determine which species, or their incidence, might be driving community differences, for example by removing species and recalculating the *R* or multivariate ANOVA statistic.

2.2 Methods of estimating communities

It is worth briefly considering the methods that are available to estimate species incidence in microbial communities. The classic approach relies on culturing individuals from sub-samples in the laboratory in order to identify the colonies that arise. Originally, this identification was based on morphological and biochemical traits, which are unreliable due to their plasticity, and, more recently, molecular approaches rely on some area of the genome being amplified with polymerase chain reaction (PCR) and analysed via restriction fragment length polymorphism (RFLP) (Esteve-Zarzoso et al. 1999), or by directly sequencing this area and comparing it to databases (Kurtzman and Robnett 1998). For yeasts and fungi in general, the ribosomal repeat internal transcribed spacer (ITS) region or the divergent domains of the rDNA 26S are commonly used, as these contain enough information to differentiate between species.

Other than direct microscopic examination of samples, culture-based approaches were originally all microbial ecologists had at their disposal. Culture-based approaches, while powerful, have two significant drawbacks. First, not all microbes may grow on the particular artificial media a researcher chooses to use. It is well documented that ~99% of soil bacteria are unculturable under standard conditions (Hugenholtz et al. 1998). A researcher may spend a life's work elucidating the media nuisances that certain microbial species require for growth. This would be an endless and unfulfillable task as, by definition, one does not *a priori* know the complete list of species present in a sample, and thus when they have all been recovered, and so when to stop trying new media. Second, culture-based approaches put practical limits on the number of individuals sampled: even the most fastidious of researchers will probably not identify more than 200 colonies for any given sample. This means that rare species will likely be missed. For example, there is a 63% chance that a species present at 1/200th of the community will be missed if 200 colonies are analysed.

Recent significant advances in massively parallel DNA sequencing technologies now mean that a culture step may be circumvented. Now, nearly all DNA may be extracted directly from a sample of interest, a pair of PCR primers be extracted that amplify a diagnostic region from the range of taxa of interest, and hundreds of thousands to millions of the resulting PCR molecules may be sequenced. Such deep community sequencing most certainly affords a deeper insight into the communities under study. However, while the sample size is three to four orders of magnitude greater than those of culture-based approaches, such samples are still sub-samples from much larger populations and thus require the same replicate and random sampling considerations and appropriate statistical treatments discussed above.

Taylor et al. (2013) have used this approach to examine the very same Chardonnay samples from West Auckland and the Hawkes Bay that Gayevskiy and Goddard (2012) analysed, and included additional replicate samples from Marlborough and Central Otago. DNA was extracted from these 24 samples, the D1/D2 26S rDNA region amplified, and the resulting PCR products were pooled (with sample identifiers) and sequenced on a 454 Life Sciences GS FLX instrument. As the data gathered are DNA sequences (reads), they require an amount of bioinformatic processing before statistical analysis. One of the first challenges is to elucidate the number of species. At the D1/D2 26S region there is about a 2% variance in sequence within a species (Kurtzman and Robnett 1998). Thus, if sequences are clustered into cohorts with 98% similarity then this is a proxy for delimiting species in these data, and these are labelled operational taxonomic units (OTUs). Taylor et al. recovered 95,170 reads and they clustered into 253 98% OTUs from ripe Chardonnay fruit in New Zealand; 59 and 67 OTUs were seen in the West Auckland and Hawkes Bay samples respectively, some tenfold more species than seen in culture-based approaches (4 and 6 species respectively – see table 1 in Gayevskiy and Goddard 2012) implying that culture-based approaches may miss ~90% of species. Taylor et al. went on to conduct MANOVA and revealed that overall fungal communities differ by region ($F[3,19] = 4.17$, $P<0.0001$), with all regional pairwise comparisons being significantly different ($P<0.004$), apart from Hawkes Bay with both West Auckland and Marlborough. This alternative deep community sequencing approach is in line with the traditional culture-based approaches, and shows a much greater difference between these New Zealand regions that one expects to see by chance. That is to say, they differ.

3. The Limit of Community-level Analyses and the Way Forward

The analyses described above show that differences exist between these fungal communities, but these analyses afford no insight into the factors driving these differences. In principle, there are three broad mechanisms to explain why communities differ: history, selection or neutral processes (Hughes Martiny et al. 2006). These concepts are fundamental to evolution, and are the main processes that explain how species and thus communities may come to be differentiated. First, historical events may explain why these communities differ: if, at some point in the past, the fungal communities in each region were seeded by differential collections of species, this may explain the differences. Second, whether different regions were seeded by different ancestral fungal communities or not, the various climatic and physical properties of each region will induce differential selection pressures and adaptive evolution will mean those species, and thus communities, will better adapt to the prevailing conditions in each region and therefore prevail. Third, by chance different species of fungi may have come to be in different places, and further chance events may mean that some species persist while others do not. If there is sufficient dispersal of fungi among different regions, this will serve to homogenize these communities and overshadow this effect. However, if there is a lack of dispersal of fungi among these regions, this would give rise to different communities in different regions over time.

Determining which of these processes is operating is not trivial, especially as, in reality, it will be some combination of them (Hanson et al. 2012). Phylogenetic analyses may provide insights into the historical patterns of species assemblage, and would require the sequencing of a large amount of DNA from various communities. The advent of next-generation sequencing technologies means this is now possible. In principle, one may evaluate whether different fungal species are differentially adapted to the environmental conditions that vary between regions. This is easily achieved by determining the growth rate of various fungi in the laboratory, where all is held constant, apart from the factor of interest. One obvious environmental condition could be temperature. The difficulty is first defining the value of the parameter to be measured: Should it be mean temperature or the lowest or highest temperature? A similar problem might arise for any other environmental parameter. The researcher might strike it lucky and determine that there is a positive correlation between the degree to which fungal communities from different regions are adapted to environmental conditions that also differ between regions. If this is the case, it may be inferred that at least selection has a hand in defining community differences by region. On the other hand, if no correlation is found, that would mean the research can supply no evidence that selection for that particular environmental variable defines community difference by region, but it does not mean that selection for some other variable not measured is important. Tests for neutral processes may be conducted from the assumption that, under a neutral framework, different species from different communities are functionally equivalent (Volkov et al. 2003, and Bell 2000). Once again, this requires the fitness of different fungi from different regions to be evaluated and compared. Again, the problem is the conditions under which to assess fitness: they need to be as realistic as possible, but achievable under conditions where fitness may be evaluated. Our laboratory is currently working on testing each of these aspects.

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ENZYME-CATALYZED MODULATION OF THE TYPICITY OF TOURIGA NACIONAL AROMA AND FLAVOUR

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Abstract

The typicity of Touriga Nacional (TN) wine aroma is known to be characterized by a bergamot-like, fruity/citrus/floral aroma, attributed to the terpenol linalool and its acetate. Whereas commercial enzyme preparations with high levels of glucosidase activity are commonly used to release aromatic terpenols from their odourless precursor glycosides, the practice has been rather restricted to applications with aromatic white grape cultivars. This work focuses on the possible modulation of key odorants responsible for the bergamot aroma in TN, while investigating the effect of a commercial pectolytic enzyme preparation rich in beta-glucosidase activity. Respective treatments resulted in significant increases in the levels of the wine terpenols, particularly linalool and geraniol, along with corresponding intensifications of the associated characteristic bergamot aroma. Enzyme-treated wines maintained greater floral/citrus aroma complexity throughout the trial aging period of 2.5 years. This study demonstrates the successful enzymatic-induced aroma modulation of key odorants of wine, made from the red grape variety Touriga Nacional.

1. Introduction

Vitis vinifera Touriga Nacional (TN) is considered by many to be the top Portuguese autochthonous red grape variety (Mayson 1992, and Robinson et al. 2012). It has been suggested that its name is derived from the village of Tourigo (Galet 2000) in the district of Viseu from the Dão region; it has numerous synonyms (Robinson et al. 2012), mostly from the Dão region: Mortagua, Mortagua Preto, Touriga or Touriga Fina, Tourigo Antiguo (Dão), Tourigo do Dão (Bairrada) and Azal Espanhol (Ribeiro, Spain).

Over the past few decades, clonal selection has led to improved disease resistance, as well as superior quality, which has raised the cultivar's profile and popularity (Martins 2012). Presently, over 11,000 hectares are planted throughout Portuguese wine regions (ViniPortugal 2013). The variety has also been recognized overseas, with the emergence of plantations in Argentina, Australia, Brazil, New Zealand, South Africa, Spain and in the United States (Robinson et al. 2012).

TN grapes, which are characteristically small and black, make deep-coloured, powerful, tannic wines that retain their fruit with age (Mayson 1992). The TN aroma is complex, often characterized by the presence of dark fruit (plums, blackcurrants, blackberries), red fruit (raspberries), citrus fruit (bergamot), floral notes (violets and orange blossom), spice (black pepper and licorice) and fresh (mint and eucalyptus), along with wood-aged character (wood, resin and vanilla) and, in the case of aged tawny Ports, nutty aromas.

TN is considered the finest Port wine grape in the Douro region (Stevenson 1988), where it is traditionally used in blends. It is also gaining a reputation as a quality monovarietal DOC wine from the Dão, Douro and Alentejo regions (Antunes 2012).

2. Touriga Nacional Varietal Aroma

TN wines are often distinguished by their fragrances, such as bergamot, rosemary, rockroses or violets (Robinson et al. 2012). The following section reviews the published literature.

2.1 Sweet balsamic rockrose (*Cistus ladanifer*)

The chemicals ethyl 2,3-dihydrocinnamate (sweet, fruity, resin, balsamic-like) and 2,2,6-trimethylcyclohexanone (woody, hay-like), which were both identified as key odorants of the rockrose leaf (Ramalho et al. 1999), were subsequently identified and quantified in a single varietal: Touriga Nacional Port wines (Freitas et al. 1999). Whereas Port wine was shown to contain uninteresting sub-threshold levels of 2,2,6-trimethylcyclohexanone, significant levels were registered for ethyl 2,3-dihydrocinnamate (2.3 to 6.7 µg/L; threshold = 1.9 µg/L, Freitas et al. 1999). The chemical 2,2,6-trimethylcyclohexanone is an important impact chemical, which contributes a sweet, balsamic, rockrose-like aroma to Port wine. Douro winemakers associate rockrose aroma with both monovarietal DOC table wines and Port wines.

2.2 Floral: Violets

The floral violet aroma note of wine has been attributed principally to the presence of the norisoprenoid compound β-ionone (Etievant et al. 1983, and Kotseridis et al. 1999). Despite monovarietal TN wines often expressing floral violet descriptors (Martins 2012, and Antunes 2012), to our knowledge, there has been no dedicated study investigating β-ionone.

2.3 Floral: Terpene alcohols

Monoterpenes are the C-10 representatives of the terpenoid family of compounds. The typical cultivar bouquet found in varietal Muscat wines is characterized by floral aromas, composed essentially of terpenols, including linalool, α-terpineol, citronellol, nerol, geraniol, hotrienol and oxides of linalool (Usseglio-Tomasset 1966, Ribereau-Gayon et al. 1975, Bayonove et al. 1971, and Williams et al. 1981). Floral non-Muscat varieties, such as Gewürztraminer and Riesling, contain significant but lower levels of monoterpenols, while more "neutral" cultivars, such as Chardonnay, contain only trace amounts, which are thought to not contribute to flavour (Versini et al. 1981, Strauss et al. 1986, and Sefton et al. 1993).

Whereas free terpenol levels have been shown to make important floral contributions to single varietal Portuguese white wines (Guedes de Pinho 1991, Rogerson and Silva 1994, Rogerson et al. 1995, and Rogerson and Silva 1996), few studies have targeted red varietals. The first investigation identified cultivar Touriga Nacional as the richest of seven Douro varietal wines with 110.5 µg/L total free terpenols (Rogerson 1998, and Rogerson et al.1999). A second group (Barbosa et al. 2003) made a similar observation, this time ranking TN first in a comparison of monovarietal wines made from five different Portuguese red varieties from different regions.

Monoterpene alcohols also exist in grapes and wine as bound odourless forms, linked to β-D-glucose, which, in turn, is often linked to one of the sugars β-apiose, α-L-rhamnose or β-L-arabinofuranose (Williams et al. 1982, and Brillouet et al. 1989). Varietal aroma can be enhanced through aglycone monoterpene release, catalyzed by endogenous glycosidase enzymes (grape, yeast and lactic acid bacteria in origin), and also by acid hydrolysis; however, neither is capable under normal winemaking conditions of liberating all bound aromas (Canal-Llauberes 1993, Colagrande et al. 1994, and Ugliano 2009). Further enhancement is achieved through the addition of suitable glycosidase activities (Günata et al. 1988, and Voirin et al. 1990).

Whereas commercial enzyme preparations (*Aspergillus niger* in origin) containing high glucosidase activity are commonly used for the release of aromatic terpenols from their odourless precursor glycosides, the practice has been rather restricted to applications with aromatic white grape cultivars, such as Muscat, Gewürztraminer and Riesling. The application of a glycosidase enzyme preparation to a red wine made from TN grapes almost tripled the total free terpenol content (110.5 to 293.4 µg/L), indicative of a large untapped reserve (Rogerson 1998, and Rogerson et al. 1999).

2.4 Citrus/floral/bergamot aroma

Higher quality single varietal TN wines, which command higher prices in the market, often present a characteristic aroma recognized by experts as bergamot-like (Guedes de Pinho et al. 2007). Aroma Extraction Dilution Analysis (AEDA) of a bergamot essential oil identified 13 compounds responsible for the characteristic citrus/lemon/lime aroma, including linalool (Flavour Dilution [FD] = 512), and linaloyl acetate (FD = 256). Gas-Chromatography-Olfactory (GCO) and GC-Mass Spectrometry (MS) analysis of a TN wine extract successfully identified linalool and its acetate present in the second of three target

zones, which had been described as the most bergamot-like, with an Earl Grey descriptor (Guedes de Pinho 2007).

Building on initial findings (Symington et al. 2011), the present study focuses on the possible modulation of the key odorants/flavorants responsible for TN bergamot aroma/flavour, investigating the effect of commercial pectolytic enzyme preparations, rich in glycosidase activity.

3. Materials and Methods

3.1 Wine production (harvest years 2007, 2010, 2011 and 2012)

A standard industrial fermentation procedure was used for all Symington TN wines. A commercial pectolytic enzyme preparation was applied at berry crushing, with the objective of improving berry maceration and pigment extraction. On completion of both the alcoholic and malolactic fermentations, a 20 litre aliquot of wine was removed, sulphited (100 mg/L), and divided into two 10 L portions. The first portion underwent no further treatment (the control); the second received a glycosidase-rich pectolytic enzyme preparation (table 1). The wines were subsequently bottled and stored in the company's cellars. The 2012 trial compared the performance of the glucosidase-rich Prozym enzyme to three other commercial preparations, Enz A, Enz C and Enz D, using recommended dose levels.

Table 1. Glucosidase-rich enzyme dose levels

Year	Lallzyme beta	Prozym Aroma V
2007	10 g/hL	-
2010	-	10 g/hL
2011	-	5 g/hL
2012	-	5 g/hL
Note: 2007 and 2010 trials used twice the recommended dose		

3.2 Gas Chromatography-Mass Spectrometry

Quantitative analysis of the odorants linalool, α-terpineol, citronellol, nerol and geraniol was performed, either by SPME-GC-MS (Silva Ferreira et al. 2003a) or by liquid-liquid extraction and GC-MS (Silva Ferreira et al. 2003b).

3.3 Sensory evaluation: 2-Alternative Forced Choice for bergamot intensity (2007 and 2010 wines)

Wines were evaluated by an industrial panel comprising six expert tasters. Each taster had been familiarized with the typical bergamot aroma using 1) two drops of a bergamot essence extract (Segredo da Planta, Produtos Naturais e Biológicos Lda, Seixal, Portugal) diluted in 100 mL 20% aqueous alcohol, or 2) Earl Grey tea (bergamot infusion).

Each enzyme-treated/non-treated wine pair was ranked for bergamot aroma intensity (1 = lowest and 2 = highest) according to the 2-Alternative Forced Choice (2-AFC) test using a randomized complete block design (Meilgaard et al. 1999). Each panellist was asked to rank four pairs of 50 mL wine aliquots presented in tulip-shaped Port-wine glasses.

3.4 Sensory evaluation: 2-Alternative Forced Choice hedonic preference (2007 and 2010 wines)

The 2-AFC ranking test was repeated, this time asking the panellists to express hedonic preferences based on global aroma and taste attributes, and ranking 1 = first and 2 = second.

3.5 Bergamot intensity evaluation (0-10)

The wines, which were presented to panellists in coded format in tulip-shaped Port glasses, were classified for bergamot aroma intensity using a discontinuous 0-10 scale. Panellists were also asked to score the following TN varietal descriptors: red/black fruit, orange blossom, floral violets, rockrose and spicy.

3.6 Sensory analysis: Effect of spiked terpenol addition

Four 50 mL aliquots of the two-year-old 2010 TN control wine were spiked with a terpenol mixture (62% linalool, 21% terpineol, 12.5% citronellol, 2% nerol and 2% geraniol) to give the following incremental increases in total terpenols: 100 µg/L, 200 µg/L, 300 µg/L and 400 µg/L. The control and enzyme-treated wines (without spiked terpenols) were arbitrarily assigned fixed bergamot aroma intensity scores, respectively 0 and 10. Panellists were asked to rate the four spiked control TN wines in terms of bergamot intensity, using the fixed limits as reference.

3.7 Odour activity units

Odour activity values (OAV) were calculated as the ratio of a compound's concentration to its odour threshold value (Grosch 1994). Threshold levels were as reported in the literature (table 2).

4. Results and Discussion

The present study targets the quantification of the classical terpenols: linalool, α-terpineol, citronellol, nerol and geraniol in TN wines, investigating both the free and glycosidase-enhanced content. The impact of enzyme treatment on the characteristic bergamot-like aroma is explored.

4.1 Touriga Nacional: Free terpenol levels

Based on OAV, linalool, citronellol and geraniol all make important odour active contributions (OAV > 1, table 3) to the aroma of young TN wines (<1 year old). The individual contribution made by either α-terpineol or nerol is less interesting, a consequence of their higher respective

Table 2. Selected terpene sensory descriptors and threshold limits

Terpenol	Aroma Descriptors	Threshold Detection Limit (mg/l)	Threshold Reference
Linalool	sweet, floral, rosewood, woody, green, blueberry, citrus[1, 2]	25	Ferreira et al. 2000
α-Terpineol	pine, terpene, lilac, citrus, woody, floral[1]	250	Ferreira et al. 2000
Citronellol	floral, rosebud, leather, waxy, citrus[1, 3]	18	Darriet 1993
Nerol	sweet, neroli, citrus, magnolia[1]	400	Ribéreau-Gayon et al. 1975
Geraniol	sweet, floral, rose, waxy, fruity, citrus[1]	20	Escudero et al. 2007
Hotrienol	sweet, citrus-like[2]	-	-
Linalyl acetate	sweet, green, citrus, bergamot, lavender, woody[1]	-	-
α-Terpenyl acetate	herbal, bergamot, lavender, lime, citrus[1]	-	-
α-Pinene	resiny, pine-like, turpentine[4, 5]	6	Leffingwell & Associates
Limonene	lemon, orange[4]	10	Leffingwell & Associates

[1] Good Scents Company; [2] Leffingwell & Associates; [3] Darriet 1993; [4] Flavournet; [5] Guedes de Pinho 2007

sensory thresholds (table 2). Whereas OAV gives a useful indication for the aromatic impact of a particular chemical, it does not give an indication of possible synergic or masking effects caused by other compounds in the wine matrix. Furthermore, odour families make a collective contribution, for example, the terpene alcohols contribute a sweet floral bouquet, with citrus nuances (table 2).

Winemaking procedures play an important role on the levels of extracted terpenols. For example, the shortened skin-contact time experienced in rosé production resulted in the extraction of less than half the content (58 µg/L) of the fuller-bodied red (135 µg/L) made from the same batch of TN grapes (2010 vintage, table 3).

The highest level of free terpenols was recorded in a 2011 TN Douro wine, totalling 257.7 µg/L, with significant contributions from linalool (112.6 µg/L, 4.5 OAV), citronellol (30.3 µg/L, 1.7 OAV) and geraniol (41.1 µg/L, 2.1 OAV).

In terms of total terpenol content, TN (58.0-257.7 µg/L) outranks other Portuguese red cultivars (Rogerson 1998, Rogerson et al. 1999, and Barbosa et al. 2003) and even compares favourably with the Portuguese floral non-Mus-

Figure 1. Linalool impact on Touriga Nacional aroma: selected study wines without glycosidase treatment (descriptor thresholds based on Ferreira 2009)

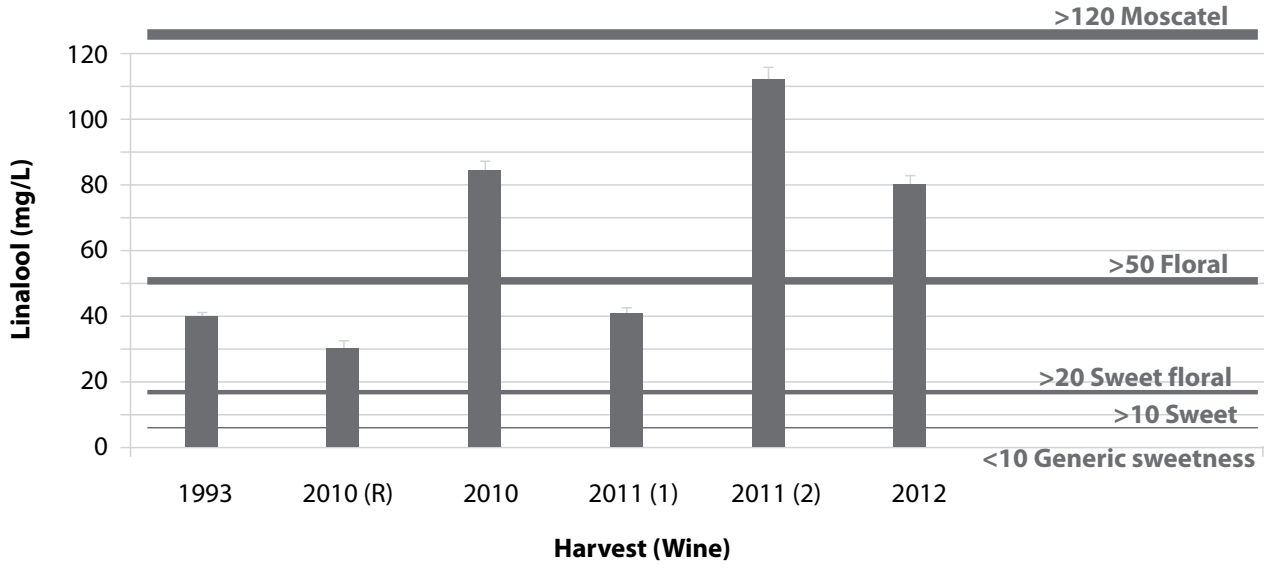


Table 3. Monovarietal Touriga Nacianal terpenol levels (µg/L) and odour activity values (OAV)

Harvest	n	Age (months)	Linalool[a]		α-Terpineol[a]		Citronellol[b]		Nerol[b]		Geraniol[c]		Total
			Concentration	OAV	µg/L	OAV	µg/L	OAV	µg/L	OAV	µg/L	OAV	µg/L
1993[d]	1	6	40.2 ± 0.6	1.6	16.5 ± 1.3	0.07	29.8 ± 0.3	1.7	3.5 ± 0.1	0.01	20.5 ± 0.9	1.0	110.5 ± 3.2
2003[e]		?	26.0 - 95.0	1.0 - 3.8	ND - 49.1	0 - 0.2	11.0 - 24.0	0.6 - 1.3	11.5 - 36.2	0.03 - 0.09	18.0 - 33.8	0.9-1.7	66.5 - 238.1
2010R[f]	1	6	30.9 ± 1.4	1.2	5.1 ± 1.7	0.02	10.3 ± 5.6	0.6	4.7 ± 3.1	0.01	7.0 ± 5.8	0.4	58.0 ± 17.6
2010[f]	2	6	73.4 - 83.9	2.9 – 3.4	4.4 – 8.1	0.02 – 0.03	17.0 - 22.3	0.9 – 1.2	4.8 – 6.5	0.01 – 0.02	2.3 – 28.4	0.1 – 1.4	116.1 – 135.0
2011[f]	5	8	41.4 - 112.6	1.7 – 4.5	28.7 - 67.3	0.11 – 0.27	27.8 - 44.1	1.5 – 2.5	3.6 – 7.6	0.01 – 0.02	21.7 – 41.1	1.1 – 2.1	130.8 – 257.7
2012[f]	1	4	48.0 ± 3.3	1.9	28.2 ± 4.0	0.11	16.3 ± 2.4	0.9	10.0 ± 0.2	0.03	14.4 ± 0.5	0.7	116.9 ± 10.4

[a, b, c] Threshold limits: [a]Ferreira 2000 (linalool – 25 µg/L; α-terpineol 250 µg/L); [b]Darriet 1993 (citronellol – 18 µg/L; nerol – 400 µg/L); [c]Escudeiro et al. 2007 (geraniol – 20 µg/L)

[d]Rogerson 1998; [e]Barbosa et al. 2003; [f]Symington Wines; R: Rosé; Note: Data for harvests with multiple wines reported as range

cat, white varieties Loureiro (222.7 µg/L) and Alvarinho (131.3 µg/L) (Rogerson 1998).

4.2 Linalool: Aroma impact compound and aroma enhancer

Linalool has been shown to be both an aroma enhancer and an important aroma impact compound (Ferreira 2009). At levels below 10 µg/L it acts as an aroma enhancer, increasing the generic sweetness. As concentration levels increase, its impact becomes increasingly floral (table 4, Ferreira 2009). The typically high levels of linalool found in TN wines emphasize the importance of its floral contribution to the varietal aroma (figure 1).

Table 4. Linalool concentration and aroma impact (Ferreira 2009)

Concentration (mg/L)	Aroma Characteristics
<10	Not detectable, but contributes to generic sweet note
10 to 20	Possible to detect if present with similar aroma based chemical, resulting in generic non-specific floral/sweet; e.g.: ethyl cinnamate
20 to 50	Detected independently in the presence of other compounds (sweet/floral)
50 to 120	Specific clear floral note
>120	Moscatel-like

4.3 Touriga Nacional bergamot aroma

While the classic terpene alcohols contribute principally floral aromas, linaloyl acetate is reported to have a sweet, green citrus, bergamot character (Luebke 1982). Although both linalool and linaloyl acetate were recently identified as key odorants in the bergamot flavour of TN wines

Table 5. Bergamot aroma impact compounds

Terpenol	FD5	Threshold Limit (mg/L)	Threshold Reference	Aroma Descriptors
α-pinene	1024	6	Leffingwell & Associates	resiny, pine-like, turpentine[4]
linalool	512	25	Ferreira et al. 2000	sweet, floral, rosewood, woody, green, blueberry, citrus[1, 2]
linaloyl acetate	256	-	-	sweet, green, citrus, bergamot, lavender, woody[1]
(E)-β-ocimene	256	-	-	sweet, herb[4]
γ-terpinene	256	-	-	gasoline, turpentine[4]
limonene	8	10	Leffingwell & Associates	lemon, orange[4]
α-terpineol	4	250	Ferreira et al. 2000	pine, terpene, lilac, citrus, woody, floral[1]
β-phellandrene	4	-	-	mint, terpentine[4]
citronellol	1	18	Darriet 1993	floral, citrus[1, 3]

[1] The Good Scents Company; [2] Leffingwell & Associates; [3] Darriet 1993; [4] Flavornet;
[5] FD – Flavour Dilution (AEDA; Guedes de Pinho 2007)

(Guedes de Pinho et al. 2007), the present study identified only linalool. The absence of linaloyl acetate indicates that it is not an essential contributor to TN bergamot character.

Linaloyl acetate was not the only aroma-active citrus-flavoured compound identified in the bergamot oil extract examined in the original study (Guedes de Pinho et al. 2007). A total of 13 compounds were shown to contribute to the typical citrus, lemon and lime character. Aroma Extract Dilution Analysis (AEDA) placed linaloyl acetate in the third most potent grouping with flavour dilution (FD = 256), ranked behind both linalool (FD = 512) and α-pinene (FD = 1024), the latter being the strongest contributor with a pine-like character (table 5). This latter terpene had also been identified as present in a targeted GCO odour zone of a TN extract, characterized with a pineapple/pine/fruity aroma (Guedes de Pinho et al. 2007). Rather confusingly, the associated figure in the same paper had characterized α-pinene as "floral." The pine-like descriptor, given in the text, appears to be more accurate.

The fact that α-pinene was found to be the most odour-active component of the bergamot oil extract emphasizes the key contribution made by its balsamic pine-like aroma. Furthermore, the fact that α-pinene was also detected in their TN wine suggests that both linalool and α-pinene are essential contributors to TN bergamot character.

Unfortunately, α-pinene was not investigated in the current study, which focused only on the quantification of selected terpenols with known precursor glycosides. Future work should focus on bergamot character contributions made by both the floral, citrus and resin/balsamic families of compounds.

It is interesting to note that the three characteristic varietal aromas identified for TN are to some extent interlinked, containing either or both floral and balsamic components: floral (violets), floral/citrus-resin (bergamot), and sweet fruity resin (rockrose). Variations in the concentration of these key aroma-active compounds, or families of compounds, will define which varietal character dominates.

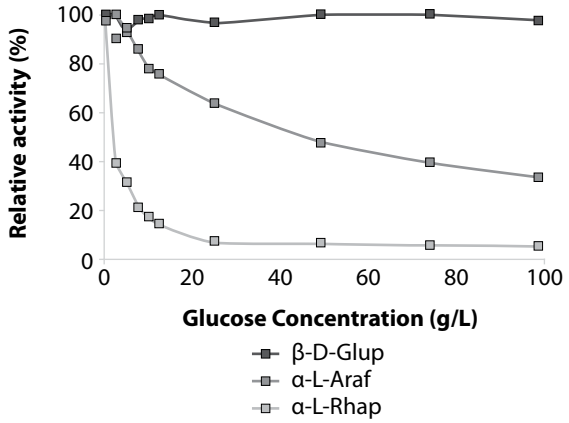
4.4 Important processing properties of glycosidase

Pectolytic preparations rich in glycosidase activity have traditionally been used to enhance aroma in the production of white wines. Relatively few studies appear to focus on the associated benefits in red winemaking.

In respect to red-wine processing, *A. niger* glycosidase activities express several important characteristics:

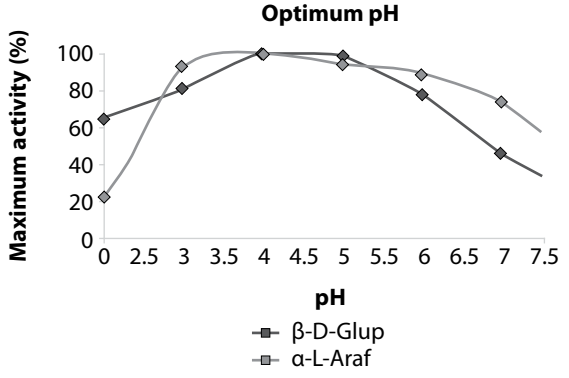
- The glucosidase activity is known to be strongly inhibited by glucose (figure 2), which implies that it will not be particularly effective for the processing of sweet dessert wines such as Port, which typically contain around 50 g/L each of glucose and fructose.

Figure2. Effect of glucose concentration on *Aspergillus niger* glycosidase activity (pH 3.5; 20ºC) (Rogerson 1998)



- Red winemaking pH (~pH 3.5), compared to more acidic white wine pH (~pH 3.0), has been shown to favour greater levels of glycosidase activity (figure 3).

Figure 3. Effect of pH on *Aspergillus niger* glycosidase activity (20ºC) (Rogerson 1998)

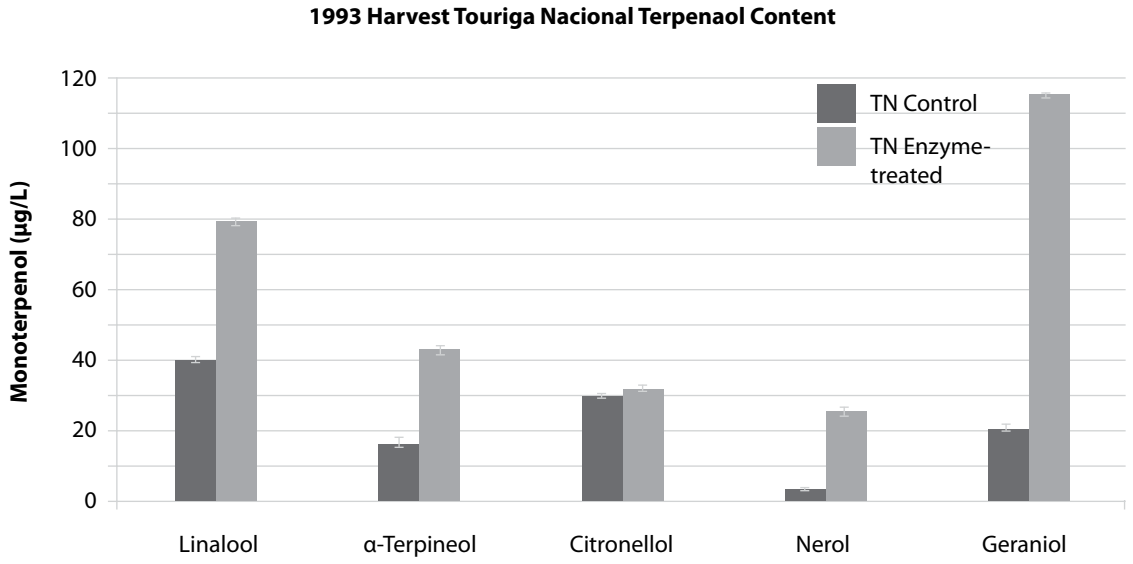


- Glycosidase activities are not inhibited by SO_2 levels typically encountered in winemaking (Rogerson 1998). Wines can therefore be processed following sulphiting, after completion of both the alcoholic fermentation (AF) and malolactic fermentation (MLF). This option minimizes the opportunity for *Brettanomyces* to metabolize cinnamic acids into unfavourable aroma active phenols, such as 4-hylphenol. Cinnamic acid would effectively be released only by residual *A. niger* cinnamoyl esterase, following sulphiting, with a zero *Brettanomyces* impact.

4.5 Effect of glycosidase treatment on Touriga Nacional wine terpenol levels and varietal aroma

The first study to investigate the effect of a glucosidase-rich pectinase treatment on a monovarietal TN wine demonstrated significant levels of terpenol release (110.5 µg/L

Figure 4. Effect of an experimental glycosidase-rich *Aspergillus niger* preparation on monovarietal Touriga Nacional wine from the 1993 vintage, aged 6 months in bottle (Rogerson 1998)



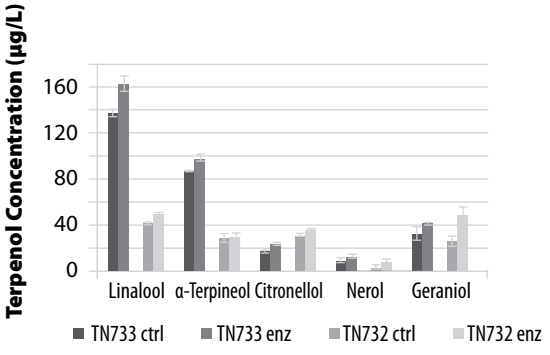
to 293.4 µg/L), with principle contributions from geraniol (+94.0 µg/L) and linalool (+38.7 µg/L) (figure 4). It would seem that glycosides of geraniol, a primary alcohol, are fairly abundant and also favourably released. Glycosides of the tertiary alcohols, linalool and α-terpineol are known to not be good substrates for *A. niger* glucosidases (Günata et al. 1990), which may explain the slower release rates.

Similar studies performed using wines from the 2007, 2010, 2011 and 2012 vintages demonstrated similar trends, always with significant levels of released monoterpenols, as well as a considerable perceived enhancement in bergamot aroma intensity (figure 5).

A comparison of the effect of different commercial glucosidase preparations on the level of released terpenols, as well as the associated bergamot intensity, demonstrated superior processing results for both the Lallemand Prozym preparation and Enzyme C (figure 6).

The terpenol content recorded for the two 2011 control wines, TN733 (283.8 µ g/L) and TN732 (130.8 µg/L) (figure 5), emphasizes the large intra-harvest variation. Whereas the TN733 control wine was characterized with a fairly intense bergamot aroma, the TN732 wine presented only a subtle citrus nuance. Enzyme treatments resulted in considerable floral/citrus aroma enhancement of both wines (figure 5). The evaluation of OAV once again highlights the key role played by linalool: TN733 enzyme (6.5) > TN733 control (5.5) > TN732 enzyme (2.0) > TN732 control (1.7). Important but lesser contributions were made by citronellol (OAV 0.9 to 2.0) and geraniol (OAV 1.3 to 2.5). Wine aging effects on terpenol content and the associated impact on aroma is discussed in the following section.

Figure 5. Effect of the glucosidase-rich *Aspergillus niger* preparation Prozym on Touriga Nacional wine monoterpenol levels and bergamot aroma intensity; 2011 Vintage aged 16 months in bottles



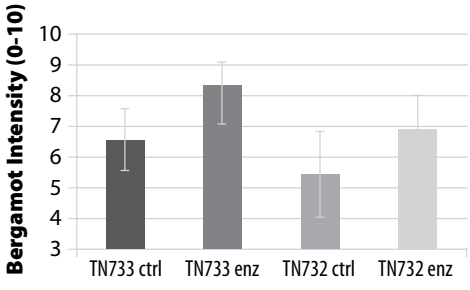
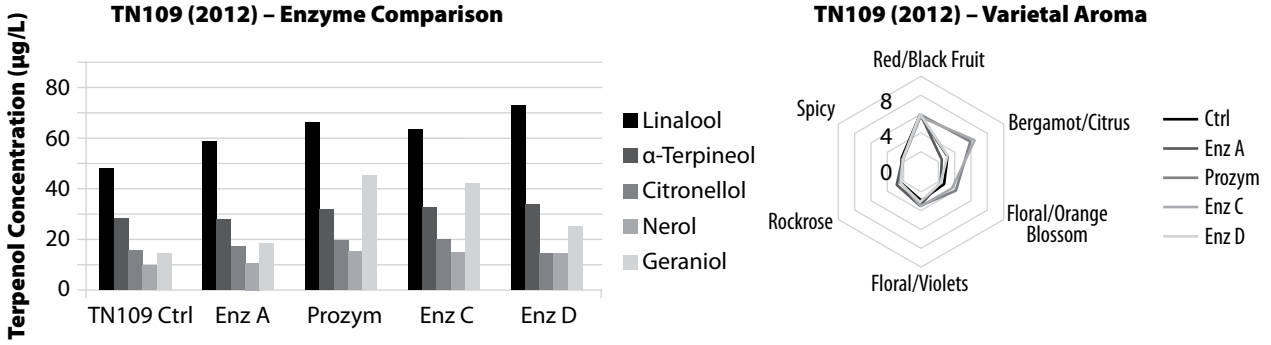


Figure 6. Effect of four commercial enzyme preparations on terpenol content and wine aroma



4.6 Age impact on terpenol varietal aroma

The stability of the monoterpene alcohols, as well as their associated aging mechanisms, need to be adequately understood if the winemaker is going to fully master TN aroma modulation. This has implications for the timing of glycosidase processing, as well as for the wine's projected shelf-life and marketing release date.

Two mechanisms are of particular interest:

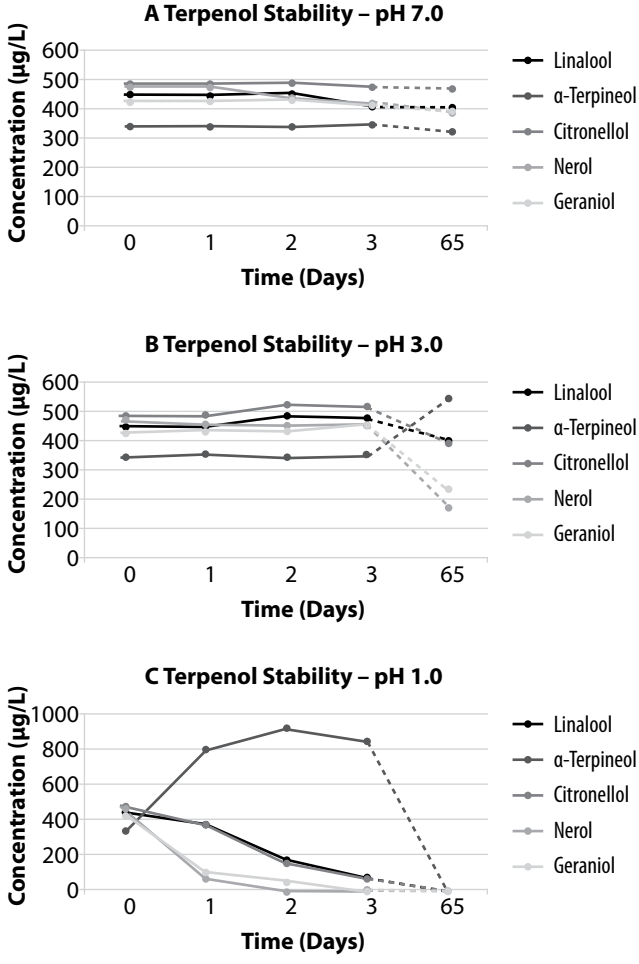
- The oxidation of monoterpene alcohols, e.g., linalool to its oxide forms. The latter compounds have much higher sensory thresholds and consequently lower aroma impact (Ribéreau-Gayon et al. 1975). Red wines, compared to white wines, contain much greater levels of phenols/polyphenols, implying greater antioxidant capacity, and possibly as a consequence, greater terpenol stability.
- The second mechanism involves acid-catalyzed monoterpenol inter-conversion through hydration/dehydration, rearrangement and cyclization reactions, resulting in multiple products, including terpene diols. Red wines should present greater monoterpenol stability, a consequence of their lower relative concentration of hydrogen ions, with typical pH 3.5, compared to white wines with pH 3.0.

Terpenol stability has been well documented in model wine solutions at pH 7.0, pH 3.0 and pH 1.0 (figure 7, Rogerson 1998). Whereas terpene alcohols are stable when stored in synthetic wine solution at pH 7.0, 65 days at a typical white winemaking pH 3.0 resulted in significant increases in levels of α-terpineol, apparently formed at the expense of other terpenols. The same trend was observed with exaggerated acidic pH 1.0, this time with greater rates of transformation (figure 7).

The relative stability of terpenol, when stored under acidic conditions, helps explain aged character:

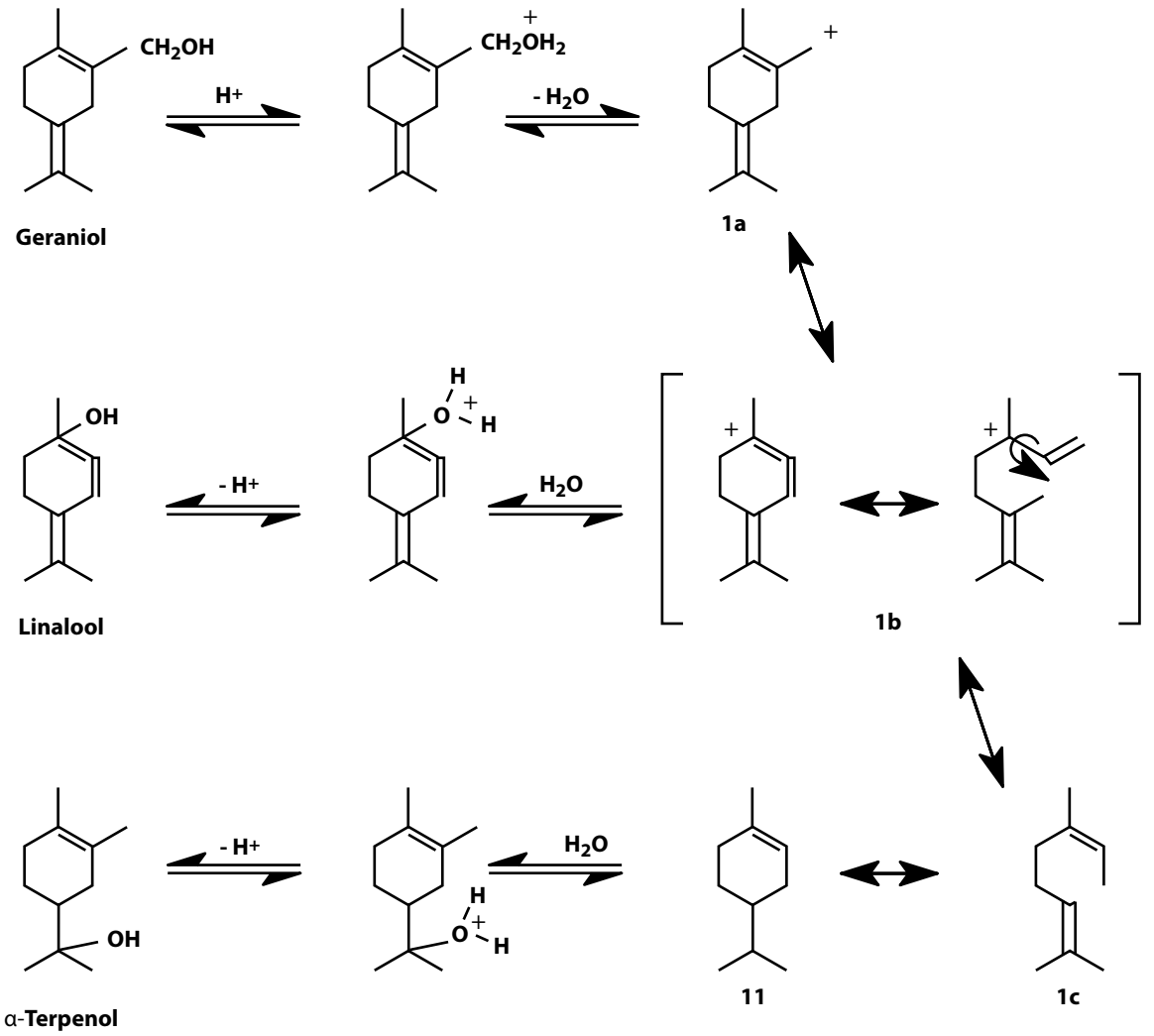
α-terpineol > linalool and citronellol > geraniol > nerol

Figure 7. Monoterpenol stability in model wine solutions: A pH 7.0, B pH 3.0, C pH 1.0 (Rogerson 1998)



Several authors (Rapp et al. 1985, Di Stefano 1989, and Rogerson 1998) identified α-terpineol as a major reaction product of the three principle monoterpenol isomers, linalool, geraniol and nerol. The mechanism for α-terpineol formation proceeds with carbocation formation through hydroxyl protonation, and water loss (figure 8). Linalool forms the tertiary carbocation (Ib), which is more stable than the primary carbocations (Ia) and (Ic), formed respectively from geraniol and nerol. The stereochemistry of (Ic)

Figure 8. α-Terpineol formation from geraniol and linalool (Rogerson1998)



favours ring closure, which, when followed by rehydration yields α-terpineol. The mechanism explains the relative instability of nerol and why its levels quickly diminish during wine aging. The stereochemistry of the geraniol intermediate tautomer (Ia) appears to favour the rearrangement to the more stable tertiary carbocation (Ib). Reaction can proceed either through rehydration to give linalool, or by C_2-C_3 bond rotation and rearrangement to give the less stable primary carbocation (Ic), which cyclizes, leading to formation of α-terpineol (Rogerson 1998).

A separate study examined the stabilities of linalool, geraniol and nerol during storage in 0.025M citric acid (Baxter et al. 1978). After 20 days, nerol and geraniol both yielded α-terpineol and linalool as major products, whereas linalool yielded only very small levels of geraniol and zero nerol (Baxter et al. 1978). The following levels of the respective starting terpenols remained unreacted: 44% linalool > 35% geraniol > 14.5% nerol. These observations once again emphasize the important role of linalool having greater stability than its isomers.

The same authors carried out similar tests, also looking at the terpenyl acetates (Baxter et al. 1978). After 20 days stored in 0.025M citric acid, linaloyl acetate had completely disappeared, being hydrolyzed to linalool and partially interconverted into other products. These observations help explain the absence of linaloyl acetate from aged TN wines.

Indeed, α-terpineol is itself not particularly stable, known to transform during long-term wine aging to its cis- and trans-α-terpin isomers (Sefton et al. 1994).

Figure 9A. Monoterpenol aging profiles for 2010 monovarietal Touriga Nacional wines with and without Prozym treatment

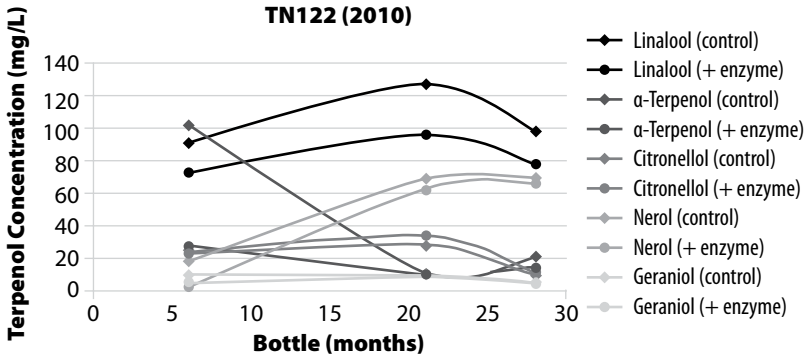
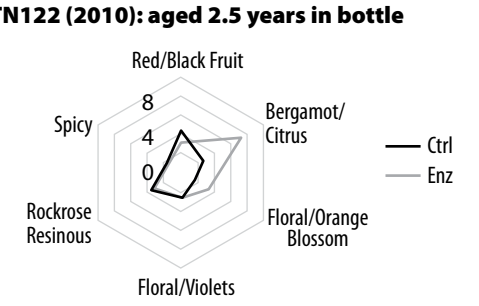


Figure 9B. Effect of Prozym treatment on varietal Touriga Nacinal aroma



Wine-aging kinetics favour a move away from the high concentrations of the lower threshold aromatic terpenols, linalool and geraniol, favouring greater levels of the lower impact compound α-terpineol. The floral/citrus notes associated with geraniol and linalool will diminish with prolonged wine aging, nevertheless, linalool considerably out-paces geraniol. Aged character is an important consideration for TN aroma modulation, and introduces the marketing concept of ideal shelf-life.

4.7 Monoterpenol stability during aging of monovarietal Touriga Nacional wines

Monoterpenol levels were followed in the 2010 TN control and enzyme-treated wines during 28 months of bottle aging. The glycosidase-treated wine maintained both higher levels of free terpenols (figure 9A), and greater bergamot intensity (figure 9B) throughout the study period. Terpenol aging trends were similar to those observed in model studies (figure 7), with levels of linalool and α-terpineol increasing at the expense of both geraniol and nerol.

Whereas young enzyme-processed wines (six months old), typically have greater aroma contribution from the terpenol geraniol (TN2010 $OAV^{6\ mo}$: geraniol [5.1] > linalool [3.7]), wines with greater age are dominated by linalool (TN2010 $OAV^{21\ mo}$: geraniol [0.6] << linalool [5.1]).

The increase observed in α-terpineol is rather mute, since its higher sensory threshold (table 2), implies only limited aroma impact. In contrast, linalool, due to its superior stability, high relative concentration and lower sensory threshold, remains a key contributor to the bergamot descriptor in both young and medium-term aged wines. Although linalool levels peaked above 120 µg/L (figure 10), the TN wine did not reveal a Muscat-like character (Ferreira 2009), but had citrus/floral/bergamot dominance instead.

Figure 10. Floral impact of linalool in control and enzyme-treated Touriga Nacional 2010 wines

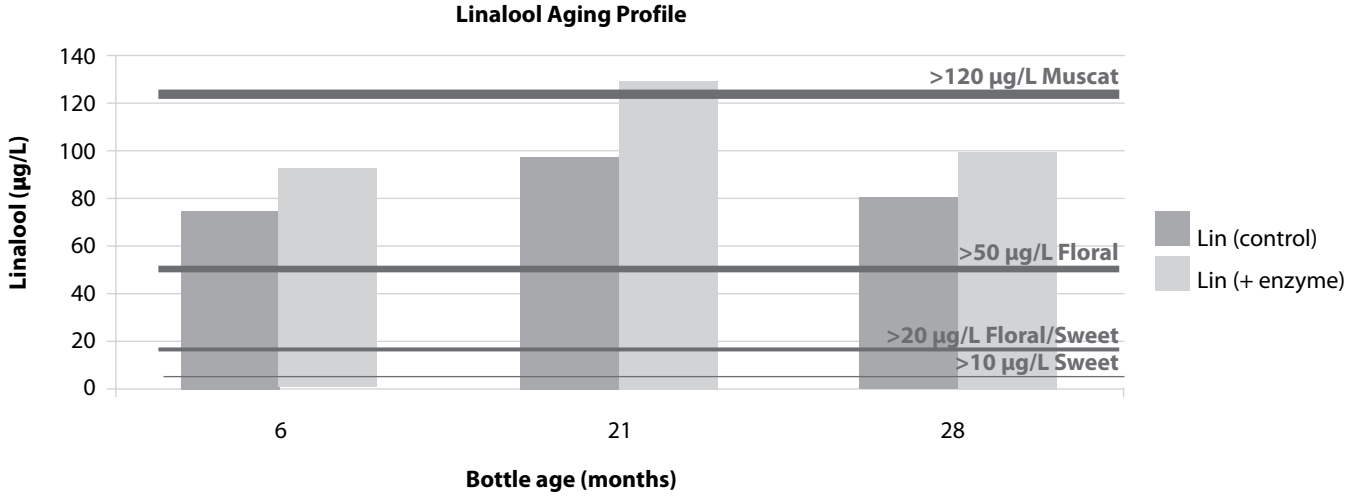
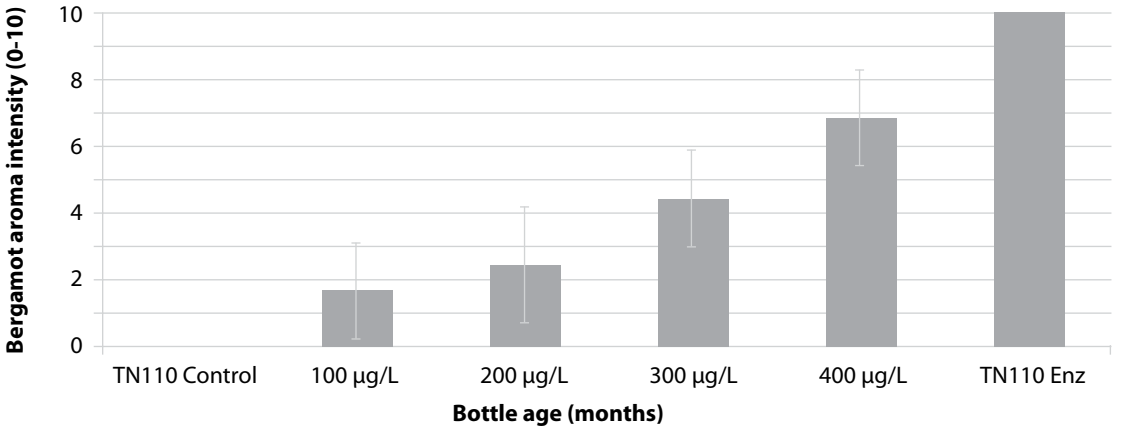


Figure 11. Investigation of the impact of spiked terpenol standard additions on Touriga Nacional 2010 bergamot intensity



4.8 Standard addition: Aroma reconstruction test

Whereas increased levels of spiked terpenols added to the 2010 control wine resulted in increased bergamot intensity (figure 11), the associated aroma was both less intense and less complex than the non-spiked enzyme-treated wine. This observation is particularly relevant when one notes that the 400 µg/L spiked control wine (621 µg/L) contained more than double the level of the terpenols present in the enzyme-treated wine, which had only 266 µg/L.

Enzyme catalysis appears to release other aglycone compounds, which add far greater complexity than the contribution from the classic terpenols alone. This implies that classic terpenols, although key to the bergamot aroma, are not the only contributors.

4.9 Sensory tests

4.9.1 Alternative forced choice (AFC) evaluation for bergamot intensity and hedonic preference

Paired comparison tests (2-AFC) were performed on the 2007 wine (aged three years in bottles) and the 2010 wine (aged six months) taken from duplicate control and enzyme-treated bottles. Panellists easily differentiated them, rating the enzyme-treated wines with greater bergamot intensity ($\alpha = 0.001$, table 6A).

The 2007 treated wine was hedonically preferred, both for aroma ($\alpha = 0.01$) and on the palette ($\alpha = 0.01$). The panellists were, however, rather indifferent about the 2010 control wine (2-AFC, table 6B), noted by several tasters as having an excessively sweet floral/bergamot character. Only the 2010 treated wine gave a possibly significant preference on the palette ($\alpha = 0.1$). The improved sensory attributes observed for the 2007 treated wine emphasize the potential of this enzyme processing technique. However, the 2010 results suggest that the enzyme dose level and application time may need to be optimized in order to temper aglycone release. Blending operations may also be required to achieve the desired complexity.

Table 6A. 2-AFC sensory tests for bergamot intensity

Harvest	Bottle Age	n	Bergamot Intensity Enzyme > Control
2007	3 years	24	$\alpha = 0.001$
2010	6 months	24	$\alpha = 0.001$

Table 6B. 2-AFC hedonic preference test for enzyme-treated wines

Harvest	Bottle Age	n	Aroma	Palette	Global
2007	3 years	24	$\alpha = 0.01$	$\alpha = 0.01$	$\alpha = 0.01$
2010	6 months	24	no preference	$\alpha = 0.1$	$\alpha = 0.2$

4.10 Palette evaluation: Touriga Nacional 2010 (0-10 non-continuous scale)

Palette evaluation of the 2010 wines, this time with 2.5 years of aging in bottles, resulted in preferred scores for the enhanced bergamot character, as well as a reduction in astringency (figure 12).

Figure 12. Palette evaluation examining the impact of enzyme-treated Touriga Nacional wine. Sensory Evaluation Scale (0-10).

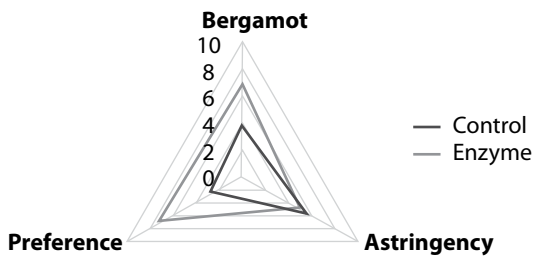
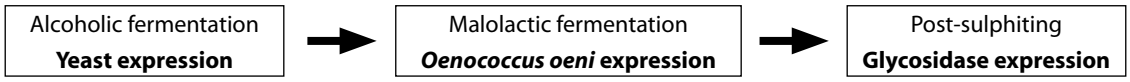


Figure 13. Wine aroma expression



This latter characteristic has also been noted in tests carried out looking at other monovarietal wines (data not presented). Furthermore, it is known that charged polysaccharides play an important role in disrupting the aggregation between procyanidins and bovine albumin serum. The following inhibition constants were determined in model solutions (Freitas et al. 2003):

polygalacturonic acid (24225) > pectin (1610) > arabic gum (329) > arabinogalactan (1.5) > glucose (0.77)

This implies the greater the level of pectin de-esterification, the greater the associated inhibition to aggregation between, for example, salivary proteins and wine phenolics, which would result in a reduction in perceived astringency.

The pectin methyl esterase activity found in commercial pectinases is often considered negative in that it is responsible for the release of small, possibly negligible levels of methanol. The de-esterification of pectin may, however, result in a considerable improvement in mouthfeel, a consequence of lower perceived wine astringency. Further investigation is merited.

4.11 Bergamot modulation options

The winemaker has numerous tools available to achieve the desired TN bergamot complexity.

- **Selection of the vineyard harvest date**

  Fruit ripeness should be followed in the vineyard through berry tasting, facilitating the selection of the ideal harvest window.

- **Winemaking options for improved terpenol extraction**

  In the winery, the winemaker has the option to enhance potential wine terpenol levels through on-skin maceration and the application of fruit maceration enzymes.

- **Optimal timing for glycosidase treatment**

  Aroma expression is driven by the favourable selection of both yeast and bacteria strains, and also whether a glycosidase-rich enzyme is used. Glycosidase application can be made before AF, during AF, prior to MLF or after MLF. The first three options do not permit the evaluation of yeast and/or bacteria aroma expression. The suggested enzyme addition time is only following wine sulphiting, i.e., post-AF and post-MLF, as that gives greatest flexibility (figure 13). This option also minimizes the effect of glucose inhibition on the glucosidase activity.

Aroma expression solely by the selected yeast and bacterial strains may lead to the desired varietal TN aroma (rockrose or violet character). Application of a glucosidase-rich enzyme preparation should be performed only if greater bergamot complexity is desired. The choice is the winemaker's, and depends upon the target wine-style. Either way, the treatment of a small test volume, using a glucosidase-rich enzyme preparation, performed post-sulphiting, permits a rapid assessment of the aroma impact of the released aglycone fraction, facilitating a decision as to whether to treat the main lot.

- **Wine pH and storage temperature**

  Wine pH and storage temperature should be carefully considered with respect to wine-style and desired shelf-life.

- **Bentonite treatment**

  The oenologist should sensory evaluate wines during glycosidase treatment. Bentonite (20 g/hL) should be used to remove the protein at the desired aroma complexity.

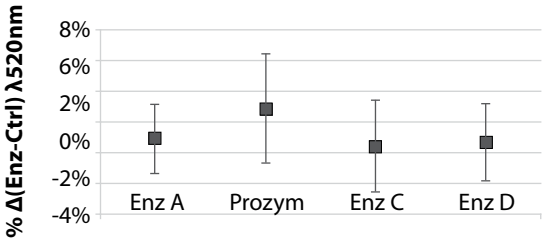
- **Blending options**

  Enzyme-treated lots (or fractions of lots) can be blended to achieve the desired complexity.

4.12 Anthocyanase activity

It has been suggested that glucosidases degrade anthocyanins, resulting in colour loss (O'Kennedy and Canal-Llauberes 2013). Tests investigating the impact of the four glucosidase-rich enzyme preparations on ten 2012 harvest wines (including two TN), resulted in minimal evidence indicative of detrimental anthocyanase colour loss. On the contrary, treated wines on balance indicated fractionally greater red colour intensity (figure 14).

Figure 14. Effect of commercial enzyme treatment on red colour intensity. Results express average for ten 2012 wines, including two Touriga Nacional, with 95% probability.



5. Conclusions

The aroma typicity of Touriga Nacional (TN) wine is complex, often characterized by either balsamic rockrose, floral violets or citrus floral notes.

Aroma modulation through the application of glycosidase-rich enzyme preparations to monovarietal red wines made from TN grapes leads to significant enhancements in free terpenol content, as well as the associated increase in bergamot aroma intensity. Enzyme-treated wines maintained greater bergamot aroma complexity over a period of at least 2.5 years of aging in bottles. The superior stability of the terpenol linalool relative to its geometric isomers confirms its key role in the expression of the bergamot aroma in both young and medium-aged TN wines. In contrast, and contrary to previous observations, linaloyl acetate was not detected and is not consequently considered an essential contributor to the TN citrus nuance. Other known key flavorants of bergamot essential oil, particularly α-pinene and limonene, which are also known wine flavorants, need to be targeted and quantified in TN wines.

Increased bergamot intensity was also observed in a TN control wine spiked with roughly a twofold excess of terpenol standards. However, the associated aroma neither matched the intensity nor expressed the complexity of the enzyme-treated wine. The results suggest that not only terpenols, but other precursor aglycone units are released and play an important role in the TN varietal complexity.

Increased floral/citrus aroma complexity was hedonically preferred in most, but not all, wines. With regards to aroma modulation, the winemaker will need to achieve the desired balance through the correct timing of glycosidase application, as well as blending operations. Bentonite treatments should be carried out only at the conclusion of the desired enzyme process time, as this fining step strips out proteins.

This study demonstrates the successful enzymatic-induced aroma modulation of key odorants of wine made from the Touriga Nacional red grape variety. Winemakers will need to learn the potential of this new tool, directing the appropriate enzyme dose level at the appropriate time in order to target a particular wine-style with a specific shelf-life aroma. TN aroma modulation sets new challenges to both the winemaker and the marketing team, with potential commercial benefits.

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CONTROLLING DIMETHYL SULPHIDE LEVELS IN BOTTLED WINES

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Abstract

Dimethyl sulphide (DMS) is a versatile aroma compound that can have significant effects on the sensory properties of wine. Depending on its concentration and the type of wine, DMS can be responsible for various aromas, including truffle, herbaceous notes, undergrowth, cabbage and fruity sensations. Long considered exclusively as a fault, by association with other light sulphur compounds responsible for reduction aroma, recent studies clearly show that DMS can enhance the fruity notes of various red wines, such as Shiraz, where its positive contribution was seen even at low concentrations. Even near its olfactory perception threshold (about 20 µg/L), DMS has been shown to influence wine aroma.

Although DMS has several origins in wine, at the moment the most important is its chemical release from the S-methylmethionine in the wine during aging. This precursor, called PDMS (potential DMS), is present in the grapes and partially transferred to the corresponding wine. The challenge for wine producers is, first, to pilot the PDMS level accumulated in grapes and transfer it into the must, and, second, because yeasts appear to degrade it, to preserve it during fermentation. The blending strategy and the control of the aging conditions could also be means to manage DMS levels in wine, with the support of predictive models.

1. Introduction

Dimethyl sulphide (DMS) is a light sulphur compound identified in numerous foods and beverages (Ségurel et al. 2004, Jensen et al. 2002, Spinnler et al. 2001, Carbonell et al. 2002, and Anderson et al. 1975). Also present in wine, DMS can contribute to the aroma, positively or negatively depending on its concentration and the typology of the wine (Ségurel et al. 2004, Ségurel 2005, Ugliano et al. 2010, Ugliano et al. 2009, De Mora et al. 1986, Anocibar Beloqui 1998, Du Plessis and Loubser 1974, and Escudero et al. 2007). With a perception threshold under 10 µg/L in water and from 10 to 160 µg/L in wine (Mestres et al. 2000), DMS contributes to the wine aroma through a wide range of odours, including truffle, cabbage, vegetal, undergrowth and green olive. Recent research (Ségurel et al. 2004, Ségurel 2005, Ugliano et al. 2010, Ugliano et al. 2009, Escudero et al. 2007, and San-Juan et al. 2011) has confirmed the capacity of DMS to reinforce fruity aroma notes, as well as its involvement in synergistic effects, corroborating older research (De Mora et al. 1986, and Anocibar Beloqui 1998).

Studies on different types of red wine, including Grenache and Syrah wines from the Rhône valley (Ségurel et al. 2004), Spanish wines (Escudero et al. 2007, and San-Juan et al. 2011), and wines from southern Australia (Ugliano et al. 2010), have shown that DMS is an exhauster of fruit aromas. The interaction between DMS and other aroma compounds changes the aromatic perception of wines

(Ségurel et al. 2004). Associated with ethylic esters and β-damascenone, it intensifies fruity notes (Escudero et al. 2007), and in the presence of methionol and hexan-1-ol, DMS brings vegetal notes (San-Juan et al. 2011). The contribution of DMS to the aroma of wine is undeniable, but complex.

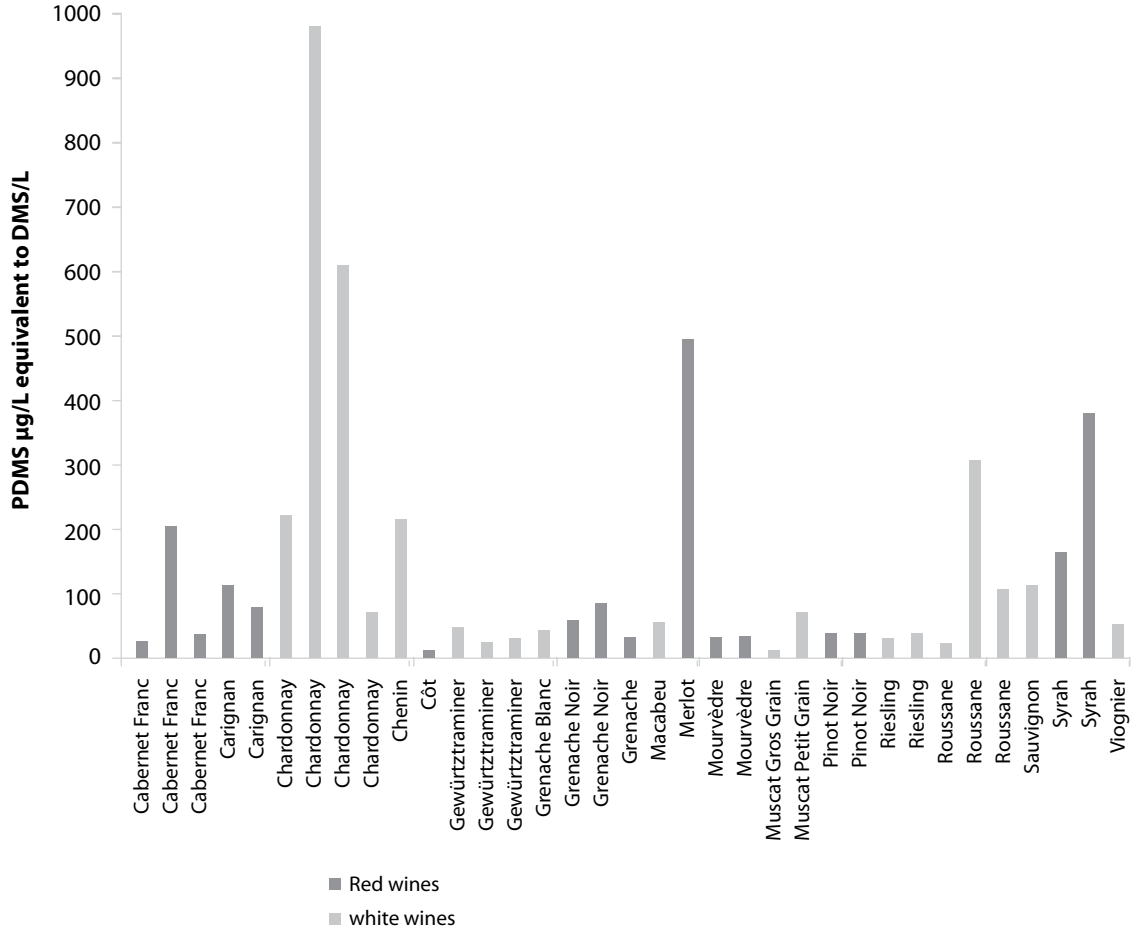
DMS is produced during fermentation from different amino acids and amino acid derivatives (Carbonell et al. 2002, Rauhut 1993, and Starkenmann et al. 2008). But the essential DMS is lost with the CO_2 produced by the yeasts, which explains why the DMS levels at bottling are generally under 10 µg/L. Indirect analysis of the potential for DMS (PDMS) by heating in an alkaline medium demonstrated the presence of precursors at the origin of DMS during storage of wines in the bottle (Ségurel et al. 2005). Indeed, S-methylmethionine (SMM) was identified in a must, where it was shown that it represents the essential DMS precursors analyzed for PDMS by heating in an alkaline medium (Loscos et al. 2008). PDMS is already present in the grapes and the musts, but a major portion of this aroma potential is lost during vinification (Ségurel et al. 2004, and Dagan 2006).

Much research has been carried out on the problems associated with the origin and the process of DMS and PDMS:

- A dissertation on the aroma of Petit Manseng and Gros Manseng (IFV Midi-Pyrénées, Syndicats des Côtes de Gascogne, UMR SPO INRA Montpellier);
- An R&D project carried out in partnership with Lallemand (2006-2008) on Syrah;
- Experiments carried out as part of the UMT Qualinov (INRA Montpellier, IFV, 2006-2011);
- Experiments carried out by the IFV on Petit Manseng and Gros Manseng, and on Malbec.

The objective of this research was to identify and evaluate the impact of viticultural factors (e.g., hydric stress, maturity, production site, leaf spraying and varietals), pre-fermentation stages (skin maceration, lees stabulation), and fermentation conditions (e.g., yeast strain, nitrogen, fermentation temperature, and fermentation adjuvants) on PDMS in order to determine new elements for controlling the levels of DMS in wines, in a pilot project.

Figure 1. Concentrations of potential dimethyl sulphide in the grapes of different white and red varietals



2. Results and Discussion

2.1 Parameters that influence potential dimethyl sulphide levels in the grapes

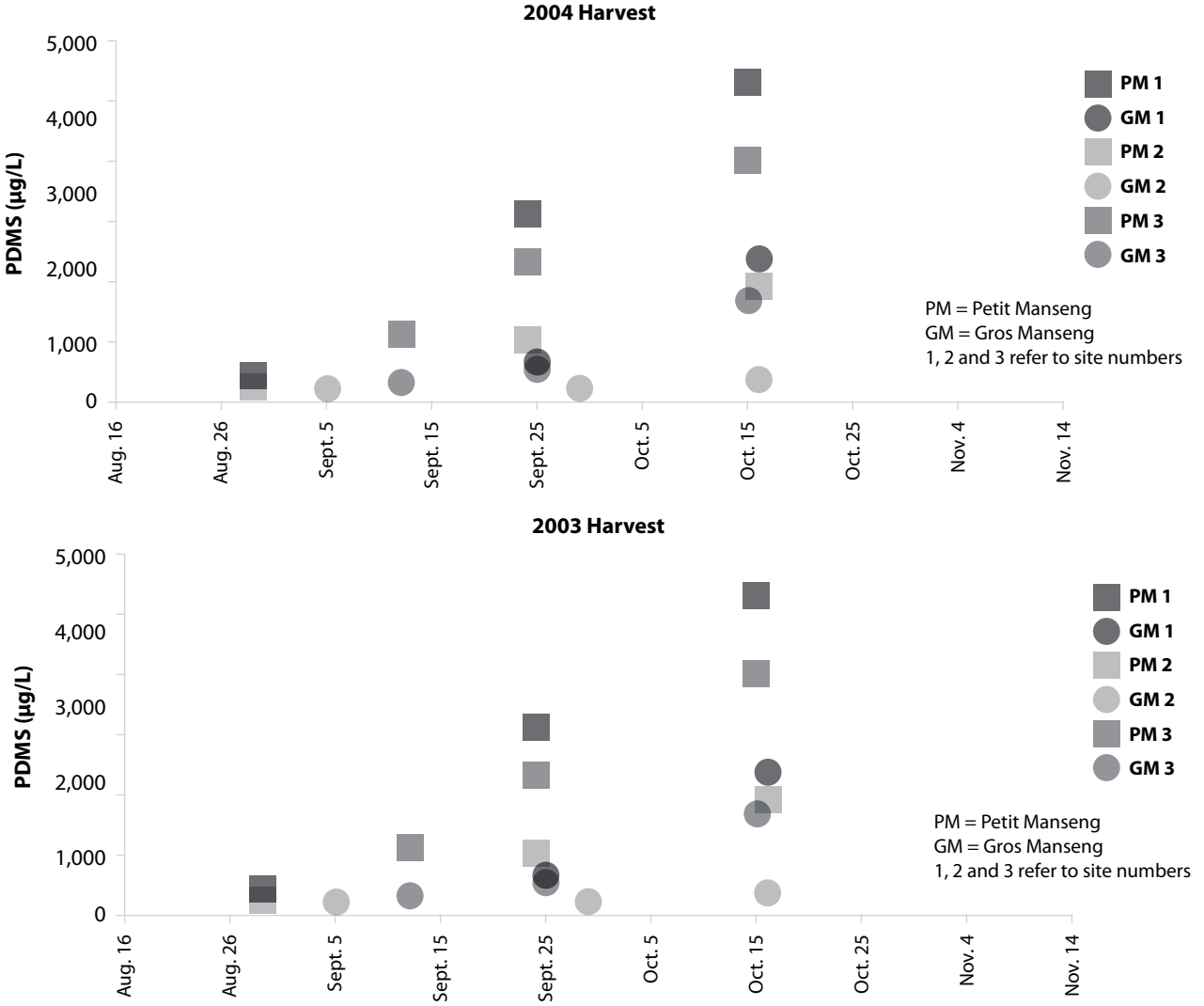
2.1.1 Studying potential dimethyl sulphide in different grape varietals

Samples of grapes from diverse varietals and diverse viticultural regions in France from the 2007 harvest were taken and preserved at -20°C. For this and the following trials, the PDMS analyses were carried out in the previously described conditions (Ségural et al. 2004). The main results of this screening demonstrated the presence of PDMS in the majority of the grapes of the varietals studied (figure 1), sometimes in concentrations far superior to those initially observed in Syrah and Manseng grapes (Ségural et al. 2004, and Dagan 2006), varietals for which DMS constitutes one of the principal aromatic markers. These results also showed some variability among the samples for a given varietal, such as Roussane or Chardonnay. This difference could be explained, notably, by differences in the maturity of the grapes, which was shown to be a predominating factor in the variations in PDMS (see 2.1.2). On the whole, the results of the screening support the further study of the sensory contribution of DMS in the wines of other varietals.

2.1.2 Impact of maturity, production site and vintage on the concentrations of potential dimethyl sulphide in Petit Manseng and Gros Manseng grapes

PDMS was measured in 2003 and 2004 on grape samples on three harvest dates, taken from three Gros Manseng vineyard sections and three Petit Manseng vineyard sections, grouped on three sites with different soil and climate characteristics (figure 2). For each of the sections,

Figure 2. Potential dimethyl sulphide concentrations in Petit Manseng and Gros Manseng grapes during ripening



40 kg of grapes were taken on three harvest dates to be vinified in experimental and standardized conditions (20 litres).

The results of analyzing the variance of this data show that maturity and the varietal are the main factors in the variations of PDMS ($P<0.001$), followed by the soil and climate environment and the vintage ($P<0.05$).

For the majority of these vineyard sections, we observed a strong increase in PDMS during the ripening and overripening of the grapes, which confirmed the hypotheses of Duplessis and Loubser published in 1974. We have observed that the progression in the PDMS levels during the ripening of the grapes was variable, depending on the varietal (figure 2). As the essential PDMS was represented by the SMM (Escudero et al. 2007), we drew a parallel with its role in other plants, because the origin and the role of SMM in grapes is still poorly known. However, among certain flowering plants, such as *Wollostonia biflora*, SMM is produced in the cytosol then transported into the chloroplasts to be metabolized into 3-dimethylsulfoniopropioaldehyde (Trossat et al. 1996). This could explain the increase in SMM during the ripening and overripening of grapes, which, as the chloroplasts disappear, will accumulate in the cytosol without being metabolized. On one site, Petit Manseng grapes systematically accumulated more PDMS than the Gros Manseng grapes. The difference in the weight of the grapes alone could not explain this variation, which shows there are real varietal differences.

The 2003 harvest was sunny and marked by high levels of hydric stress, and was associated with higher levels of PDMS than the samples as a whole from the six vineyard sections studied. On the other hand, rainy years showed low levels of PDMS on Grenache and Syrah grapes (Ségurel et al. 2004). These observations led us to study the impact of hydric stress on PDMS levels in grapes, as in certain seaweeds SMM plays a role as antifreeze and osmolyte (Karsten et al. 1992). In much lower levels, adapted to the osmotic conditions of the vine, SMM could play a similar role, notably in response to hydric stress.

2.1.3 Impact of hydric stress on potential dimethyl sulphide in Syrah and Chardonnay grapes

For three years (2008, 2009 and 2010), two vineyard sections growing Syrah and Chardonnay with each section having different hydric stress zones (non, medium or strong) were studied by measuring leaf water potential (LWP) and the grape samples underwent PDMS analysis. All the data were processed through principal component analysis (PCA), with the years, water constraint levels and varietals combined (figure 3). This PCA clearly showed that the PDMS levels were higher when the water constraints were weaker after *véraison*: on the horizontal axis, the PDMS vectors and the hydric potential after *véraison* (absolute value) are opposed. The effect of the year is very clear: the different zones corresponding to 2010 (the least dry of the three years studied) are on the left of the histogram, with the most important PDMS levels. The hydric state before *véraison*, located on the vertical axis, explains mainly the difference between the levels of water constraints for each varietal and for each year, and, to an extent, the PDMS levels.

Figure 3. Principal component analysis for different variables indicative of the hydric state of vines and the level of potential dimethyl sulphide in different vineyard sections (all years, water constraint levels and varietals combined)

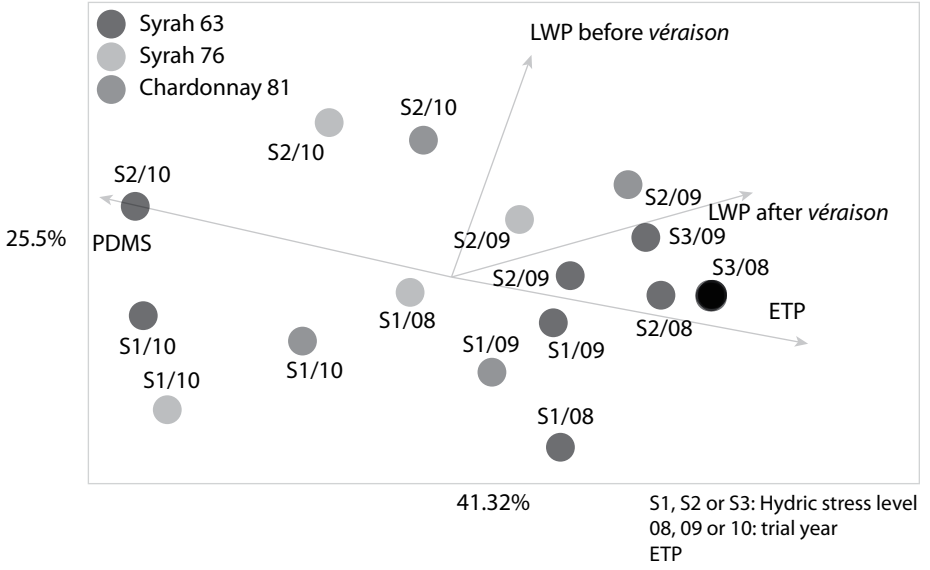
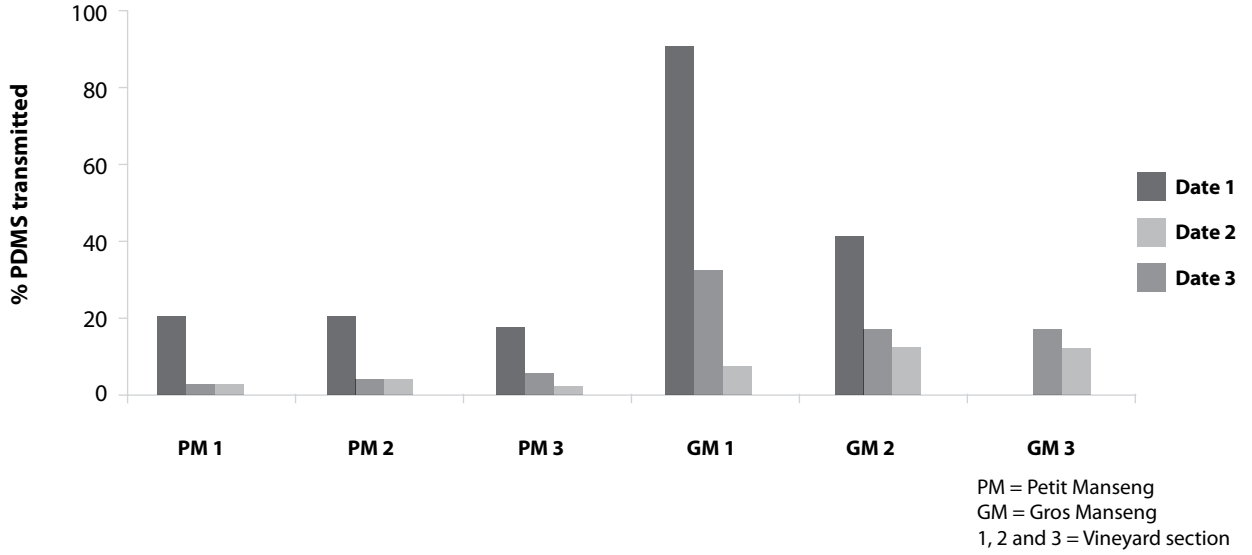


Figure 4. Percentage of potential dimethyl sulphide transmitted from the grapes to the wines (2004 harvest)



2.2 Impact of vinification on the potential dimethyl sulphide in Petit Manseng and Gros Manseng grapes

The differences between PDMS concentrations in Petit Manseng and Gros Manseng grapes and those measured in the corresponding wines obtained through mini-fermentations (20 L) showed a major decrease – an average of 80% (figure 4) – in accord with the preceeding results obtained with Grenache and Syrah (Ségural et al. 2004). Whatever the concentrations of PDMS were on the grapes, the concentrations in the corresponding wine are close, approximately 45 µg/L.

Several hypotheses could explain this significant loss of PDMS during vinification:

- The utilization of a mini-press associated with gentle extraction conditions could limit the PDMS extraction;
- The SMM representing the essential PDMS (Mestres et al. 2000), has a chemical structure that confers great reactivity vis-à-vis nucleophile groupings, which could lead to its degradation;
- Yeast is capable of assimilating SMM during cheese-making (Schreier et al. 1974), and *Saccharomyces cerevisiae* has two permeases capable of transporting SMM (Rouillon et al. 1999). The oenological yeast could be capable of consuming the SMM during fermentation.

To try to answer these questions, the rest of the research focused on the impact of pre-fermentation and fermentation parameters on the disappearance of the PDMS during vinification.

2.3 Impact of vinification parameters on potential dimethyl sulphide

2.3.1 Impact of pre-fermentation operations on potential dimethyl sulphide in Gros Manseng musts

Grapes harvested in 2009 on a section of Gros Manseng were utilized for the experiment with four pre-fermentation sequences: four-hour or 16-hour skin maceration, direct pressing, pressing then lees stabulation for 14 days.

The utilization of skin maceration and lees stabulation increased the extraction of PDMS (figure 5). A short, four-hour maceration at 18°C led to a 39% increase compared to the control, and a 65% increase with a maceration four times longer. As for the lees stabulation, it led to a 25% increase in PDMS levels.

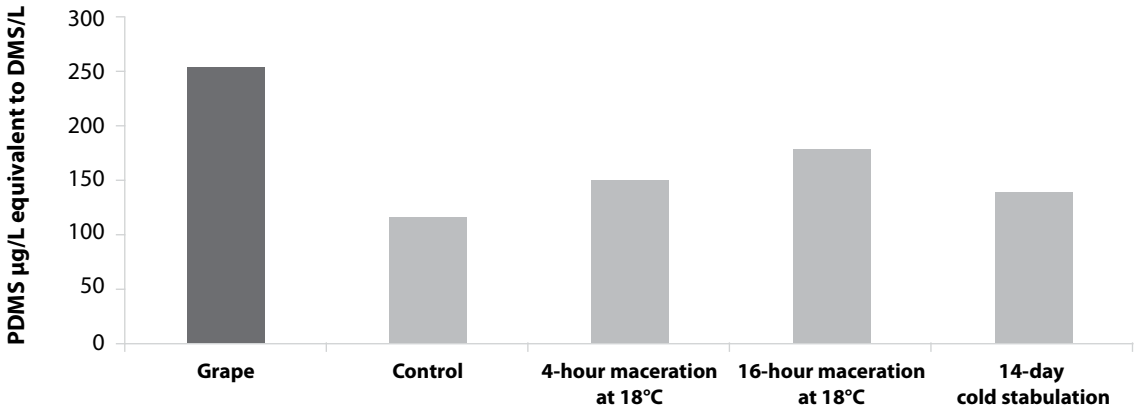
Although the pressing yield was approximately 70% (in juice volume) for the control, only 41% of the PDMS in the grapes was extracted in these conditions, which appears to show that most of the PDMS is located in the grape skin.

The pre-fermentation operations tested here could potentially enhance the extraction of PDMS and increase its concentration in musts.

2.3.2 Influence of the fermentation parameters on potential dimethyl sulphide

The objective of this trial was to evaluate the role of yeast on SMM during the fermentation of synthetic musts enriched with SMM. For the four yeast strains tested on synthetic musts (MS300) in micro-fermentation conditions (1 litre), we observed important SMM consumption levels

Figure 5. Influence of skin maceration and lees stabulation on potential dimethyl sulphide levels in Gros Manseng musts



(table 1). Residual SMM varies from 21% to 39%, demonstrating the different capacities to assimilate SMM by different yeast strains. However, this appears to be independent of the kinetics and the length of fermentation. The L4 yeast strain was also tested in duplicate on a synthetic must low in nitrogen (MS70), containing 70 mg/L total nitrogen instead of 300 mg/L. Almost all the SMM was consumed in these conditions. When the nitrogen is low, the yeast diversifies is sources of nutrients, a phenomenon at the origin of the disappearance of the SMM.

The same yeast strains in the same conditions were utilized on a Syrah must. PDMS consumption was observed as on the synthetic musts. The strains presented virtually identical aptitudes for the consumption of PDMS, which appears to confirm their specific capacity to assimilate PDMS (table 2). Nevertheless, the percentages of the remaining PDMS are lower than in the synthetic musts, which could be explained by the lower levels of total nitrogen than in the MS300 synthetic must. Also, the fermentation temperatures appear to have an impact on the assimilation of PDMS; 20°C and 28°C are the least favourable temperatures for the preservation of PDMS.

During the fermentations of the L3 and L4 strains, SMM follow-up kinetics analysis was carried out and showed that the yeasts assimilated SMM rapidly during their exponential growth phase. As soon as the maximum rate of CO_2 release was reached, the PDMS concentration did not change significantly.

In identical conditions, two yeast strains, L5 and L6, were tested in a Syrah must with and without the addition of Fermaid E. The utilization of Fermaid E corresponded to an addition of nitrogen, capable of correcting an eventual lack of nitrogen in the must. For the L5 strain, the addition of Fermaid E (5 g/hL) allowed a part of the PDMS to be preserved (figure 6). The percentage of remaining PDMS increased from 12% to 29%. This confirmed the role of nitrogen nutrition in the consumption of PDMS by yeasts. For the L6 strain, the same phenomenon was observed, but the PDMS was preserved to a lesser degree (increasing from 9% to 17%), which confirmed the specificity of certain strains to assimilate PDMS.

The assimilable nitrogen in the yeasts can be modified by spraying foliar nitrogen in the vineyard section. Trials with spraying foliar nitrogen (N10) on Gros Manseng

Table 1. Variations in the consumption of S-methylmethionine by different yeast strains during micro-fermentations (1 litre) in synthetic musts low in nitrogen (MS70) or not (MS300)

			Length of fermentation (hours)	SMM (µg eq. DMS/L)	% of SMM remaining
Synthetic must with SMM				2265	
Yeast strain	**Temperature**	**Synthetic must**		**Final wine**	
Yeast 1	24°C	MS300	120	655	29
Yeast 2	24°C	MS300	189	873	39
Yeast 3	24°C	MS300	120	473	21
Yeast 4	24°C	MS300	146	628	29
Yeast 4	24°C	MS70	427	66	3
Yeast 4	24°C	MS70	427	70	3

Table 2. Variation in potential dimethyl sulphide consumption by different yeast strains during micro-fermentations (1 L) of a Syrah must

			Length of fermentation (hours)	SMM (µg eq. DMS/L)	% of SMM remaining
Syrah must				464	
Yeast strain	**Temperature**	**Must**		**Final wine**	
Yeast 1	24°C	Syrah	209	69	15
Yeast 2	24°C	Syrah	209	91	20
Yeast 3	24°C	Syrah	209	53	11
Yeast 4	24°C	Syrah	329	113	24
Yeast 4	20°C	Syrah	351	85	18
Yeast 4	28°C	Syrah	212	89	19

vines raised assimilable nitrogen levels by 81% in the musts made with the grapes harvested on the first date (dry wine), and by 38% in those made with the grapes harvested later (sweeter wine). The utilization of a nitrogen-sulphur mixture (N10S5) brought an even greater increase (figure 7). The increase of assimilable nitrogen in the musts following the directions for N10 preserved the PDMS in the wines by 23% and 74% for date 1 and date 2, respectively. But with N10S5, while the increase in nitrogen was less important, the consumption of PDMS was identical to what was observed for the control. Spraying sulphur appears to annul the protective effect of increasing assimilable nitrogen on the preservation of PDMS (figure 7).

2.4 Wine conservation: Correlation between the percentage of freed dimethyl sulphide and the age of the wine

DMS is considered an aging aroma, but no relation exists between the concentration of DMS and the age of the wine. However, the percentage of DMS released (the ratio of free DMS over the initial PDMS) is linearly correlated to the age of the wines (Ségural et al. 2004, and Dagan 2006). New results obtained with Malbec wines aged from three to 33 years allowed to us complete the preceding correlations (Ségural et al. 2004, and Dagan 2006). Thus, the correlation reaches a plateau beyond 10 years of aging, at which point the percentage of DMS released tops out at about 75% (figure 8). Two hypotheses could explain this observation. The first would be the presence of a chemical balance between the SMM and the DMS not exceeding 75% for the DMS in oenological conditions. The second would be that among the molecules measured by the PDMS analysis method, the 25% not from the SMM could not free the DMS during the conservation of the wine. Thus, the PDMS measured in those conditions would overestimate the quantity of DMS actually releasable, and the SMM would be the only DMS precursor during conservation.

Figure 6. Influence of the addition of Fermaid E on the consumption of potential dimethyl sulphide by different strains of yeast during alcoholic fermentation

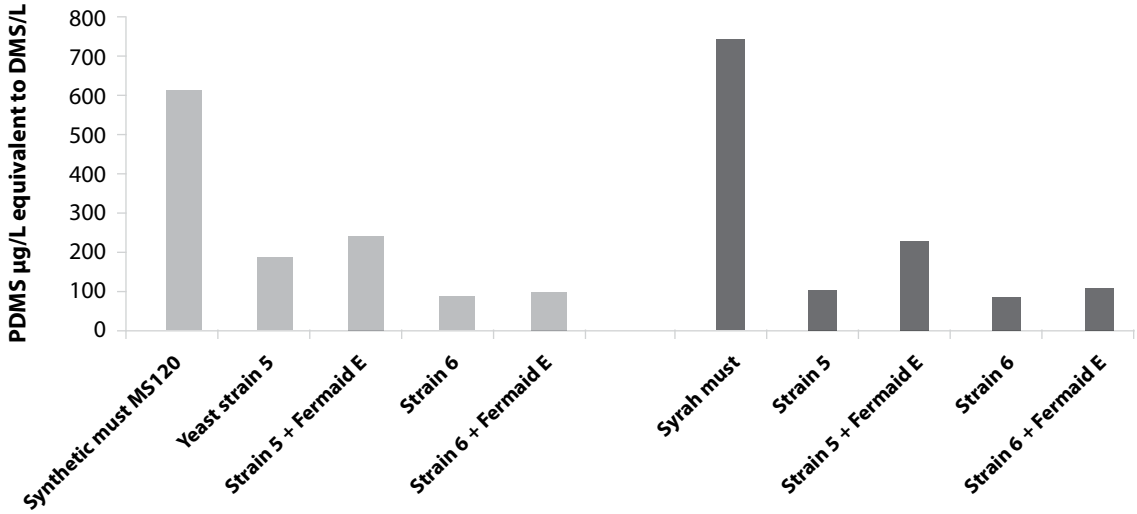
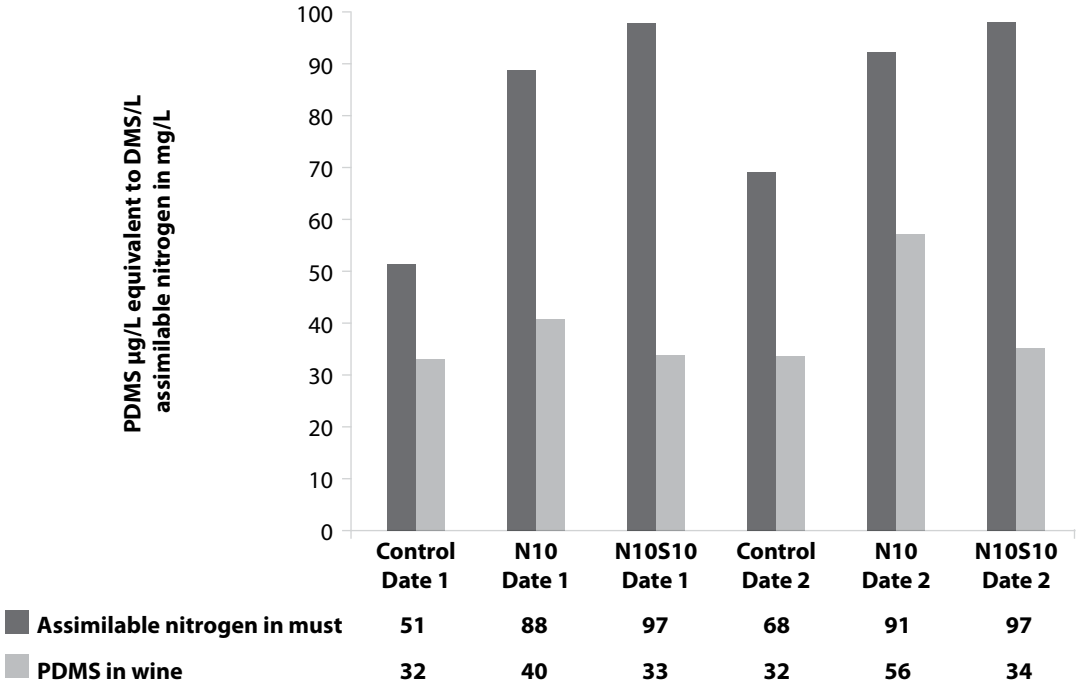


Figure 7. Influence of foliar spraying of nitrogen and sulphur on potential dimethyl sulphide consumption during yeast fermentation

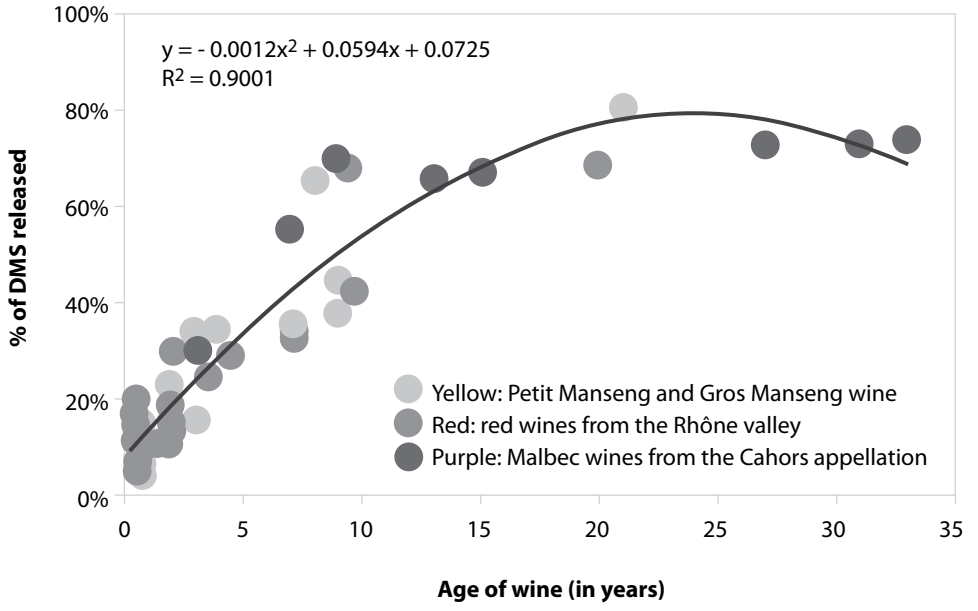


Indeed, the variability of the percentages of freed DMS observed for the wines of the same vintage shows that other parameters influence the release of DMS. The conditions of conservation constitute, therefore, a tool to modulate the forming of DMS in wines. Given the heat sensitivity of SMM, the conservation temperature could be the principal parameter to explain this variability.

3. Conclusions

Dimethyl sulphide (DMS) is an aroma exhauster and, although complex, its contribution to wine aroma can be qualitative. Thus, the presence of potential dimethyl sulphide (PDMS) in numerous varietals implies specific sensory studies regarding the contribution of DMS to different wine typologies. The identification of PDMS and, more

Figure 8. Correlation between the percentage of freed dimethyl sulphide ([free DMS]/([free DMS]+[PDMS])) and the age of wines



precisely, of S-methylmethionine (SMM) in the grapes and the must as the principal precursor of DMS during wine aging, has opened up new opportunities for study to master DMS in wines through the upstream management of its potential.

Among the winegrowing parameters studied here, some have a strong influence on the PDMS, but the fermentation parameters appear to be the determining factors to master the PDMS at racking. The choice of the yeast strain and the management of the nitrogen nutrition are two key parameters for limiting the assimilation of the PDMS during fermentation. Such pre-fermentation operations as maceration on skins and lees stabulation still need to be studied to confirm their involvement in the extraction of the PDMS, which appears to be located mainly in the grape skin.

Beyond fermentation, it is foreseeable that the management of DMS in wine will be through the management of PDMS at racking and through the length of conservation. PDMS management could also be optimized through blending. As for the length of conservation, the correlations obtained allow us to predict the approximate percentage of DMS that can be released, but this model must be further refined with a better understanding of the influence of storage conditions on the percentage of freed DMS.

A set of parameters for the production and accumulation of PDMS in the grapes, and its appreciation in the wines, can permit us to imagine integrated and better adapted production processes for different wines.

Acknowledgments

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GLUTATHIONE: RECENT DEVELOPMENTS IN OUR KNOWLEDGE OF THIS IMPORTANT ANTIOXIDANT

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Abstract

The main focus of this study was to obtain a better understanding of the evolution of glutathione (GSH) during alcoholic fermentation (AF) and to ascertain the effects of various oenological factors on its levels in Sauvignon Blanc wine. The influence of different combinations of O_2 and SO_2 additions to Sauvignon Blanc must on the GSH content in the must and wine was investigated. Wine made from oxidized juice without sulphur dioxide protection contained significantly lower levels of GSH. Twenty commercial *Saccharomyces cerevisiae* wine yeast strains were evaluated in chemically defined grape juice for differences in GSH content after AF. Significant differences were observed between strains, with some strains resulting in a sevenfold higher wine GSH content. In Sauvignon Blanc grape juice with a range of initial GSH concentrations, the concentrations fluctuated during fermentation. After AF, however, GSH concentration was generally lower than that initially present in the juice. Commercial glutathione-enriched inactivated dry yeast preparations (GSH-IDY) were also assessed in terms of the GSH concentration released into model solution. The GSH levels in grape juice fermentations supplemented with GSH-IDY were also assessed in relation to different addition times during fermentation. The GSH-IDY addition could lead to elevated wine GSH levels, provided the supplementation is done early during AF. The data have broadened our knowledge of several oenological factors, influencing GSH levels in wine and provided a new baseline for future research studies.

1. Introduction

Sauvignon Blanc is globally one of the most important cultivars and is the second most widely planted white cultivar after Chardonnay. However, wine made from this cultivar is sensitive to oxidation, which has detrimental consequences on wine quality, resulting in a loss of characteristic aroma, the development of an atypical aging flavour character and visual browning. Strategies to improve and preserve wine quality would confer a competitive advantage to the wine producer. Increasing the glutathione (GSH) levels in wine could assist in obtaining such an advantage, considering the quality-preserving function this natural antioxidant plays in wine. Apart from limiting oxidative colouration in grape juice and wine (Vaimakis and Roussis 1996, and Dubourdieu and Lavigne 2004), during wine aging GSH exerts a protective effect on various impact aroma compounds, including volatile thiols (Lavigne-Cruège and Dubourdieu 2002, Dubourdieu and Lavigne 2004, and Ugliano et al. 2011), esters and terpenes (Papadopoulou and Roussis 2001 and 2008, and Roussis et al. 2009). It has also been shown that the development of atypical aging flavour characters, including sotolon and 2-aminoacetophenone, is hampered by the presence of GSH (Dubourdieu and Lavigne 2004). High levels of this natural antioxidant may also permit the use of lower sulphur dioxide (SO_2) dosages in wine, partially addressing health-related concerns regarding the use of SO_2 in wine (Freedman 1980, and Jackson 2008). Although factors affecting GSH content in grapes (Cheynier et al. 1989, Choné et al. 2006, Lacroux et al. 2008, and Kritzinger et al. 2013a) and grape juice have been

Table 1. Code and description of different oxygen and SO_2 treatments in Sauvignon Blanc must (adapted from Coetzee et al. 2013)

Code	Treatment	Oxygen concentration in must	SO_2 additions to must
A	$-SO_2/-O_2$	<0.5 mg/L	0 mg/L
B	$+SO_2/-O_2$	<0.5 mg/L	60 mg/L
C	$-SO_2/+SO_2$	4 mg/L	0 mg/L
D	$+SO_2/+O_2$	4 mg/L	60 mg/L

elucidated (du Toit et al. 2007, Maggu et al. 2007, and Patel et al. 2010), literature on the effect of winemaking practices on GSH levels in wine is scant or contradictory. Similarly, the evolution of GSH during alcoholic fermentation (AF) is an unexplored field of study. While work done by Lavigne et al. (2007) suggests that the specific wine yeast strain may influence the GSH levels present after AF, Fracassetti (2010) regarded the influence of the yeast strain as insignificant. GSH can be assimilated by the yeast (Penninckx 2002), which would lead to reduced GSH levels in the wine. When compared to GSH levels initially present in grape juice, the levels in wine have been reported to be either lower (du Toit et al. 2007, Patel et al. 2010, and Coetzee 2011) or higher (Park et al. 2000a and 2000b, Fracassetti 2010, and Andújar-Ortiz et al. 2011). A wide range of glutathione-enriched inactive dry yeast products (GSH-IDY) are currently available on the market that claim to enhance the sensory stability of wines due to their ability to lead to higher wine GSH levels (Pozo-Bayón et al. 2009). However, little to no independent and published research on the influence of GSH-IDY on GSH levels in wine is available. Surprisingly, the single published study, by Andújar-Ortiz et al. (2011), reported that no significant difference in GSH content was observed between a control and GSH-IDY supplemented wine. Uncertainty also exists as to when these products should be added during AF. The main focus of this study was to obtain a better understanding of the evolution of GSH during AF and to ascertain the effects of various oenological factors on GSH levels in Sauvignon Blanc wine. Ultimately, the identification of factors resulting in high GSH levels in wine would be highly beneficial to wine quality and at the same time would possibly permit the use of lower SO_2 dosages in wine.

2. Methods and Materials

2.1 Influence of different combinations of O_2 and SO_2 additions to Sauvignon Blanc must on the glutathione content in the must and wine

Sauvignon Blanc juice pressed hyper-reductively using Bucher Inertys® apparatus, which excludes air during pressing by replacing it with nitrogen, was obtained from a cellar. The juice was divided into 4.5 litre glass bottles, previously sparged with CO_2 gas until inert atmosphere was reached, corresponding to O_2 concentration below 1%. Oxygen concentration was checked using an Oxi 330i handheld oxygen meter with a cell-ox 325 probe (Wissenschaftlich-Technische Werkstätten). All juices were treated with 0 or 60 mg/L SO_2 additions and 0 or 4 mg/L O_2 additions. In treatments where no O_2 was added, the O_2 levels were kept <0.5 mg/L. The different treatments and abbreviations used in this article are listed in table 1.

In the relevant treatments, the SO_2 was first added to the bottle that was then filled with juice. Oxygen levels were achieved by racking the juice into a plastic 20 L bucket to encourage O_2 pickup with continuous measurement of the oxygen until the required values were reached. Dissolved oxygen measurement was done using the Oxi 330i. A pectolytic enzyme (Rapidase® Vino Super, DSM Oenology) was added to the juice, the bottles were sealed with plastic screw caps and parafilm, and settled for one day at 15°C and the following day at 4°C. After the two days, about 3.5 L of the juice was racked from the grape lees under CO_2 pressure into another 4.5 L glass bottle (also previously filled with CO_2). All juices were inoculated with rehydrated *Saccharomyces cerevisiae* VIN 7 (Anchor Yeast Biotechnologies) at 0.3 g/L according to the supplier's recommendations, and fermentations were performed at 15°C. All treatments were performed in triplicate and the results reported are the means of the three trials. Samples destined for GSH analysis were taken before and after AF (Coetzee et al. 2013).

2.2 Screening of yeast strains in synthetic medium

The commercial wine yeast strains used in this study are listed in table 2. Chemically-defined grape juice (CDGJ) (Bely et al. 1990, Henschke and Jiranek 1993) was used for synthetic wine fermentations; the protocol of Henschke and Jiranek (1993) was followed, with the exception of the amino acid stock which was based on Bely et al. (1990). The yeast-assimilable nitrogen (YAN) content of this medium was 300 mg/L in the form of free alpha amino nitrogen and NH4Cl; 90 mL CDGJ was transferred into 100 mL glass bottles and spiked with GSH (Sigma-Al-

Table 2. Commercial *Saccharomyces cerevisiae* wine yeast strains used in this study, listed according to manufacturer (Kritzinger et al. 2013b)

Strain	Company
VIN7, VIN 13, Alchemy 1, Alchemy 2, NT 116	Anchor
LALVIN ICV® D21, LALVIN QA23 YSEO®, LALVIN DV10®, Lalvin® Rhône 4600, LALVIN® V1116, Lalvin® BA11 YSEO, LALVIN R2®, Cross Evolution® YSEO, LALVIN EC-1118®	Lallemand
CK S102, UCLM S325	Springer Oenologie
X16, X5, VL3	Laffort
ES181, Top Essence	Enartis

drich, St. Louis, MO, USA) to a concentration of 40 mg/L. Active dry yeast strains were rehydrated as recommended by the suppliers and inoculated into the CDGJ to give a cell concentration of 1 x 10^6 cells/mL. The bottles were sealed with fermentation locks and fermented at 20°C. Fermentations were conducted in triplicate. Samples destined for GSH analysis were taken directly before inoculation and after the completion of AF.

2.3 Screening in grape juice

Settled Sauvignon Blanc juice was obtained for commercial cellars and all measures were taken to protect the wine against oxidation during collection and transport. The juice was divided into 4.5 L glass bottles that had been saturated with CO_2 gas prior to filling. For the 2010 harvest, three juices were used, which will be described shortly. The GSH content of juice A was 20 mg/L. The bottles containing juice A were then divided into two groups. One group was left as is, while the other group was spiked with GSH (Sigma-Aldrich) to 80 mg/L, subsequently referred to as juice B. The GSH content of juice C was 10 mg/L and also left as is. For the 2011 harvest, settled Sauvignon Blanc juice was obtained and exactly the same protocol was followed as for the 2010 harvest. This juice is referred to as juice D. The free SO_2 concentration of each treatment was adjusted to 30 mg/L. The juice was then inoculated with commercial preparations of *S. cerevisiae* at 0.3 g/L according to the suppliers' recommendations; juices A, B and C were inoculated with strains VIN7, LALVIN QA23 YSEO®, Cross Evolution® YSEO and VL3, and juice D was inoculated with these strains plus three additional strains, LALVIN R2®, X16 and LALVIN EC-1118® (see table 2). The bottles were all sealed with airlocks and weighed until fermentation was completed. This experiment was conducted in quadruplicate in the 2010 season and in triplicate in the 2011 season. Samples destined for GSH analysis were taken five times during fermentation according to the weight loss, which corresponded to 0, 25%, 50%, 75% and 100% sugar loss. Juice samples were taken before inoculation after it had been divided into the separate bottles (Kritzinger et al. 2013b).

2.4 Determination of GSH released from various GSH-IDY

By using a model solution, the GSH, oxidized glutathione (GSSG) and total GSH concentrations (GSH + 2 X GSSG as molar equivalents) released by five different commercial GSH-IDYs were evaluated. The GSH-IDY were supplied by four different manufacturers. The model solution consisted of 5 g/L tartaric acid adjusted to pH 3.3 using 5 M NaOH (Merck Chemicals). To attain an O_2 concentration < 1 mg/L, N_2 gas (Afrox, South Africa) was bubbled through the solution for several minutes; 1 g GSH-IDY was transferred quantitatively into a 100 mL volumetric flask, filled to the mark with model solution, and stirred for 10 minutes when sampling for GSH analysis was done. This experiment was performed in triplicate (Kritzinger et al. 2012).

2.5 Influence of GSH-IDY added at different fermentation stages on GSH concentration in wine

Settled Sauvignon Blanc juice was obtained from a commercial cellar. Several measures were taken to prevent the oxidation of the juice during collection and transport; 2 L glass bottles were used as fermentation units. These bottles were first filled with water, which was then displaced with CO_2 gas (Afrox SA) to achieve inert atmosphere. The juice was then displaced with CO_2 gas into the 2 L glass bottles. The free SO_2 concentration of each treatment was adjusted to 30 mg/L. The juice was inoculated with LALVIN QA23 YSEO® (Lallemand) *S. cerevisiae* yeast at 0.3 g/L; the yeast had been rehydrated in GoFerm Protect® (Lallemand). OPTIWHITE® (GSH-IDY) addition at 0.3 g/L was made to the different treatments, as listed in table 3, the bottles were sealed with airlocks and weighed to monitor the progress of fermentation which took place at 15°C. FermaidK® yeast nutrient (Lallemand) addition at 0.25 g/L was made after 5° Brix had fermented out. This experiment was performed in triplicate. Samples were

Table 3. Time of OPTIWHITE® additions to Sauvignon Blanc must during alcoholic fermentation

Code	Description
Control	No addition made
Juice	Addition made to settled juice directly before inoculation with yeast
1/3	Addition made a third of the way through fermentation (at 14.5° Brix)
2/3	Addition made two thirds3 through fermentation (at 7.3° Brix)

taken three times during the course of the experiment; juice samples drawn before inoculation, must samples in the middle of alcoholic fermentation and wine samples after completion of AF (Kritzinger et al. 2012).

The evolution of GSH in Sauvignon Blanc juice during fermentation was studied, where no GSH (C), 5.5 mg/L GSH (5.5 GSH), 80 mg/L GSH (80 GSH), GSH-IDY (YE) and 80 mg/L GSH (YE+ 80 GSH) were added to the juice in another experiment. We obtained the clear juice from a commercial cellar and added the products 10 minutes after yeast inoculation (LALVIN QA23 YSEO®) according to the supplier's recommendations. Wine samples were taken at the end of AF.

2.6 Sampling procedure, sample preparation and glutathione analysis for juice and wine sampling

Samples for GSH analysis were drawn at the stages described above. The required sample volume was transferred under CO_2 gas into plastic sampling bottles that had been previously filled with CO_2 gas; 1000 mg/L SO_2 and 500 mg/L ascorbic acid were also added to the sampling bottles prior to sampling. Additional CO_2 gas was blown on the headspace after sampling. The samples were then immediately frozen at -20°C until analysis.

2.7 GSH analysis of juice and wine samples

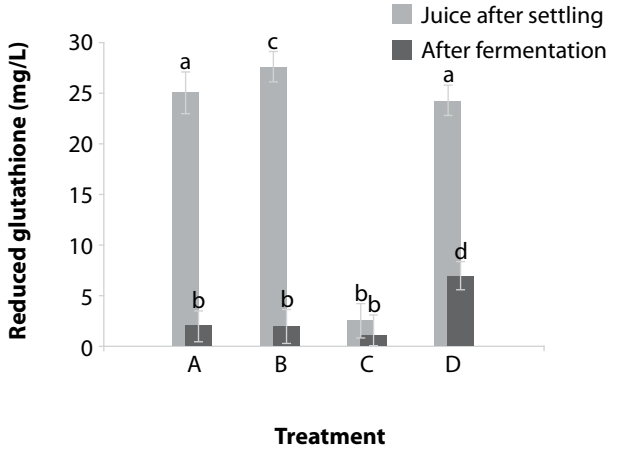
GSH in the must and wine was detected and quantified by the method described by Fracassetti et al. (2011) using ultra performance liquid chromatography (UPLC). Samples from the GSH-IDY experiments were detected and quantified by the method described by Kritzinger et al. (2012) using UPLC.

3. Results and Discussion

3.1 Influence of different combinations of O_2 and SO_2 additions to Sauvignon Blanc must on the glutathione content in the must and wine

Figure 1 displays the GSH concentrations in grape must and the corresponding wines exposed to different SO_2 and O_2 treatments. There was little or no difference in GSH concentrations among treatments A, B and D. A significant decrease in GSH concentration is observed in treatment C, where oxidation took place as a result of oxygen exposure without the protective effect of SO_2. Although treatment D was exposed to the same amount of oxygen, sufficient SO_2 was present to inhibit the oxidation of GSH. This highlights the importance of SO_2 in inhibiting grape polyphenol oxidase, which catalyzes the oxidation of phenols to ortho-quinones with the subsequent incorporation of GSH to form the Grape Reaction Product (GRP).

Figure 1. Reduced glutathione (GSH) concentration in juice and wine submitted to different SO_2 and O_2 treatments



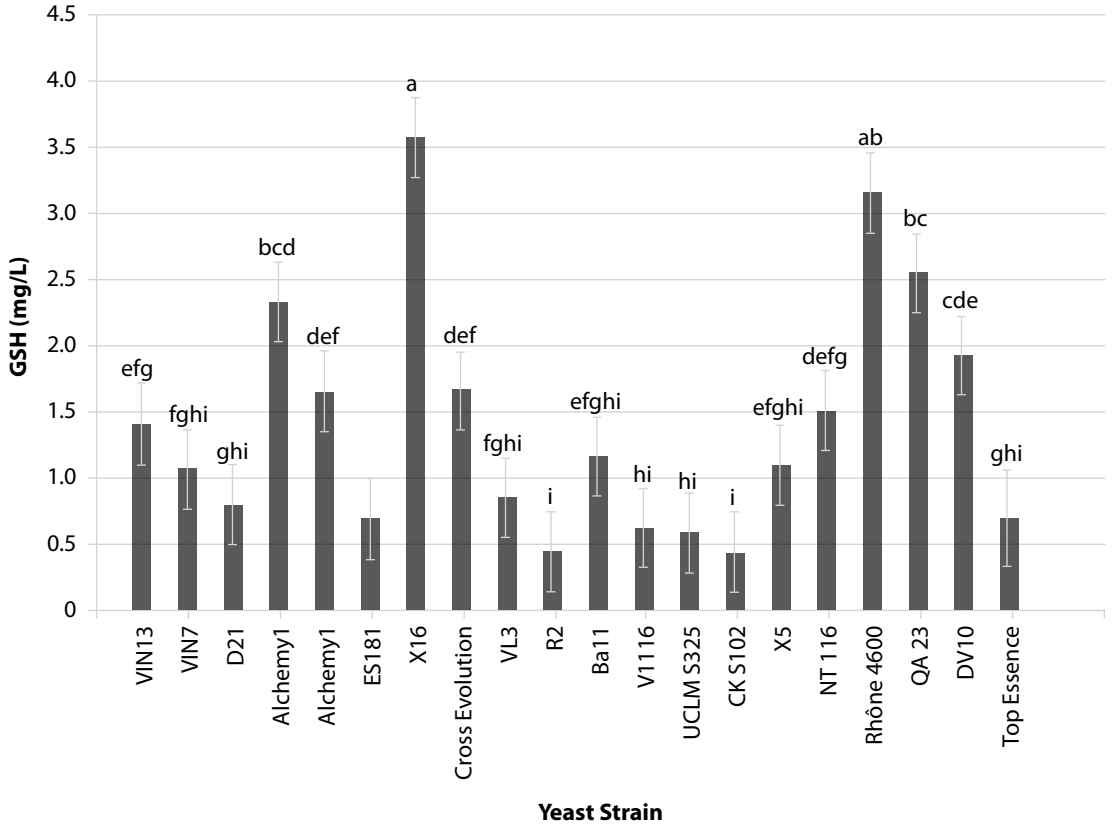
A (-SO_2/-O_2), B (+SO_2/-O_2), C (-SO_2/+O_2) and D (+O_2/+SO_2)

Error bars indicate 95% confidence intervals for the means. Letters indicate significant differences on a 5% ($p<0.05$) significance level.

Although not measured, more GRP would have formed in treatment C, resulting in less GSH available for further protection against oxidation and more ortho-quinones present in the juice.

It is evident that GSH concentrations decreased during AF. There were no significant differences in GSH concentrations between the different treatments after AF, with the exception of treatment D. Uncertainty remains as to what exactly led to the increased GSH concentration in treatment D, and this observation necessitates further investigation (Coetzee et al. 2013).

Figure 2. Reduced glutathione (GSH) concentration at the end of alcoholic fermentation for 20 different commercial *Saccharomyces cerevisiae* strains.



3.2 Screening of yeast strains in synthetic medium

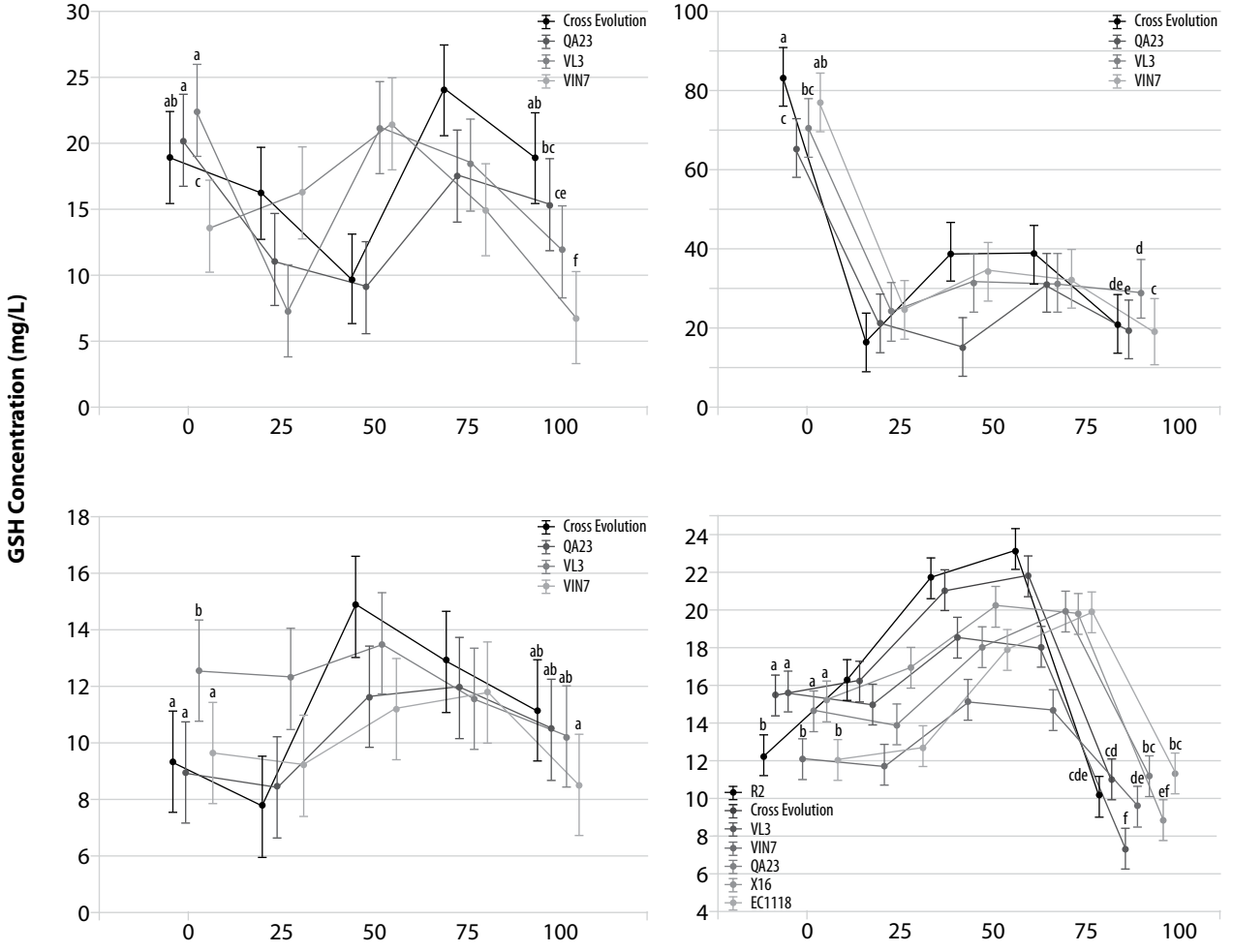
Twenty commercial yeast strains were screened in a CDGJ containing 40 mg/L GSH to resemble a natural grape must. During AF, a drastic decrease in GSH concentration was observed, with end concentrations ranging from 0.5 mg/L to 3.5 mg/L (figure 2). Marchand and de Revel (2010), Janes et al. (2010), and Fracassetti et al. (2011) found similar GSH concentrations in several white wines after AF. The formation of ortho-quinones with the subsequent incorporation of GSH to form 2-S-glutathionyl caftaric acid (GRP) was impossible, as the synthetic medium contained no phenolic compounds or polyphenol oxidase. Therefore, the decrease in GSH concentration cannot be attributed to the incorporation of GSH into GRP. Statistically significant differences ($p<0.05$) in final GSH content were observed among the different treatments. Synthetic wines fermented with strains X16 and Lalvin® Rhône 4600 displayed a sevenfold higher GSH content compared to those fermented with strains LALVIN R2® and CK S102. It therefore seems the yeast strain may indeed influence the GSH concentration present after AF, which agrees with the results of Lavigne et al. (2007). It would be interesting to investigate whether this observation is linked to the nitrogen demand of yeast strains, and whether strains with high nitrogen demands would result in wines with lower GSH concentrations (Kritzinger et al. 2013b).

Vertical bars denote 95% confidence intervals for the means. Letters indicate significant differences on a 5% ($p<0.05$) significance level (Kritzinger et al. 2013b, reproduced with permission of the Australian Journal of Grape and Wine Research).

3.3 Screening in grape juice

Four yeast strains were selected for further evaluation in grape juice A, B and C in 2010. In juice D, during 2011, three additional strains were implemented. Figure 3 displays the GSH concentration during AF for the four musts. The GSH concentration during AF for the four musts varied considerably, depending on the initial GSH concentration present in the must and the yeast strain used to conduct fermentation. This highlights the variability in GSH evolution under different conditions. In general, the GSH concentration in wines was similar or lower than initially present in the grape juice. The decrease in GSH in the early stages of fermentation may be due to the incorporation of GSH to form GRP. However, several measures were taken to prevent the formation of GRP and the subsequent loss of GSH. The juice was treated reductively by means of dry ice and CO_2 gas to prevent the oxidation

Figure 3. Reduced glutathione (GSH) evolution during alcoholic fermentation for different yeast strains for juice A, B, C and D
Vertical bars denote 95% confidence interval for the means. Letters indicate significant differences on a 5% ($p<0.05$) significance level (Kritzinger et al. 2013b, reproduced with permission of the Australian Journal of Grape and Wine Research).

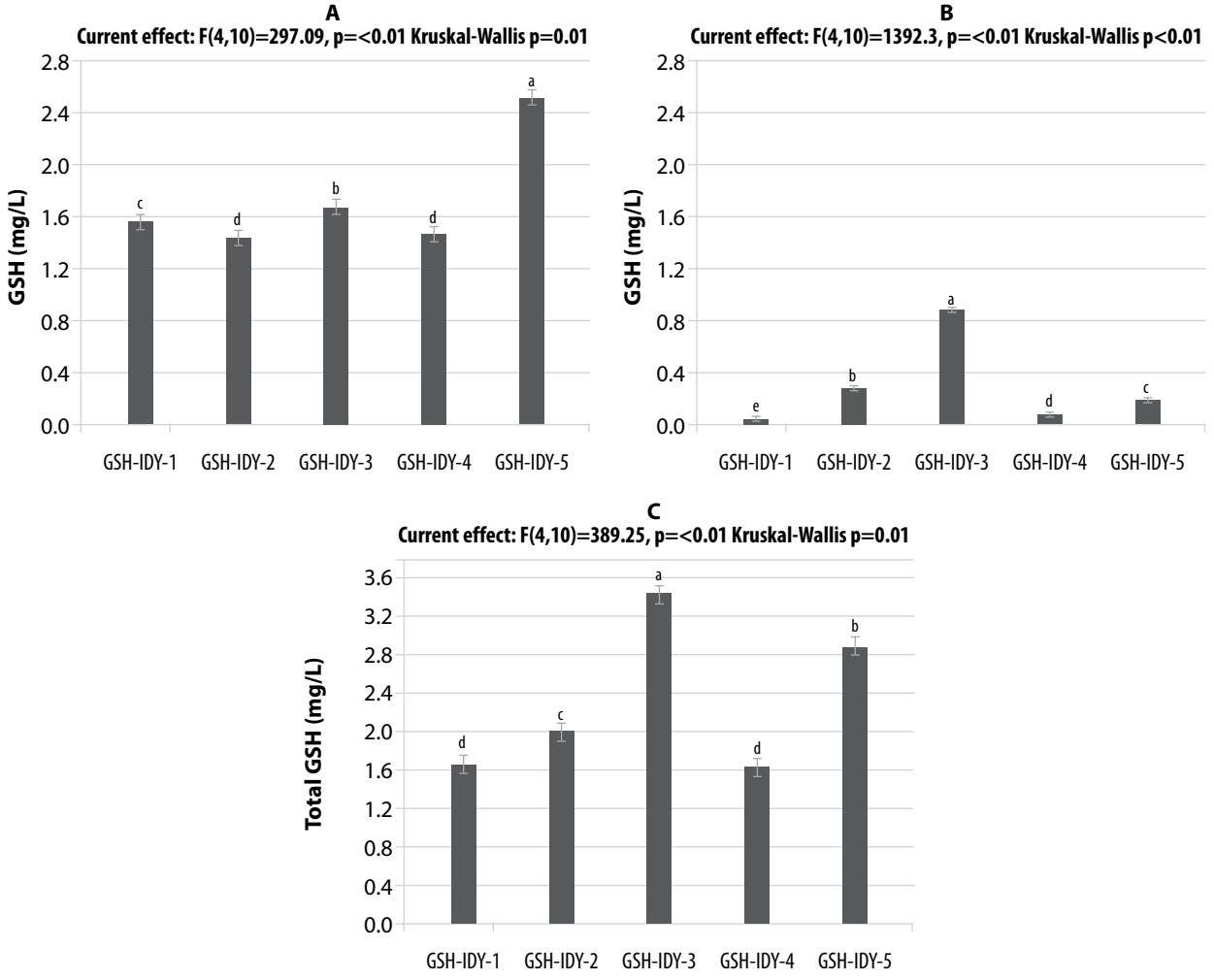


of polyphenols. In addition, the free SO_2 concentration was also adjusted to 30 mg/L free and 60 mg/L total SO_2. According to Dubernet and Ribéreau-Gayon (1973), the addition of 25 to 75 mg/L SO_2 to clarified juices led to an inhibition of 75% to 97% in polyphenol oxidase activity, respectively.

An additional explanation for the decrease in GSH concentration at the onset of AF is the possible uptake by the yeast through the ATP-driven, high-affinity GSH transporter, Hgt1p (Bourbouloux et al. 2000). According to Penninckx (2002), GSH is implicated in many stress response mechanisms, such as sulphur and nitrogen starvation, oxidative stress and the detoxification of heavy metals and xenobiotics. An interesting observation is that, in some instances, the GSH concentration increased to a concentration exceeding that originally present in the must. This may possibly be ascribed to the de novo synthesis of GSH by yeast with the subsequent secretion into the must. This hypothesis was also made by Park et al. (2000a and 2000b) who ascribed the increase in GSH concentration during fermentation to the formation of GSH by *S. cerevisiae*. Perrone et al. (2005) showed that endogenously produced GSH in the yeast cytosol can be secreted under normal growth conditions. Moreover, the secreted glutathione was predominantly in the reduced form (GSH) and this GSH could again be taken by the yeast GSH transporter. It was thus shown that intracellular GSH may cycle with the extracellular GSH present in the medium, which might explain the fluctuation of GSH observed during AF in this study. Although the exact mechanism of GSH export is not known, a novel GSH exchanger, Gex1, was recently identified in *S. cerevisiae* (Dhaoui et al. 2011). It is unclear what led to the decrease in GSH concentration in the last quarter of AF. Park et al. (2000a), however, reported the same observation in fermenting Palomino grape juice, which was not explained by the authors. According to Lavigne and Dubourdieu (2004), the YAN content of

Figure 4. (A) Reduced (GSH), (B) oxidized (GSSG) and (C) total glutathione content released by various GSH-IDY

Vertical bars denote 95% confidence interval for the means. Letters indicate significant differences on a 5% significance level (Kritzinger et al. 2012, reproduced with permission of F*ood Additives and Contaminants. Part A: Chemistry, Analysis, Control, Exposure and Risk Assessment*).



the juice may influence the yeast's ability to release GSH. They also reported that a content of 200 mg/L is needed to allow GSH release during fermentation. The YAN contents of all four juices were, however, was above 300 mg/L, excluding the possibility that a limiting nitrogen source could have potentially influenced the data. Nevertheless, it is evident that variable GSH evolution was observed during AF as a result of the different strains used, which corroborates data by Lavigne et al. (2007). Juices with initial high GSH concentrations would not necessarily result in wines with high GSH concentrations, which corroborates work done by du Toit et al. (2007). It should also be mentioned that the GSH concentration at the end of AF could be subject to variability due to the variable length of fermentation of the various strains. However, further research is necessary to elucidate this hypothesis. For further details, Kritzinger et al. (2013b) can be consulted.

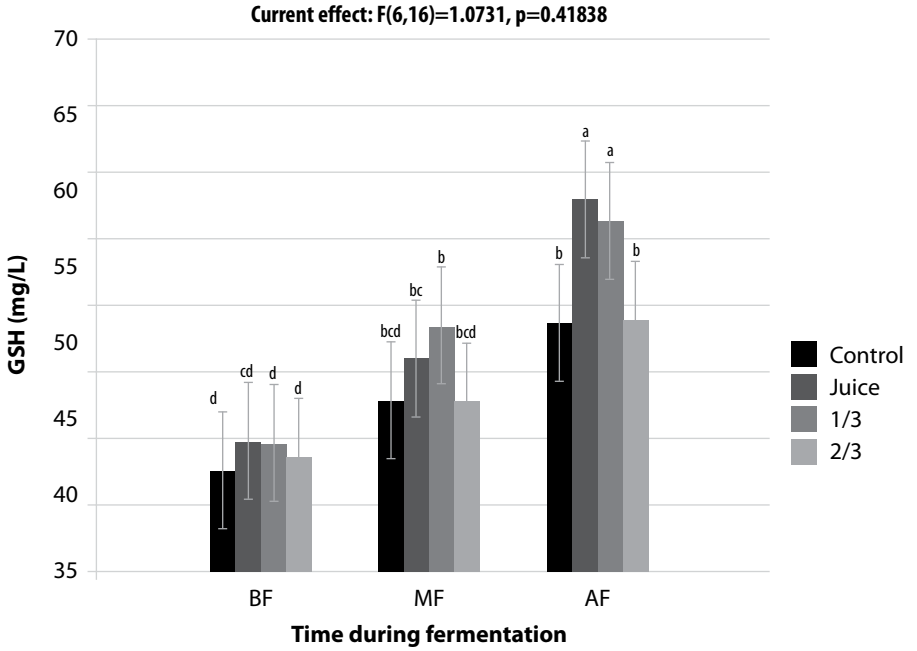
3.4 Determination of GSH released from various GSH-IDY

The GSH and GSSG contents in this section are reported as a 0.3 g/L GSH-IDY addition. Figure 4 displays the amount of GSH released by the different GSH-IDY. The GSH concentrations released by the various preparations differed significantly, ranging from 1.45 mg/L to 2.53 mg/L. This is in line with data by Andujar-Ortiz et al. (2011), who reported four GSH-IDY preparations released from 1 mg/L to 2 mg/L GSH into synthetic wine solutions. According to the authors, the differences in the amounts released might be ascribed to different manufacturing processes, especially with regards to the nutrients provided during the growth of the yeast culture.

Other factors of variance that may influence the GSH released from the GSH-IDY could be strain differences and the extent of thermal damage that takes place during the

Figure 5. Reduced glutathione (GSH) evolution during alcoholic fermentation for Sauvignon Blanc juice supplemented with GSH-IDY-4 at different stages during fermentation

Vertical bars denote 95% confidence interval for the means (Kritzinger et al. 2012, reproduced with permission of *Food Additives and Contaminants. Part A: Chemistry, Analysis, Control, Exposure and Risk Assessment*).



drying process (Tirelli et al. 2010, and Andujar-Ortiz et al. 2011). The latter may also account for the large variation reported for the GSSG contents (0.04 mg/L to 0.88 mg/L GSSG). The total GSH contents released ranged from 1.63 mg/L to 3.44 mg/L, which is similar to results by Andujar-Ortiz et al. (2011) reporting total GSH levels in the range of 1.82 mg/L to 2.72 mg/L. GSH-IDY-3 released the highest total GSH concentration, but this was attributed to the high GSSG content of this product. The data illustrate the variation that exists among GSH-IDY in terms of GSH content, and it underlines the importance of distinguishing between GSH and total GSH contents as only the reduced form can act as an active antioxidant in wine. For further details, Kritzinger et al. (2012) can be consulted.

GSH-IDY-5 (Lallemand OPTIMUM WHITE®) released considerably more GSH compared to other the products. It would be interesting to investigate whether this product would be more efficient in reducing the oxidation phenomena in wines when compared to the other GSH-IDY in this study.

3.5 Influence of GSH-IDY added at different fermentation stages on GSH concentration in wine

Figure 5 presents the GSH concentrations during AF for wine supplemented with OPTIWHITE® at different stages. The GSH concentration increased during fermentation, regardless of the treatment applied. The GSH concentrations for treatments "juice" and "1/3" were 59.9 mg/L and 58.5 mg/L, respectively, which were considerably higher than those of the control and "2/3" treatment (51.6 mg/L and 51.8 mg/L, respectively). This correlates to a 7-8 mg/L difference in GSH concentration between the control and the "juice" or "1/3" treatment, which is rather interesting, taking into consideration the 1.5 mg/L GSH released from GSH-IDY-4 (OPTIWHITE®) into the model solution as determined in the previous section (figure 4). Several soluble nitrogenous compounds are released by inactive dry yeast preparation (Pozo-Bayón et al. 2009) with some stimulating GSH synthesis by the yeast (Wen et al. 2004, and Andujar-Ortiz et al. 2011). The nutrients supplied by OPTIWHITE® (free amino acids, peptides, etc.) at the early stages of fermentation might have led to increased GSH synthesis and release. We hypothesize that the GSH-IDY supplementation of the "2/3" treatment was made too late during AF for the yeast to benefit from the increased nutrients to synthesize and release GSH. Indeed, it has been shown that the hydrogen ion-coupled import of amino acids is inhibited by ethanol (Bisson 1996). For further details, Kritzinger et al. (2012) can be consulted.

We also found in preliminary results that the addition of different levels of GSH and GSH-IDY to the must may influence the GSH content in the final wine, as indicated in table 4.

Table 4. Average GSH levels of wines treated with different GSH and GSH-IDY treatments

	Average	STD
C	10.0	0.7
80 GSH	67.3	3.8
YE	13.2	1.3
5.5 GSH	11.8	0.4
YE+80 GSH	69.8	4.1

STD: Standard deviation

This study demonstrates that the specific GSH-IDY (OPTIMUM WHITE®) can often lead to increased GSH concentrations in wine. Furthermore, this increase can exceed the GSH concentration present in the product itself. It remains unclear whether this is as a result of increased GSH synthesis and secretion by the yeast or whether it can be ascribed to the preferential uptake of additional nutrients supplied by GSH-IDY over GSH.

4. Conclusions

This study has shown that glutathione (GSH) concentrations in juice can be protected if sufficient amounts of SO_2 are used, even in the case of exposure to oxygen. SO_2 limits the oxidation of polyphenols, especially transcaftaric acid, which results in the formation of the very reactive ortho-quinones. GSH reacts with ortho-quinones to form the grape reaction product (GRP) (Singleton et al. 1985, and Cheynier et al. 1986).

Differences in GSH content were observed for synthetic wines fermented with different strains, with some strains resulting in a sevenfold higher synthetic wine GSH content. However, when these strains were inoculated into Sauvignon Blanc juice, they did not necessarily result in the highest wine GSH concentration. Important trends regarding GSH evolution during alcoholic fermentation were observed. GSH levels fluctuated during fermentation, depending on several factors, such as the yeast strain and the initial GSH concentration of the juice. It appears from this experiment that the GSH concentration in some instances increases to levels on par or higher than those initially present in the juice, suggesting the de novo synthesis and secretion of GSH by the yeast. Differences in GSH content for wines fermented with different yeast strains could be observed, albeit small.

The commercial GSH-IDY tested differed significantly in the amount of GSH and oxidized glutathione (GSSG) levels released into a model solution, which highlights the variability among the products in terms of their antioxidant potential. OPTIWHITE® supplementation could result in elevated wine GSH levels, provided the supplementation is made within the first third of alcoholic fermentation. Moreover, the difference in GSH content between the control and the OPTIWHITE® supplemented wine was fivefold higher than the GSH content released into a model solution. Further investigation into GSH-IDY, especially with regards to their influence on yeast metabolism, is needed to elucidate the exact mechanism by which GSH-IDY leads to increased GSH levels in wine. Future research will also benefit from a comprehensive sensory evaluation of the wine to establish the influence GSH-IDY supplementation has on the sensory profile of wines.

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CHASING VARIETAL AROMAS: THE IMPACT OF DIFFERENT LACTIC ACID BACTERIA AND MALOLACTIC FERMENTATION SCENARIOS

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Abstract

Lactic acid bacteria (LAB) are responsible for malolactic fermentation (MLF), first to degrade L-malic acid to L-lactic acid, and second to contribute to the wine aroma and flavour by the production of volatile metabolites and the modification of aroma compounds derived from grapes and yeasts. The yeast strains used for alcoholic fermentation have been shown to impact MLF through the production of ethanol and sulphur dioxide, as well as the competition for nutrients, etc. Most commercial MLF starter cultures still consist of *Oenococcus oeni*, but recently the focus has shifted towards the use of *Lactobacillus plantarum* alone or in mixed cultures with *O. oeni*. In the past decade, several studies have shown that co-inoculating MLF and alcoholic fermentation starter cultures has several advantages, especially in warmer climate regions that produce high alcohol levels. Those advantages include reduced overall fermentation duration, positive aroma modifications with fruitier wines and reduced risk of spoilage.

Studies of the impact of yeasts on MLF and wine aroma show that the volatile aroma profiles differ in their ratios of esters, higher alcohols and carbonyl compounds, depending on the yeast strain used. Therefore it is important to select and pair the yeast strains to ensure the desired wine style can be obtained.

Different MLF inoculation strategies can be used to change the wine style – a major trend for the fresh and fruity wine styles. In a study of two different *O. oeni* strains on cool-climate Riesling wines and four different inoculation times that compared wines produced by sequential inoculation with co-inoculation wines, the results tend to show higher concentrations of ethyl and acetate esters, including acetic acid phenylethyl ester, acetic acid 3-methylbutyl ester, butyric acid ethyl ester, lactic acid ethyl ester and succinic acid diethyl ester, in the co-inoculated wines. Another investigation studied the influences of pH and ethanol on MLF, and the volatile aroma profile of the subsequent white wines from Riesling and Chardonnay inoculated with two different *O. oeni* strains. The wines showed significant differences in total higher alcohols and in the esters and acids that are important for the sensory profile and quality of wine. This work demonstrated that the wine matrix, the pH and the alcohol concentration affect MLF and the final volatile aroma profile. The results indicate that changes in volatile aroma composition are not necessarily related to complete MLF, and that partial MLF already has distinct influences on the aroma profile of white wines.

The changes in volatile aroma composition can also be driven by using different LAB strains. The major difference between using *O. oeni* and *L. plantarum* regards esters and monoterpenes, and lies within the arsenal of enzymes which *L. plantarum* has and, therefore, the capacity to produce a greater diversity of compounds that can contribute to varietal aromas.

1. Introduction

Alcoholic fermentation (AF) is the primary fermentation in wine, carried out by yeast, mainly the more alcohol tolerant *Saccharomyces cerevisiae* that convert sugar to etha-

nol and CO_2. Other yeast genera frequently associated with wine include *Torulaspora, Candida*, *Hanseniaspora*, *Brettanomyces*, *Pichia*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Willopsis* and *Kloeckera*, to name a few (Zott et al. 2010, and Comitini et al. 2011). AF, and especially the choice of yeast strain, contributes to the aroma profile of the wine by producing compounds such as esters, higher alcohols, aldehydes and fatty acids (Styger et al. 2011).

Malolactic fermentation (MLF) is a secondary fermentation conducted by lactic acid bacteria (LAB), mainly *Oenococcus oeni*, in most red wines and some white and sparkling wines. It is a decarboxylation process where L-malic acid is converted to L-lactic acid with the production of CO_2. The three main reasons for conducting MLF in wine are to deacidify the wine, to improve the microbial stability of the wine by removing malic acid (malate) as a possible carbon source, and to modify wine aromas (Lerm et al. 2010). MLF can modify wine aroma via the production or modification of flavour-active compounds (Swiegers et al. 2005, Boido et al. 2009, and Michlmayr et al. 2012). In cooler climate countries, such as New Zealand and Canada that produce high-acid wines, MLF is conducted mostly for the purpose of deacidification (Liu 2002). In warmer regions, where deacidification is of less importance as lower malic acid concentrations are present in the grapes, MLF is conducted mainly for the purpose of changing the sensory profile of the wine (Lerm 2010).

The main LAB associated with wine are in the *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* genera. Of the four LAB genera found in wine, *O. oeni* is the best adapted to overcoming the high ethanol levels, low pH conditions and fermentation temperatures, as well as SO_2, all of which make wine a harsh environment. This explains the use of *O. oeni* as the predominant LAB in MLF starter cultures today. However, *Lactobacillus plantarum* has also proven its resilience, and is therefore now included in MLF starter cultures, especially for high pH wines, and for co-inoculation with yeast (Lerm et al. 2010, and du Toit et al. 2011).

2. Factors that Influence Lactic Acid Bacteria Growth and Malolactic Fermentation

In the complex, harsh wine environment that contains different microorganisms competing for survival, many factors can influence LAB growth and, therefore, the successful completion of MLF. These factors include high ethanol concentration (exceeding 15% v/v), low pH (less than 3.2), low temperature and SO_2 concentration (more than 50 mg/L), lysozymes, phenolic compounds, medium-chain fatty acids, yeast-bacteria interactions and nutrient availability (Alexandre et al. 2004, Lerm et al. 2010, and Bartowsky and Borneman 2011).

Ethanol plays a critical role in the success of MLF, because it can disrupt bacterial membranes and affect many membrane-associated processes, including malolactic activity and the processes involved in stress resistance (Zapparoli et al. 2009). According to Rosi et al. (2003), ethanol and pH are the most important wine parameters impacting on bacterial activity. In their study, they found that pH values below 3.2 lowered *O. oeni* viability. Ethanol has shown synergistic interactions with temperature, inhibiting LAB growth (Lerm 2010). High ethanol concentrations lower the optimal growth temperature of LAB, whereas increased temperatures lower the ability of LAB to tolerate higher ethanol concentrations (Henick-Kling 1993).

The effect of SO_2 on LAB is dependent on such factors as yeast strain and wine composition, specifically wine pH (Alexandre et al. 2004). It has been found that the molecular form of SO_2 is toxic to wine yeasts and bacteria. It was also suggested that molecular SO_2 inhibits bacterial growth by reducing maximal biomass and malic acid activity.

Yeast can produce medium-chain fatty acids, such as decanoic acid, that impact the growth rate and malolactic activity of LAB, depending on concentration, and the pH of the medium as well (Carreté et al. 2002, and Alexandre et al. 2004). Therefore, not only can medium-chain fatty acids cause yeast-bacterial antagonism, they can reduce the malic acid degradation abilities of the bacteria (Alexandre et al. 2004). The impacts of the yeast on MLF fall into three categories: inhibitory, neutral or stimulatory.

3. Inoculation Scenarios

Spontaneous MLF is generally considered to be carried out by the indigenous LAB present in the wine and/or on the winemaking equipment, making the outcome of the process very unpredictable. The risks involved with spontaneous MLF include the possible presence of spoilage microorganisms that can produce undesirable off-flavours, and/or biogenic amines that can affect human health and postpone the onset or completion of MLF. All these risks can diminish the quality of the wine (Alexandre et al. 2004, Lerm 2010, and López et al. 2011).

Inoculation for MLF traditionally occurs after the completion of AF (in sequential inoculation) using commercial starter cultures. The inoculation with LAB and yeast at the beginning of AF (co-inoculation/simultaneous inoculation) is now an alternative for especially high pH and high

alcohol wines. MLF can also be induced during alcoholic fermentation (Knoll et al. 2012).

3.1 Sequential inoculation

Some of the literature suggests that sequential inoculation could be a means to avoid such problems as antagonistic yeast-bacteria interactions potentially associated with simultaneous inoculation (Lerm 2010). Due to the completion of AF, the lower residual sugar concentrations that reduce the risk of acetic acid production are another advantage of sequential inoculation (Costello 2005).

Risks involved with sequential inoculation include sluggish or stuck MLF due to LAB viability problems caused by high ethanol concentrations, low pH, SO_2, or other microbial compounds produced by the yeast and nutrient depletion (Larsen et al. 2003). Massera et al. (2009) stated that inoculation with starter cultures after AF does not always result in the dominance of the selected strain and the desired contribution.

3.2 During alcoholic fermentation

Some winemakers implement this inoculation regime to overcome high ethanol concentrations, as is the case with sequential inoculation, so the LAB inoculated into the wine can still adapt to the increasing ethanol concentrations. Another reason why mid-AF inoculation may be implemented is because most of the free SO_2 is bound, thereby reducing the possible inhibition of LAB by SO_2. Moreover, the heat generated from the on-going AF will aid in inducing the growth of the LAB and therefore MLF. A study by Rosi et al. (2003) showed an immediate and extreme decrease in LAB cell counts when the wine was inoculated midway through AF, declining as low as 10^4 CFU/mL in the first six to eight days after inoculation then increasing again to 10^6 CFU/mL, at which point malic acid degradation began.

3.3 Co-inoculation

Co-inoculation of LAB and yeast is a helpful time-saving tool that can be used to overcome high ethanol concentrations and reduced nutrient availability, which is often associated with the conditions after the completion of AF leading to incomplete MLF (Jussier et al. 2006). The gradual adaptations of the bacteria to the increasing ethanol concentrations enhance their performance (Zapparoli et al. 2009). Co-inoculation allows an early dominance of the selected strain and better control over the outcome of MLF (Massera et al. 2009). A study by Nehme et al. (2008) found improved bacterial growth and malic acid consumption using co-inoculation.

As previously discussed, the possible yeast-bacterial interaction that might occur during co-inoculation is an important factor to consider when making decisions regarding inoculation time. Homofermentative LAB, such as *L. plantarum*, produce lactic acid as the major end product; whereas heterofermentative LAB (such as *O. oeni*) produce lactic acid, CO_2, ethanol and/or acetic acid (Zúñiga et al. 1993). The risk of increased volatile acidity due to sugar metabolism by bacteria is negligible if AF is successfully carried out by yeasts (Azzolini et al. 2011). This statement is in agreement with studies done by Nehme et al. (2010) and Knoll et al. (2012) that showed no risk of increased volatile acidity during co-inoculation. The fear of this possible increase in volatile acidity is the reason for the infrequent utilization of co-inoculation in the industry currently (Nehme et al. 2010). Studies show that co-inoculation reduces the overall fermentation time without affecting the growth of the yeast or the rate of AF (Massera et al. 2009, Abrahamse and Bartowsky 2012, and Knoll et al. 2012). Shortened fermentation times provide the opportunity to stabilize wines earlier, thereby reducing the risk of microbial spoilage (Abrahamse and Bartowsky 2012). In the study done by Massera et al. (2009), co-inoculated MLF completed in 10 to 26 days without an increase in biogenic amine production. A study done by Knoll et al. (2012) showed that co-inoculation tended to increase ethyl and acetate esters.

Co-inoculation is therefore a handy tool which can be used to overcome possible problematic wine conditions, like high initial sugar content of the grapes (often associated with such warm-climate countries as South Africa) leading to high alcohol levels and insufficient nutrient availability that, in turn, may lead to sluggish or stuck MLF when the wine is inoculated after AF. Co-inoculation can also be used for better tank utilization in the cellar, as well as improved microbial stability, because it reduces overall fermentation time without the risk of off-flavours (Jussier et al. 2006, and Nehme et al. 2010).

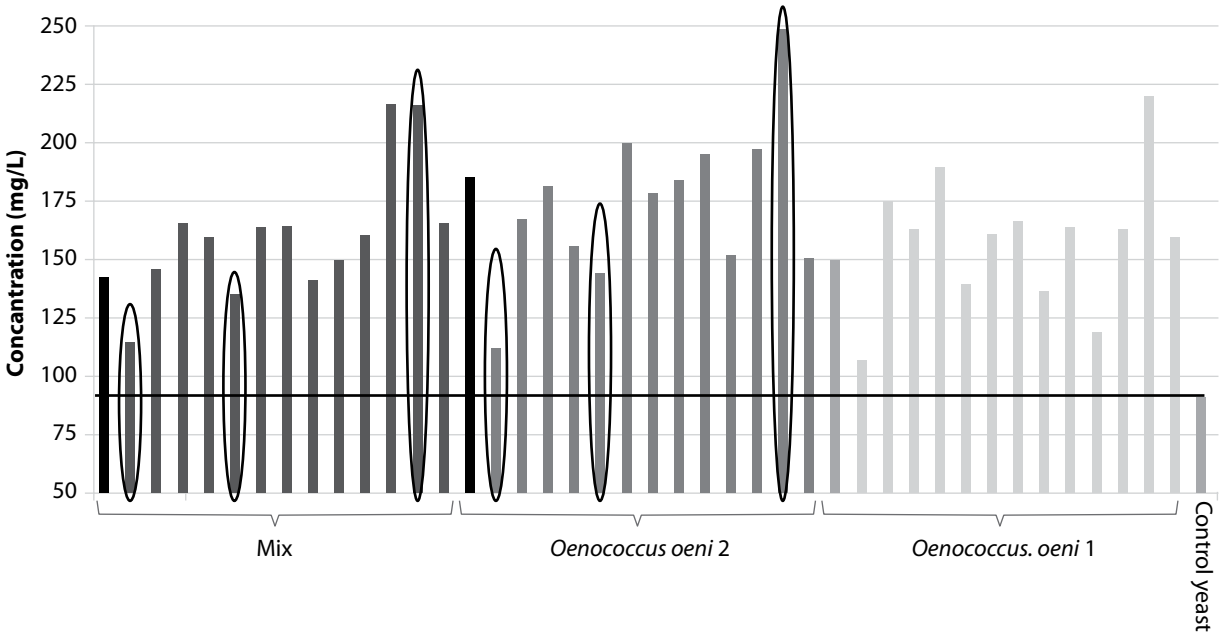
4. Aroma Modification

The aroma and flavour of wines are influenced by the LAB strain as well as the MLF inoculation scenario utilized. The production of flavour and aroma compounds is a result of the metabolism of grape constituents, such as sugars, amino acids and organic acids, and/or the modification of grape- and yeast-derived aroma compounds (Swiegers et al. 2005, and Bartowsky and Borneman 2011).

4.1 Influence of the yeast strain

The amount of total esters produced after MLF differed according to the yeast strain used (figure 1). The esters pro-

Figure 1. Total ester concentration using 14 different yeast strains in co-inoculation in Merlot with three different malolactic fermentation starter cultures and a control that was not inoculated for MLF, in 2011 Merlot (Schöltz 2013)



duced that differed significantly were ethyl lactate, ethyl acetate, ethyl caprylate, ethyl-3-hydroxy butanoate, ethyl phenyl acetate and diethyl succinate. Variations in higher alcohols are more apparent between yeast treatments than between MLF treatments. MLF resulted in higher concentrations of diacetyl and acetoin, independent of the yeast strain used. Therefore, the selection of yeast strain with MLF is important as it will impact the final aroma and style of the wine (Schöltz 2013).

4.2 Influence of the lactic acid bacteria strain

Malherbe et al. (2012) evaluated the influence of different *O. oeni* MLF starter cultures on the volatile aroma composition, using Pinotage and Shiraz grapes. Changes were observed in ester concentrations after the completion of MLF. The synthesis and hydrolysis of esters during MLF were evident. Ethyl lactate, diethyl succinate, ethyl octanoate, ethyl 2-methylpropanoate and ethyl propionate

Figure 2. Graph of the ester contribution imparted by four different malolactic fermentation starter cultures during MLF in 2008 Pinotage (adapted from Malherbe et al. 2012)

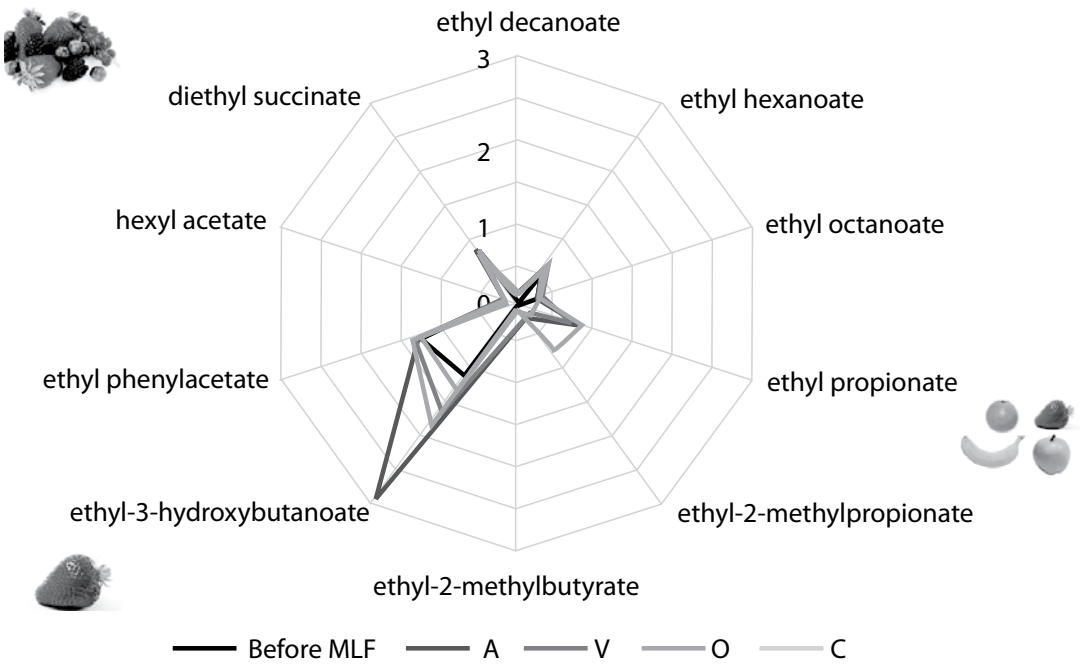
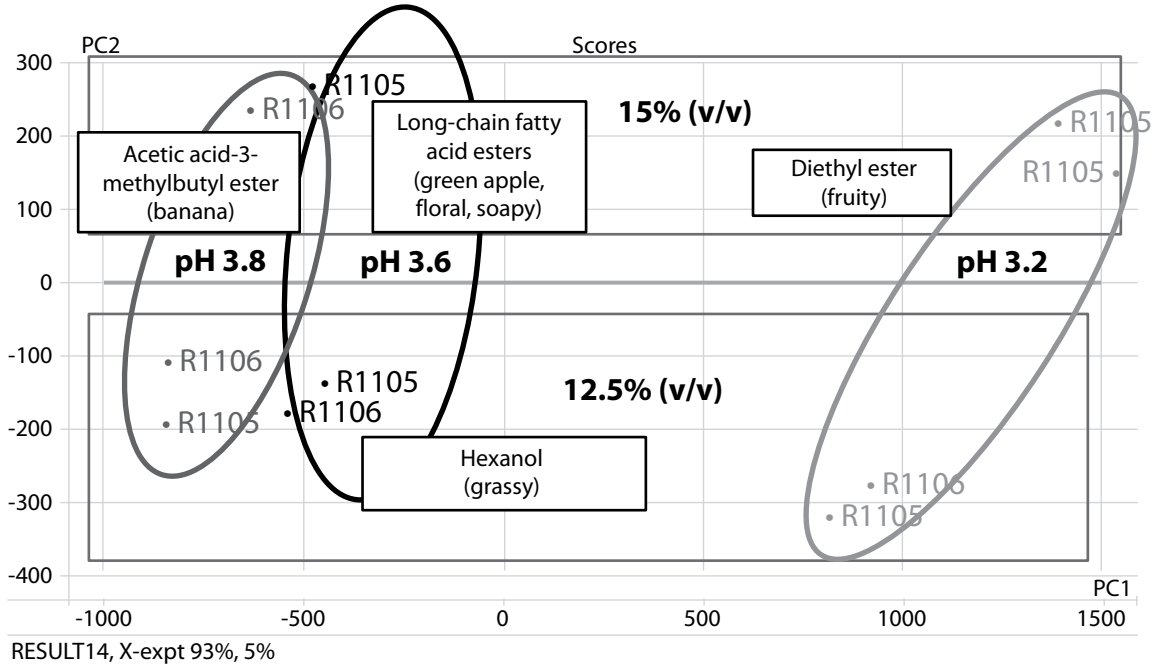


Figure 3. Principal component analysis plot showing the impact of pH and ethanol on the shift in the flavour profile of all the Riesling wines (adapted from Knoll et al. 2011)[1]



concentrations were increased during MLF, compared to the control wine, for all four *O. oeni* strains. Increases in the concentrations of most of the higher alcohols were observed in MLF wines. Isoamyl alcohol, isobutanol, 2-phenylethanol, propanol, butanol, hexanol, 3-methyl-1-pentanol, and 3-ethoxy-1-propanol concentrations were significantly increased by MLF, which indicates their potential contribution to specific characteristics in wine (figure 2).

4.3 Impact of pH and ethanol

Knoll et al. (2011) investigated the influences of pH and ethanol on MLF and the volatile aroma profile in Riesling and Chardonnay wines using two different *O. oeni* strains. The pH was 3.2, 3.6 and 3.8 respectively, and ethanol concentrations of 12.5 and 15% (v/v) were evaluated.

The results demonstrate that even if the MLF was incomplete, the ester concentrations were impacted. Increases in such fruity esters as ethyl acetate, ethyl propionate and ethyl butyrate were observed. Acetic acid ethyl ester, acetic acid 3-methylbutyl ester, succinic acid diethyl ester and lactic acid ethyl ester were most affected by wine pH and ethanol. Lower pH resulted in greater increases in total fruity esters (figure 3). For monoterpenes, the content of trans- and cis-linalooloxide and α-terpineol increased with lower pH values, and the linalool content increased with higher pH.

4.4 Impact of malolactic fermentation inoculation scenario

Knoll et al. (2012) evaluated four different MLF inoculation scenarios, from co-inoculation, 40% of AF, 60% of AF to sequential, using two different *O. oeni* starter cultures. The results show that the different inoculation scenarios were driven by different esters and resulted in different wine aroma profiles. When compared to sequential inoculation, co-inoculated wines showed higher concentrations of ethyl and acetate esters, including acetic acid phenylethyl ester, acetic acid 3-methylbutyl ester, butyric acid ethyl ester, lactic acid ethyl ester and succinic acid diethyl ester.

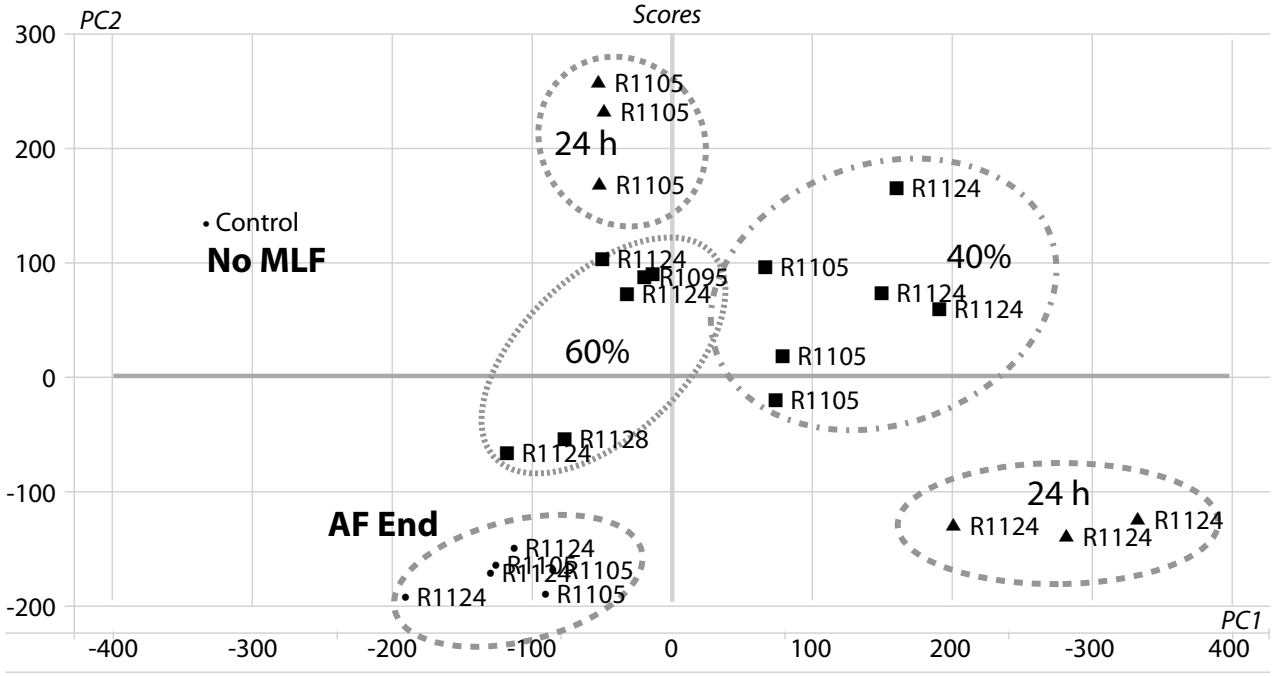
The strain differences were more profound in co-inoculation and 40% of AF where there was a clear separation between the two strains and the esters they produced.

4.5 Impact of the lactic acid bacteria used for malolactic fermentation

It was shown in different studies that *L. plantarum* can, under specific conditions, perform MLF just as well as *O.*

[1]Reprinted from *LWT - Food Science and Technology* , Vol. 44, Caroline Knoll, Stefanie Fritsch, Sylvia Schnell, Manfred Grossmann, Doris Rauhut, Maret du Toit Influence of pH and ethanol on malolactic fermentation and volatile aroma compound composition in white wines, Pages No. 2077-2086, Copyright (2011), with permission from Elsevier.

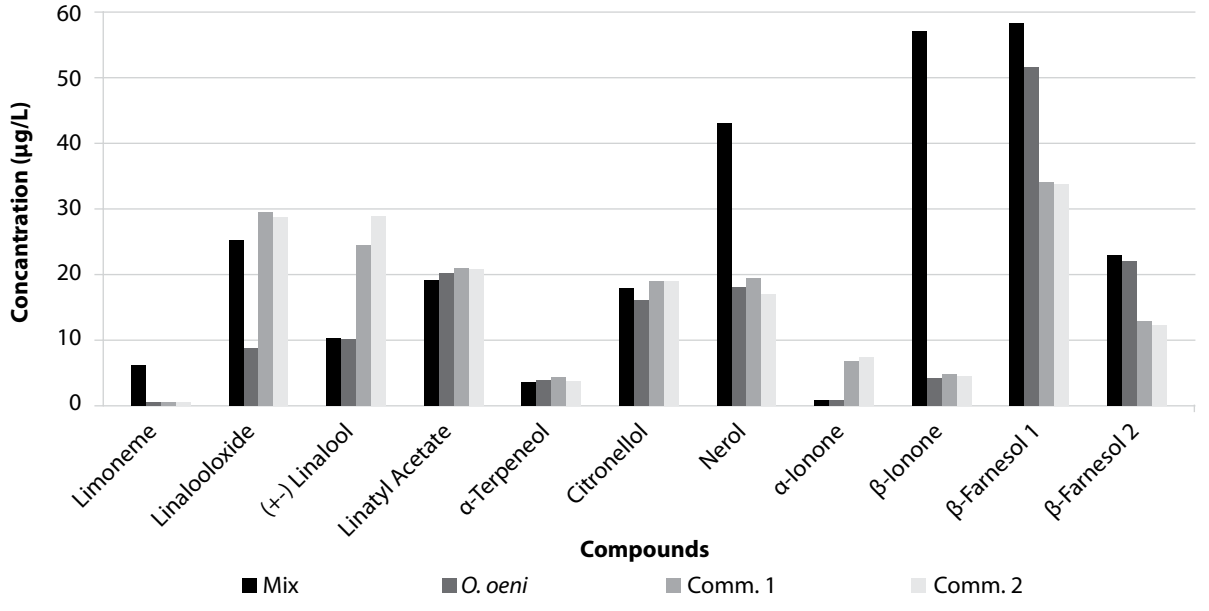
Figure 4. Principal component analysis score plot derived from volatile aroma compounds of all Riesling wines following MLF and the control wine with no MLF (Knoll et al. 2012)[2]



RESULT14, X-expt 44%, 37%

oeni. *L. plantarum* possess different genes and enzymes that can lead to different wine aroma profiles (Du Toit et al. 2011, and Lerm et al. 2011). Apart from producing different ester ratios, the biggest impact from using *L. plantarum* is related to the release of monoterpenes due to β-glycosidase activity (figure 5) (Lerm et al. 2012).

Figure 5. Comparison of the monoterpene production (excluding geraniol) of the mixed culture containing *Oenococcus oeni* and *Lactobacillus plantarum*, the individual *O. oeni* strain from the mix, and two commercial *O. oeni* cultures during co-inoculation in 2011 Shiraz (Lerm et al. 2012)



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Malherbe et al. (2013) assessed the link between consumer liking, chemical and sensory attributes in Pinotage wines that were made over two vintages by four different *O. oeni* MLF starter cultures, and a control wine where MLF was prevented. The results show that consumer liking was influenced by the sensory attributes. The main chemical and sensory correlations found for MLF-treated wines were related to 2,3-butanedione (diacetyl) with the buttery character and various esters with fruity aromas. Most important was that all consumers preferred the MLF wines over the control wine, which indicates the importance of MLF and how it can be used to meet consumer demand in a globally competitive market.

5. Conclusion

The modifications in flavour profiles during malolactic fermentation are dependent not only on the bacterial strain conducting MLF, but on the grape cultivar as well, especially in regards to the precursors available, the specific wine parameters (especially pH and ethanol content), and the timing of inoculation. These factors can all be utilized to tailor the wine style.

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CHEMICAL SYSTEMS BEHIND WINE AROMA PERCEPTION

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Abstract

This paper presents a review of our knowledge and understanding of the role played by the different aroma chemicals in the positive aroma attributes of wine, and presents a systematic approach to classifying the different aroma chemicals of wine. One basic idea is that all wines share a common basic aromatic structure formed by ethanol and 27 different aroma compounds, most of them by-products of fermentation. Combined, the mixture of these products has the typical wine aroma and exerts an aroma-buffering effect with the ability to suppress the effect of many odorants added to it, particularly those with fruity characteristics. The ability of the different odour chemicals to break such a buffer, and hence transmit a different aroma nuance to the wine, and the relationship between the transmitted aroma nuance and the aroma of the chemical, is used to define the different roles played by aroma compounds on wine aroma. These roles can be as high-impact compounds, major contributors, net contributors, subtle aroma compounds, aroma enhancers and aroma depressors.

The factors can be individual aroma chemicals or well-defined mixtures of molecules sharing chemical and odour properties (aroma families). Different examples of the aroma chemistry behind some of the most relevant wine aroma nuances from simple or complex wines are also presented and discussed.

1. Introduction

The aim of this presentation is to show the latest findings regarding the chemical interpretation of wine aroma and how the aroma chemicals interact to form the aroma nuances of wine.

Understanding wine aroma initially requires aroma extraction by gas chromatography-olfactometry analysis. Through gas chromatography-mass spectrometry (GC-MS) we can conduct qualitative and quantitative analyses to obtain quantitative lists. Sensory tests then compare the wine glass aromas.

Our work has two parts: the aroma deconstruction and the aroma reconstruction. The originality of the procedure is that we track the key odour molecules perceived in the glass of wine, analyzing only the key odorants perceived in the glass, not all the odorants.

2. Aroma Buffer

Ethanol and the other major fermentation volatiles form a sort of "aroma buffer" which is not easy to break. Note that some aroma compounds are present in all wines,

independent of the wine's origin or type. They are the groups of the volatile aroma compounds produced by fermentation in relatively well-defined proportions, and all these compounds are present at concentrations well above the perception threshold in nearly all wines, forming a particular aroma mixture often described as vinous – slightly sweet, pungent, alcoholic and a little bit fruity (Escudero et al. 2004). Although not formed by yeast during fermentation, another compound, β-damascenone, can be included in this group because it can also be found in nearly all wines at concentrations above the perception threshold.

3. Classification of Wine Odorants

The aroma buffer can be broken only by certain molecules, particularly by groups of molecules acting synergistically. The following classifications are a new proposal based not only on observation, but on well-established concepts of flavour chemistry (Belitz and Grosch 1999).

3.1. Genuine impact compound

This role is played by individual compounds which, in a given wine, are in concentrations high enough to transmit their specific aroma nuances to that wine, i.e., the aroma of the compound can be recognized in the wine.

3.2. Major contributors

This role is played by individual compounds or families of aroma compounds present in the wine in a concentration high enough to transmit a primary generic descriptor of its aroma (e.g., red fruit, citric, minty, etc.), but not the specific descriptor of the compound (i.e., the compound cannot be clearly recognized in the wine). The transmitted descriptor in the wine is nearly entirely due to the compound, so that if the compound or family of compounds were removed the sensory aspect would be affected both qualitatively and quantitatively.

3.3. Secondary or subtle contributors

This role is played by those individual compounds or families of aroma compounds present in the wine in a concentration below the level required to transmit individually one of its generic descriptors. However, such an aromatic descriptor (usually very general, such as sweet or fruity) is noted because of the concerted action of many aroma molecules or families.

Therefore, if the compound or family of compounds were removed from the wine, the sensory effect would be very weak or even null.

3.4. Aroma enhancer

This role is played by those individual aroma molecules or families of aroma compounds which fail to transmit their specific or generic descriptors, but nonetheless enhance the specific aroma of some other molecule or group of molecules present in the wine. In some cases, the enhancement brings about a new aroma quality as a consequence of the mixture of the odours, while in others the effect of the enhancement is merely an increase of the aroma intensity. In any case, if the enhancer were removed a decrease in the intensity of an aroma nuance not directly related to the aroma of the enhancer would be noted.

3.5. Aroma depressor

This role is played by those individual aroma molecules or families of aroma compounds whose presence in the wine causes a decrease in the intensity of an odour note. If the depressor were removed from the wine, an increase in the intensity of the depressed odour nuance would be noted.

4. Wine Aroma Formation

The most relevant notes of great wines are caused by complex associations of aroma compounds playing different roles (i.e., as contributors, suppressors or enhancers).

Red wines are, by nature, much more complex as, among many other factors, they contain quite large amounts of volatile phenols which exert a suppression effect on fruity notes (Atanasova et al. 2004). This phenomenon is even more intense when the wines are aged in oak casks, increasing the concentrations of volatile phenols and adding whisky lactones. In this chemical environment, the perception of the different notes, particularly fruity ones, is extremely complex. Furthermore, great red wines do not have explicit or specific odour nuances, but a large palette of many subtle odours instead. It is not surprising then, that we usually do not find genuine impact compounds in red wines, aside from the whisky lactones, but we do find relatively large groups of compounds which contribute to the different odour nuances.

To date, we have identified several major contributors to the fruity notes of red wines:

- The concerted action of ethyl esters, including several recently discovered branched ethyl esters, with norisoprenoids (β-damascenone and β-ionone) and with the enhancing effect of dimethyl sulphide, that can impart berry fruit notes to the wine (Escudero et al. 2007);
- The concerted action of five γ-lactones (γ-octa-, γ-nona-, γ-deca-, γ-undeca- and γ-dodecalactones) that can be

responsible for the peach notes of some reds, particularly those from certain areas of Spain and Portugal (Jarauta et al. 2006);

- The concerted action of furaneol, homofuraneol, maltol, sotolon, norisoprenoids and methional that can be responsible for some cherry and chocolate notes in some reds (Ferreira et al. 2005).
- The concerted action of volatile fatty acids as contributors to the fruity notes and suppressors of 4-ethylphenol (San-Juan et al. 2011).

It is very interesting to note that it is better to work in terms of aroma profiles rather than aroma intensities: wine fruitiness is a concept, and odour concepts seem to be linked to the existence of well-defined ratios of odorants.

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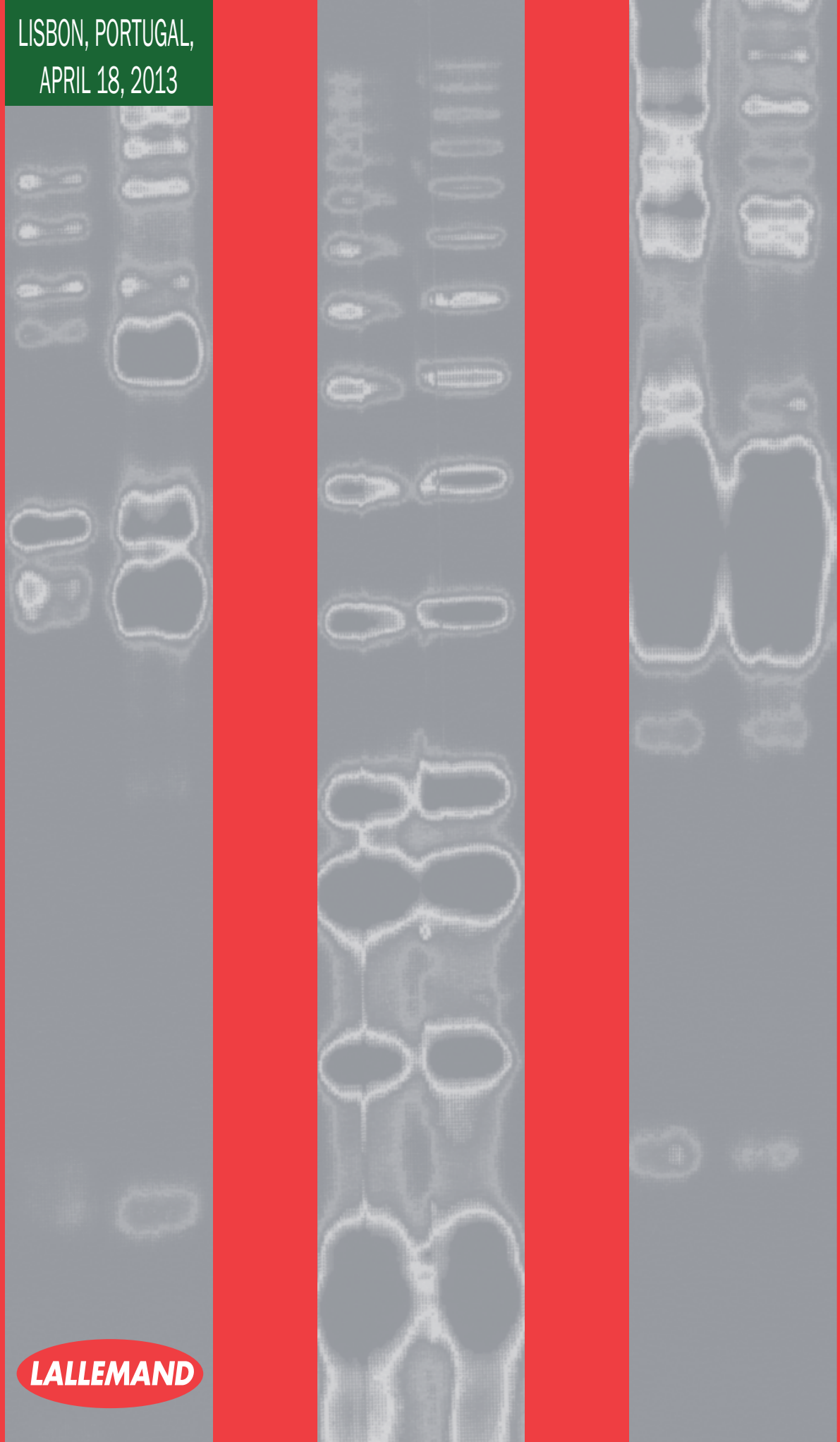

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