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Nitrogen management is critical for wine flavour and style

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"Winemaking begins in the vineyard" is a mantra that has widespread support amongst winemakers. It conveys the concept of vineyard or, in French jargon, *terroir* as an intrinsic property of grape, and consequently the corresponding wine. There is no doubt that many great wines are associated with great vineyards. So, where do yeast fit in?

The perception that fermentation yeast faithfully transform grape must into wine has been changing in the detail over the past decades. This is a result of science uncovering the many roles that yeast perform, and the wider selection of strains available that promote these various attributes. For example, whereas most strains produce a relatively similar, generic, fermentation bouquet only some strains possess a strong ability to hydrolyse cysteine conjugates responsible for Sauvignon Blanc character, meaning that only selected strains can enhance varietal expression (Swiegers *et al.* 2006). Winemakers today have many options through fermentation management to enhance the varietal characteristics of their wine, or to express further regional attributes. Furthermore, yeast strongly respond to their environment. It is well known that temperature affects the rate of fermentation, that grape solids enhance survival and that high osmotic stress, as imposed by a *Botrytis*-affected must, leads not only to increased glycerol production but also to higher volatile acidity. The latter example highlights the remarkable ability of yeast to adapt to stressful (i.e. high sugar) environments. However, there is an accompanying metabolic adaptation which can have positive or negative flavour implications.

At the time of inoculation, yeast are subjected to a range of stresses to which the cell must adapt in order to exploit its new environment. Some of the known stresses are osmotic pressure, oxidative conditions, sulphite toxicity and temperature shock (Bauer and Pretorius 2000). Nutrients, whether present in sub- or super-optimal concentration, can also induce stress and metabolic responses. The primary response is aimed at protecting the cell from committing to reproduction when key nutrients are lacking or dealing with potential toxicity when the concentration is outside the normal range. The metabolic response often involves a cascade of biochemical reactions, some of which can lead to altered metabolism of nutrients such that the yeast will secrete end-products in different amounts (Albers *et al.* 1998). Some of the end products that have sensory properties can lead to changes in the flavour profile of the wine. H_2S formation is an all-too-well known example relating to nitrogen depletion stress.

Clearly, the vineyard environment and intervention by the viticulturist shape the development of the vine and especially the composition of the grape. Because the viticulturist attempts to balance a long list of priorities in order to produce fruit to specification, most attention will focus on those factors that cannot be modified once the fruit has been harvested. Therefore, yeast nutrients, especially nitrogen, might not be optimised for fermentation, largely in the belief that nutrients can be easily corrected in the winery. Given that we estimate that up to 500t of diammonium phosphate (DAP) could be used each year to produce Australian wine, is this winemaking input being used effectively?

Our current state of knowledge on the implications of controlling vineyard nitrogen as opposed to fermentation nitrogen on fermentation performance and wine composition has been recently reviewed by Bell and Henschke (2005). In this article, we will focus on the role that fermentation nitrogen has in modulating metabolism and some of the changes that this can have on wine flavour. We will first summarise current best practice for managing fermentation nitrogen and then describe the main flavour changes that are affected by nitrogen. Finally, we will consider the flavour implications of nitrogen for white and red wine fermentations.

CURRENT BEST PRACTICE FOR MANAGING FERMENTATION NITROGEN

A common practice amongst winemakers is to make a standard addition of DAP to the juice or must (100-300mg/L) at inoculation without measuring the nitrogen concentration. This article will show that DAP addition has significant flavour consequences and that measuring the initial nitrogen concentration provides the opportunity to adjust DAP addition not only to achieve an adequate fermentation rate, but also to more reliably guide the flavour profile and style of wine required. This work is still in a conceptual stage based on studies with Chardonnay and Shiraz; however, it should stimulate winemakers to experiment with these and other varieties.

1.1 Measuring YAN

Grapes contain a variety of nitrogenous compounds of which the most important are the primary or alpha amino acids, ammonium ion and small peptides. Proline, a dominant secondary amino acid in many grape varieties, cannot be assimilated under anaerobic conditions (Ingledew *et al.* 1987). These nitrogenous compounds, excluding proline, constitute what is commonly referred to as yeast assimilable nitrogen (YAN).

Because amino acids are chemically-diverse molecules, the most convenient measure of assimilable nitrogen relates to assaying the free or alpha-amino group of the primary amino acids, which is commonly referred to as free amino nitrogen (FAN). Proline, a secondary amino acid, and protein are excluded in FAN assay methods. Of the several chemical, enzymatic and physical methods available (Shively and Henick-Kling 2001; Bell and Henschke 2005; Filipe-Ribereiro and Mendes-Faia 2007) the method of choice is the *o*-phthaldialdehyde/N-acetyl-L-cysteine (NOPA) method (Dukes and Butzke 1998). An additional enzymatic method is needed to determine ammonia, of which 82% is nitrogen. Summation of these two nitrogen measurements yields YAN (Figure 1). This procedure is used by NATA-accredited laboratories such as AWRI's Analytical Service laboratory (http://www.awri.com.au/analytical_service/analyses/yeast_assimilable_nitrogen/), which can provide a timely service during vintage.

Figure 1. Summation of free amino nitrogen and ammonia nitrogen levels provides a useful estimate of the yeast assimilable nitrogen level.

The so-called Formol Titration is a simpler, rapid method for measuring YAN (Shively and Henick-Kling 2001; Gump *et al.* 2002; Filipe-Ribereiro and Mendes-Faia 2005), although the use of formaldehyde, a toxic volatile reagent, requires a well-trained analyst and suitable laboratory. YAN determination with mid-infrared (MIR) spectrometry, which is most rapid, has recently been developed by the AWRI (Dambergs *et al.* 2005).

YAN measurements, ideally, should be performed directly on juice or must samples at the point of inoculation to avoid over-estimation due to processing losses which inevitably occur between vineyard and the fermentor. Furthermore, juice samples taken from grape musts can under-estimate total berry YAN due to an important proportion of amino acid contained in the grape skin. Refer to the review by Bell and Henschke (2005) for a detailed discussion of these points. Nevertheless, an early warning for low YAN can be achieved by sampling in the vineyard one to two weeks prior to harvest, such as during maturity sampling.

1.2 Supplementing must YAN

The YAN content of Australian grape juices varies widely from approximately 50-350mg/L, with a mean value of around 200mg/L. As a benchmark, it is generally agreed that maximum yeast biomass yield and fermentation rate results when YAN exceeds 400mg/L, whereas 150mg/L YAN marks a transition zone, below which the risk of slow or stuck fermentation notably increases (Henschke and Jiranek 1993; Blateyron *et al.* 2003). Since much of the background research work to establish these benchmarks has been carried out in synthetic and filtered grape juices, this risk value is technically only valid for highly clarified, anaerobic, juice fermentations. Nonetheless, it represents a worst-case scenario and is a useful guide for other types of fermentation.

In general, in order to achieve an adequate rate of fermentation to dryness, a cellar bright juice containing <150mg/L YAN should be supplemented with nitrogen to at least 150-200mg/L when the respective vineyard has a history of low YAN fermentation problems or a high nitrogen-demanding yeast has been selected. Nitrogen supplementation should be increased to the higher end of the range for higher °Brix juices, whereas juices containing grape solids, or fermentations that are aerated, are less susceptible to low YAN difficulties (Ribéreau-Gayon *et al.* 2000; Blateyron *et al.* 2003; Eglinton *et al.* 2005). Later on in this article, when we consider the flavour consequences of juice YAN content, some winemakers might choose to supplement low YAN juices up to a final concentration of 250-300mg/L YAN so as to produce a cleaner, fruitier style.

DAP is widely used as a YAN supplement for this purpose. DAP contains 21% N, therefore, for convenience we can consider 100mg DAP to contain 20mg YAN. By way of an example, it will be necessary to add 500mg/L DAP to a juice to increase its YAN concentration from 100mg/L to 200mg/L. While this figure seems a large addition of DAP, the YAN equivalent of 1.5g DAP would be needed to reach the point at which maximum fermentation rate would be achieved. Australian winemakers can visit the AWRI website to access the calculator to estimate DAP additions: http://www.awri.com.au/practical_solutions/calculators/.

One disadvantage of DAP as a supplement is the acidification that can result in some juices, leading to a lower-than-expected wine pH. Utilisation of the ammonium cation by yeast leaves a notable proportion of the phosphate anion, which can lower pH, depending on initial pH and must titratable acidity (TA). Furthermore, large additions of DAP can lead to excessive wine phosphate content. In practice, the maximum addition of DAP is limited by the concomitant concentration of soluble phosphate remaining in the wine, which is set at 400mg P/L (Australian and New Zealand Food Standard 4.5.1). This concentration of phosphate-P would correspond to a maximum of 1.7g/L

DAP (equivalent to 360mg/L YAN) if we assume that the juice/must contained no phosphate; in practice a lesser amount of DAP can only, therefore, be added.

Overuse of DAP can also stimulate overproduction of acetate esters, especially ethyl acetate, resulting in the perception of volatile acidity (VA) and suppression of varietal character. As discussed in the following sections, high YAN (exceeding 450-500mg/L YAN) can stimulate ethyl acetate production by many yeast strains.

When working with very low YAN juices, we have observed that other nutrients can similarly be low. Thus, when YAN is low and other nutrient deficiencies are suspected, it can be useful to add a proprietary yeast food that contains more complex forms of N, as well as vitamins, lipids and minerals. Indeed, continued H_2S production after DAP addition suggests a general vitamin deficiency (Henschke 1996; Wang *et al.* 2003), though other causes are also possible. Most yeast suppliers can advise on the use of yeast foods, which are generally produced from inactivated yeast.

GENERAL METABOLIC RESPONSES OF YEAST TO YAN

The principal role of sugar metabolism in yeast is to generate energy and carbon skeletons for building all the components of the cell. These metabolic activities result in the accumulation of several by-products, including esters, higher alcohols and polyols, carbonyls, acids and thiols which contribute to the aroma and flavour of wine. Nitrogen metabolism, which is involved in the assimilation of nitrogen for the synthesis of protein and nucleic acids, also contributes to the pool of aroma and flavour compounds. Because nitrogen metabolism is central to cell growth, it regulates other pathways, including sugar and sulphur metabolism. Consequently, nitrogen availability can significantly impact on the production of many flavour-active metabolites. The nitrogen status of a juice or must, therefore, contributes to wine flavour as well as affecting yeast growth and the fermentation of sugars.

2.1 Major fermentation products

In addition to ethanol and CO_2, other major products of sugar metabolism are the polyols, such as glycerol and butanediol and the organic acids, especially acetic and succinic acids and, to a lesser extent, the ketoacids, such as pyruvic acid and α-ketoglutaric acid. The production of many of these primary metabolites of sugar metabolism is modulated by YAN, although the magnitude of changes has been observed to depend on the yeast strain under consideration. Furthermore, the type of nitrogen source used, DAP or amino acids, affects metabolite production (Albers *et al.* 1996). Because low YAN juices are typically supplemented with DAP, only the impact of ammonium ion concentration on the production of yeast metabolites will be discussed in this article.

Ethanol is the major product of sugar fermentation. However, while DAP addition increases yeast growth and the rate of fermentation, it has little to no practical effect on final ethanol yield. Theoretically, DAP-grown yeast are forced to synthesise amino acids for cell growth when compared with amino acid grown yeast. This decreases the proportion of sugar available for ethanol production (Albers *et al.* 1996), but in our experiments this has a minor affect on ethanol yield.

Glycerol and acetic acid, which are important to wine composition and flavour, respond relatively strongly to juice YAN concentration (Albers *et al.* 1998; Torrea *et al.* 2005; Vilanova *et al.* 2007). Figure 2 summarises general trends observed in synthetic juice and white wine fermentations. Both glycerol and acetic acid production depends strongly on the yeast strain used. For example, when using

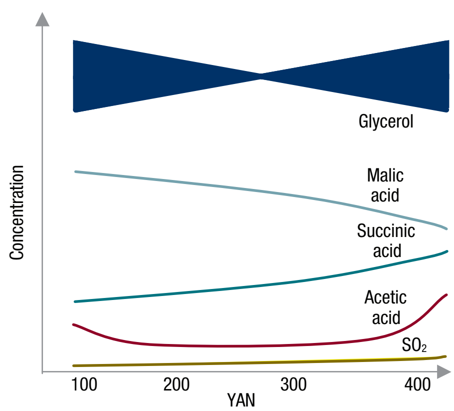

Figure 2. Effect of yeast assimilable nitrogen on production or utilisation of major metabolic products of sugar fermentation and sulphur assimilation.

yeast Vitilevure M05 DAP addition increases glycerol production whereas the reverse is the case for AWRI 796 (Vilanova *et al.* 2007). Both yeast, however, produce the lowest concentrations of acetic acid at moderate YAN concentrations (range of 200-250mg/L) while higher concentrations are produced at both lower and higher concentrations of YAN. Malic acid consumption does, however, increase with increasing DAP concentration, irrespective of yeast strain. On the contrary and depending on the strain, succinic acid concentration can increase with increasing DAP addition (Coulter *et al.* 2004). In general, YAN can affect TA and the balance of organic acids which can affect flavour (Sowalsky and Noble 1998).

Sulphur dioxide production during fermentation can also be stimulated by initial YAN concentration, but the response seems to be yeast strain dependent. Experimental work in synthetic media and wort suggests that low SO_2 is produced in low YAN media but increases when initial YAN availability is higher (Duan *et al.* 2004; Osborne and Edwards 2006). SO_2 production contrasts with H_2S production, which is generally lowered by increasing YAN. Increased risk of MLF inhibition has also been associated with high YAN addition but this inhibition has not been conclusively correlated with SO_2 production (Osborne and Edwards 2006). Nevertheless, until better information is available, consideration should be given to limiting high YAN conditions when malolactic fermentation (MLF) is required.

2.2 Volatile aroma compounds

Among the various yeast metabolic pathways that are influenced by the nitrogen composition of the juice, those leading to volatile compounds are of particular importance due to the primary role played by fermentation-derived volatiles in the aroma character of wine (Smyth *et al.* 2005). Several studies have indicated that both the total available nitrogen and the balance of amino acids and ammonia can significantly affect the production of different groups of fermentation-derived volatile compounds.

From a practical point of view, the problem of juice nitrogen composition is primarily linked to the frequent occurrence of juices with suboptimal concentrations of nitrogen, and higher risk of slow or stuck fermentation. As this problem is frequently corrected in the winery through the addition of DAP, several studies have investigated the implications of this common winery practice on the volatile composition of wine (Ayrapaa 1971, Rapp and Versini 1991; Carrau 2003; Torrea and Henschke 2004; Hernandez-Orte *et al.* 2005, 2006; Vilanova *et al.* 2007). Due to the variety of yeast strains and fermentation conditions employed, it is somewhat difficult to extrapolate from the literature definitive conclusions concerning the effect of DAP addition on wine aroma. Nevertheless, some general trends relating to DAP supplementation and wine volatile composition are summarised in Figure 3.

Higher alcohols, which are directly related to amino acid metabolism in the cell, exhibit a characteristic behaviour. Therefore, when total nitrogen is increased by adding ammonium to a medium containing very low levels of YAN, the production of higher alcohols is initially increased, but then tends to decrease after a peak between 200-300mg/L YAN. This activity depends on various factors, including yeast strain and fermentation conditions. Higher alcohols are characterised by fusel-like odours, and are generally thought to contribute to the complexity of wine fermentation bouquet. However, when present in very high concentrations they can have a negative impact on wine aroma, mainly because they mask fruity characters. Several authors have reported that ammonium supplementation can improve wine sensory quality by lowering higher alcohols production (Rapp and Versini 1991). However, based on the trend shown in Figure 3, this advice has to be taken cautiously as it might apply only to fermentations with initial YAN in the range included in the descending part of higher alcohols production pattern, i.e. YAN >200mg N/L.

The production of fatty acids ethyl esters, as well as of acetate esters, including ethyl acetate, is generally increased when DAP is added to the juice prior to alcoholic fermentation (Figure 3). This can have interesting implications for wine flavour as fatty acids ethyl esters and acetates are generally responsible for the fruity character of wine (Guth and Sies 2002). However, ethyl acetate, one of the dominant yeast-derived volatile metabolites, when present at very high concentrations, can give unwanted sensory characteristics, often described with terms like nail lacquer/solvent and volatile acidity. Branched chain esters are, from a quantitative point of view, the less abundant volatiles produced during fermentation. Although their contribution to wine flavour has still to be clarified, tentative evidence is available in the literature for these compounds to be important contributors to the red berry fruit character of some red wines (Diaz-Maroto *et al.* 2005). Their concentration appears to decrease with increased DAP additions, however.

The existence of a variety of different responses for the various groups of yeast-derived volatile compounds to DAP supplementation arises from the fact that each group of volatiles is derived from a different metabolic pathway, each of which respond differently to DAP supplementation. However, from a practical point of view, understanding the potential of DAP supplementation as a tool to modulate wine sensory characteristics cannot be based simply on compositional data. The various volatiles or groups of volatiles illustrated in Figure 3 occur in wine over an extremely broad range of concentrations. However, this figure does not represent the actual quantitative relationship between different chemical species. For example, higher alcohols, characterised by herbaceous, fusel-like odours, typically occur in concentrations that can be up to 400 times higher than ethyl fatty acid esters, characterised by fruit-like odours. Nevertheless, relatively small variations in the concentration of ethyl fatty acid esters, such as those introduced by variations in YAN content, are more likely to affect the aroma of wine than if proportionally similar variations would occur for higher alcohols. This is due to the fact that some of the possible sensory modifications associated with changes in the concentration of specific aroma compounds depend, among other factors, on the ability of that aroma compound to generate an olfactory stimulus at a given concentration.

This complex relationship is often simplified by means of the concept of odour threshold, defined as the minimum concentration at which a given compound can be detected by the sense of smell (Guth 1997). This is referred to as the odour activity value (OAV). Some of the fermentation-derived volatile compounds, such as esters, that are generally associated with the fruity character of wine, are extremely powerful odourants (i.e. have a very low odour threshold) and can, therefore, impart specific sensory attributes even when present in low concentrations. On the contrary, compounds like higher alcohols possess a much higher odour threshold and, therefore, are likely to generate variations in the aroma profile of a wine only when their concentration varies to a very large extent. In Figure 4, a theoretical relationship between the OAV of selected volatile compounds, belonging to the chemical classes of Figure 3, and DAP supplementation is illustrated. It appears clear then that the range of variations potentially introduced by DAP

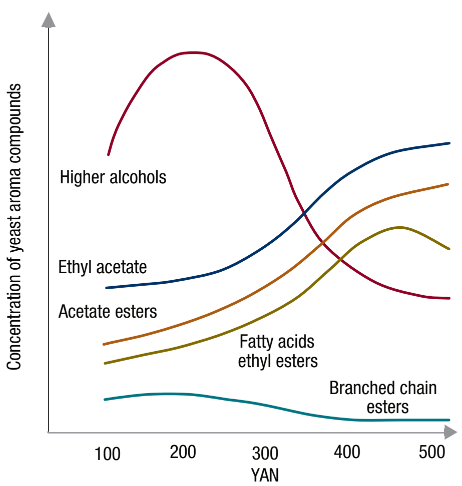

Figure 3. Relationship between initial YAN concentration and final concentration of volatile compounds after fermentation.

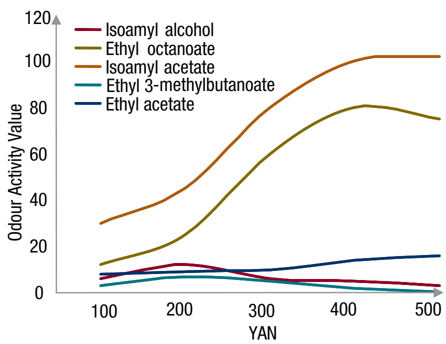

Figure 4. Theoretical relationship between initial YAN concentration and Odour Activity Values of selected yeast-derived aroma compounds.

in the concentration of acetates and fatty acid ethyl esters (isoamyl acetate and ethyl octanoate are used as reference compounds for these two classes) can have a dramatic impact on the volatile character of wine, whereas variations in compounds such as higher alcohols (isoamyl alcohol), although quantitatively extremely large, are likely to have a limited impact. Although it has to be stressed that OAVs only give a projection of the potential of a given compound to contribute to the overall aroma of a wine, the trends shown in Figure 4 provide a good indication of which one of the compositional changes associated with DAP supplementation is likely to have a greater impact on wine aroma.

IMPLICATIONS OF NITROGEN FOR WINE FERMENTATIONS

3.1 Implications of nitrogen for white wine fermentations

Interestingly, the results obtained in various winemaking trials conducted at the AWRI with sub-optimal YAN juices have indicated that, under typical winemaking conditions, DAP supplementation is an extremely powerful tool for modulating the production of esters which, based on the previous discussion, are probably the most sensorially-interesting group of compounds generated during fermentation. Figures 5 and 6 show the variations in volatile compounds and the sensory profile of Chardonnay wines made at different DAP concentrations. In good agreement with the trends shown in Figure 3, DAP had a positive effect on ester production, while it lowered the formation of higher alcohols. However, the wines obtained with moderate nitrogen supplementation of the juice were preferred by panellists compared with those obtained without or with high DAP addition. This preference might be due to a combination of higher acetates, ethyl fatty acid ester concentrations and moderate levels of ethyl acetate, the latter being associated with unwanted, solvent-like characteristics when present at very high concentrations. DAP addition to low YAN juices also suppresses the production of H_2S and mercaptans by many wine yeasts, which although not quantified in this study, no doubt contributed to the preference of the moderate YAN wines. The impact of DAP addition on the production of fruity thiols, such as 4MMP, 3MH and 3MHA, still needs to be determined.

These results highlight the complexity of predicting wine aroma from compositional data. They also underline the importance of measuring YAN and adding the appropriate amount of DAP, if necessary, before or during fermentation in order to reduce the potentially negative effects that inadequate or excessive DAP supplementation can have on wine aroma. Particularly, the risk of excessive formation of ethyl acetate has to be considered as this ester is relatively stable during wine ageing, compared with other acetate and ethyl fatty acid esters, which tend to decrease significantly after several months of bottle storage.

3.2 Implications of nitrogen for red wine fermentations

More recently, researchers at the AWRI have investigated the effect of DAP supplementation on the volatile composition of Shiraz wine (Ugliano *et al.* 2007). It is generally believed that the conditions normally adopted for the production of red wine (i.e. higher temperatures, aeration of the fermenting must during cap management operations, extraction of YAN and other nutrients from skin during maceration) render fermentations less susceptible to slow or stuck fermentations, even when YAN concentrations approach the sub-optimal range. Nevertheless, several surveys have shown that YAN levels in red grapes can be well below optimal (Gockowiak and Henschke 1992; Butzke 1998; Nicolini *et al.* 2004; Ugliano and Henschke, unpublished data).

Although during red wine fermentations YAN deficiencies are likely to have a more moderate effect on fermentation kinetics, they can still negatively affect the formation of important aroma compounds. From the results of a trial which was carried out on a low YAN Shiraz must (YAN 100mg/L) with *S. cerevisiae* AWRI 796, it is again clearly evident that DAP supplementation is a powerful tool for modulating the volatile composition of red wine. This confirms some of the trends observed during experiments with model substrates and white grape juices. As can be seen in Figure 7, DAP supplementation resulted in higher production of ethyl fatty acid esters and acetate esters, while higher alcohols were scarcely affected.

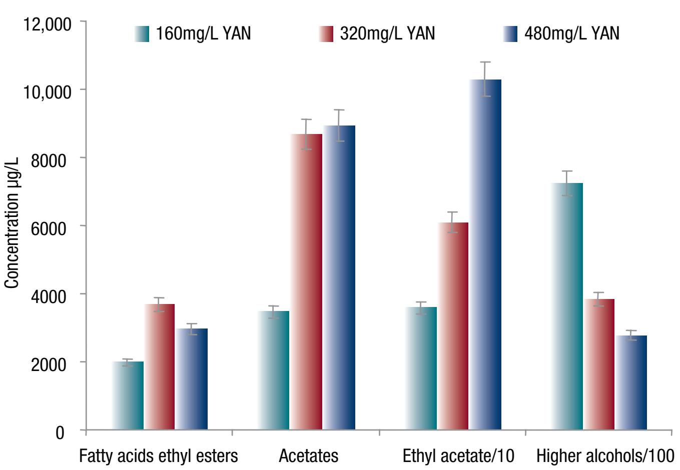

Figure 5. Volatile compounds of wines obtained from a low YAN (160mg/L) Chardonnay juice supplemented with two increasing concentrations of DAP, to a final YAN of 320mg/L and 480mg/L, respectively. Fermentations were carried out at 18°C using *S. cerevisiae* AWRI 796.

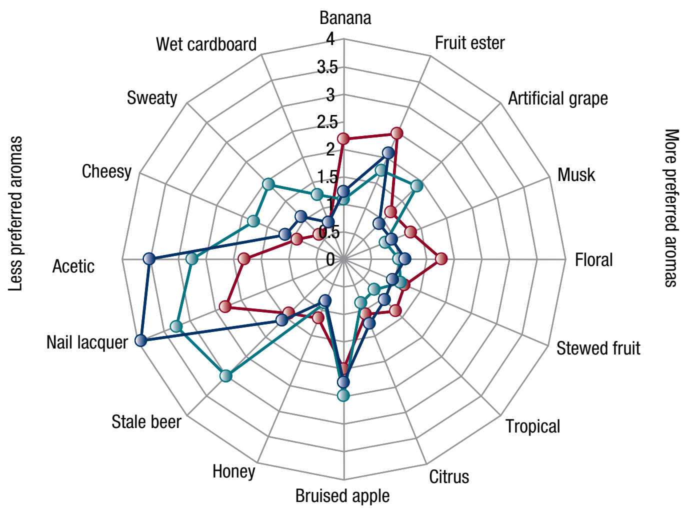

Figure 6. Sensory characteristics of wines obtained from a low YAN (160mg/L) Chardonnay juice (green line) supplemented with two increasing concentrations of DAP, to a final YAN of 320mg/L (red line) and 480mg/L (blue line), respectively. Fermentations were carried out at 18°C using *S. cerevisiae* AWRI 796.

Preliminary results also indicated that YAN supplementation of must can have an impact on red wine colour composition. Analytical parameters related to colour intensity and hue were indeed found to vary with DAP supplementation (Ugliano *et al.* 2007). The factors responsible for this effect are currently being investigated at the AWRI. The effect might be ascribable to various aspects of yeast metabolism that are known to modulate wine colour and phenolics composition. Factors include variations in the rate of ethanol production, absorption of anthocyanins on yeast cell walls (Morata *et al.* 2003) or reactions with yeast-derived metabolites such as pyruvic acid and acetaldehyde to form pigmented polymers (Romero and Bakker 1999).

CONCLUSION

This work shows that the concentration of yeast assimilable nitrogen is not only important for ensuring that adequate yeast growth and fermentation kinetics are achieved, but also can affect the production of the major metabolites arising from sugar fermentation. Whereas ethanol concentration is little affected, that of glycerol and various carboxylic acids can be markedly modulated. These changes are likely to affect wine flavour. Most importantly, however, is the finding that YAN can strongly influence production of some of the volatile metabolites, especially the acetate and ethyl esters, which are known to be positive to wine aroma when in balance. The impact of higher alcohols, which can be negative when present in high concentration, can also be modulated by YAN. These various yeast metabolites were also found to vary in red wine fermentations, suggesting that, as in white wines, must YAN can affect the development of wine flavour. Our preliminary data suggest that wine colour and phenolics composition can also be influenced by YAN.

Overall, these results suggest that, at least for Chardonnay, the flavour and style of wine is dramatically modulated by the initial YAN concentration of the grape juice. Low YAN level juices favour the production of more complex wines with less fruity aromas, whereas moderate YAN levels produce cleaner and fruitier wines. However, high YAN levels can lead to excessively estery wines. Similar effects can be expected in other varieties, excepting for those varieties that depend on thiols, for which no information is presently available. Clearly, more wine sensory studies need to be undertaken to better understand the effects of must YAN and amino acid profile on wine flavour.

A red wine trial is currently in progress to understand better the impacts of managing nitrogen in the vineyard compared with that in the winery on wine flavour and quality. This research can be expected to provide grapegrowers and winemakers with better information for optimising wine style and quality according to consumer preferences and other desired outcomes.

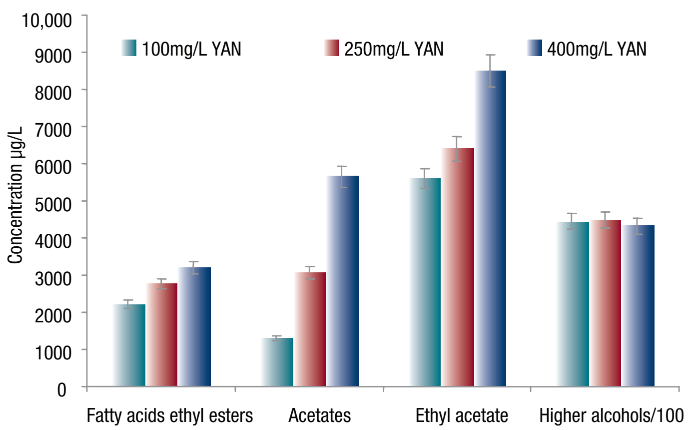

Figure 7. Volatile compounds of wines obtained from low YAN (100mg/L YAN) Shiraz grapes supplemented with two increasing concentrations of DAP, to a final YAN of 250mg/L and 400mg/L respectively. Fermentations were carried out at 22°C using *S. cerevisiae* AWRI 796, with cap plunging three times per day.

ACKNOWLEDGEMENTS

We thank our many colleagues for supporting this project, especially Tracey Siebert and Dimitra Capone for help with analysis of fermentation volatiles; Mariola Kwiatkowski and Meagan Mercurio for analysis of phenolic compounds; Kate Lattey, Belinda Bramley and Dr Leigh Francis for carrying out the sensory analyses; Industry Development and Support and Analytical Service team members for help with chemical analyses; and Dr Paul Chambers, Dr Cristian Varela and Biosciences team members for many helpful discussions. Professor Francisco Carrau, of Uruguay, and visiting scientists Drs Diego Torrea and Mar Vilanova, from Spain, have made important contributions to this project. We are especially grateful to Mike Farmilo, of Boar's Rock winery, for supplying a large number of juice samples and donating must for the Shiraz fermentation trials, and Russell Johnstone and Inca Pearce, of Orlando Wines, and Louisa Rose and Simon Dillon of The Yalumba Wine Company, for supplying white wine juices. Current work on nitrogen management also involves collaboration with Dr Sally-Jean Bell and Marcel Essling. Rae Blair is thanked for her editorial assistance. This project is supported by Australia's grapegrowers and winemakers through their investment agency, the Grape and Wine Research and Development Corporation, with matching funds from the Australian Government. The AWRI is a member of the Wine Innovation Cluster.

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